ENCYCLOPEDIA OF DAIRY SCIENCES

JOHN W. FUQUAY
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ENCYCLOPEDIA OF DAIRY SCIENCES SECOND EDITION

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John W. Fuquay, Professor Emeritus of Dairy Science at Mississippi State University, served on the faculty there from 1969 to 1999. His areas of emphasis in teaching and research were environmental physiology and reproductive physiology. He received his BS and MS degrees from North Carolina State University and his PhD degree from Pennsylvania State University, all in the area of dairy science. After completing the PhD degree in 1969, he accepted a teaching and research position at Mississippi State University, where he progressed through the ranks from assistant professor to professor before retiring in 1999. Professor Fuquay served as Coordinator for the Graduate Program in Animal Physiology from 1986 to 1999. He was a Visiting Professor in the Animal Sciences Department, University of California-Davis in 1979 and in 1985–86.

Professor Fuquay was active in his professional society, The American Dairy Science Association. He was a member of the editorial board of *Journal of Dairy Science* for seven years, an editor for four years, and served as the first Editor-in-Chief for six years (1997–2002). For his professional contributions and service to the Association, Professor Fuquay was recognized as a Fellow in the American Dairy Science Association in 2001 and received the Association's Award of Honor in 2002. Other recognitions include the World Association of Animal Production Jean Boyazoglu Award in 2003, the Distinguished Dairy Science Alumnus Award from Pennsylvania State University in 2003, and several teaching and research awards from his university.

Professor Fuquay has participated in a variety of international activities. He has presented short courses and lectures as well as provided consultations in a number of countries, primarily in Asia and Latin America. In addition to his research publications, he is the coauthor of a textbook, *Applied Animal Reproduction* (Prentice Hall), that has been widely used by universities in the United States and internationally. The first edition was published in 1980 and the last (sixth) edition in 2004. In 2010, he published a memoir, *Musings of a Depression-Era Southern Farm Boy* (Vantage Press), which reflects on how the experience of growing up on a farm in the southern United States during the great depression instills one with an understanding of the importance of strong family bonds and a sound work ethic in meeting the challenges of the adult world.

Patrick F. Fox was Professor and Head of the Department of Food Chemistry at University College, Cork (UCC), Ireland, from 1969 to 1997; he retired in December 1997 and is now Emeritus Professor of Food Chemistry at UCC. Prof. Fox received his BSc degree in Dairy Science from UCC in 1959 and PhD degree in Food Chemistry from Cornell University in 1964. After postdoctoral periods in Biochemistry at Michigan State University and in Food Biochemistry at the University of California, Davis, he returned to Ireland in 1967 to take up a research position at the Dairy Products Research Centre at Moorepark before moving to UCC in 1969.

Prof. Fox's research has focused on the biochemistry of cheese, the heat stability of milk, physicochemical properties of milk proteins, and food enzymology. He has authored or coauthored about 520 research and review papers, and authored or edited 25 text books on Dairy Chemistry. He was one of the founding editors of the *International Dairy Journal.*

In recognition of his work, Prof. Fox has received the Research & Innovation Award of the (Irish) National Board for Science and Technology (1983), the Miles-Marschall Award of the American Dairy Science Association (1987), Medal of Honour, University of Helsinki (1991), the DSc degree of the National University of Ireland (1993), the Senior Medal for Agricultural & Food Chemistry of the Royal Society for Chemistry (2000), the ISI Highly Cited Award in Agricultural Science (2002), the International Dairy Federation Award (2002), Gold Medal of the UK Society of Dairy Technology (2007), and an autobiography published in Annual Review of Food Science & Technology (2011).

Prof. Fox has been invited to lecture in various countries around the world. He has served in various capacities with the International Dairy Federation, including President of Commission F (Science, Nutrition and Education) from 1980 to 1983.

Paul McSweeney is Professor of Food Chemistry in the School of Food and Nutritional Sciences, University College, Cork, Ireland (UCC). He graduated with a BSc degree in Food Science and Technology in 1990 and a PhD degree in Food Chemistry from UCC in 1993 and also has an MA in Ancient Classics. He worked for a year in the University of Wisconsin (1991–92) as part of his PhD and as a postdoctoral research scientist in UCC (1993–94). He was appointed to the academic staff of UCC in 1995. The overall theme of his research is dairy biochemistry with particular reference to factors affecting cheese flavor and proteolysis during cheese maturation including the role of non-starter lactic acid bacteria and smear microorganisms, the ripening of hybrid and non-Cheddar varieties, the specificity of proteinases on the caseins, proteolysis and lipolysis in cheese during ripening, and characterization of enzymes important to cheese ripening (proteinases, peptidases, amino acid catabolic enzymes). He is the coauthor or coeditor of eight books, including the third edition of *Cheese: Chemistry, Physics and Microbiology* (Amsterdam, 2004) and the *Advanced Dairy Chemistry Series* (New York, 2003, 2006, 2009), and has published numerous research papers and reviews. Prof. McSweeney is an experienced lecturer and researcher and has successfully managed research projects funded through the Food Industry Research Measure and its predecessors administered by the Irish Department of Agriculture and Food, the EU Framework Programmes, the US–Ireland Co-operative Programme in Agriculture/Food Science and Technology, and Bioresearch Ireland and Industry. He was awarded the Marschall Danisco International Dairy Science Award of the American Dairy Science Association in 2004 and in 2009 a higher doctorate (DSc) on published work by the National University of Ireland.

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GUIDE TO USE OF THE ENCYCLOPEDIA

STRUCTURE OF THE ENCYCLOPEDIA

The material in the Encyclopedia is arranged as a series of entries in alphabetical order. Some entries comprise a single article, whilst entries on more diverse subjects consist of several articles that deal with various aspects of the topic. In the latter case the articles are arranged in a logical sequence within an entry.

To help you realize the full potential of the material in the Encyclopedia we have provided three features to help you find the topic of your choice.

1. CONTENTS LISTS

Your first point of reference will probably be the contents list. The complete contents list appearing in each volume will provide you with both the volume number and the page number of the entry. On the opening page of an entry a contents list is provided so that the full details of the articles within the entry are immediately available.

Alternatively you may choose to browse through a volume using the alphabetical order of the entries as your guide. To assist you in identifying your location within the Encyclopedia a running headline indicates the current entry and the current article within that entry.

2. CROSS REFERENCES

All of the articles in the Encyclopedia have been extensively cross referenced. The cross references, which appear at the end of an article, have been provided at three levels:

i. To indicate if a topic is discussed in greater detail elsewhere.
ii. To draw the reader's attention to parallel discussions in other articles.
iii. To indicate material that broadens the discussion.

Example

The following list of cross references appear at the end of the entry entitled **Bacteria, Beneficial** | Lactic Acid Bacteria: An Overview

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; *Streptococcus thermophilus*; Taxonomy and Biodiversity. **Pathogens in Milk**: Enterobacteriaceae.

3. INDEX

The index will provide you with the volume number and page number of where the material is to be located, and the index entries differentiate between material that is a whole article, is part of an article, or is data presented in a table or figure. Detailed notes are provided on the opening page of the index.

4. COLOR PLATES

The color figures for each volume have been grouped together in a plate section. The location of this section is cited in the contents list. Color versions of black and white figures are cited in figure captions within individual articles.

5. CONTRIBUTORS

A full list of contributors appears at the beginning of each volume.

6. GLOSSARY

A glossary of terms used within the work is provided in Volume Four before the Index.

PREFACE

We are pleased to present the second edition of the *Encyclopedia of Dairy Sciences*. The first edition was published in 2003 by the Major Reference Works Division of Academic Press, now part of Elsevier Sciences, and it comprised 427 articles. The objective was to satisfy the need for an authoritative source of information for people involved in the integrated system of production, manufacture, and distribution of dairy foods. It was realized from the beginning that a program of revision would be needed to keep the Encyclopedia up to date. This goal has been met in the second edition through 503 articles, of which 121 are new articles and 382 are revised articles. We express appreciation to the Editorial Advisory Board for its role in evaluating articles for needed revision, reviewing new and revised articles, and for help in identifying new topics to be included along with appropriate authors. Likewise, we are grateful for the contributions of the many authors who have either revised their articles or prepared new articles.

The main topics related to milk production and dairy technology are addressed in addition to providing information on nutrition, public health, and dairy industry economics including aspects of trade in milk and dairy products. All species that produce milk for human consumption have been included in this work. Some of these species are of regional significance only, but they have been included because of the essential role that their milk plays in the nutrition of people inhabiting various regions of the world. A significant addition to the second edition is four introductory articles addressing the history of Dairy Science and Technology. A synopsis has been prepared for each article in the second edition and will appear with the online listing of the articles in this publication.

The primary aim of the Encyclopedia is to provide a complete resource for researchers, students, and practitioners involved in all aspects of the dairy sciences as well as those involved with economic and nutritional policy and members of the media. We have tried to do this with a writing style that is easily comprehended by persons who are not highly trained in the technical aspects of the Dairy Sciences. Users should be able to access information on topics that are peripheral to their areas of expertise.

We express appreciation to the staff of the Major Reference Works Division, responsible for this Encyclopedia, for their timely responsiveness to the needs of the editors and their essential administrative role in keeping this major reference work on-track toward a satisfactory completion within the desired time schedule. We remember Nancy Maragioglio, Senior Life Sciences Editor, who initiated the work and was ever responsive to queries by the editors, as well as Sera Relton, Esmond Collins, Milo Perkins, and Claire Byrne, Development Editors, and Charlotte (Charlie) Kent, Publishing Administrator, who kept things moving through their communication with editors, authors, and reviewers and who exhibited almost flawless administrative skills. Sera Relton was particularly helpful as she assisted us in moving through the final submission and review stages. Laura Jackson is recognized for her contributions as Production Manager of the Encyclopedia.

Special recognition is due to Ms Anne Cahalane, Senior Executive Assistant, School of Food & Nutritional Sciences, University College, Cork, whose stylized representation of a cow, a milk can, and a wedge of cheese adorns the cover of the first and second editions of the Encyclopedia of Dairy Sciences.

John W. Fuquay
Patrick F. Fox and
Paul L. H. McSweeney

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FOREWORD

The cow is the foster mother of the human race. From the days of the ancient Hindoo to this time have the thoughts of men turned to this kindly and beneficent creature as one of the chief sustaining forces of human life.

William Dempster Hoard (1836-1918)
Former governor, state of Wisconsin, USA (1889-1891)
Founder of **Hoard's Dairyman** (1885)

We must never forget that milk and milk products are and will always be important sources of basic food nutrients for humans both young and old. The more scientific facts we can discover, understand, and apply related to producing, processing, and marketing milk and milk products, the better we will serve the nutritional needs of humanity throughout the world.

More than 2000 years ago Aristotle noted, *Everyone honors the wise and excellent.* We are indebted to those *wise* enough to conceptualize and envision the favorable global impact that is certain to follow by bringing together this exhaustive, rich collection of 503 pertinent articles written and reviewed by more than 700 world-renowned disciplinary experts representing 50 countries – persons each of whom bears the mark of *excellence.* Happily these timely topics are now recorded in four informative, important, engaging volumes. We thank, commend, and salute the prodigious efforts of the *wise* and *excellent* authors who generated, compiled, and put the spotlight on the useful information and data, and who now share them through their well-written articles.

One noteworthy value and enduring virtue of these articles is bringing into clear perspective the context of both the state-of-the-art and the future of dairy sciences. When the history and contributions of scholarly publications related to the all-important global dairy industry are recorded, the second edition of the *Encyclopedia of Dairy Sciences* will be cited often and with great respect and appreciation.

Fundamental to continued progress and success in the dairy industry have been the signal service, cooperation, and collective contributions of dedicated scientists, teachers, agricultural advisors/extension workers, and representatives of governments and industries. Additional exciting breakthroughs in applying new findings and developments in research and technology to the production and processing of milk are sure to follow as we move surefootedly through the twenty-first century. This continued growth and success will be aided immensely by the vast and extraordinarily useful knowledge base made available by the idea-rich, insightful authors, editorial advisory board members, editors, and publisher of the second edition of the *Encyclopedia of Dairy Sciences.*

Indeed, by perusing the comprehensive and authoritative articles of this greatly needed and monumental encyclopedia, readers will be made even more aware of the tremendous progress that has occurred in the basic and applied sciences underpinning the global dairy industry.

Ours is an internationally competitive and incredibly technological world. And unless talented, creative scientists continue to work together in researching and applying the most effective and economical ways and means of providing

an abundant, safe supply of milk and milk products for an ever-increasing world population, we will never reach our noble goal of adequately feeding all the earth's people.

May we utilize the comprehensive scientific knowledge base made available through this second edition of the *Encyclopedia of Dairy Sciences* as we pledge to realize advances in the health and well-being of the undernourished millions – including many who need and deserve to be rescued from the ugly grip of hunger – by increasing the availability of nature's most nearly perfect food – milk!

Pure milk from healthy animals is a luxury of the rich, whereas it ought to be the common food of the poor.

Mohandas Gandhi (1869-1948)
Indian nationalist leader

John R. Campbell

John R. Campbell, Ph.D., D.Sc. (Hon.)
President Emeritus and Professor of Animal Science
Oklahoma State University

Dean Emeritus and Professor Emeritus of Animal Sciences
College of Agriculture, University of Illinois

Professor Emeritus of Animal Sciences
University of Missouri

Past President, ADSA (1980-81)

April 2010

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INTRODUCTION

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History of Dairy Science and Technology
History of Dairy Farming
History of Dairy Products and Processes
History of Dairy Chemistry
History of Dairy Bacteriology

History of Dairy Science and Technology

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As described in the entry **Milk**: Introduction, milk is the characteristic secretion of female mammals, intended for use as the sole source of nutrients for their young. Mammals evolved from certain reptiles, synapsids (therapsids), during the Early Jurassic period, about 200 million years ago; however, milk was not used in the human diet until the domestication of certain species, initially sheep and goats (about 10 000 years ago), and then cattle and later buffalo, camels, and horses. Since then, milk and dairy products have become major items in the human diet, especially in Europe, North and South America, the Indian subcontinent, Australia, and New Zealand.

Presumably, some fresh milk was consumed from the beginning of dairying but since milk is very susceptible to microbial spoilage, there was a major incentive to develop more stable products. Initially, the principal products were as follows:

1. Butter, produced from fat-rich cream, obtained by gravity creaming and preserved by salting (since butter can be produced simply by agitating milk, it is likely that it was produced accidentally shortly after the domestication of milk-producing animals, probably initially from sheep and goat milk). Although butter was known to the Classical Greeks and Romans, it was not popular as a food in Greece and Rome because it spoils relatively easily; however, it was popular with the 'Barbarians' of cooler northern Europe. In India, ghee, which is more stable than butter, was, and still is, the principal high-fat dairy product.
2. Fermented milk products and cheese, preserved by a low pH and salt; these products were also probably discovered accidentally and were well established in Ancient Egypt, Classical Greece, and Rome. Several Roman authors, Cato, Varro, Pliny the Elder, and especially Columella, have described cheesemaking in considerable detail.

Although a few research papers on the chemistry of milk were published at the end of the eighteenth and early nineteenth centuries, milk processing was entirely farm-based until about 150 years ago and was not scientifically based. Although butter, cheese, and fermented milks had been produced since prehistoric times and Nicolas Appert attempted to produce heat-sterilized milk in 1809, milk processing was not industrialized until the second half of the nineteenth century. Thus, although dairying has a long history (~10 000 years), it has been industrialized for only about 150 years. These articles attempt to describe the key events in the history of the dairy industry, under four sections – (1) dairy farming, (2) dairy products and processes, (3) dairy chemistry, and (4) dairy bacteriology – and provide an introduction to the articles that follow in this encyclopedia.

History of Dairy Farming

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Introduction

Cows, goats, sheep, and camels are mentioned in the Old Testament of the Bible as sources of milk for humankind. There is evidence that cheese and yogurt were made as far back as 7000 BC. Goats and sheep were probably the first domesticated animals used for milk, meat, and skins. Cows were domesticated in the Fertile Crescent by 4000 BC. From these early beginnings, cattle moved into northern Europe (*Bos primigenius*) and southeastern Asia (*B. namadicus*). These cattle were the ancestors of the *B. taurus* and *B. indicus* cattle, respectively, of today.

Cattle account for over 90% of the world's milk production. The most popular dairy breeds within *B. taurus* include Holstein-Friesian, Jersey, Brown Swiss, Guernsey, Ayrshire, and Milking Shorthorn. Some dual-purpose breeds, for example, Montbeliarde and Simmental, are used for both milk and meat production. A brief description of each of the major dairy breeds is given below.

Breeds of Dairy Cows

Ayrshire

The foundation of Ayrshire cattle originated in the County Ayr, Scotland, between 1750 and 1780. It is the only breed of dairy cattle developed on the main Isles of Great Britain. The mature cow is of medium frame and averages 550–600 kg. Ayrshire cattle were first imported into the United States in 1822.

Brown Swiss

Brown Swiss were developed in the Alps of Switzerland. Brown Swiss began as small- to medium-framed cattle – its larger frame size of today was probably influenced by the Pinzgauer, a dual-purpose breed in the Austrian Alps. The summer tradition of grazing in the high meadows of the Alps, which contributed to its vigor and ruggedness, continues to this day. The first Brown Swiss appeared in the United States in 1869.

Guernsey

The Guernsey breed was developed by monks more than 1000 years ago on the island of Guernsey in the English Channel. The breed developed from the crosses between cattle from Brittany and Normandy. Guernsey milk has a golden hue attributed to β-carotene, a precursor of vitamin A, which is transferred directly from the diet into milk. Admiral Byrd took Guernsey cows with him on his expedition to Antarctica in 1933. Guernsey cattle appeared in the United States in the early 1830s.

Holstein-Friesian

The Holstein-Friesian is the largest of the dairy breeds; it produces the largest amount of milk and is the most popular. The breed has its origin in the northern provinces of North Holland and West Friesland of the Netherlands. In the early stages, the cattle were either black and white or red and white. The black and white characteristics of the breed developed after 1750 as farmers selected only black and white bulls for herd sires; however, the gene for red color remains in the breed to this day. The cattle grazing the lush polders of North Holland contributed to the selection of cows with high genetic merit for milk production. Several Holstein-Friesian cows have produced over 27 000 kg of milk in a 305-day lactation period in the past 30 years.

Jersey

The Jersey breed was developed on the island of Jersey in the English Channel. Like the Guernsey, the Jersey breed developed from the crosses between cattle from Brittany and Normandy. The Jersey breed ranks the highest among all major breeds in milk fat and protein percentages. It is prized by cheese makers for its rich creamy texture and outstanding yield of cheese. The jersey breed is the smallest and the earliest maturing dairy breed; it is more heat-tolerant than its counterparts and is recognized for its reproduction and longevity.

Milking Shorthorn

The Milking Shorthorn developed from the same foundation stock as the Beef Shorthorn. In England and Australia, the breed is called the Dairy Shorthorn. The breed developed in northeastern England in the middle of the previous millennium. Milking Shorthorns are a mix of red, white, and roan in color, possess a medium frame size, and generally tolerate cold temperatures better than warm temperatures. Shorthorn genes are found in the ancestry of some 30 breeds of cattle worldwide.

Zebu Cattle

Zebu (*B. indicus*) cattle, characterized by a large hump above the shoulders, originated in Southeast Asia and are dual purpose. Zebu is more heat tolerant and resistant to insects because of its thicker skin and is found primarily in tropical climates. Breeds for dairying include Gir and Red Sandhi.

The Industrial Revolution – The Beginnings of Modern Agriculture

Agriculture was a huge benefactor of the Industrial Revolution. Steam power reduced dependence on animal power and iron replaced wood as the material for implements. Several key inventions and processes developed between 1850 and 1900 expanded the utilization of milk beyond the farm. The cream separator, invented by G. P. de Laval, and the production of condensed milk by Gail Borden, were key in the early expansion of dairy farming. Pasteurization, machines for filling and capping bottles of milk, and refrigeration brought the demand for milk to urban areas. Milk was moved to urban areas by trains for the making of cheese and butter. These events expanded the opportunities for dairy farming as an economic enterprise. By 1900, there were over 5 million farms and 18 million cows in the United States.

Systems of Dairy Farming

Dairy production methods around the world are a continuum of feeding and housing practices. Pasture has been the basis for milk production from the beginning and remains as the primary source of nutrients for milk production in many parts of the world today. In some countries, most notably New Zealand, over 90% of the milk produced is based solely on pasture. The annual production cycle and length of milk production by the cow are superimposed on a geography that provides a moderate climate for a long growing cycle of rich grasslands. At the other extreme, cows are confined in environmentally facilitated dry lots and barns in hot, dry areas such as the western United States, portions of Mexico, and the Middle East. Cows in this system depend solely on stored feeds. Between the extremes are an assortment of dairy farms in Europe, Australia, Central and South America, and parts of North America. Cows on these dairy farms may be at pasture during parts of the year and receive stored forage concurrently and in winter months, along with varied levels of supplemental concentrates throughout the year.

Milk production requires an excellent forage base. Grasses, cool-season and warm-season annuals and perennials, and legumes, for example, alfalfa and clover, are a must for achieving high production and maintenance of a healthy cow. Grasses and legumes grown in temperate and desert areas support a diversity of farm sizes. Grasses are the major source of forage for dairy cattle in northern areas of the northern hemisphere and southern areas of the southern hemisphere. These are harvested as grass silage for the most part, because of unfavorable drying conditions. High-moisture silages (less than 30% dry matter) are susceptible to clostridial organisms that produce butyric acid. These silages suffer considerable spoilage and may reduce animal performance. Corn silage is a recent introduction on the timeline of dairy production brought about by mechanization in harvesting. However, the length of the growing season that is required for corn silage limits the geography where it can be planted and harvested successfully.

Confinement Systems for Dairy Cattle

Dairy cattle are provided some degree of protection from the elements in all but the total pasture-based systems. Total confinement systems began as stanchion and later tie-stall barns where cattle were tethered to individual stalls. Stalls were bedded for comfort with straw or other adsorbent materials. Cows were fed and milked while in the stall and were allowed an exercise period for 2–6 h a day in a nearby lot or pasture. While cows were outside, stalls were cleaned and rebedded. Animal waste was removed by hand labor or mechanically with scrapers. This system of housing was labor intensive and generally limited the size of the dairy herd. Confinement housing of this type remains the primary management system of dairy production in terms of the number of farms in many countries throughout the world. In northern Europe, the family house and dairy barn was often a single unit. The lowest level housed the cows in stalls or cubicles especially during winter months. Family living quarters occupied the upper levels.

The introduction in the 1950s of free-stall barns and corral dairying in hot, dry climates changed dramatically the face of dairy farming. These innovations allowed managing cows in groups. Cows could be fed in groups and milked in parlors. Dairy cow numbers on farms began to increase. The innovations created a demand for newer approaches to managing and feeding dairy cows.

The first free-stall barn built in the United States was in Washington in 1958. Free-stall barns enabled cows to lie down and gave them free access to feed and water. Individual stalls had three elements to control where the cow laid within the stall. A partial barrier at the front of the stall called a brisket board and a bar (neck rail) positioned forward in the stall about the height of the cow's withers and perpendicular to the long axis of the stall regulated how far cows could lay in a stall. Extensions from the front of the stall, often called loops, defined the lateral boundaries of the stall. Cows were positioned in stalls so that urine and feces fell into the alley of the barn. Feeding was either in a center lane running through the middle of the barn or on each side of the barn. Cows were moved to a milking parlor for milking. Since that time, the majority of new housing has incorporated free-stalls in the barn design.

Dry lot dairying began in the western United States in the late 1950s. This method of confining dairy cows worked well in hot, dry climates with low humidity and was the beginning of dairy farming in traditionally non-dairy areas throughout the world. Cows were free to move in a corral that often had only a structure that provided shade from direct solar radiation. Feeding was by fence-line bunk and milking occurred in a parlor.

The introduction of dairying into hot, dry climates posed special challenges to lactating dairy cows, which are exacerbated by high relative humidity (RH). The term temperature humidity index measures the relative levels of the effects of the environment on cow welfare. At any given temperature, increases in RH have a curvilinear effect on animal welfare. For example, a temperature of 30 °C (86 °F) at 0% RH has little effect on animal welfare. At the same temperature and an RH of 50%, the impact on welfare may be borderline to severe.

Heat abatement for dairy cows is a necessary strategy for animal well-being in hot climates and in areas that experience high temperatures for a few weeks. Air movement, water (soaking or high-pressure mists), and minimization of the transfer of radiant energy into a facility are used alone or in combination for heat abatement. These strategies are strategically located over free-stalls and feeding areas and in the holding areas of milking facilities. Newer, long and narrow barns incorporate tunnel ventilation where air is blown through tubes with holes for air exhaust into the living space of the cows. Barns with three sides enclosed and sprinklers located at intervals throughout the length of the barn have fans in the closed end that pull air through the barn for heat abatement. Cross-ventilation barns employ the same strategies for cooling but air movement is across the short axis of the barn. These barns have the capability of having all sides of the barn enclosed. Cross-ventilated barns are more popular in areas that may experience temperatures well below 0 °C.

Feeding Practices

The evolution of feeding practices followed improvements in the genetic ability of dairy cows to produce milk, increases in land prices, and the transition from numerous small dairy production units to larger production units. For many years, dairy cows were bred to calve in the spring and reach peak milk production coincident with peak grazing conditions. Limited concentrate (primarily a mixture of cereal grains and oilseed by-products) was offered at milking time. Hay and corn silage were harvested and stored for use during the winter months when milk production was at its lowest point. However, increased demand for milk during the school year resulted in monetary premiums for milk during the fall and winter months and encouraged producers to shift their breeding programs such that cows reached peak production during late summer or early fall. This change reduced the use of pasture in the United States and increased the use of harvested and stored forages.

The use of confinement housing facilities and automated forage and concentrate feeding equipment became the standard on modern dairy farms. Corn silage and alfalfa hay were favorites with dairy producers because corn silage served as a high-energy feedstuff, while alfalfa served as a high-quality and high-quantity protein source. Concentrated feedstuffs containing additional energy, protein, vitamins, and minerals were offered in granular or pellet form in the milking parlor. The increased use of proven bulls via artificial insemination (AI) led to rapid advances in the milk-producing capability of dairy cows, which increased their nutrient requirements. The average dairy cow consumes approximately 0.2–0.3 kg of concentrate per minute in the parlor and spends about 15 min per milking. This time frame limited feed consumption to 6–9 kg concentrate per day. To ensure adequate nutrient intake, dairy producers with stanchion or tie-stall facilities provided additional concentrate on an individual cow basis in accordance with the amount of milk produced. Producers that utilized free-stall or loose housing facilities either retained high producers in the parlor longer or top-dressed the forage offered in an outside bunk. Both methods of supplementation increased labor and feed costs because of longer time in the parlor (a slow milking cow in a parlor also allowed extra time for other cows to eat) and consumption of concentrates by cows managed in groups. Hence, the computer feeder was developed so that producers could provide additional concentrate to only high producers.

The complete ration, later called the total mixed ration (TMR), concept was developed following the movement of cows into group housing situations. The premise of the TMR was that each mouthful of feed contained a balanced mix of feed that supplied all the nutrients required for high milk production. Initially, cows were maintained in groups of similar production and several rations varying in nutrient density were fed. In time, the single mix TMR was fed to all cows with the assumption that cows would consume feed according to their level of production. In other words, low producers would consume less than high producers. The was important because overfeeding of concentrated feedstuffs could lead to fat cows and increased feed cost. The use of a TMR provided the opportunity to offer all feedstuffs in a bunk located outside the milking parlor. Parlor feeding increased cow time in the parlor, feed wastage, and parlor cleanup time compared to a TMR offered in an outside bunk.

Automatic feeders came on the market in the mid-1970s. The principle of automatic feeders was that managers identified high-producing cows that required supplementation beyond the herd average. The first automatic feeders utilized a magnet on a chain around the neck of the cow. As she approached the feeder, the magnet engaged a mechanism that dispensed concentrate into a bowl for the cow. These types of feeders were followed by computer feeders that identified individual cows by a transponder hanging from the neck. The amount of concentrate available for each cow over a 24 h period was programmed from an office and often limited meal size to ensure several feeding bouts during the day. The era of automatic feeders lasted for about 10 years.

Changes in the way cows were fed had a dramatic influence on the feed industry. Feed companies, local, regional, and national, provided complete feeds to complement on-farm forages. A farmer might grind his corn and mix with a supplement that contained protein, energy, minerals, and vitamins. Belt and auger delivery systems for silages were replaced by mixer wagons and trucks. Producers were able to purchase by-products, for example, hominy, citrus pulp, beet pulp, and soybean meal, from milling and food-processing industries. These commodities were delivered and stored on the farm. The producer purchased a concentrated supplement of minerals, vitamins, and specialty feedstuffs such as buffers, bypass fats, and bypass protein from a feed company. In some cases, commodity feeds might be contracted ahead for a full year, ensuring a constant source of feed ingredient at a constant price.

A major advancement in dairy cattle husbandry occurred in the 1980s with the demonstration of the importance of the dry period (non-lactating state) with respect to health and production during the lactation period. Health issues such as milk fever, ketosis, displaced abomasum, and retained placenta were found to be related to improper feeding programs during late lactation and the dry period. For example, milk fever or hypocalcemia, long thought to be caused by excess calcium in the diet of pre-partum cows, was associated with elevated dietary potassium. These findings led to significant improvements in the dry cow feeding program: limited potassium, increased vitamin E and selenium, and a two-stage feeding program during the dry period. Non-lactating cows were separated into far-off (60–30 days pre-partum) and close-up (29 days pre-partum to parturition) groups and offered diets containing different nutrient concentrations.

Feed Storage

Early settlers harvested hay with a hand scythe. The Industrial Revolution introduced the horse-drawn mowing machine and hay rake. Cut hay was stacked and shaped around a tall pole such that the stack would shed water and the hay would retain most of its quality. Early barns were constructed with a hay mow or loft for hay storage during winter months. Many barns were equipped with a rail down the centerline of the roof to accommodate a pulley with a harpoon-like hay fork attached by ropes to the rail. The center rail extended out from the barn loft such that stacked hay from the field could be moved by ropes and pulleys into various areas of the loft for storage. The process was reversed during hay feeding.

Tractors, mowers, rakes, and the hay baler further reduced the time and labor associated with haymaking and storage. The small square bales from the early hay balers could be stacked in the barn loft, and the bale density increased the hay storage capacity of the loft. Haymaking still required considerable labor, but became easier with the invention of the large round baler in the early 1970s. This machine wrapped the hay in a large round bale that could be stored outside until fed. Outside storage of large round bales was more successful in the drier western states than in wetter climates, so barns were constructed without lofts for storage in wetter areas. Large round bales reduced the amount of hay that could be stacked on trucks, so the equipment industry responded with large square balers that provided bales weighing 700–1000 kg (1540–2200 pounds). Large plastic bags and plastic sheeting were introduced around 2000 for storage of high-moisture forages and wet by-products.

Corn silage was first used in the United States by F. Morris in 1876. The use of fermented corn silage was not favored by many producers because it contained alcohol – it was thought that it would be harmful to cows and that the resulting milk would be hazardous to humans. By the early 1900s, corn silage was a staple on many progressive dairy farms.

Concrete stave and glass-lined oxygen-limiting tower structures or silos were developed as storage units. Unloaders within the silo moved silage onto belts or augers for feeding or mixing with other ration components.

As herd size increased, a move to large bunker silos allowed low-cost storage of huge amounts of feed. Feeding became even more mechanized with front-end loaders and mixer wagons fitted with load cells for rapid, accurate feed preparation. More recently, large plastic bags have gained popularity for storing corn silage and hay crop silage. The bags are filled by dumping the chopped material in a self-propelled bagger that packs the material tightly in the bag. Many of these long, white cylinders can be viewed as one travels through farm country.

In summary, advances in dairy cattle nutrition, feeding practices, and feed storage and handling techniques have dramatically improved feed efficiency and milk production capacity of the dairy cow while reducing operational cost associated with feeding on dairy farms throughout the world. These advances have allowed producers to remain in business while supplying consumers with a relatively low-cost, highly nutritious product.

Harvesting of Milk

Milking cows by hand was a twice-daily task that likely limited the size of early dairy farms. Milking was a chore that included all family members. Early farms had several pieces of equipment that enabled dairy farm families to harvest and process milk. The three-legged stool, milk bucket, milk strainer, and milk can were essentials for the task of milking. The cream separator operated by centrifugal force separated milk into cream and skim milk. Cream was churned into butter or sent to market. Skim milk was often fed to young stock.

Many patents were granted for milking machines between 1850 and 1900. However, it was not until about 1918 that milking machines became commercially available. Several milking machine companies provided milking equipment to the dairy industry. A Hinman milking machine was used for milking approximately 10 Jersey cows at the North Carolina dairy farm on Biltmore Estate in 1919. de Laval introduced a milking system in 1922 that was powered by a gasoline engine. The Alpha Dairy Power Plant generated electricity for the milking equipment and cream separator, heated water for washing, and provided electricity for lighting the milking area. Babson Brothers introduced the Surge Milker in 1922. Many of the principles of the early milking machines, for example, detachable pulsators and flexible liners, continue in use today.

The side-opening milking parlor was introduced in the mid-1920s. The rotolactor was a rotary milking parlor installed in 1930 in Plainsboro, NJ. An observation deck allowed guests to watch milking unobtrusively as cows rode on the platform below. The Rural Electric Administration of the Roosevelt presidency provided electricity to farms in the mid-1930s. Still, by 1950, only about 50% of the dairy cows in the United States were milked by machine and mostly through pipelines mounted above the stalls where the cows were housed.

The herringbone parlor was introduced in the late 1950s at a time when new housing methods began to appear. The central area or pit of the parlor was the operation center. Cows entered for milking on either side of the pit on an elevated platform. As the parlor filled, cows turned approximately 30° to the right or left to position them for milking. The number of milking units was equal to the number of milking positions on a side or in total for the parlor. When all cows were milked, cows exited from the front of the parlor. Milking efficiency in terms of the number of cows milked per hour increased dramatically.

The rapid exit parlor has similar characteristics of a herringbone parlor with three differences: cows turn 90°, milking units are attached to the udder between the hind legs, and all cows are released simultaneously when milking is finished.

Rotary or carousel parlors designed after the rotolactor became popular as herd size expanded. Rotating platforms capable of milking 60–80 cows per turn appeared on large dairies. These became popular in New Zealand and Australia before moving to large dairies in the northern hemisphere. A 106-cow rotary parlor was installed in Texas in 2009.

On-Farm Storage of Milk

Milk at the farm was transferred to galvanized cans usually about 10 gallon (approximately 40 l) on the farm. Milk was not cooled mechanically except for the occasional instances where a nearby spring supplied water for cooling. Much of the milk was still transported in cans to ‘dumping stations’ where milk from many farms was collected. Refrigerated milk coolers for bulk and can storage appeared on farms as electricity became available in the 1930s. Trucks with insulated tanks replaced rail cars during this time for transporting milk to factories for processing into ice cream, butter, and cheese. Improvements in on-farm cooling of milk and passage of strict laws on pasteurization greatly enhanced the use of fluid milk and dramatically reduced the number of cases of food poisoning caused by fluid milk.

Bulk tank storage grew rapidly on dairy farms. The growth was spurred when on-farm plate coolers were installed to lower milk temperature below 4 °C for storage. The growth of on-farm bulk tanks outpaced trucks with tanks such that some bulk tanks were elevated and after cooling, milk was emptied into cans for transport to milk plants. Tanks large enough for 2 days of milking became the norm. Tank size grew along with farm size. On many large farms today, milk is cooled and pumped directly into waiting tanker trucks capable of holding 22 000 kg and then transported immediately to the milk plant.

Udder Health

Mastitis remains as the most costly disease of the dairy cow. Losses occur through treatment cost, lost milk production, and culling. Prior to 1950, control and treatment programs varied widely, often contained conflicting elements, and were based on inadequate scientific studies.

Major advances in the understanding of the causes of mastitis, for example, environment and milking machines, and use of hygienic procedures during milking occurred between 1950 and 1980. The California Mastitis Test, introduced in 1957, was a cow-side test that identified infected quarters. Milk from each quarter was stripped into individual wells of a paddle and a solution was added. Coagulation of the milk indicated the presence of infection. Additional cow-side practices implemented during this period included cleaning teats prior to milking, use of sanitizing solutions for hands and teat cups, and post-milking teat dips. Injection of long-acting antibiotics at the beginning of the dry period reduced new infections at calving by 75%.

Mastitis control programs were introduced in the 1980s. The use of somatic cell count (SCC) in milk as a measure of overall herd and individual quarter and udder health became a cornerstone of these programs. New dry cow antibiotic therapies, introduction of vaccines against certain classes of bacteria, and the use of teat-end sealants placed emphasis on the dry period as a control point for mastitis control. Finally, new classes of antibiotics were introduced for the treatment of clinical mastitis.

Breeding and Reproduction

The pregnant cow remains the most valuable animal on the dairy farm. Breeding by natural service was the norm for many years. However, genetic progress in dairy breeds was slow. AI was one of the first technologies that began to change the dairy farm. The first AI organization began in the United States in 1938 and gained acceptance quickly. Fresh semen was delivered to farms by mail, automobile, rail, and even by air using parachutes to drop canisters of semen to AI technicians. By 1950, almost 100 AI organizations existed in the United States. During this time, J. L. Lush of Iowa State University introduced the science of statistics to the science of genetic selection.

A major breakthrough in AI occurred in 1950 with the discovery in England that sperm remained viable after freezing with the addition of glycerol to semen extenders. By the mid-1950s, AI organizations made the switch to frozen semen. During this time, automated systems were developed for processing Dairy Herd Improvement Association (DHIA) records. Animal geneticists developed quantitative methods to enhance genetic progress made possible through the widespread use of bulls brought about by the technologies of the AI industry and DHIA records.

Frozen semen led to a consolidation of the AI industry. It also allowed international transport of semen for genetic improvement of dairy cattle throughout the world. Semen from superior bulls remained in service long after the bull was deceased.

While much attention on animal breeding was focused on the male, getting cows pregnant by AI in a timely manner remained a major challenge in herds regardless of size. As herd size increased, heat (estrus) detection became the bottleneck. Programs for identifying cows in heat included chin-ball markers (an apparatus beneath the jaws of the bull that spread an ink-like substance on the tail-head of a cow when ridden by the bull), grease markers applied to the tail-head, and patches that released a dye onto the tail-head when ridden by another animal. In some cases, time-lapse photography that recorded nighttime activity was viewed the next morning to identify cows in heat.

In the early 1970s, two compounds, prostaglandin $F_{2\alpha}$ (PGF) and gonadotropin-releasing hormone (GnRH), were recognized as causing regression of the corpus luteum and growth and release of ovarian follicles, respectively. Research led to approval of these compounds by regulatory agencies worldwide for use in estrus synchronization in cattle.

It is now possible to manage estrus in cows to improve reproductive efficiency of the dairy herd. Protocols have been developed that allow 'synchronization' of the estrous cycle of multiple animals and to reasonably predict the occurrence of ovulation. Several variations of these protocols exist. The basics are to administer PGF to a group of animals and breed on observed heats. If no heat is observed, the cow receives a second PGF injection 14 days later and bred on an observed heat.

More sophisticated synchronization programs called Ovsynch and Cosynch combine the effects of GnRH and PGF. In both programs, GnRH is administered on day 0. Seven days later, cows receive a PGF injection followed by a second GnRH injection 48 h (Ovsynch) or 48–64 h (Cosynch) later. Cows are bred without heat detection 8–18 h following GnRH in Ovsynch and at the time of GnRH injections in Cosynch. Overall, pregnancy rate may be 50% or greater on first inseminations. Cows that fail to settle will return to estrus in a predictable 19–21 days later.

Sexed semen is the most recent technology introduced on the dairy farm. Sperm are separated in the laboratory into sperm with the X chromosome and sperm with the Y chromosome. The X chromosome sperm are preserved like non-sorted sperm for AI. The fertility of sexed semen is lower (60–70% of normal) than that of non-sorted sperm and is used most successfully on virgin heifers. About 90% of calves born from sexed semen are female.

Disease Control

The veterinary profession had a major impact on the control, treatment, and eradication of diseases in dairy cattle during the twentieth century. The profession initially dealt with eradication of infectious diseases such as foot-and-mouth disease (FMD) and tuberculosis (TB). These diseases had a significant impact on Brown Swiss herds in the United States between 1910 and 1920. Many parts of the world are FMD-free. An outbreak of FMD occurred in the United Kingdom in 2001, which required the slaughter and burning of carcasses. The disease remains a threat to cattle in Asia, Africa, and parts of South America. Bovine TB remains a threat to cattle populations in many countries. It may be transferred to cattle by a number of species including deer, badgers, and opossums.

Antibiotics and vaccination programs were developed for treatment and prevention of many diseases from pneumonia to brucellosis in the 1940s to the 1960s. As confinement systems changed, so did the role of the veterinarian. The profession became proactive in developing whole herd health programs and in making regular farm visits although emergency calls to the farm for cesarean delivery or for intravenous therapy for milk fever remained a significant part of the dairy veterinary practice.

Production medicine became the emphasis of veterinary practice in the past 30 years. Interpretation of records to identify bottlenecks in reproduction, metabolic disease, or aspects related to parturition continues to change the on-farm role of the veterinarian. In some countries, the cost of a veterinary education, the long hours of the practitioner, pay, and other casual factors reduce the number of veterinarians that enter large animal practice.

Raising Replacement Animals – Calves and Heifers

Calf rearing was an occasional topic of writers for newspapers in New Zealand at the beginning of the twentieth century. One article in 1902 describes a protocol where over a 6-year period, 110 out of 112 newborn calves survived to maturity. The calf was removed from the calving area immediately following birth, 'rubbed down' with and then covered in straw, and fed 'about a pint (~0.5 l) of its mother's first milk.' Calves were fed increasing amounts of milk 3 times a day until 8 weeks of age. Hay was offered in the third week and a linseed soup in the fifth week. The linseed soup was prepared by soaking linseed in 4 US gallons (~15 l) of water, overnight. The next day, the soup was boiled for 30 min and one half pound (225 g) of flour (source not given) added in the last 5 min of boiling. A tablespoon (~12 g) of sugar and a half teaspoon (~5 g) of salt were added to the soup prior to feeding. Gilruth Calf Food was advertised as 'a perfect substitute for whole milk and much cheaper' in New Zealand papers during this time.

Calves and heifers are the foundation for the future dairy herd. This statement is important today and during the beginning of the dairy farm. The issue in that era revolved around the increased use of whole milk for human consumption as being immediately more profitable than for feeding a calf. Calves were left to survive on minimal amounts of skim milk daily and on a mash of grains soaked in water and introduced at an early age. Hay and pasture were offered but poorly utilized. A weight gain of 1 pound (454 g) per day for the first 6 months of life was acceptable.

Many protein sources were tried as substitutes for milk protein in milk substitutes or replacers. Calf performance and appearance from non-milk protein sources never matched that of milk protein-based substitutes. Even a small percentage of substitutions for milk proteins reduced gains because of the inability of the proteolytic enzymes of the stomach and small intestines to digest these 'foreign proteins'.

For several decades, when a heifer survived to 3 months of age, her growth and development were assumed rather than managed. Rapid growth was disdained because of the fear of creating 'fat heifers' and decreased lifetime production. It was not uncommon, in the 1980s, for heifers to calve the first time at 30 months of age or older.

Several factors that changed the landscape of dairy farming had a dramatic influence on heifer rearing. First, nutritionists recognized that heifers required adequate amounts of protein for lean tissue growth, especially prior to puberty for normal growth of the mammary gland, a major consideration ignored by early feeding of high-grain diets to heifers. Goals for age at first calving by 22–24 months were deemed reasonable and produced the most profitable cow based on the metric, milk per day of life. Second, as dairy farms grew in cow numbers, it became more profitable to raise heifers away from the farm and dedicate limited resources of feed, labor, and capital to the lactating herd.

Many former dairy farmers began utilizing older facilities for custom-raising of heifers from as early as 2–3 days after birth until a couple of months before calving. The fad became a profession as heifer growers developed protocols and benchmarks for all phases of heifer rearing. The heifer in many instances had performance records, medical histories, and improved quality when they returned to the home farm.

Computers and information technology

The development of the computer opened the door to the application of technology to all phases of dairy production. As mentioned earlier, sire evaluations benefited greatly from the computerization of records. Ration balancing allowed for the solution of rations based on least-cost ingredients. This technology was limited to university personnel with access to mainframe computers until the introduction of programmable handheld calculators. Ultimately, the personal computer became a necessary tool for the feed sales person or the consulting nutritionist. Computer programs, for example, Spartan, developed by Michigan State University, for ration balancing were relatively simple and easy to operate. These programs greatly improved ration quality and resulted in overnight improvements in animal productivity. The programs replaced pencil, paper, and calculators for ration development. New complex models were developed that accounted for environmental factors, kinetics of digestion, and rates of passage of different feeds based on feed composition and a host of other factors that affect animal productivity.

Computers began to show up at the dairy manager's office and changed his role from managing cows to managing people and tasks. Milk weights were transferred from the milking parlor to the office computer. Later, technology allowed electronic capture of the weight of individual feeds going into TMR mixes. Software was developed that enabled monitoring of rations for consistency of ration preparation from feeding to feeding.

Instrumentation in the milking parlors permitted individual animal observation of indicators of cow health, reproductive status, and worker performance. Management by exception or by deviation from a cow's recent historical data was built into software, which produced output that enabled the manager to identify cows in the early stages of disease based on their previous production. Pedometers attached to the cow's ankle recorded walking activity between milkings. Activity reports identified cows with increased walking since the last milking, an indicator of estrus. In many cases, heat detection periods throughout the day were abandoned and pregnancy rate improved because of the accuracy of the pedometers. Increased electrical conductivity of milk identified cows in the early stages of mastitis.

Technology challenges old paradigms. During the authors' careers, raising calves has progressed from twice a day feeding of individual calves in strict isolation to housing calves in groups and feeding a milk substitute from an automated, programmable community milk feeding unit. The unit consists of a mixing station that mixes a prescribed amount of milk replacer and water for each calf. As a calf enters a feeding station, she is identified by the ear tag or neck tag. The information is transferred to a computer that passes instructions for mixing the proper amount of milk and water for dispensing at the feeding station. It most cases, the station protects the calf from other calves trying to steal a meal. A mixing station properly constructed can feed from about 40 – 140 calves daily. The units are self-cleaning throughout the day and greatly reduce labor and cleaning supplies. In properly maintained units, calf health is not compromised.

The milking center is one of the most costly components of the dairy enterprise in terms of capital investment, labor, and daily operating costs. Large operations justify costs by spreading the total operational cost across more cows. Farms of 150 cows or less may have 2–4 times the cost associated with milking as operations of 400 or more cows. The question, 'can the technology of robotics be applied to milking equipment such that machines replace human labor in the harvest of milk' was addressed by the Dutch in the 1980s. These efforts led to the introduction of the robotic milking machine in the early 1990s. Several milking equipment companies now offer robotic milkers for dairies. The initial cost of a robotic milking unit may exceed (US) $100 000 for a unit capable of milking 60–100 cows. Although this cost may seem expensive, payback time for a robotic milking unit may be less than half the time required to pay for a properly sized milking parlor because of labor and other cost savings. The robot is also more dependable than a person since it stands ready to milk a cow 24 h a day, 7 days a week, and the potential for human error is eliminated once programmed properly.

The robotic milker is a fixed station consisting of a milking unit attached to a swing arm, a stall for the cow to stand (and be fed in some models), and a control center that governs all the actions during milking. Cows entering the stall are identified electronically to the control center. The milking unit swings into place and the udder is prepared in the usual way of cleaning, predipping, and massage for milk letdown. Individual teat cups of the milk cluster are attached to teats using laser technology to identify teat location. During milking, milk moves for cooling and storage as with conventional systems. Indicators of abnormal milk cause the milk to be stored in a holding compartment. Managers can choose to add to the bulk tank or withhold when truly abnormal. Systems record each visit, milk weight, components, milk temperature, SCC, and milk conductivity on individual quarters and read pedometers for activity for identifying cows in heat. Reports also list cows that are not milked as often as desired.

Each station is capable of handling between 60 and 100 cows per day. Cows must be trained to enter the stall, a process that may take a few days for heifers or 2–3 weeks for older cows. Some cows never train to enter and have to be removed from the herd. Cows choose when they want to be milked and average between 2 and 5 times per day. As with any system, changing of filters and cleaning must be done a couple of times per day by farm personnel.

An Efficient Industry

Statistical information on the dairy industry by countries was sourced from the Food and Agriculture Organization (FAO) of the United Nations. Milk production and cow numbers in 2008 and change in numbers from 1961 (first year of available data) are presented in **Table 1** for six countries.

India and Brazil are world leaders in the total number of dairy cows. Most cows in India and Brazil remain on small dairy farms (total daily production of less than 50 kg per day) but as with other countries consolidation of farms characterizes the dairy industry. For example, the number of farms in Brazil has decreased by over 600 000 in the past 15 years. Both countries have increased milk output by over 400% since 1961, a testimony to improved management and new technology adopted by the dairy industry in the past 50 years. India had about the same number of buffalos and cows in 2008. In India in 2008, the buffalo produced 60.9 million metric tonnes of milk, for an average of 1584 kg per lactation.

New Zealand has shown the smallest increase in production per cow between 1961 and 2008. This statistic is somewhat misleading because a severe drought plagued that country in 2008. For example, milk output per cow in 2000 averaged 465 kg more than in 2008. These numbers highlight two constraints to New Zealand dairy production: timeliness of rainfall and the near 100% dependence on pasture for supply of nutrients to the cow. Forage production in other countries has similar constraints but forage provides only 40–70% of the nutrient requirements of the cow in many countries.

From the 1960s to present, dairy farm numbers in Japan have decreased from approximately 380 000, with an average of 3–4 cows per farm, to about 26 000 farms, with 60–65 cows per farm. Dairy farming is second to rice in terms of value of agriculture crops in Japan. Production per cow ranks behind world leaders, Israel and the United States. The industry in Japan is based on mostly home-grown forages and importation of concentrate feeds such as corn and soybean meal. Hokaido, the northern-most major island, is home to over 40% of the nation's cows. Production is pasture-based with supplementation.

The Israel dairy industry is relatively small with about 1000 farms and 128 000 cows. Israel leads all countries in average production per cow. It has achieved this ranking through innovation in equipment and facilities, especially in the area of heat abatement, use of technology, and management of information. The Israel Dairy Board regulates annual milk production through a quota system and government grants designed to improve the efficiency of the total industry.

A shift in milk marketing to a quota system in the European Union in the mid-1980s resulted in a major shift in the direction of milk production in the European Union. Information in **Table 2** uses 1980 as the baseline and compares the impact of the quota on dairy production in Germany with that of the United States in 2008.

Total annual milk output in Germany decreased by 10.6% after the introduction of the milk quota in the European Union in the mid-1980s. Total cow numbers decreased by 44% while output per cow increased 60% during this period. Dairy farm output increased by 48.1% in the United States during this time, aided by the introduction of a new 'free-market' system for milk pricing in 2000. Cow numbers decreased by a modest 14.6% but output per cow increased 73.2% in the United States. Interestingly, the impact of technology and expanding herd size had similar effects on milk per cow.

Table 1 Total milk production, number of cows, and milk yield per cow for selected countries in 2008 and percentage change from 1961

Country	*Annual milk production (MMT)*	*Number of cows (×1000)*	*Milk yield per cow (kg yr^{-1})*
Brazil	27.80	21 198	1309
Percentage change from 1961	431	187	85
India	44.1	38 500	1145
Percentage change from 1961	404	86	170
New Zealand	15.2	4347	3499
Percentage change from 1961	192	125	29
Germany	28.7	4218	6792
Percentage change from 1961	16	−47	119
Japan	7.98	1081	7382
Percentage change from 1961	277	92	97
United States	86.2	9224	9340
Percentage change from 1961	51	−47	183
Israel	1.3	128	10 105
Percentage change from 1961	343	88	135

MMT, million metric tonnes.

Data from FAO (Food and Agricultural Organization of the United Nations).

Table 2 Total milk production, number of cows, and milk yield per cow in Germany and the United States in 1980 and 2008

	Germany			*United States*		
Item	*1980*	*2008*	*Percentage change*	*1980*	*2008*	*Percentage change*
Annual milk production (MMT)	32.1	28.7	−10.6	58.2	86.2	48.1
Number of cows (millions)	7.57	4.22	−44.3	10.8	9.22	−14.6
Milk yield per cow (kg yr^{-1})	4234	6792	60.4	5392	9340	73.2

MMT, million metric tonnes.

Conclusion

Dairy farms have undergone tremendous change in the past century. Many areas in the world now have a modern industry where none was present at the beginning of the twentieth century. The ability of the dairy cow to transform plants with little or no value for human consumption into one of Nature's most versatile food products will promote future growth and development of dairy farms in many countries, especially in Asia and Africa, where sustainable animal protein production is limited.

Mechanization drove initial growth in the size of dairy farms. Output per cow on dairy farms was limited in the first half of the twentieth century until the sciences of genetics, nutrition, control of diseases, and reproduction helped expand the milk production potential of the dairy cow. New methods of housing and management complemented the science of dairy farming. These, coupled with computers and information management, offered the ability to successfully manage cows in large holdings often at the demise of the smaller dairy farm. Automation of milking and other labor-intensive tasks, such as feeding, potentially enables the smaller dairies to become more cost-competitive with their larger counterparts.

See also: **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses; Major *Bos taurus* Breeds; Minor and Dual-Purpose *Bos taurus* Breeds. **Dairy Farm Management Systems**: Dry Lot Dairy Cow Breeds; Non-Seasonal, Pasture Optimized, Dairy Cow Breeds in the United States; Non-Seasonal, Pasture-Based Milk Production Systems in Western Europe; Seasonal, Pasture-Based, Dairy Cow Breeds. **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis; Infectious Diseases: Foot-and-Mouth Disease; Infectious Diseases: Leptospirosis; Infectious Diseases: Tuberculosis. **Feeds, Ration Formulation**: Dry Period Rations in Cattle; Lactation Rations for Dairy Cattle on Dry Lot Systems; Lactation Rations in Cows on Grazing Systems; Models in Nutritional Management; Transition Cow Feeding and Management on Pasture Systems. **Gamete and Embryo Technology**: Artificial Insemination; *In Vitro* Fertilization; Multiple Ovulation and Embryo Transfer; Sexed Offspring. **Genetics**: Selection: Concepts; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk; Management Control Options; Medical Therapy Options; Role of Milking Machines in Control of Mastitis. **Milk**: Introduction. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Milking Machines**: Principles and Design; Robotic Milking. **Milking Parlors**. **Replacement Management in Cattle**: Breeding Standards and Pregnancy Management; Growth Diets; Pre-Ruminant Diets and Weaning Practices. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Mating Management: Artificial Insemination, Utilization; Mating Management: Detection of Estrus.

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History of Dairy Products and Processes

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Although some dairy products, for example, butter, cheese, and fermented milks, have been produced since at least 3000 BC, milk processing was not industrialized until the mid-nineteenth century. Various factors created the environment for this to occur, including the general move to industrialization at that time, but the key event was the development of the centrifugal separator for the continuous removal of fat from milk by Gustav de Laval in 1878. Within a few years, butter making began to change from a farm-based operation to factory scale. Gradually, the production of traditional dairy products became industrialized and new products were developed. Today, the whole business of producing milk and dairy products, from the producing farm to the consumer, is highly organized and operates on an international scale; the milk is processed in very large highly mechanized and automated factories. The objective of this article is to describe the development of the principal dairy products: concentrated and dehydrated milks, cheese and fermented milks, butter and spreads, sterilized milks, infant foods, ice creams, and milk fractions (proteins, fats, and lactose) to be used as food ingredients. Three processes, centrifugal separation, pasteurization, and homogenization, that facilitated industrialization are also described. The principal technological innovations are described more or less in chronological order.

Sweetened Condensed Milk

Nicolas Appert attempted unsuccessfully to produce heat-sterilized milk in 1809. Apparently, an attempt to produce a sweetened condensed milk (SCM)-like product was made by Dr. Heine (in England) as early as 1810 – a mixture of milk and sugar was heated in an open vessel but the product was not successful. SCM was first produced commercially by Gail Borden at a factory at Wolcottville, CT, in 1856. This venture failed but Borden opened a second (successful) factory at Wassiac, NY, in 1858. Borden had, in fact, attempted to produce heat-sterilized concentrated (evaporated) milk but the product frequently coagulated on heat sterilization and he switched to SCM, which is preserved by reducing its water activity by the addition of sucrose rather than by heat sterilization. Initially, the milk for SCM was concentrated in a steam-jacketed kettle at atmospheric pressure. Severe heating was required, which caused damage to the organoleptic characteristics of the milk and was expensive in terms of energy. Borden used a vacuum kettle, which solved both problems; apparently, Borden got the idea of using a vacuum pan from the Shakers, who used such pans to concentrate fruit juices. The Borden Company prospered in the United States and still survives as Eagle Brand Foods, now owned by J. M. Smucker Company, Orrville, OH. The vacuum evaporators have been improved over the years. SCM had become a major product by the end of the nineteenth century and remained so during the first half of the twentieth century; it then declined as pasteurized and ultrahigh-temperature (UHT)-sterilized milks and milk powders became more readily available. SCM is still produced for certain niche markets. The modern production protocol for SCM is described in the entry **Concentrated Dairy Products**: Sweetened Condensed Milk.

Dulce de Leche is quite similar to SCM; it is produced in South America by traditional methods, which involve severe heating, causing extensive caramelization and browning. This product is described in the entry **Concentrated Dairy Products**: *Dulce de Leche.*

Evaporated Milk

Apparently, Nicolas Appert attempted to produce heat-sterilized concentrated milk in 1809. He concentrated the milk by boiling it in a water bath over an open fire and attempted to sterilize the concentrate in glass bottles by heating in a boiling water bath for 2 h. The process was very inefficient in terms of energy input but the principal problem was that the concentrate frequently coagulated during sterilization. Heat-sterilized concentrated (evaporated) milk was first produced successfully by John B. Meyenberg, from Switzerland, who emigrated to the United States, where he patented a production process in 1884. He established the Helvetia Milk Condensing Company at Highland, IL, in 1885. Evaporated milk was a major product in the early twentieth century but since about 1950 has been replaced by milk powders for many applications and by UHT-sterilized milk for others. Today, it represents about 2% of total milk production. The production of evaporated milk is described in the entry **Concentrated Dairy Products**: Evaporated Milk.

Pasteurization

On a domestic level, the preservation/conservation of foods by heat treatment has a long history; presumably, housewives had always boiled milk at home. Attempts to industrialize the process were made by Nicolas Appert in 1809 but it was not successful owing to coagulation. Thermal sterilization of milk was not applied commercially until 1884 (see above). The sub-sterilization heat treatment of foods was introduced by Louis Pasteur in the period 1860–64 for the preservation of wine and beer, a process now known as pasteurization. In his article 'Heating milk for microbial destruction: A historical outline and update' (*Journal of Food Protection* 4: 122–130, 1978), D. C. Westhoff stated that N. J. Fjord (Denmark) applied pasteurization to milk as early as 1870. Other early pioneers of pasteurization of milk during the period 1880–90 were F. Soxhlet, N. Gerber, P. Wieske, and A. Fesca. Fjord used pasteurization to improve the quality of cream for butter making and Soxhlet recommended that for public health reasons milk used to feed infants should be heated. In 1890, it became compulsory in Denmark to pasteurize milk at creameries to reduce the risk of spreading TB through skimmed milk returned by creameries to farmers for feeding animals. It was recognized that milk was a potential vector for the spread of pathogens from animals to humans, and the pasteurization of milk for human consumption was studied in New York by Henry Koplik around 1890, and in 1893, Nathan Straus, a philanthropist, established depots in New York City for the distribution of pasteurized milk for infant feeding. The pasteurization of liquid milk became mandatory in Chicago in 1908 and the practice spread gradually; however, raw milk was fairly widely sold for consumption until the 1960s and it probably still is to a limited extent.

The equipment for pasteurizing milk has changed over the years; 'flash' pasteurizers, in which milk was heated at about 80 °C for a 'flash', were used initially and their use for cream continued until the 1960s. Flash pasteurization was followed by low-temperature–long-time (LTLT; ~63 °C × 30 min) pasteurization and finally by high-temperature–short-time (HTST; 72 °C × 15 s) pasteurization. HTST pasteurization, developed in England by Dr. Richard Seligman of the APV Company in 1923, is very efficient in terms of energy utilization (owing to extensive (95%) regeneration) and causes very little damage to the physicochemical and organoleptic properties of milk. The HTST process has become the usual method for pasteurization but LTLT pasteurization is still used by small-scale operators. The modern equipment used for HTST pasteurization and its control are described in the entry **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk.

The original objective of pasteurization was to kill *Mycobacterium tuberculosis*, then considered to be the most heat-resistant pathogen in milk; today, conditions have been broadened and range from ~65 °C × 15 s (thermization, applied to kill all heat-sensitive bacteria and extend the shelf-life of raw milk; to meet legal requirements, thermization must be followed by HTST pasteurization) to 76 °C × 15 s (superpasteurized milk, designed to kill recently emerged pathogens and to extend the shelf-life of pasteurized milk).

The introduction of pasteurized milk created the need for packaging to avoid post-pasteurization recontamination. Initially, the container of choice was the recyclable glass bottle, which was the only container used until the introduction of paperboard containers by Ruben Rausing (Sweden), who established the TetraPak Company in 1951. The triangular TetraClassic was introduced in 1951 and the TetraBrick in 1963; the latter is now probably the most popular milk package. Blow-molded high-density polypropylene plastic containers are also popular in some countries but have never dominated the market.

Centrifugal Separation of Milk

Owing to the difference in the specific gravity of milk fat and the aqueous (skim milk) phase, the fat globules rise (cream) to form a cream layer, which can be removed and used for butter production. It is also possible to adjust/modify the fat content of milk by this method, as is still done in the manufacture of Grana cheeses. However, gravity creaming is slow, inefficient, and not amenable to industrial-scale operations. During the mid-nineteenth century, it was recognized that the fat could be removed from milk by centrifugation and a number of attempts were made during the period 1864–77 to develop a commercially operable separator; the early machines were mainly batch, bucket-type centrifuges. A successful continuous mechanical separator was developed in 1878 by Gustav de Laval, whose company (Alfa-Laval) is still in business. The centrifugal separator permitted the continuous separation of milk and facilitated the industrialization of milk processing; within a very short period, creameries dominated the dairy industry, and soon monopolized it. Of course, the mechanical separator was applied to all aspects of the dairy industry for standardizing the fat content of milk as well as removing it totally. Over the past 130 years, separators have become larger and more efficient.

Homogenization of Milk

As discussed above, the fat globules in milk form a cream layer. Until quite recently, the presence and depth of a cream layer in beverage milk was regarded as a quality attribute. However, creaming was undesirable in SCM and evaporated milk and when opaque packaging of beverage milk became common, concomitant with the perceived undesirable nutritional aspects of consuming milk fat, a cream layer in beverage milk assumed a negative image. From an early stage, it was recognized that the rate of creaming was influenced, *inter alia*, by the diameter of the fat globules, according to Stokes' Law. It was realized that the rate and extent of creaming could be reduced/prevented by reducing the size of the fat globules; in 1899, Auguste Gaulin, a Frenchman, developed a machine called a valve homogenizer for doing this. Today, all milk, except that for cheesemaking, is homogenized (homogenization damages the rennet coagulability of milk). The valve homogenizer of the type developed by Gaulin is still the usual type of homogenizer used in the dairy industry but other types, for example, ultrasonic, microfluidizer, or agitation-type homogenizers, are also used occasionally for certain applications and a high-pressure (up to 400 MPa) homogenizer has been developed but is not used commercially for milk at present. The homogenization of milk is described in the entries **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers.

Cheese Manufacture

Cheese has been produced since at least 3000 BC and now represents about 40% of total milk production. Milk is converted to cheese curd by one of three methods: by limited proteolysis using rennets (~70% of all cheese), acid-induced coagulation (~30% of all cheese), or by acid–heat (very little). These methods have been used since prehistoric times, and until about 1860, cheese production was a farm-based operation. Although it has been traditional to ripen cheese curd for certain varieties at central/community facilities, the first cheese factory was established at Rome, NY, in 1851 and the first in the United Kingdom at Longford, Derbyshire, in 1870. Since then, the size of cheese factories has increased and the process has become more highly mechanized and automated, as described in the entry **Cheese**: Mechanization of Cheesemaking. However, farm-based cheesemaking persists and is particularly important in southern Europe.

Although cheese has been made since prehistoric times, the production of many well-known varieties was standardized only recently; for example, Cheddar cheese was standardized by John Harding in the mid-nineteenth century. The manufacturing protocol for most varieties has changes substantially over time.

Cheese is the most diverse group of dairy products – there are at least 1000 varieties. They are also very complex diverse systems – in contrast to other dairy products, which are 'finished' products at the end of manufacture and are stable if properly made and stored, the characteristic, flavor, texture, and functionality of cheese develop during ripening/maturation, which may last for more than 2 years. Consequently, cheese has attracted much research attention during the past 100 years, which is described in the entries **Cheese**: Accelerated Cheese Ripening; Cheese with Added Herbs, Spices and Condiments; Current Legislation for Cheeses; Mechanization of Cheesemaking; Overview; Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Raw Milk Cheeses.

The majority of cheese is consumed as a 'natural' product but 'processed' cheese, which was developed by Walter Gerber and Fritz Stettler (Switzerland) in 1911, fills certain niches and represents ~12% of total cheese production (i.e., ~2 000 000 tonnes per annum). The principal advantages of processed cheese are a very long shelf life, good storage stability without refrigeration, and uniformity of quality.

Milk Powders

Sun-dried milk was produced by the Mongols at the time of Genghis Khan, in the thirteenth century, and probably by other people living in low-humidity regions. Milk powder was first manufactured commercially by T. S. Grimwade in England in 1855; the process involved drying a mixture of milk, sugar, and Na_2CO_3 in open pans and grinding the residue. The process was slow and cumbersome and the product did not find a ready market.

A process known as 'the dough-drying system' was introduced by O. B. Wimmer (Copenhagen) and J. H. Campbell (New York) in 1901. Milk was concentrated to ~70% solids in a vacuum pan and the very viscous 'dough' dried by hot air at atmospheric pressure.

In 1902, J. R. Hatmaker (London) and J. A. Just (Syracuse, NY) introduced hot roller dryers, which were used with considerable success during the first half of the twentieth century. Roller drying is quite harsh and causes considerable damage to the proteins, fats, and organoleptic properties of milk; roller dryers are now rarely used except for the production of milk powders for the chocolate industry. The severity of drying was reduced by the use of rollers placed within a vacuum chamber but the system was never used widely.

Spray-drying, which involves spraying a finely divided ('atomized') liquid into a stream of hot air, was introduced to the chemical industry by Samuel Percy in 1872. A spray dryer for milk processing was patented by

Merrell Brothers and W. B. Gere in 1907, but the use of the process was limited until the 1950s. Since then, the capacity and efficiency of spray dryers has been increased and the range of milk powders widened in terms of properties and functionality. Spray-drying can be a relatively mild process and the organoleptic properties of the reconstituted powder are quite acceptable as a beverage product and for cheese production. Today, approximately 15% of total milk production is spray-dried (as whole or skimmed milk) for a wide range of applications.

Infant Formulae

For a variety of reasons, it is not possible for all women to breast-feed their babies, many of whom have been fed on bovine milk or the milk of other species. For centuries, such non-human milk was unmodified, except perhaps for the addition of water and sucrose. The first attempt to develop a milk-based formula for infant nutrition was commercialized by Justus von Liebig, who introduced 'Soluble Food for Babies', considered the perfect infant food, in 1867; the product was prepared by drying a mixture of whole milk, wheat flour, malt flour, and K_2CO_3 in heated trays. It was claimed that the product simulated human milk but the composition does not support this claim; nevertheless, the product was commercially successful and was copied by other companies. In the United States, H. Gerstenberger introduced a modified milk-based infant formula, 'synthetic milk adapted' (SMA), in 1915; it contained 4.6% fat, 6.5% sugars, and 0.9% protein. The SMA brand was produced for many years by Wyeth Nutrition, which was purchased recently by Pfizer.

Evaporated milk, which was cheap and sterile, was widely used in infant nutrition during the late nineteenth century, with a great reduction in infant mortality. Later, roller-dried whole milk was widely used and promoted for infant nutrition. The composition of ruminant (cow, buffalo, sheep, or goat) milk is quite different from that of human milk, especially with respect to lactose content and the amount and type of proteins. With the development of membrane technology for the fractionation of milk proteins from about 1960 onward, it became possible to modify the composition of bovine (or other) milk to simulate that of human milk more closely.

Butter and Spreads

As described above, butter has been produced for several thousand years, essentially by destabilizing the lipid emulsion in milk or, usually, cream, by agitation to create a foam, traditionally in a hand-operated wooden dash or barrel churn; the latter was sometimes operated by a water-wheel or by a harnessed horse or donkey. When butter making was industrialized after 1880, the barrel churns were increased in capacity and operated by a steam or diesel engine and eventually by electric motors, but the principle remained the same. About 1950, cubical or conical or barrel-shaped stainless steel churns were introduced and soon dominated the industry but these were soon replaced by 'continuous' butter making machines, which are now normally used in large creameries although stainless steel churns are still used in small operations.

Traditionally, butter was made from cream that had soured naturally due to the growth of adventitious lactic acid bacteria (LAB). Under these conditions, the LAB were variable and acidification was unpredictable and, consequently, the quality of the butter was variable. Cultures (starters) of selected strains of LAB were introduced around 1890 by V. Storch in Denmark and by H. W. Conn in the United States to improve the rate and consistency of acid production; the cultures contained citrate-fermenting strains of *Lactococcus lactis*, which produce diacetyl and give the product its characteristic aroma. Butter made from acid cream is known as ripened/lactic butter – it is the usual product in most of Europe, but in English-speaking countries, butter is now usually made from sweet (unripened) cream.

Butter has a relatively high content of high melting point triglycerides and consequently has poor spreadability ex-refrigerator. Approaches to solve this problem involve (1) fractionation of milk fat and blending fractions to give the desired melting profile, (2) feeding protected polyunsaturated oils to the cows to increase the content of polyunsaturated fatty acids and reduce the melting point, and (3) blending cream and vegetable oils in the desired proportions and manufacture into a water-in-oil emulsion using technology used in the margarine industry. These blended products now dominate the spreads market.

Butter, which contains ~16% moisture, is relatively unstable, especially to lipid oxidation. A more stable product is produced by removing the water. The traditional form of this product is ghee, which has been produced in India since prehistoric times; the modern version of this product is anhydrous milk fat, which is widely used as a food ingredient.

Fermented Milks

Fermented milk products, acidified through the growth of adventitious LAB (*Lactobacillus* spp., *Lactococcus* spp., *Streptococcus thermophilus*), have been produced, inadvertently, since prehistoric times and are substantial products in most dairying countries. The principal types of fermented milk are yogurt, kefir, and koumis, with lesser and regional amounts of other products.

Although fermented milks were created as a result of the ability of LAB to ferment lactose, by reducing the pH of milk LAB inhibited the growth of many spoilage bacteria; consequently, fermented milks have a shelf-life of several weeks, even at ambient temperature. Since the observation of Ilya Ilyich (Elie) Metchnikoff, in the early twentieth century, that people in the Balkan countries who consumed large quantities of fermented milk had a very long life, there has been interest in fermented milks as health-promoting products; this aspect is particularly important today and fermented milks containing probiotic bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. are attracting research and commercial interest

UHT Milk

Heating milk at a very high temperature (130–140 °C) for 3–5 s renders the product sterile with less concomitant damage to the organoleptic quality of milk than in-container sterilization (115 °C × 12 min); the process is referred to as UHT treatment. Research on UHT sterilization commenced at the USDA laboratories in Washington, DC, in the mid-1940s and was continued at the NIRD, Reading, UK, and INRA, France, in the 1950s. Aseptically packaged UHT milk became available in the 1960s and now dominates the market for liquid milk products in southern Europe. However, the flavor of UHT milk is inferior to HTST milk, which is preferred in northern Europe and North America. The principal attraction of UHT milk is its convenience and long shelf life, even under adverse environmental conditions.

Developments in the UHT process include direct heating by steam injection, which, because of the very short come-up time, is milder than indirect heating and consequently causes less collateral damage. A combination of UHT heating and a short in-container sterilization is used by some manufacturers; the principal advantage is that aseptic packaging is not required, thereby reducing the processing costs. However, the total heat load is increased with a consequent loss of flavor quality.

Ice Cream

Apparently, wine and other drinks cooled with snow or ice were popular in Classical Rome and ancient China. The art of making ices was brought from China by Marco Polo in the thirteenth century and their production soon spread throughout Europe and eventually to North America, probably in the eighteenth century. However, before the development of mechanical refrigeration by Jacob Perkins in 1834, ensuring a supply of ice throughout the year was a major problem; natural ice was transported over quite long distances in winter and stored in insulated 'ice-houses' for use in summer. Therefore, frozen products were expensive and ice cream and other frozen deserts did not become widely available and affordable until the mass production of sucrose from sugar cane or beet in the nineteenth century, the development of industrial refrigeration by Carl von Linde in the 1870s, the development of a continuous ice cream freezer in 1926, and the development of the domestic refrigerator, which was introduced in the United States in 1915 and became widespread after about 1930 and especially after 1950.

Today, a range of frozen deserts are readily available (regular, high-fat, low-fat ice cream, in hard frozen and soft-serve forms, frozen yogurt, sherbets, frozen ices). They are available in a variety of flavors and novelty forms. Consumption, especially of high-quality and novelty products, continues to increase and farm-scale production is significant and increasing in some countries. *Per caput* consumption (liters) in the leading countries is New Zealand, 27; the United States, 24; Australia, 18; Ireland, 13; the United Kingdom, 8; and Spain, 5. Owing to the wide variations in the composition of ice cream, it is difficult to calculate what proportion of total milk production is used for ice cream. If we consider the case of Ireland as 13 l of standard ice cream (12% milk fat, 13% milk solids-non fat), this is equivalent to about 3% of total milk fat produced; this is almost certainly an overestimate, as it assumes that all of the fat in ice cream is milk fat.

Current ice cream research focuses on ingredients, especially milk proteins and hydrocolloids.

Milk Protein Products

The casein fraction of milk protein, prepared by rennet-induced coagulation, has been used commercially since the end of the nineteenth century as an industrial material for the production of plastics, glues, and synthetic textiles. By the 1950s, casein had to compete in these applications with cheap, recently developed synthetic polymers and the value of casein was low. This resulted in a search for more lucrative applications for casein and research, mainly at the CSIRO in Australia, led to the upgrading of casein, mainly acid-precipitated product, as a food ingredient. These developments coincided with the increased demand for 'functional' proteins, with good emulsifying, foaming, water-binding, and gelling properties; acid casein serves these functions very well and is the premium functional protein. Rennet-coagulated casein is now used mainly in the production of Pizza cheese analogues, for which heat-induced melting and stretchability are the principal properties of interest.

About 20% of the total protein in bovine milk is in acid or rennet whey. Historically, a little of this was recovered as heat-coagulated cheese, for example, Ricotta, Manouri, and Ziger, or as heat-precipitated lactalbumin, but most of it was recovered as a constituent of whey powder, including that used for baby foods, or in whey fed to pigs. The development of membrane technology in the early 1960s enabled undenatured whey proteins to be recovered as whey protein concentrates or isolates, and used as functional proteins or used in humanized infant formulae. Methods have been developed to isolate many individual whey proteins on an industrial scale, for example, α-lactalbumin, β-lactoglobulin, lactoperoxidase, lactoferrin, immunoglobulins, and caseinoglycomacropeptide; these are value-added products.

Membrane technology also permits the production of micellar casein or total milk protein, for the fortification of cheese milk to increase plant capacity or the isolation of β-casein.

Lactose

Lactose is a reducing disaccharide unique to milk. It has been available commercially since the eighteenth century, but on a small scale until recently. Although the market for lactose has increased, only about 10% of the lactose potentially available in whey is recovered as lactose; most of the remainder is present in whey powders. Lactose can be modified to several value-added products, which are now attracting considerable attention.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat-Based Spreads; Modified Butters; The Product and Its Manufacture. **Cheese**: Accelerated Cheese Ripening; Cheese Analogues; Cheese with Added Herbs, Spices and Condiments; Current Legislation for Cheeses; Mechanization of Cheesemaking; Overview; Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Pasteurized Processed Cheese Products; Raw Milk Cheeses. **Concentrated Dairy Products**: *Dulce de Leche*; Evaporated Milk; Sweetened Condensed Milk. **Dehydrated Dairy Products**: Dairy Ingredients in Non-Dairy Foods; Infant Formulae; Milk Powder: Types and Manufacture. **Fermented Milks**: Types and Standards of Identity. **Heat Treatment of Milk**: Thermization of Milk; Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems. **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Manufacture; Ice Cream and Frozen Desserts: Product Types. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk. **Milk Lipids**: Fat Globules in Milk; Lipid Oxidation. **Milk Protein Products**: Bioactive Peptides; Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Membrane-Based Fractionation; Whey Protein Products. **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins. **Nutrition and Health**: Nutritional and Oral Health-Promoting Properties of Dairy Products: Caries Prevention and Oral Health. **Plant and Equipment**: Centrifuges and Separators: Types and Design; Evaporators. **Vitamins**: Vitamin C.

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History of Dairy Chemistry

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Introduction

The developments in dairy technology described in the preceding article were made possible by advances in the chemistry of milk and its products, and many of the new processes and products were subject to defects or changes, which required chemical investigation. The technological and economic success of dairy processing operations depends on chemical analysis. The first part of this article will trace the development of methods for the chemical analysis of milk and dairy products while the second part will describe the isolation and characterization of the principal constituents of milk.

Determination of Milk Composition

When milk was consumed or processed on the producing farm, information on the chemical composition of milk was not very important because the farmer was paid for the finished product rather than the raw material. However, when a farmer sold his milk to a creamery that produced and sold the finished product, information on the composition of the milk became critical for both the farmer and the creamery. Industrialization of milk processing on a large scale created the need for scientifically based technology to guarantee maximum yields of product of consistent quality; chemical analyses of raw materials and finished products are necessary to meet these objectives. Until relatively recently, fat was the most valuable constituent of milk and, therefore, was the focus of considerable effort to develop a rapid, sensitive quantitative assay.

Determination of Fat Content

Lipids (fats and oils) are the fraction of tissue or food soluble in an apolar solvent, usually diethyl ether, diethyl ether + hexane, or chloroform + methanol. The first method for the determination of the fat content of foods, in general, was developed by Franz Soxhlet in 1879, based on the continuous extraction of the food sample with diethyl ether for ~8 h. The Soxhlet method is not applicable for the determination of fat in liquids, including milk; also, it is very slow and not suitable for the analysis of many samples. A method based on the same principle but applicable to milk was published by B. Roese in 1884 and modified by E. Gottlieb in 1892. However, this method is also slow and not suitable for determination of the fat content in many samples of milk at a creamery. An apparatus that facilitated the determination of fat in dairy products (primarily concentrated milk) by the Roese–Gottlieb method, and also total solids (TS) by drying, was developed by T. Mojonnier and H. C. Troy in 1925 and commercialized by Mojonnier Brothers, Chicago. The Roese–Gottlieb method, probably as modified by Mojonnier and Troy, is still the standard reference method for determination of the fat content of milk and dairy products.

Analytical methods used initially at creameries involved measuring the amount of butter produced from a sample of the milk offered for sale or the depth of the cream layer formed on centrifuging a sample of milk in a graduated tube under standard conditions. The latter procedure was modified in several ways: (1) addition of concentrated alkali to a sample of milk in a special tube to dissolve the protein and liberate the fat, which was collected in the graduated neck of the tube by centrifugation; (2) using concentrated H_2SO_4 instead of alkali to dissolve the protein and butanol to clarify the fat column, developed by N. Gerber (Switzerland), in 1892, and widely used in Europe and elsewhere until about 1970; (3) a similar method to the second method, but without butanol, developed by S. Babcock (United States) in 1890, and widely used in North America and elsewhere; and (4) a method using alkali and the anionic detergent dioctyl sodium phosphate to disperse the casein micelles and then adding a strong hydrophilic nonionic detergent, sorbitan monolaurate; this method was used to a limited extent in the United States, mainly because it was less corrosive than concentrated H_2SO_4, but it was soon superseded by more rapid physical methods.

The first of these was based on light scattering by the fat globules; scattering by the casein micelles was eliminated by using EDTA to disperse the micelles and variations in fat globule size (and hence in the intensity of scattering) were eliminated by homogenization. This method was commercialized by the Foss Electric Company (Denmark) and was widely used for a period in the 1960s and early 1970s but it was replaced by infrared (IR) absorption, which is still the most commonly used principle for determination of the concentration of fat, protein, and lactose in milk.

Determination of Total Solids (TS) or Moisture Content

For certain situations, it was desirable to determine the TS (dry matter) content of milk, and indirectly the solids-not-fat (SnF) content and the moisture content, which in some cases are defined by law. The solids content can be determined by drying a sample under standard conditions in a hot air oven to constant weight, for example, at 102 °C for about 4 h or, preferably, in a vacuum oven at 70 °C. Oven-drying is slow; it can be speeded up by using the equipment developed by the Mojonnier Company, but the method is still tedious.

Indirect methods for determining the TS or SnF content of milk are based on determining the specific gravity of the sample using specially designed hydrometers, lactometers, calibrated to indicate the % TS. Several empirical formulae were developed to relate the specific gravity of milk to its composition:

Babcock's formula:	$\% \mathrm{TS} = \mathrm{L}/4 + 1.2\% \text{ fat}$
Richmond's formula:	$\% \mathrm{TS} = \mathrm{L}/4 + 1.2\% \text{ fat} + 0.14$
Fleischmann's formula:	$\% \mathrm{TS} = \mathrm{L}/4 + 1.2\% \text{ fat} + 0.24$

where TS is the total solids and L is the lactometer reading in degrees.

This approach was used commercially until relatively recently (1970s) but is no longer used in practice.

The moisture content of low-moisture products, for example, milk powders, may be determined by azeotropic distillation with toluene or by Karl Fischer titration.

Determination of Protein Content

Determination of the protein content of milk and dairy products is now a standard operation, and in most cases, a factor for protein is included in calculating the price of milk; this has become possible through the development of rapid, accurate methods for the determination of protein content, which were developed only relatively recently. Since proteins are the principal nitrogen-containing compounds in biological materials and foods, the early methods for determining the protein content of foods were based on determining its nitrogen content. A typical protein contains ~16% N and the conversion factor is %N × 6.25 (since milk protein contains ~15.7% N, protein conversion factor is %N × 6.38). The first method, developed by Jean Baptiste Dumas in 1833, involved combustion of the sample, absorbing the CO_2 and H_2O formed, and measuring the N_2 volumetrically. Because the assay was difficult to perform, it was not used in food factories until a suitable apparatus was developed by the LECO company in about 1970; since then, it has enjoyed limited application, especially for milk powders. The second method, developed by Johan Kjeldahl in 1883, essentially involves wet-ashing a sample with concentrated H_2SO_4 in the presence of a catalyst, for example, Se, Cu, or Hg; the N is converted to $(NH_4)_2SO_4$, from which NH_3 is liberated by making the solution strongly alkaline. The NH_3 liberated is recovered by distillation and quantified by either titration or colorimetry (using Nessler's reagent). The classical Kjeldahl method, which is slow, complicated, and dangerous, has been modified regularly to speed up oxidation and to facilitate execution of the procedure by the development of specialized glassware and heating and distillation equipment but it is still a difficult method. However, it is the standard reference method for protein determination.

Several rapid empirical methods have been developed and used more-or-less successfully:

1. the formal titration, which essentially involves reacting the -NH_2 group of protein-bound lysine with formaldehyde, HCHO, which reduces its $\mathrm{p}K_\mathrm{a}$ to within the phenolphthalein end-point range; the difference in the amount of NaOH required to titrate a sample of milk to the phenolphthalein end point before and after adding HCHO is proportional to the concentration of lysine, and therefore to the protein concentration, in the sample.
2. Absorbance of a sample at 280 nm; the A_{280} of a 0.1% solution of a typical protein in a 1 cm cuvette is 1.0. This principle is widely used in biochemistry for the rapid estimation of protein concentration but is not used in the dairy industry, possibly because of interference by non-protein components.
3. Reaction of the Folin–Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) with phenolic groups, that is, with tyrosine in proteins, yields a product with a $\lambda_{\max}$ of 750 nm. A modification of this method is that developed by O. H. Lowry in 1951, in which alkaline Cu^{2+} is reduced to Cu^{+} on reaction with the peptide bond, giving a blue color and thereby increasing sensitivity; neither method is applied in dairy laboratories.
4. In acidic solutions, proteins bind Coomassie Brilliant Blue G250, causing a shift in the absorbance maximum from 465 to 595 nm; A_{595} is a measure of the protein content of a sample, a principle used in the Bradford method, which is not used for food analysis.
5. Cationic dyes, for example, Amido Black or Acid Orange G, bind to the cationic groups of proteins at acidic pH; the amount of dye bound is proportional to the concentration of protein. Following fundamental research by Fraenkel-Conrat in 1944, dye-binding methods were introduced for the determination of the protein content of milk in 1956 and were widely used for a few years but were superseded by IR methods.
6. IR absorbance; the amide group of the peptide bond absorbs IR radiation at 6.46 μm, the absorbance being proportional to the concentration of protein. IR instruments are now the norm for protein determination in milk, simultaneously with fat and lactose.

Determination of Lactose Content

Since lactose was not very important in the dairy industry until recently, there was little incentive to develop analytical methods for its determination in large numbers of samples. However, the concentration of lactose in milk or dairy products can be determined by (1) redox titration using alkaline $CuSO_4$ (Fehling's solution, developed in 1850) or Chloramine-T, (2) polarimetry, (3) colorimetry after reaction with anthrone or phenol under strongly acidic conditions, (4) enzymatic reactions, or (5) IR absorbance at $\sim 9.4\,\mu m$, simultaneously with fat and protein.

Determination of Ash and Individual Elements

As for other foods, the total ash content of milk and dairy products may be determined gravimetrically after ashing a sample to constant weight at 500 °C. Individual elements may be determined by flame spectroscopy or atomic absorption spectrophotometry, or by titration or colorimetry, or by ion-selective electrodes.

Isolation and Characterization of Milk Constituents

Until the eighteenth century, milk was considered to consist of fat, curd (protein), and whey. We now know that in addition to the principal constituents, water, lipids, proteins, and lactose, there are at least 100 minor constituents in milk. In this section, the isolation and characterization of lactose, lipids, and proteins will be described.

Lactose

Whey (milk serum) has been considered to be a useful food since ancient times. In 1761, F. Hoffmann (physician to the king of Prussia) wrote *A Treatise on the Virtues and Uses of Whey.* We now know that the principal constituent of whey is a sugar called lactose, which was originally called 'the essential salt of milk' (sugars were generally called 'salts', presumably because they could be crystallized in a manner analogous to salts. The modern definition of a salt as 'the reaction product of an acid and a base' was made by Guillaume Francois Rouelle, in 1744).

Lactose, a reducing disaccharide, composed of galactose and glucose linked by a $\beta\ 1 \rightarrow 4$ glycosidic bond, is unique to milk and is the principal sugar in the milk of most mammals, with the exception of a few species of seal. It has some unusual characteristics, as discussed in the entry **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties. Lactose was first isolated, from whey, by F. Bartoletti in 1663 and became a commercial commodity in the eighteenth century, mainly for medicinal uses. Work on its structure, involving mainly Justus von Liebig, Emil Fischer, C. S. Hudson, and W. N. Haworth, commenced in 1812 and was essentially completed by 1930. Lactose can cause problems in concentrated, dehydrated, and frozen dairy products, which were the subject of considerable research throughout the twentieth century.

Until recently, lactose, which was difficult to isolate and crystallize, had a limited market. However, these difficulties have been overcome and lactose has found valuable niche markets, especially in the baby food sector and many value-added derivatives can be produced from lactose.

In the small intestine, lactose is hydrolyzed by β-galactosidase (lactase) to glucose and galactose, which are absorbed and metabolized. Intestinal β-galactosidase activity decreases with age and becomes insufficient in many humans after the age of about 10 years and such individuals are lactose intolerant; exceptions are people of northwestern European descent. This topic has been the subject of considerable research since it was first observed in studies by Holzel and by Durand in 1959.

Milk Lipids

Until relatively recently, fat was the most valuable constituent of milk and therefore attracted considerable research attention. Relative to lactose and especially proteins, there was considerable early progress on the chemistry and properties of milk lipids but progress stalled owing to the lack of analytical procedures until the development of gas chromatography (GC) by A. J. P Martin in 1950. In 1823, M. E. Chevreul showed that both fats and oils are esters of organic acids (now referred to as fatty acids (FAs)) and the trihydroxy alcohol, glycerol. Determination of the FA profile of a lipid was a formidable/impossible task and the first fairly accurate profile of milk fat was published by C. A. Browne in 1899, based on the resolution of FAs by fractional distillation and fractional crystallization, techniques that were used until the development of GC. Mainly for the purpose of detecting and quantifying the adulteration of milk fat with cheaper animal or vegetable fats, methods were developed that, in effect, estimated the concentration of certain FAs in a sample of fat, that is, volatile, water-soluble FAs (Reichert–Meissl number, 1879), volatile, water-insoluble FAs (Polenski number; Salkowski, 1887; Polenski, 1904), butanoic acid (Kirschner number, 1905), saponification number (inversely related to the mass of FAs; Koettstofer, 1879), iodine number (a measure of the degree of unsaturation; Hubl, 1884; Wij, 1898); these characteristics are still used.

The use of GC showed that bovine and other ruminant milk fats contain ~400 FAs, mostly at trace levels, and are much more complex than other animal and plant lipids. They have a high level of $C_{4:0}$, a medium level of $C_{6:0}-C_{10:0}$, a low level of polyunsaturated FAs, and a relatively high level of conjugated linoleic acid (CLA). GC has been improved greatly since 1950 and FA analysis is now a relatively simple operation.

Initially, it was believed that glycerides contained one, two, or, usually, three molecules of a single FA, that is, that they are simple triglycerides (TGs). The first strong evidence that lipids are mixed TGs, that is, that they contain two or three different FAs, was presented by G. Amberger (1910–20) and this is now universally accepted. However, it was not possible to determine the position of an FA on a TG until about 1970, when a complex technique, stereospecific analysis, was developed. The structure of milk TGs of several species has been determined. Useful information on the structure of TGs is provided by GC, which resolves the intact TGs according to the number of acyl carbons, that is, the sum of the number of C atoms in the three FAs present.

The spreadability of milk fat is one of its most important characteristics and is determined by its melting characteristics, which can be determined by dilatometry, differential scanning calorimetry, or nuclear magnetic resonance, which have been used to study the influence of various compositional and processing factors on the melting of milk lipids. Further information on the spreadability of milk fat can be obtained from rheological measurements that have been made for at least 50 years.

The fat in milk occurs as spherical globules, which were visualized by Antonie van Leeuwenhoek in 1674 using his recently developed microscope. Subsequent workers (M. Bouchardat and T. A. Quevenne (1857) and E. L. Sturtevant (1873)) showed that the globules ranged in diameter from ~1 to ~20 μm. The size of the fat globules and factors that affect it were an important subject in the early twentieth century (see Campbell, 1932). Lipids are insoluble in aqueous systems and there is an interfacial tension between the phases, which reduces the interfacial area to a minimum. Unless the surface tension is reduced, the lipids will form a continuous layer at the surface, owing to the lower specific gravity of the fat (0.90) than that of the aqueous skim milk phase (1.036). If the surface tension is reduced by surface-active agents (emulsifiers), the fat globules will remain discrete. The nature of the emulsifier in milk, the milk fat globule membrane (MFGM), has been the subject of research since 1840 when F. M. Ascherson proposed that the globules are surrounded by a thin membrane of polymerized albumin. Around 1890, S. M. Babcock proposed that the membrane consisted of 'lactofibrin' (fibrin is a blood protein). Systematic study of the MFGM commenced with the work of V. Storch in 1897, who prepared membrane from washed cream, pretty much as is done today. Over to next 50 years, data accumulated on the chemical composition of the MFGM – it was found to consist mainly of phospholipids and other polar lipids (~30%) and specific proteins (~40%). Based on polarity considerations, N. King, in 1955, proposed a speculative model of the MFGM. The seminal study on the structure of the MFGM is that of W. Bergman (about 1960), who used electron microscopy to show that the fat globules are expressed from the mammocytes through the apical cell membrane and are surrounded initially by the cell membrane, which has the typical trilaminar, fluid mosaic structure. This membrane is not very robust and is partially lost during the storage and processing of milk. The composition and structure of the MFGM has been refined over the past 50 years and is now known in considerable detail.

Apart from the chemical composition of the TGs in milk lipids and the structure of the MFGM, there have been five major research themes during the past 100 years: (1) creaming of milk, (2) lipid oxidation, (3) hydrolytic rancidity, (4) rheological properties, and (5) nutritional aspects.

Owing to the difference in specific gravity between the fat (0.90) and aqueous skim milk phase (1.036), the fat globules rise to the surface, forming a cream layer consisting, at least initially, of fat globules. The creaming of milk was academically interesting and commercially important until relatively recently and therefore was the subject of considerable research. The creaming of milk and factors that affect it were the subject of considerable research during the first half of the twentieth century and still attract some academic attention.

Creaming was important (1) as a means of separating the fat from milk for butter making until the development of the mechanical separator by Gustav de Laval in 1878, and (2) as an index of the fat content, and hence the quality, of milk. The rate of creaming can be predicted by Stokes' equation:

$$v = 2r^2(\rho_1 - \rho_2)\frac{g}{9\eta}$$

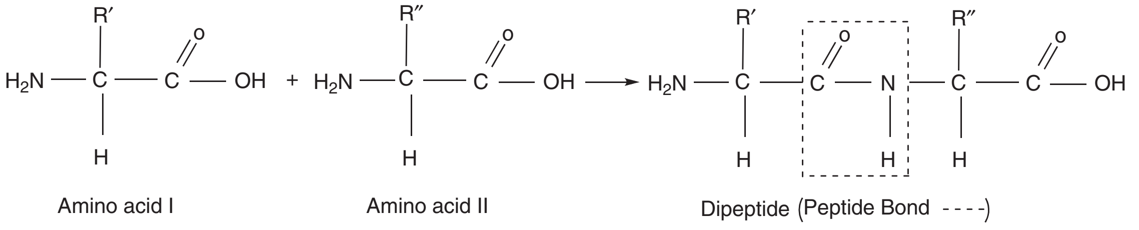
where v is the velocity of creaming; r the diameter of the globules; ρ_1 and ρ_2 the specific gravity of the fat and aqueous phases, respectively; g the acceleration due to gravity; and η the viscosity of the system.

The typical values of r, ρ_1, ρ_2, g, and η indicate that a cream layer will form in milk in about 60 h, but in fact, a cream layer is evident after ~30 min. The faster than predicted rate is due to the clumping of fat globules due to immunoglobulin M (called cryoglobulins because they are insoluble at low temperatures and precipitate onto fat globules). The clusters of globules rise rapidly owing to the large value of r. Of the animal species that have been studied, only bovine milk contains cryoglobulins.

Milk Proteins

The proteins are the most diverse, complex, and, technologically, the most important constituents of milk. The name 'protein', meaning 'of first rank', was introduced in 1838 by G. Mulder, at the suggestion of J. J. Berzelius. Based on the results of compositional analysis, Mulder proposed that proteins are composed of different combinations of the 'radical' $C_{40}H_{62}N_{10}O_{12}$ with S and/or P.

Gradually, it was realized that proteins consist of amino acids, the first of which, glycine, was isolated from gelatin by H. Broconnot in 1820. By 1904, 17 of the 20 commonly occurring protein amino acids had been isolated (see Vickery and Schmidt, 1931) and, in 1902, Emil Fischer and Franz Hofmeister proposed that in proteins, the amino acids are linked together by 'peptide' bonds between the α-amino and the α-carboxyl groups, an idea that was not accepted initially but eventually became universally accepted.



Probably, the first publication on milk proteins was in 1814 by J. J. Berzelius, who prepared *kase* (curd) from skimmed milk by acidification. H. Braconnot (1830) studied some properties of acid-precipitated milk protein, which he called 'casein' (apparently the first to do so). G. Mulder (1838) studied the elementary composition of proteins, including casein (at this time, the term 'casein' was used for all acid-precipitated proteins, regardless of source). During the period 1885–89, O. Hammersten (Sweden) improved the method for preparing acid (isoelectric) casein (milk diluted 1:5 with water and made to 0.1% with acetic acid – now known to give a pH of ~4.6, i.e., the isoelectric point of casein) and claimed that it was a homogeneous protein (in 1880, it was claimed by Danilewsky and Radenhausen that casein is heterogeneous); casein isolated by this method is still frequently called *casein nach Hammersten.* At this time, it was known that casein contains ~0.86% P and ~0.77% S (a low value), that it reacts with, and is precipitated by, Ca^{2+}, occurs as a coarse colloid (retained by porcelain Chamberlain filters), is very stable to heat, and is coagulated by rennet.

Hallaburton (1890) proposed the name 'caseinogen' for the acid-insoluble protein in milk that was converted to 'casein' by chymosin (rennet) (*ogen*, Greek for begets, i.e., caseinogen begets casein, analogous to fibrinogen ⇒ fibrin, pepsinogen ⇒ pepsin, etc.; this proposal obtained some support in the United Kingdom but not generally, and was abandoned).

Based on solubility in water and salt solutions, acid whey was shown to contain two proteins, lactalbumin (Bouchardat and Quevenne, 1847) and lactoglobulin (Sebelein, 1885).

Hammersten considered acid casein to be a homogeneous protein but, gradually, evidence accumulated (T. B. Osborne and Wakeman, 1918; K. Linderstrom-Lang, 1925–29; K. Pedersen, 1936; O. Melander, 1939) that it is heterogeneous and consists of three proteins, α-, β-, and γ-caseins. During the 1940s, attempts were made to fractionate these proteins, the first successful method being that of N. J. Hipp and colleagues in 1952. In 1956, the α-casein fraction was shown by D. F. Waugh and P. H. von Hippel to contain two proteins, which were called α_s- and κ-caseins. α_s- and β-caseins were shown to be precipitated by Ca^{2+} but κ-casein was soluble and can stabilize 6–10 times its mass of α_s- and β-caseins to Ca^{2+}; these properties were lost on treatment with chymosin (rennet). This was a seminal study and opened a new era in the study of the colloidal stability of casein.

Electrophoresis has been the principal tool for unraveling the complexity of the milk protein system. Free-boundary electrophoresis, developed by A. Tiselius in 1937, was applied to milk proteins by O. Mellander in 1939. Since then, the resolving power of electrophoresis has been improved progressively and each new development was soon applied to the study of milk proteins with continuously improved results. Free-boundary electrophoresis was soon replaced by zone electrophoresis on various supporting media – paper, cellulose acetate, and finally gels (starch, polyacrylamide, or agar). Gel electrophoresis using starch gels, which was developed by Oliver Smithies in 1955, was applied to the study of casein by R. G. Wake and R. C. Baldwin in 1961. Starch was soon replaced as the gelling medium by polyacrylamide gels, which was first applied to casein by R. F. Petersen in 1963, and quickly became the usual medium for gel electrophoresis and numerous systems have been reported. Resolution is greatly improved by using a dissociating agent, 6 mol l^{-1} urea for the caseins and SDS for the whey proteins, and mercaptoethanol to reduce disulfide bonds. Isoelectric focusing (IEF) has been used occasionally to resolve milk proteins and 2D electrophoresis (SDS-PAGE in one direction and IEF at right angles) is now used routinely in proteomic studies with excellent results.

Urea-PAGE resolved isoelectric casein into about 20 zones. There are several reasons for this level of heterogeneity: (1) αs-casein was shown to consist of two distinctly different proteins, α_{s1} and α_{s2}; (2) variations in the degree of phosphorylation; (3) variation in the degree of glycosylation (κ-casein); (4) genetic polymorphism; and (5) proteolysis by the indigenous proteinase, plasmin, especially of β-casein to γ-casein. All the caseins in bovine, ovine, caprine, porcine, equine, and human milk have been isolated and characterized, including primary structure.

It had been recognized since at least 1870 that the casein in milk occurs as coarse colloidal particles containing Ca and P_i, and now called casein micelles. These particles were retained by porcelain (Chamberlain) filters, scattered light (giving milk its white color), and could be 'seen' in the ultramicroscope. The colloidal stability of casein micelles has attracted attention for at least 100 years. It was suggested by Jerome Alexander in 1910 that the casein was stabilized/protected by the whey proteins, an idea arising from the fact that human and equine milk, which do not coagulate on renneting, contain a high level of whey proteins. The 'protective colloid' idea was developed by Linderstrom-Lang (1925–29), but with only very limited knowledge of the milk protein system then available, it was not possible to describe a realistic model of casein micelle structure until the isolation of κ-casein in 1956, which functions as the protective colloid.

The first model of the casein micelle was presented by D. F. Waugh in 1958, and since then, aided by advances in electron microscopy, the model has been refined progressively but there is not yet unanimous agreement.

The only known function of the caseins is nutritional, to supply amino acids, Ca, and P_i, although it has been shown recently that the caseins, and also the whey proteins, contain cryptic peptides which when released, for example, during digestion, display various biological activities (*see* **Milk Protein Products**: Bioactive Peptides), the significance of which *in vivo* is uncertain. However, most of the whey proteins have a definite biological role, in addition to supplying amino acids. The whey proteins are typical globular, highly structured proteins, most of which have been isolated, crystallized, and well characterized. The lactoglobulin fraction contains immunoglobulins G, A, and M, which are present at very high levels in colostrum and play very important protective roles during the first few days post-partum.

The lactalbumin fraction contains two main proteins, α-lactalbumin and β-lactoglobulin, and several minor proteins, including blood serum albumin, lactoferrin, and vitamin-binding proteins; it contains several peptide hormones and about 70 enzymes.

Milk was a popular subject for early studies on enzymology, possibly because it is relatively rich in several enzymes, is susceptible to spoilage by these enzymes, and since it is a liquid, it is relatively amenable to the assay of enzymes. Several enzymes were identified in milk before the nature of enzymes was recognized. Today, more than 70 enzymes have been identified in milk and the principal ones have been isolated and characterized and their significance in milk and dairy products studied in considerable detail.

The indigenous enzymes in milk have been the subject of research since 1881, when the first report on an indigenous enzyme (lactoperoxidase) appeared. Enzymes in milk originate from the animal's blood plasma, leukocytes (somatic cells), and the apical membrane or cytoplasm of the secretory cells. By the early twentieth century, seven indigenous enzymes had been identified in milk: (lacto)-peroxidase, catalase, xanthine oxidase, proteinase, lipase, salolase (arylesterase), and amylase. These enzymes were relatively easily assayed and were technologically important; they have now been isolated and well characterized.

Between 1924 and about 1970, nine more important indigenous enzymes were identified in milk, and isolated and characterized. These were important as indicators of pasteurization of milk (alkaline phosphatase, γ-glutamyl transferase) or of mastitis (*N*-acetylglucosaminidase, acid phosphatase) or were considered to be important for the stability of milk (superoxide dismutase, sulfhydryl oxidase). Milk is also rich in ribonuclease and lysozyme. In addition to these major enzymes, about 50 other enzyme activities have been detected in milk but have not been isolated and are considered not to be important.

Reflecting changes in dairy technology and other factors, the focus of attention in dairy enzymology has varied over the years. Although dairy enzymology has a long history, many questions about the nature and significance of milk enzymes remain to be answered definitively. Most of the research has been on bovine milk and to a lesser extent on human milk. There has been only occasional work on enzymes in the milk of other species but this has shown that there are large interspecies differences in the level of many enzymes.

Technologically Important Properties of Milk Proteins

The colloidal stability of the casein micelles is central to the success of many dairy processes and consequently has attracted the interest of researchers for more than 100 years. Casein micelles are stable to most of the processes to which milk is subjected, including normal and high-pressure homogenization, high-pressure treatment, centrifugal sedimentation (sedimented micelles can be redispersed with little change in properties), dehydration by hot air, or freeze-drying (rehydrated micelles show little change in properties). However, they are destabilized by any of three treatments: acidification, severe heating (e.g., 140 °C × 20 min), or limited proteolysis.

The acidification of milk to pH ~4.6 through the fermentation of lactose by adventitious lactic acid bacteria (LAB, *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp.) has occurred since the dawn of dairying and results in the production of fermented milks and fresh acid-curd cheese (~25% of total cheese production). Initially, fermentation was uncontrolled but since the introduction of cultures of LAB about 1880 (see the following article by T. M. Cogan), lactic fermentation processes have become highly controlled. The health benefits, probiotic effects, of consuming fermented milks were recognized by Elie Metchnikoff more than 100 years ago and have attracted much interest in recent years. The organoleptic properties, especially the texture, of fermented milks have been improved progressively and the rheology of these gel-like products has been the subject of much research.

The preparation of casein by direct acidification to pH ~4.6 was standardized by O. Hammersten in 1883 and acid casein soon became an important raw material for the production of glues, plastics, and synthetic textiles after polymerization with formaldehyde or for paper glazing; early work in this area is described in the book *Casein and Its Industrial Applications*, by E. Sutermeister and F. Browne (1939). The uses mentioned above remained the principal uses of casein until the 1960s when they were replaced by synthetic polymers, which were better and cheaper. Around this time, a need arose in the food industry for 'functional' proteins for use in fabricated foods; required properties included surface activity (for foams and emulsions), water binding, gelation, and bodying effect (viscosity). Casein was found to be an excellent protein for such applications and as a result of research undertaken mainly in Australia in the 1960s, the principal applications of casein became as functional proteins, a situation that still applies.

The development of membrane technology in the 1960s made it possible to prepare undenatured whey proteins from acid or rennet whey, and whey protein concentrates and isolates soon became a second family of functional proteins, complementary to the caseins.

Unconcentrated milk is a very heat-stable system but the stability of concentrated milk is marginal for the production of evaporated milk, which was introduced unsuccessfully by Gail Borden in 1856 and successfully by John Meyenberg in 1884. Evaporated milk became a major product during the first half of the twentieth century and heat stability became a major research topic; the first scientific study was published in 1919 and since then there has been a steady flow of research findings on the compositional and processing factors that affect heat stability and on the mechanism of heat-induced coagulation.

As described earlier, rennet-coagulated cheese has been produced for at least 2500 years. Throughout most of this period, cheesemaking was a farm-based enterprise using rennet produced in-house (usually strips of calf vells) and acidification by adventitious LAB. Probably the first attempt to explain the rennet-induced coagulation of milk was by Edmond Fremy, who, in 1839, proposed that rennet converts lactose to lactic acid, which coagulated the casein, that is, rennet-induced coagulation was essentially acid-induced coagulation (fermentation was one of Fremy's interests). This view was generally supported until the work of O. Hammersten, around 1885, who showed that proteolysis preceded coagulation, a view that proved to be correct.

By the end of the nineteenth century, it was known that the rennet-induced coagulation of milk involved alteration of the casein-calcium phosphate particles (now known as casein micelles, a term first used in 1921). The idea that the coagulation involved the destruction of a 'protective colloid' by rennet was suggested by Jerome Alexander in 1910, although the precise nature of the protective colloid did not become clear until the discovery of κ-casein by Waugh and von Hippel in 1956. Since then, the rennet-induced coagulation of milk has been a major research topic and is now fairly well understood at the molecular level.

Milk Minerals (Salts)

Although quantitatively relatively minor constituents of milk, the inorganic elements are important from at least three viewpoints, technologically (especially the colloidal stability of the casein system), as pro-oxidants, and nutritionally (*see* **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance). Most of the elements occur as salts in solution but Ca and P_i exceed their solubility and occur mostly in the colloidal phase, associated with the caseins. The soluble salts are mainly ionized and their form can be determined or calculated. However, in spite of considerable research since the 1930s, the form and structure of colloidal calcium phosphate (CCP) and the nature if its association with casein are still uncertain. The CCP is responsible for the integrity of the casein micelles and has a major role in their colloidal stability, heat stability, rennet coagulability, and alcohol stability. The concentrations of soluble Ca^{2+}, P_i, and citrate also have major effects on the stability of the casein system.

Many of the minor elements are also associated with proteins, for example, lactoferrin (Fe), and many enzymes such as xanthine oxidoreductase (Mo, Fe), lactoperoxidase (Fe), catalase (Fe), alkaline phosphatase (Zn), superoxide dismutase (Cu, Zn), and glutathione peroxidase (Se), some of which are located mainly in the MFGM.

See also: **Analytical Methods**: Differential Scanning Calorimetry; Nuclear Magnetic Resonance: Principles; Proximate and Other Chemical Analyses; Rheological Methods: Instrumentation; Principles and Significance in Assessing Rheological and Textural Properties. **Cheese**: Rennet-Induced Coagulation of Milk. **Enzymes Indigenous to Milk**: Lipases and Esterases; Other Enzymes. **Heat Treatment of Milk**: Heat Stability of Milk. **Husbandry of Dairy Animals**: Sheep: Reproductive Management. **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Crystallization; Lactose: Derivatives; Lactose: Production, Applications; Lactose Intolerance; **Milk Lipids**: Analytical Methods; Conjugated Linoleic Acid; Fatty Acids; General Characteristics; Lipid Oxidation; Milk Fat Globule Membrane; Nutritional Significance; Phospholipids; Rheological Properties and Their Modification. **Milk Proteins**: Casein Nomenclature, Structure, and Association; Casein, Micellar Structure; Immunoglobulins; Interspecies Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity; α-Lactalbumin; Lactoferrin; β-Lactoglobulin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; Proteomics. **Milk Protein Products**: Bioactive Peptides. **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance. **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Centrifuges and Separators: Types and Design.

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History of Dairy Bacteriology

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Introduction

Milk, as synthesized in a healthy udder, is sterile but it soon becomes contaminated with microorganisms from the cow herself, her environment, and particularly the milking machine. Milk is a good growth medium for many contaminating microorganisms, which will cause spoilage if not controlled. The development of modern bacteriology coincided with the industrialization of dairying and played a major role in the success of the industry. Dairy bacteriology can be considered from three aspects, milk as a vector for pathogens, spoilage of milk and dairy products, and the production of fermented dairy products, through the growth of, and acid production by, lactic acid bacteria (LAB). The early development of each of these aspects is considered below.

Milk as a Source of Pathogens

Raw milk can be contaminated with the causative agents for various infectious diseases, which affect man, including typhoid, scarlet fever, tuberculosis, and diphtheria. Although milk was considered to be quite important as a source of pathogens at the beginning of the twentieth century, this is no longer so because of improvements in the health of cows, hygienic milking of cows, refrigerated storage of raw milk, and improvements in processing technologies.

The first indication that milk could be a source of pathogens (typhoid fever) was that of Michael Taylor, a country doctor in Penrith, Scotland, in 1857. Bacteria had not yet been shown to cause disease, so his observations were based mainly on inference and deduction. He showed that raw milk was a source of typhoid in a family running a small dairy business. Ten years later, he was also instrumental in showing that raw milk was the source of scarlet fever in an outbreak, also in Penrith.

In 1882, *Mycobacterium tuberculosis* was identified as the cause of human tuberculosis (TB) by Robert Koch (1843–1910), who was convinced that *M. bovis*, the cause of bovine TB, was not the cause of human TB. The latter observation was announced at the British Congress on Tuberculosis in 1901 and caused immediate consternation, ultimately resulting in a Royal Commission being appointed to inquire and report with respect to TB: (1) whether the disease in animals and man is one and the same; (2) whether animals and man can be reciprocally infected with it; and (3) under what conditions, if at all, the transmission of the disease from animals to man takes place, and what are the circumstances favorable or unfavorable to such transmission. The Commission undertook research and showed that human TB can be transmitted to animals, and that the consumption of cows' milk containing the organism was a cause of TB, especially among infants and children.

Raw milk can also be contaminated with *M. bovis*, the cause of TB in cattle but which also causes TB (mostly non-pulmonary) in humans. An early compilation of data by Briscoe and McNeal in 1911 showed that 6.8% of milk samples from various countries were positive for this organism. According to Grange, in 1945 in the United Kingdom, *M. bovis* was isolated from 8% of in-can samples and from almost all samples of bulk tanker milk.

The nomenclature of the causative agents of human and mammalian TB has been a cause of confusion for over a century and is still not completely clear; *M. bovis* is considered to be the cause of TB in cattle and *M. tuberculosis* the cause in humans but both should be considered to be the same species since they share 99.5% similarity in genome sequence.

Raw Milk Quality

At the end of the nineteenth century, the keeping quality of milk was a major problem and, in many parts of the world, it still is today. One of the earliest papers on the production of milk with a low bacterial count was that of Henry Ayers *et al.* in 1918, who showed that sterile utensils, clean cows with clean udders, the use of small-top milking buckets, and holding milk below 10 °C were the four essential factors in the production of good quality milk. These factors are still appropriate today.

Refrigerated storage had not been developed at the end of the nineteenth century and so milk was kept at room temperature or simply water-cooled. Methods were needed to assess the quality of raw milk. Robert Stanley Breed (1877–1976), who made a major contribution to *Bergey's Manual of Determinative Bacteriology*, also contributed in a major way to dairy bacteriology by developing a microscopic procedure for evaluating the hygienic status of milk – the Breed smear, in which a known volume of milk was spread over a particular area of a glass slide, dried, and stained with methylene blue. Then, the number of bacteria in several fields was counted and the average determined and multiplied by the microscopic factor of the microscope to give the number of bacteria per ml. Orla-Jensen was responsible, along with the French scientist Barthel, for introducing the methylene blue test to assess the bacteriological quality of milk. Breed was also interested in public health problems and the imperfections inherent in many methods for estimating the bacteriological quality of milk; for many years he served as Chairman of a Committee on Standard Methods for the Analysis of Dairy Products. He was President of the American Society of Bacteriology in 1927. In 1905, at the laboratory section of the American Public Health Association Meeting, a committee was formed to study the various methods used for the bacteriological examination of milk. Preliminary reports were made in 1906 and 1907 and the standard plate count was adopted in 1910. Jezeski (1956) has compiled a detailed history of the development of the standard plate count.

Psychrophiles and Psychrotrophs

According to Eddy, Schmidt-Nielsen in 1902, was probably the first person to use the word 'psychrophile', which he used to describe bacteria that grow at 0 °C. This was subsequently challenged by Müller in 1903 on the grounds that many such bacteria would have an optimum temperature between 20 and 30 °C. The word 'psychrotrophic' (from the Greek meaning to thrive or to grow) was coined by David Mossel (1918–2004) to describe bacteria that can multiply at 5 °C or below, regardless of their optimum temperature. Such bacteria came to dominate the microflora when refrigerated storage of raw milk became common in the 1950s in the United States and in the 1960s in other major dairying countries. The dominant organisms are *Pseudomonas, Achromobacter*, and *Alcaligenes* spp. Their growth can result in off-flavor development in milk, mainly due to the activities of lipases and proteinases produced by them during growth. Many of these enzymes are very heat resistant and so they can also affect pasteurized and even sterilized milk, although the bacteria producing them are inactivated by pasteurization. In 1946, Thomas and Sekhar were probably the first workers to report this when they found no bacteria capable of growing at 3–5 °C in laboratory-pasteurized milks (see Thomas), while Kishonti and Sjostrom were the first to report the production of heat-stable proteinases and lipases by psychrotrophs. For reviews of different aspects of the heat resistance of the enzymes of psychrotrophs in foods, see McKellar.

Thermophiles and Thermoduric Bacteria

Thermophilic and thermoduric bacteria (those that withstand pasteurization without growing during the process) in milk were the subject of early studies, particularly since high numbers of thermoduric bacteria are a good indicator of poor hygiene in milking buckets and machines. The first comprehensive review was that of Robertson who identified the pinpoint colonies that appeared on plating pasteurized milk as *Microbacterium lacticum* and *Sc. thermophilus. Microbacterium lacticum, Sarcina lutea, Sc. thermophilus, Sarcina rosea,* and *Micrococcus conglomeratus* were all shown to survive pasteurization.

Yeast and Molds

Yeast and molds attracted much less attention than bacteria during the early years of microbiology, presumably because they are non-pathogenic and heat-labile, and, at least in the case of molds, they are aerobic, and cause spoilage only on the surface of foods, for example, butter and cheese. Because they are acid-tolerant, yeasts and molds can grow on the surface of most cheeses, which may be a desirable or an undesirable property. This is particularly true for washed-rind cheeses, where they play a major role in deacidifying the cheese and increasing the pH to a point where coryneform bacteria can grow and develop the red smear characteristic of these cheeses. Information on early research on yeast in dairy products is scarce but recent information has been reviewed by Fleet who noted that knowledge about the occurrence and growth of yeasts in dairy products remains incomplete. Two species of molds are important in developing the flavor of cheese, namely, *Penicillium camemberti*, in Brie and Camembert, and *P. roqueforti* in Blue cheese.

The Lactic Fermentation

In 1780, lactic acid was isolated from sour milk by Carl Wilhelm Scheele (1742–86), and in 1857, Louis Pasteur studied the production of lactic acid by fermentation in which he showed that the lactic fermentation was caused by living organisms, which were able to grow in a medium composed of yeast extract, sugar, and chalk ($CaCO_3$) and produced lactic acid. The chalk was used to keep the solution neutral. In his 1857 paper, Pasteur also described the first production of yeast extract, which is commonly used today in microbiological media.

Although Pasteur and others had studied lactic fermentations before 1878, the history of dairy bacteriology may be considered to begin in 1878 when Joseph Lister, a Scottish surgeon best known for developing antisepsis in surgery, isolated an organism, *Bacterium lactis*, in pure culture, by diluting sour milk with sterile milk so that every 1/100 of a 'minim' contained on average one cell (1 minim is equivalent to 0.062 ml). One-hundredth of this mixture was then added to each of five 'glasses' of boiled milk. On incubation, only one of these glasses showed evidence of growth and the other four remained sterile, even after 13 days incubation. This is a variation of the most probable number technique, which is still used to estimate the numbers of coliform bacteria in milk, and especially in water. As improvements were made in bacterial taxonomy, the name of *B. lactis* was changed several times. Löhnis called it *Streptococcus lactis* in 1909 and Schleifer changed it to a new genus in 1984 and called it *Lactococcus lactis*.

Butter Cultures

At the end of the nineteenth century, commercial production of butter had just begun with the development by Gustaf de Laval (1845–1914), in 1878, of the centrifugal separator, which could separate cream from milk. The cream was allowed to stand and became sour naturally in what was called 'spontaneous souring'. A major breakthrough occurred in 1890 when three independent workers, Herbert Conn (1859–1917) at Storrs, CT, USA, Vilhelm Storch (1837–1918) in Denmark, and Hermann Weigmann (1856–1923) at Kiel, Germany, showed that the ripening of cream was brought about by bacteria. Conn made the observation that hardly two creams in the same dairy are ripened by the same bacteria! These observations laid the foundation for the development of starter cultures, and the situation in Denmark in 1895 is described in considerable detail, in English, by Knudsen (1931). These cultures soon became commercially available and their use expanded rapidly; for example, in 1897, 802 out of 866 butter plants in Denmark were using commercial cultures. Photomicrographs of some of the strains isolated by Storch in 1890 are shown in **Figure 1**. In particular, strains 4 and 18 produced very good butter.

Storch was a chemical engineer who graduated from the Danish Technical University. He became an officer in the Danish Army, took part in the Danish–Prussian war in 1864 and became a prisoner of war. From 1879 until his death in 1918, he was employed at The Royal Veterinary and Agricultural University in Copenhagen, and from 1883 was leader of the chemical laboratory at the Laboratory for Agricultural Experiments.

Pasteurization of cream for butter making was also developed in the 1890s, and pasteurization of cream and the use of starter cultures were taken up very quickly by the Danish dairy industry. According to Leisner, in 1897, 93% of creameries in Denmark were using commercial starters and 97.5% were also pasteurizing the cream; in contrast, in 1897, very few creameries in the United States were pasteurizing and only 9% were using commercial cultures although the Danish firm, Chr. Hansen, expanded into the United States in 1893 and began supplying Danish cultures from a facility in New York.

Conn had an interest in all aspects of applied bacteriology and was the first microbiologist to isolate an enzyme from a bacterium, probably a *Bacillus* sp., which curdled milk, like rennin, and subsequently digested the coagulum. He was also responsible for determining that bacteria in the nodules of clovers are responsible for fixing N, and became Director of the famous summer school at Cold Spring Harbour, Maine.

The year 1919 was very important for dairy bacteriology for two reasons: (1) the publication of *The Lactic Acid Bacteria* by Sigurd Orla-Jensen (1870–1949), in which *Sc. cremoris, Sc. thermophilus, Lb. helveticus,* and *Lb. bulgaricus* (now called *Lb. delbrueckii* subsp. *bulgaricus*) are partially described; and (2) Storch in Denmark, Boekhout and de Vries in the Netherlands, and Hammer and Bailey in the United States independently showed that two different organisms, now known as lactococci and leuconostocs, are present in mixed-strain starter cultures.

According to Orla-Jensen, the credit for having first observed that the best *Streptococcus* species for use in 'starters' is *Sc. cremoris* rather than *Sc. lactis* should go to Storch rather than to Yawger and Sherman. However, the latter authors did isolate *Sc. lactis* from excellent commercial cultures. Orla-Jensen separated *Sc. cremoris* from *Sc. lactis* on the basis that *Sc. cremoris* grew as chains of considerable length in milk and broth, produced less acid in milk, and grew at lower temperatures than *Sc. lactis*, frequently failing to grow at 37 °C; however, tests to categorically differentiate *Sc. cremoris* from *Sc. lactis* were not developed until much later. Yawger and Sherman separated *Streptococcus lactis* from *Sc. cremoris* by the ability of the former to produce NH_3 when grown in 4% peptone and its ability to grow at 40 °C, in the presence of 4% NaCl, and at pH 9.2. In fact, these two organisms are now considered to be subspecies of *Lc. lactis* and have been renamed *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*.

Considerable work was done, especially in Hammer's laboratory, in showing that the leuconostoc component of starters was responsible for the production of diacetyl, the important flavor component in cultured butter, from citrate present in the milk. Hammer isolated two species of *Leuconostoc*, which he called *Sc. citrovorus* (now called *Ln. mesenteroides* subsp. *cremoris*) and *Sc. paracitrovorus* (now called *Ln. dextranicum*). According to Garvie the belief that Hammer isolated strains of *Ln. mesenteroides* subsp. *cremoris* has become accepted but has no basis in fact. Hammer was more interested in flavor production by butter cultures than in bacterial taxonomy. Two of Hammer's cultures were deposited with the American

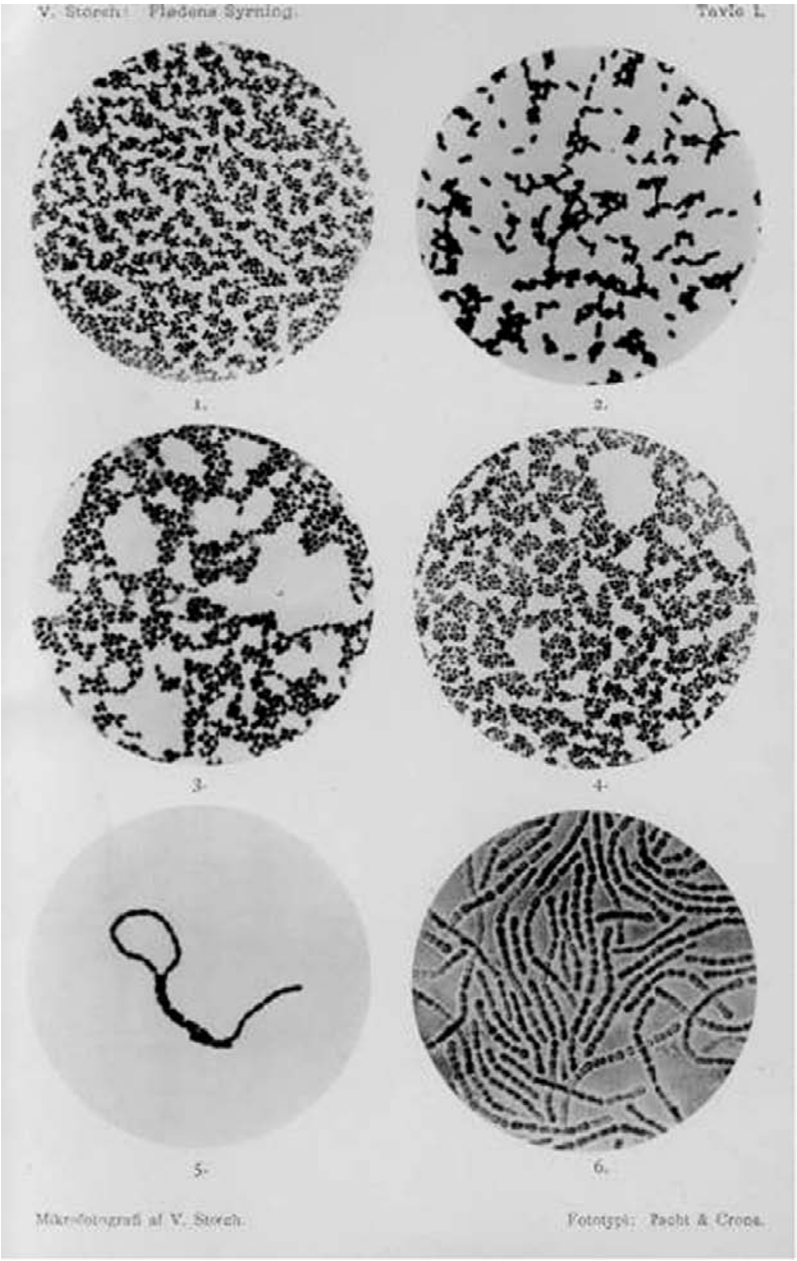

Figure 1 Photomicrographs of various lactic acid bacteria isolated by V. Storch. 1. Lactic acid bacterium no. 4, isolated from a butter sample (no. 33); 2. lactic acid bacterium no. 5; 3. lactic acid bacterium no. 6; 4. lactic acid bacterium no. 8; 5. lactic acid bacterium no. 18, isolated from a sample of acidified (fermented) cream, August 1889; 6. lactic acid bacterium no. 18, pure culture in whey; isolate nos. 4 and 18 produced very good quality butter. Source: *Nogle Undersøgelser over Flødens Syrning*. Eighteenth report from the Royal Veterinary and Agricultural University, Laboratory for Agricultural Experiments, Copenhagen (1890).

Type Culture Collection (ATCC); one of these, ATCC 8081, is a strain of *Pediococcus cerevisiae* and the other, ATCC 8082, is a strain of *Ln. dextranicum.* The exact species of *Leuconostoc* in mixed-strain mesophilic cultures remain to be identified.

Streptococcus diacetilactis, which also had the ability to metabolize citrate, was described by Matuszewski *et al.* but it was another 43 years before Kemplar and McKay showed that the ability to metabolize citrate by citrate-positive strains of *Lc. lactis* subsp. *lactis* is borne on a piece of extra-chromosomal DNA, called a plasmid. Thus, this organism no longer has separate species status but is classified as a strain of *Lc. lactis* that can metabolize citrate. Many other traits of starter cultures are also plasmid-encoded, including the ability to hydrolyze lactose, to produce proteinase, and to resist attack by bacteriophage, but these were discovered only during the past 30 years and will not be discussed further here. The chromosomes of all species of both mesophilic and thermophilic starter cultures, including several strains of *Lc. lactis* and *Lb. helveticus*, have now been sequenced. Sherman reviewed the early developments in starter cultures, including all streptococci known at that time in 1937.

Bacteriology of Cheese

Although LAB play critical roles in cheese manufacture and ripening (acid production during the conversion of milk to cheese curd, and flavor development during cheese ripening), the bacteriology of cheese attracted little attention during the early days of bacteriology. Cheese makers depended on the endogenous (adventitious) LAB or on slop-back cultures for acidification and perhaps the microflora of cheese was too complex for the techniques then available. The pasteurization of cheese milk did not become common until the 1940s and many cheeses are still made from raw milk, especially in southern Europe.

Cheese cultures are much more complex than butter cultures. Depending on the cheese variety, the primary (acid-producing) culture may be *Lactococcus* spp., *Lactobacillus* spp., with or without *Sc. thermophilus*. In addition, secondary cultures are used in many other varieties, for example, citrate-positive *Lactococcus, Leuconostoc, Propionibacterium, Brevibacterium linens, P. roqueforti,* or *P. camemberti.*

In 1919 Orla-Jensen described the response of many species of LAB, besides *Lc. lactis,* including other streptococci, leuconostocs, and lactobacilli, to temperature, their ability to use various C and N sources, and their systematics. He divided the genus *Lactobacillus* into three separate genera, *Betabacterium* (obligate heterofermenters), *Streptobacterium* (facultative heterofermenters), and *Thermobacterium* (strains with an optimum temperature of ~45 °C). The properties used to separate these genera are still used to classify lactobacilli into three main groups.

Streptococcus thermophilus was commonly found in Emmental cheese and also in milk ‘preserved’ at 50–60 °C, but it was not until 1937 that clear differentiation of strains of *Sc. cremoris* and *Sc. lactis* was made by Yawger and Sherman. In 1936 Orla-Jensen *et al.* also elucidated the vitamin and nitrogen requirements of the LAB which were expanded by Esmond Snell in 1948. While not a dairy microbiologist, Snell’s group made major contributions to the vitamin and amino acid requirements of LAB.

Cheese starters appear to have been relatively unimportant, compared with butter cultures, in the early days of dairy bacteriology. Although the mesophilic starters in use today in both cheese and butter production are similar, research on butter cultures dominated the early years of dairy bacteriology with only 2 out of 28 pages concerned with cheese starters in the earliest review, in English, of starter cultures which was written by Knudsen in 1931 and none at all in the review by Hammer and Babel in 1943, who reviewed butter cultures. The reason for this is not clear but may reflect the large number of small butter manufacturing plants throughout the dairy world and that, traditionally, whey cultures were used in cheese manufacture in many countries. Curiously, Knudsen states that the methods of cheesemaking have undergone various changes in the course of time, but these only represent a slow development of the old empirical methods of working. He claimed that cheese is not so good when it is made from perfectly fresh milk as when the milk or part of it had undergone a certain ‘ripening’ and it has gradually become clear that ripening consists essentially of the growth of LAB. This suggests that in the early days, natural contamination of milk with LAB was relied upon to produce the lactic acid necessary for the manufacture of good cheese and that starters were not added deliberately. This also implies that many cheeses may have been made from raw milk, which was incubated to allow the growth of contaminating LAB. Much the same system (i.e., no deliberate addition of starter cultures) is still used in the production of many Spanish cheeses.

At the beginning of the twentieth century, a lot of work appears to have been done on the biochemistry of cheese ripening, especially on cold aging of cheese, but little on the starters themselves with some notable exceptions. For example, Rogers reported on the relationship of bacteria to the flavor of Cheddar cheese in 1904 while Evans studied the role of streptococci in cheesemaking in 1918, and Gorini studied the relationship of lactic bacteria to cheese ripening in 1923, also in 1923 Sherman studied the use of bacterial cultures for controlling fermentation in Swiss cheese.

Orla-Jensen also worked with Eduard von Freudenreich at Berne, Switzerland, on the microbiology and biochemistry of Emmental cheese; they identified propionic acid bacteria as a major component of the microflora of this cheese. The names of prominent species of propionic acid bacteria (PAB), *Propionibacterium freudenreichii* and *P. jensenii*, reflect the eminence and contribution of these two scientists. As a result of this work, PAB were isolated and their importance in eye formation and flavor development of Swiss cheese elucidated. On the death of von Freudenreich, Orla-Jensen became the Director of the Swiss Experimental Station for Dairying at Liebefeld until 1906, when he took up a special chair in the Technical University of Denmark.

Another eminent microbiologist who made a major contribution to the study of PAB was C. B. van Niel, whose Ph.D. thesis in 1928, entitled *The Propionic Acid Bacteria*, was an expose of the biochemistry and microbiology of these organisms at that time. It should still be required reading for anyone studying these microorganisms, for the wealth of detail it contains.

Bernard Hammer (1886–1966) worked with Hastings at the University of Wisconsin from 1908 to 1911. He had a distinguished and very productive career at Iowa State University from 1911 to 1943, when he took up a position with the Golden State Co., in San Francisco, where he was in charge of Dairy Bacteriology. Much of his research career was devoted to studying the various organisms found in mesophilic cultures, especially those used as butter cultures.

Hammer was also involved in developing American Blue Cheese. Fred and Robert Maytag inherited a farm from their father and looked for ways to increase the value of the milk from their herd of Holsteins. Fred approached the Dairy Science Department at Iowa State University, where food chemists were working on a recipe for a Roquefort-type cheese made from cows' milk, rather than from sheep's milk. Fred offered to build the cheese plant and pay royalties to Iowa State in return for the opportunity to create an American blue cheese. In 1937, Experiment Station dairy microbiologists, Lane and Hammer, developed a process for making blue cheese from homogenized milk, which became the standard for the blue cheese industry, and was first used by the Maytag brothers to produce their Maytag Blue Cheese. The patent for the blue cheese process developed by Lane and Hammer netted $201 366 for the Iowa Agriculture and Home Economics Experiment Station.

Non-starter Lactic Acid Bacteria

As far as is known, all ripened cheese initially contains low levels, perhaps 100–1000 per gram of non-starter lactic acid bacteria (NSLAB), predominantly *Lb. casei* and *Lb. plantarum.* These grow during ripening, reaching in excess of 10^7–10^8 per gram in the ripened cheese. von Freudenreich and Thoni studied these organisms in 'small' cheeses in 1905 but the first major publication showing the growth of lactobacilli in Cheddar cheese was that of Hastings *et al.* in 1912. NSLAB are responsible for the conversion of L(+)-lactate to D(−)-lactate in Cheddar cheese during ripening, but despite considerable research since then, their role in cheese flavor development is still not completely understood.

NSLAB reach much higher numbers in raw milk cheese than in pasteurized milk cheese and are considered to be responsible for the stronger flavor of the former. However, NSLAB are uncontrolled and are considered to be responsible for the variability in the quality of raw milk cheese. To overcome the lack of flavor in pasteurized milk cheese, it is becoming increasingly common to inoculate pasteurized milk with selected NSLAB, which will dominate the NSLAB microflora.

Bacteriophage

Viruses that attack bacteria are called bacteriophage (phage), and cannot replicate outside their host cell. Phage for *Sc. cremoris* were first described by Whitehead and Cox in 1935. Essentially, phage are composed of DNA, or in some cases RNA, surrounded by a protein coat. The phage attach themselves to the outside of the cell and inject their DNA through their tail into the cell, where it takes over the biosynthetic machinery of the host cell to make more phage particles. Phage are much more problematic in cheeses made with mesophilic than thermophilic cultures and can ultimately result in complete failure of acid production by the starter. The early history of phage in cheese manufacture was published by Whitehead in 1953.

Fermented Milks

Milk soured by the growth of endogenous (adventitious) LAB has been consumed since the dawn of dairying. The low pH extended the shelf life of the milk and, at least for many people, improved its taste. During the past century, fermented milks have assumed new significance owing to their presumed probiotic effect. This interest arose from the work of the Russian scientist Elie Metchnikoff (1840–1916), who made a significant, though probably erroneous, contribution to dairy bacteriology. He shared (with Paul Ehrlich) the Nobel Prize for Medicine of 1908 for his discoveries of immunity, including phagocytes, phagocytosis, and cell immunity, and joined the Institute Pasteur with Pasteur, where he died. One of his students, Roux, described him as 'a 45 year old from the heart of Europe, with an impassioned face, blazing eyes, untidy hair, definitely with the air of a demon of science.' He was born in a region bordering Bulgaria where people regularly lived to over a 100 years of age at a time when life expectancy was probably no more than 50 years. In a population of 3 million people, there were some 3000 centenarians. He associated this with the large amounts of yogurt consumed by the population and spent a lot of time promoting this idea, the so-called 'sour milk cure'. In 1949 Orla-Jensen *et al.* reexamined Metchnikoff's hypothesis and rejected it; they concluded that it is not the undesirable flora found in the aged that causes impaired digestion, but rather the impaired digestion that causes the undesirable intestinal flora.

Several fermented milk products are now produced around the world and are described in several articles in this encyclopedia. The LAB used in the acidification of these products may be *Lactococcus* spp., but *Lactobacillus* spp., sometimes with *Sc. thermophilus*, are more common and probiotic lactobacilli are attracting much attention. Apart from Metchnikoff, the pioneer bacteriologists did not work on fermented milk.

Conclusion

In this article, some of the important early historical developments and early workers in dairy bacteriology are described. Three major points are considered, namely, pathogens, spoilage of milk and milk products, and the production of fermented dairy products through the action of starter cultures. The article should not be construed as being exhaustive but it tries to give the historical perspective and highlights the scientists who made major contributions.

Acknowledgments

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See also: **Analytical Methods**: Microbiological. **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology; Lactic Acid Bacteria: An Overview; Probiotics, Applications in Dairy Products; *Propionibacterium* spp. **Bacteriophage**: Biological Aspects; Technological Importance in the Diary Industry. **Butter and Other Milk Fat Products**: Modified Butters; Properties and Analysis; The Product and Its Manufacture. **Cheese**: Microbiology of Cheese; Overview; Raw Milk Cheeses; Secondary Cultures; Starter Cultures: General Aspects; Starter Cultures: Specific Properties. **Fermented Milks**: Asian Fermented Milks; Health Effects of Fermented Milks; Kefir; Koumiss; Starter Cultures; Types and Standards of Identity; Yoghurt: Types and Manufacture; Yoghurt: Role of Starter Culture. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria. Enterococcus in Milk and Dairy Products; Genomics, Genetic Engineering; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; Streptococcus Thermophilus; Taxonomy and Biodiversity. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose. **Pathogens in Milk**: *Bacillus cereus*; *Brucella* spp.; *Campylobacter* spp.; *Clostridium* spp.; *Coxiella burnetii*; *Enterobacter* spp.; *Enterobacteriaceae*; *Escherichia coli*; *Listeria monocytogenes*; *Mycobacterium* spp.; *Salmonella* spp.; *Shigella* spp.; *Staphylococcus aureus* – Molecular; *Yersinia enterocolitica*. **Psychrotrophic Bacteria**: *Arthrobacter* spp.; Other Psychrotrophs; *Pseudomonas* spp. **Yeasts and Molds**: *Aspergillus flavus*; *Geotrichum candidum*; *Kluyveromyces* spp.; Mycotoxins: Aflatoxins and Related Compounds; Mycotoxins: Classification, Occurrence and Determination; *Penicillium camemberti*; *Penicillium roqueforti*; Spoilage Molds in Dairy Products; Yeasts in Milk and Dairy Products.

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Relevant Website

http://www.biodiversitylibrary.org – Biodiversity Heritage Library.

A

ADDITIVES IN DAIRY FOODS

Contents
Types and Functions of Additives in Dairy Products
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Legislation
Safety
Emulsifiers

Types and Functions of Additives in Dairy Products

B Herr, Leatherhead Food Research, Leatherhead, UK

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Introduction

The use of additives in dairy products varies in different continental regions. Though the addition is regulated under Codex Alimentarius, country-specific provisions must always be considered. In accordance with the provisions laid down in the European Union, additives such as colors and sweeteners as well as stabilizers such as sodium and calcium phosphates, ascorbic acid and carrageenan, and calcium chloride are commonly used for dairy products except butter and unflavored fermented milk products, for which the use of additives is not permitted or is restricted. The use of preservatives and antioxidants in dairy products is also subject to restriction.

Functions of Additives in Dairy Products in the European Union

Colors

The use of colors is currently regulated by Directive 94/36/EC. Unflavored milk, semi-skimmed/skimmed milk, fermented milks, dried and condensed milk, buttermilk, cream, and cream powder as well as chocolate milk may not contain colors unless specifically expressed to the contrary in the directive. However, flavored dairy products may contain the following colors to a level required to achieve a technological effect in the product (*quantum satis* level):

- riboflavin, riboflavin-5′-phosphate;
- chlorophylls and chlorophyllins;
- copper complexes of chlorophylls and chlorophyllins;
- plain caramel, caustic sulfite caramel, ammonia caramel, sulfite ammonia caramel;
- vegetable carbon;
- carotenes;
- paprika extract, capsanthin, capsorubin;
- beetroot red, betanin;
- anthocyanins;
- calcium carbonate;
- titanium dioxide;
- iron oxides and hydroxides.

Other colors, among them artificial colors, are restricted for use in dairy products (**Table 1**).

Sweeteners

The use of sweeteners is acceptable in energy-reduced or no-added-sugar dairy products. Though bulk sweeteners such as sorbitol, sorbitol syrup, mannitol, isomalt,

Table 1 Restricted dairy products and colors

Foodstuffs	*Permitted color*		*Maximum level*
Butter (including reduced-fat butter and concentrated butter)	E160a	Carotenes	*Quantum satis*
Sage Derby cheese	E140	Chlorophylls Chlorophyllins	*Quantum satis*
	E141	Copper complexes of chlorophylls and chlorophyllins	
Ripened orange, yellow, and broken-white cheese; unflavored processed cheese	E160a	Carotenes	*Quantum satis*
	E160c	Paprika extract	
	E160b	Annatto, bixin, norbixin	15 mg kg^{-1}
Red Leicester cheese	E160b	Annatto, bixin, norbixin	50 mg kg^{-1}
Mimolette cheese	E160b	Annatto, bixin, norbixin	35 mg kg^{-1}
Morbier cheese	E153	Vegetable carbon	*Quantum satis*
Red marbled cheese	E120	Cochineal, carminic acid, carmines	125 mg kg^{-1}
	E163	Anthocyanins	*Quantum satis*
Edible cheese rind	E180	Litholrubine BK	*Quantum satis*
	E160b	Annatto, bixin, norbixin	20 mg kg^{-1}
Flavored and unflavored processed cheese	E160b	Annatto, bixin, norbixin	15 mg kg^{-1}
Flavored processed cheese	E100, E102, E104, E110, E120, E122, E124, E129, E131, E132, E133, E142, E151, E155, E160d, e, f; E161b	Curcumin, tartrazine, sunset yellow FCF, orange yellow S, cochineal, carminic acid, carmines, azorubine, carmoisine, ponceau 4R, cochineal red A, allura red AC, patent blue V, indigotine, indigo carmine, brilliant blue FCF, green S, brilliant black BN, black PN, brown HT, lycopene, beta-apo-8′-carotenal (C30), ethyl ester of beta-apo-8′-carotenic acid (C30), lutein	100 mg kg^{-1}
Desserts including flavored milk products			150 mg kg^{-1}
Edible ices	160b	Annatto, bixin, norbixin	20 mg kg^{-1}

maltitol, lactitol, xylitol, and erythritol are permitted in milk- and milk derivative-based preparations, and edible ices to a *quantum satis* level, artificial sweeteners are subject to level restrictions. **Table 2** shows a few examples of energy-reduced or no-added-sugar dairy products.

Stabilizers, Emulsifiers, and Thickeners

Hydrocolloids

For stabilizing and thickening purposes, hydrocolloids such as alginates, agar, carrageenan, and cellulose, also soybean hemicellulose, pectin, and the alginate-derived

Table 2 Restricted dairy products and sweeteners

Sweetener	*Milk- and milk derivative-based drinks (mg l^{-1})*	*Milk- and milk derivative-based preparations (mg kg^{-1})*	*Milk-based sandwich spreads (mg kg^{-1})*
E950 Acesulfame K	350	350	1000
E951 Aspartame	600	1000	1000
E962 Salt of aspartame/acesulfame*	350[a]	350[a]	1000[b]
E962 Salt of aspartame/acesulfame*	250	250	500
E954 Saccharin and its Na, K, and Ca salts	80	100	200
E959 Neohesperidine	50	50	50
E955 Sucralose	300	400	400

*Maximum usable doses for the salt of aspartame/acesulfame are derived from the maximum usable doses for its constituent parts aspartame (E951) and acesulfame-K (E950). The maximum doses for both aspartame (E951) and acesulfame-K (E950) must not be exceeded by use of the salt of aspartame/acesulfame, either alone or in combination with E950 or E951. Limits in this column are expressed either as [a]acesulfame-K equivalent or [b]aspartame equivalent.

propylene glycol alginate (PGA) are used in dairy products such as predominantly fermented milk drinks, milk drinks, dairy desserts, cream, and ice cream (**Table 3**). The use of such additives is product-specific and dependent on the chemical composition as well as pH of the various dairy products; for example, processed euchema seaweed (PES) is used mainly in chocolate milk applications. The use of additives in partially dehydrated and dehydrated milks as well as in pasteurized cream is restricted.

In the European Union, the use of polysorbates (E432, E433, E434, E435, E436) is restricted to ice cream, milk-based desserts, and milk/cream analogues only.

Phosphates

Monophosphates such as sodium and calcium phosphates, and also di-, tri-, and poly-phosphates function as stabilizers in dairy products, though their predominant use in the dairy sector is as emulsifying salts for processed cheese, cheese preparations, and cheese-based sauces. Dicalcium phosphate is also used as a calcium source to form gels with alginates, thus aiding the stabilizing or thickening process.

Coagulation Agents

In the manufacture of cheese a few additives such as citric acid, calcium chloride, and glucono-delta-lactone are used to aid the process of coagulation or curdling of milk. There is no limit to which these additives may be used; however, the use of additives is restricted for specific cheeses (**Table 4**).

Preservatives

The use of preservatives takes place predominantly in cheese manufacturing. Permitted for use is sodium nitrate up to a level of $150\,\mathrm{mg\,kg^{-1}}$ cheese, and the addition may take place in the milk or after coagulation of the casein. Other permitted preservatives include lysozyme, nisin, and hexamethylenetetramine, as well as natamycin and propionates for surface treatment of cheese (**Table 5**).

Also the sorbates and benzoates listed in **Table 6** are acceptable for use in cheese and milk.

Table 3 Restricted additives in dried milk and cream

Foodstuff	*Additive*		*Maximum level*
Partially dehydrated and dehydrated milk as defined in Directive 2001/114/EC	E300	Ascorbic acid	*Quantum satis*
	E301	Sodium ascorbate	
	E304	Fatty acid esters of ascorbic acid	
	E322	Lecithins	
	E331	Sodium citrates	
	E332	Potassium citrates	
	E407	Carrageenan	
	E500(ii)	Sodium bicarbonate	
	E501(ii)	Potassium bicarbonate	
	E509	Calcium chloride	
Plain pasteurized cream	E401	Sodium alginate	*Quantum satis*
	E402	Potassium alginate	
	E407	Carrageenan	
	E466	Sodium carboxy methyl cellulose	
	E471	Mono- and diglycerides of fatty acids	

Table 4 Restricted additives in cheese

Foodstuff	*Additive*		*Maximum level*
Ripened cheese	E170	Calcium carbonate	*Quantum satis*
	E504	Magnesium carbonates	
	E509	Calcium chloride	
	E575	Glucono-delta-lactone	
	E500(ii)	Sodium hydrogen carbonate	*Quantum satis* (only for sour milk cheese)
Mozzarella and whey cheese	E270	Lactic acid	*Quantum satis*
	E330	Citric acid	
	E575	Glucono-delta-lactone	
	E260	Acetic acid	
	E460(iii)	Powdered cellulose	*Quantum satis* (only for grated and sliced Mozzarella and whey cheese)

Table 5 Permitted preservatives for dairy products

Name	*Foodstuff*	*Maximum level*
Nisin (1)		
	Ripened cheese and processed cheese	12.5 mg kg^{-1}
	Clotted cream	10 mg kg^{-1}
	Mascarpone	10 mg kg^{-1}
Natamycin	Surface treatment of hard, semihard, and semisoft cheese	1 mg dm^{-2} surface
Hexamethylene tetramine	Provolone cheese	25 mg kg^{-1} residual amount, expressed as formaldehyde

Table 6 Permitted sorbates and benzoates in dairy products

	Maximum level (mg kg^{-1})	
Foodstuff	*Sorbates*	*Sorbates + benzoates*
Cheese, prepacked, sliced	1000	-
Unripened cheese	1000	-
Processed cheese	2000	-
Layered cheese and cheese with added foodstuffs	1000	-
Non-heat-treated dairy-based desserts	-	300
Curdled milk	1000	-

Lactic acid is widely used for its preservative function. Often it is used together with acetic acid to inhibit the growth of yeast. Applications in the dairy sector would be processed cheese, ricotta, and brined cheeses.

Antioxidants

When their use is not explicitly excluded by legislation, ascorbates and tocopherols are used for their antioxidant properties in dairy products.

The use of gallates, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) is permitted only in milk powder for vending machines.

Functions of Additives in Dairy Products in the United States

The Federal Food, Drug and Cosmetic Act (FFDCA) lays down the framework for food safety at the Federal level in the United States. This includes the definitions and principles of the use of food additives. The provisions of the Act are enforced by the Food and Drug Administration (FDA) through more detailed regulations laid down in Title 21 of the Code of Federal Regulations (21 CFR). Substances intended for use in the manufacture of foodstuffs for human consumption are classified into three categories:

- food additives, defined as substances the intended use of which results or may reasonably be expected to result, directly or indirectly, either in their becoming a component of food or otherwise affecting the characteristics of food;
- prior-sanctioned food ingredients, which are substances that received official approval for their use in food by the Food and Drug Administration (FDA) or the US Department of Agriculture (USDA) prior to 1958; and
- substances generally recognized as safe (GRAS), which are substances recognized by the experts experienced and trained in evaluating the safety of food substances to be safe for their intended use in food.

21 CFR parts 170–190 lay down provisions for the use of additives and substances in foodstuffs. These regulations cover all substances permissible in foodstuffs.

In addition to the substances listed or affirmed in the regulations as GRAS, there are also some substances that have been ‘notified’ as GRAS. These are also covered by the regulations.

Colors

Most dairy products, including cheese, may contain permitted colors provided they do not impart a color simulating that of milk fat or butterfat.

The following colors are exempt from certification and may be used in dairy products in accordance with good manufacturing practice (GMP) unless otherwise indicated:

- annatto extract;
- dehydrated beets (beet powder);
- canthaxanthin, to a maximum of 30 mg lb^{-1} (1lb = 454.6 g) in solid or semisolid foods, and a maximum of 30 mg pint^{-1} (1 pint = 568.3 ml) in liquid foods;
- caramel;
- beta-apo-8-carotenal, to a maximum of 15 mg lb^{-1} in solid or semisolid foods, and a maximum of 15 mg pint^{-1} in liquid foods;
- β-Carotene;
- cochineal extract, carmine;
- ferrous gluconate and ferrous lactate, only for ripe olives in accordance with GMP;
- toasted, partially defatted, cooked cottonseed flour;
- grape color extract (for nonbeverage foods only);
- grape skin extract, only for still and carbonated drinks and 'ades' and beverage bases;
- fruit juice;
- vegetable juice;
- carrot oil;
- paprika, paprika oleoresin;
- riboflavin;
- saffron;
- sodium copper chlorophyllin, only for citrus-based dry beverage mixes to a maximum of 0.2% in the dry mix;
- synthetic iron oxide, only for sausage casings to a maximum of 0.10% by weight of finished food;
- titanium dioxide, to a maximum of 1%;
- mica-based pearlescent pigments (titanium dioxide coated), to a maximum of 1.25% in cereals, confections and frostings, gelatin desserts, hard and soft candies (including lozenges), and chewing gum;
- tomato lycopene extract, tomato lycopene concentrate; and
- turmeric and Turmeric oleoresin.

The following colors are subject to certification:

- FD&C Blue No. 1;
- FD&C Blue No. 2;
- FD&C Green No. 3;
- FD&C Red No. 3;
- FD&C Red No. 40;
- FD&C Yellow No. 5; and
- FD&C Yellow No. 6.

A number of other colors are listed provisionally and await reevaluation. These include the aluminum and calcium lakes of the substances listed above, with the exception of the lakes of FD&C Red No. 3.

Cheese and cheese products have standards of identity defined under Title 21 of the Code of Federal Regulations (21 CFR) part 133. For some cheeses such as Asiago fresh, soft, medium, and old cheeses; blue cheese; Caciocavallo Siciliano cheese; Gorgonzola cheese; Provolone cheese; and Romano cheese, the use of blue or green color is permissible in an amount sufficient to neutralize the natural yellow color of the curd.

Other cheeses such as Edam, Gouda, and cold-pack cheese food (processed cheese), or cream cheeses with added foods may contain harmless artificial coloring.

Individual standards of the US CFR apply for milk, different kinds of cream, acidified milk, cultured milk, and concentrated and dehydrated milks, as well as yogurt. For these products, the only optional ingredients that may be used in milk include characterizing flavoring ingredients with or without coloring. However, the color additives used must not impart a color simulating that of milk fat or butterfat.

Sweeteners

The CFR distinguishes between nutritive and non-nutritive sweeteners. Non-nutritive sweeteners are substances having less than 2% of the calorific value of sucrose per equivalent unit of sweetening capacity. The following substances are permitted: acesulfame K; aspartame; saccharin and its sodium, ammonium, and calcium salts; sucralose; and neotame.

Nutritive sweeteners are substances having more than 2% of the calorific value of sucrose per equivalent unit of sweetening capacity. The substances mannitol, sorbitol, and xylitol are permitted for this purpose.

All artificially sweetened products are considered to be special dietary foods. With the exception of mannitol (max. 2.5%) and sorbitol (12%) all sweeteners can be applied to GMP unless otherwise specified in the CFR.

Most of the plain dairy products are not permitted to contain sweeteners. Flavored milks may contain generally permitted nutritive sweeteners as a result of their use in characterizing flavoring ingredients; saccharin, ammonium saccharin, calcium saccharin, and sodium saccharin are permissible up to a combined maximum of 12 mg fl.oz^{-1} (1 fl.oz = 29.57 ml), calculated as saccharin.

Non-standardized dairy-based drinks, non-standardized yogurt-type products, and non-standardized cheeses may contain generally permitted nutritive and non-nutritive sweeteners. Pasteurized cream, sterilized cream, UHT cream, whipped and whipping cream, and reduced-fat creams are allowed to contain generally permitted nutritive sweeteners. Sweeteners are not used in milk and cream powders.

Stabilizers, Emulsifiers, and Thickeners

Pure milk, buttermilk, and condensed milk are not allowed to contain additives. However, milk products such as yogurt, acidified milk, curdled milk, and eggnog may be stabilized with safe and suitable generally permitted stabilizers such as carrageenan and potassium chloride to the GMP level. The use of other stabilizers and thickeners such as guar gum and karaya gum is restricted.

Pasteurized cream (including half-and-half, heavy cream, light cream), sterilized cream, UHT cream, whipped and whipping cream, and reduced-fat creams may contain safe and suitable generally permitted emulsifiers and stabilizers.

Non-standardized dairy-based drinks and fermented milk products are allowed to contain safe and suitable generally permitted miscellaneous additives, whereas for these products the use of guar gum and karaya gum is restricted.

Stabilizers used in cream cheese are calcium acetate, carob gum and guar gum. Dioctyl sodium succinate can be used to a maximum of 0.5% of the other stabilizers used.

In the manufacturing of most of the standardized cheeses, the use of stabilizers, emulsifiers, or thickeners does not play a role and is also legally not permitted.

However, for non-standardized cheeses, the stabilizers, emulsifiers, and thickeners listed in **Table 7** are used.

For processed cheeses, monosodium phosphate, disodium phosphate, dipotassium phosphate, trisodium phosphate, sodium hexametaphosphate, sodium acid pyrophosphate, tetrasodium pyrophosphate, and sodium aluminum phosphate are employed, though their predominant function is that of an emulsifying salt. In combination with sodium citrate, potassium citrate, calcium citrate, sodium tartrate, and sodium potassium tartrate, singly or in combination, emulsifying salts should not exceed 3%.

Acetic, citric, lactic, and phosphoric acids should only be used in such a way that the pH of the final product does not exceed 5.

Coagulation Agents

Calcium chloride is used as a coagulation aid in cheese production but must not exceed 0.02% (calculated as anhydrous calcium chloride) of the weight of the dairy ingredients.

Enzymes of animal, plant, or microbial origin are also used in curing or flavor development.

Antioxidants, Antimycotics, and Preservatives

Dairy products, including cheese, may not contain antioxidants with the exception of non-standardized cheeses, dairy-based drinks, fermented milks (other than yogurt), and preserved milks. Where they are permitted, the usually applied antioxidants are ascorbic acid, calcium ascorbate, sodium ascorbate, ascorbyl palmitate, erythorbic acid, sodium carbonate, and tocopherols, which can be added according to GMP.

Similar is the case of preservatives. Dairy products, here excluding cheese, may not contain preservatives with the exception of non-standardized cheeses, dairy-based drinks, fermented milks (other than yogurt), and preserved milks.

Safe and suitable antimycotic agents are generally employed but their use must be verified with the individual standards, as for some cheeses they may be applied only on the surface of the cheese. Non-standardized cheeses may contain safe and suitable generally permitted preservatives as well as the antimycotics calcium propionate and sodium propionate to the GMP level. Antimycotic agents such as natamycin may be applied to the surface of slices or cuts in consumer-sized packages or to the surface of the bulk cheese during curing up to a maximum of 20 mg kg^{-1}.

Preservatives employed in processed cheeses such as cold-pack and club cheese; cold-pack cheese food; cold-pack cheese food with fruits, vegetables, or meats; and pasteurized processed cheese spread are restricted to sorbic acid, potassium sorbate, and sodium sorbate, singly or in combination, or sodium propionate and calcium propionate, singly or in combination, whereas the levels must not exceed 0.3%.

Table 7 Permitted additives for non-standardized cheeses

Additive	*Maximum level*
Calcium acetate (also as a firming agent, pH control agent, sequestrant and texturizing agent)	0.02%
Calcium chloride (also as an antimicrobial agent, curing and pickling agent, firming agent, flavor enhancer, humectant, pH control agent, surface active agent, synergist and texturizer)	0.2%
Carob gum (locust bean gum)	0.8%
Guar gum	0.8%
Propylene glycol alginate	0.9%
Sodium tartrate and sodium potassium tartrate	GMP

Nisin preparations are used in pasteurized processed cheese spread and pasteurized cheese spread, including these products with fruits, vegetables, or meats, up to $250\,\mathrm{mg\,kg^{-1}}$.

Bleaching agents

For most cheeses, benzoyl peroxide or a mixture of benzoyl peroxide with potassium alum, calcium sulfate, and magnesium carbonate is used to bleach the dairy ingredients; the use is restricted, and the weight of the potassium alum, calcium sulfate, and magnesium carbonate, singly or in combination, must not constitute more than 0.002% of the weight of the milk being bleached.

See also: **Additives in Dairy Foods**: Legislation; Safety.

Further Reading

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Consumer Perceptions of Additives in Dairy Products

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Introduction

Amid a variety of health scares in the late 1970s and early 1980s, the consumer very quickly became convinced that additives were dangerous chemicals to be avoided at all costs. The additives debate was both emotional and controversial, with experts openly disagreeing, leaving consumers feeling angry and suspicious.

By the mid-1990s, things appeared to have calmed down somewhat, and although many consumers were still concerned, interest in organic and natural foods had begun to rise and other health issues started coming to the fore, particularly food scares such as bovine spongiform encephalopathy (BSE) in beef and salmonella in chickens and eggs. Consumers also started becoming more aware of some of the potential benefits of additives, including improved appearance of food and increased storage time and less risk of food mold/microbial growth. However, there are still high levels of concern over the potentially harmful effects of additives in children. This article describes the consumer perception of and attitude to food additives and looks at trends and developments in the dairy market.

Key Trends in the International Dairy Market

Virtually all innovative new product development in the international dairy industry can be classified under at least one of four key themes, each of which continues to play a significant role in influencing the ways in which modern consumers eat.

These four broad trends are (1) health and wellness; (2) premiumization and indulgence; (3) convenience and snacking; and (4) lifestyle and ethics.

In health and wellness, the focus had until recently been shifting to the addition of ingredients rather than removal (i.e., fat and calorie reduction in dairy products increasingly being replaced by functionality as the prime route to a health and wellness positioning). However, there has been a slight shift away from this trend in some segments over the last year or so, with emphasis now more on naturally made products with as few additives as possible.

Functional ingredients, though, have become the mainstay of some segments of the dairy sector such as yogurts and milk, as these are considered very good carriers of such ingredients and there is an ongoing trend toward increasing the focus on specific health benefits. Although many functional ingredients have a generally positive image among consumers, the increasing trend – certainly for new-generation functional products – is to pay more attention to the ultimate effect of all the ingredients on health rather than to a specific ingredient. As a result, combining of several functional ingredients is an increasingly common practice, as manufacturers target a specific health issue or promote their products as general wellness foods.

Functional ingredients and other healthy ingredients that have a strong 'natural' image are generally performing the best. For example, in the functional arena, this means good growth potential for the likes of probiotics, which can boost the levels of 'friendly' bacteria already found in the body, and omega-3 fatty acids, which have a 'natural' image, thanks to their close association with fish and other natural marine sources. The naturalness issue is also prompting increased interest in superfood ingredients, particularly the many antioxidant-rich superfruits. Superfood ingredients generally have a dual benefit in that they give a more 'premium' image to end products as well as a healthier profile.

Although functional dairy products can command higher prices, offer better margins to suppliers, are a good way to establish strong brand awareness, and receive high levels of customer loyalty, their development does also require higher levels of investment to create and establish new concepts. As a result, the market is generally being driven by world's larger branded dairy companies, which can afford this initial outlay. With a current focus on more economy variants in the recession and with the health claims procedure in the European Union adding further pressure, it is possible that future investment in R&D might be cut back, adversely affecting the level and diversity of new-product activity.

With regard to the premiumization and indulgence trend, more unusual and upmarket ingredients and flavors are adding value to products in the dairy beverages, yogurts and desserts, cheese, and ice cream sectors in particular. Meanwhile, there has also been a shift toward targeting more products at adults. In yogurts and desserts, the development of superpremium products is attracting an older consumer base, while the ice cream market is constantly moving upmarket. Regionality and sourcing of ingredients from specific countries are other routes to adding value, and such strategies have successfully been adopted in the milk, yogurt, cheese, and ice cream categories to date.

The convenience and snacking trend is driven by an emphasis on portability and portioning (single-serve formats). Flavored milks in lidded on-the-go cups represent a prime example of portability, as do spoon-free yogurts and desserts and hand-held ice creams. Cheese snacks and single-serve dessert formats are other sectors that make full use of portioning. The development of 100-calorie portions in the United States is another trend impacting the market in both the convenience and the health-and-wellness categories. These single-serve formats allow consumers to monitor their calorie intake at the same time as providing convenient, single-serve formats for snacking.

In the lifestyle and ethics category, the organic revolution has been central to new product development (NPD) (despite the recent downturn in the fortunes of the organic sector owing to economic recession). The success of organics is also very much linked with premiumization issues, as consumers often consider organic foods to be of better quality than standard lines and, in response to this, many of the organic developments (particularly in the yogurt and desserts, and ice cream sectors) now primarily use premium ingredients as well as organic milk. Fairtrade, most closely linked with sectors such as coffee and chocolate, is also beginning to make its mark in a much wider range of markets, and the dairy sector has not escaped its attention. To date, most of the Fairtrade developments have been found in the ice cream market, where coffee, vanilla, and chocolate flavors are most prominent, but there is scope for further development in other dairy markets in the future. The Fairtrade movement also tends to be closely linked to the organic industry and, as a result, many of the Fairtrade ice cream products appearing also contain organic ingredients.

New-Product Launches as an Indicator of Trends

Reviewing recent product launches in the UK market, one can see that health, convenience, and premiumization remain the key trends. Health is the driving force behind the yogurt category, which is increasingly being spearheaded by functional brands. Numerous products have been relaunched on a health platform in the last two years, often highlighting calcium content (especially children's products), as well as promoting the versatility of yogurt. However, although probiotic ingredients are now par for the course, functional ingredients as a whole are still an area of confusion for consumers and not all guarantee success (as evidenced by Müller removing omega-3 from its Vitality brand).

The luxury end of the yogurt market also continues to witness high levels of innovation. Danone has attempted to bridge the gap between functionality and luxury with its Activia Intensely Creamy launch in 2008. Also at the premium end, the Swiss dairy company Emmi launched muesli yogurts in the market in 2007. This launch also reflected another growing trend: positioning of yogurt as a specific breakfast product.

Highlighting the origin or type of fruit/flavor has been a marked feature of the market in recent years, for example, Madagascan Vanilla, Senga Strawberry, and Champagne Rhubarb. This development meets the growing consumer demand for more authentic flavors and tastes and for provenance in food. Several Lassi products have also been launched in the market on this type of platform.

In the cheese sector, provenance has been a key trend with growing demand for cheese produced from local milk and with local ingredients. The health drive also has impacted this area, though, with numerous lower-fat cheese variants appearing. Dairy Crest and Lactalis have introduced lighter Cheddar versions, within their Cathedral City and Seriously ranges, respectively. The UK reduced-fat Cheddar market was worth £56 million in 2008, according to TNS, and growing strongly, up by 36%. The key challenge that producers are trying to address is how to improve the taste of lower-fat cheeses.

Children's cheeses have also been a focus area, particularly healthier variants. Kerry Foods launched a light version of its Cheestrings brand in 2007 and then relaunched the full range in 2008 with a greater emphasis on the nutritional aspects of the product. Kraft Foods relaunched Dairylea Lunchables in 2007 with less fat and reduced salt levels as well as its Philadelphia Light snack brand. Dairylea Bites also no longer contains artificial colors, flavors, or preservatives.

Continental cheese has also been a growing area of interest with British consumers becoming more adventurous with both their cooking and their eating habits. Retailers have been reporting strong sales of Feta, Emmental, Parmesan, Mozzarella, and goat's cheese, and new products are appearing in these sectors.

Cheese with added fruit (e.g., Wenslydale with cranberries, Stilton with apricots) continues to emerge, while several smoked cheeses have appeared on the market. A general trend toward more premium and mature varieties, such as the vintage Cheddar, and strong flavors has also been witnessed indicating that taste has become a key area of focus with a more adventurous consumer base emerging in this area.

Convenience also continues to remain a key issue influencing development of the UK cheese market, with mini portions and lunchtime snack products being a focus, along with presliced, resealable, and grated formats to save preparation time. Fondue has also made a return to the market as more consumers turn to comfort food and entertaining at home instead of eating out.

Consumer Perception

Food has its principal nutritional function in all cultures. In addition, food is a source of basic pleasure, of aesthetic experiences, and of medicine–poison dimension.

We perceive food-and-drink products using our five human senses – sight, smell, taste, touch, and hearing – and it is thus important to consider how the senses influence our perception.

1. With our sense of sight we measure the appearance of food-and-drink products. The appearance is in most instances the first information we obtain on a product, and we thus make our first judgements. The main elements of the appearance of food or drinks are likely made up by the following:
   - Product packaging
   - Product color
   - Product size and shape
   - Clarity of beverages
   - Composition of foods
   - Surface texture of products
2. Smell is the sense of volatile stimuli perceived by our nasal cavity. We perceive smell through our nose (orthonasal) and via our mouth (retronasal), when consuming food-and-drink products.
3. Taste is the sense of dissolved involatile stimuli perceived with our taste buds on our tongue, palate, and in our throat. We recognize five different basic tastes: sweet, salt, sour, bitter, and umami.
4. With our sense of touch, we mainly feel the product texture. Touch comprises two components: the tactile surface response from skin (somaesthetic sensations) and kinesthesis, a deep response from muscles and tendons.
5. Hearing may be an important sense for some food-and-drink products. Sound is often linked with the freshness of products, for example, the sounds a soda drink makes when opening the bottle, the snap of breaking chocolate, or the crunch of fruits and vegetables.

Our senses interact with each other. The appearance of a product will, for example, have an influence on how we perceive the product's flavor, with increase in color giving an increased flavor perception.

Consumer Perception of Food Additives

Besides the taste, the most important aspect about foods for the American people is what it contains. The American people appear to think that natural foods are better. A similar conclusion on the inclination to believe that foods are better when they are natural was arrived at in an online research study by Leatherhead Food Research in 1996, where two-thirds of the respondents perceived the pack claim 'natural' as important in determining their food or drink product choice. In line with this, additives identified as 'artificial' evoke strident criticism. This emotional focus on artificial additives gives the perception that man-made chemicals are more dangerous to health than chemicals naturally present in our foods – an erroneous perception.

In a study on what people think about contributors to a healthy life, reduction in additives came in at the ninth place, with reduction in smoking, increasing the consumption of fresh fruit and vegetables, and regular exercise covering the top three (**Figure 1**).

In the same study carried out in the United Kingdom, consumers accepted that, overall, additives in foods and drinks had both advantages and disadvantages. Nearly two-thirds agreed that additives extended the shelf life of foods and drinks, and almost as many agreed that some additives were essential for the shelf life of processed foods. However, despite additives being accepted as necessary in food and drink to some extent, the automatic assumption was that additives were 'bad', and not that they might be there to make the food safer, and about three-quarters of the respondents agreed that additives should be universally reduced in food products.

Less than a quarter of the respondents recognized that an E number is an indication of European safety approval of the food product; E numbers were generally seen as the 'baddie'. When told in the discussion groups that E numbers were intended to be informative and reassuring in that all foods containing E numbers had been tested to the highest safety standards, there was sincere astonishment among the consumers.

Although consumers' response was generally unfavorable toward additives, their knowledge of additives was limited. Less than two-thirds of the respondents recognized at least half of the 19 additives shown. This is an important consideration for manufacturers especially with regard to food product labeling, for not only will the consumers not understand the relevance of its inclusion, but they will not even recognize the word.

Most of the ingredients with the word 'artificial', synthetic, or some sort of technical/scientific reference in their description were automatically assumed to be an additive. Conversely, those with the word 'natural' or functional in them were far less likely to be classed as additives.

Although salt is technically not an additive, consumers were most concerned about salt. This concern was undoubtedly fueled by the Food Standards Agency advertisements. Most concerns about additives were regarding artificial flavors, synthetic colors, monosodium

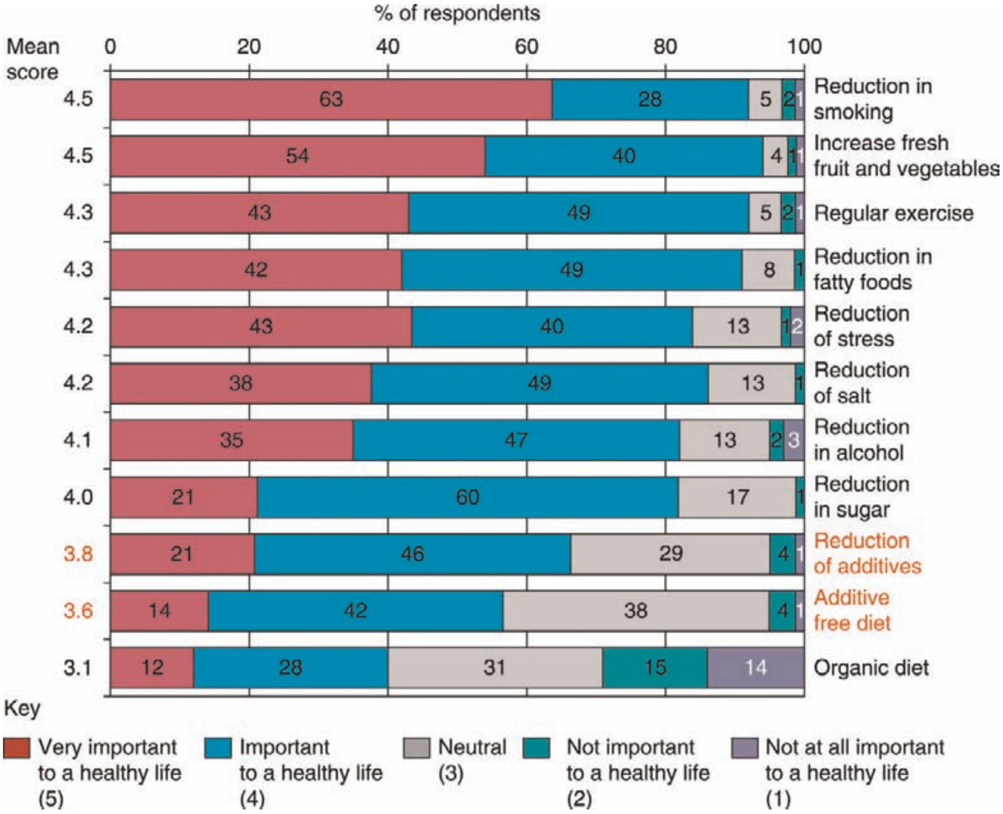

Figure 1 Importance placed on contributors to a healthy life ranked in the order of importance (determined by mean score). From Leatherhead Food Research (2006) *Additives and Attitudes, a UK Consumer Perspective*. Leatherhead ed. Leatherhead Food Research.

glutamate (MSG), and artificial sweeteners; respondents were least concerned with natural flavors, functional foods, and vitamins and minerals (**Figure 2**).

In a study by Rozin and colleagues covering food attitudes of Japanese, French, Belgian, and American people, it appeared that women showed a greater concern about the food–health link and were relatively more interested in nutrition than were men. In general, it was found that among the four studied groups, Americans associated food most with health and least with pleasure, and the French people were most food pleasure oriented and least food health oriented.

Overall, 25% of the respondents claimed to check product labels for additive information either all or most of the time when they were shopping. However, just over 1 in 10 admitted to never checking the labels for additive information. Of the respondents who did check labels, 39% to some extent confessed to finding the additive information on food product labels difficult or very difficult to understand (**Figure 3**).

Looking at product sectors (**Table 1**), respondents perceived the soft drinks category to have the highest levels of food additives. Processed cheese was perceived by the consumers as the dairy product with the highest level of additives. **Figure 4** shows the perceived additive levels for each of the dairy products questioned about.

Sensory Panel and Consumer Evaluation of Dairy Products

Sensorial responses to food products can be measured using trained sensory panels or untrained consumers. Consumers will give their hedonic response toward food products; for example, they are able to tell which products are liked, which one is preferred in a sample set, and whether specific sensory characteristics are acceptable. Consumers are unlikely to give feedback on specific sensory characteristics, such as the sourness intensity, firmness or unripe aftertaste. For this detailed feedback on product characteristics trained assessors should be utilized. Trained assessors are usually screened in basic tastes recognition, odor evaluation, ability to discriminate stimuli, and ability to verbalize and quantify sensory characteristics. In addition, they are trained in specific test methods and products.

Specifically for the evaluation of dairy products, some consideration should be given to the serving

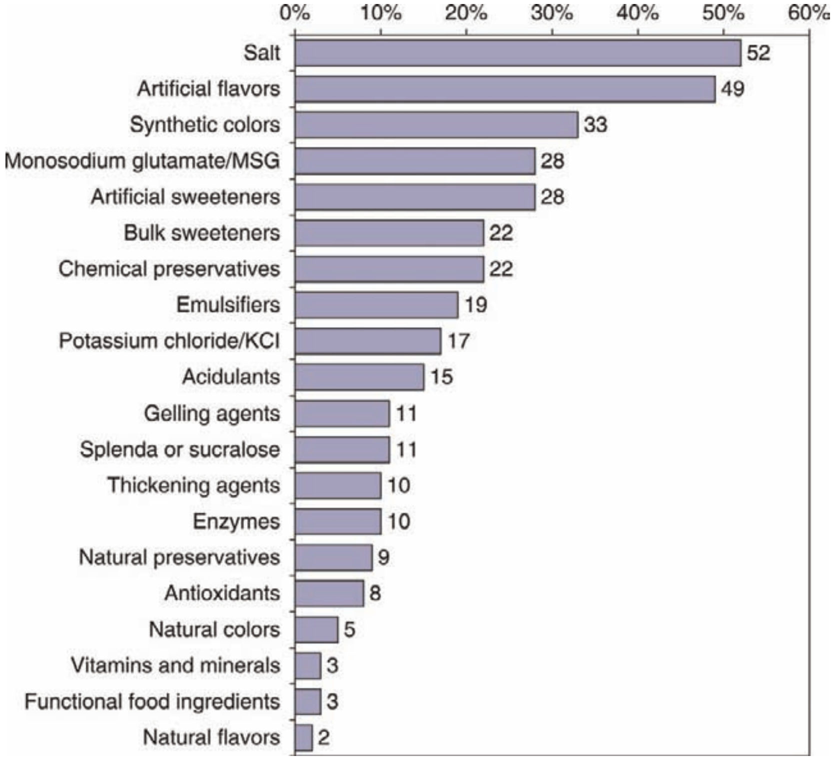

Figure 2 Consumer concern for specific additives. From Leatherhead Food Research (2006) *Additives and Attitudes, a UK Consumer Perspective*. Leatherhead ed. Leatherhead Food Research.

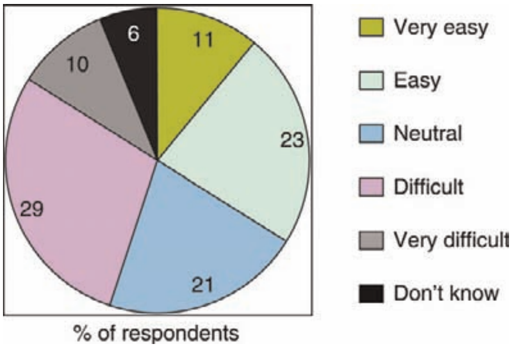

Figure 3 Understanding of additive information on food product labels. From Leatherhead Food Research (2006) *Additives and Attitudes, a UK Consumer Perspective*. Leatherhead ed. Leatherhead Food Research.

temperature and the sourness of the products, as these may influence and, if not correctly presented, bias the perception.

Important attributes of dairy products include color and texture on appearance; aroma and flavor characteristics such as dairy, creamy, and sour milk; sweet and sour taste; and the texture and mouthfeel attributes such as smoothness, thickness, creaminess, and viscosity. Also the aftertaste and afterfeel should be taken into account, as dairy products may often give some aftereffects too.

Table 1 Food and drink categories ranked by perceived additive levels

	High	*Low*	*Do not know*
Category	*% of respondents*		
Soft drinks	81	8	11
Crisps/savory snacks	77	9	14
Ready meals	75	10	15
Diet soft drinks	73	17	10
Sweets/chocolates	70	16	14
Biscuits/cakes	69	18	13
Sauces	60	19	22
Processed cheese	58	20	22
Processed meat	52	34	14
Ice cream complements	51	22	27
Milk shakes	45	35	21
Ice cream	43	31	26
Cereal bars	35	51	14
Bread	24	65	12
Fruit juice	23	69	8
Breakfast cereals	18	67	15
Yogurts	15	72	13

From Leatherhead Food Research (2006) *Additives and Attitudes, a UK Consumer Perspective*. Leatherhead ed. Leatherhead Food Research.

When specific attributes are being evaluated, it is imperative that the attributes be well understood by the assessors. Some examples of dairy attributes and their definitions are given in **Table 2**.

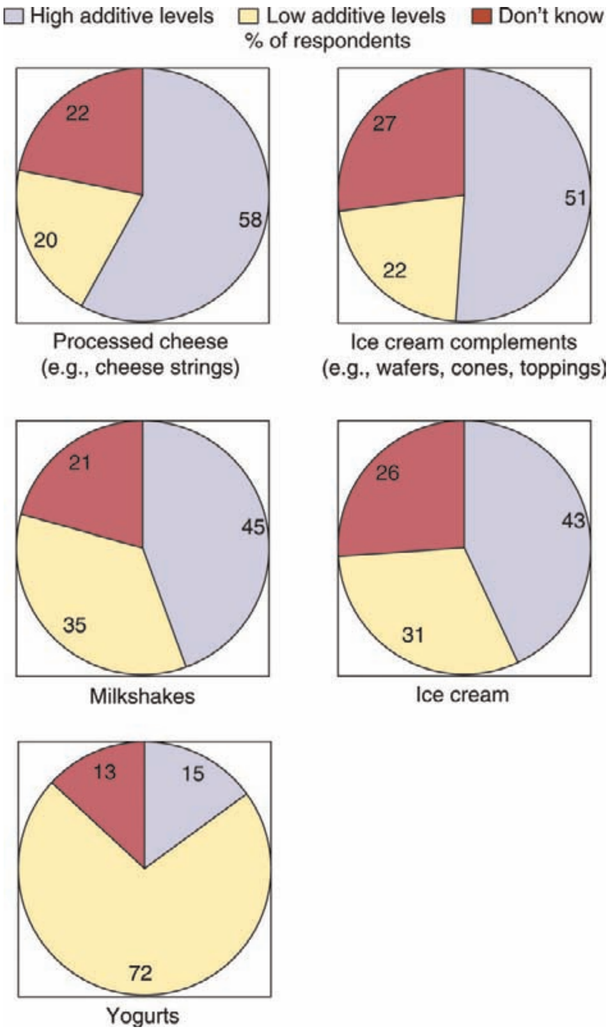

Figure 4 Dairy products by perceived additive levels. From Leatherhead Food Research (2006) *Additives and Attitudes, a UK Consumer Perspective*. Leatherhead ed. Leatherhead Food Research.

Table 2 Examples of dairy attributes and their definitions

Attribute	*Definition*
Visual thickness	Perceived thickness of sample upon dropping off spoon
Dairy	Flavor of cream, milk, Greek yogurt
Thickness	Thickness of yogurt in mouth
Creamy mouthfeel	Creamy mouth-coating effect

Consumption of Dairy Products

The major European dairy markets of Germany, France, Italy, and the Netherlands offer their consumers a wide array of dairy products and ranges, and dairy products are viewed very favorably by most consumers as a key part of their diet. These markets are characterized by particularly high levels of cheese and butter consumption.

For UK and Spanish consumers, on the other hand, most of their dairy intake is in the form of liquid milk. US consumers take less dairy products in their diet in general than do European consumers, while the dairy markets of Asia, Latin America, and the Middle East/Africa, although fast growing, are significantly less developed.

The dairy product range on offer in France is particularly extensive, and French consumers take great pride in their own expertise in dairy production. France produces over 1000 cheese types, and almost all French households purchase cheese. With an average per caput consumption of 23.7 kg yr^{-1}, France is the world's second largest cheese consumer (only behind Greece). French consumers have a strong knowledge of dairy products and of 'terroirs' (regional specialties) and traditional products in particular.

A survey for the Dairy Council in the United Kingdom in April 2008 found that 77% of consumers agreed or strongly agreed with the statement 'Dairy products are healthy' (this figure rose to 88% just for yogurt and 93% just for milk, but declined to 49% for cheese and 31% for butter). Of the consumers, 88% agreed or strongly agreed with the statement 'Dairy products are good for children'. This survey also found that dairy products were the most common source of food allergies, although only 55% of the people claiming to have a food allergy were actually medically diagnosed as having one.

With 12 million Germans reportedly being lactose-intolerant (according to the website of OMIRA Oberland–Milchverwertung Ravensburg GmbH), the high levels of dairy consumption in Germany reflect the importance of the category to the rest of the population (**Table 3**).

Table 3 Per caput consumption levels of dairy products, 2007 (kg per capita)

	Liquid milk	*Butter*	*Cheese*
Germany	64.2	6.4	20.5
France	67.1	7.9	23.7
Italy	60.1[a]	2.9[a]	22.6[a]
The Netherlands	79.4[b]	5.5[b]	21.5[c]
Spain	107.6[d]	1.0[c]	9.3[c]
The United Kingdom	115.6	2.6	10.1
The United States	86.1	2.2	15.1

[a] 2006.
[b] 2005.
[c] 2004.
[d] 2003.
EU figures from CNIEL, L'Economie Laitière en Chiffres 2009, US figures converted from University of Wisconsin (Brian Gould, *Agricultural and Applied Economics*, UW Madison).

Consumer Demand for Clean Labels

Removal of artificial colors, flavors, and preservatives has been a key feature within many food categories in recent years, as consumers have become more aware of the presence of these substances and have taken a greater interest in nutrition and the link between diet and health. Consumers are becoming more sophisticated and knowledgeable, whilst food markets are becoming increasingly global – trends in the West are swiftly reflected elsewhere, as a result of which it is no longer possible for manufacturers to treat regional markets differently. Historically, removing things from foods has reduced their taste and mouthfeel quality. It is therefore the task of the additives industry to develop effective ways to counter act this.

Recent emphasis has been on moving to foods with inherent goodness. Hence 'natural' was the top claim on all new food and drink products launched globally in 2008 (accounting for 23% of all new product launches, according to Mintel's Global New Products Database). Words such as 'naturally rich in' (e.g., antioxidants), 'wholesome', and 'nutritious' are describers that consumers increasingly understand and wish to see.

The superfood trend has led to significant interest in fruits, nuts, seeds, and cereals that offer added health benefits owing to their antioxidant, mineral, or fiber content. Throughout the food industry, fruits rich in antioxidants, including pomegranate, blueberry, cranberry, and açai, are becoming particularly popular and, although their use is perhaps most pronounced in the soft drinks industry, the yogurt sector has not been immune to the superfruit phenomenon.

In general, superfruit flavors are most commonly used in yogurt ranges that already offer some kind of natural (e.g., organic) or healthy (e.g., heart healthy) positioning. Innovation in the yogurt market has involved products containing fruits such as cranberry, acerola, pomegranate, açai, and blueberry.

In terms of the leading health-positioning categories for new dairy products, between January and July 2009, Innova Market Insights recorded the following as the top positioning claims globally: (1) digestive/gut health (14% of all launches); (2) low fat (14%); (3) allergen-free (9%); (4) vitamin/mineral fortified (8%); and (5) no additive/preservative (7%).

An example of a dairy product tapping into the natural, clean label trend is Häagen–Dazs with its ice cream brand Five™ launched in early 2009, which contains just five ingredients: skim milk, cream, sugar, egg yolks, and a flavor (e.g., mint extract for the 'mint' version).

Natural and Greek-style yogurts are experiencing something of a resurgence in several countries at present, prompted perhaps by an increasing demand for more natural foodstuffs and by an interest in new ways of eating yogurt, for example, with honey for breakfast or in cooking. The UK yogurt market has seen particular strength in recent times and own-label suppliers have recently got into the act with the launch of their own breakfast yogurts combining natural yogurt with honey or granola, and even their own organic natural and Greek-style yogurts. It was reported by Danone in 2009 that natural and Greek-style yogurts represented 11% of the healthier yogurt and yogurt drinks market (which itself was 42% of the total yogurt and pot desserts market) in the United Kingdom and were experiencing strong growth.

However, there are significant regional differences in the performance of natural and Greek yogurts. In many Continental European countries, for example, natural yogurt has a fairly mature image and is attracting little significant NPD. In general, Greek-style yogurts appear to have a more widespread appeal and are performing fairly well throughout the world. Greek-style yogurts generally contain more fat than do standard yogurts but, despite this, consumer interest is increasing, thanks to their positive image of quality. In addition, leading suppliers have offered reduced-fat versions to appeal to the health-conscious consumer, while organic versions are becoming more widespread.

The Future

The maturing dairy markets of Western Europe and North America will not be the prime drivers of future growth in the industry; that will come from developing dairy markets such as those of Eastern Europe, China, and India.

Cheese sales in Western Europe for example are forecast to decline by 0.1% per annum in volume terms between 2008-12, versus growth of 4.2% per annum in Eastern Europe, and 8.3% growth per annum in Asia.

Health looks set to remain the most dominant of the megatrends. Healthy options in dairy have significantly outperformed the rest of the dairy sector in recent years. For example, healthy dairy options grew 36.2% in value in Germany between 2003 and 2008 versus only 7.3% growth for dairy sales as a whole.

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Legislation

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Introduction

The legislation on food additives is broadly based on safety and more specifically aims to ensure the protection of public health regarding the use of additives in foodstuffs as well as informing consumers about their presence in food products.

However, additive legislation differs significantly between different regions of the world, in terms of the way it is structured, the specifications of the additives, and the acceptability of certain additives in specific foodstuffs. This makes it very difficult for additive suppliers and food manufacturers to be able to market the same food product globally.

One of the challenges to international organizations such as the Codex Alimentarius Commission is to try to achieve legislative harmonization in the use of food additives throughout the world. The European Union, the United States, and Japan will be covered specifically below.

Definition of a Food Additive

Codex Alimentarius – International Standard

The Codex standard on food additives defines food additives as

> Any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities.

The Codex standard on food labeling lists the different classes of food additives as follows (nonexhaustive list):

1. acidity regulator,
2. flavor enhancer,
3. acids,
4. foaming agent,
5. anticaking agent,
6. gelling agent,
7. antifoaming agent,
8. glazing agent,
9. antioxidant,
10. humectant,
11. bulking agent,
12. preservative,
13. color,
14. propellant,
15. color retention agent,
16. raising agent,
17. emulsifier,
18. stabilizer,
19. emulsifying salt,
20. sweetener,
21. firming agent,
22. thickener, and
23. flour treatment agent.

European Union

The additives directives, namely 94/35/EC on sweeteners, 94/36/EC on colors, and 95/2/EC on food additives (other than colors and sweeteners), are about to be repealed by Regulation (EC) 1333/2008, which is part of the so-called European Food Improvement Agents Package (FIAP) published in December 2008.

The FIAP includes the following regulations:

- Regulation (EC) 1331/2008 of 16 December 2008 establishing a common authorization procedure for food additives, food enzymes, and food flavorings;
- Regulation (EC) 1332/2008 of 16 December 2008 on food enzymes;
- Regulation (EC) 1333/2008 of 16 December 2008 on food additives; and
- Regulation (EC) 1334/2008 of 16 December 2008 on flavorings and certain food ingredients with flavoring properties for use in and on foods.

Regulation (EC) 1333/2008 on food additives applies from 20 January 2010. This regulation simplifies the old legal framework on food additives by grouping food colors, food sweeteners, and other food additives under the same piece of legislation, while these were controlled since 1994 under three different EC directives the implementation of which differed slightly between member states. This regulation, however, will apply directly in all member states.

According to Regulation (EC) 1332/2008, additives must be

– safe when used,
– used for a technological need,
– used without misleading the consumer, and
– used for benefiting the consumer.

The EU legal definition of food additive is laid down in Commission Regulation (EC) 1333/2008 on food additives, which applies from 20 January 2010. This definition is very similar to the Codex definition:

> Any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods.

The regulation specifically excludes

- processing aids (see section 'Processing aids versus food additives');
- substances used for the protection of plants and plant products in accordance with community rules relating to plant health (e.g., pesticides, herbicides, insecticides);
- substances added to foods as nutrients (e.g., minerals or vitamins);
- substances used for the treatment of water for human consumption falling within the scope of Council Directive 98/83/EC on drinking water quality;
- flavorings as they are regulated under Regulation (EC) 1334/2008 on flavorings and certain food ingredients with flavoring properties; and
- food enzymes as they are controlled under Regulation (EC)1332/2008 on food enzymes.

Extraction solvents are also not considered as food additives in the European Union and are subject to specific legislation on both their use and residual levels, under Directive 2009/32/EC.

Processing aids versus food additives

A substance not normally consumed as a food by itself, used intentionally in the processing of food, only remaining as a residue in the final food, and without any technological effect in the final product is a processing aid.

Many food additives may also be used as processing aids. The difference between food additives and processing aids is quite often misunderstood as they are both used for their specific technological functions.

The key differences are

– processing aids are used only during treatment or processing of a food product and
– processing aids may result in the non-intentional but unavoidable presence of residues or derivatives, which might not have any technological effects on the finished product.

Therefore, to determine whether a chemical substance is a food additive or a processing aid, one needs to determine whether it continues to function in the final food. For example, residues of a mold-release agent for a confectionery product are unlikely to have a technological effect on the final product, whereas an antioxidant or preservative added to protect ingredients during processing could still exert a technological function if carried over into the finished product. The former would be considered a processing aid while the latter could be considered a food additive.

The Regulation (EC) 1333/2008 contains the following five annexes:

Annex I	Functional classes of food additives and their definitions
Annexes II and III (see section 'European Union' under 'Approval of Food Additives')	Contain the three community lists of – approved food additives in foods and their conditions of use – approved additives for use in food additives, enzymes, and flavorings, and their conditions of use – nutrient carriers and conditions of use
Annex IV	List of traditional foods produced on their territory for which certain named member states may prohibit the use of certain categories of food additives
Annex V	List of food colors for which additional labeling declaration is needed

Annex I contains the following 26 food additive categories:

1. sweeteners,
2. colors,
3. preservatives,
4. antioxidants,
5. carriers,
6. acids,
7. acidity regulators,
8. anticaking agents,

9. antifoaming agents,
10. bulking agents,
11. emulsifiers,
12. emulsifying salts,
13. firming agents,
14. flavor enhancers,
15. foaming agents,
16. gelling agents,
17. glazing agents (including lubricants),
18. humectants,
19. modified starches,
20. packaging gases,
21. propellants,
22. raising agents,
23. sequestrants,
24. stabilizers,
25. thickeners, and
26. flour treatment agents.

United States

The US definition of a food additive is very different from the EU and Codex ones and is quite unique.

The Federal Food, Drug and Cosmetic Act (FFDCA) lays down the framework for food safety at a federal level in the United States. The current US legislation on food additives is based on the Food Additives Amendment, which was enacted to the FFDCA in 1958. It defines the terms 'food additive' and 'unsafe food additive' and established a premarket approval process for food additives.

The term 'food additive' means any substance the intended use of which results, or may reasonably be expected to result, directly or indirectly, in its becoming a component of any food or otherwise affecting the characteristics of the food. This includes any substance intended for use in manufacturing, processing, treating, or holding food and any source of radiation used.

The above definition excludes

- substances that are generally recognized as safe (GRAS) (the view that a substance is GRAS may be based on scientific procedures or, for substances used in food prior to January 1958, on experience derived from its common use in food) and
- color additives (controlled by separate provisions in the FFDCA).

Therefore, in the United States, substances intended for use in the manufacture of foodstuffs for human consumption, excluding colors, can fall under one of the three following categories: (1) food additives; (2) GRAS substances; or (3) prior-sanctioned food ingredients (substances that received official approval for their use in food by the Food and Drug Administration (FDA) or the US Department of Agriculture (USDA) prior to the Food Additives Amendment in 1958).

There is a US list of GRAS substances; however, the FDA has stated that it is impracticable to list all substances that are GRAS in the regulations, including common food ingredients such as salt, pepper, and monosodium glutamate. The FDA has affirmed a number of substances as GRAS in the regulations through petition by manufacturers. The listing of GRAS substances in the regulations is not exhaustive, and it is the manufacturer's responsibility to ensure the safety of substances used in a food.

Title 21 of the Code of Federal Regulations (21 CFR) Parts 170–189 lays down regulations on food additives and GRAS substances in detail, including the procedures for their approval, labeling requirements, specifications, and purity criteria. In order to clarify the provisions of their use, 43 general food categories and 32 physical or technical functions have been established.

Depending on how it is used, a food additive may be defined as

- direct,
- secondary direct, or
- indirect.

There are eight categories of direct food additives: (1) food preservatives; (2) coatings; (3) films and related substances; (4) special dietary and nutritional additives and anticaking agents; (5) flavoring agents and related substances; (6) gums; (7) chewing-gum bases and related substances; and (8) other specific usage additives and multipurpose additives.

Secondary direct food additives are components used in ingredients of processed foods that may become additives in the final food. These are divided into four categories: (1) polymer substances and polymer adjuvants for food treatment; (2) enzyme preparations and microorganisms; (3) solvents, lubricants, release agents, and related substances; and (4) specific usage additives.

Indirect food additives are materials that may become part of a food as a component of packaging material, adhesives, food-processing equipment, surfaces and containers used for food handling, and certain production aids and sanitizers.

Colors are regulated separately from food additives, under 21 CFR Parts 73 and 74. Any substance deliberately used for its coloring effect is classified as a color additive in the United States and all color additives are classed as 'artificial'. Some colors need to be certified while others need not. All synthetic organic colors are subject to certification. Colors that are not subject to certification include annatto extract, dehydrated beets, canthaxanthin, caramel, beta-apo-8′-carotenal, beta-carotene, cochineal extract, carmine, toasted partially defatted cooked cottonseed flour, grape color extract, fruit juice, vegetable juice, carrot oil, paprika, paprika oleoresin, riboflavin, saffron, titanium dioxide, turmeric, and turmeric oleoresin. It is important to note that foodstuffs for which a standard is laid down may contain color additives only if specifically permitted by that standard. Non-standardized foodstuffs, in general, may be colored with permitted color additives. However, the use of color additives is not permitted if it conceals damage or inferiority or if it makes the product appear better or of greater value than it is.

Japan

The Japanese legislation on food additives differs from the above. Provisions on the use of additives are laid down in the Specifications and Standards for Food and Food Additives (Ministerial Announcement No. 370) under the Food Sanitation Law by the Ministry of Health, Labor, and Welfare in Japan.

Food additives are defined in the first chapter of the Food Sanitation Law as

– substances used in or on food in the process of manufacturing food or
– substances used for the purpose of processing or preserving food.

A positive list system for food additives was introduced in 1947 and only additives designated as safe by the Ministry of Health, Labor, and Welfare may be used in foodstuffs. Additives designated as safe are listed in the List of Designated Additives. Currently, 403 additives including some flavoruings and flavouring groups are designated as approved by the Ministry of Health, Labor, and Welfare under Article 10 of the Food Sanitation Law.

The Japanese Standards for Use of Food Additives specify foodstuffs to which designated additives may be added and maximum levels of use. These standards also specify the major functional classes for permitted additives.

In addition, the following three categories of substances are exempted from the designation system and permitted for use as additives in Japan:

1. Existing additives: These substances were already marketed or used on the date of the amendment of the Food Sanitation Law and appear in the List of Existing Food Additives. This list came into force in April 1996. Existing additives may be used in foodstuffs to a level in accordance with good manufacturing practice, provided their use is technologically justified and they are not specifically restricted from use by a standard of composition. The names and simple details of definition, manufacturing process, and quality of the additives are given in the list.
2. Natural flavoring agents, for example, aloe, dill, green tea.
3. Substances that are both generally provided for eating or drinking as foods and are used as food additives, for example, paprika, gelatin.

Approval of Food Additives

Codex

The Codex Committee on Food Additives and Contaminants (CCFAC) is obtaining scientific advice on the safety of food additives from the Joint Expert Committee on Food Additives (JECFA). This committee is administered jointly by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) and is composed of international independent scientists.

The JECFA is responsible for providing reports and monographs on food additives as well as specifications. Based on this information, the CCFAC would formally approve or reject a substance as food. The Codex General Standard for Food Additives (Codex STAN 192-1995, as last amended in 2009) sets the conditions under which permitted food additives may be used in all foods. This standard is available online.

European Union

At present, food additives are authorized under Directives 89/107/EEC (framework directive) and 94/36/EC (colors), 94/35/EC (sweeteners), and 95/2/EC (miscellaneous food additives) and their amendments. These additives are currently under review as required by the new Regulation (EC) 1333/2008 on food additives repealing the aforementioned EC additives directives (for more information, see section ‘European Union’ under ‘Definition of a Food additive’). This reevaluation applies to all food additives authorized before 20 January 2009 based on a risk assessment carried out by the European Food Safety Authority (EFSA).

Annexes II and III of the Regulation (EC) 1333/2008 will contain the community list of approved additives in foods, as well as the conditions of use of the additives in foods and additives in food additives, food flavorings, and enzymes and nutrient carriers, together with the labeling of additives sold as such.

When a food additive is already included in a community list and there is a significant change in its production method or in the starting materials used, or there is a change in particle size, for example, through the use of nanotechnology, such change will require reauthorization. Also, any genetically modified (GM) food additives will need to be approved under Regulation (EC) No. 1829/2003 on GM foods, as well as under this regulation, to be included in the community list.

The review of food additives for entry into Annexes II and III will be completed by 20 January 2011. Until this review is completed, the annexes to the existing additives directives, namely 94/35/EC, 94/36/EC, and 95/2/EC, will still apply or will be amended as required.

Regarding the submission of applications for the approval of new food additives, Regulation (EC) 1331/2008 covers the common authorization procedure for food additives in the EC. It also lays down procedural arrangements for updating lists of substances, the marketing of which is authorized in the community due to Regulation (EC) No. 1333/2008 on food additives.

The procedure for approving a new food additive may be started at the initiative of the commission or on receipt of an application from a member state or an interested party to the commission. The interested party who submits the application may represent more than one other interested parties. The EFSA will give its opinion on each submission within 9 months of receipt. This may be extended if further information from the applicant is required. An urgent procedure can be set in motion if required. The commission may also require further information for risk management purposes within a stated period. The commission has then a further 9 months to submit a draft regulation to include a new substance in the community list. Certain information in an application will be considered as nonconfidential, for example, name and address of the applicant; name and clear description of the substance; justification for use in or on specific foods/food categories; relevant information for safety assessment; methods of analysis where applicable. Applicant will need to justify which information they wish to remain confidential; the commission will make the final decision.

Between them, Regulations (EC) 1331 and 1333/2008 introduce changes to the regulation of food additives in the European Union and will have positive implications for companies intending to submit food additive dossiers. Under the current set of additive directives, the authorization of a new additive is subject to the codecision procedure involving votes by the European Parliament and the Council of the European Union before the commission and member states can make a decision. This procedure is very lengthy and it may take up to 3 years for an additive to be authorized. The new regulation allows a more efficient and simplified procedure for authorization of food additives by comitology involving only votes by member states in a committee meeting chaired by the commission.

The commission will adopt the European reevaluation program for food additives by 20 January 2010 and it will be some time afterward (timings not yet known) before EFSA completes its evaluation of all additives.

During this transition period from the current directives to the two new regulations concerning food additives, food additive dossiers can be submitted under either the current or new regulatory procedures until January 2011.

Details of how to submit an additive dossier under the current procedure have been published by the commission (Administrative guidance for the request of authorization of a food additive). Additionally, an opinion (July 2001) from the former Scientific Committee of Food provides guidance on submissions for food additive evaluations.

Regarding the new procedures for application dossier for new food additives, the European Food Safety published in July 2009 a scientific document on the data requirements for the evaluation of food additive applications. This will be considered by the commission when finalizing legislative measures concerning applications submitted for the evaluation and authorization of food additives. The commission is due to complete these measures by the end of 2010 following a public consultation.

United States

Food additives and food colors must be approved via petition to the FDA. A specific procedure for color additive petitions is laid down in the 21 CFR.

Japan

Approval of a food additive is normally carried out in accordance with the Food Sanitation Law and an application should comply with the Standards for Use of Food Additives.

Labeling of Food Additives

Codex

The Codex standard on food labeling requires that authorized food additives must be declared in the ingredient list with their class titles together with the specific name or recognized numerical identification as required by national legislation. Any food additive carried over into foods at a level less than that required to achieve a technological function and processing aids are exempted from declaration in the list of ingredients unless they have allergenic properties. Another Codex standard provides for the labeling of food additives when sold as such.

European Union

According to Directive 2000/13/EC as amended on food labeling, food additives must be declared in the ingredient list by declaring their category names, followed by their E number or legal names, for example, emulsifier (lecithin) or emulsifier (E322). As in Codex, carried-over additives and processing aids are exempt from declaration except when they are allergenic.

Following on from recent research, a new labeling requirement has been introduced for six colors (azo dyes) in Annex V of the new Regulation (EC) 1333/2008 on food additives. These colors are

- E110 (sunset yellow),
- E104 (quinoline yellow),
- E122 (carmoisine),
- E129 (allura red),
- E124 (Ponceau 4R), and
- E102 (tartrazine).

From 20 July 2010, the declaration “name or E number of the colour(s): may have an adverse effect on activity and attention in children” would have to be labeled on food products containing any of the above azo dyes.

Foods placed on the market before 20 July 2010 that do not comply with this new labeling requirement will be able to be marketed until their date of minimum durability or use-by date expires. This labeling requirement does not apply where the colors have been used for health or other marking of meat or the stamping or other decoration of eggshells.

United States

Unlike in Codex or in the European Union, most additive functions do not need to be declared in a list of ingredients, except for chemical preservatives, leavening agents, and firming agents for which they must be stated. Some warning statements are also required, for example, foods providing more than 50 g sorbitol day^{-1} to consumers must carry the following sentence on their label: “excess consumption may have a laxative effect”.

Japan

The specific name and class name must be declared for colorants, bleaching agents, and antifungal agents. Other additives can be declared by using only their specific name. Generic terms may be used instead of the specific names, such as seasoning, flavoring, gum base, and bittering agent. Carried-over processing aids, nutrients/dietary supplements, and additives need not be declared. An important thing to note about labeling of food additives in Japan is that the use of the term ‘natural’ or any equivalent term implying ‘natural’ is not permitted.

Emerging Issues

In the European Union, the new legislation on food additives is a hot topic and has already provided for new labeling warning on some azo dyes. In November 2009, the EFSA revised its scientific advice on the six azo dyes listed in Annex V of Regulation (EC) 1333/2008. It advised to reduce the acceptable daily intake (ADI) for three – quinoline yellow (E104), sunset yellow FCF (E110), and Ponceau 4R (E124) – and that consumption of these colors could exceed the new ADIs for both adults and children. EFSA also concluded that for five of the six colors (quinoline yellow, sunset yellow FCF, Ponceau 4R, azorubine/carmoisine, and allura red AC) there was no causal link between the individual colors and intolerance reactions such as irritations to the skin or nose. Tartrazine, however, may bring about intolerance reactions in a small part of the population. For all six colors, EFSA concluded that the evidence currently available did not substantiate a causal link between the individual colors and possible behavioral effects. This conclusion may or may not have an impact on the development of the new regulation on food additives as to its recommended warning for these azo dyes and their respective currently authorized levels of use in foodstuffs.

See also: **Additives in Dairy Foods**: Safety; Types and Functions of Additives in Dairy Products.

Further Reading

http://www.codexalimentarius.net/web/more_info.jsp?id_sta=4 – Codex general standard for food additives (CODEX STAN 192-1995, as last amended in 2009).

http://www.codexalimentarius.net/web/more_info.jsp?id_sta=32 – Codex general standard for the labelling of prepackaged foods (CODEX STAN 1-1985 as last amended in 2008).

http://ec.europa.eu/food/food/chemicalsafety/additives/flav16_en.pdf – Commission’s Administrative guidance for the request of authorisation of a food additive.

http://www.efsa.europa.eu/en/scdocs/scdoc/1188.htm – European Food Safety Scientific Document – Data requirements for the evaluation of food additive applications (July 2009).

http://www.efsa.europa.eu/en/press/news/ans091112.htm – European Food Safety – updated safety advice on six food colours (November 2009).

http://www.mhlw.go.jp/english/topics/foodsafety/foodadditives/index.html – Ministry of Health, Labour, and Welfare website – food additive section.

http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:354:0001:0006:EN:PDF – Regulation (EC) 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:354:0016:0033:EN:PDF – Regulation (EC) 1333/2008 of 16 December 2008 on food additives.

http://ec.europa.eu/food/fs/sc/scf/out98_en.pdf – Scientific Committee of Food Opinion – guidance on submissions for food additive evaluations (July 2001).

http://www.codexalimentarius.net/web/more_info.jsp?id_sta=2 – The General Standard CODEX STAN 107, 1981 (as last amended in 2003) provides for the labelling of food additives when sold as such.

http://www.fda.gov/Food/FoodIngredientsPackaging/ucm082463.htm – US Code of Federal Regulations (21 CFR) citations for color additives, food ingredients and packaging.

Safety

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Introduction

Food additives have been used for centuries to enhance the quality of food products. Smoke, alcohol, vinegar, oils, and spice were commonly used to preserve food. Today, they are used to preserve the nutritional quality of food, to enhance the keeping quality or stability of food, to improve the organoleptic properties of food, or to provide an aid in the manufacturing, processing, storage, and transport of food. Additives such as food colors are used to restore the original appearance of food whose color has been affected by food processing to make foodstuffs more visually appealing and to help identify flavors normally associated with particular foods.

In Canada, more than 400 food additives are approved for use, while in the United States and the European Union, their number is approximately 3000 and 300, respectively.

Although there are several legal definitions of a food additive, as defined by the *Codex Alimentarius*, the European Parliament and Council, and the US Food and Drug Administration (FDA), for example, food additives are generally defined as substances that are added to food to produce a desired technical effect. In the United States, the definition is somewhat broader and incorporates two different kinds: direct food additives (substances incorporated directly into the food supply) and indirect additives (substances used in various types of food-contact applications, such as packaging and food machinery, that may be incorporated into food unintentionally).

In the European Union, substances proposed as food additives may be authorized for use only if a reasonable technological use can be demonstrated, if they present no hazard to consumer health at the level of proposed use based on existing scientific evidence, and if they do not mislead consumers.

Risk Assessment

The underlying objective of a food additive risk assessment is to provide a scientific basis for the control and management of potential adverse health effects that may result from human exposure to the food additive. A risk assessment can be carried out at pre-market stage or as a post-market reevaluation. Risk assessments of all food chemicals are characterized by four stages: hazard identification, hazard characterization, exposure assessment, and risk characterization. Hazard identification involves the identification of potential hazards with the potential to cause an adverse health effect. Hazard characterization quantifies potential adverse effects (e.g., reduced weight gain, organ enlargement) that may result from human exposure to a chemical using dose–response studies that are usually conducted in laboratory animals. Results from hazard characterization studies are used to derive an acceptable daily intake (ADI) of a food additive. Hazard characterization is closely linked to hazard identification since it is often based on evaluation of the same toxicological studies. Exposure assessment of a food additive entails the qualitative and/or quantitative evaluation of the likely intake of an additive via food as well as exposure from other sources (e.g., medicine, food supplements), if applicable. The final stage in the risk assessment process, risk characterization, combines results from an exposure assessment and hazard characterization to estimate the likelihood that an additive may cause harm and how severe the effect may be. Risk characterization provides the primary basis for risk managers to make decisions about how to manage the risk in different situations (e.g., whether to reduce or limit the maximum permitted level of a food additive or whether to provide advice to susceptible subgroups). This section focuses in particular on the first three steps of a food additive risk assessment: hazard identification and hazard characterization, which culminate in the derivation of an ADI, and exposure assessment, which results in estimated intakes of a food additive by humans.

Acceptable Daily Intake

The ADI of a food additive is a toxicological end-point that is internationally accepted as the basis for the estimation of safety for humans. This concept was first proposed by the joint FAO/WHO Expert Committee on Food Additives (JECFA) and is defined as the amount of a food additive, expressed on a body weight basis, that can be consumed daily over a lifetime without an appreciable health risk. It is expressed as a range from zero to an upper limit, which is considered to be the zone of acceptability of the substance.

Derivation of the ADI of a food additive is based upon a series of toxicological studies using laboratory animals (mostly rodents) that have been bred specifically for that purpose and which can be exposed at all stages of life within a reasonable time span. The tests are designed to establish which type(s) of effect (e.g., reduced weight gain, organ enlargement, embryo toxicity) a particular food additive may cause and the relation between the dose (intake) of the substance and the occurrence of that effect (risk characterization). Toxicity studies typically use a small number of doses (expressed as $\text{mg}\,\text{kg}^{-1}$ body weight) over a given range. The maximum dose at which no effect is observed is defined as the no observed adverse effect level (NOAEL). This effect is typically referred to as an 'observed' effect because assumptions cannot be made about effects that are not detectable by the test methods used. Some effects observed in toxicity studies may represent adaptive responses and would not be considered to have a negative health effect as such. Determining whether a particular effect is an adverse effect or an adaptive response to the study protocol is an important aspect of expert judgment. It is important to note that the NOAEL is not an inherent property of an additive. It is an experimental observation that is dependent on the design of the study used and does not necessarily coincide with the threshold dose. The numerical value of the NOAEL is dependent on the selection of dose levels during study design and on the ability of the study to detect adverse effects.

To account for differences between animals and humans (inter-species variability) and for intra-individual variability between humans, the NOAEL is divided by a safety (uncertainty) factor to establish an ADI. A default safety factor of 100, based on expert judgment, is typically used based on the assumption that humans are 10 times more sensitive to a substance than experimental animals and that there is a 10-fold range in sensitivity within the human population. The NOAEL is a reference point that is commonly used in the risk assessment of non-genotoxic substances and is applicable to all toxicological effects considered to have a threshold. There may be several NOAELs from a range of toxicity studies. In this case, the risk assessment will focus on the most sensitive relevant study producing the lowest NOAEL.

An alternative to the NOAEL approach is the benchmark dose (BMD) approach. This approach makes use of all dose–response data to estimate the shape of the overall dose–response relationship for a particular end point. It refers to the dose level, derived from the estimated dose–response curve, that corresponds to a low but measurable change in response, denoted the benchmark response (BMR). The lower confidence limit of the BMD is the value normally used as the reference point (or point of departure as used by the US Environmental Protection Agency (EPA)). Similar to the NOAEL approach, the ADI derived using the benchmark approach is derived by dividing the reference point by a safety factor. The benchmark approach is of particular value in situations where the identification of the NOAEL is uncertain. BMD software programs are available to facilitate its use. Outside the United States, there has not been widespread use of the BMD for non-cancer risk assessments. However, it is likely that it may be used as the method of choice by the European Food Safety Authority (EFSA) (the European Union's risk assessment body) when dealing with food chemical risk assessments in the future.

Thus, the overall approach for the derivation of an ADI contains a number of safety margins. Given that in most cases data are extrapolated from lifetime animal studies, the ADI relates to lifetime exposure and provides a margin of safety large enough for toxicologists not to be particularly concerned about short-term exposure above the ADI provided that the average intake over longer periods does not exceed it. Therefore, periodic short-term excursions above the ADI are not considered to pose a health risk depending on the circumstances. With regard to prolonged excursions above the ADI, it is not possible to determine a general frequency or degree of excursion that may pose harm, since the NOAEL does not indicate the dose level (threshold) at which an effect is observed. As the level of exposure above the ADI increases, the risk of adverse effects also increases. Therefore, the significance of prolonged excursions above the ADI needs to be considered on a case-by-case basis, with reference to the toxicological study that leads to the derivation of the safety statement. For some food additives of low potential toxicity, evaluation of the available toxicological data may lead to the conclusion that the total potential intake from all sources does not represent a hazard to health. In this situation, the term 'not specified' is used in relation to the ADI and the additive must be used in accordance with good manufacturing practice.

The ADI of a food additive relates to daily ingestion because accepted additives should not accumulate in the body. The amount is expressed per kilogram body weight to allow for differences in body weight between test animals and humans and for variability in human body size (e.g., children compared with adults). The concept is based on the premise that for most compounds there is a discrete threshold of exposure above which adverse effects may be produced. However, the concept of thresholds may not hold for substances that are potentially carcinogenic. In the case of genotoxic substances, which interact with DNA, it is assumed that there is no threshold in their mechanism of action (i.e there is no dose without a potential effect). Consequently, known or suspected carcinogens are not permitted as food additives.

Toxicological Assessment of Food Additives

Before embarking on toxicity testing of food additives, it is important to make maximum use of any available prior knowledge concerning the nature, chemical structure, intended biological activity, mechanism of action, and toxicokinetics of the additive.

Toxicity tests on animals encompass a wide variety of tests ranging from relatively simple *in vitro* studies to complex multigeneration animal studies. Several international regulatory authorities such as the Organization for Economic Cooperation and Development (OECD), the US FDA, the EFSA, and JECFA have issued guidelines and advice for the appropriate conduction of toxicity tests. The OECD guidelines have been endorsed by member countries, allowing the acceptance of data between all countries belonging to the OECD. In addition, the concept of good laboratory practice (GLP) is important in assuring that testing laboratories are able to generate valid data, and some regulatory bodies publish principles of GLP. The above-mentioned guidelines are not strictly defined protocols and allow flexibility in the methods selected. Given that food additives are ingested by humans over a lifetime, long-term animal studies assessing chronic exposure are usually required. These are usually preceded by short-term studies to identify early developing effects and to help in the selection of appropriate doses for longer-term studies. In some cases, interpretation of data derived from these studies may reveal the need for further testing, which may not have been apparent at the outset, to address specific concerns. Other toxicological tests contribute to the overall understanding of the possible adverse effects of a substance on biological systems.

Core Toxicity Tests

Metabolism and toxicokinetic tests

Metabolism and toxicokinetic studies provide data on the rates of absorption, distribution, metabolism, and excretion of an additive, which determine the concentration of an additive and its metabolites in a particular tissue at a particular time after ingestion. These tests are useful in understanding the mechanism of toxicity and the subsequent selection of appropriate test species, dose levels, and duration of toxicity testing.

Subchronic toxicity tests

The primary objective of sub-chronic toxicity studies (repeated-dose toxicity testing for a period of at least 90 days) is to determine the *in vivo* effects of repeated daily exposure to food chemicals over periods of 1 month or longer. Sub-chronic toxicity tests should reveal targets for toxicity (e.g., organs, tissues, cells) resulting from exposure to the test substance. These tests are useful in determining the appropriate dose levels for chronic (long-term) toxicity studies and can identify the need for additional studies on particular effects, such as neurotoxic or immunological effects. Preceding feeding studies conducted for 14 or 28 days can provide an indication of target organs and help in the selection of appropriate doses for 90-day studies.

Genotoxicity tests

Food additives should be evaluated for genotoxicity in order to assess their mutagenic and carcinogenic potential. The objectives of genotoxicity testing include the detection of both germ cell mutagens and somatic cell mutagens. Animal-based *in vivo* assays are not normally required for the initial detection of a genotoxic hazard, which in most cases can be detected by a suitable set of *in vitro* tests. However, following a positive result from an *in vitro* assay, further testing *in vivo* is normally required. In general, a battery of three *in vitro* genotoxicity tests is required for food additives: (1) a test for induction of gene mutations in bacteria; (2) a test for induction of gene mutations in mammalian cells *in vitro*; and (3) a test for induction of chromosomal aberrations in mammalian cells *in vitro*.

Chronic toxicity and carcinogenicity tests

The objective of chronic toxicity testing of a food additive is to provide information on gross and histopathological changes other than neoplasia in organs and tissues, and changes in blood, urine, and serum chemistry following long-term exposure. This type of test may reveal new effects that were not apparent in sub-chronic toxicity tests and are often pivotal in defining NOAELs for setting an ADI.

The main objective of carcinogenicity testing is to identify substances that may cause an increase in cancer by observing test animals for the development of neoplastic lesions as a consequence of chronic exposure. Since chronic toxicity as well as carcinogenicity is usually required for food additives, a combined protocol for studying chronic toxicity and carcinogenicity in the same experiment (18–24 months) is often recommended to maximize the information from the animals used.

Reproduction and developmental toxicity tests

Tests for reproductive effects take into account that food additives are consumed by men and women throughout the reproductive stages of their lives including pregnancy and lactation. These tests usually comprise multigeneration and developmental toxicity tests. A major objective is to provide information on the effects on male and female libido, potency, and fertility, the female's ability to carry pregnancy to term, maternal lactation, pre- and post-natal survival, growth, development, and reproductive capacity of the offspring, and major target organs for toxicity in the parents and offspring. A multigeneration reproduction study, including assessment of end points relevant to endocrine disrupter potential, should be conducted in at least two generations and one litter per generation. Developmental toxicity studies should not only cover the period of embryogenesis but also continue to the end of gestation in order to ensure detection of effects such as endocrine disrupter potential.

Other Toxicity Tests

In addition to the core studies, other tests may be helpful or necessary, depending on the chemical structure or class and known or predicted toxicological properties. Examples of these tests include immunotoxicity, neurotoxicity, allergenicity, food intolerance, human volunteer, and predictive mechanistic studies.

Acute toxicity tests

As human exposure levels tend to be much lower than the high doses employed in acute toxicity tests, acute toxicity testing of animals is not particularly useful for hazard identification and risk.

Acute toxicity tests are useful in identifying major target organs for toxicity and provide a rough guide for the selection of doses in subsequent repeated-dose toxicity tests (subchronic toxicity tests).

Exposure Assessment

Exposure assessments constitute an integral part of a risk assessment process and entail the provision of information on whether an additive is present in a food and, if present, the level at which it is present and the amount of food consumed. The primary purpose of a food additive exposure assessment is to assess whether exposure to an additive is below the ADI for the ultimate protection of the consumer.

Available methods range from crude 'screens' based on theoretical concentration and consumption data (e.g., the budget method) to more sophisticated methods employing food consumption data at individual level and analytical chemical concentration data pertaining to representative samples from target populations (e.g., total diet studies). Since most food additives are considered to be consumed at acceptable levels, employing a sophisticated analysis to begin an exposure assessment may not be cost-effective. In general, a stepwise approach is employed and that begins with crude screening methods based on worst-case assumptions and then proceeds to more refined methods, if results from crude methods dictate the need to do so. Such a prioritization system is of accepted internationally. Refining of crude data can be achieved by means of more precise food intake data or more precise additive concentration data. When comparing results from a food additive exposure assessment with an ADI, the estimation of high-level intakes (e.g., upper percentiles of exposure) is usually required. This ensures that the majority of consumers are protected from potential adverse effects associated with a particular additive. Consideration should also be given to the presentation of data as 'consumers-only intake' or 'total population intakes', and to 'at-risk' groups. At-risk groups may include individuals who may have higher additive intakes compared to the general population on the basis of their dietary habits (e.g., diabetics may have a higher intake of intense sweeteners compared to the general population).

With the exception of duplicate diet studies, exposure assessments do not have consumption, occurrence, and concentration data related to the same individuals in the population. Therefore, some degree of modeling is usually required in assessments of exposure to food additives.

In the point estimate (deterministic) approach, a fixed value for food consumption (e.g., mean population value) is multiplied by a fixed value for the concentration of an additive in a food (e.g., mean concentration or maximum permitted level (MPL)). The additive intake from all foods is then summed to estimate total dietary exposure. A limitation to this approach is that it assumes 100% occurrence of an additive in a food category, which is rarely the case in practice, and it ignores the presence of variability in food consumption and additive concentrations and does not provide a range of food additive intakes. The estimated daily intake (EDI) method as defined by the FAO/WHO is an example of this approach.

A more refined approach to the EDI employs a distribution of food intake data, taking into account variability in food consumption data, but uses a fixed value for the additive concentration value (e.g., MPL) and assumes a 100% probability of the presence of an additive in a food category. Typically, a point estimate of exposure is presented (e.g., mean or upper percentile) for comparison with the ADI.

There is increasing interest in probabilistic approaches for quantifying variability and uncertainty in food additive exposure assessments, especially for refined assessments. The application of probabilistic modeling to food additive intake assessments uses distributions in place of fixed values for food consumption, additive occurrence, and additive concentration data. Several software programs are available to facilitate its use. The approach allows variability (i.e., natural variation in a model input that is irreducible) and uncertainty (i.e., lack of knowledge about the true values of a given model input, which may be reduced by further measurement) to be quantified and provides more refined and realistic estimates of food additive exposure compared with traditional point estimates, which are conservative in nature. Although the US EPA has developed guidance on the application of probabilistic modeling, there is currently no established guidance for its use by the EFSA and it is not yet employed as a standardized approach to food additive exposure assessments at present.

The approach used to estimate dietary food additive exposure generally depends on the type of food consumption and additive concentration data available. As with all aspects of a risk assessment, it is important to clearly document any assumptions used in a food additive exposure assessment to ensure transparency for risk managers.

Emerging Issues

Concern regarding the use of animals for experimental purposes has led to the development of alternative approaches to animal testing in food chemical risk assessments to take account of animal health and welfare. While complete replacement of all animal experiments in toxicology tests with alternative methods is difficult, without affecting the level of food additive safety, a number of methods based on the three R's approach (replacement, reduction, and refinement) are undergoing development. This is an ethical experimental framework comprising three methodological principles: replacement of living sentient animals in scientific procedures, reduction in the number of animals used, and refinement to cause less pain and suffering during testing. Replacement methodologies include improved storage, exchange and use of available information on animal experiments, mutual acceptance of data generated according to recognized guidelines, mutual recognition by international regulatory authorities, predictions based on physical and chemical properties of a substance, computer modeling, and *in vitro* methods. Reduction relates to the number of animals and the number of tests and can be applied by careful selection of species and study design, and refinement relates to the use of methods that aim to enhance their well-being during routine husbandry and care. To improve the acceptance of alternative methods at international level, the OECD published guidance on the validation and international acceptance of new or updated test methods for hazard assessment.

Following the publication in 2007 of the so-called Southampton study, which concluded that exposure to two mixes of six synthetic colors (tartrazine, quinoline yellow, sunset yellow FCF, Ponceau 4R, allura red, and carmoisine) and a preservative (sodium benzoate) in the diet resulted in increased hyperactivity in 3-year-old and 8- to 9-year-old children in the general population, there has been increased attention on the effect of synthetic food additives and children's behavior. As a result, a voluntary ban on the use of the colors was imposed in the United Kingdom. Although the EFSA indicated that there was limited evidence that the mixtures had an effect on the hyperactivity of children and that there were no grounds for altering the ADI of any of the colors based on the study, the European Commission (risk manager) proposed labeling rules to accompany the use of the Southampton colors in foodstuffs to indicate that the additives may have adverse effects on activity. As part of an ongoing post-market reevaluation of all food additives permitted in the European Union from a food safety perspective, colors have been prioritized. To date, following a comprehensive review of toxicity data, the EFSA has lowered the ADIs for the artificial food colors quinoline yellow, sunset yellow FCF, and Ponceau 4R. As a result, exposure to these colors could exceed the new ADIs for both adults and children. EFSA's advice, issued in 2009, will be used by risk managers to inform their decision on any follow-up action to ensure consumer safety.

See also: **Additives in Dairy Foods**: Legislation; Types and Functions of Additives in Dairy Products.

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Emulsifiers

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Introduction

The physical definition of emulsions states that they are dispersions of one liquid in another, both of which are otherwise practically insoluble in each other. Oil-in-water emulsions are typically liquids (e.g., milk, cream), but may be highly viscoelastic, such as mayonnaise. Water-in-oil emulsions such as butter or margarine are semisolid, plastic products.

The stability of emulsions is controlled by various factors, among which the function of surface-active lipids or proteins is an important one. Emulsions are thermodynamically unstable and may undergo various physical changes, such as flocculation, creaming, and coalescence, as described below.

Emulsification

Food emulsions can be either water-in-oil (W/O) types, such as butter, margarine, or low-calorie spreads, or oil-in-water (O/W) emulsions such as milk and cream. When making an emulsion, the two liquid phases are mixed by a strong mechanical action that disperses one liquid phase in the other. The type of mechanical mixers or homogenizers used varies according to the type of emulsions produced. W/O emulsions are made by adding the water phase to the oil phase while agitating with a low-energy propeller-type stirrer, resulting in a rather coarse distribution of water droplets ranging from 5 to 50 μm or more in size.

Most O/W emulsions, such as dairy-based emulsions, are produced using high-energy homogenization where oil or fat droplets are formed under turbulent flow conditions. This results in a dispersed phase with a particle size ranging from 0.3 to 3 μm, typically around 0.5 μm on average. The particle size distribution of the dispersed phase (e.g., fat globules or water droplets) depends on energy input, interfacial tension, and mass density as described by Kolmogorow's equation:

$$d \infty \ E^{-2/5} \ \gamma^{3/5}\rho^{-1/5}$$

where d is the minimum droplet size, E the energy density, for example, 10^4–10^{12} W m^{-3}, γ the interfacial tension, for example, 5–25 mN m^{-1}, and ρ the mass density, which is usually constant.

The relative contributions to droplet disruption by energy density, interfacial tension, and mass density are about 400:4:1; thus, droplet disruption is highly dominated by energy density. A reduction in interfacial tension by adding emulsifiers, on the other hand, is of minor importance, although a decrease in interfacial tension will reduce the surface energy needed to break up the dispersed oil droplets into smaller ones. The energy density may vary by as much as 8–10 orders of magnitude from low-energy paddle mixers to high-pressure homogenizers, whereas interfacial tension of oil–water interfaces, which is ~25 mN m^{-1} without the addition of emulsifiers, may be reduced to 5–15 mN m^{-1} in the presence of emulsifiers.

In practical terms, then, the addition of emulsifiers to O/W emulsions made by high-pressure homogenization does not have any significant effect on the particle size distribution obtained. In the case of W/O emulsions, which are made by low-energy paddle stirring, the addition of an emulsifier results in a finer water droplet distribution.

However, the effect of emulsifiers on protecting the water droplets against recoalescence during the emulsification period may play a greater role in the reduction of particle size of the final emulsion than played by the influence of the emulsifier on interfacial tension.

Physical Properties of Emulsions

Emulsions are thermodynamically unstable, and their kinetic stability is affected by various factors such as (1) formation of a strong viscoelastic interfacial film around the droplets of the dispersed phase, (2) particle size distribution of the dispersed phase, and (3) ion concentration, pH, and viscosity of the continuous phase.

A number of physical changes may take place during the storage of O/W emulsions, affecting their relative stability. These changes are discussed briefly in the following sections.

Flocculation

Flocculation of aggregated particles may be a precursor to creaming and in some cases even coalescence, but flocculation may also be a desired phenomenon as, for example, in whippable emulsions. Many emulsions stable in terms

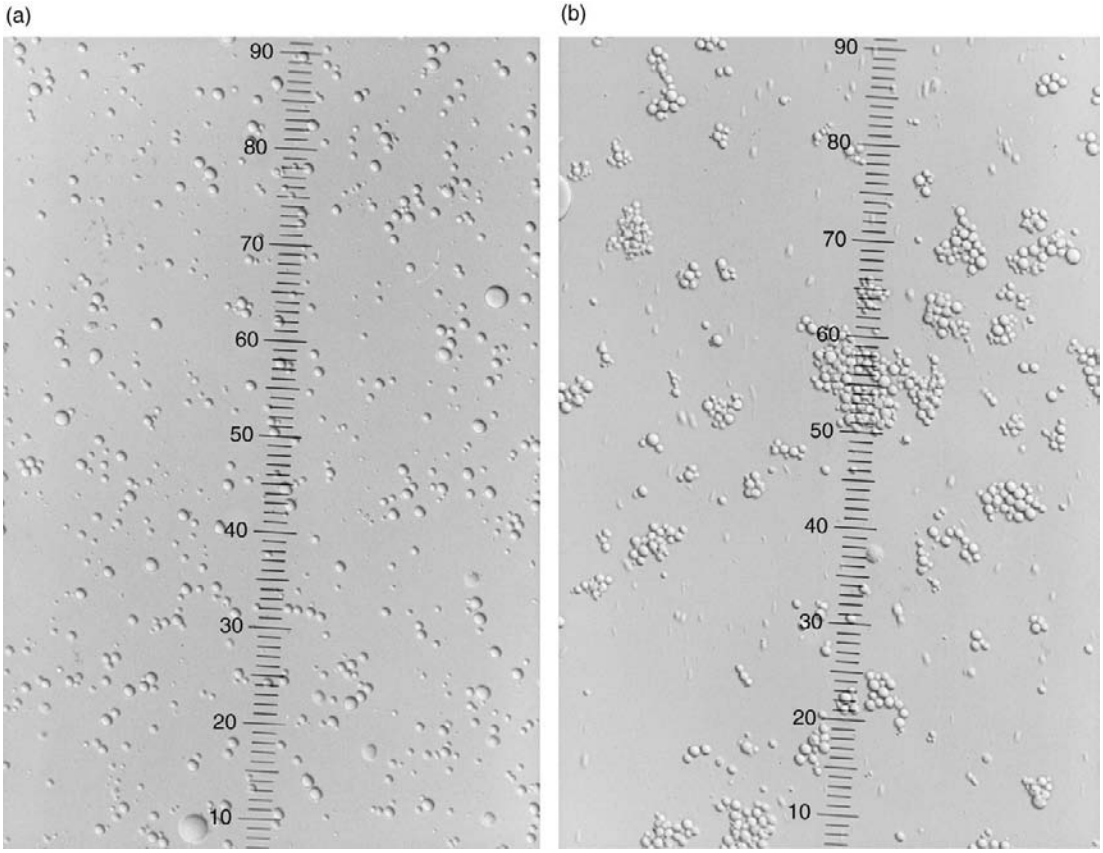

Figure 1 Photomicrographs of (a) O/W emulsion stabilized with milk proteins, and (b) O/W emulsion with flocculated oil droplets forming large aggregates. 1 unit = 1.25 μm.

of coalescence are susceptible to flocculation. This is particularly the case with protein-stabilized O/W emulsions such as dairy emulsions. An example of flocculated oil droplets in an O/W emulsion is shown in **Figure 1**. The so-called feathering of coffee cream in hot coffee is due to the flocculation of oil droplets caused by a combination of the high temperature and the lower pH of coffee than that of the cream itself, which induces net particle attraction between the protein-stabilized oil droplets.

The tendency to flocculate is influenced by the amount of proteins present in relation to the amount of emulsified oil or fat. If the protein present is insufficient to cover the entire surface of the fat/oil droplets during homogenization, a so-called bridging flocculation may take place.

Adjusting the pH to the isoelectric point of the emulsion or increasing the ionic strength by adding calcium ions strongly affects the flocculation of dairy emulsions.

Creaming

The rise of dispersed particles to the surface of an emulsion is referred to as creaming, which occurs due to density differences between the dispersed particles and the serum phase.

The creaming rate (*Cr*) of particles in a dilute system follows Stokes's law and is given by

$$\text{Creaming rate. Cr} = R^2(\rho_c - \rho_o)/\eta_c$$

where *R* is the radius of the particle, ρ_c is the density of the continuous phase, ρ_o is the density of the dispersed phase, and η_c is the viscosity of the continuous phase.

Creaming is a reversible process, and although Stokes's law is inadequate for predicting the creaming of concentrated emulsions, it predicts that creaming can be reduced by the following factors:

1. Reduction of particle size. When the particle size of the milk fat globules, for example, is reduced to <1 μm by homogenization, creaming is practically eliminated due to Brownian motion, which keeps the globules in suspension. Ideally, <3% of all oil droplets should exceed 0.8 μm in size. Furthermore, the particle size should be monodisperse, which is not the case with food emulsions.
2. Reduced density difference between the continuous and dispersed phases. This is not possible to obtain in food emulsions. Although the natural density difference between water and edible oils or fats may be

reduced by the use of high-density additives, such additives are not permitted for use in foods. A thick adsorbed layer of proteins increases the density of small fat droplets (<0.3 μm), even to an extent where they may precipitate.

3. Increased viscosity of the continuous phase. The rate of creaming can be reduced effectively by adding hydrocolloids (gums) or carbohydrates, which increases the viscosity of the continuous phase. Chocolate milk is stable due to high zero shear viscosity created by a network of milk proteins, hydrocolloids, and cocoa particles formed at high temperatures.

Other factors, such as a high volume of the dispersed phase, reduce the speed of creaming; for example, mayonnaise is an O/W emulsion that does not cream off. On the contrary, polydispersity increases flocculation in low- to medium-concentration O/W emulsions, thus contributing to creaming.

Coalescence

Coalescence is an irreversible process in which oil droplets merge into larger droplets owing to rupture of the protecting interfacial film. Such a process eventually leads to a total breakdown of the emulsion, resulting in total separation of the oil and water phases.

Coalescence is promoted by a large droplet size, weak repulsion between droplets, and too low an interfacial tension. This is why proteins are effective emulsion stabilizers. Surface shear rheology of the adsorbed protein films is a significant factor influencing the coalescence kinetics of protein-stabilized O/W emulsions.

Solid fat droplets cannot merge as liquid oil droplets do, but form clumps of aggregated fat globules. This is referred to as partial coalescence and is a desired phenomenon in O/W emulsions that are to be aerated to a foam (e.g., whipping cream, ice cream mix). Controlled destabilization in the form of droplet flocculation (aggregation) and partial coalescence improve whippability and foam stability, as is described in detail later.

In the most common W/O emulsions (butter, margarine), the water droplets are fixed in a semisolid fat phase. This means water droplets usually do not coalesce, unless the fat phase is liquefied owing to the melting of the fat crystal networks.

Interfacial Films in Emulsions

The interfacial film around milk fat globules is a complex physiological biomembrane consisting of proteins (enzymes, glycoproteins), phospholipids, glycolipids, and mono-, di-, and tri-acylglycerols. The structure of the membrane is poorly understood. It is assumed that a lipid monolayer adsorbed from the cytoplasm is surrounded by lipid bilayers, interspersed with proteins, which may protrude into the milk plasma. The thickness of the membrane varies from 10 to 20 nm, and the interfacial tension is very low (1–1.5 mN m^{-1}).

When milk is homogenized to avoid creaming, numerous new fat globules are formed and their interfaces covered by adsorbed milk proteins and fractions of the native milk fat globule membrane (also referred to as 'milk lipid globule membrane'). The surface-active macromolecules in milk are the proteins: the whey proteins β-lactoglobulin and α-lactalbumin together with the casein proteins (α_{S1}-, α_{S2}-, β-, and κ-caseins). The casein fraction may adsorb either as entire casein micelles or as fragments thereof. Added lipid emulsifiers may co-adsorb with milk proteins, or emulsifiers and proteins may compete for adsorption at the interface of milk-based emulsions. The composition of the resulting interfacial film depends on the concentration and chemical structure of the added emulsifier, which determines its polarity (hydrophilic/lipophilic balance). Low-polar, oil-soluble emulsifiers (monoacylglycerols) tend to form mixed lipid–protein interfacial films, whereas high-polar, water-dispersible emulsifiers (e.g., polysorbates) tend to displace most of the interfacially adsorbed proteins and dominate the interfacial structure. In some cases the adsorption of low-polar emulsifiers depends on the temperature of the emulsion, resulting in increased adsorption of the emulsifier – followed by a decrease in the amount of adsorbed protein – when the emulsion is cooled. This results in destabilization of the emulsion, making it more sensitive to shear-induced flocculation and partial coalescence of fat globules, which is needed for whippable emulsions (whipping cream, toppings, or ice cream mix) to obtain a satisfactory foam structure and stability.

Figure 2 shows schematic models of the interfacial films of adsorbed proteins and emulsifiers. A protein film exerts high viscoelasticity and provides a strong barrier to fat globule coalescence. Mixed emulsifier–protein films are less coherent, and the viscoelasticity is reduced, especially by emulsifiers that form liquid-condensed types of monolayers (e.g., unsaturated monoacylglycerols). Mixed emulsifier–protein films may provide increased stability or cause destabilization of emulsions, depending on the ratio between the emulsifier and protein concentrations at the interface and on the type of emulsifier used.

Generally, anionic-active emulsifiers increase stability by complex formation with interfacial proteins, whereas nonionic emulsifiers displace proteins from the fat globule surface.

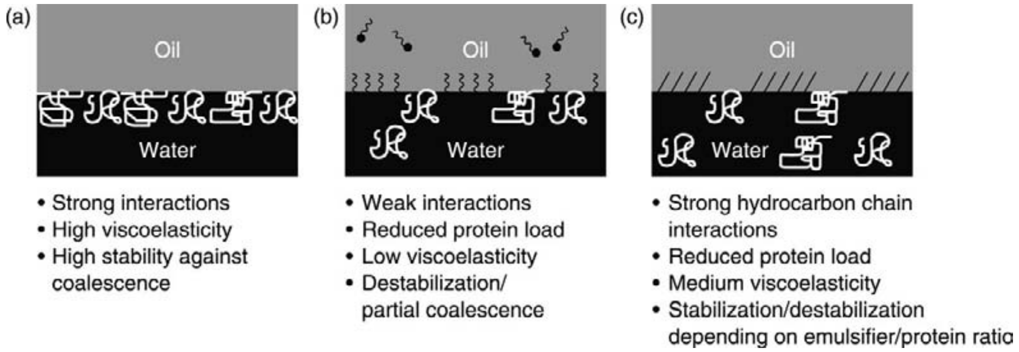

Figure 2 Schematic models showing interfacial films of (a) pure protein, (b) mixed lipid–protein film with emulsifiers forming liquid-condensed monolayers, and (c) mixed lipid–protein film with emulsifiers forming solid-condensed monolayers.

Emulsifiers and Their Applications

Naturally Occurring Emulsifiers

Proteins

Proteins are amphiphilic compounds, containing both hydrophilic and lipophilic segments. The lipophilic segments are shielded by hydrophobic segments in aqueous solutions, but in the presence of oil droplets they partly unfold and adsorb strongly at the O/W interface.

Milk proteins are highly valued for their emulsion-stabilizing properties. The adsorption of milk proteins at the O/W interface, in the form of casein micelles together with whey proteins, β-lactoglobulin and α-lactalbumin, takes place during the homogenization process. The proteins form strong interfacial membranes, providing steric stabilization against coalescence, creaming, or flocculation of the fat globules. The large negative free-energy change associated with protein adsorption means that the process is irreversible with respect to the dilution of the continuous phase. However, some of the adsorbed proteins may be partially or entirely displaced from the interface by more surface-active protein molecules or lipid-based emulsifiers. Therefore, the overall stability of protein-stabilized emulsions is strongly affected by the dynamic aspects of interfacial protein–protein or lipid–protein interactions. β-Casein is considered a better steric emulsion stabilizer than the other caseins, probably because it is a more distinctly amphiphilic molecule. Furthermore, among the milk proteins in general, the caseins are preferentially adsorbed at the O/W interface during emulsification in contrast to whey proteins.

Phospholipids and Glycolipids

Milk contains small amounts of phospholipids and glycolipids, which belong to a group of surface-active vital lipids present in the cell membranes of all living organisms. Phospholipids are diacylglycerols with a phosphate group at 3 position, which is esterified either with an amino alcohol (choline, ethanolamine, serine) or with a polyol (inositol, glycerol). The chemical structure of these major phospholipids is shown in **Figure 3**. They are amphiphilic compounds and considered natural emulsifiers. In milk, phospholipids associate with water and proteins and form the so-called lipoproteins. They also form a vital part of the native fat globule membrane. The phospholipids, mainly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), together with sphingomyelin (SPM), are the major lipid constituents of the milk fat globule membrane. The other type of membrane lipids found in milk is glycolipids (cerebrosides and gangliosides).

Table 1 shows the concentration of phospholipids and glycolipids in milk. About 65% of these polar lipids are present in the fat globule membrane, and 35% are found in the milk serum in the form of lipoproteins or vesicles.

Phospholipids may be hydrolyzed enzymatically to form the so-called lyso-phospholipids, which are monoacyl phospholipids. Such components are more hydrophilic than the corresponding diacyl phospholipids.

The predominant sources of commercially available phospholipids are vegetable oil seeds (soybean, rapeseed, sunflower seed) and egg yolk. **Table 2** shows the typical composition of the commercially available phospholipids, usually referred to as ‘lecithins’.

Synthetic Emulsifiers

This group of polar lipids consists of esters of fatty acids and various polyols (glycerol, polyglycerol, propylene glycol, sorbitol/sorbitan, and sucrose), their organic acid or ethylene oxide derivatives, and lactic acid esters of mono- and di-glycerides. The majority of these emulsifiers or surfactants are produced by interesterification of fats and oils with glycerol or direct esterification of fatty acids with selected polyols. **Table 3** shows food emulsifiers with their identity numbers according to the food regulations of different countries.

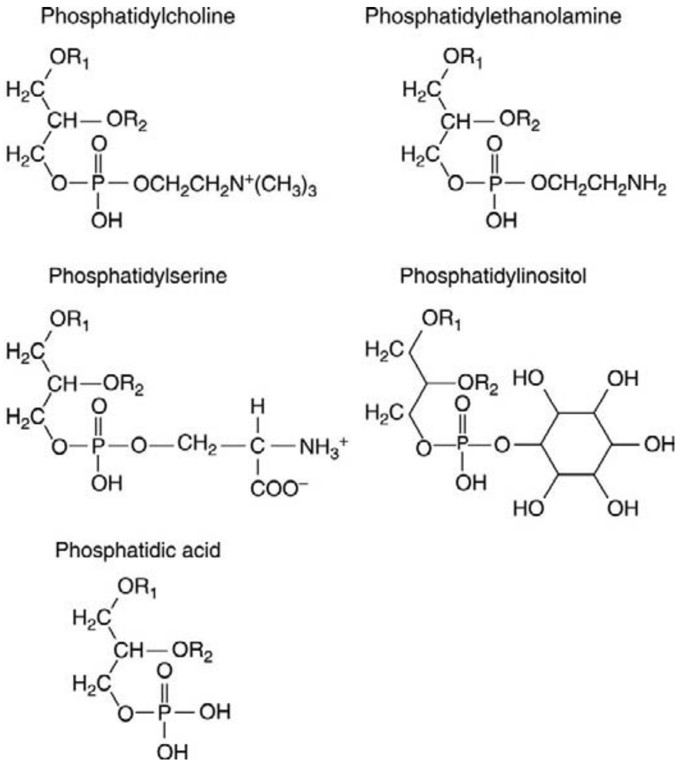

Figure 3 Chemical structure of commercially available lecithin components. R_1, R_2, fatty acid residues.

Table 1 Average composition of lipids in cow's milk

Lipid class	*Abbreviation*	*Total lipid (%)*	*Fat globule membrane lipids (%)*
Triacylglycerols	TAG	98.3	
Diacylglycerols	DAG	0.3	1–3
Monoacylglycerols	MAG	0.1	1–3
Phospholipids (total)[a]	PL	0.8	
Phosphatidylcholine	PC		29–36
Phosphatidylethanolamine	PE		28–31
Phosphatidylserine	PS		4–6
Phosphatidylinositol	PI		4–7
Sphingomyelin	SPM		18–22
Glycolipids		0.1	10–12
Sterols		0.3	2–4
Free fatty acids	FFA	0.1	
		100	100

[a] Approximately 65% of total phospholipids are present in the fat globule membrane and 35% are in the milk plasma. Data compiled from Walstra P, Geurts TJ, Noomen A, Jellema A, and van Boekel MAJS (1999) *Dairy Technology: Principles of Milk Properties and Processes*. New York: Marcel Dekker and Schlimme E and Buchheim W (1995) *Milch und ihre Inhaltstoffe: Chemische und physikalische Eigenschaften*. Gelsenkirchen, Germany: Verlag Th. Mann.

Mono- and di-acylglycerols

Mono- and di-acylglycerols and their organic acid derivatives are the emulsifiers most commonly used by the global food industry. They are produced by interesterification (glycerolysis) of fats or oils with glycerol. The most commonly used fats as raw materials are hydrogenated vegetable oils (soybean, rapeseed, cottonseed, etc.) or animal fats (lard, tallow). The composition of the equilibrium mixture obtained after glycerolysis is typically 40–50% monoacylglycerols, 30–40% diacylglycerols, and 10–20% triacylglycerols, which is the standard composition of many commercial mono- and di-acylglycerols.

Table 2 Composition (wt. %) of commercially available lecithins

Class	*Soybean*[a]	*Rapeseed*[a]	*Sunflower seed*[a]	*Maize*[a]	*Egg*
Phosphatidylcholine	34	25	41	43	74
Phosphatidylethanolamine	21	22	18	6	19
Phosphatidylinositol	18	15	31	23	
Phosphatidylserine			1	1	
Phosphatidylglycerol/diphosphatidylglycerol	3		2	2	1
Phosphatidic acid	9		5	13	
N-Acyl-phosphatidylethanolamine	4		2	4	
Phosphosphingolipids: ceramide phosphocholine (sphingomyelin)					2
Lyso-phospholipids	5	19		8	3
Others	6	19	-		1

[a]Figures are based on the acetone-insoluble constituents of commercially available lecithins, which also contain certain amounts of glycolipids. Adapted from Schneider M (1997) Phospholipids. In: Gunstone FD and Padley FB (eds.) *Lipid Technologies and Applications*, pp. 51–79. New York: Marcel Dekker.

Table 3 Food emulsifiers, their legal status, and typical applications

Chemical name	*EU no.*	*United States*[a]	*Typical uses in food*
Lecithin	E322	184.1400[a]	O/W and W/O emulsions, bakery products, cereals, confectionery, ice cream and other dairy products, coffee whiteners, margarines, spreads, etc.
Mono- and diglycerides (distilled monoglycerides)	E471	184.1505[b]	O/W and W/O emulsions, bakery products, cereals, confectionery, ice cream and other dairy products, coffee whiteners, margarines, spreads, and so on
Acetic acid esters of monoglycerides	E472a	172.828	Bakery products (cakes), dessert products, nondairy cream, toppings
Lactic acid esters of mono- and diglycerides	E472b	172.852	Bakery products (cakes), dessert products, nondairy cream, toppings
Citric acid esters of mono- and diglycerides	E472c	GRAS (self-affirmed)	O/W and W/O emulsions, margarines, meat products
Diacetyl tartaric acid esters of monoglycerides	E472e	184.1101[b]	Bakery products (bread, rolls, buns), O/W emulsions, coffee whiteners, nondairy creams, sauces
Succinic acid esters of monoglycerides		172.830	Bakery products (bread)
Ethoxylated mono- and diglycerides		172.834	Bakery products (bread)
Salts of fatty acids (Na, K)	E470a	172.863	Co-emulsifiers
Polyglycerol esters of fatty acids	E475	172.854	O/W emulsions, bakery products, dessert products, margarine, spreads, confectionery products
Polyglycerol polyricinoleate	E476		Chocolate, confectionery, cake margarine, low-fat spreads
Propylene glycol esters of fatty acids	E477	172.856	Cakes, dessert products, toppings, shortenings, etc.
Sodium stearoyl lactylate	E482	172.844	Bakery products, O/W emulsions, nondairy creams, coffee whiteners
Calcium stearoyl lactylate	E481	172.846	Bakery products
Sucrose esters of fatty acids	E473	172.859	O/W emulsions, bakery products, dessert products, nondairy creams, toppings, coffee whiteners
Sorbitan monostearate	E491	172.842	O/W emulsions, confectionery products, nondairy creams, toppings
Sorbitan tristearate	E492	GRAS (petition filed and accepted)	Confectionery and chocolate products, margarine, low-fat spreads
Polysorbate 60	E435	172.836	O/W emulsions, ice cream, bakery products
Polysorbate 65	E436	172.838	O/W emulsions, ice cream, bakery products
Polysorbate 80	E433	172.840	O/W emulsions, ice cream, bakery products

[a]US Food and Drug Administration Regulation 21 CFR.

[b]Generally recognized as safe (GRAS). O/W, oil-in-water; W/O, water-in-oil.

Monoacylglycerols can exist in different polymorphic crystal forms (sub-α-, α-, and β-crystals) similar to those of triacylglycerols.

Monoacylglycerols can be separated from di- and triacylglycerols by a process referred to as 'molecular distillation', comprising a thin-film, high-vacuum technique. The total content of monoacylglycerols in distilled products is 93–97%, the content of 1-monoacyl esters being a minimum of 90%. Unlike the mixtures of mono- and di-acylglycerols, distilled monoacylglycerols may be dispersed in water above their Krafft temperature to form mesomorphic, liquid-crystalline phases. This is important in applications where the emulsifier must be added to the aqueous phase in order to interact with carbohydrates or proteins.

Organic acid esters of monoacylglycerols

Organic acid derivatives of monoacylglycerols are important emulsifiers used in many food emulsions. The most commonly used types are lactic, diacetyl tartaric, and citric acid esters, but acetic and succinic acid esters are also commercially available for specific applications.

The physical properties of the organic acid derivatives of monoacylglycerols, such as melting point, crystallization behavior, and polarity, are strongly modified as compared to those of the monoacylglycerols themselves.

The melting point of the derivatives is generally lower than that of the monoacylglycerol, and the crystallization properties have become monomorphic. All organic acid esters of monoacylglycerols are stable in the α-crystal form. In contrast to the nonionic properties of the monoacylglycerols, the diacetyl tartaric, citric, and succinic acid esters are anion-active, and this makes such emulsifiers considerably more hydrophilic than the corresponding monoacylglycerols.

Other fatty acid esters of polyols or lactic acid

Polyol acyl esters commonly used in emulsions are based on polyglycerol, propylene glycol, sorbitan, or sucrose esterified with palmitic and stearic fatty acids.

Glycerol dehydration leads to polyglycerol, which can be esterified with fatty acids, mainly palmitic and stearic acids. The polyglycerol esters can vary in composition both with respect to the degree of polymerization and esterification, but generally such products are more hydrophilic than monoacylglycerols.

Propylene glycol monostearate can be concentrated by molecular distillation so that it contains more than 90% monoacyl esters. Propylene glycol esters are stable in the α-crystal form and are often used in combination with monoacylglycerols in whippable emulsions.

Sorbitan esters exist in various types having varying degrees of esterification. Monoacyl sorbitan esters are used mainly in emulsions, and monoacyl esters may be reacted with ethylene oxide forming polyoxyethylene sorbitan esters (polysorbates), which are highly hydrophilic and water-dispersible emulsifying agents. Triacyl sorbitan esters are oil-soluble, lipophilic compounds with low surface activity and function mainly as crystal modifiers in fat-based products.

Fatty acid esters of lactic acid, in the form of sodium stearoyl lactylates, are anion-active, water-dispersible emulsifiers, which are used in many food products, including dairy emulsions. **Figure 4** shows the chemical structure of some commonly used synthetic emulsifiers.

Hydrocolloids

Hydrocolloids or gums are macromolecular biopolymers often used in emulsions and foams to modify appearance, texture, stability, and taste, on account of their ability to form gels or increase the viscosity of aqueous solutions. Most hydrocolloids are of natural origin and are manufactured by isolation from seaweeds, plant seeds, or cells of bacterial sources, as indicated in **Table 4**.

The function of hydrocolloids in emulsions is generally to modify the viscosity to avoid creaming or sedimentation. Xanthan gum is often used in O/W emulsions to decrease the rate of creaming, but it may increase the rate of creaming by depletion flocculation at very low concentrations, for example, below 0.1%. The inhibition of creaming at higher xanthan concentrations is due to immobilization of the dispersed oil droplets in a weak gel-like network with a high low-stress shear viscosity.

Some amphiphilic hydrocolloids may even adsorb on the surface of protein-stabilized fat globules, thus contributing to the stability against coalescence. **Table 5** gives an overview of the typical applications of hydrocolloids in dairy emulsions. This should not be regarded as a complete list, but shows the main types of hydrocolloids in use.

Applications of Emulsifiers in Dairy Products

Commercial emulsifiers are used in the production of ice cream, milk ice, and so on, or in vegetable fat-based emulsions such as imitation dairy creams, toppings, coffee creams (liquid or powder), and recombined milk manufactured from vegetable fat and skimmed milk powder. The function of emulsifiers in such emulsions is either to extend the shelf life of the emulsion or to improve whippable emulsions with regard to aeration time, foam stability, and creaminess.

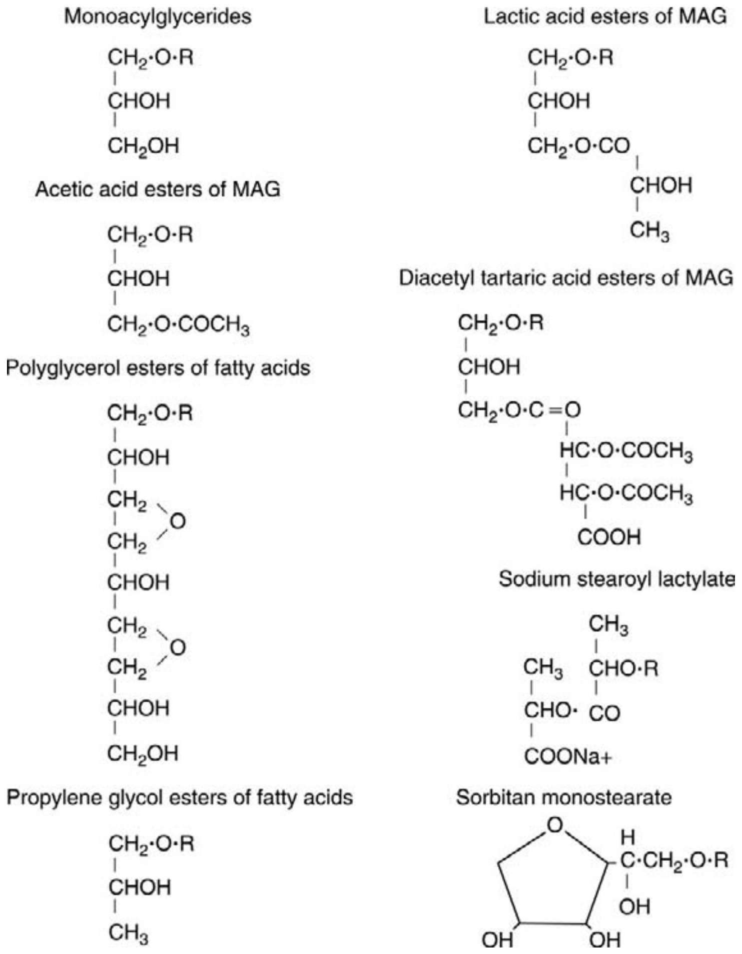
Monoacylglycerides
Acetic acid esters of MAG
Polyglycerol esters of fatty acids
Propylene glycol esters of fatty acids
Lactic acid esters of MAG
Diacetyl tartaric acid esters of MAG
Sodium stearoyl lactylate
Sorbitan monostearate

Figure 4 Chemical structure of food emulsifiers. MAG, monoacylglycerides.

Emulsion Stability

In imitation dairy emulsions based on milk proteins and vegetable fats, emulsifiers improve the shelf life stability. In the case of non-dairy coffee creams, coemulsifiers improve the resistance to 'feathering' in coffee, which is the flocculation of fat globules due to the low pH of coffee. This can be eliminated by using anionic-active emulsifiers, often in combination with monoacylglycerols or lecithins. In recombined milk there is normally enough protein to provide stability, but monoacylglycerols are often added in combination with gums to improve flavor properties (creaminess). The enriched mouthfeel obtained is due to a slight degree of enhancement in fat globule flocculation (self-bodying effect) brought about by the emulsifiers.

Destabilization of Emulsions

Emulsifiers are used as destabilizing agents in whippable dairy emulsions that are to be aerated into a foam, such as ice cream mix, ice milk, and nondairy creams and toppings. The function of emulsifiers in such emulsions is not to provide stability, but to improve whippability, foam stability, and creaminess. This is achieved by controlled destabilization of the fat globules under shear during the whipping process, forming clusters of aggregated fat globules that stabilize the air cell structure.

The destabilization process is initiated by a low temperature (5–10 °C), which promotes fat crystallization and partial protein desorption from the fat globule surface, reducing the interfacial film viscoelasticity, resulting in partial coalescence of fat globules.

The rheological properties of the fat globule surface film play an important role in the physical properties of emulsions. A thick, viscoelastic protein film provides a strong barrier against partial coalescence, whereas a mixed emulsifier–protein film, resulting in reduced protein load at the interface, makes the surface film less viscoelastic and easier to rupture under shear, particularly at low temperatures.

During the aeration of an emulsion, the fat globules lose some of their protective surface film membrane, which increases their hydrophobicity and results in adsorption at the air–serum interface.

Table 4 Properties of hydrocolloids in food systems

	EU no.	*Ionic charge*	*Solubility, cold*	*Solubility, warm*	*Thickening*	*Gelation*	*Thermo reversible gels*	*Shear reversible gels*	*Acid stability*	*Origin*
Gelatin	–	+	–	+	+	+	+	+	+	Animal source
κ carrageenan	E407	+	(–)	+	(+)	K^+ κ-casein +	+	–	–	Seaweeds
ι carrageenan	E407	+	(–)	+	(+)	Ca^{2+}	+	–	–	Seaweed
Sodium alginate	E401	+	+	+	+	Ca^{2+}	–	–	–	Seaweed
Locust bean gum	E410	–	Partial	+	+	–	–	–	+	Plant seeds
Guar gum	E412	–	+	+	+	–	–	–	+	Plant seeds
HE pectin	E440	+	+	+	(+)	Sugar acid	–	–	+	Plant cells
LC pectin	E440	+	(+)	+	+	Ca^{2+}	–	(+)	+	Plant cells
LA pectin	E440	+	(+)	+	(+)	Ca^{2+}	+	(+)	+	Plant cells
Xanthan gum		+	+	+	+	–	–	(+)	+	Bacterial source
Carboxymethyl cellulose	E466	+	+	+	+	–	–	–	Partial	Synthetic

HE, high-ester; LA, low-ester, amidated; LC, low-ester, conventional.

Table 5 Applications of hydrocolloids in dairy emulsions

Dairy emulsions	*Gelatine*	*Carrageenan*	*Alginate*	*Pectin*	*Guar gum*	*Locust bean gum*	*Xanthan gum*	*Carboxymethyl cellulose*	*Microcrystalline cellulose*
Ice cream, etc.	−	+	+	+	+	+	+	+	+
Sorbet, sherbet	+	−	−	+	+	+	+	+	−
Gelled milk desserts	+	+	+	+	+	+	+	+	+
Mousse	+	+	+	+	+	+	−	−	−
Creams, dairy/ vegetable	−	+	+	−	−	−	+	+	+
Recombined milk	−	+	+	−	+	−	−	−	−
Chocolate milk	−	+	−	−	+	−	−	+	+
Yogurt drinks	−	−	−	+	+	−	−	+	+
Yogurt	+	−	−	+	−	−	−	−	−
Sour cream	+	+	−	−	+	+	−	−	−
Cream cheeses	+	+	+	−	−	+	−	−	−

Table 6 Applications of emulsifiers in dairy emulsions

Type of emulsion	*Type of co-emulsifiers*
Recombined milk	MAG
Coffee cream (liquid or powdered)	MAG, DATEM, SSL, sorbitan esters, polysorbates, sucrose esters
Milk desserts	MAG, SSL
Ice cream, etc.	MAG, polysorbates, sucrose esters
Mousse	MAG, LACTEM
Whipping cream (liquid or powdered)	MAG, LACTEM, PGMS, lecithin, polysorbates, SSL, sucrose esters, polyglycerol esters
Sour cream, yogurt	MAG
Recombined butter, cheese	MAG

DATEM, diacetyl tartaric esters of MAG; SSL, sodium stearoyl lactylate; LACTEM, lactic acid esters of MAG; MAG, monoacylglycerols; PGMS, propylene glycol esters of fatty acids.

In aerated emulsions (ice cream, whipped cream, toppings, etc.), the air cells are covered with clusters of partially coalesced fat globules and adsorbed fat crystals together with proteins, stabilizing the foam. The melting stability and creaminess of ice cream are related both to the amount of fat globules adsorbed around the air cells and to the formation of clusters of fat globules between air cells, linking them together in a structural matrix.

Table 6 shows the type of emulsifiers commonly used in dairy emulsions. Often two or more different emulsifiers are used in combination to obtain optimal results. The types of emulsifiers shown are not to be considered a complete list, as other emulsifiers not mentioned may also be applicable.

The types of emulsifiers used in vegetable fat-based creams or toppings include monoacylglycerols together with their lactic acid esters or propylene glycol esters of palmitic/stearic acid blends. In liquid imitation dairy creams the formulation requires a careful balance between proteins and emulsifiers providing stability against creaming and flocculation during transport and storage and, at the same time, inducing destabilization under shear when aerated to a foam. Therefore, lecithin or anionic emulsifiers are often used in combination with low-polar emulsifiers. It should be noted that the destabilizing effect of unsaturated monoacylglycerols (e.g., glycerolmonoolein) is considerably stronger than that of saturated monoacylglycerols (e.g., glycerolmonostearin).

The choice of emulsifier for a specific food emulsion is often governed by the local food laws combined with consumer preferences for texture, mouthfeel, or appearance. Quality standards for foods (e.g., ice cream) vary from country to country and thus create a need by the manufacturer to meet these requirements by different formulations, including the type of emulsifiers.

Among the wide range of emulsifiers available to the food industry, many different types may be used for a given application with equally good results. However, some reformulation will often be necessary when changing from one type of emulsifier to another.

See also: **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Product Types. **Imitation Dairy Products**. **Liquid Milk Products**: Recombined and Reconstituted Products. **Milk Lipids**: Milk Fat Globule Membrane; Phospholipids.

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ANALYTICAL METHODS

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Sampling

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Introduction

The sampling of any dairy food must be done by qualified and trained technicians. All necessary paperwork must be completed correctly, dated, and signed so that one copy can accompany the sample. In most situations, duplicate or triplicate samples are collected. The extra samples are stored correctly in a locked room within the plant in the event of legal or disputed results.

Whatever container is used to store a sample of dairy food must be inert relative to potential interaction with the product. Glass, plastic, and stainless steel make suitable sample containers if the lid can be tightly sealed. If there is any possibility that samples may be the subject of litigation, then paperwork and validated traceability and security for samples are legally mandated.

Sample size is also important. Generally, unless requested to take an additional portion of a particular dairy food, the sample size should be 10–20 times that needed for analysis.

Liquid Dairy Foods

Liquid dairy foods include all varieties of milk, cream, whey, and fermented and condensed milks. Similarly, liquid dairy foods are held in a wide variety of containers from farm tanks to individual milk cans to tank trucks. Moreover, it may not be possible to agitate the contents of the milk tank to achieve an acceptable degree of analytical homogeneity between the contents and the sample. Some samples, for example, from farm tanks, must be taken only by a certified sampler because such samples will be used as the basis of payment for the raw milk. Other samples, for example, cream for butter manufacture, often involve only the shipper and the receiver.

Liquid dairy foods can be sampled using one of several devices. Following sufficient agitation of the tank contents, a sanitary dipper is adequate to remove the product. For tank trucks, where no agitation is available, a proportional sampler is used to take small samples at set time intervals during pump-out. This device is particularly necessary where the tank truck has not been moving and stratification has occurred. The size of the sample should not vary with head pressure on the sampler. Agitators mounted in the central manhole of tank trucks are insufficient to ensure adequate mixing, and samples taken from such tanks should not be relied on for uniform composition. The third type of sampler is a straight stainless-steel tube that reaches to the bottom of the container. This core sample is often taken from tank trucks where no automatic sampler is available or where agitation is incomplete or nonexistent.

Pasteurized dairy foods in retail containers should not be opened prior to sampling. Containers selected at the start, middle, and end of processing of a single lot of product represent the sample. Sample quantity can be reduced for short process runs; however, the first container off the line is usually indicative of the remainder of the product.

Samples should be stored under refrigeration in the dark. For storage of more than a few days, the addition of a preservative is necessary, but this must not interfere with subsequent analysis. When a preservative is used, it must be indicated on paperwork or a label associated with the sample.

Canned Dairy Foods

Retail containers can be selected randomly and form a sample when unopened. Larger containers and drums can be sampled using a dipper following manual agitation to create uniform composition. A stainless-steel tube going to the bottom of the container can be used if there is minimal adhesion of the product to the exterior of the tube. This core sample will be representative of the container, and the entire core sample must be placed in the sample container. In the case of high-viscosity products such as sweetened condensed milk, manual or mechanical agitation, followed by collection of a sample with a dipper, is practiced. Before sampling, it is important to ascertain, by scraping the walls during mixing, that no product has adhered to the container walls.

Frozen Dairy Foods

Small containers of frozen dairy foods up to 2 l may be taken as a sample. More than one container may be taken if long process runs are the practice at the plant. Stop, start, and flavor changeover are points where cautious sampling should be practiced. The samples taken must be representative of the whole production lot.

From larger containers, a frozen core is taken using a cylindrical device with a sawtooth edge powered by an electric drill. The sample container must encompass the entire sample. Depending on the weight of the cylinder of product, more than one core may be needed to constitute a representative sample. Such frozen dairy foods contain air, if labeled frozen desserts, and hence more than one core may be needed.

The samples should be stored frozen until preparing for analysis.

Butter and Similar Products

When collecting samples of butter and similar products for subsequent analysis, the sample must represent the churning; often it is difficult on a time basis to separate churnings, particularly during the change-over from one silo of cream to another or from unsalted to salted butter using a continuous churn. In such cases, it is important to collect before, during, and after the changeover so that the analyst gets a true picture of the whole product. With batch churns, one churning is a lot, regardless of the amount of cream in a silo.

In butter production plants, at least 0.5 kg from each lot of cream churned should be collected. If a continuous churn is used, it is necessary to collect a sample during change-over of silos on a time-period basis. If 25-kg boxes are being filled, then the sample should be collected using a trier with a blade length of 30.5 cm. The entire sample less the outer 2.5 cm of butter is the sample and must fit into a sample container. In sampling from larger amounts of butter and similar products, one sample should be collected from each churning up to 25 000 kg, two samples from each churning of 25 000–50 000 kg, and four samples from each churning of 50 000–150 000 kg. All samples must be clearly marked showing the amount of product represented by the sample and its lot number.

All samples should be refrigerated until preparing for analysis.

Milk and Whey Powders

Samples collected from bags or totes (portable bins) of milk or whey powders are taken using a ‘powder thief’. This is a tube within a tube, close-fitting, with numerous side openings on the inner and outer tubes to allow for inflow of product. The thief must reach near the bottom of the bag or tote. The thief is inserted in the closed position, the tubes opened, gently shaken, closed, and withdrawn. This core sample will represent the product from top to bottom of the bag or tote. The entire sample is placed into a container and stored refrigerated.

To collect samples from large lots of dried products, the sampling plan given for large lots of butter should be used: one sample from one lot up to 25 000 kg, two samples from one lot of 25 000–50 000 kg, and four samples from one lot of 50 000–150 000 kg. All samples must be clearly marked, showing the volume represented by the sample.

Cheese and Cheese-Like Products

In sampling cheese and cheese-like products, the sample taken often depends on the size and shape of the cheese. Sharp knives or triers with blades 14.6 or 30.5 cm in length are used to take samples. Wheels or balls of cheese are either cut to isolate a wedge representing the product from the outside to the center or sampled using a trier of suitable length to produce a core from the outside to the center. The trier is inserted either perpendicularly to one face and passed through the center to the other face or obliquely toward the center or horizontally in the middle of the block. The outer 2 cm of the plug is returned to fill the hole left by sample removal.

Sampling from random weights or uniform weights of cheese cut from larger blocks involves selecting one piece of 0.5 kg maximum weight at random. However, the sample-taker must be aware that cheese classically lacks analytical uniformity from outside to the center, for example, due to rind formation or brine salting. Temperature is a factor in moisture migration during storage. Moisture always migrates to a colder temperature until temperature equilibrium is reached; then the migration stops and reverses until steady-state conditions are reached. This is true even in salt-brined cheeses as long as salt is at equilibrium across the cheese.

Plugs or wedges of cheese can be held in individual storage containers or wrapped in aluminum foil before placing in a container. The foil reduces migration of moisture from the sample to the environment of the container. Small pieces of cheese weighing 0.5 kg or less constitute a sample. If wrapped or waxed, the necessary paperwork should be attached to each piece of a sample.

Cottage cheese is sampled by taking one or two containers representing each lot or vat. Cheese in salt brine is sampled in a similar fashion: one or two containers or one or two small cheeses per lot or vat of cheese are retained as a sample.

Sampling Dairy Foods for Microbiological Analysis

Some precautions must be followed when a sample is to be evaluated for its microbiology. The first sample collected should be used for microbiological evaluation. All equipment and the container must be sterile. With some products, the sample must be collected subsurface. For example, powders, cheese, and butter may require a subsurface sample. Using a sterile scoop, the sampler should move powder from the surface and then collect the sample using a ‘thief scoop’ or tubular sampler. With butter and cheese, the sampler should remove a core using a sterile trier, and then with a sterile spatula or knife remove the upper 1–2 cm and return it to the hole in the block of product. Wedges of cheese can be trimmed from the surface using a sterile knife. In this instance, the amount removed is a piece 1–2 cm thick.

However, in the event that the surface of any product may harbor microbes of interest, no such surface removal is practiced. A rule to follow is that when sampling any product for subsequent microbiological analysis, the sampling procedure must not influence the result.

Samples collected for microbiological analysis must be appropriately labeled and stored in an environment that will not alter the sample or its microflora. Subsequent analysis must be performed as soon after sampling as possible. Sampling date, storage and shipping conditions, and analysis date must be indicated on the final report form.

Samples for Sensory Evaluation

When a sample is needed for sensory consideration, it should be as fresh as possible. Even core samples of cheese or butter when wrapped in aluminum foil lose flavor intensity and quality over time. The sample collected should be large enough to minimize changes in flavor, body and texture, and appearance. Furthermore, the sample must be stored to minimize changes in flavor, body and texture, and appearance.

Samples for sensory analysis are collected when needed. Generally, when a sample is collected for analytical assay, a duplicate sample could be used for sensory evaluation. Thus, dairy liquids, frozen desserts, high-moisture cheeses, and powders present little difficulty in that consumer-sized packages can be used or products placed in a sterile container. Cheese and butter pose potential problems in that the attributes of small pieces will change over time and will not represent the original lot of product. Samples should be evaluated immediately for best results.

Further Reading

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Proximate and Other Chemical Analyses

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Introduction

Compositional analysis of dairy products is of interest for a number of reasons: the requirement for accurate nutritional labeling; quality assurance in processing; ensuring compliance with any legal compositional requirements; and payment of primary producers. The aim of this article is to give the reader an overview of the various methods that may be applied to the compositional analysis of dairy products. The focus will be on the traditional wet-chemical methods used for the determination of the various macroconstituents, for example, solids/moisture, ash, protein, fat, and lactose – often referred to as proximate analysis. While many of these methods have been largely superseded by instrumental analyses, they remain reliable methods of long standing and in many cases are the standard reference methods used for the calibration of automated analytical methods (*see* **Analytical Methods**: Infrared Spectroscopy in Dairy Analysis).

Moisture and Total Solids

The moisture content of a dairy product is of interest as it is inversely related to the total solids content and thus the nutritive and economic value. In addition, the level of moisture is often an important determinant of the microbial and chemical stability of products such as cheese and milk powders. A broad range of direct and indirect methods has been developed for the determination of moisture content in foods, a number of which may be applied to moisture determination in dairy products.

Conventional Oven Drying

The simplest method for the estimation of moisture is by direct gravimetric determination following oven drying at ambient pressure and a temperature of ~100 °C (102 ± 2 °C is generally recommended). The sample size may vary depending on the product (2–5 g) but should be such as to allow accurate weighing, particularly of the dried residue. The weighed sample is placed in a static or forced air circulation oven and dried to constant weight, which typically takes ~4 h. The dried sample is then cooled in a desiccator and weighed, and the loss in weight on drying is interpreted as the moisture content (MC).

$$\text{MC}\ (\%,\ \text{w/w}) = \frac{\text{Weight loss on drying}}{\text{Sample weight}} \times 100$$

Samples with high moisture levels, such as liquid milk, may boil over in the oven and it is recommended that they be subjected to a pre-evaporation step over a steam bath for ~30 min prior to being placed in the oven. While oven drying is simple and requires relatively unsophisticated equipment, it suffers from certain disadvantages, such as a long analysis time and inadequate sensitivity for low-moisture products. In addition, products with high carbohydrate content, such as sweetened condensed milk (SCM) and whey powders, can undergo chemical decomposition – for example, caramelization or Maillard reactions – during drying; this is usually reflected in a continuous loss of weight and sample discoloration during heating. In these instances, it may be preferable to conduct the moisture determination in a vacuum oven, which allows evaporation of moisture from the sample at temperatures in the range 65–80 °C, thus avoiding the problems encountered at higher temperatures. To address the long analysis time, a number of rapid moisture analyzers have been developed, which comprise a high-temperature infrared or microwave heating system with an integrated accurate balance. These give analysis times of minutes rather than hours but are not suitable for use with products that are subject to thermal decomposition.

Moisture is routinely determined in butter for quality control purposes by heating a weighed sample in a metal cup over a hotplate until foaming ceases; the sample is then cooled and the weight loss expressed as the moisture content.

Karl Fischer Titration

The Karl Fischer method is based on the reduction of iodine by sulfur dioxide in the presence of water:

$$2H_2O + SO_2 + I_2 \rightarrow H_2SO_4 + 2HI$$

In its simplest form, the sample is dispersed in anhydrous methanol and is subsequently titrated with the Karl Fischer reagent, comprising iodine, SO_2, and an organic base such as pyridine, imidazole, or diethanolamine dissolved in methanol. The SO_2 and iodine react, consuming the water in the sample; the end point is signaled by the appearance of free-iodine brown colors. For the most part, the Karl Fischer method is performed using specially designed automatic titrating systems in which the iodine is generated electrochemically and the end point is detected amperometrically. The main advantage of the Karl Fischer method is that it is more sensitive than most other methods of moisture determination and can be used to quantify water at the level of mg kg^{-1}. Its main application is for very-low-moisture products such as dried products, tablets, and purified oils and fats.

Densitometric Methods

The total solids content of milk may also be estimated using densitometric methods; indeed a specific densitometer (lactometer) is available that covers the range of specific gravity (sp.gr 1.025–1.035) expected for milk samples. The specific gravity of milk depends on the levels of the major constituents – milk non-fat solids (sp.gr. 1.614), butterfat (sp.gr. 0.93), and water. The specific gravity measurement is made by floating the lactometer in a milk sample at 20 °C and reading the specific gravity directly from the scale. If the fat content of the sample is determined separately, then the total solids (TS) may be calculated from the following empirical formula:

$$\mathrm{TS}\ (\%,\ \mathrm{w/w}) = 0.25D + 1.22F + 0.72$$

where D is the lactometer reading and F the fat content (%, w/w).

Densitometers are also used for in-line monitoring of total solids and fat content during processing operations such as separation or evaporation.

Cryoscopic Methods

While not considered a method for moisture determination *per se*, cryoscopic methods may be used to detect the adulteration of milk by addition of water. The general principle is that the aqueous phase of milk is a solution and the solutes that it contains depress the freezing point (by ~0.54 °C) relative to that of the solvent (water). The addition of water to milk dilutes the solutes and thus reduces the depression of the freezing point, which increases toward that of the solvent with increasing level of dilution. Typically, milk of normal composition has a freezing point of −0.55 to −0.53 °C and samples having a freezing point depression less than 0.53 °C may be considered to have been adulterated with water. The level of added water can be calculated as follows:

$$\text{Added water}\ (\%,\ \mathrm{w/w}) = \left(\frac{\mathrm{FP_{av}} - \mathrm{FP_s}}{\mathrm{FP_{av}}}\right) \times (100 - \mathrm{TS})$$

where $\mathrm{FP_{av}}$ is the average freezing point depression observed for unadulterated milks (0.545), $\mathrm{FP_s}$ the freezing point depression of the sample, and TS the total solids (%, w/w).

Water Activity

The water activity (a_w) of a food is a measure of the availability of the water that it contains and thus can often be more informative about its likely stability than the moisture content *per se*. Water activity may be defined as follows:

$$a_\mathrm{w} = \frac{P}{P_0}$$

where P is the vapor pressure of the food and P_0 the vapor pressure of pure water.

A number of devices that allow 'direct' determination of a_w are commercially available. Most involve equilibration of the food sample within a sealed chamber at a specified temperature, and subsequent measurement of the equilibrium vapor pressure in the headspace using a range of techniques – for example, capacitance, dew point measurement, or even hair hygrometers. In other instances, it may be more instructive to explore the relationship between moisture content and a_w more fully, and this most commonly involves constructing the moisture sorption isotherm (MSI) for the food. Typically, weighed samples are chemically dehydrated – for example, over P_2O_5 – and are subsequently equilibrated to a range of a_w values – usually in sealed containers containing various saturated salt solutions – at a specified temperature. The increase in the weight of the samples is expressed as moisture content (on a dry basis) and plotted as a function of a_w. The moisture content increases with increasing a_w but not in a linear fashion and the shape of the MSI for a product can yield useful information about its likely stability over a range of storage conditions (*see* **Water in Dairy Products**: Analysis and Measurement of Water Activity).

Ash Content

Dry Ashing

The ash content of a food is literally the residue that remains once the organic constituents have been burned off. What remain are the oxides, phosphates, and sulfates of the various elements present, which in turn reflects the salts in the sample, although it should be remembered that organic salts such as citrates are destroyed by the ashing process. The determination usually involves accurately weighing a sample (2–10 g) into a silica crucible and heating in a muffle furnace, typically at 525 ± 25 °C. Heating is continued until the ash is a uniformly white or light gray in color, which typically takes 3–6 h. The crucible is then cooled and the weight of the ash residue is determined. To avoid samples with a high organic content bursting into flames, they must first be slowly carbonized or charred over a low flame until smoking ceases, prior to placing in the furnace. In addition, liquid samples such as milk must first be evaporated to dryness on a steam bath prior to charring.

Wet Ashing

Where specific mineral constituents are to be determined, it is often desirable to remove the organic matter by wet rather than dry ashing. This involves heating the sample in the presence of a strongly oxidizing mixture of acids such as H_2SO_4, HNO_3, and/or $HClO_4$. Heating is continued until a clear digest is obtained, which may then be suitably diluted and the specific analyte of interest determined by appropriate techniques (*see* **Analytical Methods**: Atomic Spectrometric Techniques).

Protein and Nitrogen Determination

A variety of methods may be used to determine the protein content of dairy products. Some measure protein directly, for example, colorimetric determination of peptide bonds or amino groups, whereas others involve calculation of protein content based on the determination of nitrogen in the sample. For clean solutions of pure proteins, direct methods are usually preferred because of their precision and rapidity. However, most dairy products are neither clean, pure (in terms of their protein content), nor in many cases soluble and so indirect methods tend to be used. Again it should be remembered that only wet-chemical methods of analysis are described in this article, and that automated rapid determination of protein in the dairy industry is routinely performed by IR spectroscopy (*see* **Analytical Methods**: Infrared Spectroscopy in Dairy Analysis).

Kjeldahl Method

The 125-year-old Kjeldahl method for the determination of organic nitrogen has long been the international reference method for the determination of the protein content of dairy products. Although it involves a long analysis time and the use of hazardous reagents at high temperatures, the method is extremely reliable and precise, which is probably why it has stood the test of time so well.

In the procedure, a weighed sample of the food is heated together with concentrated H_2SO_4 in the presence of K_2SO_4 and a Se, Hg, or Cu catalyst in a heat-resistant tube at a temperature of ~400 °C. This step is essentially a wet oxidation of the sample, and the organic constituents are initially carbonized as evidenced by the appearance of a dirty black color. Continued heating results in complete oxidation to CO_2 and the digest becomes clear. This digestion step converts sample N into a soluble nonvolatile form, $(NH_4)_2SO_4$. The clear digest is cooled, suitably diluted, and made alkaline by the addition of an excess of strong base (NaOH), which releases the N in a quantifiable form as free NH_3. The neutralized digest is then subjected to steam distillation, which separates the volatile NH_3 from the other constituents, and the condensed NH_3 is trapped in dilute boric acid. The final step involves quantification of the trapped NH_3 by titration with a standard acid. The amount of acid used in titration can easily be related to the N content of the sample:

$$\mathrm{N}\,(\%,\ \mathrm{w/w}) = \frac{\text{Volume of acid (ml)} \times \text{molarity of acid } (\mathrm{mol\,l^{-1}}) \times 14\,(\mathrm{g\,mol^{-1}})}{\text{Weight of sample (g)} \times 1000} \times 100$$

To obtain the protein content of a food, the N content must be multiplied by a factor that reflects the percentage of N in the sample protein. Dairy proteins contain an average of 15.67% N, giving a correction factor of 6.38 (100/15.65); thus,

$$\text{Protein}\,(\%,\ \mathrm{w/w}) = \mathrm{N}\ (\%,\ \mathrm{w/w}) \times 6.38$$

For samples where the exact protein profile is not known, a general conversion factor of 6.25 may be applied. As the Kjeldahl method determines N rather than protein *per se*, the protein content so determined is termed the crude protein value. The main source of inaccuracy in the method arises from the contribution of non-protein nitrogenous species, for example, urea, nucleic acids, and organic bases, to the N value obtained; for most dairy products – with the exception of products that have undergone extensive proteolysis, such as ripened cheese – this error is relatively small.

Dumas Method

The Dumas method is even older (1831) than the Kjeldahl method. It is based on N determination by combustion analysis, but in its earlier forms was a difficult and imprecise method and so remained relatively little used. However, in recent decades, instruments that allow accurate and rapid protein determination by this method have been devised and, the best known is the Leco N analyser. A small sample of food (typically <0.5 g) is sealed in a foil container and burned at ~800 °C in a nitrogen-free oxygen atmosphere. The combustion gases are collected in a ballast tank and a sample aliquot is carried in a He stream through a series of traps and scrubbers that remove H_2O vapor and CO_2. All that remains in the carrier gas is N_2 and various oxides of N; the final step is passage of the gas through a heated (~400 °C) column containing metallic Cu, which reduces the N oxides to elemental N_2. The N_2 in the He carrier stream is then determined by a thermal conductivity measurement. Again, this is a crude protein determination, and the protein content is calculated from the N content using a suitable conversion factor. The main advantages of the Dumas method over the Kjeldahl are (1) an analysis time of less than 5 min per sample in the modern analyzers and (2) the absence of any requirement for hazardous reagents and associated waste disposal protocols. While it is extremely useful for dried samples and those containing relatively high N levels, it is less suitable for more dilute samples, especially liquid samples that may require careful pre-evaporation prior to combustion. In addition, the small sample size may make representative sampling of heterogeneous and particulate foods difficult.

Dye Binding

Dye-binding methods are based on the principle that certain strongly anionic dyes, for example, amido black, form insoluble complexes with proteins at low pH (<2.5). Under these conditions, the protein is typically below its isoionic pH and so is positively charged whereas the dye is negatively charged, resulting in the formation of an insoluble dye–protein complex. Techniques for protein estimation by this method involve the addition of an excess of the dye to the sample under acidic conditions, followed by a filtration step to remove the insoluble dye–protein precipitate. The filtrate contains the unbound dye, the concentration of which is inversely related to the protein content and may easily be measured using a spectrophotometer. Such methods are highly empirical and require the construction of a calibration curve and strict adherence to the assay conditions, and are limited to cases where the protein is in a soluble form. However, for routine analysis of liquid samples, for example, in a process control laboratory, these methods offer a relatively rapid method of analysis – certainly relative to the Kjeldahl. The Pro-Milk Tester®, a semiautomated instrument for determination of protein in milk by dye binding, has been available for many years.

Fractionation of Milk Protein or N

The separation of the nitrogenous constituents of milk into the casein N, the non-casein N, and the non-protein N fractions is well known (**Figure 1**) and quantification of the distribution of N between these fractions is of significant interest to the dairy industry.

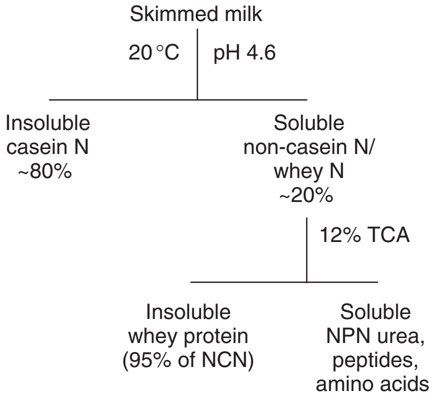
Skimmed milk
20 °C
pH 4.6
Insoluble casein N ~80%
Soluble non-casein N/ whey N ~20%
12% TCA
Insoluble whey protein (95% of NCN)
Soluble NPN urea, peptides, amino acids

Figure 1 Approximate distribution of milk N between various fractions.

Most methods involve an initial determination of total milk N by Kjeldahl. The casein in another sample is precipitated at pH 4.6 by the addition of a sodium acetate/acetic acid buffer. Subsequent filtration allows separation of the soluble non-casein N fraction, which is also determined by Kjeldahl. If desired, an aliquot of the soluble noncasein or whey fraction can be mixed with an equal volume of 24% (w/v) trichloroacetic acid (TCA), which will precipitate the whey proteins, after which the soluble non-protein N fraction may be separated by filtration and quantified by Kjeldahl.

Fat

Historically, fat has been considered the most valuable of the major milk constituents, in no small part because of the importance of butter manufacture and trading to many countries. The fat or lipid content of a food may be defined in analytical terms as that portion of the food that is soluble in low-polarity solvents. Given such a broad definition it is not surprising that the fat fraction contains many different classes of compounds, for example, triglycerides, fatty acids, phospholipids, sterols, and hydrocarbons. It should be remembered that qualitative differences will occur between extracts obtained using solvents of different polarities, and while this is not so significant in general compositional analysis, it is an important consideration when preparing extracts for further, more detailed characterization, for example, fatty acid or vitamin analysis. In the case of milk products, neutral triglycerides and associated species – free fatty acids, monoglycerides, and diglycerides – account for ~99% (w/w) of the lipid fraction, with most of the remainder accounted for by the phospholipids. While milk fat contains many other lipid species that are nutritionally and functionally important, they are quantitatively insignificant. A range of methods has been developed for the determination of fat in milk and dairy products, including various spectroscopic methods discussed elsewhere in this work (*see* **Analytical Methods**: Infrared Spectroscopy in Dairy Analysis; Nuclear Magnetic Resonance: Principles). The most widely used wet-chemical methods involve gravimetric determination of solvent-extracted fat, or volumetric determination of centrifugally separated fat.

Gravimetric Solvent Extraction Methods

Direct admixture of solvents such as diethyl ether or petroleum spirit with dairy products extracts only a small portion of the fat. This is because most of the fat is inaccessible to the solvent, either because it is in an emulsified form, for example, milk, cream, or ice cream, or because it is trapped within a dense protein matrix, for example, cheese or casein. In order to quantitatively extract the fat from such products, it must first be liberated from the sample matrix.

The international reference method for determination of fat in milk is the Rose–Gottlieb method, which involves gravimetric determination of solvent-extracted fat liberated from the sample matrix. A sample of milk (~10 g) is weighed into a specially designed extraction bottle such as a Mojonnier flask (**Figure 2**) and mixed with 1 ml of concentrated ammonia, which solubilizes the milk protein and aids in destabilizing the fat globules. Ethanol (10 ml) is then added and mixed to complete release of the fat from the globules. The liberated fat may then be recovered by extraction with a mixture of diethyl ether and petroleum spirit. After standing, the solvent layer containing the fat is decanted into a round-bottom flask; the extraction is repeated twice to ensure complete extraction of the fat. The solvent is then evaporated from the pooled extract and the weight of the fatty residue determined; this may be expressed as a percentage of the sample weight. This method may also be used for cream, ice cream, and most milk powders.

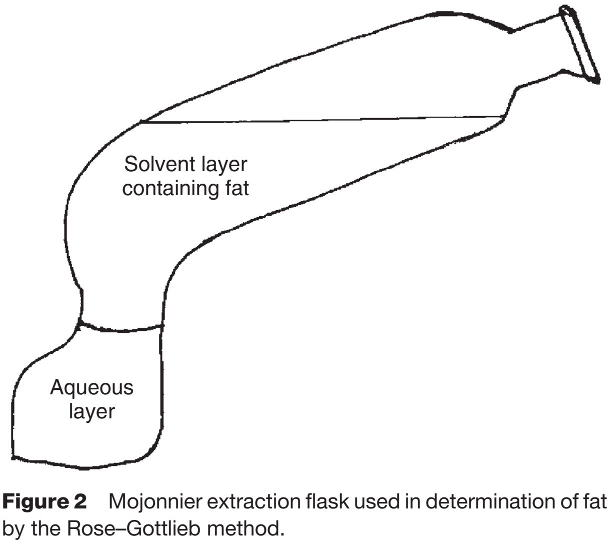

Figure 2 Mojonnier extraction flask used in determination of fat by the Rose–Gottlieb method.

To liberate the fat from dense protein matrices such as cheese or casein, a more aggressive approach must be used. One such approach is the Schmidt–Bondzynski–Ratzlaff method, in which the cheese or casein sample is first heated with $6\,\mathrm{mol\,l^{-1}}$ HCl until fully dissolved; the digest is then cooled and mixed with ethanol, and the liberated fat extracted and determined as per the Rose–Gottlieb method. For special applications, a number of variants have been developed, such as the Weibull–Berntrop method for products with high nondairy content and the Evers method for butter and spreads.

Volumetric Methods

Alternatives to the gravimetric approach of the above methods are the volumetric methods: the Gerber, which has currency in Europe, and the Babcock, which is used in the United States. In the Gerber method, a prescribed sample weight or volume is mixed with concentrated H_2SO_4 and amyl alcohol in a specially designed sample tube called a butyrometer. The heat of solution and the corrosive nature of the H_2SO_4 result in the digestion of the sample matrix and liberation of the liquid fat. The samples are then centrifuged and the less-dense fat phase rises into the calibrated neck of the butyrometer where the fat percentage may be read directly, following equilibration of the sample at 65 °C. A range of butyrometer types has been designed for specific dairy products and sample sizes (**Table 1**). The method is relatively rapid but accuracy is dependent on strict adherence to standard protocol, particularly with respect to the sample size, the butyrometer type, and the temperature at which the butyrometer scale is read (65 °C).

The Babcock method uses a larger sample size (e.g., 18 g of milk) and a larger sample bottle of different design, and the amyl alcohol is omitted; however, the basic principle of the method is the same.

Lactose

Lactose is the principal carbohydrate in all milks and the only one that is of quantitative significance in most dairy products. In proximate analyses, carbohydrate was traditionally determined by difference, that is,

$$\text{Carbohydrate (\%)} = 100 - (\text{fat (\%)} + \text{protein (\%)} + \text{moisture (\%)} + \text{ash (\%)})$$

However, this approach can lead to inaccuracies, for example, in the case of milk, where a relatively minor error in moisture determination can lead to a far more significant error in estimated lactose content.

Table 1 Sample size and butyrometer fat percentage scale for determination of fat in different dairy products by the Gerber method

Product	*Sample size*	*Butyrometer scale (%)*
Milk	10.94 ml	0–7
Skimmed milk or whey	10.94 ml	0–1
Cream	5 g	0–70
Cheese	3 g	0–40

Because of the requirement for more accurate compositional analysis in the modern food and dairy industries, it is now more common to determine carbohydrates, including lactose, directly. Approaches to the direct determination of lactose exploit the fact that it is a reducing sugar or that it is optically active; it may also be determined enzymatically. A number of spectroscopic and chromatographic methods may also be used, and are discussed elsewhere (*see* **Analytical Methods**: Chromatographic Methods; Infrared Spectroscopy in Dairy Analysis).

Polarimeter Method

Solutions of lactose rotate the plane of polarization of plane-polarized light. An equilibrium mixture of the α- and β-anomeric forms has a specific rotation (α_{D}^{20}) – that is, the angular rotation of a 1 g ml^{-1} solution, using the D-line of the Na spectrum at 20 °C – of +55.4°. In a clear solution of lactose, the concentration may readily be calculated from the observed angular rotation (α) of the sample:

$$\text{Lactose }(\%,\ \text{w/v}) = \frac{100\alpha}{\alpha_{\mathrm{D}}^{20} L}$$

where L is the light path in decimeters.

For polarimetric determination of lactose in milk, the sample must first be clarified by precipitation of protein and fat, for example, by addition of Wiley's or Carrez reagent. The rotation of the clarified sample is then determined in the polarimeter and the lactose content calculated taking account of the volume of fat and protein removed by clarification.

A modification of this method – the so-called Clerget inversion – may also be used to determine the sucrose level in Sweetened Condensed milk (SCM). Briefly, an initial polarimeter reading is taken on a clarified SCM sample, the rotation reflecting the lactose and sucrose levels. A parallel sample is subjected to an acidification step that hydrolyzes (inverts) the sucrose to glucose and fructose, leaving the lactose unchanged. The rotation of the inverted sample is then determined and the change in rotation occasioned by inversion – due to the levorotatory nature of fructose – can be related to the sucrose content.

Chloramine-T Method

The Chloramine-T method for lactose determination exploits the reducing properties of the sugar and is an internationally recognized method for lactose determination in milk. A deproteinized sample of milk is mixed with Chloramine-T and KI, and a series of reactions generate KIO within the sample, which reacts with the lactose. Acidification of the sample liberates I_2 from the unreacted KIO and allows its estimation by titration with $Na_2S_2O_3$ using a starch indicator. A blank determination is similarly performed, and the difference between the blank and sample titers represents the amount of KIO required to react with the lactose in the sample.

Enzymatic Methods

A number of kits that allow the determination of lactose by enzymatic methods are available. Among the advantages of this approach is its specificity, which allows the determination of lactose in the presence of interfering substances, including other sugars, for example, galactose in fermented milk products. Most kits involve two enzymatic steps, the first of which hydrolyzes lactose into galactose and glucose using β-galactosidase. The amount of galactose is then determined in a NAD/NADH$_2$-coupled spectrophotometric assay using the second enzyme, galactose dehydrogenase:

$$1.\ \text{Lactose} \xrightarrow{\beta\text{-galactosidase}} \text{glucose} + \text{galactose}$$

$$2.\ \text{Galactose} + \text{NAD} \xrightarrow{\text{galactose dehydrogenase}} \text{galactonic acid} + \text{NADH}_2$$

The amount of NAD reduced is proportional to the amount of galactose present, which in turn reflects the amount of both lactose and galactose in the original sample. Omitting the first step allows the determination of the galactose alone, and the difference between the two determinations gives the lactose content.

Titratable Acidity

The titratable acidity of milk is often determined as a rapid and simple quality (and, on occasion, rejection) test. Briefly, a sample of milk (yogurt or whey) is titrated to the phenolphthalein end point (faint pink color) using a standard solution of a strong base such as NaOH. Since lactic acid is the acid associated with milk souring, it has been traditional to titrate a 10 ml sample aliquot using 0.11 mol l^{-1} NaOH, as under these conditions 1.0 ml of 0.11 mol l^{-1} NaOH ≡ 0.1 g lactic acid per 100 ml milk.

Table 2 Summary of methods commonly used for the compositional analysis of milk and dairy products

Constituent	*Method*	*Principle*	*Reference(s)*
Fat	Rose–Gottlieb	Gravimetric determination of ether-extracted fat following emulsion disruption by NH_4OH and EtOH	International Dairy Federation standard method. FIL-IDF standard 1D (1996)
	Gerber	Volumetric determination of fat in calibrated butyrometer tube following emulsion disruption with H_2SO_4 and amyl alcohol	Irish standard method IS 69 (1955). British standard method BS 11870 (2009)
Protein	Kjeldahl	Digestion of sample in H_2SO_4 to convert sample N to $(NH_4)_2SO_4$ and subsequent determination by titration	FIL-IDF standard 20B (1993)
Lactose	Chloramine-T	Multistep redox reaction, final quantification by titration with $Na_2S_2O_3$	BS 1741, part 7, section 7.2 (1988)
	Polarimeter	Precipitation of fat and protein followed by direct determination of rotation in polarimeter	BS 1741, part 1, section 7.1 (1988)
	Enzymatic	Two-enzyme (galactose dehydrogenase and lactase) NAD/$NADH_2$-coupled spectrophotometric method	Various manufacturers' manuals, for example, Boehringer® (1989)
Total solids	Oven drying	Dry at 102 ± 2 °C to constant weight	FIL-IDF standard No. 21B (1987)
	Lactometer	Float lactometer in milk, determine density, and calculate total solids (TS) using the formula $TS = 0.25D + 1.22F + 0.72$, where D is the lactometer reading and F the fat content (%, w/w)	BS 734 (1985)

Fresh milk samples containing no lactic acid have a titratable acidity – expressed as %,w/v, lactic acid – of 0.14–0.16% due to the buffering action of milk salts and protein, and only samples having acidities >0.17% are normally considered sour. When monitoring acid production during cheese or yogurt fermentation, it is necessary to perform a blank determination on the milk or yogurt mix to allow the determination of the developed lactic acid (**Table 2**).

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Chromatographic Methods; Infrared Spectroscopy in Dairy Analysis; Nuclear Magnetic Resonance: Principles. **Water in Dairy Products**: Analysis and Measurement of Water Activity.

Further Reading

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Relevant Websites

www.codexalimentarius.net – CODEX alimentarius
www.fil-idf.org – FIL-IDF

Statistical Methods for Assessing Analytical Data

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Introduction

When we use an analytical method during an experiment or in quality control or assurance practice, we expect our measurement to be as accurate, reproducible, and sensitive as made possible by the measurement procedure we are using. When a measurement instrument is used for the analysis, we expect that it provides an unbiased response that remains stable over time. Unfortunately, except when the measurand is a reference standard, its true value is unknown and the measured quantity value is only an estimate of the true quantity value and it is affected by random, uncontrollable, errors and, possibly, by systematic errors. In order to estimate how reliable our measurement is, we need to use a range of statistical techniques to characterize the measurement process. This article will present a brief account of statistical methods used in measurement system characterization. Only basic knowledge of statistics is needed to understand the material presented. The interested reader is encouraged to refer to one of the many excellent textbooks or review articles available on this subject for further details. Some of the terms used in this entry (accuracy, repeatability, reproducibility, bias, uncertainty, etc.) have been defined in slightly different ways. Here, the definitions provided by *International Vocabulary of Metrology – Basic and General Concepts and Associated Terms (VIM)*, some of which are shown in **Table 1**, will be used.

Basics

Measurements and Variables

The result of a measurement procedure is the attribution of a quantity value to a measurand. The value can be on either a continuous or a discrete scale. A variable is continuous if it can, at least theoretically, assume any value on the scale on which it is measured. Examples are weight, concentration, and length. However, our ability to measure a quantity on a continuous scale may be limited by the resolution or by the reproducibility of the measurement procedure.

Continuous variables can be measured on ratio or on interval scales. Ratio scales are divided into equal intervals and have a fixed 0: an example is the thermodynamic temperature in Kelvin. Interval scales are divided in equal units, but the 0 is arbitrary. Examples are temperatures measured in degree Celsius or degree Fahrenheit. Although 25 and 50 °C are separated by exactly the same number of units as 298.15 and 323.15 K, milk at 50 °C is definitely not twice as hot as milk at 25 °C, because the 0 in the Celsius scale is arbitrary.

In sensory evaluation (**Analytical Methods**: Sensory Evaluation), one further problem is that even when apparently continuous quantitative scales are used by assessors, numbers are actually only ranks and the distance between the points of the scale is not constant. When panelists are asked to grade the perception of a descriptor for a cheese (e.g., bitterness) from 0 'no perception of the descriptor' to 9 'very intense', they may tend to use more frequently the central part of the scale and a score of 4 may not actually be 4 times as large as 1. This is an example of an ordinal scale in which ranks, not distances, are important. Semi-quantitative methods for milk quality based on color comparators (resazurin test, rapid phosphatase tests) also provide ordinal scales. Conventional parametric statistical tests may not be adequate for rank-order data of this kind, although they may provide in some instances a reasonable approximation.

In several cases, a single measured quantity may be insufficient to characterize the product and the measurement procedure may result in a small (e.g., L^*, a^*, and b^* values in colorimetric measurements using the CIE $L^*a^*b^*$ scale) or very large (absorbances, reflectances, emission at different wavelengths in a variety of spectroscopic techniques) number of variables that must be handled by appropriate multivariate statistical methods (**Analytical Methods**: Multivariate Statistical Tools for Chemometrics).

In a few instances, the results of analytical procedures are expressed in terms of nominal properties. Measurands can fall into two or more mutually exclusive categories: a test item may pass or fail a conformity test; a cheese sample may be negative or positive in testing for *Salmonella* using a given sampling plan; a cheese may be classified on the basis of its color in a number of mutually exclusive categories. Data in this format can only be enumerated and classified but most of the methods described in this article do not apply to nominal properties.

Table 1 Definitions from the *International Vocabulary of Metrology – Basic and General Concepts and Associated Terms (VIM)*, JCGM 200:2008

Term	*Definition*
Calibration	Operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication
International measurement standard	Measurement standard recognized by signatories to an international agreement and intended to serve worldwide
Measurand	Quantity intended to be measured
Measured quantity value	Quantity value representing a measurement result
Measurement	Process of experimentally obtaining one or more quantity values that can reasonably be attributed to a quantity
Measurement accuracy	Closeness of agreement between a measured quantity value and a true quantity value of a measurand
Measurement bias	Estimate of a systematic measurement error
Measurement error	Measured quantity value minus a reference quantity value
Measurement precision	Closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions
Measurement repeatability	Measurement precision under a set of repeatability conditions of measurement, i.e., condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time
Measurement reproducibility	Measurement precision under reproducibility conditions of measurement, i.e., condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects
Measurement standard	Realization of the definition of a given quantity, with stated quantity value and associated measurement uncertainty, used as a reference
Measurement trueness	Closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value
Measurement uncertainty	Non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used
Primary measurement standard	Measurement standard established using a primary reference measurement procedure, or created as an artifact, chosen by convention
Quantity	Property of a phenomenon, body, or substance, where the property has a magnitude that can be expressed as a number and a reference
Random measurement error	Component of measurement error that in replicate measurements varies in an unpredictable manner
Reference measurement standard	Measurement standard designated for the calibration of other measurement standards for quantities of a given kind in a given organization or at a given location
Secondary measurement standard	Measurement standard established through calibration with respect to a primary measurement standard for a quantity of the same kind
Reference quantity value	Quantity value used as a basis for comparison with values of quantities of the same kind. A reference quantity value can be a true quantity value of a measurand, in which case it is unknown, or a conventional quantity value, in which case it is known
Resolution	Smallest change in a quantity being measured that causes a perceptible change in the corresponding indication
Selectivity of a measuring system	Property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measurands such that the values of each measurand are independent of other measurands or other quantities in the phenomenon, body, or substance being investigated
Sensitivity of a measuring system	Quotient of the change in an indication of a measuring system and the corresponding change in a value of a quantity being measured
Systematic measurement error	Component of measurement error that in replicate measurements remains constant or varies in a predictable manner
True quantity value	Quantity value consistent with the definition of a quantity
Working measurement standard	Measurement standard that is used routinely to calibrate or verify measuring instruments or measuring systems

Measurement Error

Even when a single trained operator takes repeated measurements on a measurement standard (**Table 1**) using an accurate instrument over a short interval of time, the results of the replicate measurements will be slightly different due to random error. Therefore, each individual result will be the sum of the true quantity value (estimated by the mean of the population of measurements) of the measurand and random measurement errors:

$$x_i = \mu + \varepsilon_i \qquad [1]$$

In most instances, x_i will be distributed as a normal random variable and the random measurement errors will be normally distributed, with 0 mean and standard deviation σ and independent.

Due to cost constraints, the number of replicate measurements that can be performed is limited and the true quantity values (the population mean, μ) and the repeatability standard deviation, σ, can only be estimated from the sample mean, $\bar{x}$, and standard deviation, s. The difference between the sample mean and the true quantity value will, in turn, provide an estimate of the accuracy of the measurement, while the standard deviation is an estimate of repeatability (**Table 1**).

Other, non-random, sources of errors may affect the measurement results: by performing repeated measurements of the same measurand or standard by different operators, using different instruments, on different days, and using an appropriate statistical design, we can estimate the different error components and the reproducibility of the measurement and identify sources of bias (**Table 1**).

The issues of repeatability, reproducibility, and bias and the use of some descriptive graphing techniques for evaluating the distribution of measurement results are illustrated in **Figures 1–3**. Let us assume that a single trained operator has taken five repeated measurements on a check standard (see section 'Check standard methodology' for a definition) every day for 50 days. Individual data and day means are shown in the scatter plot in **Figure 1**. The data were actually generated by extracting 50 independent samples of 5 measurements from a normal distribution with $\mu = 5$ and $\sigma = 0.3$. Day-to-day variability is shown by the position of day means, while within-day variability is shown by the scatter of individual measurements for each day. Although the mean (5.000 1) and standard deviation (0.304 8) for the whole experiment are very close to the values of the parameters of the parent distribution, means and standard deviations calculated for single days are significantly more variable and range from 4.763 1 to 5.350 3 and from 0.055 9 to 0.508 7, respectively. With a small number of replicates, standard deviations calculated from individual measurement days are poor estimates of the repeatability and reproducibility of the measurement. The procedure for calculating repeatability (0.31) and reproducibility (0.13) standard deviations are described in section 'Check standard methodology'.

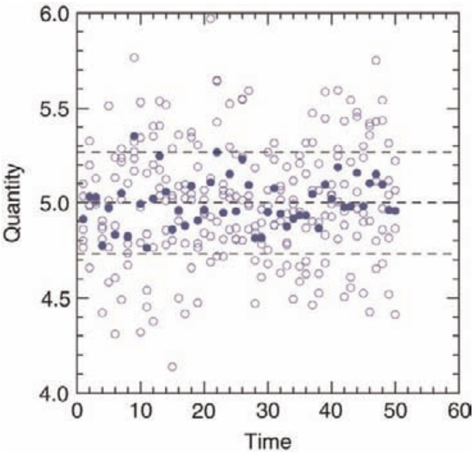


Figure 1 Simulated within-day and between-day variation of a measurement process: 250 simulated measurements were obtained by generating 50 samples of 5 elements from a normal distribution with $\mu = 5$ and $\sigma = 0.3$. This simulates the results obtained by a single trained operator who has taken five repeated measurements on a check standard (see section 'Check standard methodology' for a definition) every day for 50 days. Open circles show values for individual measurements, while filled circles are the means of the five simulated measurements taken each day. Horizontal lines show the grand mean ± standard error.

The distributions represented by the histograms in **Figure 2** simulate 50 measurement results for a measurand with a true value of 5 obtained by 3 operators. The data were actually generated by extracting 50 random numbers from normal distributions with $\mu = 5$, $\sigma = 0.3$ (D1); $\mu = 5.5$, $\sigma = 0.3$ (D2); and $\mu = 5.5$, $\sigma = 0.6$ (D3). D1 simulates an operator using a correctly calibrated instrument with a repeatability standard deviation of 0.30: the estimated mean and standard deviation obtained from the 50 replicate measurements are $\bar{x} = 4.97$, $s = 0.30$. D2 simulates an operator using an incorrectly calibrated instrument, which results in a theoretical bias of 0.5. As a result, the estimated mean and standard deviation obtained from the replicate measurements are $\bar{x} = 5.47$, $s = 0.30$. D3 simulates an operator using an instrument that is incorrectly calibrated and shows a random drift, resulting in biased measurement results with a larger repeatability standard deviation (0.60): the estimated mean and standard deviation are $\bar{x} = 5.45$, $s = 0.60$. The three histograms provide a good representation of the location and spread of the measurements. Popular alternatives to histograms are box and whiskers plots. In these nonparametric displays, the box shows the interquartile

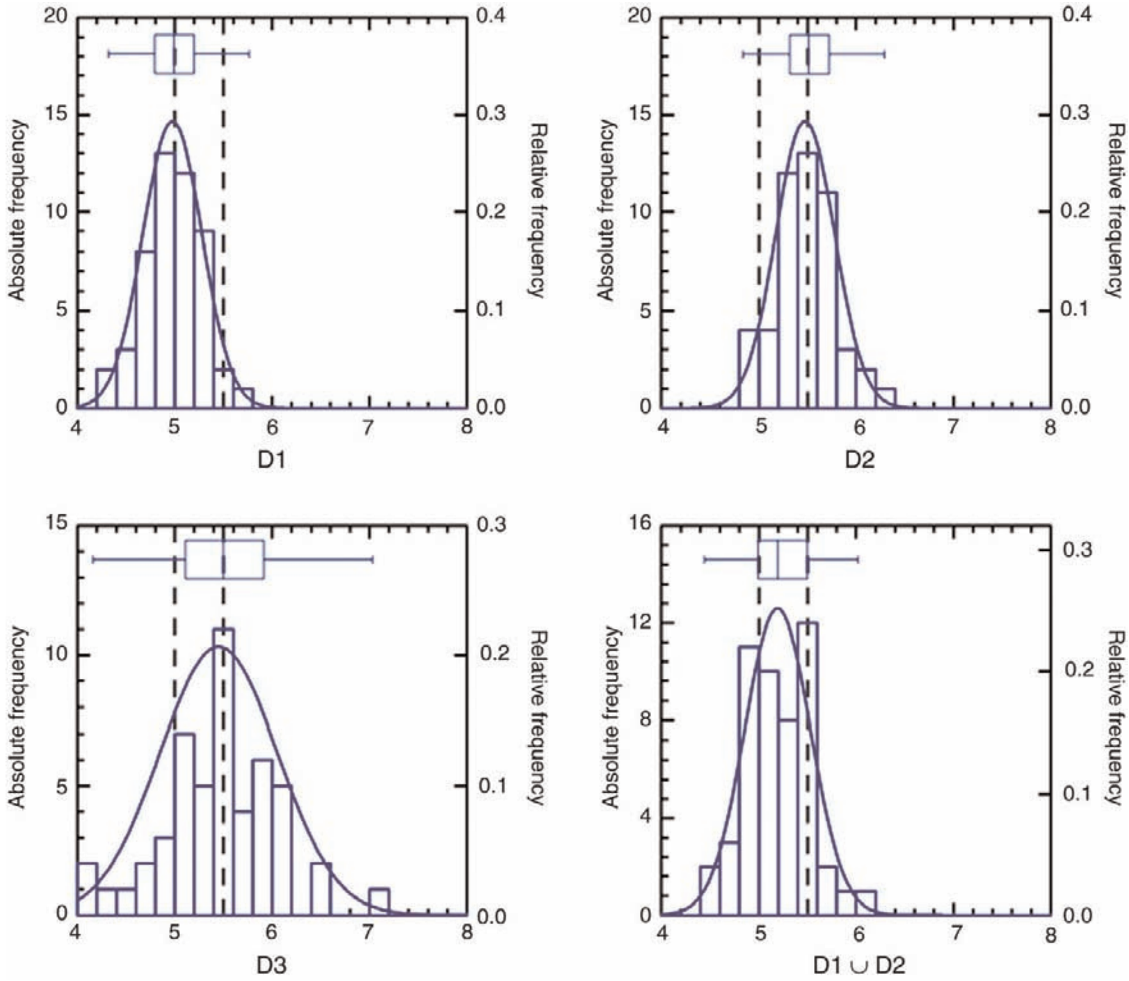


Figure 2 Histograms and box and whiskers plots for simulated measurement distributions. The data were generated by extracting 50 random numbers from normal distributions with $\mu=5$, $\sigma=0.3$ (D1); $\mu=5.5$, $\sigma=0.3$ (D2); and $\mu=5.5$, $\sigma=0.6$ (D3). Dashed vertical control lines mark the means of D1 and of D2 and D3. Box and whiskers plots for the same distributions are also shown. D1 ∪ D2 is the distribution resulting from the union of D1 and D2.

range (IQR, range between the first and the third quartiles of the distribution, $x_{0.25}$ and $x_{0.75}$, respectively), with a middle line marking the median ($x_{0.50}$). Whiskers extend from $x_{0.25}$–1.5IQR to $x_{0.75}$–1.5IQR. The mean may be marked by a dot within the box. Measurements located 2IQR or 3IQR beyond the central box are marked as outliers or extreme outliers.

Most statistical procedures related to measurement process characterization require the assumption that the measurements are normally distributed. This can be checked by comparing the values of kurtosis and asymmetry with those expected for a normal distribution, or by fitting a normal probability density function (**Figure 2**) on a histogram or by using a normal probability plot (**Figure 3**). In the latter, ordered measurement values (usually on the y-axis) are plotted against the order statistic medians (usually on the x-axis, expected values). Data will be distributed approximately along a straight line if the distribution is normal. Deviations from normality can be identified by bends or curvatures. The plot can be adapted easily to check if the data fit distributions other than normal (log-normal, Weibull, etc.). Deviations from normality may also result when samples obtained from different statistical populations are merged. Suppose, for example, that D1 and D2 in **Figures 2** and **3** had been pooled (e.g., by continuing to collect data after the bias was introduced by an incorrect calibration). This is shown, for example, by the bimodal nature of the histogram and by the asymmetry of the box plot for D1 ∪ D2 in **Figure 2** and by the pronounced bend in the probability plot in **Figure 3**.

Although slight deviations from normality may be tolerable, violations of the assumptions on error distribution may severely affect the significance of statistical test.

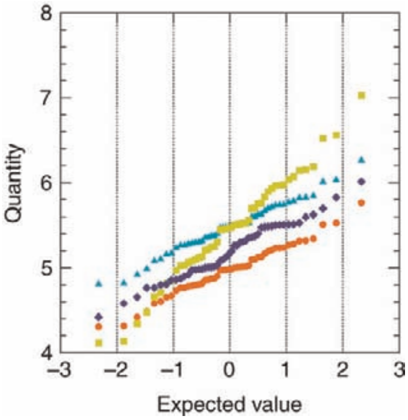


Figure 3 Normal probability plots for the distributions shown in **Figure 2**, D1 (●), D2 (▲), and D3 (■). D1 ∪ D2 (♦, which is the union of D1 and D2) shows an indication of nonnormality. Means can be estimated by finding the *y*-value corresponding to an expected value of 0. The slope of the lines is correlated with standard deviation: D3, which has a higher standard deviation, has a higher slope.

Transformation of raw data may alleviate the problem. Common transformations are

$$\text{logarithmic transformation: } y_i = \log_n(x_i + c) \qquad [2]$$

$$\text{power transformation: } y_i = (x_i + c)^n \qquad [3]$$

$$\text{Box–Cox: } y_i = \frac{(x_i^n + c)}{n} \qquad [4]$$

where y_i is the transformed value corresponding to x_i, and c and n are constants. c is needed when the measurements to be transformed include 0 or negative values. Logarithmic transformations (with $n = 10$) are appropriate for microbial counts and particle size distributions, although for small numbers a square-root transformation (i.e., a power transformation with $n = 0.5$) would be more appropriate. The Box–Cox transformation provides a more general approach: the optimal value of n is estimated by finding the value that maximizes the coefficient of correlation between the x- and y-axis in a normal probability plot of the transformed data.

Measurement Process Characterization

The objective of measurement process characterization is to identify and understand the sources of random or systematic error that affect the process. While components associated with random errors vary in an unpredictable manner and affect the repeatability and reproducibility of the process, components related to systematic errors vary in a predictable manner, causing biases and thus affecting the measurement accuracy (**Table 1**).

In a purely instrumental procedure, the position of the measuring instrument, microclimate changes, or vibrations may all affect the short-term repeatability or long-term reproducibility of the measurement. On the other hand, lack of, or improper, calibration and instrument wear may cause bias and drift in the measurement, producing results that are inaccurate. Bias may differ throughout the measuring range of the instrument, thus affecting the linearity of the response. In a procedure requiring the intervention of an operator, both the gauge (a thermometer, a caliper, a spectrophotometer, etc.) and the operator may contribute to measurement errors. When characterizing a measurement process related to aspects of production, part-to-part variation must be identified and its contribution separated from the other causes.

In addition, the issue of data integrity may be relevant and must be addressed prior to any measurement system characterization. Typical examples are errors in the registration of an analytical result in a database, or loss of data due to instrument failures or due to improper storage of a biological sample.

The characterization of ongoing processes in which items produced in a relatively constant production process can be repeatedly measured (either in process control or in certification laboratories) or for which measurement standards are available for calibration is relatively easy. Check standard methodology can be used to evaluate repeatability and reproducibility of the process and to identify the occurrence of biases or drifts. Gauge repeatability and reproducibility (R&R) studies are used to evaluate the contribution of operator, gauge, and parts being measured to the total variability. Finally, calibration is used to correct for bias and lack of linearity.

Measurement procedures that require sampling or use destructive tests or those that are based on one-time tests are more difficult to characterize. Due to the intrinsic variability of milk composition and of processes based on fermentation and due to the potential deterioration of physical, chemical, sensory, and biological properties of dairy products over storage, it may be actually difficult to carry out repeated measurements on the same sampling unit over different days: this may confound the effect of operator or measuring instrument and product variability. In some cases, all we can do is to assume that products obtained on different days are reasonably similar.

Check Standard Methodology

Check standard methodology is used to evaluate if a process is in statistical control prior to performing further studies. According to the NIST/SEMATEC *e-Handbook of Statistics*, a check standard “can be defined as an artifact or as a characteristic of the measurement process whose value can be replicated from measurements taken over the life of the process". A check standard must be stable and continuously available for the measurement process.

A check standard database is built by taking replicate measurements under repeatability conditions (**Table 1**; e.g., measurements taken by a single operator using the same measurement procedure over a short period of time) on the check standard over several days. An example is provided in **Figure 1**, which shows 250 simulated measurements taken on 50 days (5 replicate measurements per day).

Before analyzing the data, their distribution should be evaluated by using histograms and normal probability plots, and data transformations should be carried out to normalize the measurement results, if needed. As an alternative, the parent probability density function for the data should be identified and evaluation of uncertainty of control limits on control charts should be calculated accordingly.

If I is the number of replicates for each day and J the number of days for which the check standard data have been collected, the within-day mean ($\bar{x}_{j}\cdot$) and standard deviation (s_j) for the jth day can be calculated as follows:

$$\bar{x}_{j}\cdot = \frac{1}{I}\sum_{i=1}^{I} x_{ij} \qquad [5]$$

$$s_j = \sqrt{\frac{1}{I-1}\sum_{i=1}^{I}\left(x_{ij}-\bar{x}_{j}\cdot\right)^2} \qquad [6]$$

Both will be quite variable and will be poor estimates of the true quantity value of the check standard and of the measurement process repeatability and reproducibility, unless the number of degrees of freedom (I–1) is larger than 10. An estimate of the repeatability of the measurement process can be obtained by pooling the within-day variances in an estimate of the level-1 standard deviation:

$$s_1 = \sqrt{\frac{1}{J}\sum_{j=1}^{J} s_j^2} \qquad [7]$$

The corresponding estimate for the measurements described in **Figure 1** is 0.31 with $J(I–1) = 200$ degrees of freedom.

The grand mean of the measurements is an estimate of the true quantity value of the check standard:

$$\bar{x}\cdot\cdot = \frac{1}{J}\sum_{j=1}^{J} \bar{x}_{j}\cdot \qquad [8]$$

The level-2 standard deviation, with J–1 degrees of freedom, is an estimate of the day-to-day variability:

$$s_2 = \sqrt{\frac{1}{J-1}\sum_{j=1}^{J}\left(\bar{x}_{j}\cdot-\bar{x}\cdot\cdot\right)^2} \qquad [9]$$

For the data in **Figure 1**, the values of $\bar{x}\cdot\cdot$ and s_2 are 5.00 and 0.13, respectively. Both s_1 and s_2 are measures of the precision of the measuring process, obtained at two different levels, which should be indicated when making statements on the uncertainty of the measurement.

Measurement uncertainty is stated in terms of standard deviation or a multiple of standard deviation (a value of 2 is often used) or as an interval containing the true value with a stated probability. An estimate of the reproducibility of the measurement appropriate to the experiment described above is

$$\begin{aligned} s_R &= \sqrt{\frac{1}{J-1}\sum_{j=1}^{J}\left(\bar{x}_{j}\cdot-\bar{x}\cdot\cdot\right)^2+\frac{I-1}{I}\frac{1}{J}\sum_{i=1}^{I}\left(x_{ij}-x_{j}\cdot\right)^2} \\ &= \sqrt{s_2^2+\frac{I-1}{I}s_1^2} \end{aligned} \qquad [10]$$

The standard measurement uncertainty (i.e., uncertainty expressed in terms of reproducibility standard deviation) for the example in **Figure 1** is denoted as 5.00 ± 0.38 or as 5.00(38). Relative standard uncertainty is standard measurement uncertainty divided by the absolute value of the measured quantity value.

The evaluation of the precision of a measurement process should be carried out only for measurements that are in statistical control, that is, for which variation is caused only by random errors and not by assignable causes (bias). To evaluate if this is the case, Shewart's x-bar and s control charts can be used (**Figure 4**).

In an x-bar chart, the within-day means ($\bar{x}_{j}\cdot$) are plotted and the center line is the grand mean. Upper and lower control limits can be calculated in a number of ways. A common way is to use $\bar{x}\cdot\cdot \pm 2s_2$. However, using limits appropriate for testing the null hypothesis H_0: $\bar{x}_{j}\cdot = \bar{x}\cdot\cdot$ against the alternative hypothesis H_1: $\bar{x}_{j}\cdot \neq \bar{x}\cdot\cdot$ with a type I error probability α of 0.05 or 0.01 is more appropriate. In **Figure 4**, which uses the latter approach, with $\alpha = 0.01$, no out-of-control measurements are evident. Once the control limits are established, x-bar charts can be used to identify bias and drifts in measurements. An example is presented in the x-bar chart in **Figure 5**. After day 50, a simulated drift is introduced in the data and becomes evident with many out-of-control within-day means. Drifts or nonrandom patterns are also evident in lag plots, in which within-day means are plotted against themselves with a 1-day delay (**Figure 6**).

s control charts are used to evaluate if the variation of the process is in control. The center line is calculated as an unbiased estimate of the population standard deviation and is of little interest here. Instead, the upper control limit, calculated to test the null hypothesis H_0:$\sigma_1 = \sigma_2$ against the alternate hypothesis H_1:$\sigma_1 > \sigma_2$ at a type I error probability α of 0.05 or 0.01, is important to identify increases in the variability of the measuring process. An

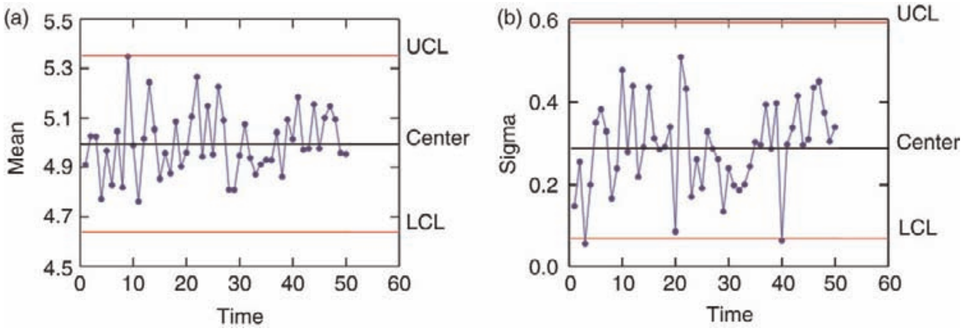


Figure 4 x-bar (a) and s (b) control charts for simulated check standard measurements ($I = 5$ replicates per day, for $J = 50$ days). Details on the procedures used for the calculation of the center line and for the upper (UCL) and lower (LCL) control limits are provided in the text. Each point is a within-day mean (x-bar chart) or standard deviation (s chart). Points that are outside the control limits represent means or standard deviations that are significantly different ($p < 0.01$) from the estimated measurement value or from the repeatability standard deviation, respectively, at a 99% confidence level.

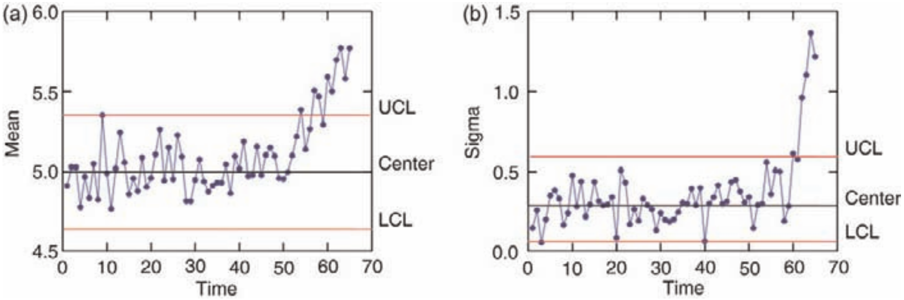


Figure 5 (a) x-bar control chart for simulated check standard measurements ($I = 5$ replicates per day, for $J = 65$ days). After 50 days of in-control measurements, 15 further days in which a mean drift occurred are shown. (b) s control chart ($I = 5$ replicates per day, for $J = 65$ days). After 50 days of in-control measurements, 15 further days in which a standard deviation increases are shown. Each point is a within-day mean (x-bar chart) or standard deviation (s chart). Details on the procedures used for the calculation of the center line and for the upper (UCL) and lower (LCL) control limits are provided in the text. Points that are outside the control limits represent means or standard deviations that are significantly different ($p < 0.01$) from the estimated measurement value or from the repeatability standard deviation, respectively, at a 99% confidence level. Center lines and limits are calculated from the in-control data (first 50 days).

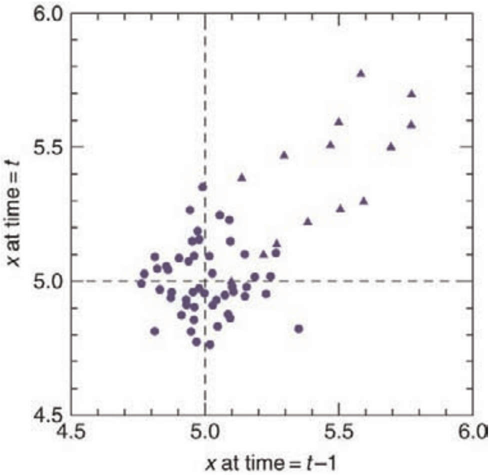


Figure 6 Time-lag plot for the simulated check standard experiment of **Figure 5(a)**. Circles are the means of the measurements obtained at days 1–50 (in-control data) which show random scatter, with contribution from random errors only. Triangles are the means of the measurements obtained at days 51–65. A drift is clearly evident.

example is presented in the s chart in **Figure 5**, which shows a simulated increase of s_1 after day 50.

In both x-bar and s charts, the same number of replicate measurements should be used each day, since unequal subgroup size will normally result in variable control limits. In addition, subgroups of measurements that are not in statistical control because of bias or increased variability should be excluded from the calculation of center line and control limits. x-bar charts are not sensitive enough to small deviations: Exponentially weighted moving average (EWMA) charts are far more effective, but the details of the calculation of center line and control limits for these charts are beyond the scope of this article.

Gauge R&R Studies

Once the measurement process is in statistical control, the components of the variability of the measurement can be estimated by gauge R&R studies.

The variance of a measurement system is usually the sum of several components. When the role of operators is important (as in measurements involving a visual assessment, e.g., when measuring parts using a caliper or measuring the diameters of fat globules using a micrometer), the measurements carried out by each single operator must be repeatable and measurements between operators must be reproducible. The sum of the two components, repeatability due to the gauge (σ_{e}^2) and reproducibility due to the operator (σ_{o}^2), gives the total measurement variance (σ_{m}^2).

$$\sigma_{\mathrm{m}}^2 = \sigma_{\mathrm{e}}^2 + \sigma_{\mathrm{o}}^2 \qquad [11]$$

In most instances when taking instrumental measurements, where the role of the operator is minimal, short-term variability (repeatability), day-to-day variability (reproducibility), and run-to-run stability over long periods of time are of interest:

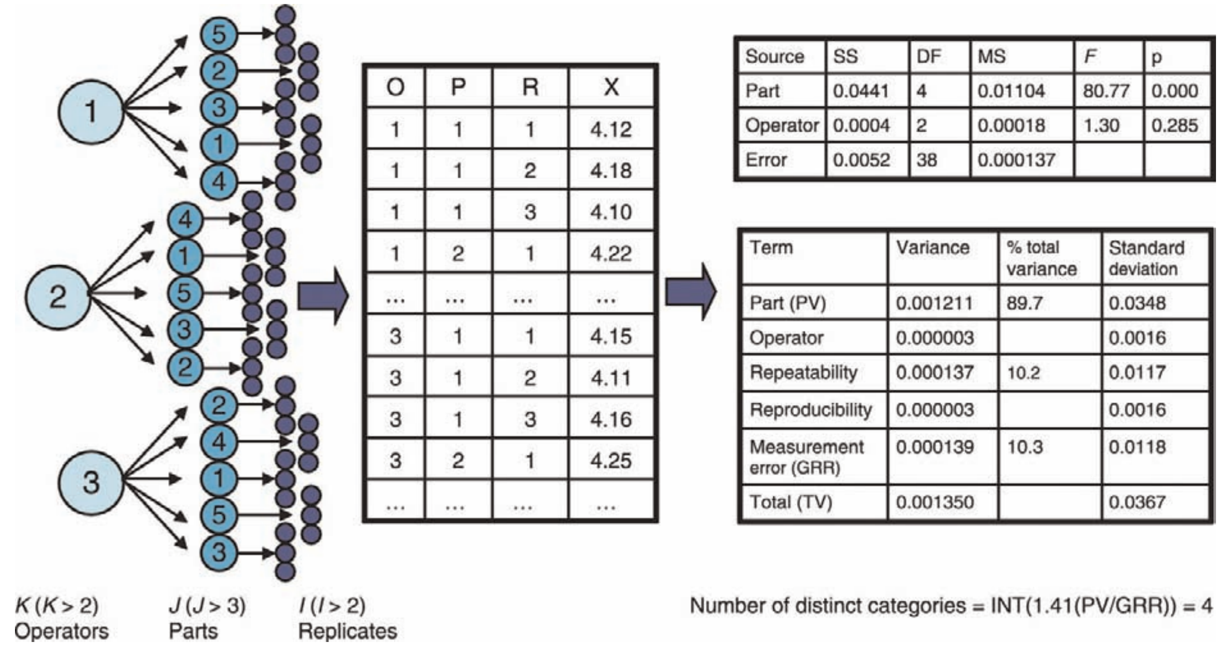
$$\sigma_{\mathrm{m}}^2 = \sigma_{\mathrm{run}}^2 + \sigma_{\mathrm{day}}^2 + \sigma_{\mathrm{e}}^2 \qquad [12]$$

If several instruments have to be used or if locations are thought to affect the results, further variance components need to be estimated.

Measurements can be repeated in this fashion only if appropriate (i.e., stable) test items or standards are available, or if the test is nondestructive. When carrying out repeated measurements on a check standard, a single item is being measured, and the variance of the measured properties is due only to the measurement process itself. On the other hand, in any production process, different parts (packages, sampling units, test items) will have variable properties because of either random or systematic (assignable) causes. If a measurement process has to be used in statistical process control (the application of statistical techniques to explore, measure, characterize, model, and improve processes), part-to-part variation has to be estimated and the variability of the measurement process must be a small fraction of the total variation.

A detailed description of experimental designs and calculations for gauge R&R studies is beyond the scope of this article. Interested readers can refer to statistical process control textbooks. A simple nested experimental design to identify part-to-part and operator variation is described in **Figure 7**.

Let us assume that three different operators are used to assess the variability of the measurement process for the assessment of the pH of yogurt in cartons. Five cartons (parts) are sampled using a design adequate to the level of variability that is of interest. Five cartons from a single batch obtained from a single vat in a single day, sampled over a short time immediately after packaging, would likely be less variable than five cartons randomly sampled from the storage facility and address different levels of the variability of the production process. Given the destructive nature of the test (the carton must be opened to perform the measurement) and the relative instability of the property to be measured (pH may change if the samples are not stored properly), nine smaller subsamples are taken from each carton and stored over a reasonably short time at low (0–4 °C) temperature. Three trained operators



O	P	R	X
1	1	1	4.12
1	1	2	4.18
1	1	3	4.10
1	2	1	4.22
...	...	...	...
3	1	1	4.15
3	1	2	4.11
3	1	3	4.16
3	2	1	4.25
...	...	...	...

Source	SS	DF	MS	*F*	p
Part	0.0441	4	0.01104	80.77	0.000
Operator	0.0004	2	0.00018	1.30	0.285
Error	0.0052	38	0.000137		

Term	Variance	% total variance	Standard deviation
Part (PV)	0.001211	89.7	0.0348
Operator	0.000003		0.0016
Repeatability	0.000137	10.2	0.0117
Reproducibility	0.000003		0.0016
Measurement error (GRR)	0.000139	10.3	0.0118
Total (TV)	0.001350		0.0367

Number of distinct categories = INT(1.41(PV/GRR)) = 4

Figure 7 Gauge repeatability and reproducibility for operator and part variation. A nested design is used to compare three operators, each of whom takes a pH measurement on five yogurt cartons (parts) sampled over one production day with three replicates. The effect of interaction of part × operator is not significant and was eliminated from the analysis. See text for details.

are asked to perform calibration of a single pH meter and then to randomly remove and measure three subsamples for each yogurt sample and to record the results.

The output (**Figure 7**) shows an analysis of variance table, with significant effect for parts ($p < 0.001$) and nonsignificant effect for operator, and a table with estimation of variance and standard deviation components including part variation (PV), total measurement variation (GRR), and total variation (TV). To evaluate the ability of the measurement process to identify small part-to-part variations, an estimate of the distinct number of categories that can reliably be separated by the measurement process is provided by most software packages used in gauge R&R studies. The number of distinct categories should be in the range 5–10 for the measurement process to be suitable for statistical process control. Lower values produce only coarse estimates and may be adequate to provide only categorical statements on the quality of the product (pass–fail, poor–average–good, etc.). Output may also include x-bar charts by operators and by parts and range or s charts for operators, in order to pinpoint problems related to significant operator differences.

An important issue in measurement process analysis is discrimination, that is, the largest change in the value of a quantity that causes no detectable change in the quantity value provided by the instrument being used. If the measurement is to be used in process control, discrimination should be small and lower than one-tenth of 6 times the total process variation measured as standard deviation.

Calibration

Calibration is used in analytical laboratories and in process control to reduce or eliminate the bias in measurement. A formal definition of calibration is provided in **Table 1**. The relationship between the actual measurement standard used in calibration and the reference base for the quantity of interest should be traceable through a chain of calibrations.

Once a calibration relationship is established, it can be used repeatedly to obtain measurement values from new measurands, as long as the measurement process is shown to be in statistical control, that is, does not provide evidences of drifts or increased variability (see above). This is often the case for calibration of sensors and instruments used in process control, which are used routinely for the same measurement process and may not require day-to-day calibration. On the other hand, for most measurement procedures carried out in analytical laboratories, calibration is carried out more frequently.

Single-value calibration of artifacts or instruments requires direct comparison of an unknown with a reference standard of the same nominal value using an appropriate calibration design to separate the effect of random errors from the effect of bias.

In research and quality control laboratories, calibration is most frequently used to relate indications provided by instruments to quantity values provided by reference standards (**Table 1**) over a range of values using an appropriate model. The instrument may read either in the same units of the reference standard (i.e., pH meters, balances, viscometers, RTD thermometers, colorimeters, etc.) or in different units (as is most often the case in spectrophotometric, spectroscopic, chromatographic, and other methods). In both cases, reference standards of different values are measured and a calibration curve is built by least square fit between the unknown response and the reference standard values. At least 2 reference standards are required for calibrations over short ranges, at least 5 for linear calibrations, and 10 for nonlinear calibration models. In addition, replicate measurements (at least two, preferably four) should be carried out on each reference standard especially if the uncertainty of the measurement and the deviation from the calibration model must be calculated. The value of unknown measurands is then obtained from the inverse of the calibration curve.

The most common relationships used in calibration and their inverse are shown in **Table 2**. The three models shown in **Table 2** are linear in their parameters or can be transformed into a linear model. Nonlinear calibration models can be used in some applications and their parameters can be estimated by nonlinear regression methods. However, estimation of the coefficients and of their uncertainty is more complicated, and correction of the bias is usually more difficult.

Table 2 Simple calibration models

	Model	*Inverse model*	*Notes*
Linear	$Y = a + bX + \varepsilon$	$X' = \dfrac{Y' - \hat{a}}{\hat{b}}$	
Quadratic	$Y = a + bX + cX^2 + \varepsilon$	$X' = \dfrac{-\hat{b} \pm \sqrt{\hat{b}^2 - 4\hat{c}(\hat{a} - Y')}}{2\hat{c}}$	The choice of the root depends on practical considerations
Power	$Y = aX^b\varepsilon$	$X' = e^{\left(\frac{\log_e(Y') - \log_e(\hat{a})}{\hat{b}}\right)}$	The model can be linearized as $Y = \log_e(a) + b\log_e(x) + \log_e(\varepsilon)$

Y is the measured reference standard value or the response of the instrument being calibrated, *X* the reference standard value, *Y′* a future measurement, *X′* the calibrated value associated to *Y′*, *a*, *b*, and *c* the coefficients of the regression model, and ε the error. Estimates are denoted by a hat (ˆ).

To obtain reliable estimates by calibration curves calculated by least square regression, the following assumptions must hold: the values of the reference standard must be known without error; the errors must be independent and come from a normal distribution with 0 mean and a common variance.

Although simple calibration curves may be obtained in most spreadsheet packages in a rather straightforward way by adding a regression line directly to a scatter plot, which rapidly provide the coefficients of the model and R^2, further information is needed to evaluate the adequacy of the model. With a little more effort, using spreadsheet functions in Microsoft Excel or in OpenOffice, estimates of coefficients and of their standard errors, residual sum of squares, and F statistics for testing if regression sum of squares is significantly different from residual sum of squares can be obtained. Furthermore, statistical software packages offer a range of diagnostic tools to test for the validity of assumptions underlying the estimation of regression coefficients: residual plots, autocorrelation plots, identification of outliers, and others.

In several instances, univariate calibration provides unsatisfactory results. This may depend on lack of selectivity of the analytical procedure and on the occurrence of noise. Multivariate calibration makes full use of the ability of many analytical procedures to generate multivariate data (**Analytical Methods**: Multivariate Statistical Tools for Chemometrics) to solve both these problems. In addition, multivariate calibration methods may provide further descriptive information on the structure of the data.

In contrast to univariate calibration, multivariate calibration makes use of a profile of n (with n typically >100, especially for spectroscopic methods) variables to create a calibration model for one or more analytes in a complex mixture. Discrimination of different analytes can be achieved if they have different response profiles. Multiple linear regression is not usually suitable, because the high collinearity of the variables in the profile does not allow one to obtain reliable estimates of the coefficients in the model. Principal component regression, partial least square regression, and artificial neural networks have been applied successfully in multivariate calibration in dairy science in a variety of contexts (**Analytical Methods**: Multivariate Statistical Tools for Chemometrics). Depending on the complexity of the calibration problem, the number of reference standards (or reference standard mixtures) required to establish the multivariate calibration model is typically much higher than with univariate calibration. While in relatively simple situations, 10–20 measurements may be sufficient, when using complex models with many input variables, hundreds of measurements may be necessary to establish a reliable model.

The application of partial least square regression to the prediction of cheese age from spectroscopic data presented in **Figure 5** in the article **Analytical Methods**: Multivariate Statistical Tools for Chemometrics is an example of multivariate calibration.

Software

A nonexhaustive list of the most frequently used general and specialized software packages for statistical analysis is presented in the article **Analytical Methods**: Multivariate Statistical Tools for Chemometrics. Most of these packages would provide functions and routines that are appropriate for the assessment of analytical data or for process control. One further option with more specialized functions for quality control is Minitab, a general program with emphasis on statistical process control and quality improvement (runs only under Windows environment).

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Biosensors; Chromatographic Methods; Differential Scanning Calorimetry; DNA-Based Assays; Electrophoresis; Infrared Spectroscopy in Dairy Analysis; Light Scattering Techniques; Mass Spectrometric Methods; Microbiological; Multivariate Statistical Tools for Chemometrics; Nuclear Magnetic Resonance: Principles; Sampling; Sensory Evaluation; Spectroscopy, Overview. **Rheology of Liquid and Semi-Solid Milk Products**.

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Multivariate Statistical Tools for Chemometrics

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Introduction

The quality of dairy products is defined by complex, sometimes large, sets of features, including microbiological, chemical, physical, and sensory properties. For example, pH, moisture, salt-in-moisture, a_W, protein and fat content, ethanol- or pH 4.6-soluble nitrogen, free fatty acid, elastic modulus, firmness, and counts of pathogenic, indicator, or starter organisms may all be important in determining or measuring the quality of a cheese or of a cheese-making process. Modern process monitoring techniques provide, in an automated way, large amounts of data collected over time by process sensors (pH, temperature (°C), pressure, etc.), which are critical for process control, documentation, and optimization. Although each of these parameters can be treated as a single variable by using univariate statistical techniques, multivariate approaches are necessary to obtain a complete representation of the product or of the process. This is especially true for the characterization of dairy products by electrophoretic, chromatographic, spectroscopic, and spectrometric techniques or by electronic noses/tongues. These methods provide complex data patterns, which need extensive preprocessing and statistical treatment to extract and visualize the relevant information. Finally, traceability and authenticity of dairy foods are of foremost importance in local and international trade. The identification of the geographical origin of foods and/or the confirmation that a quality labeled food (protected denomination of origin, protected geographical indication, traditional specialty guaranteed) has been produced using the raw materials and process stated in the standards of identity is essential in fraud prevention in the European Union and most often requires the use of multivariate statistical treatment of complex chemical, physical, or sensory data patterns.

According to the International Chemometrics Society "Chemometrics is the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods." Chemometric approaches have been used for the treatment of analytical data in dairy science for more than 30 years. An overview of the application of multivariate statistical techniques to analytical data in dairy science is presented in **Table 1** and examples related to quality and process control and authenticity testing will be provided, with emphasis on interpretation of some common graphs and data displays. The interested reader is encouraged to refer to one of the many excellent textbooks or review articles available on this subject for further details.

Multivariate Data

Multivariate data are represented as a two-dimensional (or multidimensional) ordered rectangular array (a matrix), with n rows (observations) and p columns (variables) (**Figure 1**). Each data point is univocally identified by its row and column coordinates, i and j: for example, x_{22} is the second observation of the second variable. Often we are interested in finding quantitative relationships between **X** variables (predictors) and **Y** variables (responses). In this case, measurements may be taken on a single y variable or on a multivariate response matrix **Y** (**Figure 1**). A data matrix may contain both categorical and quantitative variables (**Analytical Methods**: Statistical Methods for Analytical Data) and depending on the objective of the analysis, different approaches are appropriate. In some instances, two or more than two multivariate **X** data matrices may be available for the same samples because of the use of different measurement methods (e.g., when different spectroscopic techniques are used to characterize the same sample). Examples of combinations of categorical or quantitative **X** and **Y** matrices relevant to dairy science are presented in **Table 2**.

The data matrices may contain missing data because of malfunctioning of instrumentation, accidents, lack of response in a test, and other reasons. This can significantly reduce the amount of information in the matrix and prevent or complicate the calculation of relevant statistics. Missing value analysis is important in evaluating the occurrence of systematic patterns (or lack thereof) in missing values and to identify the best method to handle them. Two common alternatives are deleting observation or variables containing missing data or replacing them with estimates obtained by a variety of approaches.

Preprocessing of Analytical Data

In most instances, the assignment of analytical data to variables is straightforward: if different gross composition

Table 1 An overview of multivariate statistical methods in research and development and quality control in dairy science

Objective	*Type of data (see Figure 1)*	*Method*	*Relevant to*
Data mining, exploratory statistical analysis: finding patterns, generating hypotheses, exploring the relationship among variables, data reduction	A rectangular **X** data matrix containing p quantitative continuous variables or a covariance (**S**) or correlation (**R**) matrix	PCA: the variance in the data set is explained and summarized by a set of uncorrelated variables (the PCs), which are linear combinations of the original variables. The percentage total variance explained by the first few PCs provides an indication on how effectively the original variance is summarized. Component scores and component loadings are plotted (often as a biplot) to explore the relationship within and among observations and variables (see text and **Figure 2** for details) EFA: similar to PCA but postulates that observed variables are linear combinations of unobservable latent variables, the factors. Factor scores and factor loadings are plotted and examined to evaluate the relationships among objects and variables	Data reduction for CHR, ELP, SPS, SPM, and SAM methods prior to calibration and inferential analysis. Exploratory analysis in authenticity studies
	A contingency table containing frequencies or counts for categorical **Y** and **X** variables	CoA: decomposes the χ^2 statistics of a table and provides a graphical representation of row (predictor) and column (outcome) variables in a plot that bears analogies to a PCA biplot	Exploratory analysis in ES
	A similarity or dissimilarity matrix obtained from an **X** matrix of quantitative continuous and/or discrete variables	MDS: a map in m (with $m << p$) dimensions is built in such a way that distances between observations in the map match as closely as possible the observed dissimilarities. Points (observation) that are close in the map are also close in the input space. Variables do not appear in the map although it is usually possible to associate one or more variables with one of the dimensions (see text and **Figure 2** for details)	Exploratory analysis in authenticity studies and characterization studies

	An **X** matrix containing quantitative continuous and/or discrete variables and/or a similarity or dissimilarity matrix calculated between variables or observations	HCA: the relationships among observations and/or variables are shown as a dendrogram, a tree-shaped graph in which groups of objects are hierarchically organized in larger groups on the basis of their interobject or intergroup similarities or dissimilarities (see text and **Figure 2** for details) NHCA: objects are partitioned in a predefined number of mutually exclusive groups by optimization of a clustering criterion. No hierarchical relationship is assumed among clusters (see text and **Figure 2** for details) KSOMs: objects are assigned to artificial neurons in a two-dimensional square grid during an unsupervised learning process. Objects that are close in the input space are also close on the grid and neurons mark cluster of data in the input space (see text for details)	Exploratory analysis in authenticity studies and characterization studies. Grouping of cases and/or variables prior to hypothesis testing. Identification of 'natural' groups prior to analysis of their properties
	Two or more rectangular **X** data matrices containing quantitative continuous variables	CCA: PCs are calculated for two (or more) data tables, standardized, and linear combinations (called canonical variates) of the components for each table with a procedure that maximizes the correlation between couples of canonical variates, one (*k*) for the first table and one (*l*) for the second table: The couples are arranged in descending order of correlation and are orthogonal to each other. With this procedure, data obtained from two analytical procedures can be plotted in a common space	Exploratory analysis of results from different spectrometric techniques (e.g., NIR + fluorescence spectra) for cheese authenticity studies
Supervised pattern recognition (i.e., assignment of objects to predefined groups)	A binomial or multinomial categorical Y variable and a rectangular **X** matrix containing *p* quantitative continuous and/or discrete variables	LDA, QDA: a linear or quadratic discriminant function of the variables that allows individuals to be classified into one of two or more mutually exclusive groups is found by maximizing the ratio of between-group and within-group covariance matrices. Selection of the subset of **X** variables that allows the best discrimination is performed by stepwise regression (see text and **Figure 3** for details)	Product authenticity studies based on CHR, ELP, SPS, SPM, and SAM methods. IDM

(*Continued*)

Table 1 (Continued)

Objective	*Type of data (see Figure 1)*	*Method*	*Relevant to*
		Supervised artificial neural networks (MLP; BN; RBF network): a multilayer network made of simple processing units (neurons and/or radial basis functions) is trained during a supervised process. During the training step, network parameters are adjusted iteratively while the network is presented with multivariate inputs and the corresponding outputs. A properly trained network becomes able to generalize and to correctly classify input not used during training (see text for details)	
		CTs: a tree structure (usually with binary splits) is built in analogy with a dichotomic classification key. Variables used at each node and the cut values are selected during an iterative training step. If the response variable is quantitative, an RT is used	
Prediction, modeling, process optimization	A **Y** vector or a **Y** matrix and the corresponding two-dimensional or multidimensional rectangular **X** matrix, both containing continuous quantitative variables	MLR: estimates a linear model of the form $y = \mathbf{x}'\boldsymbol{\beta}$, where $\boldsymbol{\beta}' = [\beta_0, \beta_1, \ldots, \beta_p]$ is the vector containing the $p + 1$ (including the constant) model coefficients, which are estimated by least squares. Some nonlinear relationships can be handled by transformation of variables. Stepwise regression may aid in the selection of $k < p$ variables. Sensitive to violation of assumptions	Calibration in SPS and SPM methods. Prediction of product properties or composition from complex data. Exploration of the relationships among and within **X** and **Y** variables.Multivariate statistical process control
		PCR: PCA is performed first on the **X** matrix and linear regression is used to relate the y variable(s) and the PCs	

		PLSR: used to estimate in a parsimonious and effective way the relationships between a single *y* variable and **X** variables (PLS1), and among and between **Y** and **X** variables (PLS2). Unlike PCR, the new *t* variables (latent variable estimates) obtained from **X** are estimated to be good predictors of **Y**. The optimal number of **X** components is estimated by cross-validation. The **X** and **Y** scores contain information on similarities among objects, while weights are used to evaluate how variables combine in the relationship between **X** and **Y** (see text and **Figure 4** for details)	
		Supervised artificial neural networks (MLP; BN; RBF network): similar to the use in pattern recognition, but the response variables are quantitative	
	As above but with categorical **X** variables	MANOVA: similar to MLR. Coefficients and statistics are estimated by least square regression. Useful in designed experiments for the evaluation of differences in responses or response profiles (e.g., when repeated measures are available for each case) between two or more groups. In analysis of covariance, **X** includes both categorical and continuous variables	Optimization, test of hypothesis, comparison of treatments
	A contingency table containing counts or frequencies for categorical **Y** and **X** variables	LR, LL models: the coefficients of a linear model are calculated by maximum likelihood estimation to evaluate the effect of change in the level of the predictor variables on the log(odds) of the response variable(s)	Shelf life studies, epidemiological and reliability studies
	A binomial or multinomial Y variable and a rectangular **X** matrix containing *p* quantitative continuous and/or discrete variables	LR, SA: an **X** matrix containing noncensored or right-censored data is used to predict the probability of failure or survival in a product or subject	

Data: **X** refers to input, explanatory variables, predictors; Y refers to output, response variables.

BN, Bayesian network; CCA, canonical correlation analysis, CoA, correspondence analysis; CT, classification tree; EFA, exploratory factor analysis; HCA, hierarchical cluster analysis; KSOM, Kohonen self-organizing map; LDA, linear discriminant analysis; LL, log-linear: LR, logistic regression; MANOVA, multivariate analysis of variance; MDS, multidimensional scaling; MLP, multilayer perceptron; MLR, multiple linear regression; NIR, near-infrared region; NHCA, nonhierarchical cluster analysis; PC, principal component; PCA, principal component analysis; PCR, principal component regression; PLSR, partial least square regression; QDA, quadratic discriminant analysis; RBF, radial basis function; RT, regression tree; SA, survival analysis.

Methods: CHR, chromatographic methods; ELP, electrophoretic methods; ES, epidemiological studies; IDM, identification and characterization of microorganisms; SAM, sensor array methods (electronic noses, electronic tongues); SPM, spectrometric methods; SPS, spectroscopic methods.

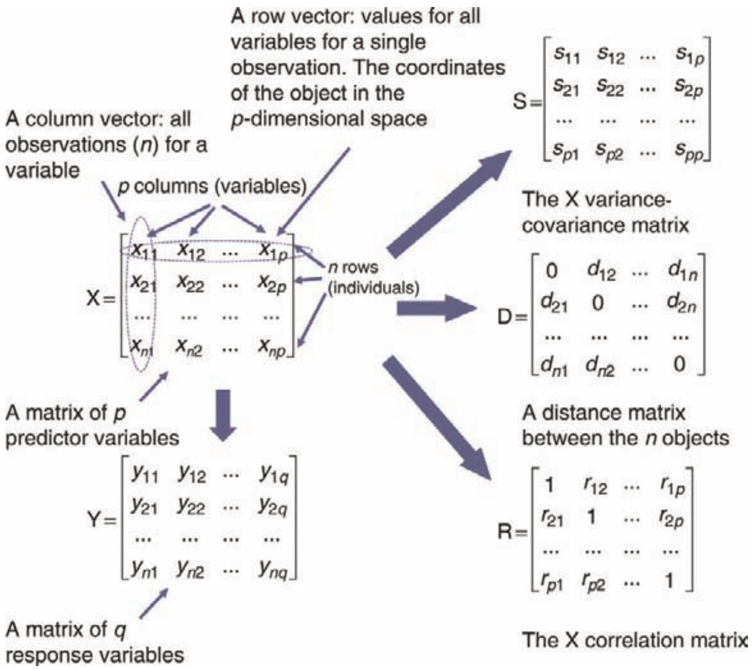

A row vector: values for all variables for a single observation. The coordinates of the object in the p-dimensional space
A column vector: all observations (n) for a variable
p columns (variables)
n rows (individuals)
X=
A matrix of p predictor variables
Y=
A matrix of q response variables
S=
The X variance-covariance matrix
D=
A distance matrix between the n objects
R=
The X correlation matrix

Figure 1 Data matrices in multivariate statistical analysis. Each of the *n* lines in both **X** and **Y** matrices corresponds to an observation, that is, an object (a cheese or milk sample, a point in a process, etc.) for which measurements have been taken. Matrix **X** groups the independent (predictor) variables that may be used to predict the values of the dependent (response) variables in matrix **Y**. Examples of predictor and response variables are presented in **Table 2**. The variance–covariance matrix (**S**), the correlation matrix (**R**), and distance matrices are calculated from the **X** (and **Y**) matrix. They describe the relationships among variables and/or observations and are used as input in multivariate statistical analysis.

Table 2 Some examples of **X** and **Y** data matrices relevant to dairy science

Type of analysis	***Y** data matrix*	***X** data matrix*
PCA, MDS, HCA, NHCA, KSOM	None	Normalized heights of *p* peaks in RP-HPLC chromatograms of 70% ethanol-soluble nitrogen for *n* cheese samples
	None	Preprocessed heights of *p* peaks of NIR, MIR, or front face fluorescence spectroscopy (FFFS) spectra of cheese for *n* cheese samples
LDA, MLP	A single binomial or multinomial categorical variable with origins or age of cheese samples	Preprocessed (PCA) peak heights of NIR, MIR, or FFFS spectra of cheese; combination of chemical, spectroscopic, and microbiological data for cheese samples
PLS1 (a single *y* variable)	Concentration of selected free amino acids in cheese during ripening	Preprocessed NIR spectra
	Rheological parameters of cheese	Fluorescent spectra of tryptophan and riboflavin
PLS2 (a multivariate **Y** data matrix)	Sensory properties of cheese	Preprocessed NIR spectra of cheese, composition of microbiota measured by single strand conformation polymorphism of rRNA and rDNA

HCA, hierarchical cluster analysis; KSOM, Kohonen self-organizing map; LDA, linear discriminant analysis; MDS, multidimensional scaling; MIR, mid-infrared region; MLP, multilayer perceptron; NHCA, nonhierarchical cluster analysis; NIR, near-infrared region; PCA, principal component analysis; PLS, partial least square; RP-HPLC, reversed-phase high-performance liquid chromatography.

parameters have been measured on a cheese sample, each (pH, moisture, salt-in-moisture, fat, protein, nonprotein nitrogen, free amino acids, etc.) will be attributed directly to a variable. On the other hand, for spectroscopic, spectrometric, chromatographic, and electrophoretic techniques, more or less extensive preprocessing of raw data may be required prior to statistical analysis. When the identity of a peak or a band in a chromatogram, spectrum, or electropherogram is known (i.e., it can be attributed to a specific molecule), the process of attributing it to a variable is simple, since each identified peak or band will be considered as a variable. With chromatograms or electropherograms of the soluble or insoluble fractions in cheese, no information on peak or band identity is usually available for several peaks or bands and band/peak matching has to be performed in order to create variables. This usually requires a number of steps (often performed by the data acquisition software assisted by manual intervention of the operator) related to background subtraction/baseline removal, identification of peaks or bands, calculation of their height or area, creation of a band or peak set (including all bands or peaks in all the electropherograms/ chromatograms of the experiment or a relevant subset), and automated or manual matching of bands/peaks in each electropherogram or chromatogram with individual bands/peaks in the band or peak set. Alternative approaches are based on the partition of the chromatogram or electropherogram in a number of classes of retention time or molecular mass and on the accumulation of peaks or band intensities in the classes by use of membership functions.

The pretreatment of spectroscopic data requires even more extensive preprocessing: near infrared (NIR) region, mid infrared (MIR) region, and fluorescent spectra contain hundreds or thousands of absorbances, reflectances, or emissions measured at different wavelengths, sometimes on several replicates of the same sample. Band positions, band intensities, and bandwidths are all related to chemical information on the sample, and averaging spectra and wavelengths, baseline removal, normalization, and extraction of first or second derivatives are usually performed before multivariate statistical analysis.

Data Transformation and Standardization

Transformation and/or standardization of variables may be necessary to make scales of measurements of different variables in a given data set comparable or to correct for departures from assumptions related to hypothesis testing. Several multivariate statistical techniques are extremely sensitive to differences in scales of variables in the data set, and variables with the largest values may 'dominate' the analysis (especially with methods that use Euclidean distance or covariance matrices, see below), dwarfing the effect of variables measured on different scales. Common standardization methods are standardization based on range standardization (eqn [1]), total information (eqn [2]), and normalization to z scores (eqn [3]):

$$x_{ij} = \frac{x_0 - \min(x_j)}{\max(x_j) - \min(x_j)} \qquad [1]$$

$$x_{ij} = \frac{x_{ij}}{\sum_{j=1}^{p} x_{ij}} \qquad [2]$$

$$x_{ij} = \frac{x_{ij} - \overline{x}_j}{s_j} \qquad [3]$$

where x_{ij} is the value of observation i for variable j, $\min(\mathbf{x}_j)$ and $\max(\mathbf{x}_j)$ are the minimum and maximum values of variable j, and $\bar{x}_j$ and s_j are the sample mean and standard deviation for variable j. Equations [1] and [2] are frequently used for the standardization of chromatographic or electrophoretic data. Standardization performed using eqn [3] is referred to as autoscaling and it is often carried out prior to several statistical techniques, including partial least square regression (PLSR).

Square root, logarithmic, logistic, and angular transformations are sometimes needed to apply linear models to data that are not normally distributed and/or for which mean and standard deviation are not independent (**Analytical Methods**: Statistical Methods for Analytical Data).

Exploratory Multivariate Statistical Analysis

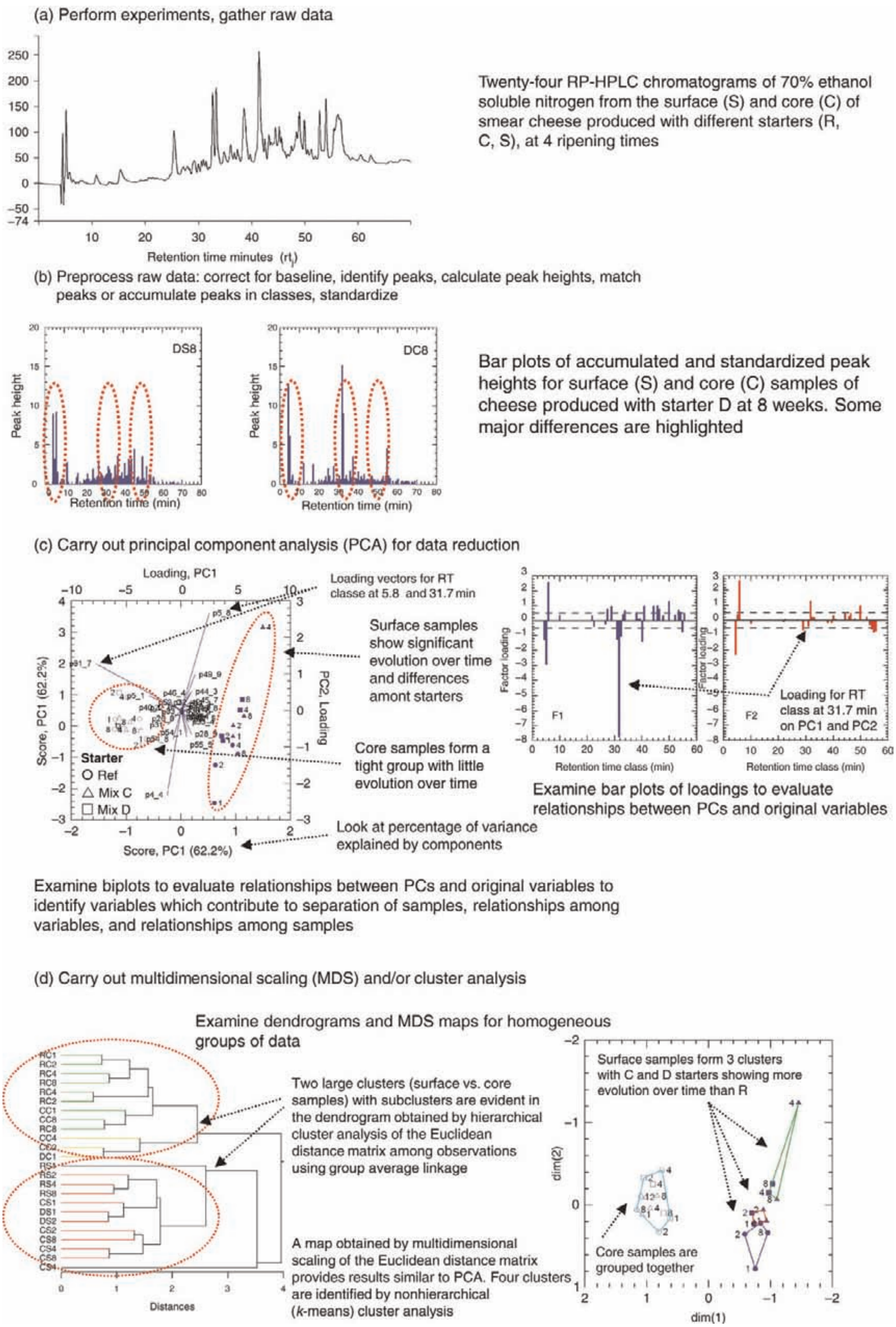
In the first phase of a study, researchers are often concerned with exploring the data in order to recognize relationships among variables or grouping of observations prior to the formulation of hypotheses. Several exploratory techniques have been applied to analytical data for different purposes (**Table 1**). An example of workflow in exploratory analysis is shown in **Figure 2**.

Principal Component Analysis and Factor Analysis

Principal component analysis (PCA) is arguably the most frequently used data reduction technique in chemometrics. PCA is applied to a matrix of continuous variables. No assumptions are made on the existence of explanatory (**X**) and response (**Y**) variables but the evaluation of the relationships between both variables and observations is of interest. Either the covariance (**S**) or the correlation (**R**) matrix can be used in the analysis and the total variance in the data set is explained and summarized by a set of uncorrelated variables (the principal components (PCs)), which are linear combinations of the original variables:

$$y_i = \mathbf{a}_i{}'\mathbf{x} \qquad [4]$$

where y_i is the ith component (there are as many components as there are variables) and $\mathbf{a}_i$ is the vector of coefficients for component i. Components are extracted in such a way that they provide a new set of coordinates



(a) Perform experiments, gather raw data

Twenty-four RP-HPLC chromatograms of 70% ethanol soluble nitrogen from the surface (S) and core (C) of smear cheese produced with different starters (R, C, S), at 4 ripening times

(b) Preprocess raw data: correct for baseline, identify peaks, calculate peak heights, match peaks or accumulate peaks in classes, standardize

Bar plots of accumulated and standardized peak heights for surface (S) and core (C) samples of cheese produced with starter D at 8 weeks. Some major differences are highlighted

(c) Carry out principal component analysis (PCA) for data reduction

Loading vectors for RT classe at 5.8 and 31.7 min

Surface samples show significant evolution over time and differences amont starters

Core samples form a tight group with little evolution over time

Look at percentage of variance explained by components

Loading for RT class at 31.7 min on PC1 and PC2

Examine bar plots of loadings to evaluate relationships between PCs and original variables

Examine biplots to evaluate relationships between PCs and original variables to identify variables which contribute to separation of samples, relationships among variables, and relationships among samples

(d) Carry out multidimensional scaling (MDS) and/or cluster analysis

Examine dendrograms and MDS maps for homogeneous groups of data

Two large clusters (surface vs. core samples) with subclusters are evident in the dendrogram obtained by hierarchical cluster analysis of the Euclidean distance matrix among observations using group average linkage

A map obtained by multidimensional scaling of the Euclidean distance matrix provides results similar to PCA. Four clusters are identified by nonhierarchical (*k*-means) cluster analysis

Surface samples form 3 clusters with C and D starters showing more evolution over time than R

Core samples are grouped together

250
200
150
100
50
0
−50
−74
10
20
30
40
50
60
Retention time minutes (rt$_i$)
DS8
DC8
Peak height
Retention time (min)
0
10
20
30
40
50
60
70
80
Loading, PC1
−10
−5
0
5
10
Score, PC1 (62.2%)
PC2, Loading
Starter
○ Ref
△ Mix C
□ Mix D
p5_8
p1_7
p49_9
p46_4
p44_3
p28_9
p55_5
p4_4
Factor loading
F1
F2
Retention time class (min)
dim(1)
dim(2)
Distances
RC1
RC2
RC4
RC8
RC4
RC2
CC1
CC8
RC8
CC4
CC2
DC1
RS1
RS2
RS4
RS8
CS1
DS1
DS2
CS2
CS8
CS4
CS8
CS4

Figure 2 An example of workflow in multivariate exploratory data analysis of a data set composed of reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms of 70% ethanol-soluble nitrogen of surface-ripened cheeses produced with different starters. From Piraino P, Parente E, and McSweeney PLH (2004) Processing of chromatographic data for chemometrical analysis of proteolytic profiles from cheese extracts: A novel approach. *Journal of Agriculture and Food Chemistry* 52: 6904–6911.

that are independent (uncorrelated with each other, orthogonal) and explain progressively lower proportions of the sample variance. The percentage of total variance explained by the first few PCs provides an indication on how effectively the original variance is summarized. Component scores (the rescaled coordinates of the original data points in the new axis system defined by the PCs) and component loadings (the covariances, if **S** was used, or correlations, if **R** was used, of the variables with the PCs) are shown in the output, which usually takes the form of a biplot, as in **Figure 2(c)**. Use of bar plots for loadings is also common.

Since there are as many components as there are original variables in the data set, a decision on the number of PCs that satisfactorily summarize the variance must be taken. One possibility is retaining the number of components that explain a predetermined amount of variance: reasonable targets in data sets with a low or moderate number of variables are 75–85% of the variance. A common alternative when factoring the correlation matrix is retaining the components whose eigenvalue is higher than 1.

PCA has been used in exploratory analysis in a large variety of situations (authenticity studies, comparison of complex chromatographic or electrophoretic patterns, etc.) and in data reduction for chromatographic, electrophoretic, spectroscopic, and spectrometric techniques prior to calibration and inferential analysis. In fact, variables in these data sets are usually numerous and strongly correlated, which prevents or complicates the use of multiple linear regression (MLR) and analysis of variance (ANOVA). The use of the first PCs allows circumventing these problems by providing a parsimonious set of uncorrelated variables, which hopefully summarize most of the variability of the data and can be related to response variables.

Factor analysis is another technique that seeks to explain the variability in the data set in a lower number of dimensions. Exploratory factor analysis postulates that existing variables can be expressed in terms of a linear combination of unobservable k latent variables with $k < p$:

$$\mathbf{x} = \Lambda \mathbf{f} + \mathbf{u} \qquad [5]$$

where Λ is the factor loadings matrix, **f** the latent variables or factors vector, and **u** the residuals vector. The model obtained by factor analysis is more easily interpretable than PCA in terms of relationships among the original variables and the factors, and the two techniques frequently bring to similar results.

Multidimensional Scaling

Multidimensional scaling (MDS) provides a way for representing $n \times n$ dissimilarity matrices by a simple geometrical model (or map) in d dimensions (with $d < n$ and hopefully close to 2 in order to facilitate plotting) such that the distance between data points in the new set of coordinates is as close as possible to the original dissimilarity. The dissimilarity matrices can be generated directly (e.g., by asking assessors to evaluate how dissimilar two products are) or from the original data matrix. Classical, metric, and nonmetric MDSs use different iterative algorithms that minimize a fit criterion (Kruskal's stress formula is frequently used) to calculate the coordinates in d dimensions. Increasing d improves the fit, and scree plots (stress vs. d) can be used to identify the values that provide the best compromise.

The interpretation of MDS maps is quite straightforward since observations that are closer in the map should also be close in the original space. PCA and MDS often provide comparable results: in fact, the grouping of data in the PCA biplot and MDS plot in **Figure 2** is strikingly similar. A shortcoming of MDS is that it is usually difficult to associate positions in the map with values of original variables, although it may be possible to identify a few variables that are associated with the main directions of variations: in **Figure 2(d)**, the first dimension obviously contrasts surface versus core samples while the second dimension is associated with ripening time.

Applications of MDS are similar to those of PCA with focus on the graphical representation of data and on the detection of natural groups of observations. As such, it can be used as a preliminary step in cluster analysis or to graphically represent the distance relationships of clusters obtained from nonhierarchical cluster analysis (NHCA) (**Figure 2**).

Cluster Analysis

The objective of cluster analysis is to partition (i.e., to classify) observations in a number of groups that share common features. Variables, rather than observations, or both variables and observations can also be clustered. The researcher may or may not know *a priori* the number of groups. When group memberships are not known a *priori*, cluster analysis provides a form of unsupervised pattern recognition, as opposed to discriminant analysis (DA), in which group memberships are known, and the objective of the analysis is to define the rules to allocate unclassified objects among the groups. The term 'groups' is here loosely used as a proxy for cluster. There is probably no single, all-purpose definition for this term. A cluster is usually recognized on the basis of internal cohesion (homogeneity) and external isolation (separation): in a p-dimensional space, a cluster may be defined as a continuous portion of the space containing a relatively high density of points separated from other clusters by regions of space containing a relatively low density of points. Clusters of data are clearly evident in the score plots and MDS plots in **Figure 2**.

Clustering can be divided into hierarchical (i.e., cases belong to clusters, which in turn belong to larger clusters: the clusters are organized in a hierarchy) and nonhierarchical techniques (there is no assumption of the hierarchical relationships among clusters).

Hierarchical clustering

Hierarchical cluster analysis (HCA) is frequently used in taxonomy (e.g., in the classification of bacterial strains or species on the basis of their phenotypic or genotypic features). Given the hierarchical nature of taxa (strains belong to species, which belong to genera, which belong to families, etc.), the application of agglomerative methods that progressively group entities (species, strains, isolates) into larger groups is justified. Clustering proceeds in two steps. First, a matrix of distances or similarity measures is calculated (the choice of the metric depends on the data and the objective of the analysis). In the second step, a clustering algorithm is used to group cases (and/or variables) together, and then to group the clusters into larger clusters. The choice of similarity or dissimilarity measure is critical and different metrics are appropriate to different data sets. Pearson's *r* and Euclidean distance are frequently used for continuous variables. With the former, cases with a similar pattern of variables will be close together, but scale becomes of foremost importance when Euclidean distance is used. Therefore, standardization often becomes an issue, especially when the variables in the data set are measured on very different scales: if no standardization is applied, variables that have large scales will dominate the clustering.

While calculation of similarities or dissimilarities among cases is straightforward, once cases are joined in clusters, different methods can be used to calculate intergroup distances. Commonly used methods are the simple linkage, the complete linkage, and the group average linkage method. In the single linkage method, the distance between two clusters is defined as the minimum distance between a pair of objects, one in one cluster and one in the other, while the maximum distance is used in the complete linkage methods. The group average linkage method uses the average distance between all pairs of objects, one in one group and one in the other. Different clustering methods often produce different grouping of the data, and it is not always easy to determine the best method for all situations.

The results are represented as a dendrogram (see **Figure 2**). Since the number of clusters is not known *a priori* and eventually all observations will be grouped in a single cluster, some sort of rule must be found to determine how many clusters are present in the data set. Although formal criteria exist for this purpose, previous knowledge on data structure or heuristic rules are used to determine the number of clusters.

Nonhierarchical clustering

In most cases, the assumption of the hierarchical organization of clusters in untenable nonhierarchical cluster analysis (NHCA) should be used rather than HCA. Several nonhierarchical clustering techniques use optimization of a clustering criterion to allocate *n* objects (observations) to a predetermined number (*g*) of clusters.

The criteria are based on lack of homogeneity or separation, that is, on the maximization of between-group variance and/or on the minimization of within-group variance. The number of different combinations of *n* observations grows very rapidly with *n* and *g*. For example, with 5 objects to be partitioned in 2 groups, 15 different combinations are possible, while with $n = 10$ and $g = 3$, the number of combinations to be considered is 9330. Therefore, checking all the possible allocations of one individual in groups becomes unpractical even with relatively low values of *n* and *g*. The algorithms used in NHCA operate iteratively by allocating observations to different groups and evaluating the effect on the clustering criterion until convergence is obtained (i.e., the change in the criterion is lower than a specified tolerance) or the number of iterations specified by the user is exceeded.

Optimization methods based on the search of maxima or minima (hill climbing or hill descending) are used to reduce the number of allocation steps, and convergence is usually achieved within 25 iterations. The most common hill-climbing method is the *k*-means algorithm, which iteratively allocates observations to the cluster based on the distance between the object and the group mean vector (the centroid of the group). The *k*-means method tends to find clusters of approximately the same shape and it is very sensitive to scale: unless standardization is used, variables with the larger scale will have the largest influence on allocation of observations.

A number of formal or informal criteria can be used to evaluate the number of natural clusters present in the data prior to NHCA. Examination of PCA score plots or MDS maps can help in finding compact groups of points (**Figure 2**). Formal criteria are based on the calculation of a numerical index and on the evaluation of the effect of adding a further group on the value of the index.

After clustering, descriptive statistics can be calculated on clusters and a number of univariate or multivariate displays can be produced to examine the data graphically. Cluster analysis can also be used prior to inferential univariate (ANOVA, analysis of contingency tables) or multivariate techniques (DA).

Inferential Methods

None of the methods described above leads directly to hypothesis testing, although they can be used by the investigator prior to hypothesis testing. For example, after performing the analysis described in **Figure 2**, the investigator may be interested in modeling the relationship between time and one or more chromatographic peaks, or to test formally for differences between two or more treatments, or in relating the reversed-phase high-performance liquid chromatography (RP-HPLC) pattern to one or more response variables (sensory properties, time of ripening, mode of production) represented by categorical or quantitative variables. However, data matrices generated by modern analytical techniques often have more variables than observations. This may be due to the cost of the analysis, the number of samples available, or, more simply, the fact that many analytical methods generate a large number of variables. For example, a single electropherogram of 70% ethanol-insoluble cheese nitrogen may contain intensities for more than 50 bands, a single RP-HPLC profile of 70% ethanol-soluble nitrogen may contain areas for >100 matched peaks, and an NIR or MIR spectrum of cheese may contain hundreds of spectral features. In addition, in most instances, variables will be highly correlated. This may severely limit the application of traditional inferential tests based on linear models.

Linear Models: Multiple Linear Regression and Multivariate Analysis of Variance

MLR is often treated in the context of multivariate statistical analysis because of the multivariate nature of the **X** matrix, but it is a simple extension of the univariate linear regression and the same constraints apply. Estimation of the coefficients ($\boldsymbol{\beta}$) of the linear model

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta}+\boldsymbol{\varepsilon} \qquad [6]$$

where **y** is the vector of the response variable for the n observations, **X** is the $n \times p$ predictor variable matrix, $\boldsymbol{\beta}$ is the vector of the p coefficients, and $\boldsymbol{\varepsilon}$ is the vector of residuals obtained by least squares in MLR, ANOVA, and analysis of covariance (ANCOVA). The estimates obtained with this approach are the best unbiased estimates only if the classical assumptions of linear models (independent errors with a common variance, lack of correlation between errors and the predictors, accurate measurement of predictor variables) hold, which is unlikely in many practical situations. Moreover, since researchers are often interested in a parsimonious model with $m < p$ explanatory variables, stepwise methods are used to remove variables that do not add to the predictive value of a model. Except in designed experiments in which the predictor variable vectors are uncorrelated (i.e., orthogonal) by design and the number of variables is relatively low, collinearity leads to a situation in which the coefficients are not independent and their estimated value depends on the order in which the variables are entered in the model. An obvious alternative is to perform a PCA on the **X** matrix to reduce the p variables to $k < p$ uncorrelated components and then perform MLR or ANOVA on the components. This is usually referred to as principal component regression (PCR).

In several situations, a multivariate **Y** response variable matrix is available either because measurements have been taken on a number of variables or because repeated measurements have been taken on a single sample or both. Multivariate ANOVA uses extensions of the classical univariate hypothesis tests for testing the significance of the ratio between hypothesis sum of squares and cross-products matrix **H** and the error sum of squares and cross-products matrix **E** to test differences among groups.

Discriminant Analysis

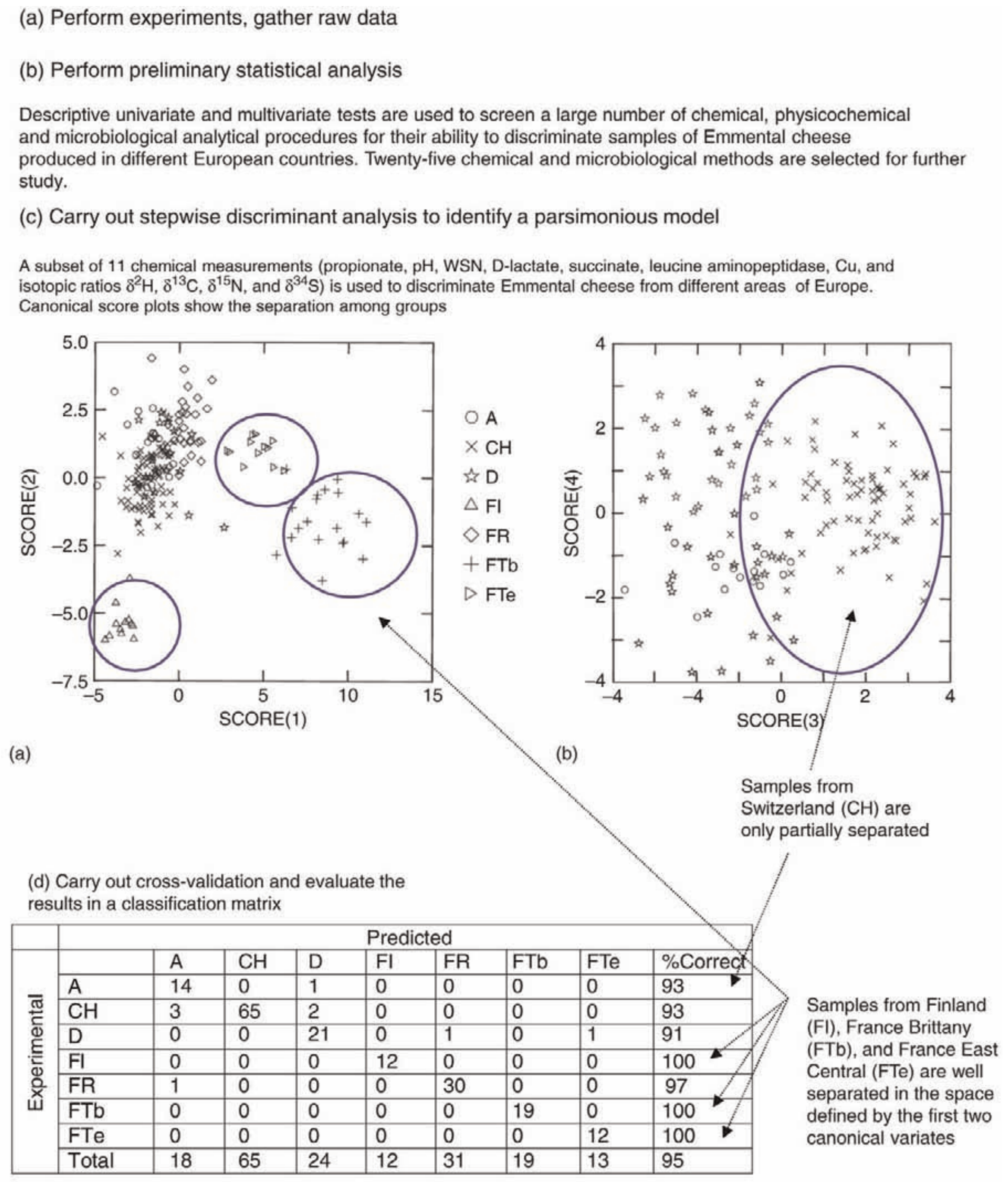
The purpose of DA is to find a classification rule to allocate unknown objects to one of two or more (g) groups the properties of which are known in advance. In the classical version of linear discriminant analysis (LDA), a training set composed of observations whose group membership is known *a priori* and for which p quantitative variables have been measured is used to build one (for two groups) or more ($\min(p,g-1)$) discriminant functions:

$$z = a_1x_1 + a_2x_2 + \cdots + a_px_p \qquad [7]$$

The coefficients of these functions allow the best discrimination by maximizing the ratio of **B** (between group) and **W**(within group) covariance matrices. New cases from a run set for which group memberships are unknown are allocated to the group to the mean of which the value of the discriminant function is closer. Stepwise DA can be performed to retain in the model only the variables that are more valuable in the discrimination. When more than two groups are present, canonical score plots of the training set observations on the best-separating dimensions (canonical variates) can be used to evaluate the output (**Figure 3**).

The performance of DA is evaluated on the basis of the misclassification rate for different classes. In most cases, the frequency of misclassifications calculated on the training set is overly optimistic and a more reliable estimate can be obtained by cross-validation. The latter can be performed either by a leave-one-out approach (jackknife), in which n classification functions are built by leaving each case out in turn and then calculating the misclassification rate on all the results, or by using more effective resampling techniques (like bootstrapping, in which m subsamples containing an adequate number of observations, usually 80%, are extracted from the original sample with replacement, and the process is repeated k times).

LDA is moderately robust toward violations of the assumption of multivariate normal distribution, but is



(a) Perform experiments, gather raw data

(b) Perform preliminary statistical analysis

Descriptive univariate and multivariate tests are used to screen a large number of chemical, physicochemical and microbiological analytical procedures for their ability to discriminate samples of Emmental cheese produced in different European countries. Twenty-five chemical and microbiological methods are selected for further study.

(c) Carry out stepwise discriminant analysis to identify a parsimonious model

A subset of 11 chemical measurements (propionate, pH, WSN, D-lactate, succinate, leucine aminopeptidase, Cu, and isotopic ratios δ^2H, δ^{13}C, δ^{15}N, and δ^{34}S) is used to discriminate Emmental cheese from different areas of Europe. Canonical score plots show the separation among groups

Samples from Switzerland (CH) are only partially separated

(d) Carry out cross-validation and evaluate the results in a classification matrix

		Predicted							
		A	CH	D	FI	FR	FTb	FTe	%Correct
Experimental	A	14	0	1	0	0	0	0	93
	CH	3	65	2	0	0	0	0	93
	D	0	0	21	0	1	0	1	91
	FI	0	0	0	12	0	0	0	100
	FR	1	0	0	0	30	0	0	97
	FTb	0	0	0	0	0	19	0	100
	FTe	0	0	0	0	0	0	12	100
	Total	18	65	24	12	31	19	13	95

Samples from Finland (FI), France Brittany (FTb), and France East Central (FTe) are well separated in the space defined by the first two canonical variates

Figure 3 An example of application of inferential multivariate methods for the discrimination of cheese samples. Classification of Emmental cheese by discriminant analysis is presented. A, Austria; D, Germany; FR, France Savoil; WSN, water-soluble nitrogen. From Pillonel L, Bütikofer U, Schlichtherle-Cerny H, Tabacchi R, and Bosset JO (2005) Geographic origin of European Emmental. Use of discriminant analysis and artificial neural network for classification purposes. *International Dairy Journal* 15: 557–562.

very sensitive to violations of the assumption of a common covariance matrix for the groups. Quadratic DA can be used when the covariance matrices of the groups are known to differ.

DA has been used in cheese authenticity studies with some success. An example of the discrimination of Emmental cheeses from different areas of Europe is presented in **Figure 3**.

Partial Least Square Regression

The use of PLSR has been increasing steadily in chemometrics since its introduction in 1975–80 as a tool for modeling the relationships between two data matrices (**X** and **Y**) and the relationships among variables within the matrices. The name derives from the original algorithm (nonlinear iterative partial least squares) used for the estimation of the model parameters. When used in chemistry to estimate the relationship among the properties of a molecule from its composition or structure, the terms quantitative structure–activity relationship (QSAR) or quantitative structure–property relationships (QSPRs) are frequently used. PLS models are extremely versatile and can be efficiently used to estimate the parameters of a large variety of models that were traditionally approached by MLR, multivariate analysis of variance (MANOVA), MANCOVA, or DA. In contrast to MLR and multivariate analysis of covariance (MANOVA), PLS can easily handle highly collinear **X** matrices.

A detailed illustration of PLS is beyond the scope of this article but some of the elements of the analysis are illustrated in **Figure 4**. Briefly, starting from an **X** matrix of p predictor variables, and a matrix **Y** of q response variables, the researcher is interested in modeling the relationships within the two data sets and between the **X** and **Y** variables by using a parsimonious model containing a new set of a (with $a << p$) latent variables. Prior to analysis, both the **X** and **Y** variables are centered and autoscaled by subtracting the mean and dividing by the standard deviation. Then, a PLSR algorithm is used to calculate the **X** score, weight and loading matrices, the **Y** score and weight matrices, the PLSR coefficients matrix and the **X** residuals, and **Y** residuals. PLSR differs from PCR because the scores, weights, and loadings are calculated from the combined **X** and **Y** variance–covariance matrix, rather than from the **X** variance–covariance matrix. The optimal number of latent variables is evaluated by cross-validation: the training data set is divided in smaller data sets (e.g., by bootstrapping) and parallel models are developed on all groups by sequentially removing components and evaluating the effect on the predictive ability of the model. The results can be finally validated against an independent test set. For interpretation of results, the PLSR coefficients are used to calculate predicted **Y** values from **X**, the **X** and **Y** scores are used to evaluate similarities among the observations (objects), and the weights are used to evaluate how variables combine in the model. As in other regression models, the analysis of the residuals is of paramount importance in diagnosing the model.

PLSR has been successfully used in a variety of applications in dairy science: prediction of sensory properties from chemical, rheological, and microbiological data; multivariate calibration for the prediction of chemical and rheological parameters from rapid nondestructive spectroscopic techniques; and prediction of age of cheese from spectroscopic data.

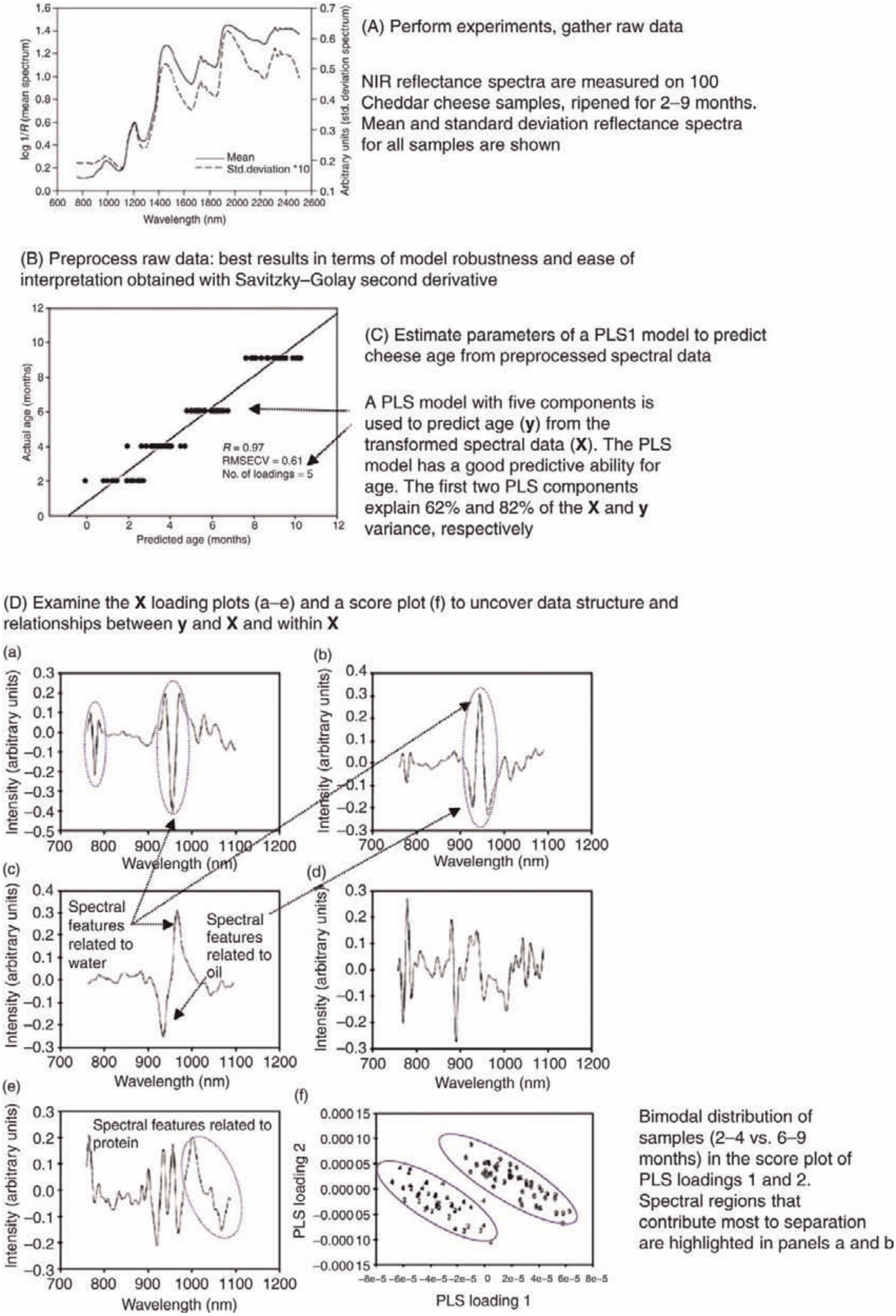
Artificial Neural Networks

Basics

Artificial neural networks (ANNs) are a valuable alternative to classical multivariate statistical methods for prediction and classification purposes. ANNs simulate in software biological neural networks: like their biological counterpart, they are made of simple processing units, the neurons, which are connected with the external environment and/or with other neurons. In one of the most common network architectures, the multilayer perceptron (MLP), there are three or more layers of neurons: input neurons, which receive and process discrete or continuous inputs from the data set; output neurons, which return the output; and one or more hidden neuron layers. The artificial neurons are connected by synapses and mimic the behavior of biological neurons: they receive a (weighted) input from the environment or from other neurons, and use a transfer or activation function to process the sum of the inputs and transfer it to other neurons or to generate results.

In analogy with biological neurons, artificial networks 'learn' their task (pattern recognition, prediction, etc.) during an iterative training process in which the synaptic weights are adjusted using a variety of algorithms. Training can be supervised or unsupervised. In supervised training, input signals are paired with the desired response, while in unsupervised training, no response is paired to the input patterns and the network creates its own representation of the data. If the network is properly designed and trained, it becomes able to generalize, that is, to provide reasonable outputs for inputs to which it had not been exposed during training.

ANNs can be used to analyze continuous, categorical, and symbolic data and are often relatively robust toward missing, fuzzy, or inconsistent data. Moreover, supervised networks can handle complex nonlinear classification and prediction problems due to the presence of multiple layers of neurons and the use of nonlinear activation functions. On the other hand, ANNs behave as black boxes, that is, it is extremely difficult, if not impossible, to establish why a given input is generating the corresponding output. As such, they are extremely useful when the level of prior knowledge on the problem is poor or when the problem is too complex to be handled by mechanistic modeling, but they do not increase knowledge on the behavior of the system. Critical issues in the development of ANNs are the identification of the network architecture and training algorithm that is more suited to handle a given problem, data standardization,



(A) Perform experiments, gather raw data

NIR reflectance spectra are measured on 100 Cheddar cheese samples, ripened for 2–9 months. Mean and standard deviation reflectance spectra for all samples are shown

(B) Preprocess raw data: best results in terms of model robustness and ease of interpretation obtained with Savitzky–Golay second derivative

(C) Estimate parameters of a PLS1 model to predict cheese age from preprocessed spectral data

A PLS model with five components is used to predict age (**y**) from the transformed spectral data (**X**). The PLS model has a good predictive ability for age. The first two PLS components explain 62% and 82% of the **X** and **y** variance, respectively

(D) Examine the **X** loading plots (a–e) and a score plot (f) to uncover data structure and relationships between **y** and **X** and within **X**

Bimodal distribution of samples (2–4 vs. 6–9 months) in the score plot of PLS loadings 1 and 2. Spectral regions that contribute most to separation are highlighted in panels a and b

log 1/R (mean spectrum)
Arbitrary units (std. deviation spectrum)
Wavelength (nm)
Mean
Std.deviation *10
Actual age (months)
Predicted age (months)
R = 0.97
RMSECV = 0.61
No. of loadings = 5
(a)
(b)
(c)
(d)
(e)
(f)
Intensity (arbitrary units)
Wavelength (nm)
Spectral features related to water
Spectral features related to oil
Spectral features related to protein
PLS loading 2
PLS loading 1

Figure 4 An example of application of inferential multivariate methods for the discrimination of cheese samples. Prediction of age of Cheddar cheese from near-infrared region (NIR) reflectance spectra by use of partial least square regression is presented. From Downey G, Sheehan E, Delahunty C, O'Callaghan D, Guinee T, and Howard V (2005) Prediction of maturity and sensory attributes of Cheddar cheese using near-infrared spectroscopy. *International Dairy Journal* 15: 701–709.

and the size and composition of the training, validation, and test sets.

Unsupervised Artificial Neural Networks

The best example of unsupervised ANNs is Kohonen self-organizing maps (KSOMs). KSOMs are built in analogy with the organization of some areas of the brain that process external stimuli (tactile, olfactory, visual), and in which neurons responding to the same stimulus are topologically close. Training is carried out through a competitive learning process. KSOMs have some analogies with PCA, MDS, and *k*-means and can rapidly process very large data matrices. Although they are used mostly for data partition, they can be used with symbolic outputs to produce multilayered maps (one layer for each symbolic output) and, once trained, can be used in run mode to identify the node that responds more strongly to a new input. There are several examples of the use of KSOMs for processing chromatographic or electrophoretic patterns in chemotaxonomy of microorganisms but, to date, they have not been used in specific dairy science applications.

Supervised Artificial Neural Networks

Supervised ANNs can be classified on the basis of their function (pattern recognition, prediction, forecasting), the number of layers, the extent to which neurons are connected in the network, the direction of flow of information (recurrent and nonrecurrent), the training algorithm, and the learning rule. A common network architecture is the MLP, a multilayer, fully connected, nonrecurrent feed-forward network: the network has at least three layers (input, hidden, and output layers) and neurons in each layer are connected to every other node in the forward layer immediately adjacent to it; information flows from the input to the output without recurrent loops. It is trained using a back-propagation algorithm that iteratively minimizes the squared difference between expected (i.e., defined by the training set) and observed output by adjusting network weights. Typically, the data are divided in three sets. The training set is used during the training step to adjust the network weights; the validation set is used during the training step to control training performance and avoid overfitting and loss of generalization; the test set is used to evaluate network performances at the end of training. During the training step, the network is initialized with random weights and it is exposed sequentially to all input values for the training set and to the corresponding output values. Signals travel from the input layer to the output layer and generate the network output, which is compared to the desired output. An error measure is calculated (usually the sum of squared differences between desired and calculated output) and the weights of the layers are adjusted in a backward fashion (i.e., starting from the uppermost hidden layer) to reduce the error. The process is iteratively repeated until the error measure becomes lower than or equal to a tolerance measure or the increase in error for the validation set shows that the network is losing generalization ability. Two alternative network architectures that have provided excellent results in a number of applications are Bayesian networks and radial basis function networks.

A critical issue in the development and use of supervised ANNs is the size and composition of the training set: for optimal performance and to ensure that the network is able to generalize, the training set should include at least W/ϵ cases, where W is the number of weights to be estimated and ϵ is the desired error rate. For example, an MLP with 10 neurons in the input layer, 5 neurons in the hidden layer, and 1 neuron in the output layer, and which has 61 synaptic connections (including bias), would require, to achieve an error rate of 5%, $61/0.05 = 1220$ cases in the training set. However, it has been shown that acceptable performances can be obtained even with relatively small training sets, if the training sets are well balanced and provide a complete representation of the input space. Normalization of inputs is also essential to avoid inputs with large scales dominating the training process.

Performance of networks used in supervised pattern recognition is usually evaluated by cross-tabulation of true and predicted identifications, while residual sum of square or similar measures are used to evaluate ANNs for regression problems.

Supervised ANNs have been used in a variety of applications in dairy science: prediction of milk yield, prediction and control of acidification processes, prediction of microbial growth, identification of microorganisms on the basis of genotypic or phenotypic data, sample recognition in conjunction with electronic noses, and cheese authenticity applications.

Software

This is a nonexhaustive list of the most frequently used general-purpose and specialized software packages for statistical analysis.

R is a powerful programming environment that is available as free software for a variety of platforms. Although it has a very steep learning curve and may be difficult to use by nonspecialized users, it offers the most comprehensive selection of graphical and statistical routines and it is continuously improved by a large scientific community. It is available for Windows, MacOS, and Linux/Unix.

SAS (available for Windows, Linux/Unix), STATISTICA (available for Windows only), and SPSS (available for Windows, MacOS, Linux/Unix) offer extensive data manipulation, statistical analysis, and graphing procedures; a variety of software modules for specialized applications are available.

SYSTAT is a general-purpose program with excellent graphing facilities and a large selection of univariate and multivariate statistical tools. SYSTAT Inc. offers MYSTAT, a (much) simplified version of SYSTAT as free software for the use of students in academic environments. SYSTAT and MYSTAT run only under Windows environments.

The Unscrambler is a specialized software that offers a large selection of multivariate statistical techniques and design of experiments. It runs only under Windows environments.

NeuroSolutions offers a variety of packages for analyzing statistical problems by using ANNs, working under different environments.

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Biosensors; Chromatographic Methods; Differential Scanning Calorimetry; DNA-Based Assays; Electrophoresis; Infrared Spectroscopy in Dairy Analysis; Light Scattering Techniques; Mass Spectrometric Methods; Microbiological; Nuclear Magnetic Resonance: Principles; Sampling; Sensory Evaluation; Spectroscopy, Overview; Statistical Methods for Analytical Data. **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health. **Rheology of Liquid and Semi-Solid Milk Products**.

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Spectroscopy, Overview

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Introduction

The term 'spectroscopy' encompasses a range of techniques for acquiring information on atomic and molecular structure. In all cases, there is absorption, emission, or scattering of electromagnetic radiation, but the basic processes by which these occur vary considerably, as does the nature of the information that can be obtained. This article provides a broad overview of the important processes that give rise to the most important spectroscopic methods used in food and nutrition.

The Electromagnetic Spectrum

When light interacts with matter, it may stimulate transitions between quantized energy levels. Light is considered to interact with matter in discrete packets (quanta) of energy called photons. The exact type of transition stimulated in the sample depends upon the energy (E) of the photon, which in turn is related to the frequency (ν) by

$$E = h\nu \quad [1]$$

where h is Planck's constant.

The energy of the quanta is highest at the gamma-ray end (10^6 eV) and lowest at the radiofrequency end (10^{-8} eV) of the electromagnetic spectrum. The full electromagnetic spectrum is shown in **Figure 1**. In this discussion, a more detailed examination will concentrate on the region between the ultraviolet and the radiofrequency ends of the spectrum, as this is where most routine chemical spectroscopy is carried out. Spectroscopy is usually concerned with the measurement of a specific spectrum, which is a measure of energy absorbed or emitted as a function of wavelength (or frequency) across a limited region. The objective is to use the spectrum to obtain information on molecular structure or for quantitative analysis. The types of transitions normally stimulated range from electronic to vibrational through rotational to the low-energy nuclear transitions examined in nuclear magnetic resonance (NMR). There are also high-energy nuclear transitions that can be stimulated by gamma radiation and are used for Mössbauer spectroscopy, but this will not be considered in this article.

As the energy required to stimulate an electronic transition is more than that for a vibrational transition, which in turn is more than that for a rotational transition, it is found that more than one transition is usually stimulated so that, for example, pure vibrational spectra are not seen and are nearly always complicated by rotational transitions.

The normal way the interaction of the radiation occurs is through one of four processes: (1) absorption; (2) emission; (3) elastic scattering, although there is no net energy absorption; and (4) inelastic scattering. These mechanisms are described in more detail as the different spectroscopic methods are discussed in the following sections. The first method to be discussed is ultraviolet–visible (UV/VIS) spectroscopy, which involves transitions between electronic energy levels.

Electronic Transitions

In UV/VIS spectroscopy, absorption of radiation is the result of the excitation of electrons between electronic energy levels. This can occur for atoms as well as molecules. Electrons in molecules are considered to reside in molecular orbitals, which can be bonding, non-bonding, or antibonding orbitals. Therefore, the transitions between electronic energy levels can be described as transitions between the molecular orbitals of the molecule. The part of the molecule that gives rise to electronic absorptions is known as a chromophore. The chromophore electrons involved in the transition are either those directly used in bond formation or those that are the non-bonding or unshared outer electrons of an electronegative atom such as oxygen, nitrogen, or sulfur. The general mechanism in a chromophore such as C=C, in which the chromophore electrons originate in the so-called π bond, involves the promotion of an electron from the bonding π orbital to an antibonding $\pi*$ orbital (the so-called $\pi-\pi*$ transition). Such a transition typically requires about 7 eV of energy – an amount of energy that corresponds to a wavelength of about 180 nm. In other chromophores, it is also possible for

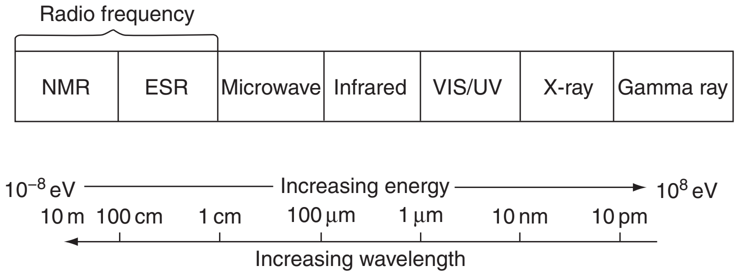
Radio frequency
NMR
ESR
Microwave
Infrared
VIS/UV
X-ray
Gamma ray
10^{-8} eV
Increasing energy
10^{8} eV
10 m
100 cm
1 cm
100 µm
1 µm
10 nm
10 pm
Increasing wavelength

Figure 1 The electromagnetic spectrum. NMR, nuclear magnetic resonance; ESR, electron spin resonance; VIS/UV, visible and ultraviolet. Reproduced with permission from Wilson RH (1993) Spectroscopy: Overview. In: Macrae R, Robinson RK, and Sadler MJ (eds.) *Encyclopedia of Food Science, Food Technology and Nutrition. London: Academic Press*.

a nonbonding (n) electron to be promoted to a $\pi*$ orbital (an $n-\pi*$ transition). These two kinds of transitions are the most common, although similar ones exist for single (σ) bonds, including $n-\sigma*$ and $\sigma-\sigma*$ transitions. However, because the latter require much higher energies, they are seen in the vacuum UV and are harder to observe. The observed UV/VIS absorption frequencies can be influenced by solvents and by the delocalization of electrons in conjugated systems.

Transition metal ions absorb in the UV/VIS region, and the transitions responsible involve 4f and 5d electrons. Alternatively, in some inorganic complexes, the process of charge transfer absorption occurs.

Most UV/VIS spectroscopy involves absorption processes, and normally, a spectrophotometer is used to measure the spectrum. The major components of the spectrophotometer are a source, a dispersing system, and a detector. Normally, light from a suitable source is passed through a prism or grating where it is dispersed into its component frequencies. The dispersing element may be rotated so that each frequency passes in turn through a narrow slit. This light may be divided so that one half passes through a channel containing the sample and the other half through a reference channel. The intensities of the emerging beams are measured with a detector and the ratio of these signals is used to determine the absorbance. In this manner, as the dispersing element is moved, the absorbance of the sample as a function of frequency (the spectrum of the sample) is determined. Modern instruments use diode array detectors (essentially a series of detectors) and a slightly different experimental set up to obtain absorption spectra more quickly than dispersive UV/Vis instruments.

It should be noted that UV/VIS spectra do not consist of discrete lines. The reason is that the high energy of the UV/VIS region can be transferred into the vibrational and rotational substates so that both types of transitions are simultaneously stimulated with the electronic transition. In **Figure 2** the energy level diagram for a chromophore is shown. E_0 and E_1 represent the ground

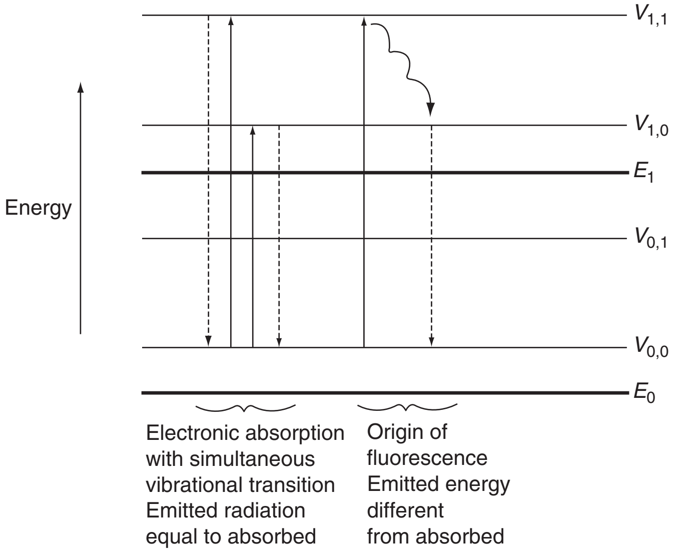
$V_{1,1}$
$V_{1,0}$
E_1
Energy
$V_{0,1}$
$V_{0,0}$
E_0
Electronic absorption with simultaneous vibrational transition Emitted radiation equal to absorbed
Origin of fluorescence Emitted energy different from absorbed

Figure 2 Energy levels for a chromophore, showing electronic and vibrational levels. (Reproduced with permission from Wilson RH (1993) Spectroscopy: Overview. In: Macrae R, Robinson RK, and Sadler (eds.) *Encyclopedia of Food Science, Food Technology and Nutrition*. London: Academic Press.

and excited electronic energy levels of the molecule. Each electronic level has associated with it various vibrational sublevels $v_{0,1}$, $v_{0,2}$, and so on, where the first subscript indicates the electronic level, and the second indicates the vibrational level within that electronic state. The vibrational levels, in turn, have rotational sublevels associated with them. An electron may be promoted from the E_0 to E_1 electronic state but may go from the $v_{0,0}$ state to the $v_{1,0}$ or the $v_{1,1}$ state as part of this transition. In the latter case, there is a simultaneous vibrational transition (**Figure 2**) that accompanies the electronic transition. The range of vibrational subtransitions possible, combined with rotational transitions, means that there is no discrete frequency at which transition occurs, but rather a range of absorption frequencies that generally form a bell-shaped profile in the spectrum.

Lastly, if an electron is promoted from the $v_{0,0}$ state of E_0 to the $v_{1,1}$ state (or higher) of E_1, it may lose energy through collision with neighboring molecules, for example, and become lowered into the $v_{1,0}$ state. During subsequent relaxation to the ground electronic state, E_0, a photon is emitted with an energy different from that absorbed, and this process is called fluorescence.

Vibrational Spectroscopy

The transitions between the vibrational energy levels are the basis of infrared spectroscopy. The infrared region is divided into near-, middle- (or mid-), and far-infrared. This division is on the basis of instrumental factors as well as the types of vibration that occur in each region. It is easiest to consider first the middle infrared, which is usually considered to lie between 2.5 and 25 μm in wavelength. It is common practice, however, for vibrational spectroscopists to use the wavenumber unit (reciprocal of the wavelength in centimeters, or cm^{-1}) rather than wavelength. Expressed in this unit, the middle infrared then stretches from 4000 to 400 cm^{-1}. As they are reciprocal to wavelength, wave numbers, like frequency, are directly proportional to energy.

The bond between two atoms can be considered rather like a spring that has a certain strength of force constant (k). The bond, or spring, can be stretched and caused to oscillate. It will do so at some natural frequency, f, which depends upon k and the masses of the atoms according to Hooke's law:

$$f = \left(\frac{1}{2\pi}\right)\sqrt{\frac{k}{\mu}} \qquad [2]$$

where μ is the reduced mass defined as

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \qquad [3]$$

with m_1 and m_2 being the masses of the individual atoms constituting the bond. Equation [2] shows that a particular bond will give rise to a characteristic frequency that depends upon the masses of the atoms and the strength of the bond. Therefore, a C=O bond, which has a larger force constant than a C–O bond, will have a larger vibrational frequency. In practice, different functional groups give rise to characteristic vibrational frequencies. These characteristic vibrational frequencies underlie a key use for vibrational spectroscopy; it is a highly useful probe for the identification of functional groups and for structural determination. It has greater selectivity than UV/VIS in this respect.

Equation [2] is derived from classical physics, but of course the actual process for molecules is quantized and eqn [2] should be written as

$$f = \left(\frac{h}{2\pi}\right)\sqrt{\frac{k}{\mu}} \qquad [4]$$

In general, an infrared spectrum is generated by absorption using a similar arrangement to that used for a UV/VIS spectrum but with different source, detector, and dispersing optics. The process is illustrated in **Figure 3**. The energy level diagram shows the ground ($v_{0,0}$) and excited ($v_{0,1}$) vibrational states. Note that both states occur in the ground electronic state, as infrared energies are not sufficient to excite electronic transitions. Also shown are the various rotational substates (J' and J'). Excitation can occur from $v_{0,0}$ ($J' = 0$) to $v_{0,1}$ ($J' = 0$), corresponding to the band center of the absorption. However, excitation

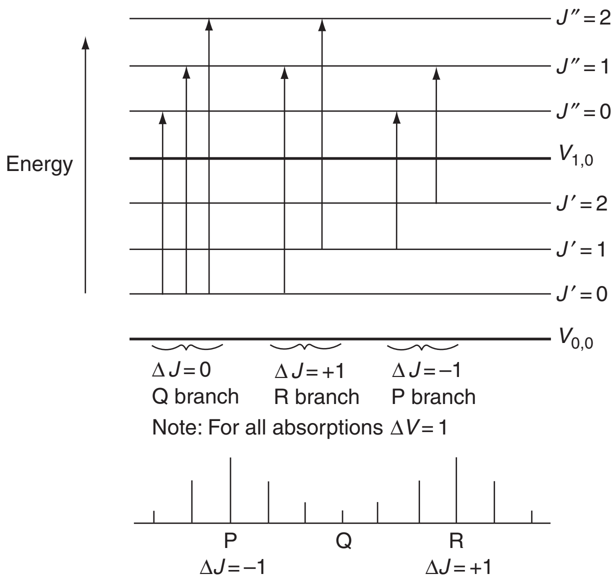

Figure 3 Energy levels for infrared transitions, showing vibrational and rotational levels. Reproduced with permission from Wilson RH (1993) Spectroscopy: Overview. In: Macrae R, Robinson RK, and Sadler MJ (eds.) *Encyclopedia of Food Science, Food Technology and Nutrition*. London: Academic Press.

from the $' = 1$ to $J' = 1$ rotational substates (i.e., $\Delta J = 0$) will produce a slightly different frequency than the $J' = 0$ to $J' = 0$ transition as the rotational sublevels are not equally spaced. It is also possible for ΔJ to be ± 1, giving rise now to a complicated absorption spectrum comprising a central absorption (Q branch, from the $\Delta J = 0$ transitions) with equally spaced lines to either side called the P branch ($\Delta J = -1$) and the R branch ($\Delta J = +1$). This structure is seen as such only in the gas phase. In solid or solution state, the result is that a broad absorption rather than a sharp series of lines is seen. However, no absorption will be seen at all unless there is a change in dipole moment during the vibration. This is a fundamental rule of infrared vibrational absorption spectroscopy. Consequently, homonuclear bonds do not absorb, as their dipole moments do not change with vibrational motion.

At normal room temperature, most molecules will be in the ground vibrational state. However, as the temperature is increased, a more significant population will develop in the first excited state. As a result, transitions from the v_1 to v_0 state can occur with the emission of a photon. This is the process of infrared emission which is, albeit rare, an alternative to absorption spectroscopy. In this case, the (heated) sample acts as the infrared source.

Infrared spectroscopy had been of little use for industrial, biological, and food analysis purposes owing to the difficulties of sample handling and the time required for data acquisition. However, the advent of Fourier transform methods, which use an interferometer rather than a dispersing element, provided increased speed, throughput, and frequency reproducibility as compared to infrared absorption experiments. When coupled with new methods of sample presentation, this method has enabled the useful application of middle-infrared spectroscopy to food science.

The most common absorptions in the middle infrared are fundamental transitions, which originate from the ground vibrational state (v_0) and involve an excitation to the next higher vibrational level (v_1). However, various overtones and combinations of the fundamental transitions can arise. For example, a molecule with two fundamental transitions at frequencies ν_1 and ν_2 may give overtones at $2\nu_1$, $3\nu_1$, $4\nu_1$ or $2\nu_2$, $3\nu_2$, and so on, or combinations at, say, $\nu_1 + \nu_2$ or $2\nu_1 + \nu_2$. In practice, not all fundamentals give rise to overtones, but usually only bonds in which a heavy atom such as N or O is coupled to hydrogen. The overtone and combinations often occur in the near-infrared (2.5–0.7 μm), which, despite the apparent complexity of the spectra, has found considerable application in food problems.

Raman Spectroscopy

If a sample is illuminated with monochromatic visible light, it is found that much of the light is scattered and that this scattered radiation is of the same frequency as the illuminating light. This occurrence is known as elastic or Rayleigh scattering. However, analysis shows that a small amount ($<10^{-6}$) of the incident radiation is scattered at a different frequency. This process is called inelastic or Raman scattering. Raman scattering leads to a series of lines appearing at frequencies less than that of the incident light (Stokes lines) and a weaker series of the same type of lines at higher frequencies than that of the incident light (anti-Stokes lines). Though they are measured in a different manner to an infrared spectrum, these lines also correspond to vibrational transitions of the sample. When a set of these lines is presented as a spectrum of intensity versus the frequency shift away from the Rayleigh frequency, the result is called a Raman spectrum. Typically, a shift of about 20–4000 cm^{-1} from the Rayleigh frequency is depicted in a Raman spectrum, representing a similar range to that presented in an infrared spectrum. The Stokes lines are typically chosen to illustrate the Raman spectrum, as they are the more intense of the two sets of transitions.

In the Raman effect, the electric field of the incident radiation interacts with the electrons in the sample so that molecules are momentarily excited to a virtual state, virtual in the sense that it does not represent a defined energy level of the molecule. Most molecules in the sample relax from this virtual state by the emission of energy of the same frequency as that absorbed (Rayleigh scattering). In this instance, the molecule relaxes back to its original vibrational state, which in the great majority of cases is the ground vibrational state. In a few cases, however, the sample will relax to a vibrational state above the ground state, shifting the frequency of the scattered light to lower values. The difference between this value and the Rayleigh scattered frequency is equal to the frequency of a vibrational motion and represents the Raman shift. The transitions that occur in this way from the ground vibrational state lead to the Stokes lines. Much fewer molecules will be in non-ground vibrational states before excitation, and when they relax from the virtual state, the emitted photon will be of higher energy than the incident radiation, leading to the anti-Stokes lines.

In contrast to infrared spectroscopy, there must be a change in the electronic polarizability during the vibration of the molecule for that vibrational transition to be observed in the Raman spectrum. There is thus a distinct difference in the two kinds of spectra, and vibrations that may be weak or absent in infrared spectra are present and perhaps strong in the Raman. The two spectroscopic methods are thus complementary and together provide a complete picture of the vibrational states of a molecule.

Far-Infrared/Microwave

To complete the picture, at lower energy there is the far-infrared region (10–400 cm^{-1}), which has major applications in inorganic chemistry because herein appear bonds between metals and organic ligands as well as the skeletal vibrations of molecular backbones. This region is of limited application in food and nutritional studies.

In the microwave region, at even lower energy, pure rotational spectra can be produced. However, they will not be addressed here, as it is also of limited application.

At the radiofrequency end of the spectrum is NMR spectroscopy, which involves transitions between the magnetic quantum levels of atomic nuclei. Nuclei have properties of spin and magnetic moment. Splitting of the energy levels can be induced by placement in a magnetic field, and transitions can be induced by the application of radiofrequency radiation. This is usually achieved by irradiating the sample exposed to a high magnetic field with a pulse of broadband radiation. After excitation, the nuclei reemit energy at their resonance frequencies. The observed signal is a combination of these frequencies and decays with time. A spectrum can be produced by the Fourier transformation of this decaying signal. The usefulness of the technique lies in the fact that the resonance frequency of a given nucleus depends upon its chemical environment. The range of NMR experiments possible is very large indeed and it is a powerful method for structural analysis.

In the food industry, the use of NMR spectra as such is limited. Instead, relaxation time measurements are more important, particularly in the determination of solid/liquid ratios. The relaxation rate from the excited state depends on environmental factors and molecular mobility.

Absorption Laws

In UV/VIS, near-infrared, and mid-infrared absorption spectroscopy, the fundamental law governing absorption is the Beer–Lambert relationship. For a sample illuminated by radiation of intensity I_0, the amount transmitted, I, is given by

$$I = I_0 e^{-\varepsilon cl} \qquad [5]$$

where c is the concentration of absorbing species, l is the pathlength through which the light passes, and ε is the molar absorptivity.

For quantitative analysis, spectra are usually presented in absorbance units, where absorbance, A, is defined as

$$A = -\log\left(\frac{I}{I_0}\right) = \varepsilon cl \qquad [6]$$

so that absorbance is directly proportional to the concentration at constant pathlength.

Practically, optical spectroscopy requires that ε be determined for any absorbing species. This is achieved by calibration. Solutions of the sample to be determined are prepared at various known concentrations and their absorbance values are measured. When the absorbance values are plotted against the concentration, a linear plot is produced with a slope equal to ε. This plot is known as a calibration curve. Unknown concentrations can then be calculated by measuring the absorbance and using the equation of the line from the calibration curve.

Deviations from the Beer–Lambert relationship can occur if too wide a range of concentrations is chosen so that solute–solute interactions occur, or if there is chemical interaction between the solution components.

A particular problem that exists in the near- and mid-infrared is where a significant overlap of absorbance peaks occurs. Clearly, the absorbance at a given wavelength may then depend upon more than one concentration, so that

$$A = \varepsilon_1 c_1 + \varepsilon_2 c_2 + \varepsilon_3 c_3 + \cdots \qquad [7]$$

Hence, more complicated solutions to the Beer–Lambert relationship may be required for multicomponent analysis. Such methods include *p* and *k* matrix, partial least squares, or principal-components regression.

In NMR single-pulse experiments, the signal observed is directly proportional to the number of nuclei, provided sufficient time is allowed between pulses for the reestablishment of equilibrium. Under such circumstances, the NMR experiment is quantitative and requires no calibration. Double-resonance experiments can, however, lead to enhanced signals for certain nuclei (nuclear Overhauser effect) so that some form of calibration is necessary. In relaxation measurements, the magnetization decay can be broken down into contributions from fast (solid) and slow (liquid) components, the relative magnitude of each reflecting the relative concentrations.

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Infrared Spectroscopy in Dairy Analysis; Nuclear Magnetic Resonance: An Introduction; Nuclear Magnetic Resonance: Principles.

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Infrared Spectroscopy in Dairy Analysis

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Introduction

Use of spectroscopic methods in dairy laboratories has become common in recent years. Several spectroscopic techniques, including ultraviolet-visible (UV-Vis) spectroscopy, atomic absorption spectroscopy, hyperspectral imaging, and nuclear magnetic resonance spectroscopy, have been applied to analysis and study of dairy products. The principles of these techniques and their applications to dairy products are discussed in detail in other articles in this encyclopedia. Among all the spectroscopic techniques, infrared (IR) spectroscopy has received special attention and is being used increasingly by the dairy industry and research institutions. The advantages of IR spectroscopy that make it an excellent analytical tool include:

1. Simplicity, sensitivity, and speed of detection
2. High throughput (ability to analyze several samples in a short time)
3. Possibility of non-destructive analysis depending on the application
4. Requirement of relatively low sample volumes
5. Possibility of simultaneous analysis of multiple analytes
6. Less use of hazardous solvents that pose environmental and health hazards
7. Relatively low operational cost
8. Capability of on-site (using hand-held spectrometers) and in-line analysis.

Although Fourier transform IR (FT-IR) spectroscopy is almost a century old, its application to dairy products analysis and research received attention only during the past 2–3 decades. The areas of application of IR spectroscopy in dairy analysis include raw materials control, process control, and analysis of finished products. Readers are encouraged to refer to the publications listed in the 'Further Reading' section of this article for more details on specific topics. This article describes the theory of IR spectroscopy and provides an overview of various laboratory and industrial applications reported to date on analysis of composition and quality of dairy products.

Infrared Spectroscopy

Electromagnetic waves can interact with materials in different ways. The wave can pass through the material (transmission), or it can be reflected at the surface (reflection), with some part of the wave energy being absorbed by the material. Absorption of energy by a molecule can cause the molecule to translate or rotate, or it can cause specific groups within the molecule to vibrate or some electrons of the molecule to get excited. IR spectroscopy monitors the vibrations exhibited by molecules under IR light. In stretching vibrations, the atoms move in the same direction (symmetric) or opposite directions (asymmetric). Bending vibrations include scissoring, rocking, twisting, and wagging. To induce these vibrations, (1) the energy of the radiation must exactly match the energy difference between the ground state and excited state, and (2) the dipole moment of the molecule must change. These two conditions are called resonance conditions for unpolarized light.

The IR spectrometer produces IR light over a range of wavelengths and monitors the vibration of molecules. A schematic diagram of an FT-IR spectrometer is shown in **Figure 1**. Essentially, it consists of a source to produce IR light, an interferometer to generate a range of wave numbers, and a detector to record the signal. The interferometer is the heart of a spectrometer and consists of a fixed mirror, moving mirror, and a beamsplitter. The beamsplitter splits the IR beam and recombines it to produce different IR wavelengths. A series of mirrors is used to deflect the beam. The laser acts as a time reference for data collection. The detector records the signal as an interferogram, which is then Fourier-transformed to result in a single-beam spectrum. Fourier transform is a mathematical function to convert data to a more meaningful absorbance/transmittance over a frequency/wavelength form. A single-beam spectrum of the background and the sample are ratioed to obtain an IR spectrum of the sample. Use of the absorption information obtained with an interferometer by FT-IR spectroscopy improves analysis speed because all frequencies are measured simultaneously. It offers simplified mechanics with only one moving part (i.e., the moving mirror) in the instrument, which reduces the risk of mechanical breakdown and makes it less sensitive to temperature variations and vibrations in field use. Spectral reproducibility and

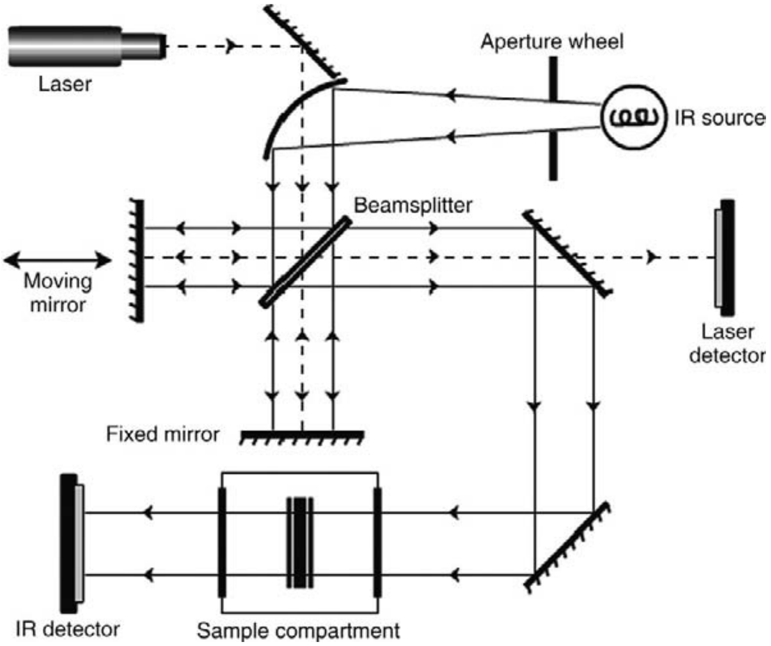

Figure 1 Optical layout of a typical FT-IR spectrometer. The moving mirror, fixed mirror, and the beamsplitter assembly are commonly referred to as the interferometer, or Michelson interferometer. Reprinted from Sun D-W (2009) Infrared Spectroscopy for Food Quality Analysis and Control. New York: Academic Press, with permission from Elsevier.

wavenumber precision are also better compared with that of dispersive instruments.

Fourier Transform Near-Infrared Spectroscopy

Fourier transform near-infrared (FT-NIR) spectroscopy involves studying the absorption of compounds in the NIR range (10 000–4000 cm^{-1}) of the electromagnetic spectrum. A typical FT-NIR spectrum consists of overtone and combination bands of fundamental vibrations. A band can be produced at frequencies 2–3 times the fundamental frequency (overtone). Majority of the overtone peaks in an FT-NIR spectrum are due to O–H, C–H, S–H, and N–H stretching modes. Two or more vibrations can combine through addition and subtraction of energies to give a single band (combination). NIR radiation penetrates the sample more than does mid-infrared (MIR) radiation, and its absorption bands are approximately 10–100 times less intense than MIR absorption bands. This can be very useful in direct analysis of highly absorbing bulk and porous samples with little or no sample preparation. NIR instruments can be deployed readily to the field or process lines for direct and simultaneous measurements of several constituents in food matrices. In contrast to MIR spectrometers, NIR instruments are more rugged and less energy-limited and offer more flexible handling options, which makes it possible to analyze samples in convenient glass vials. The relatively weak absorption provides a built-in dilution series that has faciliated direct analysis of samples that are highly absorbing and strongly light scattering without dilution or extensive sample preparation, enabling high-moisture foods to be readily analyzed.

An FT-NIR spectrum is complex and is marked by broad overlapping peaks and large baseline variations, which makes interpretation difficult. However, mathematical processing such as derivatization and deconvolution can be applied to improve spectral characteristics. Typical FT-NIR absorbance spectra of whey protein concentrate and Swiss cheese are shown in **Figure 2**. Overtone bands of C–H groups of fatty acids appear in the regions 8600–8150 cm^{-1} (first overtone) and 5950–5600 cm^{-1} (second overtone). The regions 7400–7000 cm^{-1} and 4350–4033 cm^{-1} can be attributed to combination bands of C–H, typically from fatty acids and carbohydrates. Bands of O–H groups can be identified in the spectral regions 5200–5100 cm^{-1} (first overtone) and 5190 cm^{-1} (O–H stretch). Stretching and combination vibrations of N–H and C=O of amide A/I and amide B/II of proteins absorb between 5000 and 4500 cm^{-1}. Several other overtone and combination bands are present in the FT-NIR spectrum.

Fourier Transform Mid-Infrared Spectroscopy

Fourier transform mid-infrared (FT-MIR) spectroscopy monitors the fundamental vibrational and rotational stretching of molecules, which produces a chemical profile of the sample. The MIR region (4000−400 cm^{-1}) is a very robust and reproducible region of the electromagnetic spectrum in which very small differences in composition

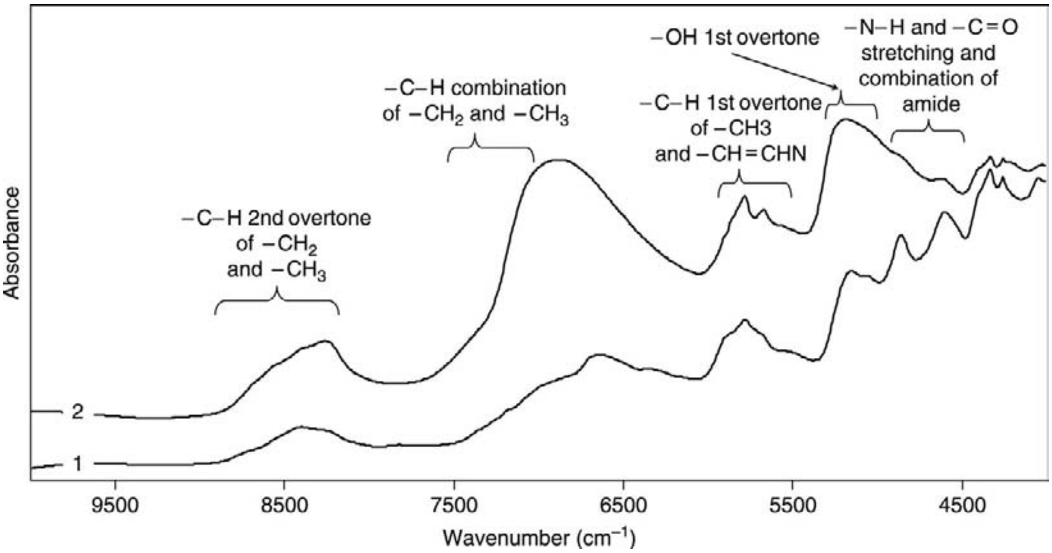

Figure 2 FT-NIR spectrum of (1) whey protein concentrate and (2) Swiss cheese.

of samples can be measured reliably. Molecules absorb MIR energy and exhibit vibrations at one or more locations in the spectra, depending on several factors including bond configuration, location, and so forth. MIR spectrum is rich in information that helps in analyzing the composition and determining the structure of chemical molecules.

FT-MIR spectroscopy enables monitoring of minor functional groups. However, such applications frequently require a sample clean-up such as extraction to reduce the effect of sample matrix. FT-MIR spectra of Cheddar cheese and its water-soluble extract are shown in **Figure 3**. The spectra of Cheddar cheese are marked by absorbance from complex fat and protein in the regions 3000–2800 cm^{-1} and 1800–1000 cm^{-1}, respectively. Asymmetric and symmetric stretching vibrations of C–H groups in methylene groups of long-chain fatty acids are observed in the region 3000–2800 cm^{-1}. A strong signal from the C=O groups of fatty acid esters is also present at 1740 cm^{-1}. Broad amide I and amide II bands of proteins peak at around ~1640 and ~1540 cm^{-1}, respectively. The spectra also include several other bands in the region 1800–900 cm^{-1}, primarily due to C–H bending, C–O–H in-plane bending, and C–O stretching vibrations of lipids, organic acids, amino acids, and carbohydrate derivatives.

Sampling Techniques

Many different sampling techniques are available for both FT-NIR and FT-MIR spectroscopy. Transmittance, reflectance, attenuated total reflectance, diffuse

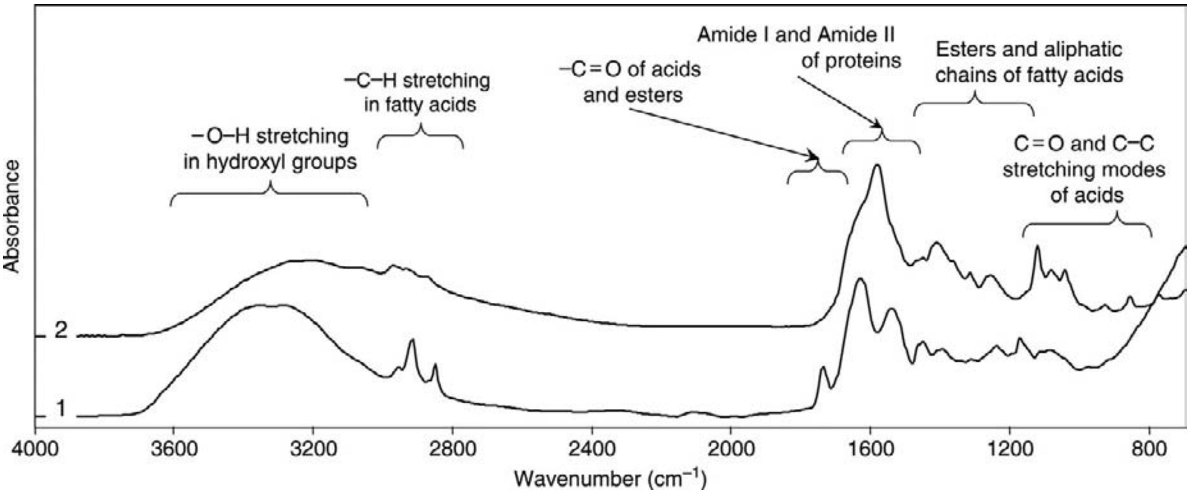

Figure 3 FT-MIR spectrum of (1) Cheddar cheese and (2) Cheddar cheese water-soluble extract. Extraction of sample may often be required to reduce the effect of sample matrix and enhance signal from analytes of interest.

reflectance, specular reflectance, photoacoustics, and integrating spheres are some examples. Selection of sampling technique depends on the type of analysis (e.g., structure, composition, etc.), sample surface characteristics (e.g., reflectivity, smoothness, etc.), sample form (solid, liquid, or gas), and requirements (e.g., speed, convenience, accuracy, details, etc.). A reflectance spectrum is the result of both absorption and scattering. Even if the sample is homogeneous with respect to chemical constitution, there will always be scattering variation due to the physical properties of the sample. Heterogeneity can be the result of particle surface, particle size, the orientation and density or compactness of material, surface roughness, distance from the sample to the probe, and sample movement (if applicable). The development of various mathematical pretreatments and multivariate calibration methods provides analysts with a means of overcoming these problems. When sample volume is a limitation, the application of FT-IR microspectroscopy may significantly improve the sensitivity, reproducibility, and differentiation capabilities of the IR technology through use of an infinity-corrected microscope. Additionally, it allows for visualizing the sample and selecting scan regions. FT-IR microspectroscopy has been applied for the characterization and identification of a few hundred microbial cells, and the technology could be useful for rapid and high-throughput screening of microorganisms.

Standardization and Spectral Data Preprocessing

Qualitative and quantitative analysis both require high-quality spectral information. Standardization of experimental conditions and sample preparation greatly reduce variation between samples and improve accuracy of analysis. However, some minor deviations may still occur due to instrumental variations between samples. Such noises and differences may be reduced by using mathematical spectral pretreatment methods such as differentiation, baseline correction, and multiplicative signal correction. Mathematical pretreatments can allow extraction of useful band information through removal of baseline variations, resolution of overlapping peaks, and correction of scatter effects. Mathematical transformations of the data are used to enhance the prediction ability of the models and the qualitative interpretation of the spectra. Standardization of the spectra by using smoothing and multiplicative scatter correction (MSC) and signal normal variate (SNV) preprocessing steps improves the signal-to-noise ratio of the data and corrects for the nonlinear light-scattering effects of the samples and baseline offsets. The Savitzky–Golay second-derivative transform allows the extraction of useful band information through the removal of baseline variations and resolution of overlapping peaks.

Statistical Analysis

Normally, IR spectra comprise absorptions from several analytes and even sample matrix. The NIR region contains weak and broad overtone and combination bands that make it difficult to identify and associate IR frequencies with specific chemical groups. Building calibration models in such situations requires monitoring more than one variable to predict the concentration of more than one analyte. Although an FT-MIR spectrum is relatively easier to interpret than an FT-NIR spectrum, it is still complex for a non-technical operator and does not provide any quantitative results by itself. Statistical or chemometric analysis (e.g., principal component analysis) is required to build prediction models, simplify interpretation, and draw meaningful information from the spectra. Modern multivariate statistical software and procedures are capable of correlating changes in multiple IR wavenumbers to one or more dependent variables (e.g., concentrations). Use of these types of software is usually required to fully tap the potential of IR spectroscopy.

Analysis of Dairy Products

Applications of IR spectroscopy in the dairy industry and in research studies are growing. The primary reason for the dairy industry to adopt IR spectroscopy is the speed and convenience it offers over traditional methods. Applications of IR spectroscopy including methods currently in use and those being tested for analysis of composition, constituents, and characteristics of some dairy products and ingredients are summarized in **Table 1**. Common industrial applications are focused primarily on determination of the concentrations of constituents and determination of purity and authenticity or adulteration of products. Research applications range from simple concentration determination to monitoring complex biochemical and structural changes in components during processing and characterization of dairy microorganisms.

Quantitative Analysis

Unlike most other analytical techniques, quantification using IR spectroscopy rarely involves monitoring a single peak or an IR absorption band. Classical least squares regression, inverse least squares regression, principal component regression, partial least squares regression, and artificial neural networks are some examples of calibration methods available for quantitative analysis of spectroscopic data.

Compositional analysis is by far the most common quantitative application of IR spectroscopy in the dairy industry. Analysis of product constituents such as moisture, solids, fat, protein, and so forth, is applied to almost

Table 1 Applications of IR spectroscopy in analysis of composition, constituents, and characteristics of dairy products

Dairy products	*Analyte/analysis*
Milk, evaporated milk, cream, coffee cream, and cocoa concentrate	Fat, protein, casein, whey proteins, lactose, dry matter, cholesterol, total carbohydrates, energetic values, calcium, and detection of antibiotics (e.g., tetracycline)
Cheese	Fat, protein, salt, pH, moisture, amino acids, organic acids, fatty acids, age, study of ripening changes, flavor quality (e.g., fermented, unclean, sour, etc.), cholesterol, characterization of microorganisms, shelf life, study of lipolysis, identification of geographic origin, sensory flavor descriptors (e.g., whey, nutty, diacetyl, fruity, sulfur, etc.), rheologic characteristics (e.g., firmness, texture, rigidity, etc.), and process control (e.g., monitoring coagulation, syneresis, ripening, etc.)
Yogurt and fermented milk products	Solids, pH, cholesterol, sugars, protein, calcium, and energetic value
Casein and whey proteins	Structural studies of casein and whey proteins and monitoring changes during processing
Butter	Moisture, fat, solids, cholesterol, authentication of organic butter, solid fat content, acid value, adulteration, and study of changes during storage
Ice cream	Viscosity, conductivity, and structural parameters (e.g., ice crystal size)
Milk powder and creamer	Moisture, protein, cholesterol, and adulteration

all dairy products including milk, cheese, yogurt, and milk powders. An official method based on IR spectroscopy, approved by the association of official analytical chemists (AOAC) International (AOAC method 972.16), exists for the determination of fat, protein, lactose, and total solids content of milk. **Figure 4(a)** shows a typical partial least squares regression (PLSR) calibration model developed using FT-MIR spectra and the Schmid–Bondzynski–Ratzlaff method for prediction of fat content in Cheddar cheese. In addition to the macromolecules such as sugars, proteins, and fats, minor compounds in dairy products such as amino acids and organic acids can also be analyzed using FT-MIR. The PLSR calibration model in **Figure 4(b)** shows the correlation between IR spectra and concentration of glutamic acid in Cheddar cheese determined by the reference gas chromatographic method. By plugging the spectra of test samples into the calibration models, it is possible to rapidly determine the parameter of interest.

Calibration models have been developed and tested for several other product characteristics: pH, salt, cholesterol, short-chain fatty acids, quantitative sensory descriptors, rheologic indices, microbial count, and so forth. Furthermore, most versions of chemometric software allow simultaneous determination of multiple characteristics (e.g., fat, moisture, solids, individual amino acids, individual organic acids, etc.) from the same spectra. These advantages make IR spectroscopy a great time-, cost-, and labor-saving analytical tool.

Qualitative Analysis

Qualitative analysis compares spectra and looks for similarities or differences. Most common applications include

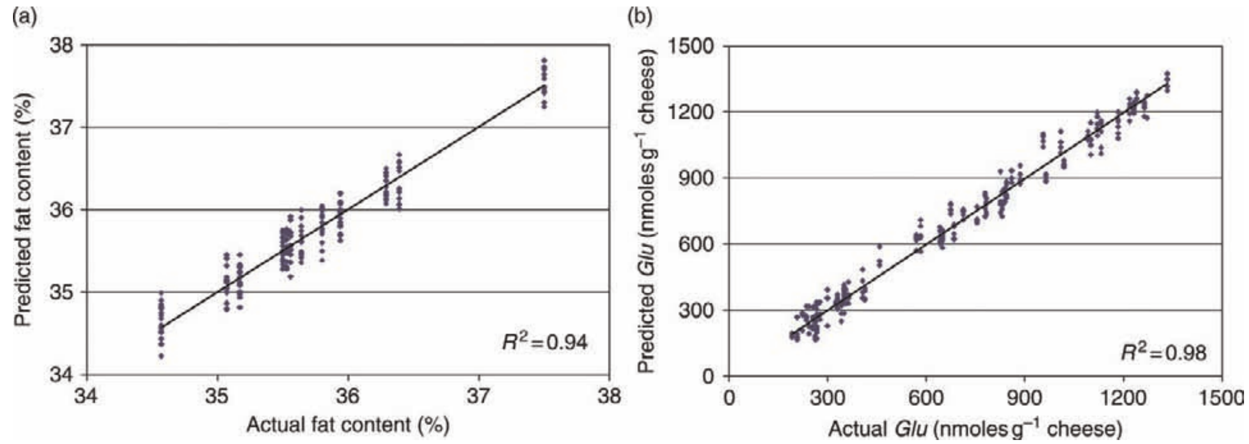

Figure 4 Partial least squares regression (PLSR) calibration models for (a) fat content (%) and (b) concentration of glutamic acid (*Glu*; nmol g^{-1} cheese) in cheddar cheese. The high R^2 values indicate a good fit of IR spectra with the reference methods (fat, Schmid–Bondzynski–Ratzlaff method; glutamic acid, gas chromatography with flame ionization detector).

identification of unknown samples or detection of differences between samples. A spectral library of known substances to which unknown spectra can be compared is required. The spectral distance is computed to determine whether or not the unknown substance has the same spectral distance as that of a spectrum in a library. If the distance is close to zero, both substances are identical. If the distance is large, it is not possible to identify the substance. Statistical procedures useful for the above types of qualitative analyses include cluster analysis, discriminant analysis, soft independent modeling of class analogy, *K*-nearest neighbors, and artificial neural networks.

Confirming the identity of ingredients, testing purity, and detection of adulteration or deviation from standards are applications that fall into this category. Apart from these, several novel applications are being developed and investigated. **Figure 5**(**a**) shows a soft independent modeling of class analogy (SIMCA) discrimination model developed for classifying Cheddar cheese samples based on their flavor quality (fermented, unclean, slight sour, good Cheddar, etc.). The distinct clusters formed by cheese samples of different flavor quality clearly show that these samples are biochemically different. The circle around each cluster represents a 95% probability cloud that can be used for predictions. When an unknown sample falls within a circle, it can be concluded with 95% confidence that the sample belongs to that category.

Another example based on the above principle is the identification and characterization of dairy microorganisms. Traditional plating methods require several days to positively identify an unknown microorganism. Furthermore, these methods require preparation of media and tedious microbiological procedures. Bacteria have shown highly specific MIR spectral patterns that may be unique for individual strains. FT-IR allows for the chemically based discrimination of intact microbial cells and produces complex biochemical fingerprints that are distinct and reproducible for different bacteria. The complex FT-IR spectra reflect the total biochemical composition of the microorganism, with bands due to major cellular constituents such as lipids, proteins, nucleic acids, polysaccharides, and phosphate-containing compounds. Advances in IR instrumentation and the development of powerful supervised chemometric methods have enabled discrimination of different bacterial taxa and differentiation of subtle physiological differences between strains of the same species of bacteria. **Figure 5**(**b**) shows the SIMCA classification plot for nine different strains of *Streptococcus thermophilus*, forming distinct clusters due to their unique FT-MIR fingerprint. In another example of microbial analysis, 56 strains from four closely related species of *Lactobacillus* (*L. sakei, L. plantarum, L. curvatus*, and *L. paracasei*) were differentiated. Spectral analysis by pattern recognition differentiated the strains into four clusters according to species. Furthermore, it was possible to recognize strains that were incorrectly identified by conventional methods prior to the FT-IR analysis.

Product and Process Monitoring

The speed of analysis enables the industry to quickly perform analysis of incoming raw materials (e.g., fat and protein in milk) and pay suppliers based on the quality of the material. Similarly, the identity, authenticity, or purity of the raw materials can be determined using a portable IR spectrometer or a remote fiber-optic probe prior to unloading the truck. This enables quick assessment of quality and acceptance/rejection of raw materials. A well-established and constantly updated spectral library of materials of interest is essential.

IR spectroscopy overcomes many limitations of conventional methods and provides the possibility of performing near to real-time analysis on-line (simultaneously with the production process) and in-line (direct measurement in the process line). Following are some instances where IR spectroscopy can help in process control:

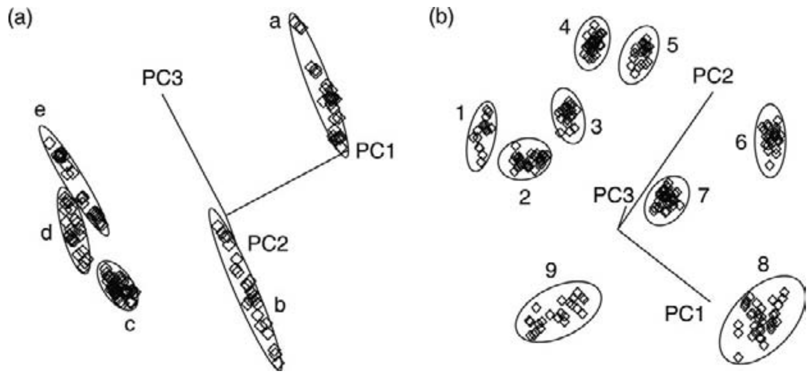

Figure 5 Soft independent modeling of class analogy models for (a) discrimination of Cheddar cheese based on flavor quality (a, fermented; b, unclean; c, slight sour; d, good cheddar; and e, slight burn) and (b) discrimination of *Streptococcus thermophilus* strains used in Swiss cheese making (1, S731; 2, S794; 3, S838; 4, S728; 5, S392; 6, S341; 7, S804; 8, S884; and 9, S869).

1. An NIR spectrometer may be used to determine the water content of milk powder directly after the powder passes through the drying chamber. Having this information, one can regulate the concentrate feed to the chamber.
2. An NIR spectrometer may be used to measure the water content of butter at the end of the butter-making process. This can be used to control the separation by pressing, which influences the water content.
3. To standardize fat and protein for cheese milk or fat for market milk, an MIR spectrometer may be used to measure these compounds for process control.
4. The formation of the coagulum during the cheese-making process may be monitored with NIR diffuse reflectance spectroscopy with fiber optics.

In addition, both qualitative and quantitative methods can be used to study chemical changes during processing. For example, cheese ripening is yet to be completely understood. IR spectroscopy offers several advantages that could simplify cheese research and provide insights into biochemical changes during ripening. This would enable ripening process monitoring and optimization to achieve cheese of uniform quality, which is currently a challenge that is difficult to overcome.

Accuracy and Reliability of IR Prediction Models

The accuracy of NIR prediction (i.e., the difference between the results obtained by the reference method and the IR prediction) is close to the repeatability of the reference method. The absolute difference of two analytical results (same person, same instruments and chemicals, short time between the analyses) with the reference method, on identical material, should be within the repeatability value of the reference method at 95% probability. In principle, IR spectroscopy cannot do better because results obtained with the reference method are used for calibration.

Limitations of IR Spectroscopy

Although IR spectroscopy offers several advantages, it may not be suitable for everything. It is essential to understand its limitations in order to design the analysis appropriately.

1. IR cannot detect atoms, monatomic ions, elements, inert gases, and diatomic molecules (e.g., N_2 and O_2). However, in certain cases this can be seen as an advantage as it eliminates the need for vacuum for analysis.
2. Calibration analysis, more often than not, is indirect and requires a reference method and constant updating of the models.
3. Almost all dairy products are complex mixtures, and hence their IR spectra are complicated with overlapping peaks and signal masking.
4. Most dairy products contain a high level of moisture (except powders), which has a strong absorption band that can mask certain important signals. Spectra of water and milk, for example, look very similar. Often, sample preparation procedures are required to reduce the effect of water.
5. Changes in environment can occur between samples, causing uncertainties in the spectra.

Good Laboratory Practices for IR Analysis

As with all methods of chemical analysis, one must check the performance of the method regularly. Because the IR method is an indirect method (requiring a calibration step) for the determination of constituent concentrations, one must set up a more-complex checking routine testing three kinds of potential problems:

1. One must ensure that the instrument operates within an acceptable error. This can be done by taking the IR spectra of inert standards over time (a certified material of known concentration is used to check whether the method is reliable). If the difference between the standard spectrum and the spectrum obtained with the instrument is unacceptable, the instrument must be readjusted.
2. If sample preparation is a necessary step for the technique, it must be tested to determine if the operators satisfy the demands of the standard operating procedure. This can be evaluated by preparation of the same material several times and predicting the constituent concentration. Limits will help to clarify this step. This is also done with other chemical methods.
3. The performance of the calibration must be monitored. This is not always possible with certified material because certified materials sometimes do not exist (e.g., for a calibration for fat in yogurt; no yogurt exists that can be used as certified material). The only way is to analyze the corresponding sample by the reference method and compare the difference between reference value and IR prediction over time. Preset limits of the difference can help to define warning and action levels.

The last point is very important because changes in the recipe can influence the IR spectrum. Difference between the results obtained by the reference method and IR predictions needs to be monitored constantly; sometimes, a new calibration is necessary. Usually, the difference between reference method and IR prediction is plotted versus time. This task can be accomplished by a control chart that also shows the warning and action levels.

Having arranged such a system, one can show that the IR method gives more accurate values than the reference method when performing multiple analyses of the same inert material. IR predictions are more constant and have smaller variations than the reference values.

Much experience is necessary to set up such a good laboratory practice (GLP) system. The fast way is to operate a network so that the performance of many instruments can be monitored simultaneously.

Networks

Many analytical spectrometers can be controlled centrally, monitored, and updated, creating a network. There are three kinds of networks, illustrated by an example from Lower Saxony, Germany.

Service Network

Since 1988, the Ahlemer Institut of the Landwirtschaftskammer Hannover has operated a service network, which has been accredited by the German Accreditation Council (DAP). There are nine dairies with 12 NIR instruments connected by telephone and modem to the Ahlemer Institut (**Figure 6**). The institute conducts feasibility studies, validates new applications, and performs the GLP procedure. The advantage is that the individual dairies do not need to employ trained and expensive personnel to perform the IR analysis. Furthermore, persons with different scientific backgrounds work together at the institute, so that any problems can be solved in less time. Nowadays, businesses generally focus on their key activities and therefore are outsourcing their other activities.

Surveillance Network

The Ahlemer Institut has been given responsibility by the Lower Saxony government to check the MIR instruments, which are used for computing the payment to the farmers according to the constituent concentrations in raw milk. This is done in the following manner:

1. On a weekly basis, calibration standards are sent to the laboratories to test the performance of instrument and calibration. After approximately 200 raw milk samples, one of the standards has to be reanalyzed. Analysis is repeated after each set of 200 raw milk samples. The

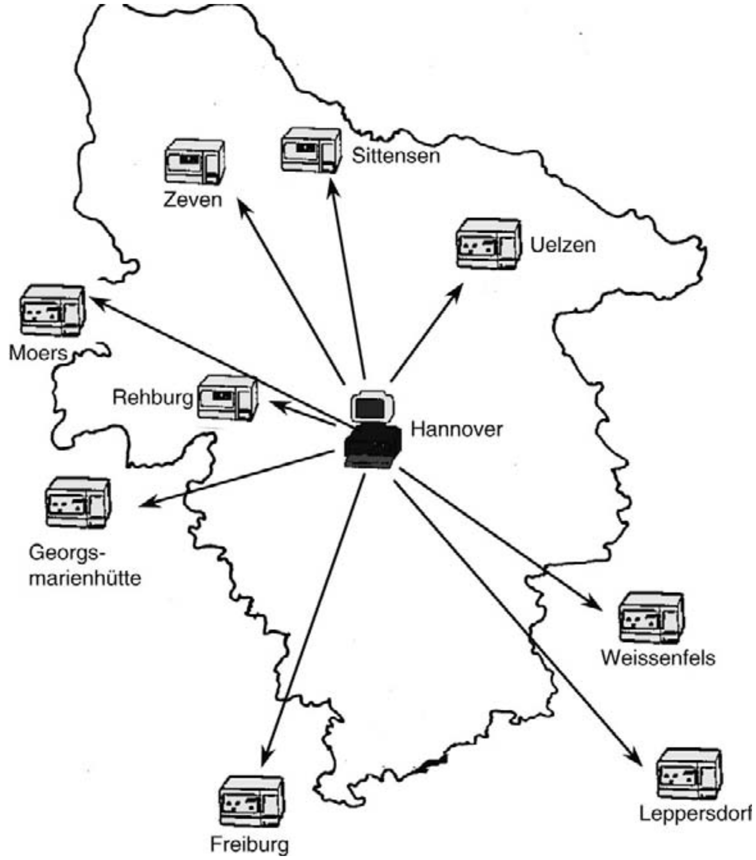

Figure 6 Map of the Milchwirtschaftliches Infrarot Netzwerk (MIRN: Infrared Instrument Network for Dairies) including Lower Saxony (a state in Germany) and the dairies connected.

results of tests on standards can be transferred by modem to the institute for further evaluation.

2. Each month, a series of standards is prepared to check the calibration over a wider concentration range. These results are transferred to the institute.

The advantages of this network are that the calibration is monitored more often, the checking is done without the necessity for traveling and is thus less time-consuming, and the institute staff can assist with their expertise. The network serves as a confidence-building measure between farmers and laboratories.

Harmonization Network

Harmonization of analytical results is an important issue in two ways:

1. Large dairies with multiple production sites, which transport milk or products from one site to another, would like to ensure that all measurements, performed on the same product at different locations, are the same or at least in good agreement.
2. Results of tests on exported products, obtained at different laboratories, should be in good agreement.

With respect to the chemical methods, standard operating procedures are defined as well as precision parameters (used for checking the results). However, the results are strongly dependent upon individual operators. Within a research and development project funded by the European Union, it has been demonstrated how this harmonization goal for IR spectroscopy can be achieved. The method is based on the concept of 'matching instruments', where one instrument is used as the 'master' (reference) instrument. Having compared the characteristics of the master and the other instruments, the spectra of the other instruments are transformed so that they match those of the master instrument. Spectra obtained in this way look as if they were obtained with the master instrument. Using the calibration of the master instrument, the correct sample composition can be predicted. This ensures that all predictions include the same information, and therefore all instruments behave in the same manner.

Conclusion

IR spectroscopy is capable of rapidly determining composition and characteristics of dairy products and can be a powerful tool for both industrial and research applications. Examples and applications presented in this article and those described in the published literature show that the technique is accurate, rapid, and reliable. To ensure the proper performance of instruments, it is necessary to establish and follow GLP guidelines. To obtain optimal results, much experience is necessary. Some dairies use the service of a network, thus outsourcing the calibration and application work. In most applications, reliable information can be obtained only with use of statistical methods.

Alternative spectroscopic techniques or coupling IR spectroscopy with other methods may also be required to achieve the desired outcome. Raman spectroscopy is not as susceptible to water as IR spectroscopy and may be suitable for some applications. Coupling IR spectroscopy with analytical techniques such as gas chromatography, liquid chromatography, and thermogravimetric analysis has extended its analytical capabilities. A relatively new IR imaging technique, which constructs a complete image of the sample with each point in the image containing a complete spectrum, can offer advanced features for dairy research. Another recent development is the miniaturization of FT-IR instrumentation (e.g., TruDefender™ FT handheld FT-IR by Ahura Scientific, Inc., shown in **Figure 7**), which would enable on-site analysis while the products are being produced. With numerous developments in FT-IR spectroscopy and several applications still unexplored, the future of IR spectroscopy in dairy analysis and control is promising.

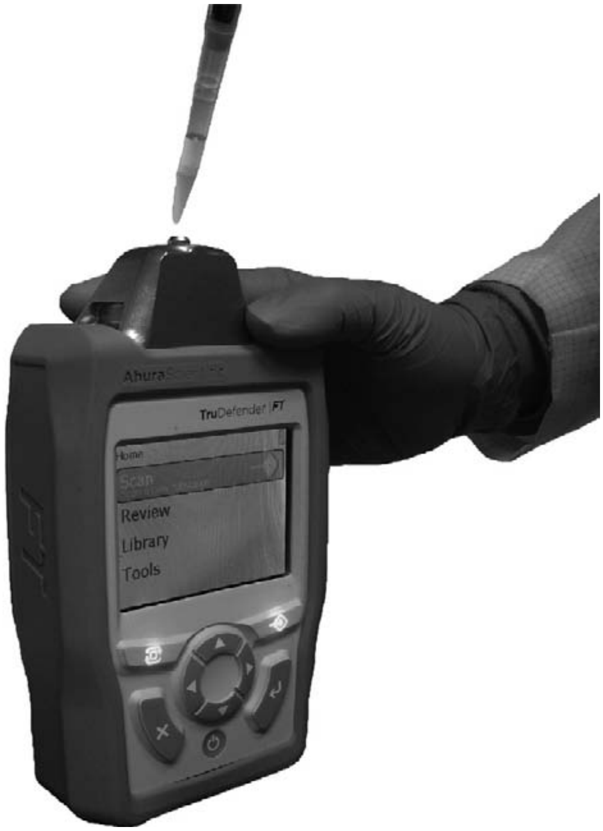

Figure 7 TruDefender™ FT handheld FT-IR (copyright Ahura Scientific, Inc., Wilmington, MA, USA).

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Hyperspectral Imaging for Dairy Products; Light Scattering Techniques; Multivariate Statistical Tools for Chemometrics; Spectroscopy, Overview; Statistical Methods for Analytical Data.

Further Reading

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Hyperspectral Imaging for Dairy Products

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Introduction

Hyperspectral imaging (HSI), also known as chemical or spectroscopic imaging, is an emerging technique that integrates conventional imaging and spectroscopy to attain both spatial and spectral information from an object. It was developed originally for remote sensing applications utilizing satellite imaging data for the Earth, Moon, and planets, but has since found applications in such diverse fields as astronomy, agriculture, pharmaceuticals, and medical diagnostics.

Hyperspectral images are made up of hundreds of contiguous wave bands for each spatial position of a target studied. Consequently, each pixel in a hyperspectral image contains a spectrum representing the light absorbing and/or scattering properties of the spatial region represented by that pixel (although it should be noted that due to various optical, instrumental, and background effects, each pixel spectrum may be influenced by its neighboring pixels; this becomes a greater problem in high-magnification imaging). The resulting spectrum acts like a fingerprint, which can be used to estimate the chemical composition of that particular pixel. Hyperspectral images, known as hypercubes, can be represented as three-dimensional blocks of data, comprised of two spatial and one wavelength dimension, as illustrated in **Figure 1**. The hypercube allows for the visualization of biochemical constituents of a sample, separated into particular areas of the image, since regions of a sample with similar spectral properties tend to have similar chemical composition.

Hyperspectral

Hyperspectral Image Acquisition and Instrumentation

It is currently not feasible to obtain information in all three dimensions of a hypercube simultaneously; one is limited to obtaining two dimensions at a time, then creating a three-dimensional image by stacking the two-dimensional 'slices' in sequence. There are two conventional ways to construct a hypercube. One method, known as the staring imager configuration, involves keeping the spatial image field of view fixed, while restricting or filtering light throughput, thereby obtaining images one wavelength after another (the use of traditional waveband filters is another option, but becomes difficult when greater than 100 wave bands are required). Hypercubes obtained using this configuration thus consist of a three-dimensional stack of images (one image for each wavelength examined; **Figure 1**), which may be stored in what is known as the band sequential (BSQ) format. Staring imager instruments incorporating tunable filters have found a number of applications in pharmaceutical quality control, their lack of moving parts representing an advantage in many situations.

Another configuration involves acquisition of two-dimensional camera frames representing complete spectral measurements acquired simultaneously from a series of adjacent linearly spaced spatial positions. Acquisition of the full hypercube thus requires relative movement between the object and the detector along the second spatial axis. Such line-mapping instruments record the spectrum of each pixel across a line of a sample simultaneously recorded by an array detector and this is known as pushbroom acquisition. This results in a hypercube, which is stored in the band interleaved by line (BIL) format. This method is particularly well suited to conveyor belt systems, and may therefore be more practicable than the staring imager configuration for some food industry applications.

Some instruments produce hyperspectral images based on a single complete spectrum – point step and acquire mode: complete spectra are obtained at single points on the sample, while the sample is moved in the *X*, *Y* spatial dimensions. Hypercubes obtained using this configuration are stored in what is known as the band interleaved by pixel (BIP) format. This is a very time consuming process, but may result in extremely high-resolution spectra with hundreds of wavelength channels. Recent advances in detector technology have reduced the time required to acquire these hypercubes.

Typical HSI systems contain the following components: focusing lens, wavelength modulator, detector, and illumination and acquisition system as shown in **Figure 2(a)**. In the case of pushbroom line-scanning HSI systems, a spectrograph is used for wavelength

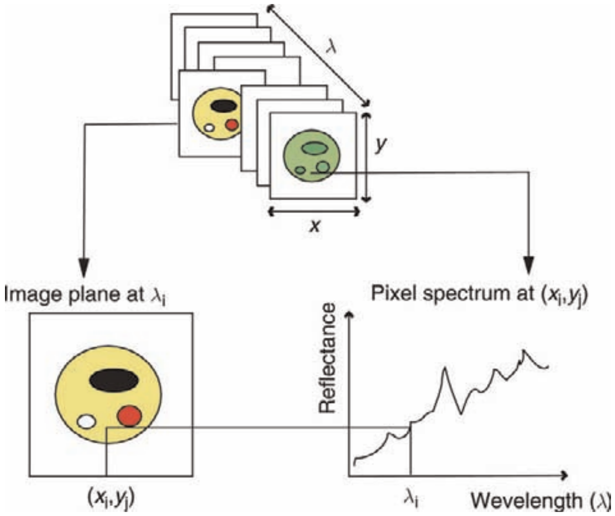
λ
y
x
Image plane at λ_i
Pixel spectrum at (x_i, y_j)
Reflectance
(x_i, y_j)
λ_i
Wevelength (λ)

Figure 1 Schematic showing hypercube structure; spatial axes *x* and *y*, and wavelength axis λ.

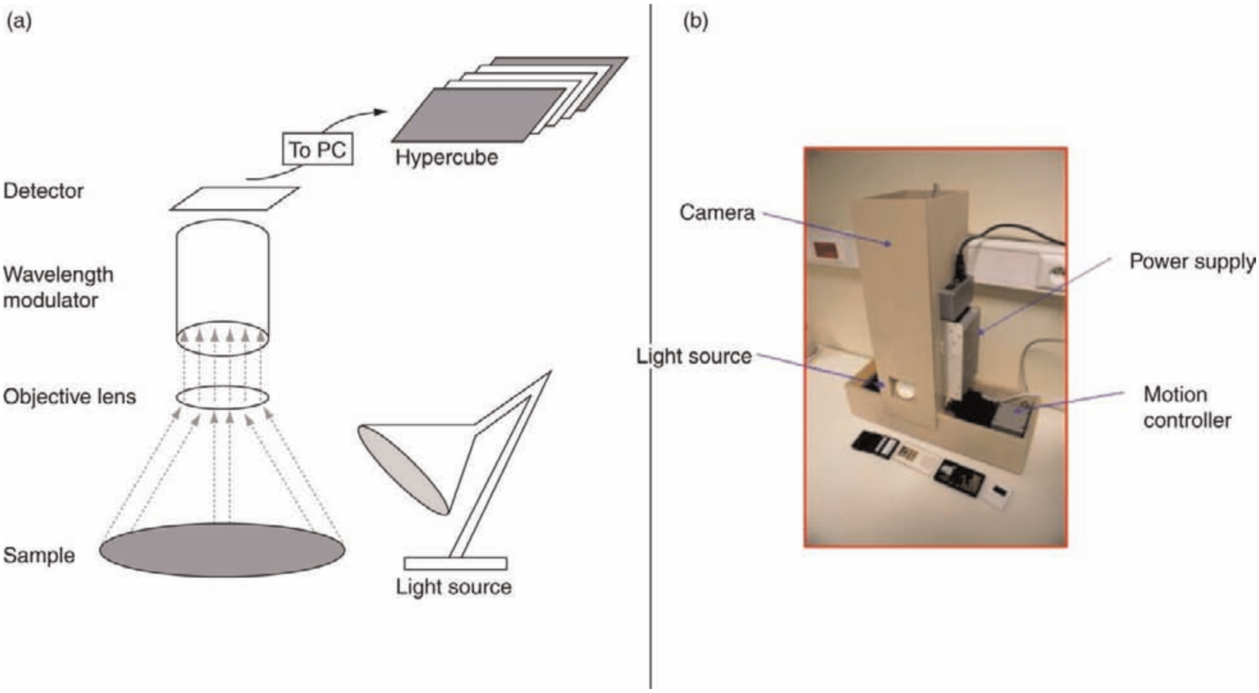
(a)
To PC
Hypercube
Detector
Wavelength modulator
Objective lens
Sample
Light source
(b)
Camera
Power supply
Light source
Motion controller

Figure 2 (a) Schematic showing typical components of a hyperspectral imaging system and (b) example of a prototype turn-key pushbroom hyperspectral imaging system.

modulation; a line of light reflected from or transmitted through the sample under investigation enters the objective lens and is separated into its component wavelengths by diffraction optics contained in the spectrograph; a two-dimensional image (spatial dimension × wavelength dimension) is then formed on the detector; two-dimensional line images acquired sequentially at adjacent positions from the sample target are stacked to form a three-dimensional hypercube, which may be processed immediately in real time or stored for further analysis. For such pushbroom systems, relative movement between the object and the detector is necessary and this may be achieved either by moving the sample (by use of a translation stage (see **Figure 2(b)**) or a conveyor belt) and keeping the hyperspectral camera in a fixed position or by moving the camera and keeping the sample fixed.

The wavelength of incoming light in the staring imager configuration is typically modulated using a tunable filter; acousto-optic tunable filters (AOTFs) and liquid crystal tunable filters (LCTFs) are the two most common types used. AOTFs have been used in the construction of commercially available HSI systems; the main advantages of AOTFs are good transmission efficiency, fast scan times, and large spectral range. On the other hand, LCTFs show greater promise for filtering of Raman images. More recently, staring imager systems that incorporate a tunable laser as the light source, thus removing the need for a wavelength modulator have been developed. Such systems can produce hyperspectral images in a fraction of the time required by conventional systems based on tunable wavelength filters, representing a significant advance in the field.

Hyperspectral images can be obtained for reflected, transmitted, or emitted light coming from the ultraviolet (UV), through the visible–near-infrared (Vis–NIR), and up to the short-wave infrared (SWIR) regions of the electromagnetic spectrum. The camera, wavelength modulator, and illumination conditions determine the wavelength range of the system. Commercially available Vis–NIR HSI systems typically range between 400 and 1000 nm, and use cameras with charge-coupled device (CCD) or complementary metal oxide semiconductor (CMOS) sensors; longer wavelength systems require more expensive IR focal-plane array detectors. The sample/target is usually diffusely illuminated by a tungsten–halogen light source. Data acquisition and data storage are a major issue in HSI; a typical image of 320 × 240 pixels in size will contain over 75 000 spectra, each with >100 spectral data points, resulting in a file containing >7 500 000 numbers; if each number is stored in floating point double precision (16 bytes), the resultant image will be >100 MB in size.

Hyperspectral Image Analysis

Numerous techniques exist to analyze HSI data, all of which aim to optimally reduce the immensity of the data while retaining important spatial and spectral information with the power to classify important chemical or physical areas of a scene. Typical steps followed in analyzing hyperspectral images are briefly described below.

Image calibration

Hyperspectral image calibration is required to account for spectral and spatial variations in light source intensity, detector response, and system optics. Calibration of spectral response can be achieved using a range of narrow-band light sources (e.g., laser 'pen lights') or calibrated standard reference materials such as NIST (National Institute of Standards and Technology) glasses, and this calibration should be verified periodically. Spatial calibration over the field of view of the HSI instrument should be carried out using a spatially and spectrally homogeneous sample (e.g., flat ceramic tile). Intensity calibration is required to compensate for changes in the detector response and should be carried out using certified reference standards (e.g., Spectralon gray scale standards). Development of suitable reflectance standards and the use of correct calibration transformations remain a challenge in HSI. Spatial and intensity calibration should, at the very minimum, be carried out on a daily basis as small changes in electrical power sources, illumination, detector response, and system alignment may result in significant changes in the detected response. Inclusion of 'internal reference' standards in each hyperspectral image acquired is recommended; this is also a good way to monitor the performance of the system over time.

Spectral and spatial preprocessing

Preprocessing is usually performed to remove nonchemical biases from the spectral and spatial information contained in a hyperspectral image (e.g., scattering effects due to surface inhomogeneities) and to prepare the data for further processing. A number of spectral preprocessing techniques exist, including polynomial baseline correction, Savitzky–Golay derivative conversion, mean centering, and unit variance normalization. Spatial operations usually carried out at the preprocessing stage include (but are by no means limited to) thresholding and masking to remove redundant background information from the hypercube, image filtering (e.g., Gaussian filtering) to decrease noise, and interpolation (e.g., bilinear interpolation) to reduce image size.

Classification and regression

Classification of hyperspectral images aims to identify regions or objects of similar characteristics using the spectral and spatial information contained in the hypercube. Various unsupervised methods, including principal component analysis (PCA), k-nearest neighbors clustering, and hierarchical clustering, can be applied in either the spectral or spatial domains to achieve classification. These methods are particularly useful in the analysis of samples of unknown composition, enabling the identification of spectral and spatial similarities within or between images that can be used further for their characterization. PCA is commonly used as an exploratory tool in HSI, as it represents a computationally fast method for concentrating the spectral variance contained in the more than 100 image planes of a hyperspectral image into a smaller number (usually <10) of principal component score images. Supervised classification methods, including partial least squares discriminant analysis (PLS-DA), neural networks, linear discriminant analysis, and spectral angle mapping, require the selection of well-defined and representative calibration and training sets for classification optimization. One of the major advantages of HSI in this respect is the sheer volume of data available in each hypercube with which to create calibration and training sets.

Hyperspectral image regression enables the prediction of constituent concentration in a sample at the pixel level, thus enabling the spatial distribution or mapping of a particular component in a sample to be visualized. Many different approaches are available for the development of regression models (e.g., partial least squares regression (PLSR), principal component regression (PCR), stepwise linear regression), all of which require representative calibration sets containing spectra with corresponding accurate reference values (e.g., fat content, protein content). This poses a problem in HSI: it is practically impossible to measure the precise concentration of components in a sample at the pixel scale and therefore impossible to provide reference values for each pixel spectrum. To overcome this, regression models may be built using mean spectra obtained over the same region of the sample (or a representative region) on which the reference value was obtained. After model optimization through training and testing, the regression models developed using the mean spectra can be applied to the pixel spectra of the hypercube, resulting in model predictions at the pixel level. This results in a prediction map in which the spatial distribution of the predicted component(s) is easily interpretable.

Image processing

Images from different planes in a hypercube may be combined using algorithms based on straightforward mathematical operators, for example, addition, subtraction, multiplication, and division. Image processing is also carried out to convert the contrast developed by the classification/regression analysis into a picture depicting component distribution. Gray scale or color mapping with intensity scaling is commonly used to display compositional contrast between pixels in an image. Image fusion or false color mapping, in which two or more images at different wave bands are represented as red, green, or blue channels and combined to form a new RGB (red, green, and blue) image, may be employed to enhance apparent contrast between distinct regions of a sample.

Applications of Hyperspectral Imaging to Dairy Products

Technological advances in spectrograph and detector design, leading to reduced cost and improved instrumentation, have enabled HSI applications to increase in number and widen in scope over the past 20 years (**Figure 3**). Reported applications of HSI in food science and technology (while not as numerous as those in established HSI disciplines such as remote sensing) are rapidly emerging, with the majority of reported research concerned with quality control of vegetable, fruit, grain, meat, and poultry products. There have been, to date, very few reported applications of HSI to dairy foods; its use as a research tool in dairy science has not yet been exploited. However, a wide range of quality and safety testing practices in the dairy industry could be complemented and potentially improved with HSI. Therefore, it is expected that research on its application in dairy science will expand in the future.

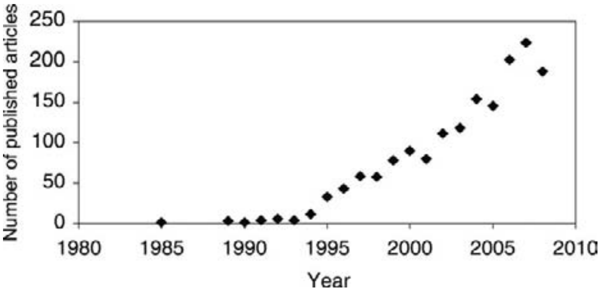

Figure 3 Number of published scientific articles with ‘hyperspectral imaging’ or ‘chemical imaging’, or ‘imaging spectroscopy’ in the title. From Web of Science Citation Reports, January 2009. Reproduced from Burger J and Geladi P (2006) Hyperspectral NIR image regression part II: Dataset preprocessing diagnostic. *Journal of chemometrics* 20:106–119.

Process Monitoring

Dairy products are subjected to numerous heat, pressure, mixing, and fermentation stages during processing to produce from milk the wide range of dairy products currently available, such as cream, butter, cheese, and yogurt. Driven by international economic, social, and legislative trends, dairy production has increasingly shifted from small-scale farm production to high-volume industrial-scale processing. Advances in computer processing technology have enabled an increased level of integration in the automation and control of dairy processes. Automated dairy processing and manufacturing units are required to consistently meet the stringent safety and quality standards set out by food regulatory agencies. Monitoring these unit process operations and characterizing their influence on the final product quality are a major challenge for dairy producers. Potential applications of HSI in the control of some typical dairy processes are described below.

Milk coagulation and curd formation

Scattering of light is intrinsically related to the size and distribution of particles in a sample; for example, smaller particles of glass tend to scatter more light and appear whiter in color than larger ones. Changes in the light-scattering properties of milk during coagulation can be used for automatic non-destructive quality control of the cheesemaking process. Monitoring changes in the optical properties of milk arising during coagulation and curd formation using Vis–NIR spectroscopy and RGB imaging is well documented. These light-scattering properties have been exploited successfully in the development of online sensors based on Vis–NIR spectroscopy for optimization of the gel-cutting step in cheesemaking. HSI may offer improved characterization of the coagulation and curd formation processes, through spatial characterization of the spectral response. The time required (typically 1–2 min for current instrumentation) for hyperspectral image acquisition may limit its use, since milk is a spatially dynamic product; hyperspectral line scanning, as mentioned previously, may also be useful here.

Dehydration

Spray drying is commonly used for the preservation of dairy products and in the production of milk powders. The resulting chemical, microbial, physical, and organoleptic properties of the dairy product are highly dependent on the drying conditions applied. The potential of HSI to monitor the drying process lies in its ability to provide spatial information on the distribution of water in a sample. Water molecules have known absorbance features in the near infrared, and absorbance patterns in this wavelength region may also be used to differentiate between free and bound water in a substance. Consequently, HSI may be used to generate moisture distribution profile maps for products during dehydration and to investigate the effects of various drying parameters on the final product quality. This would enable estimation of accurate drying end points in dairy powder production and also improve quality assurance of the final product. Moreover, HSI may be used to detect any problems in the drying process, for example, nonuniform drying due to equipment malfunctions would be detected by nonuniform moisture profiles in a product. Examination of the surface composition of milk powders, which is known to differ from their bulk composition, is another area in which HSI may potentially be applied.

Blending

The HSI technique has an added advantage over traditional bulk quality measurement techniques in its ability to detect problems arising during processing. HSI can be used to spatially map the distribution of components within a food product during manufacturing, allowing direct qualitative comparison with control products. This would be useful in monitoring the process of blending in dairy food production, which is important since the uniformity of blending directly affects the final product quality. For example, regions of ingredient agglomeration in blended dairy products may be identified and related to inadequate blending protocols. The potential application of HSI has already been demonstrated in the monitoring of blend homogeneity of powder and tablet forms in pharmaceutical processing. HSI may also be used to estimate particle size distribution during processing, enabling improved monitoring of the blending process, more accurate estimation of blending end points, and enhanced insight into the behavior of dairy products during blending.

Compositional Analysis

Dairy products are complex food matrices composed of dissolved, suspended, and emulsified substances, fat in globular and continuous forms, proteins, carbohydrates, minerals, and vitamins. Traditional wet chemistry methods for compositional analysis of dairy foods are labor-intensive, time-consuming, and require sample destruction. Numerous studies that demonstrate the effectiveness of near- and mid-infrared spectroscopy for nondestructive prediction of dairy food composition (e.g., for the prediction of fat, protein, and lactose) have been reported. HSI provides the added potential to simultaneously estimate the spatial distribution of numerous components in a sample while also predicting average compositional information. A number of authors have published work on the application of HSI to estimate the distribution and concentration of active ingredients in pharmaceutical products, and its potential for predicting the location of components such as water, fat, and protein in food products has also been demonstrated.

The first reported application of HSI to dairy products was for prediction of cheese composition from hyperspectral images in an article on NIR hyperspectral image regression. The researchers also examined the effects of various spectral pre-processing methods on the predictive ability of the developed regression models. A range of 12 commercial cheese products were tested, selected to span as wide a range as possible in terms of protein, fat, and carbohydrate content. The average composition values on the packaging labels were used as standard reference values and a parallel set of reference values for protein and fat content was determined using standard techniques. The challenges of developing accurate calibration models using hyperspectral image data were discussed. One major issue is that reference values were available only for entire bulk samples, not at the individual pixel level. To overcome this limitation, the authors used the mean spectral response from sample images to build calibration models. PLSR models were developed on mean spectra subjected to various spectral pretreatments, and (considering the prediction error of the regression models) the results suggested that applying a first-derivative Savitzky–Golay smoothing was the most effective spectral pre-treatment. Using this approach, a PLSR model with two or four latent variables could be used to satisfactorily predict fat, protein, and carbohydrate. Typical model prediction errors of 1–2% for protein and fat and 2–3% for carbohydrate were obtained, which were greater than the errors in the reference measurements (0.14% for protein and 0.41% for fat) but similar to results reported for other NIR spectrometers.

The regression models developed on mean spectra and bulk reference values were then applied to pixel spectra for each hyperspectral image studied, to generate prediction maps showing the distribution of each component as estimated by the calibration model. **Figure 4** shows the false color maps for the two latent variable PLSR models for predicting protein (green), fat (red), and carbohydrate (blue) of the 12 individual cheese samples studied. Some inhomogeneous regions are noticeable as changes in the coloration of the images, which in some cases do not appear to be uniformly distributed; these may indicate areas of non-uniform fat and protein distribution, which may in turn be related to processing parameters involved in the production of the selected cheeses. The presence of cheese eyes or holes in the cheese surface may pose difficulties in hyperspectral image analysis. In the case of thin slices, where the holes are open, it may be possible to apply thresholding operations to remove such regions from the image.

Predicting Physical Properties

Physical properties of foods are intrinsically related to their composition and structure; consequently, processing methods that alter dairy product composition and structure directly affect their physical properties. Potential applications of NIR spectroscopy and conventional RGB imaging have been reported for the prediction of physical properties of dairy products; some examples include turbidity and viscosity of milk, and free oil formation and meltability of cheese. Other optical techniques reported for monitoring structural changes related to physical properties in dairy products include confocal laser scanning microscopy, scanning and transmission electron microscopy, and magnetic resonance imaging.

Control of the physical properties of dairy products demands an understanding of where the constituent components (e.g., fat, protein) are located in relation to each other, and how they are modified during processing. HSI offers a relatively low-cost method for examining the

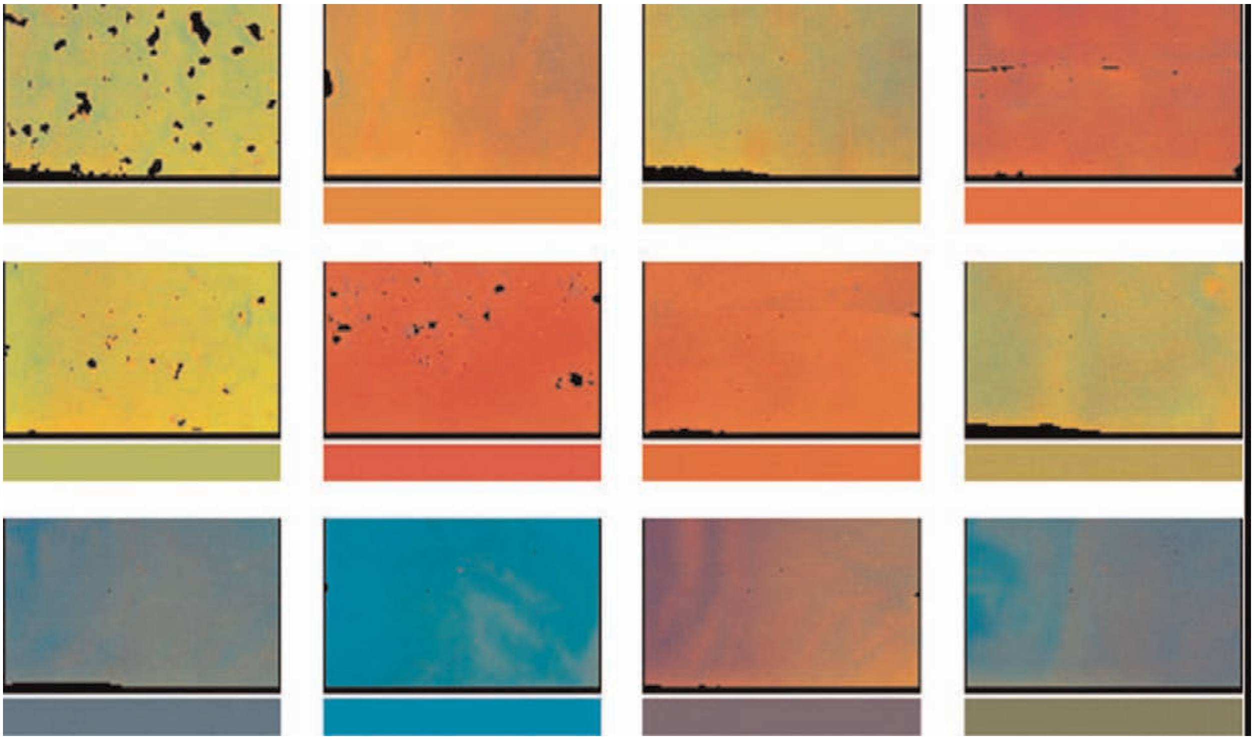

Figure 4 False color concentration prediction maps for two-component first-derivative spectral models of 12 different cheeses. A smaller rectangle below each prediction map indicates the target 'color' expected. Rectangular cheese regions are 209 × 320 pixels in size corresponding approximately to 50 × 62 mm^2. Reproduced from Burger J and Geladi P (2006) Hyperspectral NIR image regression part II: Dataset preprocessing diagnostics. *Journal of Chemometrics* 20: 106–119.

distribution of components within a sample, which may be useful in monitoring the development of physical properties during processing. HSI would enable improved image contrast not available with RGB imaging, and provide additional insights into the effect of underlying composition distribution on physical properties. One example where HSI may be particularly useful is in NIR monitoring of free oil production in cheese, as oil is semi-transparent to visible light captured in RGB imaging. Physical properties of milk powders, including flowability and particle size distribution, could also potentially be evaluated using HSI, since these properties directly relate to the concentration and size of constituents in the powders expressed as light-scattering differences, which may be examined using HSI.

Classification

Accurate classification is critical for pricing, authentication, and categorization of dairy products. Products may be classed based on the manufacturing methods used in their production and based on their geographical origin, composition, physical properties, and maturity; for example, milk and cream products are priced based on their fat content. Therefore, rapid and accurate classification techniques would represent an economic benefit for producers. The multivariate nature of dairy product classification, combined with the knowledge that many dairy food components exhibit characteristic light absorbance and scattering behavior in the near infrared, makes NIR spectroscopy well suited for many classification tasks. Multispectral imaging has been used in the classification of blue cheeses based on product type and producer using a custom-made system capable of recording images at eight wave bands in the UV–Vis–NIR wavelength regions.

HSI offers exciting new opportunities in object classification, based on spatial and spectral properties of samples. This method is particularly well suited to the classification of blue cheese products, where distribution and concentration of ingredients are the key grading parameters. In order to demonstrate the potential of HSI in the classification of cheese products, a hyperspectral image of 12 pieces of high-fat and low-fat cheese slices arranged on a piece of black cardboard was obtained using a pushbroom HSI system operating in the wavelength region of 400–1000 nm. For comparison, an RGB image of the cheese samples studied (obtained using a digital camera) was acquired (**Figure 5(a)**); all cheese samples appear similar in color and appearance. However, looking at the mean HSI spectral profiles of each product, it is evident that the full-fat product reflects more light in the visible (500–950 nm) wavelength range. These spectral features may be used to classify each pixel of the hyperspectral image into one of two or more groups. In the present case, a spectral angle mapping algorithm, which compared the similarity of the spectrum of each pixel in the hyperspectral image with the mean spectra shown in **Figure 5(b)**, was applied and each pixel was classified as full-fat or half-fat depending on its similarity to each mean spectrum. Although the algorithm correctly classified most pixels, some edge regions in the half-fat samples were misclassified, possibly due to lighting inhomogeneities at edge regions.

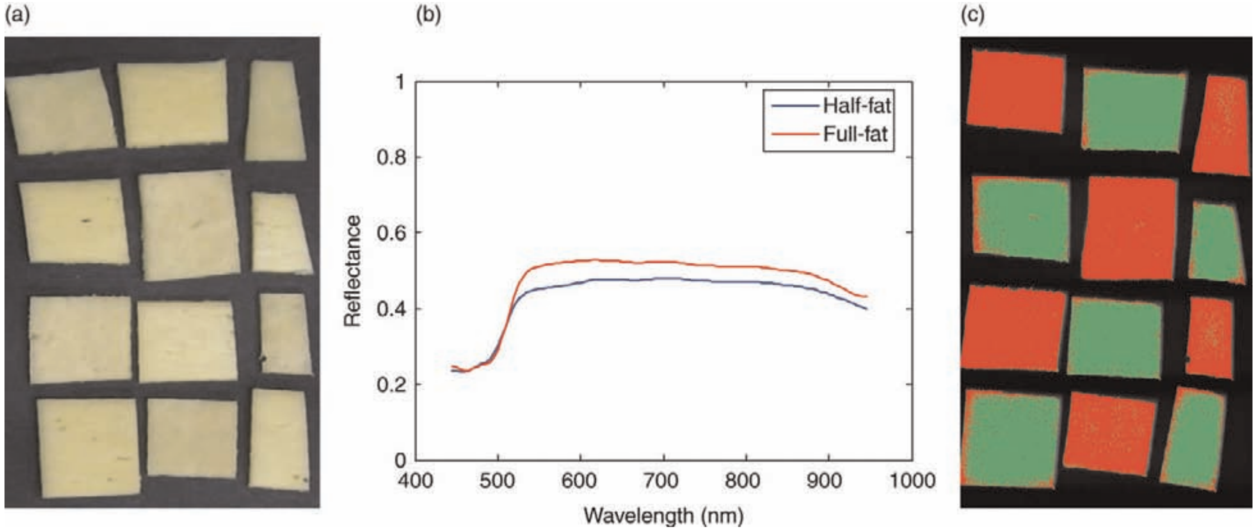

Figure 5 (a) RGB image of cheese samples studied; (b) mean spectra of half- and full-fat cheese samples; and (c) cheese classification map (red = half-fat, green = full-fat) obtained using spectral angle mapping algorithm.

Conclusion

HSI offers new possibilities to researchers and producers in food science; by combining spectroscopy and imaging, this tool can be used to map the distribution of constituents over the surface of a sample. This capability may be useful for dairy food analysis at the laboratory-scale research and for the development of both laboratory and online monitoring technologies in the dairy industry. Future improvements in the precision and speed of HSI are likely to arise with improved lighting systems, higher-quality photometric sensors, and faster hardware. As HSI continues to emerge as a tool for food quality and safety analysis, and with new systems offering faster image acquisition and processing times, the potential role of this technology in the monitoring and control of dairy food processes seems very promising. It is anticipated that the number of applications of this technology to problems in the dairy industry will increase rapidly in the coming years.

Further Reading

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Light Scattering Techniques

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Introduction

Fluid dairy products, milks, homogenized milks, creams, and dairy emulsions, are white because the dispersed species, be they casein micelles, fat globules, or emulsion droplets, are efficient scatterers of light. We can gain quantitative information on the sizes and size distributions of those scattering species using the light scattering techniques to be described in this article. All analysts know that the particles that constitute real samples do not consist of any single type but instead will generally exhibit a range of shapes, more so for casein micelles than emulsion droplets, and sizes. Particle size determinations therefore are undertaken to obtain information about the size characteristics of an ensemble of particles. Furthermore, because the particles being studied are not the exact same size, information is gained only about the average particle size and the distribution of sizes about that average. Nevertheless, these data are useful in assessing the success of product preparation, monitoring stability, or identifying the effects of recipe or process changes, among other benefits.

Background Principles

The interaction of light with matter is ubiquitous. We are able to see and register objects, near and far, because essentially all materials scatter and/or absorb light. The essential main elements of an apparatus to observe the scattering process are a light source, a scatterer (essential), and a detector. First, we require a well-focused or collimated stable source of light. A laser fulfills these requirements as well as providing coherence, an essential requirement for dynamic light scattering. The well-collimated beam enables the direction of scattering to be established with precision; stability in the light source is always important when comparing samples under different conditions. Emission over a narrow linewidth or wavelength range is another important attribute of laser light sources allowing the wave vector to be defined precisely.

Next we have a scatterer contained in a thermostated cuvette, preferably cylindrical. The light source should be focused on the center of this cuvette. The scatterer should be of a size that light scattering theories (to be described) are applicable. The volume fraction of the scatterer should be low enough to ensure that only single scattering occurs, that is, any photon received at the detector is scattered only once in its passage through the cuvette. Multiple scattering is an avoidable complication in static light scattering and in conventional dynamic light scattering, where size distributions might be sought, although diffusing wave spectroscopy (DWS), an extension of dynamic light scattering, exploits it. It should also be realized that only the scattering particles of interest should be present in the sample. This means the diluting suspending medium should be filtered through micropore filters to remove dust and other contaminants.

The third component is the detector. This always used to be a photomultiplier but nowadays avalanche photodiodes are frequently employed. The detector may be mounted on a goniometer arm centered on the scattering center to allow a variable scattering angle or scattering wave vector, but again frequently the scattering angle is fixed at 90°. In some static light scattering instruments, a range of annular detectors at fixed angles to the direction of scattering are employed to maximize the amount of scattered light detected.

Static Light Scattering

Theoretical Background

If the photon counts measured by the photomultiplier are averaged over time, a static light scattering experiment is performed. If the scatterers are small in size, a, compared to the wavelength, λ, of the light employed ($a < \lambda/20$), the scattering is largely independent of the angle of scattering but the data can be used to obtain information on the molecular weight and interaction behavior of the scatterer through the equation

$$\frac{Kc}{R_\theta} = \frac{1}{M_W} + 2A_2c \qquad [1]$$

where K is an instrument constant defined below, c the concentration of the scatterer, M_W the molecular weight, A_2 the second virial coefficient (a measure of intermolecular interactions), and R_θ the Rayleigh ratio.

$$R_\theta = \frac{r^2 i_\theta}{I_0} \qquad [2]$$

where r is the distance of the detector from the scattering volume, I_0 the incident intensity of the light, and i_θ the excess scattered intensity at angle θ. To avoid determining the primary light intensity, the instrument is usually calibrated by a standard of known scattering power, usually benzene or toluene. The Rayleigh ratio is then written as

$$R_\theta = \frac{i_{\text{solution}} - i_{\text{solvent}}}{i_{\text{toluene}}} I_{\text{abs.toluene}} \qquad [3]$$

where $I_{\text{abs.toluene}}$ is tabulated in the instrument literature.

The optical constant, K, is given by

$$K = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 \qquad [4]$$

where n_0 is the refractive index of the medium, λ_0 the wavelength of the light *in vacuo*, N_A Avogadro's number, and dn/dc the refractive index increment of the scatterer. The presence of the latter means that, if there is no refractive index difference between the scatterer and the medium, there is no scattered light signal. It should always be remembered that innocuous additives, such as sucrose, change the background refractive index and can even in sufficient concentration remove any refractive index difference, a technique known as contrast matching.

For larger particles, measured scattered intensity becomes a function of the angle of observation and follows the equation

$$\frac{Kc}{R_\theta} = \left(\frac{1}{M_W} + 2A_2c\right)\frac{1}{P(\theta)} \qquad [5]$$

Scattered photons from different parts of the particle differ in total scattering pathlength on arrival at the detector; they differ in phase and accordingly destructively interfere. This is taken into account by the Rayleigh–Gans–Debye form factor or scattering factor, $P(\theta)$, or more properly $P(x)$, where $x = qa$, q being the scattering wave vector defined as $q = (4\pi n/\lambda)\sin(\theta/2)$. As $x \to 0$, $P(x) \to 1$, so that for either very small angles or very small particles, the form factor is unity, scattering becomes independent of angle, and eqn [5] reduces to eqn [1]. Analytical expressions are available for several particle geometries, for example, sphere, ellipsoid, disc, or for polymeric species where the reciprocal form factor is written as

$$\frac{1}{P(\theta)} = 1 + \frac{16\pi^2}{3\lambda^2}\langle s^2\rangle \sin^2\left(\frac{\theta}{2}\right) + \cdots \qquad [6]$$

which, on substitution in eqn [5], gives the following expression as a function of concentration and scattering angle:

$$\frac{Kc}{R_\theta} = \left(\frac{1}{M_W} + 2A_2c\right)\left(1 + \frac{16\pi^2}{3\lambda^2}\langle s^2\rangle \sin^2\left(\frac{\theta}{2}\right)\right) \qquad [7]$$

This yields the well-known Zimm plot.

Plotting Kc/R_θ as a function of $\sin^2(\theta/2)$ at constant concentration, c, gives a straight line with slope M and intercept I as

$$M = \left(\frac{1}{M_W} + 2A_2c\right)\left(\frac{16\pi^2}{3\lambda^2}\langle s^2\rangle\right) \qquad [8a]$$

$$I = \left(\frac{1}{M_W} + 2A_2c\right) \qquad [8b]$$

If the intercept values obtained at different concentrations are plotted as a function of c, we obtain a new straight line with slope $2A_2$ and intercept the reciprocal of the average molecular weight. Back substitution then allows calculation of the mean square radius of gyration $<s^2>$.

By seeking to reduce or remove the effects of particle size and concentration by extrapolating to zero angle and zero concentration, only weighted averages are obtained for size and interaction parameters. This throws away most of the information content of the data. Theoretically, the effect of polydispersity on the second virial coefficient, A_2, is unclear.

Instruments are available, however, that take the measured scattered intensity as a function of q and, using a Fourier transform approach, deconvolute from that the particle size distribution giving rise to that light scattering spectrum. These instruments incorporate multidetector arrays to accumulate the intensity data and their software employs Mie theory, the light scattering theory that allows calculation of the angular distribution of light scattered by a sphere with no underlying assumptions or approximations, apart from considering the particle as spherical. From these instruments, particle size distributions are obtained, but little research seems to have been done on using them to study the influence of particle concentration, which might lead to information on the effect of particle interactions on the scattering behavior.

Comments and Caveats

A size distribution has two coordinates. The size, which is, invariably in light scattering, an equivalent spherical diameter, is plotted on the x-axis; and the amount in each size class is plotted on the y-axis. The amount is usually given as either the number or volume or mass of particles. If the particle density is the same for all sizes, then the volume and mass descriptions are equivalent. This would not be true if we had two populations of differing densities, as for example, casein micelles and fat globules in a suspension, as in whole milk. Resolving the two is not easily done and the best approach for obtaining the size distributions of both particle groups is to remove one or the other from the suspension. For the fat globules, where the smaller micelles actually present less difficulty for reasons to be explained below, the casein micelles can be dispersed by adding an excess of a calcium sequestrant such as ethylenediaminetetraacetic acid (EDTA). For the casein micelles, the milk should be skimmed thoroughly and filtered to remove the larger fat globules. Even then, perhaps the preferred technique is dynamic light scattering rather than the static technique, although advances in static light scattering methodology may have obscured the advantages that dynamic light scattering once manifested for particles in the micellar size range.

Each particle sizing technique weights the amount observed differently. For example, light scattering by really small particles is weighted by the intensity of scattered light, which varies in that size range as the 6th power of the diameter. As larger particles are encountered, the power law dependence drops smoothly to a second power dependence, as for Fraunhofer diffraction. For most particles of interest, however, the power law dependence remains sufficiently high that a few large particles can dominate the scattered light signal, obscuring the presence of small particles. This is the reason why dust is such a problem in light scattering. It also explains why the determination of the size distribution of larger fat globules can tolerate the presence of much smaller casein micelles but not vice versa.

Dynamic Light Scattering

Theoretical Background

Far from being constant and subject only to random noise generated in the response of dynodes to photons or the presence of stray background light, the intensity of light scattered by a colloidal suspension or a hydrocolloid solution fluctuates rapidly due to the Brownian motion of the scattering entities (**Figure 1**).

Dynamic light scattering analyzes these fluctuations in terms of correlation functions computing the intensity correlation function, $G_2(t)$, as

$$G_2(t) = \langle I(0).I(j\tau)\rangle \qquad [9]$$

where the angle brackets denote a time average. The delay time, t, is given by $j\tau$, where j is the number of delay channels ($j = 1, 2, 3, \ldots, N$, the last being the number available in the correlator) and τ the time span of each channel, assuming a linear spacing of channels. The correlation procedure is illustrated in **Figure 1**, where the fluctuating light scattering intensity $I(t)$ is plotted as a function of t. The correlator performs the operations $I(0).I(t = \tau)$, $I(0).I(t{=}2\tau)$, $I(0).I(t{=}3\tau)$, ..., $I(0).I(t{=}N\tau)$, repeating this procedure over a period of time (minutes to hours) and accumulating the autocorrelation function $G_2(t)$. This is a monotonically decaying function that decays from an initial value equal to the time average of the intensity squared, $<I^2>$, to the square of the average intensity, $<I>^2$. Emphasizing this point, the product collected in the first correlator channel is that at $t = 0$ and its immediate neighbor, where intensity has hardly changed; hence, the product is effectively I^2, and integrating this over the period of accumulation gives $<I^2>$. Also, in multiplying $I(0)$ by $I(t = N\tau)$, the two values are uncorrelated and may take any value around the mean $<I>$, so that their accumulated product is effectively $<I>^2$. Note that the time span of the delay channel is selected to achieve both these events; that within one channel, intensity is slowly changing and over the complete delay span of the correlator, the intensities are completely uncorrelated. The amplitude of the correlation function is therefore the variance of the intensity fluctuations, $<I^2> - <I>^2$.

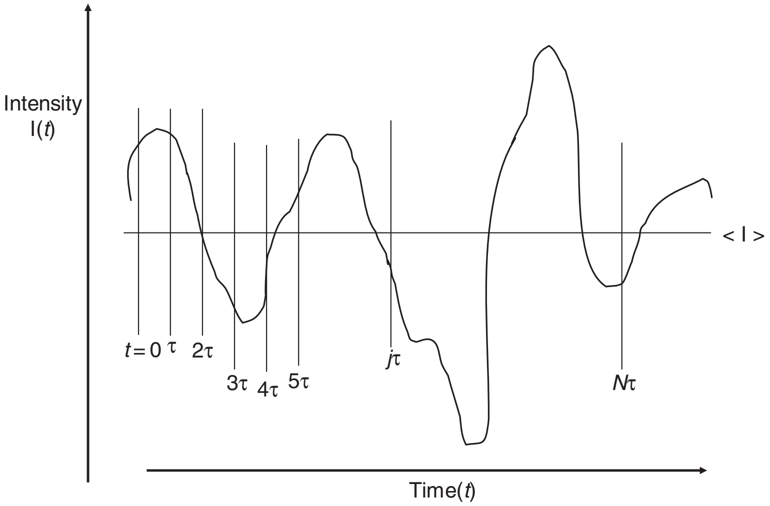

Figure 1 Fluctuating light scattering intensity as a function of time and the derivation of the correlation function. After one cycle has been completed, the time grid shifts up one channel (1 becomes 0, 2 becomes 1, etc.) and the cycle is repeated.

The intensity correlation function computed above is generally normalized as

$$g_2(t) = \frac{G_2(t)}{\langle I \rangle^2} \quad [10]$$

The correlation function that carries the information regarding particle mobility is the electric field correlation function, $g_1(t)$, and the two are related by the Siegert relation,

$$g_2(t) = 1 + b|g_1(t)|^2 \quad [11]$$

where b is an instrumental constant, of order 1, whose value depends on scattering volume, detector efficiency, and apparatus geometry.

If we have a dilute suspension of noninteracting particles, all of the same size (monodisperse), the electric field correlation function decays as a single exponential.

$$|g_1(t)| = \exp(-\Gamma t) \quad [12]$$

A plot of the logarithm of this correlation function against decay time will therefore be a straight line of gradient Γ. The result of the experiment will therefore be a mobility, a measure of the scatterer's ability to move through the solution, or in some examples, the inverse mobility, the relaxation time. For a dilute suspension, where single scattering prevails, this mobility is related to the translational diffusion coefficient of the particle (D) by the equation $\Gamma = Dq^2$, q being the previously defined scattering vector, emphasizing that the actual relaxation time will be a function of the angle of observation and that the ruler against which the measurements are made is the scattering vector. For spherical particles, the translational diffusion coefficient is directly related to the particle radius, a, by the Stokes–Einstein relation

$$D = \frac{k_B T}{6\pi\eta a} \quad [13]$$

Here k_B is the Boltzmann constant, T the absolute temperature, and η the viscosity of the suspending medium. Entering a cautionary note here, anything that changes the viscosity, be it temperature, solvent, or solution composition such as addition of sucrose or polysaccharide, will impact on the mobility of the particle and be computed as an apparent change in particle size unless viscosity is corrected for that change.

Rod-like and plate-like particles can also undergo rotational movement, which introduces another term into the correlation function. Flexible particles like polymer chains have internal motions, which also give rise to correlation terms. In both instances, specialized data treatments are required to extract useful information on such systems. These are beyond the scope of this introductory article, but further information may be found in texts on dynamic light scattering.

Uses of Dynamic Light Scattering

Again, most samples of interest are not monodisperse but encompass a range of sizes. This polydispersity manifests itself as a range of decay times in the correlation function where each size contributes according to its intensity-weighted mass fraction in the system and produces a nonlinear decay in the semilog plot versus decay time. Smaller particles introduce faster decay and larger particles a slower decay.

Mathematically, the correlation function can be expressed as

$$g_1(t) = \int G(\Gamma)\exp(-\Gamma t)\ d\Gamma \quad [14]$$

where $G(\Gamma)$ is the intensity-weighted distribution of decay constants, which must be determined to obtain the underlying particle size distribution.

Over the years, much effort has been devoted to the problem of deconvoluting or inverting this integral equation, and most manufacturers of dynamic light scattering equipment provide software for this exercise, based for example on the exponential sampling technique or the CONTIN package. Space does not permit extensive description or discussion of these methods, but it should be remembered that these inversion problems are ill-conditioned, that the solutions are not necessarily unique, and that as many independent pieces of prior knowledge as available should be employed in justifying any particular selection. Knowing what the solution will look like is a great help in finding it! For particles in a suitable size range where the intensity of scattering is angular dependent, one particular approach is to compare solutions for the particle size distribution obtained from correlation function data recorded at different scattering angles. Although the correlation functions differ, the ultimate particle size distributions should be identical within the error of the experiment. Cummins and Staples also exploited angular dependence of correlation function behavior but used a forward calculation of intensity-weighted correlation functions to evaluate the distribution of sizes. This procedure also works well with multimodal mixtures in predicting both sizes and relative scattering distributions.

More generally, the first estimates of average particle size in a polydisperse system are obtained by a so-called cumulants analysis of the correlation function. In essence this fits the logarithm of the correlation function to a polynomial in the decay time t. Generally, this series is truncated at the quadratic or cubic term. It is readily demonstrated that the coefficient of the linear term yields the average hydrodynamic size and that the quadratic coefficient gives a measure of the variance or width of the size distribution, hence an indication of the polydispersity. Higher order coefficients can also be used to learn more about the shape of the distribution but the quality of the data frequently limits their usefulness.

Finally, a word of caution is necessary. Many of the modern methods of particle size analysis purport to give complete size distribution information. Often they do not. They often attempt to fit noise in the data resulting in the introduction of spurious peaks. With the exception of, perhaps, image enhancement, rarely can a computer improve resolution in particle sizing applications.

Laser Doppler Electrophoresis

Imposing movement onto the random Brownian motion can also be detected in light scattering behavior. Thus, the imposition of an oscillating electric field at a suitable frequency can be picked up as a damped oscillation in the decaying correlation function. This is the principle behind the dynamic light scattering technique known as laser Doppler electrophoresis. The magnitude of the response can be related to the electrophoretic mobility of the charged particle and hence by a suitable model to its zeta potential, a measure of electrostatic repulsion between particles and an indicator of their long-term stability.

Light Scattering from Opaque Concentrated Dispersions

The extension of dynamic light scattering to opaque systems presents a series of problems, both theoretical and experimental. Conventional dynamic light scattering demands operation under single scattering conditions. This is because the inverse scattering wave vector is the ruler against which diffusional motion is measured. In a multiple scattering situation, knowledge of the scattering vector is lost. Several approaches have been employed to try to rectify this deficit.

One approach is to reduce the level of scattering by matching the refractive index of the scattering particle to that of the medium, generally by judicious choice of solvents. This should not markedly change the particle size but may influence the particle–particle interactions. Another approach employs two lasers of different wavelengths and cross-correlates the light at angles chosen in such a way that the scattering vectors for both beams coincide. This is still limited to fairly low particle concentrations as the light still has to traverse a scattering volume. This latter problem of light collection was ingeniously circumvented in one apparatus, previously marketed by employing a single optical fiber to carry light into the sample with the same fiber collecting the back-scattered light. The constraints of this geometry ensured that the back-scattered light was dominated by single scattering and therefore the theories and procedures for data treatment for single scattering, as described above, still applied. However, the apparatus was not a commercial success and was subsequently withdrawn.

Diffusing Wave Spectroscopy

DWS approaches the problem of analyzing the dynamic light scattering behavior of an opaque suspension by turning multiple scattering into an absolute necessity. In strongly scattering media, the propagation of light can be described very well by treating the passage of light as a diffusion process, that is, by assuming that each detected photon has executed a random walk through the suspension. The detected light is the incoherent sum of contributions scattered along all possible paths and this fluctuates in intensity, as always, due to the motion of the scatterers. Considering only one such path, physically, the decay of the correlation function reflects the time it takes for the pathlength to change by one wavelength. This wavelength change results from the contributory movements of the large number of particles encountered along that scattering path. All paths are possible. None are excluded. Paths involving large numbers of scattering events decay the most rapidly because each individual particle along the route needs to move only a very short distance and takes a short time to do so. In contrast, paths made up of only a few scattering events decay more slowly as the lower number of particles must move relatively further to reach an accumulated pathlength change of one wavelength. Because all such paths are sampled, the overall correlation function obtained is markedly nonexponential.

To obtain the correlation function, the contributions from all paths are summed, each weighted by the probability, $P(s)$, that the light follows that path or random walk. This is where the light diffusion approximation is at its most powerful because it allows the problem of summation, really integration, to be reduced to the solution of a diffusion equation for the light subject to boundary conditions set by the geometry of the experiment. Size, shape, and positioning of sample, source, and detector all play a part. The back-scattering geometry of a point detector and planar source is particularly favorable in this respect and solution results in a reasonably simple analytical expression for the electric field correlation function (eqn [15]), which simplifies even further to a stretched exponential with exponent 1/2 when sample depth (L) is much greater than the diffusion mean free path for light through the suspension (l^*):

$$g_1(t) = \frac{1}{\left(1-\frac{\gamma l*}{L}\right)} \frac{\sinh\left[\left(\frac{L}{l^*}\right)\left(\frac{6t}{\tau}\right)^{1/2}\left(1-\frac{\gamma l*}{L}\right)\right]}{\sinh\left[\left(\frac{L}{l^*}\right)\left(\frac{6t}{\tau}\right)^{1/2}\right]} \qquad [15]$$

$$g_1(t) = \exp\left[-\gamma\left(\frac{6t}{\tau}\right)^{1/2}\right] \quad \text{for } L >> l^* \qquad [16]$$

Here, τ is the relaxation time and L is the thickness of a slab of infinite lateral extent; light is incident on its front face where the point detector is also located. The parameter γ is introduced as a multiplicative factor to locate the conversion plane, parallel to the front face, within the sample at which the passage of light can be considered diffusive. This provides a physically meaningful boundary condition that allows analytical solution of the light diffusion equation. In experiments using monodisperse polystyrene latices of known diameter, a value of 2.0 provided a good approximation for the value of γ. Similar equations involving hyperbolic functions can be developed for the alternative transmission geometry, although the diffusion mean free path of the light, l^*, remains to be determined.

An apparatus that approximates to the ideal back-scattering geometry described above uses a bifurcated bundle of optical fibers in a Y-shaped configuration. Half the fibers bring light from an HeNe laser down one arm of the Y into the common central leg where they are randomly mixed over the front face. When this common leg (the probe) is dipped into the scattering suspension, the other half of the fibers carry light back toward the detector. There, masking by slit and pinhole ensures that light from only a few fibers impinges on the detector. Launching and receiving fibers in the immersed face of the common leg are physically separated by a finite distance. Light can reach the receiver only through the suspension and only after having followed a multiple scattering path through that suspension. The signal received at the photomultiplier is then processed in the conventional way in a digital autocorrelator.

Equation [16] predicts that for any significant depth of suspension, the logarithm of the correlation function measured in this back-scattering configuration should be linear in the square root of the delay time, the gradient of the line being $2\gamma(\tau/6)^{-1/2}$, from which we can derive the relaxation time for the mobility of the particle, τ. Note again that we do not measure size by this technique but only particle mobility, which in favorable circumstances can be related to particle size through the Stokes–Einstein relationship (eqn [13]), as for conventional single scattering dynamic light scattering.

Because the correlation function is built up of the summation of contributions for all possible scattering paths and there is no means of dictating or interrogating the size of the particle encountered at each of the many scattering events along each path, it is physically impossible to obtain any direct information on particle size distribution from a DWS experiment. However, in studies of mixtures of pairs of lattices in predefined composition, it was possible to demonstrate that the average size was a weighted sum of individual contributions with a weighting factor proportional to the cross-sectional area of the particle.

DWS Relaxation Time and Particle–Particle Interactions

When particle concentration is increased, particle mobility is constrained by the presence of interacting neighbors. This is observed by DWS as an increase in the relaxation time with increasing volume fraction, φ. For fluid emulsions, it has been demonstrated that the Stokes–Einstein equation still held at these volume fractions but now the appropriate viscosity term was the viscosity of the emulsion itself and not simply that of the suspending solvent. This dependence of the relaxation time on particle concentration means that in an emulsion undergoing creaming, an apparent increase in relaxation time will be recorded with the probe located in the cream layer, irrespective of any size increase due to droplet coalescence. Such behavior was noted, for example, in a study of Ostwald ripening where anomalous increases in particle size, beyond those due to ripening, were encountered at high volume fractions.

DWS Behavior in Aggregating and Gelling Systems

Relaxation time is lengthened as particle motion is hindered or slowed. A logical progression of this would be toward particle entrapment and complete immobility when the particle becomes part of a gel network. With milk as a substrate, we have monitored the changes seen in DWS relaxation time as gel formation progresses, either due to enzyme action with chymosin or due to acidification. Such data may be utilized at several levels of sophistication. Initial increases in relaxation time beyond those recorded for the fluid milk have been ascribed to aggregation and the formation of aggregating micellar clusters. Since the relaxation time rapidly rises by 2–3 orders of magnitude from this point on, the reaction time at which this departure occurs has been identified as the clotting time. As a critical time in the progress of the reaction, this clotting time can be usefully related to many reaction parameters for the testing of postulated mechanisms. Also, the ability to measure clotting time with such a non-perturbing, non-destructive, non-invasive probe marks DWS as an extremely useful technique for online monitoring of these reactions and attempts have been made to market such methodology for this purpose. However, the above usage falls well short of the full potential of DWS in the study of gelling systems.

DWS and Microrheology

Groups have recognized that this light scattering behavior is a probe of the (micro)rheology of the maturing network. As we have already noted, the dynamic light scattering behavior interrogates the particle mobility. This random motion uses only the thermal energy of the system, $k_B T$. In the viscous limit, the mean-squared displacement, $<\Delta r^2>$, increases linearly with time, the gradient of this line being the translational diffusion coefficient. In the case of a particle moving in a pure elastic homogeneous medium, a plateau in the mean-squared displacement occurs when the thermal energy density of the probe particle equals the elastic energy density of the network that is being deformed by the displacement of the particle.

$$\left\langle \Delta r^2 (t \to \infty) \right\rangle = \frac{k_B T}{\pi G' a} \qquad [17]$$

Using a generalization to finite frequencies of the Stokes–Einstein relation, based on the description of the dynamics of the particle by the Langevin equation (18), the solution of which gives a relationship between the Laplace transform of the relaxation modulus, $\tilde{G}(s)$, and the Laplace transform of the mean-squared displacement, $\langle \Delta \tilde{r}^2(s) \rangle$, where s is the Laplace frequency.

$$\tilde{G}(s) = \frac{s}{6\pi a} \left[\frac{6 k_B T}{s^2 \langle \tilde{r}^2(s) \rangle} \right] \qquad [18]$$

Implementation of this scheme to yield real and imaginary components of the complex modulus, G^*, requires an algebraic functional description of their dependence on frequency ω. This is frequently not known and assumptions have to be made. Nevertheless, the technique has been used to measure the rheological properties of polymer and biopolymer gels, successfully overlapping rheological data transformed from light scattering measurements with results obtained by conventional mechanical rheometry.

These experiments were carried out monitoring the motion of a probe particle in a continuous background network. An important limitation is that the dimensions of the probe particle should be much greater than the network mesh size. When the particle is comparable to or smaller than the length scale of the structures in the medium, the tracers can move within small cavities. Motions are then a measure of not only the viscoelastic response of the network but also the effect of steric hindrance caused by the cavity walls. Also, if the surface chemistry of the particle modifies the structure of the material around the bead, the particle displacement will be a reflection of the local environment rather than the bulk rheology, which the experiment is seeking to probe. All of this relationship between DWS behavior and microrheology is an area of active research, offering the promise of widening our understanding of the role of interactions in defining rheology and structure.

In another empirical approach, it was recalled that for a Maxwellian body consisting of a single spring and dashpot in series, the rheological phase angle is given by the relationship $\tan \delta = 1/\omega\tau$, where ω is the oscillation frequency. In parallel rheology and light scattering experiments monitoring the acid-induced gelation of skim milk, this relationship was obeyed through the experiment when DWS relaxation time was substituted in this expression, $\tan \delta$ being taken from the rheology data. In another experiment using latex particles as scattering tracers during the sol–gel transition of gelatin, this correlation was obeyed for phase angle covering the range 85–5°, that is, from a viscous solution through the gel point and beyond. In these same experiments, it was noted that the amplitude of the correlation function decayed beyond the rheologically observed gel point. Correlation function amplitude, as we noted earlier, is the variance of the intensity of the fluctuations in the intensity of scattered light due to particle mobility. As the scatterers become locked up into the gel network, it is intuitively expected that such fluctuations should decay. Indeed, an inverse correlation was observed between the rate of decay of correlation function amplitude and the rate of increase of elastic modulus for a wide range of gelation kinetics in the formation of acidified milk gels. Experiments continue to expand on these observations and on the factors controlling the magnitude of the correlation function amplitude itself, but it seems obvious that information is available from these data on the bulk rheological properties of soft gels. The difficulty lies in relating this information to the structure and interactions that give rise to it, but this is a problem faced in the interpretation of mechanical bulk rheological data also.

Conclusion

This article has attempted to give a summary of the state of the art in static and dynamic light scattering techniques. To most readers, light scattering will be used as a tool, a means to an end. Hopefully, those readers will no longer see it as a black box and that the information given will help them avoid obvious pitfalls in the interpretation of their data. To others, may they see it as a vibrant technology the uses of which continue to grow and expand, perhaps through their own contributions.

See also: **Analytical Methods**: Microscopy (Microstructure of Milk Constituents and Products); Physical Methods; Principles and Significance in Assessing Rheological and Textural Properties. **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Liquid Milk Products**: Liquid Milk Products: Flavored Milks; Liquid Milk Products: Membrane-Processed Liquid Milk; Liquid Milk Products: Modified Milks; Liquid Milk Products: UHT Sterilized Milks; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects; Recombined and Reconstituted Products. **Milk**: Physical and Physico-Chemical Properties of Milk. **Milk Lipids**: Fat Globules in Milk. **Milk Proteins**: Casein, Micellar Structure.

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Atomic Spectrometric Techniques

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Atomic Spectrometric Analysis

In atomic spectroscopy, the sample is placed in an environment hot enough to break molecular bonds and produce free atoms. The atoms can be identified and their concentration measured by the emission or absorption of specific wavelengths of characteristic radiation. In atomic absorption spectrometry (AAS), light from a source emitting radiation characteristic of a specific element (usually a hollow cathode lamp) is passed through the atomized sample and the transmitted radiation is measured as a decrease in intensity. In atomic emission spectrometry (AES), the sample is heated to sufficiently high temperatures so that the electrons of the atoms are excited from their ground electronic state to an excited state. As the electrons return to ground state, they emit radiation characteristic of the element present. In conventional AAS and AES, photon detectors are used to detect the radiation transmitted through/emitted from the atomizer. Increasingly, however, mass spectrometry is used as a detector and very high sensitivity is achieved.

Minerals Analyzed in Dairy Products Using Atomic Spectrometry

All the 15 elements for which United States Recommended Dietary Allowance (RDA) and Adequate Daily Dietary Intake values exist (Ca, P, I, Fe, Mg, Cu, Zn, Se, Cr, Mo, Mn, F, Na, Cl, and K) can be determined using some type of atomic spectrometric technique. In addition, many of the elements considered essential, but have no defined requirement levels for humans, can also be determined using atomic spectrometry. This latter group includes As, Ni, Si, B, Cd, Pb, Li, Sn, V, and Co. A number of elements in both of these categories can be toxic at high levels, so their analysis in food products is all the more important. The atomic spectrometric method of choice for the determination of minerals in dairy products depends, of course, on the element to be determined, but also on the type of product to be analyzed.

Sample Preparation

Atomic spectrometric techniques conventionally are optimized for handling liquid samples. Some notable exceptions to this rule (e.g., arc and spark emission and laser ablation techniques) exist, but those are not utilized traditionally for the analysis of foods. As a result, the first step in most analyses is the digestion or decomposition of the sample to facilitate liquid sample handling. It is important that the entire sample is digested to ensure that the element(s) of interest is(are) dissolved. A sample such as milk can be analyzed after simple dilution with dilute nitric acid, but for solid samples acid digestion or fusion may be used. Milk and dairy products are normally acid digested using either wet-ashing or dry-ashing procedures. These procedures destroy the organic material and release minerals into the solution in a form suitable for analysis. Dry ashing involves combustion of the sample in a furnace followed by dissolution of the ash in dilute acid. Wet ashing can be achieved on a hot plate or heating block, or it may be carried out in a sealed Teflon vessel in a microwave oven. The increased pressure provided in sealed microwave digestion can significantly reduce digestion times. For example, 0.25 g of animal tissue can be digested in a Teflon vessel containing 1.5 ml of 70% nitric acid and 1.5 ml of 96% sulfuric acid at a microwave power of 700 W in ~1 min. The commercially available microwave digestion systems allow temperature and pressure control, and a variety of digestion methods are available in the literature. Microwave technology has greatly improved in recent years, and fully automated systems are readily available.

It is possible to analyze solid samples directly using the common forms of atomic spectrometry. The strategies used for this application are unique to each technique and are discussed below (see 'Techniques and Applications').

An important consideration in the determination of trace elements is the choice of reagents used in sample preparation. The acids used should be of high purity to ensure that contaminants if any are present at low levels. Sub-boiling distilled or double-distilled acids are the preferred choices. The laboratory environment in which samples are prepared is equally important. In an ideal situation, samples should be prepared under clean-room conditions under laminar flow to reduce contamination. Sources of contamination are many and include the analyst, vessels and containers used, ovens, and the air inside the laboratory.

Choice of Instrumentation

The most commonly used systems for the analysis of trace elements in milk and dairy products are as follows:

- flame atomic emission spectrometry (FAES)
- flame atomic absorption spectrometry (FAAS)
- electrothermal atomic absorption spectrometry (ETAAS), also called graphite furnace atomic absorption spectrometry (GFAAS)
- inductively coupled plasma atomic emission spectrometry (ICPAES)
- inductively coupled plasma mass spectrometry (ICPMS)

A summary of the analytical performance and the characteristics of the above systems are given in **Table 1**. Analytical performance involves limit of detection (LOD) in solution (ng ml^{-1}), robustness of the method (freedom from matrix interferences), selectivity (degree of spectral interferences), and precision. The analytical figures of merit are only indicative as developments and improvements in commercial instrumentation are continual.

In all analyses, several factors affect the choice of the instrumental detection method. The method selected depends on (1) the substances present in the sample, (2) the expected concentration of the analyte, (3) the accuracy required, (4) the number of samples, (5) cost, and (6) the time, equipment, and expertise available. For each application, all factors should be considered, but often detection capability is the top priority. A general rule is to use the simplest method available that provides the detection required. The vast body of literature in analytical chemistry serves as the most important and most useful source of information on sample preparation and choice of detection method. Modern instruments now provide a library of methods for determining different elements with a list of potential interferants.

Techniques and Applications

The five techniques listed above are used for the vast majority of analyses involving milk and dairy products. A brief description of each technique follows, together with a mention of their applications in dairy product analysis. To keep informed, the reader is directed to the annual Atomic Spectrometry Updates in Clinical and Biological Materials, Foods and Beverages in the *Journal of Analytical Atomic Spectrometry*.

Flame Atomic Emission Spectrometry

This method is also known as flame photometry and is a robust, low-cost technique used for the determination of Na, K, Li, Ca, and Ba. Simple instruments with digital readout use air and methane flames to atomize and excite the elements listed in a liquid sample. The strong emission lines of Na, K, Li, Ca, and Ba are isolated using optical filters. Flame photometers are routinely used in clinical laboratories and are very useful for determination of the relatively high concentrations of Na and K present in dairy products.

Flame Atomic Absorption Spectrometry

Like FAES, FAAS is a simple, robust, and easy-to-use tool for the analysis of liquid samples. Common flame types are air–acetylene, which can reach a temperature of ~2500 K, and nitrous oxide–acetylene, which can reach a temperature of ~3000 K. The sample is passed in a continuous flow through the flame, where atomization takes place. Calibration can normally be achieved using aqueous standard solutions. LODs are in the low range of micrograms per milliliter, so the method is useful for the detection of a number of elements in dairy products, including Ca, Cu, Fe, Mg, Mn, and Zn.

Direct analysis of milk can be achieved using FAAS, eliminating the need for sample preparation. Although the sample introduction system is optimized for liquid samples, the interface in FAAS is quite forgiving to high levels of dissolved solids, and to suspended solids. Several groups of workers have reported excellent sensitivities and LODs achieved through direct introduction of suspensions of milk powder and milk-based infant formulae into FAAS. Milk fat can be emulsified with sodium dodecylbenzosulfate and directly analyzed by FAAS. Of paramount importance in direct analysis, particularly of suspensions and emulsions, is the homogenization of the sample prior to and often during introduction into the flame. This can be achieved by placing the sample-containing vessel in an ultrasonic bath or on a vortex mixer immediately before analysis.

Table 1 Summary of analytical performance of the atomic spectrometric techniques most commonly used in analysis of milk and dairy products

Technique	*Sample volume (ml)*	*LOD (ng ml^{-1})*	*Multielement*	*Matrix effects*	*Spectral interferences*	*Precision (% relative standard deviation)*
FAES	5–10	1–100	Yes	Large	Significant	0.5–1
FAAS	5–10	1–10^{3}	Possible	Large	Few	0.5–1
ETAAS	0.01–0.1	10^{-2}–0.1	Possible	Moderate	Few	3–5
ICPAES	1–10	0.05–10	Yes	Small	Large	0.5–1
ICPMS	1–10	10^{-3}–10^{-2}	Yes	Moderate	Significant	1–3

Electrothermal Atomic Absorption Spectrometry

ETAAS provides low and sub-nanogram-per-milliliter detection limits using microliter-sized sample volumes. The sample is placed onto a small graphite platform situated inside a resistively heated graphite tube. The furnace is heated to temperatures above 3000 K. Over the past two decades, stabilized temperature platform furnace (STPF) conditions established by M Slavin have become adopted widely. Methods developed according to the STPF conditions have allowed direct quantitation using aqueous standards.

In addition to the higher temperatures obtainable in ETAAS as compared with FAAS, the temperature is controllable and various steps are incorporated into a program, to facilitate drying, charring, or ashing, and atomization of the sample. In modern instruments, these steps can be followed via a built-in video camera placed to look into the graphite tube as the program is executed. Judicious choice of a temperature program can allow *in situ* sample preparation, so that solid samples can be analyzed directly. The most successful solid sampling ETAAS analyses have been achieved through the use of slurries or suspensions of powdered sample in aqueous media. Preparation of a slurry means that conventional liquid sample handling equipment can be used. Much of the work in the area of slurry analysis, particularly for food analysis, has been carried out by N J Miller-Ihli.

Inductively Coupled Plasma Atomic Emission Spectrometry

Inductively coupled plasma (ICP) reaches much higher temperatures than a combustion flame (>8000 K). Argon plasmas are conventionally used to provide a high-temperature, high-stability excitation source in ICPAES. The inert atmosphere of the argon plasma eliminates a significant proportion of the interference encountered in combustion flames. The photon detectors used in ICPAES have become increasingly sophisticated; charge-coupled devices and charge injection devices are now commonplace. These detection systems allow both fast sequential and simultaneous multielement analyses with ever-decreasing LODs. A very desirable feature of ICPAES for the analysis of milk and dairy products is the wide linear dynamic range of calibration achievable. ICPAES allows both trace and macro elements to be determined simultaneously. Because of these features, ICPAES is replacing AAS as the elemental analytical technique of choice in many laboratories. However, it should be remembered that capital and running costs of ICPAES are far higher than those of FAAS or ETAAS though the detection capabilities are similar. The number of elements to be determined routinely or otherwise must be borne in mind when investment in ICPAES is considered.

Inductively Coupled Plasma Mass Spectrometry

The high temperature and relative freedom from interference offered by ICP renders it an excellent source not only for atomic emission spectrometry, but also for mass spectrometry. Ion detection by a mass spectrometer provides both enhanced sensitivity over ICPAES and the possibility of isotopic analysis. ICPMS provides multielement, isotopic data with detection at the sub-nanogram per milliliter level, also permitting the detection of non-metals of interest in milk and dairy products, such as phosphorus and iodine. ICPMS permits the use of isotope dilution in trace and major element analysis. Isotope dilution uses isotopes as internal standards for quantitative analysis; so no external calibration is required.

The costs involved in purchasing and operating ICPMS equipment are significant and commensurate with the analytical performance, and the use of the technique is increasing.

Method Validation

Analytical methods are validated by demonstrating that accurate results have been obtained. This can be achieved by a number of approaches:

- analysis of standard reference materials
- comparison of results obtained using a second analytical method
- comparison of results obtained from another analytical laboratory

One or more of the above should be carried out when a new method is developed. A number of standard reference materials are available for validation of mineral analysis of milk and dairy products. Some examples are given in **Table 2**. Use of standard reference materials is essential for quality control in method development. However, as these materials are expensive, analysts should consider production of in-house quality control materials. These materials can be characterized using the approaches listed above.

Conclusions

Various techniques of atomic spectrometry have been used for decades for the analysis of trace and major elements in milk and dairy products. Advances in both

Table 2 Commercially available reference materials for milk and dairy products analysis

Supplier	*Reference material*	*Certified constituents*
Agricultural Research Centre, Finland	ARC/CL-1 Skim milk powder	Cd, Ca, Cu, Fe, Pb, Mg, Mn, Hg, Mo, Se, Zn
BCR, European Commission	CRM063R Skim milk powder (natural)	Ca, Cl, Cu, I, Fe, Pb, Mg, N, P, K, Na, Zn
	CRM150 Skim milk powder (spiked)	Cd, Cu, I, Fe, Pb, Hg
	CRM151 Skim milk powder (spiked)	Cd, Cu, I, Fe, Pb, Hg
National Institute of Standards and Technology (NIST), USA	SRM1549 Nonfat milk powder	Cd, Ca, Cl, Cr, Cu, I, Fe, Mg, Mn, Hg, P, K, Se, Na, S, Zn
	SRM8435 Whole milk powder[a]	Al, As, Ba, B, Br, Cd, Ca, Cl, Cr, Co, Cu, F, I, Fe, Pb, Mg, Mn, Mo, Ni, N, P, K, Rb, Se, Na, Sr, S, Ti, W, Zn
	SRM1846 Milk-based infant formula[a]	Ca, Cu, I, Fe, Mg, Mn, P, K, Se, Na, Zn, Cl

[a] Values for these reference materials are noncertified but indicative.

sample preparation and instrumental detection techniques have resulted in improved sensitivity and ease of use in many routine analyses.

See also: **Analytical Methods**: Infrared Spectroscopy in Dairy Analysis; Sampling.

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Nuclear Magnetic Resonance: An Introduction

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Introduction

Nuclear magnetic resonance (NMR) spectroscopy is a technique used to determine the detailed, three-dimensional structure of molecules and, more broadly, to study the physical, chemical, and biological properties of matter. It uses a strong magnet that interacts with the natural magnetic field in atomic nuclei. In addition, unlike other techniques for determining molecular structures and physicochemical properties, NMR allows the user to carry out experiments non-destructively. The rheological and functional properties of food components are related to their molecular structure, morphology, and atomic mobilities. NMR provides a powerful tool for elucidating chemical structures, molecular conformations, and interactions of components in food systems. NMR spectroscopy uses the interactions of radio frequency waves with the nuclei of certain atoms in a magnetic field to probe the electronic environment of the nucleus, and from this determine the molecular structure of the sample being examined. NMR and X-ray crystallography remain the only methods for structural determination, with NMR being the only method available to determine the structure of a molecule in solution. For compounds in a mixture, while there may be some small changes due to solvent and other effects, in general the NMR signals remain characteristic of each compound and useful information may be gained even from complex biological samples, although full structural elucidation may not be possible.

A fully accurate description of the principles behind NMR will tend to become complicated beyond the point of any clarity for readers not already extensively familiar with the technique. As a first stumbling block, the theory of NMR is based on an intrinsic quantum mechanical property of the nucleus; attempts to explain this in classical terms will be incomplete while accurate descriptions quickly become exercises in complex mathematics and models. In addition, the procedure giving rise to the spectrum involves timed and calibrated radio frequency pulses, superconducting magnets with additional tuning coils for uniform magnetic fields, and Fourier transforms from time to frequency domains. While all of these are integral to NMR, the complex theory stands in sharp contrast to the ease of use and interpretation associated with most modern systems where a large part of the more theoretically complicated aspects is handled by computerized systems during normal use. However, awareness at some level of the theory behind the technique is always an advantage even for the most casual NMR user and will enable users to better plan and execute their experiments. In the following, we will attempt to give a brief introduction to the basics of NMR background, techniques, and use, without straying into the overly technical or theoretical aspects. More complete accounts can be obtained from the list of recommended reading provided in Further Reading.

Nuclear Spin as the Basis for NMR

The nuclei of atoms possess a characteristic called spin. In atoms with odd mass numbers, the spin is described with half-integral spin quantum numbers. In atoms with even mass and atomic number, the spin is zero, and in atoms with even mass number and odd atomic number, the spin is described using integral quantum numbers. Only nuclei with non-zero spin quantum numbers are NMR active. In those atoms that are NMR active, the atoms possess a magnetic moment due to the spinning electric charge associated with the protons in their nucleus. In the absence of a magnetic field, there is no energy difference between the possible alignments of these states. In the presence of an applied magnetic field, protons may align with or against the applied field depending on the spin state. Transitions between the spin states of the atoms in a magnetic field from a lower to higher level require energy. The energy required can be supplied by electromagnetic radiation in the radio frequency region, with the energy required being proportional to the strength of the magnetic field. NMR spectroscopy studies the absorbance of this energy in order to determine the structure of the compounds examined.

The energy (and hence wavelength) of radiation absorbed is a function of both the applied magnetic field and the properties of the nucleus. An additional complication is the magnetic field caused by the electrons surrounding the nucleus. Electrons in an atom in a magnetic field also produce an independent magnetic field, which opposes the applied magnetic field. This results in alterations of the magnetic field experienced at the nucleus and alters the absorption frequency of the nucleus by effectively shielding the nucleus. Therefore, each nucleus of the isotope being studied in a particular environment will absorb radio waves at a distinct wavelength. As the electronic environment of a nucleus depends on its neighboring atoms and its bonding to these atoms, the examination of the wavelengths absorbed and the intensity of the absorption gives an indication of the structure of the atoms in question.

While the spectrum obtained by an NMR spectrometer can be thought of as the signal obtained due to absorbance of radiation while a magnetic field is varied, modern NMR systems do not use this method. Instead, the sample is held in a strong magnetic field, while being irradiated with a short pulse of radio frequency radiation. This excites all the nuclei at once, with the resulting signal being a free induction decay (FID) signal as a function of time. This is converted to a frequency signal by the Fourier transform to give the NMR spectrum. A series of short pulses is usually used with the final spectrum being an average of the spectra obtained at each pulse.

Practical Requirements for NMR

A simple cross-sectional diagram of a typical NMR spectrometer is shown in **Figure 1**. The principal requirement for an NMR spectrometer is a strong magnet. In nearly all systems in current use, this is in the form of a superconducting electromagnet. The core of these systems consists of a current flowing through a superconducting loop; as there is no resistance in the circuit, the current flows without external driving energy, and so as long as the temperature remains below the temperature at which the magnetic material behaves as a superconductor, the magnetic field remains constant over a prolonged period of time. This low temperature is generally achieved by placing the magnet in a dewar of liquid helium at 4 K, which is then surrounded by a dewar of liquid nitrogen to limit the boiling of the helium. The center of the system contains the bore for introduction of samples and probes.

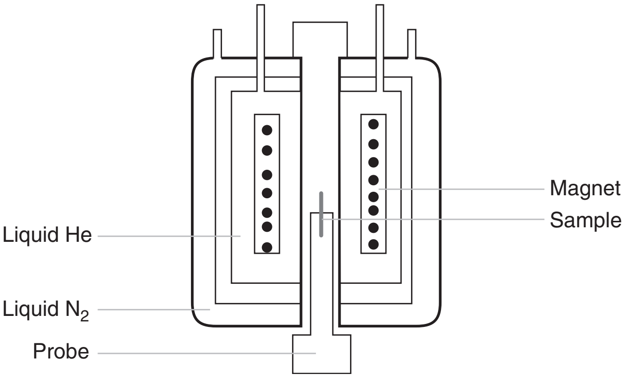

Figure 1 Cross-sectional diagram of an NMR magnet.

The probes consist of the radio frequency transmitters and receivers, in addition to localized temperature controls. The sample is generally introduced into the bore using a pneumatic system with the lower end of the sample tube (containing the sample solution) sitting in the probe surrounded by the transmitter and receiver coils.

In general, for a spectrum, the sample is dissolved in deuterated solvents, that is, common solvents where hydrogen atoms are substituted with deuterium, for example $CDCl_3$ in place of $CHCl_3$ (chloroform). The use of deuterated solvents is necessary as the signal from protons in solvents would effectively mask the signals from the sample being analyzed. It also allows the use of the deuterium in the sample as a lock signal. This is used to compensate for small changes in the magnetic field due to external factors.

Most standard NMR sample tubes are 5 mm in diameter, although both larger and smaller diameter tubes are also used depending on requirements and the particular system in use. In all cases, the tubes are made from high-purity glass and manufactured to high standards to minimize interference and artifacts.

Once dissolved and filtered into a tube, the sample can be introduced into the magnet. Small frequency and sensitivity adjustments are made to obtain optimum results for each sample. In addition, small changes to the magnetic field are usually necessary, a process known as shimming. This involves making small (in comparison to the main magnet) changes to the current flowing in special shim coils. The purpose of this is to ensure that the sample experiences a uniform magnetic field, which results in sharper peaks and greater resolution. Shimming can be carried out manually, although automatic systems simplify the process and provide good results. Once all necessary adjustments are made, the spectrum can be obtained. A number of experiments can be run on the same sample and the spectrometer systems are generally highly automated. While programs exist to aid analysis of complex spectra, in general simpler spectra are interpreted manually.

Uses and Information Obtained

In essence, any element that has an isotope of non-zero nuclear spin can be used for NMR, with the usefulness of information obtained varying due to many conditions. In practical terms, NMR is not a very sensitive technique, and relatively large amounts of compounds are required for good results with the exact amount varying from system to system and with varying experimental requirements; however, the sample can be recovered at the end of the experiment as NMR is a non-destructive technique. The recommended amounts required for a sample will vary according to the type of tube, probe, strength of magnet, and isotope being examined.

The most commonly examined nuclei are ^{1}H, the most common isotope of hydrogen, and ^{13}C, the only isotope of carbon active in NMR terms. As the frequencies of absorption vary with magnetic field, a scale has been developed for the easy comparison of results. The scale used is the ppm scale and is described by the equation

$$\delta \text{ (in ppm)} = (\text{observed shift from TMS in Hz}) \times \frac{10^{6}}{(\text{operating frequency of the instrument in Hz})}$$

Hydrogen will in general give rise to peaks between 0 and 15 on this scale. The scale is referenced to tetramethyl silane (TMS) (at 0), which contains protons in a region of high electron density (highly shielded protons). For most common uses, the spectra are referenced to the residual solvent peak position rather than using TMS in every sample, although where this results in ambiguity or where greater accuracy is required, TMS can be added to the sample solution. As a rule, protons in an electron-rich environment will absorb toward zero, while those in an electron-poor environment (due to bonding or the presence of strongly electronegative atoms) will absorb at the 7–15 end of the scale. An additional complication is seen in aromatic compounds, where the presence of delocalized electrons increases rather than decreases the magnetic field felt at the nucleus, resulting in most aromatic species absorbing at 7–9 ppm. Deshielding is seen in protons attached to doubly bonded carbons, while particularly strong deshielding is seen in aldehydes due to both the presence of the double bond and the electron withdrawing effect of the oxygen. In addition to providing information as to the particular environment of the proton, the integration of the peak obtained will give the relative amount of that particular proton to another (with an error of approximately 10%). This allows the distinction between, for example, CH_3, CH_2, and CH in a molecule.

The carbon of TMS can also be used in a similar fashion, with the scale in this case extending over a wider range, and with the positions of the carbon signal being dependent on the same types of deshielding and shielding seen in proton spectra.

There are two further contributions toward the signal obtained. These can broadly be defined as the influence of atoms that are connected through bonds to the signal obtained and the influence of atoms that are near in space to the observed atom.

The influence of atoms that are closely bonded gives rise to splitting patterns, as the transfer of energy through bonds gives rise to small energy level differences. Therefore, an adjacent CH_3 will split a CH_2 into a quartet, while a CH_2 will split a CH_3 into a triplet. The splitting pattern depends on the number of equivalent protons, with in general *n* equivalent protons giving a splitting of $n+1$. Equivalent protons for the purposes of splitting are defined as those that have the same chemical shift and are chemically equivalent. For instance, for CH_3 or CH_2 in alkyl chains, including those substituted with heteroatoms or other groups, all protons are considered equivalent. Non-equivalent protons are frequently seen in saturated ring systems (such as carbohydrates) and in alkene groups. This gives rise to more complicated splitting patterns in these groups. Note that protons in different enantiomers will be equivalent, and therefore splitting will not in general be different in these groups, while for other structural isomers, the protons will not in general be equivalent in the absence of other symmetry factors.

Splitting intensities are in general described by Pascal's triangle (shown below) so, for example, a single proton will split an adjacent set of protons into a doublet, with the ratio of the intensities of the peaks of the doublet to each other being 1:1.

0 – singlet 1

1 – doublet 1 1

2 – triplet 1 2 1

3 – quartet 1 3 3 1

4 – quintet 1 4 6 4 1

Where the relative intensities of the splitting pattern are significantly different from this, it can be assumed that the splitting pattern is caused by more than one set of equivalent protons; for example, in the case of CH_3-CH_2-CH_2CN, the splitting pattern will consist of a CH_3 triplet, a CH_2 triplet while the central CH_2 will be split into a 1:3:3:1 quartet with each peak further split into a 1:2:1 triplet by the CH_2 to give 12 peaks, which will not have a relationship defined by Pascal's triangle and will have two distinct splitting constants.

Splitting constants (the distance between the peaks in a multiplet, usually expressed in Hz) are in general reciprocal, where one group splits a group by $J = 6$ Hz and that group will in turn be split by $J = 6$ Hz. This is one way of determining connectivity in complicated spectra. Splitting patterns also vary with angle according to the Karplus equation, although there are some exceptions, and this can be useful for the determination of the geometry of ring systems and other constrained structures. In addition, more complex splitting patterns are seen in aromatic molecules, with substituted aromatic ring systems giving distinctive splitting patterns depending on the substitution pattern. These can help determine substitution patterns and give distinct and easily recognized signals for key groups.

The presence of protons or other atoms nearby in space gives rise to the nuclear Overhauser enhancement (NOE). This is an enhancement of the signal due to the presence of atoms nearby in space. It is used in NOE spectroscopy, which can be used to probe the three-dimensional structure of molecules and also gives rise to small enhancements in ^{13}C spectra.

Carbon-13 NMR

As we have already seen, for an atom to be useful for NMR purposes, it must possess uneven nuclear spin. In the case of the carbon, this leads to a significant disadvantage, as the most common isotope (^{12}C) has even nuclear spin. The stable isotope ^{13}C has uneven spin, but is present at only 1% of total natural abundance. In addition, it is less sensitive to NMR than the proton. In general, this would make it unsuitable for NMR analysis; however, as it is such an important component of most organic systems of interest, methods have been developed to compensate for these disadvantages.

The acquiring of ^{13}C spectra must also be carried out using pulse Fourier transform-NMR (FT-NMR) spectrometers using signal averaging. Instrumental parameters are used to decouple proton–carbon interactions, to prevent splitting of carbon signals, which would result in loss of sensitivity and a more complicated spectrum. In general, higher concentrations of samples are required with a far greater number of scans needed when compared to proton NMR and therefore acquiring of a spectrum will take a far greater length of time (over an hour compared to roughly a minute).

In a ^{13}C spectrum, the ppm scale runs from 0 to 200 ppm, and in addition no splitting due to adjacent carbons is seen due to the low probability of two ^{13}C atoms being adjacent in a molecule. Peaks will generally be narrow and well defined, and peaks due to carbons having protons attached will usually be higher than those with no attached carbons. In the case of carbon spectra, integration of the peaks gives no meaningful information about the relative number of carbons responsible for a signal. Additional pulse experiments distortionless enhancement by polarisation transfer (DEPT) can give information about the number of protons attached to a carbon giving a signal at a particular position.

Sample Spectra

Ethanol can provide an example of the use of an NMR spectrum of a molecule to solve structure (**Figure 2**). The ^{1}H spectrum of ethanol consists of one peak at 1.17 ppm and one peak at 3.65 ppm, with a third peak visible due to the solvent D_2O. When examined closely, as in the insets next to both peaks above, the peak at 1.17 ppm is split into a triplet, while the peak at 3.65 ppm is split into a quartet. Depending on several factors, including temperature and solvent used, an additional broad OH peak may be seen at varying locations. Integration of the areas of the peaks will give a ratio of 3:2 for the peaks 1.17:3.65. This allows us to determine that the peak at 1.17 is due to CH_3, from both the integration and its position toward 1 due to the absence of any directly connected heteroatoms. The only other significant peak at 3.65 is due to CH_2; integration shows that it contains two protons, and the position shows the connection to the OH group, resulting in slight deshielding due to the electronegative oxygen.

The CH_3 peak is split into a triplet by the adjacent CH_2, with the CH_2 peak being split into a quartet by the CH_3. The splitting constants are reciprocal, both being in the region of 7 Hz. This confirms the assignments made above, and it also confirms that the two carbons in question are bonded and adjacent, confirming that the structure of the molecule in the sample tube in this case is CH_3CH_2OH.

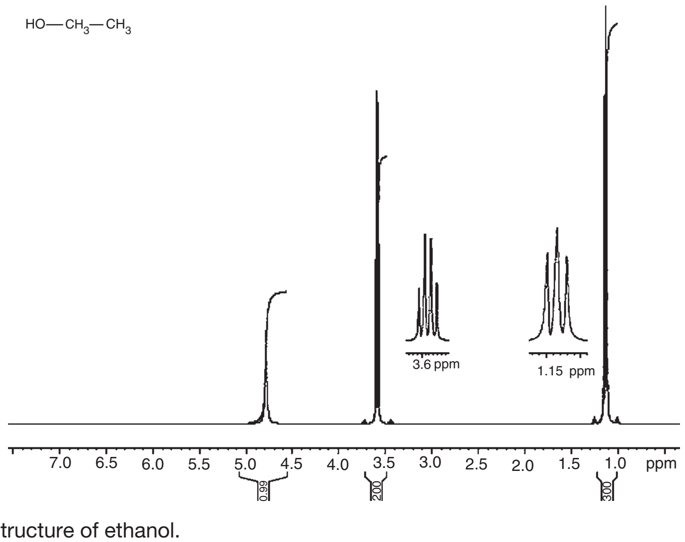

Figure 2 ^{1}H spectrum and structure of ethanol.

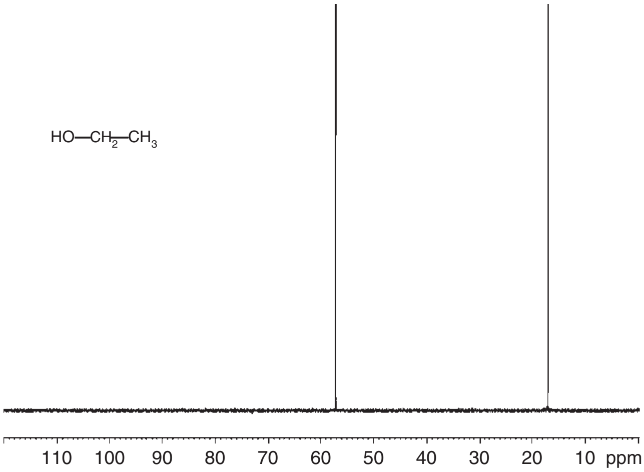

Figure 3 ^{13}C spectrum of ethanol.

The broadness, variability, or occasional absence of the OH peak demonstrates another characteristic of NMR. In terms of molecular motion and reactions, it is not a particularly fast technique, and is in effect taking an averaged spectrum of species in constant fast interactions. The OH peak in alcohols, phenols, and carbohydrates is subject to exchange with other hydrogens, whether in solvents or other alcohols. If D_2O is used as a solvent, the ethanol will exchange D for H frequently enough to remove the OH signal. The exchange also leads to the observed signal broadening. Techniques have been developed to take advantage of these effects to probe reactions and movements on a molecular scale . The ^{13}C spectrum of ethanol is relatively simple, consisting of two peaks, one at 17.5 corresponding to the shielded CH_3 and the other at 58.1 corresponding to the CH_2 attached to the OH (**Figure 3**).

Multidimensional Spectra

The spectra described above are one dimensional. This means that we have irradiated the sample and observed the FID, which is transformed into our spectrum. Additional information can be gained by two-dimensional and multidimensional methods. In these, the molecules in the sample are irradiated, leaving the molecules with an altered energy level distribution. Before the molecules return to their original energy level distribution, a further irradiation is carried out and a two-dimensional spectrum obtained.

Variations on the excitation methods used can further probe the structure of the molecules, with various interactions giving rise to altered distributions; for example, relaxation through bonds or through space will give altered absorption compared to isolated systems. Examples of two-dimensional methods include COSY (correlation spectroscopy) and HMQC (heteronuclear multiple-quantum coherence). A COSY spectrum will display cross-peaks where peaks are due to protons on adjacent carbons, and supplements information available from splitting patterns. In particular, this can be useful in aromatic systems for distinguishing long-range and short-range splitting patterns.

An HMQC is used to correlate the carbon and proton signals, confirming that a particular carbon peak is the same carbon that a particular group of hydrogens producing a given signal are attached to.

Once again, we can return to the spectrum of ethanol to demonstrate some of these two-dimensional spectra . In the COSY spectrum (**Figure 4**), the cross-peaks between the signals due to the adjacent carbon can be seen clearly, confirming the conclusions made with regard to the splitting pattern. In the HMQC spectrum (**Figure 5**), the peaks show the correlation between the carbon at 17.5 ppm and the proton at 1.17 ppm, and the carbon at 58.1 ppm and the proton at 3.65 ppm. While these results

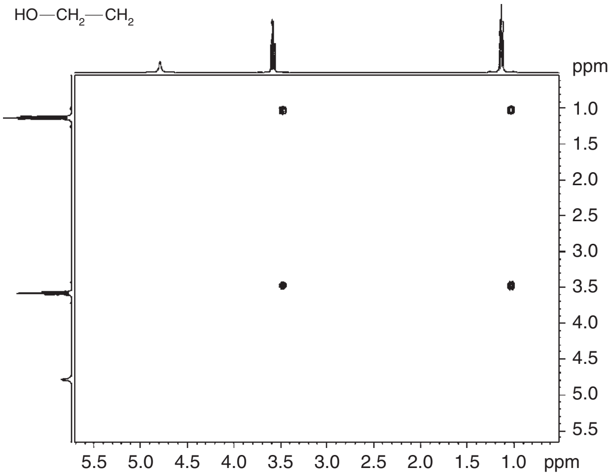

Figure 4 COSY spectrum of ethanol.

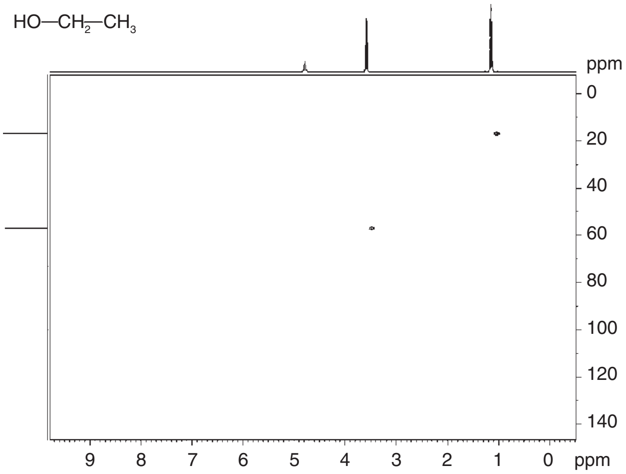

Figure 5 HMQC spectrum of ethanol.

seem trivial when applied to a small molecule, they can be invaluable in solving more complex molecules and for confirming results for unknown compounds.

In addition, methods have been developed such as DOSY to correlate diffusion constants of mixtures with particular signals in mixtures, HETCOR (heteronuclear correlation spectroscopy) for correlation with heteroatoms, TOCSY (total correlation spectroscopy) for all long-range and short-range coupling, and a wide variety of variations on each of these methods are used depending on the required results and systems.

Applications of NMR in Food Science

NMR spectroscopy has many applications in food science. Some of these are not unique to food science. For example, structure elucidation of new and novel compounds derived from foods is one of the most common applications of NMR to foods. However, unlike conventional organic chemistry where molecules of interest can be synthesized in large enough quantities to acquire an NMR spectrum, food science researchers are often faced with the considerable challenge of extracting and purifying compounds from their natural sources. It is, however, not always necessary to extract and purify a particular component to obtain useful information by NMR from a food. In fact, NMR has been used extensively on whole foods and crude extracts thereof in techniques such as stable isotopic analysis. This technique is an excellent tool for origin assessment as the ratio $^{13}C/^{12}C$ gives straightforward responses concerning the primary photosynthetic metabolism of plant products, and the ratios of the stable isotopes of oxygen ($^{16}O/^{18}O$) and hydrogen ($^{2}H/^{1}H$) are good indicators of environmental conditions. Using NMR, the natural abundance of isotopomers may be precisely and accurately quantified, thus allowing the user to obtain vital information about the geographical origin and authenticity of foodstuffs. The application of ^{1}H and ^{13}C and site-specific natural isotope fractionation (SNIF) NMR studies to the practical problems of food authenticity, adulteration, and geographic origin has increased significantly over the last decade. The SNIF technique allows measurement of the relative amounts of deuterium and hydrogen in a particular sample, and in cases where an alcohol has been derived from a plant-produced sugar allows determination of the type of plant used, for example, sugar beet versus grape. This is used extensively for the determination of the adulteration of grape must with sucrose derived from sugar beet.

To date, NMR analysis of solid foods has usually been carried out on crude or purified components dissolved in deuterated solvents. However, modern NMR probes using magic angle spinning allow the application of NMR to whole solids, which is of considerable importance to food science as many foods are solids at room temperature. This approach results in a very complex spectrum; however, using chemometric and statistical methods, these spectra can provide insights into the structures and dynamics of constituents in complex food systems. This in turn allows the user to gain a better understanding of attributes governing quality, stability, and safety such as appearance, feel, smell, and taste. For example, signal peaks indicative of lipid oxidation and important quality index governing flavor can be determined against a background of normal lipids and other components in complex systems.

Metabolomics is the study of the metabolome, which is the entire metabolic content of a cell or organism at a given moment. The most extensively used analytical approach for metabolomics is NMR spectroscopy. This technique allows the user to develop a metabolomic fingerprint of a sample. This fingerprint can then be used to look for compositional similarities and explore the overall natural variability. NMR spectroscopy of biofluids and tissue extracts can rapidly generate vast quantities of spectral data and therefore advanced bioinformatic approaches are necessary to obtain useful information. While most of the above discussion has focused on carbon and proton spectra, it should not be forgotten that in principle NMR can be used for the study of any atom with an isotope that has nonzero spin. Nitrogen-15 spectroscopy can be used in the determination of peptide and some protein structures; in general, the samples used must be enriched with ^{15}N. Other isotopes of interest include isotopes of oxygen and phosphorus. While specific difficulties for each isotope and particular requirements may be present, in general an understanding of the principles involved in carbon and proton NMR and the interpretation of their spectra will be useful in understanding a wider range of techniques.

From the above discussion, it is clear that NMR can be used to determine the structure of molecules in solution, and is the only method capable of doing this. It can be used to probe the composition of complex mixtures and can be an important tool for the determination of the quality and purity of a range of samples. With careful measurement and techniques, it can be used in a semi-quantitative fashion. It also complements and can be complemented by various other analytical techniques; for example, it can be used in conjunction with mass spectrometry to determine the structure of an unknown plant isolate. The further examples and descriptions in this encyclopedia will give a clear demonstration of the various uses to which NMR can be put, in particular focusing on the area of food science.

See also: **Analytical Methods**: Nuclear Magnetic Resonance: Principles.

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Nuclear Magnetic Resonance: Principles

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Background

The many applications of nuclear magnetic resonance (NMR) to dairy products can be divided into three groups, according to the NMR technique used. The first group uses high-resolution NMR spectroscopy for the molecular study of dairy compounds such as casein, α-lactalbumin, β-lactoglobulin, lactose, and triacylglycerol. All possible nuclei (^{1}H, ^{2}H, ^{17}O, ^{13}C, or ^{31}P) can be used to obtain molecular structures. The second group involves the application of magnetic resonance imaging (MRI) to dairy processing and the third group concerns studies based on NMR relaxation and diffusion measurements. Only MRI and NMR relaxation and diffusion applied to dairy products will be described in this article. We first discuss NMR and MRI techniques as such, with special emphasis on relaxation time parameters, and provide some examples of how NMR and MRI can be applied to dairy products and dairy processes.

NMR Technique and Parameters

NMR spectroscopy is based on the fact that certain nuclei possess a permanent nuclear magnetic moment. When placed in an external magnetic field, they take on a certain well-defined state, which corresponds to a distinct energy level. Transitions take place between neighboring energy levels due to the absorption of electronic radiation at radiofrequencies. The energy difference between the energy levels is proportional to the magnetic field B_0:

$$\Delta E = \frac{\gamma h}{2\pi} B_0 = h\nu \qquad [1]$$

where γ is the gyromagnetic ratio of the respective nucleus, h the Planck's constant, and ν the resonance frequency. The signal in NMR is obtained by simultaneous excitation of all transitions with radiofrequency pulses at the Larmor frequency ω_0, followed by detection of the radiation emitted as the system returns to the equilibrium state. The signal recorded after excitation is called the relaxation signal, and two relaxation signals can be measured according to the excitation scheme: spin–lattice (or longitudinal) relaxation, designated T_1, and spin–spin (or transverse) relaxation, designated T_2. The time constants describe these exponential relaxation processes and are known as relaxation times. Relaxation is a function of the spin species and the chemical and physical environments surrounding the spins. Analysis of the T_1 and T_2 of a sample will therefore allow study of the chemical and physical properties of the sample.

The mechanisms involved in T_1 and T_2 are different. T_1 is the interaction of a nuclear spin dipole with the random, fluctuating magnetic fields caused by the motion of the surrounding dipoles: the closer the frequencies of the fluctuating magnetic fields to the resonance frequency, the more efficient or faster the spin–lattice relaxation. There is a distribution of frequencies in the sample and only the frequencies at the Larmor frequency will affect T_1. T_1 will therefore vary as a function of the strength of the magnetic field since the Larmor frequency is proportional to B_0 ($\omega_0 = \gamma B_0$) and will vary as a function of the molecular motion τ_c. For slow motion ($\omega_0\tau_c >> 1$), the system is in the solid regime and T_1 can be very long. This is the situation encountered for crystals. In liquids, when rapid motion occurs ($\omega_0\tau_c << 1$), the molecular motion is very rapid and T_1 is also very long. For cases with $\omega_0\tau_c \cong 1$, T_1 is very short.

T_1 is measured by the inversion recovery (IR) pulse sequence or the saturation recovery (SR) sequence (**Figure 1**). The signal is generally described by the sum of exponentials:

$$Y(t) = \sum_{i=1}^{n} I_i \left(1 - \alpha \exp\left(-\frac{t}{T_{1i}}\right)\right) \qquad [2]$$

where α is a constant that depends on the NMR sequence used, that is, IR or SR, and on the imperfection of the pulses. For an IR sequence, α is close to 2, and for an SR sequence, α is close to 1. I and T_1 are the NMR signal intensity and the spin–lattice relaxation time, respectively. The number of exponential terms (n) will vary according to the chemical composition of the sample and according to the microstructure. Specific examples are given below for dairy products.

Unlike spin–lattice relaxation, spin–spin relaxation is an adiabatic process and the redistribution of energy among the spins does not change the number of nuclei at the higher energy level. Moreover, T_2 is independent of the resonance frequency. T_2 will thus vary only as a function of the molecular motion. For solids or crystals, T_2 values are approximately several microseconds, and for liquids, T_2 values are in the seconds range. Two pulse sequences should be used according to the T_2 value for the measurement of T_2. A single 90° pulse is used for short T_2 values and the signal recorded is named free

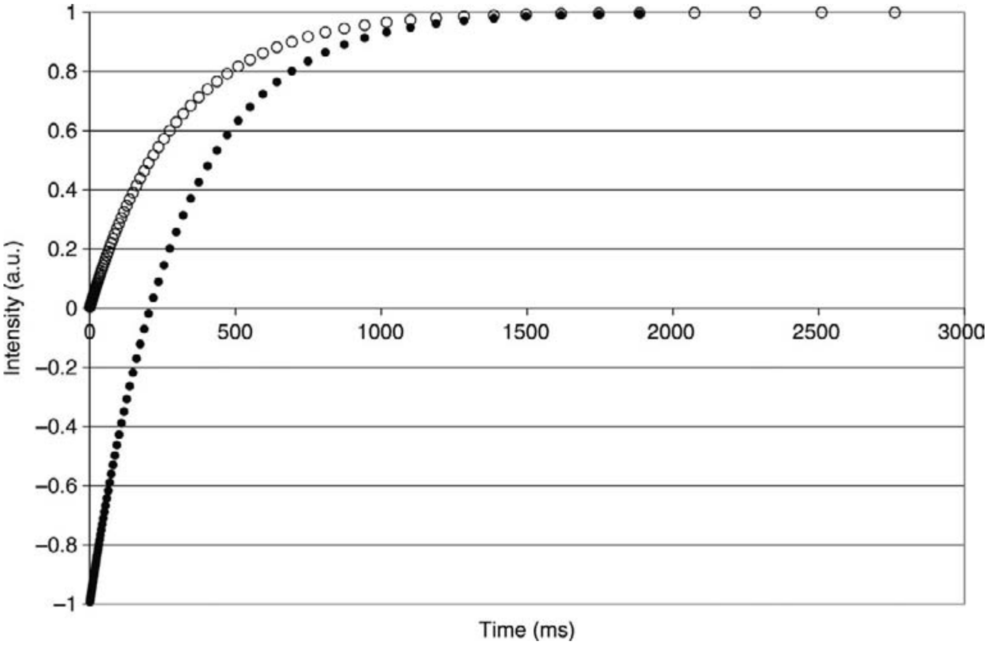

Figure 1 Spin–lattice relaxation signal after a saturation recovery NMR sequence (O) and an inversion recovery sequence (●).

induction decay (FID). However, for long relaxation T_2, the spin–spin relaxation measured is much longer than the T_2 value expected because of the inhomogeneity of the magnetic field. This magnetic field inhomogeneity induces a faster signal decrease. The most common approach used to reduce this effect is a Carr–Purcell–Meiboom–Gill (CPMG) sequence. This sequence is a 90° pulse followed by a train of 180° pulses. Each of the 180° pulses is used to reduce the effect of the magnetic field inhomogeneity. Nevertheless, the shortest relaxation time that can be measured with a CPMG sequence is around the millisecond range because of the slow acquisition rate compared to the FID. Thus, both signals have to be acquired when the sample is a mixture of compounds with short and long relaxation times, that is, the FID and the CPMG signal, respectively (**Figure 2**). For a

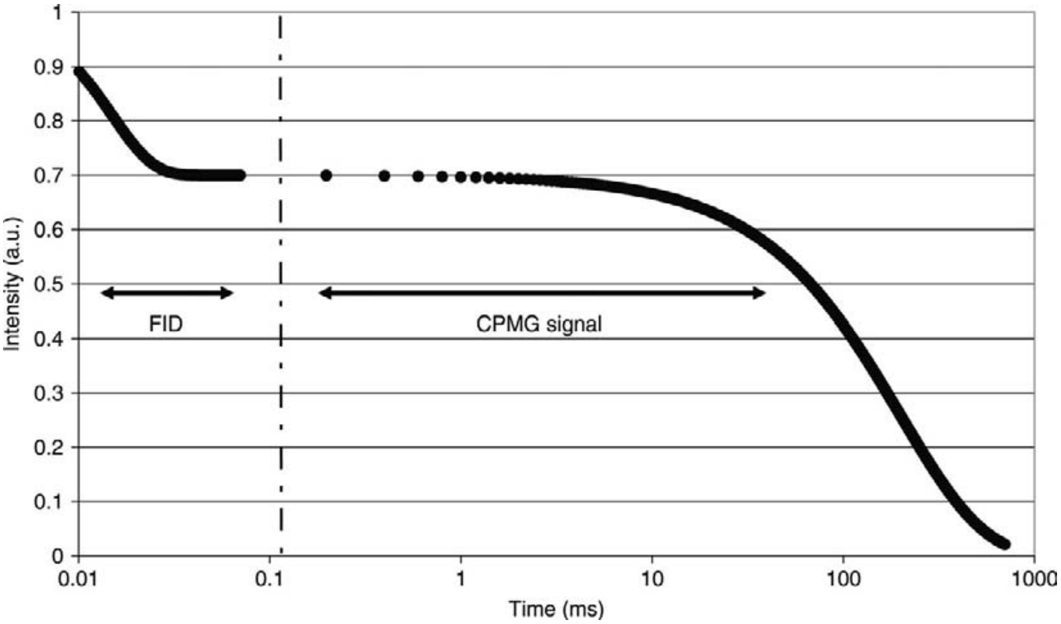

Figure 2 Spin–spin relaxation after a free induction decay (FID) and a Carr–Purcell–Meiboom–Gill (CPMG) sequence.

solid and liquid mixture, the spin–spin relaxation decay signal can be described as

$$Y(t) = \sum_{n=1}^{n} S_n \exp - \left(\frac{t}{T_{2Sn}}\right)^2 + \sum_{p=1}^{p} L_p \exp\left(-\frac{t}{T_{2Lp}}\right) \quad [3]$$

with a Gaussian function for protons from molecules in the solid state and an exponential function for protons from molecules in the liquid state. The signal intensities S and L are proportional to the amount of solid and liquid and T_{2S} and T_{2L} are the spin–spin relaxation times for the solid and liquid molecules, respectively. As already mentioned for T_1, the numbers of Gaussian terms n and exponential terms p will be modified according to the compositional and structural characteristics of the product.

NMR Diffusion

An alternative way to study water states in macromolecular solutions and gels is self-diffusion measurement of water mobility. This can be achieved by means of the pulsed field gradient NMR (PFG NMR) techniques. These techniques are all based on the use of well-defined linear gradient pulses, which change the strength of the magnetic field probed locally by the molecule's protons. Consequently, if a molecule diffuses spatially in this magnetic field gradient, the NMR signal is reduced: the faster the diffusion rate, the greater the NMR signal reduction. In its simplest version, the method consists of two equal and rectangular gradient pulses of magnitude g and length δ, one on either side of the 180° pulse after the first 90° pulse. For a molecule undergoing free diffusion characterized by a single diffusion coefficient of magnitude D, the NMR signal intensity is given by

$$I(\Delta, \delta, g) = I_{g=0} \exp\left[-\gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) D\right] \quad [4]$$

where Δ represents the time between the two gradient pulses. By varying g, or δ or Δ, D can be obtained by fitting eqn [3] to the observed NMR signal intensities.

MRI Technique

MRI can be regarded as an extension of NMR. Compared to NMR, MRI provides spatial NMR information. The spatial information is obtained with magnetic field gradients superimposed onto the main magnetic field. Indeed, the proportionality between magnetic field and resonance frequency in NMR can be used for spatially selective excitation of spins in a magnetic field gradient and for computing the spatial localization of the nuclei from a resonance signal acquired in the presence of a magnetic field gradient. The combination of these options makes MRI feasible. It is possible to obtain two-dimensional slice images with a resolution in the millimeter range, or even three-dimensional images. A resolution in the submillimeter range is achievable but requires a longer acquisition time. The signal intensity in a given voxel (a three-dimensional volume element) in the sample depends on the choice of the parameters in the imaging sequence applied. For example, for a standard spin echo sequence (a 90° pulse followed by a 180° pulse), the signal is given by

$$s(x,y) = s_0(x,y)\exp\left(-\frac{t_e}{T_2(x,y)}\right)\left[1 - \exp\left(-\frac{t_R}{T_1(x,y)}\right)\right] \quad [5]$$

where s_0 is the signal intensity extrapolated to $t_e = 0$ and $t_R = 5T_1$ (usually called spin density and proportional to the number of excitable nuclei in the voxel), and t_e is the echo time (2× the delay between the 90 and 180° pulses).

NMR Relaxation

Dairy Protein and Skimmed Milk

Most of the NMR relaxation studies on milk and dairy protein systems are performed with low magnetic field spectrometers typically operating at frequencies of less than 25 MHz. Since the introduction of the benchtop low magnetic field NMR equipment in the 1970s, the electronic and computational specifications have improved dramatically. Short, powerful excitation pulses now permit sampling of the relaxation decay curve from 11 μs up to several seconds. The number of data points available (several hundreds for FID and several thousands for the CPMG sequence) and the fast sampling rate (20 MHz) are used for the simultaneous acquisition of the solid- and liquid-like relaxation. Moreover, the use of continuous inverse Laplace transform methods has improved the understanding of relaxation behavior in complex food products.

For T_2 relaxation in milk, the signal is therefore directly fitted as the sum of a Gaussian function and an exponential function:

$$Y(t) = I_{\text{n-exch}} \exp - \left(\frac{t}{T_{2\text{n-exch}}}\right)^2 + I_{\text{exch}} \exp\left(-\frac{t}{T_{2\text{exch}}}\right) \quad [6]$$

The Gaussian function describes the NMR behavior of the non-exchangeable protons from the protein and lactose protons (CH_2 and CH_3), with $I_{\text{n-exch}}$ the signal intensity and $T_{2\text{n-exch}}$ the relaxation time of the nonexchangeable protons. The exponential function describes the behavior of the water protons, including the exchangeable protons of the protein and the lactose. I_{exch} is the signal intensity and $T_{2\text{n-exch}}$ the relaxation time of the water proton and exchangeable protons.

In milk and in dilute dairy protein systems, the amount of non-exchangeable protons is generally too low to be detected and eqn [6] becomes

$$Y(t) = I_{\text{exch}} \exp\left(-\frac{t}{T_{2\text{exch}}}\right) \qquad [7]$$

For non-exchangeable proton relaxation T_2 or T_1, the changes in relaxation can be related directly to a change in the mobility of the molecule, explained by either composition or a structural modification. For the relaxation of exchangeable protons, the interpretation can be more difficult since this parameter involves different molecules in interaction, for example water and protein. Several studies have been undertaken to understand the mechanisms that govern the variations in $T_{2\text{exch}}$ relaxation in dairy products. The general model considers three exchanging fractions of protons: free water protons, water protons in the casein micelles, and exchangeable protons of the soluble proteins and lactose. At low field, the transverse relaxation rate is given by

$$\frac{1}{T_{2\text{exch}}} = \frac{P_{\text{water}}}{T_{2\text{water}}} + \frac{P_{\text{water-casein}}}{T_{2\text{water-casein}}} + \frac{P_{\text{lactose-soluble prot}}}{T_{2\text{lactose-soluble prot}} + k^{-1}_{\text{lactose-soluble prot}}} \qquad [8]$$

where T_{2i} is the relaxation time of different proton states, with P_i their relative population. k_c is the rate of proton exchange. Therefore, because of the chemical exchange mechanism, $T_{2\text{exch}}$ measured experimentally is not a true measurement of water mobility, as was often assumed, but a probe of the protein mobility.

In a skimmed milk of pH 6.6 and with 9% of dry matter, quantitative analysis of the relaxation value has revealed that 58% of the proton relaxation rate comes from water protons in the casein micelles ($P_{\text{water-casein}}$) and 34% from the soluble whey proteins and lactose protons ($P_{\text{lactose-soluble prot}}$). The hydrating water proton has been explained by the porous structure of the casein micelle, which contains a large amount of water. This water is still highly mobile in fast exchange with the bulk water. Consequently, any physicochemical treatments that modify the structure of the micelle and hence the hydration properties would be monitored through changes in NMR relaxation time.

This sensitivity is illustrated by the investigation of the effect of pH on micellar structure (**Figure 3**). When the pH is decreased from a natural value of 6.6 to a value around 5.3, the micellar calcium and phosphorus (MCP) content decreases and induces considerable modification of the micellar voluminosity. The amount of water inside the micelle then decreases and the overall relaxation rate of the water decreases independent of the temperature. Above pH 5.3, the concentration of MCP is nil, and gel formation starts. At this pH range, the relaxation time of water reflects the change in gel structure induced by the temperature used during the acidification kinetics. The advantage of the NMR technique to monitor pH changes

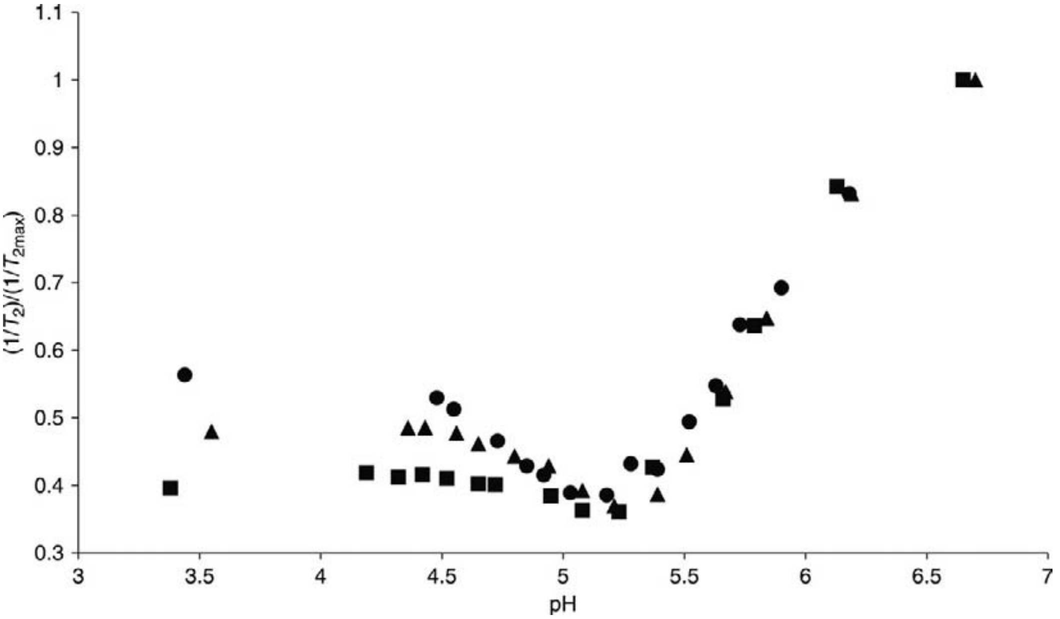

Figure 3 Variations in normalized relaxation rate $1/T_2$ of reconstituted skimmed milk at 20 MHz with pH at different temperatures: ● 5 °C, ▲ 20 °C, ■ 30 °C. Reproduced with permission from Mariette F, Tellier C, Brule G, and Marchal P (1993) Multinuclear NMR study of the pH dependent water state in skim milk and caseinate solutions. *Journal of Dairy Research* 60(2): 175–188.

is that the method can be used for whole milk and skimmed milk and it allows continuous investigation of the pH-decreased kinetic effect on micellar destabilization. Indeed, it has been demonstrated from NMR measurements performed during an acidification process that the micellar organization for the same pH values differs according to the acidification rate. A continuous change in the micellar structure is observed for a slow acidification rate, whereas no variation in the micellar structure is observed when the acidification rate is fast, until instantaneous destabilization of the micelles occurs.

Moreover, relaxation parameters are insensitive to gel formation with rennet, and thus micellar structural changes induced by lactic fermentation after renneting can be followed selectively. Many experimental conditions have therefore been studied, for example high-pressure treatment of milk. In this case, variations in the relaxation time were measured as a function of pH for milk samples treated at different high-pressure intensities. Between pH 6.8 and 5.2, changes in transverse relaxation time were enhanced by the pressure treatment and they demonstrated that the amount of micellar calcium phosphate was less than that in untreated milk.

The sensitivity of the water relaxation T_2 to protein structure has also been used to investigate thermal denaturation of whey proteins. In this case, the main mechanism involved in the relaxation rate is the chemical exchange between water and whey protein. Equation [8] becomes

$$\frac{1}{T_{2\text{exch}}} = \frac{P_{\text{water}}}{T_{2\text{water}}} + \frac{P_{\text{soluble prot}}}{T_{2\text{soluble prot}} + k^{-1}_{\text{soluble prot}}} \qquad [9]$$

and the relaxation rate measured is now sensitive to changes in the amount of labile protons from the whey proteins and to the local mobility of the whey proteins. When aggregation occurs, the local mobility is reduced and the relaxation rate ($1/T_{2\text{soluble prot}}$) increases and dominates the relaxation mechanism.

The relaxation mechanism described above is valid only when the fast diffusive exchange limit is verified. In this case, because the exchange through thermal diffusion is rapid, the different water pool, the bulk and the hydration or internal water pool, and the labile biopolymer are in exchange and the relaxation is the weight average of the three. Nevertheless, the T_2 from water can also be complicated by the effect of translational mobility characterized by the diffusion coefficient. Consider two different environments (1 and 2) with a size a, where the NMR characteristics of water T_{21} and T_{22} are different. If the diffusion coefficient rate (D (m^2 s^{-1})) is rapid compared to the difference in the relaxation rate ΔR_2 (with $R_2 = 1/T_2$), water relaxation will be average and a single monoexponential signal will be measured. The NMR signal is given by eqn [7]. On the other hand, if the diffusion coefficient rate is slow compared to the difference in the relaxation rate, then the water molecule will not be exchanged and the NMR signals will be characterized by several components for water relaxation, each with its own relaxation time. The NMR signal then becomes

$$Y(t) = A_1 \exp\left(-\frac{t}{T_{21}}\right) + A_2 \exp\left(-\frac{t}{T_{22}}\right) \qquad [10]$$

In this case, each exponential term will be an NMR signature of the two local environments. The limit between the fast and slow diffusion regimes is given by $a^2\,\Delta R_2/D << 1$.

Biexponential behavior has been reported during the syneresis of milk gel after renneting. A biexponential decay relaxation curve was observed and each exponential term was attributed to the water expelled from the gel and to the water confined in the shrunken gel, respectively. According to eqn [10], the intensity A_1 describes the whey fraction expelled from the gel. The intensity is therefore directly related to the amount of whey expelled. The T_{21} is given by

$$\frac{1}{T_{2\text{ whey}}} = \frac{P_{\text{water}}}{T_{2\text{water}}} + \frac{P_{\text{lactose}-\text{soluble prot}}}{T_{2\text{lactose}-\text{soluble prot}} + k^{-1}_{\text{lactose}-\text{soluble prot}}} \qquad [11]$$

Since the composition of the whey is constant during syneresis, the $T_{2\text{whey}}$ is time-independent of the syneresis.

The intensity A_2 describes the water confined in the gel. The intensity decreases during syneresis in proportion to the water content of the gel, and the evolution of the relative intensity $A_2/(A_1 + A_2)$ during the time course of the syneresis is related to the gel volume.

Finally, the T_{22} is given by eqn [8]. Because the whey concentration is constant, the relaxation terms for water proton and exchangeable proton from lactose and soluble proteins are constant and a linear variation can be observed during the relaxation rate $1/T_{22}$ and the Pwater-casein which is related to the casein concentration. Three kinds of information are available from the NMR method: the curd volume-decreased kinetics, the curd moisture-decreased kinetics, and the curd relaxation rate. An example of the kinetics of the curd shrinkage is given in **Figure 4**. Three replicate syneresis experiments were performed and the results showed a good level of reproducibility of the method.

The method has been used to study the effects of chemical factors on syneresis such as ionic strength and pH, or physical factors such as temperature. After modeling of the shrinkage kinetics with an exponential equation, the effects of ionic strength and heat treatments were highlighted (**Table 1**). For example, the curd shrinkage kinetics appeared to be sensitive to the ionic strength of milk and to the heat treatment. Indeed these two factors are known to be important factors for the protein network through the protein–protein interactions, which explains

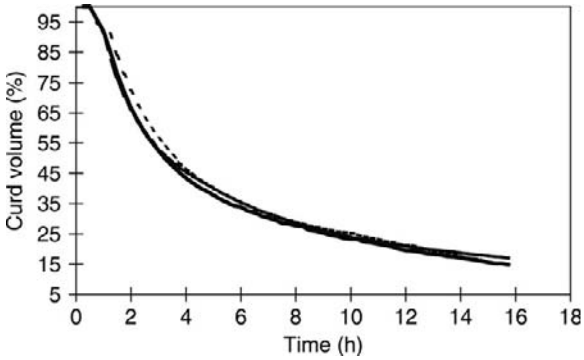

Figure 4 Curd volume according to time during syneresis. Three replicate kinetics were compared. Reproduced with permission from Mariette F (2003) NMR relaxometry and MRI for food quality control: Application to dairy products and processes. In: Webb A, Belton PS, Gill AM, and Rutledge DN (eds.) *Magnetic Resonance in Food Science: Latest Developments*, p. 209. Cambridge: Royal Society of Chemistry.

their effects on the kinetics and also on the amount of residual water at the end of the syneresis. The casein level in milk affects only the shrinkage rate through an increase in the probability of a protein–protein bond: the greater the increase in the casein level, the faster the shrinkage. Finally, the NMR method has also been used to monitor simultaneously the kinetic effects of lactic fermentation on casein destabilization and the consequences on water retention properties of yogurts.

Concentrated Dairy Products: Cheese Products and Retentate

NMR relaxation time analysis of concentrated dairy products is complicated by the presence of fat and protein. For example, the T_2 relaxation curve for cheese has been described by the following equation:

$$Y(t) = A_1 \exp\left(-\frac{t}{T_{21}}\right)^2 + A_2 \exp\left(-\frac{t}{T_{22}}\right) + A_3 \exp\left(-\frac{t}{T_{23}}\right) + A_4 \exp\left(-\frac{t}{T_{24}}\right) + cte \qquad [12]$$

For such a complex signal, with numerous relaxation parameters, the challenge is to validate the attribution of each exponential and Gaussian term to a specific proton molecule fraction before using T_2 as a probe of the matrix structure. For example, attribution was undertaken using eqn [12] to fit the T_2 relaxation curve for soft and hard cheeses. The first A_1 component was explained by the fat crystal fraction, whereas the second was attributed to the amorphous fraction. The other two components, A_3 and A_4, were attributed to the liquid fat and to water. Care should be taken using T_2 NMR on cheese because of the multiexponential behavior of the liquid component. This multiexponential behavior and the ratio between A_3 and A_4 are in fact very sensitive to the amount of water and liquid fat. Moreover, the water T_2 relaxation depends on the pH and microstructure of cheese, whereas the liquid fat signal depends on the fat composition. Although the sensitivity of the water T_2 relaxation allows detection of changes in the microstructure of cheese, quantification can be difficult because of the similarity of the fat and water T_2 values. Lastly, since water diffusion between water compartments induces a complex modification of the relaxation decay, transition from a single exponential behavior to a multiexponential behavior for water relaxation only can occur. Despite this difficulty in the attribution of the relaxation component, several investigations of cheese microstructure using NMR findings have been performed and they demonstrate the high sensitivity of the technique.

For example, the effect of pH on the casein structure in retentate has been investigated according to NMR relaxation time measurements. As observed for milk, a reduction in pH from 6.6 to 5.5 induced a decrease in the water relaxation rate ($1/T_2$). Moreover, when the casein concentration increased, the variation in relaxation time according to the change in pH decreased (**Figure 5**). Two mechanisms should be considered. First, when the casein concentration increases, the buffering power of the sample increases and the MCP released is lower at a constant pH value. Consequently, the effect of pH on the casein structure is lower and a small variation in T_2 is registered. Second, the sensitivity of the relaxation is explained mainly by the considerable difference between the relaxation time of water inside the micelle and the relaxation time of bulk water. For concentrated casein dispersion, the amount of water outside the micelle decreases to the benefit of the water inside the micelle.

Table 1 Statistical analysis of the effects of casein concentration, heat treatment, and ionic strength and curd shrinkage kinetics

Parameter of the model	*Casein effect*	*Ionic strength effect*	*Heat treatment effect*
y = **a** + bexp(-k × t)	ns	+	++
y = a + **b**exp(-k × t)	ns	+++	+++
y = a + bexp(-**k** × t)	++	++	ns

Casein concentrations were 27 and 37 g kg $^{-1}$. The ionic strength was reduced to 0.5 compared to that of unmodified milk. Untreated milk and milk heat treated at 72 °C for 20 s were compared. The curd shrinkage kinetics were modeled with an exponential equation. ns, nonsignificant.

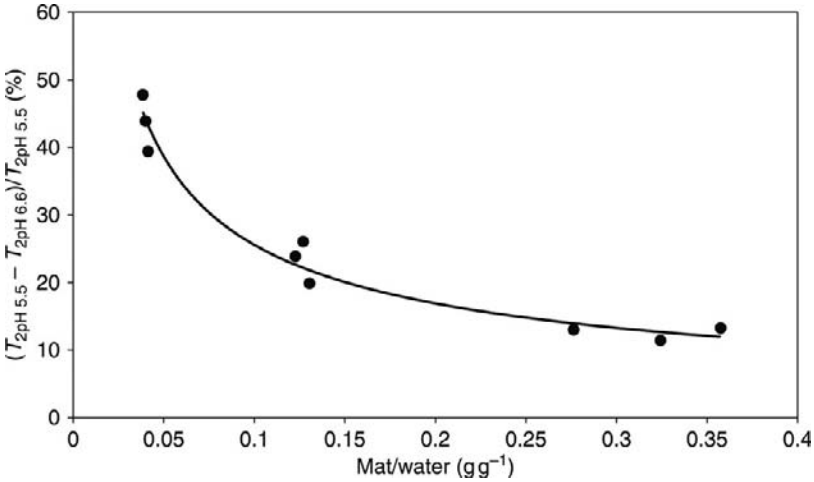

Figure 5 Variations in the T_2 relaxation time for casein retentate at pH 6.6 and 5.5 according to casein concentration. Reproduced with permission from Mariette F (2003) NMR relaxometry and MRI for food quality control: Application to dairy products and processes. In: Webb A, Belton PS, Gill AM, and Rutledge DN (eds.) *Magnetic Resonance in Food Science: Latest Developments*, p. 209. Cambridge: Royal Society of Chemistry.

The difference between the water relaxation rates in the two compartments is then small. The water relaxation time thus becomes less sensitive to acidification.

If the effect of pH could be monitored when it decreases, the water relaxation time could also be used to monitor increased pH. The latter has been demonstrated when considering the ripening of soft cheese. During ripening, the lactate produced by the lactic acid bacteria during the acidification period is oxidized. This lactate metabolism causes an increase in pH. The combined action of pH and the precipitation of salt (especially calcium) induces modifications of the protein gel structure and softening of the cheese. In turn, these modifications induce an increase in the water relaxation time. For example, the transverse relaxation time of the water phase was shown to be 35 ms for a commercial non-mature soft cheese and reached 60 ms when the cheese was mature (**Figure 6**). This variation in T_2 relaxation is dependent on the dry matter in the cheese: the lower the dry matter, the greater the variation.

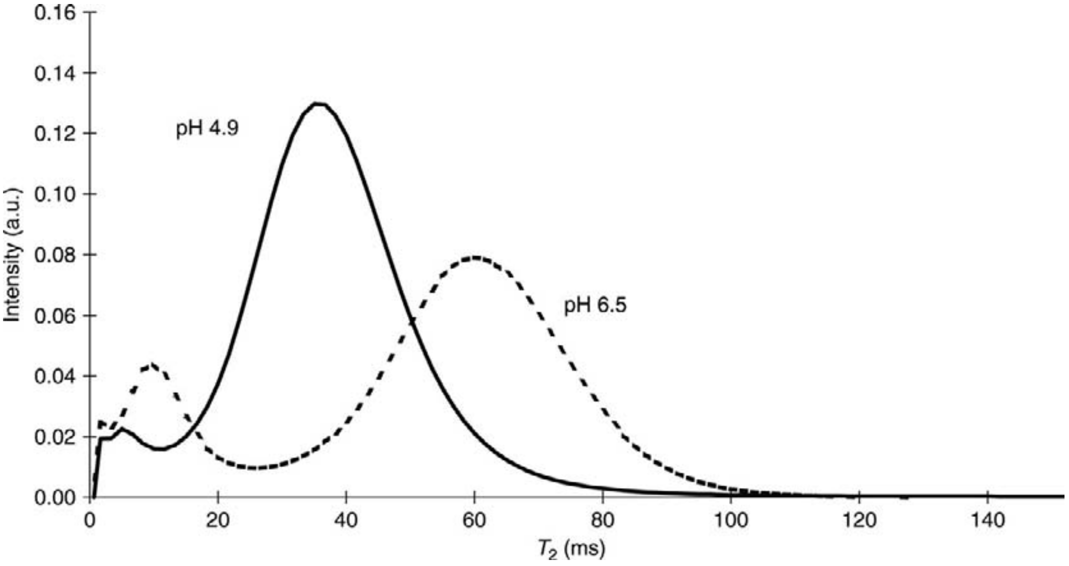

Figure 6 T_2 distribution from a Carr–Purcell–Meiboom–Gill (CPMG) signal. Samples were soft cheeses, one non-ripened with pH 4.9 and the other ripened with pH 6.5. The first peak could be attributed to the liquid fraction of the fat and the larger peak to the water phase. Reproduced with permission from Mariette F (2003) NMR relaxometry and MRI for food quality control: Application to dairy products and processes. In: Webb A, Belton PS, Gill AM, and Rutledge DN (eds.) *Magnetic Resonance in Food Science: Latest Developments*, p. 209. Cambridge: Royal Society of Chemistry.

Although the multiexponential behavior of the T_2 relaxation in cheese is explained mainly by cheese composition, certain technology can induce multiexponential behavior for water relaxation only. Indeed, when a limitation to averaging of the water pools through diffusion occurs, two or even three components can be detected for water. For example, three water relaxation components have been identified in Mozzarella cheese: one for serum water, the water accumulated in the large open channels of the protein network; one for the water inside the meshes of the casein gel-like network; and one for water trapped within the casein matrix. A decrease in the T_2 from the serum was observed during aging, attributed to a change in the microstructure of the cheese, induced by the rearrangement of the protein network. The effects of the composition of the fat globule surface on water holding capacity have been studied in rennet-derived retentates. Reconstituted fatty retentates were prepared from a fat-free retentate mixed with different fat-in-water emulsions stabilized with native phosphocaseinates (NPCs) or sodium caseinates. The coagulation of retentate reconstituted with native fat globules (fresh cream) and industrial fatty retentate was also investigated. The results showed that fatty products reconstituted from fresh cream and the industrial retentate presented lower water holding capacity than that obtained with NPCs or sodium caseinate emulsions.

The water holding capacity can also be measured using a method based on washing out of water by heavy water. The T_2 experiments were performed before and after washing with D_2O. The reduction in the NMR signal is therefore proportional to the amount of water replaced and is related to the water-holding capacity of the sample, assuming that the NMR signal for liquid fat protons is negligible.

Dairy Fat and Dairy Emulsion

Solid-Fat Content Measurements

The most widespread application of NMR for dairy products is the solid-fat content (SFC) method. Since NMR-SFC has become an international ISO method, the method will not be detailed in this article, and only certain key points will be mentioned and some recently proposed improvements will be described. The general determination of SFC is based on the sampling of two points from the FID signal, one after the dead time of the probe (11 μs) and the next at 70 μs. This method is valid only for anhydrous products. If measurements are performed on aqueous products such as butter or cream, the contribution of the water signal should be subtracted from the signal at 70 μs. Indeed, at 70 μs, the NMR signal intensity is the sum of the intensities of the liquid fat signal and the water signal. Moreover, this method supposes that the relaxation time of the solid part is temperature-independent and also independent of fat composition when using an external reference. These assumptions have not always been verified and a precise analysis of the free induction signal of milk fat at 5°C has shown that an intermediate phase characterized by relaxation $T_2{}^*$ (varying from 50 to 250 μs) range can be detected. This intermediate phase represents about 4% of the whole fat and thus should be considered when absolute SFC is required. One solution to overcome the choice of sampling time is to fit the complete NMR signal. It was recently demonstrated that greater accuracy in the measurement of the SFC could be achieved from combined FID and CPMG acquisition. Moreover, this method takes into account the α and β polymorphic forms of the fat crystal. In anhydrous products, the FID of the α polymorph was described by a monotonically decaying Gaussian function such as eqn [3], while for the β and β' polymorph, an 'Abragam sinc' function has to be added (eqn [15]). The results obtained demonstrated that the NMR method was potentially the first technique to provide simultaneously the amounts of the solid and liquid phases and the polymorphic state of the lipids. Therefore, the FID and CPMG relaxation decay curve is fitted with the sum of terms for the liquid and solid phases. The liquid phase is fitted by

$$[\mathrm{Liquid}] = \sum_{n}^{l_1} L_1(n)\exp\left(-\frac{t}{T_{2n}}\right) + \sum_{i}^{l_2} L_2(i)\exp\left(-\frac{t}{T_{2i}}\right) \qquad [13]$$

and the solid phase is fitted by

$$[\mathrm{Solid}]_\alpha = \sum_{k}^{g} G(k)\exp\left(-\frac{0.5t}{T_{2k}}\right)^2 \quad \text{for the } \alpha \text{ form} \qquad [14]$$

$$[\mathrm{Solid}]_\beta = \sum_{l}^{k} P(l)\exp\left(-\frac{A^2t^2}{2}\right)\frac{\sin(Bt)}{Bt} + \sum_{j}^{l_3} L_3(j)\exp\left(-\frac{t}{T_{2j}}\right) \quad \text{for the } \beta \text{ form} \qquad [15]$$

with L_1, L_2, L_3, G, and P the proportions of the different phases and T_{2i} their T_2 distribution. A and B are the terms of the Abragam sinc function. This method simultaneously provides the SFC and the ratio between the α and β polymorphic forms for the crystal phase. An extension of this method has also been proposed in order to adapt the method to emulsions.

Polymorphism and Crystal Organization

Parallel to the development of this T_2-based approach, an investigation into the behavior of the spin–lattice T_1 relaxation time from the crystallized fat phase was also carried out. The study was based on the effects of storage time on the fat crystal network. The T_1 of a model mixture consisting of a liquid and a solid triacylglycerol was followed over time (**Figure 7**). The evolution of T_1 with storage time followed a power-law model which was related to the Ostwald ripening phenomenon, which corresponds to the melting of smaller crystals to form larger ones. At the same time, it was verified that polymorphism and SFC remained constant. The changes in the thickness of the crystals were determined by X-ray measurements in order to establish the correlation between T_1 of the crystalline phase and crystal size. The two parameters followed the same evolution (**Figure 7**), establishing that the increase in T_1 is related to growth in the thickness of the crystals.

The sensitivity of the T_1 relaxation time of the fat crystal organization has also been observed in fat mixtures with added surfactant and in food emulsions. For example, wide variations in T_1 were observed according to the cooling rate of milk fat. A shorter T_1 was observed for a faster cooling rate, and the T_1 was reduced when surfactants were added, compared to the T_1 from the dairy fat without surfactant. In dairy emulsions, a change in T_1 relaxation time of the fat crystal was observed according to the maturation temperature of the emulsion. A narrow T_1 distribution was obtained for a maturation temperature of 4 °C, whereas a bimodal distribution was obtained for a maturation temperature of 12 °C. Changes in the T_1 relaxation time of the crystallized fat phase have also been reported in ice cream mix and ice cream. The T_1 of the crystalline phase thus seems to be a very interesting parameter for investigation into the behavior of food containing significant amounts of fat, since the fat crystal network confers on food its physical properties.

Liquid Fat Phase and Crystal Network

Surprisingly, the behavior of the signal from lipids in the liquid state has had little attention. In fact, the relaxation time of a mixture of lipids is often characterized by a wide continuous distribution of relaxation times, both in T_2 and T_1, and is influenced by both the length of the carbon chain and the amount of unsaturation, which complicates the interpretation of relaxation times of the lipid phase. Nevertheless, despite this wide distribution, a mean T_2 value can be calculated and this makes it possible to explain the expected relationship between the molecular mobility (reflected by T_2) and the viscosity and chain length of the triacylglycerol. It should be pointed out that this relationship is valid only in a nonconstrained system, in other words in the absence of a crystal network. Indeed, in porous media, the spin–lattice relaxation time (T_1) and the spin–spin relaxation time (T_2) depend on the interactions with the surface of the pores. The T_1 and T_2 relaxation times at the surface are reduced by dipole–dipole interaction, crossrelaxation, and chemical exchange. Due to the surface relaxation mechanism, the relaxation times are shorter in pores with a high surface-to-volume ratio. The relaxation parameters can therefore be used to obtain information on pore geometry. The relationship between the T_2 or T_1 relaxation times and the surface-to-volume ratio can be expressed by

$$\frac{1}{T_{1,2\text{measured}}} = \frac{P_{\text{bulk}}}{T_{1,2\text{bulk}}} + \frac{S\lambda}{VT_{1,2\text{s}}} \qquad [16]$$

where $T_{1,2\text{bulk}}$ is the bulk relaxation time, $T_{1,2\text{s}}$ the relaxation of the molecule at the surface of the pore, and λ the thickness of molecules interacting with the surface of the pore. S is the total surface accessible to

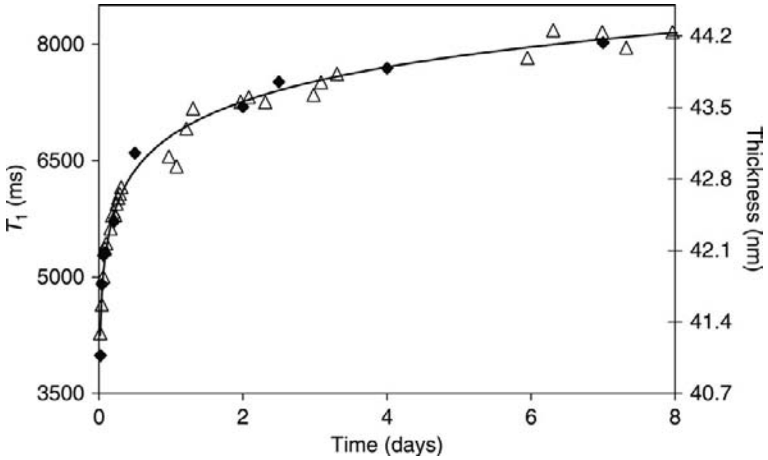

Figure 7 Evolution of T_1 (Δ) and crystal thickness (◆) measured by X-ray diffraction over time for a 50:50 (w/w) mixture of tricaprin and tristearin at 40 °C. The line corresponds to the power-law fit of T_1. Reproduced with permission from Adam-Berret M, Riaublanc A, Rondeau-Mouro C, and Mariette F (2009) Effects of crystal growth and polymorphism of triacylglycerols on NMR relaxation parameters. Part 1: Evidence of a relationship between crystal size and spin–lattice relaxation time. *Crystal Growth and Design* 9(10): 4273–4280.

the liquid phase and *V* the open pore volume. In the fat crystal network, *S* corresponds to the specific surface area developed by the crystals and *V* depends on the amount of liquid phase. The surface-to-volume ratio is inversely proportional to the mean radius of the pores and depends on pore geometry. The model presented above is valid only when the system is in the fast diffusive exchange limit, that is, when molecules are mobile enough and exchange by diffusion between the molecule in the pore and the molecule at the surface is rapid. If the porous material contains pores of different sizes, a superposition of monoexponential functions is observed. It is then necessary to determine the distributions of the relaxation times in order to obtain information about the shape of the pore size distribution. However, it is only possible to determine the average pore size.

This model was recently used to describe the behavior of the mean T_2 value of tricaprin in the liquid state in a tristearin crystal network during storage (**Figure 8**). In this situation, the amount of liquid phase remained constant and the evolution depended only on the modifications to the specific surface area developed by the crystals. It was possible to note that there was a rapid increase in T_2 during the first few days and a slower increase subsequently. The T_2 values of the liquid phase were fitted by a power-law model. It has already been proved that the specific surface area of tristearin crystals in paraffin oil decreases over time, as does the average crystal size. This change in T_2 was related to the decrease in the specific surface area of the fat crystals during storage.

Dairy Powders

The rehydration capacity of dairy powders is a crucial quality criterion for dairy companies. Nevertheless, the standard methods are crude and dedicated to milk powder and not suitable for other dairy powders. In 1997, an NMR method was proposed for monitoring rehydration. The advantage of the NMR method is that measurement is performed under stirring at a controlled temperature. Consequently, the NMR method provides complete kinetics of the rehydration process and can be used whatever the composition of the powder. Usually, the NMR method is performed during continuous stirring for 30 min and without stirring for the remaining 30 min. This method allows the identification of the rehydrated protein which exhibits low stability and high sensitivity to destabilization through decantation. This method is based on the T_2 changes induced by rehydration of the dairy protein, and can therefore be used for milk powders and for dairy protein powders. Moreover, due to the sensitivity of T_2 to protein structure, the method allows discrimination of the powder according to the denaturation of the protein that is induced by the preparation of the concentrate before drying and by the drying process itself.

Diffusion

Water Diffusion in Dairy Suspensions and Gels

The great advantage of PFG NMR is that quantitative measurement of the diffusion of water and metabolites can be performed noninvasively on a microscopic scale

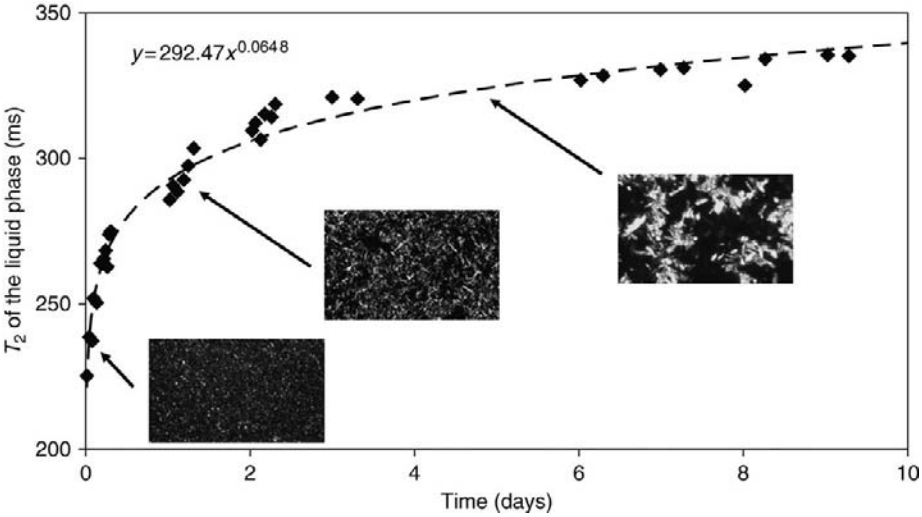

Figure 8 Evolution of T_1 from liquid fat phase and polarized light micrographs over time at 60 °C for a 50:50 (w/w) mixture of tricaprin and tristearin. Reproduced with permission from Adam-Berret M, Riaublanc A, Rondeau-Mouro C, and Mariette F (2009) Effects of crystal growth and polymorphism of triacylglycerols on NMR relaxation parameters. Part 1: Evidence of a relationship between crystal size and spin–lattice relaxation time. *Crystal Growth and Design* 9(10): 4273–4280.

and in any direction of displacement. Moreover, the technique can be applied to most samples and provides structural information about the system being investigated. The dependence of the water diffusion coefficient on the protein concentration has been reported in casein and whey protein solution and gels. In both systems, the water diffusion is reduced with increased protein concentrations in solutions and gels (**Figure 9**). The obstruction effect of the protein can be explained by taking into account two self-diffusion flows: a water flow close to the protein backbone and a water flow around the casein micelles or globular whey proteins. The effect of the change in network structure on the water diffusion coefficient was also investigated for casein gels. No difference in water diffusion was observed regardless of the method of preparing the gel, that is, acid or renneted gel. However, heat treatment of the whey protein solution induced a slight reduction in the water diffusion, explained by a change in the accessibility of the whey protein aggregates to water. For casein, it was recognized that the overall accessibility to water was not significantly affected by the formation of a gel. It was concluded that water diffusion in protein systems follows a general trend whatever the protein system studied.

The specific effects of casein and fat content on water diffusion have been outlined in a cheese model. The experiments demonstrated that the water diffusion was not explained by the water content of the cheese model alone, as had been expected. The obstruction effects induced by the casein and the fat globules on the water diffusion were different. The reduction of the water diffusion coefficient was less with fat compared with casein, explained by the internal diffusion of water molecules in casein, which induced a significant reduction in the diffusion. Moreover, a general model describing the effects of water content on water self-diffusion has been proposed. This model includes the effects of aqueous phase composition, and the obstruction effects of casein and fat droplets on water diffusion. It requires no structural information on the gel network since no effect of the structure has been observed, except when the water distribution inside the gel becomes heterogeneous and when the serum phase starts to release. This model was validated on information obtained from industrial cheese.

Droplet Size in Dairy Emulsions

Using PFG NMR, the diffusion coefficient of molecules can be measured over time (Δ). This parameter can be chosen during the experiment from a few milliseconds to several seconds. For free diffusion, the diffusion coefficient is constant over Δ. This situation has been met in the experiments in dairy gels. However, in dairy emulsions, when Δ is large enough to allow the molecule to probe the boundary of the droplet, the molecular displacement cannot exceed the droplet size and diffusion is restricted. In this case, the diffusion coefficient becomes a function of the diffusion time Δ and the droplet size distribution can be measured in oil-in-water (o/w) or water-in-oil (w/o) emulsion from a careful analysis of the PFG NMR data. The accuracy of the method has been widely debated for determination of water droplet and oil droplet size in food emulsions such as butter, margarine, and dressings. However, the method has mainly been applied to nondairy fat-containing products, because of the effects of the crystalline fat.

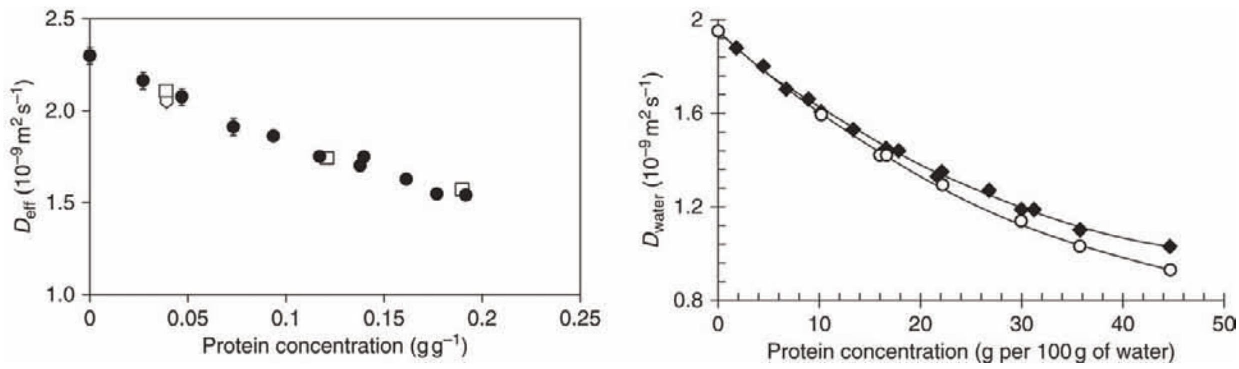

Figure 9 Water diffusion as a function of casein concentration for casein dispersion (●) in acid gels (O) and in rennet gels (□) (left) and water diffusion as a function of whey protein concentration in whey protein solution (◆) and gels (O) (right). Reproduced with permission from Métais A, Cambert M, Riaublanc A, and Mariette F (2004) Effects of casein and fat content on water self-diffusion coefficients in casein systems: A pulsed field gradient nuclear magnetic resonance study. *Journal of Agricultural and Food Chemistry* 52(12): 3988–3995 and from Colsenet R, Cambert M, and Mariette F (2005) NMR relaxation and water self-diffusion studies in whey protein solutions and gels. *Journal of Agricultural and Food Chemistry* 53: 6784–6790.

High amounts of solid fat may affect the apparent droplet size because the fat crystal network may introduce a second mechanism to hinder diffusion within the droplet. For dairy products, it has been assumed that droplet size measurement with PFG NMR can be performed only at temperatures above 15 °C, in view of the SFC for butterfat according to temperature. For example, the droplet size of fat in cheese has been determined at 37 °C.

Multidimensional NMR Relaxation

The NMR applications described above are all based on the analysis of one-dimensional conventional relaxation spectra. Although T_1-filtered T_2 or T_2-filtered acquisition has been performed for some specific applications, the data have always been processed with a one-dimensional Laplace inversion. The advent of a fast algorithm for two-dimensional Laplace inversion has recently led to the development of 'multidimensional' NMR relaxometry. These types of measurements are valuable to improve the assignment of multiexponential relaxation behavior and to study dynamic processes such as chemical exchange or diffusive exchange. For example, T_2–T_1 correlation and D–T_2 correlation experiments have been performed on milk, yogurt, cream, and cheese. For milk samples, a single peak was observed for both experiments and in both cases the two-dimensional relaxation approach did not provide more information than one-dimensional relaxation experiments. However, for the other samples, a bimodal distribution was observed for both experiments (**Figure 10**). The first peak was assigned to the water fraction and the second peak to the fat fraction. In T_1–T_2 correlation experiments, the separation between the two relaxation components was still difficult and affected by the water content of the sample. Indeed, the results showed that the fat relaxation did not change with the chemical composition of the dairy sample, while the water relaxation associated with the water phase varied from sample to sample. Consequently, for a few samples, an equal relaxation time value was observed for the two fractions, thus preventing any separation of the two compounds. In contrast, the D–T_2 distribution functions improved separation of the water and fat features and, because of the wide difference between the water diffusion and fat diffusion coefficients, this separation was independent of the chemical composition. Such methods offer many opportunities for sample characterization, but it is necessary to step back from the complexities of real food systems in order to assign the peaks to particular proton pools.

Magnetic Resonance Imaging

Evaluation of Water and Fat Content Distribution by MRI

Using MRI to obtain water and fat content distribution is one of the applications that are widely represented in the literature about food. Several NMR parameters are used to determine water content, that is, proton density and spin–spin relaxation time T_2 since both are dependent on the water content. The spin–lattice relaxation time T_1 can also be used but, because its sensitivity to water content is less than the sensitivity of T_2, the latter is preferred. The MRI method requires establishing a model between the gray level intensity of the voxel and the water content whatever the NMR parameters chosen for the experiments (proton density, T_2 or T_1). Nevertheless, the T_2- or T_1-based approach is usually preferred since the methods based on this approach are less sensitive to the variation in gray level induced by the nonuniformity of the coil used for excitation and acquisition of the MRI signal. For example, a T_2-based method has been successfully used for the determination of water distribution during the draining of curd (**Figure 11**). A linear model was previously validated between the water relaxation rate ($1/T_2$) and the water content, then the T_2 was computed for each voxel in the image, and the water content was calculated from the model. Moreover, other information can be extracted from MR images such as the size of the curd, the location of whey, and the amount of whey in the container. The same approach has been used to study the effects of freezing–thawing on the macroscopic structure and water distribution in the curd. The MRI method for water content distribution measurements has also been evaluated on fatty products such as cheese and cream.

This requires a more sophisticated MRI acquisition protocol and the complexity of the model between the gray level intensity and the water content increases. Indeed, the methods require that the gray level intensity is not polluted by the fat proton or the water proton when fat distribution methods are implemented. A sophisticated method has been proposed mainly based on the differences in resonance frequency between fat and water protons.

Macrostructure Information

Many attempts have been made to quantify the macroscopic structure or organization of cheese and to relate these features to quality criteria. This approach was first applied to Swiss cheese for quantification of the morphology of holes by MRI. The number of holes was determined, and the thinness ratio, cross-sectional area, and hole center locations were calculated. The mean gray level, angular

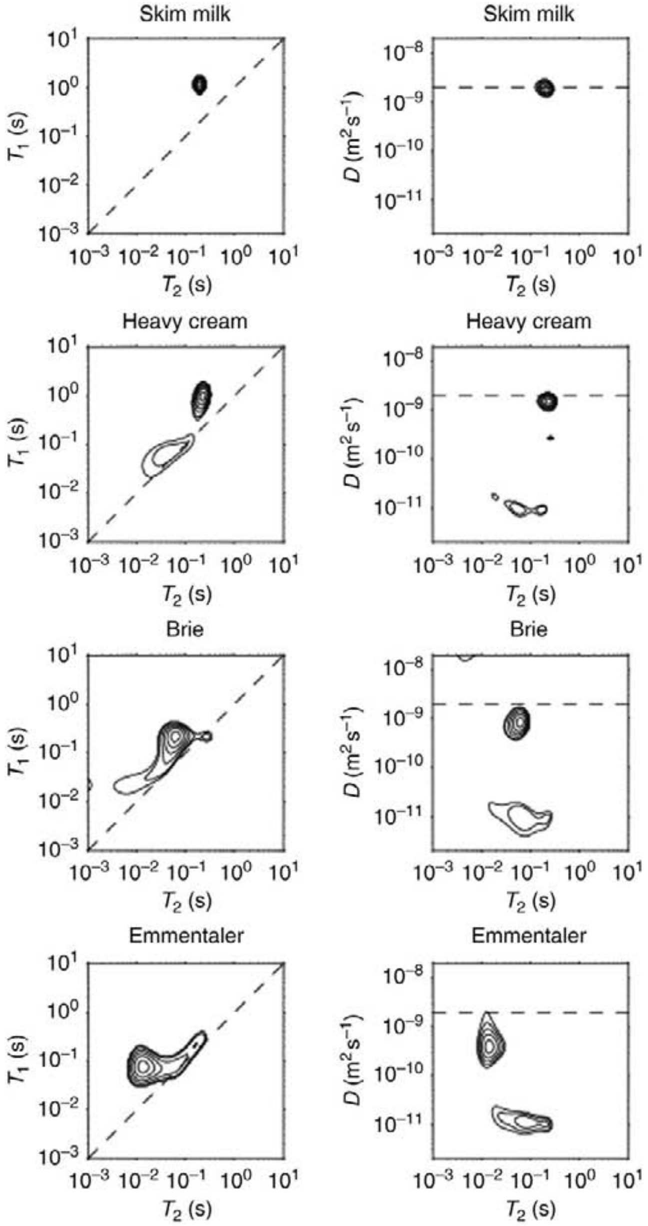

Figure 10 Comparison of $T_1 - T_2$ distribution functions (left) and D–T_2 distribution functions (right) measured on four different dairy products: skimmed milk, heavy cream, a soft cheese (Brie), and a hard cheese (Emmentaler). The dashed lines in the T_1–T_2 distribution functions indicate $T_1 = T_2$, whereas in the D–T_2 distribution functions, they indicate the diffusion coefficient of water. Contour lines are shown at 10, 30, 50, 70, and 90% of the maximum values in each panel. Reproduced with permission from Hurlimann MD, Burcaw L, and Song YQ (2006) Quantitative characterization of food products by two-dimensional *D*–*T*-2 and *T*-1–*T*-2 distribution functions in a static gradient. *Journal of Colloid and Interface Science* 297(1): 303–311.

moment, and contrast of gray scale images were calculated to define the internal texture of Swiss cheese and a classification procedure was devised using these parameters. This approach was also used for the classification of soft cheese according to the processing conditions, and to predict the sensory textural properties of the cheese. Other examples have focused on the determination of rind thickness and analysis of the structure of blue-veined cheeses (**Figure 12**).

Microstructure and Molecular Structure MRI

The sensitivity of T_2 water relaxation to changes in the molecular dynamics of protein has also been at the origin of MRI investigations into dairy processing operations. Consequently, if the voxel intensity is T_2 value-weighted (T_2-weighted images), information can be provided by MRI at a molecular level. However, since several

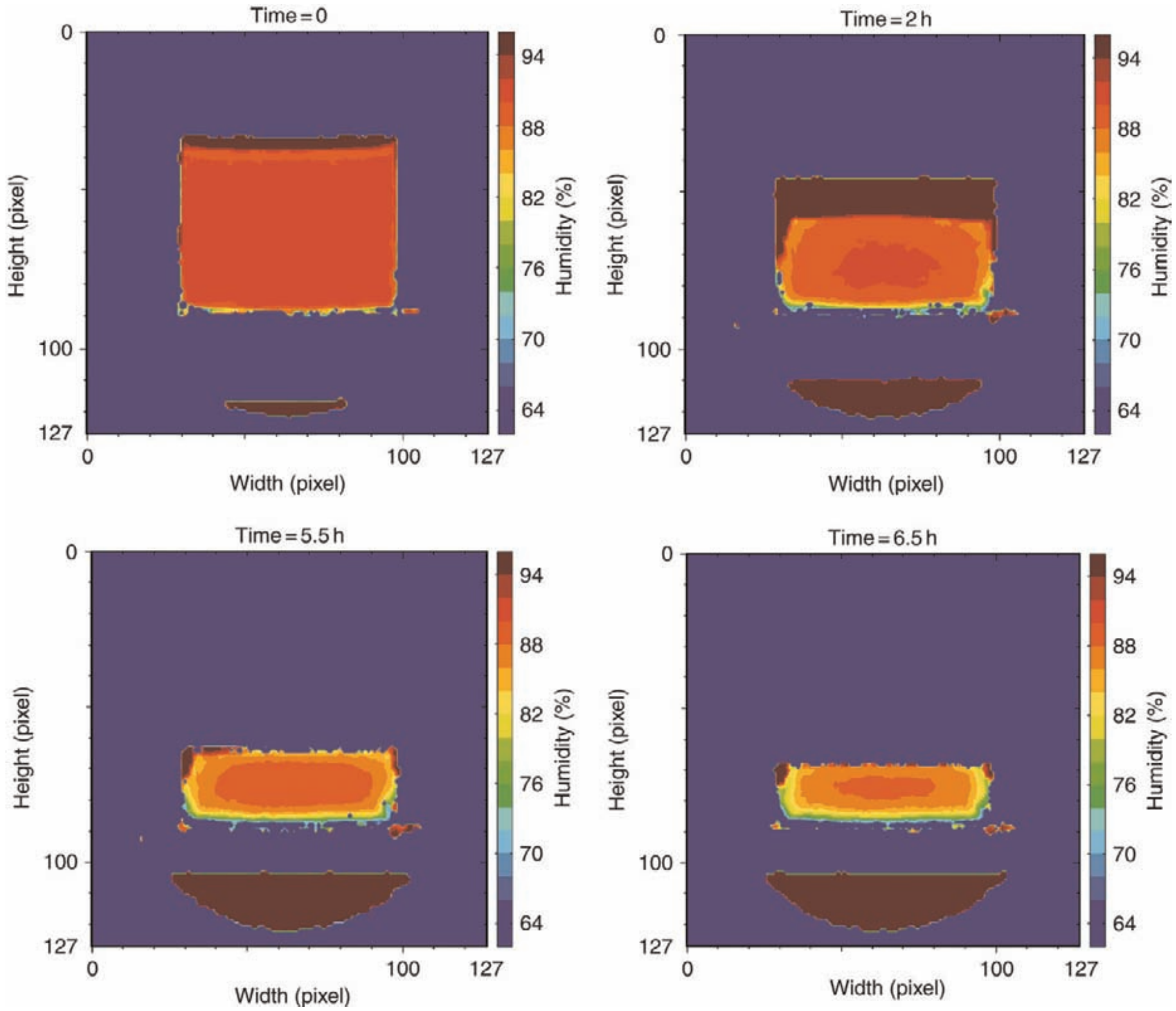

Figure 11 Water distribution in curd according to draining time. Images were acquired with an open scanner (Siemens) at 0.2 T. Reproduced with permission from Mariette F, Davenel A, Marchal P, and Chaland B (1998) A study of water by NMR and MRI in dairy processes. *Revue de l'Institut Francais du Petrole* 53(4): 521–525.

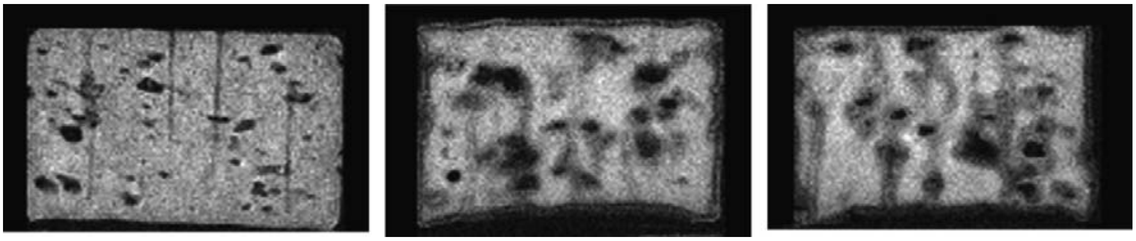

Figure 12 T_2-weighted MR images of blue-veined cheeses at *D* + 3 (left), *D* + 15 (central), and *D* + 37 (right). Reproduced with permission from Onea A, Collewet G, Fernandez C, Vartan C, Richard N, and Mariette F *et al*. (2003) Quality analysis of blue-veined cheeses by MRI: A preliminary study. *Proceedings of the SPIE, The International Society for Optical Engineering*. San Diego, CA, USA.

processing operations induce concomitant changes in water content and molecular structure, both of which affect the MR images to a certain extent, care should be taken in the design of MRI protocols in order to provide correct interpretations of the gray level intensity of the images. It is nevertheless possible, using the appropriate MRI protocol, to obtain spatial distributions on the scale defined by the voxel size (between 100 μm up to several millimeters) of structural changes on a molecular scale. This property has been widely exploited for the study of cheese ripening and freezing–thawing effects in dairy gels.

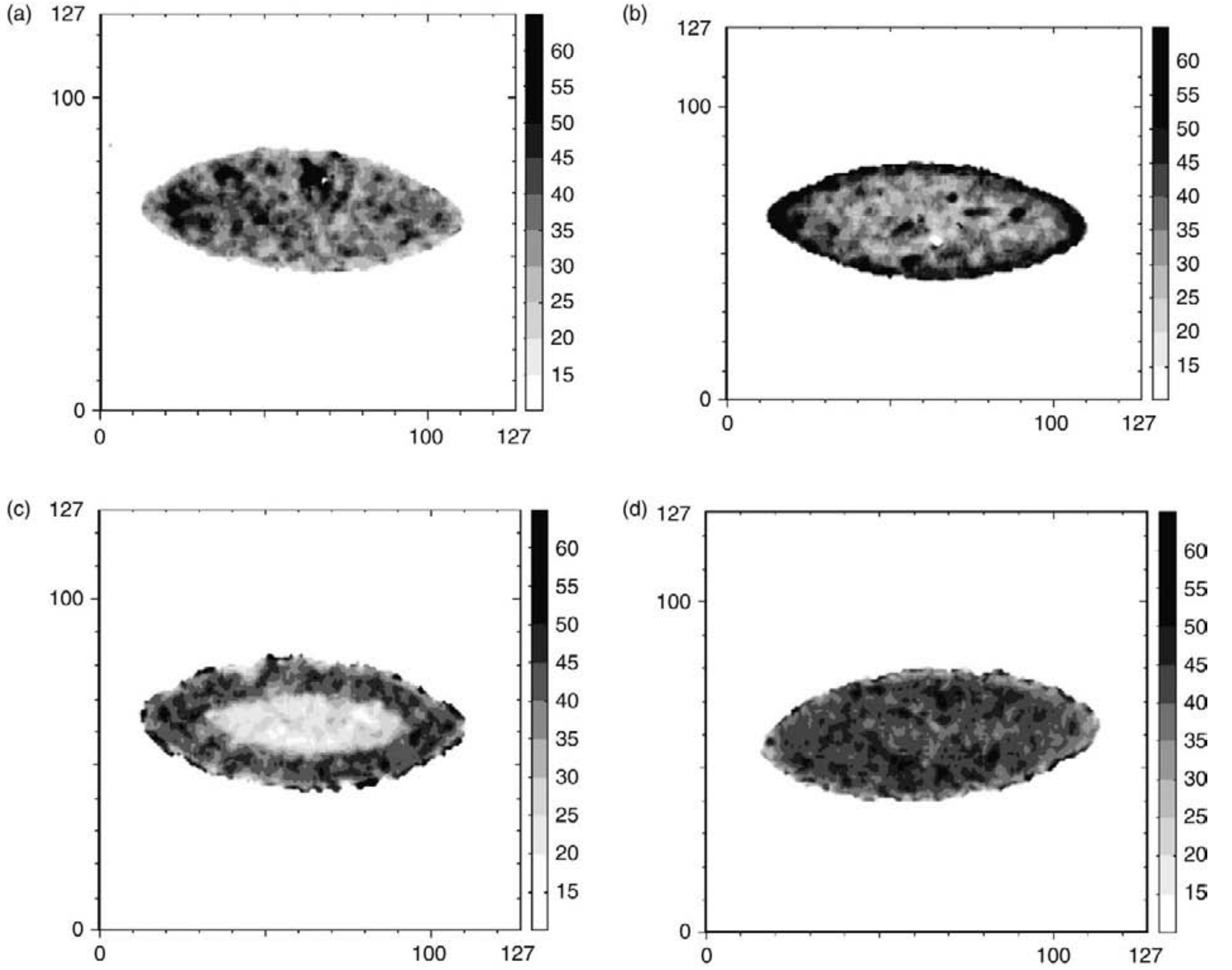

Figure 13 T_2 map of soft cheeses according to ripening time. (a) $D + 9$, (b) $D + 14$, (c) $D + 30$, and (d) $D + 44$. Images were acquired with an open scanner (Siemens) at 0.2 T.

For example, **Figure 13** represents a T_2 map of soft cheese as a function of time of ripening. As explained previously in the 'NMR Relaxation' and 'Concentrated Dairy Products: Cheese Products and Retentate' section, change in T_2 can be related to change in the protein network structure induced by an increase in pH. After 9 days, the relaxation time of the cheese was low and slight heterogeneity was observed. After 14 days, an increase in the T_2 was observed on the periphery of the cheese. Moreover, a gradient took place from the center to the cheese surface. The high T_2 value voxel number increased to the detriment of the low T_2 value voxel number as a function of time. After 44 days, all the voxels had reached the same T_2 values and the cheese was totally mature. The variation in T_2 observed during ripening was in close agreement with the expected ripening mechanism. Indeed, it is known that the deacidification occurs initially at the surface, resulting in a pH gradient from the surface to the center of the cheese. The MRI technique could thus be applied to different ripening conditions, and the kinetics of ripening could be followed.

The effects of freezing on Mozzarella have been studied according to the T_2 distribution in the T_2 map. Differences between unfrozen pasta filata and nonpasta filata were noted in the T_2 histogram. Moreover, after a freezing–thawing process, the T_2 histogram was modified and this effect was dependent on the initial structure of the cheese and also on the storage time, the differences being explained by the microstructure of the gel and by the damage to the protein structure induced by the freezing–thawing process.

Future Trends

The NMR and MRI applications described in this article demonstrate the potential for non-invasive characterization of dairy processing and how information about composition, internal structure at different scales, and molecular mobility can provide new insights into the mechanisms involved in dairy engineering and formulation. Moreover, several studies are currently in progress to improve the applications of NMR and MRI techniques to dairy sciences. Some of these, such as the use of multidimensional NMR relaxation, are related directly to ways of manipulating spin magnetization and will increase the sensitivity of NMR to changes in the molecular structure. Some involve the design of new magnet geometry such as the NMR-Mouse, and some are open access systems such as the Halbach magnet, which will allow the characterization of products directly in the factory, or through the packaging. Finally, developments are proposed for the measurement of non-NMR parameters such as flow and rheological properties, referred to as velocimetry MRI or Rheo-NMR, and others have proposed multisensor technology when NMR is simultaneously combined with other spectroscopy techniques such as near-infrared (NIR), impedance and ultrasound, or scattering techniques. These recent studies demonstrate that promising new results can be expected in the near future.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Properties and Analysis. **Cheese**: Acid- and Acid/Heat Coagulated Cheese; Biochemistry of Cheese Ripening; Blue Mold Cheese; Camembert, Brie, and Related Varieties; Curd Syneresis; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese). **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders. **Fermented Milks**: Yoghurt: Types and Manufacture. **Milk**: Physical and Physico-Chemical Properties of Milk. **Milk Proteins**: Casein Nomenclature, Structure, and Association. **Water in Dairy Products**: Water in Dairy Products: Significance.

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Chromatographic Methods

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Introduction

Chromatographic methods of analysis, which combine the separation and analysis steps, are very useful for the analysis of complex foods such as milk and dairy products, and several techniques have been developed and standardized (**Table 1**). Chromatography in general comprises separation techniques in which the analytes partition between different phases that move with different velocities relative to each other. In principle, anything that can be dissolved can be analyzed by high-performance liquid chromatography (HPLC) using different techniques to separate the analytes by size, charge, solubility, or biological activity (**Table 2**). Components that are volatile, or could be made volatile by heating or derivatization, can be analyzed by gas chromatography (GC). Thin-layer chromatography (TLC), which was developed long before HPLC and GC, is still used for specific analyses (e.g., analyses of radioactive compounds). Only the highly automatic techniques HPLC and GC will be discussed further in this article.

Because of the complex structure of milk and dairy products, some sample preparation is needed before chromatographic analysis can be performed. For liquid chromatography, fat is separated efficiently from the protein and aqueous phase by techniques such as centrifugation at high speed and low temperature. Deproteination is performed by acid precipitation followed by centrifugation or filtration. The components of the aqueous phase may be fractionated specifically using ultrafiltration, further precipitation, extraction with organic solvents, distillation, or initial chromatography on solid-phase cartridges. Solid products like cheese have to be dissolved or dispersed in a solvent as a first step of sample preparation and are then treated similar to liquid dairy products. Specific sample preparation methods used for the analysis of volatile compounds are described in the section 'Gas chromatography'.

HPLC and GC techniques that are commonly used to analyze milk and dairy products are described briefly below. Separation techniques, detection methods, and some specific sample preparation methods are described, and analysis methods currently used for different components of milk and dairy products are given as examples.

Gel Permeation Chromatography

The separation principle of gel permeation chromatography (GPC) or size-exclusion chromatography (SEC) is, in general, simple. The analytes should preferentially not interact with the stationary phase and they should be separated only by their ability to penetrate the pores of the matrix. Smaller molecules penetrate more easily into small pores of the porous stationary phase, than larger molecules do, which consequently have a shorter passage through the column and elute first. Buffers should be chosen for their ability to dissolve the sample and keep the analytes separated. To suppress interaction of the column material with the analyte, separation is usually performed at a particular salt concentration. Samples that are poorly soluble or tend to aggregate (e.g., caseins) require to be suspended in buffers containing dissociating agents like urea (typically 6 mol l^{-1}), sodium dodecyl sulfate, ethylenediaminetetraacetic acid (EDTA), mercaptoethanol, or dithiothreitol.

The choice of column packing material is crucial and the pore size determines which molecular range can be separated. The stationary phase commonly consists of polymer beads made of polyacrylamide, cross-linked dextran (Sephadex), or agarose for low-pressure applications, or for high-pressure applications, silica that may be derivatized with glycidylpropylsilane for use at neutral and acidic pH, or polystyrene beads are used.

The separating power of GPC is generally poor and the technique is used mainly as a step in purification procedures and sample preparation. In the case of dairy products, whey proteins may be analyzed successfully by GPC (**Figure 1**). Several GPC methods have been tested for describing the extent of proteolysis in cheese. This is because GPC can be used to measure the changes in the molecular size distribution of released peptides incurring during the maturation of cheese. However, the resolving power has so far been poor, and problems with association between protein and peptide molecules in the samples, as well as interaction with the stationary phase, are not easily overcome.

Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) separates molecules on the basis of their molecular charge or, in the case

Table 1 Components of milk and dairy products analyzed by chromatographic methods by IDF standards

Component	*Method*	*IDF standard*
Chymosin, bovine pepsin	IEC	110B:1997
Vitamin A	RP-HPLC	142:1990
Vitamin D	RP-HPLC	177:2002
Iodide in milk	RP ion-pair HPLC	167:1994
Cholesterol	TLC, GLC	159:1992
Sterol composition of fat	GC	200:2006
Antioxidants in butter oil	RP liquid chromatography	165:1993
Benzoic acid, sorbic acid	RP-HPLC	139:2008
Natamycin	RP-HPLC	140-2:2007
Furosine	Ion-pair RP-HPLC	193:2004
Heat treatment intensity	Gel permeation HPLC	162:2002
Lactulose	Cation-exchange HPLC	147:2007
Lactose	HPLC	198:2007
Acid-soluble β-lactoglobulin	RP-HPLC	178:2005
Organophosphorus compounds	GLC	144:1990
Pesticides, organochlorine compounds	Capillary GLC	130:2008
Aflatoxin M_1	Immunoaffinity, TLC	190:2005
Aflatoxin M_1	Immunoaffinity, HPLC	171:2007

GC, gas chromatography; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; IDF, International Dairy Federation; IEC, ion-exchange chromatography; RP, reversed-phase; TLC, thin-layer chromatography.

Table 2 Separation principles in HPLC

Molecular property	*HPLC technique*
Size	GPC
Charge	IEC
Biological activity	Affinity chromatography
Hydrophobicity/hydrophilicity	HILIC RP chromatography HIC

GPC, gel permeation chromatography; HIC, hydrophobic interaction chromatography; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; IEC, ion-exchange chromatography; RP, reversed-phase.

of large polymers and biomolecules, mainly on the basis of their surface charge. Analytes with charge opposite to that of the ion-exchange resin are retained to different extents. At a pH above the pI (pH at isoelectric point) of the analytes, they become negatively charged and will bind to an anion exchanger. Salt and pH gradients are used to elute the compounds from the column. The resolution is influenced by the pH of the eluent, which affects the selectivity and the ionic strength of the buffer, which in turn mainly affects the retention. The combined effects of ionic strength and pH are used to optimize a separation.

Principally, four groups of ion-exchange resins exist; they may exchange anions or cations and they may be weak or strong. Anion-exchange resins are prepared by attaching cations to the matrix. The attached ions are associated with negatively charged counterions, which are mobile and can be exchanged by ions in the sample. Analytes with higher affinity for the matrix than the counterions will be exchanged. The ionic charge of the analytes depends greatly on pH.

An early application of ion-exchange separation with HPLC is the ‘amino acid analyzer’. Amino acids are separated on a cation-exchange column using a complex pH gradient from acidic to basic reaction to elute the amino acids as a function of their isoelectric pH. Post-column derivatization with ninhydrin is made online and the amino acids are detected using spectrophotometry. A drawback for this technique is the high cost of the equipment, and, further, it can be used only for amino acid analysis.

Milk proteins are separated and analyzed by IEC. Typically, the proteins are loaded onto the ion exchanger in a buffer of chosen pH and low ionic strength. The proteins are commonly eluted using a linear gradient of ionic strength over 10–20 column volumes to a NaCl concentration of about 0.25–0.50 mol l^{-1} in the elution buffer.

The caseins are separated by high-performance anion-exchange chromatography. The sample solution and the elution buffers contain large amounts of a dissociating agent such as urea, and a NaCl gradient is used for elution. The γ-, β-, and κ-caseins are well separated, but α_{s1}- and α_{s2}-caseins co-elute. For analytical purposes, capillary electrophoresis offers better possibilities of separating all casein components, as well as their primary breakdown products, in cheese.

The isoelectric pH values of lactoferrin and lactoperoxidase are higher than that of any other main milk

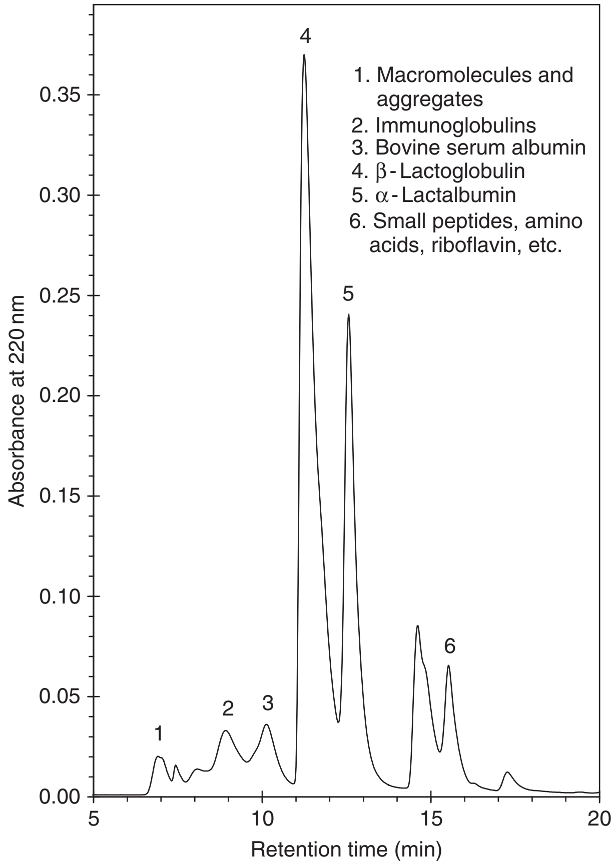

Figure 1 Gel permeation chromatography to whey proteins on a TSK Gel G2000SWXL 7.8 × 300 mm column with a 6 × 40 mm TSK guard column and a buffer of 0.01 mol l^{-1} KH_2PO_4, 0.09 mol l^{-1} K_2HPO_4, and 0.15 mol l^{-1} Na_2SO_4, pH 5.75 at a flow rate of 0.8 ml min^{-1}.

proteins and it is possible to choose a pH value where they are the only two proteins that are positively charged. Cation-exchange chromatography with a polymeric resin is an excellent technique to both analyze and purify these proteins.

Anion exchange at a high pH is useful for the analysis of carbohydrates. These analytes are at least partly ionized at high pH and can be separated using a strong anion-exchange resin (**Figure 2**). This approach cannot be used with silica-based columns, which will deteriorate at the high pH, and a base-stable polymer anion-exchange matrix is needed. A polystyrene and divinylbenzene substrate agglomerated with quaternary amine-activated latex is used for analyzing mono- and disaccharides. Attention must be paid to the instabilities of several carbohydrates at pH above 7.0, such as epimerization or deacetylation of *N*-acetyl glucosamine. Usually, the analysis is made at a sufficient short time, but whenever a longer time is needed, the stability must be investigated. High-precision detection of the carbohydrates is successfully made using pulsed amperometric detection (PAD).

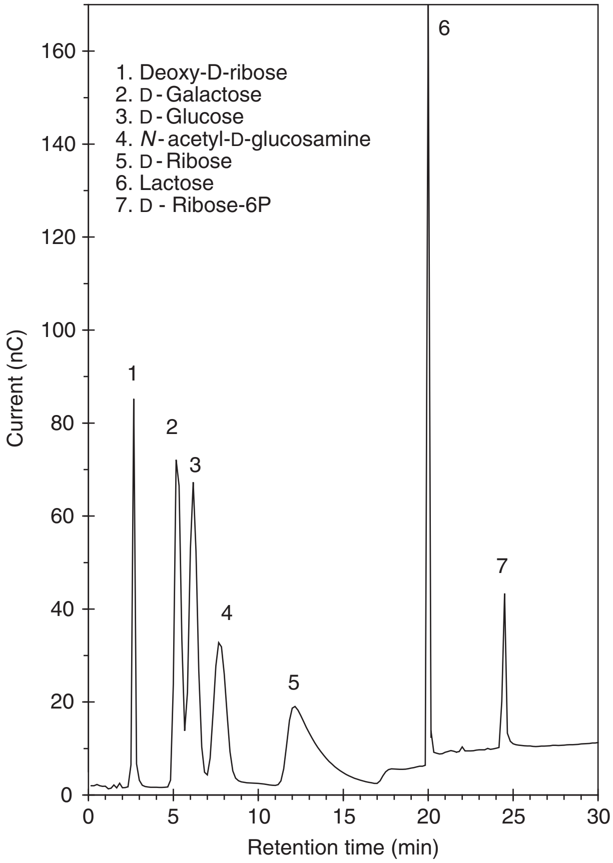

Figure 2 Anion exchange chromatography of carbohydrates on a Dionex Carbopac PA1 9 × 250-mm column with a flow rate rate of 1 ml min^{-1}. Elution gradient was 0–10 min at 5 mmol l^{-1} NaOH, 10–20 min linearly from 5 to 100 mmol l^{-1} NaOH and 0–200 mmol l^{-1} HAc, 20–25 min at 100 mmol l^{-1} NaOH and from 200 to 500 mmol l^{-1} HAc, 25–30 min from 100 to 25 mmol l^{-1} NaOH and 500–1000 mmol l^{-1} HAc. Detection was made by Dionex ED 40 electrochemical detector. Unpublished data from our laboratory by Dorte Fris Jensen.

Reversed-Phase High-Performance Liquid Chromatography

The chromatography method most commonly used for analytical purposes is reversed-phase high-performance liquid chromatography (RP-HPLC). The stationary phase of RP-HPLC columns is nonpolar and typically made of silanized silica with C_8 or C_{18} groups coupled to the silanol groups. A large variety of organic molecules may then be chromatographically separated using a polar, largely aqueous, mobile phase. The surface of silica always contains quite a lot of free silanol groups, which act as weak cation exchangers at neutral pH. At or below pH 3, the silanol groups are protonated and do not interfere with the chromatography, which could be a reason for choosing a low pH.

Casein that has been precipitated from milk or dairy products and dissolved in a buffer may be analyzed by

RP-HPLC, but β- and γ-caseins are only partly separated. Casein solutions are preferably analyzed by capillary electrophoresis.

A standard protocol for gradient elution at low pH has been developed for proteins and peptides. Water with 0.1% trifluoroacetic acid (TFA, as counterions to avoid aggregation and non-specific interactions) is used as the hydrophilic part of the mobile phase and the organic part acetonitrile (also containing 0.1% TFA) as the hydrophobic modifier. This method is used widely for the analysis of casein hydrolysates and water-soluble peptides in cheese. The peptides are detected online by measuring ultraviolet (UV) absorbance at 210–220 nm. The lower part of the interval is preferred because of a higher sensitivity. The method is used to study changes in peptide profiles during cheese ripening or to compare typical profiles of specific cheese varieties (**Figure 3**). Chemometric methods are used for evaluation of differences between the peptide profiles. However, because of independent variations in both retention time and light absorbance, quite a lot of data manipulation is needed to achieve correct results. The chromatograms represent a much higher number of peptides than there are peaks, but it is possible to identify peptides of the most dominant peaks using online mass spectrometry (MS) as well as sequencing of the amino acids using tandem MS-MS or even analysis of fragments of amino acids obtained by MS^3. UV spectra of the peaks measured online can be used as a step in the identification procedure because some amino acids differ markedly from others especially in absorbing light at 280 nm (tyrosine and tryptophan).

RP-HPLC of small organic molecules such as amino acids, amines, small carboxylic acids, and α-ketoacids is commonly performed after derivatization and at a pH where the silanol groups of the silica surfaces contribute to the separation. This group of analyses will be exemplified here by HPLC of amino acids that are typically derivatized at the primary amino group with *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid (MPA). The amino acid proline has no primary amino group and is not derivatized by OPA and a second derivatization step is needed if all amino acids are to be analyzed. The separation is then made according to the different properties of the amino acid side chains usually around neutral pH. By including an internal standard, at least 23 amino acids can be analyzed quantitatively this way (**Figure 4**). The coefficient of variation can be kept well below 5% for most amino acids, but sometimes it can reach up to 10% because of variations in the interactions between amino acids and the negatively charged column material, with the latter undergoing changes during aging. Another drawback is that the derivatives of some amino acids are not stable for a long time, making an automatic derivatization and injection system highly useful. Several other derivatization methods have been developed for amino acids and these can solve the problem of unstable derivatives; however, all have some limitations.

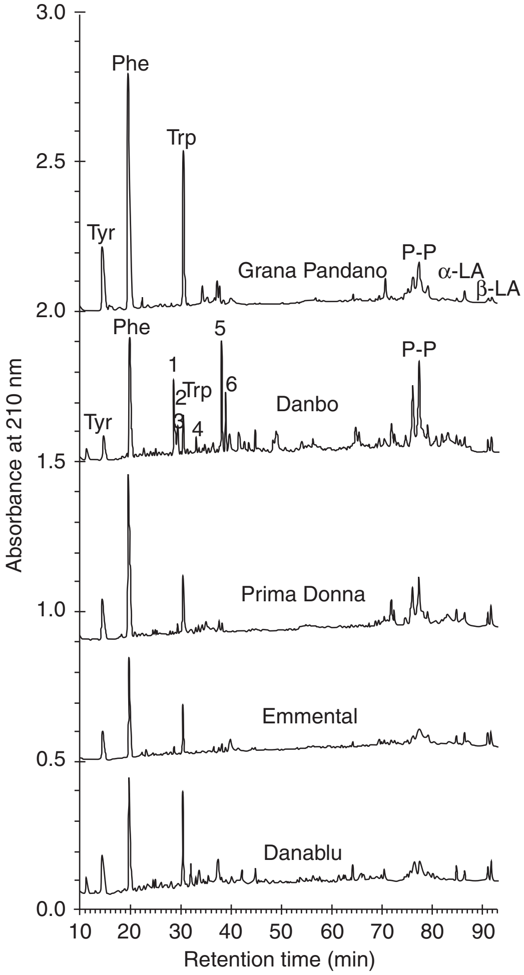

Figure 3 Reversed-phase high-performance liquid chromatography profiling of peptides in different mature cheese varieties. A silica column (Nucleosil 5 μm C$_{18}$, 250 × 4.6 mm) was used with an elution scheme in three steps at a flow rate of 1 ml min^{-1}. The gradient started with 0.1% trifluoroacetic acid (TFA) and then a linear increase from 0 to 48% acetonitrile for 80 min and finally for 10 min with 80% acetonitrile for 10 min with a constant TFA concentration. Three amino acids (Tyr, Phe, and Trp) and two whey proteins (α-lactalbumin and β-lactoglobulin) are found on all the chromatograms. P-P is proteose-peptone from plasmin activity on β-casein. Peptides from the amino end of α_{s1}-casein are peaks 1–6, (f1-9), (f1-8), (f1-7), (f1-6), (f1-13), and (f1-14), respectively.

Hydrophobic anions or cations may be added to the eluents to prolong retention of oppositely charged molecules, which speeds up the elution of the molecules with the same charge without affecting the neutral analytes at the chosen pH. This technique is named paired-ion chromatography and is used, for example, to improve resolution for analysis of water-soluble vitamins.

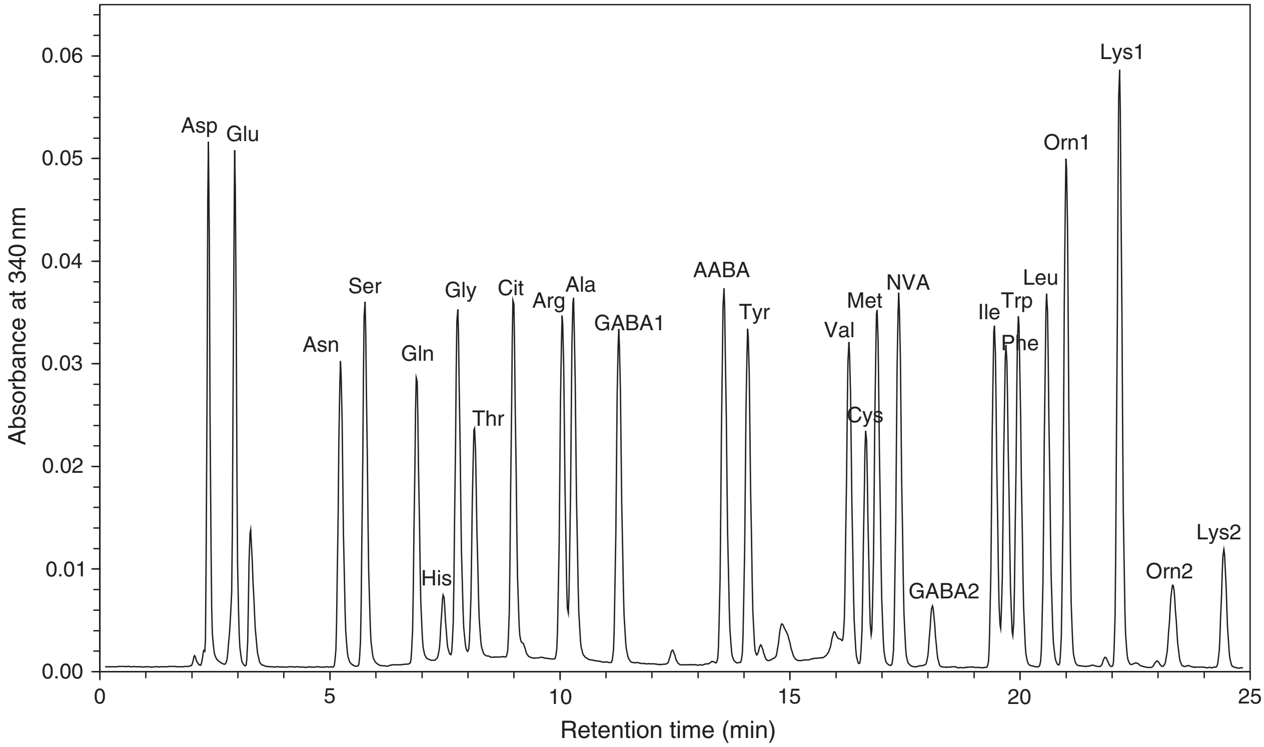

Figure 4 Reversed-phase high-performance liquid chromatography used for quantitative analysis of amino acids on a Waters XTerra™ RP18, 3.5 μ column. *o*-Phthalaldehyde derivatives were analyzed at 340 nm. The elution buffers contained tetrahydrofuran and Titriplex III as described in the Laboratory Manual in further reading section. A linear gradient was used from retention time 0 to 28 min with an increase in acetonitrile from 0 to 32% and a slight increase in NaOAc from 30 to 34 mmol l^{-1}. Using an internal standard (NVA) and reference standard curves, it is possible to obtain quantitative results. Three of the amino acids give two peaks each, but it is possible to handle quantitatively. GABA, γ-aminobutyric acid; AABA, α-aminobutyric acid; NVA, norvaline.

Other HPLC Techniques

Adsorption chromatography or normal-phase chromatography (NPC) uses a polar adsorbent (silica) and a nonpolar mobile phase such as petroleum ether and chloroform. Hydrophilic interaction liquid chromatography (HILIC), which can be considered as a modern version of NPC, uses an aqueous mobile phase for the separation of very polar analytes, such as hydrophilic peptides, sugars, oligosaccharides, and complex carbohydrates. In particular, this technique has become popular for the analysis of fluorescently derivatized *N*- and *O*-glycans (oligosaccharides).

The same separation mechanisms as for RP-HPLC are used for hydrophobic interaction chromatography (HIC), but denaturation of analytes occurs less frequently because the column material has a low population of hydrophobic groups, while those used for RP-HPLC have the entire surface covered with these groups. Proteins are injected to the column in a salt solution and then water and dilute buffers with low ionic strength are used to elute them using a salt gradient of decreasing salt concentration. HIC is important as a preparation step for biologically active molecules with a complicated structure that is sensitive to denaturation and crucial for biological activity.

Enzymes and other biologically active molecules may be isolated and analyzed by high-performance liquid affinity chromatography. Columns are available with tresyl chloride-activated silica, which can couple with amino groups of different molecules such as proteins or peptides. Components with an affinity specific to those groups may then be analyzed. Phosphopeptides are analyzed successfully in dairy products using Fe(III) affinity chromatography.

Detection Techniques for HPLC

Spectrophotometry is the most commonly used technique for online detection in HPLC. Analytes with UV absorption are very well detected without derivatization. For example, proteins are detected at 280 nm; two amino acid residues, tyrosine and tryptophan, absorb light at this wavelength. UV/visible (VIS) spectrophotometry is used for detecting small derivatized analytes that absorb light at a specific wavelength. Diode array detectors may register the spectra for a defined interval of wavelengths and can be used for online identification of specific compounds by comparing the spectrum with those in a database.

Fluorimetry measures the fluorescence at a specific emission wavelength after induction with a light pulse of a specific excitation wavelength. Combining these two wavelengths in different ways gives possibilities for very specific analysis of components or groups of components. The detection limits for fluorometric methods are typically 10 times better than for light absorbance detection methods. Some compounds have intrinsic fluorescence, for example, tryptophan, tyrosine, and phenylalanine in proteins, whereas others require derivatization with fluorescent probes, for example, 2-aminobenzamide and 2-anthranilic acid for glycan analysis.

Potentiometry measures the current produced in a sample when a defined voltage is applied to the sample. PAD methods are developed especially for analyzing carbohydrates in an effluent stream. pulsed amperometric detection (PAD) measures the current generated from electrolytically oxidized analytes on the surface of a gold electrode, after application of specific patterns of positive potential pulses over the sample to be analyzed.

Refractometry is the classical technique used to detect sugars that change the refractive index of the eluent. The method is, however, applied only to neutral carbohydrates and the sensitivity is low compared to other techniques. It is used successfully for identification of sugar peaks that may interfere with the results when analyzing other compounds in complex samples from foods like milk and dairy products.

Evaporative light-scattering detection can be used for measuring phospholipids in dairy products by atomizing the HPLC column eluent and following evaporation. Light scattering by the particles is measured at 45° to the incident beam of light. A modification of this is multiangle laser light scattering, which provides information regarding molecular dimensions and molecular mass of the eluted solute and is often used to measure aggregates in heat-treated products.

Surface plasmon resonance (SPR) in combination with HPLC utilizes electrostatic adsorption of proteins, which have positive or negative charges, onto the surface of a chemically treated SPR sensor and employs changes in the refractive index at the interface of the metal/HPLC eluent solution of the SPR sensor. This technique can be used to detect compounds that do not absorb in the UV/VIS range.

Radioimmunoassay can be used online to detect radioactivity from labeled molecules coupled to the analytes. It is also useful for studying biological activities on an added radioactively labeled component. The main problem involved in using this technique by many laboratories is the expense involved, because the whole chromatography equipment needs to be kept in rooms approved for work with radioactivity and needs to be handled with special care.

MS determines the molecular mass of the analytes online in a small amount of sample from the chromatographic column. Mainly positively charged ions are separated according to their relative masses. Large molecules may be ionized resulting in one or more charges, and may give complex spectra, which can be used for their identification. Depending on the technique used, the ions may be analyzed intact or after fragmentation (due to the ionization process). Determination of the molecular masses of the fragmented ions is useful in identification work. The mass spectra can be compared to those collected in a database and be used for identification of the analytes. In electrospray ionization (ESI), the sample and the solvent are sprayed through a needle, producing droplets, which evaporate and thereby form ions. These are fed through a capillary and into a quadrupole ion trap, which, by using a radiofrequency, selects individual ions. These may be fragmented by collision with helium resulting in a predictable fragmentation. The individual fragments are analyzed and the identity of the parent ion determined using software for this purpose.

Gas Chromatography

GC is a widely used technique to analyze thermally stable compounds that are volatile or may become volatile after derivatization. The sample is introduced to the GC system via a heated injection port and carried through a column in a continuous gas stream. Separation on the gas chromatographic column is based on volatility and interactions of the analytes with the stationary phase.

Volatile compounds in foods are commonly present at low concentrations (ng kg^{-1} to mg kg^{-1}); hence, prior to GC analysis, the volatile compounds have to be isolated from the food matrix and concentrated. Interactions of volatiles with fat and protein in dairy products have to be considered, because it may influence their release from the product during sample preparation and analysis.

Most isolation methods for dairy volatiles are based on either headspace analysis or distillation combined with solvent extraction. Static headspace sampling is quite a simple method for analyzing the headspace of a sample that has been kept in a closed container at a defined temperature until equilibrium between sample and headspace is reached. A higher sensitivity is obtained by purge-and-trap or dynamic headspace sampling in which the sample is purged with an inert gas stream above or through the sample, continuously removing the headspace and shifting the sample/air equilibrium. The stripped volatile constituents are collected in a trap, containing adsorbent material, and are desorbed from the trap prior to GC injection by either heat or a solvent. Solid-phase microextraction (SPME) is a technique developed more recently where volatiles adsorb to a thin solid-phase coated fiber that is kept in the headspace above the sample. The adsorbed volatiles are thermally desorbed in the GC injector. The composition of the adsorbents varies and it is possible to select an adsorbent for a chosen group of components. SPME and static headspace are widely used in routine analysis, and the sensitivity can be increased by increasing sampling temperature. GC samples can also be prepared by simultaneous distillation extraction using either atmospheric pressure or low pressure and a temperature around 30–40 °C to avoid artifacts from chemical reactions. Acid, neutral, and basic volatiles are extracted with high precision, but highly volatile compounds like acetaldehyde and several sulfur compounds may be lost. Solvent-assisted flavor evaporation (SAFE) is a direct high-vacuum distillation technique that is used for milk, working at ambient temperature preventing thermal changes. One drawback of methods that involve the use of solvents is the resulting solvent front in the chromatogram masking early eluting compounds.

Derivatization can be used to make compounds more volatile and this procedure is efficient for the analysis of short-chain carboxylic acids in cheese. The acids are analyzed using GC after vapor distillation and benzyl esterification, with a capillary column and a known detection limit (e.g., $10\,\mathrm{mg\,kg^{-1}}$ for butyric acid). GC methods for free fatty acids in butter and cheese, as well as for fatty acid composition of milk fat, are widely used. Also, amino acids may be analyzed with GC after derivatization.

For GC analysis of foods, flame-ionization detectors (FIDs) and MS are the most commonly used detectors, but other detectors like the nitrogen–phosphorus detector (NPD) are also available. In FID, the organic compounds are readily pyrolyzed when introduced into a hydrogen–oxygen flame and ions are produced in the process. These ions are collected at charged electrodes and the resulting current is measured by means of an electrometer amplifier. Compounds are identified by their retention time and the identity of peaks is confirmed by adding known reference substances to the sample (spiking), and FID results are successfully used for quantification. Better identification, however, is obtained by an MS detector. The concentration of many volatile compounds in dairy products is below threshold for odor activity. Those that are odor-active can be determined by GC–olfactometry, in which a human nose is used as detector of the compounds eluting from a GC column. In GC–olfactometry, several people may be used as assessors. Some substances can be detected at lower concentrations with olfactometry than with MS detection (**Table 3**).

Table 3 Flavor-active compounds analyzed by GC of a 10-month-old Prima Donna cheese sampled by dynamic headspace, given in the order they elute from the column

No.	*Odors observed by GC–olfactometry*	*Compounds identified by GC–MS*
1	Solvent, permanent marker, butyric acid	2-Methylbutanal, 3-methylbutanal
2	Caramel, diacetyl, butter, cheese	2,3-Butandione (diacetyl)
3	Fruit, pineapple, ethyl butanoate	Ethyl butanoate
4	Caramel, sweetish, diacetyl-like	2,3-Pentandione
5	Burned, burned coffee, sweetish, greenish/fermented	Unknown
6	Silage, cat food, cheese, onion, slightly green	Unknown
7	Solvent, sour, estery	2-Methylbutanol, 3-methylbutanol
8	Sweetish, fruity, nasty, fermented	Ethyl hexanoate, (*Z*)-4-heptenal
9	Dirty socks, popcorn, sweetish, baked	2-Acetyl-1-pyrroline
10	Fermented, onion, cheese-like	Dimethyl trisulfide
11	Pea pod, pyrazine, cucumber, potato, dry	Isopropyl methoxypyrazine
12	Butter, dairy, cucumber, sweetish	(*E*)-2-Octenal
13	Potato, cabbage, fermented	Methional
14	Solvent, metallic, acid	Unknown
15	Slightly green, slightly potato, carrot, gas, old	2-*sec*-Butyl-3-methoxypyrazine
16	Grass, sourish, cucumber	(*E*,*E*)-2,6-Nonadienal
17	Flower, hyacinth, cheese	Phenyl acetaldehyde
18	Vitamin tablet, soup, cheese	2-Methyl-3-(methyldithio)furan
19	Pig sty, dirty dish cloth, butyric acid, dirty feet, cheese	Isovaleric acid
20	Fruity, gas, sourish	(*E*,*E*)-2,4-Nonadienal
21	Cheese-like	Pentanoic acid
22	Sweetish baked, rice, sour, burned	2-Acetyl-2-thiazoline
23	Fresh herb, tobacco, slight potato/vegetable, newly boiled rice, dry	Unknown
24	Ozone, fruit, electric train, plastic/rubber, oat flakes, bad	(*E*,*Z*,*E*)-Nonatrienal
25	Coconut, dill, aromatic plant	δ-Decalactone
26	Fruit, sweetish, coriander	δ-Dodecalactone
27	Bad breath, cigarette smoke	Unknown

GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry.

C Varming, MA Petersen, and Y Ardö, Department of Food Science, University of Copenhagen, Denmark, unpublished data.

Future Trends

Column materials have been developed extensively during recent decades and have reached a very high technological level, and this development continues mainly for more specific applications. The improved quality of the instruments considerably facilitates chromatography. It is possible today to have stable pressure from the pumps, degassing systems giving constant composition of the buffers for each run, and efficient flow through detectors, and these improvements will continue. On the very high-standard equipment available today, several new analysis applications will be seen in the near future. Online detection of UV/VIS spectra and mass spectra (MS) is becoming available for many laboratories, and it makes the identification work much easier. Development is expected in the improvement of solid-phase extraction methodologies as well as improvement in MS techniques including refinement of Fourier transform ion cyclotron resonance. New developments of several other online detection techniques could be expected, and multivariate statistical analysis is increasingly adapted to analysis of chromatographic data.

See also: Cheese; **Analytical Methods**: Electrophoresis; Mass Spectrometric Methods; Sampling. **Cheese**: Biochemistry of Cheese Ripening; Overview. **Flavors and Off-Flavors in Dairy Foods**. **Milk Lipids**: Fatty Acids.

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Immunochemical Methods

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Terminology

Immunochemical techniques can be defined as analytical techniques based on the interaction between antigens (substances that prompt the generation of antibodies and can cause an immune response) and antibodies (proteins that are found in blood or other bodily fluids of vertebrates and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses). Antigens are usually proteins or polysaccharides. This includes parts (coats, capsules, cell walls, flagella, and toxins) of bacteria, viruses, and other microorganisms. Lipids and nucleic acids are antigenic only when combined with proteins and polysaccharides.

Immunoassays have often been used to detect admixtures (in the case of frauds) and contaminants (to avoid toxic components or food-borne pathogens) or to quantify constituents in dairy products. From a more basic point of view, immunochemical techniques can be used to obtain structural information on viruses, bacteria, supramolecular structures, and so forth. Antigens are constituted by a succession of antigenic determinants, so-called epitopes, corresponding to the part of a macromolecule that is recognized by the immune system, specifically by antibodies, B cells, or T cells. The part of an antibody that recognizes the epitope is called a paratope. Most epitopes recognized by antibodies or B cells can be thought of as three-dimensional surface features of an antigen molecule; these features fit precisely and thus bind to antibodies. Exceptions are linear epitopes, which are determined by the amino acid sequence (the primary structure) rather than by the three-dimensional shape (tertiary structure) of a protein. It is generally accepted that the contact zone of the epitope with the paratope is constituted by 3–6 amino acids; however, 20–25 amino acids on the antigen are involved in the antigen–antibody interaction.

Antibodies are of either monoclonal or polyclonal nature. Monoclonal antibodies are monospecific antibodies that are identical because they are produced by one type of immune cell, all of which are clones of a single parent cell. Polyclonal antibodies (or antisera) are antibodies that are derived from different B-cell lines. They are a mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope. More recently, technologies for producing recombinant monoclonal antibodies (known as repertoire cloning or phage display/yeast display) have been developed. Recombinant antibody engineering involves the use of viruses or yeasts, rather than mice, to create antibodies. These techniques rely on rapid cloning of immunoglobulin gene segments to create libraries of antibodies with slightly different amino acid sequences from which antibodies with desired specificities can be selected. These techniques can be used to enhance the specificity with which antibodies recognize antigens, their stability in various environmental conditions, their therapeutic efficacy, and their detectability in diagnostic applications.

Interaction between antigens and antibodies occurs via the establishment of noncovalent bonds between the two partners. Among these bonds, it is generally accepted that hydrophobic interactions account for more than 50% of the interactions between the two partners.

General Characteristics of the Immunochemical Techniques

One of the most important characteristics of immunochemical techniques is their high specificity. These techniques allow for the detection and/or quantification of a single molecule, even in a complex protein mixture. Furthermore, by using very specific antibodies, it is possible to discriminate different forms of the same molecule; for instance, an antibody can be specific to the heat-denatured form of a protein but not to its native form.

Sensitivity is also a major characteristic of immunochemical techniques. In milk, these techniques can quantify molecules at very low concentrations such as a few nanograms per milliliter. It is even possible to increase immunoassay sensitivity by using fluorescence or luminescence for the revelation of the reaction.

Finally, immunochemical techniques provide results rapidly if optimized. For instance, quantification of a protein using an immunosensor can be performed within 10 min.

Description of the Immunochemical Techniques Applied to Dairy Products

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was first described in 1971. It consists of a two-pronged strategy: (1) the reaction between the antibody and its corresponding antigen and (2) the detection of that reaction using an enzyme, labeled to the reactants, as an indicator. This technique is based on the fact that at alkaline or neutral pH, a protein can be immobilized by noncovalent binding onto a solid phase such as the polystyrene of a microplate.

The different types of ELISA are represented in **Figure 1**. Direct and indirect ELISAs are noncompetitive methods that involve the coating of the antigen onto the microplate. This step may induce conformational changes in the antigen resulting in the modification of antibody binding. Therefore, direct and indirect ELISAs are often used for qualitative tests. Another noncompetitive method is the sandwich ELISA. Here, antibodies are immobilized to trap antigens from crude extracts. This technique requires that the antigens have at least two epitopes.

Competitive methods include competitive and inhibition ELISAs. Competitive ELISAs are based on the competition of enzyme-labeled antigen with the antigen present in the test sample for the antibody on the solid phase. Inhibition (also called competitive indirect) ELISAs are based on the inhibition of the reaction of enzyme-labeled antibodies and the immobilized antigen by free antigen present in the test or calibration sample. The amount of enzyme immobilized on the solid phase is inversely proportional to the amount of free antigen present in the incubation mixture.

Quantitative assays are mostly inhibition, competitive, or sandwich ELISAs where the antigen–antibody interaction occurs in solution and keeps the antigen in its native form. On the one hand, these methods are fast and require minimum controls, but on the other hand, high antigen concentrations are needed for proper detection.

There has been some misunderstanding in the definition of ELISA sensitivity. The limit of detection or detectability of an ELISA corresponds to the lowest concentration that gives a signal that is significantly different from that of the background values (= ability to detect). In contrast, sensitivity is defined by the dose–response curve and corresponds to the change in response (dR) per unit amount of reactant (dC) and equals dR/dC as the slope of the titration curve. Thus, an ELISA can have a high limit of detection and, at the same time, be extremely sensitive.

In the same way, there is sometimes confusion between the terms ‘accuracy’ and ‘precision’. Accuracy is the conformity of a result to an accepted standard value or true value. Precision, however, is defined as the degree of agreement between replicate measurements of the same quantity and may be of very low accuracy.

Radioimmunoassays (RIAs) have also been developed for analyzing milk proteins. These techniques are very similar to the ELISAs, except that visualization of the reaction is made using radioisotopes and scintillation counters instead of a colorimetric substrate and a spectrophotometer.

Immunoprecipitation in Gel

Immunoprecipitation in gel techniques are based on the diffusion of an antigen and/or an antibody in a gel. A precipitate forms as the antigen and the antibody interact. Some of these techniques are quantitative. Single radial immunodiffusion (SRID) has been the most widely used quantitative technique. It involves the diffusion of an antigen (antibody) through an agarose gel that contains the corresponding specific antibody (antigen). This leads to the formation of circles, with diameters proportional to

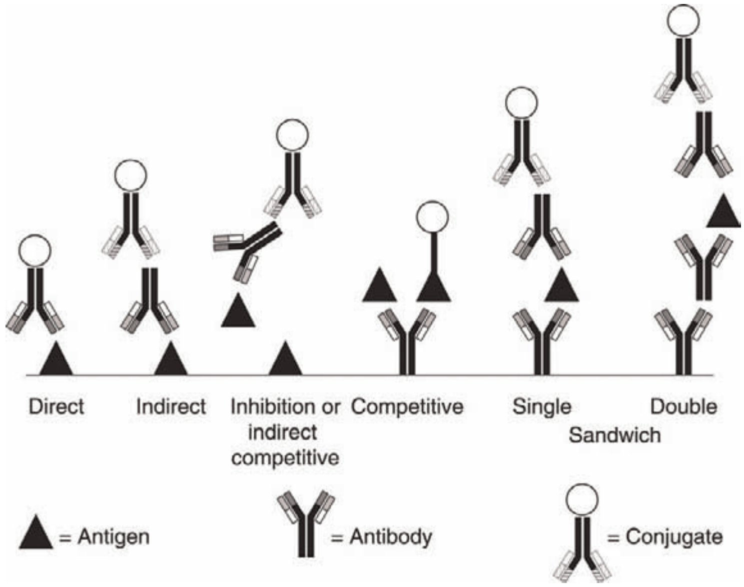

Figure 1 The different types of ELISA.

the antigen concentration. SRID is simple, easy, and rapid to perform even if one day is needed to obtain results. The most important drawbacks, however, are its lack of sensitivity (limit of detection of a few micrograms per milliliter) and the necessity to use precipitating antibodies.

Immunoblotting

Immunoblotting involves the separation of the different constituents of a mixture by electrophoresis, their transfer onto a membrane, and their revelation by an antibody. It can be a sensitive technique especially when luminescence is used for revelation of the proteins (10 pg of a protein can be detected in this case). However, this technique can hardly be quantitative and it can take a day to perform it.

Immunosensors

An immunosensor is a type of biosensor (*see* **Analytical Methods**: Biosensors) that combines a biological recognition mechanism with a transducer, which generates a measurable signal in response to changes in the concentration of a given biomolecule. One component (ligand) of the interaction to be studied is covalently immobilized to the matrix, and other interactants (analytes) are passed over the sensor in solution. Biosensors using antibodies as ligands are called immunosensors. Immunosensor technologies are quite recent and began to be used for the analysis of food around the mid-1990s. In recent years, significant progress has been made in the miniaturization of the immunosensors. Portable immunosensors for on-site detection are now commercially available, some of them allowing the simultaneous detection of several biomolecules.

Antibody Microarray

An antibody microarray is a specific form of protein microarray. A collection of capture antibodies are spotted and fixed on a solid surface, such as glass, plastic, or silicon chip, for the purpose of detecting antigens. An antibody microarray is often used for detecting protein expressions from cell lysates in general research and special biomarkers from serum or urine for diagnosis applications. Applications related to milk and dairy products are limited and involve the diagnosis of milk-related pathologies (allergy) or the detection of minor constituents of major biological interest (cytokines in colostrum).

Applications

The majority of the immunochemical techniques applied to milk and milk products aim at detecting contaminants (pathogens, chemicals, antibiotics, etc.). However, some applications for determining the protein composition of dairy products, the detection of milk allergens in food, the impact of processing on milk proteins, and the adulteration of dairy products have also been widely investigated using dedicated immunoassays.

Proteins

Quantitative techniques have been developed in the past years for determining the concentrations of caseins and whey proteins in milk, dairy products, and food using milk proteins as ingredients. These techniques were developed to determine the food composition and its evolution during storage. For example, caseins were quantified and their degradation followed during processing (cheese ripening, milk clotting, etc.). One major difficulty to overcome was that an antibody specific to a peptide will also cross-react with the mother protein. Therefore, sandwich immunoassays with antibodies specific to the N- or C-terminal extremities of the casein have been proposed to quantify only intact proteins. Similarly, α-lactalbumin, β-lactoglobulin, and lactoferrin have been the targets of many immunoassays due to their nutritional properties.

Milk proteins are also major food allergens. Immunoassays are perfect tools for detecting in complex food matrices the presence of traces of milk proteins that could result from cross-contamination. Immunoassays, particularly antibody microarrays, have also been developed as diagnostic tools for food allergy. In that case, milk proteins or peptides are spotted onto the array. Detection of these immobilized ligands by specific IgE of allergic patient sera allows a quick diagnosis using only a few microliters of biological sample. Furthermore, antibody arrays can allow the simultaneous detection of dozens of allergens, and they will therefore probably play a major part in the food allergy diagnostic market.

One of the major adulterations of dairy products consists in the fraudulent substitution of ewe’s or goat’s milk by cow’s milk due to the difference in price between them. This substitution can become a serious problem in cheese manufacture as the origin of the milk influences the sensory characteristics of the final product. Thus, for economic as well as ethical reasons, there is a need for analytical procedures that can detect the addition of cow’s milk to ewe’s or goat’s milk in order to protect ovine and caprine milk products from adulteration and to assure consumers the authenticity of the product and its quality.

Immunological techniques because of their specificity and sensitivity are well adapted for revealing dairy product adulteration. Indeed, it is quite easy to produce a species-specific antibody that will detect only a cow's marker in ewe's or goat's milk. Three different strategies can be used to obtain such a species-specific antibody:

- A polyclonal antibody can be raised against a protein and adsorbed against the protein of the other species.
- When the protein sequence of a different species is known, it is possible to select a species-specific peptide, to synthesize it, and to use it for immunization of an animal. The polyclonal serum thus obtained will recognize only the target marker to detect.
- Monoclonal antibodies raised against a protein can be directed against species-specific sequences and thus can be a valuable tool for detection of dairy product adulteration.

The different immunological techniques developed for studying dairy product adulteration are listed in **Table 1**.

Whey proteins also have different sensitivities to heat denaturation and have been widely used as markers of heat treatment. Changes in their conformation resulting from heat denaturation can be followed when antibodies specific to the native form of the tracer are available. Therefore, native α-lactalbumin-specific antibodies have been used to study the impact of ultra-high-temperature (UHT) treatments on drinking milk. More recently, antibodies specific to native milk alkaline phosphatase have been produced allowing the detection of milk pasteurization, which is a key issue for protected designation of origin (PDO) cheeses made from raw milk.

Enzymes

More than 60 different enzymes are reported in milk. Most, if any, of them are present in milk in very low concentrations. However, due to their specific activities, these enzymes can play a significant role in the quality of dairy products. For instance, proteases such as plasmin have been shown to play a critical role in the development of sensorial qualities of Swiss-type cheeses. Another example is the role played by proteases from psychrotrophic bacteria such as *Pseudomonas fluorescens* in the destabilization of UHT milk during storage. Therefore, many immunoassays detecting either the enzyme itself or the product of its activity have been developed and applied to dairy products. However, in some cases, it has been shown that immunochemical detection of the enzyme has to be completed by determination of its specific activity as the enzyme, even if present and detectable in the food matrix, can be inactivated by the other constituents.

Hormones

Different types of hormones are present in milk. Among protein hormones, some are indigenous such as prolactin, whereas others like somatotropin are administered to cows for increasing milk production. However, because the use of this hormone is not allowed in some countries, there is a strong need for detection techniques.

The operation of the milk industry is connected to good management of animal reproduction within herds. The major problem a farmer has to face is the precise determination of the cow's estrus period. Estrus corresponds to the period preceding ovulation. The quantification of certain hormones in milk, in particular progesterone, constitutes an excellent marker of the estrus period. Indeed, during this period, milk progesterone concentration, which is usually around $0.1\,\mathrm{ng\ ml^{-1}}$ increased dramatically to reach more than $6\,\mathrm{ng\ ml^{-1}}$. Therefore, several sensitive immunoassays have been proposed to quantify progesterone in milk. Among those, a very interesting approach was to develop immunosensors designed to operate online in a dairy parlor, allowing the estimation of the progesterone concentration in milk during milking.

Antibiotics, Drug Residues, and Chemical Contaminants

The use of antibiotics and drugs in animal husbandry can lead to the appearance of residues in milk. The traditional microbiological techniques used to detect these contaminants show poor sensitivity, whereas analytical techniques such as chromatography and mass spectrometry are not widespread and need high-level skills and costly investments. Therefore, immunochemical techniques became very popular for the detection of drug residues in milk, and immunoassays for the quantification of antibiotics like penicillin, streptomycin, gentamicin, chloramphenicol, and cephalexin are available. A nonexhaustive list of some techniques available for antibiotic detection in milk is given in **Table 2**.

Similarly, chemical contaminants such as pesticides and herbicides have been tracked in dairy products, and molecules like atrazine, polychlorinated biphenyls, and acetochlor can now be monitored in milk using dedicated immunoassays.

Toxins and Pathogens

Some microorganisms such as pathogenic bacteria and fungi are able to produce toxins. Staphylococcal

Table 1 Detection of milk and cheese adulteration using immunochemical techniques

References	*Product*	*Adulteration*	*Marker*	*Antibody*	*Technique*	*Limit of detection*
Hewedy and Smith (1990)	Milk	Soy/milk	Soy proteins	Adsorbed polyclonal	Indirect ELISA	1%
Moio *et al*. (1992)	Cheese	Cow/ewe	γ2-CN	Polyclonal	Immunoblotting	5%
Garcia *et al*. (1993)	Milk	Goat/ewe	Whey proteins	Adsorbed polyclonal	Sandwich ELISA	0.5%
Bitri *et al*. (1993)	Milk, cheese	Cow/ewe or goat	κ-CN f139–152	Polyclonal	Competitive ELISA	0.25%
Levieux and Venien (1994)	Milk	Cow/ewe or goat	β-lg	Monoclonal	ELISA	0.01%
Rolland *et al*. (1995)	Milk	Cow/goat	αs_1-CN f140–149	Polyclonal	Competitive ELISA	0.5%
Addeo *et al*. (1995)	Cheese	Cow/ewe or buffalo	β-CN	Polyclonal	Immunoblotting	0.5%
Haza *et al*. (1996)	Milk	Goat/ewe	αs_2-CN	Monoclonal	Indirect ELISA	0.5%
Anguita *et al*. (1996)	Milk, cheese	Cow/ewe	β-CN	Monoclonal	Immunostick ELISA	1% (milk) 0.5% (cheese)
Beer *et al*. (1996)	Cheese	Cow/ewe or goat	β-lg	Adsorbed polyclonal	Inhibition ELISA	0.1–0.2%
Molina *et al*. (1996)	Cheese	Cow/ewe or goat	β-lg	Polyclonal	Immunoblotting	1%
Haza *et al*. (1997)	Milk	Ewe/goat	αs_2-CN	Monoclonal	Indirect ELISAInhibition ELISA	0.5% 0.25%
Anguita *et al*. (1997)	Milk, cheese	Cow/ewe or goat	β-CN	Monoclonal	Competitive ELISA	0.5%
Richter *et al*. (1997)	Milk, cheese	Cow/ewe or goat	γ3-CN	Polyclonal	Inhibition ELISA	0.1%
Haasnoot *et al*. (2001)	Milk powder	Pea, soy, wheat/milk	Plant extracts	Polyclonal	Immunosensor	<0.1%
Chavez *et al*. (2008)	Milk	Milk/whey	GMP	Polyclonal	Western blotting	0.5%
Haasnoot *et al*. (2006)	Milk	Cow/ewe or goat	κ-CN	Monoclonal	Immunosensor	0.17%

β-lg, β-lactoglobulin; CN, Casein; GMP, Glycomacropeptide.

Table 2 Detection of antibiotics in milk using immunochemical techniques

References	*Antibiotic*	*Technique*	*Limit of detection*
Rohner *et al*. (1985)	Penicillin G	ELISA	
Jackman *et al*. (1990)	Penicillin G	ELISA	
Brown *et al*. (1990)	Gentamicin	ELISA (fluorescence)	
Hammer *et al*. (1993)	Streptomycin	ELISA	
Everest *et al*. (1994)	Tetracycline	ELISA	
Martlbauer *et al*. (1994)		ELISA	
Schneider *et al*. (1994)	Chloramphenicol	ELISA	
Usleber *et al*. (1994)	Penicillin	ELISA	
Kumar *et al*. (1995)	β-Lactam (penicillin G, ampicillin, cloxacillin, amoxicillin, ceftiofur, cephapirin)	ELISA (fluorescence)	
Mitchell *et al*. (1995)	β-Lactam	Immunodiffusion ELISA	70 ng ml^{-1}
Albrecht *et al*. (1996)	Spiramycin	ELISA	5.2 ng ml^{-1}
Bouksaïm *et al*. (1999)	Nisin	ELISA	1.7 ng ml^{-1}
Watanabe *et al*. (1999)	Kanamycin	ELISA	0.2 ng ml^{-1}
Haasnoot *et al*. (1999)	Gentamicin, neomycin, streptomycin, dihydrostreptomycin	ELISA	
Nadankumar *et al*. (2000)	Nisin	Immunosensor	3 μmol l^{-1}
Baxter *et al*. (2001)	Streptomycin	Immunosensor	4.1 ng ml^{-1}
Ferguson *et al*. (2002)	Streptomycin	Immunosensor	30 ng ml^{-1}
	Dihydrostreptomycin	Immunosensor	100 ng ml^{-1}
Loomans *et al*. (2003)	Gentamicin, kanamycin, neomycin	ELISA	

enterotoxins, for example, are 26- to 30-kDa proteins that are resistant to heat treatment (*see* **Pathogens in Milk**: *Staphylococcus aureus* – Molecular). Their adsorption by the consumer will result in gastroenteritis (diarrhea, vomiting). The high toxicity of these toxins needs analytical techniques sensitive enough to allow the detection of extremely low concentrations in food (around 1 ng ml^{-1}). Immunoassays, particularly ELISAs, have been widely used for this purpose. More recently, immunosensors have been used to detect staphylococcal enterotoxins in food. Milk can also be contaminated with small amounts of aflatoxin M1 (AFM1) as a consequence of metabolism by the cow of aflatoxin B1 (AFB1), a mycotoxin commonly produced by the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus* and found in certain animal foodstuffs (*see* **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds). The maximum allowed content of AFM1 in milk under the European Union directives is 50 ppt, and because of their high sensitivity, immunoassays are particularly relevant for quantifying aflatoxins in milk.

Another alternative consists in detecting the pathogenic microorganism itself, and numerous immunoassays have been proposed in the literature to reach this goal (**Table 3**). Compared with the basic culture techniques traditionally used, commercially available immunochemical techniques for pathogen detection permit one to get a quicker response. However, this still requires a pre-enrichment on selective media to obtain a sufficient amount of bacteria for immunodetection.

Table 3 Immunodetection of pathogens in milk and dairy products

References	*Pathogen*	*Product*	*Technique*	*Limit of detection*
Cohen and Kerdahi (1996)	*Escherichia coli*	Cheese	ELISA	
Waller and Ogata (2000)	*Campylobacter jejuni*	Milk	Immunocapture polymerase chain reaction	1 cell ml^{-1}
Liu *et al*. (2002)	*E. coli*	Milk	ELISA with immunomagnetic separation	
Karamonova *et al*. (2003)	*Listeria monocytogenes*	Milk	ELISA	
Waswa *et al*. (2007)	*E. coli*	Milk	Immunosensor	100 cfu ml^{-1}
Chu *et al*. (2009)	*Bacillus cereus*	Milk	Immunomagnetic beads	10 cfu ml^{-1}
Cheng *et al*. (2009)	*E. coli*	Milk	Magnetic nanoparticles	20 cfu ml^{-1}
Pal and Alocilja (2009)	*Bacillus anthracis* spores	Milk	Immunosensors	420 spores ml^{-1}

Conclusion

Because of their high specificity and sensitivity, immunochemical techniques have been widely applied to milk and dairy products and are particularly adapted for quantifying proteins, contaminants, pathogens, and so forth. In the near future, more and more specific probes such as highly specific monoclonal or phage recombinant antibodies will be needed. Novel forms of detection such as chemiluminescence and cascade amplified system will significantly improve the already high sensitivity of the immunoassays. It is also probable that the strong need for 'on-farm' control will result in the development of immunoassays easy to use for the end user. It is likely that the trend will be toward different solid-phase configurations, such as 'dipsticks' or latex bead assays. Also, biosensors based on immunological principles have the advantage of providing continuous 'online' measurements and are therefore more suited to process control than are current immunoassays and thus will show a strong development.

See also: **Analytical Methods**: Biosensors. **Pathogens in Milk**: *Staphylococcus aureus* – Molecular. **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds.

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Electrophoresis

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Introduction

Electrophoresis is defined as the migration of charged molecules under the influence of an electric field. Since only charged particles migrate in an electric field, experimental conditions are made conducive to enable the resolution of particles based on differences in their charge or charge density (i.e., by adjusting the pH or ionic strength of the solvent buffer to influence the charge on solute particles). In a solution, if a solute has a positive charge (cation), it will migrate toward the negative electrode (cathode), and a negatively charged ion (anion) will migrate toward the positive electrode (anode). Each kind of molecule travels through the medium at a rate, specific to that molecule and the rate depends on its electrical charge and molecular size. A solute may be charged due to the ionization of groups on the molecule or a charge may be induced by the electrolyte.

The first part of the article describes gel-based electrophoresis. Acrylamide gel is the medium commonly used for the electrophoresis of proteins. Native electrophoresis is realized when proteins are separated due to their native charges. Denaturing electrophoresis is realized when using sodium dodecyl sulfate (SDS), an anionic detergent. The second part of the article describes gel-free electrophoresis and multidimensional electrophoresis, involving at least two different electrophoretic separations of proteins.

Gel-Based Electrophoresis

Gel electrophoresis is a technique used for the separation of biomolecules, especially nucleic acids and proteins, using an electric current applied to a gel matrix. Polyacrylamide gel electrophoresis (PAGE) is a very useful technique that has been widely used in research and development laboratories for the separation of macromolecules from various origins. The matrix used in gel electrophoresis is a mixture of acrylamide and a cross-linking agent (usually bisacrylamide). Polyacrylamide gels are formed by copolymerization of acrylamide and bisacrylamide ('bis', *N*,*N*′-methylene-bisacrylamide). The chemical reaction is a vinyl addition polymerization initiated by a free radical-generating system. Polymerization is initiated and catalyzed by ammonium persulfate and tetramethylethylenediamine (TEMED): TEMED accelerates the rate of formation of free radicals from persulfate and these free radicals in turn catalyze polymerization. The persulfate free radicals convert acrylamide monomers to free radicals, which react with unactivated monomers to begin the polymerization chain reaction. The elongating polymer chains are randomly cross-linked, resulting in a gel with a characteristic porosity. The pore size of a gel is determined by two factors: the acrylamide concentration and the amount of cross-linker. Pore size decreases with increasing acrylamide concentration; acrylamide concentration varies from 4% for large proteins to 18.5% for peptides. A 5% concentration of cross-linking agent gives the smallest pore size; any increase or decrease in the concentration of the cross-linking agent increases the pore size. Acrylamide/bisacrylamide gels of three different ratios are commercially available: a 37.5:1 ratio of acrylamide/bisacrylamide is usually recommended for the separation of high/medium molecular weight proteins (10–250 kDa); a 29:1 ratio is recommended for the separation of small proteins or DNA, particularly for the analysis of protein/DNA interactions, or peptides; and a 19:1 ratio is used for the sequencing of small nucleic acid fragments.

Slab gel PAGE systems have found widespread application in the analysis of proteins. Continuous buffer systems have been used to resolve caseins and whey proteins; however, nearly all one-dimensional (1-D) PAGE techniques that have been used recently for the analysis of caseins and whey proteins have used discontinuous buffer systems. Gel buffers normally contain urea or SDS as a dissociating agent. Nondenaturing buffers are not useful for caseins, although they are effective for the analysis of whey proteins.

Sample preparation normally involves dissolving the sample in a buffer (which usually contains a reducing agent, e.g., 2-mercaptoethanol) prior to electrophoresis. The sample may be defatted centrifugally and a solute (e.g., sucrose or glycerol) added to increase the density of the sample and facilitate loading into the gel slots. Following an electrophoresis run, the resolved proteins/peptides are fixed in position within the gel by denaturation and/or precipitation to prevent diffusion, which would result in reduced resolution.

Fixing solutions containing trichloroacetic acid (TCA, e.g., 12% w/v), acetic acid, organic solvents, or a shift in pH are commonly used. Direct or indirect staining using Coomassie blue or amido black followed by destaining using several changes of distilled water until the background becomes clear, is the most widely used technique to visualize protein bands. However, since only relatively large peptides stain under these conditions, it is limited to the detection of whey proteins, caseins, and their primary proteolytic degradation products.

For example, peptides in the 10 kDa ultrafiltration permeate or in the 70% ethanol-soluble fraction of water-soluble extracts of cheese do not stain with Coomassie blue on urea-PAGE, but the retentate of the water-soluble extract contains several detectable peptides, as do the 2% TCA-soluble and 2% TCA-insoluble fractions of the retentate. Proteins and peptides can also be visualized using a silver staining technique, incorporating glutaraldehyde fixing, which is a very sensitive staining technique.

After staining, gel electrophoretograms are usually recorded photographically or by scanning the gels into a computer, although densitometry or excision and elution of the stained bands, followed by spectrophotometric quantitation, have also been used. The difficulty in obtaining quantitative data is a serious limitation of electrophoresis. It is recommended that several reference samples should be included in each gel and that comparisons should be made only between samples on the same gel. Band dimensions are critical for densitometry and dye uptake is a function of the protein as well as the staining and destaining protocol. Thus, PAGE should be considered only as a semiquantitative analytical technique.

SDS-PAGE

A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and SDS to denature the proteins. The method is called SDS-PAGE.

SDS is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass (1.4 mg SDS mg^{-1} protein). The negative charge on SDS destroys most of the complex structure of proteins, and the negatively charged SDS molecules are strongly attracted toward an anode (positively charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in the relative molecular mass of polypeptides. In a gel of uniform density, the relative migration distance of a protein is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between Rf and mass can be plotted and the mass of unknown proteins estimated. The Rf is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye front.

Tris/Glycine–SDS-PAGE

The most commonly used system is also called the Laemmli method after UK Laemmli, who was the first to publish a paper using SDS-PAGE in a scientific study. Laemmli gels are discontinuous and are composed of two different gels (stacking and running gels), each cast at a different pH and with a different acrylamide concentration (pH 6.8 and 4% acrylamide for the stacking gel; pH 8.8 and between 6 and 18% acrylamide for the running gel). The running gel is buffered with Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol) by adjusting it to pH 8.8 with HCl (375 $\mathrm{mmol\,l^{-1}}$ Tris, 0.1% SDS). The stacking gel is also buffered with Tris but adjusted to pH 6.8 with HCl. The sample buffer is adjusted to pH 6.8 with Tris–HCl (125 $\mathrm{mmol\,l^{-1}}$ Tris, 0.1% SDS). The electrode buffer is also buffered with Tris, but here, the pH is adjusted to a few tenths of a unit below that of the running gel (in this case 8.3) using only glycine (usually 25 $\mathrm{mmol\,l^{-1}}$ Tris, 192 $\mathrm{mmol\,l^{-1}}$ glycine, 0.1% SDS).

As electrophoresis begins, both $\mathrm{Cl^-}$ and glycinate ions begin to migrate through the stacking gel. Because the pH is several points lower in the stacking gel than the $\mathrm{p}K_{\mathrm{a2}}$ of glycine, the vast majority of glycine molecules are zwitterionic at any moment and their mobility is very low. Because the mobility of the chloride ions is greater than the mobility of glycine, the chloride ions (leading ions) begin to migrate away from the glycine (trailing ions). The chloride ions do not move far before they leave behind an area of unbalanced positive counter-ions. A steep voltage gradient develops, the Kohlrausch discontinuity, which pulls the glycine along so that the chloride and glycine ions become successive fronts moving at the same speed. The ion fronts sweep through the sample molecules. The sample molecules, being of intermediate mobility between that of chloride and glycine, are carried along, becoming stacked into very thin distinct layers in the order of electrophoretic mobility.

When the interface between the stacking and separation gels is reached by the moving boundary region, the pH changes abruptly (as well as the pore size). At the higher pH, a much higher percentage of glycine will be in the ionized state, and its mobility will be increased commensurately. The mobility of sample molecules is decreased by the sieving of the new, higher percentage matrix. The glycine accelerates past the stacked layers of sample molecules and the process of unstacking in the separating gel begins. From this point on, the electrophoretic separation occurs under conditions of constant pH and voltage that are indistinguishable from homogeneous PAGE.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative

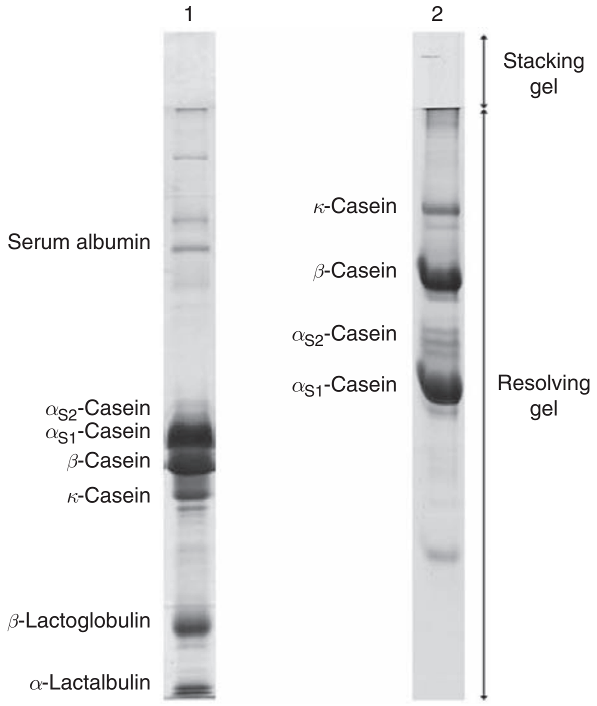


Figure 1 Glycine–SDS-PAGE and alkaline urea-PAGE (F Chevalier and AL Kelly, unpublished data). (1) Glycine–SDS-PAGE of bovine skim milk (6% acrylamide in the stacking gel and 12% acrylamide in the resolving gel); (2) alkaline urea-PAGE of bovine skim milk (6% acrylamide in the stacking gel and 12% acrylamide in the resolving gel).

abundance of major proteins in a sample. SDS-PAGE is not widely used for analysis of the caseins since they present a similar migration and thus are not as well resolved by SDS-PAGE as by alkaline urea-PAGE (**Figure 1**). β-Casein has a higher molecular mass than α_{S1}-casein, and therefore would be expected to have a lower mobility; however, in SDS-PAGE electrophoretograms of the caseins, β-casein has a higher mobility than α_{S1}-casein, as it binds more SDS and therefore acquires a higher net negative charge.

SDS-PAGE is widely used for the analysis of whey proteins as they have a much broader molecular weight range. The major whey proteins, α-lactalbumin, β-lactoglobulin, and bovine serum albumin, with molecular masses ~14.2, 18.3, and 66.3 kDa, respectively, are resolved well using SDS-PAGE.

Tris/Tricine–SDS-PAGE

Tricine is a commonly used electrophoresis buffer and is also used for the resuspension of cell pellets. It has a lower negative charge than glycine, allowing it to migrate faster. In addition, its high ionic strength causes more ion movement and less protein movement. This allows for low molecular weight proteins to be separated in lower percent acrylamide gels.

A discontinuous SDS-PAGE system for the separation of proteins in the range from 1 to 100 kDa was described by Schagger and von Jagow in 1987. Tricine, used as the trailing ion, allows a resolution of small proteins at lower acrylamide concentrations than in glycine–SDS-PAGE systems. A superior resolution of proteins, especially in the range between 5 and 20 kDa, is achieved without the need to use urea. Proteins above 30 kDa are already destacked within the sample gel. Thus, a smooth passage of these proteins from sample to separating gel is warranted and overloading effects are reduced. This is of special importance when large amounts of protein are to be loaded onto preparative gels. The omission of glycine and urea prevents disturbances that might occur in the course of subsequent amino acid sequencing.

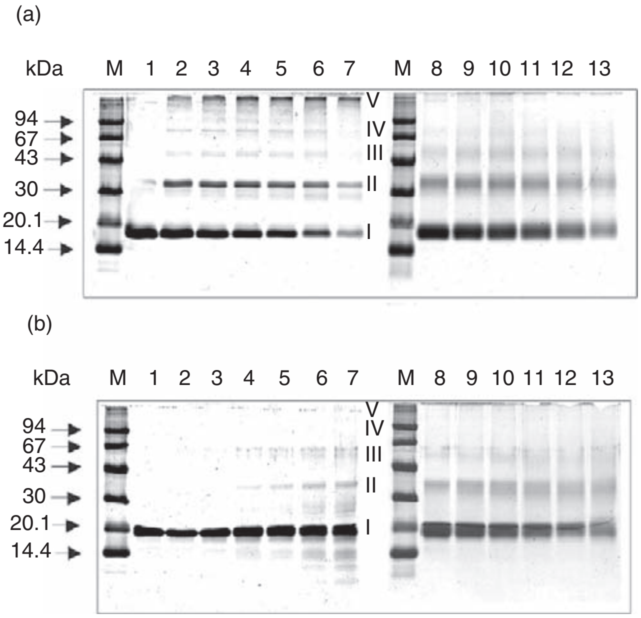
Practically, two electrode buffers are prepared. The cathode buffer (pH ~8.25) is composed of 0.1 mol l^{-1} Tris, 0.1 mol l^{-1} tricine, and 0.1% SDS (no pH adjustment needed). The anode buffer contains only 0.2 mol l^{-1} Tris–HCl at pH 8.9. The sample buffer is the same as with the Tris/glycine–SDS-PAGE protocol. In contrast, tricine and glycerol are added in the gel buffer. Tricine–SDS-PAGE was used to analyze β-lactoglobulin glycated with glucose or heated at 60 °C with or without a reducing agent (**Figure 2**).

Native PAGE

'Native' gel electrophoresis is run in the absence of SDS. In SDS-PAGE, the electrophoretic mobility of proteins depends primarily on their molecular mass, whereas in native PAGE, the mobility depends on both the protein's charge and its hydrodynamic size. The electric charge driving the electrophoresis is governed by the intrinsic charge on the protein at the pH of the running buffer. This charge will, of course, depend on the amino acid composition of the protein as well as post-translational modifications.

Thus, native gels can be sensitive to any process that alters either the charge or the conformation of a protein. This makes them excellent tools for detecting events such as changes in charge due to chemical degradation (e.g., deamidation), unfolded, 'molten globule', or other modified conformations, oligomers and aggregates (both covalent and noncovalent), and binding events (protein–protein or protein–ligand).

This method of gel electrophoresis allows one to separate native proteins according to differences in their charge density. The buffer in the gel is suitable for maintaining the protein in its native state. Thus, for enzymes, their activity can be assayed after the electrophoretic separation. All proteins present in the gel can be visualized using a general protein stain. By comparison of identical gels stained specifically for the enzyme of interest and gels stained by the general protein stain, one can evaluate the purity of an enzyme preparation. The evaluation can be quantitative by comparing relative


(a)
kDa
M 1 2 3 4 5 6 7 M 8 9 10 11 12 13
94
67
43
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20.1
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IV
III
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(b)
kDa
M 1 2 3 4 5 6 7 M 8 9 10 11 12 13
94
67
43
30
20.1
14.4
V
IV
III
II
I

Figure 2 Tricine–SDS-PAGE of heated and glycated β-lactoglobulin. Reproduced from Chevalier F, Chobert J-M, Dalgalarrondo M, and Haertlé T (2001) Characterization of the Maillard reactions product of β-lactoglobulin glucosylated in mild conditions. *Journal of Food Biochemistry* 25: 33–55. (a) Without β-mercaptoethanol; (b) with β-mercaptoethanol. M, molecular mass markers. (1–7) β-Lactoglobulin heated without sugar for 0, 24, 48, 72, 96, 168, and 264 h, respectively; (8–13) β-lactoglobulin heated in the presence of sugar for 24, 48, 72, 96, 168, and 264 h, respectively; (I) monomeric β-lactoglobulin; (II) dimeric β-lactoglobulin; (III) trimeric β-lactoglobulin; (IV) tetrameric β-lactoglobulin; (V) polymeric β-lactoglobulin.

mobilities of the enzyme-stained protein band and the protein-stained protein bands. Relative mobility is defined as the distance moved by the protein band of interest compared to the distance moved by the dye front (a low molecular weight dye that is highly charged is used to mark the electrophoretic front).

Urea-PAGE

In urea-PAGE, urea partially unfolds proteins during the solubilization process, and hence it is not a native PAGE. Urea-PAGE is mostly used only for the separation of caseins. The use of urea gives good solubilization of the hydrophobic proteins of the casein micelle. Caseins are then resolved according to the charges, and well-separated bands of α_{S1}-, β-, and κ-caseins can be observed (**Figure 1**). α_{S2}-Casein is obtained as several distinct bands between α_{S1}- and β-caseins, owing to its multiple phosphorylation status. A number of urea-containing buffer systems have been used, including Tris–HCl/glycine and Tris–ethylenediaminetetraacetic acid (EDTA)–borate buffers at acid pH, but the most common technique uses urea-containing buffers at alkaline pH, for example, Tris–glycine (pH 8.9) containing 6 mol l^{-1} urea.

Isoelectric Focusing

Isoelectric focusing (IEF) is a powerful electrophoretic technique for resolving proteins and peptides. Analytes migrate through a gel containing a pH gradient and cease to migrate through the gel at a pH value corresponding to their isoelectric point, since they have no net electrophoretic mobility at this pH. To improve protein solubilization during IEF, proteins are usually partially unfolded with urea and a reducing agent (dithiothreitol (DTT)). For more detailed information about IEF principles and chemicals, *see* **Milk Proteins**: Proteomics.

Thus, IEF separates based on differences in isoelectric point and it has been particularly valuable in separating genetic variants of milk proteins. Other applications of IEF in protein biochemistry include comparison of glycated protein isoforms (**Figure 3**).

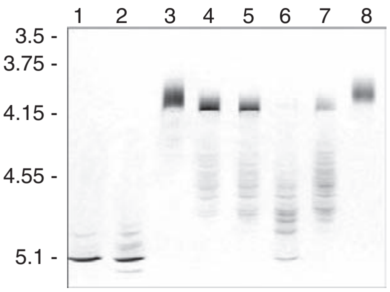

Figure 3 Isoelectrofocusing of heated and glycated β-lactoglobulin. Reproduced from Chevalier F, Chobert J-M, Choiset Y, Dalgalarrondo M, and Haertlé T (2002) Maillard glycation of ß-lactoglobulin induces conformation changes. *Nahrung* 46: 58–63. Isoelectric focusing of (1) native β-lactoglobulin, (2) β-lactoglobulin heated without sugar, and (3–8) β-lactoglobulin heated in the presence of arabinose, galactose, glucose, lactose, rhamnose, and ribose, respectively.

Blue Native Electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a native electrophoresis method that can be used for molecular weight determination of most soluble protein complexes as well as most membrane proteins. Subsequent SDS-PAGE can be used in a second dimension to resolve the complexes into their subunits.

Most, if not all, proteins require binding to other proteins to function in a regulated manner. These regulatory and functional interactions result in the formation of multiprotein complexes (MPCs). The Coomassie blue dye, which binds non-specifically to all proteins and is itself negatively charged, is used in BN-PAGE. Therefore, the electrophoretic mobility of an MPC is determined by the negative charge of the bound Coomassie blue dye and the size and shape of the complex. Coomassie blue does not act as a detergent, and it preserves the structure of MPCs. In contrast to other native gel electrophoresis systems, MPCs are separated independent of their isoelectric point and, therefore, the size of the MPCs can be estimated. In addition, the binding of Coomassie blue to proteins reduces their tendency to aggregate during the stacking step of the electrophoresis process.

BN-PAGE is advantageous for the study of MPCs in that it can provide information about the size, number, protein composition, stoichiometry, or relative abundance of MPCs. BN-PAGE was described originally by Schägger and von Jagow as a technique for the separation of enzymatically active membrane protein complexes under mild conditions. Until now, this technique has been used rarely for milk and dairy product analysis. But due to its potential for analysis of complexes, it can be, for example, a very useful strategy for the analysis of micellar and whey protein interactions.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2-DE) starts with mono-dimensional electrophoresis (separation of native proteins or complexes according to charge) and then separates the proteins by a second property (usually according to the mass by SDS-PAGE) in a direction 90° to the first.

The result is that the molecules are spread out across a 2-D gel. The gel is stained with Coomassie blue or silver stain to visualize the proteins. Spots on the gel are proteins that have migrated to specific locations. Because it is unlikely that two proteins will be similar in two distinct properties, molecules are separated more effectively by 2-DE than by 1-D electrophoresis.

IEF/SDS-PAGE

Based on two independent biochemical characteristics of proteins, IEF/SDS-PAGE combines IEF, which separates proteins according to their isoelectric point, and SDS-PAGE, which separates them further according to their molecular mass.

IEF/SDS-PAGE has been used, for example, to separate milk proteins, to detect proteolysis products in cheese and milk, to analyze post-translational modification of κ-casein, and to investigate milk fat globule membrane proteins. IEF/SDS-PAGE is now a robust and reproductive technique, widely used in biochemistry for the analysis and identification of proteins. For more detailed information about IEF/SDS-PAGE 2-DE, *see* **Milk Proteins**: Proteomics.

Other 2-DE strategies

Proteins and MPCs are separated under native conditions in a first-dimension BN-PAGE. For 2-D BN-PAGE and SDS-PAGE, the proteins and/or MPCs are denatured by SDS in the gel strip after they are separated by BN-PAGE and then applied to a second-dimension SDS-PAGE gel. The hyperbolic shape of the diagonal in the 2-D gel is due to a gradient gel in the first dimension and a linear gel in the second dimension. Monomeric proteins are located on the diagonal and the components of MPCs, below the diagonal.

Urea-PAGE/SDS-PAGE was used to explore the differences in the irreversible disulfide bond changes among the milk proteins after heat and pressure treatments of milk.

Two-dimensional SDS-PAGE or IEF/SDS-PAGE techniques under various non-reducing and reducing conditions have been used to study disulfide interactions of milk proteins after heat treatment.

Free-Flow Electrophoresis

Free-flow electrophoresis (FFE) is a highly versatile technology for the separation of a wide variety of charged analytes like peptides, proteins, or protein complexes, under native or denaturing conditions.

FFE separates charged particles ranging in size from molecular to cellular dimensions according to their electrophoretic mobilities or isoelectric points. Samples are injected continuously into a thin buffer film, which may be segmented or uniform, flowing through a chamber formed by two narrowly spaced glass plates. Perpendicular to the electrolyte and sample flow, current may be applied while the fluid is flowing (continuous FFE) or while the fluid flow is transiently stopped (interval FFE). In any case, the applied electric field leads to movement of charged sample components toward the respective counter-electrode according to their electrophoretic mobilities or isoelectric points. The sample and the electrolyte used for the separation enter the separation chamber at one end and the electrolyte containing different sample components as separated bands is fractionated at the other side.

Capillary Electrophoresis

Capillary electrophoresis (CE) encompasses a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size, or hydrophobicity.

During electrophoresis, one end of the capillary is placed in a buffer reservoir containing the anode and the other in a reservoir containing the cathode. To load the column, the positive end of the capillary is moved to a sample reservoir for the seconds needed to inject the sample and then returned to the positively charged reservoir before the electric field is applied across the column (**Figure 4**). A window is made at the end of the capillary by burning the outer protective coating of the capillary to allow optical detection directly through the capillary wall. The detection technique used most commonly is ultraviolet (UV) absorption for proteins and peptides; absorption of visible light, as well as fluorescence detection, may also be used for other applications. Online mass spectrometry (MS) detectors have recently become available for CE, and methods for qualitative analysis of several compounds that should or should not be present in milk and dairy products are to be expected. Promising examples are control methods to reveal the use of genetically modified organisms, false labeling, and environmental contaminants.

CE has great potential for the resolution of complex mixtures of peptides. It has a number of advantages over traditional electrophoretic techniques, including the choice of running buffer and the use of automated, high--performance instrumentation. The composition of running buffers can be changed easily and separation times are relatively short, although only one sample can be analyzed at a time. CE has been used successfully to study the hydrolysis of caseins by chymosin or plasmin, for example, in cheese during ripening. Plasmin activity is seen as an increase in the γ-caseins (present at low levels in fresh cheese), with a simultaneous decrease in the amount of β-casein.

CE may also be used to estimate the amount of whey proteins in heated milk that are heat denatured and coprecipitate with the caseins at pH 4.6. Other applications include determination of the effect of high-pressure treatment of milk, and enzymatic modification of whey proteins to improve functionality. CE has been used to analyze slimmed milk powder and to monitor the lactosylation of β-lactoglobulin that occurs by condensation of lactose with protein-bound amino groups through the Maillard reaction during heating (**Figure 5**).

Other analytical applications in dairy chemistry have included ion analysis in milk, milk powders, and cheese, measurement of hippuric and orotic acids in whey, fractionation of whey proteins, measurement of sorbate and benzoate in cheese slices and dips, determination of adulteration of ovine and caprine milk with cow's milk, detection of biogenic amines in dairy products, quantification of cholesterol in foods, determination of free amino acids in infant formulae, examination of peptide uptake by dairy starter bacteria, and detection of chloramphenicol in milk.

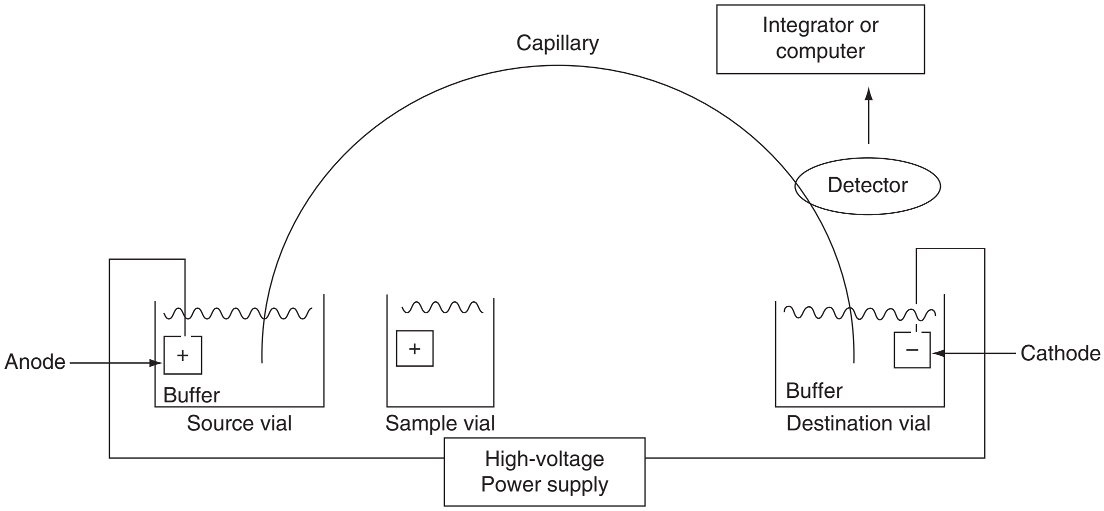

Figure 4 Principle of capillary electrophoresis.

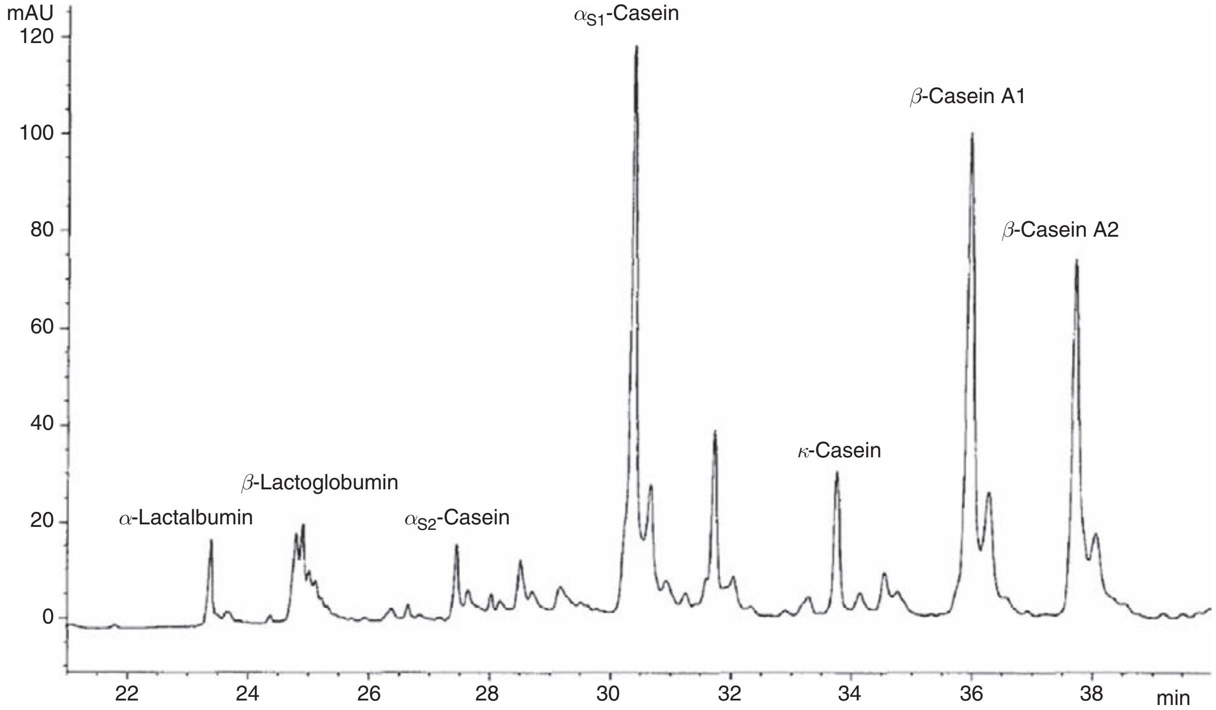

Figure 5 Capillary electrophoresis of skimmed milk powder. Reproduced from Jones AD, Tier CM, and Wilkins JP (1998) Analysis of the Maillard reaction products of beta-lactoglobulin and lactose in skimmed milk powder by capillary electrophoresis and electrospray mass spectrometry. *Journal of Chromatography A* 822: 147–154.

Chip-Based Electrophoresis

Using a combination of microfluidics and electrophoresis, chip-based electrophoresis systems enable sensitive, high-resolution separation and data analysis of protein samples. The term 'microfluidics' refers to the transport and manipulation of very small volumes of liquid. Generally, microfluidics includes not only miniaturization of the channels through which the liquid flows, but also the movement and interaction of materials of interest – dissolved or suspended in the liquid – by fluid dynamic, electrophoretic, and chemical principles on a small scale. Microfluidic separation technology brings the advantages of miniaturization and automation to a variety of different chemical and biochemical analyses. These analyses benefit from dramatically reduced time to obtain results, reduced consumption of reagents and samples, and minimization of user intervention.

Miniaturization of analytical instrumentation has many advantages over conventional techniques. These advantages include improved data precision and reproducibility, short analysis times, minimal sample consumption, improved automation, and integration of complex workflows.

This system offers a number of advantages over traditional gel electrophoresis, such as faster results, reduced sample and reagent usage, automatic calculations, reduced hands-on time, and elimination of the challenges of having to work with hazardous materials typically associated with these applications. For example, the procedure – from sample preparation through the chip run – can be accomplished in as little as 30 min compared to the 2–3 h generally required for SDS-PAGE.

See also: **Analytical Methods**: Immunochemical Methods. **Heat Treatment of Milk**: Heat Stability of Milk. **Lactose and Oligosaccharides**: Maillard Reaction. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Protein. **Milk**: Bovine Milk. **Milk Proteins**: Analytical Methods; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; Proteomics; α-Lactalbumin; β-Lactoglobulin.

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Electrochemical Analysis

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Introduction

Electrochemical analysis can be subdivided into several categories: potentiometric, voltammetric, coulometric, conductimetric, and capacitance. For dairy analysis, some of these techniques are more important than others. Given the complexity of dairy products, direct electrochemical analysis is rarely possible. Many of the techniques employed, therefore, have been combined with instrumental separation systems or rely on rigorous pretreatment prior to analysis. Despite these drawbacks, electrochemical analysis is still widely practiced in the analysis of food products.

Basic Electrochemistry

Potentiometric Analysis

Potentiometric measurements involve zero applied current. The analyte is detected by the accumulation of charge density at the electrode surface. The Nernst equation relates the potential to analyte concentration:

$$E = E^{\circ}([\mathrm{Ox}] - [\mathrm{Red}]) - \frac{RT}{nF}\ln\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) \qquad [1]$$

where E is the potential measured, E° is the formal reduction potential of the system (characteristic of each redox couple), R is the gas constant, T is the absolute temperature, F is Faraday's constant, n is the number of moles of electrons transferred in the reaction, and [Red] and [Ox] represent the reduced and oxidized species, respectively.

The interaction between the analyte and the electrode produces a potential difference relative to the reference electrode. Potentiometric analysis is an attractive method, as the analyte is not consumed. Ion-selective electrodes, such as the electrodes for the determination of pH, calcium, potassium, and fluoride, are potentiometric.

Voltammetric Analysis

Voltammetry comprises a group of widely used electrochemical techniques, where the current flowing through the working electrode cell is measured as a function of the applied potential. The voltammetric cell is composed of two or three electrodes immersed in an analyte solution. The solution usually contains inert ionic species to ensure electrical contact between the electrodes and decrease the effects of migration of other ions to the electrodes.

Polarography

The first electroanalytical technique, polarography, is based on the measurement of the diffusion-controlled current flowing in an electrolytic cell in which the working electrode is the dropping mercury electrode. The current is directly proportional to the concentration of the electroactive species, and the potential of reduction ($E_{1/2}$) is characteristic of the species. The term polarography is used when a dropping mercury electrode is the working electrode, generally for reduction. As many foods have electroactive components, this technique is widely applicable. Metals such as copper, lead, tin, and zinc and antioxidants such as vitamin C can be measured using polarography in foodstuffs. However, the use of mercury in this method has limited its development within the food industry. Polarography has been replaced by differential pulse anodic stripping voltammetry (DPASV) on solid electrodes with a mercury film. The latter method is more sensitive, does not require liquid mercury, and can easily be miniaturized.

Cyclic Voltammetry

Cyclic voltammetry (CV) is based on a potential sweep at a certain rate (scan rate). CV is a powerful qualitative technique used mostly for studying electrochemical reactions and electrodynamic properties of chemical compounds, but has no value in quantitative analysis. The initial applied potential is in the region where no reaction is occurring and is then increased in a linear way into a region where the reaction is occurring; once the potential reaches a certain value, it is switched and swept back to its initial potential. Multiple cycles are usually recorded. As the potential approaches the oxidation/reduction potential of the electroactive species, the anodic/cathodic current increases until a plateau is reached. The plateau is caused by concentration polarization at the electrode. This means that the concentration of the analyte is depleted near the surface of the working electrode, and diffusion can only replace it at a certain rate. CV can be performed only in a quiescent solution for this reason. The ratio of the anodic and cathodic peak heights gives information as to whether the electrochemical reaction of the species is reversible or not, that is, whether the oxidized species can be subsequently reduced. If the peaks are equal in height, then all the oxidized species have been reduced and vice versa. To check the system, E_0 can be calculated from the equation

$$E_0 = (E_{\mathrm{pa}} + E_{\mathrm{pc}})/2 \qquad [2]$$

The peak height of the couple is described by the Randles–Sevcik equation:

$$i_{\mathrm{p}} = \left(2.69 \times 10^5\right) n^{3/2} A D^{1/2} C v^{1/2} \qquad [3]$$

where i_{p} is the peak current, A is the area of the electrode, D is the diffusion coefficient of the analyte, C is the bulk concentration of the analyte, and v is the scan rate.

Amperometric Analysis

In amperometric analysis, a constant potential is applied and the current generated due to an oxidation or reduction reaction of the analyte is measured. This current is proportional to the concentration of the analyte of interest. This process consumes the analyte. Amperometric detectors are commonly used because of their high reliability and sensitivity. The commonly used setup of amperometry is the hydrodynamic amperometric detector in flow injection analysis (FIA) or liquid chromatography (LC). The relatively low selectivity allows the detector to be group specific.

Conductometric Analysis

Conductometric detectors measure the overall conductance of a sample. Conductance is the movement of ions in solution in an electric field and is the inverse of resistance. The practical setup relies on applying an AC voltage between two platinum electrodes and measuring the current. The conductometric cell is usually calibrated with a solution of KCl. Many reactions produce or consume ionic species, which then changes the resistance of the solution. Strong electrolytes (salts) increase the conductivity of the solution, whereas the conductivity is reduced by the presence of dissolved nonionic species (sugars, polyols, etc.). Conductometry is not a very specific method, as it will detect the sum of all ions. Immobilized salt sensors can be used to determine the water activity of canned foods, though the method is sensitive to polyols and volatile amines. Water activity, a_{w}, can be defined as the ratio of the vapor pressure of water in the product to that of pure water. Conductivity can also be determined on maple syrup, a key factor in its purity, as it naturally contains minerals. As no reagents are required for conductivity measurements, and the cell can be made of platinum electrodes and glass, probes for conductivity measurements can be dipped directly into the sample or used as an in-line analyzer.

Applications of Electrochemistry in Dairy Analysis

Karl Fischer Titrations

Karl Fischer titration is the classical method for the determination of water in low-water foods, such as dried milk, where evaporation techniques give inaccurate or inconsistent results. This method is unsuitable for foods containing a large percentage of water. The reactions occurring within the cell can be summarized as follows:

$$\begin{aligned} &\mathrm{H_2O + I_2 + 2RN + [RNH]SO_3CH_3} \\ &\quad \rightarrow \mathrm{2[RNH]I + [RNH]SO_4CH_3} \end{aligned} \qquad [\mathrm{I}]$$

A modification of this method introduces electrochemically generated I_2 in the reaction vessel. Based on the reaction scheme (eqn [I]), there is a stoichiometric relationship between the amount of iodine used and the amount of water in the sample. The end point is then detected potentiometrically. The electromotive force (EMF) of the cell rises dramatically as unreacted iodine appears, that is, when all of the water has been reacted. The quantity of water in the sample is then determined by calculating the charge passed to produce the I_2, and, in turn, the amount of I_2 used.

Examples of other foods the water content of which is regularly analyzed by the Karl Fischer method include oils, roasted coffee, and dried fruit and vegetables.

Coulometric Titration for Salt

This method is similar in theory to the Karl Fischer titration, where a reactive species is coulometrically generated (silver ions), which proceeds to react with any available chloride to form insoluble silver chloride. When all the available chloride ions have reacted, the conductance of the solution rises rapidly, thus providing an end point. The charge passed, in producing sufficient silver ions to react with chloride ions, is stoichiometrically related to the concentration of salt in the test material.

Ion-Selective Electrodes

Ion-selective electrodes (ISEs) are potentiometric electrodes that measure the activity of ions in solution. Activity (a) differs from concentration (C) by the activity coefficient (γ), especially at higher concentrations, as shown in the following equation:

$$a = \gamma C \qquad [4]$$

ISEs can be classified into several subgroups: (1) glass membrane electrodes, (2) solid-state membrane electrodes, (3) liquid membrane electrodes, and (4) gas-sensing electrodes, all of which share some common features of construction. A high-input impedance voltmeter is connected to a probe; the body of the probe is an insulated glass or plastic tube sealed with an ion-specific membrane. The tube contains a solution of the ion to be measured, and electrical contact is made via a silver/silver chloride reference electrode. The ionic activity of the analyte influences the membrane potential, which can be approximated by the Nernst equation (eqn [1]). ISEs must be calibrated with solutions of known concentration.

Glass Electrodes

This is the standard pH electrode. Acidity of samples can be measured in a variety of ways, but in most cases the use of pH electrode serves as the quickest and simplest method. It can be described as a potentiometric probe coated with a proton-permeable layer. In the most common form, the membrane is made of permeable soda glass and the internal solution is dilute HCl. The glass electrode then develops an electrical potential in response to the activity of the H^+ in the sample solution. The ionic activity of the analyte is related to this potential by the Nernst equation (eqn [1]). The pH electrode must be calibrated with solutions of known pH. The frequent calibrations necessary for this type of electrode are either a result of changes in the glass membrane with use or due to contamination of the glass membrane. The glass electrode is one of the most commonly used electrochemical analyzers. Owing to its ease of use, it has superseded titrimetry in most non-dairy analyses, though both methods are used in concert within the dairy industry. The pH electrode can also be used as a detector for titratable acidity, when the traditional colorimetric endpoint determination is not suitable. Though the pH measurements of milk are relatively easy, analysis of solid dairy products such as cheese can be more difficult, as a small amount of pretreatment is necessary. Usually the cheese is finely minced for analysis, as dilution with water can alter the pH. For very soft cheeses, it may not be necessary to pretreat. Another key application of the pH electrode in the dairy industry is in the analysis of wastewater. Cleaning frequently involves both acid and alkali detergents, which must be neutralized prior to the disposal of wastewater. The sodium ISE is another common glass electrode. Different cheeses have widely differing concentrations of salt, which contributes to their unique flavors. This requires that the salt content be monitored. The use of an ISE is easier than the Volhard test, though it still requires that the cheese be dissolved in a buffer prior to analysis.

Solid-State Electrodes

Solid-state membrane electrodes contain an insoluble crystalline material in equilibrium with the analyte of interest. The membrane potential is measured. In the case of the fluoride ISE, the migrating ion is fluoride within a crystal of lanthanum fluoride. However, most other ISEs use silver sulfide as the membrane. In the case of fluoride, total ionic strength adjustment buffer (TISAB) can be used to minimize complexation of the fluoride to polyvalent cations.

Liquid Membrane Electrodes

Modern versions of liquid membrane electrodes behave very much like solid-state electrodes. A PVC-gelled ion exchanger is used as the membrane, and the extent of ion exchange determines the membrane potential. A good example of this is the calcium electrode, which consists of the calcium salt of dialkyl phosphate and a calcium chloride internal solution. Another one is the iodide ISE. Both of these can be used to analyze milk and other dairy products.

Gas-Sensing Electrodes

In the case of gas-sensing probes, a pH probe is sealed inside a microporous hydrophobic film, through which only gases may permeate, typically polytetrafluoroethylene. A pH meter detects the change in pH, caused by the diffusion of the gas. The sulfur dioxide probe is a good example of this type of electrode. It is employed to determine the sulfur dioxide content in liquid foods. Normally, sulfur dioxide is not found in dairy products. However, it is used as a preservative in pulped fruits, and can thus be found in flavored yogurts. More recent advances in semiconductor technology have resulted in the ion-sensitive field-effect transistor (ISFET). The miniaturization of this device and its low cost of manufacture make it an attractive option in the market.

Electrophoresis

Electrophoresis is an electrochemical separation technique commonly used for dairy products to separate caseins and whey proteins. It is widely used in research laboratories throughout the entire food industry. It is based on the principle that ions are attracted to the electrode of opposite charge when situated within an electrical field. As different molecules may have different charges, a mixture will separate out according to the charge and mass of the molecules. Other factors affecting the separation are pH and ionic strength of the analyte.

A modification of this technique is sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Sodium dodecyl sulfate is bound to proteins, which results in proteins having a large negative charge. Their movement is now limited only by their mass, which results in a mass separation of the protein constituents. Urea–PAGE is also used to separate caseins. Another technique gaining wide appeal is capillary electrophoresis (CE). Small samples can be loaded onto the capillary and a very high voltage applied across it, resulting in very rapid separation (*see* **Analytical Methods**: Electrophoresis).

Biosensors

Biosensors have several advantages over traditional analytical techniques in the area of food analysis. Owing to the complex nature of most food products, a separation technique is usually required before analysis by traditional techniques. Biosensors can frequently be used without pretreatment of the product. As the biosensor systems can be small and of low cost relative to other automated analyzers, they can easily be fitted on-site. Biosensors are composed of a biorecognition agent and a transducer. This transducer is commonly electrochemical, optical, or piezoelectrical.

The use of biosensors in foods can be subdivided into two groups: (1) enzyme sensors for food components and (2) immunosensors for pathogenic bacteria or pesticides in food. Within the first group, glucose is the most common analyte. The main reasons for this are the high stability of glucose oxidase (EC 1.1.3.4) and the applicability of these sensors in the commercially lucrative blood glucose measurement. Despite the primary focus of this research being on the blood glucose market, the vast knowledge gained in the area has led to the development of several glucose sensors for food. Other food analytes detected by amperometric biosensors include L-lysine, malate, ethanol, ascorbate, and fructose. In the field of biosensor analysis of dairy products, lactose and lactate are probably the analytes of greatest interest. Lactate is by far the most widely researched, probably owing to the availability of lactate dehydrogenase (EC 1.1.1.27) and more recently lactate oxidase. The wide use of these biosensors was probably limited by the reversibility of the lactate dehydrogenase reaction, depending on pH. Lactate oxidase (EC 1.1.3.2) catalyzes an irreversible reaction, so may prove to be a much more suitable biocomponent in future research. Lactose determination by biosensors usually involves an enzyme-catalyzed sequence of reactions with glucose oxidase and β-galactosidase (EC 3.2.1.23).

Immunosensors, in the field of dairy analysis, are less common than enzyme sensors, as many of the important analytes occur in too small a concentration to produce an immune response. Their main applications in foods are to detect pathogenic bacteria such as *Listeria monocytogenes*, staphylococcal enterotoxin A, and pesticides. Owing to the small size of pesticides, the antibodies are raised against pesticides conjugated to immunogenic carriers. This increases the difficulty in producing immunosensors as many of the antibodies are against the carrier, not the hapten, and conjugation itself may produce a structural change in the hapten, making it immunologically different from the free species.

In this area, electrochemical transducers are less popular than optical detection. Amperometry and chronoamperometry are the main electrochemical transduction techniques employed in biosensors. The potential is selected to cause either oxidation or reduction of the analyte. When the analyte is introduced to the system, the current responds rapidly. In the case of amperometry, the current reaches a steady state, where the rate at which the analyte is diffusing to the electrode surface is the same as the rate at which it is being consumed at the electrode. In chronoamperometry, no such steady state is reached, as the analyte is consumed at the electrode, and no convection is employed to replenish it. An advantage of amperometry is that the current is effectively independent of time; thus, the operator can wait until the response becomes independent of charging currents or matrix effects. The advantage of chronoamperometry is that it does not require a stirred solution, and thus, the entire analysis can be performed within a membrane on the electrode surface, making the equipment small, cheap, and highly portable (*see* **Analytical Methods**: Biosensors).

Anodic Stripping Voltammetry

As the name suggests, anodic stripping voltammetry (ASV) is a voltammetric method. Its primary use in foods is to determine trace metal concentrations. This method is used where atomic absorption spectroscopy (AAS) is not suitable, for example, for cadmium and lead. Typically, solid electrodes, such as glassy carbon, are used for ASV. The food sample must be digested using microwave digestion prior to analysis. DPASV is generally regarded as having limits of detection comparable with those of AAS. Even though it is not applicable to oils and fats, it can be used for fat-containing foods, such as evaporated milk.

Polarography

Polarography, as described in the section 'Voltammetric Analysis', can be used to determine the concentration of fumaric acid in foods. Fumaric acid is an additive used to control pH in fruit juices, though it is produced to a small extent in fermentation processes.

Organophosphorus pesticide residues including malathion, diazinon, methyl parathion, and parathion can be determined using single-sweep oscillographic polarography. Peroxides can strongly interfere with this analysis, and thus may have to be tested for separately.

As with DPASV, polarography can also be used to determine the lead content of foods, most commonly of fish. Tin can interfere with this analysis; however, with the addition of chelators to the analyte, this can be overcome.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a widely used, highly developed analytical technique for separation of analytes from excipients in a complex mixture. The basic theory is the partitioning of the analyte across two different phases, one mobile and the other stationary. This will result in different chemicals being retained on the column for different lengths of time. This, however, is only half of the analysis; detection of the separated materials still remains to be done. Over the 40 years since its development, it has been coupled with a wide variety of detectors, the most common ones being UV, fluorescent, and electrochemical. The electrochemical HPLC detectors fall into two categories: conductance and amperometric. Conductivity detectors are used for ionic materials, whereas amperometric detectors can be used for oxidizable or reducible substances. Amperometric detectors are used, as they can have much lower detection limits than UV detectors, and the cells can be produced to have a very small volume, some down to the submicroliter range. In milk analysis, conductance detectors have been used to detect ammonium ions, whereas amperometric detectors have been used for a variety of electroactive vitamins such as vitamins A and D.

See also: **Analytical Methods**: Biosensors; Electrophoresis. **Milk Proteins**: Analytical Methods.

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Mass Spectrometric Methods

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Introduction

Mass spectrometry analysis alone or coupled to either gas chromatography (GC) or liquid chromatography (LC), with their characteristic high sensitivity and specificity, is becoming increasingly popular for determining lipid, protein, vitamin, and other trace elements.

Recently, new strategies for the analysis of milk proteins based on mass spectrometric methodologies have been developed. These methods, leading to the identification of most of the proteins present in milk samples, have successfully been used to set up some rapid and convenient protocols aimed at monitoring structural damage caused by industrial thermal processes, such as pasteurization and sterilization, and to reveal differences in the protein patterns of milk samples obtained from various mammalian species.

In this article, the contribution and potential of mass spectrometry-based techniques to characterize milk components in natural milk and in processed milk are reported.

Mass Spectrometry – An Overview

The application of mass spectrometry (MS) to the proteomic analysis of food samples constitutes a particularly important advance in milk protein analysis.

In the past decade, a number of advances in MS have enabled the rapid, comprehensive characterization of milk proteins. Central to these advances was the development of new methods for the ionization of large biomolecules. The 'soft' ionization techniques of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the methods used in most mass spectrometers designed for biological applications.

The coupling of two-dimensional electrophoresis (2DE) with the identification of the separated protein by MS analysis by a 'bottom-up' approach – in which protein identification is achieved after separation by 2DE, protein digestion with trypsin, and analysis by MS – offers a potent strategy for dairy research. The mass fingerprint of the peptides obtained allows comparison of a set of determined molecular mass values of a proteolytic digest against values calculated by theoretical digestion of sequences from a protein database.

Schematic Representation of a Mass Spectrometer

A mass spectrometer can be simplistically described as an ionization source coupled to a mass analyzer. Analyzers used in such devices range from the simpler quadrupole time-of-flight (Q-TOF) analyzers to more complex devices such as Fourier transform mass analyzers. Mass spectrometers have the capacity to generate immense amounts of data pertaining to both the mass and sequence of proteins. Simpler mass spectrometers are capable of delivering only mass-related information (e.g., MALDI-TOF), whereas more complex analyzers, including hybrid analyzers such as the Q-TOF, utilize tandem mass spectrometry (MS/MS) to provide sequence information. Sequence information is most commonly derived from MS/MS, in which a specific ion, selected for by an initial analysis, is fragmented in a subsequent chamber and the fragments are analyzed in a second mass analyzer.

MALDI-TOF MS

In 1988, Karas and Hillenkamp discovered that large protein molecular ions can be produced by laser desorption without much fragmentation when these biomolecules are mixed with a large excess of small organic compounds that serve as matrix for strong absorption of a laser beam. The ultraviolet (UV) laser light is absorbed by the organic aromatic matrix compound that protects the biomolecule. The UV laser energy is high enough to allow direct vaporization of the matrix and carry away the intact biomolecule in the gas phase. The masses of desorbed biomolecular ions are determined by the transit time (the TOF) between desorption and detection. Both linear and reflectron TOF devices have been used widely for biomolecule measurements depending on the signal level and resolution requirements. The typical sample preparation technique for MALDI is to dissolve biomolecular samples in solution and then prepare a matrix solution that contains small organic compounds such as sinapinic acid (SA) or α-cyano-4-hydroxy cinnamic acid (CHCA). These samples and matrix solutions are subsequently mixed, and a small amount of the mixed solution is placed on a metal sample plate to dry and co-crystallize. The sample plate is then placed in the mass spectrometer for analysis. In general, the molar ratio of matrix to biomolecule is more than 1000, depending on the mass of the biomolecule. SA is more often used for large protein detection, and CHCA is often used in small peptide detection. The ions detected are usually singly charged.

ESI MS and HPLC–ESI MS

ESI has been widely used in proteomics and other applications that need the determination of the mass of selected biomolecules. John B. Fenn, Wong's group leader, was awarded the 2002 Nobel Prize for Chemistry for the development of this technique. In ESI, a liquid is pushed through a very small capillary. A high voltage (some kilovolts) is applied between the tip and an extraction plate. This liquid contains the analyte biomolecules dissolved in a selected solvent. The liquid pushes itself out of the capillary by the strong electric field and forms an aerosol with a mist of small droplets. As the solvent evaporates, the biomolecular ions can be produced for mass spectrometric analysis. Biomolecular ions observed are quasimolecular ions created by protonation or deprotonation. Small mass ions are usually singly charged, whereas larger biomolecules are usually multiply charged and exhibit many charge states. The charge number depends on the mass and chemical properties of biomolecules as well as on the solvent used (polarity, acidity, etc.). Therefore, there are often many ion peaks for just one single biomolecule compound. For samples with a pure compound, the pattern of these multiple peaks can help to get very accurate mass determination. For a complex proteomic sample, it can add tremendous complexity for the mass spectrum. Therefore, a preseparation such as a high-performance liquid chromatography (HPLC) is often needed for ESI MS for proteomics application.

ESI is a very soft ionization, and it presents the advantages of high reproducibility; no crystallization process involved; and a high flexibility to attach to different types of mass spectrometer. By adding multiple charges to the analyzed biomolecule, the m/z ratio measured in the mass spectrum is thus lowered, so even high-mass biomolecules (e.g., proteins) can be detected. The ESI source can be fitted to all types of mass analyzer from single quadrupole, ion trap, TOF, tandem, or Fourier-transform mass spectrometers.

Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry (GC–MS) is composed of two major parts: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column (housed in an oven) with specific dimensions and characteristics as well as the phase properties (e.g., 5% phenyl polysiloxane). To inject the molecule into the gas chromatograph, the temperature is increased, thus the molecule needs to be volatile at the injection temperature. The molecules are eluted sequentially from the gas chromatograph by increasing the oven temperature. The differences in the chemical properties between different molecules in a mixture will separate the molecules as the oven temperature increases and the sample travels through the column. At the exit of the column, the separated compounds enter the ionization chamber of the mass spectrometer. The electron impact (EI) ionization at a fixed energy of 70 eV is the main technique and produces fragment ions directly in the source. The mass analyzer then separates the fragments according to their mass. The resulting chromatogram combines data from the retention time on the column and the ion fragments (the intact molecular ion is usually not or barely detected). This information is searched against large databases (e.g., Wiley mass spectral data; NIST mass spectrometry data) containing hundreds of thousands of spectra and compounds. The most common type of mass spectrometer associated with a gas chromatograph is the quadrupole mass spectrometer; other detectors may be encountered such as TOF or tandem quadrupoles (MS/MS).

Strategies of Analysis

Two fundamental strategies for biomolecule identification by MS are used in proteomics. They are classified as bottom-up and top-down approaches. In the bottom-up approach, complex mixtures are subjected to enzymatic or chemical cleavage, and the peptide products are analyzed by MS. For the top-down approach, intact protein ions are subjected to gas-phase fragmentation for mass spectrometric analysis. The top-down approach is often used for molecular imaging.

Bottom-up approach

The bottom-up technology for protein identification is mature and widely used. Many different mass spectrometers are currently used for bottom-up MS/MS analysis, including quadrupole and linear ion traps and tandem TOF (TOF/TOF) mass spectrometers. Reversed-phase (RP)-HPLC provides high-resolution separations of peptide digests suitable for ESI analysis. On-line and fully automatic commercial instruments are available for generating good data for the bottom-up approach. There are a few disadvantages to the bottom-up strategy. Only a small percentage of the total peptides of a given protein are identified. Post-translational modification and proteins from alternative splicing process are difficult to be probed completely by the bottom-up approach.

Top-down approach

In top-down proteomics, intact protein ions generated are subjected to gas-phase fragmentation. The two major advantages of the top-down approach are to study Post translational modifications (PTMs) and elimination of protein digestion. Nevertheless, there are several disadvantages for top-down approaches. Only highly purified protein samples can be used, and large proteins are still not easy to be analyzed routinely, although some recent work has passed 50 000 Da. In addition, bioinformatics for the top-down approach still needs more development. As both approaches have their niches and disadvantages, coexistence of both approaches is expected to last for quite a while until new breakthroughs in terms of time, cost, and mass range can be achieved for the top-down strategy.

Mass Spectrometry Analysis of Milk Protein

Protein Identification of Milk Proteome

Identification of proteins is usually achieved via a four-step process consisting of protein separation, protein digestion, MS analysis of resulting peptides, and comparison of observed peptides with those in a database. Protein digestion can take place either before or after separation. In the case of liquid-phase separation, proteins from sample mixtures are digested prior to separation, whereas in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), an in-gel (or *in situ*) digestion takes place. The ability to obtain a set of peptides from a single protein often allows 2D-PAGE-based techniques to assign an identity purely on the basis of peptide mass fingerprint (PMF) data. Liquid-phase-based identification is typically precluded from identification by PMF due to the presence of high numbers of peptides from multiple proteins, which obscure PMF identification. Instead, MS/MS data from individual peptides are required to achieve identification. MS/MS data can also be used to identify proteins separated by 2D-PAGE when PMF data are inconclusive (see also **Figure 2**).

Milk proteome

Milk from all species is dominated by the presence of just a few primary proteins. In bovine milk, these proteins are α_{s1}-casein (α_{s1}-CN), β-casein (β-CN), κ-casein (κ-CN), α_{s2}-casein (α_{s2}-CN), β-lactoglobulin (β-lg), α-lactalbumin (α-la), and bovine serum albumin (BSA) with relative abundances of approximately 30:30:10:12:10:4:1, respectively. Despite this fact, the milk proteome is still extremely complex. A great deal of this complexity is the consequence of abundant PTMs and the presence of numerous genetic variants of this limited list of proteins. An additional level of complexity arises from the many proteins of low abundance that require some form of enrichment to be visible against the background of abundant casein and whey proteins.

Milk fat globule membrane proteome

The milk fat globule membrane (MFGM) proteins form a unique subcategory of milk proteins (~2–4% of total protein in human milk), the content of which is still largely unknown. Highly hydrophobic in many cases, various MFGM proteins are difficult to solubilize, posing problems for their application to 2D-PAGE. These problems have been largely overcome by the application of various solubilization strategies. The most productive strategies make use of a two-step procedure, with the first step using a detergent/reducing agent solution and the second step an organic solvent extraction. Such strategies not only improve the recovery of MFGM proteins but also prepare the sample for optimum 2D-PAGE analysis. This strategy enhances MFGM protein availability and reduces the amount of several overabundant proteins, including the vast majority of caseins, allowing for more efficient detection of the MFGM proteins. Zwitterionic detergents were shown to be efficient in recovering integral and peripheral proteins from membrane material. Spots were identified by MALDI tandem time-of-flight (MALDI-TOF/TOF). The advantages of MALDI-TOF/TOF (speed, ease of analysis, good sensitivity, and high mass accuracy) were demonstrated in the MFGM proteome investigation. Identified proteins are implicated in a wide range of functions including fat secretion and transport, protein trafficking, and regulation.

Protein Modification

Post-translational modification

Milk proteins are excellent examples of the complex heterogeneity that PTMs can confer on proteins. They display significant levels of phosphorylation and/or glycosylation. In addition, constitutive levels of proteolytic activity produce a range of significant peptides. Combining these three modifications with genetic variation produces the complex milk proteome. These modifications, pertaining primarily but not exclusively to the caseins, have been studied extensively.

Phosphorylation

Phosphorylation is well characterized within the bovine caseins. The vast majority of bovine caseins exist in a phosphorylated form, with individual variants possessing as little as 1 phosphorylated residue (κ-CN) and as much as 13 for others (α_{s2}-CN). Phosphovariants are usually discernible upon 2D-PAGE with noticeable shifts along the horizontal axis corresponding with changes in the proteins' pI.

In 2003, Mamone and colleagues reported a fast and easy-to-use procedure that combines PAGE with MALDI-TOF MS and nanoelectrospray-tandem mass spectrometry (nES-MS/MS) analysis for the identification of casein components and defined phosphorylated sites. This methodology ensured identification of more than 30 phosphorylated proteins, including nonallelic, differently phosphorylated, and glycosylated forms.

Glycosylation

Glycosylation occurs naturally in numerous milk proteins, with examples of both N- and O-linked glycoproteins. N-Linked glycosylation of asparagine residues has been associated with numerous milk proteins. It is particularly prevalent in proteins of the MFGM.

Alternatively, glycosylation can occur at serine and threonine residues through O-linked glycosylation. Such modifications occur prominently within κ-CN, a major protein component of milk. Such sugar moieties are important forces in the stabilization of the casein micelle and hence overall milk protein stability.

In 2000, Moreno and colleagues analyzed ovine casein macropeptide (CMP) by anion-exchange fast protein liquid chromatography (FPLC), RP-HPLC, and on-line or off-line MS. Only 30% of ovine CMP was glycosylated. Assuming that the monosaccharide fraction of ovine CMP is composed of *N*-acetylgalactosamine, galactose, and *N*-glycolylneuraminic acid, molecular masses consistent with the presence of CMP containing tetra-, tri-, di-, and monosaccharides were identified.

In 2003, Mamone and colleagues identified the sugar motif covalently bound to κ-CN as chains, trisaccharide GalNAc, Gal, NeuGc, and tetrasaccharide 1GalNAc, 1Gal, 2NeuGc. Also identified was a biantennary chain made up of both chains of trisaccharide 1GalNAc, 1Gal, 1NeuGc and tetrasaccharide 1GalNAc, 1Gal, 2NeuGc moiety on a single κ-CN component.

In 2004, Holland and Deeth were able to resolve and identify 10 different glycoforms of κ-CN using 2D-PAGE and MALDI analysis. In this study, the loss of sugar units caused by fragmentation during MALDI analysis allowed the assignment of sugar sequences. In addition to κ-CN glycosylation, this study was also able to verify the presence and quantity of phosphorylation upon each of the glycovariants separated by 2D-PAGE.

In 2005, Mollé and colleagues proposed two methods to analyze κ-casein macropeptide. A quantification of both aglyco-casein macropeptide forms A and B (CMPAB) and total CMPAB (including both aglyco and glycosylated forms) in different types of commercial dairy product powders by using online RP-HPLC–ESI MS and/or RP-HPLC–ESI MS/MS was described. Quantitative analyses by MS were based on the detection of specific multiply charged ions from aglyco-CMPA and aglyco-CMPB forms. Due to the high degree of sensitivity and selectivity of MS or MS/MS, it becomes possible to detect characteristic fragment ions of CMP using several scanning modes, allowing their unambiguous identification in complex dairy products.

Identification of Genetic Variants/Polymorphism

In 2002, Roncada and colleagues analyzed the polymorphism of goat α_{s1}-casein using 2DE followed by MALDI-TOF MS.

In 2003, Mamone and colleagues identified 34 casein components of ovine caseins using a combined approach. Samples were analyzed by 2DE, and immunoblotting was subsequently performed to define the different casein families.

In 2000, Trujillo and colleagues successfully resolved the major caprine milk proteins and main casein variants by coupling RP-HPLC and ESI MS.

Ovine casein heterogeneity was analyzed by Ferranti and colleagues in 2001 using on-line LC-ESI MS and allowed determination of each fraction's composition by detecting 13 α_{s1}-CN, 11 α_{s2}-CN, 7 β-CN, and 3 κ-CN components.

In 2004, Galliano and colleagues performed the identification and characterization of a new β-casein variant in goat milk by HPLC with ESI MS and MALDI MS.

Identification of Species Variants – Food Adulteration Analysis

MALDI MS can be employed as a valid analytical method in the dairy industry, allowing the investigation of the most common types of adulterations of milk samples. It has been shown to be a valid and useful method for the determination of possible fraudulent addition of cow milk to ewe milk used for the production of commercial ewe (Pecorino) cheese and to water buffalo milk used in the production of water buffalo Mozzarella cheese. This technique has also demonstrated its ability to identify the possible addition of powdered milk to samples of fresh raw milk.

Protein Damage Analysis

Protein mass spectrometry techniques, such as ESI MS or MALDI-TOF MS, are effective methods to screen for protein damage such as modifications derived from the Maillard reaction and protein oxidation.

Glycation and oxidation are a major cause of food quality loss and are therefore of a great interest for detection in dairy products.

Milk protein glycation

It is generally appreciated that the glycation of proteins leads to an extraordinarily heterogeneous mixture of compounds that are classified as early or advanced modifications. In particular, the early stage of the Maillard reaction comprises the formation of the so-called Amadori product, an aminoketose formed by the condensation and subsequent rearrangement of the carbonyl group of a reducing sugar and an amino group. Milk and dairy products are affected by the Maillard reaction because of the high Lys content of the proteins and the presence of the reducing disaccharide lactose, which favor glycation reactions. At present, a plethora of different analytical approaches have been employed to determine glycation products and modification sites in proteins, milk, and dairy products.

Using an LC MS strategy, several glycation sites were identified on β-lactoglobulin glycated on aqueous state (**Figure 1**). Leucine 1, lysine 14, and lysine 47 were glycated by galactose, glucose, or lactose. Lysines 69, 75, and 135 were modified only in the case of protein glycated with glucose.

Trypsinolysis of glycated proteins, followed by RP-HPLC coupled to MS/MS in the neutral loss scanning mode, indicated that all potential reactive amino groups, except lysine 101, were involved in lactose binding to β-lactoglobulin modified in a dry state.

Free and protein-bound early and advanced glycation-induced lysine modifications in raw and processed cows' milk samples were analyzed using comparative liquid chromatography-tandem mass spectrometry (LC-MS/MS) profiling. The method developed was based on an isotope dilution LC-MS/MS to determine the glycation profiles for *N*-epsilon-fructoselysine, *N*-epsilon-carboxymethyllysine, and pyrraline in dairy products.

In a study of infant formula powders, a highly selective and specific tandem MS technique has been developed to characterize and localize up to 10 lactosylation sites in β-lactoglobulin and α_{S2}-casein. α-Lactalbumin, with five lactosylated peptides, was found to be an interesting protein marker in the milk powder sample to detect chemical modification induced by the processing/storage conditions.

Milk protein oxidation

Protein carbonylation content is widely used as a marker to determine the level of protein oxidation that is caused either by the direct oxidation of amino acid side chains or via indirect reactions with oxidative by-products. The identification of the target oxidized proteins could be obtained by bidimensional electrophoresis with immunostaining followed by MS identification. Immunochemical assays are based on the detection of the 2,4-dinitrophenylhydrazine derivatives of carbonylated proteins.

Exposure of milk to light, which induces both lipid and protein oxidation through excitation of the singlet sensitizer riboflavin, has been analyzed with MS. Carbonyl groups were detected in tryptophan (as kynurenine), histidine, and methionine by RP-HPLC using UV detection, coupled with MS detection using ESI and quadrupole detection. The tryptophan oxidation products were observed to be highest in the caseins, indicating that the proteins lacking well-defined tertiary protein structures were more readily oxidized than globular proteins.

Milk Proteins Degradation/Proteolysis

Peptides are precursors of flavor compounds or exhibit biological properties; these are essential for the development of dairy products with specific sensory characteristics or health-promoting features, respectively.

Peptides of dairy products are produced mainly by the degradation of milk proteins by various proteolytic enzymes, originating, in large part, from milk, bacteria, or mycelium. Proteolysis of caseins and whey proteins has been demonstrated by 2DE followed by MS protein identification. It was used to determine the C-terminal truncated forms of β-lactoglobulin in whey from Romagnola cows' milk. MALDI MS has been used in the determination of the changes in milk protein profiles due to the action of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Sreptococcus thermophilus* during yogurt production.

Milk Protein Polymerization

Milk protein polymerization occurs frequently after storage and/or heat treatment. Polymerization may result from many types of cross-links, including disulfide bonds. The proteins species involved in such polymers were analyzed by 2DE followed by MS (**Figure 2**). Disulfide-linked polymers of κ-casein, α_{s2}-casein, and whey proteins were identified using a nonreducing/reducing comparative analysis strategy. Intermolecular bridges between β-lactoglobulin and κ-casein were identified at the position Cys160 of β-lactoglobulin and Cys88 of κ-casein by on-line LC-ESI MS.

Mass Spectrometry Analysis of Milk Lipid

Regarding bovine milk, lipids exist in the form of globules surrounded by a complex membrane (i.e., MFGM), which contains a mixture of unsaturated phospholipids, proteins, glycoproteins, cholesterol, enzymes, and other minor components. Although MFGM accounts for a relatively small part of the milk and ranges from 2 to 6% of the mass of the fat globules, it plays an important role in stabilizing the fat globules against immediate flocculation and coalescence by acting as a natural emulsifying agent. The lipid fraction of milk is composed mainly of triacylglycerols (TAGs), with minor contributions from diacylglycerols, monoacylglycerols, sterols (predominately cholesterol), phospholipids, fat-soluble vitamins, and β-carotene.

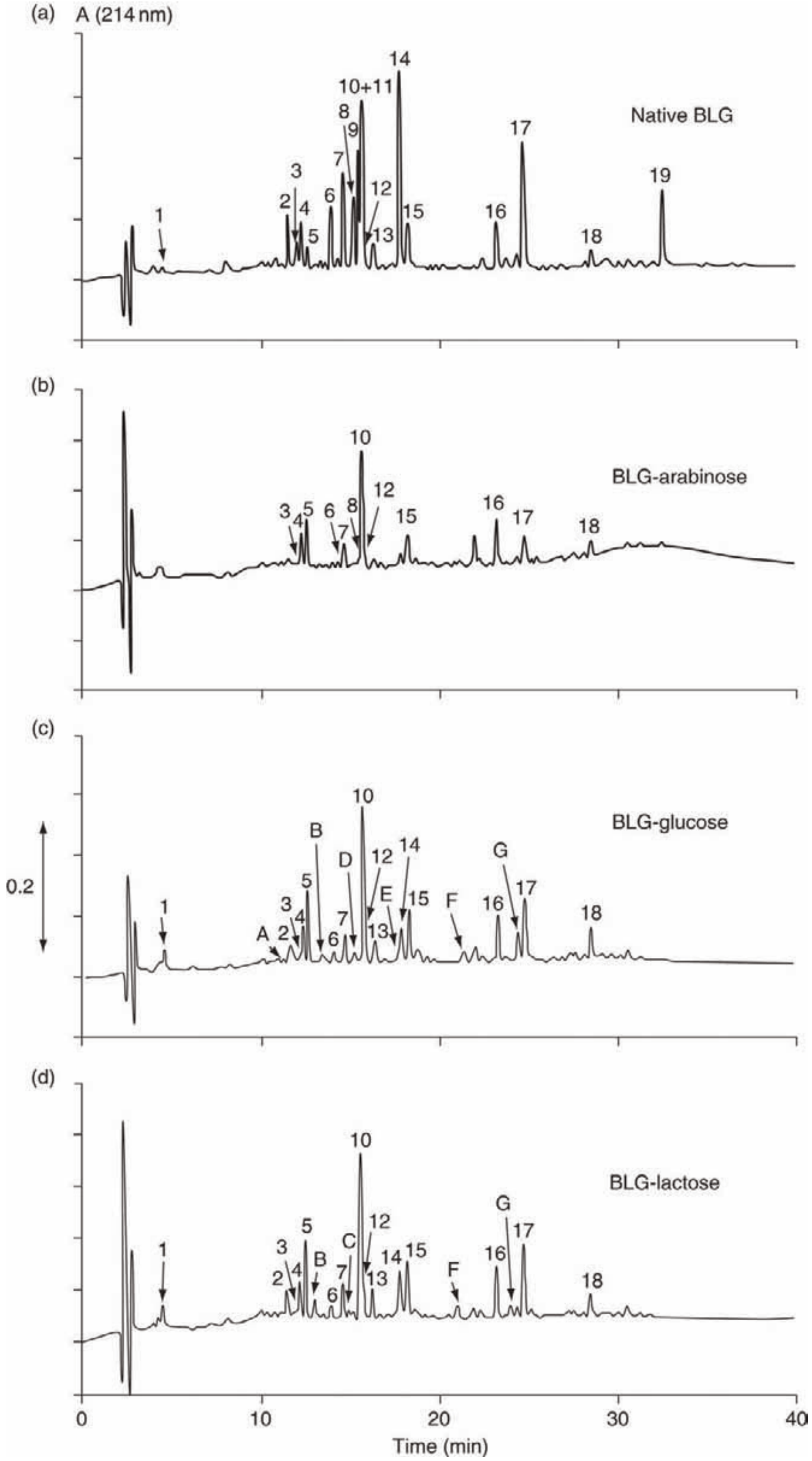

Figure 1 RP-HPLC elution profiles of the tryptic hydrolysates of (a) native β-lactoglobulin (BLG) and BLG glycated with (b) arabinose, (c) glucose, or (d) lactose. Peptides indicated with numbers were obtained from unmodified tryptic peptides. Peptides indicated with letters were obtained from modified tryptic peptides, letters A–G corresponded with a glycation of lysine 75, leucine 1, lysine 100, 135, 69, 14, and 47, respectively. Reproduced from Chevalier F, Chobert J-M, Mollé D, Haertlé T (2001) Maillard glycation of β-lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Le Lait* 81: 655–666.

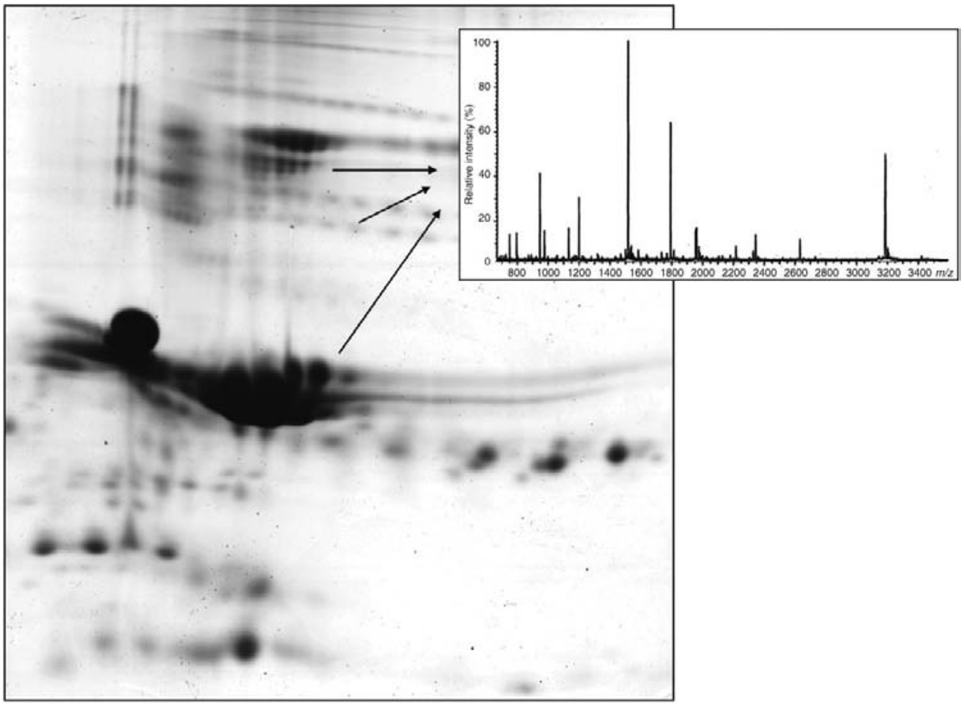

Figure 2 Two-dimensional electrophoresis of protein complexes in bovine milk under nonreducing condition. Spots with arrows were identified by peptide mass fingerprint-MALDI-TOF MS as the same protein, but with various molecular weights. On the mass spectra (top right), each peak represents peptides ionized from a crystalline structure of small organic molecules; when excited by a laser, the peptides were vaporized ('desorbed') and ionized predominately to a $+1$ charge. The masses were accurate enough to determine precisely the amino acid composition of each peptide. Taken together, all peptide masses were associated to a 'protein fingerprint', which allowed interrogation of a data bank to identify the unknown protein. Reproduced from Chevalier F, Hirtz C, Sommerer N, Kelly AL (2009) Use of reducing/non-reducing two-dimensional electrophoresis for the study of disulphide-mediated interactions between proteins in raw and heated bovine milk. *Journal of Agricultural and Food Chemistry* 57: 5948–5955.

The characterization of milk fat TAGs has been realized by coupling HPLC separation either to light-scattering detection and desorption chemical-ionization tandem MS or to atmospheric pressure chemical ionization (APCI) MS. Many (58) peaks were resolved with HPLC, determining the acyl carbon number, the number of double bonds, and the fatty acids in each TAG eluted by HPLC. The list of the TAG structures and the most abundant TAGs were identified, but without quantitative information. Moreover, although the sequence of fatty acids in the TAGs could not have been determined by the methods employed, the composition of more than 180 TAGs was determined.

Triple quadrupole (TQ or QqQ) tandem mass spectrometers coupled to an ESI source can be used for the efficient and accurate quantitation of small molecules. The strategy, called SRM (single reaction monitoring) or MRM (multiple reaction monitoring), is simple. Usually, on QqQ instruments, the first quadrupole (i.e., the first mass analyzer) selects the ion of interest, the second quadrupole (q) serves as collision cell to produce fragment ions of the previously selected biomolecule, and the third quadrupole scans the fragment masses produced. For SRM strategies, after characterization of the compounds (fragments produced with the previously described method), the first quadrupole selects the ion of interest, the second quadrupole (q) fragments the ion, and the third quadrupole, instead of scanning, is fixed to the mass value of a fragment specific to the biomolecule. The detected signal corresponds with a specific fragment of a specific ion. As no time is lost in scanning uninteresting masses, the sensitivity is increased by 2 or 3 orders of magnitude. For MRM strategies, selectivity is increased by following (in the third quadrupole) more than one (usually three) compound-specific fragment ion.

This mass scanning capability is used routinely for quantitation of vitamins (e.g., vitamins D_2 and D_3) in very different matrixes such as milk, dairy products, infant food, or even bovine serum.

Mass Spectrometry Analysis of Other Trace Elements

Several MS methods have been reported to monitor the final quantities of trace elements in milk products such as neutron activation, graphite furnace atomic absorption spectrometry, or flame and graphite furnace atomic absorption spectrometry. An inductively coupled plasma atomic emission spectrometer coupled to a gel filtration chromatography system was used to characterize the species of Cu, Zn, Mn, Mg, and Ca in human milk and milk formulae.

MS detection was also used for the analysis of trace elements with the potential of better sensitivity and multielemental analysis. A review article described examples of trace analyses by isotope dilution MS techniques with various ionization sources such as thermal ionization, spark source, EI, inductively coupled plasma, and field desorption. Indeed, the use of stable isotopes for element investigations can be for labeling experiments, determination of natural isotope variations, kinetic studies, or for a more accurate quantitative analysis.

See also: **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Protein. **Milk**: Bovine Milk. **Milk Proteins**: Analytical Methods; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; α-Lactalbumin; β-Lactoglobulin; Proteomics.

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Ultrasonic Techniques

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Introduction

Ultrasound is a versatile diagnostic technology that is finding increasing application in the dairy industry for process monitoring, determination of physical properties, level sensing, and flow metering. Ultrasound may travel through solids, liquids, or gases, and may be described as a pressure wave or a stress wave propagating through a medium in which the molecules or particles of the medium move. Ultrasound may be propagated directly in the medium of interest, or through the walls of a container, reaction vessel, or pipe, and can be used under a wide range of environmental conditions. There are several different types of ultrasonic waves; the most commonly used are termed bulk waves as they propagate through the bulk of the medium of interest, but various types of surface waves can also exist at interfaces between different material phases such as solid–liquid, solid–gas, and liquid–gas. In restricted geometries such as plates, layers, or rods, various types of guided waves may also exist and these may all be used for analytical purposes.

Properties of Ultrasound

If the motion of the particles of the medium is periodic, it may be assigned a frequency, *f*, in Hz, and this is usually related to the speed of sound, *c*, in m s^{-1}, in the medium and the wavelength, λ, in m, between regions along the path of propagation that have identical levels of pressure or stress. This relationship is usually linear, such that

$$c = f\lambda \qquad [1]$$

although some wave types are dispersive, that is, waves of different frequencies travel at different speeds in the same medium.

Bulk waves are classified by the type of stress or the direction of wave propagation relative to the motion of particles from their rest positions, as shown schematically in **Figure 1(a)** for solid media. If the particles move in the direction of the propagating wave, then a longitudinal or compression wave exists, as shown in **Figure 1(b)**, with wavelength λ_L. If the particle motion is perpendicular to the direction of wave propagation, then a transverse or shear wave exists, as shown in **Figure 1(c)**, with wavelength λ_T. Transverse waves cannot be supported in gases and most liquids, as these media cannot support shear stresses. Longitudinal waves can be supported in solids, liquids, and gases and are the most common type used for ultrasonic measurements.

Speed of Sound

The speed of sound is dependent on the wave type and the properties of the propagating medium (compressibility and mass density). For liquids and gases, which do not support shear forces, the speed of sound may be found using

$$c = \sqrt{\frac{K}{\rho}} \qquad [2]$$

where *K* is the bulk modulus in Pa and ρ the mass density in kg m^{-3}. For ideal gases such as air at standard temperature and pressure (STP), $K = \gamma P$, where γ is the adiabatic ratio of the gas and *P* is the absolute pressure of the gas in Pa. Hence

$$c = \sqrt{\gamma R T} \qquad [3]$$

where *R* is the specific gas constant in J kg^{-1} K^{-1} and *T* is the specific gas temperature in K. As there is a strong relationship between the speed of sound and temperature in gases and many liquids, this must be accounted for to prevent significant experimental error. For homogeneous solids, the longitudinal velocity c_l is typically given by

$$c_l = \sqrt{\frac{E(1-\upsilon)}{\rho(1+\upsilon)(1-2\upsilon)}} \qquad [4]$$

where *E* is Young's or elastic modulus in Pa and υ is Poisson's ratio. The transverse velocity c_t is given by

$$c_t = \sqrt{\frac{E}{2\rho(1+\upsilon)}} = \sqrt{\frac{G}{\rho}} \qquad [5]$$

where *G* is the shear modulus in Pa.

Care must be taken in mutiphase media, for example when discrete particles of one medium are dispersed throughout a continuous phase of another. A simplification of this phenomenon assumes uniformly dispersed volume fractions of the different phases, such that

$$\rho_e = \phi\rho_1 + (1-\phi)\rho_2 \qquad [6]$$

where ρ_e is the effective density and ϕ the volume fraction of medium 1 with density ρ_1 dispersed throughout

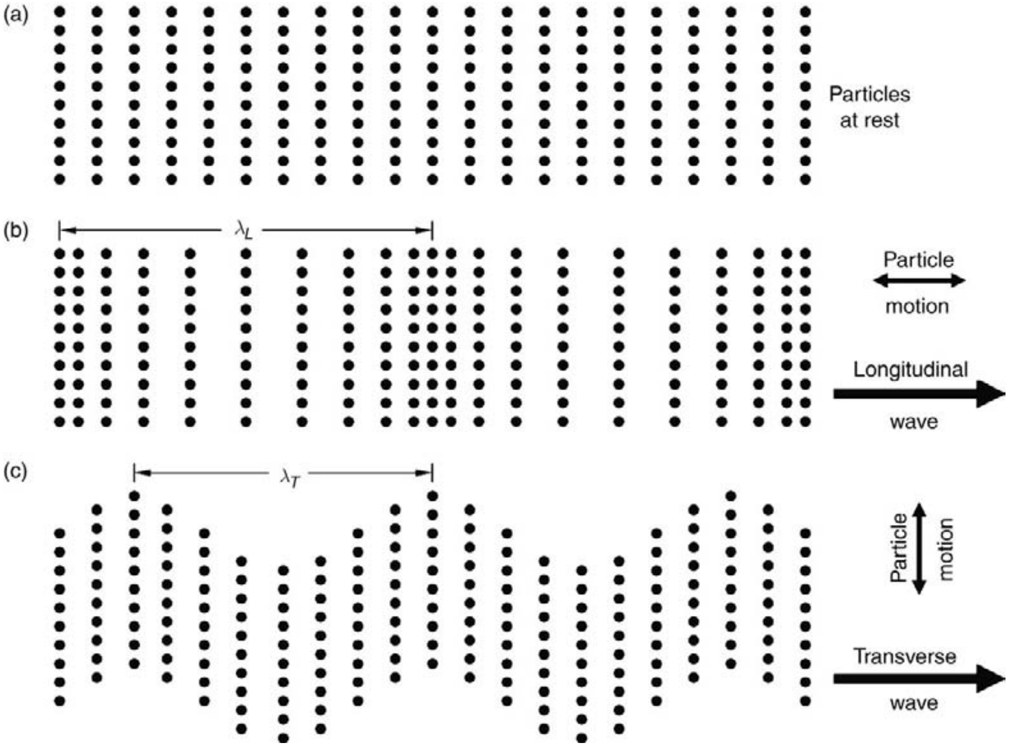

Figure 1 Particle motion in ultrasonic bulk wave propagation, showing (a) particles of a solid medium at rest and particle motion for (b) a longitudinal (compression) wave and (c) a transverse (shear) wave.

medium 2 with density ρ_2. Similarly, for the effective bulk modulus K_e

$$\frac{1}{K_e} = \frac{\phi}{K_1} + \frac{1-\phi}{K_2} \qquad [7]$$

which then leads to Urick's equation for the effective speed of sound, c_e, of the combination of phases:

$$c_e = \sqrt{\frac{c_2^2}{\left[1-\phi\left(1-\frac{\rho_1}{\rho_2}\right)\right]\left[1-\phi\left(1-\frac{c_2^2\rho_2}{c_1^2\rho_1}\right)\right]}} \qquad [8]$$

where c_1 and c_2 are the speed of sound in media 1 and 2, respectively. This simplification does not take into account the nonuniform dispersion of medium 1, for which a tortuosity parameter may be used, nor does it account for any interconnectivity in the particulate phase, for which Biot's wave propagation theory for porous media may be used.

Reflection and Refraction

The amplitude of the particle motion, ξ, in m, has a corresponding sound pressure, p, in each medium, given by

$$p = 2\pi f \xi \rho c = \omega \xi Z \qquad [9]$$

where ω is the angular frequency ($=2\pi f$) in rad s^{-1} and Z is the specific acoustic impedance of the medium, in N s m^{-3} (or rayl). Where adjacent regions of a medium have different specific acoustic impedances, for example due to a local change in sound speed or density, then for a smooth interface at normal incidence, a proportion of the incident ultrasonic intensity will be reflected with a reflection coefficient, ε_R, according to

$$\varepsilon_R = \left(\frac{Z_1 - Z_2}{Z_1 + Z_2}\right)^2 \qquad [10]$$

and the remainder transmitted through the interface according to the transmission coefficient, ε_T, given by

$$\varepsilon_T = \frac{4Z_1Z_2}{(Z_1 + Z_2)^2} \qquad [11]$$

where Z_1 and Z_2 are the two different specific acoustic impedances. If amplitudes are to be used instead of intensities, then the square root of the expressions for ε_R and ε_T must be used. The transmitted part of the ultrasonic wave is refracted, that is, it is diverted from the original path of the incident wave, according to Snell's law:

$$\frac{\sin\theta_1}{c_1} = \frac{\sin\theta_2}{c_2} \qquad [12]$$

where θ_1 is the incident angle to the interface normal in medium 1 with sound speed c_1 and θ_2 is the refracted angle to the interface normal in medium 2 with sound speed c_2. Note that as longitudinal and transverse waves have different sound speeds in the same medium, they refract at different angles.

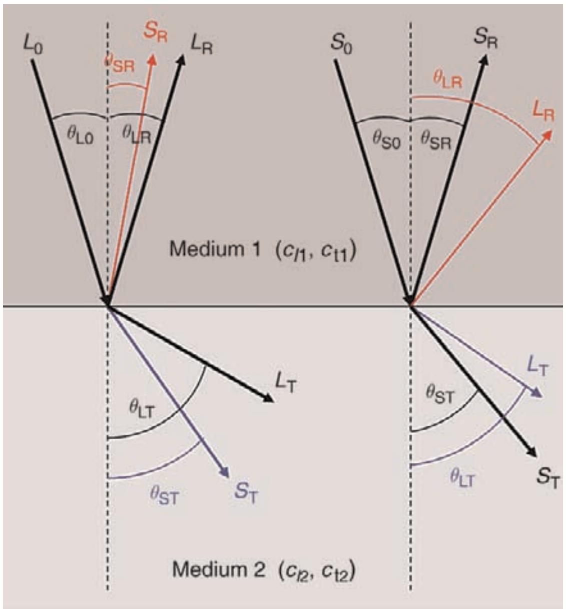

Figure 2 Reflection, refraction, and mode conversion of longitudinal (*L*) and shear (*S*) waves at an interface between two media with different sound speeds. For explanations of abbreviations, see text.

The situation is further complicated at non-normal incidence, as the different waves can also mode convert, as shown in **Figure 2**. An incident longitudinal wave, L_0, will be reflected back into medium 1 as L_R at an angle, θ_{LR}, equal to the angle of incidence, θ_{L0}, but part of the wave will be mode converted into a transverse (shear) wave S_R, which will be reflected at a different angle, θ_{SR}, determined by the transverse wave speed c_{t1}. Similarly, part of the transmitted longitudinal wave, L_T, will also mode convert into a shear wave, propagating into material 2 as S_T with a different refracted angle, θ_{ST}. For certain combinations of incident angle and sound speed, the refracted longitudinal wave, L_T, has a refracted angle $\theta_{LT} > 90°$ and does not propagate into medium 2, leaving just the refracted mode converted shear wave S_T. This phenomenon is regularly exploited to produce an angled beam of shear waves by the use of a suitably angled wedge attached to a longitudinal transducer, and it allows shear waves to be used to test a solid medium surrounded by a fluid through which shear waves cannot be directly coupled. Similar mode conversions occur for an incident shear wave S_0. For multiple reflections occurring in thin layers such as the walls of a container, the ultrasonic signals can quickly become extremely complex.

Diffraction

Straight-ray theory is regularly used to predict ultrasonic wave behavior, but under certain conditions ultrasonic waves will be diffracted, a phenomenon in which waves appear to bend around certain features. For low frequencies and large wavelengths, diffraction may not be an issue, but at smaller wavelengths, close to the size of structural features in the medium, significant scattering can occur. Diffraction must be considered when predicting the output of an ultrasonic transducer, as the pressure field in front of the transducer face is not uniform. The beam emanating from the transducer has two components, a plane wave from the element face and an edge wave diffracting from the element boundary. These then interfere both constructively and destructively at different points in front of the transducer, resulting in pressure maxima and minima until the edge and plane waves have passed each other at a distance *N*, known as the near-field distance, which may be approximated by

$$N = \frac{D^2 - \lambda^2}{4\lambda} \qquad [13]$$

where *D* is the transducer diameter and λ the wavelength in the medium. These pressure field variations can produce erroneous results if measurements are made within *N* of the transducer face, so often a delay line of length *N* that contains the entire transducer near-field is used. Diffraction also causes the beam emanating from an ultrasonic transducer to diverge; for a transducer of diameter *D* and a wavelength λ in the medium, for small values of λ/D, the angle of spread, θ, may be approximated by

$$\sin\left(\frac{\theta_{-6\,\text{dB}}}{2}\right) = \frac{0.56\lambda}{D} \qquad [14]$$

at –6 dB of the central intensity, and by

$$\sin\left(\frac{\theta_{-20\,\text{dB}}}{2}\right) = \frac{1.08\lambda}{D} \qquad [15]$$

at –20 dB of the central intensity, as shown in **Figure 3**. Thus, relative to the wavelength, small-diameter probes tend to have a wide beam and large-diameter probes tend to have a narrow beam.

Attenuation

As the ultrasonic wave propagates through a medium of interest, it loses energy by scattering from any internal microstructure such as miniature impedance interfaces, by absorption as the molecular interactions are not perfectly elastic, resulting in dissipation of heat, and in certain media such as emulsions, by relaxation of the immiscible phase. Attenuation in $\text{dB}\,\text{m}^{-1}$ is heavily dependent on frequency, and values are usually stated at a particular frequency. Two ultrasonic amplitudes A_1 and A_2 or two ultrasonic intensities I_1 and I_2 are said to differ by *n* dB if

$$n = 20\log_{10}\left(\frac{A_2}{A_1}\right) = 10\log_{10}\left(\frac{I_2}{I_1}\right) \qquad [16]$$

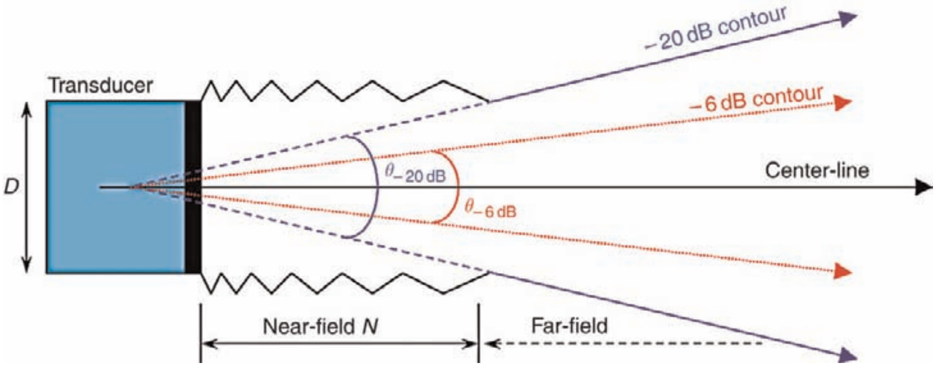

Figure 3 Beam divergence from a circular ultrasonic transducer due to diffraction.

Thus, 6 dB is an amplitude ratio of 2:1 and 20 dB is an amplitude ratio of 10:1. Attenuation is difficult to calculate from a theoretical basis and so many values are obtained experimentally. Attenuation may be described by

$$I_z = I_0 e^{-\alpha_T z} \qquad [17]$$

where I_z is the intensity at some depth z in the medium, I_0 is the original incident intensity, and α_T is the total attenuation coefficient in dB m^{-1} at the specified frequency. α_T may be further separated into absorption α_A, scattering α_S, and relaxation α_R components, as appropriate:

$$\alpha_T = \alpha_A + \alpha_S + \alpha_R \qquad [18]$$

Ultrasonic Transducers

The key apparatus in any ultrasonic system is the transducer, the vast majority of which contain an element of piezoceramic such as lead zirconate titanate (PZT), piezopolymer such as polyvinylidene fluoride (PVDF), or piezocomposite usually consisting of pillars of piezoceramic embedded in an epoxy resin matrix. These elements deform under an applied electric potential to produce ultrasound, and conversely produce a measurable electric potential when deformed by an incident ultrasonic wave when acting as a detector. A schematic of a typical ultrasonic transducer is shown in **Figure 4**, where various damping methods remove unwanted resonances and radial vibrational modes, and both electric and acoustic impedance matching are shown. Typical transducer frequencies range from 0.5 MHz to more than 50 MHz, and diameters from millimeters to centimeters. Various types (such as twin element, normal incidence, angled wedge, focused, contact, immersion, and air coupled) are available to suit the measurement application.

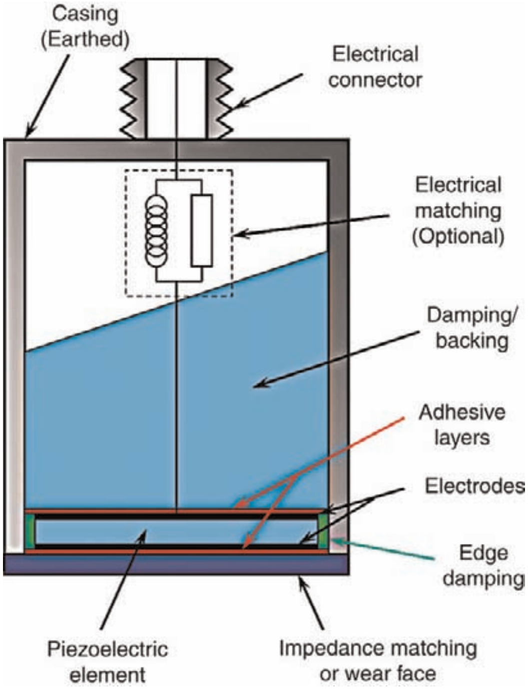

Figure 4 Internal details of a typical ultrasonic transducer.

The ultrasonic vibrations must be coupled from the transducer to the medium of interest, and due to the usually large differences in specific acoustic impedance between the transducer element (Z_1) and most media (Z_2), significant energy losses occur. In liquid media, an intermediate impedance matching layer may be incorporated into the front face of the transducer, ideally one-quarter of a wavelength in thickness at the frequency of interest, and made from a layer of material with an ideal specific acoustic impedance Z_L given by

$$Z_L = \sqrt{(Z_1^2 + Z_2^2)} \qquad [19]$$

For solid media, the transducer element is often covered with a hard wear-resistant face or a detachable delay line. An intermediate layer of liquid couplant is required between the transducer face and the surface of most solids, as this fills in any microscopic surface imperfections, which, if they contained trapped air, would prevent effective transmission of ultrasound across the interface. Transducers may also be permanently adhered to a tank, pipe, or similar structure.

Ultrasonic Measurement Systems

There are many different ultrasonic measurement methods, and the exact apparatus and configuration would ultimately be determined by the nature of the desired test, but a typical ultrasonic measurement system would operate as follows. A high-voltage electrical pulse or signal is applied to the transmitting transducer, which then converts the signal to ultrasound. The same signal triggers a timebase generator that synchronizes the start of a display. As the ultrasound propagates through the medium, any ultrasound detected by the receiver is converted into an electrical signal, amplified, and plotted against time on the display; until some ultrasound is detected by the receiver, there is usually no output and the display trace is horizontal. Ultrasonic pulses may be produced at rates up to 1000 Hz (the pulse repetition frequency (PRF)) to refresh the display, perform signal averaging, and allow sufficient time for the outgoing ultrasound to be detected by the receiver before the next transmitted wave or pulse is sent out.

Arguably, the most common system configuration is pulse-echo, in which a single transducer (T/R) is used as both a transmitter and a receiver of ultrasound as shown schematically in **Figure 5(a)**. The transducer is switched between a high-voltage signal generator and a sensitive signal amplifier to generate the ultrasound and then listen for the corresponding reflected echoes. This configuration uses only a single transducer, and requires access to only one side of the medium, but the transducer element tends to continue vibrating for a period of time after the excitation signal has stopped, during which time no signal can be received, resulting in a 'dead time' and a corresponding 'dead zone' in front of the transducer that cannot be investigated.

An alternative method is through-transmission, sometimes referred to as pitch-catch, in which separate transmitter T and receiver R transducers are used, placed directly opposite each other as shown in **Figure 5(b)**. This is less attractive as it uses two transducers and requires access to both sides of the test medium, but it allows testing of highly attenuating materials as the path taken by the ultrasound is half that of the equivalent pulse-echo system. Pitch-catch may also use two probes as shown in **Figure 5(c)** on the same side of the medium, sometimes angled and housed within the same transducer casing in a twin-element device, which allows very accurate near-surface measurements when used with a delay line.

Ultrasonic Property Measurement

The type of ultrasonic signal analysis depends on the desired property measurement. The simplest method is to measure the propagation time, t, in s, of the ultrasonic wave through a known distance, L, in m, from which the speed of sound, c, in m s^{-1}, may be measured. If a series of discrete reflections within the sample can be obtained, the attenuation coefficient may also be determined by comparing the relative heights of adjacent echoes. Care must be taken to ensure that the correct path length is used; in pulse-echo, the path traveled by the ultrasound will be $2L$ between each echo, but for through-transmission, the first signal will travel only a distance L, and the subsequent echoes travel $2L$, as shown in **Figure 5**. Sound speed may be used to estimate density, compressibility, or temperature, and details of microstructure, or particle size can be obtained from attenuation measurements.

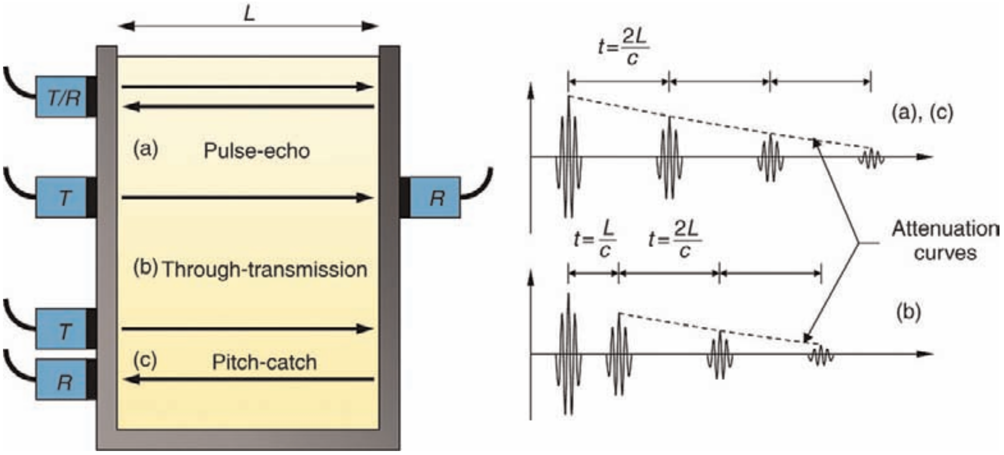

Figure 5 Typical transducer configurations (left) and idealized received signals (right) for (a) pulse-echo, (b) through-transmission, and (c) pitch-catch ultrasonic measurements.

Interferometry and Spectrometry

Another technique for measuring wavelength is a form of interferometry, in which a reflector is precisely positioned opposite a source of continuous ultrasound at a specific frequency until it is an integer number of half-wavelengths away from the transducer face. A standing wave will then be produced with maxima occurring at multiples of the half-wavelength. Any small change in the speed of sound produces a change in the wavelength and hence the position of the maxima. This technique can detect very small changes in sound speed with a higher degree of accuracy than with transit time methods such as pulse-echo.

Ultrasonic spectroscopy allows measurement of attenuation at different frequencies. Typically, the ultrasonic frequency is swept through a range of interest, where the wavelengths are of an appropriate size, and the attenuation at each frequency is analyzed using a fast Fourier transform (FFT). Attenuation at a specific frequency may be used to indicate a particular size of microstructural features in solids, fat globule or particle sizing and distribution in liquids, or the presence or quantity of a particular gas such as carbon dioxide.

Void or Inclusion Detection

One common use of ultrasound is to determine the size, shape, and distribution of hard inclusions, or the size and location of voids. These may be desired product features (e.g., nuts in chocolate, holes in Emmental cheese) or undesired contaminants and defects (e.g., machinery parts that have fallen into the product). These measurements may be achieved using pulse-echo or through-transmission configurations, but the received ultrasonic signals must be interpreted carefully. The presence of a single highly reflective region will produce multiple echoes in a pulse-echo system, and will reduce or eliminate a through-transmitted signal depending on the relative size of the region and the ultrasonic beam. However, other features further along the pulse-echo path may be obscured, and other phenomena such as poor transducer coupling may reduce the signal amplitude in through-transmission.

Testing may be automated if the transducers do not need to be directly coupled to the sample. This may be achieved using immersion transducers and placing individual samples in water, by using roller or wheel probes, or air-coupled ultrasound. Transducers may be scanned over the surface of the sample, and the received signal at each location analyzed using a technique known as C-scanning. The signals are time windowed to correspond to a particular depth, and the maximum signal amplitude within the window is represented as a colored pixel in a two-dimensional image. By recording the entire ultrasonic trace at each point in the scan, the time window may be moved and the same data set reanalyzed and used to produce multiple cross-sectional slices at different depths, which may then be reconstructed into a three-dimensional image. Other signal parameters such as frequency content may also be analyzed at each point in the scan using an FFT.

Level Sensing

A common use of ultrasound is for level sensing of both liquids and powders, as shown in **Figure 6**. For liquid level sensing, a number of different methods may be used. An ultrasonic wave is usually propagated through the liquid from the bottom of the container and reflected from the top surface of the liquid (**Figure 6(a)**), and if the speed of sound in the liquid is known precisely or measured *in situ* (**Figure 6(b)**), the liquid level may be determined. For powders, highly attenuating liquids, or where there is significant turbulence in a moving liquid such that wave propagation within the liquid itself is impractical, an alternative is to use airborne ultrasound and transmit an ultrasonic wave from the top of the container to reflect from the top surface of the liquid or powder (**Figure 6(c)**). This method relies on prior knowledge of the surface profile. Another method for liquid level measurement is to attach a series of ultrasonic sensors to the outside of the vessel; when not covered by liquid on the inside of the vessel, ultrasound will reverberate within the vessel wall; when covered with liquid, ultrasonic energy will couple into the liquid in the tank

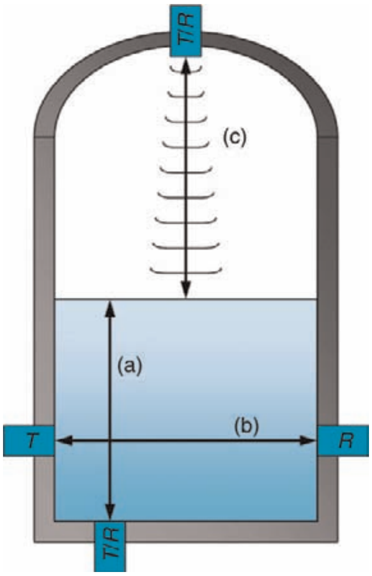

Figure 6 Ultrasonic level measurement in a tank, showing (a) the level measurement path in the liquid, (b) *in situ* speed of sound measurement, and (c) air-coupled measurement of the location of the top surface.

and the vessel wall reverberations will be significantly reduced or eliminated.

Flow Measurement

Ultrasound is ideal for measurement of fluid flow, as it can be noninvasive, can produce a negligible pressure loss in the system, and can simultaneously determine the sound speed and other properties of the fluid. There are several different designs of ultrasonic flow meter, shown schematically in **Figure 7**. Arguably the most common type uses the principle of contra-propagating transmission, in which an ultrasonic wave propagates through the fluid at some angle θ to the flow axis in both upstream and downstream directions along the same path L, in m, as shown in **Figure 7(a)**. The wave traveling upstream will take longer to travel the same path than the wave traveling downstream due to the axial component of the flow velocity, v, in m s^{-1}. The upstream travel time t_u, in s, is given by

$$t_u = \frac{L}{(c - v\cos\theta)} \qquad [20]$$

and the downstream travel time t_d, in s, is given by

$$t_d = \frac{L}{(c + v\cos\theta)} \qquad [21]$$

By combining eqns [20] and [21], the following expression can be obtained for v that is independent of the sound speed c of the fluid:

$$v = \frac{L}{2\cos\theta}\frac{t_{ud}}{t_u t_d} \qquad [22]$$

where $t_{ud} = t_u - t_d$, which means that the same meter can be used for different fluids in the same conduit. Similarly, an expression can be obtained for c that is independent of v, allowing simultaneous determination of fluid properties.

Doppler shift meters measure the change in the frequency of the ultrasound reflected back to the receiver from particles or other scatterers in the fluid, as shown in **Figure 7(b)**. The received frequency f_u, in Hz, of a wave traveling in the upstream direction will be Doppler shifted from the transmitted frequency f_0, in Hz, according to

$$f_u = \left(1 - \frac{v_s\cos\theta}{c}\right)f_0 \qquad [23]$$

where v_s is the velocity, in m s^{-1}, of the scatterer in the fluid; so care must be taken to ensure that the scatterers are traveling at the same velocity as the bulk of the fluid for accurate flow measurement. High-frequency ultrasound may be scattered from turbulent eddies or vortices in the fluid without the need for particulates.

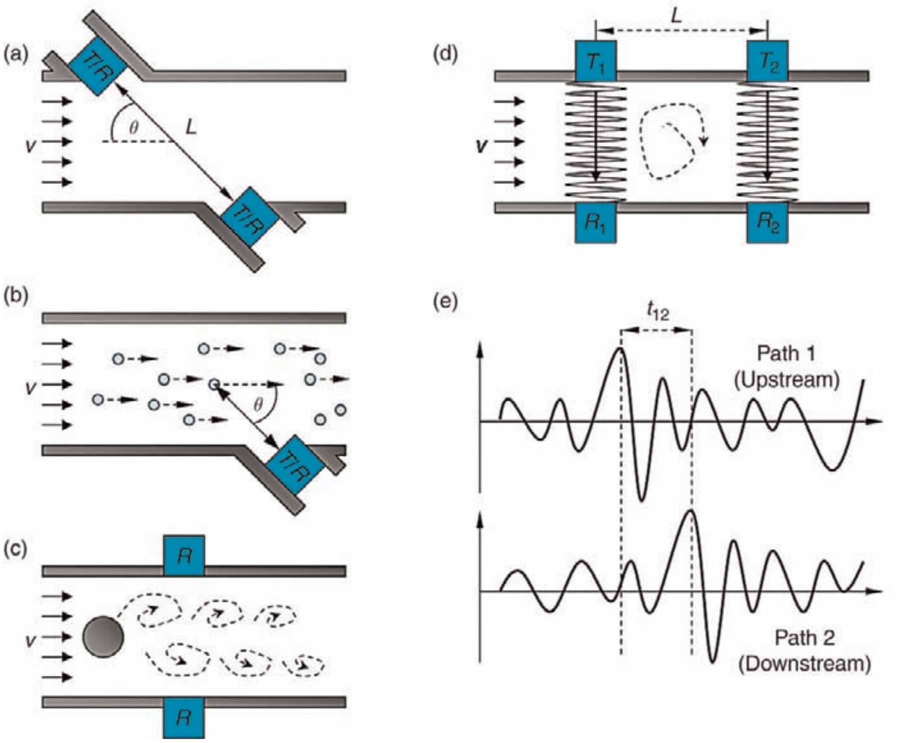

Figure 7 Ultrasonic flow meter configurations: (a) contra-propagating time-of-flight meter; (b) Doppler meter; (c) vortex shedding meter; (d) cross-correlation meter; and (e) typical signal from a cross-correlation meter.

Other types include vortex shedding meters, as shown in **Figure 7(c)**, which use a bluff body installed in the flowing fluid to produce alternating vortices downstream at a rate proportional to the fluid flow, which can be recorded using an ultrasonic transducer. Cross-correlation meters, shown in **Figure 7(d)**, use two continuous beams of ultrasound, which traverse the flow from transmitters T_1 and T_2 to receivers R_1 and R_2 a fixed distance L apart. Eddies or vortices in the flow disturb the two beams in turn as they flow down the pipe, and the two received ultrasonic signals are cross-correlated to extract the time difference t_{12} between the eddy crossing the upstream beam 1 and the downstream beam 2, as shown in **Figure 7(e)**. v is then proportional to L divided by t_{12}.

The ultrasonic transducers in a flow meter may be wetted, that is, directly exposed to the flowing fluid, requiring a metering section to be installed. Clamp-on transducers are also common; they can be installed anywhere where external access to the conduit is possible, but certain gas flows may be difficult to measure accurately due to the impedance mismatch between the pipe wall and the gas. Care must also be taken in the placement of a flow meter in a pipe or conduit; the accuracy of measurement is affected by the fluid flow profile within the pipe, and as such 15–20 diameters of straight unobstructed pipe upstream and 5–10 diameters downstream are recommended to allow a fully developed flow profile to exist in the metering section.

Tomography

One of the limitations of investigating the sample with ultrasound from one direction only is that the first highly reflective region encountered effectively occludes the presence of others further along the beam. One technique that can overcome this is tomographic imaging, often referred to as computer-assisted tomography (CAT) or computerized tomography (CT). Ultrasonic transducers are positioned around the periphery of the region of interest, which may then be investigated with many ultrasound beams from different angles. The transducers are usually regularly spaced to produce either linear data sets at regular angular intervals, or more commonly fan-shaped data sets emanating from each transmitter position T_n to a number of receiver positions R_n simultaneously, as shown in **Figure 8**. A cross-sectional image of either attenuation or slowness (the inverse of sound speed) and associated physical properties may then be reconstructed using one of two different classes of algorithm. The first of these uses the Fourier slice theorem, which requires ultrasonic data that are regularly spaced both linearly and radially and uses a straight-ray approximation. As certain rays in different fans are parallel, as shown by the dashed ray paths in **Figure 8**, sets of parallel rays may be compiled from fan-beam data using a process known as rebinning. Fourier-based algorithms include filtered back projection and back propagation, and are computationally efficient.

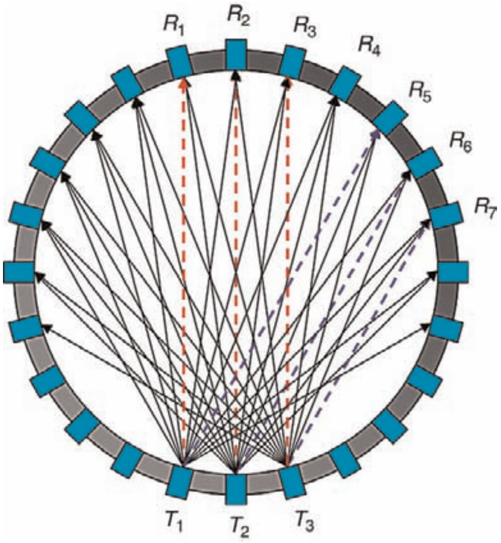

Figure 8 Fan-beam data sets for ultrasonic tomography, showing parallel rays (dashed) used for rebinning.

The second class of algorithm is iterative and has less stringent geometric requirements. The region of interest is divided into pixels, each with an initial estimate of the desired acoustic property (such as slowness). A proportion of the measured value along each ray path is then added to each pixel that the ray intersects, using a weighting factor that is usually determined by the proportion of the pixel that interacts with the ultrasonic beam. The sum of all the pixels along the ray path is then compared to the measured value along each ray, and the difference is used to update the estimate in each pixel in an iterative process until some convergence criterion is reached. Typical algorithms include algebraic reconstruction technique (ART) and simultaneous iterative reconstruction technique (SIRT). These methods tend to be computationally slower, but can correct for nonlinear effects such as diffraction and ray bending.

Applications

Aside from general level sensing, flow measurement and process tomography applications, specific examples of ultrasonic testing applied to the analysis of dairy products are too numerous to describe in full, but have included measurements of the fat content of milks and creams, characterization of fat globule size and distribution, monitoring the aggregation of milk proteins, assessing the maturity, texture, fat content, and water content of different types of cheese, identifying the curd coagulation point, monitoring the growth of gas bubbles, and determination of density or temperature variations in reaction vessels. Continuing improvements in dairy products and processes, ultrasonic transducers, electronics, signal processing methods, and computing power mean that many new applications of ultrasound in the dairy industry will be developed in the future.

See also: **Analytical Methods**: Chromatographic Methods; Infrared Spectroscopy in Dairy Analysis; Light Scattering Techniques; Physical Methods; Sampling; Statistical Methods for Analytical Data.

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Microbiological

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Introduction

Microorganisms affect milk and dairy products in unique ways. Microorganisms not only provide us with many value-added products through dairy fermentations but can also reduce the shelf life and the quality of products due to their metabolic activities. We are seeing phenomenal increases in the commercial health food segment that rely mainly on a new class of dairy organisms, generally referred to as probiotics. At the same time, it is also well established that the enzymes produced by microbial contaminants may lead to several undesirable changes in milk and dairy products. It is also important to mention here that several foodborne pathogens are transmitted through milk and dairy products and have serious public health implications. Many pathogenic microorganisms such as *Listeria monocytogenes, Campylobacter jejuni, Yersinia enterocolitica, Escherichia coli* O157:H7, *Salmonella* spp., *Mycobacterium paratuberculosis* have recently become of significance in dairy products, especially Hispanic cheeses. Some organisms have also been implicated in food-poisoning outbreaks due to the production of bacterial toxins such as staphylococcal toxins, which are heat stable and are carried over even to the final products.

Precision in analytical techniques is thus the key in monitoring these microorganisms and their metabolites to control the microbial quality and safety of milk and dairy products. Several techniques are used in microbiological analysis of dairy foods. These techniques can be divided broadly into culture and microscopic techniques. Another category of techniques that are being used increasingly nowadays is the rapid microbiological methodologies. The current article elaborates on the microbiological techniques for the enumeration and identification of different organisms of significance to dairy foods. It is imperative that the laboratory involved in microbiological analysis is adequately equipped to meet biosafety requirements and that it has proficient analysts.

Sampling

Correct sampling techniques are very important in microbiological analysis due to the heterogeneous distribution of microflora in the samples. Dairy products offer a large variation in product attributes such as liquid, concentrated, dried, frozen, or coagulated. This makes the sampling more challenging and has led to standardization of several techniques for obtaining a representative sample.

Samples are collected aseptically to avoid microbial contamination from external sources. Statistical sampling plans are used to obtain samples that are representative of the lot of food. Temperature and time of holding of samples prior to analyses are critical in obtaining accurate results. In general, perishable samples that cannot be frozen are held at 0–4.4 °C during collection and transportation and are analyzed within 6 h. Other perishable samples that can be frozen are stored at −20 °C and are analyzed within 36 h of collection.

The next important aspect is the sample size, which should describe the population accurately. The single sampling attribute plan (also known as the two-class plan) investigates an attribute such as the presence or absence of a microorganism. Similarly, multiple sampling attribute plans are based on multiple samplings. Three-class attribute plans, on the other hand, differ by having two microbiological limits to create three classes of product. Sampling procedures for low contamination levels generally involve variable sampling by either increasing the sample size or the sample units.

Sample homogenates are prepared aseptically. Liquid milk or semi-liquid product samples in containers with head space can be mixed by rapidly inverting the sample container 25 times before drawing the sample for analysis. Dried product samples are stirred using a sterile spatula to ensure a homogeneous sample, and an 11 g sample is blended with 99 ml diluent for making the homogenate. Blending is preferably done using a stomacher.

Culture Techniques

In any microbiological analysis, culture techniques are important as they help in the enumeration of viable microflora. Such counts can then be easily correlated with the product shelf life, quality, and safety attributes. In many cases, the shelf stability of dairy foods will be directly related to the number of viable cells. In addition, many fermented dairy products, especially probiotic products, are best judged based on high levels of viable lactic acid bacteria.

The number of viable microorganisms can be estimated by a plate count technique, referred to as standard plate counts (SPCs), which is a measure of aerobic and facultatively anaerobic microflora. The other culture techniques involve growth in broth tubes, referred to as most probable numbers (MPNs), and membrane filtration (MF).

Serial Dilutions

One of the basic requirements of any enumeration process is the required dilution of the sample to achieve a countable population on culture plates that is between 25 and 250 colony forming units. The most common diluent is saline water (0.85% NaCl). Others include phosphate-buffered saline (PBS) and 0.1% peptone water. Test portions of 1.0 or 11.0 ml of liquid milk and other non-viscous products are added to 9.0 or 99.0 ml of the diluent volume, respectively (1:10 dilution). In the case of semi-solid or solid dairy foods samples, test portions of 1.0 or 11.0 g are blended with respective volumes of diluent to make homogenates. Further serial dilution (1:100) is made by transferring 1 ml of 1:10 dilution to 9 ml fresh diluent, and so on. The dilution factor is generally based on the anticipated population of microflora in the test sample.

Plating Techniques

Microorganisms require a nutrient source for growth, which is provided by nutrient media. There are several types of media, of which plate count agar (PCA) is common for enumerating aerobic viable counts. The following types of techniques can be used to enumerate microorganisms using nutrient media.

Pour plate technique

For standard plate counts, 1.0 ml of the desired sample dilution is put in a sterile Petri plate. Replicate plates may be prepared for each dilution plated. This is followed by pouring 12–15 ml of liquefied agar medium adjusted to 44–46 °C into each plate, and the plates are swirled to mix the sample and the agar medium. The plates are then kept on a level surface to allow the agar to solidify. The plates are then inverted and incubated for 24–48 h at 32 ± 1 °C for mesophilic counts, or for 7–10 days at ≤7 °C for psychrophilic counts, or for 24 h at 55 ± 1 °C for thermophilic counts. For counting anaerobic bacteria, such as *Clostridium* spp., the plates are incubated in anaerobic jars. The plates having 25–250 colonies are counted on the completion of incubation, and the viable bacterial count in terms of colony forming units (cfu) per milliliter or gram is computed using a standard formula. Colony counts of less than 25 per plate are represented as the 'estimated counts' (ECs), and more than 250 colonies are represented as 'too numerous to count' (TNTC).

Dry dehydrated films (3M Petrifilms)

These ready-to-use culture media films are available as proprietary products from 3M Center (St. Paul, MN, USA). The plastic films are impregnated with the required nutrients, a cold gelling agent, and a tetrazolium indicator. The Petrifilms have been found to be quite similar to the SPC technique for milk and dairy products. The sample volumes of 1.0 ml are spread over a 20 cm^2 growth area. The obvious advantage of this method is the ready-made agar system, which saves time and sterilization effort. In addition, the indicator stains the colonies that makes it easier to read the results.

Spiral plate technique

Another prominent method that has been tested for milk and dairy products is the spiral plate method. This requires use of a spiral plater (Spiral Systems Instruments, Inc., Bethesda, MD, USA). The spiral plater has a stylus that can automatically deposit samples on a rotating plate in decreasing amounts as the spiral moves out toward the edge of the plate. The plates are then incubated in a manner similar to the SPC technique. Counting of colonies requires a specialized grid and computation for final counts.

Hydrophobic grid membrane filter technique

The hydrophobic grid membrane filter (HGMF) technique has found great use with liquid samples or sample homogenates that have very low counts and require large volumes to be tested. The hydrophobic nature of the grid coating helps to spread the bacterial colonies. Large volumes of the sample can be passed through the membranes to capture low numbers of cells. The HGMF method employs the use of a general-purpose nutrient medium with the fast green (FCF) stain. The green-colored colonies are easier to count. The enumeration is based on most probable number (MPN) determinations.

Most probable number technique

Another culture technique for enumerating low counts, especially coliforms, is based on the multiple tubes culturing method. The presumptive counts are based on MPNs. An example of this is the use of brilliant green lactose bile (BGLB 2.0%) broth tubes with inverted Durham's tubes for the enumeration of coliforms. The serially diluted sample of the liquid milk or product homogenate is added at 1.0, 0.1, and 0.01 ml volumes in a set of three or five tubes for each dilution. The tubes are incubated at 32 ± 1 °C for 24–48 h and are observed for the collection of gas in Durham's tube and for any color change in the broth. The positive tubes are compared with McCrady's probability tables for computing the MPNs.

Enumeration of Specific Groups of Microorganisms

Milk and milk products are pasteurized (high temperature short time (HTST) at 72 °C for 15 s or equivalent process), which ensures complete elimination of non-spore-forming pathogenic bacteria, *Coxiella burnetii* being the index organism for the process. Pasteurization concurrently reduces the population of non-pathogenic bacterial numbers substantially. Ultra-pasteurization further reduces the bacterial numbers, and ultra-high temperature (UHT) (140–150 °C for a few seconds) processing aims to achieve a near-sterile product. Despite these thermal treatments, some resistant bacteria are carried over to the final product. Post-process contamination and cross-contamination may also lead to an increase in bacterial numbers and types in milk and milk products. Special techniques are used to isolate and identify these specific microorganisms.

In addition to this, fermented dairy products are manufactured using desirable lactic acid bacteria. Some of these are referred to as probiotics and are used in several value-added products. Isolation and identification of these organisms requires some modification of techniques.

In the following section, we will discuss the isolation and identification of common dairy pathogens, useful lactic acid bacteria, and common spoilage bacteria.

Common Pathogens in Milk and Milk Products

Coliforms and Escherichia coli

Coliforms are often regarded as indicators of the overall food quality and the hygienic conditions prevalent in dairy processing environments. As a group, they include genera *Escherichia, Enterobacter*, and *Klebsiella*. Coliforms also include several bacteria of non-fecal origin; hence, they are not intended to be used as exclusive indicators of fecal contamination or other enteric pathogens. Incubation at 45.0 ± 0.2 °C is generally used to differentiate fecal from non-fecal coliforms. Violet-red bile agar is the selective medium of choice for the isolation of coliforms. In case of stressed or injured cells, a resuscitation step needs to be included prior to pour-plating using general-purpose enrichment media such as tryptic soy broth. Direct differentiation of *E. coli* is done on eosin methylene blue agar, on which *E. coli* forms dark colonies with a green metallic sheen. A modified fluorogenic assay involves the use of any medium containing the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). The test is based on the nearly exclusive presence of the enzyme β-glucuronidase in *E. coli*. This enzyme *cleaves* MUG in the medium to *fluoresce* under UV light (366 nm). Isolates suspected of being *E. coli* are confirmed by IMViC (indole production, Voges-Proskauer test, methyl red test, citrate utilization) pattern. It must, however, be kept in mind that this test may not help in species differentiation, as about seven species may have a pattern similar to typical and atypical *E. coli*. The Gene-Trak is an example of a DNA hybridization-based semi-quantitative test that is also being used to identify *E. coli* from food samples containing ≥3 cells per gram or milliliter using specific probes.

Staphylococcus aureus

Another pathogen of significance in milk and dairy products is *Staphylococcus aureus*. The organism has been identified frequently as a causative agent of bovine mastitis. Post-process contamination due to human contact is also a matter of serious concern in dairy production and manufacturing environments. The organism is also known to produce several thermostable enterotoxins (serotypes A–E) that have the potential to be carried over to the final products. Enrichment using tryptic soy broth containing 20% NaCl is a preferred method for recovering the stressed or injured cells. Selective plating is done on Baird-Parker (BP) agar by the surface plating procedure. Plates are incubated for 48–72 h at 35–37 °C. The typical colonies appear gray to black, surrounded by an opaque zone and an outer clear zone.

A positive coagulase or clumping factor test is generally considered as a confirmatory test for *S. aureus*. The suspect colonies from BP agar are transferred to small tubes containing 02–0.3 ml brain heart infusion (BHI) broth and incubated for 18–24 h at 35 ± 1 °C. To these tubes is added 0.5 ml reconstituted coagulase plasma with ethylenediamine tetraacetate (EDTA). The tubes are incubated at 35–37 °C and examined periodically for clot formation during a 6-h interval. A 3+ or 4+ clot formation is considered a positive reaction for *S. aureus*. The staphylococcal isolates can also be tested for enterotoxin production by semi-solid agar or micro-slide methods that are also approved by the Association of Official Analytical Chemists (AOAC).

Salmonella *species*

Salmonella species are perhaps the most frequent pathogens associated with milk and dairy products. The enumeration of *Salmonella* is a complex process that often includes selective or non-selective pre-enrichment, selective enrichment, followed by selective and differential plating. Confirmation is generally done using biochemical and serologic tests. Lactose broth is a good example of a pre-enrichment medium. Rappaport-Vassiladis (RV) medium is likely to replace selenite cystine (SC) broth as a selective enrichment process due to the known toxicity of selenium, which is formed from the reduction of sodium selenite during the incubation. For maximum recovery of cells, the preferred temperature of incubation is 42 °C for RV broth. After the enrichment process, the colonies can be isolated on one or more several plating medium such as brilliant green (BG), bismuth sulfite (BS), desoxycholate citrate (DS), xylose lysine, and desoxycholate citrate (XLD). Triple sugar iron (TSI) agar and lysine iron agar (LIA) are used in combination as biochemical tests that are based on the production of hydrogen sulfide and the decarboxylation of lysine. The confirmation of *Salmonella* is generally done by specific antigenic components including somatic (O), flagellar (H), and capsular (K) and classified by the Kaufmann White or the Edwards and Ewing scheme.

Lactic Cultures and Probiotics

Lactic acid bacteria are essential for a variety of dairy fermentations. The major genera include *Streptococcus, Lactococcus, Leuconostoc,* and homofermentative and heterofermentative *Lactobacillus* species. In addition to acid production, several of these cultures are now used to develop probiotic products that provide health benefits beyond basic nutrition. Lactic acid bacteria may also be responsible for some acid defects that may result in reducing the shelf stability of milk and dairy products. The growth requirements of these groups are diverse, so no growth medium can be applied to all samples. Enumeration of stressed or injured cells may require non-selective enrichment prior to plating. An example is the sub-lethally injured population of lactobacilli that survive milk pasteurization.

Lactococcus *spp.*

Many dairy starters constitute different species of *Lactococcus*. This important group of lactic acid bacteria can be isolated using selective media such as lactic (Elliker) agar, Rogosa SL agar, M 17 agar, and APT agar. Incubation of plates is done under aerobic conditions for 48 ± 3 h at 30 or 37 °C depending upon mesophilic or thermoduric enumeration. Fermented dairy products normally contain high numbers of starters or secondary fermentation microflora, making total counts insignificant except for products that are thermally treated to inactivate added cultures.

Lactobacillus *spp.*

Lactobacilli are considered as fastidious organisms. The homofermentative species produce lactic acid during carbohydrate fermentation, and several other products such as acetic acid, ethanol, and CO_2 are produced frequently in varying proportions by these species. The deMan, Rogosa, and Sharpe (MRS) medium supports the growth of various lactobacilli. Species of *Pediococcus* and *Leuconostoc* also grow luxuriously on this medium. To facilitate colony formation, the incubation is carried out for 48–72 h at 35 °C under anaerobic conditions. As MRS medium is not highly selective, colonies are tested for catalase-negative, Gram-positive cocci (lactococci) or rods (lactobacilli) for tentative identification as lactic acid bacteria.

Probiotic microorganisms

Several lactic acid bacteria have been placed recently under another group referred to as ‘probiotic microflora’. These organisms are claimed to provide health benefits beyond basic nutrition. In the case of probiotic microorganisms, identification of genus and species becomes critical, as several of the probiotic effects appear to be strain-specific. A working group of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) provides general guidelines for evaluation of probiotics in foods that include testing the isolates for characteristics such as resistance to gastric acidity, bile acid resistance, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogen adhesion to surfaces, and bile salt hydrolase activity.

Spoilage Groups

The microbiological quality of milk and milk products is greatly influenced by the initial microflora of the raw milk and by processing and post-process contamination. Conditions such as temperature abuse leads to rapid multiplication of these bacteria leading to several defects. In milk and dairy products, a large number of microorganisms have been shown to cause changes that will lead to spoilage. This diverse population can be categorized into several groups based on the general nature of spoilage. Some of the important groups are described in the following sections.

Proteolytic microorganisms

Many bacteria hydrolyze proteins and are responsible for a variety of flavor and odor defects, especially in refrigerated dairy products under extended storage. Proteolytic counts can thus be useful in identifying sources of contamination of such bacteria. Skim milk agar (SMA) plating is commonly used to enumerate proteolytic bacteria in a manner similar to plate counts as described earlier. The hydrolysis of casein, as reflected by a zone of clearance against an opaque background of SMA, indicates proteolysis. To confirm proteolysis, a dilute acid solution (1% HCl or 10% acetic acid) is added to the agar surface to precipitate any undigested casein. Another common test is the use of litmus milk. A well-isolated colony is transferred to a litmus milk tube and incubated at the optimum temperature of the organism for up to 14 days. Coagulation of the milk without any change in the blue color of the dye is evidence of proteolysis.

Lipolytic microorganisms

Milk and dairy products may have several microorganisms that are lipolytic. These organisms produce lipases, the enzymes responsible for hydrolyzing the carboxylic acid ester bonds of triglycerides thereby liberating free fatty acids (FFAs). The FFAs can be oxidized and they release compounds with undesirable organoleptic effects in dairy products. Victoria blue B–tributyrin agar is used to detect lipolytic activity. The organisms that produce lipases will release FFAs from tributyrin, and the Victoria blue color becomes visually brighter in the presence of FFAs. Lipolytic microorganisms on tributyrin agar without Victoria blue B are detected by a transparent zone surrounding the colonies. The recovery of psychrotrophs can be increased by incubating for 72 ± 3 h at 21 ± 1 °C.

Yeast and mold counts

Yeasts and molds form another important group of spoilage microflora. Most bacteria cannot grow at a_w levels below 0.85, whereas some yeasts and molds can grow even at a_w levels of as low as 0.61. They may cause several undesirable changes in refrigerated dairy products under extended storage. In addition, there is a large variety of mold-ripened cheeses that are processed with desirable molds. Potato dextrose agar (PDA) is acidified to pH 3.5 by adding sterile 10% tartaric acid to the pre-sterilized molten agar. The plates are prepared as described in the 'Plating Techniques' section and are incubated for up to 5 days at 25 ± 1 °C. Another plating medium that is commonly used for testing of yeasts and molds is antibiotic plate count (APC) agar. In this case, the plate count agar is supplemented with chlortetracycline hydrochloride and chloramphenicol solutions. Some other examples are yeast extract–glucose–chloramphenicol agar (International Dairy Federation (IDF) recommended), and dichloran–rose Bengal–chloramphenicol (DRBC) agar. Plates with 15–150 colonies are counted at the end of incubation, and the yeast and mold counts are reported per gram or milliliter of product.

Microscopic Techniques

Direct Microscopic Counts or Direct Microscopic Clump Counts

The direct microscopic technique is a rapid method for examining individual bacterial cells (direct microscopic counts; DMCs) and bacterial clumps (direct microscopic clump counts; DMCCs). This technique involves counting both dead and live cells; however, some of the dead cells may lose the ability to become stained. Similarly, very-low-count pasteurized milk is not suitable for evaluating compliance with product standards using this method. Hence, the method is generally applied to raw milk and dry dairy products. An 0.01 ml volume of well-mixed milk sample is spread on a 1.0 cm^2 circular area of a microscopic slide and allowed to air dry. The slides are stained with modified Newman-Lampert stain or Levowitz-Weber stain and viewed under an oil-immersion objective (×100). The individual cells and cell clumps are counted in a statistically appropriate number of fields. The DMC or DMCC per milliliter sample is expressed after multiplying by the microscopic factor (MF). The MF can be determined by the formula $\mathrm{MF} = 40\,000/\pi d^2$, where $\pi = 3.1416$ and $d =$ diameter of the microscopic field.

Direct Microscopic Somatic Cell Counts

Mastitis continues to be the most costly and perplexing disease facing dairy producers. Although clinical mastitis (abnormal milk) is easily detected, the major loss is due to subclinical mastitis when milk appears normal to the eye but the udder is still infected. Subclinical mastitis can be detected by culture techniques or the direct microscopic somatic cell count (DMSCC). When making DMSCC, only those somatic cells with an identifiable stained nucleus are counted. Milk from uninfected mammary glands generally contains <100 000 somatic cells per milliliter.

Rapid Methods

Various unconventional rapid methods are increasingly being used to evaluate quickly the microbiological quality of milk and dairy products. Some of the prominent methods are Vitek automicrobic Gram-positive and Gram-negative identification cards (BioMerieux Vitek, Hazelwood, MO, USA), API identification kits for different groups of bacteria (BioMerieux Vitek), Micro-ID$^{\mathrm{TM}}$ kit (Organon Teknika Corp., Durham, NC, USA), ELISA test kits for detection of specific antigens by polyclonal or monoclonal antibodies, TECRA visual immunoassays (Bioenterprises Pty. Ltd., Roseville, NSW, Australia), visual immunoprecipitate assays (BioControl Systems, Inc., Bothell, WA, USA), nonradioactive DNA probe test kits (Gene-Trak Systems, Framingham, MA, USA), AccuProbe$^{\mathrm{TM}}$, a chemiluminescent genus-specific assay (Gen-Probe, San Diego, CA, USA), and automated Riboprinter microbial characterization systems (Qualicon, Wilmington, DE, USA).

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DNA-Based Assays

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Introduction

The development of DNA-based methods in the past 30 years has allowed for the rapid and direct identification of lactic acid bacteria (LAB), bifidobacteria, and common dairy pathogens from enrichment cultures as well as directly from food matrices. The ability of two single-stranded DNA molecules to form double-stranded DNA by specific base pairing *in vitro* is the basis of all DNA-based detection methods. A number of these high-throughput analytical methods rely on the discriminatory power of the hypervariable regions of 16S rDNA gene sequences, allowing for identification and taxonomic placement of species into a phylogenetic framework. These DNA-based assays are fast, robust, and far better than traditional methods relying on phenotypic approaches, which often lack the resolving power to handle the complexity of bacterial populations.

Polymerase Chain Reaction

Conventional Polymerase Chain Reaction

The advent of the polymerase chain reaction (PCR) has led to an explosion of amplification-dependent DNA-based assays. PCR assays have been extremely successful because they allow selective *in vitro* amplification of a desired DNA region by mimicking the steps of *in vivo* DNA replication. In the PCR method, a specific amplification of a defined target DNA is achieved by successive cycles of three steps: denaturation of DNA to obtain single-stranded targets, annealing of short and specific primers to the target DNA, and polymerization of DNA starting from the primers by use of a thermostable DNA polymerase. The number of amplicons is doubled in each cycle, and except for the fragments produced with the original target DNA as template, all successive products will be of a fixed size, corresponding to the distance between the two primers. Detection of the positive reaction can be confirmed by demonstrating the correct DNA fragment by agarose gel electrophoresis.

Multiplex PCR

Multiplex PCR-based assays allow for the use of several primer pairs in a single reaction. Simultaneous amplification of multiple DNA regions of interest in one assay reduces work, time, cost, and the risk of cross-contamination, since sample handling is minimal. Many multiplex assays used for the taxonomic identification of microorganisms focus on the variable regions of the 16S rDNA and 23S rDNA gene sequences, as well as the 16S–23S rDNA intergenic spacer region. Multiplex assays have been successfully used in food microbiology to simultaneously detect LAB in commercial dairy products, to detect variants of enteropathogenic *Escherichia coli*, to differentiate among *Listeria* species, and to characterize strains of *Staphylococcus aureus* with respect to enterotoxin type.

Immunomagnetic Separation

In some cases, the application of immunomagnetic separation facilitates the preparation of template, that is, DNA or RNA targets. Antibodies specific for a selected pathogen are chemically bonded to coat paramagnetic beads. Therefore, when these beads are mixed with samples containing pathogenic microbes, the antigens present on the surface of these bacterial cells will bind with the antibody-coated bead. This in essence captures the targeted cells and also acts as a means to concentrate the selected pathogens. The process of concentrating the antibody-coated beads–antigen complex is accomplished by using a magnet placed on one side of the sample vessel.

Real-Time PCR

In real-time PCR assays, amplification and analysis occur simultaneously. DNA dyes and/or fluorescent probes are added to the PCR mixture before amplification, allowing for data to be collected during amplification in the same tube and in the same instrument. The power of real-time PCR techniques lies in the ability simultaneously to detect, quantify, and analyze during DNA amplification. As a result, real-time assays have a lower limit of detection, and provide results faster than conventional assays, which depend on some form of gel electrophoresis for fragment size confirmation. Moreover, the availability of multiple dyes and probes that fluoresce at different wavelengths allows for the development of multiplex assays using this rapid detection method.

Amplified Fragment Length Polymorphism

DNA templates for amplified fragment length polymorphism analyses are prepared by first cutting the bacterial genome with two enzymes. This results in DNA fragments with two different types of sticky ends. Appropriate adaptors are ligated to these ends to form templates for PCR. Primers are designed containing the adaptor sequence extended to include one or more selective bases next to the restriction site of the primer. Only fragments that completely match the primer sequence are amplified, resulting in selective amplification, which is dependent on the initial DNA structure and restriction fragments. The amplification process results in an array of 30–40 DNA fragments that can be group, species, and even strain specific, and are detected by agarose gel electrophoresis.

Restriction Fragment Length Polymorphisms

PCR-restriction fragment length polymorphism analyses involve the amplification of a target region, followed by restriction enzyme digestion. Restriction endonucleases, such as *Eco*RI and *Hind* III, cut the amplified DNA products at certain recognition sequences, usually 4–8 bp long. Sequence polymorphisms may be present in the amplified product, which are mostly a consequence of single-nucleotide substitutions, which sometimes alter the site of cleavage for the restriction enzymes. Alterations in the positions of cleavage sites result in DNA fragments of different sizes. The fragments of DNA produced by restriction analysis are then separated by size using gel electrophoresis.

Repeated Extragenic Palindrome/ Enterobacterial Repetitive Intragenic Consensus Fingerprinting-PCR

Repeating sequences are present in the genomes of all organisms. The most extensively studied repeated sequence is the repetitive extragenic palindrome (REP). This repeating sequence has a copy number of 500–1000 and consists of a 35–40 bp inverted repeat. It is found at characteristic locations in prokaryotic genomes, with successive copies arranged in opposite orientations. Enterobacterial repetitive intragenic consensus (ERIC) sequences are 124–127 bp long and have a copy number of about 30–50 in *E. coli* and 150 in *Salmonella typhimurium.* REP and ERIC sequence repeats are located in noncoding but probably transcribed regions of the chromosome and have a potential stem–loop structure. REP and ERIC PCRs have been used to identify numerous lactobacilli as the amplification generates complex and highly specific genomic fingerprints.

Randomly Amplified Polymorphic DNAs

In the case of randomly amplified polymorphic DNAs (RAPDs), arbitrary primers of 8–10 bp in length are used to amplify random genomic DNA fragments during a PCR assay. This technique focuses on existing DNA polymorphisms and has been extensively used to type LAB, to monitor population dynamics in food fermentation, and to estimate the diversity of LAB in numerous foods. The RAPD fragments are separated by size using gel electrophoresis.

Denaturing Gradient Gel Electrophoresis/ Temperature Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) methods have been developed for the analysis of microbial communities without culture, by the sequence-specific separation of PCR-amplified 16S rDNA fragments. Separation is based on the lower electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants, a mixture of urea and formamide (DGGE), or in gels subjected to a linear temperature gradient (TGGE). The members of the microbial community are amplified using 16S rDNA-specific primers. Species are then distinguished by comparing the migration distance of the PCR products in gels with those of reference strains. DGGE and TGGE have been used extensively to evaluate the microbial diversity of *Lactobacillus* communities in cheeses, sausages, and other food matrices.

Isothermal PCR

The high demand for handheld diagnostic devices that can be used to detect pathogens in the field has led to the development of a number of isothermal amplification methods. Unlike amplification reactions that require several reaction temperatures, isothermal reactions can be performed at a uniform temperature, thereby eliminating the need for expensive equipment. Several variations of this technology exist, and some are discussed below. Variations of isothermal PCR include transcription-mediated amplification, nucleic acid sequence-based amplification (NASBA), strand displacement amplification, rolling circle amplification, loop-mediated isothermal amplification of DNA, isothermal multiple displacement amplification, helicase-dependent amplification (HDA), and circular. These techniques can use DNA or RNA as target molecules for amplification and have great potential in diagnostic applications.

Nucleic acid sequence-based amplification

NASBA begins with the synthesis of a DNA molecule complementary to the target nucleic acid, which is usually RNA. This is accomplished by using a primer containing a T7 RNA polymerase binding site at one end. Following heat denaturation, a second primer binds to the newly formed cDNA and is extended, resulting in the formation of double-stranded cDNAs with one or both strands then serving as templates for T7 polymerase. The RNA portion of the RNA–DNA duplex formed during the first step of amplification is destroyed by the addition of RNase H, allowing the amplification process to be carried out under isothermal conditions. NASBA applications have been primarily marketed for the detection and quantification of viruses such as the human immunodeficiency virus and cytomegalovirus, a member of the herpesviruses.

Helicase-dependent amplification

Unlike conventional PCR assays, which require thermocycling to separate the two DNA strands, HDA allows for an isothermal reaction. The use of DNA helicase to generate single-stranded templates for primer hybridization and subsequent extension by a DNA polymerase allows HDA assays to be carried out at one temperature for the entire process. As the DNA helicase unwinds double-stranded DNA enzymatically, the initial heat denaturation and subsequent thermocycling steps required by PCR can all be omitted, resulting in a method that can be developed into a simple portable DNA diagnostic assay. **Figure 1** illustrates the basic principle behind this technology.

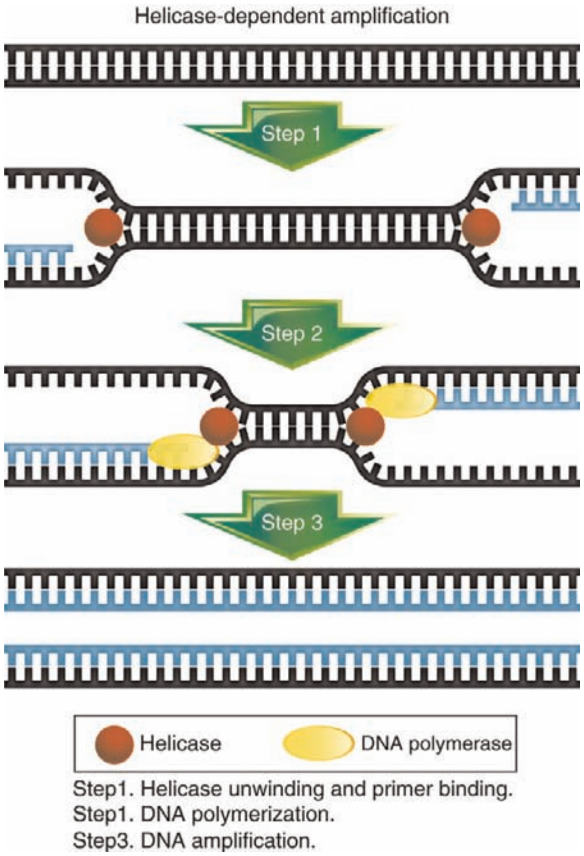
Helicase-dependent amplification
Step 1
Step 2
Step 3
Helicase
DNA polymerase
Step1. Helicase unwinding and primer binding.
Step1. DNA polymerization.
Step3. DNA amplification.

Figure 1 Helicase-dependent amplification (HDA). HDA is an isothermal system that utilizes a DNA helicase to unwind the target DNA molecule, generating single-stranded templates. Primer annealing and subsequent synthesis of complementary strands by DNA polymerase yield another copy of the original target DNA sequence. The entire amplification reaction is performed at a single temperature.

Ribotyping

Ribotyping is based on the inherent ability of single-stranded DNA molecules to hybridize with one another. Isolated microbial DNA is digested with restriction enzymes, specifically endonucleases, followed by agarose gel electrophoresis. The DNA is then transferred to a nitrocellulose or nylon membrane for hybridization with labeled 16S, 23S, or 5S rRNA gene probes. Because bacteria have multiple copies of rRNA operons in their chromosome, several fragments in the restriction digest mixture hybridize with the probe, resulting in a microbial fingerprint. Ribotyping, in general, has a greater discriminatory power at the species level than at the strain level.

Pulsed-Field Gel Electrophoresis

Much like ribotyping, pulsed-field gel electrophoresis (PFGE) utilizes restriction enzyme digestion, particularly low cutting frequency enzymes such as *Sma*I and *Not*I. PFGE is dependent on the application of alternating electrophoretic current, which is ‘pulsed’ in different directions over a gradient of time intervals, allowing a range of DNA molecules, including those of mega-base size, to be resolved (see **Figure 2**). PFGE is highly discriminatory, successfully differentiating at the strain level, is reproducible, and generates a banding pattern that is easy to interpret.

DNA Microarray

DNA microarray technology has directly evolved from conventional DNA–DNA or DNA–RNA hybridization techniques, such as Southern blotting. Microarrays

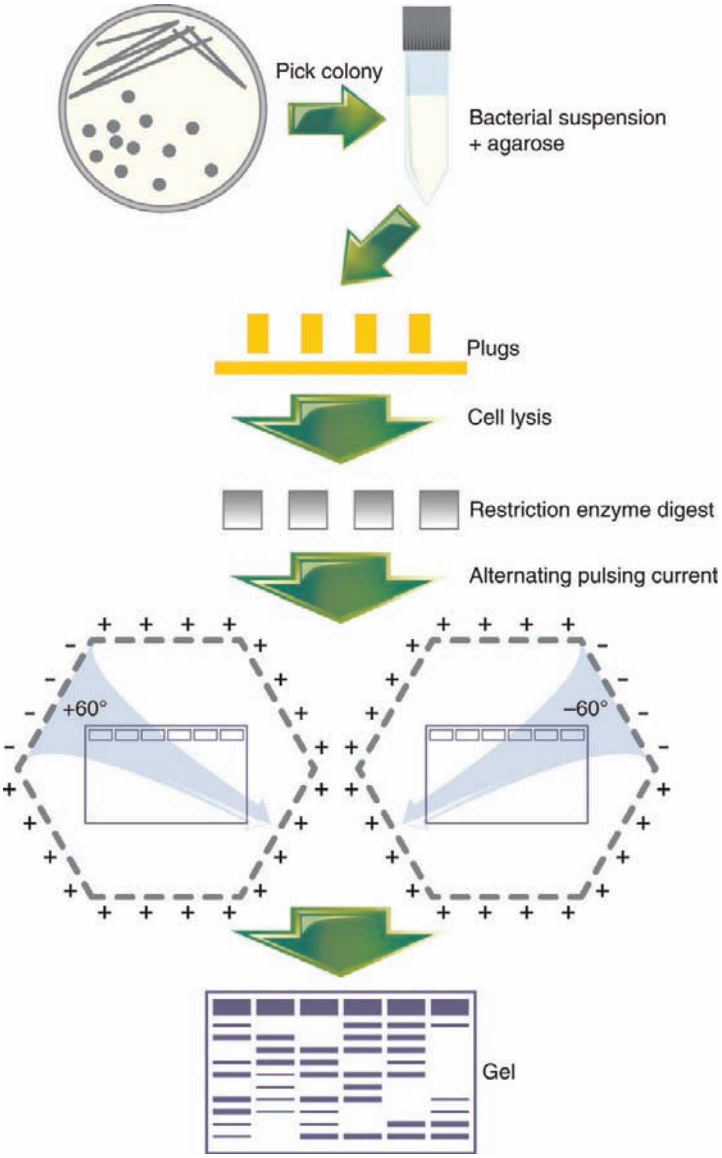

Figure 2 Pulsed-field gel electrophoresis (PFGE). DNA fragments larger than 20 kb are not able to be resolved on conventional electrophoresis platforms. PFGE uses alternating electrical fields to separate large DNA fragments in an agarose gel. This is accomplished by the pulsing action of electrical currents from spatially distinct electrode pairs enabling the DNA to reorient and move at different speeds through the gel.

consist of an arrayed series of defined oligonucleotides, commonly referred to as probes, which are covalently bound to a solid support surface, such as glass, silicon chips, or even to microscopic beads. These collections of spots (probes) are arranged in an orderly prescribed landscape so that capture of the probe–target hybridization, usually detected by means of fluorescence, can be automatically scored. The target is usually a specific gene or unique region of the bacterial chromosome or plasmid. These hybridizations can be monitored and perhaps quantified, based on the amount of fluorescence generated by the number of probe–target bound molecules. Microarray technology is revolutionary because unlike traditional DNA hybridization, which works with one probe at a time, thousands of probes can be placed into the same chip.

See also: **Lactic Acid Bacteria:** Genomics, Genetic Engineering; Taxonomy and Biodiversity. **Pathogens in Milk:** *Bacillus cereus*; *Brucella* spp.; *Campylobacter* spp.; *Clostridium* spp.; *Coxiella burnetii*; Enterobacteriaceae; *Enterobacter* spp.; *Escherichia coli*; *Listeria monocytogenes*; *Mycobacterium* spp.; *Salmonella* spp.;

Shigella spp.; *Staphylococcus aureus* – Molecular; *Staphylococcus aureus* – Dairy; *Yersinia enterocolitica*.

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Microscopy (Microstructure of Milk Constituents and Products)

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Introduction

The term microstructure generally describes structures in the length scale 0.1–100 μm. Milk constituents and dairy products exhibit a wide range of structures and physical states, including liquids, gels, foams, emulsions, plastic and thermoplastic materials, and solid powders. They range from high water content products (liquid milk) to dry products (milk powders) and from low-fat products (yogurt) to high-fat products (butter). Structuring elements may comprise fat globules, water droplets, air spaces, colloidal protein aggregates, and crystals. The textural behavior of these materials is greatly dependent on their microstructural elements and associated interactions. Dairy processing, for example, evaporation, shearing, or heating, also has profound effects on microstructure. Consequently, understanding the relationship between microstructure and a dairy product's functional and sensory attributes can aid the design of novel products.

Microstructural Analysis Techniques

Food microstructures may be studied directly using visualization techniques, such as microscopy, as well as indirectly by rheological or spectroscopic techniques. The most important microscopy techniques for the study of dairy systems are given in **Table 1**.

While some microscopy techniques may be more relevant than others for a particular application, a full understanding of the structural organization of food materials often requires complementary non-imaging techniques such as rheometry, spectroscopy, or large-scale deformation analysis.

Light Microscopy

The compound light microscope employs a series of glass lenses to produce a magnified image of the sample, showing details unavailable to the unaided eye. There are several imaging modes in light microscopy and each provides particular microstructural information:

- *Bright field*: Light is transmitted from below the specimen and requires a relatively thin sample (<100 μm). Contrast between different ingredients may be achieved by specific labeling with colored dyes. For dairy products such as cheese, frozen cryostat sections stained with Oil Red and Fast Green show fat and protein distributions, respectively.
- *Polarized light*: Two polarizing plates are arranged perpendicularly, one below the condenser and a second above the objective. If the polarized light passes through an anisotropic substance, part of the light is rotated and appears bright. Polarized light microscopy is useful for studying fat or lactose crystallization (**Figure 1**).
- *Phase contrast*: An annular phase ring situated in the condenser below the sample retards the phase of light by 1/4λ. Diffracted and non-diffracted light through the sample are recombined using a similar phase ring in the objective. Contrast is obtained due to differences in the refractive indices between the sample and its surroundings. This technique has been used to study milk fat droplets, which would otherwise be transparent in the bright field.
- *Differential interference contrast*: A polarizer and a prism are located above and below the specimen, analogous to phase contrast. Differences in refractive index are visualized in relief. This technique is particularly useful for studying phase separation and depletion flocculation in transparent mixed dairy emulsions (**Figure 2**).
- *Epifluorescence*: Most modern microscopes use epifluorescence, where a monochromatic beam of light is used to illuminate the specimen and the emitted fluorescence (either autofluorescence or dye-induced) is detected via the same optical path using appropriate optical filters. The fat and protein components of various cheese types have been studied using this technique. Confocal microscopy has largely superseded conventional epifluorescence in food studies.

Confocal Microscopy

The essential feature of confocal imaging is that both the illumination and detection systems are focused on a single volume element in the specimen. Conventional light microscopy uses wide-field illumination where the volume of sample above and below the plane of focus is uniformly and simultaneously illuminated. This necessitates a thin sample and also results in out-of-focus blur, which reduces resolution and specimen contrast. In confocal scanning laser microscopy (CSLM), a diffraction-limited illuminated spot is detected by means of a small aperture (pinhole) placed in front of the emitted light detector, greatly reducing out-of-focus information. The illuminated spot is then scanned across the specimen.

Table 1 Comparison of main microscopy techniques used to study dairy microstructure

Technique	*Incident radiation type and wavelength range (nm)*	*Approximate resolution (nm)*	*Application*
Stereomicroscopy	Photons, 400–750	5000	Overview of microstructure, gross organization of components
Compound light microscopy	Photons, 400–750	300	Ingredient localization, emulsion droplet sizing
Confocal microscopy	Photons, 350–1200	200	Ingredient localization, three-dimensional reconstruction, dynamic processes
Scanning electron microscopy	Electrons, 0.001–0.01	4	Large depth of field, simulated three-dimensional view
Transmission electron microscopy	Electrons, 0.001–0.01	0.2	Visualization of macromolecular structures

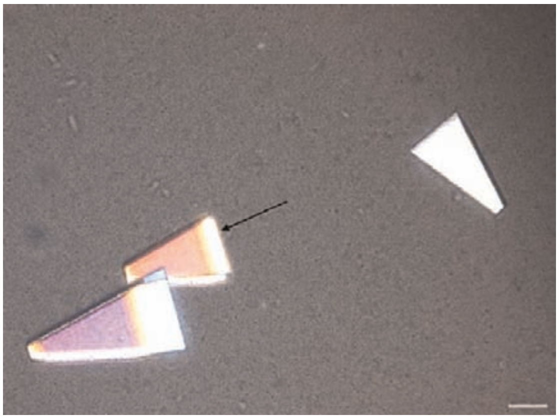

Figure 1 Polarized light micrograph of evaporated milk, showing characteristic tomahawk-shaped α-lactose monohydrate crystals (arrow). Scale = 25 μm.

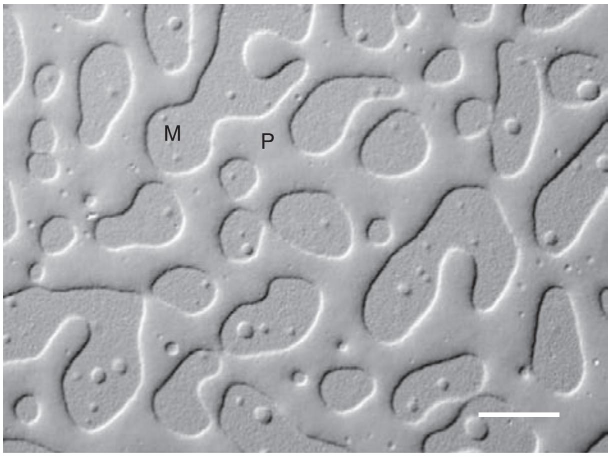

Figure 2 Differential interference contrast micrograph of a skim milk/locust been gum mixture showing phase separation between milk (M) and polysaccharide-rich (P) phases. Scale = 20 μm.

A stepping motor fitted to the sample stage enables acquisition of consecutive *x–y* planes of focus through the *z*-plane, resulting in a three-dimensional data set. Advantages of CSLM include

- three-dimensional imaging of bulk samples by optical sectioning and digital reconstruction,
- minimal sample disturbance,
- improved resolution,
- sensitive detection of fluorochrome probes, and
- study of dynamic processes under ambient conditions.

A wide variety of dairy products have been investigated using CSLM; these include yogurt, milk protein gels, dairy spreads, milk powders, and several cheese varieties. Dynamic events such as protein gelation can be monitored in real time. The effects of processing parameters – time, temperature, pH, and shear – on milk and dairy products can thus be studied directly. Visualization by CSLM requires that the object of interest is fluorescent, which is usually achieved by labeling with specific fluorochromes such as Nile red (for lipids) or Rhodamine B (for proteins). Combinations of dyes enable simultaneous labeling of dairy components (**Figure 3**). Emerging techniques such as confocal Raman microscopy may facilitate spatial mapping of food materials without the need for specific labeling.

Electron Microscopy Techniques

The resolution of a light microscope is inversely proportional to the wavelength of light – accelerated electron beams have a much shorter wavelength and consequently give greatly increased resolution. Electron microscopes (EMs) basically consist of an electron gun encased in a high vacuum. The electron beam is focused with electromagnets and an image is produced either by electrons passing through a thin section of material (transmission electron microscopy (TEM)) or by electrons impinging on the surface of a bulk sample and emitting further

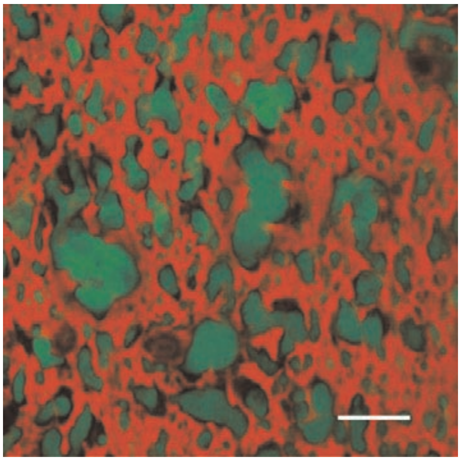

Figure 3 Confocal scanning laser micrograph of dual-labeled (Nile Red (lipids) and Fast Green FCF (proteins)) Cheddar cheese showing fat (green) and protein (red) phases. Scale = 25 μm.

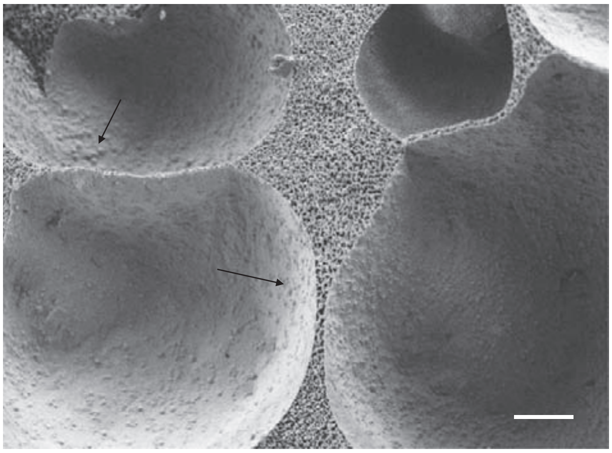

Figure 4 Cryo-scanning electron micrograph of whipped cream showing large air bubbles stabilized by the aqueous protein phase and fat droplets (arrows). Scale = 10 μm.

secondary electrons (scanning electron microscopy (SEM)). Traditionally, chemical fixation and dehydration protocols were necessary to preserve milk proteins and lipids from the harsh environment of EMs. Recent developments in cryo-electron microscopy and 'environmental' SEM have greatly reduced sample preparation. As with all microscopy techniques, interpretation of all EM images requires a thorough understanding of the effects of sample preparation, whether by chemical fixation or freezing, on the integrity of microstructural elements.

Scanning electron microscopy

In the SEM, secondary electrons emitted by the sample provide topographic information. The high depth of field gives a simulated three-dimensional view of samples. Many dairy products have been studied by SEM; these include milk gels, yogurt, food powders, and various cheese types including cream cheese, Cheddar, Mozzarella, and Gouda. Traditional sample preparation included glutaraldehyde/osmium tetroxide fixation, followed by critical point drying or freeze drying. To prevent charging of the sample surface in the electron beam, a thin coating of gold or platinum is applied under vacuum. Two major recent advances in EM technology include (1) replacement of tungsten filaments with a field emission source to facilitate low kV observation (<1 kV) and (2) variable pressure or 'environmental' SEMs that allow high-resolution imaging of hydrated samples with little or no sample preparation. Cryo-SEM, where frozen hydrated samples can be imaged, has been used successfully to study dairy spreads and ice cream, although freezing artifacts, predominantly due to ice crystal formation, can occur. Freeze fracturing allows visualization of internal structures, such as air distribution in whipped cream (**Figure 4**). Elemental mapping of milk salts can be obtained by X-ray microanalysis.

Transmission electron microscopy

TEM involves passing a narrow beam of electrons through a thin specimen at accelerating voltages in the range 60–200 kV. The sample may be prepared either as a negatively stained dispersion or in the form of a thin section or a metallic replica. For negative staining, the sample is immersed in a solution of a heavy metal salt such as uranyl acetate. When dried, the sample appears translucent, but enables observation of internal or surface structures. This technique is useful for dilute protein dispersions and has been used to study casein micelles (**Figure 5**), their subunit structure, and their association with whey proteins. For thin sections, sample preparation can be extensive and usually involves chemical fixation in glutaraldehyde and osmium tetroxide, solvent dehydration, and resin

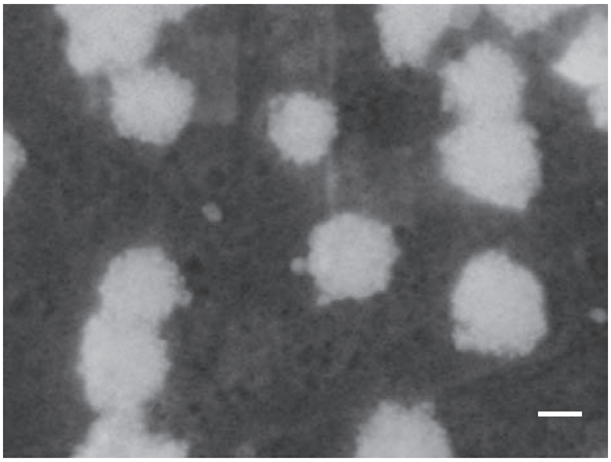

Figure 5 Transmission electron micrograph of bovine casein micelles (bright against a dark background). Phosphotungstic acid was used as a negative stain (pH 6.6). Scale = 100 nm.

embedding. Ultrathin sections ~90–150 nm thick are cut using an ultramicrotome, and the sections are post-stained to increase contrast and carbon coated to increase resistance to beam damage. A third main TEM technique is replica shadowing, usually in combination with freeze fracturing or etching. A small sample of the product is frozen rapidly and fractured to expose internal features. The fracture face may then be warmed under vacuum to allow sublimation of water molecules from the fracture surface to reveal ultrastructural details. Carbon or a heavy metal is then evaporated at an oblique angle to the sample, which is supported on a thin grid. The evaporated coating thus matches the contour features of the sample. This technique has been used extensively to study interfacial features such as fat globule membranes. More recently, frozen hydrated sections of milk proteins including casein micelles have been examined in the TEM using a specially cooled cryo-TEM stage. Localization of specific proteins or polysaccharides may be achieved using immunogold labeling. Small gold particles of a known diameter (from 1 to 25 nm) are conjugated to a ligand or receptor, which may be an antibody (for proteins) or a lectin (for polysaccharides). β-Lactoglobulin, casein, and bovine whey protein have been localized in reduced-fat cheese using this approach.

Atomic Force Microscopy

Atomic force microscopy (AFM) is being used increasingly to probe food materials, including milk proteins. An oscillating cantilever with a fine silicon nitride tip literally ‘feels’ across the sample surface to produce a topographic image with a height resolution of ~1 Å. Individual biopolymer molecules can be visualized by AFM (**Figure 6**), making it the ideal tool for characterizing nanostructures that are beyond the resolution limits of electron microscopy.

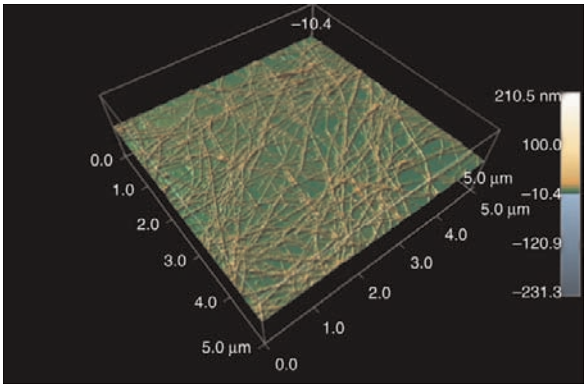

Figure 6 Atomic force topographic (height) image of β-lactoglobulin nanofibrils. Fibril diameter was measured at 3 nm. Scan width 5 µm.

Nonmicroscopy techniques

The structural organization of milk and dairy products may also be characterized using the following nonmicroscopy techniques:

- Rheological and mechanical tests: The size and shape of dairy proteins in liquids may be deduced from their rheological properties using rheometers. Small-scale deformation of viscoelastic products, such as milk gels, may be studied by low-amplitude oscillatory rheometry and it gives information on their interparticle interactions. Large-scale deformations, such as Cheddar cheese fracture, are studied by Instron-type texture analyzers and they give information relating to sensory properties, for example, hardness or fracture stress.
- Dynamic light scattering may be used to measure particle size distributions of fat droplets in dairy-based emulsions, and photon correlation spectroscopy may be used to analyze particle size distributions in dilute suspensions of milk proteins (size range 20–1000 nm). Diffusion wave spectroscopy analyzes multiple scattering phenomena associated with concentrated gelling systems.
- Nuclear magnetic resonance using D_2O can be used to study protein unfolding, while magnetic resonance imaging can be used to study mass/energy transfer phenomena.
- Fourier transform infrared spectroscopy, when combined with light microscopy, can give localized chemical characterization with a spatial resolution of ~10 µm.
- Ultrasound has been used to study creaming rates and casein micelle aggregation in milk.
- Differential scanning calorimetry provides information on phase changes of water, lipids, sugar glasses, and protein denaturation.
- X-ray diffraction is often used to characterize crystal structures (lipids, lactose, milk salts).

Processing Influences

Dairy ingredients undergo a wide range of processing influences, depending on their application. The function of processing may be to facilitate mixing of ingredients, improve shelf life, or impart texture, for example, in stretching of Mozzarella curd. Each processing step will affect the microstructure, and hence behavior, of a dairy product to a greater or lesser extent.

Microstructure of Milk

Native milk is a colloidal dispersion of proteins existing in a state of dynamic equilibrium together with lactose and

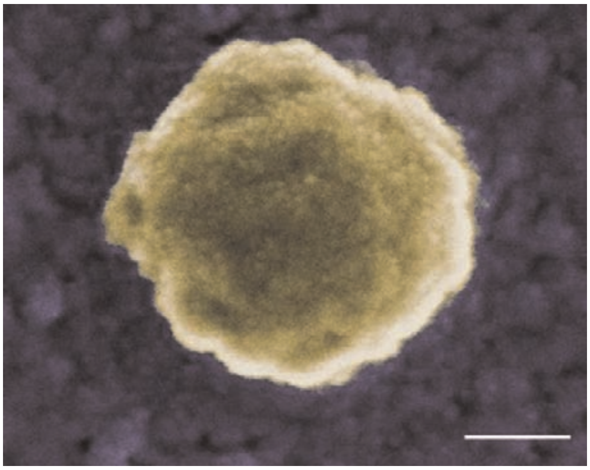

Figure 7 High-resolution scanning electron micrograph of a casein micelle. Scale = 50 nm.

fat droplets. The two main microstructural elements in native milk are casein micelles and fat globules.

- Casein micelles are protein aggregates of colloidal dimensions (50–250 nm; **Figure 7**). Casein micelles are a heterogeneous mixture of casein proteins with associated calcium phosphate. Two main models are proposed for the structure of the micelle: (1) subunit structure based on submicelles linked together by calcium bridges and (2) flexible array of casein molecules interlinking calcium phosphate 'nanoclusters'. In both models, the micelle is stabilized by a surface covering of κ-casein providing both steric and electrostatic repulsion.
- Fat globules are 0.1–10 μm in diameter, 90% of which are between 1 and 8 μm. The fat globule is composed of triglycerides present as a mixture of oil and crystals and is bounded by a membrane. Fat crystallization rate and morphology affect several processes including homogenization, churning, and creaming. The native fat globule membrane is complex, consisting of an inner phospholipid unit membrane ~0.1 nm thick with associated lipoproteins, glycoproteins, enzymes, and carotene. Although protein accounts for 2% of the weight of the fat globule, the total interfacial area of the membrane is $80\,\mathrm{m}^2\,\mathrm{l}^{-1}$ or $2\,\mathrm{m}^2\,\mathrm{g}^{-1}$ fat and can have a significant influence during subsequent processing.

The remaining serum phase contains dispersed whey proteins and dissolved lactose as major components. In milk, these components have little structural influence; however, they become more significant during subsequent processing, for example, heat denaturation of whey proteins or lactose crystallization during evaporation.

Centrifugation

Centrifugation is a separation technique that greatly accelerates the rise of fat globules from the aqueous phase, resulting in cream and skim milk. This has little effect on the microstructure of the milk components although partial coalescence of fat globules may occur.

Homogenization

Homogenization is a size-reduction process frequently applied to milk to ensure emulsion stability. Milk is forced by pressures mostly between 10 and 25 MPa through a small slit aperture at high speed causing deformation and rupture of milk fat globules. The accompanying fat globule size reduction, from 4 to <1 μm (mean volume diameter ~0.5 μm), results in an approximately sixfold increase in interfacial area. Much of the original phospholipid-based membrane remains attached to the globules, while casein micelles (preferentially), submicelles, and nondenatured whey proteins adsorb to denuded interfacial areas. The total protein loading is $\sim 10\,\mathrm{mg\,m}^{-2}$, 95% of which is casein. Homogenized milk fat globules behave differently from native globules in these respects:

- participation of fat globules in acid or enzymatic coagulation;
- clustering of fat globules at low pH;
- reduced foaming capacity of cream; and
- reaggregation of fragmented casein micelles at the fat interface.

High-pressure (HP) homogenization (>100 MPa), which causes partial breakdown of the casein micelles, has been shown to improve textural qualities of milk products as well as reduce microbiological activity.

Heating

Milk is relatively heat stable and its microstructure is not greatly affected by standard heat treatments such as pasteurization (typically 72 °C/15 s), although partial denaturation and structural reorganization of membrane proteins may occur. TEM studies, combined with immunogold labeling, have demonstrated that heating milk at higher temperatures or for a longer period results in denaturation of globular whey proteins with associated interaction of β-lactoglobulin with κ-casein on the micelle, a consequence exploited in yogurt manufacture. Heat-induced changes can be summarized as follows:

- denaturation of whey proteins and their self-aggregation and association with casein micelles;
- transfer of calcium and phosphate to casein micelles;
- aggregation of casein micelle/whey protein complexes above 100 °C; and
- denaturation of fat globule membrane proteins.

Concentrated milks are less heat stable, particularly if they are heated outside their optimum pH range of 6.4–6.6 and this can lead to calcium-mediated casein/whey protein precipitation or 'age gelation' (see below). The textural properties of heat-set whey protein gels are strongly influenced by heating rate. At slow rates, gels form coarse particulate networks with large pores, while at faster heating rates, gels contain smaller whey particles and pores as shown by light and electron microscopy techniques.

Concentration

Concentration of milk by vacuum evaporation has little immediate effect on its microstructure. However, there are two main time-dependent consequences of concentration:

- *Lactose crystallization*: As water activity decreases, lactose becomes increasingly likely to crystallize because of its relatively low solubility (22 g per 100 g water at 25 °C). Seeding lactose crystals into the cooled, supersaturated lactose solution controls this process by generating numerous small crystals undetectable in the mouth. Sweetened condensed milk, as used in caramel manufacture, contains ~44% added sucrose; the high viscosity of the product retards further crystallization and prevents fat separation.
- *Age gelation*: If evaporated milks are stored for extended periods, interactions between the casein and calcium salts can lead to gelation.

Dehydration

Dried dairy powders (<5% water) are formed either by roller drying or, more commonly, by spray drying. Roller drying whole milk results in flattened irregular plates of amorphous lactose containing protein and >90% solvent-extractable fat, making it an ideal milk ingredient for chocolate, which has a continuous fat phase. Most milk powder is spray dried, where whole or skim milk is forced through a narrow opening and into a stream of hot air, which evaporates the water. The resulting rounded particles comprise aerated amorphous lactose phase entrapping the remaining milk components (**Figure 8**). Small pores or cracks, produced during drying or cooling, increase the porosity of powders affecting their dispersibility. The dispersibility of powders is not greatly affected by particle microstructure, although agglomeration of powder particles by rewetting and redrying improves their subsequent rehydration. Amorphous lactose is highly hygroscopic, and moisture uptake during storage can lead to lactose crystallization and disruption of fat globules with concomitant release of surface fat and caking of the powder into lumps.

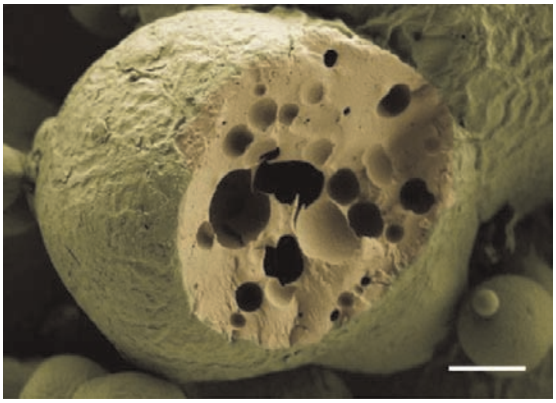

Figure 8 Scanning electron micrograph of a fractured milk powder particle showing rough surface and porous internal structure. Scale = 10 μm.

Freezing

Freezing of milk usually causes destabilization of the casein, due to increased milk salts and reduced pH in the non-frozen portion of the milk. Frozen products such as ice cream may be described as partly frozen foam containing ~50% air by volume, 50% water, 10% fat, 12% sucrose and lactose, and 0.5% stabilizers by weight. The texture and rheological properties of ice cream are strongly influenced by its microstructure in several ways:

- The amount of ice present depends on temperature; the size of crystals depends on the freezing rate and temperature history. Rapid freezing promotes nucleation and formation of small crystals, but temperature fluctuations during storage result in larger crystals (>40 μm) growing at the expense of smaller ones by a process known as Ostwald ripening. Polysaccharides such as carrageenan and locust bean gum are added as stabilizers and are thought to help immobilize water, thus retarding ice crystal growth.
- The temperature of storage relative to the glass transition temperature (T'_G) affects ice crystallization. Below T'_G, the serum phase exists as an amorphous solid, whereas above T'_G, the serum phase becomes mobile and reactive allowing ice crystal growth.
- Fat helps retard ice crystal growth and provides lubrication in the mouth.
- Excess or very large air spaces are prone to collapse causing more rapid melting.
- Emulsifiers added to the premix adsorb on the fat globule surface during cold storage of the ice cream mix. This reduces the protein load on fat droplets and, during subsequent freezing and agitation, fat globules partially coalesce and adsorb on air spaces to form an internal matrix of fat that promotes foam stability.

Rennet

The addition of rennet (chymosin) to milk cleaves the κ-casein macropeptide of the micelle, reducing its surface charge and thus promoting irreversible *para*-casein aggregation. The flocculation of *para*-casein particles eventually forms a three-dimensional gel network with pores up to 10 μm entrapping whey and fat. The size of the pores depends on the relative rates of the enzyme reaction and protein aggregation. Continued fusion of *para*-casein after gel formation results in expulsion of whey (syneresis).

pH and Ionic Concentration

Acidification of milk results in the dissolution of colloidal calcium phosphate. As the isoelectric point of casein is reached (pH 4.6), aggregation of micelles results in a continuous network. Dynamic confocal microscopy can be used to visualize this process (**Figure 9**). In yogurt manufacture, milk is heated prior to acidification to promote interaction between β-lactoglobulin and casein micelles. This results in a finer gel network and reduces syneresis. The addition of calcium promotes the fusion of casein micelles. The concentration of salts greatly influences the microstructure of heat-denatured whey protein gels, particularly during heating, with higher ionic concentration producing coarser gels as determined by light and electron microscopy studies.

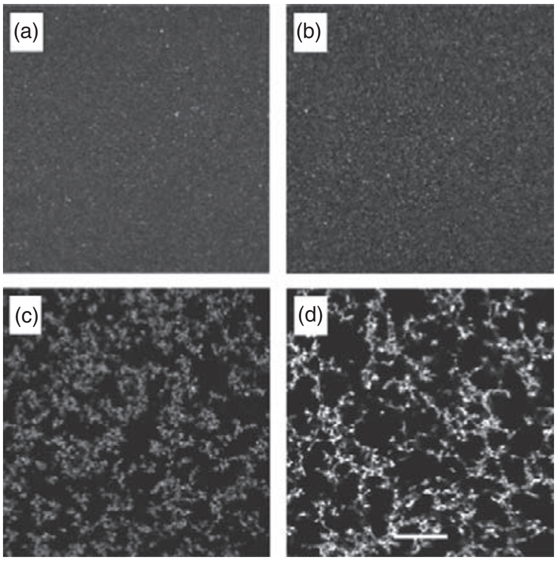

Figure 9 Confocal scanning laser micrographs showing the formation of an acid casein gel acidified with glucono delta lactone. (a) 1 min; (b) 30 min; (c) 60 min; and (d) 120 min. Protein particles (bright) are labeled with Rhodamine B. Scale = 25 μm.

High-Pressure Treatment

HP-treated milks form acid or rennet gels with increased firmness, water-holding capacity, and resistance to syneresis. HP treatment causes disintegration of the casein micelles and induces β-lactoglobulin denaturation, resulting in finer gel networks as demonstrated by SEM and TEM studies. Recently, CSLM studies of HP-treated Cheddar and Mozzarella cheese indicated swelling of the *para*-casein matrix resulting in microstructures similar to those of ripened cheeses.

Mechanical Effects

Mechanical agitation is often used to mix ingredients and move product, in addition to imparting texture, for example, in the production of aerated products such as whipped cream and ice cream. Shearing forces occur in pipes, pumps, and mixing equipment, and these may be detrimental to product quality. High turbulence may cause disruption of fat globules, leading to increased free fat, while vigorous agitation can cause foaming, leading to the denaturation of globular proteins due to interfacial forces. Microstructural observation during shear or controlled flow conditions and in large deformation regimes is technically challenging, but progress is being made using specially constructed shear cells and tensile stages (**Figure 10**).

Examples of Dairy Ingredient and Product Microstructure

Milk Proteins as Ingredients and Structuring Agents

Casein and whey proteins are highly functional and are used in a wide range of food and pharmaceutical products. Caseins add body to beverages, while globular milk proteins, such as β-lactoglobulin, are flexible biopolymers that can be engineered into a variety of structures including fine-stranded and particulate gels, encapsulants, and also fibrillar nanostructures (**Figure 6**).

Whipped Cream

Cream is oil-in-water emulsion containing ~40% fat. Whipping incorporates air into the system, thus forms a foam structure that is stabilized by the following factors, based on TEM and cryo-SEM studies (**Figure 4**):

- lowering of the surface tension of the air/serum interface by proteins and phospholipids;
- adsorption of fat globules on the air bubbles;
- flocculation–coalescence of the fat globules;

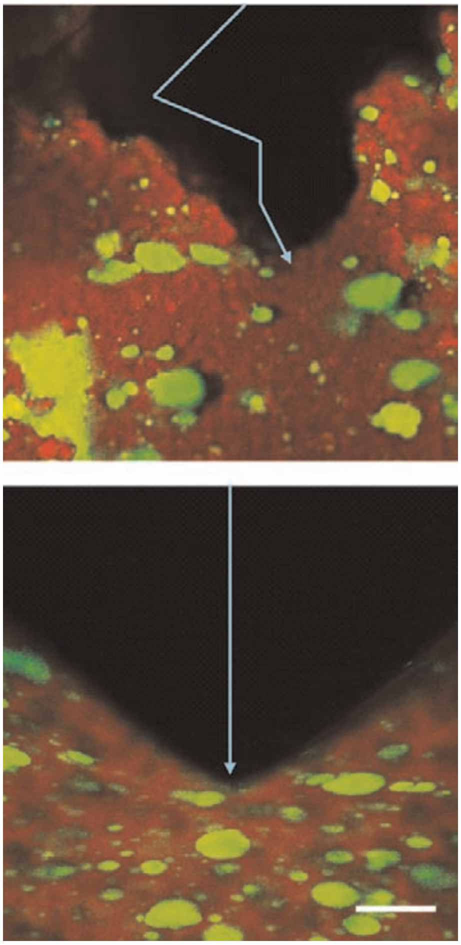

Figure 10 Confocal scanning laser micrographs of a fat-filled heat-set whey protein gel. Notch propagation technique using microtensile stage showing fracture propagation pattern (arrows): (a) fine stranded gel (pH 7.0) and (b) particulate gel (pH 5.5). The images are dual labeled as in **Figure 3**. Scale = 25 μm.

- adsorption of fat crystals to the air/serum interface; and
- formation of fat 'bridges' between neighboring air cells.

Destabilization phenomena include air bubble coalescence, creaming, and liquid drainage.

Yogurt

Yogurt is an acid gel formed by fermentation of heated milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. TEM studies were instrumental in demonstrating that heating milk creates interactions between denatured β-lactoglobulin and the κ-casein of micelles. The resulting micelle is covered with numerous appendages, which facilitate linking into fine chains ~230 nm in cross section, resulting in high water- holding capacity (**Figure 11**). Unheated milk has much smoother micelles and consequently forms a coarser gel with poor syneretic properties. Acid-induced casein gels are particulate and fractal in nature; image analysis of CSLM images has been used to relate fractal dimension to rheological properties.

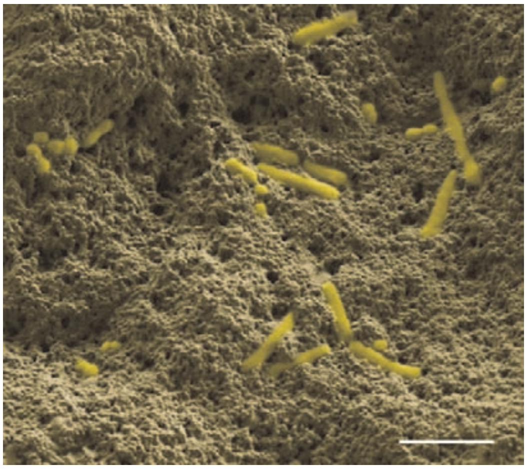

Figure 11 Cryo-scanning electron micrograph of low-fat yogurt made from heated milk showing protein aggregates and very small pores. The images are pseudo-colored to highlight bacteria (yellow). Scale = 5 μm.

Mozzarella Cheese

The microstructural development of Mozzarella cheese during manufacture and ripening can be monitored using CSLM, SEM, and TEM, and observations can be related to compositional, rheological, and functional properties. Key stages of structure development are as follows:

- *Curd formation and whey drainage*: Aggregation of *para*-casein micelles with concomitant release of whey into the surrounding spaces.
- *Cheddaring and salting*: Fusion of *para*-casein micelles into a more continuous protein phase, forming curd granules.
- *Plasticization*: Linearization on stretching of protein into layers or fibers. Fat globules partially coalesce and align between the protein fibers.
- *Aging*: Progressive swelling of protein phase and isolation of fat globules. Expressible moisture decreases as the protein hydrates. The increased water binding capacity of the curd improves flow and stretch properties.

Dairy Spreads

Margarine and butter contain ~20% water, which is present as finely dispersed droplets 0.5–10 μm in diameter. The structural properties of these products are largely governed by the continuous fat phase. SEM and CSLM studies of margarine show that the fat phase consists of a network structure of small, interconnected fat crystals and sheet-like crystal aggregates. Butter has a more complex

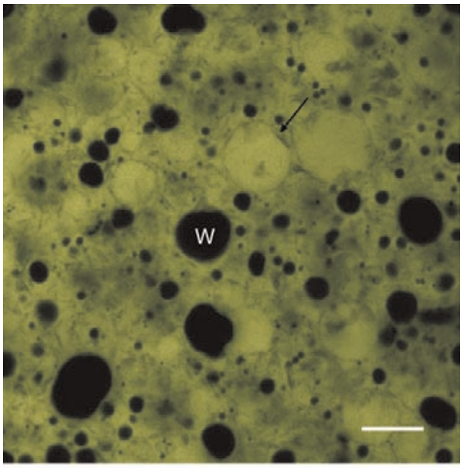

Figure 12 Confocal scanning laser micrograph of butter. Fat was labeled with Nile red. Solid fat crystals appear as fine dark lines (arrow) and water droplets as dark circles (W). Scale = 10 μm.

microstructure, comprising oil globules, globular butterfat crystals, isolated needle-like crystals, and interglobular fat (**Figure 12**). These differences are reflected in functional properties, such as hardness, mouthfeel, and emulsion stability. Product softening or plasticity is higher for margarine than butter, most likely due to the higher number of intercrystalline bonds present in margarine.

Conclusion

Since the late 1990s, dairy microstructure research has greatly increased and advances in imaging and bulk structural analysis techniques give new insight into how microstructure relates to processing and sensory properties. Dairy products are complex heterogeneous systems and characterization of microstructure will continue to play a key role in improving quality and developing innovative products.

See also: **Additives in Dairy Foods**: Emulsifiers. **Analytical Methods**: Physical Methods. **Butter and Other Milk Fat Products**: Properties and Analysis. **Cheese**: Overview. **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders. **Fermented Milks**: Yoghurt: Types and Manufacture. **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Milk Proteins**: Casein, Micellar Structure. **Milk Protein Products**: Functional Properties of Milk Proteins. **Plant and Equipment**: Milk Dryers: Dryer Design; Milk Dryers: Drying Principles.

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Biosensors

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Introduction

Biosensors are sensors that use biological material to make measurements. There are various types of biosensors, including sensors based on electrochemical, acoustical, field effect, and optical physics. Biosensors are a potentially powerful tool in the milk industry for quality control testing, analysis of product composition, production control, and management of dairy animals. Biosensors have potential use in the milk industry for analysis of the presence of toxins, pathogens, antibiotics, pesticides, and chemical contaminants.

Potential Advantages of Biosensors

Biosensors have several potential advantages over other methods of analysis including the following:

High sensitivity and *multianalyte detection*: Several types of biosensors, such as field-effect transistors (FETs), enable detection of even a few molecules, and their small size may enable integration of many such sensors into a multianalyte detector for the detection of many target molecules or microorganisms simultaneously.

Fast or real-time analysis: Fast or real-time detection provides almost immediate interactive information about the sample tested, enabling facilities to take corrective measures before a product is further processed or released for consumption.

Continuous flow analysis: Because of their flow-through design, many biosensors allow continuous flow analysis. In such biosensors, the sample flows through the flow cell or column past the recognition element. Continuous flow systems enable continuous monitoring and can enhance detection sensitivity by bringing more of the target analyte in contact with the recognition element.

Miniaturization: Biosensors can be miniaturized so that they can be integrated into various pieces of equipment such as milking systems, dairy tanks, centrifuges, and other dairy machinery.

Control and automation: Biosensors can be integrated with online process monitoring schemes to provide real-time information about multiple parameters at each production step or at multiple time points during a process, enabling better control and automation of milk and dairy facilities. Biosensors can also be integrated into hazard analysis and critical control point (HACCP) programs, enabling critical examination of the entire food manufacturing process.

Biosensor Technologies

Biosensors consist of a recognition element (ligand or receptor), a signal conversion unit (transducer), and an output interface (the electronic component for interacting with the instrument). This general design (**Figure 1**) is common to all biosensors.

Recognition Elements

The recognition element, or ligand, is the biological component of the biosensor that produces the signal. There are various types of recognition elements, ranging from whole cells to relatively small molecules. Recognition elements can be divided into two general categories: noncatalytic (**Figure 1(a)**) and catalytic (**Figure 1(b)**) elements. Noncatalytic recognition elements, such as cell receptors or antibodies, are often used in direct detection (i.e., label-free) biosensors in which the interaction is directly measured and the output signal is produced immediately. Catalytic recognition elements, such as antibodies labeled with alkaline phosphatase, initiate a secondary reaction or physical effect that is subsequently detected. Two important characteristics of a recognition ligand are affinity, which is the ligand–target binding strength, and specificity, which is the ability of the ligand to differentiate between the target molecule and other molecules in a sample. Ideal recognition ligands should have high affinity (minimizing false negatives) and high specificity (minimizing false

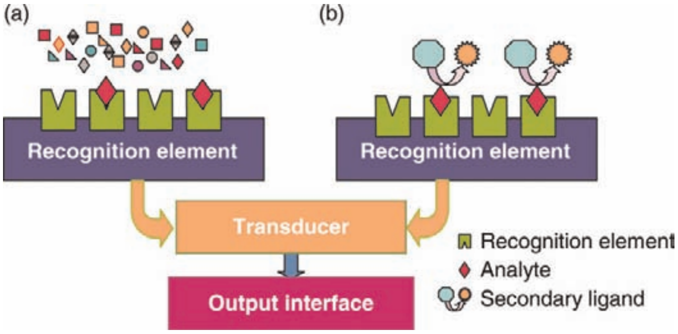

Figure 1 Schematic general design of biosensors. Biosensors consist of a recognition element (receptor), a signal conversion unit (transducer), and an output interface. The recognition elements can be divided into two general types: (a) noncatalytic (label-free), used for direct detection, and (b) catalytic, used for indirect detection. In both cases, the analyte binds specifically to the recognition element. In indirect detection biosensors, a secondary, labeled molecule then binds to the target analyte and the sensor detects the effects of the labeled molecule.

positives). The common measure of ligand affinity is the ligand–target equilibrium dissociation constant (K_D).

Antibodies

Antibodies are the best natural ligands, with equilibrium dissociation constants (K_D) on the order of 10^{-9} mol l^{-1}. Antibodies are widely used as noncatalytic recognition elements because of their specificity, versatility, and strong and stable binding to the antigen. Biosensors that use antibodies in the measured reaction are called immunosensors. Polyclonal antibodies are generated by an organism in response to a foreign molecule, but often the specificity of polyclonal antibodies is variable and can be limited. The specificity of monoclonal antibodies can be higher, but they require more sophisticated production techniques. In general, antibodies are often expensive to produce because they require either a live animal host (polyclonal) or a live animal and a technically sophisticated cell culture technique (monoclonal). This is especially problematic for high-throughput or multianalyte applications, where large numbers of diverse antibodies have to be developed.

Alternative ligands

Although antibodies have excellent affinity and selectivity, they also provide some challenges for biosensor applications. Development of antibodies is time-consuming and expensive. In addition, there are other limitations including inability to raise antibodies for highly toxic substances, variability between antibodies, and limited shelf life. To overcome the limitations of antibodies, in recent years, new types of synthetic ligands that mimic antibodies have been developed. The most common synthetic ligands are aptamers, which are single-stranded nucleic acid molecules, either DNA or RNA, that fold into a three-dimensional secondary structure with binding affinity for a target molecule (including dyes, proteins, peptides, drugs, organic and inorganic molecules, or even whole cells).

Other synthetic recognition ligands include peptides, scaffolded peptides, combined binding agents derived from low-affinity ligands and combinatorial chemistry ligands. With the exception of aptamers, many of these synthetic ligands have a major drawback, which is their low K_D ($\sim 10^{-6}$–10^{-7} mol l^{-1}), 2–3 orders of magnitude lower than that of antibodies.

Transducers

Transducers are the physical components of the sensor that respond to the signal produced by the biosensing process and output the response in a form that can be amplified, stored, displayed, and analyzed. Transducers are based on many different physical principles, including electrochemistry, electrical conductivity, fluorescence, optics, mass detection, and spectrometry. The general principles of these transducers are described here. Biosensors can be classified in a variety of ways. However, this article groups biosensors as either direct or indirect and then further describes examples of the various types of biosensors within each of these groups. Direct detection biosensors, such as surface plasmon resonance (SPR) sensors (**Figure 2**) or quartz resonator transducers (**Figure 3**), detect the physical presence of the target bound to a recognition element. Direct detection methods are label-free, a factor that simplifies and speeds up the detection, keeping down the assay cost. Indirect detection biosensors, such as electrochemical sensors, require a secondary labeled ligand for detection of the target.

Direct detection biosensors

There are several types of direct detection biosensors in which the recognition reaction is directly measured using various physical properties.

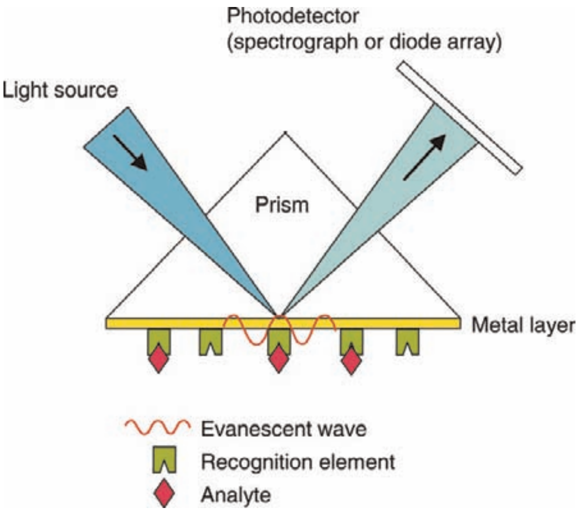

Figure 2 Surface plasmon resonance (SPR) biosensor. An SPR biosensor is based on evanescent wave physics and measures changes in light interactions on the surface of a biosensor prism. Molecular interactions (binding of the target molecule to the recognition element) in the immediate vicinity of the prism surface result in refractive index changes measured by the photodetector.

Optical transducer biosensors

While there are many types of direct optical biosensors, those that have been used in food and dairy analysis are usually based on evanescent wave physics and measure changes in light interactions with the surface of the biosensor prism (**Figure 2**). Molecular interactions in the immediate vicinity of the prism surface, such as binding of the antibody and antigen, result in refractive index changes, which can be detected in several ways.

Evanescent wave biosensors can be used for measuring proteins, such as microbial toxins in milk and dairy products, or bacteria. They allow rapid (close to real-time) detection and direct label-free measurements. The typical sensitivity is ~1–10 ng ml^{-1} for 30 kDa proteins, depending on the antibody affinity. In general, direct measurement of low-molecular-weight molecules is impractical because these methods respond to the mass of the target. However, all of the direct technologies can also be run in an indirect mode to increase sensitivity.

Mechanical biosensors

Two main types of mechanical sensors are resonant crystal (**Figure 3(a)**) and cantilever (**Figure 3(b)**), both based on sensor surface dynamics. Resonant crystal biosensors (also known as quartz crystal microbalance (QCM), piezoelectric, or acoustic wave) are relatively simple direct sensors. Resonant crystal systems measure changes in the acoustic resonant frequency of a quartz crystal due to bound mass on the crystal surface. Detection is done using an oscillator circuit and a frequency counter. The quartz crystal resonator (**Figure 3(a)**) consists of a disk of quartz (piezoelectric) with electrodes plated on it. Application of an external oscillating electric potential across the device induces an acoustic wave that propagates through the crystal.

Like the evanescent wave biosensors, quartz crystal biosensors allow rapid, direct, label-free measurement of larger antigens and indirect measurement of lower-molecular-weight antigens. In addition, they are relatively simple to use and cost-effective.

Cantilever-based biosensors (**Figure 3(b)**) are newly developed transducers measuring the mass-dependent vibrational frequency or bending stress changes on the

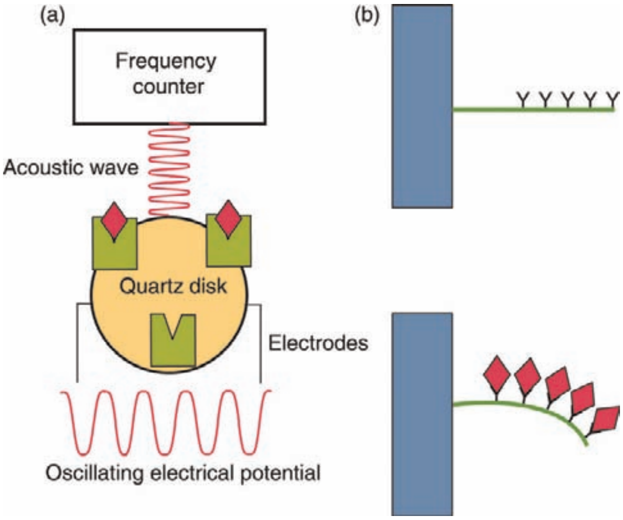

Figure 3 Mechanical biosensors. Resonant crystal biosensors (a) are based on an electrode-plated quartz crystal resonator. An external oscillating electric potential across the device induces an acoustic wave that propagates through the crystal, which is measured by the frequency counter. Cantilever-based biosensors (b) measure bending vibrational resonant frequency or stress changes on the cantilever surface.

cantilever surface. Cantilevers are typically made from silicon and can be miniaturized to a micrometer scale. Ligands can be covalently attached to the surface of the cantilever and the mass change induced by target–ligand binding events can be detected by changes in the resonant frequency or by measuring the cantilever position using optical methods (e.g., by focusing a laser beam on the tip of the cantilever to measure the position).

Field-effect transistors

FETs are transistors gated by changes in the electric field. A special class of FETs, called ion-sensitive FETs (ISFETs), are useful as biosensors. ISFET biosensors have a source, a drain, and a gate (**Figure 4**). Recently, ISFETs have been fabricated using nanomaterials (e.g., carbon nanotubes) to enhance performance. In an ISFET, the ligands are immobilized on the gate surface (**Figure 4**(**a**)). Single-walled carbon nanotube (SWNT)-based ISFETs have shown a large conductance change in response to binding events on the surface (**Figure 4**(**b**)). In addition to the single-wire effects, SWNTs can be interconnected into a submonolayer network (also fabricated by the chemical vapor deposition methods used to grow the nanotubes). These networks exhibit semiconductor-like behavior in which the conductance can be gated and surface interactions with biomolecules can be used for biosensing.

Indirect detection biosensors

In biosensors that rely on indirect detection, the target analyte is bound by a labeled secondary molecule. The secondary molecule is often an antibody conjugated to an enzyme such as alkaline phosphatase. The sensor detects the catalytic reaction carried out by the enzyme. Indirect detection biosensors are very sensitive, relatively simple, and generally less expensive than direct detection sensors, even though they use a labeled secondary molecule and typically exhibit a slower response than direct detection sensors. There are several types of indirect detection biosensors, including fluorescent-label, chemiluminometric, electrochemical, and light-addressable potentiometric sensors.

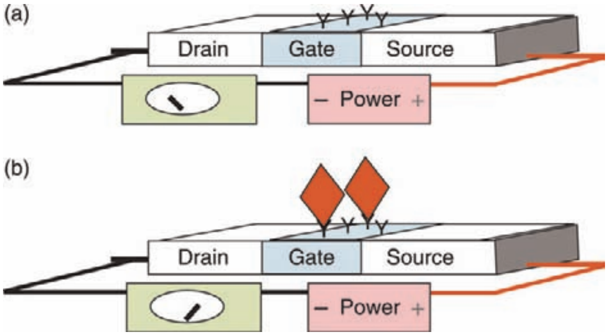

Figure 4 Field-effect transistors (FETs). FETs are field-effect transistors where the gate is sensitive to the surface charge on the semiconductor surface (a). pH changes or antibody (diamonds) binding to the SWNT gate ligands (Y) cause large changes in surface charge that result in conductance changes between the drain and the source of the FTE (b).

Fluorescent-labeled based biosensors

In fluorescent sandwich immunoassays, fluorochrome molecules are used to label the secondary antibodies (**Figure 5**(**a**)). When the fluorochrome is excited, the emissions occur, which can be detected by the biosensor transducer. Generally, fluorescent-label biosensors can detect analytes at concentrations as low as 1–10 ng ml^{-1}. Several multichannel fluorescent-label biosensors have been developed to test for the presence of multiple pathogens and toxins simultaneously. This is a versatile type of instrument suitable for antibody-based detection of microbes and toxins. Fluorescence can be measured in several ways, which include the use of photodiodes, photomultipliers (PMTs), phototransistors (**Figure 5**(**a**)), or charge-coupled devices (CCDs) (**Figure 5**(**b**)), which measure the emission intensity over an area.

Chemiluminometric biosensors

Chemiluminometric biosensors utilize bioluminescence or enzymatic light emission. The luciferase bioluminescence reaction is catalyzed by the firefly (*Photinus pyralis*) enzyme luciferase:

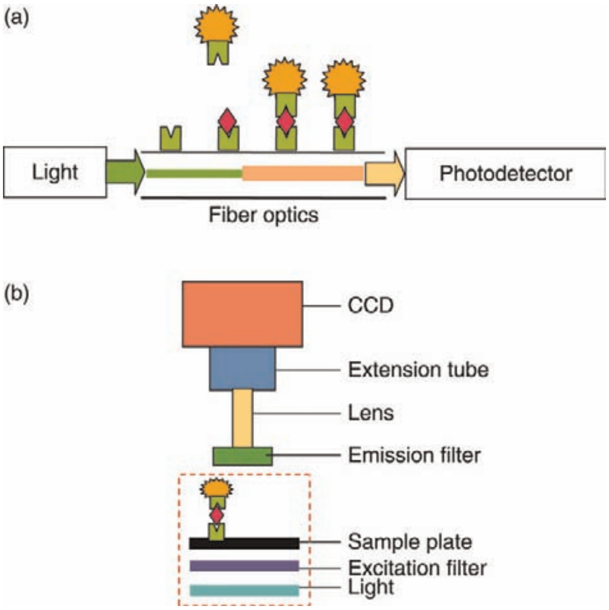

Figure 5 Fluorescence-labeled biosensors. Fluorochrome molecules are used to label secondary antibodies that bind to the antigen in a sandwich format. The fluorochrome is excited by absorbing short-wavelength light and it then emits light at a higher wavelength, which can be detected by a biosensor transducer such as photodetector (a) or a CCD detector with a filter (b).

$$\text{ATP} + \text{luciferin} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{Ppi} + CO_2 + \text{light}$$

The luciferase reaction is very rapid, occurring within seconds after the addition of the luciferin–luciferase mixture. In the presence of adenosine triphosphate (ATP) (from microbial contamination), luciferin converts the ATP to adenosine monophosphate (AMP) releasing phosphate (Ppi), carbon dioxide (CO_2) and emitting light. The amount of light emitted, centered at 562 nm, is proportional to the level of ATP present in a sample, which is an indication of the amount of microbial contamination. Approximately 1 photon of light is produced for each ATP molecule. Thus, this reaction is useful for detecting microbes because, like all living organisms, they contain ATP as the principal carrier of free energy between catabolic and anabolic reactions.

This type of biosensor has a high specificity for ATP, and that translates into a useful tool as a generic assay for the presence of microbial life. One potential application of bioluminescence methods in the dairy industry is as a tool for rapid evaluation of cleaning effectiveness as part of good manufacturing practices (GMP) and HACCP programs. However, bioluminescence-based sensors have some limitations: it is difficult to eliminate interfering substances that may alter the results, and these biosensors cannot distinguish between types of microbe.

Light-addressable potentiometric sensor

Light-addressable potentiometric sensor (LAPS) combines both electrochemical (ISFET) and electrooptical (photosensitive) detection (**Figure 6**), measuring small pH differences (~0.01 pH units) on a semiconductor through their effect on the magnitude of photocurrent. The pH-sensing region of the instrument is an ISFET. A LAPS measures the alternating photocurrent generated when a light source, such as a light-emitting diode (LED), flashes rapidly. The magnitude of the current depends on the surface potential, which in turn depends on the surface pH.

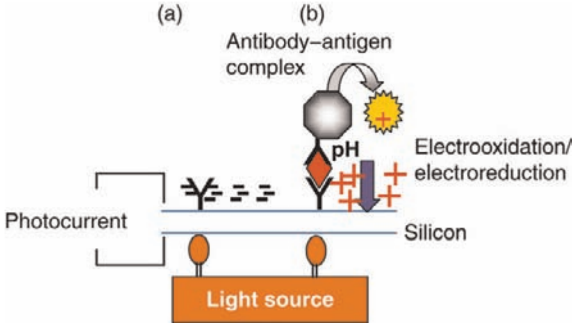

Figure 6 Light-addressable potentiometric sensor (LAPS). A LAPS sensor combines both electrochemical (ISFET) and electrooptical detection, measuring small pH differences on a semiconductor. The pH-sensing region of the LAPS consists of a silicon layer wired into an electrical circuit. An alternating photocurrent is generated by the light source. The magnitude of the current depends on the surface potential, which in turn depends on the surface pH. (a) No antigen bound to the primary antibody; the potential (−) is the potential generated by the light source. (b) Antigen-labeled secondary antibody complex bound to the primary antibody catalyzes electrooxidation/electroreduction, thereby produces a pH change that affects the surface potential (+).

In a LAPS device, the immobilized antibody captures the target antigen–secondary antibody complex. The secondary antibody is labeled with an enzyme such as urease that catalyzes the hydrolysis of urea. Inside the transducer, the hydrolysis of urea by urease produces ammonia, which causes an increase in pH in the microvolume, thereby changes the surface potential. LAPS sensors can be arrayed on a chip to allow analysis of multiple samples simultaneously during a 90-s measurement. LAPS systems are extremely sensitive due to the combination of the small sample volume and the low-noise sensor.

Electrochemical biosensors

Electrochemical detectors measure electron transfer due to oxidation/reduction reactions with an analyte at a suitable electrode (**Figure 7**). The common electrochemical transducers are amperometric devices that detect changes in current at constant potential, conductimetric devices that detect changes in conductivity between two electrodes (at constant voltage), impedimetric devices that measure the ratio of voltage to alternating current, and potentiometric devices that detect changes in potential at constant current.

Some electrochemical biosensors measure directly. However, most utilize indirect means such as a sandwich detection system where the primary antibody is immobilized on the surface of a working electrode or a membrane. The biosensor measures the electron flow catalyzed by an enzyme such as peroxidase or urease, which is conjugated to the secondary antibody. Both the

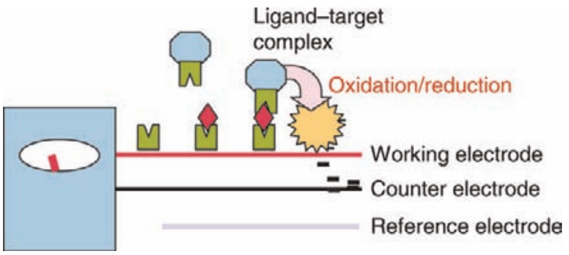

Figure 7 Electrochemical biosensors. Electrochemical detectors measure electrochemical reactions such as oxidation/reduction reactions (−) of an analyte at close proximity to the working electrode in a sandwich format. The potential generated at the working electrode is measured relative to a known potential obtained from the reference electrode. In a three-electrode electrochemical biosensor configuration, a working electrode is used to perform the electrochemical analysis, a reference electrode serves as a reference point against which the potential of working electrode is measured, and a counter electrode is used to apply current to the working electrode.

immunointeraction and the electrochemical reaction occur directly on the surface of the electrode.

Biosensor Analysis of Milk and Dairy Products

Biosensors are suitable for four important types of analysis of milk and dairy products including product safety assurance, analysis of milk composition, control of milk and dairy products processing, and improvement of dairy animal management.

Milk and Dairy Product Safety Assurance

Milk and dairy product safety analysis includes detection of antibiotics, microbial pathogens and their toxins, and insecticides and pesticides in milk, as well as detection of the causes of mastitis in milk cows.

Biosensor antibiotic analysis

Antibiotics (mainly sulfonamides, β-lactams, tetracyclines, and aminoglycosides) are frequently used in dairy husbandry. However, the presence of antibiotic residues in milk is problematic for the production of fermented milks where antibiotic residues can slow or inhibit the growth of the fermenting bacteria. In addition, some people are allergic or sensitive to specific antibiotics, and consuming food containing them can have severe health consequences. In terms of public health, frequent exposure to low-level antibiotics can lead to the emergence of resistant microorganisms. Biosensors can be used to evaluate milk for antibiotics both during milking and during milk processing.

Biosensors for multiple antibiotic detection

Various biosensors have been developed to detect major classes of antibiotics. For detection of multiple antibiotics in milk, a CCD-based immunodetector with a second antibody labeled with horseradish peroxidase (HRP) for generating enhanced chemiluminescence was used; this system could analyze 10 antibiotics in milk. The detection limits ranged from 0.12 (cephapirin) to 32 $\mu g\,l^{-1}$ (neomycin). Similarly, an SPR biosensor was used for the detection of gentamicin and neomycin in milk with sensitivity in the nanogram-per-liter range and sulfonamides at 40 $\mu g\,l^{-1}$ in milk. For rapid determination of chloramphenicol and penicillin residues, an enzymatic lactate oxidase-based amperometric biosensor was used. Dipstick-based technology was reported for rapid and precise detection of tetracycline, streptogramin, and macrolide antibiotics in milk using a competitive assay based on antibiotic-responsive protein bound to DNA, enabling detection at concentrations as low as 5–10 $ng\,ml^{-1}$. However, the majority of biosensors were developed for a single antibiotic or a class of antibiotics including the following:

Sulfamethazine (SMZ): An SPR immunosensor device that analyzes milk for sulfamethazine (SMZ) using a competitive assay for this low-molecular-weight compound was developed. The sample is mixed with anti-SMZ antibodies and then applied to a chip containing immobilized SMZ. The limit of detection (LOD) of this assay is less than 1 $\mu g\,kg^{-1}$ (ppb) and analysis time is 8–30 min per sample.

Fluoroquinolones: Biosensor-based antibiotic detection of fluoroquinolones (including enrofloxacin and ciprofloxacin) in milk has been developed, including a method based on the ability of DNA to extract fluoroquinolones from milk combined with an SPR sensor with an LOD of 3 $\mu g\,ml^{-1}$.

β-Lactams: An SPR biosensor was used for detection of β-lactam antibiotics in milk. The assay was based on the enzymatic activity of the β-lactam receptor protein carboxypeptidase (converting a 3-peptide into a 2-peptide), which was inhibited in the presence of β-lactams. The sensitivity limit for penicillin G was 1.2 $\mu g\,kg^{-1}$. A label-free impedimetric flow injection immunosensor for the direct detection of penicillin G exhibited an LOD of 3.0×10^{-15} $mol\,l^{-1}$ of the antibiotic in milk.

Streptomycin: An SPR immunobiosensor inhibition assay was used for the detection of streptomycin and dihydrostreptomycin residues in whole cow's milk with an LOD of 30 $\mu g\,kg^{-1}$.

Biosensor bacterial analysis

Pasteurization, sterile handling and storage, and other hygienic milk production practices have decreased the threat of milkborne diseases such as tuberculosis, brucellosis, and typhoid fever. Today, milkborne illnesses are generally associated with ingestion of raw milk, improperly pasteurized milk, or improperly handled milk. The major current milkborne bacterial pathogens are *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Campylobacter jejuni*.

Rapid or real-time biosensor-based microbial analysis may allow early detection and subsequent prompt treatment, potentially further reducing the health concerns and economic losses associated with bacterial contamination. While conventional (culture-based) bacterial identification methods are very sensitive, they are relatively time-consuming. Current methods require up to 72 h to produce confirmed results. Various biosensors have been developed for microbial analysis of milk, including pathogen-specific devices and some with capability for detection of multiple pathogens simultaneously. The majority of biosensors developed for microbial pathogens analysis are electrochemical biosensors.

Multipathogen biosensors

A multipathogen biosensor was developed for *E. coli*, *L. monocytogenes*, and *C. jejuni* in milk. The method is based on a flow-through immunoassay system utilizing highly dispersed carbon particles and an amperometric transducer. The system utilized a sandwich assay with HRP-conjugated antibodies, providing detection limits of 10–50 cells ml^{-1} within 30 min.

Pathogen-specific biosensors

An amperometric immunosensor with screen-printed electrodes (SPEs) was developed for *L. monocytogenes* in milk with a working range of 10^3–10^6 cells ml^{-1} and an LOD of 9×10^2 cells ml^{-1}. The assay took about 3.5 h to complete. An impedimetric immunosensor based on electropolymerized polytyramine (Ptyr) films was used for the detection of *Salmonella typhimurium* in milk with an LOD of 10 CFU ml^{-1} in milk after 3–10 h incubation. Disposable amperometric immunosensing strips fabricated using Au nanoparticle-modified screen-printed carbon electrodes were used for the detection of food-borne pathogen *E. coli* O157:H7 with a sensitivity of $>5.03 \times 10^3$ CFU ml^{-1} (i.e., ~50 CFU per strip) in milk. An amperometric immunosensor with a mercaptopropionic acid self-assembled monolayer-modified gold electrode was used for the quantification of *Staphylococcus* with a detection level of 4.8×10^3 cells ml^{-1} in milk. An impedance sensor for the detection of viable *S. typhimurium* was developed with a sensitivity of 10^5–10^6 CFU ml^{-1}.

In addition to electrochemical biosensors, some other types of biosensors have been used in studies of milk. A resonant crystal sensor was used for detection of *Francisella tularensis* with an LOD of 10^5 CFU ml^{-1}. Two optical biosensors have been developed: an SPR biosensor to detect *Salmonella* in milk with an LOD of 1.25×10^5 cells ml^{-1}, and a resonant mirror biosensor to detect *Staphylococcus aureus* in milk with a sandwich assay yielding an LOD of 4×10^3 cells ml^{-1}. One of the most sensitive biosensors for detection of *E. coli* uses a 4 × 4 array of independently operating photodiodes in an integrated circuit (IC) to enable detection of as few as 20 *E. coli* cells in a 0.4-ml reaction chamber and was used in milk analysis.

Other biosensors that have been developed for analysis of microbial pathogens in chicken carcasses, wash water, ground beef, and other foods may be adaptable for milk analysis. With few exceptions, the sensitivity of biosensors for microbial pathogens is limited, typically in the range 10^2–10^6 cells ml^{-1}. Thus, at the current stage of development, they can only detect highly contaminated milk. More work needs to be done to develop sensitive microbial biosensors for the most demanding milk industry applications.

Biosensor mastitis detection

In addition to pathogen analysis in milk, biosensors can be used to detect cow health problems such as mastitis (inflammation of the udder). Such inflammation can be caused by bacteria such as *S. aureus*, *E. coli*, and *Streptococcus* spp. and is often accompanied by increases in bacterial and somatic cell populations in the milk. Mastitis is a health concern for the cows and for the milk consumers. In general, mastitis reduces milk quality and causes economic losses.

Biosensors are being developed to assay milk (or serum) for proteins characteristic of acute inflammation. An automatic electric conductivity biosensor for potentiometric measurements using a custom-made multichannel voltmeter was developed. The multisensor system distinguished between control and clinically mastitic milk. An affinity sensor assay for haptoglobin (Hp) markers for inflammatory reactions (e.g., mastitis) in lactating cows based on their interaction with hemoglobin was developed using an SPR biosensor with an LOD of 1.1 mg l^{-1}.

Microbial toxins analysis

Another important milk safety issue is microbial toxin contamination. Microbial toxins such as staphylococcal enterotoxins (SEs), mycotoxins, and botulinum toxin are threats to the health of consumers. Milk contaminated with these toxins can cause significant health problems and economic losses.

Staphylococcal enterotoxins

Staphylococcus aureus is often found in milk and dairy products. Although it generally dies off rapidly during pasteurization and cheese ripening, the nine major serological types of staphylococcal enterotoxins are heat-stable and survive pasteurization.

Optical biosensors have been used for direct real-time analysis of SEs in milk. For example, a resonant mirror biosensor was able to detect SEA in milk at levels of ~10 ng ml^{-1} in under 5 min. An SPR biosensor was combined with mass spectrometry for multi-SE detection, and SPR biosensors detected SEB in milk at levels of 0.5–5 ng ml^{-1} in less than 2 min.

Among the many indirect biosensors developed for SEB analysis are a LAPS device that utilizes immunofiltration to detect toxins at concentrations as low as 5 pg ml^{-1}, a portable fiber-optic biosensor able to detect 5 ng ml^{-1} toxins in biological samples and food in 15–20 min, a carbon nanotube device with enhanced chemiluminescence immunoassay using CCD with an LOD of 0.01 ng ml^{-1}, and an array biosensor that combines a CCD detector and microfluidics for multianalyte detection, enabling detection of 0.5 ng ml^{-1} of SEB in milk.

Conductimetric immunosensors for the detection of SEB using immobilized anti-SEB antibodies conjugated with HRP enabled detection of SEB concentrations in the range 0.5–83.5 ng ml^{-1}. Another approach is based on a resonant crystal immunosensor for the detection of SEB with detection in the range of 2.5–60 μg ml^{-1}.

Aflatoxins

Aflatoxins are highly toxic mycotoxins that can appear in milk when dairy cattle consume feed contaminated by mold. They are a series of related compounds (e.g., AFB1, AFB2, AFG1, and AFG2) produced by some members of the *Aspergillus* mold genus. They are of particular concern because some of these toxins and their metabolic by-products (AFM1 and AFM2) are potent carcinogens. If milk containing aflatoxins is used for cheesemaking, the toxin will persist during ripening and storage. Like SEs, most aflatoxins are unaffected by pasteurization. Various biosensors have been developed for analysis of aflatoxins.

There are many biosensors designed to analyze milk for aflatoxin M1. One such biosensor uses a membrane-based flow-through system with an antibody against AFM1 conjugated to HRP. This indirect visual detection mobile biosensor can detect concentrations as low as 0.05 ng ml^{-1} of AFM1 in milk within 18 min. Another system uses an impedimetric immunosensor for aflatoxin B1 with an LOD of 0.1 mg l^{-1}. A fluorometric handheld biosensor carries out automatic immunoaffinity fluorometric assays for AFM1 present at 0.1 ng ml^{-1} in less than 2 min.

Botulinum toxin

Botulinum toxin at levels of 8 ng ml^{-1} and above has been detected using a multianalyte fluorescence biosensor. This instrument was able to detect other bacterial toxins (cholera toxin and ricin toxin) simultaneously, using an antibody array (a set of antibodies on the biosensor surface).

Insecticides and pesticides in milk

Analysis of insecticides and pesticides in milk is important because some of these compounds are very toxic to humans. A disposable biosensor was developed based on screen-printed thick-film electrodes, which is suitable for monitoring organophosphate and carbamate residues in foods from animal origin, including milk. The electrochemical biosensor based on acetylcholine esterase inhibition enables sensitive detection of several insecticides including 4 μg l^{-1} tebufenpyrad, 4 μg l^{-1} tetraconazole, and 2 μg l^{-1} bifenthrin. An SPR immunoassay for ivermectin residues (antiparasite medication) in bovine milk yielded an LOD of 16.2 ng ml^{-1}.

Milk allergens

β-Lactoglobulin (β-LG) is the major whey protein and is a known allergen. In several countries, food manufacturers are required by law to provide labeling information regarding the presence or absence of β-LG. A resonance-enhanced absorption (REA) effect biosensor was used in an immunological sandwich assay, using antibodies labeled with monodisperse colloidal gold clusters for β-LG. This system was able to detect β-LG at 100 ng ml^{-1}. An SPR biosensor with a sandwich assay enabled detection of the allergen levels down to 1–12.5 μg g^{-1}.

Biosensors for the Control of Milk and Dairy Product Quality and Processing

The quality factors for milk include several types of parameters: freshness, assessment of the effectiveness of milk heat treatment, milk component determination, and analysis of milk adulteration.

Milk freshness

Freshness of milk can be measured by amino acid composition or protein degradation.

Amino acid analysis

The amounts of both L- and D-amino acids in milk tend to decline over time, depending on the type of processing and the duration and conditions of storage. This reduction can be used to monitor milk-aging effects. Several electrochemical biosensors have been used for this application.

An amperometric sensor that uses L- and/or D-amino acid oxidase to measure the amount of amino acids present in the concentration range 150–470 μmol l^{-1} was developed. A screen-printed amperometric biosensor for the detection of D-amino acids has been constructed by the immobilization of D-amino acid oxidase on a graphite working electrode enabling measurements of D-alanine in the concentration range from 5 to 200 μmol l^{-1}. A similar sensor tests for L-tryptophan using immobilized tryptophan-2-monooxygenase. This instrument rapidly detects L-tryptophan at concentrations of 25–1000 μmol l^{-1} in a batch-mode system and 100–50 000 μmol l^{-1} in a flow injection mode. The response time was 30 s, and the total analysis time was less than 3 min.

For more general use, a screen-printed amperometric biosensor was developed for the rapid measurement of L- and D-amino acids, and it uses a rhodinized carbon working electrode incorporating L- and/or D-amino acid oxidase for the general-purpose measurement of all 20 common L-amino acids. Linear response profiles were observed for L-leucine, L-glycine, and L-phenylalanine with limits of detection of 0.47, 0.15, and 0.20 mmol l^{-1}, respectively. The devices were reproducible and exhibited stability over a 56-day test period. The biosensor compares favorably with a standard photometric amino acid test and was used to monitor milk-aging effects. The assay is inexpensive, simple to perform, and rapid, requiring only a buffer electrolyte and a small sample.

Protein degradation

SPR was used to assess milk protein degradation based on a sandwich assay for the simultaneous quantification of the N- and C-termini of the three major milk caseins (α_{s1}, β, and κ) so as to include only intact caseins (with both ends), and not their degradation products, in quantification. The fast (10 min), sensitive (LOD $\sim$0.87 μg ml^{-1}) method has been applied successfully to raw and drinking milks.

Process control – effectiveness of milk heat treatment

Lactulose, fructose, or other heat-sensitive milk components can be used as indicators for the effectiveness of milk heat treatment.

Lactulose

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose) is a disaccharide of D-galactose and D-fructose formed from lactose (4-*O*-β-D-galactopyranosyl-D-glucopyranose) in milk during heat treatment at a ultra-high temperature (UHT). Very little lactulose is formed during the normal pasteurization process. This makes it a useful indicator of the intensity of thermal processing. UHT milk generally has a lactulose content over 500 mg l^{-1}, while pasteurized milk usually has <100 mg l^{-1} lactulose.

An electrochemical biosensor for lactulose in milk uses an electrode with immobilized fructose dehydrogenase (FDH) and β-galactosidase on the electrode surface. The lactulose hydrolysis product, D-fructose, is oxidized by FDH, which is simultaneously reduced, and then directly reoxidized on the electrode. The LOD of this sensor is 1.0 μmol l^{-1} and the selectivity for lactulose is at least 1000 times higher than that for lactose.

Sulfhydryl groups

Electrochemical biosensors have also been used to evaluate the effectiveness of milk heat treatment by measuring reactive sulfhydryl groups exposed by heat treatment of proteins. While these groups are normally buried inside whey proteins, they become free and highly reactive after heat treatment. The method enables differentiation between raw or non-UHT milks and UHT milks, and the results were in good agreement with the results obtained by HPLC and colorimetry.

α-Lactalbumin

SPR analysis allows discrimination of the different milk heat treatments (pasteurization, direct and indirect UHT and sterilization), using an immunological assay with specific monoclonal antibodies for the native and heat-denatured forms of α-lactalbumin. The SPR analysis is fast (4 min), repeatable, and fully automated.

Starter culture characterization

Characterization of starter culture by measuring differences in acid production of various *Lactococcus lactis* and *Streptococcus thermophilus* inoculum was reported. Microplates with integrated optical pH sensors were developed for this application. Two fluorophores, one pH-sensitive and the other pH-insensitive, are immobilized at the bottom of each well of a polystyrene 96-well microtiter plate. The pH-insensitive fluorophore serves as an internal reference and makes calibration unnecessary. The sensor measures pH accurately in optically well-defined media.

Measurement of Milk Composition

Measurement of milk composition is used to assess its nutritional and economic value. The three major components of milk total solids are fat, protein, and lactose. Online measurements with biosensors allow analysis of each component at the level of individual cow, herd, or tanker. Such data could be used to improve management at all levels.

Determination of proteins

An amperometric sensor for the assay of the protein content of milk based on digestion of proteins by two enzymes (carboxypeptidase A and L-amino acid oxidase) was developed. The enzymes generate hydrogen peroxide, which is measured electrochemically enabling an LOD of 1.5 μmol l^{-1} of proteins in milk products. In addition to this nonspecific protein determination, immunoglobulin G (IgG) content in bovine milk during the early lactation transition from colostrum to milk was detected with an SPR biosensor with an LOD of 0.08 mg ml^{-1}. This sensor can also be used for measuring other minor proteins of bovine milk including folate-binding protein, lactoferrin, and lactoperoxidase in consumer bovine milk, colostrum, and infant formulae.

Determination of carbohydrate

Several multicarbohydrate biosensors were developed including a flow electrochemical biosensor for simultaneous detection of fructose, sucrose, glucose, galactose, and lactose using amperometric enzyme electrodes. Similarly, amperometric detection with an enzyme-catalyzed reaction for the combined detection of glucose, galactose, and lactose gave a linear response between 0.05 and 10 mmol l^{-1} for glucose, between 0.1 and 20 mmol l^{-1} for galactose, and between 0.2 and 20 mmol l^{-1} for lactose. In addition to these multianalyte biosensors, analyte-specific sensors have been developed for specific carbohydrates.

Lactose

The disaccharide lactose comprises 4.8–5.2 wt.% of milk. In addition to its nutritional relevancy, the determination of lactose is important for people with lactose intolerance. An amperometric lactose biosensor was constructed based on sequential reactions of three enzymes immobilized on a glassy carbon electrode. The first enzyme, β-galactosidase, cleaves lactose to glucose and galactose. The second enzyme, glucose oxidase, catalyzes a reaction that converts glucose into H_2O_2 and D-glucono-1,5-lactone. Finally, HRP utilizes H_2O_2 to oxidize a dye or fluorophore. Using a series of enzymatic reactions increases the selectivity and sensitivity of the sensor. The response of this sensor was linear at lactose concentrations between 0.027 and 1.00 mmol l^{-1}. A similar instrument detects lactose using only the glucose oxidase and β-galactosidase enzymes. The response of this bienzyme probe was linear at lactose concentrations between 0.02 and 3.00 mmol l^{-1}. A lactose biosensor was developed by immobilizing lactase and galactose oxidase in a polyvinyl formal membrane and was attached to the oxygen electrode of a dissolved oxygen analyzer for estimation of lactose in milk, with a linearity in the range 1–7 g dl^{-1} (30–200 mmol l^{-1}) of lactose. Another approach based on carbon paste electrodes modified with baker's yeast (*Saccharomyces cerevisiae*) was used in an amperometric biosensor for lactic acid in dairy products enabling the detection of lactic acid down to 1 mmol l^{-1}. Still another amperometric lactose biosensor was developed by immobilizing lactase and galactose oxidase on the working electrode combined with platinum as a reference electrode. The enzyme electrodes showed linearity in the range 1–6 g dl^{-1} of lactose. A direct immobilization sensor was developed based on the highly efficient direct electron transfer between two newly discovered cellobiose dehydrogenases (CDH) from the white rot fungi *Trametes villosa* and *Phanerochaete sordida*. The system uses amperometric detection and has an LOD of 1 µmol l^{-1} of lactose and a response time of 4 s.

Fat and fatty acids

The fat content of milk is of economic importance because price formulae for milk are functions of the fat content. Milk fat comprises 2.4–5.5% of raw milk, depending on the cow's species and lifestyle.

One approach to measuring fat is analysis of fat globule membrane (FGM), since more than 99% of total milk fat is found inside fat globules. These fat globules are liquid fat droplets covered by a thin bilayer membrane originating from the plasma membrane of the secretory cell, which continually envelops the fat droplets as they are secreted. The major components of the native FGM include protein and phospholipids. An SPR biosensor was developed to analyze human milk that uses monoclonal antibodies against deoxycholine-solubilized human milk FGM, making it likely that a similar instrument could be used to analyze fat in dairy milk. The detection of free short-chain fatty acids is also important for the evaluation of milk quality. A microbial biosensor using the microorganism *Arthrobacter nicotianae* was developed to determine the concentration of free fatty acids in milk. The sensor monitors the respiratory activity of the bacteria, which are immobilized in calcium alginate on the electrode surface. This instrument showed linearity over the concentration range ~10–160 µmol l^{-1} of fatty acids, and its response time was ~3 min.

Casein

Proteins make up 3.25 wt.% of milk, and 80% of the protein is casein. Hydrolyzed casein and whey protein formulae have been developed to prevent sensitization in infants at high risk of cow milk allergy and for treatment of children with cow milk allergy. Nevertheless, severe reactions have occurred in some allergic infants fed with these products. Therefore, it is important to develop *in vitro* techniques to detect residual allergenic activity in infant formula. ELISA with antibodies against casein components (α-, β-, and κ-casein) detected residual antigenic activity in partial hydrolysates and in extensive whey protein hydrolysates at levels of 0.05–0.67% of total protein. Peptic and tryptic digestion of bovine β-casein were investigated using a resonant crystal biosensor, with β-casein immobilized on the surface of the crystal sensor where its degradation caused shifts in the resonant frequency measuring the kinetics and effectiveness of digestion. Localized surface plasmon resonance (LSPR) using titania-coated gold nanoparticles immobilized on the surface of a glass slide as the sensing substrate and UV–visible spectrophotometry as the detection tool for tryptic digestion product of β-casein yielded an LOD of 50 nmol l^{-1}. Quantification of the three major caseins (α_{s1}, β, and κ) in a sandwich immunosensor enables detection of only intact caseins and not their degradation products with an LOD of about 0.9 µg ml^{-1} in 10 min. Another SPR sandwich immunosensor enabled detection of α_{s1}-casein with a detection range of 8.8–12.06 mg ml^{-1} in milk.

Calcium

Calcium is a significant component of milk (0.65 wt.%) and other dairy products. An optical biosensor for calcium analysis was developed based on aequorin, the bioluminescent protein found in the jellyfish *Aequorea* sp. The presence of calcium specifically triggers luminescence of aequorin immobilized in a porous solgel. This biosensor was used to test calcium levels in milk samples.

L-Lactic acid

The quality of Mozzarella cheese is strongly dependent on careful control of the acidity in the curd. L-Lactic acid is the major product of lactose fermentation. An electrochemical L-lactic acid biosensor with immobilized lactate oxidase was developed to control acidification during cheese processing. This assay was shown to be more sensitive than pH measurement procedures.

Folic acid in fortified infant formula

The addition of folic acid to infant formula for the prevention of prenatal neural tube defects resulting in spina bifida or anencephaly is required in several countries. An antibody-based SPR biosensor was developed to measure folic acid in milk-based infant formula using a competitive assay.

Riboflavin in milk

SPR was used for indirect riboflavin (Rf) analysis by measuring the excess of Rf-binding protein (RBP) that remains free after complexation with Rf molecules originally present in the sample solution, yielding an LOD of $70\,\mu g\,l^{-1}$.

Choline

Choline, a natural amine, is an essential nutrient that serves as an important metabolic precursor for a number of biologically significant compounds. Only recently, it was suggested that a dietary source for daily intake of choline may be required for adult humans. An electrochemical biosensor was developed based on a phospholipase D-packed bioreactor coupled to a choline oxidase-based amperometric detection scheme, with an LOD of $0.02\,mmol\,l^{-1}$.

Determination of vitamin B_{12} in milk products

A competitive binding assay was developed based on cobalamin (vitamin B_{12}) interacting with vitamin B_{12}-binding proteins haptocorrin (also known as R protein), a carrier protein that protects vitamin B_{12} from acid-peptic digestion. The assay was based on an SPR biosensor with sensitivity in the range $0.08–2.40\,ng\,ml^{-1}$.

Milk Adulteration

SPR has been used for detection of various adulterants and contaminants in milk and milk powder. Bovine κ-casein was used as a marker for bovine milk added to ewe's and goat's milk and for the detection of bovine rennet whey powder. In the assay, an SPR biosensor enabled the detection of different adulterants and contaminants in milk and milk powder with an LOD of 0.17% (v/v) for cow's milk and above 1% (w/w) for bovine rennet whey powder.

An SPR immunoassay was used for the detection of nonmilk proteins in milk powder. The system allowed for the simultaneous detection of soy, pea, and soluble wheat proteins in milk powders allowing an LOD of <0.1% of plant protein in total milk protein. Milk is sometimes adulterated with urea (called 'synthetic milk'). An electrochemical biosensor was developed with immobilized urease yielding a bacterial cell coupled to an ammonium ion-selective electrode and configured as a potentiometric transducer.

Biosensors for Management of Dairy Animals

Biosensors can be used for various management aspects of dairy animals including feed consumption, herd reproductive status, and milk yield management.

Biosensors for feed management

Feed costs are one of the largest expenses in dairy production, and protein supplements are the single most costly ingredient. Excessive intake of nitrogen in feed proteins cause elevated systemic urea levels without increasing milk production. In addition, excessive nitrogen can impair reproductive performance and excessive nitrogen in dairy waste is an environmental concern. Milk urea measurement is a useful tool that can allow dairy managers to manage feeding and health of their herds. An electrochemical biosensor has been used to measure the concentration of urea in milk using immobilized urease (see above), and other assays measure the carbon dioxide generated by the enzymatic hydrolysis of urea by urease.

Biosensor application for reproductive management

Reproductive management is a major financial concern of the dairy industry, with missed estrus detection as a main cause of lost income. Online biosensors for monitoring reproductive hormones are a powerful tool for reproductive management. Most of these biosensors measure progesterone in bovine milk to detect estrus. For example, an online fiber-optic immunosensor detected concentrations of $0.1–5\,ng\,ml^{-1}$ progesterone in milk, and was reusable for 15–20 cycles. Others have used an amperometric biosensor to detect progesterone using progesterone-conjugated alkaline phosphatase in a competitive immunoassay format. A progesterone immunosensor using a colloidal gold–graphite–Teflon–tyrosinase composite biosensor as an amperometric transducer was used for determining progesterone in milk samples in the concentration of 5.0 and $1.5\,ng\,ml^{-1}$. A total internal reflectance fluorescence (TIRF)-based biosensor was used to assay for progesterone in bovine milk with an assay time of about 5 min and it reached an LOD of $0.04\,ng\,ml^{-1}$. An SPR biosensor-based inhibition immunoassay for progesterone in cow's milk was developed with a sensitivity of 0.4–0.6 ng ml^{-1}. An electrochemical biosensor with screen-printed carbon electrodes was used for progesterone analysis in cow's milk using a competitive immunoassay with alkaline phosphatase-labeled progesterone with an LOD of 2 ng ml^{-1}.

Recombinant bovine somatotropin

Bovine somatotropin (BST) is a protein hormone produced in the pituitary gland and has been shown to increase the milk yield in lactating cows by preventing mammary cell death in dairy cattle. Recombinant bovine somatotropin (rBST) (artificial growth hormone) can be administered to the cow by injection and used to increase milk production. The use of rBST in cows increases bovine insulin-like growth factor 1 (IGF-1). In some countries, the use of rBST is controversial because of its potential effects on animal and human health and because of economic concerns. According to an EU report, rBST substantially increased health problems in cows, such as mastitis, foot problems, injection site reactions, and reproductive disorders. In several counties, rBST is not approved for use. rBST can be measured by an SPR biosensor with polyclonal anti-IGF-1 antibodies with specific binding capacity to IGF-1. Using recombinant IGF-1 in inhibition experiments, the range of measurements of IGF-1 in fresh or autoclaved cow's milk was 1–50 ng ml^{-1}. For comparison, native IGF-1 content in normal fresh raw milk is <4 ng ml^{-1} and higher levels of IGF-1 (>30 ng ml^{-1}) have been measured in the milk of cows treated with BST. In a similar inhibition assay using different antibodies, the LOD for rBST in the assay was 8 ng ml^{-1}, which is far below the expected concentration in injection preparations.

Incorporation of Biosensors into HACCP Programs

The HACCP program focuses on critical food safety areas as part of total quality assurance programs. On-farm and dairy plant HACCP monitoring requires accurate and reproducible tests that can determine the status of cows, milk, and the dairy environment. There are several possible uses for biosensors in HACCP monitoring, including screening for antibiotic residues at the level of individual cows, assaying for pathogenic organisms or toxins, and assessing the effectiveness of milk heat treatment in dairy plants.

Future Trends – Integrated Multichannel Biosensors for Milk and Dairy Products

Biosensors provide miniaturized systems that can be integrated with online process monitoring schemes to analyze samples. Because of their small size, the sensors can be incorporated into various dairy equipment including milking systems, dairy tanks, centrifuges, and fermentors.

The majority of biosensors developed to date detect a single analyte, but a future trend in biosensing is the development of multiple-sensing instruments. One example of this emerging technology is antibody arrays, in which antibodies are placed in an orderly arrangement in the two-dimensional format of the chip. Using antibody arrays, various antigens can be identified by their binding to specific antibodies at known positions. The use of such integrated biosensor chips may allow a complete analysis of the milk or dairy product for toxins, pathogens, antibiotics, pesticides, insecticides, and chemical contaminants. This could allow increased quality control of milk processing through analysis of milk and dairy product composition as well as better herd management including dairy animal reproductive hormones and feed management. These features will not only reduce the cost of milk production but will also provide greater safety and increased milk quality. Some examples of such integration were already reported including a remote monitoring system based on a piezoelectric quartz crystal sensor developed for the determination of the bacterial population of raw milk. The system can provide dynamic data monitoring on a web page via the Internet for real-time monitoring of bacterial growth in milk.

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Physical Methods

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Introduction

Milk is composed of a mixture of lipids, proteins, carbohydrates, vitamins, minerals and water. A multitude of chemical and physical methods have been developed for the analyses of these constituents and for monitoring quality and processing operations employed in the manufacture of milk and dairy products. Modern chemical and physical methods, which include spectrophotometric techniques, atomic absorption spectrometry, chromatography and rheological techniques, are discussed elsewhere (*see* **Analytical Methods**: Atomic Spectrometric Techniques; Chromatographic Methods; Principles and Significance in Assessing Rheological and Textural Properties).

In the dairy industry, measurements of the physical properties of milk and dairy products are made to obtain data necessary for the design of dairy equipment, to determine the concentration of a constituent or group of constituents (e.g. specific gravity to estimate total solids or freezing point to determine added water) or to assess the extent of a chemical or physical change (e.g. titratable acidity to follow bacterial action). Some important physical properties of milk are summarized in **Table 1**. The great advantages of physical measurements for such purposes are their speed and simplicity as well as their potential for automation. In this article, some of the important physical methods are discussed in terms of general physical principles and methods of measurement.

Titratable Acidity and pH

One of the most important physical properties of milk is its acid–base equilibrium. The pH of fresh milk varies between 6.5 and 6.7 at 25 °C. Differences in pH among individual lots of fresh milk reflect compositional variations. In general, the pH is lower (down to pH 6.0) in colostrum and higher (up to pH 7.5) in cases of mastitis than in normal milk of mid-term lactation. Bacterial deterioration of milk lowers the pH. Titration methods rather than pH measurements are used to check the acid–base equilibrium, which is usually referred to as the titratable acidity of milk.

The procedure involves the following steps. A 9- or 18-g sample is pipetted into a beaker or a white dish. Water (twice the weight of the sample) is used to rinse the pipette completely into the container. Dry samples can be analyzed by weighing accurately the prescribed amount of sample and dispersing it in water. For most samples, 1% phenolphthalein is added (0.5 ml) and the sample is titrated with $\frac{N}{9}$ sodium hydroxide to the first permanent (30 s) color change to pink. The concentration and the amount of indicator used must be kept constant in all titrations, as any variation is likely to influence the results. Also, the results are affected by the diluent used, by the speed of titration and by the temperature of the sample. Therefore, these conditions must be standardized in all titrations. The results are expressed in terms of percent lactic acid. One milliliter of $\frac{N}{9}$ sodium hydroxide used in the titration is equivalent to 0.01 g lactic acid. The normality of the sodium hydroxide must be determined exactly $(\frac{N}{9})$ using standard acid titration. Samples that are dark or colored should be titrated to a pH of 8.3 using a standardized pH meter and probe.

Freshly drawn milk contains practically no lactic acid. However, the titration of such milks from their initial pH value of about pH 6.6 to the phenolphthalein end-point (pH 8.3 or above) requires 13–20 ml of 0.1 N sodium hydroxide $100\,\text{ml}^{-1}$ of milk. This titration is a measure of the buffering capacity of the milk and is termed the ‘initial acidity’. The buffering capacity is the molar quantity of acid or base (‘strong’ meaning completely dissociated in the experimental pH range) required per unit change in pH. The most important buffering components of milk are caseins, phosphates and citrates, although quantitative assignment of the buffering capacity to these constituents is rather difficult. This problem is primarily attributed to the presence of calcium and magnesium in the system, which are present as free ions, as soluble undissociated complexes with phosphate and citrate, and as colloidal phosphate associated with the casein micelles.

Traditionally, titratable acidity has been used as an indicator of milk quality, because there is no lactic acid in fresh milk. Under current methods of handling and

Table 1 Some physical properties of milk

Osmotic pressure	*$\sim$700 kPa*
a_w	$\sim$0.993
Boiling point	$\sim$100.15 °C
Freezing point	-0.522 °C (approx.)
Refractive index, n_D^{20}	1.3440–1.3485
Specific refractive index	$\sim$0.2075
Density (20 °C)	$\sim$1030 kg m^{-3}
Specific gravity (20 °C)	$\sim$1.0321
Specific conductance	$\sim$0.0050Ωcm^{-1}
Ionic strength	$\sim$0.08 mol l^{-1}
Surface tension (20 °C)	$\sim$52 mN m^{-1}
Coefficient of viscosity	2.127 mPa s^{-1}
Thermal conductivity (2.9% fat)	$\sim$0.559 W m^{-1} K^{-1}
Thermal diffusivity (15–20 °C)	$\sim$1.25×10^{-7} m^2 s^{-1}
Specific heat	$\sim$3.931 kJ kg^{-1} K^{-1}
pH (at 25 °C)	$\sim$6.6
Titratable acidity	1.3–2.0 mmol OH$^-$ 100 ml^{-1} (0.14–0.16% as lactic acid)
Coefficient of cubic expansion (273–333 K)	0.0008 m^3 m^{-3} K^{-1}
Redox potential (25 °C, pH 6.6, in equilibrium with air)	+0.25 to +0.35 V

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distributing milk, temperatures are rarely such that lactic acid-producing bacteria can grow. If titratable acidity is used as a test to determine the acceptance of milk, factors such as temperature, odor and taste should also be noted. Measurement of acidity is influenced by any condition that causes a change in the distribution of calcium phosphate in the sample. Milk which is high in protein may also have an apparently high acidity because charged groups on the protein molecule react with alkali. The normal acidity of fresh milk is usually 0.15–0.16% (expressed as percentage lactic acid). If values significantly above normal are obtained, the milk is suspect, but other quality tests (especially taste and odor) should be performed prior to rejection. Several million lactic acid bacteria per milliliter are necessary to produce detectable developed acidity. As some common spoilage organisms in the milk supply (psychrotrophic bacteria) do not produce lactic acid, their action will not be detected by this method. Titratable acidity is useful in the manufacture of cultured products, where acid development is encouraged, yet controlled. Specifications for the titratable acidity values expected during various stages of the cheesemaking process exist. Although lactic acid is not the only acid present in fermented milks, it predominates and is used as the basis of the calculation of acidity.

Titratable acidity is obviously related to the pH of the product. Frequently, pH measurements are made because the method is nondestructive and rapid. The condition of the electrode is most crucial to the accurate determination of pH. Milk products contain fat and protein, which clog the electrode. Care must be taken to clean the electrode according to the manufacturer's instructions. The pH meter should be standardized using both buffer solution at pH 7.0 and a buffer solution as close as possible to the pH of the sample being tested (*see* **Analytical Methods**: Electrochemical Analysis).

Liquid samples do not require further preparation before measurement of pH. For skim milk powder, 100 ml of water are added to a 10-g sample, which is blended in an electric mixer for not more than 1 min (to avoid the formation of excessive foam) and allowed to stand for 1 h at room temperature. For cheese, the product is grated to provide a uniform sample and the sample is packed in a small container to ensure good electrode contact. In the case of butter, the pH is determined in the serum phase.

Oxidation–Reduction Potential

Oxidation is the loss of electrons, whereas reduction is synonymous with the gain of electrons. The loss or gain of electrons may or may not include the transfer of oxygen or hydrogen. The oxidation–reduction (redox) potential is expressed in volts and is designated as E_h. The standard potential when the oxidized [Ox] and reduced [Red] forms are at equal activity is designated as E_o. The redox potential is measured relative to the potential of the standard hydrogen electrode, which is assigned a value of 0 V at pH 0. At 25 °C and one electron transfer, E_h is defined as:

$$E_h = E_o + 0.059 \log\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) \qquad [1]$$

By convention, a large ratio of [Ox]/[Red] indicates a positive potential. The redox capacity of the system is determined by the total amount of reactants $[\mathrm{Ox}] + [\mathrm{Red}]$. E_o is an index of the potential of the system relative to other systems. When the value of E_h is near E_o, the system exhibits poising or a resistance to a change in potential similar to the buffering that occurs in an acid–base system near its pK value.

Fresh milk, produced in the normal way, exhibits a potential at a gold or platinum electrode of between +0.20 and +0.30 V, which is largely attributed to dissolved oxygen. Milk is essentially oxygen-free when secreted, but about 0.3 mmol l^{-1} O_2 are present after equilibrium with air is established. The removal of oxygen by nitrogen sweeping lowers the redox potential to about −0.12 V.

When bacteria grow in milk, the redox potential of the milk changes with time and the tendency, in general, is for the potential to change in a negative direction. A rapid change in potential occurs only after the dissolved oxygen has been consumed by aerobic bacteria, and may be identified by a change in color of certain dyes added to the milk. These dyes are oxidants of redox systems. This forms the basis of methylene blue and resazurin reduction tests for bacterial quality of milk. As the time that elapses before these dyes are reduced to the colorless reductant form is roughly proportional to the number of bacteria present, this reduction time is an index of the degree of bacterial contamination.

The major components of milk other than water, i.e. milk fat, sugar and protein, have been shown to have no influence on its redox potential. The redox systems in milk involve lactate-pyruvate, ascorbate and riboflavin.

The method for determining the redox potential varies, to some extent, with the pH potentiometer used. Generally, a platinum electrode is connected to the pH meter in place of the usual glass pH electrode and readings are obtained in millivolts. Initially, the instrument is standardized against (1) pH 4.0 phthalate buffer (0.05 mol l^{-1} potassium hydrogen phthalate) into which a little quinhydrone has been stirred (reading A) and (2) 0.1 N hydrochloric acid also with quinhydrone added (reading B). The standard potential of a calomel electrode containing saturated KCl is 0.248 V at 20 °C (0.2441 V at 25 °C). Assuming that the readings are converted to volts at 20 °C, the following values should be obtained:

$$\mathrm{A} + 0.248 = 0.471\ \mathrm{V} \qquad [2]$$

$$\mathrm{B} + 0.248 = 0.642\ \mathrm{V} \qquad [3]$$

The redox potential of the test solution (E_h) is then determined in the same way as for (1) and (2) (reading = X V), so that:

$$E_h = X + 0.248\ \mathrm{V} \qquad [4]$$

If X is negative, the electrode is reversed. If a 0.1 mol l^{-1} KCl calomel electrode is used, the standard potential is 0.336 V at both 20 °C and 25 °C.

As well as being the basis of redox titrations, measurements of E_h are useful for correlating bacterial systems with the corresponding chemical reactions. The most common applications are therefore in the dairy, fermentation, baking and meat industries.

Density

Density (ρ) is defined as mass per unit volume and is usually expressed as $\mathrm{kg\,m^{-3}}$ or $\mathrm{g\,cm^{-3}}$. Specific gravity (SG) is the ratio of density of product to that of water, i.e. $\rho_{\mathrm{product}}/\rho_{\mathrm{water}}$. The density of milk and milk products is used to convert volumetric measurements to gravimetric and vice versa, and to calculate other physical properties, such as kinematic viscosity and thermal diffusibility. Measurement of the density is also an indirect way of measuring the total solids concentration and hence checking for the adulteration of milk with water. The in-line measurement of density is commonly used for process control purposes, e.g. measurement and control of the total solids in milk concentrates from evaporators or standardization of milk for cheesemaking.

The density of milk depends on its composition and can be calculated from the density and mass fraction of individual components. Equation [5] can be used to estimate the density of the product when the apparent density and the mass fraction of each component are given:

$$\frac{1}{\rho} = \sum\left(\frac{M_x}{\rho_x}\right) \qquad [5]$$

where ρ is the density of the product, ρ_x is the apparent density of each component and M_x is the mass fraction of each component.

The density of concentrated products is obtained using the following expression:

$$\frac{1}{\rho_c} = \frac{R}{\rho_o} + \frac{1-R}{\rho_w} \qquad [6]$$

where ρ_c is the density of the concentrated product, ρ_o is the density of the initial unconcentrated milk, ρ_w is the density of water and R is the concentration ratio, i.e. the ratio of the total solids of the concentrated milk to the total solids of the initial milk.

The densities of dairy products (milk, whey, evaporated milk, sweetened condensed milk and freshly frozen ice cream) are generally measured by: (1) weighing a known volume by pycnometer or hydrostatic balance; (2) determining the extent to which an object sinks, using hydrometers or lactometers; (3) hydrostatic weighing of an immersed bulb with a Westphal or analytical balance; or (4) measuring the volume of a given weight of product, using a dilatometer. The choice of method depends on the precision required on the one hand and speed and convenience on the other. A dilatometer would seem to be the instrument of choice for determining the density at various temperatures, with a precision of 1×10^{-5} or $1 \times 10^{-6}\,\mathrm{g\,ml^{-1}}$. Pycnometers offer a precision of up to $5 \times 10^{-6}\,\mathrm{g\,ml^{-1}}$ but disadvantages are the long time required to reach temperature equilibrium and the possibility of loss of fat (with fat-containing products) at the capillary. However, the error arising due to evaporation at high temperatures, which constitutes a problem with lactometers or hydrostatic balances, can be avoided with pycnometers.

The density of milk depends on composition, temperature and temperature history. The density of fluid milk products decreases with increasing temperature because of thermal expansion. The fat content and the proportions of liquid fat (lower density) and solid fat (higher density) have the greatest influence on the density of milk. The ratio of liquid fat to solid fat depends on the temperature and temperature history of the milk, as considerable supercooling of milk fat can occur and fat crystallization can be slow. Therefore, the best approach is to pre-warm the milk to 40–45 °C, hold for a few minutes to melt the fat completely and cool the milk to 20 °C before measuring the density.

The determination of total solids from density measurements is based on the following expression:

$$\mathrm{TS} = \frac{(\mathrm{SG_{SNF}} - \mathrm{SG_{fat}})F}{SF_{\mathrm{fat}}(\mathrm{SG_{SNF}} - 1)} + \frac{\mathrm{SG_{SNF}}(100\mathrm{SG_{milk}} - 100)}{\mathrm{SG_{milk}}(\mathrm{SG_{SNF}} - 1)} \qquad [7]$$

where F is the % fat (w/w), SNF is the % solids-not-fat (w/w) and TS is the percentage total solids. At a given temperature, SG and ρ are related by the equation:

$$\rho = \mathrm{SG} \times \rho_{\mathrm{water}} \qquad [8]$$

Lactometers are special hydrometers intended for determining the milk solids-not-fat (MSNF) and total solids contents of milk. Their simplicity and rapidity have made them popular with dairy factories that do not have to analyze a large number of samples and do not have to invest in sophisticated methods of analyses of fat and total solids. The Quevenne lactometer is most commonly used for determining the MSNF or total solids of milk. Its stem is calibrated in units from 15 to 40; these units represent the last two significant digits of SG values from 1.015 to 1.040.

The formula, as originally proposed by Richmond for calculating the MSNF and total solids from SG measurements, did not take into account the increase in the SG of milk that occurs when milk fat solidifies; this is known as the Recknagel phenomenon. Errors due to the Recknagel phenomenon can be avoided by determining the SG after the milk samples have been heated to 45 °C for 30 s and cooled to 30 °C so that the fat remains in a fluid state. The MSNF and total solids are calculated using the following formulae:

$$\%\mathrm{MSNF} = 0.2537\mathrm{F} + \frac{268(L+3)}{L+1000} \qquad [9]$$

and

$$\%\mathrm{TS} = 1.2537F + \frac{268(L+3)}{L+1000} \qquad [10]$$

where F is percentage fat and L is the Quevenne lactometer reading.

For a lactometer calibrated at 102 °F (39 °C), the equation proposed for percentage total solids is:

$$\%\mathrm{TS} = 1.33F + \frac{273L}{L+1000} - 0.40 \qquad [11]$$

Dispersed gas can considerably lower the density, which then becomes dependent on pressure. Density measurement at controlled pressure is one way of determining the gas content of fluid milk products.

Freezing Point

Milk may be adulterated by the addition of water or standardized by the addition of skim milk or partially skimmed milk. Generally, when solutes are dissolved in an aqueous solvent, the freezing point of the solvent is lowered. Such lowering is usually proportional to the concentration of solutes in the solvent. The relationship between the depression of the freezing point (T_{f}) and the molality of an aqueous solution (M) is given by Raoult's equation:

$$T_f = K_f M \qquad [12]$$

where K_{f} is the molar depression constant (1.86 °C for an ideal aqueous solution). When water is added to milk, the concentration of fat, proteins, salts and lactose dissolved in the serum is reduced. As the freezing point of milk is lower than that of water, progressive dilution of milk with water gives a freezing point approaching that of water. Therefore, the adulteration of milk with water may be detected using the freezing point test. In the dairy industry, freezing point is mainly used to determine added water but it can also be used to determine the lactose content of milk, to estimate the whey powder content in skim milk powder and to determine the water activity of cheese. The use of the freezing point test demands caution and understanding of the factors that cause variations in the freezing point value.

Lactose and chloride account for approximately 75% of the freezing point depression of milk. Fat globules, casein micelles and whey proteins make negligible contributions to the freezing point depression. Most of the variations among normal milk samples have been attributed to changes in the non-chloride ash fraction of the milk. However, freezing point of bulk milk is relatively constant. There are small differences in freezing point between breeds (of the order of 0.002–0.007 °C), with Holstein milks generally having the lowest freezing point. There is a slight tendency toward lower freezing points in late lactation but it is not clear whether this effect is independent of feed effects. Similarly, seasonal differences in freezing point are probably due to feed effects. The freezing point of morning milk tends to be 0.003–0.007 °C lower than that of evening milk. Larger differences may be observed if the cattle do not have free access to water at all times. Variations in the proportion of grains to roughage and fresh forage versus dry forage have significant but small effects on the freezing point. With respect to the interpretation of freezing point for added water determinations, the most significant variables are the nutritional status of the herd and the access to water. Underfeeding causes an increase in the freezing point. Large temporary increases in freezing point occur after the consumption of large amounts of water.

The primary sources of non-intentional added water are rinse water and condensation in the milking system. Leaky coolers used to cool the milk before it enters the bulk tank may also be a problem. Recommended procedures to avoid added water, to determine the residual water in milking systems and to obtain authentic milk samples for interpreting freezing points have been reported. Soured or fermented milk is unsuitable for added water testing, because the freezing point is lowered by lactic acid and increased concentrations of soluble minerals. Heat treatment, provided it does not involve vacuum treatment, is unlikely to affect the freezing point of milk.

The Hortvet cryoscope has been used for a number of years to measure the freezing point of milk. The apparatus and the technique for determining freezing points are outlined in detail in British Standard 3059 (1959) and *Official Methods of Analysis of the Association of Official Analytical Chemists* (1970). The freezing points of sucrose solutions at 7% w/v (−0.422 °C) and 10% w/v (−0.621 °C) were prescribed by the US Bureau of Standards as reference points for calibration of Hortvet and other cryoscope thermometers. Temperatures reported on the Hortvet (H) scale were initially thought to be equivalent to Celsius (C) values. However, the freezing point of a standard 7% sucrose solution at −0.422 °H is actually −0.408 °C and that of a 10% sucrose solution at −0.621 °H is −0.600 °C. Some cryoscopes are calibrated in terms of degrees Hortvet but the results are often reported in degrees Celsius. The results obtained from cryoscopes employed using Hortvet's principle are not true freezing points. However, it is possible to correct the results from one method of calibration to the other:

$$^{\circ}\mathrm{C} = 0.96231\,^{\circ}\mathrm{H} - 0.00240 \qquad [13]$$

and

$$^{\circ}\mathrm{H} = 1.03916\,^{\circ}\mathrm{C} + 0.00250 \qquad [14]$$

where H = Hortvet reading (negative value) and C = Celsius reading (negative value).

The general procedure used to determine the freezing point of milk is as follows. The Hortvet thermometer is first standardized with water and calibrated against pure sucrose solutions. A 7% sucrose solution or salt equivalent freezes at −0.408 °C; a 10% sucrose solution or salt equivalent freezes at −0.600 °C. Calibration should be checked at least daily. The freezing point of the standard solution is determined using the same test procedure as for the milk sample.

The percentage of added water in milk is given by:

$$\frac{B-T}{B} \times 100 \qquad [15]$$

where B is the base freezing point of the authenticated sample and T is the true freezing point of the test sample. For example, if the authenticated sample freezes at −0.510 °C and the unknown milk freezes at −0.485 °C, then:

$$\%\text{added water} = \frac{(-0.510)-(-0.485)}{(-0.510)} \times 100 = 4.9\% \qquad [16]$$

Samples with large amounts of added water should also be tested for fat, solids, lactose or protein to confirm a problem. Samples that will not freeze may have a high dissolved solids content, caused by high acidity or by a contaminant, such as cleaners or chlorine. Samples that continue to prefreeze may have high bacterial counts or high somatic cell counts.

If the titratable acidity of milk exceeds 0.3% lactic acid, the freezing point test should not be applied. When the titratable acidity exceeds 0.18% lactic acid but does not exceed 0.3% lactic acid, a correction of 0.0034 °C for each 0.01% lactic acid above 0.18% shall be applied according to the formula:

$$\begin{aligned}&\text{Freezing point depression (corrected)}\\&\quad = \text{freezing point depression} - 0.34 \times (\%\text{lactic acid} - 0.18)\end{aligned} \qquad [17]$$

For routine determinations of added water, it is important to have a reliable reference point. Based on a UK study, it was concluded that fewer than 1 in 1000 samples of genuine or authentic milk (i.e. milk produced under supervised conditions and certified free of added water) will have a freezing point higher than −0.508 °C. The reference point recommended in 1970 by the Association of Official Analytical Chemists (AOAC) is −0.505 °C (−0.525 °H). This value is based on a North American study of genuine milk and is still used by most testing laboratories in North America. Milk samples with a freezing point higher than this reference point are considered to contain added water. In a study of the freezing point of milk in The Netherlands, it was suggested that the reference point should not be fixed but should vary with season and region.

Correct interpretation of freezing point data with respect to added water depends on a good understanding of the factors affecting freezing point depression. It is often necessary to conduct repeat sampling and obtain genuine samples from herds showing freezing points near the reference point in order to eliminate natural causes of abnormally high freezing points.

Added water may also be estimated from changes in osmotic pressure, as measured by vapor pressure osmometry. Vapor pressure is measured as a function of dew point depression. A thermocouple detector senses the temperature of a milk sample at vapor pressure equilibrium in the headspace of a sample chamber. The results, expressed as milliosmoles per kilogram of water, are highly correlated to freezing points and the procedure has been approved by the AOAC for the determination of added water in milk.

Polarimetry

A number of chemical compounds, with an asymmetric carbon atom, are 'optically active', as they have the ability to rotate the plane of polarized light. An optically active compound can exist in two mirror images, one of which is dextrorotatory and the other of which is levorotatory.

The angular rotation of an optically active compound in solution is directly proportional to the concentration of the compound, the length of the column of solution through which the light passes and the specific rotatory power of the substance. Specific rotation (α) is defined as the angular rotation (in degrees) of the plane of polarized monochromatic light produced by a solution containing 1 g ml^{-1} of optically active compound and with a light path of 1 dm. The basic relationship is expressed as:

$$(\alpha) = \frac{100A}{IC} \qquad [18]$$

where A is the observed rotation (plus or minus), l is the length of the tube in decimeters and C is the concentration in grams per 100 ml of solution. Specific rotation is a function of temperature as well as the wavelength of light. Therefore, to standardize data, specific rotation is indicated as $(\alpha)_D{}^{20}$, which refers to the specific rotation at 20 °C with light from the D-line of the sodium spectrum.

The optical rotatory power of many compounds is a characteristic and readily determined property, and hence can be used in both qualitative and quantitative analysis. In this section, we focus on the measurement of optical rotatory powers for the quantitative determination of sugars in milk and milk products. The instrument for measuring optical rotation is called a polarimeter. The polarimeter employs monochromatic light from a sodium source and in its simplest form has two Nicol prisms. The prism nearest the light source is in a fixed position and is known as the polarizing prism; the prism nearer to the eye of the observer is movable and is called the analyzer. If the optical axis of the analyzer is at right angles to that of the polarizing prism, the radiation will be totally absorbed. This is termed total extinction. The instrument is also equipped with a scale to indicate the number of degrees through which the analyzer is rotated. The zero point may be set at the point where the two Nicol prisms are crossed without the sample in the polarized beam. If an optically active compound is placed between the prisms, the plane of polarized light is rotated either to the right (dextrorotatory) or to the left (levorotatory). The operator rotates the analyzer until the prisms are again crossed, and the angle by which the analyzer is turned is equal to the angular rotation of the optically active compound. If it is necessary to rotate the analyser to the right to accomplish total extinction, the optically active compound is dextrorotatory (+). In contrast, if the analyzer is rotated to the left, the compound is levorotatory (−).

Although a polarimeter may be used for the quantitative determination of sugars, in practice a saccharimeter is more commonly employed. The essential differences between these two instruments are that a polarimeter employs monochromatic light and reads in angular degrees, whereas a saccharimeter employs white light and reads the sugar concentration directly, provided a single sugar is present and a normal weight of sugar is used for the reading. A normal weight of sugar is defined as the weight that, when made to a volume of 100 ml and viewed in a 200-mm tube at 20 °C, will give a reading of 100°.

The general procedure for determining lactose in milk is based on the method of double dilution. A portion of milk equal in weight to twice the normal weight of lactose (normal weight, 32.9 g 100 ml^{-1} of solution), i.e. 65.8 g, is transferred to each of two volumetric flasks, 100 and 200 ml. Both flasks are treated with a clarifying agent consisting of mercuric iodide solution and 5% phosphotungstic acid solution (5 ml are added to the 100-ml volumetric flask and 15 ml to the 200-ml volumetric flask) and diluted to volume with distilled water. This step results in the precipitation of the protein and fat in the milk. The flasks are shaken frequently during a 15-min period, the contents are filtered through dry filter paper and the percentage lactose is determined on the filtrate using a polarimeter. To determine true polarization and hence the actual percentage of lactose, it is necessary to allow proper correction for the volume of the precipitate. It is preferable to read the solution from the 200-ml volumetric flask in a 400-mm tube and the solution from the 100-ml volumetric flask in a 200-mm tube.

$$\%\text{Lactose} = \frac{R_{100} - [2(R_{100} - R_{200})]}{2} \qquad [19]$$

where R is the polarimeter reading.

The polarimetric method is very easy to use and is suitable for routine analysis. The instrument cost and the cost per run are low and multiple samples can be prepared. However, the method is not suitable for use when the concentration of lactose is low or for the determination of lactose in the presence of other optically active substances.

Total Solids Determination

The total solids represent the components that remain after the complete removal of water. A known amount of sample is weighed into a pre-weighed, clean and dry sample container. For greatest accuracy, samples are weighed to the nearest 0.0001 g. Heat is applied to the sample until a constant weight is attained, the sample is cooled and the weight is again determined. The total solids (%) are calculated as the weight of the sample after drying divided by the weight of the sample before drying multiplied by 100. Any variation in procedures is in the method of applying heat. The official procedure accepted by the AOAC, International Standards Organization and International Dairy Federation specifies dehydration under atmospheric pressure. The basic procedure is to pre-weigh 2.5–3.0 g of prepared sample into a weighed flat-bottomed dish of diameter 5 cm or greater. The sample is preheated on a steam bath for 10–15 min, and then transferred to an air oven at 98–100 °C for 3 h. The AOAC Official Method (1996) for moisture in cheese recommends heating in a vacuum oven (13.3 kPa) at 100 °C for approximately 4 h after partial drying on a steam bath.

The dish and sample are cooled in a desiccator and quickly weighed, and the results are calculated. Specific precautions apply to all weight determinations. Once the dishes are pre-dried, they should be exposed to the atmosphere for a minimum length of time and should be handled only with forceps and tongs. All cooling should be done in a clean desiccator.

There are several other procedures that are applicable to milk for rapid screening but do not serve as official test methods. In the Mojonnier method, moisture is removed by pre-drying the sample on a hot plate at 180 °C until slight browning occurs. The sample is completely dried in a vacuum oven (not less than 6.7 kPa) for 10 min at 100 °C.

When high precision and accuracy are not required, moisture may be determined using a moisture balance with an infrared heating device. The time required for heating in the moisture balance will vary depending on the amount of moisture in different types of sample. Once the correct drying time has been established for a product type, it is desirable to have the same drying time for all similar samples to be tested.

Microwave energy has also been used to remove moisture. Factors that affect drying are time, sample size, position of the sample in the oven and energy of the microwaves. As microwave ovens vary from unit to unit, each must be evaluated individually. The power setting and time may vary between units and with the age of the unit. The exact power setting and time should be determined using samples of various total solids content and comparing the results with the results of the same set of samples tested using a vacuum oven.

Both infrared heating and microwave determinations are useful for the in-process determination of moisture because the results may be obtained in minutes rather than hours.

The total solids content of products with a high amount of soluble solids may be determined using a refractometer. This is especially useful for the in-process control of moisture in whey and whey products. However, if air is incorporated in the sample, erroneous results are generally obtained. Therefore, the samples must be thoroughly but carefully mixed. The accuracy of the test is about ±0.5–0.6%. The results should be compared with those from a reference method, and a correction should be made based on multiple tests.

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Chromatographic Methods; Principles and Significance in Assessing Rheological and Textural Properties.

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Differential Scanning Calorimetry

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Introduction

Differential scanning calorimetry (DSC) is a thermoanalytical method that measures the difference in the amount of heat flow or the difference in temperatures between a test sample and an inert reference as both are raised and/or lowered in temperature, usually at some constant rate (°C min^{-1}). Two types of DSC instruments are available for laboratory use: the power compensation DSC and the heat flux DSC. For the power compensation DSC, the temperature of the sample (of low mass <1 g) in a sealed aluminum pan and that of the inert reference (i.e., a sealed sample pan without anything in it) are controlled independently to be at the same temperature by using two separate and identical furnaces. The difference of power input between these two specimens is measured as a function of time and recorded as the sample temperature is increased at a constant rate (e.g., 1, 2, 5, or 10 °C min^{-1}). In general, the temperature scanning program is designed such that the sample holder temperature increases linearly as a function of time. For the heat flux DSC, both the sample and the reference are enclosed in the same single furnace, which is much larger in mass than the furnaces used in power compensation DSC. The enthalpy or heat capacity changes in the sample cause a difference in temperature between the sample and reference. This temperature difference is related to the enthalpy change in the sample and can be converted into heat flow information through calibration experiments and mathematical equations built into most of the programs supplied by the instrument manufacturers.

The slower the temperature rise used in the instruments, the more reliable are the results, but the experiments take much longer. Thus, a scan from −40 to +140 °C at 10 °C min^{-1} takes 18 min but at 1 °C min^{-1} takes 3 h. Typically, a researcher will scan up at constant rate, dwell for 5–10 min at the upper end to anneal the sample, rapidly cool back to the original start temperature, dwell again to anneal for several minutes, and then rescan at the original rate. This then means a test time of 1–10 h depending on the scanning rate. The difference between the two scan-up curves can verify irreversible or reversible gelation of starch or denaturation of dairy proteins and enhance the ability to detect the glass transition temperature (T_g). We usually regard that any changes that take place in the sample are physical and not chemical. This is not true if we have milk protein and lactose present together as the Maillard reaction occurs, which can confound the results, especially if one goes above 80 °C. Above 100 °C, one can lose water by boiling off if the sample pan is not sealed properly, and this may cause severe chemical degradation, especially in dry or semimoist systems. Thus, it is important to measure the weight of the sample pan/sample before and after the test run and open the pan to see the condition of the sample. They should be the same; otherwise, reactions have occurred and/or moisture loss has occurred allowing for weight loss through the sample lid/pan seal.

Phase Transitions of Dairy Ingredients Measured by Differential Scanning Calorimetry

The major application of DSC is to determine various phase/state thermodynamic and transient transitions (**Figure 1**), including glass transition, crystallization, melting, and denaturation. These transitions involve heat capacity changes (*Cg*) or energy change. The major components of milk and its processed products include lactose, fats, proteins, water, and minerals. During processing and storage, changes in the physical states of these dairy components may occur, resulting in variations in the product functionality and stability. In most cases, these changes relate to first-order or second-order phase transitions, the latter being the glass transition while the former may be melting and/or crystallization of solutes like lactose and denaturation/renaturation of proteins.

Glass Transition of Lactose

Lactose is a sugar found in milk at about 4.5–5% (w/w). During the production of whole or skim milk powders, the preconcentrated milk is dried (spray, freeze, drum) into a continuous mass that consists of mainly amorphous lactose, proteins, and fat globules. The physical state of lactose is very important for the shelf life of milk powder. With a low water content (or water activity) as a result of concentration and drying at a low environmental temperature, <30 °C, the lactose has gone from a solution into an amorphous solid state called a glass (i.e., it is

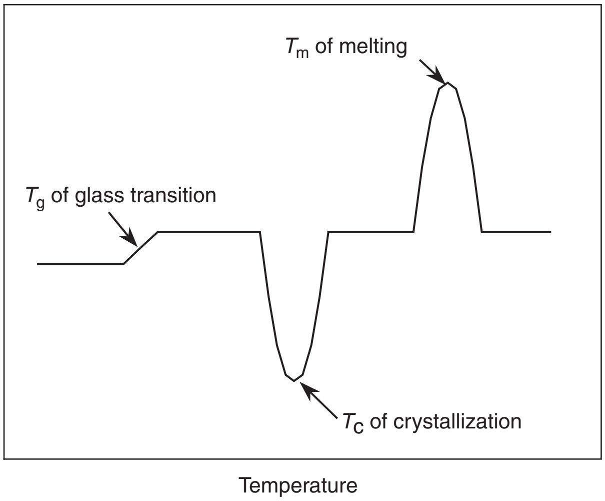

Figure 1 A schematic DSC curve demonstrating the appearance of phase transitions including glass transition, crystallization, and melting.

not crystalline as can be shown through X-ray crystallography). An amorphous state is a nonequilibrium physical state unlike the thermodynamically stable crystal, liquid, or gas states. **Figure 2** shows a hypothetical 'state diagram', which includes the solid, liquid, and gaseous state of a water/food component as well as the glass transition line. The filled circle at 23 °C represents the position on the diagram for the glassy state for a free-flowing stable lactose system such as whole milk powder or dry whey protein concentrate. An increase in either the environmental temperature or moisture content (i.e., exposure to higher humidity) or both, as represented by the dashed arrows, brings the sample above the T_g of the matrix. This causes the transition of lactose from a glassy amorphous state into a rubbery amorphous state, which leads to caking and crystallization, undesirable to the industry. Caking begins to occur at about 10 °C above the glass transition line. This transition due to the increase of moisture content occurs because water is an effective plasticizer, and the plasticization by water reduces the T_g of an amorphous powder matrix. Roos and Karel as well as Jopilla and Roos have researched this extensively in dairy powders (*see* **Water in Dairy Products**: Water in Dairy Products: Significance).

The glass transition is measured as a change in the heat capacity of the system as the matrix goes from the glassy state to the rubbery state. It is a second-order endothermic (sample takes in heat) transition, so in the DSC scanning curves, the transition appears as a step transition due to a step change (**Figure 1**) in heat capacity (C_p). Generally, there is no peak, but in some studies a small endothermic relaxation peak is found. The actual glass transition occurs usually over a temperature range of up to a few tens of degrees (i.e., it is not a point defined by one or two decimal places). Thus, T_g can be reported as a combination of the temperature at the half-height of the C_p change, which is the point where the differential slope (dT/dt) between steps is zero. The temperature at the beginning of the transition ($T_{g\ onset}$) and at the end of the transition ($T_{g\ end}$) should also be reported; unfortunately, there is no standard. It is imperative that research papers elaborate on the test conditions used and the criteria used for reporting T_g. In general, a faster scanning rate results in a higher

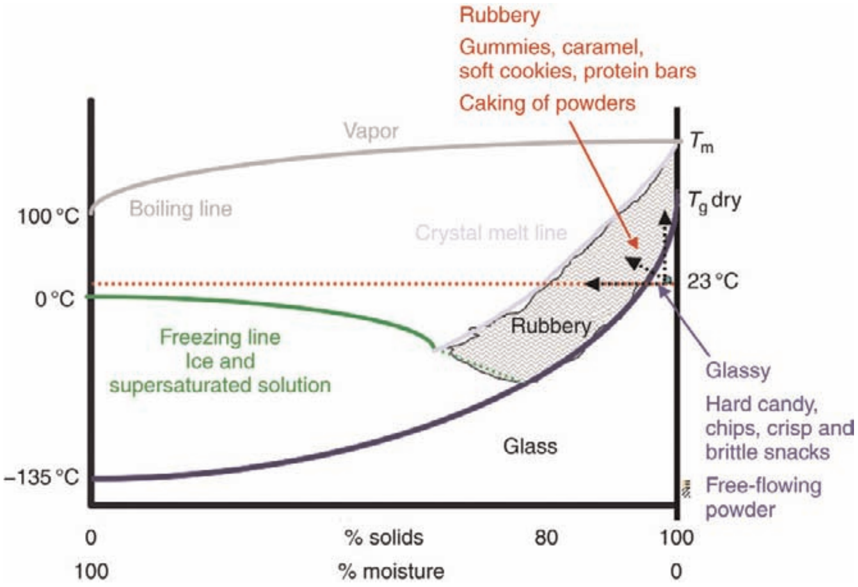

Figure 2 Hypothetical state diagram for a dry dairy powder containing lactose showing the state position as a function of % solids (or % moisture) and temperature.

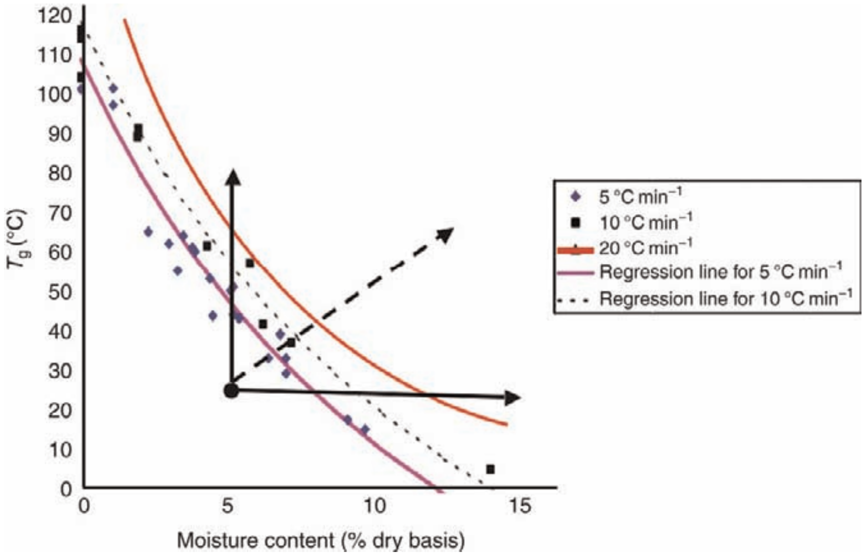

Figure 3 The glass transition curve for lactose taken from many published sources showing the effect of DSC scanning rate on measured value. As also seen in **Figure 2**, the black circle at 5% moisture and 25 °C represents a stable glassy system while the three arrows show the system transitioning over the T_g curve to enter the unstable rubbery region where caking can occur. Note that this scale of % moisture is in the opposite direction from that shown on the state diagram (**Figure 2**).

reported T_g as shown for data compiled from many studies on lactose (**Figure 3**). The difference occurs as the actual sample temperature lags behind the measured heater temperature due to heat transfer limitations in the DSC. The value of T_g measured by DSC is also affected by various external factors such as pan size, sample thickness, and moisture content. Test conditions should be kept consistent to make the final data comparable between laboratories.

Because of a possible concomitant enthalpy relaxation, it is sometimes difficult to determine the T_g of lactose in a single scanning on the DSC, so in most cases, a second scanning is required to give a clear result. The dry glass transition temperature ($T_{g\ onset}$) of lactose, as shown in **Figure 3**, is about 100 ± 5 °C depending on the scan rate. The DSC scan pattern of skim milk powder is very similar to that of lactose, suggesting that the physical state of skim milk powder is governed mostly by lactose (**Table 1**). With the increase of water content in lactose or skim milk powder, the T_g decreases sharply, indicating the significance of the plasticizing effect of water molecules on matrices containing lactose.

Crystallization and Melting of Lactose

At a temperature between the T_g and the melting temperature, the crystallization of amorphous lactose occurs depending on the water content/water activity. **Figure 4** shows the resulting crystallization and caking in whey powder held at three water activities for 1 week at 25 °C. The rate of crystallization depends on the difference between the environmental temperature and the T_g of the matrix (i.e., $T - T_g$). The crystallization of lactose can also be achieved by cooling of a supersaturated lactose solution into the rubbery zone. In addition, partial crystallization may occur during the spray drying of milk

Table 1 Glass transition temperatures of lactose and skim milk powder measured by DSC

	Lactose		*Skim milk powder*	
Water activity	*Water content (g of H_2O per 100 g solid)*	T_g *(°C)*	*Water content (g of H_2O per 100 g solid)*	T_g *(°C)*
0	0	97	0	92
0.12	2.4	64	3.7	58
0.24	4.3	43	5.6	34
0.33	5.9	33	7.1	33
0.44	8.6	15	8.8	9

Data from Jouppilla K and Roos YH (1994) Glass transitions and crystallization in milk powders. *Journal of Dairy Science* 77: 2907–2915.

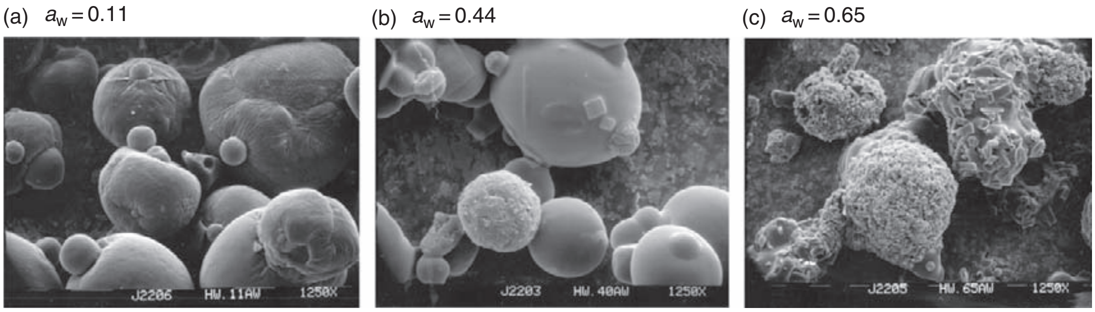

Figure 4 Scanning electron micrographs showing the influence of increased water activity and thus moisture plasticizing on the crystallization of lactose in whey powder at 25 °C.

or milk products. The degree of crystallinity can be controlled by varying the operation parameters.

In determining the degree of crystallinity of lactose in a powder by DSC, one makes a slow ($\leq 5\,^{\circ}\mathrm{C\,min^{-1}}$) upward first scan where one would first see an exothermic peak in which the amorphous noncrystalline lactose crystallizes followed by another peak at a higher temperature where all the lactose present melts. ΔH_c represents the enthalpy change of the crystallization peak of the amorphous lactose and can be determined by integrating the areas ($\mathrm{J\,g^{-1}}$) under the exothermic peak during heating scanning, and ΔH_m ($\mathrm{J\,g^{-1}}$) represents the enthalpy change of lactose under the endothermic melting peak on the upward scan. Note that a scan gives a plot of energy in heat units versus temperature or time at a constant heating rate, $\mathrm{d}T/\mathrm{d}t$; thus, the area under the peak, the integral of $\mathrm{d}Q/\mathrm{d}t$ versus T, is the total enthalpy change ΔH ($\mathrm{J\,g^{-1}}$) per gram of a material going through a state change. The term ΔH_{ms} is a reference value and represents the heat enthalpy of melting if the lactose were 100% crystalline, thus eqn [1] applies. Depending upon the sample's given thermal history, the crystallization exothermic peak may or may not be observed during the DSC experiment, it is important to use as low a $\mathrm{d}T/\mathrm{d}t$ as possible.

$$\text{Crystallinity (\%)} = \frac{[\Delta H_m - \Delta H_c]}{H_{ms}} \times 100\% \qquad [1]$$

In addition to determining the amount, one can also use the DSC to determine the time to crystallize as a function of temperature and moisture content and position above the T_g line (i.e., $T - T_g$) (**Figure 5**). This is done by rapidly bringing the sample up to the desired temperature and then waiting. Because the process may be for days at room temperature, generally higher temperatures are used to extrapolate down to a $T - T_g = 10$ where caking and crystallization can start to occur. From **Figure 3**, at 5% moisture, the T_g using the slowest scan rate is about 48 °C; thus, one would want to hold samples at >58 °C, while at 7% moisture this is reduced to >35 °C. On a final note, the work of Roos also showed that a semilog plot of time to crystallize versus water activity gives a straight line, which is very useful for both extrapolation and interpolation.

Phase Transitions of Fat

The fat in milk is a complex mixture composed mainly of triacylglycerols (TAGs), which are the esters of fatty acids and glycerol. More than 400 fatty acids have been identified from milk fat, and they vary in the length of chain and the number and location of unsaturated double bonds. Because of the diversity of TAGs and the polymorph forms (the existence of more than one crystalline modification of the same substance) for each TAG, the melting point of milk fat can vary from −40 to +40 °C. The composition and physical state of milk fat have a significant effect on the chemical and physical properties of fat-rich dairy products, such as cheese, cream, and butter. Thus, an extensive investigation of the thermal properties of milk fat in various dairy products can contribute to a better understanding of the structure, texture, and sensorial properties of dairy foods.

DSC is a suitable technique for investigating the melting and crystallization properties of milk fat; however, the melting ranges of different components in milk fat often overlap each other; moreover, for each fat component, there may exist several different polymorph forms. So the determination of melting behavior of a single component of milk fat is almost impossible by using DSC. **Figure 6** shows the superposition of DSC plots for whole milk powder previously stored at different water activities for 4 weeks. The scan rate was $5\,^{\circ}\mathrm{C\,min^{-1}}$. Peaks labeled '1' are fat melting endotherms found between 0 and 45 °C. As seen, there are three different melting curves; we did not scan below the freezing point to save time. As noted, they do not change in position with the increase of moisture content (a_w increases from 0.06 to 0.44) as would be expected. The peak labeled '2' is a protein denaturation endotherm, which shows a decrease in onset temperature with increased moisture (a_w). The peak labeled '3'

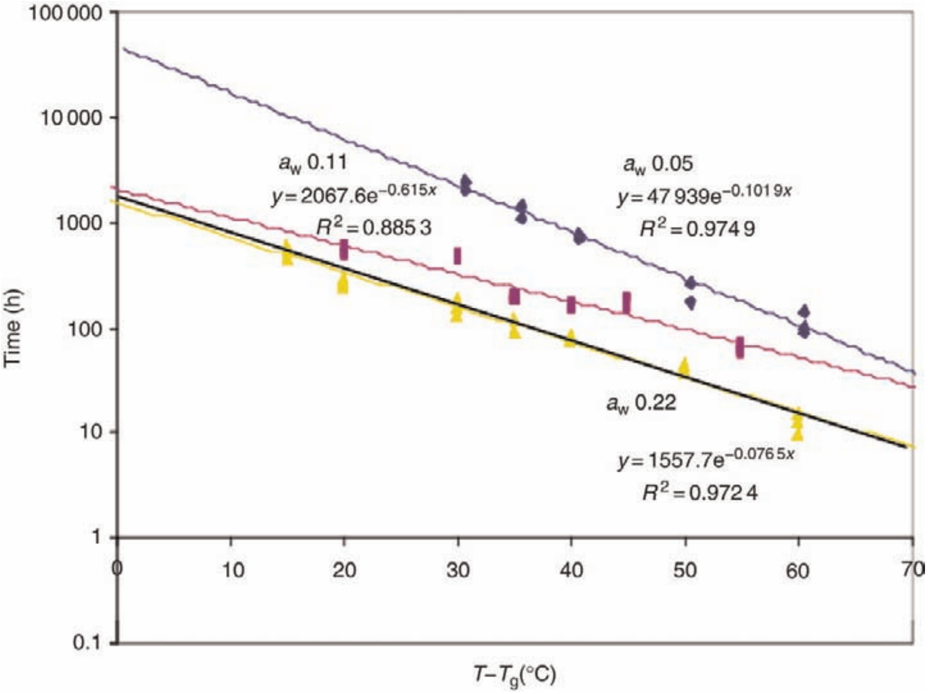

Figure 5 Effect of temperature and moisture on time to crystallize pure lactose as a function of $T-T_g$. Note that close to T_g, the line theoretically rises exponentially to infinity (not shown). The a_w 0.05 sample is ~1% water (db), the 0.11 sample is ~3% water (db), and the 0.22 sample is ~6% water (db). From Labuza TP, Roe K, Payne C, *et al.* (2004) Storage stability of dry food systems: Influence of state changes during drying and storage. In: Silva M and Rocha S (eds.) Drying, pp. 48–68. Brazil: Ourograf Grafica Campinas.

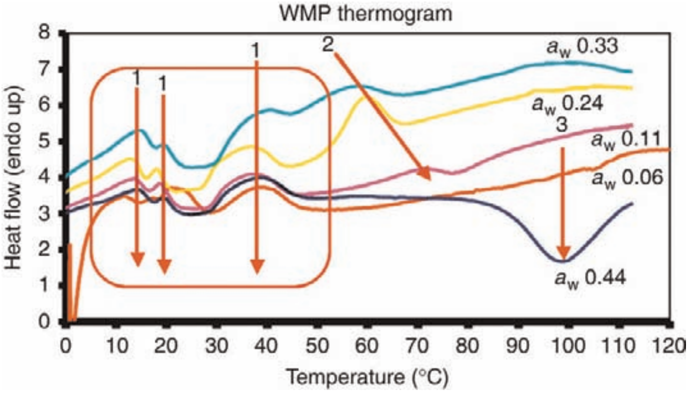

Figure 6 Superimposed DSC plots for whole milk powder (WMP) previously stored at different water activities for 4 weeks. Scan rate 5 °C min^{-1}. Peaks labeled '1' are fat melting endotherms, the peak labeled '2' is a protein denaturation endotherm, and the peak labeled '3' is a lactose crystallization exotherm. Above 115 °C, the Maillard reaction caused all peaks to be unmeasurable.

is a lactose crystallization exotherm as discussed earlier, indicating that at the lower water activities no lactose crystallized, but during storage at 44% Relative Humidity (RH) there was enough plasticizer to induce lactose crystallization. In addition, there are no protein denaturation peaks, possibly because the Maillard reaction occurred during storage, unraveling the protein. Above 115 °C, the Maillard reaction caused all peaks to be unmeasurable due to reaction during the scan (portion not shown). One should note that the DSC data may also be used to calculate the kinetic parameters and activation energy of the thermal oxidative decomposition of milk fat, although if lactose is present this is masked by the Maillard reaction.

Protein Denaturation in Processing

In milk and its dried processed products, casein and whey proteins are the major protein fractions affected depending on the degree of processing (e.g., in cheesemaking, the resulting whey solution has essentially no casein). Heat treatment is a common operation in dairy and other food processing during pasteurization, concentration, and drying. Changes in protein structures during heat treatment and storage have significant effects on their functional properties and may also lead to decreased protein quality if they undergo further protein–protein interactions by the exposed –SH groups (e.g., disulfide bond formation).

Denaturation of proteins may occur during heat processing, and the liability and reversibility of protein denaturation depend on the type and structure of proteins and environmental factors such as pH, concentration, and ionic strength. However, in the initial heat treatment (pasteurization) required to eliminate pathogens for powders and some cheeses, casein is relatively stable, while whey proteins are liable to denaturation due to their globular structures. Whey proteins are a mixture of proteins, in which β-lactoglobulin (β-L$_g$,50%) and α-lactalbumin (α-L$_a$,30%) are the two major components, with bovine serum albumin (BSA) being the third at ~10%. The denaturation temperature of whey protein isolate and β-L$_g$ shows a similar and strong dependence on the water content as shown in **Figure 7** where it decreases exponentially with increasing water content. However, the decrease in denaturation has a temperature limit based on the first law of thermodynamics; that is, the driving force for denaturation requires a negative change in free energy (i.e., ΔG, the free energy change for the phase change where $\Delta G = \Delta H - T\Delta S$ must be negative). In this equation, T is the reaction temperature in K. Both ΔH (enthalpy measured by DSC) and ΔS are positive, but as $-T\Delta S$ gets larger than ΔH, denaturation is favored, that is, ΔG becomes negative and the reaction is favored. Thus, for many proteins including dairy proteins, at high water content, >1 g water g^{-1} protein or ≥50% moisture (wet basis), the temperature limit for denaturation is about 57–58 °C, that is, below this, $-T\Delta S$ is smaller than ΔH, so ΔG is positive and the denaturation reaction is not favored. This fact has been used by several companies to pasteurize foods such as in-the-shell eggs and raw oysters by a long-time low-temperature (LTLT) process much like the 7-day, 53 °C process used for pasteurization of dry milk powder as a critical control point in the Hazard Analysis and Critical Control Points (HACCP) plan for dairy proteins. At this temperature (53 °C), the food protein is minimally affected while the DNA of the microbial pathogens possibly present is denatured, rendering the food safe. Several companies advertise pasteurized in-the-shell eggs by an LTLT process, done for nursing homes where the inhabitants can safely consume them even though they are immunocompromised.

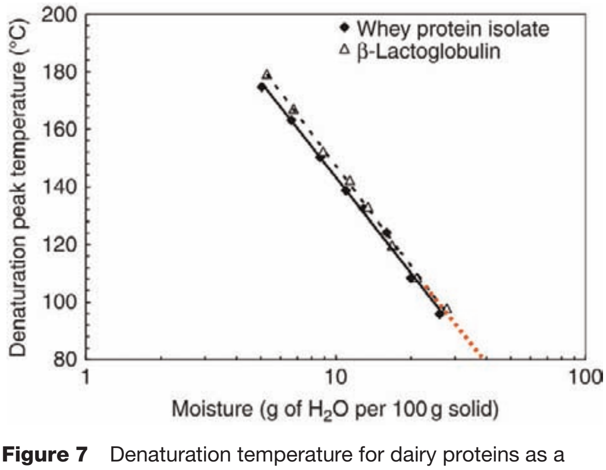

Figure 7 Denaturation temperature for dairy proteins as a function of moisture content. Adapted from Zhou *et al*. (2007).

During the storage of dry milk powders, both deleterious chemical reactions and changes in physical properties can occur over time as a function of storage temperature and relative humidity (water content). At a very low water content, dry milk powders and dairy protein powdered matrices, such as infant formula or dry protein drink mixes, generally exist in the amorphous glassy state (i.e., the T_g is well above the storage temperature). It is clear that no reactions should take place below the calculated Brunauer Emment and Teller equation (BET) (or Guggenheim Anderson and DeBeer equation (GAB)) moisture monolayer, but an increase in reaction rate with an increase in water content is possible because the liquid medium plasticizes the amorphous structure, increasing mobility resulting in a decrease in T_g. In addition, storage at a higher temperature to accelerate the reaction can cause the system to enter the rubbery state and thus also allow reactant mobility. Above T_g, the system will undergo a change from the 'glassy' state to the 'rubbery' state, converting the system from a free-flowing powder into a much more reactive system. Changes in several physical properties of food protein powders have been characterized in the rubbery state, which include stickiness, caking, and collapse. Using a DSC at low heating rate with whey protein isolate stored at different temperatures, one is able to see another effect of higher temperature storage as shown in **Figure 8** for a whey protein isolate (WPI):water ratio of 3:2. As seen, at 45 °C there is complete loss of the structure of α-La and BSA, not due directly to a physical change alone but due more to both hydrophobic interactions and irreversible disulfide bond formation making those proteins insoluble. As seen, all three protein fractions are stable at 4–35 °C, but at 45 °C the mobility induced by the temperature allowed these reactions to occur in α-La and BSA but not in β-Lg.

With respect to minerals present in milk, for α-L$_a$, it has been found that at neutral pH, this protein may be in a Ca^{2+}-bound form (holo form) or in an apo form if it is

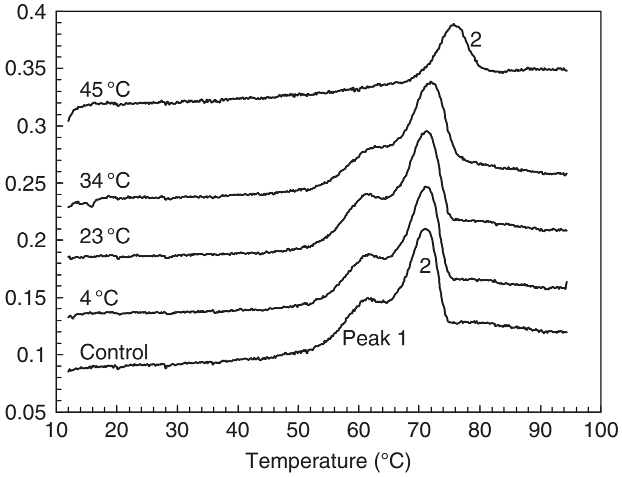

Figure 8 Effect of storage at different temperatures for 1 month on denaturation of the major proteins in whey protein isolate. Peak 1 represents the combined peak of α-lactalbumin (α-La) and bovine serum albumin (BSA), and peak 2 represents β-lactoglobulin (β-Lg).

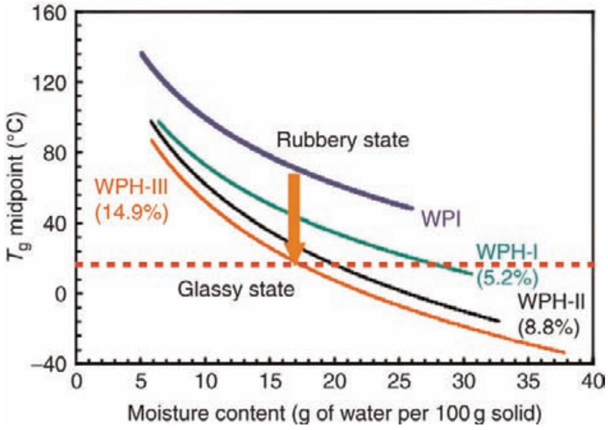

Figure 9 Effect of hydrolysis on lowering of the glass transition of whey protein isolate (<1% lactose) and its hydrolysates as a function of moisture content. Whey protein hydrolysate (WPH)-I5.2% hydrolyzed, WPH-II ~8.8% hydrolyzed, and WPH-III ~14.9% hydrolyzed.

Ca^{2+}-free. The presence of Ca^{2+} helps to stabilize the protein; thus, α-L_a in the holo form is more thermal-stable than the one in apo form and has a higher denaturation temperature and higher denaturation enthalpy as measured by DSC.

The glass transition of low-moisture dairy proteins usually cannot be observed in the first DSC scan due to the overlapping of enthalpy relaxation and denaturation peaks; however, it can be determined in the second scan by DSC. In addition, the heat capacity changes (ΔC_p) during the glass transition of globular proteins such as β-L_g and BSA are small, in the range between 0.1 and $0.2\,J\,g^{-1}\,°C^{-1}$, making it difficult to detect unless scan rates are slow. This is due partly to the complex secondary and tertiary structures of globular proteins, which results in a gradual increase in heat capacity during glass transition. After denaturation, globular proteins lose their tertiary structure, and the change in heat capacity becomes more evident. However, the T_g of denatured proteins may be only slightly different from that of native proteins, despite the changes in protein conformation and the effects of protein interactions. On the other hand, the ΔC_p of dairy protein hydrolysates (whey protein hydrolysates and casein hydrolysates) during glass transition is in the range between 0.3 and $0.4\,J\,g^{-1}\,°C^{-1}$, and the T_g of these protein hydrolysates can be easily detected by DSC. For both whey proteins and their hydrolysates, an increase in water content causes the expected exponential decrease in T_g. This is shown in **Figure 9** where the T_g of a whey protein isolate (<1% lactose) and that of three hydrolysates of it with decreasing average Molecular Weight (MW) were examined at different water contents (unpublished data from our laboratory). As seen, hydrolyzation causes significant decreases in the T_g as moisture increases; for example, at 15–17% moisture (dry basis), the difference from the original whey isolate is from 20 to 60 °C, with lower values as the percent hydrolyzed increases. Work in our laboratory has shown that using hydrolysates in protein nutrition bars ensures that the bars do not become unacceptably hard during 12 months storage at 23 °C, due basically to the lower glass transition ensuring that the system is in the rubbery state.

The Future

DSC is a powerful tool for thermal analysis. The application of DSC to illustrate the physical states and phase transitions of dairy ingredients during processing and storage can provide a better understanding of their properties and stability. New techniques such as temperature-modulated DSC (TMDSC) and micro-DSC are being developed, which will provide more choices for future dairy research. Coupling this with FT-IR, circular dichroism, and Raman spectroscopy gives the dairy protein chemist a set of tools to better understand the effects of processing and storage.

See also: **Lactose and Oligosaccharides:** Lactose: Crystallization. **Milk Lipids**: Analytical Methods. **Milk Protein Products**: Whey Protein Products. **Milk Proteins**: Analytical Methods. **Water in Dairy Products:** Water in Dairy Products: Significance.

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Principles and Significance in Assessing Rheological and Textural Properties

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Introduction

Rheological and/or textural properties of food materials are categorized within the group of physical properties, and are important for product handling, processing, and consumer acceptance. Usually, the term 'texture' is used in a very broad sense, covering not only rheological properties, but also characteristics that cannot be accessed easily by means of instrumental measures. Texture is rather linked to sensory sensations occurring during mastication, arising from the specific flow properties, structural arrangements, or even chemical properties of a particular food material. Texture properties also play a key role in consumer acceptance, and are important contributors to food quality.

Basic Concepts of Food Texture

As texture obviously means different things to different people, it is extremely difficult to achieve a final definition without ambiguity. One basic approach toward food texture is commodity oriented and relates textural properties directly to specific quality attributes of food materials. A more global definition places emphasis on interactions between food properties, originating from physicochemical characteristics, and perceptions that result from processing through the system of human senses. Generally, food texture may be defined by the following characteristics:

- Texture comprises a group of physical (partly rheological) properties, which can be derived from structural attributes.
- Texture is perceived mainly by contact, usually in the mouth (other parts of the body may be involved, too), and is not directly related to the sensations of taste and flavor.
- Objective measurements are most frequently done as a function of mass, distance, and time; some, but not all, texture properties are amenable to instrumental measurement.

Classification of Textural Properties

In the early and constitutive reports on texture properties, food texture as perceived by the human subject was classified into three categories. The haptic and kinesthetic sensation of mechanical characteristics such as firmness or brittleness was associated mainly with rheological properties of the food, and the sensation of geometrical properties was linked to perceivable macro- and microstructure; finally, other characteristics (sometimes referred to as mouthfeel) were related to residual sensations, including those of water and fat. It can be seen from **Table 1** that, besides classification into the aforementioned subgroups, the characterization of food texture in the sensory context is highly descriptive. This is very important, especially for geometrical and mouthfeel characteristics, as it is almost impossible to subject the underlying stimuli to instrumental analysis. When referring to sensory texture, it is therefore essential to consider semantic and linguistic aspects, especially when attempting to use a certain vocabulary in another geographical region or, more crucial, when attempting to translate descriptive terms. A number of collections of descriptive texture terms, partly with the underlying definitions, and translations into other languages are available in the literature. Using English and German as an example pair, it is extremely important to balance terms and descriptions in both languages to avoid a systematic bias, which, exemplarily, is evident for the translations of 'crunchy' and 'crisp' into the German language.

Much effort has been made to quantify the impact of texture properties on the total quality of foods. Although the studies show a certain variation in methodology, and size and configuration of the test panels, it may be concluded that, from the viewpoint of the untrained consumer, textural properties of foodstuffs are at least as important as flavor and aroma characteristics. It also appears that some food attributes such as crunchy, crisp, juicy, or creamy emerge irrespective of language, country, and food and eating habits, whereas the size of the vocabulary used for verbal description of texture strongly depends on the semantics of the respective language.

Table 1 Classification of texture and examples of verbal descriptors

Parameter	*Popular descriptors*
Mechanical characteristics	
Hardness	Soft → firm → hard
Cohesiveness	
Brittleness	Crumbly → crunchy → brittle
Chewiness	Tender → chewy → tough
Gumminess	Short → mealy → pasty → gummy
Viscosity	Thin → viscous
Elasticity	Plastic → elastic
Adhesiveness	Sticky → tacky → gooey
Geometrical properties	
Particle size and shape	Gritty, grainy, coarse, etc.
Particle shape and orientation	Fibrous, cellular, crystalline, etc.
Other (mouthfeel) characteristics	
Moisture content	Dry → moist → wet → watery
Fat content	
Oiliness	Oily
Greasiness	Greasy

Adapted from Szczesniak AS (1963) Classification of textural characteristics. *Journal of Food Science* 28: 385–389; Civille AV and Szczesniak AS (1973) Guidelines to training a texture profile panel. *Journal of Texture Studies* 4: 204–223.

Sensory Texture Profile Analysis

A tool to perform a complete sensory analysis of the textural properties of foods was developed in the early 1960s: the texture profile analysis (TPA). This technique is based on the classification of texture (see **Table 1**) and the sequence of mastication, which has been divided into three steps. During the first contact with a food, usually with the incisors, a person is able to identify and quantify mechanical properties such as hardness, brittleness, or viscosity, but also geometrical and mouthfeel properties. During mastication, leading to the disintegration of a food so that a swallowable state is reached, the subject is able to judge another set of mechanical properties (chewiness, adhesiveness, gumminess) and, again, geometrical and mouthfeel properties. TPA is concluded by the so-called residual sensations, which arise after the bolus has been swallowed, and include rate and type of food breakdown, moisture absorption, mouth coating, and other characteristics. It is obvious that the entire schedule of the TPA has to be adapted to the particular food under study, and some steps or criteria from the base TPA scheme may be eliminated when appropriate (e.g., it makes no sense to instruct a panel on viscosity measurements when the texture of hard cheese has to be evaluated).

For the assessment of mechanical properties, rating scales were developed. Besides physical and sensory definitions for each of the attributes, these scales comprise a varying number of rating points, with reference materials being assigned to each of them. In the case of geometrical properties and for residual sensations, verbal descriptors for the attributes must be worked out by the rating panels, and reference products should be assigned to the descriptors. An appropriate scaling and training procedure results in well-educated and experienced evaluation panels, being able to perform their assessments very reproducibly. The procedure has been adapted to various geographical regions after an appropriate adjustment of vocabularies, definitions, and underlying reference materials.

Various specific TPA methods for comparative evaluations have, among others, been published for rice, meat balls, and some cheese varieties such as Emmental, Tilsit, and Camembert. Apart from appropriate definitions for the sensory techniques of texture evaluation, there is a need to specify the parameters from both physical and sensory points of view. An example of these definitions of textural characteristics is given in **Table 2**.

Table 2 Definition of textural characteristics of hard and semihard cheeses

Parameter	*Physical definition*	*Sensory definition*
Elasticity	Ability of a substance to recover its initial shape and dimensions after being submitted to pressure	Ability of a cheese sample to rapidly regain its initial thickness after compression and deformation
Firmness	Resistance to a given deformation	Resistance of the sample to a very slight opening and shutting of the jaws
Deformability	The capacity of internal binding to be deformed and thus bring about displacements inside the substance	The ease with which the sample, once placed in the mouth, deforms in a gradual process or becomes stretched out of shape before breaking up
Friability	Characteristic of a substance that can be easily broken up into pieces	Capacity of a sample to break up into numerous pieces from the beginning of mastication
Adhesivity	The effort involved in overcoming the forces that keep two surfaces in contact	The effort needed for the tongue to detach a product stuck to the palate and the teeth

Adapted from Lavanchy P, Berodier F, Zannoni M, *et al*. (1994) *A Guide to the Sensory Evaluation of Texture of Hard and Semi-Hard Cheeses*. Paris: INRA.

Significance of Texture in Food Quality

When referring to 'quality' of materials and, particularly, foods, the reader of scientific research papers will early notice that there are at least as many definitions as papers available in the literature. The span ranges from quality concepts representative of transcendent or metaphysical approaches, wherein quality is considered to be synonymous with innate excellence resistant to analysis but recognizable only through experience, to the far other extreme of the definition spectrum, where 'objective' quality is referred as being measurable and verifiable on the basis of pre-determined 'ideal' standards. Although there might be some concern about any standard mentioned in this context, the core of this approach is formed by the hypothesis that quality can be assessed on the basis of technical, that is, both instrumental and sensory, measurements.

The 'perceived quality' approach as a global but more pragmatic attempt considers food quality as being dependent on the consumers' judgment. One important concept may be defined along the following four modalities: (1) perception, (2) product, (3) person, and (4) place.

As regards perception, the judgment of the consumer is based on experienced or associated product characteristics, especially when these characteristics are very specific (e.g., the flavor and texture of blue-veined cheeses). Both the specific product or product category under study (e.g., eye formation in cheese may be either a negative or a positive quality attribute, depending on the cheese variety) and the person factor (which is evident from different quality ratings by untrained and expert graders) may be considered to influence perceived quality as well as the place factor, which refers to context or intended purpose of use (exemplarily, a certain brand of processed cheese might be appropriate for spreading but inappropriate to be used in Hamburger preparation).

Another way to approach food quality is to distinguish between quality cues and attributes, as is evident in the quality perception quadrant (**Figure 1**). Cues are defined as product characteristics that can be observed independent of usage or consumption. Whereas intrinsic cues are strongly linked to the product itself and inaccessible to manipulations without changing the physical character of the product, the importance of extrinsic cues such as price or brand increases, especially when products are very similar in their appearance or other intrinsic cues. Experience quality attributes include taste, texture, and texture-related acoustical sensations as well as factors such as convenience or freshness and are strongly linked to the intrinsic quality cues. Finally, the group of credence quality attributes comprises a number of factors that are, in most cases, accessible only through the consumer's background or by labeling information. These parameters cannot be experienced directly but certainly play an important role within the 'perception and choice' process. If one considers the cues to be directly related to the product and, especially, the credence quality attributes to be determined by the market, a relationship such as 'the composite of those characteristics that differentiate individual units of a product, and have significance in determining the degree of acceptability of that unit by the buyer'may be constructed.

Food Texture within the Quality Complex

Other approaches to food quality distinguish between quality factors that are related to the product itself, quality factors that are related to the manufacturing process, and quality in the user-oriented context. Although these types of quality are obviously interrelated, an attempt at further differentiation of user-oriented quality may be derived from the flowchart presented in **Figure 2**. Here, the quality of food products is in conformity with consumer requirements and consumer acceptance, which are

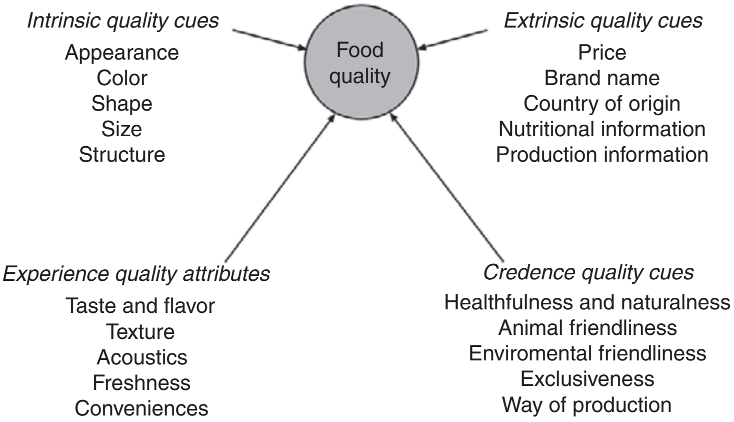

Figure 1 Effects on food quality. Compiled from Oude Ophuis PAMS and van Trijp HCM (1995) Perceived quality: A market driven and consumer oriented approach. *Food Quality and Preference* 6: 177–183.

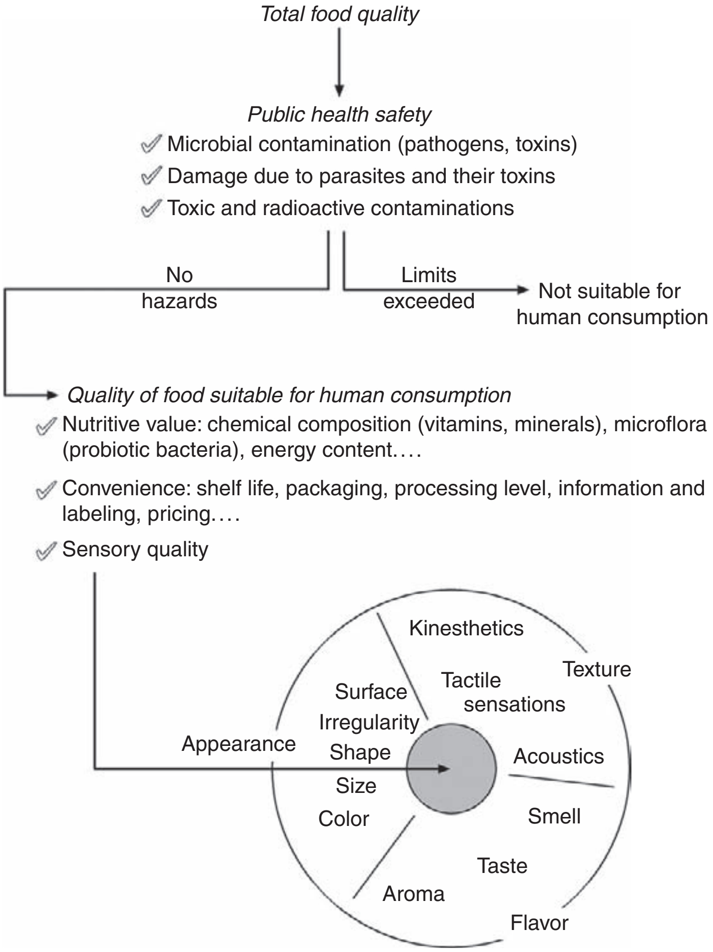
Total food quality
Public health safety
✓ Microbial contamination (pathogens, toxins)
✓ Damage due to parasites and their toxins
✓ Toxic and radioactive contaminations
No hazards
Limits exceeded
Not suitable for human consumption
Quality of food suitable for human consumption
✓ Nutritive value: chemical composition (vitamins, minerals), microflora (probiotic bacteria), energy content....
✓ Convenience: shelf life, packaging, processing level, information and labeling, pricing....
✓ Sensory quality
Kinesthetics
Texture
Tactile sensations
Surface
Irregularity
Appearance
Shape
Acoustics
Size
Smell
Color
Taste
Aroma
Flavor

Figure 2 Sensorially accessible continua within the food quality complex.

determined by the sensory attributes of a particular food, its chemical composition, physical properties, the level of microbiological and toxic contaminants, shelf life, and aspects related to packaging and labeling.

As can be seen from **Figure 2**, public health safety can be considered as being initially responsible for food quality. When food not suitable for human consumption is eliminated because of the efficient control of public health authorities or avoided by good manufacturing practices, three important groups of quality criteria remain. Whereas convenience attributes might be considered as being of secondary importance (for both the food itself and the consumer) and refer mainly to the credence quality attributes, the so-called primary quality attributes cover the nutritive value and sensory quality *per se.* It is evident for the nutritional quality that parameters including, for example, chemical and microbiological composition and energy content are important for a sufficient and balanced diet but inaccessible by the sensory system of the human being. In line with the main receptor systems responsible for perception, sensory quality may be separated into groups of 'appearance', 'texture', and 'flavor'. The arrangement of these basic criteria in a closed cycle is simply based on the fact that sensory processes involved in the perception of quality attributes are complex in nature, and overlapping occurs both within the groups and between the factors because there are different steps in quality assessment, which might be separated into 'anticipation' and 'participation'.

Special Aspects of Food Texture Sensation

When referring to the perception of texture properties, some special aspects have to be considered, which allows us to distinguish the responsible physiological processes from those of criteria grouped within the appearance and flavor sections. As a general rule, the quality appraisal of a food usually starts with an inspection of appearance properties comprising, for example, color or shape. When no attributes are out of the norm or, possibly, lead to rejection due to personal experiences, the subject goes into deeper contact with the food via tactile and kinesthetic sensations by actions such as cutting or squeezing, which still occur outside the mouth. After a decision toward acceptance is made by the consumer, part of the food will be transferred into the oral cavity where the sensations of texture properties, taste and aroma, take place. It is necessary to point out that this concept gives only a rough estimation of the sequence of processes, which depends on the type of food under study. In food materials with excessive and dominating aroma, for example, the initial appearance check will obviously occur simultaneously with an aroma scan by smelling. On the other hand, significant interactions between texture and aroma properties also occur. In certain food materials, flavor can be judged appropriately only after a destruction of the texture, which is responsible for an appropriate aroma release.

Independent of any classification, some additional remarks are necessary to characterize the process of texture sensation. Simple sensory models cover interactions between stimulus, the human organism, and a response within the general psychophysical approach. As the perception of texture is linked to functional responses of tactile and kinesthetic processes, such models must be modified because the flow of information, which results from intentional movements, which, in turn, lead to forces and deformations that can be sensed by the human being, must be considered. Besides passive stimulation, the sensory perception of food texture also covers complex stimuli arising from the motoric activity, for example, by squeezing between fingers or tongue and palate (**Figure 3**). In the corresponding model, we observe passive stimuli and motoric movements that lead to an integration of the secondary, active stimuli in the receptor system.

Basic Concepts of Food Rheology

In 1929, EC Bingham introduced the term 'rheology' (which was deduced from the Greek word 'rhein', meaning 'to flow') to the scientific community and founded the American Society of Rheology; he defined rheology as an unequivocal scientific discipline that 'is the study of deformation and flow of matter'. Although this definition is still widely accepted, some clarifying remarks are necessary to avoid confusion with other engineering sciences, especially with continuum mechanics. Continuum mechanics provides tools and equations to describe the behavior of materials in terms of stress and deformation. The results are reflected by Newtonian fluid mechanics and the elastic theory, which rely largely on a linear behavior between stress and rate of strain, and between stress and imposed strain, respectively.

There are three main aspects, which can hardly be treated in terms of continuum mechanics, that underline the importance of rheology:

1. Real materials exhibit a wide range of different and complex rheological behaviors. The linear models of Newton and Hooke may be considered as anchor points, and continuum mechanics may be expanded to describe these rheological properties by combining viscous and elastic components in order to define constitutive equations for stress–strain relationships of real, viscoelastic materials.
2. Both elastic theory and Newtonian fluid mechanics are based on the concept of solid and liquid materials, respectively. It is, however, evident that real materials exhibit solid-like or liquid-like properties, depending on the applied stress and the timescale of deformation (and observation).
3. Rheology also links flow or deformation phenomena to the (micro)structure of the material under study. Structural elements, irrespective of whether these are macromolecules, embedded particles in multiphase systems, or biological elements such as plant cells, interact with themselves, and microstructure may be subjected to certain modifications depending on motion or deformation. New techniques that allow simultaneous observation of rheological and optical properties in the broadest sense become increasingly important tools for the explanation of the macroscopic behavior as caused by microstructure or microstructural processes.

Special Aspects of Food Rheology

Among others, there are two important motivations for applying rheological techniques to food materials: the quantification of textural properties by instrumental

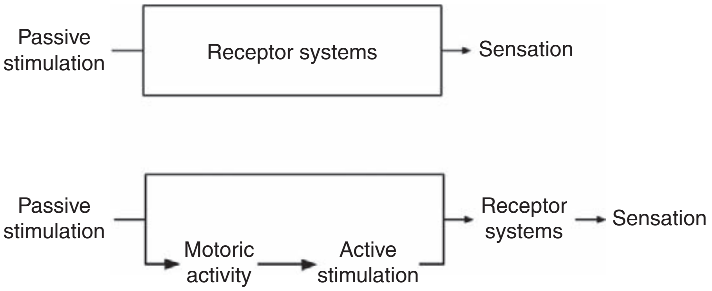

Figure 3 Stimulus–response mechanisms with passive (upper figure) simulation and both passive and active stimulations (lower figure).

methods and the acquisition of specific information related to the optimization of food processing. These two motivations differ obviously in the analytical approach, and also in the treatment of the data obtained.

Basically, rheology is meant to deal mainly with small deformation properties achieved in the linear viscoelastic region where the stresses are small enough to ensure direct proportionality to the corresponding strain (it is irrelevant here whether stress or strain is the input variable). However, it is widely agreed among food scientists that inclusion also of large deformation measurements and, especially, the measurement of fracture properties, in food rheology is necessary. In many cases, large deformation properties and, particularly, fracture properties may serve as an instrumental measure of some texture properties, mainly of those that are classified within the subset of mechanical properties. Some dairy products such as cheese or dairy-based fat spreads, which appear solid and retain their shape under atmospheric conditions, may be subjected to fracture testing using various analytical configurations such as compression (mostly preferred), tension, or bending. Measures taken from the stress–strain curves (e.g., fracture stress, fracture strain, the apparent modulus of deformability, or fracture work) may then be used to predict sensory parameters such as firmness, brittleness, or elasticity.

Important interactions between rheological properties, product microstructure, and food processing should be considered in food technology. The selection of ingredients or the adjustment of processing parameters may help to design food materials or to modify and tailor specific (techno) functional properties. Some examples related to dairy products are starter cultures with exopolysaccharide-producing bacteria, which strongly affect viscosity and shear stability of stirred yogurt, or the use of different oils for incorporation into processed cheese, which affect the firmness of the end product. It is a general rule for food materials that the importance of interactions between structure, processing, and rheology increases with increase in level of processing.

Classification of Rheological Properties

Although there are various possibilities to categorize rheological properties of food materials, it is most convenient to start at an anchor point that refers to ideal materials, that is, either to apparently liquid or to apparently solid materials. In this context, 'apparently' may refer to visual appearance under the influence of gravity. The behavior of simple fluids such as pure solvents (water, ethanol) and melts (oil), and also of dilute solutions, can be classified as almost Newtonian. The application of a particular shear rate, $\dot{\gamma}$ (s^{-1}), results in a shear stress, τ (Pa), which is proportional to the shear rate. Hence, viscosity, η (Pa s), which is defined as the ratio of stress to shear rate, is independent of the shear stress or shear rate level and can be considered as a real material constant (**Figure 4**). A commonly used tool to represent this behavior is the mechanical analog of a damping unit filled with a more or less viscous fluid (**Figure 5**). When

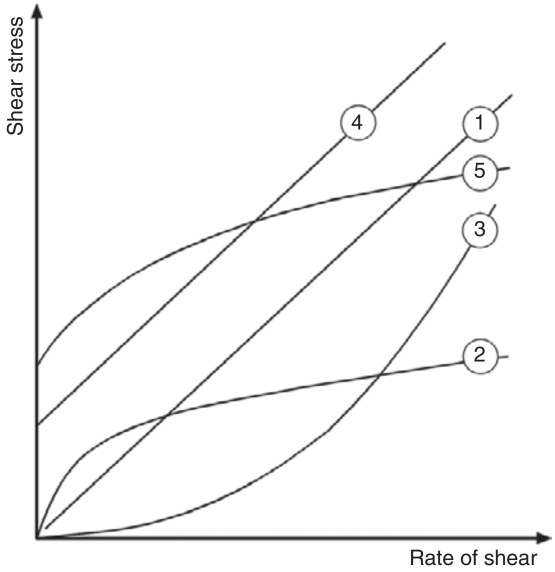

Figure 4 Stress–shear rate profiles for (1) Newtonian, (2) shear thinning or pseudoplastic, (3) shear thickening or dilatant, (4) Bingham, and (5) plastic fluids.

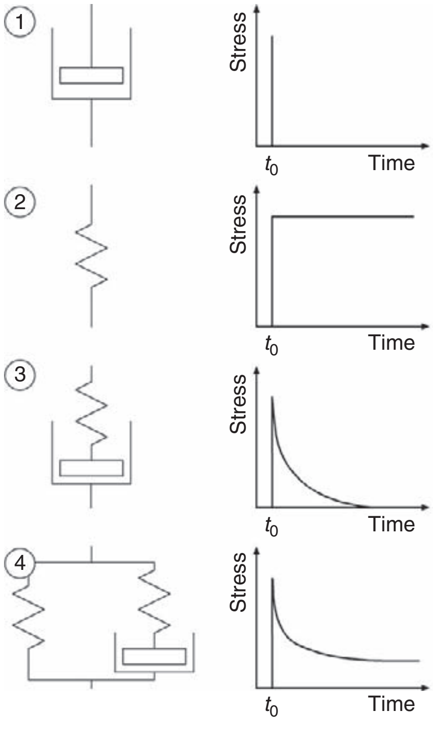

Figure 5 Mechanical analogs for the description of basic rheological properties and their corresponding stress–time reactions. (1) Newtonian fluid, (2) elastic body, (3) viscoelastic liquid (Maxwell element), and (4) viscoelastic solid.

moving the outer cylinder downward, the magnitude of the strain rate depends on the velocity of the moving part and the thickness of the sheared layer, and the resistance of the fluid is usually expressed in stress units (i.e., force divided by contact area). Subjecting a damping unit to an instantaneous deformation, which is then kept constant, results in an instantaneous stress reply, which dissipates momentarily.

Many complex foods, mainly dispersions of various types, deviate in their behavior widely from the Newtonian behavior. Frequently, the stress increase per unit strain rate diminishes with increasing strain rate, so that the dependency of shear stress on shear rate becomes nonlinear. Consequently, the viscosity of the so-called shear-thinning fluid systems is more or less far from being constant and decreases with increasing shear rate; to specify viscosity, experimental conditions must be specified, and the use of the term 'apparent viscosity' is appropriate. The viscosity decrease in shear-thinning fluids may be attributed to, for example, orientation of rod-shaped particles, fibers, and other materials in the solvent when shear rate is increased. Albeit few in number, an opposite behavior (increasing viscosity with increasing shear rate) may be observed for a more or less expanded shear rate range; this is true mainly for concentrated suspensions with irregularly shaped particles showing a trend toward wedging.

In many structured systems, including concentrated dispersions irrespective of the type of solvent and weak biopolymer gels, viscosity tends to infinity when the shear rate approaches zero. Therefore, the stress–shear rate profile starts with a finite value at zero shear rate, which is called yield stress, τ_0 (Pa), and represents the most important feature of plastic materials (**Figure 4**); below τ_0, only elastic deformation occurs. A simplified version of plastic behavior is the Bingham fluid with a yield value and a linear relationship between stress and strain rate for $\dot{\gamma} > 0$. When attempting to describe such flow curves mathematically, the most commonly used approach is a generalized power law equation, the Herschel–Bulkley model of the form $\tau = \tau_0 + k\ \dot{\gamma}^n$. For systems without a yield value, $\tau_0 = 0$, and for fluids with a linear relationship between stress and strain rate, $n = 1$.

Frequently, shear thinning is overlapped by a time-dependent behavior, which implies that shear stress and, consequently, viscosity are affected by the time a system is subjected to a particular shear rate. For thixotropic materials, the shear stress reaches a peak value after applying a particular shear rate and then decreases until an equilibrium stress (stationary state) is achieved. This type of time-dependent behavior is frequently associated with shear thinning and typical for disperse systems with structure degradation caused by, for example, the reduction of aggregate dimensions in the disperse phase. The opposite phenomenon called antithixotropy, which appears less frequently, is usually associated with shear thickening and typical for some concentrated particle suspensions.

To distinguish between thixotropic and time-dependent viscoelastic fluids, it may be considered that time-dependent behavior results from instantaneous structural changes induced by shear, whereas in viscoelastic systems, time dependency arises from a delayed reaction of stress to strain or vice versa. As an appropriate mechanical analog, the spring model for pure elastic materials can be taken into account. Here, any deformation of a spring, for example, by elongation, results in a stress, σ (Pa), which is proportional to the applied strain, $\epsilon(-)$. As long as the (micro)structure remains unaltered, stress and modulus (i.e., stress divided by strain, hence representing a material constant) remain constant as a function of time (**Figure 5**). Consequently, the Maxwell model representing a linear spring–dashpot arrangement combines properties of both an ideal fluid and an ideal elastic body, and any deformation of a viscoelastic fluid results in a stress decay asymptotically approaching zero. The relaxation time, which can be derived from the constitutive equation of the model, is then characteristic of the material under study. The simple Maxwell model can be extended in various forms, for example, by arranging two or more Maxwell units in parallel, or by including an additional spring element for the modeling of viscoelastic solids. The stress decays asymptotically until a permanently lasting stress is achieved, which corresponds to the equilibrium modulus of the single spring in the generalized Maxwell model.

Several other mechanical models have been proposed to illustrate the rheological behavior of semisolids and solids, and these are, however, beyond the scope of this short introduction. For example, a solid friction body is used frequently to describe the yield stress, and fracture elements have been used to explain large deformation behavior. Some of these models depend on whether the material under study is subjected to a stress-based or a strain-based deformation.

See also: **Analytical Methods**: Rheological Methods: Instrumentation. **Cheese**: Cheese Rheology; Gel Firmness and Its Measurement. **Milk Lipids**: Rheological Properties and Their Modification. **Rheology of Liquid and Semi-Solid Milk Products**.

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Rheological Methods: Instrumentation

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Introduction

To quantify rheological properties of food materials, in particular, dairy products, a wide range of methods (based on several underlying principles) have to be considered. The principles of some of these methods were established a long time ago and have only been subjected to minor changes. On the other hand, the progress in electronics and precision mechanics in the past two decades has resulted in the increased availability of specialized instruments on the market. Because of a significant reduction in price, some of these instruments today represent standard laboratory equipment.

One of the early classical attempts to categorize methods for rheological or textural measurements led to the postulation of some criteria for 'ideal methods', as well as to a distinction between fundamental, empirical, and imitative methods (**Table 1**). This concept may, however, be criticized from various points of view mainly because the developments in technology provide the analyst with much better access to defined measurements and also because imitative methods have vanished almost completely. This article is 'therefore' organized in a more pragmatic manner: two sections dealing with the measurement of large deformations of apparently liquid and apparently solid materials are followed by a section describing the ways to assess food materials without causing structural damage. The article concludes with a brief description of one-point methods for the simple assay of rheological and textural properties of dairy products.

Viscosity

For Newtonian liquids, viscosity, η (Pa·s), represents, per definition, a real material property that relates a particular shear stress, τ (Pa), to a corresponding shear rate, $\dot{\gamma}$ (s^{-1}), by $\eta = \tau/\dot{\gamma}$. In addition, viscosity is affected neither by the time the material is subjected to shearing (i.e., shear stress at a particular shear rate, or shear rate at a particular shear stress, is constant) nor by shear acceleration. This means that viscosity depends only on factors such as temperature, pressure, and, in case of solutions, concentration, molar mass, and molecular structure of the solute. However, only a few apparently liquid materials relevant for the food scientist may be regarded as Newtonian. Concentrated solutions as well as multiphase systems such as suspensions, emulsions, or foams usually show a much more complex behavior, which includes deviations from $\tau/\dot{\gamma}$ linearity, deviations from $\tau(\dot{\gamma})/t$ linearity, and viscoelastic effects.

As a consequence, a satisfactory characterization of these materials is possible only using sophisticated instrumentation with defined geometries and a known stress distribution. Independent of the type of instrument and the measuring geometry, it is a prerequisite to keep the flow kinematics as simple as possible. Associated with simple shear flow is, for example, steady tube flow, and also tangential shear flow or steady torsional flow, which can easily be realized in commercial instruments. For a proper description of steady simple shear flow, a fluid element between two parallel plates separated by a distance d has frequently been used (**Figure 1**). Assuming perfect adhesion of the fluid to the plane surfaces, applying a force F to one of the plates with area 'A' causes a shear stress $\tau = F/A$. The velocity, V, of the moving area depends on the resistance (or, in other words, the viscosity) of the fluid. As long as the shearing surfaces are plane and the shear lines are straight and parallel to the x_1-axis (laminar flow), the velocity v of any fluid layer at distance 'h' is $v = V\,(h/d)$. The shear rate $\dot{\gamma} = \mathrm{d}v/\mathrm{d}h = V/d$ coincides with the velocity gradient and is independent of the position; hence the flow field is homogeneous.

Measuring Devices

For analytical purposes rotation-symmetric geometries are mainly used; the most widely employed devices are outlined in **Figure 2**. Concentric-cylinder systems consist of an outer cup with radius r_a and an appropriate inner bob with radius r_i, with the sample being sheared in the annular gap, of an effective height h, by rotary motion of either the cup or the bob. In case of Newtonian fluids, the shear rate depends on angular velocity, which is a simple function of rotary speed, and on r_i and r_a. Shear stress τ depends on the torque, M, and also on the geometrical properties of the device. In non-Newtonian fluids, however, the shear rate distribution is not uniform throughout the gap. Besides some algorithms for mathematical correction, an appropriate selection of geometrical factors (e.g., $r_i/r_a > 0.95$) may help keep these deviations at a justifiable level. With concentric-cylinder

Table 1 Classification of instrumental methods

Methods	*Advantages*	*Disadvantages*
Ideal	Suitable for routine work (simple, rapid, cheap) Correlate with sensory measurements Close to mastication, characterizes texture completely	None
Fundamental	Good definition of what is measured	Lack of correlation with sensory results Measures only specific properties Slow, sophisticated, expensive
Empirical	Suitable for quality control (simple, rapid, cheap) Correlates with sensory measurements	Ill-defined and arbitrary test principle One-point measurement only Specifies texture incompletely
Imitative	Close to the 'service test' in engineering processes Correlates with sensory measurements	Physically ill-defined test (duplicates mastication or other sensory principles) Not suitable for routine work

Compiled from Bourne MC (1975) In: Rha CK (ed.) *Theory, Determination and Control of Physical Properties of Foods* pp. 131–162. Dordrecht: D.Reidel, and Mohsenin NN (1986) *Physical Properties of Plant and Animal Materials*. New York: Gordon and Breach.

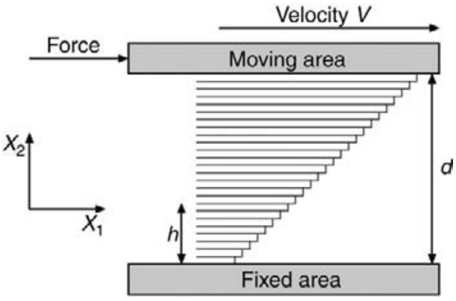

Figure 1 Model for simple shear flow.

systems, a wide variety of fluids can be measured. For fluids with low viscosity, double-gap systems are available.

The cone-and-plate device consists of a flat plate and a cone with an angle α usually ranging between 1° and 5°; usually, the cone tip is truncated so that the working distance between the plate and the cone tip ranges between 25 and 150 μm. Shear rate is proportional to angular velocity, ω, and indirectly proportional to the cone angle, and stress is a function of torque and the radius of the system. One of the main advantages is the uniform shear rate distribution between the cone and the plate; therefore, this device should be used whenever possible. In addition, only a small volume (<2 ml) is needed for viscosity determination. Problems may arise in the case of multiphase systems with relatively large particles, especially when particle diameter exceeds the working distance between the plate and the truncated cone.

For fluids with high viscosity, and also for multiphase systems with large dispersed particles, the parallel-plate device with radius r and working gap h may be used. As a rule of thumb, the gap should exceed the diameter of the largest particles at least by a factor of 5. Whereas shear rate depends on angular velocity, radius of the plate, and the working gap, stress is proportional to the torque and inversely proportional to r^3. Hence shear rate distribution is not uniform between the plates but reaches a maximum at the circumference; correction procedures for non-Newtonian materials are recommended. As long as the instruments are stiff enough, parallel-plate systems and

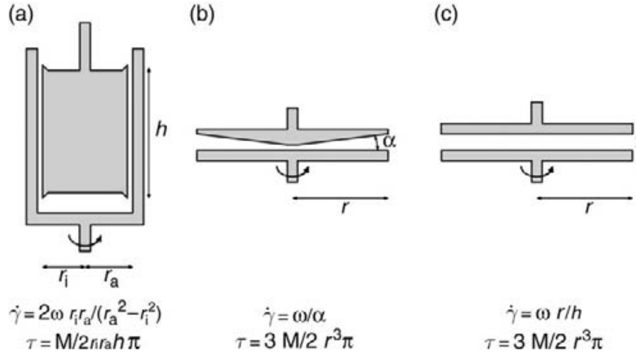

Figure 2 Test geometries used for rotational viscometry: (a) concentric-cylinder system; (b) cone-and-plate system; and (c) parallel-plate system.

cone-and-plate systems may also be used for the determination of normal stress differences, which are relevant in the analysis of viscoelastic systems.

Classification of Instruments

The rotational viscometers available on the market can be classified with respect to (1) mode of operation and (2) geometrical design. In strain-controlled instruments, a defined angular velocity, which determines the shear rate, is provided by a motor. In the Couette-type viscometers, the resulting torque is measured on the counterpart of the rotating tool; that is, at the bob in case of a rotating cup, or at the cone in case of a rotating plate. In the Searle-type viscometers the torque is measured on the rotating tool, which is, usually, the bob, the cone, or the upper plate. The design of the Searle viscometers is simpler with respect to the construction of the drive, and it is also easier to achieve an appropriate temperature control of the outer, static part of the rheometer. However, as compared to the Couette systems, the Searle viscometers have some disadvantages as regards the flow profiles in the annular gap, which may become important at higher rotational speeds. In addition, acceleration forces contribute to the torque at the beginning of the rotation of the moving part. Usually, viscometers for routine quality control are of the Searle type, whereas the Couette principle is used in high-end viscometers for research and development.

In stress-controlled rheometers, which are usually the Searle type, a particular torque is applied to the moving part of the geometry. Considering the device dimensions, this torque corresponds to a particular stress, which causes a reaction of the fluid. Any deflection of the moving tool is then measured as a function of time, thus giving angular velocity as the primary output, which can easily be converted into shear rate coordinates. Most of the instruments currently available are stress controlled, simply because they are less expensive than the comparable strain-controlled instruments. There is only one company that still provides the market with sophisticated strain-controlled instruments.

As the applications vary widely, it is hardly possible to provide any specific advice for using either stress- or strain-controlled viscometers. As the samples are not forced to move below a critical value of the applied stress, stress-controlled instruments may be of advantage in applications where it is important to measure a yield value or related properties. On the other hand, strain-controlled instruments also have some advantages, especially in applications where it is essential to have torque or stress responses at exactly defined shear rates. One of the most important features is instrument sensitivity, which is roughly a function of the elimination of friction and inertia. Even though for the measurement of highly viscous samples viscometers with mechanical bearings are still available, more sophisticated instruments have air bearings. For the past couple of years, a new generation of rheometers with magnetic bearings has been on the market, which provides the user with much more sensitivity than achieved before.

Mechanical Measurements

The aim of mechanical methods is to determine rheological or textural properties of food materials that appear in a solid or semi-solid state. Instrumentation covers the so-called universal testing machines, which were developed for non-food industries and, in some cases, adapted to fulfill the specific requirements necessary for the evaluation of food materials (e.g., temperature control).

Universal testing machines consist of three main elements: a stable frame that carries a moving crosshead and tools to attach test fixtures, a motor to induce linear motion of the crosshead, and a transducer to measure the response forces (**Figure 3**). With the high-end instruments available nowadays it is possible to cover crosshead speeds ranging from a minimum of 0.1 mm min^{-1} to a maximum of 2500 mm min^{-1} or even more. Owing to the availability of frames with different nominal capacities and transducers with different maximum loads, universal testing machines can be adjusted to measure a wide variety of products.

These instruments show the advantage of being versatile, and a number of different test devices may be attached to the instruments. Some of these devices, which are used mainly for one-point measurements, will

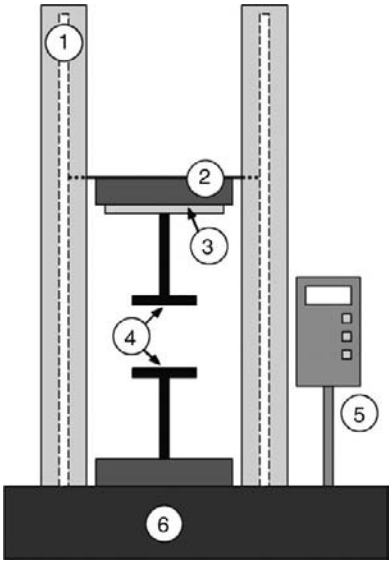

Figure 3 Core elements of a universal testing machine (simplified): (1) frame with spindles; (2) crosshead; (3) force transducer; (4) test fixture; (5) control unit; and (6) base unit including drive.

be discussed below. For a more sophisticated assessment of the mechanical and fracture properties of solid foods, tests may be configured for compression, tension, or three-point bending. Whereas tension and bending represent the state of the art in materials testing, some peculiarities have to be considered when referring to foods. In bending and tension, fracture usually starts at the outside of the sample, which allows for distinguishing between crack initiation and fracture propagation. In addition, notching may be used to control fracture and to calculate the specific energy involved in fracturing. However, several problems may occur during the preparation of samples and during fixing to the test geometries. Bending tests are not suitable for soft materials, nor for foods that deform strongly, and relatively large samples are needed for bending, which increases the possibility of inhomogeneity. Although a large strain can be applied in tensile testing, it is difficult to attach the sample to the test fixtures so that slipping or the effects of the grips on fracturing are avoided. Therefore, uniaxial compression is frequently used because (1) sample preparation is easy, (2) sample size is small, and (3) test execution is simple.

Bending, tension, and compression are usually performed in the constant displacement mode, with crosshead speeds ranging between 10 and 1000 mm min^{-1}. In addition, some of the newer instruments offer the possibility to perform constant load tests (creep experiments), where a constant force is applied to the sample and kept constant by slight movements of the test fixtures, or constant strain rate tests, where the crosshead velocity is continuously adjusted to account for the change in specimen size.

Uniaxial Compression

In uniaxial compression, cylindrical specimens of a size of approximately 10–25 mm diameter and height are compressed between parallel plates (see **Figure 3**). Specimens can easily be prepared from larger samples by means of cork borers or similar instruments, and the reduction of the cylinders to the required length is usually done by stretched wire devices or razor blades. As the samples are small, the risk of inhomogeneities within the samples is negligible, and there is no need to fix the specimens to the instrument. However, fracture during compression usually starts within the samples, so the determination of fracture parameters might be ambiguous. As a consequence, there is a common agreement within the scientific community that fracture parameters obtained by uniaxial compression should be referred to as stress or strain at apparent fracture.

During uniaxial compression, force, distance, and time data are collected by the instrument's software. As the force versus absolute deformation data depend largely on the dimensions of the specimen, and because increasing deformation during testing changes the strain coordinates significantly, it is desirable to apply appropriate correcting algorithms. There are two widely accepted possibilities to display the data: as 'engineering' stress versus the Cauchy strain, and as 'true' or 'corrected' stress versus the Hencky strain.

Engineering stress σ_e (Pa) is calculated by dividing the actual force by the initial cross section of the specimen, and the corresponding Cauchy strain ε_C (–) is a measure of the relative deformation obtained by $\varepsilon_C = (h_0 - h_t)/h_0$, where h_0 and h_t refer to the initial sample height and the height at time t, respectively. In case of foods the volume of which do not change during compression (e.g., cheese or butter), the sample dimensions get altered significantly during deformation; therefore, these measures only roughly represent stress and strain. **Figure 4(a)** depicts sample profiles of two hard cheeses, which show a relatively large difference in fracture force (or fracture stress calculated as σ_e), while the deformation (or the Cauchy strain) at the fracture peak differs only slightly.

Calculation of the true or corrected stress, σ_t (Pa), is based on the assumption that the volume of a specimen

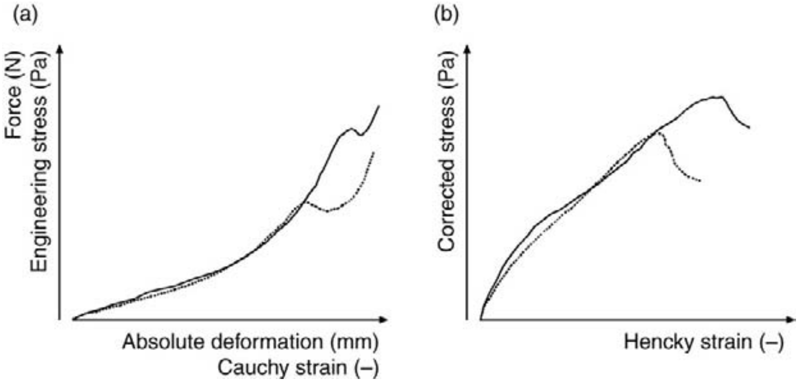

Figure 4 Illustration of results from uniaxial compression of two cheese samples depicted in terms of (a) raw data or engineering stress vs. the Cauchy strain and (b) corrected stress vs. the Hencky strain.

remains constant during compression; this can be assumed as long as it is ensured that the cylindrical specimens do not change their shape, which may be achieved by lubricating the compression plates with oil to eliminate friction or by using Teflon plates. For calculation of σ_t, the actual force, F_t, is divided by the actual cross section of the sample cylinder, which, in turn, can be calculated from the initial sample dimensions (radius and height) and the actual height at time *t*. The Hencky strain, ε_H (–), which is frequently used as an accompanying deformation measure, considers that a particular absolute deformation causes an increasing relative effect with increasing compression and is calculated by $\varepsilon_H = -\ln(h_t/h_0)$. It can be seen in **Figure 4(b)** that, after conversion of raw data into σ_t/ε_H coordinates, the relative differences between the two samples decrease for stress at apparent fracture but largely increase in case of the corresponding strain measure.

For hard and semi-hard cheeses, stress at apparent fracture has been accepted as an instrumental measure of sensory firmness, and strain at apparent fracture can be used as an instrumental measure of the elasticity of the cheese body. In addition, uniaxial compression allows the calculation of the modulus of deformability M_D (Pa) from the initial, linear part of the stress–strain curves. M_D represents an approximation of Young's modulus and is an estimate of firmness prior to any structural damages.

Dynamic Rheometry

Increasing attention has been paid to methods that allow a rheological characterization without affecting the microstructure of the sample under study. These methods include dynamic methods as well as transient tests, that is, tests where the sample's response is measured as a function of time; the results of the dynamic and the transient tests are interrelated as defined by the basic theories of linear viscoelastic behavior. Dynamic methods may be applied to apparently liquid as well as to solid-like materials by using dynamic rheometers, which can operate in different modes and are capable of applying several directions of deformation (e.g., bending or compression). However, most of the commercially available instruments are extensions of simple viscometers and, to be more versatile, operate in dynamic shear. Test geometries do not differ from those used in viscometric studies (see **Figure 2**). As in viscometers, a sample can be deformed by controlling either the strain magnitude or the applied stress. In dynamic methods, strain-controlled rheometers show some advantages, especially when the task is to determine phase transitions from low-viscous materials to complex networks (e.g., yogurt), but are usually much more expensive than stress-controlled instruments because of the higher mechanical effort.

Figure 5 depicts the principal actions of a dynamic strain-controlled rheometer. A particular strain that varies sinusoidally, and is characterized by its amplitude γ_0 (–) and a corresponding frequency ω (rad s^{-1}), causes a response stress that, in case of an 'ideal' elastic material (a Hookean solid), is also sinusoidal in nature. The maximum shear stress amplitude, τ_0 (Pa), depends on the modulus of the material and, as the highest stress is reached at the maximum strain, varies sinusoidally and in phase with the input strain. The dynamic shear strain rate amplitude, $\dot{\gamma}$ (s^{-1}), also varies sinusoidally but is zero at the maximum strain and reaches its highest at zero strain, where the angular speed is at its maximum.

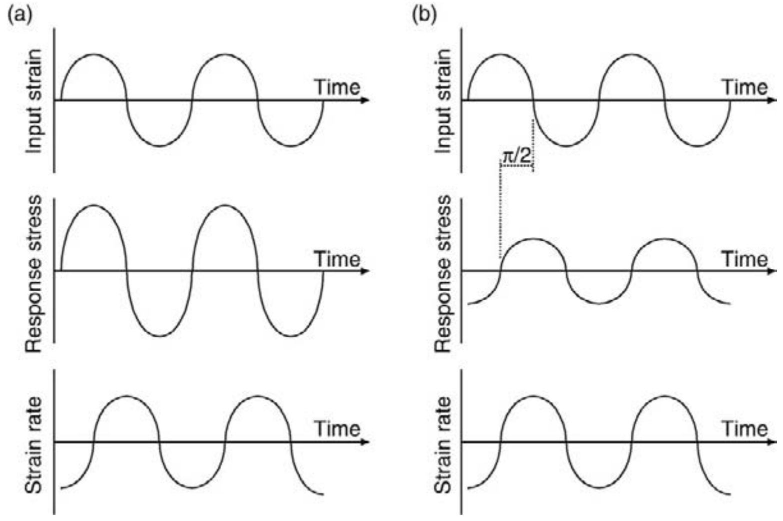

Figure 5 Schematic illustration of strain, stress, and strain rate fluctuation in dynamic rheological measurements. Behavior of (a) an elastic solid and (b) and a viscous liquid.

The corresponding phase shift between shear strain amplitude and shear strain rate amplitude is, therefore, $\pi/2$. This is, obviously, also true when Newtonian fluids are subjected to dynamic tests. However, the shear stress response runs in phase with the strain rate, that is, stress is the highest when strain rate is the highest, but it runs out of phase with the strain input; the response stress is zero at the maximum strain and vice versa. Materials that exhibit viscoelastic behavior show an intermediate reaction to a strain input, with a phase shift ranging between 0 and $\pi/2$, depending on the relation between elastic and viscous contributions to the total response.

Viscoelastic behavior is quantified using the dynamic moduli. The storage modulus G' (Pa) represents a measure of energy stored in the material (elastic contributions) and is calculated from the stress and the strain amplitudes and the sine function by $G' = (\tau_0/\gamma_0)$ cos δ. The loss modulus, G'' (Pa) $= (\tau_0/\gamma_0)$ sin δ, represents a measure of the dissipated energy (viscous contributions). The phase shift δ, or the corresponding tangent, which can also be calculated by $\tan\delta = G''/G'$, is then used as a quantitative measure of viscoelasticity.

Regarding the instrument's operating principle, there are several ways to control dynamic experiments. Increasing the strain amplitude at a constant angular frequency and plotting the evolution of stress and, consequently, G' and G'' is an appropriate way for determining the linear viscoelastic region. As long as neither G' nor G'' depends on the strain amplitude, the deformation is small enough to ensure that the microstructure of the material remains intact. Other dynamic tests such as frequency sweeps, performed by applying a constant strain amplitude, and time sweeps, performed by applying a constant strain amplitude and a constant angular frequency, also are usually executed within the linear viscoelastic region. Frequency sweeps result in the so-called mechanical spectra, that is, the evolution of G' and G'' as a function of angular frequency, and display the reactions of a material to deformations induced at different timescales. As time sweeps measure the evolution of dynamic moduli as a function of experiment time, hardening or softening effects as well as reactions to temperature changes can be monitored.

Depending on the type of rheometer, transient methods can be executed either as relaxation tests (strain controlled) or as creep experiments (stress controlled). In relaxation tests, a particular deformation is instantaneously applied and the relaxation modulus, G_t (Pa), defined as the ratio of stress to strain, is monitored as a function of time. In creep experiments, a certain stress leads to the evolution of a strain, and the corresponding ratio of strain to stress is called compliance, $\mathcal{J}_t$ (Pa^{-1}). As long as the transient experiments are performed within the linear viscoelastic region, there are several ways to calculate mechanical spectra from the transient functions, and vice versa.

Empirical Rheometry and One-Point Measurements

There are a number of simplified test setups that can be considered as 'empirical', and, therefore, are hardly interpretable in exact rheological terms, but can be useful for routine characterization of textural or rheological properties of foods. These methods are easy to perform; on the other hand, they are somewhat limited as regards the interpretation of test results. In many cases, the selection of one-point methods is based either on their correlation with more fundamental rheological tests or on empirically established interrelations with the results of sensory experiments.

Flow Time Measurements

As regards liquid food systems, a number of simple devices are available for monitoring flow time – that is, the time a particular volume of a fluid needs to pass a defined orifice – and the related parameters. These systems are derived from capillary viscometers and are appropriately modified by, for example, changing the length-to-diameter ratio of the annulus. Examples are the Ford cup and the Posthumus funnel, which have frequently been used for the determination of viscosity-related parameters of yogurt. Another example is the Hoeppler viscometer, which is based on Stoke's law; here, the time taken by a ball of a particular diameter and density to pass through a tube filled with a liquid is measured.

Generally, flow time measurements are simple to handle in case of apparently Newtonian fluids. With increasing complexity of the fluid, for example, in structured materials with shear-thinning or time-dependent behavior, flow time measurements become less reliable because handling of the sample, shear history, and other factors may affect the flow time results.

Penetration Tests

Penetration tests are frequently used to assess textural properties of apparently solid food materials. Depending on the principle of the method, that is, whether the probe is driven at a constant velocity or by a constant mass, the measured parameter is either a force value obtained after a particular time or a corresponding penetration depth, or, in case of constant mass penetrometers, the penetration depth obtained after a predefined time interval or the time necessary to achieve a particular penetration depth. A number of different-shaped probes, such as needles, plungers, and cones, may be used for penetration tests.

Several attempts have been made to take advantage of the simple test setup of penetration tests by combining the results with algorithms that can achieve clearer results. For example, an apparent yield value may be calculated from cone penetration results of plastic fats; this equation considers both the cone angle and the mass applied to the system so that the method can be executed on samples with largely differing spreadability. An apparent modulus calculated from the initial slope of force-versus-deformation curves obtained from plunger penetrations of gels such as yogurt might be useful to approximate results in the undeformed state.

See also: **Analytical Methods**: Principles and Significance in Assessing Rheological and Textural Properties. **Cheese**: Cheese Rheology; Gel Firmness and Its Measurement. **Milk Lipids**: Rheological properties and Their Modification. **Rheology of Liquid and Semi-Solid Milk Products**.

Further Reading

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Sensory Evaluation

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Introduction

The sensory properties of dairy products, categorized as aroma (orthonasal), flavor (in-mouth aromatics, basic tastes, and mouthfeel), texture, and appearance attributes, determine consumer acceptability and willingness to repeat purchase of a product, with some additional contribution from their nutritional value and wholesomeness. A majority of sensory properties are complex by definition, as they are stimulated by the integrated involvement of many different compositional and structural properties of the product, and, for this reason, they cannot be adequately detected or represented by analytical techniques. However, due to the sophisticated functioning of the human sensory systems, even a slight change in composition can be detected as a change in sensory character and, therefore, sensory evaluation, in one form or another, has become routinely applied in the dairy industry, in particular for quality assurance and more recently as a powerful research and marketing tool.

The American Society for Testing and Materials (ASTM) Committee E18 on Sensory Evaluation of Materials and Products has defined sensory evaluation as "a scientific discipline used to evoke, measure, analyse and interpret reactions to the characteristics of foods and materials as they are perceived by the senses of sight, taste, touch and hearing".

A more practical definition can also be applied: "a set of tools to accurately measure human responses to external stimuli".

These definitions have a general meaning and intent. Different techniques with very different objectives and outcomes can be used to evoke, measure, and interpret sensory characteristics, creating a key distinction between sensory evaluation and other chemical and instrumental analytical techniques, which has caused some confusion and debate. Sensory evaluation can be carried out to determine whether milk or dairy products have undesirable characteristics or defects, whether there is any perceptible difference in sensory characteristics between two or more products, how differences in sensory characteristics can be described and quantified, and whether consumers find products to be acceptable or not. These distinctions in the objectives of sensory evaluation can be classified broadly under the methodologies used for quality scoring and judging, discrimination, quantitative descriptive evaluation, and consumer acceptability evaluation. Even more broadly, sensory evaluation can be divided into three categories: quality scoring/judging, analytical sensory tests, and affective or consumer sensory tests. The latter two categories encompass a multitude of science-based and established accurate techniques for the measurement of sensory responses to foods or other stimuli.

Quality Scoring

Sensory quality has been defined as

> that complex set of sensory characteristics, including appearance, aroma, flavour, taste and texture, that are maximally acceptable to a specific audience [sic] of consumers.

Quality control is extremely important for maintaining consumer confidence and loyalty toward a product. Dairy products are susceptible to a wide range of quality defects that can originate in the feed of the milk-producing cow or arise from contamination of the milk or product during processing and storage. To test for all possible defects, including off-appearance, off-texture, and off-flavors, would be an extremely laborious task instrumentally, and may not achieve success. For example, the compounds responsible for many off-flavors are present at concentrations below the detection limit of even the most sophisticated instruments.

Application of formal sensory evaluation for quality control and quality judging in the dairy industry has a history of more than 75 years. The traditional, and still most widely used, approach is that of quality scoring for specified defects on standardized scorecards. The International Dairy Federation (IDF) has developed standard scorecards for milk and the major dairy product categories (e.g., butter, milk powder, cheese, cream, fermented milk products, and ice cream) and specifies a scale that ranges from 5, representing very high quality, to 1, representing very low quality. For each product, each point deducted from the scale is supported by a list of the defects, and the degree of some defects, that merit the deduction. These standards aim to provide objectivity to the evaluation. However, some studies have shown that such standards are used differently in different locations because of differences in the interpretation of standard defects. The American Dairy Science Association (ADSA) has established a similar quality scorecard, which is used by the US industry and for student activities and training, and the United States Department of Agriculture (USDA) also has grading standards that are established in a similar manner (pre-determined defects).

The potential sensory defects in milk and dairy products are numerous, and those documented include some transmitted from the feed of the cows and the environment in which the cows are housed (e.g., 'feed', 'barny', 'garlic/onion'), those that result from the stage of lactation of the cows (e.g., 'bitterness' may be present in late-lactation milk, increased lipolysis in late lactation milk may accelerate the development of 'rancidity') and subsequently from any number of possible manufacturing processes and storage conditions, such as agitation (agitation can cause rapid 'lipolysis' and 'rancidity'), contact with cleaning agents (e.g., 'foreign') or unsuitable equipment (e.g., 'metallic'), or exposure to sunlight (e.g., 'oxidized') or to high temperatures (e.g. 'cooked'). Unhygienic or lengthy storage can also result in flavor defects due to the activity of microorganisms (e.g., 'putrid', 'malty', 'unclean'). In products such as cheese or ice cream, defects to the physical properties of 'body', or consistency (e.g., 'corky', 'crumbly') and texture (e.g., 'gassy', 'open'), are also widely documented.

One or more expert evaluators (assessors), who have detailed product knowledge built up over many years and who maintain a mental standard of what the ideal product is in terms of sensory characteristics, carry out this quality evaluation. These experts have the ability to relate their recognition of specific defects to the cause of that defect, to weight the influence of each defect at different levels of severity, and to judge how a defect or combination of defects detracts from overall product quality. This technique provides a rapid and simple way to assess quickly the overall sensory quality, but does not adequately take into account the so-called non-quality related differences in sensory properties that give the products of individual producers, or regions of production, a distinctive taste that is now sought by discerning consumers. Also, this technique does not determine accurately the degree of difference for a given defect; therefore, further statistical analyses that determine the extent to which products differ are not appropriate and data cannot be related to those provided by other chemical and instrumental analyses. In addition, when compared with the product quality of 50 years ago, today's products are no longer viewed as commodities, and the number of defects found in products manufactured in automated facilities is extremely low. Traditional quality criteria are changing as product ranges expand, and differentiation is increasingly made by developing distinctive sensory characteristics that will appeal to consumers, such as those now given to cheeses by the use of adjunct cultures. It is also now well documented that consumer opinion of quality often differs from that held by experts. However, quality scoring evaluation is still widely used for dairy products because of activities such as the collegiate dairy judging competition sponsored by the ADSA.

Analytical Sensory Tests: Discrimination Testing

Sensory discrimination tests involve direct comparisons of products to determine whether there is a perceptible difference between them for a designated sensory characteristic. The most commonly used discrimination tests include paired comparison (ISO 5495: 1983), duo-trio (ISO 10399: 1991), triangular tests (ISO 4120: 1983), and ranking test (ISO 8587: 1988). In the paired comparison test, two products are compared against each other, generally by one characteristic at a time, and assessors are asked to indicate which product has more of the designated characteristic, such as 'rancid'or 'metallic'. In the duo-trio test, assessors are asked which of two products is most similar to a third reference product, allowing a common reference to be used repeatedly as a control. In the triangular test, assessors are asked to choose which two of three products are alike, or which product is most different from the other two. In the ranking test, a number of products are compared with each other for a single designated attribute and the assessor is asked to rank them in the order of increasing intensity of that attribute. For each of these tests, if the assessors are forced to make a choice each time, there will be no response bias. Therefore, discrimination tests are the most objective and most sensitive of all sensory tests. An additional advantage of discrimination tests is that they do not require well-trained assessors; only people who have average sensory acuity and who recognize and agree on the meaning of the designated attribute are required. When compared with the traditional quality scoring methods, these discrimination procedures are far better-suited for application to research problems because they follow good sensory evaluation principles and do not encounter problems in scaling and statistical analyses. For this reason, the best aspects of discrimination methods are now being applied for quality control of dairy products in an attempt to eliminate the subjectivity associated with expert evaluators. In addition, it is common practice to undertake sensory discrimination tests on products prior to further analyses in order to determine whether a difference that merits further examination exists. Another important note is that discrimination tests, though simple and powerful, are only one category of scientifically sound sensory tests. Discrimination tests can be easily misused like any other test if applied for an inappropriate objective. It is crucial for any sensory test to ensure that the objectives of the sensory project are compatible with the method selected.

Analytical Sensory Tests: Descriptive Sensory Evaluation

Descriptive sensory evaluation refers to a collection of techniques that seek to differentiate between a range of products based on all of their sensory characteristics and to determine a quantitative description of all the sensory attributes that can be identified, not just the defects. Unlike traditional quality judging methods that use scorecards, no judgement of 'good' or 'bad' is made because this is not the purpose of the evaluation. Using traditional methods, products that have very different sensory characteristics, but have no defects, will obtain the same quality score when standard methods are followed. Therefore, such an evaluation does not record differences in sensory characteristics that may be very important for consumer acceptance, and it does not provide sufficient information to enable a full understanding of the physical and chemical causes of sensory characteristics. Therefore, descriptive sensory evaluation methods give an additional tool for application in research, product development, and marketing. In addition, descriptive sensory evaluation methods use a panel of assessors, rather than one expert, and therefore the result obtained represents a consensus that is less subjective, and less susceptible to bias, than the result obtained when one expert performs the evaluation. A trained sensory panel operates as an instrument and provides data comparable in nature to instrumental output.

The sensory characteristics of a dairy product that can be quantified include all the aroma, appearance, flavor, texture, after-taste, and even sound properties of the product that distinguish it from other products. There are several different methods of descriptive analysis, including the flavor profile method, texture profile method, Quantitative Descriptive Analysis$^{\text{TM}}$(QDA), Spectrum$^{\text{TM}}$ method, quantitative flavor profiling, and free-choice profiling. Implementation of each descriptive method has three stages. The first involves selecting a panel of assessors to conduct the sensory evaluation; the second involves developing and defining terminology, or a vocabulary, by which to describe the sensory characteristics of the product(s); and the third involves quantifying these sensory characteristics. All of the methods referred to above reflect different sensory philosophies and approaches, but the goal is the same: to train a group of individuals to operate in unison as an instrument to identify and quantify sensory attributes. Given that the dairy industry has a strong tradition of sensory quality judging that is linked to an extensive knowledge of dairy product characteristics and their causes, a wise approach will seek to build on this knowledge rather than to reinvent the wheel. The ultimate choice of the descriptive method to be used will be determined by the product category to be tested and the potential time and financial investment allocated to the product testing program. The options available, considering the extremes of time and financial factors, range from the QDA$^{\text{TM}}$, which provides a rapid protocol for the selection of assessors, panel training, and descriptive sensory evaluation, and does not require precise definition and reference of product sensory characteristics, to the Spectrum$^{\text{TM}}$ method, which builds on existing experience and, over many meetings with assessors, aims to precisely define and reference each sensory characteristic so that the descriptive evaluation procedure is ultimately standardized. The latter approach also allows for comparison of results between panels, between laboratories, and from one time to another.

In general, when using descriptive sensory evaluation, the panel of assessors must be capable of recognizing many different sensory characteristics of a product, they must reach agreement on how these are perceived and how they should be labeled, and they must be capable of individually scoring the intensity of each characteristic on line scales in a consistent way. Therefore, descriptive sensory evaluation assessors must receive much training before the panel attains the level of objectivity and reproducibility required. If a panel is to be used long term, investment of time and money must be made to maintain and calibrate the panel.

Affective or Consumer Tests: Consumer Acceptability Testing

There is little point in producing a product unless a sufficient quantity of it can be sold to make a profit. Existing products may not be competing effectively in the market – perhaps a drop in sales has resulted from new competition – and it may therefore be necessary to reformulate the existing product to improve its consumer appeal. In addition, any company should be innovative and proactive in new-product development. Sensory appeal, or getting the sensory properties right for consumers, is a very important part of product development and is the first step to profitability.

Sensory consumer acceptance testing makes use of rating scales that measure relative dislike and like, discrimination tests that are based on preference rather than on difference (e.g., paired preference, ranked preference), and 'just about right scales' that ask a consumer how they feel about a designated sensory characteristic. The sensory methods referred to previously require trained assessors, but consumer acceptance testing must be carried out with subjective assessors or with untrained consumers. In addition, these assessors should be regular consumers of the product type under test or represent the target market for the product. It is important that such consumers apply their subjective experience to this test because, although their preferences will be based on the sensory characteristics tested, they will also be based in past eating experience. In addition, when one considers that the target markets may be children, elderly consumers, consumers in another country, or consumers from a culture virtually unknown to the producer, then it becomes clear that the internal expertise in a company or organization cannot hope to adequately predict acceptance by all. Other sensory testing methods are also available that deal with determining consumer experiences with products, which include home usage tests, focus groups, or identifying appealing product concepts (conjoint analysis).

Sensory Laboratory and Requirements for Sensory Analysis

The aim of all sensory evaluation, whether it be for quality control using experts or for acceptance using consumers, is to interpret human perceptions of foods. However, perceptions of a food may be influenced by factors other than those intrinsic to the food. Therefore, it is almost always necessary to use scientifically controlled environmental conditions and test procedures for all analyses. The minimum requirements for testing are an area in which products can be prepared for analysis under hygienic and standardized conditions and group or individual tasting areas, or booths, which are free of extraneous odors, bright, evenly illuminated, and temperature controlled.

In addition, for tests other than consumer acceptance, the most important consideration for selection of potential assessors is that they possess sensory acuity at least as good as, but preferably better than, a typical population of consumers. As a panel, they must be capable of detecting sensory properties that consumers of the product will detect. In addition, they should be motivated, have the ability to work as a team, and be prepared to offer opinions. Assessor performance should be tested and monitored as part of the training procedure, under real testing conditions, and constructive feedback given at an individual and group level, if needed. The equipment necessary for powerful and relevant sensory analysis capacity is not expensive, but the time investment and the necessary skills to select and organize tests, maintain panels, and analyze results require a serious commitment or the selection of an appropriate provider of these services.

Relationships between Sensory Evaluation and Other Analyses

The manufacturer of products with the most acceptable sensory characteristics, if aware of this, will have an advantage in the market. In addition, in order to maintain this position, they must understand the reasons why their product has particular sensory characteristics and have the knowledge needed to ensure the sensory quality of their products or to change the sensory characteristics as dictated by consumer demand. Therefore, sensory evaluation will be most effective when validated relationships are determined between the sensory evaluation measures and appropriate chemical, instrumental, or consumer choice data.

There are direct relationships between sensory properties and product composition, which can be measured by instruments. The determination of such relationships is one of the most important applications of sensory evaluation, because this knowledge can be used to considerable advantage by scientists who have knowledge of the technology, microbiology, and chemistry of the product. For example, with a knowledge of a desired composition for consumers, these scientists can set about optimizing the manufacturing procedure to achieve this composition. The difficulty encountered in obtaining good relationships between product composition and sensory characteristics is usually that of obtaining relevant physical and chemical data. For example, in the study of flavor, or more specifically the study of the stimulus of aroma quality, extraction techniques that seek to replicate in-mouth conditions, including mastication and saliva addition, have been developed recently with this goal in mind. In addition, the technique of gas chromatography-olfactometry (GCO) can be used to carry out sensory evaluation of the GC-separated eluate of a product extract and to distinguish the compounds in the extract with high odor activity from those with low odor activity based on sensory threshold. In fact, it is often the same compounds, but in different concentrations, that give flavor to a wide variety of dairy products. Examples of commonly occurring compounds that are important in dairy flavors are the fatty acids butyric and isovaleric acids (which have 'rancid' and 'sweaty' character), the fatty acid esters ethyl butanoate and ethyl hexanoate (which have 'fruity' character), the methyl ketones 2-heptanone, 2-undecanone, and 2-nonanone (which have 'spicy' and 'green' character), the compounds 1-octen-3-ol and 1-octen-3-one (which have 'mushroom' character), and the sulfur-containing compounds methional and dimethyl sulfide (which smell like 'boiled potato' and 'boiled cabbage', respectively).

'Preference mapping' is a generic term given to a collection of techniques that have emerged in recent years to quantify, analyze, and interpret consumer preferences for products. A premise can be made that the preferences of a sufficiently large group of consumers will discriminate between comparable products on the basis of their intrinsic sensory differences, and that the degree and direction of discrimination will reflect the size and type of sensory differences that can be found. Therefore, by simply quantifying and analyzing preference or acceptance for the range, a preference map reflecting sensory differences can be drawn. The preferences of individual consumers can be represented as a map and areas of minimum and maximum preference can be identified. Descriptive sensory evaluation is used most often to facilitate interpretation of the preference map. These techniques provide a powerful research tool for market analysis and new-product development. The preference map can be enhanced by seeking technical extensions or relationships between preferences, sensory characteristics, and physical and chemical properties of products. It can also be enhanced by seeking behavioral extensions or by determining characteristics of the consumers and how they have developed their preferences and make their choice decisions.

Conclusions

The objective of sensory evaluation methods is to measure the human response to product characteristics that can be perceived by the senses. However, sensory evaluation encompasses a wide collection of techniques that achieve different results for different applications. In the dairy industry, there is a strong tradition of quality judging that has provided a body of knowledge on sensory defects and their causes. These methods are valuable for quality control, but are not useful for research applications and for product innovation to meet consumer acceptance. Sensory discrimination methods, descriptive evaluation, and consumer acceptance tests that can meet these objectives, have been developed, and these are now being applied increasingly in the dairy industry. The true value of sensory evaluation is realized when sensory data are used as the means of translating consumer preferences into a product composition specification that enables consistent quality product production and innovative new-product development.

See also: **Analytical Methods**: Sampling. **Butter and Other Milk Fat Products**: Properties and Analysis. **Cheese**: Cheese Flavor; Cheese Rheology. **Flavors and Off-Flavors in Dairy Foods**. **Organic Dairy Production**.

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ANIMALS THAT PRODUCE DAIRY FOODS

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Major *Bos taurus* Breeds

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Introduction

Dairy cattle form a unique niche among animals used for human purposes. They have been selected out among cattle to perform one very specific purpose, the production of fluid milk. While many dairy cattle also have value as beef or draught animals, there are no other types of livestock that have been selected as systematically, and for as many years, as dairy cattle have been selected to produce milk. In many parts of the world, cattle are raised for dual (meat and milk) or even triple (meat, milk and draught) purposes. Breeds of cattle frequently show the effects of such multiple objectives. The dairy cattle breeds are those which have been specifically improved, especially in Europe and North America, for milk production.

This article will cover the concept of the breed and the history and background of several breeds of dairy cattle. It will also include results of experiments designed to evaluate breeds for both dairy production and for beef production.

Domestication of Cattle

Cattle have been domesticated for several thousand years. Stone Age hunters left us with pictures on cave walls of the aurochs (*Bos primigenius*) which are the progenitors of the cattle of today. There is evidence of domestication of cattle as long as 8000 years ago in central and western Asia. While early cattle may have been kept for the common uses of meat, milk and draught, it may be that the earliest domestication was for religious purposes. As cattle were brought into the human orbit, they became smaller and their conformation began to approximate to modern type fairly quickly. The aurochs finally became extinct early in the seventeenth century when poachers probably killed the last one in Europe. While there were reports of aurochs in the decades that followed, there was limited evidence to their validity.

Cattle of the world fall into two classifications. All cattle are members of the genus *Bos*. Most cattle can be assigned either to species *taurus* or *indicus*. Some authorities feel that they should not actually be separated into two species. There is no reproductive barrier between them as they are able to freely interbreed. There are physical differences and, while apparently derived from a common progenitor species, they evolved separately for several thousand years. *Bos indicus* cattle typically have a pronounced hump on their shoulders while *Bos taurus* cattle are humpless. The hump is probably for energy storage and it also serves to increase surface area which is an aid in heat dissipation. *Bos indicus* cattle remained on the Indian subcontinent for many generations and then began to migrate along the east coast of Africa and toward Southeast Asia. *Bos taurus* cattle evolved in more northern areas of Asia and in Europe. There were migrations of *Bos taurus* cattle along western Africa and the Americas with the explorations of the Spaniards. Although it is generally true that *Bos indicus* cattle are tropically adapted and *Bos taurus* cattle are adapted to temperate regions, the migration of *Bos taurus* cattle along western Africa has resulted in some tropically adapted *Bos taurus* breeds. Some early crossing of *taurus* and *indicus* cattle in Africa resulted in a subgroup referred to as 'Sanga' cattle.

The Concept of a Breed

Since domestication of cattle began, humans have attempted, through various means, to identify superior animals and retain them. The widely varying geographical areas in which cattle existed and the multiplicity of uses (meat, dairy, draught, hides, ceremonial, etc.) meant that cattle would develop in many diverse ways. It was inevitable that cattle would begin to fall into groupings which we have come to refer to as 'breeds'. The term 'breed' is a difficult one to define precisely because it means different things to different people. Breed might be defined as a group of animals with similar physical characteristics (such as color, horns, body type, etc.). It has been observed that the term breed was coined by livestock breeders and to assign a definition other than the common usage by livestock breeders would be improper.

A breed has been described as something which arises more rapidly than normal evolutionary processes would dictate but more slowly than would be true in the laboratory. Breed development probably covers almost the entire range of rates in that spectrum. Some breeds arose almost entirely through natural forces while others are developed by human managers in a highly directed fashion.

Cattle can also be subdivided by utility. Some cattle are raised for beef or draught purposes. The object here is to describe cattle that are raised for production of milk. The dairy breeds are those in which artificial selection has caused them to excel in milk production. This is especially true in Great Britain and North America where there is a fairly clear delineation between beef and dairy breeds. The dairy breeds produce quantities of milk far in excess of that which could ever be consumed by a calf and have become well adapted to a highly intensive schedule of being milked twice, or thrice, daily.

Breeds are easy to recognize in many of the developed countries because organizations have arisen to protect the purity of the breed and to pursue its improvement. These breed societies originated in Great Britain during the early part of the nineteenth century and spread to other countries, most notably the United States.

Breeds of Dairy Cattle

Various listings of breeds of cattle identify as many as 1000 breeds. In many cases, some of these breeds are local or national varieties of a breed with a wide distribution. With such a large number of choices for breeds, it is difficult to specifically identify breeds to be classified as dairy breeds. One such listing is shown in **Tables 1**, **2** and **3**. No doubt there are other breeds in the world that could be identified as dairy breeds and all of these breeds are used, at least in some measure, for other purposes.

Identifying 'major' breeds as a subset of this listing is even more difficult. 'Major' is obviously in the eyes of the beholder. However, for the purposes of this article the following will be identified as major breeds (in alphabetical order rather than order of importance): Ayrshire, Brown Swiss, Guernsey, Holstein, Jersey, Milking Shorthorn and Norwegian Red. All of these breeds are of the *Bos taurus* type. Each has been subjected to intense selection for increased milk production over the past several decades.

Ayrshire

Improvement of the cattle of Ayr County, Scotland was scant prior to 1814 when the Highland and Agricultural Society first awarded prizes for Ayrshire cattle. Several breeds probably contributed to the origin of the breed. Many of the cattle of the area were predominantly black although various browns began to appear prior to 1800. Some of the progenitors of the Ayrshire breed were

Table 1 Asian and Australian breeds of cattle used primarily for milk production

Breed	*Place of origin*	*Species*	*Distribution*	*Color*
Red Sindhi	Pakistan	*indicus*	Asia, Africa, Australia	red
Sahiwal	India	*indicus*	Asia, Africa, North America, Australia	red
Local Indian Dairy	Malaysia	*indicus*	Asia, Oceania	white
Javanese	Indonesia	*indicus*	Oceania	tan
Xinjiang Brown	China	*taurus*	Asia	variable
Illawarra	Australia	*taurus*	Australia, Asia	red, roan or white

Adapted from Buchanan and Northcutt (1999).

Table 2 European breeds of *Bos taurus* cattle used primarily for milk production

Breed	*Place of origin*	*Distribution*	*Color*
Braunvieh	Switzerland	Europe	brown
Danish Red	Denmark	Europe	red
Dexter	Ireland	North America, Europe	black, red
Dutch Belted	Netherlands	Europe	black and white
Flamande	France	Europe	dark brown to black
Montbéliard	France	Europe	red and white
Norwegian Red	Norway	Europe	red and white
Russian Black Pied	Russia	Europe, Asia	black and white

Adapted from Buchanan and Northcutt (1999).

Table 3 Breeds of *Bos taurus* cattle used primarily for milk production in North America and Europe

				Milk production		
Breed	*Place of origin*	*Body weight (kg)*	*Color*	*Yield (kg day^{-1})*	*Protein (%)*	*Fat (%)*
Ayrshire	Ayr, Scotland	550–700	red and white	19–27	2.9–3.3	3.5–4.1
Brown Swiss	Switzerland	600–800	light brown	21–29	3.1–3.5	3.6–4.4
Guernsey	Guernsey, Channel Islands	400–650	fawn and white	18–26	3.1–3.5	4.2–4.8
Holstein	The Netherlands	600–800	black and white	25–35	2.8–3.2	3.3–4.1
Jersey	Jersey, Channel Islands	350–550	fawn	19–25	3.1–3.9	4.1–4.9
Shorthorn	England	500–650	red, white or roan	17–25	2.9–3.3	3.2–3.9

Adapted from Buchanan and Northcutt (1999).

probably related to those that established the Shorthorn and Holstein breeds. Many breeders contributed to the improvement of the breed during the first half of the nineteenth century. The Ayrshire Herd Book Society of Great Britain and Ireland was established in 1877.

Introduction of Ayrshire cattle to the United States began in 1822, but the earliest imports were crossed with local cattle and the purity of the Ayrshire cattle was lost. Other importations were made during the next 40 years with the most significant and long-lasting being the importations of MH Peters of Southborough, Massachusetts in 1860. Dr E Lewis and Joseph Sturdevant established a herd in 1869 and they kept good records and distributed the results to others. By 1900 there were more than 11 000 Ayrshires in the United States. Other important early herds included the agricultural school at Ste Anne de la Pocatiere, Caverly Farm at Clinton, Maine, Androssan Farm at Villanova, Pennsylvania and Fairdale Farm of Bennington, Vermont.

The Ayrshire may be red of any shade, mahogany or brown (**Figure 1**). There should be well-defined areas of white. Black or brindle markings are not desired. Individual spots are commonly small. Ayrshire cattle are moderate in size (550–700 kg) and average milk production is intermediate among dairy breeds (19–27 kg day^{-1}) (**Table 3**).

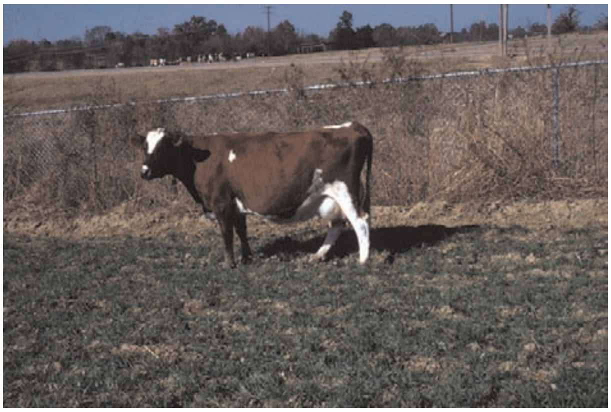

Figure 1 Ayrshire cow. (Photograph courtesy of Mississippi State University.)

Brown Swiss

The Brown Swiss originated in the cantons (states) of Schwyz, Zug, St Gallen, Glarus, Lucerne and Zurich in Switzerland. It became a prominent breed during the middle of the nineteenth century although its origin may have been much earlier. The Pinzgauer breed from Austria was a contributor to the breed although many other earlier breeds also contributed. The first importation of Brown Swiss cattle to the United States was in 1869. The importer was Henry M Clark of Belmont, Massachusetts. His imported bull was recorded in the Brown Swiss Cattle Breeders' Association (formed in 1880 in Worcester, Massachusetts) as William Tell 1. The breed did not increase in popularity in the United States until about 1920 when there were more than 8000 Brown Swiss in the country. Some prominent early US herds included Lee's Hill Farm, Morristown, New Jersey and White Cloud Farm, Princeton, New Jersey.

Brown Swiss should be, as the name suggests, some shade of brown (**Figure 2**). The actual shade may vary from a very light color to a deep, dark brown. Frequently, the back and areas around the muzzle are lighter in color than the rest of the body. The nose, hooves and switch should be black. Brown Swiss are large cattle (600–800 kg) and dairy-type Brown Swiss should display good dairy characteristics with moderate levels of milk production (21–29 kg day^{-1}) (**Table 3**). There are strains of Brown Swiss that tend more toward beef characteristics and they are used, at times, in beef crossbreeding programmes.

Guernsey

Guernsey cattle developed on the island of Guernsey, which is one of the Channel Islands lying between England and France. Progenitor stocks were probably brought to Guernsey as early as AD 960 from Normandy and Brittany, in France. There was interchange between Jersey and Guernsey which may explain some of the similarities between the breeds. Further importation into Guernsey was halted in the early nineteenth century, when ordinances were passed to limit imports in an attempt to halt diseases. This also had the effect of promoting breed purity. The first Herd Book was published in 1878 and a competing Herd Book was published in 1881. Early Guernsey breeders paid much attention to the color of milk, with a deep yellow color being preferred. In addition, record-keeping for milk and butterfat was initiated in 1907.

Early importation of Guernsey cattle into the United States is difficult to differentiate from importation of Jersey cattle. Limited distinction between the breeds was made during the nineteenth century and shippers picked up cattle from all the Channel Islands to bring to North America. There were numerous imports from Guernsey throughout the nineteenth century. After the turn of the century, more than 1000 head entered the United States in 1913 with a total of more than 13 000 imported by the time imports essentially ceased in the 1930s.

The most important early American herd of Guernsey cattle was established in 1901 at Langwater Farms in North Easton, Massachusetts. Another important herd was built by JC Penney in Hopewell Junction, New York as was Caumsett Farm of Huntington, New York. The American Guernsey Cattle Club was organized in 1877 with the aim of recording animals and issue the Herd Book Register. Provisional registration was added in 1970. This enabled registration of graded-up animals with a Guernsey sire and maternal grandsire as long as they met stringent performance requirements.

The Guernsey is of medium size and is fawn and white in color (**Figure 3**). The color markings should be clearly defined. The muzzle should be buff or flesh-colored and the switch should be white. Guernseys are intermediate to Holsteins and Jerseys in size (400–650 kg) and rate of maturity. The level of milk production is moderate (18–26 kg day^{-1}) but the fat content of the milk is high (**Table 3**).

Holstein

The Holstein–Friesian may be the single most important breed of cattle, not just dairy cattle, in the world (**Figure 4**). Members of the breed are exceedingly specialized animals. They possess an ability to convert feed

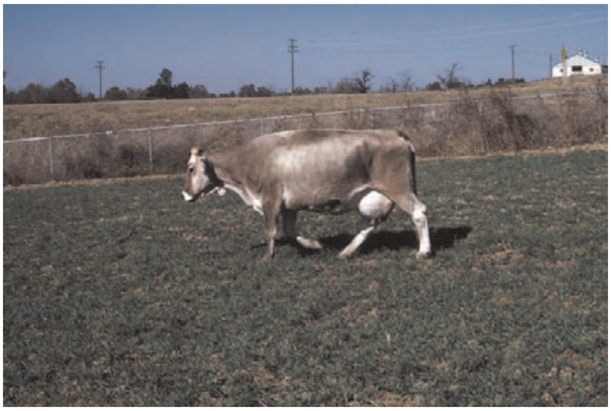

Figure 2 Brown Swiss cow. (Photograph courtesy of Mississippi State University.)

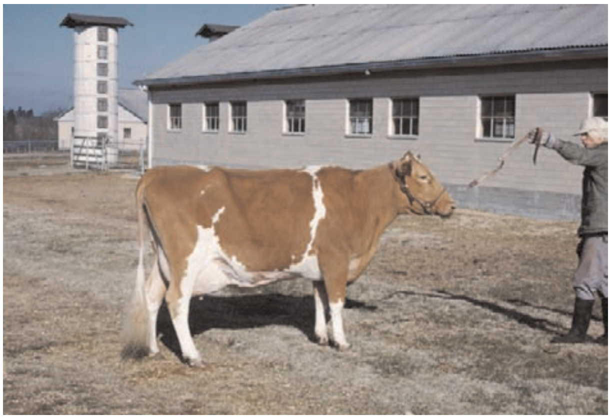

Figure 3 Guernsey cow. (Photograph courtesy of Mississippi State University.)

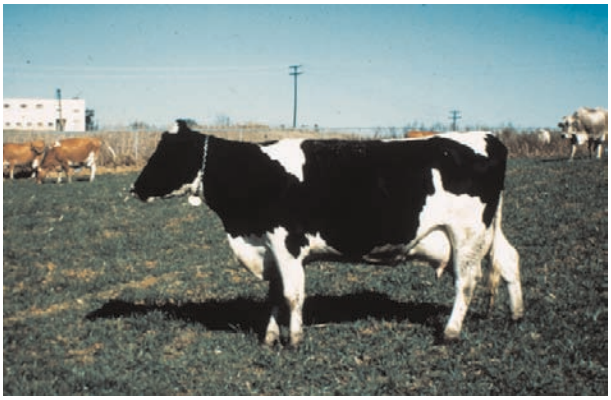

Figure 4 Holstein cow. (Photograph courtesy of Mississippi State University.)

into protein for human consumption to a degree that is unique. Their precise time of origin is uncertain but Holsteins come from The Netherlands, specifically the two northern provinces of North Holland and Friesland which lie on either side of the Zuider Zee. The original stock were the black animals and white animals of the Batavians and Friesians, migrant European tribes who settled in the Rhine Delta region about 2000 years ago. They spread into Germany as far as the province of Holstein, thus giving the breed its name.

Dutch settlers in America brought cattle for milk from their homes in Holland as early as 1621. The first permanent herd in the United States was in Belmont, Massachusetts and was owned by Winthrop Chenery. Other important early herds include those of Gerritt Miller of Peterboro, New York and Smiths and Powell of Syracuse, New York. Carnation Farms, near Seattle, Washington, was started in 1910. The Association of Breeders of Thoroughbred Holstein Cattle was formed in 1871. In the next year a competing association, the Dutch Friesian Cattle Breeders' Association, was established. In 1885, the two associations were brought together in the Holstein–Friesian Association of America in Brattleboro, Vermont. More Holsteins are registered than all the other American breeds combined.

Holstein cattle have the well-known black-and-white color pattern. The spots should well defined. The amounts of white and black may vary from almost entirely black to almost entirely white although, as in other black breeds of cattle, the red allele is present at low frequencies.

The popularity of the Holstein is due to the extremely high average milk production (25–35 kg day^{-1}). Although other breeds have a higher percentage of fat, protein and solids, the very high milk production of the Holstein means that the total quantity of milk components is also superior (**Table 3**). Holstein cattle are also very large cattle (600–800 kg).

Jersey

Like the Guernsey, the Jersey breed originated in the Channel Islands. Jersey is the largest of the islands and cattle were introduced there before 1100. Specific references to the cattle of Jersey as having high-quality milk began in the eighteenth century. Maintenance of purity of Jersey cattle was assisted by laws preventing the importation of cattle from the European continent. Jersey cattle were brought to America in the early nineteenth century but the exact time is not known because ships which carried one or a few cows for milk production frequently carried Jersey cattle because of their small size and high-quality milk. The first registered Jerseys were brought to America in 1850 by Samuel Henshaw. Some important early herds in the United States were Ewell Farms, Sporing Hill, Tennessee, Hood Farms, Lowell, Massachusetts, Meridale Farms, Meredith, New York and Twin Oaks Farm of Morristown, New Jersey. The American Jersey Cattle Club was organized in 1868.

Jersey cattle may vary in color from light gray to a dark fawn (**Figure 5**). A dark switch is common although aspects of color take second consideration to characteristics that pertain to production efficiency. Jersey cattle have milk that is high in fat and other solids and cows are small in stature (350–550 kg). The average milk production is low among dairy breeds (19–25 kg day^{-1}) (**Table 3**).

Milking Shorthorn

Milking Shorthorns (known in Britain as Dairy Shorthorns) represent a branch of the Shorthorn breed that has become specialized for milk production. The Shorthorn is one of the oldest organized breeds of cattle in the world. They originated in the valley of the River Tees in northern England. Early development took place both in England, where a dairy type dominated, and in Scotland, where the cattle were thicker and beefier. The

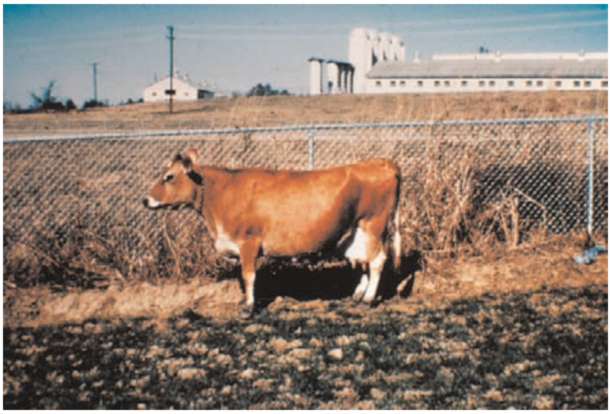

Figure 5 Jersey cow. (Photograph courtesy of Mississippi State University.)

earliest importation into America was in 1783 when milk-type Shorthorns were brought to Virginia. Importations of both beef-type and dairy-type Shorthorns continued well into the nineteenth century and the breed was well established in America. The Milking Shorthorn Cattle Club of America was organized in 1912 to work along with the American Shorthorn Breeders' Association. In 1948 the American Milking Shorthorn Society was started to handle registration and promotion of Milking Shorthorns. Improvement of American Milking Shorthorns moved forward in 1969 when importation of the Illawarra strain from Australia began. Like Beef Shorthorns, Milking Shorthorns may be red, white or roan in color. They are moderate in size (500–650 kg) and low in milk production (17–25 kg day^{-1}) (**Table 3**).

Norwegian Red

The Norwegian Red is not a breed in the same sense that defines the previous breeds. Unlike many breeds, it is not the result of closed breeding in a population with an old origin. It is a mixture of native and imported breeds including the Norwegian Red-and-White, Red Polled Eastland, Ayrshire, Swedish Red-and-White, Holstein and Friesian. The average milk production level is high. This breed is increasing in popularity, not only in Norway, but in other locations as well.

Performance of Dairy Breeds in Straightbred and Crossbred Populations

Although a huge amount of data is collected on dairy cattle in numerous countries, direct comparisons between breeds is more scarce since many herds maintain only one breed or have representatives from a second breed in very small numbers. There have been, fortunately, breed comparisons in experimental herds in several countries including the United States, Canada and New Zealand. These studies also considered crossbreeding as a system for dairy production. A brief summary of some of the results from these studies is shown in **Tables 4**, **5** and **6**. Each of the experiments illustrated the superiority of the Holstein for milk production. The high percentage fat level in the Jersey breed is also a consistent result. Crossbreeding is also shown to have some advantages, despite the widespread use of purebred Holsteins in commercial dairy herds. There is heterosis (advantage of a crossbred in comparison with the average of the component purebreds; also known as 'hybrid vigor') for milk, fat and protein production and for reproductive performance. Additionally, while Holstein cows give prodigious amounts of milk they are also very large compared to other breeds so crossbred cows may give more milk per unit of land or feed resource. While there may be some advantages to crossbreeding, the added complexity of a breeding system to develop crossbred cows may prevent crossbreeding from increasing in popularity.

Table 4 Predicted breed performance of several purebreds and two breed crosses from breed evaluation experiments in the United States

Sire breed	*Dam breed*	*Milk (kg)*	*Fat (kg)*
Purebred			
Ayrshire	Ayrshire	4874	189
Holstein	Holstein	6410	233
Jersey	Jersey	3991	202
Brown Swiss	Brown Swiss	5381	217
Crossbred			
Ayrshire	Holstein	6573	243
Ayrshire	Brown Swiss	5300	209
Holstein	Ayrshire	6731	217
Holstein	Jersey	5581	245
Holstein	Brown Swiss	6131	234
Jersey	Holstein	5793	241
Brown Swiss	Ayrshire	5215	212
Brown Swiss	Holstein	6294	236
Brown Swiss	Jersey	4949	230

Adapted from McDowell (1982).

Use of Dairy Breeds in Beef Production

Milk is not the only product produced by the dairy herd. Male calves, unselected female calves and cull cows are used for production of meat. In addition, some dairy breeds are used in commercial beef production. The high milk production of crossbred cows with quarter or half dairy blood is attractive to beef producers with sufficient feed resources to provide for high-producing cows. Breeds of beef cattle have been compared since the 1970s at the United States Meat Animal Research Center in Nebraska. Jersey, Brown Swiss and Holstein have been included in these comparisons and results are shown (in comparison with the Hereford–Angus as the control group) in **Tables 7**, **8**, **9** and **10**. The results illustrate that growth and carcass merit of Brown Swiss and Holstein crossbred calves is comparable to many breeds commonly used in beef production. Jersey crossbred calves were slow growing but had exceptional carcass quality. Reproductive performance of crossbred cows with half dairy breeding was excellent with very high calving percentage and low calving difficulty. While Brown Swiss and Holstein crossbred cows were quite large, they also weaned very heavy calves. The Jersey is an interesting candidate for beef production. Jersey crossbred cows were small, allowing for a higher stocking rate, and kept the calf birth weight low, causing

Table 5 Predicted breed performance of several purebreds and rotational crosses from breed evaluation experiments in New Zealand

	Annual production per cow			*Production (ha^{-1} year^{-1})*		
Breeding group	*Milk (l)*	*Fat (kg)*	*Protein (kg)*	*Milk (l)*	*Fat (kg)*	*Protein (kg)*
Purebred						
Holstein	3402	154	121	8194	371	291
Jersey	2706	147	107	7733	419	305
Ayrshire	3172	142	114	8318	371	299
Rotational crosses						
Holstein–Jersey	3161	156	118	8257	408	308
Holstein–Ayrshire	3350	151	120	8447	380	302
Jersey–Ayrshire	3037	149	114	8257	406	310

Adapted from Lopez-Villalobos *et al*. (2000).

Table 6 Predicted breed performance of Holstein, Ayrshire and crossbreds from breed evaluation experiments in Canada

	Annual production per cow	
Breeding group	*Milk (kg)*	*Fat (kg)*
Holstein	6502	239
Ayrshire × Holstein	6201	234
Holstein × Ayrshire	5807	221
Ayrshire	5201	207

Adapted from McAllister (1986).

minimal calving difficulty. One of the primary limitations to the use of the Jersey breed for beef production is the low value of the male siblings of the half-Jersey heifers that might enter beef herds.

Genetic Trends in Dairy Breeds

Genetic improvement for milk production is one of the true success stories in agriculture. Milk production is a trait measured in only one sex of a type of livestock which reproduces slowly. Despite these limitations, widespread use of artificial insemination and near-universal use of production records in North American and European dairy herds have combined to create staggering genetic improvement during the second half of the twentieth century. Genetic trends for six breeds is shown in **Figures 6–8**.

The Stability of Dairy Breeds

The widespread use of the Holstein breed has placed many other breeds in somewhat precarious positions. The

Table 7 Sire breed group means for birth, weaning and postweaning traits (calves out of Hereford or Angus dams) from Germ Plasm Evaluation Experiment at the US Meat Animal Research Center

Breed	*Number*	*Gestation length (days)*	*Calving difficulty (%)*	*Birth weight (kg)*	*200-day weight (kg)*
Jersey	302	283	2.9	31	184
Brown Swiss	263	286	8.4	39	205
Holstein	143	287	5.1	37	203
Hereford–Angus	962	284	2.9	36	195

Adapted from Cundiff *et al*. (1986).

Table 8 Sire breed group means for carcass characteristics (calves out of Hereford or Angus dams) from Germ Plasm Evaluation Experiment at the US Meat Animal Research Center

Breed	*Number*	*Fat thickness (cm)*	*Kidney fat (%)*	*Marbling score*	*Retail product (%)*
Jersey	134	1.17	6.2	13.3	65.5
Brown Swiss	120	0.99	4.0	10.4	69.1
Holstein	68	1.07	3.5	9.7	70.7
Hereford–Angus	472	1.63	3.9	11.3	66.3

Adapted from Cundiff *et al*. (1986).

Table 9 Sire breed group means for age at puberty and pregnancy rate in heifers (heifers out of Hereford or Angus dams) from Germ Plasm Evaluation Experiment at the US Meat Animal Research Center

Breed	*Number*	*Age at puberty (days)*	*Pregnant at 550 days (%)*
Jersey	117	308	81
Brown Swiss	126	332	93
Holstein	50	341	94
Hereford–Angus	322	357	87

Adapted from Cundiff *et al*. (1986).

Table 10 Sire breed group means for reproductive and maternal traits of crossbred cows from Germ Plasm Evaluation Experiment at the US Meat Animal Research Center

Breed	*Number of births*	*Cow weight (kg)*	*Calf crop born (%)*	*Calving difficulty (%)*	*Birth weight (kg)*	*200-day weight (kg)*
Jersey	628	484	90	7	36	189
Brown Swiss	681	563	92	8	43	206
Holstein	113	583	95	10	43	210
Hereford–Angus	1685	555	91	13	39	182

Adapted from Cundiff *et al*. (1986).

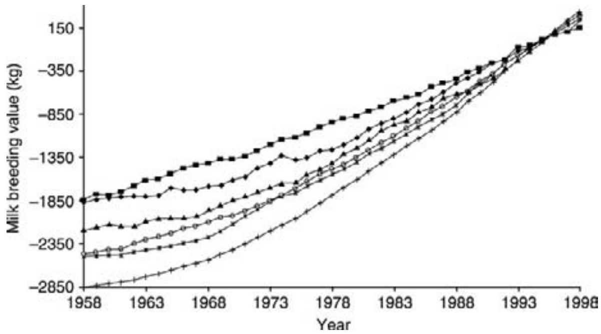

Figure 6 Milk production genetic trend for six breeds of North American dairy cattle (average genetic merit of individuals from each breed with 1995 as the base year). ■ Ayrshire, ▲ Brown Swiss, ○ Guernsey, + Holstein, × Jersey, ◆ Milking Shorthorn. (Adapted from US Department of Agriculture, Animal Improvement Laboratory: http://www.aipl.arsusda.gov.)

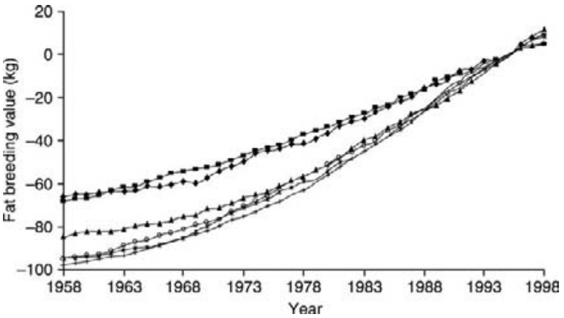

Figure 7 Fat production genetic trend for six breeds of North American Dairy Cattle (average genetic merit of individuals from each breed with 1995 as the base year). ■ Ayrshire, ▲ Brown Swiss, ○ Guernsey, + Holstein, × Jersey, ◆ Milking Shorthorn. (Adapted from USDA, Animal Improvement Laboratory: http://www.aipl.arsusda.gov.)

American Livestock Breeds Conservancy lists the Ayrshire, Guernsey and Milking Shorthorn among their “Watch” breeds indicating low numbers of registrations. Additionally, the effective population sizes of Ayrshire, Brown Swiss, Guernsey, Holstein, Jersey and Milking Shorthorn are all less than 60 at the present time. The effective population size represents the size of the genetic background of the population. It is inversely related to the rate of inbreeding in the breed. As can be seen, the genetic background of each of these breeds is not very large, even the Holstein, despite its extremely large census number. One cause is the widespread use of artificial insemination. A relatively small number of bulls sire a very large proportion of the cows in each of the breeds, even the Holstein.

The Future of Breeds of Dairy Cattle

Breeds of any type of livestock undergo changes over time. It is tempting to think that the current relative strengths among dairy breeds will remain the same. One has only to look at the history of breeds of livestock to realize how foolish that assumption may be. Many extremely popular breeds, in many types of livestock, have experienced severe declines in popularity. While it seems that the major role of the Holstein breed in dairy production will be maintained, that assumption is dependent upon the continued willingness of Holstein breeders to adapt to new opportunities.

The Holstein breed has become popular because Holstein cows produce a large amount of milk. Production

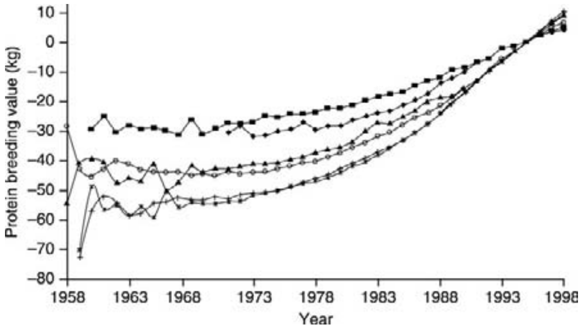

Figure 8 Protein production genetic trend for six breeds of North American dairy cattle (average genetic merit of individuals from each breed with 1995 as the base year). ■ Ayrshire, ▲ Brown Swiss, ○ Guernsey, + Holstein, × Jersey, ◆ Milking Shorthorn. (Adapted from US Department of Agriculture, Animal Improvement Laboratory: http://www.aipl.arsusda.gov.)

of milk for specialized purposes may become more important in the future. The opportunity to develop herds to meet specific marketing niches through the information gained in molecular genetics research may contribute to changes in the breed dynamics of the dairy industry.

Recognized breeds have become less important in some other types of livestock, most notably in the swine and poultry industries. Some of the reasons for the decline in the popularity of breeds in this industry do not apply to the dairy industry at this time. The structure of the dairy industry probably means that breeds will sustain their importance for the foreseeable future. Technology advances, the continuation of services by the breed organizations and the degree to which dairy production remains in the hands of private, independent producers will probably dictate future developments.

See also: **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses; Minor and Dual-Purpose *Bos taurus* Breeds. **Genetics**: Selection: Concepts; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. **Mammals**. **Reproduction, Events and Management**: Mating Management: Artificial Insemination, Utilization.

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Minor and Dual-Purpose *Bos taurus* Breeds

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Introduction

Cattle production has a long history and milk production was not always the ultimate goal. Keeping cows and oxen for draughting purposes and for the production of natural manure was as important as for milk and beef production even in Europe till the mid of the twentieth century. This is the reason that quite a number of European breeds are well muscled and have sound feet and legs. Modern breeding programs combined with artificial insemination (AI) were applied in most of the breeds, improving milk yield and beef performance at the same time. In Scandinavia, as well as in continental Europe, selection programs started around 1965 and replaced the former dominance of the show ring. Since these breeding programs have been quite successful, many dual-purpose breeds still exist and are under active improvement. Farm size and cost of land are another explanation for missing competition of beef breeds in most parts of Europe. Therefore, milk and beef are produced with the same breeds. This article also covers minor breeds, but it is impossible to describe all of them. References offer the possibility to go into further detail, and it should be mentioned that there are several activities to document the world cattle gene pool and also programs to conserve some of the original breeds. Details can be found in the FAO database on animal genetic resources.

Simmental and Related Breeds

The largest population of dual-purpose cattle is the Simmental-Fleckvieh breed, which comprises the broad-headed spotted mountain breeds of Central and Eastern Europe. The breeds, which originated from regionally different local strains, were upgraded by imports from Switzerland (region of Berne and the Simmen valley, giving the name) mainly in the second half of the nineteenth century. Most of the animals were kept for triple purpose – milk, beef, and draught – which is one explanation for the muscular development of shoulders and hindquarters.

Subpopulations in different countries are briefly described and milk production figures are summarized in **Table 1**. Beef potential is tested in performance and progeny tests, where high final weights are realized to test for growing capacity: up to 650 kg and daily gains of 1400 g can be reached. The color is quite variable, ranging from light yellow to dark red, and a white head is dominating.

In Switzerland, Simmental has been the dominant breed for a long time, but since 1967 the breed has changed through the intensive use of Red Holstein (RH) breeds. Today, only 11% of the population belong to the ‘Simmental strain’, less than 13% RH genes, 26% ‘Swiss Fleckvieh’ (14–74% RH genes), and 51% ‘Red Holstein’ (>74% RH genes). Cows show a variation in height and weight, ranging from 135 to 155 cm and from 650 to 800 kg, respectively. Production figures in **Table 1** show the yield differences of the different strains. Simmental are kept under less favorable conditions in higher mountain areas with Alpine summer pasture.

By far, the largest subpopulation is the German Fleckvieh, which was upgraded from local strains in Baden, Wuerttemberg, and Bavaria by Swiss imports, mainly between 1840 and 1880. Early application of AI and modern breeding programs led to the improvement of milk production and fattening ability, thus avoiding a larger infusion of RH genes (at present less than 1% genes). German Fleckvieh is a modern dual-purpose breed with strong feet and legs, and good muscularity in the fore- and hindquarters. Cows weigh between 650 and 750 kg and have a height of 140–145 cm. The breed has a low incidence of mastitis and low somatic cell counts. There is a high recording intensity for milk yield (**Table 1**), beef traits, calving ease, fertility, and longevity. Modern sire evaluation systems for all these traits are started to be combined in a genomic selection program.

In Austria, the Fleckvieh breed dominates and it has the same roots: local regional strains were consolidated by Swiss and German infusions. Since the environmental variation is large, there is some variation in type. The modern dual-purpose type is preferred because of the large export of fatteners to Italy. Breeding cooperation between Germany and Austria has resulted in a joint genetic evaluation and in a common total merit index.

The French Simmental family has three strains: French Simmental, ‘Montbeliard’, and ‘Abondance’. All three breeds originated from local strains and are adapted

Table 1 Milk records of Simmental cows in 2006

Country	*No. of recorded cows*	*Percentage of recorded cows in the country*	*Milk yield (kg)*	*Fat (%)*	*Protein (%)*
Austria	224 158	70.6	6464	4.18	3.43
Croatia	34 672	66.0	4459	4.07	3.35
Czech Republic	152 811	58.9	6175	4.08	3.46
France – Montbeliard	386 933	15.3	6451	3.93	3.43
France – Simmental	14 042	0.6	5702	4.01	3.49
France – Abondance	21 267	0.8	5155	3.72	3.48
Germany	877 859	26.7	6628	4.15	3.47
Hungary	3852	2.7	5166	3.80	3.32
Italy	31 692	3.9	6389	3.92	3.41
Poland	4602	0.9	4785	4.01	3.35
Slovakia	13 472	11.4	5175	4.10	3.36
Slovenia	34 714	44.3	5380	4.20	3.33
Switzerland – Simmental	15 541	4.6	5677	3.87	3.33
Switzerland – Fleckvieh	46 569	13.9	6875	3.98	3.28
Switzerland – Red Holstein	77 766	23.2	7360	4.03	3.30
Switzerland – Montbeliard	3694	1.1	7325	2.77	3.27
Vorderwald	6303	0.2	5397	4.17	3.34
Hinterwald	556	0.02	3502	4.09	3.43

Reproduced from International Committee for Animal Recording (ICAR), 2008.

to their local climatic and production conditions. The French Simmental is the largest in size and can be found in the eastern part of France. Montbeliard is smaller in size, is specialized in milk production, and has less pronounced muscularity. The milk has a high level of kappa casein B. The main distribution area is the high plateau of the Jura. The Abondance is a local strain in the French Alpine region and in the Massif Central, is the smallest in size, and has a deep red color with a white face. The strain is well known for its adaptation to unfavorable conditions and for specialty cheese products. Italy has a small Simmental population, mainly in the region of Friula and South Tyrol, but today the breed is found in most parts of the country. This population depends on regular imports. Simmental milk is especially respected for cheese specialties and breeders try to keep a high level of kappa casein B in their population.

Simmental was the dominant breed in the former Czechoslovakia with historical roots in the Bohemian, Moravian, and Slovakian local strains. Between 1965 and 1990, Ayrshire and RH were used to improve milk yield and udder quality. The dual-purpose strain is now favored and is dominant in the Czech Republic, even in larger farms. The Simmental breed is the most important breed also in Slovenia, Croatia, and Serbia. Upgrading started here in the nineteenth century. The base population consisted of Slavonian Podolic cattle. The breed is mainly kept in family-operated farms.

In Hungary, Simmental has a long history, since large estates imported cattle from Switzerland in the nineteenth century to replace the Grey Steppe cattle. Also upgrading took place, leading to the 'Magyar tarka', where the yellow color dominated. Around 1970, crossbreeding with Jersey and later with Holstein-Friesian started, leaving only a small Simmental population. Simmental is also the most numerous breed in Romania, especially in the center and the west. Upgrading was mainly on Grey Steppe and Illyrian Shorthorn with Swiss, German, and Austrian imports. Between 1980 and 2000, there was an infusion of RHs, but today the dual-purpose type is most popular.

Poland has a small Simmental subpopulation in the Carpathian mountain region, where small farms dominate. Imports from Germany and Austria have influenced this subpopulation. Ukraine is also a region with long Simmental tradition around Kharkov, Kiev, Cherkassy, and in the Carpathian region.

Russian Simmental population has a long history. First imports go back to 1850 and earlier from Switzerland and Miesbach in Germany. From the Sytchew center near Smolensk, the breed was systematically distributed throughout the former Soviet Union, giving Russia the largest number of Simmental cattle with about 1.5 million cows (13.4% of cows) in 2005. The regional strains depend on the local base populations: the Steppe Simmental in the central European steppe region, the Volga Simmental around Saratov, the Ural Simmental, the Siberian Simmental, and the Far Eastern Simmental. There have been continuous imports from Germany, Switzerland, and Austria, but RH genes were used after 1980 with varying intensity. The success of the RH introduction was not guaranteed, since the environmental conditions were unfavorable and fertility suffered. In recent years, steps back toward the dual-purpose type were taken. The Russian Simmental is variable in color (yellow to red) and in size.

Simmental was used in China and formed with local Mongolian cattle around 1900 the 'Sanhe' type, which are kept as dual-purpose animals. Recently, there have been imports from Germany and semen is used on a regular base to upgrade the local yellow cattle. In the northern part, this is especially aimed to increase productivity in small private farms.

Simmentals have been exported from Europe to all continents. In South Africa, the Simmental breed has existed for more than 100 years, is well adapted to harsh conditions, and today it is mainly kept for use as nurse cows. Exports of Simmental were made to America and Australia between 1968 and 1980 especially to increase the size and milk potential in beef herds. Several efforts to keep Simmental cows as dual-purpose cows were undertaken in South America and Australia, but on a rather limited scale. In several countries, Simmentals are used for crosses with dairy breeds to stabilize stayability and feet and legs in commercial herds. In Central Europe, only a few of the original local breeds have remained. Two of these breeds are kept in the Black Forest region: Vorderwald is a local breed at the eastern end of the mountain, somewhat influenced by Simmental and recently by RHs and Montbeliard. Cows have a height of 130–135 cm and 600 kg weight, and are of dark red pied color with white heads.

Hinterwald is the smaller strain, at home in the higher mountain region of the Black Forest. The cows have a height of 120 cm and 380–450 kg weight. They are of yellowish-red pied color with white heads and preferably white legs. Special governmental support is given to maintain this well-adapted local breed with extremely good claws.

Vosges or Vosgienne cattle have the same ancient origin as Hinterwald cattle. They are kept in Southern Alsace and are well adapted to most difficult mountain areas. The black-sided (sometimes red-sided) color is characteristic of these cattle; the tasty 'Munster' cheese is a renowned product.

Red Breeds

Red cattle were dominant in the northern part of Central Europe until the nineteenth century, ranging from France, The Netherlands, Germany, and Denmark to Poland and the Baltic States. Only a few strains from these lowland cattle are left, but these formed the origin of further distribution in Eastern Europe in the twentieth century. Information on milk production is summarized in **Table 2**. There is an intensive cooperation between countries with respect to red cattle, including a joint genetic evaluation between Denmark, Sweden, and Finland.

Red Danes are the most popular of these breeds and have developed from local strains of Seeland, Laaland, Falster, and Fünen with cattle from Angeln and Schlewig from 1840 till 1860. Red Danes have a long history of milk records, starting in 1895. Around 1970, inbreeding became a problem and crossbreeding trials with several breeds were conducted. From 1975 onward, American Brown Swiss and later Red and White Holstein and Finnish Ayrshire were used. Today, mainly Swedish Red is used for improvement. The breed is known for the high potential in fat and protein content and for its fattening performance. Carcass recording is regularly done. The breed has a sophisticated index system of selection for milk, beef, and functional traits including mastitis resistance. Cows have a height of 142 cm with 600–650 kg weight, and bulls have a height of 150 cm with 1000 kg weight. The color is of solid dark red, but through the use of foreign breeds there is a variation from brown to light red with color markings.

Swedish Red and White (SRB) originated from several local breeds (Smaland, Gotland, Oland, Hergard, and Shonen). The influence of German Red Pied Lowland and Shorthorn is known, but from 1927 Ayrshire and later Norwegian Red (NRF) cattle were incorporated. Lately, Ayrshire from Finland and Canada was used. Today, there is cooperation with Danish Red. The SRB is a dual-purpose breed with emphasis on functional traits.

Angeln is the German variety of the red lowland cattle and follows more or less the development of the Danish

Table 2 Milk records of Red cows in 2006

Country	*No. of recorded cows*	*Percentage of recorded cows in the country*	*Milk yield (kg)*	*Fat (%)*	*Protein (%)*
Belgium	3493	5.9	6582	4.24	3.37
Denmark	41 973	8.9	8560	4.25	3.50
Estonia	25 348	25.8	6303	4.29	3.40
Germany	13 989	0.4	7362	4.75	3.63
Lithuania	37 262	8.7	5243	4.51	3.49
Norway	154 221	95.4	6374	4.16	3.27
Poland	1492	0.3	4028	4.28	3.34
Sweden – SRB	146 884	46.7	8633	4.32	3.73

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Red. After the introduction of the quota system on fat yield, this breed has decreased in number.

The roots of the NRF are local breeds (Malselv, Red Tronder, Norwegian Red Pied, Hedmark, Red Polled Eastland, Dole, Lyngdal, etc.). Shorthorn and Ayrshire as well as SRB were used in early years to upgrade local strains. Recently, Black and Red Holsteins and SRB were used to establish this 'open synthetic' breed. Carcass data are collected in slaughterhouses and used to select for beef performance. The breed has quite a variation in color, and cows weigh between 550 and 600 kg with a height of 130 cm. Small local groups of West Finncattle, Eastern and Western Red Poll in Sweden, and small samples of Telemarkfe and Fjordfe in Norway still exist and are in conservation programs.

Red cattle in Estonia go back to local cattle, which were upgraded by Red Danes and Angler since 1860. There is still a small group of native Estonian cattle. The same history applies to the Latvian Brown, the dominant breed with red color. Populations of Estonia and Latvia had a large influence on the development of red breeds in Russia in Soviet times, since cattle breeding in the Baltic States was well developed.

Red cattle in Ukraine have a long history: first imports came at the end of the eighteenth century with German Mennonites from East Friesland. This was followed by the introduction of Red Highland Cattle by German immigrants after 1812 and a third infusion happened after 1870 with different sources (red, brown, and Tuxer cattle). This led to the Red Steppe breed, which was rather influential on other red breeds in Russia and Moldavia. The influence of local strains differs in different subpopulations. Polish and Ukrainian-Polish Red used to be an independent local red lowland breed. Sukzun is a Russian red strain located in the Ural region and is influenced by Red Danes and Latvian Brown. Between these strains, there was an exchange of genetic material. Different strategies have been used after 1970, using Brown Swiss, RH, and other breeds. Size and production are quite variable due to the environmental conditions and the genetic makeup.

Belgian Red and Red Flemish are two local dual-purpose breeds in Belgium, belonging to the red breeds. The Belgium Red developed from local strains in the north of Belgium in the nineteenth century and were influenced by Shorthorn. Also Normande breed from France was used around 1920 to improve milk and beef production. The Red Flemish breed developed from red local strains in Belgium and Northern France. Also some Shorthorn blood was introduced during the consolidation of the breed. Lately, Red Danish and other breeds have been used to conserve this small local red strain.

Despite their different origin, the Red Highland Cattle also belong to the family of red breeds. They originated from different local strains from Harz, Vogelsberg, Vogtland, Donnersberg, and other regions in middle Germany, and today the Red Highland Cattle breeders try to conserve the genes of the old triple-purpose red breeds. There were also varieties of Red Highland Cattle in Poland, Bohemia, Moravia, and Slovakia. With fews exceptions, these breeds are extinct or upgraded by Angler or Red Danes. There are governmental activities to maintain some of the genes, but the numbers are declining.

Pinzgau Cattle

The Pinzgau breed, which originated from local short-headed cattle in the Pinzgau, Upper Styria, and Carinthia, evolved around 1850. The influence of Bernese cattle and also that of lowland breeds have been confirmed by genetic distances. Pinzgau was originally bred for draught purposes, but today it is a typical dual-purpose breed, adapted to mountain conditions with strong claws. The coat color is mahogany red-brown, with a white stripe along the back that widens at the rump. White buttocks are typical markings.

Austria has the largest population of Pinzgau breed (**Table 3**) with the center of origin of this breed in the state of Salzburg and it had a major influence on distributing the breed in Europe. From 1975 on, RH was used to improve milk yield, but this led to a large decrease in numbers. Today, there are a number of active breeders to maintain the breed in connection with ecological farming.

Pinzgau cattle were imported to Bavaria (Berchtesgaden and Miesbach) in the nineteenth century and were kept in the southeastern edge of Germany. Numbers were decreasing, but now they have stabilized at a low level. In Slovakia, there is still a larger population of Pinzgau cattle in the Carpathians, which were upgraded by Illyrian Shorthorn-type cattle. Crosses

Table 3 Milk records of Pinzgau cows in 2006

Country	*No. of recorded cows*	*Percentage of recorded cows in the country*	*Milk yield (kg)*	*Fat (%)*	*Protein (%)*
Austria	7082	2.2	5400	3.90	3.27
Germany	533	0.02	5222	3.93	3.40
Slovakia	1108	0.9	4195	4.02	3.36

Reproduced from International Committee of Animal Recording (ICAR), 2008.

were conducted with Ayrshire and RHs, but a purebred nucleus is kept at the Zilna station. From this station, there was also an exchange with Ukrainian and Russian Pinzgau.

Transylvanian Pinzgau cattle were developed from Austrian imports and from local cattle in the highland of Transylvania, but after a flourishing period around 1960 the breed has declined. In the northeast of Romania, a substrain with black color, the so-called Dorna, is kept. Pinzgau cattle were also kept in the neighboring regions of Austria: in Italy around Trento, in Slovenia, and in Croatia. Today, there are only a few herds left, including the Cika, a dwarf strain in Slovenia.

Grey Cattle

The Tyrol Grey is one of the few surviving breeds of the Grey Mountain breeds, which were rather common in the Alps. The breed has a good potential for milk and beef production (**Table 4**), and is well adapted to higher alpine conditions. It was also used for draught purposes. Cows have a height of 125–130 cm with 550 kg weight, and bulls have a height of 135–140 cm with 900–1000 kg weight.

The Grey Alpine is the Italian variant and is kept in the neighboring province of Bolzano. There is a close cooperation with the Tyrol Grey, with an active AI service. A high level of kappa casein B is valuable to cheese factories. In Switzerland, the Raetian Grey Cattle are a restored subgroup of the former numerous Grey cattle living in the high Alps.

Another group of Grey cattle is of Podolian origin in the Balkan countries and in Southern Russia. In Croatia and Yugoslavia, the Istrian breed is still kept for draught purposes. Milk production is at a low level. There is some infusion of Romagnola, a beef strain from Italy. The Ukrainian Grey and the Romanian Steppe cattle are dual-purpose minor breeds. The Turkish Grey has the same roots and is kept for milk production. There is some infusion of Brown cattle in most of these local landrace types of cattle and the survival of all of these breeds is not guaranteed.

Further Breeds of Regional Importance

Europe

The Normande breed is a local breed of the Normandy, established around 1850 from local strains with the infusion of Shorthorn and Brown cattle. It is a typical dual-purpose breed (**Table 5**) with heavy cows (750 kg in weight and 140 cm in height) and bulls (1100 kg in weight and 152 cm in height). The color pattern varies from big splashes to small spots in an irregular dark red to brown patched coat. The Normande breed can also be found in parts of South America, such as Columbia and Uruguay.

The Tarentaise is a solid colored (reddish to wheat colored with an eel stripe) breed in Southeastern France,

Table 4 Milk records of Grey cows in 2006

Country	*Breed*	*No. of recorded cows*	*Percentage of recorded cows in the country*	*Milk yield (kg)*	*Fat (%)*	*Protein (%)*
Austria	Tyrol Grey	2878	0.9	4751	4.01	3.24
Italy	Grey Alpine	6205	0.8	4975	3.74	3.37

Reproduced from International Committee of Animal Recording (ICAR), 2008.

Table 5 Milk records of minor breeds in 2006

Country	*Breed*	*No. of recorded cows*	*Percentage of recorded cows in the country*	*Milk yield (kg)*	*Fat (%)*	*Protein (%)*
Belgium	Bleu Belge	2860	5.9	4104	3.53	3.23
Finland	Finn cattle	2641	1.1	6218	4.33	3.48
France	Normande	263 095	10.4	5901	4.31	3.62
France	Tarentaise	7727	2.9	3991	3.56	3.38
Germany	Gelbvieh	4753	0.1	5630	4.20	3.51
Switzerland	Herens	762	0.2	3246	3.60	3.43
Italy	Modicana	1706	0.2	3286	3.54	3.46
Italy	Valdotaine	11 020	1.3	3825	3.50	3.25
Australia	Illawara	6514	1.2	5910	4.08	3.43
Canada	Canadienne	203	0.01	5412	4.24	3.60

Reproduced from International Committee for Animal Recording (ICAR), 2008.

originating from the Massif Central, and is well adapted to rough conditions and is also kept in North Africa.

Gelbvieh (Yellow cattle) is a local breed in Germany. The origin is local red strains, consolidated in the second half of the nineteenth century by means of solid colored Simmental and Shorthorns. Until 1940, oxen for draught purposes were one of the main products. Today, Gelbvieh is a beef pronounced dual-purpose breed. Intensive use of AI led to genetic improvement of both milk and beef traits (**Table 5**). Local strains of Gelbvieh are Limpurger and Glan in Germany, which were recently restored. In Austria, Murbodner, Waldviertel, and Carinthian Blond belong to this family.

The Herens is an ancient Alpine breed, kept in the Swiss canton Wallis, in the Haute Savoie in France, and in neighboring Italy. Cows are of brownish-black to red-brown solid color and famous for their fighting to determine the ranking order. Substrains of the Herens are the Evolenard. The Valdotaine cattle in Italy include the Aosta Chestnut and the Aosta Black Pied. The Modicana breed is of Podolian origin and is mainly kept on Sicilia with a substrain on the Sardi Island. This breed is of unicolored red, has long horns, and is known for its heat tolerance.

The Pirenaica is a local breed in the Spanish–French Basque region with regional importance. There is a relationship to the French Béarnaise. Asturian Mountain is a local breed in the Cantabrian mountains of Northern Spain, which is well adapted to unfavorable conditions. The coat color is fawn to reddish-brown. Galician Blond is supposed to be the origin of Minorca and Canary Island cattle, which are ancestors of Criollo cattle in South America.

The Mirandesa breed is kept in Northeastern Portugal and is used for triple purpose. The cows have a brown coat and long horns, and the breed is quite large with a height of 135–140 cm. There are relationships to other breeds: Maronesa, Arouquesa, and Verinesa in Spain.

Asia

From the original breeds in the Caucasus region, only Mingrelian Red in Georgia and East Anatolian Red and East Anatolian Black in Turkey are left, but infusions with Brown Swiss, Simmental, and Friesian are quite common. In the Near East, the Baladi breed is the base of many local strains: Oksh or Arab in the Lebanon, Bedouin in Saudi Arabia, and Jaulan in Syria and the Golan. The Damascus breed in Syria has the highest milk potential. Four varieties of Egyptian cattle exist: Damietta, Baladi, Maryute, and Saidi. These are kept in distinct regions of the country and are also used for work. In most of the countries, the local breeds are used for crossings with European breeds.

Today, the Turano-Mongolian breeds of Central Asia have only a few strains: the Kalmyk near the Caspian Sea, Yakut in Siberia, and Kazakh in Kazakhstan, Uzbekistan, and Kirgisia. Mongolian, Menggu, and Tibetan are authentic breeds, kept in Mongolia and Northern China. The Caujen Red (Chinese Red Steppe) is the result of crossings with Shorthorn and the Sanhe with Simmental. Most of these breeds are kept for triple purpose. Xinjang Brown resulted from upgrading local strains with Brown Swiss from Europe.

Africa

North Africa is a region with a long history of keeping cattle. Strains of the native Brown Atlas breed exist in Morocco, Algeria, and Tunisia. There are also the Guelma breed and Lybian Shorthorn. Based on these local cattle, different European breeds (Tarentaise, Montbeliard, Brown Swiss, and Friesians) were used for upgrading.

The N'Dama is one of the native taurin breeds in West Africa, going back to ancient times. The center of development of this triple-purpose breed is Guinea and this Longhorn breed has spread all over the region because of its tolerance to trypanosomes. Guinea N'Dama is a well-known breed with brown color. It is also found in Mali, Senegal, Sierra Leone, and Liberia. The Gambian N'Dama is of white color and smaller in size. Several breeds in the region are N'Dama × Zebu crosses, like Djakore in Senegal and Bambara in Mali.

The West African Shorthorn is another taurin breed group with a long history in Nigeria, Senegal, Gambia, Liberia, Eastern Ghana, Togo, and Benin. A Savannah and a dwarf variety are found, both of which are resistant to trypanosomes. The Kuri is a taurin breed found around Lake Chad and has a long history but limited distribution.

The New World

There are some cattle breeds going back to the early settlements in the fifteenth century. They have their origin in Spain, Portugal, and the Canary Islands, and are mostly called Criollo. The Cuban Criollo cattle are still numerous and are used for triple purpose, while Haitian and Dominican Criollo are mostly absorbed by European breeds. Tropical Dairy Criollo was recently established in Costa Rica with animals from Nicaragua and Honduras. The Limonero in Venezuela, and Costeño con Cuernos, Romosinuano, and Blanco Orejinegro in Columbia are Criollos with triple purpose. Criollos are also found in the high Andes (Sierra Criollos) and in Brazil (Caracu, Yacumeno). Although the Criollos are adapted to the specific conditions, their milk production is not sufficient. This is the main reason that most of them are endangered and that they get absorbed in crosses with Holstein, Brown Swiss, and Zebu today.

The Canadienne is the oldest breed in North America, mainly located in the province of Quebec. Roots go back to imports of French settlers around 1610. Their origin is Northern France (Brittany and Normandy). The color is dark brown to black and milk production is medium (**Table 5**).

The Illawara in Australia is considered to be an authentic breed, going back to Shorthorn from Durham, originally imported around 1820. The breed was developed with some foreign genes (Ayrshire) and today semen from SRB and RH is used. The breed is of solid red color and is known for high levels of fat and protein.

See also: **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses; Major *Bos taurus* Breeds. **Mammals**.

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Bos indicus Breeds and *Bos indicus* × *Bos taurus* Crosses

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Introduction

Bos indicus breeds have special adaptations to tropical environments that may be conveniently used for dairy production. The crosses between them and *Bos taurus* show increased production while retaining adaptation. The biological, economic and practical aspects of the utilization of these animals are discussed in this article and a brief description of the main breeds is given.

Domestic cattle belong to the genus *Bos* of the order Artiodactyla, suborder Ruminantia, family Bovidae, subfamily Bovinae, tribe Bovini. *Bos taurus* L., the common cattle of temperate countries, and *Bos indicus* L., the zebu, are believed to have originated in the Neolithic from the aurochs or wild ox (*Bos primigenius*), and were probably separated by domestications in the Near East and Baluchistan.

Bos indicus is characterized by a pronounced hump, which is present in both sexes but more pronounced in the males; long, wide ears; a large dewlap; and an abdominal skinfold, including a large sheath in males. Wide variation exists both between and within breeds in the size of these features. Most breeds are horned, with horns varying in size and shape, but polled varieties exist. Most breeds are white, gray or red in color, with ample variation in shade and color pattern. Their bellowing call is very characteristic and distinct from that of *Bos taurus*.

Both *Bos taurus* and *Bos indicus* have 30 pairs of chromosomes. The 29 autosomes are acrocentric and the X sex chromosome is submetacentric in both species. However, the morphology the Y chromosome is submetacentric in *Bos taurus* but acrocentric in *Bos indicus*. Chromosome pairing abnormalities at meiosis have been reported in male hybrids. However, hybrids are fertile. A high proportion of young hybrid bulls have been considered inadequate for frozen semen production (ranging from 0.36 to 0.50 in three programs in Brazil and India). Nonetheless, the fertility of hybrid bulls in natural mating has been generally higher or equal to that of pure *Bos taurus* or pure *Bos indicus* bulls. Fertility of hybrid cows has been generally higher than that of purebreds.

Although *Bos indicus* breeds have a long gestation period (a mean of 289 days in several breeds), the cows are able to limit the birth weights of their calves, so they have practically no calving difficulties. Mean birth weights in the Gir are 24 kg for males and 23 kg for females and, in the Guzera, 28 and 27 kg. The absence of calving difficulties has been reported even for primiparous *Bos indicus* cows carrying calves sired by bulls of large Continental *Bos taurus* breeds.

Bos indicus cattle have a particular behavior. They are affectionate animals if frequently handled as, for example, in the Indian subcontinent, where they are often housed next to humans. However, in the less densely populated areas of Latin America, they may be more difficult to handle. In pastures, they tend to herd together in a very gregarious manner. Cows need to be stimulated by the presence of the calf for milk letdown.

Main *Bos indicus* Dairy Breeds

Although breed differences in dairy traits have not been well documented in comparative trials, some breeds have a reputation as dairy animals. Brief descriptions of some of them are given below. Production characteristics in India, Pakistan and Brazil are given in **Table 1**. It should be emphasized that the data for each breed are not comparative because they have been obtained from cattle in different environments; as a result, breed and environmental differences are confounded. However, the data give an indication of the mean production traits involved.

Gir

This breed is predominantly red and white in color, with patterns varying from solid to mottled and shades from yellowish to dark red (**Figure 1**). The animals display a very characteristic broad, prominent forehead, with horns curving downward, backward and outward and then upward. A herd book for a polled variety exists in Brazil. The ears are long and pendulous and the temperament is generally tranquil.

The breed is found on the central west coast of India. It has been exported to Brazil, where it is

Table 1 Noncomparative means in *Bos indicus* breeds

Breed	*Age at first calving (months)*	*Calving interval (months)*	*Lactation yield (kg)*	*Lactation length (days)*	*Milk fat (%)*
India[a]					
Gir	47	15.7	1403	257	—
Kankrej	47	16.2	1850	351	—
Rath	40	19.3	1931	331	—
Red Sindhi	42	14.7	1605	284	—
Sahiwal	40	15.0	1718	283	—
Tharpharkar	49	14.9	1659	280	—
Brazil[b]					
Gir	45.2 (6911)	16.1 (15 365)	2778 (27 431)	291 (27 431)	4.6 (16 771)
Guzera	44.2 (575)	14.9 (1040)	2400 (2298)	285 (2298)	4.9 (851)
Pakistan[c]					
Sahiwal	44.0 (4601)	15.3 (13 951)	1522 (17 292)	256 (17 469)	4.5 (293)

[a] Taneja VK and Bhat PN (1986) *Proceedings of the 3rd World Congress on Genetics and Applied Livestock Production* 9: 73–91.
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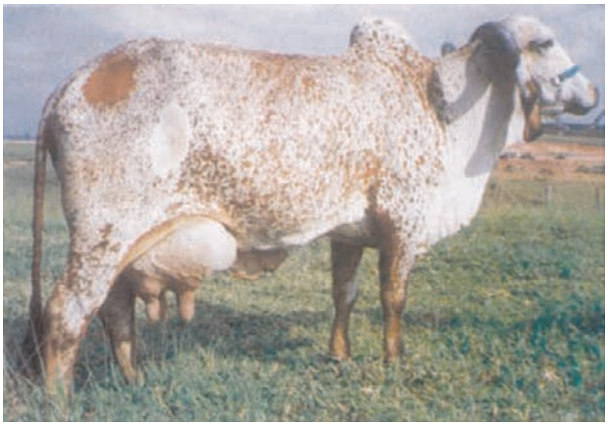
Number of observations are in parentheses.



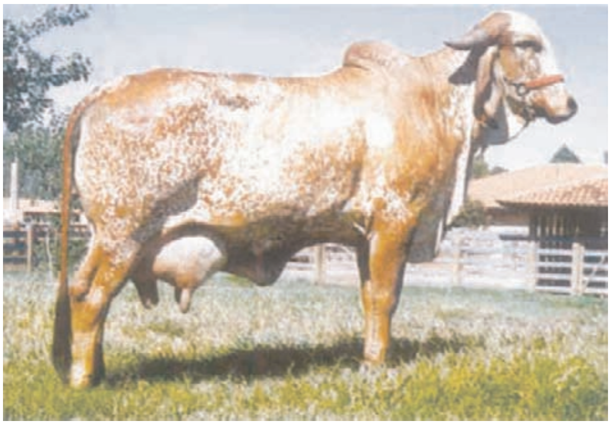


Figure 1 Gir females. Courtesy of Beef Milk Brasil Marketing, Brazil.

considered to be the main *Bos indicus* dairy breed, with some 7000 animals of both sexes being registered each year. It is also found in other countries, including Colombia and the United States. Frozen semen sales in Brazil amount to 344 000 doses per year, 76% of which is milking Gir. The Brazilian Milking Gir Breed Society (ABCGIL) and the Federal Research Organization (EMBRAPA) have run a conventional progeny-testing program of approximately 10 bulls per year since 1985. Genetic evaluations are based on animal model best linear unbiased prediction (BLUP) methods. As a matter of interest, the record milk yield was 15 126 kg in a 361-day lactation.

Guzera/Kankrej

Predominantly light to dark gray in color, this breed has characteristically lyre-shape horns (**Figure 2**). Animals are tall and the ears are shorter than in the Gir and not as pendulous. Animals of this breed display an attentive, active temperament.

The Guzera is a Brazilian breed derived from the Kankrej, an Indian breed found in Gujarat. These cattle are remarkably similar to those depicted in a famous Mohenjo-Daro seal dating from more than 2000 BC. The Guzera has been exported to other countries of Latin America and Africa. Along with the Nelore and the Gir, it was the most important founder breed of the Brahman. In Brazil, about 6000 animals of both sexes are registered per year and semen sales amount to 134 000 doses per year, 23% of which is from the dairy improvement

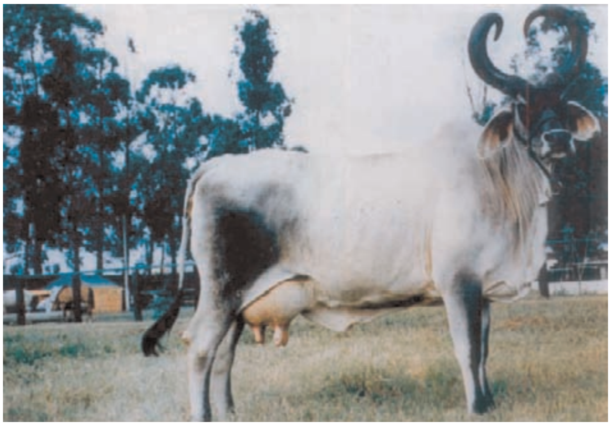


Figure 2 Guzera female. Courtesy of Beef Milk Brasil Marketing, Brazil.

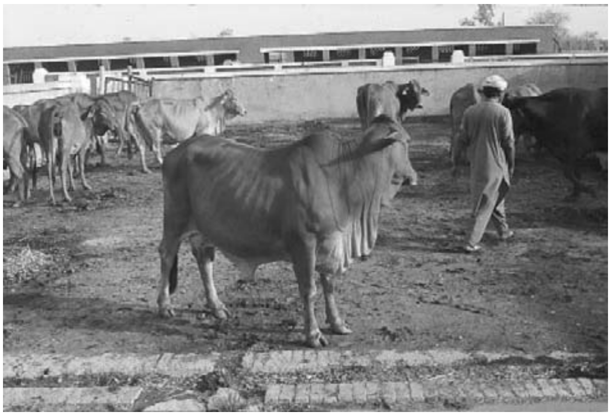


Figure 3 Sahiwal female, Punjab Province, Pakistan. (Photograph courtesy of JW Fuquay.)

program. The breed has a conventional progeny-testing of approximately six bulls per year, coupled with a multiple ovulation and embryo transfer (MOET) selection nucleus of 12 donors per year, run with technical support from the Federal University of Minas Gerais. The genetic evaluations are run by EMBRAPA, using modern statistical methods (animal model–BLUP).

Rath

This horned breed is white and gray in color and is found in Rajasthan State in India.

Red Sindhi

This breed is generally solid red, horned and of small size. The udders tend to be pendulous and the teats are large. Originally from Pakistan, it is now found in many countries. Blood-group studies suggest that it might be related to the Gir and Sahiwal. The breed has a reputation for hardiness.

Sahiwal

The Sahiwal is usually various shades of red to brown, with varying white markings (**Figure 3**). Originaly from Pakistan, it may have influences from the Gir and Red Sindhi. It is now an international breed found in many countries. The number of animals worldwide has been reported to be in the order of 15 000, of which 4000 are breeding females.

Tharparkar

Usually white or gray, with lyre-shaped horns, the Tharparkar is strongly built and short-legged. Found in Hyderabad Sindh Province, India, it has been interbred with the Kankrej, Red Sindhi and Gir. Its original habitat is arid and the breed is considered to be a good milker under poor feeding conditions; it is also used for work and has a reputation for resistance to draught. The breed has been exported to several countries.

New Synthetic *Bos taurus* × *Bos indicus* Breeds

Countless attempts have been made and continue to be made to develop new synthetic breeds from *Bos taurus* × *Bos indicus* crosses. When evaluating these efforts, it should be borne in mind that successful development of new composite breeds requires a combination of genetic soundness, operational effectiveness and commercial organization. Genetic soundness involves the appropriate choice of breeds and breed composition; the avoidance of inbreeding, which requires the use of large populations and an adequate breeding program, based on progeny testing or, more recently, MOET nucleus selection schemes; scientifically based methods of genetic evaluation, such as animal model–BLUP or formerly herd mate comparisons; appropriate definitions of economic selection objectives and criteria; and optimization of generation intervals. Operational effectiveness is rare in the public organizations of developing countries while commercial breeders have tended to place too much emphasis on traditional show-ring competitions and other ineffective methods of genetic improvement. For example, due perhaps to the influence of the Santa Gertrudis (an early new tropical synthetic beef breed that met with commercial success worldwide), several populations have been based on a $\frac{5}{8}$ *Bos taurus* × $\frac{3}{8}$ *Bos indicus* cross, although there is no experimental demonstration of that fraction being superior. The combination of large-scale investment and managerial/commercial organization required for optimal results in the

Table 2 Noncomparative means in new composite *Bos taurus* × *Bos indicus* breeds

Breed	*Number of herds*	*Number of lactations*	*Lactation yield (kg)*	*Lactation length (days)*	*Milk fat (%)*
Australian Milking Zebu[a]	—	651	1763	262	4.5
Australian Friesian Sahiwal[b]	27	269	2342	—	4.2
Brazilian Milking Hybrid[c]	14	6092	2549	—	—
Brazilian Milking Hybrid[d]	23	2321	1793	248	3.4
Carora[e]	19	13527	2701	267	—
Girolando[f]	153	12610	3335	280	—
Jamaica Hope[g]	12	2158	2737	—	4.7
Mambi[h]	—	11515	2873	300	3.5
Siboney[h]	2	8040	2606	262	3.6
Sunanini[i]	—	2072	2194	—	—

[a] Franklin I (1983) *Proceedings of the 1st Brazilian Symposium on Tropical Dairy Cattle Breeding*, pp. 331–347.
[b] Alexander GI, Reason GK and Clark CH (1984) *World Animal Review* 51: 27–34.
[c] Elite herds, mature equivalent yields.
[d] Herds for progeny-testing. Mostly first-lactations, 305-day yield. Madalena FE (2000) *Indian Committee for Animal Recording Technical Series* 3: 365–379.
[e] 305-day yield. Morales F, Blake RW, Stanton TL and Hanh MV (1989) *Journal of Dairy Science* 72: 2161–2169.
[f] Menezes CRA (2000) *Proceedings of the 3rd Brazilian Symposium on Animal Breeding*, pp. 222–225.
[g] 305-day yield. Wellington KE and Mahadeven P (1975) *World Animal Review* 15: 21–32.
[h] All lactations. Lopez D (1989) *Brazilian Journal of Genetics* 12(3)(supplement): 231–240.
[i] Unnithan NR, Kishan BN and Kishore G (2000) *Indian Committee for Animal Recording Technical Series* 3: 469–477.

development of tropical dairy breeds is not yet been available.

Some tropical dairy breed development programs are briefly described below. Noncom-parative means for dairy traits are shown in **Table 2**.

Australian Milking Zebu (AMZ)

This was the result of an experimental program, conducted by CSIRO in Badgery's Creek, northern New South Wales, that examined the possibility of developing a productive breed adapted to tropical stresses. The Australian Milking Zebu (AMZ) (**Figure 4**) was founded on a very small base of three male and five female Sahiwal and four male and four female Red Sindhi animals, introduced from Pakistan in 1952. The first stage (1956–67) consisted of producing a herd of 270 F_1 to F_3 *Bos indicus* × Jersey females at the field station. A high incidence of short lactations in the first cross (70%) was reduced to less than 10% after three generations of strict selection. A second stage was based on progeny-testing of young bulls from top-yielding cows in cooperating herds of predominantly Jersey breeding, selecting from the crossbred population. The *Bos indicus* gene fraction stabilized at around 0.25. The breeding program consisted of producing 30–40 candidate bulls per annum, after three independent successive stages of selection:

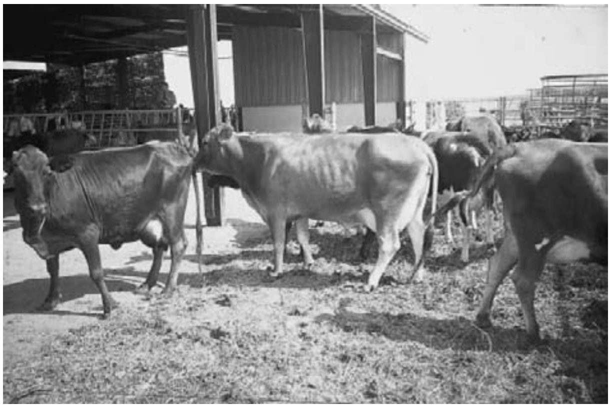


Figure 4 Australian milking Zebu females, Sultan Qaboos University, Oman. (Photograph courtesy of JW Fuquay.)

1. Approximately half (i.e. 20) were selected for heat tolerance assessed in a climatic room.
2. Approximately half of these (i.e. 10) selected for tick resistance under artificial infestation.
3. The best one on progeny-testing for milk yield, provided it was adequate for frozen semen production.

The program showed that it was possible to select simultaneously for milk yield and adaptation so that, in about 20 years, the animals had reached the milk yield of the Jersey base population but with increased heat tolerance and tick resistance.

Australian Friesian Sahiwal (AFS)

The Australian Friesian Sahiwal (AFS) was developed by the Queensland Department of Primary Industries from a closed population of half-bred Friesian × Sahiwal, the latter having the same origin as in the AMZ. Emphasis was on milk yield and tick resistance; it was not considered necessary to test for heat tolerance because the climate was considered stressful to *Bos taurus* dairy cattle. The program was initiated in 1961 by crossing Sahiwal × *Bos taurus* dairy breeds, but only the Friesian crosses were retained. A small nucleus Sahiwal herd was developed, including testing of Sahiwal sires with *Bos taurus* cross progeny. Progeny testing of AFS sires of F_2 or a higher generation commenced in 1976, also with selection for tick resistance. The AFS females were kept at the Kairi Research Station near Atherton, in Queensland, and with loan cooperating farmers. The cow population was more than 2000 strong in 1982. A MOET selection nucleus scheme was adopted at a later stage. A similar fast elimination of short lactations by selection was observed, as in the AMZ. However, such a high incidence of short lactations as found in the AMZ and AFS has not been found elsewhere, so this might have been inherent to the small sample in the *Bos indicus* founders of the Australian breeds.

Brazilian Milking Hybrid (MLB)

The Brazilian Milking Hybrid (MLB) (**Figure 5**) was the results of a research and development program conducted by the National Dairy Cattle Centre of the Federal Research Organization of Brazil (EMBRAPA), with the assistance of the Food and Agriculture Organization's United Nations Development Programme (FAO/UNDP). Its main objective was to obtain estimates of

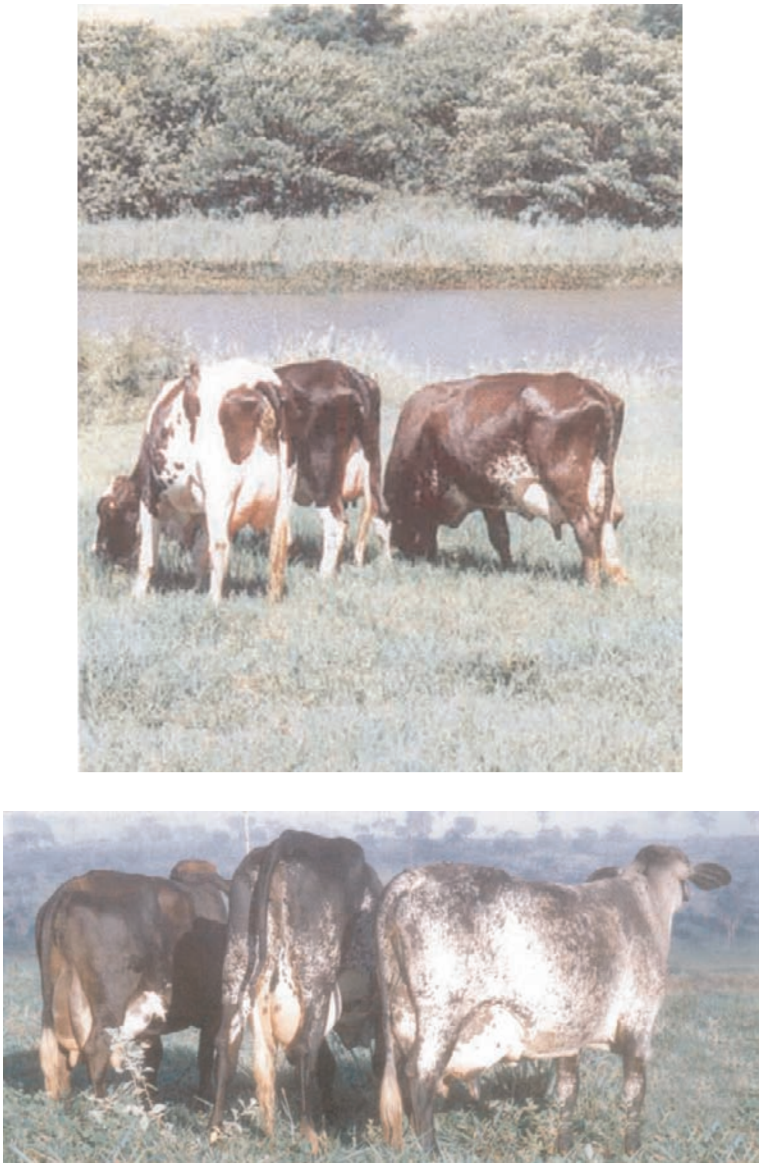


Figure 5 Brazilian Milking Hybrid (MLB) females. Courtesy of Beef Milk Brasil Marketing, Brazil.

heritabilities and genetic correlations on the dairy, reproduction, growth and adaptation traits needed to design breeding programs for synthetic dairy cattle breeds suitable for the dairy production systems of the Brazilian tropics. The program operated through the progeny-testing of approximately 10 young bulls per annum, selected for their genetic value for milk, irrespectively of breed composition, coat color or type. Thus, the population was a multibreed composite, reflecting the situation of the local cattle population of 7.5 million hybrid cows that varied widely in their *Bos taurus* and *Bos indicus* composition. The predominant breeds were the Holstein–Friesian, Gir and Guzera, but several other breeds were also represented in the gene pool. The program ceased after progeny-testing 121 bulls for milk yield and composition between 1977 and 1994. Results are being analyzed. The genetic correlations between tick burden and yields of milk, fat or protein were low (0.06 to −0.14) indicating that there is no important antagonism between yield and resistance.

Carora

Officially a breed since 1975, the Carora originated in 1935 from crosses of Brown Swiss and Criollo in a private farm in northeastern Venezuela. Other *Bos taurus* and *Bos indicus* breeds also had some influence in its formation, the later increasing its contribution over time. Carora animals have a coat color similar to the Brown Swiss. The breed is some 6000 animals strong and has a breed society responsible for registration and the conducting of a sire progeny-testing scheme with the assistance of the University of Milan.

Girolando

This commercial breed (**Figure 6**) is nominally composed of $\frac{5}{8}$ Holstein–Friesian × $\frac{3}{8}$ Gir, although, in practice, other breeds intervene in its foundation and the $\frac{5}{8}$ fraction has been somewhat relaxed. Animals are horned and usually black and white, with various color patterns, including mottled. The breed society is officially responsible for keeping the herd book and also commenced a small-scale sire progeny-testing program. It registers some 7000 animals of both sexes per year and semen sales amount to 44 000 doses per year.

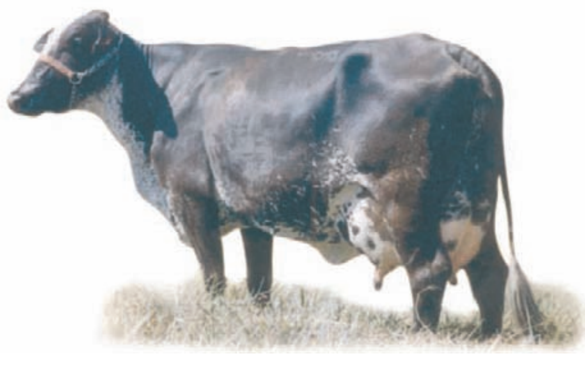


Figure 6 Girolando female. Courtesy of Beef Milk Brasil Marketing, Brazil.

Jamaica Hope

This was one of the earliest and most famous attempts to develop a tropical dairy cattle breed. The Government of Jamaica established a dairy herd at the Hope Farm, near Kingston, where several dairy breeds were tested (Ayrshire, Brown Swiss, Guernsey, Holstein–Friesian, Jersey and Red Poll and creole and zebu crosses). A Sahiwal bull was imported from India in 1920. Breeds were successively dropped. In 1943, the second phase was begun by importing Jersey bulls and using Jersey × Sahiwal crossbred bulls and cows of breed composition varying from $\frac{3}{4}$ to $\frac{1}{8}$ Jersey genes and testing sires on progeny performance. In 1952 the Holstein–Friesian breeding was terminated because grade Jerseys were considered superior in their culling rates, age at first calving and milk production per hectare. In 1950 the herd was moved to another farm, Bodles Station. In 1952 the breed received official status and a breed society was formed, including the Bodles Station herd and animals of similar genotype in private farms. The breed stabilized at about 80% Jersey inheritance, 15% Sahiwal and 5% Holstein–Friesian. A national milk-recording scheme was implemented. Selection was based on production performance and fertility, with no conscious selection for color or type. However, the program suffered from the lack of a larger population on which to test more young bulls with sufficient numbers of daughters. Some 50 breeders were involved but the enthusiasm was reported to be less than that required for stronger breed development.

Siboney

This $\frac{5}{8}$ Holstein × $\frac{3}{8}$ nondescript *Bos indicus* composite breed was developed under the national crossbreeding policy of Cuba. Some 7500 cows have been involved in this project. The breed development involved intensive selection of $\frac{1}{4}$ Holstein × $\frac{3}{4}$ *Bos indicus* females, inseminated with Holsteins of Canadian origin to produce the $\frac{5}{8}$ Holstein grades, which were then mated among themselves (*inter se*). A conventional sire progeny-testing program was superimposed onto this crossbreeding scheme. A related breed, the Mambi, *inter se* of $\frac{3}{4}$ Holstein × $\frac{1}{4}$ *Bos indicus* composition, was developed on similar lines.

Sunandini

A $\frac{5}{8}$ Brown Swiss × $\frac{3}{8}$ local *Bos indicus* was developed in Kerala State by an Indo-Swiss project started in 1963. Half-breds and $\frac{3}{4}$ Brown Swiss were crossed to produce the $\frac{5}{8}$ Brown Swiss crosses. The Brown Swiss base consisted of 33 sires and 45 cows. American Brown Swiss, Holstein and Jersey genes have also been introduced recently. The Indo–Swiss project evolved into the Kerala Livestock Development Board, which initiated a sire progeny-testing program in 1977. This required the development of field performance and pedigree recording. Some 4000–5000 animals are registered annually and milk quantity is recorded on about 2300 cows.

Bos indicus × *Bos taurus* Crosses

Breeds/Crosses and Production Systems

Bos taurus breeds, highly selected for milk yield in developed temperate countries, do not perform well under the prevailing production systems in the tropics, where they are unable to cope with the stresses of heat, humidity, parasites and low-quality forages, to the point of being unable to sustain their numbers. For example, in Venezuela, it was estimated that imported and locally born Holstein cows produced only 0.6 and 0.7 replacement females respectively in their lifetimes. *Bos indicus* breeds, on the other hand, while adapted to the environmental challenges, showed little response in milk yield to improved management. Thus, the main interest in crossing is to combine milk yield and adaptation.

Use of purebred *Bos taurus* has been advocated for dairy systems that use coolers and ponds to alleviate heat and freestalls or other types of buildings to keep the cattle indoors. However, these expensive systems are not generally economic in developing countries, where capital is scarce and unemployment severe, favoring systems with a lower input. Also, systems based on high concentrate consumption are not feasible if cows are competing with humans for cereals. On the other hand, improved systems based on pasture may be extremely efficient economically although, in these conditions, milk yield per cow is not as high as in temperate countries. As an example, a model private farm in Brazil, keeping F_1 Holstein × Guzera crosses on irrigated, fertilized pasture and feeding 3 kg concentrates day^{-1}, had a production cost US$0.08 l^{-1}, which is extremely low on a world basis. This result was due mainly to the high stocking rate of 4.2 cows ha^{-1} and to the low input in terms of machines, buildings and veterinary costs.

Solar radiation, which might be seen as a hindrance for dairy production, is in fact the main asset of tropical systems, because it allows intense photosynthesis and plant growth; however, to make good use of it, adapted animals are required.

Adaptation Traits

Bos indicus tolerates heat better than *Bos taurus*, and this is reflected in a lower rise of body temperature under hot conditions. This tolerance is due to the high heat resistance of their sleek, dense coat, which prevents heat gain from the environment; low tissue resistance to heat transfer from the body core to the surface; and high sweating competence. Humidity has no adverse effect on *Bos indicus* sweating rate, while water vapor trapped in the air spaces between the hairs of *Bos taurus* impedes evaporation. *Bos indicus* can also store heat during the day and then dissipate it nonevaporatively at night, thus reducing the need for watering.

Dry matter intake relative to body weight is higher in *Bos indicus* than in *Bos taurus* when coarse forages are fed, but the contrary holds for good-quality forages. The greater ability of *Bos indicus* to recycle urea to the rumen makes it less dependent on feed nitrogen. For *Bos indicus* net energy requirements for maintenance are lower than in *Bos taurus*, but requirements for growth are higher. *Bos indicus* is also highly resistant to parasites.

In *Bos taurus* × *Bos indicus* crosses of up to 50% *Bos taurus* gene fraction, resistance to environmental stresses approaches that of *Bos indicus*, but it is much reduced above that level of *Bos taurus* inheritance. For example, burdens of the tick *Boophilus microplus* increase exponentially with the *Bos taurus* fraction in the cross.

Survival, reproduction and herd-life are generally higher at intermediate gene fractions of both species. Some examples of the effects of crossbreeding on adaptation traits are shown in **Table 3**.

Milking Traits

The rate of milk flow decreases linearly with the *Bos indicus* gene fraction in crosses; this may be due to a tauter teat sphincter, because the ease of hand-milking follows a similar trend. The temperament of *Bos taurus* is better but crosses of up to $\frac{1}{2}$ *Bos indicus* are considered docile by milkers.

Table 3 Adaptation traits in Holstein–Friesian × Guzera crosses

	Holstein–Friesian gene fraction[a]					
	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{5}{8}$	$\frac{3}{4}$	$\frac{7}{8}$	$\geq\frac{31}{32}$
Boophilus microplus (ticks per animal)[b]	44	71	151	223	282	501
Dermatobia hominis (nodes per animal)[b]	4.2	4.3	3.9	8.8	7.3	8.4
Age at first calving (months)[c]	44.3	39.1	47.5	42.7	46.4	44.2
Heifer calf mortality to 1 year (%)[d]	12.4	8.0	18.7	8.9	13.9	21.1
Heifers died/22-month-old heifers (%)[e]	3.0	1.5	9.1	10.4	14.1	23.4
Cows died/22-month-old heifers (%)[e]	10.5	13.7	19.7	25.4	42.2	37.5
Females calving/22-month-old heifers (%)[e]	89.6	95.5	81.8	88.1	85.9	71.9
Number of lactations in 12 years[e]	3.8	6.0	3.6	4.5	3.7	3.2

[a] All crosses by purebred sires excepts for the $\frac{5}{8}$ obtained by *inter se* matings of sires and dams of that fraction.

[b] Madalena FE (1990) *Proceedings of the 4th World Congress on Genetics and Applied Livestock Production* 14: 310–319.

[c] Lemos AM, Madalena FE, Teodoro RL, Barbosa RT and Monteiro JBN (1992) *Brazilian Journal of Genetics* 15: 73–83.

[d] Based on 91–122 calves per group. Madalena FE, Teodoro RL and Lemos AM (1995) *Brazilian Journal of Genetics* 18: 215–220.

[e] Based on 64–67 heifers per group kept on commercial farms. Lemos AM, Teodoro RL and Madalena FE (1996) *Brazilian Journal of Genetics* 19: 259–264.

Short lactations are a major problem in tropical dairy production because of the consequent increase in the proportion of dry cows in the herd. Crosses with a high fraction of *Bos taurus* have a high incidence of short lactations caused by underfeeding and environmental stresses. Crosses with a high fraction of *Bos indicus* also have a high incidence of short lactations but these are genetically determined. Removal of the calf causes drying-off in these cows and gestation has a greater adverse effect on milk yield than in *Bos taurus* cows. Lactation length has a heritability of the same order of magnitude as milk yield in *Bos indicus* or *Bos taurus* × *Bos indicus* breeds and also displays important heterosis effects. In crosses with improved dairy *Bos indicus* breeds, such as the Gir or the Guzera, the F_1 generations usually show a lower incidence of short lactations when milked by hand, either with or without the calf stimulus for milk let-down, and also when machine-milked in the presence of the calf. Whether F_1 cows may be machine-milked without the presence of the calf is an open question, i.e. the proportion drying-off is not well documented nor is there consensual opinion among farmers in this respect, whereas it is accepted that *Bos taurus* grades of $\frac{3}{4}$ or higher have no problems. F_2 or other *inter se* crosses behave similarly to the high *Bos indicus* grades.

Dairy Performance

As may be seen in **Figure 7**, where milk yield per day of calving interval (MY/CI) from several studies is plotted against the *Bos taurus* gene proportion, F_1 crosses outperform the other grades in the lower performance levels, which are the commonest. Discrepancies in the slopes shown in **Figure 7** may be due to several factors, such as different editing-out of short lactations, or long CI, and to different breeds/samples being represented within both species in the various studies. However, all studies but one (Kenya) in **Figure 7** were conducted with Holstein–Friesian and all used improved *Bos indicus* breeds (Sahiwal, Gir or Guzera).

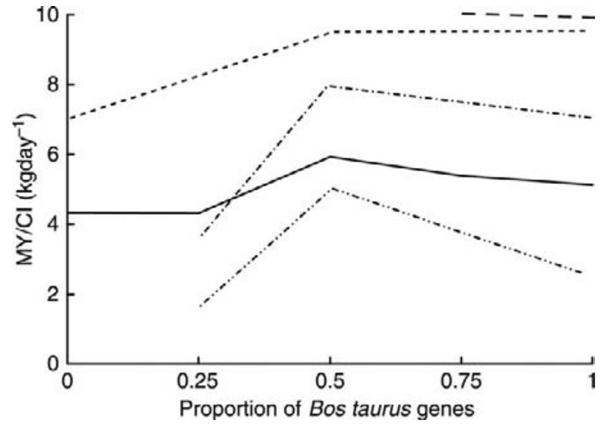


Figure 7 Milk yield per day of calving interval (MY/CI) of *Bos taurus* × *Bos indicus* hybrids by purebred sires in different tropical countries/managements. -..-. Brazil-1; -.-.- Brazil-2; - - Brazil-3; --- India; ---- Kenya. (Brazil-1 and -2: Madalena FM, Lemos AM, Teodoro RL, Barbosa RT and Monteiro (1990) *Journal of Dairy Science* 73: 1872–1886; Brazil-3: Madalena FE, Valente J, Teodoro RL and Monteiro JBN (1983) *Pesquisa Agropecuaria Brasiliera* 18: 195–200; India: Katpatal BG (1977) *World Animal Review* 22: 14–40; Kenya: Mackinnnon MJ, Thorpe W and Baker RL (1996) *Animal Science* 62: 5–16.)

As performance increases, the high and *Bos taurus* grades reach F_1 level, there are no differences among those grades at a level of approximately 10 kg MY/CI (**Figure 7**). At higher levels of MY/CI, the purebred *Bos taurus* outperformed the crosses in early studies in the southeastern United States.

Figure 7 also shows the genetic limitation for milk production of high *Bos indicus* grades, as their response to improved management is less than that observed for *Bos taurus*. This genetic × environment interaction was demonstrated in the Brazilian trial, where crosses with the same origin were evaluated in farms of high and low management (**Figure 7**, Brazil-1 and Brazil-2).

Heterosis and Profit

Favorable heterosis is present in most traits of economic importance in *Bos taurus* × *Bos indicus* crosses. As a result, when the receipts and costs associated with fat and protein yield, salvage value, survival, age at first calving, herd life, feed consumption, milking time and other costs are combined in a profit function, the superiority of the F_1 cross is enhanced over that shown in **Figure 7**, because the effects of heterosis accumulate over traits, particularly under low inputs. For example, in the commercial farms of the Brazilian trial, net profit per day of herd-life in the $\frac{1}{4}$, F_1 and $\geq \frac{31}{32}$ Holstein–Friesian groups was equivalent to 1.7, 4.4 and −1.3 kg of milk, respectively. Supporting results have been obtained elsewhere.

Heterosis is partially lost in *inter se* crosses of hybrid sires and dams. A review of 14 studies showed that the mean difference of F_2 minus F_1, expressed as a percentage of F_1 performance, was 7.0%, 5.8%, −24.4% and −3.6% for age at first calving, calving interval, milk yield and lactation length, respectively. However, the differences in the last two traits are underestimated in studies that exclude short lactations from the analysis on the grounds of them being 'abnormal'. Under the commercial conditions in the Brazilian trial (**Figure 7**, Brazil-1), the MY/CI of the $\frac{5}{8}$ *inter se* group was only 56% of the same trait in the F_1. One quarter of that difference was attributed to recombination loss of parental epistatic combinations.

Because heterosis is wasted in *inter se* crosses also for other economic traits (e.g. **Table 3**), the overall decline in profit with respect to the F_1 is high. As an example, the profit of the $\frac{5}{8}$ *inter se* under commercial conditions in the Brazilian trial was only 30% of that of the F_1.

Crossbreeding Strategies

In several tropical regions, farmers have been keeping their herds intermediate between *Bos taurus* and *Bos indicus* for decades by *ad hoc* methods. In Latin America, these involve periodical switching of the bull species, generating less productive, extreme *Bos indicus* and *Bos taurus* grades, or using common hybrid bulls that are not properly selected.

Given its economic importance, exploitation of heterosis is the major genetic consideration in strategies for maintaining hybrid *Bos taurus* × *Bos indicus* populations. A system of continuous replacement with F_1 females maximizes the economic use of heterosis. Such a system is being applied commercially in Brazil by beef cattle-type ranches, specializing in the production of F_1 heifers for sale, who use artificial insemination of *Bos indicus* dams. In Colombia *Bos taurus* females in the highlands are inseminated with *Bos indicus* semen to produce F_1 heifers which are sold to lowland farms. A similar cross is carried out in New Zealand for export to tropical countries.

The F_1 system requires the maintenance of large numbers of females of at least one of the purebred parental breeds, so that it is justifiable to exploit the breed economically, as in the situations mentioned above. Production of F_1 replacements is facilitated by generalization of embryo transfer techniques involving *in vitro* fertilization. Such a system has been announced by a prominent international breeding company. Semen-sexing would much enhance the economic value of this scheme.

Rotational crossing would be second to F_1 in genetic terms, as recombination loss is less than in *inter se* crosses. However, low usage of artificial insemination or controlled mating prevents wide use of this system. Moreover, many farms in tropical systems would not be large enough to justify keeping two bulls.

Using a hybrid bull is most practical but involves loss of heterosis. Counteracting selection would alleviate this, at least for milk yield, although mortality and fertility are unlikely to be effectively improved by selection. In any case, this option would require effective breed development programs run on a much larger scale of investment than that applied up to now.

See also: **Animals that Produce Dairy Foods:** Minor and Dual-Purpose Bos Taurus Breeds. **Dairy Production in Diverse Regions:** Africa; Latin America; Southern Asia. **Gamete and Embryo Technology:** Multiple Ovulation and Embryo Transfer. **Genetics:** Selection: Concepts; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. **Mammals**. **Stress in Dairy Animals:** Heat Stress: Effects on Milk Production and Composition; Heat Stress: Effects on Reproduction.

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Goat Breeds

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Introduction

Goats are widely distributed globally and are well adapted to tropical and temperate, arid and humid, cold and hot, and steep mountainous environments. They are valued for many different products and purposes in different parts of the world: for meat, milk, cheese, yogurt, mohair, cashmere, leather, manure, draft, nutritional and household security, brush clearing, and recreation. Some goat breeds like the Angora are valued for the fine mohair fiber, other breeds for the precious cashmere and pashmina fiber, and the Barbari is known for meat and skin, and has evolved and been bred for this purpose. The developing world is the home of about 97% of the total world population of about 782 million goats.

It is estimated that out of a total of 570 goat breeds, only 69 are single-purpose dairy types. Europe accounts for about 2% of the population, but has a sizeable number of 187 breeds, many of which are high-producing dairy breeds and have been introduced for use in other countries. Within the 69 dairy breeds, 36 breeds (about 52%) originated in Europe, followed by 25 breeds (37%) in Asia and 8 breeds (11%) in Africa. In the developing countries, the majority of goat breeds are dual-purpose breeds used mainly for meat production plus milk at a medium to low level. However, it is probable that there are even more dairy animals since dual-purpose types are variable and have often not been adequately described.

The description of indigenous goat breeds outside of Europe and their potential has not been extensive nor is it based on organized breed registries. However, many of the recognized breeds are important because of their adaptation to difficult climates, sparse feeds and water resources, and various diseases. Due to efficient breeding and management, and a more favorable climate, goats in temperate climate regions are exceeding most others in productivity, especially in milk and dairy products. For these reasons, many breeds have been exported to developing countries for upgrading their indigenous breeds through crossbreeding.

This article begins with the description of leading dairy breeds in terms of milk yield, following which dual-purpose breeds are described in the order of regions of their origin. Goat breeds have been defined by breed standards and descriptions of differences in color, ear size and type, horn size and type, face type, hair coat length, beard, wattles, body weight, and height in adult males and females. The treatment has been done in regional terms to be consistent with the area of origin and climate as well as to provide an indication of their potential. The potential of many of these breeds, with the exception of a few, seems to be not fully explored. The descriptions and tables include dairy breeds as well as dual-purpose breeds (meat and milk types). The information is drawn from key references and published proceedings of the last six international goat conferences. **Table 1** summarizes information on the different types of dairy goat breeds in terms of high and medium milk yields and country of origin including dual-purpose types. The majority of these breeds are important 'improver breeds' and are potentially useful for increasing productivity. **Table 2** summarizes approximate performance data of the different dairy goat breeds in the tropics and subtropics with indications of seasonal versus nonseasonal breeding. Performance data of goat breeds in the temperate regions are given in the breed descriptions of Europe and North America, along with some indication of their potential usefulness in improving other breeds.

Northern and Central Europe

Dairy goat populations in northern and central Europe consist mostly of well-organized breeds, while they are less well defined in southern Europe. Through centuries of select breeding in northern and especially central Europe, these dairy goat breeds have distinguished characteristics of capacious body type, well-formed and firmly attached udders, strong legs and feet, unique colors, high milk productivity, and long lactations. Their milk is sold and consumed as fluid milk, made into popular yogurt, various highly acclaimed soft and aged hard cheeses, or mixed legally, if labeled, in different proportions with sheep or cow milk in yogurt and cheese production. Especially, the dairy goat breeds developed in Switzerland have become the world's leading milk producers and for that reason have been exported to many other countries of Europe, Israel, North America, Russia, Japan, China, Australia, New Zealand, Taiwan, Mexico, and so on, where they have continued as distinct Swiss breeds, often under new domestic names, and are used for the improvement of native breeds in developing countries. In parts

Table 1 Dairy goat breeds

Specialty	*Breed*	*Country of origin*
High yield	Alpine	Switzerland; temperate, wet
	Anglo-Nubian	UK; temperate, wet
	Appenzell	Switzerland; temperate, wet
	Chamoisee	Switzerland; temperate, wet
	Malaguena	Spain; subtropical, dry
	Murciana-Granadina	Spain; subtropical, dry
	Nordic	France; temperate, wet
	Poitevine	Norway; temperate, wet
	Saanen	Switzerland; temperate, wet
	Sable	USA; temperate, wet
	Toggenburg	Switzerland; temperate, wet
Medium yield	Bach Thao	Vietnam; tropical, dry
	Barbari	India; tropical, dry
	Beetal	India; tropical, dry
	Black Bedouin	Israel and Egypt; tropical, very dry
	Bujiri	Pakistan; subtropical, dry
	Damani	Pakistan; tropical, dry
	Damascus	Syria; subtropical, dry
	Dera Din Panah	Pakistan; tropical, dry
	Jamnapari	India; tropical/subtropical, dry
	Jakhrana	India; tropical, dry
	Jarakhell	Pakistan; subtropical, dry
	Jattan	Pakistan; subtropical, dry
	Kacchan	Pakistan; subtropical, dry
	Kamori	Pakistan; subtropical, dry
	Kilis	Pakistan; subtropical, wet
	Koh-i-Ghizer	Turkey; subtropical, dry
	Labri	Pakistan; subtropical, wet
	Maltese	Malta; subtropical, dry
	Pateri	Pakistan; tropical, dry
	Sahel	West Africa; tropical, humid
	Red Sokoto	Niger and Nigeria; tropical, humid
	Shurri	Pakistan; subtropical, wet
	Sudanese Nubian	Egypt and Sudan; tropical, dry
	Zaraibi	Egypt; tropical, dry
Dual purpose		
	Boer	South Africa; tropical, dry
	Chapper	Pakistan; tropical, dry
	Chegu	India; subtropical/tropical, dry
	Criollo	South America; tropical, humid
	Gaddi	India and Pakistan; tropical, dry
	Kajli	Pakistan; tropical, humid
	Khurasani	Pakistan; tropical, humid
	Malabar	India; tropical, humid
	Ma T'ou	China; subtropical, humid
	Nigerian Dwarf	Nigeria; tropical, humid

Note: This table needs unscrambling and re-aligning in alphabetical order, besides adding missing breeds.

of Europe and other countries, they have even displaced indigenous breeds.

Saanen

The Saanen (**Figure 1**) is widely recognized as the world's best-developed and highest milk producing breed. The Saanen goats are often called Holstein Friesian and compared among goat breeds to the Holstein Friesian dairy cattle breed, because of their high level of daily milk yield and relatively low level of milk fat content. The Saanen goats derived their name from the Saanen valley of central Switzerland, where they originated. The breed has completely white, short hair with occasional black spots on the udder, ears, and nose. They have been bred for polledness, but horned goats are also used, because of less problems with infertility. The ears are erect and of medium length and they point forward. The face is straight. Horns are saber shaped and point backward.

Table 2 Milk yield and lactation length of dairy and dual-purpose goat breeds from tropical and subtropical countries

Breed	*Country*	*Lactation yield (kg)*	*Daily yield (kg)*	*Lactation length (days)*
Tropics and subtropics: Nonseasonal breeders				
Barbari	India, Pakistan	150–228	1–6	180–252
Black Bedouin	Israel		1.3–2.0	
Boer	South Africa		1.3–1.8	
Chapper	Pakistan	75	0.7	105
Chegu	India	40	0.4	100–110
Criollo	Venezuela	60	0.5	
Damani	Pakistan	104	1.0–1.2	105
Dera Din Panah	Pakistan	200	1.5	130
Jakhrana	India	122	1.0–1.2	115
Kamori	Pakistan	228	1.8–2.2	120
Maradi	Niger	75	0.5–1.5	100
Nubian (Sudanese)	Sudan, Egypt	70	1.0–2.0	
Seasonal breeders				
Beetal	India, Pakistan	140–228	1.2	208
Damascus	Cyprus	500–560	2.0	190–290
Gaddi	India, Pakistan	40–50	0.8	90–290
Jamnapari	India	200–562	1.5–3.5	170–200
Kilis	Turkey	260	1.0	260
Malabari	India	100–200	1.0	181–210
Mamber	Israel	350–450	1.5	
Marwari	India	90	0.9	106
Najd	Iran	250	1.0	150
Sirohi	India	116	0.9	134

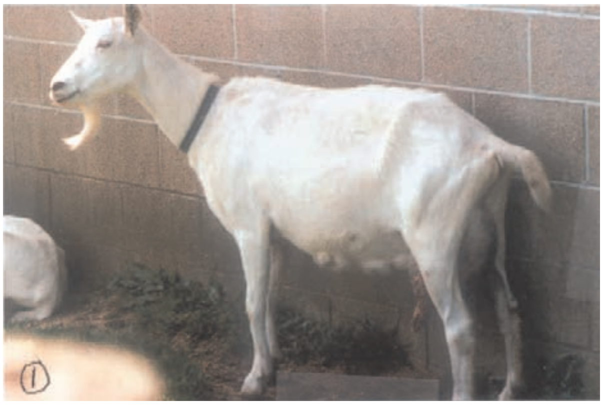

Figure 1 Saanen (USA). Photo: G. Haenlein.

Wattles, two little unique external appendices of no known function, are common, as are beards. The average expected body weight of adult males is 90 kg and that of females 65 kg. The average height at withers is 90 cm for adult males and 80 cm for females. The Saanen breed has been most widely exported around the world and has become established as French Saanen, Weisse Deutsche Edelziege (German Improved White), British Saanen, Dutch White Goat, Polish Improved White, Bulgarian White, Banat White, Improved North Russian White, and so on. Annual milk production ranges from 300 to 2000 kg in 150–300 days of lactation, depending on the country, averaging 1285 kg with 3.5% fat and 3.4% protein in 301 days in France for dams in the year 2000 and above 975 kg in other leading countries.

Alpine

This mountain goat breed from Switzerland is the other main dairy breed in terms of milk productivity and distribution, but it has many colors and color types, which led to the formation of distinctly separate breeds, the French or Italian Alpine or the American Oberhasli or Bunte Deutsche Edelziege (German Fawn), and the American Alpine or the Swiss Chamoisee in their respective countries.

Oberhasli

The French Alpine and the American Oberhasli (**Figure 2**) are mostly 'chamoisee' colored, that is, they are solid brown in color with black dorsal stripe, on face, belly, feet, and legs. They have short hair, straight face, erect ears of less than medium length, beards, wattles, and if horned of the saber type pointing backward. The body weight of males is 75 kg and that of females 50 kg; the height at withers is 80 and 65 cm for males and females, respectively. The name Oberhasli is derived from the Swiss Oberhasli Brienz strain of the Chamoisee. The French Alpine is the main dairy goat breed besides the

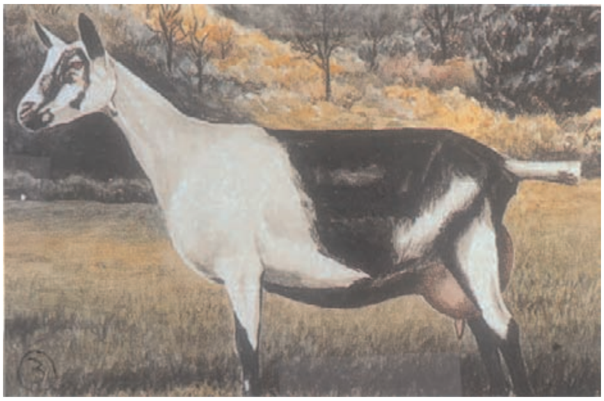

Figure 2 American Alpine (Chamoisee). Courtesy of American Dairy Goat Association.

Saanen in France and in French-speaking Mediterranean countries. Milk production of registered French Alpine dams in France averaged 1109 kg with 3.9% fat and 3.6% protein in 295 days in the year 2000.

Chamoisee

The Chamoisee and the American Alpine (**Figure 3**) are mostly of a unique color combination and patterns of white, gray, and black, the so-called cou clair with front quarters black or white and shading to gray and black hindquarters. Other color pattern variations of black, gray, and white ('cou blanc', 'sundgau', and 'pied') are also found. The other breed characteristics are the same as for Oberhasli, except that they are heavier, with males weighing 80 kg and females 60 kg; males are 80 cm in height and females 75 cm. The breed's wide distribution as Alpine around the world is due to its high milk producing reputation next to the Saanen. Milk production of the United States recorded goats averaged 950 kg in 270 days, but in other countries it is less.

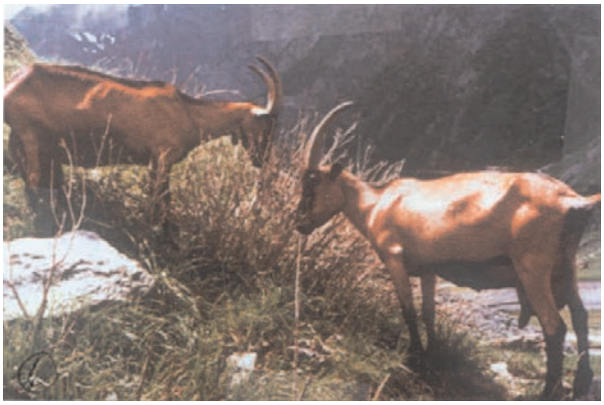

Figure 3 American Oberhasli (French Alpine). Photo: G. Haenlein.

Toggenburg

The Toggenburg (**Figure 4**) is one of the main Swiss dairy breeds from the Toggenburg valley. The hair coat may be short or long, as valued for rough climates. The color is brown to gray, with legs and the area around the base of the tail white in color. The belly may be white. The face is straight with two unique white stripes from the muzzle to the eyes and poll, called 'badger' face, which is a simple recessive genetic trait. The erect ears are less than of medium length. The breed is polled. Males weigh 70 kg and females 55 kg, with heights of 75 and 70 cm, respectively. Because of their high milk yield, Toggenburgs have also been exported next to Saanen and Alpines. Milk production of recorded goats was 650 kg in 270 days.

Minor Swiss Breeds

Dairy goat breeds with low numbers in their native Switzerland and insignificant distribution in other countries are the Appenzell, the Buendner Strahlen Goat, the Black Verzasca, and the Valais Blackneck.

The Appenzell resembles the Saanen except for long hair, slightly smaller size, and mostly polled nature.

The Buendner has scimitar-shaped horns that are turned outward. The breeds are black in color, with white limited to muzzle, area around the eyes and ears, area under the tail, and legs below the knees. The face is straight and the ears are relatively short and erect.

The Verzasca also has scimitar-shaped horns twisting outward. The black hair is of medium length. They have straight face, erect ears, beards, and tufts on the forehead.

The Valais Blackneck (**Figure 5**) is more a dual-purpose breed with long hair. The face is straight, ears are erect, and horns are saber shaped and bent backward. Their unique coat color pattern is black front quarters and white hindquarters. This and the other three breeds are relatively small. Their milk production level is medium around 380–400 kg in 200 days, except for the Appenzell

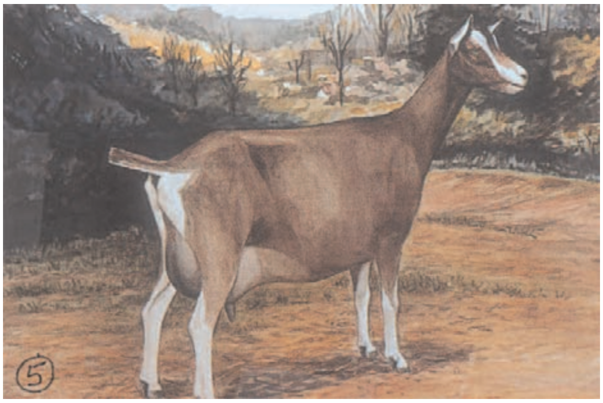

Figure 4 Toggenburg (USA). Courtesy of American Dairy Goat Association.

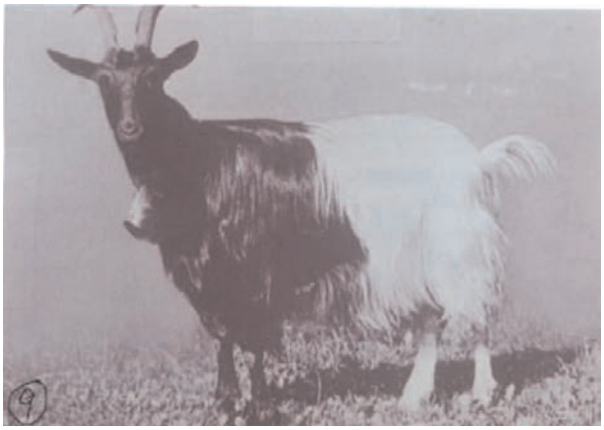

Figure 5 Valais Blackneck (Switzerland). Photo: G. Haenlein.

with 670 kg in 265 days. They are well adapted to high mountain grazing and harsh winter and summer temperatures. It is interesting for genetic resource conservation that the Appenzell, Black Verzasca, and Valais Blackneck breeds are supposed to be nonsusceptible to infectious arthritis or periarthritis.

Anglo-Nubian

The Anglo-Nubian (**Figure 6**) was derived from crossing Indian Jamnapari and Egyptian Zaraibi with British native goats and is sometimes called dual purpose. In the United States, it is known simply as Nubian and is recognized as a single-purpose dairy breed. It has more fleshiness than the Swiss breeds, but has not been used to supply the US goat meat market more than the Swiss goats, and less than the nondairy breeds the Spanish brush goats, the Angoras, and Boer goats. This breed is more long legged than the Swiss breeds and is tall. It has short silky hair with many colors, patterns, roan and white spotting; also solid black and all shades of red and brown can be seen. The breed is mostly polled, but spiral horns may be found. The face has a uniquely high arched Roman nose, often with a shortened upper jaw (brachygnathia), which is an undesirable recessive genetic trait, against which selection is practiced. The ears are hanging pendulous and are very long. The udder has good ground clearance, but is not nearly as well developed and attached as in the Swiss breeds. Nubians are the most numerous dairy goat breed in the United States and are known for high milk production, but with a much higher milk fat content than the Swiss breeds. Therefore, they are often called 'Jersey of the milk goats' and compared among goat breeds to the Jersey dairy cattle breed. Males weigh about 85 kg and females 65 kg, with an adult height at withers for males of 85 cm and for females 80 cm. The breed has been exported widely to tropical countries such as Central and South America, Philippines, Malaysia, and Africa, because of the breed's original adaptation to that kind of environment. Milk production of US Nubians averaged 770 kg in 270 days, but is less in other countries.

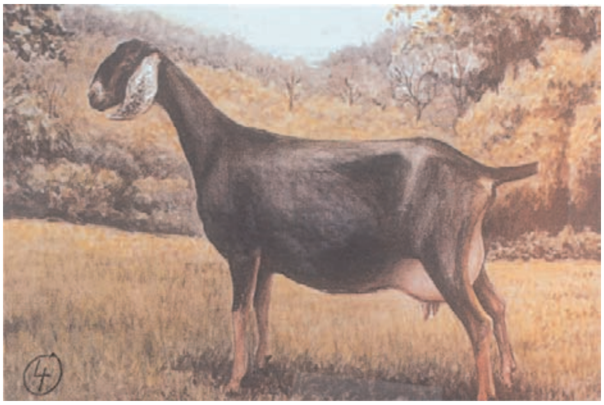

Figure 6 American Nubian (Anglo-Nubian). Courtesy of American Dairy Goat Association.

Poitevine

This French breed is of more recent origin. It is well adapted to hardy conditions of the mountains and to the subtropical Mediterranean basin including the island of Corsica. It is of medium size with brown to black short hair except for white bellies and legs, and can be white below the tail. White markings on the head similar to Toggenburg patterns are also found, but they tend to disappear with age. Horns may be absent. This breed makes up about 3% of the total French dairy goat population, with French Alpine about 70% and Saanen 20%. However, they all excel in high milk production and are an important, very well-organized part of the French dairy industry and economy. Milk production of the Poitevine breed averaged 440 kg in 230 days.

Nordic

In Norway, Sweden, and Finland, the native goats of various strains and of long-time breeding without any outside influence have become a uniform breed under the name Nordic. They are long haired of many colors but mostly brown. They are short legged, with straight face, erect ears, saber-shaped horns or polled, and are of medium size. Annual milk production in the range of 600–700 kg has been reported.

North America

LaMancha

The officially recognized and well-organized dairy breeds in the United States and Canada with purebred breed registries, pedigrees, milk performance records, sire proving, and type classification records are the Alpine, LaMancha, Nigerian Dwarf, Nubian, Oberhasli, Saanen, Sable, and Toggenburg. The dairy goat breed developed in the United States, specifically in California, from imported Spanish Murciana-Granadina and crosses with US Swiss and Nubian breeds, is the medium-sized LaMancha (**Figure 7**). Its unique breed characteristic is the near absence of external ears, which can also be found in African and Near-East goats. This trait is genetically dominant to normal ears. Two types are found. The 'gopher' ear is an extremely short appendage, with no auricle and almost without cartilage. The 'elf' ear has some cartilage and can be up to 5 cm long. Males must have gopher ears to be eligible for registration. The face is straight. The hair is short and of many colors and patterns. The breed is polled, and since this is a recessive genetic trait, no horned LaMancha goats will be found. This breed has a reputation for docility and hardiness. American and Canadian dairy goats have been widely exported as improver breeds. Milk production records in recent years averaged 800 kg in 250 days.

Sable

This breed evolved from the Saanen goats, but has some color, which breeds true and has been recently recognized as a separate and official breed in the United States. They are high milk producers similar to the Saanen. The present record holder is Sable # 1 253 232, at age 2 years 11 months producing 1923 kg milk with 3.1% fat and 2.8% protein in 305 days in 2005.

Nigerian Dwarf

As early as 1918, West African Dwarf (WAD) goats, originally also known as 'pygmies', were imported into the United States. A majority are brown, black, or gold with or without white markings. They are stocky with short legs and short, wide, straight face. The ears are medium sized and erect or horizontally carried. Horns are twisted outward and backward in males, but are more slender in females. Polled goats are also found. The hair is short. They are very prolific with 65% multiple births. They weigh between 20 and 25 kg and males are 40–50 cm high at the withers and females 30 cm. They are widespread in 15 countries of the west African humid zone, numbering about 38 million head. They are important for their immunity to the fatal trypanosomiasis disease. A US herdbook was started in 1984 by the American Goat Society (AGS). By 2008, the AGS had registered more than 41 000 US goats as Nigerian Dwarfs. They are mainly nonseasonal breeders and many have lactations more than 1 year long. Official tests have shown peak milk production averaging 1.6 kg day^{-1} and 340 kg milk with 6.5% fat and 3.9% protein in 305-day-long lactations. The present record holder is # 1 360 584, at age 3 years 10 months producing 782 kg milk with 5.3% fat and 3.7% protein in 305 days in 2006.

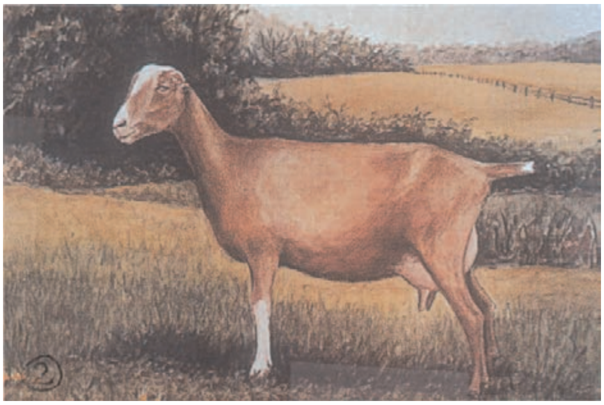

Figure 7 LaMancha: View of typical 'gopher' ear, compared to normal erect ear of Saanen and hanging lop ear of Nubian in background (USA). Photo: G. Haenlein.

Mediterranean Region

This region is rich in goat and sheep milk production compared to the rest of the world. Among 10 countries surrounding the Mediterranean Sea, Portugal, Spain, France, Italy, and Greece have the most well-organized goat breeds, milked mainly for cheese production. This region produces about one-fifth of the world goat milk production.

Girgentana

This Italian medium-sized and medium-level milk producing breed has very unique corkscrew type horns, which point vertically upward about 70 cm in males and 50 cm in females. The face is slightly convex and ears are of medium length and mainly horizontal. Males have a beard. The white hair coat is long and coarse, but ears and neck may be red or brown. Milk production is about 350 kg in 210 days.

Ionica

This Italian breed of medium size has good level of milk production and is mostly kept in smaller herds semi-intensively. It has long lop ears, is polled, and has long white hair. Milk production is around 380 kg in 210 days.

Garganica

Garganica (**Figure 8**) is an Italian mainly dual-purpose breed and it is kept for milk and meat production. It is a small, hardy breed with long black hair. The kids are born

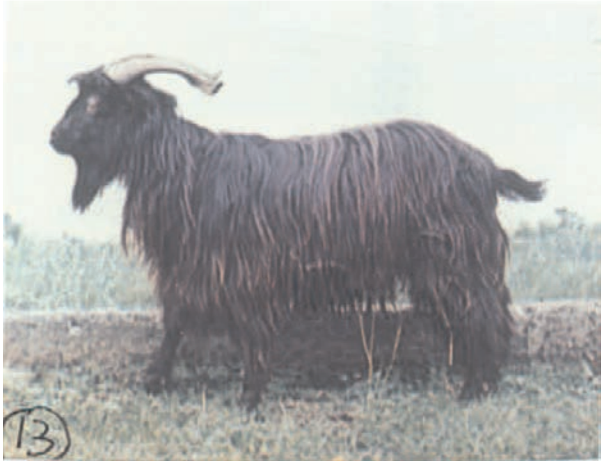

Figure 8 Garganica (Italy). Photo: G. Haenlein.

with curly hair and their skin is used for fur production. Their face is straight, and they have beards and long horns, which are flat and twisted backward. The ears are long and often carried horizontally. Milk production is about 210 kg in 190 days.

Sarda

This numerous indigenous breed on the island of Sardinia is a well-adapted breed and is managed semiextensively in larger herds. The breed has many colors, but is mostly white and gray. The ears are of medium length and are mainly horizontal. The breed has horned and polled goats. Milk production has been reported to be about 180 kg in 190 days.

Maltese

Originating from the island of Malta, the Maltese (**Figure 9**) is widely found in Italy and also in other Mediterranean countries and in urban environments. Males weigh about 65 kg and females 45 kg, with heights of 80 and 70 cm, respectively. They have short or long hair of various colors, but in Italy they have white long hair. The ears are pendulous and long with tips pointing forward. The face is straight. The breed is mostly polled, but thin, sickle-shaped horns are also found. Wattles and beards are common. Milk production averaged 300 kg in 200 days but can be as high as 900 kg.

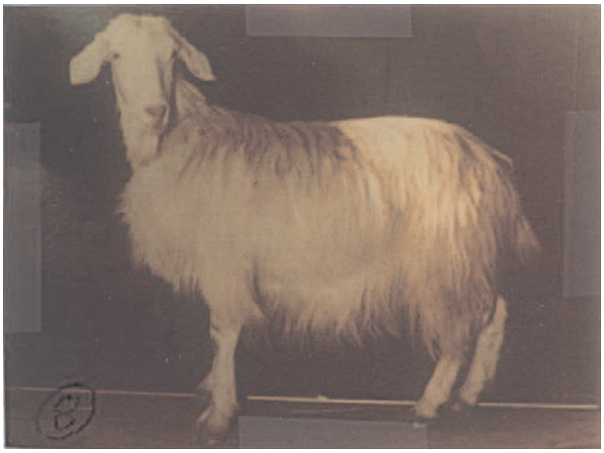

Figure 9 Maltese (Italy). Photo: G. Haenlein.

Canaria

This breed is one of several Spanish breeds known for high milk levels. They are mainly found on the Canary Islands and are managed intensively for milk production, but have not been selected for good udder conformation. They have many colors and patterns with mainly short hair. The ears are of different lengths, horns have different shapes, and the face may be straight or convex. The breed is of medium size. Milk production has been reported to be between 400 and 700 kg in 240 days but can be as much as 1200 kg in 300 days.

Malaguena

This Spanish breed is of medium size and has light red-colored short hair (**Figure 10**). The ears are long and horizontal. The face is mostly straight. Horns are saber shaped, long, and pointing backward, but polled goats are also found. Males and some females have beards. Milk production has averaged 600 kg in 260 days.

Murciana-Granadina

This Spanish breed (**Figure 11**) is a combination of two types, the mahogany-colored Murciana and the black Granadina. They have been widely exported to Central and South America because of their prolificacy, high milk production, and ability to breed at any time of the year.

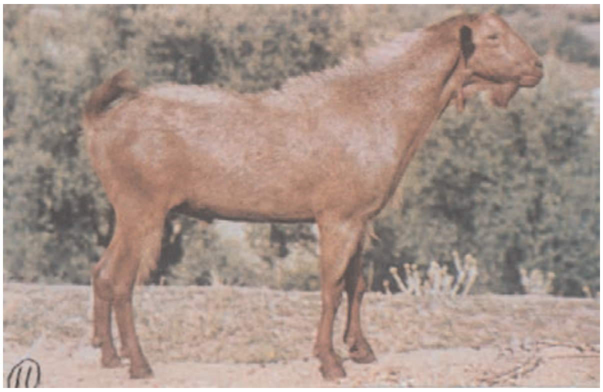

Figure 10 Malaguena male (Spain). Photo: G. Haenlein.

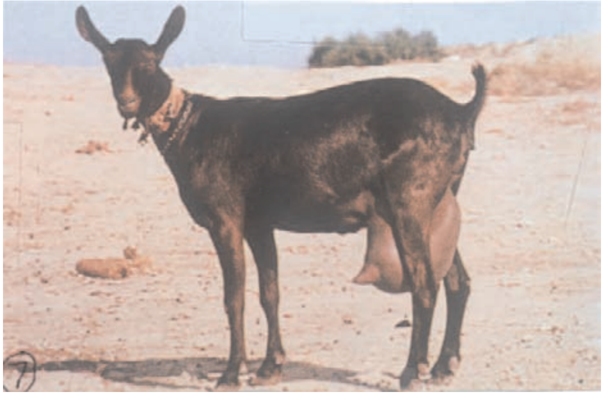

Figure 11 Murciana-Granadina: View of typical large but pendulous udders (Spain). Photo: G. Haenlein.

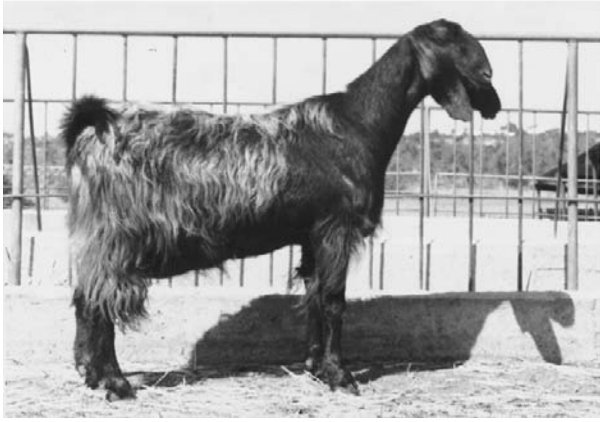

Figure 12 Damascus (Egypt). Photo: G. Haenlein.

Their face is straight or slightly concave. The ears are erect and small. Horns may be present. Males have a beard, which is a sex-linked dominant trait. Milk production is between 500 and 730 kg in 210 days but can be 1275 kg in 300 days.

Serrana

This numerous, medium-sized Portuguese breed is mostly kept in small herds. It has black or reddish-brown long hair. The ears are small and horizontal. The face is slightly concave. Horns, beard, and wattles are common. Their milk production is about 350 kg in 250 days.

Greek Native

The majority of Greek goats are of the Native breed with diversely colored long hair, but mostly black, and with different types of horns. Their face is straight and ears are horizontal or slightly hanging. These goats make an important contribution to the Greek dairy industry and economy, producing from over 6 million head over 500 000 tonnes of milk annually, which is one-third of the milk produced in Greece and 6% of the world goat milk production. The breed is well adapted to the mountainous conditions of the Balkan countries and traditionally has been managed with transhumance grazing of large herds mixed with dairy sheep. The average milk production under extensive conditions in the mountains is 120 kg in 210 days.

Damascus

The Damascus (**Figure 12**) is the most important dairy goat breed of the eastern Mediterranean countries. It is a tall breed with Roman nose, pendulous, long ears, and long shaggy hair mostly reddish brown in color. Horns, if present, are fairly long, twisted outward and backward, while in females they may be sickle shaped. At Cyprus, they are bred polled. Wattles are common. The breed is known to be quite prolific with 70% multiple birth. The average body weight of males is 85 kg and that of females 65 kg, with a height of 85 and 75 cm, respectively. The breed is known for its long lactations. Milk production has been reported from different countries to be between 250 and 560 kg in 220–300 days.

Zaraibi

This breed is also called Egyptian Nubian and has been part of the development of the Anglo-Nubian breed. It is mostly kept in small herds, tethered or stabled. The breed has long legs, is medium sized, and has Roman nose. The ears are long and lopped. Horns, if present, are short and curved backward. They are diverse in color, mainly black with white or brown spots. Hair is of medium length and often long on hindquarters. A related breed is the Sudanese Nubian with similar type. These Nubian breeds are of importance as milk and meat providers to the rural populations. Breeding for longer lactation length and higher milk yields is needed. Milk production is around 75 kg in 150 days.

Barki

This small breed is also called Black Bedouin and is mainly kept by Bedouins in the deserts of Sinai, Syria, Israel, Egypt, Jordan, and Somalia. The hair is long and black with some white spots on the head and legs. Horns are of medium length and scimitar shaped. The ears are long and horizontal. The breed is dual purpose and well adapted to the harsh desert conditions with poor feed and sparse water. They have low milk production levels of less than 100 kg in less than 100 days.

Kilis

This breed originated in Turkey and was derived from crossbreeding the Damascus and Anatolian Black goats. They are usually black with some gray. They have long hair, lop ears, and may be horned. Their udder is well developed and they are good milk producers. Adult females weigh about 50 kg. They are usually managed in small herds. Their milk production has been reported to be between 280 and 330 kg in 220–260 days.

Minor Breeds

There are several dairy goat breeds in the Mediterranean countries of north Africa and the Near East, known as the Egypt Baladi, the Lybian, Berber, Mamber, and Negev goats. They are mostly of small size, have convex faces, lopping or horizontal ears, long black hair, horns of varying size and shape, and are dual-purpose breeds with short lactations and low-level milk production, except for the Mamber, which can yield between 120 and 240 kg in 210 days under improved conditions.

Oceania

Australia and New Zealand have imported Swiss and Anglo-Nubian breeds and developed them to outstanding levels of milk production. In recent years, they have also developed the Boer breed, originally imported from South Africa. However, there are also many thousand feral goats, which are collected regularly for meat exports.

Asia

This vast region has many distinct indigenous breeds of various sizes, mostly dual purpose, therefore of medium or low milk productivity, and they are often named for their principal area. Many breeds are from India and Pakistan and are kept for nutritional and food security.

Jamnapari

A large, long-legged Indian breed, the Jamnapari (**Figure 13**) is known for fairly high milk production and is widely distributed in Asia. It is one of the ancestors of the Anglo-Nubian and is called Etawah in Indonesia. The breed originated in the Uttar Pradesh and Madhya Pradesh states near the Jamna river. It has extreme Roman nose with often undershot upper jaws (brachygnathia), which is a recessive genetic trait, and makes the goats prefer browsing over grazing. The ears are very long and pendulous, covering the eyes on grazing, which is another reason for their preference of tree and bush lopping. They may have mixed colors, but white dominates. They have long hair only on their hind legs. The short horns are scimitar shaped. Males weigh about 50 kg and are 78 cm high at withers, and females weigh 40 kg and are 75 cm high. Milk production has been reported to average 210 kg in 260 days.

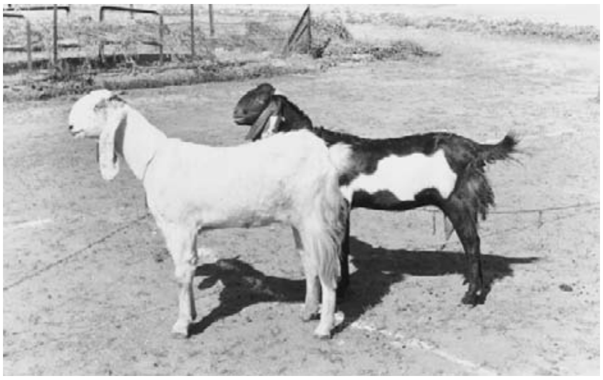

Figure 13 Jamnapari (left) and Beetal does in Agra, India. Photo: C. Devendra. Reproduced with permission from C. Devendra.

Beetal

This Indian dairy breed was derived from the Jamnapari and is widely distributed in the Punjab, Haryana, and Uttar Pradesh states (**Figure 13**). The goats are tall, have Roman nose, long droopy, curled ears, and short, mostly black or brown hair. Both sexes have thick, medium-sized horizontal horns. They have well developed udders and are prolific. Adult females weigh about 45 kg. Milk production averaged at 200 kg in 190 days.

Barbari

This is a small size breed (**Figure 14**) in India and Pakistan. Adult females weigh about 30 kg, but are good milk producers and very prolific. They have short hair of white color with tan spots. Their ears are short and erect, and horns are of medium length, twisted backward and upward. Males have large beards. Milk production has been reported to average 110 kg in 150 days.

Bach Thao

The Bach Thao is an indigenous dual-purpose breed (**Figure 15**). They originated from crossing native Deco goats with imported Saanen and Alpine goats from France and, more recently, dairy goat breeds from India. The goats are very popular for household milk consumption in central south Vietnam. The animals are mostly black and white with brown patches. They are medium-sized

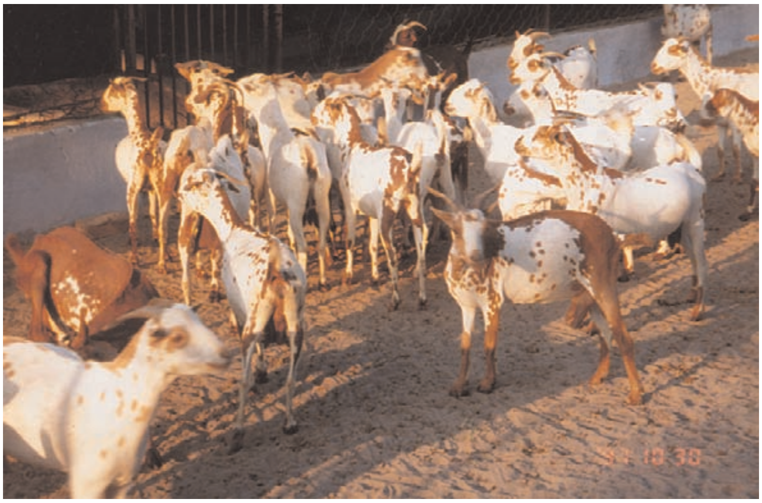

Figure 14 A flock of Barbari goats in Mathura, India. Photo: C. Devendra. Reproduced with permission from C. Devendra.

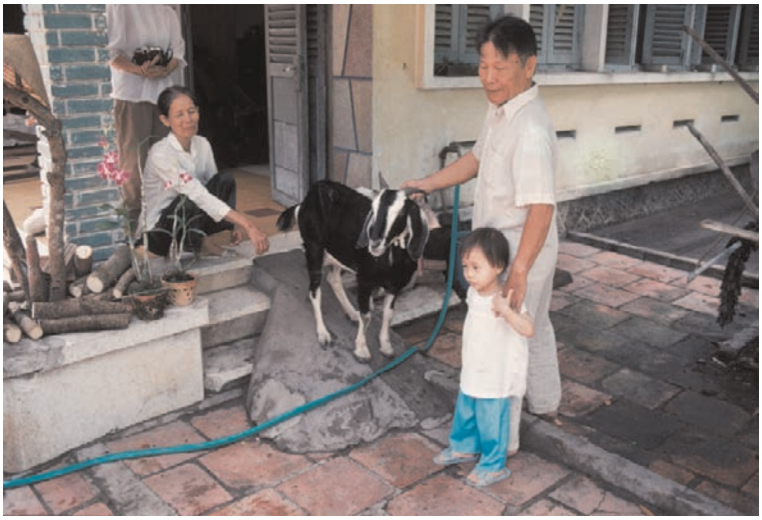

Figure 15 Bach Thao goat in Ninh Thuan province, Vietnam. Photo: C. Devendra. Reproduced with permission from C. Devendra.

animals with an average live weight of about 37 kg. The average daily milk yield is about 0.9 kg.

Bujiri

This is a medium-sized dairy breed with long white hair, found in the Sindh province of Pakistan. The face is convex and the horns are small and slender. The average live weight of does is 30–35 kg. The udder is reasonably well developed and the average daily milk yields are about 1–1.2 kg.

Chapper (Kohistani or Jablu)

The Chapper breed, also called Kohistani or Jablu, originated in the Kohistan area in Sindh, Pakistan. It is the most common breed in the Sindh province. It is mainly black in color or black and white. It is a small-sized dual-purpose breed, with small ears and with horns. Adult females weigh about 20 kg. The average daily milk yields are about 0.8–1.0 kg.

Chegu

This breed is found in the high-altitude areas of the Spiti valleys in Himachal Pradesh and in Pithoragarh district of Uttar Pradesh bordering Tibet. It resembles the Gaddi breed with a small size, lop ears, and spiral horns. It is usually white in color. The outer coat is long and coarse, but the undercoat provides pashmina cashmere. The live weight of adult females is about 20 kg. The breed is dual purpose, and milk production is relatively low.

Damani

This breed is found in the North-West Frontier Province of Pakistan. It is a dual-purpose breed, mainly black with brown on the legs, head, and ear. The animals are small in size and have small ears and short curled horn. The udder and teats are very well developed. Adult females weigh about 20 kg. The average daily milk yield is about 1.2 kg.

Dera Din Panah

This is a tall breed with a conspicuous Roman nose, and is found in the Punjab province in Pakistan. The goats of this breed have long hair and are usually black in color (**Figure 16**). The horns are thick and have two or three spiral curves. The ears are long and pendulous. The udder is well developed. The live weight of adult females is about 40 kg. The goats are good milk producers and the average daily yields are in the range of 1.6–2.6 kg.

Gaddi (Kashmiri or Chamba)

The Gaddi breed is also known as the Kashmiri or Chamba, and is owned mainly by Gaddi people in Punjab and Himachal Pradesh in India, and is also found in Azad Kashmir in Pakistan. Gaddi goats, like the Chegu breed, are also dual purpose in function, but are taller and are usually kept at lower altitudes. The goats are usually white, but gray and red colors are also common. They have long lopped ears and long spiral horns. They also have long hair and a tough skin, which may be adaptations to the high mountainous sub-Himalayan regions to which they are native. The udder is small and rounded. Adult females weigh about 40 kg and the average yields are about 0.8 kg.

Jakhrana

The Jakhrana is an important dairy breed found in Rajasthan in India. It is a medium-sized dairy breed. It is predominantly black with white spots on the ears and the muzzle and resembles the Beetal. The forehead is narrow and the ears are of medium size. The height at withers is about 62 cm. The udder is well developed. The live weight of adult females is about 44 kg, and daily milk yields are in the range of 1.0–1.2 kg. Recent field surveys in India indicated that the milk yield was 116 kg over 116 days and the kidding percentage was 75% in which 79.1% was of twins.

Jarakhell

This is a large-sized breed found in Azad Kashmir area in Pakistan. It has black and white patches and occasionally brown with white patches. The average live weight is about 40 kg. The breed has long hair and long drooping ears. This is a dairy breed and the average daily milk yields are about 1.2–3.3 kg.

Jattan

The Jattan is a large dairy breed that is found in the Sindh province of Pakistan. It is gray to fawn in color and is medium sized. The ears are of medium size and are white and spotted. Horns are present, and are twisted and swept backward. The average live weight of does is about 50 kg, and the daily milk yield is about 1.5–2.5 kg.

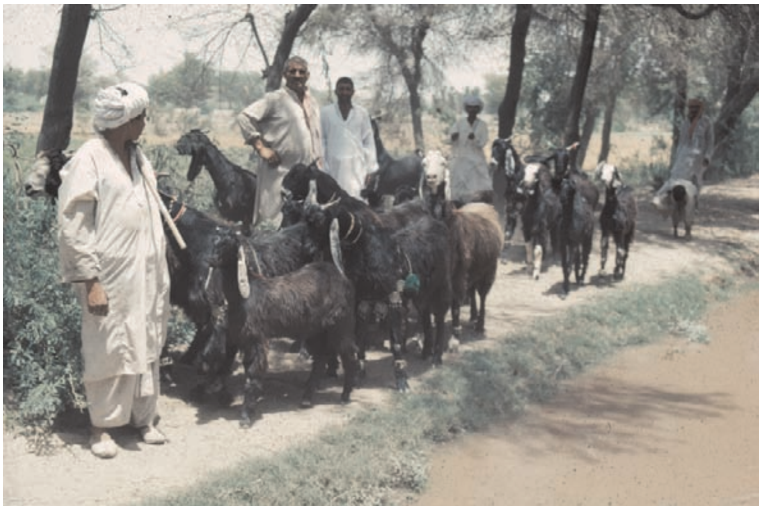

Figure 16 Flock of Dera Din Panah goats, Sindh province, Pakistan. Photo: C. Devendra. Reproduced with permission from C. Devendra.

Kacchan

This is a large-sized dairy breed that is predominantly black in color (**Figure 17**). It is found in the Sindh province of Pakistan. Brown-colored goats are also found with white markings on the ears and in the base. Horns are present and are small. The legs are long and there is a dense covering of hair around the back. The average live weight is about 45 kg and daily milk production is about 2–3 kg.

Kajli (Pahari)

The Kajli breed is also called Pahari and is found in Punjab, Pakistan. It is usually white or brown in color, but gray and black are also common. Horns are present and are swept backward. It is a dual-purpose breed. The teats and udder are medium sized. The average adult live weight of does is 25 kg and milk yield is 1 kg day^{-1}.

Kamori

The Kamori (**Figure 18**) is a popular dairy breed, found in the Sindh province of Pakistan. They are large goats and are brown in color with black or gray markings. The ears are long and lopped, and the horns are small. The udder is well developed. The average adult live weight of does is 50 kg. The average daily milk yield is in the range of 1.8–2.2 kg.

Khurasani

This dual-purpose breed is found in the border areas of Afghanistan and Iran, and in Balochistan in Pakistan. It is a small-sized dairy breed and is black, white, or gray in color. Horns are present, with one or two spirals. The average live weight of does is 25 kg and daily milk production is about 1–1.3 kg.

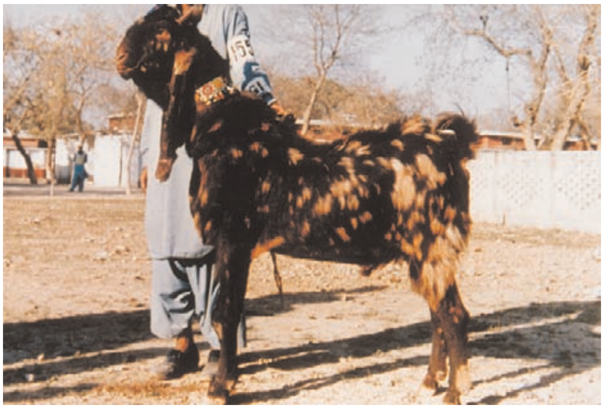

Figure 18 Kamori male, Sindh province, Pakistan. Photo: C. Devendra. Reproduced with permission from C. Devendra.

KOH-i-Ghizer

This dairy breed is tall and is found in northern Pakistan, close to the border areas with China. Black brown with white colors are common, with brown rings around the eyes. Males are horned but females are always polled. The teats and udder are relatively small. The average adult weight of does is 35 kg and daily milk yield is 1 kg.

Kurri

The Kurri is a tall medium-sized black and brown dairy breed that is found in the Sindh province of Pakistan. It has short conical ears from where it derives its name. The udder is also medium sized. The average

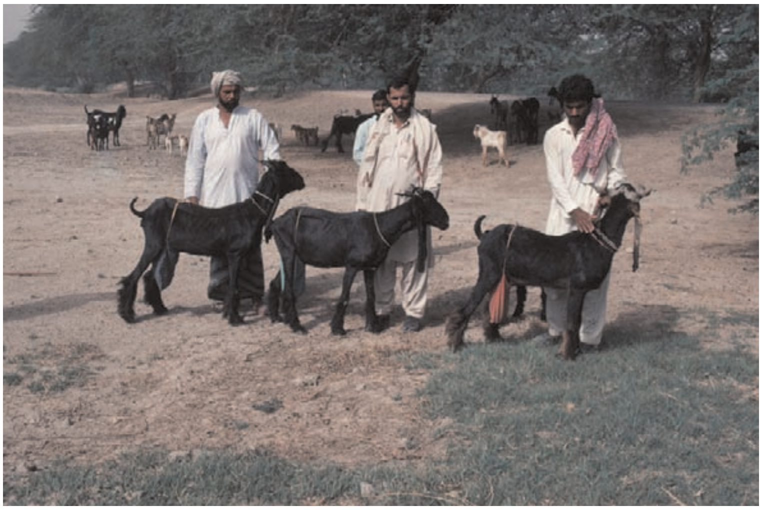

Figure 17 Kacchan goats in Sindh province, Pakistan. Photo: C. Devendra. Reproduced with permission from C. Devendra.

adult weight of females is about 35 kg and daily milk yield is about 1.0 kg.

Labri

The Labri is a dairy breed found in Azad Kashmir in Pakistan. It has long hair and is usually black, but white and gray with brown patches are also common. Horns are present in both sexes and the ends are sharp and pointed. The udder is very well developed and has long teats. The average weight of does is 35 kg and approximate daily milk yield is about 1.1 kg.

Malabar (Malabari)

The Malabar or Malabari goat breed (**Figure 19**) is widely distributed in Kerala in southeast India, where they are used for milk production. The goats are of mixed ancestry, due to crossing with dairy breeds from northern India. They are of medium size and colors are very variable from white to black. Horns are found in both sexes but polled animals are also common. Adult females weigh about 40 kg and the breed is also used for meat production. The breed is also prolific. The udder is small, and the average daily milk production is about 1 kg.

Ma T'ou

The Ma T'ou breed is found in Hupeh province in subtropical central China. The breed is a useful dual-purpose animal, and is important for meat and milk production. The goats are tall, white or white with tan, and are polled. Adult females weigh about 20–45 kg and the average daily milk yield is about 1.8 kg.

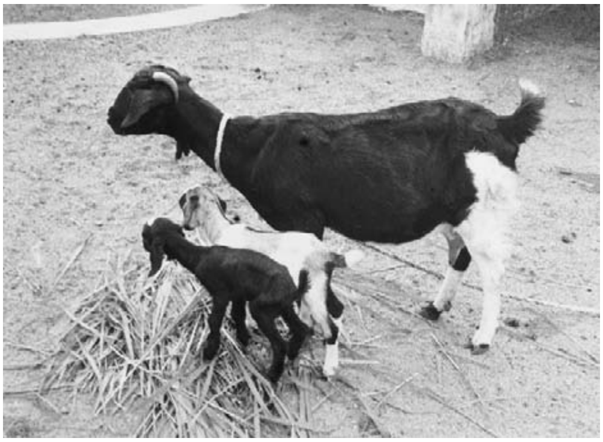

Figure 19 Malabar goat with twin kids, Malabar, India. Photo: C. Devendra. Reproduced with permission from C. Devendra.

Pateri

This is a tall, very distinctive dairy breed that is found in the Sindh province of Pakistan. It is white in color but has conspicuous reddish-brown belt that covers the head, ears, neck, and the body up to but not including the front legs. The ears are long and pendulous. The udder is well developed. Horns are present and they move sideways. Adult does weigh on average about 45 kg and produce about 1.2–2.0 kg of milk daily.

Shurri

This dairy breed is found in Azad Kashmir in Pakistan. It is medium sized and white, gray, and black with white patches are common. Horns are present in both sexes and the ears are long. The udder is well developed and has long teats. The average adult weight of does is 30 kg and the dairy milk yield is about 0.9 kg.

Africa

Boer

The Boer goat was developed principally from Hottentot stock north of the Cape Peninsula in South Africa, but also with some imported Indian Jamnapari and dairy breeds, beginning around 1930. It is essentially a single-purpose meat breed similar to beef cattle in contrast to dairy cattle, but Boer goats also have good dairy conformation and provide good milk yields to support twins (**Figure 20**). The modern Boer goat originated from three types: the common medium-size Boer goat with short glossy white coat with brown spots on its reddish-brown head and neck, which is the dominant type in the United States; the long-haired, larger, heavier, and more late-maturing Boer goats; and the multicolored polled

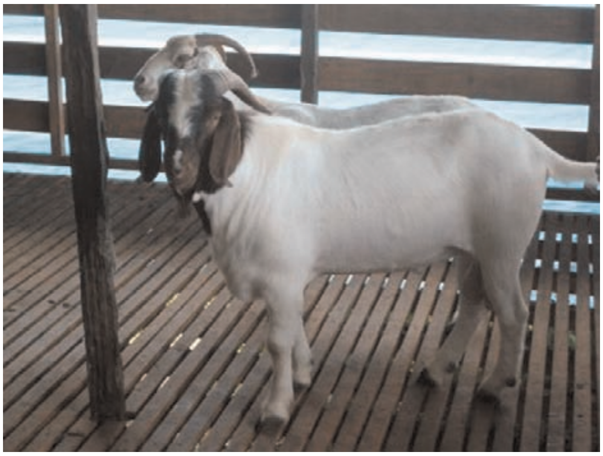

Figure 20 Boer buck imported into Sabah, east Malaysia. Photo: C. Devendra. Reproduced with permission from C. Devendra.

Boer goat with good dairy conformation. Some South African goats are solid black or dark red. Mature bucks weigh 100–120 kg and are 75–80 cm high, and females weigh 65–80 kg. Boer goats have a high kidding rate of about 180–190% in which twins are common. The average milk yield is about 1.8 kg day^{-1} and 300 kg in medium-length lactations. Boer goats have been imported widely into several countries of Europe, Asia, North America, and Australia to improve meat productivity in local goats and cover the growing popular ethnic markets.

Red Sokoto

This is a widespread goat breed in west and northwest Africa numbering about 17 million in Nigeria alone. They are of intermediate size with straight face, short pendulous or horizontally kept ears, flat outward-twisted horns, and short mahogany-colored hair. Males weigh about 30 kg and are 27 cm high at the withers. They are very prolific and grow fast, but are kept under sedentary conditions. Their skin is valued for the production of Morocco leather. Milk production has been reported at 150 kg in 105 days with 6.6% fat.

Sahel

This breed is kept by transhumance pastoralists for milk and meat on the ranges of northwest Africa, in Chad, Niger, Mali, Burkina Faso, Mauritania, and Senegal, numbering about 21 million. The face is straight or convex, ears are short and horizontal or pendulous, horns are thin and upward twisted in males but sickle shaped in females, and the hair is short with many colors. The Sahel males have a beard, long mane, and long hair on the thighs, and some are polled. Males weigh about 25–40 kg and are 75–90 cm high, and females weigh 20–35 kg and are 60–88 cm high at withers. They are also known as West African long-legged goats. Milk production is around 120 kg in 120 days.

South and Central America

Criollo

The Criollo (**Figure 21**) is of Spanish origin and is widely distributed in Central America and the West Indies, numbering about 20 million head. It is a dual-purpose goat and mainly black or brown in color with white patches. The horns are scimitar shaped and swept backward and upward. The ears are generally short. The average live weight of females is about 35–60 kg, with a height of 68–76 cm. The average daily milk yield is mostly low, about 0.5–3.0 kg, but 190 kg in 272 days has been reported, which indicates their innate potential.

Conclusions

There exists a great diversity of dairy goat breeds, involving both true dairy and dual-purpose types. They are distributed widely across tropical and temperate regions. Although India and Pakistan are the home of the largest number of breeds, improved and high milk yielding goats are found only in Australia, China, Europe, Israel, Mexico, New Zealand, and North America. These improved dairy breeds have been widely introduced into all continents without exception to improve milk production through crossbreeding. Many of the indigenous dairy breeds in Africa and Asia are potentially important 'improver breeds' for wider use in environments with similar climates,

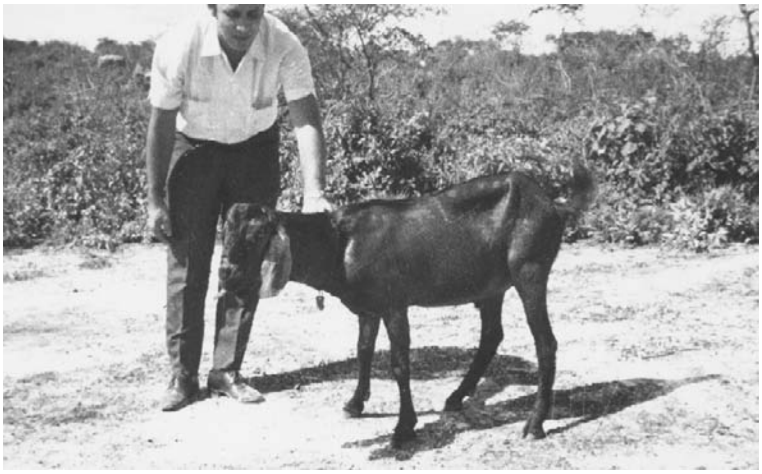

Figure 21 Criollo female in Peru. Photo: C. Devendra. Reproduced with permission from C. Devendra.

contribution to nutritional and food security, and sustainable agriculture. In order to expand this contribution further in tandem with the potential of goats, it is essential to have clear production objectives, efficient systems of management, and breed organizations with record keeping of ancestry, performance, and type data. All this can then focus on increased individual productivity, which will impact on improved nutrition and better livelihoods of rural poor, besides providing urban people goat milk and dairy products, which have become increasingly popular among discerning consumers and people with medical needs from allergies and gastrointestinal afflictions. Such concerted use of these dairy goat breeds can stimulate expanded milk production in rural areas, parallel to milk production from dairy buffaloes, yaks, camels, dairy sheep, and dairy cattle in periurban areas. It is equally important to ensure conservation of valuable and unique germplasm of the minor goat breeds.

See also: **Husbandry of Dairy Animals**: Goat: Multipurpose Management.

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Sheep Breeds

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Introduction

The definition of dairy breeds is loosely applied to all sheep producing milk whether it is used for human consumption or nursing lambs. In developing countries, milk from sheep and goats is an important source of dietary protein for millions of people, mainly smallholders, landless people, nomads and peasants. In contrast, in developed countries, milk from sheep is a raw material that is further processed into high-quality dairy products. The importance of milk production is that it is often considered as a key factor in determining the feasibility of the other production systems, i.e. meat and fiber production. In general, ewes that produce more milk tend to raise more lambs contributing to the efficiency and economic viability of the production system.

Genetically isolated populations of indigenous sheep with distinct morphological characteristics and production performance evolved over centuries as a result of natural and induced selection in a wide range of physiogeographical habitats. Wide variation in physical appearance, production and reproduction performance is known to exist among dairy breeds. The potential for milk production tends to vary considerably according to the genetic composition and the cumulative selection pressure previously applied. However, this potential is influenced by many environmental factors such as nutrition, climate, management, diseases and the socioeconomic patterns of sheep farmers.

Distribution and Classification of Dairy Sheep

Nearly half of the world's sheep milk is produced in the Mediterranean basin and the Middle East. Though not significant, milking of indigenous sheep in China and a number of countries in Southeast Asia has been frequently observed. There are many breeds of dairy sheep on the Mediterranean Islands of Corsica, Sardinia, Cyprus, Chios and Crete; on the Balearic, Ionian and Aegean islands; on the plains of Thessaly; on the plateaus of the south Massif Central, Anatolia and Iranian Azerbaijan; in the oases on the borders of Morocco, Algeria and Tunisia; and in the El Fayum region in Egypt. In 1991, Boyazoglu broadly classified sheep in this region into six distinct groups based on their origin, morphological characteristics and production performance.

1. The fat-tailed sheep from Asia include the majority of the breeds in Iran and Afghanistan, the Awassi, Mytileni, Cyprus, White and Red Karaman; the Barbary breed in Tunisia; and the Barbaresca and Laticauda breeds in Italy.
2. The Zackel/Karakachan/Ruda types include the primitive mountainous breeds from Central Europe such as the Cakiel in Poland; Karagouniko, Serres and Vlahiko in Greece; Turcana in Romania; Pleven in Bulgaria; Ruda in Albania; and Pramenka in Yugoslavia.
3. The coarse-wooled breeds of the western Mediterranean consist of the Churra in Spain; the Bordaleiro in Portugal; the Sarda in Italy; and the Corse breed in Corsica and some Island breeds.
4. The prolific breeds of the Greek Islands with increased potential for milk production include the Chios, Kymi and Skopelos; the D'man of Morocco; and the Langhe and Bergamasca of the sub-Alpine type in Italy.
5. The white and medium-wooled, average milking sheep breeds of the western Mediterranean consist of the Lacaune in France; the Manchega in Spain; and occasionally breeds with Merino ancestry.
6. The Merino type of fine-wooled populations include the Arles Merino breed; and breeds with Merino ancestry such as the Spanish and Portugese Merino; the Sopravissana, Gentile di Puglia and other Italian breeds.

The duration of suckling during lactation and the milking interval are important criteria that need to be considered in determining total milk yield. In many studies the milk yield reported represented only the amount of milk produced during lactation and did not account for the milk consumed by lambs during nursing. In addition, the milk yield reported in milk recording systems is based on a certain period during the lactation, not on the entire lactation period. Furthermore, the time of sampling influences milk composition. These factors should be taken into consideration when comparing the performance of various breeds.

In the twentieth century, there was extensive research on the evaluation of sheep breeds based on production and composition of milk. The majority of these studies were carried out under varying conditions of management, nutrition and environment, and milk recording procedures were based on limited sample size. Comparative studies involving several breeds of dairy sheep are rare. The conclusion from studies based on widely variable attributes, therefore, can only be considered as preliminary evidence of breed differences in ewe productivity.

In this article important and well-established dairy sheep breeds are classified into four distinct groups (superior, high, medium and low) according to their milk production potential. Many other breeds can fit within these classifications but are not presented here because very little information is available in the literature on their characteristics and performance. Information is also given on some of the most important newly developed breeds.

Dairy Breeds with Superior Milk Production

Breeds in this category have the potential to produce in excess of 300 kg of milk per lactation. Currently, only two breeds, East Friesian and Improved Awassi, can be classified in this category.

East Friesian

Origin and distribution

The East Friesian breed, also known as East Friesland Milch, German Milk Sheep and Milchschaf, originated from the marsh type sheep indigenous to the coasts and islands of the North Sea extending from Holland to Denmark, mainly in the East Friesland region of northern Germany and the East Friesian Islands (**Figure 1**). This breed has spread across Europe into Scandinavia and Poland, and into the high altitudes of the Alps. The East Friesian breed is recognized for its superior milking ability, and has been exported to North and South America, the United Kingdom, the Middle East and Oceania.

The East Friesian sheep, well adapted to harsh climatic conditions and mountainous terrains, appears to prosper in the outdoors, and is most common in humid areas. These sheep are not productive under dry and/or hot conditions but are suitable for farming at sea level or in areas where rainfall is frequent. The East Friesian sheep have an inherent potential for increased milk, lamb and wool production; however, sparse feed and unimproved pastures may fail to meet their nutrient requirements, resulting in lower levels of production.

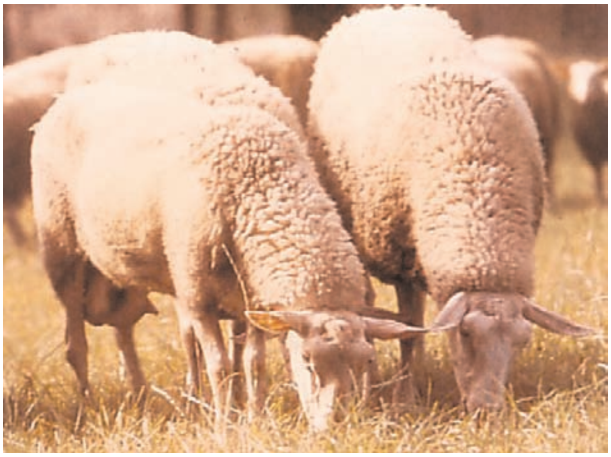

Figure 1 East Friesian ewes. (Photograph courtesy of Zofia Raczkowska.)

Physical and reproductive characteristics

East Friesian sheep are white and long-wooled with a thin, long tail bare of wool (rat tail). The head is long and polled with a slight Roman nose. The ears are thin, long and pointing forward. The head, ears, legs, inner legs and scrotum are devoid of wool. The back is broad, solid, long and firm over the loin. The udder has wide attachment with distinct quarters and strong teats pointed downward.

Wool is classified as medium type with a spinning count of 48–52 s, fiber diameter of 40 μm and staple length of 11–20 cm. Yearling ewes produce 3 kg of fleece, whereas ewes 2 years and older produce 3.6–6.2 kg. Lambs weigh 4.0–5.2 kg at birth, gain 145 g daily to 40 kg, and subsequently 286 g daily to 200 days of age. Body weights of mature rams and ewes are 90–120 kg and 57–75 kg, respectively.

Ewe lambs attain puberty at 7 months of age. Ewes continue estrous activity for approximately 5 months exhibiting an average of 8.8 estrous cycles. Under optimum environmental condition fecundity of 210–230% is common, however, under commercial conditions lower fecundity may be expected. The embryonic mortality is 12% and lamb mortality from birth to 120 days of age may reach 24% in large flocks, and 8% in small flocks. These findings confirm that East Friesian sheep need proper feeding and management in order to express their inherent potential.

Milk production traits

The estimates for milk production of East Friesian sheep tend to vary widely among countries according to prevailing nutrition and management as well as milk recording procedures. **Table 1** summarizes some recent

Table 1 Estimates of milk yield of East Friesian sheep in various countries

Country	*Milk yield*	*Lactation length (days)*
Germany	540–650 kg	—
	283–680 l	212–264
United Kingdom	450 l	—
Sweden	250–350 kg	—
Bulgaria (experimental conditions)	343 l (1st)	—
	311 l (>1st)	—
Greece	178–183 kg	140–170
Uruguay	196 l	235
Israel	161 kg	198
Saxony Province (Germany)	388–551 kg	—

estimates of milk yield from various countries. Production from outstanding ewes can reach 1500 kg per lactation. The lactation length varies between 180 and 210 days, and in exceptional cases lasts 260 days; some recent reports indicate that lactation length in some Balkan countries exceeded 300 days, or well into subsequent pregnancy. In drier regions, milk production tends to be lower and the duration of lactation is shorter compared to countries in Northern Europe.

In Germany, the fat content of East Friesian milk is 5.5–6.0%. However many published estimates are lower and vary considerably among countries. In Poland, the fat content of milk from East Friesian sheep is 4.2%, lower than in the Finnish landrace and native Polish breeds. Likewise, the protein content of 4.5–4.7% is lower, whereas the lactose content of 4.6–4.8% is consistent among breeds. Udder volume in the second month of the second lactation is estimated at 1843 ml and milking speed at 8.9 ml s^{-1}.

Improved Awassi

Origin and distribution

At the beginning of the twentieth century, indigenous Awassi sheep from Turkey and the Middle East were imported into Israel. An accelerated breeding program based on selection for increased milking ability resulted in the development of the improved Awassi breed (**Figure 2**). These sheep with potential for increased milking ability have been exported to several countries including Iran, Cyprus, Hungary, Spain, India, Turkey, Yugoslavia and New Zealand.

Physical and reproductive characteristics

The improved Awassi population, classified as fat-tailed, carpet-wool-type sheep, are medium to large in size. The predominant color is white while the head, ears and upper part of the neck may be reddish-brown. In order to eliminate rudimentary ears and colored fleece, selection against these undesirable characteristics was rigorous. The legs are totally or partially brownish in color. The rams are horned and ewes are polled or may have rudimentary horns. Ewe lambs are usually mated at 8–16 months of age. The detailed description of the physical characteristics of the breed is presented in the next section.

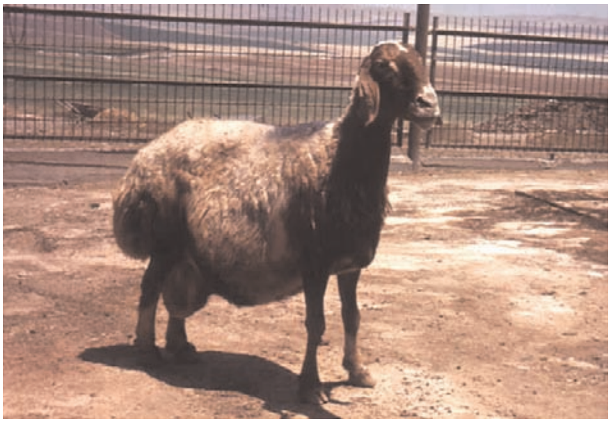

Figure 2 Improved Awassi ewe. (Photograph courtesy of the Volcani Agricultural Research Centre, Israel.)

Milk production traits

In Israel, the average production of improved Awassi ewes in 1979 was 342 l of milk in 200 days of lactation with 6% milk fat. Selection increased milk production over a period of 5 years from 378 to 444 kg in yearlings, and from 473 to 532 kg in 2-year-old ewes. In year 2000, the official milk recording program estimated milk yield at 530 l. In Spain, 22% of Awassi ewes imported from Israel produced up to 200 kg of milk. Similarly, 49% produced 201–300 kg, and 25% produced 301–400 kg of milk. In Iran, ewes produced 222 kg of milk in the first lactation, 268 kg in the second lactation and 298 kg in the third and subsequent lactations. In India, yearlings produced 231 kg of milk in 323 days of lactation. Milk fat content varied between 5.5% and 9.8%, while solids non-fat content varied between 9.4% and 10.8%.

Dairy Breeds with High Milk Production

Breeds with milk production potential of 200–300 kg per lactation are classified in this category. Milk production and length of lactation of major dairy sheep breeds are presented in **Table 2**.

Awassi

Origin and distribution

The exact origin of Awassi sheep has not been established; however, it is widely believed that their evolution occurred in the region between the rivers Tigris and Euphrates in Iraq and Syria. The name Awassi comes from an ancient Arab tribe (El-Awas) that lived near the Euphrates river in northern Syria. These sheep, classified as fat-tail, carpet-wool type, are raised primarily for milk, lambs and wool.

The Awassi sheep are well adapted to the hot and dry subtropical climate. These sheep are good walkers capable of travelling over extended distances in search for food and water. Nevertheless, it is important to provide shelter as a safeguard against cold and humidity, especially snow. In the semiarid and arid regions of southwest Asia Awassi sheep are raised under extensive management. Furthermore, these sheep are highly productive under intensive management and adequate feeding. The Awassi sheep is popular in the countries of the Middle East, southeast Turkey and southwest Asia.

Physical and reproductive characteristics

Awassi sheep are medium in size, but the body size varies among flocks in the Middle Eastern countries. The entire body is covered with wool, whereas the head and legs have short, lustrous hair, and sometimes the chest and belly are bare. The head, ears and anterior of the neck are red, brown or black in color, with these colors covering various parts of the body. Occasionally, white, gray or spotted colors have been observed. Although rams are characterized by large, spiral and strong wrinkled horns, there are a small number of polled animals. The majority of ewes are polled, however 25% of the female population may have short straight horns or scurs. The ears are long and pendulous, and sometimes small, rudimentary or absent. The tail is broad, round, medium-sized and bilobed with a fat cushion that is bare on the under surface and extends to the hocks. The middle of the tail is narrow, wooled and bent upward, ending in a short, thin appendix.

Rams and ewes produce heavily medullated fleece weighing 2.0–2.5 kg and 1.8 kg, respectively. The fleece has a spinning count of 36–46 s, staple length of 15–20 cm and fiber diameter of less than 26 μm in 43% of the fleece, and more than 36 μm in 28%. In Lebanon and Turkey, birth weights of single-born ram and ewe lambs were 4.6 and 4.3 kg, respectively. Corresponding weights for twin-born lambs were 3.7 and 3.6 kg. In Israel, birth weights of ram and ewe lambs were 4.9 and 4.5 kg for singles, and 4.1 and 4.0 kg for twins, respectively. In Lebanon, the body weight of lambs was 4 kg at birth, 27 kg at 6 months, 43 kg at 12 months and 52 kg at 24 months of age. Correspondingly, ram lambs under proper management and feeding weighed 4.9 kg at birth, 28 kg at 2 months, 37 kg at 3 months, 43 kg at 4 months and 50 kg at 5.5 months of age.

In Iraq, Awassi sheep (Shafali) raised on irrigated farms are early maturing and relatively larger in size. The rams attain sexual maturity as the body weight reaches 30–37 kg around 8 months of age. Usually, lambs reach puberty at 6 months of age with 75% of the ewes lambing as yearlings. When rams and ewes are raised together, lambing occurs in nearly 85% of the ewes between mid September and mid March, but mostly in January. Awassi ewes have the ability to lamb year-round, and when raised on good pastures can produce two lamb crops annually. Fertility tends to vary among countries: 85–90% in Iraq, 90% in Syria and Kuwait, 60% in Jordan and Lebanon, and 98% in Israel. Correspondingly, prolificacy also varied: 1.2–1.3 in Iraq, and 1.0–1.1 in Syria, Saudi Arabia, Kuwait and Israel. The proportion of twins is 5% among ewes raised by small farmers and Bedouins. Lamb mortality in sheep flocks maintained by Bedouins varies from 15% to 20% annually. The mortality rate may increase with drought or during adverse climatic conditions.

Table 2 Milk production and lactation length of various breeds with high milk yield

	Alfa-Laval (1981)		*Boyazoglu (1963)*	
Breed (country)	*Milk yield (kg)*	*Lactation length (days)*	*Milk yield (kg)*	*Lactation length (days)*
Awassi (Turkey)	—	—	175–205	—
Awassi (general)	150–500	250	—	—
Chios (Greece)	180–200	160–260	202–251	157–265
Lacaune (France)	130–200	100–210	177–182	161–170
Sarda (Italy)	110–250	170–240	122–194	—

Milk production traits

In Lebanon, Awassi ewes produced 225 kg of milk in the first lactation of 217 days. The average milk production over the following two to six lactations was 216 kg in 191 days with a peak production of 406 kg in the fifth lactation. In Syria and Turkey, ewes produced 100–185 kg of milk. In Iraq, although Awassi sheep are raised mainly for meat, ewes produced 108 kg of milk in 138 days of lactation. All the milk produced is normally consumed by the owners. The average length of lactation in the various flocks varied from 130 to 220 days. In Syria, the range was wider, from 116 to 262 days.

Udder shape and teats in Awassi ewes tend to vary, displaying many faults. Uniform udder characteristics in improved Awassi ewes are a result of selection for mechanical milking. The udder is globular shaped, well attached, moderate in depth, wide between the legs, elongated anteriorly and extends well to the rear. The teats face downward and are of fair length and moderate thickness.

Chios

Origin and distribution

The exact origin of Chios sheep is unknown. Possibly, these sheep are the descendants of crosses among sheep indigenous to the island of Chios, western Anatolia, Kivircik and Dagli. The Chios is classified as a semi fat-tailed breed (**Figure 3**). These highly productive stall-fed sheep are raised primarily for milk production. In Turkey, the Chios breed is known as Sakiz and classified as a thin-tailed sheep.

Chios sheep adapt well to a wide range of ecological and climatic conditions within the broader Mediterranean region. However, these sheep are not recommended where adverse and difficult conditions prevail. In central Turkey, fertility and milk yield declined in Chios sheep raised under dry and cold climatic conditions. Studies in Greece suggest that productivity of Chios sheep tends to decline when they are exposed to cold and windy conditions. The Chios breed is well recognized for its milking ability, and has been exported to several Mediterranean and Middle Eastern countries.

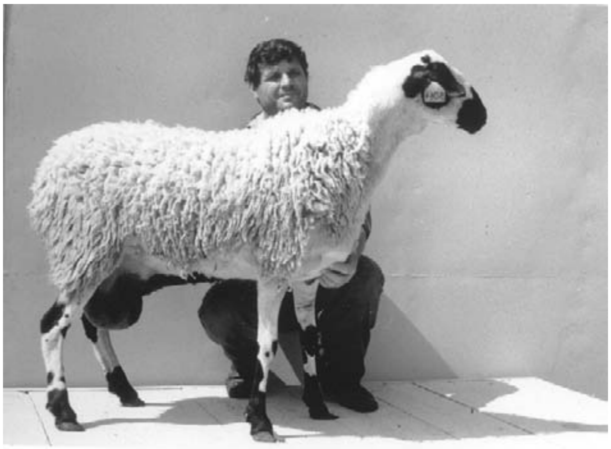

Figure 3 Chios ewe. (Photograph courtesy of Dr I. Hatziminaoglu.)

Physical and reproductive traits

Chios sheep are predominantly white with black or brown spots around the eyes, and on the extremities of the ears, nose, belly and legs. The head is relatively small and delicate with a light to moderately convex profile and sometimes completely black in color. The ears are long, horizontal and slightly droopy. In general, rams have long spiral horns and ewes are polled. There is a small proportion of ewes with small curved horns. Chios sheep have a long and narrow neck. The legs are long, and fine-boned. The tail is 24–27 cm long, cone-shaped, with a 9–12 cm broad base extending to the hocks. On the island of Chios, body weights of mature rams and ewes are 65–80 kg and 48–52 kg, respectively.

The head, legs and belly and sometimes the neck are bare. The fleece weight of rams and ewes weighs 2.2 and 1.2–1.9 kg, respectively. The wool appears to be uniform, but the quality tends to vary with a spinning count of 44–56 s, staple length of 8– 13 cm, breaking strength of 20–24 g and extensibility of 45–63%. Ram lambs weigh 3.8 kg at birth, 16 kg at weaning (42 days), 44 kg at 150 days and 51 kg at 180 days of age. Corresponding weights for ewe lambs were 3.5, 13, 35 and 37 kg.

Ewe lambs exhibit puberty at 243–290 days of age as body weight reaches 37–43 kg. The mean onset of estrus occurs in late July and the breeding season lasts from April to November. In Turkey, more than 80% of ewes were in estrus from September to March and the breeding season lasted for 116 days. Ovulation rate was 3.25, and duration of estrus was 1.8 days with an estrous cycle of 17–18 days. Fertility was 91%, while prolificacy increased from 1.7 under conventional management to 2.0 under improved conditions. Preweaning lamb mortality was 6.2% for singles, 7.2% for twins and 9.8% for multiple births.

Milk production traits

Chios ewes produce an average of 120–300 kg of milk annually. In Turkey, ewes produced 188–259 kg of milk under experimental conditions and 120–180 kg under commercial establishments. In Cyprus, ewes produced 119 kg of milk in 90 days of lactation, and 195 kg in 194 days of lactation. In Greece, ewes produced 180–200 kg of milk annually following a suckling period of 40–60 days. Results based on 4225 milk records showed 53% of the ewes produced 150–300 kg of milk annually. In a study of 4000 ewes, 28% produced 3 kg of milk or more daily, 28% produced 2–3 kg and the remaining ewes produced 1–2 kg. Outstanding ewes produced up to 500 kg of milk. The highest milk production recorded for a ewe was 597 kg in 272 days during the second lactation. On the island of Chios, ewes produced 1.8–2.0 kg of milk daily containing 5.9–6.8% fat and 5.5% protein. In general, lactation length varied between 150 and 200 days according to the prevailing management.

Chios ewes have a typical dairy conformation with a large pendulous udder and a high inclination of teats that causes problems during machine milking. Large udders may have two additional teats that are often milked. In milking ewes, the average udder circumference before and after milking was 48 and 36 cm, respectively; udder depth was 23 cm; and teat length and diameter were 4.3 and 2.3 cm, respectively.

Lacaune

Origin and distribution

The Lacaune breed was named after a town southeast of Tarn in the Lacaune mountains (**Figure 4**). In 1870, in an effort to improve the milk production potential, Merino, Southdown and Barbary breeds were crossed with Lacaune sheep. This action has had little influence on the breed. In 1947, the Camarès breed from south of the Aveyron was officially absorbed into the Lacaune breed together with the Larzac and Ségala breeds of Aveyron and the Lauraguais and Corbires breeds of Aude. The Lacaune sheep, noted for milk production, have been raised widely across central and southern France. These sheep are maintained in flocks of 50–100 ewes and almost two-thirds of the population are milked.

Physical and reproductive characteristics

Lacaune sheep are generally white or yellowish-white in color; however, some are pigmented. Wool covers the body except the head, nose, abdomen and legs. The head is long with a straight or slightly convex profile. The forehead is triangular and both sexes are polled. The Lacaune's eyes are large and light in color, and it has long, horizontal ears located sideways and drooping to a very slight degree. The face is covered with white hair while the neck is round and arched. The sheep are 70–80 cm in height with a long trunk and a straight back. The legs are medium in length.

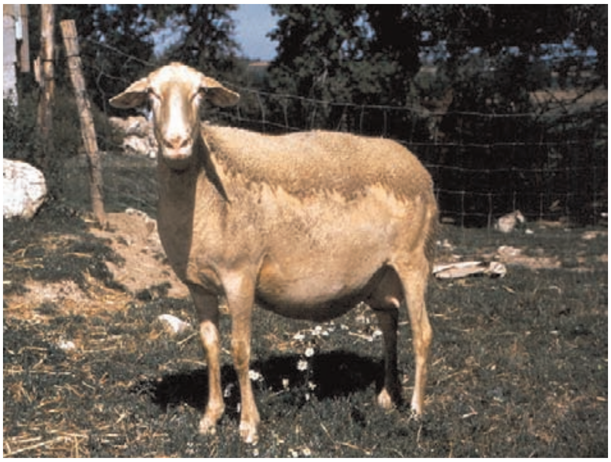

Figure 4 Lacaune ewe. (Photograph courtesy of INRA, Toulouse Research Centre, France.)

The Lacaune breed is classified as a semi-coarse-wooled sheep with short, thick, elastic locks and a more extensive semi-closed fleece. The fleece is dense, crimped, springy, strong, white in color and fairly fine weighing 2.5 kg in rams and 1.5 kg in ewes. The fleece has a spinning count of 58–60 s, fiber diameter of 20–28 μm, fiber length of 7–10 cm, and breaking strength of 12 g. Twinning is common and lambing occurs from December to March. Usually, ewes continue to lactate until July. Lambs weigh 3–4 kg at birth. On dairy farms, lambs weighing 10–16 kg are marketed at 30–45 days of age. Lambs sold for meat vary in weight from 25–30 kg at 1 month to 40–50 kg at 3 months, or 50–60 kg at 12 months. Body weights of mature rams and ewes are 95–100 kg and 70–75 kg, respectively.

Milk production traits

Lacaune sheep have been intensively selected for milk production. Results from the 2000 milk recording programme showed that yearlings up to 18 months of age produced 225 l in 146 days of lactation whereas older ewes produced 287 l of milk in 172 days of lactation. For ewes of all ages, the yield was 270 l in 165 days. The milk contains 5.2% protein and 7.1% fat. The primary product of this breed is milk, used in manufacturing Roquefort cheese. The production of meat and wool is also common. There are many specialized varieties of Lacaune sheep that have been selected for both milk and meat production.

Sardinian (Sarda)

Origin and distribution

There is no definitive evidence on the origin of the Sardinian breed. These sheep are predominantly found in the Sardegna region of Italy (**Figure 5**). The mountainous terrain on the Island of Sardinia consists of bushes and rocks on shallow and infertile land. The majority of sheep, with no access to supplementary feed, remain outdoors in an inhospitable environment facing extremes in temperature with little or no reliable source of water. The Sardinian sheep are a triple-purpose breed with milk as the primary product followed by meat and wool production. The breed has been classified into the small mountain, medium hill and large lowland varieties. The Sardinian breed, recognized for milking ability, has been exported to Tunisia, France, Spain, Greece, Cyprus and Israel.

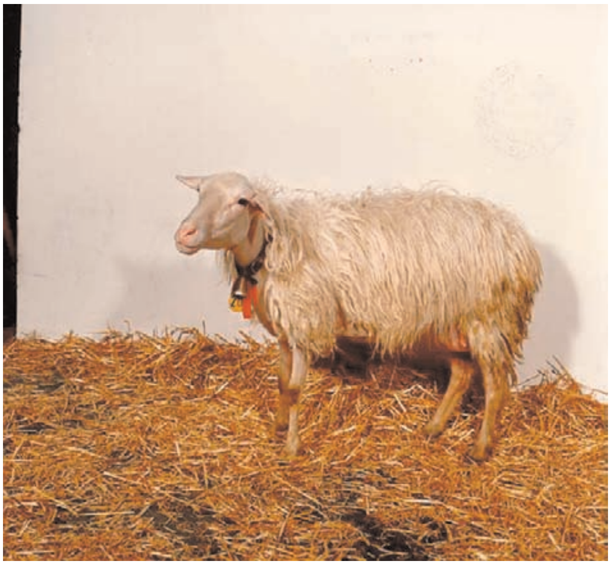

Figure 5 Sarda ewe. (Photography courtesy of Italian Sheep Breeders' Association.)

Physical and reproductive characteristics

The small mountain variety of Sardinian sheep is found in migratory flocks in the eastern highlands and the northeast and extreme southwest regions of Italy. Rams have large spiral horns while ewes have small horns curled downwards and backwards, occasionally with vestiges. The ears are medium in size, horizontal and tend to be small and upright. Body weight of mature rams and ewes averages 35–40 kg and 25–35 kg, respectively. The sheep are white in color and carry a tuft of wool on the forehead. The fleece weighs 0.6–0.8 kg. The smaller variety has a coarse outer coat and a more pronounced undercoat. The tail is long and thin, sometimes with small terminal lipoma.

The medium hill variety of Sardinian sheep is common in the province of Sassari, and in areas where the small and large varieties are not widespread. Rams are always horned while ewes rarely have horns. The ears are medium in size, horizontal with a tendency to be smaller and upright. The sheep are white in color with a long and thin tail. Body weights of mature rams and ewes are 45–60 kg and 35–45 kg, respectively. The fleece weighs 1.6–2 kg for rams and 1–1.4 kg for ewes.

Campidano sheep, known locally as Sarda, is a large lowland variety mostly confined to the area near the town of Cagliari. The introduction of Barbary and Merino breeds may have contributed to the large size, fat tail and compact fleece. The ewes are white in color and highly fertile but produce less milk. These sheep, maintained on a high plane of nutrition, have been selected for fertility. Rams are polled, but a small proportion may have scurs, while ewes are always polled. The sheep have large ears that rest slightly below horizontal. Body weights of mature rams and ewes averages 65–80 kg and 42–60 kg, respectively. These sheep have a long and thin tail, very wide at the base. The head, legs and abdomen are bare. The larger varieties are more uniform and compact. The fleece contains coarse wool fibers, open and mixed with hair. Fleece weight averages 2.3–3 kg in rams and 1.8 kg in ewes. The fleece has a spinning count of 36–40 s, fiber diameter of 37 μm with 1.1 crimps cm^{-1} and fiber length of 14 cm.

In the medium variety lambs weigh 3 kg at birth whereas in the smaller variety they weigh 10% lighter and in the larger variety 10% heavier. In general, rams are 10% heavier than ewes. At 30 days of age, slaughter weights of lambs of the small, medium and large varieties are 6–7 kg, 8–9 kg and 10–11 kg, respectively. The sheep usually attain 18–20 kg at 90 days and 27–32 kg at 150–180 days of age.

About two-thirds of ewes are exposed to rams as yearlings at 11–19 months of age, while the remaining ewes are mated at 22–26 months. The lambing season extends from September to April. Sardinian sheep raised under an accelerated breeding program have lambed at 6–8 month intervals. About 8–10% of the ewes produce twins and the proportion increases from 1% at 1–2 years to 15% at 5–7 years of age.

Milk production traits

Sardinian sheep are hand-milked, twice daily until May and once daily until July–August. In general, the average milk production in selected flocks was 100 kg for the smaller variety, 150–200 kg for the medium variety, and 120–180 kg for the large variety. Milk production of Cagliari sheep was 134 kg in the first lactation of 172 days, 188 kg in the second lactation of 225 days, and 216 kg in subsequent lactations of 248 days. The year 2000 milk recording results of over 1100 flocks showed that in 100 days of milk recording, yearlings up to 18 months of age produced 130 l compared to 203 l for older ewes. The milk contains 6.7% fat and 5.1% lactose. In another study, milk production of 63 kg in first lactation and 215 kg over the subsequent 160–200 days was estimated from weight gain of lambs suckling for 35 days.

Dairy Breeds with Moderate Milk Production

Breeds with a milk production potential of 100–200 kg per lactation are considered in this category. Recent milk yield results from the year 2000 European Milk Recording Program for the major breeds in this category are presented in **Table 3**. Milk production and length of lactation of these breeds and others in this category are presented in **Table 4**. **Table 5** gives the milk production

Table 3 Average milk yield per recorded ewe for selected breeds in 2000

		Yearlings 12–18 months old		*Ewes older than 18 months*	
Breed	*Country*	*Milk yield (liters)*	*Lactation period (days)*	*Milk yield (liters)*	*Lactation period (days)*
Castillana	Spain	101	120	116	120
Churra	Spain	105	120	123	120
Comisana	Italy	99	100	182	200
Karranzana	Spain	127	120	128	120
Langhe	Italy	108	100	150	180
Latxa	Spain	102	120	129	120
Manchega	Spain	140	120	160	120
Massese	Italy	130	100	152	120
Sarda	Italy	130	100	203	180
Valle del Belice	Italy	104	—	194	180

Data from Astruc and Barrillet (2001).

Table 4 Lactation length and milk production of several medium-yielding dairy breeds

		Alfa-Laval (1981)		*Boyazoglu (1991)*	
Breed	*Country*	*Milk yield (kg)*	*Lactation length (days)*	*Milk yield (kg)*	*Lactation length (days)*
Churra	Spain	45–75	150	150	151
Comisana	Italy	—	—	144	—
Garfagnana	Italy	150–160	180–210	—	—
Karagouniko	Greece	—	—	123–163	158–176
Kymi	Greece	—	—	141–166	153–265
Lacha	Spain	—	—	207	180
Langhe	Italy	—	—	82–150	—
Manchega	Spain	50–125	90–150	97	126
Massese	Italy	150–160	180–210	138	—
Serra da Estrela	Portugal	100–120	200–230	150	105
Sfakia	Greece	—	—	128–134	190–197
Skopelos	Greece	—	—	153–163	164–180

Table 5 Milk production of some medium-yielding breeds of minor importance

				Milk composition (%)		
Breed	*Country*	*Milk production*	*Lactation period (days)*	*Fat*	*Protein*	*Lactose*
Basque-Béarn	France	100 l	130	—	—	—
Bordaleiro	Portugal	131–155 l	120–180	7.4	5.0	—
Corsican	France	150 l	86	—	—	—
Flemish Milksheep	Netherlands	117 kg	days 10–45	6.8	5.2	5.7
Karranzona	Spain	128 l	120	—	—	—
Pinzirita	Italy	117 l	180	—	—	—
Préalpes du Sud	France	114 kg	169	6.8–7.0	5.8–6.1	—
Stara Zagora	Bulgaria	154 l	—	—	—	—
Zakinthos	Greece	160–180 kg	175	—	—	—
Zigaja	Germany	96 to 145 l	169–208	—	—	—

figures for some similar yielding breeds of minor importance.

Bergamasca

Bergamasca is the largest sheep breed in Italy, and originated in the province of Bergamo. The Bergamasca breed is also known as Gigante di Bergamo in Italy; Bergamasker in Germany; and Bergamacia in Brazil. The breed has been grouped with the lop-eared Alpine sheep. These sheep are white in color with a semi-open fleece. Sheep are large in size with a heavy-framed body (**Figure 6**). The head is large and heavy characterized with a prominent Roman nose in the ram. The forehead

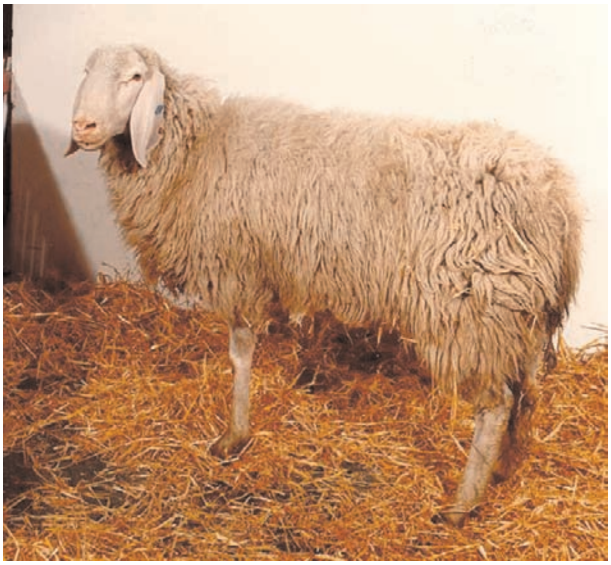

Figure 6 Bergamasca ewe. (Photograph courtesy of Italian Sheep Breeders' Association.)

and upper parts of the nose are covered with wool. The ears are wide and long, often hanging below the level of the jaws. Animals of both sexes are hornless. The cheek, ear, muzzle, abdomen and lower parts of the legs are bare. The body weight of mature rams and ewes averages 80–90 kg and 60–70 kg, respectively.

Fleece weights are 4.6–5.2 kg for rams and 3.7–4.0 kg for ewes. Staple length varies with the month of shearing from 4–6 cm in March to 8–10 cm in September. The fleece has a spinning count of 40–48 s and breaking strength of 8–10 g. The ewes are highly fecund, with 95% fertility and a prolificacy of 1.5 lambs at birth. Body weights of male and female lambs are 4.9 and 4.5 kg at birth; 15 and 12 kg at 30 days; 29 and 22 kg at 90 days; and 41 and 33 kg at 180 days of age, respectively. Milk production has been estimated at 160–180 kg per lactation.

Churro

Churro, which means coarse wool, consists of several varieties of sheep; the Bragan a Galician, Miranda Galician, Badano, Mondego, Churro do Campo and Algarve Churro types found in the northern Portugal, as well as the Cantabrian and Pyrenean, Occidental and Gallega varieties in Spain (**Figure 7**). These sheep grouped under the Portuguese coarse-wool Churro (Bordaliero) type, are raised primarily for meat and also used for milk production. The proportion of white-colored sheep has increased to about 85%. Nevertheless, there is a small proportion of brown- and black-colored sheep. Rams are horned whereas ewes may be either horned or polled. The ewes are marginally prolific, producing 1.2 lambs at birth. The body weight of lambs at birth is 4 kg for singles, 3.3 kg for twins, and 2.8 kg for triplets. Lambs weigh 29 kg at 30 week of age, and 56 kg at maturity. Fleece weight ranges from 1.35 kg at 30 weeks to 1.97 kg at 4–8 years of age.

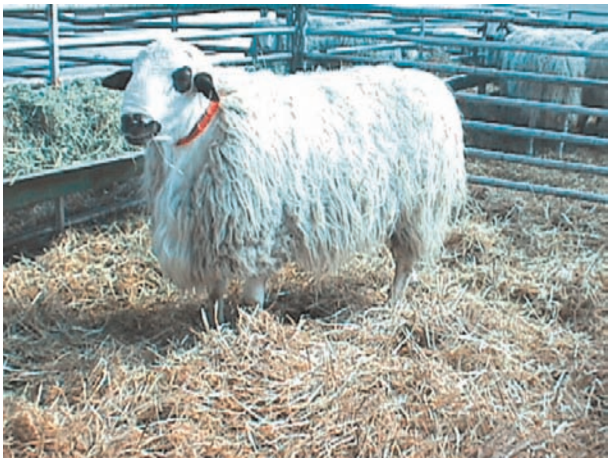

Figure 7 Churro ewe. (Photograph courtesy of Dr L.F. de la Fuente.)

In the Castilla–Leon region of Spain, ewes produce 91 ml of milk daily. The milk has a fat content of 6.8% and protein content of 6.3%. Churro da Terra Quente ewes produce 132 l of milk in 120 days of lactation. In France, milk production was lower at 68 l per lactation. The udder was 9.3 cm in depth, 12.2 cm in width and 46.6 cm in circumference. The 18% total solids in the milk includes 6.1% fat and 5.5% protein. The daily fat content of milk in the first three lactations varied from 7.1% to 8.4%.

Comisana

The Comisana breed originated in southeast Sicily from crosses between indigenous Maltese and Sicilian sheep (**Figure 8**). Subsequent introductions of rams of the Barbary breed from Tunisia have caused a reduction in milk yield. Comisana sheep, raised primarily for milk and meat produce coarse wool fleeces. The medium-sized breed, with mature ewes weighing 50 kg, is polled. Sheep are white in color with a reddish-brown face and semilopped ears. The ewes are slow maturing, with onset of estrus at 12–13 months of age. Ewes are prolific producing 1.8 lambs at birth. The first lactation milk yield of 62 kg was estimated from weight gained by lambs suckling for 35 days. In the subsequent 160–200 days of milking, 148 kg of milk was produced. The official estimate of milk production was 159 kg per lactation with 6.5% fat and 5.0% protein content. The highest record for milk production was 500–600 kg in 190 days of lactation.

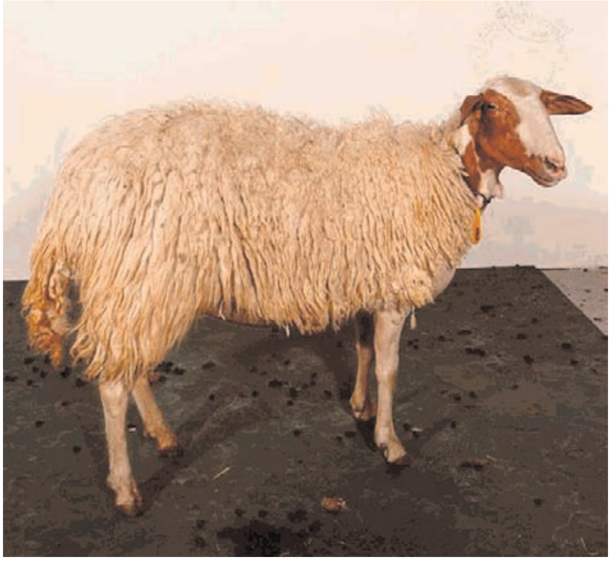

Figure 8 Comisana ewe. (Photograph courtesy of Italian Sheep Breeders' Association.)

Karaman

The fat-tailed Karaman sheep are known to have originated in Turkey. The Karaman sheep raised throughout central Anatolia are white in color while those raised in eastern Anatolia are red or brown in color. The majority of rams are polled while ewes are always polled. The white variety of Karaman sheep weighs 40–45 kg at maturity whereas the red variety is 3 kg heavier. In Turkey, the ewes are marginally prolific producing 1.3 lambs at birth. Karaman sheep are also found in Syria and Iran. In Iran, the common name of these sheep varies from region to region.

In Turkey, Karaman sheep produce 51–78 kg of milk in 130–144 days of lactation. In Iran, milk production of Ghezel sheep estimated by a combination of lambs suckling and hand-milking was 149 kg in 173 days of lactation. Daily milk yields during suckling and postweaning periods were 1.2 kg and 0.3 kg, respectively.

Lacho (Manech)

The Lacho originating in the Basque region of Spain are dual-purpose sheep raised for milk and meat production. The coarse carpet-wool type Lacho sheep resemble Churro sheep. These sheep have long wool with a black, brown or gray colored face and feet. Rams have horns and ewes are either horned or polled. In Spain, Lacho sheep produce 130 l of milk in 150 days of lactation. In France, these sheep known as Manech (**Figure 9**) produce 948 ml of milk daily. This represents 367 ml from machine milking, 375 ml from machine stripping and 206 ml of residual milk. The average milk production from 40 000 Manech ewes was 90 l in 127 days of lactation.

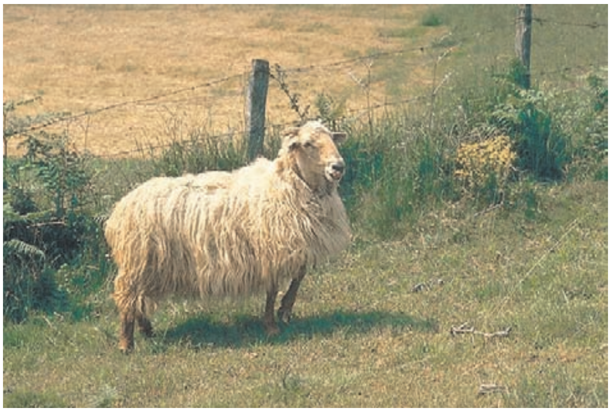

Figure 9 Manech ewe. (Photograph courtesy of Dr Vidal Montoro.)

Langhe

Langhe are a large sized, dual-purpose Italian sheep raised for milk and meat production. These sheep are white, polled, early maturing and produce coarse wool of carpet type (**Figure 10**). Ewe lambs bred at 8 months of age produce offspring between January and March. About 80% of the ewes produce offspring as yearlings, while the remaining do so at 2 years of age. In the normal breeding season, ewes have a prolificacy of 1.4, with a twinning rate of 38%, and multiple birth rate of 1%. In ewes synchronized to breed out of season, fertility and fecundity were 73% and 95%, respectively, with a prolificacy of 1.3 lambs at birth. First lactation milk production was 77 kg over a 35-day suckling period (estimated from lamb weight gain) and 172 kg over the subsequent 160–200 day period. Milk contains 6–7% fat, 5–6% protein and 5% lactose. In the

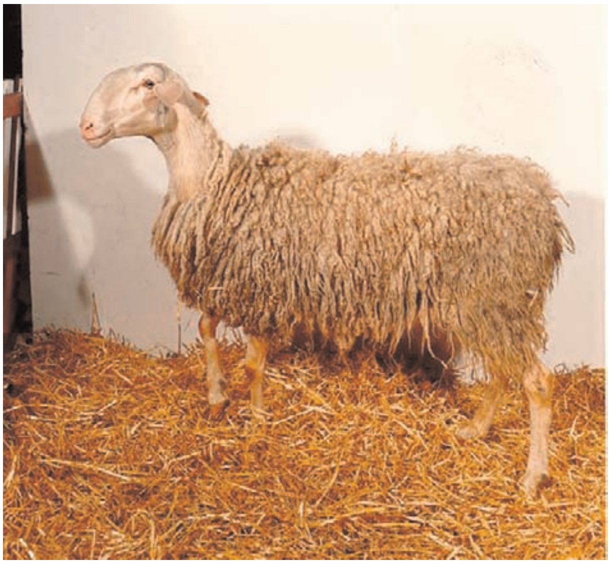

Figure 10 Langhe ewe. (Photograph courtesy of Italian Sheep Breeders' Association.)

first three lactations milk production was 198 kg for ewes with singles and 227 kg for those with twins.

Latxa

Latxa sheep are also known as Blondofaced Lacco in the Spanish Pyrenees. These sheep are similar to the Manech sheep in the French Basque country. The ewes produce 138 kg of milk per lactation, which contains 5.8% fat and 5.1% protein.

Manchega

The Manchega sheep originating in the La Mancha and New Castille regions of Spain is a dual-purpose breed raised for milk and meat production (**Figure 11**). The wool is coarse and of medium quality. These sheep are polled and usually white in color, but some are black. In the first four weeks of lactation, milk yield in ewes with singles was 43.7 kg, and in those with twins was 80.4 kg. Corresponding milk yields in the subsequent 4 weeks of lactation were 34.4 kg and 45.4 kg for totals of 78 and 126 kg for 8 weeks of lactation. The milk is composed of 8.59% fat, 4.51% protein, 5.18% lactose and 19.3% dry matter.

Massese (Massa)

The Massese breed originated in the province of Massa Carrara in Italy. It is a dual-purpose breed raised for milk and meat production (**Figure 12**). The sheep resemble the Garfagnana breed, but are smaller in size and grey or brown in color, with dark head and extremities. Rams and ewes are horned. Wool is relatively uniform and dark in color. Ewe lambs are mated at 10–11 months of age. Ewes are marginally prolific with 1.3–1.4 lambs weighing 3.6–4.5 kg at birth.

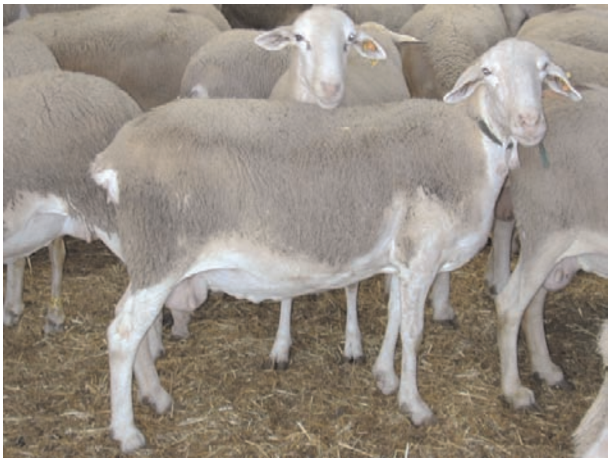

Figure 11 Manchega ewes. (Photograph courtesy of Italian Sheep Breeders' Association.)

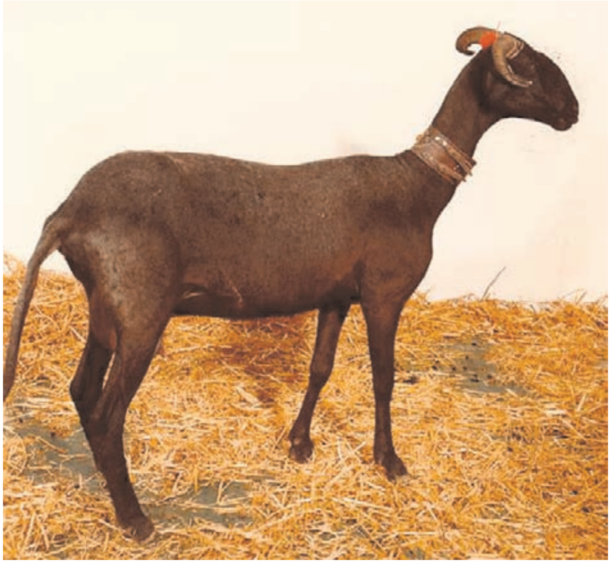

Figure 12 Massese ewe. (Photograph courtesy of Italian Sheep Breeders' Association.)

During the first part-lactation, milk yield was 73 kg (estimated from lamb weight gain) over a 35-day suckling period. Correspondingly, ewes produced 160 kg of milk over the subsequent 160–200 days of lactation. Estimates of milk production vary with season of lambing: 197 kg in 158 days of lactation (spring), and 218 kg in 232 days of lactation (winter). In general, most ewes produce 178 kg of milk in 254 days of lactation. The milk is composed of 7.3–8.4% fat, 5.7–6.0% protein, 4.3–4.9% lactose and 19.7% total solids.

Mehraban

Mehraban is a fat-tailed, coarse wool breed originating in western Iran (Hamadan) and mainly raised for meat, with milk as a secondary product (**Figure 13**). The color of the

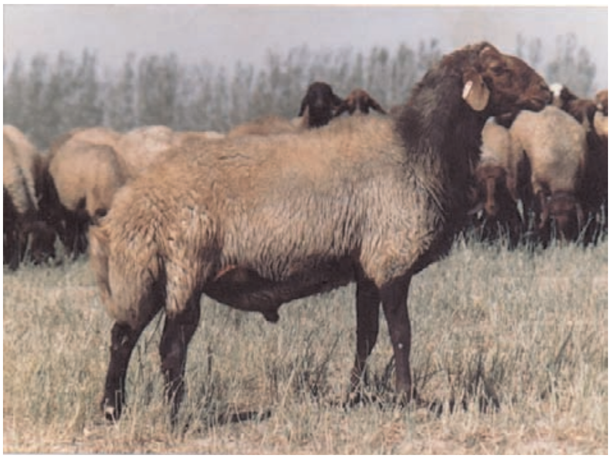

Figure 13 Mehraban ram. (Photograph courtesy of Dr Ibrahim Nooriyan.

sheep varies from light brown to creamy or gray with a dark face and neck. Milk production was estimated from a combination of suckling (15 weeks) and hand-milking. Daily milk yield was 1.2 kg during the suckling period, and 0.3 kg during the postweaning period. In general, ewes produced 142 kg milk in 177 days of lactation.

Serra de Estrela

Serra de Estrela sheep originating in northern and central Portugal are known as Bordaleiro, a term used primarily to identify different types of sheep between the fine-wool Merino breed and the coarse-wool Churro breed. Serra de Estrela is a dual-purpose breed raised for milk and meat production. The wool is coarse and of medium quality. The sheep are white in color with brown spots on the head and legs and some are completely black. The white variety of Serra de Estrela sheep produces 144 kg of milk in 205 days of lactation whereas the black variety produces 183 kg in 208 days of lactation. Ewes of 20 months or younger produce 97 l of milk in 138 days of lactation or 138 l of milk in 195 days. The maximum milk yield recorded was 497 l in 269 days of lactation.

Skopelos

Skopelos sheep are found on the islands of northern Sporades and in some areas of mainland Greece. The Kimi variety common in the lowlands was derived from the Skopelos breed. These sheep are white or yellowish white in color with black or red-brown spots on the face and the extremities. Some have white spots on the legs and at the tip of the tail, while others are completely red. Rams have short slender horns, but a small proportion are polled. Ewes are polled, and a small proportion have small curled horns. Body weights of mature rams and ewes are 58–60 kg and 40 kg, respectively. Ewe lambs are usually bred at 8–10 months of age. The lambing season extends from November to June. The ewes are prolific, producing 1.84 lambs at birth. Ewes milked after 21 to 42 days of suckling produce 233 kg in 232 days of lactation. All ewes officially recognized as a part of the Skopelos breed on the island of Skopelos produce 155 kg of milk in a lactation. The milk contains 8.4% fat. The udder is large and cone-shaped, with sizeable teats. Supernumerary teats are common.

Dairy Breeds with Low Milk Production

Breeds with a milk production potential of less than 100 kg per lactation are classified into this category. Although many breeds from around the world fall within this category, only those with accurate information are listed here. Milk production, length of lactation and milk composition of breeds with low milk yield are presented in **Table 6**.

Barbary

The Barbary breed, derived from the fat-tail sheep of the Near East and southwest Asia, is found in North Africa and southern Italy (Barbaresca). The Barbary accounts for 95% of the sheep population in Libya and Tunisia, usually kept in nomadic flocks of 200–300 sheep. Considerable variation in morphological characteristics and production performance of Barbary sheep in different countries has been reported. These sheep are generally white in color, but some are colored or have colored spots. The head is brown, black or spotted brown and sometimes white, while the legs are colored. In Libya and Algeria, rams have horns while ewes are polled. Occassionally, ewes have small straight horns. In Tunisia, the rams and ewes are usually polled. Body weights of mature rams and ewes are 40–60 kg and 35–50 kg, respectively. Barbary ewes in Libya produce 40–53 kg of milk in 160 days of lactation; in Tunisia they produce 90 kg of milk in 120 days of lactation; and in Sicily the ewes produce 92 kg of milk in 212 days of lactation. In year 2000, yearlings recorded 63 l in 100 days of milk recording in Italy.

Epirus

The Epirus sheep, indigenous to Greece, are raised in the mountains under extensive management. The ewes, following weaning at 49 days, produce 92 kg of milk in 217 days of lactation. Epirus ewes produce 0.4 kg of milk

Table 6 Other minor breeds with low milk yield potential

Breed	*Country*	*Milk yield (kg)*	*Lactation period (days)*
Florina	Greece	75	145
Karagouniko	Greece	70	135
Moscia Leccese	Italy	88	180
Roumloukian	Greece	71	139
Tsigai	Slovak Republic	82–89	131–155
Vlakhiko	Greece	76	147

daily. The milk is composed of 7.8% fat, 6.6% protein and 4.8% lactose.

Kivircik (Thrace)

The Kivircik breed, whose name in Turkish means ‘curly coat of the lamb’, is found in Thrace and northwest Anatolia. Kivircik sheep confined to western Thrace in Greece are known as the Thrace breed. This is the only breed in European Turkey identical to the Karnobat breed of Bulgaria. The morphological characteristics and production performance appear to vary considerably among populations. These sheep are generally white in color, however 10–15% may be brown or black. The tail varies from long and thin reaching the hocks, to short with a variable amount of fat in the base. Kivircik sheep produce tender and juicy meat with little subcutaneous and tail fat. Body weights of mature rams and ewes are 40–45 kg and 30–40 kg, respectively. About 85–92% of the ewes are fertile and marginally prolific producing 1.1 lambs at birth. The breed has a low potential for milk production. Ewes produce 63 kg of milk in 140 days of lactation. Another estimate of 91 l of milk in 187 days of lactation has been reported.

Rouge de l’Ouest

The Rouge de l’Ouest sheep originated in northwestern France. The head is wine-red in color. The fleece is long with medium wool. Rouge de l’Ouest sheep are kept for milk production in commercial flocks. The ewes produce 78 l of milk in 105 days of lactation following a suckling period of 48 days. The milk is composed of 7.6% fat and 6.2% protein. The udder averages 93 mm in depth with a cistern height of 14 mm. The teats are 32 mm in length with a 17 mm diameter and an inclination of 26.5 degrees.

Serrai

The Serrai sheep is a dual-purpose breed indigenous to Greece. The breed is also referred to as Seres, Serres or Seris. These sheep have coarse wool and a low milk production potential. The Serrai sheep are usually white in color with black marks on the head and legs. Rams are horned whereas ewes are usually polled. Ewes produce 71 kg of milk in 157 days of lactation.

Somosierra Blond

The Somosierra Blond, indigenous to northeastern Spain, is a dual-purpose sheep breed raised for meat and milk production. These sheep are white in color with a pale brown face and legs. Rams and ewes are usually horned. Daily milk yield of ewes recorded once a month for 4 months following weaning at 50 days was 1016, 790, 625 and 493 ml, respectively. Correspondingly, milk fat content was 5.1%, 5.5%, 6.3% and 7.7%, respectively, and protein content was 5.1%. Ewes lambing in July had lower milk yield than those lambing in November.

Newly Developed Dairy Breeds

The majority of the new breeds of dairy sheep were developed from crosses between East Friesian and indigenous sheep in the various regions. The development programs were undertaken mainly because East Friesian-cross sheep failed to sustain increased milk production potential and/or crossbreeding caused deterioration in characteristics considered desirable in the indigenous sheep.

Assaf

The Assaf breed was developed in Israel from crosses between Awassi and East Friesian breeds (**Figure 14**). In Israel, there are 40 000 Assaf sheep, on average producing 333 l of milk in 180 days of lactation. The ewes are prolific, producing 1.6 lambs at birth. Assaf sheep, recognized for milking ability, have been exported to Abu Dhabi, Jordan, Portugal, Spain and Peru.

British Milksheep

The British Milksheep was developed in England with 70% East Friesian, 10% Blue-faced Leicester, 10% Polled Dorset, 5% Lleyn and 5% from a composite of three breeds. The primary objective was to produce rams for use as terminal sires, to mate with ewes of different breeds raised in varying environments. The resulting crossbred offspring were expected to be highly productive. The British Milksheep is a robust, active, tough,

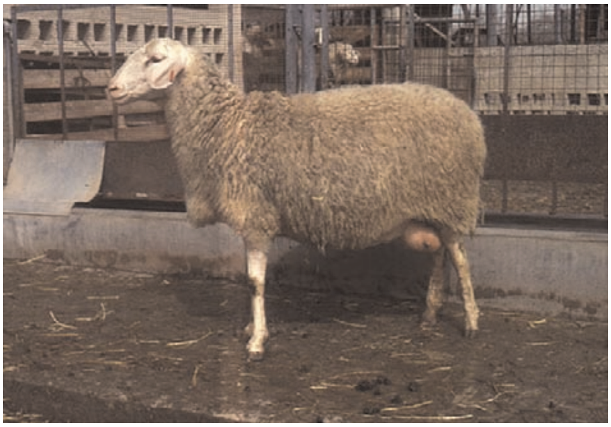

Figure 14 Assaf ewe. (Photograph courtesy of the Volcani Agricultural Research Centre, Israel.)

hardy and docile sheep that can adapt to the surrounding environment. These sheep are medium to large in size and polled, with a white face and legs. Fleece weight averages 6.5 kg in rams and 4.5 kg in ewes. The semilustre wool has a spinning count of 50–54 s with a staple length of 15–17 cm. British Milksheep ewes are highly prolific, producing 2.2 lambs as yearlings, 2.6 lambs as 2-year-olds, and 3.1 lambs as mature ewes. The ewes produce 650–900 l of milk in 300 days of lactation. The milk solids are particularly high with protein content rising from 5% in early lactation to 7.5% in late lactation, and fat content rising from 5.5% to 9%.

FSL

In France, a breeding program was initiated in 1967 with the intention of combining the East Friesian, Sarda and Lacaune breeds into a composite population, the FSL breed. The main objective was to develop sheep with potential for increased milk and meat production. The East Friesian breed, which excels in prolificacy and milk production, was crossed with the Sarda breed, noted for ease of milking, and the Lacaune breed contributing genes for hardness and meat quality. The backcrosses of $\frac{3}{4}$ East Friesian × $\frac{1}{4}$ Lacaune, and $\frac{3}{4}$ Sarda × $\frac{1}{4}$ Lacaune were mated to produce a population composed of 37.5% East Friesian, 37.5% Sarda and 25% Lacaune. During the first stage of development ewes produced 175 l of milk in 210 days of lactation.

Frisarta and Frisonarta

In Greece, a breeding program was initiated to develop the Frisarta breed by crossing East Friesian rams with a population of indigenous sheep consisting of crosses among the Chios, Karagouniko and Zakynthos breeds. The Frisarta breed was developed with the objective of improving milking potential and prolificacy of indigenous sheep. The greatest concentration of these sheep is on the plains of Arta in western Greece. The morphological characteristics of Frisarta sheep resemble those of their East Friesian ancestors. These sheep are white in color. Both rams and ewes are polled. The breed is characterized by a long thin tail. At maturity, ewes weigh 70–80 kg. Ewe lambs can be bred for the first time at 8 months of age. The ewes are highly prolific, producing 1.8 lambs at birth. The fleece weight ranges from 2.0 to 5.5 kg. Frisarta ewes produce 230–250 kg of milk in 220 days of lactation following a 42-day suckling period.

Rideau

The Rideau breed was developed in Canada to perform under intensive management. The development started in 1966 with a foundation stock consisting of ewes and rams from several British breeds, Ile de France, Finnsheep and East Friesian with a final combination consisting of 40% Finnish Landrace, 46% British breeds and 14% East Friesian. Rideau sheep are generally white, although color spots may appear on head and legs (**Figure 15**). Mature body weight for rams is 80–100 kg and for ewes 70–90 kg. Ewe lambs weighing 47 kg can be bred at 7 months to lamb at 1 year of age. Under intensive systems, ewes can lamb at 8-month intervals with conception rate of 55–57% for ewe lambs and 80–82% for adult ewes. Prolificacy is 1.7 lambs for yearlings and 2.5 for adult ewes. Multiple births account for 82% of all litters. Lambs weigh 3.4 kg at birth and about 36 kg at 118 days of age. Rideau ewes machine milked twice daily for about 120 days following weaning at 30 days produced 77 l of milk, containing 6.6% fat, 5.8% protein and 4.8% lactose.

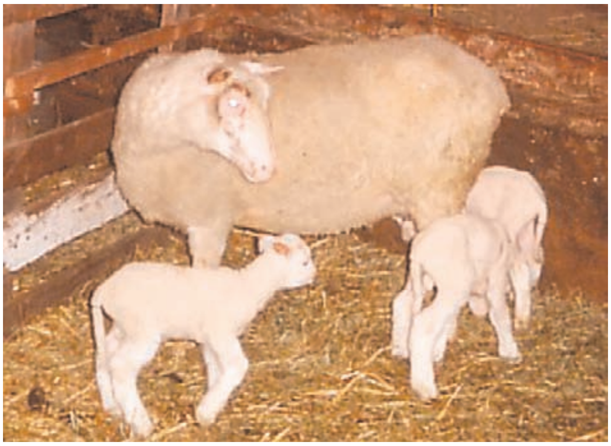

Figure 15 Rideau ewe with lambs. (Photography courtesy of Agriculture and Agri-Food, Canada.)

Tahirova

Turkey initiated a breeding program in 1964 to develop the Tahirova breed by crossing East Friesian rams with a population of indigenous Kivircik sheep. The genetic composition of the Tahirova breed is 25% Kivircik and 75% East Friesian. The breed was developed with the objective of improving milk and meat production potential. Tahirova sheep are white in color, polled, open-faced and thin-tailed. Lambs weigh 4.0–5.4 kg at birth and 28–30 kg at 90 days. Body weights of mature rams and ewes average 80–90 kg and 55–65 kg, respectively. Ewe lambs attain puberty at 290 days. The ewes are prolific producing 1.5–1.8 lambs at birth, and 60% of the lambings are multiple births. Lamb mortality to weaning is about 5%. The fleece weighs 3–4 kg with a spinning count of 50 s or better. Ewes produce 250–300 kg of milk in 200–240 days of lactation. In Turkey, a number of populations of highly fecund dam lines suitable for crossbreeding have been derived from the Tahirova breed.

Synthetic Milk Sheep

In Bulgaria, a synthetic breed yet to be named has been developed from crosses of Pleven Blackhead, East Friesian and Awassi breeds. The main objective has been to develop sheep with potential for increased milk and meat production. These sheep produce 3.25 kg of fleece with a staple length of 13.5 cm. Ewes produce 280–350 l of milk in 180 days of lactation.

Future Prospects

In the last three decades, there has been increased interest in milk production from dairy sheep with the establishment of a number of large-scale commercial farms. The depressed wool prices have forced many countries to reorient sheep production from wool toward the production of meat and milk. The growing interest in prolific sheep has been analogous to the expansion of the cheese industry based on dairy sheep. An example of this new direction is evident on the North American continent where prolificacy and milk production are gaining popularity. This is because farmers are looking for prolific ewes with milk production potential to raise more lambs. Even New Zealand, whose economy is based on sheep meat and wool production, has started moving toward alternative sources of revenue based on processed sheep milk. This phenomenon is also evident in Eastern Europe.

There are a number of factors that have previously contributed to the development of the modern dairy sheep industry. These include:

1. The role of milk recording, and the standardization of procedures for milk recording among important milk-producing nations.
2. The use of mixed model methodologies for the prediction of breeding values and the identification of sheep breeding stock with superior potential for milk production.
3. Newly designed milking machines to help minimize stripping and residual milk.
4. Improved management and feeding of milking ewes.
5. Preventive measures to control diseases.
6. Increased efforts to develop new dairy breeds with potential for increased milk production, superior udder conformation and teat placement.

It is hoped these attributes and others will continue to play an important role in the future development of the sheep milk industry.

See also: **Dairy Farm Management Systems**: Sheep. **Genetics**: Selection: Concepts. **Husbandry of Dairy Animals**: Sheep: Feeding Management; Sheep: Milking Management; Sheep: Health Management; Sheep: Multipurpose Management; Sheep: Reproductive Management. **Milk**: Sheep Milk.

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Water Buffalo

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Introduction

The water buffalo (*Bubalus bubalis*), a ruminant mammal of the Bovidae family, is a very important dairy and work animal in many countries of Asia and Africa (*see* **Mammals**). It does not live in water as the name might imply, rather it has been domesticated along the water-courses such as riversides in the Asian subcontinent. The name probably is to distinguish it from bison (*Bison bison*), which has long enjoyed the designation of 'buffalo' in the American continent. According to FAO, there were at the beginning of the third millennium about 170 million buffaloes in about 30 countries ranging from Australia through Indonesia and the Philippines: in Asia, they are found in Thailand, China, Russia, India, Pakistan, Burma, Iran, Iraq, Turkey, and Egypt; and in Europe, in Italy, Romania, and Bulgaria. Among these countries, India (55%), China (15%), and Pakistan (10%) comprise most of the population, which is only one-ninth of the bovine population of the world. There are two main types: the swamp buffalo and the river buffalo.

Swamp Buffalo

The swamp buffalo is the principal work animal of rice-growing countries of Southeast Asia. The countries that hold the greatest numbers are China and Thailand. Animals are used to plow and harrow, and to puddle the paddy fields before planting rice. Pulling carts and sledges is also common. They are rarely milked but are often eaten toward the end of their long working life. There is only one breed, the swamp buffalo, with many types and strains. These are usually stockily built with huge variation in their size or weight (250–1000 kg) and also in the darkness or lightness of their gray color. The long horns grow outward laterally and horizontally in the young animals and curve round in a semicircle as the animal gets older. The degree of horn curvature and size varies.

River Buffalo

The river buffalo is primarily a dairy animal and it differs from swamp buffalo both genetically (the river buffalo has a chromosome number of 50, while the swamp buffalo has 48) and in its physical appearance. River buffaloes are very docile and easygoing animals and they prefer to wallow in clear water compared to swamp buffaloes, which prefer a mud wallow. India and Pakistan are home to most of the river buffaloes of the world. Many breeds and varieties are found including the famous Nili-Ravi (**Figure 1**), Kundi, Murrah, Jafarabadi, Surti, and the Mediterranean. There is a wide variation in color and horn shape. The animals are mostly black, but other shades such as gray and slaty gray do occur. There may be white markings on the forehead and the lower half of the legs along with a white switch of the tail. Brown-colored water buffaloes are also not uncommon. Piebalds and albinos also exist. The horns are usually short and may curl tightly forward, downward, or backward. Some breeds possess uncurled long horns similar to those of swamp buffaloes. For example, the horns are tightly curled and set close to the head (downswept) in the Jaffarabadi, Kundi, Murrah, and Nili-Ravi breeds. In Bhadawari, Mediterranean, Mehsana, and Surti, they are sickle-shaped and upswept. Adult body weight varies widely across breeds. In the heavy breeds, males may weigh 500–600 kg, while females usually weigh 450–500 kg. There are no polled buffalo breeds and, unlike with cows, farmers do not like to dehorn them.

Crossbreeding between River Buffalo and Swamp Buffalo

River buffaloes have successfully been crossed with swamp buffaloes, with the F_1 hybrids having a diploid chromosome number of 49. *Inter se* matings and backcross matings also produce fertile individuals. The unbalanced karyotypes of crossbreds may, however, affect the process of gametogenesis in the males and may result in lower fertility. Murrah and Nili-Ravi have been the principal breeds for crossing with the swamp buffaloes.

Productivity

The river buffaloes are multipurpose animals. Milk production, however, remains the primary objective, apart from meat, draught, and manure (as fertilizer and fuel).

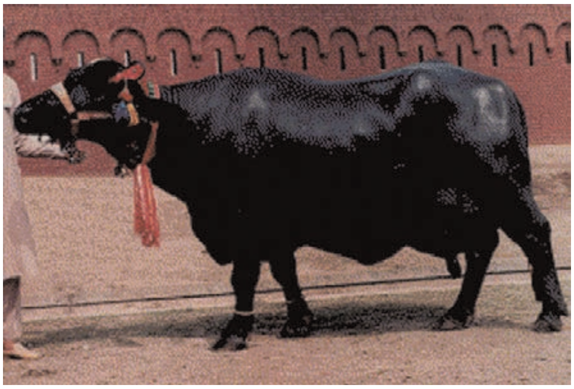

Figure 1 The Nili-Ravi buffalo of Pakistan.

Most breeds produce around 5 l day^{-1}, whereas good breeds on an average have a daily yield of 8–10 l for a lactation period of 10 months. A herd average of 2500 l in Nili-Ravi, Kundi, Murrah, Surti, and Mediterranean breeds is not unusual under commercial setups. Exceptionally good animals have been reported to produce more than 6000 l per lactation. Buffalo milk has comparatively higher milk fat and nonfat solids as compared to milk from cattle (**Table 1**). Higher fat percentage in their milk (more than 6.5%) is one of the major reasons for their popularity with the farmers. The Italian Mozzarella cheese is also a speciality made from buffalo milk (*see* **Husbandry of Dairy Animals:** Buffalo: Mediterranean Region). Buffalo milk and milk products are white because carotene is absent. They are, however, rich in vitamin A. Buffaloes have a long productive life. They may continue to give a satisfactory amount of milk up to 18 years of age or more. Growth potential is fairly good. The growth rate may range from 0.5 to 1 kg day^{-1}. Buffalo meat is generally similar to beef from cattle in structure, chemical composition, nutritive value, and palatability. It is lean and tender. The muscle fibers are thicker with more numerous cell nuclei and very little marbling. The body fat is white due to the absence of β-carotene. It has less saturated fat and cholesterol. There is no 'buffalo flavor' in the meat.

Reproduction

Buffaloes generally take longer than cattle to attain puberty under similar environments. Age at first calving is 35–50 months with a calving interval of 400–500 days. Females are generally shy in their reproductive behavior. Homosexual behavior in females in estrus is almost nonexistent. Other estrous symptoms are also not very pronounced. Therefore, determination of the optimum time for artificial insemination is sometimes difficult. Vasectomized males have been used successfully to improve detection of females in heat. Late age at puberty, at least within a breed, is more a function of feeding and management than of genetics. The reduced number of cycles after calving as compared to cattle and the onset of anestrus in the absence of conception tend to make buffaloes a seasonal breeder.

Females remain pregnant a little more than 10 months and have very strong instinct to own their young ones. Weaning is possible as is being practiced in many commercial setups especially where buffaloes are machine milked, but some females refuse to be milked without the presence of their calf.

Males start producing good-quality semen at the age of 2 years. Seminal characteristics and sexual behavior coincide with the natural breeding season in female. Semen and embryo freezing is successful. Artificial insemination in buffaloes is also being practiced successfully.

Diseases

Most of the diseases that affect cattle are also found in buffaloes. The control, prevention, and treatment of the disease also follow the same lines. The most important infectious diseases include hemorrhagic septicemia, anthrax, foot-and-mouth disease (*see* **Diseases of Dairy Animals:** Infectious Diseases: Foot-and-Mouth Disease), and rinderpest. Susceptibility to bovine tuberculosis may be similar to that of cattle, but tuberculosis lesions are less often found in the thoracic cavity than in cattle (*see* **Diseases of Dairy Animals:** Infectious Diseases: Tuberculosis). Affected buffaloes respond more markedly to tuberculin and the skin reaction tends to persist longer than in cattle. Buffaloes have also been reported to be more resistant to rinderpest than cattle, but susceptibility may vary in different parts of the world. Buffaloes also appear to be less susceptible to foot-and-mouth disease. Symptoms may be less severe and recovery after the disease outbreak is usually quicker. Mastitis resistance is also believed to be better in buffaloes, but a gangrenous

Table 1 Composition (%) of buffalo milk in comparison with cow milk

	Fat	*Protein*	*Lactose*	*Ash*	*Nonfat solids*	*Total solids*	*Water*
European cow	3.7	3.2	4.8	0.72	8.7	12.4	87.6
Zebu cow	5.3	3.3	4.7	0.76	8.8	14.0	86.0
Buffalo[a]	6.9	3.8	5.1	0.75	9.6	16.6	83.4

[a] Reproduced from Dastur NN (1956) *Dairy Science Abstract* 18: 968–1008.

condition of the udder after a severe mastitis attack may develop more quickly.

Other Attributes

Buffaloes can graze a wide variety of herbage and will take unpalatable vegetations ignored by cattle. Their unique ability to digest and assimilate cellulose and to utilize efficiently poor-quality feeds such as paddy or wheat straw and stubbles is well recognized. They can grow and sustain weight gain on less productive pastures.

The Future

The number of dairy buffaloes in the world has been increasing over the years in spite of the presence of other dairy species and in the absence of any advertising extravaganza such as is prevalent for developed dairy cattle. It is an indication of their growing importance. They remain the major milk-producing animals in India, Pakistan, and Egypt, where one-sixth of the world's human population lives. Swamp buffalo populations, on the other hand, are under much pressure due to mechanization. Most of the genetic improvement programs in dairy buffaloes are in their inception. Limited initial investments available in most of the developing countries, where sizable concentrations of buffaloes exist, is the major constraint so far in making visible genetic gains. The potential, however, is likely to be exploited in the future. Population dynamics indicate that buffalo will produce enough to emerge as a future economic entity and the chances of it becoming a 'sacred buffalo' are remote.

See also: **Diseases of Dairy Animals:** Infectious Diseases: Foot-and-Mouth Disease; Infectious Diseases: Tuberculosis. **Fermented Milks:** Asian Fermented Milks. **Genetics:** Selection: Concepts. **Husbandry of Dairy Animals:** Buffalo: Asia; Buffalo: Mediterranean Region. **Mammals**.

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Yak

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Introduction

The yak is one of the world's most remarkable bovines, adapted to living on 'the roof of the world' in conditions so harsh that few other domestic animals can survive. Yak have many uses, milk production being only one of those. Although many sheep, a smaller number of goats, and the herdsmen's horses coexist with yak in many areas, the yak is the mainstay of life for the several million people – the herders and their families – in the remote regions of western China and other parts of Asia. The yak is integrally associated with the culture, religion, and social life of the peoples and communities of these regions and not only with the economy. It may be of interest therefore to provide a broad background to the characteristics and uses of the yak and its distribution before concentrating on the aspects related to milk.

The yak was listed as *Bos grunniens* by Linnaeus (*grunniens* because of the characteristic grunting noises made by the animals). More recent scholarship suggests a classification as *Poephagus grunniens*, which may be more appropriate, although it is not universally accepted.

Different names are often used for male and female yak and this can vary between countries and even localities. There is a profusion of names also for the hybrids of yak with other species of cattle, the names varying among regions and depending on the direction in which the cross is made and the generation of hybridization (backcrosses of various types). For simplicity, in this article, only the name 'yak' will be used (in both singular and plural sense) and will be prefaced, where necessary, by 'male' or 'female'. Crosses of yak with other species of cattle will be referred to as 'hybrids' and no distinction will be made between those where the yak is the dam and where it is the other species of cattle, except when required. Hybrids contributing to milk production will most often be the first crosses of the two species and most often with the yak as the dam.

Distribution, Adaptation, and Characteristics

Distribution

The domestic yak and its wild ancestor have a long history, with fossil remains dating back to the Pleistocene period. About 10 000 years ago, the yak was domesticated from its wild ancestor. Since then, it has spread from its original location on the Tibet–Qinghai plateau of China northward across the rest of this vast plateau (exceeding 2.5 million sq. km) to the Altai, southward over the Himalayas to their southern slopes, and from the Pamir in the west to the Minshan mountains in the east. In more recent times, yak have been introduced for commercial use to the Caucasus and other countries of the Russian Federation. A small population (around 2000) has grown up in North America from tiny introductions of yak since the late nineteenth century up to the 1920s and possibly later. There are also small herds in several parts of Europe and the rest of the world, including, for the past 50 years or so, a successful small herd on the Dunstable Downs in England (Whipsnade Wild Animal Park) in addition to yak in zoos and some reserves.

The majority of yak – and there are probably around 14 million in total – are found at high altitudes (between 3000 and 5000 m – though yak trek to higher elevations) and in cold climates, but at more northern latitudes colder climate to some extent compensates for lower altitude (around 2000 m). Sichuan Province of China may now have the largest yak population among the Chinese provinces with over 4 million, followed not far behind by Qinghai Province and the Tibet Autonomous Region. (It is possible that the major earthquake of 2008, which affected parts of the yak territory in Sichuan Province, may have impacted the numbers if some herders have moved elsewhere. This is not known at the time of writing.) Among the countries outside of China, Mongolia has the largest number with around 0.6 million. The great majority of herders and their families in the regions of China are ethnic Tibetan, and a majority of them live outside the Tibet Autonomous Region.

Environment

Almost without exception, the yak in China and the other parts of Asia live in a harsh environment where for several of the winter months conditions of near starvation are the norm. Extreme cold, thin air at high altitudes, often treacherous terrain, and very variable feed availability over the year have led to the development of adaptive characteristics of the yak to these harsh conditions. Vegetation on the mountain plateau is mixed but ample for a few short summer months, most often from the end of May to the end of August, resulting in rapid weight gain and fat deposition by the animals over that period. This is followed by progressive shortage of feed for most of the rest of the year, resulting in severe weight loss by the animals over winter and into early spring. Supplementary feed, such as conserved forage, arable residues, or grain is not usually provided to yak – it is simply not available. Generally, crops do not grow in these regions though advances in genetic technologies may allow limited changes in that respect in the future. Yak associated with some of the steeper mountain valleys, where crops are grown at lower elevations, sometimes benefit from some supplementary feed in winter – but that is an exception. As a rule, the feed in winter consists of the wilted herbage remaining on the pasture after the end of the growing season. The amounts decline as winter advances and, prior to the onset of new growth, the amounts of herbage reach critically low levels. The loss of body weight of yak over this period is frequently on the order of 20–25%. Yak will cope with light snow cover, scraping through this to the underlying herbage, but periodic severe snowfalls can lead to starvation and widespread deaths among the animals in the affected areas.

Adaptation

Adaptive characteristics of the yak to cold include its compact body with short neck, relatively short limbs, no dewlap, short ears, and short tail. The scrotum of the male and the udder of the female are small and hairy. Heat is conserved by a thick fleece comprising an outer coat of long hair ending in a long 'skirt' around the animal and, in the cold season, a dense undercoat of fine down. Vasoconstriction and a lack of sweating assist in conservation of heat. To cope with the low oxygen content at the high altitudes, the yak has a relatively large thorax containing large lungs and a large heart. Evidence that yak blood has a higher erythrocyte count and higher hemoglobin levels than the blood of other cattle is equivocal, with some studies showing this and the others not. However, there is some evidence that yak hemoglobin has a higher affinity for oxygen than hemoglobin of other cattle and that this attribute together with a high air intake allows adequate oxygen to be absorbed from the thin air.

Yak can graze both like other cattle, using their tongues to wrap round the herbage or shrub material, and like sheep to use the short grass and creeping stems – thus exploiting the often-sparse vegetation to best advantage (**Figure 1**). The yak is also exceptionally sure-footed to cope with difficult terrain, and this makes it a valuable pack animal. Thus, the animals help in the transport of camps and belongings of nomadic or seminomadic peoples, as the herds move across the summer pastures to exploit the available grazing and also move between the summer and winter pastures at higher and lower elevations, respectively. Yak were used, particularly in the more distant past, to carry goods on trade routes across the region and the passes over the Himalayas to countries on the southern slopes of this, the highest mountain range in the world. With the growth of tourism and mountain-climbing expeditions, yak are being used for trekking – the Sherpa people of Nepal being the best known among those in this trade. To cope with predators, wolves in particular, yak have also developed protective habits and postures. Unless specifically trained for human contact, yak are shy of people and readily panicked.

As a consequence of these adaptive characteristics, the pure yak are generally kept at the highest elevations or in the coldest parts of the region. Hybrids of yak with other species of cattle (discussed later) are often kept at

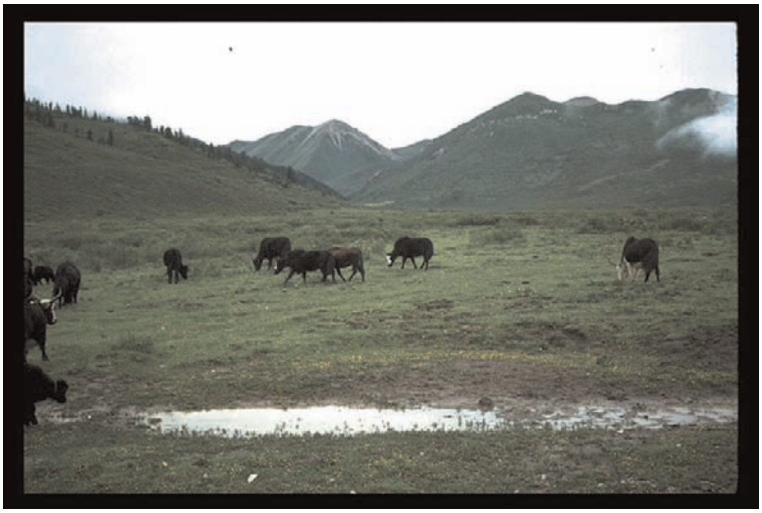

Figure 1 General view of yak grazing (Qinghai Province). Photograph courtesy of Dr. Gerald Wiener.

intermediate altitudes – below those of the pure yak – and cattle at levels below that.

While the yak is undoubtedly adapted to the harsh circumstances of its native region, its more recent introductions to temperate or even warm parts of the world suggest that the yak can cope with a wide variety of environmental and climatic conditions, contrary to the received wisdom about yak. The distribution of yak in North America illustrates this point. Many of the 90–100 yak herds there are within or adjacent to the Rocky Mountain range – an environment quite 'normal' for yak. However, a significant proportion of the herds are near the West Coast close to sea level, where snow and frost are unusual, or deep inland where climate is also far from traditional for yak.

Size of Yak

Relative to most dairy breeds in the west (e.g., Holstein cattle), yak are very small. Typically, adult females (6 years old and above) weigh, at the end of the summer grazing, around 200–300 kg depending on breed and perhaps 25% less at the end of winter. Bulls are typically between 50 and 100% heavier than the females. At birth, calves are only about 5–7% of the maternal weight.

Wild Yak

These proud beasts were once numerous, but have been hunted close to extinction, although they are now a protected species and conservation is intended. Estimates of the numbers remaining in small groups, mostly in parts of Tibet, vary, but the total may already be down to a few thousand animals. Wild yak are larger than their domestic cousins. They have been hunted for their meat by local people and, less excusably, by trophy hunters and 'sportsmen' from other countries.

Types and Breeds of Yak and Hybridization

Types

The two principal types of yak are the 'plateau' or 'grassland' type and the 'Alpine' or 'valley' type. The former is the most numerous and inhabits the central part of the Qinghai–Tibet plateau including the cold grasslands of Qinghai Province and Tibet, the western parts of Sichuan Province, and southern Gansu, as well as the Qilian mountain area. Yak in Mongolia and countries of the Russian Federation are probably of this general type. The 'Alpine' type is distributed over the Henduan mountain range of the southeastern part of the plateau, but also in parts of the Yushu Tibetan autonomous region, in the mountains and valleys of the western Sichuan and the northern Yunnan Provinces, and in similar locations.

Breeds

Several breeds are officially recognized and described as the main breeds. The largest in number among the plateau type are the Qinghai plateau and the Maiwa of Sichuan Province. The Tianzhu White of Gansu Province is the most distinctive because of its color. The majority of yak have dark-colored coats as protection against intense solar radiation at high altitude in the more southern latitudes. The principal breeds of the Alpine type are the Jiulong of Sichuan and the Alpine of Tibet. However, many more breeds are named, some very localized, some more widely distributed. With the relative isolation of many of the communities keeping yak, it is not surprising if different breeds develop. However, in strictly scientific terms, there is very little to show how different some of these breeds are from each other in their genetic makeup. Until now this would have been detectable only if the different 'breeds' were reared alongside each other in the same locations (which they are not) and crossed with each other. In future, information on the extent of genetic differences between the breeds may come from DNA profiles. Crosses of domestic yak with wild yak (achieved nowadays by use of AI with semen from captive wild yak bulls) have found favor with herders because of the larger size and hybrid vigor shown by the cross relative to the domestic yak. It has led to the development of a new breed – the Datong yak – recently recognized officially as a new yak breed.

Hybrids

For the most part, yak are bred pure. There is, however, a long-standing practice of hybridization of yak with other species of cattle, both *Bos taurus* and *Bos indicus* breeds, which are kept at lower altitudes. The hybrids generally inhabit the intermediate altitudes with a somewhat less stressful environment than that of the pure yak. The hybrids benefit from the hardiness and special adaptive qualities of the yak and the higher potential for productivity, especially in terms of milk production, of the other cattle. More recently, semen from breeds like the Holstein – developed in the West for high milk yield – has been used to inseminate yak cows to meet a higher demand for milk in selected districts. The availability of artificial insemination services is, however, restricted at present to a few readily accessible yak-rearing areas. Live bulls of these dairy breeds do not survive in the conditions of the region. Moreover, such 'improved' hybrids are more demanding in their nutritional and management requirements than pure yak or the hybrids with local hill cattle. Hybrids of yak with highly improved dairy breeds of cattle have therefore only a limited role to play over the whole region – in spite of much official enthusiasm for them. Male hybrids are sterile (though the female hybrids can be bred), and thus the hybrid type cannot be 'fixed' to create a permanent combination of high-yielding cow with hardy yak. This further limits the extension of hybridization as a means of meeting the demand for milk.

Uses of the Yak

Yak are used for milk, meat, transport, fur, and hide (clothes and tents), dung for fuel, and a host of by-products, some of cultural or medicinal significance. The yak are also – as is common among many pastoral people, with regard to other animals – a form of security for the herdsman and his family, with numbers representing wealth. High productivity of milk or meat from the animals is not the main objective of most of the people, although that may be the aim of advisory (extension) services, where these exist, and of the scientists aiming for 'improvement'. In areas of tourist interest, trekking with yak will be the main source of income (but such activity is mainly restricted to the 'Sherpa' country). The undercoat of the yak, the fine down, is used to make very high quality, cashmere-like, garments, but this is a minority trade.

Importance of Milk

The importance of milk relative to other products from the yak varies considerably with the location of the herd. In the more remote areas, milk and products from it are used mainly for consumption within the households. Maximum income, in such cases, is likely to come from the sale or barter of surplus animals, mostly castrated males, usually for meat. In an increasing number of areas with reasonable access to roads leading to markets, or perhaps to a dried-milk factory, milk becomes the source of a much more important component of the herder's income from yak and may account for 60% of the total. Nepal is the only country at present, among those rearing yak, with an established, active industry based on converting milk from yak and yak hybrids into cheese and other products through a government-sponsored scheme that set up cheese factories.

More recently, an ambitious enterprise was started in 2003–04 in Hongyuan County of Sichuan Province, P.R. China, an area where there is a large population of yak, with the aim of improving the income of the herders by exporting 'standardized' yak milk and milk products to both the Chinese and the international niche markets. Finance was made available from both Chinese government and international commercial sources. Roads were constructed or improved to assist in milk collection from the herders (**Figure 2**). Large-scale milk pasteurizing and processing plants were erected. The enterprise also promoted a breeding scheme to improve milk yield from yak and yak hybrids and introduced a program for producing winter fodder. The expectation was to get higher-than-normal financial returns for the products. However, at the time of writing (July 2010) reports have suggested (Han Jianlin and Xiang-Dong Zi, personal communications) that the export potential and financial advantages hoped for by the enterprise have not yet materialized. Most of the products are still marketed locally. As this scheme, which is likely to appeal to 'planners', might well presage similar production and marketing schemes for yak milk elsewhere, it will be useful to note some potential problems that might arise. To facilitate milk collection, there is likely to be a greater concentration of animals in the

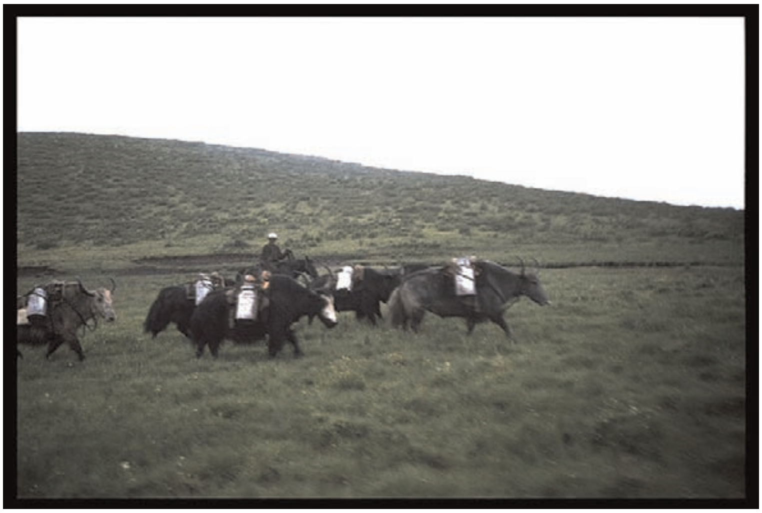

Figure 2 Milk collection (Nong Ri farm, Sichuan Province). Photograph courtesy of Dr. Gerald Wiener.

more accessible areas, that is, closer to the roads and hence more likely to be on the relatively lower ground of the winter pastures. This in turn could lead to overstocking of these areas, additional pressure on these pastures to provide winter feed, and additional range management problems affecting both winter and summer pastures. Moreover, a greater reliance on hybrid yak for larger milk yields leads to increased requirements for animal feed. It is not known whether these potential problems have as yet arisen in the present scheme or what solutions might be envisaged to resolve them, apart from the efforts to grow additional winter feed.

Milk Production

Milk yield per yak cow is low, judged by the modern dairy cattle standards. Total yields in the range of 200–400 l are quoted for the first 6 months following calving, for groups of yak of different breeds in China, with little or no production after 6 months. Such estimates make an adjustment for milk taken by the calf and are based on the groups under investigation, as milk recording is not practiced as a rule. Higher yields (400–700 l) have been claimed for former state-controlled and cooperative farms in Mongolia and Russia. Yak lactate again in a second season without a further calving and produce about two-thirds of the amount of milk as produced in the first season. (This second year of production is generally referred to in the literature from China as 'half milk' and is not to be confused with semiskimmed milk.) The vast majority of yak calve only once every 2 years. Hybrids can give substantially more milk depending on the cattle breed involved and the management system. There is a seasonal trend in lactation performance, rising to a peak in July and declining thereafter. There is no clear evidence of a lactation peak in relation to date of calving.

Milking

Pure yak females (and most hybrids) need the presence of the calf to initiate milk letdown. In traditional yak-keeping, the cow is tied up for milking – generally outdoors – and the calves are tethered and then set free, in turn, to suckle their dam for a short period. Thereafter the calf is removed, the yak cow is hand-milked (**Figure 3**), and finally the calf is allowed back to strip the udder. Hand milking stops in early winter (after October), but the calf may be allowed to continue to run with its dam. But whether the calf receives much milk from its dam owing to nutritional deprivation and weight loss in winter and early spring is open to question. An estimate from Bhutan suggests that the amount obtained may be 0.25 kg day^{-1} from December to March.

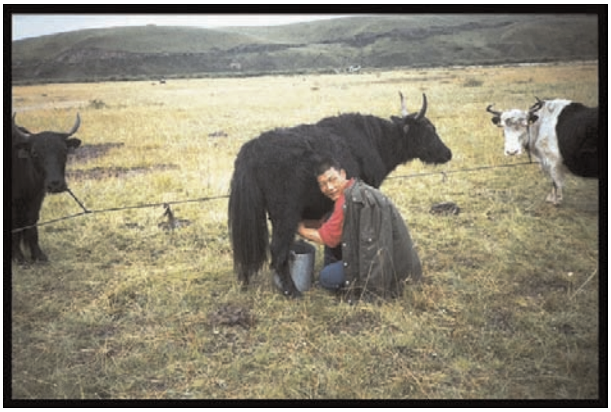

Figure 3 Man milking a yak tethered to a rope (Nong Ri farm, Sichuan Province). Women usually do the milking. Photograph courtesy of Dr. Gerald Wiener.

National Production

In China, the main country involved, total milk production from cattle and yak was estimated for 1996 at over 6 million metric tonnes (Information Exchange Department of China Ministry of Agriculture). Extrapolation from yak numbers and yield and the data from two provinces where yak predominate suggest that perhaps 10% of the total was attributable to yak (i.e., around 600 000 metric tonnes) – but this is only a rough estimate. Another crude method of estimation, from the total number of yak in China and their average milk yield, suggests 800 000 metric tonnes. Since 1996, milk production from cows has hugely increased in China, whereas there is no evidence that the total milk output from yak has altered similarly.

In Nepal, where milk production from yak and yak hybrids is encouraged, these accounted for about a quarter of the total liquid milk output of 62 000 metric tonnes in the year 1999–2000.

Although yak and yak hybrids with beef cattle in the United States and Canada at present represent only a small niche market, it may be of interest to note that the yak calves (and hybrid calves) are left entirely with their dams. Therefore, marketing yak milk or milk products has not yet become a commercial venture.

Milk Composition

Fat percentage varies seasonally but is in the range 6–7% on average. In the second year of lactating, if the female yak has not calved again, fat percentages are typically 1% higher – in line with the negative correlation between

Table 1 Yak milk composition

Sources	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Fat (%)	6.5	7.1	6.5	6.5	8.6	6.2 (7.4)	
Protein (%)	5.5		5.4	5.8		5.0 (6.4)	
Lactose (%)	5.5	5.0	4.6	4.6	3.3	4.9 (4.7)	
SNF (%)		8.8	10.9		7.1		
Ash (%)	0.8	0.7			0.4		
K (mmol l^{-1})							27.6
Na (mmol l^{-1})							20.8
Ca (mmol l^{-1})							36.8
P (mmol l^{-1})							24.8
Mg (mmol l^{-1})							2.5
α-Lactalbumin (% in protein)						3.8 (3.4)	
β-Lactoglobulin (% in protein)						15.3 (15.6)	
Serum albumin (% in protein)						2.2 (2.2)	

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6. Zheng Y, *et al*. (2000) *Personal Communication*. Milk from Jiulong yak in first summer season after calving and, in parentheses, milk in second summer season after calving without calving again (‘half milk’).
7. Zheng Y, *et al*. (2000) *Personal Communication*. Milk from Maiwa yak.

milk quantity and fat percentage found in other cattle. Other constituents of milk are shown in **Table 1** and are based on data from a variety of studies.

Utilization of Yak Milk

Traditional Uses

Raw milk

Whole milk is usually drunk only by people who are ill or weak, but is also given to children and old people. Generally, milk is boiled first, on health and hygiene grounds. Yak milk has a fragrant, sweetish smell and whole milk tastes somewhat sweet – so when drunk by herdsmen sugar is never added.

Raw milk is used mainly for a beverage called ‘milk tea’ – a mixture of tea and milk – and is a staple part of the diet. In the warm season, when there is plenty of milk available, or when served to guests, the brew will contain 20% milk or even more; the color of the drink is yellow. Herdsmen and their families more usually drink a lighter colored tea with only 5% milk added. Milk tea is brewed from tea leaves (cut from a tea brick) added to water and boiled for a few minutes; milk is added as required and boiling continues for a few more minutes. Sometimes a little salt is added. Also, mostly among Tibetan people, zanba may be added to the brew, making it both a food and a drink. Zanba (also zangba or tsampa) is the staple food of Tibet. It consists of roasted oat or barley flour, or a mixture of the two, made into a paste with yak butter and, for serving, usually rolled into balls. Though whole milk is normally used in the tea, skim milk is also used so that an increased amount of butter can be produced.

Milk boiled up with mushrooms and added salt is regarded as a delicacy by the herdspeople. Milk powder plants have been built in recent years to process raw milk into powder and limited amounts of butter and other milk products. Only little milk is sold for direct consumption in towns and villages in the upland areas (but note the potential commercialization of marketing in some areas – as mentioned earlier).

Butter

Butter is the main product from yak milk and represents one of the staple foods of the local people. Raw butter contains 12–15% water, 1% protein, and the rest fat. (Butter after a long period of storage contains about 3% water.) Butter yield is used by the herdsmen as a yardstick for the ‘quality’ of the yak.

Making yak butter

The traditional method in China is to squeeze the butter in a bag made of hide, or to make it in a churn. Milk separators are coming into use in some areas. Cream separated in this way before churning produces a better quality of butter with less water and a longer storage life than achieved with the older methods.

When butter is made by churning, the milk is allowed to stand for a day to ferment and is then heated to about 20 °C. The warm milk is poured into a churn up to 80 cm high and 60 cm in diameter. A stick for stirring is held in the center of the churn by the lid. The herdsmen (or, more usually, the women) rotate the stick until the fat solidifies. Churning becomes difficult after 1–4 h, depending on the size of the churn and the quantity of milk. The lumps of milk fat floating on the surface are then removed by hand and washed in water. Next, water is squeezed out and the butter is made into cylindrical or cube-shaped blocks by using a plank of wood. Finally, the butter is packed in bags made of calf hide or yak rumen for storage, each bag weighing ~50 kg. The butter will keep for 1 or 2 years without going moldy.

When butter is made in a hide bag, the milk is first heated, as before, and poured into a bag made of calf or goat hide. The herdsman inflates the bag by blowing into it and closes the opening. The bag is then shaken until the fat solidifies into globules, after which the contents are emptied into another container. Thereafter, the procedure continues as described above.

When using a milk separator to make butter, the milk is first heated to 30–35 °C and then filtered. The separator is operated by turning a handle until the fat separates. The fat and skim milk are put in separate containers, and the rest of the butter-making process is as described before. Sometimes a small amount of sour milk is added as a starter to the raw milk to increase the amount of butter that can be made.

Uses of butter

Butter is used in a number of foods including zanba, pancakes, and dishes fried in butter. It is also added to milk tea and consumed salted or unsalted according to the area. In some areas, butter is used in tea when milk is not available, but butter tea is also the preferred choice, particularly, it is said, of herdsmen in northwest Sichuan and in Tibetan pastoral areas.

In another use of butter it is melted and mixed, in equal quantities, with roasted flour. The mixture is kneaded and stored. When required, this dough is melted into salted or sugared water and either eaten as it is, or further mixed with seeds such as peanut, sesame, walnut, or soybean, or with Chinese dates. These ingredients provide extra flavor and make the food a favorite among Tibetan people, especially for serving to their guests.

Butter is used also for many purposes other than as food, including its use for tanning and for polishing fur coats. It is used as a fuel in domestic lamps and by lamas in sacred lamps and on family altars. Women also use butter on their skin and as hair grease and as a lubricant to assist in hand milking. Butter is a component of some Tibetan medicines. Mixed with different coloring materials, butter is also used to make molded sculptures – some very large – for religious ceremonies and New Year celebrations.

Toffee

A product with the consistency of toffee (korani, in Sherpa) is made by boiling milk very slowly to evaporate the water.

Milk residue

Products called ‘milk residue’ by herdsmen in China come in more than one form. They are made mostly from skim milk after the preliminary separation of the fat to make butter, but can also be made from whole milk. Milk residue can come in either hard or semisoft form.

To make the milk residue, milk is heated to 50–60 °C and sour milk is added for curdling. The mixture is then poured into a wicker basket or gauze bag and the whey strained off. The remaining curds are spread on a cloth to dry. Milk residue prepared from skimmed milk is white and hard and when made from whole milk yellow and brittle. The protein content is ~55%, and the lactose content ~21%.

A softer form of milk residue sometimes has a little sugar added to aid fermentation and is akin to a fermented cottage-type cheese, but drier than the Western variety. It is served as a snack on its own or mixed with butter zanba.

Milk cake

This is usually made from whole milk from which between one-third and two-thirds of the water has been evaporated by natural drying, but it remains relatively soft. Starch is sometimes added to give a cake consistency. It is usually eaten with butter and sugar and is one of the dishes offered to guests.

Sour milk (or yogurt)

Sour milk is a favorite among the herdspeople throughout the year, but availability is more during the summer season when a maximum amount of milk is being produced. For preparing yogurt, freshly boiled milk is poured into a pail, and when the temperature has dropped to 50 °C, a small amount of sour milk is added to it. The mixture is allowed to cool to 40 °C. The pail is then covered and wrapped in wool to maintain its warmth. After 5 or 6 h in summer, or longer in winter, the milk sours. Either whole or skimmed milk can be used to make this product, with the whole-milk product having richer color and taste. The sour milk is drunk or eaten alone or sometimes mixed with zanba. The product is similar to natural yogurt made from cow’s milk in the west. Fruit or other flavors are not added.

Whey

The whey remaining after butter and milk residue have been separated is rarely used in the main pastoral regions for yak. In agricultural–pastoral areas whey can be given to pigs. The whey is, however, also used in the traditional processes for making leather.

Alcohol

In Mongolia, fermented yak milk is also distilled to make alcoholic drinks.

Manufactured Products

Dried milk

Factories for the production of dried milk exist in proximity to yak-rearing areas in several of the provinces of China, as well as in Nepal and possibly elsewhere. Dried milk is widely available in the towns of the region.

Cheese

Local production of cheese from yak and hybrid yak milk has been traditional in the remote northern parts of Nepal and northwest India. This is a hard casein product called chhurpi, special to the Himalayan region, prepared by drying the soft cheese produced first; a typical quantity of 200 l milk used for this purpose yields 4.5% chhurpi containing 8–10% moisture, 8–9% fat, and 80% protein on a dry matter (DM) basis.

Since the 1950s, production of a Swiss-style (gruyere-type) cheese has been developed in Nepal based on Swiss technology. There were 11 cheese factories run by the Nepalese Dairy Development Corporation and a number of privately run cottage factories producing, in 1998–99, 150 metric tonnes of yak cheese from yak and hybrid yak milk (out of a total cheese production of 350 metric tonnes).

Processing

Ten liters of milk from yak or yak hybrids produces 1 kg of cheese. The 16-stage process includes standardizing the fat content of the milk, pasteurizing, adding culture and rennet, curd formation, and cooking followed by molding and pressing. The cheese blocks are brined and stored for ripening. After 5 months of ripening, a good flavor develops.

Chemical composition

The composition of a 3-month-old Nepalese yak cheese is 31.8% water and 68.2% total solids. On a DM basis, the butterfat is 49.4%; salt 1.37%; and pH 5.75. Corresponding figures for a 3-year-old cheese are 23.1% water and 76.9% total solids, and on a DM basis, 46.8% fat and 3.12% salt. There may be some variations around these estimates.

Economics

Among the conclusions of a published assessment of the cheese factories, their operation and the economics involved were the following: (1) the demand for yak cheese outstripped the supply, (2) more investment was needed for the rehabilitation of most of the factories, and (3) the production chain from farm to marketing should be improved. Of the four factories studied in detail, three were in profit, with income exceeding costs, but one was running into deficit.

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See also: **Fermented Milks**: Asian Fermented Milks.

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Camel

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Introduction

The dromedary and the Bactrian camel are known for their ability to produce milk, in comparison to other species of Camelidae. In Africa, dromedaries are still considered as an important livestock species for milk and meat production, while unfortunately in the Arabian Peninsula more attention is directed toward camel racing than milk and meat production. For hundreds of years, camels have been used as multipurpose animals. Therefore, little selection for specific traits has taken place. They are raised for transport, milk, and meat, as well as for draft use.

Milk production from camels is mainly practiced in pastoral migratory systems. Camel raising is conducted outside the agrosystem. Most camel herds are kept in natural pastures with little or no supplemental feeding. Differences in the composition of camel milk reported from different countries may reflect differences in breeds, nutrition, and stage of lactation at sampling. Most camel milk is consumed as fresh milk. Camel milk is not as sweet as cows' milk, which is a major constraint in marketing fresh camel milk to consumers in cities located in the arid zones, but camel milk can play an important role in providing high-quality protein to the people living in the arid and semiarid areas of the world.

Geographical Distribution

Camels are pseudoruminant mammals and they first evolved in North America. In time, the camels evolved into a number of species of the camel family Camelidae (camelids). The Camelidae belongs to the order Artiodactyla (even-toed ungulates) and the suborder Tylopoda (pad-footed animals). The Camelidae family consists of two genera: *Camelus* and *Lama*. The genus *Camelus* comprises two species: *Camelus dromedarius* (also known as the one-hump camel or Arabian camel) and *Camelus bactrianus* (also known as the two-hump camel or Bactrian camel). The *Lama* genus comprises four species: *Lama glama* (llama), *Lama pacos* (alpaca), *Lama guanicoe* (guanaco), and *Lama vicugna* (vicuna).

The dromedary was first domesticated in southern Arabia at about 4000–3000 BC mainly for its milk. Camels form an important part of the domestic livestock population in most of the dry areas of the world. The camel is considered as an animal of the desert (hot or cold). It is found in areas where there is low rainfall, occurring in a relatively short period of time. The success of the camel in desert climates that other domestic farm animals cannot tolerate is due to its peculiar physiology.

Most dromedaries are found in the arid and semiarid areas of north and east Africa, the Indian subcontinent, and throughout the Arabian Peninsula. These areas are characterized by low rainfall and a long dry season with high ambient temperatures (in the range of 5–45 °C). On the other hand, Bactrian camels are mainly distributed through the deserts and semideserts of the north and northwest of China and throughout Mongolia. The temperature in areas where Bactrian camels are raised ranges from −20 to 40 °C. They are multipurpose animals, mainly used for working and producing wool. There are an estimated 18 million *Camelus* in the world, of which 2 million are Bactrian camels. Of the 16 million dromedaries, approximately 13 million are found in Africa, with the largest population in east Africa (Somalia and Sudan). The remainder of dromedaries are found mainly in the Arabian Peninsula and the Indian subcontinent.

Breeds and Genetic Groups

Camels may be classified based on their function (racing, draught, or milk) or their habitat (lowland and mountain type). Only recently an extensive breeding program selecting for speed took place in the oil-rich Arabian countries in the Arabian Peninsula. The racing camel can be considered as a separate breed, and within this breed there are different types of racing camels.

Even though many breeders do not accept the classification of dairy camels into breeds, it is very common to use this classification among camel owners in the many countries where camels are an important livestock species for milk and meat production. Dairy camels can be classified into three groups, high, medium, and low, based on their milk production. Only the high and medium milk-producing camels can be considered as true dairy camel types. Lactating camels with an annual milk production of more than 3000 l will be considered as high-producing dairy camel types, while camels with an annual milk yield of more than 1500 l but less than 3000 l will be considered as medium-producing dairy camel types.

Group 1: High-Producing Dairy Camel Types (Dromedaries)

Dairy camels in this group are characterized by large body size but less beefy body conformation, relatively big abdomen, well-developed humps, prominent milk veins, and well-developed udder. Camels belonging to this category are mainly black and light to dark brown in color (**Figure 1**). Average milk production is more than 3000 l yr^{-1}. Known types in this group are as follows:

1. Marecha: This type originated in Pakistan, and is named after Marecha tribe from the desert of Pakistan. This type has real production potential for milk.
2. Al-Majahim Al-Arabia: This is also called Al-Njdeiah. It originated in Saudi Arabia especially in Najd and Dawaser Valley in the north and northeast of Saudi Arabia.
3. Sirtawi: This is found mainly in the Sirt area in the middle coastal zone in Libya.
4. Fakhreya: This is found in the southern and western areas of Benghazi in Libya.
5. Arvana: This was developed in Turkmenistan in the Kara-Kum Desert, and can also be found in Uzbekistan, Kazakhstan, Afghanistan, and Iran.

Group 2: Medium-Producing Dairy Camel Types (Dual Purpose)

Camels in this group are characterized by medium body size and a medium-sized hump. Most of the pack and riding types are dual-purpose camels. These breeds are mainly white, gray, light brown, and brown to reddish in color (**Figure 2**). Average milk production ranges from more than 1500 l to less than 3000 l. Known types in this group are as follows:

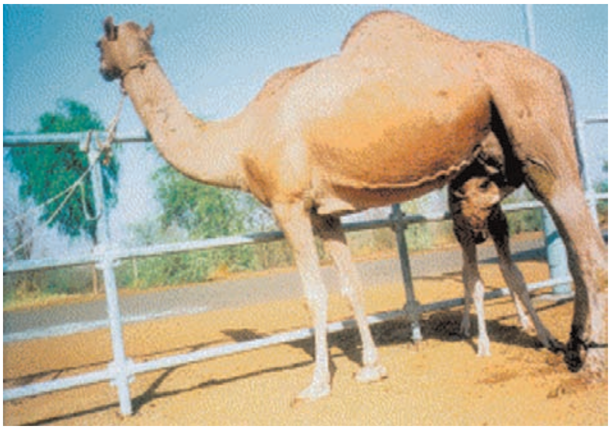

Figure 1 High-producing dairy camel.

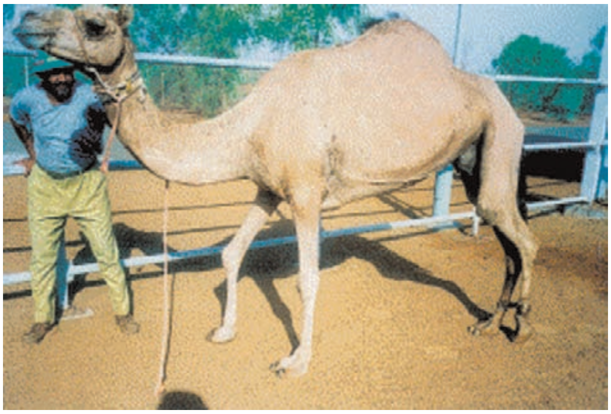

Figure 2 Medium-producing dairy camel.

Dromedaries

1. Hor (Godir): This can be found in central Somalia. It is the most common type of camel distributed in different parts of Somalia.
2. Rashaida: This is found in the Kasala area of eastern Sudan and is raised by the Rashaida tribe.
3. Ould Sidi Al-Sheikh: This is found in the northeast area of Mauritania and the southeast of Morocco.
4. Al-Hmor: This is found in many areas of the Arabian Peninsula and is especially popular in certain parts of Saudi Arabia.
5. Seifdar: This is found in Somalia. Camels belonging to this type are good producers of milk and they have the characteristics of racing camels.
6. Al-Khawar (also called Atfateir): This type is found in the northern steppes of Syria and the western steppes of Iraq.
7. Al-Shameya: This is found in the Syria steppe, north of Jordan, west of Iraq, and north of Saudi Arabia.
8. Pishin: This type is named after the Pishin district of Baluchistan in Pakistan. Camels belonging to this group have body structure of a typical mountain camel and can carry heavy weights.
9. Brela or Thalocha: This breed can be found in Punjab province, Pakistan. Camels of this breed are tall and have a big strong body and big head and neck with a broad chest and a wide girth.
10. Benadir: This Somali dairy camel belongs to the heavy baggage type.
11. Birabish: This can be found in Mauritania.
12. Al-Tilal: This can be found in Morocco.
13. Al-Tibawi: This can be found in Libya. Camels of this breed are small in size and have the ability to withstand a long period of water deprivation.

Bactrian camels

1. Alxa: This can be found in the Alxa county in China. Alxa camels can produce from 1.5 to 2 l of milk per day in

addition to the amount suckled by the young. Nowadays, this breed can be found in northern Afghanistan, Tajikistan, and parts of China.

Husbandry

Considering the harsh climate of the desert, with very low annual rainfall and very hot climate, the economic importance of the camel is based on its physiological and anatomical adaptation to such harsh conditions prevailing in the arid zones. The camel has the ability to regain body water losses of up to 30% of its body weight within 10 min without producing intravascular hemolysis.

Reproduction

Puberty of the female camel usually occurs at 4 years of age. The dromedary is a seasonal polyestrous animal. The mean duration of the estrous cycle is 17.2–23.4 days. Ovulation occurs at coitus (as in the rabbit). The placenta is the diffuse and noninvasive epitheliochorial type (as in the mare). Pregnancy duration is 12–13 months. Lactation length in the dromedary and Bactrian camel varies from 6 to 18 months. Russian investigators have reported that on rare occasions when Bactrian camels calve every year, a lactation period of 7 months is normal. In the case of dromedaries, there is no report in the literature indicating that dromedary camel can calve once every year without cessation of milk production. However, if camels do not become pregnant, the lactation may continue for up to 24 months. The udder (mammary glands) of the camel consists of four glandular quarters (**Figure 3**), each with its own teat. The left and right halves are separated from each other by a double sheet of fibroelastic tissue taking origin from the linea alba and prepubic tendon. Arterial blood supply is mainly by the external pudendal artery and the venous drain is mainly by the superficial thoracic vein, the external pudendal vein, and the femoral vein.

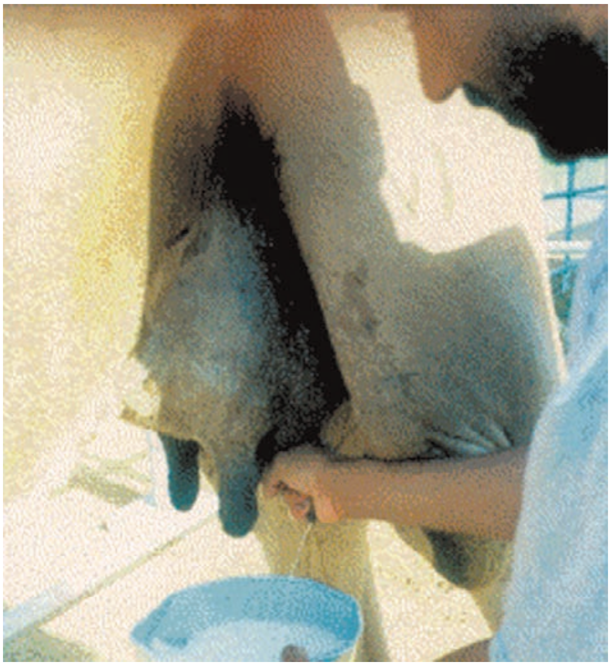

Figure 3 Well-developed udder of lactating dromedary.

Mastitis

Mastitis is one of the main problems in lactating camels. Major factors that contribute to the occurrence of mastitis infection in lactating camels are poor milking hygiene, bad management, and teat injuries. Infection by bacterial or mycotic pathogens is the main cause of mastitis in camels. These include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Corynebacterium pyogenes*, *Streptococcus agalactiae*, *Pasteurella haemolytica*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Different types of mastitis in camels have been reported from different countries. These include peracute, acute, chronic, and subclinical forms. Staphylococcus mastitis appears to be the more prevalent form. *Staphylococcus aureus* is found in both peracute (gangrenous) and chronic mastitis cases. In acute form, the udder is swollen, hot, and painful. Changes in milk composition are obvious. The milk is watery with flakes. If the udder is not treated, the affected gland becomes hard and less productive. Multiple abscesses with complete fibrosis of the affected portion can be noticed. In subclinical mastitis, no obvious changes in the mammary gland can be seen. However, milk secretion is not normal.

Diagnosis of mastitis can be based upon clinical examination of the udder, bacteriological isolation of the causative agent, and physical examination of the milk. The California mastitis test (CMT) and milk cell counts are of value in the diagnosis of mastitis in camels. A cell count of less than 400 000 cells ml^{-1} is normal. However, in mastitic milk, cell counts of between 800 000 and 1 600 000 cells ml^{-1} have been reported. Locally and parenterally administered antibiotics have been used to treat mastitis infection in camels. The antibiotic is infused into the affected quarter every 12–24 h for 3–5 treatments. The affected gland is infused after emptying the gland of milk. Administration of oxytocin (10 IU) intravenously will help in emptying the milk.

Udder Edema

Udder edema is another problem that affects milk production in lactating camels. It is characterized by an excessive accumulation of fluid in the interstitial spaces of the udder that occurs at the time of parturition. High-producing camels are more susceptible. The exact cause of udder edema is unknown. However, recent physiological and pathological studies have shown that udder edema at parturition results from a decreased mammary blood flow associated with an increase in the venous blood pressure in the cranial superficial epigastric veins (milk vein). Clinically, a typical case of udder edema usually involves all four quarters of the udder. The condition has two distinct stages. During the first stage, there is a gradual congestion of the skin of the udder. The udder becomes greatly distended, swollen, and filled with colostrum. During the second stage, digital pressure produces pitting of the edematous areas. The udder skin also becomes thick and hard on digital palpation. The base of the teats is also edematous. This makes milking difficult. In most cases, udder edema does not need any treatment. The swelling gradually decreases after calving. However, physical massage of the udder during milking together with the alternate application of hot and cold water will help to remove the fluid. Diuretics, especially furosemide, may have some beneficial effects by reducing the edema. A dose of 5 ml of a 5% solution twice daily for up to 3 days can be used to treat udder edema. Other minor diseases that effect the udder of the camels are teat stenosis and udder wounds.

Milk Harvesting

In the extremely hot arid areas of Arabia and all the drought-stricken areas of the world, where continuous drought decimates the cattle, sheep, and goat populations, only the camels survive and continue producing milk and calves. Progress in improving camel milk production has been almost impossible, because the socioeconomic constraints where camels are raised are rather complex. One major constraint to milk production is that camels stop lactating within 4 weeks' gestation. This means a longer calving interval, which means higher costs of production. Another constraint that hinders improving milk production in camel is hand milking. Unlike dairy cows, lactating camels are almost always hand-milked. Hand milking of camels is still practiced because the presence of the calf is important in ensuring satisfactory milk letdown before the camels are milked. Milking machines are available but are not widely used because of many problems associated with machine milking in camels.

Intensive Systems

Camel milk production from intensive systems has started to become a reality, and has shown promising results. The settlement of Bedouin in the oil-producing countries in the Arabian Peninsula and the distribution of wealth there has made it possible for the Bedouin to keep their camels in confinement for the purpose of milk production and racing.

Milk Yield

Recently, animal scientists have begun collecting data on camel milk yield and composition but a lack of organized research efforts and the socioeconomic constraints hinder the efforts of scientists to improve camel milk production. The potential for high milk production from camels has prompted scientists to attempt to improve local husbandry methods. Unfortunately, camels have seldom been selected for high milk production as cattle are in developed countries. Some nomadic tribes in Pakistan and east Africa have selected camels for milk production but the methods of selection used are primitive compared to those employed in the developed countries.

Generally, there are some factors influencing the milk yield, including nutrition, water availability, genetics, reproduction, health status, breeds, stage of lactation, milking frequency, and presence of the calf. Thus, frequency of suckling or milking is likely to have a considerable effect on milk production. The frequency of milking lactating camels depends on the customs of the people. Some people milk their camels once a day, others up to six times. Even though frequency of milking tends to increase milk yield in camels, it is not a consistent practice. The Afar people in Ethiopia sometimes milk their camels six times a day, while at other times they may leave their animals for a whole day without milking them. This practice may hinder improving milk production in camels.

Data on camel milk yield in different regions vary greatly. This may be due to differences in breeds and camel management systems. The dromedary, like most mammalian species, gives most milk during the early stage of lactation. However, the peak of lactation in camels tends to decline more steeply than in dairy cows. It has been reported in the literature that some camels in the Punjab district of Pakistan immediately after calving can produce up to 30 kg day^{-1} of milk with an average of 17.4 kg day^{-1} over a 10-month period. This means a total yield of 5300 kg per 305 days. A report from Saudi Arabia indicates that Al-Majahim camels can produce up to 10 l day^{-1} of milk under intensive systems. Also, the milk production of Somali camels on pasture during the rainy season has been estimated at an average of 10 l day^{-1} during the lactation period. It appears that the milk yield of camels is low, especially if compared with the yield of Holstein cows. Nevertheless, camels are much better providers of milk than cows, sheep, and goats in the arid areas of the world.

Milk Composition

The composition of camel milk is similar to that of cattle and goat milk (**Table 1**). Camel milk is generally opaque white and low in carotene. It has a sweet and sharp taste, but sometimes can also be salty. The type of fodder and the availability of drinking water affect the taste of camel milk. The pH of camel milk ranges from 6.5 to 6.7, titratable acidity is 0.03 after 2 h and 0.149 after 6 h, and the specific gravity ranges from 1.025 to 1.032.

Water

It has been stated that water is the most important factor affecting the composition of camel milk. The water content of camel milk fluctuates from 84 to 90%. Published results on the effect of lack of drinking water on camel milk indicated that when water was freely accessible to the lactating camels, the water content of the milk was 84–86%. But when water was restricted, the water content of milk rose to 90%. It would appear that the lactating camel loses water to the milk in times of drought. This could be a natural adaptation in order to provide necessary fluid to the calf. It is also of benefit to the Bedouin, who are in need of fluid to sustain themselves in the desert where water is not easily accessible. It has been reported that antidiuretic hormone (ADH) secretion is elevated in the dehydrated camels and thus the loss of water into the milk is due to the action of this hormone.

The average content of lactose in camel milk is around 3.7%. Ash content of camel milk ranges from 0.6 to 0.8% and the fat content varies between 2.5 and 5.4% (**Table 1**). It has been noticed that the hydration status as well as the type of feeding can determine the fat content of the milk. With the increase in water content of milk produced by thirsty camels, there will be a drastic decrease in the fat content. The bulk of the fat in milk exists in the form of very small spherical globules (1.2–4.2 μm in diameter). The surface of these fat globules is coated with a thin layer known as the fat globule membrane, which acts as an emulsifying agent for the fat suspended in milk. Compared with cows' milk, camel milk shows a very slow creaming rate. Creaming layers vary from 0.5 to 2 ml at 4 °C. The ratio of fat to total solids averages 31.6%.

Table 1 Average composition (%) of camel milk compared to that of milk from other farm animals

Constituents	*Camel*	*Cow*	*Ewe*	*Goat*
Moisture	86.9	87.0	80.8	87.7
Protein	3.4	3.5	5.5	3.3
Fat	4.1	3.9	5.3	4.1
Lactose	3.7	4.9	4.5	3.9
Solids-not-fat	8.9	9.1	11.8	8.2
Ash	0.7	0.7	0.9	0.8

Fatty Acids

Compared to the fat in cows', buffaloes', and ewes' milk, the fat in camel milk contains fewer short-chain fatty acids, but similar long-chain fatty acids. Data on the composition of the major fatty acids in camel milk are listed in **Table 2**. In general, short-chain fatty acids (C_4–C_{12}) are present in very small amounts in camel milk compared to cows' milk. But the concentrations of $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ are relatively high. Also, camel milk has high concentrations of linoleic acid and polyunsaturated fatty acids. Phospholipids are a small but important fraction of the lipids of milk and are found mainly in the milk fat globule membrane. Also, it has been noticed that phospholipid fatty acids of camel milk are not entirely characteristic of the ruminant herbivores. Camel milk phospholipid fatty acids have high amounts of linoleic acid ($C_{18:3n-3}$) and long-chain polyunsaturated fatty acids.

Minerals and Vitamins

Mineral and vitamin contents of camel milk are shown in **Table 3**. The mineral content includes mainly chlorides, phosphates, and citrates of sodium, calcium, and magnesium. Few data are available on the vitamin content of camel milk. It appears that vitamin A, vitamin E, thiamin, riboflavin, folic acid, and pantothenic acid contents in camel milk are lower than that found in cows' milk, while the contents of pyridoxine and vitamin B_{12} are about the same. The contents of niacin and vitamin C are substantially higher than that found in cows' milk. In

Table 2 Proportion of major fatty acids in camel milk fat

Fatty acid	*g per 100 g total fat*
$C_{4:0}$	0.85
$C_{6:0}$	0.37
$C_{8:0}$	0.28
$C_{10:0}$	0.37
$C_{12:0}$	0.69
$C_{14:0}$	10.90
$C_{14:1}$	1.50
$C_{15:0}$	1.14
$C_{16:0}$	29.87
$C_{16:1}$	9.60
$C_{17:0}$	0.88
$C_{17:1}$	0.55
$C_{18:0}$	12.90
$C_{18:1}$	23.50
$C_{18:2}$	3.10
$C_{18:3}$	1.40
$C_{20:0}$	0.70

Table 3 Content of minerals and vitamins in camel milk

	mg l^{-1}
Minerals	
Calcium	1060.0–1570.0
Inorganic phosphate	580.0–1040.0
Copper	13.0–1.8
Iron	1.3–2.5
Magnesium	75.0–160.0
Manganese	0.1–0.2
Sodium	360.0–620.0
Zinc	4.0–5.0
Vitamins	
Retinol (A)	0.10–0.15
Thiamin (B$_1$)	0.33–0.60
Riboflavin (B$_2$)	0.42–0.80
Pyridoxine (B$_6$)	0.52
Cobalamin (B$_{12}$)	0.002
Niacin	4.6
Folic acid	0.004
Pantothenic acid	0.88
Tocopherol (E)	0.53
Ascorbic acid (C)	24–36

particular, the high level of vitamin C in camel milk (25–60 mg l^{-1}) is of significant nutritional relevance in the arid areas where fruits and vegetables containing vitamin C are scarce.

Milk Products

Camel milk is one of the most valuable food resources for the people living in arid and semiarid zones. Most of the camel milk is consumed as fresh milk. However, surplus milk is fermented naturally at 25–30 °C until it turns sour. A naturally fermented product called 'susa', which has a long shelf life and is pleasant to drink, is produced in Kenya, Somalia, and Sudan. Susa is made by incubation of camel milk in smoked wooden buckets for 1–3 days. Kenyan researchers have shown that the quality of susa could be improved using selected mesophilic starter cultures rather than spontaneous fermentation; the resulting fermented milk has a uniform taste and a longer shelf life. Another fermented camel milk product is 'shubat'. It is of snow-white color and its fat content reaches 8%. It can be preserved for some time without losing its properties. Some researchers have claimed that shubat can be used to cure tuberculosis and some gastric and intestinal diseases.

Butter

The method of making butter from cows' milk cannot be applied to camel milk. Production of butter from camel milk cannot be achieved easily because camel milk shows little tendency to cream up. It takes a large quantity of camel milk to produce a small amount of butter. To obtain a reasonable amount of butter, cream from camel milk must be churned at a higher temperature (22–25 °C) than the temperature (8–14 °C) used with cream from cows' milk. The reason for this may be due to the high melting point (40 °C) of camel milk fat. The average moisture content of butter from camel milk is lower (12–13%) than the moisture content of butter from cows' milk (15–16%), which may explain the sticky texture of butter from camel milk. Butter made from camel milk is white and waxy.

Cheese

Cheese can be made from camel milk, but it is difficult to make. Cheese yield from camel milk is very low, in the range of 35% of dry milk compared to 85% for cows' milk. This may be due to lower amounts of total solids, smaller fat globules, and poor rennet capability. Soft cheese made from camel milk requires a much larger amount of rennet (50 times more) than the normal amount used in making cheese from cows' milk. Addition of 2% calcium chloride will increase cured firmness slightly. If a cow cheese whey culture is added to the milk before adding rennet, the coagulation time of camel milk will be shortened and a firm, consistent coagulum will form and a hard cheese can be made. Also, hard cheese can be made from camel milk combined with yogurt. This type of cheese, which is called 'kadchgall', is produced mainly in Afghanistan.

Milk Processing

Most Bedouin and other camel owners prefer to consume camel milk in raw form. There is a strong belief among camel owners in many parts of the world that pasteurizing camel milk will change its taste. There are very legitimate concerns regarding human health risks due to the consumption of camel milk. The presence of pathogenic bacteria in raw camel milk has been a major concern to health authorities in many countries. Researchers at the United Arab Emirates University are developing and testing a new sterilization system for camel milk. It is based on a new concept in raw milk germicidal control using an electromagnetic orthogonal ripple field.

Marketing

In order for the camel dairy industry to grow, the acceptance of camel milk among consumers in cities located in the arid zones needs to be encouraged. The taste of camel milk is a major constraint in marketing fresh camel milk; it is perceived to have an unpleasant taste. Camel milk is not as sweet as cows' milk. Even though the percentage of lactose in camel milk varies from 3.4 to 5.8%, most of the time the lactose percentage in camel milk is low and does not exceed 4%. A study conducted at the United Arab Emirates University on the acceptance of camel milk showed that school children preferred camel milk to be flavored with chocolate.

Future Developments

Camel dairy farming has not yet been developed in a proper way. However, in certain countries, such as Saudi Arabia, Mauritania, and the United Arab Emirates, large-scale camel dairy farms have been established. The future of the camel lies in its ability to produce meat and milk. Camel milk can play an important role in providing a high-quality source of protein to the people living in the arid and semiarid areas.

See also: **Fermented Milks:** Middle Eastern Fermented Milks. **Mammals. Mammary Gland:** Anatomy. **Mastitis Pathogens:** Contagious Pathogens; Environmental Pathogens. **Mastitis Therapy and Control:** Management Control Options; Medical Therapy Options.

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Horse

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Introduction

It is thought that 30 million people throughout the world drink horse milk more or less regularly. Horse milk consumption is a very ancient practice, which was mentioned by Homer in the *Iliad*. The traditional use of mare milk for human consumption, first by nomadic people, then for therapeutic uses in the area of production, is now completed by different luxe products in some economically developed countries. Besides horses, asses were also milked for a few years. The milking routine is very specific, due to physiological particularities in mares. Equine milk composition will be compared to cow's milk as a reference and to human milk because the composition of horse milk is often considered to be very close to that of human milk. The different uses of horse milk, raw or fermented as koumiss, are reviewed.

Geographic Distribution and Breeds

Although horses are found throughout the world, dairy herds are mainly located in Central Asia, from the Ural Mountains to Mongolia. Dairy horses are found in Kazakhstan, Kirghizia, and, to a lesser extent, in Tadzhikistan, Uzbekistan, in some parts of Russia near Kazakhstan (Kalmukia, Bashkiria), and in Mongolia and its periphery (Buryatia in Siberia and Inner Mongolia in North China). Dairy horses are also found in Tibet and Xinjiang, China. Dairy herds are also present, to a lesser extent, in Eastern Europe (Belarus, Ukraine), Central Europe (especially Hungary, Austria, and Germany), and Italy. Few horse dairy herds are located in other countries.

In Central Asia, native horse breeds are used. Most of them weigh 500–600 kg. They also have been used for different purposes, including riding. The Kazakh breeds are probably the most important and have been selected for a long time for milk production. Among them, Jade Kazakh and Draft Kazakh produce more milk than Saddle Kazakh, owing to a better lactation persistency. Kushum horses are also found in Kazakhstan. They are mainly devoted to meat production but are also used for dairy purposes. Bashkir horses are present in the south of the Ural mountains. Russian draft horses, weighing 600 kg, and Soviet or Lithuanian draft horses, weighing 650–700 kg, are used in Russia for milk and draft. Lokai horses in Tadzhikistan and Novokirghiz horses in Kirghizia are traditionally used for riding (herd survey) and now for milk production. In Mongolia, crossbreeds are generally used.

In Western Europe, the main dairy breed is Haflinger, a small-sized (500 kg for adult mares) breed from Austria. It is famed for its dairy characteristics, but, in fact, any breed can be milked. The essential condition is the acceptance of milking by mares and this factor is likely taken into account in dairy horse selection.

Milk Harvesting

Due to the low capacity of the udder (less than 2 l, with 75–85% alveolar milk) compared to milk potential (more than 12 l per day), maximum milk production requires frequent milkings. Five or six milkings per day every 3 h are often practiced, with eight milkings done sometimes at the peak of lactation; at the end of lactation, mares are often milked 4 times a day. The main constraint is the necessity to regularly empty the udder in order to avoid a decrease in milk secretion; an interval between two milkings that is longer than 3 h may be detrimental for total milk yield. It is necessary to maintain the foal with the mother, so that sucklings are possible when the mare is not milked (**Figure 1**). A high proportion of alveolar milk requires a good conditioning of mares to milking routines, so that milk ejection allows maximal recovery of secreted milk.

Milking generally begins between 20–30 days and 1.5 month after foaling because this is when suckling frequency by the foal decreases and their grazing time increases. At this time, a foal is able to eat enough concentrate and forage so that it can withstand a decrease in milk intake without negative consequences on its growth. Lactations last for 5–6 months. Because the amount of milk harvested per milking is not dependent on the hour of the day or the day of milking, it is possible for the dairy farmer to modulate the number of milkings per day and the number of days of milking, provided the foal is allowed to suckle.

Mares are milked using milking machines designed specifically for them. The recommended vacuum level is the same as for other species, and it is no higher than

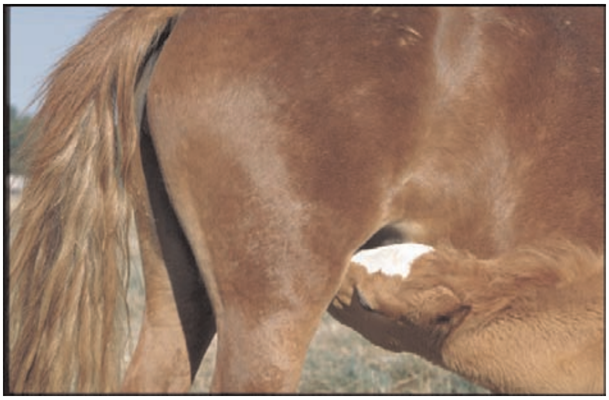

Figure 1 The presence of the foal is useful to remove milk when the milking interval extends beyond 3 h and is beneficial for milk ejection when the mare is being milked.

40–50 kPa. The advisable pulsation rate is between 80 and 180 pulsations per minute (120 on average). This high value, justified by the small part of milk that is cisternal, is similar to the value for camels and some ewe breeds. The recommended pulsation ratio is near 50%.

With machine milking, each milking lasts 1 min as compared with 2–3 min for hand milking. With mechanical milking, the cisternal milk flow lasts less than 20 s followed by rapid removal of alveolar milk in 30–35 s. This rapid removal is probably due to the level of stimulation of the udder. The presence of the foal in front of its mother often favours oxytocin release, which causes ejection of milk from the alveoli.

The amount of milk harvested depends on the individual ability of mares to be milked. It is likely that selection of dairy mares has been made mainly for this feature or for udder characteristics (and thus the proportion of cisternal milk) than for the effective milk potential, so that the difference in milk production between dairy and nursing mares is low (**Figure 2**). Experiments in nursing mares have shown that milk production is proportional to body weight (about 25 g daily per kilogram of body weight). This may be similar in dairy mares. Although most dairy mares in Eastern Europe and Asia weigh 500–600 kg, heavier draft breeds are milked in the former Soviet Union and Lithuania. They produce more milk than do lighter breeds. In Western Europe, heavy mares such as the French Auxois breed (800 kg) can produce, when well adapted, 30% more milk than breeds such as the Haflinger (500 kg). Nevertheless, between-breed and intrabreed differences exist, although most dairy mares in the world produce between 2500 and 3200 l per lactation, of which half is milked and half is sucked by the foal. The amount of milk consumed by the foal is calculated from the relationships between milk intake and daily weight gain.

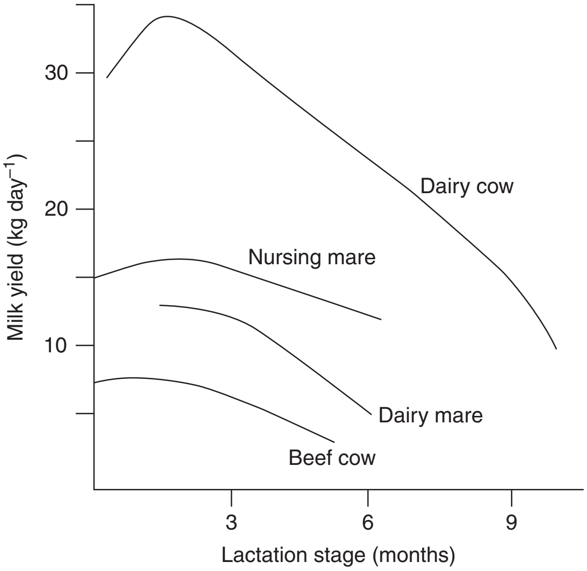

Figure 2 Comparison of milk yield by dairy and nursing mares and cows.

Husbandry and Feeding

Feeding dairy mares follows the same rules as for nursing mares with respect to energy and nitrogen requirements. Feedstuffs given to dairy mares can vary. Nevertheless, the fat composition of milk depends on feeding. Horses can eat large amounts of forages, even forages of low nutritive value, contrary to ruminants. The husbandry of mare herds takes advantage of this ability. If mares are fed *ad libitum*, feed intake may rise from 2 to more than 3 kg dry matter per 100 kg body weight between the first and the sixth week of lactation with voluntary intake remaining high.

Daily energy and nitrogen requirements for milk production in early lactation (until 3 months) are 2.3–2.0 and 3.5–3.0 times the maintenance requirement, respectively. In late lactation (4 months and over), energy and nitrogen requirements decrease to between 1.6 and 2.4 times the maintenance requirement. Daily energy and nitrogen requirements are provided by the nutrients in the ration, and by body reserves when the amount of feed nutrients supplied is not sufficient and if the body condition score of the mare is not too low (more than 2.5 on a 0–5 scale). If the body condition score is very low, *ad libitum* feeding can rebuild body reserves without negative effects on milk yield. Milk yield is very similar for mares with low and high body condition score, when fed *ad libitum.* Milk yield can be significantly improved by increasing concentrate supplementation, but it is not decreased by a shortage in feed nitrogen, even in early lactation.

The decrease in milk intake by foals must be compensated for by solid feed supplementation. Forage and concentrates are given to cover about half of their requirements.

It is necessary to provide a shed for mares and foals, as they are not managed together. Management must be compatible with repeated milkings during the day, and thus with a limited time of intake and a limited access to grass during the diurnal period. It is advisable to allow the mare to see the foal during milking, with the milking parlor located close to the pastures. The presence of a pen with feed racks near the milking parlor is recommended so that forage and concentrate distribution can be ensured during the lactation period. These constraints result in the need for specific installations for dairy mares.

Milk Composition and Quality

The mechanisms of milk synthesis in the horse are similar to mechanisms described in ruminants. Lactose comes from glucose. Fatty acids have a double origin: *de novo* synthesis and a direct uptake by the udder. The precursors of *de novo* synthesis are acetate and 3-hydroxybutyrate, the latter arising from butyrate. Acetate and butyrate are the end products of carbohydrate digestion in the large intestine. The other end products, glucose in the small intestine and propionate in the large intestine, are not precursors of milk fatty acids. All fatty acids from 4 to 14 carbons come from *de novo* synthesis, and palmitic acid is derived almost equally from *de novo* synthesis and fatty acid uptake. However, 18-carbon fatty acids, which come mainly from direct uptake (diet and body reserves), originate to a limited extent from *de novo* synthesis, in contrast to ruminants.

Average Composition of Milk

Horse milk has a dry matter content of 10–12%. Fat, protein, and lactose contents are generally in the range of 10–20, 20–35, and 55–65 g kg^{-1}, respectively. Their variation according to lactation stage is shown in **Figure 3**. Horse milk is low in fat compared to other mammals, and similar to another perissodactyl species, the rhinoceros. Values lower than 5 g kg^{-1} have been reported. Horse milk is often thought to have the same composition as human milk. Compared with human milk, horse milk contains less lactose, as much protein, and much less fat. Compared with other milks for human consumption (cow, ewe, goat, camel), horse milk is richer in lactose, lower in protein, and especially lower in fat. Fat accounts for only 25% of the energy in horse milk as compared to 50% for human milk or cow's milk. The energy value of horse milk is between 2.0 and 2.5 MJ kg^{-1}, which is lower than that of other farm animals and humans. Horse milk contains approximately 5 g kg^{-1} of minerals. Mineral and trace element contents are summarized in **Table 1**. The pattern of vitamins in horse milk is characterized by a high content of vitamin C, compared to other dairy species. Colostrum

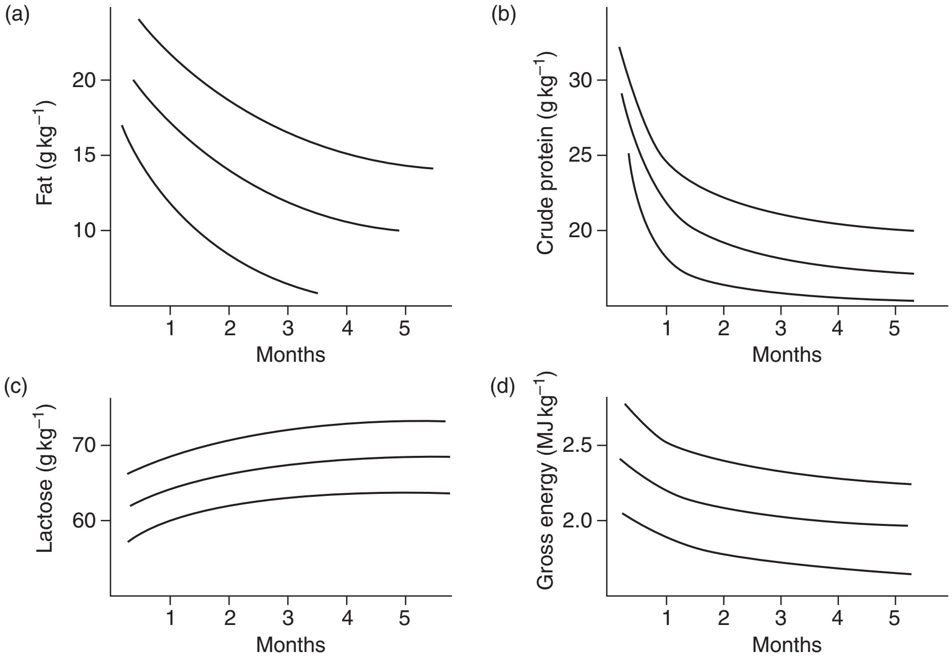

Figure 3 Variation in (A) fat, (B) crude protein, (C) lactose and (D) gross energy content in horse milk throughout lactation (mean and extreme values found in the literature).

Table 1 Range of variation of mineral and trace element concentration in horse milk (after the colostral period)

Minerals (g kg^{-1})	
Calcium	0.5–1.3
Phosphorus	0.2–1.2
Magnesium	0.04–0.11
Sodium	0.07–0.20
Potassium	0.3–0.8
Chloride	0.2–0.6
Sulfur	0.22
Trace elements (mg kg^{-1})	
Aluminum	0.12
Barium	0.08
Boron	0.10
Copper	0.2–1.0
Iodine	0.004–0.042
Iron	0.22–1.46
Lead	<0.05
Lithium	0.01
Manganese	0.01–0.05
Molybdenum	0.02
Silicon	0.16
Strontium	0.44
Titanium	0.14
Zinc	0.9–6.4

contains more proteins, especially immunoglobulins, and less fat than milk. Colostrum composition is close to milk composition by 24 h after foaling.

Fat globules are 2–3 μm in size. Contrary to cow's milk, which is almost totally made of triacylglycerols, horse milk contains less than 80% triacylglycerols, the rest being mainly composed of free fatty acids and phospholipids; the respective amounts of these fractions are not clearly established. Total phospholipid content (at least 5% of lipids) is higher than that of cow's milk and similar to or higher than that of human milk.

The fatty acid composition of milk is related to dietary fatty acids because they are absorbed in the small intestine, without the preliminary hydrogenation that occurs in ruminants. The percentage of fatty acids with less than 16 carbons is variable: 15–35% according to the experiments. The range of variation of the main fatty acids is given in **Table 2**. Horse milk is characterized by a low amount of stearic acid (less than 2%) but contains significant amounts of palmitoleic acid. This suggests a high activity of the mammary Δ9-desaturase. A high content of linolenic acid when on forage-based diets is characteristic of horse milk, unlike monogastric species, which do not eat forages, the main dietary source of linolenic acid. Ruminants also consume significant amounts of linolenic acid, but hydrogenation occurs before absorption. Compared to other species, horse milk is lower in stearic acid but higher in linoleic and linolenic acids because horse feeds are rich in these fatty acids, with linoleic acid coming from concentrates and linolenic acid from forages. It appears that *trans* monounsaturated fatty acids and conjugated linoleic acid are not present in significant amounts in horse milk, although *trans* octadecenoic acid has been found at a level of 1% of fatty acids in colostrum. These acids are specific for ruminants, due to ruminal hydrogenation. They may be produced in the large intestine of the horse, but are not absorbed.

Table 2 Fatty acid composition of horse milk (extreme variations from 25 publications)

	% (w/w)
$C_{4:0}$	0.3–0.9
$C_{6:0}$	0.3–1.4
$C_{8:0}$	0.8–6.1
$C_{10:0}$	2.3–16.7
$C_{12:0}$	3.8–14.6
$C_{14:0}$	4.7–19.2
$C_{14:1}$	0.1–2.6
$C_{16:0}$	12.4–28.5
$C_{16:1}$	2.2–9.7
$C_{18:0}$	0.3–3.0
$C_{18:1}$	9.4–31.6
$C_{18:2}$	3.6–20.3
$C_{18:3}$	2.2–26.2

Nonprotein nitrogen constitutes approximately 10% (8–15%) of the total nitrogen of horse milk, which is double that of cow's milk and half that of human milk. Approximately one-half of nonprotein nitrogen is associated with urea nitrogen, with the remaining mostly associated with amino acids and peptides. The free amino acid fraction is especially rich in serine and glutamic acid. Proteins are made of 40–60% caseins, close to the proportion in human milk (40%) and much less than the proportion in the milks of other dairy animals. The proportion of these fractions do not vary to a large extent through the lactation period, but there is high variability among experiments (**Table 3**), which cannot be explained by methodological errors owing to the simplicity of analytical determination.

Caseins contain β-casein and γ-like casein, which represent 40–50% and less than 10% of total caseins, respectively. Several fractions of β-casein have been identified, differing in the number of phosphate groups.

Table 3 Whey protein composition of horse milk (extreme variations from 13 publications)

	% (w/w)
β-Lactoglobulin	25–63
α-Lactalbumin	25–49
Immunoglobulins	4–21
Serum albumin	2–19
Lactoferrin	6–10
Lysozyme	5–11

Numerous isoforms of casein α_{S1} (40–60% of total caseins) have been isolated, and α_{S2} has been recently found in small amounts. It had been thought that mare milk did not contain κ-casein, but recently it has been identified (6–7% of total casein). It is likely that the functions of κ-casein could be their use in micelle stabilization by some fractions of β-casein. Caseins are lower in proline and glutamic acid and higher in aspartic acid than are caseins in cow's milk. The amino acid composition of horse's milk casein is closer to that of human milk casein than to that of cow's milk casein.

After the colostral stage, whey proteins contain 2–19% serum albumin, 25–50% α-lactalbumin, 28–60% β-lactoglobulin, and 4–21% immunoglobulins. This large range of variation could be due in part to methodological problems. Compared to other dairy species, horse milk is rich in lysozyme and lactoferrin. In particular, the lactoferrin content of horse milk (0.2–2 g kg^{-1} milk) is more than 10 times higher than the content of cow's milk and slightly lower than that of human milk. Lysozyme concentration in horse milk (0.8 g kg^{-1} milk) is similar to that in human milk and much higher than that in cow's milk.

Variations in milk composition according to breed have not been extensively studied. They appear to be of lower magnitude than intrabreed variations. Contrary to observations in cow, the negative correlation between milk yield and butterfat has not been evidenced in mare, although there have been few experiments with a large number of mares. Fat mares produce less protein and more fat in milk in early lactation due to the mobilization of lipid reserves so that the proportion of 18-carbon fatty acids is increased.

More and more data on ass milk are found in the literature. The composition of ass milk is very similar to that of horse milk; however, low fat content is more frequently observed in asses than in horses (*see* **Animals that Produce Dairy Foods**: Donkey).

Modulation of Milk Composition by Feeding

An increase in concentrates in the diet of mares decreases butterfat significantly. The decrease in *de novo* synthesis is a consequence of the decrease in the precursors acetate and butyrate. The decrease in 18-carbon fatty acids is less easy to understand. The highest content of linolenic acid is observed when mares are on pasture in summer (more than 20% in several cases), a period when grass is both rich in fatty acids and contains the highest proportion of linolenic acid to total fatty acids. Linolenic acid proportion is the lowest with concentrate diets, which contain little linolenic acid. Few data are available on the effect of nature of concentrates (rich in fiber, starch, or fat) on milk composition. However, it has been shown that lipid supplementation with corn oil rich in oleic and linoleic acids increases these fatty acids in milk. Feeding fish oil, rich in 20- and 22-carbon fatty acids, leads to a very limited incorporation of eicosapentaenoic and docosahexaenoic acids in milk, showing a very low transfer from diet to milk, as in cows.

High-concentrate diets decrease milk protein content, contrary to what happens in cows. This could be in part due to a dilution effect resulting from a higher milk yield. A decrease in protein content in the diet, leading to a shortage of amino acids, decreases crude protein in milk. Urea in milk also decreases, due to a lower uremia because amino acid catabolism for energy purpose is reduced.

Lactose content is known to be independent of feeding, due to its contribution to the osmotic pressure of milk. However, it has been observed that lactose content was lower in hay-fed mares than in concentrate-fed ones. This could be due to the negative effect of an all-forage diet on precursors of lactose (i.e., glucose and gluconeogenic products such as propionate).

Milk Quality

The bacteriological quality of horse milk is higher than that of cow milk. In the same way, the number of somatic cells is limited. Consequently, mastitis does not seem to be a limiting factor for milk production in mares. Although this problem has not been studied extensively, it is likely that the small size of the mare's udder limits the exposure of teats to infection.

The high level of fatty acid unsaturation could make horse milk oxidizable. In fact, it is much less sensitive to oxidation than expected, perhaps due to natural antioxidants present in milk, although this has not been confirmed. On the other hand, lipolysis probably occurs, as horse milk tends to become rancid, making it necessary to preserve milk by freezing, lyophilization, or spraying if it is not consumed or fermented immediately. The characteristics of the milking machine could contribute to induced lipolysis, but no experimental data are available. The effects of feeding on the organoleptic qualities of milk have not yet been studied.

Milk Products

Horse cheese does not exist because it is not possible to coagulate horse milk with chymosin. The ability of horse milk to coagulate in acidic conditions suggests that the low amount of casein in milk (10 vs. 25 g kg^{-1} for cow's milk) is not a limiting factor for coagulation by chymosin. The absence of coagulation may be due to the low concentration of κ-casein in horse milk because the action of chymosin on κ-casein allows coagulation to be initiated. It has also been suggested that the absence of coagulation was due to the low interaction between calcium and caseins. The pH value, which sometimes reaches 7.0, could also limit chymosin activity.

Koumiss is a fermented horse milk that is popular in Russia, Kazakhstan in west Asia, and Mongolia. Koumiss is widely consumed, especially by elderly people, by tradition, or for its reputed therapeutic value. Koumiss contains between 0.6 and 3% alcohol, at a mean of 2%, and it is slightly gaseous. The end products of fermentation include both lactate and ethanol, due to a consortium of bacteria and yeast that seed the milk. Bacteria mainly belong to the *Lactobacillus* and *Streptococcus* species. Among yeast species, different genera of *Saccharomyces*, *Torula*, *Torulopsis*, and *Candida* are found. This flora is not standardized and differs from one production site to another. Koumiss processing generally lasts 4–6 h. After milk seeding, processing includes sequences of mixing and maturation, sometimes with the addition of milk in the course of the process, to reach the expected acidity and alcohol content. The main problem caused by poor control of the process is the unpleasant taste due to either the proliferation of yeast or an excess of acidification. The name 'koumiss' is now often used, especially in Russia and Germany, for a product based on cow's milk, using the same fermentation process.

Milk Utilization

Logically, horse milk is consumed in countries where traditional breeding of horses for dairy production is maintained. Recently, the use of horse or ass milk appeared in some parts of Western Europe where it is sold as a luxury product.

Uses for Human Consumption

Over time, horse milk has been widely used as a replacer of human milk. It is known to be close in composition to human milk. Lactose and protein content and protein composition are similar to human milk. However, horse milk is much poorer in fat, and the milk fatty acid composition differs. Nevertheless, the allergenicity of horse milk and ass milk is low. Studies on the allergenicity of horse milk in children with IgE-mediated cow's milk allergy have clearly shown that horse milk is much better tolerated. On the other hand, an occasional case of allergy to horse milk has been mentioned. Horse milk is thus used as a substitute of human milk for premature newborns and in case of allergy to infant formulae. It is clear that infant formulae, which are based on cow milk, are less adapted than horse milk. Moreover, the digestibility of horse milk, measured *in vitro* or on rats, is higher than cow's milk and similar to human milk, probably because whey protein is more digestible than casein. Horse milk is also evacuated more rapidly from the stomach than cow's milk. However, (1) horse milk does not have the same composition as human milk and, if possible, it is better that human milk be fed to children; and (2) recent progresses in infant formulae, especially with hypoallergenic milks, restrict the possible uses of horse milk.

For centuries, horse milk has been thought to be beneficial for human health. There is an abundant literature on this subject from the former USSR, but the experimental procedures are seldom rigorous. Empirical knowledge must, however, be taken into account. Horse milk, similar to camel milk, is more efficient than cow's milk in treating chronic hepatitis and peptic ulcers. The higher content of phospholipids and vitamin A is assumed to favor healing; horse milk also has antacid properties. Tuberculosis has been often treated with horse milk, which increases the number of erythrocytes and lymphocytes and restores a normal sedimentation rate of erythrocytes. Long stays in Russian sanatoriums, which dispensed lasting treatments, confirm this.

Anemia, nephritis, diarrhea, gastritis, and other digestive diseases have been treated with horse milk and/or koumiss, and they have been used for postoperative care. Koumiss could be more efficient than raw milk because, in addition to milk components, some products of microbial metabolism, such as peptides or bactericidal substances, could act. A stimulation of the immune system has been suggested. The presence of fatty acids of the n–3 series could promote the synthesis of prostaglandins. Moreover, horse milk contains more lysozyme and more lactoferrin than cow's milk. These proteins are known for their antibacterial activity. However, the specific activity of lactoferrin from horse milk is not higher than that observed in cow, goat, or ewe milk. Among the hypotheses, it has been suggested that peptides coming from the hydrolysis of β-casein could have a positive action on human health. However, specific research on biologically active peptides from horse milk is very scarce. It has recently been shown that koumiss contains peptides with hypotensive activity, but the effect of koumiss on hepatitis or tuberculosis has not been attributed to these peptides. Although the positive effects of horse milk on human health have been widely described in scientific reports and in commercial literature, the mechanisms involved remain unknown.

In economically developed countries of Western Europe, horse milk is often sold in shops that specialize in organic foods, as frozen milk or capsules of lyophilized milk, for people who want to maintain good health. Advertising is based on the good and 'natural' image of the product, on the high content of polyunsaturated fatty acids and A, B, and especially C vitamins, and on the high protein digestibility of proteins, as it is known that albumins are more digestible than caseins. The higher digestibility of horse milk compared with cow's milk is a reality. However, one can wonder whether the consumption of horse milk for this purpose is rational because there are many other natural sources of vitamins and good-quality fatty acids in the human diet that are less expensive than horse milk.

Other Uses

Horse milk is used in cosmetology in Europe. No scientific information is available about the specific properties of horse milk, compared to other milks. However, horse milk has a very good image. Queen Cleopatra bathed in donkey milk. A panel of cosmetic products made with horse milk, such as creams containing about 10% horse milk, soaps, and moisturizers, are commercialized. These products remain marginal, due to the absence of demonstration of the specificity of horse milk compared to cow's milk, which is less expensive.

A minor use of horse milk is the distribution of frozen or lyophilized colostrum to orphan foals, or to newborn foals that refuse to suck their mother's colostrum, or if their mother's colostrum is insufficient in quality or quantity. Indeed, the foal must ingest high-quality colostrum in the first 10 h of life. Colostrum banks of horse milk, or colostrum-concentrated preparations, help ensure adequate colostrum intake by foals and are of interest for owners of high-priced horses, although mother's colostrum is always the best.

Conclusion

Horse milk is often considered to have numerous qualities, mainly related to human health. This is partly because its composition is close to that of human milk, and because there is, in some countries, a long tradition of using horse milk either as a substitute for human milk or as medicine. In fact, there are numerous differences between horse milk and human milk, especially in fat content and protein composition. Moreover, further research is necessary to prove the efficacy of horse milk in treating different illnesses, compared to other treatments. If increased use of horse milk is to be considered, it should be understood that the constraints of horse breeding and the expenses associated with the high frequency of milking result in a high production cost that must be regained through the products being produced.

See also: **Animals that Produce Dairy Foods**: Donkey.

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Donkey

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Introduction

Donkeys are domesticated asses that constitute their own species, *Equus asinus* (class Mammalia, order Perissodactyla, family Equidae); in particular, two populations of wild asses are reported to have been domesticated in Africa *c.* 5000 years ago. The tame and humble species has been involved in human history, mainly as pack and riding animals, contributing to a cultural shift toward more extensive trade. This species remains important for rural economies in semiarid and mountainous areas for light cultivation tasks and transportation. Because of their origins and history, domestic donkeys represented an interesting example of animal biodiversity around the world until the late twentieth century, when the asinine species suffered substantial decline in many countries, as a result of the mechanization of transportation and agriculture.

Donkeys as a dairy species have also been known since the Roman age not only for the nutritional value of their milk but also for its beneficial properties in skin care. For a long time, it was recognized as a common remedy and, in the late nineteenth century, donkey's milk was successfully used for feeding orphan infants in Paris.

More recently, the use of ass's milk has been revalued as an alternative food for infants with cow's milk protein allergy (CMPA), the most common food allergy in childhood. Clinical data obtained by Italian researchers suggest that infants with CMPA could tolerate donkey's milk up to an age at which bovine milk could be reintroduced to some patients' diets without complications. The results also showed high palatability, which is of great importance because of the young age of the consumers. However, such milk requires appropriate nutritional modification before administration to infants, and its safety profile must be carefully evaluated.

Based on these results, studies on donkey management and milk production were carried out in Italy and China, providing more in-depth information on lactation and milk characteristics in donkeys.

Milking Dairy Donkeys

Ass's milk production differs greatly from that of traditional dairy species in terms of both consumer demand and milk supply. However, the feasibility of an innovative dairy donkey enterprise has been demonstrated. Dairy donkey farms are located in Europe, mainly in Italy, France, Spain, and Belgium. Dairy donkeys are also raised in the Xinjiang and Shanxi provinces of China, sharing the largest donkey stock worldwide with Ethiopia and Pakistan.

Different milking strategies have been studied in terms of anatomical, physiological, and ethological characteristics of donkeys, while observing the legal milk quality standards. In particular, European Community legislation defines specific health requirements for the primary production of raw milk from one or more farmed animals, without species restriction (Regulations EC 853/2004 and EC 1662/2006, *Official Journal of the European Union* L 226 of 25.6.2004 and L 320 of 18.11.2006, respectively). In short, milking and storage at 6 °C must be carried out in clean places, designed and equipped to avoid the risk of contamination.

Similar to mares, donkey's milk is essentially alveolar. Therefore, jennies must be milked frequently following 3 h of physical separation from their foals. Natural weaning occurs at 7 months of age or later.

In different experimental conditions, the observed average milk yield ranges from 350 to 850 ml per milking. This variability is a result of many factors such as management of foals and dams, individual milkability, milking procedure, stage of lactation, body size and condition, and feeding.

There are no studies available in the scientific literature on the dairy aptitude of different donkey breeds around the world. Therefore, milk production in jennies is assumed to be proportional to body weight, as is the case for mares. Moreover, some Italian breeds (Martina Franca, Ragusano, Romagnolo, and Sardo) have been defined by microsatellite marker analysis as genetically similar despite their morphological differences. However, a remarkable genetic dissimilarity was noted for the white Asinara donkey.

After teats cleaning, milk from jennies is harvested manually or using a milking machine. Although manual milking could be as efficient as machine milking in terms of quantity of milk harvested, production may vary less when donkeys are machine milked (**Figure 1**). Moreover, the risk of contamination can be significantly reduced when compared to manual milking.

Presence of the foal during milking does not affect milk ejection in jennies adapted to the milking procedure, but when foals are not present the milking routine is more manageable for the animals and for optimal milk harvest.

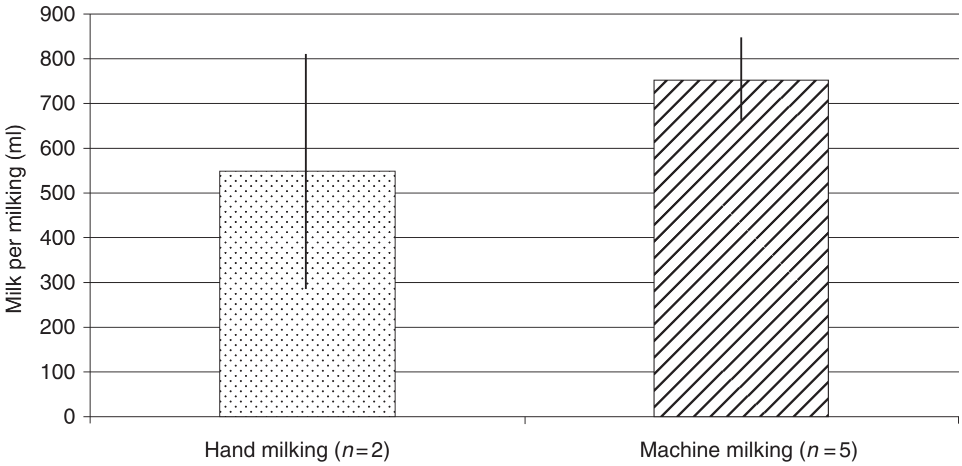

Figure 1 Milk yield per milking according to the scientific literature. Standard deviations about the means are represented by bars. n is the number of publications reviewed. Adapted from Salimei E and Chiofalo B (2006) Asses: Milk yield and composition. In: Miraglia N and Martin-Rosset W (eds.) *Nutrition and Feeding of the Broodmare*, 1st edn., pp. 117–131. Wageningen, The Netherlands: Wageningen Academic Publishers; Guo HY, Pang K, Zhang XY, *et al*. (2007) Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Journal of Dairy Science* 90: 1635–1643.

The essential components of a milking plant consist of a rotary vacuum pump (140 l min^{-1} at 50 kPa) with a 15 l vacuum tank, a 150 ml claw, a stainless steel mastitis detector for visual evaluation of milk, a sheep cluster, and a graduated jar (3 l). Optimized machine milking parameters are very similar to those used for dairy mares (42 kPa of vacuum level, 50:50 pulsation ratio, and a pulsation rate of 120 cycles min^{-1}). In dairy jennies, average milk ejection varies from 40 to 90 s per milking and is directly related to the amount of milk harvested (**Figure 2**).

Recent studies on donkey's milk for human consumption have shown that jennies are usually milked starting 1 month after foaling in order to support the neonatal growth of the foal. The average milk yield per milking in nonpregnant Martina Franca jennies (average body weight 280 kg) shows an initial decline up to the fourth to sixth month of lactation. Subsequently, milk production stabilizes at 600–800 ml up to the 11th month of lactation (**Figure 3**). Individual variation is high, suggesting that adequate genetic selection programs and reproductive technology will lead to a substantial increase in milk production, as already experienced with dairy mares.

In jennies adapted to milking procedures, no differences were observed between morning and evening milkings, consistent with the absence of circadian rhythm for suckling in horses. Moreover, intervals between milkings do not influence the amount of milk harvested by machine milking.

Basic Characteristics of Milk

Data reported in the scientific literature on the gross composition of donkey's milk differ from that of cow's milk and partially confirm the compositional resemblance to human and mare's milk. For example, if lactose, protein, and ash levels are similar to those in human milk (**Figure 4**), the low and variable content of both dry matter (81.0–110.0 g kg^{-1}) and fat (2.8–18.2 g kg^{-1}) is similar to equine milk.

Manual or mechanical milking does not affect the fat content of donkey's milk. However, oxytocin release during machine milking is insufficient for complete udder emptying, consistent with data on dairy cows. Moreover, milk fat is the major component in residual milk and averages 17 g kg^{-1}.

Variables that are associated with changes in milk fat content include (1) lactation stage, with a reduction in milk fat content during lactation; (2) daily rhythms, with peaks occurring at night; and (3) interval between mechanical milkings, with milk fat content showing an increase with longer intervals.

The diameter of milk fat globules varies from 1 to 10 μm, showing a dimensional distribution consistent with that reported for bovine milk (**Figure 5**). Notwithstanding the low fat content and the incomplete knowledge of its composition, the free fatty acid profile of donkey's milk shows, on average, a higher level of total unsaturated fatty acids than that reported for ruminants' milk. Moreover, the high levels of total $n-3$ polyunsaturated fatty acids (PUFAs) (7.4–9.6 g per 100 g fatty acids) are well balanced by the total $n-6$ PUFA content, ranging from 10 to 13 g per 100 g fatty acids. The triglyceride fraction is qualitatively similar to that of human milk and differs from that of cow's milk.

As far as the aromatic composition is concerned, 30 flavor components have been identified in donkey's milk. Besides fatty acids and terpenes most likely of dietary

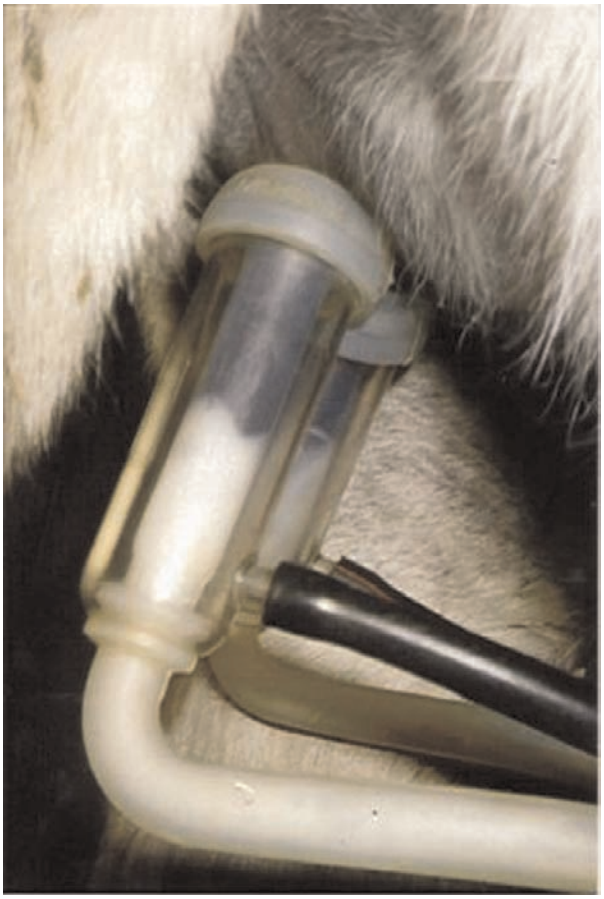

Figure 2 A quick adaptation of donkeys to the management of milking, also including a 3 h separation from the foal, is reported in the literature. Courtesy of Roberta Belli Blanes.

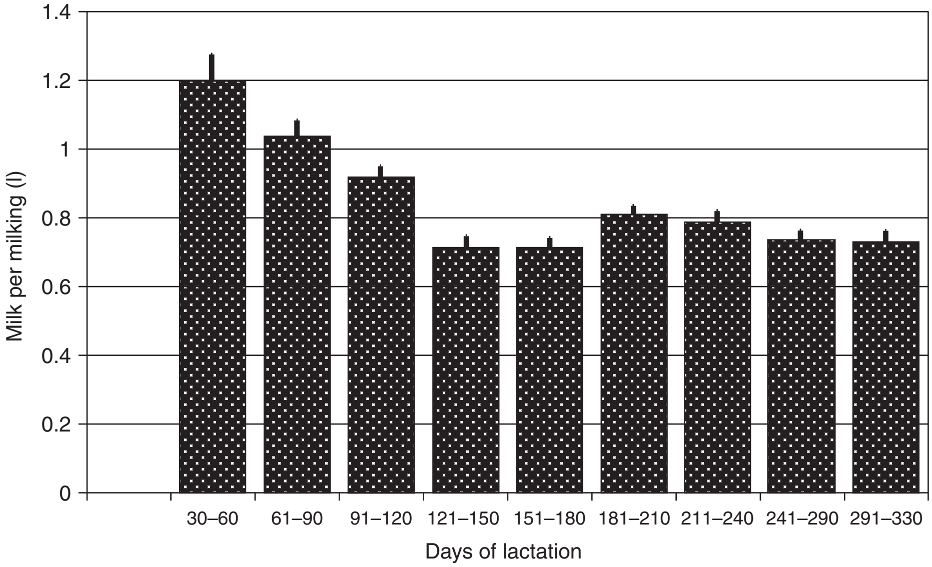

Figure 3 Milk yield per milking of nonpregnant jennies. Bars represent standard errors about means. Adapted from Salimei E and Chiofalo B (2006) Asses: Milk yield and composition. In: Miraglia N and Martin-Rosset W (eds.) *Nutrition and Feeding of the Broodmare*, 1st edn., pp. 117–131. Wageningen, The Netherlands: Wageningen Academic Publishers.

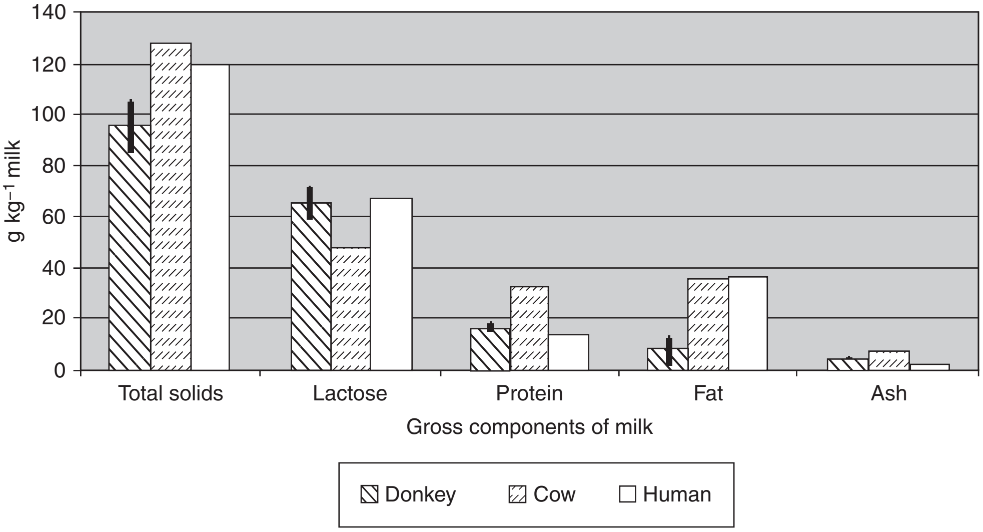

Figure 4 Gross composition of donkey's milk reported in the scientific literature compared to cow's and human milk. Standard deviations about the mean of donkey's milk components are represented by bars. Data from Miraglia N, Polidori M, and Salimei E (2003) A review of feeding strategies, feeds and managements of equines in Central-Southern Italy. In: Pearson RA, Lhoste P, Saastamoinen M, and Martin-Rosset W (eds.) *Working Animals in Agriculture and Transport*, 1st edn., pp. 103–112. Wageningen, The Netherlands: Wageningen Academic Publishers; Salimei E and Chiofalo B (2006) Asses: Milk yield and composition. In: Miraglia N and Martin-Rosset W (eds.) *Nutrition and Feeding of the Broodmare*, 1st edn., pp. 117–131. Wageningen, The Netherlands: Wageningen Academic Publishers.

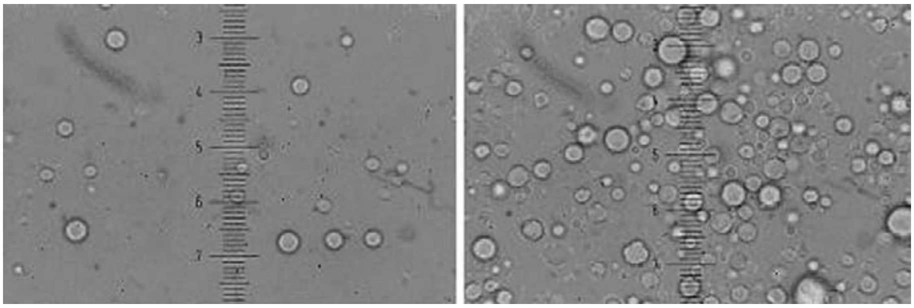

Figure 5 Milk fat globules (×100) from donkey's (left) and cow's milk (right). Reproduced with permission from Maglieri C (2007) Valorizzazione di aree marginali appenniniche mediante un approccio di filiera controllata: Il contributo della nutrizione animale. PhD Thesis, Università degli Studi del Molise, Campobasso. Permitted by Università degli Studi del Molise.

origins, alcohols, ketones, and aldehydes may be derived from biochemical modifications of feed in the animal's digestive tract.

Despite the high lactose content (51–72.5 g kg^{-1}), it must be noted that the energy content, averaging 1750 kJ kg^{-1}, is lower compared to *c.* 2800 kJ kg^{-1} for both human and cow's milk. Therefore, modifying donkey's milk to conform to human milk composition is usually carried out through supplementation with medium-chain triglycerides (4 ml 100 ml^{-1} milk).

The protein content of donkey's milk ranges from 13.6 to 19.0 g kg^{-1} (**Figure 4**), according to the scientific literature. A daily rhythm has been observed, showing a peak during the day, while a gradual decline is reported during lactation, similar to findings from mare's milk.

The average urea content (*c.* 34 mg per 100 g) of donkey's milk is consistent with data from mare's and cow's milk but slightly lower than those from human milk. Moreover, the reported non-protein nitrogen levels, ranging from 14 to 16% of total nitrogen, are intermediate between the lowest values reported for mare's milk and the highest values for human milk.

Current data underline the interesting amino acid profile of the ass's milk protein fraction, showing percentages of some essential amino acids higher than those of both mare's and bovine milk. However, no tryptophan was recovered in donkey's milk protein and cysteine was found at lower levels than in human milk.

It must also be noted that the protein content is characterized by an N casein:N whey protein ratio close to 1, which is less than that in mare's milk but higher than in human milk. Furthermore, the size of casein micelles in donkey's milk ranges from 0.1 to 1 μm, consistent with data from bovine milk.

The weak cross-reactivity observed between donkey's milk and cow's milk proteins is due to the content of beta-lactoglobulin in donkey's milk, which is absent in human milk. However, several studies have demonstrated that the casein fractions may also play an antigenic role. In this regard, proteomic studies highlight the complexity of both casein and whey protein fractions of different milk types, clearly illustrating the similarity of equine and human milk when compared to other dairy species.

Milk, which is the most complete dietary source of macronutrients for growing newborns, should be considered as a source of not only antigens but also bioactive and functional compounds, that is, substances that foster either proper growth and nutrition in newborns or proper secretion in mammary glands. Among the functional proteins detected in donkey's milk, there are molecules active in antimicrobial protection, for example, lysozyme (molecular weight (MW) ~17 kDa) and lactoferrin (MW ~75 kDa): compared to the low level observed in cow's milk, lysozyme and lactoferrin account, on average, for 21 and 4% of whey proteins, respectively.

Bioactive peptides recovered in human milk, such as leptin, ghrelin, and insulin-like growth factor I (IGF-1), are also receiving great attention from nutritional researchers because of their potential direct role in regulating food intake, metabolism, and body composition of infants. In this regard, the human-like leptin content of donkey's skimmed milk ranges from 3.2 to 5.4 ng ml^{-1}, consistent with data obtained from other mammals. Moreover, milk leptin levels did not vary during lactation in nonpregnant dairy donkeys. Human-like ghrelin and IGF-1 have also been measured in donkey's milk: the average values reported for ghrelin and IGF-1, 4.5 pg ml^{-1} and 11.5 ng ml^{-1}, respectively, were similar to those observed in milk from conventional dairy species.

The average ash content in donkey's milk (3.2–5.0 g kg^{-1}) is consistent with data reported for mare's milk but differs from the highest mineral content in cow's milk and the lowest value in human milk. In particular, when compared to human milk, the higher absolute levels of both Ca and P (averaging 680 and 490 mg kg^{-1}, respectively) are evident. However, the average Ca:P ratio (~1.5) observed in donkey's milk is intermediate between the values of bovine and human milk.

Physicochemical characteristics of donkey's milk are summarized in **Table 1**.

Table 1 Range of variation in pH, density, and freezing point reported for donkey's milk

	Min–Max
pH	7.00–7.35
Density (g l^{-1})	1029–1037
Freezing point (°C)	−0.55 to −0.49

Adapted from Salimei E and Chiofalo B (2006) Asses: Milk yield and composition. In: Miraglia N and Martin-Rosset W (eds.) *Nutrition and Feeding of the Broodmare*, 1st edn., pp. 117–131. Wageningen, The Netherlands: Wageningen Academic Publishers; Guo HY, Pang K, Zhang XY, *et al*. (2007) Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Journal of Dairy Science* 90: 1635–1643.

Milk Hygiene and Mammary Status of Jennies

Current data show that donkey's milk is suitable for human consumption when hygiene standards are observed during milking, even though the total bacteria count was found to vary from 3.7 to 5.9 log CFU ml^{-1}. This highlights the importance of cleaning and disinfecting in both manual and mechanical milking. The adoption of adequate hygiene procedures in milking allows a prolonged storage of raw milk at 4 °C, as the total microbial count illustrates after 2 weeks (6 log CFU ml^{-1} milk).

Donkey's milk is considered an optimal growth medium for lactic acid bacteria and a rare presence of undesirable groups like enterobacteria and fecal and total coliform as well as the absence of *Listeria monocytogenes* and *Clostridium* spp is reported in the scientific literature. The observed values could also be related to the presence of lysozyme, ranging from 84 to 375 mg per 100 ml, depending on the analytical method used.

Lysozyme activity is not affected by heat treatments up to 100 °C for 5 min. However, other functional and bioactive compounds of milk may be inactivated by thermal processes.

The low somatic cell count (SCC) of donkey's milk, ranging from 3.5 to 4.5 log SCC ml^{-1}, suggests that mammary health is good with both manual and mechanical milking. Although the incidence of mastitis in equines is low, teat injuries are the main cause when it does occur and may be increased by a more intensive exploitation of dairy jennies.

Husbandry

Donkeys ($2n = 62$) and horses ($2n = 64$) differ only by one pair of chromosomes, but extensive chromosomal rearrangements occurred during their speciation. The jenny is very similar to the mare in many productive and reproductive features, while showing some differences and specific characteristics.

As far as the reproductive behavior is concerned, donkeys (1 jack for 10–15 jennies) are reported to have a mainly territorial social system although jennies can also stay in a semi-harem territorial breeding group. It is therefore advisable to group dairy jennies by age and stage of lactation. Moreover, it is recommended not to breed jennies before 30 months of age.

The estrous cycle lasts on average between 23 and 30 days, the estrous period being 6–9 days. It is important to note that dairy jennies raised in Italy do not cease reproduction during the year although they may show irregularity of the estrous cycle during winter and early spring.

Gestational length in donkeys is on average 372–374 days but both the wide variation reported (11–13 months) and the absence of clear and constant signs of impending foaling may complicate the attended delivery operations. Foaling, on the other hand, is a critical event in the dairy donkey enterprise and management of the puerperal period is crucial, as 'foal heat' occurs as early as 6–10 days postpartum. In pregnant jennies, ultrasonography studies indicated that features of the conceptus resemble those of mares. Artificial insemination has also been successfully investigated in jennies. However, when frozen semen was used, the pregnancy rate was much lower than the level obtained for mares.

During lactation, housing elements in a dairy donkey farm must provide a healthy environment for jennies stabled with their foals on straw bedding, usually in barns with a large external paddock and a covered feeding alley (**Figure 6**). The unit also includes additional boxes where jennies are gathered 3 h before each milking.

In order to improve management of both animals and milk, a specific facility has been developed including a milking parlor and a milk room (**Figure 7**). The unit, equipped with a movable platform for ergonomic purposes when differently sized donkeys are milked, is divided into two areas connected only by the milk pipeline in order to minimize the environmental contamination of raw milk. Jennies show quick adaptation to the facility-related milking routine that requires 3 min per head.

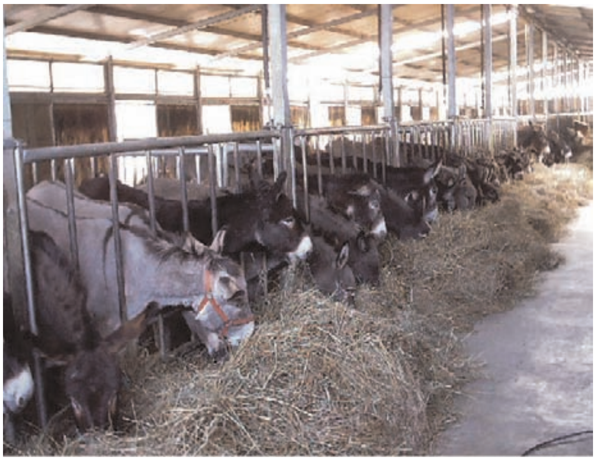

Figure 6 A dairy donkey barn. Courtesy of Davide Borghi.

After weaning, foals are usually moved to pasture provided with movable shelters, feeders, and hay mangers (for adequate supplementation when pasture is poor), while dams are moved to stalls with large paddocks, isolation boxes, and other facilities.

Feeds and Nutrition

Donkeys originated in environments of sparse food supplies and adapted as nonruminant herbivores and hindgut fermenters. They are therefore at an advantage in adverse environmental conditions when compared with cattle, which are ruminants and foregut fermenters. Besides a low daily water requirement and efficient recycling of urea, donkeys can also tolerate very high-fiber diets by increasing their feeding selectivity and/or intake to meet their nutrient requirements. This ability may allow the dairy donkey enterprise to exploit marginal and low-production land, where summer dryness reduces considerably the productivity of pastures and their nutritive value, and hay is characterized by a relatively low protein content and a high content of lignified fiber.

The dietary fiber sources, on the other hand, should be evaluated while considering the structural components. Colic, mainly due to impacted ingesta in the large intestine, is a clinical condition of serious concern, affecting donkeys raised as companion animals. However, in dairy donkeys, little is known about the incidence and associated risk factors of this and other conditions that recognize a nutritional etiology. Obesity may be a problem for donkeys in areas where food is relatively abundant and of good quality. Specific techniques have been developed for assessing both body condition and live weight, as useful indicators of nutritional status and well-being. Moreover, indices of both energy and protein metabolisms have been investigated (**Table 2**) in dairy jennies.

Nutrient requirements for milk production are not yet defined for donkeys. However, based on a high feed intake (3.0–3.2 kg dry matter per 100 kg body weight), the dairy jenny rations reported in the scientific literature are on average characterized (dry matter basis) by a 70:30 forage-to-concentrate ratio, a protein content of 10–13 g per 100 g, and a digestible energy value of 8.5 10.0 MJ kg^{-1}.

Digested dietary fats, soluble carbohydrates, and protein, besides minerals (except for phosphorus), are mainly absorbed by the small intestine of equines; the absence of biohydrogenation before absorption suggests the direct influence of the diet on the fatty acid composition of milk. Moreover, the transfer of *n*−3 PUFAs from blood to milk was found to be more efficient in jennies than that of *n*−6 PUFAs: for a nutritionally correct ratio of

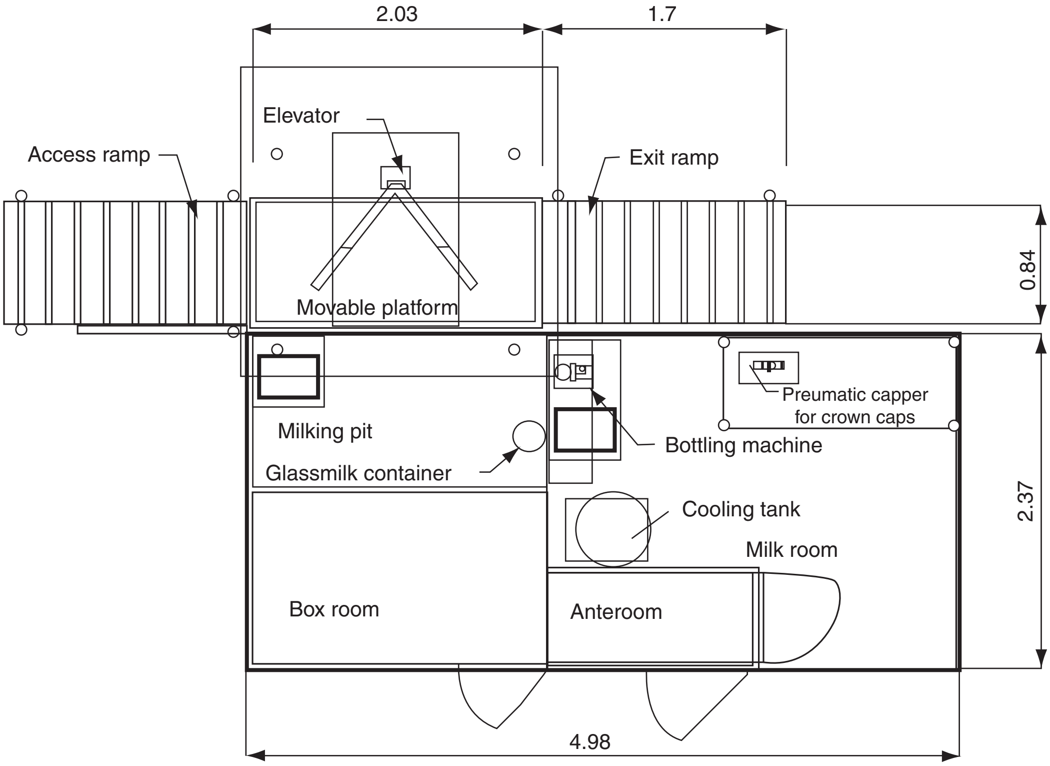

Figure 7 Plan of a milking facility (milking parlor and milk room) for donkeys. Dimensions are given in meters. Reproduced with permission from Simoni A, Salimei E, and Varisco G (2004) Milking parlour and routine and characteristics of ass's milk yield, an hypoallergenic food for infants. In: Martin-Rosset W, Miraglia N, Salimei E, Pilla F, and Bergero D (eds.) *Proceedings of the 6th Congress on New Findings in Equine Practices*, pp. 85–91. Università degli Studi del Molise, Campobasso. Permitted by Università degli Studi del Molise.

Table 2 Nutritional profile of lactating jennies

	Min–Max
Glucose (mmol l^{-1})	3.4–4.5
Triglycerides (mmol l^{-1})	0.2–0.7
Cholesterol (mmol l^{-1})	1.6–2.9
Nonesterified fatty acids (mmol l^{-1})	0.13–0.17
Total protein (g l^{-1})	63–73
Albumin (g l^{-1})	25–32
Urea (mmol l^{-1})	4.1–6.2
Creatinine (μmol l^{-1})	97–138

Adapted from D'Alessandro AG, Martemucci G, Palazzo M, *et al.* (2006) Values on serum metabolites in lactating jennies of Martina Franca breed. In: Miraglia N and Martin-Rosset W (eds.) *Nutrition and Feeding of the Broodmare*, 1st edn., pp. 171–174. Wageningen, The Netherlands: Wageningen Academic Publishers; Fantuz F, Maglieri C, Palazzo M, Chiofalo B, and Salimei E (2007) Nutritional status of dairy asses managed with different machine milking strategies. *Italian Journal of Animal Science* 6(supplement 1): 647–649.

$n-3$:$n-6$ PUFA in milk, dietary lipid sources should be evaluated with regard to the fatty acid profile.

Dietary factors can also influence the palatability of donkey's milk: among the flavors with a distinctive sensorial character, C6 and C9 alcohols and aldehydes were identified, accounting for the 'green' aromatic notes of milk fat. Also, beta-pinene, beta-myrcene, limonene, *p*-cymene, and gamma-terpinene, related to the 'green-grassy' flavor, were identified in milk when jennies were fed fresh forage.

Finally, it must be considered that a highly digestible creep feed should be provided to nursing foals until 1 month of age while complementary feeds should be administered to foals until weaning, when dams are milked. Constant access to water and salt blocks is highly recommended for both foals and dams.

Health Issues in Dairy Donkeys

Dairy donkeys must be negative for glanders, tuberculosis, brucellosis, and equine infectious anemia. According to the European normative, raw milk must also come from donkeys that do not show any symptoms of infectious diseases and are in a good general state of health, presenting no sign of disease that might result in the contamination of milk.

Moreover, animals must not be affected by any infection of the genital tract or enteritis with diarrhea and fever; udders with apparent inflammation or wounds cannot be milked.

When authorized products or substances have been administered, the withdrawal periods prescribed for these products or substances must be observed.

Besides adequate parasite control for the animals, it is also recommended to carefully avoid not only mycotoxin-contaminated grains in the dairy donkey ration but also moldy feedstuffs. These may affect the microenvironmental parameters of housing in an intensive farming system, predisposing the animals also to respiratory and eye diseases.

Utilization of Milk

Based on clinical evidence on the hypoallergenicity of its protein fraction, donkey's milk represents an interesting alternative food for infants with allergies. The rationale treatment of this disease may require changing food regularly because of allergenic sensitization: when breast milk is not available or not advisable, any hypoallergenic and palatable substitute should resemble the nutritional characteristics of human milk. In this regard, formulae based on extensively hydrolyzed proteins or soybean-derived formulae are preferred in the treatment of CMPA. However, these may have drawbacks, for example, bitter taste and risk of cross-reactions in highly sensitive infants.

The organoleptic characteristics have certainly contributed to the success of ass's milk used as infant food, its high palatability being due to its flavors as well as high lactose content. However, the 'virtues' attributed to donkey's milk could also be referable to its health-promoting properties, likely related to the presence of functional and bioactive components of recent interest in human nutrition.

In such an agro-medical chain, management of animals and milk should be carefully evaluated and regulated in specific traceability systems for obvious safety reasons.

As previously highlighted, the nutritional deficiencies of donkey's milk must be corrected through appropriate nutrient supplementation to optimize its role in infant nutrition. Moreover, immediately prior to consumption, donkey's milk should be heated to 72 °C for 15 s, considering that its heat stability is very weak when compared to both cow's and mare's milk. The low level of vitamin E (on average 5 μg per 100 g raw milk) is also significantly reduced by heat treatments.

In addition to its role in infant nutrition, the low fat content and its fatty acid profile, resulting in low atherogenic (on average 0.80) and thrombogenic (on average 0.32) indices, candidate donkey's milk as possible food for the prevention of pathologies in elderly people, such as atherosclerosis.

Donkey's milk could also be placed among the new generation of fermented milk drinks, such as koumiss derived from mare's milk, and would allow for an effective combination of the advantageous properties of the raw ingredient with lactic acid bacteria of probiotic interest.

In recent years, dairy products such as ice cream and desserts have been developed from pasteurized donkey's milk.

Besides its potential role in human nutrition, historically donkey's milk has also been used in cosmetology, most likely due to its lysozyme content, effective in smoothing skin and scalp inflammations.

Conclusion

Donkey's milk represents a recent niche product in human nutrition, and for people with food allergies. Food scientists may exploit its natural attributes and help expand the dairy donkey enterprise, especially for those marginal and hilly areas that are increasingly abandoned, resulting in increased soil instability and fire damage. The recovery of these areas using sustainable agriculture could be included in promoting rural tourism with the donkey as a feature of cultural and historical education. Besides these cultural relapses, the renewed interest toward donkeys could help in preserving animal biodiversity, counteracting the negative trend generally occurring in donkey populations of industrialized countries.

Finally, besides their dairy aptitude, donkeys could also play an important social role as companion animals and could also be used in an emerging form of pet therapy known as onotherapy.

See also: **Animals that Produce Dairy Foods:** Horse. **Milk**: Equid Milk. **Nutrition and Health:** Milk Allergy.

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Reindeer

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Introduction

The reindeer (*Rangifer tarandus*) is the only cervid with a long and continuous history of domestication. Reindeer husbandry probably originated in southeastern Siberia around Lake Baikal as a result of resource depletion. The extinction of the megafauna in the early Holocene left reindeer as the main meat source for many hunter and gather societies, and the increased harvesting pressure led to a decline in the wild populations. As a result, these societies could no longer rely on the efficient and affluent seasonal harvesting of migrating reindeer. The shrinking resource base induced a more nomadic lifestyle following the herds year-round. Gradually, the domestication process started, characterized by small tame multipurpose herds, where milking was an integrated part of the production system. Indeed, hunting, fishing, and gathering contributed substantially to these subsistence-based societies and using domestic reindeer for transportation was crucial for their mobility – a key factor for efficient acquisition of clumped but scattered resources. This form of reindeer husbandry is at least 2000 years old. A similar milking practice evolved independently around AD 1500, among the westernmost reindeer herding ethnic group – the Saami.

Today, reindeer husbandry is practiced in about 20 ethnic groups spread all over the northern part of the Eurasian continent, from the Saami of Fennoscandia in the west throughout Siberia to the Koryak of eastern Siberia. The industry engages about 25 000 people owning around 2 million semidomestic reindeer, roughly 1.2 million in Russia (half of them are located in the Yamal-Nenets Autonomous Region) and almost 700 000 distributed quite evenly between Finland, Sweden, and Norway. The industry in Fennoscandia and Russia is dominated by extensive meat production. Also, in some parts of Russia, soft antlers are an important product. In other parts of Russia, a subsistence-based economy is still prevailing, but only remnants of traditional milking are practiced by some ethnic groups in southeastern Siberia.

This article briefly describes the traditional reindeer milking regime, the ecological and life history constraints set by the reindeer, and key production parameters including chemical properties of the milk, and discusses challenges of establishing a new niche-based milk industry.

Past

Where

According to Chinese annals, reindeer pastoralism with milking as an integrated part evolved at least 2000 years ago in the Taiga region of eastern Siberia around Lake Baikal and spread to the nearby groups. Cultural exchange, facilitated by the pastoral nomadic practices of the Asian steppe, probably triggered this development. This small-scale subsistence-based economy, with hunting and fishing as integrated parts, persisted relatively unchanged until the Socialist Revolution in 1917. The collectivization and rationalization in the 1930s transformed these societies into market-oriented economies and the close bond between the herders and their households and the animals, a prerequisite for keeping tamed milking animals, was weakened. However, in the late 1920s, researchers started exploring the industrial potential of reindeer milking, but in the late 1930s the production was shut down. The main objection to commercial milk farming was said to be the negative effect milking had on calf development. Indeed, the low yield and labor-intensive production made it not profitable. In addition, the tight herding and confined grazing made the animals susceptible to contagious diseases and parasites, which also contributed to the setback. Today, only remnants of traditional milking are practiced among some groups of Tofalars, Evenks, and Tuvins centered in the Baikal area.

The milking regime evolved independently, although at a later stage (probably around AD 1500), among the westernmost reindeer herding ethnic group, the Saami, influenced by the Nordic milking husbandry culture. The early Saami reindeer husbandry was based on small tame herds as in southeastern Siberia and had many resemblances to the herding practice in that area.

In the early 1800s, a transition period started, lasting for about hundred years and ending up in an extensive pastoralism based on meat production. Still, in the late 1800s, many Saami reindeer herding families living in the southern area of their range practiced small-scale reindeer pastoralism where milking was an integrated part of the production system and milk was used to manufacture cheese and butter for domestic consumption as well as for sale. However, milking was abandoned in the early 1900s. Milking was occasionally practiced up to the 1960s, but not as an integrated part of the production system.

Management Practice

The reindeer pastoralists adopted methods of milking and processing from their southern pastoralist neighbors and developed it further as part of their nomadic reindeer husbandry. However, all kinds of transition forms existed, from sporadic to regular milking and manufacturing, reflecting ecological adaptations to natural resources, topography, and climate, as well as cultural traditions and external influences. The nomads had to protect their small semidomestic herds from mixing with wild reindeer by intensive herding. This favored close bonds between the herders and their reindeer and a mutual relationship evolved characterized by multiple use of the animals primarily based on living products (decoys for hunting, transportation, hair, milk, and antlers), whereas the herders provided protection against predators and insects and occasionally supplied feed. These multipurpose production systems also influenced management practices including herding techniques, herd size and composition as well as selection regimes. We here shortly describe the most intensive form found in the two core areas: in southeastern Siberia among groups of Tofalars, Evenks, and Tuvins and in Fennoscandia among Saami groups.

The first weeks after parturition the calves were given first priority to the milk produced by the females. Indeed, raising calves, for recruitment and traction as well as meat production, was an integrated part of the milk production regime. Milking started in the middle of June and continued until the rut in early October. During this period, the calves were allowed to suckle their mother only part time. Females and calves were physically kept separated by tethering or penning during part of the day. Techniques limiting the calves' access to the udder were also used by covering the whole udder or parts of it. The most efficient form of Saami milking regime, but also the most labor intensive, was to 'kjevle' the calf. The calves were caught in the afternoon and equipped with a small wooden stick in the mouth, which makes them unable to suckle, but does not hamper grazing seriously. The females were milked to empty the udder and the herd released for grazing. The next morning, the animals were herded into pens where the females were milked and the calves demounted the 'kjevle'. This routine was normally repeated 2–3 times per week. An extensive form of milking took advantage of the synchronous activity pattern of the animals. At the end of a long resting period (normally around noon), the flock was herded into pens, onto snow patches, or into natural delimited areas, for example, a peninsula, not allowing the calves to suckle before the females had been milked.

Interestingly, all these techniques are known from other pastoralist systems. They were flexible and easily adjustable while securing the growth of the calves and a descent milk output. The production systems were based on the selection of good and healthy milking females, which were home bound and easy to herd and handle. The females were easily trained and stayed calm during milking, which for a trained worker took 10–15 min depending on udder characteristics and quantity of milk. In some systems, the back teats were covered allowing the calf to approach and suckle the front teats for a short period, thus priming the udder with its butting. In other systems, the milkers patted the udder in imitation of the calf's butting to induce the letdown. But the most common practice was to mimic the calf's butting by striking the udder vigorously by hands. Often the animals were given salt and during periods of insect harassment smoking fires were burned to protect and calm the animals.

Milking was labor intensive. Not only the milking *per se*, but also the handling of the animals, the intensive herding, and the frequent migrations including establishing new camps and construction of pens were very time consuming. Indeed, processing the milk was also labor intensive.

Products and Processing

Reindeer milk was important in the pastoralists' late summer and early fall diet and provided milk both for immediate consumption and for processing. The milk products were used primarily within the household economy. However, the most advanced milking societies also used cheese and butter as trading goods. The processing techniques were very much the same as practiced by the pastoralist neighbors to the south, developed and adapted according to local conditions.

During the lactation period, the chemical composition of the milk changes substantially, reaching a fat content of 15–20% in late lactation. This influences the processing properties of the milk. Normally milk from mid-June was consumed fresh; midlactation milk was used for cheese production, while milk from the last stages was more appropriate for churning butter. In mid- and late lactation, milk was stored in cool sites in wooden containers for use next winter and spring; occasionally, milk was also stored frozen.

Lactation

Life History Perspective

Semidomestic reindeer is the only domesticated species whose wild ancestor evolved in the harsh northern environment. This suggests that semidomestic reindeer are adapted to a 'low-input–low-output' life history strategy, which limits the milk yield. Lactation is the major energetic component of maternal investment in mammals, implying allocation trade-offs influencing milk yield and chemical composition as well as reproductive and behavioral traits. The short summers favored a rapid and affluent transmission of energy and protein from mother to calf to optimize lifetime reproductive success. The reindeer is a typical capital breeder, relying on body reserves to satisfy energy needs of early lactation. Accordingly, lowered milk production and decreased suckling time have been observed when the mother is low in body reserves. The milk production and accessibility are under maternal control, and, given the short summer, it is in the mother's best interest to control the availability and to time the weaning, which normally occurs before rut in late September/early October, so that she is able to improve her body condition to ovulate in due time during the upcoming rut. This secured an optimal timing of the birth in spring (normally in early May) – an important fitness parameter in seasonal environments.

Does produce only one offspring per reproductive event, reflecting the limitations in feed resources. Reindeer have a follower-type mother–young relationship because of their migratory habits. The relatively small udder of reindeer compared with other ungulates is advantageous considering the great need for mobility. Indeed, the secretion of concentrated milk, high in protein and low in lactose, is appropriate for both body growth and energy requirements of the calf in a harsh environment.

The suckling pattern is characterized by frequent bouts of short durations. The daily nursing time drops from about 1 h at peak lactation to less than 10 min after 8 weeks. Peak lactation occurs 3–4 weeks after calving, and is estimated to be about 1 l day^{-1}. Production declines linearly after the peak is reached, and is reduced to around 50% of peak production after 12 weeks (midlactation) and to 25% after 20 weeks (late lactation) (**Figure 1**). However, the increase in milk nutrient content during the same period partly compensates the decline. This also underlines that during the last part of the lactation the nutritional transfer of resources from doe to offspring is energetically demanding.

Milk Composition

Although reindeer milk has been extensively analyzed, it is difficult to characterize its composition, due to limited sample sizes, different sampling techniques as well as feeding regime and variation in lactation stage examined.

The milk of reindeer from peak lactation is relatively high in fat (11–15%) and protein (7–10%) and moderately low (about 3.5%) in lactose (**Table 1**), as compared to the milk of most ungulates. The progressive rise in dry matter during lactation as compared to other cervids is especially pronounced in *Rangifer*. Especially the increase in fat content is prominent as lactation progresses, and in late lactation the milk is creamy with a fat content of around 20%. The ratio of protein to fat throughout the lactation follows the interspecific relationship typical of

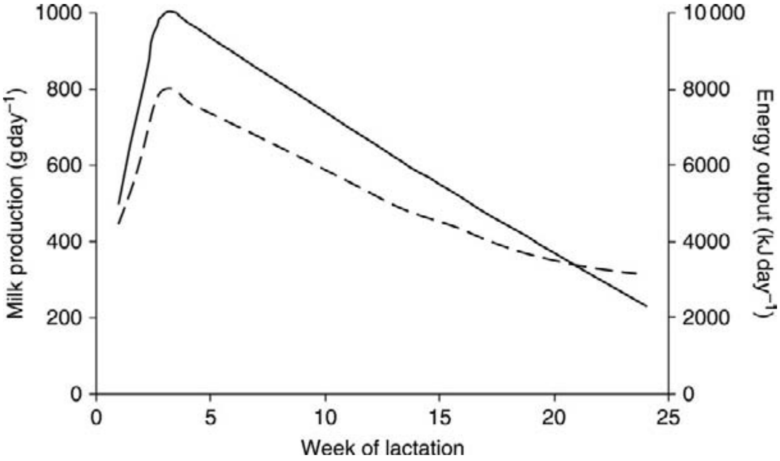

Figure 1 Schematic curve of daily mean potential milk production (continuous line) and energy output (dotted line) of an average female (75 kg) through lactation. Reproduced with permission from Holand Ø, Gjøstein H, and Nieminen M (2006) Reindeer milk: Composition, yield and utilization – challenges for establishing a modern reindeer dairy industry. In: Park YW and Haenlein GFW (eds.) *Handbook of Milk of Non-Bovine Mammals*, pp. 355–370. Blackwell Publishers.

Table 1 Gross composition (% of wet weight) of reindeer milk

Week of lactation	*No.*	*Dry matter*	*Fat*	*Crude protein*	*Lactose*	*Ash*
4–5	8	27.1	11.1	11.1	3.0	1.5
4	3	23.7	10.2	7.5	3.7	1.2
6	5	26.0.	12.4	8.8	3.1	1.4

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ruminants. The decreased ratio during the course of lactation is probably due to the high demand for protein for growth in calves during the early stage of life, whereas fat deposition to meet the harsh winter probably becomes more important as the lactation progresses.

Protein

Casein is the predominant protein fraction in reindeer milk with a content of around 8%, whereas β-lactoglobulin is the main whey protein. The relative composition of the different amino acids in reindeer milk is rather constant throughout the lactation, although the protein content increased from around 9% in early lactation to around 12% in late lactation. The amino acid profile resembles that found in small ruminants, except for relatively low value of cysteine and high value of tyrosine (**Table 2**). However, the absolute content of almost all amino acids, as compared to most dairy animals, is high.

Table 2 Mean amino acid composition (weight % of the milk protein fraction) in reindeer milk based on samples from (1) and (2)

Amino acid	*(1)*[a]	*(2)*[b]
Alanine	3.1	3.26
Arginine	2.6	2.75
Aspartic acid	6.7	6.70
Cysteine	0.8	0.73
Phenylalanine	4.5	4.63
Glutamic acid	20.6	18.97
Glycine	2.3	2.34
Histidine	2.5	2.54
Isoleucine	4.6	4.38
Leucine	9.5	9.69
Lysine	7.9	8.08
Methionine	2.9	2.71
Proline	9.2	9.93
Serine	5.7	5.58
Threonine	4.7	4.73
Tryptophan	1.3	1.52

[a] Eight animals fed a pelleted concentrate (Poronherkku) (Malinen *et al*. 2002).

[b] Four animals during three lactational stages (early, mid, and late) fed a pelleted concentrate (Formel Favør 20) (Holand *et al*., unpublished data). Reproduced with permission from Holand Ø, Gjøstein H, and Nieminen M (2006) Reindeer milk: Composition, yield and utilization – challenges for establishing a modern reindeer dairy industry. In: Park YW and Haenlein GFW (eds.) *Handbook of Milk of Non-Bovine Mammals*, pp. 355–370. Blackwell Publishers.

Fat

Fat is the major energy component, representing two-thirds of the energy content at peak lactation and three-quarters at late lactation. The fat composition of reindeer milk is similar to that of cow's milk, containing high levels of palmitic acid (16:0), accounting for one-third of the total fat, and stearic (18:0), oleic (18:1), and myristic (14:0) acids, each accounting for around 13% of the fat, along with smaller amounts of short-chain fatty acids (**Table 3**). The content of *de novo* short-chain fatty acids, especially butyric (4:0) and caproic (6:0) acids, seems to be higher in reindeer (**Table 3**) than in most ungulates, but will depend on how much of the substrate for fat synthesis is based on forage resources versus body reserves. The studies on the fatty acid composition of reindeer milk are based on supplementary feeding. Indeed, the fat composition of milk of reindeer on natural pastures will probably differ slightly including a larger proportion of conjugated linoleic acid (CLA), as their precursors, especially linoleic acid (18:2), are abundant in natural forages.

Lactose

The average lactose content in reindeer milk at peak lactation is 3–3.5% on a wet weight basis (**Table 1**), which is slightly lower than what is found in other wild ungulates. In reindeer, the lactose content is reported to decrease during the lactation cycle, confirming its role as an osmotic regulator.

Minerals

The mineral content of reindeer milk is moderate to high (1–1.5% per unit of fresh milk) as compared to most other ungulates, whereas the ash content of the dry matter is more or less similar, accounting for around 5%. The relative concentration of the main minerals, namely, calcium and phosphorus, is quite similar to that of other ungulate species, accounting for around one-fourth and one-fifth of the ash, respectively, whereas sodium and potassium are markedly less concentrated in cervids, including reindeer, compared to cow's ash. Increased concentration of osmotic active salt may compensate for the decrease in the lactose content during the lactation cycle, as suggested by the increased content of ash in reindeer milk as the lactation progresses.

Table 3 Fatty acid composition in reindeer milk based on six females fed a pelleted concentrate (Poronherkku) during midlactation (Holand *et al*. unpublished data)

Fatty acid	*Weight % of FAME*
4:0	6.77
6:0	2.42
7:0	0.09
8:0	0.47
10:0	0.79
11:0	0.06
12:0	0.99
13:0	0.07
iso-14:0	0.11
14:0	12.68
iso-15:0	0.21
anteiso-15:0	0.36
14:1	0.22
15:0	0.78
iso-16:0	0.24
16:0	35.47
iso-17:0	0.32
16:1n-9/anteiso-17:0	0.48
16:1n-7	0.90
17:0	0.41
iso-18:0	0.05
17:1n-8	0.07
18:0	13.87
trans4,18:1	0.04
trans5-6,18:1	0.17
trans7-8,18:1	0.30
trans9,18:1	0.27
trans10,18:1	0.33
trans11,18:1	0.88
trans12,18:1	0.30
cis9,18:1	13.38
trans15,18:1	0.17
cis11,18:1	0.54
cis12,18:1	0.19
cis13,18:1	0.07
trans16,18:1/cis14-18:1	0.35
18:2n-6	2.26
20:0	0.26
18:3n-6	0.05
20:1n-7	0.08
20:1n-9	0.07
18:3n-3	0.27
cis9,trans11,18:2	0.31
22:0	0.09
20:4n-6	0.19
22:5n-3	0.08
Others	1.52

FAME, fatty acid methyl ester.

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Vitamins

Reindeer milk is rich in both fat-soluble vitamins and water-soluble vitamins. Ten days after birth, vitamin A, E, and D levels of 51–141, 19–88, and 0.07–0.14 µg per 100 g, respectively, have been reported, whereas vitamin C content was around 2 mg per 100 ml. Reindeer milk is also rich in D_3 (0.5–2.0 mg kg^{-1}) as compared to cow's milk and its K_3 content is nearly twice that of cow's milk (0.06–0.08 mg kg^{-1}).

Milk Yield

Domesticated species are adapted to releasing their milk under human-induced stimuli. However, the biological potential of a species as a milk producer should be assessed by the potential yield rather than the actual yield. Indeed, convergence of the two measurements could be a measure of degree of domestication.

Potential yield

The shape of the lactation curve in reindeer (**Figure 1**) is similar to curves found in most wild ungulates exposed to highly seasonal environments. A daily output at peak lactation, which occurs during weeks 2–4, of 1–1.5 l has been reported. The total milk yield during lactation has been estimated to about 100 kg. Estimated milk yield of does on a low plane of nutrition equaled only 57 kg, confirming that variation in the nutritional state of the mother has a marked effect on milk production.

Actual yields

Previously published estimates of milk yield in reindeer are difficult to interpret and should be handled with caution, basically because of lack of information about milking practice, including separation time, lactation interval, stimulation practice, regularity of milking, and stage of lactation, as well as the nutritional status of the cows. Reliable Russian measurements from the 1930s reported an average daily yield of 250–300 g with calves partly separated. In a series of experiments we have measured the actual yield to be 100–200 g 2–3 months after parturition with a separation time of 8–12 h.

Energy content and output

In reindeer milk, the energy content at peak lactation is around 7 kJ g^{-1}. With a milk yield of around 1 l for a female weighing 75 kg, the daily output at peak lactation will be about 7000 kJ, comparable to the interspecific regression of ungulates with single young: $\approx$300 kJ kg$^{-0.75}$. Indeed, the daily energy output at peak lactation is remarkably high considering the harsh winter conditions and that the females have to draw on their own body reserves early in lactation to meet the calves' need. However, the short lactation period ($\approx$4.5 months) and the rather rapid decline in milk production after peak lactation can be seen as an adaptation to the short arctic summer. This compares with the somewhat lower total energy output during the entire lactation period per unit metabolic weight in reindeer (about 30 MJ kg$^{-0.75}$) as compared to most other cervids. Although the content of fat and protein increases with the stage of lactation, the total production of fat, protein, and lactose decrease, because of the decline in milk yield. From peak lactation the energy output decreases markedly, whereas from midlactation the energy output declines at a somewhat slower rate than the milk yield (**Figure 1**). This is mainly attributed to a marked increase in milk fat content.

Present

In the mid-1990s, an initiative was undertaken for establishing reindeer dairy milking as a niche production in Fennoscandia. A milking machine for reindeer was therefore developed and tested. The udder health of the reindeer was unaffected by long-term regular machine milking. Basic lactation physiology and behavioral studies were carried out and different management regimes tested, but in the early experimental years the main challenge was to induce efficient milk ejection.

We first attributed the low actual yield of 100–200 g in midlactation using a separation time of 8–12 h to stress. This was in line with the high residual milk volume of around 75% reported, although habituation reduced the volume to around 50%, in late lactation. The importance of ejection for milk yield is well documented, and various stressors may inhibit or suppress milk letdown, even among traditional dairy animals. However, the reindeer rapidly habituated to the milking environment. We did not observe any negative reaction during the stimulation, put-on, milking, and offtake phases and concluded that the females showed low level of stress during the milking sessions, which was also confirmed by only a slight increase in heart rate. Actually, the female's heart rate was in the same range as that when the calves were suckling, increasing from around 75 beats to 100–125 beats per minute. Furthermore, we observed that after a short stimulation of calves butting, the most efficient ejection stimulus for inducing ejection reflex, we were able to empty the udder completely. We therefore concluded that the milk ejection problems in reindeer were not related to degree of domestication, but a question of appropriate stimulation. The field season 2004 was a breakthrough for an efficient and complete milk letdown in reindeer. The proportion of residual milk was reduced as lactation progressed, from around 40% at the start of milking in mid-June to around 20% in late August (**Figure 2**), confirming that appropriate stimulation of the udder and also habituation and training are important factors for triggering milk ejection.

We were able to keep up a high output throughout August (**Figure 2**) by supplementary feeding and partial separation of mother and offspring. Permanent separation also worked, although the total milk production was one-third less as compared to females having calves at foot part of the time. The growth curve of calves that had been separated in mid-June did not differ from the growth curve of calves that had been partly separated. This suggests that the calves are able to compensate for the reduced milk intake by increasing their intake of forage. However, the separated calves were kept in a 'kindergarten' together with females suckling their own offspring, and they were able to 'steal' milk from these does by allosuckling.

Future

Maintenance of milk secretion requires repeated exposure of the mammary cells to hormones released during milking, in addition to a continuous removal of milk. In semidomestic species, it may be hard to maintain normal secretion rate in the absence of young, unless the milking frequency is high during the first days after separation of

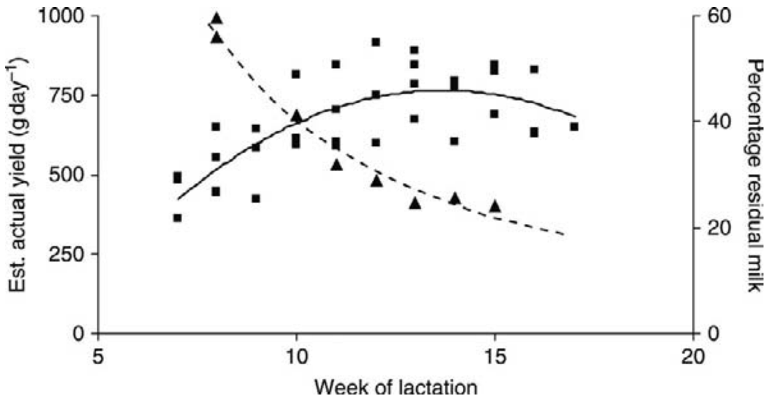

Figure 2 Estimated actual daily milk yield (■) of reindeer does milked by machine after 8–14 h separation from their calves and percentage of residual milk (▲) through mid- and late lactation (Holand *et al*., unpublished data).

mother–offspring. Hence, permitting limited suckling by calves may be a viable strategy for keeping up the production. This will secure a flexible management system, for example, in periods of shortage of labor force or insect harassment, the milking can be reduced without jeopardizing future yield. Also from an ethical perspective, calves partly at foot would be preferable.

Reindeer milking is an intensive form of reindeer husbandry, and is hardly feasible within the extensive free roaming meat production system practiced today, as selection for milk yield and training of the milking does would be severely constrained. But in isolate areas, a partly free-ranging milking system may be viable if a herdsman and his dog(s) manage the roaming animals during the daytime and pen them close to the camp at night. The animals should be milked both morning and evening. To secure the females' homing, the calves could be kept separated close to the milking camp and allowed to join their mothers after the milking session. The homing could be further enhanced by supplementary feeding, which also will contribute to a positive energy balance and hence a sufficient milk output. The summer milking farm has to be highly mobile to ensure high-quality natural forages and reduce the parasite load and risk of contagious diseases.

Reindeer milk is and will be a valuable niche product, but a commercially sound production is dependent on continuing high returns. The marked potential lies in an exclusive niche within the tourist industry as well as in the gourmet and cosmetic market. A new milking industry would need to adapt new techniques and management regimes within the biological and ecological constraints of the animals and the environment and within an acceptable ethical framework.

See also: **Milk**: Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.).

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B

BACTERIA, BENEFICIAL

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Bifidobacterium spp.: Morphology and Physiology

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Introduction

Fermentation with lactic acid bacteria is one of the oldest methods of food processing and preservation. Fermented foods containing selected strains of *Bifidobacterium* are claimed to provide several prophylactic, probiotic, and therapeutic benefits. Fermented dairy foods containing *Bifidobacterium* spp. are well established in a number of countries in Europe and also in Japan, and the products are gaining popularity in North America and Australia. As a result, several brands of yogurt now sold in these countries contain *Bifidobacterium* spp. This article will consider the taxonomy and occurrence, gastrointestinal ecology, growth characteristics, and carbohydrate metabolism of *Bifidobacterium* spp.

Taxonomy and Occurrence

Bifidobacterium was first isolated in 1899 from the feces of breast-fed infants by Tissier of the Pasteur Institute. When first isolated, the organism was named *Bacillus bifidus*, based on the morphology, as the organism typically exists in a Y-shaped or 'bifid' form. An Italian scientist also discovered a bacterium in similar conditions as described by Tissier and classified the organism as belonging to the genus *Lactobacillus*. Although, differences existed between these two bacteria, a common name, *Lactobacillus bifidus*, was proposed. In 1924, Orla-Jensen was responsible for a decisive shift in the direction of the history of taxonomy of lactic acid bacteria. The classification and identification of microorganisms are now based on other criteria, such as genetic physiology, metabolic and enzymatic characteristics, and nutritional requirements for energy metabolism. In 1967, de Vries and Stouthamer demonstrated the presence of fructose-6-phosphate-phosphoketolase (F6PPK; EC 4.1.2.22) in bifidus and the absence of two enzymes, aldolase and glucose-6-phosphate dehydrogenase, which are found in lactobacilli. The presence of other enzymes, such as α-galactosidase (EC 3.2.1.22) and α-glucosidase (EC 3.2.1.20) in *Bifidobacterium*, could be used for rapid differentiation and identification.

The taxonomy of *Bifidobacterium* has been revised since it was first described; at different times, the organism has been assigned to the genera *Bacillus*, *Bacteroides*, *Nocardia*, *Lactobacillus*, and *Corynebacterium*. Recent techniques in strain identification of *Bifidobacterium* include use of DNA probes and pulsed-field gel electrophoresis. The percentage of guanine and cytosine (G+C) in the DNA of *Bifidobacterium* differs from that of *Lactobacillus*, so the classification of bifidobacteria in the genus *Lactobacillus* was not justified. *Lactobacillus* contains <50 mol% G+C in DNA, whereas all members of the genus *Bifidobacterium* contain >50 mol% G+C. Based on the mol% G+C content, all lactic acid producers have been allocated into two subdivisions: *Clostridium* and *Actinomycetes*. The Actinomycetaceae family consists of five genera: *Bifidobacterium*, *Propionibacterium*, *Microbacterium*, *Corynebacterium*, and *Brevibacterium*. The mole percentage of G+C bases of the DNA of the genus *Bifidobacterium* is 58 for the human-type strains. The rest of the species have a mole percentage of G+C ranging between 55 and 66, based on melting temperatures.

Today, the *Bifidobacterium* genus includes 32 described species grouped according to their ecological origin, with 14 occurring in humans, 12 in animals, and 3 in honeybees (**Table 1**). Presently, five species of *Bifidobacterium* have attracted attention in the dairy industry for manufacturing probiotic milk products: *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium longum*. In the manufacture of fermented milks, *Bif. bifidum* is the species most commonly used, followed by *Bif. longum* and *Bif. breve*.

Bifidobacterium species are natural inhabitants of the gut of many animals including man. The groupings of the species are heterogenous. There is no test that permits the determination of origin of a strain, and thus classification as 'human' or 'animal' origin

Table 1 Species of the genus *Bifidobacterium* and their mole percentages of G+C content

	Species	*% G+C*	*Habitat*
1	*Bif. adolescentis*	58.9	Vagina and feces of humans, bovine rumen, sewage
2	*Bif. angulatum*	59.0	Human feces, sewage
3	*Bif. animalis*	60.0	Animals (pig, cow, chicken)
4	*Bif. asteroides*	59.0	Honeybee
5	*Bif. bifidum*	60.8	Vagina and feces of humans, suckling calf
6	*Bif. boum*	60.0	Pig, cattle
7	*Bif. breve*	58.4	Human infants, human vagina, sewage, suckling calf
8	*Bif. catenulatum*	54.0	Human infants, human adult, sewage
9	*Bif. choerinum*	66.3	Pig, sewage
10	*Bif. coryneforme*		Honeybee
11	*Bif. cuniculi*	64.1	Feces of rabbit
12	*Bif. dentium*	61.2	Human dental caries and oral cavity, feces of human adult
13	*Bif. gallicum*	61.0	Human feces
14	*Bif. gallinarum*	65.7	Chicken cecum
15	*Bif. globosum*	63.8	Pig, cow, chicken
16	*Bif. indicum*	60.0	Honeybee
17	*Bif. infantis*	60.5	Feces of human infants, suckling calf
18	*Bif. longum*	60.8	Vagina and feces of human adults and infants, suckling calf, sewage
19	*Bif. magnum*	60.0	Feces of rabbit
20	*Bif. mericicum*	59.0	Rumen of cattle
21	*Bif. minimum*	61.6	Sewage
22	*Bif. pseudocatenulatum*	57.5	Feces of infants, suckling calf, sewage
23	*Bif. pseudolongum*	59.5	Feces of cattle, pig, and chicken
24	*Bif. pullorum*	67.5	Feces of chicken
25	*Bif. ruminatium*	57.0	Cattle rumen
26	*Bif. saeculare*	63.0	Feces of rabbit
27	*Bif. subtile*	61.5	Sewage
28	*Bif. suis*	62.0	Feces of piglets
29	*Bif. thermophilum*	60.0	Chicken, pig, bovine rumen
30	*Bif. lactis*	61.5	Fermented milk
31	*Bif. thermacidophilum*	56.85	Anaerobic digester
32	*Bif. denticolens*	55.0	Human dental caries

Adapted from Scardovi V (1986) In: Sneath PH, Nair NS, Sharpe ME, and Holt JG (eds.) *Bergey's Manual of Systematic Bacteriology*, 9th edn., Vol. 2. Baltimore, MD: Williams and Wilkins; Sgorbati B, Biavati B, and Palenzona D (1995) The genus Bifidobacterium. In: Wood BJB and Holzapfel WH (eds.) *The Genera of Lactic Acid Bacteria*, pp. 279–303. London: Blackie Academic and Professional; Biavati B, Vescovo M, Torriani S, and Bottazzi V (2000) Bifidobacteria: History, ecology, physiology, and applications. *Annals of Microbiology* 50: 117–131.

is arbitrary. *Bifidobacterium* species found in humans are *Bif. adolescentis*, *Bif. angulatum*, *Bif. bifidum*, *Bif. breve*, *Bif. catenulatum*, *Bif. dentium*, *Bif. infantis*, *Bif. longum*, and *Bif. pseudocatenulatum. Bifidobacterium breve*, *Bif. infantis*, and *Bif. longum* are found in human infants. *Bifidobacterium adolescentis* and *Bif. longum* inhabit human adults. *Bifidobacterium pseudolongum* type A and *Bif. thermophilum* occur in cattle and pigs, whereas *Bif. pseudolongum* types A, B, and C and *Bif. thermophilum* are found in chicken. Other species occur in the intestinal tract of various animals and insects including honeybees (**Table 1**).

Bifidobacteria in Gastrointestinal Ecology

A diverse and complex bacterial population is found in the intestinal microflora of human beings, and approximately 400 types of bacteria have been isolated in the feces of humans. Intestinal contents have a viable microbial count of about 10^{12} cfu g^{-1}. In addition to *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*, there are other genera such as *Bacteroides*, *Clostridium*, *Eubacteria*, *Peptococcus*, and *Fusobacterium* (**Table 2**). The stomach has the lowest population of microorganisms, and its contents have counts in the range of 10^0–10^3 cfu ml^{-1}. *Bifidobacterium* spp. is present in the colon of the intestinal tract. *Bifidobacterium* constitutes a major part of the natural microflora of the human intestine, and when present in sufficient numbers, these organisms create a healthy equilibrium between beneficial and potentially harmful microorganisms in the gut. *Bifidobacterium* is predominant in the large intestine, especially in the proximal colon, whereas lactobacilli are found mainly in the distal end of the small intestine. The intestine of the fetus is sterile, but immediately after birth, colonization by various organisms begins. In 1 or 2 days, coliform, enterococci, and clostridia enter. Initially, a wide range of genera colonize the large intestine, and within 48 h of birth, the stool of infants may contain up to 10^{10} cfu g^{-1}. *Bifidobacterium* appears in the intestine and rapidly becomes the dominant flora.

Bifidobacterium infantis is the predominant species in the stools of breast-fed infants. Formula-fed babies normally have 1 log count fewer *Bifidobacterium* present in fecal samples than breast-fed babies. Formula-fed infants have higher numbers of *Enterobacteriaceae*, *Streptococcus*, *Bacteroides*, and anaerobes other than *Bifidobacterium. Bifidobacterium* grows better in human milk than in bovine milk; this may be the reason that breast-fed infants have a higher *Bifidobacterium* population than that of formula-fed infants. The fecal microflora of formula-fed infants are similar to that of adults. Breast feeding should be promoted, as such feeding practice encourages the establishment and maintenance of a high number of *Bifidobacterium* in the gut.

The walls of the colon provide special ecological sites for the proliferation of *Bifidobacterium.* Lipoteichoic acids associated with the walls of Gram-positive bacteria can initiate adhesion to the epithelial cells of the intestine. The most likely sites for interaction between epithelial cells and bifidobacteria are proteins or glycoproteins, which can bind fatty acid fractions of lipotechoic acids. Some strains of *Bifidobacterium*, such as *Bif. infantis,* secrete polysaccharides, which can initiate adhesion to the epithelial cells of the intestine.

The level of *Bifidobacterium* decreases with age and changes in diet. In adults, the microbial flora of the intestine is present in the following order:

Table 2 Distribution of human gastrointestinal flora in different segments of the gastrointestinal tract

Number of organisms per gram of intestinal contents				
	Stomach	*Jejunum*	*Ileum*	*Colon*
Total microbial count	0–10^3	0–10^5	10^3–10^7	10^{10}–10^{12}
Aerobic or facultative anaerobes count				
Enterobacter	0–10^2	0–10^3	10^2–10^5	10^4–10^{10}
Streptococcus	0–10^3	0–10^4	10^2–10^6	10^5–10^{10}
Staphylococcus	0–10^2	0–10^3	10^2–10^5	10^4–10^7
Lactobacillus	0–10^3	0–10^4	10^2–10^5	10^6–10^{10}
Anaerobes count				
Bacteroides	Rare	0–10^2	10^3–10^6	10^{10}–0^{12}
Bifidobacterium	Rare	0–10^3	10^3–10^7	10^8–10^{12}
Peptococcus	Rare	0–10^3	10^3–10^4	10^8–10^{12}
Clostridium	Rare	Rare	10^2–10^4	10^6–10^{11}
Fusobacterium	Rare	Rare	Rare	10^9–10^{10}
Eubacteria	Rare	Rare	10^3–10^5	10^9–10^{12}
Veillonella	Rare	0–10^2	10^3–10^4	10^3–10^4

Adapted from Ballongue J (1993) Bifidobacteria and probiotic action. In: Salminen S and von Wright A (eds.) In: *Lactic Acid Bacteria*, pp. 357–428. New York: Marcel Dekker, Inc.

Bacteroidaceae (up to 86% of the total flora), eubacteria (6–19%), *Bifidobacterium* (6–36%), Peptococcaceae (2–14%), Enterobacteriaceae (trace–5.3%), and *Streptococcus* and *Lactobacillus* (low numbers). In the elderly, the populations of *Bifidobacterium* decrease, and an increase in the number of pathogenic bacteria, such as some coliforms, enterobacteria, and clostridia, occurs. This is due to a diminished secretion of gastric juices in the elderly. As a result, the elderly often suffer from constipation. Studies have shown that *Bifidobacterium* has a beneficial effect in reducing constipation in the elderly by replacing the microflora present in the gut with helpful bacteria.

In children, bifidus milk products are found to have more beneficial effects than most other fermented milk products, as *Bifidobacterium* produces only L(+)-lactic acid, which infants less than 1 year of age are able to metabolize easily.

Morphology

All members of genus *Bifidobacterium* show a bacillar form. Some strains develop ramifications giving V, Y, X, or other shapes. However, their polymorphism depends mainly on the culture medium and growth conditions. The levels of *N*-acetylglucosamine, which is involved in the synthesis of peptidoglycan (a component of the cell wall), affect the branching of *Bifidobacterium.* While lower levels of *N*-glucosamine and amino acids produce more highly branched shapes, rich and favorable growth conditions produce longer and *Bacillus*-form morphology.

Colonies formed by *Bifidobacterium* are smooth, convex, cream or white, glistening, and of soft consistency. Cells of *Bif. angulatum* show the V or palisade arrangement, whereas *Bif. animalis* cells show an enlarged middle portion. *Bifidobacterium asteroides* exhibits unusual starlike arrangements.

Most species of *Bifidobacterium* of human origin produce vitamins such as thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), folic acid (B_9), cobalamin (B_{12}), ascorbic acid (C), nicotinic acid (PP), and biotin. Ability to synthesize these vitamins could be important to the animal or human hosts as the vitamin supplies for the requirement of the host may not be affected negatively as the demand for the vitamins by these bacteria would be minimum or nil within the gastrointestinal system.

Growth Characteristics

Bifidobacterium is a Gram-positive, nonmotile, nonspore-forming, and anaerobic organism. Bifidobacterial cells often stain irregularly with methylene blue. Some species can tolerate oxygen, some are obligate anaerobes, and some species can tolerate oxygen in the presence of carbon dioxide. These organisms are catalase-negative; however, some species, such as *Bifidobacterium indicum* and *Bif. asteroides*, possess weak catalase activity when grown in the presence of air. Catalase can break down hydrogen peroxide produced by other lactic acid bacteria, including *Lactobacillus delbrueckii* ssp. *bulgaricus*, one of the two yogurt bacteria. Hydrogen peroxide is claimed to inactivate F6PPK, a major enzyme in bifidobacteria responsible for metabolism of sugar.

Bifidobacterium produces higher levels of acetic acid than lactic acid, usually in the ratio of 3:2. These bacteria produce formic acid, ethanol, and succinic acid. Some reports suggest that butyric acid and propionic acid are not produced. However, studies in the authors' laboratory have shown the production of butyric acid and hippuric acid by these microorganisms.

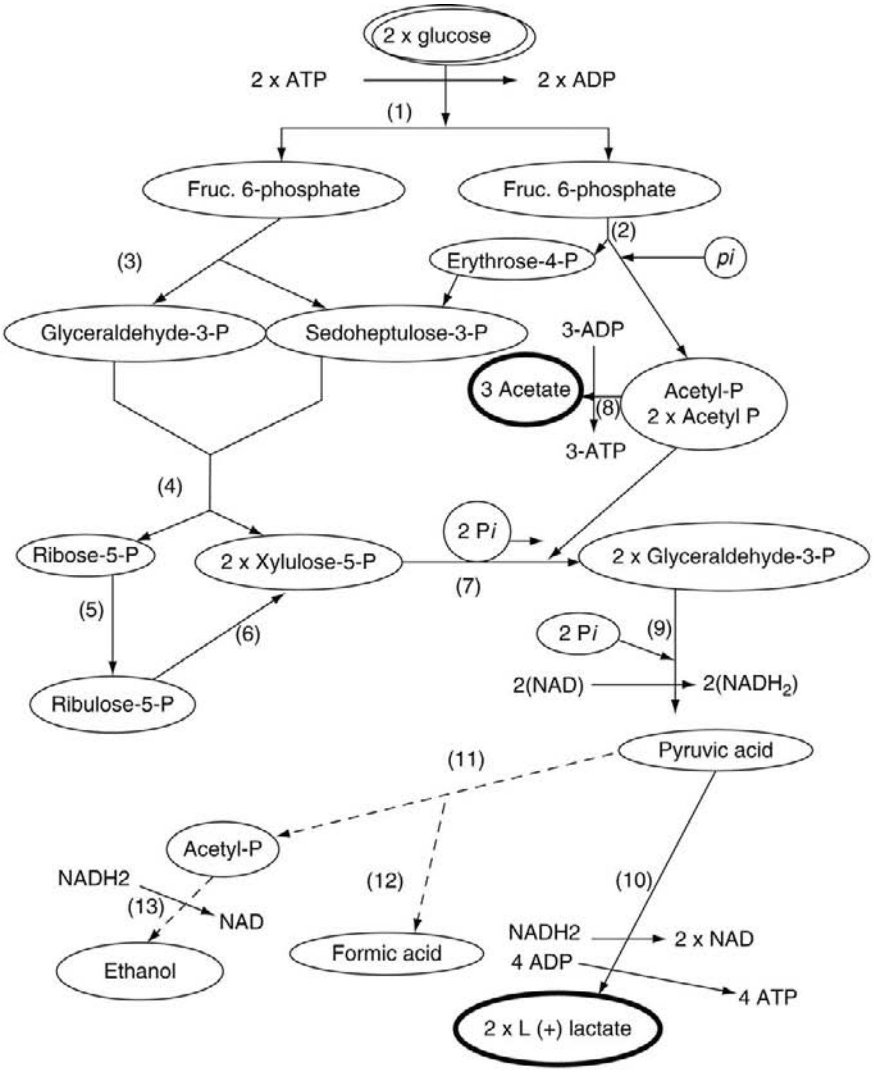
Bifidobacterium can grow in the temperature range of 25–45 °C, with a maximum growth temperature of 43–45 °C and a minimum growth temperature of 25–28 °C. The optimum temperature for growth of *Bifidobacterium* of human origin is between 36 and 38 °C, whereas for those of animal origin this is between 41 and 43 °C. Growth of *Bifidobacterium* does not occur below 20 °C, and these organisms do not have thermoresistance above 46 °C. The optimum pH for growth of *Bifidobacterium* is 6.5–7.0. No growth occurs below the pH of 4.5–5.0 or above 8.0–8.5. Below pH 4.1, most species die in less than a week even at 4 °C, and below pH 2.5 most species die in less than 3 h. Carbonate or bicarbonate can be readily used by *Bifidobacterium* as carbon sources. However, *Bifidobacterium* cannot utilize fatty acids or organic acids as carbon sources.

Growth Requirements

After early observations that the growth of *Bifidobacterium* is stimulated by human milk, numerous nutritional studies have been designed to elucidate the properties of bifidus factor(s) present in human milk or to find a substitute for it. The essential factor in human milk (i.e., the bifidus factor) that is lacking in cow's milk was subsequently identified as *N*-acetyl-D-glucosamine-containing saccharides. Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) also has growth-promoting effects on *Bifidobacterium.* Lactulose is not metabolized by humans and can pass on to the colon, and is used selectively by *Bifidobacterium.* Lactulose has been isolated from human milk. This may be one of the reasons why breast-fed infants have higher populations of *Bifidobacterium.* Various oligosaccharides known as prebiotics also stimulate the growth of *Bifidobacterium* spp. Oligosaccharides including raffinose, stachyose, and inulin (polyfructose) are used by *Bifidobacterium*, but not by other intestinal bacteria, including *Escherichia coli* and *Enterococcus faecalis.* Fructooligosaccharides are polymers of fructose containing 2–35 fructose units, which are not degraded by human digestive enzymes nor by the colonic flora. Thus, they are used as selective food for *Bifidobacterium*.

Maximum growth and acid production occurred for several strains of *Bifidobacterium* cultured in each of bovine milk, bovine milk with 20% pepsin-digested milk and bovine milk with 2% lactulose syrup. Increases in cell biomass of *Bifidobacterium* in whey digested with pepsin, whey plus whey proteins digested with pepsin, whey proteins digested with pepsin, and skim milk digested with pepsin have been reported.

Growth of *Bif. bifidum* also occurs in sterile skim milk supplemented with 1% dextrose and 0.1% yeast extract. Acid production can be increased by addition of 0.05%


2 x glucose
2 x ATP
2 x ADP
(1)
Fruc. 6-phosphate
Fruc. 6-phosphate
(2)
(3)
Erythrose-4-P
pi
Glyceraldehyde-3-P
Sedoheptulose-3-P
3-ADP
3 Acetate
(8)
Acetyl-P
2 x Acetyl P
3-ATP
(4)
2 P*i*
Ribose-5-P
2 x Xylulose-5-P
2 x Glyceraldehyde-3-P
(7)
(5)
(6)
2 P*i*
(9)
2(NAD)
2(NADH$_2$)
Ribulose-5-P
(11)
Pyruvic acid
Acetyl-P
NADH2
(13)
NAD
(12)
(10)
NADH2
2 x NAD
4 ADP
4 ATP
Ethanol
Formic acid
2 x L (+) lactate

Reaction	Enzyme
1	Hexokinase and glucose-6-phosphate isomerise
2	Fructose-6-phosphate phosphoketolase
3	Transaldolase
4	Transketolase
5	Ribose-5-phosphate isomerise
6	Ribose-5-phosphate epimerase
7	Xylulose-5-phosphatecetolase
8	Acetate kinase
9	Glycerladehyde-3-phosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase
10	L (+)-Lactate dehydrogenase
11	Phosphoroclastic enzyme
12	Formate dehydrogenase
13	Alcohol dehydrogenase

Figure 1 Bifidus pathway (fructose-6-phosphate shunt).

Table 3 Sugar fermentation patterns of *Bifidobacterium* spp.

	Ribose	*Arabinose*	*Cellobiose*	*Sorbitol*	*Xylose*	*Mannose*	*Fructose*	*Sucrose*	*Maltose*	*Mellibiose*	*Lactose*
Bif. adolescentis	+	+	+	±	+	+	+	+	+	+	+
Bif. angulatum	+	+	−	±	+	−	+	+	+	+	+
Bif. animalis	+	+	±	−	+	±	+	+	+	+	+
Bif. asteroides	+	+	+	−	+	−	+	+	+	+	−
Bif. bifidum	−	−	−	−	−	−	+	±	−	±	+
Bif. boum	−	−	−	−	−	−	+	+	+	+	±
Bif. breve	+	−	±	±	−	+	+	+	+	+	+
Bif. catenulatum	+	+	+	+	+	−	+	+	+	+	+
Bif. choerinum	−	−	−	−	−	−	−	+	+	+	−
Bif. coryneforme	+	+	+	−	+	−	+	+	+	+	−
Bif. cuniculi	−	+	−	−	+	−	−	+	+	+	−
Bif. dentium	+	+	+	−	+	+	+	+	+	+	+
Bif. gallicum	+	+	−	+	+	−	+	+	+	N	−
Bif. gallinarum	+	+	±	−	+	±	+	+	+	N	+
Bif. globosum	+	±	−	−	±	−	±	+	+	+	+
Bif. indicum	+	−	+	−	−	±	+	+	+	+	−
Bif. infantis	+	−	−	−	±	±	+	+	+	+	+
Bif. longum	+	+	−	−	±	±	+	+	+	+	+
Bif. magnum	+	+	−	−	+	−	+	+	+	+	+
Bif. mericicum	+	+	w	−	+	−	w	+	+	N	+
Bif. minimum	+	+	−	−	−	−	+	+	+	−	−
Bif. pseudocatenulatum	+	+	±	±	+	+	+	+	+	+	+
Bif. pseudolongum	+	+	±	−	+	+	+	+	+	+	±
Bif. pullorum	+	+	−	−	+	+	+	+	+	+	−
Bif. ruminatium	+	−	−	−	−	−	+	+	+	N	+
Bif. saeculare	+	+	−	−	+	+	+	+	+	N	W
Bif. subtile	+	−	−	+	−	−	+	+	+	+	−
Bif. suis	−	+	−	−	+	±	±	+	+	+	+
Bif. thermophilum	−	−	±	−	−	−	+	+	+	+	±

N, not known; W, when positive, it is weakly fermented.

Adapted from Scardovi V (1986) In: Sneath PH, Nair NS, Sharpe ME, and Holt JG (eds.) *Bergey's Manual of Systematic Bacteriology*, 9th edn., Vol. 2. Baltimore, MD: Williams and Wilkins; Sgorbati B, Biavati B, and Palenzona D (1995) The genus Bifidobacterium. In: Wood BJB and Holzapfel WH (eds.) *The Genera of Lactic Acid Bacteria*, pp. 279–303. London: Blackie Academic and Professional.

cysteine and/or 0.2% yeast extract or 0.2% tryptone. Cysteine appears to be essential for the growth of *Bifidobacterium.* Cysteine and other compounds containing sulfhydryl groups help lower redox potential and thus seem to be beneficial for the growth of *Bifidobacterium.* Although *Bifidobacterium* are considered anaerobic, lowering redox potential may not necessarily improve the viability of *Bifidobacterium.* However, an additional nitrogen source in the form of peptides or amino acids is required to improve the viability of *Bifidobacterium.* The slow growth of this organism is due to the lack of proteolytic activity. Enhanced acid production also occurs in whole milk on addition of peptides and amino acids obtained from culture-free filtrates of proteolytic organisms including *Lb. delbrueckii* ssp. *bulgaricus* and *Lactobacillus casei.*

Mucins (glycoproteins of mucus) are considered as growth factors for *Bifidobacterium* spp. Mucins are secreted by the mucous cells of the salivary gland and gastrointestinal tract. Mucins are oligosaccharides of galactose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid linked to peptides such as proline, serine, and threonine. Glycoproteins isolated from colostrum appear to be effective in promoting the growth of *Bifidobacterium.*

Carbohydrate Metabolism

The bifidus pathway is a major pathway of carbohydrate metabolism occurring in all *Bifidobacterium.* This pathway is also known as fructose-6-phosphate shunt. In the genus *Bifidobacterium*, hexoses are metabolized exclusively and specifically by the fructose-6-phosphate pathway. Many researchers regard the bifidus pathway as a marker for the genus *Bifidobacterium* (**Figure 1**).

The key enzyme in the bifidus pathway is F6PPK, which hydrolyzes hexose phosphate to erythrose-4-phosphate and acetyl phosphate. From tetrose and hexose phosphates through subsequent action of transaldolase and transketolase, pentose phosphates are formed. The final fermentation products are formed by the action of transaldolase (EC 2.2.1.2), transketolase (EC 2.2.1.1), xylulose-5-phosphate phosphoketolase (EC 4.1.2.9), and enzymes belonging to the Embden–Meyerhof pathway (glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase), which act on glyceraldehyde-3-phosphate. In the bifidus pathway, fermentation of 2 mol of glucose leads to 3 mol of acetate and 2 mol of lactate. Phosphoroclastic cleavage of pyruvate to formic and acetic acids and the reduction of acetate to ethanol can often alter the fermentation balance of end products to a great extent.

Although F6PPK is found in *Bifidobacterium*, aldolase and glucose-6-phosphate dehydrogenase are not present in these organisms. Absence of F6PPK in other Gram-positive anaerobic bacteria such as *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacterium*, and *Actinomycetaceae*, which could be morphologically confused with *Bifidobacterium*, is important for identification purposes of the members of the genus *Bifidobacterium.*

Bifidobacterium adolescentis can utilize a wide range of carbohydrates, whereas *Bif. breve*, *Bif. infantis*, *Bif. longum*, and *Bif. infantis* can utilize only a limited number of carbohydrates, including fructose, galactose, and lactose. All *Bifidobacterium* species of human origin are able to utilize glucose, galactose, lactose, and generally fructose as the carbon source. Among these bacteria, utilization of carbohydrates varies from species to species (**Table 3**). Species such as *Bif. bifidum* can ferment only 4 carbohydrates, whereas *Bif. adolescentis* can ferment 19 carbohydrates. Several species of *Bifidobacterium* can utilize lactose. Many strains of *Bifidobacterium* sp. are able to use ammonium salts as the nitrogen source. *Bifidobacterium suis* and *Bifidobacterium cuniculi* require some organic nitrogen.

Bifidobacterium species of animal origin have high urease activity; for example, *Bif. suis* is strongly ureolytic, whereas *Bif. bifidum* of human origin is weakly ureolytic. In addition, *Bifidobacterium* does not reduce nitrate, nor does it form indole, and tests for liquefaction of gelatine and fermentation of glycerol are also negative.

See also: **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; Taxonomy and Biodiversity.

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Bifidobacterium spp.: Applications in Fermented Milks

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Introduction

Because of the potential health benefits, *Bifidobacterium* is increasingly incorporated into fermented dairy foods including yogurt. *Bifidobacterium* grows slowly in milk and the usual practice is to incorporate yogurt cultures, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, along with *Bifidobacterium* for making yogurt. In order to realize health benefits, the suggested level for *Bifidobacterium* is $>10^6$ viable cells per gram of a product. In general, *Bifidobacterium* used for therapeutic benefits should originate from human sources, be capable of surviving in fermented products, and be abundant in the product prior to consumption. Several factors affect the survival of *Bifidobacterium* in such products; however, proper strain selection and improvement in technology of production may improve their survival. Viability of probiotic bacteria is important in order to provide health benefits and can be improved by appropriate selection of acid- and bile-resistant strains, microencapsulation, and incorporation of micronutrients such as cysteine, peptides, and amino acids. A number of health benefits have been claimed including antimicrobial, antimutagenic, and anticarcinogenic properties, reduction in serum cholesterol, improvement in lactose tolerance in lactose-intolerant individuals, and improvement in adherence to intestinal cells.

Bifidus Products

A number of fermented foods employing *Bifidobacterium* are well established in the market. Among fermented foods, yogurt is the most popular vehicle for incorporation of bifidobacteria. *Bifidobacterium*-containing yogurt is known by different names in various parts of the world. Products containing bifidobacteria include Biogarde, Bioghurt, Bifidus milk, Bifighurt, Milmil, and Progurt.

The trend has shifted from incorporation of *Lb. acidophilus* to *Lb. acidophilus* and *Bifidobacterium*. Yogurts containing *Lb. acidophilus* and *Bifidobacterium* are referred as 'AB' yogurt. Recently, the trend has been to incorporate *Lb. casei* in addition to *Lb. acidophilus* and *Bifidobacterium* and such products are known as 'ABC' yogurt. Traditionally, yogurt is manufactured using *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* as starter cultures. These yogurt organisms have been claimed to offer some health benefits; however, they are not natural inhabitants of the intestine and cannot survive under the acidic conditions and bile concentrations usually encountered in the gastrointestinal tract. Therefore, for yogurt to be considered as a probiotic product, *Lb. acidophilus* and/or *Bifidobacterium* and *Lb. casei* are incorporated as dietary adjuncts. A number of fermented milks containing one or more strains of probiotic organisms including *Bifidobacterium* are already available in the market (**Table 1**). Fermented milk with only *Lb. acidophilus*, *Bifidobacterium*, and *Lb. casei* could be manufactured; however, the longer incubation period and product quality are the two main factors that are sacrificed when fermenting milk with only *Bifidobacterium*. Thus, the normal practice is to make yogurt with both yogurt bacteria and probiotics, *Lb. acidophilus*, *Lb. acidophilus* and *Bifidobacterium*, or *Lb. acidophilus*, *Bifidobacterium*, and *Lb. casei*, as adjunct starter culture. This work will focus on fermented foods containing *Bifidobacterium* as adjunct starter culture (*see* **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products).

In order to obtain the desired therapeutic effects, the *Bifidobacterium* must be available in sufficient numbers. The organism should be present in a food to minimum levels of $10^6\,\mathrm{cfu\,g^{-1}}$. Such high numbers have been recommended to compensate for the possible reduction in the numbers of probiotic organisms during passage through the stomach and the intestine.

Several countries including the United States and Australia do not specify any requirements regarding the numbers of *Bifidobacterium* in yogurt. However, in other countries, standards have been developed regarding the requirement of the numbers of probiotic bacteria in fermented products. In Japan, the Fermented Milks and Lactic Acid Bacteria Beverages Association has established a standard, which requires a minimum of 10^7 viable *Bifidobacterium* cells per milliliter to be present in fermented dairy foods.

Several reports have shown that the viability of *Bifidobacterium* is often low in yogurt. A number of brands of commercial yogurts were analyzed in Australia and Europe for the presence of *Bifidobacterium*. Most of the products contained very low numbers of *Bifidobacterium*. A variety of factors have been claimed to affect the viability of *Bifidobacterium* in yogurt, including inhibitory substances such as lactic acid produced during fermentation and cold storage, strains used, interaction between

Table 1 Main fermented milks containing *Bifidobacterium* and lactic acid bacteria available in the market

Product	*Country of origin*	*Organism(s)*
ACO-yogurt	Switzerland	*Str. thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. acidophilus*
Cultura-AB	Denmark	*Lb. acidophilus*, *Bif. bifidum*
AB-yogurt	Denmark	*Lb. acidophilus*, *Bif. bifidum*, *Str. thermophilus*
Biogarde	Germany	*Lb. acidophilus*, *Bif. bifidum*, *Str. thermophilus*
Bifighurt	Germany	*Bif. longum*, *Str. thermophilus*
Gefilac	Finland	*Lb. casei* GG (rhamnosus)
Yakult	Japan	*Lb. casei*
Miru Miru	Japan	*Lb. acidophilus*, *Lb. casei*
Biokys	Slovakia	*Bif. bifidum*, *Lb. acidophilus*, *Pediococcus acidilactici*
Ofilus	France	*Bif. bifidum*, *Bif. longum*, *Lb. acidophilus*, *Str. lactis*, *Str. cremoris*
Gaio	Denmark	*E. faecium*, *Str. thermophilus*
LC1	Europe	*Lb. acidophilus* La1
Symbalance[a]	Switzerland	*Lb. reuteri*, *Lb. casei*, *Lb. acidophilus*
Probiotic plus oligofructose	Germany	*Lb. acidophilus*, *Lb. bifidus* LA7
ProCult3	Germany	*Bif. longum* BB536
Actimel Orange	Germany	*Lb. acidophilus*
Fysiq[a]	Netherlands	*Lb. acidophilus* Gilliland
DanActive Immunity	USA	*Lb. casei Immunitas*™
Activia	USA	*Bif. animalis*
Morinaga BB536 Nomu Drinking Yogurt	Japan	*Bif. longum* BB536
Megumi series	Japan	*Lb. gasseri* SP, *Bifidobacterium* SP
Actimel (Danone)	France	*Lb. casei* Immunitass DN-114001
Yakult Light	Netherlands	*Lb. casei* Shirota
Vitality	UK	*Bifidobacterium* sp., *Lb. acidophilus*

[a] Contains fructooligosaccharide.
Bif., *Bifidobacterium*; *E. faecium*, *Enterococcus faecium*; *Lb.*, *Lactobacillus*; *Str.*, *Streptococcus*.

species present, final acidity, availability of nutrients, growth promoters and inhibitors, dissolved oxygen, and oxygen permeation through the package.

Growth Requirements of *Bifidobacterium*

Cow's milk is considered less than an optimum medium for the growth of *Bifidobacterium*. However, the growth of *Bifidobacterium* is stimulated by human milk. The essential factor in human milk, known as bifidus factor, which is lacking in cow's milk, has been identified as *N*-acetyl-D-glucosamine-containing saccharides. Bifidus factor is reported to stimulate the growth of *Bifidobacterium* spp. Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) also has a growth-promoting effect on *Bifidobacterium*. Bifidus factors, which promote the growth of *Bifidobacterium*, are termed 'prebiotics'. A number of bifidus factors are incorporated in fermented foods, including inulin, lactulose, lactitol, raffinose, and fructooligosaccharides, to maximize the effectiveness of bifidus products (*see* **Prebiotics**: Functions; Types).

Since *Bifidobacterium* is an anaerobic microorganism, oxygen toxicity is an important and critical problem. During yogurt production, oxygen can easily permeate and dissolve in the milk. To exclude oxygen during the production of bifidus milk products, special equipment is required to provide an anaerobic environment. Oxygen can also enter the product through packaging materials during storage. Lack of nutrients appears to affect the population of *Bifidobacterium*. Satisfactory growth of a variety of *Bifidobacterium* spp. without the use of anaerobic conditions in a medium containing L-cysteine and yeast extract has been reported.

Improving Viability of *Bifidobacterium*

Selection of Acid- and Bile-Resistant Strains

One of the most important characteristics of *Bifidobacterium* spp. is their ability to survive in harsh acid conditions such as that encountered in yogurts and tolerate the acidic conditions in the human stomach as well as bile concentrations in the intestine in order to colonize the gut. Many strains of *Bifidobacterium* spp. intrinsically lack the ability to survive harsh conditions in yogurt and in the gut and may not be suitable for use as dietary adjuncts in fermented foods. *Bifidobacterium longum* survives best in acidic conditions, and tolerates bile concentrations as high as 4.0%. Selection of appropriate strains on the basis of acid and bile tolerance would help improve viability of these probiotic bacterial strains.

Incorporation of Peptides as Micronutrients

During yogurt making, *Str. thermophilus* dominates the early stage of yogurt fermentation. As redox potential of milk medium is reduced and the pH lowered from 6.5 to 5.5, growth of *Lb. delbrueckii* ssp. *bulgaricus* is stimulated during yogurt fermentation and the organism produces acetaldehyde and lactic acid, yielding the green apple flavor characteristic of yogurt. Continued acid production lowers yogurt pH to near 4.6, which induces clotting. Fermentation is terminated at pH 4.5.

Bifidobacterium grows slowly in milk because of a lack of proteolytic activity, and the usual practice is to add yogurt bacteria to reduce the fermentation time. *Lactobacillus delbrueckii* ssp. *bulgaricus* produces essential amino acids owing to its proteolytic nature, and the symbiotic relationship of *Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* is well established; the former organism produces amino nitrogen for the latter. However, *Lb. delbrueckii* ssp. *bulgaricus* also produces lactic acid during refrigerated storage. This process is known in the industry as 'postacidification'. Acid produced during refrigerated storage (i.e., postacidification) is found to cause loss of viability of *Bifidobacterium.*

To overcome the problem of postacidification, the present trend is to use starter cultures that are devoid of *Lb. delbrueckii* ssp. *bulgaricus*, such as ABT (*Lb. acidophilus*, *Bifidobacterium*, and *Str. thermophilus*). Such starter cultures may necessitate the incorporation of peptides and amino acids as micronutrients typically in the form of casein hydrolysate for reducing the fermentation time and improving the viability of *Bifidobacterium. Streptococcus thermophilus*, which is less proteolytic than *Lb. delbrueckii* ssp. *bulgaricus*, is the main organism responsible for fermentation in ABT cultures. ABT starter cultures increase fermentation time significantly (up to 8 vs. 4 h with both yogurt bacteria) as there is no symbiosis without *Lb. delbrueckii* ssp. *bulgaricus* and the fermentation is carried out primarily by *Str. thermophilus*. Longer incubation times are undesirable, given the rigid schedule in modern yogurt manufacture.

Addition of whey protein concentrate and acid casein hydrolysate has been found to improve the viability of *Bifidobacterium* particularly in yogurt made from ABT starter cultures. A nitrogen source in the form of peptides and amino acids from whey protein concentrate and acid casein hydrolysate is responsible for improved viability of *Bifidobacterium.* Thus, it is important to provide adequate peptides and amino acids for improved viability of these bacteria.

Application of *Bifidobacterium* in Fermented Foods

One of the earliest probiotic products, developed in the 1940s, was bifidus milk, which was used in the treatment of infants afflicted with nutritional deficiencies. Later, it was established that the intestinal flora could be positively modified with cultures of *Bifidobacterium*, and by the 1970s, products containing viable *Bifidobacterium* began to emerge on a commercial basis.

The consumption of dairy products containing *Bifidobacterium* spp. has increased tremendously. Japan is the world leader in the production of bifidus products. Bifidus product was first commercialized in Japan in 1971. This product was a fermented milk containing *Bif. longum* and *Str. thermophilus*. In 1977, Morinaga Milk Industry Company in Japan launched a home-delivered bifidus milk, which contained $10^7\,\mathrm{ml}^{-1}$ of *Bif. longum* and $10^7\,\mathrm{ml}^{-1}$ of *Lb. acidophilus*. The following year, the same company launched a firm-set bifidus yogurt. Today, there are over 70 bifidus-containing products produced worldwide, including sour cream, buttermilk, yogurt, powdered milk, cookies, and frozen desserts. More than 53 different types of milk products that contain *Bifidobacterium* spp. are marketed in Japan. The species of *Bifidobacterium* commonly found in fermented milk and yogurt products include *Bif. bifidum*, *Bif. breve*, and *Bif. longum*. Two main factors have prevented rapid commercialization of *Bifidobacterium* in North America: suspicion about the beneficial effects of these cultures in the intestine, and difficulty in propagating the cultures on an industrial scale.

Bifidobacterium species are difficult to propagate as they are not acid tolerant. The growth of most strains of *Bifidobacterium* is significantly retarded below pH 5.0. In most cases, the pH of the yogurt must be maintained below pH 4.6. This means that the population of *Bifidobacterium* will decline more rapidly in yogurt. The pH of the product declines further during storage due to postacidification. *Bifidobacterium longum* and *Bif. pseudolongum* are more tolerant to acid than other strains of *Bifidobacterium. Bifidobacterium pseudolongum* is of animal rather than human origin and may not provide therapeutic benefits. Therefore, this organism could not be recommended for the manufacture of fermented products. *Bifidobacterium bifidum* is commonly used by many yogurt manufacturers, but this strain has been found to be poorly tolerant to levels of acid and bile that may be encountered in the gastrointestinal tract. Thus, the use of *Bif. longum* for the manufacture of fermented products should be encouraged. It is important to select the strains of *Bifidobacterium* that may survive in yogurts during the projected shelf life of the product. *Bifidobacterium bifidum* and *Bif. longum* are widely employed in combination with *Lb. acidophilus* and yogurt culture organisms. A survey of fermented milk products containing *Bifidobacterium* in Germany, France, and Japan found that *Bif. longum* was widely used in Germany.

The recent trend toward products containing high counts of viable bifidobacteria could possibly lead to a replacement of *Bif. longum* with *Bif. animalis*. A survey has found that four out of five products contained *Bif. animalis* in yogurt sold in Europe. However, *Bif. animalis* being of animal origin may not survive and colonize in the human intestine. The survival of *Bifidobacterium* in products may also depend on improvement in the technology of yogurt production to provide anaerobic conditions or more suitable packaging materials.

Therapeutic Properties of *Bifidobacterium*

Lactobacillus and *Bifidobacterium* are considered probiotic organisms. In general, *Lactobacillus* scores higher in several therapeutic properties than *Bifidobacterium*. Probiotic organisms are defined as "live microorganisms, which can produce beneficial effects to the host and contribute to maintain a healthy equilibrium in the microflora of the gut of the host humans or animals". There is sufficient experimental evidence to support the view that oral administration of *Bifidobacterium* is able to maintain the normal balance of microbial population in the intestine. In addition to their established role in gastrointestinal therapy, *Bifidobacterium* species are also claimed to offer various other nutritional and therapeutic benefits (**Table 2**). These benefits include antimicrobial and antimutagenic properties, anticarcinogenic activity, reduction in serum cholesterol, improvement in lactose tolerance, and improvement in adherence properties.

Antimicrobial Properties

One of the important properties of *Bifidobacterium* spp. is their ability to produce antimicrobial substances such as organic acids (e.g., lactic and acetic acids), hydrogen peroxide, and bacteriocins to suppress the multiplication of pathogenic and putrefying bacteria. Because of these virtues, *Bifidobacterium* shows stronger antimicrobial properties against Gram-positive bacteria such as *Staphylococcus aureus* and *Clostridium perfringens* than against Gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli*. Hydrogen peroxide in the presence of organic acids such as lactic acid is more inhibitory to bacteria.

Two isomers of lactic acid, L (+) and D (−), are produced during fermentation by lactic acid bacteria in

Table 2 Some of the established and potential health benefits of *Bifidobacterium*

Health effect	*Mechanism*
Scientifically established	
Alleviation of lactose intolerance	Delivery of intracellular β-galactosidase into human gastrointestinal tract
Prevention and reduction of symptoms of rotavirus- and antibiotic-associated diarrhea	Competitive exclusion Translocation/barrier effect Improved immune response
Potential	
Treatment and prevention of allergy (atopic eczema, food allergy)	Translocation/barrier effect Immune exclusion, elimination, and regulation
Reduction of risk associated with mutagenicity and carcinogenicity	Metabolism of mutagens Alteration of intestinal microecology Alteration of intestinal metabolic activity Normalization of intestinal permeability Enhanced intestinal immunity
Hypocholesterolemic effect	Deconjugation of bile salts
Inhibition of *Helicobacter pylori* and intestinal pathogens	Competitive exclusion Barrier effect Production of antimicrobial compounds
Prevention of inflammatory bowel diseases	Competitive exclusion Improvement of epithelial tight junctions Modification of intestinal permeability Modulation of immune response Production of antimicrobial products Decomposition of pathogenic antigens
Stimulation of immune system	Recognition by toll-like receptors – induction of innate and adaptive immunity: • Downregulation of proinflammatory cytokines and chemokines • Upregulation of phagocytic activity • Regulation of Th1/Th2 balance

general. Some species of bacteria, including *Lb. delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis*, produce only D (−) lactic acid, whereas some lactic acid bacteria such as *Streptococcus* and *Lb. casei* produce L (+) lactic acid. *Lactobacillus helveticus* and *Lb. acidophilus* produce a racemic mixture of L (+) and D (−) lactic acid. D (−) lactic acid is metabolized only slowly to pyruvic acid in the body due to a lack of hydroxy acid dehydrogenase. The L (+) isomer is completely harmless. *Bifidobacterium* produces L (+) lactic acid. Thus, the lactic acid produced by *Bifidobacterium* is easily metabolized, while providing antimicrobial properties.

Antimutagenic Properties

Antimutagenic activity of fermented milk has been demonstrated *in vitro* against a large spectrum of mutagens and promutagens in various test systems based on microbial and mammalian cells. Epidemiological evidence has indicated a negative correlation between the incidences of certain cancers and consumption of fermented milk products. Although there is no direct evidence regarding antimutagenic or anticarcinogenic properties of *Bifidobacterium* in human subjects, studies conducted using human cell lines have shown that certain strains have positive effects that could lead to prevention of cancer. As most of the probiotic organisms produce various short-chain fatty acids such as acetic and butyric acids, these acids may be responsible for the antimutagenic effect observed in *Bifidobacterium.*

In general, live *Bifidobacterium* cells have shown higher antimutagenicity against mutagens. This suggests that live cells may metabolize or bind mutagens. Inhibition of mutagens and promutagens by *Bifidobacterium* appeared to be permanent for live cells and temporary for killed cells. Killed cells released mutagens and promutagens when extracted with dimethyl sulfoxide, emphasizing the importance of consuming live *Bifidobacterium* and of maintaining their viability in the intestine in order to provide efficient inhibition of mutagens.

Anticarcinogenic Activity

Fermented foods made using *Bifidobacterium* have potential anticarcinogenic activity. Oral supplementation of the diet with viable cells of *Bifidobacterium* decreased the levels of bacterial enzymes, β-glucuronidase, azoreductase, and nitroreductase. These enzymes catalyze the conversion of procarcinogens to carcinogens. Potential anticarcinogenic effects of *Bifidobacterium* may be due to direct or indirect removal of procarcinogens or activation of the body's immune system.

Direct removal of procarcinogens by *Bifidobacterium* may involve reduction in the rate at which nitrosamines are produced. *Bifidobacterium* may remove the sources of procarcinogens or the enzymes that lead to the formation of carcinogens. It has been shown that *Bifidobacterium* can greatly reduce the mutagenicity of nitrosamines. This may be due to the fact that certain species of *Bifidobacterium*, such as *Bif. breve*, have a high ability to absorb carcinogens, such as those produced upon charring of meat products. A reduction in excreted carcinogens and bacterial procarcinogenic enzymes has been observed in mice fed with *Bif. breve* and fructooligosaccharides.

In the presence of *Bifidobacterium*, the proliferation of tumors is reported to decrease considerably. Tumor suppression via the body's immune response system has been reported. Injection of cell wall fractions into growing tumors caused regression of the tumors and activation of the immune response. Cell wall fractions of *Bif. infantis* are claimed to contain active antitumor constituents. Enhancement in the body's defenses may be due to increased production of IgA antibody by *Bifidobacterium.*

The presence of *Bif. longum* in the gut of gnotobiotic C3H/He male mice has been found to reduce the incidence of liver tumors. The effect is claimed to be due to stimulation of immune response of the host or due to decreased activity of some fecal bacterial enzymes by *Bifidobacterium.* However, further research is needed in this area and more evidence is required to verify these claims.

Reduction in Serum Cholesterol

Cholesterol-lowering effects of fermented milks and their culture organisms have been the subject of a number of studies. Feeding of fermented milks containing very large numbers of *Bifidobacterium* (10^9 bacteria per gram) to hypercholesterolemic human subjects has resulted in a lowering of serum cholesterol levels from 3.0 to 1.5 $\mathrm{g\,l^{-1}}$. The role of *Bifidobacterium* in reducing the serum cholesterol is not completely understood. The effect may be due to the production of hydroxymethylglutarate, which inhibits hydroxymethylglutaryl-CoA reductases required for the synthesis of cholesterol. Uric acid inhibits cholesterol synthesis and orotic acid and hydroxymethylglutamic acid reduce serum cholesterol.

Bifidobacterium can actively assimilate cholesterol and other organic acids. The organisms themselves may take up cholesterol during their growth in the small intestine and make it unavailable for absorption into the bloodstream.

Improvement in Lactose Tolerance

Lactose malabsorption is a condition in which lactose, the principal carbohydrate of milk, is not completely digested into its component monosaccharides glucose and galactose. Since lactose is cleaved by the enzyme β-D-galactosidase, lactose intolerance results from a deficiency of this enzyme. The traditional cultures used in making yogurt, that is, *Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus*, contain substantial quantities of β-galactosidase, and it has been suggested that the consumption of yogurt may assist in alleviating the symptoms of lactose intolerance. *Bifidobacterium* is resistant to bile, which gives the above species an increased chance of colonizing the gut, and delivering the enzyme to its site of action.

Many Oriental and African people are lactose intolerant. This is due to the deficiency of β-D-galactosidase in the small intestine. People who are unable to digest lactose often complain of 'gastric distress' after consuming fresh, unfermented products, such as milk. The gastric distress is due to the formation of hydrogen gas from the unfermented lactose as a result of microbial action.

It is well accepted that lactose digestion from yogurt made with live *Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* is significantly improved in lactase-deficient individuals as compared with that of milk or heated yogurt. Mechanisms of improved lactose digestion are not well understood, but at least three factors have been suggested. One possible reason for an improvement in the lactose tolerance level could be due to the fermentation of lactose by yogurt bacteria. Another reason could be that the enzyme autodigests the lactose intracellularly before reaching the intestines, and the third reason may be slower oral–cecal transit time, allowing more time for residual β-galactosidase in the intestine to hydrolyze lactose. Slower oral–cecal transit time has been observed for yogurt as compared with milk.

Although there are limited studies conducted on the efficacy of bifidus products in the management of lactose malabsorption, products containing *Bifidobacterium* were less effective in alleviating the symptoms of lactose intolerance. The contribution of *Bifidobacterium* to the alleviation of lactose intolerance remains speculative.

Adherence

Adherence is one of the most important criteria for selection of strains of probiotic bacteria. The desirable effects of these organisms will be produced only if they are able to adhere, multiply, and colonize in the intestine. The ability of probiotic bacteria to adhere to the intestine will improve their chances of winning the competition against 'unfriendly bacteria' in order to occupy the intestinal 'niches'. Adherence to the intestinal cell is an important prerequisite for colonization in the gastrointestinal tract. However, thus far, only a few *Bifidobacterium* spp., *Bif. breve*, *Bif. longum*, *Bif. Bifidum*, and *Bif. infantis*, have been studied for their adherence properties. In a study carried out in the author's laboratory on adherence properties of *Bifidobacterium*, *Bif. infantis* and *Bif. longum* showed the highest level of adherence. An Ht-29 colonic carcinoma cell line is normally used for *in vitro* adherence studies. Proteins or peptides were involved in adherence of probiotic bacteria to Ht-29 cells. However, involvement of polysaccharides from bacteria and Ht-29 cells in adherence varied from strain to strain of probiotic bacteria. Polysaccharides produced by Ht-29 cell surfaces contributed to adherence than those originating from the bacterial cells. In *Bif. infantis* and *Bif. longum*, polysaccharides of both bacterial and Ht-29 origin were involved in adherence.

Conclusion

Probiotic products, in particular bifidus products, are becoming increasingly popular. Several health benefits have been claimed for *Bifidobacterium*; however, not all *Bifidobacterium* species are effective in providing health benefits. Proper strain selection should be carried out in order to incorporate these strains for providing the claimed health benefits. The survival and viability of *Bifidobacterium* could be improved by incorporating peptides or amino acids in probiotic foods. The combination of prebiotics along with probiotics, particularly *Bifidobacterium*, to modify gastrointestinal flora has the potential to provide a complete fermented food for maximum health benefits.

See also: **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products. **Prebiotics**: Functions; Types.

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Brevibacterium linens, Brevibacterium aurantiacum and Other Smear Microorganisms

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Introduction

Bacterial surface-ripened cheeses can be classified as hard (e.g., Gruyère and Comté), semihard (e.g., Tilsit, brick, and Limburger), or soft (e.g., Münster, Livarot, and Reblochon). Typically, hard, surface-ripened cheeses are made with thermophilic starter cultures, and the semihard and soft cheeses are made with mesophilic starter cultures. Cheeses made with thermophilic cultures can be cooked to temperatures in excess of 52 °C, whereas only limited cooking (~35 °C) is given to cheeses made with mesophilic cultures, which consequently have relatively high moisture contents. A red or orange color develops on the surface of most surface-ripened cheeses during ripening due to the production of pigments by the yeasts and bacteria growing on the surface.

Immediately after manufacture, the surfaces of these cheeses have a low pH (4.8–5.2) and high levels of salt because they are brine salted. After pressing, the surfaces of many of these cheeses are deliberately inoculated with various combinations of *Arthrobacter arilaitensis*, *Brevibacterium aurantiacum*, *Brevibacterium linens*, and/or *Geotrichum candidum*, either directly or by washing them with smear from old cheese. The latter is called the 'old-young' method of smearing and can be a source of pathogens, particularly of *Listeria monocytogenes*. These cultures are sometimes added directly to the milk. The cheeses are ripened under conditions that promote microbial growth (e.g., relative humidities of >90% and temperatures of 10–20 °C). This results in the development of a smear on the surface of the cheese, which is composed of yeasts and bacteria that grow at high salt concentrations, low pH, and at these temperatures. During ripening, many of these cheeses are washed with a dilute brine solution to spread the growing organisms more uniformly on the surface. For this reason, these cheeses are also called washed-rind cheeses.

The old theory was that, in these cheeses, yeasts grow first and metabolize the lactic acid – produced by the starter lactic acid bacteria during manufacture – to CO_2 and H_2O. This is called deacidification and, together with NH_3 production from deamination of amino acids, causes the pH on the surface to increase from an initial level of 5.2 to greater than 7.0, where the bacteria can grow. The yeast also produce growth factors that stimulate the growth of the bacteria. More recent studies suggest that many of the bacteria isolated from the surfaces of these cheeses are also capable of metabolizing lactate and can grow at the same time as the yeasts. In the well-ripened cheese, the bacteria and yeasts reach numbers of $\sim 10^8$ and $\sim 10^6$ colony forming units (cfu) cm^{-2}, respectively. The relationship between the yeasts, bacteria, and pH on the surface of a smear cheese is shown in **Figure 1**. The surface flora is thought to be very important in promoting the garlic or boiled cabbage flavor, which characterizes the flavor of many of these cheeses. This is mainly due to catabolism of methionine to different S-containing aldehydes and ketones, particularly methanethiol, by the surface microflora.

Smear Organisms

The smear on the surfaces of these cheeses is an ill-defined consortium of salt-tolerant bacteria and yeasts. The main reason for the poor definition is that the bacteria are dominated by coryneform bacteria, which are difficult to identify by classical techniques. *Agrococcus*, *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Leucobacter*, *Microbacterium*, *Mycetocola*, and *Rothia* spp. are often found in the smear of different cheeses and are all considered to be coryneform bacteria. This is based primarily on their morphology under the microscope; they are all Gram-positive, irregularly shaped, non-sporeforming rods. Species of some of these genera, e.g., *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, and *Curtobacterium*, also undergo a distinctive rod/coccus transformation during growth, with rod-shaped organisms dominating early exponential growth and coccoid organisms dominating the late-exponential or stationary phase of growth. All coryneform bacteria have high G+C contents and are found in the actinomycete branch of the Gram-positive bacteria. Many of them are only distantly related and are very difficult to differentiate from each other on the basis of simple phenotypic tests. Instead, their cell walls are analyzed for the type of peptidoglycan, including the diamino acid present and acyl type, and the different fatty acids, sugars, menaquinones, and polar lipids that are present. This type of identification is called chemotaxonomy and is very tedious where large numbers of organisms have to be analyzed. In modern taxonomy, chemotaxonomic

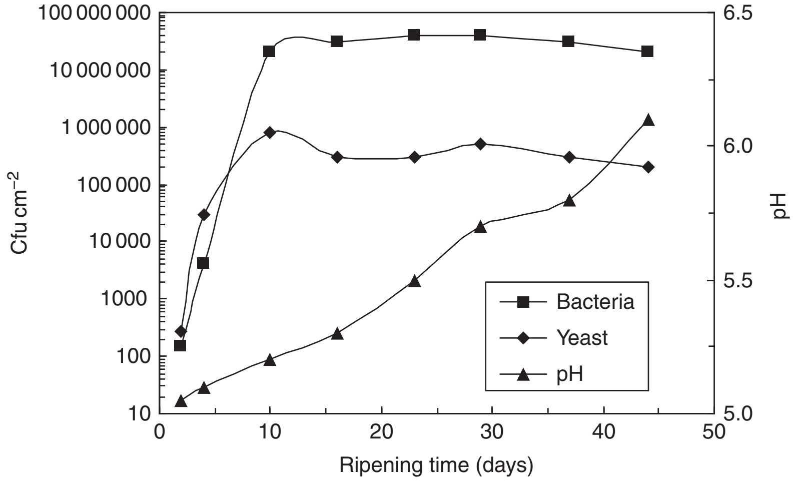

Figure 1 Relationship between pH and bacterial and yeast counts on a smear cheese (unpublished data).

techniques have been superseded by molecular techniques, and several of these techniques were used in the major study referred to later.

For a long time, it was considered that *B. linens* was the major bacterium on the surfaces of these cheeses. *Brevibacterium linens* produces pale yellow to deep orange or brown colonies. The orange color of the organism is considered to be responsible for the orange color of the cheese, and it is thought that growth in the presence of light promotes color formation by the organism, but this may also be related to the length of the incubation time. *Brevibacterium linens* has an optimum growth temperature of 25 °C and generally grows poorly, if at all, at pH 5 or 37 °C. It grows well at pH values between 6.5 and 8.5, but in the presence of 4% salt, it can also grow well at pH 6.0 and fairly well at pH 5.5. Recently *B. linens* has been separated into two species, *B. linens* and *B. aurantiacum.*

Several new species of bacteria from the surface of smear-ripened cheese have been described recently including *Arth. arilaitensis* and *Arth. bergerei*, which were originally isolated from Reblochon and Camembert cheese, respectively, and *Staphylococcus succinus* subsp. *casei* and *Staphylococcus equorum* subsp. *linens*, which were isolated from a Swiss smear cheese made from raw milk. Sequencing of the 16S rRNA gene has also resulted in the reclassification of *Caseobacter polymorphus* (which was isolated from the surface of the Dutch cheese, Menshanger) and of *Microbacterium flavum* (which was originally isolated from a Danish cheese) as *Corynebacterium variabilis* and *Corynebacterium flavescens*, respectively. *Leucobacter komagatae* has been isolated from the surface of French and German smear cheeses.

Recently, in a trans-European project partly funded by the European Union in the sixth Framework Programme (QLK1-CT-2001-02228), the bacteria and yeasts from the surfaces of five commercial smear-ripened cheeses – Limburger, Reblochon, Tilsit, Livarot, and Gubbeen – were identified at three different times during ripening. Several modern taxonomic and molecular techniques were used to identify the organisms, including pulsed-field gel electrophoresis, repetitive sequence-based PCR, and 16S rRNA gene sequencing for the bacteria and Fourier transform infrared spectroscopy and mitochondrial DNA restriction fragment length polymorphism for the yeasts. The results are summarized in **Tables 1** and **2**. Twenty-eight species of rod-shaped and coccoid bacteria from 11 genera were identified (**Table 1**). Some of them, including *Agrococcus casei*, *Corynebacterium casei*, *Microbacterium gubbeenense*, and *Mycetola reblochonii*, were new species. All the bacteria were not found on every cheese – indeed some of them appeared to be unique to a particular cheese – and the individual contribution of many of them to cheese flavor has not been studied to any great extent. *Brachybacterium aurantiacum* was the dominant bacterium, followed, in turn, by *Staphylococcus saprophyticus*, *Arth. arilaitensis*, *Cor. casei*, *Cor. variabilis*, *Mb. gubbeenense*, and *Staph. equorum/cohnii.* The techniques used in identification could not separate the latter two species. All of the bacteria listed in **Table 1** are Gram-positive, but several Gram-negative organisms were also isolated from the French cheese Livarot, which is made from raw milk, including several Enterobacteriaceae (*Citrobacter freundii*, *Escherichia coli*, *Hafnia alvei*, *Proteus vulgaris*, and *Serratia marcesens*) and *Alcaligenes faecalis*, *Pseudomonas putida*, and several unidentified *Alcaligenes*, *Pseudomonas*, and *Psychrobacter* spp.

In the past, *Micrococcus* was considered to be a degenerate form of *Arthrobacter. Micrococcus* is a very heterogeneous genus and has recently been divided into five new genera, namely, *Micrococcus*, *Kocuria*, *Dermacoccus*, *Nesterenkonia*, and *Kytococcus*, based on phylogenetic analysis of the 16S rRNA gene and differences in

Table 1 Bacteria isolated from the surfaces of five smear-ripened cheeses

	Limburger	*Reblochon*	*Livarot*	*Tilsit*	*Gubbeen*
Rod-shaped organisms					
Agrococcus casei			+	+	+
Arthrobacter arilaitensis	+	+	+	+	+
Brachybacterium alimentarium			+	+	
Brachybacterium faecium			+		
Brachybacterium tyrofermentans		+			
Brevibacterium aurantiacum	+	+	+	+	+
Brevibacterium helvolum					+
Brevibacterium linens		+		+	+
Corynebacterium casei		+	+	+	+
Corynebacterium variabilis		+		+	+
Corynebacterium flavescens		+		+	+
Curtobacterium flaaccumfaciens					+
Leucobacter spp.			+		
Microbacterium gubbeenense		+	+	+	+
Microbacterium lacticum		+		+	
Mycetocola reblochonii		+			
Rothia spp.		+			
Coccoid-shaped organisms					
Kockuria kristiniae				+	
Kocuria varians				+	
Macrococcus spp.	+		+		
Staphylococcus aureus					+
Staphylococcus capitatis					+
Staphylococcus equorum/cohnii			+	+	+
Staphylococcus epidermidis	+	+			+
Staphylococcus hominis					+
Staphylococcus saprophyticus		+	+	+	+
Staphylococcus sciuri				+	
Staphylococcus xylosus			+		

+, indicates the organism was present on the cheese surface.
Gavrish E, Krauzova VI, Potekhina NV *et al.* (2004) Three new species of Brevibacteria, *Brevibacterium antiquum* sp. nov., *Brevibacterium aurantiacum* sp. nov. and *Brevibacterium permense* sp. nov. *Microbiology* 73: 218–225.

chemotaxonomic properties. Similarly, *Staphylococcus* has recently been split into *Staphylococcus* and *Macrococcus*. *Micrococcus*, *Kocuria*, *Dermacoccus*, *Nesterenkonia*, and *Kytococcus* spp. have a high G+C content and are found in the actinomycete branch of the Gram-positive bacteria, whereas *Staphylococcus* and *Macrococcus* spp. have a low G + C content and are found in the clostridial branch.

Kocuria, *Staphylococcus*, and *Macrococcus* spp. have been isolated from smear cheeses (**Table 1**). These bacteria are catalase-positive, Gram-positive cocci that occur in clusters. *Micrococcus* and *Kocuria* are obligate aerobes, which do not produce acid anaerobically from glucose. They can be distinguished from each other by differences in menaquinone composition and in the major aliphatic hydrocarbons in their cells. *Staphylococcus* and *Macrococcus* are facultative aerobes, produce acid anaerobically from glucose and aerobically from glycerol in the presence of erythromycin, and are sensitive to lysostaphin; however, *Macrococcus caseolyticus* is slightly resistant to lysostaphin. Macrococci can be distinguished from staphylococci based on their cell size (macrococci are larger than staphylococci) and their DNA can be hydrolyzed with the restriction enzyme *Not*I; staphylococcal DNA is not hydrolyzed with this enzyme. *Micrococcus* and *Kocuria* show none of these properties and are more closely related to the coryneform bacteria as they have a high G+C content.

There is some evidence that *Staphylococcus*, *Kocuria*, and *Macrococcus* species are found on the cheese surface early in ripening and are later supplanted by the coryneforms as ripening progresses. Except for *Staph. aureus*, the *Staphylococcus* species listed in **Table 1** do not produce enterotoxins, are coagulase negative, and are not considered to be pathogenic.

Twenty-eight species of yeast from 12 genera were also identified from the five cheeses (**Table 2**). The dominant species was *Debaryomyces hansenii*, followed, in turn, by *G. candidum*, *Candida catenulata*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Saccharomyces servazii*, and *Saccharomyces unisporus* (**Table 2**).

The surface flora of Limburger cheese showed the least diversity, comprising two recently described coryneform species, *Arth. arilaitensis* and *B. aurantiacum*, and two yeasts, *D. hansenii* and *G. candidum*, whereas that of Livarot

Table 2 Yeasts isolated from the surfaces of five smear-ripened cheeses

	Limburger	*Reblochon*	*Livarot*	*Tilsit*	*Gubbeen*
Candida anglia		+			
Candida atlantica		+			
Candida catenulata			+	+	+
Candida etchellsii				+	
Candida intermedia		+			
Candida natalensis			+		
Candida musci			+		
Candida parapsylosis					+
Candida tenuis		+			
Candida zeylanoides				+	
Clavispora lusitaniae					+
Debaryomyces hansenii	+	+	+	+	+
Geotrichum candidum	+	+	+	+	
Issatchenkia occidentalis				+	
Kluyveromyces lactis		+	+	+	
Metschnikowia fructicola				+	
Metschnikowia pulcherrima				+	
Pichia cactophila				+	
Pichia guillermondii					+
Pichia jadini			+		
Pichia triangularis				+	
Rhodotorula glutinis				+	
Rhodotorula spp.				+	
Saccharomyces cerevisiae		+			
Saccharomyces servazii		+			
Torulaspora delbrueckii		+	+		
Trochosporon ovoides					+
Yarrowia lipolytica			+	+	

+, indicates the organism was present on the cheese surface.

showed the greatest diversity, comprising 9 yeasts and 30 bacteria, though not all of them were found on every individual Livarot cheese. Reblochon cheese also supported a very diverse microflora, consisting of 11 yeasts and 13 bacterial species. The yeast flora of Tilsit was similar to that of Limburger containing mainly *D. hansenii* and *G. candidum*, but in contrast to Limburger, the bacterial diversity was very high. Gubbeen had a complex and variable bacteria and yeast microflora. *Debaryomyces hansenii* was the dominant yeast, but *C. catenulata* was found in four of the six batches and *Clavispora lusitaniae* in three out of the six batches.

Recovery and Source of the Microflora

The only known source of *B. linens* is the surface of smear-ripened cheeses, and, at one time, it was considered to be the dominant bacterium found on these cheeses. The results in **Table 1** show that *B. aurantiacum* is now the dominant organism in these cheeses. This is likely to be due to mistakes in taxonomy, as recently *B. linens* was shown to comprise two species, *B. linens* (represented by ATCC 9172) and *B. aurantiacum* (represented by ATCC 9175), which hybridize very poorly with each other. *Brevibacterium linens* is the only component of the bacterial microflora (**Table 1**) that is deliberately inoculated onto the surface of smear cheese, but this is not done in every case.

Commonly selected strains of *Arth. arilaitensis*, *B. aurantiacum*, *B. linens*, and/or *G. candidum* are inoculated either into the milk or onto the surface of the fresh cheese after brining. Despite this, these strains are not subsequently recovered during ripening except, occasionally, in the very early stages of ripening. This finding raises the issue of whether addition of such cultures in the production of these cheeses is actually necessary. It also raises a further issue, namely, where do the other organisms found on the cheese surface come from. All the other bacteria are adventitious contaminants, which originate in the cheesemaking environment (e.g., the brine or the shelving used to ripen the cheese). In addition, smear cheeses receive a lot of manual handling during ripening and so skin may also be a potent source of the microorganisms as staphylococci and brevibacteria are common inhabitants of the skin surface. Many of these cheeses are washed several times during early ripening with a dilute (2–3%) salt solution to spread the microcolonies of bacteria more evenly on the cheese surface. The bacteria and indeed the yeasts found on the surface of smear cheese are very salt tolerant (e.g., *Brachybacterium tyrofermentans* and *Brachybacterium alimentarium* can grow in the presence of 14 and 16% salt, respectively). Because of this, salt is used as a selective agent in media used to isolate the smear bacteria. The initial pH of the surface is low (~5.3), and there is very little information on how well these bacteria grow at low pH.

Enumeration

Staphylococci can be enumerated on mannitol salts agar or on other suitable medium. However, it is not clear how many of the other bacteria listed in **Table 1** grow on this medium. There is no good selective medium for *B. linens*. In the past, the medium used to enumerate the surface bacteria on cheese was a nonselective medium containing 0.5% sodium lactate and 3% salt. The total number and the number of yellow and orange colonies were enumerated separately, and the orange-colored colonies were assumed to be *B. linens*. More recently, spread-plating (all of the bacteria in **Table 1** are obligate aerobes or facultative anaerobes) on plate count agar containing 3–5% salt has been used. There is also some evidence that enumeration of the bacteria in the smear by classical plating methods underestimates the numbers present and that *in situ* hybridization with suitable probes shows the presence of much greater numbers of organisms. The number of different species found in smear cheeses is greatly underestimated, and, in several studies, organisms have been isolated that have not been identified. DNA probes have been developed to separate the genera *Corynebacterium*, *Micrococcus/Arthrobacter*, and *Microbacterium* and work well in practice. *Aureobacterium* species also react with the *Microbacterium* probe, but these two genera have recently been amalgamated into a redefined genus, *Microbacterium*. A probe for *Cor. casei* has also been developed.

Role in Cheese Ripening

Except for *B. linens*, the role of the other bacteria in cheese ripening is poorly understood. *Brevibacterium linens* produces several proteinases, peptidases, and lipases, many of which have been purified and characterized, and are also involved in ripening these cheeses. Because *B. linens* has been separated into *B. linens* and *B, aurantiacum*, some of these enzymes may have been isolated from the latter organism. The proteinases are serine proteinases with optimum pH values of 7–8.5, although one strain produced a proteinase with an optimum pH of 11. They are all active on various caseins, and, in some cases, the specificities of these proteinases on the different caseins have been determined. The peptidases had optimum pH values of ~9.5. The specificity of three of them has been reported. They were all especially active on dipeptides with leucine as the N-terminus amino acid, and two of them were also active on dipeptides with proline at the N-terminus. In addition, the latter two peptidases hydrolyzed tri- and tetrapeptides.

Metabolism of methionine to different end-products, including methanethiol, dimethyldisulfide, dimethyltrisulfide, and methional, is considered to be a major component of the garlic or cooked cabbage flavor of smear-ripened cheeses. This reaction occurs in *B. linens* and is also likely to be carried out by many, if not all, of the other coryneforms found on the surface of the cheese. Of these compounds, methanethiol is considered the most important and can be produced either directly or indirectly from methionine. The direct method involves L-methionine-γ-methiolase activity, whereas the indirect method requires an aminotransferase and an amino acceptor (e.g., α-ketoglutarate). This reaction results in the production of α-keto-γ-methylthiobutyrate, which is transformed to methanethiol or decarboxylated directly to methional. Methanethiol inhibits the germination of mold spores, and this is considered to be the major reason for the lack of mold growth on the surfaces of smear-ripened cheeses.

Hygiene

The increase in the pH of the cheese surface that occurs during ripening, from initial levels of 5.2 to 7.0 or greater, makes smear cheeses prone to growth of pathogenic and indeed other bacteria. Therefore, good hygiene is particularly important in producing smear cheeses, especially where smear from ripened cheese is used to inoculate fresh cheese in the so-called ‘old-young’ method of smearing, as the smear from old cheese is often contaminated with *L. monocytogenes* and will thus infect the young cheeses. Such practices are traditional in the production of these cheeses, particularly in Germany, and efforts are being made to develop defined strain smear starters to overcome the *Listeria* problem.

Bacteriocins

One of the most common pathogens found on the surfaces of smear cheeses is *L. monocytogenes*. This is a salt-tolerant organism that grows quite well at low pH values and at low temperatures. Consequently, the organism grows well on the surfaces of smear cheeses during ripening. Two outbreaks of listeriosis have been traced to soft cheeses – a Mexican-type cheese in the United States and Vacherin Mont d’Or in Switzerland. Several deaths occurred in both outbreaks. Because of this, there is considerable interest identifying GRAS (generally regarded as safe) organisms that produce bacteriocins. Some strains of *B. linens* that produce bacteriocins, which inhibit the growth of other bacteria, particularly *L. monocytogenes*, have been identified. Two of these have been purified and sequenced but to the author's knowledge are not used commercially in cheese manufacture. Very recently, a *Staphylococcus* species that produced a potent polypeptide antibiotic (micrococcin P, which inhibits *L. monocytogenes*) was isolated from a smear-ripened cheese. The potential of the yeasts to produce inhibitors of listerial growth also deserves to be considered.

See also: **Cheese**: Biochemistry of Cheese Ripening; Microbiology of Cheese; Smear-Ripened Cheeses.

Further Reading

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Lactic Acid Bacteria: An Overview

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Lactic acid bacterias (LAB) are characterized by the production of L(+) and/or D(−) lactic acid from the fermentation of sugars, including lactose (by most species). They are Gram-positive, microaerophilic, acid-tolerant, non-spore-forming, mainly nonmotile rods or cocci. They may be mesophilic (optimum temperature ~30 °C) or thermophilic (optimum temperature ~45 °C). LAB belong to the following principal genera: *Lactobacillus* (125 species), *Lactococcus* (5 species + 3 subspecies), *Streptococcus* (only 1 species, *Streptococcus thermophilus*, is of interest for food technology, some other species are pathogenic), *Leuconostoc* (22 species and 3 subspecies), *Pediococcus* (9 species), *Bifidobacterium*, *Carnobacterium*, and *Enterococcus*. Since most of these genera are the subject of at least one article in this encyclopedia, mainly in the entry Lactic Acid Bacteria (*see* **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; *Streptococcus thermophilus*; Taxonomy and Biodiversity. **Pathogens in Milk**: Enterobacteriaceae), the purpose of this brief article is simply to state that LAB are (probably) the most beneficial microorganisms in the dairy industry and also in other food sectors. They are responsible for acid production in all fermented foods: dairy products (cheeses, fermented milks, sour cream, and lactic butter, each of which is the subject of one or more articles in this encyclopedia), fermented meats, fermented vegetables, and sour-dough bread. They colonize various mucous membranes of the body (mouth, large intestine, and vagina) and a few strains are probiotic. However, LAB can also cause undesirable changes, for example, souring of fresh milk unless sanitary production processes and milk cooling are rigorously implemented, spoilage (by acid production) of beer and wine, and the production of exopolysaccharides (which may be desirable in some circumstances). The natural habitats of LAB are plants from which they infect the food products, via the cows and the environment in the case of milk. The first step in the metabolism of lactose is its hydrolysis to its component monosaccharides by β-galactosidase (for most species that can ferment lactose) or phospho-β-galactosidase (in lactococci). The gene that encodes phospho-β-galactosidase in *Lactococcus* is carried on a plasmid, suggesting that it has been acquired relatively recently, probably due to exposure to lactose, which is exclusive to milk and emerged following the evolution of mammals about 100 million years ago. Where information is available, lactobacilli and *Sc. thermophilus* carry the genes for lactose utilization on the chromosome. Owing to their importance in food technology, as probiotics or as enzyme or metabolite 'factories', LAB are highly characterized at the physiological, application, and genetic levels. Research on LAB has received financial support from the European Union during 30 years and has been the subject of a triennial symposium, the proceedings of which have been published (*see* 'Further Reading').

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; *Streptococcus Thermophilus*; Taxonomy and Biodiversity. **Pathogens in Milk**: Enterobacteriaceae.

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Propionibacterium spp.

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Introduction

Propionibacteria are named for their unique metabolism leading to propionic acid as a major end product of metabolism. They were first described in cheese at the beginning of the twentieth century by Orla-Jensen and von Freudenreich. The genus *Propionibacterium* is divided into two groups based on its habitat. 'Dairy' propionibacteria are found in milk and cheese, and some other fermented products, for example, silage, whereas propionibacteria of the 'acnes group' are constituted of species found on human skin, for example, *Propionibacterium acnes*.

Dairy propionibacteria are mainly used as ripening cultures in the manufacture of Emmental (also called Swiss cheese) and related cheeses. They have a long documented history of use in foods and are also more and more considered for their probiotic properties. *Propionibacterium freudenreichii* plays an essential role in the formation of the characteristic flavor and holes of Emmental and related varieties. It has a generally recognized as safe (GRAS) status in the United States for use in cheese and a qualified presumption of safety (QPS) status in Europe.

Classification and Identification

On the basis of 16S rRNA gene sequences and G + C mol% (57–68%), propionibacteria are classified in the class of Actinobacteria with other Gram-positive bacteria with a G + C content higher than 50%.

Several changes occurred in the classification of propionibacteria since the pioneer work of Van Niel in 1928. The current taxonomy of propionibacteria describes 13 species (http://www.bacterio.cict.fr/). There are four typical dairy species, *P. freudenreichii*, *P. acidipropionici*, *P. jensenii*, and *P. thoenii*, and two 'dairy' species more recently described, *P. cyclohexanicum*, isolated from spoiled orange juice, and *P. microaerophilum*, isolated from olive mill wastewater and closely related to *P. acidipropionici*. *Propionibacterium freudenreichii* is divided into two subspecies, *P. freudenreichii* subsp. *freudenreichii* and subsp. *shermanii*, on the basis of lactose fermentation and nitrate reductase activity (**Table 1**).

Molecular methods, based on PCR amplification of selected DNA fragments and/or restriction of DNA or PCR products, have been developed to identify propionibacteria at the genus, species, and strain levels. The results of classification by numerical taxonomy based on phenotypical criteria fit generally well their classification by molecular methods, although some atypical strains may be misclassified by using phenotypical methods only.

Only dairy propionibacteria will be further considered in this article.

Morphology, Envelopes, and Growth Conditions

Morphology

Propionibacteria are Gram-positive, nonmotile, nonsporing, pleomorphic rods or small cocci. They may occur singly, in pairs, in short chains, or in clumps. Their morphology varies markedly depending on the conditions and phase of culture.

Envelope Composition

Two types of peptidoglycan are found in the cell wall of propionibacteria, according to the nature and composition of amino acids in the interpeptide bridge. Both types contain alanine, glutamic acid, and either *meso*-diaminopimelic acid (DAP) or LL-DAP, depending on the species (**Table 1**). Polysaccharides are present in the cell wall of propionibacteria. Both secreted and surface polysaccharides have been described in propionibacteria. The most frequently identified monosaccharides in secreted polysaccharides are glucose, galactose, mannose, and small amounts of glucosamine, galactosamine, fucose, rhamnose, and glucuronic acid. A surface polysaccharide, containing only glucose, has been described for *P. freudenreichii*. A paracrystalline protein surface layer (S-layer) has been reported for one strain of *P. jensenii* and of *P. freudenreichii*. Lipoteichoic acids (LTAs), membrane-associated polymers characteristic of Gram-positive bacteria, are poorly documented. An unconventional LTA, lipomannan, has been described in *P. freudenreichii*.

Cell lipids mainly contain branched-chain fatty acids in cell lipids (anteiso-$C_{15:0}$ and iso-$C_{15:0}$), except *P. cyclohexanicum*, which contains ω-cyclohexyl undecanoic acid as a major cellular fatty acid.

Table 1 Similar and distinctive features of propionibacteria

Species[a]	*Pfj*	*Pff*	*Pj*	*Pt*	*Pa*	*Pm*	*Pc*	*'Acnes' group*
Color[b]	Cream		Cream or red-brown	Orange to red-brown	Cream to orange	White	White to cream	White, cream, or grayish
Opt. growth temperature (°C)	30–32		30–32	30–32	30–32	30	35	36–37
G + C mol%	64–67		65–68	66–67	66–68	68	67	57–65
DAP isomer	*meso*		LL	LL	LL	NR	*meso*	LL (or *meso*)
Whole cell sugars[c]	gal man rha		glu gal man		gal	man NR	gal man glu rib rha	Combination of glu or gal, and man
Catalase	+	+	+/–	+	+/–	–	–	+/–
Indole production	–	–	–	–	–	NR	–	+/–
Gelatin liquefaction	–	–	–	–	–	NR	+	+/–
Nitrate reduction	–	+	–	–	+	+[d]	–	+/–
Carbohydrates fermented								
Glycerol	+	+	+	+	+	+	+	+/–
Erythritol	+	+	+	+	+	+	–	+/–
Lactose	+	–	+/–	+/–	+	–	+	NR
Trehalose, saccharose, maltose	–	–	+	+	+	+	+	+/–
Rhamnose	–	–	–	–	+	+	–	+/–
L-Arabinose	–	–	–	+/–	+	+	–	–
Esculin hydrolysis	+	+	+	+	+	–	–	+/–

[a] *Pfj*, *P. freudenreichii* subsp. *jensenii*; *Pff*, *P. freudenreichii* subsp. *freudenreichii*; *Pj*, *P. jensenii*; *Pt*, *P. thoenii*; *Pa*, *P. acidipropionici*; *Pm*, *P. microaerophilum*; *Pc*, *P. cyclohexanicum*.
[b] The color may differ according to aerobic/anaerobic conditions of growth.
[c] glu, glucose; man, mannose; rha, rhamnose; gal, galactose; rib, ribose.
[d] Nitrate reduction to N_2.
DAP, diaminopimelic acid; NR, not reported.

Isolation and Growth Conditions

Dairy propionibacteria have only a few nutritional requirements. Many strains grow in the absence of organic nitrogen sources, in a basal medium containing a carbon and energy source, ammonium, minerals, and 2–4 vitamins (at least pantothenate and biotin are required). However, their growth is stimulated when a complex nitrogen source, for example, peptone or yeast extract, is provided. Dairy propionibacteria are anaerobic to aerotolerant. They grow best at ~30 °C and their optimal pH is 6–7. Growth still occurs but is markedly slowed down at pH < 5.5.

Dairy propionibacteria are classically maintained and isolated in yeast extract–peptone–lactate (yel) media at a neutral or slightly acidic pH, incubated at 30 °C under air atmosphere without agitation. They grow under these conditions with a generation time of 5–6 h. In yel–agar medium incubated under strict anaerobiosis, propionibacteria form lenticular colonies of 1–4 mm diameter within 5–6 days at 30 °C. Their color varies from light cream to orange or red-brown depending on the species (**Table 1**). Some inhibitory compounds like cloxacillin, other antibiotics, and/or lithium are added to increase the selectivity of culture media.

Genetics

The propionibacteria genome size ranges from 1.6 to 3.1 Mb. A complete genome sequence is publicly available only for a strain of *P. acnes*, and is publicly available since May 2010 for *P. freudenreichii* subsp. *shermanii* type strain CIP103027 (INRA). The genome size of this strain is 2.6 Mb. Plasmids (generally 1–2 per strain) are frequently found (in 9–41% of the strains examined). Apart from a bacteriocin-encoding gene, plasmids do not seem to harbor genes of technological interest.

The development of genetic tools is highly desirable to validate gene function. The transformation of propionibacteria is, however, made difficult for two main reasons. The first reason is the poor efficiency of transformation of propionibacteria: between 10^3 and 10^8 cfu μg^{-1} DNA. Competent propionibacteria cells are best obtained by growing them in the presence of saccharose and glycine.

The second reason is the resistance of some targeted strain to antibiotic commonly used to select transformed clones.

Cloning shuttle vectors have been developed, allowing cloning a gene of propionibacteria in *Escherichia coli* before transferring the plasmid obtained in *Propionibacterium.* They contain a replicative origin and a resistance gene for *E. coli*, and a replicative origin (generally extracted from a cryptic plasmid of *Propionibacterium*) and a resistance gene for *Propionibacterium* (**Table 2**). To develop food-grade cloning vectors, the resistance to the propionicin F bacteriocin has also been proposed (**Table 2**).

Cloning vectors can also be used as expression vectors if a high-strength promoter is introduced upstream of the coding sequence to overexpress it. Several examples of overexpression of various genes in propionibacteria are given in **Table 3**.

Gene inactivation, by site mutagenesis or allelic replacement, is another way to validate the function of a gene. No thermosensitive vector that would facilitate the integration of the mutated gene in genomic DNA is available to date. An integrative vector, however, has been successfully used to inactivate the acetate kinase gene in *P. acidipropionici.*

Only few promoter sequences are available to date. Generally they have been isolated from a library of *Propionibacterium* DNA fragments cloned into a reporter

Table 2 Cloning shuttle vectors developed for propionibacteria

Name	*Size (pb)*	*Antibioresistance genes*	*Original plasmid*	*Replication type*	*References*
pPK705	8257	HygR: *hyg*B from *Streptomyces* AmpR: *bla* from pUC18 (*E. coli*)	pRG01 = pLME106	Theta	Kiatpapan *et al*. (2000)
pMPS1 and pMPS9	8212	CmR: *cml*(A) *cmx*(A) from *Corynebacterium* AmpR: *bla* from pUC18 (*E. coli*)	pRG01 = pLME106	Theta	Miescher *et al*. (2000)
pBRESP36A	8191	eryR: *erm*E from *Saccharopolyspora* AmpR: *bla* from pBR322 (*E. coli*)	p545	Theta	Jore *et al*. (2001)
pAMT1 and pAMT2	6250	CmR: *cml*(A) *cmx*(A) from *Corynebacterium* AmpR: *bla* from pUC18 (*E. coli*)	pLME108	Rolling circle	Stierli (2002)
pSL106	3316	CmR: *cml*(A) *cmx*(A) from *Corynebacterium* AmpR: *bla* from pUC18 (*E. coli*)	pLME108	Rolling circle	Brede *et al*. (2007)

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Table 3 Experiments of over-expression or inactivation in *P. freudenreichii* and *P. acidipropionici*

Vector name	*Over-expression (O) or Inactivation (I)*	*Species*	*Target*	*References*
pPK705	O	*P. freudenreichii*	5-Aminolevulinic acid	Kiatpapan and Murooka (2001)
pPK705	O	*P. freudenreichii*	Cholesterol oxidase	Kiatpapan *et al*. (2001)
pAMT1	O	*P. freudenreichii*	Antimicrobial peptides	Brede *et al*. (2005)
pTAT	I	*P. acidipropionici*	Acetate kinase	Suwannakham *et al*. (2006)
pPK705	O	*P. freudenreichii*	Secreted esterase	Dherbécourte *et al*. (2010)
pUC:Δ*gtf*:CmR	I	*P. freudenreichii*	Polysaccharide synthase	Deutschf *et al*. (2010)

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vector. A reporter vector for *P. freudenreichii* was recently developed, enabling the determination of promoter strength by the intensity of a blue coloration due to β-galactosidase activity. Consensus sequence data for the ribosome binding site and the −10, −16, and −35 box are also available.

Metabolism

The metabolism of propionibacteria is very complex. Several interconnected pathways are used simultaneously and some of them are reversible.

Substrates and End Products

In Emmental cheese, *P. freudenreichii* uses lactate as the main carbon source. Lactose (fermented only by *P. freudenreichii* subsp. *shermanii*) and galactose can also be used if present. Many other substrates can be fermented by propionibacteria, including carbohydrates, polyols, for example, glycerol and erythritol, and some acids. *Propionibacterium acidipropionici* and *P. microaerophilum* ferment a wide range of substrates, compared to *P. freudenreichii* and *P. cyclohexanicum* (**Table 1**). The major end products of propionic fermentation are propionic, acetic, and succinic acids and CO_2.

Metabolic Pathways

Substrates are first oxidized to pyruvate via glycolysis or via the pentose phosphate pathway, generating ATP and reduced coenzymes. Pyruvate is further catabolized via two main pathways, producing either propionate, or acetate + CO_2 (**Figure 1**).

The most characteristic feature of the metabolism of propionibacteria is the reduction of pyruvate to propionate. It occurs via a specific cycle referred to as Wood–Werkman or transcarboxylase cycle, which regenerates the oxidized form of the coenzymes and produces extra ATP. One of the key reactions is a transcarboxylation reaction without the intervention of free CO_2, transferring a carboxyl group from methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA (reaction 1, **Figure 1**). The enzyme involved has been fully characterized. It is a biotin-dependent carboxytransferase (EC 2.1.3.1) composed of three subunits. The expression of the gene coding for one of the subunits has been used as a target to investigate the metabolism of propionibacteria in complex ecosystems like the colon. Another particular enzyme is methylmalonyl-CoA isomerase (EC 5.4.99.2), a dimeric coenzyme B_{12}-dependent enzyme that catalyzes the isomerization of succinyl-CoA to methylmalonyl-CoA (reaction 2, **Figure 1**). All the reactions of transcarboxylase cycle are reversible.

The second main pathway of pyruvate conversion is its decarboxylative oxidation to acetyl-CoA, via the activity of pyruvate dehydrogenase, resulting in the formation of acetate and CO_2 with a concomitant production of $NADH_2$ (reaction 3, **Figure 1**).

To maintain their redox balance, cells modulate the proportions of pyruvate catabolized to acetate + CO_2 and to propionate as a function of the amount of reduced coenzymes produced when a substrate is oxidized to pyruvate. Typically, when 3 mol of lactate (or 1.5 mol of glucose) is oxidized to 3 mol of pyruvate, 1 mol of

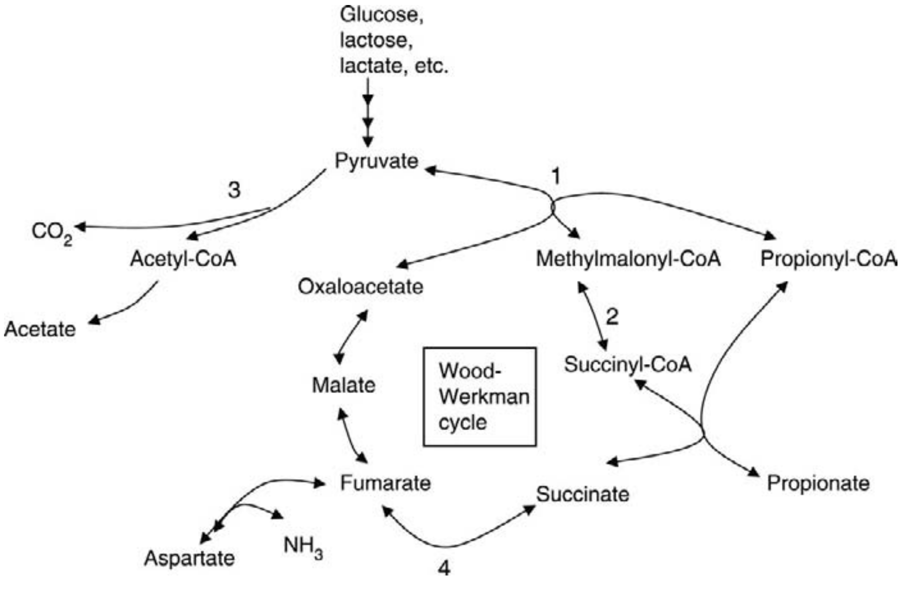

Figure 1 Schematic diagram of metabolic pathways of pyruvate catabolism in propionibacteria. 1, methylmalonyl carboxytransferase; 2, methylmalonyl-CoA isomerase; 3, pyruvate dehydrogenase; 4, fumarate reductase. Main substrates and end products are in bold.

pyruvate is further oxidized to acetate and CO_2, whereas 2 mol of pyruvate is reduced to propionate via the Wood–Werkman cycle (known as the Fitz' equation, with a molar ratio of propionate:acetate of 2:1). The oxidation of glycerol to pyruvate generates a higher amount of reduced cofactors than the oxidation of lactate or glucose. In this case, pyruvate is converted to propionate only.

Aspartate, in cometabolism with other substrates, is deaminated to fumarate by aspartase and further reduced to succinate by a membrane-bound fumarate reductase, with a concomitant regeneration of oxidized coenzymes and ATP production (reaction 4, **Figure 1**). In this case, less pyruvate is metabolized via the Wood–Werkman cycle to maintain the redox balance, thus decreasing the ratio of propionate:acetate. The conditions of pH and salt also influence the direction of metabolism. In Emmental cheese, the ratio propionate:acetate also varies widely. Aspartate, along with asparagine, is the main amino acid used by propionibacteria, but other amino acids, that is, serine, glycine, and alanine, can also be catabolized.

Adaptation to Stress

As an important component of the microflora of Emmental-type cheese and as probiotic food complement, dairy propionibacteria are exposed to technological and digestive stresses. In both applications, adaptation toward these stresses and survival is a key factor for efficacy. Furthermore, they were isolated from diverse ecological niches including soil, rumen, waste waters, indicating adaptation to various environmental parameters.

Osmotic adaptation, relevant for cheesemaking, is evident in dairy propionibacteria. In rich media such as yel or in the presence of exogenous osmoprotectants including trimethyl glycine (glycine betaine), dimethylsulfonioacetate, and dimethylsulfoniopropionate, they grow despite NaCl concentrations up to 0.5–1 mol l^{-1}. In the absence of such solutes, propionibacteria can synthesize and accumulate large amounts of trehalose, in response to osmotic or cold stress. Trehalose, as well as glycogen, can be accumulated in propionibacteria and serve as cellular carbon reserve compounds.

Heat stress adaptation, leading to survival at temperatures up to 55°C, can be triggered by a mild heat pretreatment (42°C). Thermal adaptation in *P. freudenreichii* involves overexpression of chaperones, ATP-dependent proteases, as well as proteins taking part in SOS response, metabolism of the cell wall, remediation of damage caused by reactive oxygen species, and phosphorylation of nucleotides.

Dairy propionibacteria face acid stress both during fermentation processes and during transit through the digestive tract. Acid tolerance response is evident in *P. freudenreichii*. Exposition to moderately acidic conditions, pH 4–5, leads to tolerance toward acid stress down to pH 2. Acid tolerance response depends on the overexpression of enzymes involved in DNA synthesis and repair, enzymes of the central carbon metabolism including the transcarboxylase cycle, specific to propionic fermentation in propionibacteria, ATP-dependent proteases, and chaperones.

One of the major stresses undergone in the digestive tract is the toxic effects of bile salts. While naive cells of *P. freudenreichii* exhibit a dramatic loss of viability upon exposure to physiological concentrations of bile salts present in the colon, preexposure to lesser concentrations leads to efficient adaptation. This adaptive pathway depends on the overexpression of proteins involved in stress sensing and signal transduction and enzymes involved in oxidative stress remediation and detoxification.

For all the stresses considered, it should be noticed that considerable differences exist between strains, in terms of susceptibility. The most tolerant strains were shown to constitutively overexpress a set of stress proteins.

Use as Ripening Cultures in Emmental Cheese

Occurrence and Growth in Dairy Products

Dairy propionibacteria are present in milk at populations ranging from less than 10 to 10^4 cfu ml^{-1}. This presence probably results from a contamination via the environment of dairy farm, since propionibacteria are present in the cow's rumen and feces, in grass, in soil, in milking machines, and so on.

Propionibacteria can be detected in many cheese varieties, even if they were not deliberately added, at populations ranging from 10^2 to 10^4 cfu g^{-1}. In Emmental and other Swiss-type cheese varieties, *P. freudenreichii* is added as ripening culture. It grows with a generation time of ~24–48 h and reaches populations over 10^9 cfu g^{-1} cheese. Many factors influence its growth in cheese, including the temperatures encountered during the manufacture process and the ripening, the pH of cheese, and the NaCl concentration. *Propionibacterium freudenreichii* is recognized to survive much better the 'cooking' step of Emmental cheese (~50–55°C for 30 min) than the other dairy species. The concentration of copper, originating from the copper-containing vats used for traditional manufacture, is also known to slow down growth of propionibacteria.

The contamination of Emmental cheese by bacteriophages of propionibacteria may be common. However, since propionibacteria grow during the hot-room step of ripening in a solid cheese matrix, the propagation of phages is probably very limited or absent and no growth arrest due to phage attacks has been reported. Two types of phages that infect propionibacteria have been described to date: a filamentous phage and, more classically, phages with an icosaedric head.

Role in the Formation of Cheese Flavor

Propionibacterium freudenreichii plays a key role in the formation of Emmental cheese flavor. The flavor compounds produced by *P. freudenreichii* have three main origins: propionate and acetate are produced from lactate fermentation, short branched-chain fatty acids from the catabolism of branched-chain amino acids, and free fatty acids from hydrolysis of milk fat. The last two activities occur when cheeses are placed in a warm room during the growth of propionibacteria, but continue during further cold storage of cheeses. The production of short branched-chain fatty acids (2-methylbutanoic and 3-methylbutanoic acids) is constitutive in propionibacteria. It is probably related to the synthesis of long branched-chain fatty acids of cell membrane. Fat hydrolysis occurs simultaneously with the growth of propionibacteria, in the absence of cell lysis, and would at least partly be due to the activity of a secreted esterase. All *P. freudenreichii* strains possess these activities, but large differences have been observed between strains. Propionibacteria also produce other volatile compounds (ethanol, propanol, propanal, acetoine, etc.).

Propionibacteria have very low caseinolytic activity, but possess diverse intracellular peptidases, including several enzymes specific for proline-containing peptides. However, since they do not lyse in cheese or only at a very limited extent and late during ripening, the activity of these intracellular enzymes in the secondary proteolysis of cheese appears rather small.

Propionibacteria are also considered as adjunct cultures to diversify flavor in some cheese varieties where eye formation is not expected. They produce the same aroma compounds as in Emmental-type cheese (short-chain acids and free fatty acids) and can induce detectable changes in the flavor of cheese, without a concomitant formation of eyes.

Role in the Formation of Eyes in Swiss-Type Cheeses

Propionibacteria play a key role in the formation of typical round holes ('eyes') in Swiss-type cheese. The ripening of Emmental cheese and similar cheeses includes a storage period in a warm room (22–24°C). *Propionibacterium freudenreichii* grows actively during this period and ferments lactate produced by lactic acid bacteria into propionate, acetate, H_2O, and CO_2. This results in the rapid formation of CO_2 and thus eyes. Propionibacteria produce 40% of the total CO_2 and the remaining part results from the activity of lactic acid bacteria (urea and amino acid catabolism).

The correct formation of eyes of a regular shape also depends on other factors in addition to the rate of CO_2 production: a local saturation of CO_2, the presence of nucleation sites for eye development, and an appropriate cheese structure. If CO_2 formation occurs too late during the ripening, it can lead to the formation of opening defects: formation of slits and cracks rather than regular holes.

The metabolism of aspartate by propionibacteria during propionic fermentation results in additional CO_2 being produced, thus accelerating the opening of cheese, and can also be responsible for an undesirable late fermentation in Emmental cheese. The intensity of aspartate metabolism in *P. freudenreichii* is highly strain dependent.

Propionibacteria are able to grow slowly in cheese at low temperatures (e.g., 10–12°C), and their activity under these conditions does not generally lead to a sufficient rate of gas formation to cause opening. Some strains show metabolic activities at temperatures of refrigerated storage (4–6°C).

Interactions with Lactic Acid Bacteria

The kinetics of propionic fermentation is highly affected by the species and strains of lactic acid bacteria growing in Emmental-type cheese before the development of propionibacteria. The extent of these interactions has been known for a long time, but the mechanisms are still not fully elucidated.

The strains of homofermentative thermophilic lactobacilli used as starters in Emmental-type cheese manufacture (*Lactobacillus helveticus* and/or *L. delbrueckii* subsp. *lactis*) can significantly affect the rate of propionic fermentation. Their effect is thought to result from a combination of factors, including the proportions of lactate isomers produced by the species of lactobacilli and the nature of peptides released from their proteolytic activity.

Heterofermentative mesophilic lactobacilli such as *L. paracasei/casei* and *L. rhamnosus*, can inhibit propionic fermentation. These species are present in many cheeses as natural non-starter lactic acid bacteria or can be deliberately added as adjunct cultures to modulate propionic fermentation and prevent the phenomenon of late fermentation. Their inhibitory effect on propionibacteria is strain dependent and is at least partly due to citrate metabolism by mesophilic lactobacilli. The metabolism of citrate results in the formation of inhibitory products, formate and acetate. It also leads to the release of copper in the aqueous phase of cheese, copper being previously chelated by citrate.

Interest as Probiotics

Dairy propionibacteria do not possess any known virulence factors, although all *P. thoenii* strains and some *P. jensenii* strains show β-hemolytic activity. Dairy propionibacteria exhibit a natural resistance to several antibiotics, which does not appear to be plasmid-encoded. All strains of interest should be tested prior to use for their antibioresistance profile.

Selected strains of dairy propionibacteria display remarkable robustness and efficient adaptive responses to digestive stresses, consistent with studies evidencing survival and metabolic activity within the human digestive tract. Moreover, observed adhesion to human intestinal mucus is consistent with the persistence of probiotic dairy propionibacteria during 2 weeks following ingestion.

Stimulation of the growth of bifidobacteria was evidenced in humans and the corresponding mechanisms were well established *in vitro*. Propionibacteria release 1,4-dihydroxy-2-naphthoic acid (DHNA), a bifidogenic factor favoring growth of bifidobacteria *in vitro* and *in vivo*. Alleviation of constipation was observed as a result of ingestion of *P. freudenreichii* in humans. Propionibacterial metabolites, including short-chain fatty acids, are thought to favor intestinal motility and absorption of divalent cations. Accordingly, enhanced iron absorption from the rat colon was reported in the presence of *P. freudenreichii*.

Production of β-galactosidase and hydrolysis of lactose, stimulated by exposure to bile, suggest a role in the treatment of lactose intolerance, which remains to be confirmed in humans.

A very promising area is the still poorly understood impact of propionibacteria on the human immune system. A strain of *P. granulosum*, a species of the acnes group, injected as a vaccine, stimulated the immune system and lowered wound infections and metastasis in patients treated for colorectal carcinoma. The corresponding mechanism, studied in mice, involved activation of macrophages and T-cell-mediated cytolytic activity. Such mechanisms remain to be investigated in dairy species.

Regarding carcinogenesis, ingestion of dairy propionibacteria results in lowered bioavailability and absorption of aflatoxin B1 in humans consuming food contaminated with this human hepatocarcinogen. Furthermore, a mixture of *P. freudenreichii* and *L. rhamnosus* has been shown to lower fecal activity of azoreductases, enzymes involved in the biosynthesis of carcinogens. Finally, induction of apoptosis of colon cancer cells by dairy propionibacteria, both *in vitro* in cultured adenocarcinoma cells and *in vivo* in mutagenized rats, opens new probiotic perspectives in this field.

Other Properties of Interest

Production of Nutraceuticals

The enzymes used by dairy propionibacteria to perform the Wood–Werkman cycle require cofactors including vitamin B_{12} (cobalamin), B_9 (folic acid), and B_8 (biotin). Indeed, dairy propionibacteria have been used for industrial production of food-grade vitamin B_{12}. Moreover, selected strains excrete vitamin B_9, suggesting that dairy products containing these strains may constitute a source of folate. One strain of *P. freudenreichii* was shown to produce large amounts of vitamin B_2 (riboflavin) in fermented milk, which proved efficient at balancing a riboflavin-deficient diet. Furthermore, some strains of *P. freudenreichii* convert free linoleic acid into conjugated linoleic acid (CLA), the main isomer produced being the beneficial rumenic acid (*cis*-9, *trans*-11-octadecadienoic acid). Finally, the large amounts of trehalose accumulated in propionibacteria may prove useful as a low-calorie dietetic sugar.

Antimicrobial Activities

The production of food-grade antimicrobial compounds constitutes a promising alternative to the use of chemical food preservatives. Propionic acid, widely used as an antifungal agent in the industry because of its inhibitory effects toward undesirable microorganisms, can be produced in a food-grade manner by propionibacteria instead of chemical synthesis. Propionibacteria are effective at protecting fermented milks and sourdough against spoilage by yeast and molds, with varying efficiency depending on the medium and on the species. In addition to propionic, acetic, and succinic acids, other organic acids (2-pyrrolidone-5-carboxylic, 3-phenyllactic, and hydroxyphenyllactic acids) could have a synergistic inhibitory effect on yeasts. Cocultures of lactic acid bacteria and propionibacteria are generally still more effective in avoiding these food spoilages. Propionibacterial cultures are considered in this context as biopreservatives. A commercially available product, Microgard^{TM}, consisting of a skim milk fermented by *P. freudenreichii* subsp. *shermanii*, exhibits an inhibitory activity on fungi and some Gram-negative bacteria.

Propionibacteria also produce bacteriocins (**Table 4**). Some of them are produced by different species. Propionicins SM1 and SM2, first isolated from *P. jensenii* DF1 cultures, were later also detected in *P. acidipropionici* and *P. freudenreichii* cultures. Interestingly, jenseniin P and thoeniicin 447 are active against members of the propionibacteria of the acnes group and may be useful in fighting opportunistic pathogens. To date, only a limited number of bacteriocins from propionibacteria have been fully sequenced and characterized.

Table 4 Bacteriocins of dairy propionibacteria

Name	*Size (Da)*	*Producer strain*	*Target microorganisms*	*References*
Jenseniin G		*P. jensenii* P126 (ATCC 4872), reclassified as *P. thoenii*	*P. acidipropionici*, *P. jensenii*, lactococci, lactobacilli, clostridial spores	Grinstead and Barefoot (1992), Ekinci and Barefoot (1999), and Holo *et al*. (2002)
Propionicin PLG-1	9328	*P. thoenii* P127	*P. thoenii*, *P. jensenii*, *P. acidipropionici*, lactic acid bacteria, yeasts and molds, *Listeria*, *Pseudomonas*, *Vibrio*, *Yersinia*	Lyon and Glatz (1993) and Lyon *et al*. (1993)
Propionicin SM1	22 300	*P. jensenii* DF1	*P. jensenii*, yeasts and molds	Miescher (1999) and Miescher *et al*. (2000)
Propionicin SM2	13 600			
Propionicin T1	7130	*P. thoenii* 419, *P. thoenii* LMG2792	*P. thoenii*, *P. jensenii*, *P. acidipropionici*	Faye *et al*. (2000) and Faye *et al*. (2004)
Jenseniin P	6000–9000	*P. jensenii* B1264	Propionibacteria of acnes group, lactobacilli	Barefoot and Ratnam (2001)
PAMP (protease-activated antimicrobial peptide)	6383	*P. jensenii* LMG3032	*P. acidipropionici*, *P. jensenii*, *P. thoenii*, *P. freudenreichii*, lactobacilli	Faye *et al*. (2002) and Faye *et al*. (2004)
Propionicin F	4397	*P. freudenreichii* LMGT2946	*P. freudenreichii*	Bredeet *al*. (2004)
Thoeniicin 447	7130	*P. thoenii* 447	*P. acnes*, lactobacilli	Van der Merwe *et al*. (2004)

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See also: **Bacteria, Beneficial:** Probiotics, Applications in Dairy Products; **Bacteriocins**; **Cheese:** Cheese Flavor; Swiss-Type Cheeses; **Vitamins:** Vitamin B_{12}.

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Probiotics, Applications in Dairy Products

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Introduction

According to the commonly accepted definition, probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host. The definition requires that the identity, safety, and efficacy of the strain have to be demonstrated before a microorganism can be considered as a probiotic. Historically, different probiotic cultures have been used in the form of fermented dairy foods, fermented vegetables, and fermented cereals for the treatment of gastroenteritis in adults and children. Health effects of probiotics have been reported within the oral cavity, stomach, small intestine, and large intestine. Probiotics mainly consist of lactic acid bacteria (LAB), but bifidobacteria and yeasts have also been successfully used and other types of beneficial microbes are under investigation.

Probiotic studies have been conducted in humans, pets, and farm animals. For studying the mechanisms of probiotics in humans, studies in animal models have been carried out. Some studies have suggested host specificity of probiotic strains, but many probiotics have properties that do not depend on the host species.

One of the major challenges has been defining and identifying the strains in a careful manner and depositing them in international culture collections, such as the German Collection of Microorganisms and Cell Cultures (DSMZ) or the American Type Culture Collection (ATCC). The second challenge has been conducting good clinical studies to document clearly the health effects of specific probiotics and their mechanisms. Today, we have specific strains of probiotics with scientifically proven health benefits for humans. In the future, functional foods with demonstrated health effects containing new probiotic strains and combinations of strains and other bioactive ingredients for specific treatment, management, and prevention of diseases are likely to emerge.

Gut Bacteria – the Health-Promoting Microbiota

While many are aware that there are about 10^{13} eukaryotic cells in the human body, most of us are unaware that the human body is also host to 10^{14} bacteria. The majority of these bacteria are found in the gastrointestinal tract (GIT), especially in the colon, and are known as the gut microbiota (formerly referred to as microflora). The bacteria comprising the gut microbiota are estimated to weigh approximately 1 kg and include several hundred culturable species of bacteria. However, DNA-based research indicates that there are many more species that have not been previously cultured and seem to be unculturable with current methods.

Bacterial numbers and composition vary considerably along the GIT. The density of microorganisms in the gut microbiota increases dramatically from less than 10^3 per gram content in the stomach, due to its harsh acidic conditions, to greater than 10^{10} per gram content in the large intestine (**Table 1**).

The reported numbers depend very much on the method of enumeration of bacteria. The growth and metabolic activity of the gut microbiota have a tremendous influence on our physiological and nutritional well-being. It has even been suggested that the metabolic activity of the gut microbiota is potentially greater than that of the liver. The gut microbiota therefore plays an important role in the maintenance of health: forming a healthy gut barrier, modulating the activity of the immune system, protecting the host from invading bacteria and viruses, and aiding digestion. Measures aimed at maintaining a healthy gut microbiota will therefore promote health even beyond the intestine. The applications of probiotics have been shown to be one of the methods that can positively affect the composition and activity of gut microbiota. An alternative approach is the use of prebiotics – nondigestible food ingredients

Table 1 Type and concentration of selected bacterial genera in the human large intestine

Bacteria	*Concentration (mean and range)* *log$_{10}$ per g dry wt of feces*	*Health effect*
Bacteroides	11.3 (9.2–13.5)	Vitamin synthesis, production of toxic compounds, competitive exclusion of pathogens, role in energy harvest and storage
Eubacteria	10.7 (5.0–13.3)	Competitive exclusion of pathogens
Bifidobacteria	10.2 (4.9–13.4)	Vitamin production, competitive exclusion of pathogens, aid in digestion, often anti-inflammatory
Clostridia	9.8 (3.3–13.1)	Production of toxic compounds, production of butyrate, competitive exclusion of pathogens, role in energy harvest
Lactobacilli	9.6 (3.6–12.5)	Competitive exclusion of pathogens, aid in digestion, assistance on bifidogenic properties
Ruminococci	10.2 (4.6–12.8)	Competitive exclusion of pathogens, aid in digestion
Peptostreptococci	10.1 (3.8–12.6)	Competitive exclusion of pathogens, production of toxic substances, aid in digestion
Peptococci	10.0 (5.1–12.9)	Competitive exclusion of pathogens
Methanobrevibacter	8.8 (7.0–10.5)	Reduction of gas in the intestine
Desulfovibrio	8.4 (5.2–10.9)	Production of toxic sulfur compounds
Propionibacteria	9.4 (4.3–12.0)	Aid in protein digestion, production of vitamins
Actinomyces	9.2 (5.7–11.1)	
Streptococci	8.9 (3.9–12.9)	Competitive exclusion of pathogens, production of toxic compounds
Fusobacteria	8.4 (5.1–11.0)	Aid in protein digestion, competitive exclusion of pathogens
Escherichia	8.6 (3.9–12.3)	Competitive exclusion of pathogens, enteric pathogens

that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host's health (*see* **Prebiotics**: Functions; Types).

The gut microbiota is acquired rapidly by the newborn during and shortly after birth. At first, facultative species such as *Escherichia coli* and *Streptococcus* spp. predominate, but in breast-fed infants there is a sharp increase in the number of bifidobacteria with a concomitant decrease in the number of *E. coli* and *Streptococcus*. Generally after weaning, populations of streptococci and *E. coli* further decrease and, by the second year of life, an adult-type microbiota has been established.

The method of birth has a profound effect on the development of the gut microbiota. Infants born by cesarean section tend to have a less complex gut microbiota, which develops slowly during the first 6 months, compared to that of infants born by vaginal delivery, who tend to have a more diverse microbiota resulting from contact with the mother's birth canal.

Many of the bacterial species that comprise the gut microbiota exert beneficial effects, for example, bifidobacteria and lactobacilli. However, some species can in certain circumstances have the potential to exert harmful effects, for example, clostridia, sulfate reducers, and amino acid-fermenting species. An optimum balance of the gut microbiota is one in which beneficial bacteria, such as lactobacilli and bifidobacteria, predominate over potentially harmful bacteria. Many factors, such as composition of the diet, antibiotic therapy, infections, food poisoning, environment, stress, health status, and aging, can influence the balance of the gut microbiota.

History of Beneficial Bacteria in Fermented Foods

The concept of ingesting live bacteria as a means of modulating the gut flora to maintain health and promote beneficial effects is not new. At the beginning of the twentieth century, the Nobel laureate Elie Metchnikoff was the first to propose a scientific rationale for the beneficial effects of the bacteria in yogurt. In his book *The Prolongation of Life*, he postulated that yogurt consumption played a role in health and he attributed the perceived longevity of Bulgarian peasants to their intake of yogurt containing *Lactobacillus* species. Tissier in 1906 advocated the administration of bifidobacteria to infants suffering from diarrhea in the belief that the bifidobacteria displaced the pathogenic bacteria. In Japan, in the early 1930s, Dr. Minoru Shirota focused his research on selecting beneficial strains of LAB that could survive passage through the gut. By using such strains, he developed fermented milk drinks. A long history of studies on fermented milks did not provide answers on the health effects, mainly due to poorly designed studies and unclear details of test products and strains used. In recent years, several workers have isolated well-described probiotic strains that survive the gastric conditions, tolerate bile acids, adhere to the intestinal mucosa, and lead to specific

Table 2 Current selection criteria for probiotic bacteria

Member of the normal healthy gut microbiota in man (debated at times, but provides backup on safety and possible host-specific properties)
Nonpathogenic, noncariogenic, no transferable antibiotic resistance
Acid and bile tolerant
Ability to withstand technological processes and remain viable during extended shelf life period
Production of antimicrobial substances against potential pathogens (whether such substances are produced *in situ* often remains to be determined)
Genetically stable (identification by appropriate molecular techniques)
Adherence to intestinal mucosa (mucus and enterocytes) may be used for specific applications
Evidence of potential beneficial preclinical effects later to be established in human studies

health benefits. These factors are among the most common selection criteria for potential probiotic strains (**Table 2**).

Concept of Probiotic Bacteria and Probiotic Dairy Foods

The word probiotic is derived from the Greek, meaning 'for life' (the opposite of antibiotic), and the definition of probiotics has evolved since the early 1960s. The word was used to describe substances produced by one protozoan to stimulate the growth of another; tissue extracts that stimulated microbial growth; and animal feed supplements including organisms and substances that had a beneficial effect on an animal by contributing to its intestinal flora balance. In 1989, Fuller defined a probiotic as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". This definition stressed the importance of live cells as a component of an effective probiotic. Two years later, Huis in't Veld and Havenaar redefined and expanded the definition to "a mono- or mixed culture of live microorganisms which when applied to man or animal affects beneficially the host by improving the properties of the indigenous microbiota". As part of an European Union (EU)-supported ILSI Europe coordinated concerted action project, a group of European scientists suggested that probiotics for use in human nutrition are best defined as "live microbial food ingredients that are beneficial to health". This definition takes into account results from recent research and allows for the possibility of probiotic effects not mediated by the microbiota, for example, direct probiotic effects on the immune system. Recently, a definition has been proposed that also includes nonviable microorganisms or parts of microbial cells: "probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host". However, the most commonly accepted definition today is the one developed by the WHO/FAO working group in 2002: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host." This definition

- does not restrict probiotics to a specific host (host may be human or animal),
- does not restrict probiotics to food (other application forms are not excluded),
- does not restrict the health benefits to the GIT (health benefits in, e.g., urogenital tract, oral cavity, and skin are also possible),
- does not require changes in the microbiota (e.g., immune modulation) for beneficial health effects,
- requires that probiotics are administered in sufficient amounts (although what that is, is left open), and
- requires that probiotics are live at the time of consumption (although dead microbes and parts of microbes have been implicated in health effects).

Selection of Probiotic Strains

In selecting probiotic bacteria for use in human foods, criteria outlined in **Table 2** are often followed. Probiotic bacteria used in foods for human consumption are generally of human origin and are nonpathogenic. In addition, they retain significant viability during processing and transit through the gut. Also technological properties are assessed during the selection of probiotics (see below). Probiotic bacteria are predominantly, though not exclusively, LAB; *Saccharomyces boulardii* (*nom. inval.*) is a probiotic yeast. Examples of species used as probiotics are given in **Table 3**.

Validated *in vitro* models that allow rapid screening of potential probiotic bacteria are available. It is also possible to monitor the transit kinetics of probiotic bacteria through the GIT using DNA-based technologies such as *in situ* hybridization with special 16S rRNA probes and a variety of techniques based on the polymerase chain reaction. Many critics of probiotics question whether probiotic strains remain viable during GIT transit. However, there are many studies using classical microbiological techniques and more sophisticated DNA-based

Table 3 Examples of microorganisms used in probiotic products around the world

Lactobacilli	*Bifidobacteria*	*Other LAB*	*Non-LAB*
Lactobacillus acidophilus	*Bifidobacterium animalis*	*Enterococcus faecium*	*Bacillus cereus*
Lb. casei	*Bif. breve*	*Enterococcus faecalis*	*Bacillus subtilis*
Lb. delbrueckii ssp. *bulgaricus*	*Bif. infantis*	*Lactococcus lactis*	*Clostridium butyricum*
Lb. johnsonii	*Bif. longum*	*Streptococcus thermophilus*	*Escherichia coli*
Lb. reuteri	*Bif. adolescentis*		*Propionibacterium freudenreichii*
Lb. rhamnosus	*Bif. animalis* ssp. *lactis*		*Saccharomyces boulardii*
Lb. salivarius	*Bif. bifidum*		
Lb. paracasei			
Lb. fermentum			
Lb. plantarum			
Lb. crispatus			

techniques confirming that selected probiotic strains remain viable and temporarily colonize the intestine. Following ingestion of a food containing probiotic bacteria, there is an increase in the fecal count of the these bacteria, which decreases once consumption has ceased but specific probiotics have been demonstrated in mucosal surfaces for some time after fecal recovery has ceased. However, permanent colonization is generally not observed in humans.

Technological Properties of Probiotics

In the production of fermented dairy products containing probiotic microorganisms, the need for the ability to grow in milk is obvious. However, many probiotic strains do not grow well in milk; for example, *Lactobacillus rhamnosus* GG does not ferment lactose, while *Lb. johnsonii* La1 needs additional amino acids, nucleotides, and iron. Supplementation of the milk with glucose, yeast extract, or milk protein fractions may enhance their growth. A more generally applied method is, however, the use of a supporter strain, often *Streptococcus thermophilus* and/or *Lb. delbrueckii* ssp. *bulgaricus*, which also allows shorter fermentation times. For use on an industrial scale, growth and survival during large-scale processing are important, for example, tolerance of shear forces and oxygen is essential. The latter is of particular importance when bifidobacteria are used as probiotics. As mentioned above, retaining viability during storage is important in order to maintain sufficient levels of probiotics for the desired effect. Here too, bifidobacteria are of special concern due to their sensitivity to oxygen and low pH. As with all industrially used microorganisms, strains need to keep their properties from year to year. This can be assured by avoiding repeated successive propagation. Of course, the probiotic strains need to contribute favorably to the taste and flavor of the final product, or be neutral in this respect.

Mechanisms of Probiotic Action

The efficacy of a specific probiotic strain in producing a given health effect is always unique and strain-dependent. The following mechanisms are involved: adherence to intestinal mucosa and mucus, production of antimicrobial substances, antagonism against pathogens and cariogens, competition for adhesion sites (competitive exclusion), interaction with gut-associated lymphoid tissue (immune modulation), inactivation of harmful components within the intestinal contents (binding of toxins and regulation of the metabolic activity of the intestinal microbiota), a trophic effect on the intestinal mucosa (e.g., through the production of butyrate), and overall normalization of the composition and activity of intestinal microbiota. Recently, also a direct impact on the host has been described through signaling pathways in the intestinal surfaces. All or some of these mechanisms are likely to be involved in the reported successful clinical studies quoted in **Table 4**. With regard to the modulation of the immune system, it is important to note that probiotics can both downregulate the immune response, as in allergy, or stimulate it, as in diarrheal disease or oral vaccination. However, in healthy subjects, no immune effect is usually observed; thus, probiotics appear not to modify a well-balanced immune response.

Efficacy of Probiotics

The efficacy of a probiotic strain in producing a given health effect has to be documented in well-designed human studies, which may be supported by *in vitro* studies or animal studies. These should include the hypothesis-based human studies that will fulfill the requirements listed in **Table 5**. Such studies should focus on specific proposed health effects using established and validated biomarkers. The studies should be conducted according to the guidelines set for good clinical studies and both the product and the probiotic strains should be accurately

Table 4 Reported studies and proven effects of some currently available probiotics

Strain	*Reported effects in clinical studies*	*Scientifically established effects*[a]
Lactobacillus johnsonii LJ1	Adherence to human intestinal cells, balances intestinal microbiota, immune enhancement, adjuvant effect in oral immunization	Mucosal adherence, improved oral vaccination
Lb. acidophilus NCFB 1748	Lowering of fecal enzyme activity, reduction in fecal mutagenicity, prevention of radiotherapy-related diarrhea, improvement of constipation	Reduction in mutagenicity, improvement in bowel movements during constipation
Lb. acidophilus NFCM	Lowering of fecal enzyme activity, high lactase activity, treatment of lactose intolerance, production of bacteriocins	Alleviation of lactose intolerance symptoms
Lb. rhamnosus GG (ATCC 53013)	Prevention of antibiotic-associated diarrhea, treatment and prevention of rotavirus diarrhea, primary prevention of atopic eczema with food involvement, treatment of relapsing *Clostridium difficile* diarrhea, prevention of acute diarrhea, stabilization of Crohn's disease	Shortening of the duration of rotavirus diarrhea, mucosal adherence, increase in bifidobacteria, prevention and treatment of antibiotic-associated diarrhea, alleviation of symptoms of food allergy, reduced risk of atopic diseases in infants
Lb. rhamnosus HN001	Increase in natural killer cell and phagocytic activity	Reduced risk of atopic diseases in infants
Lb. casei Shirota	Prevention of intestinal disturbances, balancing intestinal bacteria, lowering fecal enzyme activities, positive effects on superficial bladder cancer	Reduced recurrence of superficial bladder cancer, normalizing intestinal microbiota
Streptococcus thermophilus; Lb. delbrueckii subsp. *bulgaricus*	No effect on rotavirus diarrhea, no immune-enhancing effect during rotavirus diarrhea, no effect on fecal enzymes	Alleviation of lactose intolerance symptoms
Bifidobacterium lactis Bb12	Treatment of viral diarrhea including rotavirus diarrhea, balancing intestinal microbiota	Shortening of the duration of rotavirus diarrhea, treatment of atopic eczema
Bif. lactis HN019	Increase in natural killer cell and phagocytic activity	Reduced incidence of disease
Lb. gasseri (ADH)	Fecal enzyme reduction, survival in the intestinal tract	Altering intestinal metabolic activity
Lb. reuteri	Colonizing the intestinal tract, production of antimicrobial	Shortening of the duration of rotavirus diarrhea, reduced crying by infants with colic
Lb. paracasei F19	Colonizing the intestinal tract, decreasing the frequency of atopic eczema in infants	Shortening of the duration of rotavirus diarrhea
Saccharomyces boulardii	Prevention of antibiotic-associated diarrhea, treatment of *C. difficile* colitis	Prevention of antibiotic-associated diarrhea, treatment of *C. difficile* colitis

[a] Proven by at least two published, independently conducted clinical studies in humans.

Table 5 Suggestions for studying the efficacy of probiotics

Well-defined and identified probiotic strains
Well-defined study populations
Well-characterized treatments
Carefully designed model systems for the study of mechanisms
Double-blind, placebo-controlled, randomized study designs
Results confirmed by at least two independent research groups in different countries
Results published in peer-reviewed scientific journals

defined prior to starting the clinical trials. It has been concluded, in a recent European assessment of functional foods, that certain health effects of probiotic bacteria could be considered unequivocally proven for specific strains. The criterion for scientifically proven was that the effect was established in at least two well-performed independent human studies. The effects of probiotics can be divided into two groups: effects reported in scientific literature and effects scientifically established in at least two human studies with supporting information from *in vitro* studies and animal studies (**Table 4**). Such effects have been further verified for specific strains according to the guidelines for good clinical practice (which include accurate definition of both the product and the probiotic organisms) and more data become available all the time.

For the efficacy of probiotics, a sufficient intake of the microorganisms is necessary. In general, 10^9 colony forming units (cfu) per dose, or more, is often used. However, few dose–response studies have been performed and different health effects may require different doses of different probiotic strains.

Are All Probiotic Bacteria and Fermented Dairy Products the Same?

There has been some criticism regarding the composition and labeling of some probiotic products. The criticism has been mainly targeted at probiotic supplements that have been shown, using limited samples, either not to contain the number or type of bacteria stated on the labels or to contain species not mentioned on the labels. Probiotic yogurts and fermented milks have generally received favorable reviews when tested. However, these products usually do not mention the numbers of viable bacteria, and bifidobacteria are often referred to as 'bifidus'. This highlights the importance of rigorous quality control and assurance in the manufacture of probiotic products for maintaining and building consumer confidence. It is also important to safeguard the properties of probiotic strains in the long-term use of such strains in dairy technology. This creates new challenges in assuring the quality and developing quality control criteria for future fermented milks.

It cannot be assumed that all probiotic strains, even the same species, have similar or even any desirable properties. As shown previously (**Table 4**), it is obvious that the strain properties vary significantly as far as health effects are concerned. Thus, it is important to choose products that provide details of the strain(s) and ensure stability of the probiotic strains or indicate the total viable count of organisms present in the product throughout the shelf life of the product. It is also important to follow the manufacturers' instructions regarding the amount (g or ml) that has to be consumed. For gut health maintenance, probiotic products with viable numbers of approx. 10^7 cfu ml^{-1} or greater are generally recommended by studies on a number of starter cultures and probiotic strains. While supplements are convenient for delivering high numbers of probiotic bacteria, there is some evidence to suggest that these products are not consumed on a regular basis for long periods of time. Probiotic foods, for example, fermented dairy products, juices, and fermented oatmeals, in addition to providing high and stable counts of probiotic bacteria are also excellent sources of nutrients and can easily be incorporated into a balanced diet. Such products may also provide the needed buffering capacity for the strains to survive the harsh conditions in the human stomach and small intestine. Modern processing allows the production of flavorsome fermented products with high counts of probiotic bacteria that are guaranteed to remain viable over the shelf life period.

As the EU has adopted rigorous rules for assessment of health claims, probiotics also require rigorous human intervention studies on individual strains or strain combinations to have a health claim approved. The European Food Safety Authority has provided guidelines for such studies and all previously used health claims are reassessed by 2010. This procedure will set the guidelines for demonstration of health effects in humans.

Safety of Probiotics

Safety issues relating to new and novel probiotics and fermented milks should be assessed according to the EU novel food regulations. The use of LAB in foods has a long history and most strains are considered commensal microorganisms with no pathogenic potential. Their ubiquitous presence in the human GIT together with their traditional use in fermented foods and dairy products without significant problems attests to their safety. Members of the genus *Lactobacillus* are most commonly given safe or generally recognized as safe (GRAS) status, while members of the genera *Streptococcus* and *Enterococcus* contain many opportunistic pathogens. Specific GRAS notifications approved by the US FDA include *Bifidobacterium lactis* Bb12, *Lb. rhamnosus* GG, *Lb. acidophilus*, *Lb. reuteri*, *Lactococcus lactis*, *Pediococcus acidilactici,* and *Propionibacterium freudenreichii* subsp. *shermanii.* Also the European Food Safety Authority assessment based on the Qualitative Presumption of Safety of Probiotics attests the safety of currently used probiotic strains.

The safety of probiotics has been assessed in recent reviews. Clinical reports have drawn attention to a few cases of human bacteremia associated with the presence of LAB. A variety of strains of probiotic organisms have been used in the clinical treatment of gastrointestinal disorders and in the prevention of gut colonization by pathogens in both children and adults. These have included conditions where mucosal integrity was impaired by antibiotics, radiotherapy, or acute diarrhea of bacterial or viral origin. No evidence of opportunistic infections or other ill effects by probiotics has been observed in these studies. Although a recent study has associated probiotics with challenges in acute pancreatitis, no direct probiotic-associated detrimental effects were demonstrated. Animal studies indicate an absence of infectivity, and specific toxicity studies show no sign of toxic or harmful effects even at extremely high dose levels.

Table 6 Recommendations for assessing and ensuring the safety of probiotic microorganisms

The producer is responsible for supplying safe food, this may include postmarketing assessment
When a probiotic food is a novel food, it is subject to the appropriate legal approval
The best test for food safety is a well-documented history of safe use
When a strain has a long history of safe use, it will be safe as a probiotic
When a strain belongs to a species for which no pathogenic strains are known, but which does not have a history of safe use, it may be safe as a probiotic strain, but it should be regarded as a novel food
When a strain belongs to a species for which pathogenic strains are known, it is a novel food
Strains that carry transferable antibiotic resistance genes should not be marketed
Proper state-of-the-art taxonomy is required to describe the strain and the strain should be deposited in an internationally recognized culture collection
Strains that are not properly taxonomically described should not be marketed
Strains should be deposited in a recognized public culture collection (e.g., DSMZ or ATCC)

The safety aspects of probiotic bacteria can be studied using *in vitro* methods, animal models, and human subjects. Some recommendations for assessing and assuring the safety of probiotic microorganisms are listed in **Table 6**. Several human studies, both in Finland and in Sweden, have confirmed that the number of infections associated with LAB is extremely low. Survey of the literature also indicates that in those rare cases, patients are usually successfully treated with antibiotics. Generally, established probiotics have been considered safe.

Future Prospects

There are many medical conditions being investigated for specific probiotic therapy. Such areas include inflammatory bowel disease (Crohn's disease, ulcerative colitis, pouchitis), irritable bowel disease, obesity, food allergy, hypertension, oral rehydration therapy, superficial bladder cancer, and urogenital infections. As for the healthy consumer, recent studies indicate a possible reduction in the incidence and duration of specific diseases such as respiratory tract infections, and reduced antibiotic use. Methodological advances allow more sophisticated and precise studies of the role of the gut flora and of probiotics as health-enhancing ingredients. Research efforts worldwide are now focusing on the development of probiotic products containing bacteria selected for their specific health-enhancing characteristics and also on applying these to fermented dairy products, the traditional way of administering probiotics.

Conclusion

LAB have a long history of use in different fermented food products. Health effects have been attributed to many of these foods, mainly on an anecdotal basis. However, in recent years, selected probiotic microorganisms have been tested for their health effects and some of these have now been unequivocally established. Many claims of health effects still need further investigation and several new potential areas are currently under investigation. It is further important to note that only some of the proposed effects can be attributed to each strain; that is, no strain will produce all health effects. Some strains may, in fact, have no beneficial effects.

The condition of probiotic action is usually the ingestion of relatively large numbers of viable bacteria. There are no indications that this poses any risk for the consumer, not even for subjects from the so-called risk groups, for example, elderly and immune-compromised individuals.

In conclusion, specific probiotic strains have been shown to have beneficial health effects and to be safe. Potential new applications for defined age-specific, disease risk-specific, and dietary target populations are rapidly progressing creating exiting options for new products.

See also: **Prebiotics**: Functions; Types.

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BACTERIOCINS

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Antimicrobial Factors Produced by Lactic Acid Bacteria

The lactic acid bacteria (LAB), including the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus*, have long been used in fermentations to preserve the nutritive qualities of various foods. The primary function of a starter culture is the production of lactic acid at a suitable rate to ensure a consistent and successful fermentation. Other functions include production of flavor compounds such as diacetyl from citrate by mesophilic cultures and acetaldehyde from lactose by thermophilic cultures; acting as a source of proteolytic enzymes during growth in milk and ripening of many cheeses; and finally, contributing to the preservation of the fermented product through a number of inhibitory metabolites produced by the lactic cultures.

The major metabolite of LAB is lactic acid. Production of this organic acid and the associated drop in pH may be sufficient to restrict the growth of many undesirable microorganisms. In addition to a direct effect on the pH, the undissociated form of the molecule can cause collapse of the electrochemical proton gradient of susceptible bacteria, leading to bacteriostasis and eventual death. Outside of its use in food fermentations, the main application of lactic acid in the food industry is in the decontamination of meat and poultry carcasses. Acetic and propionic acids are also produced in small amounts by LAB. They act in a similar manner to lactic acid and are widely used as food additives; however, they are not usually derived from LAB fermentations for this purpose. They do play an important antimicrobial role in some fermented foods, and it is known that acetic acid has a synergistic antimicrobial effect when present with lactic acid. Other bioactive compounds produced by specific LAB include ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin.

Diacetyl and acetaldehyde, as well as imparting aroma and flavor attributes to cultured dairy products, also have an antimicrobial effect. Acetaldehyde can inhibit the cell division of *Escherichia coli*, and diacetyl inhibits yeasts as well as Gram-negative and Gram-positive bacteria. However, the use of the latter as a food preservative is precluded as a result of both its intense aroma and the relatively large amounts required for preservation.

Hydrogen peroxide is also produced by a large number of LAB lacking the enzyme catalase, but in particular by *Lactobacillus* spp. H_2O_2 inhibits other microorganisms such as *Staphylococcus aureus* and *Pseudomonas* spp. Furthermore, the antagonistic radical hypothiocyanate can then be generated by the action of lactoperoxidase on hydrogen peroxide, and the thiocyanate ion (SCN^-), by the lactoperoxidase system, a naturally occurring antimicrobial system in milk. This system has been successfully used to extend the shelf life of raw milk and Cottage cheese and to inhibit pathogens in raw and processed milk products. The potential of H_2O_2 produced by LAB for food preservation may be limited by the oxidizing nature of the molecule, and free radicals produced may have profound effects on the sensory quality, causing rancidity of fats and oils, and discoloration reactions.

Reuterin (β-hydroxypropanaldehyde) is an inhibitory compound produced by *Lactobacillus reuterii* under anaerobic conditions in the presence of glycerol. Reuterin has an inhibitory activity covering a very wide spectrum of microorganisms including Gram-positive and Gram-negative bacteria, yeasts, fungi, and protozoa. Organisms of public health significance that are inhibited by reuterin include species of *Salmonella*, *Shigella*, *Clostridium*, *Staphylococcus*, *Listeria*, *Candida*, and *Trypanosoma*.

Some LAB can synthesize antimicrobial peptides (other than bacteriocins, which are discussed below) that may also contribute to food preservation and safety. For instance, strains of *Lactobacillus plantarum* isolated from sourdough and grass silage display antifungal activity owing to the production of cyclic dipeptides (and/or organic acids and/or other low-molecular-mass metabolites). The focus of this article will be on another category of inhibitory molecules, the bacteriocins. These antimicrobial peptides are produced by many bacterial species, but of particular interest to the food industry are those produced by LAB members, as they enjoy GRAS (generally recognized as safe) status and thus have the potential to be used as preservatives in food.

Bacteriocins

Bacteriocins are ribosomally synthesized, extracellularly secreted bioactive peptides that have a bactericidal or bacteriostatic effect on other bacteria. These small, heat-stable peptides can have a narrow or broad target spectrum, and many are active in the nanomolar range. In all cases, the producer cell exhibits specific immunity to the action of its own bacteriocin and thus bacteriocin production contributes to the competitiveness of the producer cells. Such producing strains can be readily identified in a deferred antagonism assay, in which colonies of the putative producer are overlaid with a bacterial lawn of a sensitive strain. After further incubation, zones of inhibition are visible in the sensitive lawn (**Figure 1**). The term 'bacteriocin' was originally coined in 1953 specifically to define protein antibiotics of the colicin type, which are produced by *E. coli*, but is now accepted to include peptide inhibitors from any genus. They are generally considered to act at the cytoplasmic membrane of the target bacteria, frequently, though not always, through the formation of pores in the phospholipid bilayer leading to dissipation of vital ion gradients and resulting in disruption of the proton-motive force and eventual cell death.

Bacteriocins form a heterogeneous group of peptides and proteins, and although as many as five main classes of LAB bacteriocins have been mooted, the system employed here divides the bacteriocins into two distinct categories: the lanthionine-containing broad host range 'lantibiotics' (class I), and the non-lanthionine-containing bacteriocins, including the circular LAB bacteriocins (class II). **Table 1** comprises a nonexhaustive list of a selection of LAB bacteriocins. Lantibiotics are small peptides (19–38 amino acids in length) that possess the eponymous lanthionine or β-methyllanthionine residues (**Figure 2(a)**). These unusual residues are produced by posttranslational modification and form covalent bridges between amino acids, which result in internal 'rings' and give lantibiotics their characteristic structural features. Lantibiotics can also contain other unusual residues that result from posttranslational modification. The non--lanthionine-containing bacteriocins are also small (<10 kDa), heat-stable peptides but, unlike lantibiotics, are not subject to extensive posttranslational modification. Previous classification systems also included class III, which contained the larger, heat-labile murein hydrolases, but more recently it has been suggested that because of the distinctive nature of these compounds they should be renamed as bacteriolysins.

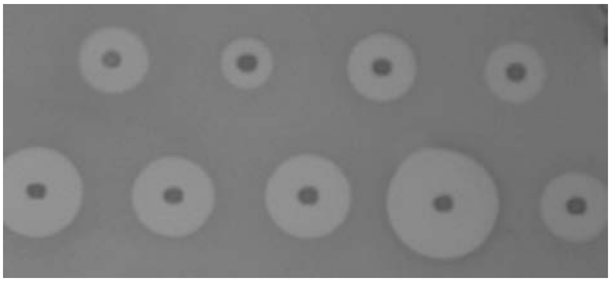


Figure 1 Deferred antagonism assay. A lawn of sensitive bacteria in which zones of inhibition are visible surrounding bacteriocin-producing colonies.

Bacteriocins are often confused in the literature with antibiotics. However, bacteriocins differ from antibiotics on the basis of synthesis, mode of action, antimicrobial spectrum, and toxicity. In addition, organisms that show resistance to antibiotics are generally not cross-resistant to bacteriocins. This distinction is important and means that bacteriocins can be used for food applications. Consequently, the term 'biological food preservatives' or the description of bacteriocins as a form of 'innate immunity in food' has been proposed. Although they have been the focus of much attention in recent years, bacteriocins, unlike antibiotics, are not currently used for clinical purposes.

Although the deliberate use of bacteriocins as preservatives in food was formally proposed in 1951, it is likely that mankind has benefited from the serendipitous production of bacteriocins in food in the 8000 years since cheese and other fermented foods were first manufactured. Studies have revealed that many of the LAB used in cheese manufacture to convert lactose to lactic acid also produce bacteriocins that can influence the composition of the complex cheese microflora and potentially inhibit adventitious spoilage/pathogenic bacteria. The successful manner in which LAB bacteriocins perform this role has resulted in a renewed interest in their use, especially owing to increased consumer concerns associated with the use of chemical preservatives in food and the resultant demand for more natural and minimally processed high-quality 'safe' food. It should be noted that the ideal natural food preservative or biopreservative should fulfill the following criteria:

- acceptably low toxicity
- economically viable
- stable to processing and storage
- no deleterious effect on the food
- efficient at low concentration

Significantly, most bacteriocins fulfill all of these criteria. Despite this, to date nisin is the only bacteriocin

Table 1 Representative bacteriocins characterized from lactic acid bacteria

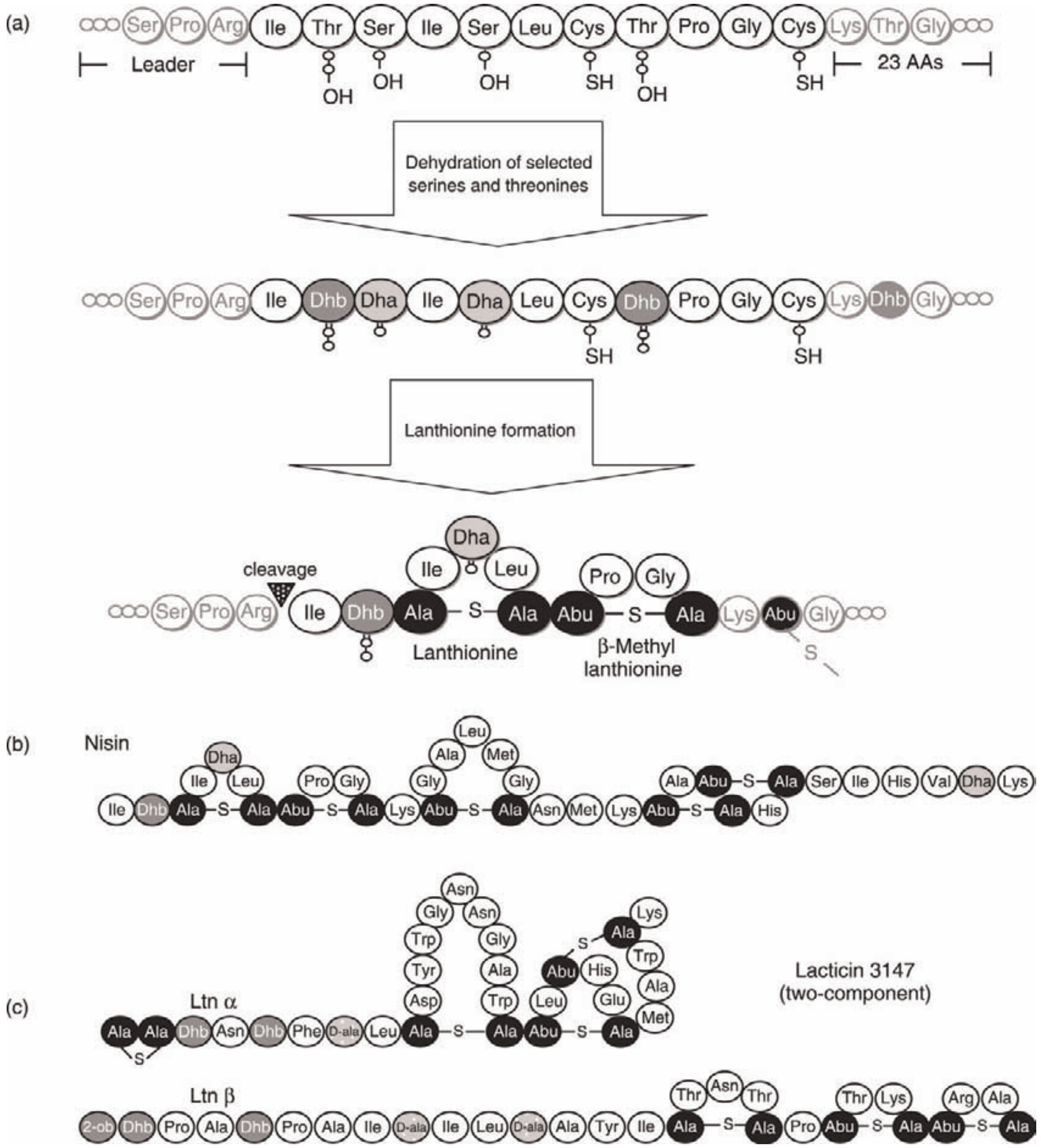
Bacteriocin	*Producer*	*Inhibitory spectrum*[a]	*Size (aa)*	*Food from which the organism was isolated*[b]
Class I: Lanthionine-containing lantibiotics				
Nisin (A and Z)	*Lc. lactis*	Broad	34	Milk
Lacticin 481	*Lc. lactis*	Broad	27	
Lactocin S	*Lb. sake*	Broad	37	Fermented sausage
Carnocin U149	*Carnobacterium piscicola*	Broad	35–37	Fish
Variacin	*Micrococcus varians*	Broad	25	Meat fermentations
Lacticin 3147	*Lc. lactis*	Broad	29 and 30	Kefir grain
Class II: Non-lanthionine-containing bacteriocins				
Lactococcin A	*Lc. lactis*	Narrow	54	Cheese
Lactococcin B	*Lc. lactis*	Narrow	47	Cheese
Lactococcin M	*Lc. lactis*	Narrow	48	Cheese
Lactacin F	*Lb. johnsonii*	Narrow	57 and 48	
Mesenterocin 52B	*Leu. mesenteroides*	Narrow	32	Raw milk
Curvaticin FS47	*Lc. curvatus*	Medium	31	Ground beef
Pediocin-like bacteriocins				
Sakacin A	*Lb. sake*	Medium	41	Meat
Sakacin P	*Lb. sake*	Medium	41	Fermented sausage
Carnobacteriocin A and B	*Carnobacterium piscicola*	Medium	53 and 48	Meat
Pediocin PA-1	*P. acidilactici*	Medium	44	Meat
Leucocin A-UAL-187	*Leu. gelidum*	Medium	37	Meat
Enterocin 1146/A	*En. faecium*	Medium	47	Fermented meat/milk
Piscicolin 126	*Ca. piscicola*	Medium	44	Ham
Mesenterocin Y105	*Leu. mesenteroides*	Medium	37	Goat's milk

[a] Narrow spectrum indicates those bacteriocins that affect only the producer genus. Medium spectrum indicates those bacteriocins that affect the producer genus and members of one or two other genera.
[b] Where known.

to be commercially exploited on a large scale. Nisin was first marketed in England in 1953 and has since been approved for use in over 48 countries. Notably, nisin was assessed to be safe for food use by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives in 1969. In 1983 this bacteriocin was added to the European food additive list as number E234, and in 1988 it was approved by the US Food and Drug Administration (FDA) for use in pasteurized, processed cheese spreads. Its success as a biopreservative has stimulated further research targeted toward identifying new bacteriocins from GRAS organisms with different spectra of activity or which exhibit enhanced activity in environments where nisin is only weakly active. Given that many bacteriocins have now been characterized as exhibiting antibacterial activity against a range of pathogenic/food spoilage bacteria, it is expected that bacteriocins and bacteriocin-producing LAB (used as starters or protective cultures) will find many roles in both fermented and nonfermented foods as a means of improving food quality, naturalness, and safety. That said, perhaps the most effective means of demonstrating the potential usefulness of these compounds is to discuss in some detail the prototypical example nisin.

Nisin

Nisin, a bacteriocin produced by certain strains of *Lc. lactis* subsp. *lactis*, is inhibitory to a wide range of Gram-positive bacteria, including strains or species of streptococci, staphylococci, lactobacilli, micrococci, *Listeria*, and most spore-forming species of *Clostridium* and *Bacillus*. Gram-negative bacteria, yeasts, and fungi are not normally affected. Nisin is a lantibiotic (class I, **Table 1**) and thus undergoes significant posttranslational modification (the mature nisin molecule is shown in **Figure 2**(**b**)). Nisin has a bactericidal effect on sensitive cells, causing a rapid death within a minute of addition. Nisin has recently been shown to have more than one mode of action in that it affects both cell wall synthesis and cell membrane integrity. It interacts with lipid II (the 'docking' molecule), a key intermediate in peptidoglycan assembly, and as a result inhibits peptidoglycan synthesis. The action of nisin then proceeds through insertion into the membrane and pore formation, leading to a rapid and a specific efflux of low-molecular-weight compounds and the depolarization of the membrane. Membrane insertion relies upon a charged membrane and does not occur in nonenergized liposomes. It is currently mooted that the membrane-associated nisin


(a)
Ser Pro Arg Ile Thr Ser Ile Ser Leu Cys Thr Pro Gly Cys Lys Thr Gly
Leader
OH
OH
OH
SH
OH
SH
23 AAs
Dehydration of selected serines and threonines
Ser Pro Arg Ile Dhb Dha Ile Dha Leu Cys Dhb Pro Gly Cys Lys Dhb Gly
SH
SH
Lanthionine formation
cleavage
Dha
Ile
Leu
Pro
Gly
Ser Pro Arg Ile Dhb Ala –S– Ala Abu –S– Ala Lys Abu Gly
S
Lanthionine
β-Methyl lanthionine
(b)
Nisin
Leu
Ala
Met
Dha
Ile
Leu
Pro
Gly
Gly
Gly
Ala Abu –S– Ala Ser Ile His Val Dha Lys
Ile Dhb Ala –S– Ala Abu –S– Ala Lys Abu –S– Ala Asn Met Lys Abu –S– Ala His
(c)
Asn
Gly
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Trp
Gly
Tyr
Ala
Asp
Trp
Lys
Ala
S
Trp
Abu
His
Ala
Leu
Glu
Met
Lacticin 3147 (two-component)
Ltn α
Ala Ala Dhb Asn Dhb Phe D-ala Leu Ala –S– Ala Abu –S– Ala
S
Ltn β
Thr
Asn
Thr
Thr
Lys
Arg
Ala
2-ob Dhb Pro Ala Dhb Pro Ala Ile D-ala Ile Leu D-ala Ala Tyr Ile Ala –S– Ala Pro Abu –S– Ala Abu –S– Ala

Figure 2 Lanthionine synthesis and lantibiotic structure. Lanthionine bridges are formed when an enzymatically dehydrated serine condenses with the sulfhydryl group of a neighboring cysteine, thus creating a ring within the modified peptide or lantibiotic (Ala-S-Ala). When the partners are threonine and cysteine, the novel residue is β-methyllanthionine (Abu-S-Ala). Many lantibiotics also contain dehydrated serines (dehydroalanine, Dha) and threonines (dehydrobutyrine, Dhb). Lantibiotics can be composed of a single peptide (nisin) or two peptides, which act synergistically to yield full activity (lacticin 3147). The dehydration and ring formation reaction can be catalyzed by two enzymes (NisB and NisC in the case of nisin, LtnM1 and LtnM2 in the case of lacticin 3147) or by a single enzyme (LcnM in the case of lacticin 481). A number of additional modified residues can be found in lantibiotics, for example, the D-alanines found in lacticin 3147.

molecules aggregate to form amphiphilic pores, through which the low-molecular-weight compounds can exit. The affected cell is unable to derive energy with which to synthesize macromolecules such as proteins or nucleic acids, and is rapidly killed. It has been observed that Gram-negative cells, normally insensitive to the action of nisin, can be sensitized by the addition of chelating agents; these disrupt the integrity of the outer membrane and allow the bacteriocin access to the cytoplasmic membrane.

Nisin was initially proposed for use as an antibiotic, but its inactivity against Gram-negative bacteria, sensitivity to digestive proteases, and instability at physiological pH rendered it unsuitable. However, nisin possesses many features that make it ideal for use as a food preservative: it is nontoxic; it is produced by GRAS microorganisms; and it is rapidly degraded in the gastrointestinal tract and thus does not negatively impact the gut microflora. Currently, it is not used clinically and there is no evidence that the development of nisin resistance could have a negative impact on antibiotic therapeutics. The physicochemical properties of nisin dictate that stability, solubility, and activity are greatest at low pH levels. It is used mainly to prevent the outgrowth of spores in a wide variety of foods, including processed cheese and canned vegetables (see below). Some species of sporeformers are more sensitive than others; for example, *Bacillus stearothermophilus* is more sensitive than *Bacillus cereus*, *Bacillus megaterium*, or *Bacillus polymyxa*, and although it is effective in preventing the outgrowth of *Clostridium botulinum* types A, B, and E, the more proteolytic types exhibit greater resistance. Sensitivity to nisin increases with lower pH, increased temperature, length of heat shocking, and lower spore load. Significantly, it is sporicidal rather than sporistatic.

Uses of Nisin

Nisin is sold under the trade name of Nisaplin®. Nisaplin® is a highly stable, powdered preparation that contains ~2.5% nisin. The balance consists of milk and milk solids derived from the fermentation of a modified milk medium by nisin-producing strains of *Lc. lactis*. Nisin has found many applications in the food industry, a few of which are presented here.

Dairy products

The potential for growth of and toxin production by *C. botulinum* in processed cheese products, particularly spreads, is of considerable significance. In addition, *Clostridium sporogenes*, *Clostridium butyricum*, and *Clostridium tyrobutyricum* are often associated with the spoilage of processed cheese. The spores of these anaerobic species can survive a heat treatment of 85–105 °C for 6–10 min, so nisin is employed to prevent their outgrowth and hence, for example, has been approved in the United States for use in cheese spreads to inhibit the outgrowth of *C. botulinum* spores. The use of nisin allows these products to be formulated with higher moisture levels and lower sodium chloride and phosphate contents than would otherwise be possible, and also allows them to be stored outside chill cabinets without the risk of spoilage. The level of nisin used depends on food composition, likely spore load, required shelf life, and temperatures likely to be encountered during storage. Nisin is also used to extend the shelf life of dairy desserts that cannot be fully sterilized without damaging appearance, taste, or texture, and is added to milk in the Middle East where shelf life problems occur owing to the warm climate, the necessity to transport milk over long distances, and poor refrigeration facilities. Its use can double the shelf life of such products at chilled, ambient, and elevated temperatures and prevent the outgrowth of thermophilic heat-resistant spores that can survive pasteurization. It can also be used in canned evaporated milk. Finally, in addition to its use to control spoilage/pathogenic bacteria, nisin has also been added to stirred yogurt postproduction to inhibit the starter culture, resulting in flavor maintenance by preventing the subsequent overacidification of the yogurt.

Canned foods

Nisin can be added to canned foods to control thermophilic sporeformers such as *B. stearothermophilus* and *Clostridium thermosaccharolyticum*, which may survive and grow in canned foods stored at high temperature. It also allows a reduction in heat processing required, without compromising food safety. It is used in canned potatoes, peas, mushrooms, soups, and cereal puddings, while its increased activity at acidic pH makes it ideally suited to preserving low-pH canned foods, for example, canned tomatoes, by inhibiting acid-tolerant spoilage flora such as *Bacillus macerans* and *Clostridium pasteurianum*.

Meat and fish

Concerns regarding the toxicological safety of nitrite used in cured meat to prevent clostridial growth have prompted the food industry to look for other methods of preservation, nisin being one of the alternatives investigated. Unfortunately, however, uneconomically high levels of nisin are required to achieve good control of *C. botulinum*, perhaps owing to nisin binding to meat particles, uneven distribution, poor solubility in meat systems, high pH, interference of meat phospholipids, or the inhibitory activity of glutathione found in raw meat. There exist, however, certain conditions under which nisin can be used effectively in meat. A commonly examined system is sausage, as its spoilage is often attributable to LAB that can be inhibited by bacteriocins. It has been established that lower fat contents correlate with higher nisin activity in the system, and it has also been determined that nisin in combination with lactic acid can inhibit Gram-negative organisms. Nisin is also effective at inhibiting *Brochothrix thermosphacta*, the predominant spoilage organism in chilled raw meats and processed meat products, when incorporated in a cold meat-binding system. With regard to fish, it is known that the growth of the psychroduric pathogen *Listeria monocytogenes* can be a problem with smoked fish, especially in fresh and lightly preserved products. Fortunately, nisin is an effective antilisterial agent in smoked salmon, especially when packed in a carbon dioxide atmosphere.

Alcoholic beverages

The insensitivity of yeasts to nisin allows it to be used to control acid-tolerant spoilage LAB of the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* in beer or wine.

It can maintain its activity during fermentation without any effect on the growth and fermentative performance of brewing yeast strains and also has no deleterious effect on taste. It can therefore be used to reduce pasteurization regimes and to increase shelf life of beers. Although nisin can also be applied to prevent the spoilage of wine, its use for this purpose is complicated owing to the potentially negative impact on the microorganisms that carry out the desirable malolactic fermentations. Here, nisin-resistant bacterial starter cultures such as resistant strains of *Leuconostoc oenos*, in conjunction with nisin, can be used to control the malolactic fermentations. Nisin can be used in the pitching yeast wash as an alternative to acid washing for the control of LAB, while it can also reduce the amount of sulfur dioxide used by winemakers to control bacterial spoilage.

Other applications

Nisin is used in the control of bacterial contamination in high-moisture, hot-baked flour products and liquid egg, and also to prevent spoilage of nonfermented, low-pH foods such as salad dressings. It has been shown to be effective in soup manufacture in preventing or delaying the outgrowth of psychroduric spoilage *Bacillus* spp. that are able to survive the pasteurization process. It is also useful in controlling the growth of Gram-positive contaminants in fermentations that depend on Gram-negative bacteria or fungi, for example, single-cell protein, organic acids, polysaccharide, amino acid, or vitamin production. Finally, whereas veterinary medicine has predominantly relied on traditional antibiotics for prophylactic and treatment therapies, bacteriocins offer an attractive natural alternative. As a result, there is considerable interest in the exploitation of nisin for the treatment and prevention of bovine mastitis, an inflammation of the udder that is both persistent and costly to treat. Two products that incorporate nisin as an active ingredient, a pre- and postmilking dip and cleaning wipes, are currently commercially available.

Lacticin 3147

Another member of the lantibiotic family of bacteriocins that has received a lot of attention in recent years is lacticin 3147. The producing organism, also a strain of *Lc. lactis*, was originally isolated from an unusual source, a kefir grain used in domestic sour milk production in Ireland. Production is plasmid-encoded and the plasmid in question can be conjugally transferred to commercial starters to create non-GMO transconjugants for various applications. Lacticin 3147 is a two-component lantibiotic (class I, **Table 1**) with a broad host range that is similar to, yet distinct from, that of nisin. Both lanthionine-containing components, known as Ltnα and Ltnβ, act synergistically to yield full activity (the mature lacticin 3147 peptides are shown in **Figure 2(c)**). A lacticin 3147-producing live starter culture has been successfully used in trials to control non-starter LAB during Cheddar cheese ripening, and to prevent safety problems in cottage and mold-ripened cheeses and in fermented meats. One of the main advantages of the incorporation of bacteriocin into foods in this way is that bacteriocin is produced during the manufacture of the product, rather than being added as a concentrate where it may be considered an additive. This lantibiotic has also been effectively used in trials in powdered form to improve the safety of various food systems, including baby formula, soups, and dairy products. Like nisin, lacticin 3147 also possesses traits that indicate that it could have veterinary and/or medical applications. Lacticin 3147 is bactericidal to all the major Gram-positive mastitic pathogens, and trials to date confirm that it could be used as an alternative antibiotic-free therapy to prevent bovine mastitis. A number of other significant Gram-positive pathogens, such as methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, and *Clostridum difficile*, are all sensitive to this antimicrobial, while the fact that *Propionibacterium acnes* has been shown to exhibit particular sensitivity to lacticin 3147 suggests that lacticin 3147 may have potential in the treatment of acne when used in topical formulations.

Pediocin-Like Bacteriocins

Although nisin is the only purified bacteriocin used commercially, others, such as pediocin, have application in food systems. Pediocin-like bacteriocins are members of the class II bacteriocins (**Table 1**), a group of bacteriocins in which there is considerable commercial interest. These are small heat-resistant peptides that are not posttranslationally modified to the same extent as the class I bacteriocins, apart from, in the majority of cases, the cleavage of a leader sequence from a double glycine site upon export of the bacteriocin from the cell, and the presence of disulfide bridges in some molecules. All of the pediocin-like (or class IIa) bacteriocins share certain features, including a 7-amino acid conserved region in the N-terminal of the active peptide (...Tyr-Gly-Asn-Gly-Val-Xaa-Cys...). Perhaps, the most extensively studied of this class is pediocin PA-1, which is produced by *Pediococcus acidilactici*. The bactericidal mode of action of pediocin PA-1 involves three basic steps: pediocin binding to the cytoplasmic membrane, insertion of bacteriocin molecules into the membrane, and the formation of pores. It is worth noting that the mannose PTS system has recently been definitively identified as the receptor for class IIa bacteriocins.

Pediococci are important in the fermentation of vegetables and meat for both acid production and flavor development. The pediocin-like bacteriocins (which are also produced by genera other than the pediococci) are active against other LAB but are particularly effective against *L. monocytogenes*, a food-borne pathogen of significant concern to the food industry. *Listeria* may be found in raw milk, dairy products, vegetables, and meat products, and can grow under such hostile conditions as refrigeration temperatures (growth has been reported as low as −1 °C), high salt concentrations (up to 10%), low pH (pH 5.0), and high temperatures (44 °C). The biopreservative potential of pediocin PA-1 has already been commercially exploited; the pediocin PA-1-containing fermentate AltaTM *2341* is a food ingredient reported to extend the shelf life of a variety of foods and, particularly, to inhibit the growth of *L. monocytogenes* in ready-to-eat meat products. Pediocin PA-1 has been observed to inhibit *Listeria* in dairy products such as cottage cheese, ice cream, and reconstituted dry milk. *In situ* production in dry fermented sausage inhibits *L. monocytogenes* throughout fermentation and drying, probably owing to a combination of the reduction in pH and bacteriocin production. *Pediococcus acidilactici* may also be used as a low-level inoculum in reduced-nitrite bacon to prevent the outgrowth of *C. botulinum* spores and subsequent toxin production.

Other ‘pediocin-like’ bacteriocins include sakacin P (*Lactobacillus sake*), curvacin A (*Lactobacillus curvatus*), leucocin A (*Leuconostoc gelidum*), and enterocin A (*Enterococcus faecium*). The relative insensitivity of starter LAB to some of the above suggests a potential role in food fermentations where normal starter activity is required. One example of this is a strain of *Enterococcus faecalis* producing enterocin A, isolated from natural whey cultures, which has been utilized as starter in the manufacture of Mozzarella cheese from water buffalo milk. It inhibits *L. monocytogenes*, but not the useful LAB. As nisin is not effective in raw meat, the use of other bacteriocins that are less susceptible to the presence of glutathione has been examined. Significantly, leucocin A and a number of enterocins, sakacins, and the carnobactericins A and B successfully prolong the shelf life of fresh meat. Leucocin A, the producer of which was isolated from meat, also shows promise in the preservation of vacuum-packed meat. Such meat is stored at low temperatures in anaerobic conditions, possibly with added organic acids. However, spoilage bacteria of meat are psychrotrophic, facultatively anaerobic, and acid tolerant. Therefore it is necessary to control them by other means, such as bacteriocinogenic LAB that must be able to compete with the relatively high indigenous microbial loads present in raw meat. Because leucocin A is stable in meat and can be produced during chilled storage without undesirable organoleptic changes under aerobic or anaerobic conditions, it can prevent spoilage occurring due to sulfide production by *Lb. sake*.

Applications of Other Bacteriocins

Many other bacteriocins with commercial potential have been isolated. The following are just some of a myriad of different bacteriocins and applications that have been researched. One successful commercial application of a bacteriocin other than nisin or pediocin PA-1 is a treatment for bad breath licensed by a biotechnology firm called BLIS Technologies. Since 2002, Throat Guard lozenges, containing the naturally occurring beneficial bacterium *Streptococcus salivarius*, have been available on the New Zealand market. The strain is a salivaricin B producer, which has been shown to assist in maintaining throat health by supporting the throat’s natural defenses against undesirable bacteria.

Powdered skim milk previously fermented by a bacteriocin-producing strain can be used as a food ingredient. This approach has been commercially developed with a strain of *Propionibacterium freudenreichii*, resulting in a product with a very wide antimicrobial spectrum including some Gram-negative bacteria, yeasts, and fungi. It is available as a liquid concentrate, spray-dried or freeze-dried preparation. It is added to a variety of dairy products, such as yogurt and cottage cheese, where it is used as an inhibitor of psychrotrophic spoilage bacteria, and a nondairy version is also available for use in meat and bakery goods. The inhibitory activity almost certainly depends primarily on the presence of propionic acid, but there has also been a role proposed for a bacteriocin-like protein produced during the fermentation. This use of milk fermented by a bacteriocin producer as an ingredient in milk-based foods may be a useful approach for introducing bacteriocins into foods at little cost.

Plantaricin S and T production, by *Lb. plantarum*, in Spanish-style green olive fermentation is beneficial in that these peptides are active against a number of natural competitors and spoilage microorganisms such as propionibacteria and clostridia, resulting in a more reliable product without completely eliminating the indigenous microflora, an occurrence that could detract from the quality of the final product. It is interesting to note that a number of enterococci, isolated from Argentinean milk samples and milk products, produce proteinaceous compounds that inhibit *Vibrio cholerae*. These compounds may already play an important role in the natural preservation of foods, especially in those regions where cholera is a concern. Similarly, many traditional African foods are fermented by LAB before consumption. Naturally occurring bacteriocin-producing strains in such products could be used to improve the quality and shelf life of other African fermented foods, some of which are plagued by problems such as inconsistent quality, hygienic risks, and premature spoilage.

An alternative potential application of bacteriocins other than in preservation and protection of food is bacteriocin-induced starter lysis to improve the quality and to accelerate the ripening of Cheddar cheese, particularly where starters undergo limited autolysis, which could result in the production of a bitter cheese. A strain of *Lc. lactis* producing lactococcins A, B, and M causes lysis of susceptible lactococci, has been used in studies as an adjunct culture to accelerate the lysis of starter lactococci, and thus is an example where a narrow- rather than broad-spectrum bacteriocin is of use.

Advantages and Disadvantages of Bacteriocins as Food Additives

One of the advantages associated with the use of bacteriocins in food is that these molecules can be said to be normal constituents of the human and animal diet, in that meat and dairy systems are particularly rich sources of bacteriocinogenic LAB. Bacteriocins are proteinaceous in nature and would therefore be expected to be inactivated by proteases of gastric or pancreatic origin during passage through the gastrointestinal tract. Therefore, such bacteriocins, if used in foods, should not alter the digestive tract ecology or result in risks associated with the use of common antibiotics.

A further advantage of bacteriocins is that most are thermostable and thus can survive the thermal processing of foods. Others can work at both low pH and low temperature and could therefore be useful in acid foods and cold-processed or cold-stored products. Bacteriocins may also have applications in minimally processed refrigerated foods, for example, vacuum- and modified-atmosphere-packaged refrigerated meats and ready-to-eat meals, which lack some of the barriers or hurdles to the growth of pathogenic/spoilage bacteria formerly conferred by traditional preservation techniques. In addition, the genetic determinants for production of, and immunity to, the more well-known bacteriocins are well characterized and thus it is possible that these could be transferred to nonproducing starter strains for *in situ* production. This is particularly true for those bacteriocins whose genes are located on naturally transmissible elements, like nisin (conjugal transposon) and lacticin 3147 (conjugal plasmid).

One possible drawback to the use of bacteriocins in foods is that they are hydrophobic molecules and therefore their use could be limited as a consequence of their being partitioned to the organic fat phase within a food matrix. However, even though most bacteriocins are indeed very hydrophobic, they are relatively small molecules and so can easily diffuse into the water phase of food products. Despite this, binding to food surfaces and poor activity are often observed when bacteriocin-producing strains are added to food systems. This may instead be due to poor solubility or uneven distribution of the bacteriocin molecules, their sensitivity to food enzymes, or the negative impact of high salt or other added ingredients on the production or activity of the bacteriocin. Although these disadvantages have been identified by many scientists, in practice bacteriocins have been shown to be effective in a number of food systems, including full-fat cheeses and meats under certain conditions.

It should be noted that for most bacteriocins, as is the case with antibiotics, there is a natural variability in the sensitivity of individual target species and even strains within a species to these antimicrobials, with some displaying much higher minimum inhibitory concentrations than others. Once a new preservative is found to be safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. Unfortunately, experiences in clinical medicine have demonstrated that the emergence of bacteria that are resistant to multiple antibiotics can be a significant issue. Even though bacteriocins are not antibiotics, there is concern that exposure to bacteriocins will render cells more resistant to antibiotics. Although bacteria exhibiting nisin resistance do not show cross-resistance to antibiotics, it is still important to investigate whether bacteriocin resistance could become a problem. Studies to date have demonstrated that nisin-resistant mutants of *L. monocytogenes* can appear at frequencies of 10^{-8}–10^{-6}; in properly processed food such high levels of pathogenic bacteria should not be encountered. In the case of lacticin 3147, resistance emerges at a lower frequency (10^{-8}–10^{-9}) and even then the extent to which these cells become more resistant is modest.

Another problem to overcome is the reluctance of industry to incorporate new methodologies over old, tried-and-tested ones, particularly if they have to embark on substantial and expensive programs of toxicological testing before the new antimicrobial can be used as a purified additive. Though the use of any new food ingredient has to undergo strict regulatory considerations, in the case of biologically derived macromolecules with well-understood pathways of digestion and metabolism such as proteins, they may be determined to be safe for consumption by utilizing available knowledge of their structure, biological activity, digestibility, and biological and compositional factors. In the case of bacteriocins, safety assessment may require characterization of the substance as completely as possible; a description of the preparation, proposed use, and proportion in food; knowledge of its effect in the food; an understanding of its metabolic fate in the gastrointestinal tract; and also perhaps an environmental impact assessment. Even though it is unclear how many detailed toxicity trials have been performed to date, no evidence of bacteriocin-related toxicity has been reported. Toxicity studies for nisin carried out using amounts far in excess of the amount that would be used in food caused no ill effects. It is rapidly inactivated in the intestine by digestive enzymes and is undetectable in human saliva 10 min after consumption. Though nisin is currently the most commercially used bacteriocin, the safety of other bacteriocins with potential applications in food has also been evaluated. Toxicity of pediocin PA-1, lacticin 3147, and a number of other bacteriocins has been tested, and in all cases they have been found to be nontoxic and susceptible to proteolysis.

Production of Bacteriocins

There are at least three ways in which bacteriocins can be incorporated into a food to improve its safety: addition of a purified/semipurified bacteriocin preparation as an ingredient in food; incorporating an ingredient previously fermented with a bacteriocin-producing strain, or using a bacteriocin producer as a starter or adjunct culture in fermented foods to produce the bacteriocin *in situ*. The use of purified bacteriocins is not always attractive to the food industry, as in this form they may have to be labeled as additives and require regulatory approval. The latter two alternatives (fermentate/starter culture) are not restricted by these requirements, and thus these options are often regarded as being more attractive. Although bacteriocins can be produced in the food matrix during food fermentation, LAB bacteriocins can be produced in much higher amounts during *in vitro* fermentations. Such higher production results owing to the absence of limiting factors such as strong diffusion limitations, inactivation by proteases, and adsorption to food particles. However, if fermentation is allowed to proceed in an uncontrolled manner, significant differences in activity yields are observed; also the influence of the process conditions on bacteriocin activity can be observed and may need to be optimized. An ideal protocol would be one which is applicable for large-scale purification with low production and recovery costs, leading to a bacteriocin yield >50% and purity >90%. The cultivation conditions influence specific bacteriocin production, and also indirectly affect levels through the amount of biomass accumulated, because bacteriocin production is a growth-dependent physiological trait and most bacteriocins are produced during the active-growth phase.

Furthermore, a lowering of pH results in a decreased adsorption of the bacteriocin molecules to the producer cells, and hence in an increased bioavailability. In addition, temperature and nutrient availability seem to play a vital role in bacteriocin production, whereas the presence of elevated amounts of sodium chloride usually decreases production levels. Maximum bacteriocin yield can be obtained by harvesting immediately when activity peaks or by using conditions that minimize adsorption. Bacteriocins could be removed from the fermentation either batchwise or continuously by using adsorbants. Fermentations should be based on cheap substrates and a suitable and low-cost downstream processing strategy devised to optimize the bacteriocin production process. Another parallel development of relevance to bacteriocin production is the use of techniques to allow one strain to produce a number of bacteriocins, thus increasing the spectrum of bacteria sensitive to that strain.

Future Prospects for Bacteriocins in Food

Bacteriocins should not be seen as a primary means of food preservation. Rather they can contribute to the ‘hurdle concept’ approach to food preservation and safety whereby a number of treatments, both intrinsic and extrinsic, are combined to preserve food more effectively by affecting microbial growth. Many bacteriocins have been observed to be more stable and effective at acidic pH, higher-than-normal temperatures (important in the case of temperature abuse), or lower-than-normal temperatures (important for refrigerated foods). Bacteriocins serve as bactericidal barriers that can help to reduce the levels of contaminating bacteria, whereas biostatic measures such as modified-atmosphere packaging or water reduction can prevent the remaining population from growing. For example, a combination of modified-atmosphere packaging and nisin has been found to be more effective than either treatment alone in preventing the growth of *L. monocytogenes*. Bacteriocins can also be used in conjunction with other antibacterial factors. The enzyme lysozyme lyses many Gram-positive cells and is used to prevent gas formation in some cheeses. Lysozyme and nisin can act synergistically to inactivate *L. monocytogenes*. Bacteriocin activity can also be enhanced by the effect of chelators; for example, even though the effectiveness of nisin against Gram-negative cells is generally low, the growth of pathogens such as *E. coli* O157:H7 and *Salmonella* can be controlled when EDTA, which disrupts the Gram-negative outer membrane, is used in combination with nisin. Plant-derived antimicrobials, some of which are already in use, such as benzoic, sorbic, acetic, and citric acid, and also phenolic compounds and essential oils such as those found in garlic, can be used in food preservation, but have disadvantages in that the food may not be considered ‘natural’ or the level required for inhibition may introduce too strong a flavor to the food. Bacteriocins may replace or permit a lower level of such inhibitors.

A promising and rapidly developing area of research is that focused on the bioengineering of lantibiotics. Because of the gene-encoded nature of lantibiotics, it has been possible to develop bioengineering systems that have given insight into the roles and importance of different amino acids in various peptides. Such systems also have the potential to generate novel derivatives with improved properties, such as increased activity, stability, and host range. There have been recent successes in this regard in that a nisin-producing strain with improved bioactivity against the Gram-positive mastitic pathogen *Streptococcus agalactiae* has been identified, as well as derivatives with enhanced bioactivity and specific activity against other Gram-positive pathogens, including *L. monocytogenes* and/or *S. aureus*, have been identified. The identification of derivatives with superior properties represents a major step forward in the bioengineering of nisin, and lantibiotics in general, and confirms that peptide engineering can deliver derivatives with enhanced antimicrobial activity against specific problematic spoilage/pathogenic microbes or against Gram-positive bacteria in general. However, such bioengineered peptides may have to overcome regulatory hurdles before they can become commercially applicable.

The future for bacteriocins does not lie in discovering, or engineering, the perfect bacteriocin for all applications. Rather it is more practical to imagine specific bacteriocins for specific tasks. Once a particular bacteriocin is deemed useful for a particular application, the economics of bacteriocin production on an industrial scale becomes the main issue. In the case of using bacteriocins to protect fermented foods, this obstacle is in general less significant in that the bacteriocin can be introduced into the product at little cost through the use of a bacteriocin-producing starter bacterium. This approach ensures an even distribution of the inhibitor throughout the food, and the low pH of fermented foods is often optimal for bacteriocin activity. Also LAB boast GRAS status, so regulatory considerations can be overcome. Addition of partially purified bacteriocin preparations to foods may be less cost effective, but has been accomplished with nisin. Perhaps a more readily acceptable means of adding bacteriocins will be through powders derived from a milk fermentate – these may be used as an ingredient in milk-based foods as a way of introducing bacteriocins at little cost. Genetic analysis of bacteriocin operons will continue to pave the way for bacteriocin applications, as the ability to overproduce the inhibitor will certainly impact its cost-effectiveness. Further research into the prevalence of natural bacteriocinogenic strains in retail foodstuffs, in conjunction with toxicological studies, may provide an even stronger case for the safe use of bacteriocins in the food chain.

See also: **Cheese**: Non-Starter Lactic Acid Bacteria. **Lactic Acid Bacteria**: *Lactococcus Lactis*; *Leuconostoc* spp.; *Lactobacillus spp*.: General Characteristics; *Pediococcus* spp.; Taxonomy and Biodiversity;

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BACTERIOPHAGE

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Biological Aspects

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Introduction

Hugh Whitehead and Geoffrey Cox in 1935 identified the first bacteriophage (phage) specific for a lactic acid bacterium (LAB) at the New Zealand Dairy Research Institute. They discovered lactococcal bacteriophage as the causal agent of the complete breakdown of acid production experienced when using single-strain starters. Since then, phages infecting every species of LAB used in the dairy industry have been isolated. The phage infecting dairy lactobacilli was first isolated from sewage water in New York City and reported in 1934. Later many *Lactobacillus* phages infecting strains of *Lactobacillus delbrueckii* (subsp. *bulgaricus* and subsp. *lactis*), *Lactobacillus helveticus*, *Lactobacillus plantarum*, and *Lactobacillus casei*/*paracasei*, among others, were isolated from traditional yogurt and other fermented foods. Likewise, the *Streptococcus thermophilus* phages were first isolated 55 years ago in Switzerland. As *Lactococcus lactis* and *S. thermophilus* are responsible for most industrial dairy fermentations, the knowledge focusing on phages infecting these species is more extensive and deeper.

Since the discovery by Whitehead and Cox, phages of LAB have been the subject of ongoing research to understand the interaction between them and bacteria, and to exploit this knowledge for the improvement of valuable LAB strains. However, the inhibitory effect of bacteriophage against lactic acid starters is still recognized as one of the major and persistent troubles in dairy fermentation processes (*see* **Bacteriophage**: Technological Importance in the Diary Industry).

Classification

According to the International Committee on Taxonomy of Viruses (ICTV), all known phages infecting LAB are tailed phages and currently members of the *Caudovirales* order. In turn, tailed phages are arranged in three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. In brief, myophages have tails consisting of a neck, a contractile sheath, and a central tube; tails in *Siphoviridae* phages are simple, noncontractile, flexible or rigid tubes; finally, *Podoviridae* members have short and noncontractile tails.

At present and based on DNA–DNA hybridization and morphology results, lactococcal phages belong mainly to the *Siphoviridae* family, with a few being *Podoviridae* members. Over a decade ago, a total of 12 lactococcal phage groups was conformed. However, one appeared extinct (P107) and three others (1483, T187, and BK5-T) were merged with the P335 group, reducing the number of lactococcal phage species from 12 to 8. Groups 936, P335, 1358, c2, P087, and 949 belong to the *Siphoviridae* family, whereas species P034 and KSY1 are included in the *Podoviridae* family. Nonetheless, additional lactococcal phage groups may exist. For example, phages 1706 and Q54, which were unrelated to the other phages, could be members of two novel groups. However, the vast majority of lactococcal phages isolated from dairy fermentations belong to one of the three main species, that is, 936, c2, and P335.

To date, 231 *Lactobacillus* phages have been reported and 186 of them have been morphologically characterized. A total of 109 of these phages were assigned to the

Siphoviridae family, 76 were myophages, and only 1 was of the family *Podoviridae*. Several attempts have been made to classify phages infecting *Lactobacillus*. However, phage diversity and the lack of uniform information on most of them complicate this purpose. The large number of species in the *Lactobacillus* genus is probably one of the reasons for phage diversity, even though a relatively well-conserved genome organization is observed among the reported complete nucleotide sequences of these phages. Before the availability of genomic sequences, the classification of *Lactobacillus* phages was based mainly on morphological observations and DNA homology, *Lb. delbrueckii* phages being the first to be classified in the 1980s as groups a, b, c, and d. Later, several completely sequenced *Lactobacillus* phages were assigned to the group Sfi21 or Sfi11, a classification scheme based on the organization of the structural gene module of the siphophages. Interestingly, this latter scheme was able to group phages infecting diverse low GC-content Gram-positive bacteria (*S. thermophilus*, *L. lactis*, and *Lactobacillus*) (**Table 1**). Additional proposals for classification of lactobacilli phage were based on the deduced proteomic trees disregarding phage morphology, but in the particular case of *Lactobacillus* phages, their underrepresentation in these schemes might distort the impact of those phylogenetic trees.

Regarding phages of *S. thermophilus* reported to date, all are members of the *Siphoviridae* family and can be assembled into only two distinct groups according to their DNA packaging mechanism (*cos* or *pac*) and the number of major structural proteins. Specifically, there exists a strict correlation between the presence of a particular set of major structural phage proteins and the mechanism of DNA packaging, demonstrating that *cos*-containing phages possessed two major structural proteins (32 and 26 kDa), in contrast to the *pac*-containing phages, which possessed three major structural proteins (41, 25, and 13 kDa).

Phage Morphology

The advent of nucleotide sequence determination has revolutionized biology and largely rationalized taxonomy, including that of viruses. Only proteins and nucleic acids compose phage particles. Proteins constitute the head, tail, spikes, and fibers. Virion capsids are not enveloped and are icosahedral or elongated in shape. Tails are helical and may have a collar, a sheath separated from the head by a neck, or fixation structures (baseplates, fibers, spikes) (**Figure 1**). The phage genome is not segmented and contains a single molecule of double-stranded DNA.

The diversity observed in phage particle dimensions is linked to the multiplicity of morphologies. Phages of *S. thermophilus* constitute the more homogeneous group. Virion heads are generally 60 nm in diameter, but abnormally small heads of 40–42 nm have been reported. According to tail length, phages seem to fall into two groups: their tails being ~220 and 330 nm long, respectively, with a few rare phages having tails of 130 and 420 nm in length.

Lactobacillus myophages possess tails ranging in length from ~120 to 272 nm and icosahedral capsids are 50–115 nm in diameter. Tails of siphophages are 115–500 nm in length, and among them the prolate ones have capsids 120–150 nm long and 40–50 nm wide, whereas the icosahedral capsids are 40–76 nm in diameter.

The widest variability in virion dimensions is observed among phages of *L. lactis*, from the highest elongated capsids (phage group KSY1) of 223 nm length and 45 nm width to the smallest one of 45 nm diameter (phage group 1358). Tail lengths are equally variable, from 32 nm (phage group KSY1) to 490 nm (phage group 949).

Lysogeny

The lysogenic cycle is one of the two methods of viral reproduction (the lytic cycle is the other one). In prokaryotes this cycle is characterized by integration of the bacteriophage nucleic acid into the genome of the host bacterium .

Lysogeny is widespread in all species of LAB, but it is best studied in the genus *Lactococcus*. Temperate bacteriophage can disturb the normal fermentation process by various mechanisms. By mutation, these phages can become virulent for the lysogenic strain and are able to overcome its lysogenic immunity. On the other hand, in commercial starters, these released temperate phages could find other sensitive strains in the same starter and infect them.

Temperate phages are also capable of mediating transduction, which, in addition to transferring plasmids encoding traits, may eventually find a role in developing chromosomal maps for various members of the LAB. Plasmid transduction has been well documented in both lactococci and lactobacilli. The efficiency of this process could facilitate the development of phage-mediated gene transfer systems for lactobacilli.

The release of temperate phages can be influenced by different factors such as temperature fluctuations, pH, osmotic pressure, and low nutrient concentration. Released temperate phages can be detected by electron microscopy, DNA extraction, or propagation on sensitive strains. This fact has a great practical importance, as they could contribute to the dissemination of active phage particles in industrial environments.

The presence of prophages in LAB strains that will be used as commercial starter components might be a fundamental criterion for the choice of the strain, together with other technological characteristics.

Table 1 LAB phages for which complete genomes have been sequenced

Phage	*Host*	*GenBank accession number*	*Genome size (kbp)*	*Life cycle*[a,b]	*Phage type*[c]
Abc2		FJ236310	34.882	V	B1; Sfi21 group; *cos*
ALQ13.2		FJ226752	35.525	V	B1; Sfi11 group; *pac*
2972		AY699705	34.704	V	B1; Sfi11 group; *pac*
5093		FJ965538	37.184	na	na
7201	*S. thermophilus*	AF145054	35.466	V	B1; Sfi21 group; *cos*
858		EF529515	35.543	V	B1; Sfi11 group; *pac*
DT1		AF085222	34.815	V	B1; Sfi21 group; *cos*
O1205		U88974	43.075	T	B1; Sfi11 group; *pac*
Sfi11		AF158600	39.807	V	B1; Sfi11 species; *pac*
Sfi19		AF115102	37.370	V	B1; Sfi21 group; *cos*
Sfi21		X95646	40.739	T	B1; Sfi21 species; *cos*
1706		EU081845	55.597	V	B1; 1706 species; *cos*
4268		AF489521	36.596	V	B1; P335 group; *cos*
712		DQ227763	30.510	na	B1; 936 group
asccphi28		EU438902	18.762	na	C2; 034 group
bIBB29		EU221285	29.305	V	B1; 936 group; *cos*
bIL170	*L. lactis*	AF009630	31.754	V	B1; 936 group; *cos*
bIL285		AF323668	35.538	T	B1; P335 group
bIL286		AF323669	41.834	T	B1; P335 group
bIL309		AF323670	36.949	T	B1; P335 group
bIL310		AF323671	14.957	T	B1; P335 group
bIL311		AF323672	14.510	T	B1; P335 group
bIL312		AF323673	15.179	T	B1; P335 group
bIL67		L33769	22.195	V	B2; c2 group; *cos*
BK5-T		AF176025	40.003	T	B1; P335 group; *pac*
c2		L48605	22.172	V	B2; c2 species; *cos*
jj50		DQ227764	27.453	na	B1; 936 group
KSY1		DQ535032	79.232	V	C3; KSY1 species
LC3		AF242738	32.172	T	B1; P335 group; *cos*
P008	*L. lactis*	DQ054536	28.538	na	B1; 936 group
P087		FJ429185	60.074	V	B1; P087 species; *pac*
P335		DQ838728.1	33.613	V	B1; P335 species; *pac*
Q54		DQ490056	26.537	V	B2; Q54 species; *cos*
r1t		U38906	33.350	T	B1; P335 group; *cos*
sk1		AF011378	28.451	V	B1; 936 group; *cos*
TP901-1		AF304433	37.667	T	B1; P355 group; *pac*
Tuc2009		AF109874	38.347	T	B1; P335 group; *pac*
uL36		AF349457	36.798	V	B1; P335 group
A2	*Lb. casei*	AJ251789	43.,411	T	B1; Sfi21 group; *cos*
adh	*Lb. gasseri*	AJ131519	43.785	T	B1; *cos*
AT3	*Lb. casei*	AY605066	39.166	T	B1; Sfi21 group; *cos*
KC5a	*Lb. gasseri*	DQ320509	38.239	T	B1
Lb338-1	*Lb. paracasei*	FJ822135	142.111	V	na
Lc-Nu	*Lb. rhamnosus*	AY131267	36.466	V	B1; Sfi21 group; *cos*
Lj771	*Lb. johnsonii*	AF195902	40.881	T	na
Lj928	*Lb. johnsonii*	AY459533	38.384	T	na
Lj965	*Lb. johnsonii*	AY459535	40.190	T	Sfi11 group; *pac*
LL-H	*Lb. delbrueckii* subsp. *lactis*	EF455602	34.659	V	B1; *pac*
LP65	*Lb. plantarum*	AY682195	131.522	V	A1; SPO1 group
Lrm1	*Lb. rhamnosus*	EU246945	39.989	T	B1; Sfi21 group; *cos*
Lv-1	*Lb. jensenii*	EU871039	38.934	T	na
phig1e	*Lactobacillus*	X98106	42.259	T	B1; *pac*
phiJL-1	*Lb. plantarum*	AY236756	36.674	V	B1; *pac*

[a] T, temperate phage; V, virulent phage.

[b] na, not available.

[c] A1, isometric head *Myoviridae* (phages with contractile tails); B1, isometric head *Siphoviridae*; B2, prolate head *Siphoviridae* (phages with long noncontractile tail); C2 and C3, isometric head *Podoviridae* (phages with short noncontractile tail); *cos*, *cos* site, genome with cohesive ends; *pac*, *pac* site, DNA packaging by the head-full mechanism; phage species designation where available is given.

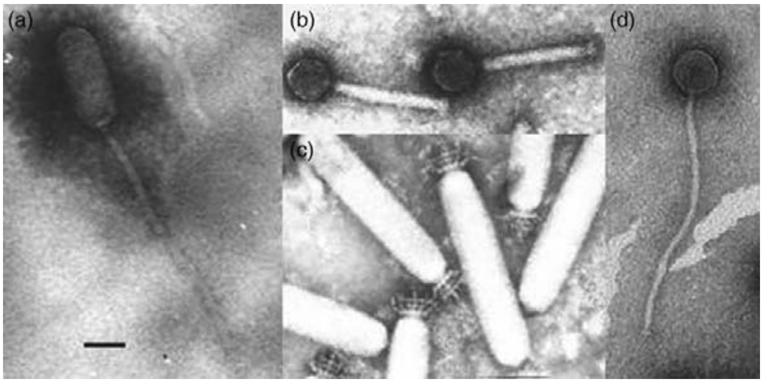

Figure 1 Electron micrographs of bacteriophage Cb1/204 (*Lactobacillus delbrueckii*) (a), ATCC 15807-B1 (*Lactobacillus helveticus*) (b), KSY1 (*Lactococcus lactis*) (c), and Abc2 (*Streptococcus thermophilus*) (d). Scale = 50 nm.

Life Cycle of Lactic Acid Bacteria Lytic Phages

The infection process (lytic cycle) involves a number of tightly programmed steps, as shown in **Figure 2**. The first step involves phage adsorption to the cell surface. This process occurs when specialized adsorption structures, such as fibers or spikes, bind to specific surface molecules on their target bacteria. Frequently, adsorption occurs first in a reversible step (binding to carbohydrate components in the cell wall), followed by an irreversible (blender-resistant) step (binding to chromosomally encoded protein embedded in the cell membrane). Adsorption velocity and efficiency are important parameters that may vary

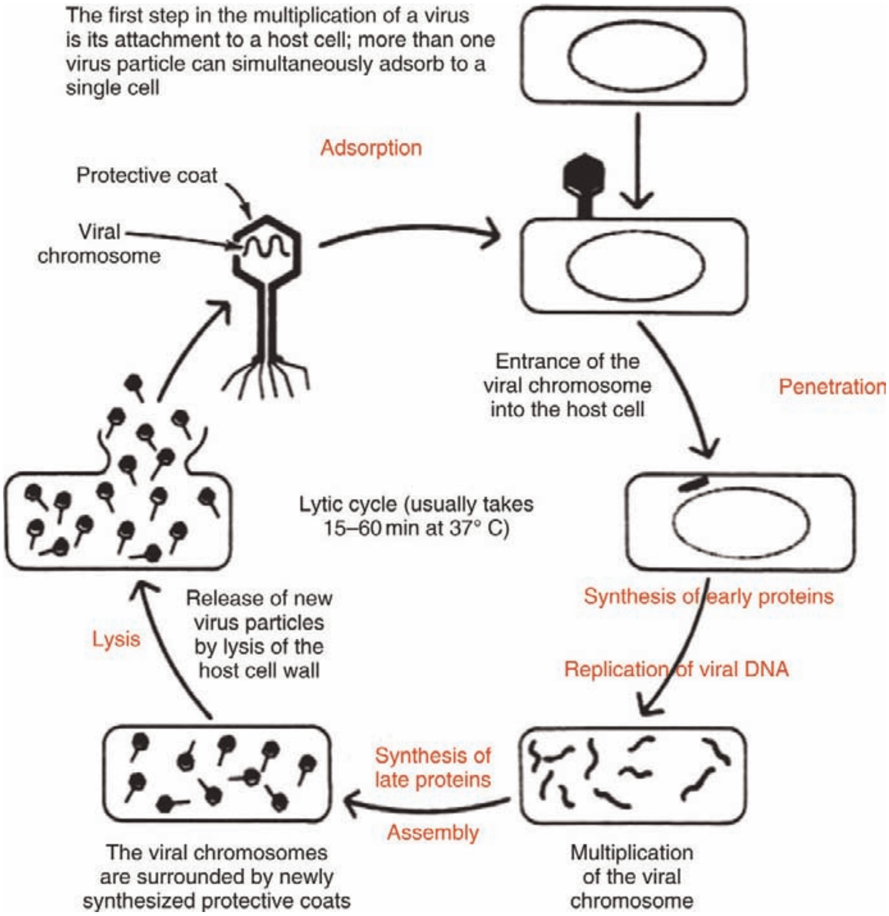
The first step in the multiplication of a virus is its attachment to a host cell; more than one virus particle can simultaneously adsorb to a single cell
Adsorption
Protective coat
Viral chromosome
Entrance of the viral chromosome into the host cell
Penetration
Lytic cycle (usually takes 15–60 min at 37° C)
Synthesis of early proteins
Replication of viral DNA
Release of new virus particles by lysis of the host cell wall
Lysis
Synthesis of late proteins
Assembly
The viral chromosomes are surrounded by newly synthesized protective coats
Multiplication of the viral chromosome

Figure 2 Life cycle of bacteriophage specific for lactic acid bacteria.

for a given phage–host system depending on external factors and the host's physiological state.

After an irreversible attachment, the phage genome passes through the tail into the host cell. This process generally requires cell energy from intact cells, and it has been suggested that this step is dependent on the protein synthesis machinery of the bacterial host. It is known that divalent cations such as Ca^{2+} and Mg^{2+} are, sometimes, indispensable to cellular lysis. The action mechanisms of these cations are diverse, but it is well determined that they act in this step of lytic cycle.

Once into the host cell, the phage DNA can be expressed and replicated. The initial step generally involves the recognition by the host RNA polymerase of very strong phage promoters, leading to the transcription of early genes. The products of these genes may protect the phage genome and restructure the host appropriately for the needs of the phage. Often, a set of middle genes is then transcribed, synthesizing the new phage DNA, followed by a set of late genes that encode structural proteins and phage lysin. After the synthesis of macromolecular components, the phage particles are assembled. The DNA is packaged into the heads. Before or during the packaging, the head expands and becomes more stable, with increased internal volume of DNA. Located at one vertex of the head is a portal complex that serves as the starting point for head assembly, the docking site for the DNA packaging enzymes, a conduit for the passage of DNA, and, for myoviruses and siphoviruses, a binding site for the phage tail, which is assembled separately.

Finally, the lysis of the host cell is a precipitous event that, for tailed phages, uses two enzymes: lysin and holin. The timing of lysis is affected by growth conditions and the genetics of strains of bacteria.

Genetics

More than 20 complete lactococcal phage genome sequences are available today in public databases, including phages belonging to the species P335, c2, and 936, as well as sequences from rare lactococcal phage species such as Q54, KSY1, P034, and 1706 (**Table 1**). The biodiversity observed in phages infecting *L. lactis* is perfectly visualized in many different-sized genomes of the morphologically different phages. The molecular mass of genomes varies from 14 510 bp for a *Siphoviridae* member of the P335 group (phage bIL311) to 79 232 bp for a member of the family *Podoviridae*, namely, the phage KSY1, which possesses a large elongated capsid (223 nm long, 45 nm wide). Comparative genomic analyses of these phages raise the knowledge on their origin, evolution, relationship with other phages, as well as their prevalence in dairy environments.

Currently, 11 complete genomes of *S. thermophilus* phages, ranging from 34 to 43 kbp in size, are available in public databases and represent the two recognized groups of *S. thermophilus* phages (**Table 1**). In addition to the distinction between *cos* and *pac* types, genomic analysis has shown that all phage genomes are similarly organized into distinct modular regions; the genes coding for DNA replication and host lysis are the most highly conserved. A comparison of these phages shows that, like their hosts, they are rather homogeneous, as they belong to one polythetic species containing both temperate and virulent phages.

Among *Lactobacillus* phages, 15 complete genome sequences are currently available, of which only 1 is a myophage and 7 are siphophages (**Table 1**). Thus, comparative genome analyses mainly focus on siphophages, the modular organization of whose genome is similar in all the cases, also in *cos*- and *pac*-type *Lactobacillus* phages. A considerable diversity is found in the genomic size of these phages, ranging from 34 659 bp (virulent phage LL-H, infective of *Lb. delbrueckii* subsp. *lactis* LL23) to 142 111 bp (virulent phage Lb338-1, infective of *Lb. paracasei* 338).

Lactic Acid Bacteria Phage Characterization

The characterization of LAB phages can be made by means of diverse criteria. The fundamental one is the study of their morphology, which permits to include them in the classification of the ICTV.

An indispensable characterization of bacteriophage is based on their physicochemical properties. Description of the adsorption step and determination of the burst size, host range, and dependence on divalent cations to replicate are obligatory items to characterize phages. The DNA restriction analysis, DNA packaging mechanism (*cos*/*pac*), and protein composition can also facilitate the differentiation of morphologically and physicochemically similar phages.

Because of the technological and economical significance of viral infections in the dairy industry, it is very important to study the resistance of phages against diverse thermal and chemical treatments usually implemented to minimize their number in the environment, as raw milk is the major source of phage entry to the processing dairy plant. There are thermal treatments normally used to sanitize the raw milk before processing: HTST (high temperature–short time, 72 °C × 15 s), LTLT (low temperature–long time, 63 °C × 30 min), and 82 °C × 5 min (milk for making yogurt) as an alternative method. For LAB phages, several studies reported that neither HTST nor LTLT treatment guarantees their complete inactivation. Instead, 82 °C is effective to inactivate phages, except for a few exceptions (some phages of *Lb. delbrueckii*).

Regarding the chemical treatments commonly used at laboratories or plants, their efficiency depends on the biocide used. The most efficient disinfectants at the laboratory level are those that include quaternary ammonium as the active compound, whereas sodium hypochlorite and ethylic and propylic alcohols have a lesser effect on phage inactivation. In general, low concentrations of these biocides (between 1.0 and 2.5% v/v) are capable of inactivating high concentrations of bacteriophage within 2 min of exposure. In dairy plants, a wide range of disinfection agents are used. Most of them were assayed against microorganisms, but they were never tested on bacteriophage. Some recent studies have demonstrated that biocides with acids (peracetic or peroctanoic acid) or quaternary ammonium chloride as active compounds were very efficient in inactivating bacteriophage specific for LAB.

Host Resistance Mechanisms

The extended cosurvival of LAB and phages within the same environment has prompted the strains to the acquisition of a variety of native bacteriophage defense systems. These mechanisms include inhibition of phage adsorption, blocking of DNA injection, restriction/modification (R/M) systems, and abortive infection (Abi). More recently, when studying *S. thermophilus* strains, CRISPRs (clustered regularly interspaced short palindromic repeats) have been demonstrated as the latest defense mechanism found in prokaryotes. As *L. lactis* and *S. thermophilus* constitute a key group of organisms of most industrial dairy fermentations, their native host resistance mechanisms have been meticulously characterized, in contrast with those of *Lactobacillus* strains. In lactococci, these mechanisms may be encoded by chromosomal or plasmid genes. Interestingly, the natural gene transfer by conjugation of plasmid DNA is a very widespread feature in lactococci. So, the conjugation of native phage-resistant plasmids has been a strategy of great profit to genetically improve dairy LAB for more than 20 years, yielding multiple dairy starter cultures that have been in commercial use for many years; many are under patent worldwide. This situation contrasts with that found in *S. thermophilus*, where, a very few plasmids are present in the strains.

Adsorption Inhibition

The first defense line against bacteriophage attack is to block the adsorption of the phage particle to the extracellular bacterial envelope. Adsorption inhibition has been proposed to occur by two distinct mechanisms. The first mechanism involves the expression of extracellular factors, including exopolysaccharides (EPSs), which presumably obstruct the interaction between phage and the cell surface, either by masking the receptors or by binding to the receptors. The second mechanism results in the reduction or complete absence of the phage receptors expressed on the cell surface. These mechanisms have been extensively characterized in lactococci; however, they have not yet been clearly identified in strains of *S. thermophilus*, although it has been unambiguously demonstrated that the production of EPSs does not confer potential protection against phage infections. The knowledge on *Lactobacillus* is even, and neither the nature of phage receptors nor the adsorption inhibition has been fully described. However, studies on phage adsorption inhibition have considerable importance, as this is a particularly effective resistance mechanism the bacterial strain may use to prevent phage DNA entry.

Inhibition of Phage DNA Injection

To date, very few host-mediated systems inhibiting phage DNA injection have been identified or reported. One study described the isolation of a spontaneous phage-resistant mutant of *Lb. casei* ATCC 27092 to which phage PL-1 adsorbed successfully but failed to inject its DNA. In lactococci, one plasmid-encoded system, namely pNP40, was able to delay or impair the internalization of phage genomes infecting *L. lactis* subsp. *lactis* biovar *diacetylactis* DRC3, which was later transferred to a variety of strains used in industrial fermentations. Chromosomally encoded phage DNA injection-blocking mechanisms were revealed in *L. lactis* subsp. *lactis* PLM-18 and in a spontaneous phage-resistant mutant of *L. lactis* C2. Another phage resistance system manifested as a DNA injection blocking, and which represents a novel superinfection exclusion mechanism, was identified, and this was linked to a lactococcal prophage gene designated *sie*$_{2009}$. The Sie$_{2009}$ protein, associated with the cell membrane, interfered with phage replication.

Restriction/Modification Systems

These systems constitute the first line of intracellular defense against bacteriophage. Their great advantage is that they stop the infection prior to the beginning of phage-directed cell death. The R/M systems are composed of two complementary enzymatic functions. The first one is a restriction endonuclease (REase), which cleaves double-stranded DNA. The second component is a modification enzyme, classically a methyltransferase (MTase) that modifies DNA at specific sequences all over the genome. Host DNA is protected from this cleavage by being modified. Likewise, if the incoming phage DNA escapes cleavage it may be modified by the MTase, thus becoming protected and able to replicate in that host and other host strains with the same R/M system. Currently, the functional classification of R/M systems includes the types I, II, III, and IV, based on the nature and complexity of their (1) target recognition sequences, (2) cleavage site(s), and (3) enzyme structure. To date, only members of the type I, II, and III R/M systems have been identified in dairy LAB. In *Lactococcus*, the functional exploitation of these systems, mainly the plasmid-encoded ones, has extended the utility of many industrially important strains. In contrast, the vast majority of R/M systems identified in *S. thermophilus* are chromosomally encoded, which makes their characterization difficult. In lactobacilli, R/M systems have been demonstrated for *Lb. helveticus* and *Lb. delbrueckii*. R/M systems are often associated with other phage resistance mechanisms, providing multiple hurdles for the phage to overcome.

Abortive Infection

The Abi is characterized by a normal start of infection (i.e., phage adsorbs and injects its DNA into the host cell) followed by an interruption of phage development, leading to the release of few or no progeny particles and to the death of the individual infected cell. As the infection of other cells in the culture is prevented, the bacterial population survives. The action of an Abi mechanism on phage typically results in reduced efficiency of plaquing (EOP), burst size, and efficiency of formation of centers of infection. To date, 22 lactococcal Abi mechanisms have been isolated in *Lactococcus*, characterized, and designated AbiA through AbiZ. Most of them appear to have a distinct mode of action, although they share some common features. Usually, plasmid-encoded Abi mechanisms provide a stronger phage resistance (due to a high gene copy number) than that provided by the chromosomally encoded, which may explain the fact that so few lactococcal Abi genes are present in the bacterial chromosome. The Abi phenotype is usually mediated by a single gene, although complex systems require two genes for expression. A single report on the possible Abi mechanism in *S. thermophilus* NST5 has appeared in the literature; however, that system has not been cloned or characterized. Likewise, the plasmid pLKS harbored in *Lb. plantarum* NGRI0101 is the only one encoding a phage resistance phenotype possibly linked to Abi in lactobacilli.

Natural Abi mechanisms have been used extensively for protection of industrial starter cultures and, as a consequence, this selective pressure has led to the emergence of phage mutants capable of overcoming the resistance barriers. Therefore, even though Abi is arguably the most efficient mechanism, it should be used in combination with other systems to provide increased phage resistance to starter cultures.

CRISPRs as a Novel Phage Resistance Mechanism

Until now, the mechanism involved in the generation of bacteriophage-insensitive mutants (BIMs) was often attributed to mutations in the phage receptors, although recent studies have demonstrated that CRISPRs play a role in the development of BIMs. It was recently proved that CRISPRs and the associated genes constitute the latest defense mechanism found in prokaryotes and particularly in *S. thermophilus*. Specifically, it was found that in response to challenges with virulent phages, an *S. thermophilus* strain was able to integrate new spacers derived from the phage genomes, generating a phage-resistant phenotype; meanwhile, a small population of phages was also able to infect the bacteriophage-insensitive mutants, suggesting that both CRISPR locus and phage genomic regions may rapidly evolve. The effectiveness and wide-ranging applicability of this antiphage system is in agreement with the fact that CRISPRs are found in a wide range of bacterial genomes. These facts clearly represent a novel and interesting approach for the development of phage-resistant bacterial strains for fermentation and biotechnological processes.

Engineered Phage Resistance Mechanisms

The understanding of dairy bacteriophage has progressed rapidly owing to the availability of at least 50 complete genome sequences in the database (**Table 1**). A wide variety of engineered phage resistance systems that target different stages in the lytic life cycle have been constructed using the information acquired from the examination of sequence data. Therefore, genomic analysis allows the identification of well-conserved genes among the genomes of industrially relevant phages, which could be the targets to construct genetic tools for use in the dairy environment. However, it is important to note that these systems have not been used in the manufacture of products for human consumption owing to legal restrictions and public concerns regarding the introduction of recombinant DNA into the food chain.

Origin-Derived Phage-Encoded Resistance

When a phage replication origin (*ori*) is provided in *trans* on a recombinant plasmid, it can act as a molecular decoy that competes for, and titrates away, both phage- and host-derived replication factors that catalyze phage genome replication. As a result, the number of phage genomes replicated during the lytic infection is reduced and, in opposition, the phage-associated replication factors might also catalyze a dramatic increase in plasmid copy numbers. Phage-encoded resistance (PER) system was first reported to be effective in *L. lactis*, but has been extended to *Lb. casei* and *S. thermophilus*.

Antisense RNA

Antisense RNAs may be used to interfere with the phage lytic cycle by inhibiting the translation of phage-encoded genes essential for normal development. Mechanistically, antisense RNA hybridizes to the sense RNA strand and creates a translationally inactive double-stranded RNA (dsRNA) molecule. Formation of the dsRNA molecule silences gene expression through the cooperative action of one or more intermolecular mechanisms. The first systems of this type were constructed in *Lactococcus* and, more recently, in *S. thermophilus*. The effectiveness of RNA-based phage defense strategies has been highly variable, exhibiting both target- and phage-specific differences. To improve the level of phage resistance, antisense RNA systems were combined with PER systems, although the effectiveness was limited.

Superinfection Exclusion and Immunity

Approximately half of the sequenced bacterial genomes contain prophage-associated sequences, signifying that prophage-containing strains abound in nature. Examination of these sequences reveals that prophage or prophage remnant sequences comprise 3–10% of the total genomic content of lysogens. This is apparently due to the fact that prophages often provide benefits to the lysogen by encoding factors that may increase the fitness of the bacterium, such as the lysogenic conversion genes. The genes encoding these functions are located between the lysin gene and the phage attachment (*att*P) site. In *S. thermophilus*, the lysogenic conversion genes are among a small handful of phage-encoded genes actively and consistently transcribed by the prophage. From a phage resistance point of view, superinfection exclusion and immunity genes are well-characterized examples of beneficial genes associated with the prophages of Gram-positive bacteria, including LAB. These genes protect lysogens from becoming infected with additional phages, and they were shown to mediate superinfection exclusion when they were expressed from a high-copy–number plasmid, which leads to a significant protection from diverse lytic bacteriophage. Examples of superinfection exclusion were reported for *S. thermophilus* and *L. lactis*.

Another efficient system is the expression of the gene encoding the repressor of the lytic cycle. The expression of the repressor gene (*cI*) of the *Lb. casei* temperate bacteriophage A2, cloned in the *attB* site of its host chromosome, confers a total phage resistance, even during milk fermentation.

Subunit Poisoning

The expression in *trans* of mutant genes encoding protein subunits can suppress, or poison, the function of native multimeric proteins in a dominant negative way. Subunit poisoning has been applied as a phage defense system in *S. thermophilus*, based on the use of multiple alignments of related target protein sequences so as to identify the critical amino acid residues involved in enzyme catalysis and/or protein subunit oligomerization. In this case, the model system was the putative primase of *S. thermophilus* phage κ3, which is a component of its Sfi21-type genome replication module. Invariant and highly conserved amino acids within a phage primase consensus ATPase–helicase domain were targeted by site-specific mutations. When expressed in *trans* from a high-copy-number vector, mutant proteins appeared to be completely inhibiting phage genome replication and significantly reducing the EOP. In a related form of resistance, *L. lactis* lytic bacteriophage of the P335 group were inhibited by overexpression of a truncated *cI* gene repressor.

Host Factor Elimination

The elimination of host-encoded factors essential for phage replication has great potential to block phage replication at one or more stages of development, without compromising the technological properties of strains. The first case reported had achieved this by means of *in vitro* mutagenesis of the PIP (the chromosomal-encoded protein known as phage infection protein) gene, which was then replaced in a c2-sensitive *L. lactis* strain. In effect, the truncated protein conferred phage resistance phenotype and the resistant strain showed technological properties similar to those of the parent strain as well as genetic stability. Similarly, a putative *S. thermophilus* transmembrane protein (functionally analogous to the lactococcal PIP), when mutated, provided complete resistance to specific bacteriophage.

Phage-Triggered Suicide Systems

Phage-triggered suicide systems represent another approach to engineer phage-derived resistance based on a genetic trap in which a strictly phage-inducible promoter isolated from a lytic phage is used to activate a bacterial suicide system after the infection. Early phage factors are required for the expression of the phage-inducible promoter, and therefore it remains silenced until the infection. The expression in a high-copy-number vector of the gene encoding an REase, under the control of late-expressed inducible promoter, without its associated MTase gene reduces the EOP of infecting phages. The induction of the gene encoding the restriction endonuclease by the phage infection kills the host and aborts phage infection by restricting both the host and the phage genome. This construction is similar to an Abi system, because the infection involves degradation of the host DNA and the consequent death of the infected cell before the liberation of the viral progeny.

Phage Counterdefenses

Industrial strains of dairy starters typically encode one or more phage resistance systems (either natural or nonnatural). As a result of these extreme selective pressures, phages have evolved a variety of genetic countermeasures that allow mutant derivatives to circumvent these defenses. Knowledge is currently being accumulated regarding the mechanisms of evolution that lead to the genetic diversity of bacteriophage in the dairy factory environment. Nevertheless, the response of a phage to a particular phage resistance mechanism cannot be easily predicted. In general, it depends on the ability of the phage to replicate and to mutate to variants that can overcome the particular phage-resistant mechanism. The mechanisms by which this is achieved vary and include acquisition of MTase genes from the host genome via recombination, incorporation of modified nucleotides into bacteriophage DNA, production of antagonistic proteins that inhibit the activity of bacterially encoded REases, and elimination of the REase recognition sites throughout the genome.

These new phages represent an economic threat to the dairy industry. Given the intensive way that starter strains are grown in the presence of phages, the dairy fermentation environment is an ideal niche to monitor phage evolution, the study of which would help the design of novel methods to counteract adaptation to phage resistance mechanisms.

Detection of Phage and Nonphage Inhibition

An efficient way to avoid phage attacks in dairy factories is the early detection of bacteriophage in milk at any point during production. There is therefore great interest in the development of techniques that can detect dairy phages. Usually, milk is examined for phages using standard microbiological methods (plaque assays, spot test, activity test, etc.) that allow the detection of active phage particles and the discrimination between phage and nonphage inhibitors. But these assays are time-consuming and require the availability of a sensitive strain. Molecular methods for the rapid detection of bacteriophage are the new choices for this kind of task, giving results in a much shorter period of time. Indeed, polymerase chain reaction- (PCR-) based assays have already been successfully used to detect and identify viruses and bacteria in a number of foods, and they have been adapted to detect dairy phages in cheese whey, cheese whey starters, and milk samples. Increasing demand for more sensitive and rapid detection procedures, such as fluorescence-based PCR, has prompted their routine use in the laboratory. Real-time PCR (qPCR) has been recently applied to the development of highly sensitive assays for the detection of *Lb. delbrueckii* and *S. thermophilus* bacteriophage in dairy samples, allowing amplifications in the range of 30 min. It is important to highlight that these molecular methods do not discriminate between active and nonactive phage particles, as they detect phage DNA.

See also: **Bacteriophage**: Technological Importance in the Diary Industry.

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Technological Importance in the Diary Industry

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Introduction

The word bacteriophage or the shortened, more common term 'phage' originates from the Greek, the literal translation of which is 'bacteria eater' or 'virus that can infect bacteria'. In 1915 the first phage attack was documented by Frederick Twort, a bacteriologist who was studying smallpox vaccines and found what he called a 'bacteriolytic agent' that destroyed colonies of staphylococcal cells. At about the same time another scientist conducting studies in France, Felix d'Herelle, found an invisible antagonistic microbe of the dysentery bacillus that caused cell lysis; he called this a bacteriophage or bacteria eater and this name has remained to the present day.

Bacteriophage are among the most common forms of life on earth and are found in all ecosystems where bacteria live. They require bacterial hosts to grow and multiply. A typical phage consists of a protein head that contains nucleic acids (DNA or RNA). In the lactic acid bacteria, commonly used in the dairy industry for fermentation, the phage genome is composed of DNA as the form of nucleic acid responsible for phage replication. The most common phage also have a collar and a tail through which the phage nuclear material is injected into the host cell. For multiplication, the host has to be actively growing, as phage are not metabolically active on their own and therefore cannot reproduce by themselves.

Reproduction of Bacteriophage

Typically, bacteriophage have two types of replication cycle. The lytic cycle is the most common. The phage attaches itself to the cell via specific receptors on the host cell surface: a process know as adsorption. These receptors are host-specific and consist of, but are not limited to, lipopolysaccharides, techoic acids, and proteins on the cell wall. This receptor is so specific that it is one of the factors that limits the host range of bacteriophage. The presence of divalent ions, such as Ca^{2+}, in the environment is also important to the adsorption process. After attachment to the cell, injection of the nucleic acid takes place followed by the takeover of the replication machinery of the cell. Once in the cell, the phage nuclear material takes over the replication and expression systems of the cell. This starts the replication process, producing numerous copies of viral nucleic acid code. These copies are then packaged into new viruses, the cell lyses, and the intact viruses are released into the environment where they continue the lytic cycle.

The other replication process is known as the lysogenic cycle. This process involves the viral genome integrating into the host cell where it replicates as part of the bacterial cell and does not, in the normal course of cell division, result in lysis. The integrated viral genome or prophage exists as one or several copies per cell and confers resistance to attack from related phage. This relationship is stable but can change due to certain environmental triggers, resulting in the phage entering the lytic cycle and releasing new phage particles. These viable phage cannot infect the other cells in the culture, which are immune to infection due to their lysogenic status. If a suitable nonrelated host strain is available in the environment, they can cause infection and lysis of the susceptible host.

Technological Importance of Bacteriophage in Milk Fermentation

Bacteriophage are of technological importance because of the large variety of pharmaceutical products, chemicals, and food products manufactured by fermentation. The fermentation process itself involves the production of the desired product using large quantities of microbes including bacteria. Fermentation can take place in a relatively sterile environment and take hours, days, or even weeks, but if infection takes place then the fermentation is slowed or halted completely, resulting in a reduction of product quality and quantity, as well as altered and disrupted production schedules. In the dairy industry the substrate used for fermentation, namely, milk, cannot be sterilized for cheesemaking and as such is a prime environment for the bacteriophage–bacterial cell interaction. On a global basis, bacteriophage are still the major cause of slowdown or failure of food fermentation processes, due to the failure to complete the acidification process. The resultant failure or slowdown in acid production has been the subject of and motivation for decades of research and partnership between industry and academia to investigate the source of bacteriophage, understand the mechanism of its interaction with the host cell, and provide solutions to ensure the success of the fermentation process.

Fermentation Process

Fermentation of milk and the production of dairy products is a process that has been recorded in history as far back as 5000 BC. The process involves the conversion of a relatively unstable, short shelf life product – milk – into a stable acidified – fermented milk – or a coagulated, dehydrated, and acidified product – cheese.

The conversion of milk into cheese involves three steps. The first step – coagulation – involves the formation of a rennet or an acid gel. The second step involves the concentration of the coagulum and removal of whey by mechanical processes, while the third step – ripening – involves the production of the finished cheese characteristics through the biochemical transformation of the cheese components, namely, protein, fat, and sugar, to various intermediates, the degree of transformation and the length of time involved giving rise to the desired cheese characteristics including texture and flavor.

Starters in Milk Fermentation

The earliest dairy fermentations were artisan-type processes, where fermentations were initiated or started using microorganisms from raw milk, an inoculum from the previous day's production. These traditional cheesemaking processes gradually evolved to suit the local fermentation conditions, so the type of lactic acid bacteria most suited to grow under these conditions were also selected. These starters are the origins of the culture systems, strains, and programs used globally today in the production of cheese and fermented milks.

These starter cultures were undefined, mixed multiple-strain blends of unknown composition. The composition of these cultures was either predominantly mesophilic blends of acid producers (*Lactococcus lactis* subsp. *lactis* or *Lactococcus lactis* subsp. *cremoris*) with or without flavor-producing strains (citrate-positive strains of *Lactococcus lactis* or *Leuconostoc* species). In parallel for thermophilic fermentations, for example, mixed cultures containing *Lactobacillus* and *Streptococcus* species evolved. Their use was limited to a small number of vats per day and the artisan nature of the cheese production meant that the culture was able to complete the acidification process as required with no constraints on the time taken for cheesemaking or the consistency of the final product. The number or type of strains was continually in flux due to the growth conditions of the culture, as well as the presence of phage, with sensitive strains being wiped out and replaced by more phage-insensitive strains within the culture itself. The key characteristics of the selected cultures were acidification rate, proteolytic activity, and bacteriophage insensitivity. Starter cultures were often exchanged between dairies which, if maintained or subcultured differently, resulted in a change in the culture balance and a change in the culture performance. Approximately 100 years ago, with the onset of the techniques to isolate and characterize strains based on their phenotypic traits, these traditional types of cultures were finally handled in a way to preserve their characteristics by moving to laboratories, where the culture program could be analyzed microbiologically. In some parts of the world today, this program of subculturing strains or using the previous day's whey to inoculate the next day's batch continues with varying degrees of success.

With the onset of modern cheesemaking processes the interaction between bacteriophage and their hosts, namely, the lactic acid bacteria, has assumed more critical importance. While some cheese varieties continue to be made in a more traditional fashion, the majority of cheese, including varieties such as Cheddar, Mozzarella or pizza cheese, and Emmental types, as well as Gouda and Edam, are made to time in large highly automated plants with multifilling of vats. In such a situation the starter culture has to produce acid consistently, resulting in cheese of standard chemical composition and water content, while reaching the required pH in a recipe, which is controlled by automated control systems. There is no tolerance for in-process delays due to the highly automated process from receiving of milk to packaging and cooling of the finished cheese.

To achieve this acidification control, a number of different starter culture systems are employed globally to ensure this level of culture performance. One such system, largely employed in Dutch or Continental cheesemaking systems, uses a highly maintained culture program of mixed-strain undefined cultures of mesophilic lactic acid bacteria. These cultures are a mixture of homofermenters that convert lactose to lactic acid, and aroma-forming bacteria. The aroma-forming bacteria can utilize citrate to produce diacetyl and CO_2. In addition to being a mix of acid and flavor producers, they can also be classified as mixed-strain cultures due to an indeterminate number of strains of the same species present in the culture. To achieve the consistency of performance required in modern cheesemaking systems, control of bacteriophage is key. So, maintenance of strain balance and prevention of strain dominance are the most important actions to ensure culture performance. Among the systems where bulk starters are employed for example, in Continental cheese, the most studied system is that of the Dutch L starter and P starter.

The sensitivity of P (practice) and L (laboratory) starters to bacteriophage was documented by the Netherlands Institute for Dairy Research (NIZO) in 1966. It was shown that transfer of cultures in laboratory environments free of phage could lead to strain dominance. When exposed to phage these cultures were more susceptible to failure due to phage attack than the so-called P starters, which were maintained at the time in the factories. P starters were phage-sensitive, but their key characteristic was a diverse strain population to continue acidific process. However, this did not mean that they could not be infected by phage, so to ensure that the cultures perform in the field on a continuous basis, NIZO along with the industry agreed on a number of steps to be taken to ensure the success of the program.

Starters are supplied by NIZO for daily inoculation of the bulk starter tanks. The starter culture is selected on the basis of consistent activity and the desired cheese flavor and eye formation. This starter material is maintained with the minimum number of transfers, to prevent strain dominance and the turning of a P starter into an L starter.

Prevention of Phage Infection in Starter Cultures

The technology employed by the cheese factories to grow bulk starter focuses on prevention of bacteriophage infection. Milk and bulk starter equipment are treated in such a way as to prevent phage infection of the starter. Milk used to grow the starter is carefully selected for quality and the ability to grow lactic acid bacteria. It is heated in place (in the starter tank) or using a high-temperature short-time (HTST) system.

If treated in place, the milk is heated above 95 °C with a holding time of up to 30 min at this temperature. The head space in the bulk starter tank, along with the ancillary pipework, is sterilized using steam to destroy phage that may be present in the system. If the milk is heat-treated using an HTST system, the temperature used is 95 °C or higher with a holding time of at least 60 s. The risk during the cooling of the bulk starter tank and the milk is that air containing phage will enter the vessel and contaminate the milk media. This is addressed using a supply of sterile air to maintain overpressure on the starter tanks.

The sterile air supply is activated automatically when the heating of the tank and its contents has been completed. High-efficiency particulate air (HEPA) filters installed on the bulk tank are extremely efficient at removing particles including phage from the air. It has been shown that less than 1 in 100 million particles of phage will penetrate these filters if well maintained. In addition, the starter tank is equipped with a special inoculation system in order to add the thawed culture contents aseptically. During the use of this culture program, proper sanitation of the totally enclosed cheese vats between fills, along with adequate heat treatment of the whey cream, ensures that the activity and performance of the starter are of the required quality.

The result is that the Dutch industry continues to have confidence in this type of program, and some of the tools used in this program, such as the use of HEPA filters and overpressurization of tanks using sterile air, have translated to other culture programs where bulk starter systems are employed.

Selection of Strains for the Starters

Traditionally all mesophilic cultures used in both Dutch-type and Cheddar-type cheese systems were undefined, mixed, multiple-strain blends of mesophilic lactococci and contained various levels of citrate-fermenting *Lactococcus lactis* subsp. *lactis biovar diacetylactis* or *Leuconostoc* species. These citrate fermenters gave, to a greater or lesser degree, some openness in the cheese due to gas production. These cheeses were also traditionally bandage-wrapped, so the gas could diffuse from the cheese. The advent of plastic barrier packaging along with the export of cheese from New Zealand to Asia/United Kingdom and the parallel increase in the processing capacity of Cheddar cheese plants led to the demand for cultures that did not produce gas and which could produce acid at consistent rates without failure even under bacteriophage attack.

Work with these undefined cultures to eliminate gas production in Cheddar Cheese began initially in New Zealand in the 1930s and 1940s, and led to the isolation of defined single strains of mesophilic, homofermentative lactococci. These strains were initially used as single starters, then as a rotation to overcome culture inhibition caused by phage in whey contamination of the cheese milk. The cause of culture inhibition and variability in acid production was ultimately identified as bacteriophage, and a number of different strategies, based on the knowledge of single strains and their properties, were employed by the cheese industry to overcome the potential failure of starters to maintain even acid production during the cheesemaking day.

Failure to produce acid consistently using single strains led to the development of paired single strains. This evened out any differences in the rate of acid production and reduced the tendency of some strains to cause bitterness in cheese. The concept of paired single strains, initially developed in New Zealand, was introduced to Australia in the 1950s and involved a 4-day rotation which was thought sufficient to allow phage levels to fall below a critical level and allow for incompatibility of strains. However, despite the proven ability of single-strain programs, their use was limited to Australia and New Zealand with the exception of some factories in Scotland and the United States until the 1970s. Failure of the American dairy industry to use single-strain starters was due to the fact that they were not under pressure to make closed textured cheese. In addition laboratories, such as the Dairy Research Institute (DRI) in New Zealand and the Commonwealth Scientific and

Industrial Research Organisation (CSIRO) in Australia did not make cultures available to the industry outside their home countries.

An extension of the paired strain system, called the multiple-strain system, was introduced to the United States in the late 1970s primarily due to the increase in the size of cheese factories, multifilling of vats, and the tendency toward shorter manufacturing times. The use of this culture program containing 2–6 strains was pioneered in the United States by researchers at Oregon State University and Utah State University. The program was also introduced in Ireland in the early 1980s.

In multiple-strain systems, all the strains are propagated separately and blended on inoculation into the bulk tank. If a fast-replicating phage is encountered, the susceptible strain is withdrawn and replaced by a different one. Initially, multiple-strain programs contained up to six strains, and this was the most common number used commercially in New Zealand, America, and Australia. However, developments in the understanding of phage–host interactions, phage resistance at a molecular level, and limits to the isolation of completely new strains have led to the evolution and successful use of other systems based on 2–3 strains used without or in limited rotation.

The overriding factor in the development of defined strain systems has been the understanding of individual strains and their role in flavor and acid production, along with their ability to withstand bacteriophage attack. Developments in classical microbiology, plating techniques, and, in the 1970s, the discovery that plasmid DNA was an important factor in the stability of many of the most desirable traits of lactic acid bacteria allowed the study of the bacteriophage–host interaction and the development of strategies to make the cultures more robust in industrial applications.

When a defined strain shows susceptibility to phage in the factory environment, it can be replaced using a naturally occurring phage-insensitive isolate or by a bacteriophage-insensitive mutant (BIM) of the original strain. The isolation of new phage-insensitive strains is favored by some workers, as BIMs may have slower acid production, may revert to phage sensitivity during use, or be attacked by new disturbing phage in the cheese vat. Such phage that attack the BIM may be more virulent and have an extended host range.

During the development of single-strain programs the focus of the cheesemaker was to make sure that the culture performed consistently, and to do so he had to monitor the performance of the culture as close to the cheese vat as possible. This meant the development of routine phage testing at the cheese production site to predict phage buildup and to be able to ensure that the starter produced by the bulk starter was active. In collaboration with support organizations (both academic and commercial), this monitoring program was also used to isolate factory-derived cheese starters.

Exposure of a growing culture to whey containing phage will, if phage is present that can infect the strain, result in lysis of the majority of the cells in the population. Some cells that are genetically resistant to the phage can then grow; derivatives can be isolated, purified, and tested as to their suitability as cheese starters. This program of factory-derived phage-resistant mutants was successful in increasing the reliability of single-strain culture programs in Australia in the 1970s. The selection program along with the introduction of tests such as the Heap Lawrence test, 7-cycle test to indicate robustness of the selected isolates, increased the reliability of the culture program by extending the lifetime of the strains in the manufacturing environment from an average 2–3 weeks to several months.

Historically, and even today in some countries, bulk starter cultures are being propagated in cheese factories by successive transfer of the culture from stock, usually freeze-dried or liquid, to build up a big enough inoculum for use in the cheese vat. This process is especially prone to bacterial contamination, strain imbalance, and, most importantly, bacteriophage attack.

The objective of the culture supplier is to make available highly active cells free from contaminating bacteria and bacteriophage. Most starter cultures used globally are nowadays supplied by commercial companies. While the source of cultures has changed little, advances in growth, harvesting, and stability of cultures have led to a diverse range of forms, either as frozen liquid or as freeze-dried, being commercially available. The liquid forms can come as frozen solid concentrates to be thawed prior to inoculation, or as pellets that can be added directly to the cheese milk without thawing. This minimizes the risk of contaminating the culture with phage or other microorganisms. These cultures can be used to prepare bulk starters on site or, as is increasingly common, to supply direct vat set (DVS) concentrates directly to the cheese plant for direct inoculation into the cheese milk.

Preparation of bulk starters free of phage is necessary to assure the cheesemaker that they can have a culture free of contaminating bacteriophage for inoculation into the cheese vat. Failure to prevent even very low numbers of phage from entering a bulk tank and surviving pasteurization of the starter medium, or gaining access during cooldown or inoculation, can cause the starter to lyse, either partially or completely, resulting in a slow or dead starter culture. If the quality assurance program does not test the starter, this will lead to lost cheese vats, before the lack of activity is discovered.

To control bacteriophage contamination and growth in the bulk tank, investments have been made in the technology of both the starter medium and the tank itself. Most modern bulk tanks are stainless-steel vessels with key air inlets protected by HEPA filters. Most tanks are steam sterilized and the medium is either heat treated using an HTST system or in-place in the tank. HEPA filters protect the heat-treated medium by filtering the air of most particles during cooling. The tanks are overpressured so as to prevent air entering through unprotected seals during the cooling process. As mentioned previously, the medium has to be heated to 90 °C for at least 30 min to ensure adequate phage destruction.

The inoculation of the bulk tank is also a critical step in the protection of the starter culture from bacteriophage infection. In the ideal situation, most factories will attempt to locate their bulk starter tanks in a room separate from the cheesemaking site. Ideally this involves not just a separate air supply, but also a positive pressure in the room as well as in the tanks. In New Zealand, dedicated personnel with their own changing areas and canteen facilities are considered standard. Inoculation of bulk tanks with liquid culture using the Lewis needle was standard in the United Kingdom until the early 1980s when the use of these cultures eventually gave way to frozen concentrates, unsuitable to this type of inoculation system.

Even though starter inoculation and the maintenance of a phage-free starter are considered critical to cheesemaking operations, the frustration of finding an economical and efficient way of inoculating the bulk tank has led to reliance on the cultures themselves and the growth media rather than on the inoculation system to prevent bacteriophage attack.

Growth of lactic acid bacteria for cheesemaking is a balancing act between maximizing cell populations and minimizing lactate inhibition, while maintaining a phage-free environment to allow the cells to grow. Overripe starters with a developed acidity of greater than 1.0 mEq KOH 100 ml^{-1} will increase the lag time on inoculation into the cheese milk by 30–60 min, which also makes the culture more susceptible to bacteriophage infection.

To prevent acid injury and to increase cell populations, neutralization of the milk starter was employed initially in New Zealand and Australia, using a single-step or a multistep approach in which an external neutralizer such as sodium hydroxide is used. This increased the activity of the starter culture, effectively doubling the starter capacity of the cheese plant, but did nothing to prevent phage proliferation if contamination occurred.

Alternative bulk starter systems had to be developed both to increase cell numbers and to minimize bacteriophage proliferation. It was discovered in the 1950s that bacteriophage required calcium for growth and, when combined with the knowledge that phosphates bind calcium, this led to the development of phage inhibitory media (PIM). These media were milk- or whey-based and also contained growth stimulatory factors such as yeast extract, as well as phosphates and citrates.

While these media have been widely used in the United States, Canada, the United Kingdom, and Ireland, either stand-alone or as concentrates in combination with skim milk, their effectiveness has been problematic as phage have evolved that do not require calcium for replication, and the presence of phosphate salts can inhibit the primary growth of phosphate-sensitive strains of mesophilic lactococci or also result in inhibition on inoculation into cheese milk due to dissociation of the cell wall-associated proteinase. In addition, the protective effect of calcium chelation is no longer effective on addition of the starter to the cheese milk.

Levels of phosphate required to chelate calcium and control the growth of bacteriophage have had to be balanced against inhibition of the starter culture. This balance was achieved using reduced phosphates combined with external pH control where aqueous ammonia is added to a lactose-limiting starter medium.

This method of external pH control of bulk starter has been successful in the 1980s and 1990s for the production of both mesophilic and thermophilic cultures, primarily in the United States and Canada, but to a lesser extent in the United Kingdom and Ireland. It resulted in a starter that was more active in the cheese vats as compared to conventionally grown starter and could be used at reduced inoculation rates. In addition attempts using different pH values could be made to control the rod-to-coccus ratio of thermophilic cultures for the Italian cheese industry.

The use of complicated and high-maintenance external pH control equipment, allied to the safety concerns over the use of aqueous ammonia, led concurrently to the development of internally pH-controlled media, where buffers were formulated to maintain the pH, thus minimizing acid injury.

These media, based on the same principle as acid control in the stomach, made use of an initially insoluble buffer system composed of ammonium, alkali metal, and trimagnesium phosphates. The insoluble buffer had to be kept suspended by constant stirring, and it dissolved as acid was produced, preventing the pH from dropping, increasing the cell biomass without the consequence of acid injury. The culture was not overexposed to high phosphate concentrations, so there was no acid injury, but the system did not prevent phage from multiplying and the constant agitation inhibited some strains of lactic acid bacteria.

Despite the advances in the technology of bulk culture preparation and the implementation of good manufacturing practices and other quality systems focused on food safety, it has become increasingly obvious to the dairy and in particular to the cheesemaking industry that the production of culture is not their major focus and the elimination of bacteriophage remains difficult to achieve in practice. Consolidation of the cheese and fermented milks industry has resulted in operations where the investment in bulk starter capacity is not a priority and the need for predictable, quality starter culture performance is a necessity, not an option.

Modern cheesemaking facilities capable of processing up to 5 million liters of milk per day in 24-h continuous operations need a culture system that has been quality controlled, is of consistent activity, and is economical. Holding a bulk culture for 48 h until quality checks are complete is not economically viable. The risk of bacteriophage infection of the bulk tank and not having the assurance that it is phage-free would result in severe economic losses, even before the failure is detected in the cheesemaking operation.

This, allied to the knowledge that has been accumulated on the production of cell concentrates of high and cost-effective activity, the increased understanding of the mechanisms of phage evolution to select robust strains, and the use of nontraditional blends of bacteria in making cheese, has resulted in an increasing reliance on DVS concentrates in large-scale milk fermentation.

The use of concentrates assures the cheesemaker that the culture is bacteriophage-free and will perform as required in an environment where correct plant sanitation is routine. The resources of the culture supplier and the increasing knowledge base on combining strains along with an understanding of the mechanisms of phage–host interaction and phage evolution have resulted in a decrease in the number of major starter failures in practice. This is primarily due to the improved selection of bacteriophage-insensitive cultures and the combining of strains that are difficult to grow consistently as bulk cultures. A classic example of this has been the development of culture blends for Cheddar cheesemaking using mesophilic lactococci and *Streptococcus thermophilus*. This technology exploits the Cheddar cheese production profile, which is a hybrid of temperatures ideal for both lactococci (30–34 °C) and *Str. thermophilus* (38–40 °C).

Initially documented in Australia in a modified Cheddar process, this combination of bacteria has been exploited globally in the production of Cheddar cheese using concentrates. The cultures have been shown to perform consistently in large multifill operations with regard to economy of use, acidification rate, cheese flavor, and phage resistance. The bacteriophage resistance of such cultures has not yet been fully explained on a genetic level.

Conclusions

It could be argued that while starter systems have come a long way with regard to their ability to perform consistently and that the role of bacteriophage has been diminished, the understanding of phage and culture genomes and its application in the use of starter bacteria on an industrial scale is yet to be fully exploited. There remains the challenge of developing flavor and other functional properties desired in finished dairy products known to be associated with individual strains, but considered to be too phage-sensitive to allow the exploitation of these bacteria and their properties on an industrial scale.

The importance of bacteriophage in dairy fermentation has resulted in the production of cultures moving away from the dairy to a more high-tech environment with the support of advances in the fundamental understanding of bacterial genetics and fermentation technology.

The technological importance of bacteriophage in dairy fermentations will continue to drive cooperation between scientists and technologists in academic institutions, culture supply houses, and manufacturing operations in developing the tools, systems, and procedures to stay one step ahead of bacteriophage – a constantly evolving threat to the dairy industry.

See also: **Bacteriophage**: Biological Aspects.

BIOFILM FORMATION

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Introduction

Biofilms are communities of microorganisms found everywhere, living on surfaces in natural, industrial, and clinical environments. In dairy manufacturing plants, the presence of biofilms in pipelines, around seals, and on product contact surfaces in processing equipment such as heat exchangers and evaporators is believed to be a significant source of product contamination. Biofilm formation is becoming more problematic as processing plants increase in complexity, providing more niches for microbial growth, and as manufacturing run times lengthen, allowing more time for cell growth. Bacteria attached to surfaces are more difficult to kill than free-living cells, and contamination from biofilms increases the microbial load and potentially reduces the safety and quality of dairy products. Minimizing biofilm growth is increasingly a challenge for dairy manufacturers. This article provides a description of the problems caused by biofilms, development of biofilms, detection of biofilms, and some of the latest information on controlling biofilms.

Definition of a Biofilm

Biofilms are communities of microorganisms attached to and living on a surface. This state is believed to be the predominant and natural form of microbial growth and offers ecological advantages for the attached cells by facilitating interspecies and intraspecies interactions. The biofilm provides mutual protection from antimicrobial agents and environmental stress and the environmental conditions in the biofilm are distinct from the conditions pervading in the overlying fluid.

Biofilms can form on most surfaces in dairy manufacturing plants, commencing when microorganisms present in the milk, from the environment or from elsewhere in the plant, come into contact with the surface. Initially, this contact may be a transient association, as the cells may leave the surface and settle elsewhere. This is known as the reversible stage. If conditions are suitable, bacterial cells will form tenacious bonds with the surface (irreversible stage), grow, reproduce, and form a biofilm. The presence of colonizing cells can encourage the settlement and growth of other species and eventually a complex consortium of cells develops on the surface, which inevitably releases cells into the product stream flowing past.

In dairy manufacturing plants, planktonic (free-floating) bacteria do not usually get the chance to increase in numbers, as the residence time of the milk is too short between control steps such as pasteurization, acidification, salting, drying, or cooling.

The biofilms often comprise single species that are well adapted to survive the extrinsic (heat, cooling) and intrinsic (pH, salt) factors associated with milk processing.

Problems Caused by Biofilms in the Dairy Industry

Biofilm formation can occur in many stages of milk processing, including raw milk transportation and treatment, production of commodity products such as cheese and milk powder, secondary processing such as the production of whey powder, and in the environment. The main problems caused by biofilm formation are poor quality product and reduced run length. However, the formation of pathogenic biofilms can occur in the environment resulting in a product safety issue.

The functional properties of the final product may be decreased, while product losses and milk deposition may increase as a result of biofilm development. Production run times are shortened and the proportion of time expended in cleaning increases as attempts are made to control the fouling and consequent decreased heat transfer coefficients. The buildup of deposits requires an increase in pumping energy to maintain given flow rates.

Raw Milk

Raw milk contains many species of microorganisms that can form biofilms. Poor raw milk quality is believed to be because of biofilm formation that occurs in the tankers during milk transportation as well as on pipelines and other processing surfaces before the milk is treated. In addition, there are other species that seem to prefer surfaces to which other cells have already attached. For example, the growth of *Listeria monocytogenes* on a surface has been shown to be enhanced 10-fold in the presence of a *Flavobacterium* species. Typical species from raw milk forming biofilms on stainless steel milk delivery pipelines and tanker trucks include *Pseudomonas*, *Streptococcus*, and *Bacillus* species. While vegetative cells such as *Pseudomonas* species are generally not a problem in milk powder plants due to the fact that they are readily killed by pasteurization, their occurrence in large numbers can cause concern if they are allowed to increase unchecked. They produce heat-stable enzymes (lipases and proteases) that survive pasteurization and have the potential to spoil the milk and products manufactured from it. Other bacterial species found in raw milk, such as *Streptococcus thermophilus* and *Bacillus* species, are more heat tolerant and can survive pasteurization. These cells can then form biofilms on processing plants downstream of the pasteurizers and their occurrence in large numbers in the final product can result in the generation of off-flavor or off-odors and a reduction in the quality of the final product.

Cheese

Biofilms of non-starter lactic acid bacteria (NSLAB), for example, *Lactobacillus* species, on the surfaces of cheese manufacturing plants have been shown to survive cleaning and provide a source of contamination of cheese. Their presence in high numbers in cheese can indicate poor hygiene in the manufacturing plant and may cause flavor defects and flaws in composition: calcium lactate crystals and gas. Interestingly, not all NSLAB cause defects and some actually provide desirable flavors to certain types of cheese. Additionally, biofilms consisting of a mixture of yeast and bacteria actually assist in the ripening of some traditional French cheeses, and some of these natural biofilms found on ripening shelves have been reported to inhibit pathogenic bacteria.

Bacteriophage of lactic acid bacteria (LAB) are a serious concern in cheese manufacture, as their presence threatens the viability of starter bacteria. The presence of bacteriophage in cheese plants has, in part, been attributed to their ability to survive within biofilms, although this has not been well studied.

Milk Powder

Growth of thermophilic bacilli as biofilms (**Figure 1**) and the consequent contamination of milk is a significant factor limiting the processing run length during the production of milk powder. Optimal conditions for the growth of these microorganisms, mainly *Geobacillus* and *Anoxybacillus* species, are found in the preheating section of milk evaporators. The presence of high numbers of thermophilic bacilli in milk powder is a quality rather than a safety issue. When the milk powder is reconstituted into a consumer product, flavor and compositional defects may arise if the liquid product is stored at temperatures suitable for the growth of thermophiles. Milk powder destined for ultrahigh-temperature (UHT) milk is tested and graded on the number of thermophilic spores it contains, and milk powder for general use is tested and graded on the total number of thermophiles. Understanding the factors influencing the development of biofilms of thermophilic bacilli and the knowledge of how to control them is therefore of key economic importance for milk powder manufacturing plants as they strive to increase run lengths.

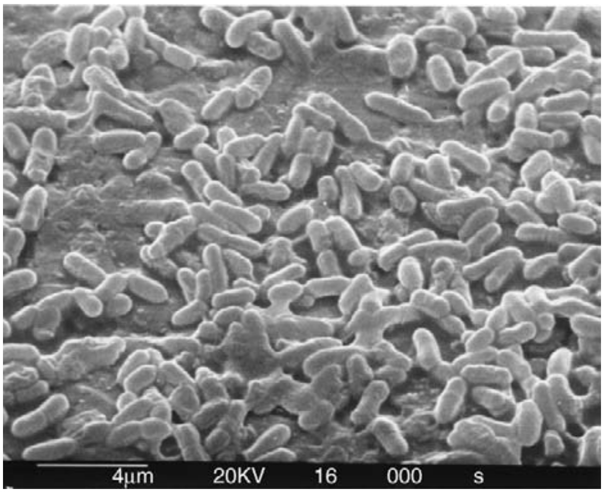

Figure 1 A biofilm of thermophilic bacilli isolated from a dairy manufacturing plant, growing on a stainless steel surface. Source: D. Hopcroft, Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North, New Zealand.

Whey

Maintaining the microbiological quality of whey products is a challenge to manufacturers. Cheese whey originates from a manufacturing process that introduces LAB to the product. Biofilms of thermophilic streptococci can grow in the heat exchangers designed to heat-treat the incoming whey and further contaminate the product stream. Spiral-wound ultrafiltration membranes used to concentrate the whey proteins can also act as a source of contamination, as they provide a large surface area for biofilm development. It has been shown that a variety of thermophilic and spore-forming bacteria can contaminate membrane surfaces, and that these bacteria can survive standard cleaning. It appears that the water used for the diafiltration step may also be a source of contamination, since heat-sensitive bacteria, mostly Gram-negative bacteria, are also found in biofilms on ultrafiltration membranes. As with milk powder, high levels of bacteria in the final whey products decrease their value and under conditions of secondary manufacture may result in spoilage. In addition, the Gram-negative bacteria found on ultrafiltration membranes have the potential to produce enzymes that cause sensory problems with the final product.

Pathogens

Pathogens in the general environment of a dairy manufacturing plant also grow as biofilms that can persist following regular cleaning and act as a potential source of contamination. Conveyor belts are often difficult to clean and provide a source of pathogens close to the product line. Biofilms of pathogens such as *L. monocytogenes* may be harbored in other parts of the general factory environment, in particular drains. These biofilms are potential sources of product contamination through aerosols created during routine cleaning operations. For example, the contamination of infant formula with *Cronobacter sakazakii* is believed to have originated from biofilm growth in the plant environment, resulting in contamination of the product in the final stages of manufacture. The risk to infants is believed to be exacerbated by growth of these microorganisms as biofilms on the nasogastric tubing used to feed premature neonates.

Development of Biofilms in the Dairy Industry

Biofilm development in the dairy industry is similar to biofilm development in other environments (**Figure 2**). An initial adhesion stage occurs where microorganisms become attracted to a surface through complex interactions involving charge, hydrophobicity, and physical interactions. The initial attachment is often mediated by the microbial cell surface proteins, which interact with material already present on the processing surface. Such a buildup of fouling material is frequently termed a conditioning film and rapidly develops on surfaces exposed to product streams. The conditioning layer alters the physicochemical properties of the substrate and can have a large impact on attachment.

The initial attachment step can be a transient association, as the cell may leave the surface and settle elsewhere (reversible stage). If conditions are suitable, bacterial cells will form tenacious bonds with the surface (irreversible stage), associated with the production of extracellular polysaccharides and extracellular DNA. Cell division results in colonization of the surface where nutrients are readily available and when temperature is suitable for growth. Growth occurs rapidly under ideal conditions, with maximum cell numbers reaching $10^6\,\mathrm{cm}^{-2}$ within 12h. Microorganisms enter the product stream from the biofilm, either as a steady release of cells, or through clumps of biofilm sloughing from the surface when the biofilm reaches a mature state. The unpredictable shearing of large clumps of cells results in an uneven distribution of microorganisms in the product stream. The release of microorganisms from the biofilm into the food has been referred to as the biotransfer potential of the biofilm and is key to the contamination of dairy products.

In dairy manufacturing plants, the product normally passes through several processes that limit microbial growth. Heat treatment is a common example of a microbe

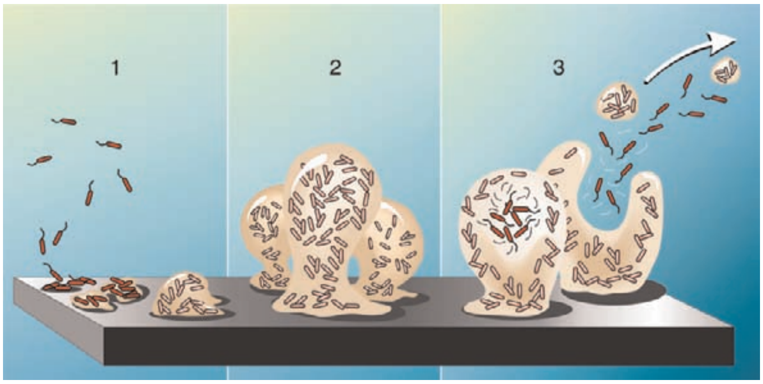

Figure 2 Key stages in the formation of a biofilm. Stage 1, adhesion; stage 2, growth; stage 3, release. The structures represent how a biofilm is believed to form in most environments. Source: P. Dirckx, MSU-Center for Biofilm Engineering.

limiting factor; the stress selects for microorganisms that can survive under different processing conditions. As a result, biofilms in dairy manufacturing plants are often limited to a single dominant species. These biofilms have been termed 'process biofilms' and are quite distinct from most biofilms in the general environment, which consist of multiple species. This has implications when considering controlling biofilms in dairy manufacturing plants, as control measures need to be targeted to specific microbial groups. Typical zones that are prone to biofilm growth are plate heat exchangers, certain sections of evaporators, and ultrafiltration plants, where there are large surface areas exposed to specific conditions that favor the growth of particular microorganisms.

The microorganisms typically found forming biofilms and being released into dairy fluids are heat-tolerant bacteria. Thermophilic or thermotolerant species predominate in the warm zones of dairy manufacturing plants. *S. thermophilus*, *Anoxybacillus flavithermus*, and *Geobacillus* species are the main thermotolerant or thermophilic species commonly associated with many dairy products. Mesophilic thermoduric bacteria, such as *Bacillus* strains, are also a concern, but they have received less attention, probably as they are slower growing than the thermophilic bacteria.

Some bacterial species can produce spores and, while spore production is traditionally associated with adverse limiting conditions, there is some evidence that spore production for dairy *Bacillus* spp. occurs optimally when conditions are favorable for growth. Spores seem to form readily in biofilms, although the mechanisms and conditions promoting spore development and release in a biofilm are unknown. Spores do attach readily to dairy plant surface; so it is likely that they are more important than vegetative forms of the same species in their role in initiating biofilm development.

Biofilms can form on all substrates used in dairy manufacturing. Stainless steel, while being visibly smooth, contains numerous crevices on the microscopic level that protect microorganisms from shear forces during production and cleaning. Cells that are not removed can initiate the regrowth of biofilms. The importance of surface roughness in bacterial attachment is debatable, but it is clear that established biofilms within crevices are difficult to remove.

Flow rate may also affect biofilm growth, although there is little information on dairy biofilms. Films formed under high flow rate tend to be thinner and stronger, while release of bacteria into the flowing medium is believed to be higher under high flow, possibly due to a faster growth rate.

Detection of Biofilms

Confirming a biofilm problem in a dairy manufacturing plant can be difficult. Comparison of the numbers of microorganisms entering and leaving a processing line can provide a good indication of a problem, as the residence time of a product in a processing line is easy to calculate. If the number exceeds what could possibly grow during the residence time in the plant, then it is likely that those numbers resulted from a biofilm.

Where the surfaces of a manufacturing plant can be accessed for inspection, a mature biofilm may be seen on the surface. This may be associated with foulant, making the contamination more clearly visible. Without obvious fouling deposits, biofilms can appear as a discoloration of the surface that may be slimy to touch. Such contamination is important, as a visibly fouled surface will release large numbers of cells into food passing over that surface.

Swabbing to recover viable microorganisms from surfaces is often used to confirm a suspected biofilm. Only a small area of a manufacturing plant can be sampled in this way and it is estimated that only about 10% of the biofilm cells can be recovered; so the results are not representative of the overall contamination level, but merely an indication. The use of swabs to detect ATP provides a much faster indication of contamination than attempting to recover the microorganisms through culture. However, it is no more accurate than culture methods.

In order to study biofilms more accurately, sampling coupons can be inserted into a manufacturing stream. The most commonly used system is a Robbins device, which replaces a section of the pipe in an area where biofilm growth is suspected. Coupons removed from the device can be examined microscopically and the microorganisms making up the biofilm can be effectively recovered through sonication, vortexing or mixing with beads, and subsequent culturing. Impedance measuring systems have also been used to provide a good estimate of the numbers of viable cells colonizing the surface.

Controlling Biofilms

Cleaning and Sanitizing

Control of biofilms in dairy manufacturing plants is generally achieved through cleaning. Unfortunately, standard cleaning systems do not always eliminate biofilms from the surfaces of manufacturing equipment.

A standard clean-in-place (CIP) system used to clean dairy manufacturing plants consists of five stages. The first is a rinse to remove gross residual dairy products. This is followed by an alkaline wash (e.g., 1.5% sodium hydroxide) recirculated through the plant for 60–90 min at temperatures above 70 °C. Much of the remaining dairy fats and proteins (food soils) are removed. The third stage is a rinse to remove the alkaline cleaner and emulsified soil, followed by the fourth stage consisting of an acid wash (e.g., 1% phosphoric or nitric acid) at 55–80 °C for about 30 min. The final stage is a cold water rinse that may include a sanitizer.

The effectiveness of CIP systems is determined by the cleaning time, strength and temperature of cleaning chemicals, and turbulence. The age and nature of the food soil will determine the most appropriate cleaning chemicals and cleaning time. Temperature and chemical strength must be optimized for the type of soil. Excessive temperatures and very high chemical strengths can change the structure of the food soil and make cleaning more difficult. Other factors that affect cleaning are the microtopography of the food contact surface and aspects of the plant design that influence the ability of the cleaning solutions to reach surfaces at the temperature and velocity needed for an effective clean.

Cleaning is designed to remove dairy soils including microbial cells attached to surfaces. The biofilm matrix and product residues protect microbial cells from cleaning chemicals. While the removal of soil is associated with the removal of most microorganisms, any remaining cells that survive the cleaning process may seed the next manufacturing run. Often inadequate cleaning is a result of some failure in the CIP process. Low temperatures, inadequate flow, wrong chemical strength, and reduced cleaning time, once identified, can be easily solved. However, even with a well-designed CIP program, there is evidence that residual microorganisms recolonize manufacturing equipment surfaces. Manufacturing plants that have not been used for several months can often run for longer between cleans than a plant in daily use. This is believed to be due to plant surface drying and inactivation of residual microorganisms.

Sanitizers are sometimes used to inactivate microorganisms remaining on plant surfaces after cleaning. In some cases, sanitizers have been ineffective in reducing bacterial numbers associated with biofilms. This can be explained by protection of the cells within the biofilm matrix, which may inactivate the sanitizer, or use of an inappropriate sanitizer for the microbial contaminant. The bacteria may possess a specific phenotype that confers resistance and the metabolic state of biofilm bacteria may slow the uptake of sanitizer. There are reports of biofilms developed in the presence of milk being more resistant to sanitizers than biofilms produced in other environments, such as meat media or culture media.

A relatively recent, more novel approach to cleaning is the use of pigging. In the oil industry, a bullet-shaped device called a ‘pig’ is forced through pipes by the fluid flow to scour foulant from the walls. This is limited to straight pipes and largely unsuitable for a dairy manufacturing plant. However, the use of a pig consisting of a slug of crushed ice avoids problems with curved pipes and has potential for use particularly in restricted spaces, such as plate heat exchangers.

Incoming Milk Quality

Even with the ideal cleaning system, there are limits to the effectiveness of cleaning in controlling biofilm development. Milk is not a sterile product and bacteria as well as milk components attach rapidly to completely clean surfaces, initiating biofilm development.

Attempts to control biofilms in dairy manufacturing systems have focused on the manufacturing plant. While this may seem a logical approach, biofilms developing in the raw milk during transportation and storage might be responsible for initiating biofilm development in manufacturing plant, either directly through seeding the plant with microorganisms or indirectly through alteration of milk composition by microbial by-products, mediating microbial attachment. Although raw milk quality is regarded as important, the effect of raw milk quality, in particular the effect of biofilms in raw milk systems, on manufacturing run lengths and the final product quality remains speculative.

Plant Surface Modification

While stainless steel is generally accepted as a highly hygienic and easily cleaned material for construction of the manufacturing plant, biofilm colonization of stainless steel suggests that there is potential to improve surfaces to resist microbial attachment. Modifications to stainless steel, including hydrophobicity and charge adjustment through metal ion impregnation and surface coatings, have been reported with variable success in defending against biofilm development. This is largely due to the highly complex process of microbial adhesion, the variation between different microorganisms, and the condition of microbial cells (spores, vegetative, young, old). As soon as a biofilm gains a foothold, the characteristics of the surface are changed by the production of extracellular polymeric material, possibly negating the treatment. However, surface modifications are believed to have potential in controlling biofilm development.

There are some surface modifications that have shown potential in preventing bacterial attachment to surfaces. These include Ni–Cu–P–PTFE-coated surfaces that reduce calcium phosphate and protein binding. As microbial attachment is often mediated by protein on the cell surface or a conditioning film from the environment on the substrate, this coating is likely to have some success in slowing the initiation of biofilms. Molecular brushes produced with polyethylene glycol use steric hindrance to block bacterial attachment and have shown some success. However, these are neither permanent nor desirable in a food system. Toxic chemicals used to coat nonfood surfaces have been successful in preventing biofilm formation on surfaces such as ship hulls. Many of these are unsuitable for food systems. Incorporating silver ions into or onto surfaces has potential in controlling biofilms and if cost effective could be considered in select parts of dairy manufacturing plants. Bacteriocins, such as nisin, have shown potential in preventing microbial colonization of surfaces, but are effective only for a limited time. Antimicrobial enzymes, such as lysozyme, are highly effective in preventing the attachment of viable bacteria to surfaces. Unfortunately, the cost of such treatments, interference from dairy components, their limited life and heat sensitivity, and regulatory concerns with the source of these enzymes (e.g., eggs) limit opportunities for their use in dairy manufacturing plants. Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), also known as Irgasan, is an antimicrobial used in toothpastes and soaps and incorporated in some general food manufacturing environment surfaces. It is active against a limited range of Gram-negative bacteria, but is prohibited in food and therefore cannot be used on surfaces that come into contact with food. Although the antimicrobial surfaces that have been developed have limitations, developing this concept further is worth pursuing.

Disruptive Technologies

Finally, altering the running conditions in dairy manufacturing plants to limit biofilm growth is, in theory, a simple engineering solution to a biological problem. Termed ‘disruptive technologies’, the simplest example is the manipulation of temperature to disrupt microbial growth in a specific area of a dairy plant. This has been used to limit the growth of *S. thermophilus* biofilms in plate heat exchanger pasteurizers. In order to develop this concept fully, the microbial contaminants need to be fully understood and, ideally, modeled to optimize the disruptive element (e.g., temperature or flow) in terms of degree, length, and frequency of change in conditions needed to prevent biofilm growth.

Future Developments

The dairy industry is likely to continue to support initiatives that aim ultimately at controlling biofilm development, improving product quality, and increasing the operational efficiency of dairy manufacturing plants. Future developments will be in understanding the genetic basis of biofilm development, the process of sporulation in a biofilm, the effect of microbial interactions (e.g., quorum sensing), the effect of biofilm architecture on biofilm development, and modeling of biofilm growth under different conditions during dairy manufacture.

See also: **Liquid Milk Products:** Pasturizatuion of Liquid Milk Products: Principles, Public Health Aspects. **Milking and Handling of Raw Milk**: Milking Hygiene. **Milk Protein Products**: Membrane-Based Fractionation. **Plant and Equipment:** Pasteurizers, Design and Operation.

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BIOGENIC AMINES

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Introduction

Biogenic amines are low-molecular-weight organic bases that possess biological activity. They consist of an aliphatic, aromatic, or heterocyclic structure to which one or more amino groups are attached. The number of reactive amino groups determines whether the amine is a monoamine, a diamine, or a polyamine (**Table 1**). Monoamines and diamines (tyramine, phenylethylamine, histamine, tryptamine, cadaverine, and putrescine) are formed either as the result of endogenous amino acid decarboxylase activity in raw food materials or by the growth of decarboxylase-positive microorganisms under conditions favorable to enzyme activity. Polyamines (spermidine and spermine) can also be formed by 'deureation', an alternative metabolic pathway consisting in the incorporation of aminopropyl groups originating from methionine. Owing to their origin and specific physiological role, polyamines are now included in a distinct group. Putrescine shares characteristics of both biogenic amines and polyamines. Diamines such as putrescine and cadaverine can react with nitrite to form carcinogenic nitrosamines. Polyamines and diamines may be converted into stable carcinogenic *N*-nitroso compounds and enhance the growth of chemically induced aberrant crypt foci in the intestine. For this reason, it is recommended that their accumulation be prevented in cured and fermented food products.

The main biogenic amines in cheese, and in most foods, are tyramine and histamine. Tyramine is a potent vasoconstrictor, primarily responsible for hypertension. Its effect on healthy individuals is usually limited to headache or migraine. Histamine is also a vasoactive substance, which may cause urticaria, hypotension, headache, flushing, and abdominal cramps. The detoxification mechanism for tyramine and histamine in the human organism consists in the oxidative deamination catalyzed by monoamine oxidase (MAO). In patients lacking normal amino oxidase activity, because of treatment with MAO inhibitors (antiparkinsonian drugs and antidepressants), the detoxification mechanism is no longer active. MAO inhibitors prevent the oxidative catabolism of amines, thus maintaining high levels of neurotransmitters such as dopamine, serotonin, tryptamine, and phenylethylamine. As MAO inhibitors do not discriminate between the different MAOs, they also maintain high concentrations of tyramine, which may cause hypertensive crises leading to brain hemorrhage and heart failure.

There is a large variation in the sensitivity of individuals to biogenic amines. Levels above 500 mg kg^{-1} histamine and 100 mg kg^{-1} tyramine are considered potentially dangerous for human health, but cheeses with lower histamine contents have also been involved in outbreaks. A tyramine intake of only 6 mg is sufficient to cause negative health effects in susceptible individuals or in those being administered MAO inhibitors. The effect of potentiating factors such as alcohol consumption must also be taken into account. For this reason, acceptable levels of biogenic amines have not been established for most foods, although an official upper limit of 100 mg kg^{-1} histamine has been set for certain fish and fish products.

Biogenic Amine Producers and Degraders in Cheese

During cheese manufacture and ripening a wide range of biochemical changes take place, affecting milk proteins, lipids, and carbohydrates. Primary proteolysis results in peptide formation, and secondary proteolysis in the accumulation of free amino acids. Thereafter, the biogenic amines tyramine, phenylethylamine, histamine, tryptamine, cadaverine, and putrescine may be formed, respectively through microbial decarboxylation of tyrosine, phenylalanine, histidine, tryptophan, lysine, and ornithine or arginine via agmatine.

The prerequisites for the formation of biogenic amines are availability of free amino acids, presence of decarboxylase-positive microorganisms, and environmental conditions allowing decarboxylase activity. Contaminating microbiota is generally responsible for biogenic amine formation in cheese. Traditionally, heterofermentative lactobacilli have been considered as the main histamine formers in cheese, and enterococci as the main tyramine formers, but other lactic acid bacteria (LAB) and some Gram-negative bacteria may also be involved in biogenic amine formation.

In a study on 129 strains of enterococci, mostly isolated from dairy products, 124 produced tyramine from tyrosine, though none of them decarboxylated histidine, lysine, or ornithine. During a survey on the tyramine- and histamine-forming microorganisms present in 20

Table 1 Characteristics of the main biogenic amines and polyamines

Amine	*IUPAC name*	*Molecular formula*	*Molar mass*	*Classification*
Tyramine	4-(2-Aminoethyl)phenol	$C_8H_{11}NO$	137.18	Aromatic, monoamine
Phenylethylamine	Phenylethan-2-amine	$C_8H_{11}N$	121.18	Aromatic, monoamine
Histamine	2-(1H-Imidazol-4-yl)ethanamine	$C_5H_9N_3$	111.15	Heterocyclic, monoamine
Tryptamine	2-(1H-Indol-3-yl)ethanamine	$C_{10}H_{12}N_2$	160.22	Heterocyclic, monoamine
Cadaverine	Pentane-1,5-diamine	$C_5H_{14}N_2$	102.18	Aliphatic, diamine
Putrescine	Butane-1,4-diamine	$C_4H_{12}N_2$	88.15	Aliphatic, diamine
Spermidine	*N*-(3-Aminopropyl)butane-1,4-diamine	$C_7H_{19}N_3$	145.25	Aliphatic, polyamine
Spermine	*N*-*N*′-Bis(3-aminopropyl)butane-1, 4-diamine	$C_{10}H_{26}N_4$	202.34	Aliphatic, polyamine

Spanish cheese varieties, 117 Gram-positive isolates, out of which 92 were enterococci, formed tyramine, whereas histamine was produced only by 10 Gram-positive isolates, out of which 3 were lactobacilli and 6 were lactococci. Within the Gram-positive microbiota of Pecorino Abruzzese cheese, lactobacilli decarboxylated tyrosine, phenylalanine, and ornithine (9, 1, and 1 positive isolates, respectively, out of 42), enterococci also decarboxylated the same amino acids (15, 5, and 2 positive isolates, respectively, out of 25), lactococci decarboxylated only tyrosine (3 positive isolates out of 11), and none of the isolates was able to decarboxylate histidine. In a study on the microbiota of Montasio cheese, 151 out of 1237 LAB isolates decarboxylated tyrosine and only 13 decarboxylated histidine, with *Streptococcus thermophilus* followed by enterococci and leuconostocs being the prevailing biogenic amine formers. Tyramine production of up to 400 mg l^{-1} by *Lactococcus lactis* strains, and up to 800 mg l^{-1} by *Leuconostoc mesenteroides* strains, in a tyrosine-supplemented culture medium has been observed. Decarboxylation of tyrosine and tryptophan, but not of phenylalanine, by some *L. lactis* strains has also been reported.

Regarding Gram-negative bacteria, only 2 isolates obtained during the survey on biogenic amine formers present in Spanish cheeses produced tyramine, whereas histamine was formed by 43 isolates. All 104 *Enterobacteriaceae* isolates from an Italian blue-veined cheese were able to decarboxylate at least 2 amino acids, with 104 being able to form cadaverine; 100 putrescine; 71 tyramine; and 60 histamine. *Enterobacteriaceae* from Pecorino Abruzzese cheese decarboxylated lysine, ornithine, phenylalanine, tyrosine, and tryptophan (66, 33, 8, 3, and 1 positive isolates, respectively, out of 80), but they were not able to decarboxylate histidine. Out of 200 *Enterobacteriaceae* isolates from Montasio cheese, 169 degraded ornithine and 134 lysine.

Some foodborne microorganisms may degrade biogenic amines in buffer systems, mostly through the activity of amine oxidases under aerobic conditions. Thus, 25 out of 64 LAB strains degraded histamine and 1 tyramine; 12 out of 32 *Brevibacterium linens* strains degraded histamine and 16 tyramine; 12 out of 44 *Micrococcus* sp. strains degraded histamine and 15 tyramine; whereas none of the 20 *Staphylococcus carnosus* strains degraded histamine or tyramine. Histamine and tyramine degradation by two *B. linens* strains in a buffer system and during Munster cheese ripening has been demonstrated. Most yeast isolates from raw goat milk Armada cheese, belonging to the species *Geotrichum candidum*; *Candida lambica*, *Candida krusei*, *Candida lipolytica*, other *Candida* spp.; *Kluyveromyces lactis*; *Kluyveromyces marxianus*; *Saccharomyces unisporus*; and *Trichosporon beigelii*, were capable of histamine and tyramine assimilation and might play an important role in the degradation of biogenic amines during ripening. However, decarboxylation of some amino acids by yeasts isolated from Pecorino Crotonese cheese, in particular by *Debaryomyces hansenii* and *Yarrowia lipolytica* strains, has also been reported.

Factors Influencing the Formation of Biogenic Amines in Cheese

Milk Treatment

Pasteurization scarcely influences biogenic amine formation through its direct effect on milk components and enzymes (**Table 2**). This was demonstrated when Gouda cheese made from raw aseptically drawn milk inoculated with a *Lactobacillus buchneri* histamine-producing strain was compared with cheese made from the same aseptically drawn milk after pasteurization, the former cheese showing a 25% higher mean histamine content after a 12-month ripening period.

However, pasteurization of milk obtained under standard milking conditions affects biogenic amine formation by reducing the population of biogenic amine-forming microorganisms, which are not

Table 2 The effect of some cheese manufacturing and ripening factors on biogenic amine formation

Factor	*Effect*	*Mechanism*
Milk pasteurization	Decrease	Elimination of decarboxylating bacteria
Milk thermization	Increase	Unclear
Milk bactofugation	Decrease	Elimination of decarboxylating bacteria
Milk high-pressure homogenization	Decrease	Elimination of decarboxylating bacteria
Milk pressurization	Increase	Higher free amino acid content in cheese
Starter culture	Variable	Effect on proteolysis and pH, inhibition of decarboxylating bacteria
Proteolytic enzymes	Increase	Increased level of precursor amino acids
High ripening temperature	Increase	Increased level of precursor amino acids, increased activity of decarboxylases
Ripening time	Increase	Increased level of precursor amino acids
Cheese pressurization	Increase	Increased level of precursor amino acids

particularly thermoresistant. Idiazábal cheeses made from raw ewe's milk contained after a 6-month ripening period up to 3.5 times higher tyramine levels, and slightly higher histamine concentrations, than in cheeses made from pasteurized milk. Semisoft cheeses made from raw milk showed after ripening for 5 months 28.3-, 1.8-, 2.6-, 5.8-, and 1.8-fold higher concentrations of histamine, tyramine, cadaverine, putrescine, and phenylethylamine, respectively, than pasteurized milk cheeses made using the same starter culture. These results are ascribed to the lower bacterial load of pasteurized milk. Cheeses made from raw goat milk contained on day 90 of ripening 29.8-, 6.8-, 3.1-, 1.6-, 6.0-, and 5.9-fold higher levels of tyramine, histamine, phenylethylamine, tryptamine, cadaverine, and putrescine, respectively, than the cheeses made from pasteurized milk. During ripening of Manchego cheeses made from pasteurized milk, biogenic amines remained below detection level, whereas in 6-month-old raw milk cheeses histamine, tyramine, and tryptamine concentrations averaged 125.8, 466.5, and 4.8 mg kg^{-1}, respectively.

Heating of 3% fat milk up to 70°C increased total biogenic amines in Ras cheese, and further heating up to 75 and 80°C decreased them, whereas in the case of 2% fat milk heating up to 80°C was needed to reduce the content of total biogenic amines in cheese. Similarly, a 25% increase in the content of total biogenic amines in 4-month-old Montasio cheese caused by milk thermization at 67°C has been reported.

Nonthermal treatments of raw milk such as bactofugation, pressurization, and high-pressure homogenization (HPH) considerably reduce its bacterial load, including biogenic amine-forming bacteria. The use of bactofugated milk for the production of Emmental cheese resulted in ~24 times less putrescine and 7 times less cadaverine with respect to raw milk cheese, although it did not influence histamine and tyramine formation. When goat cheese was made from milk pressurized at 500 MPa for 15 min at 20°C, total biogenic amines increased in comparison with pasteurized milk cheese, by 48% on day 30 and by 16% on day 45, a result that was ascribed to the higher total free amino acid content of the former cheese. HPH of milk reduced the counts of yeasts, lactobacilli, and *Micrococcaceae*, but not of enterococci, in Caciotta cheese with respect to cheese made from raw or pasteurized milk, a fact that resulted in lower levels of putrescine, cadaverine, and histamine, but not of tyramine. In the same work, the authors reported higher yeast counts, and lower enterococci counts, in Pecorino cheese made from HPH milk, which showed lower concentrations of putrescine, cadaverine, and tyramine than those present in cheese made from raw or pasteurized milk.

Initial Level of Biogenic Amine Producers and Presence of Inhibitory Bacteria

In a Gouda cheesemaking experiment carried out adding different *Lb. buchneri* St2A inocula (0.01, 0.2, and 5 cfu ml^{-1}) to milk, it was observed that higher inocula resulted in higher populations of the biogenic amine producer in 3-month-old cheese (3×10^5, 6×10^6, and 1×10^8 cfu g^{-1}, respectively). Control cheese from noninoculated milk did not contain histamine (<10 mg kg^{-1}) after 3 months of ripening, whereas experimental cheeses contained increasing histamine concentrations (35, 410, and 1060 mg kg^{-1}), which were related to the inoculum size but not directly proportional to it.

Complete inhibition of histamine formation in cheese by *Lb. buchneri* St2A occurred when LAB producing bacteriocins inhibitory to the biogenic amine producer were added to milk, even though histidine was present at concentrations of 167–475 mg kg^{-1}. One of the bacteriocin producers used was a mutant of *Enterococcus faecalis* lacking tyrosine decarboxylase, selected to circumvent tyramine production when used as adjunct culture.

Type and Level of Starter Culture

The type of starter culture may influence biogenic amine formation because of its effect on the accumulation of free amino acids in cheese, the pH value of cheese, and the inhibition of biogenic amine formers by bacteriocins, as seen above, or by other mechanisms. Histamine concentration in 12-month-old Gouda cheeses made from milk inoculated with *Lb. buchneri* St2A using six different mesophilic starters was in the range 500–667 mg kg^{-1}, independent of the starter culture used, but addition of a proteolytic *E. faecalis* adjunct culture resulted in a higher pH value (5.31 vs. 5.18 after 3 months) and a histamine concentration of 1800 mg kg^{-1} in 12-month-old cheese.

Semisoft cheeses made from raw milk using a commercial lyophilized culture composed of only lactococci had 2.5- to 3.8-fold higher concentrations of histamine, tyramine, cadaverine, putrescine, and phenylethylamine than the cheeses made using a whey culture composed of *S. thermophilus*, *Lactobacillus delbrueckii*, and lactococci, even though enterococci counts were 6.6 log cfu g^{-1} in the former cheese and only 2 log cfu g^{-1} in the latter. Idiazábal cheeses made from raw ewe's milk using a commercial starter composed of lactococcal strains contained higher levels of tyramine and putrescine, and lower levels of histamine, cadaverine, spermidine, tryptamine, and phenylethylamine, than if an indigenous starter composed of lactococci and *Lactobacillus casei* was used. Total biogenic amines in 4-month-old Montasio cheese made from thermized milk reached 207 mg kg^{-1} when only a natural milk culture was used, but were lowered to 30 mg kg^{-1} by the combination of the natural milk culture with a commercial starter culture. The use of a starter culture composed of lactococci, leuconostocs, *S. thermophilus*, and *Lactobacillus helveticus* in the manufacture of Dutch-type cheese resulted in a higher total biogenic amine content than if a starter culture composed only of lactococci was used, independent of the manufacturer and the cheese fat content, whereas the polyamine concentration was not affected.

The level of starter culture (0.1 or 1.0%) used in the manufacture of raw ewe's milk Manchego cheese influenced biogenic amine concentration in 3-month-old cheeses, with 24% more tyramine and 20% more histamine found in cheese made with 1.0% starter, even though counts of decarboxylase-positive bacteria did not vary. The results were ascribed by the authors to the slightly higher pH of this cheese, which might have favored both the formation of free amino acids and the decarboxylation reactions. A lower biogenic amine formation was recorded in raw cow's milk Hispánico cheese, not influenced by the level of starter culture, probably because of the low counts of decarboxylase-positive bacteria.

Addition of Proteolytic Enzymes

If proteolytic enzymes are added to milk or curd to accelerate cheese ripening, the enhanced formation of free amino acids is expected to increase the biogenic amine content of cheese. However, a proteolytic enzyme added to milk in the manufacture of pasteurized milk Gouda cheese did not by itself increase the formation of biogenic amines, which only occurred when decarboxylating *Lactobacillus* strains were inoculated into cheese milk.

Addition of a *Bacillus subtilis* neutral proteinase to milk increased by itself the formation of tyramine and histamine during ripening of ewe's milk Manchego cheese and cow's milk Hispánico cheese. However, the authors reported a lower formation of both amines when a *Micrococcus* sp. cysteine proteinase was added to milk, a result that was ascribed to a possible contamination of the enzyme preparation by amine oxidase.

Ripening Conditions

As ripening temperature affects cheese proteolysis, higher free amino acid concentrations are to be expected in cheeses held at higher temperatures. Gouda cheeses made from milk inoculated with *Lb. buchneri* St2A and ripened for 12 months at 9, 14, 18, and 21 °C contained 245, 378, 622, and 756 mg kg^{-1} histamine, respectively. Even short periods of storage at higher-than-normal temperatures may enhance the formation of biogenic amines, as shown for 1-month-old Azeitão cheese, which had a total biogenic amine content of 838 and 1078 mg kg^{-1} after further storage for 48 h at 4 and 25 °C, respectively.

The concentration of biogenic amines usually increases with cheese age. In the Gouda cheese experiment described above, histidine concentrations increased from month 3 to month 12 by factors of 4.4, 4.9, 5.6, and 4.3 at ripening temperatures of 9, 14, 18, and 21 °C, respectively. A more pronounced accumulation of tyramine in the edge than in the core during ripening of Dutch-type cheese, with linear regression slopes of 1.19 and 0.19 mg kg^{-1} per day, respectively, has been reported. In some cheese varieties, the increase of total biogenic amine concentration with age is far from linear, depending on manufacturing and ripening procedures. Concentration of total biogenic amines in 3-day-old Feta cheese were 21 mg kg^{-1}, and increased to 273 mg kg^{-1} on day 15, and to 330, 501, and 617 mg kg^{-1} after 2, 3, and 4 months of ripening, respectively.

A decline in the levels of some biogenic amines during ripening may occur, as reported for tryptamine, phenylethylamine, and histamine in Idiazábal cheese. These biogenic amines were no longer detected after day 30, whereas cadaverine level remained fairly constant, and tyramine and putrescine levels increased by factors of 8.2 and 1.9, respectively, from day 30 to day 180.

High-Pressure Treatment of Cheese

Application of high-pressure treatment to cheese causes bacterial lysis and release of intracellular enzymes, which may result in an enhancement of proteolysis and thereafter in an increase in the biogenic amine concentration. When goat cheese was pressurized at 400 MPa for 5 min, 50 MPa for 72 h, or 400 MPa for 5 min + 50 MPa for 72 h, in all cases at 14 °C, and ripened at this temperature for 28 days, tyramine and histamine increased 2.8- and 2.9-fold in the 50 MPa cheese with respect to the control untreated cheese, whereas they declined in the other cheeses. Putrescine, cadaverine, spermidine, and spermine increased 22.7-, 7.0-, 1.2-, and 3.9-fold, respectively, in the 50 MPa cheese, and 14.0-, 4.3-, 1.4-, and 2.8-fold, respectively, in the 400 + 50 MPa cheese. The increases in biogenic amines were considerably higher than the increases in free amino acids or in nonprotein nitrogen caused by high-pressure treatments.

Detection and Quantification of Biogenic Amines and Biogenic Amine-Forming Microorganisms

The interest in the determination of biogenic amines in food is mainly on account of their potential toxicity, but biogenic amines can also be considered as chemical indicators of the hygienic conditions of the raw material, manufacturing practices, and, in general, food quality. Cheese is a complex matrix with high contents of fat, protein, peptides, free amino acids, and inorganic cations that render biogenic amine determination more difficult than in other matrices. The analytical efficiency may be affected by the high variability in the biogenic amine concentration owing to the cheese type and origin, the manufacturing and ripening conditions, and the microbiota and enzymatic activities present in cheese.

Extraction of biogenic amines from food matrices is a crucial step in analytical procedures. Different solvents and solutions (hydrochloric, trichloroacetic, perchloric, sulfosalicylic, and acetic acids, borate buffer, methanol, and ethanol) have been used in the extraction of biogenic amines from cheese. Analytical techniques used for the separation and quantification of biogenic amines in cheese are mainly chromatographic methods that include thin-layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE), and high-performance liquid chromatography (HPLC). Determination of biogenic amine content by these techniques requires sample pretreatment and relatively long analysis times. Their application in foods has been reviewed.

Numerous HPLC techniques have been developed to separate and quantify biogenic amines. Most methods require derivatization of amines before detection by UV–VIS absorption or fluorescence. Different chemical reagents have been used for derivatization, mainly dansyl chloride, dabsyl chloride, and *o*-phthaldehyde (OPA) with precolumn, on-column, or postcolumn derivatization. The recently developed ultra-HPLC (U-HPLC) methods increase speed, resolution, and sensitivity by using sub-2 μm particles, shorter columns, and higher flow rates. A rapid method of U-HPLC coupled with an online OPA postcolumn derivatization and fluorescence detection allowed the determination of 12 biogenic amines in cheese in less than 7 min of chromatographic elution. In different varieties of cheeses, U-HPLC with precolumn derivatization with 6-aminoquinolyl-*N*-hydroxy-succinimydil carbamate (AQC) separated 20 primary and secondary biogenic amines within 9 min, whereas 24 min was necessary for HPLC separation. The authors reported limits of detection from 0.4 to 16.2 mg kg^{-1}, and limits of quantification between 1.6 and 60.9 mg kg^{-1}. A fast nuclear magnetic resonance method has also been developed, with a limit of detection of 0.6–1 mg kg^{-1} for histamine.

Biogenic amine production by bacteria is strain dependent. The detection of bacterial strains possessing amino acid decarboxylase activity is a useful tool to estimate the risk of biogenic amine production and to prevent biogenic amine accumulation in foods. Detection of biogenic amine-producing bacteria by culture techniques on differential culture media including the precursor amino acid of biogenic amines and a pH indicator has been extensively used. Identification and quantification of biogenic amines in bacterial cultures have been accomplished by HPLC and TLC. Cultural assays require 2–3 days to complete, and sometimes yield unreliable results. False-positive reactions owing to the formation of other alkaline compounds, and false-negative reactions as a result of the fermentative activity of some bacteria, have been described. Also, loss of decarboxylating activity by cultures after prolonged storage or cultivation may occur.

Molecular methods for the early and rapid detection of biogenic amine-producing bacteria are becoming an alternative to traditional culture methods. PCR methods targeting the genes encoding for the decarboxylating enzymes are fast and sensitive, and allow the identification of biogenic amine-producing bacteria in food before the compound is produced. Multiplex PCR assays have been developed for the detection of potential producers of histamine, tyramine, putrescine, and cadaverine by a wide range of Gram-positive and Gram-negative bacteria, achieving the simultaneous detection of several genes encoding amino acid decarboxylases. An overview of the molecular methods proposed for the detection of biogenic amine-producing bacteria has been published.

A method for the direct detection and quantification of potential histamine-producing LAB in milk and cheese by real-time quantitative PCR has been proposed. The authors reported a good correlation between the presence of tyramine-producing bacteria determined by PCR and tyramine concentration in cheese determined by HPLC. Molecular tools allow early and rapid detection of potential biogenic amine-producing bacteria in food before the amine is produced in cheese, and are also of interest in the selection and evaluation of decarboxylase-negative strains of LAB for use as starter cultures in cheese industry.

See also: **Bacteria, Beneficial**: *Brevibacterium Linens, Brevibacterium Aurantiacum* and Other Smear Microorganisms; Lactic Acid Bacteria: An Overview. **Cheese**: Biochemistry of Cheese Ripening; Non-Starter Lactic Acid Bacteria.

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BODY CONDITION

Contents

Measurement Techniques and Data Processing

Effects on Health, Milk Production, and Reproduction

Measurement Techniques and Data Processing

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Introduction

Mammals have evolved a system of storing energy as fat, primarily triacylglycerols, in order to sustain energy needs during periods of feed scarcity or increased energy need. No other animals have such a highly evolved and regulated system of energy storage, although birds and fishes do store energy as fat and adapt to seasonal food supply. This adaptation is likely because most mammals evolved at the mercy of seasonal variations in food supply. However, when nursing young, mammals have a tremendously increased energy requirement for milk production. This adaptation has allowed tremendous ability to produce milk, for which humans have selected many animals, including dairy cattle. For food-producing animals (milk production and litter production), we now routinely measure and manage the body fat of the animal in addition to feed intake and milk production. The importance of properly managing body fat and amino acids has led to the development of a system of monitoring body fat and to a lesser extent, muscle, now known as 'body condition score'. Body condition is a general term used to describe 'fleshiness' or outward signs of fat and muscle in many species used in food production as well as companionship, pleasure, or work. Today, it is used widely in the health and nutritional management of dairy cattle.

Importance of Body Fat in Dairy Cattle

Optimal metabolism of lipid and amino acids in dairy cattle, as in other mammals, is essential to overall health, production, efficiency, and longevity. Animals such as whales and seals give birth to a calf, which doubles its weight in 4–6 months, while the mothers eat very little or none at all. The milk fat and indeed the energy requirement of the mother are supplied from the mother's adipose tissue. In rodents, a litter can increase in total body weight over 10 times while the dam loses almost all of its stored body fat, even though feed intake increases 300–400%. The dairy cow also undergoes periods of tremendous metabolic demand, and proper management of body fat is key to the overall efficiency of these animals.

The dairy cow births a calf and starts to milk at rates several fold greater than the calf requires. Dairy cattle can produce more than 45 kg (100 lbs) of milk per day (in many cases much more), or over 120 megajoules (MJ) of energy per day within a week after calving and sustain these rates for months. The adaptation in feed intake is impressive, often increasing 2–3-fold in 2 weeks. However, the increase in nutrient intake in most dairy cattle is usually slower than the increase in milk production. The deficit in energy and in fatty acids for milk fat is overcome by the adipose tissue. The amount and use of body fat is a function not only of diet, but also of milk production rates and environmental variables such as temperature. In addition, body fatness is a genetic trait with a high heritability. The use of body fat can affect the overall efficiency of the cow, including reproductive fertility and resistance to disease. The integration of genetic selection, dietary management, environment, and health and reproduction is fundamentally related to management of body fat as measured by body condition.

An average cow consuming a well-balanced ration may lose over 25 kg of body fat in about 6 weeks, and many will lose 50–75 kg. Then, she can restore this energy reserve during the next 3–5 months, even while still secreting more than 50 MJ of milk energy a day. Because most dairy cattle require significant fat stores at the start of lactation, and also because too rapid fat loss can cause or lead to several metabolic diseases, it is important to have a useful system to estimate changes in body fat. The body condition scoring system to estimate body fat was developed to have a quantifiable measure of energy reserves in the cow. Although changes in body fat in adult cattle makes up a large portion of changes in body weight, monitoring of body weight alone has proven inadequate to monitor and manage body fat. Thus, more specific systems to estimate body fat have been actively developed and tested over the years.

Body condition score has been of scientific interest since the late 1800s' assess 'external' measures of body size and composition. There have been many different measurements on animals such as body weight, height at the withers or rump, length, girth, and even surface area. Many of these basic traits of animal growth (weight, height, width) were and still are used in breeding systems for beef cattle. These measures have utility, and improvement in efficiency and understanding of animal growth has resulted from relating these measures to more specific ones such as body composition, feed efficiency, and meat and milk production. However, none of these measures or combinations of them has fully been able to capture the essence of changes in body fatness in dairy cattle.

It is difficult to pinpoint the first use of the term 'body condition'; however a quote from Dr. W. D. Hoard, a founder of modern dairy science, in 1885 reads: "...a cow always gives the best milk, and is in the best condition, when she is feeding on succulent, juicy food." The phrase 'body condition' has been in use for at least several decades to describe the general physical strength, endurance, or composition of animals and humans.

Methods of Body Condition Scoring

Development of the Body Condition Scoring System

Systematic approaches to monitor body fat have been used for several decades, and in the past three decades, major efforts have quantified and compared body condition across a range of nutritional, genetic, physiological, and environmental situations. The high cost and technical demands of direct determination of body fat and protein led to the development of several indirect methods. One method is the assessment of the amount of body water, which is negatively related to the amount of body fat. This method was used in research trials, but is fairly expensive and intensive in use of equipment, materials, and time. Ultrasound images may also be used to measure thickness of subcutaneous fat depots and this technique is used more often in meat-type animals. However, the anatomy and dynamic nature of the subcutaneous body fat in lactating cattle have fairly well precluded useful application of this technology. Therefore, the primary practical methodology for indirectly assessing body fat content, and to a lesser extent, body protein content, is use of a body condition score system.

There have been a few different systems of body condition scoring developed and tested, although all have the basic purpose of estimating and comparing body fatness. All the systems are based on visual and/or tactile evaluation of body fatness or fleshiness over the ribs, spine, and hindquarters of the animal. All use an 'average' value that tries to capture the characteristics of a cow that has sufficient body fat to respond to potential energy shortages but not so much to cause potential problems with calving, or that would negatively affect feed intake or increase the potential for metabolic diseases. Some systems have used a scoring value from 1 to 9 or 10, with average being 4 or 5; other systems use a 1–5 point scale, with 3 being average. Variations on the latter system have included 'half points', 1, 1.5, 2 ... 4, 4.5, 5, for example. While there are advantages and disadvantages to either approach, in all cases the actual characteristics of the animal to which the number is applied must be agreed upon. The 1–9 point scale tends to be used in the beef and in the horse, while the 1–5 scale has become the basic standard for dairy cattle in most situations and in research.

Use of the Body Condition Scoring System

The 5-point system

Most scientists and practitioners would now agree that the system in common usage, for the field or for research, is the 5-point system, with usually gradations of 1/2 points, and has been developed simultaneously in the United States, Europe, Australia, and New Zealand. The system (**Table 1**) would show that a value of 3 indicates an amount of body fat that was neither too little nor too excessive, based on a generally agreed-upon standard. A body condition score of 1 would indicate 'emaciated'; 3 is an average, while a score of 5 would be 'obese'. An example of visual descriptions is given in **Figure 1**. The basic premise of using a visual and tactile system to assess mass or percentage of body fat is that the measurements are taken independent of frame size, milk production, health, or other factors. The use of such a system can then be applied to identify strengths and weaknesses of nutritional and feeding management, of genetic selection schemes, and for management of individual animals.

Table 1 A 5-point body condition score system for dairy cattle

	Approximate		
	Body fat (600 kg cow)		*Descriptions*
Body condition score	*(kg)*	*(%)*	
1 Emaciated	25–45	5–10	Individual bones prominent; sharp look and feel to bones; gaunt; deep depressions between vertebrae and between pelvic bones
2 Below average	65–80	10–15	Individual bones evident, sharpness, prominent depressions between vertebral and pelvic bones
3 Average	105–120	18–22	Smoother ridges, individual bones not prominent, vertebral processes just evident, moderate flesh covering
4 Above average	140–160	25–30	Flat over vertebrae; smooth, rounded edges over bones; no depressions between bones of vertebrae or pelvis
5 Obese	180–210 or more	30 plus	Vertebrae buried in fat; rounded over back; bulging fat deposits, rounded appearance over pelvis and tailhead

Adapted from Waltner SS, McNamara JP, Hillers JK, and Brown DL (1994) Validation of indirect measures of body fat in lactating dairy cattle. *Journal of Dairy Science* 77: 2570–2578. Edmonson AJ, Lean IJ, Weaver LD, Farver T, and Webster G (1989) A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* 72: 68–78. Wildman EE, Jones GM, Wegner PE, Boman RL, Trout HF, Jr., and Lesch TN (1982) A dairy cow body condition scoring system and its relationship to selected production characteristics. *Journal of Dairy Science* 65: 495–501.

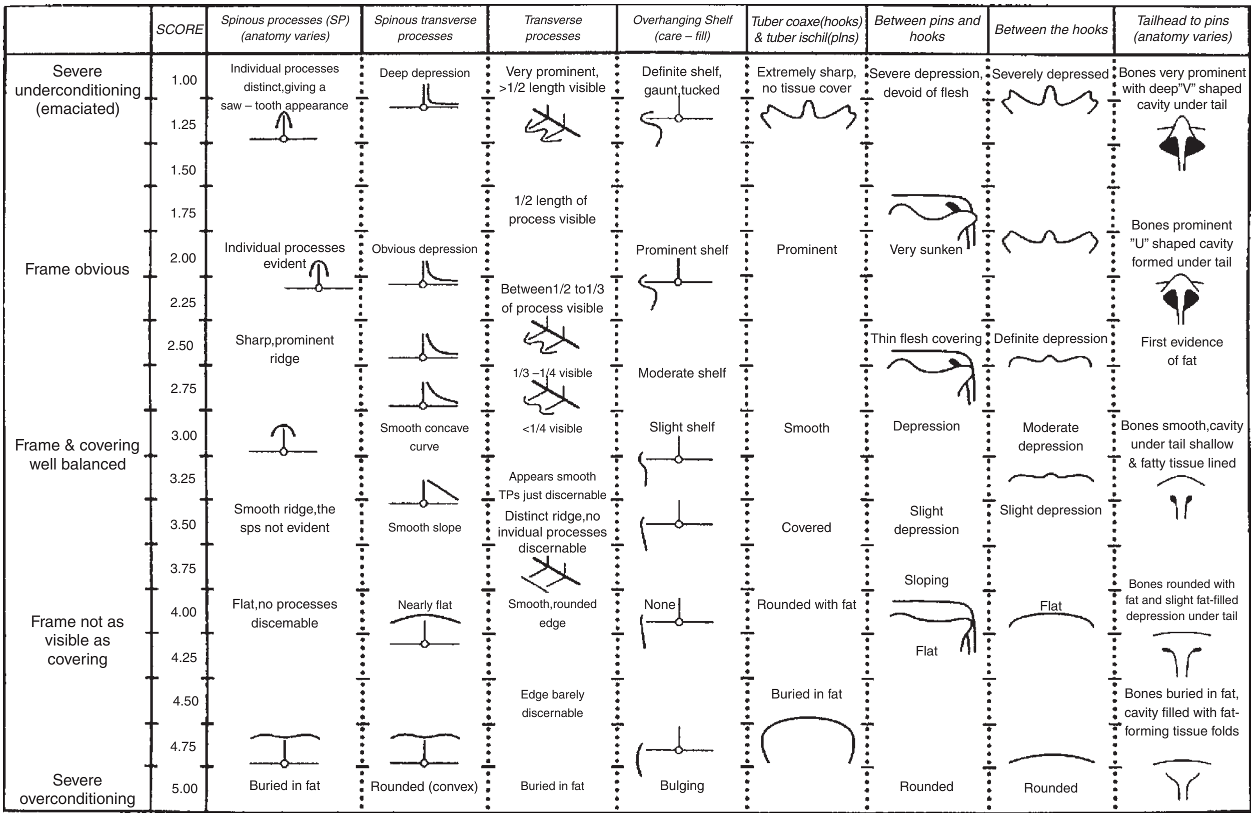
SCORE
Spinous processes (SP) (anatomy varies)
Spinous transverse processes
Transverse processes
Overhanging Shelf (care – fill)
Tuber coaxe(hooks) & tuber ischil(plns)
Between pins and hooks
Between the hooks
Tailhead to pins (anatomy varies)
Severe underconditioning (emaciated)
1.00
Individual processes distinct,giving a saw – tooth appearance
Deep depression
Very prominent, >1/2 length visible
Definite shelf, gaunt,tucked
Extremely sharp, no tissue cover
Severe depression, devoid of flesh
Severely depressed
Bones very prominent with deep"V" shaped cavity under tail
1.25
1.50
1.75
1/2 length of process visible
Bones prominent "U" shaped cavity formed under tail
Frame obvious
2.00
Individual processes evident
Obvious depression
Prominent shelf
Prominent
Very sunken
2.25
Between1/2 to1/3 of process visible
2.50
Sharp,prominent ridge
Thin flesh covering
Definite depression
First evidence of fat
2.75
1/3 –1/4 visible
Moderate shelf
Frame & covering well balanced
3.00
Smooth concave curve
<1/4 visible
Slight shelf
Smooth
Depression
Moderate depression
Bones smooth,cavity under tail shallow & fatty tissue lined
3.25
Appears smooth TPs just discernable
3.50
Smooth ridge,the sps not evident
Smooth slope
Distinct ridge,no invidual processes discernable
Covered
Slight depression
Slight depression
3.75
Sloping
Frame not as visible as covering
4.00
Flat,no processes discemable
Nearly flat
Smooth,rounded edge
None
Rounded with fat
Flat
Bones rounded with fat and slight fat-filled depression under tail
4.25
Flat
4.50
Edge barely discernable
Buried in fat
Bones buried in fat, cavity filled with fat-forming tissue folds
4.75
Severe overconditioning
5.00
Buried in fat
Rounded (convex)
Buried in fat
Bulging
Rounded
Rounded

Figure 1 Schematic diagram of body condition scoring elements and examples at different scores. Reproduced with permission from Edmonson AJ, Lean IJ, Weaver LD, Farver T, and Webster G (1989) A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* 72: 68–78.

Using the system

Body condition scoring usually includes both a visual and tactile appraisal. The focus is on the rear half to two-thirds of the animal, with focus on the ribs, vertebral processes, and rear quarters. Care must be taken not to let body girth, rumen fill, or pregnancy status influence the assessment. Appraisers should look over the animal from right, rear, and left. Note the prominence of individual vertebrae, and the transverse processes. Note the prominence of the pelvic bones ('hooks' and 'pins'; ischium and ilium); note the depression between the pelvic bones and the head of the tail. Very sharp, clear distinctions between individual bones, a 'sharp' sense in the visual appraisal and upon tactile appraisal, and the flesh just covering the bones would be a score of 1. Moving up the scale, as flesh is added, when individual bones are still prominent, but not as sharp, with depressions in all the areas between the bones would be a 2. A score of 3 is an 'average' condition cow: individual ribs and vertebral bones are not clearly visible, but may be palpated easily. Pelvic bones have more covering and appear more rounded, and the tail head region is beginning to have more flesh, but the animal still has an appearance of skeletal structure with good flesh covering. With a condition score of 4, individual ribs and vertebral bones are neither evident by sight nor with easy palpation. The rear quarters are taking on a flat to rounded appearance, with the normal depression between the pelvic bones flat to slightly rounded. The tail head region is full with fat. An obese (condition score = 5) animal appears rounded, individual bones are buried in fat and flesh bulges along the rear quarters; the tail head is also buried in fat.

Regardless of whether on the farm or for research purposes, the use of any body condition scoring system is best when it is used comparatively, not as a 'one-time measure' or as a means to quantify exactly how much body fat an animal has (although with cautious application, body condition does relate to actual body fat). As noted by Wildman in 1982, "On a practical basis, neither dairy farmers nor advisors agree on what constitutes desired body condition for a dairy cow." This is still true today, thus it is best not to focus on what might be ideal, but to use the system to monitor and manage body fat for the most optimal patterns of body fat changes that indicate the most efficient animals and feeding systems. Finally, on individual farms or in research trials, the actual appraisers should spend significant time 'norming', that is to come to agreement on the animal characteristics for each scale point, so that variation is minimized among appraisers and between farms and trials.

Automated body condition scoring

In recent years, there has been interest on the potential of using automated cameras and imaging systems to routinely measure body condition. Presently, most commercial dairies do not employ a consistent practice of measuring and monitoring body condition score. Several of these dairies will in fact notice if a cow or cows are getting 'too thin' or 'too fat', but not in a systematic way. The reasons for lack of adoption include the time and labor involved, lack of a readily accepted and easy-to-use data collection and analysis system, and the perception of 'we are doing enough already'. To help aid the adoption of body condition scoring on commercial dairies, as well as in research trials, there have recently been attempts reported to automate body condition scoring through use of digital cameras and data recording and compiling systems. The process is relatively simple, using multiple cameras to capture each cow, usually as they come out of or into the milking parlor. Specific points along the surface of the animals are noted and recorded, and through use of mathematical algorithms, the size, shape, and depth can be calculated. From this a score is assigned, and then, a user can access and use the information. In many ways the technology is not difficult; however, completing the loop of actually digitizing and quantifying the image, and the management of data to calculate a score and/or compare scores over time are more of a challenge.

Presently, research on such a system has shown promise. Cows are scanned with one or more cameras as they come out of the milking parlor. Specific areas of each cow are digitized and a number is entered for the cumulative score (**Figure 2**). A database for each cow is built up over time, and the data can be summarized as changes in body condition score over key times (early lactation, peak lactation, late lactation). The managers must still, of course, use the data and make a decision as to how to make dietary changes or to move animals from pen to pen based on changes in body condition and milk production.

Use of Body Condition Score Data (Data Processing)

Body condition score in dairy cattle is used to monitor the overall nutritional management of groups of cows. Because the body fatness of cattle is associated with several important production traits, disease states, or reproductive success, standardized collection, analysis, and interpretation of condition score data have had a large amount of interest. Because of the nature and variation of the measure, caution must be practiced in its use. Data processing is the accumulation, analysis, and interpretation of data. This article cannot cover all the various processes and analytical procedures tested. Readers desiring further instruction in statistical analysis of the data collected should refer to the Further Reading section.

The collection, analysis, interpretation of the data, and implementation in a management decision, as always, are functions of the objective of the activity – is it to monitor feeding systems on a single farm, groups of farms, or for individual or groups of animals? Is the purpose to ask research questions about effects of genetic selection, diet, or management on body fatness in a herd or herds? Each objective will dictate how often to collect data, on

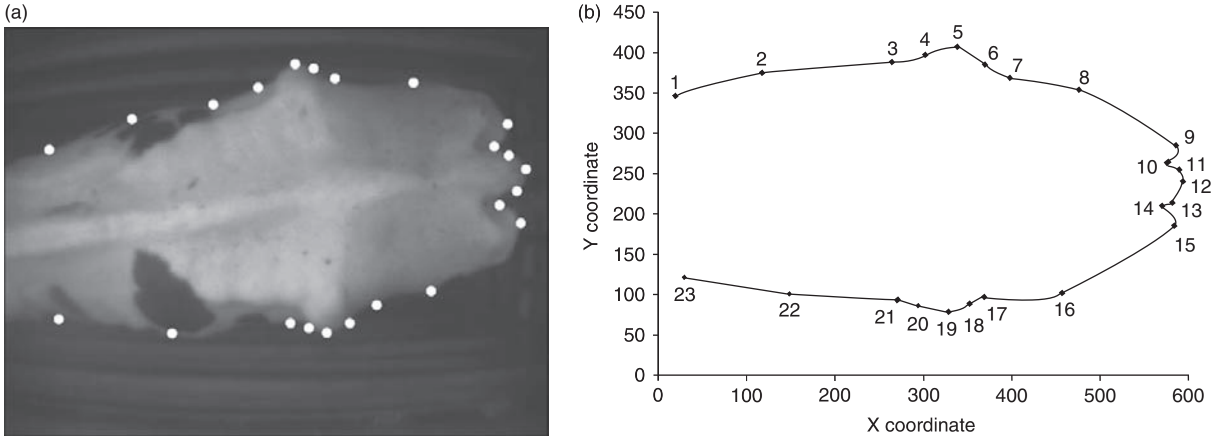

Figure 2 Example of digital imagery of a body condition scoring camera, data capture, and analysis program with potential on-farm use. Reproduced with permission from Bewley IM, Peacock AM, Lewis O, *et al*. (2008) Potential for estimation of body condition scores in dairy cattle from digital images. *Journal of Dairy Science* 91: 3439–3453.

how many animals, and what procedures would be best for analysis and interpretation.

The use of BCS in research requires sufficient replication to properly estimate error variation. Based on published studies, the standard deviation of BCS will be at least 0.5 units, so that replicates of 15–20 animals per group are really required to be precise enough to make statistical inferences. The system can be of great help in answering research questions, as long as caution is applied in the interpretation of data.

Estimates published from a number of studies have put a value of approximately 30–45 kg of body fat on 1 condition score unit. That is, the difference between a body condition score of 2.5 and 3.5 would be 30–45 kg of body fat. There are a limited number of studies that have actually done the research to measure body fat of dairy cattle at the same time as body condition scoring; therefore, we are limited by the lack of precision. Thus, for most research, the technique is limited practically to discriminating differences no less than 15–20 kg of body fat. This is in fact sufficient for some research questions; however, the lack of precision limits our ability to improve our quantitative understanding and prediction of body fat use in dairy animals.

Most dairy cattle will lose somewhere between 25 and 75 kg of body fat (0.5–1.5 body condition score units) in the first 4–8 weeks of lactation, some much more and a few less. The few decision support systems that incorporate body condition score use a value of 35–50 kg per BCS unit. Most recommendations then would be to manage for a BCS loss of no more than 1–1.5 units prior to peak lactation. Thereafter, it usually takes 4–6 months to gain back that amount (to have a body condition score at or near that at time of calving). Thus, this would be only a change in BCS of 0.25–4 units per month, a difference that most scorers would not be able to differentiate. Therefore, the implementation of a BCS system must be seriously evaluated so that the minimal effective number of measures is taken.

There is general agreement that a reasonable system would be that body condition score data are generally collected at important points in the reproductive cycle: when heifers (nulliparous females) are ready to breed (about 12–15 months of age); when pregnant heifers first enter the milking herd and then when they calve; when any cows are checked for reproductive readiness after calving (usually 1–2 months) or about the time of peak milk production (2–3 months); at reproductive service; when cows are ready to be moved to a different nutritional regimen; at time when cows are dried off from lactation; and during the dry period, usually 3–5 weeks prior to expected calving date.

Decisions using individual cow data may include things such as delaying breeding, deciding on the time to start or stop use of bovine somatotropin, or switching the animal to a less-energy and protein-dense ration. These are decisions for which historical data and experience for that specific herd are much more important than collective averages.

Starting with heifers, first breeding should occur when body condition score is 3 or above; at first calving, condition score should be 3–3.5 and will drop 0.5–1 unit by peak lactation, and should be returned to 3–3.5 by the time the cow is dried off. It is more energetically efficient (and practical) to allow lactating animals to regain body condition rather than trying to achieve this during the dry period. Animals should not increase too much in body condition score during the dry period, with the exception of those that, for whatever reason, were dried off below a score of about 3. In those cases, a slight increase in energy intake should be allowed so that the animal can achieve a score of at least 3 by next calving. In the 'old days' there was a greater concern about cows being overly fat. In most production situations, this is no longer the case and the real challenge is in getting cows to have sufficient stores to meet the need of the next lactation. In many herds, a score of 3.5–4 is desired, as those animals have the genetic capacity to make the milk, which will demand the use of those extra kilograms of fat and protein.

Very few herds would condition score at all these points. Minimal use would likely include first breeding, calving, first breeding after calving, after peak lactation, and at dry-off. It must be pointed out that the use of body condition score data does not supersede feed intake or milk production data in decision making on milk production and cow health. Rather, a sense of the body fatness of the animal(s) complements these two important measures, so that one can make a more specific decision on the overall nutritional program. Also, body condition score aids the producer in managing particular cows, which may have much less or much more body fat than the majority of cows on the same feeding program. It is hoped that economical use of digital imaging can increase the adaptation and use of body condition scoring to improve overall dairy efficiency.

Body condition scoring can be used to judge the overall effectiveness of the nutritional program. Body condition scores below average for several cows in a group may indicate inadequate feed intake or an improperly balanced ration. If several cows in a group have a high body condition score (for example, 3.5–4) and still have a few months of lactation remaining, they may be receiving too much energy. Data can also be used to select individual cows that should be changed to a less or greater energy-dense ration.

Body fatness is an integral part of mammalian biology. In dairy animals, body fat is directly tied to the overall efficiency of milk production. Body condition score systems are used to help producers better manage the production and health of their animals, and it is used by scientists to improve our understanding and prediction of nutrient use by dairy cattle.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Diseases of Dairy Animals**: Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Ketosis. **Feeds, Ration Formulation**: Models in Nutritional Management; Models in Nutritional Research.

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Effects on Health, Milk Production, and Reproduction

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Introduction

Mammals have evolved a system of storing energy as fat in order to sustain energy requirements during periods of feed scarcity or increased energy need. No other animals have such a highly evolved and regulated system of energy storage, although birds and fishes do store energy as fat and adapt to seasonal food supply. For food-producing animals (milk production and litter production), we measure and manage body fat because it is a marker of body energy storage and because of the relationships of body fatness to resistance to certain diseases or propensity for metabolic diseases or reproductive problems. During late pregnancy and early lactation, dairy cattle may experience several problems related to food intake control or supply of critical nutrients. Many of these problems are correlated with the amount of body fat of an animal in late pregnancy. In addition, the amount of body fat is related to milk production of the cow. Therefore, a system to monitor the amount of body fat known as body condition score (BCS) has been developed to help monitor and manage body fat reserves so as to minimize disease and metabolic, and reproductive problems in dairy cattle. Managing body fat in dairy cattle has improved overall production efficiency and reduced metabolic and reproductive disease incidence and severity.

Relation of Body Condition Score Measures to Production and Health Indices

There is a wealth of information relating body fatness or 'condition' to various disease states, metabolic problems, and reproduction. Observations earlier in this century included the noting and cataloging of phenomena such as animals that became infertile when body fat increased dramatically or when body fat was extremely low. Lactating animals in 'poor condition' were less likely to be fertile than lactating animals in 'good condition'. Obesity is also linked to reduced reproduction as well as a whole syndrome of associated metabolic and degenerative diseases. There are many different physiological and environmental situations in which the amount of body fat relates to seasonal food supply, reproductive processes, nursing of young, and resistance to disease. The role of body fat in production, health, and disease is complex, but in general terms, very low body fat or very high body fat is associated with the increased presence or risk of several diseases, or metabolic or reproductive problems. Today, the need for monitoring and managing body fat content for the optimum health and nutritional management of dairy cattle is widely recognized.

The amount of body fat in itself is not always the direct cause of increased presence or incidence of problems. This is because the amount of body fat or changes in the body fatness of an animal are a cumulative effect of the underlying changes in physiology or metabolism that also lead to various reproductive or health problems.

It is true, however, that excess body fat does increase the incidence of many problems. Examples include increased difficulties in birthing (dystocia) in overly fat animals, increased metabolic and reproductive diseases in early lactation of cattle, pigs, and sheep breeds, as well as increased blood pressure (hypertension), diabetes or diabetes-like symptoms, arteriosclerosis, and heart disease in overly fat humans and some other animal species. The adipose tissue can produce a number of endocrine or cytokine factors that can affect health. Much of this work has been done in human or rodent models, and specific data on dairy cattle are lacking. Yet several examples exist in dairy cattle as discussed below and certainly the next decade will bring more discoveries.

Body Condition, Feed Intake, and Milk Production

Many of the problems associated with milk production in early lactation are a result of rapidly changing feed intake. This is often a vicious cycle that does not always have an easily identifiable 'starting point'. Let us use the following example: a cow with no easily identifiable problems, for one or more of several reasons (weather, feed delivery, competition, diet composition, stress, subclinical metabolic or other disease), has a depressed appetite for 1 or 2 days in early lactation. The demands of the mammary gland do not immediately diminish; therefore, the increasing deficit of nutrients elevates the chances of developing subclinical or clinical ketosis, acidosis, or calcium deficiency. These imbalances may lead to a further diminished appetite, worsening the problem. The cow now is in serious trouble. The low intake with associated metabolic disease state can reduce the cow's ability to handle other stressors, and infertility, decreased production, or increased mastitis can easily result.

Body condition is a major part of the overall food intake, production, and health system. There is a link between the amount and rate of use of body fat stores and feed intake. Scientists still disagree on the exact nature of this relationship and the underlying metabolic causes. But in brief, it was observed long ago that cows with excessive body fat during late pregnancy or those that gained a large amount of fat during late pregnancy usually ate less feed than animals that maintained 'body condition' during this time. This reduction in feed intake from the expected rate (to meet milk production and other requirements) then resulted in decreased rates of milk production and increased incidences of postpartum metabolic and reproductive diseases. Overly fat animals also had increased difficulty in delivery of the young (dystocia), due to excess fat deposits altering the shape or position of the young leading to inability to deliver the calf. The term 'fat cow syndrome' described the problems in late pregnancy and early lactation that are associated with overly fat animals. It was the desire to reduce the incidence of this syndrome and minimize associated loss of profitability and increased health problems of the cow that led to a large effort in basic and applied research in nutrition and physiology over the last 30 years. This research has done much to define the role of body fat in postpartum problems in cows. In most situations in commercial production, there is no longer a problem with 'fat cows'. We now strive for an even better management of body fat.

Significant research effort has been expended to identify the physiological connections between body fat and feed intake. Quite early on, it was hypothesized that there was some 'message' from the body fat stores to the feeding control centers in the brain. The general phenomenon was that over the long term (days, weeks), the brain was monitoring in some way the amount of body energy stores and adjusting feed intake to keep them in balance. This was in addition to the well-known shorter-term homeostatic regulation to maintain blood glucose (and other nutrient) concentrations within a specific range.

In general, BCS at calving is related quadratically to milk production (**Figure 1**). There is a general, broad level of body fat that is related to optimum milk production. Above or below this, milk production is compromised. One hypothesis put forth, substantiated by large numbers of data sets and field observations, is that cows that are not 'fat enough' may be sufficiently nutritionally challenged for a long enough period of time that their mammary function is diminished. Those cows that are too fat are in the situation of greater rates of fatty acid release from the fat stores inhibiting the maximal potential feed intake. There has been a wealth of research since this figure was created to further define the situation.

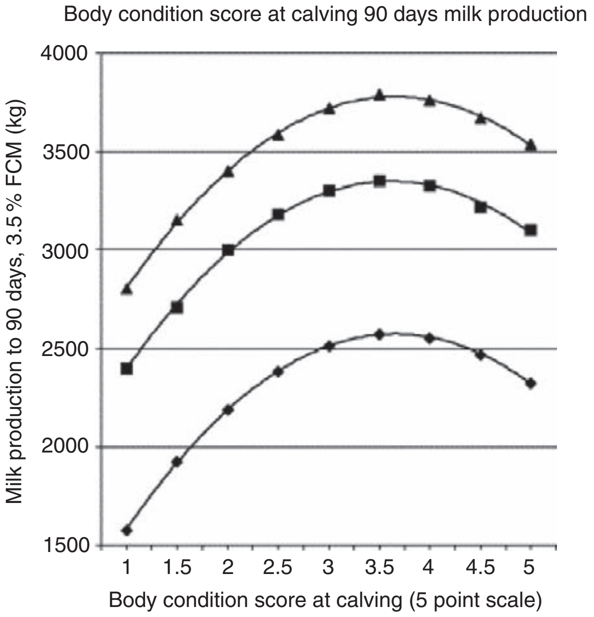

Figure 1 Relationship of body condition score at calving with the fat-corrected milk (FCM) production to 90 days in milk. ◆ lactation 1; ■, lactation 2; ▲, lactations 3 and 4. In general, one needs to have animals between a score of 3 and 4 to maximize milk production.

A detailed summary of the research on signaling molecules between body fat and feed intake is far beyond the scope of this article. Many books have been written on this topic over the last 30 years and there is still a lack of agreement among researchers in this field. However, several factors were tested early on, including estrogens and other steroid hormones and fatty acids. Although general trends existed, until the last 15 years or so no signal was ever really identified. Additionally, there were a number of exceptions to the rule – in that large percentages of 'fat' or 'thin' animals had no problems. However, there were data that clearly showed that animals that were fatter at calving ate less and therefore gave less milk than those that were not.

One finding was that increased circulating concentrations of blood fatty acids or hormones related to increased free fatty acids could diminish feed intake. Simultaneously, research into the biochemistry and molecular biology of food intake control discovered that specific pathways of glucose and fatty acid metabolism in brain centers had direct effects on feeding behavior; however, in some situations, this was independent of the body fatness of the animals.

In the mid-1990s, a major discovery led to a clear understanding on the relationship between body fat and intake, and opened up new research areas. The protein hormone leptin, produced by the adipose tissue, was discovered after a long search to find the 'obesity gene' that would explain the heritable obesity from mutations in rodent populations. The discovery that one of these mouse strains was making a defective gene product led to the discovery of the gene product, named 'leptin' (after the Greek word leptos, meaning thin). Animals that were genetically obese did not produce the proper hormone; therefore, they were always hungry, ate too much, and became fat. Later, it was found that there was a leptin receptor in the feeding centers of the brain, and mutations in the receptor also altered feeding. So the question arose – could leptin be involved in feed intake in dairy cattle?

The role of leptin in potentiating feed intake in lactating cattle has been studied; however, no clear picture has emerged that leptin is a major regulator of food intake in this situation. Research in this area in dairy cattle is very difficult, as circulating concentrations of the hormone vary tremendously and it is not easy to measure receptor concentrations or activity in neural tissues of farm animals. It is, however, likely that leptin is one of the signals that help the animal monitor the stores of body fat.

Since the discovery of leptin, however, much newer research has led to the possibilities of other signaling molecules. One of these is the protein hormone ghrelin, which is made in the cells of the abomasum, the true stomach. Ghrelin may modulate feed intake in dairy cattle. There is ongoing research and discussion on the multifactorial nature of the relationship of body fat to feed intake in dairy cattle. Because there are so many factors involved at metabolic, hormonal, and physiological (neural) levels, it will be awhile before the complex system is defined. In the interim, in practice we know that selecting for, proper housing, feeding balanced rations at the right amounts, and a consistent monitoring of body fat through condition scoring have and will minimize problems with feed intake and resultant loss of production and increased health problems.

Body Condition Score and Metabolic Diseases

There are several metabolic diseases associated with the end of pregnancy and beginning of lactation. Although the diseases range from calcium deficiency, to energy deficiency, to mammary inflammation, and to other infections, the amount of food intake and body fat are intricately involved with all of these problems. Because body fat, food intake, and milk production are so completely intertwined, a problem with any one of them can have several acute and chronic effects on the animal. Because lower food intake may reduce resistance to infection, a seemingly 'unrelated' infection may be exacerbated by diminished intake. Alternatively, a bacterial infection that causes a reduction in feed intake can depress milk production and at the same time increase loss of body fat, leading to ketosis and milk fever. It gets a little confusing. In any case, much research effort has gone into defining the role of body fat (condition) and its use in the prevention of many diseases.

The relationship to milk fever is straightforward. Any situation that decreases intake, even of a diet sufficient in calcium, could increase the likelihood of acute calcium deficiency. If excess body fat (high condition score) translates to diminished feed intake in early lactation, then the chance of milk fever also increases.

Another major metabolic disease is ketosis. This is primarily a shortage of glucose, which is required by the brain and the central nervous system at all times, and is demanded by the mammary gland for lactose synthesis. This is a critical need for the early lactation cow. If glucose precursors (propionate and amino acid carbons) are not present in sufficient amounts, certain biochemical pathways are limited, causing energy generation from free fatty acids released from the adipose tissue. This causes at least two problems: one is hepatic lipidosis, better known as fatty liver. The other is ketosis, caused by a buildup of ketones: acetoacetate, beta-hydroxybutyrate, and acetone. This gives the characteristic 'sweet' or acetone smell. This indicates that the liver cannot process the excess fat sufficiently fast, and the circulating fatty acids and resultant endocrine responses usually decrease feed intake even more, worsening the problem. Utilization of body fats at fast rates is a normal adaptation in early lactation; however, excess body fat at calving usually results in an excessive rate of body fat loss, resulting in ketosis. If feed intake is diminished, because of increased amounts of fatty acids released from the adipose tissue, this compounds the problem. This vicious cycle is a major problem in the balance between proper amounts of body fat and stimulation of optimal feed intake.

Another major metabolic problem associated with BCS is fatty liver. The excess fatty acids released from body fat are transported to the liver. If the mammary gland's needs are already met and oxidation is maximal (ketones are building up), the liver tries to store the extra fat. Ruminant liver is genetically limited in how fast it can export triglycerides from the liver. This is probably because ruminants did not 'evolve' in a situation of high-fat diets as other mammals did, or with long periods of rapid body fat loss as now occurs during early lactation. For whichever reason, the fast rate of fatty acid release from the adipose tissue coupled with the slow rate of release from the liver can cause liver fat content to rise from just a few percent in the weeks before calving to over 20 and even 30% in early lactation. This in turn diminishes normal liver function such as amino acid metabolism, glucose synthesis, and ammonia detoxification to urea. This leads to further problems with subclinical ammonia toxicity and acid–base balance, also affecting feed intake, and thus the cycle continues.

Thus, we see that the connection between body fat, milk production, and liver metabolism in early lactation is a cyclic and complex system. The goal of feeders of dairy cattle is to find the optimal balance between milk production, feed intake, and body fat use to minimize problems. Monitoring and management of BCS is a tool to achieve this goal.

Body Condition Score and Reproductive Diseases

The immediate postcalving period and early lactation is also a time during which problems associated with the reproductive tract can occur. These would include dystocia or calving difficulty and retained placenta – the improper or delayed sloughing of the placenta, causing excess bleeding, putrification, and infection, known as metritis. Additionally, several reproductive insufficiencies or failures occur in lactation related to body fat and fatty acid and glucose metabolism. These would include delayed return to the ovulatory cycle, delayed ovulation, inhibition of ovarian follicular growth, reduced fertility, and early embryonic death. The scope of all these problems is too great to go into much detail; there are several excellent reviews in Further Reading. Nevertheless, the amount of body fat in lactating dairy cattle is a central factor in all these reproductive areas.

The immediate calving and postcalving problems most likely relate to excess fatness interfering with calf movement, uterine motility, and uterine contractions. All these problems affect birthing. The relationship with retained placenta and subsequent metritis is possibly due to a lack of muscle tension in the uterus, caused by the excessive work done during parturition. The uterine muscle cannot expel the placenta and infection may occur. Retained placenta may also be caused in part by other factors such as selenium, which may also act through altering smooth muscle strength. Thus, one part of the 'fat cow' syndrome is problems with calving and immediate postcalving uterine function. However, as noted above, because of changes in dietary management, and genetic improvements in milk production, the incidence of cows that are too fat has greatly diminished.

A strong connection exists between body fat amount and rate of use, fatty acid and glucose metabolism, and reproductive cyclicity, follicular growth, conception, and pregnancy. Many observational studies have directly related excess or insufficient body fat, and fat cow syndrome with diminished reproductive success. However, attempts to improve reproduction by manipulating body fat in dairy cattle have been inconsistent. The amount or use of body fat is not the only factor that controls reproduction. The amount and rate of use of body fat are both effectors of and affected by the intake and use of glucose and fatty acids or their precursors, and the amount of removal as milk. Although the amount of body fat does affect the release of different hormones and cytokines that affect reproduction, the amount of body fat can never be fully separated from the intakes and outputs of nutrients.

Several related metabolic and physiological pathways do connect body fat with reproduction. The amount and use of glucose, and possibly fatty acids, in the brain affect the release of luteinizing hormone-releasing hormone, which directs the pituitary to release luteinizing hormone to stimulate ovulation. The relation with body fat is circular in the sense that, for example, if an animal calves with too little body fat to support milk production, she is already at risk of diseases. In this case, if there is not enough energy coming from fatty acids in the adipose tissue, most of the glucose will be used for milk lactose, and luteinizing hormone-releasing hormone may diminish and the animal does not cycle. Also, there may be an insufficient or incorrect pattern of fatty acids to supply the ovary. In addition to glucose, related hormones such as insulin and insulin-like growth factor-I (IGF-I) also affect ovarian function, and are linked to body fat use, glucose use, and food intake.

In a different situation, an animal that calves with too much body fat may have a diminished food intake and the same result is obtained – incorrect cycling or follicular development.

Fatty acid availability and type of fatty acids (omega-3 or omega-6) may also play a role in follicular development; however, these are discussed in **Feed Ingredients**: Feed Supplements: Fats and Protected Fats.

Therefore, because of the direct connection between body fat amount and use, glucose and fatty acids, and the resultant hormonal changes, it is imperative in practice that we select for, feed, and manage animals that can produce milk at fast rates, eat to maintain those rates, and manage body fat changes efficiently. In practice, there is a need to avoid rapid loss of fat (certainly shoot for no more than 1 BCS unit in the first 2 weeks of lactation). It must always be noted, however, that individual cows may in fact lose that much fat, eat a tremendous amount of food, make prodigious amounts of milk, and cycle early and conceive! Others will 'crash and burn', as we say, and that is where research needs to focus – what is it about the 'top cow', the cows that 'can do it all', that is different? If one looks at all the available data, in fact, many dairy cattle of high production rates when managed correctly have little problem in reproducing a calf in a timely fashion.

Conclusion

Mammals have evolved a system of storing energy as fat, primarily triacylglycerols, in order to sustain energy requirements during periods of feed scarcity or increased energy need. We measure and manage body fat because of its relationship to resistance to certain diseases or propensity for metabolic diseases or reproductive problems. Several problems in dairy cattle have been associated with food intake control or supply of critical nutrients. The amount and use of body fat is an integral link between nutrition, metabolic diseases, and reproductive problems. A system to monitor the amount of body fat known as BCS has been developed to help monitor and manage body fat reserves so as to minimize disease and metabolic and reproductive problems in dairy cattle. Managing body fat in dairy cattle has improved overall production efficiency and reduced metabolic and reproductive disease incidence and severity. Continued research and field applications in body fat metabolism and management will allow us to pinpoint more specific mechanisms by which to optimize the overall metabolic and reproductive efficiency of milk production.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Ketosis. **Feeds, Ration Formulation**: Dry Period Rations in Cattle. **Feed Ingredients**: Feed Supplements: Fats and Protected Fats. **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity.

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BULL MANAGEMENT

Contents
Artificial Insemination Centers
Dairy Farms

Artificial Insemination Centers

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Introduction

The management of dairy bulls resident in artificial insemination centers (AICs) has changed during the last five decades. Twenty to 50 years ago, numerous small AICs each housed fewer than a hundred bulls. Now, most dairy bulls reside in a few commercial AICs each housing over a thousand bulls. In the early era of the artificial insemination (AI) industry, most semen was cooled and inseminated within 3 days of its collection and most was used domestically. However, when technology enabled the successful cryopreservation of bull semen, the international exchange of semen became commonplace (*see* **Gamete and Embryo Technology**: Artificial Insemination; **Reproduction, Events and Management**: Mating Management: Artificial Insemination, Utilization). Subsequently, the need arose to document that the health status of semen donor bulls and the AIC herd complied with an array of international regulations. Accordingly, extensive herd health programs were developed. This article will present information on several aspects of a complete bull management program including nutrition, bull and herd health programs, evaluation of bulls for general and reproductive health, and semen collection management.

Nutrition and Feeding

A nutritionally balanced feeding program is an important component of any bull management system. Feedstuffs should be of good quality and clean water should be regularly available. Dairy bulls should be fed to gain weight gradually (**Table 1**). An average daily gain in body weight of about 1.0 kg between 6 and 18 months of age is a reasonable goal. Thereafter, the average daily gain should slowly decline (**Table 2**). An optimum weight for adult, large breed, dairy bulls is 1000 kg. Adult bulls need to be fed to maintain body condition and not gain excessive weight. Adult bulls that carry excessive weight relative to their body frame may be more reluctant to mount and exhibit reduced libido.

The daily dry matter intake (DMI) for a yearling dairy bull should be 2.0–3.0% of body weight; the daily DMI for a 1000 kg dairy bull should range from 1.2% to 1.4% of body weight. The ration for a yearling bull should provide relatively high energy and crude protein compared to the ration of an adult dairy bull. While DMI values provide a guide for the quantity of nutrients to feed, the suggested DMI values are not necessarily an estimate of voluntary intake. In fact, limit feeding may be required, especially in adult bulls and particularly if highly digestible feeds are available. Fiber length of nutrients is also an important consideration because relatively long fiber length stimulates chewing and the secretion of saliva. Because saliva contains high levels of bicarbonate, it is an important buffer for ruminal contents (*see* **Nutrients, Digestion and Absorption**: Fermentation in the Rumen).

Special consideration should be given to the levels and ratio of calcium and phosphorus in the rations of bulls. In the early years of AI, bulls were often fed dairy cow rations and there were concerns that feeding excessive calcium had contributed to thyroid hyperplasia, calcification of specific soft tissues, and excessive bone deposition along the

Table 1 Recommended weights for large breed dairy bulls

Age (months)	*Weight (kg)*	*Weight per day of age (kg)*
6	200	1.1
12	380	1.0
18	550	1.0
24	700	1.0
30	800	0.9
36	900	0.8
48	1000	0.7

Adapted with permission from Jorgensen (1988).

Table 2 Suggested average daily weight gain for large breed dairy bulls

Age (months)	*Average daily gain (kg)*
0 to 6	1.1
6 to 12	1.0
12 to 18	1.0
18 to 24	0.7
24 to 30	0.5
30 to 36	0.5
36 to 48	0.3

Adapted with permission from Jorgensen (1988).

thoracolumbar vertebrae leading to ankylosing spondylitis and associated neurological deficits. As rations were corrected toward a calcium:phosphorus ratio of 1.5 : 1, the observations of thyroid problems and soft tissue calcification declined. However, the observations of hind limb neurological deficits in ageing bulls associated with the presence of ankylosing spondylitis continued. Research determined an association between the major histocompatibility complex and development of spondylitis associated with neurological deficits of the hind limbs in older bulls. Balancing the ratio of calcium and phosphorus in a bull nutrition program continues to be important. But because nonnutritional factors play a role in the gradual development of ankylosing spondylitis, reducing the intake of calcium and phosphorus will not eliminate the associated neurological problems infrequently observed in older bulls.

Because the availability of feedstuffs in different geographic regions is variable as is the quality of feeds and water, a list of specific feeding recommendations is not provided. Analysis of the feed ingredients is suggested, followed by consultation with a local animal nutritionist.

Health and Disease Control

The importance of a disease control program for semen donor bulls was recognized as early as 1947. Epidemiologic evidence reported that *Brucella abortus* and *Tritrichomonas foetus* could be transmitted to cattle bred by AI if the donor bull was infected (*see* **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis). These reports provided the impetus for establishing bull health programs. A herd health program may be basic, or it may be complex when the export of semen is a part of the AIC business strategy. It is important for managers to establish goals for an AIC health programme relative to the efforts required and the benefits received.

Establishing Goals

The herd health program should provide clinical surveillance and diagnostic testing to detect the presence or incursion of disease. The goal is to prevent transmission of diseases within the herd and keep all bulls in optimal health.

Personnel responsible for bull health should be familiar with the medical science pertinent to seminal transmission of disease agents. This second goal of the health program is to prevent contamination of semen by specific bacterial, viral or protozoan pathogens, thereby precluding the seminal transmission of disease to cattle bred by AI.

The health program may also be designed so that semen donor bulls and the herd in which they reside comply with international regulations. The goal is to provide an opportunity for the AIC to market semen internationally. While a preventive medicine program will provide many of the items necessary to comply with international regulations, additional requirements for segregated bull housing, semen collection locations and semen processing laboratories may be necessary to produce semen for some countries.

General Considerations

The foundation for a bull health and disease control program is regular surveillance of the animals by direct observation and repeated testing of specimens by a diagnostic laboratory. This is managed by dividing the health program into an entry isolation interval lasting 30 to 60 days, followed by a long-term residence interval.

Most dairy bulls entering commercial AICs are the result of a contracted mating and they enter as calves. A better understanding of their health status and diagnostic testing needs is enabled if the diseases to which they may have been exposed prior to entry are recognized. This includes the gestation interval. Diseases transmitted *in utero* can have a negative impact on the future health of a bull or its acceptability to comply with AIC health requirements.

Health Concerns during Gestation and for the Young Calf

The pathogen of greatest concern during the gestation interval is bovine viral diarrhea virus (BVDV). It can be transmitted across the placenta to the fetus if the cow is persistently infected (PI) with BVDV, or when the cow is acutely infected during gestation. When infection of the fetus occurs during the 1st trimester of gestation, the fetus becomes PI with BVDV because its immune system is not adequately matured. Such BVDV-PI calves are stated to be immunotolerant because they are infected with virus but cannot mount an immune response against it. A BVDV-PI calf may die, be physically unthrifty, or survive and reach breeding age. Those BVDV-PI bulls that survive will shed BVDV in all secretions, including semen. It is important, therefore, to conduct a virus isolation test, or other viral antigen detection test, on all bulls designated to enter an AIC. Any bulls that test positive should not be transported to an AIC.

Another disease for which transplacental transmission is possible is bovine leukosis virus (BLV). Most calves born from BLV-infected cows will not be infected, but those calves infected *in utero* will remain infected for life. Bulls are typically tested for evidence of BLV antibodies prior to transport. If a bull tests positive prior to 6 months of age, it could be infected or have passively acquired antibodies. A positive retest after 6 months of age is usually indicative of infection. Bull calves infected with BLV are often rejected by AICs because many international regulations require bulls to test negative, even though research has shown that BLV is not transmitted via semen used for AI.

A bacterial disease that may be transmitted *in utero* is *Mycobacterium avium* subsp. *paratuberculosis*, the cause of Johne's disease (*see* **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease). Infection of a neonatal calf is also possible from exposure to a contaminated maternity pen, when the calf nurses a contaminated teat, or from contaminated colostrum.

The Entry Isolation Interval

Bulls should be tested for several diseases at the farm of origin and again during the entry isolation interval. All bulls over 6 months of age should be tested for BVDV by virus isolation or other antigen detection test during the isolation interval. Bulls that test positive should be segregated and retested. Bulls that have two positive tests at least 3 weeks apart should be classified as PI and must be removed from the AIC isolation facilities. A highly unusual and infrequently detected form of BVDV infection presumably occurs prior to or during puberty wherein the virus becomes sequestered in the testicles. Such bulls are not PI in the classical manner, but the testicles are apparently persistently infected with BVDV. Health programs include conducting a virus detection test on processed semen, and perhaps only from those bulls from which antibodies have been detected.

Most countries require bulls to be tested negative for tuberculosis (*Mob. bovis*) (*see* **Diseases of Dairy Animals**: Infectious Diseases: Tuberculosis) and brucellosis (*Br. abortus*). Bulls are also routinely evaluated for evidence of prior exposure to BLV, infectious bovine rhinotracheitis virus (IBRV) and leptospiral serotypes of importance in the country. Testing for bluetongue virus or vesicular stomatitis virus may be conducted in those countries or regions where bluetongue virus is enzootic or where a regional incursion of vesicular stomatitis virus infrequently occurs.

Bulls should also be tested for the pathogens causing classic venereal diseases of cattle, namely *Tritrichomonas foetus* and *Campylobacter fetus* subsp. *venerealis*. A sample collected from the upper region of the prepuce is the preferred specimen. The sample should be inoculated into an appropriate transport enrichment media and promptly forwarded to a diagnostic laboratory. The number of weeks a preputial specimen is collected and found negative is dependent on the age of bull at the time of entry to the AIC and local regulations. Specific antibiotics are added to the semen and/or to the extender to control the potential for seminal transmission of bovine genital campylobacteriosis. Because a second line of control for bovine genital trichomoniasis is not available, it is important that the bull and the AIC herd be tested negative for *Ttm. foetus*.

Control of disease by vaccination is not an important component of an AIC health program. Vaccination of yearling bulls to prevent clostridial diseases or vaccination to reduce the incidence of penile fibropapillomas may be conducted. However, vaccination to control diseases like IBRV and leptospirosis is not advised. Antibody titers subsequent to vaccination would interfere with interpretation of serologic test reports.

Health Programs for Resident Bull Herds

Bulls that continue their residency at an AIC should be periodically retested to confirm their freedom from disease and provide assurance that the semen distributed for breeding is not the source of disease. A semi-annual retest is advocated by some countries; others advocate annual testing. A semi-annual test interval reduces the time during which a subclinical disease could spread through a bull population. Periodic retesting also provides regulatory support when documentation is required for the international transport of semen.

Evaluation for General and Reproductive Health

Bulls are selected by AIC personnel because of their potential to genetically improve the breed, either in milk production parameters or conformational characteristics. For a bull to have a genetic impact, semen of acceptable quality must be regularly collected. Bulls must be in good health and physical condition, have sound feet and legs, and their genitalia must be of acceptable size and health.

General Health Considerations

Bulls must be well managed, adequately housed and correctly fed to achieve and maintain good physical condition. They should have access to shelter to provide protection from wind and precipitation and be provided with a cushioned, dry area on which to lie. Facilities should have adequate lighting and be designed to reduce the risk of injury to bulls and bull handlers.

Monitoring bull health is an important responsibility of barn personnel. Often the first indication of a problem is a bull's reduced appetite. Concern increases if there is little or no fecal material observed in its pen or detected during rectal palpation. Medical treatment administered upon the advice of a veterinary surgeon is often adequate, but occasionally surgery is required to correct a gastrointestinal problem.

Bull health can also be monitored with hematologic and serum biochemical tests. Normal reference values for yearling bulls and bulls 4 years of age and older are shown in **Tables 3** and **4**. Monitoring these variables is helpful in adult bulls that develop a chronic respiratory tract infection. Affected animals may cough and have mucoid nasal discharge or fetid breath. Bulls with chronic pneumonia will often have decreased albumin levels, elevated immunoglobulins and a lower than normal albumin : globulin ratio, and may be anemic. Systemic antibiotics and anti-inflammatory medications are warranted, along with treatment of the anemia with cyanocobalamin and iron dextran.

Table 3 Recommended reference ranges of hematologic variables for Holstein bulls

Variable	*Yearling bulls*	*Adult bulls*
Erythrocytes		
Red blood cells × 10^{6} μl^{-1}	6.3–9.0	5.9–8.9
Hemoglobin (g dl^{-1})	8.7–13.1	10.5–16.2
Packed cell volume (%)	23–34	28–43
Mean cell volume (fl)	33–41	44–54
Mean cell hemoglobin concentration (g dl^{-1})	36–41	36–39
Leucocytes		
White blood cells μl^{-1}	7 600–16 400	3 300–8 000
Band neutrophils μl^{-1}	0–500	0–200
Segmented neutrophils μl^{-1}	1 700–9 000	1 900–5 900
Lymphocytes μl^{-1}	3 100–9 200	500–2 400
Monocytes μl^{-1}	0–1 100	0–600
Eosinophils μl^{-1}	0–3 900	0–900
Basophils μl^{-1}	0–300	0–100

Reproduced with permission from Monke *et al*. (1998).

Table 4 Recommended reference ranges of serum biochemical variables for Holstein bulls

Variable	*Yearling bulls*	*Adult bulls*
Alkaline phosphatase (IU l^{-1})	110–310	30–80
Aspartate transaminase (IU l^{-1})	50–120	50–190
Bilirubin (total; mg dl^{-1})	0–0.2	0.1–0.3
Blood urea nitrogen (mg dl^{-1})	8.0–15.0	13.0–24.0
Calcium (mg dl^{-1})	9.3–10.5	8.0–9.7
Chloride (mEq l^{-1})	92–103	87–102
Creatine kinase (IU l^{-1})	100–340	70–470
Creatinine (mg dl^{-1})	1.0–1.6	1.9–3.0
γ-Glutamyl transferase (IU l^{-1})	11.0–35.0	23.0–47.0
Glucose (mg dl^{-1})	45–90	45–80
Magnesium (mg dl^{-1})	1.5–2.3	1.6–2.2
Phosphorus (mg dl^{-1})	7.0–9.6	5.0–8.1
Potassium (mEq l^{-1})	3.8–6.3	4.1–6.0
Protein (plasma; g dl^{-1})	6.1–7.3	7.3–8.5
Protein (serum; g dl^{-1})	5.9–7.1	7.1–8.6
Albumin (g dl^{-1})	3.0–3.6	3.0–3.7
Globulin (g dl^{-1})	2.6–3.8	3.8–5.3
Albumin to globulin ratio	0.8–1.3	0.6–0.9
Sodium (mEq l^{-1})	133–142	127–143
T3 (ng dl^{-1})	90–290	60–225
T4 (μg dl^{-1})	4.0–8.0	3.2–6.8

Reproduced with permission from Monke *et al*. (1998).

Health of Upper Limbs and Hooves

A bull's physical condition, presence of degenerative osteoarthritis from prior injury, and its conformation may impact its ability to mount regularly for semen collection. Such bulls may need to receive anti inflammatory medication and may benefit from additional bedding in their stalls. Management of hoof health is also an important component of a bull health program. Disease or injury to a foot or hoof causes pain, which may make the bull reluctant to mount. It is important, therefore, that AICs have access to some form of animal restraint to permit examination and treatment of a bull's hooves.

While hoof disease can occur in any foot, observations suggest that most hoof problems are in the hind feet and most problems occur in the lateral hooves. Typical problems are overgrown hooves, separation of the junction of the hoof wall and sole (the white line region), cracks in the heels and an ulcer of the sole at its junction with the heel. Routine reshaping of overgrown hooves reduces excessive weight bearing on the heels, which may reduce the incidence of heel cracks. Separations along the white line region should be explored, particularly in a bull exhibiting lameness, because the hoof may be abscessed. Opening a hoof abscess and alleviating the pressures on the sensitive laminae can markedly improve a bull's locomotion. A serious hoof disease is ulceration of the sole. If left untreated, the ulceration may extend into the foot permitting infection and tissue damage of the deeper tendons and ligaments. Treatment involves careful but extensive debridement of the affected hoof tissue, application of an antibacterial ointment followed by bandaging, and finally application of a wooden or plastic block to the opposite healthy hoof. The hoof block reduces the need for the affected hoof to bear weight, and thereby reduces pain.

While regular hoof examinations are important, it is equally important to monitor the affects of the AIC environment on the bulls' feet. Concrete is commonly used to provide an impervious flooring capable of disinfection. However, it can be abrasive to hooves and cause excessive wear, particularly if the finish is rough. On the other hand, bulls continually resident on deep bedding may have excessive hoof overgrowth. Care must also be given when walking bulls across wet concrete. Unless the floor is grooved or otherwise provided a nonskid surface, the bulls may slip or fall, resulting in a sprained joint or muscle injury.

Reproductive Health

The reproductive capability of adult dairy bulls is one consideration determining the success of an AIC business. It also directly determines the extent to which any bull's genetic characteristics may be distributed. To optimize the eventual harvest of spermatozoa from adult dairy bulls, the health and size of the reproductive tract of yearling bulls must be carefully evaluated (*see* **Bull Management**: Dairy Farms).

Scrotal circumference

Meaningful information about a young bull's testicles and epididymides can be acquired between 6 and 18 months of age. Measurement of the scrotal circumference (SC) is an important criterion for evaluating testicular size and growth. Scrotal circumference is a meaningful predictor of semen production in yearling bulls and is strongly correlated with a bull's SC at 5 years of age. Healthy bull calves provided a ration for growth without overconditioning will have an increase in SC of about 2 cm per month between 5 and 10 months of age and about 1 cm per month between 11 and 15 months of age. This is shown in **Table 5** for Holstein bulls; these measurements would also provide an acceptable guide of SC growth for Ayrshire, Brown Swiss, Guernsey and Dairy Shorthorn bulls. The normal SC of yearling Jersey bulls is slightly smaller at specific age increments (**Table 6**).

Table 5 Average scrotal circumference measurements of young Holstein bulls

Age (months)	*Scrotal circumference (cm)*
5	18.9 ± 1.6
6	21.9 ± 2.5
7	24.3 ± 2.4
8	26.6 ± 2.4
9	28.6 ± 2.3
10	30.5 ± 2.1
11	31.7 ± 2.2
12	32.7 ± 2.0
13	33.7 ± 2.0
14	34.7 ± 2.1
15	35.2 ± 2.4
16	35.9 ± 2.3
17	36.1 ± 2.2
18	36.4 ± 2.1

Reproduced with permission from Hueston *et al*. (1988).

Table 6 Average scrotal circumference measurements of young Jersey bulls

Age (months)	*Scrotal circumference (cm)*
4.0–5.9	16.9 ± 1.3
6.0–7.9	20.8 ± 2.9
8.0–9.9	24.9 ± 2.6
10.0–11.9	28.9 ± 2.4
12.0–13.9	31.1 ± 2.3
14.0–15.9	32.3 ± 2.2
16.0–18.0	33.7 ± 1.7

Reproduced with permission from Monke (1988).

Regular measurement of SC between 6 and 12 months of age may be used to determine when semen collection may commence. For example, a 12-month-old bull with an average SC for its age will typically be a good candidate for semen collection. On the other hand, a young bull with a SC that is 2 or more standard deviations below the mean for its age is considered to have testicular hypoplasia. A determination of testicular hypoplasia at 12 months of age or older suggests the bull will have low sperm production capabilities as a yearling and as an adult. Such bulls should be culled.

Incomplete testicular descent

When conducting a reproductive examination, the conformation and symmetry of the scrotum should be carefully assessed. One cause of testicular asymmetry is incomplete testicular descent, an uncommon congenital defect in bulls. The affected testicle is retained in the inguinal canal or is only partially descended into the scrotal neck. Complete descent into the scrotum of a retained testicle is uncommon after 8 to 12 months of age. The incompletely descended testicle does not grow normally or have normal spermatogenesis. Such bulls should be rejected from an AIC breeding program.

Testicular degeneration

Testicular degeneration is associated with a dysfunction of spermatogenesis. Its development may be suggested by a decline in the percentage of normally motile spermatozoa, or by palpation of a testicle that has less than normal resilience. A common feature of testicular degeneration is an elevation of testicular temperature. Testicular degeneration may be transitory or permanent.

The most common cause of transitory testicular degeneration is 'summer infertility', that is, the inability of individual bulls to adequately regulate testicular temperature during the months when there is elevated ambient temperature and humidity. Similarly, some bulls with a marked febrile response to systemic infection may develop a transitory testicular degeneration. The degeneration in these bulls is typically bilateral.

Unilateral testicular degeneration associated with traumatic injury to the scrotum is infrequently observed in dairy bulls maintained in groups. Blunt trauma to a testicle results in hemorrhage and a large scrotal hematoma. Initially the scrotum is markedly distended on the affected side. Resolution of the injury occurs during the next several months by resorption of the clot, followed by fibrosis and complete degeneration of the affected testicle. Transitory degeneration is probable in the opposite testicle from the heat of local inflammation. Interestingly, however, the unaffected testicle may eventually increase in size, a phenomenon referred to as compensatory hypertrophy.

Pathology of the epididymis

The epididymis is a long, convoluted tubule in which spermatozoa mature and are stored prior to ejaculation. A congenital abnormality or acquired pathology may block passage of the spermatozoa from the associated testicle. For example, segmental aplasia of the epididymis is an absence of a part or all of one epididymis. It is usually unilateral, occurs infrequently, and is readily palpable when it involves the tail of the epididymis. Another possible problem is a congenital defect of the efferent ductules, which are located near the dorsum of the testicle. A congenitally blocked efferent ductule may result in a sperm granuloma, or severe inflammation of the head of the epididymis. Yearling bulls affected with either problem should be culled.

Vesiculitis

The primary accessory genital organ in bulls is the seminal vesicle (vesicular glands). Infection or inflammation of the vesicle usually results in contamination of semen with pus, or infrequently with blood. Treatment with broad-spectrum antibiotics and anti-inflammatory medications may provide transitory benefit, but a long-term cure is difficult to achieve. For this reason, some AICs cull bulls affected with vesiculitis. An alternate treatment is surgical resection of the vesicles; however, treatment success is at best 50%.

A congenital defect of the vesicular ducts at their termination in the pelvic urethra has been reported to be associated with vesiculitis in yearling bulls. This noninfectious etiology suggests that affected yearling bulls that do not promptly respond to medical treatment should be culled.

Semen Collection Management

The primary goals of a semen collection program are to optimize seminal quality and maximize spermatozoal harvest. While maximizing the quantity of spermatozoa collected on any one day is important, bull handling and semen collection techniques should be conducted to maximize production over the life of the bull. This includes preventive measures to reduce the risk of injury to the bull's musculoskeletal system and to its penis and prepuce. The program must also include provisions for safety of the semen collector and bull handler.

A bull's experiences in the semen collection area should be favorable. The bull should be encouraged to mount a similarly sized stimulus animal, preferably a steer or a docile bull, directly from the rear. Negative behavioral factors such as excessive movement of the stimulus animal or violent jerking of the lead rope should be avoided as much as possible. Good footing for the bull is also important so its feet do not slip when it mounts. A clay floor provides good footing, but some managers prefer shavings on concrete floors, or rubber mats.

To maximize the collection of spermatozoa the bull is stimulated to mount, but during its initial mount the bull is not collected. This is a false mount. Subsequently, the bull will again be stimulated to mount but actively restrained from mounting. These actions of false mounting and active restraint are repeated once or twice. When the bull next mounts, a properly prepared and lubricated collection device (artificial vagina) is applied to the bull's penis. Following a brief resting period, the procedure is repeated. This method of sexual preparation and semen collection has been demonstrated to markedly increase spermatozoal harvest.

Seminal quality is optimized by having the bull service, only once, a clean semen collection device. The collection device should be constructed to provide optimal temperature (56–60 °C) and sensitivity to the penis of the bull, but permit the semen to be ejaculated into a cone-shaped liner positioned beyond the heated region of the collection device. To prevent cold shock of the semen, an insulated jacket that has been previously warmed to 35 °C is applied over this liner. Following collection of the semen, the collector removes the tube with the semen, applies a label with the bull's name and number, and passes the semen to the laboratory technicians who initiate the seminal evaluation and processing procedures.

Precautions to reduce the risk for transmission of genital disease among bulls include the single use of a semen collection device on any day, after which it is dismantled, washed, disinfected, rinsed and dried. The collector should also wear disposable gloves, which are discarded after each seminal collection. Finally, the area of the stimulus animal that may have been contacted by a bull's penis should be disinfected between each bull.

For the better management of bulls, a form to record sexual behavior data should be used. The form should include items such as date, time from initial stimulus to each seminal collection, stimulus animal, location, and bull health observations. Maintaining maximal seminal harvest from each bull will require adjustments in how each bull is prepared for semen collection. Accordingly, a record of previous events provides an important database from which accurate decisions can be made.

See also: **Bull Management**: Dairy Farms. **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis; Infectious Diseases: Johne's Disease; Infectious Diseases: Tuberculosis. **Gamete and Embryo Technology**: Artificial Insemination. **Nutrients, Digestion and Absorption**: Fermentation in the Rumen. **Reproduction, Events and Management**: Mating Management: Artificial Insemination, Utilization.

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Dairy Farms

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Introduction

While artificial insemination (AI) is widely used in most dairy herds, bulls are commonly used to mate cows that fail to conceive to AI after several cycles. In many seasonally calving dairy herds, herd sires may be used to breed a few selected animals during the artificial breeding season (e.g., cows that are to be culled at the end of the season) and then will be run freely with the herd for periods varying between 6 and 15 weeks after the end of the artificial breeding program. In some production systems associated with seasonally calving dairy herds, many of the cows that conceive to natural service will be induced to calve prematurely; hence, the breeding merit of the natural sire is of little relevance. In many dairy herds, heifers are not mated by artificial breeding and bulls are used to serve the maiden heifers. Furthermore, in a few herds, AI is not used at all and bulls are used to mate all of the cows.

With year-round calving herds, bulls are commonly used as a supplement to an AI program. Individual cows are separated from the herd when in estrus and placed with the bull. Bull matings may be used for cows that have repeatedly failed to conceive to AI, or for cows of lower genetic merit whose offspring will not be retained for herd replacement purposes.

It is often found that satisfactory reproductive performance is obtained in the herd during the artificial breeding period, but that the reproductive performance of the cows that fail to conceive to artificial breeding is poor during the subsequent bull mating period. One possible reason is that among the cows that are not pregnant at the end of the artificial breeding period, there is a core of ‘difficult to breed’ cows. Another reason may be that bull management has not been optimal. Thus, there is a need to pay due attention to the health and management of bulls when they had turned out with the herd.

The breeding management of herd sires must ensure that they detect the few animals that come into estrus within a herd of mainly pregnant cows, that they can competently serve these estrous cows, and that they can achieve satisfactory pregnancy rates at these services.

While there are a range of definitions of the word ‘fertility’, the Australian Association of Cattle Veterinarians has adopted the definition most appropriate to the bovine: ‘an animal is fertile when it is able to reproduce prolifically’.

It is recognized that there is a spectrum of fertility from very high to very low.

- Fertile bulls can impregnate (pregnant at day 42 of gestation) by natural service at least 60 and 90% of 50 normal, cycling, disease-free females within 3 and 9 weeks, respectively.
- Subfertile bulls can achieve pregnancy by natural service, but not at the rate achieved by fertile bulls when the opportunity exists.
- Infertile bulls cannot achieve pregnancy.

Bull Nutrition

Low energy intake during the first year of a bull’s life may reduce testicular growth with a subsequent delay in the onset of puberty, delay the onset of semen production, and may result in a smaller-sized bull at puberty. Sperm production is also lower in bulls that are underfed up to 2 years of age. Conversely, overfeeding energy to young bulls can reduce reproductive performance due to increased fat deposition on the scrotal wall or in the neck of the scrotum around the spermatic cords with a deleterious effect on the efficiency of testicular thermoregulation. In one study, 12-month-old bulls fed high-energy diets until 21 months of age had reduced epididymal sperm reserves, lower percentages of progressively motile sperm, and higher percentages of sperm abnormalities compared with bulls fed a medium-energy diet. Severe reduction of protein in rations fed to young bulls may decrease sperm production capacity.

With mature bulls, continued high energy intakes can result in excessive weight gain leading to reduced sexual activity and additional strain on the bull’s feet and legs. Overweight bulls may also accumulate fat at the base of scrotum and this can adversely affect thermoregulatory function in the testes. In mature bulls, severe undernutrition reduces sperm production potential, and this reduction can persist for some time after nutrient restrictions are removed.

Gossypol, the primary toxic principle found in whole cottonseed and cottonseed meal, may cause testicular damage and subsequent reduced reproductive performance in bulls.

Evaluation of Health and Fertility

On many dairy farms, bulls are not subject to a breeding soundness examination of any type before they are run with the cows or heifers. The true test of a bull's breeding soundness is the demonstration of his ability to cause conception in adequate numbers of fertile cows.

A bull soundness examination attempts to estimate a bull's suitability for breeding. In the dairy farm situation, a large percentage of abnormal bulls will be detected by a basic examination consisting of

- measurement of scrotal circumference,
- clinical examination of the genitalia,
- assessment of the conformation of the bull (with emphasis on legs and feet), and
- observation of coitus.

Scrotal Circumference

Scrotal circumference is measured using a metal scrotal tape that is formed into a loop and slipped upward around the scrotum and then tightened snugly around its greatest diameter. The testes are pulled firmly into the lower part of the scrotum by encircling its base with the hand and pulling down on the testes. The thumbs and fingers should be located on the side of the scrotum rather than between the testes so as to prevent separation of the testes and inaccurate measurement. The tension of the sliding tape should be enough to bring the testicles close together and to cause contact of the tape and skin for the entire circumference (the scrotal skin will be slightly indented by the tape).

Scrotal circumference is highly correlated with testicular weight, which in turn reflects sperm production in normal testicles. Testicular size has a high heritability, and is also affected by age, breed, and nutritional conditions. In bulls over 18 months of age, a scrotal circumference less than 30 cm indicates that adverse influences have affected testicular growth. Under conditions of moderate-to-good nutrition and in *Bos taurus* breeds, a 2-year-old bull with a scrotal circumference of less than 30 cm has a high probability of having testicular hypoplasia or degeneration, poor semen quality, and low fertility.

Recommendations for scrotal circumference in *Bos taurus* bulls are as follows:

- A scrotal circumference of 30 cm is recommended as a minimum for 12- to 15-month-old bulls.
- A scrotal circumference of 32 cm is recommended as a minimum for 18-month-old bulls.
- A scrotal circumference of 34 cm in 2-year-old bulls is considered as the minimum standard acceptable for sale.
- A scrotal circumference of 34 cm or greater in mature bulls indicates that testicular development is adequate.

The scrotal circumference measurement is a useful method for eliminating bulls whose sperm-producing capacity is likely to be too low to achieve adequate results in the field.

Physical Examination of the Scrotum and Its Contents

Testes should be symmetrical and nearly identical in size. Asymmetry of the testicles may be due to one testis being enlarged, atrophied, or hypoplastic.

Testicular consistency should always be assessed. Consistency reflects the composition of the parenchyma and the enclosing tunics. It has two components, firmness and resilience. Normal testicle tissue is firm and resilient (springy). Abnormally soft or flabby testicles are usually associated with testicular dysfunction or degeneration. Abnormally firm testicles, and testicles with a low degree of resilience, may be indicative of fibrosis or calcification.

The head, body, and tail of the epididymis on each side should be palpated as well as the spermatic cords and the scrotal skin. The head of the epididymis may be enlarged as a result of inflammation or sperm granulomas, which may prevent sperm transport and result in a small, flaccid, empty tail of the epididymis. The tail of the epididymis of a normally functioning testicle is turgid and prominent at the base of the testis. Differences in size and consistency between the left and right epididymis may indicate inflammation or may indicate a blockage of sperm transport. The spermatic cords should be palpated from the body wall down to the top of the testis to assess scrotal fat and to detect abscesses or the presence of a scrotal hernia. The scrotal skin should be thin, cool, and pliable.

Examination of the internal genitalia involves a rectal examination whereby the seminal vesicles, the ampullae, the prostate, and the inguinal rings can be palpated. The most common abnormal finding on rectal palpation is enlargement and excessive firmness or loss of lobulation of the vesicular glands.

Physical Examination of the Penis

The level of examination of the penis varies between

1. Palpation of the penis from the sigmoid flexure to the tip through the skin of the sheath. Gross lesions, if present, will be detected.
2. Protrusion of the penis and inspecting it in an extended position. This can frequently be achieved by massaging the seminal vesicles per rectum or during the process of electroejaculation. Most lesions of the penis and prepuce will be detected, but not deviations that are manifest only in the erect state.
3. Examination of the erect penis during serving. This allows the function of the penis to be assessed and will

allow the detection of deviations of the penis and difficulties with intromission. This is the preferred level of examination. Two types of penile deviation may be observed – premature spiral deviation of the penis (corkscrew penis) or ventral deviation of the penis. Premature spiral deviation is the most common penis defect in polled beef bulls. With ventral deviation of the penis, the free part of the penis curves downward and prevents intromission.

The protrusion of the penis can be effected in the great majority of bulls if an assistant passes a gloved hand and forearm into the rectum. Relaxation of the retractor penis muscles generally occurs. This facilitates a two-handed action by the operator, pushing the preputial skin back, grasping the penile shaft through the skin between the preputial opening and the scrotum, and pulling the shaft forward. While the shaft is held firmly in that position, the skin of the sheath close to the preputial orifice is pushed back and the protruding glans of the penis can then be grasped with the other hand using cotton gauze to prevent slipping.

Semen Examination

Semen examination may allow the detection of bulls that are producing abnormal semen, but which are normal on clinical examination. However, the cost-effectiveness of semen testing of all dairy bulls to find the fairly small number of bulls that fit into this category can be regarded as debatable.

Semen collection can be done in four ways:

1. artificial vagina;
2. electroejaculation;
3. massage of the seminal vesicles and ampullae; and
4. collection of a postservice drip sample.

While electroejaculation is usually preferred for bulls not trained for the artificial vagina, the artificial vagina is recommended as the method most likely to provide a representative semen sample. The results of the other methods of semen collection need to be interpreted carefully. When any of them provides a sample of good quality, then this can be accepted as a representative sample from the bull. When the quality is not good, then further efforts should be made to ensure that a sample is collected that is representative of the bull at the time. This will usually involve the collection of a further sample immediately.

Crush-side examination of semen allows for evaluation of semen density, color, and motility. Experience in the United States, Canada, and Australia suggests that an acceptable minimum threshold for bulls used for natural service is more than 30% progressively motile sperm. There is good evidence that sperm morphology is related to fertility, and thus, examination of sperm morphology is a useful additional test that can be applied.

Observation of Coitus

It is essential that coitus is observed. It is preferable that this observation be undertaken by the veterinarian examining the bulls, but in many cases this will be done by the herd manager. The manager should be instructed to continue observing the mating behavior of bulls throughout the joining period to check that they continue to be able to perform coitus (injuries may occur at any time during the breeding season, which may render a bull incapable of coitus). The observation of coitus provides information about the libido of bulls and also allows the detection of the presence of anatomical defects of the penis (e.g., penile papillomas and penile deviations).

Serving behavior can be assessed in two ways:

1. Observation of a bull's serving is called a serving ability examination and includes an assessment of libido, erection of the penis with respect to stiffness and protrusion, seeking movement, ejaculatory thrust, and body position.
2. The serving capacity test is the term used for a quantitative assessment of a bull's serving performance during a set time period after sexual stimulation and over restrained females. It assesses libido, serving technique, and the capacity for sustained serving activity in a herd situation. The serving capacity test has been widely used in the beef industry, but the smaller number of bulls available for testing on many dairy farms makes this test more difficult to undertake in the dairy farm situation.

Management of Bulls during the Mating Period

Because of the relatively limited use of bulls in many dairy herds, bulls may not be as carefully managed as may be appropriate.

Selection of bulls should be made at least 60–90 days before the breeding season. Appropriate vaccination, anthelmintic treatment, nutritional acclimatization, and breeding soundness examination should be included in this adaption process.

Because spermatogenesis requires 60 days for completion, this is the minimum period that should be allocated for preparing bulls for the breeding season. To facilitate establishment of a social pecking order among the sires, exposure to other sires in the herd is essential before bulls are turned out together. If a pecking order is not allowed to develop before the breeding season, the first 21 days of the season can be consumed in establishing the pecking order rather than in servicing the cows.

Young bulls need to be fed so that they are well enough grown to mate when required. Older bulls need to be managed so that they do not become overfat between mating seasons. They should receive similar vaccination programs as other animals in the herd and receive routine anthelmintic treatments as appropriate.

Yearling and 2-year-old bulls do not have the semen capacity for use in estrus synchronization programs, nor should they be relied upon as 'follow-up bulls' after AI where large numbers of females are expected to return to estrus.

Sexual activity of bulls is greater when several sires are run with a group of cows; however, dominant bulls will tend to serve more cows than the subdominant bulls. In multisire breeding situations, the sexual activity of bulls can be strongly influenced by their social position in the bull group. More dominant males may monopolize estrous females even though they may have poor libido, mating ability, or semen quality. A major consequence of dominance is that low conception rates can occur in group-mating systems when the dominant bull is subfertile. Fighting among bulls is reduced if the bulls are of similar size and age, and have been run together before they are turned in with the cows or heifers.

In general, the reproductive performance of bulls increases between 1 and 3 years of age, but then begins to decline beyond 4 years of age. This decline appears to be due to decreased libido and the detection of fewer animals in estrus. Loss of efficiency of bull use undoubtedly occurs when bulls of different ages are run together.

The breeding management objectives that require the use of herd bulls will be best achieved using a team of young sexually active bulls that have each received a veterinary examination for breeding soundness before running with the cows in the herd. At least two bulls should always be with the herd, and implementing a rotational roster will contribute to maintaining sexual interest.

Selection of Bulls for Use

Selection of breed of sire for use with heifers, or for mating those cows not pregnant at the end of the AI program, should be carefully considered. Larger-framed breeds of bulls may be associated with increases in dystocia due to fetopelvic disproportion. This may compromise the viability of their calves or may increase the risk to the dam. A number of studies have shown that even moderate degrees of dystocia have been associated with subsequently decreased reproductive performance of the affected animals. Thus, it is preferable that sires should be selected so as to minimize the risk of dystocia due to fetopelvic disproportion.

In situations where the value of the newborn calf is low relative to the value of the potential milk production of the cow, the use of a bull that will sire small calves is economically justifiable. In this situation, Jersey bulls are very often used on heifers, and as 'follow-up' bulls for use with the herd after the end of the AI program.

Where the value of calves is much higher, there may be more justification for using a sire that will produce a larger calf, which is of more value as a replacement animal in the production system or as a beef animal in an alternate production system.

Numbers of Bulls Required

The workload required to be performed by the bulls when they had turned out with the dairy herds at the end of the artificial breeding season depends on the success of the artificial breeding program and whether or not any form of estrus synchronization was used in the herd.

There is a lack of information regarding the ratio of bulls:cows that should be used in dairy herds. A general recommendation is to allow 1 bull per 30 nonpregnant cows or heifers. Although data from a number of studies with beef cattle indicate that the ratio of 1 bull per 30 cows can be exceeded, care needs to be taken where estrus synchronization programs have been employed in large herds. In such herds, a large number of animals can return to estrus on the same day and this may place unreasonable pressure on the available bull team.

Many farmers with larger herds prefer to have a number of herd sires available in reserve. These are commonly used on a rotational basis with groups of bulls being removed and replaced with fresh bulls. It is commonly recommended that groups of bulls should be replaced every 7–10 days with rested bulls if the procedure is to have any significant effect on reducing the risk of subfertility; changing at 3-week intervals has little or no beneficial effect. Some dairy herd owners have found that changing groups of bulls as often as every 3 days is of benefit. A potential benefit of bull rotation is that there is a succession of sexually rested bulls available for mating. As frequent ejaculation over a period of weeks may reduce sperm density in the ejaculate, regular provision of rested bulls minimizes the risk of cows not being adequately inseminated.

Heat Stress and Bull Fertility

Heat stress during summer months can be severe enough to impair bull fertility, as an adverse effect of increased ambient temperature on semen quality has been determined in many studies. In one study, two Guernsey bulls were exposed to 37 °C and 81% relative humidity for 12 h per day for 17 consecutive days. Approximately 30–40% of the spermatozoa were morphologically abnormal (mostly coiled tails and detached heads) and the total number of spermatozoa, sperm concentration, and sperm motility decreased profoundly. In another study, an ambient temperature of 40 °C at a relative humidity of 35–45% for as little as 12 h reduced semen quality. *Bos taurus* bulls are more susceptible to high ambient temperatures than *Bos indicus* bulls.

Heat stress impairs bull fertility by decreasing sperm concentration, lowering sperm motility, and increasing the percentage of morphologically abnormal sperm in an ejaculate. Individual bulls may produce very acceptable semen in the face of heat stress, while others are more severely affected. The deleterious effect of heat stress on semen quality reaches its peak 2–3 weeks after the period of heat stress. Once heat stress occurs, semen quality does not return to prestress levels for approximately 2 months. The effect of prolonged heat stress on bull fertility may interact with reduced conception rates of cows experiencing heat stress to further decrease herd fertility.

The effect of heat stress on libido is less well documented. In one study, semen quality in dairy bulls was reduced by continual exposure to an ambient temperature of 86 °F for 5 weeks or 100 °F for 2 weeks with no apparent effect on libido. Other workers believe that extended periods (weeks) of heat stress may reduce bulls' libido.

The effect of heat stress on bulls may be minimized by attention to good breeding management. This includes the provision of adequate supplies of cool water, providing access to shade, maintaining bulls in good, but not excessively obese, body condition, and ensuring that bulls are not overworked during hot periods of the year.

Monitoring Bull Performance during Their Mating Program

The producer must continually observe bulls during the breeding season to monitor libido and serving ability of bulls. Social pecking order and physical incapability to perform service (as a result of hematoma or spiral or ventral deviation of the penis) can be detected. Penile deviation develops in some 3- and 4-year-old bulls. After 1–2 years of satisfactory service, such bulls can develop deviations that prevent intromission. These defects, and similar problems, can be detected only by observing mating.

Injuries that render a bull incapable of service are not an uncommon occurrence during the mating season – it is essential that these injured bulls are detected at an early stage so that they can be removed from the herd and replaced by a bull that is capable of performing service. Careful observation of the cows during the mating period should detect if there are an excessive number of cows returning to estrus after service.

Lameness in Bulls

Lameness in bulls can be a particularly important issue. If bulls are run with the herd, and allowed into the concrete milking yard, they frequently suffer excess hoof wear and severe lameness can result. It is strongly recommended that bulls be drafted from the herd at milking time and not allowed into the concrete yards.

Severe lameness will impair the bull's ability to serve and can result in testicular degeneration and reduced fertility. Similarly, malformation of the claws may result in discomfort during mounting so that the bull either refuses to mount or may dismount without ejaculating.

Health and Disease Control

The individual herd bull can be exposed to all of the communicable diseases within the herd and can serve to transmit specific venereal diseases such as campylobacteriosis (vibriosis) and trichomoniasis. Bulls should not be forgotten when vaccination programs are being developed for dairy farms and their vaccination schedules should be strictly maintained.

As a means of controlling disease, only virgin bulls should be introduced into a herd. Visual examination of normal and abnormal conformation characteristics of the hind legs and hooves greatly contributes to a bull's longevity and usefulness. Traveling ability is an important component of activity in large dairy herds at pasture.

Campylobacter fetus subspecies *venerealis* and *Trichomonas fetus* infections are well known as the major venereal diseases of cattle. Furthermore, there are a number of other conditions that, while not behaving as true venereal diseases, are nevertheless sexually transmitted causes of impaired fertility. Examples of such diseases include infectious bovine rhinotracheitis–infectious pustular vulvovaginitis (IBR-IPV), bovine virus diarrhea (BVD), and infections caused by *Histophilus somni.*

Purchase of virgin bulls only reduces the risk of introduction of such diseases. Vaccination against infectious agents such as *C. fetus* subspecies *venerealis* further reduces the risk of such diseases causing economic loss on farms.

There are advantages in checking the BVD status of bulls prior to their introduction onto a property. Persistently or freshly infected bulls may infect cows at the stage of pregnancy at which they are vulnerable to BVD-induced early embryonic death. It is commonly recommended that tested-negative bulls should be fully vaccinated against BVD prior to the start of mating.

See also: **Bull Management:** Artificial Insemination Centers.

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BUSINESS MANAGEMENT

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Roles and Responsibilities of the Manager

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Introduction

This article provides an overview of business management for a commercial dairy farm. Topics discussed include the roles and responsibilities of a manager, namely, planning, implementing the plan effectively, and monitoring and evaluating dairy herd and farm financial performance. The information presented describes recommended practice for commercial dairy farms in developed countries and similar farms in developing countries. However, the underlying principles apply to dairy farms of any type, size, or geographical location.

The Role and Responsibilities of the Dairy Farm Manager

Modern dairy farming is a business and the primary decision makers of a dairy farm, the managers, have three essential roles or responsibilities. These are developing a plan for the business, implementing that plan effectively, and 'controlling', a term used to describe monitoring and evaluating the performance of the business. If the farm is to achieve the owners' goals, these responsibilities must be discharged regardless of farm size or business structure and organization. Furthermore, given the continuous change that occurs in dairy farming, these are ongoing responsibilities.

Clearly, the planning, implementing, and controlling functions of management are interrelated. To successfully achieve the goals of the business, there must be a sound plan that is implemented effectively, producing results that are measured and compared to the performance standards set in the plan. If the results are unsatisfactory, then both the soundness of the original plan and the effectiveness with which it is being implemented must be evaluated and changes must be made to enhance the performance. Therefore, managers must also be skilled at problem solving and decision making.

Managers of dairy farms typically must fulfill the above three roles in three broadly different aspects of the farm operation: production, marketing, and finance. Each of these requires different skills and knowledge. Production management is concerned with what the farm will produce, how much it will produce, and how and where those products will be produced. The marketing of raw milk is regulated in many countries and marketing management may be limited to choosing a milk buyer and managing price risk. However, the scope of marketing activities is much broader for farms that process their milk and sell dairy products. Financial management refers to the overall financial performance and health of the business, including profitability, solvency, liquidity, and debt management.

Dairy farming is a demanding occupation. The trend in developed countries is for fewer but larger farms. In many countries, the nonfarm population is demanding changes in farming practices to protect or enhance the quality of the environment. Consumers are becoming more concerned about farming practices and how their food is produced. These changes in social and consumer concerns have led to restrictions being placed on some farmers and have created new market opportunities for others. Changes in trade policies and increased globalization have created new or expanded markets for some and new competition for others.

New production technology provides both new opportunities and new challenges. The Internet provides access to huge amounts of information and places farmers who do not use it effectively at a disadvantage.

Managers need to have knowledge and skills in many areas, including the various aspects of animal husbandry, crop husbandry, human resource management, nutrient management, information management, record keeping and analysis, problem solving, and decision making. Accurate and timely information is needed to monitor the important aspects of farm performance and for planning purposes. This information must include farm production, marketing, and financial data. High levels of interest, knowledge, and skills in animal husbandry and related areas of farm production are essential for success, but, by themselves, they are not sufficient to ensure success. To be effective, the manager must either possess the necessary knowledge and skills for planning, implementation, and control or obtain them from sources outside the business. Management specialization is an option in a large herd. The operator of a small farm is often responsible for all the roles and activities of a manager and may have additional responsibilities that compete for time and energy, such as herd management and farm work.

Planning

Planning begins with setting clear business goals and then determining the type of business needed to achieve these goals. Public stock companies focus on financial goals such as profitability and share value. However, most dairy farms are family owned and operated, and each of the family members has personal, family, and business goals. Some of these goals will be financial in nature, including an income for family living, both for the present and the future, and building family wealth and financial security. However, there are likely to be many other goals related to lifestyle, community service, and involvement in farm or nonfarm organizations. Any differences or conflict among these goals must be reconciled and priorities set before an effective business plan can be devised. Any plan to achieve the agreed-upon goals must recognize and incorporate certain financial realities and the demands they place on the farm business. These include profit potential, cash flow feasibility, and the level of family income desired for personal and family use.

In broad terms, the plan specifies what the farm will produce, how much it will produce, where and how those products will be produced and marketed, and how the farm finances will be structured in order to achieve the desired results. The farm plan must take account of the economic, social, and political environment within which it must operate, the available resources, and reasonable expectations for the financial performance of the various farming alternatives. The specifics of the farm plan imply certain levels of performance or targets that must be achieved if the farm is to succeed.

In an existing business, planning may simply involve making routine decisions about farming activities day-to-day or for a period of weeks or months to ensure that the farm operates efficiently. Similarly, making financial plans and projections for the coming year based on expected production levels and prices in order to determine expected profits and credit needs should be routine. Short-term planning of this type can also help determine the expected financial contribution of the farming operation to family living needs and as an aid in income tax planning. This type of planning to enhance the smooth running of an existing farm is called tactical planning, which is borrowed from military terminology.

In contrast, from time to time all farm businesses make major decisions that set the course of that business for years to come. Long-term or strategic planning may be stimulated by new opportunities, by serious problems with an existing business, or by changes in family circumstances. Plans may include major investment decisions such as starting a new dairy operation, expanding an existing dairy operation, remodeling an existing dairy facility, and buying land. Changes in the enterprise mix, whether by adding or reducing the number of farm activities, can cause major changes in income, expenses, investments, debt load, and cash flow. Retirement planning and planning for the intergenerational transfer of farm assets also imply major changes in the financial structure and performance of the farm business.

There are several steps to effective strategic planning: (1) setting clear business goals; (2) taking an inventory of farm, financial, and human resources; (3) analyzing past and projected performance; (4) identifying alternatives; (5) assessing the external business environment; (6) evaluating the production, marketing, and financial feasibility of alternative courses of action, including a risk assessment; (7) making a decision; (8) specifying how the plan will be implemented; and (9) specifying how farm performance will be evaluated.

Goal setting is discussed above. Farm resources are likely to be unique to each farm situation. The planning and controlling functions of management are discussed in some detail in the article **Business Management**: Management Records and Analysis. The alternatives open to a particular farm family or business will be influenced by the resource base and the skills and interests of the primary decision makers. The external business environment is affected by many factors including national economic, trade, environment, farm, and food policies and regulations. Laws governing property ownership, business transactions, and banking contribute to the business environment. Other factors include consumer preferences, the availability and prices of competing foods, and the structure of the dairy and food industries.

All of these factors have a major impact on the nature of the operation and profit potential of a farm business.

Establishing production, marketing, and financial feasibility requires a lot of information. The choice of production technology drives farm and financial performance.

There is great diversity among dairy farming systems, including farm and herd size, breed of cow, and feeding, housing, and milking systems. Assessments of long-term profitability and competitiveness include farm and financial performance expectations for one farm type relative to competing technologies when viewed from a local, national, and global perspective. It should be noted that there are wide variations in performance among similar types of farms and between farms of different types and farms located in different regions. Research data and measures of performance from other farms provide data on the reasonableness of performance assumptions. When available, measures of a manager's past performance provide some information about the level of performance to be expected under a new system. In some plans, production and marketing feasibility must be considered simultaneously.

Marketing decisions include what product or products to sell, where to sell those products, and at what price those products should be sold. Most dairy farmers in developed countries sell raw milk in markets that are heavily regulated. Marketing decisions may be limited to the choice of milk buyer. Factors to consider when choosing a buyer include prices paid relative to competitors, market security, and the financial health of the buyer. Some farmers will opt to sell to a producer cooperative over a proprietary firm for philosophical reasons and to increase their market and political power. Other decision factors include any ancillary services and products provided by a milk buyer.

Dairy farmers who process their milk face additional marketing challenges. Market research is required to identify a customer base and their preferences. Research on competing firms and products helps define the opportunities for various types of products, package types and sizes, and market channels. Most importantly, this research helps determine the size of the potential market, competitive price levels for the various products, and whether specific market opportunities are likely to be profitable.

Risk is inherent in agriculture and in decision making. The sources of risk are many and varied. Adverse weather and other natural phenomena affect production on an individual farm, but on a global scale they affect world production and farm prices. Changes in technology can render production methods and farm assets obsolete, modify costs of production, and drive changes in farm structure and regional shifts in production. Social attitudes and consumer preferences affect the market for dairy products and influence the regulations governing production practices. Government policies and institutions affect farm prices for both milk and purchased inputs and influence production practices. Individuals and their behavior can enhance farming operations or create problems for the farm and the family. These risks, occurring alone or in combination, can have a dramatic effect on individual farm performance and viability.

Risk management involves prioritizing risk based on the likelihood that a specific event will occur and the financial impact on the business if that event did occur. The highest priority is given to the risks that are more likely and could have a greater adverse impact on the farm. Risk management strategies can attempt to reduce the likelihood of an event, for example by diversifying crop activities or spreading production geographically, by building excess equipment capacity, and by carrying excess inventory. Investing in skills, knowledge, and information can also be a type of risk management strategy. A second approach to risk management is to transfer the risk. Examples include buying insurance against potential loss or damage and using futures markets to manage price risk. Risk management alternatives incur costs and provide different levels of protection. Therefore, the costs and benefits of alternative strategies must be weighed.

Decision making is an essential component of planning. Various tools can be used to evaluate the likely financial consequences of a potential decision. Budgeting is used to evaluate profitability. Partial budgets look only at changes in income and costs between alternatives that do not fundamentally change the business. Enterprise budgets look at the revenue, costs, and net returns associated with adding, dropping, or modifying significantly a specific farm enterprise. Whole farm budgeting is used to evaluate the profitability of a proposed new farm operation or when major restructuring of an existing business is being contemplated. Net present value is another technique for analyzing the profitability of major new investments and it is particularly useful when the costs and returns vary considerably from year to year over the life of the project. Cash flow projections are needed in addition to any assessment of profitability. Projecting the timing and amounts of required investments and expected revenue and expense is needed to determine credit needs, debt repayment capacity, and overall feasibility.

Sensitivity analysis is a method of assessing the riskiness of a farm plan. It involves calculating and evaluating the impact of different assumptions about important production levels, prices, and costs on farm financial performance and feasibility. Other risk assessment techniques use the probabilities associated with different production and price levels and the farmers' risk preferences as an aid to decision making. The 'best' decision will depend on family goals and preference, ability to bear financial risk, and attitude toward risk.

One aspect of planning concerns the legal form or structure of the business. Several factors affect this decision, including laws and regulations governing legal liability and taxation. Other aspects include the effects on the availability and cost of capital, operating efficiency, and the equitable treatment of investors.

Strategic planning requires a great deal of skill, information, and time. For these reasons, and because strategic planning is undertaken infrequently, many dairy farmers use outside advisers to assist them.

Implementation of the Plan

Implementing the farm and business plan effectively means acquiring the necessary farm and financial resources, staffing the farm, training and supervising the workforce (including family members), scheduling the work, using outside advisers and services effectively, making decisions, and solving problems as they arise. These activities consume the largest part of a farm manager's time.

Dairy farming requires various types of farming and financial resources. Farming resources include land, facilities, livestock, machinery, and equipment, which may be owned, rented, or leased. People are needed to operate the farm. Financial resources include investment and working capital. Several factors affect decisions about the asset structure of the farm business and how those assets are controlled and financed. These include the initial equity position of the primary operator or investor, the returns expected from investing in various types of farm assets, the availability and cost of borrowed money, risk, potential tax liability, laws affecting various aspects of asset ownership and farming operations, and the specific financial performance goals of the business.

There is a trend toward a larger farm size, and human resource management is becoming increasingly important. Staffing a farm begins with defining the various job responsibilities, levels of authority and responsibility, and lines of communication. Specific job descriptions and a compensation package must be developed for each position. Finding qualified workers begins with effective recruitment to develop a sufficiently large pool of candidates. Effective interviewing techniques are needed to identify the most qualified individual and to convince that candidate that this is a desirable position. The manager is responsible for knowing the provisions of laws and regulations that apply to hiring and employment. As the dairy industry becomes increasingly global in nature, managers may need foreign language skills and a cultural awareness of and sensitivity to nonnative employees.

Employees, including family members, require training to develop or improve their skills and increase their effectiveness and productivity. For new employees, this training should begin with an orientation session on arrival at the farm. Effective training involves several steps, including explaining the purpose of the training, a clear demonstration and explanation of the desired procedures, and a trial period under supervision. Those in supervisory positions have the responsibility of monitoring and evaluating employee performance, conducting corrective interviews, providing retraining and, when necessary, terminating employment.

Management skills and knowledge are learned and the manager must set aside time for personal development. This requires a broad understanding of all the management functions and a self-assessment of areas of strength and weakness. However, it is not necessary that the manager must possess all the knowledge and skills and, increasingly, dairy farm managers rely on outside advisers and services to help them plan and operate the farm effectively. Veterinarians, nutritionists, engineers, extension educators, business consultants, and other dairy farmers can supplement the manager's knowledge and skills in specific areas of herd, farm, and business management. Lawyers, accountants, and government agency personnel provide specialized services.

Controlling

Controlling means setting standards or targets for various important aspects of farm performance and then measuring and comparing actual performance with these standards. Record keeping for this purpose is discussed more fully in the article **Business Management**: Management Records and Analysis. There are wide variations in herd and farm financial performance, even among herds of a similar type in a geographical region. Therefore, performance measures should include both important production and financial aspects of the farm operation. When performance fails to meet the standards or targets that have been set, this should prompt the manager to investigate the cause or causes of the problem. The cause of a problem may lie with unanticipated changes in the farm economy, flaws in the original plan, or the effectiveness with which the plan is being implemented. Regardless of the cause, if outcomes are unacceptable, the manager must take corrective action to resolve or work around the problem.

Problem Solving

Corrective action is called for when production or financial performance does not meet expectations and the farming operation is not achieving the business goals set for it. There are several distinct steps to effective problem solving. Sound problem solving calls for clearly identifying the problem and then diagnosing the root cause or causes. The cause may be external to the business, such as bad weather or poor milk prices, in which case the manager must find ways to work around the resulting situation. If the cause is internal to the business, then the problem must be the result of a person who failed to act or who acted improperly. The next step in problem solving is to identify alternative courses of action that can solve the problem and prevent a recurrence. This may be simple for routine or common problems, but for others it may take considerable effort and creativity. Serious problems may entail developing an entirely new farm plan. Evaluating alternatives may require seeking out, assembling, and analyzing a great deal of information. Incomplete and incorrect information will result in poor decisions. Making a decision should be guided by the goals set for the business and a set of criteria or procedures that will ensure that the chosen solution is the most appropriate one for achieving those goals.

Concluding Remarks

Dairy farms operate in an ever-changing business environment, which creates new challenges, problems, and opportunities. The management cycle of planning, implementation, and control must be a continuous process if business goals are to be achieved consistently. The knowledge and skills to be an effective manager can and must be learned and applied if a dairy farm is to thrive and prosper. Individuals wishing to learn more can obtain assistance from many sources, including educators, advisers, consultants, farmers, agribusiness people, and published information.

See also: **Business Management**: Management Records and Analysis. **Labor Management on Dairy Farms**. **Risk Analysis**. **Welfare of Animals, Political and Management Issues**.

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Management Records and Analysis

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Introduction

This article describes the information needed to effectively manage a dairy farm business and its importance and use in monitoring and evaluating farm performance. Few dairy farmers enjoy record keeping and analysis, but, like many other chores, it is an essential task and 'If you don't measure it, you can't manage it'. Dairy farm business management includes production, marketing, and financial management. However, all three areas are interrelated and farm record keeping and analysis must accommodate this.

Animal Identification

There are several reasons for identifying individual animals in a dairy herd, including proof of ownership, as an aid to herd management, to maintain animal pedigrees, as a marketing aid, and for regulatory compliance. Effective animal identification systems have several essential characteristics: they uniquely identify each animal, are easily seen and read, and are durable and tamperproof.

Permanent identification can be achieved through branding (heat or freeze branding, or tattooing), ear or neck tags, or implanted electronic devices. However, heat brands can be modified, freeze brands can fade, and tags can be lost, so these systems are not completely foolproof. Breed registries may require sketches or photographs in addition to other methods of identification. Implanted electronic devices are a relatively new technology and are not in widespread use. However, early indications suggest that they can be a powerful component of a herd information management system. The implanted devices can be reliable and permanent, but, because they require electronic equipment to 'read' the encoded information, they cannot fulfill all the functions required of animal identification systems. Cost is a concern for smaller herds.

Temporary identification systems for individual animals or special groups are used for specific purposes, including reproduction management, nutrition management, and identifying sick and treated cows. Paint, markers, tape, and patches can be used. Special purpose methods include electronic identification for computerized recording of production and health status, for nutrition management, and as reproduction aids.

Outbreaks of zoonotic diseases and bioterrorism threats have created interest among policy makers in national animal identification systems and databases to permit rapid traceback to the original source of infection or contamination. A growing proportion of consumers in developed countries are concerned about the origin of food products and the practices used (or not used) in production systems. The food industry has responded by imposing requirements on some suppliers. It is increasingly likely that dairy producers will be required or induced to participate in regulatory or market-driven animal identification programs in future.

There is no single or ideal identification system capable of meeting every need. The choice of identification system or systems depends on the managers' perspective on the purposes and benefits, availability, reliability, and cost. Compatibility between regulatory or marketing requirements and management needs is highly desirable.

Production Information

Production records have five main uses: to verify specific characteristics of cattle for sale (e.g., genetics, milk production, health, reproductive status, and history); to monitor individual animals and overall herd performance in order to be able to identify problems promptly; to assess the effectiveness of management changes; to provide information for sound decision making; and, for planning purposes, to provide a factual basis for making predictions of animal, herd, and business performance.

Verifying individual cattle attributes usually involves participating in third-party programs such as those administered by breed associations, herd improvement associations, and the like.

Each manager must determine what information is needed to effectively manage the various farming activities, and this, in turn, determines the record-keeping needs. Both current and historical data are needed. Current data are needed to monitor herd performance to identify problems in a timely manner and to evaluate changes in herd management. Historical data provide longer-term and more representative performance measures as a basis for decision making and planning. Information is needed on individual animals, on groups of animals, on land use, and on supplementary and nondairy farm enterprises.

Livestock records typically include a count of the number of animals currently in the herd, showing the number of animals in milk, dry cows, mated heifers, yearlings, and calves. Useful records and measures of various aspects of milking herd performance include milk production per cow, milk composition (fat and solids-not-fat or protein levels), stage of lactation, and calving and drying off dates. Herd reproduction records include cows identified in estrus, treatments to induce estrus, breeding dates, sire information, pregnancy status, and calving dates. These records allow measures of reproductive efficiency to be calculated, including heat detection rates, conception rates, and calving intervals. Records of herd health and longevity include incidences of specific disease and health events, treatment of those health events, routine preventive treatments and procedures, and death losses. Routine measures of milk quality and udder health are also needed for herd management and to meet regulatory standards. Production, reproduction, and health information on individual cows guides culling decisions. These records yield herd turnover rates and identify the reasons for cows leaving the herd, thereby helping to identify economically significant problem areas. Nutrition records include analyses of forages, feedstuffs, and rations, quantities fed and discarded, and body condition scores. This information is needed to assess the adequacy of the ration relative to animal performance goals, to help diagnose the causes of performance problems, and for calculating feed costs.

When replacement animals are raised on a farm, it is important to monitor their growth rates, body condition scores, reproduction performance, health problems, and death losses. Performance in these areas affects rearing costs and the productivity of replacement animals in the milking herd.

Crop production costs can be a significant component of total operating costs for farms producing forages and other feeds. Furthermore, the quantity and quality of homegrown feeds affects total ration costs, herd performance, and income. For these reasons, it is important to have accurate records of crop acreage(s), operating and fixed input use, crop yield(s), and analyses of crop quality, including dry matter, energy, protein, and fiber. Information on harvest, storage, and feeding losses is useful when formulating rations to ensure they are adequate to meet animal needs and when calculating feed costs accurately. An assessment of the economic value of homegrown crops should consider cost and profitability relative to purchased forages and feeds.

Resource use and output records comparable to those discussed above should be kept for all supplementary and value-added enterprises, for example, dairy steers kept for beef, on-farm processing, and cash cropping.

Financial Information

Financial records are required for management purposes and as part of normal business operations, such as payment of bills, tracking income and expenses, preparing a payroll, and making tax payments. Payroll and tax requirements vary from country to country and will not be discussed here. However, effective office procedures are needed to ensure financial transactions are handled in a timely and efficient manner and appropriate records are kept.

The record-keeping system must accommodate all routine accounting needs, but the following discussion emphasizes records and analysis for management purposes. Financial management is of particular importance in the planning and controlling functions of management (*see* **Business Management**: Roles and Responsibilities of the Manager). Financial data collected from individual farms typically show a wide variation in financial performance and health, even among farms of a similar type in the same geographical area. Furthermore, the correlation between production measures and financial performance is weak – financial performance and health cannot be inferred from production measures. This emphasizes the need for each farm to keep and use financial records for management purposes. Unfortunately, there is no global standard for the measurement of financial performance and definitions and procedures vary somewhat from country to country. However, there are general principles that have universal application.

The information needed for effective monitoring and evaluation includes both specific measures of financial performance and a basis for comparison. Just as there is no single measure that adequately describes dairy herd performance, information is required on several different aspects of farm financial performance. At the very least, a healthy dairy farm business is able to pay its bills and debts on time, make a profit, and, over time, increase the wealth (equity) of the owners. Each of these aspects – cash flow, profitability, and wealth creation – should be evaluated at least annually.

Net worth or owner's equity is the difference between the value of the farm assets and the farm liabilities on a specific date. Over time, a series of these statements show trends in asset values, liabilities, and net worth, which will help measure the progress of the business in achieving financial goals.

An annual statement of assets, liabilities, and net worth should be prepared on the first day of the financial accounting year. Assets normally are grouped based on their useful life or liquidity. Current assets include cash on hand, business savings, market livestock, crops and feed on hand, accounts receivable, and prepaid expenses.

Noncurrent assets include breeding livestock, machinery and equipment, buildings and facilities, and land. Current liabilities include accounts payable, operating loan balances, accrued interest and taxes, and principal payments due within 12 months on longer-term debt and capital leases. Noncurrent debt includes the remaining balance on longer-term debt and capital leases.

Assets should be valued both on a cost basis and at current fair market value. Valuing assets based on cost is a more conservative approach that is often used by nonfarm businesses. Asset values at cost should reflect the initial acquisition or production cost, adjusted for any depreciation or depletion. However, there can be marked differences between the values assigned on a cost basis and the prevailing market value of assets.

When assets are valued at fair market value, the net worth statement describes the solvency of the business and the amount of collateral available to support additional borrowing. Lack of creditworthiness is one measure of the vulnerability of the farm to periods of low earnings or other sources of financial stress. A comparison of asset values at cost and at fair market value shows the contribution changes in asset values have made to the owner's net worth. It also gives some indication of gains if assets are sold, which may trigger tax liabilities.

There is an element of uncertainty involved in estimating fair market value because true market value cannot be determined unless there is an actual sale. Furthermore, if farm assets were to be sold, there would be sales costs and, possibly, taxes to be paid on some of the proceeds. These so-called contingent liabilities should be accounted for to accurately estimate the solvency of the business and the equity position of the owners. Contingent liabilities should be listed along with other farm liabilities.

Dairy farm cash inflows and outflows can be uneven throughout the year because of seasonality in milk production, milk prices, and crop expenditures; because new investments were needed or assets were sold; as a result of financing decisions; and (for a family farm) because of family expenditures. An annual summary of the sources and uses of cash provides important information about this dimension of farm financial performance.

Cash flows are typically and most usefully categorized as (1) operating flows, that is, cash income and expenses associated with the day-to-day operations of the farm; (2) investing flows associated with the purchase or sale of farm assets; and (3) financing or debt-related flows, including new borrowing and loan principal repayments. For family farms, a fourth category, nonfarm income and expense flows, is helpful in completing the picture, although these items should be clearly separated from farming activity. The cash flow summary should show both the gross inflows and outflows in each category and the net flows in each category. This summary should reconcile to the change in the cash balances on hand at the start and end of the accounting period.

If the farm business is sound, the net flow from operations is positive, with the farm producing more cash in sales than it spends on expenses. Net investment flows normally are negative because the farm facilities and equipment wear out or become obsolete and must be replaced periodically. If the farm debt is to be reduced over time, a common goal, the net flows from financing will be negative. If family members draw against the farm for family living needs, then the net nonfarm flows will also be negative. Therefore, it follows that in a healthy business the positive net cash flow from operations must be large enough to offset the negative net flows associated with investing, financing, and nonfarm uses. Cash flows should be monitored on a monthly or quarterly basis and compared with projections or benchmarks in order to detect emerging problems promptly.

Farm income and profitability measures describe farm earnings and returns to farm resources. The cash flow summary considers only actual cash transactions during the accounting period, whereas profitability measures consider the value generated by the farm operation and the associated expenses incurred during the accounting period. This gives a more accurate picture of the productivity of the farm business.

Profitability measures use accrual accounting concepts, measuring the value of production at the time it is produced, not when it is sold. Expenses are recorded when incurred, not when paid for. Typically, the timing of the value produced and the expenses incurred do not correspond to the actual cash flows and the difference may be positive or negative. For example, an increase in the number of replacements being raised creates value but not cash income. Supplies of feed on hand may be used and not replaced, incurring an economic cost but not a cash cost. If the primary record-keeping system is cash based, some adjustments will be needed to calculate farm earnings and expenses. These include changes in the value of feed and livestock inventories, in prepaid and unpaid bills, and in accounts payable and receivable. An annual depreciation charge should be made to reflect the decrease in the value of depreciating farm assets. Opportunity costs of unpaid family contributions of labor and capital may be included in some profit measures.

A statement of owner equity shows the sources of changes in net worth and ensures accuracy in the preparation of the other three statements.

Cash flow, profitability, and net worth statements, individually and in combination, provide an overview of financial health and performance. These may reveal strengths or weaknesses in any one or in some combination of these three areas. This overview is just the beginning, however, because the manager also needs to know why these results are what they are and whether performance can be improved upon. A comparison with other farms helps assess the competitiveness of the farm and can help identify specific areas of strength and weakness, but additional, more detailed, performance measures are required.

Additional financial performance measures can be generated from the net worth, cash flow, and income statements. For example, solvency measures derived from the net worth statement can be compared to industry norms. These include the debt-to-asset ratio, defined as total liabilities divided by the total fair market value of assets, which measures the overall level of solvency and indicates the level of risk associated with the current level of debt. Cash flow or liquidity measures look at the adequacy of working capital and the farm's ability to repay debts and replace farm assets. Profitability measures, which should really be thought of as measures of economic efficiency, include rate of return on assets (investment) and on equity. These measure how efficiently the farm investment is being used and in a profitable business these rates will exceed the rate of interest on borrowed money. Net income or total expense as a percentage of gross farm revenue measures cost control and the efficiency of the production process. These are examples of measures to help evaluate the financial performance of an entire farm, but it is also desirable to examine the contributions of individual enterprises or activities, particularly when the overall performance is weak.

Many dairy farms consist of several farm enterprises or profit centers. Revenue and expenses should be allocated to each of these in order to evaluate the contribution each one is making to the overall financial performance of the farm. This information guides decisions about changing the enterprise mix, scale of operation, or production practices to enhance profitability.

More detailed whole farm and enterprise efficiency measures help explain farm performance and help identify specific strengths or problem areas. These measures should include production efficiency (e.g., yield per cow or per hectare), financial efficiency (e.g., the relationship between certain expenses and gross revenue), and combinations of production and financial information (e.g., revenue and cost per cow, per unit of milk sold, and per hectare). To some extent, the relevance, value, and interpretation of specific performance measures will depend on the type of dairy farm. For example, targets for milk per cow will differ for pasture-based and confinement systems, and labor cost targets will differ for a large corporate farm and a small family farm. Nevertheless, the central idea is to have all of the necessary information to be able to evaluate the essential aspects of farm performance and identify areas that fall short of expectations.

Benchmarking

For a farm's production and financial management data to have meaning, there must be some benchmark or yardstick against which to measure various aspects of farm performance. Some of these may be targets established by the manager and some may be based on comparisons with other farms. In general, the most useful benchmarks are comparative and time specific. Weather, pasture and crop development, milk prices, and input prices vary unpredictably from one year to the next, so an acceptable benchmark one year may be unattainable the following year. Genetic progress and improvements in farming technology and management mean production benchmarks change over time. Fortunately, many government agencies, universities, and private concerns develop benchmark data. When local data for similar farms are available, they help a dairy farm manager to assess the efficiency of his or her farm relative to other farms operating under similar conditions. National benchmarks help evaluate the competitiveness of one farm relative to other types of farms and to farms in other regions. An annual assessment is recommended.

Financial and production standards should be related clearly and specifically to the goals of the business. Examples of internally set targets are a reduction in the farm debt load by a specified amount over a stated period and a particular amount of money to be available for family living needs. A rate of return on farm assets (investment) greater than the bank lending rate and annual production costs lower than the average of a group of other dairy farms are examples of comparative targets or standards.

For benchmarks to be useful and valid, the participants must use common terminology, definitions, and methodology, both for record keeping and for summarization. This applies both to the farms contributing data to the benchmarking project and to those using benchmarks to evaluate their farms. Group record-keeping programs also contribute data for other purposes that benefit the dairy industry, such as research and genetic improvement programs.

Record Keeping for Management Purposes

The information needed to effectively manage the various components of a dairy farm will determine the record-keeping needs. Both current and historical data are needed. Current data are needed to monitor herd performance to identify problems in a timely manner and to evaluate changes in herd management. Information is needed on individual animals, on groups of animals, on land use, and on other farm enterprises. Historical farm and financial data provide longer-term performance measures and form the basis for decision making and planning. Participating in group record- keeping and analysis programs or the desire to use comparative benchmarks adds additional requirements.

The foregoing discussion provides some insight into the challenges and complexity of business management and there is no single, integrated record-keeping system that is capable of meeting all these information needs. When selecting a record-keeping system or component, the first and most important step is to define precisely what information the manager needs to be able to manage effectively. The most appropriate system cannot be selected until this issue is resolved.

The specific capabilities of a particular component or system must match the management requirements. In general, these capabilities include the ability to generate information for timely monitoring of both farm and financial performance, to incorporate farm production and financial data, to create information for the periodic analyses of performance, to aid in effective decision making, and to contribute to tactical or strategic planning. Note that, in general, record-keeping systems designed or intended for income tax reporting are not suitable or are of limited value for business management purposes. However, systems can be designed that will meet both tax reporting and business needs, and that incorporate both farm and financial information.

Farmers and their employees must collect the basic information that goes into the farm record-keeping system. Most dairy farmers find they need a combination of methods, including pocket record books or personal digital assistant devices to record events that occur around the farm. These data must then be transferred to other systems periodically to develop individual cow, herd, individual field, and crop records. Group programs are available to most dairy farmers in countries with well-developed dairy industries, for example, the Dairy Herd Improvement Association program in the United States. The installation of on-farm computer systems that produce comparable management data on a more frequent basis can be justified in larger herds. These systems often have an interface with an electronic identification and automatic milk recording system.

Raw information may be of value as collected or may require further summarization or analysis to be useful. Managers may choose to keep and use all the information on the farm, using farm staff or family, or they may contract out some part of the work, for example to an accounting professional or service. Larger herds require a more structured record-keeping system because the volume of data is correspondingly greater and the manager must rely on these records to a greater extent.

Several other factors should be considered when choosing or developing a record-keeping system. These include the ease and timeliness with which information can be collected and entered, the ability or power of the system to generate the reports and other outputs needed to meet management needs, reliability, and cost. Information may be stored on paper or electronically. More and more farmers are finding that the power of computer-based systems outweighs the initial cost and the time required to learn to operate the hardware and software (**Figure 1**). Computer records may be kept on a purchased software package specifically designed for the purpose or on systems that have been created on the farm using generic software such as a spreadsheet. Most vendors of commercial software provide demonstration versions free to potential buyers. It is prudent to seek advice from other users, look up published evaluations, and consult other knowledgeable individuals before making a purchase. The availability and quality of technical support is another factor to consider.

In short, an effective record-keeping system must be capable of yielding information for monitoring and evaluating farm performance and for problem solving and decision making based on these performance evaluations. These systems must incorporate financial as well as production information.

Market Information

Individual dairy farmers selling raw milk to a processor have no control over the general level of milk prices. However, milk prices received and prices paid by competing milk buyers should be monitored because of the importance of price on total farm revenue for a

Figure 1 Farmer and his wife at a computer. Copyright North Carolina State University, Department of Communication Services, Raleigh, NC, USA.

specialized dairy farm. Farmers making dairy products also need to monitor their prices relative to those of competing products and suppliers. The unit prices paid for the inputs used in production should be monitored in a comparable manner, for example, purchased feed ingredients and fertilizers.

One important aspect of marketing involves monitoring milk and input markets for impending changes that might create problems or new financial opportunities. This includes monitoring the factors that affect global and national supply and demand conditions, including changes in economic conditions, consumer behavior, government policies (economic, trade, environmental, and farm), industry structure, and technology. Market information is essential for forecasting near-term prices and trends in the price received for milk and the price paid for purchased inputs and for managing price risk. Producers in some countries have the opportunity to forward contract or use futures markets to modify the price of the milk they sell or the inputs they buy in cash or spot markets.

Other Records

Records are also required for legal and regulatory purposes. Legal records are required for a number of reasons, which include proof of ownership of farm assets and property; supporting information that ensures the accuracy of financial statements; business proposals; and tax filings on income, property, and payroll. Other legal documents include bills of sale, contracts related to business dealings, property insurance contracts, and the like. Wills, records of gifts, and similar documents are essential if property is to be transferred to others according to the owner’s wishes. The form of these documents will depend on local laws, but, in general, all important business documents should be in writing and stored in a safe place.

Farmers are increasingly required to comply with various types of government regulations. Records may be required to demonstrate compliance with safety and environmental regulations governing pesticide and animal drug use, nutrient management, farm labor laws, animal health and welfare rules, licensing requirements, and the like. Usually, the farmer has little discretion over the type and form of these records, how and where they are kept, and who has access to them. These regulations vary both from country to country and within an individual country. The old adage ‘ignorance of the law is no excuse’ means the burden of compliance lies with the farmer.

A third area of record keeping concerns evidence of compliance with specifications required by buyers of farm products. These conditions may be imposed to guarantee the presence or absence of specific characteristics that affect the value of the product. Increasingly these requirements are imposed in response to customer concerns about production practices, for example, related to use of pesticides or genetically modified organisms, animal welfare concerns, and organic certification.

Concluding Remarks

Dairy farming is a challenging and dynamic business, whether the farm in question is a small family farm or a large corporation. Effective business management requires both farm production and financial information, to be used separately for some purposes and in combination for others. The responsibilities of a dairy farm manager and the principles of sound management are common to all types of dairy farms. However, the specific applications will depend on individual farm factors and the external business environment, including the rules and regulations imposed by government. The principles summarized here are well established and additional information and support are available in most parts of the world.

See also: **Business Management**: Roles and Responsibilities of the Manager. **Genetics**: Selection: Evaluation and Methods. **Milk Quality and Udder Health**: Test Methods and Standards. **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy. **Replacement Management in Cattle**: Growth Standards and Nutrient Requirements.

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BUTTER AND OTHER MILK FAT PRODUCTS

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The Product and Its Manufacture

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Introduction

Production of butter is a very old way of preserving milk fat, which is an essential part of the nutritional value of milk. Butter can be made from milk of different animal species, for example, sheep, goats, camels, buffaloes, and cattle, but the dominant source for production of butter today is bovine milk.

Throughout the centuries, butter was manufactured at farms in small quantities with considerable variation in quality. In the nineteenth century, industrialization of the production started through centralization and mechanization, and the quality of the product was improved substantially. Development of new production technology has since then further promoted centralization, and production volumes of 100 000 tonnes annually, including blends and low-fat spreads, are not unusual at modern butter factories today.

According to the International Dairy Federation, the world production of butter is ~4.1 million tonnes annually. However, it should be noted that production figures for anhydrous milk fat and ghee converted into butter equivalent are included in this volume. The European Union (EU) is by far the largest manufacturer of butter, with approximately half of the world production. The largest butter-producing countries are the United States, Germany, France, New Zealand, and Russia.

According to the Codex Alimentarius Commission under the Joint FAO/WHO Food Standards Programme, butter is a fatty product derived exclusively from milk. A 100 g portion of butter must contain a minimum of 80 g fat and a maximum of 16 g water and 2 g nonfat milk solids. A similar definition is used within the EU as stated in Council Regulation No. 2991/94 regarding standards for spreadable fats, such as butter, blends, and spreads.

Butter Varieties

Butter is manufactured in four varieties with different flavors and tastes.

1. Sweet cream, unsalted butter is produced in many countries around the world. The product, which is typically used within the confectionary and bakery industry and in the production of recombined milk, is rather vulnerable to microbial deterioration.
2. Sweet cream, salted butter is the main variety produced in countries like the United States, Canada, New Zealand, Australia, and Great Britain. This type of butter is often produced with a relatively high salt content, for example, 2 g 100 g^{-1}, added in order to give a distinct taste, and also to function as a preservative. Extra salted varieties containing 3–4 g salt 100 g^{-1} are also marketed.
3. Cultured, unsalted butter is a common variety produced in countries like the Netherlands, Germany, and France. Its pH is typically between 4.6 and 5.2 and this acidification inhibits microbial deterioration.

4. Cultured, salted butter is the main variety produced in Scandinavia. The combination of salt and low pH value inhibits spoilage microorganisms, but at the same time it enhances oxidative deterioration. A salt content below 1 g 100 g^{-1} is normally preferred in this type of butter.

There are no specifications concerning salt content and pH in either the Codex standard for butter or the EU Council Regulation No. 2991/94, but such specifications may be found in national legislation.

Butter Characteristics

Butter is a water-in-oil emulsion in which fat globules, fat crystals, water droplets, and air bubbles are dispersed. There are normally two fat phases in butter, a continuous fat phase, which has been squeezed out from the fat globules during churning and working, and a globular fat phase consisting of more or less intact fat globules originating from the cream. The globular fat phase will normally be about one-third of the total fat content of butter, but the balance between the two phases depends on the intensity of the working of the butter, as a very strong mechanical processing, especially at high temperature, will result in an almost homogeneous structure with a highly diminished globular fat phase.

Milk fat is liquid at temperatures above 40 °C, but when it is cooled, fat crystals are quickly formed. Fat crystals occur in different polymorphic forms (*see* **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee), but this is probably of little practical importance as polymorphism seems to have minor influence on the consistency and texture of butter. The crystals formed will eventually aggregate and grow into a three-dimensional network in the continuous fat phase. The mechanical strength of this network has a major influence on the rheological attributes of butter such as firmness, spreadability, and mouthfeel. In butter with a very homogeneous fat phase, an extensive and rigid crystal network is formed by high-melting triacylglycerol molecules squeezed out from damaged fat globules, and the product will therefore be very firm with poor spreadability at low temperature.

When butter is exposed to pressure, for instance by a knife, a small, reversible deformation takes place in the beginning, but then the structure of the product breaks and the deformation becomes larger and irreversible and when the deformation speed is sufficiently high butter will flow like a liquid. This rheological characteristic is typical of a pseudoplastic material with the so-called yield stress, which is the point where the three-dimensional crystal network yields and the product flows. The spreadability of butter is highly dependent on the yield stress.

Butter contains many small water droplets dispersed in the fat matrix. The size of these aqueous droplets, where the water-soluble flavor components are located, depends on the intensity of the working of the product. Most of the water droplets should have a diameter in the range of 1–5 μm, which results in a good microbiological keeping quality, as microorganisms cannot multiply in so small water droplets. However, it is still desirable that a few larger droplets (e.g., 10–20 μm) are present in order to give a better flavor release in the mouth when the product melts.

The typical flavor of sweet cream butter arises from many different flavor components, for example, short-chain free fatty acids, aldehydes, ketones, and lactones. Their intensity is typically very strong in freshly produced butter, but decreases during storage. The flavor in cultured butter is due to a mixture of these components and flavor components formed during the fermentation. One of the most important fermentation components is diacetyl (2,3-butanedione), and lactic acid, acetic acid, and acetic aldehyde also contribute significantly to the cultured flavor, which reaches a maximum level several weeks after production.

The quality of the raw material used in the production of butter is very important, and milk and cream with a distinct off-flavor or with an inferior microbiological quality should not be used for production. In butter with an optimal water distribution, where microbial deterioration is normally not an issue, keeping quality is mainly influenced by lipolysis and oxidation.

Raw milk contains lipoprotein lipase, which can hydrolyze the ester linkage between the fatty acids and the glycerol core of the triacylglycerol molecules in milk fat causing rancid taste. Lipolysis occurs when the fat emulsion is damaged by intense mechanical treatment, for example, in pipelines, if these are not correctly constructed or contain leakages, for example, around damaged sealing rings, where air can be sucked in, which results in foam formation. Milk lipase is almost completely inactivated by pasteurization, but this is not the case with bacterial lipases, mainly produced by psychrotrophic bacteria. These very heat-stable enzymes are transferred from milk and cream into butter in an active form causing lipolysis during storage, although the bacteria that produced the enzymes are killed during pasteurization. The only way to avoid this problem, which normally originates from the raw milk, is to improve the hygienic standard of the milk production and to reduce the storage time and temperature of the raw milk.

Another typical off-flavor that limits the storage stability of butter is metallic, fishy, or oily flavor caused by lipid oxidation, which involves complex reactions between unsaturated lipids and oxygen. Oxidation is influenced by a variety of catalysts, for example, copper and iron ions, but also light, especially of short wavelength, which often occurs in refrigerated counters in food shops, has an activating effect, similar to the effect of low pH of the serum phase of the butter, especially in salted butter.

Manufacturing of Butter

There are two completely different methods for manufacturing butter: the churning method and the emulsification method. In the churning method, crystallization of the fat takes place in cream, followed by phase inversion where the oil-in-water emulsion of the cream is turned into a water-in-oil emulsion by strong mechanical treatment. The fat content is then concentrated by draining of the buttermilk, and the butter is finally plasticized by mechanical working. In the emulsification method, the first three subprocesses are carried out in reverse order. First the fat emulsion is concentrated to a fat content corresponding to the composition of the final product, then a phase inversion is carried out followed by crystallization, and finally a coherent fat mass is formed and plasticized. (**Figure 1**).

The Churning Method

The basic principle of the churning method is that air is mixed into cream where it forms a foam. Simultaneously some of the fat globule membranes are disrupted, and liquid fat is squeezed out from the damaged fat globules and spread at the interface of the foam making fat globules stick to the lamella of the foam. By further agitation the foam collapses, and the fat globules are forced so closely together that they coalesce into small lumps, which are further pressed into small butter granules. Churning can be accomplished in either continuous or batch processes after a certain pretreatment of the cream.

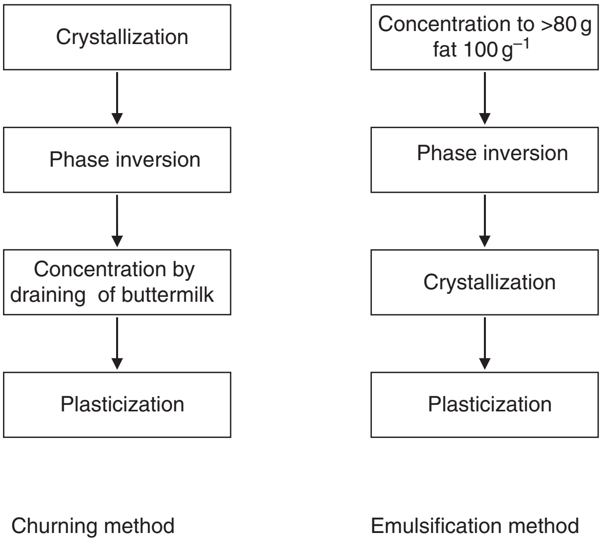

Figure 1 Block diagram showing the principle of butter manufacture by the churning and emulsification methods.

Pretreatment of the cream

Prior to churning, it is necessary to concentrate the fat emulsion in milk to a fat content about 35–42 g 100 g^{-1} or even higher in a centrifugal separator. The cream is then heated to 85–110 °C for 10–30 s in a plate heat exchanger in order to kill any pathogenic or spoilage microorganisms. It is possible to combine this high-temperature short-time treatment with vacuum deodorization, also called vacreation, as a vacuum chamber could be installed after the heating unit. Such treatment might have a positive effect on the flavor of the butter, for instance if off-flavors originating from feeding of the cows occur. This system is mainly used in countries where dairy cows are fed on pasture with strong tasting weeds, which could cause off-flavors in the milk.

The cream is cooled immediately after heat treatment as churning is impossible unless the milk fat is partly solidified. One cooling procedure could be that the cream is cooled directly to a low temperature, 4–5 °C, kept overnight, and churned. This treatment results in the formation of mixed fat crystals, also called compound crystals, where a considerable part of the low-melting triacylglycerols, due to the fast cooling, is trapped in the crystal lattice formed by high-melting triacylglycerols. Butter churned from such cream will have a low liquid fat content and therefore a very firm consistency and a rather poor spreadability.

When the milk fat is relatively soft, that is, the content of low-melting fat is high (iodine value above ~35 g I_2 100 g^{-1} fat), spreadability of butter can be improved by stepwise cooling of the cream, for example, cooling after heat treatment to ~20 °C, holding for a few hours prior to cooling to ~16 °C, holding for further 2–3 h, and finally cooling to the churning temperature. This warm–cold–cold ripening method segregates high-melting triacylglycerols from low-melting triacylglycerols, which results in the so-called ‘overlaid crystals’, which have a sort of laminated structure in which high-melting triacylglycerols form the center on which layers of low-melting triacylglycerols are successively built up concurrently with the decrease in temperature. Butter churned from stepwise cooled cream will have a higher content of liquid fat than butter churned from fast cooled cream and thereby a softer and more spreadable consistency.

However, if the content of low-melting fat is low (iodine value below ~35 g I_2 100 g^{-1} fat), the butter will become rather firm if the cream is cooled stepwise, and in this case it would be advantageous to use a cold–warm–cold ripening method often called the Alnarp method after the location in Sweden where the method was developed. A commonly used procedure is to cool the cream to 8 °C

Table 1 Examples of cooling of cream

Iodine value	*Temperature sequence (°C)*
28	8–22–12
31	8–20–12
34	8–18–12
37	8–16–12
40	8–14–12

after heat treatment, hold at this temperature for 2 h, and thereafter heat to ~20 °C. After holding for a further 3–4 h the cream is finally cooled to the churning temperature. This method will further favor the formation of a laminated crystal structure, increase the liquid fat content, and reduce the firmness of the butter by ~25% compared to the warm–cold–cold method.

If the target is churning of sweet cream, a cooling procedure often used is initiated by cold storage at 8 °C for at least 2 h independent of the composition of the milk fat. The cream is then heated to a temperature determined by the composition of the fat (iodine value). A temperature of about 20–22 °C is chosen if the iodine value is low, and a lower temperature if the iodine value is high. Finally, the cream is cooled to the churning temperature. Examples of temperature sequences in such cooling procedure are shown in **Table 1**.

Fermentation

The traditional way of fermenting cream for production of cultured butter is to use a starter culture containing the lactic acid-producing bacteria *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, and citric acid fermenting strains of *Lc. lactis* subsp. *lactis* and *Leuconostoc mesenteroides* subsp. *cremoris*.

Normally, such starter cultures are delivered from commercial laboratories as concentrated, deep-frozen or freeze-dried cultures. A traditional culturing procedure used at butter factories is to prepare a bulk starter from the commercial culture and add it to the cream in the storage tank. The amount of inoculation depends on the culturing temperature and on the time available for the fermentation. An inoculation of ~5% of the amount of cream and a culturing temperature of ~20 °C are commonly used. When the pH value has dropped to ~5.2, the cream is cooled to ~16 °C, which slows down the lactic acid-producing bacteria and favors the growth of the aroma bacteria. After some hours, the cream is cooled to the churning temperature.

This fermentation method has three disadvantages: first, it results in cultured buttermilk of which there is limited utilization; second, the cooling procedure of the cream for regulation of the consistency is restricted by the culturing process; and third, the method is not suitable for large-scale production. This was the reason why the NIZO Food Research in the Netherlands in the 1970s developed an alternative method for manufacturing cultured butter from sweet cream, where the fundamental principle is that sweet cream is churned, the buttermilk is drained, and the pH of the butter is lowered by direct addition of a lactic acid concentrate and a starter culture.

Continuous butter manufacturing

Continuous butter manufacturing by the churning method is often called the Fritz method after the German scientist who constructed the first machine. The different butter machines available today are rather similar, although some minor differences may be found. One example in **Figure 2** shows the different sections in the machine. These sections are the churning section (1), the separation section (2), and the two working sections (4 and 7) divided by a vacuum chamber (6). At the outlet from the machine (8), butter is conveyed to packaging. Modern continuous butter machines have capacities from 500 to 15 000 kg h^{-1}.

Churning

The pretreated cream is transferred to the churning section (1) by a pump with adjustable capacity. This section consists of a horizontal cylinder with a rotating beater having a high and adjustable speed. The phase inversion occurs within a few seconds, and the rotation speed of the beater regulates the size of the butter grains formed, which again influences the loss of fat in buttermilk. Both too low and too high speed will increase the loss of fat. A general rule is that the lowest speed that will result in butter grains with a diameter of 2–4 mm should be applied.

The optimal churning speed depends on the fat content and the temperature of the cream. Low speed is used if the fat content and the temperature are high. The churning temperature is very important as phase inversion will take place only if sufficient liquid fat is present. If the content of liquid fat is too low, high rotational speed is needed in order to increase the temperature of the cream until sufficient fat is melted. Increasing the temperature by mechanical agitation, where the energy input is converted into heat, will increase the energy consumption of churning dramatically. If the churning temperature is too high, the loss of fat in the buttermilk and the water content of the butter will increase substantially. Normally, a churning temperature around 10–12 °C is used.

As much as possible of the fat in the cream should be converted into butter, minimizing the loss of fat in the buttermilk. The fat content in buttermilk should not be higher than 0.3–0.5 g 100 g^{-1} if the churning is optimal. However, sweet buttermilk can be used in the production

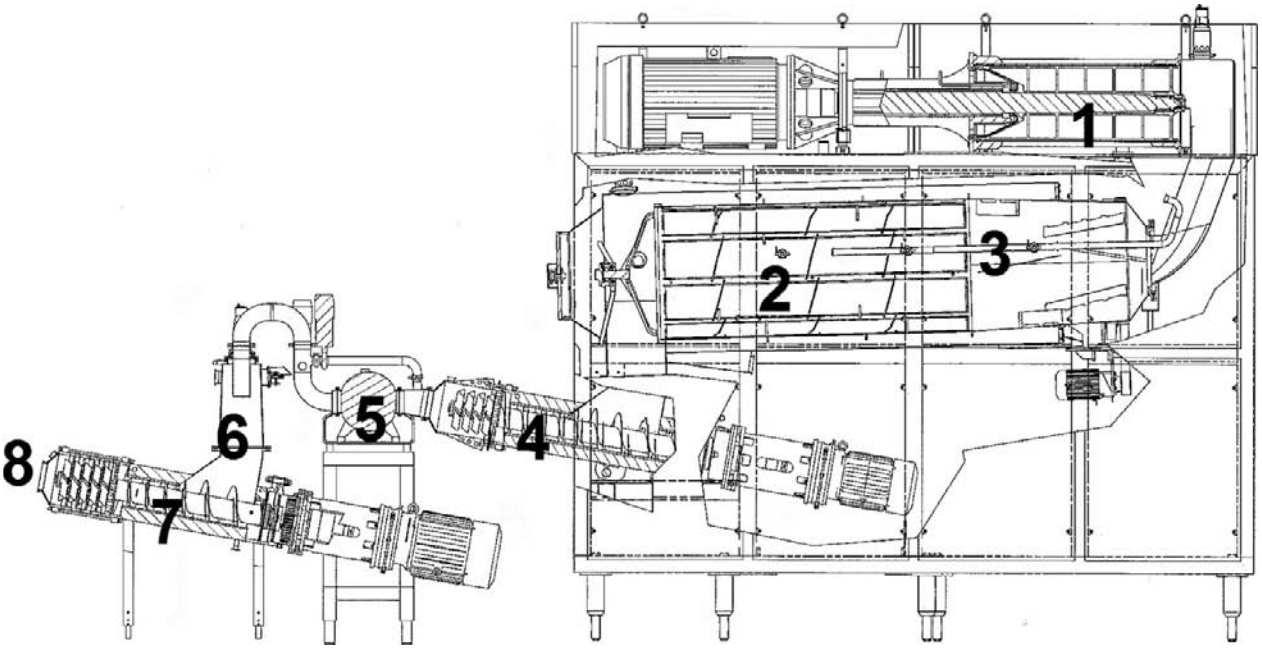

Figure 2 Diagram of APV's continuous butter making machine. 1, churning section; 2, separation section; 3, buttermilk spray; 4, first working section; 5, butter pump; 6, vacuum chamber; 7, second working section; 8, butter outlet. Courtesy of SPX/APV Danmark A/S, Silkeborg, Denmark.

of other dairy products like cheese, cream cheese, or milk powder, and in this case the fat in buttermilk is reutilized.

Separation

After phase inversion has taken place, the mixture of butter grains and buttermilk slides from the churning section into the separation section (2). This section consists of a horizontal, relatively slow-rotating sieve drum with adjustable speed where the butter grains are detained, while the buttermilk passes through a finely meshed wire screen.

It is very important to keep the temperature of the butter low through the whole process, and an efficient way to do that is to cool the butter grains in the separation section before they gather into bigger lumps. This can be done by spraying the butter grains either with cold water or even better with recirculated cooled buttermilk (3), which will not cause dilution. Another way of controlling the temperature of the butter grains is circulating chilled water in the jacket of the separating section, but this is not as efficient as spraying of the butter grains.

Working

The butter grains, now gathered in larger lumps, are then transferred into the first working section (4) in which a pair of parallel contra-rotating augers transports the butter forward and squeezes most of the remaining buttermilk out of the product in order to adjust the water content so that culturing and/or salting can be carried out if required without exceeding the statutory limit of $16\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ water in the final product. Different parameters influence the water content, and the most important parameters having an increasing effect are listed in **Table 2**. It should be noted that the interaction between these parameters is very strong, so careful adjustment of their effect has to be done.

Some butter machines are equipped with a couple of working units consisting of perforated plates interspersed with mixing vanes where an intensive working of the butter is performed. Another possibility is to install a medium-shear mixer after the first working section for the same purpose. From the first working section the butter is conveyed either directly or indirectly via a butter pump (5) to the second working section (7), where the final working takes place. It is important

Table 2 Parameters increasing the water content in butter

Lower fat content in cream
Higher churning temperature
Too low and too high churning speed
Too small and too large butter grains
Higher rotation speed of the separation cylinder
Too low and too high rotation speed of the augers in the first working section
Higher working temperature

that the working intensity is high enough to ensure a homogeneous texture in the butter. Too low intensity will result in loose or free moisture in the product, and too high intensity in a greasy and sticky consistency. It is also very important that the working temperature is kept low (14–16 °C), as this temperature determines the size and the composition of the continuous fat phase, and thereby the extent of the three-dimensional crystal network. The working temperature can be controlled by circulating chilled water in the jacket of the two working sections.

Salting

Salt can be added in the last part of the first working section by a dosage pump with adjustable capacity, and mixed into the butter by the working units. Subsequent working of the butter is accomplished in a very short time that is insufficient for dissolving large salt grains. Undissolved grains will attract moisture during storage, which will result in loose water droplets in the butter and reduced keeping quality.

It is therefore necessary to use very fine-grained salt (average particle size around 15 μm), which could be added as a suspension (e.g., 100 g salt in 100 g water). It is crucial that the salt is not contaminated, especially with copper and iron, as this will reduce the oxidative stability of the butter dramatically.

Culturing

A mixture of lactic acid concentrate and a starter culture is added with a dosage pump simultaneously with the salt suspension if the target is cultured butter manufactured from sweet cream. As butter has a very limited buffering capacity, considerable dosage accuracy is required in order to avoid unwanted variations in the pH of the final product, which is often adjusted to ~5.2.

Vacuum treatment

In order to reduce the air content in the butter and to create a more homogeneous texture, a vacuum treatment can be introduced after the butter leaves the first working section. On entering the vacuum chamber (6) the butter passes through a perforated plate or a similar device where it is pressed out into thin layers, which increases the surface of the product so much that air can be sucked out under a pressure of ~20 kPa. Butter worked without vacuum normally contains 7–8% (v/v) air, which often results in a loose and crumbly texture. Vacuum treatment will reduce the air content to 0.5–1.0% (v/v) or even lower, which also helps avoid laminations in the final product.

Packaging

From the outlet of the second working section (7) the butter is conveyed to a butter silo or a similar container, which acts as a buffer between the continuous churn and the packaging process. This provides a break in the product flow, so a short interruption in packaging will not imply that the churning has to be stopped. On its way the butter passes in-line measuring equipment, which constantly monitors the water content in the final product. Naturally, the statutory limit of 16 g water 100 g^{-1} has to be respected, but on the other hand, for economical reasons, the water content should be adjusted as close as possible to this limit. A commonly used monitoring system is based on near-infrared (NIR) methodology, which measures directly the stream of butter leaving the machine. Based on the result, the water content can be adjusted by a dosage pump at the end of the first working section regulated manually or by the computer monitoring and controlling the whole manufacturing process.

From the silo or a similar buffer vessel the butter is pumped directly to packaging, which could be either retail packaging or packaging in bulk cartons (e.g., 25 kg cartons).

Batch manufacturing of butter

Before the technology for manufacturing butter in a continuous process was developed, the traditional procedure was batch production and this is still used especially for small-scale production.

A batch churn consists of a barrel made of stainless steel having the shape of a cylinder or a single or double cone. Pretreated cream is transferred to the churn, and the churning process is started by rotating the barrel at high speed. The time it takes to convert the cream into buttermilk and butter grains depends on the fat content of the cream, the filling of the churn, and the churning temperature. Normally, the process takes from 30 min to ~1 h.

The buttermilk is drained, and working of the butter starts at low rotation speed with open bottom valve, so more buttermilk can be squeezed out from the butter and drained away. When the water content is sufficiently low, the bottom valve is closed and salt is added if salted butter is desired. As the working time in this process is much longer than in continuous butter making, salting can be done as dry, wet, or brine salting. By wet salting, the salt is wetted with water before it is added to the butter as a salt mash or slurry. This method results in a rapid solution of the salt in the butter. An even more effective way is brine salting. This, however, requires that the water content of the butter is low before the salting. If cultured butter is produced, the cream could be fermented prior to churning, or lactic acid concentrate and starter culture could be added together with the salt after draining of the buttermilk.

The first part of the working is typically carried out under a reduced pressure of about 20 kPa. After adjustment of the water content, the working is continued, but now the vacuum is reduced to half of what it was during the first part of the working. The reason for this is that too high vacuum during the final working increases the risk of liquid fat being drawn out from the butter mass, resulting in butter containing free drops of butter oil.

When the butter appears dry, the working is stopped, the water content is measured and adjusted, and the working is continued until all loose water has been absorbed. Finally, the butter is transferred to packaging.

The Emulsification Method

This method is based on a two-step separation process where milk in the first separator is concentrated to cream with 30–40 g fat 100 g^{-1}, which again is concentrated to ~82 g fat 100 g^{-1} in a second separator. The concentrated cream is then sheared, for instance, using a homogenizer, in order to destroy the fat globule membranes and the mixture of butter oil and butter serum is separated in a third separator. At this stage, the butter oil might be washed with water and treated under vacuum (deodorization) in order to remove unfavorable flavors. The process could be expanded further by a fractionation step in order to make the final product more spreadable. The manufactured butter oil is then standardized with skim milk to the fat content required in the final product, and the mixture is emulsified in a high-pressure pump and crystallized in a scraped surface heat exchanger (*see* **Butter and Other Milk Fat Products**: Milk Fat-Based Spreads). Finally, the resulting butter is worked in a special blending unit and transferred to packaging. It should be mentioned that it is possible to use anhydrous milk fat as starting material instead of cream. The main principle of the emulsification method is shown in **Figure 3**.

Several processes based on this principle were developed in the middle of the twentieth century, but have been abandoned because of quality problems. However, the Meleshin process, which is based on a similar principle, is still used in some of the countries of the former Soviet Union.

Butter manufactured by the emulsification method has a very fine water distribution and a good keeping quality, but the product has a very temperature-dependent consistency, which is extremely firm and brittle at low temperatures, and very soft and greasy at high temperatures. This is caused by the completely homogeneous structure of the fat phase, which, as earlier explained, results in a very extensive and rigid three-dimensional crystal network. An attempt has been made to improve the spreadability of the butter by adding cream containing globular fat immediately before crystallization. This is done in the AMIX process developed in New Zealand, but it has not complexly solved the consistency problems.

Final Remarks

Once upon a time, butter making was a trade where product knowledge, technical skills, and long experience were required. Today, the skilled craftsman has more or

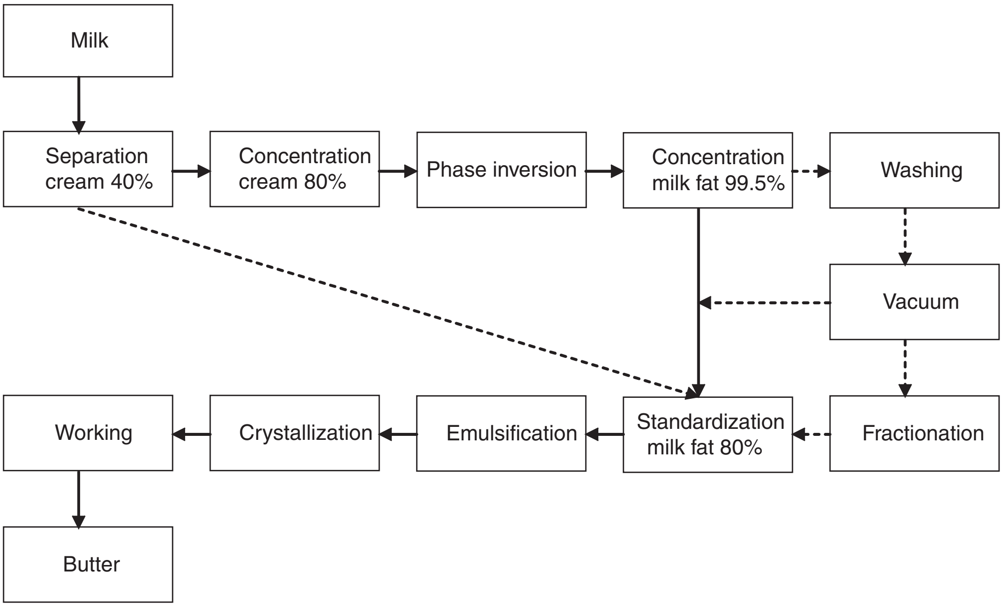

Figure 3 Block diagram showing the principles of butter manufacture by the emulsification method.

less been replaced by computers, and in the highly mechanized and automated butter plants of today an operator can monitor and control the running of several continuous churns producing many tonnes of butter per hour from a control room.

See also: **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview. **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat-Based Spreads; Properties and Analysis. **Milk Lipids**: Lipid Oxidation; Lipolysis and Hydrolytic Rancidity; Rheological Properties and Their Modification. **Flavors and Off-Flavors in Dairy Foods**.

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Modified Butters

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Introduction

Butter is an ancient product with roots reaching several thousand years back in the history of mankind, and many modern consumers, no doubt, look at butter as a very traditional and standardized commodity where wide-ranging variations in composition, flavor, and texture are not desired. The possibility for modifying the product and thereby expanding the market could therefore seem rather limited. Nevertheless, many modifications of butter have been suggested over the years; few have, however, resulted in marketed products and even fewer have survived for a longer period on the marketplace. There are no supranational standards concerning composition and designation of modified butters, but such regulations might be found in national legislation.

In this article, modified butters will be discussed under three groups: products with modified functionality, products with modified flavor and taste, and nutritionally modified products.

Products with Modified Functionality

The functionality of butter can be modified either by changing the chemical composition of the milk fat or by altering the physical structure of the fat phase.

Changed Milk Fat Composition

The rheological properties of fatty products are very much dependent on the chemical composition of the fat. The easiest way of changing the composition is of course to add fat or oil of another origin and composition, preferably vegetable oils (*see* **Butter and Other Milk Fat Products**: Milk Fat-Based Spreads), but if this is not an option more complicated methods have to be used.

Feeding

It is well known that changes in milk fat composition can be achieved by supplying dietary fat to dairy cows. In order to understand the mechanism behind this and be able to utilize this possibility in an optimal way, it is important to distinguish between the origins of the different fatty acids in the triacylglycerols (glycerol esterified to three fatty acids) in milk fat. Short-chain fatty acids with a carbon chain of 4–14 carbon atoms are produced in the bovine mammary gland by *de novo* synthesis using acetate and β-hydroxybutyrate, synthesized by bacteria in the rumen, as building blocks.

Fatty acids with more than 16 carbons in the chain are transferred from the dietary fat via circulating blood lipids, while fatty acids in the intermediate group (16 carbons in the chain) are partly synthesized in the udder and partly transferred from the blood. This means that the composition of the long-chain fatty acids in milk fat can be manipulated through the dietary fat. However, there is a limit, as hydrogenation in the rumen of the unsaturated fatty acids results in a lower content of these fatty acids in the milk fat than that found in the dietary fat. Despite this limitation, it is well known that supplementary feeding of dairy cows with concentrates, such as oilseeds, will increase the content of unsaturated fatty acids in milk fat and improve the spreadability of butter. This is in fact a procedure used commercially with success, and today several dairy companies are marketing spreadable butter with a fat composition that is manipulated through feeding.

It has been shown that the effect of feeding unsaturated fats and oils to dairy cows can be further enhanced if the fatty acids are protected against hydrogenation in the rumen using a technique originally developed in Australia several years ago in which vegetable oil with a high content of polyunsaturated fat was encapsulated in formaldehyde-treated casein. By this method, it is possible to produce soft butter that is spreadable at low temperature. However, the keeping quality of the product is reduced substantially because of oxidative instability and this, combined with the increased production costs, has limited the commercial utilization of this feeding procedure.

Fractionation

Milk fat consists of a mixture of triacylglycerols with different melting points, which implies that it can be split up into fractions with different melting properties suitable for various applications. The most commonly used procedure in the dairy industry today is to split milk fat into two fractions by crystallization from the melt (*see* **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee). The outcome is a high-melting part called the stearin fraction and a low-melting part called the olein fraction. Butter and dairy fat spreads can be modified and made substantially more spreadable by incorporating 25–40 g of the olein fraction per 100 g milk fat. This can be done either by emulsifying the fraction into the cream before churning or by mixing the fraction directly into butter or low-fat spread during manufacturing. From a technical point of view, this is a straightforward procedure and the method has been commercialized.

Addition of the high-melting fraction is also an opportunity as this will improve the stand-up properties of the butter and reduce the oiling-off tendency (separation of liquid oil), which might be convenient in tropical areas.

A more complicated procedure used to obtain an optimal melting curve in the butter is to recombine the high- and the low-melting fractions in such a way that the intermediate middle-melting part of the milk fat is diminished. Technically this is feasible, but the substantial increase in the production costs has limited the utilization of this procedure.

Interesterification

Another possibility of modifying the rheological properties of butter is application of interesterification, where the distribution of the fatty acids among the triacylglycerols is altered. Interesterification, in which anhydrous milk fat is mixed with vegetable oil, implies that existing ester linkages are broken and new ones created, resulting in an exchange of fatty acids between the triacylglycerol molecules. The resultant product has the same total fatty acid composition as the starting material, but the composition of the triacylglycerols and thereby their physical properties are changed. Sodium methoxide can be used as a catalyst in the process, which will result in randomization of the fatty acids, but enzymatic interesterification with lipase is a milder reaction, which might be less harmful to the butter flavor. Depending on the lipase used, the result could be random, regiospecific, or fatty acid-specific replacement of the fatty acids. However, both chemical and enzymatic interesterifications give rise to off-flavors due to the release of short-chain fatty acids and oxidation. In both cases, removal of off-flavors by deodorization has to be done. Several laboratory experiments have shown that the spreadability of butter can be significantly improved by interesterification of milk fat with rapeseed oil, although problems with off-flavors and loss of butter flavor have so far prevented industrial exploitation of the method.

Changed Physical Structure

Work softening

A considerable increase in the firmness of butter, a phenomenon called setting, takes place after manufacturing and cooling due to crystallization in the continuous fat phase where a three-dimensional crystal network is formed (*see* **Butter and Other Milk Fat Products**: The Product and Its Manufacture). If butter is worked mechanically after setting, for example, in a continuous butter mixer or in the working section of a continuous butter machine, it loses much of its firmness due to disruption of the crystal network. It has been reported that up to 50% reduction in firmness can be obtained by reworking. Naturally, the effect is increased if a strong crystal network has been formed prior to the reworking, meaning that the setting of the butter should be in an advanced stage. Thus, reworking of butter immediately after production has almost no effect on the firmness. Under practical conditions this means that butter should be stored at low temperature for a couple of days before the reworking. It is very important to avoid a major temperature increase during the process as this will cause melting of part of the crystal network followed by recrystallization during further storage, which causes restoration of the firmness.

When butter is stored after work softening, it will regain some but not all of its original firmness. A good portion of the effect is permanent, so only part of the crystal network seems to regenerate; besides, the recovery of firmness is quite slow and can still be observed after several months' storage.

However, it should be mentioned that the effect of reworking is partly eliminated if the butter is later exposed to a temporary temperature increase, for example, caused by taking the butter from the refrigerator, leaving it at ambient temperature for some time, and then returning it to the refrigerator. This resetting phenomenon indicates that the crystal network is partly re-created.

Storage of freshly made butter for a couple of days followed by reworking before packaging is of course a costly process, and it has normally been used only when it is impossible to obtain an acceptable spreadability by other means. In the highly mechanized butter production of today, work softening is not a commonly used method.

Whipped butter

Butter produced in the old traditional way will normally contain 5–7% (v/v) air. However, if the product is worked under vacuum, which is an often-used method in industrialized butter production today, the air content is reduced to approximately 0.5% (v/v), resulting in a more homogeneous texture and appearance. On the other hand, high content of air will make the product less hard, but as it often crumbles it would not be spreadable straight from the refrigerator. It could be argued that a high air content will reduce fat consumption because the larger volume per gram implies that a smaller amount of butter is used per serving.

Whipped butter is a special type of butter containing a considerable amount of air (e.g., up to 50% (v/v) or even more) made by incorporating air or an inert gas into the product. This modified butter was originally developed in the United States, and it has been widely used in North America for many years. Whipped butter can be manufactured simply by incorporating air into butter during mechanical working in a batch mixer until the product has reached the desired increase in volume. However, whipped butter can also be manufactured in a continuous process where the butter is softened in a mixing device like a pin-worker or an in-line mixer, while a controlled amount of air is introduced through a gas-injecting system. The product is then cooled in a scraped surface heat exchanger (*see* **Butter and Other Milk Fat Products**: Milk Fat-Based Spreads) and packed, for example, in plastic cups. Nitrogen is often used for the 'swelling' of the product in order to increase the oxidative stability. It is very important that the whipping temperature is not too high (max. 13–16 °C), as higher temperature will result in a product with a very firm consistency and a crumbly texture.

The water dispersion of whipped butter is similar to that found in ordinary butter, but the appearance of the product is very pale unless color is added. Whipped butter has a solid, foam-like texture, which makes it easier to spread on bread than regular butter, although the product is rather crumbly and brittle and not plastic like butter. The product has good stand-up properties and is quite stable at higher temperatures.

Whipped butter is marketed in both salted and unsalted versions and could also be produced with a lactic acid flavor. The product is used instead of traditional butter as a spread on bread or crackers, but can also be used as toppings on baked potatoes, pancakes, and different hot dishes. The product is widely used in the catering sector for restaurants and airlines.

Butter powder

Milk fat can be transformed into powder form, commonly called butter powder, with the aim of increasing its microbial stability and making it easier to handle because of its free-flowing properties and mechanical stability at ambient temperature without being greasy or sticky. Butter powder has often the same fat content as butter, but only approximately 0.5 g water per 100 g or even lower.

The main principle in manufacturing butter powder is to melt butter and mix it with a carrier such as nonfat milk solids or sodium caseinate dissolved in hot water. Suitable additives like antioxidants, emulsifiers, flow agents, and stabilizers might also be added. The next production step is creation of an oil-in-water emulsion by homogenization, as it is important that the diameter of the fat droplets is small (<0.6 μm). The emulsion is then spray-dried, and the powder is after-treated in a fluidized bed cooler for further crystallization. The carrier can also be added after spraying, but in this case special equipment is required in which the melted milk fat is sprayed into a stream of cold air or nitrogen. The shock-cooled fat droplets created are then further cooled to promote after-crystallization and finally mixed with a powdery carrier material.

In many countries, especially in tropical areas, there is an interest in butter powder due to its easy handling compared to normal butter. Butter powder has many of the same functional and sensory attributes as butter such as a full buttery flavor and a light yellow color. The product has many applications in which the use of butter is impractical or where the dry form offers some functional advantages. It is for instance very handy in dry mixes as it blends easily with the other dry ingredients, and a typical application is as an ingredient in the food industry in products such as coffee whiteners, bakery products, and soup, sauce, pudding, and pancake mixes. Butter powder is a commercial product on the market, but despite its many potential applications the interest for butter powder seems to be modest.

Products with Modified Flavor

Spiced butter

Addition of different herbs and spices to butter intended for cooking purposes has been known and used for many years, and a huge variety of different recipes are available from different areas of the world, for example, from the Mediterranean, Indian, and Latin-American kitchens.

Blending spices and fresh herbs with butter is often used when preparing spiced butter, also called savory butter, in private households where long shelf life is not an important issue. But if spiced butter is manufactured on an industrial scale, a number of factors need to be considered.

Fresh or freeze-dried herbs are not suitable for manufacturing spiced butter in industrial scale because they often contain enzymes, for example, lipases, which very rapidly result in an extensive fat hydrolysis in the butter. Many fresh herbs might also cause serious discoloration in the butter caused by color components penetrating into the butter mass. Besides, it is well known that herbs could be heavily contaminated with microorganisms, and freeze-drying is not sufficient to reduce such contamination. Often the best result is achieved by using heat-dried herbs; however, the content of microorganisms in different batches might vary considerably and could be as high as several millions per gram, so careful monitoring of the microbiological quality of the ingredients is vital. The increased keeping quality achieved by using heat-dried herbs is to some extent obtained at the expense of the flavor, as dried herbs do not give the same flavor as fresh herbs. Industrial manufacture of spiced butter therefore often requires addition of flavors, for example, different herbal oils. Spices could also be heavily contaminated, and decontaminated products should therefore be used. This could be spices treated by ionizing radiation or by gas such as ethylene oxide. Such procedures are technically feasible but might be restricted by national legislation.

In small-scale production, herbs, spices, and supplementary flavoring agents, for example, lemon concentrate, mild mustard, and different types of vinegars such as estragon vinegar, could be added to butter in a batch mixer where the ingredients are blended in order to obtain a homogeneous product. In industrial manufacture, the blending could be carried out in a conventional batch churn where herbs and other ingredients are added to the butter after draining of the buttermilk. The working and blending is carried out at a low rotational speed until the product is sufficiently homogeneous.

The keeping quality of spiced butter depends very much on the quality of the ingredients used, but keeping quality around 6 weeks at a storage temperature not exceeding 5 °C is not unusual.

A number of commercial products are available in different countries. Among the most popular varieties are butter spiced or flavored with parsley, garlic, sun-dried tomatoes, basil, ginger, rosemary, lemon, lime, pepper, chili, paprika, and horseradish either as single ingredients or in different combinations. This type of modified butter might be used as toppings on potatoes and on dishes like grilled fish and steaks both in private households and in the catering and ready-meal sectors.

Confectionery butter

A great variety of recipes are available concerning mixing of butter with puree or small pieces of fig, walnut, pumpkin, peach, cherry, strawberry, and apple, just to mention a few possibilities. This type of modified butter, which can be blended in a food processor, is often called confectionery butter and used as a spread combining butter and jam in one product.

Commercial products of this type are well known in a number of countries, for example, Russia, Germany, and Japan. Several products such as butter mixed with raisins, strawberries, cherries, nuts, almonds, honey, or banana have been on the market for varying lengths of time. The ingredients are often combined with a rather high content of sugar, which improves the microbiological keeping quality of the products. There are few technical problems involved in manufacturing products of this type, as the necessary ingredients are easily worked into butter either in a conventional churn or in small-scale production in a batch mixer.

A special product within this category is the so-called ‘Christmas butter’ marketed as a special delicacy every year around Christmas, especially in England. It is butter with alcohol, for example, cognac, calvados, liqueur, or rum. The content of sugar in these products also is normally very high. A typical example of the composition (gram per 100 g) could be 40 butter, 40 sugar, 10 alcohol, and 10 water. The manufacturing process is very simple. Sugar, alcohol, and water are mixed and blended into softened butter until a smooth and homogeneous mass is achieved. The mixture is then cooled and packaged. The product is very tasty when spread on scones, Christmas pudding, or mince pies.

Nutritionally Modified Products

Reduced cholesterol content

When the link between blood cholesterol and coronary heart disease was established years ago, it was recommended that people in the high-risk category should avoid foods containing cholesterol. This caused an interest for reduction or removal of cholesterol from milk fat, which contains ~0.3 g cholesterol per 100 g. This can be done by biological, physical, or chemical means. Many different methods have been tried mostly based on extraction, distillation, adsorption, enzymatic conversion, or combinations thereof.

Cholesterol can be extracted with organic solvents like methanol in a single-stage or multiple-stage batch extraction process followed by deaeration with steam under vacuum. As the use of organic solvents in food processing is problematic, these methods are of minor commercial interest. Supercritical extraction using carbon dioxide as a solvent under high pressure and moderate temperatures has been suggested for removal of cholesterol from milk fat, but due to the large investment and considerable operational costs linked to this method real commercial success has not been reported.

Another method for removal of cholesterol from milk fat is distillation. Although cholesterol has a low volatility, it is more volatile than most triacylglycerols found in milk fat and it can, therefore, be removed by steam distillation. Different distillation techniques, such as vacuum steam distillation and short-path molecular distillation, have been applied, and some of these methods seem to be rather effective. However, the problem is that most of the typical butter flavor is removed together with the cholesterol. The commercial interest has been modest in these rather complicated and expensive distillation methods.

Cholesterol can also be removed by adsorption onto activated carbon, diatomaceous earth, or specially coated glass, ceramics, or plastic. Melted anhydrous milk fat is brought into contact with the absorbent material either in a batch process or in a more or less continuous process based on columns. Absorption methods based on complex formation between cholesterol and cyclodextrin, which is a family of cyclic oligosaccharides, have found some application in industrial scale. In this method, melted milk fat is blended with an aqueous solution of cyclodextrin and when the complex with cholesterol is formed, it is removed from the milk fat by centrifugation. The process is relatively simple; it does not require large investment and the reduction in butter flavor is less than that by the distillation methods. However, large quantities of cyclodextrin must be used, and the production costs are consequently high. Nevertheless, this process has been commercialized.

The enzyme cholesterol reductase can convert cholesterol into forms that cannot be absorbed by humans. Several laboratory trials have been reported, but the process seems complicated and combined with high production costs. Reports on industrial use are lacking.

A variety of modified butters and spreads made from reemulsified, cholesterol-reduced milk fat have been developed and marketed in many countries, but the commercial success is difficult to assess. Many products seem to have had a short life on the marketplace before they were withdrawn again, but often quickly replaced by new products in the same category.

Increased conjugated linoleic acid content

Milk fat is an important source in human diet of bioactive conjugated linoleic acid (CLA), which is claimed to exhibit a number of health benefits such as anticarcinogenic, antiatherogenic, immunomodulating, and antiobesity effects, and during the past two decades CLA has been much debated. However, most of the studies of the health effects of CLA have been carried out on animals, and the effects on humans are still not conclusive.

Typical concentrations of CLA in milk fat are 4–7 mg g^{-1} fat, but the level can vary widely among herds and individual cows. The level of CLA is influenced by feeding, and milk fat from grass-fed cattle normally has a several-times higher content than milk fat from stall-fed animals. A number of methods have been described for the production of CLA-enriched milk fat and the use thereof based on grass-feeding or feeding a low-forage diet supplemented with oil, for example, sunflower seed oil, which results in an incomplete biohydrogenation of polyunsaturated fatty acids in the rumen and greatly enhances the concentration of CLA in the milk. Another possibility of enhancing the CLA level is fractionation of anhydrous milk fat, as CLA will be concentrated in the olein fraction.

Butter rich in CLA is on the market as a specialty in several countries, but so far the commercial success seems to be limited probably because of the rather high prices caused by increased production costs.

Increased omega-3 fatty acids content

Long-chain polyunsaturated n–3 fatty acids (popularly referred to as omega-3 fatty acids) comprise α-linolenic acid ($C_{18:3}$, ALA), eicosapentaenoic acid ($C_{20:5}$, EPA), and docosahexaenoic acid ($C_{22:6}$, DHA). The most widely available source of omega-3 fatty acids is cold water oily fish like salmon, herring, and mackerel. The interest in these fatty acids originates in studies of Greenland's Inuit population conducted in the late 1970s. The low occurrence of coronary heart disease in the Eskimo population was attributed to their traditional diet of marine animals and fish. This was later confirmed by similar studies in other cultures with high fish consumption, for example, in Japan and Alaska. Today, health authorities normally recommend a higher intake of these fatty acids as they are considered important for normal growth and development and for prevention of a number of diseases like hypertension, diabetes, cancer, and coronary heart disease, although contradictory information can be found in the literature.

The content of omega-3 fatty acids in milk fat is low and only to some extent dependent on feeding. So the simplest way to increase the content in butter seems to be direct addition of fish oil. This oil has to be refined before it can be used in butter and spreads, and special precautions like addition of antioxidants and/or storage and shipment under modified atmosphere should be taken to prevent oxidation of the highly unsaturated oil. If this is done, fish oil can easily be added to butter and spreads in the same way as vegetable oils.

Modified butter products like blends and spreads fortified with fish oil have been and still are marketed, but apparently with modest success, probably because of the relatively high price due to the costly refining and shipment of the fish oil.

Final Remarks

A large number of modified butters have been developed and marketed over the years. Compared to the world production of traditional butter, the volume of these butter products is quite insignificant, but they contribute to the impression of a multiple, dynamic, and innovative product category, which is a very important aspect in relation to the retail trade. At the same time, they give the consumers a wider range of free choice, which undoubtedly plays a role in the always ongoing competition between butter and other edible fats and oils.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat-Based Spreads; The Product and Its Manufacture. **Feed Ingredients**: Feed Supplements: Fats and Protected Fats. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat. **Milk Lipids**: Conjugated Linoleic Acid; Removal of Cholesterol from Dairy Products.

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Properties and Analysis

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Introduction

Butter is a high-energy concentrated natural dairy product consisting mainly of milk fat, water and nonfat solids (proteins, lactose, minerals, vitamins). It has a unique delicate flavor, which in combination with its characteristic and pleasant mouthfeel is an unparalleled selling point enabling butter to command premium prices in the market-place. On the other hand, its poor spreadability at refrigeration temperature makes butter less attractive to consumers. Butter suffers further from having been labeled as 'saturated' and of a high cholesterol content; both of these properties have been associated with vascular and heart disease. At the same time, margarine developed from a cheap substitute to a well-accepted health-promoting convenience product ('from plants only', 'polyunsaturated', 'spreadable from the fridge'). As a consequence, over the last decades butter consumption has fallen drastically worldwide. Despite a slight rebound since 1997, world butter consumption in 1999 was about 12% below its level in 1990. Recent new findings, however, of the nutritional significance of conjugated linoleic acid – a rather specific milk constituent – as well as of the potentially different impact on human health of milk-inherent versus processing-related positional *trans* fatty acid isomers may show butter in a new light and, therefore, might contribute to a stabilization of butter consumption close to its present level.

Overall Composition

Standards pertaining to butter composition are similar in most countries. It contains about 80% fat and 16% water. The rest, referred to as solids-not-fat (SNF), primarily contains constituents such as proteins (0.6–0.7%), lactose (0.7–0.8%), salts (~0.2%) and traces of all other skim-milk substances. Water, β-carotene and salt may be added.

Water, fat and SNF are determined according to International Dairy Federation (IDF) Standard 80 (1977). In principle, moisture is removed from the butter sample by oven drying. The weight loss of butter through a particular heating process is defined as moisture. The fat is then removed from the dried butter using a suitable solvent. The retained SNF portion is dried and weighed. An IDF standard of a direct determination of fat in butter is currently under preparation.

Chemical Composition of the Fat Phase

The lipids in butter consist mainly of triacylglycerols (roughly estimated to be 98% w/w); the rest is diacylglycerols (~0.3%), monoacylglycerols (traces), phospholipids (~0.3%), sterols (~0.3%), free fatty acids (FA) and traces of waxes, squalenes and carotenoids. Free FAs occur in milk and amount to approximately 0.1% of milk lipids. Because of their polarity they are partitioned in the fat phase and in the plasma. The shorter the chain length and the higher the pH, the more the partition equilibrium is shifted toward the plasma. Therefore, the free FA level in butter is rather variable. The phospholipids originate from the milk fat globule membrane (~0.9% of the milk lipids). During churning they are partly retained in the moisture droplets of butter in the form of lipid–protein complexes or as integral parts of membrane fragments. The latter may also – possibly with absorbed water – be suspended in the fat phase. The major part of the phospholipids is left in the buttermilk, where it constitutes some 20% of total lipids.

Fatty Acid Composition

FA analysis is accomplished by chromatographic means, mainly by gas chromatography (GC) on capillary columns; in the case of minor FAs it is also achieved in combination with thin-layer or high-performance liquid chromatography (HPLC). Altogether, more than 400 different FA species have been found in milk fat. However, the class of major FA, which are present at 1% each of total mass of FA, comprises only 15 FA species – eight even-numbered, unbranched, saturated FAs ($C_{4:0}$–$C_{18:0}$), three monoenic ($C_{14:1}$, $C_{16:1}$, $C_{18:1}$), two polyenic ($C_{18:2}$, $C_{18:3}$) and two odd-numbered saturated ones ($C_{15:0}$, $C_{17:0}$). These amount to more than 85% weight of all fatty acids. The minor FA class of butterfat includes *cis*/*trans* isomers as well as keto and hydroxy FAs.

[†] Deceased.

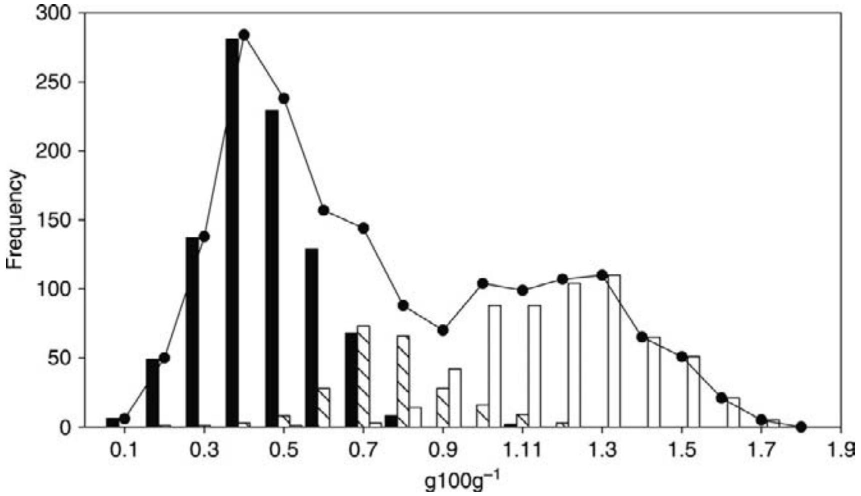

Figure 1 Frequency distribution of conjugated linoleic acid (CLA) contents derived from German milk fats ($n = 1756$) subdivided into samples from the barn feeding (filled columns), the pasture feeding (open columns) and the transition (spring or late autumn) period (cross-hatched columns). Dots show all milk fats. (Reproduced with permission from Wolf L, Precht D and Molkentin J (1999) Occurrence and distribution profiles of *trans* $C_{18:1}$ acids in edible fats of natural origin. In: Sebedio JL and Christie WW (eds.) Trans *Fatty Acids in Human Nutrition*, pp. 1–33. Dundee: Oily Press.)

The content of butterfat FAs is subjected to major seasonal and regional variations. For example, oleic (*cis*-9-$C_{18:1}$) and palmitic acid ($C_{16:0}$) may vary from approximately 20% to 28%, and from 22% to 37% mass of total FAs, respectively. **Figure 1** shows the frequency distribution in butterfat of the FA which is currently most discussed, namely conjugated linoleic acid (CLA), *cis*-9-*trans*-11-$C_{18:2}$, which occurs almost exclusively in ruminant fats like butterfat. Based on recent animal tests, CLA has been recognized as being highly bioactive. Conversely, adverse effects have so far been attributed to *trans*-$C_{18:1}$ isomers in general. However, the difference in frequency of *trans* isomers in butter and margarine is striking (**Figure 2**): vaccenic acid (*trans*-11-$C_{18:1}$) predominates in butter, whereas the main *trans*-$C_{18:1}$ FA in margarine is elaidic acid (*trans*-9-$C_{18:1}$).

There have been some indications that the metabolism of elaidic acid is different from that of vaccenic in that the latter may be converted to CLA by $\Delta 9$ desaturase and thus the effects of *trans* FA on human health will have to be assessed in a more differentiated way.

Another unique characteristic of butterfat is its high level of short-chain fatty acids, from butyric ($C_{4:0}$) to capric acid ($C_{10:0}$). Because of their particular metabolic pathway (directly to the liver via the portal vein), and several features, such as more rapid lipolysis and resorption, these FA are considered valuable nutrients.

On average these FA total some 20–25 mol%. Approximately 50% of all butterfat triacylglycerols contain a short-chain fatty acid residue in the nutritionally significant *sn*-3 position.

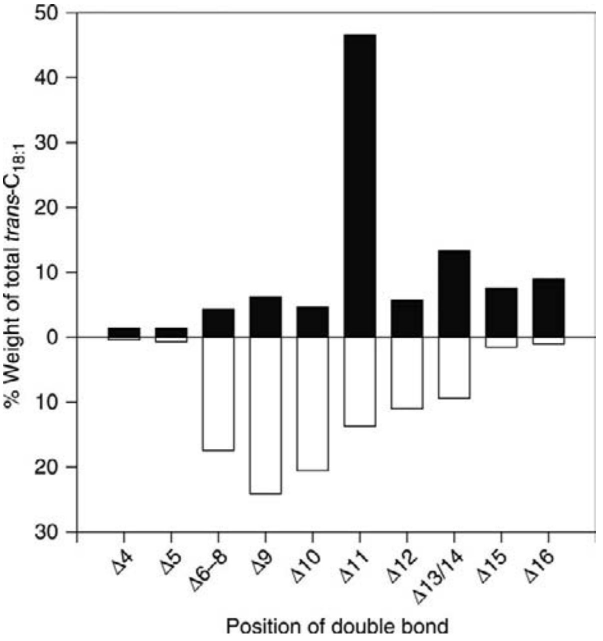

Figure 2 Relative isomeric distribution of *trans*-$C_{18:1}$ positional isomers in German milk fats (filled columns: $n = 1756$) and in German margarines and shortenings (open columns: $n = 62$; % weight of total *trans*-$C_{18:1}$ isomers). (Reproduced with permission from Precht D and Molkentin J (2000) Frequency distribution of conjugated linoleic acid and *trans* fatty acids contents in European bovine milk fats. *Milchwissenschaft* 55: 687–690.)

Triacylglycerol Composition

Because of large fluctuations in FA composition, the contents of triacylglycerols (TAG) also vary considerably, as shown in **Figure 3**. The contents of the TAGs,

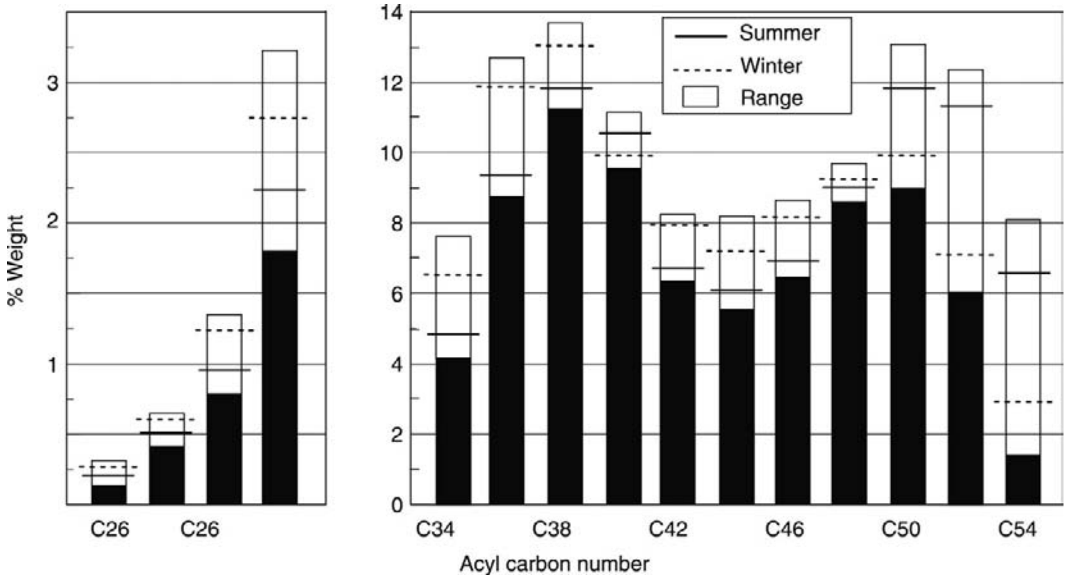

Figure 3 The triacylglycerol composition of German milk fats ($n = 45$, average values and ranges). (Courtesy of Dr H Timmen, Kiel.)

classified by their acyl carbon numbers, were determined by GC on packed columns. The TAG classes from C26 to C48 fluctuate synchronously, apart from one exception, C40, which varies along with C50–C54.

The frequencies of the TAG groups C26–C54 in any milk fat are strongly related to each other. Mathematical relationships which characterize butterfat in general, and which show even small additions (down to 2%) of foreign fats, were established in 1980 in Australia. They were further developed in Germany and recently promoted to the reference method for all European Union (EU) member countries to determine precisely butterfat adulterations by GC analysis on packed columns.

Melting Behavior of Butterfat

The compositional variations in butterfat are reflected by its thermophysical properties, which in turn have a strong effect on the quality and manufacturing process of butter. **Figure 4** shows three sets of melting curves of three butterfats. They originate from pasture feeding, barn

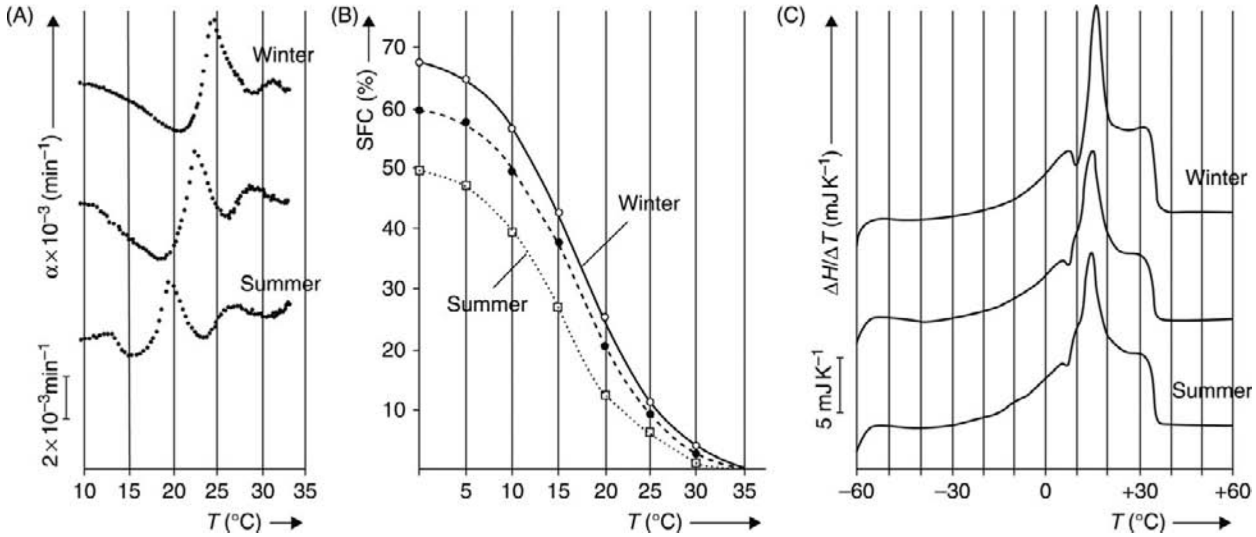

Figure 4 Melting curves of three different milk fats (pasture and barn feeding and transition period) (A) Obtained by the Shukoff flask method, where the minima of the curves indicate maximum melting; (B) obtained by nuclear magnetic resonance; and (C) obtained by differential scanning calorimetry. SFC, solid fat content. The Shukoff flask containing the cooled fat is immersed in a thermostatted heating bath. α is computed from the heating speed of the fat, dT/dt, and the bath temperature, T_B, as well as $\alpha = (\mathrm{d}T/\mathrm{d}t)/(T - T_B)$.

feeding and a transition period (in line with most regions of central/northern Europe). Three different measuring techniques were applied:

1. The Shukoff flask method (**Figure 4**), where the minima in the curves indicate temperature ranges of maximum melting. This method has been developed for controlling the fat crystallization in cream for buttermaking. It is essential, for instance, when applying a cold–warm–cold ripening for making a butter softer to set the temperature of the warm stage between the first minimum and the following maximum of the melting curve.
2. Low-resolution nuclear magnetic resonance (**Figure 4**), yielding the solid fat contents (SFC) at temperatures ranging from 0 to 35 °C. The characteristic courses of the melting profiles (SFC versus temperature) have an impact on the texture and mouthfeel of butter.
3. Differential scanning calorimetry (**Figure 4**) supplying the differential energy absorption by the fat during heating. The curves clearly display the wide melting range of butterfat (from −40 to 37 °C). The high-energy absorption above 10 °C is responsible for the pleasant cooling sensation caused by butter in the mouth.

The influence of individual feedstuffs included in the barn ration on the hardness of the butterfat is shown in **Figure 5**.

The Microstructure of Butter

Figure 6 is a schematic drawings of the microstructures of butter (**Figure 6A**) and margarine (**Figure 6B**). As is common to most high-fat spreads, their overall structure is characterized by a continuous oil phase in which crystallized fat, water and (eventually) air are dispersed. The striking difference with margarine is the presence of many more or less damaged fat globules in butter (globular microstructure). They originate from the cream and are characterized by curved stacks of fat crystals at their periphery. These crystal shells consist primarily of high-melting TAG. They impart rigidity to the fat globules, thus enabling them to some extent to withstand the strong shear forces during churning and working. As a result, numerous cream fat globules have survived the churning process, whether damaged or not. Their number depends on the process conditions, being smaller under continuous than under batchwise churning. While globular fat structures are missing in margarine, which has a homogeneous microstructure, another population of globular structural elements is found in both products. These are water droplets which are masked by thin fat crystal plates densely arranged at the water–oil interface. Normally, the water droplets in margarine are smaller than in butter. The nonglobular crystalline fat phase in butter mainly consists of platelet-shaped aggregates. They are smaller in size than the fat crystals present in margarine (0.1–5 versus 0.1–20 μm). Air cells are occasionally found in butter, varying in number and size depending on the

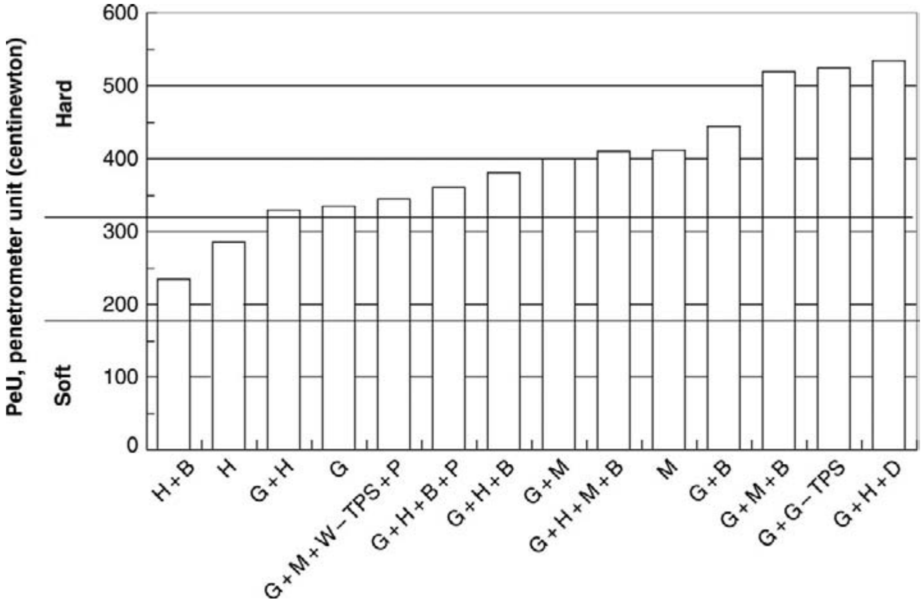

Figure 5 The hardness of butterfat (measured by cone penetrometry at 15 °C) as influenced by the cows' ration. Cone angle 40°, penetration speed 1 mm s^{-1}. B, beet; D, dried pulp; G, grass silage; H, hay; M, maize silage; P, potato; TPS, total plant silage; W, wheat. (Courtesy of Dr K Pabst, Kiel.)

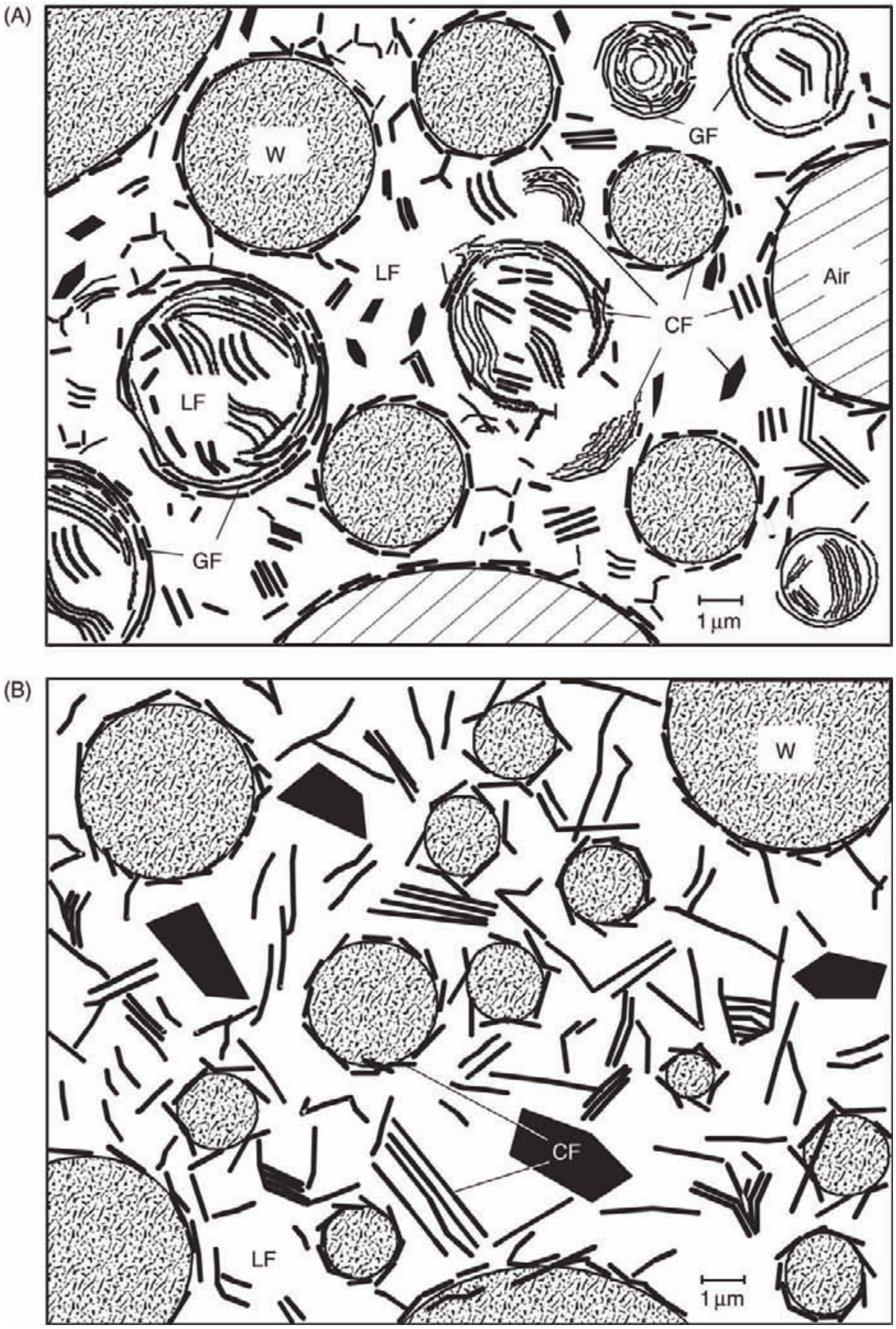

Figure 6 Schematic view of two microstructures at ambient temperature: (A) butter; (B) margarine. F, fat; W, aqueous phase; CF, crystallized fat; GF, globular fat; LF, liquid fat.

manufacturing method (e.g. vacuum treatment). There is also an arrangement of fat crystals along the air–fat interface.

Most of our former knowledge about the microstructure of butter came from light microscopic investigations undertaken in the first half of the last century, but since about 1960 electron microscopic studies have allowed direct detailed visualization of the individual structural elements. **Figure 7** shows freeze-fracture transmission electron micrographs of butter and margarine, respectively, corresponding to **Figure 6**. Incidentally, broken globules are easily identified as either fat globules or as water droplets, by using a special preparation technique (freeze-etching, **Figure 7A**). Fat globules with sizes ranging from about 2 to 8 μm were determined at 30–60% (v/v) in butter, depending on the manufacturing method. The ratio of crystalline to liquid fat inside the fat globules may be greater than that outside, because during churning the liquid fat will be partly squeezed out of the fat globules. A considerable part of the continuous oil phase is

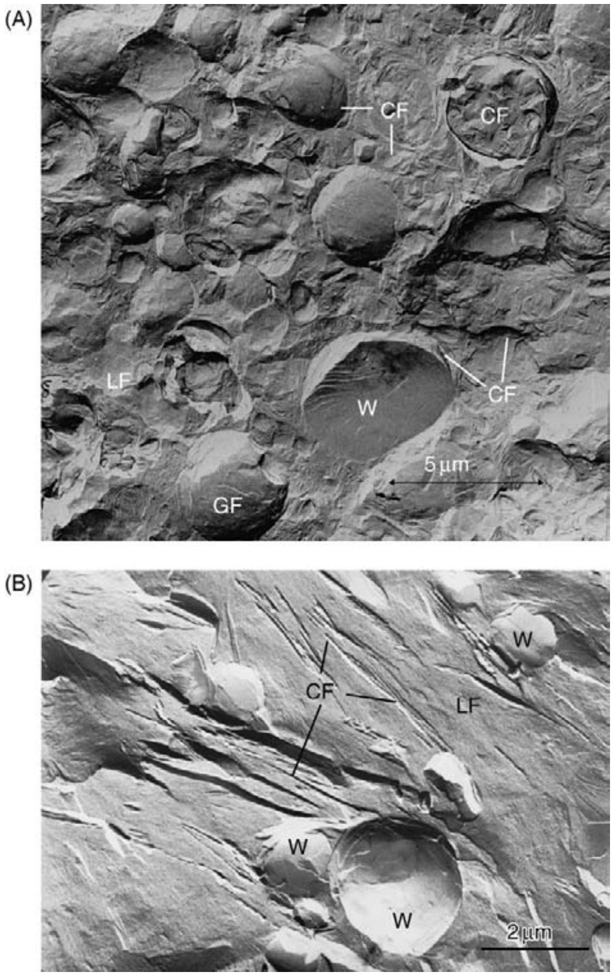

Figure 7 Electron micrographs of (A) butter and (B) margarine at 15 °C. W, aqueous phase; CF, crystallized fat; GF, globular fat; LF, liquid fat. (Courtesy of Dr W Buchheim, Kiel.)

not free-flowing, but instead adheres to the surface of the fat crystals. These in turn interact with each other, forming a continuous network in which water droplets and fat globules may participate. Since the number and sizes of the fat crystals (roughly estimated at $10^{13}\,\text{ml}^{-1}$ and up to 5 μm in length, respectively) greatly depend on the temperature treatment of the cream prior to churning, the same applies to the number of 'knotting points' of the network as well as to the degree of immobilizing the oil phase. The fat crystals inside the fat globules are not involved in building up the network which, therefore, is (theoretically) assumed to be weaker than that of margarine (under equal conditions, i.e. the same number and sizes of fat crystals, which is difficult to achieve).

Macroscopic Properties of Butter

The main consumer-perceived properties may be divided into three categories – color and appearance, flavor (odor and taste) and consistency. Consistency may be subdivided into texture, mouthfeel, firmness (after storage and working) and spreadability. A further property, keeping quality, concerns both consumers and trade.

Color and Appearance

The natural yellow color of butter, in combination with its smooth, slightly matt surface appearance, gives a particular impression of richness, which is rarely matched by margarine. A 'faultless' butter cuts cleanly when sliced and does not appear greasy or shiny. The yellow color mainly results from β-carotene (provitamin A) which, dissolved in butterfat, originates from green plant nutrients in fresh or silaged feed. When animals are fed with low-carotene feedstuffs, the butter has a pale and even whitish color. Typically, the natural β-carotene level in summer butter may be 10 times higher than in winter butter (approximately 1000 versus 100 IU $100\,\text{g}^{-1}$). Therefore, a limited enrichment of butter with β-carotene (E160) may be permitted. Both the intensity and the spectrum of the butter color differ from those of anhydrous butterfat. This results from the way light interacts with fat crystals, water droplets and air bubbles which contribute to the microstructure of the butter surface. For example, vacuum treatment leads to a significantly more intense dark-yellow color of the butter.

Flavor

The unique flavor of butter in combination with its pleasant mouthfeel is the main reason for consumer acceptance despite its higher selling price compared to vegetable fat-based spreads. Hence in official quality tests for butter, flavor is an important factor. A long list of identified flavors and off-flavors exists (IDF Standard 99C: 1997). Butter flavor is made up of a large number of chemical compounds; the total physiological response is a composite of these constituents. They are derived mainly from the butterfat itself and, in the case of cultured cream butter or soured/indirectly cultured sweet cream butter, also from a starter culture or from flavor/culture concentrates, respectively.

The flavor compounds of the lipid fraction comprise free volatile FA, lactones, methyl ketones, phenolic and sulfurous compounds, all in very low quantities (close to their sensorial thresholds). Partly, these are already present in raw milk; partly, they are formed and released from precursors (e.g. β-ketoacid or hydroxyacid triacylglycerols) by virtue of heat. Hydroxyacid triacylglycerols rearrange spontaneously to give the highly flavorsome γ- and δ-lactones. The conversion rate depends on temperature: half-lives at −10, 60 and 150 °C are approximately 1 year, 1 week and 1 h, respectively. Another group of butter flavors originates from the oxidation of unsaturated fatty acids, the most important being arachidonic ($C_{20:4}$), linolenic ($C_{18:3}$), linoleic ($C_{18:2}$) and oleic ($C_{18:1}$) and their isomers. Oxidation of these acids can be initiated enzymically by xanthine oxidase (the main protein of the milk fat globule membrane) or chemically by $OH^{\cdot}$ radicals produced from ascorbic acid and copper. Because an appreciable part of the copper present in butter (by contamination) moves to the fat globules during souring, this metal is found to a larger extent in cultured cream butter than in sweet cream butter. Several flavor defects are related to oxidation, e.g. 'metallic', 'fatty', 'trainy', 'fishy' or 'tallowy'. On the other hand, slight oxidation may contribute to the desirable flavor. Hept-*cis*-4-enal, for instance, is claimed to impart a creamy flavor to butter at a level of $1\,\mu g\,kg^{-1}$. Oxidized butter may produce perfectly satisfactory baked goods.

The flavor of cultured cream butter originates from a bacterial starter culture converting skim milk ingredients into a series of flavor compounds, e.g. lactose into lactic acid and citrate into diacetyl. In the case of soured/indirectly cultured butter, flavor concentrates and/or starter culture concentrates are injected into the working section of the buttermaker. As an example, the Netherlands Dairy Research Institute (NIZO) concentrate comprises 20 compounds, the dosages ranging from $2\,\mu g\,kg^{-1}$ (ethyl butyrate) to $57\,mg\,kg^{-1}$ (acetic acid). Lactic acid is dosed to pH 4.6.

For an analytical distinction of the three butter types – cultured cream butter, sweet cream butter and soured/indirectly cultured butter – three suitable compositional parameters (e.g. lactic and citric acid and adenosine or uridine contents) must be determined.

Despite many efforts it has so far not been possible to match the organoleptic qualities of butter by incorporating synthetic flavor compounds into margarine. This is particularly striking when considering butter as an ingredient for cooking and baking. In order to reach the optimum level of lactones in a cake the fresh margarine must contain an overpowering level of lactones, whereas butter contains the lactones in the form of flavorless precursors, which are slowly released on baking. Another difficulty may be that the specific melting properties of butterfat affect the rate of release, and hence the profile of the flavor as well as the interaction between mouthfeel and flavor.

Consistency

Texture and mouthfeel

The texture of butter is rather specific. At temperatures above 12–14 °C (depending on the individual milk fat) butter exhibits plasiticity and body; both features are perceived as pleasant when the butter is cut or spread. Upon tasting, a particular characteristic haptic impression (a property related to the sense of touch) and mouthfeel are created, which is not really imitated by any nondairy spread. When butter comes into contact and interacts with the gustatory system in the first moment its firm consistency is felt by the tongue and the palate. Subsequently, the butter turns liquid, thereby being readily inverted to an oil-in-water emulsion and releasing its full potential of volatile and water-dissolved flavors. The heat which is required for melting the fat is drawn from the mouth, thereby causing a pleasant cooling sensation. Finally, the emulsion is cleanly swallowed without causing an unpleasant greasy (e.g. tallow-like) aftertaste.

These features are all primarily derived from the composition, and thus the physical characteristics of butter, which may be influenced or determined by the method of manufacture. The firm plasticity and smooth appearance are due to the high SFC level at (sub)ambient temperatures as well as to the microstructure. The rapid meltdown in the mouth and the strong cooling effect are directly derived from the melting characteristics monitored by differential scanning calorimetry or nuclear magnetic resonance (**Figure 4**). Phospholipids present in butterfat at levels of approximately 0.1% as well as proteins aid in inverting the butter from a water-in-oil to an oil- in-water emulsion in the palate.

In most countries texture and mouthfeel are officially graded. Numerous texture or body defects have been identified (e.g. IDF Standard 99C: 1997 or US Department of Agriculture 1989), including 'crumbly' (lacks cohesion), 'mealy or grainy' (a granular consistency when butter is melted on the tongue), 'short' (lacks plasticity and tends toward brittleness), 'sticky' (butter adheres as a smear) 'soft' or 'hard'.

Setting and work softening

Freshly produced butter, on keeping, increases in firmness, initially at a fast rate and after that slowly and asymptotically, following an exponential saturation function. This process, which may last several weeks, is called setting. It may be significantly accelerated or retarded by elevating or lowering the temperature. Setting occurs even when the butter is raised permanently to a higher temperature than that at which it was manufactured. Therefore, a steady increase in crystallized fat cannot be the cause of setting. Instead, setting has been attributed to the reversible build-up of a network structure previously described, i.e. to the thixotropic properties of butter. Three main features can be directly related to thixotropy: (1) reduction in firmness on mechanical working and the subsequent recovery, though not up to the initial level; (2) the accelerating effect of temperature; and (3) the exponential course of setting. By working, the crystal network is broken down and the butter becomes soft (work softening). On keeping, the crystals are rearranged and attracted and reversibly bound to each other by weak Van der Waals forces, so that the network recovers and the firmness increases accordingly (step 1). The higher the temperature, the more liquid fat is available, the more mobile are the crystals and the faster they rearrange (step 2). Many more crystals can rearrange at the start of setting than at the end, when open places are scarce and the probability of occupying them is low (step 3). The well-known phenomenon that after work softening butter does not regain its initial firmness within a reasonable time has been attributed to strong irreversible bonds which are formed when fat crystals grow together locally, thus forming a strong network. This may occur immediately after churning when supercooled liquid fat is present or – on a much larger time-scale – together with recrystallization processes.

Spreadability

The texture and mouthfeel, two highly valued features of butter, are due to the specific melting properties of the butterfat. However, where spreadability is concerned, the same properties, i.e. mainly the high SFC (48–66% at 5 °C; **Figure 4**) and its steep fall with increasing temperature, have an adverse effect on this characteristic. Spreadability begins to be acceptable below solid fat levels of 45% (and down to about 13%). Because the poor spreadability at refrigerator temperatures has been one of the reasons for the steady decline in butter consumption over the last decades, by far the most research work worldwide invested in butter has concerned its spreadability. This property is mainly affected by three factors:

1. Seasonal diet and lactation stage: the periodically varying melting properties of milk fat (**Figure 4**) lead to corresponding changes in butter spreadability. In the northern hemisphere the terms 'summer butter' (soft because of pasture feeding) and 'winter butter' (hard because of high starch concentrates) are frequently used. The influence of lactation (maximum oleic acid level, i.e. softest fat, about 8 weeks after calving) is normally masked by that of feeding. Several methods to compensate for these variations by modifying the fat composition are currently in use (e.g. rapeseed feeding).
2. Physical cream ripening: by special temperature–time treatments of the cream prior to churning, either alone (sweet cream) or together with microbial ripening (cultured cream), a physically unfavorable (regarding spreadability) fat composition can be efficiently counteracted. Changes in the firmness of the butter by 30% are possible in both directions, softer and harder. The basic approach of such temperature treatments is to control the microstructure of the butter in terms of the spatial arrangement of solid and liquid fat, the size, number and shape of the fat crystals and the overall ratio of solid to liquid fat.
3. Work softening: as outlined earlier, set butter after working will not regain its initial firmness. In some creameries, it has proved successful practice to let the freshly produced butter rest before packing and set for several hours and after subsequent cooling to about 12 °C to work it soft again in a butter homogenizer.

Butter spreadability is graded sensorially or instrumentally by using penetrometric, extruder or sectilometric methods (by which the force required to cut through a standard piece of butter, under standard conditions, is measured). Numerous variants of these methods are in use, partly defined by national standards. Common to all methods is that they yield physical quantities which are rather closely related to the sensorially evaluated spreadability of butter. For instance, the German standard DIN 10331 defines the 'hardness' of butter by the force (measured in newtons) needed to cut a butter cube (edge length 25 mm) by a wire (diameter 0.3 mm) when cutting speed is 0.1 mm s^{-1}. An IDF standard for measuring the firmness of butter on the basis of a modified cutting-wire method is currently being developed.

Keeping Quality

Well-made butter can be kept for at least 2 years, 20 days or 10 days and retain acceptable flavor if stored at −20, 10 or 20 °C, respectively. In any case butter must be protected from moisture evaporation and light-induced photooxidation reducing nutritional value and spoiling flavor and appearance. Butter defects caused by lipases or bacteria present, though initially not perceptible, may cause deteriorations during storage and thus reduce the shelf life. On cold storage, autoxidation is the main limiting factor which depends on the copper present, the level of which greatly depends on the conditions of manufacture.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat Based Spreads; The Product and Its Manufacture. **Milk Lipids**: Conjugated Linoleic Acid; Fatty Acids; General Characteristics; Triacylglycerols.

Further Reading

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Anhydrous Milk Fat/Butter Oil and Ghee

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Introduction

Milk fat is an essential part of the nutritional value of milk, but in the form of milk, cream, or butter, it has a limited keeping quality owing to microbiological deterioration, lipolysis, or oxidation. Because most of these spoilage processes take place in the water phase or at the interface between the water and the fat phase, it is well known that removal of the water phase extends the keeping quality of milk fat considerably. Butter surplus in many dairy-producing countries, combined with production fluctuations during the year and the unequal allocation of milk production in the world, has encouraged production of more stable forms of milk fat products.

This is by no means a modern innovation because such products have been known for several thousand years by various names in Asia, the Middle East, and in milk-producing areas of Africa. Considerable quantities of anhydrous milk fat products are still produced in these parts of the world, often by indigenous methods, mainly for use in cooking.

During World War II, large quantities of anhydrous milk fat was produced and used as spread by the Allied troops, but it was not until the 1960s that the production of anhydrous milk fat increased rapidly, especially in areas where large surplus of butter made it an obviously good idea to convert milk fat into products with higher storage stability, making export to new markets feasible.

Specifications

The Codex Alimentarius Commission under the Joint FAO/WHO Food Standards Programme has established a standard (CODEX STAN 280-1973) with later revisions and amendments, for milk fat products intended for further processing or culinary use. The standard applies to products designated anhydrous milk fat, milk fat, anhydrous butter oil, butter oil, and ghee, defined as fatty products derived exclusively from milk and/or products obtained from milk by means of processes that result in almost total removal of water and nonfat solids. Ghee is furthermore defined as a product with an especially developed flavor and physical structure.

The main specifications in the standard concerning the composition of the products and the designations to be used are shown in **Table 1**. It is further stated in the standard that many food additives acting as antioxidants can be added. These additives, and the quantities allowed to be used, are listed in Codex General Standard for Food Additives (CODEX STAN 192-1995) with the remark that the designation ‘anhydrous milk fat’ is reserved for products where no additives are used.

Finally, it should be mentioned that CODEX STAN 280-1973 also contains a list of quality factors intended for voluntary application by commercial partners but not for application by governments. These factors are listed in **Table 2**.

Products

It is difficult to estimate the production volume of the various types of milk fat products as no official statistics are available. Normally the figures for all product types are pooled under the names AMF or butter oil independent of their composition, and without consideration for the specifications established by the Codex Alimentarius Commission. Furthermore, production figures for anhydrous milk fat and ghee are often converted into butter equivalents and included in the butter data. In the following text the term ‘AMF’ is used for both anhydrous milk fat/anhydrous butter oil, milk fat, and butter oil because both the production technology and the application of these products are quite similar, and AMF is furthermore the term commonly used in both industry and international trade. However, ghee is discussed separately.

AMF

Major producers of AMF are New Zealand and Australia, each with big annual production volumes, but many countries with an industrialized dairy industry produce minor quantities. AMF is mainly exported to countries lacking sufficient production of fresh milk, especially in Asia, the Middle East, the South and Central Americas, and the Pacific, for production of recombined milk and other dairy products. Another important market for AMF is the food industry in which it is used for production of ice cream, bakery products, confectionery, and such. Furthermore, AMF is often an important commodity in different food aid programs.

Table 1 Composition of milk fat products (CODEX STAN 280-1973)

	Anhydrous milk fat/ Anhydrous butter oil	*Milk fat/butter oil/ghee*
Minimum fat (g 100 g^{-1})	99.8	99.6
Maximum water (g 100 g^{-1})	0.1	

Product characteristics

AMF consists of a mixture of triacylglycerols (glycerol esterified to three fatty acids) with different physical properties, for example, melting point, which means that AMF melts over a wide temperature range. Normally AMF is completely melted at temperatures higher than 40 °C where it appears as a transparent liquid with a light yellow color caused by the natural content of β-carotene. When AMF is cooled it starts to crystallize and transform into a pale whitish yellow solid with a soft, slightly grainy texture.

Like most fats, milk fat crystallizes from the melt in different polymorphic forms, of which only one is stable, whereas the others are metastable. On rapid cooling, metastable α-crystals with a rather simple and loose structure usually form first, but these crystals are later rearranged irreversibly into the more stable β'-form, and finally into the most thermodynamically stable, compact-structured β-form. However, it seems that most of the milk fat crystals remain in the β'-form even after prolonged storage. The α-form has the lowest melting point, and the β-form normally has the highest. The β'-form is preferred as it provides a fine arrangement of the fat crystals. The β-form is often not desirable because the large crystals of this arrangement may result in a coarse and sandy texture. Over the years, polymorphism of milk fat has been studied thoroughly, but it is still unclear whether polymorphism has any importance under practical conditions and most costumers are probably not very concerned about the crystal structure in AMF.

AMF has a relatively high-average melting point, and that means that the functionality of AMF is not optimal for all applications. That milk fat consists of a mixture of triacylglycerols with different melting points implies it can be split into fractions with different melting properties suitable for different applications. The main procedure is to split AMF into two fractions: (1) a high-melting part called the stearin fraction and (2) a low-melting part called the olein fraction.

Milk fat has a unique flavor mainly attributed to components like short-chain free fatty acids, aldehydes, ketones, and lactones, and this is the main advantage of using AMF instead of cheaper fats and oils.

Unpleasant off-flavors, however, can occur in AMF, one of which is the rancid flavor originating from the milk or butter used as starting material for production (*see* **Butter and Other Milk Fat Products**: The Product and Its Manufacture). Rancid flavor is caused by lipolysis (enzymatic hydrolysis of the ester linkage in the triacylglycerols) resulting in release of free fatty acids (FFA); in particular, increased concentration of free short-chain fatty acids give a distinct off-flavor. It is possible to remove, or at least reduce, this off-flavor during manufacturing of AMF as described later. As stated in CODEX STAN 280-1973 the content of FFA should not be higher than 0.3 or 0.4 expressed as the percentage of oleic acid, depending on the designation of the product.

Another typical off-flavor that limits the storage stability is metallic, fishy, or oily flavor caused by lipid oxidation, which involves complex reactions between lipids and oxygen promoted by catalysts such as copper and iron ions. As stated in CODEX STAN 280-1973 the extent of the oxidation should not be higher than 0.3 or 0.6 milliequivalents of oxygen per kilogram fat, depending on the designation of the product. Stored butter used for production of AMF could be the origin of this off-flavor but it can also arise during prolonged storage of AMF. The oxidative stability of AMF can be improved considerably if different precautions are taken during processing. Contamination with copper and iron ions, which could originate from the processing equipment or from metal containers used for packaging of the

Table 2 Recommended quality factors (CODEX STAN 280-1973)

	Anhydrous milk fat/anhydrous butter oil	*Milk fat/butter oil/ghee*
Maximum free fatty acids (FFA) expressed as oleic acid (g 100 g^{-1})	0.3	0.4
Maximum peroxide value (milli-equivalents of oxygen kg^{-1} fat)	0.3	0.6
Maximum copper content (mg kg^{-1})	0.05	0.05
Maximum iron content (mg kg^{-1})	0.2	0.2
Taste and odor	Acceptable for market requirements after heating a sample to 40–45 °C	
Texture	Smooth and fine granules to liquid, depending on temperature	

product, should be avoided, dissolved oxygen in the product should be reduced during processing, and the product should be package in light- and airtight containers and stored at low temperature. Also, addition of antioxidants to delay the oxidation is a possibility, depending on the designation of the product (CODEX STAN 192-1995). AMF will normally have a shelf life of several months, if these precautions are taken even if stored at ambient temperature, which will often be the case.

Applications

An important application of AMF is production of recombined liquid milk. Low-heat- or medium-heat skim milk powder is mixed with water and AMF. The AMF is added at a temperature higher than 40 °C and has to be properly dispersed and emulsified into the product. In large-scale production, the melted AMF is injected in-line in a continuous operation and emulsified in a high-shear mixer. The mixing could also take place in a batch operation, in which the melted AMF is added to a solution of milk powder in water followed by emulsification, for example, in a homogenizer or a mixing device. It might be necessary to add suitable emulsifiers to create a stable emulsion. Also cream, cultured milk products, cheese and butter could be manufactured in a recombination process if the fat content of the mix is adjusted accordantly.

AMF can also be used in production of blends, low-fat dairy spreads and blended spreads and an obvious application for low-melting fractions of AMF is in butter and dairy fat spreads in order to make these products more spreadable at low temperatures (*see* **Butter and Other Milk Fat Products**: Milk Fat-Based Spreads). Addition of high-melting fractions could also be an option with the aim of making butter more stable at high temperatures, which could be desirable in tropical countries. One disadvantage of using fractions of AMF is the increased production costs.

Considerable quantities of AMF are used for production of ice cream in which it contributes not only to the flavor of the product, but also to a smooth, full, rich and creamy mouthfeel. One of the advantages of using AMF instead of cream or butter is that AMF is easier to handle in an automatic process line for preparing the ice cream mix.

The advantages of using milk fat in the bakery industry are the buttery flavor and the creamy mouthfeel it imparts in products such as puff pastry, croissants, and Danish pastry and the functional properties, especially that of the stearin fraction of AMF with a higher melting point and solid fat content, that are useful in such products. AMF, also used in butter cookies where the main concern is flavor, has shortening properties such as crunchiness. A serious problem in butter cookies is fat blooming, which are mold-resembling pale stains, on the surface of the cookies formed by crystals of high–melting point fat; this can be avoided using the olein fraction of AMF.

Milk fat in different forms, often AMF, is also used as an important ingredient in a variety of confectionery products despite its relatively high cost compared with most other fats and oils. Milk fat is primarily used for its buttery flavor, but another important property of milk fat is its ability to form part of the continuous fat phase in chocolate as a result of its compatibility with cocoa butter at most levels of addition. In dark chocolates, small amount (2–3 g $100\,g^{-1}$) of milk fat is used to soften the product and to control texture formation. In milk chocolates, higher addition (often up to 20–30 g $100\,g^{-1}$) is used. Milk fat has also the ability to inhibit the formation of fat bloom in chocolates, which appears as a grayish powdery discoloration on the surface of the chocolate composed of large fat crystals formed by phase transition in cocoa butter crystals at low ambient temperature.

Ghee

Ghee is the name used in India for a variety of milk fat products mainly made from cows' or buffaloes' milk by methods that can be traced back to ancient times. Similar products are produced in the Middle East and Africa commonly from goat, sheep, or camel milk. In these areas, the products are mostly known as *maslee* or by some variant of the Arabic word *samn*. These products are discussed in the following paragraphs under the common name ghee.

The products were traditionally manufactured in rural areas where cream or butter was heated over open fire until the water had boiled off. During this treatment, considerable browning of the nonfat milk solids occurs that develops a special taste and a strong antioxidative effect. Today, ghee is often factory made by more industrialized methods. India is the major producer of ghee, but the product is now also gaining popularity in the Western world and can be purchased in many ethnic shops.

In the Codex Alimentarius Commission standard applying to milk fat products (CODEX STAN 280-1973) the only difference in the specifications between ghee and other AMF products is that ghee is characterized as having a special flavor and physical structure. This is a rather unspecific and not very useful definition, but the problem is of course that the specification tries to cover all the varieties of ghee made in different countries. However, the main point is that ghee is an almost anhydrous milk fat product obtained by a high-temperature process that leaves part of the nonfat milk solids in the product.

Product characteristics

The flavor of ghee is very dependent on the manufacturing process. Much of the typical flavor is the result of a very complex mixture of compounds including carbonyls, lactones, FFA, and esters generated during processing. The typical flavor of burnt nonfat milk solids combined with some component derived from oxidation of the milk fat is also important. Lactones, which have a coconutlike flavor, seem to be especially important for the characteristic flavor of ghee. The levels of these are increased by increasing temperature during the production, and it is often twice as high in ghee as in butter. The role of phospholipids in ghee has been much debated. Some studies have shown that high content of phospholipids results in poor keeping quality, whereas others have indicated that phospholipids dissolved in fat, as is the case in ghee, have distinct antioxidative properties. This needs further investigation.

Ghee has an intense yellow color, unless produced from buffaloes' milk, which contains much less β-carotene than cows' milk. Ghee should have a soft and granular texture, with a large proportion of crystalline fat suspended in sufficient liquid fat so the product can be poured at room temperature. Such a texture could be promoted by slow cooling under constant stirring, but textural changes might occur during storage depending on the temperature.

Ghee normally has a shelf life of 6–8 months even at ambient temperature, but considerably longer storage stability has been experienced probably because of the antioxidative effect created during processing. However, ghee eventually deteriorates during storage; the limiting factor is normally the development of oxidized flavor.

Applications

Ghee is primarily used for cooking and frying and as dressing or toppings for various foods. It is also used in the manufacture of snacks and sweets often mixed with vegetables, cereals, fruits, and nuts. In some parts of the world, ghee is considered as a sacred product and is used in religious rites. It could also be mentioned that ghee is used in Ayurveda, which is a system of traditional medicine developed in India several thousand years ago and now also practiced in other parts of the world as alternative medicine.

Manufacturing Technology

The overall principle in the manufacturing technology for the different milk fat products is removal of water, leaving an almost pure fat phase. The industrialized process for manufacturing of AMF is described later, followed by a separate discussion of the manufacturing process for ghee.

AMF Technology

AMF is produced from either cream or butter in a process where centrifugal separators are the essential equipment as shown in **Figure 1**.

Manufacture of AMF from cream

The process for production of AMF directly from cream is shown in **Figure 2**. Cream is concentrated in a special separator (4) to a fat content of 70–80 g 100 g^{-1} after which the highly concentrated fat emulsion is homogenized (7), which results in disruption of the fat globule membranes causing a phase inversion. The mixture of butter oil and serum is then separated in a centrifugal separator or concentrator (9), where the fat content is concentrated to approximately 99.5 g 100 g^{-1}.

It might be necessary to wash the oil phase with water to obtain a clear transparent, bright shining product (**Figure 1**). This production step, called polishing, involves addition of 20–30 g water 100 g^{-1} to the oil. It is important that the water temperature remain the same as the oil temperature. After mixing and a short holding time, the water is removed again in a separator together with the water-soluble components. Polishing could be combined by neutralization if the content of FFA in the oil is too high. Neutralization implies that a sodium hydroxide solution is injected continuously into the oil stream coming from the concentrator (9) after which the mixture is held for some seconds in a holding cell to allow the reaction to take place followed by addition of the

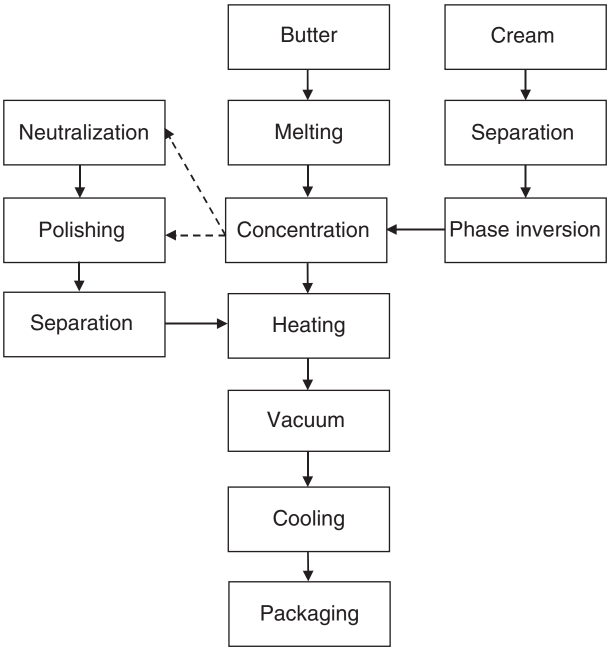

Figure 1 Block diagram showing principles of production of anhydrous milk fat (AMF) from cream or butter with possibilities for polishing and/or neutralization.

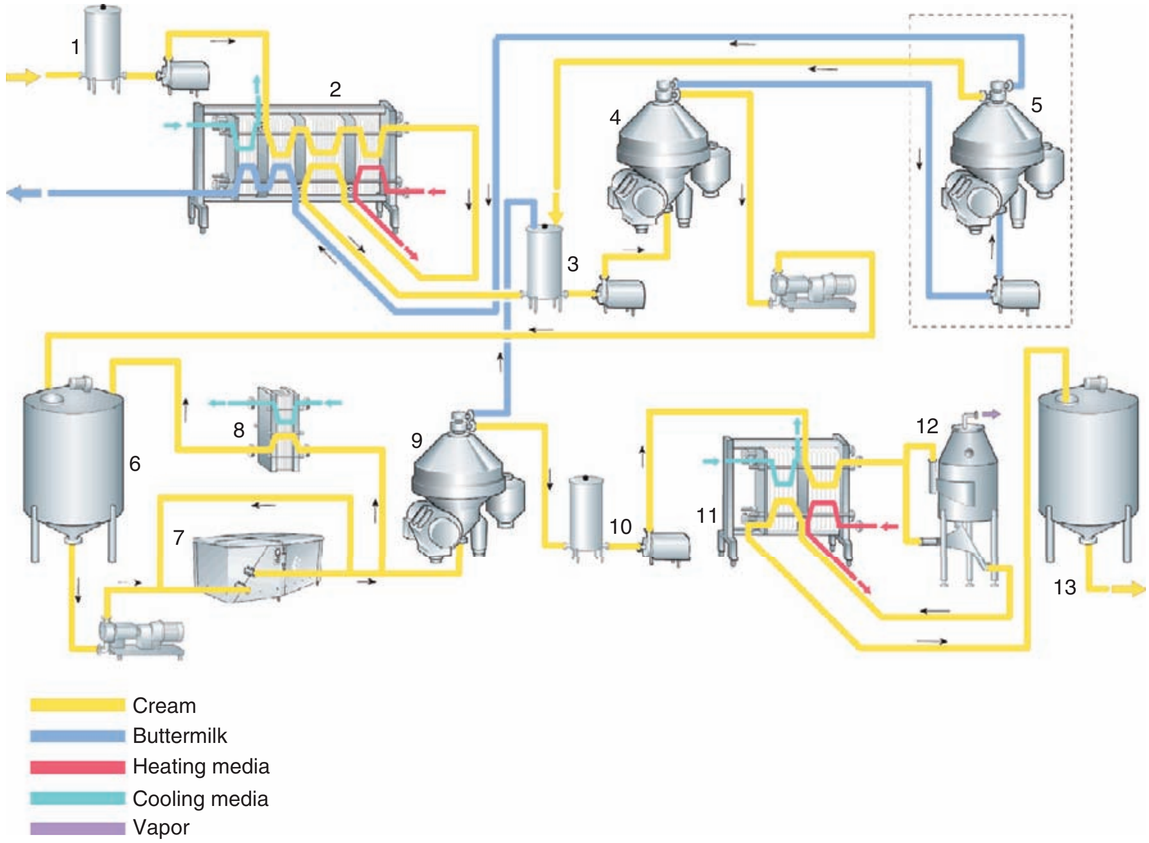

Figure 2 Production line for anhydrous milk fat (AMF) from cream. 1, balance tank; 2, plate heat exchanger; 3, balance tank; 4, preconcentrator; 5, separator (optional) for 'buttermilk' from the preconcentrator (4); 6, buffer tank; 7, homogenizer for phase inversion; 8, plate heat exchanger for cooling; 9, final concentrator; 10, balance tank; 11, plate heat exchanger for heating/cooling; 12, vacuum chamber; 13, storage tank. Reproduced with permission from Dairy Processing Handbook by permission of Tetra Pak A/B, Lund, Sweden.

polishing water. When the added water is removed again in a separator, the saponified FFA will be removed simultaneously.

After the final concentration the butter oil is heated to 90–95 °C in a plate heat exchanger (11) and pumped to a vacuum chamber (12), where the oil is introduced either as a thin film or as a spray so the increased surface area makes it possible to further remove water and dissolved air. The oil, now having a fat content of higher than 99.8 g 100 g^{-1}, is finally pumped back to the plate heat exchanger, where it is cooled to approximately 40 °C and pumped to a storage tank (13), where flushing with an inert gas and addition of antioxidants is a possibility before packaging.

Manufacture of AMF from butter

If it has already been produced, butter is the starting material for the production of AMF; the process, which is shown in **Figure 3**, is initiated by melting of the butter. Both sweet cream and cultured butter as well as salted and unsalted butter can be used, often in the form of cold stored or frozen butter stored for some time. The melting process often starts by heating the butter in a temperature-controlled room, but microwave thawing can also be used. After warming, further heating follows in a special melting equipment (1) by indirect heating with steam or hot water to a temperature well above the melting point of the fat. If the production of AMF takes place immediately after the butter manufacture without any intermediate storage, the butter is pumped directly to the melting equipment.

After melting the now liquid blend of milk fat and butter serum is pumped to a holding tank (2), where it is held for about 30 min to ensure complete melting of the fat and aggregation of the proteins in the aqueous phase. From the holding tank, the blend is pumped to a special separator or concentrator (3). The separation is a critical stage in the process as it is very dependent on the type of butter used. The more or less undenaturated proteins in the serum phase of sweet cream butter will often result in a certain degree of emulsification of the fat, which will complicate the separation. This can be counteracted by lowering the pH-value of the serum phase to about 4.5 for example by adding citric acid during melting. This will denaturate the proteins and consequently reduces their emulsifying ability.

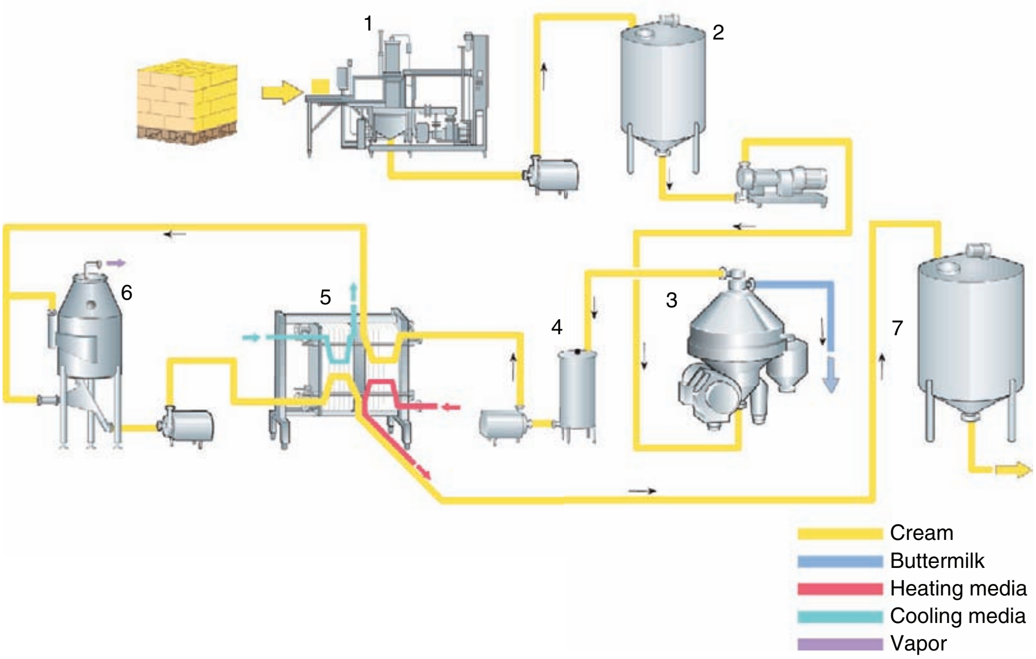

Figure 3 Production line for anhydrous milk fat (AMF) from butter. 1, melter and heater for butter; 2, holding tank; 3, concentrator; 4, balance tank; 5, plate heat exchanger for heating/cooling; 6, vacuum chamber; 7, storage tank. Reproduced with permission from Dairy Processing Handbook by permission of Tetra Pak A/B, Lund, Sweden.

After separation, the fat phase is further treated in a similar process as described when using cream as starting material.

Fractionation of AMF

As already mentioned, AMF is not suitable for all applications, but its functional properties can be altered by using a fractionation process where the milk fat is split into fractions with different melting points. Different methods of fractionation have been developed. One of the simplest and most efficient methods is crystallization from a solvent, such as acetone, but the use of organic solvents in food production and the consequent loss of butter flavor are problematic and have prevented commercial exploitation of this method.

The most commonly used fractionation process (**Figure 4**) in commercial operation today is one in which liquid AMF is crystallized in a tank, where it is taken through a controlled temperature program. During this process triglycerides with high melting points will crystallize, and afterwards the slurry is divided into a liquid and a solid fraction by vacuum or pressure filtration, by centrifugation, or by a combination of these methods. The applied cooling program strongly influences the composition of the fat crystals, and the cooling rate influences the quantity of the crystallized fat and the size of the crystals. This so-called dry fractionation or crystallization from the melt is relatively cheap and is based on a physical process not involving solvents. Furthermore, this process has the least effect on flavor and is generally accepted by legislation.

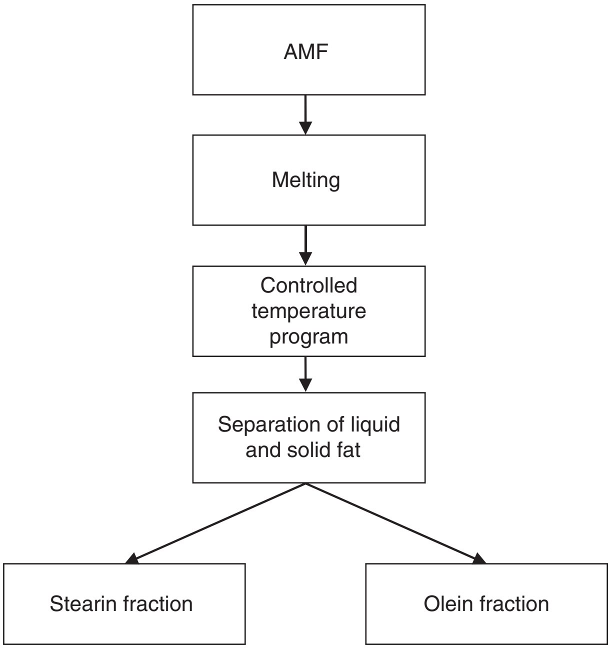
AMF
Melting
Controlled temperature program
Separation of liquid and solid fat
Stearin fraction
Olein fraction

Figure 4 Block diagram showing the principle of fractionation of anhydrous milk fat (AMF).

The result of the fractionation is a high-melting fraction, the so-called stearin fraction, and a low-melting fraction, the so-called olein fraction. These two fractions can be further fractionated by a multistep fractionation process into even harder and softer fractions, respectively.

Packaging of AMF

An often used procedure in connection with packaging of AMF is to flush the containers with an inert gas before, during, or after filling and use airtight containers. The traditional way of packaging AMF is in large iron drums or barrels containing 190–200 kg and enameled on the inside or lined with a plastic foil, a so-called inner liner, to prevent direct contact between the metal and the AMF. A certain headspace in the barrels is required to control the drop of pressure arising from the reduction in volume caused by crystallization of the AMF. Nowadays a commonly used packaging is cardboard boxes with an inner liner of plastic foil, the so-called bag-in-box system. Different size of packaging is used, for example, 25 kg. Also consumer packaging in cans containing 0.5, 1, or 2 kg seems to be gaining popularity.

For very big customers, shipping often takes place in road tankers or very large bulk tanks suitable for transport with containerships.

Ghee Technology

From ancient times, ghee has been prepared in households by heating fermented milk or butter in open pans causing the water to boil off. The fat is then separated through a strainer, and finally cooled slowly to give a coarse texture of crystallized fat and liquid oil. Nowadays ghee is often factory made from milk, cream, or butter using different technologies in which heat treatment is the essential process step.

If milk is the starting material, it is normally separated into cream and skim milk, and the cream is then subsequently churned into butter, melted, and processed into ghee in a process where the butter is heated to about 100–104 °C until the residual water content is lower than $1\,\mathrm{g}\,100\,\mathrm{g}^{-1}$. After this the temperature is increased to 110–115 °C, and the product is held at this temperature until the desired flavor has developed. The advantage of this method is that most of the water is removed by the skim milk and by the buttermilk, which is a much more energy-efficient method than removal by heating. In an even more efficient method the butter is heated to about 85 °C, held for 30 min to allow the aqueous phase to form a bottom layer of residual buttermilk, which is then removed. Finally the fat phase is further heated to about 110 °C.

One of the industrialized methods used for production of ghee is a batch process in which butter or cream is boiled in a stainless steel vessel by indirect heating with steam. India has a large production of ghee, and because of the commercial importance of the production, considerable improvements in the manufacturing process have been developed over the years in that country. Thus a continuous ghee manufacturing process has been developed employing scraped surface heat exchangers and centrifugal separators and clarifiers. A method based on heating with microwave technology has also been developed.

Ghee is normally packed in metal cans and containers of various sizes internally lined with a nontoxic lacquer. Cardboard boxes with an inner liner of plastic foil are also used.

Final Remarks

AMF and similar products have many applications, and in some parts of the world, they are of vital importance for the economy of the dairy industry. An essential characteristic of the products is their prolonged keeping quality caused by the very low water content, which makes the products suitable for acting as a buffer during fluctuations in milk production over the year. It also makes the products suitable for shipment to faraway destinations to help leveling the unequal allocation of milk production in the world.

See also: **Butter and Other Milk Fat Products**: Milk Fat Based Spreads; The Product and Its Manufacture. **Enzymes Exogenous to Milk in Dairy Technology**: Lipases. **Enzymes Indigenous to Milk**: Lipases and Esterases. **Flavors and Off-Flavors in Dairy Foods**. **Milk Lipids**: Fatty Acids; Lipid Oxidation; Lipolysis and Hydrolytic Rancidity.

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Milk Fat-Based Spreads

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Introduction

Butter has a unique flavor and a natural image, but ordinary butter also has some shortcomings like high fat content, high content of saturated fat, and reduced spreadability when taken directly from the refrigerator.

The high content of saturated fat in butter has bothered many for many years from a health point of view and at the same time vegetable oils with a high content of polyunsaturated fatty acids have attracted many for their positive effect in relation to cardiovascular diseases. This has, combined with recommendations for reduction in the total amount of fat in the human diet, resulted in a decline in the consumption of full-fat butter and a growing interest in low-fat spreads and blends produced from milk fat, and fats and oils of other origin, especially vegetable oils. Addition of vegetable oils will at the same time improve the spreadability of the product.

Legislation aimed at protection of agricultural produce prohibited for many years mixture of milk fat and other fats and oils and restricted product development within the butter sector. However, this situation has changed, and nowadays food legislation in most countries permits manufacturing and marketing of a multitude of butter-related products varying in both fat content and fat composition. The driving force behind this development has been and still is the dairy industry's attempt to maintain or even increase its share of the yellow fat market by meeting the consumer's desire for more healthy products and products with improved functionality.

Specifications

The Codex Alimentarius Commission under the Joint FAO/WHO Food Standards Programme has established a standard (CODEX STAN 253-2006) for low-fat butter-like products, where milk fat is the only fat source. In this standard, such products are designated 'dairy fat spreads' although other names may be used if allowed by the national legislation in the country of retail sale. The products are defined as milk products relatively rich in fat in the form of a spreadable emulsion principally of the water-in-oil type that remains in the solid phase at a temperature of 20 °C. The raw materials, which should be milk and/or products obtained from milk, including milk fat, may have been subjected to any appropriate processing (e.g., physical modifications including fractionation) prior to their use. The milk fat content should not be less than $10\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ and not more than $80\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ and must represent at least two-thirds of the dry matter. The standard also comprises a list of food additives that may be used, including colors, emulsifiers, preservatives, stabilizers/thickeners, acidity regulators, antioxidants, antifoaming agents, and flavor enhancers.

Similar specifications are established in the Codex Alimentarius standard for fat spreads and blended spreads (CODEX STAN 256-2007) concerning products in which the fat phase is a mixture of milk fat and fats or oils of vegetable, animal, or marine origin. This standard applies to fat products, containing not less than 10 and not more than $90\,\mathrm{g}$ fat $100\,\mathrm{g}^{-1}$, intended primarily for use as spreads. The fat content must represent at least two-thirds of the dry matter. The designation 'blend' is used for products with $80\,\mathrm{g}$ fat $100\,\mathrm{g}^{-1}$ or more of which milk fat is more than $3\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ of the total fat content, and the designation 'blended fat spreads' is used for products with less than $80\,\mathrm{g}$ fat $100\,\mathrm{g}^{-1}$. However, a higher minimum percentage of milk fat may be specified in accordance with the requirements of the country of the retail sale.

Within the European Union, Council regulation No. 2991/94 provides standards for spreadable fats principally of the water-in-oil emulsion type. In this standard, it is stated that products in which the fat phase derives exclusively from milk and fat content is less than $80\,\mathrm{g}\,100\,\mathrm{g}^{-1}$, a spread is designated 'dairy spread *x*%', if *x* is the actual percentage of fat. The name 'three-quarter-fat butter' is used for products with fat content between 60 and $62\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ and the name 'half-fat butter' is used for products with a fat content between 39 and $41\,\mathrm{g}\,100\,\mathrm{g}^{-1}$. In the same regulation, rules are also established for production and marketing of blends between milk fat and fat of other origin where the milk fat content is between 10 and $80\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ of the total fat content. For such products, the designation 'blend' is used instead of butter and 'blended spread' is used instead of 'dairy spread' (**Table 1**).

Products

Dairy Fat Spreads

The idea of producing butter-like products with a reduced fat content is by no means new; it was tried in the United States during World War II when there was a

Table 1 EU standards for spreadable fats (EU Council regulation No. 2991/94)

Fat content	*100 g milk fat 100 g^{-1} of total fat content*	*Milk fat between 10 and 80 g 100 g^{-1} of total fat content*
Not less than 80 g 100 g^{-1}	Butter	Blend
But less than 90 g 100 g^{-1}		
Not less than 60 g 100 g^{-1}	Three-quarter-fat butter	Three-quarter-fat blend
But not more than 62 g 100 g^{-1}		
Not less than 39 g 100 g^{-1}	Half-fat butter	Half-fat blend
But not more than 41 g 100 g^{-1}		
– Less than 39 g 100 g^{-1}	Dairy spread *x*%	Blended spread *x*%
– More than 41 but less than 60 g 100 g^{-1}		
– More than 62 but less than 89 g 100 g^{-1}		

fat shortage, but the products, which were mainly of the oil-in-water emulsion type, were obviously very different from butter and were not successful. Renewed interest for low-fat products in the 1960s intensified product development, often based on margarine technology applying scraped-surface heat exchangers and stirred crystallizers. However, the quality of most of these products did not meet the expectations of the consumers, and many of the products were quickly withdrawn from the market.

From the beginning, the ambitious target was spreads with only half the fat content of full-fat butter, and this created a lot of quality problems as it is difficult to combine 40 g fat 100 g^{-1} and the corresponding high water content into a stable product. Later, the efforts were focused on products with a higher fat content (e.g., 60 g 100 g^{-1}), which from a technical point of view is a much easier product to manufacture, and new production technologies were eventually developed. Several products of this type are produced today.

Dairy fat spreads with even lower fat content based solely on milk fat will have a very firm consistency and poor spreadability unless the composition of the fat is adjusted by adding a low-melting fraction of milk fat. This will, however, increase the production costs considerably, and only few products of this type have been marketed with success.

Blends

In consequence of the debate about saturated versus unsaturated fat and the criticism of the spreadability of ordinary butter, it was an obvious idea to mix butter and vegetable oils with a high content of unsaturated fatty acids. However, legislation in most countries prohibited production and marketing of such blends as already mentioned, and it was not until the product Bregott® containing 80 g fat 100 g^{-1}, of which 80 g 100 g^{-1} fat was milk fat and 20 g 100 g^{-1} fat soybean oil, was launched in Sweden in 1969 and obtained a considerable market share that the dairy industry and the farmers in other countries realized that it was in their own best interest to market such products and started lobbying for a change in legislation. Today, a variety of full-fat blends are on the market in many countries, and these products have in some cases obtained a higher market share than regular butter.

The preferred vegetable oil used today in the production of blends seems to be canola oil, which is rapeseed oil low in erucic acid and high in oleic acid, but sunflower seed oil and olive oil (high in monounsaturated fatty acids) are also used. Blends with olive oil seem to be especially popular within certain consumer segments probably caused by association with the claimed health benefits of the ‘Mediterranean diet’. Also blends containing refined marine oils rich in omega-3 fatty acids are on the market, but high production costs have made it difficult to increase sales of such products.

Blended Fat Spreads

As mentioned elsewhere, it is costly to change the composition of milk fat by fractionation and therefore adding vegetable oil when manufacturing low-fat spreads looks obvious alternative. Again the development was pioneered by the Swedish dairy industry, and the product Lätt & Lagom was launched in 1975, which was quickly followed by similar products in other countries. The original Swedish product had a fat content of 40 g 100 g^{-1} of which 60 g 100 g^{-1} fat was milk fat and 40 g 100 g^{-1} fat was soybean oil, and it had a water content of 48 g 100 g^{-1}.

A broad range of blended fat spreads have been marketed since then, and the oils added today are similar to those used in the manufacture of full-fat blends. Products with 40 g fat 100 g^{-1} or even lower are not unusual, but the majority of blended fat spreads on the market seem to be products with 60 g fat 100 g^{-1}, often marketed as lighter spreadable products.

Product Characteristics

The microstructure of milk fat-based spreads of the water-in-oil emulsion type depends on the manufacturing process. A butter-like, partly globular, structure is obtained if a method based on churning or phase inversion of cream is used. Addition of low-melting vegetable oils or milk fat fractions will of course increase the continuous fat phase and diminish the globular fat phase proportionally, but it also results in the formation of a less extensive three-dimensional crystal network in the continuous fat phase during cooling. If an emulsification method based on margarine technology is used, a homogeneous margarine-like structure is obtained. This structure results in the formation of a very extensive crystal network, which, even at low fat content, causes a very firm consistency and poor spreadability at low temperatures if the composition of the fat phase is not modified substantially by addition of low melting point fat.

As in butter, microscopic moisture droplets are distributed in the continuous fat phase. The droplet size is most important for the product's ability to release flavor during melting in the mouth, and for its stability in respect of microbiological spoilage during storage. In butter, most of the water droplets have a diameter in the range 1–5 μm, but in low-fat spreads with high water content, the droplet size might increase dramatically, and the droplets come in close contact with each other, separated by only a thin film of fat, and tend to coalesce into larger droplets. Every state between water-in-oil and oil-in-water emulsion types has been observed in spreads with very low fat content. It is therefore important to stabilize or immobilize the dispersed aqueous phase against coalescence by adding suitable stabilizers. However, a balance exists and a too stable water phase will result in a very slow flavor release in the mouth. On the other hand, if the aqueous phase is too unstable, syneresis might occur, meaning that water tends to leak out of the product. If spreads with a poor binding of water are spread on a slice of bread and the sandwich is left for a couple of hours, part of the water will be absorbed into the bread, which then obtains an unpleasant pasty and spongy consistency, and a thin and transparent film of fat is left on the bread.

Concerning the flavor and taste of the products, addition of other fats and oils will inevitably dilute the characteristic butter flavor, and if the content of milk fat is below 25–30 g 100 g^{-1} of the total fat content, it might be necessary to add flavoring substances and flavor enhancers.

Unsaturated lipids may undergo spontaneous autooxidation during storage, and the addition of vegetable or marine oils will therefore decrease the oxidative stability of blends and blended spreads. It is well known that most vegetable oils contain natural antioxidants, for example, tocopherols, which delay oxidation at the beginning of storage, but when the antioxidative effect is exhausted the oxidation accelerates as a consequence of the high content of unsaturated fat. Addition of antioxidants during the manufacturing process will decrease the reaction rate and prolong the induction of the oxidation, but it cannot completely prevent autooxidation during storage.

Manufacturing Technology

Many experiments concerning manufacturing of milk fat-based spreads have been carried out over the years, and many procedures have been patented claiming to result in products with the desired spreadability, mouthfeel, and flavor. The technologies available today are mainly based on margarine technology, churning technology, and technology involving direct inversion of cream.

Margarine Technology

The main principle in margarine technology is that the fat phase and the aqueous phase are prepared separately, mixed, emulsified, crystallized, and then subjected to an intensive mechanical working (**Figure 1**).

The fat phase has to be prepared in a temperature-controlled tank where the temperature should be at least 40 °C in order to avoid partial crystallization resulting in a grainy texture in the final product. The composition of the fat phase depends on the type of product in question. Anhydrous milk fat, often mixed with a low-melting fraction of milk fat, is normally used if dairy fat spread is the target. In the production of blends and blended fat spreads, a mixture of anhydrous milk fat and vegetable oil is normally prepared. In all cases, it is necessary to add a certain level of emulsifiers such as distilled monoacylglycerols or lecithin to the melted fat phase; otherwise, it is not possible to create a stable emulsion that is imperative in the following production steps. However, there is a limit on the level of emulsifiers added, as addition of high levels of emulsifiers will result in too stable a product, difficult to destabilize in the mouth during eating, which most consumers will experience as an unpleasant waxy mouthfeel. Also, flavors and antioxidants might be added during the preparation of the fat phase.

The aqueous phase is prepared in another tank where stabilizers are added to water in order to immobilize the moisture phase in the final product so that phase separation and release of water from the product are prevented. The lower the content of fat in the final product, the more important the composition and properties of the aqueous phase become. Initially, the most common approach, based on experience from the margarine industry, was to prepare a protein-free aqueous solution stabilized by addition of high amounts of hydrocolloids. Later in the 1970s, another course was taken when the Swedish product Lätt & Lagom® was developed, in which the aqueous phase was stabilized by increased viscosity obtained through addition of high amounts of soluble proteins

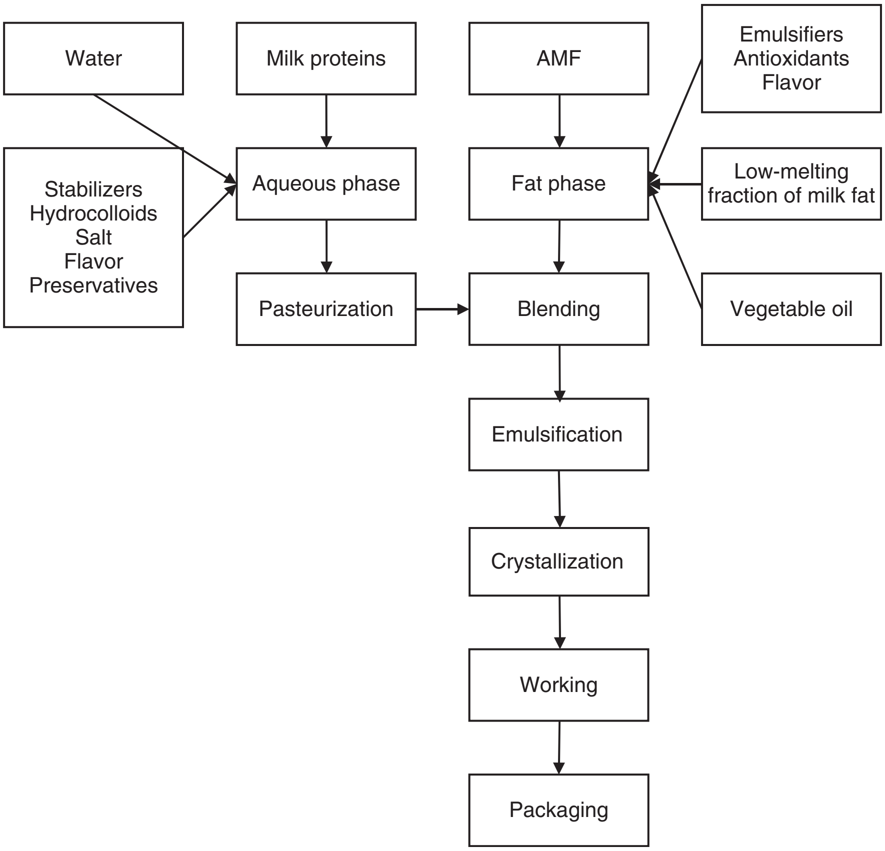

Figure 1 Block diagram showing the principle of production of milk fat-based spreads by the margarine method. AMF, anhydrous milk fat.

from buttermilk, skim milk powder, whey protein concentrates, or sodium caseinate. When the price of milk proteins increased during the 1980s and 1990s, spreads with low and medium level of protein were developed, where the protein content was supplemented with thickeners and stabilizers such as starch, gelatin, alginate, pectin, inulin, or carrageen. However, as already mentioned, there is an upper limit for the use of stabilizers, as too strong binding of the aqueous phase will result in poor flavor release when the product is used. Also salt, flavors such as lactic acid and diacetyl (2,3-butanedione), and preservatives such as sodium, calcium, or potassium sorbate might be added to the aqueous phase, and finally the whole mixture is pasteurized.

After preparation of the fat and aqueous phases, the two are mixed in a blending tank at a temperature above the melting point of the fat phase. The blend is then emulsified either by agitation or by pumping through a homogenizer at moderate pressure or through an emulsification pump where the creation of a water-in-oil emulsion takes place. A crucial point at this step is the stability of the emulsion. An unstable emulsion increases the risk of phase inversion back to an oil-in-water emulsion in the following production step, which will result in an inhomogeneous final product. It is therefore vital that the composition and the properties of both the fat and the aqueous phases are carefully monitored and controlled.

The emulsion created is pumped through a scraped surface heat exchanger, where it is crystallized in the form of a water-in-oil emulsion under mechanical working and fast cooling. The cooling rate and the in-line pressure in the cooler are most important and should be controlled carefully, as too high shearing forces might break the emulsion. A scraped surface heat exchanger (**Figure 2**) designed for heating or cooling of viscous products consists of a number of cooling tubes or cylinders linked in series or parallel where the product is pumped through in countercurrent flow with the cooling medium, often ammonia, circulated in the surrounding jacket.

The cylinders are equipped with knives or blades mounted on a central rotor continuously scraping the crystallized emulsion formed on the cold inner surface of the tube and mixing it in order to ensure uniform heat transfer (**Figure 3**).

Between or after the cooling tubes, the product is exposed to mechanical kneading or working in a special unit, which could be a sort of mixer or pinworker where further crystallization takes place while the emulsion is intensively sheared by a central rotor with a number of pins (**Figure 4**). The aim of the mechanical working is to

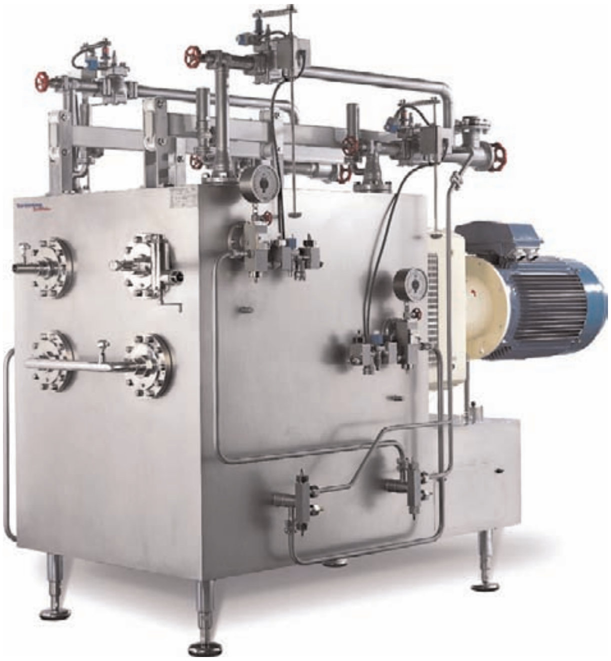

Figure 2 Scraped surface heat exchanger. By permission of Gerstenberg Schröder A/S, Brøndby, Denmark.

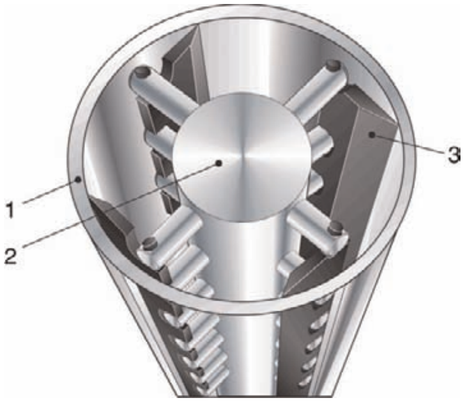

Figure 3 A cross-sectional view of a scraped surface heat exchanger. 1, Cylinder; 2, rotor; 3, blade. Reproduced with permission from (2003) *Dairy Processing Handbook*, 2nd revised edn. Lund, Sweden: Tetra Pak Processing Systems AB.

enhance crystallization and to break the already formed crystal network in order to ensure a homogeneous product with optimal plasticity and spreadability.

Crystallization takes time and the emulsion will therefore still contain a certain amount of supercooled fat when leaving the scraped surface heat exchanger. In order to leave time for further crystallization, the product could be led through resting cylinders. These are often large diameter jacketed tubes cooled with water, which allow the product to crystallize further under milder shear conditions. After the final treatment, the product is transferred to packaging.

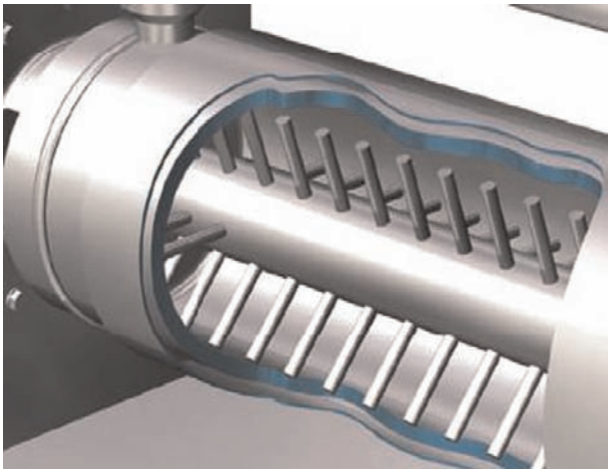

Figure 4 A cross-sectional view of a pin rotor. By permission of Gerstenberg Schröder A/S, Brøndby, Denmark.

Churning Technology

In the traditional process of manufacturing butter based on either batch churning or continuous churning (the Fritz method), water content higher than $16\,g\,100\,g^{-1}$ can be retained in the product by choosing higher churning speed, higher churning temperature, and/or increased dosage of water. However, there are limitations, as it will not normally be possible in this way to increase the water content more than $25–30\,g\,100\,g^{-1}$ corresponding to a reduction in the fat content to about $70\,g\,100\,g^{-1}$.

Higher water content and a corresponding lower fat content can be obtained by adding a moisture phase with an increased viscosity. Butter with a normal fat content of approximately $80\,g\,100\,g^{-1}$ is mixed with a pasteurized and chilled viscous aqueous solution of sodium caseinate. The mixing is carried out in a sort of blender or mixer where a controlled amount of the aqueous solution is added to the butter. Also preservatives, stabilizers, and flavor enhancers can be added if necessary. The product can then be cooled to the packaging temperature in a scraped surface heat exchanger and transferred to packaging.

When the technology for production of blends was first developed more than 40 years ago, it was based on batch churning where the vegetable oil was added either in the cream storage tank or in the churn prior to churning. During the churning process, the vegetable oil is emulsified into the cream forming small fat globules, and when the formation of butter grains takes place, these small fat globules are incorporated. The grains formed in this way are very soft, and it is often necessary to lower the temperature by washing the grains with cold water after draining the buttermilk. Furthermore, the working time has to be shorter and less intensive than working of normal butter; otherwise, the product will be overworked, which results in a very greasy product.

Later, when the production was based on continuous churning, vegetable oil was either added in the cream storage tank or injected directly into the pipeline transferring cream from the storage tank to the continuous churn. In order to obtain a low churning temperature, it is necessary to cool the cream as well as the oil to about 5–7 °C before churning. However, it is vital that the churning temperature is chosen according to the type of vegetable oil, as it is important that the oil does not start to crystallize at the churning temperature. The mechanical working of the blend must be sufficient to obtain a homogeneous structure in the product. However, it is also important that the working is not overdone as this will result in a greasy consistency in the final product.

Addition of vegetable oil to the cream before churning has one big disadvantage as residues of the oil will inevitably end up in the buttermilk. This will greatly limit the application of the buttermilk in other dairy products such as cream cheese and milk powder. This can be avoided if the vegetable oil is added downstream from the separation section in the continuous butter machine after the buttermilk has been drained. The vegetable oil is added at the end of the first working section or in a mixer between the two working sections. Other additives such as lactic acid concentrate, salt, and starter culture can also be added.

When the product leaves the second working section, the temperature will typically be 13–16 °C. It is then cooled to 10–12 °C in a scraped surface heat exchanger, which makes the product firmer and easier to handle during packaging.

This technology can also be used for the production of blended spreads with reduced fat content if a higher amount of aqueous phase with increased viscosity is incorporated simultaneously with the vegetable oil. The increased viscosity in the aqueous phase could be obtained by adding proteins like sodium caseinate.

Another variant of the manufacturing technology for production of blended spreads involves mixing butter, vegetable oil, and an aqueous phase in a medium-shear mixer or blender. The added vegetable oil can be liquid, semisolid, or solid. When a homogeneous mix is obtained, it is cooled in a scraped surface heat exchanger before packaging. A significant increase in temperature might occur in the mixer and again it is important to keep the temperature low during the processing.

Inversion of Cream

As emphasized earlier, a globular microstructure in the fat phase of low-fat spreads is preferable as this will result in a more butter-like consistency compared to products produced by the margarine technology. The main principle in direct inversion of cream is that cream with 40 g fat 100 g^{-1} is heated to 70 °C, stabilized by adding a suitable stabilizer such as gelatin or carrageen, and mixed with an emulsifier. The mixture is then cooled to 18–20 °C in a scraped surface heat exchanger and phase inverted in a high-shear mixer. The water-in-oil emulsion formed is worked in a pinworker, crystallized during cooling to 14 °C, and finally transferred to packaging. Addition of a soft fraction of milk fat or vegetable oil to the mixture before phase inversion is also a possibility.

Blends can be produced in a similar process where pasteurized cream with a fat content of approximately 40 g 100 g^{-1} is heated to 60 °C in a plate heat exchanger and further concentrated to a fat content of approximately 80 g 100 g^{-1} in a special separator capable of boosting the fat content of the cream without shattering the fat globules. The highly concentrated cream is then cooled to 20 °C and led to a holding and precrystallization tank where it is left overnight. In the following step, the cream is blended with an appropriate amount of vegetable oil and other additives such as salt, starter culture, and lactic acid concentrate, and the mixture is pumped through a scraped surface heat exchanger where the oil-in-water emulsion is turned into a water-in-oil emulsion and crystallized.

Conclusion

The main conclusion concerning milk fat-based spreads is that full-fat blends have been marketed with considerable success and have been widely accepted by consumers. The situation concerning low-fat products is less clear. Spreads with about 60 g fat 100 g^{-1} have been rather successful, while products with lower fat content have had difficulties in the marketplace as the overall quality perception seems to be that they still lack the texture, mouthfeel, and flavor of full-fat products although significant improvements have been made over the years caused by optimization of the production technology. However, the consumers' interest in low-fat products is steadily increasing, and the production technology will undoubtedly be further developed in the future.

See also: **Additives in Dairy Foods**: Emulsifiers. **Butter and Other Milk Fat Products**: Fat Replacers; Properties and Analysis. **Milk Lipids**: Lipid Oxidation.

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Fat Replacers

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Introduction

The prevalence of obesity and overweight in most Western countries has increased in recent decades. High dietary fat intake has been implicated in the etiology of obesity and certain cancers. Obesity, in turn, is a risk factor for type 2 diabetes and hypertension. Furthermore, high intake of saturated fats has been shown to elevate total blood cholesterol levels, and LDL-cholesterol in particular. High total- and LDL-cholesterol levels are key risk factors for coronary heart disease and ischemic stroke.

Many expert groups worldwide have recommended a reduced intake of dietary fat, in particular, saturated fats. For example, the 2005 United States Dietary Guidelines recommend reducing total fat intake to not more than 30% of the total daily caloric intake, with saturated fats reduced to not more than 10% of the daily caloric intake.

Fat intake in Western countries is tending to decline in recent years when expressed as a percentage of energy consumption from a high of approximately 40–42% of the total daily energy consumption. However, actual daily fat intake in grams in countries such as the United States has increased slightly in recent decades due to higher total energy intakes. The prevalence of overweight and obesity has also increased partly due to lower exercise levels.

The principal sources of over 90% of total fat in Western diets are fats and oils, meats, dairy products, confectionary, and snack foods. Among dairy products, intake of whole milk has tended to decrease in Western countries, though at the expense of reduced-fat or skimmed milk. However, cheese and yogurt intakes have tended to increase, and intakes of cream and ice cream have remained relatively constant.

In addition to its contribution to energy intake, fat's role in enhancing palatability, texture, and lubricity of foods makes it a key food constituent. It contributes to a feeling of satiety following food intake. Fat is a source of essential fatty acids, which are precursors for eicosanoids in the body. It is also a source of fat-soluble vitamins.

Dietary surveys in Western countries typically indicate that relatively small proportions of the population meet the dietary guidelines regarding fat intake. For example, it has been reported that only 29% of adult males and 25% of adult females in the United States consume less than or equal to the recommended 30% of energy intake from fat. Surveys have also shown that a much higher percentage of the population is interested in reducing fat intake. However, many consumers find low- or reduced-fat varieties of common foods to be relatively unpalatable and, hence, long-term adherence to diets containing these foods may be poor. For example, low- or reduced-fat cheeses are commonly described by consumers as bland, rubbery, bitter, or defective in color.

An alternative approach has been the development of fat replacers, which can be incorporated into traditional food products instead of all or part of the fat and which yield food products with the sensory and consumer acceptability characteristics of their full-fat counterparts. A large variety of fat replacers have been developed over the last two decades. Some expert groups and professional associations, for example, the American Dietetic Association, have acknowledged that the fat content of foods may be safely reduced or replaced by fat replacers. They advise that individuals who choose such foods should do so within the context of a diet consistent with the dietary guidelines. Of course, the degree to which adherence to the dietary guidelines regarding fat intake will result in health benefits for any one individual is controversial and unclear, as many other factors influence an individual's long-term health, such as genetic constitution, smoking, exercise level, and intakes of other dietary constituents such as fruit and vegetables. Furthermore, the extent to which consumption of foods containing fat replacers will result in a reduction in total energy intake and weight loss is unclear. Available evidence suggests that energy compensation and increased food intake may occur when fat replacers are consumed.

Types of Fat Replacers

Fat replacers can be divided into two categories: fat substitutes and fat mimetics. Fat substitutes are molecules that possess the physical and functional characteristics of conventional fat molecules, for example, triglycerides. Fat substitutes can directly replace conventional fat molecules in foods on a weight-for-weight basis. They are typically synthetic molecules that provide no energy (calories) or structured lipid molecules that provide reduced energy. Fat substitutes can successfully maintain the palatability of foods, as they can reproduce the texture and mouthfeel of fat. They are generally heat-stable and suitable for high-temperature cooking and frying applications. They may not reproduce the taste properties of fat, as fat itself provides flavor to foods and is a carrier of other fat-soluble flavor compounds in foods.

Fat mimetics are substances that can mimic some of the organoleptic and physical properties of conventional fat molecules. However, they cannot replace fat molecules in foods on a weight-for-weight basis. Fat mimetics are typically protein- or carbohydrate-based molecules that may be modified to mimic some of the properties of conventional fats. Their energy contribution to the diet ranges from 0 to 4 kcal g^{-1}. Fat mimetics are generally not suitable for high-temperature applications, such as frying, as they are susceptible to denaturation or caramelization. Fat mimetics are generally polar water-soluble compounds. Thus, they cannot replace some of the nonpolar functional characteristics of fats, such as lipid-soluble flavor-carrying capacity. However, their polar nature facilitates water binding, which helps generate a sense of creaminess and lubricity in foods similar to that found in full-fat products.

Fat Substitutes

A range of fat substitutes have been developed and are commercially available while others are still in a developmental, noncommercial phase.

Sucrose Fatty Acid Polyesters

The best-known sucrose polyester fat substitute is Olestra, produced by the Procter and Gamble Company (Cincinnati, Ohio). Olestra is a mixture of hexa-, hepta-, and octaesters of saturated and unsaturated fatty acids, of chain length greater than or equal to 12 carbons, with sucrose. The physicochemical characteristics, functionality, and applications of Olestra in foods are influenced by the nature of the fatty acids esterified to sucrose.

The product is synthesized by preparing fatty acid methyl esters (FAMEs) from edible fats and oils. The FAMEs are transesterified to sucrose or interesterified to sucrose octa-acetate using alkali metal catalysis under high vacuum in an anhydrous environment. The crude product of transesterification or interesterification is subsequently processed to remove free fatty acids, unreacted FAMEs, off-odors, and sucrose esters with less than six fatty acids. The resulting pure mix of hexa-, hepta-, and octaesters is termed Olestra. It has the mouthfeel, appearance, flash point, heat stability, and oxidative stability of conventional fats. It is suitable for deep frying or baking applications on account of its flavor-carrying ability.

The United States Food and Drug Administration (FDA) finally approved Olestra as a food additive in 1996 for use in replacing up to 100% of the fat in savory snack foods (e.g., crisps, chips, crackers, puffs) and for frying of savory snack foods. It is likely that further applications of Olestra will be approved at a future date in the United States and possibly elsewhere, such as its use as a fat substitute in shortenings and oils, spreads, salad dressings, and dairy products. Work has been published in which sucrose polyesters with physicochemical characteristics of milk fat were synthesized from milk fat fatty acids and incorporated into reduced- and low-fat Cheddar cheese. The firmness and texture of the sucrose polyester-containing Cheddar did not differ significantly from conventional Cheddar. However, flavor problems were reported, which were attributed to inadequate cleanup of the sucrose polyesters following synthesis.

Due to its molecular size and shape, Olestra passes through the gastrointestinal tract without being hydrolyzed by lipases or absorbed and hence contributes no energy to the diet. It has been reported, however, to have the potential to give rise to gastrointestinal side effects such as abdominal cramping, loose stools, and reduced absorption of fat-soluble vitamins. Accordingly, Olestra-containing foods were originally required by FDA to carry a label indicating the potential for gastrointestinal side effects, though this requirement was rescinded in 2003. Olestra is supplemented with prescribed regulatory amounts of vitamins A, D, E, and K. There is some evidence that Olestra reduces carotenoid absorption. However, it does not affect the absorption of other macronutrients, water-soluble vitamins, and minerals. The FDA concluded that, based on available data, Olestra is not toxic, genotoxic, carcinogenic, or teratogenic. However, consumer groups in the United States have mounted a vigorous campaign against the use of Olestra in the food supply. It has been argued, however, that the use of Olestra as a fat substitute may benefit obese individuals, reduce the risk of coronary heart disease, and benefit colon cancer patients. Olestra is permitted for use only in the United States at present.

Structured Lipids

These reduced-calorie fat substitutes are triacylglycerides containing two short-chain fatty acids (SCFAs) and/or medium-chain fatty acids (MCFAs), and one long-chain fatty acid (LCFA).

Caprenin (caprocaprylobehenic triacylglycerol) is a structured lipid produced by the Procter and Gamble Company (Cincinnati, Ohio). It consists of caprylic (8:0), capric (10:0), and behenic (22:0) fatty acids esterified to glycerol. Because 22:0 is poorly absorbed, and 8:0 and 10:0 are absorbed and metabolized differently from LCFAs, caprenin contributes only 5 kcal g^{-1} energy to the diet compared to 9 kcal g^{-1} for conventional fats. Its functional properties are similar to those of cocoa butter and, hence, it has potential applications in soft candy and confectionery coatings.

Salatrim (short and long acyl triglyceride molecule) was developed by the Nabisco Foods Group (Parsippany, New Jersey) and is a group of structured triacylglycerides containing at least one SCFA (2:0, 3:0, or 4:0) and at least one LCFA (usually 18:0) randomly esterified to glycerol. The amounts and types of SCFAs and LCFAs attached to glycerol influence the functional and physical properties of Salatrim such as hardness. Its applications include use in chocolate-flavored coatings, fillings for confectionery and baked goods, savory dressings, dips and sauces, and dairy products, including frozen dairy desserts, cheese, and sour cream.

It contributes only 5 kcal g^{-1}, as 18:0 (stearic acid) is incompletely absorbed and the SCFAs have a lower caloric value than LCFAs.

Other Fat Substitutes

Several other fat substitutes have been patented but have not yet achieved regulatory approval and commercial availability. These include dialkyl dihexadecylmalonate (DDM), which has been patented by Frito Lay, Inc. (Dallas, Texas) for use in replacing oil in food formulations or in frying. It is a noncaloric fat substitute, as it is not digested or absorbed. It is an ester of a fatty alcohol and a dicarboxylic acid, malonic acid and alkylmalonic acid.

Esterified propoxylated glycerols (EPGs) were patented as fat substitutes by ARCO Chemical Company (Wilmington, Delaware). They are produced through the formation of a polyether polyol from glycerol and propylene oxide. EPGs are then produced by esterifying fatty acids to the polyether polyol. They are very resistant to lipase and hence have low caloric value. Depending on the fatty acids used, EPGs have different functional properties and potential applications in frozen desserts, salad dressings, baked goods, and spreads. They are suitable for cooking and frying.

Fat Mimetics

Fat mimetics imitate the physical and organoleptic properties of conventional fat molecules in foods. They can be divided into protein-based fat mimetics and carbohydrate-based fat mimetics.

Protein-Based Fat Mimetics

This category of fat mimetics is suitable for use in dairy products, salad dressings, frozen desserts, and table spreads. Nutritionally, this category of fat mimetics is digested and absorbed as protein and hence has a caloric value of 4 kcal g^{-1}. They possess the antigenic properties of the protein. Typically, they cannot be used in cooking oils or in products subject to frying conditions, as the proteins are denatured and lose their functionality, namely, the creamy, fat-like organoleptic properties. However, they are generally suitable for use as ingredients in foods that undergo less strenuous cooking regimes such as baking, retorting, and ultra-high temperature processing. Protein-based fat mimetics are typically produced from egg, milk, whey, soy, or wheat proteins. These proteins are often microparticulated, a process that involves heating the proteins to produce a gel structure. Heat-coagulated proteins normally form large gel particles, which are perceived as rough in the mouth. However, if a high shearing force is applied during heating of the proteins, very small spherical (0.1–2.0 μm diameter) protein gel particles are produced. These microparticles are too small to be perceived as individual rough particles in the mouth. Instead, they are perceived in the mouth and taste buds as similar to fat with a creamy, smooth texture.

One of these protein-based fat mimetics is Simplesse®, which is produced by microparticulation of whey protein concentrate by the NutraSweet Kelco Co. (San Diego, California). It has been used since 1990 in frozen dessert products and since 1994 in yogurt, soft cheeses and cheese spreads, and sour cream. It can also be used in dips, frostings, sauces, mayonnaise, salad dressings, and baked goods. Hydrated gel forms of Simplesse provide as little as 1 kcal g^{-1}. Simplesse provides body, texture, and mouthfeel to the food containing it. It contributes to creaminess, viscosity, and opacity of the food; inhibits syneresis; and increases water-binding capacity.

Other protein-based fat mimetics include Dairy Lo, which was developed by Pfizer Inc. (Groton, Connecticut) from whey protein, and K-Blazer developed by Kraft Foods (Chicago, Illinois). Dairy Lo incorporated at 5% by weight has been used to develop an ice cream containing 1% fat. It has also been used in reduced- and low-fat Cheddar cheese.

Carbohydrate-Based Food Mimetics

Many carbohydrates can be used as mimetics to replace fat in foods either partially or totally. Digestible carbohydrates provide 4 kcal g^{-1}, whereas nondigestible complex carbohydrates and hydrocolloid gums provide essentially no calories. Carbohydrate-based fat mimetics include gums, maltodextrins and dextrins, polydextrose, cellulose derivatives, starch derivatives, and oat flour derivatives. Carbohydrate-based fat mimetics typically perform a fat replacement role by stabilizing water in the food product in a gel-like matrix. This results in increased viscosity and body as well as a creamy mouthfeel similar to that of full-fat products. However, these mimetics typically are not suitable for frying applications. Furthermore, the water activity of foods in which they are incorporated is typically high, resulting in increased potential for microbial growth and hence reduced shelf life. The flavor profile of the product can be influenced by the fat mimetic used. Gum-based fat mimetics tend not to influence flavor, whereas starch- and cellulose-based mimetics tend to reduce flavor intensity.

Gums

Gums are high-molecular-weight, negatively charged carbohydrate hydrocolloids. At a low concentration (0.1–0.5%), gums increase the viscosity of food systems and act as stabilizers and gelling agents. Xanthan, guar and locust bean gums, gum Arabic, carrageenan, and pectins can all be used as fat replacers either on their own or in a combination, or with other categories of fat replacers. Their applications include use in dairy products, salad dressings, soups, sauces, ice cream, icings and glazes, and baked foods.

The principal factors that require consideration in choosing a gum for use as a fat mimetic include the rheological properties of the gel it forms, the effects of temperature and shearing forces on the functional properties of the gum, and its compatibility with other ingredients in the food.

Maltodextrins and dextrins

These substances are nonsweet starch hydrolysates, typically produced by partial hydrolysis of maize or potato starch. They can also be derived from oat, rice, wheat, or tapioca starch. They usually have a dextrose equivalence (DE) of less than 20. (DE is an index of reducing sugar content expressed as glucose.) These substances increase viscosity and contribute to mouthfeel and body. One part of a 20–35% aqueous solution of these substances is considered a suitable fat mimetic for one part of oil. Thus, if 1 g of a 25% solution of maltodextrin is used to replace 1 g of oil, a reduction of 8 kcal g^{-1} is achieved. These substances are used as fat mimetics in table spreads, sour cream, frozen desserts, frostings, and salad dressings.

Polydextrose

Polydextrose is defined as a randomly bonded polymer of glucose, sorbitol, and citric or phosphoric acid. It is partially metabolizable but contributes only 1 kcal g^{-1}. Its primary use in foods is as a bulking agent, but it also has a fat-sparing effect. However, at higher intakes, it may induce a laxative effect. It can be used in foods such as baked goods, frozen dairy desserts, salad dressings, puddings, and frostings.

Cellulose

Several types of cellulose and cellulose derivatives can be used as fat mimetics, usually in combination with other hydrocolloids. Microcrystalline cellulose (MCC) is a noncaloric fat replacer. It mimics fat in aqueous systems by contributing to body, mouthfeel, and viscosity. Typically, 60–70% of the cellulose microcrystals are less than 0.2 μm long. These microcrystals form an insoluble dispersion in water. Other ingredients, for example, gums, hold the insoluble microcrystals together in a network, which gives a creamy mouthfeel and body similar to that found in full-fat products. Other forms of cellulose such as powdered cellulose, methylcellulose, and hydroxypropylmethylcellulose also have application, as fat mimetics in baked goods, frozen desserts, sauces, and salad dressings.

Starch

A wide range of modified starches are available that can act as fat mimetics by enhancing the creaminess and moisture retention of foods. Potato, maize, oat, rice, wheat, and tapioca starches modified by chemical and/or physical means have found applications as fat mimetics. Starch-based fat mimetics generally are relatively inexpensive and readily available. However, their freeze/thaw, heat, acid, and shear stabilities may not be sufficient for some applications. They may also mask, to some degree, the flavor of the foods in which they are incorporated. They tend to perform well as fat mimetics in high-moisture foods such as salad dressings, sauces, baked goods, frostings, and sausages.

Oatrim

Oatrim is a fat mimetic with a mouthfeel very similar to that of triglycerides. It is produced by partial enzymatic hydrolysis of oat flour and consists of 5% β-glucan, soluble fiber, and amylodextrin. It can be used as a dry powder (4 kcal g^{-1}) or as a gel hydrated with three parts of water (1 kcal g^{-1}). It can withstand high-temperature short-time (HTST) processing and retorting but not frying conditions. It has been used to produce a fat-free, cholesterol-free milk, which has been reported to have a taste similar to 2% fat milk. Oatrim may also be incorporated into confectionery, frozen desserts, baked goods, and meat products.

Conclusion

Several categories of fat replacers are available: either lipid-based fat substitutes or protein- or carbohydrate-based fat mimetics. None of the currently available fat replacers can provide all the functional and sensory advantages of conventional fats.

Ideally, fat replacers should be safe, inexpensive, low- or noncaloric, suitable for frying as well as cooking applications, yet maintain the texture and flavor of the foods in which they are incorporated and not interfere with the absorption of fat-soluble nutrients or drugs. Combinations of different categories of fat replacers may help to reproduce the organoleptic and functional properties of conventional full-fat foods. The use of fat replacers may be helpful to meet dietary guidelines to reduce fat intake and, in conjunction with other lifestyle and dietary changes, may contribute to improving public health status.

See also: **Additives in Dairy Foods**: Consumer Perceptions of Additives in Dairy Products; Legislation; Safety; Types and Functions of Additives in Dairy Products. **Cheese**: Low-Fat and Reduced-Fat Cheese; **Milk Lipids**: General Characteristics.

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CHEESE

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Overview

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Introduction

Cheese is the generic name for a group of fermented dairy products, produced throughout the world in a great diversity of flavors, textures, and forms; there are more than 1000 varieties of cheese. It is commonly believed that cheese evolved in the 'Fertile Crescent' between the Tigris and Euphrates rivers, in what is now Iraq, about 8000 years ago, during the so-called 'Agricultural Revolution', when certain plants and animals were domesticated as sources of food. Milk is a rich source of nutrients for neonatal mammals, and adult humans soon exploited it as a valuable component of the diet. However, milk is also a rich source of nutrients for bacteria, which contaminate milk and grow well under ambient conditions. Some contaminating bacteria (lactic acid bacteria (LAB), including the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus*) catabolize milk sugar, lactose, as a source of energy, producing lactic acid as a by-product. When sufficient acid is produced to reduce the pH to ~4.6, the caseins, the principal proteins in milk, coagulate to form a gel, which occludes the fat and aqueous phases of milk. An acid-induced milk gel is quite stable if left undisturbed and is consumed as such in a wide range of fermented milk products. However, if cut or broken, the gel separates into curds and whey. The shelf life of the curds can be extended by dehydration and/or salting to yield acid-coagulated cheeses, for example, Cottage cheese, Cream cheese, and Quark, which represent ~25% of total cheese production and in some countries are the principal varieties; they are consumed fresh and are used widely in other food products, for example, cheesecakes and cheese-based dips and sauces.

An alternative mechanism for coagulating milk was also recognized in prehistoric times. Many proteinases from bacteria, molds, plants, or animal tissues (referred to as rennets) can modify the casein system, causing it to coagulate under certain circumstances. Rennet-coagulated curds have better syneretic properties (ability to exude whey) than acid-coagulated curds, which makes it possible to produce lower-moisture, more stable cheese. Therefore, rennet coagulation has become the principal mechanism for cheese manufacture; most modern cheese varieties and ~75% of total world production of cheese are produced by this mechanism. During the storage of rennet-coagulated curds, various bacteria grow and the enzymes in milk and rennet and the enzymes from bacteria continue to act, resulting in changes in the flavor, texture, and functionality of cheese. When controlled, this process is referred to as ripening (maturation), during which a great diversity of characteristic flavors and textures develop. The principal traditional rennets used for the manufacture of long-ripened cheeses are extracts from the stomachs of young mammals, in which the principal enzyme is chymosin. However, increased production of cheese led to a shortage of such rennets, necessitating the introduction of rennet substitutes.

History of Cheesemaking

The advantages accruing from the conversion of milk to cheese are apparent from the viewpoints of storage stability, ease of transport, and diversification of the human diet. Cheese manufacture accompanied the spread of civilization through Egypt, Greece, and Rome. There are several references to cheese in the Old Testament, in the tombs of ancient Egypt, and in classical Greek literature. Cheese manufacture was well established during the Roman Empire and was described by many Roman writers, for example, Cato (about 150 BC), Varro (about 40 BC), Pliny (AD 23–89), and especially Columella (about AD 50). Movements of Roman armies and administrators contributed to the spread of cheese throughout the Roman world. The great migrations of peoples throughout Europe after the fall of the Roman Empire probably promoted the spread of cheese manufacture, as did the Crusaders and pilgrims of the Middle Ages. However, the most important contributors to the development of cheese 'technology' and to the evolution of cheese varieties during the Middle Ages were the monasteries and feudal estates. Monasteries were major contributors to the advancement of agriculture in Europe and to the development and improvement of food commodities, including cheese; many current cheese varieties were developed in monasteries, for example, Wensleydale, Port du Salut or Saint Paulin, Fromage de Tamie, Maroilles, Munster, and Trappist.

The great feudal estates of the Middle Ages were self-contained communities, which, in the absence of an effective transport system, relied on locally produced foods. Surplus food was produced in summer and preserved to meet the requirements of the community throughout the year. Fermentation and/or salting, two of the classical principles for food preservation, were used to preserve meat, fish, beer, wine, vegetables, fermented milks, butter, and cheese. Cheese was traded when amounts surplus to local requirements were available. Within large estates, individuals acquired special skills, which were passed on to succeeding generations. Because monasteries and feudal estates were essentially self-contained communities with limited intercommunity travel, numerous varieties of cheese evolved. Traditionally, many cheese varieties were produced in limited geographical regions, especially in mountainous areas of France, Spain, Italy, the Balkans, and Greece. The localized production of certain varieties is now protected and encouraged through the Appellation d'Origine Contrôlée program, which legally defines the region and manufacturing technology for certain cheese varieties.

Almost certainly, most cheese varieties evolved by accident because of particular local circumstances, for example, species or breed of dairy animal, local vegetation, a peculiarity in the chemical composition or microflora of the milk, or an 'accident' during the manufacture or storage of the cheese, for example, growth of molds or other microorganisms. Presumably, those accidents that led to desirable changes in the quality of cheese were included in the manufacturing protocol, which underwent a series of evolutionary changes and refinements.

The colonization of North and South America, Oceania, and Africa by Europeans introduced cheesemaking to these regions, and cheese, mainly European varieties, sometimes modified to meet local conditions, has become an item of major economic importance in the United States, Canada, Argentina, Brazil, Mexico, Australia, and New Zealand.

Cheesemaking remained a craft until relatively recently. The first attempt to standardize Cheddar cheese was made in England by John Harding in the mid-nineteenth century. Prior to that, 'Cheddar cheese' was that produced around the village of Cheddar, in Somerset, England, and probably varied considerably, depending on the cheese maker and other factors. Cheese manufacture was a farmstead enterprise until the mid-nineteenth century – the first cheese factory in the United States was established near Rome, New York, in 1851 and the first in Britain at Longford, Derbyshire, in 1870. There were thousands of farm-scale cheesemakers and there must have been great variation within any one general type; even today, there is very considerable inter- and intrafactory variation in the quality and characteristics of well-defined varieties, in spite of the very considerable scientific and technological advances. The curds for many famous varieties of cheese, for example, Parmigiano Reggiano, Grana Padano, Emmental, and Roquefort, are produced in many farm-level dairies under the supervision of a producer consortium and the cheeses ripened and marketed by central facilities.

Research on the microbiology, chemistry, and technology of cheese commenced toward the end of the nineteenth century and continues today, as a result of which cheese science and technology are quite well understood. However, there are still large gaps in our knowledge, for example, the complete description of cheese flavor, cheese structure, and physicochemical properties. With the gradual acquisition of knowledge on the chemistry and microbiology of milk and cheese, it became possible to control the changes involved in cheesemaking. Although relatively few new varieties have evolved as a result of this improved knowledge (notable examples are Jarlsberg and Maasdammer), existing varieties became better defined and their quality more consistent. There has been a marked resurgence of farmhouse cheesemaking in recent years; many of the cheeses being produced on farms might be regarded as new varieties.

Overview of Cheese Production

The production of all varieties of cheese involves a generally similar protocol (**Figure 1**), various steps of which are modified to give a product with the desired characteristics. The principal features of the manufacture and characteristics of the principal cheese varieties will be described in the following articles; the objective of this article is to present an overview of the cheesemaking process so that the operations described in later articles can be seen in an overall context.

Selection of Milk

The composition of cheese is strongly influenced by the composition of the milk, especially the content of fat, protein, and calcium, and pH. The constituents and composition of milk, which are described in various articles throughout this encyclopedia, are influenced by several factors, including species, breed, individual animal variations, nutritional status, health, and stage of lactation of the milk-producing animals. Owing to major compositional abnormalities, milk from cows in the very early or late stages of lactation and those suffering from mastitis should be excluded. Somatic cell (leukocyte) count is a useful index of milk quality. Some genetic polymorphs of the milk proteins have a significant effect on cheese yield and quality and there is increasing interest in breeding for desirable polymorphs, especially in the case of sheep and goats. Cheese milk should be free of chemical taints and free fatty acids, which cause off-flavors in the cheese, and antibiotics, which inhibit bacterial cultures.

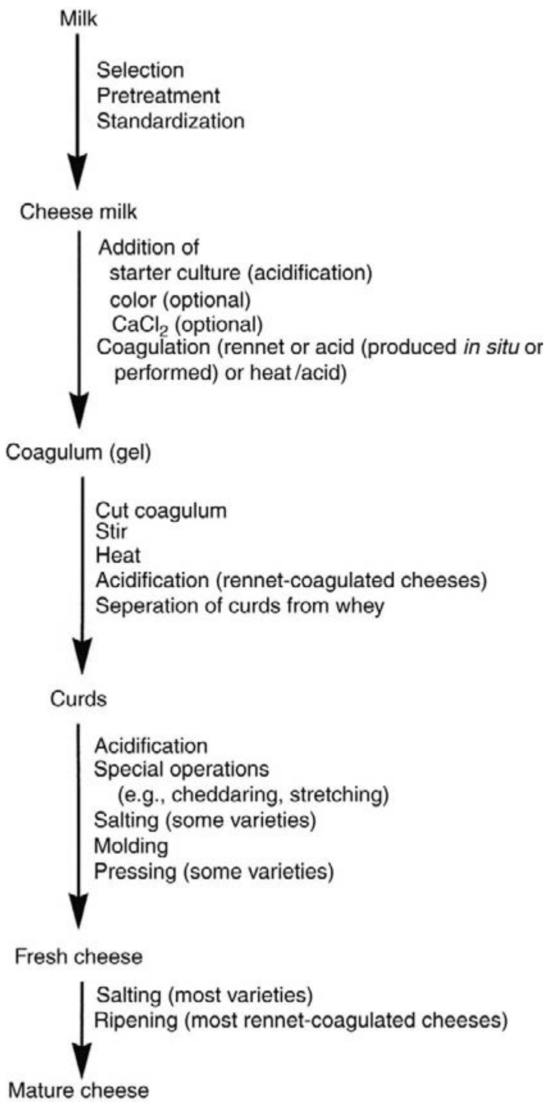
Milk
Selection
Pretreatment
Standardization
Cheese milk
Addition of
starter culture (acidification)
color (optional)
CaCl$_2$ (optional)
Coagulation (rennet or acid (produced *in situ* or performed) or heat/acid)
Coagulum (gel)
Cut coagulum
Stir
Heat
Acidification (rennet-coagulated cheeses)
Seperation of curds from whey
Curds
Acidification
Special operations
(e.g., cheddaring, stretching)
Salting (some varieties)
Molding
Pressing (some varieties)
Fresh cheese
Salting (most varieties)
Ripening (most rennet-coagulated cheeses)
Mature cheese

Figure 1 General protocol for cheese manufacture. Reproduced from Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers.

A major cause of variation in the characteristics of cheese is the species of dairy animal from which the milk is obtained. The principal dairying species are cattle, water buffalo, sheep, and goats, which produce 85, 11, 2, and 2% of commercial milk, respectively. Goats and sheep are significant producers of milk in certain regions, for example, around the Mediterranean, where their milk is used mainly for the production of fermented milks and cheese. Many world-famous cheeses are produced from sheep's milk, for example, Roquefort, Feta, Pecorino Romano, and Manchego; traditional Mozzarella (Mozzarella di buffalo) is made from buffalo milk. There are very significant interspecies differences in the composition and physicochemical properties of milk, which are reflected in the characteristics of cheese produced therefrom. There are also significant differences in milk composition between breeds of cattle, which influence cheese quality. The milk of yak and reindeer is used for local small-scale cheesemaking, but the milk of camel, horse, and donkey yields a very weak, or no, gel and cannot be used for cheese production.

The milk should be of good microbiological quality, as contaminating bacteria are concentrated in the curd and may cause defects or public health problems. However, cheese milk is normally pasteurized or subjected to one or more of the treatments described below (see section 'Heat treatment of milk'), to render it free of pathogenic, food poisoning, and certain spoilage bacteria.

Standardization of Milk Composition

The composition of cheese is prescribed in '**Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products' with respect to moisture and fat-in-dry-matter, which in effect defines a certain fat:protein ratio. The moisture content of cheese, and hence the level of fat and protein, is determined mainly by the manufacturing protocol, but the fat:protein ratio in cheese is determined mainly by the fat:casein ratio in the milk. Depending on the ratio required, it can be modified by

- removing some fat by natural creaming or centrifugation,
- adding skimmed milk,
- adding cream, and
- adding milk powder, evaporated milk, or ultrafiltration (UF) retentate; such additions also increase the total solids content of the milk and hence increase the yield of cheese curd per unit volume.

Calcium plays an essential role in the coagulation of milk by rennet and in the subsequent processing of the coagulum; hence, it is common practice to add $CaCl_2$ (e.g., 0.01%) to cheese milk.

The pH of milk is a critical factor in cheesemaking. The addition of 1.5–2% starter culture to cheese milk reduces its pH by 0.1 units; starter concentrates (direct - to- vat set (DVS)) have little or no direct acidifying effect. Previously, it was standard practice to add the starter to the milk 30–60 min before rennet addition. The objective of this operation, referred to as 'ripening', was to allow the starter bacteria to enter the exponential growth phase and become highly active during cheesemaking; ripening is not necessary with modern high-quality starters. Some acid was produced during ripening, which favored rennet action and gel formation. However, ripening increases the risk of bacteriophage infection of the starter because phage become distributed throughout the liquid milk; following coagulation, bacteriophage are fixed in position and therefore their destructive effect becomes localized and reduced. Although ripening is still practiced for some cheese varieties, it has been discontinued for most varieties.

The pH of milk on reception at the dairy is higher today than previously owing to improved hygiene during milking and the widespread use of refrigeration at the farm and factory. In the absence of acid production by contaminating bacteria, the pH of milk increases slightly during storage due to the loss of CO_2 to the atmosphere. The natural pH of milk is $\sim$6.7 but varies somewhat (e.g., it increases in late lactation and during mastitic infection).

As an alternative to ripening, the preacidification of milk by 0.1–0.2 pH units, either through the use of gluconic acid-δ-lactone (GDL) or by limited growth of a lactic acid starter, followed by pasteurization (referred to as prematuration), is recommended and is claimed to result in better and more uniform rennet coagulation characteristics and cheese quality.

Heat Treatment of Milk

Traditionally, cheese was made from raw milk, a practice that was almost universal until the 1940s. Although cheese made from raw milk develops a more intense flavor than that produced from pasteurized milk, the former is less consistent and poses a public health risk. When cheese was produced from fresh milk on farms or in small, local factories, the growth of contaminating microorganisms was minimal but as cheese factories became larger, storage of milk for longer periods became necessary and hence the microbiological quality of the milk deteriorated and varied. Thermization (*see* **Heat Treatment of Milk**: Thermization of Milk) of cheese milk is fairly widely practiced on receipt at the factory to reduce the microbial load and extend the storage period. For public health reasons, it became increasingly popular from the beginning of the twentieth century to pasteurize milk for liquid consumption (*see* **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Plant and Equipment**: Pasteurizers, Design and Operation). The pasteurization of cheese milk became widespread about 1940, primarily for public health reasons, but also to provide a milk supply of more uniform bacteriological quality. Although a considerable amount of cheese is still produced from raw milk, on both an artisanal and factory scale, especially in southern Europe (including such famous varieties as Swiss Emmental, Gruyère de Comte, Parmigiano Reggiano, and Grana Padano), pasteurized milk is now generally used, especially in large factories (*see* **Cheese**: Raw Milk Cheeses).

There are four alternatives to pasteurization for reducing the number of microorganisms in milk, but it is important to realize that these methods may not produce pathogen-free milk:

1. Treatment with H_2O_2: not practiced in developed dairying countries.
2. Activation of the lactoperoxidase–H_2O_2–thiocyanate system (*see* **Enzymes Indigenous to Milk**: Lactoperoxidase): very limited or no application in the cheese industry.
3. Bactofugation: frequently used to remove clostridial spores as an alternative to the use of nitrate to prevent late gas blowing in cheese.
4. Microfiltration: very effective for removing bacteria and spores from milk but not yet widely practiced in the cheese industry (*see* **Cheese**: Membrane Processing in Cheese Manufacture. **Liquid Milk Products**: Liquid Milk Products: Membrane-Processed Liquid Milk).

Cheese Color

The principal pigments in milk are carotenoids, which are obtained from the animal's diet, especially from fresh grass and clover. Cattle transfer carotenoids to adipose tissue and milk, but goats, sheep, and buffalo do not. Therefore, bovine milk fat and high-fat products, including cheese, are yellow to an extent dependent on the carotenoid content of the animal's diet, whereas their counterparts made from sheep, goat, or buffalo milk are very white in comparison. The yellowish color of dairy products produced from cows' milk may make them less acceptable than products produced from sheep's, goats', or buffalo milk in regions where the latter are traditional. The carotenoids in bovine milk can be bleached by treatment with H_2O_2 or benzoyl peroxide, or masked by chlorophyll or titanium oxide, if these additives are permitted.

At the other end of the spectrum are individuals who prefer highly colored cheese, which is usually achieved by adding annatto, extracted from the seeds of *Bixa orellana*, a native of Brazil, which contains two apocarotenoid pigments, bixin and norbixin. Alternatively, synthetic or natural carotenoids may be used.

Conversion of Milk to Cheese Curd

After the milk has been standardized and pasteurized or otherwise treated, it is transferred to vats (or kettles), which vary in shape (hemispherical, rectangular or cylindrical, vertical or horizontal), may be open or closed, and may range in size from a few hundred liters to 30 000 l or more, where it is converted to cheese curd by a process that involves three basic operations: acidification, coagulation, and dehydration.

Acidification

Acidification is usually achieved through the *in situ* production of lactic acid by the fermentation of lactose by LAB. Initially, the indigenous milk microflora was relied upon to produce acid, but since this was variable, the rate and extent of acidification were variable, resulting in cheese of variable quality. Cultures of LAB (starters) for cheesemaking were introduced about 100 years ago and since then have been improved progressively and refined. The science and technology of LAB and starters will be described in several articles in this encyclopedia (*see* **Cheese**: Starter Cultures: General Aspects. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactococcus lactis*). The acidification of curd for some artisanal cheeses still relies on the indigenous microflora.

Direct acidification using acid (usually lactic acid or HCl) or acidogen (GDL) is an alternative to biological acidification and is used commercially to a significant extent in the manufacture of Cottage, Quark, Feta-type cheese from UF-concentrated milk, and Mozzarella. Direct acidification is more controllable than biological acidification and, unlike starters, is not susceptible to bacteriophage infection. However, enzymes from starter bacteria are essential in cheese ripening and hence chemical acidifcation is used mainly for cheese varieties for which texture is more important than flavor.

The rate of acidification depends on the amount and type of starter added and on the temperature profile of the curd and ranges from 5 to 6 h for Cheddar and Cottage cheese and from 10 to 12 h for Dutch and Swiss types. The ultimate pH of the curd for most rennet-coagulated cheeses is 5.0–5.3 but the pH of acid-coagulated varieties, for example, Cottage, Quark, and Cream, and some soft rennet-coagulated varieties, for example, Camembert and Brie, is ~4.6.

The production of acid at the appropriate rate and time affects several aspects of cheese manufacture and is critical for the production of good-quality cheese; it affects

- coagulant activity during coagulation,
- denaturation and retention of the coagulant in the curd, which influences the rate of proteolysis during ripening and may affect cheese quality,
- gel strength (curd tension), which influences cheese yield,
- gel syneresis, which controls the moisture content of cheese curd and hence regulates the growth of bacteria and the activity of enzymes in the cheese; consequently, it strongly influences the rate and pattern of ripening and the quality of cheese,
- colloidal calcium phosphate in the casein micelles, which dissolves as the pH decreases, as a result of which the susceptibility of the caseins to proteolysis during ripening is increased and the rheological properties, meltability, and stretchability of the cheese are modified, and
- the growth of many non-starter bacteria in cheese, including pathogenic, food poisoning, and gas-producing microorganisms; properly made cheese is a very safe product from the public health viewpoint.

Some cheese varieties, for example, Cheddar, are salted by mixing dry salt with chips of curd at the end of manufacture; since the level of salt in the cheese moisture rapidly reaches a value (5–6%) that halts the growth of starter bacteria, the pH of curds for these varieties at salting must approximate the ultimate value (pH 5.1). However, most varieties are salted by immersing the formed cheese in brine or by surface application of dry salt; the diffusion of NaCl into the interior of the cheese is relatively slow and therefore there is ample time for the pH to decrease to ~5.0 before the concentration of salt becomes inhibitory throughout the cheese. The pH of the curd for most cheese varieties is 6.2–6.5 at molding and pressing but decreases to 5.0–5.2 during or shortly after pressing and before salting (*see* **Cheese**: Salting of Cheese).

In a few cases, for example, Domiati, a high level of NaCl is added to the milk, traditionally to control the growth of adventitious microorganisms. This NaCl has a major influence on acid development, rennet coagulation, gel strength, and curd syneresis.

Secondary Cultures

The starter LAB dominate the microflora of cheese initially, but after reaching a maximum of $\sim 10^9$ per gram, they die off and lyse, and are replaced by a secondary microflora, which consists of

1. non-starter lactic acid bacteria (NSLAB), mainly adventitious mesophilic *Lactobacillus* spp., which are normally contaminants from the milk, equipment, or environment, and hence are variable in type and number. Because NSLAB dominate the viable microflora of long-ripened cheese and because of their variability, they are responsible for much of the inconsistency of cheese quality; therefore, it is becoming increasingly common to add selected strains of *Lactobacillus* as part of the starter and these will outcompete the adventitious NSLAB.
2. cultures of non-LAB, which are added to perform specific functions. These include *Propionibacterium*

freudenreichii in Swiss-type cheeses, *Brevibacterium linens* in surface smear-ripened varieties, *Penicillium roqueforti* in blue cheeses, and/or *Penicillium camemberti* and *Geotrichum candidum* in surface mold-ripened varieties. These are very active microorganisms and dominate the ripening of cheese in which they are used. They may be adventitious (from milk and environment) or added as a culture to the milk or curd (becoming increasingly common).

Coagulation

The essential step in the manufacture of all cheese varieties involves coagulation of the casein of milk to form a gel, which entraps the fat, if present. Coagulation may be achieved by

- limited proteolysis by selected proteinases (rennets),
- acidification to pH 4.6, or
- acidification to pH 5.2–5.5 and heating to 90 °C.

Most cheese varieties, and about 75% of total production, are produced by rennet coagulation but some acid-coagulated varieties, for example, Quark and Cottage cheese, are of major importance (*see* **Cheese**: Acid- and Acid/Heat Coagulated Cheese; Rennet-Induced Coagulation of Milk). The acid/heat-coagulated cheeses are relatively minor varieties, which are usually produced from rennet cheese whey or a blend of whey and skim milk and evolved as a means for recovering the nutritionally valuable whey proteins; they are usually used as food ingredients. Important varieties are Ricotta (Italy), Anari (Cyprus), and Manouri (Greece).

A fourth, minor, group of cheeses is produced, not by coagulation, but by thermal evaporation of water from a mixture of whey and skim milk, whole milk, or cream and crystallization of lactose, for example, Mysost and Gjetost. These cheeses, which are almost exclusive to Norway, bear little resemblance to rennet- or acid-coagulated cheese.

Postcoagulation Operations

Rennet- or acid-coagulated milk gels are quite stable under quiescent conditions, but if cut or broken, they synerese extensively, expelling whey. Syneresis concentrates the fat and casein of milk by a factor of 6–12, depending on the variety. The rate and extent of syneresis are influenced, *inter alia*, by milk composition, especially the concentrations of Ca^{2+} and casein, pH, cooking temperature, rate of stirring of the curd–whey mixture, and time. The composition of the finished cheese is determined mainly by the extent of syneresis, which initiates the differentiation of cheese varieties, although the type and composition of the milk, the amount and type of starter, and the amount and type of rennet are also significant in this regard.

The liquid expressed from the curds in the vat or in the molds is called whey, which contains about 50% of the solids in milk (98% of the lactose, 25% of the protein, and 10% of the fat). In the past, whey was regarded as essentially worthless, to be disposed of as cheaply as possible. However, whey is now the source of valuable food products (*see* **Whey Processing**: Demineralization; Utilization and Products).

A more or less unique protocol has been developed for the manufacture of each cheese variety. These protocols differ mainly with respect to postcoagulation operations. The manufacture of the principal families of cheese will be described in the articles **Cheese**: Blue Mold Cheese; Camembert, Brie, and Related Varieties; Cheddar-Type Cheeses; Cheeses Matured in Brine; Dutch-Type Cheeses; Hard Italian Cheeses; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Smear-Ripened Cheeses; Swiss-Type Cheeses.

Salting

Most, probably all, cheeses are salted at the end of curd manufacture by

- mixing dry salt with curd chips, for example, Cheddar and related varieties,
- submersion in NaCl brine, for example, Gouda, Emmental, and Camembert, and
- rubbing dry salt on the surface of pressed cheese, for example, blue cheese.

Salt, which varies from about 2 to 10% in the moisture phase, has a major influence on various aspects of cheese ripening, quality, and safety (*see* **Cheese**: Salting of Cheese).

Applications of Ultrafiltration in Cheesemaking

Since cheese manufacture is essentially a dehydration process, it was obvious that UF would have applications, not only for standardizing cheese milk with respect to fat and casein, but more importantly for the preparation of a concentrate with the composition of the finished cheese, referred to as ‘precheese’. Standardization of cheese milk by adding UF concentrate (retentate) is now common but the manufacture of precheese has to date been successful commercially for only certain cheese varieties, most notably UF Feta and Quark (*see* **Cheese**: Membrane Processing in Cheese Manufacture).

Ripening

Acid-coagulated cheeses, which constitute a major proportion of the cheese consumed in some countries, are ready for consumption at the end of curd manufacture. Although rennet-coagulated cheese may be consumed as fresh curd, and a little is, most of these varieties are ripened (matured) for a period ranging from about 3 weeks to more than 2 years; generally, the duration of ripening is inversely related to the moisture content of the cheese. Many varieties may be consumed at any of several stages of maturity, depending on the flavor preferences of consumers and economic factors.

Although curds for different cheese varieties are recognizably different at the end of manufacture (mainly due to compositional and textural differences), the unique characteristics of each variety develop during ripening as a result of a complex set of biochemical reactions. The changes that occur during ripening, and hence the flavor, aroma, and texture of the mature cheese, are predetermined by the manufacturing process, especially by the levels of moisture and NaCl and pH, residual coagulant activity, the type of starter, and, in many cases, by the secondary microflora (added or adventitious).

The biochemical changes that occur during ripening are caused by one or more of the following agents:

- coagulant,
- indigenous milk enzymes, especially proteinase and perhaps lipase,
- starter bacteria or their enzymes, and
- secondary microorganisms (NSLAB and secondary cultures) and their enzymes.

Ripening is a very complex series of biochemical reactions, which may be divided into three principal groups:

- Catabolism of lactose, lactic acid, and, in some varieties, citric acid; this results in changes in flavor and texture (through changes in pH) and in the production of CO_2, which causes eyes and other openings.
- Lipolysis and the catabolism of fatty acids; in some varieties, for example, blue cheeses, these reactions dominate ripening.
- Proteolysis and modification of amino acids, which are the most complex, and perhaps the most important, reactions in cheese ripening, especially in internal bacterially ripened varieties; they affect flavor, texture, and functionality.

An overview of the biochemistry of cheese ripening is presented in the article **Cheese**: Biochemistry of Cheese Ripening.

Cheese as an Ingredient and Processed Cheese Products

About 50% of cheese is consumed as such, referred to as 'table cheese'. A considerable amount of natural cheese is used as a food ingredient, for example, Parmigiano Reggiano or Grana Padano on pasta products, Mozzarella on pizza, Quark in cheesecake, and Ricotta in ravioli (*see* **Cheese**: Cheese as a Food Ingredient). In addition, cheese is used in the production of a broad range of processed cheese products, which in turn have a range of applications, especially as spreads, sandwich fillers, or food ingredients. Other cheese-based products include cheese powders and enzyme-modified cheese, both of which are becoming increasingly important as food ingredients (*see* **Cheese**: Cheese Analogues; Enzyme-Modified Cheese; Pasteurized Processed Cheese Products).

Cheese Production and Consumption

World production of cheese is $\sim 16 \times 10^6$ tonnes per annum ($\sim$35% of total milk production) and is increasing at a rate of 2–3% per annum. Europe, with an annual production of $\sim 8 \times 10^6$ tonnes, is the principal producing region, followed by North America (**Table 1**). Cheese consumption, which varies widely between countries (**Table 2**), has increased consistently in most countries for which data are available; along with fermented milks, cheese is the principal growth product within the dairy sector. There are many reasons for the increased consumption of cheese, including a positive dietary image, convenience and flexibility in use, and a great diversity of flavors and textures. Cheese can be regarded as the quintessential convenience food: it can be used as a major component of a meal, as a dessert, as a component of other foods, or as a food ingredient; it can be consumed without preparation or subjected to various cooking processes. The most rapid growth in cheese consumption in recent years has been as a food ingredient (*see* **Cheese**: Cheese as a Food Ingredient).

Classification of Cheese

There are at least 1000 varieties of cheese, ~500 of which are recognized by the International Dairy Federation. For various reasons, a number of attempts have been made to classify cheeses into meaningful groups. Traditional classification schemes have been based principally on moisture content, that is, extra-hard, hard, semihard/semisoft, or soft. Although used widely, this scheme suffers from serious limitations since it groups cheeses with widely different characteristics, for example, Cheddar and Emmental are

Table 1 Production of cheese ($\times 10^3$ tonnes)

Europe			
Austria	137	Belgium	67
Bulgaria	63	Czech Republic	109
Denmark	324	Estonia	36
Finland	107	France	1765
Germany	2032	Greece[a]	230
Hungary	73	Ireland	140
Italy	1064	Latvia	40
Lithuania	107	Netherlands	724
Norway	84	Poland	637
Portugal	57	Romania	65
Russia	425	Slovakia	34
Slovenia	20	Spain	119
Sweden	114	Switzerland	179
United Kingdom	339	Ukraine[b]	346
North, *Central*, and *South America*			
Canada	402	Mexico[b]	154
United States	4463	Argentina	491
Brazil[b]	580	Chile	65
Uruguay[b]	48		
Africa, *Asia*, and *Oceania*			
Australia	343	New Zealand	355
Iran[b]	230	Israel[b]	112
Japan[b]	125	South Africa[b]	42

[a] From BarryWilso's dairy Newsletter 21(13): 2009.
[b] From Dutch Dairy Industry, 2008.
Data are mainly from the Dutch Dairy Board.

Table 2 Consumption (kg per head per annum) of cheese (in 2007)

Greece[a]	30.0	Argentina	11.2
France	24.3	Poland	10.7
Iceland	23.5	Portugal[a]	10.5
Norway	23.5	Hungary[a]	10.4
Germany	22.2	Slovakia[b]	10.2
Switzerland	22.2	Estonia	10.0
Italy	20.5	Slovenia[b]	9.4
Finland	19.1	Croatia	8.2
Austria	18.8	Spain	7.2
Sweden	18.4	Russia[a]	6.2
Netherlands	17.3	Ireland[b]	6.1
Czech Republic	17.0	New Zealand	6.1
Denmark[b]	16.5	Bulgaria[b]	5.6
Israel	16.5	Lithuania[b]	4.9
United States	16.0	Chile	4.0
Belgium[b]	14.6	Latvia	3.7
Canada	12.6	Mexico[b]	2.2
Luxembourg[b]	12.6	Japan[a]	2.0
United Kingdom	12.2	South Africa	1.6
Australia	11.9	Ukraine	1.1

[a] 2006.
[b] From Dutch Dairy Federation.
Data are from *Bulletin of the International Dairy Federation*, no. 432/2008, Brussels, unless otherwise indicated.

classified as hard cheeses although they have quite different textures and flavors, are manufactured by very different technologies, and the microbiology and biochemistry of their ripening are very different. In addition, cheeses traditionally developed a rind through which moisture evaporated; hence, the composition of cheese changes as it ages and there is a moisture gradient from the surface to the center; the moisture content of long-ripened cheese may decrease by 5–10% during ripening. The composition-based scheme is made more discriminating by

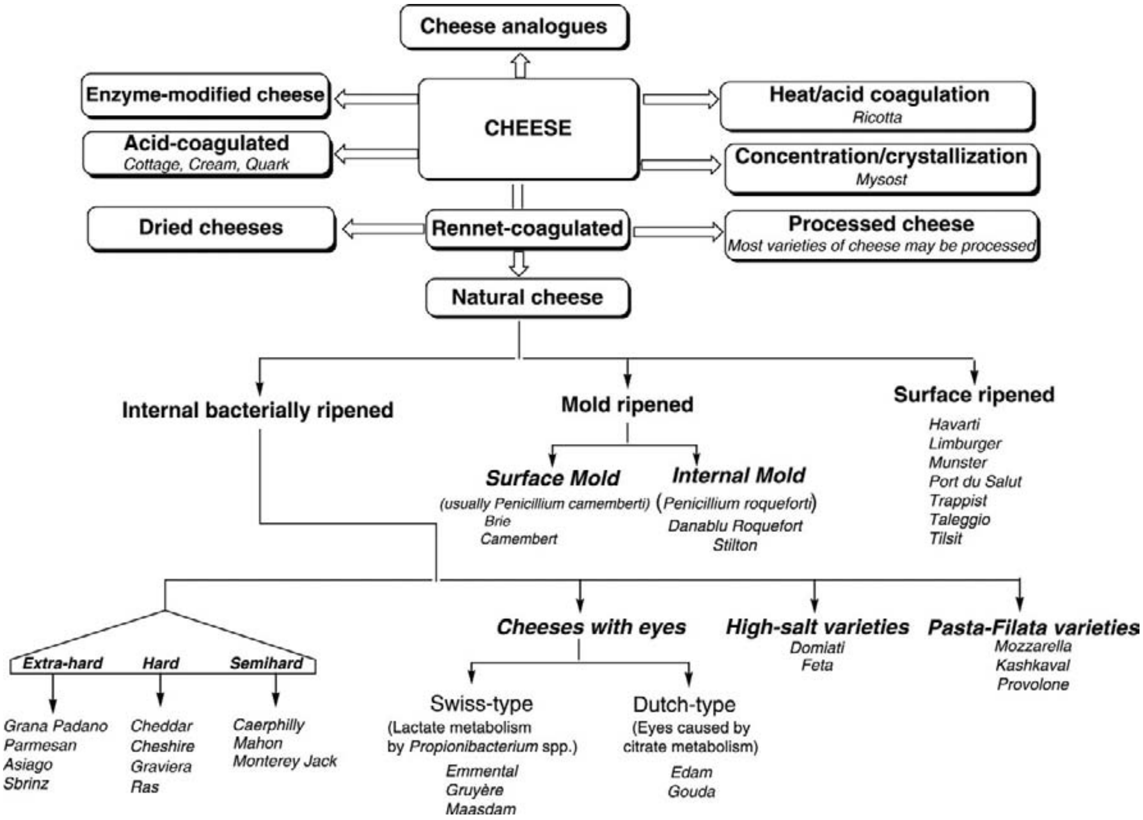
Cheese analogues
Enzyme-modified cheese
CHEESE
Heat/acid coagulation
Ricotta
Acid-coagulated
Cottage, Cream, Quark
Concentration/crystallization
Mysost
Dried cheeses
Rennet-coagulated
Processed cheese
Most varieties of cheese may be processed
Natural cheese
Internal bacterially ripened
Mold ripened
Surface ripened
Havarti
Limburger
Munster
Port du Salut
Trappist
Taleggio
Tilsit
Surface Mold
(usually Penicillium camemberti)
Brie
Camembert
Internal Mold
(Penicillium roqueforti)
Danablu Roquefort
Stilton
Cheeses with eyes
High-salt varieties
Domiati
Feta
Pasta-Filata varieties
Mozzarella
Kashkaval
Provolone
Extra-hard
Hard
Semihard
Grana Padano
Parmesan
Asiago
Sbrinz
Cheddar
Cheshire
Graviera
Ras
Caerphilly
Mahon
Monterey Jack
Swiss-type
(Lactate metabolism by Propionibacterium spp.)
Emmental
Gruyère
Maasdam
Dutch-type
(Eyes caused by citrate metabolism)
Edam
Gouda

Figure 2 A scheme for the classification of cheese. Reproduced from Fox PF, McSweeney PLH, Cogan TM, and Guinee TP (2004) *Cheese: Chemistry, Physics and Microbiology*, 3rd edn., Vols. 1 and 2. San Diego, CA: Elsevier.

including information on the source of the milk, the coagulant, principal ripening microorganisms, and the cook temperature. On the basis of the method of milk coagulation, cheeses may be divided into four superfamilies:

- rennet-coagulated cheeses: most major cheese varieties;
- acid-coagulated cheeses: for example, Cottage, Quark, Cream;
- heat/acid-coagulated cheeses: for example, Ricotta; and
- ‘brown cheese’, prepared by concentration/crystallization of whey/skim milk blends fortified to varying degrees with cream: for example, Mysost.

Owing to the great diversity of rennet-coagulated cheeses, these can be classified further based on the characteristic ripening agent(s), for example, internal bacteria, internal mold, surface mold, or surface smear (bacteria), or manufacturing technology; such a scheme is shown in **Figure 2**.

See also: **Cheese**: Acid- and Acid/Heat Coagulated Cheese; Biochemistry of Cheese Ripening; Blue Mold Cheese; Camembert, Brie, and Related Varieties; Cheddar-Type Cheeses; Cheese Analogues; Cheese as a Food Ingredient; Cheeses Matured in Brine; Dutch-Type Cheeses; Enzyme-Modified Cheese; Hard Italian Cheeses; Membrane Processing in Cheese Manufacture; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Pasteurized Processed Cheese Products; Raw Milk Cheeses; Rennet-Induced Coagulation of Milk; Salting of Cheese; Smear-Ripened Cheeses; Starter Cultures: General Aspects; Swiss-Type Cheeses. **Enzymes Indigenous to Milk**: Lactoperoxidase. **Heat Treatment of Milk**: Thermization of Milk. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactococcus lactis*. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Plant and Equipment**: Pasteurizers, Design and Operation. **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products. **Whey Processing**: Demineralization; Utilization and Products.

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Preparation of Cheese Milk

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Introduction

Milk may undergo several processing steps once it is taken from the animal and before it is added to the cheese vat. These practices vary widely throughout the world and even within countries and between cheese plants. This article will focus on common practices used in large-scale cheesemaking facilities and will start with milk as received at the cheese factory. It should not be inferred that the processes mentioned in this chapter are universally practiced. Consequences of preprocessing steps on milk and their impact on cheese quality will also be discussed.

First Steps: From Milk Truck to Silo

Milk is generally sampled at the farm for eventual determination of antibiotics, somatic cells (indication of inclusion of milk from diseased animals), composition (payment), bacteria (milk quality), added water (adulteration), and the amount of extraneous material (called sediment) that may be in it (general cleanliness). Milk must receive a negative result in a test for antibiotics before it can be off-loaded into the cheese-manufacturing facility. Any milk testing positive for antibiotics must be tagged and disposed of. It is also illegal to dilute milk known to contain antibiotics with uncontaminated milk to obtain blended milk with a negative antibiotic test result.

As trucks hauling milk often pick up milk from several farms, samples are also taken at the individual farms. In this manner, if a sample returns a positive result for antibiotic test, the source of the antibiotic can be located. The culprit farm is liable for lost value of the entire load of milk. Consequently, many large farms have taken a proactive approach to test their milk for antibiotics before it is picked up. A potential issue with antibiotic testing is that the test used may not detect all types of antibiotics or may not detect very low levels of individual antibiotics it was designed for. Antibiotics are used to treat a variety of illness or infections in animals. Normally, there is a clearly defined withdrawal period stated for the antibiotic. During this prescribed time, milk from treated animals must be dumped, including milk from quarters of the udder not treated. Owing to poor record keeping, inappropriate treatment practices (overdosing), or negligence, milk from treated animals may inadvertently get mixed into milk from untreated animals. Antibiotics may inhibit the growth of starter bacteria and induce shock in humans who are sensitive to them.

Testing for antibiotics in milk is a mandatory first step, but other tests are also preformed. There are laws in the United States that prohibit the off-loading of milk that arrives at the factory at temperatures higher than mandated, generally ~7 °C. In addition, milk is often smelled to assess the likelihood that the milk has soured or picked up undesirable flavors often derived from the animal feed, and inspected visually for the presence of butterballs (indicator of excessive agitation) and color (pink indicating blood). Pink-colored milk may result from the introduction of colostrum into the milk, from injured teats, or from mastitic animals. After testing, the milk is pumped into storage vessels called raw milk silos. As milk may have undesirable volatile flavors and may have absorbed air during pumping and agitation, it is often deaerated while being pumped into the silos or horizontal tanks. And often it is deaerated again while being prewarmed during the pasteurization process. Excessive oxygen content can be detrimental to starter growth. In addition, dispersed air can cause fouling in pasteurizers or heaters, reduce efficiency in separators, and may result in inaccurate volumetric measurements of milk and loss of precision in automatic in-line standardization.

There may be some stratification (creaming) of the fat during storage of milk in the silo. This can cause variability in the casein-to-fat ratio and consequently lead to variability in the fat-in-dry matter and moisture content of the cheese unless the milk is standardized.

Clarification or Filtration

While milk is pumped from the silos into the cheese plant it is often clarified (mechanical treatment) or filtered (stainless mesh or wedge-wire filters) to remove extraneous material from milk. This material could be straw, hay, hair, clumps of somatic cells, or insects (flies) inadvertently incorporated into the milk during milking or which entered the milk storage tanks on the farm. It could also include O-rings and gaskets. Clarifiers are two-phase centrifuges designed to remove solids heavier than the proteins and fat found in milk. The use of clarifiers has been cyclic: at one time they were common, but were later removed from many facilities because better milk production practices led to great reduction in the level of extraneous material and because many cheese manufacturers thought the practice was an unnecessary expense. Clarifiers periodically must be flushed to remove the built-up sludge removed from the milk. The loss of milk solids during this process was substantial enough to warrant not using clarifiers. However, recently, there has been a periodic problem of material from petroleum-based dry cow treatments entering the milk supply and eventually getting into the cheese. Bismuth used in one of these treatments reacts with sulfur produced in cheese during extended ageing of cheese and results in black spots in the cheese. Clarifiers are being reintroduced into factories as they are able to remove any remnants of petroleum-based dry cow treatment.

As an alternative to clarifiers, some cheese manufacturers have opted to use filters (called filter socks or filter bags). These fine-mesh filters (made of cotton cloth or synthetic materials such as Dacron, Rayon, Polyester, or Nylon) are designed to be tied or clamped on to the discharge end of milk pipelines. They are sometimes used as prefilters before clarifiers and are more commonly used at the point where the milk enters the cheese vat. Filters are also used on the farm as milk is pumped from the milking machines to the refrigerated bulk storage tank. Stainless-steel filters or strainers are also used in pipelines to protect equipment (including clarifiers and reverse-osmosis and ultrafiltration membranes) from larger objects such as broken gaskets or metal parts.

Separation

After clarification, milk may be separated. This is common in plants that produce cheeses with a fat on a dry basis (FDB) of less than 50%. FDB is largely determined by the ratio of casein to fat. The higher the casein-to-fat ratio is, the lower the FDB of the cheese will be. Whole milk generally has a casein-to-fat ratio of ~0.65–0.70. This ratio will result in a cheese that has at least a 50% FDB. Cheeses such as low-moisture-part skim Mozzarella (average FDB of 42%) and hard grating cheeses (average FDB of 40%) require higher casein-to-fat contents than that naturally occurs in whole milk. Consequently, a portion of the fat must be removed or casein must be added.

The main use of a separator is to produce milk with a prescribed fat content. A separator is a three-phase centrifuge designed to separate milk into liquids of three phases: light phase (cream), heavy phase (skim milk), and a third phase that includes solids such as hair, clumps of bacteria, dirt, and even clumps of somatic cells. When cleaning out the separator, the operator may notice the telltale pink-to-red (bloody) sludge that results from milk with added colostrum or obtained from injured and mastitic animals.

Separators can be used in lieu of a clarifier, but they are not as efficient. The efficiency of the separator can be adjusted to produce skimmed or partially skimmed milk and cream. The percentage of fat in the cream that is removed can also be adjusted. It is usually to the advantage of the cheesemaker to produce cream with the highest feasible fat content. Butter makers often dictate the fat level in cream, as they have a strong economic interest in the skim milk solids. However, they are also concerned that if the cream is too viscous (higher than 40% fat) they may have difficulty pumping the cream without causing damage to the fat. Typically, cheese manufacturers will run only a portion of the milk through the separator. This skimmed milk will later be blended with other milk or cream to produce the desired casein-to-fat ratio in the standardized milk. In this manner, only a portion of the milk is separated, thus saving time and costs.

For many full-fat varieties such as Cheddar, the casein-to-fat ratio of the milk received from the farm is often slightly too low to produce cheese of desired quality (cheese may become too soft, sticky, or pasty with age). Hence, a portion of the fat must be removed or skim milk must be added. It is often difficult to obtain the precise casein-to-fat ratio desired by removing cream via a separator or by simply adding a prescribed amount of skim milk (or other milk solids, e.g., membrane- or heat-condensed milks).

Bactofugation

Bactofugation is a special centrifugation process that removes spores from milk. Germinated spores, especially *Clostridium* species, can cause undesirable flavors (putrid, rancid, and sewer-like), and the gas that they produce causes splits and cracks in the cheese or blown packages. Similarly to a clarifier, sludge is produced and is discarded, making it a potentially expensive process (on account of solids loss). But the loss of cheese quality is an important factor that must also be considered. Modern advances in equipment design have reduced the amount of sludge produced, and thus more factories are installing bactofugation equipment. Bactofugation is still mainly used in Swiss and Gouda cheese plants. These cheeses are often low in acid and salt and have a relatively high pH. These conditions make them vulnerable to growth of clostridia.

Standardization

Standardization is the process of changing the solids composition of milk from that which is received from the producer. It includes the removal or addition of cream (fat) and/or addition of casein via noncondensed or condensed milks (whole milk, nonfat dry milk, or skim milk). It also includes just water removal. The addition of milk solids is also known as fortification. Standardization is often used to reduce the inherent day-to-day variability in milk composition and changes in milk composition owing to weather conditions, stage of lactation, feeding practices, and health of the animal. Many cheese varieties require more fat or more casein than is naturally present in milk to produce cheese with desired composition for legal or quality issues. The desired or legal composition of cheese is sometimes given in terms of solids composition, that is, FDB. The FDB of cheese is almost entirely determined by the milk composition. For most cheeses, more than 94% of the solids of cheese are casein and fat; thus the ratio of casein to fat in milk and the recovery of each component as cheese are the factors that influence the FDB of the cheese.

Reasons Encouraging Standardization of Milk for Cheesemaking

1. Variability in milk composition particularly casein content will result in differences in clotting rate, coagulum firmness, and rate of pH drop owing to buffering by casein. Variability in casein content of the milk may require that the cheesemaker adjust the rate and extent of acid development. Failure to do so may result in production of cheese that does not meet quality standards related to functionality and body characteristics. It is often undesirable, difficult, and not feasible for a cheesemaker to make process adjustments during cheesemaking. Standardization lessens the need for 'on the fly' process adjustments.
2. Standardization of milk composition reduces variability in cheese composition, that is, FDB and moisture content, and pH. Thus standardization can reduce variability in cheese quality.
3. Fortification, or standardization by adding milk solids, improves productivity; that is, more cheese per vat, more cheese per man-hour, and improved productivity of whey processing.
4. Standardization of milk is necessary to produce cheese that meets legal standards in terms of FDB and moisture content.

Milk is generally standardized to obtain predetermined casein (protein)-to-fat ratio. Some manufacturers still practice standardization with the only objective of achieving a consistent milk fat content. Standardization of milk to milk fat content alone is not effective in reducing variability in cheese composition because the casein content of the milk can also vary. Milk of average composition will produce a cheese with an FDB of ~52–54%. Whole milk cheeses generally do not require that the milk be standardized, but at times standardization is necessary. This is especially the case during hot weather or if milk collected during late lactation is used. Cheeses with desired FDB values of less than 50%, such as part skim Mozzarella, Parmesan, and reduced-fat cheeses, require that fat be removed from the milk or casein be added. Because of the variability in whole milk composition, which consequently results in variability in cheese FDB, some manufacturers of whole milk cheeses also are beginning to see the value in standardization of milk for their cheeses as well. Although the emphasis of standardization is on achieving the desired FDB, it is also necessary during cheese production to adjust the moisture content to make it compatible with the desired FDB. One of the main causes of defective cheeses or shortened shelf life (excessive softening, pasty body) is excessive moisture content for the FDB of the cheese. A benefit of standardization is that it is easier for the cheesemaker to manufacture a cheese with both a consistent FDB and a consistent moisture content if the milk composition is consistent.

The optimal standardization objective is not only to modify milk composition in terms of casein-to-fat ratio, but also to reduce variability in the total amount of casein and fat in the milk. Thus milk is also standardized to a prescribed total solids level. Additional objectives may be to standardize whey protein, minerals, and lactose content although this is rarely practiced.

The precept of standardization is quite simple. It is all about manufacturing cheese with a consistent composition, a key parameter in producing cheese of consistent quality. To achieve this goal, the cheesemaker must use milk of the same composition and follow a consistent manufacturing protocol. Standardization of milk also helps to equalize the amount of cheese produced from each vat of milk. This is extremely important with Swiss- and Gouda-type cheeses: A consistent curd depth during pressing is critical to produce a consistent block size, which, in turn, is critical for subsequent handling and eye development.

What Are the Processes Involved in Standardization?

Step 1. Establish the desired FDB required for the particular type of cheese to be manufactured. This may be dictated by regulations and is often predetermined by the desired physical attributes of the cheese.

Step 2. Determine the casein-to-fat ratio of the milk that will produce a cheese with the desired FDB.

Step 3. Determine the means by which the casein-to-fat ratio can be changed. This involves either addition of casein/cream or removal of cream. Once this is established, it may also be necessary to set limits on the total amount of casein and fat in the milk. Different sources of casein may contain different levels of lactose. Excessive addition of lactose to the milk may result in a cheese of very low pH if the lactose is fermented and may also lead to browning of cheese during storage.

Step 4. Determine the constraints on total fat and casein (or total solids) content of the milk. Excessive amounts of casein and fat in the milk may produce too large a quantity of cheese for the cheesemaking equipment to handle properly.

Step 5. Perform an economic analysis of standardization. This includes analyses of the whey volume, composition, and products made from the whey, as well as of productivity in terms of cheese yield and the cost of equipment, time, and manpower necessary to implement standardization.

Step 2 in the standardization process is best accomplished through the use of an appropriate predictive cheese yield formula. Cheese yield formulae are used to predict the cheese yield from milk of known composition, but they can also be used to predict cheese composition in terms of FDB prior to cheesemaking. The cheese yield formula is used to predict how much of the fat, casein, and other solids (plus added salt) in the milk will be captured as cheese. Thus the ratio of fat to all solids can be predicted, that is, FDB of the cheese. The casein-to-fat ratio of the milk required to produce a cheese with the desired FDB can then be determined. An appropriate cheese yield formula must be used for each individual cheese variety. This requirement extends to individual cheese factories and even individual cheese milks. Even though it is often inappropriate to apply a cheese yield formula developed for one cheese or cheesemaker to another cheese plant, standardized yield formulae are generally available for most cheeses. An appropriate cheese yield equation can be developed for each cheese plant by adjusting the recovery factors to numbers obtained from cheesemaking trials. Cheese yield formulae are generally developed independent of actually measuring the cheese yield; instead they are based on milk and cheese composition data. An example of determining the casein-to-fat ratio needed to produce the desired cheese FDB using the Van Slyke predictive cheese yield formula is given below.

$$\%\text{ Cheese yield} = \frac{[\text{RF}\times\ \%\text{ Fat in milk} + \text{RC}\times\ \%\text{Casein in milk}]\times \text{RS}}{\%\text{Solids in the cheese}/100}$$

RF = ratio of fat in milk to fat recovered in cheese
RC = ratio of casein in milk to casein recovered in cheese

$$\text{RS} = 1 + \frac{(\%\text{ Total cheese solids} - \%\text{Fat in cheese} - \%\text{ Casein in cheese})}{\%\text{Fat in cheese} + \text{Casein in cheese}}$$

$$\text{FDB} = \frac{\text{RF}\times\%\text{Fat in milk}}{[\text{RF}\times\%\text{Fat in milk} + \text{RC}\times\%\text{ Casein in milk}]\times\text{RS}}$$

If RF is 0.92, RC is 0.95, RS is 1.10, and milk is 3.70% fat and 2.50% casein:

$$\text{FDB} = \frac{0.92\times 3.70}{[0.92\times 3.70 + 0.95\times 2.50]\times 1.10}$$

Predicted FDB = 0.535 and casein-to-fat ratio = 0.676

If the desired FDB is 0.51, what must the casein-to-fat ratio of the milk be in order to achieve it? There are two unknowns (casein and fat); however, only the ratio of casein to fat is needed, not the actual amounts of casein and fat. If the %fat is given as 1.0, casein can be calculated. The casein required will be the actual casein-to-fat ratio of the milk required to produce the cheese with an FDB of 0.51.

$$\text{FDB} = 0.51 = \frac{0.92\times 1.0}{[0.92\times 1.0 + 0.95\times\text{Casein}]\times 1.10}$$

Hence, Casein = 0.76

Once the casein-to-fat ratio is established, the cheese maker can now determine the best or the most economical means to accomplish it.

Changing Milk Composition

The cheesemaker potentially has at least four objectives when changing milk composition:

1. standardize to fat content only;
2. standardize to casein-to-fat ratio (or protein-to-fat ratio);
3. standardize to total amount of casein and fat in addition to the casein-to-fat ratio;
4. standardize to total solids content; and
5. standardize lactose content in serum phase of milk

Standardization of lactose content is not widely practiced currently, but can be accomplished with membrane filtration and diafiltration of retentates. It has been used in the manufacture of Mozzarella to ensure that the entire mass of cheese prior to the pasta filata (cooking–stretching) step is at the desired pH (i.e., reduces variability in cheese pH from the first batch of curd that enters the mixer from that of the last batch to enter). Standardization of lactose content of the milk eliminates the need for whey dilution or curd rinsing, both of which are used during the manufacture of certain cheeses to prevent excessive acid development. In addition, it also aids whey processing in that there is no extra water that must be removed during whey processing as there would be if water were added during cheesemaking.

The cheesemaker has two options to change the casein-to-fat ratio of the milk: add casein, or add or remove fat. Combinations are often used. The actual amount of casein or fat to be added or the amount of cream to be removed is determined mathematically and is fortunately part of computer-based programs designed for this purpose. In most countries, it is illegal to add casein as a purified product. Thus casein can only be added as nonfat dry milk, reconstituted nonfat dry milk, condensed milk, or concentrated or membrane-separated milk. There are differences among countries in the regulations governing the use of membrane-separated milks, that is, ultrafiltered (UF) or microfiltered (MF) milks. Use of milk concentrated by reverse osmosis (RO) is a generally accepted practice albeit not as popular as other forms of casein addition because it introduces extra lactose to the standardized milk. Milk concentrated by RO is a nonthermal alternative to milks condensed using heat. Ultrafiltration and microfiltration can be performed on either whole or skimmed milk. Standardization of lactose content (almost always done to decrease lactose in the serum phase of milk) requires the use of diafiltration as part of UF or MF processes, or addition of water to the cheese milk. Addition of water to milk or to curd/whey mixture is generally an undesirable process because it requires the eventual removal of water and thus adds to the cost if the whey is processed later. Addition of heat-condensed or nonfat dry milk directly to the cheese milk also is practiced; however, it is used only in cases where excessive lactose will not cause a quality problem (acidic cheeses such as Feta). In most cheese varieties where they are used, it is important to add water to the milk to dilute the serum lactose concentration or reconstitute the nonfat dry milk prior to addition to the cheese vat. Excessive lactose in milk may lead to excessive lactic acid in the cheese, which in turn leads to defects associated with low pH and high acid content (acidic flavor, loss of serum in packaged cheese, brittle body, and calcium lactate crystals). Diafiltration is usually carried out in a continuous mode, with water being added as serum is being removed as the permeate stream. It can also be done in a batch process.

With diafiltration, the lactose content of membrane-separated milks can be reduced with a concomitant increase in the relative solids proportion of fat and protein. Ultrafiltration of milk results in the partial separation of water, lactose, dissolved minerals, and low-molecular weight nitrogenous compounds from other milk components. Consequently, the proportion of the larger serum proteins increases relative to the other serum solids such as lactose. However, the serum concentration of lactose, mineral, or other low-molecular weight components that remain may actually increase very slightly as compared to the native milk serum concentrations, unless diafiltration is used. Microfiltration of milk is used to separate dissolved serum solids from colloidal solids. It is primarily used to remove a portion of the large serum proteins and dissolved caseins (mostly β-casein) from milk. For cheesemakers, the choice between using UF and MF is determined by the value of the extracted serum proteins as unique products relative to their value as cheese or as an ingredient in other food products, or to the value of serum proteins extracted from cheese whey. The choice of the source for added casein is based on price, continuity of availability, legality, heat treatment (amount of denatured serum proteins), and lactose content. Establishing an exact lactose-to-protein ratio can enable the cheesemaker to produce a cheese with a more uniform functionality.

The method or objective of standardization will have an impact on whey composition and value, and these factors must be considered in the economic evaluation of standardization. Addition of solids to milk or removal of water (serum) will increase the solids of the whey and will alter its solids composition. Both factors will determine the value of the whey and the type and quality of the finished products derived from it.

How Is Standardization Performed?

There are two general methods of standardization: batch and continuous. Both require precise measurements of composition of the initial milk as well as of the standardization agents. In batch standardization, the prescribed amounts of standardization agent, that is, condensed milk, nonfat dry milk, or cream, are blended with a prescribed amount of milk. This method requires sufficient holding vessels both for the solids to be added and for the blended milk. The cheese vat may be the blending vat. A version of batch standardization is to run the milk through a separator to a prescribed fat content.

It is often difficult to obtain the desired precise casein-to-fat ratio by removing cream via a separator or by simply adding a prescribed amount of skim milk (or other milk solids, e.g., membrane- or heat-condensed milks). In many larger cheese plants a continuous standardization process is used. It is called ‘in-line’ standardization. In this process, milk composition (fat, protein, and total solids) is rapidly and continuously measured using special sensors as the milk is pumped through pipelines. The sensor is linked to other equipment. The cheesemaker sets the desired protein-to-fat ratio and solids level into a computerized system that takes the information from the sensors and automatically opens valves of tanks containing the standardizing agent. In this method the standardization agent is blended 'in-line' with the milk as it flows through the pipes. In many cheese plants it is common to first remove a portion of the fat of the milk in excess of what is required to produce the desired protein-to-fat ratio. As the milk is lower in fat than required, cream must be added back.

A potential problem with standardization is the requirement for accurate analytical measurement of milk components. Often, these analytical tests are based on expensive and time-consuming chemical methods. Consequently, faster nondestructive methods are necessary for more efficient and productive standardization processes.

Homogenization

Homogenization of milk or cream prior to cheesemaking is done mainly for blue-veined cheeses, but it is not used by all manufacturers of these cheeses. Homogenization of milk is very rarely done for other cheese varieties. Homogenization of fat results in increased fat hydrolysis and consequently increases the development of fat-derived flavors, especially methyl ketones. Excessive concentrations of methyl ketones can lead to harsh medicinal or chemical notes in blue-veined cheeses. To some consumers this is a negative, but by others this is exploited in blue cheese intended for use in salad dressings. Most farmstead cheesemakers do not use homogenization, and their blue cheese tends to develop sweeter, earthy, and less-chemical flavor notes.

Homogenization of fat can result in increased fat recovery, increased moisture, and smoother or creamier bodied cheese. Consequently, it has been used by some manufacturers of reduced-fat cheese varieties. Use of homogenized milk may result in cheese that is excessively sticky or has the mouthfeel similar to that of processed cheese. It has been used to restrict flow of cheeses with a low pH (5.2–5.4) when they are heated. The lack of flow is maintained for a few weeks, but, as proteolysis occurs during ageing, these cheeses will eventually exhibit flow. Homogenization has also been used to increase whiteness or opacity of cheese, especially in blue-veined cheeses where the contrast between the blue veining and whiteness of the cheese is desirable.

Heat Treatment

Prior to cheesemaking, milk is usually heat-treated not only to kill pathogenic bacteria but also to lower the total bacteria count. A few cheesemakers have chosen not to use any heat treatment prior to cheesemaking and often never heat the milk or cheese curd to temperatures higher than the temperature of the animal from which the milk was obtained. Heat treatment is thought of in terms of three approaches:

1. No heat treatment (raw)
2. Pasteurization
3. Some level of heat treatment (thermization), but short of pasteurization (milk is still considered raw)

Debate on the benefits (safety and consistency of cheese quality) or detractions (lack of desired flavor attributes) of using any heat treatment of milk for cheesemaking can be intense, but the cultural side of this argument cannot be ignored.

Pasteurization can be used to increase the moisture content of cheese, and the degree to which this occurs is determined both by the heating temperature and by the heating time used. Serum proteins are denatured and become associated with casein at the surface of the micelles. This association will inhibit clotting and syneresis. If severe enough, it will also result in shattered curd during cutting and inhibition of the curds knitting (curdy body) when pressed, and can restrict the melting of young cheeses. Of particular concern is the use of excessively heat-treated condensed skim or nonfat dry milk for standardization.

High-heat treatment of starter media containing serum protein and a small amount of casein exploits denaturation. If denatured proteins are added to milk that itself has not been overheated, the denatured proteins will not interfere with clotting, nor cause curd shattering, impede curd fusion, or inhibit flow of cheese. However, incorporation of denatured protein complexes will inhibit syneresis and result in increased moisture content of the cheese. This practice is often used in Mozzarella and other high-moisture cheeses. If pasteurization is used, manufacturers of naturally ripened aged cheeses, such as Cheddar or hard Italian types, will use only sufficient temperatures and times to meet the regulatory requirements.

Many large-scale manufacturers of aged cheeses prefer to use thermization. It is believed that thermization (if temperature and time are sufficient) will kill most types of pathogens that may be in the milk or at least reduce their numbers. It is thought that with proper acidity in the cheese and with proper ripening times, any pathogen that remains will eventually die. If the initial numbers of pathogens are high and if the pH of the cheese is high, pathogens can remain viable in the cheese for extended periods of time. It is thus imperative that cheesemakers that use thermized milk ensure that the milk is of high bacterial quality (low numbers of bacteria including low to no pathogens). It is also necessary, as with any cheesemaking facility, that they take precautions not to infect the cheese during or after manufacture and to manufacture cheese that has the hurdles (acid, pH, salt) necessary to inhibit the growth of potentially harmful bacteria.

Preacidification

Preacidification is the introduction of acid, generally diluted lactic or acidic acids, or directly injected carbon dioxide, to milk prior to rennet addition. It is used to speed up the cheesemaking process. Preacidification is commonly used in the manufacture of low-fat cheeses or when the casein content of the milk has been increased by removal of water or by addition of casein. It is always used in conjunction with added starter to complete the acidification process. It should not be confused with direct acidification, which is a means of manufacturing cheeses without added starter (as is sometimes the practice for Cottage, fresh, or whole milk Mozzarella) and where the final pH of the cheese is obtained solely by the amount of added acid.

A key requirement in the manufacture of any cheese is to control the level of insoluble calcium phosphate dissolved from casein. Standardization practices that result in an increased casein content of the milk often require that more acid be developed prior to rennet addition than in case of milk with lower casein content. Adjustments to the extent of acid development at key manufacturing steps may have to be made to account for the increase in concentration of the total amount of insoluble calcium phosphate (colloidal calcium phosphate). The equilibrium between soluble and insoluble calcium is due in part to pH of the milk or cheese, the acid content of the serum, and the concentration of soluble or ionic calcium in the serum. With an increase in casein a more acidic, or a lower, pH may have to be attained to solubilize adequate amounts of calcium. The most effective means to accomplish this is to use preacidification.

Because of time constraints, large-scale manufactures will opt for the addition of lactic or acetic acid to the milk rather than allowing additional time for the starter to develop sufficient acid. If the acid is added prior to pasteurization, it is critical that the pH of the milk be higher than ~6.3, or proteins may 'burn on' or foul the pasteurizer plates. Regardless of when it is added to the milk, the acid is diluted. Another option is to add lactic acid in the form of a bulk starter (or adjunct cultures) grown without pH control, that is, pH is ~4.6–4.8, rather than using a neutralized culture (pH 6.0). In this method the lactic acid is added after pasteurization of the milk (no burn on), is diluted, and is consequently much more quickly and evenly dispersed in the milk. The level of preacidification (pH) is determined by the amount of culture used.

Preacidification is often the preferred method to solubilize calcium phosphate rather than develop acid after the whey has been separated from the curd. Preacidification usually results in an increase in moisture content of the cheese and is thus used primarily in the manufacture of high-moisture cheeses. Development of acid by the starter in the curd after cutting will increase syneresis (decrease moisture content of the cheese). The greater the drop in pH necessary after the addition of starter, the greater the loss in moisture. In addition, acid development in curd after cutting the coagulum is less efficient at removing insoluble calcium phosphate from the casein. Consequently, without preacidification, it is often necessary to develop more acid in the cheese to achieve the desired physical attributes. This may result in an excessively acidic cheese.

Preacidification has potential drawbacks. Insoluble calcium phosphate is the major buffer in milk, and it is lost as a result of preacidification. Excessive loss of this buffering agent may result in a lower pH in the cheese than desired. To compensate for the loss of buffering, it is often necessary to add water to the whey after cutting (whey dilution) or to rinse the curd (curd washing). These steps reduce the concentration of lactose and lactic acid in the curd. The need to add water also depends on the temperature and salt sensitivity of the starter culture used.

As preacidification lowers the pH prior to rennet addition, the coagulum will form more rapidly and become firmer faster. The rapid clotting can result in a more open or coarse casein network and a firmer coagulum. As the coagulum forms, the pores or spaces between aggregates of casein become larger and the aggregates or strands of casein become thicker (firmer). The pores are filled with fat and serum. A more open coagulum (larger pores) can lead to higher fat loss after cutting but can result in a higher-moisture cheese. Generally, higher moisture content in cheese more than compensates for the loss in cheese yield owing to lower fat recovery. Preacidification is a common practice in lower-fat cheeses because it results in an increase in moisture content of the cheese and greatly reduces the level of colloidal calcium phosphate. Both of these parameters, along with proteolysis as the cheese ages, greatly reduce the firmness of reduced-fat cheeses.

Conclusion

It is often said by cheesemakers that cheesemaking begins at rennet addition. Others go further back and state that cheesemaking begins at the farm. Regardless, milk may go through many processing steps prior to entering the cheese vat. These processing steps are either required or are necessary to produce the desired cheese quality on a consistent basis. The challenge of modern cheesemaking is to manufacture cheese of consistent quality and composition, and preprocessing of milk helps obtain that goal.

See also: **Cheese**: Membrane Processing in Cheese Manufacture. **Heat Treatment of Milk**: Thermization of Milk. **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Influence on Free Fatty Acids; Milking Hygiene. **Milk Protein Products**: Membrane-Based Fractionation. **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Centrifuges and Separators: Types and Design; Flow Equipment: Principles of Pump and Piping Calculations; Flow Equipment: Pumps; Flow Equipment: Valves; Process and Plant Design. **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products.

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Starter Cultures: General Aspects

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Introduction

Modern cheesemaking makes use of advanced engineering, biotechnology, and food science. Even so, cheesemaking is fundamentally an ancient process and many of the standard cheesemaking procedures are based on traditional practices. It was not until 1873 that Lister isolated the first pure starter cultures and thereby confirmed bacterial involvement in cheesemaking. The handling and manipulation of bacterial cultures emerged as a technology by the 1890s, through Conn in the United States, Storch in Denmark, and Weigmann in Germany. Advances in the selection, propagation, and handling of starter cultures were essential in enabling cheesemaking to become industrialized and continue to influence cheesemaking practice today. Growing understanding of the biochemistry, physiology, and genetics of lactic acid bacteria has allowed more targeted use of specific cultures and promises even greater control over culture performance and cheese flavor.

This article addresses general concepts surrounding the use of starter cultures. Further details can be found in articles addressing specific cheese types and specific starter species.

Functions of Starter Cultures

Production of Lactic Acid

Cheese starter cultures are predominantly composed of lactic acid bacteria (*see* **Lactic Acid Bacteria**: Taxonomy and Biodiversity), although other bacteria and yeasts may also be involved. In cheese manufacture, the primary role of starter cultures is the production of lactic acid from lactose at a predictable and controlled rate (*see* **Cheese**: Overview). It is the consequent decrease in pH that then affects a number of aspects of the cheese manufacturing process and ultimately cheese composition and quality.

Effect of pH on Cheese Flavor and Texture

Acid alone can lead to coagulation of the milk caseins to form curd, but for most cheese types a preparation containing a proteolytic enzyme (rennet or, in more general terms, coagulant) is used to induce curd formation. During the vat stage of cheese manufacture, the decrease in pH directly affects the proteolytic activity of both coagulant and natural milk proteinase. At the point of separation of curd from whey, the pH can influence the amount of coagulant retained in the curd. This retained coagulant will continue to carry out proteolysis within the maturing cheese, releasing peptides and amino acids (which may impart brothy or bitter flavor defects or act as precursors for more complex flavor development) and causing a gradual breakdown of the curd structure and softening of the cheese.

Different coagulants display different properties, with some showing greater pH dependency than others. For example, the coagulant chymosin (either in traditional calf rennet or in a purified form) operates optimally at low pH (e.g., pH 2–4) and retention within the curd is also greater at lower pH. Cheese milk is typically set at a pH of 6.5–6.7 and the pH at which the whey is drained influences the amount of chymosin retained in the curd. Consequently, cheeses with a high acid level, particularly those that are not highly cooked, may develop a bitter flavor due to specific peptides originating from caseins as a result of coagulant activity. Plasmin, an indigenous milk proteinase with an alkaline optimum pH, also plays a role in cheese ripening but has little activity in low-pH cheeses.

The rate of curd contraction and whey expulsion (syneresis) is enhanced as the pH decreases, and this directly affects the final cheese moisture content and subsequently cheese texture and the rates of the various biochemical reactions involved in flavor compound formation. Cheese texture is also influenced by the level of pH-dependent dissolution of colloidal calcium phosphate from the casein micelles, particularly if this occurs prior to whey removal. Generally, cheeses with low pH tend to be crumbly while those of a higher pH tend to be more elastic.

It is important to note that these effects are not simply related to the final pH of the cheese but depend on the pH of the cheese at crucial points during manufacture, especially the pH at the point of curd/whey separation and during syneresis. Predictable acid production by the starter culture throughout the process can be crucial to predictable control of cheese texture and flavor development.

Production of Flavor and Aroma Compounds

Flavor development in cheese is largely dependent on the combined proteolytic activity of a number of proteolytic agents, including natural milk proteinases, coagulants, starter bacteria, adventitious non-starter bacteria, and adjunct organisms. Starter cultures possess an array of predominantly intracellular peptidases that degrade peptides formed by proteolytic agents to amino acids, which then act as precursors for a range of volatile flavor compounds (*see* **Cheese**: Biochemistry of Cheese Ripening; Starter Cultures: Specific Properties). When starter culture cells lyse in cheese, the intracellular peptidases are also available to act upon peptides in the cheese matrix itself.

Starter culture metabolism can also directly affect cheese flavor development by forming various compounds from lactose and citrate. One of the key branch point metabolites in lactose metabolism is pyruvate, the vast majority of which is converted under normal growth conditions to lactic acid by the enzyme lactate dehydrogenase. However, when starter bacteria are fermenting galactose or fermenting glucose or lactose at growth-limiting rates, products other than lactic acid can be formed from pyruvate. A number of strains of starter bacteria can also metabolize citrate, which is present at low concentrations in milk and cheese, to form pyruvate and acetate. The pyruvate can then be converted to various flavor compounds. For example, production of diacetyl and CO_2 is responsible for the characteristic aroma and eyes of Gouda cheese.

Control of Adventitious Organisms

The growth of many acid-sensitive pathogenic organisms in cheese is inhibited to some extent by the reduced pH as well as by the undissociated lactic acid molecules. However, as many pathogens can still grow at the pH of cheese, pH acts as one part of a hurdle system that serves to inhibit the growth of pathogens, operating in cheese along with low temperature, low water activity, salt concentration, organic acids other than lactic (e.g., acetic acid), and low oxygen availability. Both organic acids and reduced oxygen tension are by-products of starter metabolism. Water activity is discussed below.

Water Activity

Lactic acid production contributes to the depression of water activity in cheese through the formation of solutes either directly (e.g., lactic acid) or indirectly through the increased dissolution of colloidal calcium phosphate with decreasing pH. The influence of lactic acid production on the moisture content of cheese also significantly affects water activity. In Cheddar cheese, decreasing water activity has been shown to inhibit lactate formation and bacterial growth while there is an increase in the production of the flavor compound diacetyl.

Lactate Metabolism

Lactose is converted principally to L-lactate by starter bacteria, but some starter bacteria and many adventitious non-starter lactic acid bacteria present in cheese can produce D-lactate from pyruvate or racemize L-lactate to D-lactate. The calcium salt of the racemic DL-lactate is less soluble than calcium L-lactate and can precipitate in cheese, causing white specks of calcium lactate. These are undesirable in most cheese types. Control of the lactose and lactate content of cheese depends on predictable starter activity throughout cheesemaking.

L-Lactate can be oxidized to acetate, a contributor to cheese flavor, by non-starter lactic acid bacteria but this is dependent on the availability of O_2. Lactate oxidation also contributes to the reduction in redox potential, which in turn affects the growth of potential pathogens and possibly the rate of formation of various flavor compounds via nonenzymic pathways. In Swiss-type cheeses, the propionibacteria metabolize lactate to propionate and acetate, both flavor contributors, and CO_2, which is responsible for eye development.

Redox Potential

Little information is available on the effect of redox potential on the various chemical and enzymic reactions within maturing cheese. However, in Cheddar cheese, the E_h decreases during ripening (reported in one study to be $-104\,\text{mV}$ initially, decreasing to $-217\,\text{mV}$ after 5–6 months), and this appears to be necessary for the development of good flavor. The initial decrease in E_h has been attributed to starter bacteria as they remove oxygen during lactose metabolism, but once cell numbers become limiting, the E_h rises again before slowly returning to a low E_h level, presumably due to the increasing numbers of non-starter lactic acid bacteria.

Starter Types

Starter cultures can be classified in several ways. Historically, they have been described in terms of the procedures used to prepare them, the form in which they are delivered to the vat, the products made using them, the flavors, aromas, or other product qualities generated by them (i.e., the biochemical properties of the starter), the incubation and manufacturing temperatures under which they are used, and, most recently, in terms of the species of microorganisms present or the number of strains used. These various overlapping classifications address different aspects of the traditional art and the modern science of cheese starters.

Defined and Undefined Cultures

Traditionally, artisan cheesemakers prepared starters without any formal microbiological knowledge. Although there are many variations (some purely regional, others depending on the cheese being made), there are essentially two approaches. One approach relies on incubating milk under selective incubation conditions (without the addition of any culture) to encourage the dominance of temperature and acid-tolerant organisms already present in the milk. The other approach involves using some of a successful product batch (or whey derived from it, often after a selective incubation) as starter for the next batch (see **Table 1**).

Traditional starter preparation methods are still in use for some European regional cheeses, and have been adapted to limited industrial scale. However, industrial-scale cheese production requires starters that give reproducible performance and are free of undesirable organisms. These goals are difficult to achieve using traditional methods. Industrial starters are produced by two different approaches: propagating carefully from preserved, tested archive stocks of good artisanal cultures or the use of defined mixtures of purified, characterized bacterial strains.

Mesophilic and Thermophilic Cultures

Traditional preparation of an undefined starter, using some of a successful product batch (or whey from it) as the starter source for the next batch, results in selective enrichment of microorganisms that survive and multiply under cheesemaking conditions. Thus, traditional starters for any particular cheese contain bacteria suited to the traditional manufacturing process (especially the manufacturing temperature profile) for that cheese. This interrelation also applies in industrial-scale cheesemaking.

Mesophilic cheese cultures grow and produce lactic acid at a moderate temperature, whereas thermophilic cultures function at higher temperatures. There is no universally recognized exact definition of these terms, but the cultures are easy to distinguish in practice. Mesophilic undefined cultures, dominated by strains of *Lactococcus lactis*, have a typical optimum growth

Table 1 Culture types and their preparation

Culture type	*Description*	*Comments*
'Natural' milk cultures	Artisanal. Milk is incubated under conditions that favor the growth of naturally occurring thermophilic lactic acid bacteria, and then used as starter	Low cost. Undefined composition. Highly variable composition and performance. Prone to undesirable contamination; microbiologically hazardous
Back-slopped starter	Artisanal. Use some of a previous product batch as starter	Low cost. Undefined composition. Highly variable composition and performance. Microbiologically hazardous
Whey cultures	Artisanal. Starter is produced by incubating cheese whey under conditions that favor the growth of desirable (typically thermophilic) lactic acid bacteria	Low cost. Undefined composition. Variable composition and performance. With careful handling and quality control testing, whey cultures are used on an industrial scale for making some traditional Italian and Swiss cheese types
Undefined mixed cultures (propagated in cheese factory)	Cultures (typically descended from artisanal cultures with desirable properties) are sequentially subcultured at the cheese factory	Undefined composition. Variable composition and performance, but less variable than whey cultures. With careful handling and some quality control testing these are still in limited use, but have largely been replaced by laboratory-maintained cultures
Undefined mixed cultures (maintained in laboratory)	Cultures (typically descended from artisanal cultures with desirable properties) are preserved and propagated under controlled laboratory conditions. Supplied to the cheese factory in frozen or freeze-dried form	Undefined composition. Variability greatly reduced through maintenance of stable stocks (usually frozen) and production of each batch with minimal sequential subculturing. Used as bulk starter or direct vat inoculum
Defined-strain starters	Laboratory-purified selected strains, free of microbiological contaminants, are preserved and propagated under controlled laboratory conditions, and then blended to give a mixed culture with desired properties. Supplied to the cheese factory in frozen or freeze-dried form	Defined composition, usually of only a small number of strains. This gives a high degree of control over starter performance parameters and product properties, as long as strains are carefully selected and managed. Novel blends with specific properties can be made. Used as bulk starter or direct vat inoculum

temperature of about 30 °C and are capable of acid production (although not necessarily growth) at a cheese cook temperature of 38–40 °C. Some strains (especially *Lc. lactis* subsp. *cremoris*) are more temperature-sensitive than others, and this has been exploited extensively when choosing strains for use in defined-strain cultures for mild Cheddar manufacture.

Cheeses manufactured using a high fermentation temperature (such as Italian and Swiss cheese types with temperatures from about 37 to over 50 °C) typically use undefined mixed starter cultures dominated by thermophilic bacteria with optimum growth temperatures of around 42 °C, mainly *Streptococcus thermophilus* and various *Lactobacillus* species. Other bacteria (e.g., *Lc. lactis*, *Enterococcus faecium*) may also be present at lower numbers. Defined-strain starter cultures for making these cheeses usually contain *Sc. thermophilus* and *Lactobacillus helveticus*.

Matching the Starter to the Product

The primary function of all cheese starter cultures is acidification (metabolism of lactose to lactic acid by lactic acid bacteria). However, other starter properties have important effects on the characteristics of the cheese. For example, the dominant characteristics of Emmental depend on the production of gas and propionic acid by strains of *Propionibacterium*. Gouda relies on gas and diacetyl production by citrate-utilizing (Cit$^+$) *Leuconostoc* and Cit$^+$ *Lc. lactis*. The texture and stretch of Mozzarella are affected by the level of proteolytic activity of starter strains. Proteolysis and amino acid metabolism are precursors of complex Cheddar flavor. For any cheese, the starter (in combination with any added 'adjunct' secondary cultures or adventitious non-starter bacteria) must have the correct microbiological components to bring about the biochemical conversions typical of that cheese.

Traditional cultures (including industrial undefined mixed starters derived from artisanal precursors) typically contain many strains of many microbial species, sometimes including yeasts and molds as well as bacteria. These all contribute biochemically to the complexity (and to the variability) of the final product. Cheeses made using defined-strain starters containing only a few strains of one or two key species (the dominant species of the traditional product) usually have a 'cleaner' flavor and aroma, that is, they are less likely to suffer from flavor defects but may seem bland to some consumers. In response to this issue, users of defined starter cultures are making increasing use of flavor-enhancing adjunct cultures. These cultures, which may themselves be defined or undefined, are added at low levels to the starter or separately to the milk in the vat, leading to enhanced flavor development and increased control over the nature of that flavor.

Choosing Starter Cultures

A starter culture must satisfy three basic criteria for being used in industrial-scale cheese manufacture: reproducible rate of acid production under cheesemaking conditions, predictable characteristics of the cheese produced, and absence of pathogens or spoilage organisms. Artisanal starter preparations are inherently undefined and variable, and pose greater microbiological risks than the use of well-tested cultures propagated by specialist laboratories and culture suppliers. A general idealized scheme for selection and management of starter cultures is presented in **Figure 1**.

Cheesemaking characteristics

Starter cultures are often chosen on a historical basis; that is, they are chosen for use in a particular factory or for a particular product application because they have been used successfully previously. This is especially true of undefined mixed cultures. Defined-strain cultures allow control over the composition and properties of the culture. Examination of key properties of each strain (e.g., genetic or biochemical features as well as growth and acid production characteristics) can lead to rational mixing of strains to form a culture with a desirable set of properties. However, this requires a high level of specialist laboratory testing backed by fundamental research to define the key properties. In the current state of the art, strain selection is guided by a mix of laboratory testing and historical cheesemaking data. Strains are usually subjected to cheesemaking trials before committing to large-scale production.

Sensitivity to bacteriophages

Viruses that multiply by infecting bacteria are known as bacteriophages (or phages) (*see* **Bacteriophage**: Technological Importance in the Diary Industry). The most sophisticated modern cheese factories use air filtration systems to exclude airborne phages and thorough factory sanitation to inactivate phage particles that might be present within the factory. However, the milk used in cheesemaking may contain phages (associated with their host bacteria) and these phages survive pasteurization. Phages can also enter the factory in lysogenic (carrying temperate phages) starter strains or in phage-contaminated starter cultures.

Infection, resulting in the death of infected bacterial cells, leads to a reduced rate of lactic acid production by the starter culture, which may necessitate an extended fermentation or make it difficult to achieve the desired pH and moisture levels in the cheese. Low levels of phage can often be tolerated without effect on cheese production, especially if good vat and pipeline cleaning systems are operated between vat fills and whey from previous vats is not permitted to contaminate a newly filled cheese

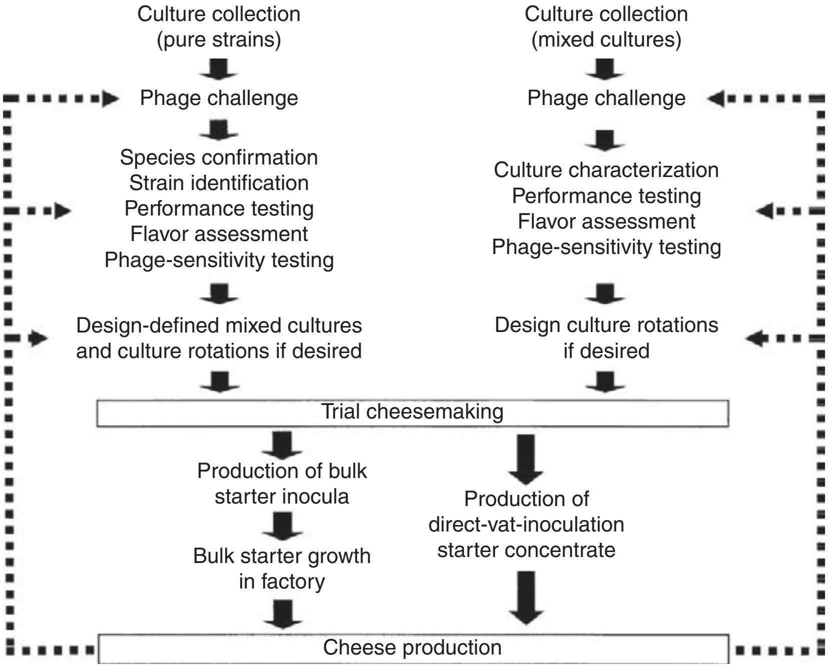
Culture collection
(pure strains)
Phage challenge
Species confirmation
Strain identification
Performance testing
Flavor assessment
Phage-sensitivity testing
Design-defined mixed cultures
and culture rotations if desired
Culture collection
(mixed cultures)
Phage challenge
Culture characterization
Performance testing
Flavor assessment
Phage-sensitivity testing
Design culture rotations
if desired
Trial cheesemaking
Production of bulk
starter inocula
Bulk starter growth
in factory
Production of
direct-vat-inoculation
starter concentrate
Cheese production

Figure 1 A simplified scheme for selection and management of starter cultures. Dotted lines represent feedback (e.g., culture performance information and whey samples for phage testing) from cheese factory to culture supplier.

vat. Most importantly, the starter inoculum into the vat must be free of phage.

When a starter culture containing a mixture of bacterial strains (and/or a mixture of species) is used, it is unlikely that all strains will be infected by the phage present in the cheese factory environment. However, killing of one or more components of the culture will affect acid production, and could also alter the metabolic balance of the culture, which might significantly alter the flavor characteristics of the cheese.

Using the same culture continuously provides ideal conditions for phage numbers to increase to a potentially disruptive level. The most widely used strategy to prevent this is culture rotation. A set of cultures is used in which the cultures (i.e., the strains in the cultures) differ in their phage sensitivity. These cultures are used in rotation (i.e., one after the other). Each culture is used for only a short time (depending on the effectiveness of factory sanitation systems and the virulence of extant phages, this might be just a few cheese vats or it might be a day's production or more) so that phage levels remain relatively low. Regular cleaning procedures will reduce the phage numbers before the next use of each culture.

Before a culture is used in a factory, it should be tested for sensitivity to phages that are already present in the factory environment and it should be ascertained whether strains in the culture are infected by phages that infect any other strains that are likely to be used in that factory. Ideally, no two strains used should share any phage sensitivity, but this is often difficult to achieve.

Selection of phage-resistant variants

A supplier of undefined mixed cultures might have many different cultures to choose from, each with different performance, flavor, or phage-sensitivity characteristics. Cultures with resistance to particular phages can be selected (this is selection in the Darwinian sense) by incubating a mixed culture in the presence of the phage. Only strains resistant to the phage will survive. Selection of phage-resistant strains also takes place within artisanal mixed cultures propagated within the cheese factory and exposed to any phages that may be present. In both cases, the composition and characteristics of the derived culture may be different from the original culture in ways that alter its suitability for cheesemaking.

With defined-strain mixed cultures, it is possible to identify which of the culture component strains is infected by phage. As part of the culture management strategy, this one strain can be replaced by another with similar cheesemaking properties but different phage sensitivity (leaving the rest of the culture unchanged). Alternatively, a natural variant of that strain that is resistant to the phage can be selected. Variants must be checked to ensure that their cheesemaking properties are not impaired.

Some starter strains carry naturally occurring genetic factors (usually genes carried on plasmids) that diminish their sensitivity to phage infection by cleavage (restriction) of phage DNA or by interfering with intracellular phage replication. Strains with these genetic factors are now well known in the species *Lc. lactis*, but relatively little research has been done on phage resistance in other starter species. In many examples, the genes can be transferred between strains by the natural mating process known as conjugation so that starter strains with enhanced (but not absolute) resistance to phage infection can be obtained.

Genetically modified starter strains

Extensive research on the genetics of starter bacteria has made it possible to use the techniques of *in vitro* genetic manipulation to construct strains with particular characteristics such as phage resistance or altered metabolic properties. These genetically modified organisms have been used extensively in laboratory research and have contributed to much of our understanding of the details underlying starter characteristics. Whether and when they will be used in industrial cheesemaking will depend on their usefulness (they must satisfy a need) and cost-effectiveness (the research required to generate a strain can be very expensive) as much as on regulatory approval and market acceptance (*see* **Lactic Acid Bacteria**: Genomics, Genetic Engineering).

Starter Delivery Systems

The conditions under which starter cultures are preserved, propagated, and distributed for industrial cheesemaking are chosen to maximize consistency of starter composition and acid-producing activity, and to minimize the opportunities for phage infection or other microbial contamination. Primary stocks of mixed cultures and single strains are typically stored deep-frozen (expensive, but most strains survive well) or freeze-dried (cheap long-term storage, although some strains do not survive the process well) on a laboratory scale. Propagation is standardized (each culture producer will have their own method, but consistency is important) and the number of subcultures is kept to a minimum. This is particularly important for undefined cultures in which the relative cell numbers of different component strains might change (some strains might even be lost) if conditions are not controlled carefully.

The size of the starter culture inoculum required to make cheese will depend on the manufacturing process (i.e., the cheese type), on milk quality (i.e., milk composition and inhibitory substances), and on the inherent acid-producing activity of the starter culture, but may be as high as 10^9–10^{10} cells for each liter of cheese milk. Laboratory stock cultures are used as the initial inoculum for sequential growth of larger and larger cultures until a sufficient inoculum for cheese manufacture is obtained.

Two alternative systems are employed for delivering this inoculum to the vat: growth of a large volume of fresh culture (bulk starter) in the cheese factory or prior preparation of a culture concentrate (frozen or freeze-dried culture for direct inoculation of the cheese vat) by an external culture supplier.

Bulk Starter Cultures

The inoculum for bulk starter production can be prepared in-house, but it is now much more common to use an inoculum (in frozen or freeze-dried form) prepared by an external specialist culture supplier. The size of the bulk starter growth vessel will depend on factory requirements, but 10 000 l vessels are not uncommon in large factories. Various growth media are used, including milk (full-fat or skimmed, fresh or reconstituted, with or without growth-enhancing supplements) and a range of whey-based media with supplements (e.g., yeast extract) and buffering agents. Media formulated to minimize opportunities for phage infection during bulk starter growth are typically whey-based and have added phosphate or citrate, which will sequester calcium (infection by most phages of lactic acid bacteria requires free calcium ions). The growth medium is sterilized by heat treatment, either in the vessel or through an external ultra-high temperature (UHT) heat exchanger.

The lactic acid produced during growth of the bulk starter culture lowers the pH of the medium and is a major factor in limiting the bacterial cell numbers obtained. Greater cell numbers can be reached if the pH is held higher during at least part of the culture growth. This can be achieved using 'internal' pH control (addition of buffering agents such as phosphate or carbonate to the medium) or 'external' pH control (manual or automated addition of alkali such as sodium hydroxide, potassium hydroxide, or ammonium hydroxide) to raise the pH of the medium during growth.

Reliable preparation of consistent bulk starter is beyond the technical and economic reach of many small-scale cheesemakers. Modern bulk starter growth units are fully enclosed stainless steel vessels with thorough cleaning-in-place systems, temperature control (heating and cooling via a water jacket), pH control (pH monitoring and alkali dosing systems), and an internal positive pressure of sterile (filtered and/or UV-treated) air to exclude entry of airborne contaminants (bacteria and phages). To prevent entry of phages from the cheese factory, starter growth should take place in a room with its own treated air supply and isolated from the cheese vats and any whey handling systems.

Growth temperature and optimum pH will depend on the starter being used. Typically, after 16–20 h of growth, the culture is chilled using the cooling jacket or a heat exchanger. The culture will retain good activity for at least 24–48 h at 4 °C, as long as the pH is not too low (typically pH 5.0–5.2 for mesophilic cultures; thermophiles will generally tolerate more acidic conditions).

If desired, this holding time can be used to check the activity and purity of the culture before use, or one bulk starter batch can be used for cheese production on sequential days. The starter is delivered to the cheese vats via calibrated dosing pumps or through a weight-based dosing system.

Cultures for Direct Vat Inoculation

Cultures for direct vat inoculation (DVI) are grown in much the same way as bulk starter cultures, except that they are grown by a remote culture supplier (this guarantees isolation from the cheese factory), concentrated (typically by centrifugation), and then frozen or freeze-dried for storage and transportation to the cheese factory. The concentrated culture is added directly to the cheese vat or, in some applications, to a holding tank of chilled medium for later dosing to the vats.

DVI is the obvious choice for factories that lack the specialized facilities and trained personnel necessary for reliable in-house bulk starter preparation. The external supplier checks culture purity and activity before delivery to the cheese factory. In some cases, the cultures as supplied are blended from separately grown components, allowing defined combinations that are difficult to achieve by growth as a mixed culture. Secondary (adjunct) cultures, which are used to control or enhance flavor, aroma, and eye formation in cheese, are ideally suited to be supplied in DVI form.

Commercial frozen and freeze-dried cultures

Frozen DVI cultures have historically suffered from two main drawbacks: bulkiness and a short shelf life. The advent of pelletized frozen cultures has improved the convenience of culture blending, transport, and use.

Freeze-dried cultures are more expensive to produce but are more compact and convenient to store. Shelf life at ambient temperature is typically more than 6 months, but can be much longer if the cultures are stored refrigerated or frozen. However, the bacterial physiology associated with freeze-drying is not well understood and the freeze-drying process is not applicable with the same efficiency to all strains, even within the same species. For example, there are only a few strains of *Lb. delbrueckii* subsp. *bulgaricus* that can be freeze-dried with high levels of survival and activity. Because the number of strains that can be freeze-dried economically is restricted, it can become difficult to maintain a proper bacteriophage control rotation.

All types of cheese can be made using bulk or DVI starter cultures, but DVI starters can be relatively expensive for the manufacture of cheeses that require rapid acid production (e.g., Cheddar). This cost has been reduced in recent years by the use of blends of *Lc. lactis* and *Sc. thermophilus* instead of pure *Lc. lactis*, but this is not without impact on cheese flavor development. In general, larger cheese factories are more likely to find a long-term cost advantage in bulk starters, but the relative costs of bulk and DVI starters will differ depending on the scale of cheese production and the mix of cheese varieties being produced.

See also: **Bacteriophage**: Technological Importance in the Diary Industry. **Cheese**: Biochemistry of Cheese Ripening; Microbiology of Cheese; Overview; Starter Cultures: Specific Properties. **Lactic Acid Bacteria**: Genomics, Genetic Engineering; *Lactobacillus* spp.: General Characteristics; *Lactococcus lactis*; *Streptococcus thermophilus*; Taxonomy and Biodiversity.

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Starter Cultures: Specific Properties

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Introduction

The bacteria used in cheese starter cultures perform one or more of the following functions in cheese:

1. lactose metabolism, producing lactic acid, which is central to conventional cheese manufacture and also minimizes the growth of spoilage and pathogenic organisms in the cheese;
2. production of a wide range of enzymes and metabolic products that play an active role in generating a flavor profile and enhance organoleptic properties during maturation of the cheese;
3. production of other antimicrobial substances that reduce the risk of survival and proliferation of pathogens; and
4. enhancement of the health-promoting properties of the cheese.

These properties have a significant impact on cheesemaking and flavor development. For instance, the rate and amount of lactic acid produced during cheesemaking will determine the amount of moisture lost, the final pH, and residual lactose in the curd, which in turn will have a strong influence on the maturation rate and the final flavor profile of the cheese. The biochemistry, physiology, and genetics of some of the major starter bacteria will be discussed in this article.

Starter Species Involved in Cheesemaking

Taxonomy of the bacteria involved in cheesemaking has made steady and meaningful progress since the mid-1980s (*see* **Lactic Acid Bacteria**: Taxonomy and Biodiversity). The transfer of *Streptococcus lactis* and *Streptococcus cremoris* to the new genus *Lactococcus* and the reorganization of lactobacilli are notable and significant steps. The microflora of cheese is a mix of the added culture, the flora of the cheese milk (especially when unpasteurized milk is used), and the flora acquired from the manufacturing environment (*see* **Cheese**: Microbiology of Cheese). Current trends in industrial cheesemaking are toward achieving greater control over cheese microflora by using pasteurized milk and culture blends designed for customized flavor control. Thus, the bacterial flora of cheese may comprise the following:

1. Starter (primary) culture, added mainly for its acidification properties but often with significant impact on cheese flavor (*see* **Cheese**: Starter Cultures: General Aspects).
2. Adjunct (secondary) cultures, added mainly for their effect on flavor, eye formation, and so on (*see* **Cheese**: Non-Starter Lactic Acid Bacteria; Secondary Cultures; Starter Cultures: General Aspects. **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development).
3. Adventitious organisms (*see* **Cheese**: Microbiology of Cheese; Non-Starter Lactic Acid Bacteria).

Various authors and culture suppliers use these terms in somewhat different ways, depending on culture composition and whether or not this composition is microbiologically defined. Many cultures used in cheesemaking combine both the primary and secondary aspects, and so in this article they are collectively described as starter cultures.

Bacteria belonging to the genera *Lactococcus*, *Lactobacillus*, and *Streptococcus* are the most commonly used microorganisms in starter cultures. These lactic acid bacteria perform the primary acidification of the milk and participate in the maturation process via production of flavor compounds or their chemical precursors, either directly by cellular metabolism or indirectly by the release of enzymes.

An overview of the bacterial species commonly used in modern cheesemaking is presented in **Table 1**. *Leuconostoc* (e.g., typical of Gouda and Edam flora) and *Propionibacterium* (Gruyère and Emmental) are well known for their role in creating characteristic flavors. Various lactobacilli are found in ripened cheese, and their use as adjunct cultures is increasing because they can offer some control in specific flavor profile development. Brevibacteria are important in many smear-ripened cheeses. Enterococci occur in artisanal starters but are not widely used in defined cultures.

Starter Biochemistry

Flavor and texture development of cheese are dependent on biochemical degradation of milk components including lactose, lactate, citrate, milk fat, and caseins to a large

Table 1 Main bacteria associated with cheesemaking

Species/subspecies[a]	*Morphology, main uses, and other comments*[b]
Lactococcus	
Lc. lactis ssp. *lactis*	Cocci (spherical or ovoid) in pairs and chains; 0.5–1.2 × 0.5–1.5 μm. Mesophilic starter for many cheese types.
Lc. lactis ssp. *lactis* biovar diacetylactis	Cocci (spherical or ovoid) in pairs and chains. Gouda, Edam, sour cream, lactic butter. No longer regarded as a separate species or subspecies. Capable of citrate metabolism.
Lc. lactis ssp. *cremoris*	Cocci (spherical or ovoid) in pairs and chains. Mesophilic starter for many cheese types.
Streptococcus	
Sc. thermophilus	Cocci (spherical, ovoid, or elongated) in pairs and chains; 0.5–2.0 μm diameter. Thermophilic starter for yogurt and many cheese types, especially hard and semihard high-cook cheeses.
Lactobacillus	
Lb. acidophilus	Rods. Probiotic adjunct in cheese and yogurt. Homofermentative.
Lb. delbrueckii ssp. *bulgaricus*	Rods; 0.6 × 4.5–10 μm. Thermophilic starter for yogurt and many cheese types, especially hard and semihard high-cook cheeses. Homofermentative.
Lb. delbrueckii ssp. *lactis*	Rods; 0.5 × 5 μm. Fermented milks, high-cook cheeses. Homofermentative.
Lb. helveticus	Rods; 0.8–1.0 × 4.0–5.0 μm. Thermophilic starter for fermented milks and many cheese types, especially hard and semihard high-cook cheeses. Homofermentative.
Lb. paracasei	Rods; 0.4–0.6 × 1.0–1.5 μm. Cheese ripening adjunct culture. Facultatively heterofermentative.
Lb. rhamnosus	Rods; 0.6–0.8 × 1.0 μm. Cheese ripening adjunct culture. Facultatively heterofermentative.
Leuconostoc	
Le. mesenteriodes ssp. *cremoris*	Cocci (spherical or ovoid) in pairs and chains; 0.5–0.7 × 0.7–1.2 μm. Mesophilic. Gouda, Edam, fresh cheese, lactic butter, sour cream.
Enterococcus	
E. faecium	Cocci (spherical or ovoid) in pairs and short chains; 0.6–2.0 × 0.6–2.5 μm. Raw milk cheese.
E. faecalis	Cocci (spherical or ovoid) in pairs and short chains. Raw milk cheese.
Brevibacterium	
B. linens	Irregular rods; single cells, pairs, and in V shape; 0.6–1.2 × 1.5–6 μm. Camembert, Stilton, Limburger.
Propionibacterium	
Pr. acidipropionici	Pleomorphic rods, often club shaped or branched; 0.5–0.8 × 1.5 μm. Gruyère, Emmental.
Pr. freudenreichii ssp. *shermanii*	Pleomorphic rods. Gruyère, Emmental.

[a] Taxonomic information can be found in the article **Lactic Acid Bacteria**: Taxonomy and Biodiversity.

[b] Sizes are indicative only, and may vary greatly depending on strain and growth conditions. Data from Holt JG, Kreig NR, Sneath PHA, Staley JT, and Williams ST (1994) *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore, MD: Williams and Wilkins and from Dellaglio F, de Roissart H, Torriani S, Curk MC, and Janssens D (1994) Caractéristiques générales des bactéries lactiques. In: de Roissart H and Luquet FM (eds.) *Bactéries Lactiques, Aspects Fondamentaux et Technologiques*, Vol. 1, pp. 25–116. Uriage, France: Lorica.

range of chemical compounds. Many compounds have been implicated in cheese flavor, but no individual compound is totally responsible. The characteristic cheese flavor appears to be dependent on many compounds present in the correct ratios and concentrations, a concept often referred to as the 'component balance theory'.

Carbohydrate Metabolism

A summary of the principal pathways in lactic acid bacteria for the metabolism of lactose (the major milk sugar) and citric acid is shown in **Figure 1**. The transport of lactose into the cell requires energy. In the lactococci, this energy is sourced via energy-rich phosphoenolpyruvate (PEP), an intermediate of the glycolytic pathway. This is part of a transport mechanism, referred to as the phosphoenolpyruvate phosphotransferase system (PEP-PTS), in which the lactose is phosphorylated as it is transported across the cell membrane. Once inside the cell, phosphorylated lactose is hydrolyzed by the enzyme phospho-β-galactosidase (EC 3.2.1.85) to glucose and galactose-6-phosphate.

In other dairy starter bacteria, including *Streptococcus thermophilus*, leuconostocs, lactobacilli, and the bifidobacteria, lactose transport appears to be via a specific protein (a permease) that translocates the lactose into the cell without modification although in many of these organisms the exact nature of the system used is still unclear. The lactose is then hydrolyzed by the enzyme β-galactosidase (EC 3.2.1.23) to glucose and galactose. The energy required for this system is provided in the form of proton motive force (PMF) or other transmembrane potential effects. This usually involves the simultaneous transport of a biochemical moiety either into or out of the cell. In a number of organisms (*Sc. thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lb. delbrueckii* ssp. *lactis*) that are noted for their inability to metabolize galactose, the molecule simultaneously exported from the cell is galactose.

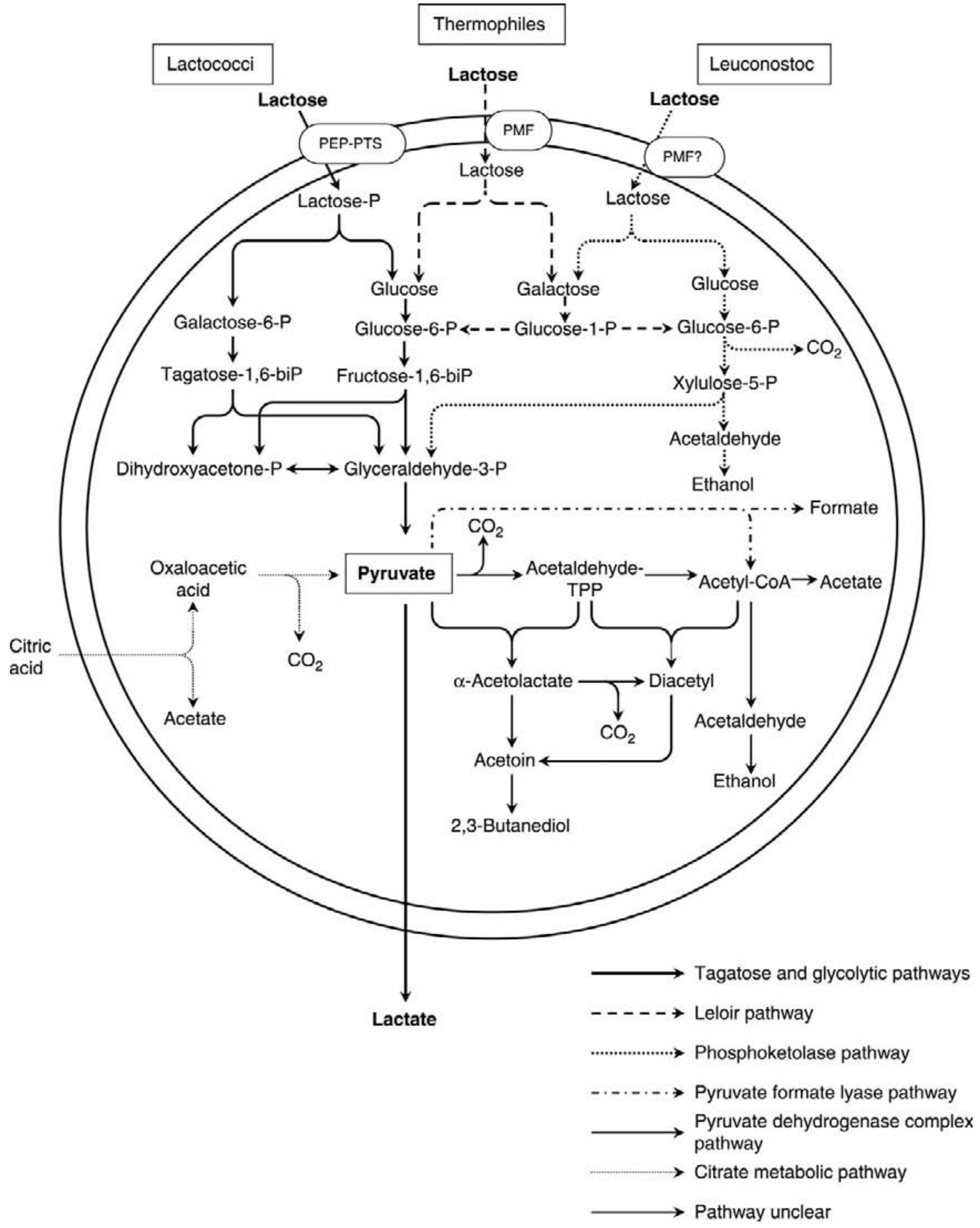
Thermophiles
Lactococci
Leuconostoc
Lactose
Lactose
Lactose
PEP-PTS
PMF
PMF?
Lactose
Lactose-P
Lactose
Glucose
Galactose
Glucose
Galactose-6-P
Glucose-6-P
Glucose-1-P
Glucose-6-P
CO_2
Tagatose-1,6-biP
Fructose-1,6-biP
Xylulose-5-P
Acetaldehyde
Dihydroxyacetone-P
Glyceraldehyde-3-P
Ethanol
Formate
CO_2
Oxaloacetic acid
Pyruvate
Acetaldehyde-TPP
Acetyl-CoA
Acetate
Citric acid
CO_2
α-Acetolactate
Diacetyl
Acetate
CO_2
Acetaldehyde
Acetoin
Ethanol
2,3-Butanediol
Lactate
Tagatose and glycolytic pathways
Leloir pathway
Phosphoketolase pathway
Pyruvate formate lyase pathway
Pyruvate dehydrogenase complex pathway
Citrate metabolic pathway
Pathway unclear

Figure 1 General pathways for carbohydrate catabolism by lactic acid bacteria. PEP-PTS, phosphoenolpyruvate phosphotransferase system; PMF, proton motive force or other transmembrane potential.

Under normal growth conditions in milk, lactococci metabolize galactose-6-phosphate and glucose via the tagatose and glycolytic pathways, respectively, to pyruvate, the vast majority of which is converted to lactic acid by the enzyme lactate dehydrogenase (EC 1.1.1.27). However, where lactococci are fermenting galactose or lactose at growth-limiting rates, products other than lactic acid can be formed from pyruvate. The enzyme pyruvate formate lyase (EC 2.3.1.54) is able to convert pyruvate to formate, acetate, acetaldehyde, and ethanol under anaerobic conditions and at high pH (>7.0). Under aerobic conditions and at pH 5.5–6.5, pyruvate can be converted to acetate, acetaldehyde, ethanol, and the minor products acetoin, diacetyl and 2,3-butanediol via the multienzyme pyruvate dehydrogenase complex.

Starter cultures that use a permease system to transport lactose into the cell also metabolize the glucose moiety via the glycolytic pathway to lactic acid. The galactose portion is either excreted, as in strains of *Sc. thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Lb. delbrueckii* ssp. *lactis*, or metabolized via the Leloir pathway present in *Lactobacillus helveticus* and a few strains of *Lb. delbrueckii* ssp. *lactis* to glucose-6-phosphate and, ultimately, lactic acid. In *Leuconostoc*, the glucose portion of lactose is metabolized to carbon dioxide (CO_2), ethanol, and lactic acid via the phosphoketolase pathway, while galactose appears to be converted to glucose-6-phosphate and, finally, lactic acid by the Leloir pathway.

Citric acid metabolism occurs in the citrate-fermenting strains of *Lc. lactis* ssp. *lactis* and *Leuconostoc*. In lactococci, citrate is converted to acetate, CO_2, and pyruvate, which is then converted to acetate, diacetyl, acetoin, 2,3-butanediol, and CO_2. In *Leuconostoc*, the pyruvate produced from citrate is converted to lactate although at low pH and in the absence of glucose (or lactose) *Leuconostoc* will produce diacetyl and acetoin (*see* **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria). Acetate is also formed via the heterofermentative metabolism of lactose during cometabolism with citrate.

Lipolysis

The limited enzymatic hydrolysis of milk fat triacylglycerides to free fatty acids and glycerol, monoacylglycerides, or diacylglycerides is essential for the development of the typical flavor in cheese. The enzymes involved, lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1), originate from a number of sources, including the milk, starter bacteria, non-starter bacteria, and, in certain cheeses, the coagulant. Esterases are arbitrarily defined as the enzymes that hydrolyze substrates (soluble esters of short-chain fatty acids) in solution. Lipases are arbitrarily defined as the enzymes preferentially hydrolyzing acylglycerol esters in emulsion. Both are present in starter bacteria and appear to be intracellular. In Cheddar- and Dutch-type cheeses made from pasteurized milk, the starter bacteria appear to be the prime lipolytic agents. However, most starter cultures do not possess high lipolytic activity although should they grow to high numbers and survive in cheese for an extended period they can impact the degradation of fats in cheese. Free fatty acids can contribute directly to cheese flavor; react with alcohols or free sulfhydryl groups to form esters and thioesters, respectively; or act as precursors to a number of other flavor compounds, such as lactones.

Protein Degradation

As approximately 90% of the growth of lactococci in milk is supported by casein-derived peptides, these organisms possess a complex proteolytic system (**Figure 2**). The system consists of one cell envelope-associated proteinase, which hydrolyzes casein into oligopeptides (4–8 amino acid residues), an ATP-dependent oligopeptide transport system, di/tripeptide transport systems for hydrophilic and hydrophobic substrates, and at least 10 amino acid transport systems. Located within the cell is an array of peptidases with both broad and narrow specificities that degrade the peptides to amino acids, which are utilized for the synthesis of the cell's own proteins.

In maturing cheese, primary starter bacteria such as *Lc. lactis* typically decrease slowly in numbers, while many of their proteolytic enzymes are still active and can be released into the cheese medium as the cells lyse. It is these enzymes, particularly the peptidases, along with other proteinases and peptidases (originating from the milk, the coagulant, and the non-starter lactic acid bacteria), that play a significant role in releasing amino acids that are in turn the precursors for specific cheese flavor compounds.

The catabolism of amino acids by starter cultures generally involves an initial transamination reaction catalyzed by an aromatic or branched-chain aminotransferase that transfers the α-amino group of the amino acid to a keto acid acceptor, usually α-ketoglutarate. In maturing cheese, a limiting factor of the transamination reaction is the supply of α-ketoglutarate although this can experimentally be overcome either by adding α-ketoglutarate to the cheese or by utilizing starter culture strains that have glutamate dehydrogenase activity. This allows the cultures to form α-ketoglutarate from glutamate, which is an abundant amino acid in cheese. Generally, glutamate dehydrogenase appears to be widespread in *Lb. plantarum* (an adventitious non-starter bacterium) and *Sc. thermophilus*; however, in other lactic acid bacteria, including *L. lactis*, the presence of the enzyme is a variable trait.

The α-keto acids formed from transamination can then be converted into hydroxyl acids, alcohols, aldehydes, carboxylic acids, and esters by a range of other enzymes. For example, methionine is transaminated to form 4-methylthio-2-oxobutyrate, which appears to be degraded either nonenzymically or enzymically to methanethiol. Methanethiol, in turn, is the precursor to a number of potential flavor compounds such as dimethyl sulfide, dimethyl disulfide, and *S*-methylthioesters. Methionine can also be converted to α-ketobutyrate and methanethiol via a second minor pathway catalyzed by cystathionine β-lyase (EC 4.4.1.8) and cystathionine γ-lyase (EC 4.4.1.1). (*see* **Cheese**: Cheese Flavor for further information.)

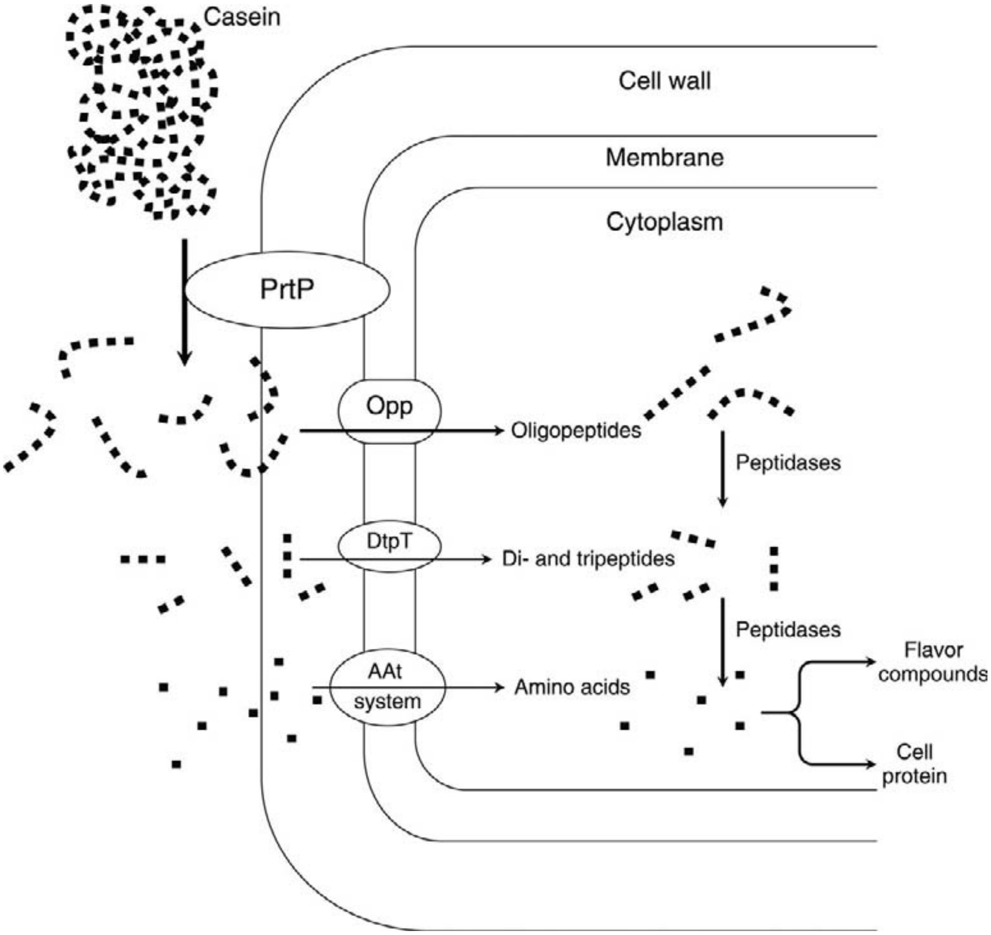

Figure 2 General pathways for casein catabolism by lactic acid bacteria. AAt, amino acid transport system; DtpT, di/tripeptide transporter; Opp, oligopeptide transport system; PrtP, cell wall–bound extracellular proteinase.

Physiological Factors Affecting Starter Activity during Cheesemaking

The growth of starter bacteria in milk during cheesemaking and in other fermented milk products is dependent on physiological factors, which must be well understood by the manufacturer to properly control the entire process, including maturation.

Milk itself is not a uniform substrate, being a changeable metabolic product of the cow and dependent on the breed of the cow, its individual genetic traits, the stage of lactation, the adequacy of the feed, and the climate and environment. The bulking of large volumes of milk at the manufacturing site in large silos (often more than 300 000 l) has been a key factor in providing some uniformity in milk composition and hence consistency in cheese make and composition. The factors relating to the cheesemaking process that affect starters during growth in milk must be considered in this context to allow a thorough understanding of the concomitant physical changes occurring in the curd.

Setting Temperature

The setting temperature of the cheese milk and (for many cheese types) its rate of increase to reach the cooking temperature are very important parameters in the cheesemaking process. The setting temperature usually matches the optimum growth temperature of the culture used (about 32 °C for mesophiles and about 37 °C for thermophiles). This will allow the starter bacteria to reach their exponential growth phase in the shortest possible time and hence will determine the overall length of the cheesemaking process. The cooking temperature is usually reached over a 30–40 min period. Too rapid an increase can affect starter activity in the postdrain period; hence, a gradual increase in temperature is normally adopted.

Lactose Metabolism

Metabolism of lactose (producing lactic acid) by the starter bacteria is another crucial factor that affects the syneresis of the curd, the level of moisture in the curd, and the level of residual lactose in the curd after pressing. During most cheesemaking processes, the initial stage is set at an optimum temperature for the starter bacteria so that lactose metabolism and lactic acid production rate are at the maximum. During this time, the coagulum is usually cut into small cubes and heat is gradually applied to just beyond the optimum when sometimes growth is uncoupled but lactose metabolism is still proceeding. The bulk of the whey is usually removed at that stage, while lactic acid production continues within the aqueous phase of the curd particles. This will lead to the accumulation of high levels of lactate, which in turn affects the growth of the starter bacteria. Different starter species have different optimal pH (e.g., pH 5.5–6.2 for lactobacilli and 6.3–6.5 for lactococci and leuconostocs). The basis for the different pH optima that can be tolerated by the starter stems from the internal cell pH sustainable by a particular strain or species. Lactobacilli can tolerate a significantly more acidic cytoplasm pH of 4.4 (which they can maintain if the external pH falls to 3.4) than tolerated by lactococci (minimum cytoplasmic pH of 5.7 at an external pH of 4.5).

Effect of Salting

Addition of salt (NaCl), a common practice in most cheeses, will also affect the growth of the starter bacteria. Most lactic acid bacteria are partially or fully inhibited by a level of NaCl higher than 5%. However, different strains have different salt tolerances; hence, this criterion in starter selection is important to ensure that cheese pH can be controlled after the cheese has been made. Starter bacteria in cheese salted by direct addition of salt to the curd (dry salting) will encounter a rapid rise in salt concentration with the resultant inhibition of acid production. By contrast, cheese salted by immersion in brine will take a relatively longer time to control the acid development. Judicious selection of starter strains with different responses to pH and salt can therefore be used to control the cheese pH that will be obtained after maturation.

Starter Cultures and Cheese Flavor

Starter cultures are one of the principal ripening agents in cheese along with natural milk enzymes, the coagulant, secondary cultures, and adventitious non-starter bacteria. The combined action of these agents on milk proteins is probably the most important biochemical event in cheese ripening leading to the changes in texture and the development of flavor. In this system, the major role of starter cultures is the degradation of peptides generated by the coagulant to small peptides and amino acids. Starter cultures are also capable of degrading caseins and converting amino acids to a range of flavor compounds. To carry out this function, particularly in relation to protein metabolism, starter cultures rely on a broad range of enzymes with varying activities. However, as many of these enzymes are intracellular, flavor development in maturing cheese also depends on the release of the enzymes from starter cultures into the cheese matrix through cell lysis.

Bitterness

During cheese maturation, a bitter flavor defect can occur, associated mainly with the production (usually by the coagulant but with some contribution from the starter) of short bitter peptides containing predominantly hydrophobic amino acid residues. The extent to which these bitter peptides accumulate is dependent on a balance between their production by the coagulant and their degradation by various peptidases. This in turn is influenced by factors controlling retention of the coagulant in the cheese during manufacture (type of coagulant, drain pH, cooking temperature) and the environmental conditions present in the cheese that affect enzyme activity (salt concentration, ionic strength, pH, temperature). One of the more successful strategies to counteract the defect involves the use of lactic acid bacteria with high peptidase activities, particularly Pep N. However, within starter cultures there is a great strain-to-strain diversity in peptidase activities and consequently there has been considerable interest in the development of miniaturized high-throughput screening assays for the determination of peptidase activity to select starter cultures with increased debittering potential. Such assays can also be used to characterize starter cultures for a range of other enzymes associated with the development of cheese flavor.

Autolysis

As starter culture peptidases and other ripening enzymes are mainly located intracellularly, the rate at which the cells lyse and release their enzymes into the cheese matrix is an important characteristic for both general protein degradation and also the control of bitterness. Autolysis results from the enzymatic degradation of the bacterial cell wall by indigenous peptidoglycan hydrolases released into the growth medium although it is still unclear how the process is controlled in the cell. The process is highly strain dependent and is also influenced by factors such as the nutrient status of the growth medium and environmental conditions. For example, cultures grown under pH-controlled conditions are more sensitive to lysis than cells grown under acidifying conditions, and during cheese maturation increasing salt-in-moisture levels, decreasing pH, and higher maturation temperatures influence the rate at which starter cultures lyse.

Generally, in maturing cheese there is a positive relationship between the extent of starter culture autolysis and the flavor forming reactions involving proteolysis and lipolysis. Consequently, various screening assays using buffers or model cheese and milk solutions have been proposed to select highly autolytic strains for use in cheese manufacture. The extent of lysis can be assessed by the enumeration of viable cells on selective media, by the determination of specific intracellular enzyme activities, or simply by monitoring the decrease in absorbance over time of starter cultures suspended in a buffer. However, even with these screening procedures, it is still difficult to predict the extent to which starter cultures will lyse in cheese, and there remain many questions regarding the mechanisms involved, autolysis control in cheese, and its impact on cheese ripening.

Accelerated Ripening and Attenuated Starter Cultures

Cheese maturation under controlled conditions is a slow and expensive process; therefore, there is considerable interest in processes that can accelerate cheese ripening. One of the approaches has been to increase the level of starter culture enzymes using attenuated starters (*see* **Cheese**: Accelerated Cheese Ripening). Attenuated starter cultures are unable to produce lactic acid during cheese manufacture but still retain the activity of enzymes associated with the production of flavor compounds. Attenuated starter cultures are produced by heat shocking, freeze/thawing, lysozyme treatment, and spray or freeze drying. Naturally occurring and easily isolated lactose-negative starter culture variants that are unable to contribute to acid production, but still retain an active proteolytic (peptidolytic) system, can also be utilized.

The advantages of attenuated starter cultures over other accelerated ripening approaches are that they are mostly retained in the cheese when added with the normal starter culture, they contain a wide range of enzymes, and they are subject to few legal barriers. Furthermore, the addition of attenuated starter cultures to cheese milk has a largely positive effect, with the reduction of bitterness being the most frequently reported observation.

Starter Genetics and Genomics

The properties of starter bacteria are, of course, dictated by their genomes. Little was known about the genetics of starter bacteria until the emergence of the techniques for DNA analysis. Examination of plasmid-encoded traits began in the 1970s, followed by studies (mostly using recombinant DNA techniques) on chromosomal genes, comparison of strains using methods such as pulsed-field gel electrophoresis of genomic restriction fragments, and, later, whole-genome sequence analysis and gene expression studies. The continual refinement of the taxonomy of lactic acid bacteria in recent decades has been largely on the basis of DNA hybridization studies and, more recently, comparative analysis of homologous gene sequences.

Genome sequences are publicly available for an increasing number of species, though it should be noted that often the genome sequence of only one strain of each species is known. As sequencing costs fall, it is becoming increasingly feasible for private companies (rather than publicly funded collaborations) to determine the partial or complete genome sequences of strains of commercial interest. However, not all this information enters public databases.

Chromosome sizes vary for different species, in the approximate range of 1.7–3.4 Mbp (megabase pairs), and variation is also seen within species. A larger genome size generally indicates a greater number of genes and, therefore, a broader range of biochemical and physiological responses available to the organism.

Genome sequences and comparative genome hybridization analyses can be used to infer the total gene complement of a bacterium. Though the functions of many of the inferred genes are not known, it is possible to predict the enzyme activities and metabolic pathways that the bacterium is likely to express, and to hypothesize the influence that the bacterium might have on cheese flavor. Sequence information can also be used to design microarray systems for use in gene expression studies. Prediction of properties such as growth rate, responses to temperature changes and salt addition, or sensitivity to bacteriophages is currently limited due to insufficient knowledge of the mechanisms involved.

Plasmids

Plasmids are relatively small, usually circular, DNA molecules. Most plasmids that have been found in lactic acid bacteria are cryptic, that is, their presence or absence has no apparent effect on growth, survival, or other observed properties of the bacterium. Plasmids are remarkably common in strains of *Lc. lactis*. Most strains have several plasmids; sizes range from about 2 to over 100 kbp. Some of these plasmids are important for the dairy exploitation of lactococcal starters, because they carry the genes necessary for various properties, including lactose transport and metabolism, production of the major cell-surface proteinases, citrate transport, production of and resistance to bacteriocins, and enhanced resistance to bacteriophage infection through DNA restriction/modification systems and 'abortive infection' systems of generally unknown mechanism. Though less well studied, it seems that a similar range of properties is encoded by plasmids in at least some strains of various species of *Lactobacillus*. Plasmid genes associated with lactose metabolism, citrate metabolism, and bacteriocin production have been reported in strains of *Leuconostoc*. However, plasmids are rare in *Lb. delbrueckii* ssp. *bulgaricus*. Also, most strains of *Sc. thermophilus* have no plasmids, and most of the plasmids that have been studied are cryptic. Other bacteria associated with dairy products (e.g., *Bifidobacterium*, *Propionibacterium*, *Enterococcus*) also have plasmids, and most are cryptic.

DNA rearrangements (deletion of part of a plasmid or recombination events between plasmid molecules or plasmid and chromosome) and sequence mutations result in plasmid changes that can alter the genes carried or the way in which the genes are expressed. Rearrangements via DNA recombination between plasmids carrying related DNA sequences (replication regions, repeated sequences, or other genes) are not uncommon.

Plasmids, especially large plasmids that usually exist as only one or a few plasmid molecules per bacterial cell, are sometimes lost during cell division. This is usually a low-frequency event but, with some plasmids in some strains (e.g., the proteinase plasmid in some lactococcal starters), causes an observable rate of plasmid loss that results in significant changes in the properties of a culture.

Genetic Improvement of Starter Strains

Conjugal transfer of plasmids and transposons (especially those carrying phage resistance genes) via natural mating of strains has been used to enhance the industrial usefulness of lactococcal strains. It is also possible to introduce plasmids into strains using membrane permeabilization techniques, such as electroporation. Development of *in vitro* genetic manipulation techniques has also made it possible to construct strains with particular modified characteristics. These strains have been very useful in laboratory studies to define desirable starter properties for cheesemaking. Further information can be found in **Lactic Acid Bacteria**: Genomics, Genetic Engineering.

See also: **Bacteriophage**: Technological Importance in the Diary Industry. **Cheese**: Accelerated Cheese Ripening; Cheese Flavor; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Secondary Cultures; Starter Cultures: General Aspects. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Genomics, Genetic Engineering; Lactic Acid Bacteria in Flavor Development; Taxonomy and Biodiversity.

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Secondary Cultures

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Introduction

Secondary cheese cultures may be defined as those cultures used for the manufacture of cheese and their principal function is to develop and control the flavor, color, and texture of the cheese. The growth and development of secondary cheese cultures is normally preceded by the fermentation of lactose to lactate by the primary cheese starter culture, which consists of mesophilic or thermophilic lactic acid bacteria. The contribution of secondary cheese cultures to acidification of milk is limited or nonexistent, due to their aerobic metabolism or inability to utilize lactose. The main secondary cheese cultures and their respective roles in cheese ripening are summarized in **Table 1**. For Camembert cheese, in addition to the primary cheese starter culture, a secondary culture consisting of *Penicillium camemberti* is used. The metabolic activities of *P. camemberti* have a significant effect on the final flavor, color, and texture of the ripened cheese. In contrast, for Cheddar cheese, no secondary cheese culture is normally employed, and flavor generation is dependent on the metabolic activity and enzyme complement of the primary cheese starter culture and on adventitious non-starter lactic acid bacteria (NSLAB). Cheese varieties such as Edam and Gouda are manufactured using a starter culture consisting of homofermentative lactococci, citrate-fermenting lactococci, and *Leuconostoc* spp. Despite the fact that the citrate-fermenting lactococci and the *Leuconostoc* spp. produce significant amounts of CO_2, diacetyl, acetoin, acetate, ethanol, and butanediol, such cultures are considered as primary cheese starter cultures, rather than secondary cultures, due to the fact that they also ferment lactose to lactate, and contribute directly to the acidification of the milk.

Secondary cheese cultures are used in cheese manufacture due to their distinctly different and unique physiological or biochemical properties that are either absent or severely limited in the primary cheese starter culture. These properties may include, for example, halotolerance, growth at low pH, lactate utilization, carbon dioxide formation, proteolytic/peptidolytic activities, lipolytic/esterolytic activities, or amino acid catabolic activities. Secondary cheese cultures consist of a variety of species of yeasts, molds, and bacteria. During the early stages of ripening, the surface microflora of mold surface-ripened cheeses, such as Camembert or Brie, and bacterial surface-ripened cheeses, such as Limburger or Saint Paulin, may be very similar, but at the end of the ripening period, molds, such as *P. camemberti*, are the dominant flora found on the surface of the mold surface-ripened cheeses, while bacteria, such as *Brevibacterium* spp., *Arthrobacter* spp., *Micrococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp., are the dominant microorganisms on the surface of bacterial surface-ripened cheeses.

Geotrichum candidum

Geotrichum species are commonly found in nutritionally rich, semisolid or liquid substrates, such as decaying plant material and industrial effluents, and in a wide variety of foods. Numerous species are currently accepted in the genus *Geotrichum*. The cheese relevant species is *Geotrichum candidum* that may also be referred to as *Galactomyces geotrichum* (teleomorph form). *Geotrichum candidum* is commonly found on the surface of mold and bacterial surface-ripened cheeses. Its growth temperature range is 15–25 °C, with a pH growth range of 4.5–7.5. *Geotrichum candidum* strains are much more sensitive to high NaCl concentrations than *Penicillium* and *Brevibacterium* spp. Growth rate is severely reduced at NaCl concentrations in excess of 5%. Based on morphological and biochemical characteristics, *G. candidum* can be divided into three distinct types of strains. Type 1 consists of cream-colored yeast-like strains, with low proteolytic activity; type 3 consists of white-colored strains with a true mycelium and with high proteolytic activity; and type 2 consists of strains that cannot be clearly classified as type 1 or type 3.

Biochemical Activity of *Geotrichum candidum* Relevant to Cheese Ripening

Geotrichum candidum rapidly colonizes the surface of mold and bacterial surface-ripened cheeses; colonization normally occurs within 1–2 days after manufacture and numbers continue to increase up to 7–10 days after

Table 1 Principal secondary cheese cultures used for the ripening of a variety of cheese types

Cheese type	*Variety*	*Principal secondary culture*	*Function of secondary culture*	*Major flavor compounds*
Mold surface-ripened	Camembert, Brie	*Penicillium camemberti*	Flavor, texture, color	Ammonia, phenylethanol, thioesters
Bacterial surface-ripened	Münster, Limburger, Tilsiter	*Brevibacterium linens*, *Brevibacterium casei*, *Corynebacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Arthrobacter* spp.	Flavor, color	Methanethiol, hydrogen sulfide, ammonia
Blue-veined	Danablu, Roquefort, Gorgonzola	*Penicillium roqueforti*	Flavor, color	Free fatty acids, methyl ketones, esters, lactones
Swiss-type	Emmental, Gruyère, Appenzeller	*Propionibacterium freudenreichii* ssp.	Flavor, eye formation	Propionate, acetate, proline

manufacture. During this colonization period, *G. candidum* metabolizes lactate in the curd and deacidification at the surface of the cheese takes place. Amino acid catabolism by *G. candidum* and, in particular, the deamination of glutamate and asparate, with the production of ammonia, contribute further to the deacidification of the curd. *Geotrichum candidum* colonization of the cheese surface precedes colonization by *Penicillium* spp. The degree of colonization of the cheese surface by *G. candidum* requires careful control. Excessive growth of *G. candidum* inhibits the subsequent colonization by the other members of the surface microflora; on the other hand, insufficient growth of *G. candidum* leads to lower flavor complexity in the final cheese. Selection of the specific *G. candidum* strain is of critical importance regarding flavor development. Some *G. candidum* strains can inhibit contaminating molds such as *Mucor* spp., while other strains have no effect. The degree of growth of *G. candidum* on the surface of the cheese is controlled by exploiting the differences between the relatively low NaCl tolerance of *G. candidum* and the much higher NaCl tolerance of *Penicillium* spp. and *Brevibacterium linens*.

Geotrichum candidum produces both intracellular and extracellular proteinases, which have pH optima at 5.5–6.0. The extracellular proteinase is active against casein, with β-casein being preferentially hydrolyzed. *Geotrichum candidum* also produces an extracellular aminopeptidase. The extracellular proteinase activity of *G. candidum* is somewhat less than that of most *P. camemberti* strains; in contrast, its extracellular aminopeptidase activity is generally higher than that of *P. camemberti*. *Geotrichum candidum* produces two extracellular lipases, known as lipase A and lipase B; both enzymes have pH optima of 6.5. Lipase A is fairly nonspecific and releases medium-chain length fatty acids, such as C8, C10, C12, and C14. Lipase B is highly specific for long-chain length unsaturated fatty acids. The high level of free oleic acid found in Camembert cheese may be due to lipase B activity. The lipases of *G. candidum* have been studied and characterized extensively.

Penicillium camemberti and *Penicillium roqueforti*

Penicillium is a very large genus, with over 200 different species recognized, but only a few of these, in particular, *P. camemberti* and *P. roqueforti*, are directly involved in cheese ripening. *Penicillium camemberti* produces gray-white colonies and is used as a secondary cheese culture for mold surface-ripened cheeses such as Brie, Camembert, and Coulommier. *Penicillium camemberti* is also sometimes referred to as *P. candidum*, *P. caseicolum*, or *P. album*. *Penicillium roqueforti* produces green-blue colonies and is used for the production of blue-veined cheeses such as Danablu, Gorgonzola, Stilton, and Roquefort. *Penicillium roqueforti* has also been referred to as *P. glaucum* or *P. gorgonzola*. *Penicillium camemberti* grows at 5 °C, but not at 37 °C, and has a pH growth range of 3.5–8.5; *P. roqueforti* grows over a similar temperature range, but has a wider pH growth range of 3.0–10.5. *Penicillium roqueforti* is characterized by its ability to grow normally at 2% O_2, with slower growth at 0.5% O_2; *P. roqueforti* can also tolerate high levels of CO_2. These properties make it particularly suited for growth at the low O_2 and high CO_2 levels present in the internal cracks and holes of blue-veined cheeses. *Penicillium camemberti* and *P. roqueforti* can grow at high salt concentrations, with a concentration of about 20% NaCl being the upper limit for growth.

Biochemical Activity of *Penicillium camemberti* and *Penicillium roqueforti* Relevant to Cheese Ripening

The pH of the fresh unripened curd 1 day after manufacture of mold surface-ripened and blue-veined cheeses is typically in the range of 4.6–5.2; the pH may drop a little further over the next few days due to the continued activity of the primary starter culture. However, as the ripening period progresses, initially yeasts, followed by molds, begin to colonize the surface and utilize the lactate, which results in deacidification of the curd. The utilization of lactate by *P. camemberti* is well documented, but the situation with *P. roqueforti* is less clear. In the case of Camembert cheese, the surface and internal pH values at the end of ripening are about 7.0 and 6.0, respectively. An additional effect of the deacidification of the curd is softening of the cheese texture.

In addition to deacidification of the cheese curd, the proteolytic, lipolytic, and amino acid catabolic activities of *P. camemberti* and *P. roqueforti* result in significant increases in the concentration of free amino acids, free fatty acids, amines, ammonia, methyl ketones, aldehydes, esters, and lactones in the curd; all of these compounds influence the final characteristic aroma and flavor of the ripened cheese. *Penicillium camemberti* produces two extracellular proteinases: a metalloproteinase with a pH optimum of 6.0 and an aspartyl proteinase with a pH optimum of 5.0. The pH optima for the production of the metalloproteinase and the aspartyl proteinase are 6.5 and 4.0, respectively. Both proteinases are highly active toward casein, and contribute significantly to the increase in total pH 4.6-soluble nitrogen observed over the ripening period; no free amino acids are released by these proteinases. *Penicillium camemberti* also produces an extracellular alkaline aminopeptidase and an extracellular carboxypeptidase, with pH optimum at 8.0–8.5 and 3.5, respectively. These two peptidases are responsible for the release of significant quantities of free amino acids from the casein and casein-derived peptides. *Penicillium roqueforti* has a proteolytic system similar to that of *P. camemberti*, and also produces a metalloproteinase, an aspartyl proteinase, an aminopeptidase, and a carboxypeptidase.

Penicillium camemberti and *P. roqueforti* have high lipolytic activities; the lipolytic activity of *P. roqueforti* is critically important in determining the characteristic piquant flavor associated with blue-veined cheeses. *Penicillium roqueforti* synthesizes an extracellular alkaline lipase (pH optimum 7.5–9.0) and an extracellular acid lipase (pH optimum 6.0–6.5). *Penicillium camemberti* produces one extracellular lipase, with a pH optimum at 9.0. The lipases of *P. roqueforti* and their ready accessibility (in the cracks and holes in the curd) to their milk fat substrate result in the release of very high levels of free fatty acids. In the case of a well-ripened Roquefort or Danablu cheese, the concentration of free fatty acids can exceed 30 000 mg kg^{-1} cheese, which is about 10-fold greater than the free fatty acid concentration of mold surface-ripened cheeses, such as Camembert or Brie. The piquant flavor associated with blue-veined cheeses has been correlated with the high concentration of free fatty acids and especially their oxidation products, the methyl ketones; 2-heptanone and 2-nonanone are the most abundant methyl ketones in blue-veined cheeses. However, excessive ripening can result in the production of styrene in the cheese, via the catabolism of phenylalanine by *P. camemberti*, and thus resulting in a cheese with a plastic-like off-flavor.

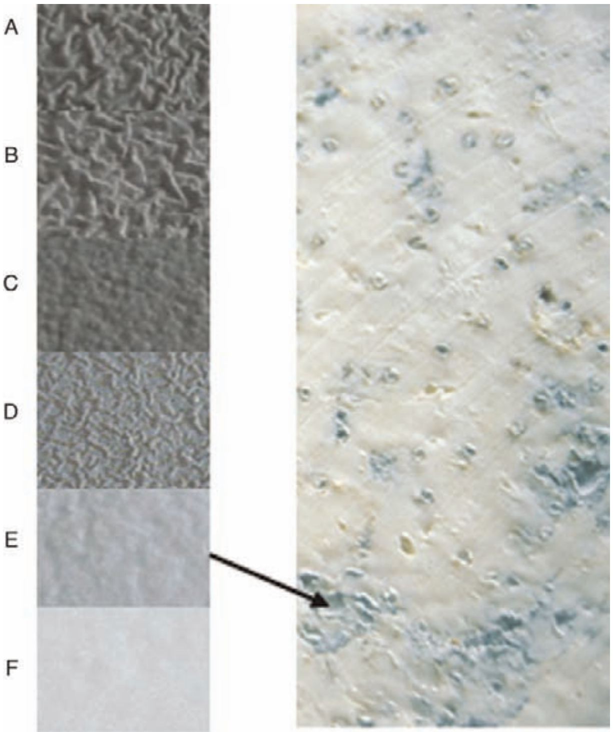

Figure 1 Color differences between various *Penicillium roqueforti* strains A–F. Gorgonzola cheese made with light blue *P. roqueforti* strain of color type E.

Penicillium camemberti and *P. roqueforti* show considerable strain variation in their proteolytic and lipolytic activities. Furthermore, differences between strains in terms of color, halotolerance, germination, growth characteristics, and their response to external stresses are also common. For example, the significant differences in the color of six *P. roqueforti* strains are indicated in **Figure 1**. Clearly, careful strain selection and application is necessary to control the color quality of the final cheese.

Brevibacterium linens

Brevibacterium linens is a strictly aerobic microorganism with a rod–coccus growth cycle, and has temperature and pH growth optima at 20–30 °C and 6.5–8.5, respectively. Slow growth of this organism occurs in cheese-ripening conditions, such as 12 °C and pH 5.5. It is a halotolerant microorganism, and can grow in the presence of 15% NaCl. The growth of *B. linens* on the surface of bacterial surface-ripened cheeses, such as Saint Paulin, Limburger, and Münster, is preceded by the growth of yeasts and molds. The yeasts and molds utilize the lactate present in the curd, and deacidification of the surface occurs. This pH increase enables the growth of *B. linens* and other bacteria, including *B. casei*, *Arthrobacter* spp., *Corynebacterium* spp., *Micrococcus* spp., and *Staphylococcus* spp.

Biochemical Activity of *Brevibacterium linens* Relevant to Cheese Ripening

Brevibacterium linens produces extracellular aminopeptidases and proteinases, the number and properties of which depend to a large extent on the strain. The extracellular aminopeptidases produced by the organism have a pH optimum in the range of 7.0–9.5 and have a strong preference for leucine at the N-terminus of peptides. The extracellular proteinases produced by *B. linens* are serine proteinases and are highly active on α_{s1}- and β-casein. In addition to these extracellular enzymes, the presence of intracellular peptidases and proteinases has also been reported for *B. linens*; however, these intracellular activities are low compared to the extracellular activities. The production of extracellular lipolytic and esterolytic activities by *B. linens* has not been determined unambiguously, with a number of reports presenting conflicting data. However, intracellular esterases have been detected and a number of them have been purified and characterized.

One of the most interesting and important properties from a cheese-ripening perspective is the production of various volatile sulfur compounds, in particular methanethiol, by *B. linens*. L-Methionine-γ-demethiolase is the principal enzyme responsible for the production of methanethiol by *B. linens*. This enzyme catalyzes the α,γ-elimination of methionine to produce methanethiol, α-ketobutyrate, and ammonia. Methanethiol is a very volatile compound with a low odor threshold, and has a characteristic ‘cabbage-like’ or ‘rotten egg-like’ odor, which is typical of the many cheese varieties in which *B. linens* is a component. *Brevibacterium linens* also synthesizes various *S*-methyl thioesters, which are also important cheese flavor compounds. The production of these various volatile sulfur compounds is highly strain dependent. *Penicillium camemberti* and *G. candidum* also have the ability to produce methanethiol and various other sulfur-containing compounds.

Brevibacterium linens is also characterized by its ability to produce various bacteriocins and antimicrobial substances. The biochemical properties of the bacteriocins produced by *B. linens* appear to be strain dependent, but at least some of them have been shown to be inhibitory toward foodborne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes*. Another important property of *B. linens* is its unique yellow-orange aromatic carotenoid pigmentation. The red-orange color of the surface of cheese varieties such as Saint Paulin, Münster, and Limburger is due primarily to the pigments produced by *Brevibacterium* spp., *Corynebacterium* spp., *Micrococcus* spp., and *Arthrobacter* spp. The precise contribution of any of these species to the final color of the surface of the smear is difficult to establish due to the complexity of the surface flora, but *B. linens* is recognized as a contributor. Pigment production by *B. linens* is related to its growth conditions, and is dependent on the dissolved oxygen and methionine concentrations of the growth medium.

Yeasts

There is a tremendous degree of diversity in terms of the species of yeasts associated with mold and bacterial surface-ripened cheeses. Even within the same cheese variety, considerable differences exist in the yeast microflora isolated from cheeses produced at different factories. In general, the most commonly found yeasts associated with mold and bacterial surface-ripened cheeses belong to the *Kluyveromyces*, *Debaryomyces*, and *Saccharomyces* genera. A number of detailed studies on specific cheese varieties have been carried out and report the isolation of *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, and *Issatchenkia orientalis* from Tilsiter cheese, *Candida famata*, *C. zeylanoides*, *Cryptococcus laurentii*, and *Saccharomyces dairensis* from Reblochon cheese, and *D. hansenii*, *Torulopsis sphaerica*, *T. candida*, *Kluyveromyces lactis*, *Candida sake*, *C. intermedia*, and *Y. lipolytica* from St. Nectaire cheese. The number of different yeast species involved in the ripening process makes the study of their contribution to ripening difficult.

Most of the yeasts isolated from mold and bacterial surface-ripened cheeses are halotolerant and can grow in the presence of 10–15% NaCl. The most distinguishing feature of *D. hansenii* is its ability to tolerate a very high concentration of NaCl (up to 24% for some strains). The yeasts colonize the surface of the cheese 1–2 days after manufacture and reach levels of 10^7–10^9 cells per gram of cheese. Similar to *Penicillium* spp. and *G. candidum*, the yeasts utilize residual lactate and deacidify the cheese surface. A rapid growth of yeast is desirable in order to eliminate any possible contamination on the surface of the cheese. In addition to the deacidification process, certain yeasts also produce vitamin and vitamin precursors (niacin, riboflavin, *p*-aminobenzoic acid, and pantothenic acid), which stimulate the subsequent growth of *B. linens* on the cheese surface. The yeasts *K. lactis* and *K. marxianus* (formerly *K. fragilis*) are important components of the cheese flora due to their ability to ferment residual lactose in the cheese curd. The fermentation of lactose by these yeasts results in the production of CO_2, which may help in creating the open structure required in Roquefort cheese.

Propionibacteria

Propionibacteria are pleomorphic rods, often diphtheroid or club shaped, but may also exist as single cells, as pairs, or as branched cell aggregates; they are anaerobic to aerotolerant and generally catalase-positive. The principal propionibacteria associated with cheese are *Propionibacterium freudenreichii*, *P. thoenii*, *P. jensenii*, and *P. acidipropionici*, often referred to as the dairy propionic acid bacteria (PAB). *Propionibacterium freudenreichii* consists of two subspecies, *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii.* The propionibacteria have temperature and pH growth optima at 25–32 °C and 6.5–7.0, respectively. They are generally more sensitive to conditions of high acidity than the lactic acid bacteria. Propionibacteria can grow in the presence of 6–7% NaCl under optimum conditions, but at the low pH found in cheese (pH 5.2–5.4) their growth rate in the presence of NaCl is further reduced.

Biochemical Activity of Propionibacteria and Their Role in Cheese Ripening

Propionibacteria are essential for the development of the characteristic flavor and eye formation in Swiss-type cheeses such as Emmental, Gruyère, and Appenzeller. Unlike *P. camemberti*, *G. candidum*, and *B. linens*, which grow on the cheese surface, the propionibacteria grow internally in the cheese matrix. Propionibacteria are naturally present in the raw milk, but this natural source is lost or severely reduced if the cheese milk is microfiltered or bactofugated. Therefore, the addition of propionibacteria to the cheese milk is often required. Propionibacteria grow in the cheese curd during warm room ripening (18–24 °C) and can reach a level of 10^9 cfu (colony-forming units) per gram of cheese in 4–8 weeks. The lactate produced by the growth of the primary starter culture is utilized by the propionibacteria as a fermentable energy source for growth. Lactate is principally fermented to propionate, acetate, and CO_2, and is described by the Fitz equation;

$$3 \text{ lactate} \rightarrow 2 \text{ propionate} + 1 \text{ acetate} + 1 \ CO_2 + 1 \ H_2O$$

However, in Swiss-type cheese, these theoretical proportions are rarely found, and propionibacteria can also utilize free amino acids, in particular asparate, which may explain some of the variations in the relative concentrations of propionate, acetate, and CO_2.

The proteolytic activity of the dairy PAB is generally low, with a clear species and strain variability. They grow poorly in milk, but addition of casein hydrolysate to milk enables growth to significantly higher cell numbers. This is in contrast to the situation for most lactic acid bacteria, which are capable of growing to high cell numbers in milk without the addition of a casein hydrolysate. The propionibacteria are not as well characterized as the lactic acid bacteria in terms of their proteolytic activity. The proteinase activity of *P. freudenreichii* has been shown to have both cell wall and intracellular locations. A general aminopeptidase, an iminopeptidase (PepI), an X-prolyl dipeptidyl aminopeptidase (PepX), an endopeptidase, and two different oligopeptidase activities have been purified from dairy PAB and are located intracellularly. The dairy PAB are distinguished from the lactic acid bacteria by the presence of carboxypeptidase activity, which is believed to be cell wall located.

The high proline content (1500–6000 mg kg^{-1} cheese) present in Swiss-type cheese contributes to the sweet flavor of these cheeses; furanones are also believed to contribute. The high level of proline is believed to be due to the peptidolytic activity of the propionibacteria peptidases on casein-derived peptides, rather than due to *de novo* synthesis. PepI and PepX are the key enzymes involved in the release of proline and proline-containing dipeptides from casein and casein-derived peptides. The PepI activity of the dairy PAB is much higher than that of the lactic acid bacteria, with *P. freudenreichii* having the highest PepI activity among the dairy PAB.

The lipolytic and esterolytic activities of dairy PAB are less well studied than the proteolytic activity. Extracellular lipase and esterase activities have been detected for *P. freudenreichii*; a number of intracellular esterases have also been detected, and the gene for one of these esterases has been cloned and sequenced. Recently, using a combined genomic and cloning approach at least six active esterases have been detected in *P. freudenreichii.* The lipolytic activity of the dairy PAB is about 100-fold greater than the lipolytic activity of the lactic acid bacteria and it has been shown that PAB significantly influence the degree of lipolysis and the free fatty acid profile of Swiss-type cheeses. Growth of PAB in cheese may be stimulated by certain *Lactobacillus helveticus* strains, and it is believed that this stimulation is due to the proteolytic activity of *Lb. helveticus.* In contrast, *Lb. casei* and *Lb. rhamnosus* are inhibitory toward PAB due to the production of the inhibitory compounds such as diacetyl, acetate, and formate, which are produced as a result of citrate fermentation by these species.

Commercial Secondary Starter Cultures

Traditionally, the development of the secondary microflora of mold surface-ripened, bacterial surface-ripened, or Swiss-type cheeses has relied on the indigenous populations of yeasts, molds, and bacteria present in the cheese ripening rooms, cheese manufacturing equipment, or cheese milk. However, the reliance on these sources for the successful development of the necessary microflora may not be practical for large-scale production and control of ripening may also be difficult with these indigenous sources of secondary microflora. Additionally, if a spoilage or pathogenic outbreak occurs in the cheese plant, decontamination of the entire cheesemaking process is required, with the subsequent loss of the indigenous plant microflora.

The introduction of defined primary cheese starter cultures possessing desirable industrial traits, such as rapid acidification, high phage robustness, or proteolytic activity, has resulted in a dramatic improvement in the reproducibility and reliability of cheesemaking. Similar developments, although not to the same extent, have been seen for secondary cheese starter cultures. Well-characterized strains of yeasts, molds, surface-ripening bacteria, and propionibacteria with defined properties have been developed by various commercial culture producers. These defined secondary cheese cultures have been selected for various important traits, such as rapid growth rates, halotolerance, pigment production, lipolytic activity, proteolytic activity, or amino acid catabolic activity. In common with the commercially available primary cheese starter cultures, these commercially available secondary cheese cultures have greatly increased the reproducibility and control of cheese ripening. Secondary cheese starter cultures are available as frozen cell concentrates, freeze-dried cell concentrates, or liquid concentrate cell suspensions, and are normally applied to the cheese by brine washing or spraying. In addition to these approaches, a portion of the surface ripening culture can be directly added to the cheese milk, in order to improve early establishment of the surface flora.

The commercial production of secondary cheese starter cultures such as *B. linens* and propionibacteria is typically performed in a fashion similar to that used for the commercial production of lactic acid bacteria. The organisms are propagated in large-scale fermentation vessels using optimized growth media (specific carbon and nitrogen sources, vitamins, and other micronutrients) and growth conditions (O_2 saturation, pH, stirring rates, etc.). Upon reaching maximum cell numbers, the cells are harvested by centrifugation and various cryoprotectants may be added prior to freezing or freeze drying.

The commercial production of *Penicillium* spores may be carried out on either solid or liquid substrates. The traditional cultivation of *Penicillium* spores is on a solid medium, such as bread, grain, or a nutrient agar-type medium. The solid substrate is first sterilized in 1 l Roux flasks, inoculated, and incubated at 20–25 °C for 10–20 days. The spores are harvested either by flushing with a sterile liquid carrier (water or saline solution) or by direct vacuum collection. For bread-cultivated spores, the spore-covered bread is simply dried, pulverized, and sieved. However, the production of spores on solid substrates is a labor-intensive and expensive process, and adequate containment of the spores within the production plant may be problematic.

The submerged cultivation of spores in liquid media using fermentation vessels presents some obvious advantages over cultivation on solid substrates. However, a difficulty with molds grown under submerged conditions is that the new cell material produced tends to consist primarily of vegetative mycelia, with relatively few spores present. In order to ensure adequate sporulation in submerged cultures, careful optimization of the growth medium and growth conditions is required. A high calcium ion concentration has been shown to be of particular importance in inducing molds to sporulate under submerged culture conditions. The carbon-to-nitrogen ratio (C/N) is also a major factor influencing sporulation; sporulation occurs mainly under conditions of nitrogen limitation. When maximum spore numbers have been reached, the spores are harvested by centrifugation and separated from mycelial growth by filtration.

See also: **Bacteria, Beneficial**: *Brevibacterium Linens, Brevibacterium Aurantiacum* and Other Smear Microorganisms; *Propionibacterium* spp. **Cheese**: Biochemistry of Cheese Ripening; Blue Mold Cheese; Camembert, Brie, and Related Varieties; Cheese Flavor; Smear-Ripened Cheeses; Swiss-Type Cheeses. **Yeasts and Molds**: *Geotrichum candidum*; *Kluyveromyces* spp.; *Penicillium camemberti*; *Penicillium roqueforti*; Yeasts in Milk and Dairy Products.

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Rennets and Coagulants

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Introduction

Since ancient times, mankind has used extracts from the abomasum (the fourth 'true' stomach) of young calves and sometimes that of lambs and kids in cheese manufacture. The abomasum contains milk-clotting enzymes, which are designed to clot the milk rapidly in the stomach of the young offspring and thereby slow down the flow rate of the milk into the small intestine. The nutrients, casein and fat (the latter trapped in the casein network), of the milk are in this way delayed in the abomasum until enough pancreatic juice is secreted to achieve optimal digestibility. The easily absorbed water, lactose (carbohydrate), and minerals of the milk, on the other hand, continue almost immediately to the small intestine. Although the origin of cheese manufacture is not exactly known, it was discovered in about 8000 BC that the addition of abomasal extracts from newly slaughtered calves to milk rapidly clotted the milk to produce a firm gel. This finding might have arisen from the discovery of pieces of milk clots (curd) in the abomasa of young calves when they were slaughtered.

Until the nineteenth century, all cheeses were produced on farms using fresh extracts from dried abomasa to coagulate the milk. In the 1850s, however, small cooperative dairies were set up, which required larger amounts of rennet (by definition, an extract of ruminant abomasa). From the name rennet was derived the word rennin for the milk-clotting enzyme, which today is called chymosin (EC 3.4.23.4) in the recommended international enzyme nomenclature. In fact, the trivial name, chymosin, was used as early as 1840 by the French pharmacist Jean Baptiste Deschamps.

The need for rennet by dairies led to the industrial production of calf rennet by the end of the nineteenth century. In fact, rennet was the first industrial enzyme preparation produced and sold with a standardized enzyme activity; this preparation was developed by the Austrian pharmacist Franz Soxhlet. Since then, the Soxhlet unit has been widely used for the characterization of rennet strength (milk-clotting activity), although it has been somewhat modified over time and today a new kind of unit is used (see section 'Analysis of rennets and rennet substitutes').

The annual and worldwide increase in cheese production during the twentieth century has resulted in a shortage of calf rennet, which is nowadays widely substituted by fermentation-produced chymosin (FPC), adult bovine rennet, or different microbial coagulants (see below). In parallel with the use of calf rennet, the abomasa of lambs and kids have also been used to prepare milk-clotting preparations in the countries around the Mediterranean Sea. In the same region, rennet paste, which contains a lipase, pregastric esterase, is used to coagulate the milk for certain cheese varieties; the lipase gives these cheeses a special sharp and peppery flavor. In Portugal, an extract from the flowers of thistles from the genus *Cynara* (mainly *Cynara cardunculus*) has been used to coagulate cheese milk.

Bovine Rennets

As discussed above, rennet was produced mainly from the abomasa of young calves, that is, calf rennet, up until the end of the twentieth century. The reason for secretion of the specific milk-clotting enzyme, chymosin, in the stomach of newborn ruminants is that they get their immunoglobulins from colostrum, that is, the milk secreted during the first 2–3 days of lactation. Chymosin has sufficient proteolytic activity to coagulate the milk, but its general proteolytic activity is too weak to extensively damage the immunoglobulins. In contrast, in other species of mammals, immunoglobulins are transferred via the blood from the mother to the fetus *in utero*.

In addition to chymosin, ruminants also secrete the milk-clotting enzymes pepsin (EC 3.4.23.1) and gastricsin (EC 3.4.23.3); the latter is a minor proteinase in ruminants and will not be discussed further. Pepsin has, in addition to its milk-clotting activity, a strong general proteolytic activity under the acidic conditions of the abomasum/stomach. Both chymosin and pepsin are produced and secreted by chief cells, and to some extent also by the mucous-neck cells, in glands of the fundic region of the abomasal mucosa. Actually, the granules from the Golgi apparatus of the cells contain both enzymes. They are produced as inactive zymogens (i.e., prochymosin and pepsinogen) and are activated to chymosin and pepsin at the low pH owing to the presence of HCl secreted by parietal cells in the fundic glands. These glands are protected against proteolytic degradation by mucins secreted by the mucous-neck cells.

The proportion of chymosin and pepsin in the abomasal mucosa depends on the feeding regime and the age of

Table 1 Influence of feeding regime and age on the proportion (%) of chymosin or pepsin (assayed by milk-clotting activity at pH 6.5) in bovine abomasal extracts

Feed	*Age (months)*	*Chymosin (%)*	*Pepsin (%)*
Suckled or milk-fed calf	<3	90	10
Pasture-fed and suckled calf	6	75	25
Concentrate-fed calf	6	30	70
Concentrate- and hay-fed cow	>24	Trace	100

the ruminant. From birth, and even from the 10th to the 20th week of gestation, chymosin is the dominant proteinase in the abomasa of calves. As long as the calf is only suckled or milk-fed, the proportion of chymosin, assayed by milk-clotting activity at pH 6.5, is around 90% (**Table 1**). The proportion of chymosin remains at a high level (75%) even in a 6-month-old pasture-fed calf if it is allowed to suckle its dam. However, if the calf is weaned and fed non-milk concentrates, the proportion of chymosin decreases to about 30% at the age of 6 months. The proportion of chymosin then decreases with age and in adult cattle only traces of chymosin are found. It could thus be concluded that milk feeding and the proportion of chymosin are highly related.

Today, animal rennets are produced mainly from frozen abomasa (earlier, dried abomasa were used), which are cut up in special grinders and their milk-clotting zymogens extracted in a 3–10 g per 100 g NaCl brine solution. The zymogens are activated to chymosin and pepsin by lowering the pH to about 2 for 1 h and then adjusted to about pH 5.5 before filtering and concentrating the extract. The extract is further filtered to remove bacteria and the concentration of NaCl is then increased to about 20 g per 100 g. Since the proportions of chymosin and pepsin in the abomasal extract depend on the age and feeding regime of the animals from which the abomasa are obtained, different batches of rennet are mixed to give a desired proportion of chymosin and pepsin. Finally, the rennet is diluted to a certain strength (total milk-clotting activity), which varies between countries (see section ‘Analysis of rennets and rennet substitutes’). Rennets are usually distributed as liquids, but they may also be in powder form. It should be noted that the production of rennets from older cattle, that is, adult bovine rennet, causes more difficulties during the filtration steps owing to a high concentration of mucins in the extract. The manufacturers of rennets have solved this problem in many different ways.

Extracts from ovine and caprine abomasa are also used as rennets, but to a much lesser extent than bovine rennet. The proportion of chymosin and pepsin in these rennets is expected to depend also on the age and feeding regime of the animals from which the abomasa are obtained.

Molecular and Catalytic Properties of Chymosin and Other Milk-Clotting Enzymes

Chymosin and the other milk-clotting enzymes belong to the group of aspartic (acid) proteinases (EC 3.4.23), which are characterized by a high content of dicarboxylic and hydroxy amino acids and a low content of basic amino acids. The molecular mass of the different milk-clotting enzymes is between 30 000 and 40 000 Da. Calf chymosin has a molecular mass of 35 600 Da based on its primary structure (323 amino acid residues). Chymosins from different species cross-react immunochemically and show 75–80% identity in the amino acid sequence of the N-terminus with calf chymosin, which in turn has a sequence identity of about 50% with pepsins.

The tertiary structure of the aspartic proteinases shows a high homology. The structure contains an extended cleft, which contains the binding site, which can accommodate at least seven amino acid residues of the substrate (κ-casein). Within the cleft, the side chains of Asp_{32} and Asp_{215} are extended from the N- and C-terminal domains and form the active site of chymosin.

The isoelectric point and proteolytic pH optimum of all aspartic proteinases are acidic, although the milk-clotting enzymes have high activity at almost neutral pH (6.5). The general proteolytic pH optimum of chymosin is ~3.8, but it has high specific milk-clotting activity at the pH of milk, that is, 6.7. Compared with pepsin, which has its general proteolytic pH optimum at about 2, the milk-clotting activity of 1 mg chymosin corresponds to that of about 5 mg pepsin at pH 6.7. There are three genetic variants of chymosin, A, B, and C, where the B variant is the dominating (allelic frequency ~50%) and the C variant the most rare (~10%) in bovine stomach extracts. Only one amino acid residue differentiates the A variant of chymosin from the B variant, while there is still no information on the primary amino acid sequence of the newest found C variant of chymosin. According to the latest report, chymosin A, with aspartic acid in position 244, has about 50% higher specific milk-clotting activity than chymosin B, which has glycine at position 244. Despite the difference in specific milk-clotting activity between the A and B variants, they are both considered to have the same cheesemaking properties.

The catalytic mechanism of the milk-clotting enzymes is to hydrolyze the Phe_{105}–Met_{106} bond of κ-casein on the casein micelle surface. Hydrolysis of κ-casein destabilizes the casein micelles, which coagulate in the presence of Ca^{2+} (*see* **Cheese**: Rennet-Induced Coagulation of Milk). Chymosin has a strong affinity for this region of κ-casein and has the highest specific milk-clotting activity of the aspartic proteinases. The other milk-clotting aspartic proteinases, that is, pepsin, *Rhizomucor miehei* proteinase (EC 3.4.23.6), *Rhizomucor pusillus* proteinase (EC 3.4.23.6), and *Cryphonectria* (formerly *Endothia*) *parasitica* proteinase (EC 3.4.23.6), have a specific milk-clotting activity that is lower than that of chymosin and it varies among them. It must also be remembered that milk-clotting conditions, such as pH, calcium content, and temperature, strongly influence milk-clotting activity; the milk-clotting activity of pepsin is especially highly pH dependent. Ovine and caprine chymosins have been shown to have a higher specificity for ewes' and goats' milk, respectively, than for cows' milk. The opposite is, of course, the case with bovine chymosin. Very interestingly, recent reports have shown recombinant camel chymosin to exhibit a 70% higher specific milk-clotting activity for bovine milk than bovine chymosin and only 20% of the general proteolytic activity of the latter.

Rennet Substitutes (Coagulants)

Up to the 1950s, calf rennet was almost exclusively used for cheesemaking. At that time, however, a worldwide shortage of abomasa from young milk-fed calves started to become a serious problem. This was due to increased milk production from individual cows, which led to a decrease in the number of cows and hence the number of calves. In addition, the demand for beef increased, which led to the postponed slaughter of the calves until they were grown to adults. The resulting shortage of abomasa from young calves led to a search for other coagulants.

Note that the word 'rennet' by definition is an extract of the abomasa of ruminants and therefore rennet substitutes should be named coagulants according to the International Dairy Federation (IDF). Adult bovine rennet, that is, an extract dominated by pepsin from the abomasa of adult cattle, could also be regarded as a 'calf rennet substitute', as its production was started as a complement to calf rennet when the number of calf abomasa decreased. However, as it could also be classified as a bovine rennet, it is described together with calf rennet (see section 'Bovine rennets'). In addition to a higher pH dependence, adult bovine rennets are considered to give a slightly lower yield of cheese.

Many rennet substitutes have been developed and evaluated. Today, only a few survive on the market (mainly two different fungal coagulants) and they may become increasingly less important in the future as FPC (see below) becomes more popular. However, its future use is dependent on the acceptance of products of genetically modified organisms (GMOs) by consumers.

The most widely used rennet substitutes are Miehei coagulant (*R. miehei* proteinase), Pusillus coagulant (*R. pusillus* proteinase), and Parasitica coagulant (*C. parasitica* proteinase). Miehei coagulant dominates the market for microbial coagulants and is produced in two different heat-labile forms, which are less proteolytic than the native form. Parasitica coagulant is very proteolytic and is normally used only for the manufacture of cheeses for which the curd is heat-treated to ~55 °C and hence the coagulant is inactivated before the cheese ripens.

One of the earliest rennet substitutes used was porcine pepsin, an extract of pig stomach. Porcine pepsin was either used alone or mixed 50:50 with calf rennet. A disadvantage of porcine pepsin coagulants is that their milk-clotting activity is very pH-dependent, even more so than bovine pepsin, making them sensitive to inactivation. At the pH (~6.5) and temperature (~30 °C) used for cheesemaking, porcine pepsin starts to denature and after 1 h only 50% of its milk-clotting activity remains. Today, porcine pepsin coagulants are seldom used.

Chicken pepsin has also been used as a rennet substitute, mainly for religious reasons, but it is too proteolytic to be suitable for most cheese varieties. Most milk-clotting enzymes of bacterial origin have been found to be unsuitable for cheese manufacture owing to their very high proteolytic activity.

Generally speaking, the rennet substitutes give a lower yield of cheese and a different cheese flavor compared with calf rennet (i.e., chymosin). However, if they are used for short-ripening cheeses, the difference is not significant.

Fermentation-Produced Chymosin

During the 1980s, recombinant DNA technology was used to develop microorganisms capable of producing chymosin, using the DNA sequence of chymosin from a calf abomasum cell. Today, there are two preparations on the market that are produced by a fermentation process involving either *Aspergillus niger* or *Kluyveromyces lactis* (both produce chymosin B). The IDF has proposed the name FPC for this type of preparation. A third FPC, produced by *Escherichia coli* (chymosin A), which was allowed for use by the US Food and Drug Administration at the beginning of 1990, is no longer on the market after the patent was bought by a competitor manufacturing FPC producer.

Since FPC is identical to calf chymosin (the same amino acid sequence), its properties are, in principle, the same as those of calf chymosin. The only difference is that the producing organism could add some residues (10% of the molecules are glycosylated by fungi) to the chymosin molecule. However, such modifications have not been observed to change the properties of FPC significantly compared to calf chymosin. Therefore, the properties of FPC are almost identical to calf chymosin, and the introduction of this 'rennet substitute' on the market has been very successful (about 50% market share in the world in 2008). One drawback is, however, consumer reactions, since the consumers might consider cheese produced by FPCs to be genetically engineered. Another drawback could be that FPCs do not contain a small proportion of pepsin as calf rennet does. Regarding the first drawback, it has to be remembered that only a small amount of rennet is added to the cheese milk (~30 ml to 100 kg of milk, i.e., 0.03%) and only about one-fifth of that amount remains in the cheese (i.e., 6 mg kg $^{-1}$ cheese). The lack of bovine pepsin in FPCs might not be a problem since bovine pepsin could easily be added and it is generally accepted that the higher the amount of chymosin in the rennet, the higher the yield of cheese. With regard to analysis, FPCs could be handled as a calf rennet containing 100% chymosin (see below).

In the near future, the recently introduced product, fermentation-produced camel chymosin, could have increased its market share significantly due to its higher specific milk-clotting activity and lower general proteolytic activity compared to bovine chymosin. However, it is still too early to predict the future for this product and how it influences the manufacture and ripening of traditional cheeses.

Analysis of Rennets and Rennet Substitutes

The characterization of rennets from around 1870, when they were first produced on an industrial scale, up to the 1950s was based on the strength (total milk-clotting activity), since the compositions of the rennets were quite similar, that is, calf rennet dominated by chymosin. The strength of the rennets was expressed in Soxhlet unit, which originally was defined as the volume of fresh milk that can be clotted by one unit volume of a rennet in 40 min at 35 °C. Owing to variations in the clotting ability of raw milk, however, this is not a satisfactory definition. An improvement on milk-clotting analysis was published by the English scientist NJ Berridge, in 1952, who proposed the use of standardized milk powder, reconstituted in 0.01 mol l $^{-1}$ $CaCl_2$, as the substrate in activity tests. The Berridge unit, or rennet unit (RU), is defined as the activity that is able to clot 10 ml of standardized milk (pH ~6.3) in 100 s at 30 °C. Until the 1990s, Soxhlet and Berridge units were used to a great extent for almost all national definitions of rennet strength and milk-clotting tests. However, the variations in the composition of rennets and rennet substitutes on the market today make the old definitions too uncertain and inaccurate. Rennets could be dominated by either chymosin (calf rennet) or pepsin (adult bovine rennet) and they could have all the different proportions of chymosin and pepsin between these extremes. Since the milk-clotting activity of pepsin increases much more than that of chymosin as the pH is reduced, the milk-clotting test must be performed at a defined pH close to that of cheese milk (~6.5). Otherwise, the activity of pepsin will be overestimated, as is the case using the Berridge substrate containing 0.01 mol l $^{-1}$ $CaCl_2$, which has a pH of about 6.3 and a very high calcium content. Furthermore, the fungal rennet substitutes contain milk-clotting enzymes of different origin and their characteristics regarding milk-clotting activity vary compared to bovine rennets (both calf and adult) and among themselves.

It was apparent that there was a need for international standard methods for determining the composition and strength of rennets and rennet substitutes. The IDF has had an expert group working on this and there are now four published international standards. One is for the composition of bovine rennets and the other three determine the strength of bovine rennets, the microbial coagulants, and the ovine/caprine rennets, respectively. It is hoped that their use will facilitate communication between producers and users regarding rennets and microbial coagulants, both within and between countries. Also, scientific papers would be improved if the composition and strength of rennets and microbial coagulants were characterized and referred to according to these international standard methods.

The composition of bovine rennets (calf and adult) can be determined with the IDF Standard 110: 2010a, which expresses the results as a percentage of chymosin and pepsin activity or milligrams of active chymosin and pepsin per liter. It is preferable to use the expression percentage of activity, owing to a better repeatability and reproducibility of the analysis. The principle of the method is that chymosin and pepsin are chromatographically separated into two fractions on an anion exchange column. The milk-clotting activity of the chymosin and pepsin fractions is then determined relative to one of two international reference rennet powders (see below) on a standardized milk substrate (containing 0.05 g per100 ml $CaCl_2$) at pH 6.5 and at 32 °C. The percentage of milk-clotting activity of chymosin and pepsin is calculated from the results of the activity tests. The total milk-clotting activity, however, should not be calculated by adding the activity of both fractions owing to some enzyme losses on the column and the difficulty of knowing exactly the amount of rennet introduced to the column.

The origin of microbial coagulants or any adulteration of bovine rennets (calf and adult) with microbial coagulants or porcine pepsin can be determined by an immunological method described in Annex A of IDF Standard 110: 2010a. If bovine rennets are found to be adulterated with non-bovine milk-clotting enzymes, the results of the chymosin and pepsin content of the rennet are not reliable, since other coagulants could have influenced the composition of the fractions.

The total milk-clotting activity of bovine rennets (calf and adult) can be analyzed by the method described in IDF Standard 157: 2007. The principle of the method is that the milk-clotting activity of the rennet is determined relative to the activity of two international reference rennet powders, that is, one calf rennet (≥98% chymosin activity) and one adult bovine rennet (≥98% pepsin activity) (see below). The Association of Manufacturers of Animal-Derived Food Enzymes (AMAFE), appointed by the IDF, has produced large batches of both the calf and the adult bovine reference standard powders. The milk-clotting activity of these batches has been adjusted to be exactly the same on a standardized milk substrate (0.05 g per 100 ml $CaCl_2$) at pH 6.5 and 32 °C: it is set at 1000 international milk-clotting units per gram (IMCU g^{-1}). The reference powders can be bought directly from AMAFE at the address given in the IDF Standard 157: 2007. When the stocks of the original large batches of reference powders are exhausted in the future, the total milk-clotting activity of the new batches will be denoted relative to the old batches.

The total milk-clotting activity of a rennet sample is thus measured relative to both reference powders at pH 6.5 and 32 °C on a standardized milk substrate (0.05 g per 100 ml $CaCl_2$). The results obtained are interpolated with respect to the composition of the rennet sample. For example, if the composition of the rennet is 75% chymosin and 25% pepsin activity, 75% of the relative milk-clotting activity against the calf rennet reference and 25% of the relative milk-clotting activity against the adult bovine reference should be summarized. The results are given in international milk-clotting units per milliliter or per gram (IMCU ml^{-1} or IMCU g^{-1}). The total milk-clotting activity of FPCs is measured relative to the calf rennet reference only, since FPCs contain 100% chymosin.

There is also an International IDF Standard (176: 2010b) for the total milk-clotting activity of the most widely used microbial coagulants (Miehei coagulant, Pusillus coagulant, and Parasitica coagulant). The principle of this method is the same as for bovine rennets (IDF 157: 2007), but standard reference powder of the *R. miehei* proteinase is used. The total milk-clotting activity of the microbial proteinase reference powder is defined according to the international calf rennet reference powder, that is, set at a fixed value relative to the calf rennet reference powder. The microbial reference powder can be obtained from the producer given in the IDF Standard 176: 2010b.

To measure the total milk-clotting activity of ovine and caprine rennets, including rennet paste, the IDF Standard 199: 2006 is appropriate. The principle and the reference standard powders are the same as for bovine rennets (IDF 157: 2007).

See also: **Cheese**: Biochemistry of Cheese Ripening; Cheese Rheology; Gel Firmness and Its Measurement; Overview; Rennet-Induced Coagulation of Milk.

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Rennet-Induced Coagulation of Milk

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Introduction

Coagulation of milk by rennet initially occurred probably by accident, as warm milk was stored in sacks made from the stomach hides of ruminant animals, which contained some residual proteinase. Crude extracts (called rennets) prepared from the fourth stomach of young calves have been used for cheesemaking for thousands of years. Today, the most popular rennet enzyme, chymosin, has been cloned into several genetically modified organisms to produce recombinant chymosin, which is widely used in many countries around the world. Recently, chymosin from camel (*Camelus dromedarius*) has been cloned and is now commercially available for cheesemaking.

Coagulation of milk by rennet is widely exploited as the first step in the production of many different cheese varieties. Rennet coagulation has several important advantages over acid coagulation; these include faster coagulation (i.e., minutes) compared to the slower rate of acid development (i.e., hours) required by starter cultures to coagulate milk for cheeses such as Cottage cheese (in contrast, milk coagulates very rapidly when acid is added directly). Renneted milk gels also undergo much more syneresis than do acid milk gels, which helps to produce cheeses with lower moisture levels. In most cheeses, starter cultures are added to reduce the pH, which helps to improve keeping quality (i.e., less perishable), as well as to alter textural attributes and to generate flavor compounds during ripening.

Coagulation of milk by rennet may be divided into primary (enzymatic hydrolysis) and secondary (aggregation) stages, although these stages normally overlap to some extent during cheesemaking (**Figure 1**). During the primary stage, κ-casein is cleaved by rennet at the Phe_{105}–Met_{106} bond (to form *para*-κ-casein and macropeptide) resulting in a reduction in both the net negative charge and steric repulsion, such that rennet-altered micelles become susceptible to aggregation and, after a lag phase, a three-dimensional gel network (called a 'coagulum') is formed (**Figure 2**). There has been considerable interest in the study of rennet coagulation owing to the commercial importance of this process as the first step in cheesemaking.

Primary (Enzymatic) Phase

The basic building blocks of rennet-induced gels are the casein micelles. Caseins constitute ~80% of the protein in bovine milk, with the four main types (α_{s1}-, α_{s2}-, β-, and κ-caseins) in combination with appreciable quantities of micellar or colloidal calcium phosphate (CCP) nanoclusters occurring in the form of large aggregates called casein micelles. Of the caseins, α_{S1}-, α_{S2}-, and β-caseins are sensitive to precipitation by Ca^{2+} in milk, as they have clusters of phosphoserine residues, which have a high calcium-binding affinity, and are protected by their association with κ-casein, which binds little calcium as it contains few phosphoserine residues. κ-Casein molecules have a predominantly surface location on micelles where the hydrophilic C-terminal part apparently acts as a 'hairy' layer, providing steric stabilization and a barrier against association with other micelles.

In the primary phase of rennet coagulation, the hydrophilic C-terminal part (residues 106–169) of the κ-casein molecule is hydrolyzed off and diffuses away from the micelle into the serum phase. This macropeptide is called caseinomacropeptide (CMP) or if it is highly glycosylated, that is, contains carbohydrate residues, such as *N*-acetyl neuramic acid, it is called glycomacropeptide (GMP). There are several different levels of glycosylation of κ-casein, and these can be distinguished by their solubility in trichloroacetic acid (TCA). Apparently, all the principal rennet types hydrolyze the same Phe_{105}–Met_{106} bond (with the exception of the coagulant from *Cryphonectria parasitica*, which hydrolyzes the Ser_{104}–Phe_{105} bond).

The peptide sequence His_{98}–Lys_{111} is the important section of the κ-casein molecule affecting the ability of chymosin to hydrolyze the Phe_{105}–Met_{106} bond. The active site of chymosin is within a cleft, so the cleavage site of κ-casein must be able to fit into this cleft. The region between residues Leu_{103} and Ile_{108} of κ-casein is strongly hydrophobic, and this region probably associates with a hydrophobic region within the chymosin cleft. Negatively charged residues on chymosin probably interact up with positively charged residues (His_{98}–His_{102} and Ile_{108}–Lys_{112}) in κ-casein. Evidence that electrostatic interactions seem to be involved comes from the findings that the addition of NaCl prolongs the rennet coagulation

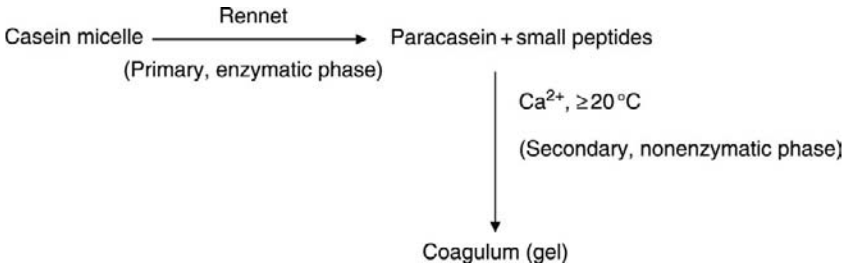
Rennet
Casein micelle
Paracasein + small peptides
(Primary, enzymatic phase)
Ca^{2+}, ≥20 °C
(Secondary, nonenzymatic phase)
Coagulum (gel)

Figure 1 Summary of the two stages involved in the rennet coagulation of milk.

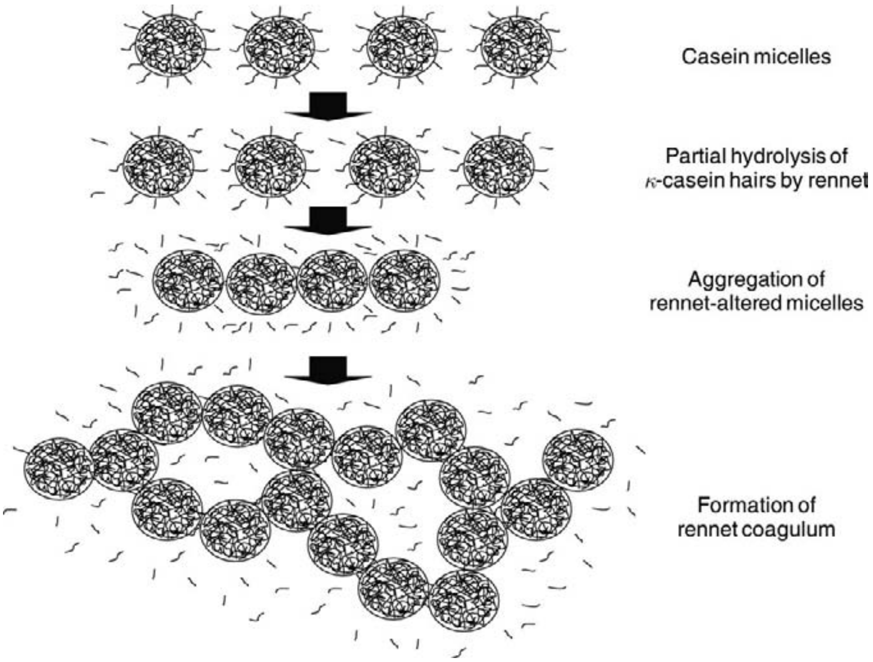
Casein micelles
Partial hydrolysis of κ-casein hairs by rennet
Aggregation of rennet-altered micelles
Formation of rennet coagulum

Figure 2 Schematic drawing of the various processes occurring during the rennet coagulation of milk.

time (RCT), although this may also be due to slower aggregation of rennet-altered particles.

The enzymatic reaction in milk seems to obey first-order kinetics. The proteolysis of κ-casein is usually described by standard Michaelis–Menten kinetics, giving the velocity of proteolysis by

$$\frac{-\mathrm{d}[\mathrm{S}]}{\mathrm{d}t} = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]} \qquad [1]$$

where [S] is the concentration of substrate, $V_{\max}$ is the maximum rate of proteolysis at infinite substrate concentration, and K_{m} is the dissociation constant of the enzyme–substrate complex. Values for K_{m} in the range of 1×10^{-4} to $5 \times 10^{-4}\,\mathrm{mol\,l^{-1}}$ have been reported for intact casein micelles. Different kinetic results, that is, reaction order and K_{m} values, have been obtained with purified κ-casein, probably due to easier accessibility of the enzyme to a soluble κ-casein substrate, as presumably the (unhydrolyzed) κ-casein hairs on casein micelles may represent a barrier, for example, physical or activation energy, to the enzyme gaining access to the cleavage site (i.e., Phe_{105}–Met_{106} bond).

The reactions leading up to aggregation of renneted micelles have also been studied using the adhesive hard sphere (AHS) theory. The AHS model views casein micelles as hard spheres (i.e., the internal structural features are not important) that are sterically stabilized by the κ-casein 'hairs', which are viewed as a charged brush. The theory has successfully predicted the (well-known) viscosity changes up to the point where aggregation occurs.

Secondary Phase

The stability of casein micelles in milk is attributed to their net negative charge and steric repulsion by the flexible macropeptide region of κ-casein (the so-called hairs), Ca-induced interactions between protein molecules, and hydrogen bonding, as well as electrostatic and hydrophobic interactions. The release of the CMP (or GMP), which diffuses away from the micelles, leads to a decrease in the zeta potential, by 5–7 mV (50%), which reduces electrostatic repulsion between rennet-altered micelles. Removal of the hairs also results in a decrease in the hydrodynamic diameter by 5 nm and a loss of steric stabilization, and causes a slight minimum in viscosity during the initial lag phase.

The nature of the attractive forces during the aggregation of casein micelles is still not completely clear, although Ca bridges, van der Waals forces, and hydrophobic interactions may be involved. Destabilized micelles will aggregate only in the presence of free Ca^{2+}, and gelation occurs only if there is sufficient CCP present, that is, it needs the native casein micellar structure to be intact. The aggregation process is also highly temperature dependent and occurs only at temperatures >15 °C (at least under normal conditions of calcium content, the use of chymosin as rennet source, and where little nonrennet casein hydrolysis has occurred).

When milk is clotted under normal conditions of pH and protein content, viscosity does not increase until the enzymatic phase is at least 87% complete and it is >60% of the (visual) RCT. Thus, there is some overlap between the hydrolysis and aggregation phases; the extent of overlap depends on the experimental conditions such as pH, temperature, and protein content. The high degree of κ-casein hydrolysis needed for aggregation may be due to the presence of other caseins on the micellar surface that also contribute to the repulsive barrier opposing aggregation. When the repulsive barrier is lowered sufficiently by the removal of the κ-casein hairs, aggregation can occur. Factors that enhance the attractive interactions between micelles (e.g., higher calcium content, higher solids) encourage the aggregation of renneted micelles. Small linear chains of micelles form initially, and these continue to aggregate to form clumps, clusters, and eventually a system-spanning network that has a fractal-like appearance (**Figure 3**).

No visible aggregation occurs at low temperatures, for example, <18 °C, which is usually taken as an indication of the importance of hydrophobic interactions. It is more likely that with decreasing temperature the activation free energy for flocculation increases, presumably because of the presence of β-casein on the outside of the micelle. There is an increase in the strength of renneted milk gels set at, say, 30 °C on cooling to a lower temperature at which hydrophobic interactions are weak, due to swelling of casein particles, and due to an increase in the contact area between aggregated particles and strands. Plant-derived coagulants appear to be able to cause aggregation at lower temperatures as compared to animal rennets, probably due to the greater nonspecific proteolytic activity of these coagulants.

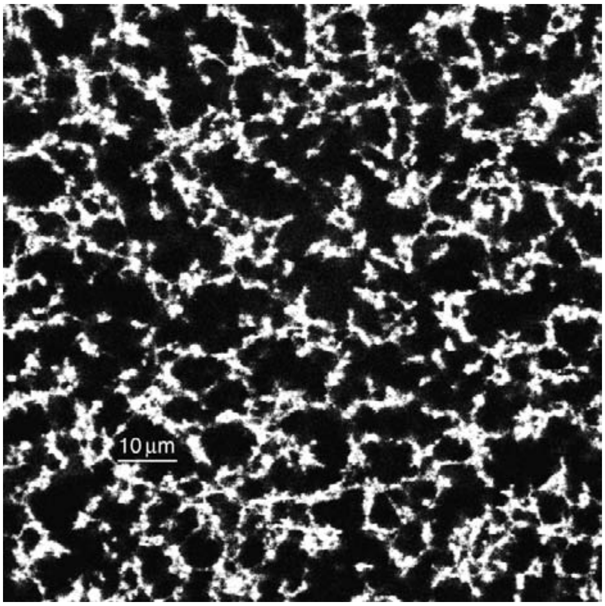

Figure 3 Confocal laser scanning micrograph of a rennet-induced milk gel. White (fluorescent) areas are protein. Scale = 10 μm.

Flocculation of renneted micelles has been modeled by Smoluchowski kinetics (modified to account for the lag phase that occurs during renneting). Some of the features that have been investigated for this approach include the similarity between predicted clotting times and those determined using the Holter equation (eqn [5]), the predicted degree of proteolysis at the coagulation time, the reaction order for the initial hydrolysis reaction, and the mode of hydrolysis of κ-casein hairs (i.e., catch-and-razor model vs. random attack).

Recently, fractal aggregation theory has been applied successfully to the formation of rennet-induced milk gels. This theory assumes that spherical particles (e.g., casein micelles) of radius a can move by Brownian motion and that they can aggregate when they encounter each other. The aggregates thus formed then also aggregate with each other. This cluster–cluster aggregation process leads to aggregates obeying the scaling relation

$$\frac{N_p}{N_0} = \left(\frac{R}{a_{\mathrm{eff}}}\right)^{D-3} \qquad [2]$$

where N_p is the number of particles in an aggregate of radius R, N_0 is the total number of primary particles that could form such a floc, D is a constant called the fractal dimensionality ($D < 3$), and a_{eff} is the radius of the effective building blocks forming the fractal cluster. The volume fraction of particles in an aggregate, φ_A, decreases as aggregation proceeds and R increases. At a certain radius R, the average ϕ_A will equal the volume fraction of particles in the system, ϕ, and all aggregates will touch, forming a continuous gel network:

$$\phi = \left(\frac{R_g}{a_{\text{eff}}}\right)^{D-3} \quad [3]$$

Rearranging gives the radius of the aggregates at the point of gelation, R_g:

$$R_g = a_{\text{eff}}\phi^{\frac{1}{D-3}} \quad [4]$$

R_g is a measure of the upper cutoff length, that is, the largest interparticle distance (length scale) at which the fractal regime exists. The structure of a rennet gel can be characterized by the parameters *a*, ϕ, R_g, and *D*. For rennet-induced gels, a *D* of ~2.3 has been observed.

The possible aggregation mechanisms involved in particle gels, for example, casein-based gels, are now being studied using Brownian dynamics computer simulations. With this type of approach, the aggregation behavior of particles that have different types of bonds and repulsive barriers can be studied. This technique is very helpful in our understanding of how particle interactions influence gelation properties.

Compositional and Processing Factors that Affect Rennet Coagulation

Temperature

Optimum temperature for the coagulation of milk by calf rennet at pH 6.6 is 45 °C, but this temperature varies depending on pH and rennet type. The temperature coefficient (Q_{10}) for the enzymatic phase is 2, and the reaction occurs down to 0 °C. The Q_{10} for the secondary phase is 11–16, and the reaction occurs very slowly at temperatures less than ~15 °C. The aggregation rate of rennet-altered micelles increases greatly with temperature. The optimum coagulation temperature for the firmness of rennet-induced gels is 30–35 °C, and the typical coagulation temperature used in cheesemaking is 31 °C. Prolonged cold storage of milk prior to renneting can result in a longer RCT and a weaker gel due mainly to dissociation of β-casein from the micelles. This can be reversed to some extent by pasteurization of the milk prior to cheesemaking.

Enzyme Concentration

There have been many attempts to describe the influence of enzyme concentration on the coagulation time of milk; one of the most widely used is the Holter equation, which describes the well-known inverse relation between coagulation time and enzyme concentration:

$$\text{CT} = \frac{K}{[\text{E}]} + A \quad [5]$$

where CT is the clotting time, *K* and *A* are constants, and [E] is the enzyme concentration. Clotting time refers to the time taken by both the enzymatic and aggregation reactions. The constant *A* in the Holter equation reflects the time needed for the nonenzymatic phase of milk coagulation. More complex equations have been developed to describe the clotting time of milk, and these attempt to incorporate features of both the enzymatic and aggregation steps. Holter plots tend to become nonlinear if the [E] range is too large or if pH or temperature is varied widely. An increase in enzyme concentration obviously results in a reduction in RCT and concomitantly the rate of increase in gel firmness is greater.

pH

Optimum pH for the action of chymosin in milk is 6.0, but the optimum pH is lower for isolated (purified) caseins or synthetic peptides. Lowering the pH of milk leads to a reduction in the RCT (due to reduced electrostatic repulsion) and a faster rate of increase in gel firmness. Several factors are involved in this phenomenon, including a reduction in the electrostatic repulsion between micelles, increased [Ca^{2+}] due to solubilization of CCP, flocculation at a lower degree of κ-casein hydrolysis, and increased rennet activity. Acidification of milk increases the strength of rennet-induced milk gels up to pH 6.3–6.0. At lower pH values (i.e., <6.0), gel strength is reduced and there is an increase in the loss tangent, tan δ, probably due to excessive solubilization of CCP, which acts as a cross-linking agent between casein molecules and casein micelles.

Calcium

Generally, it is thought that calcium does not directly affect the enzymatic phase, although addition of CaCl_2 does reduce milk pH, which accelerates the hydrolysis reaction. Addition of calcium (<50 mmol l^{-1}) reduces the RCT, even at constant milk pH, and flocculation occurs at a lower degree of κ-casein hydrolysis. Addition of calcium also increases the rate of firming of rennet-induced milk gels, mainly by charge neutralization of the negatively charged groups on the micelle surface and possibly by the formation of calcium bridges. Addition of high concentrations of calcium (e.g., >0.1 mol l^{-1}) reduces the rate of gel firming, probably by increasing the effective (positive) surface charge on the micelles. Addition of up to 10 mmol l^{-1} calcium increases the strength of rennet gels. Reduction of the CCP content of casein micelles by 30% prevents coagulation unless [Ca^{2+}] is increased. It appears that removal of CCP results in the disruption of the micellar structure, which makes it unable to participate in gel formation.

Heat Treatment of Milk

Severe heating of milk (e.g., >70 °C) impairs its rennet coagulation properties. When milk is heated, β-lactoglobulin and κ-casein form a complex via sulfhydryl–disulfide interchange and hydrophobic interactions. The RCT of milk increases with the severity of the heat treatment. The rate of enzymatic hydrolysis of κ-casein is reduced, probably due to reduced accessibility of the Phe_{105}–Met_{106} bond. In addition, it appears that some κ-casein is no longer hydrolyzed by rennet. Heating reduces the rate of aggregation of renneted micelles to a great extent. It is likely that denatured whey proteins on the surface of casein micelles sterically hinder the aggregation of renneted micelles. The renneting properties of heated milk deteriorate further during storage, that is, rennet hysteresis, probably due to some continuing structural changes in the β-lactoglobulin–κ-casein complex. Some of the effects of heating on the rennet coagulation properties of milk can be reversed, if the conditions of heating are not too severe, by addition of calcium, reducing the pH, or pH cycling (acidification and neutralization of milk). Milk has been subjected to high heat treatment mainly to incorporate denatured whey proteins in cheese as a means of increasing cheese yield, although this is becoming less attractive as whey is becoming more important as a profitable by-product from cheesemaking.

Total Solids

As the casein concentration increases, the rate of aggregation increases. This is very obvious when the milk solids content for cheesemaking is increased by ultrafiltration. Cutting and curd handling operations also become increasingly difficult when the protein content of cheese milk exceeds 4.5%. In milks with a higher solid content, coagulation occurs at a lower degree of hydrolysis of κ-casein. Often, clotting time is only slightly reduced in concentrated milk, but this depends on the concentration of rennet added (i.e., added on a milk volume or per casein basis) and the method used for detecting coagulation time.

Sodium Chloride

Salt or ionic strength also affects rennet coagulation. As addition of NaCl reduces milk pH, many of the effects depend on whether milk pH is kept constant. Addition of NaCl (>0.01 mol l^{-1}) reduces the hydrolysis reaction, presumably by inhibiting the electrostatic interactions involved in the formation of the chymosin–κ-casein complex at the active site. Adding NaCl increases the RCT and reduces the initial rate of aggregation (even if the pH is kept constant). If higher rennet levels are used to get similar RCTs, then the addition of up to 100–200 mmol l^{-1} NaCl (at a constant milk pH) gives higher storage moduli for rennet-induced gels. These results emphasize the importance of electrostatic interactions in the rennet coagulation process. Adding NaCl also results in some solubilization of CCP (probably due to substitution/exchange of Na^+ for Ca^{2+}).

Postcoagulation Processing Operations

One of the main reasons for the great interest in studying rennet coagulation is to optimize the gel cutting time. When the cheesemaker (subjectively) decides that the gel (coagulum) is firm enough, it is cut by mechanical knives in both the horizontal and vertical directions to produce curd particles. In cheesemaking, the cutting time varies from ~20 to 50 min depending on the following:

1. Concentration of rennet used; for example, 20 ml of single-strength rennet per 100 l milk, although this depends on the strength of the rennet used and the other coagulation conditions.
2. Whether CaCl_2 was added, as this accelerates clotting (the maximum legal level in many countries is 0.02%).
3. Coagulation temperature (coagulation occurs faster at higher temperatures).
4. pH (the activity of chymosin decreases with an increase in pH).
5. Seasonal changes in milk composition; for example, late-lactation milk can be slow to clot due to its high pH and hydrolysis of caseins within the mammary gland by plasmin. Low levels of plasmin hydrolysis reduce RCT and increase the initial rate of aggregation of rennet-altered micelles, although the final gel strength is reduced. Some of these problems can be minimized by maintaining cows on a high nutritional plane and drying off before the milk becomes abnormal. Standardization of the protein level in milk (e.g., using ultrafiltration retentates) also helps to minimize seasonal variations in milk composition and results in more uniform coagulation properties.
6. The quality of the dilution water used to make the rennet solution prior to addition to the cheese vat, as both excessive chlorine and a high level of water hardness can adversely affect chymosin activity.

See also: **Cheese**: Cheese Rheology; Overview; Rennets and Coagulants. **Milk Proteins**: Casein, Micellar Structure; Casein Nomenclature, Structure, and Association. **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Functional Properties of Milk Proteins.

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Gel Firmness and Its Measurement

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Role of Coagulum Firmness in Making Cheese

Cheese is a food consisting of casein (the major protein present in bovine and buffalo milk), along with some other milk constituents, mainly fat. Cheese is produced by 'destabilizing' the casein in milk, causing it to coagulate/gel in a solid/semisolid form, that is, curd. Normally, the casein in milk remains in suspension in the form of casein micelles, which are self-repelling, but these are 'destabilized' by the action of enzymes in rennet, which has the effect of removing the self-repulsion forces, leading to aggregation. Aggregation leads to a coagulum whose structure is a casein matrix (a gel) that becomes progressively firmer with time and begins to shrink and exude the serum phase (whey), a process known as syneresis, while partially immobilizing the aqueous phase (milk serum) and trapping fat globules.

The physical changes in casein, from a stable suspension to a semisolid matrix, are exploited to separate curd from whey in the manufacture of cheese, and hence an awareness of the changing rheological characteristics of curd during the renneting step is of major interest to cheesemakers. During renneting, a progressive increase in gel firmness occurs until eventually the gel reaches a firmness that allows it to withstand, without shattering, mechanical cutting in the cheese vat. Syneresis is accelerated by cutting the gel into small pieces, exposing a much larger surface area, and reducing the distance that the whey must travel to leave a curd grain. Cutting at the optimum firmness, and rate of curd firming, results in the correct curd particle size, minimum losses of fat and fines in the cheese whey, and maximum cheese yield.

'Curd strength' is a general term, arising from a cheesemaker's sensory concept of a gel that was sufficiently intact to be cut into discrete grains. While gel strength may be precisely defined as the force required to break a gel, the term has been used generically to refer to several rheological characteristics of a gel, including firmness (G') and fracture stress. Various approaches have been adopted toward a discrete determination of gel (curd) strength or firmness, which could be used in research laboratories to study factors that affect milk coagulation and in cheese factories as process control tools. In 1935, Sommer and Matsen at the University of Wisconsin measured the force on a blade as it cut the milk coagulum. Now that physical measurements of curd rheology are possible using precision rheometers, it is possible to describe curd firming in rheological terms, for example, by dynamic changes in parameters such as elastic shear modulus, G'.

Determination of Correct Gel Firmness for Cutting into Grains of Curd

For the manufacture of natural cheese, the coagulum (gel), when sufficiently firm, needs to be cut into discrete grains, which expel whey without fragmenting. For this purpose, there is an acceptable range of curd firmness or strength, occurring some time later than the point of gelation, implying a need to measure or infer the firmness of a gel as it forms and up to the point where it is ready to cut.

In traditional practice, the 'finger' or 'knife test' was used to monitor curd strength. This is an empirical assessment, based on experience, in which the cheesemaker makes a slight cut in the coagulum with a finger or knife and lifts the curd to see if there is a clean break and if clear whey is exuded, providing an indication of firmness and readiness for cutting.

As cheese manufacturing became increasingly mechanized and food safety issues became more critical, the commercial cheese factory began to operate around a series of enclosed vats with less opportunity for the cheesemaker to assess manually the gel strength or to suspend measurement devices into the vat during renneting. In addition, simultaneous operation of a suite of cheese vats required a time-based cycle where vats are filled and emptied in sequence allowing a fairly continuous flow of milk from the milk intake/pasteurizing plant. Various online devices for measuring curd formation were developed, which needed to be nonintrusive and cleanable in place.

Objective Measurements of Gel Firmness in the Laboratory

The Berridge Method

As research expanded to factors influencing milk clotting and in particular to the effectiveness and efficiency of different milk clotting enzymes, and as new clotting enzymes were isolated, whose clotting effectiveness needed to be evaluated, there was a need for more objective measurement of milk clotting. The Berridge method, also known as the rolling bottle method, published in 1952, was a more precise visual observation method than the cheesemaker's knife. A sample of milk with added enzyme was placed in a test tube and the test tube was rotated slowly in a water bath at controlled temperature until clots were observed as visible specs on the inside surface of the bottle and the time of flocculation was recorded.

Viscosity-Based Techniques

The measurement of viscosity changes accompanying milk coagulation was developed as a means of finding a method less dependent on detection by the human eye. Initially, the capillary method was used; that is, the time taken for successive milk samples to flow through a capillary tube was taken as a measure of viscosity changes. In time, the capillary method was superseded by the use of rotating spindle viscometers, giving almost instantaneous measurement of viscosity of milk up to the point of clotting. However, continuous viscometry is not considered an accurate method for monitoring coagulation because it disrupts bonds during gel formation, limiting useful measurement to some point where clotting begins to occur.

An instrument known as the Formagraph™ and which was based on the drag force technique was developed for evaluating curd-firming time in dairy laboratories. In this instrument, there were 10 wells for holding milk samples. The block containing the wells was oscillating slowly ($2\,\mathrm{min}^{-1}$) in the horizontal plane while a series of small pendulum loops were brought into contact with the milk samples. Each pendulum was part of a damped mechanical system with a means of plotting an amplified graph of the minute movement of the pendula onto a photographic paper by means of a reflecting mirror, which was part of the mechanical system. As a gel formed, increasing drag force on the pendula resulted in increased displacement of the pendula giving a graph with a bifurcated envelope, from which characteristic measurements could be obtained. Three parameters obtained using this instrument became widely used, namely rennet clotting time (RCT), which was the time from rennet addition until the milk began to gel; the 'rate of firming' (K20), which was the time from the start of gel formation until an amplitude of 20 mm was reached on the graph; and curd firmness (a30), the amplitude at 30 min. This system proved useful for research and for identifying problems with milk clotting in the cheese industry. Computerized instruments based on the Formagraph principle have recently appeared under names such as Lattodinamografo™ and computerized renneting meter (CRM™).

A minimally oscillated sensing needle was used to sense viscosity changes in milk by measurement of induced voltage; the system was named the Gelograph-M (not to be confused with the Gelograph-NT, which is an infrared system).

Viscometric methods detect changes related to the reduction in micellar size upon cleavage of κ-casein followed by an apparent increase in particle size due to agglomeration prior to gelation. The extent of flow involved in the measurement of viscosity during gelation needs to be kept very small to avoid disrupting weak structures.

Small-Amplitude Dynamic Rheometry

Computer-controlled dynamic rheometers were eventually developed, which were precision-engineered for low-amplitude oscillatory shear-testing, enabling gel firmness to be measured in the course of curd formation without exceeding the elastic limit of the gel. This approach has the advantage that milk coagulation can be monitored under quiescent conditions, corresponding to those in the cheese vat. A concentric cylinder geometry is normally used. The renneted milk sample is placed in a cylindrical cup and an inner cylindrical bob is immersed in the sample, leaving the sample occupying an annular gap of a few millimeter thickness, and a relatively large surface area through which shear stress can be applied. The cup is maintained at the renneting temperature of a cheese vat. A minute oscillatory shear stress is applied to the bob, subjecting the sample to a harmonic, low-amplitude shear stress. The resistance to shear of the sample, which increases with gelling and curd firming, controls the rotary displacement of the bob. The torque and rotary displacement of the bob are both measured, and are used to calculate the shear stress and strain on the sample in real time. As the phase angle between the oscillatory stress and shear displacement can be measured, viscous and elastic components of viscoelastic behavior can be determined. As the shear strain is kept within the elastic limit, curd firmness can be measured up to, and beyond, the point of gel cutting.

A very low-frequency vibrating probe (model Viscoprocess™ from Metravib, Lyon, France) was used to measure rheological properties of coagulating milk in laboratory trials. This instrument contains two piezoelectric ceramic devices arranged face to face, one of which oscillates at a frequency in the range of 10–50 Hz. The instrument measures a stiffness factor, which has been correlated with gel firmness. RCTs were successfully monitored over a range of temperatures.

Microscopic Analysis

Optical microscopy, combined with image analysis, has been used to follow milk coagulation. By counting protein agglomerates and measuring their average size, flocculation and coagulation were observed. The main value of this method is that it confirmed the mechanism by which a milk gel is formed.

Air Puff Technique

A noncontact device based on measurement of deformations due to puffs of air directed at the surface of the renneting milk, created by an air nozzle, was demonstrated. Surface deformation was measured using a laser displacement sensor. The device was mounted 4 cm above the milk surface. The results correlated to a high degree with reference measurements (G' and G''). The main challenges in adapting this technique commercially are ensuring hygienic operation and finding a way to have the device follow the surface of the milk when a vat is filled to different levels.

Ultrasonic Systems

Because ultrasonic wavelengths are of a similar order of magnitude to casein micelles, they are absorbed by agglomerating micelles at frequencies greater than 1 MHz, and can be used to monitor milk coagulation. Ultrasonic techniques (continuous and pulsed) have been studied for monitoring milk coagulation, using piezoelectric transducers at frequencies in the range of 1–400 MHz. It was found that gelation could be sensed either by an attenuation measurement or by a velocity measurement. While the potential of ultrasound to monitor milk coagulation was demonstrated, it was found that air bubbles in milk, and possibly fat globules, confounded some of the measurements. It was concluded that a significant amount of development work would be required to bring the technology to a stage where it could be used online in a commercial cheesemaking situation.

Electrical Conductivity

Electrical conductivity decreases during renneting, by about 0.5–1.0%, depending on the protein level and therefore could theoretically be used to monitor clotting. Changes in electrical conductivity during renneting were compared with viscosity measurements and elastic modulus measurement (by oscillatory rheometry). It was found that because of the large temperature coefficient of conductivity, it was necessary to minimize temperature gradients in the trials. Electrolyte variation due to salt also confounds conductivity changes as a measure of coagulation. Thus, this technique appears to be of limited use unless combined with other techniques.

Laboratory Techniques Based on Visible and Infrared Light

Colorimetry was originally designed to simulate the perception of the human eye. Reflection photometry was applied to the off-line measurement of milk coagulation using a Hunterlab colorimeter. Aggregation can be monitored in terms of the lightness (L^*), and blue–yellow (b^*), parameters. This can be explained in terms of Rayleigh's law of light scattering according to which diffusion of light is proportional to the cube of diameter for particles smaller than about one-fourth the wavelength of light, as would be the case for casein micelles when visible light is used (400–800 nm). For online monitoring of gel formation, colorimetry has been superseded by other optical techniques.

An instrument based on near-infrared (NIR) reflectance, and known as the CoAguLab (Reflectronics Inc., Lexington, KY, USA), has been developed for monitoring coagulation and pH during renneting. The instrument can monitor two samples simultaneously.

Rolling Metal Ball

Attempts were made to study milk clotting using a laboratory instrument based on a metal ball in a rotating sample bottle (Amelung™ coagulometer). However, this system, designed for the measurement of blood clotting properties, has not been applied commercially in milk testing laboratories. The continuous rolling motion of the metal ball must cause syneresis, and therefore limits the measurement to an early stage of coagulation. The technique is not useful for prediction of a coagulum cutting point.

Online Techniques for Monitoring Gel Formation

Various online techniques have been developed for monitoring milk coagulation, some of which have been adapted to commercial cheese vats. The development of a technique for online monitoring in a commercial vat is not trivial as several additional requirements must be met, as compared with the requirements for laboratory-based analysis. A commercial online in-vat sensor must be nonintrusive so that it does not interfere with any of the mechanical operations in the vat, and it must meet the most up-to-date dairy hygiene requirements; in particular, it needs to be cleanable in place.

Mechanical Systems

By the time that curd firmness was successfully measured at laboratory level, cheese manufacturers were expressing an interest in online measurement of curd formation, which would allow them to monitor curd formation in the cheese vat.

Several early methods for online detection of coagulum firmness were demonstrated in small cheese vats in research institutes. Some methods sensed the drag force on a suspended slowly moving body, such as a penetrometer, whereas others sensed the torque on a slowly rotating body suspended in the milk and were given names such as curd torsiometer and thromboelastograph.

One drag force device, named a 'Vatimer', was designed for the continuous measurement of coagulation of milk in open or closed cheese vats. A plunger, consisting of a 20-cm-long rod attached perpendicularly to a 10-cm-diameter disc, was suspended in the milk and oscillated vertically by a gear motor at 6 cycles per minute via a cantilever spring mechanism (maximum amplitude of motion, 2.4 cm). A strain gauge measured the increasing resistance to motion during coagulation. The plunger was removed before the cutting of the coagulum. It was shown that such instruments could be used to maximize yield by cutting curd at optimum firmness and optimizing the heal time.

Some early online systems, reported for monitoring milk coagulation, employed low-frequency compression wave transmission by means of low-speed harmonic motion of diaphragms or plungers that could be suspended in an open cheese vat. Such a system, described as a curd firmness tester, was developed by G. Vanderheiden at CSIRO and was based on opposing flexible diaphragms, to measure curd firmness. This system, and others using similar principles, contained a pressure transmission system that transmitted very low-frequency pressure waves through the cheese milk using a pair of transmitting/receiving diaphragms. As the coagulum formed and developed rigidity, the transmitter/receiver diaphragms became more effectively coupled and a graph of deformation of the receiver diaphragm represented the clotting of the milk as a sinusoidal wave with a sigmoidal envelope of increasing amplitude. This proved useful for research on cheesemaking.

All of these instruments followed rheological changes during milk coagulation in an empirical manner and were able to measure the effects of rennet concentration, added calcium, temperature, and pH of milk on curd-firming rate. Such systems, being rather intrusive, were not really suited to commercial cheesemaking as they required careful cleaning.

Vibrational Systems

Vibration probes are much less intrusive than mechanically moving systems and could be adapted to commercial cheese vats. Such systems may vibrate in a tuning-fork mode (they are sometimes known as acoustic probes) or in torsional mode, and either mode can be used for monitoring coagulation. In either mode, complex fluid viscosity can be measured by damping and phase-shift effects, which occur when a vibrating probe is immersed in a fluid such as milk. When such a probe is immersed in a cheese vat, the coagulation of milk can be followed directly by measuring the increasing viscosity prior to gelling and the increasing curd elasticity following gelling. Since vibrations can be generated and measured in such probes using the piezoelectric principle, they offer the advantage of not having mechanically moving parts, in contrast to the earlier vibrating reed viscometer. Hence, such probes are not subject to mechanical wear. It is possible to adapt such probes for insertion through the wall of a cheese vat using sanitary fittings.

It was shown that a vibration probe in torsional mode (Paar Physica Rheoswing™) at a frequency of around 8500 Hz could follow the coagulation of milk. However, the amplitude of vibration has to be controlled so as to minimize the risk of microsyneresis due to the amplitude of motion. This limits the degree of coagulation that could be measured. For this reason, a vibration probe for use in cheesemaking needs to be designed with a very low amplitude of vibration. Torsional vibration senses the product of dynamic viscosity and density (unit, N m) and this 'consistency' was found to decrease to a minimum for 10–20 min after rennet addition, the initial stage of hydrolysis of κ-casein, and then to increase progressively. The technique was used to monitor coagulation in renneted ultrafiltered milk up to a protein concentration factor of 4 and showed faster rates of increase in viscosity due to milk concentration, indicating faster setting and firmer coagulum.

Hot Wire Probe

The use of a hot wire in monitoring coagulation in cheese manufacturing is due to the principle that convective heat transfer from a heated probe immersed in a fluid is governed by the viscosity of the fluid. Changes in the viscosity of the coagulum in the course of gel formation (first decreasing as casein micelles become *para*-casein micelles, and then increasing rapidly as aggregation of the latter takes place) can thus be observed by measuring the heat transfer rate, or in practice by measuring the temperature difference between a heated probe and the milk. As the viscosity of a fluid is exponentially proportional to the temperature elevation of the hot wire, such a probe can determine a coagulation point very sensitively. The hot wire was the first technique that was suitable for in-place cleaning. In the 1980s, Tomoshige Hori at the Snow Brand Milk Products Co. in Japan applied a hot wire instrument to study the influence of curd-firming time relative to gel point on yield and quality of cheese in a commercial cheesemaking operation. He found that yield increased with the time of cutting the coagulum up to a certain point (*c.* 30 min after gel point), after which it leveled off. However, quality fell beyond that point, in terms of springiness (or elasticity), flavor, and overall acceptability. He also used the hot wire probe to verify Holter's equation, that is, that RCT is inversely proportional to enzyme concentration. He showed by addition of an acidifying agent (glucono-δ-lactone) that the constant of proportionality in Holter's equation decreases with increasing acidity, that is, with reducing pH.

While a hot wire sensor measures a gelation point quite accurately, it does not follow the curd-firming phase of coagulation. However, where coagulation rate was varied by rennet level, and where milk composition and renneting temperature were fixed, it was found that the hot wire inflection point could be used to predict the coagulum cutting point.

Optical Systems

It has been known for many decades that the coagulation of milk is accompanied by changes in optical properties (including color, absorbance, and reflectance) and this led to the use of optical measurement systems in monitoring milk coagulation.

Online Techniques Based on Visible and Infrared Light

Several techniques involving light absorption and scattering have been used to follow milk coagulation, for example, light absorbance at 600 nm. NIR has also been successfully used, for example, NIR reflectance at various wavelengths, such as 860 and 940 nm. The Gelograph NT (Gel Instrumente, Thalwil, CH 8800, Switzerland) is based on NIR transmission at 850 nm. Fiber-optic technology was exploited to develop online NIR reflectance probes for the prediction of optimum cutting times in commercial cheese vats. It was shown that the inflection point of the sigmoidal phase of the diffuse reflectance/time curve was well correlated with the Formagraph measure of cutting time, K20, when a protein correction factor was included in the prediction formula.

One such system is the CoAguLite™, a fiber-optic NIR reflectance probe that is used as an online device for predicting an optimum gel cutting point in cheese manufacture (**Figures 1** and **2**).

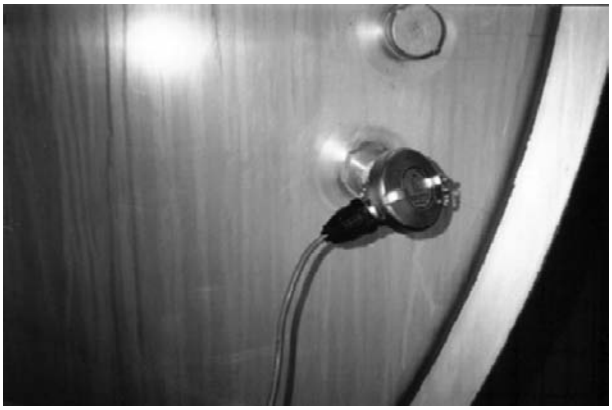

Figure 1 Fiber-optic CoAguLite™ probe inserted through the wall of a commercial cheese vat for the online monitoring of rennet-induced gelation of milk.

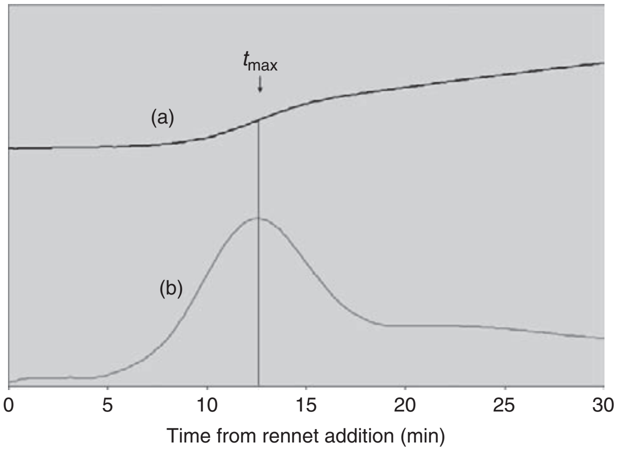

Figure 2 Typical near-infrared reflectance trace (a) and its first derivative (b) during gel formation in cheesemaking. The peak of the derivative gives the time of maximum slope (t_{max}) in the basic signal and corresponds to a maximum curd-firming rate. An optimum time for gel cutting can be predicted from t_{max}.

Diffusing Wave Spectroscopy

Dynamic light scattering (DLS), also known as photon correlation spectroscopy or quasi-elastic light scattering, can be used to determine the size distribution profile of small particles in suspension and it can, therefore, detect the aggregation of casein micelles. The principle of DLS is that light that is scattered from a moving particle experiences a Doppler shift. Because particles in a suspension experience Brownian motion, which is random in direction and amplitude, the scattered light will have a bell-shaped spectrum with a bandwidth proportional to the diffusion coefficient. This will be inversely proportional to the effective diameter of the particles by the Stokes–Einstein equation:

$$D = \frac{kT}{3\pi\eta d_{\text{eff}}}$$

where k is the Boltzmann constant, η the viscosity of the suspending liquid, T the absolute temperature, and d_{eff} an effective average particle size.

As the spectral bandwidth due to Brownian motion is very small compared to the optical frequency, autocorrelation techniques (in the time domain) have been more successful than the use of spectrum analyzers (in the frequency domain) for the practical determination of particle size. The application of digital autocorrelation techniques has given rise to the term photon correlation spectroscopy.

The application of DLS techniques in dense phase systems, such as milk, results in multiple scattering of photons and is known as diffusing wave spectroscopy (DWS). This technique is used to monitor rennet curd formation, using optic fibers to pass light into the milk and to collect backscattered light. An autocorrelation function is applied to the backscattered light to give a relaxation time from which a characteristic particle diameter is inferred, which is a measure of curd firmness. This begins to increase after a lag time, giving a coagulation time, which correlates with the visual coagulation time. The relaxation time (and hence particle diameter) increases up to the point of cutting the coagulum, showing that the technique could follow the entire gel-firming stage to which most online techniques are not well suited. The use of DLS/DWS can differentiate gels according to strength.

Up to the gel point, a linear relationship has been shown between viscosity/temperature and the DWS relaxation time, which is predicted for a dispersion where the particles are undergoing a hindered Brownian motion.

Conclusion

Considerable development has taken place in the monitoring of the renneting of milk, both at laboratory level and online. There is growing interest in the adoption of a process analytical technology approach, where the monitoring of milk coagulation is combined with the monitoring of other parameters, such as pH and milk composition, so that the combined effects of these parameters on cheesemaking can be determined and taken into account in an integrated process control system.

See also: **Analytical Methods**: Rheological Methods: Instrumentation. **Cheese**: Cheese Rheology; Curd Syneresis; Mechanization of Cheesemaking; Rennet-Induced Coagulation of Milk.

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Curd Syneresis

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Introduction

Syneresis is the loss of serum (whey) from the curd. Syneresis is a process whereby whey is separated from curd particles, and as a result of the expulsion of whey the curd particles shrink in volume. It is probably more correct to describe gel shrinkage as a process that forces or 'squeezes' whey out of the matrix through the pores. The aqueous phase in rennet gels is mostly physically trapped and not chemically bound. Rennet-induced milk gels remain apparently stable (i.e., no visible collapse) for several hours if the gels are left undisturbed but they synerese rapidly if disturbed by cutting or by wetting the gel surface. The tendency to exhibit syneresis in gels can be viewed as the reverse of the swelling behavior. Cheesemaking can be viewed as a dehydration process and syneresis is the crucial method by which most of the moisture is lost from curd particles. Since syneresis is the main method available to cheesemakers for controlling cheese moisture content, it is also the process that is mostly manipulated during cheesemaking and various dehydration approaches help to facilitate differentiation between cheese varieties (**Table 1**).

Measurement or Modeling of the Syneresis of Rennet Gels

Various (mostly empirical) techniques have been used to measure syneresis of curd (**Table 2**). Because of the complexity of modeling of shrinkage of curd particles in various dimensions, one-dimensional syneresis of thin gel slabs (where the diameter is much larger than the thickness) has been used to model the syneresis process. In these one-dimensional syneresis experiments, the gel is not cut but rather the surface is wetted, which is sufficient pressure to initiate syneresis. One-dimensional syneresis of rennet-induced milk gels is related to the flow of liquid (whey) through the network (since liquid flows out of the gel concomitantly with gel shrinkage) and is governed by the equation of Darcy:

$$v = \frac{Bp}{\eta x}$$

where v is the superficial flow velocity of the syneresing liquid, B the permeability coefficient, η the viscosity of the liquid, p the pressure acting on the liquid, and x the distance over which the liquid must flow.

The permeability of renneted gels increases with time after renneting (even when the gel is not cut or broken) due to microsyneresis or the formation of larger pores in the matrix. Initially, the rate of syneresis is very high but due to shrinkage, the gel becomes locally denser, the rheological modulus increases, and permeability decreases. Thus, after the gel has undergone considerable contraction (when shrinkage is initiated by surface wetting), further shrinkage is inhibited by the reduced permeability and the increased resistance of the matrix to further deformation. In the model of Darcy, it is assumed that the matrix allows the outflow of whey without collapsing or resisting. However, in the case of rennet gels, rapid shrinkage of curd particles occurs. In practice, syneresis of curd particles occurs in three dimensions simultaneously and is much harder to study than the one-dimensional model. Curd grains rapidly collapse once removed from (being suspended in) whey, making measurements difficult.

Mechanism of Syneresis

The initial rennet-induced gel should be viewed as a weakly stabilized, transient (dynamic) network. Hydrolysis of the κ-casein hairs on casein micelles results in the loss of both steric and charge stabilization mechanisms. The renneted micelles then aggregate and form a network. The interactions between rennet-altered micelles are weak (ionic bridges, hydrophobic interactions) and the resultant matrix has high bond mobility (or bond relaxation, as indicated by the high values for the loss tangent parameter from rheological measurements). If bonds between aggregating particles are reversible (at least for a short period after gelation), rearrangements may occur in the aggregates/clusters formed as well as in the gel network. In the initial rennet gel network, bonds are breaking and reforming, which increases the possibility of rearrangements.

Experienced cheesemakers know that if they wish to promote syneresis (or decrease the cheese moisture content) they should cut the rennet gel when it is still very weak. After gelation, there is ongoing particle fusion and the formation of additional cross-links between the caseins. With increasing time after renneting, rennet gels

Table 1 Approaches used to increase or decrease the syneresis (moisture content) of cheese curds

Decrease moisture content	*Increase moisture content*
Avoid excessive whey protein denaturation	Use high heat treatment of milk (or the use of ingredients with denatured whey proteins, such as starter media)
Avoid homogenization of cheesemilk	Use homogenization of the cheesemilk
Use milk with normal protein levels (e.g., 3.5%)	Use liquid precheese
Avoid preacidification of milk	Use preacidification of milk
Cut curd into smaller pieces	Cut curd into larger pieces
Cut the gel softer, that is, sooner after visible gelation	Cut the gel when it is firmer, that is, wait longer after visible gelation
Use higher cooking temperatures	Use lower cooking temperatures
Use longer cooking (stirring) times	Use shorter cooking (stirring) times
Use hot water for washing or rinsing curd	Use cold water for washing or rinsing curd
Use more dry-stirring of curd after whey drainage (stirred curd)	Avoid dry-stirring of the curd or use shorter stirring times
Apply pressure in forms/molds/hoops	Do not press curds in molds
Apply mechanical dewatering of curd (e.g., separators or membrane filtration)	Avoid the use of mechanical dewatering devices
Use higher salting level	Use lower salt level

Table 2 Techniques used to quantify syneresis

One-dimensional shrinkage of curd slabs
Amount of whey expelled as a result of syneresis
Monitoring of tracer dyes
Dry matter content of curd sampled during shrinkage
Density of curd grains
Light scattering properties of the curd/whey mixture
Low-resolution nuclear magnetic resonance (NMR)

increase in stiffness and in resistance to deformation, which act to reduce the ability of the network to rearrange its microstructure. Thus, waiting for the gel to become firmer before cutting makes it harder for that gel to undergo extensive syneresis and therefore the cheese has higher moisture content.

There is some tendency or driving force promoting increased casein interactions. Recently, views of syneresis suggest that this tendency could be viewed as a type of phase separation in a viscoelastic transient gel system. It is possible that the renneted micelles have surfaces that are only partly attractive (due to the high pH) and this promotes shuffling of particles to reduce repulsion (increase attraction). It could also be that the completion of the hydrolysis of all the κ-casein hairs by rennet, after the formation of a weak network, alters the attractive/repulsive balance in the system. The incorporation of additional particles in the network (i.e., micelles where hydrolysis of κ-casein was completed only after network formation) results in the formation of new physical cross-links between protein strands, which may promote tensile stresses in the system resulting in strand breakage. Micelles that are only partly attached to the network (dangling ends) at the point of gelation could become 'fully' attached to the matrix with aging.

An important aspect of the syneresis mechanism in cheese curd is the ability of the initial gel (coagulum) to retain its shape after cutting. Cheesemakers wait until they can subjectively determine that the gel can withstand the cutting process. Often they evaluate this by cutting the gel with a spatula/knife and they observe if the cut gel surface does not rapidly collapse. The retention of structure in the curd pieces is critical in the creation of a large amount of exposed surfaces through which whey/serum can easily be expelled. The weight of the curd particles and gravity and collisions between curd particles (e.g., as a result of stirring) encourage the compression/deformation of curd particles, which promotes squeezing out of whey.

In order for rennet gels to undergo syneresis, the network must be flexible enough to be able to rearrange itself into a smaller and more compact matrix. Syneresis of gels can occur either spontaneously or more commonly as a result of some physical stresses applied during cheesemaking. Syneresis can also occur in gels due to environmental changes, for example, decrease in pH or increase in temperature. It has been suggested that there is in rennet gels an 'endogenous syneresis pressure', that is, a pressure within the gel that is causing spontaneous syneresis or the syneresis of wetted gels. It has not been possible to measure experimentally this endogenous pressure since the predicted values are very low. The rate of syneresis increases initially as a function of time after renneting but decreases at longer times, presumably due to fusion of *para*-casein micelles and a reduction in the permeability of the contracting network.

The mechanism responsible for the strong tendency of rennet-induced milk gels to synerese is related to (extensive) rearrangements of the casein network, which occur after gel formation. The rearrangement process is accelerated and is more extensive at high temperatures. Aging of rennet-induced gels results in a coarsening (sometimes called 'microsyneresis') of the gel (i.e., rearrangements) and an increase in the fractal dimensionality.

In rennet-induced milk gels, low gel stiffness (elastic modulus) and high values of the loss tangent (tan δ at low frequencies) are important rheological conditions that facilitate rearrangements of bonds (when these rheological measurements are made at approximately the same timescale over which rearrangement processes related to syneresis in these gels are estimated to occur).

Rearrangements of casein particles into a more compact structure would increase the number of bonds and hence decrease the total free energy of the system. However, the particles are part of the gel network, which must be deformed or broken locally to form new junctions. In cheesemaking, conditions such as cutting, stirring, acid production, and the increase in temperature that occurs during cooking all encourage syneresis and the rearrangement processes that facilitate syneresis of the gel network.

Factors That Impact Syneresis

Syneresis is very much dependent on the pH of curd. Small adjustments of pH (i.e., decreasing pH from 6.5 to 6.3), such as in the preacidification of cheesemilk, can result in an overall reduction in syneresis. This is due to the increase in the stiffness of rennet gels that occurs with the small reduction in pH, which is due also to the reduced electrostatic repulsion (and thereby increased attractive interactions) between particles. A larger decrease in pH (e.g., from pH 6.3 to 5.2) promotes greater syneresis of rennet gels. This is presumably due to solubilization of intraparticle insoluble calcium phosphate cross-links, which increases the flexibility of casein particles. Acid-induced milk gels (pH 4.6) undergo much less syneresis than rennet-induced gels (**Figure 1**). Due to the different interactions present in acid-induced gels, the bonds are less mobile (lower loss tangent) and strands are less susceptible to rearrangements.

High heat treatment of milk results in reduced syneresis. High heat treatment (greater than pasteurization) causes denaturation of whey proteins, and at the normal pH of milk, most of these whey proteins become associated with micelle surface. The presence of denatured whey proteins on the micellar surface impedes fusion of renneted micelles and limits the rearrangement of strands and clusters, which is a requirement of the syneresis process.

Syneresis is enhanced by higher coagulation or cooking temperatures. Temperature increases the thermal motion of particles and strands in the network. The stiffness of the rennet gel also decreases with increasing cooking temperature. There is a decrease in the voluminosity (particles shrink in size) with increasing temperature, which reduces the potential contact area between clusters/strands.

Rennet gels made from highly concentrated milk, where the milk is concentrated to the required total solids content of that cheese variety (i.e., liquid precheese), hardly undergo syneresis. Presumably the resistance to deformation or breakage of strands in the matrix is too high to facilitate syneresis. In highly concentrated milk, the number of protein–protein bonds in the strands and junctions of the gel matrix becomes very high. This lack of syneresis is exploited in the so-called cast cheese (e.g., UF cast Feta), where an ultrafiltration retentate is heated and along with rennet and cultures/enzymes is filled directly into containers where it sets to a gel (cheese).

Proteolysis of caseins, for example, by enhanced plasmin activity in mastitic or late-lactation milk, results in

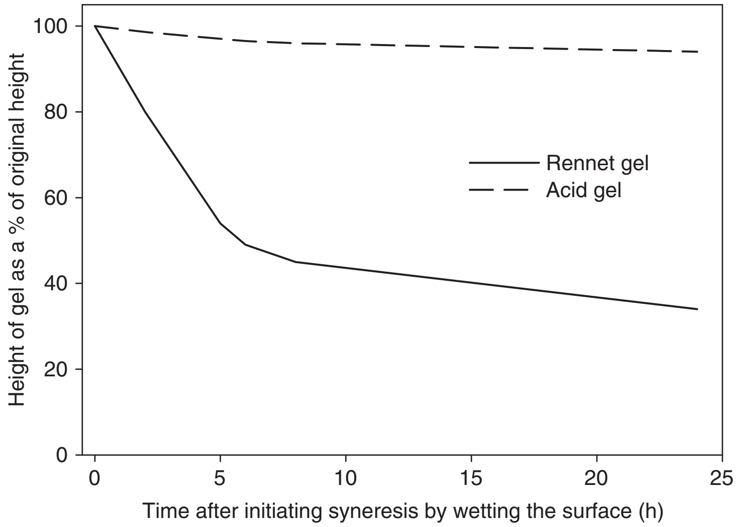

Figure 1 Syneresis of one-dimensional slabs of rennet-induced (solid line) and acid-induced (dotted line) gels as a function of time. Rennet gels were tested 1 h after rennet addition and the acid gels were tested at pH 4.6 for gelation induced by glucono-δ-lactone. Reproduced from Lucey JA (2001) The relationship between rheological parameters and whey separation in milk gels. *Food Hydrocolloids* 15: 603–608 with permission from Elsevier.

weak gels. Cheeses made from milk that has degraded caseins also have higher moisture contents. Presumably, this reflects a better contraction ability of intact rennet gels compared to gels made from hydrolyzed caseins.

See also: **Cheese**: Acid- and Acid/Heat-Coagulated Cheese; Cheese Rheology; Gel Firmness and Its Measurement; Overview; Rennets and Coagulants; Rennet-Induced Coagulation of Milk; Salting of Cheese. **Heat Treatment of Milk**: Heat Stability of Milk. **Milk Proteins**: Casein, Micellar Structure.

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Salting of Cheese

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Introduction

Salting of perishable foods is among the most ancient and widely practiced techniques of food preservation. Salt has achieved universal acceptance as a mineral of great importance in trade and industry, and, in view of its preservative qualities, it has become a peculiarly appropriate symbol of fidelity in many cultures. It is therefore no surprise that salting is a key element in the combination of techniques that has evolved for preserving the solids of milk in the form of cheese.

Most natural cheese varieties, apart from fresh, short shelf life, acid-curd types with a low pH (4.5–4.8) such as Fromage frais, Quark, and related types, contain added salt (NaCl). For practically all cheese varieties, apart from Domiati, the salt is added to the curd toward the end of manufacture. It may be: added to subdivided cheese curds, as is the case with Cheddar and related types; applied by immersion of the formed cheese in brine (aqueous solution of NaCl), as for brine-salted cheeses (e.g., Gouda, Swiss, Feta, and related types); or rubbed onto the surface after molding is complete, as in some Blue cheeses. The presence of salt in the cheese and the manner of its incorporation have a significant impact on the course of fermentation of lactose to lactic acid and on the, microbiology, biochemistry, and final characteristics of the cheese as consumed.

Role of Salt in Cheese

Salt in cheese serves two major functions; namely, it acts as a preservative and directly contributes to flavor. In addition to these functions, salt exerts a number of important effects on cheese (**Figure 1**).

Preservative Effect

The salt in cheese is held in solution in the aqueous phase, and its concentration in solution (salt-in-moisture content, S/M) is a strong determinant of the growth of microorganisms. The preservative action of NaCl derives from its depressing effect on the water activity (a_w) of the cheese:

$$a_w = \frac{p}{p_0}$$

where p and p_0 are the vapor pressure of water in a cheese and of pure water, respectively. On exposure to air of a given relative humidity, water in the cheese, which does not contribute to its vapor pressure, may be considered as not being free, and not available for microbial growth. The a_w of cheeses ranges from ~0.99 in Quark to 0.92 in Parmesan, and the minimum a_w required for growth ranges from ~0.8 for most yeasts and molds to ~0.95–0.96 for pathogenic bacteria such as *Escherichia coli* and *Yersinia enterocolitica*. The a_w of young cheese is determined principally by the concentration of NaCl in the free aqueous phase (~10% of the water being bound to proteins), and may be calculated by the relationship

$$a_w = 1 - 0.033[\text{NaCl}_\text{m}] = 1 - 0.005\,65[\text{NaCl}]$$

where $[\text{NaCl}_\text{m}]$ is the molality of NaCl, that is, moles of NaCl per liter of H_2O, and [NaCl] is the concentration of NaCl in g 100 g^{-1} cheese moisture. Solutes other than NaCl in the aqueous phase of fresh cheese also make a contribution to lowering of the vapor pressure. These include: residual lactose, lactic acid, and other by-products of fermentation; soluble minerals (mainly calcium and phosphate ions liberated from the casein as the pH falls during cheese manufacture); and nonprotein nitrogenous compounds from the cheese milk. As cheese matures, water activity falls in most varieties owing to increasing concentrations of low-molecular-weight solutes (such as peptides, free amino acids, water-soluble free fatty acids) arising from the metabolism of lactose/lactic acid, proteins, and lipids. Dehydration during ripening may lead to further reduction in a_w for those cheeses matured in their own rind. In addition to its effect on water activity, salt increases the osmotic pressure of the aqueous phase of foods, causing dehydration of bacterial cells, killing them, or, at least, preventing their growth.

Pathogenic organisms vary widely in salt tolerance, some growing best only at a salt level below 2%, some tolerating 2–6% salt, and a few able to grow at over 5–6%

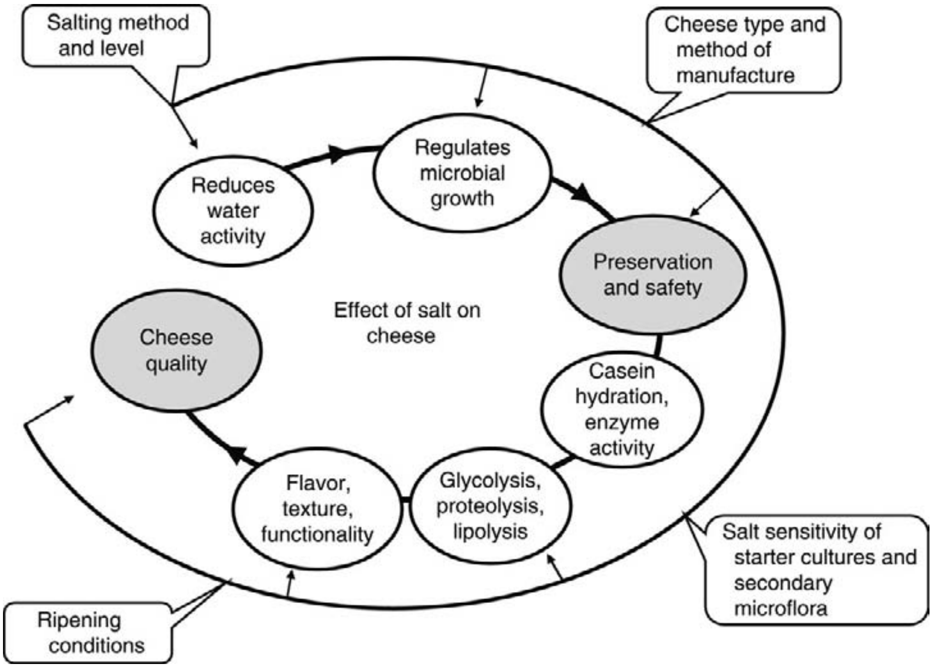
Salting method and level
Cheese type and method of manufacture
Reduces water activity
Regulates microbial growth
Preservation and safety
Effect of salt on cheese
Cheese quality
Casein hydration, enzyme activity
Glycolysis, proteolysis, lipolysis
Flavor, texture, functionality
Salt sensitivity of starter cultures and secondary microflora
Ripening conditions

Figure 1 Salt contributes to preservation, safety, and overall quality of cheeses, by its effects on water activity, microbial growth, protein hydration/confirmation, and enzymatic activities, which in turn influence biochemical changes such as glycolysis, proteolysis, and lipolysis.

salt. Thus, in cheese, salt generally does not act alone as a defense against pathogens, but must be considered as one of the 'hurdles' protecting cheese from spoilage, along with limiting infection during milk production and harvesting, effective heat treatment of cheese milk (pasteurization), active starter growth producing the desired pH drop, and plant hygiene.

Regulation of the Growth of Starter Bacteria, Nonstarter Bacteria, and Blue Mold in Cheese

The populations of starter culture bacteria and types of non-starter lactic acid bacteria (NSLAB) in cheese can significantly influence the flavor and sensory properties of cheese (*see* **Cheese**: Cheddar-Type Cheeses; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Starter Cultures: General Aspects; Starter Cultures: Specific Properties; Swiss-Type Cheeses). The growth of mesophilic lactococci strains most commonly used in cheese fermentation (e.g., *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*) is stimulated by S/M levels of 2–3% (w/w), but is inhibited at higher levels, with most strains exhibiting growth inhibition at S/M concentrations ≥5%. *Streptococcus thermophilus*, a starter bacterium frequently used in high cook temperature cheeses such as Romano and Mozzarella, is considerably less salt-tolerant than *Lc. lactis* ssp. *lactis*; its critical NaCl concentration is 0.4 mol l^{-1} (2.34%, w/w), corresponding to an a_w of 0.984, compared with 1.1 mol l^{-1} NaCl ($a_w = 0.965$) for *Lc. lactis* ssp. *lactis*. Similarly, other thermophilic bacteria, including *Lactobacillus helveticus* and *Lb. delbruechii* ssp. *lactis*, have also been found to be less salt-tolerant than mesophilic lactococci, being inhibited by 0.95 and 0.90 mol l^{-1} NaCl, respectively.

Propionibacteria are used as part of the starter culture in the manufacture of Emmental-type cheeses in which they metabolize lactic acid to produce CO_2, the gas responsible for the characteristic eyes, and propionic acid, which is thought to contribute to its sweet nutty flavor. *P. freudenreichii* was the most salt-tolerant of the propionic bacteria species investigated, its critical NaCl concentration being 1.15 mol l^{-1} (~6.7%, w/w; $a_w = 0.955$). However, as for *Lactococcus*, salt sensitivity is dependent on strain and growth medium.

It is generally agreed that NSLAB are more salt-resistant than starter bacteria. Indeed, it has been found that ~90% of NSLAB strains (*Lb. casei*, *Lb. plantarum*, and *Lb. curvatus*) isolated from commercial Cheddar grew in the presence of 6% (w/w) NaCl, whereas 58% grew in the presence of 8% (w/w) NaCl. Nevertheless, the population of NSLAB in reduced-salt Cheddar (1.0%, w/w) has been found to be significantly higher than that in the control (1.8%, w/w), suggesting that salt level has a major effect on the growth of NSLAB.

Salt significantly affects the growth of *Penicillium roqueforti*, a key organism responsible for the typical appearance and flavor of blue-veined cheese; 3–6% salt prevents germination of the spores and the associated generation of blue color. This explains the mold-free zone seen in the surface regions of brine-salted blue-veined cheese.

Enzyme Activity

Enzymes that are significant in cheese for modification of texture and formation of flavor compounds include indigenous milk proteinases (most notably plasmin), residual coagulant proteinase(s), lipases and esterases from rennet paste and/or added molds, plus proteinases and peptidases from the starter, secondary flora, and adventitious bacteria. Salting has an influence on the activity of most of these enzymes; the activity of water-soluble enzymes decreases with the decrease in water activity as salt level increases. The proteolytic activity of the residual coagulant on casein in dilute model systems ($\leq 5\%$, w/w, casein) is strongly influenced by salt concentration: hydrolysis of the α_{s1}-casein is stimulated by increasing S/M up to about 6% (w/w), whereas proteolytic breakdown of the β-casein is inhibited at 5% (w/w) S/M.

For cheeses such as Emmental, in which plasmin plays a dominant role in the primary proteolysis of the major caseins, the effect of salt is critical. This enzyme is stimulated at S/M levels up to about 2% (w/w), but its activity decreases at higher levels.

The salt level in cheese also affects microbial proteinases. Intracellular enzymes of *S. thermophilus* and some strains of *Micrococcus* show up to 80% less activity in the presence of salt. Proteolysis in Camembert-and Blue-type cheeses is markedly affected by elevated salt levels, with attendant effects on the sensory properties.

Flavor Effect

NaCl directly contributes to the 'saltiness' in cheese, a flavor that is generally highly appreciated. The flavor of salt-free cheese is insipid and 'watery', and a minimum concentration of 0.8% (w/w) NaCl is required to overcome the insipid taste. NaCl also contributes indirectly to flavor by its controlling influence on microbial and enzymatic activity, which in turn influences lactose metabolism, cheese pH, degradation of fat and casein, and the formation of flavor compounds such as peptides, free amino acids, and free fatty acids.

Casein Hydration

Casein hydration is generally considered to be the water that is more or less immobilized by a protein. Salt, together with pH and calcium level, has a large effect on the extent of *para*-casein hydration, or aggregation, which in turn affects the water-binding capacity of the casein matrix, its tendency to synerese, its rheological and textural characteristics, and its cooking properties. Generally, the addition of salt at a level in the range of 0–2% (w/w) to dilute casein dispersions (e.g., 2–10%, w/w) increases casein hydration, resulting in an increase in solubility and viscosity. This effect is probably due mainly to the increase in ionic strength, but also to a possible partial exchange of calcium attached directly to the casein for the sodium ion. Though increased hydration is desirable in many processed and formulated food products, it is generally highly undesirable during rennet gelation of milk and the early stages of curd manufacture, where the opposite effect (casein aggregation) is being promoted (e.g., via selective enzymatic cleavage of the casein, acidification, and/or heating) to enable the formation of a casein gel, gel syneresis, and curd formation/recovery. The addition of salt to cheese milk, even at low levels $>1\,\mathrm{g}\,100\,\mathrm{g}^{-1}$, severely impairs the ability of the milk to gel and the ability of the resultant gel to synerese. This is one of the main reasons why salt is added to curd at the end of manufacture rather than to the milk. Nevertheless, salt is added, at a level of 5–15% (w/w) NaCl, to the milk for Domiati cheese manufacture, but its adverse effects on curd formation are offset by the use of water buffalo milk, which has a higher casein content than bovine milk, by the fortification of milk with skim milk powder, and/or by the addition of calcium chloride. However, the addition of NaCl to milk promotes a texture/mouthfeel (short, nonchewy, 'dissolves' relatively easily) unique to Domiati, among the hard cheeses. On the contrary, apart from its contribution to flavor and as a means of controlling microbial and enzymatic activity, the positive effect of adding salt to curd/cheese at the end of manufacture (when milk casein and fat are recovered in the form of a curd with the desired moisture content) is desirable for its enhancement of casein hydration and water binding. This is essential in facilitating the knitting of dry-salted curd chips in dry-salted cheeses during pressing, and for the formation of the desired viscoelasticity in rennet-curd cheeses, which in turn (along with proteolysis, pH, and calcium control) is a central component in the development of the desired texture (smooth, cohesive, not excessively rubbery) and cooking properties (e.g., ability to melt into a cohesive mass that flows) during maturation.

Methods of Salting Cheese

There are three principal methods of salting cheese curd:

- *Brine salting* or *brining* – immersion of molded cheese curds for a period, typically ranging from ~0.5 to 5 days, in brine, which is a pH-adjusted (~5.2) salt solution (~18–25%, w/w, NaCl) containing added (0.2%, w/w) calcium (e.g., Edam, Gouda, Saint Paulin, Provolone).

- *Dry salting* – direct addition and mixing of dry salt crystals to broken or milled curd pieces for a period of ~10–30 min prior to molding and pressing (e.g., Cheddar and Cottage cheese).
- *Surface dry salting* – rubbing of dry salt or salt slurry to the surface of the molded curds (e.g., Blue-type cheeses).

Sometimes, a combination of the above methods is used.

Variations of brine salting that have been evaluated in experimental studies include brine injection under pressure (e.g., 17 MPa); high-pressure brining (e.g., at isostatic pressures up to 500 MPa); acoustic brining using high-intensity (300 W) ultrasonic (30 kHz) waves; and vacuum impregnation brining at 3.7 kPa, absolute.

Brine Salting

Brine-salted cheeses are formed into their final size and shape prior to being immersed in brine for periods ranging from a few hours to a few days in vats containing circulating or static brine. Static brine systems usually have undissolved salt at the bottom of the vats and stirring must be carried out frequently. Circulating systems have means for automatically maintaining the strength of the brine. Brine concentration typically ranges from 18 to 25% (w/w) NaCl in water, and temperature may vary from about 8 to 20 °C. The salting time depends primarily on the desired salt content, and is further influenced by the following:

- brine temperature (diffusion rate increases with temperature)
- salt concentration (higher concentration gives higher salt uptake, but causes more extreme variations in the salt level of the freshly salted cheese)
- cheese dimensions (smaller and flatter cheeses take up salt more rapidly; a spherical cheese takes up salt more evenly)
- cheese moisture and pH (both higher moisture and higher pH lead to higher salt uptake)

As the specific gravity of cheese is lower than that of brine, the cheeses float on top of the brine solution with their upper face exposed. Hence, to ensure uniform uptake of salt through all surfaces of the cheese, various means are provided, such as keeping the cheese beneath the brine surface (e.g., the placing of cheese in brine cages that are submerged), spraying the exposed surfaces with brine, and/or placing dry salt on this surface. With repeated exposure to immersed cheeses, the brine becomes equilibrated to the aqueous phase of the cheese with respect to pH and all soluble constituents (e.g., Ca, K) except NaCl. However, whenever fresh brine is prepared, it is usual to adjust both the pH and the calcium content to those of the cheese serum, thus avoiding problems of softened surfaces and high surface pH caused by leaching of calcium and lactic acid, respectively. One or more of the various methods, which include filtration, microfiltration, heat treatment, and chemical sanitation, are adopted to maintain the clarity and microbiological quality of brine.

Dry Salting

In dry-salted cheeses, the curd grains after whey drainage are fused into a cohesive mass (e.g., Cheddar-type cheeses, Stilton), which is kept warm (e.g., ~30–38 °C) for some time before salting to allow the pH to reach almost the final value (e.g., 5.4–5.1) sought for the variety in question. The curd mass is then mechanically milled into small pieces, typically about the size of a potato chip (~7 × 1.2 × 1 cm^3), in the case of Cheddar or Cheshire, or alternatively mechanically broken into relatively large irregular-shaped pieces (up to 0.25 kg) for Stilton cheese. Dry, powdered crystalline salt is then added to the milled curd on a weight/weight basis and distributed over the surfaces by thorough mixing, which is generally performed in a tumbler or on a rotating belt where the curd bed (on the belt) is raked to facilitate mixing of curd and salt. Following a period of time for salt absorption (frequently denoted by "mellowing" in the case of Cheddar cheese), the milled salted curds are formed into the shape of the final cheese by application of pressure with, or without, an associated vacuum treatment. Like all other stages/operations of manufacture, dry salting in modern cheese factories is highly automated, with precise continuous measurement of curd flow on the belts and the levels of salt added.

Salt Uptake and Moisture Loss during Salting

Brine-Salted Cheeses

When a molded cheese is placed in brine, there is a net movement of Na^+ and Cl^- from the brine into the cheese as a consequence of the concentration difference between the cheese moisture and the brine. Consequently, moisture, along with other diffusible solutes in the cheese moisture such as lactate and soluble salts, diffuses out through the cheese matrix so as to restore osmotic pressure equilibrium between the cheese moisture and the brine.

Model studies involving the brining of cheese under conditions designed to obey Fick's law for one-dimensional brine flow into cheese have concluded that the penetration of salt into cheese and the concomitant outward migration of moisture during brining occur as an impeded diffusion process; that is, NaCl and H_2O move in response to their respective concentration gradients, but

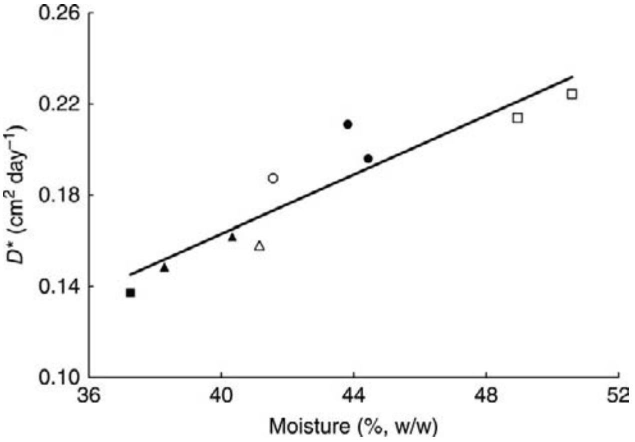

Figure 2 Relationship between the moisture content of unsalted cheese and the pseudo-diffusion coefficient (D^*) for NaCl in cheese moisture. Different cheese varieties were salted in ~20% (w/w) NaCl brine at 15–16 °C: Blue cheeses (□), Gouda cheese (•), Romano-type cheese (○), Jarlsberg (△), Emmental cheeses (▲), and unsalted milled Cheddar (■) (redrawn from Morris, Guinee, and Fox, 1985).

their diffusion rates are much lower than those in pure solution due to a variety of impeding factors. The (pseudo) diffusion coefficient for NaCl in cheese moisture, D^*, is typically 0.2 cm^2 day^{-1}, though it varies from ~0.1 to 0.35 cm^2 day^{-1} with cheese composition (**Figure 2**) and brining conditions, compared to the diffusion coefficient of 1.0 cm^2 day^{-1} for NaCl in pure water, D, at 12.5 °C. The lower diffusion rate of NaCl in cheese moisture as compared to water may be understood by viewing cheese as a concentrated protein matrix of interlocked para-casein strands. The aqueous phase is entrapped in this matrix along with a roughly equal mass of fat in the form of discrete globules with a mean diameter of 4–5 μm. On diffusing from the brine (region of high concentration) through cheese moisture (region of low concentration) held in such a matrix, the progress of Na$^+$ and Cl$^-$ is retarded by the following structural elements:

- obstructing fat globules/pools and protein aggregates around which the diffusing ions must proceed, and, thereby, travel an extra pathlength on proceeding from one region to another within the cheese
- the mechanical sieving effect of the pores of the protein matrix; this is analogous to the restriction a sphere encounters when moving through a pipe of varying aspect and diameter which at its smallest is comparable to that of the sphere
- the net outflow/loss of H_2O (for which the sieving effect is lower than for the larger hydrated Na$^+$/Cl$^-$ ion pair), which causes the plane of zero mass transfer (a plane where the average flux of all diffusing species is zero) to recede from the cheese/brine interface into the brine, thereby creating an additional pathlength through which the Na$^+$ and Cl$^-$ must migrate before reaching the cheese surface
- the high relative viscosity of cheese moisture (~1.27 times that of pure water at 12.5 °C), which contains dissolved substances that inhibit the progression of Na$^+$ and Cl$^-$ by collision and by their charged fields

Moreover, not all moisture in cheese, some of which is bound by the protein (~15 g 100 g^1 moisture), is free and available for diffusion.

As salt (from brine) and moisture (from cheese) migrate in opposite directions during diffusion, salt uptake by cheese during the brine-salting process is accompanied by a simultaneous moisture loss. Consequently, there is an inverse relationship between the levels of salt and moisture in cheese. This is most readily observed in brine-salted cheeses immediately after salting, where a decreasing salt gradient from surface to the center is accompanied by a decreasing moisture gradient in the opposite direction (**Figure 3**). The quantity of moisture lost by the cheese is related to the quantity of salt absorbed, by the proportionality factor, p, known as the flux ratio:

$$-\Delta W_x = p\,\Delta S_x$$

where ΔW and ΔS are the changes (from the unsalted cheese) in the g H_2O and g NaCl, respectively, per 100 g cheese solids-not-salt in planes of cheese at a distance of x cm from the cheese–brine interface; the minus sign indicates a net loss of water from the cheese to the brine. The value of the flux ratio has been found to be >1 and varying with location, from ~3.5 at the cheese–brine interface to ~1.5 at the salt front within the cheese mass, depending on brining

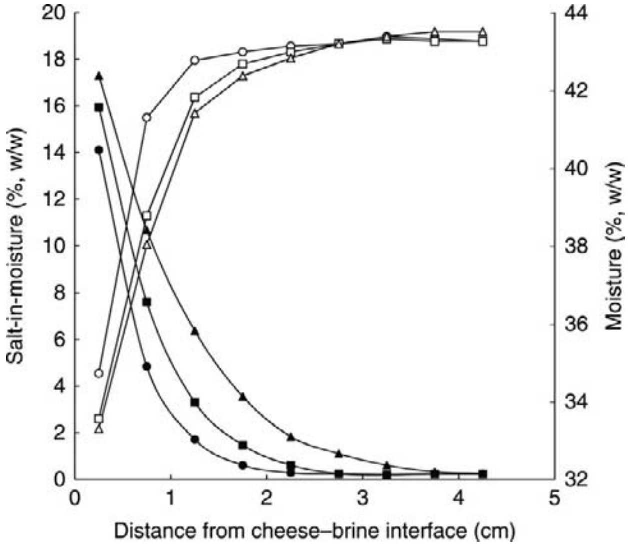

Figure 3 Moisture content (open symbols) and salt-in-moisture concentration (closed symbols) in experimental Gouda-type cheese (fat-in-dry matter, 49.1%, w/w; moisture, 43.64%, w/w; pH before salting, 5.26) as a function of distance from the salting surface after one-dimensional brine salting for 1 (○, ●), 3 (□, ■) or, 4 (△, ▲) days at 15 °C. Cheeses were suspended over the brine bath so that only a flat surface of the cylindrical-shaped cheeses was in contact with the brine, which was agitated gently at intervals of ~12 h.

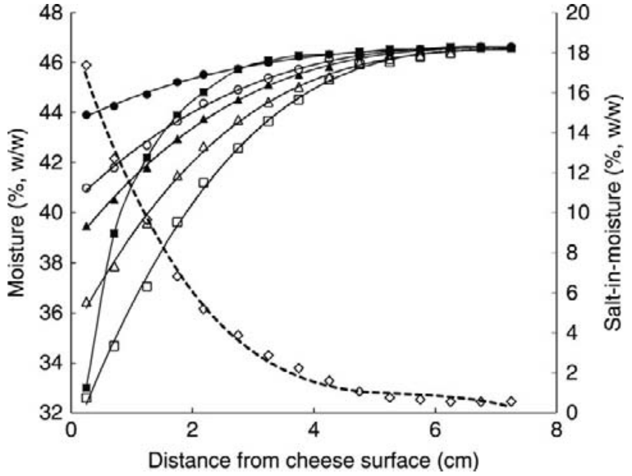

Figure 4 Experimental (■) and theoretical moisture levels (●, ○, ▲, △, □), and experimental salt-in-moisture concentrations (—) in Romano-type cheese after one-dimensional salting in brine (19.3%, w/w, NaCl) for 9 days at 20 °C. The theoretical moisture levels were calculated from the relationship, $-\Delta W_x = \quad p\,\Delta S_x$ for values of p = 0 (●), 1 (○), 1.5 (▲), 2.5 (△), and 3.75 (□).

conditions and curd composition (**Figure 4**). The average over the whole region of salt and water diffusion in cheese has been found to be ~2.0, indicating that the weight of water lost during brine salting is typically about twice the weight of salt absorbed. This trend is consistent with the fact that the size of the diffusing hydrated Na^+/Cl^- ion pair is approximately twice that of H_3O^+/OH^-.

A comparatively long contact time is needed for salt uptake because of the low surface area-to-volume ratio of the cheese. Consequently, as the surface of the cheese is in contact with concentrated brine for a long time (several days in many cases), there is considerable contraction of the curd surface (due to the salting-out of protein), which slows down the uptake of salt. After removal of the cheese from the brine, the contracted surface layer may dehydrate even further under the influence of storage conditions (surface coating, relative humidity (RH) of the store, air movement in the store), thus forming a protective rind.

Factors affecting salt uptake during brine salting

The quantity of salt absorbed during brine salting is affected by numerous factors, of which the major ones are discussed below.

Brine concentration and concentration gradient

Higher levels of NaCl in the brine give higher rates of salt absorption and increased S/M levels in the cheese. While the rate of NaCl diffusion through the cheese is scarcely affected by brine concentration in the range, of 5–20% (w/w), the rate of uptake increases at a diminishing rate with increasing brine concentration in the same range. However, increasing NaCl concentrations $\geq$25% (w/w) in brine may cause a decrease in the level of salt absorbed, depending on the pH of the cheese prior to brining and brining temperature; this effect is associated with dehydration in the surface layer that impedes salt absorption.

Salting time

The quantity of salt absorbed in brine-salted cheeses increases with brining time, albeit at an ever-diminishing rate, as the NaCl concentration gradient between the cheese moisture and the brine decreases. Experiments using one-dimensional brine flow into cheese showed that the quantity (mass) of salt absorbed per unit surface area of flat surface is proportional to the square root of brining time:

$$M_t = 2(C - C_o)\left(D^* \frac{t}{\pi}\right)^{1/2} W \qquad [1]$$

where M_t is the quantity of salt absorbed per unit surface area over time (g NaCl cm^{-2}); C, the salt content of brine (g NaCl ml^{-1}); C_o, the original salt content of the cheese (g NaCl ml^{-1}); t, the duration of the salting period (days); D^*, the pseudo-diffusion coefficient (cm^2 day^{-1}), and W, the average water content throughout the cheese at time t (g g^{-1}). Extrapolating this relationship to the whole cheese, an estimate of the quantity of salt absorbed after a given bringing time, t (Q_t) can be made using the following relationship:

$$Q_t = 100\, M_t \frac{A}{G}$$

where A is the cheese surface area (cm^2) and G is the weight of cheese (g), and where the effect of curvature is negligible.

Temperature of curd and brine

Increasing brine temperature from 5 to 20 °C increases both the diffusion rate and quantity of salt absorbed.

Cheese geometry

Salt absorption increases with increasing surface area-to-volume (SA/V) ratio of the cheese. This is most readily observed on comparing the rate of salt uptake by milled Cheddar curd chips and whole molded cheeses (Brick-, Emmental-, Romano-, or Blue-type cheeses) in brine: in the former, salt absorption occurs from many surfaces simultaneously, and the time required to attain a fixed level of salt is very much less than that for brine-salted molded cheeses. Shape also affects the rate of salt absorption via its effect on the number of directions of salt penetration from the salting medium into the cheese and on the ratio of planar to curved surface area of the cheese. The quantity of NaCl absorbed per unit area (cm^2) of cheese surface under fixed brining conditions decreases with curvature to a degree that increases with brining time, leading to a loss of the proportionality of salt uptake with $\sqrt{t}$, as discussed above. Thus, for cheeses with an equal volume and composition brined under the same conditions, the rate of salt absorption per unit surface area (and hence the cheese as a whole) is in the order rectangular > cylindrical > spherical.

Initial S/M level of curd and presalting

Brine salting of cheese can be an expensive process in terms of space, maintenance cost, and corrosiveness of brine. Consequently, presalting of cheese (e.g., by direct mixing of dry salt with the curd) is sometimes used as a means of reducing the brining time; however, it may also have other functions such as regulating curd hydration during plasticization (e.g., low-moisture part-skim Mozzarella during kneading and stretching). Presalting increases the salt level in the curd after a given brining time, but the magnitude of the increase in S/M with brining time decreases with the level of presalting, because the corresponding reduction in the salt concentration gradient between the cheese moisture and the brine.

Initial moisture content of the curd

The quantity of salt absorbed during brine salting generally increases as the prebrining moisture content of the curd increases; the effect becomes more pronounced with brining time.

pH of curd and brine

For cheeses with a similar prebrining moisture content, the level of salt uptake during brining decreases as the pH is increased from 4.7 to 5.7 in Gouda and other cheeses. The higher salt uptake at lower cheese pH coincides with a lower water loss during brining (per unit weight of salt gained), which may be attributed to a higher lactate level in the low-pH cheese. In contrast, Cheddar curd dry salted at low acidity retains more salt than curd salted at high acidity (as discussed below).

In practice, the pH of brine is adjusted to ~5.0–5.3, which is close to that of most brine-salted cheeses before brine salting. Acidification has a preservative effect and also minimizes the risk of surface defects (e.g., velvety, soft rind) associated with the loss of H^+.

Dry-Salted Cheeses

When dry salt is distributed over the surfaces of curd granules/pieces or milled curd chips, some of the salt dissolves in the surface moisture and diffuses a short distance into the curd. This initiates a counterflow of moisture from the curd, the flow being driven by the NaCl concentration gradient between the aqueous phase of the curd and the layer of brine on the surface. The expressed whey dissolves the remaining salt crystals and, in effect, creates a saturated brine solution around each curd granule or chip, provided mixing of curd and salt is adequate. Some of the brine so formed is absorbed into the curd, whereas the remainder drains away or remains on the curd surface until physically expelled during subsequent pressing. Owing to the relatively large surface area-to-volume ratio of the curd mass as a whole, salt uptake is very rapid (10–20 min for milled Cheddar curd) as compared to brine salting of molded cheeses (0.5–5 days depending on the dimensions).

Typically, the mass of water lost is about twice the mass of salt absorbed, leading to a net loss of cheese mass, a reduction in cheese volume, and the formation of a temporarily contracted surface layer on the individual salted curd chips/pieces. In dry salting, the salt-to-surface area ratio is low, and the time of contact with concentrated salt solution is short, and thus the extent of permanent contraction of the curd surface is minimal. With time, the contracted protein layer becomes virtually indistinguishable from the bulk cheese mass. Salt loss increases with increases in curd moisture and curd temperature, whereas absorption increases with increases in curd pH and surface area-to-volume ratio of the milled particles, and with increased time period between salting and pressing. Salt uptake is proportionally greater for smaller curd particles because of their higher surface area-to-volume ratio. Equilibration of salt levels within the curd chips is essentially complete within 24 h, and the key requirements for uniformity of salt level within the pressed cheese are accurate dosing of salt onto the milled curds, effective mixing of the salted curds to effectively distribute the dry salt crystals and to intimately blend the larger and smaller curd chips, and provision of sufficient time for contact between the curds and the whey brine before pressing.

Factors affecting salt uptake during dry salting

Salt uptake increases, at a diminishing rate, with the level of salt added to the curd. The less-than-proportional increase in salt uptake is due to the concomitant increases in the level of whey expressed from the curd and, hence, in the level of added salt lost.

The salt level in the curd is increased significantly by increases in the mixing time of the curd chips and added salt, the surface area of the curd (with smaller curd chips), and duration of salting period (prior to molding and pressing). In contrast, increasing the depth of the curd bed during mellowing results in lower salt levels in the curd. Increasing the temperature of Cheddar curd chips from 24 to 41 °C results in a slight increase in the percentage of added salt lost during holding and pressing, and a decrease (~11%) in salt level in the curd. Simultaneously, the level of fat lost increased markedly, for example, by ~0.6 kg fat per tonne curd per 1 °C rise in temperature in the normal working temperature range of 29–35 °C.

In contrast to brine salting, an increase in the moisture content, and a reduction in pH, of Cheddar curd chips prior to dry salting has been found to reduce salt uptake during salting/mellowing and S/M levels in the cheese. These effects are due to higher losses of whey and salt from the curds during the salting process.

Dry Surface Salting of Molded Pressed Curd

Some cheeses, such as Blue cheese, may be salted by rubbing dry salt to the molded curd over a period of days. A molded curd can be regarded as a very large curd particle or chip and, as for Cheddar, solution of dry salt at the surface layer is a prerequisite for salt absorption in this method also. The counterflow of moisture from the cheese creates a saturated brine layer on the cheese surface, and salt uptake then occurs by an impeded diffusion process, as in brine-salted cheese.

Salt Distribution and Attainment of Salt and Moisture Equilibrium after Salting

Brine- and Surface Dry-Salted Cheeses

In these cheeses, there is a large decreasing salt gradient from the surface to the center and a decreasing moisture gradient in the opposite direction at the completion of salting (**Figure 3**). Due to the slow diffusion of salt from the rind inward, these gradients disappear slowly, and the equilibrium of S/M is essentially reached at some stage of ripening (**Figure 5**). The time required for S/M equilibration depends on cheese type, composition, size and shape of cheese, and ripening conditions. Typical time periods for achieving uniform salt distribution are Camembert (0.25 kg flat disk, 55% water), 7–10 days; Edam (2.5 kg sphere, 46% water), 4–6 weeks; Gouda (10 kg wheel, 42% water), 7–9 weeks; and Emmental (60–130 kg wheel, 36% water), more than 4 months.

Though the factors affecting the rate of salt diffusion in cheese during brine salting have been investigated in detail, little research has been undertaken to determine the factors that influence the diffusivity of salt throughout

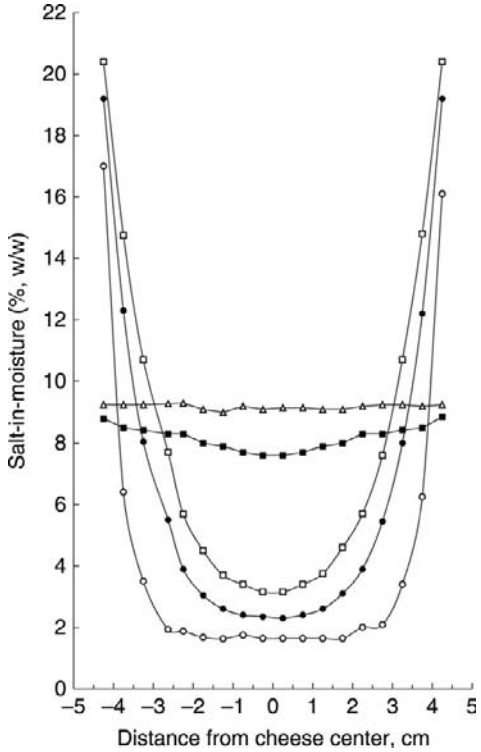

Figure 5 The mean salt-in-moisture levels in discs taken at various distances from the center of cylindrical Romano-type cheese salted in 19.5% (w/w) NaCl brine at 23 °C for 1 (○), 3 (●), and 5 (□) days or salted for 5 days and stored wrapped at 10 °C for 30 (■) and 83 (△) days (redrawn from Guinee and Fox, 1986b).

the cheese mass after removal from the brine. However, it is envisaged that the factors that affect salt diffusivity in cheese during brining or surface dry salting presumably similarly affect it postsalting, that is, during ripening, and hence have a decisive effect on the time required for equilibration of both S/M and moisture levels. However, continuing physicochemical and structural changes during ripening may alter the situation somewhat. The effects of different factors on the rate of salt diffusion in cheese moisture during the salting process are discussed briefly below.

Concentration gradient across the different zones of cheese

The concentration gradient between cheese and brine is determined by the difference in the level of salt in the brine and the S/M of the cheese, whereas the concentration gradient between neighboring regions within a cheese loaf is determined by the difference in S/M level between the two regions. The gradient changes with time both for cheeses that are matured after removal from the brine (e.g., Gouda, Blue cheese) and for those that are matured in the brine (e.g., Feta, Gaziantep) until S/M equilibrium is approached.

Though the concentration gradient has a major effect on the level of salt absorption by a cheese during salting, it scarcely affects the rate of salt diffusion, as reflected by the constancy of D^* for brine concentrations in the range 5–20% (w/w) NaCl. However, D^* decreases sharply at brine concentrations >20% (w/w) NaCl, especially at high brining temperatures, for example, 20 °C. However, as the S/M level in cheese seldom reaches >20% (w/w), except in the rind layer, the large interzonal variations in S/M level at the end of brine salting or surface dry salting should not significantly alter the rate of attainment of S/M equilibrium within a given cheese loaf or between loaves of the same variety.

Ripening temperature

Increasing brine (and curd) temperature is paralleled by an increased rate of salt diffusion, as reflected by an increase in D^*, the increase being on the order of $\sim 0.008\,\text{cm}^2\,\text{day}^{-1}\,°\text{C}^{-1}$ for Gouda-type cheeses for brine temperatures in the range 5–25 °C. Owing to the large effect of temperature on D^*, it is expected that S/M equilibration throughout a cheese loaf occurs more rapidly when the ripening temperature is increased.

Levels of fat, protein, and moisture in the cheese

It is difficult to establish the direct effect of different gross compositional parameters (e.g., fat, protein, moisture) on salt diffusion, because a change in any of these parameters is generally accompanied by changes in the others and their effects tend to be interactive. However, model studies involving altered cheese manufacturing methods and one-dimensional brine salting have helped to elucidate some of the effects of composition on the diffusion of salt in cheese moisture during salting.

It is generally accepted that the moisture content of cheese is a major factor affecting the rate of salt diffusion in cheese, with equilibration of S/M being attained more rapidly as the mean moisture content of the salted cheese increases. This is supported by the strong positive correlation between the D^* of different cheese varieties and their initial moisture content prior to brine salting (**Figure 2**), and the curvilinear increases in D^* with moisture content in Gouda-type cheeses of equal fat-in-dry matter (FDM) content. The increase in D^* with moisture content is due to the concomitant reduction in the levels of fat and protein in the cheese. However, for two cheeses of the same variety, the rate of salt diffusion is not necessarily higher in the higher-moisture cheese; D^* depends on the ratios of fat-to-SNF (solids-not-fat) and moisture-to-SNF ratios. These factors affect the relative volume fractions of the fat and protein phases, which in turn determine the degree of impedance contributed by fat globules and protein particles to the migration of salt and moisture molecules within the cheese matrix.

Fat globules in cheese impede the movement of diffusing molecules, which must travel around the obstructing globules. Nevertheless, in experimental Gouda cheeses with equal moisture (50%, w/w), D^* was found to increase with fat content, from $0.15\,\mathrm{cm^2\,day^{-1}}$ at $11\,\mathrm{g\,100\,g^{-1}}$ fat to $0.25\,\mathrm{cm^2\,day^{-1}}$ at $26\,\mathrm{g\,100\,g^{-1}}$ fat. The increase in D^* was not due to the higher fat *per se* (which actually reduces D^*), but rather to the concomitant decrease in the volume fraction of the protein matrix and the associated impedance to the movement of the hydrated $\mathrm{Na^+}$ and $\mathrm{Cl^-}$, that is, extra pathlength owing to the obstruction by protein aggregates, and the sieve effect of the pores of the matrix on the diffusing species. However, in practice, reducing the fat level of cheese, while increasing the moisture percentage *per se*, usually results in a reduction in the ratio of moisture to solids-not-fat (MNFS) and an increase in protein level. In this situation, the results of model studies on brine salting would suggest that the concomitant increase in protein level would reduce D^* significantly, unless the reduction in fat content is small (e.g., 1–3%, w/w) and the cheese-making process modified to prevent a reduction in the MNFS (e.g., by reducing the calcium-to-casein ratio of the cheese by setting the milk at lower-than-normal pH values). Hence, it can be inferred that the attainment of uniform salt-in-moisture levels in reduced-fat cheeses would generally require a longer time than in full-fat cheeses.

Other factors

Cheese geometry influences the rate of attainment of S/M equilibrium via its effect on the relative dimensions of the cheese, with the net difference in S/M concentration along planes/layers in a cheese at any time during storage increasing with layer length. Hence, S/M equilibrium is generally reached more rapidly in smaller cheeses than in larger ones. Though not investigated to date, conditions of relative humidity, rates of air circulation, and frequency of turning the cheese during storage possibly alter the rate of attainment of salt and moisture equilibrium as a result of alterations in the distribution of moisture throughout the cheese.

Cheddar and Dry-Salted Varieties

Salt is fairly uniformly distributed in Cheddar-type cheese initially, as it is mixed with the milled curd. However, significant intra- and interblock variations in S/M can occur in mature Cheddar cheese if zonal variations occur within a cheese immediately after salting as a result of compromised salting practices, such as large variations in curd chip size, inadequate mixing of salt and curd, and differences in curd composition at salting (e.g., pH, moisture level), that affect the quantity of salt absorbed by individual chips. Such variation is undesirable as it leads to considerable variations in the rate of ripening and grading quality.

Model studies, undertaken in an attempt to explain this variation, have shown that equilibration of S/M is rapidly established within individual milled curd chips, for example, 24–48 h after molding, and suggest that movement of salt and moisture across the chip boundaries, and hence within the cheese mass as a whole, even where a concentration gradient exists, is inhibited because of the following:

- the contracted protein layers (due to salting-out of protein) at the surface of individual chips, which in effect lock the salt within the individual chips
- the occurrence of microspaces between milled curd (chips) junctions, which break the continuity of the interpenetrating gel fluid/moisture (in which salt is dissolved)
- lack of a continuous S/M gradient in combination with an impeded diffusion process

Owing to the importance of salt level and salt distribution in cheese quality, salting of Cheddar curd at factory level is a carefully controlled operation, designed to ensure the application of the correct ratio of salt to curd and uniform uptake of salt by all curd chips. Recent developments in equipment design have greatly assisted in minimizing variations of salt and moisture within individual curd blocks, for example, the use of load cells to weigh the curd flow on belts, drum mixers for thorough mixing of salt and curd chips, and curd distribution tanks to ensure uniform feed distribution of salted curd chips varying in size to the pressing/molding units. Consequently, it is now possible to achieve much tighter control of salt content in Cheddar cheese, for example, 1.85 ± 0.12% NaCl in modern manufacturing plants operating at 100–200 tonnes of product per day.

Effect of Salt on the Properties of Cheese

Cheese Composition

The main factors that determine the composition of cheese are the relative proportions of fat, water, proteins, and minerals in the cheese milk, the nature and extent of fermentation, and the syneresis, as affected by the cheese-making technology/process. The addition of salt does modify the gross composition of cheese, although to a lesser extent than these primary factors do, through its direct contribution to the dry matter of the cheese and, more significantly, through the salt-induced release of whey during the course of salt absorption.

Moisture content

As a general rule, for any particular cheese, as salt content increases, the moisture content decreases for cheese made under the same conditions from the same milk. This is seen most clearly in the case of brine-salted cheese immediately at the end of the brining period (**Figure 3**). Here, there is a clearly defined compositional pattern, with a decreasing salt gradient from the surface to the center being accompanied by an increasing moisture gradient in the same direction. For brine-salted cheese, this is a transient situation as the levels of both moisture and salt tend to even out in the cheese during maturation, driven by the large and consistent concentration gradients.

With dry-salted varieties, such as Cheddar, the impact of salt on cheese moisture is most significant in the case of uneven salt levels. If salt distribution is poor, there may be randomly located regions within a single pressed cheese block in which the salt and moisture levels are significantly different from the mean, with only a limited driving force acting toward equilibration of salt and moisture throughout the cheese. This may lead to the same cheese developing significantly different sensory properties at maturity from region to region.

Levels of lactate in cheese

For most cheese varieties, other than fresh cheeses such as Cottage cheese and Quark, lactose is fully metabolized during the early stages of ripening. But the level of salt in the cheese and the method of its application have a significant role in determining both the rate of lactose metabolism and the balance between the numbers of starter and non-starter bacteria that grow in the maturing cheese and influence its sensory properties.

For brine-salted cheeses, where the salt penetrates slowly, over days, there remains ample time for virtually complete fermentation of lactose by the starter before the S/M level becomes inhibitory, although inhibition may occur in the surface layer. However, for dry-salted cheeses, such as Cheddar and related types, it is important that fermentation proceed nearly to completion prior to salting, for otherwise the cheese may not ripen correctly. Cheddar curd at salting contains 0.5–0.8% lactose, which ferments slowly during early maturation via continued activity of the starter, but this depends on the actual S/M levels and the salt tolerance of the added strains. This period of slow decline in residual lactose provides conditions suitable for the growth of salt- and acid-tolerant NSLAB, principally mesophilic lactobacilli. Excessive populations of some NSLAB can lead to flavor inconsistency and defects such as crystallization of calcium lactate associated with the racemization of L(+)-lactate (produced by lactococci) to D(−)-lactate.

pH

The pH of cheese is determined to a large extent by the ratio of lactic acid to buffering capacity, the latter being controlled mainly by the levels of casein and colloidal calcium phosphate. Salting for the purposes of pH control is seen only in those cheese types where the method of incorporation promotes rapid penetration of the salt throughout the aqueous phase. Formation of lactic acid slows or stops as the S/M level increases (depending on the salt sensitivity of the starter culture), and the pH reaches its final level. The most notable example is stirred-curd cheese, a bulk cheese type used for processed cheese manufacture. Here, continuous agitation prevents the original curd particles from fusing after whey separation, and when the pH of the still-warm curds has reached the desired value (pH range 5.0–5.4), further pH change is prevented by application of dry salt directly onto the particles. The small size of these particles (∼2–4 mm in diameter) means that salt diffusion is complete within minutes and acid production stops accordingly. The larger the size of the curd particles at salting, the more gradual the slowing and cessation of pH change. For Cheddar, where the cross-sectional area of the milled curd chips may be up to 15 mm × 15 mm, this time period may be about 24 h. In brine-salted cheeses, salting has little effect on the pH of the internal regions, as the molded cheese is relatively large and salt diffusion from the brine through the cheese mass is slow. Hence, the low internal salt level in brine-salted cheeses generally allows for continuation of fermentation by the starter organisms of practically all of the lactose to lactic acid and associated end products, thus leaving little fermentable carbohydrate to support the growth of NSLAB, resulting in a different course of maturation and different flavor profiles. In contrast, high salt levels in the outermost regions of brine-salted cheeses could lead to starter culture inhibition and a higher pH where the pH is not allowed to fall prior to brine salting, especially if the surface of the cheese cools too much by being held at inappropriately low temperatures before salting. The inhibitory effect of salt level on starter growth and the pH decrease in brine-salted cheeses are expected to increase with reductions in the size and temperature of the cheese, and the duration of holding before-brine salting.

Cheese quality

Salt level has a major influence on cheese quality through its effects on cheese composition, microflora, and enzyme activity. There is considerable direct evidence of correlations between salt level and sensory quality as judged by expert cheese graders. The general observation is that there is, for each cheese variety, an optimum range for salt content and S/M level. At levels below this, it is usual to see defects due to either the growth of undesirable bacteria or unregulated enzyme activity. An example is bitterness in Cheddar cheese associated with a low S/M level (e.g., <4.5%, w/w), which leads to excessive primary proteolysis. At salt levels above the optimal range, the defects are more likely to be due to inhibition of the primary fermentation and/or the subsequent maturation processes, or due to the direct effect on gross composition, for example, excessively firm texture caused by moisture loss. In addition, there is a direct impact of salt on taste, with too high, or too low, salt levels becoming evident organoleptically.

Low- and Reduced-Salt Cheese

With the trend toward better regulation of dietary sodium intake has come a market demand for a reduced salt level in cheese. Even though cheese contributes only up to about 5–10% of dietary sodium in the highest-consumption regions, there is a market demand for reduced-sodium cheese. A number of approaches are being exploited for this purpose, a few of which are as follows:

1. Maintaining the S/M consistently at the level required for optimum quality (e.g., 4.7–5.7%, w/w) for Cheddar cheese, by adopting a more standardized cheese manufacturing process, which carefully regulates factors such as composition of cheese milk (e.g., protein-to-fat ratio, casein concentration), ratios of starter culture and coagulant quantities to casein load, firmness of gel at cut, and pH at different stages of manufacture.
2. Partial substitution (e.g., 25%, w/w) of NaCl with KCl; however, this approach is still not widely practiced owing to reported deterioration in taste (particularly a tendency toward bitterness).
3. Adding less salt during manufacture (with attendant increased risk of bitterness and of growth of undesirable bacteria and/or pathogens); this approach would require maximizing the retention of indigenous milk salts (e.g., Ca, P, K) as a means of increasing the buffering capacity of the cheese matrix (thereby minimizing the pH reduction associated with reducing salt content in some cheeses) and increasing 'saltiness'.
4. Addition of flavor-enhancing substances (e.g., autolyzed extract, ribonucleotides) at low levels (e.g., <0.5%, w/w) to compensate for the low saltiness. Various problems have, however, been found with the addition of different materials (e.g., burnt, scorched, meaty, brothy off-flavors). Because of this, and because of legislative issues, such an approach appears to have had little success for natural cheese.

Long-term, sensory research of the effects of product factors (e.g., structure, rheology, texture) affecting the release of salty flavor during mastication of cheese, and how the perception of taste (e.g., saltiness) during mastication is affected by the presence of other taste and/or odor compounds, is likely to hasten the development of high-quality, acceptable, low-sodium cheeses.

See also: **Cheese**: Cheddar-Type Cheeses; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Starter Cultures: General Aspects; Starter Cultures: Specific Properties; Swiss-Type Cheeses.

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Mechanization of Cheesemaking

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Introduction

Cheese manufacture has been carried out for thousands of years and, for the most part, as a cottage industry. Toward the end of the nineteenth century, as industrialization progressed, cheese manufacture moved into the factory. Since then, there has been progressive development of the technology to the situation today, with large, highly automated plants employing minimal staff. A major component of this development can be described under the term cheese mechanization, which may be defined as a process where mechanical devices are used to carry out the manufacturing processes traditionally done manually.

The major developments in cheese mechanization occurred during the period from the 1950s to the 1970s. The primary drivers of cheese mechanization include reduced manufacturing cost, elimination of laborious tasks, manufacturing efficiency, labor savings, increased capacity, and improvement in quality.

Much of the early development work occurred in Australia and New Zealand, where major expansion of the cheese industry, in particular the manufacture of hard cheese varieties such as Cheddar, necessitated the development of novel approaches to equipment design. Most of this work was done under the auspices of the Commonwealth Scientific and Industrial Research Organization (CSIRO) in Australia, the New Zealand Dairy Research Institute (NZDRI), and the New Zealand Dairy Board. Both the CSIRO and the NZDRI had research groups dedicated to this task.

This article uses the developments associated with hard cheeses, in particular Cheddar, as its framework, with the focus on those developments that have survived. Consideration is then given to other cheese types and likely future directions.

Hard Cheeses – Cheddar Family

The mechanization of Cheddar cheesemaking can be reviewed by considering, sequentially, the key steps in the process, using much the same approach as that taken by those undertaking the original mechanization of the cheesemaking process. The steps discussed include milk treatment, starter culture preparation, vat process, texturing (cheddaring), milling, salting, pressing (block forming), and ripening (including packaging and storage).

Milk Treatment

Fat standardization of cheese milk has been used as a means of better controlling the composition of the final cheese for some time, but the more recent innovation in this area has been the standardization of the protein content of the cheese milk, especially where the milk composition tends to vary over a dairy season as is the case in New Zealand and Australia.

New Zealand was one of the first dairying nations to use this approach to standardize its Cheddar cheese milk in the early 1990s. The protein in skimmed milk is concentrated in a separate retentate stream to a higher level than required and the concentrated skimmed milk is fed back into the whole milk to achieve a consistent protein-to-fat ratio and a consistent protein content of between 3.7 and 4%. Nonconcentrated skimmed milk can also be added if required. The advantages of protein standardization for cheesemaking include increased throughput, improved processing, improved product consistency, less whey handling, and a clean lactose permeate stream.

Starter Culture Preparation

Neutralization of the starter culture during the growth phase resulted in increased numbers of organisms per milliliter of culture, allowing the cheesemaker to reduce starter addition volumes and to save on bulk starter capacity.

Introduced during the 1980s in New Zealand, the one-shot neutralization of mesophilic starter cultures with sodium hydroxide during bulk starter growth doubled starter numbers from 5×10^8 to 1×10^9 per milliliter. Once the pH of the starter culture had reached 4.8, a quantity of sodium hydroxide solution was added in one amount (one-shot) and was stirred in to return the pH to 6.7, allowing further starter growth before acid levels again became inhibitory. Later development of a multishot continuous system tripled starter numbers, allowing for a threefold reduction in the starter volume added to each vat. Other systems use internal pH-controlled media (buffered bulk starter media) to achieve a similar increase in starter numbers to that shown for one-shot neutralization.

Vat Process

The traditional cheese vat consisted of a long, rectangular vessel of about 4500–10 000 l, in which all the operations up to pressing were carried out. Elimination of the back-breaking labor associated with these vats was one of the main drivers of mechanization.

It was recognized early on that further development necessitated retention of the vat stage for the steps associated with primary curd formation and fermentation only. The remaining operations could be transferred to downstream equipment that had yet to be developed. The vat stage became concerned solely with the conversion of liquid milk into a coagulum, followed by cutting, stirring, and cooking, to produce a curd and whey mixture of the appropriate pH, moisture, and mineral composition, by the controlled activity of the starter cultures incorporated at the beginning of the process.

The late 1960s saw the introduction of the enclosed vat. The majority of the enclosed vat systems available contain

- one or two revolving knife panels of various designs that are used for both cutting and stirring operations, depending on their direction of rotation,
- a fully surrounding or partially surrounding (steam or hot water) heating jacket,
- whey removal systems for predraw and in-vat curd washing, and
- automated rennet addition, cleaning-in-place (CIP), and computer-controlled options for cutting/stirring speeds and cooking recipes (later models only).

Another advantage of the fully enclosed cheese vat is the reduced risk of foreign matter and airborne microbiological contamination.

The choice of equipment for the vat stage of the cheesemaking process depends on many external factors including type of cheese to be made, downstream curd processing, flexibility, cost, and throughput. Internal vat factors are also important, such as the configuration of the vat and its cutting and stirring mechanism, how the tank is heated and emptied, rennet addition, and CIP configurations.

How the curd is cut is of particular significance. The cutting operation, together with the speed of stirring following cutting, influences how large the particles will be at draining and how much of the original milk components (fat and protein) is lost to the whey.

A number of vat types are available, including OST, Damrow, Scherping, and APV CurdMaster; a number of cheese types are made using these systems, including Edam, Gouda, St. Paulin, Havarti, Cheddar, Emmental, Romano, Monterey Jack, Egmont, Mozzarella, Danbo, Raclette, Tilsit, Blue, Feta, Maasdam, Cagliata, Provolone, Norvegia, Manchego, Camembert, Pecorino, Grana, Port Salut, and Parmesan.

One of the first and most popular choices of enclosed cheesemaking vat was the Tetra Tebel OST vat. To date, five models have been produced, each model designed with and without predraw capability. Both the Alfa Laval OST I vat and the Alfa Laval OST II vat were upright, single silo-shaped tanks with one (OST I) or two or more (OST II) knife panels that were vertically mounted. The OST III vat was the first horizontally mounted vat of the OST series and its design was driven by a demand to process larger (>20 000 l) volumes of milk. The OST III vat design has been continued in OST IV and OST V models (**Figure 1**).

The vertical Damrow vat was developed in 1972 and has had two updates since then. Easily recognized with its 'double-O' configuration, the vertical Damrow vat had two vertical knife arrangements that were used to both cut and stir the curd (**Figure 2**).

The first dual-barreled horizontal cheese vat was developed by Scherping Systems in 1988. Of interest is the unique design of the vat's 'counter-rotation', dual agitator, cutting and stirring system and the staggered design of the knife arrangement of the third generation model (**Figure 3**).

The first APV CurdMaster was produced in 1993 and its design is based on the Protech CurdMaster and the Damrow Double-O vat.

Texturing

Development of equipment to replace the traditional in-vat cheddaring process presented one of the more challenging aspects of mechanization. A common device developed for dewheying prior to cheddaring was the static dewheying screen, followed by a short drying belt. Many ingenious devices were developed for texture development, including some that mimicked the process in the vat. However, the development of the cheddaring tower in New Zealand provided a revolutionary system for cheddaring that is still in use today (**Figure 4**). Another very significant development has been the use of belts for cheddaring, as demonstrated by the Damrow draining and matting conveyor two-belt system, developed in the United States, and the Alfomatic (**Figure 5**), developed in Australia by Alfa Laval (now Tetra Pak). In this system, the curd is allowed to fuse together on another belt and is then inverted as it drops on to a third belt for further cheddaring. Stretch is induced at the transfer. The belt cheddaring system has been widely adopted by other manufacturers such as APV (**Figure 6**) and Scherping. The belts are now plastic, rather than the original stainless steel, may be perforated, and may be

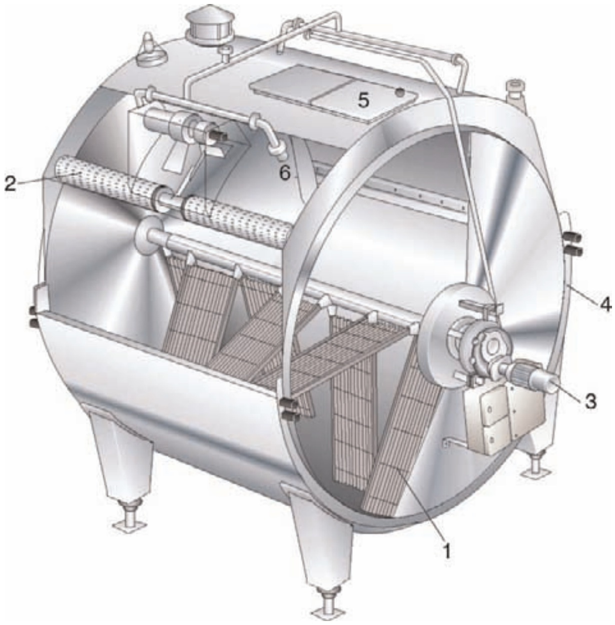

Figure 1 OST IV cheese vat. 1, Combined cutting and stirring tools; 2, strainer for whey drainage; 3, frequency-controlled motor drive; 4, jacket for heating; 5, manhole; 6, CIP nozzle. Courtesy of Tetra Pak, Sweden.

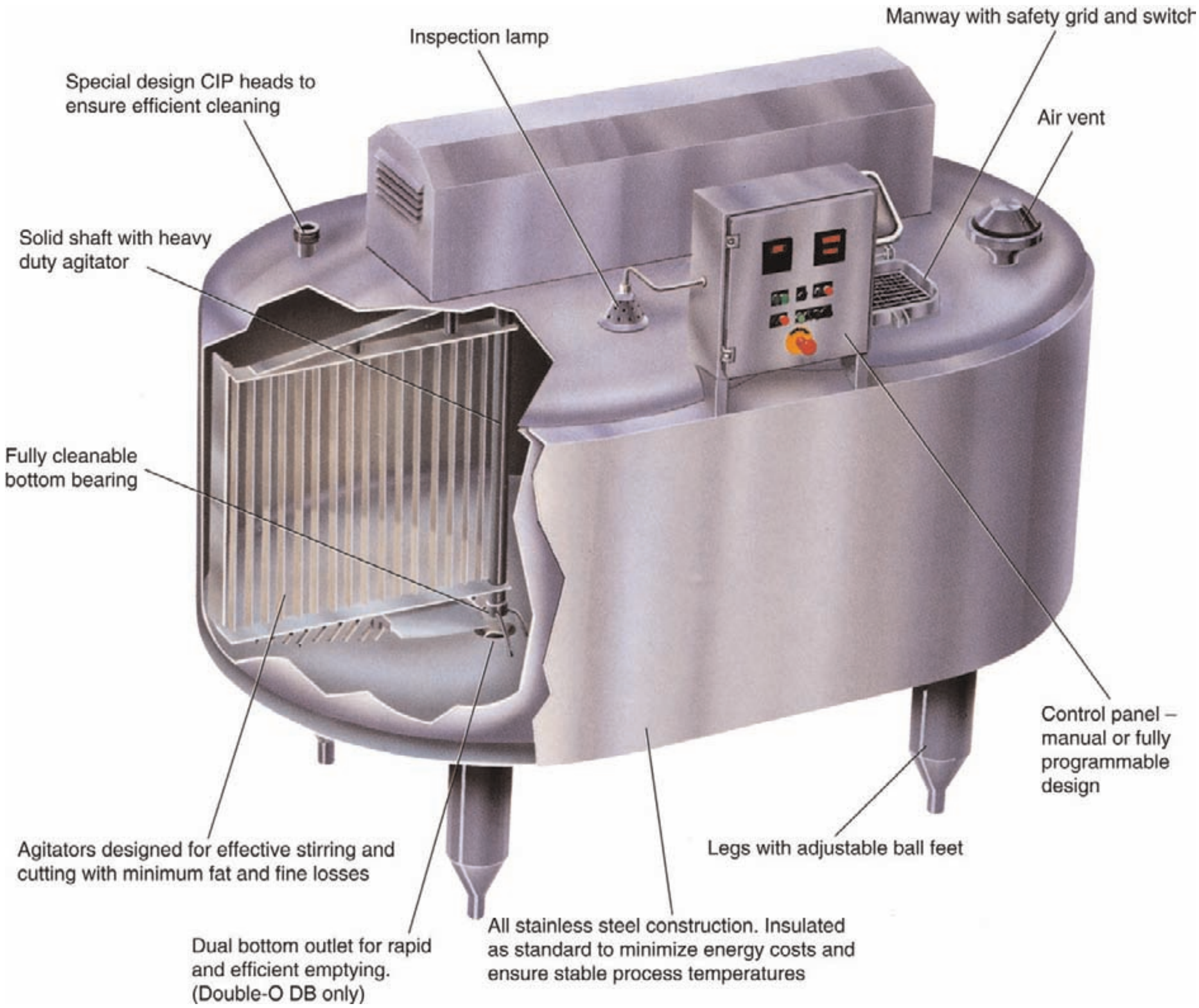
Manway with safety grid and switch
Inspection lamp
Special design CIP heads to ensure efficient cleaning
Air vent
Solid shaft with heavy duty agitator
Fully cleanable bottom bearing
Control panel – manual or fully programmable design
Agitators designed for effective stirring and cutting with minimum fat and fine losses
Legs with adjustable ball feet
Dual bottom outlet for rapid and efficient emptying. (Double-O DB only)
All stainless steel construction. Insulated as standard to minimize energy costs and ensure stable process temperatures

Figure 2 Damrow Double-O cheese vat. Courtesy of Damrow Inc., USA.

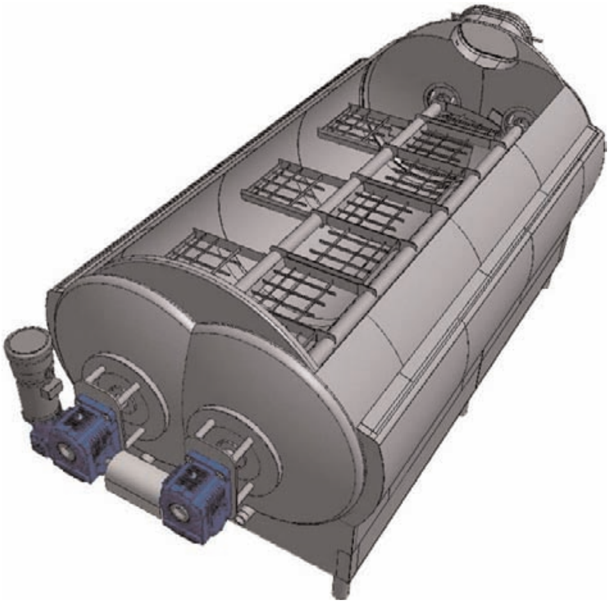

Figure 3 Scherping horizontal cheese vat. Courtesy of Scherping Systems, USA.

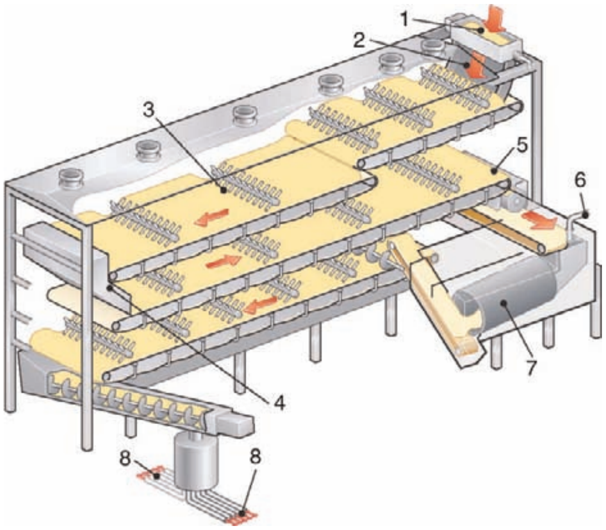

Figure 5 Alfomatic cheesemaker. 1, Curd/whey mixture inlet; 2, whey screen; 3, curd stirrer; 4, turnover chute; 5, chip mill; 6, dry salting feed; 7, salt mixing drum; 8, chips drawn to blockformer by vacuum. Courtesy of Tetra Pak, Sweden.

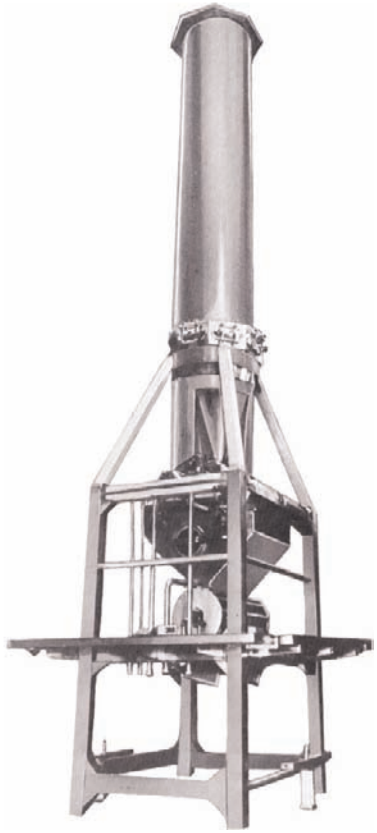

Figure 4 APV cheddaring tower, with guillotine and mill at base. Courtesy of APV, UK.

Figure 6 APV Cheddarmaster belt system. Courtesy of Fonterra Cooperative Group, New Zealand.

equipped with stirrers for curd agitation. Some plants, particularly in the United States, make Cheddar without ‘cheddaring’ and continuously dry stir the curd prior to salting.

Milling and Salting

The purpose of milling is to cut the cheddared curd into fingers (chips) of approximately 1.5 × 1.5 × 8 cm, to aid in salt uptake. Mechanical reciprocating chip mills were introduced in the earliest stages of mechanization for open vats and for equipment such as the Australian CheeseMaker 3. In contrast, the milling system developed for the New Zealand Cheddarmaster process consisted of a rotating cutting drum, which cut the curd in two directions at once using a blade and a cutting comb. Rotary mills using a similar principle but covering the width of the belt are now used at the end of the cheddaring belts on systems such as the Alfomatic and the Cheddarmaster.

A number of techniques have been used to apply and mix dry, granular salt into the curd. The CheeseMaker 3 design incorporated what became known as the trommel salter, in which the milled curd passed over a weighing belt that discharged into a rotating conical mixing drum, with a metered flow of salt being deposited on to the curd as it entered the drum. The early Cheddarmaster design utilized a salting boom that moved backward and forward across the curd on the mellowing conveyor belt. The quantity of salt applied was proportional to the curd flow, determined by a sensing fork that measured the curd depth. Following salting, the curd passed along the mellowing conveyor where peg stirrers would aid salt uptake and moisture expulsion.

Interestingly, the systems that have survived to be incorporated into modern plants consist of refined versions of the trommel design (**Figure 7**), as these give excellent control of the salt content in the final cheese.

Pressing

It was recognized early on that the traditional pressing process using metal hoops or molds was very labor intensive. About half the labor force in New Zealand factories was used in this part of the process. Sophisticated systems have been developed to fully mechanize and automate the mold filling and pressing operations, such as the APV Sanipress, but, for dry-salted cheeses, these have been largely superseded by the development of the continuous block former described below.

However, the early development of the large hoop system as part of the Cheddarmaster process deserves a mention. These vertical hoops were designed to hold 818 kg of curd. After pressing for approximately 10 h, including a vacuum stage, the large block of curd would be hydraulically pushed from the hoop through a special cutter head, producing 44 blocks of cheese (each 18.1 kg) per hoop. The labor saving with this system was significant, but one major problem with the long pressing time was the lack of curd cooling. Nonstarter lactic acid bacteria could grow to very high numbers during the overnight pressing and this could give rise to problems of flavor and gas production in the final cheese. However, barrels or large forms of cheese are still produced in the United States, normally for rapid use as ingredients in processed cheese.

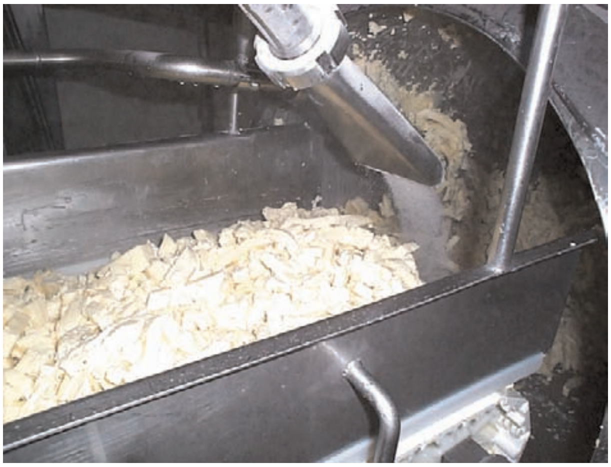

Figure 7 Trommel salting system. Courtesy of Fonterra Cooperative Group, New Zealand.

The introduction of Wincanton's continuous block former in 1980–81 in New Zealand following a 17-year development reduced holding times from 24 h to 30 min and reduced and minimized the floor space and the labor force required to carry out the pressing and block-forming part of the process. Operating principles are shown in **Figure 8**.

The modernization of the 'hanging plate' arrangement to a completely welded, perforated, rectangular-shaped, continuous inner skin and the extension of the tower to increase the capacity from 700 to 1600 kg are more recent innovations, shown in **Figure 9**.

Ripening or Curing (Including Packaging and Storage)

The initial reel feed sheet systems for wrapping rindless cheese have now been replaced by laminated barrier bags, such as those provided by Cryovac. This company has also been involved in the development of bag presenters, which automatically place bags on the cheese blocks as they exit the block-forming towers. The bagged cheese blocks are transported through vacuum chambers, where they are heat sealed, and then placed in a carton base and conveyed to a rapid cooling tunnel. The carton format varies from base-only and box and lid to the most popular wraparound style.

The cheese exiting the towers can be quite warm (30–33 °C). The rapid cooling process reduces the average block temperature to between 16 and 20 °C in 12–24 h. This solidifies the fat, firms the block, and, more importantly, dramatically reduces the growth potential of undesirable non-starter lactic acid bacteria, which can cause major problems of flavor and gas production. Fully automated, first-in/first-out, open-stacked conveyor systems operating in blast chillers are used to achieve this cooling. An example is shown in **Figure 10**.

Following this initial cooling, the cheese blocks are palletized by a robot and then transferred to controlled-temperature ripening and storage rooms. The operations,

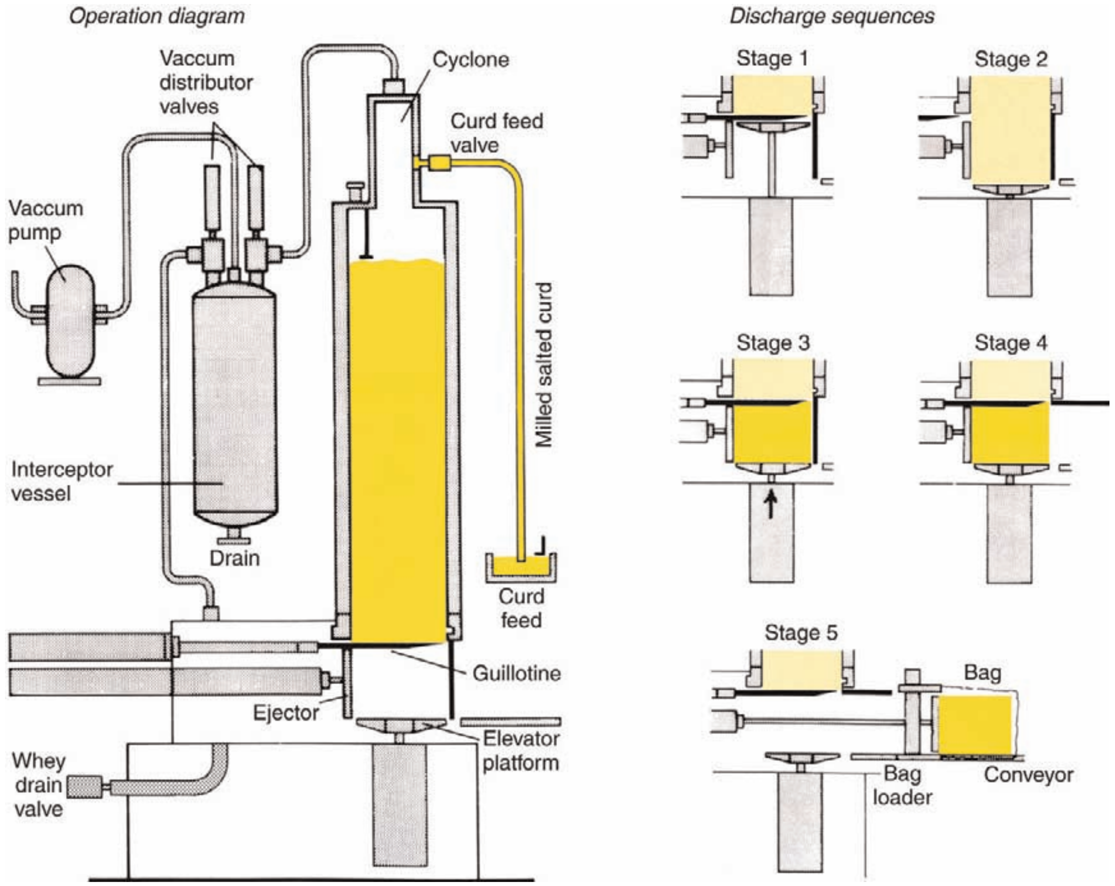
Operation diagram
Vaccum distributor valves
Cyclone
Curd feed valve
Vaccum pump
Milled salted curd
Interceptor vessel
Drain
Curd feed
Guillotine
Ejector
Elevator platform
Whey drain valve
Discharge sequences
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Bag
Bag loader
Conveyor

Figure 8 Blockformer operating principles. Courtesy of Tetra Pak, Sweden.

including cardboard packaging, are highly mechanized and automated, with minimal staff involvement.

Continuous Hard Cheese Process

Any discussion of the mechanization of hard cheeses would be incomplete without mention of the pioneering work of the Australian CSIRO team, who came very close to the successful commercial development of a semicontinuous Cheddar (Sirocurd) process. This was based on ultrafiltration of the milk, the incorporation of whey proteins, and many ingenious inventions such as a barrel system for coagulation. Two commercial plants were commissioned but are no longer in operation.

Other Varieties

The same issues of reduction in labor costs and manufacturing costs, increased throughput, and improvement in quality applied to other varieties and similar process developments were undertaken.

Semihard Cheeses

Developments associated with these cheese types, such as Gouda, Edam, and Tilsit, paralleled those for dry-salted varieties. Three manufacturing differences distinguish these varieties from hard cheeses such as Cheddar – whey drainage and curd washing in the vat, pressing of the curd under whey, and brine salting.

Vat Process

Similar vats to those developed for Cheddar varieties can be used. An additional feature incorporated into the Tetra Pak OST vat is a whey strainer that can be lowered into the vat, when stirring has been stopped, to remove part of the whey. The whey can then be replaced by hot water to wash the curd and reduce the lactose content.

Pressing

Post-vat manufacturing operation is quite different for these cheese types. It is necessary to separate the curd and whey without air incorporation, to allow for anaerobic fermentation during the ripening process. This is

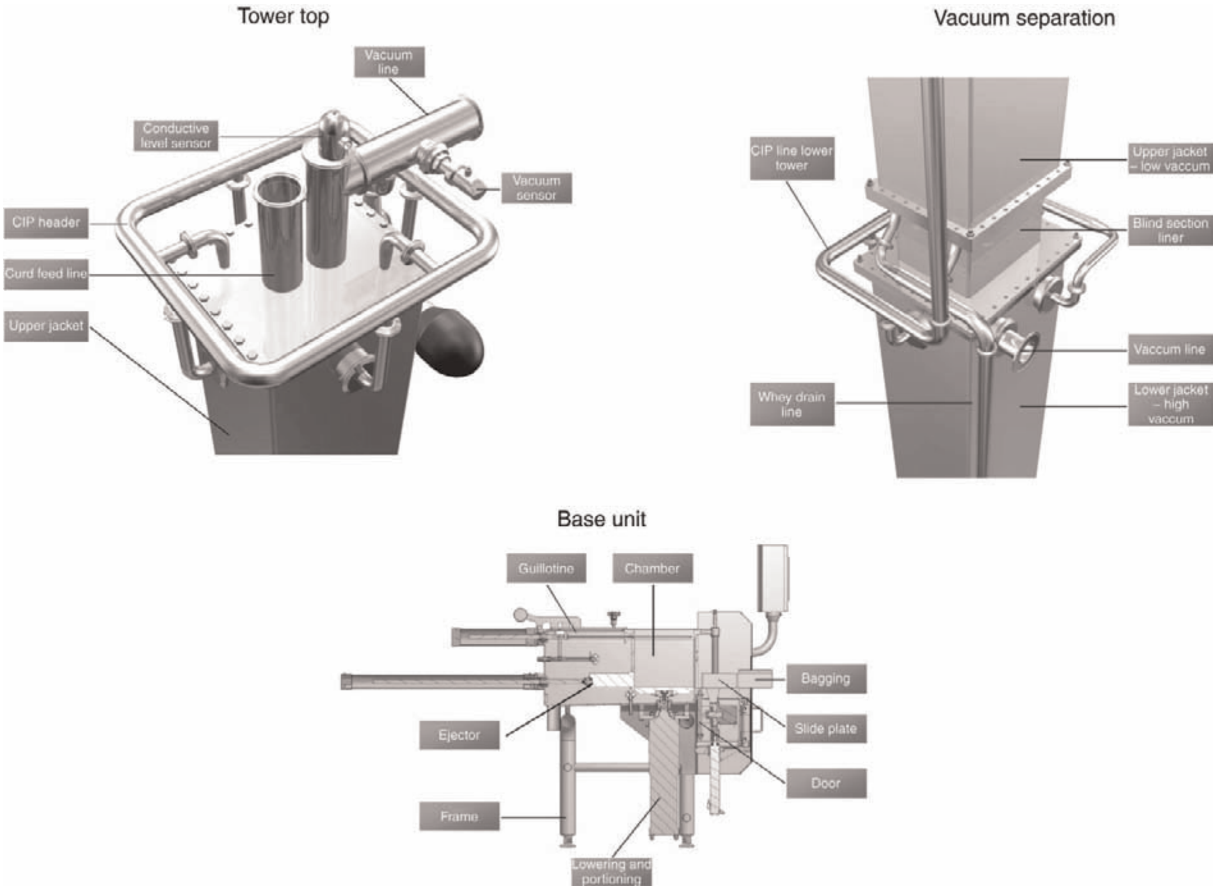
Tower top
Vacuum line
Conductive level sensor
Vacuum sensor
CIP header
Curd feed line
Upper jacket
Vacuum separation
CIP line lower tower
Upper jacket – low vaccum
Blind section liner
Vaccum line
Whey drain line
Lower jacket – high vaccum
Base unit
Guillotine
Chamber
Bagging
Slide plate
Ejector
Door
Frame
Lowering and portioning

Figure 9 Details of Blockformer 6. Courtesy of Tetra Pak, Sweden.

achieved by first forming the curd into a block (prepressing), followed by further compression into a closely pressed block while further acidification occurs. Early attempts to mechanize the first part of this process resulted in the development of prepressing vats, a modern example of which is shown in **Figure 11**.

In larger plants, the major type of equipment that is used is the Tetra Pak Casomatic (**Figure 12**). The curd and whey mixture in a 1:4 ratio is pumped to the top of the column, about 3 m in height. The curd settles below the whey to a height of about 2 m. The whey is removed via three whey drainage bands. A controlled rate of removal is essential for the formation of a block of curd at the base of the column. The curd block is formed in a dosing chamber and is then cut by guillotine and discharged into a mold to which a lid is fitted, enabling further pressing. Blocks of various shapes and sizes can be produced with minor modifications, and a multicolumn version is also available. The development of plastic microperforated molds such as the Laude mold (**Figure 13**) has been a major advance from the traditional wooden or metal cloth-lined molds.

Further pressing of the formed block is necessary to achieve the desired cheese properties and to allow ongoing acid development and whey drainage. Large table systems have been developed; the individual molds are conveyed on to tables that are fitted with individual hydraulic presses for each mold. The pressing regime is computer controlled, for example, 100 kPa for 20 min, followed by 200 kPa for 40 min. Following pressing, the blocks are discharged and the molds are washed and recycled. An example of this type of installation is shown in **Figure 14**. The APV Sanipress can also be used.

Salting

Cheeses that have been formed into blocks under the whey cannot be salted prior to molding and pressing, as further acid development is required. For some varieties, dry salt may be applied to the cheese surface, but, for most, brining is the technique that is used. Brining essentially involves the immersion of the cheese block into a refrigerated brine bath (which also cools the cheese) for the required period

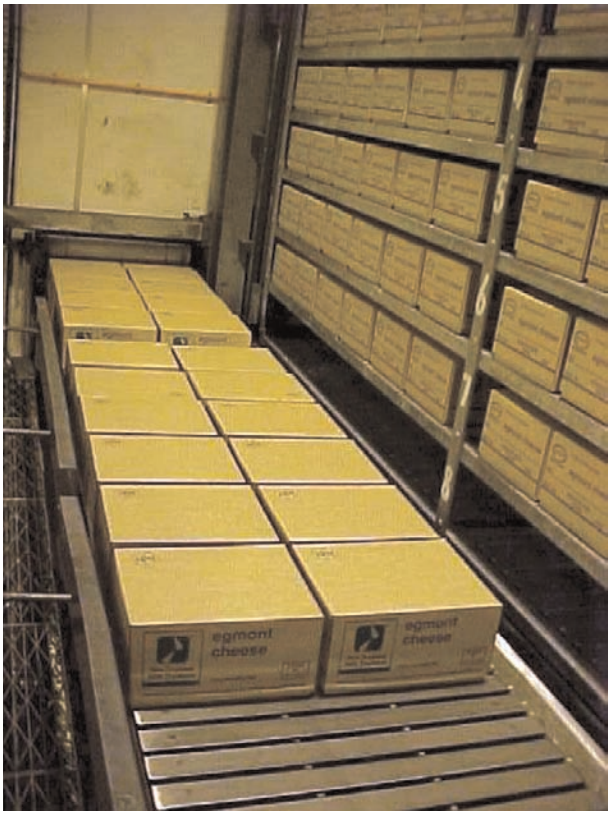

Figure 10 Rapid cooling tunnel. Courtesy of Fonterra Cooperative Group, New Zealand.

to achieve the desired salt uptake. Highly automated systems have been developed, and these often involve the use of the brine solution as a conveyor system for the demolded cheeses, which float in the brine and are directed to a racking system in deep brine tanks that, once loaded, are submerged (**Figure 15**) for the required time, for example, 36 h for a 10 kg block, before unloading, drying, and wrapping.

Ripening, Packaging, and Storage

Highly mechanized systems have been developed for the material-handling aspects of these steps, including specialized cheese-turning equipment and the use of robots.

Soft Ripened Cheeses

Many types of cheese fall into this category, such as Camembert, Brie, and Blue. Their manufacture is characterized by a high rate of syneresis and acid development, followed immediately by molding of the curd to the various final cheese shapes, under gravity, as the whey is drained from the curd. Hence, large-scale vat production is not desirable as the cheeses produced at the start and end of emptying would be quite different, due to composition and pH changes, and consistency of mold filling would be impossible.

There have been various attempts to automate and mechanize curd production using a series of mini-vats, followed by automated mold filling and handling. Systems have been developed by companies such as Tecnal and Servi Doryl in France and APV in the United Kingdom, whose Contifiller system is shown in **Figure 16**. The multimolds used to form the cheese may be in two sections to provide sufficient volume for the initial fill, with the upper layer being removed later. The filled molds can be stacked automatically, conveyed to ripening rooms, turned frequently as required to ensure even block production, brine salted (or surface dry salted), and then conveyed to ripening rooms for mold development.

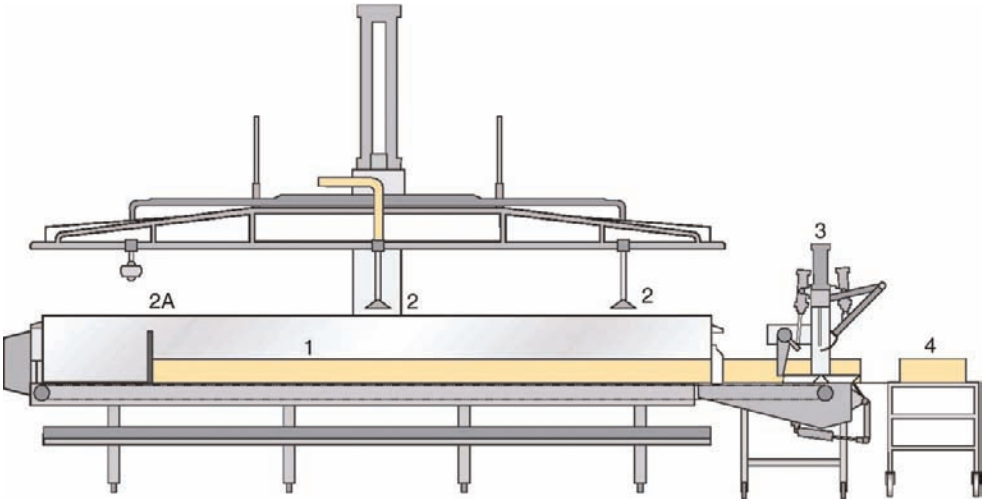

Figure 11 Prepressing vat. 1, Prepressing vat; 2, curd distributors, or CIP nozzle (2A); 3, unloading device; 4, conveyor. Courtesy of Tetra Pak, Sweden.

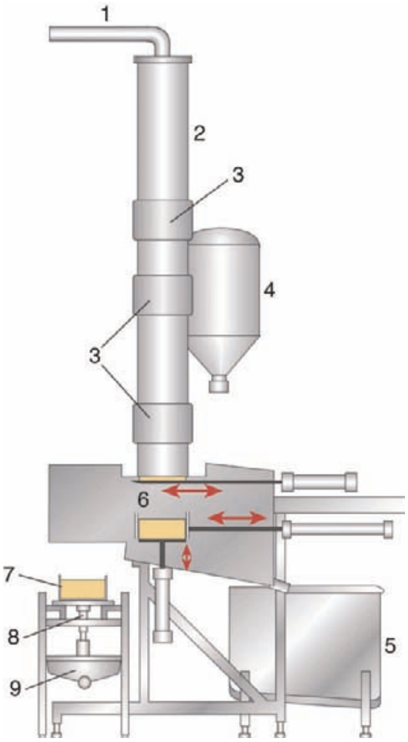

Figure 12 Casomatic operating principles. 1, Curd/whey mixture inlet; 2, column with sight glass; 3, perforated whey discharge; 4, interceptor; 5, whey balance tank; 6, cutting and discharge system; 7, mold; 8, pawl conveyor; 9, whey collecting chute.

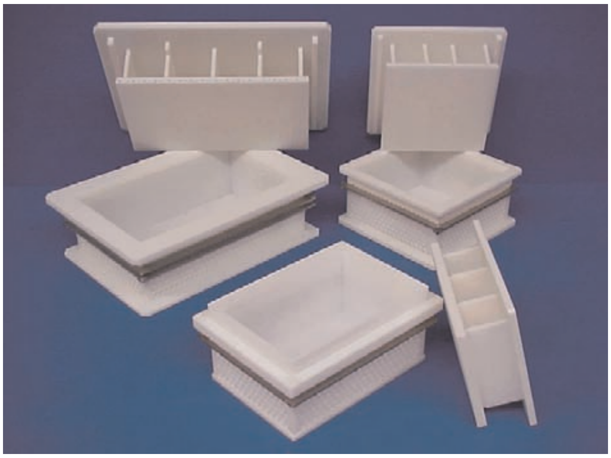

Figure 13 Laude block mold. Courtesy of Laude bv, The Netherlands.

A very successful process that comes close to a continuous vat stage has been developed by Alpma in Germany. The basis of this system is a continuous flexible belt, which is formed into a trough to hold the milk. The trough is then subdivided into a series of mini-vats by a series of semicircular plates that also move with the belt.

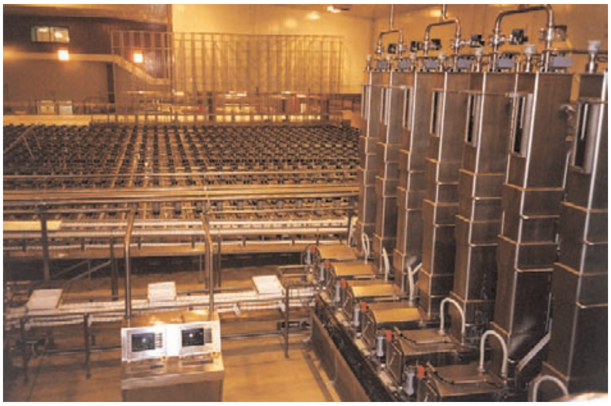

Figure 14 Conveyor pressing system, with Casomatics in the foreground. Courtesy of Fonterra Cooperative Group, New Zealand.

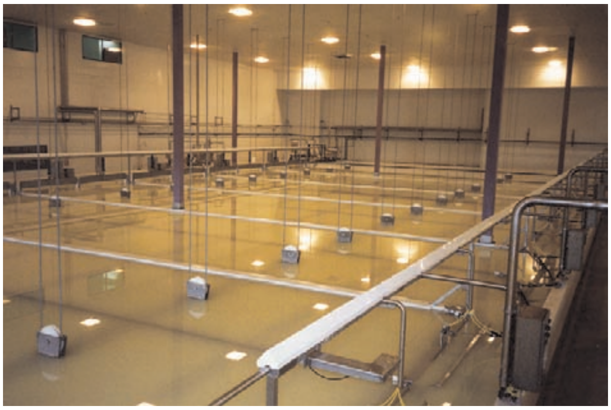

Figure 15 Deep brining system. Courtesy of Fonterra Cooperative Group, New Zealand.

The same processes of coagulation, cutting, and stirring occur as the mini-vats move along, by the use of tools that are placed alongside the belt. The curd/whey mixture discharges into molds at the end of the belt.

Soft Fresh Cheeses

Cottage cheese consists of curd particles that are packed into a container with the appropriate dressing. Specialized equipment, such as the O-Vat from Tetra Pak, has been developed to mechanize and automate production of this popular product.

Quark uses a very different process of mechanization, in which the curd is formed in special ripening vats, followed by whey separation using a specially designed centrifugal separator. The product is then blended with components such as cream and is filled directly into the final container.

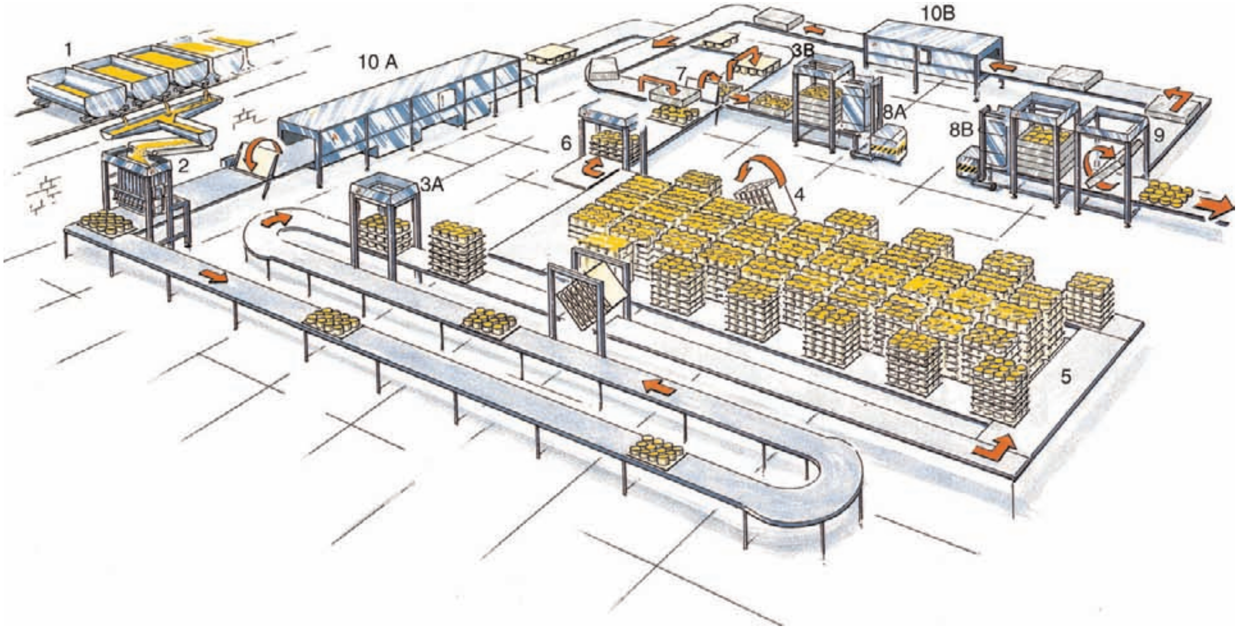

Figure 16 Process line for soft cheese with Contifiller. 1, Curdmaking; 2, curd draining and filling; 3, stacking of mold batteries (A) and trays (B); 4, turning of mold stacks; 5, acidification lines; 6, destacking; 7, transfer/turning of cheese from mold batteries to trays; 8, transport to climate room (A) and from brining (B); 9, turning/emptying; 10, washing of mold batteries (A) and trays (B). Courtesy of APV, UK.

Pasta Filata cheeses are cheeses for which the curd has been worked or stretched and molded at elevated temperatures before cooling. This process imparts a unique and characteristic fibrous structure that influences both the ripening and the functional profile of the final cheese.

Mozzarella is probably the best known of the Pasta Filata cheeses, which are mainly Italian in origin. However, the category also includes such cheeses as Provolone, Scamorza, Caciocavallo, Kashkaval, and Pizza Cheese.

Equipment designed to perform the stretching operation incorporates two essential components: cooking and stretching. Stretching describes the mechanical treatment of the curd following cooking. Cooking is the phase in which the Pasta Filata curd is transferred to the hot water section of a cooker/stretcher. At this point, the curd is immersed, heated, and worked by single- or twin-screw augers. Typical water temperatures vary between 60 and 75 °C, with cooked curd temperatures varying between 55 and 65 °C.

Future Trends

The mechanization of the cheesemaking process was the result of an intense amount of activity and development during the 1960s and 1970s. The latter half of the twentieth century saw the refinement of that development – the implementation of specific processes that enhanced the overall mechanized approach – and these advances guaranteed a more consistent, higher quality, cleaner, and more cost-effective product.

Although automation has led to reduced labor costs, improved efficiencies, and higher yields, it has also allowed for greater flexibility in what the plant produces and the ability to store and process large volumes of data that can be used to trend specific characteristics, for example, variability in drain pH, monitoring coagulation devices, temperature and pressure monitoring, and yield data analysis.

Rapid and sophisticated in-process analysis techniques, both in-line and at-line, have been developed based on spectrophotometric analyses such as the absorption of infrared energy at specific wavelengths (MilkoScan, FoodScan). The integration of these techniques and the rapid analysis of the data that they can provide have led to almost instant feedback on parameters such as fat, moisture, and salt contents. Calcium contents can now be determined using X-ray fluorescence.

Concurrent with the continued development in this area, recent published literature and patent applications would suggest that the next steps in the evolution of cheesemaking in the twenty-first century will be the development of completely new ways of making cheese, together with the continued refinement of the traditional processes that have been seen to date.

Driven by the same needs that drove the initial mechanization of the traditional processes, these 'alternative cheesemaking concepts' will become more common.

As an example, in February 2008, the New Zealand dairy industry commercialized a Mozzarella process that is an alternative approach to traditional Mozzarella manufacture. Unique to New Zealand, this process produces a functionally acceptable product directly off the line in a shredded format in less time than the traditional process and at a lower cost.

This new process takes a number of ingredients derived from milk and blends and works them in a low-shear environment at an elevated temperature to produce a homogeneous mass that is cooled, shredded, and the shred then chilled or individually quick frozen. It involves processes and equipment that are used in other milk processing operations but have not, to date, been used to make Mozzarella.

Acknowledgments

The authors are grateful to Tetra Pak AB, Sweden, specifically Bryce Griffiths (New Zealand), for information supplied and for permission to use illustrations from the *Dairy Processing Handbook* and other sales literature.

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Permission to use photographs from numerous Fonterra sites within New Zealand is gratefully acknowledged, as are the insightful discussions held with Alister Barclay and Craig Honore, Fonterra Co-operative Group.

See also: **Cheese**: Blue Mold Cheese; Camembert, Brie, and Related Varieties; Dutch-Type Cheeses; Hard Italian Cheeses; Membrane Processing in Cheese Manufacture; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Preparation of Cheese Milk; Raw Milk Cheeses; Salting of Cheese; Secondary Cultures; Starter Cultures: General Aspects; Swiss-Type Cheeses.

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Membrane Processing in Cheese Manufacture

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Introduction

Cheesemaking is a process of controlled removal of moisture from milk by acid and temperature manipulation. Ultimately, most of the casein, fat and insoluble minerals along with some water are retained in the cheese. The efficiency with which these components are retained is of great interest to cheese-makers because of the impact on cheese yield and therefore on the cost of production. Membrane processing provides the potential for improving the efficiency of cheesemaking and therefore offers capabilities that are of value in terms of economics and quality, and also provides opportunities for the development of new cheeses.

Membrane processing, including reverse osmosis (also known as hyperfiltration), nanofiltration, ultrafiltration and microfiltration, are pressure-driven separation/concentration operations which employ organic or inorganic membranes. Reverse osmosis of milk or whey will remove only water and is therefore similar to thermal evaporation (**Table 1**). Nanofiltration removes water and small monovalent ions, such as sodium, potassium and chloride. Ultrafiltration achieves greater separation; in addition to water and smaller minerals, it also removes lactose and most water-soluble minerals and vitamins. Microfiltration, on the other hand, is able to separate larger components of milk such as proteins. It is therefore possible to separate caseins and whey proteins. Bacterial cells and spores can also be removed from milk with this process.

This diverse range of separation capabilities is possible with the help of membranes of specific pore sizes and process parameters (pressure) (*see* **Milk Protein Products**: Membrane-Based Fractionation). Membrane-processed milk or whey possesses unique compositional and physical characteristics that enable applications in the manufacture of various products. Cheese manufacture using membrane processing has been practiced commercially since the early 1970s but the manner in which it is used has evolved over time owing to experience gained by cheese-makers and the development of new membranes and applications.

Membrane processes can be used in cheese manufacture to accomplish various specific tasks. The effects of reverse osmosis of milk are similar to those of thermal concentration of milk or fortification of milk with milk powder. The objective of such methods is to concentrate all milk components equally to a predetermined level. The later two methods (thermal concentration and fortification) are practiced in commercial cheese manufacture to improve the efficiency of cheesemaking and increase cheese yield. Reverse osmosis is generally not used for such applications because current multiple-effect evaporators equipped with vapor recompression systems offer greater efficiencies of operation, although combinations of thermal evaporation and reverse osmosis have been suggested for process optimization (*see* **Plant and Equipment**: Evaporators).

Ultrafiltration and microfiltration are the most common membrane processes used in the cheese industry. Applications for nanofiltration, which is a relatively new membrane process in cheesemaking, are also being developed.

Ultrafiltration

Ultrafiltration of milk is conducted at approximately 50 °C but a lower or slightly higher temperature may also be used. The feed runs under pressure tangentially across an ultrafiltration membrane with a molecular weight cutoff of 10 000 to 100 000 Da. Low molecular weight materials, i.e. water, lactose, soluble minerals and vitamins, pass through the membrane and form the permeate stream. The membrane retains the remaining components and this mass, called retentate (or concentrate), is used for cheesemaking. The concentration of the retentate is varied by continually recycling the feed across the membrane until the desired concentration of milk proteins is achieved or by using a very large surface area of membrane, as in large commercial operations.

There are three major methods for using ultrafiltration for cheesemaking; low concentration (also known as protein standardization), medium concentration, and high concentration (precheese concept). The latter (precheese concept) paved the way for the application of ultrafiltration in cheesemaking. This process, commonly known as the MMV process after its inventors Maubois, Mocquot and Vassal of INRA, France, was originally developed for

Table 1 Composition of milk concentrated approximately threefold by reverse osmosis and ultrafiltration

Component	*Milk*	*Reverse osmosis (%)*	*Ultrafiltration*
Total solids	12.2	36.6	28.0
Fat	3.50	10.5	10.5
Total protein	3.20	9.6	9.5
Lactose	4.80	14.4	4.1
Ash	0.70	2.1	1.3

Camembert cheese in the late 1960s and has been adapted for other cheeses, such as Feta.

Certain physicochemical properties of ultrafiltered milk are particularly critical in cheesemaking applications and should be understood. These include viscosity, buffering capacity and rennet coagulation properties. As the protein content of milk is increased by ultrafiltration, there is an increase in viscosity. This aspect is of particular importance in the pumping requirements of ultrafiltered milk at high protein levels. For example, in some cheesemaking procedures where fermented milk is concentrated to a high level in a multistage ultrafiltration unit, positive displacement pumps have to be used to transport efficiently the viscous retentate across the later stages of the membrane unit. Further, mixing of ingredients such as starter, rennet and salt requires attention to prevent localized coagulation.

During ultrafiltration of milk, proteins and colloidal salts are concentrated simultaneously. This causes an increase in the buffering capacity, and hence directly influences acid production characteristics of lactic acid bacteria, the pH of cheese, ripening characteristics and rennet coagulation. Under conditions of high buffering, it is difficult to obtain the desired pH even with the production of large amounts of lactic acid by the starter bacteria (**Figure 1**). Such a reduction in the rate at which the pH falls allows lactic acid bacteria to grow to large numbers but also offers the potential for growth of spoilage and pathogenic organisms. The large amount of lactic acid produced results in an acid-tasting product and an imbalance in calcium will cause poor cheese texture and functionality, such as stretching. It is possible to lower the buffering capacity of ultrafiltered milk by removing some of the colloidal salts by solubilization. This can be accomplished by reducing the pH of milk to 5.6–6 during ultrafiltration.

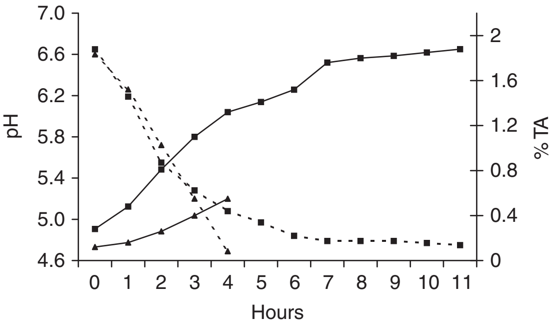

Figure 1 Relationship between lactic acid production (solid line) and pH (broken line) during lactic fermentation of unconcentrated milk (▲) and 4.3x ultrafiltered milk (■).

Another property of concern is rennet coagulation. Generally, concentration reduces the rennet coagulation time and increases the firmness of the coagulum. The firmness of rennet curd from unconcentrated whole milk, as measured by a formagraph, is approximately 8 mm after 40min while that of 6% protein ultrafiltered milk is 58 mm. This is in part because of increased protein and calcium in the retentate and also because in ultrafiltered milk (4×) hydrolysis of only 50% of the κ-casein is required for curd formation compared to 97% for unconcentrated milk. This phenomenon is useful where high temperature treatment of milk, such as UHT, is used. It is well known that severely heated milk has poor rennet coagulation properties, i.e. the coagulum is very weak. French workers have demonstrated that if milk is ultrafiltered prior to UHT treatment, its rennet coagulation properties are restored.

Low-Concentration Retentates (Protein Standardization)

At present, this is probably the most widely used application of ultrafiltration for cheesemaking because it is easily adaptable to most cheese varieties while at the same time providing economical benefits. In this method, milk is ultrafiltered to a concentration of no more than 2× and conventional cheesemaking equipment is used. A common practice is to increase the milk protein concentration to 3.7–4.5% prior to cheesemaking. This enables uniformity in the composition of milk, hence the term 'protein standardization' for this application of ultrafiltration. Other terms used include low-concentrated retentates (LCR). This method is used for various cheeses, including Camembert, Cheddar and Mozzarella. Advantages of using this procedure include uniformity in milk composition from day to day, a firm coagulum and therefore lower losses of casein to whey, increased cheese yield (approximately 6% on a protein basis), improved cheesemaking efficiency (more cheese per vat) and, importantly, there is no need for additional specialized cheesemaking equipment other than an ultrafiltration unit (**Figure 2**). The increase in cheese yield is attributed to better fat and protein recovery and the retention of some whey proteins.

For cheeses such as Cheddar, concentration of up to 1.6 to 1.7× is used. At higher levels, the rennet coagulum is extremely firm and difficult to handle and fat losses in the whey may be high. The moisture content of Cheddar

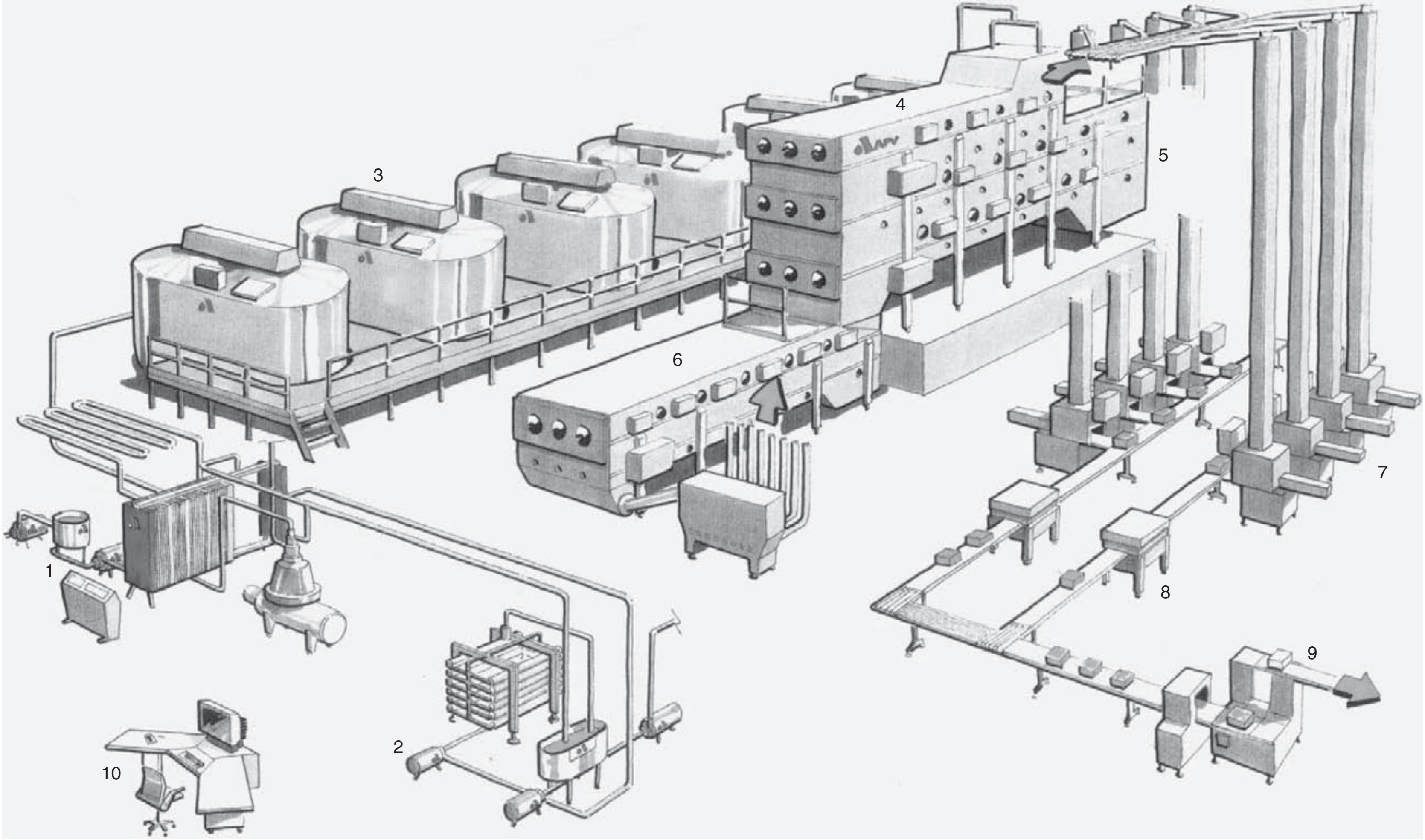

Figure 2 Typical plant layout for Cheddar cheese manufacture using ultrafiitration for protein standardization. 1, Pasteurization and fat standardization; 2, protein standardization using UF; 3, cheesemaking; 4, draining conveyer; 5, cheddaring conveyor; 6, salting/mellowing conveyor; 7, block former; 8, vacuum packaging; 9, cheese block packing; 10, main process control panel. (Courtesy APV Nordic, Aarhus, Denmark.)

cheese made with this process tends to decrease with protein content in milk; suggested reasons for this effect include rapid syneresis because of the coarser network of the protein gel. Using standard procedures such as low-temperature cooking can increase moisture content. Researchers in the United States have demonstrated that homogenization of the cream can readily increase the level of cheese moisture. This method can also be used to increase further the yield of Cheddar cheese made from ultrafiltered milk. Recovery of fat in Cheddar cheese made from milk ultrafiltered to 4.6% protein and without cream homogenization was 94.7%, whereas that with cream homogenization was 96.8%.

Maubois and colleagues in France developed the concept of ultrafiltration of milk on farms for cheesemaking in the late 1970s. This method used the LCR approach and involved the ultrafiltration of milk to less than 2× on the farm prior to transportation to the cheese factory. Permeate was fed to cows at the farm. The objective was to reduce the cost of transport of milk and to increase cheese yield. The economics of the process should also take into account the disposal of permeate that is produced on the farm. Subsequent studies in the United States suggested that this process would be economical for farms with 100 to 1000 cows. This method of ultrafiltering milk on the farm is now being used in the United States where milk is ultrafiltered cold to 3.5× at a collection centre and then transported long distances (>500 km) to cheese factories, at which the retentate is used to raise the total solids content of cheese milk to 13.5–15%.

Medium-Concentration Retentates

In this method, milk is concentrated to 2 to 5× prior to cheesemaking. In some instances, diafiltration may be adopted to adjust the mineral content and buffering capacity. Much higher quantities of whey proteins are retained in the cheese and the yield is also higher than with the LCR method. The changes in the physicochemical properties of milk are large enough to warrant the use of specially designed equipment. The rennet-induced coagulum, for example, is very firm and difficult to handle with conventional equipment.

After various industrial trials, commercial application of this method for cheesemaking is currently limited; the most notable example includes the APV-SiroCurd process for Cheddar cheese. This method was developed in Australia and involved continuous rennet coagulation of milk ultrafiltered to 40–45% solids. A small portion of the ultrafiltered milk was prefermented with lactic acid bacteria and used as bulk starter at the level of 10–12%. The continually forming coagulum was cut with specially designed wire knives and cubed curd pieces transferred into a rotating drum where syneresis took place during heating to 38 °C over a 30–40 min period. Automated cheddaring occurred at the optimum pH, followed by milling and salting. Yield increases of 6–8% were claimed with this process. After several years of operation, however, this process is no longer used because of technical difficulties and poor economics.

Production of Liquid Precheese

This method was the earliest of all ultrafiltration applications for cheesemaking. Milk is ultrafiltered to a concentration that is equal to the composition of the cheese being manufactured. It is then set with rennet, and acid development takes place, followed by additional treatments required for the specific cheese variety and there is very little whey separation. Thus, this process is unique in that practically no conventional cheesemaking equipment is required and of all the ultrafiltration methods, this method has the highest yield potential because of maximum whey protein retention in the cheese.

While the protein standardization technique can be adapted to most cheese varieties, the liquid precheese concept is more limited in its applicability because it is not possible to achieve the composition of all cheeses by ultrafiltration. The process developed for this method was originally for Camembert cheese. It has also been applied to Feta cheese. New cheeses, such as Pave d'Affinois, have also been developed using the liquid precheese concept.

For Camembert cheesemaking using this method, pasteurized milk is ultrafiltered to 5× and at 20 °C a mesophilic lactic culture and salt are added at 2% and 0.75%, respectively. After a pH of 5.5 is reached, rennet is added and the mixture transferred to forms in which the coagulum is formed. At the proper firmness, the coagulum is removed from the molds, brined for 30min, sprayed with *Penicillium camemberti* spores and ripened at 11–12 °C for 12 days at high humidity for mold growth on the surface of the cheese wheels. This process results in a yield increase of 12% to 15%.

A high level of success with the liquid precheese concept has also been achieved with Feta cheese. In this Danish procedure, 5× ultrafiltered whole milk is homogenized, blended with lactic starter, salt and a lipase-rennet mixture and poured into 18-kg tins where curd forms. The curd is then covered with salt or 6% brine and held for ripening. This process is an example in which the cheese is actually manufactured in its retail package.

Recently, a process for the manufacture of blue cheese was commercialized in France in which standardized milk is ultrafiltered to 6×. Starter and rennet are added and continuous coagulation, cutting and molding follow.

Application of Ultrafiltration for Fresh Cheeses

The manufacture of fresh acid-type cheeses, such as cream cheese, quark and Ricotta, was particularly challenging until mineral and ceramic membranes became available. These membranes made it possible to ultrafilter acid curd to a high solids level with few fouling problems. The general principle is to ferment high-heat-treated milk to pH 4.6 to 4.8 and then ultrafilter the curd to the desired concentration. Traditionally, for quark, a centrifugal separator is used to separate curd and whey. The advantage of the ultrafiltration procedure is that whey proteins are retained and therefore cheese yield is increased. For cream cheese, the Cornell procedure involves the blending of heavy cream with 27.5% solids skim milk retentate to achieve the composition of cream cheese. This mixture is pasteurized and homogenized, then fermented, mixed with stabilizers and pasteurized.

Characteristics of Cheese from Ultrafiltered Milk

While the retention of whey proteins is advantageous from the cheese yield perspective, its impact on cheese quality should also be taken into account. Whey proteins generally are inert filler materials and undergo very little proteolysis during ageing. Flavor development is therefore slow. Retarded proteolysis, along with the high water binding capacity of whey proteins, also influences the texture of cheese. Furthermore, the high buffering capacity of such cheese retards autolysis of lactic starter cells and the breakdown of casein. These effects become more pronounced as the concentration of whey proteins in cheese is increased (LCR cheese versus liquid precheese concept).

The impact of high mineral retention on cheese functionality and flavor is also of concern. Excessive retention of calcium makes it difficult to obtain optimal functionality in cheeses such as Mozzarella and may lead to bitterness in fresh acid-curd cheeses. Bitterness also arises because of increased buffering, which leads to high levels of starter cells. Preacidification during ultrafiltration can control the mineral content of cheese.

Microfiltration

Microfiltration of milk for cheesemaking is a relatively new concept but is rapidly gaining commercial acceptance because of the potential to use a wide range of membrane pore sizes (0.05–10 μm). This flexibility makes it possible to achieve the desired specific separation, as well as fractionation, of milk constituents. Ceramic microfiltration membranes are commonly used but polysulfone membranes are also available.

The current direct applications of microfiltration for cheesemaking include a process for the removal of bacteria and casein standardization of cheese milk. Both these approaches are commercialized.

Removal of Bacteria

Reducing the microbial load of milk prior to cheesemaking by processes such as pasteurization is a common practice and in some cases even mandatory. On the other hand, high heat treatment of milk is believed to alter the cheesemaking characteristics of milk and flavor characteristics of cheese. High-speed centrifugation of milk (bactofugation) was developed to remove bacteria, particularly spores of *Clostridium tyrobutyricum,* from milk but the process is not as efficient as newly developed microfiltration procedures.

The microfiltration process of Alfa Laval (now Tetra Pak) for removing bacteria and spores is called the Bactocatch® process (**Figure 3**). Raw skim milk is microfiltered using a membrane with a pore size of 1.4 μm at 35–50 °C. The retentate contains bacteria and the permeate is the bacteria-free milk. This bacteria-free milk can be blended with heated cream for standardization of fat. Bacteria removal efficiencies of 99.6–99.98% are reported, i.e. almost sterile milk is obtained. In the original Alfa Laval (Tetra Pak) procedure, the retentate (which contains bacteria and some milk components) was heated to a high temperature to kill the bacteria and blended with cream. In a modification developed by

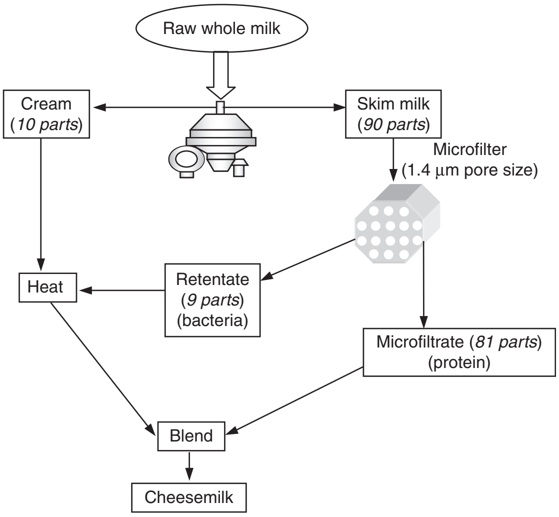

Figure 3 Process for removal of bacteria from milk by microfiltration.

APV, this bacterial concentrate is recycled through the self-desludging separator prior to microfiltration. Hence, bacteria are removed as sludge and milk components are recovered.

This process is particularly suitable for fluid milk production *(see* **Milk Protein Products**: Membrane-Based Fractionation) but is attractive for the manufacture of cheeses such as Swiss because of the possibility of removal of spores of *Cl. tyrobutyricum* without using nitrates or excessively high heat. On the other hand, French researchers have demonstrated that under normal circumstances, microfiltered milk is not ideal for eye formation in Swiss cheese because of the removal of non-starter lactic acid bacteria by microfiltration. This has been overcome by modifying the starter system. Specific heterolactic strains with mesophilic, thermophilic and propionic starters are recommended.

Casein Standardization

Separation of casein and whey proteins can be accomplished by using a microfiltration membrane of 0.1 μm pore size. When skim milk is microfiltered in this manner, casein is concentrated and whey proteins are in the permeate. The casein content of milk is increased from 2.5% to 3.5% and hence cheese yield is increased. Furthermore, the microfiltrate (permeate) generated from this process contains whey proteins but no glycomacropeptide, which is normally found in whey from conventional cheesemaking procedures. With this method, it is therefore possible to standardize the casein content of cheese milk while producing an 'ideal whey' that has better functional characteristics than whey containing glycomacropeptides.

In recent work, French workers have used this approach in combination with ultrafiltration to improve the cheesemaking properties of dried milk. In this patented process, whey proteins are completely or partially removed from milk by microfiltration, as above. The microfiltration permeate, which contains the whey proteins, is ultrafiltered and the permeate from ultrafiltration, which contains lactose, minerals and water, is blended with the retentate of microfiltration. The microfiltration retentate contains casein, and when blended with ultrafiltration permeate yields milk with low or no whey protein. This milk is evaporated and spray-dried. The spray-dried product can be used for cheesemaking after reconstitution without the typical difficulties encountered with conventional powder, which contains denatured whey proteins because of the heat treatments employed during manufacture.

Nanofiltration

Dairy applications of nanofiltration are very recent and major use is currently in the area of whey processing. Such use includes demineralization and concentration of whey and reduction of salt from salt whey *(see* **Whey Processing**: Demineralization). Interesting applications for cheese are also emerging. High permeability of monovalent ions (40–90%) and low permeability of polyvalent ions (5–20%) typically characterize nanofiltration. Consequently, it is possible to concentrate milk by nanofiltration to obtain an altered mineral balance. Experimental work suggests that this would offer potential for soft cheese manufacture.

Future Potential

Over the past 35 years, membrane processing of milk has allowed the introduction of many innovations for cheesemaking. Not only has cheesemaking efficiency improved but also new cheeses have been developed. The process that led the development of membrane applications in cheesemaking, MMV process for Camembert cheese, is no longer used for this cheese because of difficulties in meeting consumer expectations for the appearance of the cheese. However, that process inspired the development of applications for other cheeses, namely, Feta, Pavé d'Affinois, Le Petit Moulé, La Roche (blue cheese) and others. Since the early days, significant improvements have been made in membrane design and processes that have further enhanced cheesemaking applications. As new membrane processes and applications are developed, innovations in cheesemaking will continue. Unfortunately, not all countries have taken advantage of the applications of membranes for cheesemaking because the standards of identity pertaining to membrane-processed milk have not been fully resolved in individual countries but Codex Alimentarius regulations do permit the use of such milk and progress will continue.

See also: **Milk Protein Products**: Membrane-Based Fractionation; **Plant and Equipment**: Evaporators; **Whey Processing**: Demineralization.

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Microbiology of Cheese

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Introduction

Cheese is essentially a microbial fermentation of milk by selected lactic acid bacteria (LAB), whose major function is to produce lactic acid from lactose, which, in turn, causes the pH of the curd to decrease. It is a complex microbial ecosystem consisting of bacteria and, in the case of smear- and mold-ripened cheese, yeasts and molds. During ripening, the various microorganisms produce enzymes, particularly proteinases and lipases, which hydrolyze the protein and fat to amino acids and fatty acids, which, in turn, are the precursors of the compounds that impart flavor to cheese. In this article, an overview of the different bacteria found in cheese and the factors controlling their growth will be presented.

Starter Bacteria

In most cheeses, selected strains of LAB, are previously grown in milk or another medium and added deliberately to the milk at the beginning of cheese manufacture. In some countries, for example, France and Italy, with a long tradition of cheesemaking, whey starters are used. In this case, fresh whey is incubated under carefully controlled temperatures for use in the next day's production. They are called 'starters' because they start lactic acid production and are divided into two types: mesophilic and thermophilic.

Mesophilic starters comprise lactococci and sometimes leuconostocs, and thermophilic cultures comprise *Streptococcus thermophilus* and thermophilic lactobacilli. Mesophilic starters are used in Cheddar cheese, soft cheeses, and most Dutch cheeses. The cultures are generally selected for their ability to produce lactic acid at a rate appropriate for the cheese being made, to resist attack by bacteriophage, and to produce a cheese with the desirable flavor. Many artisanal cheeses, especially Spanish varieties, are made without the deliberate addition of a starter. In these cheeses, lactococci, which are present in milk as natural contaminants, also make up the major part of the microflora at the beginning of ripening. Nowadays, mixtures of mesophilic and thermophilic cultures, particularly *Str. thermophilus*, are used in, Cheddar cheese, which in the past was made solely with mesophilic cultures. In these mixtures, the function of the thermophilic culture is to improve the flavor and combat the effect of bacteriophage (phage).

The initial number of starter bacteria in cheese milk ranges from 10^5 to 10^7 cfu ml^{-1}, depending on the cheese type. They grow relatively rapidly during manufacture, reaching numbers of $\sim 10^9$ cfu g^{-1} in almost all cheeses within a few hours of being added to the milk. Therefore, starter bacteria are the dominant organisms in cheese at the beginning of ripening. As they grow, they produce lactic acid in direct proportion to the amount of lactose used by them. It is this action that causes the decrease in the pH of curds and whey.

Most of the starter bacteria lyse relatively rapidly during ripening, releasing their intracellular enzymes, which, together with chymosin, help the development of cheese flavor. The ability to lyse varies; some strains lyse relatively quickly, whereas others lyse slowly. Faster-lysing strains generally produce cheese that develop flavor more rapidly than slower-lysing strains. Lysis is caused by an intracellular muraminidase that hydrolyzes the bacterial cell wall. Generally, *Lactococcus lactis* subsp. *cremoris* strains lyse faster than *Lactococcus lactis* subsp. *lactis* strains, which partly explains why the former organism is thought to produce a better flavored cheese than the latter. Lysis is influenced by several factors, including the level of salt in cheese and the presence of prophages in the starter bacteria, which are induced by the high cooking temperature used during the manufacture of some cheeses. The presence of a small number of lytic phage is also likely to play a role in lysis, though this aspect has not been studied extensively.

During the early days of ripening, the high numbers of starter bacteria continue to metabolize any residual lactose in the cheese to lactic acid (**Figure 1**), at a rate very much dependent on the salt-in-moisture (SM) in the cheese. In cheese, all the salt is dissolved in the moisture component and, therefore, the salt is generally measured as the SM, which is defined as

$$\frac{\text{Salt, g } 100\text{ g}^{-1} \times 100}{\text{Moisture, g } 100\text{ g}^{-1}}$$

This figure has no units, even though it is routinely expressed as a percentage.

Enterococci

Enterococci are found at high numbers ($>10^7$ g^{-1}) in many artisanal cheeses, particularly those produced in southern Europe. They are not deliberately added to the milk for cheesemaking except as probiotic cultures. They may originate from the milk used in cheesemaking, but

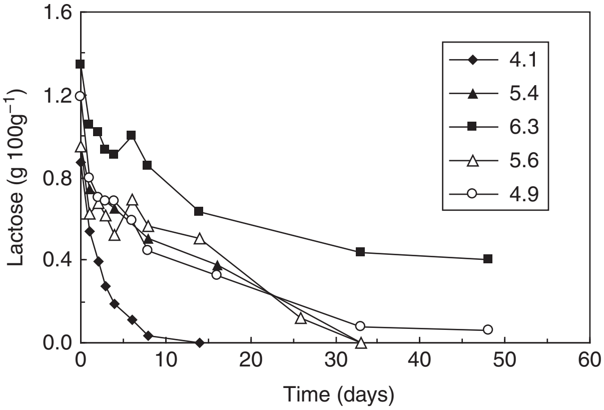

Figure 1 Effect of salt-in-moisture (SM) levels (as reflected by the symbols in the inset) on lactose metabolism in Cheddar cheese made with *Lactococcus lactis* ssp. *cremoris* C13 and C266 and ripened at 12 °C. Redrawn from Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers.

are also found in significant numbers in many milk and whey starters used for traditional cheese manufacture. For this reason, they are often considered to be starter cultures. Many of the cheeses in which they are found are made from raw milk, but they are also found in cheeses made from pasteurized milk, as they withstand pasteurization. Most of the evidence suggests that they play a significant role in flavor development in the cheeses in which they are found.

Enterococci are considered to be emerging pathogens; in recent years, they have been incriminated as the cause of several hospital-acquired infections. They are promiscuous, easily picking up plasmids encoding resistance to antibiotics, especially vancomycin, which is usually the antibiotic of choice in treating these infections. Therefore, their presence in cheese is being questioned.

Nonstarter Lactic Acid Bacteria

All cheeses ripened for any length of time – fresh cheeses are an exception – contain non-starter lactic acid bacteria (NSLAB). They mainly comprise facultatively heterofermentative lactobacilli, especially *Lactobacillus casei* and *Lactobacillus paracasei*, but *Pediococcus* spp. and obligately heterofermentative *Lactobacillus* spp., for example, *Lactobacillus brevis* and *Lactobacillus fermentum*, are occasionally found. NSLAB are also called mesophilic lactobacilli to distinguish them from the thermophilic lactobacilli used as starters. Raw milk and/or the factory environment are the major sources of NSLAB in cheese. Small numbers of lactobacilli survive pasteurization and the high cooking temperature (52 °C) used in making some hard cheeses, like Emmental, which is traditionally made from raw milk. Most NSLAB are salt-and acid-tolerant, facultative anaerobes and therefore grow quite well in cheese. They need a fermentable carbohydrate for energy production and growth, but the energy source used by them in cheese is unclear, since at the time of exponential growth of NSLAB, no lactose is present. A likely source is the sugars present in the glycoproteins of the milk fat globule. Other suggested sources include citrate and amino acids, but the evidence for this is not strong.

In contrast to the starter, the initial number of NSLAB in cheese is relatively low (~100 cfu g^{-1}), but they grow relatively quickly to high numbers (~10^8 cfu g^{-1}), within the first few days of ripening (**Figure 2**). Their growth rate depends primarily on the particular strains present, the ripening temperature, and the moisture content of the cheese. Growth will be more rapid in high-moisture than in low-moisture cheeses. The generation time of the NSLAB in the cheese in **Figure 2** was ~8.5 days.

Despite extensive studies, the role of NSLAB in the development of cheese flavor is not clear. There is considerable interest in inoculating cheese milk with carefully selected mesophilic lactobacilli with the objective of accelerating ripening and/or intensifying flavor. In contrast to starter cells, NSLAB die off very slowly in hard cheese and their intracellular enzymes are probably not released into the cheese matrix. Nevertheless, cells of NSLAB are viable and, at the high cell densities found in cheese, have considerable metabolic activity, for example, in transforming L-lactate to D-lactate.

Other Microorganisms in Ripening Cheese

Many cheese varieties contain a secondary (nonlactic acid bacteria) microflora of other bacteria, yeasts, and or molds, the function of which is to produce some specific

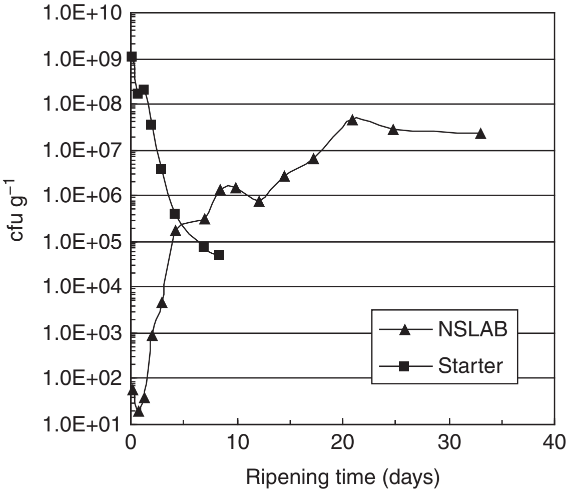

Figure 2 Growth of starters and non-starter lactic acid bacteria (NSLAB) in Cheddar cheese ripened at 6 °C.

characteristic changes in the cheese. Examples include smear- and mold-ripened and Swiss-type cheeses. In these cheeses, flavor development is dominated by the metabolic activity of the secondary flora and, except in Swiss- and Blue (mold)-type cheese, the activity is confined to the surface of the cheese. The secondary flora also produces proteinases, peptidases, and lipases; the lipases are especially important in Blue cheese.

Micrococci and Staphylococci

Micrococcus and *Staphylococcus* are found in large numbers ($>10^6$ cfu g^{-1}) on the surface of hard, semihard, and soft smear-ripened cheeses. Microscopically they are very similar, but they are phylogenetically unrelated to each other. *Staphylococcus* has a low GC content (30–39%) and occurs in the clostridial branch of Gram-positive bacteria, whereas *Micrococcus* has a high GC content (65–75%) and occurs in the actinomycete branch of the Gram-positive bacteria. They are easily distinguishable from each other; for instance, staphylococci produce acid aerobically and anaerobically from glucose, and are oxidase-negative and sensitive to lysostaphin, whereas micrococci produce acid only aerobically from glucose, when they do it at all, and are oxidase-positive and resistant to lysostaphin. These genera were probably confused with each other in the past, and it is often unclear whether cheese isolates were genuinely staphylococci or micrococci. The dominant staphylococci in cheese are *Staphylococcus saprophyticus*, *Staphylococcus equorum*, *Staphylococcus vitulus*, and *Staphylococcus xylosus*, all of which are coagulase-negative.

Recently, the genus *Micrococcus* has been split into five genera, *Kocuria*, *Nesterenkonia*, *Kytococcus*, *Dermacoccus*, and *Micrococcus*, and the strains isolated from the surface of cheese include *Kocuria rosea*, *Kocuria varians*, *Dermacoccus sedantarius*, *Micrococcus lylae*, and *Micrococcus luteus*. The exact role of staphylococci and micrococci in cheese is not very clear, but many of them produce proteinases and lipases.

Coryneform Bacteria

Coryneform bacteria are mainly found on the surface of smear-ripened cheeses and, for a long time, *Brevibacterium linens* was thought to be the most important one, being responsible for the red or orange color on the surface. For this reason, it is often deliberately inoculated onto the surface of the cheese after brining. Recent evidence shows that other coryneform bacteria are also important, including *Arthrobacter*, *Agrococcus*, *Brachybacterium*, *Corynebacterium*, and *Microbacterium* spp. Generally, the staphylococci are the first to grow on the surface of the cheese, followed by the coryneforms.

The sources of these bacteria include the brine and shelving; manual handling of cheeses is also an important source, as coryneforms, micrococci, and staphylococci are a major part of the skin microflora.

Propionic Acid Bacteria

Propionic acid bacteria (PAB) are found in Swiss-type cheese, for example, Emmental and Comté. Their major function is to metabolize the lactate produced by the starter bacteria to propionate, acetate, and CO_2:

$$3 \text{ lactate} \rightarrow 2 \text{ propionate} + 1 \text{ acetate} + 1CO_2$$

CO_2 is responsible for the large holes, called 'eyes', characteristic of these cheeses. *Propionibacterium freudenreichii* is the most important species. PAB, although catalase-positive, are essentially anaerobes and grow only within the cheese. Traditionally, natural contamination of the milk was relied upon as the source of PAB in the case of Emmental and Comté cheeses, but, nowadays, selected strains are generally deliberately added to the milk with the starter culture. Development of PAB in Italian cheeses, for example, Parmigiano-Reggiano and Grana, is considered to be a defect.

Yeasts

Yeasts form a large part of the surface flora of smear- and mold-ripened cheeses, for example, Comté, Tilsit, Limburger, Blue, and Camembert. They are very tolerant of the low pH and high salt concentrations on the surface of the cheese and, during the early days of ripening, grow rapidly to perhaps 10^6–10^7 cfu g^{-1}. Simultaneously, they oxidize lactate to CO_2 and H_2O, and produce NH_3 by deaminating amino acids, both of which result in an increase in pH from an initial value of ~5.0 to >7.5. This process is called deacidification and the increase in pH also promotes the growth of bacteria, which are much less tolerant of low pH values.

A diverse group of species are involved. The most common ones are *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica*. Generally, yeasts are considered to be adventitious contaminants though some of them, particularly the first two mentioned above, are often deliberately inoculated onto the surface of smear-ripened cheese after brining.

Geotrichum candidum has characteristics of both yeasts and molds and, in the past, was often called a yeast-like fungus; it is commonly known as the dairy mold. Its natural habitat is soil, where it is involved in the decay of organic matter. *Geotrichum candidum* can grow at pH values in the range 2.5–8.1 and in environments with low levels of O_2.

Yeasts may also cause defects in cheese, particularly gas production, but nowadays this is very rare because of improved hygiene and better quality control in cheese manufacture.

Molds

The dominant molds in cheese are *Penicillium roqueforti* in Blue cheeses (e.g., Stilton, Roquefort, and Gorganzola) and *Penicillium camemberti* in surface mold-ripened cheeses (e.g., Camembert and Brie). *Penicillium roqueforti* grows in the air spaces between the incompletely fused curd particles and is responsible for the blue veins that run throughout Blue cheese, whereas *P. camemberti* grows as a compact, fluffy mass on the surface of Camembert and Brie cheese. Molds are obligate aerobes and, therefore, require O_2 for growth. *Penicillium roqueforti* grows well at much lower O_2 levels than those required by other molds, and, for this reason, Blue cheeses are generally pierced after brining to allow a small amount of O_2 to diffuse into the center of the cheese to promote mold development. Hydrolysis of fat to mono- and diacylglycerols and fatty acids and subsequent production of methyl ketones through β-oxidation of the fatty acids are the major biochemical activities of molds in Blue cheese. Butyric (C_4) and caproic (C_6) acids and 2-heptanone are the major compounds responsible for the strong, piquant flavor of Blue cheeses.

Starters containing high numbers of citrate-utilizing *Lc. lactis* and *Leuconostoc* species are normally used in the manufacture of Blue cheese because they produce an open-textured curd through the production of CO_2 from citrate, which helps the development of *P. roqueforti.*

Penicillium roqueforti and *P. camemberti* also produce proteinases, and proteolysis, especially by *P. camemberti*, is the dominant activity in Camembert and Brie cheeses. These cheeses soften from the outside to the inside, and originally it was thought that this was due mainly to the proteolytic activity of *P. camemberti.* Nowadays, it is felt that the softening of these cheeses is due principally to the increase in pH on the surface early in ripening, which causes the curd to solubilize and soften.

Different combinations of *B. linens*, *G. candidum*, *D. hansenii*, *P. roqueforti*, and/or *P. camemberti* are deliberately added either to the milk or to the cheese after brining in smear- and mold-ripened cheeses. They are not considered starter cultures, as they have no role in acid production. Several methods of inoculation are used. The milk for the mold-ripened varieties, namely, Blue, Camembert, and Brie, is inoculated with pure cultures of the relevant species of *Penicillium* at the same time as the starters. The curd for Blue cheese is subsequently pierced to allow limited entry of O_2 to promote growth of *P. roqueforti.* Surface-ripened cheeses like Tilsit, Münster, and Limburger are dipped, sprayed, or brushed with aqueous suspensions of different combinations of *B. linens*, *D. hansenii*, and *G. candidum* soon after the cheeses are removed from the brine. Smear from ripened cheese, the so-called 'old' smear, may also be used, but this can also be a source of pathogens, especially listeria. Both mold- and bacterial-ripened cheeses are then ripened at 10–15 °C to promote microbial growth and activity, and at a high relative humidity to prevent loss of moisture from the cheese surface.

Yeasts and molds grow much better than bacteria at the pH of cheese, and for this reason they are the first microorganisms to grow on the cheese surface. The low pH of freshly made cheese is therefore partially selective for the growth of yeasts and molds. *Brevibacterium linens* does not grow at a pH value below 6.0; whether this is true of the other bacteria found on the surface of cheese has not been determined. Yeasts and molds are generally heat-sensitive and are killed by pasteurization.

Factors Controlling Microbial Growth

The factors controlling the growth of microorganisms in cheese include water activity, concentration of salt, oxidation–reduction potential, pH, NO_3^-, temperature, and, perhaps, the production of bacteriocins by some starters. These factors are called 'hurdles'. The effect of the individual hurdles may not be significant, but all of them acting together lead to considerable control. Other compounds produced during curd manufacture and ripening, for example, H_2O_2 and fatty acids, also inhibit microbial growth, but the concentrations of these produced by the starters in cheese are not sufficiently high to have a significant effect on the bacteria.

Water Activity

All microorganisms require water for growth, but it is the availability of the water, rather than the total amount present, that is the important factor. Water availability is measured by water activity (a_w), which is defined as the ratio of the vapor pressure over the cheese to the vapor pressure of pure water at that temperature. The value of a_w ranges from 0 to 1.0. NaCl and organic acids (lactate, acetate, and propionate) dissolve in the moisture in the cheese and reduce the vapor pressure. The greater the concentration of these compounds, the lower the a_w.

Cheese, unless vacuum packed, loses moisture by evaporation during ripening. This results in a gradient in the a_w of the cheese (lower on the outside than on the inside). The a_w gradient is generally much greater in large cheeses than in small ones. Proteins in cheese are hydrated, and this 'bound' water is not available for bacterial growth. Hydrolysis of proteins to amino acids and peptides and lipids to acylglycerols and fatty acids during ripening reduces the availability of water, as one molecule of water is added at each bond hydrolyzed.

Bacteria grow at higher a_w values than yeasts, which, in turn, grow at higher a_w values than molds. Most bacteria require an a_w >0.92 for growth. The limit for most yeasts is ~0.83, whereas that for molds is ~0.75; osmophilic yeasts grow at a_w values <0.60. An a_w value of <0.92 is equivalent to a salt concentration of 12.4%. Growth of microorganisms at low a_w is characterized by a long lag phase, a slow rate of growth (i.e., a long generation time), and a reduction in the maximum number of cells produced. Each of these factors helps to limit the multiplication of microorganisms.

Salt

The use of NaCl as a food preservative is probably as old as food production itself. The concentration required depends on the nature of the food, its pH, and moisture content, but, generally, <10% is sufficient. The action of salt is intimately connected with the reduction in a_w that occurs when salt (or any solute) gets dissolved in water. The relationship between salt concentration and the decrease in a_w is almost linear. In cheese, the salt concentration varies from perhaps 0.4% in Emmental cheese to 5% in Blue cheese. In calculating the inhibitory effect of salt in cheese, it is the amount of salt dissolved in the water (SM) of the cheese, rather than the actual concentration of salt, that is the important parameter. The SM in Cheddar cheese varies from 4 to 6.

Most cheeses are brine-salted – Cheddar is an exception and is dry-salted. In brine-salted cheeses, a salt gradient (outside higher, inside lower) exists at the beginning of ripening, which decreases relatively slowly during ripening. Salt concentration is influenced by the size of the cheese (the larger the cheese, the longer it is brined), the concentration of salt in the brine, the temperature of the brine, and the length of time that the cheese is immersed in the brine. Generally, the brine used contains 21% NaCl, has a pH of 5.2 (adjusted with lactic acid), and contains 0.2% Ca to prevent leaching of lactate and Ca from the cheese surface.

All brined cheeses contain a high level of salt in the surface layers; therefore, the secondary microorganisms growing on the surface must be salt-tolerant. Most coryneforms, micrococci, and staphylococci can grow in the presence of 10–15% NaCl. The growth of *P. camemberti* is largely unaffected by 10% NaCl, and some strains of *P. roqueforti* can tolerate 20% NaCl. *Geotrichum candidum* is quite sensitive to salt. A slight reduction in its growth occurs in the presence of 1% NaCl and it is completely inhibited at ~6%. Therefore, too much brining will prevent its growth on the cheese surface. Its intolerance to salt may explain why *G. candidum* is generally deliberately added in the manufacture of surface-ripened cheeses, the hope being that some cells will grow.

Oxidation–Reduction Potential

Oxidation–reduction potential (E_h) is a measure of the ability of chemical/biochemical systems to oxidize (lose electrons) or reduce (gain electrons). A positive value indicates an oxidized state, whereas a negative value indicates a reduced state. The E_h of milk is about +150 mV and that of cheese is about −250 mV. The exact mechanism of the lowering of E_h when cheese is produced from milk is not clear but is probably related to the fermentation of lactose to lactic acid by the starter during growth. At the low E_h, cheese is essentially an anaerobic system, in which only facultatively or obligately anaerobic microorganisms can grow. Therefore, obligate aerobes, like *Brevibacterium* and *Micrococcus* spp., do not grow within the cheese, even when other conditions for growth are favorable.

pH and Organic Acids

Most bacteria require a neutral pH value for optimum growth and exhibit poor growth at pH values <5.0. The pH of cheese curd after manufacture generally lies within the range 4.5–5.3, so pH is a significant factor in controlling bacterial growth in cheese. LAB, especially lactobacilli, generally have a pH optimum below 7, and *Lactobacillus* spp. can grow at pH values of ~4.0; most yeasts and molds have an optimum pH of 5–7 but can grow at pH values <3.0. Coryneforms and micrococci are thought to be unable to grow below pH 5.5 or 6.0.

The efficacy of organic acids as inhibitors of microbial growth is thought to depend on the amount of undissociated acid present and therefore on the dissociation constant (pK_a) and pH. The principal acids found in cheese are propionic, acetic, and lactic, and these have pK_a values of 4.87, 4.75, and 3.08, respectively, so that at the same concentration lactic acid is the least and propionic the most effective inhibitor. However, the concentration of the acid is also important and, in cheese, lactate is invariably present in young cheese curd at much greater concentrations than those of the other two acids. The pH of many soft cheeses characteristically increases during ripening, particularly on the surface, and this will reduce the inhibitory properties of the surface. Propionic acid is very effective at repressing the growth of molds.

Nitrate

Nitrate (NO_3^-), as KNO_3 (saltpeter) or $NaNO_3$, is added to the milk ($20\,g\,100\,l^{-1}$) for some cheeses, especially Dutch-type cheeses like Gouda and Edam, to prevent early and late production of gases by coliforms and *Clostridium tyrobutyricum*, respectively. The real inhibitor is NO_2^-, which is formed from NO_3^- by xanthine oxidoreductase in the milk or curd. The exact mechanism by which NO_2^- prevents microbial growth is not clear. NO_2^- is an effective inhibitor of clostridia, but it does not inhibit coliforms. Instead, NO_3 acts as an alternative electron acceptor, reducing the formation of gases (H_2 and CO_2) from pyruvate by pyruvate–formate hydrogen lyase. NO_2^- can also react with aromatic amino acids in cheese to produce nitrosamines, many of which are carcinogenic.

Temperature

The temperature at which cheese is ripened is dictated by two opposing forces. On the one hand, there is the need to control the growth of potential spoilage and pathogenic bacteria and, on the other hand, the need to promote the ripening reactions and the growth of the secondary microflora in the case of surface-ripened and Swiss-type cheeses. Higher temperatures promote faster ripening by the starter and non-starter microorganisms but also allow the growth of spoilage and pathogenic bacteria. Generally, Cheddar cheese is ripened at 6–8 °C, whereas mold- and smear-ripened cheeses are ripened at 10–15 °C. Emmental cheese is ripened initially for 2–3 weeks at a low temperature (~12 °C), after which the temperature is increased to 20–24 °C and maintained for 2–4 weeks to promote the growth of PAB and the fermentation of lactate to propionate and acetate; the temperature is then reduced again to ~4 °C. For soft cheeses, the humidity of the environment is also controlled to prevent excess evaporation of moisture from the cheese surface.

Microbial Spoilage of Cheese

The most common microbial defects of cheese are the development of early and late gas, but neither is common in cheese made today, thanks to better hygiene in milk production and better quality control in cheese plants.

Early gas formation generally occurs within 1 or 2 days after manufacture. It is characterized by the appearance of many small holes in the cheese and is caused by coliform bacteria and/or yeasts. The gas produced by coliforms is mainly H_2, whereas that produced by yeasts is CO_2; both are produced from lactose. Early gas production is more problematic in soft and semisoft cheeses than in hard cheeses because of the higher a_w of the soft cheeses. An effective way of controlling early gas formation is to add nitrate to the milk.

Late gas formation, also called 'late blowing', does not occur until late in ripening and is due to fermentation of lactate to butyrate, CO_2, and H_2 by *Cl. tyrobutyricum* and *Clostridium butyricum.* The butyrate is responsible for off-flavor development, and the gases, for the production of large holes in the cheese. Late gas production can be particularly prevalent in Swiss-type cheese, as clostridia can grow during the hot-room ripening period. Silage is a potent source of these bacteria and, in Switzerland, it is forbidden to feed it to cows whose milk is used for cheesemaking. In addition, many thermophilic cultures stimulate the growth of clostridia, through the production of peptides and amino acids. Late gas production can be controlled by NO_3^- or by bactofugation of the milk to remove the clostridal spores, but the latter can result in inferior quality cheese. Increasing the level of salt, lowering the pH of the cheese rapidly through the use of an active starter, addition of NO_3^-, and addition of lysozyme are also effective in preventing late gas production. Lysozyme, which occurs naturally in milk, saliva, tears, and other body fluids, hydrolyzes the cell walls of sensitive bacteria, like *Cl. tyrobutyricum*, causing them to lyse. It is commonly used in Italian cheeses and is added to the milk with the starter at a level of $25\ \mathrm{mg\,l^{-1}}$.

The bacteriocin, nisin, produced by some strains of *Lc. lactis* subsp. *lactis* is effective in controlling the growth of clostridia and is used for this purpose in processed cheese. However, it is not suitable for use in natural cheese because many starters are sensitive to it.

Other microorganisms have occasionally been implicated in spoilage. Citrate-metabolizing lactobacilli have been incriminated as the cause of open texture in Cheddar cheese owing to the production of CO_2 from citrate. The optimum pH for uptake of citrate ranges from 4 to 5, and a significant metabolism of citrate occurs in the absence of an energy source at pH 5.2, the pH of many semihard and hard cheeses. *Enterococcus malodoratus*, which, as its name implies, causes the production of bad flavors, has been found in Gouda cheese. The surface of cheese, especially when it is moist, for example, an unwrapped soft or semisoft cheese, is an ideal environment for the growth of molds and yeasts. These cause little damage to the cheese but are unsightly. They can be washed off the cheese surface with a dilute brine solution.

Microbiological Analysis of Cheese

Traditionally, the various groups of microorganisms in cheese are detected by plating decimal dilutions of the cheese, emulsified in trisodium citrate, on selective media followed by incubation and counting. Such methods give very useful information especially if they are conducted on a time basis. Recently, these culture-dependent methods have been augmented by culture-independent, molecular methods, for example, direct extraction of DNA and RNA followed by amplification of a portion of the genes encoding 16S rRNA and analysis of the products by single-stranded conformation polymorphism (SSCP), temporal gradient gel electrophoresis (TTGE), denaturing gradient gel electrophoresis (DGGE), and restriction fragment length polymorphism (RFLP). Such techniques have been used to examine the yeasts and bacteria in several cheeses. Fluorescence *in situ* hybridization (FISH) has also been successfully used. These techniques are giving very useful information and complement the data obtained by conventional microbial analysis.

In addition, strain differences between isolates from cheese are being studied by molecular techniques including randomly amplified polymorphic DNA (RAPD), 16S rRNA sequencing, use of species-specific probes, polymerase chain reaction (PCR) and multiplex-PCR, and pulsed-field gel electrophoresis (PFGE).

Real-time PCR has been used to quantify *Corynebacterium casei*, based on primers that target the 16S rRNA gene.

See also: **Cheese**: Avoidance of Gas Blowing; Blue Mold Cheese; Camembert, Brie, and Related Varieties; Cheddar-Type Cheeses; Dutch-Type Cheeses; Non-Starter Lactic Acid Bacteria; Overview; Public Health Aspects; Salting of Cheese; Secondary Cultures; Smear-Ripened Cheeses; Starter Cultures: General Aspects; Starter Cultures: Specific Properties; Swiss-Type Cheeses.

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Use of Microbial DNA Fingerprinting

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Recent Trends in Dairy Microbiology

It is known that the quality of cheese and other fermented foods is influenced by microorganisms appearing during the various stages of production. As different microbial species have different metabolic potentials, it is not surprising that the study of the structure of cheese microbiota and its role in the achievement of desired sensorial properties has been an important target of dairy microbiology.

The study of microbial ecology of complex natural ecosystems has dramatically changed in the last decades. This is due to the availability and use of novel approaches to perform detection, identification, and typing of microorganisms. Novel scenarios have opened in the field of environmental microbiology after molecular techniques based on DNA and RNA analysis have been introduced and profitably employed. On the basis of the interesting achievements and advance of knowledge in the field of environmental microbiology, other microbiological disciplines, amongst which is food microbiology, have updated their systems of investigation and moved forward with improved tools of analysis. The science and approach to study microorganisms in food is completely different as compared to those of the past. The present day is the age when functional genomics, transcriptomics, proteomics, and metabolomics are establishing the overall role of bacteria in food fermentation. Nevertheless, during the last decade, much research effort has been dedicated to the development and optimization of molecular methods for the detection, reliable identification, and monitoring of microorganisms involved in food fermentation. Owing to the development of molecular techniques, important changes have been introduced into research laboratories and also into the thinking of researchers who are now more used to approaching through nucleic acids from bacteria than bacteria themselves in their studies of the microbial ecology of foods. The availability of such methods has made food scientists shift from a more traditional isolation and biochemical characterization of microbes from food to a direct detection of microbes not as microbes anymore but rather as their DNA or RNA. Within food microbiology, the field of dairy microbiology is the one that has adapted more rapidly to the new trends and made use of the novel approaches for the specific purposes of dairy scientists. The principal interest of dairy microbiologists is to study the diversity and dynamics of microorganisms in dairy production and possibly to correlate the occurrence of certain microbial species and strains with a desired quality of the cheese.

Traditional culture-based procedures to study cheese microbiota remain the official and the most commonly employed approach to study cheese microbiology. However, more and more laboratories now possess the appropriate equipment and scientific and technical expertise to study microorganisms from cheese by molecular approaches. Cultivation of microorganisms on appropriate, even selective, culture media is at the basis of traditional microbiology. Therefore, the first approach to study the microbiology of a cheese is to get representative samples and plate them out on as many substrates as possible to pick up the desired microbial flora. After an estimation of the viable microbial loads of specific microbial groups, dairy microbiologists usually go through isolation, purification, and identification of some target microorganisms, the last step often being based on biochemical assays. Depending on the scope of the research/analysis, a further step is to undertake a functional characterization and/or a genotyping to deepen the knowledge of the identity of microbial biotypes within the identified species and to finalize the experimental work to gain the desired information for the dairy scientist.

An acknowledged problem with the culture-dependent study of microorganisms is the lack of knowledge of the real conditions under which most of the bacteria grow in their natural habitat and the difficulty in developing media for cultivation accurately resembling their natural environment. Therefore, the culture-independent techniques, widely used nowadays, are intended to overcome this pitfall of the traditional approach and to study the microorganisms by directly extracting their nucleic acids from the original sample. In other words, the original cheese sample is subjected to direct and total extraction of microbial DNA and/or RNA and further techniques are employed to gather information from the extracted mixture of nucleic acids. As a matter of fact, according to the current literature, the occurrence of uncultured bacteria in cheese is limited; indeed, several different media have been widely used to target the diverse dairy microbial populations, and cases of unculturable microbial taxa have rarely arisen.

Culture-Independent Microbial Fingerprinting of Cheese

Culture-independent molecular methods not only overcome the problems associated with culture-based methods, but are also versatile and rapid, and provide reliable results. In the case of dairy products, genetic fingerprinting techniques provide a profile representing the genetic diversity of the microbial community occurring in a particular cheese, raw material, or intermediate of production.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and temporal temperature gel electrophoresis (TTGE) are the most commonly used among the culture-independent fingerprinting techniques; these are based on the separation of PCR amplicons of the same size but of different sequences. PCR-DGGE of ribosomal RNA genes has been in use in microbial ecology since 1993, though the first application to food microbiology happened in 1999. So far, many studies have benefited from the use of these techniques and they are currently well-established tools for investigating microbial diversity in many laboratories worldwide. The literature describing the application of PCR-DGGE in microbiology is extremely wide. This fingerprinting technique is in fact very versatile and has been successfully used in many fields of microbial ecology. It is employed to assess the structure of microbial communities in various food samples (including cheese) without cultivation and to determine the community dynamics in response to environmental or technological variations.

Technically, DGGE is an electrophoretic method capable of differentiating mixtures of DNA fragments of the same size but with different sequences. These fragments can be separated in a denaturing gradient gel where the denaturing conditions are achieved by using chemicals such as urea and formamide. A combined use of denaturants and temperature make double-strand DNA fragments partially melt in discrete regions called melting domains, which makes the fragments migrate more slowly in the gel. Therefore, DNA fragments of the same size but of different base pair compositions will show different responses to the denaturing gradient and migrate different distances in the gel according to their sequence. As a result, a mixture of different sequences of the same size will take the shape of a fingerprint after DGGE. The fragments loaded on DGGE gels are usually PCR products. An optimal resolution is obtained when the molecules do not completely denature. The addition of a 30–40 bp GC clamp to one of the PCR primers ensures that the fragment of DNA will remain partially double-stranded and that the region screened is in the lowest melting domain.

The most commonly employed target for PCR amplification prior to DGGE is the DNA encoding the ribosomal RNA. This is because it is a very conserved region of the genome that also includes variable regions that have different sequences according to the microbial species, which can be separated by DGGE.

A fingerprint can be obtained from a cheese sample through the procedure depicted in **Figure 1**. After the extraction of bacterial DNA from the sample of cheese, raw material, or intermediate of production, a mixture of genomic DNA from the different species occurring in that specific sample is obtained. A PCR is then performed to amplify variable regions of the DNA encoding rRNA genes, resulting in a mixture of amplicons that are of the same size but have different sequences according to the specific microbial species. These amplicons can be separated by DGGE giving a fingerprint with a number of bands correlated, in most but not all cases, with the number of microbial species present in the original sample. Overall, the procedure allows a rapid linking of the cheese sample with a fingerprint, which is a microbiological identity card showing the species populating that cheese at the time of sampling. Further analysis can be carried out by cutting the bands out of the gel and sequencing the fragments after appropriate and repeated steps of purification, and obtaining information on the homology of each band sequence from the closest relative species whose sequence is published in public databases. At the end of a complete analysis, the dairy microbiologist will not only have a fingerprint of the cheese but will know the identity of the microbial species occurring in that specific sample.

After PCR amplification of variable regions of the 16S rRNA gene, an alternative to DGGE/TTGE separation of the amplicons is to use denaturing high-performance liquid chromatography (DHPLC). Based on the same principles, it is an automated alternative to DGGE. PCR amplicons are loaded on the HPLC cartridge with triethylammonium acetate (TEAA), which more strongly binds the double-stranded molecules. Partially (thermally) melted amplicons are eluted more rapidly, and also in this case there is a sequence-specific separation of amplicons from different microbial species, which have different retention times. The final output is a fingerprint in the form of a chromatogram (**Figure 1**). The use of the DHPLC may have advantages because it is rapid, allows an entire batch of samples to be automatically processed without human intervention, and avoids time-consuming steps of gel preparation and electrophoresis and the use of toxic staining agents.

Single-strand conformation polymorphism (SSCP)-PCR analysis detects sequence variations between different DNA fragments, usually PCR-amplified from variable regions of the 16S rRNA gene. This technique is essentially based on the sequence-dependent

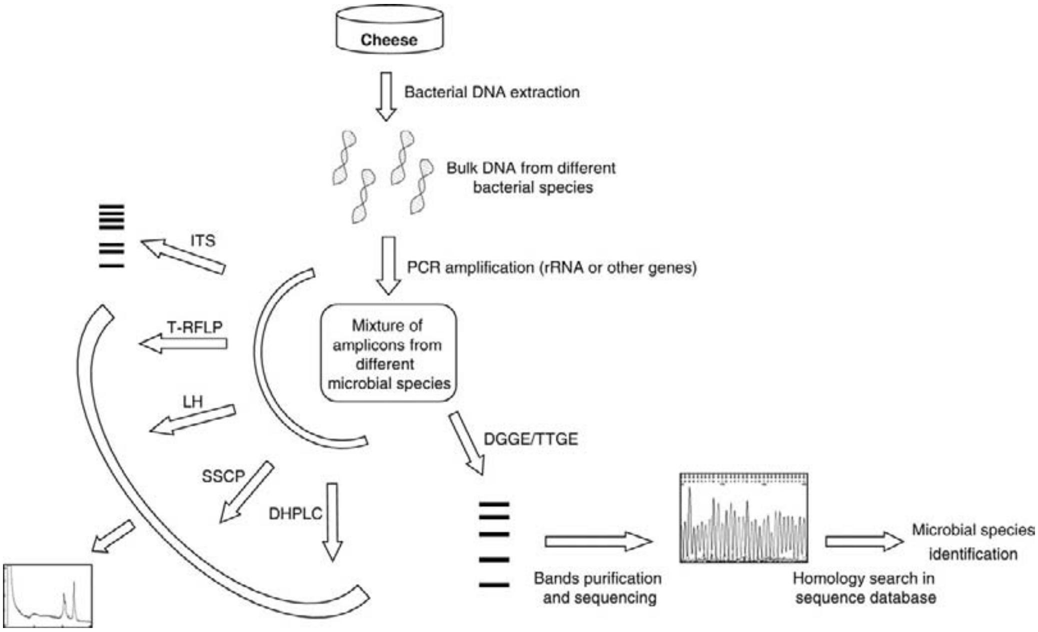
Cheese
Bacterial DNA extraction
Bulk DNA from different bacterial species
PCR amplification (rRNA or other genes)
Mixture of amplicons from different microbial species
ITS
T-RFLP
LH
SSCP
DHPLC
DGGE/TTGE
Bands purification and sequencing
Homology search in sequence database
Microbial species identification

Figure 1 Scheme of different possibilities of a culture-independent analysis of cheese. Starting from a cheese sample, different fingerprinting options and the corresponding operational steps are indicated. ITS, intergenic transcribed spacer; T-RFLP, terminal-restriction fragment length polymorphism; LH, length heterogeneity; SSCP, single-strand conformation polymorphism; DHPLC, denaturing high-performance liquid chromatography; DGGE/TTGE, denaturing gradient/temporal temperature gel electrophoresis; PCR, polymerase chain reaction.

differential intramolecular folding of single-strand DNA, which alters the migration speed of the molecules, which is usually detected by capillary electrophoresis (**Figure 1**); it constitutes a valid alternative to DGGE for culture-independent fingerprinting of dairy products.

A number of other methodologies have been developed coupling PCR amplification with automated sequencing systems for laser detection of amplified, fluorescently labeled DNA fragments, which can be used for DNA fingerprinting of microbial communities. Terminal-restriction fragment length polymorphism (T-RFLP) analyzes variations within the 16S rRNA genes from different species giving information on structure of the microbial community. It is based on the restriction endonuclease digestion of fluorescent end-labeled PCR products separated by gel (or capillary) electrophoresis and the quantification of fluorescence signal intensities (**Figure 1**). Length heterogeneity-PCR (LH-PCR) is another PCR-based fingerprinting technique similar to T-RFLP. Whereas T-RFLP highlights PCR fragment length variations based on variability of restriction sites, LH-PCR analysis can differentiate organisms based on species-specific variations in the length of 16S rRNA gene sequences. In LH-PCR, a fluorescently labeled oligonucleotide is used as a forward primer coupled to a reverse primer to amplify variable regions of the 16S rRNA gene, and labeled fragments are separated by gel (or capillary) electrophoresis and detected by laser-induced fluorescence with an automated gene sequencer. In the case of both T-RFLP and LH-PCR, a database is needed on the electrophoretic profiles of microbial species possibly occurring in cheese to be able to identify the different peaks after separation.

Another fingerprinting option is the PCR analysis of the bacterial intergenic transcribed spacer (ITS) region located between the 16S and 23S ribosomal genes. It is a viable fingerprinting technique based on the species-specific length and sequence polymorphisms of the spacer region between the 16S and 23S ribosomal genes. This technique can be applied to the DNA extracted from the cheese sample, and the fingerprinting can be obtained by conventional agarose electrophoresis (**Figure 1**).

Creation of Databases of Bacterial Fingerprints

As an alternative to band sequencing in fingerprinting profiles, databases of microbial species migrations can be created. Construction of such databases consists of analyzing variable regions of the 16S rRNA gene of as many species as possible that usually occur in a dairy product and storing the migration distance data. Further, the individual bands in a cheese profile can be identified by comparison of the migration distances of the band in the cheese fingerprint with the migrations of the microbial species in the database. Moreover, in samples where bands cannot be recognized as falling in the migration field of the species available in the database, identification can still be performed by band sequencing. The databases developed so far include DGGE and TTGE separation of variable regions of the 16S rRNA of more than 150 microbial species of dairy interest, including lactic acid bacteria, and spoilage and pathogenic microorganisms.

What Information Can Be Obtained from Microbiological Fingerprinting of Dairy Products?

Once a fingerprint of a dairy product has been obtained, different ways may be used to get the information it contains. This will basically depend on the scope of the analysis or research and on the type of sample analyzed.

Assessment of Microbial Diversity of Cheeses

The dairy microbiologist is certainly interested in the identification of the microbial species occurring in the cheese under study. This can be achieved by sequencing of PCR fragments from the profiles as described above. Thus, species identification in cheeses is made possible without isolation of the microorganisms from the cheese. This gives a great opportunity to the dairy microbiologist to study the microbiota of different cheeses or different samples of the same type of cheese with the possibility to assess the species diversity rapidly and reliably. The procedure has been applied in the past years to study the microbial diversity of many types of cheese. As can be expected, very interesting results have been obtained by examining very complex cheese ecosystems like those of artisan productions made of raw milk or with the use of natural starter cultures.

Even in cases where sequencing and species identification are not needed, the fingerprint alone can give some information. In fact, with the help of appropriate statistical methods and software for image analysis (*see* **Analytical Methods**: Multivariate Statistical Tools for Chemometrics), relationships between microbial diversity in different cheeses can be evaluated. During the first years of exploitation of culture-independent fingerprinting techniques, a possible use of microbial fingerprinting of cheeses for classification purposes was hypothesized – in other words, a method to rapidly determine the identity (category) of a cheese on the basis of its microbial flora evaluated by DNA fingerprinting such as PCR-DGGE. However, microbial diversity can be extremely variable, and different samples of cheese of the same variety display differences in microbial profiles due to different species composition. For example, different samples of Emmental cheese, though characterized by a common part of the fingerprint with several common species, will probably differ by the presence/absence of some bands belonging to species occurring only in some samples and not in others. Such sample-specific variability discourages the use of such fingerprinting to recognize the type of cheese.

Monitoring of Microbial Populations during Manufacture and Ripening

Cheese microbiota may arise from the raw milk or from the environment and production equipment, or can be added as selected starter cultures under controlled conditions. Another interesting possible source of bacteria consists in natural starter cultures, often employed in traditional cheesemaking, where fermentation is assured by the back-slopping of milk or whey cultures from previous preparations.

The studies of selected as well as natural starter cultures are extremely important not only because they drive the manufacturing process but also because they are often responsible for ripening, thus contributing to the principal sensorial attributes of the cheese. Such starter microbial flora can interact with the background microorganisms of the raw materials such as raw milk and such interactions often lead to premium quality cheeses with desirable characteristics. In addition, the study of the accessory microbiota is also important as the bacteria occurring at lower numbers, along with the dominant bacteria, can potentially contribute to development of product flavor and taste thanks to specific metabolic pathways. Based on the above premise, the study of the fate of starter cultures or accessory microflora during cheese manufacture and ripening is of relevance for the monitoring of population dynamics and ripening activities important for the quality of the final product. Such monitoring can be performed by culture-independent fingerprinting methods applied to bacterial DNA extracted from samples of raw milk, starter cultures, and intermediates of production through ripening. This procedure consists of collecting a series of microbial fingerprints of all the samples during manufacture and ripening; analyzing differences and similarities between samples, and occurrence and succession of microbial species; and monitoring the presence of the starter species through the whole process. An example of such procedure is represented in **Figure 2**. By analyzing the series of fingerprints showing the microbial species present during cheesemaking, the fate of the starter can be determined. The starter has its own profile and it is supposed to dominate the system; therefore, the progress of the microbial species of the starter during the manufacture can be monitored by observing the presence of the starter-specific bands in the fingerprint. In some cases, it could be noted that the starter species dominate during the early as well as the late phases of the manufacture and ripening, whereas in other cases the starter could be outcompeted by other microbial species

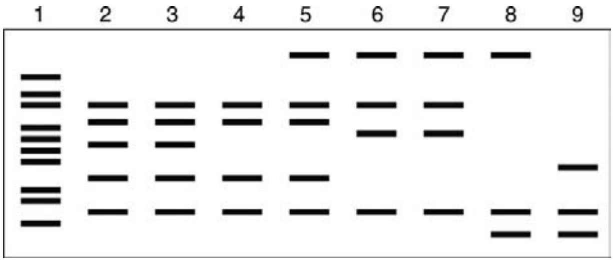

Figure 2 Schematic representation of PCR-DGGE fingerprints of dairy samples obtained during the production of a cheese from raw milk using natural starter cultures. 1, raw milk; 2, natural starter culture; 3, curd at the beginning of ripening; 4, curd at the end of ripening; 5, cheese after molding and brining; 6, cheese after 1 day of ripening; 7, cheese after 1 week of ripening; 8, cheese after 1 month of ripening; 9, cheese after further ripening. The scheme shows how it is possible to have an overall idea of the microbiology of a specific dairy production by fingerprinting the intermediates of production. In this example, the raw milk microbiota is very complex with many different species; however, it is clear that the microflora of the natural starter dominates the ecosystem and drives the ripening of the curd. However, after molding and brining, other microbial species develop, which can play a role during the ripening of cheese depending on the condition and time of ripening. In all cases, identification of the microbial species in each sample can be achieved by band purification and sequencing.

during later phases of cheese-ripening. All these information can be rapidly collected by fingerprinting samples from the whole process (**Figure 2**). In addition, the presence of possible contaminants can also be detected by the appearance of additional bands in the profiles.

The contribution of bacteria to cheese-ripening can also be studied by the same approach. Cheese samples during ripening can be used to obtain microbial fingerprints, and thus the species involved in ripening can be established. This is particularly interesting for long-ripened cheeses where the initial microbiota of cheese is different from the one occurring at later stages of ripening. An example of such succession is that of long-ripened cheeses such as Grana or Parmigiano Reggiano cheeses, which are initially dominated by thermophilic species (such as *Streptococcus thermophilus*, *Lactobacillus helveticus*) of the starter cultures, which are later taken over by mesophilic, adventitious species (mainly mesophilic lactobacilli) that play a different role in the overall ripening process.

Assessment of Mode of Production and/or Geographical Origin

Many cheeses in the world have quality and designation labels and such productions most of the times have territory and typicality claims. The microbiota of traditional cheeses is strongly linked to the production environment and to the traditional tools and procedure of manufacture. Such link to a specific territory or geographical area and to traditional heritage in cheese-making is the basis for the assignment of the protected designation of origin (PDO) in Europe. In some cases it has been demonstrated that the microbial diversity of the cheeses or of the natural starter cultures for traditional cheeses can be a marker of authenticity of such products. It is known that traditional cheeses made from raw milk or by using natural starter cultures are characterized by a very complex microbiota. Such feature makes traditional and artisanal cheeses easily distinguishable from industrial cheeses. In most of the cases, the latter are in fact produced from heat-treated milk and by using selected starter cultures and are characterized by a narrow microbial diversity. On several occasions it has been demonstrated that fingerprinting techniques such as PCR-DGGE and ISR analysis could be used for the differentiation of artisanal and industrial Mozzarella cheeses (**Figure 3**). The complexity of the microbial diversity and the related complexity of the profiles in the fingerprints in terms of number of bands increases with increasing use of traditional manufacturing techniques (4–8 species on average), whereas industrial Mozzarella cheeses are characterized by simple microbial profiles with a maximum of one or a couple of species (**Figure 3**). Thus, cheese fingerprinting can help in assessing the mode of production of cheeses.

Traditional cheeses are also characterized by a strong link with their respective territory of production. By collecting fingerprints of raw milks or natural starter cultures used in the production of various cheeses and by analyzing the data using appropriate statistical tools several works have demonstrated that the microbial diversity (in terms of fingerprints) was very much dependent on the geographical origin of the samples. This is important also because a specific geographical area can be identified as a particular source of certain microorganisms and therefore as the potential source of some properties of the final product such as aroma, taste, and texture.

Identification of Culturable Microbial Species without Isolation

Some of the fingerprinting techniques described above can also be used to check rapidly the diversity of the bacterial community after cultivation of microorganisms from cheeses on selective or nonselective culture media.

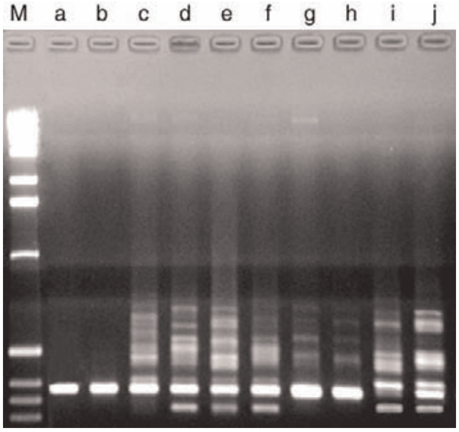

Figure 3 Intergenic transcribed spacer (ITS) analysis of industrial and artisanal pasta filata cheeses. Ethidium bromide-stained 2% (w/v) agarose gel of PCR products were obtained with primers G1 and L1 targeting the 16S–23S rDNA spacer region. DNA of microbial origin was extracted directly from the dairy samples and used as templates in PCR amplification. Lanes a, b: industrial Mozzarella cheeses; lanes c to f: water-buffalo Mozzarella cheeses of Campania; lanes g, h: Fior di latte cheeses; lanes i, j: traditional Fior di latte cheeses. M, 1 kb DNA ladder (Gibco BRL, Gaithersburg, MD, USA) used as molecular weight marker. Reproduced from Coppola S, Blaiotta G, Ercolini D, and Moschetti G (2001) Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese. *Journal of Applied Microbiology* 90: 414–420.

PCR-DGGE has often been employed for this purpose. After a traditional colony counting has been performed, the colonies from the plates can be collected in bulk and subjected to DNA extraction and PCR-DGGE analysis. Consequently, a DGGE fingerprint can be obtained for each plate, each dilution, and each culture medium. This method constitutes an alternative to the traditional tools used for the identification of dominant species when bulk cells from countable plates are analyzed. Identification of the dominant species can be achieved by sequencing the DGGE bands arising from the patterns corresponding to the highest dilutions. The PCR-DGGE analysis of bulk cells after cultivation can also support and integrate the analysis of the microbial diversity in cheese done through direct DNA extraction. Also, it is worthwhile to find out whether there is agreement or not between the results obtained by PCR-DGGE from direct DNA extraction and from species detection after cultivation. Sometimes it can be observed that the microbial diversity detected by direct DNA extraction underestimates the number of species occurring in the specific cheese sample, as more species can be revealed after cultivation.

This procedure can also investigate the selectivity of some culture media. In some cases, it has been shown that the selectivity of some culture media for the viable counts and isolation of lactic acid bacteria (LAB) was not satisfactory.

Identification of Microbial Isolates by Fingerprinting Techniques

There is a wide range of molecular methods available for the identification, genotyping, and monitoring of microbial isolates from dairy products. The problem of unreliability of biochemical methods of identification, even though associated with the miniaturized easy-to-handle kits or devices, makes their use inadvisable as the only tool for identification of microbial taxa from cheese. For this reason, significant efforts have been made to develop alternative identification methods combining speed, reliability, and low cost. Molecular techniques focus on the unique nucleic acid sequence of the microorganisms rather than on the phenotypic expression of products that are encoded by the respective genes. These methods vary from PCR-based techniques such as RAPD-PCR (and similar techniques such as REP-PCR, AP-PCR, BOX-PCR) to restriction profile analysis such as REA-PFGE, AFLP, and RFLP, or to protein-based fingerprinting like SDS-PAGE of whole cell proteins. Several of these methods are described in the article **Analytical Methods**: DNA-Based Assays. Moreover, the literature on the matter is very exhaustive and more than one paper suggested in the Further Reading section will deal with this important aspect of microbial ecology of microorganisms of interest in the dairy industry.

Future Perspectives: Molecular Microbiology in Dairy Science

Fingerprinting of cheeses and the fingerprinting techniques have proven to be fundamental tools in the study of structure and dynamics of microbial populations in dairy ecosystems. The studies of microbial diversity will soon benefit from high-throughput sequencing facilities and metagenomic analysis of bacterial populations in cheese. However, it is desirable that much effort be employed in the study of functional genomics of microorganisms of interest in the dairy industry. The study of dairy microorganisms should shift from genetic diversity to functional diversity. The expression of the activities of LAB in cheese, through studies of gene expression of technologically relevant activities, can be the timely target of dairy microbiologists. It is also desirable that such molecular study of microbial activity be performed *in situ* in cheese after development of suitable and effective methods of RNA and enzyme isolation from dairy matrices. Such approaches will benefit from the use of other fundamental molecular tools such as reverse transcription real time PCR and microarray technology, which can shed light on the actual role that LAB play in dairy fermentation and on the interactions between different microorganisms in cheese. Therefore, possibly the next task of dairy scientists will be to combine LAB, technology, and processing conditions with the final aim of improving quality and safety of dairy products.

See also: **Analytical Methods**: DNA-Based Assays; Electrochemical Analysis; Electrophoresis; Microbiological; Multivariate Statistical Tools for Chemometrics; Statistical Methods for Assessing Analytical Data. **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview. **Cheese:** Dutch-Type Cheeses; Hard Italian Cheeses; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Raw Milk Cheeses; Starter Cultures: General Aspects; Starter Cultures: Specific Properties; Swiss-Type Cheeses. **Lactic Acid Bacteria:** Taxonomy and Biodiversity.

Further Reading

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Non-Starter Lactic Acid Bacteria

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Introduction

The process used to convert drab, flavorless curd into lively, palatable, and well-bodied cheese is termed maturation, curing, or ripening. Maturation of many cheese types, including Cheddar, Swiss, and Gouda, depends on the action of microorganisms and enzymes that are trapped in the curd and therefore these cheeses are referred to as 'bacterial-ripened' cheeses. To attain their characteristic flavor and body properties, many such cheese varieties must be stored ('ripened') at low temperature for several months to two or more years. During this period, bacteria and enzymes in the cheese gradually break down milk sugar (lactose), lipids, and proteins. The breakdown of proteins and metabolism of their amino acid subunits is of particular importance in many cheese varieties as it influences cheese flavor both directly through production of flavor compounds and indirectly by softening the body of the cheese and enhancing the release of volatile and nonvolatile flavor compounds.

The most important microorganisms in the manufacture of bacterial-ripened cheeses are lactic acid bacteria (LAB), a diverse group of Gram-positive, acid-tolerant, and strictly fermentative cocci and bacilli that produce lactic acid as the major metabolic end product of sugar catabolism. Although LAB can be isolated from raw milk, modern cheese manufacture usually involves deliberate addition of one or more LAB species to ensure a proper fermentation. These deliberately added species include the so-called 'starter bacteria', which acidify milk via the conversion of lactose in to lactic acid, as well as 'adjunct' cultures that are sometimes added with the starter to provide a secondary function such as flavor enhancement. Because they are present at very high cell densities in the matrix of young cheese, LAB starter cultures and adjuncts are generally thought to make the strongest contributions to cheese flavor development.

Since flavor development is known to require LAB, cheese microbiology has long been a focal point of research to accelerate or enhance flavor reactions. Interestingly, microbial examination of ripening curd has shown that in addition to deliberately added LAB, bacterial-ripened cheeses also contain large populations of adventitious ('contaminant') bacteria that gain access to cheese through milk or the milk-processing environment. In cheese made from pasteurized milk, these contaminants are always nonpathogenic species of LAB, termed non-starter LAB (NSLAB), that pose no threat to human health. Modern sanitation practices help keep NSLAB to very low levels in young cheese, but these bacteria inevitably begin to grow and will reach high numbers within a few months of ripening. In Cheddar cheese, for example, number of *Lactococcus lactis* starter bacteria commonly exceed 10^9 colony-forming units (cfu) per gram when ripening begins (**Figure 1**). As maturation proceeds, the harsh cheese-ripening environment (no residual lactose, acidic pH, presence of added salt, and low ripening temperature) gradually takes its toll and starter cells begin to lose their viability. At the same time, NSLAB populations (whose initial numbers are typically well below 10^2 cfu g^{-1} in cheese made under good sanitary conditions with high-quality milk) begin to multiply and will eventually plateau at cell densities of 10^7–10^8 cfu g^{-1} after 3–9 months of aging (**Figure 1**). Microbiological characterization of NSLAB populations in bacterial-ripened cheeses has shown that they may be quite diverse, but are usually dominated by facultatively heterofermentative species of lactobacilli or, far less frequently, *Pediococcus pentosaceus.*

Population Dynamics of Nonstarter Bacteria

While the types and numbers of starter or adjunct bacteria that are present in cheese can be readily controlled, the complexity of NSLAB populations is still largely a matter of chance. The NSLAB composition of cheese is influenced by several factors, but especially by milk heat treatment and equipment sanitation in the manufacturing plant. As noted above, NSLAB levels commonly reach cell densities above 10^7 cfu g^{-1} in Cheddar and other bacterial-ripened cheese varieties with ripening times longer than 3–4 months. It is interesting to note that the final numbers of NSLAB in cheese appear relatively constant, and are not significantly affected by their initial levels in fresh curd, by the level of residual lactose in the curd, or by the typical range of pH or salt-in-moisture content found in Cheddar cheese. As was noted previously, NSLAB populations in bacterial-ripened cheeses may be quite diverse but are most commonly dominated by facultatively heterofermentative species of

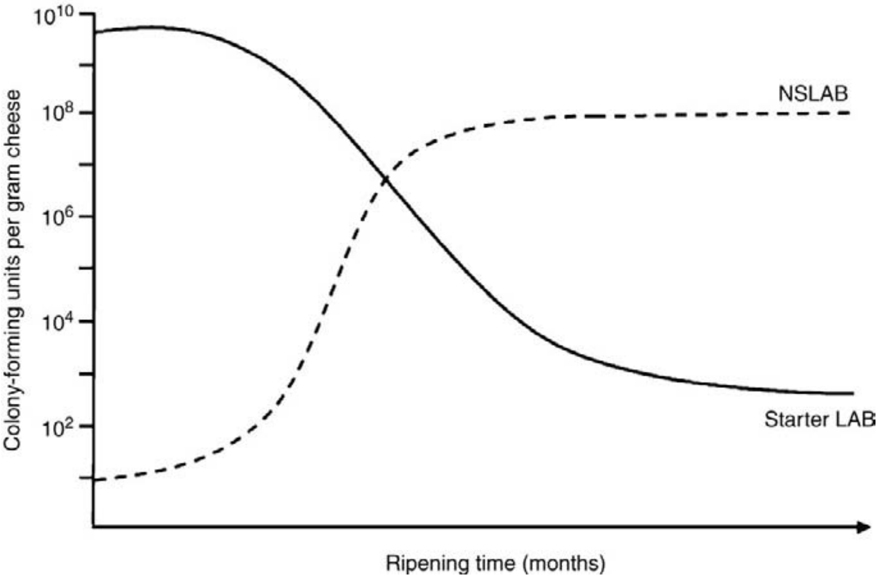

Figure 1 Changes in the populations of starter LAB and NSLAB that typically occur in Cheddar cheese during the ripening period.

lactobacilli, especially *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus curvatus*, *Lactobacillus rhamnosus*, or *Lactobacillus plantarum*. As an aside, the term 'facultatively heterofermentative' means that these cells ferment six-carbon sugars such as glucose to lactic acid, but convert five carbon pentose sugars into a mix of lactic and acetic acids.

Although lactobacilli are the most prominent component of the NSLAB biota, considerable heterogeneity has been detected among the *Lactobacillus* species, and especially strains, that dominate in a given cheese over the course of ripening. Recent use of molecular strain typing techniques to monitor NSLAB population dynamics in Cheddar cheese has also demonstrated that a succession of different *Lactobacillus* species and strains may dominate the NSLAB biota during aging. Species diversity among non-starter lactobacilli is generally highest during the early weeks and months of ripening, before strains of one particular species (most often *Lb. casei* but sometimes others) emerge to dominate the NSLAB biota as maturation proceeds. The complexity of NSLAB population dynamics is further compounded by the finding that the number and ratio of strains of a particular species can also vary dramatically during ripening, even among cheeses that are made in the same processing facility.

Impact of NSLAB on Cheese Quality

Addition of *Lactobacillus* spp. NSLAB isolates to milk during Cheddar cheese manufacture has indicated that these bacteria may influence flavor in at least three ways: they may intensify or accelerate typical flavor development; they may impart atypical but nonetheless desirable flavor notes; or they may promote the development of undesirable off-flavors. Of course, strains that consistently impart desirable flavor changes have value as adjunct cultures, and many of today's commercially used adjunct cultures were originally isolated as NSLAB from high-quality cheese.

In addition to their potential impact on flavor, NSLAB can also promote cheese quality defects such as open body (via gas production) or the formation of calcium lactate crystals, which consumers sometimes mistakenly regard as evidence for mold contamination. Since the influence of NSLAB on cheese quality will ultimately be determined by the metabolic activities of strains and species that dominate during ripening, the complex and dynamic nature of 'wild' NSLAB communities is widely viewed as a major source of cheese flavor inconsistencies and random defects. As a result, efforts by the dairy industry to consistently produce uniform, high-quality cheese ultimately require technology to control the composition and growth of NSLAB communities throughout ripening.

One of the most commonly proposed strategies to control the composition and diversity of NSLAB in cheese is to employ adjunct cultures that can also grow in ripening cheese and somehow suppress the emergence of 'wild' NSLAB populations. Such cultures should preferably also make desirable contributions to flavor, but at minimum should have no deleterious impact on flavor or other quality attributes of cheese. The general principle behind this strategy is, of course, the same one applied with great success through starter technology; that is, one can effect more consistent fermentation of a nonsterile food substrate by adding microorganisms that are known to impart the desired effects at levels high enough to suppress the contribution from adventitious microbes naturally present in the unprocessed food. The concept of using adjuncts to control cheese NSLAB is supported by a few academic trials that showed adjunct addition helped reduce the susceptibility of cheese to culture-related quality defects. However, real success in this strategy ultimately rests on whether or not the selected adjuncts can effectively suppress the growth of 'wild' NSLAB populations during ripening. While some studies have reported promising results, others have shown that 'wild' NSLAB frequently grow to numbers that are equal to – or even higher than – those of the adjunct as aging proceeds. However, the collective data from these studies do support the premise that adjunct cultures have promise as a tool to control the NSLAB biota and improve overall cheese quality, but they also suggest that effective realization of this technology will require a more fundamental understanding of the factors that determine which species or strains will proliferate in ripening cheese.

Metabolism of NSLAB

The ability of bacteria and other microorganisms to grow in cheese will be determined, as is the case with any other food, by the intrinsic (e.g., pH, substrate availability, and oxidation–reduction potential) and extrinsic (e.g., temperature, atmosphere, and relative humidity) properties of the cheese itself. However, the environment of ripening cheese is dynamic, and the additional variability that is imparted by differences in cheese composition (fat, protein, and moisture), the types of cultures or enzymes that are used, and manufacturing or ripening regimens that are applied makes cheese an especially complex microbiological subject.

As we contemplate factors that may determine the relative abundance of a particular NSLAB strain, it is helpful to conceptualize the cheese matrix in ecological terms, wherein the potential contribution of a particular species or strain to the overall population of bacteria will be influenced by its ability to exist within the available ecological niche (i.e., the cheese matrix). This capability will in turn be determined by the physical and biological conditions required by the bacterium to sustain a stable or growing population, by the extent to which those requirements are satisfied within the niche, and by the cell's ability to compete against other organisms for any resource that may be limiting in the niche.

At the surface, the intrinsic and extrinsic factors that appear most likely to influence growth and dominance of NSLAB in cheese include ripening temperature, curd pH and lactate content, percent salt (sodium chloride) in moisture, levels of residual lactose or other carbohydrate availability, and oxidation–reduction potential. However, the impact of these factors on species and strain succession is unknown, and studies suggest that the pH, moisture, and salt-in-moisture content of ripening Cheddar cheese have little or no effect on the growth rate or final number of NSLAB. The growth rate of NSLAB also appears to be independent of the residual lactose content in cheese.

Like other LAB, NSLAB possess a strictly fermentative metabolism and so must generate energy (ATP) via substrate level phosphorylation. Despite a marked substrate preference for simple carbohydrates, growth of NSLAB in cheese is typically noted several weeks after all of the residual lactose has been consumed by the starter bacteria. This observation has fueled considerable interest and debate on the nature and specific identity of substrates used by these bacteria to attain populations of 10^7–10^8 cfu g^{-1} in cheese. In theory, NSLAB may derive energy from a wide variety of compounds present in cheese, including lactic acid, citric acid, fatty acids, glycoproteins, glycolipids, amino acids, and even nucleotides that are released into the cheese matrix by dying starter bacteria. However, efforts to identify the specific substrates whose availability impacts NSLAB growth and strain dominance have not yet proved fruitful.

To address this need, researchers have recently begun to use aqueous extracts from ripening cheese as a medium to study the growth and metabolism of NSLAB. This work has shown that different substrates may be used in sequence to support the growth of particular NSLAB. One NSLAB strains of *Lb. casei*, for example, initiates growth in cheese extracts via concurrent metabolism of a phosphopeptide fraction and residual lactose, then cometabolizes galactose and citrate, then galactose alone, before it finally begins to catabolize another phosphopeptide fraction. Knowledge of specific substrates used to support NSLAB growth and of the sequence in which they are utilized by these bacteria is very important because it should eventually allow the dairy industry to develop adjunct strains that rapidly and consistently utilize key substrates during cheese ripening and thereby suppress the emergence of 'wild' NSLAB.

Contribution of NSLAB to Cheese Flavor

NSLAB can exert a positive, neutral, or negative influence on cheese flavor development due largely, but not exclusively, to their ability to catabolize amino acids during cheese ripening. The catabolism of amino acids generally includes a transamination step where α-ketoglutaric acid is the amino group receiver. Subsequently, the resulting α-ketoacid is used by a dehydrogenase thereby regenerating NAD$^+$. These pathways lead to the production of numerous volatiles and result in the regeneration of NAD$^+$. In particular, the metabolism of methionine, aromatic amino acids, and branched-chain amino acids (BCAAs) results in compounds that have a strong effect on cheese flavor. Methionine has a positive effect via the production/formation of methanethiol, a highly reactive compound associated with the development of 'cheesy' flavors. Methanethiol may be produced from methionine via a combination of enzymatic (transamination pathway) and nonenzymatic (chemical degradation) reactions, or directly in a single-step reaction catalyzed by cystathione β-lyase or L-methionine γ-lyase. Further reactions of methanethiol may result in thioester formation (i.e., methylthiobutyrate), catalyzed by thioesterases, and/or in di- and trimethyldisulfides, which arise by chemical oxidation of methanethiol. Depending on the amount formed, each of these sulfur-containing compounds may be beneficial or detrimental to cheese flavor, making the metabolic diversity of NSLAB a key variable to their formation in ripening cheese.

In contrast, catabolism of aromatic amino acids is more commonly associated with the development of off-flavor notes such as floral and pungent aromas. For example, phenylalanine can be converted to phenylpyruvate via transamination, and the phenylpyruvate formed can be further catabolized into phenylacetaldehyde and 2-phenylethanol (floral note) through a series of enzymatic decarboxylation and dehydrogenation reactions, or by chemical degradation.

The metabolism of BCAAs (leucine, isoleucine, and valine) results in a wide range of flavor compounds, including various acids, aldehydes, and, alcohols, which contribute to the complexity of a fermented food. As an example, 2- or 3-methyl butanal is associated with good mild Cheddar cheese flavor giving a 'dark chocolate' note. Short-chain organic acids are generated by either metabolism of BCAAs or degradation of milk triglycerides (i.e., butyrate). Butyrate (butanoate) has been associated with a 'cheesy' aroma note and is formed by the degradation of triglycerides. Caproate (hexanoate), commonly described as 'goaty' aroma, propionate (propanoate), valerate (pentanoate), and isovalerate (2-methyl-propanoate) all come from the sequential degradation of BCAAs into hydroxyacids, aldehydes, alcohols, and finally carboxylic acids. Metabolism of BCAAs is extremely varied in NSLAB, and the outcome of their degradation pathway is closely dependent on the NAD^+/NADH equilibrium.

Species of heterofermentative NSLAB such as *Lactobacillus brevis* can also produce a variety of end products when readily available carbohydrate is limiting (as is the case in ripening cheese). Potential end products include methylglyoxal, lactate, acetate, ethanol, formate, diacetyl, acetoin, 2,3-butanediol, and succinate. Methylglyoxal, a dicarbonyl, is a by-product of glycolysis, formed by the nonenzymatic dephosphorylation of the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The reactivity of methylglyoxal with amino acids (cysteine and BCAAs) can form furanones that are associated with beefy and malty flavor defects in cheese.

Compounds associated with a more positive impact on cheese flavor include diacetyl and succinate. Diacetyl, which has a butter-like flavor, is a flavor compound found in young to mild Cheddar cheese preferred by the US consumers. Succinate is a flavor enhancer described as 'savory' in cheeses. Acetate, a semivolatile organic acid, imparts acidity and a vinegar note. It can be a flavor enhancer at low levels but at higher levels it is detrimental to flavor quality. Further metabolization of acetate can lead to the formation of ethanol, which has a detrimental effect on cheese flavor. Formate has an irritant smell; however, its esters (formyl esters) are normally associated with fruity notes (apple, peach, prune, raspberry, and strawberry) and may therefore play positive or detrimental roles in cheese flavor quality depending on their intensity. Finally, lactic acid isomers (D and L), although not exclusively related to NSALB metabolism, impart acid flavor in cheese. Lactic acid metabolism by NSLAB is related to the production of D-lactate and/or further oxidation of L-lactic acid into acetate and carbon dioxide, partially reducing the acid flavor of cheeses.

NSLAB Genomics

Historically, one of the great challenges to effective control of NSLAB has been the fact that traditional microbiological methods (e.g., phenotypic characterization of NSLAB isolates) cannot provide the details needed to identify and understand factors that drive NSLAB growth and strain dominance in ripening cheese. Technology, and circumstances, has changed dramatically. One important example involves the development of molecular phylogenetic techniques such as denaturing gradient gel electrophoresis, which can be used to identify bacteria in a complex material such as soil or cheese, without any need to grow these cells ('culture-independent' methods). This technology has been used to demonstrate that dominant NSLAB species cannot always be detected by traditional bacteriological isolation and culture methods. Even more impressive, however, is the advent of genome sequence information and genomic tools for cheese starters and NSLAB species.

Compilation of entire genome sequences, and the development of associated microarray and proteomics tools that permit whole cell or 'global' research perspectives, has revolutionized bacteriology and created unprecedented opportunity to examine cell biology. Thus, genome sequence information of starter LAB and NSLAB has endowed industry and academia with the power to determine the means by which both LAB

Table 1 Public genome sequence information of seven bacterial species that have been associated with NSLAB populations

Species	*Strain(s)*
Lactobacillus brevis	ATCC 367, ATCC 27305, DSM 20054
Lactobacillus buchneri	ATCC 11577, NRRL B-30929
Lactobacillus casei	ATCC 334, BL23, 8700:2, ATCC 25302, Zhang
Lactobacillus fermentum	ATCC 14931, IFO 3956, 28-3-CHN
Lactobacillus plantarum	WCFS1, ATCC 14917, JDM1
Lactobacillus rhamnosus	GG, HN001, LMS2-1, Lc 705, MSUIS1
Pediococcus pentosaceus	ATCC 25754

populations interact with, and respond to, one another as well as the microenvironment of cheese.

In 2001, the complete genome sequence of the starter bacterium *Lc. lactis* became one of the first publicly accessible LAB genomes. Since that time, genome sequences of several important NSLAB species and strains have also become available (**Table 1**), and many more appear each year. As can also be seen from **Table 1**, it is becoming increasingly common for researchers to determine the genome sequence of more than one strain of a particular species. This outcome is extremely important because bacterial genomics has demonstrated that there is no such thing as a 'representative strain' for any species, and access to multiple sequences of a species of interest allows researchers to perform detailed comparative genetics studies that reveal both the unique and the conserved features of particular cells. This knowledge helps researchers understand what makes a particular strain different or special, and ultimately will allow industry and academia to use these microorganisms more effectively in biotechnological processes. With respect to cheese technology, access to genomics tools is expected to provide invaluable insight into the biology of starter LAB and NSLAB, as well as the genetic diversity within strains and species of starter LAB and NSLAB. Research on these topics is under way with strains of *Lb. casei*, a common species of NSLAB. DNA microarray technology has been utilized to evaluate the gene content of 21 strains of *Lb. casei* and the results have demonstrated that strains of *Lb. casei* can differ from another strain by as much as 515 genes (19% of the total number of genes present in the test strain). These same strains have been evaluated in a model cheese ripening system to determine their growth parameters and the volatile compounds that they produce. Correlating gene content with growth parameters or volatile compound production will allow for the development of hypotheses concerning which genes, hence metabolic pathways, are essential for competitiveness and volatile compound formation in ripening cheese. These hypotheses will need to be tested by the construction of isogenic strains that differ in the activity of a single gene, hence enzyme. It is anticipated that these results will result in the identification of factors that allow particular strains of NSLAB to emerge and dominate in ripening cheese, and the molecular mechanisms by which these cells influence cheese quality.

In summary, the dynamic and complex nature of NSLAB populations in ripening cheese is an ongoing source of cheese flavor inconsistencies and quality defects. Technology to control NSLAB populations during ripening is needed in the dairy industry to secure greater uniformity in the quality of bacterial-ripened cheeses such as Cheddar, Gouda, Swiss, and Italian cheeses. Although *Lactobacillus* adjuncts hold promise as a tool for this purpose, their efficacy is still challenged by the ecological diversity that exists in cheese because of differences in gross composition as well as manufacturing and ripening processes. To overcome this limitation, additional research is needed to understand the relationship between cheese microenvironments and the growth or dominance of individual NSLAB.

See also: **Cheese**: Accelerated Cheese Ripening; Biochemistry of Cheese Ripening; Cheese Flavor; Microbiology of Cheese.

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Public Health Aspects

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Introduction

There have been only 30 confirmed outbreaks of food poisoning due to the consumption of cheese in Western Europe, Canada, and the United States in the period 1970–97 during which it is estimated that about 235 000 000 tonnes of cheese was consumed. These figures tell us one important fact: food poisoning due to the consumption of cheese is rare and consequently cheese is a very safe product. No outbreaks of food poisoning due to cheese have been reported in Australia or New Zealand, the other major cheese-producing countries. A diverse group of Gram-positive and Gram-negative organisms was involved in the cheese-related food poisoning outbreaks, including *Staphylococcus aureus*, *Bacillus* sp., *Clostridium botulinum*, *Listeria monocytogenes*, enterotoxigenic *Escherichia coli*, and *Shigella*, *Salmonella*, and *Brucella* spp. About one-third of the outbreaks were caused by soft cheese made from raw milk or cheese containing low levels of acid; Cheddar cheese was incriminated in six of the outbreaks.

As a general rule, cheese is a very safe product when it is properly made. In cases where it has been incriminated in food poisoning, confounding factors have often played a part in the outbreak. These include improper storage of the milk (no refrigeration for several days prior to manufacture), poor starter activity with consequent production of cheese with a high pH, poor starter activity due to inhibition of acid production by phage and/or antibiotic residues in the milk, poor plant hygiene, gross environmental contamination, faulty pasteurization, shedding of the causative organisms by plant personnel, or different combinations of these.

Source of Pathogens

Raw milk, water, and improperly cleaned utensils can be sources of pathogens in cheese. *Staphylococcus aureus* is a common organism in raw milk because it is an important cause of mastitis, the major disease of the mammary gland. It can colonize the teats of the udder from where it is washed off by the milk during milking and so milk itself is a primary source of the organism. The cow can shed other pathogens in her milk, for example, *Brucella abortus*, the cause of brucellosis in cattle and undulant fever in man, and salmonella. *Brucella abortus* was a major cause of disease in the past but is very infrequent today in milk in developed countries where brucellosis in dairy cows is controlled. An outbreak of food poisoning in Canada in 1984, involving Cheddar cheese, was traced to a farm where one cow in the herd was shedding ~200 salmonella per ml of milk.

Cows' feces generally contain *E. coli*, salmonella, and enterococci and can be a source of contamination of milk, although recent evidence suggests that fecal contamination of milk is a minor source of contamination. Contamination may also occur from water and improperly cleaned milking equipment, where growth can occur in milk residues between milkings.

Infective Dose

The infective dose (ID) of pathogens varies. *Salmonella heidelberg* has an ID of 100–500 cells while that of *E. coli* O157:H7 is thought to be only 10 cells. The ID of *L. monocytogenes* has not been determined but it is generally considered to be low – perhaps a few hundred cells. In contrast, the ID of *Staph. aureus* is high because the actual cause of food poisoning is not the organism itself but a number of closely related enterotoxins produced by it. These toxins are heat-stable proteins, which withstand 100 °C for >30 min. To obtain sufficient toxin to cause food poisoning, it is generally believed that growth to a minimum of 10^6 cells g^{-1} of food is necessary. The exact number will depend on the particular toxin being produced and the amount produced by each cell. About 20% of *Staph. aureus* strains that cause mastitis in cows produce enterotoxins.

Growth of Pathogens during Cheese Manufacture

The major factors involved in controlling the growth of bacteria during cheesemaking are

- pasteurization of the milk,
- the time and temperature of cooking of the curd/whey mixture, and
- the rate of pH decrease in the curd.

All vegetative pathogens are inactivated by pasteurization. Therefore, pasteurization of the milk is the most significant factor in ensuring there are no pathogens in cheese. Despite this, significant amounts of cheeses are produced from raw milk, particularly in France, Germany, Switzerland, and Italy, which command premium prices in the market.

The time/temperature combination used in cooking the curd, including the rate of heating, the final cooking temperature, the length of time it is held at the cooking temperature, and the subsequent rate of cooling of the curd are also important parameters in controlling the growth of pathogens during cheese manufacture. Many cheeses are cooked at a temperature from 34 to 40 °C. Such temperatures will promote the growth of pathogens if they are present in the cheese curd. However, the cooking temperature plays a major role in preventing the growth of pathogens in cheeses like Emmental, Gruyère, Comté, and Parmigiano-Reggiano, in which the curd is cooked to >50 °C for ~1 h and where the temperature remains above 45 °C for several hours. These cheeses are made from raw milk.

If growth of the starter is rapid (i.e., an active starter is being used), the pH of the curd decreases quickly and growth of the pathogens will be inhibited. The reverse also occurs, that is, if growth of the starter is slow due to phage contamination and/or antibiotic residues in the milk, considerable growth of pathogens may occur during the first hours of manufacture. Therefore, the use of a fast acid-producing starter is a good way of controlling the growth of pathogens in cheese. Fast acid production occurs in Cheddar cheese manufacture but growth of pathogens in the curd of soft cheese would probably be greater than that in Cheddar, because of slower acid production in the soft cheese curd. The cooking temperature would also be a factor because Cheddar curd is cooked to a relatively higher temperature (~40 °C) than the soft cheese curd (~35 °C).

Many starter cultures may also produce bacteriocins. These are peptides that have the ability to inhibit other bacteria. Deliberate use of such cultures could be useful in inhibiting the growth of pathogens but they often inhibit other starter bacteria also.

Examples of the growth of *E. coli* O157, *L. monocytogenes*, and *Staph. aureus* in Cheddar cheese during manufacture are shown in **Figure 1**; *E. coli* and *Staph. aureus* multiplied, but *L. monocytogenes* did not. During cheesemaking, the moisture content of the curd decreases continuously, which causes an apparent increase in bacterial numbers in the curd. Based on this argument, a small decrease in the number of *L. monocytogenes* probably occurred during manufacture. Considerable growth of *E. coli* occurred between the beginning of manufacture and cutting the coagulum, when little acid production or change in curd moisture would have occurred.

Growth of Pathogens during Ripening

The factors controlling the growth of pathogens in cheese during ripening include moisture, salt, nitrate, pH, and temperature of ripening. All of them are not effective against the growth of all pathogens. For example, in cheese, the salt is dissolved in the moisture, so a cheese containing 1.5% NaCl and 38% moisture has an actual salt concentration of ~4% in the moisture of the cheese. This level of salt will prevent the growth of many pathogens but not *Staph. aureus* or *L. monocytogenes*, which can grow in the presence of 6.5 and 10% NaCl, respectively. Salt and moisture contents are intimately connected because the salt is dissolved in the moisture and reduces

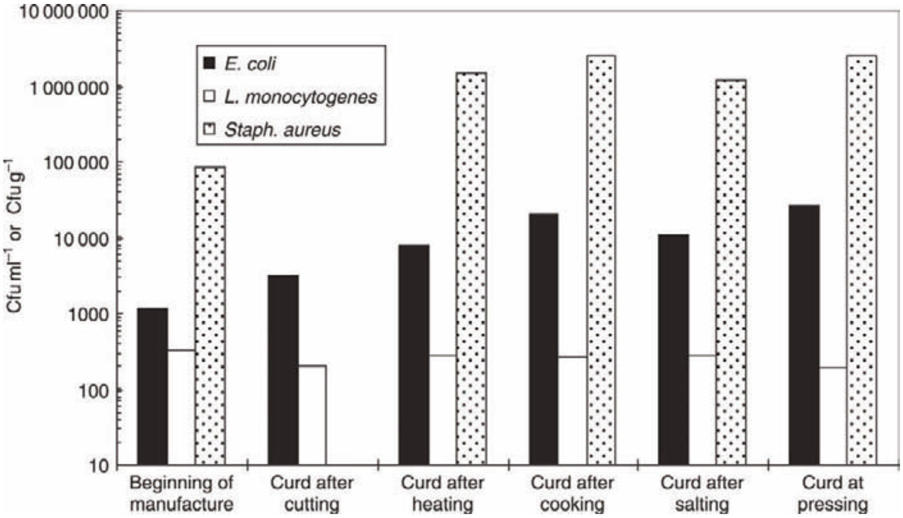

Figure 1 Growth of *Escherichia coli* O157, *Listeria monocytogenes*, and *Staphylococcus aureus* in Cheddar cheese during manufacture. Redrawn from Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers.

the water activity. The amount of moisture is also important, which, in turn, determines whether a cheese is hard or soft. A good rule of thumb is that the lower the moisture content of the cheese, the lower is the likelihood of any problems occurring due to the growth of pathogenic or indeed spoilage bacteria. Hard and semihard cheeses, if made properly, are safe because almost all pathogens die off during the relatively long ripening period at a low temperature. In contrast, significant growth of pathogens can occur in soft cheese.

The pH of most hard and semihard cheeses at the beginning of ripening is ~5.3 and may increase very slowly to 5.5 during the long ripening time. Such pH levels will have a significant inhibitory effect on the growth of pathogens. In contrast, the pH of mold- and surface-ripened cheeses, for example, Camembert, Brie, Blue, Tilsit, and Münster, increases during ripening. The pH increase, which is much greater on the surface than in the core of the cheese, is due to oxidation of lactate to H_2O and CO_2 and production of NH_3 through deamination of amino acids by the yeasts, particularly *Debaryomyces hansenii*, *Geotrichum candidum*, and *Yarrowia lipolytica* on the surface of the cheese, and is termed deacidification. The effect of pH on the growth of *Salmonella* spp. during the ripening of Cheddar cheese is shown in **Figure 2**. At pH 5.03 and 5.23, the salmonellae died off quickly, while at pH 5.7 they did not die at all. A pH of 5.23 is typical of well-made Cheddar, while a pH of 5.7 could indicate poor starter activity, either as a result of phage contamination or due to antibiotic residues in the milk.

The temperature at which cheese is ripened is generally well below the optimum for growth of pathogens and indeed other microorganisms, for example, 8 °C for Cheddar and ~12 °C for some surface-ripened cheeses. Therefore, growth of pathogens, if they are present, will be slow. However, *L. monocytogenes* is a psychrotroph and can grow at 4 °C, which is well below the ripening temperature of most cheeses. Moreover, Swiss-type cheeses, for example, Emmental and Comté, are ripened at 22 °C for several weeks to promote the propionic acid fermentation. Despite this, little growth of pathogens occurs in these cheeses because of the high cooking temperature, low pH, and low moisture content of these cheeses.

All of these factors acting together are much more restrictive of bacterial growth than each of them individually. In recent years, there has been a major effort to develop models to predict the growth of pathogens in food based on their growth responses to different combinations of these inhibitory factors, particularly salt, temperature, and pH. These predictive models have been developed mainly from experiments carried out in complex media and the results are thought to reflect the worst-case scenario in foods because growth in foods at the same temperature, salt concentration, and pH value is generally considered to be less than in model systems. For example, soft cheeses often have a salt concentration of 1.5%, a pH of 6.5 and are stored at 5 °C. Using these figures, one of the models predicts that 10 *L. monocytogenes* cells per gram would multiply to 10 000 cfu g^{-1} in 10 days, which is equivalent to a generation time of ~1.5 days.

The oxidation–reduction potential of cheese is low (~ −250 mV) due to the growth of the starter lactic acid bacteria. However, this factor is of little importance in controlling the growth of pathogens because all of them are facultative anaerobes.

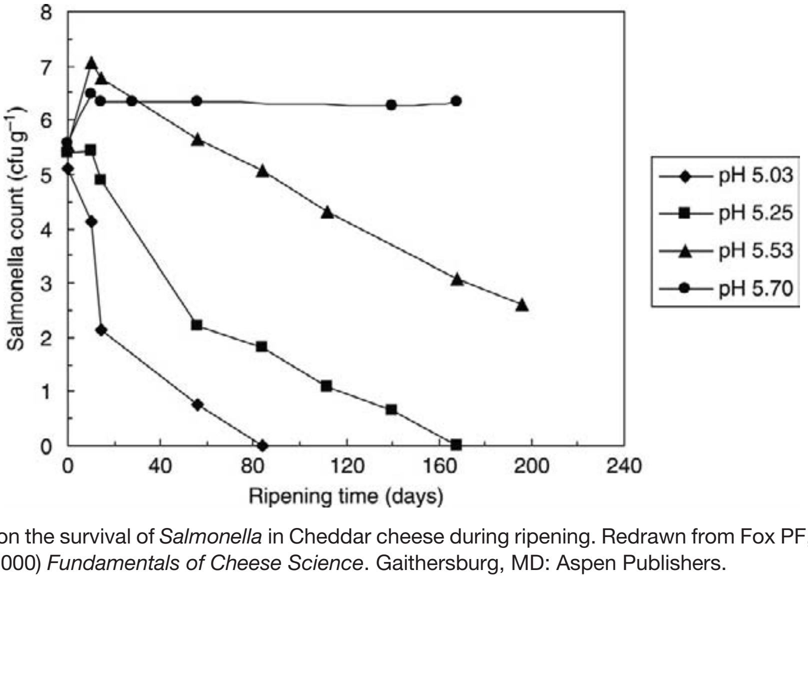

Figure 2 Effect of pH on the survival of *Salmonella* in Cheddar cheese during ripening. Redrawn from Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers.

Hard and Semihard Cheeses

The fate of several potential pathogens in Cheddar cheese during ripening is shown in **Figure 3**. *Enterococcus faecalis*, *Staph. aureus*, *E. coli*, and *Salmonella* spp. all decreased during ripening, and the Gram-negative bacteria decreased at a faster rate than the Gram-positive organisms. Coliform bacteria die off at a rate of 0.3 and 0.7 log cycles per week in Cheddar and Gouda cheese, respectively. A note of caution is necessary in interpreting the *Staph. aureus* data. Even though their numbers decrease significantly during ripening, sufficiently high numbers may have been present during the early stages of ripening to produce enterotoxins, which may still be present in the cheese at the time of consumption. Whether the enterotoxins are hydrolyzed by chymosin or starter proteinases during ripening is not clear. In the United States, storage of cheese at 2 °C for 60 days has been used as a safety precaution instead of pasteurization. This is now being questioned and is being actively researched.

Soft and Semisoft Cheeses

The situation in soft and semisoft, mold- and smear-ripened varieties like Camembert, Brie, Limburger, and Münster is quite different and many pathogens grow readily on the surface of these cheeses.

These cheeses contain a high level of moisture and the pH of the surface increases during ripening due to the deacidification activity of the yeasts. The pH increase during ripening is significantly greater on the surface than in the interior of the cheese and will be conducive to the growth of many pathogens. In addition, many soft cheeses are made from raw milk, which may contain pathogens; moreover, the frequent washing of the surface of smear cheeses with dilute brine during ripening is conducive to the spread of any pathogens that may be present. Consequently, soft cheeses are more prone to the growth of pathogens than hard or semihard cheeses. Growth of *L. monocytogenes*, an enterotoxigenic strain of *E. coli*, *Enterobacter aerogenes*, and *Hafnia alvei*, on the surface of Camembert cheese during ripening is shown in **Figure 4**. The number of *L. monocytogenes* decreased initially during ripening but increased again once the pH increased above 6. The increase also occurred in the core but not to the same extent, probably because the pH increased more slowly. In contrast, the numbers of *E. coli* and *Ent. aerogenes* increased during manufacture but began to decrease once the pH of the curd reached 5.0 and continued to decrease during ripening. This is probably true for all coliforms but *H. alvei* is an exception to the rule. The numbers of *H. alvei* increased until the pH decreased to ~5, and then remained constant and decreased to 10 cfu g^{-1} during the first week of ripening. The numbers began to increase again as soon as the pH began to increase, reaching final cell numbers of 10^8 cfu g^{-1}.

The rate of increase in the pH of the four Camembert cheeses in **Figure 4** varied. This was probably due to differences in manufacturing procedure and differences in the rates of growth of the different strains of yeast and *Penicillium camemberti* used in manufacture.

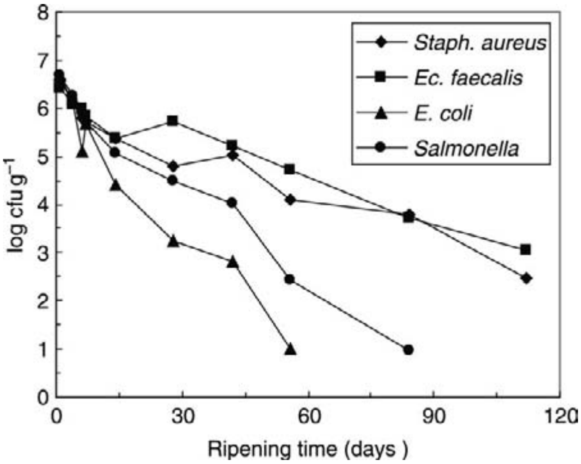

Figure 3 Decrease in the numbers of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and a *Salmonella* species in Cheddar cheese during ripening. Redrawn from Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers.

Raw Milk Cheeses

Today, much commercial cheese is made in automated systems from pasteurized milk but significant amounts of cheese are also made from raw milk in France, Switzerland, and Italy. Small-scale farmhouse production involving raw milk, and manual manipulation of the curd during manufacture, molding, and ripening, is also practiced.

Cheese made from raw milk has a much better taste than the cheese made from pasteurized milk and this is considered to be an important marketing advantage for raw milk cheeses. Nevertheless, from the foregoing, it is clear that growth of pathogens can be problematic in soft cheeses made from raw milk. *Staphylococcus aureus* is a common cause of mastitis in dairy cows and, therefore, is probably present in most raw milks. *Escherichia coli* O157:H7, the major source of which is bovine feces, and *L. monocytogenes* can also be present in raw milk and grow during cheese manufacture and ripening. In addition, many soft, mold- and smear-ripened cheeses have a high moisture content, and the high-moisture condition, together with the pH increase on the surface during ripening, is potentially hazardous, especially when the

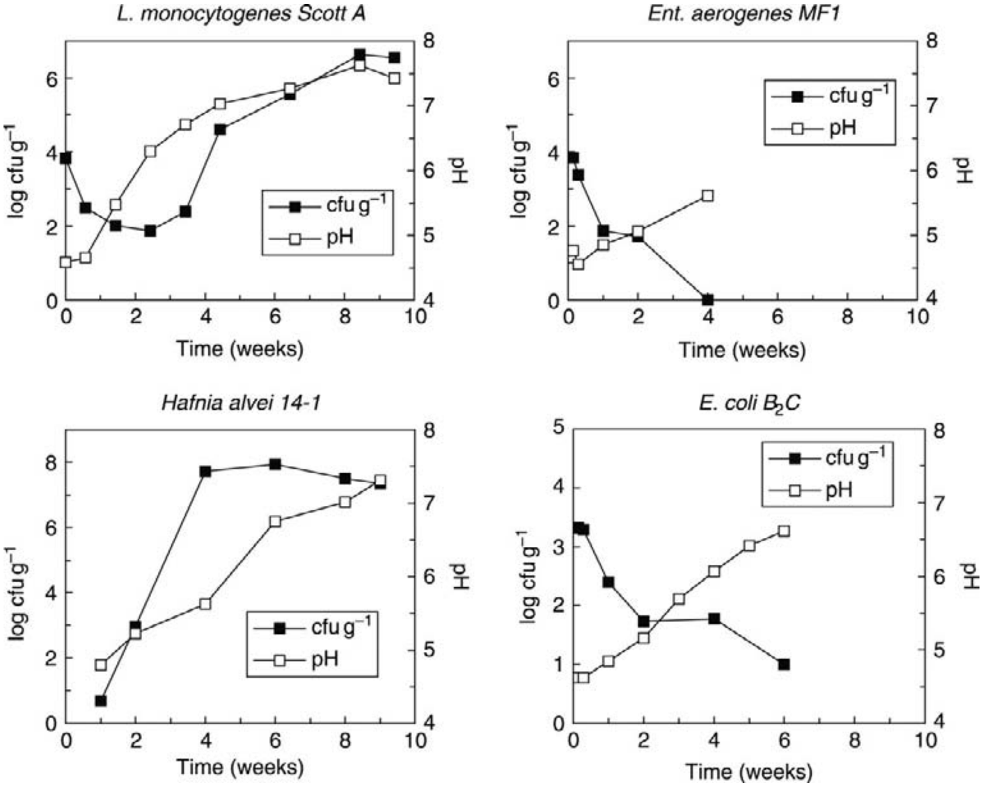

Figure 4 Growth of *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter aerogenes*, and *Hafnia alvei* and the increase in pH on the surface of Camembert cheese during ripening. Redrawn from Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers.

cheeses are made from raw milk. Despite this, relatively few food poisoning outbreaks (~10) have been traced to the consumption of raw milk cheese; those that did cause problems contained a variety of microorganisms, including *E. coli* O157, *L. monocytogenes*, *Salmonella typhimurium*, *S. dublin*, and *S. paratyphi*, and in most cases there were also problems with plant hygiene.

Control of Pathogens

Prevention of contamination of the milk and meticulous attention to good hygiene during cheese production and ripening will reduce the incidence of pathogens. The use of a good acid-producing culture is also helpful.

Implementation of good hazard analysis and critical control point (HACCP) systems is also very effective in preventing the growth of pathogens in cheese. The use of an active, phage-free starter and pasteurization are major critical control points. The activity of the starter can be determined by measuring the pH of each batch of cheese at a preset time after starter inoculation each day. Recording the temperature of the cheese at that point is also useful. Comparisons of the data on a continuing basis will allow one to determine if starter activity is normal on a particular day. Soft cheeses are small and cool quickly; therefore, it is necessary to keep the ambient temperature high to encourage growth and acid production by the starter when the cheese curd is in the molds. Good hygiene is particularly important in smear cheeses, especially where smear from ripened cheese is used to inoculate fresh cheese, in the so-called 'old–young' method of smearing because smear from old cheese may be contaminated with *L. monocytogenes* and will thus infect the young cheeses. Such practices are traditional in the production of these cheeses, particularly in Germany, and efforts are being made to develop defined-strain smear starters to overcome the problem. For example, much attention is being focused on identifying smear bacteria that produce bacteriocins active against *L. monocytogenes*; application of such cultures to the cheese surface should be very useful in helping to prevent the growth of *Listeria* on cheese.

Enterococci

Enterococci are found at high numbers ($>10^7\,g^{-1}$) in many cheeses, particularly those made from raw milk around the Mediterranean and they are considered to be important in the development of flavor in these cheeses. They also form part of the microflora of natural whey and milk starters. Enterococci can metabolize lactose and grow in the presence of 6.5% salt and at 10 °C, so one would expect them to grow in cheese during ripening. In Cheddar cheese, they grow during manufacture to high cell numbers and remain constant during ripening

There is considerable debate as to whether enterococci should be considered to be pathogenic. During the past few decades, they have been incriminated as the cause of several diseases, including bacteremia, urinary tract infections, and endocarditis. The incidence of vancomycin-resistant enterococci (VREs) in hospitals has increased dramatically in recent years. Many enterococci are promiscuous and easily pickup plasmids, which encode antibiotic resistance, particularly vancomycin. Many of these plasmids are conjugative and can be transferred naturally from cell to cell by sexual combination. Vancomycin is a glycopeptide antibiotic and it acts by inhibiting cell wall biosynthesis in bacteria. It is often the antibiotic of choice in controlling enterococcal infections.

The use of avoparcin, which is also a glycopeptide antibiotic, as a growth promoter in animal feed has been incriminated in the increased occurrence of VREs in farm animals, including pigs and poultry. Because of this, the use of avoparcin has been banned in several European countries. Many VREs are difficult to deal with because they are also resistant to other therapeutic antibiotics, implying that alternative antibiotic therapy may not be available. However, many bacteria, including many of those found in cheese, for example, mesophilic *Lactobacillus*, *Pediococcus*, and *Leuconostoc* spp., are intrinsically resistant to vancomycin; indeed, media containing vancomycin are used to selectively count these bacteria in cheese.

Staphylococcus aureus

Staphylococcus aureus can infect bovine teats and is the most common cause of mastitis in dairy cows. Thus, the organism can be a natural contaminant of raw milk. It is estimated that about 20% of isolates also produce enterotoxins and such strains if they grow to high numbers could cause food poisoning. Numbers in excess of $10^6\,\mathrm{g}^{-1}$ are necessary to do this because it is the enterotoxin that is the actual cause of food poisoning.

Listeria

Listeriosis is caused by *L. monocytogenes* and mainly affects pregnant women, the immunocompromised persons, and the elderly. Three major outbreaks of listeriosis have been traced to cheese, two in the United States, involving Mexican-style cheese, and the other in Switzerland, involving Vacherin Mont d'Or cheese, which is a soft cheese made from raw milk. Two of the outbreaks involved deaths, 48 in the United States and 34 in Switzerland. The third outbreak involved still births, premature babies, and infected babies. Poor hygiene was the major factor in one of the US and the Swiss outbreaks and improper pasteurization was also implicated in the case of the US outbreak. However, the fact that both cheeses also had a low salt level and that the Mexican-style product was a low-acid cheese, made without the deliberate addition of a starter culture, while Vacherin was a surface-ripened variety, in which the pH increases during ripening, also contributed to the outbreaks. In the second US outbreak, pulsed field gel electrophoresis (PFGE) patterns of isolates of *L. monocytogenes* from 10 patients, from raw milk and cheese bought from a local vendor, and from cheese bought from two Hispanic markets were indistinguishable, confirming that the incriminated cheese was produced in a single dairy.

Pathogenic *Escherichia coli*

The normal habitat of *E. coli* (and indeed enterococci and salmonellae) is animal feces from where it may contaminate raw milk, particularly if the animals have been lying in their own dung and the udders have not been properly washed before milking. *Escherichia coli* strains are differentiated from each other on the basis of their somatic (O), flagellar (H), and capsular (K) antigens. To date, 174 O antigens, 56 H antigens, and 80 K antigens have been detected. Most strains of *E. coli* are harmless, commensal organisms but some of them are pathogenic.

These strains are generally subdivided into enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohemorrhagic (EHEC), depending on the type of infection they cause. *Escherichia coli* O157:H7 is an EHEC strain and has been implicated in several severe food poisoning outbreaks involving meat products. The cytotoxin of *E. coli* O157:H7 can be produced in Vero or African Green Monkey kidney cells, and hence this strain is also called a verotoxigenic strain. A major outbreak of food poisoning in cheese due to this organism has the potential to be very serious because *E. coli* O157:H7 can result in the death of affected victims. This organism does not survive in Cheddar cheese during ripening.

Four outbreaks of food-borne disease due to pathogenic *E. coli* have been traced to the consumption of soft, mold-ripened cheeses. These outbreaks involved ETEC O27:H20, EIEC O124:B17, and EHEC O157. The outbreak due to *E. coli* O124:B17 occurred in the United States but involved French Cambembert, Brie, and Coulommiers cheeses made by the same procedure in the same plant over a 2-day period; no deaths were reported. In the outbreak involving *E. coli* O27:H20, the cheeses were from two lots, made 46 days apart, suggesting that contamination was intermittent. It is not clear if the cheeses in these outbreaks were made from raw or pasteurized milk. Two outbreaks of food poisoning due to *E. coli* O157 have been traced to cheese. The first occurred in France and involved fromage frais, made from raw milk. Four children were involved and one died. The second outbreak occurred in Scotland and involved a raw milk Lancashire cheese. Twenty people were involved but no deaths occurred.

Salmonella

Several outbreaks of food poisoning due to *Salmonella* spp. have been traced to cheese including Cheddar, Mozzarella, and Mexican style cheese in the United States, Cheddar in Canada, goats' milk cheese and Morbier cheese in France, a soft cheese in Ireland, and Vacherin Mont d'Or and Doubs cheese in France. In two recent cases, multidrug-resistant serotypes were involved with resistance to five antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) in one case. The ultimate source of these strains was not determined. In more recent outbreaks, PFGE of isolates from patients and cheese confirmed that the organism causing the infection was also present in the cheese but in many cases it is not clear if the cheese involved was compromised by poor manufacturing methods and/or poor hygiene in the plant or other factors (see above). It is important where cheese is suspected of causing food poisoning outbreaks that the pH, salt, and moisture are also measured to determine if the cheese was of a satisfactory composition.

Biogenic Amines

Cheese has been incriminated in several outbreaks of biogenic amine poisoning. These amines can cause a variety of responses in man, including rashes, diarrhea, hot flushes, sweating, heart palpitations, headaches, and hyper- and hypotension. They are produced by organisms, particularly lactobacilli, that contain enzymes that decarboxylate the corresponding amino acids to the amine. Other organisms, for example, enterococci and propionibacteria, can also produce these amines. A variety of amines such as cadaverine (from lysine), putrescine (from ornithine), tryptamine (from tryptophan), phenylethylamine (from phenylalanine), histamine (from histidine), and tyramine (from tyrosine) have been implicated but histamine and tyramine are the most important. The amounts required to cause problems are not known with any degree of accuracy, but a histamine level of $40\,\mathrm{mg}\,100\,\mathrm{g}^{-1}$ was found in a Cheddar cheese involved in a food poisoning incident in Canada and $85\,\mathrm{mg}\ 100\,\mathrm{g}^{-1}$ in a Gouda cheese involved in another incident in the Netherlands.

See also: **Cheese:** Microbiology of Cheese. **Pathogens in Milk:** *Escherichia coli*; *Listeria monocytogenes*; *Salmonella* spp.; *Staphylococcus aureus* – Molecular.

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Raw Milk Cheeses

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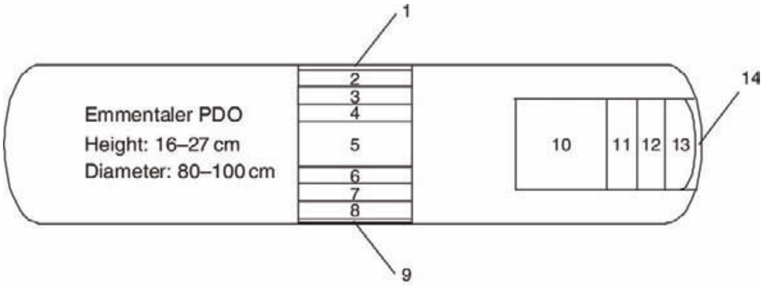
Introduction

For the production of cheese, the use of raw milk was common until a few decades ago. With the industrialization of cheese manufacture, the factories became larger, storage time of milk increased, and, as a consequence, the microbiological quality of raw milk and of the corresponding cheeses often varied considerably from batch to batch. Since the 1940s, raw milk was being subjected more and more to heat treatments to improve the process control of cheesemaking as well as for public health reasons. However, both the heat-induced changes in milk and the elimination of the raw milk microflora considerably affect the cheesemaking and cheese-ripening processes, yielding cheeses with altered sensory characteristics. It is generally accepted that cheese made from raw milk develops a more intense flavor than that made from pasteurized milk. A considerable amount of cheese is therefore still produced from raw milk. The present article covers the background of today's raw milk cheese production, describes the particular requirements for the manufacture of raw milk cheeses, and highlights the advantages and disadvantages with respect to sensory characteristics, possible cheese defects, safety, and marketing.

Definition of Raw Milk and Raw Milk Cheese

According to the code of hygienic practice for milk and milk products (CAC/RCP 57-2004), raw milk is defined as milk that has not been heated beyond 40 °C or undergone any treatment that has an equivalent effect. The same document defines pasteurization as a microbiocidal heat treatment aimed at reducing the number of any pathogenic microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard (*see* **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects). Pasteurization conditions are designed to destroy effectively the organisms *Mycobacterium tuberculosis* and *Coxiella burnetii*.

National food regulations in general define pasteurization as a rapid heating of milk to a temperature of no less than 72 °C (161 °F) and retaining it at that temperature for no less than 15 s. The same process performance can be obtained by using other temperature and holding time combinations (e.g., heating to not less than 63 °C (145 °F) for not less than 30 min). In the early 1930s, it was noted that the enzyme alkaline phosphatase, present in raw milk, is destroyed at a time–temperature combination of 63.5 °C × 30 min, which is sufficient for the inactivation of *Mycobacterium turberculosis* (*see* **Enzymes Indigenous to Milk**: Phosphatases). Measurement of alkaline phosphatase activity therefore offers a means of checking whether milk has been subjected to pasteurization or not. This method has been adapted to a large number of other dairy products as well as cheese. However, its application to raw milk cheese may result in confusing results. The typical scalding conditions applied for the manufacture of traditional raw milk cheeses such as Swiss Emmentaler PDO cheese (heating 52–54 °C × 40 min, dry stirring 53–51 °C × 30 min) or Gruyère PDO cheese (heating 57 °C × 40 min, dry stirring 57 °C × 0–15 min) result in an almost complete inactivation of the alkaline phosphatase in the curd. **Figure 1** shows the residual activity of alkaline phosphatase found in different zones of Emmentaler PDO cheese. The residual activity of alkaline phosphatase may be below the detection limit in samples from the core region. In contrast to this, clearly positive test results are obtained in samples from the rind owing to faster cooling during manufacture. The detection of residual alkaline phosphatase activity in cheese is therefore of limited use in assessing whether raw milk was used for cheese production. For food safety reasons, thermization of the cheese milk at 57–68 °C for 10–20 s has been introduced in the manufacture of various semihard and soft cheeses (*see* **Heat Treatment of Milk**: Thermization of Milk). Such heat treatments of raw milk reduce markedly the number of bacteria, with a minimum of collateral heat-induced changes in milk. Depending on the manufacturing prescriptions of the individual cheese association, substitution of raw milk with 0–100% thermized milk became common for the production of a large number of traditional soft and semihard cheese varieties. In practice, thermization is often applied only to the evening milk prior to storage, whereas the freshly collected morning milk is made into cheese without heat treatment. This example illustrates that a correct definition or declaration of raw milk cheese is difficult to achieve. Nowadays, partial or full



Zone no.	Activity (IU)
1	1408
2	527
3	162
4	114
5	77
6	129
7	324
8	819
9	1382

Zone no.	Activity (IU)
10	59
11	385
12	1427
13	2853
14	3639

Figure 1 Residual activity of alkaline phosphatase measured in different zones of Emmentaler PDO cheese using *p*-nitrophenol phosphate as substrate.

use of raw milk for the manufacture of cheese has to be declared in most countries, whereas a declaration of the heat treatments (thermization, pasteurization) of cheese milk is voluntary.

The Tradition of Raw Milk Cheeses

The initial cheesemaking process included only a spontaneous fermentation of raw milk with native lactic acid bacteria and subsequent pressing and salting of curdled milk to preserve it. On the basis of records of monasteries, the manufacture of cheese can be tracked back to the eleventh century CE in different areas of Europe. The use of rennet for cheesemaking emerged around the sixteenth century. Most of today's popular cheese varieties made from bovine milk, such as Cheddar, Parmigiano Reggiano, Gruyère, Gouda, Camembert de Normandie, and Emmentaler, were first mentioned in records of the seventeenth and eighteenth centuries. These varieties obtained a high international recognition due to the increasing trade in cheese. The long history of cheesemaking in several European countries is responsible for the still prevailing tradition of consuming raw milk cheeses. Recently, more than 166 cheese varieties have obtained a registration as products with a protected designation of origin (PDO). Most of these registered cheeses originate from a few countries with a long tradition of cheesemaking such as France (42), Italy (35), Spain (22), Greece (20), and Switzerland (11), and were initially manufactured from raw milk.

The use of high-quality milk, the use of adequate starter cultures, the application of appropriate manufacturing and ripening conditions, and last but not least the know-how of the cheesemaker are important requirements for the manufacture of high-quality raw milk cheeses. The existing know-how has been passed down from generation to generation. During the past decades, the procedures of cheesemaking have been greatly modified for almost all cheese varieties: In the Mediterranean areas, several traditional sheep and goat milk cheeses were progressively substituted by analogues made from cow's milk; the use of traditional whey cultures was replaced to a great extent by well-defined cultures; and in the course of the last 50–60 years, several milk pretreatments such as thermization, pasteurization, bactofugation, and microfiltration were introduced in order to obtain better process control. Nowadays, the protocols of a large number of registered PDO cheeses allow such treatments and do not require implicitly the use of raw milk. Nevertheless, there are numerous well-known cheese varieties in Europe still being made exclusively from raw milk (**Figure 2**). The two best-selling PDO cheeses in France, Comté and Roquefort, but also Camembert de Normandie and Brie de Meaux, or Parmigiano Reggiano in Italy, as well as Swiss cheese varieties such as Emmentaler and Gruyère, and various alpine cheeses (e.g., L'Etivaz and Berner Hobelkäse) are still made fully from raw milk. Moreover, individual cheese manufacturers have retained or reintroduced the tradition of using raw milk in various other countries (e.g., Boerenkaas or Boeren-Leidse met sleutels from the Netherlands; West Country farmhouse Cheddar cheese

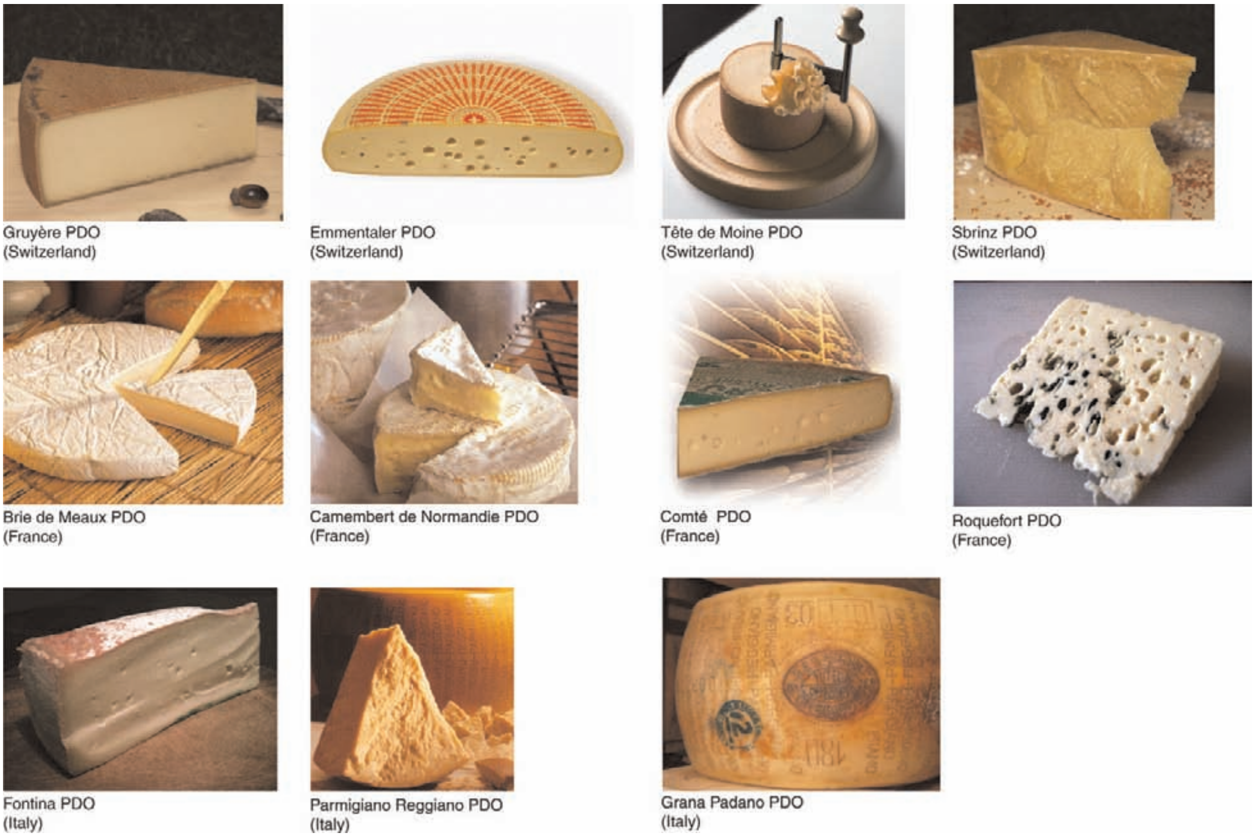


Figure 2 Examples of European cheese varieties made exclusively from raw milk.

and Exmoor Blue Cheese from the United Kingdom; or Manchego, Idiazábal, Roncal, or Cabrales from Spain). In recent years, it has been recognized that artisanal raw milk cheese constitutes an important economic niche in which its producers enjoy competitive advantage. Besides the aspects of product quality, sociocultural factors also are of growing importance for the marketing of raw milk cheeses. Raw milk cheeses meet the expectations of an increasing number of consumers who prefer minimally processed foods and natural products without additives as well as authentic and artisanal specialities with well-defined origins.

Particular Requirements for the Production of Raw Milk Cheeses

The manufacture of raw milk cheeses is a challenging task. Both the production of high-quality raw milk and its accelerated processing into cheese are essential requirements for a successful manufacture of raw milk cheeses. The quality of the raw milk received by the processing plant depends on a number of factors: health of the animal, feed consumed, milking conditions and procedures, cleanliness of equipment, temperature control, duration of intermediate storage, and appropriate conditions for transport (*see* **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene). Disregard of such factors endangers food safety and inevitably leads to the loss of product quality, which may become manifest in the form of cheese defects. Thus, the manufacturers of raw milk cheeses and their suppliers are forced to maintain a constant high quality of raw milk in order to minimize the risk of production losses. For the production of raw milk cheese, the milk is usually processed within 12 h after collection. Only if the milk is cooled down to 4–12 °C immediately after milking, processing of the milk within 24–48 h is possible. Fast controls of the quality of raw milk and full traceability back to the individual milk suppliers are other important features. Owing to such logistic constraints, raw milk cheeses are preferably produced in small, peripheral plants, which in general are certified according to the international standard ISO 22000. Good agricultural practice (GAP) and good manufacturing practice (GMP) are overarching principles for the production, processing, and handling of all milk products. However, the requirements for the production of raw milk cheeses go far beyond those for pasteurized milk cheeses: Advanced hygienic practices throughout the whole production and processing chain as well as additional control measures from raw material production to the point of consumption are essential for ensuring a good product quality and food safety.

Comparison of Raw and Pasteurized Milk Cheeses

Apart from eliminating pathogenic bacteria, milk pasteurization modifies the biochemistry and microbiology involved in ripening, as well as the flavor and texture of the final cheese. The following effects are known to be associated with milk pasteurization (*see* **Heat Treatment of Milk**: Heat Stability of Milk):

- reduction of bacteria including non-starter lactic acid bacteria (NSLAB)
- activation of the plasmin–plasminogen complex
- inactivation of lipoprotein lipase (LPL) and alkaline phosphatase
- slight denaturation (7%) of serum proteins
- impaired rennetability of heated milk due to the formation of complexes of κ-casein with β-lactoglobulin
- modification of the activity of starter LAB

Although impaired rennetability of heated milk can be compensated by adding $CaCl_2$, cheeses made from pasteurized milk tend to have a higher water content and a more elastic texture. However, the main effect in pasteurized milk cheeses, independent of the variety, is a milder and more uniform flavor, the maturity reaching its optimum at a later stage. Raw milk microflora is considered to be responsible for the wide diversity of cheese aromas of traditional cheese, especially in the case of semihard, hard, and extra-hard cheese varieties that are ripened from a few months up to 2–3 years and that undergo an intense in-depth proteolysis and lipolysis during ripening. Consequently, higher levels of free amino acids, free fatty acids, and volatile compounds are usually found in raw milk cheeses. Various attempts have been made to overcome the sensorial disadvantages of pasteurized milk cheese by the addition of adjunct cultures. However, application of such NSLAB adjunct cultures is not common in practice. A significant comparison of cheeses made from raw milk with similar cheeses made from pasteurized milk is difficult to achieve, because in practice pasteurization of cheese milk is usually only one among many differing factors during processing. As an example, the production of a generic Emmental (see CODEX STAN 269-1967; *see* **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius; Standards of Identity of Milk and Milk Products) and Camembert cheese in a large-scale factory may differ considerably from the artisanal manufacture of an Emmentaler PDO cheese and a Camembert de Normandie PDO cheese, respectively, as shown in **Tables 1** and **2**. Treatments such as pasteurization, thermization, bactofugation, and microfiltration allow

Table 1 Typical differences in the manufacture of Swiss Emmentaler PDO made from raw milk and generic Emmental cheese made from pasteurized milk

Cheese characteristics	*Swiss Emmentaler PDO cheese*	*Generic Emmental cheese*
Production of milk	Within a radius of 30 km around the cheese factory, about 10–30 milk suppliers	Larger number of milk suppliers and longer transportation distances
Feeding of cows	Without silage	With silage
Intermediate storage of milk	Transformation within 24 h after milk collection	Transformation frequently within 72 h after milk collection
Heat treatments of cheese milk	Not allowed	Pasteurization
Other milk treatments	Not allowed	Bactofugation or microfiltration
Additives	Not allowed	• Colors • Preservatives (e.g., lysozyme, nitrate, nisin) • Acidity regulators • Ripening enzymes
Equipment for cheesemaking	Copper vessels or copper vats	Stainless-steel vats
Heating of the curd	52–54 °C for 30–60 min	Typically 48–50 °C
Dimensions of cheese loaves	Wheels of 75–120 kg	Typically wheels or blocks of 40 kg or more
Firmness type	Hard cheese (dry matter >620 g kg^{-1})	Semihard or hard cheese (dry matter >600 g kg^{-1})
Ripening	Only dry ripening; minimal ripening time is 4 months for mild cheese, >8 months for fully flavored cheese, and >12 months for cave-aged cheese	Usually ripening in a film, normally 6–8 weeks
Size of cheese factory	2–4 persons producing 100–200 tonne yr^{-1}	Usually large-scale factories producing >10 000 tonne yr^{-1}

Table 2 Typical differences in the manufacture of Camembert de Normandie PDO made from raw milk and generic Camembert cheese made from pasteurized milk

Cheese characteristics	*Camembert de Normandie PDO cheese*	*Generic Camembert cheese*
Production of milk	Normande cows required in herd (minimum 50% in 2020)	No requirement
Feeding of cows	Pasture minimum 6 months per year	No requirement
Intermediate storage of milk	Transformation within 48 h after milk collection	Transformation up to 96 h after milk collection
Heat treatments of cheese milk	Not allowed	Pasteurization, thermization
Other milk treatments	Not allowed	Microfiltration
Equipment for cheesemaking	Coagulation in vats	Continuous coagulation processing
Processing	Primary ripening of milk	Facultative primary ripening of milk with less mesophilic LAB starter or no primary ripening
	Slight stabbing of the curd or none	More stirring of the curd
Molding	Ladle molded, generally 5 fillings	Multimolds system, 1 filling
Curd type	More acidic curd	More rennet curd
Green cheese characteristics	Calcium/nonfat dry matter: 0.6–0.7%	Calcium/nonfat dry matter: 1.0–1.5%
	Dry matter: 39–40%	Dry matter: 42–43%
	pH 4.6–4.7	pH 4.9
Secondary starter	*Geotrichum candidum* (GC) and *Penicillium camemberti* (PC)	GC+PC and other secondary starter (bacteria and yeasts)
Ripening	12 days minimum in the ripening room at 10–18 °C;	Usually more quickly ripened (15 days before sale)
	Minimal ripening time is 21 days before sale	
Package	Paraffin film	Multilayer film
Size of cheese factory	Small factories with slight mechanization	Usually large-scale factories with large-scale mechanization

additional control of bacteriological cheese milk quality, offer more flexibility regarding the storage time and transportation distances, and thus enable the production of cheese in large-scale factories.

Sensorial Characteristics of Raw Milk Cheeses

The real contribution of raw milk to sensory properties of cheeses has been evaluated via experiments in which only heat treatment of milk varied, despite the conditions being not really the same as in commercial cheeses, where adaptations are made to the processing of pasteurized milk. Available data are presented in **Table 3**.

Independent of the cheese variety, raw milk cheeses have a more diversified and 'stronger' flavor than pasteurized milk cheeses, which give them typical characteristics. On the one hand, raw milk cheeses present a higher degree of maturity at the same age; on the other hand, pasteurized milk cheeses fail to develop certain flavors throughout ripening. Besides the overall flavor intensity, pungency is often associated with raw milk cheeses, and so are acid, sour, rancid, and animal attributes. These flavor characteristics are linked to the earlier and more extensive development of raw milk microflora, to differences in the patterns of proteolysis, to higher lipolysis, and to a generally larger extent of formation of volatile compounds (*see* **Cheese**: Biochemistry of Cheese Ripening). Changes in proteolytic and lipolytic activities involve nonmicrobial as well as microbial enzymes. Both earlier production and higher levels of volatile compounds occur in raw milk cheeses. In particular, end products such as volatile fatty acids and esters are higher in raw milk cheeses in all cases, and alcohols and sulfur compounds in most cases. Methyl ketones and aldehydes have a variable distribution because they are intermediary metabolites and the balance between their production and degradation depends on the ripening stage at which the cheeses are analyzed. Diacetyl and/or acetoin are higher in pasteurized milk cheeses, because they are present in young cheeses and are further degraded during ripening in raw milk cheeses.

Differences in texture are less obvious, because texture settling is dependent more on water retention and binding, calcium equilibrium, and primary proteolysis due to nonmicrobial enzymes, and less on the activity of raw milk microflora. Raw milk cheeses seem in general more firm and grainy, and less elastic or rubbery, which indicates a higher maturity at the same age of consumption.

The determinants of flavor differ according to the cheese variety, due to different structures, physicochemical compositions, and microorganisms involved, so variations due to pasteurization may differ between cheese varieties. For example, in hard-cooked cheeses, unlike in others, pasteurized milk cheeses are more bitter

Table 3 Comparison of sensory characteristics and composition of raw/pasteurized milk cheeses (compilation of 11 studies)

	Types of experimental cheeses	*Hard-cooked cheese (Comté, Bergkäse)*	*Cheddar*	*Semihard cheese*	*Hard ovine cheese (Roncal, Idiazabal)*	*Lactic goat cheese*
Flavor	Raw milk cheeses (R)	Overall intensity, pungent, salty	Overall intensity, pungent, acid, sour, bitter, creamy, fruity, sweet, sulfur, rancid, unclean	Intensity, acid milk, rind, spiced, animal, garlic, chemical, rancid, bitter, pungent	Characteristic, overall intensity, aftertaste, pungent, sour, salty, acid, animal, milk, fruit, cream	More defects
	Pasteurized milk cheeses (P)	Acid, bitter, salty		Milk, fruit, fresh milk	Roasted, toasted, bitter, sour, milk, sweet	Reduced overall flavor and odor
Texture	Raw milk cheeses	Firm, granular	Crumbly, grainy		Firm, grainy	
	Pasteurized milk cheeses		Rubbery		Elastic, creamy	
Lipolysis	Comparison R/P		Lipolysis: R > P	Lipolysis: R = P		Lipolysis: R > P
Volatile compounds	Comparison R/P	Volatile fatty acids (VFA) acetic, propionic, isovaleric: R > P	Acetoin: P > R Methyl ketones: R > P or P > R Aldehydes: P > R or var. Alcohols: var. Esters: R > P Sulfur: R > P or var.	Ketones: P > R Aldehydes: P > R Esters: R > P Sulfur: R > P	VFA: R > P Ketones: R > P Alcohols: R > P Esters: R > P Sulfur: R > P Aldehydes: var.	
Proteolysis	Comparison R/P	α_{S1}-cas: P > R β-cas: R > P WSN: P > R or R > P TCASN: R = P PTASN: R = P or R > P FAA: P > R	α_{S1}-cas: R = P β-cas: R = P WSN4.6: R = P FAA: R > P WSN4.6: P > R or R = P TCASN: P > R PTASN: P > R (240 *d*) R > P (470 *d*)	α_{S1}-cas: R = P β-cas: R = P WSN: R = P PTASN: R = P	α_{S1}-cas: R > P (90 *d*) β-cas: R > P (180 *d*) WSN: P > R TCASN: P > R (180 *d*) FAA: R > P	

var.: variable.

α_{S1}-cas(ein), β-cas(ein): native caseins, indicators of less primary proteolysis.

WSN (water-soluble nitrogen), WSN 4.6 (pH 4.6-soluble nitrogen), TCASN (trichloroacetic acid-soluble nitrogen), PTASN (phosphotungstic acid-soluble nitrogen), FAA (free amino acids): nitrogen fractions characteristic of secondary proteolysis.

d: days of ripening (when results differ according to the age of cheese).

than raw milk cheeses, which shows that proteolysis patterns are affected by pasteurization. In hard-cooked cheeses, the raw milk microflora is composed of NSLAB, and of propionic acid bacteria or some thermophilic lactobacilli if not used as starter. In Cheddar or semihard cheeses, mainly NSLAB are involved, whereas in hard ovine Spanish cheeses, enterococci also develop (*see* **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development).

Sensory differences between raw and pasteurized milk cheeses are modulated by technological aspects such as the starter composition or the temperature of ripening. As raw milk cheeses ripen more rapidly than pasteurized milk cheeses, due to enhanced enzymatic activities, the optimum age of consumption is more critical than for pasteurized milk cheeses, especially in high-moisture varieties with faster ripening. Moreover, as raw milk microflora diversity and levels are not standardized, raw milk varieties present a higher sensory diversity between cheeses than exhibited by pasteurized milk varieties (*see* **Cheese**: Cheese Flavor).

Cheese Defects Observed in Raw Milk Cheeses

A number of defects occurring in cheeses made from raw and pasteurized milk are directly related to insufficient milk quality. However, traditional raw milk cheeses are especially susceptible to cheese defects due to the lack of corrective milk treatments that reduce the number of total bacteria and spores or inactivate indigenous milk enzymes. The time of appearance and the symptoms of most cheese defects that occur frequently are quite characteristic and allow quick diagnosis. In addition, analytical determination of important metabolites such as short-chain carboxylic acids helps to distinguish unambiguously between various types of faulty fermentations even in the early stages of ripening. For example, the defect of early gas blowing occurs already within 2 days after cheese production and is characterized by formation of numerous small holes in the curd. This defect is more common in cheeses with low scalding temperatures and results from a combination of weak acidification by the starter and poor bacteriological quality of raw milk. A slow and incomplete degradation of lactose by lactic acid bacteria enables the rapid multiplication of coliform bacteria and yeasts that produce CO_2 and H_2. Furthermore, elevated concentrations of formic and acetic acid are found in the curd at an early stage. Poor hygiene at milking and of plant equipment is considered to be the main reason for contamination with coliforms and yeasts. In contrast to early gas blowing, the defect of late blowing occurs after several weeks of ripening. This defect is characterized by excessive gas production in cheeses due to the anaerobic fermentation of lactate to butyrate, CO_2, and H_2 by *Clostridium tyrobutyricum* and *Clostridium beijerinckii*. This undesired secondary fermentation is often accompanied by an off-flavor resulting from an elevated level of butyric acid, which becomes perceivable at a level of >1 mmol kg^{-1}. Despite the use of raw milk from cows fed without silage and contaminations by spores of *Cl. tyrobutyricum* at levels as low as 25 cfu l^{-1}, sporadic cases of butyric acid fermentation occur in practice.

In addition to the aforementioned blowing defects, there are a number of other cheese defects that are quite specific for raw milk cheeses: The natural presence of propionic acid bacteria in raw milk is not unusual for cheese varieties such as Comté cheese. However, the production of raw milk cheeses without propionic acid fermentation requires the concentration of propionibacteria in raw milk below 30 cfu ml^{-1}. Strains of *Propionibacterium freudenreichii* and spp. *shermanii* are rather heat-resistant and survive even harsh scalding conditions. During prolonged ripening, the undesired growth of colonies of propionibacteria leads to the formation of reddish-brown spots in the curd, an atypical sweet flavor, opening defects, and a poor storage quality in semihard, hard, and extra-hard cheeses such as Raclette, Gruyère, and Sbrinz, respectively. Similarly, for the production of Emmentaler PDO, too, low concentrations of propionibacteria in raw milk are preferred, because the naturally present strains in raw milk may progressively replace the added culture strains during ripening and reduce storage quality at advanced stages of ripening.

The incidence of elevated concentrations of biogenic amines in cheeses made from raw milk is higher than that in cheeses made from pasteurized milk. Enterococci and non-starter lactobacilli such as *Lactobacillus buchneri* play an important role in the formation of biogenic amines in raw milk cheese (*see* **Biogenic Amines**). The most abundant biogenic amines in cheese are tyramine and histamine, whereas elevated concentrations of cadaverine and putrescine are characteristic of contamination with enterobacteria and occur mainly in raw milk cheeses with obvious flavor and opening defects or in pasteurized milk cheeses in case of serious recontaminations. However, raw milk with low total bacterial counts (<5000 cfu ml^{-1}) sporadically contains tyramine- and histamine-producing strains in low concentrations (<100 cfu ml^{-1}) yielding cheeses with high contents of biogenic amines in the range of 1000–2000 mg kg^{-1} after ripening periods of 6–12 months. The formation of high amounts of biogenic amines may cause adverse toxic reactions and considerably affects the storage quality of cheese due to the continuous decarboxylation of amino acids and the related production of CO_2. Such cheeses typically exhibit slits and cracks and a burning and pungent taste. However, in raw milk cheeses with high scalding temperatures such as Gruyère cheese (56–58 °C) or Comté cheese (54–56 °C), only low concentrations of biogenic amines were detected even after a ripening period of 12 months.

Rancid flavor of cheese originates from hydrolysis of milk fat by lipases and subsequent accumulation of butyric acid and caproic acid in the product. In raw milk cheese, indigenous milk LPL is usually responsible for the development of rancid flavor (*see* **Enzymes Indigenous to Milk**: Lipases and Esterases). Elevated LPL activity in milk has been related to short milking intervals, malnutrition or hormonal disorder of the cow, high somatic cell count, and late lactation. In addition, physical damage of milk fat globules makes the milk fat accessible for the enzyme and results in an increased rate of lipolysis. As LPL is heat labile, high-cooked cheese and cheese made from thermized milk are less prone to rancidity. Moreover, in highly proteolyzed cheese (e.g., Blue cheese), rancidity is less perceptible due to the high pH. Cheeses undergoing butyric acid fermentation may develop a similar off-flavor, but that can be distinguished from rancidity on the basis of molar ratio of butyric acid to caproic acid, which exceeds 4:1 in case of butyric acid fermentation.

Food Safety Aspects

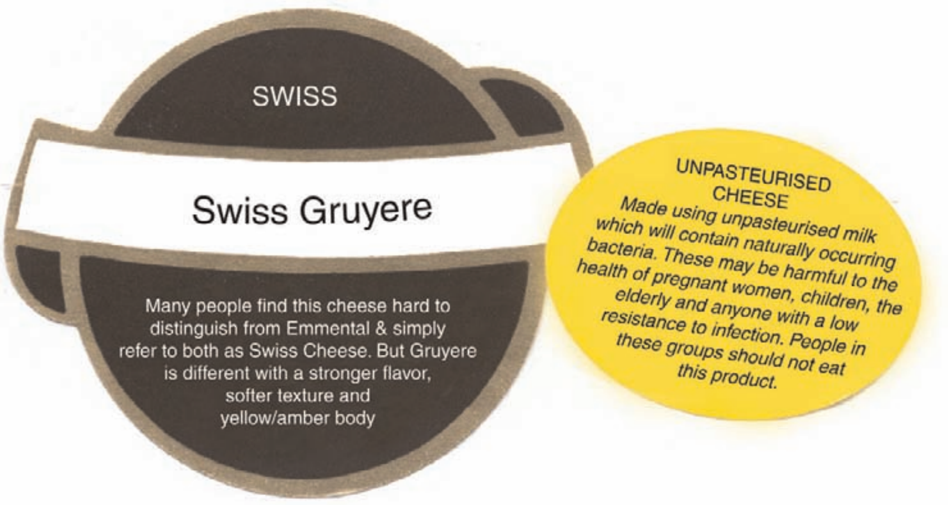
Besides beneficial microorganisms, raw milk may contain a variety of pathogenic bacteria and bacteria having a controversial safety status, for example, enterococci exhibiting antibiotic resistances and bacteria producing biogenic amines (*see* **Microorganisms Associated with Milk**). Survival of pathogens present in raw milk is greatly dependent on the technology applied to cheese processing. The acidification rate and the temperature–time profile of the whole process including ripening as well as the moisture and salt content constitute the main factors affecting the survival and growth of pathogens in cheese (*see* **Cheese**: Public Health Aspects). As a consequence of their prevalence in raw milk, and their survival and growth potential during cheesemaking and cheese ripening (*D*-value, growth temperature range, tolerance to acids and salt), the pathogens most frequently found in raw milk cheeses are enterotoxin-producing *Staphylococcus aureus*, verotoxin-producing *Escherichia coli* (VTEC), *Listeria monocytogenes*, and *Salmonella* spp. (*see* **Pathogens in Milk**: *Escherichia coli*, *Listeria monocytogenes*; S*almonella* spp.; *Staphylococcus aureus* – Molecular). Contamination of milk with *S. aureus* is usually associated with udder infections. Poor-quality raw milk may contain several thousands per milliliter, and, consequently, fresh curd produced from such milk may have an *S. aureus* count of more than 100 000 g^{-1} due to growth and passive enrichment in the cheese matrix. If the count of *S. aureus* exceeds 100 000 cfu g^{-1}, significant amounts of staphylococcal enterotoxins (SET) may be formed, which will persist in the curd for months, though the bacteria themselves substantially decrease in number during cheese ripening. The European Regulation (EC) No. 2073/2005 has set down process hygiene criteria for coagulase-positive staphylococci, requiring examination of the product at the times during the manufacturing process when the number of staphylococci is expected to be highest.

Extra-hard and hard-cooked cheeses made from raw milk, such as Parmigiano Reggiano, Gruyère, Comté, and Emmentaler, are considered to be free from viable pathogens, as hard-cooked cheeses combine a series of hurdles that prevent their survival, namely, high temperature applied during cooking and pressing, low pH and moisture content, and minimum ripening periods in the range of 4–18 months. Nevertheless, in the rind of extra-hard and hard-cooked cheeses, inactivation of pathogens (e.g., *L. monocytogenes*) may be incomplete.

Semihard cheeses are more susceptible to contamination with pathogens as the temperatures applied during production are close to their growth optimum. Pathogens such as *S. aureus*, *E. coli*, or Salmonellae can be controlled if the contamination of the vat milk is kept low and if rapid acid production is ensured by the addition of an active starter. *Listeria monocytogenes*, which may be present in raw milk, is hardly inactivated during the manufacture and ripening of semihard cheese. Therefore, regular examination of milk as well as of smear samples is an important measure to control *L. monocytogenes* in semihard raw milk cheese.

Soft cheeses are very susceptible to contamination by *L. monocytogenes*, *Salmonella* spp., and *E. coli*. As a result of high moisture content, soft cheeses can allow survival or growth of these pathogens. This is a great safety concern, as consumption of even low numbers of *Salmonella* spp. or pathogenic *E. coli* and medium numbers of *L. monocytogenes* may induce disease. In particular, surface-ripened soft cheeses are the most susceptible to *L. monocytogenes* contamination on their surface, due to deacidification of the surface by yeasts and molds.

As a primary means of food safety, milk is often treated by pasteurization, thermization, or microfiltration in order to eliminate or reduce the concentration of pathogenic bacteria present in the vat milk. However, the incidence of outbreaks associated with cheese consumption and the occurrence of contaminated cheese on the market are not higher for raw milk cheeses than for pasteurized milk cheeses. In Europe, the high safety level is primarily achieved by the implementation of comprehensive HACCP concepts in cheese factories processing raw milk and monitoring programs based on legally defined food safety criteria and process hygiene criteria (*see* **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health). Some countries disregard the fact that a high food safety standard can be obtained in raw milk cheeses, and require the labeling of all cheeses made from



Many people find this cheese hard to distinguish from Emmental & simply refer to both as Swiss Cheese. But Gruyere is different with a stronger flavor, softer texture and yellow/amber body

UNPASTEURISED CHEESE

Made using unpasteurised milk which will contain naturally occurring bacteria. These may be harmful to the health of pregnant women, children, the elderly and anyone with a low resistance to infection. People in these groups should not eat this product.

Figure 3 Example of labeling of raw milk cheeses with health alerts.

unpasteurized milk with special health alerts (**Figure 3**). In the United States, the regulations of the Food and Drug Administration (FDA) require all cheeses made from unpasteurized milk to be aged at least 60 days before being sold. As a consequence, traditional soft cheeses such as Brie de Meaux and Camembert de Normandie are available on the US market in the form of analogues made from pasteurized milk.

Heat treatments reduce the risk of occurrence of pathogens in the vat milk but simultaneously reduce the biodiversity of the flora present in the final cheese. Raw milk cheeses harbor a complex flora acting as a protective barrier against pathogens, whereas pasteurized milk cheeses are much more susceptible to postpasteurization contamination, for example, to contamination by *E. coli* and *L. monocytogenes*, typically occurring in ripening facilities or during cutting at the retail premises. Moderate heat treatments such as thermization may select for heat-resistant microorganisms (e.g., Enterococci, *M. tuberculosis*, *Coxiella burnetii*), by enabling their survival while reducing the competitive flora.

See also: **Biogenic Amines**. **Cheese**: Biochemistry of Cheese Ripening; Camembert, Brie, and Related Varieties; Cheese Flavor; Public Health Aspects; Swiss-Type Cheeses. **Enzymes Indigenous to Milk**: Lipases and Esterases; Phosphatases. **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health. **Heat Treatment of Milk**: Heat Stability of Milk; Thermization of Milk. **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Microorganisms Associated with Milk**. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Pathogens in Milk**: *Escherichia coli*; *Listeria monocytogenes*; *Salmonella* spp.; *Staphylococcus aureus* – Molecular. **Plant and Equipment:** Safety Analysis and Risk Assessment. **Policy Schemes and Trade in Dairy Products:** Codex Alimentarius; Standards of Identity of Milk and Milk Products.

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Avoidance of Gas Blowing

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Gas Blowing in Cheese

Development of gas in cheese is evident by the occurrence of eyes, cracks, slits, fissures, and holes in the cheese mass and by accumulation of gas within the cheese packaging. The number, size, shape, and distribution of gas holes within the structure of cheese are influenced by the type of gas produced (CO_2 or H_2; H_2 has a particularly low solubility in the aqueous phase of cheese), the amount of gas produced, the cheese texture, and the effect of cheese temperature on curd texture and on gas solubility, and pressure within the curd.

Gas production during cheese ripening may occur within the first few days of ripening (early gas) or at a more advanced stage of ripening (late gas). Early gas defects in cheese are manifest within the first 24–48 h of manufacture and may occur prior to or during brining/salting of the cheeses. These defects are manifested as numerous small holes in the cheese and are associated with growth of coliforms, yeasts, and sometimes heterofermentative lactic acid bacteria in the cheese. Early gas is more problematic in soft and semisoft rennet curd cheeses than in hard cheeses, as the former tend to have higher pH, shorter ripening periods, higher water activity (a_w), and lower salt concentration than the latter. However, gas spoilage may also occur in acid curd soft cheeses such as Quarg or Cottage cheese. Late gas defects are detected in cheese from a few weeks after cheese manufacture to 4–6 months into cheese ripening. They are associated with the spores of *Clostridium tyrobutyricum* or the growth of heterofermentative or salt-tolerant lactobacilli, heat-resistant streptococci, or undesired propionic acid bacteria (PAB), and can range from mild defects to extreme gas blowing of cheeses. Swiss- and Dutch-type cheeses are particularly susceptible to this defect.

Early Gas Defects

Coliforms

Coliforms are Gram-positive, oxidase-negative, non-spore-forming rods that grow aerobically or facultatively anaerobically in the presence of bile salts and ferment lactose to produce acid and gas within 48 h at 37 °C. They include the genera *Escherichia, Enterobacter, Klebsiella, Proteus, Serratia,* and *Citrobacter.*

Coliforms may find their way to cheese from raw milk or, as they are killed by pasteurization, through contamination of pasteurized milk by raw milk, or as a result of poor sanitation of the cheese manufacturing plant. Coliforms require lactose for growth as they cannot metabolize lactate, and thus rapid growth occurs during the early stages of cheesemaking particularly where temperatures and pH are favorable. After curd formation and during syneresis, coliforms are concentrated in the curd and thus can increase to high numbers after removal of the whey. Where excessive growth occurs, cheeses may develop off-flavors including yeasty and unclean flavors and early blowing due to the formation of CO_2 and, in particular, H_2. Such gas defects are evident in cheeses where coliform levels are $\sim 10^7$ cfu g^{-1}. However, growth of coliforms may not necessarily result in early gas formation as strains can vary in their potential to produce H_2 in cheese.

Avoidance of Gas Blowing by Coliforms

Measures to avoid early gas defects due to coliform growth include hygiene practices, heat treatment of cheese milk, active starters for effective acidification of cheese milk, and also the addition of oxidizing salts during cheese manufacture.

Coliform counts in cheese milk should be minimized by following hygienic milking practices and through storage of milk at low temperatures prior to cheese manufacture. This is of particular importance in the manufacture of raw milk cheeses. Coliforms are sensitive to chemical sanitizers, and thus appropriate and effective sanitation of equipment and facilities from milking machines to the cheese manufacturing plant and equipment is effective in minimizing coliform numbers. After pasteurization or other heat treatments, the milk should be rapidly cooled to inoculation temperatures or to lower temperatures should inoculation be delayed, and the period between heat treatment of cheese milk and inoculation with starter cultures should be minimized.

Growth of coliforms may be prevented by using active and fast acidifying starter cultures that rapidly metabolize lactose and decrease curd pH. Coliforms are acid sensitive, and as the cheesemaking process progresses this sensitivity is further enhanced with increased salt concentration and decreased a_w. Cheeses in which acid production is delayed are more susceptible to coliform growth and gas formation; thus, starter cultures used in cheesemaking should be free from phage attack. Similarly, the cheese milk should be free from antibiotics as coliforms are not inhibited by antibiotics such as penicillin, while different starter cultures have varying sensitivities to different antibiotics, potentially resulting in increased coliform numbers in the curd with associated gas defects. Also, rapid fermentation during cheese manufacture is beneficial as metabolites of the citric acid fermentation react with H_2 produced, and thereby reduce the risk of blowing.

Where permissible, oxidizing salts such as sodium or potassium nitrate may be added to cheese milk to inhibit development of gas by coliforms. Nitrate suppresses the formation of the enzyme system involved in the production of H_2 (conversion of lactose to formic acid and formic acid to H_2 under anaerobic conditions) and promotes the formation of nitrate- and nitrite-reducing enzymes. Nitrate and nitrite act as hydrogen ion acceptors and H_2 is not produced from formic acid. However, growth of coliforms is not prevented, CO_2 is still produced, and development of off-flavors is not inhibited.

Yeasts

Yeasts are defined as unicellular fungi that reproduce by budding or fission (excluding the fungus *Geotrichum candidum*) and include the genera *Candida* and *Kluyveromyces*. Growth of yeasts in cheese may be attributed to their ability to grow at low temperatures and low pH values and their resistance against low a_w values, high salt concentrations, cleaning compounds, and sanitizers. Production of early gas in cheese by yeasts is not frequent and is often due to *Kluyveromyces* and its imperfect forms, which ferment lactose to produce CO_2.

Yeasts developing during cheese manufacture and ripening processes can originate from raw milk, particularly where farm sanitation practices are poor, or as yeasts are heat sensitive and killed by pasteurization, through contamination of pasteurized cheese milk or curds. High numbers of yeasts may occur on surfaces of manufacturing equipment and in the air of the processing environment, particularly where hygiene levels are low. It has also been reported that the dominant species of yeasts associated with dairies (e.g., *Debaryomyces hansenii, Candida versatilis, Torulaspora delbrueckii*) are resistant to commercial sanitizers and cleaning compounds, and it is possible that these yeasts may colonize equipment during cleaning and sanitation cycles. High numbers of yeasts (10^5 cfu ml^{-1}) in whey cultures have been shown to cause blowing in Argentinean hard cheeses, and yeasts originating from whey have led to gas spoilage of Parmesan cheese. Similarly, high numbers of yeasts (10^2–10^6 cfu g^{-1}) have been observed in Cottage cheese, and gas defects associated with yeasts have been reported for Cheddar, Swiss, and pickled Feta-type and processed cheeses.

Avoidance of Gas Blowing by Yeasts

As with coliforms, yeast counts in cheese milk can be reduced by adherence to hygienic milking practices, and as yeasts are heat sensitive they may be inactivated by pasteurization of cheese milk. Maintaining hygiene and sanitation of manufacturing plant and equipment is also important. Where curd washing is employed during cheese manufacture, it should be ensured that the water used should not be a source of contamination by yeasts, and where whey cultures are utilized, yeast numbers should be limited ($<10^5$ cfu ml^{-1}) to avoid any potential gas blowing in the cheeses produced. Of high importance is the requirement for active cultures and lactose metabolism during cheese manufacture, thus avoiding any subsequent yeast fermentation of residual lactose and associated gas formation in the cheese.

Citrate-Positive Lactococci or Leuconostoc spp.

Starter bacteria such as *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* or *Leuconostoc mesenteroides* subsp. *cremoris* may lead to early gas defects during cheese manufacture and ripening by production of CO_2 from citrate. While these starter bacteria are usually added to achieve an open texture and/or a particular flavor profile, any imbalance developed between different species or strains, for example during preparation of bulk cultures or during the initial stages of cheese manufacture, may lead to excessive gas formation in the curd. Excessive gas production may also occur during the production of Cottage or fresh curd cheeses where citrate levels may be increased due to addition of milk powder.

Avoidance of Gas Blowing by Citrate-Positive Lactococci or Leuconostoc spp.

Avoidance measures include maintaining correct propagation temperatures to ensure that the desired balance between bacterial species and strains is maintained when preparing bulk cultures and also ensuring that citrate-fermenting cultures do not inadvertently contaminate bulk starter cultures where undesired.

Late Gas Production

Butyric Acid Bacteria

Butyric acid bacteria are anaerobic, spore-forming bacteria that ferment lactate to produce acetic and butyric acids, CO_2, and H_2. This gas production, particularly that of H_2 (which has a low solubility in the aqueous phase of cheese), results in late blowing of cheeses, manifested by the appearance of cracks, abnormally shaped or excessively big eyes or holes and blow holes, or the bursting of the cheese in extreme cases. The blowing of the cheeses is also accompanied by off-flavors due to butyric acid. It usually occurs a few weeks to many months after manufacture and may be evident through swelling of the cheese or in other cases may not be detected until after the cheese is cut. The defect is predominantly due to germination of spores of *C. tyrobutyricum*, although *C. butyricum* may also be responsible; spores of *C. beijerinckii* or *C. sporogenes* have also been associated with secondary causes of gas production. The spores germinate in the anaerobic environment of cheese, producing vegetative cells that are responsible for the butyric acid fermentation.

Swiss-type cheeses such as Emmental are particularly susceptible to butyric acid fermentation due to their high ripening temperatures (>20 °C) and low acid and salt contents. However, butyric acid fermentation is also prevalent in other hard cooked cheeses such as Parmesan-type cheeses and in brine-salted, low-acid Dutch-type cheeses such as Gouda. Its occurrence has also been reported in processed cheeses that had been gently heated. Spores of butyric acid bacteria in cheese usually originate from cheese milk sourced from lactating animals fed on grass or maize silage. Silage, particularly of low-quality fermentation, contains high numbers of butyric acid bacteria spores; these spores survive the animals' digestive tract and are transferred into the milk via fecal contamination of the udder. The degree of contamination of the milk by spores is dependent on the lactating animals' diet and on hygiene standards during milking. Spore numbers in the milk from cows that are not fed silage are usually <200 l^{-1}; however, spore numbers can increase to $10^5\,\mathrm{l}^{-1}$ milk, particularly where silage has been subject to low-quality fermentation. Spore numbers of 200–1000 l^{-1} milk can result in butyric acid and gas defects in Dutch- and Swiss-type cheeses, and where spore numbers reach 10^4–$10^5\,\mathrm{l}^{-1}$ milk, more intense blowing occurs in cheeses.

Although the clostridia spores are not strongly heat resistant, they survive high-temperature–short-time (HTST) pasteurization and are concentrated in the cheese curd, where they germinate and can grow at temperatures exceeding 7 °C. Growth and gas production by these bacteria are dependent on the pH, salt content, and a_{w} of the cheese and the temperature and duration of ripening. The degree of blowing is dependent on the level of gas production and on the textural characteristics of the cheese.

Avoidance of Gas Blowing by Butyric Acid Bacteria

Avoidance of gas blowing by butyric acid bacteria may be achieved by avoiding milk contaminated with *Clostridium* spores, removal of spores from the cheese milk, and/or inhibition of germination of the spores in the resultant cheese.

Avoidance or minimization of milk contamination by spores

This may be achieved by avoiding cheese milk produced from silage-fed herds and using milk produced from pasture- or hay-fed herds (in certain regions, silage-fed herds are not permitted for manufacture of hard cooked and Swiss-type cheeses). Hygiene practices during milking are also important to minimize contamination of milk by spores present on teat surfaces. Where milk from silage-fed cows is used, care should be taken to minimize soil contamination and to ensure rapid and adequate fermentation in the ensiling process. However, limiting the number of spores in milk is not sufficient to eliminate butyric acid fermentation and additional methods to remove spores or inhibit germination are also required.

Removal of spores from cheese milk

This may be achieved through bactofugation or microfilteration (MF). 'Bactofugation' is a centrifugation process (of ∼9000 ***g***) that can be used to reduce substantially the number of anaerobic, spore-forming bacteria in cheese milk and is carried out at temperatures of 55–60 °C in series with, and usually downstream of, milk separation and standardization. This process removes 80–90% of total bacteria and 98–99.5% of *Clostridium* spores to the centrifugate (i.e., the concentrate produced containing bacteria and spores) and this constitutes ∼0.15–0.2% of the milk feed, which is either discharged periodically or diluted with bactofuged milk to 2–3% of the feed volume and subsequently sterilized (e.g., 120 °C for 60 s or 140 °C for 3–4 s) and recombined with the bactofuged milk stream. The 'Bactocatch' process involves prior separation of milk into cream and skim milk streams. The skim milk is subjected to MF using 0.8–1.4 μm pore size membranes at 37–50 °C, resulting in a bacteria- and spore-rich concentrate (retentate) comprising ∼5% of the flow (or ∼0.5% if a second MF process is incorporated) and a bacteria- and spore-reduced stream (permeate). The retentate may be added to the cream stream prior to ultra-high temperature (UHT) treatment of the latter at ∼120 °C, resulting in the inactivation of bacteria and Clostridia spores; the high-heat-treated stream is then mixed with the permeate stream and HTST-pasteurized before cheese manufacture. Reductions of 4–5 log cycles of spores are possible using this system.

Inhibition of spore germination and/or bacterial growth

This may be promoted by ensuring use of cheese starter cultures with high acid-producing activity, by avoiding inadequate pressing of the curd, which may result in weak areas in the curd or unexpelled whey pockets, which could establish conditions for the formation of gas holes, and by the use of compounds (e.g., nitrate, lysozyme, nisin), although not universally permitted, that inhibit spore germination and/or bacterial growth.

Nitrate

Nitrate addition is used to delay germination of spores in cheese (although the actual mechanism may be more complicated) until the salt, which is concentrated mainly in the outer regions of the cheese immediately following salting of the molded cheeses in brine, has equilibrated within the cheese. Inhibition of germination of spores by nitrate requires the presence of xanthine oxidoreductase (EC 1.2.3.2), which reduces nitrate to nitrite. The inhibitory action is subsequently taken over by salt, if present at sufficient concentrations, as it equilibrates throughout the cheese block. The use of nitrate has been particularly adopted in cheeses with an initial pH suitable for butyric acid fermentation (optimal growth of *C. tyrobutyricum* is at pH 5.8) and with slow equilibration of salt from the cheese surface inward such as in Dutch- and Swiss-type cheeses. Traditionally, nitrate was added to the cheese milk although a more recent practice is to add nitrate to a mixture of curds and whey (when most of the whey has already been removed from the curd).

The quantity of nitrate required to prevent butyric acid fermentation is dependent, among other factors, on the number of spores present in the cheese milk and on the conditions such as pH and salt-in-moisture levels in the cheese. It has been reported that the use of an L-type (containing citrate Cit^+ *Ln. mesenteroides* subsp. *cremoris*) or O-type (containing Cit^- *Lc. lactis* subsp. *lactis* and *cremoris*) starter bacteria in comparison to a DL-type (containing Cit^+ *Ln. mesenteroides* subsp. *cremoris* and Cit^+ *Lc. lactis* subsp. *lactis*; *see* **Cheese**: Starter Cultures: General Aspects) culture may result in a decreased risk of butyric acid fermentation, probably because of the production of acetate. Conversely, high coliform numbers (e.g., $>10^5\,\mathrm{cfu\,g^{-1}}$) may result in an increased risk of butyric acid fermentation due to degradation of nitrate to nitrite early in cheese ripening. A similar effect has also been attributed to some strains of mesophilic lactobacilli.

Lysozyme (muraminidase)

This enzyme hydrolyzes peptidoglycan, a component of the bacterial cell wall, and promotes lysis. Certain spores are quite resistant to lysozyme, whereas others are relatively inhibited. The enzyme is added to cheese milk and retains 90–99% of its activity in the cheese curd; the dose levels added to cheese milk should be sufficient to prevent butyric acid fermentation without inhibiting acidification by lactic acid bacteria and, where relevant, propionic acid fermentation. Use of lysozyme is limited to applications where spore numbers are not excessive (e.g., $<500\,\mathrm{spores\,l^{-1}}$), as small numbers of Clostridia can survive lysozyme and the higher doses required for high spore numbers can impact negatively on desirable lactic and propionic acid fermentations in the cheeses. Lysozyme is frequently used in combination with bactofugation and as an alternative to nitrate addition. Where nitrate addition is permissible, lysozyme may be used in combination to reduce the dose of nitrate required.

Nisin and other approaches

Nisin (an antibacterial peptide with 34 amino acid residues) is a broad-spectrum bacteriocin effective against many Gram-positive organisms, including *Clostridium*. It may be used to prevent spoilage in processed cheeses that are produced from blends of natural cheeses and other ingredients and heated to ~80 °C. This heat treatment does not inactivate spores and when processed cheeses are stored at up to ambient temperature and with suitable pH and a_w, germination of the spores and growth of vegetative cells can occur leading to butyric acid fermentation and gas blowing.

Addition of nisin in the case of natural cheese manufacture is more problematic as added nisin is lost in whey and is also inhibitory toward lactic acid bacteria. The ability of bacteriocin-producing *Lactobacillus* adjunct cultures to prevent late blowing in brine-salted cheeses has been evaluated in recent years. It is necessary that these cultures do not inhibit starter cultures during cheese manufacture and should reach sufficient numbers post cheese manufacture to produce levels of bacteriocin that are inhibitory to germination of spores. These bacteriocin-producing cultures should also not have any deleterious effect on cheese flavor during ripening. Long-chain polyphosphates have also been shown to inhibit the growth of *C. tyrobutyricum* in processed cheese spreads.

Lactobacilli

Heterofermentative lactobacilli: Gas blowing in Cheddar-type cheeses

Late gas and slit defects in Cheddar may be attributed to the formation of CO_2 from lactose or galactose during ripening by heterofermentative lactobacilli such as *Lactobacillus brevis* or *Lb. fermentum*. Heterofermentative lactobacilli are more representative of the adventitious non-starter lactic acid bacteria (NSLAB) in cheeses made from raw milk and will not usually grow in cheeses made from pasteurized milk under hygienic conditions. Even when present early in ripening, they are usually outgrown by other NSLAB such as *Lb. paracasei*.

Avoidance of Late Gas Blowing in Cheddar-Type Cheese Caused by Heterofermentative Lactobacilli

This may be achieved by avoiding the use of starter cultures with slow activity, which can result in high levels of residual lactose and galactose, and by ensuring appropriate levels of salt-in-moisture in the cheese, as this influences starter activity and also affects lactose utilization and lactate production by NSLAB. Other causes of late gas blowing in Cheddar-type cheeses may include

- contamination of cheese milk or cheese by heterofermentative bacteria, resulting in CO_2 production by metabolism of citrate, and
- growth of *C. tyrobutyricum* in the cheese (however, this is rare in Cheddar-type cheese due to the rapid equilibration of salt-in-moisture levels in the cheese).

Lactobacilli: Gas blowing in brine-salted cheeses

Gas blowing in brine salted cheeses has been attributed to growth of common mesophilic lactobacilli (e.g., *Lb. plantarum, Lb. casei, Lb. brevis*), salt tolerant lactobacilli (related to *Lb. plantarum, Lb. casei* but with differing salt resistance) or to growth of *Lb. bifermentans* (a facultatively heterofermentative lactobacillus associated with formation of slits in Dutch-type cheeses through production of CO2 and H2 from lactic acid). Gas blowing has been associated with the formation of slits in Dutch-type cheeses through production of CO_2 and H_2 from lactic acid. Lactobacilli may find their way to cheese plants through raw milk or through contaminated equipment and plant personnel; they can survive in biofilms on plant surfaces, and even where present in low numbers ($\sim 10\,ml^{-1}$) in cheese milk may potentially reach counts of $>10^7$ cfu g^{-1} cheese within 4–6 weeks.

Salt-tolerant lactobacilli colonize cheeses that have been salted in reduced-strength brines and some strains can survive in brines of >15 g 100 g^{-1} NaCl. They differ from other strains of lactobacilli in that they possess a highly active amino acid metabolism, which may result in excessive production of CO_2 and a range of off-flavors (e.g., putrid, fruity, or H_2S) in 4- to 6-month-old cheeses. These lactobacilli enter cheeses during brining, and counts $>10^3$ cfu ml^{-1} in brine are considered to be potentially problematic. Although they colonize the brine, they do not actually grow in the brines (even where brine strength is weak) but grow as deposits on the surface of brine tanks just above the brine levels. Conditions within these deposits, for example, increased pH due to the growth of salt-tolerant yeasts, reduced salt content due to water absorption, and also higher temperatures than that of the brine, are more favorable for the growth of salt-tolerant lactobacilli, which may subsequently enter the brine and thus contaminate cheeses placed in the brine.

Avoidance of Late Gas Blowing in Brine-Salted Cheeses Caused by Lactobacilli

Although it is difficult to control common lactobacilli in raw milk cheeses, they are killed by pasteurization of cheese milk and, as with Cheddar-type cheeses, it is necessary to avoid the use of starter cultures with slow activity, which can result in high levels of residual lactose and galactose in cheeses. Furthermore, the absence of stagnant spots within continuous industrial cheese manufacture plants is also necessary. Avoidance of gas defects due to salt-tolerant lactobacilli may be achieved by avoiding contamination of cheese milk by the bacteria, achieving good rind formation through adequate pressing, avoiding damage to the rind, which may otherwise allow these bacteria to penetrate during brining, maintaining brine concentrations at ≥ 16 g 100 g^{-1} salt, reducing brine pH to ≤ 4.6 (where suitable) and temperature to ≤ 13 °C, and ensuring adequate sanitation and hygiene of the brine facilities, including removal of surface deposits.

Heat-Resistant Streptococci

When present in raw milk, heat-resistant streptococci can grow at temperatures up to 45 °C and survive heat treatments, including HTST pasteurization. During heat treatment of cheese milk, these bacteria may become attached to the walls of the regeneration section of the plate exchanger and may start to multiply very rapidly (minimum generation times, ~ 15 min) to numbers partly dependent on the initial numbers present in the raw milk. Continuous use of the plate heat exchangers for too long a period without cleaning may result in heavy contamination of the cheese milk with streptococci ($\sim 10^6$ cfu ml^{-1}). Concentration in the subsequent cheese curd and growth during the early stages of cheesemaking may increase their number to $\sim 10^8$ cfu g^{-1} cheese, resulting in the development of unclean and yeasty off-flavors. Furthermore, CO_2 production by these bacteria can lead to cheese with excessively open texture after ~ 5 weeks of ripening, particularly when the starter culture used in cheese manufacture has a high CO_2-producing capacity. Avoidance of gas blowing by heat-resistant streptococci includes avoiding the use of plate heat exchangers for excessively long processing runs without cleaning and ensuring correct sanitation of the processing plant.

Propionic Acid Bacteria

Although PAB are central to the development of a sweet nutty flavor and characteristic eyes in many Swiss-type cheeses, their growth in other cheese types such as Gouda can result in undesirable gas formation. PAB grow in cheeses with low salt-in-moisture levels and low acid levels and their growth rate increases with increasing ripening temperature. They ferment lactic acid to propionic and acetic acids, CO_2, and water. While the pH and salt-in-moisture levels in Gouda-type cheeses are favorable for PAB growth, the lower ripening temperatures and in particular the absence of a warm room ripening step (~22 °C) result in gas formation in Gouda-type cheeses only after a prolonged ripening time and thus can be associated with late blowing. Avoidance of gas defects due to PAB is achieved by pasteurization of cheese milk as PAB are inactivated by pasteurization and by control of ripening temperatures.

Other Sources of Gas Blowing in Swiss-Type Cheeses

Although not an extreme form of gas blowing, the development of splits and cracks in Swiss-type cheeses may arise due to excessive gas production, an unsuitable cheese body that cannot accommodate the gas produced, or a combination of both factors. Excessive gas production may be due to inappropriate storage temperatures, due to particular strains of PAB with high aspartase activity or an ability to grow at low storage temperatures, and also possibly due to stimulation of PAB by peptides and amino acids released by the proteolytic activity of certain lactic acid bacteria. Other sources of CO_2 may also be a contributory factor such as that produced by decarboxylation of amino acids. A poor cheese texture, lacking the desired elasticity (*see* **Cheese**: Cheese Rheology), or storage of cheeses in cold rooms with inadequate temperature control may also be problematic. During cold storage, cheese loses elasticity and may also become supersaturated with CO_2; an unintended increase in temperature at this point decreases gas solubility, increases gas pressure, and may result in splitting of the cheese.

Avoidance of Gas Blowing Due to Other Sources in Swiss-Type Cheeses

Avoidance of this defect may be achieved by using appropriate strains of PAB and lactobacilli, controlling cheese manufacture to achieve desired cheese composition and texture, and maintaining consistent cold storage temperatures. Other approaches may include water addition to the curd during manufacture to improve elastic properties by removal of unfermented carbohydrate, and the use of facultatively heterofermentative lactobacilli to control the growth of PAB and thereby prevent production of excessive gas.

See also: **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview; *Propionibacterium* spp. **Bacteriocins**. **Bacteriophage**: Technological Importance in the Diary Industry. **Biofilm Formation**. **Cheese**: Biochemistry of Cheese Ripening; Cheddar-Type Cheeses; Cheese Rheology; Cheeses Matured in Brine; Dutch-Type Cheeses; Hard Italian Cheeses; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Overview; Preparation of Cheese Milk; Raw Milk Cheeses; Starter Cultures: General Aspects; Swiss-Type Cheeses. **Contaminants of Milk and Dairy Products**: Contamination Resulting from Farm and Dairy Practices.

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Biochemistry of Cheese Ripening

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Introduction

Acid- and acid/heat-coagulated cheeses are ready for consumption immediately after processing of the curds (*see* **Cheese**: Acid- and Acid/Heat Coagulated Cheese; Overview) but the curds for rennet-coagulated cheeses are ripened (matured) for a period ranging from 2 to 3 weeks to >2 years. The objective of cheese ripening is to convert the fresh curds, which differ only slightly, mainly in moisture content, between varieties, to one of many cheeses that differ characteristically in appearance, taste, aroma, texture, and functionality. Conversion of curd to cheese involves three primary events: (1) metabolism of residual lactose and of lactate and citrate; (2) lipolysis and metabolism of fatty acids; and (3) proteolysis and amino acid catabolism, the products of which are modified via various biochemical, and perhaps chemical, reactions. The primary reactions are principally responsible for changes in texture and functionality, while flavor is probably generated mainly through modification of products of the primary reactions.

Metabolism of lactose, lactate, and citrate and related events are caused by living microorganisms (starter and/or non-starter), while lipolysis and proteolysis are catalyzed mainly by enzymes from the coagulant, milk, starter bacteria, adventitious non-starter bacteria, and, usually, secondary (adjunct) cultures. A general overview of the biochemistry of cheese ripening is given below.

Metabolism of Lactose, Lactate, and Citrate

The production of lactic acid from lactose by the starter bacteria is a major and essential event in the manufacture of cheese curd (*see* **Cheese**: Starter Cultures: General Aspects). Most (~98%) of the lactose in milk is removed in the whey as lactose or lactic acid but fresh cheese curd contains 1–2% lactose. For most cheese varieties, the pH of the curd is 6.2–6.4 at molding and since the curds are not salted at this point, the starter bacteria completely metabolize the residual lactose within about 12 h. If the curds are washed during manufacture, as practiced in the manufacture of Dutch-type cheeses, the concentration of lactic acid in the cheese at the end of manufacture is ~1%, but if the curds are not washed, as in the manufacture of Emmental or Parmigiano Reggiano, the fresh cheese contains ~1.5% lactic acid.

The pH of curd for Cheddar-type cheese is lower than that of many varieties (~5.4) at milling as the curds are dry-salted before molding and the salt-in-moisture level of the curd rapidly increases; the low pH and the rapid penetration of salt into the curd retard lactose metabolism and lactose is metabolized slowly, normally by residual starter activity, to L(+) lactic acid. However, if the salt concentration is too high, the starter bacteria are inhibited and residual lactose is metabolized by non-starter lactic acid bacteria (NSLAB) to DL-lactic acid; young Cheddar cheese contains ~1.5% lactic acid. Complete metabolism of lactose (and monosaccharides derived therefrom) is essential for cheese quality; the presence of a readily fermentable carbohydrate in cheese can lead to undesirable secondary fermentations during ripening.

Modification of Lactic Acid

The fate of lactic acid in cheese is characteristic of the variety. In Cheddar- and Dutch-type cheeses, L(+) lactate is isomerized to a racemic mixture (D/L-lactate). Racemization has no effect on cheese flavor but if the concentration of D(−) lactate is too high, it may favor the formation of undesirable crystals of calcium lactate on the cheese surface. Oxidation of lactic acid to acetic acid occurs to a very limited extent, dependent on the concentration of O_2 in the curd, which is strongly affected by the permeability of the packaging material to oxygen.

In surface mold- and smear-ripened cheeses, lactic acid in the surface layer is catabolized to CO_2 and H_2O by surface molds or yeasts, causing an increase in pH and the diffusion of lactic acid from the interior to the surface. At maturity, a pH gradient exists in Camembert cheese from the surface (~pH 7.5) to the center (~pH 6.5), with a concentration gradient of lactic acid in the opposite direction. The increase in pH contributes to softening of the texture of surface mold-ripened cheese and for the growth of coryneform bacteria on surface smear-ripened cheese.

The pH of Dutch- and Swiss-type cheeses also increases during ripening, perhaps to as high as 5.8, but the pH of Cheddar changes little during ripening. Cheese has a strong buffering capacity at ~pH 5.2 and, consequently, a low initial pH is difficult to alter. The presence of lactose in Cheddar cheese during the early stages of ripening appears to be a contributing factor: if the concentration of lactose is reduced by washing or whey replacement, residual lactose in the curd is metabolized rapidly and the pH increases when the supply of lactose has been exhausted. In contrast, the pH of high-lactose curd continues to decrease as long as lactose is present. Low-lactose Cheddar cheese has a clean, mild flavor, whereas high-lactose cheese develops a strong, harsh flavor, probably due to the low pH.

In Swiss-type cheeses, lactic acid is metabolized to propionic and acetic acids, CO_2, and H_2O:

$$3CH_3CH(OH)COOH \rightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$$

The CO_2 is responsible for the characteristic eyes in Swiss-type cheese, while propionic and acetic acids contribute to flavor.

In many types of cheese, lactate may be metabolized by *Clostridium* spp. to butyric acid and CO_2 and hydrogen gas, which cause off-flavors and late gas blowing. Contamination of cheese curd with clostridia is controlled by good hygiene, removal of the spores by bactofugation or microfiltration, or preventing their growth by the addition of KNO_3 or lysozyme to the cheese milk (*see* **Pathogens in Milk**: *Clostridium* spp.).

Thus, the metabolism of lactose and the resulting lactic acid in cheese is understood at the molecular level and is summarized in **Figure 1**. Acid production, and hence lactose metabolism, during curd manufacture is important for several reasons (*see* **Cheese**: Overview). It is also critical that lactose in the fresh curd is metabolized rapidly and completely by the starter; otherwise, catabolism by NSLAB may lead to off-flavors and gas, and lactose or its constituent monosaccharides may participate in Maillard browning, especially if the cheese is heated, for example, Mozzarella, or stored in a semi-dry form (low water activity, a_w), for example, Parmigiano Reggiano. Consequently, it is important to ensure that a galactose-positive strain of *Lactobacillus* is used in the manufacture of cheeses made using a thermophilic starter culture.

Metabolism of Citrate

Milk contains $1.8\,g\,l^{-1}$ citrate, about 94% of which is soluble and is lost in the whey. In Dutch-type cheeses, the colloidal citrate, which is retained in the curd, is metabolized by citrate-positive strains of *Lactococcus lactis* subsp. *lactis* and/or *Leuconostoc* spp., which are included in the starter, to diacetyl, acetoin, 2,3-butanediol, and CO_2. Diacetyl contributes to flavor while the CO_2 is

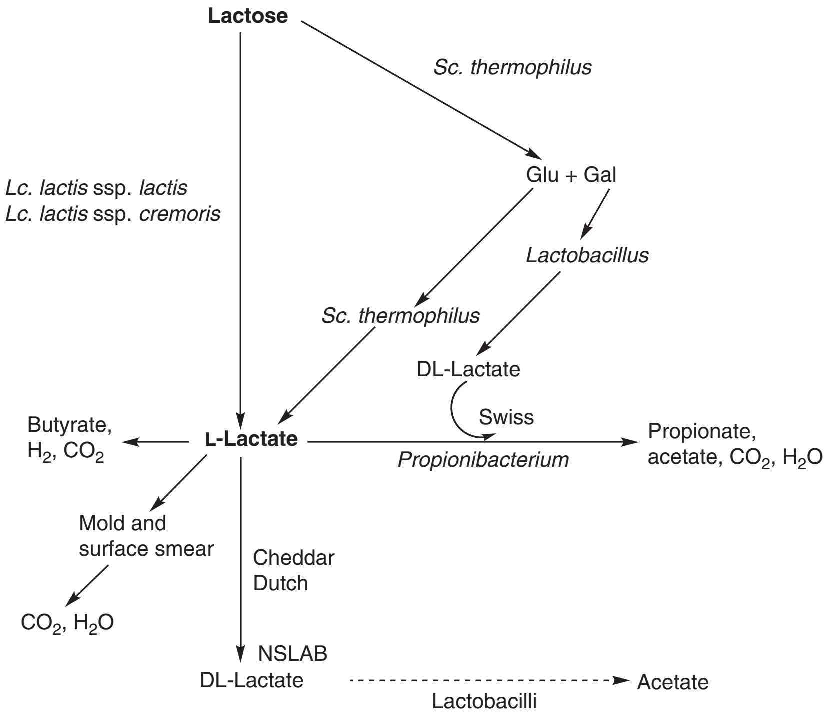

Figure 1 Summary of lactose metabolism in cheese. NSLAB, non-starter lactic acid bacteria; *Lc.*, *Lactococcus*; *Sc.*, *Streptococcus*.

responsible for the small eyes characteristic of Dutch-type cheese. The metabolism of citrate is well characterized (*see* **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria).

In Cheddar-type cheese, citrate is fermented slowly by some mesophilic lactobacilli of the NSLAB, to products including CO_2, which may cause an undesirable open texture.

Lipolysis

Lipolysis is limited in most cheese varieties. Exceptions are some Italian varieties, for example, Pecorino varieties and Provolone, in which rennet paste containing pregastric esterase (PGE) is used as coagulant, and Parmigiano Reggiano, probably due to the use of raw milk and its long ripening period. Levels of lipolysis may also be high in surface bacterial (smear)- and mold-ripened cheeses. Very extensive lipolysis occurs in blue-mold cheeses, in which *Penicillium roqueforti* secretes potent lipases. Although lactic acid bacteria (LAB) are weakly lipolytic, they do possess esterases/lipases, which release a low level of fatty acids during a long ripening period. Lipases/esterases from a number of strains of *Lactococcus* and *Lactobacillus* have been isolated and characterized.

Even slightly excessive lipolysis causes rancidity or unbalanced flavor in Cheddar-, Dutch-, and Swiss-type cheeses, but low concentrations of volatile, short-chain fatty acids, which are strongly flavored, are important, probably essential, for the flavor of many varieties. Considerably more lipolysis occurs in raw milk cheese than in corresponding cheeses made from pasteurized milk, as the indigenous lipoprotein lipase in milk is largely inactivated by pasteurization; changes to the indigenous microflora of milk may also be significant.

PGE is well characterized at the molecular, biochemical, and genetic levels. Lipases from *Rhizomucor miehei* and some *Penicillium* spp. have been reported to be satisfactory substitutes for PGE. Fungal lipases are also used extensively in the production of enzyme-modified cheese (*see* **Cheese**: Enzyme-Modified Cheese).

Penicillium roqueforti and *P. camemberti* secrete potent lipases, which have been isolated and characterized. Lipolysis is not of major significance in the ripening of surface mold-ripened cheeses but it is essential in blue-mold cheeses, the flavor of which is dominated by methyl ketones, which are produced by β-oxidation of fatty acids liberated by lipases produced by *P. roqueforti* (*see* **Cheese**: Blue Mold Cheese; Camembert, Brie, and Related Varieties).

Compared to blue cheese, lipolysis is limited in surface smear-ripened cheeses and fatty acids probably make relatively little contribution to the flavor of these strongly flavored cheeses. The surface microflora of these cheeses is very heterogeneous and develops in a sequence, that is, yeasts (various species), which grow initially, increase the pH, permitting various coryneform bacteria (especially *Brevibacterium* spp. and *Arthrobacter* spp.), staphylococci, and micrococci to grow. Many of the smear microorganisms secrete lipases/esterases, some of which have been isolated and characterized.

Catabolism of Fatty Acids

Free fatty acids, especially volatile short-chain acids, make at least some positive contribution to the flavor of most, probably all, cheeses. They may also be modified in various ways to produce other sapid compounds, including methyl ketones, esters, thioesters, lactones, aldehydes, and alcohols (**Figure 2**).

Proteolysis

Proteolysis is the most complex and perhaps the most important of the three primary events that occur in most cheese varieties, especially internal bacterially ripened cheeses such as Cheddar, Swiss, or Gouda. It is primarily responsible for changes in cheese texture and functionality and makes a significant contribution to flavor, especially background flavor, via the formation of amino acids and small peptides. Catabolism of amino acids leads to many sapid and aromatic compounds that are major contributors to cheese flavor, especially of surface smear-ripened varieties.

Proteolytic Agents in Cheese

Cheese contains a broad range of proteinases and peptidases, which originate from the coagulant, milk, starter LAB, adventitious NSLAB, and secondary cultures (e.g., *Propionibacterium*, *Brevibacterium*, *Arthrobacter*, *Penicillium* spp.). The proteinases and many peptidases from these sources have been isolated and characterized and their contribution to cheese ripening has been established.

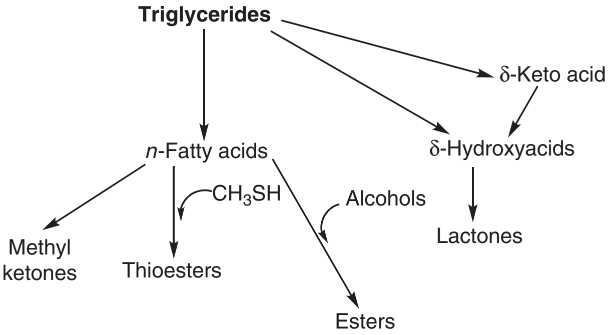

Figure 2 Summary of lipolysis in cheese.

Coagulant

Most of the rennet added to cheese milk is either lost in the whey or, depending on the variety, denatured during cooking. Rennet is extensively or completely denatured in curds cooked to a high temperature (~55 °C), for example, those for Emmental and Parmigiano-Reggiano cheese, and during the later cooking–stretching step of the manufacture of Mozzarella. Cheeses cooked to a lower temperature, for example, <40 °C, retain 5–30% of the added rennet activity, the amount being directly related to the moisture content; the proportion of chymosin or pepsin retained in the curd increases as the pH is reduced, but the retention of fungal rennet substitutes is independent of pH.

The coagulant is mainly responsible for primary proteolysis, that is, for the formation of water- or pH 4.6-soluble nitrogen. In mature Cheddar, Dutch, or similar cheeses, ~30% of total N is soluble in water. The specificity of chymosin (natural or produced by fermentation) in its action on isolated α_{S1}-, α_{S2}-, and β-caseins in solution under different conditions of pH and ionic strength is known. Some of the principal cleavage sites identified in model systems are also hydrolyzed in cheese but some are not. The principal cleavage site in α_{S1}-casein is Phe_{23}–Phe_{24}, which is completely hydrolyzed in Cheddar and similar cheeses within 4 months. The second most susceptible bond in that protein is Leu_{101}–Lys_{102}, which is extensively hydrolyzed in mature Cheddar. However, the Trp_{164}–Tyr_{165} bond, which is the second most susceptible bond in solution, does not appear to be hydrolyzed rapidly in cheese. The action of chymosin on α_{S2}-casein in cheese has not been elucidated but it is likely to be limited. Although the Leu_{192}–Tyr_{193} bond of β-casein in solution is very susceptible to chymosin, especially at very low ionic strength, chymosin has very little action on β-casein in cheese, especially in dry-salted varieties, for example, Cheddar. The C-terminal region of β-casein, which contains the chymosin-susceptible bonds, is very hydrophobic and probably undergoes intra- or inter-molecular hydrophobic interactions in the cheese environment; these interactions are promoted by NaCl – even in solution, hydrolysis of β-casein by chymosin is strongly inhibited by 5% NaCl. Inhibition of β-casein hydrolysis is probably significant for cheese flavor since the peptide β-CN (f193–209) and fragments thereof are very bitter.

The specificity of pepsins is generally similar to that of chymosin but has not been established precisely. Bovine pepsin cleaves the Leu_{109}–Glu_{110} bond of α_{S1}-casein quite rapidly; this bond is cleaved relatively slowly by chymosin.

The specificity of the fungal rennet substitutes is quite different from that of chymosin. The principal cleavage sites of *Rm. miehei* proteinase in α_{S1}-casein in solution are Phe_{23}–Phe_{24}, Met_{123}–Lys_{124}, and Tyr_{165}–Tyr_{166}, while those in β-casein are Glu_{31}–Lys_{32}, Val_{58}–Val_{59}, Met_{93}–Gly_{94}, and Phe_{190}–Leu_{191}. *Cryphonectria parasitica* proteinase is much more active on β-casein in cheese than chymosin, pepsin, or *Rhizomucor* proteinases, but its specificity on the caseins has not been determined; since it does not cause significant bitterness in cheese, its primary cleavage sites in β-casein may be in the hydrophilic N-terminal rather than in the hydrophobic C-terminal region.

Indigenous milk proteinases

Milk contains a number of indigenous proteinases, of which plasmin is the most significant. The acid proteinase, cathepsin D, and the other indigenous proteinases are of relatively little significance.

Plasmin is a well-characterized blood-derived serine proteinase with a pH optimum at ~7.5 (*see* **Enzymes Indigenous to Milk**: Plasmin System in Milk). Plasmin, plasminogen, and plasminogen activators are associated with the casein micelles and are incorporated into rennet-coagulated curd. The inhibitors of both plasmin and plasminogen activators are present in the serum of milk and are lost in the whey. The inhibitors suppress plasmin activity in milk, and the activity increases greatly if the inhibitors are removed.

The specificity of plasmin in its action on α_{S1}-, α_{S2}-, and β-caseins has been established (*see* **Enzymes Indigenous to Milk**: Plasmin System in Milk). β-Casein is the most susceptible of the caseins; its hydrolysis yields γ-caseins (γ^1-CN (β-CN f29–209), γ^2-CN (β-CN f106–209), and γ^3-CN (β-CN f108–209)) and some proteose peptones (PPs) (PP5 (β-CN f105/107), PP8 slow (β-CN f29–105/107), and PP8 fast (β-CN f1–28)). α_{S2}-Casein is also quite susceptible to plasmin action and eight bonds are hydrolyzed rapidly. Although α_{S1}-casein is less susceptible to plasmin than α_{S2}- and β-caseins, it is readily hydrolyzed in solution; the large primary products are the λ-caseins.

Plasmin is mainly responsible for the limited hydrolysis of β-casein that occurs in internal bacterially ripened cheese and its contribution is particularly important in high-cook cheeses in which the coagulant is extensively or completely denatured or in cheeses in which the pH increases markedly during ripening, for example, Camembert; some γ-caseins are formed in all cheeses. Plasmin is probably also responsible for the hydrolysis of α_{S2}-casein in cheese during ripening.

Proteolytic enzymes of lactic acid bacteria

The LAB are weakly proteolytic in comparison with many other microorganisms, but they do possess a very diverse proteolytic system that is essential to support their growth in milk. LAB are auxotrophic for many amino acids; although milk is rich in readily degraded caseins, it contains a low concentration of small peptides and amino acids. The proteolytic system of LAB, especially *Lactococcus*, is very well characterized at the physiological, biochemical, and genetic levels. It consists of a serine proteinase anchored to the cell membrane (cell envelope proteinase (CEP)) and protruding through the cell wall, giving it ready access to extracellular substrates, peptide transport systems, several amino acid transport systems, intracellular proteinases, and a battery of intracellular peptidases, including oligopeptidases (PepO$_1$, PepO$_2$, PepF$_1$, PepF$_2$, i.e., endopeptidases that hydrolyze large peptides, up to at least 30 amino acid residues, but not the parent caseins), a number of aminopeptidases (PepN, PepA, PepC, PepL, pyrolidonyl carboxylyl peptidase), an iminopeptidase (PepP), a dipeptidyl aminopeptidase (PepX), general tri- (PepT) and di-peptidases (PepV, PepD), and proline-specific dipeptidases (prolidase and prolinase, PepQ and PepR, respectively), but apparently no carboxypeptidase. Acting together, the proteolytic system can completely hydrolyze all the caseins to amino acids.

The specificity of the CEP of several strains of *Lactococcus* on all the individual caseins has been characterized. Although the lactococcal CEP readily hydrolyzes the caseins in solution and in milk, it appears to have little effect on the caseins in cheese; instead, it is mainly responsible for the hydrolysis of oligopeptides produced from α_{S1}- and β-caseins by chymosin and plasmin, respectively, to smaller peptides.

A number of studies on proteolysis in, and the quality of, Cheddar cheese made using a proteinase-negative (Prt$^-$) starter have been reported. Although some differences in quality were found between Prt$^+$ and Prt$^-$ cheeses, the quality of the Prt$^-$ cheeses was good, indicating that although the CEP is active in cheese during ripening, it is not essential. Perhaps its function can be served by other lactococcal enzymes, for example, intracellular proteinases or oligopeptidases or proteinases of NSLAB or perhaps the lactococcal or NSLAB exopeptidases can act on chymosin- or plasmin-produced peptides without the intervention of CEP.

Bitterness, which is usually due to short hydrophobic peptides, is a common problem in Cheddar, Gouda, and other internal bacterially ripened cheeses. The caseins are relatively hydrophobic proteins in which the hydrophobic residues are concentrated in certain sections; the C-terminal region of β-casein is especially hydrophobic. Consequently, casein hydrolysates have a high propensity to bitterness and several bitter peptides have been isolated, including some from cheese.

The cause of bitterness in cheese has not been fully established. Plasmin is probably not responsible, but excessive chymosin activity can cause bitterness; the peptide β-CN (f193–209) is very bitter. Certain *Lactococcus* strains have a propensity to cause bitterness, either because they reach very high cell numbers, for example, the culture grows too well at the cooking temperature of the cheese, or because they have some deficiency in the proteolytic system, for example, a CEP with the incorrect specificity or insufficient peptidase activity to degrade bitter peptides produced, for example, by chymosin action. The results of recent studies suggest that both the lactococcal CEP and peptidase systems are significant with respect to bitterness and cheese quality in general. Blending bitter and nonbitter strains or using a *Lactobacillus* adjunct culture with a bitter strain produces nonbitter cheese.

The proteolytic system of *Lactobacillus* spp. is less well studied than that of *Lactococcus*, but both systems are generally similar with respect to the distribution and properties of proteinases and peptidases. The thermophilic lactobacilli, which are used as starters for fermented milks and several cheeses, grow well in milk, indicating that they can readily obtain their amino acid requirements from casein. However, mesophilic lactobacilli grow poorly or not at all in milk, which appears to be due to a lack of proteolytic activity since they grow readily in milk supplemented with a source of small peptides or amino acids.

The role of the CEP of thermophilic lactobacilli in cheese ripening is probably similar to that of the CEP of *Lactococcus*, that is, they hydrolyze mainly oligopeptides rather than intact caseins. However, the situation is less clear because the action of the *Lactobacillus* proteinases on the caseins has not been established in the same detail as that of the lactococcal proteinases and proteolysis in cheese in which they are used as starters has not been characterized in the same detail as that in low-cook cheeses. Moreover, primary proteolysis in cheeses in which lactobacilli are used as starters is mainly due to plasmin, since chymosin is extensively or totally inactivated during cooking.

Both the *Lactococcus* and thermophilic *Lactobacillus* starters die off rapidly after curd manufacture. The dead cells lyse (at strain-dependent rates) and release their intracellular peptidases and other enzymes. Although the intracellular enzymes diffuse to a very limited extent in cheese, small peptides may diffuse to dead and damaged cells; in any case, dead and lysed cells are well distributed in cheese and their intracellular peptidases are active during ripening, resulting in the formation of very small peptides and amino acids.

In contrast to *Lactococcus* sp. and thermophilic *Lactobacillus* spp., mesophilic lactobacilli grow in cheese during ripening, from very low numbers (<100 cfu g^{-1}) in fresh curd to 10^7–10^8 cfu g^{-1} within about 3 months. Thereafter, their numbers remain more or less constant, although some species decline slightly on extended ripening; there may be a balance between growth and death of NSLAB in cheese (the carbon source(s) for the growth of NSLAB in cheese is not known with certainty but may be the sugar moieties of glycoproteins of the milk fat globule membrane). Thus, mesophilic lactobacilli dominate the viable microflora of long-ripened cheese during most of its ripening. NSLAB do not contribute significantly to the formation of either large or small peptides in pasteurized milk Cheddar but they do contribute to the formation of amino acids. However, the NSLAB in raw milk Cheddar, which are more numerous and more diverse than in pasteurized milk cheese, do contribute to the formation of small peptides as well as amino acids.

Raw milk was used universally for the production of cheese until relatively recently. It is still used for most artisanal cheeses and for many well-known varieties, for example, Roquefort, Swiss Emmental, Gruyère de Comté, Parmigiano Reggiano, and Grana Padano, which are produced in small units by a well-defined labor-intensive protocol. Today, the large-volume varieties, for example, Cheddar, Gouda, Mozzarella (Pizza), and most Camembert, are made in large automated factories from pasteurized milk. Emmental and Parmigiano Reggiano, which are produced from raw milk in their region of origin, are produced from pasteurized milk in other countries under the same or similar names.

It is generally accepted that cheese made from raw milk ripens faster and develops a more intense flavor than that made from pasteurized milk. Although a number of heat-induced changes could explain the reported differences between raw and pasteurized milk cheese, studies on Cheddar and Gruyère de Comté suggest that the indigenous NSLAB in raw milk, which are incorporated into the cheese, are primarily responsible, although heat-induced inactivation of the indigenous lipase in milk, lipoprotein lipase, is also of significance.

For public health reasons and because pasteurization of cheese milk facilitates the production of cheese of consistent quality, it is very unlikely that large cheese-making facilities will revert to the use of raw milk. Instead, attempts are being made to simulate the flavor characteristics of raw milk cheese in the pasteurized milk product by using an adjunct starter, usually selected *Lactobacillus* strains. While the adjuncts currently available may modify and intensify the flavor of cheese, they do not simulate closely raw milk cheese, probably because they contain only one or a few strains of lactobacilli, while the NSLAB of raw milk cheese are very heterogeneous and originate from various sources. As work on NSLAB adjuncts continues, it is very likely that improved adjuncts will be developed.

There is a general perception that the flavor of modern Cheddar cheese has become excessively mild owing to the generally improved microbiological quality of cheese milk, pasteurization of the milk, which renders it essentially free of LAB and largely inactivates its indigenous lipoprotein lipase, use of a highly active starter containing only a few strains of *Lactococcus*, and the manufacture of cheese in enclosed equipment, which reduces contamination from the environment. While most consumers today prefer mild Cheddar, there is a niche market for more mature, intensely flavored cheese. The use of culture adjuncts is considered to be the most effective way of achieving this. Several studies have shown that mesophilic *Lactobacillus* adjuncts intensify the flavor of Cheddar cheese and that thermophilic lactobacilli are more effective than mesophilic lactobacilli, possibly because the former die rapidly in cheese, releasing their intracellular enzymes, while the latter do not.

Since the NSLAB population reaches 10^7–10^8 cfu g^{-1}, regardless of various environmental and compositional factors, *Lactobacillus* adjuncts may be added to control the indigenous NSLAB, which are uncontrolled and hence may be responsible for some of the variability in cheese quality.

A number of studies on cheese with a controlled microflora have shown that starter LAB are essential for flavor development, for example, cheese acidified with gluconic acid-δ-lactone (GDL) instead of starter does not develop a typical flavor (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus casei* Group; Lactic Acid Bacteria in Flavor Development). In contrast, NSLAB-free cheese develops a typical and acceptable flavor and GDL-acidified Cheddar curd inoculated with a culture of *Lactobacillus casei* subsp. *casei* failed to develop typical Cheddar flavor, suggesting that NSLAB are less significant than starter in flavor development in Cheddar and, probably, similar cheese varieties.

Characterization of Proteolysis in Cheese

There has been interest in characterizing the extent and pattern of proteolysis in cheese, for several reasons: as an index of maturity and perhaps of quality and to quantify and characterize the contribution of each agent to proteolysis in cheese.

Most of the water- or pH 4.6-insoluble peptides (large peptides) are produced from α_{S1}-casein by chymosin or from β-casein by plasmin; they represent the C-terminal portions of the molecules, especially α_{S1}-CN (f24–199), α_{S1}-CN (f102–199), α_{S1}-CN (f33–199), β-CN (f29–209), β-CN (f106–209), and β-CN (f108–209). Many of the water-soluble peptides are produced by the lactococcal CEP from larger N-terminal peptides produced by chymosin or plasmin from α_{S1}- or β-casein. The reverse-phase high-performance liquid chromatography (RP-HPLC) profile of both the ethanol-soluble (short peptides) and ethanol-insoluble (intermediate-sized) fractions reflects the specificity of the lactococcal CEPs. Many of the peptides correspond to reported lactococcal CEP cleavage sites, but some are shortened by one or a few amino acids compared with those that would be expected based on known lactococcal CEP specificity, suggesting aminopeptidase activity. Some of the identified peptides are partly dephosphorylated, suggesting acid phosphatase activity (from milk or LAB).

Proteolysis in other low-cooked cheeses has not been studied extensively, but urea-polyacrylamide gel electrophoresis (PAGE) shows that primary proteolysis, by chymosin and plasmin, in Gouda and Edam and in the interior of surface mold- and surface smear-ripened cheese is generally similar to that in Cheddar.

In high-cooked cheeses, for example, Emmental and Parmigiano-Reggiano, the enzymes in the coagulant are completely or extensively denatured and primary proteolysis is mainly due to plasmin. Relatively limited proteolysis occurs in Emmental; a number of water-soluble and water-insoluble peptides have been identified. Considering the long ripening period (~2 years), relatively limited primary proteolysis occurs in Parmigiano-Reggiano, but secondary proteolysis, that is, production of very small peptides and amino acids, is extensive.

Catabolism of Amino Acids

Amino acids and some small peptides contribute to the savory background flavor of cheese and to its taste (some amino acids have sweet, sour, or bitter taste). However, a wide range of sapid and aromatic compounds are produced by the catabolism of amino acids, including amines, acids, carbonyls, ammonia, and sulfur compounds. Amines are particularly important for the characteristic flavor of smear-ripened cheese, while sulfur compounds, especially methanethiol (CH_3SH), are considered to be very important for the flavor of Cheddar.

The catabolism of amino acids has been an active area of research in recent years but much work remains to be done. Catabolism of most amino acids is initiated by the action of an aminotransferase, which transfers the amino group of the amino acid to an acceptor molecule (usually α-ketoglutarate) and produces an α-keto acid corresponding to the substrate amino acid. α-Keto acids are unstable and they degrade through enzyme-catalyzed or chemical reactions to hydroxyacids, aldehydes, carboxylic acids, alcohols, and other compounds. LAB (and probably other organisms) also have a number of amino acid lyases that act upon the amino acid side chain. The coryneform bacteria in the smear of surface-ripened cheese actively catabolize amino acids, but the enzymes involved have not been studied in detail. A C–S lyase capable of producing CH_3SH from methionine has been isolated from *Lactococcus*. It is likely that the catabolic activities of LAB will be the focus of research in the immediate future as it now appears that amino acid catabolism is a major pathway through which volatile flavor compounds are produced in cheese.

Proteolysis, amino acid catabolism, and related events in cheese are summarized in **Figure 3**. Certain strains of LAB and other bacteria may produce decarboxylases that act upon amino acids to produce amines. Amines from Tyr, His, and Trp ('biogenic amines') are the cause of a particular worry and at high levels can cause adverse physiological effects in susceptible consumers.

Outstanding Aspects

Considerable progress has been made in recent years toward elucidating the principal features of the biochemistry of cheese ripening, but the finer points remain to be described, especially the catabolism of amino acids.

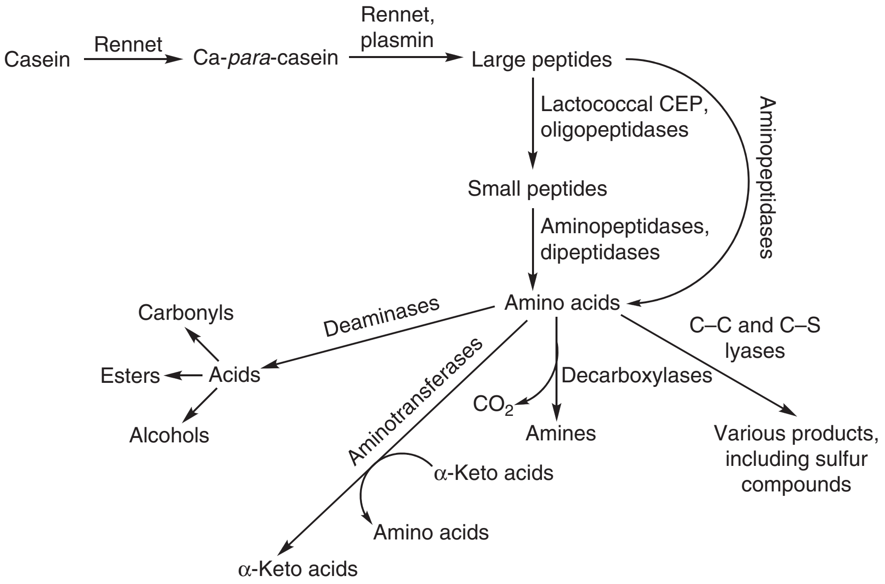

Figure 3 Summary of proteolysis and amino acid catabolism in cheese. CEP, cell envelope proteinase.

At present, many of the catabolic changes have been described only qualitatively and quantitative and kinetic data are required. The most important outstanding issue is unraveling the key reactions leading to cheese flavor; this has been a very intractable problem on which research dates from the beginning of the twentieth century. Numerous sapid and aromatic compounds have been isolated from cheese and identified, but the key compounds, their concentrations, and how they are produced have not been described precisely (*see* **Cheese**: Cheese Flavor).

Another key area requiring further research is the development of objective (chemical and/or physical) indices of cheese quality, which would replace the subjective sensory grading of cheese, as currently practiced.

See also: **Biogenic Amines**. **Cheese**: Accelerated Cheese Ripening; Acid- and Acid/Heat Coagulated Cheese; Blue Mold Cheese; Camembert, Brie, and Related Varieties; Cheese Flavor; Cheese Rheology; Enzyme-Modified Cheese; Non-Starter Lactic Acid Bacteria; Overview; Rennet-Induced Coagulation of Milk; Starter Cultures: General Aspects. **Enzymes Indigenous to Milk**: Plasmin System in Milk. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: *Lactobacillus casei* Group. **Pathogens in Milk**: *Clostridium* spp.

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Cheese Flavor

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Introduction

Many attempts have been made to define precisely what is meant by flavor. A general definition, validated by the British Standards Institute, describes flavor as the combination of taste and odor, influenced by sensations of pain, heat and cold, and touch. Therefore, the flavor of foods when eaten comprises volatile compounds sensed in the nose at the olfactory epithelium either via the orthonasal route (odor) or via the retronasal one (aroma), nonvolatile compounds sensed on the tongue (taste), and compounds perceived as mouthfeel and texture. Analyses of flavor have concentrated on the analysis of volatile components because of the major importance of aroma in the overall flavor, which is easily demonstrated by the difficulties encountered by subjects to identify a particular flavor if airflow through the nose is prevented. Moreover, the volatile components are more amenable to conventional instrumental analysis. Therefore, this article will be mainly devoted to the volatile flavor (aroma) compounds that contribute to cheese flavor. However, recently some significant efforts have been made to develop instrumental procedures to characterize nonvolatile components in cheese responsible for cheese taste. Part of this article will focus on advances made in the study of taste-active components present in the water-soluble fraction of cheese.

Sensory characteristics are an important determinant in the choice of foods by the consumer, and flavors play an important role in this context. Sensory analysis is the most direct and therefore the most valid means of measuring flavor characteristics. Applied to cheese, sensory evaluation is a descriptive tool used widely in the dairy industry and research. Some sensory information on aroma compounds can also be obtained from analyses in which sniffing the gas chromatographic eluate of the cheese volatile extract is used to identify the odorous compounds. This combined gas chromatography–olfactometry (GC–O) technique has been developed into a powerful analytical method to identify key aroma components. Cheese flavor components result mainly from lipolysis and proteolysis that occur during ripening, which makes these biochemical events important for the development of cheese flavor. The aroma compounds are mainly hydrophobic, or lipophilic, and as a consequence they tend to concentrate in cheese fat according to their water–fat partition coefficient. This is of considerable importance for their study, because, to be amenable to instrumental analyses suitable for volatile compounds, they have to be separated from the cheese fatty matrix by an extraction method.

Relationships between all the flavor compounds identified in a food and the sensory perception experienced by consumers when eating this food are still not entirely understood. It is particularly difficult to predict a flavor perception, as it is still not known how the various components combine to produce an overall sensory impression. Methods that allow direct analysis of flavor molecules released in the mouth during consumption have been developed in recent years.

The development of efficient analytical procedures and tools for the study of volatile flavor compounds has allowed research on off-flavors. For cheese, this research has covered all the steps from raw milk to the final cheese, including cheese manufacture, packaging, and storage.

Methodology for Accessing Cheese Flavor

Modern methodology used to study flavor compounds includes various steps, the ultimate aim of which is the identification of the compounds that are really relevant to the flavor. For instance, it is now well recognized that it is not necessary to identify the total volatile content of foods in order to understand the aroma. Not only the extraction and concentration procedures used prior to analysis by gas chromatography–mass spectrometry (GC–MS) may lead to the formation of artifacts, but the total volatile content in most cases is very difficult to be related to the flavor profile determined by a panel in sensory evaluation. While this determination can provide useful information in some circumstances, it appears much more efficient to concentrate the identification efforts on those compounds that make a significant contribution to the flavor. This is particularly true for taste components.

Obviously, the first step in studying cheese flavor should be sensory analysis of the cheese using descriptive tests, such as quantitative descriptive analysis (QDA).

This evaluation allows establishment of the flavor profile of the samples by measuring the sensory flavor descriptors, which might become pertinent targets for the subsequent instrumental analyses. The second step is the extraction procedure that allows separation of the relevant compounds from the cheese matrix. Many extraction procedures are available for cheese flavor studies; however, they do not give the same results in terms of quality and composition, which could be detrimental to the positive identification of key flavor compounds.

For aromas, the most reliable results will be obtained if the odor of the extract resembles closely that of the cheese submitted for extraction. Different sensory methods can be used to check the representativeness of the cheese extract odors. Here again, it is necessary to have a trained sensory panel able to perform this task. Once the best extraction method has been chosen, the next step involves analysis of the volatile compounds. The methods used to analyze volatile compounds include GC as the main tool, combined with various other techniques such as GC detectors (either universal, like the flame ionization detector (FID), or specific, like sulfur or nitrogen detectors), mass spectrometry and/or Fourier transform infrared (FTIR) spectrometry for identification purposes, two-dimensional GC (2D-GC) for chirality studies or coelution problems, and the so-called sniffing port that allows construction of aromagrams and performance of GC–O experiments. Various GC–O methods have been developed, the ultimate objective being the determination of those volatile compounds, the key odorants, that contribute significantly to the food flavor.

The next essential step in the strategy used for the analysis of food flavors is a precise quantification of all the key flavor components. For volatiles, two main routes may be used for this task. The first one combines a common standard addition method with increasing quantities of the compounds to be quantified, generally in conjunction with dynamic headspace sampling. The second route, the stable-isotope dilution assay, uses standard compounds, labeled with stable isotopes, similar to the unlabeled components to be quantified. These labeled internal standards, the physical and chemical properties of which are identical to those of the substances of interest, are added to the matrix prior to extraction, and the quantification is effected by measuring the ratio of the unlabeled to the labeled compound by mass spectrometry. The next step is the olfactory threshold determination for all the substances of interest, preferably in a matrix not very different from the starting material. For cheese, it could be advantageous to use a cheese model developed for this purpose, or a rather odorless cheese. As a final validation step where the components of interest are incorporated in amounts equivalent to their measured quantities and evaluated together in a matrix context is compulsory, these cheese models may serve as bases for this sensory validation analysis.

For nonvolatiles, the general procedure involves extraction of grated cheese by water, followed by a fractionation scheme generally adapted from the fractionation protocol used to isolate cheese nitrogen fractions in studies conducted on cheese proteolysis. The only requirement for the fractionation scheme is to use food-grade solvents when the fractions have to be sensorially evaluated.

As already outlined, poor correlations are often found between the overall levels of flavor compounds (volatile and nonvolatile) and the sensory perception experienced by the consumer. Perception of flavor is, in fact, a dynamic process. Sensory methods such as the time–intensity analysis have been used to study the time-related aspects of flavor perception. Techniques that measure flavor compounds directly in the mouth or in the nose have been developed to obtain data that reflect the pattern of flavor molecules released from food in real time. For sampling aroma from the nose (nose space), techniques based on mass spectrometry operated at atmospheric pressure (atmospheric pressure chemical ionization (APCI) or proton transfer reaction (PTR)) give robust results. Techniques to follow the *in vivo* release of nonvolatiles in the mouth have also been developed, but are essentially off-line techniques: saliva sampling at different times followed by direct liquid mass spectrometry or ionic liquid chromatography procedures.

Sampling

Flavor compounds in dairy products, like in other foodstuffs, are present in small amounts or even traces ($<10\,\mu g\,kg^{-1}$), and they are generally distributed in a heterogeneous manner. Their analysis in cheese requires grating the sample into very fine particles to maximize the surface area for subsequent extraction.

A common practice involves freezing the cheese in liquid nitrogen, removing the rind, and then grating the remainder into a fine powder in a blender at low temperature. An aliquot of this powder is then used for the subsequent steps. For some extraction procedures, the powder can be dispersed and homogenized in pure water. The resulting slurry or an aliquot of it can then be submitted to headspace extraction or vacuum distillation after pH adjustment, or fractionation, when required.

Extraction

All the extraction procedures used to isolate the volatiles from the cheese matrix should be adapted to trace analysis, minimizing losses and preventing modification or formation of artifacts.

Although steam distillation techniques, or the derived simultaneous steam distillation extraction (SDE) methods, are still used for dairy products, they present several problems. Highly volatile compounds may have very low recoveries; thermally sensitive components may decompose; and artifacts may appear unless distillations are carried out under reduced pressure. Working at a reduced pressure of ~100 Pa, an aqueous distillate may be obtained by heating the homogenized slurry just above room temperature, and adding a condenser cooled to ~5 °C above the aqueous distillate also maintained at that temperature; under these conditions, losses of highly volatile components may be limited. However, as it is necessary to add a large amount of pure water (~900 ml 100 g^{-1} cheese) to have a homogeneous suspension, the distillate obtained is in fact a dilute aqueous solution of the volatiles, which should be concentrated by liquid–liquid partitioning with a suitable solvent or by cryoconcentration. High-vacuum distillation techniques, on the contrary, produce small volumes of concentrated aqueous distillates (equivalent to cheese moisture content only). In a typical experiment (**Figure 1**), the grated frozen cheese is transferred to a cone-shaped flask connected to a vacuum statically maintained at ~10 Pa. The flask maintained at subambient temperature is rotated slowly from time to time to break up the continuously dehydrating surface of the sample. The volatiles distil with the cheese water and condense in traps held in liquid nitrogen. When enough water has evaporated, the flask can be rotated continuously to prevent complete amalgamation of the sample and the temperature can be raised to 35–40 °C. Alternatively, a gas stripping technique may be combined with high-vacuum distillation by purging the volatiles with an inert gas, such as nitrogen.

When the water of the sample is completely evaporated, a molecular distillation technique can be applied. In this technique, also called ‘cold-finger molecular distillation’, the remaining volatiles are transferred directly from the matrix to a cold (liquid nitrogen temperature) condenser maintained at a very short distance from the sample surface under a higher vacuum (~10^{-2} Pa). The trapped ice layer on the condenser contains a fraction of less volatile and more lipophilic compounds, which is claimed to be complementary to the distillate obtained in the first step of the high-vacuum procedure. A compact and versatile distillation unit, designated solvent-assisted flavor evaporation (SAFE), operated under a high vacuum (~5×10^{-3} Pa) was developed for fast isolation of volatiles from complex food matrices. It is claimed that higher yields with less artifacts are obtained with SAFE than with other distillation techniques, even for fatty matrices such as cheese.

All vacuum distillation methods need a substantial amount of cheese sample (~50–150 g) and are time-consuming (long distillation periods of 1 h to a few hours). Moreover, they all give rise to aqueous distillates that have to be extracted with a suitable solvent (e.g., dichloromethane) to be analyzed further. By adjusting the pH of the aqueous phase to a suitable value, it is possible to separate the organic extracts into acid, neutral, and basic fractions, which are more easily analyzed separately. Alternatively, cheese volatiles may be extracted directly from cheese samples by a solvent (e.g., diethyl ether). As organic solvents are also very good solvents for triglycerides, it is necessary to distil off the volatiles from the nonvolatile material under a high vacuum. The same applies for supercritical fluid extraction, as carbon dioxide used as solvent is also a very good solvent for apolar fats. Therefore, these direct extraction techniques do not present any advantage as compared to the other vacuum distillation methods.

Headspace methods, either static or dynamic, also denoted ‘purge and trap’, may also be used to study cheese volatiles. In the static method, the analyzed sample is the volume of air in equilibrium above the cheese, and this method should be restricted to the most volatile

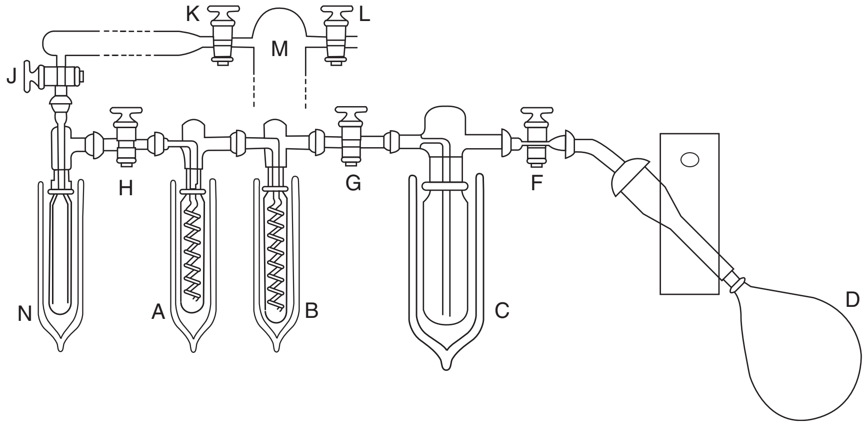

Figure 1 Apparatus used for high-vacuum distillation of grated frozen cheese. A, B, C, traps for volatiles cooled with liquid nitrogen; D, cone-shaped flask to receive grated cheese; F, G, H, J, K, L, high-vacuum stopcocks; M, connection to the high-vacuum pumping system; N, guard trap cooled with liquid nitrogen.

compounds. The dynamic method is more sensitive, and uses stripping of the cheese sample, sometimes dispersed in water, with an inert gas; concentration of the volatiles on a cold trap or onto an inert support; and subsequent thermal desorption or elution by a solvent. The main advantages of the headspace techniques are the small amount of sample necessary to perform the analysis (typically 20 g) and their rapidity. The main drawbacks are the relatively poor sensitivity as compared to other extraction methods, and the impossibility of testing the quality and representativeness of the extract. However, this technique is really suited for the study of the most volatile flavor compounds, as, no solvent being used, the most volatile compounds are not masked with the solvent peak on the gas chromatograms.

Solid-phase microextraction (SPME) techniques, first developed for the extraction of volatile organic compounds in water, have been applied to the extraction of flavor compounds with some success. SPME uses the partitioning of analytes between a solution or a vapor phase and a thin solid-phase surface made of adsorbing polymeric material coated on fused silica fibers, generally associated with a syringe, which serves directly as an injection device. It is an equilibrium technique that can be automated easily, but the extraction of the analytes depends on polarity, volatility, partition coefficients, volume of sample, temperature, and finally the nature of the fiber-coating material. While these parameters make the technique selective, it is, nonetheless, sensitive, easy to use, does not require solvent, and uses small sample volumes. As for headspace techniques, the sensory representativeness of the SPME extracts is not accessible.

For a long time, the water-soluble extract (WSE) of cheese has been reported to possess a strong flavor. Apart from some water-soluble volatile components responsible for aroma, the WSE of cheese contains mainly nonvolatile components that have been considered to be responsible for the taste of cheese. The study of taste-impact compounds in cheese, or more precisely in its water-soluble fraction, involves the study of soluble low-molecular-weight material (e.g., small peptides, amino acids, organic acids, minerals) dispersed in a very complex mixture. As it is necessary to assess the relative sensory impact of potential taste-active compounds, a fractionation scheme suitable for subsequent sensory evaluation is needed, and non-food-grade solvents or buffers must be rejected. Commonly used procedures involve extraction of grated cheese with water, possibly achieved by precipitation of caseins and large peptides at pH 4.6. The fractionation scheme that follows is generally adapted from the fractionation protocol used for isolating cheese nitrogen fractions for the study of proteolysis. The subsequent steps involve ultrafiltration using membranes with a 1, 3, or 10 kDa molecular weight cutoff or precipitation with 70% ethanol. The ultrafiltered water-soluble or 70% ethanol-soluble extracts are then subjected to gel filtration chromatography generally using pure water as the eluent. The fractions obtained by gel permeation chromatography may be evaluated sensorially after freeze-drying and redissolution in water, possibly with pH adjustment.

Gas Chromatography and Hyphenated Techniques

All the techniques used to analyze aroma extracts, whatever be the method performed to obtain them, are based on separation using high-resolution GC, combined with universal or selective detectors. A great variety of GC stationary phases are available, which provide a solution to almost all separation problems. Among the universal detectors, certainly the most popular is the FID, which combines the advantages of low cost, universality, sensitivity, and a wide linear range, making it an excellent candidate for both qualitative and quantitative determinations. Mass spectrometry is another powerful universal GC tool for both qualitative and quantitative analyses, and it adds the specific advantage of the identification of the separated compounds through their fragmentation pattern. The complementary use of libraries of thousands of reference spectra adds to the extreme usefulness of the GC–MS technique in aroma research, making it ubiquitous. Moreover, bench-top instruments with excellent performance are now available at a reasonable price. FTIR spectrometry is another identification technique that may be used combined with GC in aroma research. Despite its universal character, giving rise to the knowledge of the chemical functions present in the volatile molecules, GC–FTIR has the drawback of being less sensitive than GC–MS, with detection limits varying with chemical classes, and the molecular absorptivities depending on the chemical functions present in the molecules. Nevertheless, GC–FTIR must be considered as an excellent complement to GC–MS for identification purposes.

Among the selective detectors used with GC, the nitrogen–phosphorus detector (NPD), when operated in the nitrogen mode, is very selective for nitrogen-containing compounds and operates in a way similar to the FID. In order to detect sulfur-containing components selectively, the flame photometric detector (FPD) may be used. Although reliable and sensitive, it has several drawbacks, including its nonlinear response to the concentration of sulfur species. The sulfur chemiluminescence detector (SCD) is an interesting alternative, at an additional cost, however. Nevertheless, it is highly sensitive and selective, and has a large dynamic range with a linear response to the concentration of sulfur components. The atomic emission detector (AED) appears as the best choice, although at a further additional cost. For the detection of sulfur compounds, the AED presents a highly selective extended linear dynamic range with lower and upper limits not attainable with the other sulfur detectors. Moreover, the AED may be adjusted to detect any other element, making it a universal detector.

Another selective detector has to be cited here: the sniffing port, where the human nose is used, the selectivity being now based only on the odorous properties of the individual volatile compounds separated by the GC column. This GC detection mode represents an interesting interface to sensory analysis, as odor descriptors of odor-active compounds considered as key flavor components are generated and can be compared to descriptors generated by a sensory panel. Quantitative approaches based on odor detection thresholds or on odor intensity have also been developed (GC–O). Although interesting for tracing individual odor-active volatiles that can be considered as key flavor compounds to a certain extent, the method is based on sensory evaluation of individual compounds separated in a GC experiment and sniffed sequentially at the GC sniffing port. This method is not equivalent to sensory evaluation of cheese itself, where the flavor is evaluated in a mixture context, where interaction with the cheese matrix occurs, and where masking or enhancing effects may influence the overall sensory perception. Therefore, a validation step where the key odorants determined via GC–O are incorporated in amounts equivalent to their measured quantities and evaluated together in a mixture and matrix context is essential.

Dynamic Methods

Perception of flavor is a dynamic process. During the consumption of food, the concentration of aroma compounds at the olfactory epithelium and of sapid compounds at the taste buds on the tongue varies with time as flavor components are released progressively from food during chewing. Sensory methods such as the time–intensity analysis may be used to address the time-related aspects of perception. Mass spectrometers equipped with atmospheric pressure sources such as APCI or PTR devices have been used to monitor aroma release during chewing. Air from the nose (nose space) is sampled directly into the atmospheric pressure device through an interface, making real-time breath-by-breath analysis possible. By combining time–intensity sensory studies with nose space analysis, it is thus possible to relate the temporal parameters of aroma release to perception. It has been recently demonstrated for some French soft cheeses that simultaneous time–intensity scoring and nose space analysis of a 'sulfury' note (one of the four main sensory descriptors revealed by a trained panel) allowed a perfect superimposition of the time–intensity curve with the release curves of the main sulfur compounds (*S*-methyl thioacetate, dimethyldisulfide, and dimethylsulfide) obtained with nose space analysis. However it should be noticed that interindividual differences in terms of physiological factors (chewing force and rate, saliva flow) may present huge discrepancies.

Only a limited number of studies have been devoted to the release of nonvolatile compounds in the mouth. Saliva sampling using cotton buds coupled to liquid mass spectrometry and/or ion chromatography seems to produce the most robust results. Thus course of release versus time curves for minerals, amino acids, organic acids, and phosphoric acids can be obtained and compared to sensory measurements. For *in vivo* nonvolatile release curves also, individual physiological parameters (mastication behavior and salivation rate) play an important role.

Flavor release and perception are dynamic processes and must be studied using dynamic methods. Dynamic physicochemical methods have been developed to study the parameters of flavor release from eating foods. Together with an increase in the applications of dynamic sensory methods, they provide a better understanding of food flavor, with important results obtained for cheese. However, further work, including the study of various interactions arising at different levels in the process of food consumption, is needed to improve our knowledge of flavor perception.

Global Analyses

The methods currently used to evaluate and control the quality of food flavor are still based on sensory evaluation by a panel of experts. These panels are able to handle quality monitoring through descriptive tests, to detect defects (off-flavors), and to compare samples for classification purposes (e.g., to ascertain conformity to a standard). It has long been recognized that it would be interesting for such a task to substitute the use of humans by instruments, which could give quicker answers at lower cost. Evaluation of the odor intensity of the volatiles emitted from food using gas sensors is now theoretically feasible. A number of commercial instruments, including the so-called electronic nose, are now available. They are all developed around arrays of nonspecific gas sensors (based on different physical principles such as conductivity of metal oxides or organic polymers), giving rise to a response with a typical pattern. After data acquisition, the final step of data processing uses pattern recognition algorithms with either standard statistics or artificial neural network technology. Despite partial success in some classification tasks, none of the commercial instruments meets the food industry requirements for quality control in terms of precision, reproducibility, sensitivity, and stability. Moreover, these instruments can be hardly used to identify single odorants or to differentiate samples differing only on subtle distinctive notes. Therefore, in their present state, they are recommended neither for off-flavor studies, where identification is a prerequisite to any corrective action to be carried out, nor for quality control assessment.

For classification purposes, two other global analytical methods based on mass spectrometry seem more powerful and reliable. The first consists of a global analysis of a total headspace sample by a mass spectrometer, without any prior GC separation. This method is often described as an extension of the electronic nose. The mass patterns obtained need extensive data treatment, either by conventional statistics or by artificial neural networks. The other method is the pyrolysis mass spectrometry technique, where a tiny sample is pyrolyzed up to 500 °C and the resulting volatile fraction, characteristic of the flavor but also of the matrix, is analyzed by a mass spectrometer. Here again, a mass pattern, or fingerprint, is obtained for each sample and extensive data handling is necessary.

Flavor Compounds

Aroma

The numerous compounds involved in cheese aroma are derived mainly from three major metabolic pathways occurring during cheese ripening: (1) catabolism of lactose, lactate, and citrate, (2) lipid catabolism (**Figure 2**), and (3) protein catabolism (**Figure 3**). The agents of these metabolisms are the endogenous enzymes of milk, clotting enzymes, and enzymes from the microorganisms used in cheese manufacture and ripening. The molecules derived from these metabolic processes are principally fatty acids, ketones, alcohols, lactones, esters, aldehydes, sulfur compounds, amines, and pyrazines. This array of compounds, as a result of their presence or absence, concentration, and proportions, are often characteristic of particular cheese types.

Fatty Acids

Fatty acids are important for the aroma of cheese. Not only are they aromatic compounds by themselves, but they are also precursors of methyl ketones, alcohols, lactones, and esters (**Figure 2**). Milk fat contains 98% of glycerol esters, neutral lipids composed of a wide variety of fatty acids. The free fatty acids (FFAs) in raw milk cheese come from the hydrolysis of glycerol esters by milk's indigenous lipase. Fat hydrolysis, mainly due to mold activity, is particularly important in soft cheeses. Some carboxylic acids, having generally between 2 and 6 carbon atoms, are produced from the degradation of lactose and amino acids. Long-chain FFAs (>12 carbon atoms) play a minor role in flavor, owing to their high perception thresholds. Short- and medium-chain, even-numbered FFAs (4–12 carbons) have much lower

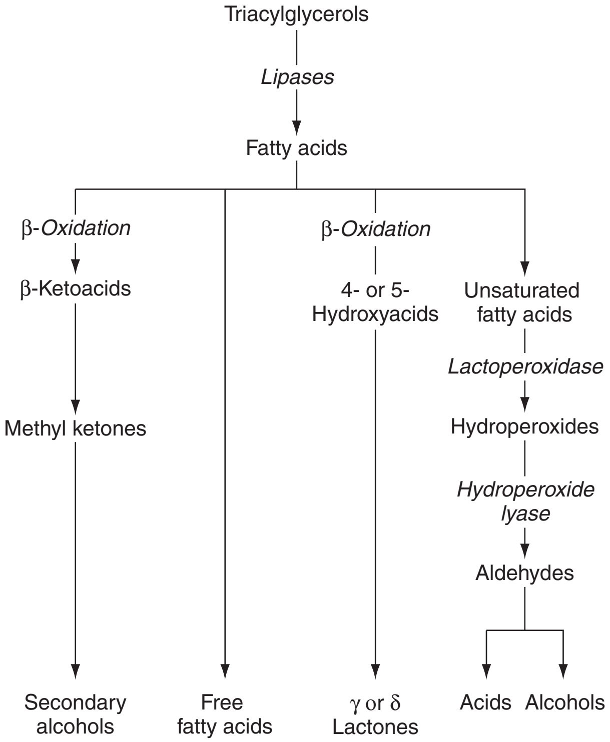
Triacylglycerols
Lipases
Fatty acids
β-*Oxidation*
β-Ketoacids
Methyl ketones
Secondary alcohols
Free fatty acids
β-*Oxidation*
4- or 5- Hydroxyacids
γ or δ Lactones
Unsaturated fatty acids
Lactoperoxidase
Hydroperoxides
Hydroperoxide lyase
Aldehydes
Acids
Alcohols

Figure 2 Formation of flavor compounds from lipids. Reproduced with permission from Molimard P and Spinnler HE (1996) Review: Compounds involved in the flavor of surface mold-ripened cheeses: Origins and properties. *Journal of Dairy Science* 79: 169–184.

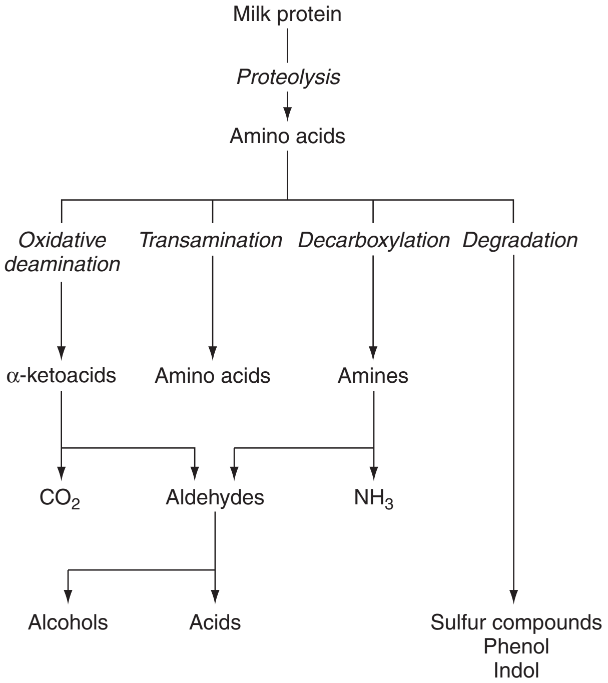
Milk protein
Proteolysis
Amino acids
Oxidative deamination
Transamination
Decarboxylation
Degradation
α-ketoacids
Amino acids
Amines
CO$_2$
Aldehydes
NH$_3$
Alcohols
Acids
Sulfur compounds
Phenol
Indol

Figure 3 Microbiological catabolism of amino acids during cheese ripening. Reproduced with permission from Molimard and Spinnler (1996) Review: Compounds involved in the flavor of surface mold-ripened cheeses: Origins and properties. *Journal of Dairy Science* 79: 169–184.

perception thresholds, in the ppm (i.e., mg kg^{-1}) range. Octanoic, 4-methyloctanoic, and, especially, 4-ethyloctanoic acids have goaty notes. The latter have a very low detection threshold (0.001 8 mg kg^{-1} in water), which certainly makes it important in goat's milk cheese. According to their concentration and perception threshold, volatile FFAs can contribute to the aroma of the cheese or to a rancid defect.

Ketones

The homologous series of odd-chain alkan-2-ones, from C_3 to C_{15}, constitutes one of the most important aroma fractions of surface mold-ripened cheeses, where the major component is nonan-2-one, and of blue cheeses, where heptan-2-one is the major component. Branched-chain (such as 3-methylpentan-2-one and 4-methylpentan-2-one) and unsaturated (such as nonen-2-one and undecen-2-one) ketones are also found in cheese. The amounts of nonan-2-one, heptan-2-one, and undecan-2-one increase steadily during ripening. Methyl ketones with even-numbered chains, except for butan-2-one, appear later during ripening and are never present in large amounts, except in very ripe cheeses. This is also true for unsaturated methyl ketones. Diacetyl (butan-2,3-dione) and acetoin (3-hydroxy-butan-2-one) are formed from citrate present in milk. Their production is mainly due to the activity of lactic acid bacteria.

The perception thresholds of the ketones found in cheeses are in the ppm (mg kg^{-1}) range in water, except for oct-1-en-3-one, which is detected in air at a concentration of 0.005 μg kg^{-1}. They generally give off floral or fruity odor notes, except diacetyl, heptan-2-one, and oct-1-en-3-one, which emit the typical buttery, Blue-cheese, and mushroom odors respectively.

Alcohols

Many metabolic pathways are involved in the synthesis of the alcohols found in cheese. Lactose metabolism leads to ethanol formation by the pentose phosphate pathway and by the mixed acid pathway; the mixed acid pathway also leads to butane-2,3-diol. Diacetyl and acetoin can also be transformed rapidly to butane-2,3-diol by the action of butan-2,3-dehydrogenase. Methanol can also form from the reduction of acetaldehyde by an alcohol dehydrogenase. Some alcohols are produced either via the Erhlich's amino acid metabolic pathway or from the reduction of aldehydes. Primary and secondary alcohols, along with ketones, are considered to be the most important compounds in the aroma of soft and mold-ripened cheeses. Of the primary alcohols, 3-methylbutan-1-ol is present in relatively large quantities in cheeses, and gives an alcoholic, floral note. The principal secondary alcohols are heptan-2-ol and nonan-2-ol, which represent, like the methyl ketones from which they are derived via a reductase activity, up to 20–30% of all aroma compounds in Camembert-type cheese. Phenyl-2-ethanol, with a perception threshold of 9.1 mg kg^{-1} in a cheese base and a characteristic rose floral note, and its ester, phenylethylacetate, play an important role in raw milk mold-ripened cheeses, where they are always present in substantial amounts. In fact, phenylethanol and its esters have cumulative effects that give the perceptible floral note in certain Camembert cheeses. This alcohol is produced mainly during the first week of ripening, because it is a metabolic product produced from phenylalanine by yeast.

Linoleic and linolenic acids are precursors of 8-carbon aroma compounds, particularly oct-1-en-3-ol, oct-2-en-1-ol, octa-1,5-dien-3-ol, and octa-1,5-dien-1-ol. Oct-1-en-3-ol is well known for its raw mushroom odor. Its low perception threshold, 0.01 mg kg^{-1}, gives a characteristic note to mold-ripened cheese aroma. This compound, together with its ketone equivalent described above, is a key compound in the global aromatic note of Camembert cheese. Because they are produced by *Penicillium camemberti* metabolism, they appear only late in the cheese-ripening process.

Lactones

The lactones found in cheese are principally γ-decalactone, δ-decalactone, γ-dodecalactone, and δ-dodecalactone. Lactones are generally characterized by very pronounced fruity notes (peach, apricot, and coconut). δ-Lactones generally have a higher detection threshold than those of γ-lactones. These thresholds are relatively low for γ-octalactones, γ-decalactones, and γ-dodecalactones (7–11 μg kg^{-1} in water) and are still lower for shorter-chain lactones.

The precursors of lactones are hydroxylated fatty acids. Cyclization of the hydroxyacids occurs by the influence of pH and/or the action of microorganisms. The action of microorganisms on lactone production in cheese has not been elucidated clearly. Hydroxyacids can be present as triacylglycerols in milk. Lipases can liberate them, and then they are cyclized. Nevertheless, hydroxylated fatty acids can come from the normal catabolism of fatty acids and can be generated from long-chain unsaturated fatty acids (18:1 and 18:2) by the action of lipoxygenases or hydratases.

Esters

There is a great diversity of esters in cheeses. Esterification occurs either directly between alcohols derived from lactose (ethanol) fermentation or amino acid catabolism and short- to medium-chain fatty acids, or via a transesterification reaction between ethanol and a partial glyceride. These reactions are well known as media detoxification reactions, enabling the elimination of toxic alcohols and fatty acids. The most common of esters are the acetates formed from acetyl-CoA and an alcohol. A wide variety of enzymes may be involved in esterification reactions, such as carboxylesterases, having a very wide range of substrates, and arylesterases present in most of the microorganisms that contribute to cheese ripening. Esters appear during the early stage of ripening, and the microorganisms involved in their formation seem to be mainly yeasts. Most of the esters encountered in cheese are described as having fruity odors, except for those bearing a phenyl group, such as 2-phenylethyl acetate or 2-phenylethyl butyrate, which have floral notes. Some of these esters have a very low perception threshold. Thus, isoamyl acetate is detected in water at a concentration of $2\,\mu g\,kg^{-1}$. Low carbon number esters have a perception threshold $\sim$10 times lower than that of the corresponding alcohols.

Aldehydes

Straight-chain aldehydes are generally produced via the catabolism of fatty acids (**Figure 2**). Other aldehydes, mainly branched, originate from amino acids by transamination, leading to an imide that can be decarboxylated. This reaction is simple and can occur without enzymatic catalysis during ripening. Aldehydes are transitory compounds in cheese because they are transformed rapidly to alcohols or to the corresponding acids. Acetaldehyde can be derived from threonine with the help of a threonine aldolase. This reaction occurs in lactic acid bacteria and ends with the cleavage of threonine to form acetaldehyde and glycine. Some lactic acid bacteria can also produce acetaldehyde from glucose. Yeasts also contribute to acetaldehyde production when alcohol dehydrogenase is less active than pyruvate decarboxylase. The benzaldehyde biosynthesis pathway is still not very well known. It could come from an α-oxidation of phenylacetaldehyde or from a β-oxidation of cinnamic acid. The main aldehydes in cheese are hexanal, heptanal, nonanal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and benzaldehyde. Hexanal and (*E*)-hex-2-enal give the green note of immature fruit. Their perception thresholds in water are, respectively, 9.18 and $24.2\,\mu g\,kg^{-1}$. Octanal, nonanal, decanal, and dodecanal are described as having an aromatic note resembling orange with perception thresholds of 1.41, 2.53, 1.97, and $0.53\,\mu g\,kg^{-1}$, respectively, in water. Benzaldehyde is described as having an aromatic note of bitter almond and a detection threshold of $350\,\mu g\,kg^{-1}$ in water.

Sulfur Compounds

It has been shown that the sulfur flavors in ripened cheese comprise a structurally diverse class of molecules that provide a whole range of characteristic aromatic notes (e.g., cheesy and garlic) in a particular cheese as evident from the analysis of Cheddar, Limburger, Camembert, Blue, and other mold-ripened varieties. In addition, the sensory properties of these sulfur compounds are pronounced at very low concentrations due to their low odor thresholds.

The origin of many sulfur flavors in cheese is associated with the production of methanethiol by the bacterial cultures used in the preparation of cheese. Numerous bacteria such as lactobacilli, lactococci, and *Brevibacterium linens* produce significant quantities of this compound. The direct metabolic pathway responsible for the generation of methanethiol involves the bacterial degradation of methionine. Significant efforts have been made to isolate and characterize methionine-γ-demethiolase, which is the intracellular enzyme responsible for this bioconversion. Other bacterial enzymes involved in methionine metabolism include cystathionine-γ-lyase and cystathionine-β-lyase, which are also implicated in the production of methanethiol; however, their role in the development of cheese flavor remains tentative at present. Further bacterial metabolism of methanethiol leads to the generation of a range of sulfur compounds that contribute significantly to the aroma of cheese, including dimethyldisulfide and dimethyltrisulfide, and some thioesters such as *S*-methylthioacetate and *S*-methylthiobutyrate.

Amines

Numerous volatile amines have been identified in dairy products: methylamine, ethylamine, *N*-propylamine, isopropylamine, *N*-butylamine, 1-methylpropylamine, *N*-amylamine, isoamylamine, anteiso-amylamine, *N*-hexylamine, ethanolamine, dimethylamine, diethylamine, dipropylamine, and dibutylamine. For example, dimethylamine is present in Camembert and Blue cheeses at a concentration of up to $1.6\,mg\,kg^{-1}$. Nitrosamines have also been found in Camembert cheese at a concentration of $25\,nmol\,10\,g^{-1}$, but not in Blue cheeses. It should be remembered that ammonia, derived from deamination of amino acids, is also an important element of cheese aroma. Nonvolatile amines have also been identified and quantified in dairy products: tyramine, histamine, tryptamine, and putrescine. Numerous volatile amines are described as imparting fruity, alcoholic, and varnish flavor notes. Ethylamine and butylamine have basic perception thresholds ranging from 0.83 to 3.63 and from 0.24 to $13.9\,mg\,kg^{-1}$, respectively, in water. Methylamine, dimethylamine, and propylamine have perception thresholds of 182, 34.4, and $62.4\,mg\,kg^{-1}$, respectively, in water.

Tertiary amines have much lower perception thresholds. Triethylamine, with a fishy odor, is perceptible at a concentration of 0.47 $\mu g\,kg^{-1}$ in water. Amines are produced by amino acid decarboxylation, which leads to the production of carbon dioxide and free amines. This reaction needs the presence of pyridoxal-phosphate as a coenzyme. Leucine thus gives isobutylamine, phenylalanine gives phenylethylamine, and tyrosine gives tyramine. A low oxygen pressure favors these reactions. However, amines are not the final products; they can undergo oxidative deamination, resulting in the formation of aldehydes. They can also be the precursors for compounds like *N*-isobutylacetamide found in various types of ripened cheeses, presumably by reaction with acetic acid.

Taste

It has been recognized for a long time that bitterness, which can limit cheese acceptability if too intense, is due to an excessive concentration of low-molecular-weight, mainly hydrophobic peptides, which accumulate during ripening as a result of proteolysis. Apart from bitterness, no clear sensory properties have been attributed to any specific nitrogen-containing compounds (small peptides and amino acids), although it is likely that they contribute to the background flavor of cheese. Among the mineral salts, NaCl is responsible for the salty taste, the other salts being essentially bitter rather than salty. Acid taste is caused by H_3O^+. It has also been hypothesized that short- and medium-chain fatty acids might contribute to the acid taste of cheese. Although this assumption seems reasonable for the abundant short-chain acids (e.g., formic, acetic, or propionic), their principal contribution to cheese flavor is to its aroma in their unionized form RCOOH. In Comté cheese, the umami taste was clearly associated with a substantial amount of monosodium glutamate, which was found at a concentration 10 times above its threshold value. For Emmental cheese, acetic, propionic, lactic, succinic, and glutamic acids have been found to be important taste contributors together with sodium, potassium, calcium, magnesium, ammonium, phosphate, and chloride ions. A study on Camembert cheese led to the conclusion that the important taste contributors are acetic, butyric, 3-methylbutyric, caprylic, and succinic acids, monosodium glutamate, ammonia, and NaCl. It was also found that the biogenic amine, cadaverine, and the rare amino acids ornithine and citrulline, when present, contribute to the bitter taste of Camembert; bitter taste is, however, essentially due to small ($MW < 1000$ Da) peptides accumulated during ripening.

Conclusion

Cheese is a biochemically active dynamic product that undergoes many changes during ripening. Cheese flavor development is one of the consequences of these chemical changes occurring during this period. Flavor compounds are produced through the principal biochemical degradation pathways: glycolysis, lipolysis, and proteolysis. Depending on the variety, technology, microflora, and ripening conditions, flavor compounds are produced to give unique sensory characteristics to each cheese variety. Though a background flavor seems common to all cheese varieties, as a result of basic biochemical transformations, the characteristic aroma of most cheeses results from the subtle combination of a large number of odorous volatile compounds present in the correct concentration ratios. In this context, off-flavors may possibly result from the breaking of the fragile equilibrium that constitutes the right flavor balance.

Among the numerous compounds present in the volatile fraction of cheese, besides FFAs, methyl ketones, alcohols, and esters are quantitatively the most important. These components, along with sulfur compounds, are important for the aroma notes of cheese, but the aromatic importance of most of the molecules present in cheese in trace amounts is only poorly understood. Modern methods allow for detailed analyses of volatile and nonvolatile compounds, and some pertinent sensory information can be obtained by combining GC with olfactometry. Considerable progress has been made in recent years in understanding the flavor of foods. Nevertheless, the relationships between the flavor compounds present in a particular food and the sensory perception of that food by a consumer are not so easy to establish. Analyzing the total flavor composition of food does not reflect the sensory profile experienced when eating that food, because it is still not well understood how the various flavor-active components combine to produce a particular sensory perception within a food matrix. In order to further advance cheese flavor research, it is imperative to investigate the interaction of flavor ingredients with the food matrix, the flavor of mixtures of individual flavor components, and flavor release within the mouth when masticating, with an emphasis on sensory perception.

See also: **Analytical Methods**: Electrochemical Analysis; Sensory Evaluation. **Flavors and Off-Flavors in Dairy Foods**.

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Cheese Rheology

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Introduction

The rheology of cheese may be defined simply as the study of its deformation and flow when subjected to stresses and strains, where stress may be elementarily defined as the force applied per unit surface area of the material, and strain as the fractional displacement of the material that occurs under the applied stress. The measurement of rheology involves deforming or straining a sample of material, for example cheese, by applying a force or stress in some sense, for example by compression, shear, extension, or pressure. The displacement in response to the force at the point of application is known as deformation. The term deformation implies a change in shape (i.e., form) of the cheese that may be temporary, permanent, or partly recoverable. A series of instantaneous measurements of force and associated displacement describe the rheological characteristics of the cheese under the measurement conditions. Conditions that affect the force (stress)–displacement (strain) response include temperature, type of stress applied, level of deformation in relation to elastic limit and fracture point of the material, rate of deformation, and previous history of deformation. The output variables from these tests (e.g., deformation curve, creep compliance curve, stress relaxation), which may include change in dimensions over time, ratio of stress to strain for certain strain levels, and stress or strain required to induce fracture, enable the determination of the rheological properties of the material such as elastic modulus, shear modulus, fracture stress, fracture strain, and firmness. For cheese, these properties are a function of

- composition (e.g., moisture, fat, and protein contents);
- microstructure, which represents the spatial distribution of its compositional components and the level of intra- and intermolecular attractions between the components;
- macrostructure, which represents the arrangement of, and attractions between, the different macrocomponents (e.g., curd particles, gas pockets, veins, and/or rind) and determines the presence of heterogeneities such as curd granule junctions, cracks, and fissures; and
- the physicochemical state of its components (e.g., ratio of solid-to-liquid fat as affected by temperature; degree of aggregation and hydration of the protein matrix).

The rheological properties are of interest to the manufacturer, prepacker, distributor, retailer, industrial user, and consumer, since they influence several properties of cheese:

- texture and eating quality, as they determine the level of mastication achieved for a given level of chewing, which in turn may influence the flavor/aroma properties of the cheese and its suitability for different consumer groups (e.g., children, elderly persons);
- physical behavior (e.g., tendency to fracture, crumble, bend and be flexible) when subjected to different size-reduction methods (e.g., grating) or blending/shearing operations such as those in the preparation of pasteurized processed cheese products, sauces, and coextruded products with cheese;
- ability to retain a given shape when stacked; and
- gas retention and formation of eyes or cracks.

The rheological behavior of cheese on cooking involves changes in fat liquefaction and coalescence, and protein solvation (*see* **Cheese**: Cheese as a Food Ingredient)

Definitions

Rheologically, cheese is classified as a viscoelastic material, that is, its stress–strain relationship exhibits of characteristics both solids and liquids. Hence, defining cheese rheology requires an examination of the two forms of ideal rheological behavior, that is, elastic behavior and viscous behavior.

Ideal Elastic Solid

Application of a force to a solid material generally results in deformation, which is manifested as a change in sample shape and dimensions. Stress, denoted σ or τ, is the force applied per unit surface area of the material. It may be applied to the surface in a normal direction (σ), resulting in compression (e.g., reduction in the sample height) or extension (e.g., increase in the sample height) (**Figure 1**(**a**)). Alternatively, the stress may be applied tangentially to the surface (τ), causing adjoining layers of the material to slide relative to each other in a direction parallel to their plane of contact (**Figure 1**(**b**)). Deformation is the displacement relative to a fixed plane

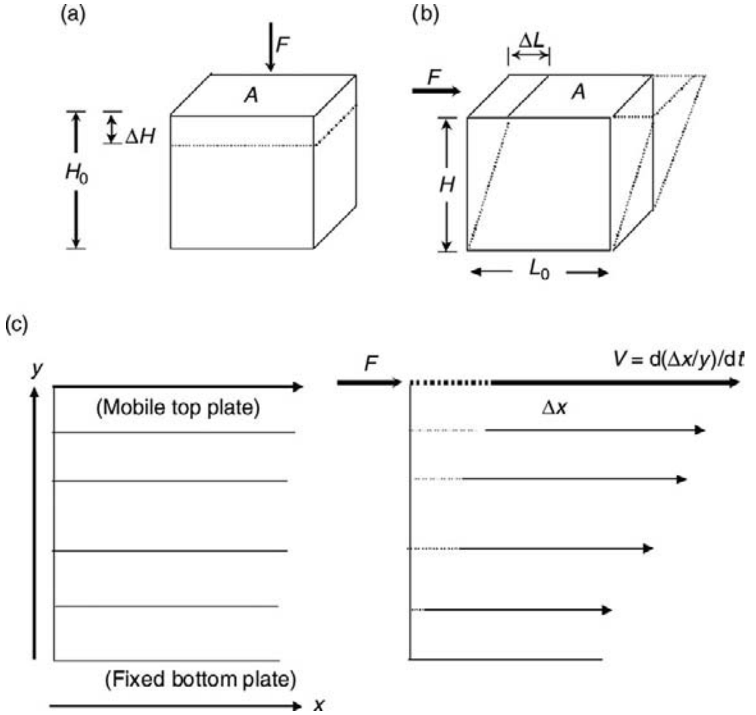

Figure 1 Application of stress (i.e., force, *F*, per unit surface area, *A*) to a solid (a, b) or liquid (c) material. For the solid material, the stress may be applied (a) in the direction normal to the surface resulting in compression deformation (Δ*H*) or (b) tangential to the surface resulting in shear deformation (Δ*L*). For the liquid material, confined between two parallel plates (a fixed bottom plate and a mobile top plate) separated by a distance *y*, application of a shear force to the top plate results in movement in the direction *x* at a velocity *V*.

in the material as stress is applied, and strain is the displacement as a fraction of a particular dimension (e.g., height). On application of σ, the resultant strain (denoted by ε) is defined as $\Delta H/H_0$, where H_0 corresponds to the original height of the sample and ΔH to displacement. On application of τ, the ensuing strain or shear (denoted by γ) is defined as the distance (ΔL) through which the point of application moves divided by the distance (H) between the moving and stationary planes of the sample (**Figure 1(b)**).

A material is described as an ideal elastic or Hookean solid if the relationship between σ and ε, or τ and γ is linear, with the σ versus ε curve passing through the origin (**Figure 2(a)**). Two types of moduli (i.e., proportionality constant between σ and ε) are obtained for an ideal solid based on the method of stress application:

1. modulus of elasticity or Young's modulus (E), where the stress is normal to the stress-bearing area, $\sigma = E\varepsilon$, and $\varepsilon = \Delta H/H$;
2. elastic shear (or storage) modulus, where the stress is tangential to a fixed plane, $\tau = G\gamma$, and $\gamma = \Delta L/H$.

The moduli E and G for an ideal solid are independent of time and the rate at which the stress is applied; hence, the stress–strain curve is always linear.

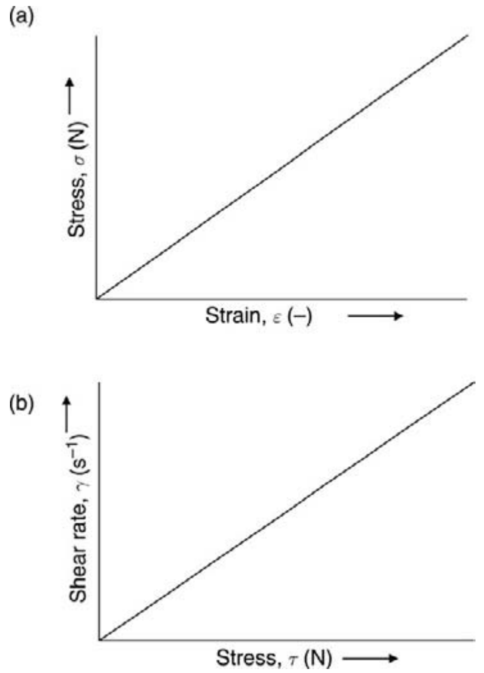

Figure 2 Relationships between stress, δ, and strain, ε, for an elastic solid (a) and between shear stress, τ, and strain rate, $\dot{\gamma}$, for an ideal liquid (b).

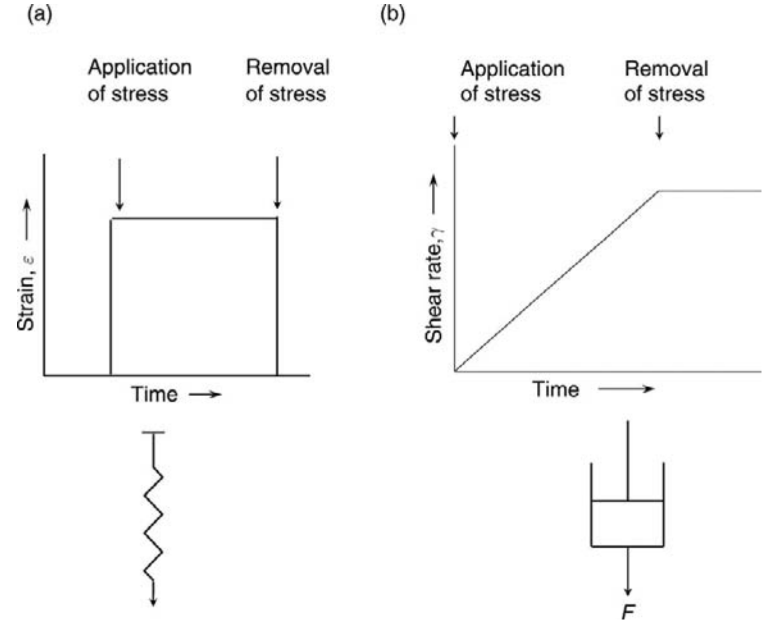

Figure 3 Creep–recovery curves showing time-related changes in the strain of an ideal elastic solid (a) and in the shear (strain) rate of an ideal liquid (b) on application or removal of a constant stress. The rheological behavior of the ideal elastic solid is mechanically represented by a single spring and that of the ideal liquid by a dashpot.

A plot known as creep–recovery curve shows the variation of ε with time on instantaneous application of a constant σ to a solid and its removal some time later (**Figure 3(a)**). An elastic solid deforms instantly on application of σ and recovers instantly to its original shape and dimensions on removal of σ. During the application of σ, the stress energy is absorbed and stored by the structural elements of the material and no breakage of the bonds between the structural elements occurs. On removal of σ, the stored energy is immediately released and enables the material to counteract the deformation and regain its original dimensions instantly. This rheological behavior may be represented mechanically by the simple spring (**Figure 3(a)**), where the degree of extension (i.e., ε) is directly proportional to the weight (and hence, σ) hanging from the spring. At low strains, some cheeses (e.g., a young – medium-aged low-moisture part-skim Mozzarella cheese) exhibit simple linear relationships between σ and ε, which can be expressed in terms of the above moduli.

Ideal Viscous Liquid

In contrast to an ideal solid, a liquid does not support a permanent stress; the strain changes constantly as long as the stress is maintained. On application of shear stress, τ, to a liquid confined between two parallel plates (upper floating plate and lower stationary plate) separated by a distance y (**Figure 1(c)**), the top plate moves a distance (Δx) in the x-direction at a velocity (v), given by $v = \Delta x/y$. Assuming that the liquid remains in contact with each plate by surface tension, the rate of change of strain (denoted shear rate and abbreviated $\dot{\gamma}$ is thus given by the differential change in v with position, since $\dot{\gamma} = \mathrm{d}(\Delta x/y)/\mathrm{d}t = \mathrm{d}v/\mathrm{d}t$. A material is defined as an ideal or Newtonian liquid if $\dot{\gamma}$ is directly proportional to τ, with the τ versus $\dot{\gamma}$ curve passing through the origin (**Figure 2(b)**). The proportionality constant between τ and $\dot{\gamma}$ is known as the coefficient of viscosity or kinematic viscosity, η, where $\eta = \tau/\dot{\gamma}$.

A creep curve for a Newtonian fluid shows that it starts to flow instantly at a constant $\dot{\gamma}$ on application of a constant τ, and immediately ceases flow (but is permanently deformed) on its removal (**Figure 2(b)**). Unlike a solid material, the energy due to stress is not stored but is dissipated (due to breakage of the interactions between the structural elements of the liquid) in the form of flow, with the strain being proportional to the time over which the stress is applied. This rheological behavior may be represented mechanically by a dashpot (i.e., a piston enclosed in a cylinder filled with a viscous liquid) (**Figure 3(b)**), where the rate of movement of the piston ($\dot{\gamma}$) is directly proportional to the applied τ.

Rheological Concepts Applied to Cheese

Cheese Microstructure

Microstructurally, cheese consists of a three-dimensional particulate gel matrix of calcium phosphate–*para*-casein (rennet curd cheeses) or calcium phosphate–casein

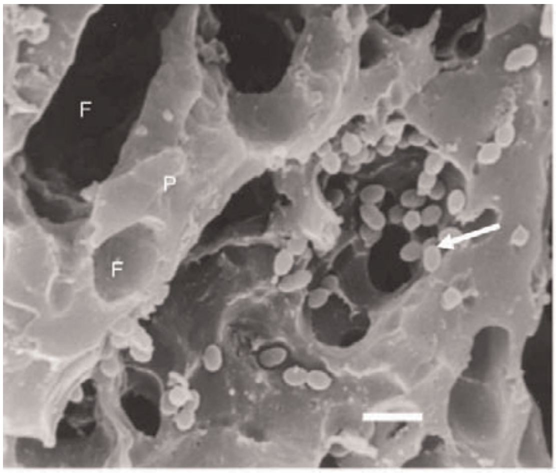

Figure 4 Scanning electron micrographs of full-fat (33.0%, w/w) Cheddar cheese showing a network of fused protein (*para*-casein micelles, gray areas marked **P**) enclosing fat (black and indented areas marked **F**), bacteria (arrow), and free serum (cannot be seen; embedded in the protein). Bar = 2 μm.

(acid-curd cheeses), which occludes fat globules, moisture, other substances (e.g., minerals, peptides, and enzymes), and starter culture bacteria within its pores (**Figure 4**). The matrix is composed of interconnected and overlapped chains of fused casein or *para*-casein aggregates. The integrity of the matrix is maintained by interprotein linkages mediated by calcium (attached to acidic amino acid residues such as glutamate and aspartate), colloidal calcium phosphate (attached to calcified serine phosphate groups), other electrostatic interactions between charged groups, and hydrophobic interactions between uncharged amino acid residues. The matrix is essentially continuous although some discontinuities exist at the micro- and macrostructural levels. Discontinuities at the microstructural level are due to differences in the distributions of structural components and bacteria (**Figure 4**), while at the macrostructural level they exist in the form of curd granule junctions and curd chip junctions in Cheddar and related dry-salted varieties (that have a higher fat-to-protein ratio than the interior of the curd particles), air pockets, and/or fissures.

The fat globules 'fill' the openings (pores) in the casein network and limit its aggregation and contraction to a degree dependent on their volume fraction and size distribution. Some clumping and coalescence occur in most cheeses as a result of deformation incurred during manufacturing operations such as molding, pressing, and/or plasticization. This in turn leads to free fat, which lubricates movement of different planes of the cheese during subsequent deformation testing (of the cheese), to an extent that increases in the range 0–40 °C as the ratio of liquid-to-solid fat increases.

Creep and Stress Relaxation in Cheese

Creep is the time-related change in strain on subjecting a material such as cheese to a constant stress that is sufficiently small so as not to break the structure. Practical examples of creep can be seen when curd or cheese is gradually compressed under its own weight during the molding/pressing stage of manufacture (e.g., Camembert), or when stacked during transport and retailing. Creep ($\mathcal{J}$) may be expressed in terms of strain or creep compliance, which is the ratio of strain to applied stress. The typical change in γ with time on application of constant shear stress, τ, to a cheese is shown in a creep curve (**Figure 5**) where creep compliance at any time, t, is given by $\mathcal{J}(t) = \gamma(t)/\tau$, and $\gamma(t)$ is the strain at time t. The creep curve for cheese shows three distinct regions:

- elastic deformation (region A–B), where γ is instantaneous and fully reversible; the creep compliance is referred to as being elastic ($\mathcal{J}_0$);
- viscoelastic deformation (region B–C), where γ is partly elastic and partly viscous; the creep compliance is referred to as being retarded elastic ($\mathcal{J}_R$) and the elastic component of γ recovers slowly on removal of τ;
- viscous deformation (region C–D), where γ increases linearly with time and is permanent; the creep compliance is referred to as being Newtonian ($\mathcal{J}_N$) and γ is not recoverable.

On removal of the stress at point D, the strain recovery curve shows three identifiable regions: an instantaneous elastic recovery (D–E), a delayed elastic recovery (E–F), and an eventual flattening. The vertical distance from the flat portion of the recovery curve to the time axis is the

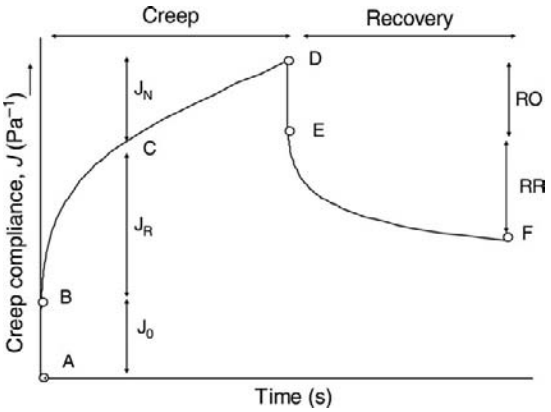

Figure 5 Creep compliance and recovery curve of a 3-month-old Cheddar cheese showing the following regions: elastic creep compliance (J_0, AB), retarded elastic compliance (J_R , BC), Newtonian compliance (J_N, CD), elastic recovery (RO, DE), and delayed elastic recovery (RR, EF); the creep compliance J ranged from 0 to 187 × 10^{-6} Pa^{-1} and time from 0 to 1000 s. The various terms are discussed in the text.

nonrecoverable strain per unit stress, which is related to the amount of structural damage of the sample during the test.

In the elastic region of the creep curve, the strands of the cheese matrix absorb and store the stress energy, which is instantly released on removal of τ, enabling the cheese to regain its original dimensions. The extent and duration of the elastic region depends on the magnitude of τ and the structural and compositional characteristics of the cheese. At $\gamma >$ critical strain, the structure of the cheese is altered via the breaking of bonds between structural elements, which are stressed beyond their elastic limit. Eventually when the stress-bearing structural casein matrix has fractured, the cheese flows. At short timescales and low τ, most cheese varieties (apart from some fresh acid, soft curd varieties) are essentially elastic, whereas after a long time, they flow, albeit very slowly. However, even hard cheeses flow eventually when stressed and do not recover to their original shape on removal of the stress. Failure to appreciate this characteristic can often lead to loss of shape (e.g., manifested by bulging, inclined surfaces) during storage, distribution, and retailing, especially if cheeses of different consistencies are haphazardly laid upon each other.

A stress relaxation test generally involves instantaneous application of a constant deformation or strain (ε; typically 0.10–0.20), by compression of the cheese sample between two parallel plates of a texture analyzer (e.g., TA HDi Texture Analyser, Stable Micro Systems; Instron Universal Testing Instrument (UTM); Instron Corporation). On application of ε, σ increases instantaneously to σ_0 (i.e., zero-time value) but decays exponentially with time (t) to σ_t, with the ultimate difference in the magnitude between σ_0 and σ_t being indicative of the stress energy dissipated by the viscous components of the cheese structure. The resultant σ–time curve is used to determine the stress relaxation time, t, which may be defined as the time required for σ to decrease to a fraction of σ_0, for example, t at which $\sigma = \sigma_0/e$, where e is the base of the natural logarithms.

Mechanical Models of Cheese Rheology

Cheese is a viscoelastic material, as can be inferred from its creep and stress-relaxation behavior. It exhibits some characteristics of both an ideal solid and an ideal liquid, but it also differs from the latter materials in that the relationship between stress and strain depends on the magnitude of the applied stress/strain and the duration of application. On application of a low stress, which is sufficiently small so as not to induce permanent damage or fracturing (breaking of bonds between structural elements) of the microstructure, for short times, cheese behaves as an ideal elastic solid. However, a low stress applied over a relatively long timescale results in an increasing strain, a gradual failure of the structure, and eventual flow. Hence, the relationship between τ (or σ) and γ (or ε) is linear only at very low τ and short timescales. The γ at which linearity between τ and γ is lost is referred to as the critical strain (i.e., linear viscoelastic range), which for most solid-like foods, including cheese, is relatively small (e.g., 0.02–0.05.)

The viscoelastic behavior of cheese may be modeled by various mechanical models that contain different arrangements of dashpots (representing the fluid element) and springs (representing the elastic element) in series and/or in parallel, for example, Maxwell (spring and dashpot in series), Kelvin (spring and dashpot in parallel), or Burger body. The shape of the creep curve for the Burger body (**Figure 6**), which consists of a Maxwell and Kelvin body in series, indicates that it affords a much closer approximation of the rheological behavior of cheese than that obtained for the model of either the ideal elastic solid (simple spring) or ideal liquid (dashpot) on its own (**Figure 3**).

Methodologies for Measurement of Cheese Rheology

The methods used for assessing the rheological characteristics of cheese may be broadly classified as sensoric or instrumental; the instrumental methods can be further categorized as empirical or fundamental.

Sensoric Methods

The aim of sensoric methods, which are routinely performed by cheese graders, is to acquire an impression of how the texture of the cheese is perceived during consumption. Cheese texture may be defined as a composite sensory attribute resulting from a combination of physical properties that are perceived by the senses of touch (including kinesthesis and mouthfeel), sight, and hearing.

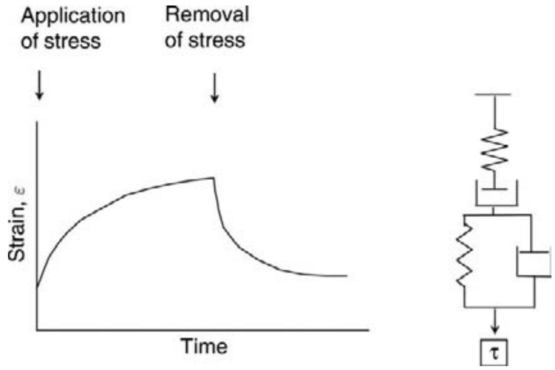

Figure 6 The Burger body as a mechanical model and its corresponding creep and recovery curve, to simulate the viscoelastic behavior of cheese.

The test conditions are arbitrary and frequently involve subjecting the cheese to a deformation that results in visual fracture, for example, as when rubbing cheese between the fingers until it becomes pliable, and finger bending of a cylindrical cheese plug and mentally gauging the force required to bend or break it. Alternatively, the cheese may be assessed by application of forces or deformations that cause no visible fracture, for example, pressing the ball of the thumb into the surface of a whole cheese and mentally measuring the degree of indentation or the force exerted on the fingers. In all cases, a mental impression is formed and the grader assigns a score, based on one or more criteria, such as test conditions and response.

Instrumental Empirical Methods

The aim is to measure a parameter that experience indicates or suggests is related to the textural characteristics of the cheese. While the test conditions are arbitrary and the stresses and strains involved may not be well defined, a value is obtained that gives some indication of the textural characteristics of the cheese and differentiates one sample from another. However, the tests provide only single datum values that are an overall measure of the many different facets of rheological behavior.

These tests involve subjecting a cheese sample to a stress or strain using various instrumental devices. The different test types include

- imitative compression tests using various instruments (e.g., bite tenderometer, texturometer) that attempted to simulate the sensory evaluation of texture during consumption (cutting, biting, and/or chewing action of the teeth (incisors, molars)) by measuring the forces involved when subjecting the cheese sample to compression by various devices (e.g., tooth-like jaws, or wedges, plungers),
- compression tests, where the extent of compression (deformation) under a constant load (force) for a specified time is measured (e.g., the ball-compressor test) to provide an index of hardness,
- penetration (puncture) tests, where the force required to insert a cone or probe a given distance into the cheese, or alternatively the depth of penetration of a cone/probe or needle under a constant load for a given time, is measured (penetrometer test),
- cutting tests, where the resistance to the passage of a wire through a cheese is measured (e.g., Cherry-Burrell curd tension meter).

The texturometer (General Foods) was designed to simulate the biting of food by the jaws and the teeth. A cheese sample was loaded onto a plate attached to a beam, and was then subjected to a deforming force provided by a tooth-shaped plunger, which was moved by a wheel device in a motion designed to simulate the vertical action of the jaw. When the plunger deformed the sample, strain gauges attached to the sample-holding beam detected the movement of the beam *per se* and a force–time trace was recorded and became known as a texture profile. The sample was subjected to two successive deformations (referred to as bites) in the texturometer.

In penetration tests, the penetration of the surface by the probe results in fracturing and forcing apart of the cheese in its path. The progress of the probe is retarded to an extent depending on the hardness of the cheese in its path and the adhesion of the cheese to its surface (which depends on the depth of penetration into the cheese and the thickness of the needle, or angle of the cone, used). Penetration tests are especially suited for soft cheeses such as Camembert, Coulommiers, and Munster from which it is difficult to obtain a sample of fixed dimensions (e.g., a cube or cylinder as required for other tests such as compression testing under standard conditions) and which tend to deform easily. Moreover, a significant correlation has been found between measurements obtained using a cone penetrometer (30°), or a 2 mm needle, and textural characteristics of a wide range of commercial cheeses (e.g., Colby, Edam, Cheddar, Mozzarella, and Cream cheese) as measured by a sensory panel.

Fracture energy during cutting is calculated by measuring the force required to push wires of different diameters at constant velocity through a cheese mass. A typical force–time curve shows an initial increase in force, which reaches a maximum as the wire penetrates and fractures the sample surface. Once the surface is fractured (broken), the force rapidly drops to a constant level as the wire ‘ploughs’ through the sample. Since fracture develops around a crack, a specific fracture energy can be defined as the energy needed per unit area (of crack) to cause a fracture to spread. The fracture energy obtained with the wire-cutting method may give a more accurate prediction of the behavior of cheese during cutting (e.g., portioning, slicing) than that obtained using large strain shear or compression deformation tests.

Fundamental Methods

There are a number of fundamental instrumental methods that can be used to measure the rheological characteristics of cheese. These can be classified as low strain or large strain.

Low-strain deformation tests involve the application of strains that are within the linear viscoelastic limit of the cheese (e.g., <0.05), while large strain deformation methods involve strains that result in fracture of the structure at least at the microstructural level and sometimes also at the macrostructural level. The various tests are summarized in **Table 1** and those most commonly used are discussed below.

Table 1 Fundamental instrumental methods for measurement of the rheological properties of cheeses

Test type	*Mode*	*Typical sample shapes*	*Apply*	*Measure*[a]	*Output parameters*	*Relevance*	*Test characteristics*
Stress relaxation	Compression or shear	Cylinder, rectangle, cube	Fixed strain (γ)	Stress (σ) vs. time (t)	Relaxation time: time for σ to reach a fraction of the original value (σ_0)	Index of intrinsic elastic and viscous character	Low deformation; may not be very predictive of large strain deformation properties
Creep/recovery Creep	Shear	Disc	Fixed τ	γ vs. t	Creep compliance: ratio of γ/σ vs. t Level of deformation at time, t	Index of intrinsic elastic and viscous character	Low deformation; may not be very predictive of large strain deformation properties
Recovery	Shear	Disc	Remove τ	γ vs. t	Creep compliance: ratio of γ/σ vs. t Degree of recoverable deformation at t, and permanent deformation	Index of intrinsic elastic and viscous character	Low deformation; may not be very predictive of large strain deformation properties but very useful to predict changes in fluidity/elasticity during melting and cooling of heated cheese
Dynamic low-amplitude stress Rheometry	Oscillating shear	Disc	Low-amplitude oscillating sinusoidal γ or τ at an angular frequency ω	τ vs. t or γ vs. t	Elastic shear modulus (G'), $G' = (\tau/\gamma)\cos\delta$, where δ is the angle by which σ and γ are out of phase Viscous or loss modulus (G''), where $G'' = (\tau/\gamma)\sin\delta$ Loss tangent = tan δ	Index of intrinsic elastic and viscous character	Dynamic, noninvasive; may not be very predictive of large strain deformation properties
Dynamic low-amplitude strain Rheometry	Oscillating shear	Disc	Low-amplitude oscillating sinusoidal γ or σ at an angular frequency ω	τ vs. t or γ vs. t	Elastic shear modulus (G'), $G' = (\tau/\gamma)\cos\theta$, where δ is the angle by which σ and γ are out of phase Viscous or loss modulus (G''), where $G'' = (\tau/\gamma)\sin\theta$ Loss tangent = tan δ	Index of intrinsic elastic and viscous character	Low deformation; may not be very predictive of large strain deformation properties
Deformation							
Uniaxial compression	Compression	Typical sample shapes	Strain (displacement)	Stress vs. strain	Fracture stress (σ_f) Fracture strain (ε_f) Stress at the end of displacement (firmness) (σ_{max}) Others	Indices of fracturability, hardness, 'longness', or 'shortness' of cheese body	Large deformation (e.g., $\varepsilon \sim 0.7$); widely used. Cheeses usually undergo fracture during the test; predictive of behavior during size-reduction processes where large ε and σ are applied

(*Continued*)

Table 1 (Continued)

Test type	*Mode*	*Typical sample shapes*	*Apply*	*Measure*[a]	*Output parameters*	*Relevance*	*Test characteristics*
Uniaxial extension	Extension	Slices, strings	Strain (displacement)	Stress vs. strain	σ_f, ε_f, σ_{max}	Indices of tensile strength or susceptibility to tearing of processed cheese slices or tensile strength of cheese strings; stretchability of melted cheese	Large deformation; rarely used, probably because of the difficulties associated with gripping a sample. Could be useful for assessing tensile strength of processed cheese slices and their susceptibility to tearing during the casting stage of manufacture
Torsion gelometry	Shearing	Capstan	Torsion/shear stress	Stress vs. strain	τ_{max}, γ_{max}	Index of shear fracture stress and strain, especially in highly deformable cheeses	Large strain deformation in shear mode; increasingly used in research studies; gives a measure of the level of stress and strain that can be applied to cheese when twisting into helical strings
Three-point bending	Compression and elongation (extension)	Fingers (long cylinders)	Strain (displacement)	Stress vs. strain	σ_f, γ_f	Index of fracturability	Large deformation; gives a measure of the ease of fracture during manual handling of edible-size portions Rarely used, especially for heated cheese because of the difficulty in gripping the cheese

[a] Symbols: σ, normal (compression) stress; τ, shear stress; ε, normal strain; γ, shear strain; v, velocity; η, viscosity; J, creep compliance; t, time; E, elastic modulus; G', elastic shear modulus; G'', viscous or loss modulus; δ, phase angle between stress and strain; σ_f, fracture stress; ε_f, fracture strain; σ_{max}, firmness.

Low-strain deformation tests

Low-strain oscillation rheometry

Linear viscoelastic measurements involve the application of a dynamic low oscillating strain to a sample and the measurement of the resultant stresses within the sample. Alternatively, a small stress is applied to the sample and the resultant strain is measured. The former is referred to as controlled strain rheometry, whereas the latter is known as controlled stress rheometry.

Several geometries are possible for applying torsion, but the most convenient for a solid material, like cheese, is parallel circular plates, where the plates have serrated surfaces to minimize the risk of slippage, associated with fat liquefaction. The disc-shaped cheese sample is gripped between the plates, one of which is fixed, while the other applies a low-amplitude torsional harmonic motion. At any time *t*, the angle of rotation, θ, of the oscillating plate is defined by

$$\theta = a \sin \omega t$$

where *a* is the maximum angle of rotation and ω is the angular velocity. The shear applied by the plate varies throughout the cheese disc, from zero at the central axis to a maximum at the edge. At a point on the oscillating plate and at a radial distance *r* from the axis, the displacement due to rotation by an angle θ is $r\theta$, and the shear strain γ is given by $\gamma = r\theta/l$, where *l* is the thickness of the cheese disc. This displacement results in a strain $\gamma(t)$ at any radius *r*:

$$\gamma = (ar/l) \sin \omega t = \gamma_0 \sin \omega t$$

where γ_0 is the amplitude of $\gamma(t)$. In general, the resultant oscillating stress is out of phase with the applied shear by a phase angle δ. The stress wave can be reconstructed as the sum of two sine waves, one in phase and the other out of phase (by 90°) with the strain wave. Thus,

$$\tau = \tau' + \tau'' = \tau_0{}' \sin \omega t + \tau_0'' \cos \omega t$$

where $\tau_0{}'$ and $\tau_0{}''$ indicate the stress components, that are in phase and out of phase with the strain γ and are related by the phase angle δ: tan $\delta = \tau_0{}''/\tau_0{}'$. Two dynamic moduli, elastic shear modulus (or storage modulus), G', and viscous modulus (or loss modulus), G'', may be defined from the relationship between τ and γ, where $G' = \tau_0{}'/\gamma_0$ and $G'' = \tau_0{}''/\gamma_0$. Hence, the tan of the phase angle, frequently denoted the loss tangent, is tan $\delta = G''/G'$.

This system is frequently used in assessing the changes in the viscoelasticity of cheese during heating and the fluidity of the melted cheese mass. A typical profile for Cheddar cheese (**Figure 7**) on heating from 20 to 80 °C shows a decrease in G' and an increase in the phase angle, δ. The changes in G' and δ indicate a phase transition from an unheated cheese, largely elastic in rheological response ($\delta \approx 12$–15° at 20 °C), to a melted cheese, which is more viscous in character. At a practical level, these trends represent a decrease in the stiffness and an increase in the fluidity of the cheese mass as it is heated, for example on a pizza. Conversely, the changes in G' and δ on cooling the heated cheese mass back to 20 °C may be used as a measure of the degree to which the molten cheese mass congeals on cooling, as occurs when a baked pizza is left at room temperature for some time before eating.

Creep

A creep test generally involves instantaneous application of a low constant τ (e.g., 10–50 Pa) and measurement of the resultant γ as a function of time (e.g., **Figure 5**). Testing typically involves placing a disc-shaped test

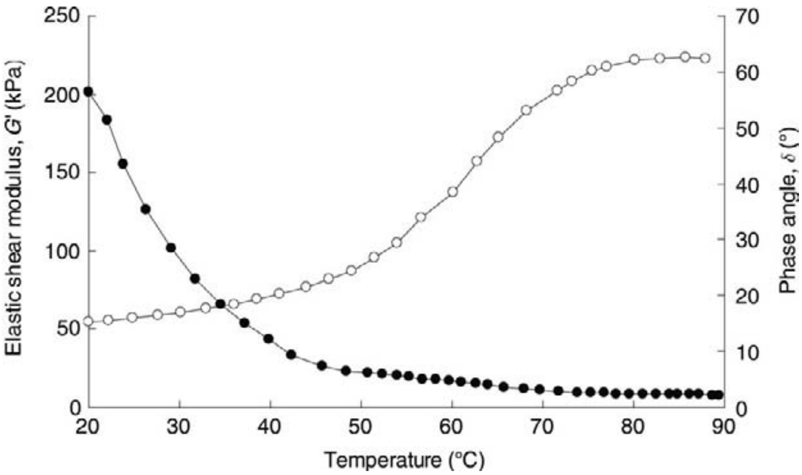

Figure 7 Changes in the elastic shear modulus (●) and phase angle (○) as a function of heating full-fat Cheddar cheese from 20 to 88 °C.

sample between the two parallel plates of a rheometer cell, that is, a stationary bottom plate and a top plate, which applies the shear. The results are expressed as creep compliance ($\mathcal{J}$) at a particular time (t): $\mathcal{J}_t = (\gamma/\tau) = 1/G_t$, where G_t is the ratio of τ/γ at time t. The test may be useful in providing an accurate determination of the elastic shear modulus of Emmental-type cheeses, as an indicator of its elasticity and ability to retain carbon dioxide in the form of eyes. It may also have application in predicting the behavior of cheese on stacking.

Large strain deformation

In practice, cheese is subjected to large stresses and strains (i.e., >>0.05) which usually result in visible fracture, that is, when interactions and/or bonds between the structural elements (e.g., strands in the *para*-casein matrix) in a given macroscopic plane fail. Fracture is clearly evident as a result of compressive and shear forces applied during typical size-reduction operations undertaken at commercial level, for example, precutting of large blocks and/or comminution (e.g., by a conveying auger crushing and forcing precut cheese through die plates with narrow apertures during processed cheese manufacture), shredding, dicing/or cubing, and slicing. Cheese is also fractured during mastication, which reduces it to a pulp capable of being swallowed. Thus, prediction of how cheese behaves under large σ and γ is desirable in many instances.

A number of different techniques are applied to evaluate the large strain deformation behavior of cheese, including

- large strain uniaxial compression using texture analyzers (e.g., Instron or Sintech 1/G universal testing machines, Stable Microsystems TA-HDi texture analyzer, Stevens Response Compression Analyser),
- large strain shear (e.g., torsion gelometer),
- vane rheometry,
- bending tests (e.g., using texture analyzers).

Of these, large strain compression and shear testing have found most application; these are discussed briefly below.

Uniaxial compression

This typically involves subjecting a sample of cheese, typically in the form of a cube (e.g., $25 \times 25 \times 25\,\text{mm}^3$) or a cylinder (30 diameter × 29 mm high), to a large strain (e.g., $\varepsilon \sim 0.8$) by uniaxial compression of the cheese sample between two parallel plates of a texture analyzer, and dynamically measuring the force and displacement. The cheese sample is placed on a base plate and is compressed at a fixed rate (e.g., $500\,\text{mm min}^{-1}$) to a predetermined level (e.g., 25% of its original height) by the mobile plate (crosshead). The force (F) developed during compression is recorded as a function of distance (or displacement); alternatively, the force may be converted into σ and the displacement into ε. The resultant σ versus ε curve (**Figure 8**) for mature Cheddar shows a number of distinct regions and enables the determination of a number of rheological parameters:

- A–B, σ increases proportionally with ε. The slope of this linear region defines the compression modulus, E (i.e., $E = \sigma/\varepsilon$). While E is of little practical significance

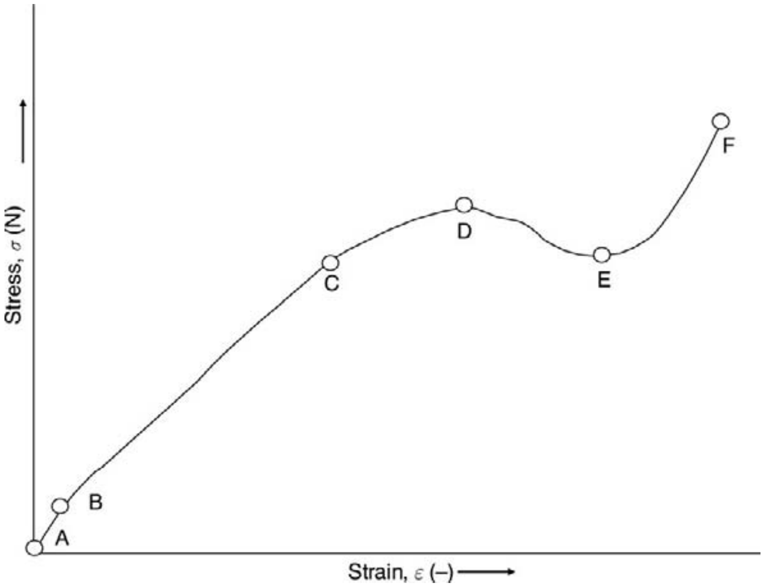

Figure 8 Large strain deformation test: typical shape of a stress–strain curve of a 6-month-old mature Cheddar cheese sample compressed to 25% of its original height; several regions of the curve are identifiable (e.g., AB, BC) based on the mean slope (see text for details).

in relation to cheese behavior during processing and consumption, where strains >>0.05 (e.g., shredding, breaking), its magnitude may be indicative of the degree of springiness or elasticity as detected during commercial grading of cheese (e.g., where a grader sensorically monitors the resistance to small deformation, as in pressing the thumb into the outside of the cheese block; the force applied during this hand deformation is typically 18 N or a σ of ~40 kPa).

- B–C, σ increases less than proportionally with ε. The slightly lower slope of the curve in this region compared to that in A–B is probably due to the formation of microcracks that do not spread throughout the sample but which allow some stress to be dissipated.
- C–D, the slope of the σ/ε curve decreases markedly. The cheese begins to fracture at C, as cracks grow and spread throughout the entire sample at an increasing rate. Eventually, at D, the rate of collapse of the stress-bearing *para*-casein matrix overtakes the buildup of σ within the matrix through further compression and a peak σ, denoted as the fracture stress, is reached. The fracture stress, σ_f, and strain, ε_f, are measures of the stress and strain, respectively, required to cause complete fracture of the sample.
- D–E, σ decreases with further compression due to the collapse of the stress-bearing structure. The decrease in σ may be attributed to (1) the shattering of the samples into pieces that spread over the base plate, resulting in an increased surface area, and (2) the probable loss of contact between some of the pieces of cheese and the base plate, which results in dissipation of stress energy stored within the individual pieces.
- E–F, σ increases as the crosshead begins to compress the fragmented pieces of cheese. σ at the end of the compression (point F) is a measure of firmness, as judged in the first bite of mastication.

The various quantities obtained from the stress–strain curve and their interpretations are given in **Table 2**. The magnitude of these rheological parameters is significantly influenced by testing conditions, including the rate of deformation (compression rate), test (sample) temperature, sample shape, and the interface conditions between sample and crosshead.

Large strain shear

As a method for characterization of cheese rheology, large strain shear is scarcely used commercially but is becoming more popular in research studies.

Testing typically involves subjecting a sample of cheese, in the form of a capstan (e.g., height, 28 mm; maximum and minimum diameters, 27 and 5 mm, respectively) to a torque. The sample, to which plastic discs (~1 mm thick) are glued at both ends, is placed in a viscometer cell (e.g., Haake VT 550; torsion gelometer) where it is gripped between two circular plates; the bottom plate is fixed while the top plate is rotated at a fixed speed (e.g., 0.2–0.7 rpm) giving angular velocities of ~0.013–0.524 rad s^{-1}. The applied torque results in twisting of the sample until it fractures along its minimum diameter. The maximum stress ($\tau_{\max}$) and strain ($\gamma_{\max}$) are computed dynamically from the equations

$$\tau_{\max} = 2KM/\pi r_{\min}^3$$

(where M is the torque (N cm) and K is a constant calculated from the ratio of the radius of the smallest cross section of the sample ($r_{\min}$) to the radius of curvature of the sample in a plane containing the longitudinal axis (r_c)) and

$$\gamma_{\max} = 2K\varphi_s/\pi r_{\min}^3 Q$$

(where φ_s is the angular deformation of the curved surface (rad) and Q is a constant related to the ratio of to the height to curvature of the sample).

Torsion shear is a useful technique for the determination of fracture stress and fracture strain in highly deformable cheeses (e.g., fresh low-moisture part-skim Mozzarella) that do not undergo elastic fracture (i.e., where sample breaks into distinct pieces) on compressing to strains of 0.7–0.8. The latter parameters may be important in characterizing the suitability of curds for the formation of cheese strings

Table 2 Rheological quantities derived from the stress–strain curve obtained on large strain deformation of cheese

Rheological quantity	*Abbreviation*	*Definition*	*Measure*
Elastic or compression modulus	E	Proportionality constant between stress and strain at low strain. Measure of elasticity	Elasticity and springiness at low stress and strain
Fracture stress	σ_f	Stress required for fracture and collapse of cheese mass beyond the point of recovery	Strength of cheese matrix to deforming stress
Fracture strain	ε_f	Deformation required to induce fracture	Resistance of matrix to deformation; index of brittleness, and the 'shortness' or 'longness' of cheese
Firmness (maximum stress)	$\sigma_{\max}$	Stress required to compress cheese sample to a given deformation	Index of firmness, hardness, or toughness

containing two different-colored cheeses in a twisted helical (ropelike) configuration. Otherwise, torsion shear offers few if any distinct advantages above large strain deformation in compression mode; the preparation of capstan-shaped samples requires specialized equipment and is time consuming.

Factors that Affect Cheese Rheology

Cheese Composition

The viscoelasticity of cheese results from the interactive rheological contributions of its individual constituents (i.e., protein, fat, and moisture), which are affected by their concentrations, their microstructural arrangement, and physical nature. The effects of some compositional parameters on cheese rheology (as measured in most cases using large strain deformation) are discussed below.

Protein content

On application of a stress to cheese, the matrix at first controls the deformation. The stress-bearing capacity of the matrix is dependent on its volume fraction and homogeneity, which determine the number of stress-bearing strands per unit area. As the concentration of casein in the matrix increases, the intra- and interstrand linkages become more numerous and the matrix *per se* displays more elasticity and is more difficult to deform. Hence, there are significant positive correlations between the content of intact casein and σ_f or σ_{max} (firmness), as measured using large strain compression, in Cheddar-type cheeses of varying protein content (**Figure 9**).

Fat content

The contribution of fat to the rheological properties of cheese depends on its volume fraction and temperature; the temperature determines the ratio of solid-to-liquid fat. At low temperatures (i.e., <5 °C), the milk fat is predominantly solid and augments the elasticity of the casein matrix. The solid fat globules limit the deformation of the casein matrix, as the deformation of the latter requires deformation of the enmeshed fat globules. However, the contribution of fat to the elasticity decreases rapidly as the ratio of solid-to-liquid fat decreases with increasing temperature and is very low at 40 °C, where all the milk fat is liquid. Liquid fat confers viscosity and also acts as a lubricant on fracture surfaces of the casein matrix and thereby reduces the stress required to fracture the matrix. Hence, for a given fat level, raising the assay temperature (e.g., in the range 0–32 °C) during compression testing results in a marked reduction of E, σ_f, and σ_{max}. The importance of ratio of solid-to-liquid fat is further highlighted by the fact that the elastic shear modulus, G', for half-fat Cheddar is lower than that of full-fat Cheddar at 4 °C, where the fat is solid. However, G' for both cheese types is similar at 40 °C, where the fat is liquid, even though the dry matter content of the full-fat cheese (~63% (w/w)) is higher than that of the half-fat cheese (~57% (w/w)).

Substitution of protein with fat (e.g., increasing the fat-in-dry matter level of cheese, while retaining the other compositional parameters constant) results in a reduction in σ_f and σ_{max}, with the effect becoming more pronounced as the temperature is increased.

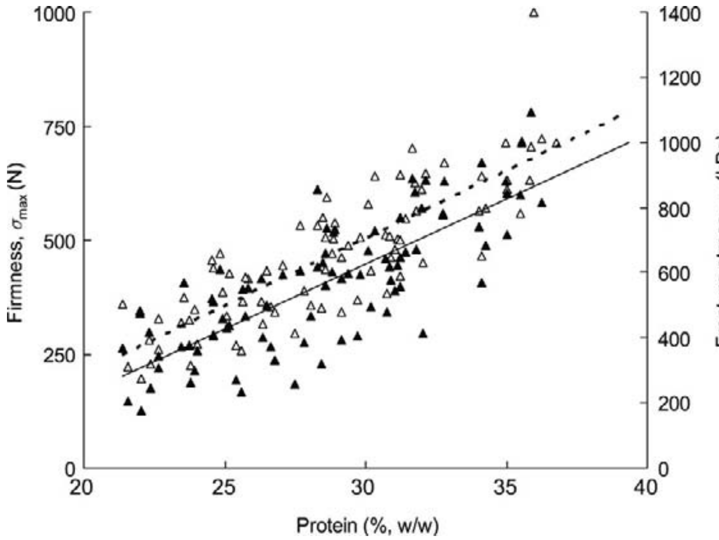

Figure 9 Change in firmness (σ_{max}, Δ) and fracture stress (σ_f, Δ) as a function of protein content in Cheddar cheeses compressed to 25% of original height.

Moisture content

Increasing the moisture content of cheese, while maintaining the other compositional parameters relatively constant, results in reductions in E, σ_f, and σ_{max}. ε_f increases slightly with moisture content to an extent dependent on cheese pH and maturity.

Salt (NaCl) content

The concentration of salt in the moisture phase (S/M) of rennet curd cheeses varies from ~2.0% (w/w) in Emmental to ~12% (w/w) in Feta. Increasing the S/M concentration of Gouda-type cheese, in the range 0–12% (w/w), while maintaining the other compositional parameters relatively constant, is associated with increases in E, σ_f, and σ_{max}. The fracture strain, ε_f, increases slightly to a maximum at S/M of 4% (w/w), then decreases sharply (to a value that is about half the maximum) and thereafter plateaus at this value for S/M levels in the range ~5–12% (w/w).

pH

Increasing the pH in the range 4.8–5.2 has been found to reduce both E and σ_f in both Cheddar and Gouda cheeses. In contrast, increasing the pH from 5.2 to 5.6 results in a marked increase in σ_f (to values much higher than those at $\text{pH} < 5.2$) and a slight increase in E. In young Gouda cheese (1 week old), the ε_f was maximum at pH 5.2 and decreased on lowering the pH to 4.8 or raising the pH to 5.6. However, the pH at which ε_f is maximum increases with ripening time, for example, from ~5.2 in 1-week-old Gouda cheese to ~5.4 in 3-month-old Gouda cheese. The differences in pH probably contribute to the differences in the rheological characteristics of different varieties. Low-pH cheeses (e.g., Cheshire, Feta) generally tend to have low values of σ_f and ε_f and crumble into many pieces on fracturing, whereas cheeses of relatively high pH (i.e., pH 5.35–5.50), such as Emmental and Gouda, exhibit higher values of σ_f and ε_f and tend to fracture into larger pieces. The effect of pH probably ensues from its influence on (1) the ratio of soluble-to-colloidal Ca, (2) the degree of *para*-casein hydration, and (3) the types of intra- and interaggregate bonds. Moreover, the effect of pH appears to be related to other factors such as the degree of casein proteolysis.

Ripening

The σ_f and σ_{max} of cheese generally decrease with ripening time due to hydrolysis and hydration of the casein strands, both of which are factors that contribute to a reduction in the integrity and elasticity of casein matrix. The structure of cheese is weakened by the early hydrolysis of α_{s1}-casein (CN) by residual chymosin, at the Phe 23–Phe 24 peptide bond. α_{s1}-CN (f1–24) is strongly hydrophobic and interacts with the hydrophobic regions of other α_{s1}- and β-CN molecules, and thus contributes to the overall continuity and integrity of the matrix.

See also: **Cheese**: Cheese as a Food Ingredient.

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Acid- and Acid/Heat Coagulated Cheese

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Introduction

Generally, acid- and acid/heat-coagulated cheeses have a mild or acidic flavor and are usually consumed fresh (i.e., without ripening). These cheeses usually do not have a rind and come in a wide range of shapes and weights. Fresh acid cheeses (sometimes called lactic cheeses) differ from yogurt and fermented milk products in having a significant amount of moisture removed after coagulation by techniques such as centrifugal separation and ultrafiltration (UF), which are used for Quarg and Cream cheeses, or cutting of the coagulum into granules, which is used in Cottage cheese to encourage syneresis. Soft, unripened cheeses, such as Quarg, Cottage, and Cream cheeses, are related in the common use of lactic cultures to form a smooth acid curd. They differ in the method of whey drainage, washing steps, cream incorporation, and type of curd structure. In Cottage cheese, the coagulum is cut to form curd particles of specific sizes, whereas the coagulum for the other acid- and acid/heat-coagulated cheeses is usually stirred by agitators and the original curd structure is lost. Many fresh cheeses are also blended with fruits, spices, herbs, or other foods. Fat content can be adjusted even after whey drainage in contrast to natural rennet-coagulated cheeses.

Some types of natural cheeses, for example, Mozzarella, are sometimes made by direct acidification but usually rennet is used for coagulation.

The main processing steps used in the manufacture of fresh acid- and acid/heat-coagulated cheeses are summarized in **Figure 1**. Traditional equipment for fresh cheese types consists of fermentation vessels, separation equipment, and packaging devices. However, most fresh cheeses are now made by continuous large-scale processes. The legal designations and standards for these fresh cheeses vary from country to country. Most standards are based on the chemical composition of the cheeses (**Table 1**). Mesophilic lactic acid bacteria (i.e., mainly *Lactococcus* spp. and *Leuconostoc* spp.) and sometimes probiotic species are used as cultures for most acid- and acid/heat-coagulated cheeses. In many of these cheeses, diacetyl, lactate, and acetate are important flavor compounds.

Incorporation of whey protein into fresh cheese is an important aspect of fresh cheese manufacture because of increased yield. High-heat treatment of milk is not usually practiced for Cottage cheese since heating reduces whey syneresis, which causes textural defects, including excessive softness. The shelf life of fresh acid-type cheeses can be reduced by the growth of yeasts and other spoilage organisms, which can grow due to the high moisture content, absence of a surface rind, and a high level of residual lactose.

Mechanism of Coagulation

The stability of casein micelles of milk is attributed to their net negative charge and steric repulsion by the flexible macropeptide region of κ-casein (the so-called 'hairs') (*see* **Milk Proteins**: Casein, Micellar Structure). Different types of interactions are responsible for micelle integrity, including calcium-induced interactions between protein molecules, electrostatic and hydrophobic interactions, and hydrogen bonding. On acidification, casein particles aggregate as the negative charge repulsion force on caseins is reduced. This leads to the formation of chains, aggregates, and clusters, which eventually form a three-dimensional network. During acidification of milk, the insoluble colloidal calcium phosphate (CCP) present in casein micelles dissolves and this causes the micelles to swell and become more flexible internally.

When milk is subjected to preheat treatment, denatured whey proteins associate with casein micelles and they cross-link the gel network when aggregation occurs during subsequent acidification of milk. Heat treatment increases the firmness of milk gels formed by acidification although these gels can be more susceptible to wheying-off as the gel may undergo greater rearrangement. In gels that are formed from preheated milk, gelation occurs at a higher pH (e.g., 5.2–5.4) than that for unheated milk (pH ~5.0); these pH values depend on the gelation temperature. The higher gelation pH can be attributed to the higher isoelectric pH (~5.2) of the main whey protein, β-lactoglobulin, which initiates isoelectric precipitation/aggregation at a higher pH than that observed with caseins, which have an isoelectric point of ~4.6. In acid milk gels produced from heated milk, the solubilization of CCP in casein particles that are already part of the gel network can cause a loosening of the gel network, which assists in curd syneresis. At lower pH values, electrostatic interactions are stronger and the gel becomes firmer again and exhibits less syneresis. In

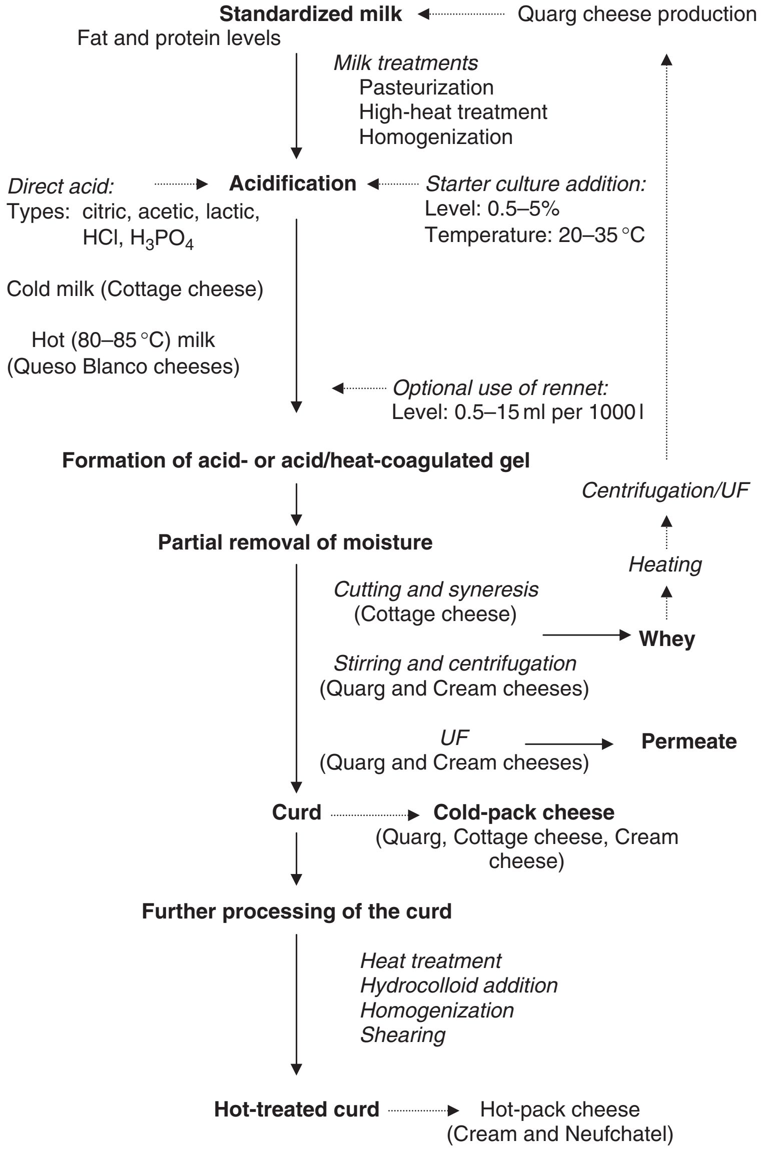
Standardized milk
Fat and protein levels
Quarg cheese production
Milk treatments
Pasteurization
High-heat treatment
Homogenization
Direct acid:
Types: citric, acetic, lactic, HCl, H_3PO_4
Acidification
Starter culture addition:
Level: 0.5–5%
Temperature: 20–35 °C
Cold milk (Cottage cheese)
Hot (80–85 °C) milk
(Queso Blanco cheeses)
Optional use of rennet:
Level: 0.5–15 ml per 1000 l
Formation of acid- or acid/heat-coagulated gel
Centrifugation/UF
Partial removal of moisture
Heating
Cutting and syneresis
(Cottage cheese)
Whey
Stirring and centrifugation
(Quarg and Cream cheeses)
UF
(Quarg and Cream cheeses)
Permeate
Curd
Cold-pack cheese
(Quarg, Cottage cheese, Cream cheese)
Further processing of the curd
Heat treatment
Hydrocolloid addition
Homogenization
Shearing
Hot-treated curd
Hot-pack cheese
(Cream and Neufchatel)

Figure 1 Main processing steps in the manufacture of acid- and acid/heat-coagulated cheeses (dotted lines are optional processing operations). UF, ultrafiltration.

general, conditions such as high incubation temperatures, fast rates of acidification, and preheat treatment of milk encourage greater rearrangements of casein particles in the gel network and the formation of a coarse network. Direct acidification of milk at a low temperature may allow solubilization of CCP prior to gelation and therefore these gels may undergo less changes in their mechanical properties (e.g., syneresis) than traditional cultured products.

The addition of rennet results in gelation at a higher pH and the mechanical properties of the curd are changed. Addition of acid to hot milk (as in acid/heat-coagulated cheeses) causes rapid precipitation of casein (and denatured whey protein), and gelation occurs at higher pH values than with acid coagulation due to the interactions between denatured whey proteins and casein micelles at the high temperature. The high temperature encourages greater collisions (due to increased thermal motion) of particles leading to faster aggregation.

Cottage Cheese

Introduction

Cottage cheese, sometimes called Pot cheese, is a creamed, acid-coagulated fresh cheese that is low in acidity and is washed during manufacture. Cottage cheese has a slight acid, salty taste with a delicate diacetyl or creamy flavor and aroma. The body of Cottage cheese should be

Table 1 Approximate composition of various acid- and heat/acid-coagulated cheeses

Variety	*Moisture (%)*	*Fat (%)*	*Protein (%)*	*Salt (%)*	*pH*
Cottage					
Low fat	79	2	14	1.0	4.8–5.0
Creamed	79	5	13	1.0	4.8–5.0
Cream					
Double	54	33–35	8–10	0.7–1.2	4.6–5.0
Single	70	14	12	0.7–1.2	4.6
Neufchatel	64	20	12	0.75	4.6
Baker's	74	0.2–0.6	19	<0.10	4.4–4.6
Quarg					
Low fat	82	0.5	13–15	0.1–0.7	4.5
Creamed	73	12	10	0.1–0.7	4.6
Fromage frais (skim)	86	1	8	0.1–1.0	4.4
Queso Blanco	48–55	15–27	19–24	2.3–3.0	5.2–5.7
Ricotta					
High fat	72	13	11.5	<0.5	5.8
Low fat	75	8	12	<0.5	5.8
Ricottone	73–82	0.5	11–19	<0.5	4.9
Paneer	50–55	25–27	16–18	0.0	5.4
Mascarpone	40–52	45–50	2.5–7.5	0.0	5.6–6.4

smooth and meaty, and curd particles should be discrete but not soft and pasty. Cottage cheese is popular in the United States (where total production was 360 000 tonnes in 2007), the United Kingdom, and several other countries, and is often used in combination with salads and desserts. Cottage cheese accounts for around 5% of the world's cheese production.

Recent innovations/developments include the addition of probiotic cultures and prebiotics, extension of shelf life through the incorporation of carbon dioxide into the dressing, smaller more convenient single-serve containers, calcium fortification, use of bacteriocin-producing cultures, fruit-flavored products, and the use of UF to increase the total solids (TS) content of the cheese milk.

Manufacturing procedures

Cottage cheese is made from pasteurized skimmed milk (in contrast to several other fresh acid cheeses where the milks are often subjected to higher heat treatments). The TS content of the skim milk is often increased to 10–13% by fortification with nonfat dry milk powder (NFDM; skim milk powder) or by the addition of UF retentate. There are at least three different procedures for the manufacture of Cottage cheese based on the length of the setting time prior to cutting: long-, medium-, and short-set methods (**Table 2**). The length of time before cutting varies from 5 to 16 h and the temperature of setting ranges from ~22 to 35 °C. A higher level of starter inoculum and high incubation temperature are used in the short-set method compared with the slow long-set method. Non-gas-producing mesophilic cultures (e.g., *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) are usually used. Recently, in the United States, there has been a trend away from bulk starter and towards direct vat inoculants.

A low level of rennet (1–3 ml per 1000 l milk) may be added, which helps to form a suitable gel after a shorter incubation period. Rennet is often added after some acidity has developed by the starter culture (e.g., after 1–2 h). When rennet is added, the gel is ready to be cut at a higher pH (e.g., 4.75) than in its absence (e.g., 4.6). After cutting, the curd is left undisturbed for 15–35 min. Curds of different sizes are produced by cutting the coagulum

Table 2 Some of the processing conditions commonly used for the production of Cottage cheese

Process step	*Short set*	*Medium set*	*Long set*
Rate of starter addition (%)			
Conventional bulk starter	5	2	1
pH-controlled starters	2	1	0.5
Time before cutting (h)	5	8	12–16
Incubation temperature (°C)	30–33	27	21–24

with different-sized knives. After this resting period, the curds are cooked slowly with gentle stirring for 1–3 h until a temperature between 47 and 56 °C is reached. Cottage cheese curd is fragile and shattering occurs if the curd is mishandled. When the same vat is used for the complete production operation, the curds are normally washed three times with water at a decreasing temperature, for example, 28–22, 16–10, and 5–2 °C. Washing removes lactose and lactic acid, and cools the curd to slow down further acid production and syneresis. The total time for washing and drainage steps is ~3 h. After all the water has been drained off, pasteurized cream (10–20% fat) at 4 °C containing a small amount of salt and stabilizers (e.g., xanthan gum, carrageenan, and/or guar gum), known as 'dressing', is added and mixed into the curd. In the United States, creamed Cottage cheese contains ~79% moisture, 16% solids nonfat, ~4% fat, and 1% salt. After a short holding time, the curds are packed into containers (e.g., vacuum-formed polystyrene tubs with a shrink film of polypropylene) and stored at 4–5 °C for retail distribution and they have a typical shelf life of up to 3–4 weeks (in the United States).

Cottage cheese can also be made by direct acidification of cold milk (≤4 °C) to pH 5.2–4.7. Milk is usually acidified to pH 5.1 with hydrochloric, phosphoric, lactic, or other acids and then warmed to a higher temperature, for example, 32 °C, when a small amount of the acidogen, gluconic acid-δ-lactone (GDL), is added to the milk to reduce the pH of milk to ~4.7. The milk then coagulates and the curd is treated as normal. Using this approach, slow acidification of milk can be achieved, whereas addition of a high concentration of GDL at a high temperature would cause a rapid reduction in milk pH.

Equipment

Most Cottage cheese is made in rectangular, open vats but the whey draining, curd washing/cooling, and creaming steps may be performed in separate equipment, which improves process efficiency (e.g., reduces water usage). The main processing equipment for Cottage cheese includes plate heat exchangers (to pasteurize the milk), milk fermentation tanks, cutting devices, stirring area with indirect or direct heating, whey drainage belts with curd washing stations/tanks, pressing belt, area for addition of dressing (in vats or by mixers), and a packaging/filling system. Several types of heating systems are used, including drawing whey from the top of the vat and passing it through a heat exchanger and 'jet cooking', which is similar to the previous system except that culinary steam is injected into the whey. Uniform heating is critical to prevent 'burn-on' during the cooking stage. Gentle agitation is required as the curd is fragile; several agitator designs have been developed including vertical stirring motion (e.g., the Vert-Stir® from Stoelting) to minimize damage.

Defects

Minor sludge formation is a defect that can occur during Cottage cheese manufacture. This 'sludge' is caused by agglutination of starter bacteria and milk proteins, which can occur during the incubation period. Homogenization of the skim milk and careful selection of starter cultures are two methods commonly used to reduce the likelihood of this defect occurring. A 'floating curd' defect can also occur during cooking of Cottage cheese curd and is usually attributed to cultures (e.g., citrate-positive strains of *Lc. lactis*) that may produce excessive amounts of carbon dioxide. Careful selection of starter cultures for both acid- and flavor-producing ability is necessary. The defect called 'major sludge formation', where all the curd seems to form a sludge at the bottom of the vat, is usually thought to be due to phage infection of the vat during fermentation.

In contrast to most other fresh cheeses, Cottage cheese has a granular, curdy texture rather than a viscous, smooth, or pasty body. Sorbates and other preservatives are often used to increase shelf life. Another approach to increase shelf life is injection of carbon dioxide (about 600–1100 mg kg^{-1}), which is injected directly in the cream line. Carbonated cream enters the cream/curd mixer and the product is barrier packaged. The increase in shelf life (up to 8 weeks) depends on the amount of carbon dioxide initially dissolved in the product and the barrier properties of the packaging system during storage.

Cream Cheese

Introduction

Cream cheese includes several closely related products including single Cream cheese, double Cream cheese, Neufchâtel (spelled Neufchatel in the United States), and Bakers' cheese. In Germany, Rahmfrischkäse (*rahm* means cream in German) is a type of single Cream cheese, while Doppelrahmfrischkäse is a type of double Cream cheese. Cheeses that are closely related to Cream cheese are produced in other countries, for example, Petit-Suisse, Gournay, Bondard, Fromage a la Crème, and Carré Frais (*carré* meaning 'square') in France (some of these cheeses have a white surface mold). In the US standards of identity, Cream cheese must contain a minimum of 33% fat and a maximum of 55% moisture. Cream cheese is very popular in the United States, with total production of Cream and Neufchatel in 2007 around 350 000 tonnes. Cream cheese was first made in the United States in 1872. Cream cheese is used on bagels, in salads, and as an ingredient in flavored spreads, frostings, and cheesecake. In some countries, a Cream cheese-type product is made by combining a Quarg-like curd with cream or butter. Imitation cream cheese involves substitution of some or all the milk fat with vegetable fats.

Neufchatel cheese must contain ≥20% but <33% fat and a maximum of 65% moisture. Bakers' cheese is produced in the United States and is widely used in the bakery and confectionary trades, hence its name. It is made from skim milk and has a soft, dry, grainy, pliable curd. Neufchatel cheese is made from milk with 5% fat but otherwise by a procedure similar to that of Cream cheese; owing to its lower fat content, it has a grainier body and is less smooth than Cream cheese. The traditional French version of Neufchâtel may be made from whole raw milk that is heated to around 20–30 °C, at which point a small quantity of rennet is added and the milk allowed to coagulate for 1–1.5 days. During this coagulation period, the milk sours due to its adventitious microflora. The curd is hung in cloth bags to drain for half a day, after which pieces of mature Neufchâtel are added and the curd put into molds and covered and salted. The curd is allowed to age for about 10 days, although it can be aged longer. The rinds develop a white surface mold.

Recent innovations in Cream cheese include nonstandard of identity (United States) products that are designed to be more spreadable, whipped cream cheese, bagels with cream cheese in one container, wheyless cream cheese made with transglutaminase (*see* **Enzymes Exogenous to Milk in Dairy Technology**: Transglutaminase), and shelf-stable cream cheeses (processed cream cheese).

Manufacturing procedures

Single (fat) Cream cheese is made from milk with a fat content of 3–3.5%, while double Cream cheese is made from milk containing 8–14% fat. Industrially, there is trend of using higher TS in cheese milks to help reduce the amount of (acid) whey that would be needed to be removed from curd during cheesemaking. Milk is standardized and homogenized (e.g., 12–17 MPa at 50 °C) and cooled to ~31 °C for a short-set (incubation time ~5 h) and ~22 °C for a long-set (incubation time 12–16 h) procedure. Then, the starter is added (e.g., 2%), the level of which depends on the incubation period and temperature. At the end of incubation, the pH is 4.7–4.8. In some processes, a small amount of rennet may be added (e.g., 5 ml of standard rennet per 1000 l milk) with the starter, but is not essential.

The gel is broken using agitators and heated to 40–55 °C (to encourage syneresis and efficient separation), when whey is separated from the curd using either a Cream cheese separator or UF (operating temperature is usually 50–55 °C to reduce viscosity while concentrating). Traditionally, whey was drained using cloth bags. There are two main product types, cold-pack and hot-pack products. In the manufacture of cold-pack Cream cheese, after whey separation, the cold curd (~10–12 °C) is salted, stabilizers are added, and the product is packaged. Typically, around 0.3% of a single stabilizer or combination of several stabilizers, such as locust bean gum, guar gum, xanthan gum, and carrageenan, is added. Stabilizers are added to help prevent syneresis and the appearance of free moisture on the surface of the product during storage. In some Cream cheese products, whey protein concentrates are also added as a stabilizer. In the hot-pack process, the curd is mixed with salt and stabilizers in kettles or scraped-surface heated vats and heated to 65–70 °C. The hot curd (~65–70 °C) is then pumped into packages and subsequently allowed to cool. The curd is sometimes heated to temperatures as high as 80 °C to aid mechanical separation of whey and this further heat treatment of the curd may be carried out in a tubular heater. Instead of a tubular heater, the hot (~70–75 °C) product may be homogenized at 12–15 MPa. Cream cheese is also made by direct acidification (using organic acids) of milk.

Equipment

Essential equipment for cold-pack Cream cheese and (United States) Neufchatel includes basic mix pasteurizing vats, homogenizer, plate surface or tubular heat exchangers, fermentation vats, balance tanks, mechanical separator or UF unit, centrifugal and positive displacement pumps, fillers, blenders, and packaging lines. Tubular heat exchangers are often used to cool the viscous cheese for cold-pack products. For hot-pack Cream cheese, large jacketed vats with agitators or a scraped-surface heat exchanger and an additional homogenizer for the hot mix are also required. Double Cream cheese separators have a design different from that of Quarg separators because the higher fat content results in ejection of the whey (being heavier) toward the bowl wall, and ejection of the cheese product toward the center.

Texture and defects

If the pH of the cheese is too high (i.e., >4.7), the texture will be soft and the cheese will lack flavor. At a very low pH (<4.6), the texture may become too grainy and the flavor too acidic. Defects in these cheeses include whey separation from the product during storage and a grainy chalky texture, especially in the lower fat types. Hot-pack cheese has a more brittle texture than the cold-pack product due to the additional heating and shearing treatments. Cream cheese should be spreadable as it is commonly used on bagels and in cheesecakes. Cream cheese is often sold in plastic tubs, in cartons, or wrapped in metal foil, and is often blended with various flavors, herbs, and spices. The shelf life of the cold-pack product is only a few weeks, whereas the hot-pack cheese has a shelf life of up to 3 months on refrigerated storage. Improperly stored or packaged Cream cheese can undergo lipid oxidation, which can limit its shelf life.

Quarg

Introduction

Quarg (Quark) is another fresh cheese that is often mixed with cream, fruit, or seasonings. Quarg is a smooth, soft, spreadable paste, which is used in many home-prepared foods and dishes. This cheese is popular in central Europe (e.g., Germany, Poland, and Austria). Other names for this type of product in different countries include Kvarg, Tvarog, Tworog, Twarog, Sauermilchquark, and Speisequark. Chakka, a product related to Quarg, is popular in India; it is a fermented curd formed by acid coagulation of milk at 28–30 °C and drainage of whey using muslin bags. Shrikhand (also spelled Srikhand) is a fresh cheese made in India by mixing sugar and spices to Chakka. Fromage frais (fresh cheese) is a cheese related to Quarg and is produced in France and some other European countries. Fromage frais products are produced with a range of fat contents and are often blended with fruits. These cheeses are marketed as chilled (dairy) desserts. Similar products are produced in other parts of the world. In Germany, several cheeses (e.g., Harzer, Mainzer, and Handkaese) are made from an acid curd (Quarg-like) product by allowing a surface flora (smear) to develop and therefore this type of cheese undergoes ripening.

Manufacturing procedures

The dry matter content of nonfat Quarg varies from 14 to 24%; for example, in Germany, the minimum TS and protein contents of soft cheese are 18 and 12%, respectively. The protein content of nonfat Quarg varies from 12 to 18%. The higher levels of solids are harder to achieve using UF or a Quarg separator than by the traditional process. Traditional Quarg was separated from whey using filter cloths or muslin bags (i.e., in batches). Quarg-like products with many different fat contents are manufactured in European countries.

In the traditional manufacture of Quarg, the milk is pasteurized just before fermentation. However, severe heat treatment of milk, which denatures whey proteins, is now commonly used in the manufacture of Quarg. There are two main processes for the manufacture of Quarg, thermoquarg and UF Quarg. In the thermoquarg process, milk is subjected to a high-temperature–long-time pasteurization at 85–95 °C for 3–15 min (using a long tubular holding section in the heat exchanger) and further treatment (56–60 °C for up to 3 min) of the acidified milk before cooling to ~40 °C for centrifugal separation. Both heat treatments are considered to give a higher yield (~10%) because of the incorporation of denatured whey proteins. In the Centriwhey and Lactal processes, Quarg whey is heat-treated (e.g., 95 °C) and concentrated (these two methods differ in the TS content of the denatured whey product), and the denatured protein is then added back to cheese milk used to make the next batch of Quarg. This process is used to alter the texture and increase cheese yield. The main difference between thermoquarg and UF Quarg processes is that whey is separated by UF in UF Quarg.

After heat treatment, milk is cooled to 20–28 °C and a starter culture (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and/or *Leuconostoc* spp. and citrate-positive strains of *Lc. lactis*) is added (0.3–4%) together with a small amount of rennet. The level of rennet added is usually about 10% of that used in the manufacture of rennet-coagulated cheese, that is, 5–15 ml standard rennet per 1000 l milk. Often, some acidity (e.g., pH 6.3) is allowed to develop (e.g., 60–90 min after the addition of starter cultures) before rennet is added. This gives a firmer coagulum in high-heat-treated milk. After ~16 h, when the gel is at pH ~4.6, the coagulum is stirred. In a short-set method, a temperature of 27–30 °C is used and the incubation time is ~5–6 h. The mix is then heated (e.g., 60 °C for 1–6 min) and separated using either a Quarg (centrifugal) separator (operating temperature 32–44 °C) or UF membranes (operating temperature 40–45 °C), both working in a continuous mode. If the Quarg is creamed, an appropriate amount of sweet or cultured cream is added prior to packaging. The shelf life is usually 2–4 weeks with refrigerated storage. For a long-life Quarg, the final product is heat-treated, which helps to reduce bacterial numbers. Suitable stabilizers are added to a buffer tank after separation and prior to final heating.

Equipment

A Quarg production line is similar to that for Cream cheese and contains fermentation tanks, plate heat exchangers, Quarg separator/UF unit, positive displacement pumps, plate coolers, creaming tank or mixer (if fat is added), and a filling/packaging system. Quarg separators are somewhat similar to cream separators but the bottom part of the bowl is equipped with nozzles through which curd is discharged continuously.

General properties

Quarg from skim milk is smooth and white with a mild clean, acid flavor. Addition of cream gives extra flavor and smoothness. Excessively high calcium and rennet levels are considered to be associated with a 'bitter' taste defect. Quarg can also be made using partially concentrated milk, followed by acidification, coagulation, and UF separation. Acidification of the partially concentrated milk prior to UF helps to reduce the calcium content of the final cheese. Gelatin or other hydrocolloids are sometimes added to Quarg as stabilizers.

Acid/Heat-Coagulated Cheeses

Queso Blanco or Hispanic Cheeses

Several types of fresh acid/heat-coagulated cheeses are made in Latin American countries under the general name Queso Blanco (also called Hispanic or white cheeses). Many types of Queso Blanco cheeses, for example, Queso Fresco, Queso de Prensa, Queso de Puna, Queso de Hoja, Llanero, and Quesillo, are usually coagulated with rennet. The manufacturing procedure (e.g., the use of rennet or acid/heat to coagulate the milk) for Queso Blanco cheeses can vary from country to country and also from the traditional (farm) process to that used in modern factories. The acid/heat-coagulated Queso Blanco cheeses (such as Queso del Pais, Queso de la Tierra, and Queso Sierra) are usually made from skimmed, partially skimmed, or whole milk that is pasteurized at ~80–85 °C. Acidulants (e.g., acetic, lactic, or citric acid) are added to the hot milk to bring the pH to ~5.0–5.4 and then stirred, dewheyed, and salt and flavors added. The mixture is then pressed, packaged, and stored. In traditional methods for the production of these types of cheese, the milk is acidified by the addition of vinegar, lemon juice, or other fruit juices that are available locally. For some cheeses, after curd has been precipitated and cooled to ~32 °C, a lactic starter culture (mainly *Lactobacillus* spp.) may be added to give additional flavor to the cheese; the cheese is then packaged as usual.

Ricotta and Ricottone Cheeses

Ricotta cheese was traditionally made in Italy from the whey from sheep's milk cheese, which was heated to denature and coagulate the whey proteins; the coagulated protein was scooped from the whey. The traditional process has been modified in some countries due to the increasing popularity of Ricotta, which is now often made from milk or milk/whey mixtures. Dried whey protein powders have also been used in the manufacture of Ricotta. In the traditional (batch) process, whey (or milk/whey mixtures) is acidified to pH 5.6–6.0 with starter culture or acid (e.g., acetic or citric acid) and heated to 80 °C by stream injection or by indirect heating of the vats. The flocculated protein rises to the surface where it is collected and separated from the whey and placed in molds for further drainage. Calcium chloride is sometimes added to improve flocculation. The curd may be homogenized to produce a smoother consistency. UF is now often used to concentrate the whey (or milk/whey mixtures) prior to or after acidification and the retentate is concentrated to ~30% TS. The UF retentate is then heated and hot packaged. Acidification can also be carried out by the addition of acid whey powder to milk. The whey derived from Ricotta cheese can be acidified to pH 5.4 with citric acid and heated to 80 °C to recover additional whey proteins and is used to make Ricottone cheese (sometimes whole milk is added to the whey). In a recently developed continuous Ricotta process, blends of full-fat milk and whey are heated to 90 °C and directly acidified with acid to pH 5.3–5.5, which results in precipitation of the protein. The curd is then separated from the whey on a conveyor belt and hot packaged. Ricotta is often used in baking and confectionary applications. Requesón is a spreadable Hispanic cheese that is similar to Ricotta.

Mascarpone

Mascarpone is a traditional soft Italian cheese that is made from hot (85–90 °C) cream (e.g., 25–35% fat) that is acidified with acid (e.g., acetic, citric, or tartaric acid) to pH 5.7–6.4. The curd formed is drained in cloth bags (for long periods, e.g., 20 h), mixed or whipped, and packaged. The cheese is consumed fresh (unsalted or lightly salted) and it is pale or cream-colored, with a mild creamy (slightly tangy) flavor and a thick, spreadable consistency. This cheese is often used in cakes or to make desserts (e.g., Tiramisu). Membrane filtration or separation has replaced the cloth bags. Recently, higher fat creams have been used in conjunction with UF and hot filling in order to speed up the long curd drainage time required with the traditional process; this process also increases yield and improves shelf life (up to 45 days at temperatures ≤4 °C).

Paneer

Paneer, another acid/heat-coagulated cheese, is popular in India and the Middle East. It is made from cows' or buffaloes' milk. The milk is heated to 85–90 °C, cooled slightly (e.g., to 72 °C), and lemon juice, citric acid, or sour whey is added to coagulate the milk (which usually takes only a few minutes). Sometimes a small quantity of calcium chloride is added to the hot milk to aid coagulation. The coagulated milk is stirred gently and the curds are separated from the whey by straining through cheese cloth. The curds are placed in hoops and lightly pressed, cooled using chilled water, packaged, and stored (usually no salt is added). The moisture content of the cheese is typically 50–55% and the pH is ~5.4. In India, the regulations for Paneer indicate that it should not contain more than 70% moisture and the milk fat content should not be less than 50% of the dry matter. Versions of this cheese are made in India by different methods, including using rennet to coagulate the milk (e.g., Panir), allowing milk to sour naturally before heating, or adding sour buttermilk to freshly boiled milk. Chhanna or Channa (which is also produced in India) is very similar to

Paneer cheese except that the curd is not pressed. In India, the regulations for Chhana indicate that it should not contain more than 70% moisture and milk fat should not be less than 50% of the dry matter. The shelf life is usually no more than about 6 days with refrigerated storage.

Derived Products

Acid whey is the major by-product from acid- and acid/heat-coagulated cheeses. The composition of the whey depends on the processing treatment used during cheese manufacture, in particular the heat treatment of the milk. In Cottage cheese production, acid cheese whey is produced, which contains undenatured whey proteins, nonprotein nitrogen, lactose, and some salts. In Cream cheese production, the whey separated in the cream separator can contain a higher fat content than cheeses like Quarg (since Quarg is made from skimmed milk). The fat content of the whey can be reduced by reseparating the whey using a standard milk separator. Acid whey is sometimes added back to the separated curd in order to standardize the final moisture content of cream cheese. In the traditional production of Quarg, acid whey is produced as a by-product of centrifugal separation. In the thermoquarg process, where whey is removed using a separator, the liquid stream contains nonprotein nitrogen and non-heat-precipitated whey proteins. When UF is used to dewater the curd, the resulting permeate contains nonprotein nitrogen but no whey proteins. Quarg can also be made from partially concentrated milk (by nanofiltration or UF), followed by acidification, coagulation (as a result of acid production), and UF separation. Acidification of the partially concentrated milk prior to UF helps to reduce the calcium content of the final cheese, which may reduce the possibility of bitterness. The (sweet) permeate produced during concentration of milk is easier to process than acid whey and reduces the volume of acid whey produced during curd/whey separation.

See also: **Enzymes Exogenous to Milk in Dairy Technology**: Transglutaminase. **Milk Proteins**: Casein, Micellar Structure.

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Cheddar-Type Cheeses

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Introduction

Cheddar is a hard ripened cheese produced by acidification and concentration of bovine milk following gel formation with rennet. The unique stage of cheddaring, which gives the cheese its texture and functional character, involves the incremental application of pressure to curd, which encourages cohesion and flow of curd particles. The process of cheddaring was traditionally carried out by piling, turning, and repiling slabs of curd in open vats. Although this practice continues today in some small factories, this labor-intensive process has now been replaced by highly mechanized conveyor systems and cheddaring towers in large-scale commercial manufacture.

Legal standards for Cheddar stipulate that the cheese should contain $\leq$39% moisture and >48% fat in dry matter. Commercial Cheddar produced for the mature or extra-mature market would be expected to have a moisture content of 33–35%, a fat in dry matter content of 52–54%, a salt content of 1.6–1.8%, and a pH of 4.95–5.25.

Technology

Milk Pretreatment

Milk for Cheddar manufacture is standardized to a casein to fat ratio between 0.69 and 0.71. The absolute value is determined by the recovery of fat and casein in individual factories. Standardization is carried out to minimize the natural seasonal variation in milk composition, thereby ensuring efficient production of a uniform cheese in terms of its manufacturing characteristics and final composition. The yield of Cheddar is determined by the total fat and casein content of the milk. Both yield and quality of Cheddar are influenced by changes in milk composition relating to the breed of cow, differences between individual cows, for example, genotype, stage of lactation, seasonal changes in climate and feed, the age of cows, mastitis, and frequency of milking. Extended cold storage of milk prior to Cheddar manufacture can encourage the growth of psychrotrophic bacteria and induce physicochemical changes in the caseins. Both factors have potential to reduce cheese yield and impair cheese quality. Milk for Cheddar manufacture in commercial plants is pasteurized to eliminate pathogenic bacteria. At certain times of year, calcium chloride may be added to the milk to optimize coagulation of the casein.

Basic Steps in Cheddar Manufacture

An outline of the basic steps in Cheddar production is given in **Figure 1**. Manufacture begins with coagulation of starter-acidified milk with rennet. The rennet may be a chymosin of bovine origin or the chymosin may be fermentation-derived. Chymosin substitutes such as proteases from *Rhizomucor miehei*, *Rhizomucor pusillus*, or *Cryphonectria parasitica* are also used commercially. The chymosin destabilizes the colloidal suspension of casein micelles by hydrolysis of κ-casein causing the micelles to aggregate and form a gel. The resulting coagulum, which is formed at 30–32 °C, comprises a continuous casein network, which entraps fat. Once formed, the coagulum is cut into small cubes, which are heated and stirred in whey to facilitate expulsion of moisture. As the temperature is increased from 30 to 39 °C over a period of approximately 45 min, the curd particles begin to contract and expel moisture by syneresis. Moisture expulsion is further enhanced by continued acid production by the starter and maintenance of the temperature while stirring at 39 °C for a further 45 min. At an appropriate level of acidification, the whey is separated from the curds by drainage. Following drainage, the process of cheddaring begins. During this period, the curds are encouraged to fuse by application of pressure. As acid development by the starter culture continues, the curd particles fuse together to form a solid mass in which a central channel is first cut to allow whey drainage. The curd mass is cut into slabs, which are turned and stacked at regular intervals over a period of about 90 min. In modern mechanized cheese plants, the process of cheddaring is highly automated and employs a continuous conveyor belt or tower system. The perforated belt allows whey drainage as the curd particles fuse together and incorporates a mechanism for turning the curd. When the optimum acidification level is obtained, the curd is milled and dry salted. Addition of salt facilitates further reduction of moisture and inhibits acid production by the starter. The salted curds are transferred to molds for overnight pressing in traditional cheesemaking. However, in highly automated manufacturing systems, the milled curd particles are vacuumed into tower block formers. The tower acts as a pressing system by virtue of the weight of curd in the

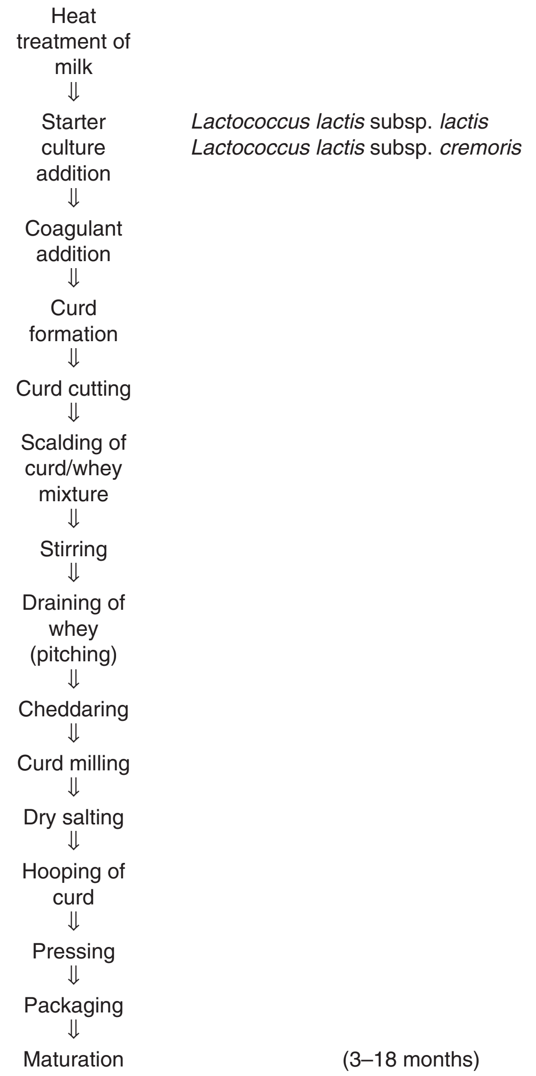

Figure 1 Basic steps in Cheddar manufacture.

vertical column. Whey is continually removed as the curd mass proceeds down the tower. A guillotine system at the base of the tower, called a 'block former', cuts a 20 kg block from the stack at regular intervals and the block is transferred to a vacuum packing system. The pressed cheese is matured at a temperature of approximately 10–12 °C for 3, 6, 9, and 12 months or longer depending on the flavor intensity required in the final product.

Starter Cultures

The primary function of starter bacteria in Cheddar manufacture is to produce acid consistently during the cheesemaking process, and the rate at which acid is produced is critical in determining cheese quality. The starter bacteria also contribute to flavor development during Cheddar maturation by releasing a complex mixture of enzymes that are responsible for proteolysis of caseins to peptides, degradation of peptides to amino acids, and conversion of amino acids to potent volatile flavor and aroma compounds. The starter culture in freshly pressed Cheddar cheese is present at a level of 10^8 cfu ml^{-1}; during maturation, the starter cells lyse and release a mixture of intracellular enzymes into the curd that are responsible for flavor and aroma development.

The lactic acid bacteria used in Cheddar manufacture are homofermentative mesophilic type cultures with an optimum growth temperature of 30 °C. Generally, they are mixtures of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. In commercial Cheddar manufacture in large mechanized plants, consistency of acid production throughout the cheesemaking process is essential and strains must be selected rigorously for their resistance to phage, acid-producing capabilities, and their contribution to flavor development. Two types of starter systems, bulk starter and direct vat inoculation (DVI) or direct vat set (DVS) cultures, are used widely in Cheddar manufacture.

A high proportion of Cheddar produced worldwide is made with a multiple-strain starter comprising a mixture of 2–3 strains. The strains are grown separately and blended prior to inoculation into a bulk starter tank. If a fast-replicating phage is detected attacking one of the strains, that strain is then withdrawn and replaced by a phage-unrelated strain. This multiple-strain approach incorporating strain replacement is currently practiced in New Zealand, Australia, the United States, and Ireland.

In the 1980s, the large-scale culture producers introduced DVS or DVI cultures for Cheddar manufacture. These are concentrates of starter cells added in frozen or freeze-dried form directly to the cheese vats, thereby eliminating the need for preparation of bulk starter. Usually, these contain three or four strains, which again have been selected for their phage robustness, consistency of acid production, and contribution to flavor development. DVI allows for flexibility in cheese manufacture, since this type of culture can easily be adapted to changes in milk volume or other changes in production planning.

With the introduction of DVI cultures in Cheddar manufacture, a new development was the blending of mesophilic and thermophilic strains. Compared with freeze-dried pure mesophilic blends, it was shown that the inclusion of *Streptococcus thermophilus* allowed lower inoculation levels (30–50% lower cell numbers) and much enhanced phage durability. However, the acidification profile during cheesemaking shows some fundamental differences from that obtained with a bulk starter culture.

Cultures in DVI freeze-dried form generally have a longer lag phase in culture growth and observable acid production compared to bulk starters due to the time required for rehydration and commencement of growth and metabolic activity. This results in an overall slower acidification profile. In commercial Cheddar production where a milling acidity of 0.45–0.55% titratable acidity (pH 5.2–5.4) is required within 4.5–5 h total elapsed time, this increase in lag phase is significant and would have to be compensated by the use of a high inoculation level to increase the rate of acid production to the required commercial parameters. Approximately 5×10^7 cells ml^{-1} of milk of mesophilic freeze-dried cultures are required compared to 5×10^6 cells ml^{-1} of milk for mesophilic bulk starter systems. By introducing *St. thermophilus* into the blend, it was possible to reduce the inoculation level to 2×10^7 cells ml^{-1} of milk.

During Cheddar manufacture with freeze-dried DVI, there is little observable acid production during the ripening, coagulation, and scalding stages. Acidity at the pitching (whey removal) stage would be expected to be 0.13% compared with 0.17% with bulk starter. These differences in the acidification profile cause a higher retention of moisture in curd, which is overcome by the introduction of a 5–10 min dry stir stage prior to cheddaring. Changes in the acidification profile also require adjustment of renneting procedures since the acidification profile influences the retention of rennet in the curd, which impacts on flavor and texture development.

Non-Starter Lactic Acid Bacteria

Adventitious non-starter lactic acid bacteria (NSLAB) dominate the viable microflora of Cheddar cheese for most of the ripening period. The source of adventitious NSLAB in Cheddar made from pasteurized milk has been the subject of much debate. High-quality raw milk contains approximately 10^2 cfu lactobacilli per ml. Factory-made Cheddar cheese contains NSLAB populations at levels of 10^1–10^2 cfu g^{-1} immediately on removal from the press. The population increases to 10^6–10^7 cfu g^{-1} within about 3 months and remains at this level throughout maturation. The source of NSLAB contamination is either postpasteurization contamination from the factory environment or failure of pasteurization to fully destroy NSLAB present in raw milk.

Studies in New Zealand identified the majority of adventitious NSLAB strains in Cheddar as belonging to two species, *Lactobacillus paracasei* and *Lb. rhamnosus*, but the composition of the adventitious NSLAB populations was shown to vary between factories, with the day of manufacture, and throughout the course of ripening. Similar observations have been made in the United Kingdom and Ireland. In UK commercial Cheddar, *Lb. paracasei*/*Lb. casei* and *Lb. plantarum* were the dominant species in cheeses ranging in age from 6 to 9 months; however, *Lb. plantarum*, *Lb. curvatus*, *Lb. brevis*, *Lb. helveticus*, *Lb. fermentum*, *Lb. bifermentans*, *Lb. buchneri*, *Lb. parabuchneri*, *Lb. farciminis*, and *Lb. kefir* were also isolated. Population dynamic studies on Irish Cheddar matured for 9 months showed a mixed population of *Lb. paracasei*, *Lb. plantarum*, and *Lb. rhamnosus* up to 6 weeks of maturation. Thereafter, *Lb. paracasei* was dominant and evidence was found for the appearance, disappearance, and recurrence of different strains of this species.

Although Cheddar cheese can develop full mature flavor in the absence of NSLAB, as evidenced by cheesemaking trials in aseptic vats, the NSLAB are considered to add desirable flavor notes such as savory, sulfur, fruity, and nutty, and also attenuate the harshness and bitterness associated with some starter cultures, which have been selected solely for rapid, robust acidification. Selected strains of NSLAB for Cheddar are now commercially available from several culture houses as adjunct cultures. If strains are carefully screened for suitability within an individual factory, these adjunct cultures can be used to improve flavor quality, impart specific flavor notes, and accelerate the rate of maturation. Given the complexity of the origin and nature of the ripening enzyme complement in Cheddar, and the variability between starter systems and milk supplies, adjunct cultures have to be rigorously screened for effective application.

Cheddar Maturation

Flavor and texture in Cheddar develop over an extended maturation period. Maturation period can vary depending on the type of cheese: 3 months for a mild cheese, 6 months for medium mature, 9 months for mature, 12–18 months for extra mature, and 18–24 months or more for vintage Cheddar. Proteolysis is the most extensive biochemical event occurring during Cheddar maturation, while lipolysis is extremely limited. In mature Cheddar, approximately 25% of the total nitrogen is soluble in water, and small peptides and amino acids that contribute to flavor generally partition into this fraction.

The proteolytic process is initiated by the hydrolysis of α_{s1}-casein by chymosin originating in the coagulant and the degradation of β-casein by the casein micelle-associated plasmin. Changes to the equilibrium of casein-bound and soluble calcium and the initial degradation of the caseins in the first weeks of ripening result in the softening of the rubbery texture of young Cheddar curd but the large peptides generated are tasteless. Further degradation of the casein-derived peptides by the starter lactic acid bacteria and NSLAB results in the formation of small peptides, amino acids, and volatile compounds derived from amino acid catabolism. These volatile compounds contribute to aroma and flavor perception.

Chymosin

Approximately 6% of the chymosin added to milk for Cheddar manufacture is retained in the curd. Chymosin retention in Cheddar is determined by the pH of whey at drainage and its activity during ripening is controlled by the pH of the curd. Residual chymosin in Cheddar rapidly hydrolyzes α_{s1}-casein at the Phe$_{23}$-Phe$_{24}$ bond producing α_{s1}-casein (f1–23) and α_{s1}-casein (f24–199). In mature Cheddar (>6 months), this hydrolysis is complete. During the early stages of ripening, the concentration of α_{s1}-casein (f24–199) increases initially, but as ripening progresses it is further hydrolyzed by chymosin predominantly at the Leu$_{101}$-Lys$_{102}$ and to a lesser extent at the Phe$_{32}$-Gly$_{33}$ and Leu$_{109}$-Glu$_{110}$. Peptides resulting from the cleavage of these bonds, including α_{s1}-casein (f24–199), α_{s1}-casein (f33–199), α_{s1}-casein (f102–199), and α_{s1}-casein (110–199), have been identified in the water-insoluble fraction of Cheddar. Further hydrolysis of the α_{s1}-casein peptides is achieved by the action of plasmin and the lactococcal cell envelope-associated proteinase (CEP).

Plasmin

The indigenous milk proteinase, plasmin, is stable to pasteurization and is almost completely retained in curd during Cheddar manufacture to give a concentration of approximately 3–4.5 $\mu g\,g^{-1}$ curd. The main role of plasmin in Cheddar ripening is in the degradation of β-casein. β-Casein is more resistant to hydrolysis than α_{s1}-casein and only 50% of β-casein is degraded in mature Cheddar. The primary cleavage sites of plasmin on β-casein are Lys$_{28}$-Lys$_{29}$, Lys$_{105}$-Gln$_{106}$, and Lys$_{107}$-Glu$_{108}$. Hydrolysis at these sites yields the peptides β-casein (f29–209), β-casein (f106–209), and β-casein (f108–209), all of which have been identified in the water-insoluble fraction of Cheddar. Plasmin is also responsible for the cleavage of α_{s1}-casein (f24–199) at the Lys$_{103}$-Tyr$_{104}$ and Lys$_{105}$-Val$_{106}$ yielding α_{s1}-casein (f104–199) and α_{s1}-casein (f106–199). These peptides are also found in the water-insoluble fraction of Cheddar.

Lactic Acid Bacteria

The lactic acid bacteria are weakly proteolytic, but release a complex well-characterized proteinase/peptidase system into the curd that contributes to flavor development. This enzyme complex is capable of hydrolyzing oligopeptides produced by chymosin and plasmin activity on caseins to small peptides and amino acids. Proteolysis by lactic acid bacteria contributes to Cheddar aroma and flavor mainly by producing free amino acids, which act as precursor compounds for further catabolic reactions. Catabolism of amino acids results in the production of potent volatile compounds. Starter bacteria contribute to protein breakdown to a greater extent than NSLAB. NSLAB activity is focused primarily on peptidolysis and the release of free amino acids.

Proteolytic Activity in Starter and Non-Starter Lactic Acid Bacteria

The starter lactic acid bacteria make a significant contribution to cheese proteolysis. The proteolytic system of *Lc. lactis* consists of a CEP (lactocepin, Prt) and a diverse range of intracellular peptidases in addition to peptide and amino acid transport systems. The specificity of lactococcal CEP on caseins and casein-derived peptides shows characteristic strain-specific differences. The enzyme is plasmid-encoded and spontaneous proteinase-negative mutants occur through plasmid loss. Intracellular peptidases include oligoendopeptidases (PepO and PepF), at least three general aminopeptidases (PepN, PepC, and PepG), glutamyl aminopeptidase (PepA), pyrrolidone carboxyl peptidase (PCP), leucylaminopeptidase (PepL), X-prolyldipeptidyl aminopeptidase (PepX), proline iminopeptidase (PepI), aminopeptidase P (PepP), prolinase (PepR), prolidase (PepQ), general dipeptidases (PepV, PepD, and PepDA), and a general tripeptidase (PepT).

Some water-insoluble peptides produced from α_{s1}-casein by chymosin or from β-casein by plasmin are degraded further by the lactococcal CEP. The peptide α_{s1}-casein (f1–23) is hydrolyzed rapidly by the lactococcal CEP at the bonds Gln$_{9}$-Gln$_{10}$, Gln$_{13}$-Glu$_{14}$, Glu$_{14}$-Val$_{15}$, and Leu$_{16}$-Asn$_{17}$. Many of the small peptides originating from this activity have been identified in Cheddar and some have been shown to be partially hydrolyzed by an aminopeptidase, releasing free amino acids. The lactococcal CEP also hydrolyzes α_{s1}-casein (f24–199) and the oligoendopeptidases (PepO and PepF) contribute to further degradation. The lactococcal CEP actively hydrolyzes peptide bonds in the N-terminal half of β-casein. The bonds Gln$_{56}$-Ser$_{57}$ and Asn$_{68}$-Ser$_{69}$ are the preferred cleavage sites. The absence of an intact plasmin cleavage site in these peptides suggests that the lactococcal CEP hydrolyzes proteose peptones rather than intact β-casein. Most of the water-soluble peptides of Cheddar are derived from the N-terminal portion of α_{s1}-casein, especially from residues 53–91, with a smaller number from the N-terminal region of β-casein.

The proteolytic activity of the NSLAB supplements that of the starter. NSLAB produce peptides with similar molecular weights as those generated by the starter culture and also add to the amino acid pool.

Amino Acid Catabolism

The free amino acids generated as a result of proteolysis may be catabolized by starter lactic acid bacteria and NSLAB to flavorful volatile compounds comprising aldehydes, alcohols, esters, and thiols. The catabolism of amino acids is mediated by aminotransferases (ATs, EC 2.6.1). ATs are intracellular enzymes and their activity has been demonstrated under Cheddar ripening conditions (pH 5.0, 10–13 °C, 4% NaCl). Amino acid degradation is a major process for aroma formation in Cheddar, and the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and branched-chain amino acids (leucine, isoleucine, and valine) are the major precursors of these aroma compounds.

Aroma compounds originating from amino acid catabolism and which have been isolated from Cheddar include 3-methylbutanal and 3-methylbutanoic acid from leucine and phenylethylacetate from phenylalanine. Methional and methanethiol are produced by the catabolism of methionine and may be oxidized subsequently to dimethyldisulfide (DMDS) and dimethyltrisulfide (DMTS). As the final flavor of cheese depends on the respective concentration of various key aroma compounds, the control of amino acid degradation during cheese ripening provides a potential means of controlling and manipulating the flavor profile of Cheddar.

Textural Characteristics

Freshly pressed Cheddar cheese comprises a casein matrix interspersed with fat globules and water. The cheddaring process leads to the formation of a fibrous protein network that produces a very close-textured Cheddar cheese. The relative levels of fat, protein, and moisture affect the rheological properties of the curd, and the rate of texture development is associated with the degradation of the casein and the solubilization of casein-bound calcium.

Taste and Aroma

The basic or background taste of Cheddar is associated with the water-soluble nonvolatile fraction. This comprises small- and medium-sized peptides, free amino acids, and organic acids and their salts. Peptides and amino acids contribute to the background savory flavor of Cheddar, while specific peptides can impart undesirable bitter flavors. Volatile aroma compounds add specific character notes to the basic Cheddar flavor. These volatile compounds are derived mainly from products of proteolysis but also to a limited extent from lipolysis. Volatile compounds isolated from Cheddar include fatty acids, aldehydes, ketones, alcohols, esters, and sulfur compounds.

Sulfur compounds are key components of Cheddar flavor. Methanethiol, DMDS, and DMTS add garlic notes to mature Cheddar. The aldehyde 3-methylbutanal at high concentrations will impart unclean harsh flavors, but at low levels gives a pleasant fruity flavor. 3-Methylbutanoic acid, derived from leucine catabolism, gives a rancid, cheesy, sweaty odor. Butyric acid, a product of lipolysis, also contributes a cheesy, sweaty odor to Cheddar. Ethylbutyrate, the ester derived from butyric acid, will contribute to a fruity flavor defect if present at excessively high levels in Cheddar. Indole and skatole, products of the catabolism of phenylalanine, and other aromatic amino acid metabolites are associated with unclean ‘barnyard’ flavor notes in Cheddar.

Bitterness

Bitterness in Cheddar is associated with the accumulation of hydrophobic peptides, which are formed by the action of the coagulant and starter proteinases. Bitter peptides are derived from both α_{s1}- and β-caseins. Bitter peptides from α_{s1}-casein are predominantly from the region of residues 14–34, 91–101, and 143–151. Bitter peptides from β-casein are mostly from the sequence of residues 46–90, and particularly from the hydrophobic C-terminus. Chymosin is important in the production of bitter peptides, since residual coagulant in Cheddar releases extremely hydrophobic peptides from β-casein. Cutting the coagulum at lower than optimum pH in Cheddar manufacture results in excessive retention of chymosin in curd and the development of bitterness during maturation. A low salt content in the final cheese enhances chymosin activity and results in the accumulation of bitter peptides. The overall level of bitterness in Cheddar depends on the relative rates at which bitter peptides are formed and degraded to nonbitter peptides. Certain *Lc. lactis* strains are associated with the development of bitterness in Cheddar cheese. These strains survive well at the scald temperature used in Cheddar manufacture and their presence at high numbers in the fresh curd results in high proteolytic activity during ripening. The total proteolytic activity of the starter strains can have a significant effect on the development of bitterness. Strains for Cheddar production in New Zealand are carefully selected for specificity of lactococcal CEP, and mixed strain starters normally include one proteinase-negative strain in order to limit total proteolytic activity and control bitterness.

Defects

Defects in Cheddar cheese arise through contamination of milk with undesirable microorganisms, inadequate control of acidification during manufacture, and failure during manufacture to attain the optimum compositional factors of pH, salt, and moisture. Additionally, physical damage to packaging of vacuum-packed Cheddar can allow contamination and growth of undesirable microorganisms on the surface of the cheese.

Gas Production

Open texture and slits in Cheddar have been attributed to the growth of obligate and facultative heterofermentative lactobacilli. Fermentation of residual lactose and galactose resulting in the production of CO_2 is associated with this defect. Slow starter activity and a high level of residual lactose in the curd are the main cause. Late gas blowing, a rare defect in Cheddar, is associated with contamination of the cheese milk with *Clostridium tyrobutyricum*.

Surface Deposits

Crystallization of calcium lactate on the surface of Cheddar causes a white deposit with an appearance similar to white mold. The crystals detract from the appearance of the cheese and reduce consumer acceptability. The deposit is calcium-D-lactate, formed by the racemization of L(+)-lactic acid to D(−) lactic acid through the action of certain strains of NSLAB, particularly lactobacilli and pediococci. Accumulation of whey at the surface of vacuum-packaged aged cheese, coupled with increased growth and metabolism of NSLAB, facilitates rapid racemization of lactic acid and subsequent crystal formation. White crystalline deposits can appear in young and mature Cheddar and both on the surface and the interior. In addition to calcium lactate, white deposits have been associated with calcium phosphate and tyrosine.

Color Defects

Color plays an important role in consumer acceptability of Cheddar and can influence grading. Bleaching of color in Cheddar is sometimes observed shortly after manufacture. Localized areas of high acid production associated with whey entrapment between curd particles can cause bleaching during the early stages of maturation. This effect diminishes as the cheese matures and the pH increases.

Color defects ranging from pink to mud-brown have been observed sporadically in both annatto-colored and white mature Cheddar. Studies on the mechanisms of the formation of these defects in Cheddar have not been conclusive. Oxidative browning may be associated with the activity of tyrosinase, which catalyzes the oxidation of monophenols, particularly the amino acid tyrosine, to quinones. The red-colored dopaquinones and dopachromes formed from tyrosine via DOPA (3,4-dihydroxyphenylalanine) are then converted through a series of chemical reactions to melanin. Although evidence for the mechanism behind the development of color defects in mold-ripened cheeses exists, the sporadic nature of the defect in Cheddar has discouraged full investigation.

Mold Growth

Most commercially produced Cheddar is vacuum packaged and as molds require oxygen to grow and sporulate they are found where pockets of air exist between the cheese surface and packaging. Growth is limited by the amount of residual oxygen.

Penicillium spp., especially *Penicillium commune*, a blue mold, and *Cladosporium* spp., especially *Cladosporium cladosporioides*, a black mold, are found most frequently in vacuum-packed Cheddar.

See also: **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview. **Cheese:** Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Rennet-Induced Coagulation of Milk; Rennets and Coagulants; Starter Cultures: General Aspects; Starter Cultures: Specific Properties.

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Swiss-Type Cheeses

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Introduction

Swiss-type cheeses were originally manufactured in the Emmen valley (Swiss German: Emmental) in Switzerland; their precursors were mountain cheeses. The cheese type Emmental is probably the best-known Swiss-type cheese and is frequently referred to simply as Swiss cheese; it comprises cheeses with the desired propionic acid fermentation. Today, Emmental is produced in many countries, and a great variety of other Swiss-type cheeses is also available on the market, including Svenbo, Jarlsberg, Greve, Maasdamer, Leerdamer, and Swiss cheese. Their body and texture correspond to those of hard and semihard cheeses.

There is an internationally recognized Codex standard for Emmental, developed by the Codex Alimentarius, which differentiates Emmental from other cheese varieties. Codex describes Emmental as follows:

> Emmental is a ripened hard cheese ... The body has a ivory through to light yellow or yellow color and an elastic, sliceable but not sticky texture, with regular, scarce to plentiful distributed, mat to brilliant, cherry to walnut sized (or mostly from 1 to 5 cm in diameter) gas holes, but few openings and splits are acceptable. Emmental is typically manufactured as wheels and blocks of weights from 40 kg or more ... The typical flavor is mild, nut-like and sweet, more or less pronounced. (Codex Stan. 269/1967)

However, descriptions and analytical values presented in this article focus on Swiss Emmental cheese, which is produced from raw milk (**Figure 1**). To achieve the characteristic eyes and nutty flavor, selected cultures of *Propionibacterium* sp. are used.

The characteristics of Swiss-manufactured Emmental are

- cylindrical shape
- firm dry rind
- weight: 60–130 kg
- 1000–2000 round eyes, diameter 1–4 cm
- flavor: mild, nutty, slightly sweet, becoming more aromatic with increasing age
- cheese body: ivory to light yellow, slightly elastic

Production Statistics

In 2008, the annual production of Emmental in Switzerland is ~29 000 tonnes (2008). Swiss-type cheeses make an important part of cheese production in the European Union. The production of Emmental in France was 245 000 tonnes in (2006), Germany 90 000 tonnes (2006), Finland 24 000 tonnes (2004), and Austria 35 000 tonnes (2006). The production in the United States in 2007 amounted to 142 000 tonnes.

Technology

In Switzerland, Emmental cheese has to be manufactured from raw milk of cows fed with a silage-free diet; therefore, the bacteriological requirements are particularly stringent. Swiss Emmental is cooked at 52–54 °C after cutting. During pressing, the temperature remains around 50 °C for many hours. At this temperature, the curd dries and undesirable microorganisms are eliminated. The cooking temperature is, therefore, an important element of the hurdle technology for ensuring the hygienic safety of Swiss Emmental cheese produced from raw milk. An important step in the technology of Swiss Emmental cheese is the addition of water (12–18%) to the milk or the curd. This leads to a high pH value after the lactic fermentation (5.20–5.30) and consequently accelerates the propionic fermentation. Furthermore, the cheese texture remains soft and elastic due to a high calcium level. A soft and elastic texture is crucial for a regular eye formation.

Because propionic acid bacteria (PAB) are sensitive to salt, brining is less intensive than for other cheese varieties. To initiate the typical propionic acid fermentation, the ripening temperature must be raised to ~20–24 °C for a certain period of time. As soon as sufficient eye development is accomplished, propionic acid fermentation is retarded by storing the cheese at a lower temperature (10–13 °C).

Table 1 shows the average composition of Swiss Emmental at different stages of ripening. The very low salt content is typical for Swiss Emmental cheese. Swiss-type cheeses are always cooked. But nowadays, Swiss-type cheeses are manufactured in many countries

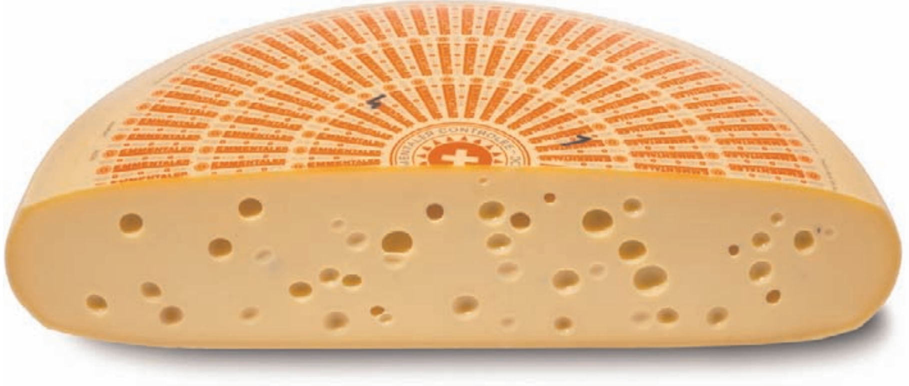

Figure 1 Emmental cheese from Switzerland – the original Swiss-type cheese. From www.emmentaler.ch.

Table 1 Average composition of Swiss Emmental cheese (mean values ± SD for $N = 30$)

Parameter		*Cheese age*				
	Unit	*1 day*	*20 days*	*3 months*	*6 months*	*1 year*
Water	g kg^{-1}	376.1 ± 5.8	365.8 ± 4.7	353.0 ± 6.3	347.5 ± 7.2	350.2 ± 6.1
Protein	g kg^{-1}	nd	nd	283.7 ± 6.3	284.8 ± 5.9	284.2 ± 6.4
Fat	g kg^{-1}	nd	nd	nd	322.2 ± 9.7	321.0 ± 9.0
Salt	g kg^{-1}	nd	2.28 ± 0.50	4.19 ± 1.01	3.69 ± 0.7	nd
pH value		5.30 ± 0.04	5.52 ± 0.04	5.65 ± 0.03	5.78 ± 0.03	5.70 ± 0.07

nd, not determined.

by using technologies that are different from the traditional Swiss procedures. However, treatment of milk, the extent of mechanization, weight and shape of a cheese loaf, average composition (hard and semihard varieties have different fat contents), ripening time, and shelf life are frequently very different from the original.

Cultures

Lactic Acid Bacteria

Thermophilic lactic acid bacteria starters are usually mixed cultures of Lactobacilli (*Lactobacillus delbrueckii* subsp. *lactis*) and Streptococci (*Streptococcus thermophilus*). Some decades ago, *Lactobacillus helveticus* was also a major component of starter cultures in the manufacture of Swiss Emmental. Due to its intensive proteolytic activity, which promotes late fermentation, it was replaced by *Lb. delbrueckii* subsp. *lactis*. Nonetheless, *Lb. helveticus* is still used in the manufacture of many other Swiss-type cheeses.

Starter cultures guarantee the homofermentative catabolism of lactose to >90% lactic acid. Lactose is generally metabolized via the fructose-1,6-diphosphate (Embden–Meyerhof–Parnas scheme) pathway and is fully hydrolyzed within 4–6 h after the addition of lactic starters. Lactic acid fermentation is completed after 24 h. Galactose from lactose breakdown is not utilized by the streptococci, but further metabolized by the lactobacilli. For streptococci, the optimum temperature for growth is between 38 and 42 °C and their optimum pH is between 6.0 and 6.5. Streptococci produce only L(+)-lactic acid, whereas *Lb. delbrueckii* subsp. *lactis* converts lactose entirely to D(−)-lactate. Lactobacilli grow best between 38 and 45 °C and in the pH range of 5.0–5.5. Lactic acid not only inhibits the development of undesirable microorganisms but also influences syneresis and thus the texture of and proteolysis in the cheese. In certain areas (where the cheese milk is collected twice daily), a mesophilic culture of *Lactococcus lactis* is added to preripen the evening milk.

During cheese ripening, the proteinases and peptidases of lactobacilli play a major role in the breakdown of casein.

Propionic Acid Bacteria

Propionic acid fermentation is essential in all Swiss-type cheeses to obtain the characteristic eyes and the nutty flavor. Fermentation is initiated by the addition of PAB cultures, usually containing the species *Propionibacterium freudenreichii*. These organisms are Gram-positive and appear under the microscope as short rods. Their pH optimum for growth is between 6.0 and 7.0 (maximum 8.5, minimum 4.6). The optimal growth temperature is 30 °C, but growth also occurs at 14 °C. PAB are anaerobic to aerotolerant. For Swiss Emmental, the inoculation size is small (only a few hundred colony-forming units (cfu) per milliliter of milk). Propionic acid fermentation begins about 30 days after the start of manufacture at about 20–24 °C for roughly 7 weeks and then continues at a slower rate at 10–13 °C. In cheeses ready for consumption, about 10^8–10^9 cfu g^{-1} PAB are present.

The lactic acid produced by the lactic starters is broken down by the classic metabolic pathway to propionate, acetate, and CO_2 as follows:

$$3\ \text{lactate} \rightarrow 2\ \text{propionate} + 1\ \text{acetate} + 1\ CO_2 + 1\ \text{ATP}$$

When aspartate is present, this fermentation may be combined with the deamination of aspartate to succinate:

$$1\ \text{lactate} + 2\ \text{aspartate} \rightarrow 1\ \text{acetate} + 1\ CO_2 + 2\ \text{succinate} + 2\ NH_3 + 1\ \text{ATP}$$

Thus, it appears that in the presence of aspartate more lactate is converted to acetate and CO_2 than to propionate. Strains capable of deaminating aspartate ferment higher amounts of lactate and are thus present in cheese at higher levels than are strains that do not utilize aspartate (**Table 2**). The higher number of PAB is responsible for increased amounts of acetate, propionate, succinate, and CO_2, and therefore for more intensive flavor and larger eyes. The capability of strains to utilize aspartate is a very important factor when selecting new cultures. A very high aspartase activity will increase the amount of CO_2 and therefore the risk of late fermentation. However, moderate aspartase activity may have a positive effect on the quality of Emmental as regards eye formation and flavor intensity.

Another metabolic pathway may also be used by the propionibacteria, namely, the formation of succinate by fixation of CO_2, which leads to a decrease in CO_2 levels. This metabolic pathway may play a minor role, but is not yet fully understood. The proteolytic activity of PAB is of little importance; the lipolytic activity, however, may influence the development of flavor during the ripening of the cheese.

Strain diversity of the natural propionibacterial flora in milk is great, which, fortunately, has not been influenced by the widespread use of commercially available cultures. Recent results have shown that wild-type strains could be as good as commercially available cultures of PAB.

Facultatively Heterofermentative Lactobacilli

Facultatively heterofermentative lactobacilli (FHL), Gram-positive microaerophilic rods, ferment hexoses almost exclusively to lactic acid via the fructose-1,6-diphosphate pathway. But at low levels of glucose, they also ferment hexoses to lactic acid, acetic acid, ethanol, and formic acid. Pentoses are catabolized to lactic acid and acetic acid by an inducible phosphoketolase.

This group of microorganisms contains, among others, *Lactobacillus casei* and *Lactobacillus rhamnosus*, which are indigenous to raw milk. Both species are able to grow at 15 °C; *Lb. rhamnosus* is the only species within the FHL that is able to grow at 45 °C.

By adding cultures of *Lb. casei* or *Lb. rhamnosus*, the intensity of propionic acid fermentation can be controlled and the risk of late fermentation minimized. The exact mechanism is not yet understood, but it is known that bacteriocin production is not involved. It is presumed that competition between the different organisms for available substrates plays a role. Cultures of FHL are widely used by producers of Swiss Emmental. These cultures are added to the cheese milk together with the starter cultures.

Cultures Used for Other Swiss-Type Cheeses

Propionic acid fermentation is common for all Swiss-type cheeses. Usually, thermophilic lactic starters

Table 2 Comparison of Swiss Emmental produced with a culture of high aspartase activity (P-culture) and Swiss Emmental produced with a culture of low aspartase activity (prop96)

		Composition at 3 months		*Composition at 6 months*	
		(mean ± SD)		*(mean ± SD)*	
Parameter		*P-culture (N = 20)*	*Prop96 (N = 10)*	*P-culture (N = 20)*	*Prop96 (N = 10)*
Aspartic acid	mg kg^{-1}	40 ± 98	174 ± 138	48 ± 101	377 ± 145
Asparagine	mg kg^{-1}	167 ± 275	317 ± 197	0 ± 0	295 ± 114
Aspartic acid + asparagine	mg kg^{-1}	208 ± 369	491 ± 303	48 ± 101	673 ± 115
Lactic acid	mmol kg^{-1}	52.8 ± 15.4	62.6 ± 10.6	32.6 ± 17.6	57.4 ± 11.1
Propionic acid	mmol kg^{-1}	60.1 ± 10.1	55.4 ± 5.6	75.0 ± 10.9	60.1 ± 4.6
Acetic acid	mmol kg^{-1}	48.8 ± 5.1	45.8 ± 2.2	56.5 ± 5.3	48.4 ± 1.4
Succinic acid	mmol kg^{-1}	9.5 ± 1.6	3.6 ± 0.6	12.2 ± 1.7	3.9 ± 0.6
Keeping quality (3 = good, 1 = bad)		2.3 ± 0.7	2.9 ± 0.1	1.9 ± 0.7	2.9 ± 0.2

Adapted from Bachmann HP (1998) Emmentaler-Käse: Aspartat-Vergärung erhöht Nachgärungsrisiko. *Agrarforschung* (4): 161–164.

such as *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, and *S. thermophilus* are also used. Some Swiss-type cheeses are semihard cheeses made from pasteurized milk. Therefore, mesophilic lactic acid bacteria such as *Lc. lactis* may also be used. Propionic acid fermentation is also carried out with *P. freudenreichii.* An exception is Comté fruité in France, which is produced without the addition of PAB to the cheese milk but with a spontaneous fermentation.

Eye Formation

The characteristic eye formation of Emmental cheese is due mainly to the presence of CO_2 produced by PAB during lactate breakdown. As shown in **Figure 2**, CO_2 diffusion begins prior to propionic acid fermentation, as small quantities of CO_2 are already produced during lactic acid fermentation and the degradation of citrate. In the initial stages of ripening, the fermentation of citrate leads to a higher number of eyes as a result of CO_2 production, but in the mature cheese, citrate fermentation leads to a lower number of eyes because of its retardant effect on propionic acid fermentation. The steep rise in the production of CO_2 coincides with the onset of propionic acid fermentation. After 60–70 days, CO_2 production and diffusion rates become identical. The diffusion rate drops as soon as the cheeses are transferred from the warm (22 °C) to the cold ripening room (10–13 °C).

Eye formation is a long process. In the beginning, that is, about 30 days after manufacture, only a few eyes appear; thereafter, the number of new holes increases progressively. The maximum rate is attained after about 50 days, which is also the time of rapid eye enlargement. The appearance of new eyes declines with decreasing CO_2 production and the simultaneous hardening of the cheese body in the cold ripening room. Nevertheless, eye formation sometimes continues in the cold room.

The number of eyes may be increased by the inhomogeneity of the curd, physical openness (entrapped air), and hydrogen-forming microorganisms. Centrifugation and thermization of the milk or application of vacuum after filling the curd into molds is performed to control the number of eyes. In a cheese loaf of 80 kg, total CO_2 production is about 120 l before the cheese is sufficiently aged for consumption. About 60 l remains dissolved in the cheese body, ~20 l is found in the eyes, and ~40 l diffuses out of the cheese.

CO_2 pressure passes through two major phases. The first covers the period of proper eye formation in the warm ripening room. During this period, the CO_2 pressure remains relatively low, between 1500 and 2500 Pa, because of the low resistance of the soft cheese mass to deformation at 22–24 °C. During storage, that is, the second stage, the CO_2 pressure increases to 4000–8000 Pa. The differences in pressure between various cheeses are higher in the second stage than in the first stage. The pressure increase in the second stage is explained by the higher resistance of the cheese mass to deformation, which is due to a decrease in temperature from 22 to 12 °C and by continued gas production. During the first stage, there is a marked pressure increase within the eyes.

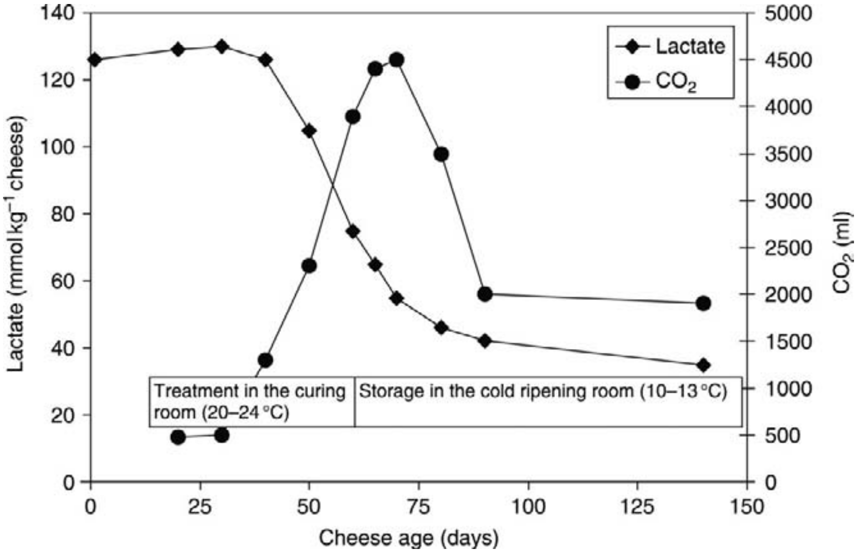

Figure 2 Lactic acid fermentation, lactate breakdown, and CO_2 loss during the ripening of Emmental cheese. Adapted from Steffen C, Eberhard P, Bosset JO, and Rüegg M (1993) Swiss-type varieties. In: Fox PF (ed.) *Cheese: Chemistry, Physics and Microbiology*, pp. 83–110. London: Chapman & Hall.

Very young (<3 months), fat-reduced or semihard Swiss-type cheeses are also available on the market these days. No rind or only a very thin one is present if the cheeses are ripened wrapped in plastic film; therefore, the diffusion of CO_2 is increased and eye formation is significantly reduced. Even cheeses without eyes may be sold nowadays as Swiss-type cheeses, which would not be in conformity with the Codex standard for Emmental.

Ripening Patterns

The relative humidity in the ripening room is rather low (70–80%). This, and also the brining, leads to a firm and dry rind, which reduces the loss of CO_2 and leads to the sturdy shape of the cheeses. Proteolysis and the consequent reactions, propionic acid fermentation, and lipolysis are important factors for ripening and flavor development.

In Cheddar- and Gouda-type cheeses rennet plays an important role in proteolysis. In Swiss-type cheeses, however, rennet is usually largely inactivated during the heating of the curd and does not play a significant role in proteolysis. In these cheeses, indigenous milk proteinases and the proteolytic enzymes of lactic acid bacteria are mainly responsible for protein breakdown. Generally, thermophilic lactobacilli with primarily proteinase activity exert a stronger proteolytic effect than mesophilic lactococci, which usually have more peptidase activity, whereas thermophilic streptococci have very little influence on protein breakdown. However, in recent years, selection and use of lactococci with increased peptidase activity became common practice to facilitate fermentation by PAB in Swiss-type cheeses made at low cooking temperatures. The proteolytic activity of PAB is not significant. Raw milk is used for Swiss Emmental, and the indigenous flora of milk may possibly be involved in proteolysis. The proteolytic enzymes of psychrotrophs from milk after prolonged cold storage sometimes influence ripening and flavor development. Proper selection of strains of lactic acid bacteria for starter cultures and the application of appropriate measures during manufacture to obtain the desired number of lactobacilli in the young cheese are the best means of controlling proteolysis. The activity of proteolytic enzymes in cheese further depends on water content, lactic acid concentration, pH, salt concentration, water activity, copper content, storage temperature, and time.

Common indices of proteolysis are the concentration of water-soluble nitrogen (WSN), 12% trichloroacetic acid-soluble nitrogen (TCA-SN), and levels of free amino acids. The amino acids are decomposed enzymatically by decarboxylation, deamination, and transamination, but nonenzymatic reactions may also be involved. The products arising from the catabolism of amino acids include aldehydes, ketones, short-chain acids, alcohols, aromatic acids, α-keto acids, hydrocarbons, amines, ammonia, and sulfur compounds. Lactic acid bacteria are the main contributors to amino acid catabolism, but PAB also have a high ability to convert branched-chain amino acids (isoleucine, leucine) to branched-chain fatty acids.

The formation of WSN and TCA-SN increases during storage in the warm ripening room and reaches 19.1 and 12.1% of total nitrogen (TN), respectively, after 3 months. After 12 months, WSN and TCA-SN are typically 28 and 21% of TN, respectively. The sum of free amino acids is 5.7% after 3 months and 12.5% of TN after 12 months. The content of arginine and of α- and γ-amino butyric acid after 12 months is typically below $200\,\mathrm{mg\,kg^{-1}}$. The median concentration of tyramine and histamine is $12\,\mathrm{mg\,kg^{-1}}$ (range $1–200\,\mathrm{mg\,kg^{-1}}$) and $45\,\mathrm{mg\,kg^{-1}}$ (range $1–400\,\mathrm{mg\,kg^{-1}}$), whereas the other biogenic amines such as tryptamine, putrescine, isopentylamine, and cadaverine are present only at trace levels below $10\,\mathrm{mg\,kg^{-1}}$ after 12 months.

The sum of free amino acids measured after 1 day with the Cd-ninhydrin method and the TCA-SN after 20 days allow an early prediction of flavor and texture development in Emmental cheese. These two parameters correlate well with the index of maturity, intensity of aroma, saltiness, and sourness of 3-month-old cheese. The higher the content of free amino acids after 1 day, the lower the stress and strain at fracture at 3 and 6 months.

Swiss Emmental can be found today on the market in a wide range of maturation: from very young cheese (at least 4 months) with TCA-SN of less than 12% of TN up to extra mature cheese (>1 year) with TCA-SN of 23% of TN. Other Swiss-type cheeses are normally consumed at a very young age and do not show intensive proteolysis.

Table 3 shows typical ripening parameters of Swiss Emmental cheese at 1 day, 20 days, and 3, 6, and 12 months. The lactate concentration is maximum at 20 days at $133\,\mathrm{mmol\,kg^{-1}}$. Due to propionic acid fermentation, lactate is decomposed to CO_2, acetic acid, and propionic acid, and the lactate concentration reduces very fast within the first 3 months. After 60–70 days, the cheeses are transferred to the cold ripening room and the lactate consumption is much slower; after 12 months $40\,\mathrm{mmol\,kg^{-1}}$ of lactate are still traceable (**Figure 3**).

Lactic acid fermentation produces about $20\,\mathrm{mmol\,kg^{-1}}$ acetate in the first 20 days. By propionic acid fermentation, the acetate and propionate concentration increases to 48 and $63\,\mathrm{mmol\,kg^{-1}}$, respectively.

Lactic acid bacteria have only limited lipolytic activity, with streptococci having the highest activity. PAB, in contrast, have comparably high strain-dependent lipolytic activity, 10–100 times more than lactic acid bacteria.

Table 3 Ripening parameters of Emmental cheese (mean values ± SD for $N = 10$)

Parameter		*Cheese age*				
	Unit	*1 day*	*20 days*	*3 months*	*6 months*	*1 year*
L-Lactic acid	mmol kg^{-1}	62.7 ± 4.8	67.1 ± 4.1	33.2 ± 8.7	31.1 ± 9.3	25.4 ± 8.1
D-Lactic acid	mmol kg^{-1}	63.9 ± 6.0	65.8 ± 6.0	29.4 ± 4.9	26.3 ± 4.3	21.6 ± 6.7
Succinic acid	mmol kg^{-1}	nd	nd	3.6 ± 0.6	4.0 ± 0.6	5.1 ± 2.8
Acetic acid	mmol kg^{-1}	nd	20.0 ± 1.9	45.8 ± 2.0	48.4 ± 1.3	47.6 ± 2.5
Propionic acid	mmol kg^{-1}	nd	0.6 ± 1.1	55.4 ± 5.3	60.1 ± 4.4	63.2 ± 4.2
Butyric acid	mmol kg^{-1}	nd	0.2 ± 0.1	0.8 ± 0.2	1.1 ± 0.2	1.7 ± 0.9
Water-soluble nitrogen	mmol kg^{-1}	nd	218 ± 17	610 ± 31	693 ± 33	901 ± 28
12% trichloroacetic acid-soluble nitrogen	mmol kg^{-1}	nd	90 ± 9	386 ± 39	469 ± 47	683 ± 60
Sum of free amino acids	g kg^{-1}	nd	2.2 ± 0.5	16.1 ± 3.7	22.7 ± 3.2	35.6 ± 4.8

nd, not determined.

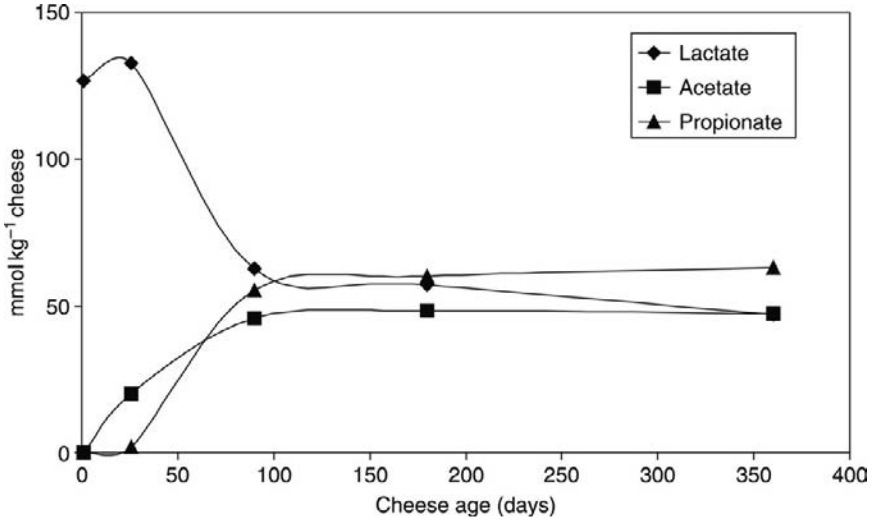

Figure 3 Lactate breakdown and production of propionate and acetate during the ripening of Emmental cheese.

Lipolysis is generally recognized as necessary to produce the typical Emmental cheese flavor. The amount of free fatty acids varies from 2 to 7 g kg^{-1}. Higher contents, however, give flavor defects. The release of free fatty acids starts in the warm room simultaneously with the growth of PAB.

By the use of milk of cows fed with a silage-free diet, butyric acid fermentation (late gas blowing) can be avoided without the addition of lysozyme or nitrate, or bactofugation, and typical butyric acid concentration after 12 months is <2 mmol kg^{-1}.

Textural Characteristics

Cheese body and texture are very important quality aspects for both traders and consumers. Variations from what is considered normal in terms of body and texture within the same cheese variety are not tolerated, as there is a close relationship between body and texture and other qualities such as eye formation, flavor, and shelf life. The structure depends to a great extent on the microstructure inside the curd particles, whereas the body consistency is characterized by the reaction of the cheese mass to compression.

The structure of the cheese body can be firm or soft and the consistency short (coarse, brittle) or long (tough, elastic). Universal tests are used to determine some important body characteristics. **Table 4** shows the development of rheological parameters and penetrometry during ripening from 3 to 12 months. The texture of Emmental changes during ripening from elastic and relatively soft to less elastic, friable, and firmer.

Figure 4 shows the typical texture profile of a 6-month-old Emmental cheese. Because of the low water content, Swiss Emmental melts at relatively high temperatures. The average softening point measured with an automatic dropping point apparatus is at 74 °C.

Table 4 Results of penetrometry and uniaxial compression test of Emmental cheese during ripening (mean values ± SD for $N = 10$)

		Cheese age		
Parameter	*Unit*	*3 months*	*6 months*	*1 year*
Penetrometry	mm	3.7 ± 1.0	2.5 ± 0.3	4.6 ± 0.6
Strain at fracture	%	68.9 ± 3.2	63.7 ± 2.5	46.5 ± 5.4
Stress at fracture	kN m^{-2}	614 ± 121	437 ± 58	319 ± 48
Stress at 33% deformation	kN m^{-2}	147 ± 16	157 ± 20	244 ± 30

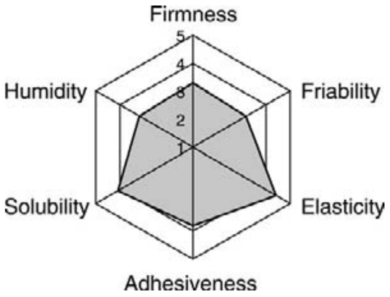

Figure 4 Texture profile of a 6-month-old Emmental cheese. Adapted from Lavanchy P and Bütikofer U (1999) Caractérisation sensorielle de fromages à pâte dure ou mi-dure fabrigués en Suisse. *Mitteilungen aus Lebensmitteluntersuchung und Hygiene* 90: 670–683.

Flavor Characteristics

The indigenous flora of milk is generally composed of unwanted microorganisms, which can influence the flavor directly by their fermentative activity or indirectly by other enzymatic reactions; the desired lactic acid bacteria must be added to the cheese milk in the form of starter cultures. The addition of rennet and the different operations involved in cheesemaking and ripening influence flavor development. In Switzerland, Emmental cheese is made from raw milk, which results in a stronger flavor as compared to cheese made from pasteurized milk. The high temperatures applied during the early stages of manufacture and pressing of Emmental cheese are essential for flavor development. Other important factors are of course the already discussed fermentative and ripening processes, and even the size and shape of the cheese.

For analytical reasons, the flavor components are generally divided into two major groups: volatile and nonvolatile compounds.

The volatile compounds include volatile short-chain acids, primary and secondary alcohols, methyl ketones, aldehydes, esters, lactones, alkanes, aromatic hydrocarbons, and different sulfur- and nitrogen-containing compounds. Methional, acetic acid, and propionic acid are the most important volatile compounds for the typical Emmental flavor. Ethyl butanoate, ethyl 3-methyl butanoate, and ethyl hexanoate contribute to the fruity odor note. Furanones are responsible for caramel-like flavor in Emmental cheese.

The nonvolatile group is composed of peptides, free amino acids, amines, organic acids (**Table 3**), salt (**Table 1**), and minerals (**Table 5**).

The peptides and free amino acids contribute to the background flavor of cheese. Free glutamic acid is mainly responsible for its umami taste. Salt (NaCl) and other minerals influence directly the saltiness and indirectly the total aroma intensity of cheese.

During ripening from 3 to 12 months, the intensity of odor and aroma increases from 3.0 to 3.6 and 2.5 to 3.7, respectively (**Table 6**). Saltiness and sourness increase in the same period by about 0.5 units. Sweetness and bitterness slightly decrease during ripening. Due to propionic acid fermentation, the sweet flavor is about 1–1.5 units higher than in other hard-type cheese varieties without propionic acid fermentation.

Off-flavors of cheeses depend quite often on the properties of the cheese milk. Certain plants and feedstuffs such as bulbous plants, leeks, vegetable waste, herb mixtures, and different mineral salt mixtures fed to dairy cows can influence the taste of milk and produce off-flavors. Certain milk enzymes such as lipases can induce flavors.

Swiss-type cheese can be found today on the market in a wide range of maturation: very young, elastic cheese with the typical sour lactic aroma and sweet taste up to very long ripened cheese (matured in humid caves) with a more intensive flavor and a nutty and spicy note.

Table 5 Concentration of nonvolatile components (g kg^{-1} dry matter) in Emmental cheese (mean values ± SD for $N = 4$)

	Cheese age		
Component	*3 months*	*6 months*	*1 year*
Glutamic acid	5.4 ± 0.6	8.1 ± 0.3	11.6 ± 1.2
Sodium[a]	5.2 ± 0.6	4.5 ± 0.2	4.5 ± 0.7
Potassium[a]	1.3 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
Magnesium[a]	0.7 ± 0.1	0.6 ± 0.1	1.0 ± 0.1
Calcium[a]	6.6 ± 0.8	6.5 ± 0.6	10.6 ± 0.7
Phosphate[a]	10.6 ± 1.2	13.9 ± 1.5	13.2 ± 0.6

[a] Concentration in the aqueous extract.

Table 6 Flavor of Emmental cheese (mean values ± SD for $N = 10$)

Parameter		*Cheese age*		
	Scale	*3 months*	*6 months*	*1 year*
Odor intensity	0–7	3.0 ± 0.3	3.1 ± 0.2	3.6 ± 0.3
Aroma intensity	0–7	2.5 ± 0.3	3.1 ± 0.2	3.7 ± 0.4
Sweetness	0–7	2.5 ± 0.1	2.3 ± 0.2	2.5 ± 0.3
Saltiness	0–7	1.9 ± 0.2	1.9 ± 0.3	2.3 ± 0.2
Sourness	0–7	2.1 ± 0.3	2.0 ± 0.2	2.6 ± 0.3
Bitterness	0–7	2.0 ± 0.3	1.8 ± 0.4	1.8 ± 0.4

Defects

Excessive proteolysis gives an overripe and sharp taste and a shorter body consistency. This defect becomes particularly evident when a large amount of casein is decomposed into low-molecular-weight compounds (high TCA-SN level). The additional CO_2 from aspartate fermentation, but also released by decarboxylation, clearly reduces keeping quality and leads to oversized and oval eye formation and taller cheeses. The cheese body often cannot withstand the pressure of the gas, and cracks or splits appear (see **Figure 5**). This defect is called late or secondary fermentation.

Frequently, the course of proteolysis in a cheese varies from one zone to the other, a phenomenon that occurs due to temperature changes in the cheese during lactic acid fermentation. As the outer zone cools faster, it often develops a bacterial flora that is proteolytically more active than the microorganisms in the center of the cheese. This usually leads to cheese defects such as short and firm body, sharp taste, or development of white color under the rind.

Butyric acid fermentation (late gas blowing) is totally undesirable, as lactate fermentation by *Clostridium tyrobutyricum* into butyric acid, acetic acid, CO_2, and hydrogen causes the cheese loaf to blow. Even in small amounts, butyric acid is unfavorable to flavor development.

Feeding cows with silage of low microbiological quality is the primary cause of contamination of the milk with clostridial spores. In many countries, either the spores are eliminated by physical treatment (e.g., centrifugation or microfiltration prior to processing) or germination is suppressed by additives like nitrate, lysozyme, or nisin. In Switzerland, however, none of these treatments or additives is permitted in the manufacture of Emmental cheese. Therefore, Swiss Emmental cheese has to be manufactured from the milk of cows fed a silage-free diet.

A particularly serious defect results from the eventual presence of *Clostridium sporogenes*. This sporeformer brings about nonspecific and very intense proteolysis, leading to putrid spots.

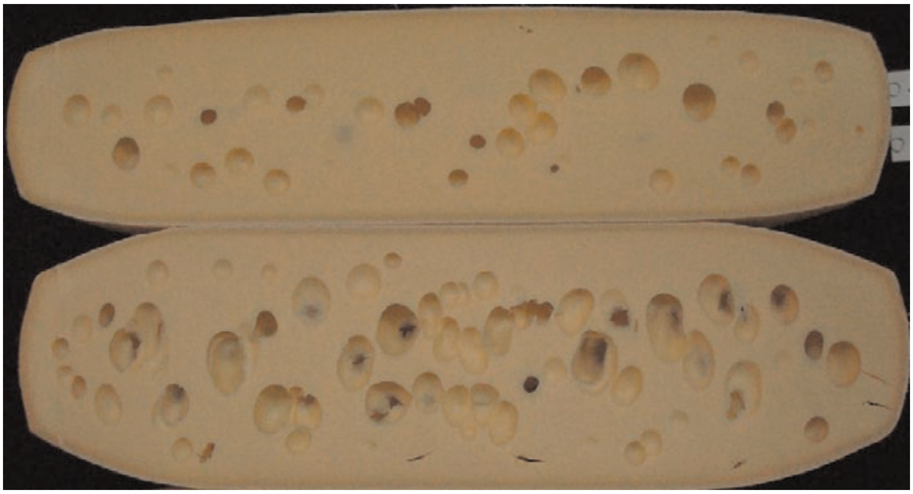

Figure 5 Emmental cheese with late fermentation (lower loaf).

Hygienic Safety

Contamination of raw milk by pathogenic microorganisms can never be completely excluded, despite intensive hygienic efforts. Infectious diseases in dairy cattle and contamination of milk during milking, storage, transport, or processing present potential hazards. Research has shown that the hygienic safety of Emmental cheese made with raw milk is comparable to that of cheese made from pasteurized milk; pathogens are generally absent after 1 week of ripening. Already after cooking, there is a remarkable decrease in the number of pathogenic microorganisms. The reason for the decrease of pathogens in Emmental cheese is the so-called hurdle technology: each step of the manufacturing procedure of Emmental cheese is a hurdle for pathogens to survive and grow. The synergistic effect of these steps is responsible for a hygienically safe product. The following technological steps pose such hurdles:

- high milk quality
- short milk storage
- antagonistic starter culture flora
- rapid acidification
- complete conversion of sugars into lactic acid
- antimicrobial effect of lactic acid
- high cooking temperatures
- brining
- ripening at an elevated temperature
- long ripening period (more than 120 days)

Thus, a Swiss Emmental cheese consumed only after 4 months is hygienically safe and is comparable to a cheese made from pasteurized milk.

See also: **Cheese**: Cheese Flavor; Cheese Rheology; Secondary Cultures; Starter Cultures: General Aspects.

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Dutch-Type Cheeses

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Introduction

Dutch-type cheeses represent the most important kinds of semihard cheese. They are manufactured from bovine milk, although small amounts of similar cheeses are made from ovine or caprine milk. Other semihard cheese types are also manufactured according to a largely similar technology but their characteristics are different. For this reason, Jarlsberg or Maasdam and Tilsit or Havarti cheeses will not be treated here.

Gouda and Edam cheese are the primary Dutch-type cheese varieties and they are made all over the world. However, in many countries, a similar technology is used for related cheese types manufactured under various names. The majority of all these cheese varieties contain 40–52% fat in dry matter, and the water content in the (unripened) fat-free cheese ranges from 55 to 63%. They are made in sizes between 0.2 and 20 kg. Their shape can be a flat cylinder often with somewhat bulging sides, a sphere, a rectangular block, or a loaf. The rectangular and loaf-shaped cheeses are often used for foil ripening (rindless cheese). The standard cheeses are naturally ripened under drying conditions.

Dutch-type cheeses are ripened for some weeks or longer, sometimes for more than a year, and are often covered with a plastic coating. The consistency varies from semisoft to rather firm and smooth, and changes during natural ripening to a firmer and more brittle structure. The flavor also changes from mild to strong during such long ripening times. The interior of the main cheese types shows some round eyes about the size of a pea. Substances like cumin seeds or other spices may be added. There is essentially no surface flora.

Production Statistics

The worldwide production volume of Dutch-type cheeses can only be estimated because from many countries there are no official figures for individual cheese types. Moreover, all related cheese types are not always well described in different countries. So a worldwide picture can only be estimated.

The world cheese production in 2004, excluding processed cheese but including fresh cheese and quark, is estimated as $14\,400 \times 10^3$ tonnes. The share of the Dutch-type varieties is estimated to be approximately 15% of this volume, which is based on a mix of real figures for various European countries and estimates for others. The production of these cheese types in the Netherlands in 2004 was 514×10^3 tonnes and in Germany 353×10^3 tonnes as the two countries with the highest production, while the whole European Union produced approximately 1460×10^3 tonnes. Dutch-type cheese is usually made from pasteurized milk; only 7.6×10^3 tonnes, mainly Gouda cheese, is farm-made from raw milk in the Netherlands and will not be considered further.

Technology

A typical manufacturing scheme for Gouda cheese is given in **Figure 1**.

The milk is thermized and standardized for its fat:protein ratio in order to control the fat content in the dry matter of the cheese. It is cold-stored until cheesemaking can take place. Then, the milk is pasteurized and pumped into the cheese vat. During one of these heat treatments, the milk is often bactofugated to reduce the number of spores of butyric acid bacteria. The total heat load must be sufficient to inhibit pathogenic microorganisms and some spoilage organisms but not xanthine oxidase (EC 1.2.3.2). The denaturation of whey proteins should be limited.

For the manufacture of Dutch-type cheeses, the milk is coagulated at approximately 30 °C by the action of renneting enzymes, usually a chymosin (EC 3.4.23.4) preparation (calf rennet). This is added together with the mesophilic starter and calcium chloride to control the renneting process. Other additives such as nitrate and coloring agents, like annatto (E160b) or carotene (E160a), are often used. The resulting coagulum consists of a continuous network of strands of (para)casein micelles that extends throughout the milk volume (*see* **Cheese**: Rennet-Induced Coagulation of Milk). In general, this gel is not as firm when cut as is the case for soft

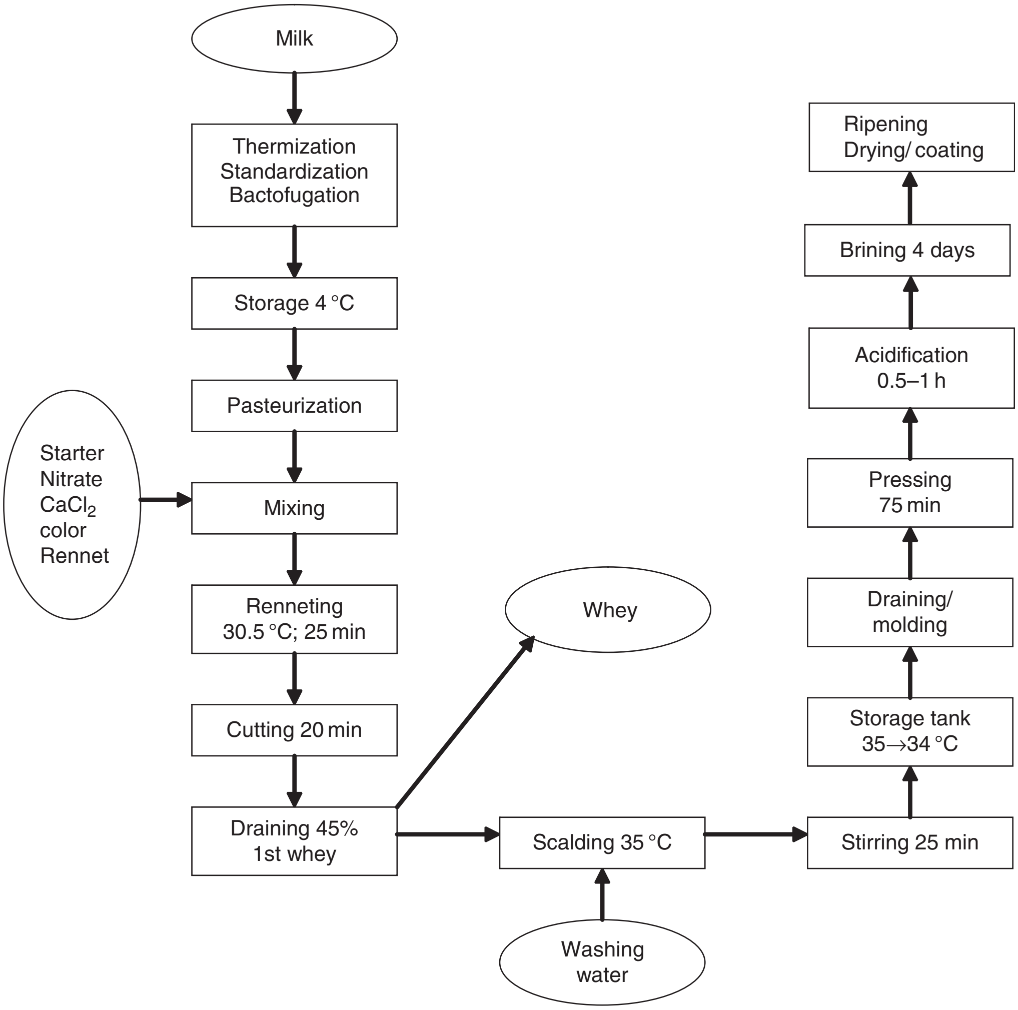
Milk
Thermization
Standardization
Bactofugation
Storage 4 °C
Pasteurization
Starter
Nitrate
$CaCl_2$
color
Rennet
Mixing
Renneting
30.5 °C; 25 min
Cutting 20 min
Draining 45%
1st whey
Whey
Scalding 35 °C
Washing
water
Stirring 25 min
Storage tank
35→34 °C
Draining/
molding
Pressing
75 min
Acidification
0.5–1 h
Brining 4 days
Ripening
Drying/ coating

Figure 1 Main process steps of a modern manufacturing scheme for 12 kg Gouda cheese.

cheesemaking. It is cut into fairly small particles but not so fine as is the case for hard-cooked cheese.

After cutting, a part of the (first) whey already liberated from the curd particles by syneresis (shrinkage of the gel and expulsion of whey) is removed and warm water is added. This washing and scalding of the curd serves to reduce the lactose content and to enhance syneresis during further stirring. The amount of the curd wash water is related to the desired pH of the final cheese. The pH of cheese is governed by the ratio of the amount of lactose that is fermented to lactic acid and the amount of the (para)caseinate–phosphate complex (the acid buffering substance). So in this respect, the lactose content of the milk and the moisture content of the nonfat matter after pressing/before brining (being equal to that in the center of the cheese when all lactose has been fermented) are important. The higher these parameters, the more lactose remains in the curd and the more curd wash water has to be added to maintain a certain pH.

The scalding temperature is primarily used to adjust the dry matter content of the cheese, as it affects syneresis. Syneresis is an intrinsic property of the (para)casein gel that involves breaking of protein strands and the formation of new bonds between casein micelles elsewhere. This leads to a rearrangement of the protein network while moisture with dissolved components (whey) is expelled. Increasing the surface of the coagulum (cutting), acidification, temperature increase, and stress (by stirring the curd or, during a successive stage, by taking the curd out of the whey) all enhance syneresis. The scalding temperature is limited to about 36 °C, as mesophilic starters are used.

After curd making, the curd particles are collected under the whey, in order to avoid inclusion of air. Subsequently, the free whey between the curd particles must be expelled by drainage in order to obtain a closed texture in the final cheese. However, even after pressing, some whey still remains in the small interstices between the curd particles. During later stages, the temperature drops and this whey is readsorbed by the curd so that fusion can be completed. In fact, a cross-section of the cheese will have a closed texture only after some days.

The blocks of curd are pressed in perforated molds covered with a lid, allowing whey drainage. A rind of a few millimeters thick is created by deformation of the curd around threads of the liner or edges of perforations in the mold. This rind formation is due to strong local syneresis and further fusion of the curd particles, and more bonds between the casein strands are created than elsewhere in the cheese. In essence, syneresis, fusion, and rind formation are similar processes.

A closed rind is necessary, for the natural ripening cheese in particular, to withstand the tension caused by brining and to protect the cheese from penetration of undesired microorganisms during brining and from molds during ripening (for brining, *see* **Cheese**: Salting of Cheese).

During curing, the integrity of the rind has to be maintained. Coating of the cheese makes the maintenance of the rind much easier and gives a smooth and glossy appearance when optimal climatic conditions, air flow, and cheese treatment scheme in the ripening room are applied. The polyvinylacetate-based coating layer reduces water evaporation from the cheese surface to some extent. However, too strong drying will make this hydrophilic coating layer too brittle and mold may penetrate into cracks. On the other hand, too little drying will easily allow undesired development of bacteria rendering the surface sticky. The presence of natamycin will then not be sufficient to prevent the growth of yeasts and molds. The normal ripening temperature is rather moderate, approximately 13 °C. In the case of ripening in foil or in wax, this temperature, as well as the moisture content of the cheese, is often somewhat lower to keep the cheese in better shape and to prevent it from being too soft after curing.

It is obvious that cheese composition has to be controlled carefully. During the manufacture of Gouda cheese, the casein is concentrated at the end of curd preparation by a factor of approximately 4.5 and in the final cheese by approximately 10. In parallel with this, the dry matter content of the curd mass increases to approximately 32% at the end of curd preparation, to 42% after drainage, to 53% after pressing, and to 57% after brining. The composition of the cheese directly influences the product yield. However, milk composition is the primary factor to reckon with. Of the components to be concentrated, casein is the most expensive one. The fat in the whey can be recovered in the cheese by adding the whey cream to the cheese milk whereas the casein (curd fines) cannot without compromising cheese quality. So it is good to express yield as kg per kg casein of the milk. However, in a quantitative sense, water is the most important cheese component. To obtain high-quality cheeses, an optimal water content, before brining in particular, is required. It is important for the desired consistency of the young cheese and for the conditioning of the ripening processes.

Modern cheese factories for Dutch-type cheeses manufacture 3–10 tonnes of cheese per hour, and mechanization and automation based on a good understanding of the technology are well developed.

Starters and Lactose Fermentation

Starter bacteria are essential for the conversion of lactose and casein in cheese. The functional role of starter bacteria in acid production and cheese ripening is discussed elsewhere (*see* **Cheese:** Starter Cultures: General Aspects). The starter bacteria for Dutch-type cheese traditionally have been mesophilic lactic acid bacteria (LAB). The commonly used starters consist of combinations of acid-producing *Lactococcus lactis* subsp. *lactis* and *cremoris* strains and citrate-fermenting (and carbon dioxide-producing) *Leuconostoc lactis* and/or *Ln. mesenteroides* subsp. *cremoris* (L-starters) or *Lc. lactis* var. *lactis* biovar *diacetylactis* and *Leuconostoc* strains (DL-starters). DL-starters are normally used when stronger eye formation is desired because they ferment citrate faster and to a greater extent than L-starters do. When in particular cases eye formation is not desired, a starter without citrate-fermenting bacteria is often used (O-starter).

(Undefined) mixed-strain starters are mainly used in Europe for Dutch-type cheese. This is the case for L- and DL-starters, but also for O-starters. Such starters have to be well selected because of their major influence on cheese quality. Manufacturers, especially the bigger factories, prefer to use their own bulk starter, to realize lower starter costs and a better yield in comparison with the use of a DVS (direct vat set) system. Such a bulk starter system is provided with means to avoid bacteriophage contamination, such as a bacteriophage-free mother starter concentrate and a starter propagation tank with an overpressure of bacteriophage-free air.

The amount of bulk starter of normal activity used to inoculate the cheese milk is 0.5–1.0%. During cheesemaking, starter LAB convert lactose to lactic acid and they grow until a level of 10^9 cfu g^{-1} has been reached. In Gouda and Edam cheese, the pH at that time is approximately 5.7 and the cheese has been pressed already. The usual course of pH during cheesemaking is given in **Figure 2**.

Figure 2 shows that, during curd preparation, the pH remains rather high until the major part of the whey is separated by drainage, in order to obtain the desired cheese consistency and yield. In addition, binding of chymosin to the casein is restrained at higher pH values and thus the risk of development of bitterness during ripening is reduced. Before lactose fermentation is

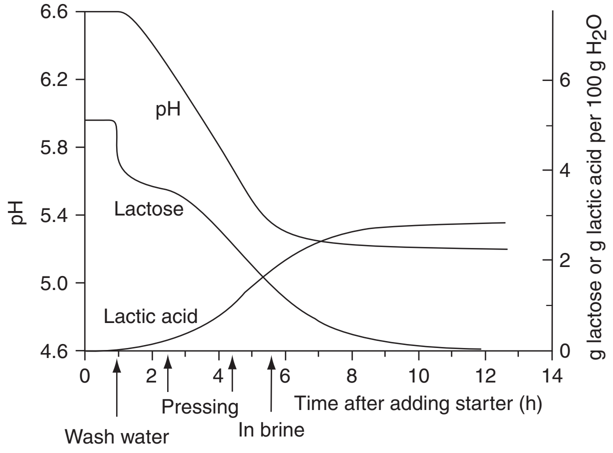

Figure 2 Lactose fermentation, acid production, and the pH of curd and cheese during the manufacture of Gouda cheese after starter addition.

completed, the cheese is already put into the brine. During brining, lactose conversion continues, whereby the pH of the cheese is lowered to about 5.2–5.4. The moisture diffusing out of the cheese still contains lactose and so does the brine, which is used for long periods of time. As a result, after brining, the rind of the cheese still contains some lactose, which normally disappears within the first few weeks of ripening. For foil-ripened block cheese, in particular, the time between pressing and brining is often even shorter and the diffusion effects concerning lactose are stronger.

Gouda cheese ripening, and resulting flavor formation, is a complex process, which primarily involves breakdown of carbohydrate and casein. A major role is played by the starter LAB present during cheese production and ripening. There is a strong interest in acceleration of ripening, and also in product differentiation (in packaging, shape, especially in flavor). For this, attenuated LAB starters (adjunct cultures) are introduced as a means to enhance flavor development. These adjuncts are often thermophilic and propagated or pretreated in such a way that they practically do not grow during cheesemaking and do not contribute to acidification. Thus, normal manufacturing procedures can be maintained. In the case of low-fat cheese, the ability to enhance protein degradation is of special interest in order to achieve a more acceptable consistency and a stronger flavor of the final product.

Ripening

Ripening is the result of numerous changes occurring in the cheese. The structure and composition, and consequently the organoleptic properties of cheese alter greatly during ripening. Next to fermentation of lactose and citric acid, proteolysis followed by amino acid degradation is the main phenomenon during the maturation of Dutch-type cheese. Within 24 h after production, the pH of the cheese is 5.1–5.2, thereafter increases during the first 2 weeks by approximately 0.15, and increases only slightly during further maturation. The redox potential in the cheese is reduced by the lactic acid fermentation to approximately −140 mV. In the center of Gouda cheese, the original water content in the fat-free cheese is approximately 65%, but it decreases gradually by salt diffusion and water evaporation. Under these conditions, casein is degraded by residual chymosin, to a lesser extent by plasmin, and by proteinases of LAB into larger peptides. These are further degraded, finally yielding free amino acids, by peptidases of the starter LAB, which also provide amino acid-converting enzymes (AACEs) for the production of various volatile (flavor) components (for further details on the degradation pathways, *see* **Cheese**: Biochemistry of Cheese Ripening; Cheese Flavor).

During normal Gouda cheese ripening, protein and amino acid conversion occurs very gradually. The degradation of the (total) casein during ripening can be described quantitatively by the level of increase in soluble nitrogen (SN) and amino nitrogen (AN). The AN fraction contains only very small peptides, amino acids, and further converted components, whereas the SN fraction contains larger, water-soluble peptides. These nitrogen-containing fractions can be separately quantified.

Figure 3 gives the average increase of the nitrogen-containing fractions (expressed as percentage of total nitrogen (TN)) in normal Gouda cheese during ripening at 13 °C. Generally, formation of the SN fraction is mainly dependent on chymosin activity and formation of the AN fraction, on activities of enzymes associated with the starter bacteria. However, the production of peptides by the former activity stimulates the conversion

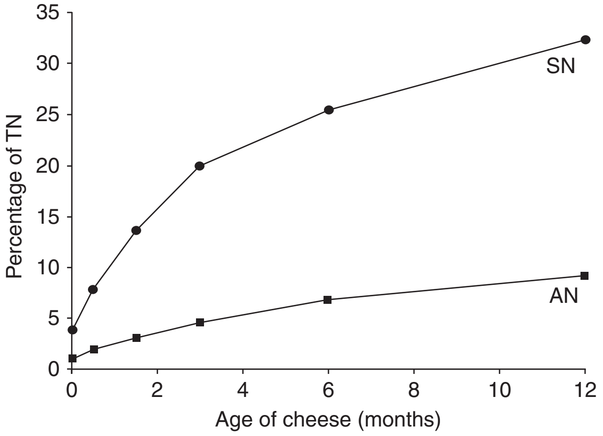

Figure 3 Increase of the soluble nitrogen-containing fraction (SN) and the amino acid-containing fraction (AN) during the ripening of normal Gouda cheese as a percentage of total nitrogen fraction (TN) in the cheese.

by the latter enzymes. The further conversions of amino acids yield various aldehydes, alcohols, acids, esters, and sulfur compounds, facilitating specific flavor developments.

As noted, **Figure 3** gives an average image of the entire cheese. In the center of the cheese, the level of proteolysis is higher because of the higher moisture content and the (initially) lower salt content. In the rind, proteolysis stops within 3 months because of the low water content. It will be obvious that when water evaporation is prevented by packaging in foil or wax, one may speak of rindless cheese, because then the ripening processes equally proceed in the interior and in the rind zone. Such cheese is usually ripened at a low temperature.

The use of attenuated thermophilic cultures in addition to the normal starter culture in general causes a strong increase of the AN fraction while the SN fraction is hardly affected. So, peptidase activity and also AACE activity are primarily increased, leading to increased flavor formation.

Lipolysis is generally limited in Dutch-type cheese. The formation of short-chain and long-chain free fatty acids, lactones, esters, and 2-ketones, all potentially affecting flavor, is however occurring in Gouda-type cheese. The formation of these flavor components is, in part, a result of nonenzymatic events, but cheese (secondary) flora also plays a role.

Textural Characteristics

Figure 4 gives an impression of the desired eye formation in normal Gouda cheese.

For eye formation, a certain gas pressure in the very young cheese is necessary when the consistency of the cheese is still very elastic. Partial saturation of the milk with air, in particular with nitrogen because oxygen disappears due to the activity of the starter bacteria, and citrate fermentation by the starter bacteria during the first weeks after manufacture are crucial. Typically, the partial pressure of nitrogen in the young cheese is approximately 0.9 bar and that of carbon dioxide 0.4 bar, together being sufficient for eye development in Gouda cheese. While these are prevailing conditions in Gouda cheese, considerable variation may be observed. Without the nitrogen, the cheese would be 'blind'. However, without 'nuclei', eye formation does not take place either, because then the pressure necessary to start the development of an eye can never be reached. Various irregularities in the structure of a curd block may serve as such 'nuclei', like tiny air bubbles or remaining whey pockets, but also foreign particles with apolar surface properties. Faster citrate fermentation by DL-starters will lead to more or bigger eyes, dependent on the 'nuclei' available, because of an increased gas pressure before the carbon dioxide can diffuse through the rind. A sufficiently elastic texture is necessary; if it is too short (too low fracture stress), cracks will be formed instead of eyes.

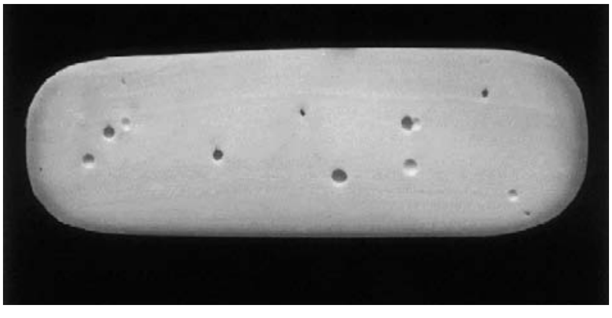

Figure 4 Section through a normal Gouda cheese.

Such a short consistency is to be expected at low pH. However, the desired consistency also depends on the correct moisture and fat content and on the presence of sufficient calcium phosphate. Therefore, the pH before pressing must be high. Due to proteolysis and moisture loss during ripening, the consistency of Gouda cheese changes within 1–2 months from rather elastic to smooth and later to short. In the meantime, the water content decreases considerably and the consistency will also be rather hard and brittle after a year. The rind zone increases in thickness during ripening; it is tougher and harder than the interior and becomes somewhat translucent. The increase in 'shortness' of Gouda cheese during ripening is illustrated in **Figure 5** by the shift in the stress–strain curves (*see* **Cheese**: Cheese Rheology).

In Gouda cheeses ripened for more than 1 year, tyrosine crystals are often visible as a result of the progress of proteolysis. If attenuated thermophilic starters are added, these crystals are present already after a shorter ripening period.

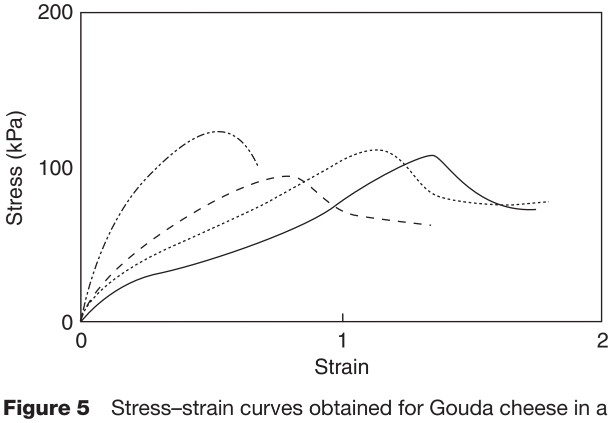

Figure 5 Stress–strain curves obtained for Gouda cheese in a compression test at 2 (—), 6 (······), 13 (– – –), and 26 (–··–) weeks. From P. Zoon, NIZO Food Research.

Flavor Characteristics

Primary products formed during cheesemaking, like lactic acid, diacetyl, and carbon dioxide and related fermentation products, are essential for the basic flavor of the young cheese. The same is true for the very small amount of free fatty acids initially present. A strong mature flavor in the cheese, however, develops during ripening that starts immediately after manufacture. During ripening, numerous conversions take place and many compounds contributing to flavor are produced. For a proper flavor, a well-balanced formation of compounds is essential. The flavor of the young cheese depends rather directly on the type of starter. Cheeses made using an O-starter have a less creamy flavor than those with an L-starter and certainly less than those with a DL-starter. In the more mature cheese, these differences disappear but other, also starter-related, differences become apparent such as sweetness or fruitiness.

The effect of proteolysis, including amino acid conversion into a great number of volatile flavor compounds, is dominant in Gouda cheese. In good quality cheese, made with a mixed-strain starter, amino acid conversion and flavor compound formation are well balanced. The steadily increasing knowledge regarding flavor formation pathways enables design of (adjunct) starters and development of new Gouda-type cheese variants. An example is Proosdij cheese (nowadays marketed under various brand names, such as Parrano®). Proosdij cheese is prepared with a mesophilic starter culture in combination with a thermophilic (adjunct) culture. This cheese has a flavor profile with characteristics between Gouda and Parmesan cheeses. One of the key aroma compounds in the cheese is 3-methylbutanal. **Figure 6** shows gas chromatograms with the most important differences, regarding volatile flavor compounds, between a Gouda cheese and a Proosdij cheeses, both after 6 months' ripening.

The flavor of Proosdij cheese has more sweet, caramel, bouillon, and fruity notes than the flavor of Gouda cheese. This difference in flavor between Gouda and Proosdij cheeses is due to differences in the concentration of certain key components, such as the already mentioned 3-methylbutanal. Proosdij cheese contains higher amounts of this compound (peak 12), but also of butanone (peak 8), 2-pentanone (peak 14), and 2-heptanone (peak 22), while Gouda cheese contains more diacetyl (peak 7), 2-methylpropanol (peak 11), 1-butanol (peak 13), 3-methylbutanol (peak 16), and ethyl butyrate (peak 21).

Defects

Process control is necessary to avoid a number of defects in the cheese. The most important ones are listed here, with the main measures required to avoid them.

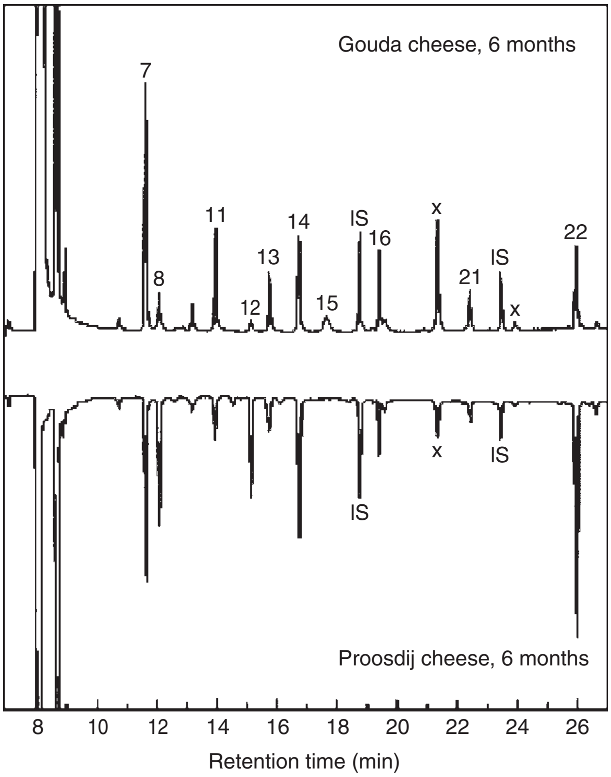

Figure 6 Gas chromatograms (relevant parts) of Gouda cheese (positive) and Proosdij (negative), both after 6 months' ripening. IS, internal standard; x, contaminant. Intensities of peaks have been normalized upon the first IS. Reproduced with permission from Neeter R, De Jong C, Teisman HGJ, and Ellen G (1996) Determination of volatile components in cheese using dynamic headspace techniques. In: Taylor AJ and Mottram DS (eds.) *Flavour Science, Recent Developments*, Special Publication No. 197, pp. 293–296. Cambridge, UK: The Royal Society of Chemistry (by the courtesy of The Royal Society of Chemistry).

Butyric acid fermentation: This 'late blowing' (large eyes and/or cracks with a sweet and butyric off-flavor) is caused by the growth of the anaerobic bacterium *Clostridium tyrobutyricum*, originating from spores in the milk, in the 1- to 5-month-old cheese. The use of nitrate and bactofugation of the milk mainly prevent it.

Mesophilic lactobacilli: Various strains of *Lactobacillus plantarum*, *Lb. casei*, or *Lb. brevis* are able to grow in cheese and to produce various off-flavors (gassy, putrid, fruity, etc.) and/or carbon dioxide, causing cracks in the mature cheese. Contamination and growth during cheesemaking must be avoided as much as possible. Some of these bacteria are salt tolerant and may contaminate the cheese during brining. So the rind has to be closed during pressing and the bacterial counts of the brine have to be controlled.

Thermoresistant streptococci: In particular, urease-producing strains of *Streptococcus thermophilus* may be responsible for excessive carbon dioxide production and off-flavor in 1- to 2-month-old cheese due to conversion of urea originating from the milk. Since *Sc. thermophilus* can grow on the surface of the plates in the regeneration section of pasteurizers, relatively high levels of this thermophile may occasionally occur in pasteurized milk. Counts of this organism in the cheese milk have to be controlled, especially by regular cleaning of the plate heat exchangers.

Slimy rind: Growth of coryneform bacteria on the surface of the cheese should be avoided by a good acidification during cheesemaking and by good curing conditions. Otherwise, they may cause gas production under the wax layer applied before selling the cheese. A slimy rind without bacterial growth is possible when the calcium content of the brine is too low.

Mold growth: Mold growth on the surface of the cheese must be avoided by the right curing conditions and turning and coating program. Under normal conditions, the use of natamycin in the coating inhibits mold growth.

Bitterness: This is a ripening defect that can be caused by using too much rennet or a low pH before pressing, which leads to more rennet retention in the cheese. The main cause, however, is using a starter with insufficient peptidase activity to degrade bitter peptides.

Texture defects: Besides the formation of cracks or too large holes, it also happens that too many small eyes or even pinholes are formed. To avoid the latter defects, the renneting milk should not contain many very fine air bubbles and air inclusion during drainage of the curd must be avoided. Curd lumps that do not lose their whey properly during drainage mainly cause nesty holes.

See also: **Cheese**: Biochemistry of Cheese Ripening; Cheese Flavor; Cheese Rheology; Rennet-Induced Coagulation of Milk; Salting of Cheese.

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Hard Italian Cheeses

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Introduction

Extra-hard cheese varieties originated in Italy. Parmigiano Reggiano, Grana Padano, and Pecorino Romano rank among the most famous international cheeses and have maintained their traditional features over time in spite of marked changes in cheesemaking technology.

The Codex Alimentarius FAO/WHO defines hard cheeses as those cheeses having values of moisture on a fat-free basis (MFFB) and fat on a dry basis (FDB) lower and higher than 56 and 45%, respectively. Overall, hard cheeses also have hardness, elasticity, and springiness factors, expressed on a logarithmic scale, higher than 8.0, 5.8, and 2.0, respectively. The classification of Italian cheeses is complicated by the use of different names for the same or very similar cheeses in different regions, and especially because many varieties are consumed after different degree of ripening.

Of the *c.* 1 004 918 tonnes of cheese produced in Italy in 2007, *c.* 459 466 tonnes was of cheeses that have a Protected Denomination of Origin (DOP). Of the latter, 356 342 tonnes was hard cheeses. **Table 1** shows the production of the more important hard Italian cheeses. All the cheeses listed in the table have a DOP, with the exception of the Fossa (pit) cheese. It should also be noted that in addition to the hard cheeses listed in the table, others (e.g., Bagoss, Bitto, Monte Veronese, and Pecorino Lupa) are produced on a small scale but deserve attention since they have ancient traditions. In particular, semihard or hard *pasta filata* Italian cheeses (e.g., Ragusano, Caciocavallo, and Provolone) are described in another article (*see* **Cheese**: Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese).

With the exceptions of Grana Padano, Parmigiano Reggiano, Asiago, and Montasio cheeses, all the others are, or may be, produced from ewes' milk alone or mixed with cows' milk. In particular, Pecorino is the name given to most Italian cheeses made from ewes' milk. The manufacture of Grana Padano, Parmigiano Reggiano, Asiago, Montasio, and Castelmagno is concentrated in the north of the country (Po valley and sub-Alpine districts), while Pecorino Romano and the other ewes' milk cheeses are of great economic significance particularly in central and southern Italy, Sardinia, and Sicily.

Main Chemical and Technological Features

The main chemical and technological features of the more representative hard Italian cheeses are shown in **Tables 2** and **3**, respectively. The use of raw milk and natural thermophilic starters, cooking of the curd to high temperature, long ripening times, and very low moisture content are features common to most hard Italian cheeses. Nevertheless, since they are linked to ancient tradition, the technology differs markedly. Some of the main relevant technological traits of the more famous hard Italian cheeses are described below.

'Grana' Cheeses (Parmigiano Reggiano and Grana Padano)

'Grana' cheeses, Parmigiano Reggiano and Grana Padano, derive their name because of the grainy texture of the ripened cheese. In addition to these, there are Grana Bagozzo and Grana Lodigiano, which, because of their limited production, have almost disappeared from the market. 'Grana' type cheeses use cheese milk produced from farms in a restricted area of the Pianura Padana (northern Italy). Milk from different areas is used for Parmigiano Reggiano and Grana Padano. For the manufacture of Parmigiano Reggiano, the feeding of the cows is subjected to the following restrictions: (1) the ratio between forage and other feeds must be ≥ 1; (2) $\geq 25\%$ of the dry matter (DM) of the forage used must be produced on the same farm where cheese is manufactured; (3) $\geq 75\%$ of the DM of the forage used must be produced within the district where the cheese is legally manufactured; and (4) the feeding of silage is not allowed to minimize the number of gas-producing bacteria in the milk. The addition of lysozyme (2 g hl^{-1}) to cheese milk is allowed for the manufacture of Grana Padano cheese as anticlostridial agent. A mixture of milk from two consecutive milkings is used for Parmigiano Reggiano and the milk from the evening milking is partially skimmed after overnight creaming. Grana Padano is manufactured from a single milking and the milking is skimmed by creaming for *c.* 8 h. During creaming, the microbial content of the milk is decreased. The fat contents of the milk for Parmigiano Reggiano and Grana Padano cheeses are

Table 1 Production of the principal hard Italian cheeses, 2007

Cheese	*Milk species*	*Milk quantity (tonne)*	*Cheese production (tonne)*	*Cheese yield (kg milk per kg cheese)*
Grana Padano	Cow	2270583	158017	14.36
Parmigiano Reggiano	Cow	1773504	117044	15.15
Asiago	Cow	209989	22649	9.27
Montasio	Cow	67502	7144	9.45
Pecorino Romano	Sheep	192796	33425	5.77
Pecorino Siciliano	Sheep	187	15600	0.012
Pecorino Sardo	Sheep	11124	1200	9.27
Fiore Sardo	Sheep	3198	600	5.33
Canestrato Pugliese	Sheep	364	112	3.25
Castelmagno	Cow/sheep and goat	2277	201	11.33
Fossa	Sheep/cow	10	350	0.028

Data from Assolatte (2007). http://www.assolatte.it.

Table 2 Gross chemical composition of the principal hard Italian cheeses (average data)

Cheese	*Moisture (%)*	*Total protein (N × 6.38) (%)*	*Fat (%)*	*Ash (%)*	*Soluble N/total N*
Grana Padano	32.0	33.0	27.0	4.9	34.0
Parmigiano Reggiano	30.8	33.0	28.4	4.6	32.0
Asiago	36.0	32.0	34.0	5.0	28.5
Montasio	32.0	26.0	34.0	3.0	26.5
Pecorino Romano	31.0	28.5	29.0	8.5	22.5
Pecorino Siciliano	31.5	32.5	28.0	na	26.5
Pecorino Sardo	31.0	27.2	35.0	na	24.0
Fiore Sardo	26.5	30.0	32.5	na	25.5
Canestrato Pugliese	34.5	26.5	30.0	na	30.0
Castelmagno	35.0	26.0	33.0	5.0	26.5
Fossa	32.0	27.0	35.0	na	32.0

na, data not available.

c. 2.4–2.5 and 2.1–2.2%, respectively. The natural whey cultures used as starters for 'Grana' cheeses are produced from the whey of the previous cheesemaking, which is held under a gradient of temperature from *c.* 50 to *c.* 35–20 °C for *c.* 18–24 h. The microbial composition of the natural starter is very complex and is subjected to environmental factors. It is dominated by thermophilic lactobacilli (*c.* 10^9 cfu ml^{-1}) such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lactobacillus fermentum.* The ratio between obligately homofermentative and heterofermentative species is generally maintained at *c.* 10:1. Curds are cooked at 54–56 °C and the time from rennet addition at 33–35 °C to the end of cooking is 22–23 min. The vat (special copper tanks) used for the manufacture of 'Grana' cheeses have a capacity of 10–12 hl; therefore, from each vat, two ripened cheeses, each weighting 33–35 kg, are manufactured. Parmigiano Reggiano is ripened for 20–24 months and Grana Padano for 14–16 months. 'Grana' cheeses have a cylindrical shape with a diameter of 33–45 cm and a height of 18–25 cm. 'Grana' cheeses have a very low moisture content (*c.* 30%) and a typical compact texture, with or without many very small eyes, and melt in the mouth with a sweet and pronounced flavor, which is the result of a very slow ripening, during which proteolysis is the main biochemical event. As indicated in the protocols for manufacture, the textures are of Parmigiano Reggiano and Grana Padano are defined as 'fine, brittle granules' and 'fine, grainy, and radially fracturing into slivers', respectively.

Asiago

Several varieties of Asiago cheese are manufactured, which differ mainly in the duration of ripening. Asiago d'Allevo is the hard cheese variety, ripened for 6–12 months, typically manufactured in the Veneto region. Previously, the cheese was manufactured from ewes' milk, but currently only cows' milk is used. Raw milk from one or two consecutive milkings is partially skimmed by a creaming protocol similar to that described

Table 3 Main ripening characteristics of the principal hard Italian cheeses

Cheese	*Type of milk*	*Starter*	*Type of rennet*	*Cooking*[a] *(°C)*	*Salting*	*Ripening*	*Proteolysis*	*Lipolysis*	*pH*
Grana Padano	Cow, raw, partly skimmed	Natural whey culture (thermophilic, rod-shaped lactic acid bacteria)	Calf, powder	53–54	22–24 days in saturated brine	12–16 months at 18–20 °C	Deep, slow	Weak	5.5–5.6
Parmigiano Reggiano	Cow, raw, partly skimmed	Natural whey culture (thermophilic lactic acid bacteria)	Calf, powder	54–55	20–23 days in saturated brine	18–24 months at 18–20 °C	Deep, slow	Weak	5.4–5.5
Asiago	Cow, raw, partly skimmed	None or natural culture in whey or milk	Calf, powder or liquid	40–46	Dry salting for 10–12 days	12–24 months at 15–16 °C	Fairly deep	Weak	5.5
Montasio	Cow, raw, partly skimmed	Natural whey culture	Calf, powder or liquid	48–50	Dry salting for 10–12 days or 4–7 days in saturated brine	12 months at 15–18 °C	Fairly deep	Moderate	5.0–5.5
Pecorino Romano	Sheep, raw or thermized, whole	Natural culture in 'scotta'	Lamb, paste	45–46	Dry salting for 30–60 days	8–12 months at 10–14 °C	Deep	Strong	5.3–5.4
Pecorino Siciliano	Sheep, raw or thermized, whole	None or natural culture in whey or milk	Lamb, paste	40–45	Dry salting for 10–20 days	6–8 months at 12–15 °C	Fairly deep	Strong	5.2–5.5
Pecorino Sardo	Sheep, raw or thermized, whole	Natural whey culture	Calf, paste	40–45	Saturated brine and/or dry salting	2–12 months	Fairly deep	Strong	5.2–5.5
Fiore Sardo	Sheep, raw, whole	Natural whey culture	Lamb or goat, paste	No heat treatment	Saturated brine and/or dry salting	3–6 months at 12–16 °C	Fairly deep	Strong	5.1–5.3
Canestrato Pugliese	Sheep, raw	None or natural culture in whey or milk	Calf, powder or liquid	No heat treatment or 45	Dry salting for 4–6 days	4–12 months at 11–14 °C	Fairly deep	Fairly strong	5.0–5.2
Castelmagno	Cow, mix of cow, sheep, and goat, raw, partly skimmed	None or natural culture in whey or milk	Calf, powder or liquid	No heat treatment	Dry salting for 4–6 days	6–12 months at 10–12 °C	Fairly deep	Fairly strong	5.0–5.3
Fossa	Sheep, mix of cow and sheep, raw	None or natural culture in whey or milk	Calf, powder	No heat treatment	Saturated brine, dry salting for 1 day	6–8 months at 12–14 and 17–25 °C	Deep	Moderate	5.0–5.2

[a]The time of cooking is variable; for details, see section 'Main Chemical and Technological Features'.

for Parmigiano Reggiano cheese. The natural whey culture used as starter is dominated by thermophilic lactic acid bacteria. Cooking of the curd is generally for 20–30 min and it is divided in two steps. After cutting, the curd is heated to 40–42 °C and held for 5–7 min; then the temperature is increased to 46 °C, and held for 15–25 min. After molding, the curd is pressed for *c.* 12 h. Exceptionally, some cheeses are ripened for 2 years. The cheese is cylindrical in shape; it is 9–12 cm in height and 30–35 cm in diameter, and weighs 8–12 kg. The texture is rather compact and the flavor is slightly sweet.

Montasio

The cheese derives its name from the homonymous place located in the Julian Alps. Currently, its manufacture has been extended to the Friuli region and to several provinces of the Veneto region. A mixture of cows' milk from two consecutive milkings is used; the milk from the evening milking is partially skimmed after overnight creaming. The natural whey culture used as starter is added to the milk at 31–35 °C and coagulation by calf rennet takes place in 30–40 min. After cutting, the curd is cooked at 48–50 °C for several minutes, pressed for 24 h, and dry-salted or immersed in saturated brine. Ripening of extra-hard Montasio cheese lasts 12 months. The cheese has a cylindrical shape; it is 8–10 cm in height and 30–40 cm in diameter, and weighs 5–9 kg. The mature cheese has a brown rind, a granular texture with very small eyes, and a pronounced and moderately piquant flavor.

Pecorino Romano

Legally, the manufacture of Pecorino Romano is limited to the regions around Rome and to Sardinia. It is the best known hard ewes' milk cheese. Usually, Pecorino Romano is made from raw or thermized milk that is inoculated with a natural culture (*scotta fermento*). The natural culture is produced by acidifying the 'scotta', the whey obtained from the manufacture of ricotta. Thermophilic lactic acid bacteria such as *Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. helveticus* dominate the microbiota of this natural starter. The milk is coagulated at 37–39 °C using lamb rennet paste, and after cutting, the curd is cooked at 45–46 °C. After removal from the vat, the curd is placed in mold, pressed manually, and pierced with fingers or with a stick for increasing whey drainage. The cheese takes 8–12 months to develop the characteristic flavor. The cheese has a cylindrical shape; it is 25–32 cm in height and 25–30 cm in diameter, and weights 22–32 kg. The sensory characteristics of Pecorino Romano cheese depend mainly on salt concentration (5%) and lipolysis by enzymes (pregastric esterase (PGE)) contained in the lamb rennet paste, and flavor intensity is related to the content of free butyric acid. Proteolysis may show wide variations but the soluble nitrogen is always less than 30% of total nitrogen.

Pecorino Siciliano

This variety of Pecorino cheese is manufactured only in Sicily, between October and June, when whole ewes' milk is available. Only natural whey cultures, containing mainly thermophilic lactic acid bacteria, are used as starter. Lamb rennet paste is used for coagulation, which occurs within 40–60 min. The coagulum is broken up using a traditional wooden tool, known as 'rotella', to the size of peas. The curd is partially cooked at 40 °C for *c.* 10 min by adding hot water (*c.* 70–80 °C). Molding takes place in a circular vessel, traditionally called 'canestro', in which the curd is pressed slightly. The cheese is ripened for at least 6–8 months to develop the moderate piquant flavor. The cheese has a cylindrical shape; it is 12–18 cm in height and 35 cm in diameter, and weights 4–15 kg. Pepato (peppery) is a variant of Pecorino Siciliano cheese that differs by the addition of black pepper to the curds during molding.

Pecorino Sardo

Legally, the manufacture of Pecorino Sardo is limited to Sardinia. Raw or thermized ewes' milk, natural whey or milk cultures, and calf rennet paste are used in cheesemaking, which does not differ substantially from that of Pecorino Siciliano cheese. Ripening of the hard variety may last 12 months. The cheese is cylindrical in shape; it is 10–13 cm in height and 15–20 cm in diameter, and weights 1.7–4.0 kg. The straw-yellow rind is smooth and springy initially, but later it becomes darker and harder. The mature cheese has a pleasant pungent flavor and a firm, hard, fairly granular texture.

Fiore Sardo

The manufacture of Fiore Sardo is strictly limited to some provinces of Sardinia. Traditionally, it was manufactured by shepherds in their cottages. Raw, whole ewes' milk from a single milking is used. A large part of the milk is produced from an indigenous breed of sheep. Starters are not used and lamb rennet paste is used to coagulate the milk. The curd is not cooked and it is pressed slightly during molding. Treatment of the curd with hot water is necessary to make the rind thick and resistant. The cheese is ripened for *c.* 6 months, and during the early phase of ripening, it may be smoked slightly by exposure to smoke from the wood of Mediterranean scrub. During ripening, the cheese is often rubbed with a mixture of olive oil and sheep fat. The cheese has a cylindrical or wheel shape with rounded sides; it is 13–15 cm in height and weighs 1.5–5.0 kg. The flavor is pronounced, aromatic, moderately spicy and the rind varies from deep gold to dark brown with a sour smell.

Canestrato Pugliese

Canestrato Pugliese is manufactured only in the Apulia region. The cheese derived its name and traditional shape from the rush basket 'canestro', in which the curd is ripened. Raw, whole ewes' milk of 1 or 2 days' milking is generally used, but thermized or pasteurized milk may also be processed. Natural whey cultures, consisting of mainly thermophilic lactic acid bacteria, may be added and liquid or powder calf rennet, or, exceptionally, lamb rennet paste, is used. After cutting, the curd/whey mixture is heated to 45 °C and held for 5–10 min. The cheese is dry-salted for *c.* 2 days and, during ripening (4–12 months) in the 'canestro', is turned regularly and rubbed with a mixture of oil and vinegar. Colonization of the cheese surface by environmental molds frequently becomes evident during ripening. Molds are removed by brushing after few months. The cheese has a cylindrical shape with a height of 10–14 cm and a diameter of 24–35 cm , and weights 7–14 kg. The rind is brown to pale yellow, and the interior is compact with small eyes. The flavor is pronounced and tends to a moderate piquancy.

Castelmagno

Traditionally, this cheese has been manufactured in the Grana valley, near Cuneo (Piedmont), since the twelfth century. Raw cows' milk is partly skimmed according to the protocol described for Parmigiano Reggiano cheese. Rarely, a mixture of cows', ewes', and goats' milks is used. The traditional technology does not involve the use of starters but the milk is acidified by its natural microflora. Liquid or powdered calf rennet, which may be combined with a small amount of lamb rennet paste, is used for coagulation. After cutting and removal of most of the whey, the curd is traditionally harvested in a cloth bag, which is hung for 10–12 h at room temperature, allowing the removal of further whey. Ripening is carried out in natural caves at 10–12 °C and 85–90% relative humidity for at least 6 months. The cheese has a cylindrical shape with a height of *c.* 20 cm and a diameter of 20–25 cm, and weights 4–6 kg. A mycelium of environmental *Penicillium* sp. colonizes the cheese surface, and occasionally the interior of the cheese. Castelmagno cheese has a compact but friable texture and moderately piquant flavor.

Fossa

The tradition of Fossa (pit) cheeses originated in the Emilia-Romagna region (northern Italy) in the Middle Ages. The typical feature of this cheese involves ripening in flask-shaped pits that are dug in the tufa soil of this region. Cheese is typically made from raw ewes' milk, but in some cases, mixed ewes' and cows' milks are used. Natural thermophilic organisms acidify the milk. The curd is not cooked, and after molding, it is held at *c.* 28 °C for 4–8 h. The curd is generally ready for ripening in pits after a period (up to *c.* 3 months) of maturation in rooms, which is necessary to achieve a certain degree of consistency, which eliminates the risk of whey losses during further pressing in pits. Before they are placed and pressed in the pits, the curds are wrapped individually in cloth. The sides of the flask-shaped pits are covered with straw, which is fixed by canes, horizontally linked with wooden rings. The pits are open during August and when completely filled with curds, they are hermetically closed. The humidity inside the pits is close to saturation and the temperature ranges from 17 to 25 °C. Traditionally, the pits are opened on the 28th of November; at this time, the cheeses are ripened for at least 6 months, including maturation in rooms. Due to the pressure inside the pits, the shape of the cheeses varies, from cylindrical to very irregular, and the weights ranges from 1.0 to 1.5 kg. The flavor is generally full, sharp, balanced, and moderately piquant.

Ripening

Although all the hard Italian cheese varieties previously described have a large market popularity and are of a marked economic relevance, only some of them have been studied extensively. Besides, similar hard cheeses may be manufactured that differ with respect to type of milk, season of milking, technology, and ripening may be manufactured. As a consequence, the results on cheese characterization may differ markedly.

Owing to the large size and prolonged brine and/or dry salting, most of the hard Italian cheese varieties are commonly characterized by a decreasing NaCl gradient from the surface to the center and by an opposite gradient of water activity (a_w). A gradient of temperature from the center to the cheese surface may also be present. All these gradients persist for a considerable period and consequently ripening may show variations, which depend on the cheese layer.

Thermophilic lactic acid bacteria mainly used as natural whey or milk starters represent a large part of the microbial biomass (*c.* 10^9 cfu g^{-1}) in the curds during the first period of ripening. Their growth and the hydrolysis of residual lactose depend mainly on the rate at which the curd cools after removal from the vat. At this time, the temperature at the center of the curd remains relatively high (e.g., >50 °C for Parmigiano Reggiano) for a rather long period, while the exterior of the cheese cools rather suddenly (e.g., *c.* 42 °C in *c.* 2 h for Parmigiano Reggiano). Consequently, bacterial growth starts earlier and is more intense in the exterior layer. This is the common behavior that characterizes the major part of the hard cheese varieties that are cooked to high temperature and have a rather large size. While the residual lactose is consumed throughout the curd within 8–10 h, bacterial numbers, pH, and lactic acid concentration do not attain equal values in the center and exterior of the curd for long time. These differences are another relevant factor that affects the cheese during subsequent ripening.

Proteolysis

Proteolysis in hard Italian cheeses does not differ substantially from the other hard/semihard internal bacterially ripened cheeses. The low moisture, high salt concentration, persistence of gradients of NaCl and temperature, and the absence of molds, which are evident only on the surface of Canestrato Pugliese and Castelmagno, are the factors that influence proteolysis during cheese ripening. The main proteolytic agents in the curd are coagulants, depending on the source and intensity of the cooking treatment, proteinases and peptidases of starters and autochthonous microorganisms, indigenous milk proteinase (plasmin), and perhaps cathepsins. Proteolysis in 'Grana' cheeses (Parmigiano Reggiano and Grana Padanao) has been studied using different analytical approaches. During the first month of ripening, gel electrophoresis shows rapid hydrolysis of α_{s1}-casein (CN) by chymosin to the primary degradation product α_{s1}-CN f24–199 and the formation of γ-CNs from β-CN by plasmin. Very low levels of β-CN (f1–192) and β-CN (f1–189), the primary products of hydrolysis of β-CN by chymosin, are also found. Overall, hydrolysis of β-CN by chymosin during ripening is limited by 5% NaCl, and during cooking of the curd most of the chymosin activity is destroyed. The same pattern for β-CN was found in Pecorino Romano cheese. Compared to commercial preparation, proteolysis by chymosin during ripening of Pecorino Romano cheeses proceeded more intensively when lamb rennet paste was traditionally manufactured. Overall, β-CN is almost totally hydrolyzed during ripening of Parmigiano Reggiano, Grana Padano, and Pecorino Romano cheeses, while α_{s1}-CN undergoes relatively less proteolysis. These findings confirm that chymosin, which is the primary proteolysis agent for α_{s1}-CN, is not very active in these cheeses. Although Fossa cheeses are characterized by large variability in the urea-polyacrylamide gel electrophoresis (urea-PAGE) profile of their pH 4.6-insoluble fraction, they are commonly characterized by the complete degradation of α_{s1}-CN after 6 months of ripening. Much of the β-CN persists unhydrolyzed in the ripened cheeses. The manufacture of Fossa cheese does not include cooking of the curd. Almost the same was found for Canestrato Pugliese cheese. In this case, since the cheese may be manufactured from either raw ewes' milk or thermized or pasteurized milks, Reverse Phase-Fast Protein Liquid Chromatography (RP-FPLC) analysis of the water-soluble nitrogen (N) fraction revealed a more complex peptide profile in raw milk cheese, which was positively linked to more intense proteolysis.

Hydrolysis of caseins leads to an increased proportion of water-soluble N, which has been used as a ripening coefficient for Parmigiano Reggiano cheese. **Figure 1** shows the changes in the percentage ratio of soluble N to total N for Parmigiano Reggiano cheese during ripening. The increase is very fast during 8–10 months, after which hydrolysis proceeds very slowly. At the end of ripening, the water-soluble N is *c.* 34% of the total N. Similar values (*c.* 32%) are found in Grana Padano cheese. Since the pH of almost all hard Italian cheese varieties is in the range of 5.0–5.5, the values of water-soluble and pH 4.6-soluble N do not differ significantly. Values of pH 4.6-soluble N/total N ranging from 19.0 to 28.7% found in Pecorino Romano cheese approximately coincided with those for the 12% trichloroacetic acid

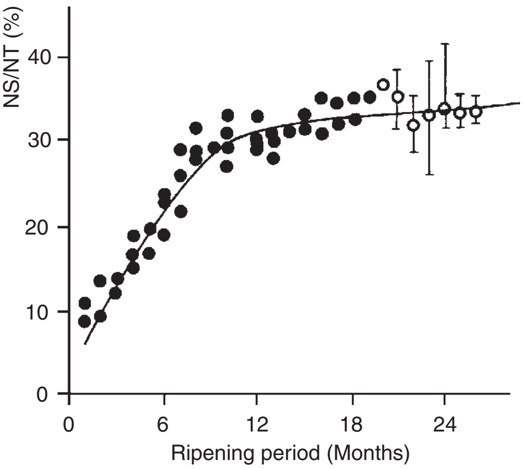

Figure 1 Level of the ratio of water-soluble nitrogen (NS) to total nitrogen (NT) (%) in Parmigiano Reggiano cheese during ripening. Open circles are the average of a few cheeses from the same ripening. Reproduced with permission from Battistotti and Corradini (1993).

(TCA)-soluble N. Since pH 4.6-soluble N is produced mainly by chymosin, while starter and non-starter bacterial enzymes are principally responsible for the formation of 12% TCA-soluble N, these data support the view that chymosin is not very active in this cheese and that once it produces soluble peptides, bacterial peptidases hydrolyze them relatively rapidly. Proteolysis of Pecorino Romano cheese seemed to vary depending on the region of manufacture and source of lamb rennet paste used. At the beginning of ripening, in some cases, proteolysis was higher in the interior of the cheese, but from 40 days onward proteolysis was more extensive in the surface layer due to the inward diffusion of NaCl. Owing to the high concentration of NaCl (5%), salt gradients are particularly persistent for Pecorino Romano cheese. In some other cases, no differences were found in the level of water- and pH 4.6-soluble N at various locations in the cheese throughout ripening. When using traditional lamb rennet paste, the diet of the lambs and slaughtering conditions should be carefully standardized to produce rennet that preserves the traditional characteristics of Pecorino Romano cheese.

The ratio of pH 4.6-soluble N to total N is very high also in Fossa cheese, ranging from 30 to 39%. The ratio of water-soluble N to total N may vary from 13 to 30% in Canestrato Pugliese cheese, depending on whether heat-treated or raw ewes' milk is used.

Variations in the concentration of free amino acids (FAAs) during ripening are other indices to compare hard Italian cheeses (**Table 4**). FAAs accumulate in Parmigiano Reggiano cheese until 15 months of ripening, after which their concentration remains relatively constant. At the end of ripening, the average concentration of FAAs is *c.* 230 mg g^{-1} protein, which corresponds to *c.* 23% of the total protein content. Consequently, Parmigiano Reggiano is one of the cheeses rich in FAAs. The same trend, with similar values, was found for Grana Padanao cheese, showing that extension of ripening to more than 18 months did not produce a significant increase in FAAs. In the last decade, a chemometric model was proposed to estimate the age of Parmigiano Reggiano cheese based on the concentration of serine, glutamine, arginine, and ornithine. The concentration of FAAs in Fossa varies markedly between cheeses but with an average value of *c.* 108 mg g^{-1}, which may be considered relatively high compared to Cheddar cheese, which typically has *c.* 3 mg g^{-1}, and with internally mold-ripened cheese such as Gorgonzola, which contains a concentration of *c.* 15 mg g^{-1}. A similar average value of *c.* 104 mg g^{-1} was found in Canestrato Pugliese cheese manufactured with raw ewes' milk. Apart from the high concentration of threonine, isoleucine, and phenylalanine in Parmegiano Reggiano cheese, glutamic acid, proline, valine, leucine, and lysine are the FAAs commonly present at high concentrations in Parmigiano Reggiano, Pecorino Romano, Canestrato Pugliese, and Fossa cheeses.

Cheese flavor is the result of several nonenzymatic and enzymatic reactions. The catabolism of FAAs

Table 4 Concentration of individual and total free amino acids (mg g^{-1} cheese) in Parmigiano Reggiano, Canestrato Pugliese, and Fossa cheeses

Amino acids	*Parmigiano Reggiano*	*Canestrato Pugliese*	*Fossa*
Histidine	8.20	3.82	2.44
Arginine	2.50	5.01	0.25
Serine	13.60	8.85	3.09
Aspartic acid, asparagine	18.60	2.99	4.09
Glutamic acid, glutamine	45.50	15.34	19.19
Threonine	12.30	3.23	2.07
Glycine	6.40	2.55	1.80
Alanine	6.90	2.87	5.83
Tyrosine	6.30	1.66	2.02
Proline	nd	8.65	5.60
Methionine	7.20	3.25	3.97
Valine	18.40	8.33	9.56
Phenylalanine	13.20	5.88	5.42
Isoleucine	15.90	6.54	6.24
Leucine	22.20	10.99	13.83
Cysteine	nd	1.57	5.00
Ornithine	3.80	nd	nd
Lysine	30.80	13.31	13.09
Tryptophan	nd	0.03	nd
Total free amino acids	231.80	104.87	103.49

The values indicated represent the average of several determinations made by different authors in cheeses that had a slightly different ripening time.
nd, not determined.

seems to be the most important process for generating cheese flavor. Decarboxylation, deamination, transamination, desulfuration, and cleavage of side chains convert FAAs to aldehydes, alcohols, and acids, which together with other compounds derived from other routes (e.g., lipolysis and catabolism of fatty acids) compose the volatile profile of extra-hard cheeses. Overall, large variations for the same cheese variety were found to be related to cheesemaking practices, season of manufacture, duration of ripening, and type of autochthonous microorganisms. Ketones are the most abundant volatile compounds in Parmigiano Reggiano cheese, representing *c.* 26% of total headspace chromatographic area. Also in the Fiore Sardo cheese, ketones are the dominant volatile flavor compounds. Esters are the main neutral constituents in the aqueous distillate of Grana Padano cheese, constituting *c.* 41% of the total neutral volatiles. Esters are also the main volatile components of Canestrato Pugliese cheese, while alcohols dominate in Pecorino Romano cheese.

Some studies have been carried out aiming at combining microbiological and biochemical characterization of hard Italian cheeses. Although an important role in primary proteolysis is variously attributed to chymosin, plasmin, and enzymes from thermophilic lactic acid bacteria starters, the extensive secondary proteolysis and catabolism of FAAs are related to peptidase and other enzyme activities of non-starter lactic acid bacteria (NSLAB), which, in the hard Italian cheeses, derive mainly from the raw milk used. The characterization of the biodiversity of this population was carried out by culture-dependent and, more recently, culture-independent analyses. Although a low number of *Lb. buchneri* and *Lb. parabuchneri* may be found, *Lb. casei*, *Lb. casei* subsp. *pseudoplantarum*, and *Lb. rhamnosus* dominate in Parmigiano Reggiano cheese. *Pediococcus* spp. are also found during ripening of Parmigiano Reggiano cheese. Pediococci seem to be fundamental for maintaining equilibrium within the cheese-related microbial community. A heterogeneous population of enterococci, together with *Lactobacillus plantarum*, *Lactobacillus curvatus*, and *Lb. fermentum*, was found in the ripened Pecorino Romano cheese. *Lactobacillus plantarum* and *Lb. curvatus* were the species isolated most frequently, with fewer numbers of *Lactobacillus paracasei* subsp. *paracasei*, from Fossa cheese. *Lactobacillus plantarum*, *Lb. casei*, *Lb. curvatus*, and *Lb. paracasei* subsp. *paracasei* were also found in Fiore Sardo cheese at the end of ripening. The NSLAB population of Canestrato Pugliese cheese is more heterogeneous and consists of *Lb. plantarum*, *Lactobacillus pentosus*, *Lb. curvatus*, *Lactobacillus brevis*, *Lb. paracasei* subsp. *paracasei*, and *Enterococcus* sp. Ripened Castelmagno cheese mainly contains *Lb. plantarum* and *Lb. paracasei* subsp. *paracasei*. In most hard Italian cheeses, NSLAB reach *c.* 10^7–10^9 cfu g^{-1} after few months, which is generally maintained up to the end of ripening.

Lipolysis

The ripening of most cheeses is accompanied by a low level of lipolysis but extensive lipolysis occurs in some hard Italian varieties. The length of ripening strongly influences lipolysis and since ripening varies markedly within the same variety, cheeses ready for the market may differ greatly. Several hard Italian cheeses are probably unique in that an exogenous lipase is normally added. The desirable flavor that characterizes the Pecorino cheeses (Romano, Siciliano, and Sardo) and Fiore Sardo is due mainly to the activity of PGE in rennet paste, which is used as the source of both coagulant and lipolytic agents in cheese manufacture. Rennet pastes are prepared by macerating the engorged stomachs, including curdled milks, of young calves, kid goats, or lambs that are slaughtered immediately after suckling or pail-feeding. The stomachs and contents are generally held for *c.* 60 days prior to maceration. PGE, the physiological role of which is to aid in the digestion of fat by the young animals, which have limited pancreatic lipase activity, is secreted during suckling and is carried into the stomach with ingested milk. The strong, balanced piquant flavor that characterizes Pecorino cheeses and Fiore Sardo is due primarily to the relatively high levels of short-chain free fatty acids (FFAs), especially butyric acid. Although there are some interspecies differences, lamb, calf, and kid PGEs preferentially hydrolyze fatty acids esterified at the *sn*-3 position of glycerol, which explains the relatively high rate of release of butyric acid from milk fat, in which 90% of the butyric acid is esterified at the *sn*-3 position. The procedure for preparation of rennet paste (e.g., rennet paste from suckling lambs slaughtered immediately after suckling or from suckling lambs not fed for 12 h) has a marked influence on the level of lipolysis during ripening. Nevertheless, the accumulation of short-chain FFAs also characterizes the ripening of Parmigiano Reggiano, Canestrato Pugliese, and Fossa cheeses, for which rennet paste is not used. **Table 5** shows the FFA profile of some hard Italian cheeses. The average values that are reported refer to ripened cheeses with a high popularity on the market, but in general there is no standard flavor for such hard Italian cheeses that is particularly acceptable to all segments of the population. For Pecorino Romano cheese, there is a direct relationship between flavor intensity and the butyric acid content but the relationship between flavor desirability and butyric acid concentration is more variable. Flavor desirability is influenced mainly by the relative proportion of the various FFAs. A strong, balanced, piquant Pecorino Romano cheese may be characterized by *c.* 10 500 mg kg^{-1} of total FFAs, mainly butyric (C_4), together with caproic (C_6), myristic (C_{14}), palmitic (C_{16}), and oleic ($C_{18:1}$) acids (**Table 5**). The total FFA content of Parmigiano Reggiano approaches 20% of that generally found in

Table 5 Concentration of individual and total free fatty acids (mg kg^{-1} cheese) of Parmigiano Reggiano, Pecorino Romano, Canestrato Pugliese, and Fossa cheeses

Fatty acid	*Parmigiano Reggiano*	*Pecorino Romano*	*Canestrato Pugliese*	*Fossa*
Butyric ($C_{4:0}$)	172	3043	425	247
Caproic ($C_{6:0}$)	48	1428	178	123
Caprylic ($C_{8:0}$)	44	429	42	55
Capric ($C_{10:0}$)	107	1009	98	84
Lauric ($C_{12:0}$)	107	690	46	35
Myristic ($C_{14:0}$)	225	778	85	62
Palmitic ($C_{16:0}$)	565	1306	172	137
C_{18} congeners[a]	1033	1843	322	251
Total free fatty acids	2301	10 526	1368	994

[a] C_{18} congeners refer to stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$) acids.

Pecorino cheeses, with variations in the proportions of FFAs. Congeners of C_{18} fatty acids dominate at the end of ripening. Grana Padano cheese contains high concentrations of butyric and caproic acids, which represent 50 and 35% of total FFAs, respectively. Methyl ketones in Parmigiano Reggiano have also been determined. Although the total concentration (*c.* 0.075 μmol g^{-1} fat) is quite low compared to blue cheeses (*c.* 5.18 μmol g^{-1} fat for Roquefort cheese), their ratio is similar to that found in cheese fat, suggesting the spontaneous formation of methyl ketones during ripening. Butyric, caproic, caprylic (C_8), and capric (C_{10}) acids are the dominant FFAs in Pecorino Sardo and Fiore Sardo cheeses. Canestrato Pugliese and Fossa cheeses show a very similar profile of FFAs, although the former has a higher total concentration of FFAs. Butyric acid, which occurs at the highest concentration, caproic, capric, palmitic, and congeners of C_{18} acids dominate. Probably due to the lipolytic activity of molds, which colonize the cheese surface during early ripening, Canestrato Pugliese cheese also shows a rather high proportion of oleic and linoleic ($C_{18:2}$) acids.

Hard Italian cheeses manufactured without the use of rennet paste may vary markedly in the concentration of FFAs depending on the use of raw milk. Several studies have reported a higher concentration of FFAs in cheese made from raw milk compared to cheese made from pasteurized or thermized milks. Notwithstanding the effect of the indigenous milk lipase, such differences were attributed mainly to the lipase and esterase activities of the autochthonous microorganisms, especially NSLAB, and were emphasized when the time of ripening increased. Studies on NSLAB revealed that *Lb. plantarum* contains lipase and esterase with a substrate specificity comparable to that of rennet paste and pancreatic lipase and since there is a very large population of NSLAB during cheese ripening, a contribution to lipolysis was suggested.

See also: **Cheese**: Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese.

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Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese)

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Introduction

The category of cheese known as low-moisture part-skim (LMPS) Mozzarella has grown in worldwide production because of its suitability for use in American-style pizza and the growing popularity of pizza restaurants around the world. In the United States, this includes home-delivery pizza stores, retail stores of frozen pizza, and 'take-and-bake' pizza stores. Since LMPS Mozzarella is an essential ingredient of pizza, the production of this type of cheese far exceeds that of other pasta-filata cheeses and LMPS Mozzarella is what is usually meant when people in the United States refer to 'Mozzarella cheese'. After decades of steady growth in the United States, consumption of pizza has leveled off and represents about a quarter of all cheese consumption, with pizzerias representing nearly one-fifth of all restaurants. As its name implies, LMPS Mozzarella is made from partially skimmed milk that contains about 2% milk fat and, in the United States, its fat content can be from 30 to 44% on a dry basis (FDB), while its moisture content is from 46 to 52%. For the pizza industry, moisture contents toward the upper end of the allowable range are used, with the fat content depending on preferences for cheese performance during baking. Higher fat content cheese has advantages during high-temperature baking conditions because of its increased ease of melting and release of fat when heated. To meet the need for rapid melting in quick service restaurants, some franchise restaurant chains may even select low-moisture Mozzarella with a fat content of 45% FDB or higher.

Since the production of LMPS Mozzarella is coupled so strongly with the sales of pizza, it is manufactured to meet functional performance when heated. The cheese must melt, flow, and brown under conditions specified by each pizza company. The one exception to this is the more recent development of the related product, 'string cheese', which is typically consumed cold. String cheese is LMPS Mozzarella that is hot extruded with a diameter of about a 1.5 cm, brined, cut into finger-length pieces, and then packaged as individually wrapped pieces, which makes it popular as a snacking cheese. The extrusion process orients the cheese fibers formed during the cooking/stretching process into parallel strands such that it is easy to pull off strings of cheese.

Manufacture

The initial manufacturing process of LMPS Mozzarella is similar to other cheeses and it is made on a large scale, with cheese vats containing up to 40 000 l of milk (**Figure 1**). As for other semihard cheeses, the conversion of milk from a fluid suspension of fat globules and casein micelles into a coagulum formed by aggregation of the renneted casein micelles followed by shrinkage of the curd and whey syneresis occurs when making LMPS Mozzarella (**Figure 2**). The characteristic texture of pasta-filata cheeses comes about as the curd is heated and mechanically stretched causing the protein to be formed into fibers. The milk is standardized to a casein-to-fat ratio of about 1.2 either by removing cream or by adding additional solids (such as a liquid or dried skim milk concentrate from ultrafiltration). A starter culture is added to the milk to generate lactic acid. It is possible to use chemical acidification instead of starter cultures. Nowadays, citric acid is often added to the milk for making Mozzarella of a more traditional moisture content (e.g., 55–60%) that is consumed fresh; however, it is not used extensively for making LMPS Mozzarella.

Rennet is added to coagulate the milk (**Figure 3**), and after the casein micelles have aggregated and formed a three-dimensional network (**Figure 4**), the coagulum is cut into particles to allow syneresis. Since thermophilic cultures are used for LMPS Mozzarella, the milk is set at about 35 °C and the curd and whey are stirred and heated to about 40 °C. In comparison, pasta-filata cheeses that are designed for aging and have a lower moisture content are cooked to about 50 °C, while a high-moisture fresh Mozzarella cheese receives little, if any, cooking.

After sufficient stirring and acid development, the whey is drained (at pH 6.2–6.3) and the curd matted or stirred to allow for further acidification and syneresis of whey. In large operations, this takes place on a draining, matting, and cheddaring belt system, after which the curd is cut into finger-size pieces (**Figure 5**). The curd may be partially salted and then heated in hot water (or a hot brine solution) and mechanically worked in a cooker/stretching machine (**Figure 6**) to produce the plastic consistency characteristic of

Figure 1 Large-scale manufacture of LMPS Mozzarella cheese in a series of enclosed cheese vats.

pasta-filata cheeses. Curd is usually ready to be sent to the cooker/stretcher when it has reached a pH of 5.3. Calcium phosphate acts as a bridging agent within the protein matrix of the cheese curd, and as the acidity of the curd increases during cheesemaking, the solubility of calcium phosphate increases, releasing it from the proteins. At a pH of about 5.3, sufficient calcium has been removed from the protein matrix, enabling the proteins to move in relation to each other when heated and subjected to mechanical shear. The casein proteins then become aligned into fibers as the cheese is stretched, with fat globules accumulating in the channels between the protein strands (see **Figures 2**). If curd is stretched before sufficient calcium has been released from the proteins (such as at too high a pH), the cheese is too rubbery.

The stretching process takes place in single- or twin-screw cookers. The hopper of the cooker is filled with hot water (65–70 °C) so that as the curd is heated, it undergoes mixing and stretching while being pushed down the barrel of the cooker/stretcher by the rotating screw(s). Cheese exits the cooker/stretcher at about 55–65 °C as a smooth plastic mass (**Figure 7**) with a fibrous structure. The screw speed determines the residence time of the cheese in the cooker/stretcher and is balanced against the water temperature. If the curd temperature is too low, the curd will not melt sufficiently to allow proper fiber formation, whereas at too high a curd temperature, the curd can melt too much and not provide the proper flow along the cooker or have the desired stretch characteristics. The hot mass of cheese is either filled under pressure into molds (**Figure 8**) or extruded as a continuous ribbon, which is cooled in cold water, cold brine, or in a cooling tunnel.

Traditionally, there was no salting of the curd or stretched cheese until after it had been cooled. Today, salting of the curd is carried out prior to stretching (by adding salt), as part of the stretching process (by using a 5–10% hot brine solution in the cooker/stretcher), or as the hot cheese mass exits the cooker/stretcher prior to being formed into blocks (by adding salt). Mechanization of the cheesemaking process allows virtually any shape of block to be formed, with 3–4 kg blocks being the most common. After cooling and brining (**Figure 9**), the cheese block is dried and vacuum packaged. There is some loss of shape during cooling and brining, but since LMPS Mozzarella is primarily shredded within a few weeks, there is no loss of yield by having different shapes of

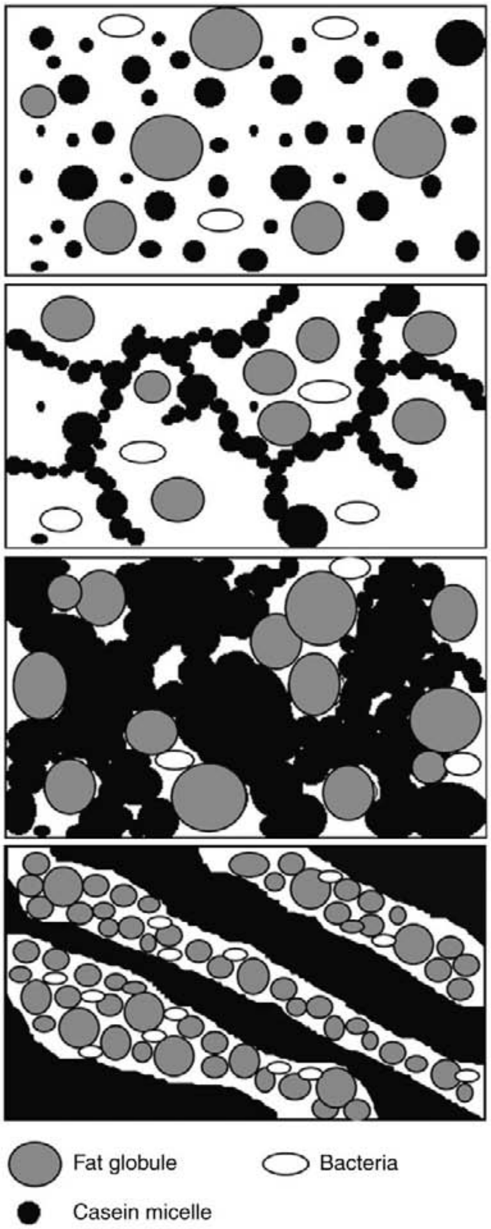

Figure 2 Schematic representation of changes in microstructure that occur in the manufacture of LMPS Mozzarella cheese showing (from top) milk prior to renneting, initial milk coagulum, curd after whey draining, and hot cheese mass after the cooking/stretching process.

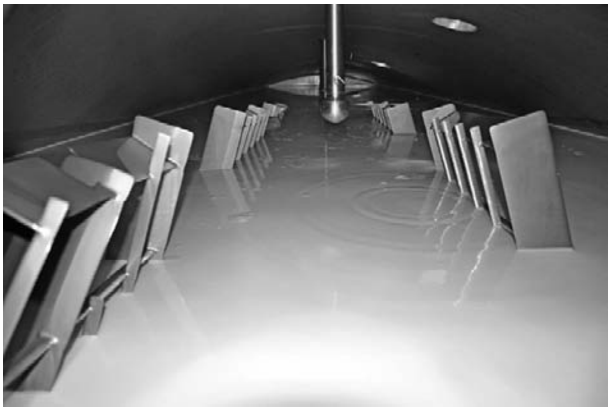

Figure 3 Formation of the initial curd after renneting of milk inside an enclosed cheese vat.

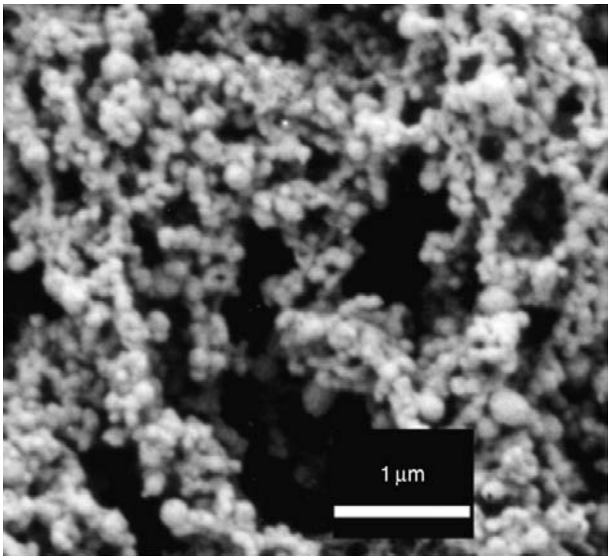

Figure 4 Scanning electron micrograph of the milk coagulum just prior to cutting, showing the network of aggregated *para*-casein micelles (fat globules were removed during sample preparation).

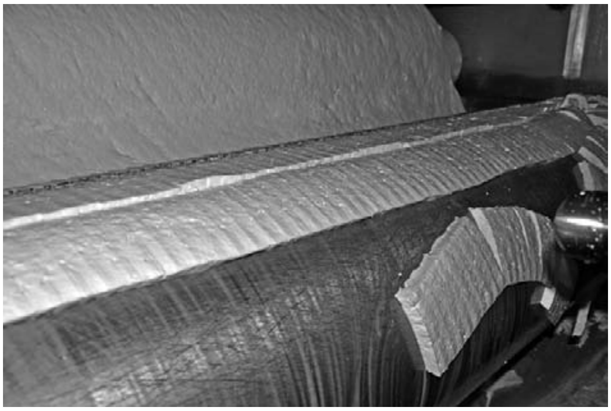

Figure 5 Curd mass being cut into pieces as it exits the draining, matting, and cheddaring belt.

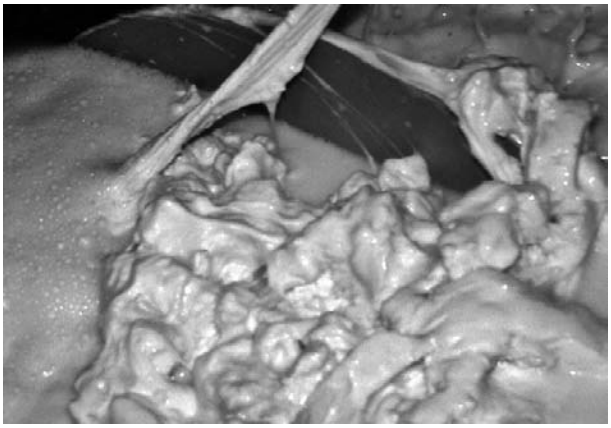

Figure 6 Curd pieces being mechanically stretched in hot water and moved by a rotating single screw into the barrel of the cooker/stretcher.

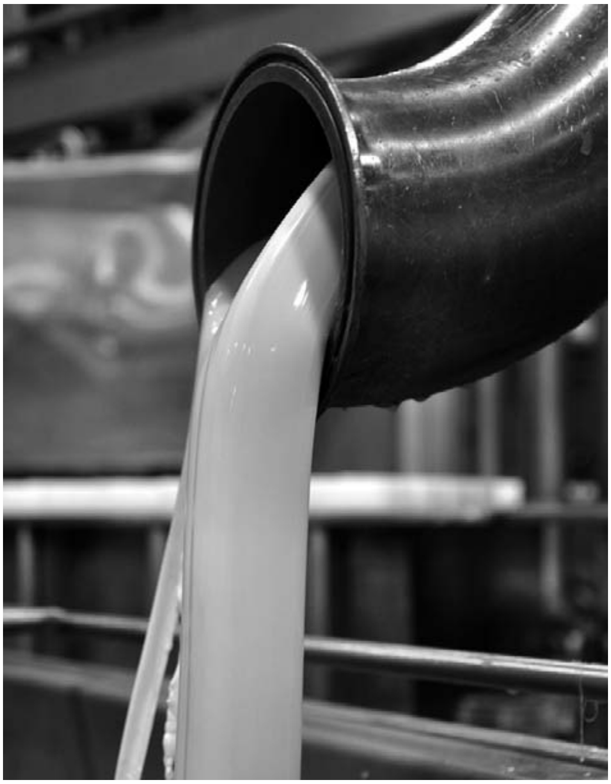

Figure 7 Hot homogeneous mass of cheese as it exits the cooker/stretcher.

cheese or rounded corners on the cheese block. Some LMPS Mozzarella is shredded on the day of manufacture, with the cheese shreds being individually quick frozen and packaged, stored, and sold frozen for use by pizza companies and in the food service industry. In the United States retail market, a small proportion of LMPS Mozzarella is sold as block cheese, with more being sold as packages of shredded cheese or as string cheese.

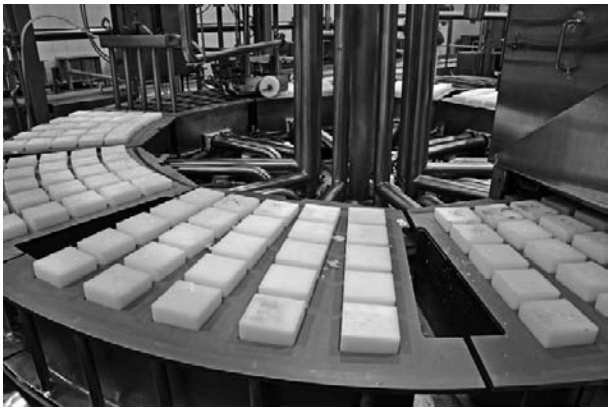

Figure 8 Mechanical molding of hot cheese into rectangular blocks.

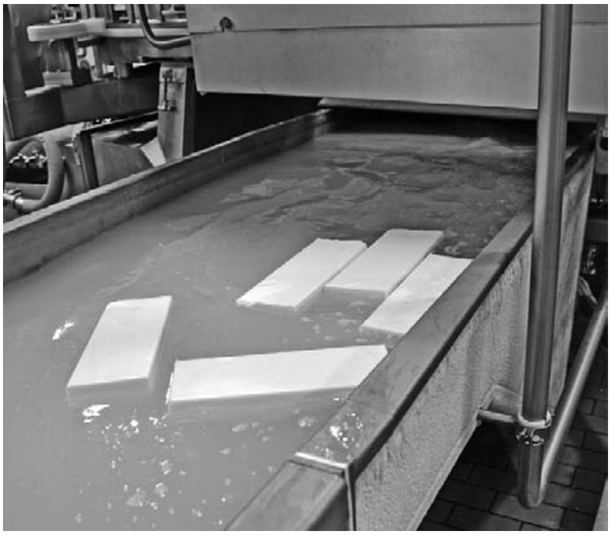

Figure 9 Blocks of LMPS Mozzarella cheese entering the brining system for cooling and salting.

Cultures

Thermophilic lactic acid bacteria are usually used for the production of LMPS Mozzarella although mesophilic cultures can be used if the manufacturing method is altered accordingly so that a cheese with the required physical and chemical properties is produced. *Streptococcus thermophilus* alone or in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus* can be used, and in the United States these are predominantly prepared as bulk cultures grown in the same tank. Symbiosis and competition occur simultaneously during culture growth and yield a particular ratio of coccus (*St. thermophilus*) to rod (*Lactobacillus*). The rate of acid production and proteolytic activity from such mixed coccus and rod cultures is generally greater than the sum of the activities of individual cultures. Lactobacilli degrade casein, supplying peptides and amino acids to the weakly proteolytic streptococci, which in turn produce formic acid and carbon dioxide (possibly through catabolism of urea) to stimulate the growth of lactobacilli.

LMPS Mozzarella has only a mild flavor, so the prime function of the starter culture is rapid production of acid during cheesemaking, which will bring about solubilization of protein-bound calcium so that the curd will have proper melting and stretching properties during the hot cooking/stretching process. *Streptococcus thermophilus* strains hold promise in this regard as they are capable of rapid acid production in which only the glucose portion of lactose is converted into lactose acid, leaving residual galactose in the cheese. Using *Lb. helveticus* as part of the starter culture can reduce these galactose levels. Fast acid-producing cultures are important in large cheese factories where manufacturing times have been reduced to 150 min or less. This requires the use of culture medium that stimulates culture growth and favors the use of coccus-only starter cultures. Having a very short manufacturing time helps to minimize bacteriophage problems even though the large-scale manufacturing facilities provide increased opportunities for bacteriophage development. In addition to a fast acid production capacity, *St. thermophilus* also has a tendency to stop producing acid in curd when pH reaches 5.25–5.30 (i.e., the desirable pH range for stretching). This helps prevent the curd from becoming overacidic if cheese production is interrupted while the curd is still in the vat and helps minimize financial losses from mechanical breakdowns in high-throughput cheese factories.

Proteolysis and Aging

The lower proteolytic activity of *St. thermophilus* also results in LMPS Mozzarella staying firm for a longer time during storage, thus extending its optimum use time for subsequent shredding and baking. Cheese made using coccus-only starter culture has less browning during baking because of decreased production of amino acids and small peptides that can participate in heat-induced browning reactions during baking, even when residual reducing sugars are present.

Overall proteolytic activity of the starter culture is not the only criterion affecting the functional properties of the cheese. Bacteria such as *Lb. helveticus* show a wide range of proteolytic activity, and while typical strains of *St. thermophilus* make little contribution to proteolysis in cheese, there are atypical strains that exhibit considerable proteolytic activity. Proteinase specificity can also influence cheese functionality and produce cheeses with different firmness, melting, and stretching properties during storage. Proteolysis occurring in the cheese is generally initiated by the residual coagulant, which when chymosin is used is mainly limited to α_{S1}-casein. Initial cleavage occurs at Phe_{23}–Phe_{24} to primarily yield α_{S1}-casein (f1–23) and *Lactobacillus delbrueckii* (f24–199), with up to 90% conversion into α_{S1}-casein (f24–199) occurring within the first month of storage depending on cheese composition and manufacturing methods. Unless the coagulant used was derived from *Cryphonectria parasitica*, proteolysis of β-casein proceeds very slowly with most of the β-casein remaining intact. The initial cleavage of α_{S1}-casein has minimal effect on the melting properties of the cheese, while cleavage of β-casein results in increased meltability. The level of correlation of melting with proteolysis during storage is given by overall proteolysis > loss of intact β-casein > loss of intact α_{S1}-casein.

After initial cleavage of the proteins by the coagulant enzymes, subsequent proteolysis through the combined action of the coagulant, indigenous milk enzymes (particularly plasmin), and bacterial enzymes occurs at a slower rate. Large peptides such as α_{S1}-casein (f24–199) are hydrolyzed into medium and small peptides. The starter culture influences the rate of secondary proteolysis and LMPS Mozzarella made using only *St. thermophilus* undergoes less proteolysis than when cheese is made with *St. thermophilus* combined with *Lb. helveticus*. Individual lactobacilli strains show a wide range of proteolytic activity, and even the specificity of their proteinases can influence changes in functional properties during cheese storage.

Rate of proteolysis will vary depending on the amount of residual coagulant in the cheese and the moisture content of the cheese. Cheeses with higher moisture typically undergo faster proteolysis. Increasing or decreasing the residual coagulant level in the cheese has a corresponding effect on the rate of initial cleavage of the caseins. The extent of such proteolysis varies widely depending on the temperature and time of hot water cooking/stretching and the consequent thermal inactivation of the coagulant and starter cultures. Thermophilic starters are generally inactivated during stretching at about the same temperature as the coagulant enzymes. Small differences in stretching temperature can cause large changes in proteolysis during cheese storage. Curd that is stretched at a high curd temperature will have limited residual coagulant activity and will require a longer period of storage to develop desirable functional properties than cheese that is stretched at a low curd temperature. If a highly proteolytic coagulant is used and it is not inactivated during stretching, there can be excessive proteolysis, which can lead to rapid softening of the cheese body and onset of poor shredding characteristics, and loss of stretch, elasticity, and chewiness.

Only a brief ripening period (usually less than 1 month at 4 °C) is required for LMPS Mozzarella cheese to develop the desired functional properties for use as a pizza ingredient. Typically after manufacture, the cheese is not suitable for shredding. It contains a considerable amount of water, which can easily be released as a watery serum onto the cut surfaces of shredded cheese. When cheese is maintained at cold temperatures, the caseins become more hydrated, allowing free water to be absorbed into the protein matrix (**Figure 10**) and the water-holding capacity of the cheese increases. After 2 or 3 weeks, the proportion of expressible water drops to zero. The cheese then shreds well and develops the desired baking characteristics of good melting with some elasticity of the melted cheese, so it stretches when pulled apart. With extended storage and continuing breakdown of the proteins, the cheese loses its optimum baking properties and produces too thin a melt to stretch well.

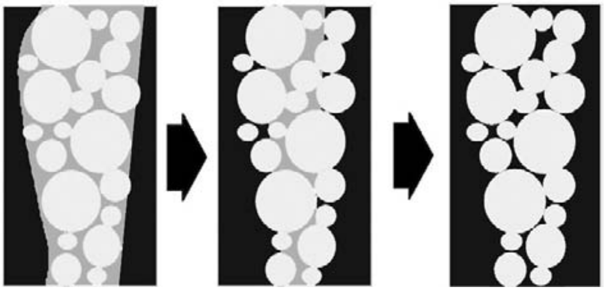

Figure 10 Schematic representation of transfer of water from the fat/serum channels into the protein matrix during cold storage of LMPS Mozzarella cheese and concomitant swelling of the protein matrix.

Cheese Structure

The texture of LMPS Mozzarella is typical of the pasta-filata family of cheeses as the stretching process transforms the curd from an open-celled structure consisting of a network of protein strands about 2–6 μm thick containing interspersed serum and fat globules (**Figure 11**) into parallel protein fibers separated by long channels of accumulated fat and free serum (**Figure 12**). After the protein strands cool, the channels separating the protein fibers are filled with close-packed fat globules. During the pasta-filata process, as the cheese melts and is mechanically mixed, the protein strands fuse together except where this is prevented by the fat globules. When fat is removed from Mozzarella cheese, it results in less fiber formation and consequently fewer pools of free serum formed during cooking/stretching. In the extreme case of a nonfat Mozzarella cheese, the result is a homogeneous amorphous structure virtually devoid of fibers because there are no fat globules to prevent coalescence of the protein strands.

After the cheese has been cooled and brined, the fat globules solidify and the pliable protein presses around

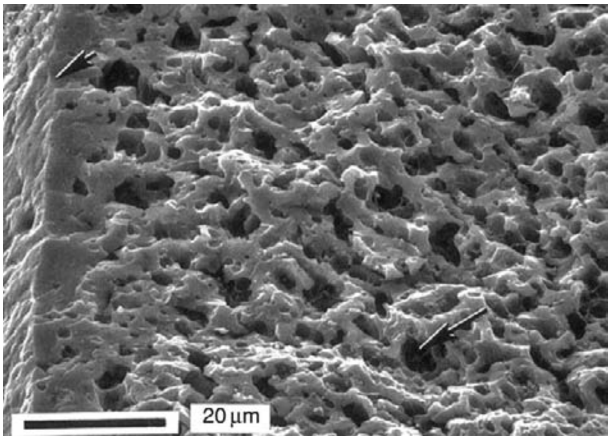

Figure 11 Scanning electron micrograph of curd particles after draining of whey showing the surface membrane (small arrow) and vacuoles within the protein network where fat globules and free serum are located (fat globules were removed during sample preparation). Reprinted from Oberg CJ, McManus W, and McMahon DJ (1993) Microstructure of Mozzarella cheese during manufacture. *Food Structure* 12: 251–258.

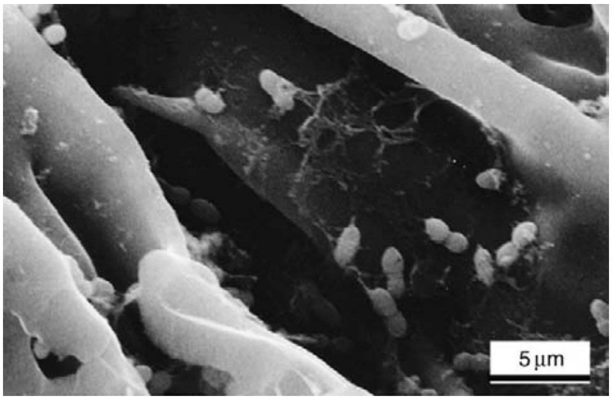

Figure 12 Scanning electron micrograph of hot LMPS Mozzarella cheese showing the formation of protein network into parallel strands separated by serum channels where fat globules and bacteria are located (fat globules were removed during sample preparation). Reprinted with permission from McMahon DJ, Fife RL, and Oberg CJ (1999) Water partitioning in Mozzarella cheese and its relationship to cheese meltability. *Journal of Dairy Science* 82: 1361–1369.

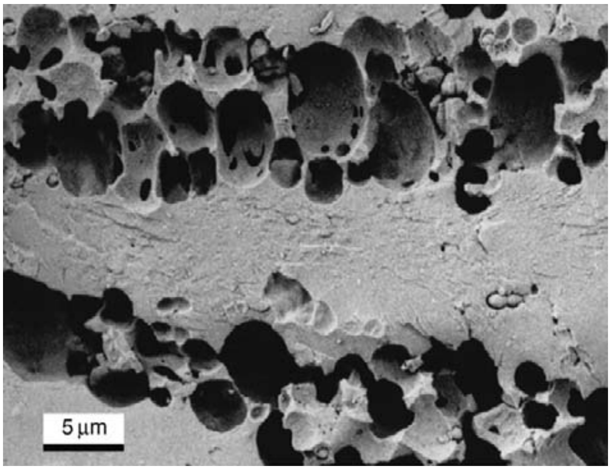

Figure 13 Scanning electron micrograph of LMPS Mozzarella cheese after 4 weeks of cold storage showing the protein network fully encasing the fat globules (fat globules were removed during sample preparation). Reprinted with permission from McMahon DJ, Fife RL, and Oberg CJ (1999) Water partitioning in Mozzarella cheese and its relationship to cheese meltability. *Journal of Dairy Science* 82: 1361–1369.

them. During the initial cold storage, the influence exerted on proteins by hydrophobic interactions is diminished and the proteins have less tendency to aggregate, allowing moisture to be adsorbed into the protein matrix. The protein strands become more hydrated and expand as moisture is transferred from the serum/fat channels (see **Figure 10**). After 2–3 weeks, the serum that can be expressed from the cheese drops to zero and the protein matrix completely encases the fat globules (**Figure 13**). Simultaneously, there is a slight increase in the proportion of soluble calcium, with a corresponding decrease in calcium bound to the caseins. The decrease in hydrophobic interactions and calcium bridging, and the increase in protein hydration in combination allow greater movement of the proteins in relation to each other. When LMPS Mozzarella is then heated, the cheese can flow as it melts and, for pizza, the desired balance between melting and stretching can be achieved. As more aging occurs, protein–protein interactions that bind proteins to each other are diminished through proteolysis, so the cheese melts too quickly and loses its stretch characteristics.

Cheese Functionality

For LMPS Mozzarella to melt well when heated, but not form excessive pools of oil and have just enough elasticity to form strands of melted cheese when slices of pizza are separated and eaten requires a balance between proteins interacting strongly with each other (elasticity) and proteins moving freely past each other (meltability). This balance is controlled primarily by the amount of calcium available for forming cross-links between the casein molecules that form the protein matrix of LMPS cheese. Proteolysis of casein appears to play a secondary role. This relationship becomes apparent during cheese manufacture because if the curd has too much calcium interacting with the caseins, it will tear and break during cooking/stretching and not produce a homogeneous plastic mass. If too much calcium is removed prior to cooking/stretching, there is insufficient protein–protein interaction, so the cheese becomes too soft and fluid when heated and the resultant cheese melts readily but lacks the proper stretch attributes when subsequently cooked on a pizza.

Traditionally, pH is used for determining when curd is ready for the stretching process, but this is actually an indication of the extent of calcium solubilization in the curd and transfer of calcium into the whey during acidification. This relationship becomes apparent when cheese is made using direct acidification in which the milk is acidified prior to renneting. In these cheeses, the desired melting and stretching properties are obtained at a higher pH when a calcium-binding acid, such as citric acid, is used. Similarly, if the calcium content of the cheese is kept constant, then the functionality of the cheese is independent of pH, although below pH 5.0, the caseins lose solubility and the increased protein–protein interaction limits the flow and meltability of the cheese.

The amount of calcium bound to the caseins can be reduced to overcome problems encountered when insufficient melting occurs, such as when making lower fat cheeses. In LMPS Mozzarella, butterfat accounts for about 20% of the weight (and volume) of the cheese, which when heated initiates the softening process as it liquefies. The consistency of the melted cheese then becomes solely dependent on the interactions between the proteins within the protein matrix. A common strategy used for making lower fat cheeses is to partially acidify milk to pH 6.2–6.4 prior to renneting. The amount, and type, of acid added depends on the extent of calcium solubilization needed. In addition to improving cheese meltability, the decrease in calcium-mediated protein–protein interactions allows for increased hydration of the protein matrix and hence a higher moisture cheese is also produced.

In general, factors that decrease protein–protein interactions in the protein matrix or increase protein–water interactions will increase melting behavior. Salt addition (as little as 0.5% NaCl to about 2%) increases casein solubility, while very high salt levels or very low pH can bring about a reduction in protein solubility and cheese melting. As a result, the desired melting and stretching properties of Mozzarella cheese can be achieved by balancing interactions of casein molecules with each other and with water. The use of chemical acidification for making cheese without any starter culture can produce a cheese that has good melting properties immediately after manufacture even though very little proteolysis has occurred. Such cheeses also exhibit less change in functionality during storage.

Browning of cheese during pizza baking is a property of LMPS Mozzarella that can be controlled based upon the concentration of amine and aldehyde compounds (amino groups on proteins/peptides/amino acids, and reducing sugars, respectively). LMPS Mozzarella cheese made using direct acidification has less browning during pizza baking. Without proteolysis by starter culture enzymes during cheese aging (especially those from lactobacilli), there is a decrease in the amount of reactive amino groups and less brown pigments due to Maillard reactions are generated during heating even in the presence of lactose. Similarly, if there is no residual sugar (lactose or galactose) in the cheese, then the cheese will remain white during baking. Typically, all of the lactose is utilized by starter cultures either during cheese manufacture or during the initial storage period, but the levels of residual galactose vary depending on the starter cultures used and the curd temperatures reached during the cooking/stretching process. The majority of *St. thermophilus* strains do not generally utilize the galactose moiety of lactose during the generation of lactic acid, and, typically, neither does *Lb. delbrueckii* subsp. *bulgaricus*. This combination of cultures can result in galactose accumulation producing a cheese that browns easily. Using *Lb. helveticus* as the rod portion of the starter culture will produce a cheese with less browning because it utilizes galactose produced by *St. thermophilus* as an energy source when all the lactose has been used. Washing of the cheese curds before cooking/stretching can also be used to reduce lactose and galactose content as a method to reducing browning. If an increase in browning is desired, then sugars, such as in the form of milk powders, can be added into the hot cheese mass after cooking/stretching. When such a cheese is baked on a pizza, there is an increase in browning and blistering.

Numerous variations for making LMPS Mozzarella cheese have been developed, including leaving out the cooking/stretching step, resulting in an equivalent pizza cheese with the same chemical composition and properties but without the pasta-filata cheese microstructure. The traditional brining process can be shortened or eliminated by adding salt to the curd prior to cooking/stretching, by performing the cooking/stretching in a hot brine solution, or by adding salt to the hot curd mass as it exits the cooker/stretcher. The functional properties of LMPS Mozzarella cheese can also be modified by adding starches, gums, surfactants, and calcium-binding emulsifying salts into the curd mass before or after heating.

See also: **Cheese**: Cheeses Matured in Brine; Past a Filata Cheeses: Traditional Pasta-Filata Cheese; Rennet-Induced Coagulation of Milk. **Lactic Acid Bacteria:** *Lactobacillus* spp.: *Lactobacillus helveticus*; *Streptococcus thermophilus*.

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Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese

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Introduction

Pasta-filata cheeses are a diverse group of cheese varieties manufactured from the milk of cows, water buffalo, goats, or sheep. Pasta-filata cheeses originated in northern Mediterranean areas, encompassing Italy, Greece, the Balkans, Turkey, and eastern Europe. The term pasta-filata derives from an Italian phrase that literally means 'spun paste' or 'stretched curd'. Some cheeses are soft or semisoft and are consumed fresh or after a short period of ripening (e.g., high-moisture Mozzarella, low-moisture Mozzarella or Pizza cheese, and Scamorza). Other cheeses are hard or especially semihard and undergo extensive ripening before consumption (e.g., Caciocavallo, Ragusano, Kashkaval, and Provolone). All pasta-filata cheeses share a unique processing step toward the end of manufacture, when the curd is immersed in hot water or salt brine and manually or mechanically worked (stretched) to a semiflowable plastic consistency that may be formed or molded into a variety of shapes. The main chemical composition of some pasta-filata cheeses is shown in **Table 1**.

Before describing the texture, microbiology, and ripening aspects, brief details of the specific technological traits of the main pasta-filata cheeses will be given.

Mozzarella di Bufala Campana

Mozzarella di Bufala Campana has a protected designation of origin (PDO) and is manufactured from only raw milk of water buffalo (*Bubalus bubalis*) in southern Italy (Campania and Lazio regions). An optimal ratio of protein (~ 4.3–4.7%) and fat (~7.0% or above) is required to get optimal functional characteristics of the cheese. Natural whey cultures are used as starters (~2.5% of the cheese milk). The whey from previous cheesemaking is incubated at room temperature until it reaches an acidity of 40–60° SH 100 ml^{-1}. Liquid calf rennet is added to the milk at 34–38 °C and coagulation takes ~30 min. After cutting into grains of dimensions of a nut (2–3 cm), the curd is allowed to ripen in the whey for a variable time period, in most of the cases ~5 h from the addition of rennet. Stretching of the curd is initiated by adding hot water (~95 °C) to raise the curd temperature to ~68 °C. Stretched curd is then immersed in cool water and further in brine, before packaging. Mozzarella di Bufala Campana has a round shape, although other typical shapes (e.g., bocconcini, trecce, and nodini) are manufactured. It weighs 20–800 g, depending on the shape. The color is porcelain white, with very thin rind (~1 mm) and smooth surface. The flavor is very characteristic and delicate, mainly originating from the lactic acid fermentation by starter organisms.

High-Moisture Mozzarella

High-moisture Mozzarella cheese is recognized in Italy as traditional speciality guaranteed (TSG). Since it is traditional and not specific to one region, the cheese is manufactured throughout Italy, mainly using cows' milk. In Italy, the annual production of cows' milk high-moisture Mozzarella cheese is noticeably higher than that of water buffalo Mozzarella and it is estimated to be ~160 000 tonnes, and is increasing yearly. The fat-in-dry matter (FDM) content of high-moisture Mozzarella cheese is standardized in the range 13–20%. The technology reflects the protocol described for water buffalo Mozzarella but pasteurized (71.7 °C for 15 s) cows' milk may also be used for the manufacture and natural milk cultures are also frequently used as starters. Low-Moisture Mozzarella (Pizza Cheese)

During recent decades, the global production of low-moisture Mozzarella cheese has markedly increased and now it exceeds that of all other pasta-filata cheeses because of premier status as a pizza topping. The FDM content of low-moisture Mozzarella cheese is standardized in the range 30–45%. Therefore, the cheese milk is characterized by a higher casein to fat ratio either by adding nonfat milk solids or, less often, by removal of cream. The standardized milk is pasteurized and then inoculated with starter cultures. The inoculated milk is coagulated with rennet and the coagulum is cut and held at ~41 °C, especially if a thermophilic starter is used, after which part of the whey is drained off. Curd is then subjected to further draining, matting, and cheddaring until the pH reaches 5.3–5.1. In most of the cases, curd is dry-salted and plasticized and stretched mechanically in hot water. The hot plastic curd is forced under pressure into a chilled mold, which gives the cheese its shape and which precools the block so that it retains its shape when removed from the mold.

Table 1 Gross chemical composition of some pasta-filata cheeses

Cheese	*Moisture (%)*	*Fat (%)*	*Total protein (%)*	*NaCl (%)*	*pH*	*pH 4.6-soluble N /(total N)*
Mozzarella di Bufala Campana	58.0	21.0	19.0	0.8	5.3	4.5
High-moisture Mozzarella	54.0	18.0	22.0	0.7	5.7	4.6
Low-moisture Mozzarella	47.0	24.0	21.0	1.5	5.4	7.5
Caciocavallo Silano	40.0	27.0	33.0	3.9	5.4	37.0
Caciocavallo Pugliese	38.0	24.0	23.5	2.5	5.4	18.0
Ragusano	38.0	30.0	30.0	2.5	5.3	16.0
Kashkaval	38.0	32.0	21.0	3.0	5.2	16
Provolone Valpadana	38.0	28.5	24.0	3.2	5.3	n.a.

The values indicated represent the average of several determinations made by different authors in cheeses that had a slightly different ripening time.
n.a., not available.

Caciocavallo Silano and Related Varieties

Legally, the manufacture of Caciocavallo Silano is limited to the regions of Calabria, Campania, Molise, Puglia, and Basilicata (southern Italy), where the mountain chains of the 'Sila' are located. It is a semihard pasta-filata cheese with PDO status and is manufactured from raw or mildly thermally treated (~58 °C for 30 s) cows' milk. Thermophilic natural whey cultures are used as starters. Milk coagulation is achieved using calf rennet paste at 36–38 °C. The curd is cut at dimensions of a nut (~2–3 cm). Curd grains are allowed to ripen under whey for a time period ranging from 4 to 10 h, depending on acidity, temperature, and size of the cheese. Manual stretching under hot water (70–80 °C) results in the typical flask-like shape of the cheese with the head closed by strings. After cooling in water, the curd is salted in brine for at least 6 h. The heads of the curds are tied with a slipknot to poles to favor aeration and allowed to ripen for 15 days to several months. The cheese weighs 1–2.5 kg, with smooth, thin, and yellow rind. The flavor is delicate with a tendency toward sweet for young cheeses, while ripened cheeses tend to become piquant.

In the southern Italy, large amounts of other types of Caciocavallo cheeses are manufactured (e.g., Caciocavallo Pugliese and Podolico), the typical feature of which is the use of natural whey starter cultures. These starters are obtained by incubating the fresh whey, derived from a previous cheesemaking, at 40–42 °C for ~24 h. The protocols for manufacture of these varieties of Caciocavallo mainly reflect those described for Caciocavallo Silano cheese.

Ragusano

Ragusano, previously named Caciocavallo Ragusano, is a semihard PDO pasta-filata cheese manufactured in Sicily from raw cows' milk. It is produced from cows reared mainly on natural pastures of the Hyblean plain region of the province of Ragusa. Cows' milk from one or more milkings is coagulated at ~34 °C using lamb or kid rennet paste. Coagulation generally takes 60–80 min. The curd is progressively cut to dimensions of rice grains (~0.7 cm). After draining of almost all the whey, the curd is pressed and hot water is added; the curd is held under these conditions for ~85 min. The curd is further subjected to drying for ~20 h and cut in slices. Stretching is carried out manually by the addition of hot water (~80 °C), and giving to the curd a parallelepiped shape with a square section. Salting is carried out in brine. Traditionally, the cheese is ripened in caves for 6–12 months, depending on the varieties. During ripening, the curd may be treated with olive oil several times. The cheese weighs 10–16 kg, with smooth and yellow rind. The flavor is delicate and sweet for young cheeses, while extensively ripened varieties tend to become moderate piquant.

Kashkaval

Several varieties of Kaskaval-type cheese are manufactured, generally in Mediterranean areas other than Italy: Kashkaval Balkan, Kashkaval Preslav, Kashkaval Vitosha (Bulgaria), Kackavaly (Croatia), Kachekavalo (Russia), Kasseri (Greece), Kasar (Turkey), and Cascaval Dobrogen (Romania). These varieties of Kashkaval are manufactured from raw or pasteurized milk from cow, sheep, or goat, or from mixed milk. Traditionally, Kashkaval cheese is manufactured from raw milk, which is generally of poor microbiological quality, without the addition of starter cultures. During the last decade, the use of pasteurized milk and starter cultures has been introduced gradually to standardize the quality of Kashkaval cheese. Calf rennet is generally used as the coagulant. The coagulum is usually cut finely into pieces of 6–8 mm and stirred at ~32 °C for ~5 min. Scalding (e.g., 42 °C for 35 min) of the curd may or may not be practiced, depending on the varieties. The curd is then ladled into molds and allowed to drain and press under its own weight for ~30 min. The bed of fused curds is then sliced into blocks and cheddared to allow the lactic fermentation to continue. The ripened curd is texturized, which is accomplished by soaking the blocks of curd of different dimensions in hot (72–75 °C) brine. The curd mass is agitated with a strong wooden stick in order to obtain a compact structure. The hot curd is then hand-kneaded like a dough, which makes it more plastic and elastic. In traditional manufacturing, Kashkaval cheese is partially dry salted during kneading of the texturized curd. Kashkaval is generally ripened for 3–4 months. The typical form of Kashkaval is flat cylindrical with a smooth, amber-colored rind, and the cheese is 30 cm in diameter, 10–13 cm in height, and 7–8 kg in weight. The flavor is generally full, sharp, balanced, and moderately piquant.

Provolone Valpadana and Related Varieties

Although certainly derived from the tradition of pasta-filata varieties from southern Italy, Provolone Valpadana is a semihard PDO cheese manufactured from raw cows' milk in the north of Italy (Lombardia, Emilia-Romagna, Veneto, and Trentino regions). Thermophilic natural whey or milk cultures are used as starters. Milk coagulation is achieved using calf rennet or calf rennet paste, depending on the variety of cheese (sweet or piquant) to be manufactured. After cutting, the curd is allowed to ripen under whey until the desirable acidity is reached. Stretching is carried out manually in hot water giving the curd the typical flask-like shape with the head closed by strings. Salting is carried out in brine and the cheese is ripened for different time periods, depending on the variety (in some cases up to 6 months); during ripening, the cheeses are bound by strings and hung. The cheese weighs 500 g to 100 kg, and for the ripened cheeses the flavor is rather piquant.

Provolone del Monaco is another semihard pasta-filata cheese traditionally manufactured in the Lattari mountains area of Campania region from raw cows' milk and without starter addition. Currently, it has transitory PDO recognition. The protocol for manufacture of this variety of Provolone cheese mainly reflects that described for Provolone Valpadana.

Texture

The stretching process is the main characteristic feature of pasta-filata cheeses and profoundly influences their texture. Stretching transforms the amorphous three-dimensional protein matrix of the curd into an oriented, quasilaminated structure, consisting of parallel-aligned protein fibers separated by long channels containing accumulated fat and serum. This ability of the curd seems to be governed primarily by the amount of casein-associated calcium (calcium phosphate) that is available to cross-link the amorphous *para*-casein matrix at the time heat is applied to the curd. The hydration of *para*-casein increases as the level of casein-associated calcium decreases, which probably contributes greatly to the ability of the curd to plasticize. Curd that contains too much casein-associated calcium fails to attain a smooth, stretchable consistency upon heating, while curd with too little casein-associated calcium becomes excessively soft and fluid-like during stretching. Two parameters determine the amount of casein-associated calcium in the curd at the time of stretching: (1) the total calcium content of the curd and (2) the distribution of total calcium between the soluble and insoluble (e.g., casein-associated) states. Casein-associated calcium dissociates from the *para*-casein matrix to the water phase as the pH of the curd decreases and syneresis progresses. Calcium losses are marked when acidification occurs before the onset of syneresis, as in the manufacture of preacidified and directly acidified Mozzarella cheeses. Overall, curd that contains a high total calcium content at stretching (e.g., ~30 mg g^{-1} protein, as for low-moisture Mozzarella cheese) must have a low pH, in the range of ~5.1–5.3, in order to attain a casein-associated calcium level that is low enough to allow the curd to plasticize and stretch. On the contrary, curd with a low total calcium content (e.g., 22 mg g^{-1}protein, as for directly acidified Mozzarella cheese) is optimally stretched at a higher pH value (e.g., 5.6–5.7). Therefore, controlled acidification, demineralization, and dehydration, coupled with attaining a critical curd pH at the time of stretching, are the key technological parameters for all pasta-filata cheeses.

This unique curd architecture gives rise to a number of important functional characteristics. For example, traditional high-moisture Mozzarella cheese, which is typically shaped into balls and packed in a dilute salt solution, retains a tender, slightly springy and chewy texture despite having a very juicy body that is very high in moisture. The surface of high-moisture Mozzarella can be peeled off in layers, reminiscent of peeling an onion.

Similarly, low-moisture Mozzarella (Pizza cheese) that is extruded continuously to form string cheese is extremely springy and elastic and peels easily into layers along the axis parallel to the fiber direction, making it well suited as a snacking cheese for children. The elastic fibrous structure also lends itself well to breading and deep frying, for use as an appetizer or snacking cheese. The fibrous structure of low-moisture Mozzarella enables it to melt on pizza to a stringy, elastic, comparatively chewy consistency that has come to be accepted as the standard for Pizza cheese. Textural defects are particularly problematic in Pizza cheese because the quality of

Pizza cheese is largely defined by its shredding and melting properties. Pizza cheese with a soft body due to high fat or moisture content, low calcium content, or excessive proteolysis may gum-up the shredding equipment and take longer to process through the equipment. The resulting cheese shreds may be deformed in shape, sticky, and prone to matting. Such cheese when melted may flow excessively (soupy) and lack stretch, elasticity, and chewiness. At the other extreme, Pizza cheese with an abnormally firm body due to low moisture or fat content may also take longer to shred and shatter and form fines during shredding. Such cheese may melt to a very tough and elastic consistency that is overly chewy.

In long-ripened pasta-filata cheeses, such as Caciocavallo and Provolone, the unique stretched curd architecture manifests itself as a flaky texture.

Microbiology

Thermophilic lactic acid bacteria such as *Streptococcus thermophilus*, alone or mainly in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lb. helveticus*, are used as starters for most pasta-filata cheeses. However, low-moisture Mozzarella for pizza may also be manufactured using mesophilic starters (e.g., *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) or some varieties of Kashkaval cheese also include in the starter formulation *Leuconostoc* sp. and *Lb. casei*; this is because the high temperature used in cheesemaking is more tolerated by thermophilic starters. *Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. helveticus* survive and remain metabolically active when the curd temperature at stretching is ~55 °C. However, the activity of thermophilic starters is substantially decreased at the higher stretching temperature of the curd (e.g., 62–66 °C). Besides, thermophilic starters more easily allow to attain the range of moisture desired for Pizza cheese (~48–52%). Nevertheless, in several cases, natural starter cultures have a very heterogeneous composition. In addition to thermophilic lactic acid bacteria, natural whey starter cultures used for the manufacture of high-moisture Mozzarella cheese contain large numbers of mesophilic lactic acid bacteria such as *Lb. plantarum*, *Lb. casei* subsp. *casei*, *Lc. lactis* subsp. *lactis*, and enterococci (mainly *Enterococcus faecium* and *Ec. durans*). A study on a large number of natural whey cultures for Caciocavallo Silano cheese revealed mainly thermophilic lactic acid bacteria, even though the mesophilic *Lc. lactis* subsp. *lactis* was also present in several preparations. Natural whey cultures for the manufacture of Caciocavallo Pugliese are dominated by strains of *Sc. thermophilus*, *Lb. delbrueckii* ssp., *Lb. helveticus*, *Lb. fermentum*, and *Lb. gasseri*.

Modifications in the composition of the microbial population are generally seen during ripening of semihard pasta-filata cheeses. Although the thermophilic lactic acid bacteria from the natural whey cultures dominate during early ripening, Caciocavallo Pugliese harbors a heterogeneous population of non-starter lactic acid bacteria (NSLAB) during late ripening, which is dominated by *Lb. parabuchneri* and *Lb. paracasei* subsp. *paracasei*. *Lactobacillus paracasei* subsp. *paracasei*, *Lb. fermentum*, and *Lb. plantarum* generally dominate in Caciocavallo Silano cheese during late ripening. Ripening of Provolone del Monaco, made without the use of deliberately added starters, is typically characterized by the dominance of thermophilic lactic acid bacteria (*Sc. thermophilus*, *Sc. macedonicus*, *Lb. delbrueckii* spp., and *Lb. fermentum*), together with enterococci and NSLAB of the *Lb. casei* group, especially *Lb. rhamnosus*.

The main role of starter cultures during the manufacture of pasta-filata cheeses is to synthesize enough lactic acid to demineralize and transform the curd into the state that undergoes stretching in hot water at the target pH. Furthermore, microbial acidification has to proceed at a rate that allows an adequate syneresis during manufacture to achieve the target moisture content. Rapid acidification allows the manufacturing time to be shortened, which reduces syneresis and enables a high moisture content to be achieved in the final cheese. Starter cultures may be eliminated altogether and replaced by direct chemical acidification of the milk during manufacture of high- or low-moisture Mozzarella, provided that an appropriate level of demineralization in combination with an appropriate pH at stretching is achieved. The secondary role of starters in ripened pasta-filata cheeses, including Pizza cheese, is concerning secondary proteolysis. Nevertheless, the significance of microbial proteolysis is largely influenced by the temperature of stretching. The synthesis of small peptides and free amino acids (FAAs) by starters is also important in low-moisture Mozzarella because they markedly influence the browning properties of the cheese during melting and baking in pizza making, which is an important functional attribute. Furthermore, Mozzarella cheeses that are manufactured using thermophilic starters generally have a characteristic yogurt-like note resulting from the synthesis of acetaldehyde by *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Mozzarella that is manufactured without starter cultures through direct acidification will assume the flavor of the chemical compounds used. For example, when vinegar is used as the acidulant, the resulting cheese will possess a mild acetic acid flavor note. On the contrary, if citric acid is used, the cheese will be insipid, due to the lack of flavor other than that arising from milk constituents.

Ripening

Some pasta-filata cheeses such as high-moisture Mozzarella and Mozzarella di Bufala Campana are eaten immediately after manufacture without ripening. On the contrary, low-moisture Mozzarella (Pizza cheese) undergoes a brief but essential ripening period (less than 1 month at 4 °C) to develop the desirable functional characteristics. Immediately after manufacture, Pizza cheese is generally difficult to shred because it contains free moisture at the cut surfaces. Besides, unripened Pizza cheese melts to an excessively tough and fibrous consistency with limited ability to flow and releases a considerable amount of watery serum. After 2 or 3 weeks of ripening, the shredding and melting characteristics of the cheese improve markedly. This is due to both proteolytic and physicochemical changes. Proteolysis is initiated by the residual coagulant and plasmin. Hydrolysis of α_{s1}- and β-caseins results in an increase of meltability (e.g., capacity to flow) and a decrease in the toughness and fibrous consistency of the melted cheese. The hydrolysis of caseins leads to an increased proportion of pH 4.6-soluble nitrogen (N). The ratio of pH 4.6-soluble N/total N is higher for low-moisture Mozzarella than for high-moisture Mozzarella and Mozzarella di Bufala Campana (**Table 1**). The rate of primary proteolysis will depend on the period of ripening and the degree of thermal inactivation of the coagulant during stretching. Curd that is stretched at a high temperature (e.g., >65 °C) has limited residual coagulant activity and requires a longer ripening time to develop functional characteristics than curd that is stretched at a low temperature (e.g., 55 °C) with a relatively high residual coagulant activity. Peptides that are produced through primary proteolysis by coagulant serve as the substrate for further hydrolysis to smaller peptides and FAAs by starter bacteria, provided that they survive during stretching. Besides primary proteolysis, physicochemical changes occur during the first few weeks. Initially, much of the water within the cheese structure exists in the form of serum pockets that are loosely held within open channels that separate the network of parallel protein fibers. Nevertheless, under the influence of the sodium chloride that is dissolved in the water phase of the curd, casein aggregates that serve as the building blocks of the protein fibers interact and bind free water through a process of casein solvation and solubilization. As a consequence, protein fibers become more hydrated and the water-holding capacity of the cheese increases, giving rise to improved shredding and melting characteristics.

The development of flavor is of limited concern during the brief ripening of Pizza cheese with low concentrations of total FAAs and total free fatty acids (FFAs) (~500 and 360 mg kg^{-1} cheese, respectively), as the cheese is expected to be very mild in flavor. Strong flavor development is essential during the ripening of long-ripened pasta-filata cheeses such as Caciocavallo Silano, Ragusano, Provolone Valpadana, and Kashkaval. Proteolysis and lipolysis are considered as the two primary biochemical processes of long-ripened pasta-filata cheeses, which involve many chemical, physical, and microbiological changes under controlled environmental conditions. During ripening, the distribution pattern of FAAs and FFAs changes due to the complexity of the maturation process, resulting in the formation of the characteristic flavor of long-ripened pasta-filata cheeses (**Tables 2** and **3**). Proteolysis of long-ripened pasta-filata cheeses is influenced by chymosin, plasmin, and peptidases from starters and NSLAB, pH, moisture of curds, temperature and time of storage, salt, and humidity. Urea-polyacrylamide gel electrophoresis (urea-PAGE)

Table 2 Concentration (mg kg^{-1} cheese) of individual and total free amino acids in some pasta-filata cheeses

Amino acid	*Caciocavallo Silano*	*Caciocavallo Pugliese*	*Ragusano*	*Kashkaval*	*Provolone Valpadana*
Aspartic acid	660	646	800	182	1000
Cysteic acid	530	43	0	0	0
Threonine	670	449	800	109	30
Serine	1790	612	1100	41	700
Glutamic acid	3860	1969	6000	401	2000
Glycine	430	410	500	67	300
Alanine	690	640	540	191	800
Cysteine	140	109	120	0	0
Valine	1500	991	210	104	1000
Methionine	460	395	650	66	200
Isoleucine	1200	992	156	81	0
Leucine	2430	1552	2800	260	3000
Tyrosine	680	458	430	130	0
Phenylalanine	1100	1286	1900	295	0
Histidine	160	267	850	62	30
Lysine	2560	1414	3000	126	3000
Arginine	0	53	0	80	0
Proline	2320	1632	2000	173	0
Total free amino acids	21 180	13 918	21 856	2368	12 060

The values indicated represent the average of several determinations made by different authors in cheeses that had a slightly different ripening time.

Table 3 Concentration (mg kg^{-1} cheese) of individual and total free fatty acids in some pasta-filata cheeses

	Butyric ($C_{4:0}$)	*Caproic ($C_{6:0}$)*	*Caprylic ($C_{8:0}$)*	*Capric ($C_{10:0}$)*	*Lauric ($C_{12:0}$)*	*Myristic ($C_{14:0}$)*	*Palmitic ($C_{16:0}$)*	*Stearic ($C_{18:0}$)*	*Oleic ($C_{18:1}$)*	*Linoleic ($C_{18:2}$)*	*Linolenic ($C_{18:3}$)*	*Total free fatty acids*
Caciocavallo Silano	4	2	540	985	875	924	11 650	300	2600	680	170	18 730
Caciocavallo Pugliese	320	100	35	70	50	95	160	20	145	25	18	1038
Ragusano	1093	437	161	309	317	597	123	1285	371	1232	40	5965
Kashkaval	5	5	3	5	17	168	554	180	456	45	16	1454
Provolone Valpadana	782	308	81	172	122	120	199	334[a]				2118

[a] C_{18} congeners refer to stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$) acids.

The values indicated represent the average of several determinations made by different authors in cheeses that had a slightly different ripening time.

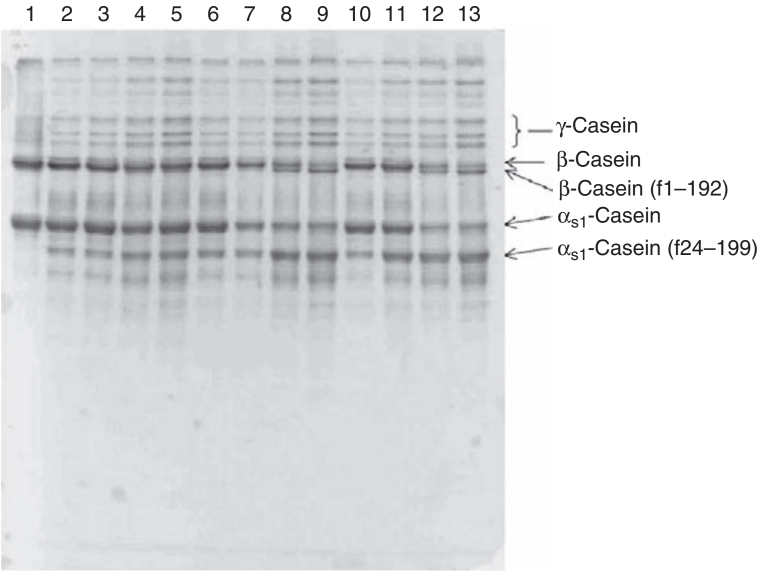

Figure 1 Urea-PAGE of pH 4.6-insoluble nitrogen fraction from different sections of Caciocavallo Pugliese cheese during ripening. Lane 1, sodium caseinate standard; lanes 2–5, outer section at 1, 21, 42, and 60 days; lanes 6–9, middle section at 1, 21, 42, and 60 days; and lanes 10–13, inner section at 1, 21, 42, and 60 days.

indicated that both α_{s1}- and β-casein are hydrolyzed during ripening. Owing to the large size of most of the long-ripened pasta-filata cheeses, persistence of gradients of temperature, and especially of NaCl between the center and rind of the curd, may influence proteolysis during cheese ripening (**Figure 1**). The action of both plasmin and chymosin on α_{s1}- and β-casein is clearly evident from their characteristic proteolytic products. Chymosin readily hydrolyzes α_{s1}-casein with the concomitant formation of a peptide identified as α_{s1}-casein (f24–199). The primary cleavage site of chymosin in β-casein is Leu_{192}–Tyr_{193}. In solution, chymosin cleaves β-casein at seven sites. The resulting large peptides are β-casein f1–192, f1–189, f1–163/4/5, and f1–139. Plasmin has been shown to be active in long-ripened pasta-filata cheeses since it is heat stable and not inactivated under the time, temperature, and pH conditions that occur during stretching. Evidence of plasmin activity in Caciocavallo Pugliese cheese is shown by the degradation of β-casein with the concomitant formation of γ-caseins. The primary proteolysis may also differ for different pasta-filata varieties depending on the stretching temperature. For instance, the high stretching temperature used during the manufacture of Provolone cheese results in limited residual coagulant activity and, therefore, proteolysis proceeds slowly. On the contrary, Kashkaval is stretched at a lower temperature, which results in higher residual coagulant activity and greater primary and further secondary proteolysis during ripening. The same is generally found in Ragusano and Caciocavallo Pugliese cheeses. The ratio of water-soluble N/total N may vary from 13 to 37% in long-ripened pasta-filata cheeses (Caciocavallo Silano, Caciocavallo Pugliese, Ragusano, Kashkaval, and Provolone Valpadana), depending on the specifics of manufacture (**Table 1**). The concentration of FAAs in the water-soluble extract of cheeses may vary from ~2368 to 21 856 mg kg^{-1}, indicating a very different level of secondary proteolysis among the different cheeses (**Table 2**). At the end of ripening, the average concentration of FAAs in Caciocavallo Silano and Ragusano cheeses is ~21 180 mg kg^{-1} (corresponding to ~6.6% of the total protein content), which may be considered relatively high compared to Caciocavallo Pugliese (~13 918 mg kg^{-1}), Provolone Valpadana (12 060 mg kg^{-1}), and especially Kashkaval, which typically has ~2368 mg kg^{-1} (corresponding to ~1% of the total protein content). Glutamic acid, valine, leucine, and lysine are the FAAs commonly present at high concentrations in all long-ripened pasta-filata cheeses. The other FAAs are present at different concentrations among the cheeses.

Long-ripened pasta-filata cheeses are characterized by a different level of lipolysis. The concentration of total FFAs may vary from ~1388 to 18 730 mg kg^{-1} (**Table 3**). Probably also due to the use of calf rennet paste, Caciocavallo Silano contains high concentrations of FFAs (~18 730 mg kg^{-1}), the principal of which are caprylic, capric, lauric, palmitic, myristic, and oleic acids. During the ripening of Ragusano cheese, lipolysis by lamb or kid rennet paste liberates ~5965 mg kg^{-1} of free FFAs. Compared to proteolysis, lipolysis seems to proceed at higher rates on the exterior portion of the curd blocks. The opposite characterizes ripening of Caciocavallo Pugliese where the main liberation of FFAs (e.g., butyric, caproic, palmitic, and oleic) decreases from the middle and inner to outer sections of the cheese.

See also: **Cheese:** Cheese Flavor; Hard Italian Cheeses; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese). **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics.

Further Reading

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Smear-Ripened Cheeses

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Introduction

The surface of smear-ripened cheeses, for example, Limburg, Romadour, Chaumes, Tilsit, Gruyère, Parmigiano Reggiano, and some acid-curd cheeses (Harzer, Quargel), is covered by a layer of yeasts and bacteria. These so-called secondary cheese cultures have a strong impact on appearance and flavor. Smear cheeses are generally known for their intense sulfurous and ammoniacal smell. This article reviews their production, ripening, and flavor characteristics. Special emphasis is put on new developments in the surface starter technology, an area neglected for long. Thus, over years, there are periodical reports of contamination of smear cheeses with *Listeria monocytogenes*, sometimes leading to food poisoning incidents and, consequently, to image damage and to large economic losses for cheese manufacturers. The data presented on novel starters were obtained from studies on semisoft cheeses (Tilsit-like), semihard cheeses (Caractère-type, Cave cheese-type), soft cheeses (Limburg type), and yellow-type acid-curd cheeses (Germany: 'Harzer'; Austria, Czech Republic: 'Quargel'). Surface culture studies were performed in several research projects and in an EU demonstration project; the latter project confirmed the functionality of the proposed starters for semihard smear cheese types.

Production Statistics

Cheese production and consumption is growing worldwide; the worldwide exports increased from around 1.25 million tonnes in 2000 to >1.5 million tonnes in 2005 (source: FAO). The annual production of cheese in the European Union (EU-27) exceeded 9 million tonnes in 2007 with a per capita consumption of 17.7 kg (forecast 2007, EU-27, source: ZMP). The production of smear cheeses has a long tradition all over Europe. However, due to the intense volatile sulfurous and ammoniacal smell, smear cheeses are not mass produced as are Gouda or Cheddar cheese. The available statistics give no information on the percentage of smear-ripened cheeses; current estimates indicate that smear-ripened cheeses account for 5–15% of the European cheese market. Smear cheeses are traditionally produced on a small scale in a farmhouse environment in many European countries. Production is more industrialized in countries like France, Germany, Denmark, and The Netherlands; however, most cheese companies are still small- or medium-sized enterprises.

Technology

Smear-ripened cheeses can be produced from any kind of rennet curd. They can be divided into (semi)-soft (moisture 45–60%), semihard (moisture 40–50%), hard (moisture 30–40%), and very hard cheeses (moisture <30%). The classification schemes commonly used are rather inconsistent; in this article, Limburg-type cheese will be classified as 'soft' and the unpressed Tilsit-type cheese as 'semisoft'. Some well-known cheese varieties are listed in **Table 1** and shown in **Figure 1**. Apart from the influence of starter and non-starter lactic acid bacteria, cheese ripening is influenced by enzymatic activities of the surface microflora. Typical 'wet ripening' (during which the smear develops) times are 2–3 weeks for the soft Limburg/Romadour-type cheeses (200–500 g), 1–6 months for semisoft Tilsit-type cheeses (2.5–4.5 kg) and semihard varieties, and 6–12 months or more for large hard and very hard cheeses such as Gruyère and Parmigiano Reggiano.

The wet ripening of 'modern' smear cheeses is significantly reduced. After 2–4 weeks, cheeses are waxed and/or wrapped in foils with product and company logos before further ripening is continued. This leads to an odor reduction without considerable loss of taste, and protects the cheeses from further microbial contamination in the ripening room.

Quite different from all rennet-type cheeses are the low-fat (<1%) acid-curd cheeses, which have a tradition in Germany (Harzer), Austria (Quargel), and some eastern European countries. They are produced from quarg (>30% dry matter), which is acidified and coagulated with yogurt cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). Wet ripening of acid-curd cheese is usually restricted to 1–3 days in the factory before packaging in oxygen-permeable foil. Ripening progresses during transportation and cold storage in food markets over the shelf life of about 45 days after packaging. When no white quarg core is visible (~14–21 days), the cheeses are called mature.

Table 1 Examples of some well-known smear-ripened cheese varieties

Cheese varieties	*Origin*	*Ripening period (months)*
Soft		
Limburg, Romadour	Germany	<1
Chaumes	France	<1
Semisoft/semihard		
Münster	France	1
Brick, Monterey	United States	1–2
Saint Paulin	France	1–2
Havarti	Denmark	1–3
Taleggio	Italy	2
Tilsit	Germany	1–5
Bel Paesa	Italy	4–5
Hard		
Danbo	Denmark	1–2
Gruyère	France	4–12
Very hard		
Grana Padano	Italy	>12
Parmigiano Reggiano	Italy	>24
Acid-curd cheese		
Harzer Roller, Quargel	Germany	<1

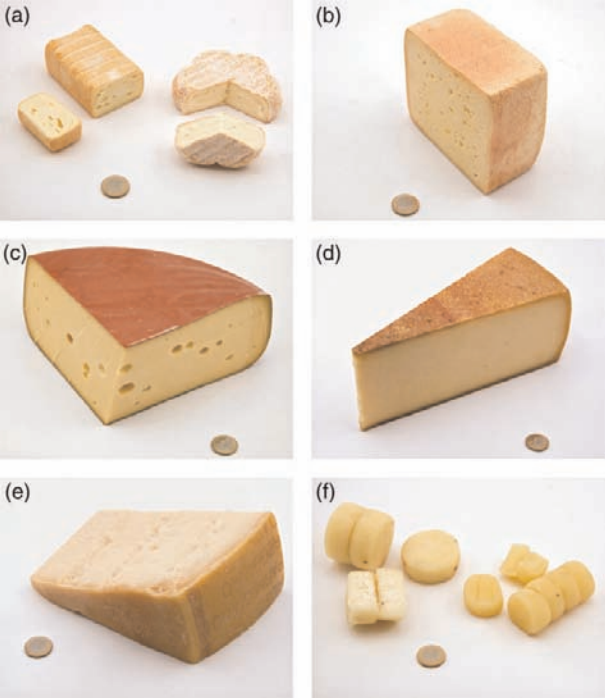

Figure 1 Surface ripened cheeses. (a) German Limburg cheese (left) and French soft cheese (right) with orange smear and white patches by *Geotrichum candidum* growth; (b) typical German Tilsit cheese with pink areas and a very smeary surface; (c) bottom side of a modern semihard smear cheese with limited wet ripening; the surface is covered by wax; the top side shows brand name and company logo; (d) Swiss raw milk hard cheese with smear; (e) very hard Italian Parmigiano Reggiano cheese; (f) yellow-type acid-curd cheese; left: traditional size, age 2 weeks with a white core of unripened acid curd; right: convenient 'party-size' product, fully ripened, without a white core of acid curd. A €1 coin is shown for size comparison.

Salting

In general, cheeses are salted by brining in sodium chloride solution of ≥18% salinity (**Figure 2**). Small soft cheeses like Limburg or Romadour are brined for 1.5– 4 h, and the larger semisoft and hard cheeses are brined for 24 h and more depending on the size and moisture content of the cheese. An alternative traditional manual method for small soft cheeses is wiping the surface repeatedly with cloths soaked in brine over one or more days; similarly, dry salt can be applied manually or by machines spraying salt vapors. Salting of acid-curd cheeses is performed by mixing acid curd with so-called ripening salts (NaCl, $NaHCO_3$, $CaCO_3$) to obtain the appropriate salinity and pH (**Figure 2**). At the start of ripening, the acidity of a smear-ripened cheese surface is around pH 5.2.

Brine Microflora

Cheese brines that are not pasteurized regularly develop a typical salt-tolerant microflora with a composition depending on the house microflora of the factory. In a study involving 48 commercial cheese brines from northern Germany, the brine microflora showed a large degree of variation between cheese plants; however, there were no major differences between brines used for different cheese types (smeared, unsmeared, or mold-ripened cheeses; cheeses produced from raw or pasteurized milk). Apart from many fungal and bacterial species that can be found at low cell counts ($<10\,\text{cfu ml}^{-1}$), salt-tolerant yeasts (*Debaryomyces hansenii*) and food-grade staphylococci (*Staphylococcus equorum*) are detected at 10^2–$10^5\,\text{cfu ml}^{-1}$ in aged brines. In factories with low hygienic standards or in times of the year with warm and humid weather conditions, cheese brines can be contaminated with *Mucor* and other molds.

Since the surface of smear cheeses is not covered by wax or foil after brining, it can be imagined that the brine microflora has a significant impact on the development of the surface microflora and product quality of smear cheeses. It was shown in experiments that high concentrations of *D. hansenii* and *S. equorum* efficiently inhibited mold growth (**Figure 3**).

Obviously, no effect of the brine microflora is present when dry salting is used or for acid-curd cheese when quarg is mixed with solid ripening salts. For acid-curd cheese, a comparable early microbial 'contamination' of the cheese surface before smearing is caused by the house microflora, that is, fermenting yeasts *Kluyveromyces marxianus* (syn. *Candida kefyr*) and *Issatchenkia orientalis* (syn. *Candida krusei*), present in the cheese milk and quarg.

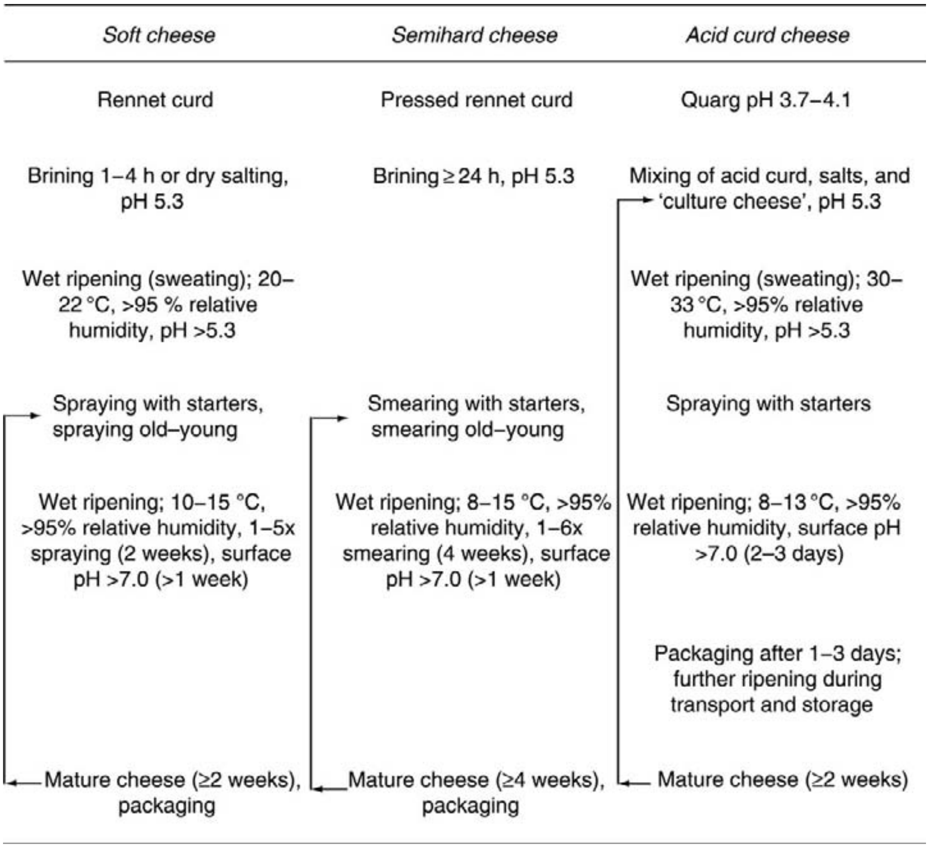
Soft cheese
Semihard cheese
Acid curd cheese
Rennet curd
Pressed rennet curd
Quarg pH 3.7–4.1
Brining 1–4 h or dry salting, pH 5.3
Brining ≥ 24 h, pH 5.3
Mixing of acid curd, salts, and 'culture cheese', pH 5.3
Wet ripening (sweating); 20–22 °C, >95 % relative humidity, pH >5.3
Wet ripening (sweating); 30–33 °C, >95% relative humidity, pH >5.3
Spraying with starters, spraying old–young
Smearing with starters, smearing old–young
Spraying with starters
Wet ripening; 10–15 °C, >95% relative humidity, 1–5x spraying (2 weeks), surface pH >7.0 (>1 week)
Wet ripening; 8–15 °C, >95% relative humidity, 1–6x smearing (4 weeks), surface pH >7.0 (>1 week)
Wet ripening; 8–13 °C, >95% relative humidity, surface pH >7.0 (2–3 days)
Packaging after 1–3 days; further ripening during transport and storage
Mature cheese (≥2 weeks), packaging
Mature cheese (≥4 weeks), packaging
Mature cheese (≥2 weeks)

Figure 2 Production and ripening scheme of smear cheese varieties. The arrows indicate the traditional old–young step, that is, recycling of smear liquid for rennet cheeses (recycling of whole 'culture cheeses' for acid-curd cheese). Conditions given in the flowchart can vary between cheese manufacturers.

Smearing/Spraying Technology

Salted, pressed cheese curds left in humid atmosphere will develop a surface flora consisting of bacteria, yeasts, and molds. The sources for these microorganisms are the milk, cheese brines, surfaces (wooden ripening shelves), air of cheese factories, and the human skin. When the humidity in ripening rooms is high enough (>95% relative humidity) and cheeses are brushed with salt water (~3%) repeatedly over days, growth of molds is suppressed by growing yeasts and bacteria, producing a sticky, smeary surface. By maintaining suitable temperatures and humidity during ripening (8–15 °C, >95% relative humidity), a typical red smear microflora develops because natural selective pressures favor the growth of these microorganisms. A fully developed smear for semisoft or semihard cheese types shows bacterial counts of $\sim 10^9\,\mathrm{cfu\,cm^{-2}}$ and lower yeast counts, usually with *D. hansenii* dominating. Soft cheese types showing growth of *D. hansenii* and *Geotrichum candidum* possess lower surface bacterial counts.

Without using any commercial starters, the ripening of young cheeses can be initiated efficiently by mature (old) cheeses: typical smearing machines are equipped with a brush in the form of a belt in a loop (semisoft and hard cheeses) wetted in a tank containing salt water. When mature cheeses are brushed, a part of the surface flora is retained in the salt water and transferred to (young) green cheeses treated thereafter. This is called 'old–young' smearing. Traditional smearing employs a high degree of manual labor. Today, automatic machinery is available and is used even by many small producers. For the smaller-sized soft cheeses, high-pressure spraying is used instead of brushing. However, the recycling of dripping smear liquid (old–young step) is used for these cheese types too. For acid-curd cheeses, a different 'old–young' step is included with the same effect: a special batch of cheeses is wet-ripened for 2 weeks instead of 1–3 days (**Figure 2**). This so-called 'culture cheese' is added to quarg (2–4% w/w) together with ripening salts to initiate the ripening of acid-curd cheeses, which represents the equivalent of smearing.

It is still general practice to use old–young smearing for ripening of rennet-type smear cheeses (**Figure 2**). In the case of acid-curd cheese, the 'old–young' step

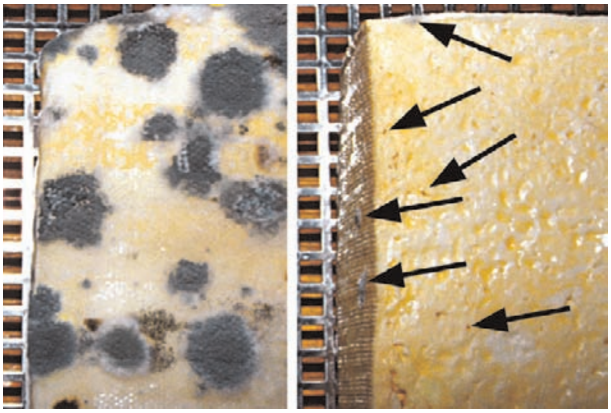

Figure 3 Mold-inhibiting properties of the brine microflora: (left) semisoft cheeses salted in 18% NaCl under sterile conditions (24 h) with a strong mold contamination after ripening at 14 °C and 97% relative humidity for 2 weeks; (right) cheese brines inoculated with yeasts and staphylococci (*Debaryomyces hansenii*, *Staphylococcus equorum*, each species at 10^7 cfu ml^{-1}) showed fewer fungal colonies with smaller diameter (arrows). No smear bacteria (coryneforms) were applied for both cheese batches, thus a typical smear development was not observed. The surface flora consisted mainly of yeasts and staphylococci. (Modified from Jaeger *et al*., 2002).

becomes critical when ripening of a special batch of 'culture cheese' is initiated by the addition of aged 'culture cheese' (=contamination cycle). The associated hygienic problems of old–young cycles are obvious: saprophytic or pathogenic bacteria as well as molds can become part of the house microflora and can persist on the cheese surface over long periods of time. A certain level of contaminating enterobacteria and enterococci is usually found on the surface of traditionally old–young smeared cheeses (**Table 2**). For reasons largely unknown, pathogens (*L. monocytogenes*) sometimes grow to high cell numbers posing a risk for consumers. Therefore, old–young smearing is more and more criticized and efforts have been initiated to establish alternative methods, that is, use of defined surface starter cultures.

Surface Ripening Cultures

The Microflora of Smear Cheeses

To define the requirements of a functional surface starter, essential components of the surface microflora have to be identified, and the role of the species detected has to be understood. In contrast to mold-ripened cheeses, such as Camembert, the composition of the surface flora of commercial smear cheeses still depends largely on the specific house microflora of the cheese manufacturer. The smear cheese flora is described in many papers and reviews as extremely diverse and variable even for a single cheese variety. However, some general compositional similarities can be found for all smear cheese varieties. The surface microflora mainly consists of fungi (*D. hansenii*, *G. candidum*), food-grade staphylococci (*S. equorum*), and the so-called 'coryneform bacteria', which is not an accepted taxon but a useful descriptor for irregular, club- or V-shaped rods belonging to the smear bacteria *Corynebacterium* spp., *Brevibacterium* spp., *Microbacterium* spp., *Arthrobacter* spp., and *Brachybacterium* spp. (**Figure 4**).

Bacterial surface cell counts are around 10^9 cfu cm^{-2} for mature smear cheeses of any kind (age >1–2 weeks). Yeast and *Staphylococcus* counts are high at the start of ripening because of their acid and salt tolerance; however, counts usually drop during ripening, when coryneforms become predominant (**Table 2**).

Soft cheeses (Limburg, Chaumes, St. Albray, etc.)

The surface microflora shows two dominating fungal species, *D. hansenii* and *G. candidum*. Apart from beige corynebacteria (e.g., *Corynebacterium casei*), a high percentage of yellow coryneforms (*Microbacterium gubbeenense*, *Arthrobacter arilaitensis*) is observed (**Table 2**). They usually constitute 20–50% of the surface microflora, but the percentage can be near 100% of total coryneform counts for some varieties. *Brevibacterium linens* counts are usually low (1–10% of coryneforms), and sometimes higher counts can be found; the orange cheese color of soft cheeses is not related to *B. linens* counts (see below). Staphylococci are often missing in the flora. One reason may be the frequent pasteurization of cheese brines, applied by some cheese plants to prevent excessive growth of house flora yeasts. Typical for a French soft cheese over several years were orange *Micrococcus* sp. and *Kocuria* sp., microscopically being large diplococci (not shown). The presence of a high percentage (30–40% of coryneforms) of salt-tolerant, motile rods (*Halomonas* sp.) was typical for a German Limburg cheese producer, always detected on cheeses in analyses over a period of 2 years. The degree of contamination with enterobacteria and enterococci is usually higher for soft cheeses than for harder cheese varieties.

Semisoft cheeses (Tilsit) and harder varieties

Due to their presence in cheese brines, *D. hansenii* and beige or orange *S. equorum* are always found on semisoft cheeses, especially at early ripening stages (**Table 2**). If commercial '*Micrococci-Preparations*' (trade name) are used for smearing, *Staphylococcus xylosus* will also be detected on the cheese surface. A certain percentage of yellow-pigmented coryneforms (*M. gubbeenense*, *A. arilaitensis*) is present with maximum counts usually observed after

Table 2 Surface microflora of smear cheeses

	French soft cheese	*German Limburg*	*German Tilsit semisoft*	*Semihard cheese*[b]	*Swiss hard cheese*	*Italian very hard cheese*	*Yellow-type acid-curd cheese*
Surface microflora[a] *(cfu cm^{-2})*	*~3 weeks*	*~3 weeks*	*~10 weeks*	*~15 weeks*	*~12 months*	*~36 months*	*~3 weeks*
YGC agar							
Debaryomyces hansenii	4.2×10^{8}	4.5×10^{5}	3.2×10^{4}	1.8×10^{4}	7.9×10^{2}	3.3×10^{2}	3.0×10^{6} (KM)[c]; 4.9×10^{6} (CK)[c]
Geotrichum candidum	1.0×10^{7}	2.6×10^{6}	8.2×10^{4}	-	1.0×10^{2}	-	1.0×10^{5}
Molds	-[d]	-	-	8.0×10^{2}	1.2×10^{5}	1.0×10^{2}	-
Modified milk agar							
Beige-red coryneforms[b] (*Corynebacterium*)	8.2×10^{8}	5.8×10^{7}	7.6×10^{8}	1.2×10^{6}	3.5×10^{7}	2.5×10^{4}	1.9×10^{4}
Yellow coryneforms (*Microbacterium/ Brachybacterium*)	-	1.1×10^{7}[e]	1.0×10^{7}[e]	-	1.2×10^{7}[f]	4.0×10^{4}[f]	-
Orange coryneforms[g] (*Brevibacterium*)	3.8×10^{5}	-	-	-	4.9×10^{8}	-	1.7×10^{8}
Orange motile rods[h] (*Halomonas* spp.)	-	1.3×10^{7}	-	-	-	-	-
Other rods	-	-	-		1.4×10^{5}	1.0×10^{4}	-
Staphylococcus spp.[i]	1.8×10^{3}	-	3.3×10^{7}	4.5×10^{6}	1.4×10^{5}	1.3×10^{4}	5.1×10^{8}
Enterococcus spp. (KEA)	3.5×10^{5}	1.0×10^{2}	1.0×10^{5}	-	-	-	4.8×10^{4}
Enterobacteria (VRBD)	2.0×10^{4}	2.0×10^{2}	4.7×10^{4}	-	-	-	3.8×10^{2}
Pseudomonads (CFCD)	1.0×10^{3}	-	-	-	-	-	1.0×10^{2}

[a] Cheeses shown in **Figure 1** were analyzed for coryneforms and staphylococci (modified milk agar), yeasts and molds (yeast extract glucose chloramphenicol agar, YGC), enterococci (kanamycin esculin azide agar, KEA), enterobacteria (violet red bile dextrose agar, VRBD), and pseudomonads (cetrimide fucidin cephaloridine dextrose agar, CFCD). A thin slice (10 g) was cut from the surface and homogenized in 90 ml quarter-strength Ringer's solution (10:1 dilution). Counts were divided by the area of the cheese slice (cm^{2}) to give surface counts (cfu cm^{-2}).

[b] Mainly *Corynebacterium casei*; *Corynebacterium variabile* was identified on hard cheese.

[c] Acid-curd cheese yeasts: KM, *Kluyveromyces marxianus*; CK, *Candida krusei*.

[d] -, not detected, either <100 (detection limit) or <1% of total counts on agar and thus not detectable among other microorganisms that are grown (e.g.,on modified milk agar).

[e] Mainly *Microbacterium gubbeenense*.

[f] *Brachybacterium* spp. identified.

[g] *Brevibacterium linens* or *Brevibacterium aurantiacum*.

[h] *Motile Halomonas* sp. typical of this cheese variety (always detected over several years).

[i] Mainly *Staphylococcus equorum*; on acid-curd cheese *Staphylococcus saprophyticus*.

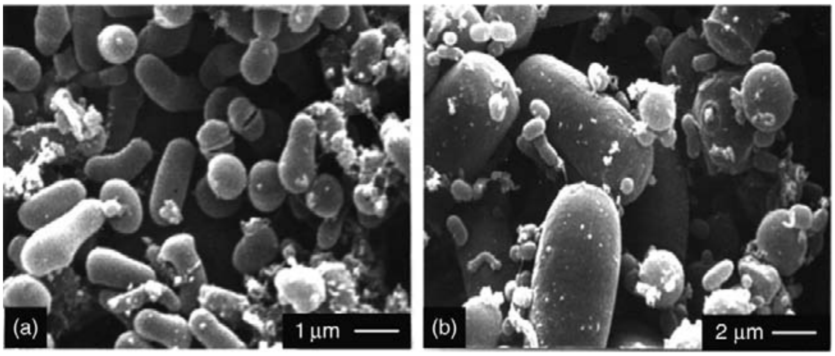

Figure 4 Scanning electron micrograph of the surface of a smeared soft cheese (Limburg/Romadour). (a) The photo shows 'coryneform bacteria', that is, Gram-positive, aerobic, nonmotile, irregularly shaped rods (club- or V-shaped) belonging to *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, or *Microbacterium*. The fissures seen in some cells indicate the 'postfission snapping movement' of cells typical for coryneforms. (b) The photo shows large cells, most likely *Geotrichum candidum*, and large oval cells, probably *Debaryomyces hansenii*, coexisting with club- or V-shaped cells ('coryneforms') and with cocci (*Staphylococcus equorum*, *Staphylococcus xylosus*). Courtesy of H. Neve, Department of Microbiology and Biotechnology, Max Rubner-Institute, Kiel, Germany.

2 weeks. Dominating throughout ripening are usually beige-pigmented corynebacteria, with *C. casei* being the most frequently identified species. *Corynebacterium variabile* and other species are also found. *Brevibacterium linens* is usually considered the 'typical red smear bacterium' before all other species because of the bright orange pigments. It is one of the best studied cheese bacteria; its strong sulfur metabolism and bacteriocins specific for *L. monocytogenes* were studied in detail. However, counts of *B. linens* on the surface of semisoft and semihard cheese are rather low (**Table 2**). A certain level of contaminating enterobacteria and enterococci can be expected on cheeses that are old–young smeared.

Acid-curd cheeses (yellow-type Harzer, Quargel)

Differences in the technologies used for cheese production from quarg result in significant differences in appearance, flavor, and surface microflora. Yeasts are detected on the surface and in the core in high concentrations ($>10^7$ cfu cm^{-2} and $>10^7$ cfu g^{-1}, respectively). *Candida krusei* and *K. marxianus* are most frequently found; both species were found essential for typical cheese ripening. *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and other species are often detected. *Trichosporon asahii* is a common yeast contaminant. The species is undesirable because it is related to human skin diseases. The spoilage yeast *Candida lipolytica* is frequently detected on cheeses with defects (liquefied surface).

Usually, beige coryneforms (*C. casei* or *C. variabile*) are important parts of the smear (**Table 2**). In contrast to all other smear cheeses, *B. linens* can account for nearly 100% of the surface smear bacteria, growing to $>10^9$ cfu cm^{-2}. The same counts can be observed for *Staphylococcus saprophyticus*, a typical contaminant of acid-curd cheese, which is frequently identified. If these high counts are formed by orange-pigmented strains, acid-curd cheeses show an untypical orange surface smear instead of a yellow-brown layer.

Classification

The taxonomy of smear bacteria and yeasts undergoes frequent changes impeding starter culture development. Depending on the classification system used, authors give different names for isolates. The teleomorph form of the well-known yeast culture *G. candidum* was named *Galactomyces geotrichum* several years ago, which was recently changed to *Galactomyces candidus*. In this article, the trade name *G. candidum* will be used.

Until the mid-1970s, all clump-building, Gram-positive, and catalase-positive cocci that did not metabolize glucose under anaerobic conditions were grouped into the genus *Micrococcus*. Species were considered 'food-grade' in contrast to staphylococci, such as *Staphylococcus aureus*. Thus, 'micrococci' preparations were commercially available containing *S. xylosus*. Later, when morphological, biochemical, and genetic differences were used to distinguish between the two genera, part of micrococci were grouped in the genus *Staphylococcus*. Today, cultures of food-grade *S. xylosus* and *S. equorum* are available.

In older papers, yellow coryneforms were classified as *Arthrobacter nicotianae*. Later, yellow coryneforms were divided into *A. nicotianae* and *Microbacterium barkeri*, the latter being reclassified as the new species *M. gubbeenense*. Recent studies indicate that perhaps most or all *Arthrobacter* isolates may belong to the new species *A. arilaitensis*. Some yellow coryneforms, which were found on Camembert and smeared hard cheese varieties, were classified as *Brevibacterium alimentarium* and *Brevibacterium tyrofermentans*.

For a long time, all orange coryneforms were classified as *B. linens*. Recently, among the isolates from smear cheeses, orange *Arthrobacter casei* was identified. The percentage of *A. casei* in the population of orange coryneforms is unclear. *Brevibacterium linens* was described as a genetically heterogeneous species based on 16S–23S rDNA restriction patterns (obtained by amplified ribosomal DNA restriction analysis (ARDRA)). By ARDRA, one of the genotypes cannot be distinguished from the recently described species *Brevibacterium aurantiacum*. Perhaps some *B. linens* strains, described in the literature including some commercial strains, belong to this new species.

Among beige coryneforms, *Corynebacterium ammoniagenes* was described as an essential component of the smear cheese flora. Isolates were later reclassified as *C. casei*. The species seems to be predominant on semisoft and harder varieties. In addition, *C. variabile* is frequently isolated. The species name *Corynebacterium mooreparkense* is no longer used; available strains were reclassified as *C. variabile*.

Methods for identification: Biochemical identification (e.g., API 32C, bioMérieux is suitable for yeast identification in combination with analysis of pseudomycelium formation and cell morphology; for the identification of *Staphylococcus*, the kit *ID32-Staph* (bioMérieux is helpful, but it often gives wrong classification of the food-grade species *S. xylosus* and *S. equorum*. The corresponding API kit for coryneforms is not helpful to identify any food-grade coryneforms, since the database concentrates on species with clinical importance. A simple molecular method, established for yeasts, staphylococci, and coryneforms, is ARDRA. When unknown restriction patterns are observed, sequencing of the 16S rDNA and Fourier transform infrared (FT-IR) spectroscopy of microcolonies serve as easy and fast methods to obtain reliable classification. Excellent databases including species from the food environment and cheese are available on the Internet or at institutions offering classification service (e.g., for FT-IR: ZIEL, University of Munich, Germany).

Species classification is time consuming and can be used only for a small number of isolates. A fast approach to characterizing the bacterial smear composition is plating of cheese surface extracts on a nutrient-rich growth medium. On plate count agar containing additional casein hydrolysate, vitamins, sodium chloride, and skim milk powder (i.e., modified milk agar (mMA)), colony morphology (color, shape) combined with phase contrast microscopy (cocci, coryneform rods) helps to group plated colonies. Orange, beige, and yellow colonies containing irregular rods indicate the presence of brevibacteria, corynebacteria, and microbacteria or *Arthrobacter*, respectively. Staphylococcal colonies are quite typical (larger diameter) and easy to distinguish from coryneforms by microscopy. It is not possible to distinguish food-grade *S. xylosus* or *S. equorum* from frequently occurring undesirable *S. saprophyticus*. Cheese isolates of *S. equorum* and *S. saprophyticus* can grow in white or orange colonies. A commercial *S. xylosus* strain offered by two major starter suppliers shows quite unusual colony morphology (cauliflower), which makes identification easy. Of course, this simple plating method has its limitations, but with repeated analyses of a certain cheese variety a good picture of the surface flora (and possible changes) is obtained, which can be verified by molecular classification of typical isolates.

Commercial Surface Starter Cultures

The trade with the so-called secondary cheese cultures is small compared with lactic starters for cheese and fermented milks. Therefore, culture development has been rather poor. Today, one or more strains of *D. hansenii*, *G. candidum*, *B. linens*, *S. xylosus*, and *S. equorum* are offered by major starter companies. The important species *C. casei* and *M. gubbeenense* will probably soon be available. Cultures for smear cheeses and information on their application can be obtained from starter culture suppliers (in alphabetical order): Cargill Inc., Chr. Hansen A/S, Danisco A/S, Sacco Srl. Apart from the suppliers mentioned, other companies with local importance can also probably provide surface ripening cultures.

New Developments: Defined Surface Starters

The variety of commercially offered smear strains is clearly expanding; still, a functional house microflora is essential as long as culture compositions do not reflect a complete, mature surface microflora. The requirements for complete smear starters were studied over the last 15 years in several research projects and in a demonstration project (EU projects 1993–2005, German FEI projects 2001–03). Experimental cheese ripening was performed in laboratories not possessing an adequate house microflora. First, a minimal smear starter for Tilsit cheese was proposed. For semisoft cheeses, five species were found necessary for smear development: *D. hansenii*, *S. equorum*, *M. gubbeenense*, *B. linens*, and *C. casei*. Single strains of the species showed fast growth on Tilsit-like cheese as well as development of red-brown color and volatile sulfurous flavor compounds typical for smear cheeses. The minimum concentration of the strains in the smear was around 10^7 cfu ml^{-1} (**Table 3**).

For smeared soft cheeses (e.g., Limburg cheese), the presence of a second yeast species was essential for typical color and aroma development. A complete surface starter tested in real cheese environment (Limburg) on laboratory and pilot scale consisted of *D. hansenii*, *G. candidum*, *M. gubbeenense* or *A. arilaitensis*, and *B. linens*. Cultures were sprayed at a concentration of 10^7 cfu ml^{-1}. Addition of

Table 3 Development of the surface flora of semisoft experimental cheeses (Modified from Bockelmann *et al*., 2007).

	Defined surface starter			*Old–young smeared*
Surface microflora[a] *(cfu cm^{-2})*	*1 week*	*2 weeks*	*6 weeks*	*6 weeks*
Yeast (Debaryomyces hansenii)	9.1×10^6	6.9×10^5	3.9×10^5	2.9×10^5
Total bacterial counts	1.7×10^9	8.1×10^8	2.6×10^9	3.3×10^9
Beige coryneforms	76%	74%	90%	90%
Yellow coryneforms	7.5%	12%	1%	2%
Orange coryneforms	3%	2%	1%	6%
Staphylococci	13.5%	12%	8%	2%
Contaminants (coliforms, etc.)	<0.01%	<0.01%	<0.01%	<0.01%

[a] Cheeses were either old–young smeared or brushed with a suspension of *D. hansenii*, *Brevibacterium linens*, *Microbacterium gubbeenense*, *Corynebacterium casei*, and *Staphylococcus equorum* (>10^7 cfu ml^{-1}). The maximum counts of yeast and *Staphylococcus* were observed after 1 week of ripening, and the counts of *M. gubbeenense* (yellow coryneforms) decreased after 2 weeks. *Brevibacterium linens* (orange coryneforms) was present in low numbers throughout ripening. The cell counts of beige coryneforms (most likely *C. casei*) increased during ripening. Similar flora development could be found for most experimental cheese batches produced in the last years (>20) with a variation of counts and composition as described in Table 2. Strain identification on numerous experimental cheese trials showed that surface starter strains can form at least a significant part of the surface microflora.

S. equorum to soft cheese brines (>100 cfu ml^{-1}) improved deacidification during the first days of ripening. *Corynebacterium casei* could be used in the starter, but was found to be not essential for typical aroma development.

Two different yeast species, *K. marxianus* and *C. krusei*, were found essential for the ripening of acid-curd cheese. They were inoculated into the milk used for quarg production (~100 cfu ml^{-1}) and developed to high counts in the quarg during transport and storage (anaerobic ripening) and later on the cheese surface (aerobic ripening). A culture mix (KM-mix) became available recently and is used by several quarg producers to guarantee the presence of these technologically important yeasts among other yeast species present in the house flora. At the cheese plant, addition of *S. xylosus* or *S. equorum* to the quarg (10^6–10^7 cfu g^{-1}) together with ripening salts helps to suppress naturally occurring *S. saprophyticus* contaminants and may have a beneficial effect on cheese texture (higher elasticity). *Brevibacterium linens* and *C. casei* cultures sprayed onto the surface (~10^7 cfu ml^{-1}) form the smear together with yeasts and staphylococci.

Functionality of Surface Starters

Bacterial strains (*B. linens*, *M. gubbeenense*, *C. casei*, *S. equorum*) used at a concentration of 10^7–10^8 cfu ml^{-1} for smearing of experimental Tilsit-type cheeses were identified in the complex cheese surface microflora by pulsed field gel electrophoresis (PFGE) after 2–8 weeks of ripening (17 of 20 isolates matched starter strains). Recovery of starter strains from mature smear cheeses was studied more thoroughly in a EU demonstration project (2003–05) in cooperation with two European cheese suppliers (Bel-Leerdammer, The Netherlands; Arla Foods, Denmark). Several hundred strains of *S. equorum*, *B. linens*, *C. casei*, and *M. gubbeenense* isolated from cheese after 2–15 weeks of ripening were analyzed by PFGE. Starter strains generally formed a significant part of the surface microflora. As expected, strains of all species of the house flora were also detected to some degree. When cheese brines with a natural *S. equorum* flora (>100 cfu ml^{-1}) were used, most *Staphylococcus* strains isolated from mature cheese matched strains from the cheese brines and not the starter strain smeared on the cheese. This demonstrated clearly the importance of cheese brine microflora for smear cheese ripening.

Brevibacterium casei and *C. variabile*, which were used in some cheese trials, were also detected on mature cheese. This underlines that other species can also contribute to a more diverse microflora when used at appropriate concentrations (>10^7 cfu ml^{-1}) in the smear.

The results of the EU demonstration project clearly showed that smear bacteria present in cheese brines were also found on the surface of mature cheeses at significant concentrations. This indicates that smear starter strains should be used not only for smearing but also for inoculating cheese brines, especially after pasteurization or cleaning (inoculation: yeasts and staphylococci >100 cfu ml^{-1}; coryneforms >10 cfu ml^{-1}).

Ripening Patterns

The ripening patterns described in the following are based on many microbiological analyses of mature commercial cheese varieties and on cheese trials with defined surface starters used for surface ripening. Most of the cheese trials were performed on a laboratory scale

(2–12 kg per batch) and involved smeared soft, semisoft, semihard, and acid-curd cheeses.

Deacidification

Deacidification is a prerequisite for smear development of all smear cheese varieties (**Figure 5**).

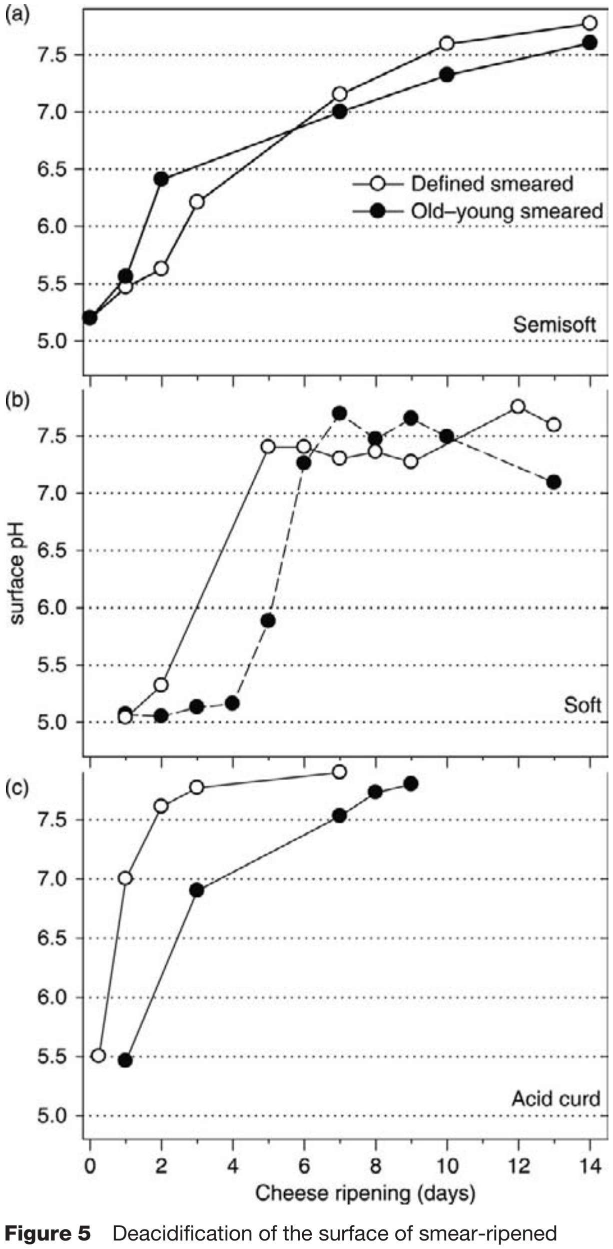

Figure 5 Deacidification of the surface of smear-ripened cheeses. The deacidification (i.e., smear development) of experimental Tilsit (a), Limburg (b), and acid-curd cheese (c) was compared with old–young smeared commercial cheeses (filled symbols). Surface pH data of commercial cheeses were provided by cheese manufacturers. Experimental cheeses were smeared with appropriate starters described in the text. pH values were taken with a flat surface electrode.

Semisoft and semihard cheeses

Lactate degradation by *D. hansenii* leads to an increase in surface pH from ~5.3 to >7 within 1 week of ripening when old–young smearing is used (**Figure 5(a)**). Deacidification was comparable when a defined starter consisting of *D. hansenii*, *S. equorum*, *B. linens*, *C. casei*, and *M. gubbeenense* was used; however, a certain lag phase at days 1–2 was observed (**Figure 5(a)**). The lag phase was avoided when *D. hansenii* and *S. equorum* were added to the cheese brines (>10^5 cfu ml^{-1}) instead of the smear liquid (not shown). Several laboratory-scale cheese trials showed that the cheese brine inoculation with yeast and staphylococci was a good strategy when using defined surface starters (**Figure 3**).

Soft cheeses

For Limburg-type cheeses, initial deacidification is mediated by *G. candidum* and *D. hansenii.* Surprisingly, old–young smeared Limburg cheeses from a German producer showed a lag phase of deacidification of 1–3 days (**Figure 5(b)**). A possible explanation was that cheese brines of the producer were pasteurized frequently to avoid too high yeast concentrations (*D. hansenii*). Consequently, no staphylococci were detected in the brines. When freshly prepared cheese brines in experimental cheese trials were inoculated with *D. hansenii* and *S. equorum* (~10^5 cfu ml^{-1}), a lag phase of deacidification was not observed (**Figure 5(b)**); *B. linens*, *M. gubbeenense*, and *C. casei* were used for smearing (10^7 cfu ml^{-1}), and *G. candidum* was inoculated into the cheese milk (100 cfu ml^{-1}).

Acid-curd cheese

For yellow-type cheeses, the yeasts *K. marxianus* and *C. krusei* are important for deacidification of the cheese surface. They are introduced into the process via inoculation of the milk used for acid-curd production with the appropriate commercial culture mix (100 cfu ml^{-1}). Yeast concentrations reach 10^7 cfu ml^{-1} under anaerobic conditions before cheeses are produced. Experimental cheese trials showed fast deacidification of the cheese surface without the addition of 'culture cheese' (old–young step) at the beginning of production (**Figure 5(c)** and **Table 2**); for the cheese trials, *S. equorum* and *B. linens* were used for smearing (10^7 cfu ml^{-1}).

In later ripening stages of all smear cheeses (>1–2 weeks), coryneforms also contribute to deacidification, for example, by liberating ammonia. The surface pH of all mature smear cheese varieties (measured with flat surface electrodes) is around 7.5–8.5.

Development of the Microflora

Experimental Tilsit-like, semisoft cheeses were 'old–young' smeared or smeared with a defined starter once at the start of ripening (**Table 3**). To ensure a good distribution of surface microorganisms, cheeses were brushed with salt water (3% NaCl) after 1 week. No fungal contamination was observed in any of the batches throughout ripening (4 weeks). Repeated experiments showed similar results, with maximum bacterial counts of 1–5 × 10^9 cfu cm^{-2}. Using the defined surface starter culture described for Tilsit-type cheeses, the highest counts of yeasts and staphylococci were found in the first week of ripening, and at later stages the counts dropped to lower values. The microflora was already dominated by corynebacteria (*C. casei*), which showed maximum counts over the whole ripening period. A complete smear layer was developed within 4–7 days, with surface counts of 10^9 cfu cm^{-2} or more. No typical sulfurous smell was observed at this time (all batches). The importance of yeasts and staphylococci for mold inhibition at the start of ripening is shown in **Figure 3**.

After 2 weeks of ripening, a typical mature surface microflora was present with high counts of yellow coryneforms (*M. gubbeenense*), which dropped to lower values after 6 weeks of ripening (**Table 3**). *Brevibacterium linens* counts were variable; generally, low values were observed throughout ripening and the highest counts were observed at later stages of cheese ripening. A possible reason for the low *B. linens* counts was found in liquid milk model systems where *B. linens* showed slower growth under cheese ripening conditions (13–16 °C) compared to *C. casei* and *M. gubbeenense.*

Microbacterium gubbeenense and *A. arilaitensis* constitute a much higher percentage of the microflora on soft cheeses than on semisoft cheeses. A possible explanation could be that the old–young recycling step is introduced at 2 weeks of ripening when the counts of yellow coryneforms are still high; an enrichment of smear bacteria of a 'young' mature composition can be assumed (**Table 3**).

High yeast counts characterize the surface flora of acid-curd cheeses – *K. marxianus* is a rather early-developing species with preference for galactose as the carbon source, whereas *C. krusei* is a late-developing species metabolizing lactate. High bacterial counts of corynebacteria, brevibacteria, and staphylococci are observed. Sometimes, 100% of smear bacteria plated on mMA can be *B. linens* (from the sprayed starter) or *S. saprophyticus* (from contaminated 'culture cheese') (**Table 2**). Yellow coryneforms are usually not found.

Color Development

Smear cheeses possess a dry and sticky surface of light-brown (semisoft and hard cheeses) or more orange color (soft cheeses), sometimes with streaks of white from *G. candidum* mycelium. The mechanisms of color development of smear cheeses are still largely unclear. Orange-pigmented *B. linens* strains can be responsible for orange cheese color if counts are at 10^9 cfu cm^{-2} or above, which is rather an exception. Such high *B. linens* counts are not observed for semisoft or semihard cheese and are rarely observed for soft cheese or acid-curd cheese (yellow type). In the case of acid-curd cheese, orange appearance is considered a defect. Acid-curd cheese can also show contamination with orange *S. saprophyticus* (>10^9 cfu cm^{-2}), which leads to a similar orange discoloration of the cheese surface.

The orange, red-brown, or pink color of smear cheeses is most likely due to the high counts of yellow-pigmented *M. gubbeenense* or *A. arilaitensis* in the surface flora (**Table 4** and **Figure 6**). Under proteolytic conditions, for example, the presence of *B. linens*, the yellow color is somehow transformed to red. In liquid cheese model systems, the addition of casein hydrolysate to *M. gubbeenense* cultures instead of *B. linens* had the same coloring effect, indicating

Table 4 Surface microflora of smeared semisoft cheeses with different surface colors shown in **Figure 6**

Surface microflora *(cfu cm^{-2})*	*Danish Tilsit beige-brown*	*German Tilsit pink*
YGC agar		
Debaryomyces hansenii	4.3 × 10^2	2.2 × 10^5
Molds	-	1.4 × 10^6
Modified milk agar		
Beige-red coryneforms (*Corynebacterium casei*)	1.5 × 10^8	7.3 × 10^8
Yellow coryneforms (*Microbacterium gubbeenense*)	1.9 × 10^7	6.6 × 10^8
Orange coryneforms (*Brevibacterium linens*)	1.3 × 10^7	2.7 × 10^8
Other rods	4.5 × 10^8	5.4 × 10^8
Staphylococcus equorum	1.0 × 10^6	2.6 × 10^8
Enterococcus spp. (KEA)	1.3 × 10^2	1.0 × 10^2
Enterobacteria (VRBD)	1.8 × 10^3	2.0 × 10^2

Single isolates of typical colonies were identified by amplified ribosomal DNA restriction analysis.
See Table 2 footnote for an explanation of YGC, KEA, and VRBD.

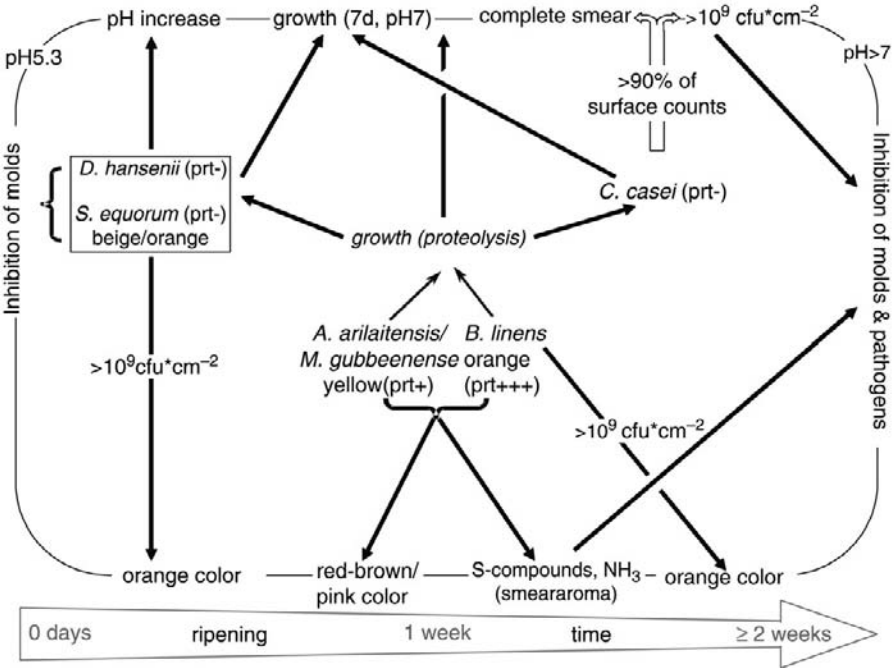
pH5.3
pH increase
growth (7d, pH7)
complete smear
>10⁹ cfu*cm⁻²
pH>7
>90% of
surface counts
Inhibition of molds
D. hansenii (prt-)
S. equorum (prt-)
beige/orange
C. casei (prt-)
growth (proteolysis)
Inhibition of molds & pathogens
>10⁹cfu*cm⁻²
A. arilaitensis/
M. gubbeenense
yellow(prt+)
B. linens
orange
(prt+++)
>10⁹ cfu*cm⁻²
orange color
red-brown/
pink color
S-compounds, NH3
(smeararoma)
orange color
0 days
ripening
1 week
time
≥ 2 weeks

Figure 6 Typical color of Tilsit-type cheese. Pink color tones are desirable for a certain north German Tilsit cheese, side color 68_L, 17_a, 15_b, top side 68_L, 13_a, 29_b; for a similar cheese from Denmark, this would be called a defect. The cheese is supposed to have more yellow-brown color tones (side color 66_L, 5_a, 27_b, top side 62_L, 13_a, 38_b). Cheeses were photographed under identical lighting conditions and gray balanced with Gretag Macbeth Color Checker. Color values are expressed as '*Lab*' values, that is, luminance (*L*, scaling 0 (=black) to 100 (=white)), green/red (*a*, scaling −100/100), and blue/yellow (*b*, 100/−100). The pink colors are most likely due to high concentrations of *Microbacterium gubbeenense* in combination with strong proteolysis (*Brevibacterium linens*). See **Table 4**.

that free amino acids contribute to color development. Reversed-phase high-performance liquid chromatography (HPLC) of a crude red pigment extract showed a number of yellow- and pink-colored fractions. It can be speculated that the ratio of yellow and pink components determines the different cheese colors, orange, red-brown, and pink, as comparable colors can be produced by inkjet printers by mixing yellow and magenta dyes. The results indicate that *B. linens* is indeed contributing to cheese color, which can be attributed rather to its proteolytic abilities than to its own orange pigments (**Table 4** and **Figure 6**).

For some bright orange soft cheeses, the use of food colorants (carotenoids) is known. The white areas, streaks, and patches of smeared soft cheeses are usually caused by *G. candidum*; a few varieties show growth of *Penicillium camemberti.* The very peculiar light-brown color of smeared acid-curd cheese (surface and core) is due to chemical reactions of ripening salts with the quarg matrix (solubilization of caseins) with an additional influence of the yeasts *K. marxianus* and *C. krusei.* Visible *G. candidum* growth ($>10^7$ cfu cm^{-2}) is considered a defect for yellow-type acid-curd cheeses; however, limited (invisible) growth of *G. candidum* may be desirable, because the surface tends to be drier and less sticky, which makes handling more pleasant.

Aroma Development

The typical volatile sulfurous smear cheese smell of soft, semisoft, and acid-curd cheeses is clearly detected after ~2 weeks of ripening when a mature surface microflora has developed. In a liquid milk model, pure cultures of *D. hansenii*, *S. equorum*, *B. linens*, *M. gubbeenense*, and *C. casei* (complete Tilsit surface starter) did not produce typical smear cheese-like smell, that is, a complex aromatic profile dominated by sulfur compounds and ammonia. Interactions between *M. gubbeenense* and *B. linens* were shown to liberate typical smear aroma in the model system; a similar effect was observed when methionine was added to pure cultures of *B. linens.* This demonstrates the importance of *B. linens* for aroma development, even at the low proportions (sometimes <1% of total cell counts) found in the surface microflora. *Geotrichum candidum*, present on smeared soft cheeses, liberates an aroma profile similar to *B. linens.* Both species show high variation with respect to flavor characteristics between strains.

In a project testing defined surface cultures for Limburg-type cheese, it was possible to produce typical aroma without *B. linens* but not without *G. candidum. Geotrichum candidum* is often not added deliberately as culture for Limburg cheese production. Natural levels of the yeast in the cheese milk (~100 cfu ml^{-1}) are sufficient for fast growth during warm ripening before smearing (**Figure 2**) and determine the company-specific volatile flavor of the mature product.

Volatile aromatic sulfur compounds originating from methionine and cysteine are probably key components of smear cheese flavor and contribute to the garlic note. The thioesters (*S*-methylthioacetate, thiopropionate, thiobutyrate, etc.) are important for the overall aroma. *Brevibacterium linens* is responsible for the conversion of methionine to methanethiol, α-ketobutyrate, and ammonia. These bacteria actively produce the very aromatic volatile H_2S, methanethiol, dimethyldisulfide, *S*-methylthioacetate, 4-trithiapentane, and ethional – it may be beneficial for the consumer acceptance of smear cheeses that *B. linens* occurs at low numbers in the surface microflora only.

The role of *Corynebacterium* species in aroma development is unclear. The contribution of a selected fast-growing *C. casei* strain to aroma development was not detected in liquid milk model systems. Due to the predominance of corynebacteria on the surface of many smear cheese varieties, an effect on aroma development is likely.

The typical aroma of smeared acid-curd cheese is mainly produced by the yeasts *K. marxianus* and *C. krusei.* When pure cultures are added to acid curd, *K. marxianus* liberates alcoholic, fruity, and ester notes, whereas *C. krusei* liberates more cheesy, sulfurous notes. In combination of both, the alcoholic, fruity, and ester notes are reduced to give a full smear cheese flavor, which shows distinct differences from all rennet-type cheeses.

Surprising results were obtained in an EU demonstration project where defined surface cultures were tested on three semihard smeared cheese varieties of two European producers (Bel Leerdammer, Arla Foods). Even with strains isolated from the commercial cheeses under study, typical aroma development was not possible when the green cheeses were produced in facilities of the scientific project partner Max Rubner Institute, Kiel, Germany. For successful studies on defined surface cultures, green cheeses had to be produced at the sites of the industrial partners. For smear culture trials, cheese were transported submerged in cheese brine containers (defined microflora), then smeared with defined starters, and ripened in facilities in Kiel, Germany.

In the project, it was found that the selection of *B. linens* strain had the biggest influence on aroma development. A defined surface starter consisting of *D. hansenii*, *S. equorum*, *B. linens*, *C. casei*, and *M. gubbeenense* was able to develop the different typical aromas of the three selected cheese varieties. Another surprising result was that large differences of the surface flora of experimental cheeses after 15 weeks of ripening (90% *S. equorum* vs. 5% *S. equorum*) did not lead to a different aroma profile assessed by sensory analysis. It can be concluded that the composition of surface flora of smear cheeses has to be analyzed several times before a typical composition can be described; a high degree of compositional variation is probably found for all smear cheese varieties.

The results indicate that the aroma profiles of mature cheeses depend on the company-specific production of green cheeses to a large degree and that it is impossible for competitors to copy unique cheese varieties by just isolating and using strains from the surface flora of other products. Thus, it may not be necessary for the connoisseur to fear that defined surface cultures will lead to uniform aroma of smear cheeses. As for all other cheese varieties, the main factor leading to flat aroma and 'uninteresting' cheeses is still the reduction of expensive ripening time. Interactions of species on semisoft cheese leading to a complete smear, high pH, typical aroma, and color are shown in **Figure 7**.

Aroma of Cheeses Ripened with Defined Starters

When defined starters were used for Tilsit cheese ripening on a pilot scale (750 kg), headspace gas chromatography–mass spectrometry (GC–MS) analysis of cheeses showed that a similar spectrum of volatile

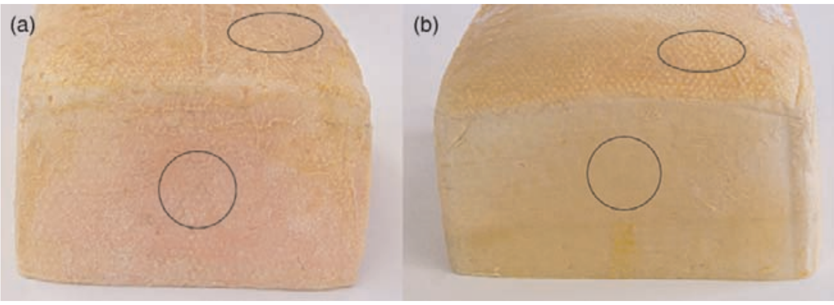

Figure 7 Surface ripening of smear-ripened semisoft cheeses. The putative interactions of major species of the smear flora are shown, leading to fast growth, typical aroma, color, and protection of the surface from microbial contamination. The picture is based on results obtained with defined surface starters described in the text and on analyses of commercial smear cheeses.

aromatic compounds was liberated compared to old–young smeared control cheeses. However, some of the highly aromatic key sulfur components (methanethiol and derivatives), some alcohols, and aldehydes were produced at lower levels by the defined starters. Amino acid conversion leading to aromatic compounds was also weaker for defined starters; lipolysis by the defined starter preparations was comparable to the old–young smeared controls. The first successful steps to better surface starter development have been made. Since the functionality of the concept of defined surface starters has been demonstrated, future work can concentrate on strain selection regarding improved aromatic properties.

Defects

Defects specific for smear-ripened cheeses usually relate to an imbalance of the surface microflora caused by various factors. Among other factors, microbial contamination of cheese milk, inappropriate temperature and especially humidity control, residual cleaning agents used in ripening rooms, and seasonal factors (e.g., high mold concentrations in warm and humid summers) can disturb the smear microflora. Traditional smear cheese production does not allow a thorough disinfection of a whole cheese plant including cheese brines. An imbalance of the surface flora is usually observed for months rather than weeks after cleaning and disinfection.

The first visible defect is mold contamination often caused by *Penicillium commune* or *Mucor* sp. For some cheeses (semisoft, hard), the mold-like *G. candidum* is undesirable. Mold contamination is especially important for the longer ripened varieties, for example, Tilsit (>4–6 weeks). It spoils the appearance, can impart musty flavors, and may produce mycotoxins. Mold contamination will most likely occur during the first 2 weeks of ripening. Mold growth can be inhibited by more frequent, if necessary daily, smearing or spraying of cheese with 3% salt. The use of added smear cultures (yeasts, staphylococci, and coryneforms) will help in protecting the cheese from mold growth.

Important bacterial contaminants of smear cheeses are enterococci and enterobacteria, especially for softer cheese varieties. Both can be easily detected by plating surface bacteria on selective agars (kanamycin–esculin–azide agar, violet-red bile dextrose agar). The main problem of these contaminants is not pathogenicity but their transferable multiple antibiotic resistances, which may interact with the human gut microflora. Recent studies showed that practically all enterobacteria isolated from smear cheese surfaces possessed multiple antibiotic resistances. In one study, >50% of the isolates showed resistance toward five antibiotics or more (maximum 10). The level of surface contamination may be more important for health when the cheese rind is consumed. This is more common for the smaller-sized soft cheeses (200–500 g) than for Tilsit-like cheeses (2–3 kg per piece) or harder varieties.

Listeria monocytogenes

It is common knowledge that *L. monocytogenes* is an important pathogen sometimes associated with soft cheese varieties. Food poisoning incidents are rare but have been reported repeatedly. Important sources of contamination are smearing machines with brushes which are difficult to clean and disinfect. An existing low level of *Listeria* contamination is established in cheese plants by the traditional old–young smearing. For reasons largely unknown, *L. monocytogenes* sometimes grows to high cell densities on cheeses posing a risk for consumers.

For efficient control of *L. monocytogenes*, old–young smearing should be avoided. A typical smear house microflora is found not only on the surface of mature cheeses but also in the cheese brines. Available commercial surface cultures, used not only in the smear but also in the cheese brines, can supplement the house microflora. Smearing of young cheeses with surface cultures in freshly cleaned machines before mature cheeses will probably require more frequent treatments but the protection from contamination by *Listeria*, enterobacteria, enterococci, molds, and other organisms will be much better than with old–young smearing.

A last alternative of *Listeria* control can be antilisterial cultures. Bacteriocin-producing *B. linens* cultures were not very successful because *B. linens* does not grow in the acidic cheese environment (pH 5.3) at the start of ripening. When *B. linens* starts to grow (pH >6) after several days of ripening, bacteriocin production is too late to inhibit *L. monocytogenes* efficiently. In a German FEI research project, acid-tolerant yeasts and staphylococci were screened as sources of antilisterial activity. One yeast strain (*Pichia norvegensis*) and one *Staphylococcus* strain (*S. equorum*) were found with excellent *L. monocytogenes* inhibition in cheese model systems. However, both strains were not able to inhibit *L. monocytogenes* strains on experimental cheeses (semisoft, soft, and acid curd) contaminated with 10–50 cfu cm^{-2}.

Two recently introduced commercial antilisterial preparations, based on a *Lactobacillus plantarum* bacteriocin or on *Listeria* phages, were also tested in the research project. The preparations were used in combination with defined smear starters on experimental semisoft, soft, and acid-curd cheeses. Both preparations reduced *Listeria* counts (contamination level: 10–50 cfu cm^{-2}) under the detection limit of 100 cfu cm^{-2} for about 2 weeks. However, the protection was not complete; upon prolonged ripening (additional 2 weeks) low *Listeria* counts were observed again. For the experiments, a single dose of protective cultures was used at the start of ripening. A second dosage after 1–2 weeks may have shown complete inhibition. Depending on the level of *Listeria* contamination, both cultures seem to be a good choice in the battle against *L. monocytogenes*. More experiments are needed to demonstrate the potential and limits of the protective cultures.

See also: **Bacteria, Beneficial**: *Brevibacterium linens, Brevibacterium aurantiacum* and Other Smear Microorganisms. **Bacteriocins**. **Cheese**: Public Health Aspects; Salting of Cheese; Starter Cultures: General Aspects. **Microorganisms Associated with Milk**.

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Blue Mold Cheese

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Introduction

The typical appearance of the blue mold cheese varieties (also called blue-veined cheeses or simply blue cheeses) is due to the growth and development of the mold *Penicillium roqueforti.* Many countries and regions have developed their own types of blue mold cheeses, each with different characteristics and involving different manufacturing procedures. The best-known types worldwide are the Italian Gorgonzola, the French Roquefort, the British Stilton, and the Danish Danablu, all of which have been granted the status of protected designation of origin/protected geographical indication (**Table 1**), together with a number of other European blue mold cheeses. Production of blue mold cheeses has a long history: the manufacture of Gorgonzola and Roquefort was documented more than a thousand years ago; Stilton is mentioned in literature from the seventeenth century; and production of Danablu started in the 1870s. In 1916, a method for homogenization of cream was invented and used in the production of Danablu to make this cheese from bovine milk as white as the traditional Roquefort, which is made from ovine milk.

Manufacture

Bovine, caprine, ovine milk, or a mixture of two or three of the milk types is used for the production of blue mold cheese. The milk is used raw or after thermization (e.g., 62 °C × 15 s) or pasteurization (72 °C × 15 s). A mesophilic or thermophilic starter is commonly added as well as conidia of *P. roqueforti* and finally rennet. Cheese milk is left to coagulate before cutting and stirring. The curd is placed in molds, and whey is drained off during ~10–48 h without the application of external pressure, and the molds are turned frequently. The cheeses are brine- or dry-salted for about 24–48 h. Piercing of the cheeses may be performed one or more times during ripening for typically between ~6 weeks and 6 months at a temperature of around 8–15 °C and a relative humidity of 85–95%.

Microstructure

The structure of blue mold cheese is in general very heterogeneous with blue and yellow/white areas containing sporelized mold (**Figure 1**) and with pronounced gradients of salt, pH, and water activity. The considerable structural differences influence the levels and distribution of oxygen and carbon dioxide, which have a great impact on the growth and biochemical activity of the various microorganisms present in blue mold cheese.

As compared to many other cheese varieties, the minimum pH in blue mold cheese is low, for example, around 4.6–4.7, as in Danablu and Stilton. In the production process of blue mold cheese, the curd produced is not pressed by external forces, and the cheeses typically have a high moisture content and contain much lactose that may be transformed into lactic acid by the lactic acid bacteria (LAB) starter. During ripening from week 1 to week 5, the pH in Danablu may increase in the core from 4.4 to 6.4 and in the surface layer from 4.4 to 5.0, and similar values have been shown for other variants of blue mold cheese. The pH in the interior rises more rapidly than on the surface, as the level of NaCl is lower and thereby allows a faster growth of the mold. The rise in pH is due to the catabolism of lactic acid to CO_2 and the oxidative formation of NH_3 from amino acids. The overall NaCl content in ripened blue mold cheeses ranges from 2 to 5%. The high content of salt is obtained by a fairly long salting period, for example, 2 days brine salting for Danablu, a high moisture content, the loose structure of the cheese matrix (no pressure applied during whey draining), and the rather small sizes of the cheeses. The diffusion of NaCl into the cheese core is faster in the piercing channels and in areas with fissures, creating an even more inhomogeneous salt distribution. The NaCl concentration measured in Danablu cheese after 8 weeks of maturation may be around 2.0% (w/w) in the core and 4.0% (w/w) in the surface layer.

Growth of the mold is affected by the concentrations of O_2 and CO_2. The level of oxygen decreases rapidly in the cheese, and a close-to-anaerobic environment is evident already after 3 weeks of ripening, except for within small areas in the cheese (fissures) and up to ~0.25 mm below the surface layer. *Penicillium roqueforti* is, however, well adapted to growth inside blue mold cheese, where a low level of O_2 is combined with a high level of CO_2 (20–40%).

Microflora

The several groups of microorganisms that make up the complex microflora of blue mold cheeses contribute at different levels to ripening. The primary and secondary

Table 1 Examples of blue mold cheese varieties

Name	*Origin*	*Milk*	*Moisture (%)*	*FDM (%)*
Danablu	Denmark	Thermized bovine milk	44	55
Gorgonzola	Italy	Pasteurized bovine milk	48	55
Roquefort	France	Raw ovine milk	44	52
Stilton	England	Pasteurized bovine milk	41	58

FDM, fat in dry matter.

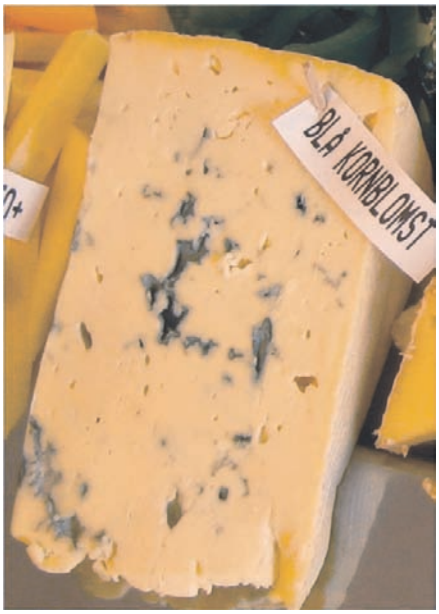

Figure 1 Typical inhomogeneous structure of blue mold cheese.

starter culture, LAB and *P. roqueforti*, respectively, are the most important, but yeast and non-starter LAB, even though they are not added to the cheese milk deliberately, may influence the ripening as well. Some varieties of blue mold cheeses are ripened naturally, with no addition of cultures during manufacture; however, still the same groups of microorganisms contribute to the ripening.

Mesophilic and thermophilic LAB are used as the primary starter culture for the production of different varieties of blue mold cheeses. A mesophilic, undefined mixed LAB culture will typically contain *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris*, and sometimes also limited amounts of citrate-positive strains of *Lc. lactis* subsp. *lactis* and *Leuconostoc* species, which produce CO_2 and open up the structure that facilitate development of the mold. The thermophilic starters used in blue mold cheese usually contain *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The primary role of the LAB starter is to acidify the curd by converting the lactose into lactate. The starter LAB may grow to numbers around 10^9 cfu g^{-1} after salting, and then they decrease during maturation to 10^7 cfu g^{-1}.

Penicillium roqueforti has previously been known under other names, but in 1980 several species, including *Penicillium stilton*, *Penicillium italicum*, *Penicillium gorgonzola*, and *Penicillium aromaticum*, were found to belong to the same species and collected under the taxon *P. roqueforti*. Conidia of *P. roqueforti* may be added directly to the cheese milk, be sprayed on the curd, or colonize the cheeses naturally. Addition of conidia is crucial for the quality of the blue mold cheese varieties made from pasteurized milk. *Penicillium roqueforti* assimilates lactose, glucose, and galactose; utilizes lactate and citrate; and grows well at the pH value and temperature of the blue mold cheese during ripening. *Penicillium roqueforti* grows at low levels of O_2 in the presence of 25% CO_2. Growth and sporulation of *P. roqueforti* occur at 20% CO_2 in an atmosphere with 0.5% O_2. *Penicillium roqueforti* grows in fissures and piercing channels in the cheese. The color of the mold varies from white through several nuances of blue and green to brown, depending on the strain and its age. The growth rate of *P. roqueforti* is strongly affected by increasing concentration of NaCl and is stimulated by up to 3.5% NaCl (strain dependent), whereas higher concentrations cause a decrease in the growth rate. Germination of *P. roqueforti* conidia is stimulated by 1–3% NaCl for most strains. In fresh blue mold cheese, the water activity in the core is optimal for the germination and growth of *P. roqueforti*, and the concentration of NaCl is in the range where *P. roqueforti* is stimulated. During the first 3 weeks of ripening, the NaCl concentration in the core increases to a level that induces sporulation and reduces the germination rate and growth of mycelia. These changes influence the appearance of the cheese, as the blue-green color is due to the conidia, and also prevent the growth of too thick a mycelium in fissures and piercing channels. Due to an NaCl gradient, the development of *P. roqueforti* decreases from the interior to the exterior part of the cheese. Commonly, mycelial growth does not occur in the surface layer because of too high a concentration of NaCl.

Yeasts develop spontaneously during the manufacturing, ripening, and storage of several blue mold cheese varieties. They are tolerant to low pH and high salt concentrations, and they grow at low storage temperatures using lactate, residual unfermented carbohydrates, and small amounts of citric and acetic acids. In blue mold cheeses like Roquefort made from raw milk, yeast may reach a population of 10^7–10^8 and 10^5–10^6 cfu g^{-1} on the surface and in the interior, respectively, before salting. The yeast population on the surface decreases after brine-salting, which causes changes in the yeast population toward asporogenous yeast forms. Yeast start to multiply on the surface of the cheese after a short adaptation period. Most investigations show the predominance of *Debaryomyces hansenii* (*Candida famata*) in blue mold cheese.

Nonstarter LAB grow in blue mold cheese typically from 10 to 100 cfu g^{-1} after brining to $\sim 10^7$ cfu g^{-1} in ripened cheese, and they are assumed to originate from the raw milk and the dairy plant environment. As in many other cheeses, the non-starter LAB flora in blue mold cheese are commonly facultative heterofermentative strains of *Lactobacillus* and mainly of the *Lactobacillus paracasei/casei* complex and *Lactobacillus plantarum.* Other NSLAB found in blue mold cheese are *Lactobacillus fermentum*, *Lactobacillus brevis*, *Pediococcus* spp., and *Leuconostoc* spp. From blue mold cheeses made from raw milk, also a high level of *Enterococcus* spp. has been isolated. Any important role of the non-starter LAB in blue mold cheese has, however, not been demonstrated.

Interactions between LAB and *P. roqueforti* have been shown to be strain-specific for both species and may be both stimulating and inhibiting. The positive interactions may result in a faster growth and more pronounced sporulation of *P. roqueforti* as well as a thicker and more velvet-like mycelium. Negative interactions may cause a reduction in, or put an end to, the growth of *P. roqueforti.* Selecting the right combinations of LAB and *P. roqueforti* cultures may stimulate the growth and sporulation of *P. roqueforti* and thereby enhance ripening and improve the general appearance of the cheese.

Detrimental Microbial Activities

Spoilage fungi can colonize and grow well on the surfaces of blue mold cheeses and cause the formation of off-flavors or mycotoxins or discoloration of the cheese. Important spoilage fungi are *Penicillium* spp., including *Penicillium commune* and *Penicillium nalgiovense*, and of special interest is the recently discovered species *Penicillium caseifulvum*, which is frequently found on the surfaces of blue mold cheeses, where it causes discoloration in the form of brown spots. Another contaminant frequently found in blue mold cheese is *Geotrichum candidum*, which can cause considerable inhibition of the growth of *P. roqueforti. Geotrichum candidum* has shown a growth potential similar to that of *P. roqueforti* in the absence of salt, indicating a possible overlap of the two species in the interior of the cheese during the initial ripening stage. Contamination of blue mold cheese by *G. candidum* can cause inhibition of growth and sporulation of *P. roqueforti* resulting in 'blind spots', which affect the quality of the cheese significantly. This emphasizes the importance of good manufacturing practice in the production of blue mold cheese to prevent contamination by *G. candidum.*

Some strains of *P. roqueforti* may produce a range of mycotoxins, like PR toxin (*Penicillium roqueforti* toxin), roquefortines, sofumigaclavines, and mycophenolic acid. The optimal conditions for the production of the most toxic of them, PR toxin, are a high sugar concentration, pH close to 4.5, and aeration, which are far from the conditions prevailing in blue mold cheese. PR toxin has never been detected in commercial blue mold cheeses or even in experimental blue mold cheeses made with known toxin-producing strains. Taking into account the very low levels and the relatively low toxicity of the various mycotoxins found in some cheeses, consumption of even large amounts of blue mold cheese should not pose any risk to the health of the consumer.

Proteolysis and Amino Acid Release

Proteolysis is intense in blue mold cheeses, and casein is hydrolyzed at a larger number of sites than in most other cheeses, and a large number of different peptides are produced (**Figure 2**). A large amount of amino acids are released from the peptides as a result of high levels of peptidases produced by the mold and LAB working in concert.

The enzymes that contribute to the complicated proteolysis in blue mold cheese originate mainly from *P. roqueforti*, and also from the milk, rennet, starter and non-starter bacteria, and sometimes yeast (**Table 2**). A significant increase in proteolysis has been observed when the mold has become visible in the cheese, typically after 2–5 weeks of maturation, depending on the cheese variety. However, while *P. roqueforti* is growing out, breakdown of the caseins is performed mainly by proteinases in rennet such as chymosin. The main activity of chymosin in cheese is on α_{s1}-casein to produce α_{s1}-casein (f24–199) and the peptide α_{s1}-casein (f1–23). The milk protease, plasmin, hydrolyzes β-casein into γ-caseins and proteose-peptones mainly during the first day before the pH in the cheese becomes too low. The cell wall protease of the *Lactococcus* or *Lactobacillus* starter culture hydrolyzes mainly the peptides produced from casein by rennet and plasmin. A limited release of amino acids by the starter aminopeptidases occurs during the first weeks of ripening.

After a couple of weeks, *P. roqueforti* dominates proteolysis, liberating both peptides and amino acids (**Table 2**). *Penicillium roqueforti* expresses two extracellular proteases, a metalloprotease and an aspartic protease, which are at maximum concentrations in blue mold cheese at the stage when *P. roqueforti* has grown out and begins to sporulate. The metalloprotease is active at pH 4.5–8.5, and it has an

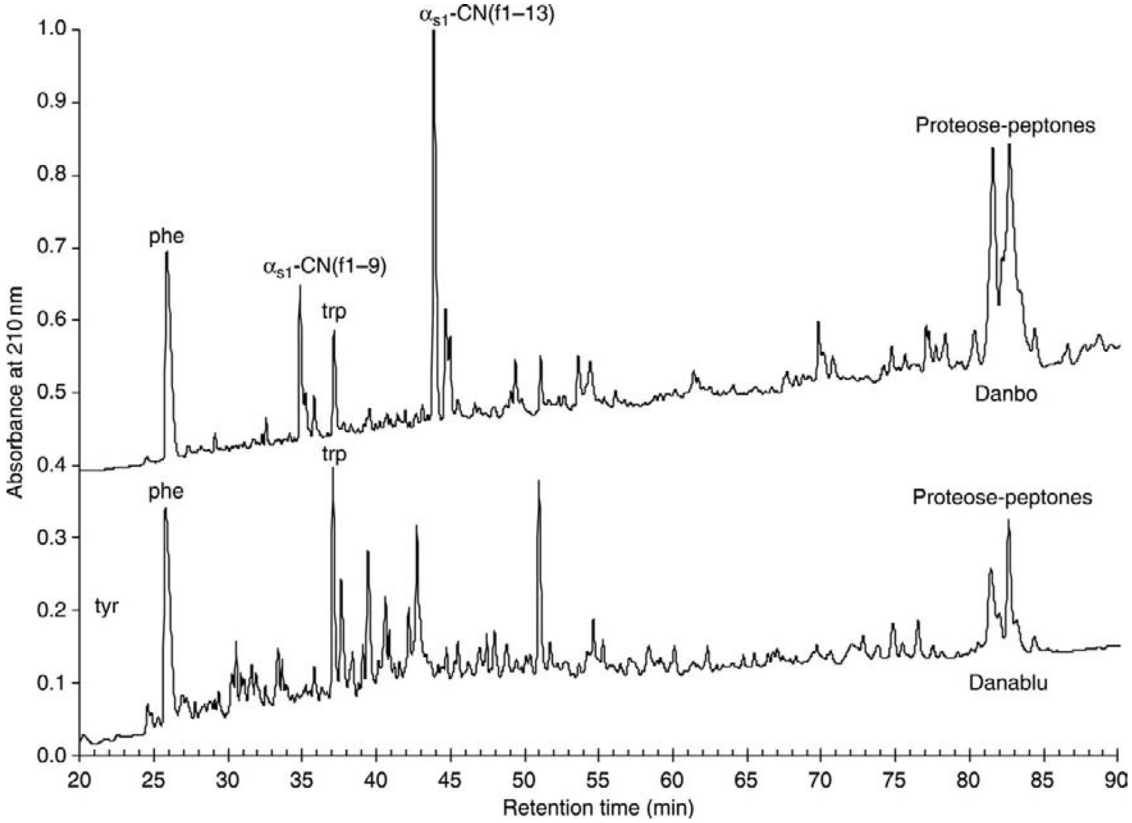

Figure 2 Peptide profiles of Danablu and Danbo made with mesophilic starter and analyzed by reverse-phase-HPLC.

Table 2 Main enzymes involved in proteolysis and amino acid release during ripening of blue mold cheese without surface microflora

Enzyme	*Specificity in cheese*
Plasmin	Cleaves β-CN and α_{s2}-CN after basic amino acids (Arg, Lys) Hydrolyzes β-CN at 28–29, 105–106, 107–108 to produce γ-CN
Rennet	Hydrolyzes α_{s1}-CN to α_{s1}-CN (f24–199) and α_{s1}-CN (f1–23)
LAB lactocepin	Hydrolyzes peptides produced from casein by the action of plasmin, rennet, or *Pencillium roqueforti*
LAB peptidases, for example, PepN, PepC, PepX	Releases amino acids from smaller peptides. Broad aminopeptidase and dipeptidase specificity
P. roqueforti aspartic protease	Hydrolyzes β-CN preferentially to produce β-CN (98–209, 30–209, 1–29, 100–209, 1–97/99); hydrolyzes α_{s1}-CN
P. roqueforti metalloprotease	Broad specificity
P. roqueforti serine carboxypeptidase (extracellular, acid)	Releases acidic, basic, and hydrophobic amino acids
P. roqueforti metallo-aminopeptidase (extracellular, alkaline)	Releases apolar amino acids, however, not next to Gly

optimum for casein hydrolysis at pH 5.5, which corresponds to the pH often found in blue mold cheese during ripening. The metalloprotease has a broad specificity and hydrolyzes both α_{s1}- and β-caseins. Of special interest is that the metalloprotease cleaves β-casein at Pro90–Glu91, which is not often hydrolyzed by proteases because of the proline residue, and that, like plasmin, it cleaves a bond close to Lys28–Lys29. The aspartic protease is stable at pH 3.5–6.0. Casein is hydrolyzed into mainly high-molecular-weight peptides, and the primary specificity on α_{s1}-casein is similar to that of chymosin. The aspartic protease hydrolyzes β-casein into several large peptides, including β-casein (f98–209), (f30–209), and (f1–29). A relationship has been established between the development of *P. roqueforti*, the activity of the aspartic protease, and the release of bitter peptides.

Those bitter peptides, as well as other peptides, are broken down by *P. roqueforti* using several exopeptidases. An extracellular acid carboxypeptidase, with a broad specificity, releases acidic, basic, and hydrophobic amino acids and may be important in the debittering process. It is a serine enzyme stable at pH 5.0–5.5. *Penicillium roqueforti* also produces an extracellular alkaline metalloaminopeptidase with a pH optimum of 8.0. It is specific for hydrophobic amino acids, and, consequently, the debittering activity of *P. roqueforti* may increase with pH in blue mold cheese. Several intracellular peptidases have also been detected, among them alkaline carboxy- and aminopeptidases, but their contribution to ripening is not obvious. The proteolytic activity, as well as the level of proteases and peptidases produced by *P. roqueforti*, varies widely between strains.

Growth of mold within a pierced blue mold cheese leads to an increase in pH, which stimulates the activity of other proteolytic enzymes in the cheese, such as the LAB cell wall protease, lactocepin, and the milk protease, plasmin. Salt inhibits the development of *P. roqueforti* and therefore also its proteolytic activity, which explains the hard and rather tasteless zones close to the rind typical for blue mold cheeses.

Proteolytic activity of yeast in blue mold cheese is limited and is not important in most varieties. An excessive contribution to proteolysis by some strains may be detrimental. Aminopeptidase activity on branched-chain amino acids is common in both *D. hansenii* and *Yarrowia lipolytica*, and because yeasts grow to large numbers in some blue mold cheeses, this activity may contribute to the release of amino acids during ripening.

Nonstarter LAB have been isolated from blue mold cheese and could, as in other cheeses, be expected to take advantage of the large amount of small peptides produced by the other microorganisms present and produce mainly similar aroma compounds from amino acids as the starter bacteria.

Lipolysis

Much higher amounts of free fatty acids are released during ripening of various kinds of blue mold cheeses than observed in many other cheeses (**Table 3**). This extensive lipolysis would cause rancidity in many other cheeses, but in blue mold cheeses the free fatty acids are neutralized because of a large increase in pH during ripening. The total level of free fatty acids tends to increase faster after the mold has sporulated, and a decrease at the end of ripening has been observed, which could be explained by conversion of the fatty acids to methyl ketones. *Pencillium roqueforti* releases larger amounts of long-chain fatty acids ($C_{12:0}$–$C_{18:3}$) than of short-chain fatty acids ($C_{4:0}$–$C_{10:0}$), in contradiction to the milk lipoprotein lipase, which is especially active in cheeses from raw milk. Metabolism of lipids in blue mold cheeses is performed mainly by enzymes from *P. roqueforti*, including two extracellular lipases, one acidic and one alkaline, and intracellular lipase activity has also been reported.

Table 3 Typical concentration of free fatty acids (FFA) in different cheese varieties

Variety	*FFA (mg kg^{-1})*	*Variety*	*FFA (mg kg^{-1})*
Cabrales	33200	Gruyere	1500
Danablu	32600	Brie	1300
Roquefort	32400	Cheddar	1000
Parmesan	5000	Camembert	700
Provolone	2100	Mozzarella	360

The acidic lipase has a pH optimum at 6.0, temperature optimum at 35–40 °C, and it retains 37% of maximum activity at 5 °C. Optimum pH for the alkaline lipase is ~9.0, but activity is retained at pH 4.5. Even though the pH of blue cheeses in general favors the activity of the acid lipase, it should be noted that the alkaline lipase has the higher activity on milk fat.

Almost all yeasts isolated from blue mold cheese have lipase or esterase activity, which is the ability to hydrolyze short-chain fatty acids from triglycerides. Lipolysis of long-chain fatty acids has been demonstrated for some strains of *Y. lipolytica* and *G. geotrichum.*

Aroma Formation

A wide range of volatile and nonvolatile aroma compounds are produced in blue mold cheese during ripening, primarily by *P. roqueforti*, influencing both taste and aroma of the final product. The varying proportions of these compounds determine the specific flavor profiles obtained for different blue mold cheeses.

Amino acids, of which the most commonly found in blue mold cheese are glutamic acid, leucine, valine, and lysine, are released in large amounts and may contribute to a background flavor, and further catabolism of them produces several aroma compounds. The metabolic pathways of LAB starting with aminotransferase activity are not very well studied in blue mold cheese; however, compounds resulting from different pathways of amino acid catabolism have been found in blue mold cheese. Oxidative deamination of amino acids may be performed by *P. roqueforti* within the cheese fissures and by the microbial flora that grow on the surfaces of some blue mold cheese varieties. This activity produces ammonia in amounts that contribute to the flavor of blue mold cheese varieties. Glutamic acid is decarboxylated to γ-aminobutyric acid and CO_2, and other amino acids are decarboxylated to amines and CO_2 by *P. roqueforti* as well as by adventitiously growing microorganisms. The concentrations of amines vary largely, and tyramine is usually observed in larger amounts than tryptamine and histamine. Catabolism of arginine to ornithine and citrulline has been shown in blue mold cheese. The complex amino acid catabolism in blue mold cheese varieties needs more research to be understood.

The characteristic flavor compounds in blue mold cheeses originate mainly from lipid degradation and formation of free fatty acids and methyl ketones. Hexanoic and octanoic acids are especially important flavor compounds. Methyl ketones are major aroma compounds in blue mold cheeses; they constitute 50–75% of the total amount of volatiles, and their concentration is correlated to the intensity of the characteristic flavor of blue mold cheese. The methyl ketones found at highest concentrations are 2-heptanone and 2-nonanone, but 2-pentanone and 2-undecanone may also be important. The flavor of the methyl ketones are, in general, described as fruity, floral, and musty, and especially 2-heptanone contributes to the typical flavor of blue mold cheese. As increasing concentrations of free fatty acids have been shown to inhibit the growth of *P. roqueforti* and thereby retard lipolysis, the formation of methyl ketones from free fatty acids has been proposed to be a detoxifying mechanism. Methyl ketones with one less carbon atom are produced via the β-oxidation pathway from the corresponding fatty acids. The majority of methyl ketones are derived directly from their fatty acid precursors, but certain methyl ketones, for example, 2-heptanone and 2-nonanone, can also be derived from longer-chain fatty acids. Both conidia and mycelia are capable of producing methyl ketones.

Alcohols have been reported to represent between 15 and 30% of the total volatile flavor compounds in blue mold cheese. Methyl ketones can be reduced to secondary alcohols, under anaerobic conditions, including mainly 2-heptanol, 2-nonanol, and 2-pentanol, depending on the cheese type and the strain of *P. roqueforti* used. Their flavor is more or less similar to that of the corresponding methyl ketones, but at higher concentrations they contribute to musty or moldy flavor notes.

See also: **Flavors and Off-Flavors in Dairy Foods. Milk Lipids**: Fatty Acids.

Further Reading

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Camembert, Brie, and Related Varieties

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Introduction

Mold-ripened soft cheeses include internally ripened (e.g., Blue cheeses and Roquefort) and surface-ripened cheeses (e.g., Camembert de Normandie, Brie de Meaux, and Brie de Melun). They also include 'related varieties', which are Camembert- and Brie-type cheeses that, for the most part, do not come under the French Protected Designation of Origin (PDO) regulation, or similar cheeses made from goats' milk (e.g., Garroxta in Spain) or ewes' milk (e.g., 'Azeitão' or 'Serpa' in Portugal). The colonization of *Penicillium camemberti* and/or *Geotrichum candidum* on the cheese surface gives these cheeses their specific appearance. The rind of Camembert-type cheeses is usually composed of what is called a 'bloom' formed by *P. camemberti*, which is roughly 3 mm thick and white or light gray in color. Immediately underneath this surface layer is a very dark orange or brown layer (~0.5 mm) formed by other microorganisms (yeasts and surface bacteria).

A wide range of manufacturing procedures are used to produce mold-ripened cheeses. Indeed, there are as many procedures as there are factories and even cheeses. However, all of them have several aspects in common.

1. *Coagulation of milk.* A good balance between acid and enzymatic activities must be ensured to produce a mixed curd. To that end, the acidification of lactic acid bacteria (LAB) is moderated: first by restricting the quantity to be inoculated to 5×10^6 cfu ml^{-1} and second by adding, as soon as the pH falls between 6.25 and 6.35, the coagulant agent (chymosin at 520 mg l^{-1} or rennet) at a concentration between 18 and 22 ml for 100 l milk. The temperature throughout this step (32–35 °C) favors both the growth of LAB and the enzymatic activity of the coagulant agent. The overall length of coagulation varies from 30 to 90 min, depending on the cheese. This coagulation produces a mixed curd whose stiffness and elasticity rank as medium, in relation to cottage cheeses, on the one hand, and hard cheeses, on the other hand. The syneresis power and permeability of a mixed curd are low.
2. *Draining stage of the curd.* Draining occurs spontaneously, and it is facilitated by cutting the curd into cubes (0.5–5 cm) and molding, according to the cheese under production and the desired shape. Acidification continues to take place during this stage, leading to a pH on day 1 (the end of draining) between 4.6 and 4.7. The mineralization of the curd is low. The calcium level varies from 0.3 to 0.5%, and dry matter content varies from 40 to 45%. Cohesiveness is reduced and the cheeses are medium sized (9–20 cm in diameter and 3–6 cm in height).
3. *Salting of young cheeses.* Either a dry salt is used or brining with sodium chloride is carried out for what are referred to as young cheeses (day 1). Surface mold-ripened soft cheeses require between 1.5 and 2.0% salt (g per 100 g wet cheese (WC)). Salting plays three main roles: (1) it encourages further draining; (2) it prevents the development of pathogenic or spoilage microorganisms; and (3) it gives the cheese its specific taste. Salt intensifies or hides aromas that are produced during ripening.
4. *Ripening of surface mold-ripened soft cheeses.* Ripening is the result of overall enzymatic reactions taking place in cheese, mainly due to microbiological flora (which is either native or provided in the starter). The starter is a mixture of LAB, yeasts, some surface bacteria, and *P. camemberti.* A part of this starter is added to milk before coagulation, but *P. camemberti* is often sprayed onto cheese surfaces after salting. Ripening occurs in two steps: ripening in chamber and ripening under packaging. During ripening in chamber, according to the cheeses under production, the temperature varies from 10 to 14 °C, the relative humidity from 90 to 95%, and the length of stay can range from 9 to 30 days. After this first step, the cheeses are wrapped and stored at 4–6 °C for ripening under packaging. According to the water permeability and other characteristics of the chosen wrap, ripening under wrapping can vary greatly. This second step is of the utmost importance because it determines whether a cheese remains or not within the range of characteristics (appearance of rind, thickness of rind, and hardness of the underrind), which must be met if the cheese is to be marketed.

Surface mold-ripened cheeses are classified in three groups of production in relationship with the coagulation method, acidification intensity, and molding technique, as summarized in **Table 1**.

Surface mold-ripened cheeses are characterized by a high surface/volume ratio and a moisture content that ranges between 50 and 60 g of water per 100 g WC. The

Table 1 Classification of surface mold-ripened cheeses into three groups, in relationship to the coagulation method, acidification intensity, and molding technique

Coagulation method	*Acidification strength*[a]	*Whey–curd treatment*	*Example*	*Quantity produced*
Rennet ($pH_{renneting} \approx 6.5$)[a]	Strong	Without working the curd	PDO Camembert de Normandie PDO Brie de Meaux or de Melun	The smallest
Rennet ($pH_{renneting} \approx 6.3$)	Medium	With a slight working of the curd[b]	Carré de l'est Industrial Camembert[c]	
Rennet with LAB action ($pH_{renneting} \approx 6.1$)	Very strong	Without working the curd	Saint-Marcellin	The largest

[a] Acidification is due to a lactic acid bacterial starter.
[b] A slight mixing of whey and curd grains before molding.
[c] What the French call industrial Camembert, a Camembert-type cheese produced on a large scale.
LAB, lactic acid bacteria; $pH_{renneting}$, pH at renneting.

surfaces of these cheeses are formed by a high concentration of microorganisms, which grow in a thin solid surface layer. This layer consists of large steric clusters as well as tangles of these microorganisms. In the last 10 years, in an effort to better understand the ripening process, studies have been dedicated to acquiring insight into the interactions among the surface microorganisms themselves, between microorganisms and the curd, and between microorganisms, the curd, and ripening agents.

A New Mind-Set for Microbiological, Physicochemical, and Sensorial Studies of Cheeses during Ripening

Until a few years ago, studies involving Camembert-type ripening and/or the microorganisms involved did not account for these various types of interactions. It had been shown that deacidification by *G. candidum* in the presence or absence of *Kluyveromyces marxianus* did not occur when a cheesy medium (a mixture of cheese and water) was experimented with. However, it was already known that deacidification does take place in the surface layer of real cheeses. Indeed, even if several strains of different species are used in the starter, the results obtained under optimum conditions are very different from (1) the results obtained for each strain and (2) the results observed in a real cheese. Consequently, and within the framework of ever-evolving laboratory technology and industrial cheesemaking demands, a new mind-set was developed to account for these interactions. First, to study a microorganism, each strain under study was incubated in a cheese-based solid model medium to reproduce the same composition as on the surface of a cheese on day 1. Growth was then studied on this solid medium in relationship to other strains under defined conditions. Otherwise, studies focused on microbiological, biochemical, physicochemical, or sensorial changes during ripening without accounting for how the cheese was made, what microflora were involved, or the given ripening conditions, relying only on two or three samples taken throughout ripening. Finally, the necessity to make a 'real' cheese with a defined starter was deemed necessary if scientists were to study inner and outer dynamics of the cheese throughout ripening under defined and controlled ripening conditions (temperature, relative humidity, and gaseous composition of the atmosphere). To study the dynamics, it has been shown that it is best to sample one cheese on a daily basis from day 1 to 14, and on a weekly basis after wrapping (day 15). Using this sampling technique, and more complete and systemic analyses of cheeses, some underlining phenomena that had merely been inferred until then were revealed.

To define kinetic parameters and maximum specific growth rates of microorganisms, statistical and mathematical models have been proposed. In order to determine the minimum number of experiments, factorial experimental design and correlation matrixes, in addition to other methods, have been developed. Results based on this new mind-set and procedures are detailed as follows, and future trends are outlined.

Microorganisms Involved in Surface Mold-Ripened Soft Cheese Ripening

Ripening microorganisms found in cheeses generate enzymes that are agents in the ripening process. The composition of these ecosystems and their evolutions are complex. *Penicillium camemberti* is the main ripening microorganism of Camembert-type cheeses and has received the most attention. Ripening microorganisms also include LAB starters, yeasts, and surface bacteria.

Penicillium camemberti

Penicillium camemberti is strictly aerobic; therefore, it grows only on the cheese surface. It is generally agreed that (1) this mold plays an important proteolytic and lipolytic role in the overall ripening process, (2) its proteolytic action varies little in the case of given strains, and (3) its lipolytic activities are generally significant, but they vary greatly from one strain to another.

To follow the growth of *P. camemberti*, over time, two techniques are used today involving a visual estimation of *P. camemberti* mycelium in addition to the test for spore concentration.

Lactic Acid Bacterial Starter

The most commonly used starters include mesophilic and lactate-producing strains of *Lactococcus lactis* because they grow readily in milk. However, *Leuconostoc mesenteroides* is added to limit curd acidification during the first days of ripening (postacidification phenomenon). The LAB starters metabolize lactose to lactate, and their growth takes place from inoculation to salting (the first 24 h of cheesemaking). The LAB starters set the stage for the production of a firm and drained demineralized curd with a pH of 4.6–4.8 on the first day of ripening. There are three phases of LAB growth in relation to ripening time: (1) significant growth from starter inoculation to the end of the first cheesemaking day. On day 1, growth reaches between 3 and 5×10^9 cfu g^{-1} WC. (2) Thereafter and until day 10, growth remains constant at this value. (3) Then, and until the end of ripening, growth decreases to around 5×10^8 cfu g^{-1} WC on day 41.

Yeasts

In practice, yeasts are divided into two different groups according to their glycolytic enzymatic activities: (1) yeasts for deacidification, essentially composed of *K. lactis* or *K. marxianus* and *Debaryomyces hansenii* and their imperfect forms (*Candida*) and (2) strains of *G. candidum.*

The main role of yeasts in deacidification is to consume lactic acid, particularly at the cheese surface, leading to an increase in the surface pH above 6.0, at which point surface bacteria can begin to grow. Moreover, it was recently shown that deacidification could also be due to the direct production of NH_3 by these yeasts. The impact of these species of yeasts on proteolysis is due to their limited endopeptidase system and this proteolysis can occur only after wrapping (on day 15) and around the end of ripening (day 41). *Kluyveromyces marxianus* produces only short- or medium-chain free fatty acids (FFAs) during its growth phase and has a minor influence on C6:0–C12:0 acid production during its late-log, stationary, and death phases.

Currently, *G. candidum* is systematically added to ripening starters due to better knowledge about its growth and development on the cheese surface as well as the capacity of *G. candidum* to metabolize bitter peptides produced by *P. camemberti.* This yeast contributes to the overall flavor of cheeses due to its significant proteolytic and lipolytic enzymatic systems. *Geotrichum candidum* generates short- and medium-chain FFAs (C4:0–C12:0) during its exponential and late-log growth periods and only long-chain FFAs (C14:0–C18:0) during its stationary phase.

Surface Bacteria

These bacteria are found in milk, salt, or brine, and come from industrial staff as well as the environment of the ripening room (e.g., atmosphere, walls) and are also added as ripening starters. Although their optimum temperature is around 25 °C, they can grow at low temperatures (8–14 °C). Surface bacteria start growing only after deacidification, when the pH is above 6.0. Their morphology depends on culture and environmental conditions. These bacteria belong to either Micrococcaceae or other bacterial families. The well-known species are *Brevibacterium linens*, which constitute the main surface bacteria for Camembert-type cheeses. Although *B. linens* do not make up the majority of the overall microflora, they do contribute to its overall characteristic flavor.

Evolution of a Simple Flora of Camembert-Type Cheeses during Ripening

A flora, representing a simple starter for Camembert-type cheeses, composed of four strains (*K. marxianus*, *G. candidum*, *P. camemberti*, and *B. aurantiacum*), was used to ripen experimental cheeses. The trends in growth of the four strains are clearly seen in **Figure 1** and **Table 2**. After day 3, *K. marxianus* reached 90% of its maximal viable cell concentration. The growth of *G. candidum*, *P. camemberti*, and *B. aurantiacum* involved three phases: a lag phase that occurred during the first days of *K. marxianus* growth; an exponential phase for which the specific growth rate depends on the environmental conditions (temperature, relative humidity, and atmospheric gaseous concentrations); and a stationary phase for which maximum viable cell concentration was related to ripening conditions.

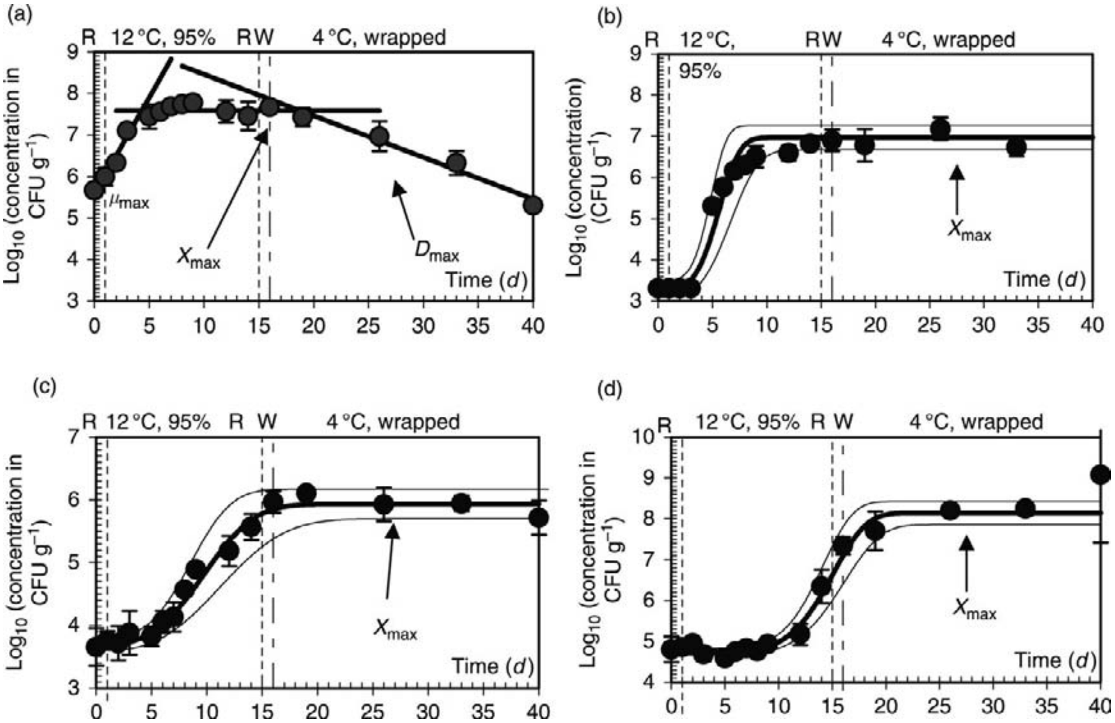

Figure 1 Changes in viable cell counts on the cheese surface during ripening (44 days) according to Leclercq-Perlat M-N, Picque D, Riahi MH, and Corrieu G (2006) Microbiological and biochemical aspects of Camembert-type cheeses depend on atmospheric composition in the ripening chamber. *Journal of Dairy Science* 89: 3260–3273. *Kluyveromyces marxianus* (a), *Geotrichum candidum* (b), *Penicillium camemberti* (c), and *Brevibacterium aurantiacum* (d). Each evolution represents the mean of four trials, and — the standard deviation. ● experimental measurements, and — modeling estimation with its confidence interval at 95% (– –).

Table 2 Modeling used and values[a] of main kinetic descriptors of microorganism evolutions obtained at 13 °C, 94 ± 1% of relative humidity, $CO_2 = 0\%$, and $O_2 = 21\%$

		Exponential growth phase			*Stationary phase*
Microorganisms	*Modeling used*	*Period (day)*	*μ_{max} (day^{-1})*	*d for μ_{max} (day)*	*X_{max} (cfu g^{-1} DM)*
K. marxianus	Linear	1–5	0.46 ± 0.04	ND	4.0×10^7
G. candidum	Weibul	4–9	0.7 ± 0.2	6 ± 1	1.0×10^7
PC spores	Weibul	7–20	0.23 ± 0.05	9 ± 3	8.5×10^5
B. aurantiacum	Weibul	13–19	0.46 ± 0.07	15 ± 1	1.4×10^8

[a] Values are the means of duplicate runs carried out under the same conditions. μ_{max}, maximum specific growth rate determined by modeling; day for μ_{max}, time when specific growth rate is equal to μ_{max}; X_{max}, mean cell concentration obtained under stationary growth phase. ND, not determined.

According to Leclercq-Perlat M-N, Picque D, Riahi MH, and Corrieu G (2006) Microbiological and biochemical aspects of Camembert-type cheeses depend on atmospheric composition in the ripening chamber. *Journal of Dairy Science* 89: 3260–3273.

During the first 12 days of ripening, *B. aurantiacum* remained constant at its initial level, but, surprisingly, no growth was observed between days 5 and 12. This period corresponded to the important change in rind pH from 4.8 to 7.4, which was linked to mycelial development of *P. camemberti*. After day 12 and until day 20, growth was exponential with a generation time around 1.5 days.

The growth of these microorganisms was highly related to carbon substrates in the case of *K. marxianus* and to carbon source and nitrogen substrate availability for the other strains. Interactions between microorganisms have been shown. These interactions could be either direct (via viable cells of two species) or indirect (after the death of cells).

Biochemical and Physicochemical Changes during Standard Condition Ripening

Lactose and Lactate Metabolism and Changes in pH

In **Table 3** and **Figure 2**, the decrease in lactose concentration at the surface coincides with the initial growth of *K. marxianus*. In this case, when lactose concentration in the rind was negligible (by day 6), this yeast consumed lactate available in the rind. *Geotrichum candidum* consumes lactate but not lactose for its growth, and it was supposed that this yeast consumed lactate diffusing from the core to the rind until day 12.

During the first 6 days, postacidification in the core and in the rind of the cheese – the result of LAB activity – may have slowed down the ripening process. After the first 6 days, until the end of ripening, the pH increased, following (1) the decrease in lactate concentration and (2) the increase in ammonia concentration in the cheese. The pH of the rind increased rapidly. This was correlated with fungal growth but also with its proteolytic activity. The assimilation of lactate by yeasts and *P. camemberti* led to a reduction in the acidity of the rind. The lactate concentration in the rind was related to the growth of *P. camemberti* mycelium and spore population. The lactate concentration observed on the rind is a balance between the activities of the fungal flora (which consumed lactate) and the LAB (which produced lactate). *Kluyveromyces marxianus* on the cheese surface does not ferment lactose and does not produce any lactate. However, most LAB are only partially aerotolerant. Therefore, the lactate present on the surface most likely originated from diffusion from the core toward the outside. This depends on the dry matter content of the cheese. At the end of the acidification phase, lactate accumulation was reduced and consumption of nitrogen substrates was rapid.

Carbon substrate concentrations in the core were highly related to those of the rind. Moreover, the concentrations of these carbon substrates related well with the number of viable microorganisms. The change in carbon substrates, which was related to the microbial growth in the rind, may well be due to the migration from the core to the rind. The lactose concentrations were negligible after 10 days of ripening, and changes in lactate quantities were related to fungal flora. Surface pH was significantly related to fungal growth and ammonium concentration, whereas the core pH depended on the concentration of ammonium.

Proteolysis

The 4.6 pH acid-soluble nitrogen (ASN) and nonprotein nitrogen indices and ammonium concentration of the rind were low until day 6 and then increased rapidly to follow the fungal concentrations until day 45 (**Figure 3**). The ASN and NPN indices and ammonium concentration in the core were lower than in the rind, and they displayed the same evolution. *Geotrichum candidum* and *P. camemberti* populations have a major effect on proteolysis; nevertheless, lysis of *K. marxianus* and *B. aurantiacum* cells also had an impact on proteolysis; the increase in these observed

Table 3 Correlations established between viable cell counts ($\log_{10}$ cfu g^{-1} dry matter) of ripening microorganisms (*Kluyveromyces marxianus*, *Geotrichum candidum*, *Penicillium camemberti*, *Brevibacterium aurantiacum*) and physicochemical variables measured during the ripening of a surface mold-ripened soft cheese

	Microorganisms related with biochemical component evolution and pH	*Diffusion phenomena highlighted*
$[\text{Lactose}]_{\text{rind}}$	*K. marxianus* in the rind	
$[\text{Lactose}]_{\text{core}}$	*K. marxianus* in the rind	Diffusion from the core to the rind
$[\text{Lactate}]_{\text{rind}}$	*G. candidum*; *P. camemberti*	
$[\text{Lactate}]_{\text{core}}$	*B. aurantiacum*	Diffusion from the core to the rind
$[\text{ASN}]_{\text{rind}}$	*B. aurantiacum*; *P. Camemberti*	
$[\text{ASN}]_{\text{core}}$	*K. marxianus* in the core	
$[\text{NPN}]_{\text{rind}}$	*G. candidum* in the rind; *B. aurantiacum*	
$[\text{NPN}]_{\text{core}}$	*G. candidum* in the core	
$[\text{NH}_3]_{\text{rind}}$	*G. candidum* in the rind; *P. camemberti*	
$[\text{NH}_3]_{\text{core}}$		Diffusion from the rind to the core (under pH_{inner} influence)
pH_{rind}	*B. aurantiacum* via lactate and NH_3 concentrations in the rind	
pH_{core}		Lactate diffusion from the core to the rind + NH_3 diffusion from the rind to the core

[Lactose], [Lactate], concentration (g kg^{-1} dry matter); [ASN], acid-soluble nitrogen index (g per 100 g total nitrogen); [NPN], nonprotein nitrogen index (g per 100 g total nitrogen); [NH_3], ammonium concentration (g kg^{-1} dry matter). Indexes 'core' for inner cheese mass and 'rind' for surface of cheese. According to Leclercq-Perlat MN, Latrille E, Spinnler HE, and Corrieu G (2004) Controlled production of Camembert-type cheeses: Part II. Aroma component evolutions. *Journal of Dairy Research* 71: 355–366.

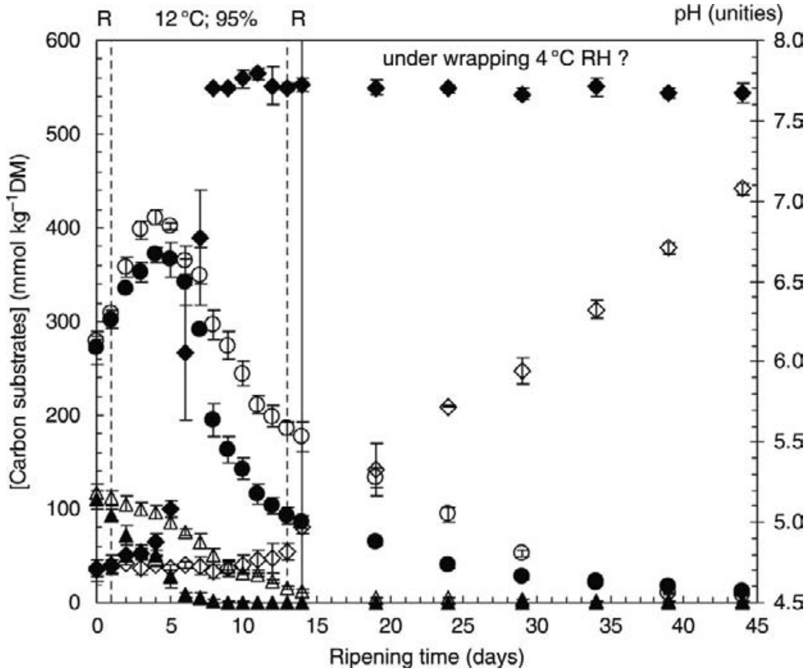

Figure 2 Changes in lactose and lactate concentrations and in cheese pH during ripening (days 0–44). ▲ lactose in rind, △ lactose in core, ● lactate in rind, and o lactate in core. ◆ surface pH and ◇ core pH. Each change represents the mean of four trials, and — the standard deviation. *R* surface drying (12 °C, 85% RH).

fractions was the balance between the amount of protein hydrolyzed and the amount of peptides consumed. After day 6 and until the end of ripening, proteolysis was highly related to the growth of *G. candidum* and *P. camemberti* known for their proteolytic and peptidolytic activities. The accumulation of NPN in the rind was highly correlated with the growth of *G. candidum*, whereas the ASN was more related to the development of *B. aurantiacum.*

The production of free NH_3 can be understood as the final step of proteolysis. The concentration of NH_3 in the rind is highly correlated with the growth of *P. camemberti*, well known for its proteolytic activities but less so for its amino acid catabolic capacity. Its growth is negatively correlated with the ASN, revealing that it has the capacity to degrade long-chain peptides.

Due to the surface microflora and the higher pH, proteolysis in the rind is more extensive and more rapid than in the core; the proteolytic enzymes do not diffuse from their production site.

Given the growth sequence of the different microorganisms, we can infer that during their extremely rapid growth these microorganisms consumed peptides more quickly than they hydrolyzed proteins. This is consistent with the initial and basic NH_3 properties. Diffusion of NH_3 from the outside to the core has been highlighted. Ammonia production by *P. camemberti* and *B. aurantiacum* in the cheeses seemed higher than that of *G. candidum.* In the case of *P. camemberti*, ammonia was produced by growth and sporulation. However, it must be remembered that these results are based on an experimental cheese; in an industrial cheese, the mechanisms associated with ammonia production could be more complex.

Lipolysis and Free Fatty Acids: Important Aroma Precursors

Biochemical pathways liberating FFAs are significant in the ripening of Camembert-type cheeses. Concentrations of butanoic, caproic, and capric acids are higher than their flavor threshold determined in a fatty medium and thus probably contribute directly to its overall flavor intensity. However, the products of catabolism generated by microorganisms are more important for flavor than the FFAs produced. In the rind and after day 6, short- and medium-chain FFAs are in their dissociated form, that is, they are much less volatile than when in their nonionized form, due to the rind pH being higher than their pK_a (close to 5.0). The FFAs in their dissociated form diffuse easily. This may significantly change the aroma impact of short-chain fatty acids (**Table 4**).

Geotrichum candidum and *P. camemberti* spores tend to produce long-chain FFAs of minor olfactive importance. *Geotrichum candidum* plays a significant role in Camembert flavor. The lipolytic enzymes of *P. camemberti* are mainly produced by the mycelium and do not diffuse to the core, whereas the spores of *Penicillium* are efficient in converting FFAs into methyl ketones or octen-1-ol-3.

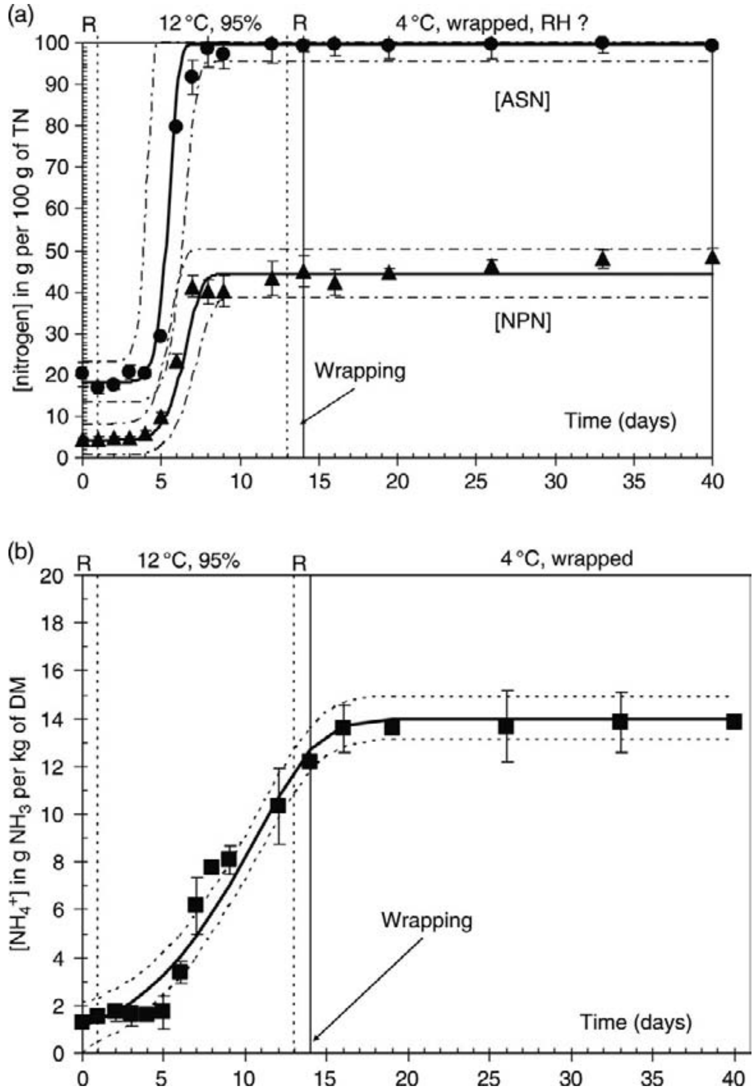

Figure 3 Changes in (a) acid-soluble nitrogen (ASN) indices and nonprotein nitrogen (NPN), as well as (b) free ammonia concentrations [NH_4^+] vs. ripening time (44 days) in cheese surface. ● ASN indices in the surface, ▲ NPN indices for A, and ■ NH_4^+ for B. Each evolution represents the mean of four trials, and — the standard deviation. TN, total nitrogen.

In the experiment under discussion, the differences in the concentrations of some medium-chain FFAs and stearic acid between the rind and the core revealed diffusion from the rind where these acids are produced to the core. β-Oxidation of FFAs on the surface takes place rapidly in the presence of oxygen, breaking down residual FFAs in the rind.

Pelargonic (C9:0) and tridecanoic (C13:0) acids, which are rarely found, were produced mainly during the growth of *G. candidum* and *P. camemberti* mycelium (days 7–14) from unsaturated FFAs disrupted by lipoxygenase and hydroxide lyases. C9:0 was produced in the rind and migrated to inside of the cheese, whereas C13:0 was produced at the same rate in the rind and the core. Some tridecanoic acid could have been due to a chemical auto-oxidation in the presence of peroxides of biological origin and not due to the enzymatic activities of *P. camemberti* alone.

Aroma Production in Camembert Cheeses

Table 5 summarizes microorganisms responsible for the formation of some aroma compounds.

Ester formation is associated with the growth of *K. lactis* and *G. candidum*, which occur during the first days of ripening. The differences in butyl and isoamyl acetate quantities observed between the rind and the core were due to diffusion from the core, where they were produced in anaerobiosis, to the surface. Evaporation of esters from the surface to the atmosphere explains the alcoholic fruity odor in the ripening chamber during this period. *Geotrichum candidum* is probably the main microorganism

Table 4 Correlations established between viable cell counts ($\log_{10}$ cfu g^{-1} dry matter) of *K. marxianus*, *G. candidum*, and *P. camemberti* to determine microorganisms that can be involved in the formation of FFAs under investigation, classified by their number of carbon atoms (short-, medium-, or long-chain FFA), and the phenomena highlighted by Leclercq-Perlat *et al*. (2007)

FFA	*FFA classes*	*Microorganisms related to FFA formation*	*Highlighted phenomena*
Butanoic acid (C_4)	Short-chain	*K. marxianus*; *G. candidum*	Lipolysis did not explain this production
Caproic acid (C_6)	Short-chain	[*K. marxianus*]; *G. candidum*	Diffusion from the rind to the core
Caprylic acid (C_8)	Medium-chain	[*K. marxianus*]; *G. candidum*	
Pelargonic acid (C_9)	Medium-chain	*G. candidum*	Diffusion from the rind to the core
Capric acid (C_{10})	Medium-chain	*K. marxianus*; *G. candidum*	
Lauric acid (C_{12})	Medium-chain	*K. marxianus*; *G. candidum*	Diffusion from the rind to the core β-Oxidation in the rind
Tridecanoic acid (C_{13})	Long-chain	*G. candidum*; *P. camemberti*	Chemical auto-oxidation
Myristic acid (C_{14})	Long-chain	*G. candidum*; *P. camemberti*	
Palmitic acid (C_{16})	Long-chain	*G. candidum*; *P. camemberti*	
Stearic acid (C_{18})	Long-chain	*G. candidum*; *P. camemberti*	Diffusion from the rind to the core

[ ] a microorganism appears involved in a minor way. *B. aurantiacum* ATCC9175 was not demonstrated to be lipolytic.

Table 5 Correlations established between viable cell counts ($\log_{10}$ cfu g^{-1} dry matter) of *K. marxianus*, *G. candidum*, and *P. camemberti* to determine microorganisms that can be involved in the formation of aroma compounds under investigation, metabolism involved in these aroma compounds, and phenomena involved in their formation or their disappearance and/or highlighted. Leclercq-Perlat MN, Latrille E, Spinnler HE, and Corrieu G (2004) Controlled production of Camembert-type cheeses: Part II. Aroma component evolutions. *Journal of Dairy Research* 71: 355–366

Aroma compounds	*Microorganisms related to volatile aroma formation*	*Metabolism involved*	*Highlighted phenomena*
Ethyl acetate	*K. marxianus*	Glycolysis	Diffusion from the core to the rind + evaporation from the rind to atmosphere
Butyl acetate	*K. marxianus*; [*B. aurantiacum*]	Glycolysis	Diffusion from the core to the rind + evaporation from the rind to atmosphere
Isoamyl acetate	*G. candidum*; *P. camemberti*	Glycolysis + proteolysis	Diffusion from the core to the rind (esterification between methyl-3-butanol and acetic acid)
3-Methyl-butanal	*P. camemberti*	Proteolysis	Oxidation of metyl-3-butanol in the rind
3-Methyl-butanol	*B. aurantiacum*; *P. camemberti*; *G. candidum*	Proteolysis	No diffusion
DMDS	*B. aurantiacum*; [*G. candidum*]	Proteolysis	Diffusion from the rind to the core
Styrene	*P. camemberti*]	Proteolysis	Diffusion from the rind to the core
Ethyl butanoate	*P. camemberti*; [*G. candidum*]	Lipolysis	ND
Ethyl heptanoate	*P. camemberti*	Lipolysis	ND
2-Pentanone	*P. camemberti*	Lipolysis	Diffusion from the rind to the core A conversion into its secondary alcohol
2-Heptanone	*G. candidum*	Lipolysis	No diffusion highlighted A conversion into its secondary alcohol
2-Octanone	*P. camemberti*; [*G. candidum*]	Lipolysis	Intrachain oxidation of unsaturated fatty acids A conversion into its secondary alcohol

[ ] a microorganism appears involved in a minor way; ND, not defined.

involved in the production of fatty acid esters. *Kluyveromyces lactis*, *G. candidum*, and *B. aurantiacum* were associated with the production of methyl-3-butanol and methyl-3-butanal. The more oxidizing an environment, the higher the production of methyl-3-butanal; thus, this compound is present in the rind and much less so in the core. Alcohol was present at a similar concentration in the core and in the rind. Accumulation of dimethyl disulfide was related to the growth of *B. aurantiacum* and *P. camemberti* sporulation but not to *G. candidum*, as previously thought. Methyl ketones are some of the components that provide the characteristic flavor of Camembert-type cheeses. 2-Pentanone is highly dependent on the time and on the portion of the cheese analyzed, and its evolution was associated with the populations of *B. aurantiacum*, *P. camemberti*, and *G. candidum.* Before packaging, *G. candidum* produced this methyl ketone during its growth phase and after the packaging of the cheese. *P. camemberti* spores generated it. The main agents of methyl ketone formation were probably *P. camemberti* and *G. candidum*. Methyl ketones are not synthesized uniformly during ripening. As the production of methyl ketones is aerobic, most of the methyl ketones found inside the cheese diffused from the rind.

Volatile compound diffusion phenomena were clearly highlighted: from the surface to the core (e.g., ethyl acetate) and the others from the core to the rind (e.g., methyl-3-butanol).

Ripening Atmospheric Conditions of Camembert-Type Cheeses

All factors involved in microbiological growth and enzymatic activities significantly influence cheese ripening, both internally and externally.

Influence of Temperature and Relative Humidity

Recently, to evaluate the effects of temperature and relative humidity (RH) on the ripening kinetics of Camembert cheeses, the growth of the four microorganisms and physicochemical changes in cheese flavor and texture were investigated under different ripening temperatures (9, 12, and 16 °C) and RH (88, 94, and 98%). This study clearly showed that (1) microbiological growth and physicochemical kinetics greatly depend on ripening temperature; (2) *P. camemberti* sporulation and *B. aurantiacum* growth, in addition to the under-rind thickness and appearance of cheeses, were also related to RH; and (3) it is feasible to determine chamber temperature and relative humidity for proper microorganism growth and physicochemical characteristics so that any and all flaws in the finished product are avoided.

Atmospheric Gaseous Composition of the Ripening Chamber

It was found that whatever the atmospheric conditions in the ripening chamber, the presence of CO_2 resulted in numerous changes in the microbiological and biochemical aspects of the cheeses. CO_2 seemed to set in motion enzymatic dynamics related to *G. candidum* and *P. camemberti* cells or mycelia. The higher the CO_2 concentration, the higher the *G. candidum* cell concentrations, and the poorer the appearance of *P. camemberti* mycelium and cheese (under-rind thickness and color). Moreover, ripening was accelerated in the presence of CO_2 on day 0. The best ripening condition to obtain an optimum balance between microbial growth and biochemical dynamics, as well as appearance of the cheese, was around 2% of CO_2 in the presence of an O_2 concentration of 17–18%.

Future Trends

An understanding of certain aspects of ripening processes is still elusive. What are the best chamber ripening conditions (temperature, relative humidity, and CO_2/O_2 gaseous concentrations)? What happens to cheeses after wrapping and storage under various sets of conditions? How does the wrapping change the quality of cheeses? How can the knowledge of industrial experts and scientists be integrated best? The long-term objective would be to determine mathematical and mechanistic models to help industrialists best manage their work and more easily solve problems that emerge throughout the ripening in chamber as well as under wrapping.

Acknowledgments

The author is grateful to Mme Tanis-Plant for fruitful discussions and editorial advice on English.

See also: **Husbandry of Dairy Animals:** Buffalo: Asia; Buffalo: Mediterranean Region. **Yeasts and Molds**: *Penicillium camemberti*.

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Cheese with Added Herbs, Spices and Condiments

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Introduction

Spices and herbs are defined as aromatic parts of the leaves, flowers, or other parts of a plant that are used to impart an aroma and taste to food. Cheeses with additives (i.e., herbs, spices, and other condiments) continue to increase in popularity among consumers seeking variety and robust flavors. Added herbs and spices also give color to the cheese, and improve presentation and attractiveness of the cheese to consumers. In addition, some herbs and spices are added as a source of health-promoting compounds for consumer health and well-being. However, spices and herbs may be sources of contaminating microorganisms that are undesirable in cheese – particularly because most cheeses are consumed without further processing. Therefore, only the highest quality spice or herb must be added to cheese.

The United States Code of Federal Regulations (CFR) defines three types of spiced cheeses: (1) spiced cheeses (21 CFR 133.190); (2) part-skim spiced cheeses (21 CFR 133.191); and (3) spiced, flavored, standardized cheeses (21 CFR 133.193) (**Table 1**). While the concentration of spice/herb is specified for spiced and part-skim spiced cheeses, there is no requirement for standardized cheese. However, there is a practical limit such that the spice/herb shall not be used to simulate the flavor of any cheese and variety. Also, when the cheese is made from milk that is not pasteurized, it must be ripened at not less than 1.67 °C for at least 60 days (21 CFR 133.190).

Herbs, spices, and other condiments are added to cheeses to impart unique flavors. These cheeses, often regarded as specialty cheeses, are often artisanal and hand crafted with little mechanization or automation.

While most spices impart a specific spice/herb flavor to cheese, some may influence the microbiological quality of the cheese. Common herbs/spices and other flavors added to cheeses include red and green peppers (paprika, habanero, chipotle, jalapeño, cayenne), black peppercorns, horseradish, thyme, cloves, cumin, caraway, parsley, tarragon, nutmeg, basil, onion/garlic, and sundried tomatoes (**Table 2**). Other flavors added to cheese include liquid and natural smoke, soot/ashes, beer, wine, and nuts like almonds and walnuts.

Quality of Spices and Herbs

Generally, spices and herbs added to cheese are dried or lyophilized. They may be also fresh and moist. They may be added whole, chopped, or ground. Sometimes, extracted juices or oils are added to the cheese to give color and flavor. It is important that spices and herbs added to cheese do not break down or undergo any changes during storage or maturation of the cheese and should not adversely affect the natural flavor of the cheese unless such effects are intended. The presence of yeast and molds exceeding 10^3 cfu g^{-1} was found to have a significant effect on the properties of cheese flavored with bush tomato compared with control cheeses and those flavored with native mint and lemon myrtle.

Microbial quality of many spices has been studied. Aerobic bacterial counts of some commercial spices exceed 10^8 cfu g^{-1}. Hence, it is important to select carefully spices and herbs to minimize microbial contamination of cheese. The microbial load of most commercial herbs and spices is reduced by irradiation or chemical sterilization.

Generally, the additives are mixed into curd granules after whey drainage, although there are reports of batch pasteurization (low-temperature long-time (LTLT)) of milk plus spices and herbs prior to cheesemaking. This process is obviously not conducive to continuous high-temperature short-time (HTST) pasteurization. Also, adding spices and herbs to milk prior to cheesemaking will influence the quality of resultant whey and is therefore not practiced in large mechanized cheese plants. Other considerations in adding herbs/spices to cheese are their influence on starter and non-starter lactic acid bacteria. It is important that herbs/spices do not negatively affect metabolism and activities of starter and non-starter lactic acid bacteria in the cheese.

Examples of Specific Cheeses and Methods of Manufacture

Turkish Cheeses

Otlu cheese

Otlu (meaning with herb) cheese is an herb-flavored cheese produced in eastern Turkey for over 200 years. Similar cheeses produced in southern Turkey are called Carra or Surk. The cheese is manufactured from raw or

Table 1 Standards for spiced and flavored cheeses in the United States

Cheese type	*CFR title, number*	*Moisture and fat contents*	*Allowable spice content*
Spiced cheese	21 CFR 133.190	50% FDB min.; no moisture limit	0.09 g per 100 g
Part-skim spiced cheese	21 CFR 133.191	20–50% FDM; no moisture limit	0.09 g per 100 g
Spiced, flavored standardized cheese	21 CFR 133.193	Must comply with the moisture and fat requirements of standardized cheese	Portion reasonably required to accomplish intended effect

Table 2 Common herbs, spices, and other condiments used in cheeses

Herb/spice/vegetables	*Botanical name*	*Examples of cheese with herb/spice*
Spices		
Chili	*Capsicum frutescens* var. *chili*	Cheddar, Monterey Jack
Red pepper	*Capsicum frutescens* var. *faseiculatum*	Cheddar, Monterey Jack
Green pepper	*Capsicum frutescens* var. *grosum*	Monterey Jack, process cheese
Paprika	*Capsicum frutescens* var. *tetragonium*	Boulette d'Avesnes
Jalapeño	*Capsicum annuum*	Monterey Jack, process cheese
Chipotle (smoke-dried jalapeño)	*Capsicum annuum*	Monterey Jack, process cheese
Habanero	*Capsicum chinense*	Jack, process
Black pepper (black peppercorn)	*Piper nigrum*	Gouda
Cayenne	*Capsicum frutescens*	Monterey Jack
Aniseed	*Pimpinella anisum*	
Mustard seeds	*Brassica sinapis alba*	Moutardier
Caraway seeds	*Carum carvi*	Gouda, Liptauer
Cloves	*Eugenia aromatica*	Gouda
Cumin	*Cuminun cyminum*	Gouda, Leidsekaas
Garlic	*Allium sativum*	Cheddar, Monterey Jack
Horseradish	*Armoracia rusticana*	Process cheese products
Herbs		
Dill	*Anethum graveolens*	Process cheese products
Thyme	*Thymus vulgaris*	
Basil	*Ocimum basilicum*	Feta, Cheddar, Monterey Jack
Fenugreek	*Trigonella foenum graecum*	Gouda
Tarragon	*Artemisia dracunculus*	Boulette d'Avesnes
Sage	*Salvia officinalis*	Cheddar
Chives	*Allium schoenoprasum*	Cheddar
Rosemary	*Rosmarinus officinalis*	Raclette
Other condiments, fruit, vegetables		
Sundried tomatoes		Feta, process cheese products
Strawberries		Cream cheese; Le Roule

pasteurized sheep's milk (or sometimes blended with goat or cows' milk) using *Lactococcus lactis* subsp. *lactis* plus *L. lactis* subsp. c*remoris* as starter. After coagulation of the milk with calf rennet (usually 60–120 min at 30–35 °C), the coagulum is cut and the curd transferred into muslin bags into which the herbs are mixed and then pressed for 3–4 h to allow whey drainage. The drained curd is cut into blocks (~7 × 7 × 2 cm) and brined (14–16% (w/v) NaCl) for 6–10 h or dry-salted. When curd is dry-salted, heated whey or defatted yogurt, respectively called 'lor' or 'cacik', is filled into the spaces between the cheese blocks in plastic or earthenware containers. Following salting, the cheese is ripened for at least 1 month. In traditional practices, the cheese in its container is ripened underground (~50 cm beneath the soil surface) for 2–3 months.

About 25 different herbs (**Table 3**) are used in the manufacture of Otlu cheese (**Figures 1** and **3**). The most common herbs used are *Allium*, *Thymus* (thyme), *Anthriscus*, and *Ferula.* The herbs may be added singly or as a mixture of two or more to curds at levels of 0.5–2 kg per 100 kg milk (optimal level is 1%). In addition to providing characteristic aroma and flavor of cheese, the herbs used in Otlu cheese do not negatively affect starter or starter adjunct activities but have antimicrobial properties against *Listeria.* Ethanol or methanol extracts of some herbs, including *Allium vineale, Chaerophyllum macropodum*, and *Prangos ferulacea,* have been reported to exhibit antimicrobial activity against *Listeria monocytogenes* serovars. See 'Further Reading' for chemistry and biochemistry of ripened Otlu cheese.

Table 3 Some herbs used in the manufacture of Otlu peynir (see **Figure 3** for pictures of selected herbs)

Latin name of the herb		*Local name of the herb*
Liliaceae	*Eremurus spectabilis*	Çiriş
	Allium schoenoprasum	Sirmo
	Allium fuscoviolaceum	Yabani soğan (wild onion), sirmo
	Allium scorodoprasum	Çatlangıç, sirmo
	Allium aucheri	Sirmo, sirim
	Allium paniculatum	Sirmo, handuk
	Allium akaka	Kuzukulağı
	Allium cardiostemon	Sirmo
Lamiaceae	*Ocimum basilicum*	Reyhan
	Ziziphora clinopodioides	Kekik (thyme)
	Mentha spicata	Yarpuz
	Thymus migricus	Kekik, zater
	Thymus kotschyanus	Kekik, zater
Apiaceae	*Ferula rigidula*	Heliz
	Ferula orientalis	Heliz
	Ferula L. sp.	Hitik, hiltik
	Prangos ferulacea	Heliz
	Prangos pabularia	Heliz, kerkur
	Anethum graveolens	Dereotu
	Carum carvi	Tarakotu
	Anthriscus nemorosa	Mendo
Caryophyllaceae	*Silene vulgaris*	Siyabo
	Gypsophila L. sp.	Çöven
Brassicaceae	*Nasturtium officinale*	Tere
Ranunculaceae	*Ranunculus polyanthemos*	Çünk

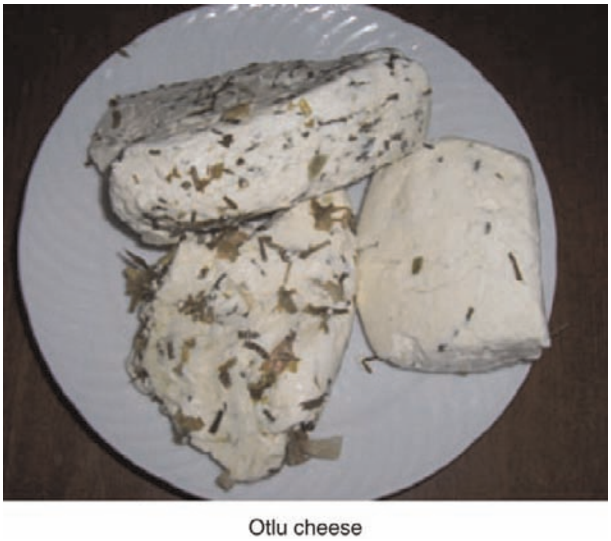

Figure 1 Otlu cheese.

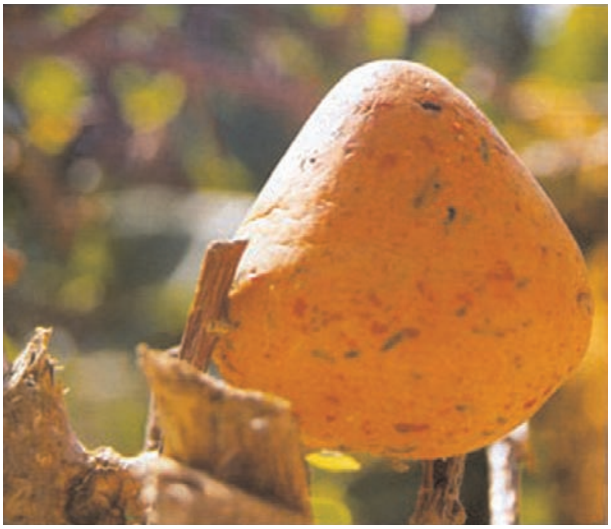

Figure 2 Surk cheese.

Other types of Otlu cheese

Otlu Cacik

Otlu Cacik is a product obtained by heating 'ayran', which is produced from a 1:1 dilution of nonfat yogurt in water and may contain salt. To manufacture Cacik, milk is first converted to yogurt to which water is added before churning into butter. After removing milk fat (butter), the remaining liquid called ayran is boiled for 5–10 min or until a white coagulum floats to the surface. The coagulum is collected and transferred to a cheese cloth for whey drainage. After whey removal, herbs similar to those used in the manufacture of Otlu cheese are added at 1–2% and mixed well to produce Cacik, which contains 16.5–20.8% total solids, 1.5–4.3% fat, 8.1–13.9% protein, 0.3–3.2% salt, and a pH of 3.2–4.2. Cacik is consumed fresh or used as filler in the manufacture of Otlu cheese.

Otlu Lor

Otlu Lor is manufactured from whey (especially from Kasar or Kashkaval production) by boiling and straining as for Otlu Cacik. The curds obtained are mixed with salt (2–8%) and herbs (2–10%) and ripened for about 2–3 months in plastic barrels or used as filler in the manufacture of Otlu cheese. The mean chemical composition of Otlu Lor is 33.7% total solids, 7.3% fat, 17.3% protein, and 5.1% salt with a titratable acidity of 1.5%.

Cheeses containing spices

Surk cheese

'Surk' means fat-free cheese in Arabic and is also called 'cokelek' in Turkish. Surk is produced by heating defatted yogurt or 'ayran' (in Turkish) to boiling for ~30 min to precipitate milk proteins. Because ayran is the liquid remaining after the manufacture of butter from yogurt, its pH is similar to that of yogurt. Therefore, it precipitates readily on heating to boiling. The precipitate is pressed for 5–6 h to remove excess whey and mixed with spices (e.g., peppermint, thyme, mint, cumin, black pepper, cinnamon, and ginger (at 0.1–0.3% each), chili pepper (2%), and sometimes garlic (1%)). After kneading with added salt (5%), the mixture is made into a conical strawberry-like shape, weighing 150–200 g and 5–7 cm in diameter (**Figure 2**), and air-dried in a shaded environment. Surk is consumed fresh or wrapped in parchment paper and placed in a jar for ~30 days at ambient temperature to promote mold growth. Alternatively, to prevent mold growth, the cheese may be dipped or smeared with olive oil prior to wrapping with stretch film. The mean chemical composition of Surk is 55.7% moisture, 9% fat, 19% protein, 8.4% salt, and a pH of 4.9. A photograph of Surk cheese is shown in **Figure 3**.

Carra cheese

Carra or Testi (meaning pottery or clay pot) cheese is a traditional product of Hatay, southern Turkey. It is manufactured from raw goats' milk and heated acid curd (cokelel). To manufacture Carra, first cokelek is produced by heat treatment (90 °C) of skim milk yogurt and draining curd in a cheese cloth and pressing overnight at ambient temperature. Separately, goats' milk is coagulated with rennet (60 min) at 30–32 °C. The coagulum is cut into cubes (1–3 cm^3) and curd transferred into vats lined with cheese cloth for drainage and pressing for ~30 min. Then, the block of curd is sliced into pieces about 4–5 cm thick and coarse salt is sprinkled between the slices and held for 2–3 days. Next, the salted curd is mixed with dried black cumin and thyme (5% each) and filled in clay pots using alternating layers of rennet curd and salted (4%) cokelek until the pot is full. The filled pot is inverted and stored in a cool place for a few days. Then, the cheese is covered with a piece of cloth and sealed with a paste made up of a mixture of wood ash, salt, olive oil, and water. After the paste is dry, the pot is covered tightly with another piece of cloth. Then, the jug is buried in the ground (about 1.5 m deep) to ripen for at least 3 months. The mean chemical composition of Carra cheese is 46.6% moisture, 24.9% fat, 18.9% protein, 8.8% salt, and a pH of 5.6. Similar spiced cheeses sold in clay pots in Armenia are Kateh and Yeghegnadzor (also called 'buried' cheese).

Spiced and Herby Cheeses in European Union and Other Countries

Switzerland and France

Swissalp Panorama and Bellevue cheeses are manufactured in the Alpine region of Switzerland. The outer

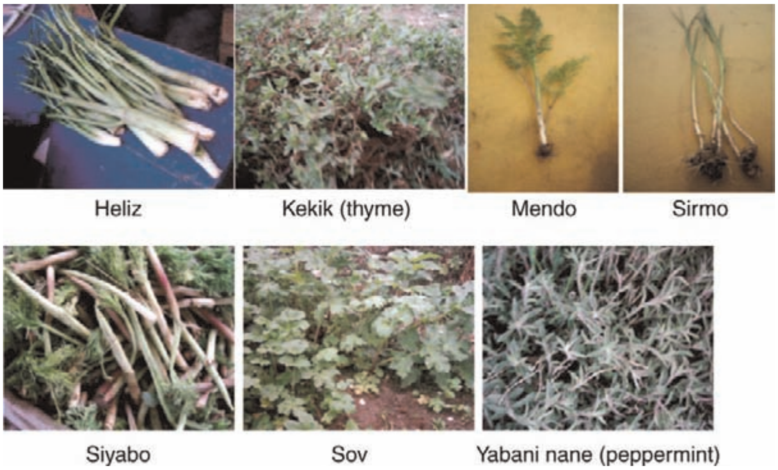

Figure 3 Herbs used in the manufacture of Otlu cheese in Turkey.

surfaces of the cheeses are coated with sage, thyme, or basil, which also provide aroma to entire cheese. Moisture levels are under 40%, protein 25–28%, and fat-in-dry matter 55% in these cheeses. Don Olivo is a Swiss-type cheese with olives and Moutardier contains mustard seeds. Sap Sago is skim milk cheese mixed with powdered melilot – a pale green clover.

Raclette is a semihard Swiss or French Alpine cheese made from cows' milk. The cheese may be smoked or contain white wine, pepper, rosemary, thyme, garlic, and other herbs. Although Raclette originated from Switzerland (Swiss canton of Valais), it is currently produced in the French regions of Savoie and Franche-Comté.

Le Roule is a French cows' milk cheese with a distinctive swirl of herbs and garlic. It may also contain salmon, dill, chives, and strawberry. Morbier is two cheeses separated by a layer of ash (soot or charcoal). It originates from the Jura Mountains in France. Gaperon is a cheese made in Auvergne, France. It contains pink garlic and pepper and is inoculated with *Penicillium candidum.* Coeur de Camembert au Calvados is a soft white ripened Camembert-type cheese that is soaked in Calvados, and fresh breadcrumbs are pressed on it and garnished with walnut. Because the cheese absorbs Calvados, it is an excellent dessert cheese. Munster is a cow's milk cheese with caraway seeds from Alsace in the Voges Mountains in France.

Boulette d'Avesnes is a soft-textured cone or pointed pear-shaped cheese with a dark red-colored rind obtained from annatto or paprika. It originates from Flanders and northern France. The cheese is mixed with paprika, parsley, tarragon, and pepper and shaped by kneading into a pointed pear. It is matured for 2–3 months.

Netherlands

Kanterkaas is a traditional hard Dutch cheese flavored or not with cloves and/or caraway. Kanternagelkaas is flavored with cloves, while Kanterkomijnekaas is flavored with caraway. According to an application for PGI (protected geographical indication) or PGO (protected designation of origin) in the Official Journal of the European Communities, Kanterkass is traditionally linked to the Friesland region and the Westerkwartier area in the Netherlands. It has a flat cylindrical shape, the side forming a sharp edge with the flat base and rounded edge with the flat top. It is ripened for at least 4 weeks at a temperature of over 12 °C. Leyden or Leidse kaas is another Dutch cows' milk cheese that contains cumin or caraway seeds. It contains 40.6% moisture, 13.5% fat, and 37.3% protein.

Spain

Afuega'l Pitu is manufactured by traditional methods in Asturias, northern Spain, and is an artisanal, acid-coagulated cheese; its body is characteristically pasty, and flavor is sharp and aromatic. Two variants, atroncao (troncoconic shape) and de trupo (pear shape), are distinguished. The latter variant is red because paprika is added to the curd. Afuega'l Pitu is made from raw bovine milk and without starter. Milk is preripened to 0.2% acidity before renneting and coagulation process takes about 16 h at ~22 °C. At the end of this process, acidity reaches 0.8–0.9% and the whey begins to separate from the curd. In the pear-shaped variant, drained curd is mixed thoroughly with salt and paprika and/or cayenne peppers and then poured into a muslin cloth, which is tied and hung until drainage is complete. The cheese is then ripened in a well-ventilated room at 15–16 °C and 85–90% relative humidity. Troncoconic pieces weigh 200–300 g, and pear-shaped pieces weigh 500–600 g.

Brin d'Amour or Fleur du Maquis is an artisanal sheep milk herb-encrusted cheese from Corsica. The cheese has a thickly coated rind covered with rosemary, thyme, savory coriander seeds, and juniper berries. It is sometimes garnished with a few tiny red chili peppers for color. This cheese is ripened for up to 2 months. San Simon is a cow's milk cheese from the northern coast of Spain that is smoked with hardwood to a rich walnut color.

Italy

Some Italian cheeses are made with herbs while some are ripened unconventionally under herbs. Examples of those ripened unconventionally under herbs are Casciotta di Urbino (ripened under walnut leaves), Barricato San Martino (ripened under herbs, mainly mint, thyme, or rosemary), Vento d'Estate (ripened under hay), and Ubriaco di Raboso (ripened under wine by-products, i.e., grape marc). The manufacturing protocols for the cheese are given in the flow diagram in **Figure 4**.

Other Italian cheeses with spice include Pecorino pepato, Romano pepato, and Romano/Asiago blends with pepato and less salt. Pepato is a Sicilian word for 'peppery', suggesting that pepato cheeses are made with black peppercorns. Piacentinu Ennese is a unique sheep's milk Sicilian cheese containing saffron (6 g 100 l^{-1} milk), which gives the cheese a bright yellow color and distinct flavor.

United States

There are several cheeses on the US market containing herbs and spices. The most popular are soft cheese (e.g., cream cheese) and soft goat cheese (chevrè). These cheeses are flavored with various herbs and spices including garlic/onion, basil, sundried tomatoes, and chives. Also in the United States, some Cheddar cheese, Monterey Jack, Feta cheeses (made in the United States), cold-pack cheese, and process cheeses are most commonly flavored with herbs and spices.

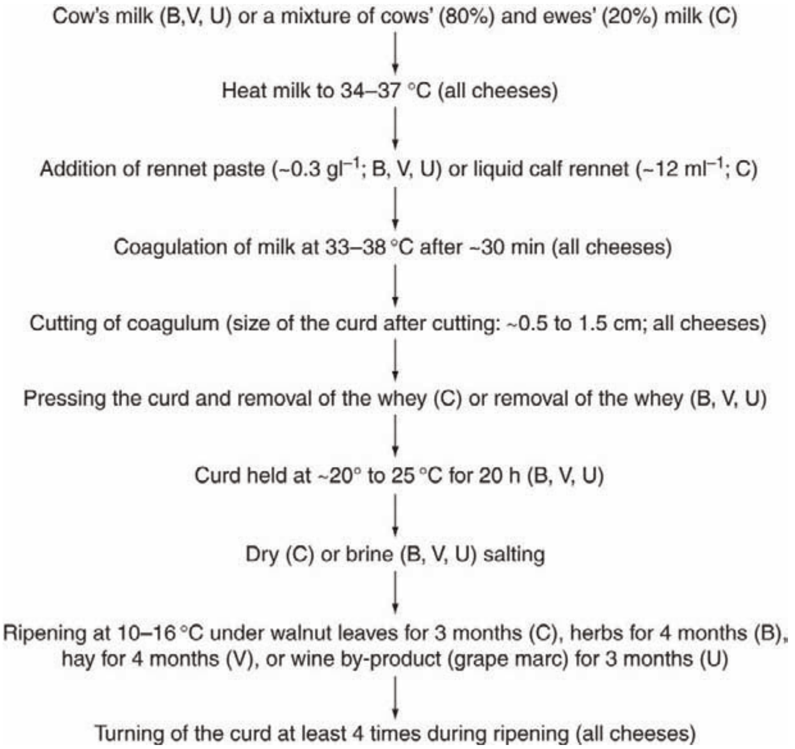
Cow's milk (B,V, U) or a mixture of cows' (80%) and ewes' (20%) milk (C)
Heat milk to 34–37 °C (all cheeses)
Addition of rennet paste (~0.3 gl^{-1}; B, V, U) or liquid calf rennet (~12 ml^{-1}; C)
Coagulation of milk at 33–38 °C after ~30 min (all cheeses)
Cutting of coagulum (size of the curd after cutting: ~0.5 to 1.5 cm; all cheeses)
Pressing the curd and removal of the whey (C) or removal of the whey (B, V, U)
Curd held at ~20° to 25 °C for 20 h (B, V, U)
Dry (C) or brine (B, V, U) salting
Ripening at 10–16 °C under walnut leaves for 3 months (C), herbs for 4 months (B), hay for 4 months (V), or wine by-product (grape marc) for 3 months (U)
Turning of the curd at least 4 times during ripening (all cheeses)

Figure 4 Protocols for the manufacture of four Italian cheeses ripened under walnut leaves, herbs, hay, or wine by-products. C, Casciotta di Urbino; B, Barricato San Martino; V, Vento d'Estate; U, Ubriaco di Raboso. Adapted from Di Cagno R, Buchin S, de Candia S, De Angelis M, Fox PF, and Gobbetti M (2007) Characterization of Italian cheeses ripened under nonconventional conditions. *Journal of Dairy Science* 90: 2689–2704.

Other countries

Bruder Basil, Rauchkase, Sebastian, and Caram are German smoked Trappist-style cheeses. Dambo (also called King Christian and Christian IX) is a popular Danish cheese that occasionally contains caraway seeds. Liptauer is a Hungarian cheese with added onions, caraway seeds, capers, anchovies, and paprika. Nokkelost (also known as Kuminost) is a Norwegian cheese containing caraway seeds, cumin, and cloves. Egyptian Karish and Syrian Shankalish cheeses are soft cheeses made with different types of herbs and spices.

Acknowledgments

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See also: **Cheese**: Cheese as a Food Ingredient; Cheese Flavor; Current Legislation for Cheeses; Overview; Pasteurized Processed Cheese Products; Salting of Cheese.

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Cheeses Matured in Brine

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Introduction

Brined cheese originated in East-Mediterranean counters about 8000–9000 years ago. More than 1000 varieties of cheese are produced around the world. They may be considered as the ancestor of the various types of cheese known today. Brined cheeses are produced under various names in all countries of the region: Feta (Greece); Telemea or Telemes (Romania, Greece); Bjalo salamureno sirene (Bulgaria); Mohant (Slovenia); Sjenicki, Homoljski, Zlatarski (Serbia); Pljevaljski, Polimsko-Vasojevaski, Ulcinjski (Montenegro); Travnicki (Bosnia-Herzegovina); Beyaz peynir (Turkey); Liqvan, Iranian white (Iran); Akawi (Lebanon); Domiati (Egypt), Brinza or Brynza (Russia, Ukraine); Halloumi (Cyprus) and addition to many other names.

Knowledge about brined cheeses remained sparse until recent years. In most cases, brined cheese varieties are still produced on small scales and consumed locally, which may explain the limited information about them. Only Feta cheese has achieved real popularity outside its land of origin. There are several reasons behind the interest in Feta cheese as a multipurpose food. The degree of mechanization that has been used in the manufacture of Feta cheese, particularly ultrafiltration techniques, is one reason for the worldwide manufacture of this cheese variety.

Cheeses matured and preserved in brine (6-12% NaCl), and in most cases without the need for refrigeration. This method of cheese storage has effecting biochemical, texture, structural and flavor changes during cheeses ripening.

Basically, cheeses matured in brine are rindless and vary in moisture content from soft to semihard type. Traditionally, brined cheeses are made mainly from sheep's, goat's, and buffalo's milk. Therefore, they retain the white color of these milks. Some brined cheeses are called white soft cheese. Cheeses matured in brine, which are quoted in different reports, are listed in **Table 1**.

Statistics

It is difficult to obtain an exact breakdown of the worldwide production of cheeses matured in brine. However, the following points are relevant:

1. Based on available data, Feta is the major brined cheese produced worldwide, followed by Domiati cheese. Other brined cheeses are produced in much smaller amounts.
2. A product similar to Feta cheese is produced from cow's milk. The largest producer of this cheese is Denmark, followed by Germany. Denmark produced 123 000 tonnes of this cheese from cow's milk in 1986.
3. Global production of Feta-type cheeses in 2009 is about 1 000 000 tonnes, which is about 7% of total global cheese production. Greece is the main Feta cheese producer from sheep's and goat's milk, followed by France and, to a much lesser degree, Hungary.
4. It is estimated that about 230 000 tonnes of Domiati cheese was produced in Egypt in 1999. The annual growth in Domiati cheese production amounts to 2.3%.
5. White brined cheeses are also widely made in Bulgaria; an estimated 105 000 tonnes of different brined cheeses was produced there in 1989.
6. Based on the combined production of Feta, Domiati, and other varieties, brined cheeses represent ~5% of the total production of cheese in the world.
7. Feta cheese and Feta-like cheeses are the most significant types of brined cheeses exported and marketed in the world. About 125 000 tonnes of cow's milk cheese similar to Feta is exported mainly to the Middle Eastern countries.
8. Feta is the main type of cheese made by ultrafiltration. About 55% of milk processed by ultrafiltration is converted into Feta cheese.
9. About 75% of the Feta cheese consumed in the EU countries is made from sheep's and goat's milk, and the market of this product is growing by 5% annually.

Characteristics

The color of brined cheeses is pure white (porcelain white, marble white, or snow white) when they are made from sheep's, goat's, or buffalo's milk. However, their color ranges from off-white to yellowish when they are made from cow's milk. When cow's milk is used and as the consumers expect a white-colored cheese, in some countries (when permitted) some 'decolorants'

Table 1 Types of cheese matured in brine

Cheese	*Milk used*
Akiavi	Sheep, goat
Bajalo (Belo Salamurene Sirene)	Sheep, cow
Bli-Sir-O-Kriskana	Sheep
Brinza	Cow
Chenakh	Sheep
Dani	Sheep
Domiati	Buffalo, cow, recombined
Feta	Sheep, sheep/goat
Halloumi	Sheep, goat
Medaffara/Magdola	Sheep, buffalo, cow, recombined
Mish	Skim milk, buffalo, cow
Shenkalish	Sheep
Teleme	Sheep, buffalo, cow
White pickled	Sheep, cow
UF-Domiati	Cow, recombined
UF-Feta	Cow, recombined

UF, ultrafiltration.

(e.g., chlorophylls, titanium dioxide) or other treatments are used to cover or eliminate the yellowish color.

Brined cheeses have no rind, and no gas holes or other openings should be present in the cheese mass, except for the small mechanical openings; the presence of small or large gas holes is taken as a defect. The texture of brined cheeses is smooth, soft, and crumbly but still sliceable, and some of them may become brittle when old.

The shape of brined cheeses is associated with that of the container, and usually cheese blocks are rectangular or cubic (250–1000 g). The cheeses are packed in containers of various sizes. The most common are rectangular tinned or lacquered metal or plastic containers holding 15–18 kg each. The containers are usually filled with brine, but in some countries (especially in small artisanal units), cheese whey containing 8–12% salt is used for filling. An old traditional practice, which still survives in some countries, was to mature and keep the cheeses in wooden barrels holding about 40–50 kg. In that case, the cheese blocks have a sphenoid shape. For retail marketing, cheese blocks (500–1000 g) may also be packed after ripening in plastic bags under vacuum without brine or in plastic containers with brine, which keeps the freshness of the cheese.

Technology

The increased demand for cheeses matured in brine, particularly Feta cheese, has created the need for standardized techniques for their manufacture. Therefore, Feta cheese, for example, is manufactured nowadays by traditional, standardized traditional, and mechanized techniques. The technology of Feta and Domiati cheese should represent the major types of this group and have undergone much development, and Halloumi cheese, which is unique in brine-salted cheeses, is described in this section.

Traditional Feta cheese is produced mainly in the mountainous regions of Greece from mixtures of sheep's milk and cow's and/or goat's milk. The milk is heated to 32–34 °C and traditional rennet extract from lamb's/kid's abomasa is added to give a coagulum ready for cutting after 50 min. The coagulum is then cut into cubes, left for 5–10 min in whey, and then transferred gradually into circular molds, placed on an inclined table, and turned occasionally to facilitate whey drainage. The curd is then removed from the molds, cut into slices, and rubbed with granulated salt. The cheeses are left until a slimy layer has formed (1–2 weeks); then the surfaces are cleaned and the cheeses placed into barrels, leaving no space between slices. Brine of 6–8% (w/w) NaCl is added and the barrels are closed. The cheese slices remain in ripening rooms until their pH reaches 4.4–4.6 and are then transferred to cold stores at 3–4 °C.

The traditional method for making Feta cheese has been modified and standardized for large-scale production. The main modifications are as follows:

1. Milk is heated to 68–70 °C and then cooled to renneting temperature (32–34 °C).
2. A mixture of commercial (calf) and traditional (lambs/kids) rennets (3:1) is used.
3. The curd–whey mixture is transferred by gravity from the vat to rectangular molds placed on a moving belt. The molds are left on the moving belt for 2 h for drainage, and then placed on a table for another 4 h to complete draining. The curd is removed from the molds, cut into cubes, and salted with granular salt, and the next day it is transferred to tins, the bottom of which is covered with salt. Salt is sprinkled between cheese layers. The cheeses are stored at 14–16 °C for 4–5 days, and the released whey is then removed. The tins are closed, kept at 14–16 °C for 2 weeks, filled with brine (6–8% NaCl), and then transferred to cold stores.

Several technologies have been developed for mechanization of Feta cheese manufacture. However, the most important is that based on the use of milk concentrated by ultrafiltration (UF). In this respect, two versions of Feta-like cheese have been developed: cast and structured. For cast Feta, cheese milk is concentrated by UF to give a retentate with the chemical composition close to that of the final cheese, whereas for the structured version, milk is partially concentrated to allow for whey drainage from the curd.

Cast Feta

The manufacture of cast Feta cheese in tins can be summarized as follows: cow's milk is standardized to 3.5% fat, pasteurized, homogenized (optional), concentrated (fivefold) by UF, heated (80 °C × 1–2 min), homogenized

(12 MPa), and cooled to coagulation temperature. Starter and rennet are dosed continuously and mixed with the retentate before filling into tins on a moving belt. The tins return to the filling station after 30 min, during which coagulation takes place. Another portion of retentate, starter, and rennet are added to the tins, and when coagulated, a third portion is added. Each of the three layers of coagulum is cut into nine cubes using a special cutting device. The top layer is covered with paper; salt is sprinkled on top; and the tins are closed, inverted, left for 3 days, and then transferred to a cooled room. Cast Feta cheese can also be made in Tetra bricks: retentate is prepared as before; salt is added at a level of 3–4%; and the salted retentate is dosed with rennet and an acidulent (usually glucono-δ-lactone) or culture, and packed into formed tetra bricks (200–500 g) using a Tetra pack filling machine.

Structured Feta

Structured Feta cheese is made from standardized cow's milk concentrated about threefold by UF. The retentate is heated to 90 °C for 1 min; homogenized (5 MPa); cooled to 30 °C; and then mixed with the starter, coagulant, decolorizing agent, and $CaCl_2$ using dosing devices and an in-line static mixer. The retentate is then pumped to a continuous cheese coagulator/cutting device, 'Alcurd', which achieves a continuous coagulation and cutting of the coagulum into uniform cubes. The curd cubes are then delivered to the molding system. Several systems are available for continuous molding and draining to remove the required quantity of whey followed by fusion of curd particles. The cheese is allowed to ripen for up to 24 h to develop the desired pH, cut into blocks, filled in tins (l4–16 kg per tin), and covered with brine (4–6 kg). The brine concentration is adjusted to give 3–4.5% salt in the mature cheese. The tins are sealed using the double-seam closure technique.

Domiati Cheese

Domiati cheese is unique in two aspects:

1. Salt is added directly to milk prior to coagulation; large amounts of salt are normally used as compared to all other cheese varieties.
2. It can be consumed fresh or after maturation in brine for different periods; ripening in brine can take place either at room temperature (Istamboli cheese) or at about 10 °C (Baramili cheese).

Traditionally, Domiati cheese is made from buffalo's milk or from variable mixtures of buffalo's and cow's milks. One-third of the milk is heated to 80 °C and mixed with the remainder of the milk to which salt (8–14%) is added. The mixture, which has a temperature of about 38 °C, is renneted using nearly double the quantity of calf rennet normally used for other cheese varieties; coagulation occurs in 2–3 h. The coagulum is then scooped into wooden frames lined with cheesecloth and placed on an inclined table to facilitate whey drainage. The frames vary in size. The curds are then pressed lightly, and whey drainage is allowed to continue for 12–24 h. The blocks of curd are cut into cubes (about 500 g) and wrapped in paper for cheese to be pickled in brine. The cheese blocks (14–16 kg) are packaged into 20 kg tins and covered with salted whey obtained from the same batch. The tins are soldered and held for 4–8 months at ambient temperature or at 10 °C for maturation. For large-scale production, milk is pasteurized before cheese is manufactured following almost the same steps of the traditional method.

Domiati cheese has also been made from milk concentrated by UF. In this case, cow's milk is usually used. Milk is standardized, pasteurized, homogenized, and then concentrated 4.2-fold by UF. The retentate is then heated (80 °C for 1–2 min), homogenized (6 MPa), salted (3–5% NaCl), renneted, poured into trays lined with a plastic sheet, and left to coagulate at 40 °C. The coagulum is then removed from the trays, cut into cubes, packaged in tins, covered with brine (5%), and stored in a cooled room.

Halloumi Cheese

Halloumi is a semihard cheese made from sheep's milk and preserved in brine. The technology for the manufacture of Halloumi cheese is unique. It can be considered as a hybrid between brined and *pasta filata* cheese varieties. The milk (pasteurized in case of large-scale production) is standardized and coagulated; the coagulum is cut into small cubes (1 cm^3), stirred gently in whey, and scalded; the whey is drained and the curd is pressed and cut into pieces. The curd pieces are cooked in deproteinized whey obtained by mixing whey with 10% milk, heated at 90–92 °C, and the precipitated curd is removed by filtration through a cheesecloth. The cooked pieces are removed, salted, and garnished with dried mint (*Mentha viridis*) leaves; they are either packaged the next day in cousumer packages or preserved in brine till consumption.

Composition of Brined Cheeses

The variety of milks and technologies used for their manufacture, together with the existing official specifications for each cheese in each country, does not permit to give an average composition of brined cheeses that can be quoted. Roughly, their moisture varies between 50 and 58%, protein is higher than 17%, fat-in-dry matter is 45–50%, and salt-in-moisture is 5.5–9%. The pH value of most brined cheeses is 4.2–4.8.

Microbiology of Brined Ripened Cheese

For centuries, cheeses matured in brine were manufactured from raw milk without the addition of starter. Studies on the microbiology of cheeses matured in brine, though rather limited in number, show that the microflora is composed predominantly of microorganisms able to grow in the presence of 4% or more NaCl.

Enterococci have been isolated in high numbers, and often represent the predominant microorganisms in the ripened cheese. *Enterococcus faecium*, *Enterococcus durans*, and *Enterococcus faecalis* are the most commonly isolated species. *Lactococcus lactis* subsp. *lactis* and citrate-positive *Lc. lactis* subsp. *lactis* are the predominant lactococci, and *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* are the predominant lactobacilli. The presence of *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Streptococcus thermophilus* has also been reported.

As far as the nonlactic flora of cheeses matured in brine is concerned, the presence of *Micrococcus* and coliforms, which usually leads to spoilage, has been reported. Many genera of molds and yeasts are commonly found, and cases of mycotoxin production have been described. Pathogenic microorganisms such as *Salmonella* and *Corynebacterium* have been isolated during the first 15–30 days of ripening. The presence of *Clostridium perfringens*, *Bacillus cereus*, and *Listeria monocytogenes* in cheeses matured in brine has also been reported.

As a general rule, NaCl is the main factor controlling the types and numbers of microorganisms present in cheeses matured in brine. Therefore, the concentration of NaCl plays a crucial role in the safety of the cheese and in the metabolic pathways leading to flavor development.

Several attempts have been made to select an appropriate starter combination for cheeses matured in brine; most of the work has been on Feta cheese, with some attempts also on Teleme, Brinza, Halloumi, Domiati, and Turkish varieties. A wide variety of microorganisms have been used, including *Lc. lactis* subsp. *lactis*, *Lb. casei*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Ec. durans*, *Lactobacillus cremoris*, *Lb. delbruckii* subsp. *bulgaricus*, *Lb. lactis*, *Lb. lactis* biovar *diacetylactis*, *Lactobacillus helveticus*, *Streptococcus* spp., *Lc. lactis* subsp. *cremoris*, *Sc. thermophilus*, *Pediococcus pentosaceus*, *Ec. faecalis*, *Leuconostoc* spp., *Lb. plantarum*, *Lb. caucasicus*, and *Ec. faecalis* biovar. *liquefaciens*.

Only in the recent years and in a very limited number of cheeses matured in brine have starters been used; industrial Feta is manufactured using blends of yogurt starter: *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Yogurt microorganisms are also used in Teleme cheesemaking. For Domiati, it seems that *Ec. faecium* and a combination of mesophilic and thermophilic lactobacilli lead to the development of the characteristic flavor of the cheese.

Biochemistry of Ripening and Flavor Compounds

The biochemical changes in brined cheeses are not extensive during ripening owing to their high salt content, low pH during pickling, and short duration of ripening. The proteolysis index ((water-soluble N/total N) × 100) in most of the brined cheeses ranges from 10 to 25%, and the level of total free amino acids ranges from 1 to 7 g kg^{-1} cheese. It should be noted that, during ripening and storage in brine, some water-soluble compounds (mainly small peptides and amino acids) selectively migrate into the brine.

The lack of curd cooking, high moisture content, the low pH during drainage, which favors coagulant retention in the curd, the low pH of the cheese, and the relatively high salt content favor the activity of chymosin during ripening, contrary to that of plasmin. In brined cheeses, α_S-casein is hydrolyzed much faster than β-casein.

Lipolysis in brined cheeses is not very extensive. The level of total free fatty acids (FFAs) ranges between 2 and 4 g kg^{-1} cheese, including acetic acid. Generally, the concentration of volatile fatty acids is higher in cheeses made from sheep's or goat's milk than in those from cow's milk. Volatile free fatty acids (VFFAs, C_2–C_8) are considered essential for the flavor of cheeses matured in brine. Piquant Feta cheese is characterized by higher levels of free C_4, C_6, and C_8 than the nonpiquant Feta cheese, while the level of acetic acid is nearly the same. Similarly, the content of VFFAs in Domiati cheese increases with advanced storage. Acetic acid is the predominant VFFA in Domiati cheese and constitutes 80–90% of the total VFFAs. The VFFAs of cheeses matured in brine arise through several pathways:

1. Lipolysis of cheese fat by pregastric lipases from rennet paste used in the manufacture of traditional Feta cheese by pregastric lipases added with rennet in industrially produced cheese. Milk lipase and lipases from the cheese microflora may contribute to lipolysis in brined cheeses.
2. Fermentation of lactose during the early stages of ripening results in the production of significant quantities of acetic acid.
3. Deamination of free amino acids that arise from proteolysis; the cheese microflora is responsible for these metabolic changes.

The type and quality of milk, the heat treatment received, the starter used, ripening and storage temperature, and brine concentration are the main factors that contribute to the concentration of flavor compounds in Feta and other white brined cheeses.

In order to get a proper and balanced flavor development in Feta cheese, it is necessary that lipolysis occurs at the same time as the modification of other milk constituents, particularly proteins.

Headspace analysis of Feta cheese reveals many volatile compounds, including relatively large quantities of ethanol, butan-2-ol, and butan-2-one, which very probably contribute to Feta flavor. Although acetaldehyde is present in Feta cheese, it is not considered crucial for its flavor as it increases to a maximum at 4 months of ripening and then decreases to a minimum after 8 months.

Fifty compounds can be found in the volatile flavor fraction of mature Domiati cheese. Some compounds form and increase in quantity during ripening, such as acrolein, butan-2-one, propan-1-ol, butan-2-ol, ethyl propionate, ethyl butyrate, propyl propionate, and propyl butyrate. Domiati cheese of inferior quality is characterized by high amounts of sulfur compounds, namely, dimethyl sulfide, methylthiopropionate, methylthiobutyrate, and dimethyl trisulfide.

Texture and Structure

Information on the texture and structure of cheeses matured in brine is limited, especially for traditional and lesser-known varieties. As a new and successful version, the texture of UF-Feta cheese has received much attention. The use of high levels of rennet in the manufacture of UF-Feta leads to a firmer and less adhesive cheese. This is attributed to an increase in the rate of aggregation of altered casein micelles, leading to a denser network structure and a firmer, grittier texture. Also, the rheological properties of UF-Feta are affected by the level of proteolysis during ripening. The stress at fracture, deformability modulus, and work up to fracture decrease during ripening, which can be attributed entirely to proteolysis. However, shear measurements are not very useful for predicting sensory textural properties. Salt has a significant effect on the rheological properties of Feta cheese; a high percentage of salt results in a harder texture, a higher pH, and a lower moisture content.

Based on sensory evaluation, traditional Teleme cheese is harder, more grainy, and more brittle than UF-Teleme.

Domiati is unique among cheese varieties in the addition of large quantities of NaCl to milk before renneting. The pH of pickled Domiati cheese is close to the isoelectric point of casein, and hence its colloidal calcium phosphate (CCP) is partially solubilized, which causes shrinkage of the cheese matrix and exudation of cheese serum into the pickle. Solubilization of calcium phosphate during cheesemaking occurs as a function of NaCl added or pH reduction; as a result, the CCP dissociates from the casein micelle. The swelling, hydration, and solubility of casein micelles in cheese matrix are greatly increased in the presence of NaCl.

The textural characteristics of UF and traditional Domiati cheese are significantly different. Fresh UF-Domiati cheese is firmer and more adhesive than conventional cheese, which may be attributed to the high retention of calcium in UF-cheese curd and to the greater extent of curd firming in UF-cheese. On the other hand, conventional Domiati cheese is chewy and gummy. These differences can also be observed by sensory evaluation. The method of packaging affects the textural attributes of UF-Domiati cheese. Ripening in brine leads to less firmness, chewiness, and gumminess as compared with cheese ripened in pouches.

The ultrastructure of Domiati cheese is composed of a framework of spherical casein aggregates held by bridges and enclosing fat. The internal structure of the cheese is affected by the changes that occur during ripening. Most of these changes occur in the protein matrix, as casein aggregates dissociate into smaller spherical particles, forming a loose structure. The fat globules in the cheese undergo slight lypolysis; otherwise, they are unlikely to change during storage.

See also: **Cheese**: Overview; Salting of Cheese. **Microorganisms Associated with Milk**.

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Accelerated Cheese Ripening

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Introduction

Cheese ripening is the term used to describe the process whereby changes occur in the cheese resulting in the development of flavor, texture, and aroma in the matured product. Cheese ripening is initiated by the addition of a starter culture and coagulant to milk. The starter lactic acid bacteria convert lactose into lactic acid, reducing the pH at which biochemical reactions occur during cheese ripening. Enzymes released from the bacteria are also involved in the degradation of proteins into peptides and amino acids, and the breakdown of fatty acids released by lipolysis into keto acids, ketones, and esters. These breakdown products are important in the development of flavor, aroma, and texture. Special characteristics can also develop in cheese during ripening, for example, the blue veins in Roquefort, the holes (eyes) in Emmental, the red smear on Limburger, and the white mold on Brie. Temperature and humidity are carefully controlled to promote the development of the desirable microbial flora and the secretion of the enzymes responsible for the biochemical changes taking place during ripening. The ripening time varies from 4 weeks to 28 months depending on the variety of cheese (**Table 1**).

Ripening of cheese is a slow and expensive process not fully predictable or controllable. It has been reported that the ripening time of Cheddar cheese adds significantly to the cost of the product, ranging from 1.5 to 3% per month. The development of an efficient way to reduce the aging time would allow significant savings to the cheese industry.

It was only in the early 1950s that the economics of cheesemaking led dairy researchers to look for an appropriate method to reduce the maturation time for cheese without altering the characteristic flavor of the final product. Methods have been described during the last 50 years to achieve this goal.

The aim of the present article is not to provide an exhaustive review of the literature on accelerated ripening but rather to discuss the different technologies available and to evaluate the most promising ones.

Strategies Used for the Enhancement of Cheese Flavor

Elevated Ripening Temperature

Cheese was traditionally ripened in caves at a temperature varying from 15 to 20 °C. Today, ripening takes place in special rooms under controlled temperature and humidity, which vary according to the cheese type. Camembert, Blue cheese, and surface-ripened cheeses are kept at 10–15 °C, and Gouda and Edam are usually ripened at 10–14 °C, whereas a relatively low temperature is used for Cheddar (6–8 °C). Several attempts have been made to accelerate Cheddar cheese ripening by increasing the temperature to 13–16 °C, which led to a 50% increase in the rate of flavor formation.

Increasing the ripening temperature may offer to the industry a technologically simple method by which to speed up flavor-forming reactions in a cheese system, and the lower refrigeration costs may provide an overall saving to the producer. However, attention should be directed to the quality of the milk and the hygienic conditions used for cheese production to avoid flavor defects and the growth of pathogens.

Slurry Systems

The basis of cheese slurries production is the utilization of specific enzymes, under optimum conditions, to produce intense cheese flavors rapidly in cheese curd. The technology was developed in the late 1960s, where fresh curd and a NaCl solution were mixed to make an emulsion of approximately 40% solids. Enzymes and other additives, including trace elements and reduced glutathione, which promotes microbial growth, are added and the mixture is incubated under anaerobic and controlled conditions at 30 °C for 4–5 days with agitation, after which the characteristic strong cheese flavor is developed.

Cheese slurries are used in a wide range of products, for example, processed cheese formulations, snacks, crackers, and imitation dairy products. They are also an ingredient in the production of enzyme-modified cheeses

Table 1 Ripening time for different varieties of cheese

Cheese variety	*Ripening time (months)*
Cheddar-type cheese	6–12
Swiss cheese varieties	6–12
Blue cheese varieties	3–4
Parmigiano	24–28
Provolone	10–12
Gouda	1–2
Ras	3–4

(**Cheese**: Enzyme-Modified Cheese). The major disadvantage of this technology is the difficulty of controlling the process. Contamination due to the relatively high incubation temperature is also likely to occur.

Addition of Enzymes

Flavor formation in cheese results from the action of different agents involved in the ripening process. A great deal of attention has, therefore, been given to the different groups of enzymes that could play a role during ripening. Exogenous enzymes are relatively cheap, have specific action, and give a choice of flavor options. Proteinases, peptidases, lipases, and esterases from various sources have been added individually or in combinations to cheesemilk or to the cheese curd to speed up the ripening process. Enzymes extracted from cheese-related microorganisms have also been considered. Lipases are used commercially in cheese varieties known for their characteristic piquant flavor, for example, Provolone, Caciocavallo, some Blue-veined cheeses, Ras cheese, and Feta made by ultrafiltration. Very little attention was directed toward enzymes involved in the metabolism of carbohydrates, and it has been limited to β-galactosidase, for which the data obtained were inconclusive.

The addition of commercial proteinase preparations led in most cases to a reduction in yield and development of a bitter flavor, and a cheese with a softer body was also reported. The development of bitter flavor was considerably reduced after the addition of proteinase–peptidase mixtures.

The major limitations in the use of exogenous enzymes are related to the method of enzyme addition. When enzymes are added to milk, only a very small portion is retained in the curd, which increases costs. Reduction in cheese yield and flavor defects from proteolysis during manufacture and in the early stages of maturation, as well as whey contamination with the added enzyme, are major obstacles in the application of this technology. The addition of enzymes to the curd is efficient in the case of Cheddar-type cheeses, which enable the addition of enzymes with the salt during curd milling. Hot spots are often noticed owing to unequal distribution of the exogenous enzymes.

Entrapped Enzymes

To overcome the problems arising from the addition of free enzymes, three methods for enzyme entrapment have been evaluated. In the first method, cell-free extracts from bacteria or whole bacterial cells, with their appropriate substrates, were encapsulated in milk fat capsules, which led to the release of the reaction product in the cheese matrix. The applications described included the generation of acetyl methyl carbinol, the generation of 3-methylbutanal and 3-methylbutanol from leucine, and, finally, a system where *Penicillium* spores and milk fat led to the generation of methyl ketones. This approach could be applied for the flavor enhancement of low-fat cheeses and for the production of a flavor-enhanced cheese to be used in the snack food industry. Milk fat, however, is unstable at curd-cooking temperatures owing to its low melting point (33 °C) and hence is unsuitable for application in Cheddar-type cheeses.

In the second method, proteinases were entrapped in phospholipid vesicles (liposomes) to protect the milk proteins from the action of the enzymes during the cheesemaking process, which should limit the development of bitter flavor and weight losses. Liposome technology is a scientifically attractive method widely used in the pharmaceutical industry, but its use is limited in the cheese industry. The reasons include expensive ingredients, use of materials for liposome production that are not generally regarded as safe and edible, lack of suitable methods for large-scale production, and low encapsulation efficiency of liposomes.

In the third method, proteinases were encapsulated in κ-carrageenan or gellan. The enzyme capsules were incorporated into milk during cheese manufacture. κ-Carrageenan and gellan capsules showed higher retention than shown by milk fat capsules. Enzyme losses from gum gel capsules were also lower for gellan and κ-carrageenan gums.

Adjunct Cultures

The development of procedures leading to a reduction in the number of microorganisms in milk at the farm level resulted in a dramatic decrease in the number of non-starter bacteria known for their positive role in cheese flavor development. Adjunct cultures are non-starter lactic acid bacteria, mainly lactobacilli, which are selected for their enzyme potential and autolytic properties, and are added to cheesemilk to supplement the microflora and enhance cheese flavor and texture.

Two different approaches are followed:

1. Viable cells may be used when the emphasis is on metabolites produced by the living cells during ripening.
2. Weakened (attenuated) cells may be used, which increase the pool of bacterial enzymes in the cheese matrix without altering the rate of acid production during cheesemaking.

Different means for attenuating the cells have been described in the literature. In heat shocking, a bacterial cell suspension is subjected to a sublethal heat treatment. A critical point in heat attenuation is to define the correct temperature–time combination, so that acidification is eliminated or delayed without significant loss of activity of potential ripening enzymes. The conditions used for heat shock treatment of various microorganisms are summarized in **Table 2**. The attenuated cells are then added to cheesemilk along with the primary starter culture. *Lactobacillus helveticus* is the bacterial species most often subjected to heat treatment. An increase in proteolysis and the level of amino acids in cheese, as well as a decrease in bitterness and an enhancement of cheese flavor, has resulted from the addition of heat-shocked cells to cheese.

In freeze-shocking, whole cells washed and concentrated 10-fold are subjected to freezing overnight or longer (**Table 2**), followed by thawing at 40 °C prior to addition to milk.

The addition of freeze-shocked cells to cheese significantly increases proteolysis and amino acid formation due to cell lysis and the release of intracellular peptidase into the cheese. Attenuation by freezing is easier to achieve than attenuation by heat. Freeze-shocked *Lb. helveticus*-treated cheeses received higher flavor and aroma scores as compared to heat-shocked *Lb. helveticus*-treated cheese.

Spray-drying and freeze-drying have also been used to attenuate bacterial cells; however, the results are not conclusive because of the rather limited number of studies carried out.

Mutants unable to ferment lactose also provide an effective means for increasing enzyme potential in cheese without interfering with the rate of acid production during cheesemaking. Reduced bitterness and accelerated flavor formation were observed in Gouda and Cheddar cheese made with Lac^{-} mutants. Another attenuation method involves treatment of bacterial cells with lysozyme, which does not seem to be effective because of the difference in the sensitivity of lactic acid bacteria to lysozyme. The relatively high price of lysozyme represents another obstacle. The addition of organic solvents, such as butanol, has been reported to attenuate bacterial cells. A major drawback of organic solvents is the health concerns posed by the residual solvent in the cheese.

A critical analysis of the advantages and disadvantages of viable versus nonviable cells led to the conclusion that a combination of both is required to obtain the balanced flavor bouquet that characterizes each type of cheese.

Information on accelerated ripening of cheese is also available in patents describing the use of pulsed electric fields (Japanese patent N. 63-502877 (502877/1988)) or subjecting the cheese to a high pressure ranging from 100 to 2500 kg cm^{-2} (US Patent 5, 180, 596/1993).

Higher levels of cell lysis were apparent in cheese manufactured using high-pressure-treated strains than in the controls. Overall, the addition of high-pressure-treated starter bacteria as a secondary starter culture accelerated proteolysis in Cheddar cheese. However, the impact of such methods on flavor development has not been described.

Genetically Modified Lactic Cultures

Genetically modified (GM) versions of common cheese bacteria have been used by researchers for the past 25 years in controlled laboratory experiments to control the activity of dairy lactic acid bacteria, including acid production, protein utilization, bacteriophage resistance, extracellular polysaccharide synthesis, and flavor/aroma metabolism.

The knowledge base is now so advanced that many genetically modified lactic acid bacteria (GM LAB) have been constructed using food-grade gene vectors and markers, and then made available for contained trials in

Table 2 Conditions used to attenuate different lactic cultures

	Treatment for physical attenuation	
Strain	*Temperature (°C)*	*Time*
Lactobacillus helveticus	67	15 s
Lactobacillus delbrueckii subsp. *bulgaricus*	63	20 s
Lactobacillus casei	67	22 s
Lactobacillus plantarum	50	15 s
Lactobacillus helveticus	−24	24 h
Lactobacillus casei	−20	20 h
Lactobacillus delbrueckii subsp. *lactis*	−20	20 h

experimental cheese plants. GM LAB that can be applied to accelerate cheese ripening have been altered with proteinase specificity, peptidase balance, and/or lytic properties in cheese.

Future Perspectives

A critical analysis of the different methods used to reduce the ripening time for cheese indicates that addition of exogenous enzymes can be used only in the case of cheese that will be used in a processed cheese blend or in the snack food industry, where the severe heat treatment will inactivate the enzymes.

Acceleration of the ripening of conventional cheese can be achieved through the selection and/or improvement of cheese-related microorganisms. Isolation of microorganisms from their natural habitat and their selection based on the type of flavor required should make available to the starter industry a wide range of microorganisms that should increase the range of available adjunct cultures. Genetic engineering techniques should also contribute to the construction of new cultures that possess special characteristics not available in the strains presently available: secretion of more active proteinases and/or lipases, ability to lyse under specific pH or temperature conditions, or cultures possessing combined pathways leading to the formation of different flavor components.

Advancements in the area of accelerated cheese ripening should lead to the selection and/or engineering of cultures showing variable enzyme profiles, which may lead to the creation of cheese with new flavor characteristics; such cultures can have a significant impact on the flavor of low-fat cheeses.

See also: **Cheese**: Biochemistry of Cheese Ripening; Enzyme-Modified Cheese; Overview; Starter Cultures: General Aspects.

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Enzyme-Modified Cheese

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Introduction

Enzyme-modified cheeses (EMCs) are concentrated cheese flavors produced enzymatically from cheeses of various ages and also from blends of casein, whey powder, skim milk powder, and butterfat. EMCs are used principally as a cheese flavor ingredient in a wide range of food products, providing a cost-effective alternative to natural cheese. EMCs can be used as the sole source of cheese flavor in a product, to intensify an existing cheese taste, or to impart a specific cheese character to a more bland product. EMCs are available in a wide range of flavors, corresponding to a number of natural cheese varieties, the most important of which are Cheddar, Swiss, Blue, and Parmesan.

EMCs are consumed exclusively as a flavor ingredient in a food product, whereas natural cheese is consumed for its flavor, texture, appearance, and functional properties. The main applications of EMCs are in flavoring of processed cheese, analogue cheese, imitation cheese, cheese spreads, snack foods, soups, sauces, biscuits, dips, and pet foods. The advantages of EMCs over other sources of cheese flavor are a high degree of flavor intensity, availability of a diverse range of flavors, reduced production costs, and extended shelf life. The flavor intensity of EMC enables them to be added to foods at very low levels (typically 0.1% (w/w)) to achieve the desired impact, and consequently, EMCs are particularly suitable for inclusion in low-fat foods, a sector that has seen rapid growth in recent years. Increased demand for convenience foods that include cheese flavor in the form of EMCs has stimulated the development of customized EMCs targeted at specific end uses and market segments.

Usage of Enzyme-Modified Cheeses

The actual market size for EMC has not been disclosed in the literature; however, it would appear reasonable to assume that their production has expanded in line with the increased demand for low-fat, highly flavored, convenience foods. These convenience foods include frozen, semiprepared, and ready-to-eat meals, which typically use EMC to intensify cheese flavor and aroma, particularly in a sauce layer. Addition levels of EMC of the order of ~1% (w/w) have been reported for a number of applications, for example, Romano EMC included in a tomato-based sauce, a Swiss-type EMC added to a frozen cheese sauce, or Cheddar EMC included in a Cheddar flavored soup. Higher inclusion levels of ~2.5% (w/w) have been reported for the application of Parmesan or Romano EMC in pizza sauce formulations. Addition of 5% (w/w) of EMC pastes with differing levels of lipolysis to bland imitation cheese bases with a fat content of 24% modified both the free fatty acid (FFA) profiles and the sensory qualities of the final products. Optimal sensory qualities were reported when EMC with a medium level of lipolysis (28% total FFAs) was added to an imitation cheese base at pH 5.5. Addition of EMC at similar rates to medium- (13%) or low-fat (2%) imitation cheese bases containing added fiber indicated that EMCs with the highest level of lipolysis provided a 'mature' cheese flavor note to the medium-fat products.

EMC powders have also been added to natural Cheddar cheese in an attempt to accelerate the ripening process. The rationale behind this approach is that EMCs may act as a source of rate-limiting flavor compounds or flavor precursors, thus alleviating bottlenecks in the flavor generation pathway during ripening. For this particular application, Cheddar EMC powder was added at 0.25 or $1\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ cheese curd at the salting stage of cheese manufacture. Overall, addition of EMC powder did not affect primary proteolysis patterns. However, secondary proteolysis patterns were enhanced in experimental cheeses after 2 months of ripening. Addition of EMC powder increased the levels of free amino acids (FAAs) or individual FFAs. Sensory analysis suggested an acceleration of 2 months in flavor development over controls. This application of EMCs may also be useful for production of intensely flavored 'ingredient type' cheeses.

Combinations of EMC types (Romano, Parmesan, or Cheddar) may also be used at an overall dosage level of up to ~10% to give the correct flavor and aroma in particular product applications such as cheese dips. It is obvious that individual manufacturers have developed dosages of EMC for particular applications that may vary widely from any published or recommended levels, but because of commercial sensitivity remain confidential. Obviously, any proposed dosage rate for EMC inclusion into a product must take account of a number of factors including the processing regime (time and temperature), desired degree of cheese flavor and aroma in the final product, and the flavor intensity of the EMC itself. A novel, emerging application of EMCs is as a source of bioactive peptides; this possibility arises from the extensive enzymatic hydrolysis of milk proteins, which is a normal feature of EMC production. EMCs have been shown to contain several biologically active peptides including two angiotensin I-converting enzyme (ACE)-inhibitory peptides. These peptides were subsequently demonstrated to have antihypertensive effects in rats. Obviously, the ability to generate bioactive peptides depends greatly on the choice of enzymes used in the production of EMC; however, this ability could spur on further development of EMCs as functional food ingredients with antihypertensive properties.

Technology of Enzyme-Modified Cheese Manufacture

The basis of EMC production involves the addition of exogenous enzymes to cheese curd under controlled conditions to generate intense cheese flavors rapidly. The general production process involves incubating cheese with exogenous enzymes and/or microorganisms for short periods (24–72 h) at moderately high temperatures (30–45 °C) and then terminating the reaction by heating (70–85 °C) after the desired flavor intensity has been achieved. EMCs are produced by two methods: an integrated single-step operation in which lipolysis and proteolysis occur simultaneously and a component approach where several different flavor fractions are created separately and then blended to a final specification (**Figures 1** and **2**). It should be noted that the component approach allows greater flexibility enabling a greater diversity of products. However, the choice of manufacturing process depends on the process economics, substrates and equipment available, and the final range of products desired.

The first stage in EMC manufacture is the inactivation of enzymes in the substrate prior to the enzyme reaction, generally achieved by heat treatment of the curd, and has the added benefit of allowing a higher degree of process control. Consistency and quality of the curd substrate are critical for flavor development and, in general, most EMCs are produced from an immature cheese of the same type to give an authentic flavor. Of particular importance in the production of EMC is the emulsification of the curd substrate to a semisolid paste by heating, shearing, and the use of emulsifying salts. This step generally occurs in tandem with the heat inactivation of the substrate and is carried out to provide optimal conditions for enzyme–substrate interactions, thereby accelerating the flavor formation process. Stable emulsions for EMC manufacture are obtained by the addition of emulsifying salts, which solubilize the protein by chelating calcium. The choice of emulsifying salts is important, as each has characteristics that govern emulsion stability. The commonly available emulsifying salts are citrates, orthophosphates, pyrophosphates, and polyphosphates; these salts can also be used to control the pH, and some have additional bacteriostatic effects. Prevention of bacterial or fungal contamination is important in producing a consistent EMC product, and potassium sorbate, nitrates, and nisin are widely included in the formulation to prevent the growth of yeasts, molds, and bacteria.

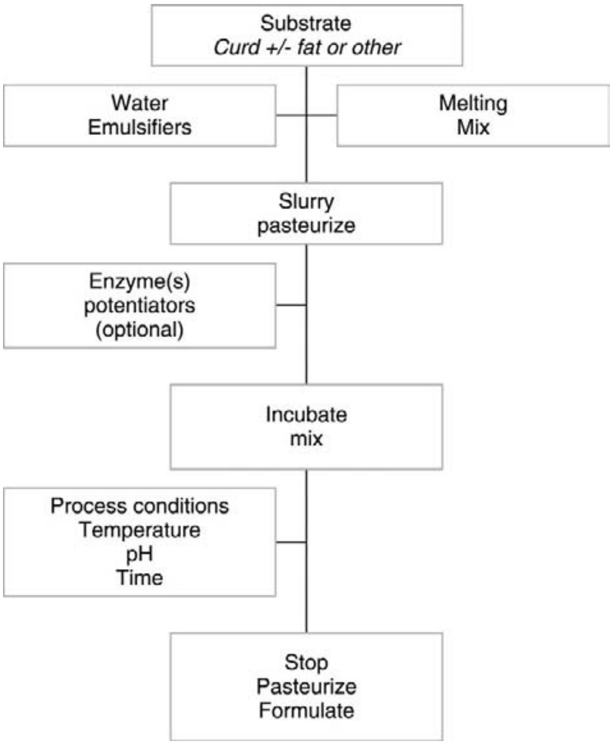

Figure 1 Enzyme-modified cheese – one-step manufacturing approach.

The process parameter values adopted for the enzymatic hydrolysis of the EMC substrate (e.g., pH, temperature, agitation, aeration, and incubation time) depend upon intensity of the cheese flavor required, nature and composition of the substrate, and specificity and dosage of the enzymes or cultures. After hydrolysis, a final heat treatment is given to the EMC to inactivate enzymatic reactions; this treatment is critical in ensuring

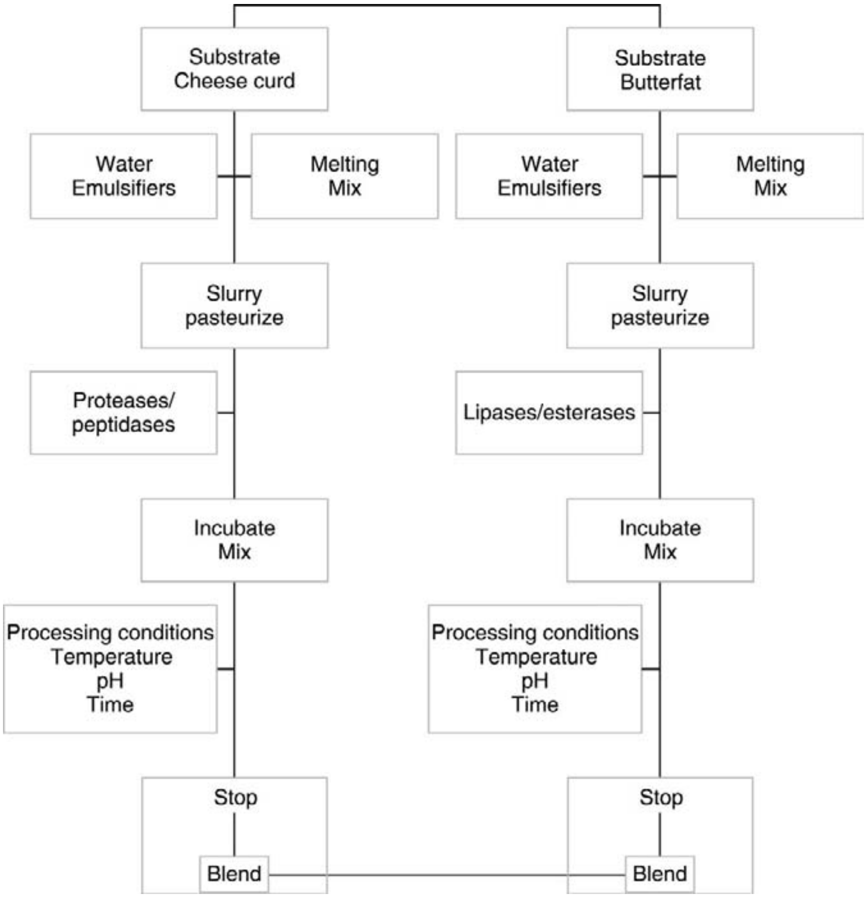
Substrate
Cheese curd
Substrate
Butterfat
Water
Emulsifiers
Melting
Mix
Water
Emulsifiers
Melting
Mix
Slurry
pasteurize
Slurry
pasteurize
Proteases/
peptidases
Lipases/esterases
Incubate
Mix
Incubate
Mix
Processing conditions
Temperature
pH
Time
Processing conditions
Temperature
pH
Time
Stop
Blend
Stop
Blend

Figure 2 Enzyme-modified cheese – component manufacturing approach.

product stability and controlling the degree of flavor and aroma. Excessive or prolonged heat treatment should be avoided, as it may destroy important volatile flavors or create unwanted off-flavors, such as Maillard-type browning. After manufacture, it is important to test EMC products for residual proteolytic or lipolytic activity (~72 h after manufacture), to ensure product stability and to avoid carryover activity in the final product application; for instance, if animal lipases are being used, it may be necessary to test for residual amylase activity, as this may pose a problem if EMCs are added to foods that contain starch.

In contrast to natural cheese, the final EMC products do not have to conform to any compositional specification. Compositional requirements for EMC are dictated only by the product format, either as a powder or as a paste. A survey of the composition of commercial Cheddar EMCs found that pastes contained approximately 40–60% moisture, 20–30% fat, and 10–25% protein, whereas powders contained approximately 5–10% moisture, 10–40% fat, and 15–40% protein. This degree of compositional flexibility allows the manufacturer of EMCs to meet customized compositional requirements and also enables the inclusion of a range of different protein and fat substrates, such as cheese curd, caseinates, butter, and vegetable fat in the formulation.

A systematic two-stage process for the production of enzyme-modified Cheddar cheese used combinations of microbial proteinases and peptidases to generate four intermediate proteolyzed products, which were ranked by sensory preference. One of the intermediates was further lipolyzed to a target acid degree value (ADV) using selected commercial lipases to generate three final EMCs. Overall, this process yielded high-quality EMC products possessing properties and sensory characteristics similar to those of a commercial Cheddar-type EMC. The method enabled greater diversification in terms of product flavors by differentiation of lipolytic and proteolytic profiles from a single substrate.

Control of manufacturing parameters is essential to achieve consistency and reproducibility in EMC production. Development of computer-based algorithmic modeling for the control of viscosity in EMC at a laboratory scale is a prelude to potential industrial-scale online process control for EMC production.

Flavor Generation in Enzyme-Modified Cheese

The flavor of EMC is principally derived from proteolysis and lipolysis, and in both cases the degree of hydrolysis is much more extensive than what occurs during the ripening of natural cheese. Extensive hydrolysis of protein and fat is primarily achieved through the addition of exogenous enzymes to the cheese curd. Proteolysis in EMC manufacture generates high levels of peptides and FAAs, which contribute to the overall background savory flavor of cheese. Indeed, the extent of proteolysis in commercial Cheddar EMC can be up to 4 times (∼20–90% nitrogen soluble in water at pH 4.6) that found in typical mature natural Cheddar cheese. A consequence of the extensive nonspecific proteolysis in EMC manufacture is the generation of high levels of bitter peptides in the final product. In common with their natural cheese counterparts, bitterness in EMCs is associated with the accumulation of hydrophobic peptides derived from β-casein by the action of proteinases. Bitter peptides contain 3–15 amino acids and are characterized by the presence of leucine, isoleucine, proline, valine, phenylalanine, tyrosine, and tryptophan; bitterness can be alleviated by their further degradation to smaller peptides and FAAs. Of particular importance is the removal of proline from a peptide, which greatly reduces bitterness by altering its three-dimensional structure, changing its solubility, and increasing its susceptibility to hydrolysis. Thus, it is not absolutely necessary to reduce bitter peptides to FAAs to eliminate bitterness, and in practice the final level of bitterness acceptable in an EMC product will be the result of a trade-off between the economics of debittering, desired functionality, and the tolerance of the final product application to the bitterness imparted by inclusion of the EMC. It should also be noted that bitterness can be prevented by a careful selection of proteolytic enzymes with substrate specificities unlikely to generate high levels of bitter peptides and/or the use of controlled levels of proteolysis to generate below-threshold levels of bitter peptides. To some degree, bitterness can also be masked by inclusion of mature cheese or glutamic acid in the EMC formulation after proteolysis.

Lipolysis plays an important role in the development of EMC flavor, particularly the production of high levels of short-chain volatile FFAs, which are associated with flavor and aroma intensity. Lipolysis is assisted by emulsification of the EMC substrate at the outset of the process, which increases the oil–water interface and provides an optimum environment for lipases, which have a strong preference for triglycerides, particularly those located at an oil–water interface, which can work up to 1000 times faster at an interface than in free solution. The extent of lipolysis in commercial Cheddar EMC can be up to 8 times that found in natural Cheddar cheese. However, a consequence of uncontrolled lipolysis is the development of rancidity or soapiness, due to an imbalance between short- and long-chain FFAs. These off-flavors can be prevented by utilizing lipolytic preparations that contain combinations of esterase and lipase activities. Flavor in EMC may also be augmented through the use of flavor potentiators, such as monosodium glutamate, yeast extracts, salt, organic acids, and starter distillates. These compounds enhance flavor perception and provide an overall savory intensity to the final product. It is also permissible to include natural, nature-identical, or artificial flavors that can convey a specific flavor note to the product. However, their use may be required to be declared on the labeling of the final product into which the EMC is added.

Enzymes in Enzyme-Modified Cheese Manufacture

Commercial enzymes used in EMC manufacture include proteinases, peptidases, and lipases derived from animal and microbial sources. Plant enzymes are not widely used in EMC manufacture due mainly to high cost and a lack of purity. Most microbial and animal enzymes contain other substances in addition to the active enzyme; the actual protein content may only constitute 1–5%, whereas sugars, salts, and preservatives make up the remainder of the preparation. Commercial enzymes are supplied as liquid preparations, which contain the active enzyme in a dilute solution, or as powdered preparations with inert fillers. Overall, the choice of enzyme preparations used in EMC production depends on the cost, substrate, processing equipment available, and the final flavor desired.

Proteinases

Broad-specificity proteinases are primarily used to rapidly develop an intense savory background cheese flavor. Many proteinases involved in EMC production are exclusively microbial, and most are derived from *Bacillus* or *Aspergillus* species. Proteinases have different pH optima, which are used to classify them as neutral, acidic, or alkaline; however, this should not be accepted as absolute. Optimum temperature is a function of the reaction time used; the longer the reaction time, the lower the optimum temperature.

Peptidases

The commercially available peptidase preparations that enhance flavor and/or reduce bitterness are derived from various *Aspergillus*, *Rhizomucor*, or *Lactococcus* species and contain key endopeptidase, aminopeptidase, and carboxypeptidase activity. The *Aspergillus* species tend to contain higher peptidase levels, which may minimize bitterness, a common problem in EMC production. A number of important peptidases have been isolated from various microbial sources and characterized. Those important in EMC manufacture include endopeptidases (PepO and PepF), which have a broad specificity and cleave large casein fragments into smaller peptides. Most commercial peptidase preparations contain aminopeptidases, such as PepN and PepC, important in debittering and flavor production, as they remove amino acids from the N-terminus of peptides. However, these enzymes alone cannot eliminate bitterness, as they are unable to cleave the imido bond (the peptide bond preceding the proline residue), which prevents them from hydrolyzing a peptide that has proline at the penultimate position from the N-terminus. Another important aminopeptidase is glutamyl aminopeptidase (PepA), which liberates N-terminal glutamic acid and aspartic acid residues from di-, tri-, and oligopeptides. As glutamic acid enhances flavor, this activity is likely to be important in EMC production. Peptidases that cleave proline-containing bonds are very important in debittering, and include proline aminopeptidase (PepP), which hydrolyzes the N-terminal amino acid from peptides that have proline in the second position; proline iminopeptidase (PepI), which removes unsubstituted N-terminal proline residues from tri-, oligo-, and polypeptides; post- (or X-) prolyl dipeptidyl dipeptidase (PepX), which releases N-terminal dipeptides from prolyl peptides; prolinase (PepR), which cleaves Pro-X dipeptides; and prolidase (PepQ), which hydrolyzes X-Pro dipeptides.

Lipases

Most lipases used in EMC production are derived from animal and microbial sources. Animal lipases act almost exclusively on the *sn*-1 and *sn*-3 positions of triglycerides and thus produce butyric acid, which is an important volatile FFA associated with cheese flavor and aroma and which is preferentially esterified at the *sn*-3 position. The most significant animal lipases are pregastric esterases (PGEs) isolated from pregastric tissues of kid goats, lambs, and calves, which are the most widely used animal lipases in the dairy industry. PGEs have an optimum pH of 5–6 and a temperature optimum of 45 °C. Different PGEs produce characteristic flavor profiles: calf PGE generates a 'buttery' and slightly 'peppery' flavor; kid PGE generates a sharp 'peppery' flavor, often called 'piccante'; whereas lamb PGE generates a 'dirty sock' flavor, often called 'peccorino' flavor.

Microbial lipases are commercially available from a wide range of fungal sources including *Rhizomucor miehei*, *Rhizopus arrhizus*, *Aspergillus niger*, *Aspergillus oryzae*, *Geotrichum candidum*, *Penicillium roqueforti*, *Achromobacter lipolyticum*, *Pseudomonas* spp., *Staphylococcus* spp., and *Candida cylindracea*. Microbial lipases tend to be cheaper than their animal counterparts and have the added advantage of being able to generate varying fatty acid profiles as they exhibit a range of specificities. The specificity of microbial lipases varies but most are *sn*-1 or *sn*-3 specific, and most display a low activity toward the *sn*-2 position. They usually have an optimum pH of 6–9 and have a temperature optimum of 45 °C. Microbial lipases have the added advantage of being suitable for vegetarian and kosher foods and are free of amylases, which, if not inactivated, can cause problems in foods into which the EMCs are added. Lipase preparations are not pure and may contain other activities that may influence their contribution to flavor generation in EMCs.

Little published information is available on the use of starter cultures in EMC production; however, a range of starter culture adjuncts have been developed to enhance the flavor of natural cheese. Inclusion of a heat-shocked *Lactobacillus helveticus* strain in a Cheddar EMC base following prehydrolysis by commercially available proteinase and lipase enzymes was reported to generate strong savory notes with enhanced secondary proteolysis. Overall, it is likely that starter or adjunct strains specifically selected for their enzyme complements or flavor-generating potential will be combined with the existing EMC technology to develop a new generation of microbial- and enzyme-modified cheeses (MEMCs).

See also: **Cheese**: Accelerated Cheese Ripening; Biochemistry of Cheese Ripening; Cheese Flavor; Starter Cultures: General Aspects; Starter Cultures: Specific Properties; Pasteurized Processed Cheese Products.

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Pasteurized Processed Cheese Products

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Introduction

Pasteurized processed cheese products (PCPs) are cheese-based foods produced by comminuting, blending, and melting one or more natural cheeses and optional ingredients into a smooth homogeneous blend with the aid of heat, mechanical shear, and (usually) emulsifying salts (ESs). Optional ingredients, which are determined by the product type, include dairy ingredients (e.g., butter oil, casein, caseinates, whey powders), water, condiments (e.g., preserved meat, fish, vegetable, salt, pepper preparations), flavors, colors, and preservatives (**Table 1**). Common packing formats for PCPs used in the food service sector include sliceable blocks, slabs, or sausage (chub) wrapped in various types of plastic film, and dips/ sauce or pastes filled into drums or buckets. Retail PCPs are available in a multitude of formats including foil-covered portions (blocks, triangles), slices (stacked or individually wrapped in plastic film), fingers, tubes, and tubs.

Attempts to increase the shelf life of cheese during the early twentieth century were inspired by the possibility of increased cheese trade, via the production of more stable transportable cheese products, and by the existence of heated cheese dishes such as Swiss Fondue, Welsh Rarebit, and Kochkäse. Many of the early approaches to extend shelf life by application of heat to the cheese were at first unsuccessful, as the heat-treated cheeses were unstable, as reflected by oiling-off and moisture exudation during cooling and storage. In 1911, the Swiss workers (Gerber and Stettler) produced a stable heat-treated Emmental cheese, known as Schachtelkäse, by the addition of a 'melting salt', sodium citrate, to the comminuted cheese before processing (i.e., heating and shearing). Subsequently, it was found that other cheeses (e.g., Cheddar) could be also processed to form stable products by the addition of other 'melting salts' (e.g., sodium phosphates) or blends of different salts. The 'melting salts' were gradually referred to as ESs when their function became known, that is, mediation of the processes of protein hydration and emulsification of free fat during processing. Initial successes were followed by numerous patents for different melting salt blends and later for inclusion of food ingredients other than cheese.

Today, global production of PCPs is estimated at ~2 million tonnes per annum, equal to ~9–10% of natural cheese production. Production in the European Union has increased from 0.53 million tonnes in 2003 to 0.7 million tonnes in 2007, with the largest producers being France, Germany, Ireland, and Poland. Moreover, production has been increasing steadily at a rate comparable to that of natural cheese since 1995. Factors contributing to the continued growth of the PCPs include

- their versatility as foods that offer a wide variety in flavor, texture (e.g., elasticity, firmness, spreadability, sliceability), cooking attributes (e.g., degrees of flowability, browning, viscosity), size and shape of the final product, and overall consumer appeal as made possible by differences in formulation and processing conditions, condiment addition, and packaging technology;
- their convenience of use in the food service sector and the home because of their excellent (stability) preservation, consistent tailor-made functionality (e.g., flowability), and convenient portion size and packaging (e.g., as slices for the beef burger and sandwiches trade);
- their popularity with young children owing to their safe ingestible consistency (for infants), mild flavors, and their attractive packaging formats and shapes, which are convenient for use in lunch boxes;
- their nutritive value, especially as a source of calcium and protein;
- their relatively low cost compared to most natural cheeses, made possible by the use of relatively cheap noncheese solids (e.g., whey, skim milk powder), lower-grade natural cheese, and offcuts; and
- the developments in manufacturing technology, ES blends, and functional dairy ingredients, which facilitate the manufacture of consistent quality products with customized attributes.

Definitions and Types

There are various types of PCPs, the category/standard of which depends on national legislation. Examples include the code of Federal Regulations in the United States, which defines three standards based on permitted ingredients and composition: pasteurized process cheese (≤43% moisture, ≥47% fat-in-dry matter (FDM)); pasteurized process cheese food cheese (≤44% moisture, ≥23% fat); and pasteurized process cheese spread cheese

Table 1 Ingredients, other than cheese, used in pasteurized processed cheese products[a]

Ingredient type	*Main function/effect*	*Examples*
***Dairy ingredient*s**		
Milk fat	Gives desired composition, texture, and meltability characteristics	Cream, butter, anhydrous milk fat
Milk proteins	Give desired compositional specification, texture. and meltability characteristics; assist the production of a physicochemically stable product	Milk protein isolates and concentrates, micellar casein powder, skim milk powder, acid casein, rennet casein, casein, sodium or calcium caseinates, whey protein isolates and concentrates, milk protein hydrolysates, ultrafiltered milk
Lactose	Low-cost filler; may affect texture (e.g., fluidity) and taste (sweetness)	Whey powder, lactose powder, skim milk powder, evaporated milk, liquid whey
Stabilizers	Assist in the formation of a physicochemically stable product; give desired texture and meltability characteristics	Emulsifying salts: sodium phosphates and sodium citrates Hydrocolloids and gums: sodium alginate, κ-carrageenan, locust bean gum, guar gum, xanthan gum
Acid regulators/ pH controlling agents	Assist in the control of the pH of the final product	Food-grade organic acids, for example, lactic, acetic, citric, phosphoric acid
Flavors	Impart flavor, especially where much young cheese is used	Enzyme-modified cheeses, hydrolyzed butter oil, hydrolyzed milk proteins, autolyzed yeast extracts, paprika, starter culture distillate, smoke extracts
Flavor enhancers	Accentuate flavor	Salt (NaCl), yeast extract
Sweetening agents	Increase sweetness, especially in products targeted to young children	Sucrose, dextrose, corn syrup, hydrolyzed lactose
Colors	Impart desired color	Colors in Table 1: annatto extracts, β-carotene, paprika, curcumin (turmeric), riboflavin, chlorophyll preparations
Preservatives	Retard mold growth; prolong shelf life	Nisin, sodium and/or potassium salts of sorbic acid or propionic acid
Condiments	Impart variety to appearance, aroma, and taste; contribute to product differentiation	Sterile preparations of meat, fish, vegetables, nuts, and/or fruits

[a]The ingredients permitted are subject to the prevailing regulations in the region of manufacture.

(44–60% moisture, ≥20% fat). International standards for processed cheese are defined by the Codex Alimentarius Commission; these standards, summarized in **Table 2**, include

- – named variety of process(ed) cheese and spreadable process(ed) cheese (Codex-Stan A-8(a)-1978);
- – process(ed) cheese and spreadable process(ed) cheese (Codex-Stan A-8(b)-1978); and
- – process(ed) cheese preparations – process(ed) cheese food and process(ed) cheese spread (Codex-Stan A-8(c)-1978).

According to these standards (**Table 2**), the minimum content of natural cheese must be such that it contributes greater than or equal to 51% of the dry matter of the final PCP in the case of process cheese foods and spreads, and about 82–96% of the dry matter in process cheese or spreadable process cheese depending on the blend of the cheeses, the amount of milk fat required to standardize to the minimum FDM content, and the levels of added ESs and product flavorings.

Manufacturing Protocol and Equipment

The manufacture of PCPs involves the following major steps:

- *Formulation*: This involves selecting the different types and levels of ingredients to give the desired end-product characteristics.
- *Size reduction of added cheese*: This is brought about by shredding, grating, or mincing to maximize the surface area of the cheese and facilitate heat transfer to the blend during subsequent processing.
- *Blending of formulation ingredients*: This is carried out to ensure homogeneity of all materials and uniform end-product quality.

Table 2 Ingredient specifications for processed cheese products as defined by Codex Alimentarius

Product category	*Permitted ingredients*
Named variety of processed cheese and spreadable processed cheese	One or more varieties of cheese, with added cheese accounting for ≥51% (w/w) of the final product; milk fat (cream, butter oil) for standardization of milk fat in the final product; water; salt, vinegar, spices, seasonings, flavoring condiments at dry matter levels ≤16.7% (w/w) of the dry matter of finished processed cheese; starter culture bacteria; enzymes; emulsifying salts (sodium, potassium, and calcium salts of citric and phosphoric acid at levels ≤0.4% (w/w)); pH regulators (food-grade organic acids), colors at ≤0.06% (w/w); mold inhibitors (sorbic acid, potassium/ sodium sorbate, and/or sodium propionates at levels ≤0.3% (w/w) or nisin at ≤0.001% (w/w))
Processed cheese and spreadable processed cheese	As for named variety of processed cheese and spreadable processed cheese, except that there are prescribed levels for any one cheese variety in the cheese blend
Processed cheese preparations – processed cheese foods and spreads	As for processed cheese and spreadable processed cheese, but with the following extra optional ingredients: Other dairy ingredients (milk, skim milk, buttermilk, cheese whey, whey proteins, caseins – in wet or dehydrated forms) Selected hydrocolloids and gums Taste intensifiers – sodium glutamate

Summarized from Codex Alimentarius Standards, CODEX-STAN A-8(a), A-8(b), and A-8(c). Reproduced from FAO/WHO (2010) http://www.codexalimentarius.net/web/standard/list.

The compositions of the various product categories are detailed in Codex Alimentarius Standards, and relate to minimum contents of dry matter and fat-in-dry matter.

- *Processing the blend*: This involves heating by direct or indirect steam injection in a cooker (kettle) typically to ~75–85 °C for 1–5 min while constantly agitating/ shearing. This step is carried out to
  - kill any potential pathogenic and spoilage microorganisms, and thereby extend the shelf life of the final PCP; and
  - facilitate the physicochemical and microstructural changes that transform the blend to an end product with the desired characteristics and physicochemical stability.
- *Homogenization of the hot molten blend*: This is an optional step and is carried out typically at first and second stage pressures of 15 and 5 MPa, respectively, to
  - assist further mixing, and size reduction of any coarse particles or nondissolved particles (e.g., ESs, dry ingredient, cheese rind); and
  - promote a finer dispersion of fat droplets, which leads to a smoother and creamier hot blend and thicker and firmer consistency in the final processed cheese.
- *Cooling and storage of the hot molten processed cheese*: This step is carried out to
  - promote fat crystallization;
  - regulate protein–protein interactions; and
  - give 'setting' of the end product, to a degree regulated by cooling rate, and impart the desired texture and functional characteristics (e.g., firmness, spreadability, heat-induced flow).

Cleaning generally involves the removal of surface contamination (e.g., any adventitious mold growth) or rind using rapid motor-driven scrapers. The cheese is cut, using hydraulically operated blades, into segments, which are finely minced by passing through high-speed shredders or large mincing machines. The rind of the cheese may also be size-reduced, using counter rotating stainless-steel rollers, to particles sufficiently small (<1 mm) to enable adequate uptake of moisture during subsequent processing.

In batch cooking, the finely ground cheese is conveyed directly to the cooker where it is blended with ESs, water, and optional ingredients. Alternatively, the cheese may be premixed at room temperature with ESs (and some or all of the water and optional ingredients) using various types of preblenders. Some cheese cookers, with interchangeable bowls, allow one batch to be filled and premixed while another is simultaneously being cooked (processed). Premixing has two main effects:

- achieves a greater breakdown/dispersion of particles (e.g., cheese, powders) prior to cooking;
- reduces cooking time by enabling reactions that occur during cooking to be initiated at lower temperature; and
- reduces batch-to-batch variation in composition and physical properties.

For a given cheese variety, variations in cheese composition (e.g., pH, calcium-to-casein ratio, and the level of intact casein) can occur owing to variations in milk composition, cheesemaking conditions, and degree of maturation. In turn, such variations can affect its processability, for example, how readily the *para*-casein in the cheese becomes hydrated and emulsifies free fat.

Premixing evens out the effects of differences in the composition and proteolysis, and thus processability, of the raw materials (e.g., cheese) on the consistency of the final product. The efficacy of premixing depends on the type and capacity of the preblender and the capacity of the cooker. However, the capacity of the preblender should not be so great as to cause a considerable difference in the premixing time between the first and the last subbatch withdrawn for processing. Otherwise, time-related differences in the degree of physicochemical changes (as discussed below) between the first and the last lot of a given premix at processing could lead to differences in processability and ultimately in the consistency of the end product. The degree of physicochemical change in a blend after a given time depends on the type of preblender, which influences the level of shear applied, the shear rate, and the degree of mixing and interaction between the different ingredients.

Processing of the blend may be performed in batch cookers or continuous cookers connected to water, steam, and vacuum. The temperature–time treatment in batch processing varies (e.g., 70–95 °C for 4–15 min) depending on the formulation, extent of agitation, the desired product texture, body, and shelf life characteristics. Bacteria in the vegetative state are killed by these temperature treatments. However, a temperature >130 °C is required to kill some spores. A temperature of 140 °C can be achieved in continuous cookers by virtue of their design, for example, scraped surface tubular heat exchangers, which maximize the surface area of contact between the heating medium (e.g., steam, oil, or hot water) and the blend and ensure sufficient agitation to prevent burn-on of the blend on the heat transfer surface. In continuous cookers, the blend is, typically, heated to and held at 140 °C for 5 s and then cooled to 70–95 °C by flash evaporation of moisture due to a pressure drop, or by passing through scraped surface tubular coolers. The product is then held at this temperature for 4–15 min to allow adequate time for interaction of the different blend ingredients, the desired physicochemical changes to occur, and the development of the desired textural characteristics.

Heating and shearing contribute to successful processing by aiding

- dissolution of the ESs and their interaction with the casein or *para*-casein present in the blend, and their partial conversion to soluble sodium caseinate or *para*-caseinate;
- dispersal of free fat, created by shearing of the native fat globule membranes in the natural cheese, into droplets and their emulsification by the partially hydrated protein; and
- structural transformation from a cheese consisting of a casein or *para*-casein gel with occluded fat globules and moisture to a processed cheese that may be described as a concentrated oil-in-water emulsion.

Role of Emulsifying Salts

In natural rennet-curd cheeses (e.g., Gouda, Cheddar, Mozzarella), protein occurs as *para*-casein aggregates, rendered insoluble by interprotein linkages mediated by calcium (attached to acidic amino acid residues such as glutamate and aspartate) and colloidal calcium phosphate (attached to serine phosphate groups), and hydrophobic interactions between uncharged amino acid residues. The calcium content (mg g^{-1} *para*-casein) of natural rennet-curd cheeses ranges from ~15 in Bavarian Blue cheese to 35 in Emmental, and most of it is insoluble (~50–60% of total at pH ~5.2). In acid-curd cheeses (e.g., Quark, Cottage cheese), protein occurs in the form of insoluble casein/casein–whey protein aggregates. In contrast to natural rennet-curd cheeses, the levels of calcium are relatively low (e.g., ~100 mg 100 g^{-1}, ~5.8 mg g^{-1} protein), owing to the low pH of the gel at whey separation (~4.7 compared to 5.8–6.5 for most rennet-curd varieties) and the ensuing solubilization of colloidal calcium phosphate and its loss in the whey. Moreover, most of the remaining calcium in the product is soluble. Hydrophobic, electrostatic, and covalent disulfide bonds (where the cheese milk has been subjected to high heat treatment) maintain the integrity of the aggregates in acid-curd cheeses.

In both cheese types, the aggregates are fused into a network (matrix) of entangled strands. The network encases fat and moisture within the pores of the network. The fat in natural cheeses generally occurs as globules enclosed by the native milk fat globule membrane (NMFGM) comprised mainly of proteins and phospholipids; the NMFGM prevents the leakage of free fat, 'sweating', and/or greasiness of the cheese. The moisture in cheese is mainly physically entrapped by the capillary effect of the protein matrix. Water chemically bound to the protein by hydrogen bonding or dipole–dipole interactions accounts for only ~0.15 g g^{-1} protein but increases somewhat with aging because of proteolysis and an increase in pH.

The process of heating (to temperatures applied to processed cheese manufacture) and shearing of natural cheese usually results in the formation of a heterogeneous, gummy, pudding-like mass that undergoes extensive oiling-off and moisture exudation during manufacture and on cooling. These defects arise from

- partial dehydration/aggregation and shrinkage of the *para*-casein/casein matrix as affected by

- increased hydrophobic interactions as induced by the relatively low pH of cheese (for most cheeses, ~4.6–5.6) and high temperature applied during processing,
- the precipitation of soluble (serum) calcium and phosphate, leading to further calcium phosphate-mediated interactions between the *para*-casein molecules (especially, in rennet-curd cheeses), and
- the consequential decline in pH and negative charge;

- physical damage to, and removal of, the NMFGM, resulting in the formation of nonglobular fat, followed by its liquefaction and coalescence.

The above defects can be prevented by the addition of ESs, at levels of 1–3% (w/w), to the cheese blend prior to processing. The ESs usually contain a monovalent cation and a polyvalent anion, with the sodium salts of citric acid and/or phosphoric acid being the most common type(s). While these salts are not emulsifying agents *per se*, they promote, with the aid of heat and shear, a number of physicochemical changes in the blend, which partially convert the insoluble casein of acid-curd cheeses or *para*-casein in rennet-curd cheeses to hydrated sodium caseinate or sodium *para*-caseinate, respectively. The hydrated proteins bind water and emulsify the free oil during processing, and thereby contribute to the formation of a smooth, homogeneous, physicochemically stable PCP. Hence, the resolubilized cheese protein is the primary stabilizing agent in the formation of PCPs, even though other materials such as hydrocolloids and starches may also have stabilization and texture-modifying functionalities.

The changes brought about by the ES include upward adjustment and stabilization (buffering) of the pH of the cheese blend, calcium sequestration, partial demineralization and hydration/dispersal of the cheese protein, emulsification of coalesced fat, and structure formation. These changes are discussed briefly below.

Upward Adjustment/Stabilization of pH and Calcium Sequestration

Owing to the buffering capacity of ESs, the use of the correct blend of ESs increases the pH from typical values of ~4.6–5.5 in the natural cheese used for processing to values of 5.6–6.1 in the PCP. The increase in pH increases the calcium-sequestering ability of the ES and the negative charge on the caseinate/*para*-caseinate.

At the pH values of PCPs, the ESs are sufficiently dissociated to sequester a large portion of the calcium attached to the *para*-casein. This results in subsequent deposition of calcium as insoluble calcium phosphate and/or calcium citrate inclusions.

Dispersion of Casein/*Para*-Casein

The partial demineralization of casein at the elevated pH leads to a more open reactive caseinate/*para*-caseinate conformation with superior water-binding capacity compared to that in natural cheeses. The protein matrix of the natural cheese is thereby dispersed and transformed to a sodium caseinate or *para*-caseinate dispersion (sol) in acid- and rennet-curd cheeses, respectively. These changes are confirmed by the large increase in the level of water-soluble protein during processing (from ~5–20% of total protein in the natural cheese to ~60–80% in processed cheese) and by the high levels of water-insoluble calcium (~60–90% of total) in PCPs (**Figure 1**).

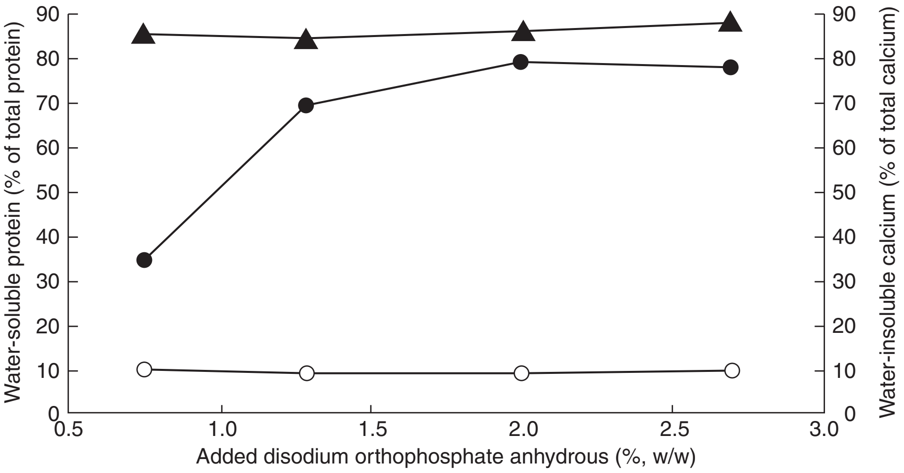

Figure 1 Changes in water-soluble protein (●) and water-insoluble calcium (▲) in processed Cheddar cheese as a function of disodium orthophosphate concentration. Identical processed cheese blends (cheese, water, and emulsifying salt) were formulated and processed to 80 °C while continuously shearing; the resultant PCPs were, packed, stored for 36–48 h at 4 °C, and analyzed. The water-soluble protein of the cheese in each blend prior to processing (○) was also analyzed.

Fat Emulsification

Under processing conditions, the dispersed hydrated caseinate/*para*-caseinate contributes to emulsification of the free fat by coating the surfaces of dispersed free fat droplets, resulting in the formation of an artificial (recombined) fat globule membrane. The relatively high water-binding capacity of the *para*-caseinate also enhances emulsion stability as it leads to high viscosity of the aqueous phase and thus a reduction in the collision frequency of emulsified fat particles.

Structure Formation during Cooling

On cooling, the homogeneous molten viscous mass sets to form a characteristic body, which, depending on blend formulation, processing conditions, and cooling rate, may vary from firm and sliceable to soft and spreadable. Factors thought to contribute to the structure formed on cooling include fat crystallization and protein–protein interactions; the formation of an amorphous structure (or gel) based on the aggregation of calcium phosphate complexes may also contribute to the structure.

Microstructural studies on PCPs indicate that the structure is an emulsion of discrete, rounded fat droplets of varying size (typically 0.3–5 μ*m*) in a continuous protein matrix. The matrix consists of strands that are finer than those of natural cheese, with the degree of fineness increasing as the pH is raised from 5.4 to 6.1, an effect attributed to a change in the proportions of different types of protein interactions: hydrophobic, electrostatic, hydrogen bonds, and residual calcium cross-links. In contrast to natural cheese, the fat and *para*-casein are more uniformly distributed, the matrix is less compact and fused, there is less clumping/coalescence of fat globules, and the mean fat globule size is generally much smaller than that of natural cheese.

The incorporation of emulsified *para*-caseinate-coated fat globules, which can be considered as fat-filled protein particles, into the new structural matrix may be considered to increase the effective protein concentration of the PCP. The positive correlations between the degree of emulsification and firmness or elasticity, and the inverse relationship between the degree of emulsification and flowability of PCPs support this suggestion.

Properties of Different Emulsifying Salts

The salts most commonly used in cheese processing and their effects in promoting the different physicochemical changes during processing are summarized in **Table 3**.

Properties of Processed Cheese Products

The textural properties of the unheated PCP and cooking characteristics (especially softening and flowability) of the heated PCP are the major determinants of quality. Consequently, numerous studies have investigated the effects of different factors on these product attributes. Despite some interstudy discrepancies (probably due to differences in factors other than the treatment investigated), definite trends are evident. The studies show that the quality of PCPs is influenced by many factors, including the type and level of ES, composition and degree of maturity of the cheese used in the blend, the type and level of optional ingredients, processing conditions, and the interactions between the different factors. These are summarized in **Table 4** and are discussed briefly below.

The firmness and elasticity of PCPs made with different ESs generally decrease in the following general order: tetrasodium pyrophosphate (TSPP) > disodium phosphate (DSP) > trisodium citrate (TSC) > sodium aluminum phosphate (SALP). In contrast, the mean fat globule diameter and the meltability on heating generally show the opposite trend, being largest with SALP and smallest with TSPP. The effects of different salts on firmness reflect differences in their calcium sequestration and pH buffering characteristics, which in turn result in differences in casein hydration and degree of emulsification. Increasing the level of ES in the range 0.5–3.0% (w/w) leads to a progressive increase in firmness and a decrease in meltability. These changes coincide with increases in the levels of water-soluble protein and water-insoluble calcium phosphate (**Figure 1**).

Increasing the pH of the PCP in the range 5.7–6.2 is accompanied by decreases in the hardness and elasticity (G', storage modulus) of the unheated processed cheese and by increases in the flowability and fluidity (loss tangent) of the melted product. These changes are paralleled by an increase in casein hydration, a finer protein network, and a more uniform structure.

Cheese is the major ingredient in all PCPs. The level of intact casein in most rennet-curd cheeses decreases during maturation as a result of hydrolysis of the casein by various proteinases such as residual chymosin and plasmin into peptides that are more soluble than the parent protein. An increase in the intact casein content from ~75 to 95% of total casein in Cheddar cheese leads to a significant increase in the firmness and a reduction in the flowability (on heating) of the resultant processed Cheddar cheese. Hence, it has long been recognized at commercial level that block processed cheese with good sliceability and elasticity requires predominantly young cheese (75–90%

Table 3 Emulsifying salts commonly used in pasteurized processed cheese products and their general properties in relation to physicochemical changes during cheese processing

			Physicochemical changes during processing			
Group	*Forms commonly used*	*Chemical formulae*	*Calcium sequestration*	*Buffering action*	Para-*casein hydration*	*Fat emulsification*
Citrates	Trisodium citrate	$2Na_3C_6H_5O_7 \cdot 1H_2O$	Low	High	Low	Low
Orthophosphates	Disodium phosphate Trisodium phosphate	$Na_2HPO_4 \cdot 12H_2O$ Na_3PO_4	Low	High	Low	Low
Condensed phosphates						
Pyrophosphates	Disodium pyrophosphates Trisodium pyrophosphates Tetrasodium pyrophosphates		Medium	Medium	Very high	Very high
Polyphosphates	Pentasodium tripolyphosphate Sodium tetrapolyphosphate Long-chain polyphosphates	$Na_{n+2}PnO_{3n+1}$ ($n = 10$–25)	High ↓ Very high	Low ↓ Very low	High ↓ Low	Very high ↓ Low

Compiled from Carić M and Kaláb M (1993) Processed cheese products. In: Fox PF (ed.) *Cheese, Chemistry, Physics and Microbiology, Vol. 2: Major Cheese Groups*, pp. 467–505. London: Chapman and Hall; and Fox PF, Guinee TP, Cogan TM, and McSweeney PLH (2000) *Fundamentals of Cheese Science*. Gaithersburg, MD: Aspen Publishers, Inc.

Table 4 General effects of various factors on the textural and heat-induced flowability of pasteurized processed cheese products

	Firmness	*Elasticity*	*Spreadability*	*Heat-induced flowability*
Formulation				
Emulsifying salt concentration				
Increasing level in the range 0.0–0.5%, w/w	↑	↑	NA	↑
Increasing level in the range 0.5–3.0%, w/w	↓	↓	NA	↑
Cheese				
Increasing degree of proteolysis	↓	↓	↑	↑
Increasing content of intact casein	↑	↑	↓	↓
Substitution of rennet-curd cheese by				
Reworked processed cheese[a]	↑	↑	↓	↓
Cheese base[b]	↑	↑	↓	↓
Acid- and heat-coagulated cheeses	↑	NA	NA	↓
Dairy ingredients				
Whey proteins	NA	NA	NA	↓
Total milk proteins	NA	NA	NA	↓
Milk ultrafiltrates	NA	NA	NA	↓
Calcium coprecipitate	↑	NA	NA	↓
Calcium caseinate	NA	NA	NA	↑
Skim milk powder	NA	NA	↑	NA
Composition of PCP				
Increasing moisture content	↓	↓	↑	↑
Increasing pH	↓	↓	↑	↑
Processing conditions				
Increasing temperature	↑	↑	↓	↓
Increasing time at maximum temperature	↑	↑	↓	↓
Increasing degree of shear	↑	↑	↓	↓

[a] Rework refers to pasteurized processed cheese product that is not packaged for sale; it is obtained from the 'leftovers' in cookers and filling machines, damaged packs, and batches that have 'overcreamed' (thickened) and are too viscous to pump/fill.

[b] Cheese base refers to milk ultrafiltrate that is diafiltered, inoculated with a starter culture (and sometimes with rennet also) until the pH reaches ~5.2–5.8, pasteurized, and concentrated to a dry matter content of ~60%, w/w.

The general effects of the different parameters, as summarized from a review of the published literature, are presented. However, the precise effects of changing any parameter may depend on the particular formulation and processing conditions and the effects of their interaction.

NA, data not available or limited; ↑, magnitude of the factor (e.g., firmness) increases; ↓, magnitude of the factor decreases.

intact casein), whereas predominantly medium-ripe cheese (60–75% intact casein) is required for spreads. *Ceteris paribus*, reducing the calcium of natural cheese in the range 30–18 mg g^{-1} casein results in PCPs that are softer and shorter (more brittle), and that become more fluid and spreadable on heating.

Apart from cheese, a wide variety of other ingredients may be included in PCPs (**Tables 1** and **2**). Milk protein preparations (e.g., sodium and calcium caseinates, rennet casein, milk protein concentrates, whey protein powders) are widely used in PCPs partly as cheaper substitutes for cheese protein and to impart certain functional characteristics to the PCP, for example, increased fracture stress in the unheated PCP, or stretch or flow in the heated PCP. The functionality of milk protein preparations in PCPs is influenced by their initial solubility, pH, and mineral composition.

Processing conditions have a marked effect on the properties of PCPs, with increases in processing time, shear, and temperature generally coinciding with increases in firmness and shortness of the unheated PCPs but a reduction in the degree of flow on heating. These changes are associated with, *inter alia*, higher degrees of fat emulsification and protein aggregation in the finished PCP. During processing, the blend thickens progressively with holding time in the cooker. The thickening, frequently referred to as 'creaming' or 'creaming effect', may be attributed to ongoing interaction of the ESs with the casein and the consequent increases in *para*-casein hydration and degree of emulsification. Both factors contribute to an increase in the apparent viscosity of the molten blend. However, extending the holding time of the molten product at high temperatures results in a gradual dehydration of the dispersed sodium *para*-caseinate, an occurrence that has the effect of imparting elasticity and firmness to the final product. Indeed, prolonged holding results in excessive protein dehydration and aggregation, and a concomitant destabilization of the emulsion, as reflected by the exudation of 'beads' of free oil on the surface of the product on cooling. This defect, known as 'overcreaming', is also accompanied by a tendency of the product to express some free water in the cooker or on subsequent cooling.

Applications

PCPs are used in many applications, in both the raw and heated forms. The suitability for particular applications depends primarily on the textural and flavor characteristics of the raw cheese and the cooking properties of the heated cheese. In the raw form, PCP may be used as a table product with a spectrum of consistencies ranging from firm, elastic, and sliceable to creamy, smooth, and spreadable. The variation in consistency makes PCP suitable for a range of uses, for example, substitute for natural sliceable or shredded cheese (e.g., on bread, crackers, or in sandwiches), table spread, sauces, and dips. PCPs are also used as an ingredient in several cookery applications, for example, as slices in burgers, toasted sandwiches, pasta dishes, au gratin sauces, and cordon-bleu entrée in poultry products. PCPs may also be dried, as cheese powders, which are then dry blended with other ingredients in the preparation of formulated foods such as dry soup or sauce mixes, ready prepared meals, and snack coatings (*see* **Cheese**: Cheese as a Food Ingredient).

See also: **Cheese**: Cheese as a Food Ingredient.

Further Reading

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Cheese Analogues

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Introduction

Cheese analogues (CAs) are cheese-like products manufactured by blending various edible oils/fats, proteins, other ingredients, and water into a smooth homogeneous blend with the aid of heat, mechanical shear, and emulsifying salts (ESs). The array of ingredients used in CAs is listed in **Table 1**. CAs were developed in the United States in the early 1970s, the main impetus being the desire to create cheaper cheese substitutes for the industrial and catering cheese sectors, where they have numerous applications: frozen pizza toppings, slices in beef burgers, and as an ingredient in salads, sandwiches, cheese sauces, cheese dips, and ready-prepared meals. While CAs are now also produced in Europe (~40–60 000 tonnes per annum), they are mainly manufactured in the United States where the annual production is ~300 000 tonnes. The most common types of partial dairy CAs produced are of low-moisture Mozzarella, Cheddar, Monterey Jack, or pasteurized processed Cheddar. Some advantages of CAs over natural cheeses include

- lower cost owing to the use of vegetable oils instead of milk fat, partial substitution of protein by starch and/or other hydrocolloids, and reduction in manufacturing cost (lower capital cost of manufacturing equipment, no maturation period required);
- simplicity of manufacture from readily available raw materials;
- amenability to be relatively easily formulated with customized textural and functional attributes, and/or with nutritional attributes (low salt, fat, and cholesterol; mineral enrichment) to meet specific dietary requirements;
- ability to meet special dietary needs and to act as a vehicle for health benefits/supplements, for example, lactose free, low in calories, low in saturated fat, vitamin enriched;
- relatively high stability of their textural and cooking properties during storage at refrigerated temperature.

Nevertheless, compared to natural cheese, CAs have a much inferior (not natural) market image, and generally tend to be second grade in some functional characteristics such as stretchability/stringiness on heating, an effect that tends to be accentuated as the protein content decreases.

Definitions and Types

CAs may be classified arbitrarily as dairy, partial dairy, or nondairy, depending on whether the fat and/or protein components are from dairy or vegetable sources. Dairy analogues are made using dairy proteins and butterfat, partial dairy analogues using dairy protein and vegetable oil, and nondairy analogues using vegetable oil and vegetable proteins. CAs may be categorized further as imitation or substitute, depending on the nutritional status. FDA regulations (Food and Drugs Administration Regulation 101.3, Identity Labelling of Food in Packaged Form (e)) in the United States specify that a CA is deemed to be imitation if it substitutes and resembles another cheese but is nutritionally inferior. Nutritional inferiority implies a reduction in the content of an essential nutrient(s) present in a measurable amount but does not include a reduction in the caloric or fat content. A CA may be classified as a substitute cheese if it substitutes and resembles another cheese and is not nutritionally inferior. Other cheese-like products that may be classified as imitation or substitute include Tofu and Filled Cheeses.

Applications

The principal application of CAs in the United States is as a cheese topping in frozen pizzas where low-moisture Mozzarella cheese analogue (LMMCA) is the main type used. Cheddar-type analogue is also common and is used mainly as slices in cheeseburgers. In Europe, CAs are being used increasingly in the industrial sector as ingredients in formulated foods such as processed meat products and combined 'cheese'-filled coextruded products, savory snack toppings, and pasta meals (*see* **Cheese**: Cheese as a Food Ingredient). Other applications of CAs include salads, sandwiches, spaghetti sprinkling, cheese sauces, cheese dips, ready-prepared meals, and as an ingredient of grated cheese blends. In addition to analogues of the cheese varieties mentioned, the successful manufacture of analogues of other cheese varieties (e.g., Parmesan, Romano, Blue, Cream cheese, low-fat Cheddar) has been reported in the trade literature. The use of enzyme-modified cheeses (EMCs) (**Cheese**: Cheese as a Food Ingredient) with flavor profiles close to those of specific to individual varieties (e.g., Emmental, Cheddar, and Blue flavor) has facilitated the development of CAs of different varieties.

Table 1 Ingredients used in the manufacture of cheese analogues

Ingredient type	*Main function/effect*	*Examples*
Fat	Gives desired composition, texture, and meltability characteristics; butter oil imparts dairy flavor	Butter, anhydrous milk fat, native or partially hydrogenated soybean oil, corn oil, palm kernel oil
Milk proteins	Give desired • composition • semihard texture with good shreddability • flow and stretch characteristics on heating Assist in the formation of physicochemically stable products	Casein, caseinates, whey protein[a]
Vegetable proteins	Give required composition Lower cost due to low cost relative to casein Rarely, if ever, used commercially owing to product defects	Soybean protein, peanut protein, wheat gluten
Starches	Substitution for casein and cost reduction	Native and modified forms of maize, rice, potato starches
Stabilizers		
Emulsifying salts	Assist in the formation of physicochemically stable products; affect textural and functional properties	Sodium phosphates and sodium citrates
Hydrocolloids	Enhance product stability; affect textural and functional properties	Guar gum, xanthan gum, carrageenans
Acidifying agents	Assist control of pH of the final product	Food-grade organic acids, for example, lactic, acetic, citric, phosphoric
Flavors and flavor enhancers	Accentuate flavor	Enzyme-modified cheese, starter distillate, smoke extracts NaCl, yeast extracts, monosodium glutamate
Sweetening agents	Add sweetness, especially in products targeted on young children	Sucrose, dextrose, corn syrup, hydrolyzed starches, hydrolyzed lactose
Colors	Impart desired color	Annatto, paprika, artificial colors
Preservatives	Retard mold growth; prolong shelf life	Nisin, potassium sorbate, calcium/sodium propionate
Minerals and vitamin preparations	Improve nutritive value	Magnesium oxide, zinc oxide, iron, vitamin A palmitate, riboflavin, thiamine, folic acid

[a] Whey proteins are mainly used in products intended for cooking applications where flow resistance is required.
The ingredients permitted are subject to the prevailing regulations in the region of manufacture.

Technology and Manufacture

Formulation

The manufacturing technology for CAs is very similar to that for pasteurized processed cheese products (PCPs). It involves the following major steps:

- formulation, deciding on the different types and levels of ingredients to be included;
- blending of ingredients;
- processing (heating and shearing) of the blend;
- homogenization of the molten blend (optional; mainly for high-moisture products);
- hot packing and cooling.

A typical manufacturing protocol is presented in **Figure 1**.

Analysis of commercial LMMCA indicates large intra- and interfactory variations in composition (**Table 2**). Such variations indicate marked differences in formulation, which suggest that formulation change is a key approach used by manufacturers in the production of LMMCAs with customized nutritional, textural, and/or functional (cooking) characteristics.

A typical formulation for LMMCA, which gives cheese with a typical composition, is presented in **Table 3**. The functions of the different ingredients used in CAs are summarized in **Table 1**.

Proteins used

The major protein source in dairy-based CAs is casein, usually rennet casein, especially in semihard block CAs; caseinates are used mainly in spreadable CA products.

Caseins are formed by coagulation of the casein, as induced by selective hydrolysis of κ-casein by rennet (rennet casein) or pH adjustment to the isoelectric point (acid casein). Following coagulation and whey

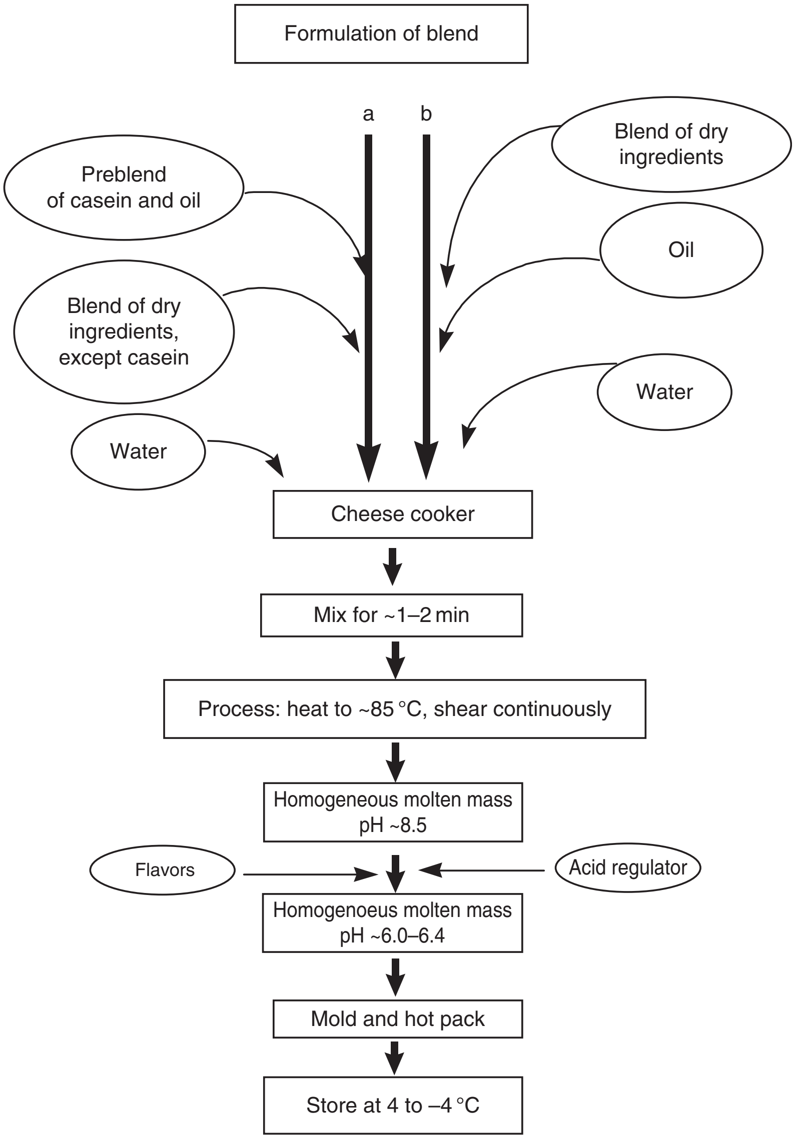
Formulation of blend
a
b
Preblend of casein and oil
Blend of dry ingredients, except casein
Water
Blend of dry ingredients
Oil
Water
Cheese cooker
Mix for ~1–2 min
Process: heat to ~85 °C, shear continuously
Homogeneous molten mass pH ~8.5
Flavors
Acid regulator
Homogenoeus molten mass pH ~6.0–6.4
Mold and hot pack
Store at 4 to –4 °C

Figure 1 Typical manufacturing procedure for low-moisture Mozzarella cheese analogue. Different protocols for preparation and adding ingredients prior to processing are shown as a and b.

separation, the insoluble curds are washed (to remove residual whey), pasteurized, concentrated by centrifugation, and dried to moisture levels of $\sim\leq 10\,\text{g}\,100\,\text{g}^{-1}$. Rennet casein is ground and separated into powders of different mean particle sizes (*see* **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Functional Properties of Milk Proteins). In both rennet and acid casein powders, the casein is more aggregated and dehydrated than casein in rennet-curd or acid-curd cheeses owing to the relatively high temperatures applied during curd pasteurization and drying, and the lower moisture levels ($\leq 0.1\,\text{g}\,\text{g}^{-1}$ protein compared ~ 1.25–$6.25\,\text{g}\,\text{g}^{-1}$ protein in cheese, depending on the variety). At a microstructural level, a powder particle of each casein type may be considered as a portion of dried skim milk (rennet-curd or acid-curd) cheese, with the casein in the form of highly fused aggregates of *para*-casein or casein. Both caseins are insoluble in water.

Despite these similarities, they differ in composition. Similar to rennet-curd cheese, the protein in rennet casein is calcium phosphate *para*-caseinate (at pH ∼6.8–7.1), where the caseino-macropeptide region of the casein micelle-stabilizing protein (κ-casein) has been cleaved by the addition of rennet (e.g., chymosin). The remainder of the casein (referred to as *para*-casein) coagulates via calcium cross-linking and hydrophobic interactions, and retains the full complement of micellar calcium and phosphate. In contrast, acid casein contains the full complement of caseins (including the κ-casein

Table 2 Mean compositional analysis of wholesale samples of low-moisture Mozzarella cheese and low-moisture Mozzarella analogue

	Cheese type	
	Low-moisture Mozzarella cheese	*Low-moisture Mozzarella cheese analogue*
Compositional parameter		
Moisture (%, w/w)	46.4 (44.1–49.5)	49.3 (40.4–52.5)
Fat (%, w/w)	23.2 (20.5–25.0)	25.6 (22.0–29.9)
Protein (%, w/w)	26.0 (24.04–29.8)	17.4 (13.8–21.3)
Salt-in-moisture (%, w/w)	3.1 (2.0–3.6)	3.7 (2.5–4.9)
Fat-in-dry matter (%, w/w)	44.6 (40.6–52.1)	50.6 (44.5–56.8)
MNFS (%, w/w)	60.5 (57.3–60.3)	66.3 (55.0–70.3)
Ca (mg g^{-1} protein)	27.3 (22.6–31.1)	33.9 (31.4–38.2)
P (mg g^{-1} protein)	20.6 (17.1–23.6)	26.9 (23.47–36.0)
pH	5.53 (5.33–5.69)	6.36 (6.1–6.62)
Proteolysis		
pH 4.6-soluble N (% total N)	4.73 (1.05–9.75)	3.04 (1.18–7.61)
5% phosphotungstic acid-soluble N (% total N)	0.45 (0.26–0.76)	0.21 (0.07–0.41)

The values presented are the means of data from 9 samples of low-moisture Mozzarella and 16 samples of low-moisture Mozzarella analogue. Values in parentheses represent the minimum and maximum values.

MNFS, moisture in the nonfat substance.

Compiled from Guinee TP, Harrington D, Corcoran MO, Mulholland EO, and Mullins C (2000) The compositional and functional properties of Mozzarella, Cheddar and analogue pizza cheeses. *International Journal of Dairy Technology* 53: 51–56; Guinee *et al*., unpublished data.

Table 3 Typical formulation of low-moisture Mozzarella cheese analogue

Ingredient	*Level of addition (%, w/w)*
Casein and caseinates	24
Vegetable oil	22
Starch	3
Emulsifying salts	2
Flavors and flavor enhancers	3
Stabilizers	0.50
Acidifying agent	0.36
Color	0.04
Preservative	0.10
Water and condensate	45

caseino-macropeptide), but none of the micellar calcium and phosphate all of which was solubilized during the acidification (to pH ~4.6) step of manufacture. Owing to the differences in casein and mineral composition, the potential of acid casein to hydrate and bind water is much higher than that of rennet casein when the pH is reversed (from 4.6) to that of native milk (i.e., ~6.7) because of its low calcium level and the presence of the highly hydrated caseino-macropeptide.

Hence, acid casein is converted to sodium or calcium caseinate to enhance its functionality. This essentially involves treatment of the 'dewatered' acid-casein curd (~40% moisture) with water, formation of a slurry (~25% dry matter) by passing through a colloid mill, addition of alkali and readjustment of the pH of the casein back to values of 6.8–7.0, heating at 75–90 °C, and drying. The type of alkali depends on the type of caseinate, for example, NaOH for sodium caseinate, $Ca(OH)_2$ for calcium caseinate, and NH_4OH for ammonium caseinate. Sodium caseinate is highly dispersible and forms highly viscous solutions at concentrations >~15% (w/w) protein; similarly, calcium caseinate is readily dispersible but not as soluble as sodium caseinate.

Despite its insolubility, rennet casein is the preferred type of casein for LMMCA and other block/shreddable CAs. By choosing the appropriate blend of calcium-sequestering (emulsifying) salts, the concentration of calcium cross-linking the *para*-casein molecules can be reduced to the desired level to give textural and cooking characteristics tailor-made to suit the envisaged application of the product. In contrast, caseinates (especially sodium caseinate) find most application in spreadable CAs (e.g., sauces, fillings, semiliquid toppings) where their high water-binding capacity and good emulsifying properties promote a desired creaming effect (*see* **Cheese**: Pasteurized Processed Cheese Products). It is noteworthy that the cooking properties (e.g., flowability, stretchability) of cheeses involve partial displacement of contiguous layers of the casein/*para*-casein on application of stress. Stress is heat induced (arising from deformation of fat globules and coalescence of free fat, and/or changes in the nature of protein interactions) and can also be applied externally by stretching of the molten cheese mass, for example, with a fork. The level of displacement for a given stress determines the suitability of cheese for a given application. A relatively high displacement coincides with a high flowability, which is suited to applications such as gratins and chicken cordon bleu, whereas a lower displacement gives a moderate flowability and higher stretchability and is more suited to pizza. For a given heat-induced stress, the level of displacement is influenced by various factors including *inter alia* the degree of calcium cross-linking and hydration of proteins.

Acid casein is generally not used in CA manufacture because of its insolubility, low pH, and its depressing effect on the pH of the CA blend, which in turn reduces the degree of dissociation of the ESs and their ability to sequester calcium from other proteins (e.g., rennet casein). Consequently, the use of acid casein at quantities of 1–3% (w/w) of the CA formulation can markedly extend the product make time, unless the pH of the blend is increased to its normal value prior to final pH adjustment (e.g., ~7.0–8.0) via addition of alkali and/or an ES blend with the desired pH-buffering effect.

Casein substitution

Owing to the high cost of caseins/caseinates, much effort has been vested in their partial replacement by cheaper casein substitutes. Vegetable proteins from various sources (soy, cottonseed, peanut, pea) have, in general, been found to give CAs that are inferior to those made using casein only, common defects being lack of elasticity, an adhesive/sticky body, and impaired flow and stretchability. Whey proteins are not generally used in CAs intended for cooking applications owing to the negative impact on flowability, except in applications where flow-resistant products may be needed (e.g., cheese insets in burgers) when ~1–3% (w/w) whey protein is added. The adverse effect on flowability is due to heat-induced denaturation and aggregation and/or gelation of added whey proteins or whey protein/*para*-casein complexes on heating the CA, during preparation or subsequent cooking.

To date, starch has been the most effective low-cost casein substitute and is used frequently, typically at levels of 2–4% (w/w) (**Tables 1** and **3**). Native maize starch appears to be the main type used commercially, with starches from other sources and with different types of modification (pregelatinized and/or chemically or enzymatically modified) being used less frequently. Following cooking, as in the manufacture of CA, starches (e.g., maize, wheat) with a high ratio of amylose to amylopectin tend to retrograde and undergo gelation more readily than those (e.g., waxy maize, rice, potato) with a lower amylose content. Continued regtrogradation during storage leads to contraction of the starch gel and a concomitant expression (syneresis) of the entrapped moisture. Owing to retrogradation, substitution of casein by starch with a high amylose-to-amylopectin ratio generally coincides with a decrease in the fluidity and flowability of the melted cheese (**Figure 2**). Moreover, on shredding, the unheated cheese tends to fracture more easily to form curd fines and also tends to exude free moisture, which often leads to sticking and balling. Conversely, starches with a high amylopectin-to-amylose ratio, such as waxy maize starch, are not as prone to retrogradation but can give CAs that are soft, sticky, and unsuitable for shredding, especially when included in the formulation at levels >2–3% (w/w). The above defects occur to a degree dependent on the type and level of added starch (**Figure 2** and **Table 4**), cooking temperature and time, degree of agitation, and cooling rate. However, with the typical processing regimes, native starches are used successfully commercially at a level of 2–4% (w/w) to replace ~10–15% of total casein.

Hydrocolloids may be also used in CAs, especially for the manufacture of high-moisture, low-protein (e.g., <14% (w/w) in LMMCA) products. Different hydrocolloids may be chosen, including anionic (κ- and ι-carrageenans, alginates, pectins) and nonionic/neutral (guar gum, xanthan, locust bean gum, konjac, or agar combinations) types, depending on the texture and physical properties (e.g., hardness, spreadability, flow, and stringiness on cooking) required in the final CA. Added κ-carrageenan or low methoxy pectin may react with free ionic calcium (in the presence of added calcium chloride) to form gel structures that compensate for the dilution of the casein matrix (in high-moisture products) and the concomitant reduction in elasticity; they thereby enhance the texture of the high-moisture composite CA product. Blends of neutral hydrocolloids may be chosen to give varying degrees of water binding (e.g., xanthan, guar gum) and gelation (agar, konjac, locust bean gum). However, care is required in the selection of appropriate hydrocolloid types/combinations and concentrations to ensure optimum balance of properties in the unheated (e.g., appropriate levels of elasticity to impart desired level of shreddability, sliceability, and/or spreadability) and heated (e.g., viscosity, fluidity, flow, and/or stringiness) CA products under the conditions of product manufacture (temperature, shear, time, and cooling rate).

Emulsifying salts

As for pasteurized PCPs, the blend includes ESs (e.g., sodium phosphates and sodium citrates) at a level of 0.5–3% (w/w). The salts promote, with the aid of heat and shear, the conversion of the insoluble acid casein or rennet casein to a hydrated sodium caseinate or sodium (calcium-phosphate) *para*-caseinate. The casein/*para*-caseinate emulsifies the dispersed oil droplets during processing and thereby contributes to the formation of a stable oil-in-water emulsion by reducing the interfacial free energy of the fat phase. The changes brought about by the ESs include calcium sequestration, upward adjustment and stabilization (buffering) of pH, and hydration of casein *para*-casein.

Calcium sequestration involves the exchange of the divalent Ca^{2+} (attached directly to the casein via the carboxyl groups of acidic amino acids and/or by attachment of colloidal calcium phosphate to serine phosphate residues) of the *para*-casein for the monovalent Na^{+} of the ES. This

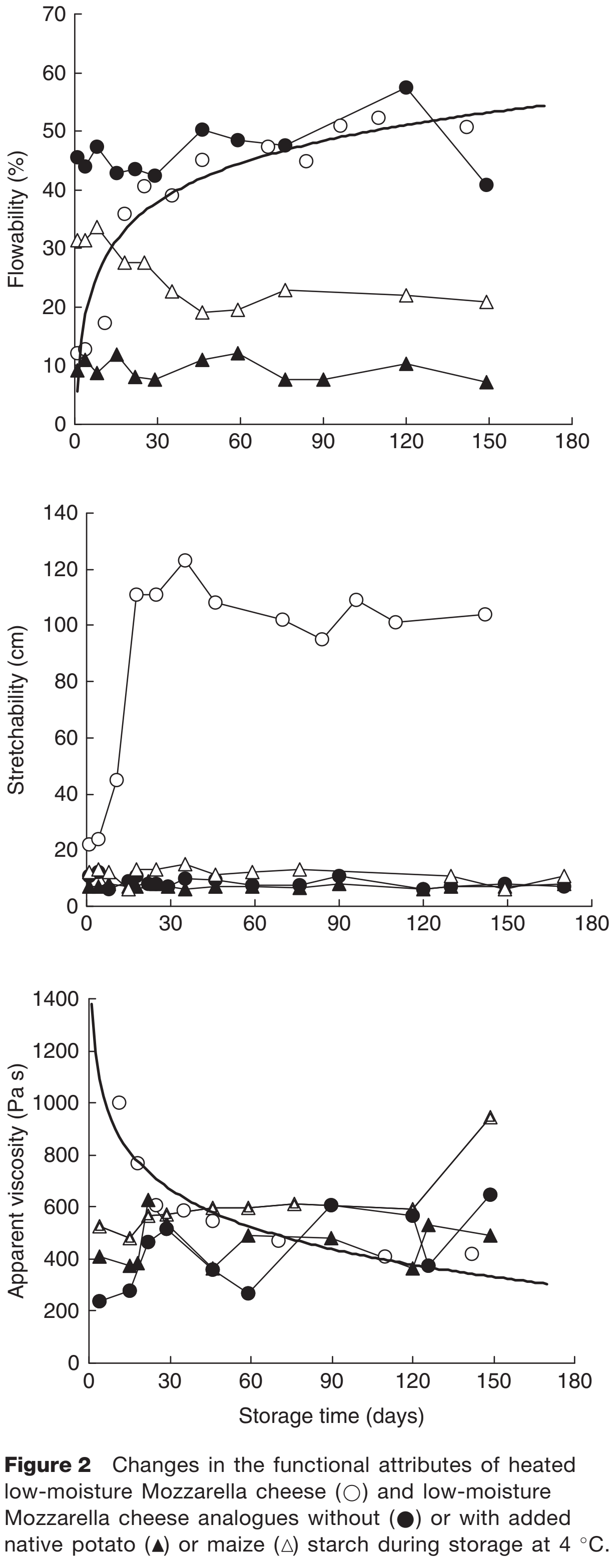

Figure 2 Changes in the functional attributes of heated low-moisture Mozzarella cheese (○) and low-moisture Mozzarella cheese analogues without (●) or with added native potato (▲) or maize (△) starch during storage at 4 °C. The results presented are the means of two replicate treatments; the composition of the low-moisture Mozzarella cheese is similar to the mean composition given in **Table 2**, while the compositions of the cheese analogues are given in **Table 4**.

ion exchange reduces the extent of calcium bridging that cross-links and aggregates the *para*-casein molecules. The use of the correct blend of ESs usually shifts the pH of the *para*-casein in the CA blend upward, typically from ~6.9 to 8.0–9.0, and stabilizes it by virtue of its high buffering capacity. Both factors (calcium sequestration and increase in pH) lead to a partial conversion of calcium phosphate *para*-casein to a negatively charged sodium *para*-caseinate and a concomitant increase in the hydration and solubility of the *para*-casein. Under processing conditions, the dispersed hydrated protein contributes to emulsification of the oil phase by coating the surface of dispersed free fat droplets. The relatively high water-binding capacity of the *para*-caseinate also enhances emulsion stability as it increases the viscosity of the aqueous phase and thus reduces the collision frequency of emulsified fat particles.

Premixing

Premixing of ingredients, which is not frequently practiced in the manufacture of CA, usually involves the blending of heated oil and casein in horizontal mixers with motorized single- or twin-shaft screws or ribbon-shaped paddles, typically for ~1 h. From an operational perspective, interaction of the oil with casein has the following potential effects:

- it reduces the processing time in the cooker during processing and thereby increases plant throughput;
- it prevents the direct contact of casein and water, as occurs on direct addition of ingredients to the cooker, and thereby minimizes the risk of lumps of undissolved casein being present in the end product, especially when the casein particles are small and the surface area is large, and the degree of agitation is low. Such lumps are formed when casein particles hydrate rapidly on impinging with the water to form a sticky plastic mass which adheres to nonwetted casein around which it forms an impervious plastic layer.

Order of Addition of Ingredients to the Cooker

Following premixing, the oil–casein blend is pumped to the cooker that already contains water, ESs, and other ingredients, added in that order. When premixing is not practiced, the ingredients are added directly to the cooker, while agitating continuously. A typical manufacturing protocol involves the following steps:

- simultaneous addition of water and dry ingredients (e.g., casein, ESs);
- addition of a portion (~90%) of the oil;
- heating to ~80–90 °C, using direct steam injection, typically at a rate of 15–20 °C min^{-1};

Table 4 Composition of LMMCA with or without starch

	Cheese		
	LMMCA without starch	*LMMCA with potato starch*	*LMMCA with maize starch*
Moisture (%, w/w)	49.6 (3.26)	50.8 (0.83)	50.1 (1.17)
Fat (%, w/w)	28.0 (2.67)	25.5 (0.62)	26.5 (2.43)
Protein (%, w/w)	16.8 (0.86)	14.6 (0.04)	14.1 (0.44)
Ca (mg g^{-1} protein)	32.6 (0.98)	33.1 (2.4)	33.8 (3.42)
P (mg g^{-1} protein)	2.4 (0.48)	27.1 (3.11)	26.4 (0.83)
Added starch (%, w/w)[a]		4.8	5.1

[a] Estimated; calculated from the difference in the contents of moisture, fat, protein, and ash.

The data presented are the means of two replicate treatments; standard deviations are in parentheses.

LMMCA, low-moisture Mozzarella cheese analogue.

Guinee *et al.* unpublished data.

- holding until a uniform homogeneous molten mass is obtained (typically 5 min);
- addition of remaining oil, flavoring materials, and acidifying agents;
- holding for a further 1–2 min;
- hot packing the molten blend.

The order of adding ingredients varies with plant practices, the hydration properties of the casein, type and level of starch in the formulation, cooker type, overall plant design, duration of cooking, and the end product characteristics. Thus, if a CA with good flowability on cooking is required, a portion of the oil may be added at an advanced stage of cooking (e.g., after casein hydration) when casein hydration is slow and a high shear cooker is used. Food-grade acids are added to adjust the pH of the CA to that required in the finished product (**Table 2**). The addition of the acid at the end of manufacture rather than at the beginning ensures a high pH (~7–9) in the blend during processing. This procedure is desirable in the manufacture of CAs where insoluble rennet casein is the major protein ingredient. A high pH during processing is conducive to greater sequestration of calcium from the rennet casein by the sodium phosphate ESs and gives a higher negative charge to the casein. Both factors mediate the conversion of the calcium phosphate *para*-casein to sodium *para*-caseinate, which binds water and emulsifies the vegetable oil. Thus, reducing the pH of the blend during processing increases the time required for the formation of the CA and probably affects its properties (e.g., firmness, meltability). The addition of flavors toward the end of processing minimizes the loss of flavor volatiles.

Processing

Processing refers to the heat treatment of the blend, indirectly or by direct steam injection, while constantly agitating. Processing contributes to the uniform distribution of all blend ingredients, the dissolution of the ESs and their interaction with the rennet casein, dispersion of fat into droplets, emulsification of fat droplets by the hydrated sodium *para*-caseinate, and, consequently, formation of the CA. It also inactivates any potential pathogenic and spoilage microorganisms, and thereby extends the shelf life of the product.

The type of cooker used for processing depends on the textural attributes of the final product and its application. Cookers that impart a high degree of shear (e.g., agitator speed of 250–1500 rpm; Stephan, Scanima, Limitech) are normally used for the manufacture of analogues of high-moisture pasteurized processed cheese spreads or dips. High shear promotes a high degree of emulsification, which coincides with a thick viscous body and creamy mouthfeel in the end product. In contrast, cookers that ensure adequate blending and a relatively low degree of mechanical shear (e.g., agitator speed ~70 rpm) are used for the manufacture of CAs in which a relatively low degree of fat emulsification is required. An example of such a requirement is a semihard (block) CA for slicing, shredding, and cooking in applications where a moderate flowability and/or a slight oiling-off (free oil) are the major quality attributes, for example, LMMCA for use in pizza, or block processed CA for use in burgers. The relatively low degree of emulsification is desirable since heat-induced flowability (meltability) and oiling-off in CAs are inversely correlated with the degree of emulsification. Typically, horizontal cookers (e.g., Blentech, Damrow) with single- or twin-screw type agitators are used for the manufacture of the latter CAs.

Homogenization

Homogenization is not normally practiced, except for analogue cheese spreads or dips. Typical first- and second-stage pressures are 15 and 5 MPa, respectively. Homogenization has a number of effects:

- it assists in further mixing and degradation of undissolved particles (e.g., ES, dry ingredient);
- it results in further shearing of the blend and interaction of blend ingredients;
- it promotes a finer dispersion of fat droplets;
- it promotes thickening.

Properties of Cheese Analogues

Composition

Analysis of commercial LMMCAs indicates large variations in composition (**Table 2**). Comparison with commercial low-moisture Mozzarella cheese (LMMC) shows that LMMCA has lower protein content, higher concentrations of moisture and fat, and higher ratios of Ca:protein and P:protein. The higher ratios of Ca:protein and P:protein reflect the use of rennet casein (which has higher concentrations of Ca and P on a protein basis than most natural cheeses) and the inclusion of sodium phosphate ES during formulation. Moreover, the mean value of the sum of moisture, fat, protein, and ash is ~96.5% (w/w), compared to ~99.5% (w/w) in the LMMC, suggesting the addition of carbohydrate-based ingredients during formulation of the CA (**Table 2**).

Functionality

Cheese is used extensively in cooking applications, mainly because of its flavor and functionality. Functionality is a composite of different attributes of the raw cheese (e.g., shreddability) and the heated cheese (e.g., ability to flow and stretch, thickness or viscosity, degree of oiling-off, surface sheen) (*see* **Cheese**: Cheese as a Food Ingredient). Shreddability is used to encompass several characteristics, including the ability of the cheese to shred into thin strips of uniform dimensions, and the ability of the shreds to resist fracture and sticking/clumping during the shredding process or when compressed. Depending on the application, one or more functional attributes may be required.

Analysis of commercial LMMCAs indicates large variations in functional attributes of importance for use on pizza. A comparison with LMMC indicates that the LMMCAs have similar mean values for flowability and apparent viscosity but markedly lower stretchability. The differences in stretchability between LMMC and LMMCA may be related primarily to differences in the composition and the degree of aggregation and microstructure of the *para*-casein, as affected by the differences in make procedure between the two products. In the production of LMMC, the heating and kneading the curd in hot water or brine at high temperatures are conducive to aggregation of the *para*-casein matrix and the formation of *para*-casein fibers with a high tensile strength. In contrast, the manufacturing process used for CA is designed to sequester calcium from the *para*-casein, disaggregate the *para*-casein aggregates, and transform *para*-casein to a hydrated sodium *para*-caseinate.

The functionality of heated LMMC, and other natural cheeses including Emmental and Gouda, generally undergoes marked changes during storage at 4 °C (**Figure 2**). Indeed, a storage period of ~5–10 days is generally required for the LMMC to attain satisfactory cooking performance, unless the calcium content and pH are adjusted, via the manufacturing protocol, to optimize casein hydration. In contrast, there is comparatively little change in the functionality of LMMCA during storage (**Figure 2**). The differences in degree of storage-related changes between CAs and natural cheeses are associated with differences in the manufacturing technology (e.g., higher temperature reached in the production of CA, i.e., ~75–85 °C compared to ≤~53 °C in the manufacture of natural cheeses), and in the microstructure, composition and degrees of protein hydration in the final products.

See also: **Cheese**: Cheese as a Food Ingredient; Pasteurized Processed Cheese Products. **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Functional Properties of Milk Proteins.

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Cheese as a Food Ingredient

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Introduction

A total of 500–800 different cheese varieties are manufactured globally; they differ to varying degrees in nutritive value, appearance, flavor, texture, and cooking properties. Consequently, cheese is capable of satisfying a diverse range of sensory and nutritional demands and, therefore, has very wide appeal. The functionality of cheese is extended when it is subjected to secondary processing to create an array of ready-to-use grated and shredded cheeses, pasteurized processed cheese products (PCPs), cheese powders (CPs), and enzyme-modified cheeses (EMCs) (**Figures 1** and **2**).

Cheese is delivered to the consumer through three main sectors, namely

- the commodity or retail sector, where cheese is mainly in the form of portions (e.g., blocks, slices) that are consumed in the home directly as a table food or indirectly as an ingredient in various dishes;
- the food service sector, where cheese is presented in the form of cheese board/cheese plate, or incorporated as an ingredient in various dishes (e.g., lasagne, pizza, omelette, cheese panini, sandwich);
- the industrial sector, where cheese is used as an ingredient in the manufacture of a vast array of assembled food products (e.g., pizza, sandwiches) or formulated foods (e.g., gratins, prepared meals, dried soups) (**Figure 3**).

The percentages of total cheese delivered via the commodity/retail, food service, and industrial sectors amount to 60, 20, and 10% respectively, in the European Union and 33, 33, and 33%, respectively, in the United States. These trends suggest that an estimated 35–45% of total cheese is consumed as an ingredient in other foods; moreover, recent market analyses indicate that the consumption of cheese as an ingredient is rapidly growing.

Definition, Types, and Applications of Cheese as an Ingredient

Cheese is used directly as an ingredient in the preparation of an extensive array of culinary dishes in the home/catering (food service) sectors and prepared foods in the industrial sector (**Figures 3–5**). Additionally, natural cheese is used extensively by the industrial sector in the mass production of so-called cheese ingredients, which include ready-to-use grated cheeses, shredded cheeses, cheese blends, dried cheeses, PCPs, CPs, and EMCs (**Figure 2**). The latter products are in turn used as ingredients by the manufacturers of formulated foods and, to a lesser extent, by the catering/food service industries (**Figures 4** and **5**).

While any cheese may be used as an ingredient, the most widely used varieties include Mozzarella (in pizza), Cheddar (in CPs), fresh acid-curd varieties (in cheesecake and dairy desserts), and pasteurized processed Cheddar (as slices in burgers).

The major cheese ingredients are defined in more detail below.

Pasteurized Processed Cheese Products

PCPs are produced by comminuting, melting, and emulsifying, into a smooth homogeneous molten blend, one or more natural cheeses and optional ingredients using heat, mechanical shear, and (usually) emulsifying salts (*see* **Cheese**: Pasteurized Processed Cheese Products). PCPs may be consumed directly as table cheeses or spreads, as a culinary ingredient, or as an ingredient in assembled (e.g., pizza, salads) and formulated (e.g., soups, cheese sauces, gratins) food products.

Dehydrated Cheeses Ingredients

Dehydrated cheeses were first developed during World War II as a means of preserving cheese solids under unfavorable conditions to which natural cheese would not be subjected, for example, holding at >21 °C for a long period of time. Obviously, the advantages of dried cheese in terms of convenience and application soon became obvious, and other dehydrated cheese-based products were developed over time, including CPs (processed plus dried cheese products) and dried EMCs. Today, dehydrated cheese ingredients (DCIs) are a product category of major economic importance because of their ubiquitous use as flavoring agents and/or nutritional supplements in a wide range of foods, including bakery products, biscuits, dehydrated salad dressings, sauces, snack coatings, soups, pasta dishes, savory baby meals, cheese dips, *au gratin* potatoes, and ready-prepared meals (**Figure 3**). They may also be included in processed

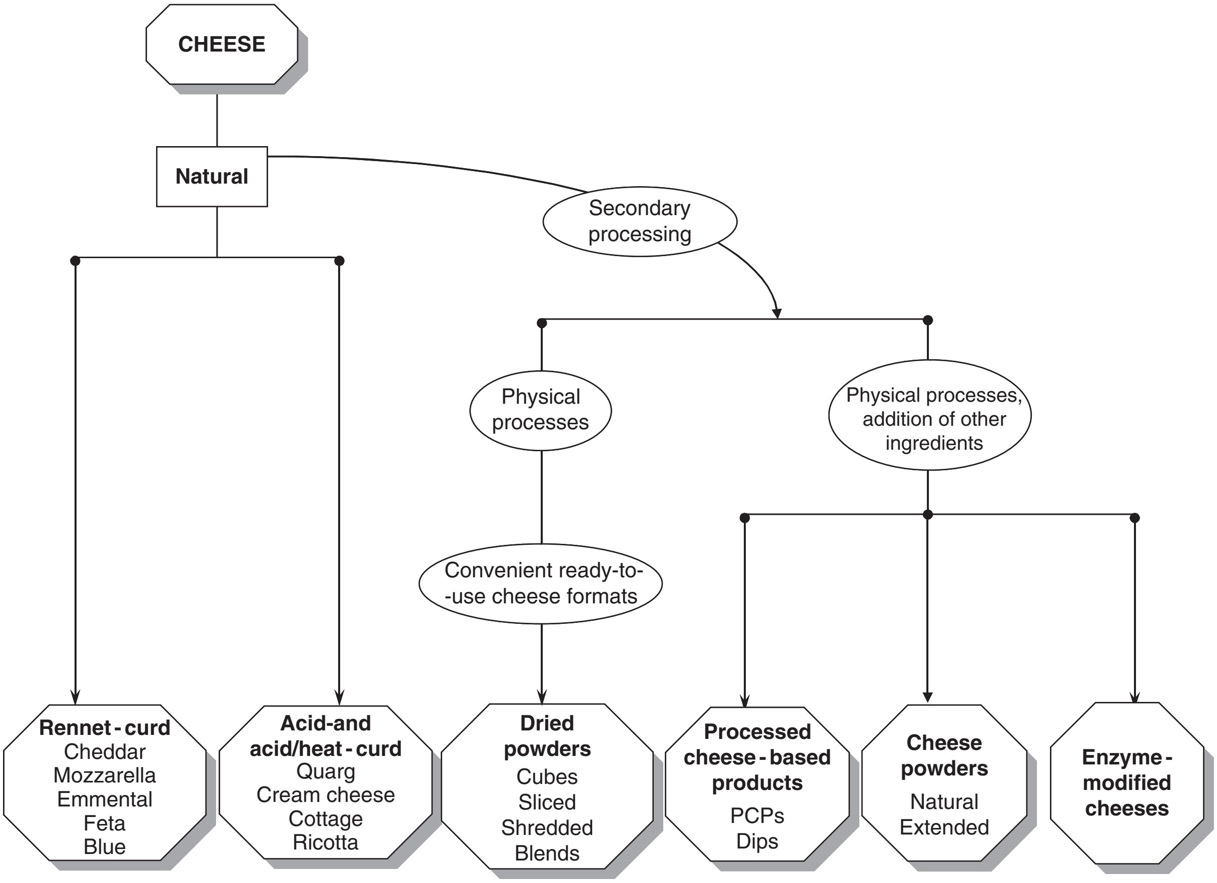
CHEESE
Natural
Secondary processing
Physical processes
Physical processes, addition of other ingredients
Convenient ready-to--use cheese formats
Rennet-curd
Cheddar
Mozzarella
Emmental
Feta
Blue
Acid-and acid/heat-curd
Quarg
Cream cheese
Cottage
Ricotta
Dried powders
Cubes
Sliced
Shredded
Blends
Processed cheese-based products
PCPs
Dips
Cheese powders
Natural
Extended
Enzyme-modified cheeses

Figure 1 Types of cheeses and industrial cheese ingredients. PCP, pasteurized processed cheese products.

Galtee
Galtee Redi-Slices
FALLIN!
1000g

Figure 2 Cheese ingredients, including pasteurized processed cheese products in different formats (slices, block, cylinders), grated cheese, and shredded cheese.

and analogue products as flavoring agents or as a functional ingredient in powdered instant cheese preparations, which can be reconstituted by the consumer for the preparation of instant functional cheeses (e.g., pizza type) for domestic use. Dehydrated cheese products have certain advantages over natural cheese, including

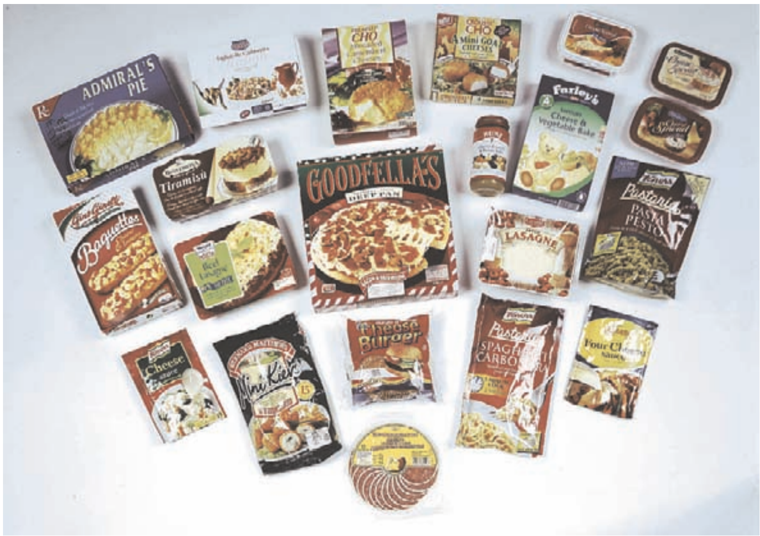

Figure 3 Selection of common retail foods in which cheese is an ingredient: pasteurized processed cheese spreads, dry cheese sauces, pizza, prepared meals (pie, lasagne, cheese-filled kiev, pasta), cheeseburger, cheese-coated salami, desserts (breaded Camembert, tiramisu), and infant meals.

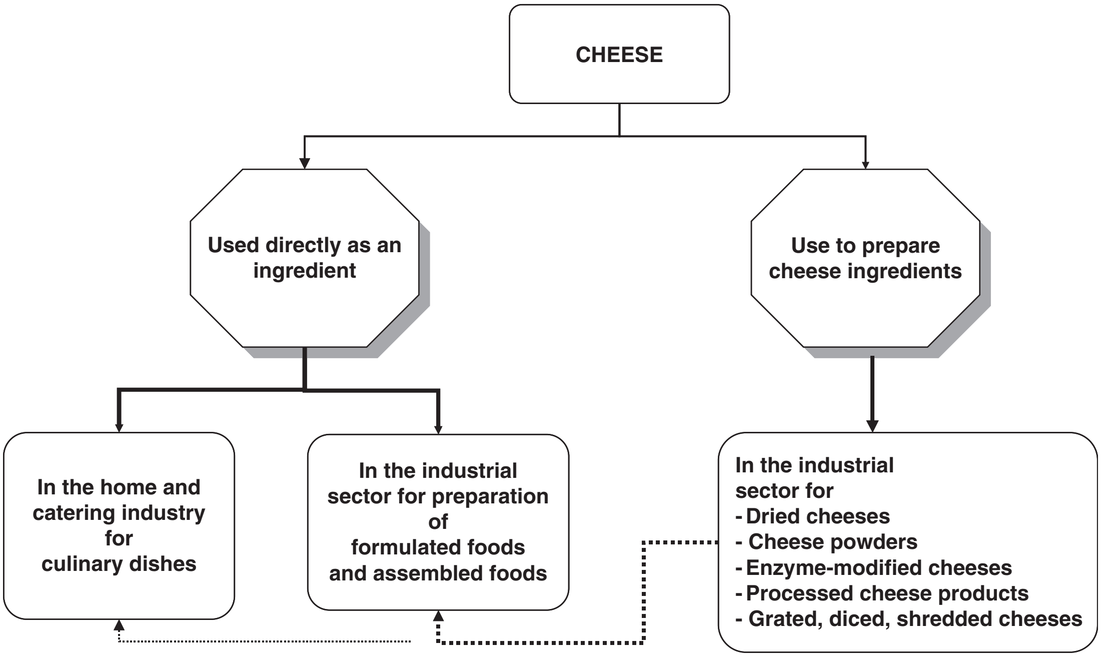
CHEESE
Used directly as an ingredient
Use to prepare cheese ingredients
In the home and catering industry for culinary dishes
In the industrial sector for preparation of formulated foods and assembled foods
In the industrial sector for
- Dried cheeses
- Cheese powders
- Enzyme-modified cheeses
- Processed cheese products
- Grated, diced, shredded cheeses

Figure 4 Sectors in which cheese is used as an ingredient in other foods or for the preparation of cheese ingredients.

1. Convenience of use by formulated food manufacturers. DCIs can be applied easily to the surface of snack foods (e.g., popcorn, potato crisps, nachos) or incorporated into formulated foods, for example, by dry mixing with other ingredients such as skim milk powder (e.g., as in dried soup, sauce, or cake mixes) or by blending into wet formulations. Natural cheeses require size reduction prior to their use in these applications. Moreover, CPs lend themselves to easier inventory management, set-manufacturing methods, and end products with consistent quality in large-scale manufacturing operations.
2. Longer shelf life than natural cheese, owing to their lower water activity (a_w, 0.2–0.3 compared to 0.91–0.99). The relatively high stability of CPs allows them to be stored for a long period without alteration or deterioration in quality. In contrast, the changes that occur in natural cheese during storage may influence its processability and flavor profile and intensity.

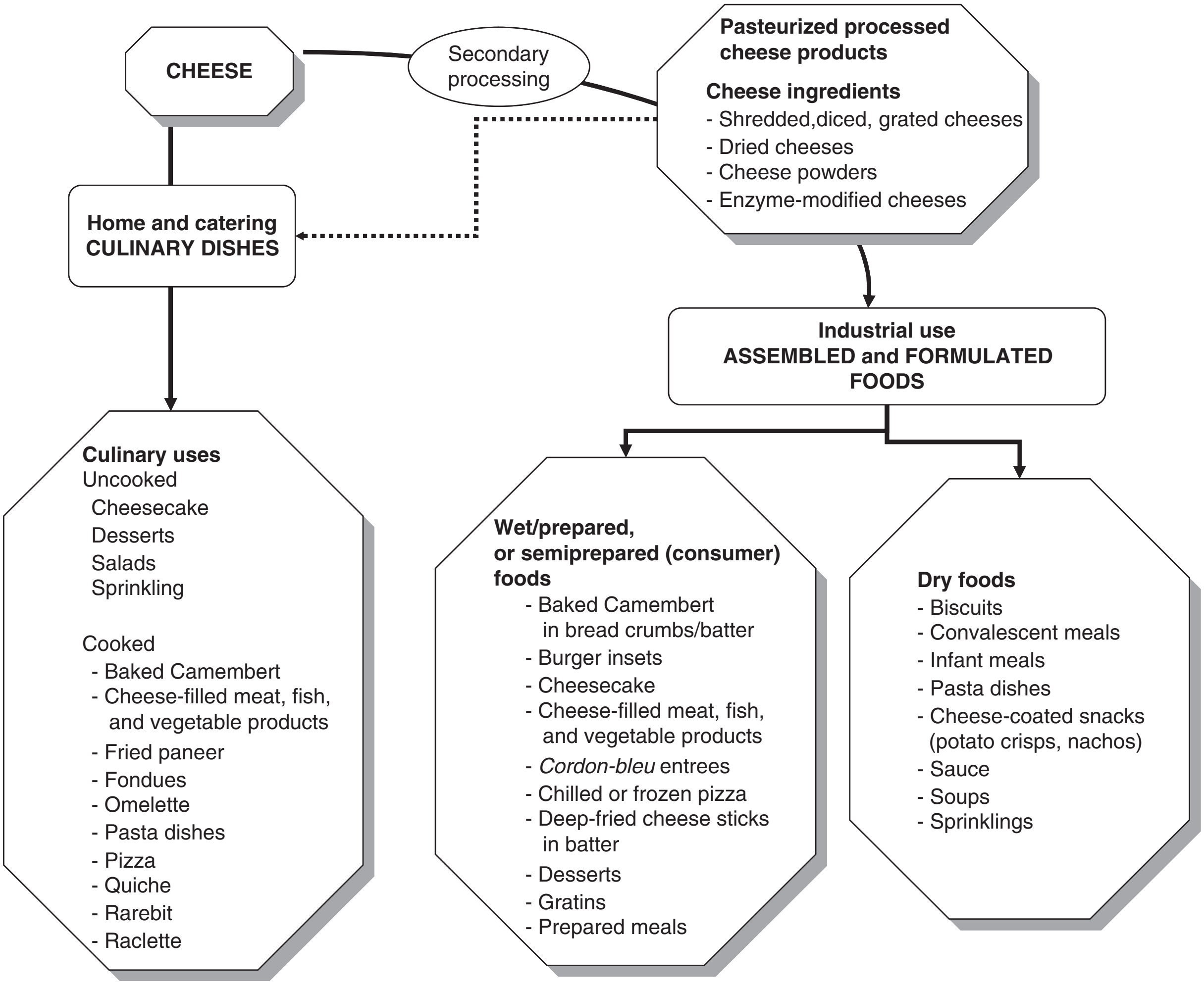
CHEESE
Secondary processing
Pasteurized processed cheese products
Cheese ingredients
- Shredded,diced, grated cheeses
- Dried cheeses
- Cheese powders
- Enzyme-modified cheeses
Home and catering CULINARY DISHES
Industrial use ASSEMBLED and FORMULATED FOODS
Culinary uses
Uncooked
Cheesecake
Desserts
Salads
Sprinkling
Cooked
- Baked Camembert
- Cheese-filled meat, fish, and vegetable products
- Fried paneer
- Fondues
- Omelette
- Pasta dishes
- Pizza
- Quiche
- Rarebit
- Raclette
Wet/prepared, or semiprepared (consumer) foods
- Baked Camembert in bread crumbs/batter
- Burger insets
- Cheesecake
- Cheese-filled meat, fish, and vegetable products
- Cordon-bleu entrees
- Chilled or frozen pizza
- Deep-fried cheese sticks in batter
- Desserts
- Gratins
- Prepared meals
Dry foods
- Biscuits
- Convalescent meals
- Infant meals
- Pasta dishes
- Cheese-coated snacks (potato crisps, nachos)
- Sauce
- Soups
- Sprinklings

Figure 5 Uses of cheese as an ingredient.

3. Greater diversity of flavor that can be obtained compared to natural cheese, made possible by the use of different cheese types, EMCs, and other ingredients in their preparation.

Dehydrated cheese products may be arbitrarily classified into four categories:

1. Dried grated cheeses (e.g., Parmesan, Romano), which are used as highly flavored sprinklings (e.g., for pasta dishes, soups) and in bakery products (e.g., biscuits).
2. CPs, which may be natural (made using natural cheeses, emulsifying salts, and, optionally, natural cheese flavors) or extended, which incorporate other ingredients, such as dairy ingredients (e.g., skim milk solids, whey, lactose), starches, maltodextrins, flavors, flavor enhancers, and/or colors. Alternatively, CPs can be classified according to the proportion of cheese solids, as a percentage of total dry matter: high cheese solids (~95%, w/w), medium cheese solids (>50%, w/w), or low cheese solids (<50%, w/w). CPs are used in numerous applications, especially in formulated foods prepared by dry blending different ingredients (e.g., dry soups, sauces, cake mixes) and as snack coatings (e.g., popcorn, nachos, tortillas).
3. Dried EMCs, which are essentially cheese curds that are modified, through the addition of water, enzymes, starter culture, and/or other ingredients (e.g., lyophilized butter oil), and by processing (as for PCPs), to accelerate the development of intense cheese flavors that mimic those of specific natural cheese varieties (e.g., Cheddar cheese). Following flavor development, the curd (usually referred to as paste) is pasteurized and dried. Compared to natural cheeses, EMCs offer certain advantages as flavoring agents: (1) high flavor intensity, which enables small quantities to impart strong cheese flavor, (2) high flavor consistency and stability, and (3) the suitability of dried EMCs for dry blending makes them suited for an array of applications, for example, bakery and snack foods.

Technology and Manufacture of Cheese Ingredients

Dried Cheeses

Production involves fine grinding of hard cheeses and conveyance of the ground cheese to a dryer (usually fluidized bed type) where it is exposed to low-humidity air (e.g., 15–20% relative humidity) at air inlet temperatures <30 °C. Under these conditions, the vibrated cheese is dehydrated rapidly and evaporatively cooled, thereby reducing the risk of fat exudation and the tendency to balls/clumps. The dried grated cheese (typically 17% moisture) is generally pulverized and packaged under nitrogen to reduce the risk of oxidative rancidity during distribution and retailing. The natural cheeses most suitable for drying are those that are hard and brittle and have an intense flavor and low contents of moisture and fat in dry matter, for example, Parmesan and Romano. These properties lend themselves to efficient size reduction, a low susceptibility to fat exudation and to sticking of the grated cheese particles, efficient drying, and a homogeneous product free from lumps. The moisture content of dehydrated grated cheese may be further reduced to ~3% (w/w) by placing it on trays that are conveyed through a drying tunnel, where it is exposed to hot air, that heats the cheese particles to 63 °C.

High-moisture (82%, w/w) cheeses, such as Cottage cheese, may also be dried directly to 3–4% (w/w) moisture by first pulverizing and then subjecting them to specialized spray-drying operations (e.g., Silo spray drying using the Birs dehydration process). These low-moisture, dried natural cheeses are generally used for nutritional supplementation of foods, for example, dried baby meals.

Freeze-dried formats of a number of different cheeses such as mature Cheddar, Gloucester, Stilton, and oak-smoked Cheddar have been developed since the late 1990s. Their manufacture involves size reduction to the required format (e.g., cubes), layering onto trays, freeze-drying to ~3% (w/w) moisture, and packaging.

The benefits of freeze-drying, compared to air-drying or spray-drying, include

- higher retention of volatile flavor compounds (e.g., methyl ketones, dimethyldisulfide); and
- ability to dry cheeses in the form of shapes (croutons, cubes) that convey an image of cheese pieces and more naturalness compared to powder

The crunchy light texture of the dried cheese pieces makes them easy to disperse (by rubbing between the fingers) as a topping and to rehydrate, allowing fast flavor release in the mouth.

Cheese Powders

The manufacture of CPs essentially involves the production of a pasteurized processed cheese slurry (40–45%, w/w), which is then spray dried. A generalized manufacturing protocol, as described in **Figure 6**, involves the following steps:

- formulation, selecting the different types and levels of ingredients (including cheese, water, emulsifying salts, and others) to give the desired powder composition and characteristics;
- processing the blend, involves heating the blend to ~80 °C while constantly shearing to form a hot molten slurry, with a dry matter content of ~45% (w/w);
- homogenization of the hot molten blend, typically at first- and second-stage pressures of 15 and 5 MPa, respectively; and
- drying of the hot molten blend, using any one of several spray-drying processes (single-stage or two-stage) and dryer configurations (e.g., tall-form, Filtermat) available.

The blend usually consists of comminuted natural cheese(s), water, emulsifying salts, flavoring agents, flavor enhancers, colors, antioxidants (e.g., propyl gallate or butylated hydroxyanisole), and/or filling materials such as whey, skim milk solids, starches, maltodextrins, and/or butterfat. The flavor profile and intensity of the final CP are determined by the characteristics of the cheese (variety, degree of maturity) and the types and concentrations of different flavoring agents. Generally, mature cheese with an intense flavor is used to impart a strong flavor to the final product. Young cheeses are generally not used because of their lack of flavor and high concentration of intact casein, which imparts high viscosity to the slurry, making it difficult to atomize and dry efficiently.

Filling materials in extended CPs are usually added to replace cheese solids and thereby lower the formulation costs. However, they may influence flavor, wettability, and mouth-coating characteristics of the product in which the cheese powder is used. The type and level of ingredients are determined by the type of CP (natural, extended, flavor intensity) and its application (e.g., whether intended for sauce, soup, snack coating, or cheese dips).

The processing of the blend is similar to that for PCPs in both principle and manufacturing technology (*see* **Cheese**: Pasteurized Processed Cheese Products). The blend is heated and sheared until the hot molten blend (sometimes denoted ‘slurry’) is homogeneous in color and consistency and free from lumps or nonhydrated material. The temperature is typically maintained at <85 °C to minimize the loss of volatile flavor compounds and reduce the risk of browning, especially in formulations containing high levels

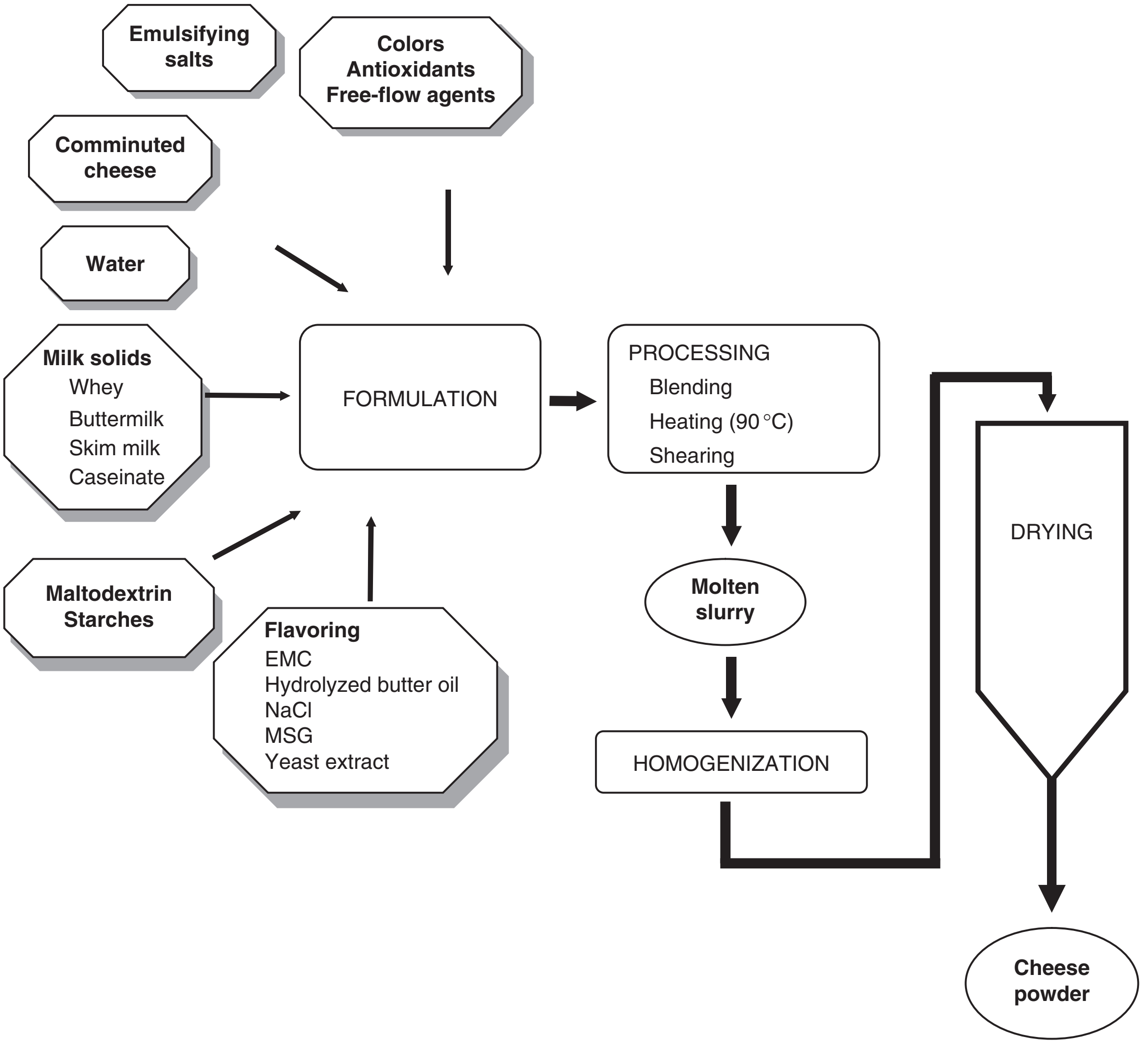
Emulsifying salts
Colors
Antioxidants
Free-flow agents
Comminuted cheese
Water
Milk solids
Whey
Buttermilk
Skim milk
Caseinate
FORMULATION
PROCESSING
Blending
Heating (90 °C)
Shearing
DRYING
Maltodextrin
Starches
Flavoring
EMC
Hydrolyzed butter oil
NaCl
MSG
Yeast extract
Molten slurry
HOMOGENIZATION
Cheese powder

Figure 6 Production process for cheese powder. EMC, enzyme-modified cheese. MSG, monosodium glutamate.

of reducing saccharides (e.g., lactose or maltodextrins with a high dextrose equivalent). Following processing, the hot blend is usually homogenized, and then dried typically at an inlet air temperature of 180–200 °C and an outlet air temperature of 85–90 °C, depending on the dryer type. The physical characteristics (e.g., bulk density, wettability, solubility, dispersability) and flavor of the powders are influenced by the drying conditions, for example, atomization type, pressure, and air inlet and outlet temperatures; thus, the instant characteristics may be varied by alteration of drying conditions. The physical properties are important in applications requiring reconstitution of the CP, for example, prepared soups, sauces, and baby foods.

Enzyme-Modified Cheeses

Natural cheeses may have certain limitations as a food ingredient, including low flavor stability due to ongoing biochemical/microbiological changes during storage, flavor inconsistency, insufficient flavor strength to enable small quantities to impart a strong cheese flavor, relatively high cost, and the need to comminute prior to their application. These deficiencies led to the development of EMCs, by exploiting the natural biochemical cheese flavor pathways through enzyme technology, which resulted in cheese flavor intensities of up to 30-fold that of the corresponding natural cheese. EMCs can be defined as concentrated cheese flavor ingredients, which offer a cost-effective alternative to natural cheese as a source of cheese flavor.

A generalized manufacturing protocol for EMC involves:

- comminution of fresh cheese curd to a slurry or paste (typically ~40–50%, w/w, dry matter), by blending with water and emulsifying salts, and subjecting the blend to heat and shear;
- pasteurization of the paste, at temperatures of 72–80 °C for 10–20 min to inactivate the existing cheese

microflora and enzymes, and thereby reduce the risk of flavor inconsistencies due to variations in the strain composition and populations of starter culture and non-starter lactic acid bacteria and in the residual enzyme activities (e.g., chymosin) in cheese curd;
- treatment of the paste with ripening agents, which involves cooling the paste and the addition of a cocktail of enzymes (e.g., lipase, peptidase, proteinase), starter cultures, and/or starter culture adjuncts to give the required flavor profile and intensity;
- incubation of the paste, at 25–35 °C for ~ 1–4 days with constant agitation and pH control (5–7) to allow the added enzymes act on the paste substrates to produce the correct balance of proteolytic (peptides, free amino acids amines, aldehydes, alcohols, and ammonia) and lipolytic (free fatty acids, ketones and alcohols) products;
- optional addition of other materials, such as flavor enhancers (e.g., NaCl);
- pasteurization of the enzyme-treated paste, to inactivate added enzymes and preserve the generated characteristics with minimum change on storage; and
- homogenization of the hot pasteurized paste, to reduce the tendency of phase separation on subsequent storage and thus to ensure product homogeneity.

The cheese paste, known as EMC paste, may then be packaged and stored at refrigeration temperatures. The paste is usually stored in opaque containers to minimize the risk of oxidative rancidity and development of associated off-flavors. The paste may be dried to give EMC powder, which has a longer shelf life than the paste and is better suited to applications involving dry blending with other ingredients (*see* **Cheese**: Enzyme-Modified Cheese).

Manufacture of Foods Containing Cheese or Cheese Ingredients

When used as an ingredient in other foods or in the preparation of cheese ingredients, cheese is subjected to an array of treatments such as comminution (e.g., portioning, shredding, grating, grinding), cooling, freezing, thawing, heating, and/or reheating. In the home and food service sector, the cheese-containing dish, once pre-prepared, is generally cooked and consumed immediately. In contrast, following assembly or formulation in the industrial sector, cheese-containing foods are frequently heated or preheated (precooked) and frozen, and are then reheated prior to consumption. The treatments of cheese during the manufacture of cheese-filled coextruded products (CFCPs) (e.g., cheese-filled croquette or meatballs) are typical of those applied during the manufacture of many formulated foods. They include

- preparation of a cheese filling by dicing, grating, blending, and/or processing (as in PCPs);
- preparation of other food materials, for example, coarse meat emulsion (burgers), fine meat emulsion (sausage), potato mash (croquette), and another cheese (e.g., where two different cheeses are coextruded);
- placing the prepared materials in two separate robotic-controlled filling machines of the coextruder unit (e.g., Vemag Verdener Maschinen-und Apparatbau, GmbH; Rheon Automatic Machinery Co. Ltd.), which enables the correct proportions of the two materials to be ejected together, one as a filling inside the other, as a continuous filled cylinder; and
- automated portioning of the cylinder (to create cylindrical or ball-shaped portions) and sealing of the inner filling.

The treatment of the filled materials varies according to the type of product. Cheese-filled sausages are filled into casings and are then cooked or smoked and subsequently cooled. Cheese-filled meatballs are typically dispatched onto a system of rotating belts and conveyed sequentially through a batter-depositing unit, a breading unit, a deep-fat frying chamber, a draining unit (to allow drainage of excess oil), a continuous freezer, and a cartoning unit.

Consideration of the various processes indicates that size reduction (comminution) and heating are the most common processes to which cheese is exposed when used as an ingredient. Hence, the behavior of the cheese during these processes is a major determinant of its functionality and its suitability as an ingredient.

Properties of Cheese as a Food Ingredient

When used as an ingredient in food applications, cheese is required to perform one or more functions, which may be arbitrarily categorized into three main headings:

- flavor,
- functional properties of raw cheese (e.g., texture), and
- functional properties of heated cheese (e.g., cooking properties).

Flavor

The flavor of cheese is an important quality factor in most applications where cheese is used as an ingredient. The importance of the flavor contribution is highlighted by

- increase in the use of highly flavored EMCs for a range of products such as PCPs, imitation cheese products and CPs, ready-prepared meals, snacks, soups, and sauces; and
- increase in the use of cheeses, such as mature Cheddar and Colby, with poor stretchability (compared to low-moisture Mozzarella) when baked, in pizza toppings.

The biochemistry of flavor generation in cheese is complex and has been reviewed extensively (*see* **Cheese**: Cheese Flavor). The flavors of most cheeses are quite similar immediately after manufacture. However, the individual character of the variety develops during ripening as a consequence of a concerted series of microbiological, enzymatic, and chemical changes, which include proteolysis, lipolysis, and the metabolism of residual lactose, lactate, and citrate. Intervariety differences in the extent of these pathways are attributable to concomitant differences in the type/composition of milk used, types of starter cultures used, cheese making conditions, composition, and/or ripening conditions.

Texture Properties

In all applications, whether as a consumer product or as an ingredient, cheese is subjected to size-reduction operations involving a combination of shear and compressive stresses that result in fracture. Cheese may be portioned (e.g., for consumer packs), sliced, crumbled (e.g., Feta, Stilton; salads), shredded (e.g., sandwiches, pizza), diced (salads) or grated (e.g., dried Parmesan), comminuted (e.g., in the preparation of sauces, PCPs), or compressed and sheared during mastication and consumption. The behavior of cheese when subjected to different size-reduction methods constitutes a group of important functional properties, which are summarized in **Table 1**. These properties are related to the rheological characteristics (**Table 2**), which determine the following parameters during size reduction:

- magnitude of the stress required to fracture (fracture stress),
- the degree of strain (e.g., change in dimensions) at fracture (fracture strain),
- the ease with which the cheese fractures, and
- the type of fracture (i.e., clean or jagged).

Table 1 Functional properties of unheated cheese

Property	*Definition*	*Examples of cheese with these properties*
Shreddability	The ability to • shred into thin strips of uniform dimensions • to resist fracture during shredding • to resist clumping/balling during shredding	Low-moisture Mozzarella, Swiss-type cheese, mild Cheddar, Gouda, Provolone, PCA
Sliceability	The ability to be cut cleanly into thin slices without fracturing or crumbling or sticking to cutting implement	Low-moisture Mozzarella, Swiss-type cheese, Provolone, analogue Pizza cheese, some PCPs
Gratability	The ability to fracture into small particles, with a low tendency to stick, on shearing and crushing	Parmesan, Romano
Spreadability	The ability to spread easily when subjected to a shear stress	Mature Camembert, cream cheese, mature blue cheese, some PCPs and CAs (depending on formulations)
Crumbliness	The ability to break down into small irregular-shaped pieces when rubbed (at low deformation)	Feta, Blue cheese, Cheshire

PCPs, pasteurized processed cheese products; PCA, pizza cheese analogue; CAs, cheese analogues.

Table 2 Some rheological properties of cheese that affect its performance as an ingredient

Rheological property	*Definition*	*Examples of cheeses with these properties*
Elasticity	Tendency to recover to its original shape and dimensions upon removal of the deforming force	Low-moisture Mozzarella, Emmental
Brittleness	Tendency to fracture at a relatively low strain	Romano, Parmesan
Firmness (hardness)	High resistance to deformation (strain) on application of a stress factor	Cheddar, especially reduced-fat types, Emmental, Gouda, Romano, Parmesan
Softness	Low resistance to deformation on application of a stress factor	Cream cheese, mature Camembert, blue cheese, some PCPs (e.g., spreads)
Adhesiveness	Tendency to stick to another material with which it comes in contact	Mature Camembert, blue cheese, some PCPs (especially spreads)
Crumbliness	Tendency to break into small, irregular-shaped pieces when rubbed	Feta, Stilton, Cheshire

PCPs, pasteurized processed cheese products.

Certain rheological characteristics of the cheese are critical so that it behaves optimally as an ingredient in the specific application. Hence, it is difficult to portion hard cheeses, which have a relatively low fracture strain (Parmesan) or which fracture in a jagged fashion (e.g., an over-acid Cheddar, Cheshire), owing to the tendency to break at the edges. Similarly, these cheeses are unsuitable in applications where shredded cheese is required (e.g., pizza) because of their susceptibility to fracture/shattering and the resultant formation of high levels of curd fines/dust, which are aesthetically unappealing. Cheeses that are semisoft and adhesive (e.g., mature Raclette, Camembert, or Brie) are also unsuitable in applications requiring shredded/diced cheese because of their tendency to stick to the shredding equipment and the tendency of the shredded cheese to form balls and clumps. However, the ability of these cheeses to undergo plastic fracture and flow under shear (i.e., spread) makes them ideal for blending with other materials in the preparation of fondues and sauces. The brittleness and tendency of hard cheeses such as Parmesan and Romano, with low levels of moisture and fat in dry matter, to undergo elastic fracturability endows them with excellent gratability and suitability as a free-flow condiment for sprinkling onto pasta dishes. Swiss cheese (e.g., Emmental) is capable of being sliced quite thinly making it suitable for use in sandwiches. Low-moisture, part-skim (LMPS) Mozzarella shreds particularly well to give pieces of uniform size that are relatively nonadhesive; these properties facilitate its distribution over pizza base. Other varieties (particularly Feta, Cheshire, and Caerphilly) are very crumbly and are principally used in tossed salads.

There is relatively little information in the literature concerning the functional properties of unmelted cheese (e.g., spreadability, brittleness, sliceability) and the factors affecting them. However, owing to the envisaged dependency of these functional properties on the rheological characteristics of cheese, it is likely that they are also influenced by factors such as cheese macrostructure, composition, temperature, and extent of ripening (*see* **Cheese**: Cheese Rheology).

Cooking Properties

Definitions of functional attributes

Cheese is used extensively in cooking, that is, baking, grilling, frying, and microwave cooking. A key aspect of the cooking performance of cheese is its heat-induced functionality, which is a composite of different attributes, including softening (melting), stretchability, flowability, apparent viscosity, and tendency to brown. The different functional attributes are given in **Table 3**. The application determines the intensity of, and number of, attributes required; for example, a high flowability is desirable for chicken *cordon bleu*, while the absence of flowability is a characteristic of fried Paneer.

Heat-induced flow (spread) and stretchability involve strain displacement of adjoining layers of the protein matrix as a result of stresses on the *para*-casein matrix. Factors that contribute stress include liquefaction of the fat globules/pools encased within the protein matrix and a change in the interactions between the protein molecules. At temperatures >40 °C, liquefaction of the fat phase results in coalescence of free fat and softening of the cheese mass; most of the fat (~55–70% of total) is solid at lower temperatures (4–10 °C) and contributes to the rigidity of the matrix. On further heating to 60–70 °C, there is an alteration in the localized balance of hydrophobic and electrostatic interactions between the casein molecules forming the matrix, with hydrophobic interactions increasing. These changes result in greater contact

Table 3 Some functional attributes of heated cheese that influence its performance as an ingredient

Property	*Definition*	*Examples of cheeses with these properties*
Meltability	Ability to soften to a molten mass on heating	Most cheeses after a given storage period, PCPs, APCs, cream cheese
Flowability	Ability of the melted cheese to flow	Most cheeses after a given storage period, some PCPs, CAs (some), cream cheese
Flow resistance (melt-resistance, controlled flow)	Resistance to flow of melted cheese	Paneer and other acid-heat coagulated cheeses; some PCPs and CAs (especially if whey protein is added), natural cheeses from high-heat-treated milk
Stretchability	Ability of the melted cheese to form cohesive fibers, strings, or sheets when extended	Low-moisture Mozzarella, Kashkaval, string cheese
Limited oiling-off	Ability of cheese to express a little free oil on heating	Most natural cheeses (if not very mature or very young), some PCPs
Desirable surface appearance	Desired degree of surface sheen with few, if any, dry, scorched black or brown particles	Most natural cheeses when aged

PCPs, pasteurized processed cheese products; APCs, analogue pizza cheeses; CAs, cheese analogues.

between the casein polymers, a contraction in the casein matrix, and an overall softening of the resultant cheese mass resulting in a limited flow. As the temperature is raised further to 90–100 °C (which is typical for most cooking applications), an increase in electrostatic repulsion and a weakening of hydrophobic interactions occur resulting in a partial solvation of the protein phase and some release of free oil. These changes lead to a greater degree of fluidity and flow of the melted cheese mass. The extent of the above changes and hence melt properties are controlled by many characteristics of the unheated cheese, including of the degree of casein hydration, the type of cheese, which affects the protein composition (e.g., the presence of intact κ-casein in acid-curd cheeses compared to *para*-κ-casein in rennet-curd cheeses), the presence of whey proteins in the cheese (which self-associate or interact with caseins to form complexes, which impede flow), cheese composition (e.g., pH, levels of moisture and calcium, protein-to-fat ratio, ratio of soluble to colloidal calcium), and the degree of free fat in cheese (as affected for example by processes such as homogenization of cheese milk and secondary handling of curd).

Further displacement can occur in the form of strings or sheets when the molten cheese mass is pulled and extended during consumption, as in the case with molten cheese on freshly cooked pizza. The degree to which the cheese stretches when pulled appears to be controlled by the same factors that affect flowability.

Effects of different parameters

The effects of different parameters on the functional attributes are summarized in **Table 4**. The functionality of most rennet-curd cheeses changes markedly during ripening (**Figure 7**). These changes are mediated by various biochemical changes including age-related increases in casein hydrolysis, casein hydration and level of free fat. The increases in casein hydration and free fat reduce the level of moisture loss during heating and thereby minimize defects associated with excessive dehydration, for example, crusting, surface discoloration, low flowability and stretch, and lack of surface sheen. Lowering the fat content markedly impairs the functionality, an effect attributable to the concomitant increase in the concentration of intact casein and the lower level of casein hydration. The presence of whey proteins generally impairs flowability, because of the thermal-induced aggregation and/or gelation of denatured whey proteins or denatured whey protein/casein complexes on heating the cheese product. Thus, flow resistance in cheese is generally conferred by the addition of whey proteins, which may be included by

- direct addition of whey powders (e.g., whey protein concentrates and isolates) to milk, for cheese manufacture, or to the blend for PCPs and cheese analogues;
- the *in situ* denaturation of the whey proteins in milk by high-heat treatment, as in acid-heat-coagulated cheeses, for example, Queso-blanco types and Paneer;
- high-concentration ultrafiltration of cheese milk; and
- the inclusion of acid-heat-coagulated cheeses in blends for PCPs.

Cooking properties of different varieties

There are marked differences in the functional characteristics of different cheese types (**Figure 7**), due to differences in make procedure, degree of fat emulsification, composition, and maturity. However, some varieties are noted for their functional attributes. The stretchability and stringiness of *pasta filata*, or stretched-curd, varieties (e.g., Mozzarella, Provolone, Kachkaval, and string

Table 4 General effects of some parameters on the functional attributes of rennet-curd cheeses on heating

Parameter	*Flowability*	*Stretchability*	*Oiling-off, surface sheen*	*Main cause*
Milk homogenization	↓	↓	↓	Inadequate fat coalescence and free fat
Ultrafiltration	↓	na	na	Interaction of whey protein and casein
Increasing the heat treatment of milk	↓	na	na	Interaction of whey protein and casein
Increasing the degree of proteolysis	↑	doa	↑	Increase in protein hydration
Increasing the content of intact casein	↓	doa	na	Increased degree of casein aggregation
Increasing fat content	↑	doa	↑	Increased level of fat coalescence and free fat
Increasing Ca content	↓	↓	na	Lower degree of casein hydration

Data presented give the general effects of the different parameters, as summarized from a review of the published literature.
↑, the magnitude of the attribute increases; ↓, the magnitude of the attribute decreases.
na, data not available or limited; doa, depends on age.

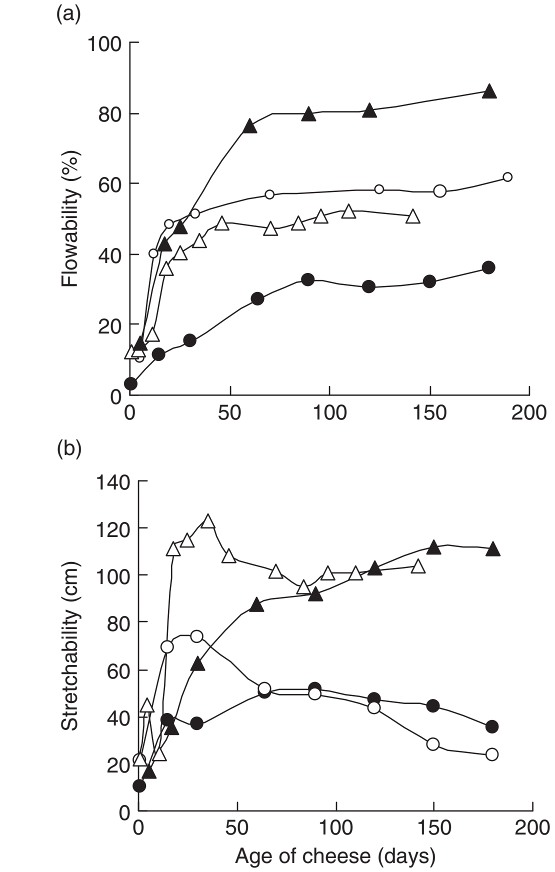

Figure 7 Age-related changes in flowability (a) and stretchability (b) of half-fat Cheddar (•), full-fat Cheddar (O), low moisture Mozzarella (▲), and Kashkaval (△).

cheese) are generally superior to those of other varieties. The high stretchability of these cheeses is largely attributable to plasticization (heating, to ~58 °C, and kneading) of the fermented curd in hot water or dilute brine at ~80 °C, which results in

- aggregation and contraction of the *para*-casein matrix, and the resultant formation *of para*-casein fibers of relatively high tensile strength;
- planar orientation of fibers; and
- a limited degree of fat coalescence and free fat.

The functional properties of *pasta-filata* cheeses make them very suitable for use in pizzas but these properties are particularly unsuitable for use in other applications such as sauces, cheese casserole, or pasta-cheese dishes.

Some cheeses are characterized by high flowability (e.g., mature Cheddar and raclette cheeses, double cream cheeses), while others (e.g., acid–heat-coagulated cheeses such as Queso-blanco types and paneer) are noted for their low flowability or flow resistance. The former cheeses are particularly suited to applications where flavor and flowability are important quality criteria, for example, sauces, omelettes, chicken *cordon bleu*, and baked Camembert in breadcrumbs. Flow-resistant cheeses are more suited for applications where softening of cheese and maintenance of visual identity and shape of the cheese on cooking are desirable, for example, fried cheese pieces, curries, casseroles, and deep-fried cheese sticks, and cheese insets in burgers.

See also: **Cheese**: Cheese Flavor; Cheese Rheology; Enzyme-Modified Cheese; Pasteurized Processed Cheese Products.

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Low-Fat and Reduced-Fat Cheese

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Introduction

Nutritionists are recommending that people consume less fat, yet at the same time, some are against giving up dairy products because they provide generous amounts of protein, calcium, and phosphorus, which are vital nutrients in the human diet. Many consumers want to follow these guidelines but do not want to give up what they like about cheese: flavor and functionality. Consumers would prefer that low-fat cheeses be identical to the full-fat versions of these cheeses in all sensory attributes, used as a 'table cheese', 'consumed as is', or as an ingredient in cooked or baked foods. They are not willing to compromise much in terms of flavor or functionality either. Consumer expectations can be met successfully in some low-fat and fat-free cheeses in the soft or semisoft varieties; however, fat reductions of greater than 50% for hard or semihard cheese varieties have not had the same success, except in certain cases where the cheeses were used as ingredients or as condiments.

Since low-fat cheeses are often so different (and sometimes made by substantially different manufacturing protocols, including the addition of nondairy ingredients) compared to their full-fat counterparts, some people think that the low-fat versions of higher-fat cheeses should have a unique name. There are also those who want to maintain strict standards for what could be called cheese, for example, by limiting the addition of nondairy ingredients. Others advocate the development of low-fat products outside of any set of varietal standards, for example, allow the use of whatever ingredients are necessary to produce the desired product as long as it contains dairy ingredients. The latter thinking is a different direction than cheese as cheese per se; instead, they advocate developing new food products that incorporate dairy ingredients, as well as nondairy components, so that the final product has the nutritional benefits (low fat, calcium, and protein), and flavor and functionality consumers are looking for.

This article will examine the principles behind the manufacture of rennet-coagulated low-fat cheeses. Successful low-fat and reduced-fat cheeses like Cottage and Cream (acid-coagulated cheeses), processed Cheddar cheese products, and string cheese (Mozzarella) will be addressed in other articles.

Definition of Low-Fat Cheese

The CODEX Commission on International Trade has set a maximum limit of 50% reduction of fat (on a dry matter basis) from a referenced variety, for a cheese to be labeled as reduced fat. Consequently, zero-fat Cheddar would not be a valid name for the purpose of international trade. Individual countries of course can set their own internal standards. In the United States, a low-fat cheese must contain 6 g or less of fat per 100 g of cheese while a reduced-fat cheese requires at least a 25% reduction in fat level from the traditional fat level of the referenced variety. Fat-free cheese is defined as that with less than 0.5 g fat per 100 g of cheese. In this article, low-fat cheese refers to at least a 25% reduction in fat from a referenced variety and includes fat-free cheeses.

Currently, most of the insurmountable defects associated with low-fat cheeses are at fat reductions of 50% or greater. In many cheese-judging contests, all low-fat versions of cheese are grouped together into one category making for a somewhat unfair competition. That is, low-fat versions of soft cheeses compete with low-fat versions of semisoft or semihard cheeses. It has been far easier to engineer low-fat versions of mild-tasting, high-moisture cheeses, such as low-fat Cream cheese or Mozzarella, than it has been to produce a full-flavored, low-fat Cheddar or Gouda cheese.

Traditional Cheesemaking Techniques: Developing Desired Physical Characteristics through a Combination of Compositional and pH Control

Standardization of Milk for Lower-Fat Cheeses and Consequences for Cheese Yield

In order to achieve the desired fat level in low-fat cheeses, the casein-to-fat ratio of the milk must be increased. This can be accomplished by the removal of fat (cream) or the addition of casein. The latter is accomplished by adding nonfat dry milk (NDM) (usually reconstituted), condensed skim milk, or ultrafiltered skim milk retentates to whole or partially skimmed milk. Very low-fat or no-fat cheeses are made from skimmed or partially skimmed milk since the needed casein-to-fat ratio is so high that it can only be accomplished by cream removal. Condensed or ultrafiltered skim milk is often used in these cheeses to fortify the casein content, which will also result in higher productivity and cheese yields.

The addition of NDM (∼52% lactose) or condensed skim milk can potentially add excess lactose, which could lead to excess acid production if starter metabolism is not stopped. To prevent excessive acidity, the starter metabolism can be stopped by reducing the cheese temperature rapidly after manufacture, by using a combination of salt-sensitive strains and increased salt-in-moisture contents, or by reducing the lactose level in the milk or curd. The last one is accomplished by adding appropriate amounts of water (generally after some whey is first removed) or by rinsing the curd to remove some of the lactose. Rinsing or washing the curd often results in less-desired flavor development in the cheese but this depends upon the cheese variety and whether or not other steps are taken to ensure the development of the desired flavor. Rinsing or adding water after whey removal also results in a shift of some colloidal calcium phosphate (CCP) to the serum. Since colloidal calcium plays a major role in the physical properties of cheese, solubilization of CCP will result in a cheese with a softer and smoother body. However, high-moisture cheeses may become too soft and gummy (pasty) during aging. This is primarily due to enhanced proteolysis occurring in cheeses with low CCP. If an ultrafiltered retentate, for example, 27% total solids, 4.0% lactose, is used to standardize milk, the lactose content of the blended milk is not increased. Consequently, there may be less need to add additional water or to rinse the curds.

The yield of cheese for skimmed or partially skimmed milk is lower when compared to the yield of whole milk used in the manufacture of the traditional full-fat cheeses and this is a major economic concern to the cheesemaker and potential deterrent to the manufacture of low-fat cheese. To compensate for the higher cost of production because of the lower productivity, the prices of the low-fat cheeses are generally higher than the full-fat versions. This can be especially detrimental to sales of low-fat cheese to institutions such as schools where the food budget is often limited. Increasing moisture content of low-fat cheese improves yield, but it can also be detrimental to quality and shelf life.

Fat and casein contents, and of course the moisture content of the cheese, determine the cheese yield obtained from milk. Therefore, the method of milk standardization plays a critical role in cheese yield. Removal of cream results in not only the loss of fat but also in the loss of some casein and, thus, milk standardized by cream removal will produce lower cheese yields compared to the same quantity of milk standardized by adding a nonfat source of casein. Economic evaluation must be done to ascertain the impact of the standardization method used. This analysis often influences whether or not the cheesemaker will even attempt to make low-fat cheeses.

Cheese Composition and Chemistry, and Their Relationship to Physical Properties

As the fat content of cheese decreases, casein becomes a more dominant component of cheese. In cheese made from full-fat milk, more than 50% of the solids in the cheese will be fat, while less than 40% will be casein. In a low-fat cheese, the casein content will be about 60% or more of the solids. Ultimately, the chemistry of the casein network must be considered if the firmness of a low-fat cheese is to be reduced and the melt, stretch, and mouthfeel characteristics improved. There are three major approaches that the cheesemaker can use to compensate for the increase in casein content in a low-fat cheese:

1. Dilute the casein network (increase moisture, add fat mimetics, starches, and denatured serum proteins).
2. Weaken the interactions between casein molecules (solubilize CCP, lower the pH below 5.1, add surfactants).
3. Disrupt the continuity of the casein network through proteolysis (aging).

Increasing Moisture in Low-Fat Cheese

To compensate for fat reduction, low-fat cheeses are manufactured with higher moisture contents to produce a quality cheese with the desired shelf life. If the moisture content is too high, the cheese will have a shorter shelf life because as it ages it gets too pasty, too soft for shredding, and melts or flows excessively when heated. On the other hand, a high-moisture cheese may be ready for consumption at a much earlier age than a low-moisture cheese.

Well-established methods to increase moisture content in cheese include the following:

a. Increase the firmness of the coagulum at cutting or delay cutting.
b. Decrease the colloidal calcium content (reduce the pH at time of coagulation).
c. Increase curd size.
d. Lower cook or scald temperatures.
e. Reduce the amount of pH drop between cutting and whey separation.
f. Reduce stir-out time before whey drainage and also after whey drainage.
g. Allow the curd to mat after whey separation.
h. Rinse the curd in cold water.
i. Use less salt if the curd is direct salted.
j. Brine for less time or dilute the brine if the cheese is brine salted.
k. Use less pressure and less time during block formation.
l. Cool the cheese block rapidly.

Other methods to increase moisture include increasing heat treatment of the milk and homogenization of the milk, but if improperly done, both can have a negative impact on the flow or melt of the cheese. Excessive heat treatment of the milk can also produce a cheese with a less smooth mouthfeel and a crumbly body while homogenization often results in a smoother mouthfeel but restricted melt. Other methods that are being used to increase moisture content are the addition of heat-denatured serum proteins, introduced as a separate ingredient via the starter media or introduced into the milk as an aggregate, use of exopolysaccharide producing cultures, and use of nondairy ingredients such as starch, hydrocolloids, and emulsifiers such as citrates and phosphates (melting salts). The latter three also directly influence the chemistry of the casein network and their impact is not only due to their ability to increase moisture content but also due to the disruption of interactions between casein molecules.

Unless the lactose content is reduced during manufacture, higher moisture content often results in an increased level of lactose in the cheese. In most cases, lactose will eventually be fermented to lactic acid and this could result in an excessively low pH, an undesirable acid-flavored cheese, and increased potential for formation of calcium lactate crystals. To prevent these situations, the cheesemaker will remove a portion of the whey and add water as the remaining curd is stirred, or the curd is rinsed or soaked in cool water after whey is removed. The latter results in a cooler curd that retains more water. However, it also produces a curdy cheese, especially in the early stages of ripening. Both methods result in a cheese with lower lactic acid content and higher pH. The pH will also increase more rapidly than in an unwashed cheese (very little residual sugar to ferment). But there is another potential benefit. The technique also results in a softer cheese and this is not just because of increased moisture content. Adding water not only dilutes the lactose content but also dilutes the calcium and calcium phosphate in the serum phase of the cheese. This enhances solubilization of casein-bound calcium to the serum, which results in an increased electrostatic repulsion between casein molecules and produces a softer, smoother-bodied cheese and at higher pH values than in an unwashed cheese. In addition, the loss of CCP makes the casein network more susceptible to proteolysis, which contributes to the softening and improved melt characteristic of the cheese as it ages. However, as the cheese is softer at an earlier age, the cheese may become excessively soft and pasty if it is aged for too long due to increased proteolysis. The pH of the cheese also increases more rapidly during aging than in a cheese made without lactose reduction.

Higher pH and lower lactic acid content can alter the microbiological ecology of the cheese. Since both pH and acid content are important factors in controlling growth and metabolism of microorganisms, which in turn contribute to the flavor of the cheese, the flavor quality of the cheese may also be affected. This may not be apparent in cheese varieties that have traditionally been manufactured with whey dilution or curd rinse (e.g., Gouda) as the flavor is typically mild. However, the development of the desirable strong flavor may not be achieved in aged low-fat Cheddar cheese. A current theory explaining this phenomenon is the concept of redox potential. As microorganisms ferment residual lactose in cheese, oxygen is consumed and this lowers the redox potential. The less lactose fermented, the more oxygen will remain in the cheese and the higher will be the redox potential. Both chemical and enzymatic reactions, as well as microbiological growth, are influenced by the redox potential of the cheese. Thus, the species and concentration of flavor compounds formed in cheese with initially less residual lactose may be different compared to a cheese that initially had higher residual lactose content.

Low-fat cheese can also be made without reducing the lactose content. To compensate for the higher level of residual lactose in the cheese, and therefore potentially high acid content in the cheese, the manufacturing protocol is adjusted to reduce lactose fermentation in addition to retaining buffering capacity. For example, a version of low-fat Cheddar cheese is made with more salt-sensitive starters (usually slow acid formers) and with high pH at rennet addition and salting. The reason for this strategy is twofold: first, with more lactose in the cheese, fermentation must be slowed and eventually stopped before all the residual lactose can be fermented and second, even though lactose fermentation will eventually be stopped before an excessively low pH is attained, there will be more lactic acid formed and it will be compensated for by buffering capacity to prevent the cheese from having an excessively low pH. The cheese manufacturing protocol for a no-wash curd cheese is therefore designed to prevent an excessively low pH but at the same time create an environment within the low-fat cheese that is as close as possible to the full-fat version in terms of acidity, salt-in-moisture, and pH. This has ramifications for both flavor and body development during aging.

CCP is a major buffer in cheese, and high pH values at rennet addition (~6.45), whey separation (~6.35), and salting (~5.9) help to retain more CCP in the cheese. CCP is released from the casein molecules during manufacture as lactic acid is developed and the phosphate ions bind some of the H^+ ions produced by the lactic acid, thus buffering the cheese against low pH. If sufficient buffering capacity is retained, a cheese with a high level of lactic acid (1.9–2.0%) can be produced without an excessively low pH (usually pH 4.95–5.05). Buffering capacity of curd may be insufficient when the moisture content is very high (>50%) or the salt-in-moisture content is too low (<5.0) to slow the starter culture. While it is usually advised to use salt-sensitive starter strains in the manufacture of low-fat Cheddar when using a no-wash procedure, it is not advisable to try to stop acid development completely post manufacture. Insufficient acid development can result in tough, curdy cheese that will not flow when heated. Attaining the desired pH of the finished cheese (~5.0) will depend upon the amount of salt added and sensitivity of the starter strains toward salt. Consequently, some manufacturers have chosen to add more salt but at a low salting pH (5.3) rather than less salt at higher pH (~5.9). Slowing or stopping lactose fermentation poses a potential problem since residual lactose will undoubtedly be fermented by some type of bacteria present in the cheese. If the cheese is contaminated with heterofermentative microorganisms and they ferment the lactose, the cheese could exhibit gassy defects, such as splits or blown packages.

Developing Desired Body Characteristics

Even with a substantial increase in moisture, the casein content is greatly increased in a low-fat cheese compared to its full-fat counterpart. For example, Cheddar cheese contains about 24% casein (37% moisture), while a 50% reduction in fat produces a cheese with about 30% casein (50% moisture) and even lower fat cheese (~6% fat, 56% moisture) still has about 32% casein. Depending upon the cheese variety, increasing the moisture content of cheese in order to reduce the casein content is a strategy that has its limitations. Although high moisture can be beneficial for cheeses with short shelf-life, excessive moisture levels may produce a cheese that will become very soft, especially if aged for more than a few months.

To improve the physical characteristics of low-fat cheese, more modifications need to be done than just increasing the moisture content. The integrity and chemistry of the casein network itself must be altered. The physical characteristics of any cheese are influenced, in part, by composition (moisture, fat, casein, salt) but more directly by how and to what extent the casein molecules interact with each other. From the moment the coagulum is formed the casein network can be considered a continuous or integrated network periodically interrupted by inclusion of the much larger fat globules. The casein network develops around the fat globule but does not interact with it (i.e., fat globules form weak spots). Consequently, as the fat content is decreased, the casein network is denser. The denser the casein network, the firmer the cheese is unless the interactions between casein molecules are disrupted.

The cheesemaker first ‘manipulates’ the degree of interaction between casein molecules by controlling cheese composition and then, and perhaps most importantly, by the rate and extent of acid development. The most important control points for the manufacture of low-fat cheese are the pH at coagulant addition, pH when most of the serum is squeezed out of the curd particles (usually the first 30 min after cutting the coagulum), and the lowest pH obtained during manufacturing. Acid development during manufacturing is critical, as it, and the pH obtained in the finished cheese have a major influence on the physical characteristics of cheese. However, cheeses with different pH histories but with similar pH values at the end of manufacture and with similar chemical composition and degree of intact casein do not necessarily have the same physical characteristics. A cheese is softer, less chewy, and more meltable and flowable if the pH of the milk is lower at the time of coagulant addition than a cheese in which the coagulant is added at a higher pH, even though the lowest pH obtained in each cheese were similar. Rather than wait for a lower pH value produced by starter metabolism, many cheesemakers will add lactic or acetic acid directly to the milk prior to rennet addition. This process is called preacidification and is a common practice in the manufacture of low-fat cheeses, as well as in any cheesemaking protocol where extra casein has been added to the milk for standardization or for increased productivity. If the pH of preacidification is below 6.4, a wash or curd rinse step is almost always used since preacidification to this pH may remove too much of the buffering capacity of the cheese (CCP).

How Does pH Affect the Physical Properties of Low-Fat Cheese?

As acid is developed by the starter during the cheesemaking process, insoluble CCP dissolves. CCP has a number of important consequences for the physical properties of cheese. CCP is linked to phosphoserine residues on casein molecules forming large nanoclusters. CCP is a bridging point between 3–4 casein molecules. As CCP dissolves, the negatively charged phosphoserine groups are now exposed and the local increase in negative charge causes greater electrostatic repulsion between casein molecules. As a result the casein network becomes more open and porous. The free serum trapped within the casein network at coagulation is eventually absorbed by the casein network during the initial stage of ripening. Concomitantly, the cheese becomes softer and more pliable, and the color of low-fat cheese becomes translucent and the melt improves. The shift of insoluble CCP to soluble forms is not instantaneous and may require a few days to a few weeks to reach some type of equilibrium depending upon the pH, ionic strength, calcium content of the serum, and fermentation of residual sugar. As calcium is lost from CCP the phosphate groups associate with H^+ and this is the reason for the early increase in pH that is often observed in some cheese varieties (especially washed-curd and pasta-filata cheeses where there is little fermentation of residual sugar during storage).

In directly acidified cheeses (coagulant is added at a low pH, e.g., Mozzarella), solubilization of CCP is more efficient and thus the cheese becomes suitable for stretching at a higher pH than a cheese produced from a milk in which the coagulant is added at a higher pH. By controlling the rate and extent of acid development the cheesemaker can influence the solubilization of CCP and thus the degree of bridging as well as electrostatic repulsion within and between casein aggregates. The change in CCP to soluble form is a key parameter for modifying physical characteristics such as firmness, toughness, curd fusion, stretch, and melt during ripening. However, at very low pH values (<5.0), electrostatic repulsion between casein molecules is reduced and as the pH approaches the isoelectric point there is an increase in electrostatic attraction between oppositely charged groups. The cheese no longer exhibits melt or stretch properties due to strong attraction between casein molecules and water held within the casein network is more readily released. Melt and stretch properties require the casein aggregates to have a high degree of bond mobility, which is lost at low pH values. The casein molecules aggregate into larger clusters. The casein aggregates are separated by serum and the cheese has a tendency to lose serum rapidly as the cheese is warmed. Cheeses with a low pH may have a grainy or rough mouthfeel due to the size of the clusters. The color of the cheese also changes. Cheese is white at low pH (<5.0) and translucent at higher pH (~5.0–5.3) if no colorant has been added.

Hydrophobic attraction between casein molecules is another factor that influences casein micelle stability and interactions between micelles, which means hydrophobic interactions have a major impact on the physical properties of cheese. Hydrophobic interactions become stronger as temperature is increased. The ratio between the attractive forces (hydrophobic and electrostatic attractions) and repulsive forces (electrostatic repulsions) must be controlled to produce the desired melt (flow) and stretch properties of cheese. Hydrophobic interactions also influence the color (translucency) of cheese, moisture migration within the cheese, and moisture absorption by the casein network. Since the hydrophobic interactions are governed largely by temperature and not by pH or loss of CCP, it is the pH, electrostatic interactions, and proteolysis that will determine cheese flow or stretch properties as the temperature is increased. Stronger hydrophobic interactions tend to decrease melt; however, if the electrostatic repulsive forces are dominant, cheese will flow when heated. At low pH (<5.0), the electrostatic repulsion decreases (electrostatic attraction increases) and together with the hydrophobic interactions prevent flow and stretch when the cheese is heated.

Hydrophobic interactions are necessary for cheese to exhibit stretch since they provide attractive forces to allow the casein network to hold together sufficiently. As the cheese is pulled, electrostatic repulsion keeps the caseins apart allowing the molecules to slip over each other but the hydrophobic interactions allow the casein molecules to still associate with other molecules so that the net result is an elongation of the casein network, that is, stretch. However, a dominant factor influencing whether a cheese will flow and stretch when heated is the amount of intact casein molecules. Proteolysis of intact casein softens the body, smoothens the mouthfeel, and increases the flow of the cheese (however, often sacrificing the stretch properties). The structural integrity of the network depends upon intact casein molecules. Proteolysis (hydrolysis) results in cleavage of exposed peptide bonds. Two peptides are formed as a result of hydrolysis of an intact casein molecule, at least one of which becomes separated from the casein network and becomes associated with the serum phase. This soluble peptide is likely metabolized further by microbiological entities, and a new surface on the casein network is exposed, which may further be hydrolyzed by enzymes in due course. This new surface may not interact with other molecules within the network. Thus, proteolysis weakens the interactions between casein molecules, and the rigidity of the network is weakened. Consequently, the cheese becomes softer and smoother, and flows more when heated, but may become pasty or sticky especially if the cheese is also high in moisture (as a low-fat cheese generally is). However, unlike loss of CCP, or an increase in moisture, proteolysis also contributes to the development of flavor in cheese, which can have both desirable and undesirable consequences for the quality of the cheese.

Color of Low-Fat Cheese

In skim milk, casein micelles are responsible for the white color. Casein aggregates are very effective at scattering light and in low-fat cheese (without added colorants) the cheese may appear translucent. The color and translucency of low-fat cheese depend upon the amount of fat, the pH, ionic strength, temperature, and level of intact casein. Casein molecules form dense clusters at very low or high pH and at high salt content (regardless of temperature). These casein clusters scatter light. Generally, at pH 5.0–5.4 and low temperature (<10 °C) the electrostatic repulsion between casein molecules is strong and the hydrophobic interactions are weak, and this set of conditions results in a finely structured network that appears translucent or straw colored. The straw color is derived from serum constituents. Concurrently, serum is absorbed by the casein network, a phenomenon referred to as swelling.

Translucent cheese will turn white when warmed if there is enough intact casein to form clusters. This transformation will begin to occur at ~10 °C but the degree of protein association is dependent on the level of intact casein molecules. Proteolysis disrupts the casein network and produces peptides which cannot form dense aggregates capable of dispersing light. The remaining intact casein will disperse light to a lesser degree when heated than fully intact casein (young cheese). Titanium dioxide has been added by cheesemakers to enhance the whiteness of low-fat cheese but since it is not very water soluble, much of it sediments to the bottom of the cheese vat and causes problems for the cheesemaker. Traditionally, homogenization of fat has also been used to increase the white appearance of low-fat cheese but this requires sufficient fat to be effective. The milk fat globules are broken apart into many smaller globules and a new membrane, predominantly consisting of casein, is formed around them. The increase in number of fat globules (with their new membrane material) results in an increase in the number of light-scattering particles and consequently the cheese appears whiter at a pH where cheese made from unhomogenized milk would otherwise have been translucent or straw colored. Since the new membrane consists of casein, the homogenized fat globules can also interact with the casein network and this can also decrease flow and stretch of the cheese when heated.

Nontraditional Approaches to the Manufacture of Low-Fat Cheeses: Homogenization and the Use of Added Ingredients, Fat Replacers, and Fat Mimetics

Homogenization

Homogenization often results in softer and creamier cheese but the impact is related to the level of fat in the milk and the increase in cheese moisture that often occurs. The higher the fat content, the softer and creamier the cheese is after homogenization. Homogenization may have less impact on low-fat cheese (>50% fat reduction) since the fat globule is already smaller in the low-fat milk (<1% fat) due to cream removal.

As fat is homogenized, casein and to some extent serum proteins are incorporated into the new membrane that forms around the small fat globules produced. As a result, casein in the membranes can interact with and become linked with the casein network. Homogenized fat tends to reduce cheese firmness due to increased moisture content and alteration to the structure of the cheese matrix. Syneresis of curd particles depends upon rearrangement of casein molecules; the presence of homogenized fat globules inhibits this syneresis process and thus the curd undergoes less shrinkage and has a higher moisture content and a smoother body. However, the melt of the cheese is impaired since the homogenized fat globules are now part of the casein network but unable to function as ‘real’ casein particles.

Fat replacers or mimetics

Fat replacers or mimetics also disrupt or alter the casein matrix in cheese. They are added to the milk and do not interact with the casein network but do fill spaces that otherwise would have been filled with fat in full-fat versions. They are usually used when the fat reduction is ≥50% and these materials are usually starches or denatured whey protein aggregates. They usually have higher moisture holding capacity than casein, so fat mimetics promote higher moisture contents in cheese.

Fat replacers and fat mimetics do increase the smoothness and soften the body; however, excessive use can increase stickiness, decrease shreddability, and may impart undesirable flavors to the cheese. While their use also increases the flowability of the cheese when heated, the cheese also releases more serum into the food matrix, making the food product soft or soupy.

Fat replacers or mimetics, of course, cannot be used in cheeses where the standard of identity (e.g., regulations) does not specifically allow for their use and consequently their use generally requires that the cheese be named creatively.

Adding surfactants or melting salts

Adding surfactants and adding melting salts are two other approaches to soften low-fat cheese. Sodium citrate is added to decrease colloidal calcium content and adding surfactants decreases hydrophobic interactions between casein molecules. They are permitted in the manufacture of some types of process cheese but regulations may not be allowed in natural cheeses. Sodium citrate is usually added to the milk (preacidification) or added to the curd at salting (e.g., low-fat Cheddar) or as phosphates and surfactants, added to the curd after a hot water mixing step as is the case of low-fat Mozzarella.

Surfactants such as mono- and diglycerides, lecithin, and phospholipids are added to disrupt the casein network and interfere with hydrophobic interactions between caseins. A good source of phospholipids is sweet cream buttermilk. They are especially useful in the manufacture of process cheese for improving the whiteness of cheese, flow when heated, moisture retention, and decreasing stickiness of cheese. There has been some discussion regarding the cheeses that are produced with these additives. Are they process or natural cheeses?

Another nontraditional approach to the manufacturing of low-fat cheese is to blend cheeses of different fat contents in the right proportion to achieve the desired fat content. This process is similar to the manufacture of cold-pack cheese (where the idea originated). This approach more directly impacts the flavor issues. It has been used to make blends of totally different cheeses, that is, blue-veined cheese with Cheddar, but it can also be used to achieve a softer, low-fat cheese with a more desired flavor. By blending sharp, aged Cheddar with milder-flavored, low-fat Cheddar, a lower-fat, flavorful Cheddar cheese is produced. The process involves a short ripening time to allow the curds to reknit or fuse together.

Flavor Enhancement of Low-Fat Cheeses

Probably the most problematic barrier to the success of low-fat cheeses is achieving acceptable flavor and on a consistent basis. There are commercially produced reduced-fat cheeses (up to 50% reduction in fat) that are comparable in flavor to their full-fat cheese counterparts; however, with further fat reduction, the gap in flavor quality and intensity between low-fat and full-fat cheeses widens. This appears to be cheese specific. It is very difficult to emulate the flavor of full-fat cheeses (aged Cheddar and Gouda) where the flavor is mostly derived from the starter and naturally occurring enzymes and microflora. In these cheeses, loss of flavor quality appears to follow not only the degree of fat reduction but also the degree of change in the cheese environment. The manufacturing processes (whey dilution, curd rinse, or wash) for many low-fat versions of Cheddar and other typically nonwashed-curd varieties produce cheeses in which the chemical composition (particularly lactic acid and salt) is dramatically different than from that of full-fat versions. These compositional differences are thought to lead to changes in microflora and biochemical processes, which consequently have a negative impact on flavor development. The desire for these particular low-fat cheeses to have stronger, more typical aged flavor has inspired much research.

Low-fat versions of cheeses, such as mold-ripened (Blue and Camembert), surface-ripened (smeared) cheese (such as Limburger), or cheeses in which the flavor is largely derived from deliberately added lipases (e.g., Asiago and Romano), are much easier to emulate with the desired flavor attributes of the higher-fat versions of these cheeses. This is because the development of flavor is derived from the activity of overwhelmingly dominating microflora and enzymes deliberately added to produce a specific and distinctive flavor profile. Flavor components derived from naturally occurring microorganisms and enzymes, while active, are usually no match for the flavor components derived from these added entities.

At least two theories have been advanced to explain the reasons for the lack of flavor development, or reduced flavor quality, in low-fat cheeses like Cheddar. The first and perhaps the most supported suggestion is that the 'chemical' environment is not conducive for the formation of the required amount of the desired flavor compounds. This involves three major aspects: microbiological metabolism, chemical or nonenzymatic reactions, and enzymatic processes. A major hurdle in developing strategies to enhance flavor development in low-fat cheese is the gaps in knowledge of the chemical basis for those components of flavor. If they are known, the complete biochemistry of their formation may not be. It is not surprising that cheese manufacturing technologies that produce low-fat cheeses emulating the chemical environment of the full-fat cheese also produce a low-fat cheese more similar to the full-fat version both in body and texture as well as flavor. Low-fat Cheddar cheese is often made with a substantial wash treatment to reduce acidity but this technique is not used in the full-fat version. Wash treatment reduces the lactic acid level and increases the moisture of the low-fat cheese. Consequently, the chemical environment is not the same as a traditionally produced Cheddar cheese. It is unlikely that a washed low-fat curd Cheddar cheese will emulate the sharp, aged flavor of full-fat nonwashed cheese. Indeed, flavor profiles of aged, nonwashed (higher-acid), higher-salt (salt in moisture 4.0–4.3%) low-fat Cheddar cheese are often closer in flavor to that of aged full-fat Cheddar than low-fat washed, low-salt versions.

Several factors influence the cheese environment, namely, redox potential, moisture, salt-in-moisture, and lactic acid-in-moisture contents. The latter two are often much lower in low-fat cheeses due to manufacturing practices. These parameters, in part, strongly influence microbial growth (including strain selectivity) and metabolism, and both enzymatic and nonenzymatic reactions, which in turn influence the development of cheese flavor. Flavor defects that are found in low-fat cheese, that is, meaty-brothy, unclean, and flat flavor are also found in whole-milk, high-moisture, washed-curd, reduced-sodium cheeses. The latter cheeses also have lower lactic acid than nonwashed whole-milk cheeses. Perhaps it is not the lack of fat per se that limits the desired flavor development but the influence of the altered chemical environment within the cheese, through its influence on microbial metabolism and enzymatic and nonenzymatic activity. This suggests that desired flavor components of an aged Cheddar cheese can be developed in low-fat Cheddar if the acidity and salt-in-moisture contents are similar to the full-fat Cheddar.

A second theory, which is built on the first theory, suggests there is enough substrate (fat and casein) even in a low-fat cheese to develop the concentration and type of flavor compounds needed to emulate full-fat cheese flavor. Thus, key flavor components do not develop because there is a lack of (or inhibition) of the required enzymatic and microbiological activity. Also, the altered cheese environment might favor development of undesirable flavor components. This has five additional aspects:

1. There may be insufficient numbers of desired microbiological species.
2. There may be substantial growth and metabolism of undesirable species.
3. There may not be enough of the desired enzymes or activity of these enzymes.
4. There may be a lack of substrate accessibility by either the microorganisms or enzymes.
5. There is an imbalance between desirable and undesirable metabolic activity, leading to an imbalance between desirable and undesirable flavor components.

Another component of both theories is a potential role of pH (acidity), fat, and serum in the oral perception of certain flavor components. Particular flavor components have lower threshold perception concentrations in serum than in fat but flavor perception is also dependent on whether the component is protonated or not. Consequently, a lower concentration of an undesirable or desirable flavor may be more easily detected in high-moisture, low-fat cheese. It also complements the idea that desirable flavors are not present in sufficient quantities, either in total or in comparison to the undesirable flavors. While the levels of undesirable flavor components that are observed in low-fat cheese may not be perceived in full-fat cheeses, they are present at concentrations sufficient to cause undesirable sensory responses in low-fat cheeses.

Based on the latter model, researchers have focused on the addition of selected starters, adjunct bacteria, enzymes, and the use of higher ripening temperatures in an attempt to moderate any shortcomings in flavor development due to lack of the desirable agents or their activities. Success has been mixed but with a better understanding at the molecular level of the biochemistry needed for the desired flavor development, major improvements are anticipated.

Currently, and in the context of aged cheese flavor development, there has been some degree of success in cheeses with 50% or less reduction in fat, but marginal or little success with higher level of fat reduction using these methods. Results depend upon the cheese variety and are also influenced in part due to the nature of the low-fat cheese business. One issue often neglected is that in order to achieve the desired strong, aged flavor, the cheese must be aged for an appropriate period. It has often been expected that low-fat cheeses will develop the desired flavor sooner than their full-fat counterpart. Due to factors (high moisture, low salt, low acid) that often accelerate negative chemical reactions, low-fat cheeses are generally sold without much aging and consequently develop much less flavor, which of course includes undesirable flavors. Low-fat cheeses are often made with higher moisture for economic and sensory (body) considerations and hence an extended ripening period would lead to excessive proteolysis and consequently excessive softening and deformation of the packaged cheese. Many very high-moisture, low-fat cheeses are therefore packaged in plastic containers that will allow the cheese to maintain its shape.

Herein lies the dilemma; extended aging of low-fat, high-moisture cheese, low in salt-in-moisture and lactic acid contents may still not produce the desired aged flavor without first developing undesirable flavor notes or a deteriorating body. On the other hand, lower-moisture cheeses with higher salt and lactic acid often develop flavor too slowly for commercial application. Consequently, most low-fat cheeses are sold as mild cheeses and are rarely aged beyond 6 months.

Manufacturing Low-Fat Cheese for Specific End-Use Applications

There are four basic applications for low-fat cheese. They dictate cheese manufacture and also influence consumer acceptability. Unfortunately, consumers, both retail and institutional, often expect the cheese to have all the desired attributes, for all applications, in one cheese. This mindset has impeded the commercial success of low-fat cheeses. The four applications for cheese are as follows.

Table Cheese

Table cheeses are eaten as they are and not manipulated further by heating. They are natural cheeses. Examples include eating a slice of cheese on a piece of bread or cutting a small piece of cheese from a block and eating it directly or putting it in a salad. The cheese maintains its original identity. A premium is placed on flavor and body characteristics such as firmness, chewiness, and mouthfeel or smoothness. Most published research efforts on low-fat cheese have been on the manufacture of a low-fat table-type cheese. These are also the most difficult versions of low-fat cheese to make, particularly when the desired product quality including flavor, body, and mouthfeel attributes must be maintained throughout ripening.

Condiment Cheese

Condiment cheeses are cheeses that will be heated or used as an ingredient in another food but still allowing the cheese to maintain its identity as cheese. Examples include Mozzarella on pizza, or cheese on a cheeseburger or in foods such as lasagna or chili. A premium is placed on flow and chew characteristics. Flavor may or may not be as important, depending upon the intensity of the other flavors in the food. These cheeses are often made with added ingredients to enhance functionality and cheese yield (to lower the cost of manufacture) and have relatively shorter shelf-life requirements. A premium is often placed on the cost of manufacture since this cheese is used in foods where price is a major concern and competition with alternative or substitute ingredients is common.

Ingredient Cheese

Ingredient cheeses are cheeses that lose their visual identity when used in another food product. Examples include cheeses typically used as a source of flavor in salad dressings or as an ingredient in the dough for cracker or bread manufacture. A premium is placed on flavor attributes but machinability or ability to work the cheese into a dough or paste is very important.

Process Cheese

Process cheese has two components: cheese used as an ingredient in the manufacture of the process cheese and the process cheese made from it. Low-fat or skim-milk cheeses are often used as ingredients in the manufacture of higher-fat process cheeses. Typically, a premium is placed on machinability, and physical–chemical attributes of the cheese rather than on flavor. The level of intact casein and amount of residual CCP are the key parameters of this form of low-fat cheese. Flavor of process cheese is often manipulated by adding flavorful ingredients rather than by developing flavor in the low-fat cheese base.

Low-Fat Cheese and Its Application

Considering low-fat cheese and its application, flavor may not be as important as in a table cheese and, similarly, physical attributes such as machinability and flow when heated may or may not be the key desirable characteristics. Other flavors in the food may mask any shortcomings of flavor but nothing can substitute or mask deficiencies in flow or machinability. Depending upon the cheese variety and needs, condiment cheeses are often the easiest cheese in which to develop the desired flavor. However, in many condiment applications, flavor is the key to successful application and as long as the cheese is also machinable, firmness and mouthfeel attributes are not as demanding.

Low-fat cheese for use as an ingredient has been the most successful. Low-fat flavorful versions of Cheddar, Blue, smear-ripened, Asiago, and Romano are cheese varieties often used as the desired flavor is easily developed. It is probably second in volume of cheese produced (excluding Cottage cheese) only to low-fat or no-fat cheese used as an ingredient for the manufacture of process cheese. Flavor of these cheeses is often of unacceptable quality (bitter, unclean, acid, and rancid) as a table cheese but as an ingredient these flavor characteristics tend to be muted and promote the desired sensory attributes in the manufactured food they are used in. This is similar to the use of enzyme-modified cheese for use in process cheese manufacture. The ability to blend the low-fat cheese into the food product is of utmost importance. Often this requires prolonged ripening to soften the cheese for ease of blending, which allows for the desired flavor attributes to develop.

Process low-fat or no-fat cheese has advantages over natural low-fat cheeses. Body and melt characteristics can easily be manipulated by the judicious use of melting salts, acids, or emulsifiers and other dairy (whey proteins) and nondairy ingredients, such as starch and gums, and desired flavors can be added. Also, expectations for flavor are not as demanding as in natural low-fat cheeses. Considering the cost of manufacture, low-fat process cheese is more in line with the cost of manufacture of a whole-milk process cheese, than a low-fat natural cheese is to a whole-milk process cheese. Consequently, it is often the cheese of choice for commercial or industrial use.

Future of Low-Fat Cheese

Nutritional awareness has led to the interest in low-fat cheeses. Additionally, there is strong interest in sodium reduction, addition of probiotics and prebiotics, and nutritional enhancement with omega-3 fatty acids, inulin, calcium, and other minerals. Can all these be done in one cheese simultaneously? Research is progressing on all fronts but will consumers be accepting these new cheeses with deletions and additions, which are undoubtedly produced at higher cost? Research on low-fat cheese has exposed major gaps in our understanding of the biochemistry of cheese flavor development. For any future success with low-fat cheese, these gaps must be closed. This is especially important since this knowledge will aid in the development of better-reduced sodium cheeses as well. Research, both from an academic and commercial point of interest, has mainly focused on specific table cheeses (e.g., Cheddar and Gouda) and condiment cheeses (Mozzarella); a potential growth area for low-fat cheeses with specific flavor profiles (Blue, Asiago, hard-grating, smear-ripened, and Provolone) appear to be cheeses used as ingredients.

See also: **Butter and Other Milk Fat Products**: Fat Replacers. **Cheese**: Cheddar-Type Cheeses; Cheese as a Food Ingredient; Cheese Flavor; Cheese Rheology; Microbiology of Cheese; Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Pasteurized Processed Cheese Products.

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Current Legislation for Cheeses

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Introduction and Background

Although from prehistoric times cheese making has been used to preserve the nutritional value of milk, production was largely for home or local use only until the emergence of larger cheese factories during the last decades of the nineteenth century. Many individual varieties have geographical names from towns, districts, regions, monasteries, and so on, which indicate their origins; see **Table 1** for some examples.

However, this is not the case with all cheeses, for example, Cream cheese, Pizza cheese, extra hard grating cheese and Cottage cheese, where the variety name is more generic or descriptive in nature. As peoples moved from one region to another, individuals or groups tended to bring their native cheesemaking skills with them, which they utilized in their new homelands. This is particularly true in case of the United States, Canada, and Argentina, where cheeses that originated in other lands are being produced and have been standardized. In quite a few cases, the original manufacturing techniques were adapted or changed to suit local conditions and the resultant cheeses, even when they retain their traditional names, are not necessarily identical to the original. One such example is Neufchâtel/Neufchatel, where the original cheese, as produced and protected by Apellation d'Origine Contrôlée (AOC) in France, is a ripened cheese, while the cheese with this name produced in North America is a Cream cheese-type unripened cheese; however, it should be mentioned that in both France and North America this cheese variety is made from milk enriched with cream.

By the middle of the nineteenth century, concerns about adulteration, purity, and wholesomeness of foods led to the development of food legislation, including standards for certain products, in different jurisdictions. Nowadays, the basis for food legislation is given as for food safety, consumer protection, and fair trade. The words may differ, but the fundamental reasons have not really changed.

Because of the diversity of varieties and types, with local and regional variations, even among the more generic varieties, development of internationally accepted standards for cheese has proved to be a challenge. The concept of protection of authenticity and diversity of certain traditional food products may be traced to the Convention of Paris for the Protection of Industrial Property in March 1883. This Convention formed the basis of the Agreement on Trade-Related Aspects of Intellectual Property Rights (the TRIPS Agreement), which is now administered by the World Trade Organization, and also led to the development of the AOC system in the legislation of France, with the enactment of the Law for the Protection of the Place of Origin in May 1919. This system specified the place (region or department) in which certain products must be manufactured. Roquefort was the first cheese granted a French AOC in the law of 26 July 1925.

The concept behind the AOC system also spread to other European countries. An international convention was signed at Stresa, north of Milan, Italy, on 1 June 1951, ratified by France, Italy, Switzerland, Austria, the Scandinavian countries, and Holland, on the use of designations of origin and names of cheeses. This was a series of multilateral agreements and is commonly referred to as the Stresa Convention. This stated that "only cheese manufactured or matured in traditional regions, by virtue of local, loyal and uninterrupted usages", may benefit from designations of origin governed by national legislation. Article 1 of the convention prohibits the use of descriptions that contravene this principle. In a 1988 ruling by the European Court of Justice (ECJ) in the 'Deserbais' case (ECJ Case 286/86), relating to the fat content of Edam cheese, the ECJ noted that the Stresa Convention was signed before the EEC Treaty entered into force and when only some member states were party to it, and hence, the ECJ did not feel that it was bound to take the treaty's requirements as limiting. However, shortly thereafter, in the European Community, the various national denominations of origin systems and conventions led to a number of regulations on the protection of designations of food products, including cheeses. These shall be outlined later.

International Standards for Cheese Developed by the Codex Alimentarius Commission

In 1954 the International Dairy Federation (IDF) drew attention to the need for international agreement on terminology for milk and milk products to protect consumers and producers from misleading descriptions. In 1957 this suggestion was discussed at the ninth session of the Conference of the Food and Agricultural Organization of the United Nations (FAO) and a resolution was passed that

Table 1 Examples of the origins of certain cheese variety names

Cheese variety	*Origin of name*
Cheddar	The village of Cheddar in Somerset, England
Camembert	The commune of Camembert in Normandy, France
Emmental	The valley of the Emme river in Switzerland
Edam (Edammer in Dutch)	The town of Edam, Noord-Holland
Roquefort	The commune of Roquefort-sur-Soulzon in southern France
Port Salut	The Trappist Abbey of Notre Dame du Port du Salut in Brittany, France
Grana Padano	The Cistercian monks of Chiaravelle Abbey near Milan, Italy – with the name Padano derived from Pianura Padano, i.e., Po Plain in Italian
Parmigiano Reggiano	The Italian adjectives for Parma and Reggio Emilia in Lombardy, Italy
Neufchâtel	The town of Neufchâtel-en-Bray in the region of Haute Normandie, France

its Director General, in consultation with the International Dairy Federation (IDF) and other interested organizations, should invite all member governments to nominate experts to a committee to develop standards for certain milk and milk products. The resultant body became known as the Joint FAO/WHO Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products (the CGECPMMP). This body remained in place until 1994 when it was renamed and reestablished as the Codex Committee on Milk and Milk Products (CCMMP). The Codex Alimentarius Commission was established in 1962, to implement the joint FAO/WHO foods standards program. Through the CGECPMMP and its successor the CCMMP, the Codex Alimentarius Commission developed, *inter alia*, international standards for cheese in general and for 35 individual cheese varieties between 1963 and 1978.

The original Codex General Standard for Cheese was adopted in 1963. Work started on its revision in the early 1970s, and in 1978 the earlier standard was replaced. A further revision of the standard was completed in 1999 and amended in 2006. This contains the following definition of cheese relevant to all cheeses:

> Cheese is the ripened or unripened soft, semi-hard, hard, or extra hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by:
>
> (a) coagulating, wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partly draining the whey resulting from the coagulation, while respecting the principle that cheese-making results in the concentration of milk protein (in particular, the casein portion), and that consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made; and/or
>
> (b) processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a).

The definition outlined in (a) is intended to represent the traditional method of manufacture, while that outlined in (b) is intended to encompass evolving methods of manufacture. It remains to be seen how the term 'similar', as used in (b), applying to characteristics of the cheese is interpreted in the event of any future trade disputes. The general standard also defined unripened cheese, including fresh cheese, as "cheese that is ready for consumption shortly after manufacture".

A separate Codex group standard for unripened cheese, including fresh cheese, was adopted in 2001; in addition to specific provisions, this requires such cheese to conform to the general definition outlined above. There is also a Codex group standard for ripened cheeses in brine adopted in 1999 and amended in 2001.

As regards the 35 individual cheese standards adopted between 1963 and 1978, a review of these was commenced in the early 1990s. Following discussions, 19 of the original standards were revoked, owing to their limited, if any, involvement in international trade. Of the remaining 16 standards, 15 were revised and were adopted in 2007; the sixteenth, that for extra hard grating cheese, was retained, but not revised. One new standard (Mozzarella) was also adopted.

There are a number of differences between the new standards and the older ones; one such difference relates to the compositional requirements related to fat content of certain varieties. Lower fat contents than heretofore are now allowed for cheeses such as Cheddar and Cream cheese. In such cases, reference fat levels are specified and the relevant variety name may be used with a qualifying term such as reduced fat. Other varieties do not allow lower fat contents, for example, Emmental, Provolone, Brie, and Camembert. Standards for cheeses such as Danbo, Havarti, and Samsø already contained provisions for different fat levels, though the format of these provisions was revised and standardized. An absolute minimum fat content is specified for all cheeses, and use of the relevant variety name is not permitted below this level, even when qualifying fat content terms are used.

In 1978 three standards were adopted for processed cheese and similar products (named variety processed cheese, processed cheese, and processed cheese preparations). A review of these standards was initiated in 1994, and subsequently, the task was limited to developing a single standard for processed cheese, excluding processed cheese preparations; to date, this has not been concluded and the fate of these standards is uncertain. The existing standards are recognized as out of date, and if work on this revision is abandoned, they may well be revoked.

A list of all Codex cheese standards is given in **Table 2**. The format of Codex standards is laid down in the Codex procedural manual, and the section headings are shown in **Table 3**.

Codex standards were originally intended to be adopted by the member countries of the Codex Alimentarius Commission; however, this could only be encouraged and they were not binding in law. The formal recognition of Codex standards as reference points for facilitating international trade and resolving disputes in the World Trade Organization (WTO) has increased their significance, role, and profile.

Of course, many individual countries had developed their own national legislation and standards over the years. Such legislation on cheese shall be discussed, with particular reference to the legislation in the European Union, the United Kingdom, France, Germany, Denmark, Italy, Spain, Ireland, the United States of America, Canada, Australia, and New Zealand.

European Legislation Pertaining to Cheese

In the early 1990s, the European Community developed a series of regulations for quality, specific to the agricultural field, on the protection of geographical indications (PGI), on designations of origin (PDO), and on certificates of specific character, which apply *inter alia* to certain cheese names. Regulation 1107/96 on the registration of such protected products, as amended, contains the list in its annex; as of August 2009, there are 155 cheeses registered as PDO and 15 registered as PGI. Two others are Regulation 2082/92 on certificates of specific character (also referred to as Traditional

Table 2 List of codex general and named variety standards for cheese

Codex standard title	*Codex standard number[a] (CODEX STAN)*	*Latest revision*	*Latest amendment*
General Standard for Cheese	283-1978	Rev. 1-1999	Amd. 3-2008[b]
Standard for Whey Cheeses	284-1971	Rev. 1-1999	Amd. 2-2006
Group Standard for Cheese in Brine	208-1999		Amd. 1-2001
Group Standard for Unripened Cheese Including Fresh Cheese	221-2001		Amd. 1-2008[c]
General Standard for Named Variety Process(ed) Cheese and Spreadable Process(ed) Cheese	285-1978		Amd. 1-2008[c]
General Standard for Process(ed) Cheese and Spreadable Process(ed) Cheese	286-1978		Amd. 1-2008[c]
General Standard for Process(ed) Cheese Preparations (Process(ed) Cheese Food and Process(ed) Cheese Spread)	287-1978		Amd. 1-2008[c]
Standard for Mozzarella	262-2007		
Standard for Cheddar	263-1966	Rev. 1-2007	Amd. 1-2008[c]
Standard for Danbo	264-1966	Rev. 1-2007	Amd. 1-2008[c]
Standard for Edam	265-1966	Rev. 1-2007	Amd. 1-2008[c]
Standard for Gouda	266-1966	Rev. 1-2007	Amd. 1-2008[c]
Standard for Havarti	267-1966	Rev. 1-2007	Amd. 1-2008[c]
Standard for Samsø	268-1966	Rev. 1-2007	Amd. 1-2008[c]
Standard for Emmental	269-1967	Rev. 1-2007	Amd. 1-2008[c]
Standard for Tilsiter	270-1968	Rev. 1-2007	Amd. 1-2008[c]
Standard for Saint-Paulin	271-1968	Rev. 1-2007	Amd. 1-2008[c]
Standard for Provolone	272-1968	Rev. 1-2007	Amd. 1-2008[c]
Standard for Cottage Cheese Including Creamed Cottage Cheese	273-1968	Rev. 1-2007	Amd. 1-2008[c]
Standard for Coulommiers	274-1969	Rev. 1-2007	Amd. 1-2008[c]
Standard for Cream Cheese	275-1973	Rev. 1-2007	Amd. 1-2008[c]
Standard for Camembert	276-1973	Rev. 1-2007	Amd. 1-2008[c]
Standard for Brie	277-1973	Rev. 1-2007	Amd. 1-2008[c]
Standard for Extra Hard Grating Cheese	278-1978		

[a] The recently revised numbering system, using only numbers, is used; previously most general or group standards had a prefix 'A' and individual cheese variety standards had a prefix 'C'.

[b] The 2008 amendment of this standard involved renumbering only; otherwise the 2006 amendment contains the latest amended and revised text.

[c] The 2008 amendment of these standards involved renumbering only; other aspects were not amended or revised. The Standard for Extra Hard Grating Cheese was renumbered again in 2008.

Table 3 Overall structure of sections of codex milk product standards

Section number	*Section title*	*Subsection number*	*Subsection title*
1	Scope		
2	Description		
3	Essential composition and quality factors		
		3.1	Raw materials
		3.2	Permitted ingredients
		3.3	Composition
4	Food additives		
5	Contaminants		
6	Hygiene		
7	Labeling[a]		
		7.1	Name of the food
		7.2	Declaration of milk fat
		7.3	Declaration of milk protein
		7.4	List of ingredients
		7.5	Labeling of nonretail containers
8	Methods of sampling and analysis		
	Annex or Appendix[b]		

[a] Not all labeling subsections are used in individual standards; those used are numbered sequentially 7.1, 7.2, etc.
[b] Annexes and Appendices are included in some standards only. Appendices are included in 10 of the individual cheese variety standards where the wording is intended for voluntary application by commercial partners and not for application by governments.

Speciality Guaranteed or TSG) and Regulation 2301/97 on entry of certain names in the 'Register of certificates of specific character.' The latter has three registered cheeses – Mozzarella (Italy), Boerenkaas (The Netherlands), and Hushållsost (Sweden).

Consequently, a European cheese could not be designated as, say, Neufchâtel, as this is a protected designation, unless produced as prescribed by French legislation. However, a cheese variety name such as Cheddar or Emmental may be used, under European law, unless the name is a specific protected designation for such varieties, for example, West Country Farmhouse Cheddar or Emmental de Savoie. Similarly, a cheese can be called Mozzarella if it is produced using any suitable method of manufacture, provided it does not claim to be a traditional specialty or does not use the logo under Regulation 2082/92. In addition, decisions of the ECJ have ruled that Feta (ECJ Cases 465/02 and 466/02) and Parmesan, that is, Parmigiano Reggiano (ECJ Case 132/05), are protected designations and cannot be used for cheeses other than those produced in accordance with the prescribed requirements.

Apart from these regulations, there is no other specific vertical EC legislation on cheese or individual cheese varieties. It should be noted, however, that European legislation of a horizontal nature, related to areas such as hygiene, additives, and packaging materials apply to all food products and certain aspects related to labeling, including nutrition and health claims, contain provisions relevant to cheese in general.

As most of the individual member states of the EU have their own legislation on cheese, care must be exercised as regards the implications of such national legislation in the countries of retail sale.

Cheese Legislation in a Selection of the Member States of the European Union

United Kingdom

The first UK cheese legislation was contained in the Cheese Regulations 1965. These regulations applied to England and Wales; Scotland and Northern Ireland had separate but identical legislation. They contained a definition of cheese and specified the requirements for hard, soft, and whey cheeses, processed cheese, and cheese spread; it subdivided soft cheese into full-fat, medium-fat, low-fat, and skimmed-milk cheese based on its composition. These standards were amended on a number of occasions but in 1970 were replaced by the Cheese Regulations 1970, which reenacted, with amendments, the 1965 Regulations, and so contained many of the earlier provisions. These 1970 regulations specified compositional requirements for 28 cheese varieties and variants.

Specific ingredients were allowed in cheese in general and in certain types such as soft cheese. The ingredients that could be used in various cheese types were specified, and the list included food additives. European food additive legislation now supersedes this list, but it may be taken as an indication of the food additives in these categories that were in use at the time the legislation was enacted.

The 1970 Regulations were amended in 1974, 1984, and 1995 and were replaced by the short-lived Cream and Cheese Regulations 1995, which applied to England, Wales, and Scotland. The 1995 Regulations had a new definition of cheese, retained the old definitions of processed cheese and cheese spread, and also retained compositional requirements for Cheddar, Blue Stilton, and 10 other UK territorial cheeses; the compositional requirements for other cheeses such as Edam, Gouda, Cream cheese, double Cream cheese, and soft cheeses were not retained. The 1995 Regulations were repealed and replaced by the (UK) Food Labelling Regulations 1996, which retained the definition of cheese and the compositional requirements of the cheeses contained in the 1995 Regulations (**Table 4**); they did not retain the definitions of processed cheese and cheese spread. For the cheeses whose composition is specified in the Food Labelling Regulations, the use of a nutritional type claim in conjunction with the variety name, such as Reduced Fat Cheddar, is not allowed at this time, but discussions have been initiated, which may alter this situation. Furthermore, if terms such as reduced fat or other such nutritional claims are used for cheese, then these should be in line with the relevant EU regulation in this area.

The current definition of cheese given in the (UK) Food Labelling Regulations 1996 is as follows:

> Cheese means the fresh or matured product intended for sale for human consumption, which is obtained as follows –
>
> (a) in the case of any cheese other than whey cheese, by the combining, by coagulation or by any technique involving coagulation, of any of the following substances, namely milk, cream, skimmed milk, partly skimmed milk, concentrated skimmed milk, reconstituted dried milk, butter milk, materials obtained from milk, other ingredients necessary for the manufacture of cheese provided that those are not used for replacing, in whole or in part, any milk constituent, with or without partially draining the whey resulting from coagulation;
>
> (b) in the case of whey cheese –
>
> (1) by concentrating whey with or without the addition of milk and milk fat, and molding such concentrated whey, or
>
> (2) by coagulating whey with or without the addition of milk and milk fat.

Table 4 Cheese varieties and compositional requirements for maximum moisture and minimum fat-in-dry matter content established in the UK Legislation Food Labelling Regulations 1996

1996 Food Labelling Regulations	*Max. moisture (g per100 g)*	*Min. fat in dry matter (FDM)(g 100 g)*
Cheddar	39	48
Blue Stilton	42	48
Derby	42	48
Leicester	42	48
Cheshire	44	48
Dunlop	44	48
Gloucester	44	48
Double Gloucester	44	48
Caerphilly	46	48
Wensleydale	46	48
White Stilton	46	48
Lancashire	48	48

Compiled from HMSO (1996).

The legal status of other varieties of cheese, especially those that had minimum compositional standards established in the Cheese Regulations 1970, is unclear. It is likely that they have become customary names (i.e., a name customary in the member state in which it is sold) if they did not depart significantly from the original compositional profile. The terms full fat, medium fat, hard, and soft, in relation to a cheese, would indicate the true nature of the food as required by Regulation 8 of the Food Labelling Regulations 1996. The UK also has 9 PDO and 2 PGI cheeses registered at European level.

The UK Cheese Regulations 1970 had a very narrow definition of processed cheese, which required it be made from cheese and did not allow the use of any other milk-based ingredients. The related definition of cheese spread allowed the use of cheese and milk products, and such products could also be designated as cheese food. Probably, as a consequence of this, most processed cheese products on the UK market today are designated as either cheese food, used for the products in block or slice formats, or cheese spread, in case of spreadable products.

France

In a speech in 1961, General Charles de Gaulle despaired, “Can you govern a country in which there are 246 kinds of cheese?” At the IDF World Dairy Summit (Congrelait) in Paris in 2002 an exhibition was held entitled La France aux 1000 fromages, which displayed the stated number of cheeses; an accompanying poster illustrated these cheeses. Given such a diversity, it is not surprising that France has detailed legislation on cheese.

The definition of cheese is given in the first article of the Decree 628 of 27 April 2007, which may be translated as follows:

The name cheese is reserved for nonfermented or fermented, unripened or ripened products obtained from the following raw materials: milk, totally or partially skimmed milk, and buttermilk used as such or in a mixture and totally or partially coagulated before draining or after partial elimination of the aqueous phase. The minimum dry matter content of the product corresponding to this definition should be 23 g for 100 g of cheese. (This last criterion may vary for fresh cheeses, depending on their fat content.)

The same text describes the authorized treatments and additions, and various conditions that apply whether the product is sold under the general definition 'cheese' or under that of a cheese defined in the annex of the decree, such as Camembert, Brie, Emmental, Sainte-Maure, and Picodon.

For the general case of a product sold under the name of cheese, one or several of the following products may be used during manufacturing:

- salt
- spices and herbs
- partially or totally dehydrated milk or buttermilk, or a dairy protein preparation (without prejudice to the specific EU regulations on the use of caseins and caseinates in cheese) where required by the technological constraints. The initial protein content of the dairy raw material mixture used should not increase by more than $10\,\mathrm{g\,l^{-1}}$ (but by not more than $5\,\mathrm{g\,l^{-1}}$ for the cheese varieties defined in the decree)
- spice extracts and natural flavor
- within a limit of 30% of the finished weight, sugars and other food products conferring the finished product with a specific flavor – the addition of fat and proteins of an origin other than milk is however forbidden
- rennet, harmless lactic acid bacteria, and yeast and mold cultures
- other substances or other categories of aroma compounds depending on relevant conditions set by interministerial decrees

Processed cheese (*fromage fondue*) is also defined, and there is a new definition for *specialité fromagère fondue* (processed cheese specialty) in the 2007 decree. The annexes

- categorize cheese under ripened and unripened; fresh white, soft, hard, and semihard; goats cheese; and one particular specialité fromagère fondue
- tabulate specific requirements for 27 individual French cheeses in the various categories as regards the origin (species) of milk
- describe the product
- specify shapes and weights
- specify the fat-in-dry matter (FDM) and dry matter composition

Specific rules apply to the use of descriptive terms relating to fat content, such as 0% *de matière grasse*, *maigre*, *allégé*, *crème*, *double crème*, and *triple crème*. The fat content per 100 g should be indicated, but this is not necessary where the product has nutritional labeling that gives the fat content per 100 g.

In addition to the general requirements for cheese outlined above, specific decrees are issued as regards AOC cheeses from France. Today, France has about 45 AOC cheeses, 40 of which are protected at EU level – 36 as PDO and 4 as PGI.

Germany

German cheese legislation is detailed in the *Käseverordnung* (cheese order). This is quite an extensive piece of legislation, defining cheese and setting requirements as regards permitted raw materials, ingredients, labeling, and chemical composition for *käse* (cheese) and *erzeugnisse aus Käse* (products made from cheese). The latter products encompass *schmelzkäse* (processed cheese), *schmelzkäsezuberitungen* (processed cheese preparations), *käsezuberitungen* (cheese preparations), and *käsekompositionen* (cheese compositions).

Cheese and cheese products may only be marketed in specified fat content categories, such as double cream, cream, and full fat, based on FDM content. It should be noted that different compositional criteria apply to processed cheese and processed cheese preparations. These category names should also be used for labeling purposes, but these may be replaced by stating the FDM content on the label in the form X% f.i.tr (*fett in trockenmasse*). Individual cheeses are further classified as *hartkäse* (hard), *schnittkäse* (slicing), *halbfester schnittkäse* (semihard, slicing), *sauermilchkäse* (acid curd), *weichkäse* (soft), and *frischkäse* (fresh) based on the moisture content of the fat-free cheese mass. *Anlage* 1 (Annex 1) sets additional criteria as regards production requirements, permitted fat categories, permitted weights, minimum ripening times, appearance, texture, smell, and taste for certain cheese varieties, including Chester (Cheddar), Emmentaler, Gouda, Edam, Tilsiter, Brie, and Camembert, as well as the typical German varieties and designations of German geographical origin – Germany has four PDOs for cheeses recognized at European level. There are also requirements specified for *frishkäse* and *speisequark*.

Cheese and cheese products from other countries that do not correspond to the specifications of the German regulations are permitted to be marketed if they are produced in accordance with the legislation of their country of origin and are marketable there, but only if the deviation is clearly indicated and legible on the finished package. Such indications should be provided also in connection with the product name if misleading consumers cannot be avoided through the ingredients list. The product name of the country of origin may also be used.

Denmark

Danish cheese legislation on cheese is detailed in the *Mælkeproduktbekendtgørelsen* (which can be translated as the 'executive order on milk products'). The present version dates from 2004 and can be accessed electronically.

Cheese is defined as
The ripened or unripened (fresh), milk, prepared with or without rind, and in which the ratio of whey protein and casein does not exceed that in milk, which is achieved

1. by total or partial coagulation of milk protein in milk, skimmed milk, fat-standardized milk, cream, and buttermilk or a combination thereof by rennet or other appropriate coagulants and with partial drainage of whey; or
2. by a manufacturing process that includes the full or partial coagulation of milk in [dairy] milk and other dairy products, which gives an end product that has similar physical, chemical, and sensory characteristics of the product manufactured listed under point 1.

Cheese is further classified by texture as, for example, extra hard, hard, firm, or soft, based on specified moisture levels on a fat-free basis; by fat content as, for example, extra full-fat, full-fat, *middelfed*, *mager*, and *ekstra mager* based on specified FDM content; and also by ripening type as, for example, unripened or ripened, with mold-ripened indicated where relevant. The FDM content should also be stated on the label as the percentage fat in dry matter or simply as X+, where X is the FDM content. The name of unripened soft cheese may be replaced by names such as Frisk (fresh), Kvark, Hytteost (Cottage cheese), Ryegost (if smoked), or Flødeost (Cream cheese), subject to meeting certain specified compositional requirements.

Italy

A catalogue produced in 1977 listed more than 450 cheeses produced in Italy, and it is thought to be unlikely that a complete list exists of all Italian cheeses. A definition of cheese is given in the Decree of the President of the Republic No. 1099 of November 1953 as follows:

> The use of the term cheese is reserved for the fresh or matured products obtained by draining after the coagulation of milk, cream, skimmed or partially skimmed milk, or a combination of these; the term 'cheese' is further reserved for products obtained by the partial concentration of whey or of buttermilk, but excluding the addition of fatty matter foreign to milk.

It must be noted that the milk sources are quite limited and the use of milk powders is not included.

Recognition of the Protected Denominations of Origin (PDOs) at the national level was introduced under Law No. 125 in 1954. The latest register of the European Union lists 35 Italian cheeses protected as PDOs, and there is also a TSG for Mozzarella as the original specialty as manufactured in Italy. Consorzi have been established to protect and safeguard the protected denominations of origin of a number of Italian cheeses, for example, Asiago, Parmigiano Reggiano, and Grana Padano, and their websites contain the specifications and requirements for the particular cheeses. These Consorzi are quite proactive internationally in the defense of their cheeses.

Spain

Spanish cheese legislation is contained in the Real Decreto 1113/2006. Annex I lays down quality standards for cheese and defines cheese as follows:

> Cheese is understood to be a fresh or matured product, solid or semisolid, obtained from milk, whole milk, skim milk, milk fat, buttermilk, or a mix of some or all of these products, partially or totally coagulated by rennet or other suitable coagulants, before the draining of whey or after the partial elimination of the aqueous matter, with or without prior hydrolysis of the lactose, as long as the ratio between the casein and serum proteins is equal to or greater than that of milk.

This is quite similar to the Codex definition outlined earlier. The minimum dry-milk solids content of cheese is specified as 15%.

Essential and optional ingredients are also laid down. The latter include milk powder, for adjustment of milk solids content to a maximum of 5% of the total dry matter from milk; sugars (sucrose and glucose), alone or in combination, to a maximum of 17% of the final product; and gelatine to a maximum of $5\,\mathrm{g\,kg^{-1}}$ in *queso fresco* (fresh/unripened cheese) and *queso blanco pasterizado* (pasteurized white cheese) only. Based on their degree of ripening, descriptors such as *queso fresco* (fresh or unripened cheese), *queso blanco pasterizado* (pasteurized white cheese), *queso madurado* (ripened cheese), and *queso madurado con mohos* (mold-ripened cheese) are defined for use.

The descriptor mold-ripened cheese may be replaced by *queso azul* (blue cheese) or *queso de pasta azul* (blue paste cheese) when appropriate. The term *madurado* (ripened/matured) may be substituted by the descriptors *tierno*, *semicurado* (semicured), *curado* (cured), *viejo* (old or mature), or *añejo* (extra mature) where they meet the minimum ripening periods specified in the decree, which may differ depending on whether the weight of the cheese is above or below 1.5 kg.

Fat level descriptors based on FDM content are also defined for use such as *extragraso*, *graso*, *semigraso*, *semidesnatado*, and *desnatado*. The fat content should be stated in the labeling of the cheese (as FDM per 100 g). However, it is not required when nutrition labeling is used; furthermore, it may be replaced by the fat level descriptors, as outlined above.

Annex II of the decree addresses quality standards for *quesos fundidos* (processed cheeses) and defines them as the products obtained through grinding, mixing, melting, and emulsification of one or more varieties of cheese, with or without the addition of milk, milk products, or other food products. The addition of milk products is limited by setting the lactose content, which may not exceed 6%. The minimum dry matter of processed cheese is specified as 35%, and for processed cheese described as a spread or spreadable, the minimum level is set at 30%. The fat descriptors used for cheese in Annex I also apply to processed cheese at the same FDM levels.

Ireland

In Ireland, there is no specific national legislation on cheese. Cheeses produced in Ireland and labeled to meet UK legislation have not had problems when sold on the Irish market. This facilitated both producers and consumers; indeed, the size of the home market could create problems if this were not the situation. However, reduced fat variants of common cheese varieties can be designated 'reduced fat' with the variety name on the Irish market but not in United Kingdom; for example, Reduced Fat Cheddar is acceptable in Ireland, provided it meets the relevant EU legislation on nutrition and health claims as regards a reduced fat claim. There is one Irish cheese, Imokilly Regato, protected as a PDO by the EU.

Another possible approach for cheeses intended for the Irish market alone would be to manufacture in accordance with the relevant Codex standard. However, in such instances it would be wise to seek prior approval of the competent Irish Authority, at this time the Department of Agriculture, Fisheries and Food, as regards its status regarding manufacture, composition, and proposed product labeling. This would help avoid a potential problem if quite similar products on the market used different product names; this could be seen to be misleading or confusing to consumers.

US Legislation on Cheese

The Code of Federal Regulations (CFR) is the consolidated source of all US federal legislation as developed by the relevant US government departments and/or their administrative agencies, such as the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA), and published in the Federal Register. It is divided into 50 titles that represent all broad areas that are subject to federal regulation. For instance, Title 7 covers Agriculture, administered by the USDA, and Title 21 deals with Food and Drugs, administered by the FDA. Each volume of the CFR is updated once each year and is issued on a quarterly basis. It is published by the Office of the Federal Register, an agency of the National Archives and Records Administration. The latest CFR is also available online.

Each title of the CFR is divided into chapters; each chapter is further subdivided into parts that cover specific regulatory areas. Large parts of the CFR are subdivided into subparts. All parts are organized in sections, and most citations in the CFR are provided at the section level. The full format of a CFR citation, for example, for Cheddar Cheese is 21 CFR Part 133 Subpart B §133.113. However, the abbreviated version 21 CFR §133.113 is the form that is normally used. Standards of identity for cheese are given in 21 CFR §133. Most of the present standards date from 1977 or 1983, though there were earlier versions of many of these standards. At this time, there are 76 standards of identity for cheeses covering 36 different cheese varieties (**Table 5**), and a further 12 standards for pasteurized processed cheese, pasteurized cheese spreads, and pasteurized blended cheeses (**Table 6**).

Standards of identity for cheese are addressed typically under four main headings:

1. Description – which can be detailed and lengthy;
2. Optional Ingredients – usually subdivided into provisions on milk ingredients, clotting enzymes, and other optional ingredients, which include food additives provisions;
3. Nomenclature – the name of the food;
4. Label Declarations – which state that each of the ingredients used in the food shall be declared on the label as required by the applicable subsections of 21 CFR §101 and §130, except that enzymes of animal, plant, or microbial origin may be declared as enzymes, and that the dairy ingredients may be declared, in descending order of predominance, by the use of the terms milk fat and nonfat milk, or nonfat milk and milk fat, as appropriate.

It is permitted to use claims on nutrient content in conjunction with the names of standardized products, including cheeses. However, in a few cases, specific standards of identity address nutritionally modified cheeses, for example, low-sodium Cheddar (21 CFR §133.116) and low-sodium Colby (21 CFR §133.121). Other general requirements for foods named by use of a nutrient content claim are given in CFR §101.10. Content claims are based on reference amounts customarily consumed per eating occasion; a list of such reference amounts is to be found in CFR §101.12. For most cheeses this reference amount is 30 g, but different amounts apply for cottage cheese (110 g), certain cheeses used mainly as an ingredient (55 g), and extra hard grating cheeses (5 g). Fat-related claims on nutrient content are contained in §101.62, with the requirements for 'light' (or lite) addressed in §101.56. The nutrient claims 'reduced' and 'light' are recognized as comparative nutrient claims and the appropriate reference food must be specified. There are also requirements for labeling statements to be used on foods that make claims based on nutrient content.

In standards of identity for cheeses, food additives or certain additive functional classes may be listed as

Table 5 US cheese varieties or types with standards of identity specified in the Code of Federal Regulations (21 CFR Part 133) excluding processed cheese and processed cheese products

Variety/type[a]	*CFR reference*	*Variety/type*[a]	*CFR reference*	*Variety/type*[a]	*CFR reference*
Asiago	133.102; 133.103; 133.104	Gammelost	133.140	Nuworld	133.162
Blue	133.106	Gorgonzola	133.141	Parmesan and Reggiano	133.165
Brick	133.108; 133.109	Gouda	133.142	Provolone	133.181
Caciocavallo Siciliano	133.111	Granular and Stirred Curd Cheese	133.144; 133.145	Soft Ripened Cheeses	133.182
Cheddar	133.113; 133.114; 113.114	Grated Cheeses	133.146; 133.148	Romano	133.183
Colby	133.118; 133.119; 113.120	Gruyere	133.149	Roquefort and other sheep's milk blue-mold cheeses	133.183
Cold Pack	133.123; 133.124; 113.125	Hard Cheeses	133.150	Samsoe	133.185
Cook Cheese/Koch Kaese	133.127	Limburger	133.152	Sap Sago	133.186
Cottage Cheese	133.128; 133.129	Monterey and Monterey Jack	133.153; 133.154	Semisoft Cheeses	133.187; 133.188
Cream Cheese	133.133; 133.134	Mozzarella and Scamorza	133.155; 133.156; 133.157; 133.158	Skim Cheese for manufacturing	133.189
Washed Curd and Soaked Curd Cheese	133.136; 133.137	Meunster and Munster	133.160; 133.161	Spiced Cheeses	133;190; 133.191; 133.193
Edam	133.138	Neufchatel	133.162	Swiss and Emmentaler	133.195; 13.196

[a] Some cheeses that have similar variety names are grouped together for convenience but all individual CFR references for standards of identity of cheeses are given.

Prepared from US National Archives and Records Administration (2009) *US Code of Federal Regulations (2009)* Washington, DC: The Office of the Federal Register National Archives and Records Administration.

The Code of Federal Regulations is described in the text.

Table 6 US standards of identity specified in the Code of Federal Regulations (21 CFR Part 133) for pasteurized processed cheese and pasteurized processed cheese products

Standard of identity name	*CFR reference*
Grated American Cheese Food	133.147
Pasteurized Blended Cheese	133.167
Pasteurized Blended Cheese with fruits, vegetables, and meats	133.168
Pasteurized Process Cheese	133.169
Pasteurized Process Cheese with fruits, vegetables, and meats	133.170
Pasteurized Process Pimento Cheese	133.171
Pasteurized Process Cheese Food	133.173
Pasteurized Process Cheese Food with fruits, vegetables, and meats	133.174
Pasteurized Cheese Spread	133.175
Pasteurized Cheese Spread with fruits, vegetables, and meats	133.176
Pasteurized Neufchatel Cheese Spread with other foods	133.178
Pasteurized Process Cheese Spread	133.179
Pasteurized Process Cheese Spread with fruits, vegetables, and meats	133.180

US National Archives and Records Administration (2009) *US Code of Federal Regulations (2009)* Washington, DC: The Office of the Federal Register National Archives and Records Administration.

optional ingredients (e.g., stabilizers). A total of 32 such additive functional classes are defined in 21 CFR §170.3(o). Specific individual food additives are not necessarily all listed in the standards of identity. The US definition of a food additive is given in the Food, Drug and Cosmetic Act 1938, as amended, in Section 201(s) and (t), and in the CFR (21CFR§170.3).

There are four parts of the CFR that list and define the substances for food use:

- 21 CFR §181 – Prior-sanctioned food ingredients
- 21 CFR §182 – Substances generally recognized as safe
- 21 CFR §184 – Direct food substances affirmed as generally recognized as safe
- 21 CFR §186 – Indirect food substances affirmed as generally recognized as safe

A list of all the ingredients and substances are given at the start of each of the above parts, giving the section reference for each compound; in addition, 21 CFR §189 lists substances prohibited from use in human food.

A further useful reference point on substances for use in food in the United States is the food additive status list on the FDA website. This lists substances alphabetically and outlines their status and limitations for use. For a brief overview on this topic, a useful document is a short publication by the International Food Information Council in 2005, prepared with the assistance of the FDA.

Canadian Legislation and Standards for Cheese

Canadian food legislation, which prescribes the standards of composition, quality, and other properties of certain foods, is contained in the Canadian Food and Drug Regulations; a consolidated up-to-date version is accessible online. The provisions related to food are contained in Part B, with dairy products addressed in Division 8 thereof. The detailed provisions for cheeses may be found from Section B.08.030 to Section B08.054.

Section B08.032 covers 50 cheeses encompassing 41 individual varieties and variants, specifying their individual compositional requirements for maximum levels of moisture and minimum levels of fat, and listing their combined permitted food additives and labeling provisions. Other specific sections address Cheddar (B08.034), Cream cheese and variants (B08.035, B08.037, B08.038, B08.039), the six standards for processed cheese and related products (B08.040, B.08.041, and B.08.041.1 to B.08.041.4), and cold pack cheeses (B.08.041.5 to B.08.041.8). Many of the cheese variety names are similar to those of the United States, but the compositional requirements are not necessarily the same; see **Table 7** for just a few such examples.

An amendment to the Canadian Food and Drug Regulations in 2007 sets the minimum amounts of casein in cheese that must come directly from certain milks (i.e., milk, partly skimmed milk, skimmed milk, or cream) rather than from other milk products. The minimum amount of casein from the specified milks is expressed as percentage by weight of the total casein content of the cheese. This is set at

- 63% for Pizza Mozzarella and Part Skim Pizza Mozzarella;
- 83% for other types of Mozzarella and for Cheddar, Colby, Monterey Jack (and variants), Brick (and variants), Farmer's, and other varieties;
- 95% for 33 other named varieties and variants; and
- 100% for Traditional Cheddar Cheese.

Table 7 General overview of compositional requirements of some major cheese varieties of importance in international trade in Codex Alimentarius standards and in the legislation of certain countries

	Moisture (maximum) (g per 100 g)						*% FDM (minimum) (g per 100 g)*						*% Fat (minimum) (g per 100 g)*					
Cheese	*UK*	*US*	*CA*	*FR*	*DE*	*CX*	*UK*	*US*	*CA*	*FR*	*DE*	*CX*	*UK*	*US*	*CA*	*FR*	*DE*	*CX*
Cheddar	39	39	39	n/s	38	39	48	50	n/s	n/s	45	22[a]	n/s	n/s	31	n/s	n/s	n/s
Edam[b]	(46)[c]	45	46	n/s	51	53	(40)[c]	40	n/s	n/s	30	30	n/s	n/s	22	n/s	n/s	n/s
Gouda[b]	(43)[c]	45	43	n/s	51	52	(48)[c]	46	n/s	n/s	30	30	n/s	n/s	28	n/s	n/s	n/s
Emmental	(40)[c]	41	40	40	38	40	(45)[c]	43	n/s	45	45	45	n/s	n/s	27	n/s	n/s	n/s
Brie	(n/s)[c]	n/s	54	56	56	58	(n/s)[c]	(50)[d]	n/s	40	45	40	n/s	n/s	23	n/s	n/s	n/s
Camembert	(n/s)[c]	n/s	56	see[e]	62	62	(n/s)[c]	(50)[d]	n/s	40	30	30	n/s	n/s	22	n/s	n/s	n/s
Cream Cheese	(60)[c]	55	55	n/s	61[f]	78	(n/s)[c]	n/s	n/s	n/s	50[f]	25[a]	(45)[c,g]	33	30	n/s	n/s	n/s

[a] Codex specified both (absolute) minimum and reference values for fat content of these cheeses; only the minimum is contained in this table.

[b] Some legislation and standards specify different requirements for 'baby' and 'loaf' variants of these cheeses.

[c] These requirements are those in the UK Cheese Regulations 1970, as amended, and are included for comparison purposes. Requirements for these varieties are not included in the UK Food Labelling Regulations 1996 (HMSO, 1996).

[d] The US Code of Federal Regulations does not have individual standards for these cheese varieties; the FDM value shown is that for Soft Ripened Cheese (21 CFR §133.182) (US National Archives and Records Administration, 2009 *US Code of Federal Regulations (2009)* Washington, DC: The Office of the Federal Register National Archives and Records Administration).

[e] The 2007 French Decree on cheese specifies the dry matter of Camembert as 110 g par pièce (per piece) (Anon, 2007).

[f] These are the requirements for Rahmfrishkäse; the Käseverordnung specifies compositional requirements for both Rahmfrishkäse and Dopplerahmfrishkäse.

[g] This is the requirement for Cream Cheese in the UK Cheese Regulations 1970, as amended, now repealed, but are still followed by products on the market. Specific compositional requirements were specified for fat in both Cream Cheese and Double Cream Cheese.

CA, Canada; CX, Codex Alimentarius; DE, Germany; FR, France; n/s, not specified; UK, the United Kingdom; US, the United States of America.

Cheese Legislation in Australia and New Zealand

Historically, Australia and New Zealand had separate legislation and indeed in part that remains the situation to this day. The earlier 1987 Food Standards Code, now repealed, was developed for Australia, but later adopted and updated by the Australia New Zealand Food Authority (ANZFA), and was applicable up to the adoption of the current code in 2000. This earlier Code contained more detailed provisions for individual cheeses in Standard H9, which encompassed cheese and cheese products.

In 1995 Australia and New Zealand signed a treaty agreeing to develop and implement a single set of food standards. The Food Standards Treaty provided for Food Standards Australia New Zealand (FSANZ) to undertake food standards development for both countries. FSANZ has produced a Food Standards Code 2000, which is regularly updated and contains the joint standards developed to date.

The 2000 Food Standards Code provides a definition of cheese similar to the 1999 Revision of the Codex General Standard for Cheese and specifies a list of ingredients permitted in cheese.

The definition of cheese given in Standard 2.5.4 of the Food Code is as follows:

> Cheese means the ripened or unripened solid or semi-solid milk product which may be coated and is obtained by one or both of the following processes –
>
> (a) coagulating wholly or partly milk, and/or materials obtained from milk, through the action of rennet or other suitable coagulating agents, partially draining the whey which results from such coagulation; or
>
> (b) processing techniques involving concentration or coagulation of milk and/or materials obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product described in paragraph (a).

Part (a) of this definition is an earlier and simpler definition from that in the present Codex definition of cheese as outlined earlier.

The ingredients permitted in cheese are specified as

- water
- lactic acid-producing microorganisms
- flavor-producing microorganisms
- gelatine
- starch
- vinegar
- salt

Processed cheese is defined as a product manufactured from cheese and products obtained from milk, which is heated and melted, with or without added emulsifying salts, to form a homogeneous mass.

Standard 4.2.4A of the Code, which applies to Australia only, sets out some primary production and processing requirements for Gruyere, Sbrinz, Emmental, and Roquefort cheese; references and requirements for other individual cheese varieties are not included.

At this time the production of individual varieties of cheese in conformance with the relevant Codex standards for cheeses should be acceptable in Australia and New Zealand.

Conclusions

Although the Codex Alimentarius standards for cheese are reflected to a greater or lesser extent in cheese legislation throughout the world at this time, it is not possible to give a single definition of cheese, or of any specific variety of cheese, that will meet with full acceptance throughout the world. Some countries, among them the United Kingdom, Australia, and New Zealand, have adopted an earlier version of the Codex definition of cheese, which is a little more liberal than the current one. The approach of the United States on the other hand has been to develop standards of identity for quite a large number of individual cheese varieties.

Table 7 outlines some similarities and differences in the broad compositional requirements for seven individual varieties of cheese across six countries and the relevant Codex standards. This table should be used with caution, as it is an oversimplification of the situation. In the short term, therefore, it is still necessary to consult the specific legislation of the individual countries to get a full understanding of the requirements. It remains to be seen if the Codex cheese standards, which have been revised and updated in the recent few years, get wider acceptance in the future. This may depend on the outcome of any challenges made at the WTO in the event of disputes between countries regarding cheese denominations.

See also: **Cheese**: Camembert, Brie, and Related Varieties; Cheddar-Type Cheeses; Cheese Analogues; Dutch-Type Cheeses; Hard Italian Cheeses; Low-Fat and Reduced-Fat Cheese; Overview; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasteurized Processed Cheese Products; Swiss-Type Cheeses.

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CHOCOLATE

Milk Chocolate

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Introduction

Chocolate is almost unique as a food in that it is a solid at room temperature, yet melts easily in the mouth, so that it can be swallowed as a smooth liquid. It originated as a drink and took its present form only in the last 150 years. In most countries, milk chocolate far outsells plain and white chocolate. The physical and chemical properties of the milk components affect the making of the chocolate as well as its flavor and texture. Many plain chocolates contain milk fat in order to retard the development of a white mold-like appearance, known as chocolate bloom. Milk-derived ingredients, such as lactose and whey, are used in a wide variety of confectionery, and new products, specifically for chocolate production, are continually being developed by the milk-processing industry.

History

To be called chocolate, a product must contain some cocoa-derived ingredients. The cocoa tree (*Theobroma cacao*) will only grow within 20° of the equator. It originated in South and Central America where the Aztecs and Incas roasted and ground the beans to make them into a drink. The Spanish introduced this to Europe and cocoa drink houses became popular in London at the time of Samuel Pepys. In 1727, Nicholas Sanders is said to have been the first to add milk to the drink.

Over half of the cocoa bean consists of a fat, called cocoa butter. This fat tended to spoil the drink, and so a Dutchman called Van Houten developed a press to remove most of it and leave cocoa powder. In 1847, the UK company, Joseph Fry, combined cocoa beans, cocoa butter, and sugar to produce a plain eating chocolate.

If water is added to chocolate, it becomes soft and pasty and does not have a snap when you break it, nor the hard bite associated with chocolate. The addition of milk to chocolate was not therefore easy and it was only in 1875 that Daniel Peter in Switzerland made the first bars. He was able to do this because condensed milk had recently been made commercially available, so he had less water to remove. Also he had cheap water power available to him to carry out the remainder of the drying. Most chocolate contains about 1% of moisture.

White chocolate is an even more recent development. The main ingredients are sugar, milk powder, and cocoa butter. This means that it does not contain any of the brown cocoa material found in cocoa powder, but has more milk than the other main types of chocolate.

Composition of Milk Chocolate

Figure 1 is a schematic diagram of a section through a piece of chocolate. Just under half the weight is sugar, and about one-quarter is the non-fat cocoa and milk particles. All these particles must be milled so that the majority of them are smaller than 30 μm. Larger particles are easily detected by the tongue and would make the chocolate feel gritty. This solid material is surrounded by fat, which is a mixture of cocoa butter and milk fat (vegetable fats made from special nuts that mimic cocoa butter are also used in some countries). This fat, which normally makes up between 27 and 32% of the weight of chocolate, must coat the surface of all the solid particles, to enable them to move past one another. This is needed to produce a smooth-flowing liquid, which can be poured into molds or poured over center ingredients such as wafers, toffees, and fondants. The fat then sets to produce the products that are found in shops.

When the chocolate is placed in the mouth, the fat melts and it is now even more important that it coats the solid particles. Otherwise, instead of having a smooth liquid that can be swallowed easily, it would be much more pasty and would tend to feel gritty due to solid particles sticking together to form agglomerates.

It is not easy to coat sugar with fat, however, as the sugar surface tends to be lipophobic. In order to help this wetting process, surface-active agents are added, which

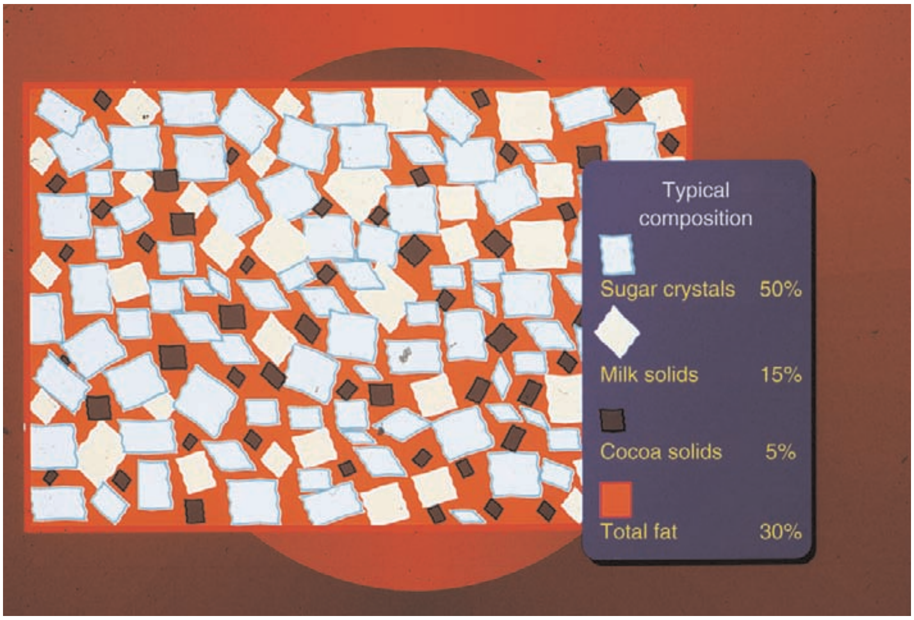

Figure 1 Schematic diagram of the solid particles and fat within a piece of milk chocolate.

form a layer on the sugar surface and help the chocolate to flow. The most widely used surface-active agent (emulsifier) is lecithin, which is normally obtained from soybeans. Milk also contains some lecithin and in addition the proteins can have an emulsifying effect, both of which can affect the flow properties of chocolate. This coating action is normally carried out in a large mixing machine, peculiar to the industry, which is called a conche and which typically holds from 3 to 10 tonnes of chocolate.

Flow Properties of Liquid Chocolate

How liquid chocolate flows has a major effect on its processing and eating properties. Incorrect viscosity can lead to misshapen or overweight products, or to poor flavor and texture. Chocolate, like tomato ketchup and non-drip paint, has a viscosity that varies depending upon how quickly it is being poured or stirred. This is known as non-Newtonian flow. It cannot be described by a single number, and traditionally the viscosity is described by two parameters: a yield value, which relates to the force required to start the mass of liquid chocolate flowing, and a plastic viscosity, which is more concerned with keeping the flow moving.

Milk affects both these parameters. The butterfat combines with cocoa butter to coat the solid particles and this helps lower both. However, if the butterfat is contained within cells within the non-fat milk solids, for example, surrounded by amorphous lactose, then it cannot affect the viscosity of the chocolate. This can be demonstrated by making a chocolate with full cream milk powder produced by roller drying and comparing it with an identical one made with spray-dried powder. The latter contains much more bound-up fat and so the chocolate is much thicker. Most milk powder producers prefer to make a product with a high level of bound fat as this has better flow and keeping properties. This is contrary to the requirements of the confectionery industry and so several methods have been patented to free the fat from the amorphous lactose. One of these involves adding crystalline lactose before spraying, to try to stop the formation of lactose glass. An alternative approach is to moisten the milk powder before redrying it in a fluidized bed. The use of high homogenization pressures during the manufacture of whole milk powder has also been shown to increase the free-fat content, although not as much as by crystallization techniques.

Small amounts of surface-active agents usually have a big effect on the yield value of chocolate. Most chocolates contain about 0.4% of lecithin and as can be seen in **Figure 2**, this amount is very beneficial in reducing both flow parameters. If more is added, however, the yield value increases again. This may be due to the formation of a bilayer around the sugar, or to the lecithin molecules joining together to form micelles. The lecithin in milk forms part of the lecithin content of the chocolate. Normally, it is at a low level, but may become important if buttermilk powder is used. Some chocolates, particularly in the United States, contain high levels of buttermilk, and in these cases the level of soy lecithin may have to be reduced to avoid the thickening effect described above.

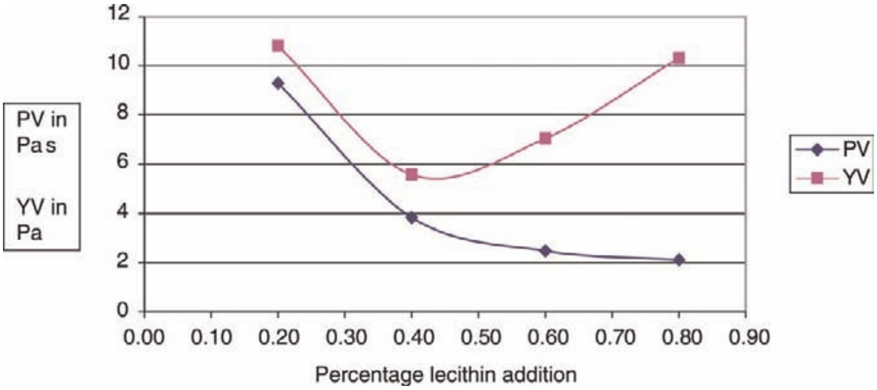

Figure 2 The effect of the addition of different amounts of lecithin upon the chocolate viscosity parameters. PV, plastic viscosity (Pa s); YV, yield value (Pa).

The proteins, both whey and caseinates, have an emulsifying effect. Their effect depends upon the amount used and the other ingredients present. The moisture of the chocolate is also important. Although this is present at around 1%, it significantly thickens the chocolate, probably by 'sticking' the sugar particles together. Lecithin and proteins, being hygroscopic, help to reduce this effect.

The processing and the structure of the various types of milk powders also play a significant role in determining the final flow properties of chocolate. Several in-depth studies have been carried out by Prof. Tscheuschner and co-workers in Germany.

Flavor of Milk Chocolate

Plain chocolate has a distinctive cocoa flavor, with some acidic/fruity notes. This comes from numerous chemical compounds, which in turn relate to the bean type, its growing conditions, fermentation, roasting, and other processing. The milk solids tend to counter the acidity and produce a more creamy taste. This is particularly true for the milk proteins. For a particular chocolate flavor, a careful balance must be maintained between these proteins and the cocoa content. A reduction by a few percent of the caseinates can produce a significant increase in cocoa flavor.

The flavor of a chocolate depends upon the speed with which the flavor components reach the different receptors in the mouth and nose. This in turn depends upon the viscosity of the chocolate and how it melts. This means that chocolates with the same amount of milk can taste very dissimilar if they flow differently or have a different particle size. Chocolate from a refrigerator will taste different from a bar that has been kept at room temperature.

The lactose in the milk does not affect this cocoa/creaminess balance, but can be used to adjust the sweetness of the chocolate. It can also be used to replace some of the sucrose, when a reduction in sweetness is required. Care must be taken, however, to ensure that the lactose is free from off-flavors. Metallic or cheesy notes can very quickly make the chocolate unpalatable.

The milk fat has little effect on the creaminess of the product compared with the non-fat components. It does, however, alter very significantly the texture and melting properties of chocolate.

The milk components have a very significant effect on the color of the chocolate, making it a much lighter brown. Other factors such as the type of cocoa bean and its roasting conditions also affect this parameter.

Effect of Milk Fat on Chocolate Texture

Chocolate obtains its characteristic texture/mouthfeel from the cocoa butter. This is a triglyceride, that is, it has three fatty acids attached to a glycerol backbone. In the case of cocoa butter, oleic (about 35%), stearic (about 34%), and palmitic (about 26%) acids make up most of the fatty acids present, with oleic acid normally (80%) being located in the central position. This in fact makes it a relatively simple fat and is the reason why it melts over the relatively narrow range of temperature between room temperature and that of the mouth. However, it can crystallize in six different forms, depending on the temperature and how it is processed (see **Figure 3**). Forms I–IV are unstable and would give chocolate a crumbly texture and a dull appearance, rather than the solid bite and gloss preferred by the consumer.

Form V is produced in the factory by machines called temperers, which take the chocolate through a cooling/heating cycle while vigorously mixing it. Although Form VI is more stable, it normally forms only by a solid-to-solid transition, unlike the other forms, which can be formed in liquid chocolate.

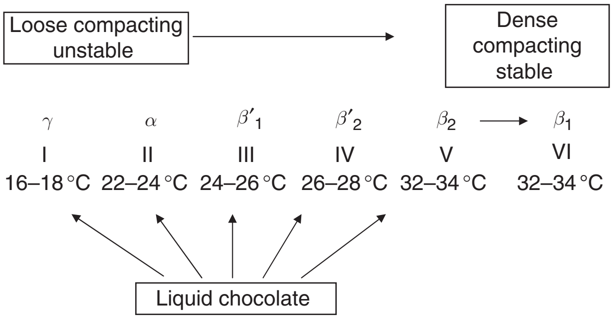

Figure 3 The temperature ranges for the stable formation of the six different forms of cocoa butter.

The different forms are due to the individual molecules of fat packing themselves together in different ways. If milk fat is present, it cannot fit exactly within this packing, so the structure formed is not so compact and the texture is much softer. For this reason, milk chocolate is normally much easier to bite into than the plain varieties and has a more easily melting creamy texture. The latter is, however, in part due to the fact that it melts more easily as there is proportionately less solid fat present at normal ambient temperatures.

Anhydrous milk fat is partially liquid at room temperature, whereas cocoa butter is largely solid. It would be expected therefore that additions of milk fat would increase the liquid fat content, but this is magnified by what is known as a eutectic effect, which limits the amount of butterfat that can be used in chocolate.

The eutectic effect arises from the fact that the two fats are not compatible in their structures. This is illustrated in **Figure 4**. At 20 °C, about 80% of the cocoa butter is solid, which is sufficient to give it a hard bite. An equal mix of cocoa butter and butterfat (20% solid) would be expected to have an average solid content of about half of this due to a dilution effect, that is, 50% solid. It is in fact less than 35% solid, which would give a very pasty product.

At 20 °C, a mixture containing 25% milk fat has a solid fat content of only about 60%. This is relatively soft and is about the limit that can be used, unless of course the fat is bound up within the lactose of the milk and so cannot affect the crystallization of cocoa butter. Chocolate contains approximately 30% of fat overall, which means that most recipes contain less than 7.5% of free milk fat or the chocolate becomes soft and easy to melt. Increasing the amount of milk fat also makes the color of the chocolate lighter.

Milk fat can be fractionated into elements with different hardnesses, usually to provide high-, medium-, and low-melting fractions. These change the hardness of the chocolate according to their different dilution and eutectic effects. The fractionation may also change the milk flavor of the chocolate. The hard fraction normally gives a harder product, although there is some evidence that extra-hard fractions may upset the crystallization (tempering) process and thereby give the opposite result by causing the cocoa butter to set in its unstable forms.

Fat Bloom on Chocolate

Chocolate bloom has the appearance of a white powder, like a frost, which can be mistaken for mold. It is in fact composed of large cocoa butter crystals that have been formed on the surface. There are three different causes of fat bloom.

Perhaps the main cause is that the fat has changed its crystal form. This may be due to the manufacturer not setting it in Form V, in which case the product may go white in a few days. Alternatively, as the chocolate ages, it changes from Form V to Form VI. This may take months or even years if the chocolate is stored correctly.

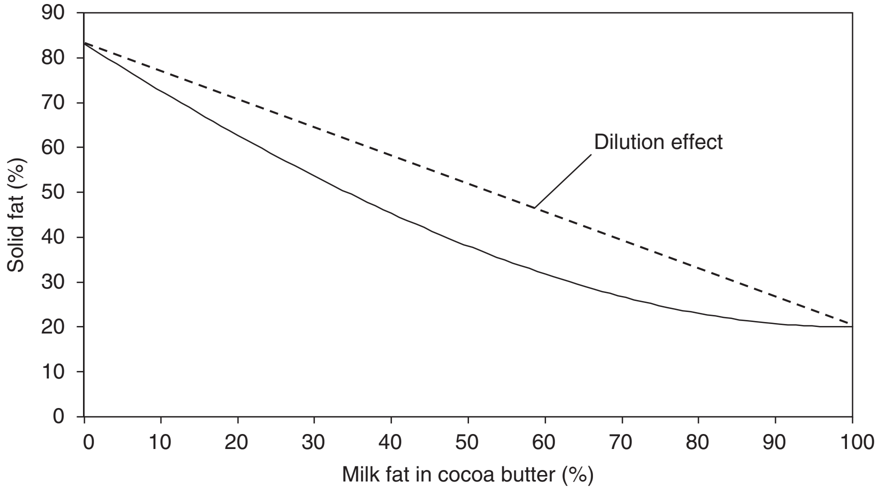

Figure 4 The relative hardness of mixtures of cocoa butter and milk fat at 20 °C.

However, the transformation is much faster at higher temperatures.

A second cause is when the product has been melted, perhaps by being left in the sun. The fat resets leaving the white crystals on the surface.

In a box of chocolates with several different types of centers, those containing nuts or nut-based pastes are usually the first to bloom. This is because the nut fat, like milk fat, is mainly liquid. This migrates through the chocolate, making it softer and pushing some of the fat onto the surface, where it crystallizes.

The addition of milk fat to the chocolate reduces the cocoa butter's tendency to change its crystalline form and thereby slows down bloom formation. This may be due to the way the different molecules of liquid fat are contained within the structure. This is also able to slow down the formation of bloom due to nut oil. The effect is independent of the other milk components and so can be applied to plain chocolates. Some plain chocolates in fact contain more milk fat than milk chocolates.

The more milk fat that is added, the stronger will be the antibloom effect. In addition, some milk fat fractions are better than others, with the harder fractions usually being the best for this purpose.

Milk-Based Ingredients Used in Chocolate Making

Whole Milk Powder

Both spray- and roller-dried powders are used to make milk chocolate. Specially processed spray-dried powders with a higher level of free fat are produced for use in confectionery.

Skim Milk Powder

This is used in combination with milk fat to make chocolate. This means that all the fat is free, so the viscosity is lower and the texture softer than in a product made with an equivalent amount of whole milk powder. The taste is also different from that of a chocolate made with whole milk powder. Sometimes, lipase-free milk powders are requested. This is because the lipase can accelerate decomposition of some of the milk fat into free fatty acids, which produce a soapy or cheesy flavor.

Chocolate Crumb

This ingredient was developed at a time when the keeping properties of milk powders were poor. The chocolate industry has peak sales at Christmas, which coincides with low milk production, so an ingredient with a long shelf life was developed.

Crumb is manufactured by drying sweetened condensed milk with finely milled cocoa beans (without the shell). Here, the sugar and the cocoa help to preserve the milk fat, which would otherwise go rancid. It is interesting that the antioxidant properties of cocoa have only been highlighted in the last few years, even though chocolate crumb was developed more than 60 years ago.

The antioxidant properties tend to be in the non-fat brown cocoa powder. This enables milk chocolate to be sold in transparent packaging if desired. White chocolate, on the other hand, must have opaque wrapping as otherwise the light will accelerate the rancidity of the milk fat.

The presence of proteins, moisture, and reducing sugars during drying is ideal for the Maillard reaction, which introduces a cooked note into the product. This is the main reason why the chocolates in countries that traditionally use crumb, like the United Kingdom, taste very different from those where milk powders are mainly used, for example, France.

Whey Powder

This powder is normally used at a level up to about 5% of the chocolate mass to increase the milkiness of the product. It is used at higher levels in coatings and chocolate-like products. In general, demineralized whey is used so as not to impart unwanted flavors into the chocolate.

Lactose

Lactose can be used as a partial alternative to sucrose in order to make the chocolate less sweet. As with whey, it must not contain any off-flavors.

High-Fat Powders

Whole milk powders are available with more than 55% milk fat. This anhydrous fat is largely in a free form and so aids chocolate flow. The high-fat powders enable the manufacturer to add all the milk fat as a powder and thereby avoid the cost and inconvenience of additional liquid fat metering systems.

Buttermilk Powder

As with whey powder, this can be used in smaller proportions to adjust the flavor and flow properties of a chocolate. It is also used in chocolate-flavored coatings and, in the United States, a special type of product known as buttermilk chocolate is manufactured.

Legislation Concerning Milk Chocolates

There are very distinct legal definitions of what can be called chocolate. These vary from country to country, although most markets apply either the European Union or *Codex Alimentarius* or United States regulations. Chocolate-like products that do not meet the appropriate criteria must be labeled as chocolate-flavored coatings or a similar name and may contain milk components, such as whey, at any level.

Most milk chocolate legislation has minimum milk fat solids and non-fat milk solids levels. The non-fat milk solids must normally be in the ratio at which they are found naturally. This means that skim milk powder cannot just be replaced by whey or lactose.

In most legislation, the minimum milk fat level is between 2.5 and 3.5% and the total milk content must be between 12 and 14% of the chocolate. Several traditional UK chocolates contain a minimum of 20% milk of which at least 5% is milk fat. Because of the higher milk content, there is normally less non-fat cocoa solids, which is regarded as inferior in some countries. This means that in all countries within the European Union, except the United Kingdom and Ireland, they have to be labeled as family milk chocolate or its equivalent.

Future Developments

Although the chocolate industry is a relatively small user of milk products, it is also a stable and slightly increasing one. It is also one where specialist ingredients can be used in an economic way. This means that the development of products with special flavors or which have components that aid liquid chocolate flow or reduce fat bloom is likely to increase. In addition, the sales of organic and fair trade products and those with ingredients from specific locations have increased dramatically over recent years and this trend is likely to continue.

See also: **Analytical Methods**: Principles and Significance in Assessing Rheological and Textural Properties. **Butter and Other Milk Fat Products**: The Product and Its Manufacture. **Flavors and Off-Flavors in Dairy Foods**. **Hazard Analysis and Critical Control Points**: Processing Plants. **Liquid Milk Products**: Recombined and Reconstituted Products. **Milk Protein Products**: Functional Properties of Milk Proteins. **Whey Processing**: Utilization and Products.

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CONCENTRATED DAIRY PRODUCTS

Contents
Evaporated Milk
Sweetened Condensed Milk
Dulce de Leche
Khoa

Evaporated Milk

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Product Description

Evaporated milk is the commercial name for sterilized unsweetened condensed milk, that is, fresh cow's milk from which a considerable portion of the water has been removed. The first person to preserve milk in concentrated form was Nicolas Appert, who, in the early nineteenth century, concentrated milk by boiling it in a water bath over a fire, then poured it into glass bottles after cooling, and sterilized the final product by heating the bottles for 2 h in a boiling water bath. Two inventions made in the second half of the nineteenth century resulted, essentially, in the process that is still used. In 1856, Gail Borden patented the evaporation of milk at reduced pressure, using the concentrate to make sweetened condensed milk. A process for sterilizing concentrated milk in tinned cans, which were rotated in an environment of pressurized steam, allowing a relatively short sterilization time, was patented in 1884 by John B. Meyenberg.

The composition of evaporated milk is regulated by the Codex Alimentarius and by the legislation of individual countries. The traditional standards are the British Standard, which requires a minimum of 9% milk fat and a minimum of 31% total milk solids, and the US Standard, with a minimum of 7.9% milk fat and 25.9% total milk solids. The standard required by the Codex is the most common in international trade, having a minimum of 7.5% milk fat and 25% total milk solids. Thus, the concentration factor ranges from 2.0 to 2.5. The Codex allows adjustment of the percentage of protein in the solids-not-fat, the minimum being 34%, by adding milk permeate, lactose, or milk retentate; however, additions that change the casein-to-whey protein ratio are not allowed. Moreover, local legislation in some countries may still forbid standardization of protein.

In addition to milk, skim milk, and cream, sweet cream buttermilk is frequently used as a source of part of the milk solids. The main reason is that sweet cream buttermilk acts as a source of membrane lipids from milk, which improves heat stability. In addition, sweet cream buttermilk is considered to improve flavor, particularly in recombined evaporated milk.

If the availability of fresh milk is limited, part or all of the solids-not-fat can be derived from milk powder and the fat from anhydrous milk fat (AMF). If only milk powder and AMF are used, the technical name by which the product is known is 'recombined evaporated milk'. As the properties of recombined evaporated milk need not be different from those of evaporated milk produced from fresh milk, it is not discussed separately here, but is discussed in the section 'Production Methods'. The Codex does not distinguish the product made from milk powders and AMF from the product made from fresh milk. However, national legislation may set limits to the use of powders; for example, EU legislation requires that not more than 25% of the milk solids in regular unsweetened condensed milk be derived from powder.

An increasing amount of evaporated milk contains vegetable fat instead of milk fat; in some countries, this is labeled 'filled' evaporated milk, and in others, with a trade name. Palm oil is used most frequently if cost reduction or local preference is the reason for not using milk fat. Oils high in polyunsaturated fatty acids, for example, sunflower or soy oil, are used in products for a blood cholesterol-lowering diet. The stability to oxidation is of utmost importance for all fats used in recombining.

Sodium orthophosphate is usually added to control heat stability. Soy lecithin, calcium chloride, or calcium carbonate may also be used for this purpose. Polyphosphates may be added to UHT-sterilized evaporated milk to retard age gelation. The Codex sets a maximum addition of 0.2% singly or 0.3% if a combination is used. κ-Carrageenan (~0.005%) is sometimes added to retard creaming.

Uses

The inventors of evaporated milk aimed to produce a product that would not perish during storage for months at ambient temperature, that could be transported easily over long distances, and that would contain most of the valuable nutrients of milk. In some countries with low internal milk production, especially in the tropics, evaporated milk still is a general-use milk product. In other markets, evaporated milk is used for specific purposes, for example, in coffee and tea, or for cooking.

As drinking milk, evaporated milk is consumed after 1:1 dilution with (boiled) water. Such a 1:1 (v/v) dilution of Codex standard evaporated milk yields a product with a slightly higher solids-not-fat content and fat:solids-not-fat ratio than those of the regular 3.5% fat full-cream milk. Some consumers, the percentage varying from country to country, actually prefer the flavor of sterilized milk obtained in this way to that of pasteurized or UHT-sterilized milk. In coffee or tea, a relatively small amount is sufficient to give the drink a milky flavor and a white appearance. Moreover, the Maillard products in evaporated milk result in coffee or tea having a yellowish hue, which is preferred over the grayish hue obtained if, for example, pasteurized or UHT milk is added.

Production Methods

Processing schemes for the production of in-container sterilized and UHT-sterilized evaporated milk are shown in **Figure 1** and for recombined evaporated milk in **Figure 2**. Essential processing steps are discussed in more detail in the subsequent sections.

Preheating

Preheating is heat treatment of the milk before it is concentrated. Its main purpose is to increase the heat stability of the concentrated milk; in fact, homogenized full-cream evaporated milk cannot be sterilized if the unconcentrated milk has not been preheated. In addition, preheating contributes to the inactivation of spores if the temperature is higher than ~110 °C. Continuous-flow preheating at 110–130 °C for about 1–3 min has been known, since the 1940s, to yield evaporated milk with the highest heat stability, and is used most commonly. It is especially suitable for the production of high-solids, for example, British Standard, evaporated milk. In addition, the traditional method for heating milk in ‘hot wells’ at 90–95 °C for about 10–15 min is still being used. For recombined evaporated milk, preheating is carried out by the powder manufacturer, before concentration and drying. This allows the powder to be reconstituted directly at the concentration required for recombined evaporated milk. An alternative method of recombining is to reconstitute standard medium-heat milk powder to ~9–10% of solids-not-fat, preheat this milk, and evaporate off water to the desired concentration. Either method yields a good-quality product; for the latter method, the benefit of using a commodity powder has to be balanced against the costs of additional equipment and higher energy use at the recombination site.

Concentration

Milk is usually concentrated in a multistage falling film evaporator, designed with emphasis on the efficient use of energy. Overconcentrating should be avoided because it may lower heat stability, and because it demands a higher energy use and lowers capacity. Therefore, the total solids content of the concentrate as it leaves the evaporator is adjusted continuously, based on the measurement of its refractive index or density. The final standardization of fat and total solids is usually carried out between concentrating and sterilizing. Concentration of milk by reverse osmosis also yields unsweetened condensed milk, but other membrane filtration processes do not, because not only water, but also salts, lactose or proteins may be removed. Although ‘evaporated’ milk produced by reverse osmosis has virtually the same composition and properties as that produced by evaporation, industrial application of reversed osmosis for this purpose appears to be very limited.

Homogenization

The purpose of homogenization is to prevent coalescence of fat globules and to reduce the rate of creaming during storage of the product. It also affects heat stability; homogenization pressures up to about 5 MPa generally have little effect or slightly increase heat stability, but higher pressures result in a large

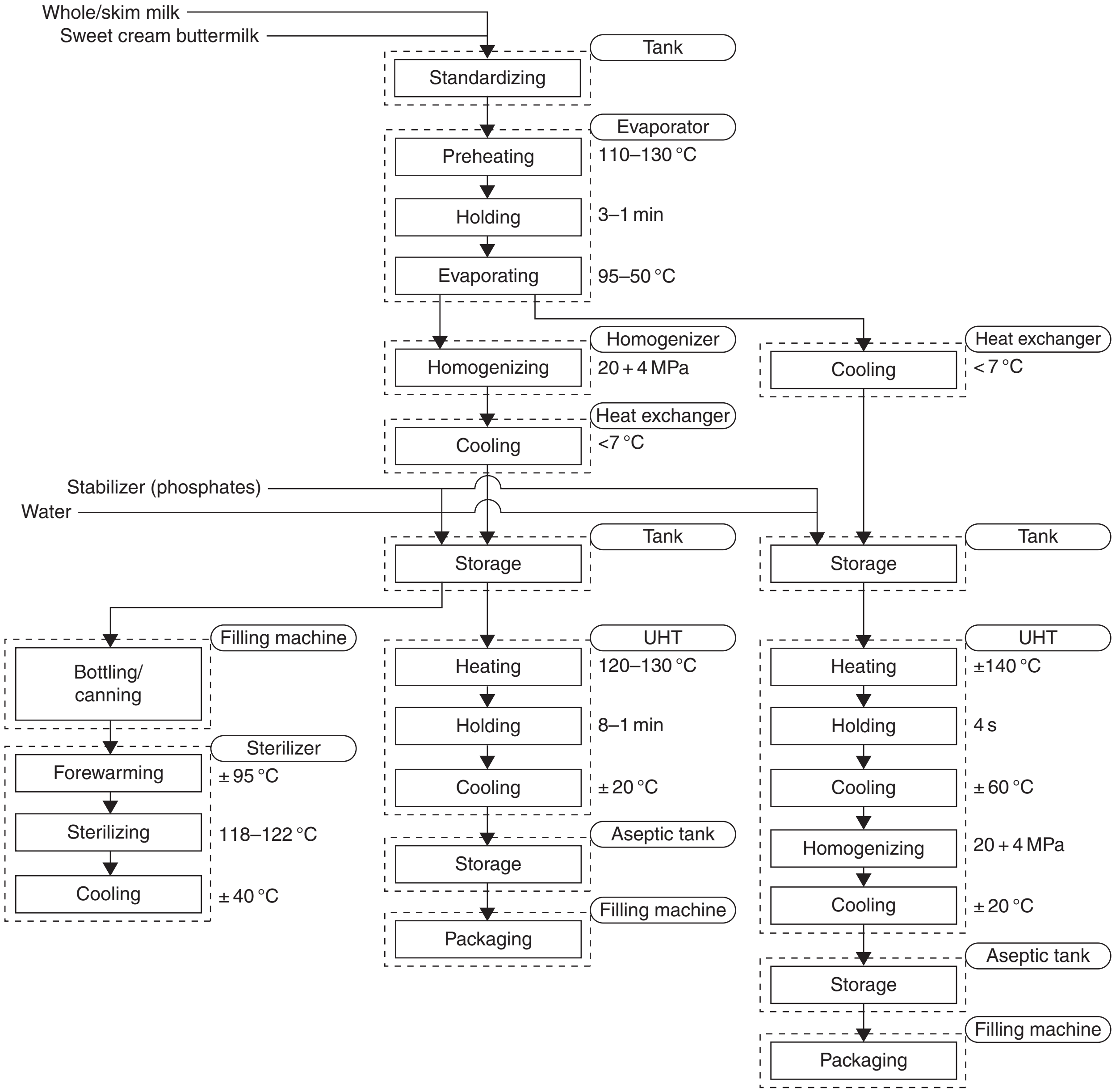
Whole/skim milk
Sweet cream buttermilk
Tank
Standardizing
Evaporator
Preheating
110–130 °C
Holding
3–1 min
Evaporating
95–50 °C
Homogenizer
Homogenizing
20 + 4 MPa
Heat exchanger
Cooling
<7 °C
Heat exchanger
Cooling
< 7 °C
Stabilizer (phosphates)
Water
Tank
Storage
Tank
Storage
Filling machine
Bottling/ canning
Sterilizer
Forewarming
± 95 °C
Sterilizing
118–122 °C
Cooling
± 40 °C
UHT
Heating
120–130 °C
Holding
8–1 min
Cooling
± 20 °C
Aseptic tank
Storage
Filling machine
Packaging
UHT
Heating
±140 °C
Holding
4 s
Cooling
± 60 °C
Homogenizing
20 + 4 MPa
Cooling
± 20 °C
Aseptic tank
Storage
Filling machine
Packaging

Figure 1 Processing scheme for evaporated milk from fresh milk.

decrease. Homogenization is always a two-stage process, in which the pressure over the second valve is ~20–30% of the total pressure, which prevents the formation of homogenization clusters; note that the risk of formation of homogenization clusters is low in evaporated milk because the fat-to-protein ratio is usually low (~1). In a standard production method, the concentrated milk is homogenized immediately after it has left the evaporator. Homogenization temperature has little effect on heat stability. However, at the same pressure, a higher homogenization temperature gives a somewhat smaller mean fat globule size. Thus, for the same mean fat globule size in the product, heat stability is better if the homogenization temperature is higher; for example, 55–60 °C is preferred to 45–50 °C.

Stability Test

A stabilizer, usually Na_2HPO_4 or a mixture of NaH_2PO_4 and Na_2HPO_4, is added to regulate heat stability. Owing to batch-to-batch variations in heat stability, the amount of stabilizer needed is not constant. The appropriate dose is determined by adding different amounts to a series of cans of the product; these are sterilized and the contents are checked for heat stability. The amount of stabilizer that gives optimum properties is added to the total batch.

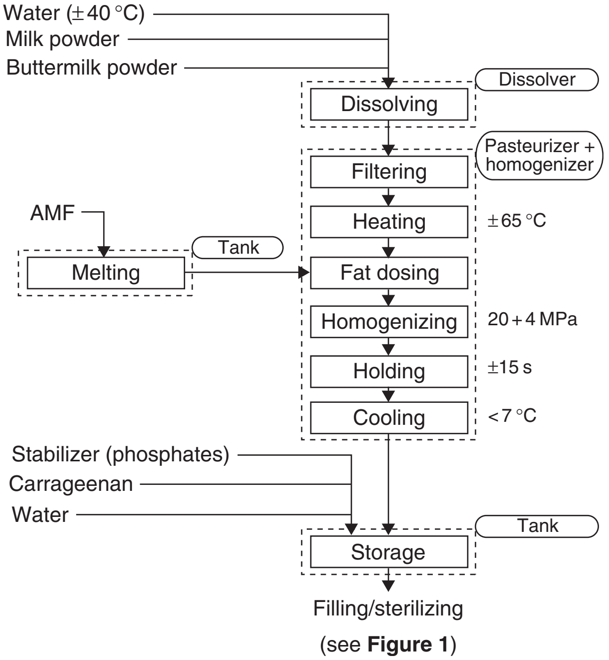

Figure 2 Processing scheme for recombined evaporated milk. AMF, anhydrous milk fat.

Cooling and Cold Storage

Because the product can be sterilized only if the results of the stability test are known and the appropriate amount of stabilizer is added, the product is cooled and stored for a while after homogenization. In addition, the final standardization of fat and total solids can be carried out at this stage, taking into account the amount of water added with the stabilizer. Long storage should be avoided to prevent bacterial growth. Of equal importance is that cold storing the unsterilized product for more than ~24 h considerably increases the risk of age gelation of the sterilized product in the market.

Packaging

The long shelf life of evaporated milk at ambient temperature places high demands on the packaging material. Mechanical resistance and permeability to water, gases, hydrophobic components, and light are important aspects, and, of course, those parts that are in direct contact with the product must be of food-grade material.

The can is the traditional, and still widely used, container for evaporated milk, with a standardized content of 170 or 411 g (6 or 14.5 oz, respectively). In the past, cans with a vent hole were used, and the hole was sealed by filling it with lead solder, which results in some risk of contamination of the product with lead. Presently, mechanically sealed cans that do not have this risk are the standard. Furthermore, modern tinned plate is coated with a layer of polymer to prevent dissolution of tin and iron into the product. In some Western European countries, glass bottles with a twist-off cap are used for packaging evaporated milk used to whiten coffee.

After continuous-flow sterilization, various types of aseptic packaging systems can be used as long as the above-mentioned criteria are fulfilled. Aluminum foil-lined ‘milk cartons’ and portion cups made of aluminum or polystyrene are widely used. Evaporated milk packaged in translucent (white) polystyrene portion cups usually develops an oxidized flavor, presumably due to the appreciable permeability of this material to both oxygen and light.

Sterilization

Sterilization kills all microorganisms and inactivates all microbial spores that may germinate under the storage conditions of the product in the market. All milk enzymes, including plasmin, are already inactivated during pre-heating. Enzymes from psychrotrophs should be absent, as these enzymes have such a high heat stability that sterilization would give insufficient inactivation.

In-container heating is usually carried out in continuously operating sterilizers: for cans, a horizontal rotating system with rotary air locks is used, and for bottles, a hydrostatic sterilizer. Both are high-capacity systems, allowing considerable heat regeneration.

Continuous-flow heating poses some problems with respect to regulating heat stability: a stability test using a continuous-flow heater is hardly possible. One solution is to aim for high heat stability during sterilization by homogenizing the concentrate aseptically afterward. Addition of a stabilizing salt is not needed for this method of processing, or a fixed amount can be used. This is done mostly for UHT-sterilized evaporated milk, which results in a relatively whiter product with a low viscosity. A product that resembles in-container sterilized evaporated milk is obtained if the sterilization temperature is 120–130 °C, for an appropriate (relatively long) holding time, for example, 10 min at 120 °C or 2 min at 130 °C, and homogenization is upstream. However, control of heat stability is more complicated for this method of processing.

Problems

Microbiology

As for all sterilized milk products, there is some risk of reinfection of the product after sterilization, for example, via microleaks in cans or during aseptic filling. One other aspect deserves specific attention: the growth of thermophilic spore-forming bacteria, in particular of *Bacillus stearothermophilus*, in the processing equipment, especially the evaporator. Many evaporators contain large stainless-steel surfaces in sections where the product temperature is 45–65 °C, that is, a temperature at which growth and sporulation of bacteria may occur. If the running time is long or cleaning of the equipment is not perfect, this may lead to an appreciable spore count in the concentrated milk. Inactivation of these spores during sterilization is limited: D_{121} value for *B. stearothermophilus* is 4–7 min, thus the sterilizing effect is, at most, 3. Residual spores may germinate and grow if the product is sold in tropical countries, where storage temperatures may exceed 40 °C.

Heat Stability

Heat stability of evaporated milk varies widely with composition and processing (*see* **Heat Treatment of Milk**: Heat Stability of Milk) for mechanisms of heat coagulation of milk and concentrated milk). Here, the focus is on optimization during manufacture. Optimization is not maximizing heat stability. Consumers prefer a so-called creamy product, that is, a product with a viscosity significantly higher than that of unsterilized evaporated milk. In addition, a higher viscosity markedly reduces creaming rate. An increase in viscosity can be achieved by incipient heat coagulation, that is, aggregation of protein particles and protein-covered fat globules during heating. Thus, some heat-induced aggregation is needed to produce a high-quality product, and the best heat stability is not the highest, but the one that gives optimum viscosity. Generally, heat stability decreases with the concentration of solids-not-fat and with homogenization pressure, although homogenization up to ~5 MPa slightly increases heat stability. For a given composition and homogenization pressure, preheating conditions and the amount of stabilizing ingredients (phosphate salts and/or soy lecithin or sweet cream buttermilk) can be varied to achieve the optimum heat stability. An example of the effects of homogenization and preheating is shown in **Figure 3**, which also shows that the heat stability of evaporated milk is highly pH dependent. Moreover, pH determines the type of aggregates formed: relatively voluminous at a pH lower than that of the maximum heat stability and relatively compact at a higher pH. As more voluminous aggregates yield a more viscous product, it is advantageous to regulate product pH such that it falls on the acidic side of the pH for maximum heat stability. Adding a phosphate stabilizer (see section 'Stability Test') is the usual method of pH adjustment. In addition, the phosphate anion has an appreciable stabilizing effect, because it lowers Ca^{2+} activity. Conversely, added $CaCl_2$ has a destabilizing effect.

Defects during Storage

The 'use by' date of evaporated milk may be as long as 18 months after production, although 6–12 months of shelf life is more common. Microbiologically, the product will keep indefinitely. The physical and chemical changes that occur during storage, however, will finally make the product unacceptable to the consumer. These are discussed below.

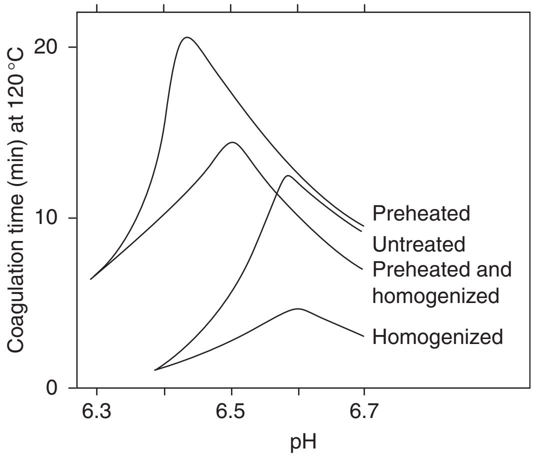

Figure 3 Influence of preheating and homogenization on the heat stability of evaporated milk as a function of pH (measured at room temperature before sterilization). Approximate examples. Reproduced with permission from Walstra P, Geurts TJ, Noomen A, Jellema A, and Van Boekel MAJS (eds.) (1999) *Dairy Technology: Principles of Milk Properties and Processes*, p. 431. New York: Marcel Dekker.

Creaming

Creaming of fat and sedimentation of protein are important quality defects, especially if dispersion of these layers is not possible. This may occur if, after a long storage time at a high temperature, the proteins in the sediment, or on the fat globules in the cream layer, fuse. Sterilized evaporated milk contains protein particles and fat globules covered with a relatively thick layer of protein, both aggregated to some extent (**Figure 4**). The closer the product is to incipient coagulation, the stronger the extent of aggregation. Large fat globules, having a relatively low protein load, and aggregates containing these globules will cream. However (aggregates of) small fat globules having a high protein load and, obviously, protein aggregates will sediment. Thus, evaporated milk that has been standing for about 6 months may contain a cream layer and a sediment layer, both of which have a higher fat and protein content than the middle layer. Of course, the proportions of fat and protein in both layers are different, and both the sediment and the middle layer have a fat content lower than the fat percentage of the fresh product.

Creaming is controlled by varying the homogenization pressure and by regulating the product viscosity. Homogenization pressure, of course, determines fat globule size. The effect of viscosity is not well understood; the viscosity of the serum surrounding the fat and protein aggregates, not the product viscosity, determines creaming and sedimentation rates. Presumably, the fact that heat coagulation gives flocs that may contain as much

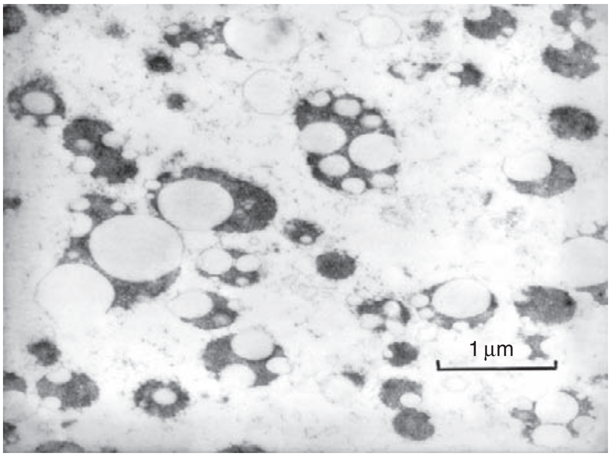

Figure 4 Protein particles and fat globules in preheated, homogenized evaporated milk after sterilization. Scale = 1 μm. Electron micrograph from Friesland archive, made by the former Technische en Fysische Dienst voor de Landbuw, Wageningen, The Netherlands.

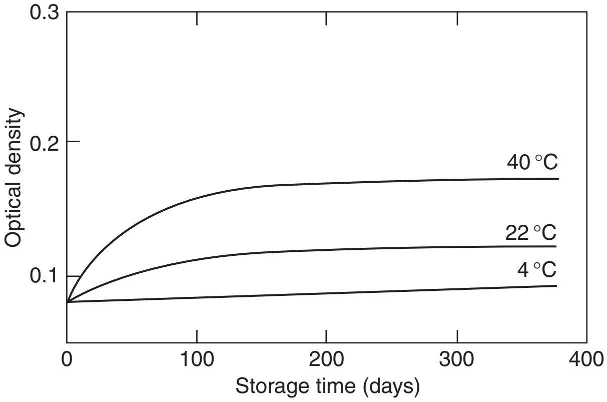

Figure 5 Optical density of evaporated milk at 520 nm as a function of storage time at storage temperatures of 4, 22, and 40 °C. Reproduced with permission from Patton S (1952) Studies of heated milk. 4. Observations on browning. *Journal of Dairy Science* 35: 1053.

protein as fat, thus having only a small difference in density from the serum, plays an important role. Mutual hindrance of the flocs, which occupy a volume fraction of roughly 0.5–0.6, may also play a part. A complicating factor, which is storage temperature-dependent, is that the aggregates in the product change during storage, resulting in a decrease in the viscosity of evaporated milk during the first weeks of storage.

Color and flavor

Shortly after sterilization, the color and flavor of evaporated milk are, after dilution to the concentration of regular milk, very similar to the color and flavor of unconcentrated milk sterilized by the same method. If the product is stored at refrigeration temperature, these properties hardly change with time. However, when stored at ambient temperature, and especially above 25 °C, Maillard reactions occur leading to color and flavor changes. Of course, this also applies to unconcentrated liquid milk products.

An example of the color change during storage is shown in **Figure 5**. The effect of temperature is clear, and browning is especially fast during the first months of storage. Thus, UHT-sterilized evaporated milk has little added value over in-container sterilized milk if the storage temperature is high: brown discoloration during storage is such that the color difference evident immediately after sterilization disappears quickly.

Age gelation

Age gelation is the formation of a gel, or a voluminous sediment or cream layer (depending on the fat content), during the storage of sterilized milk products. In-container sterilized evaporated milk does not gel during storage, unless the concentrate is cold stored for at least 1 day before sterilization. However, UHT-sterilized evaporated milk usually does gel. The main cause of the age gelation of evaporated milk appears to be an unknown physicochemical change in the casein micelles, resulting in dissociation of protein from the casein–whey protein complexes. Maillard reactions are not involved, and neither is proteolysis caused by plasmin, because plasmin is already inactivated during preheating. Proteolysis caused by enzymes secreted by psychrotrophs may induce gelation in all types of sterilized milk, due to their very high heat stability.

Gelation of in-container sterilized evaporated milk that has been cold-stored before sterilization appears as a gradual increase in viscosity; it takes at least 4–6 months for the product to gel. A gel is not always formed; some reports even mention that thinning occurs after thickening. Shortly after formation, the gel can be broken by shaking. Apart from ensuring a short cold-storage time, a somewhat more intense sterilization prevents this phenomenon from occurring; conversely, a lower heating intensity accelerates gelation. Gelation of UHT-sterilized evaporated milk usually proceeds much faster: it may take as little as 10 days for the product to gel. Breaking of the gel has been reported to cause 'wheying off'. The more intense the sterilization and the less concentrated the product, the more slowly gelation proceeds. Addition of a long-chain polyphosphate (frequently but erroneously called 'hexametaphosphate') allows storage of UHT-sterilized evaporated milk for up to 6 months, even at a tropical temperature. In contrast, an added orthophosphate (i.e., the salt used to regulate heat stability) slightly accelerates gelation.

Electron micrographs seem to indicate that the casein–whey protein particles in both cold-stored and UHT-sterilized evaporated milk develop thread-like protuberances during storage, finally linking the particles in a gel. However, some threads are always visible on micrographs of evaporated milk immediately after sterilization, and it is not clear why these sometimes seem to develop into links between the particles, sometimes develop into small protein particles in the serum, and sometimes disappear altogether, that is, fuse with the 'casein micelles'.

Crystalline sediment

Occasionally, crystals with dimensions of several millimeters consisting of tricalcium citrate are found at the bottom of the packages of evaporated milk. Formation takes some months and occurs especially at a high storage temperature. If no phosphate stabilizer is used, the formation of these crystals occurs much more frequently, especially in British Standard evaporated milk.

See also: **Heat Treatment of Milk**: Heat Stability of Milk; Sterilization of Milk and Other Products. **Homogenization of Milk**: High-Pressure Homogenizers. **Lactose and Oligosaccharides**: Maillard Reactions. **Liquid Milk Products**: Recombined and Reconstituted Products. **Plant and Equipment**: Evaporators.

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Sweetened Condensed Milk

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Product Description

Sweetened condensed milk (SCM) is one of the oldest industrially produced dairy products. It is produced by removing most of the water from fresh cow's milk and by the addition of sugar; it is preserved by its sugar content, not by sterilization.

The first record of an SCM-like product appears to be an English patent granted in 1810 to De Heine for preserving milk and sugar by heating the mixture in an open vessel. Several patents were granted in the years thereafter for variations and improvements of this process, until, in 1856, Borden was granted a US patent for a process that is essentially still used today (i.e., using a vacuum pan to remove water from milk).

The composition of SCM is regulated by the Codex Alimentarius and by the legislation of individual countries. The Codex requires a minimum of 8% milk fat and a minimum of 28% total milk solids for full-fat SCM. Sugar is generally considered to be sucrose, but a combination of sucrose with other sugars may be used, at a concentration that is between a minimum value that safeguards the keeping quality of the product and a maximum value above which crystallization of sugar may occur. The sucrose content in industrial practice is calculated as the sucrose/(sucrose + water) ratio, and should be about 62.5%, giving a sucrose content of 45% for full-fat SCM. The product contains only about 27% water, so the concentration factor of milk solids relative to water is very high, about 7.3. The Codex allows adjustment of the percentage of protein in the solids-not-fat, the minimum being 34%, by adding milk permeate, lactose, or milk retentate. Alteration of the whey proteins/casein ratio, for example, by adding whey proteins or caseinate, is not allowed. Various salts, lecithin, and carrageenan may be added to regulate viscosity, creaming, and age gelation. Legislation of individual countries usually follows the Codex, but not always in all aspects.

Part or all of the milk solids can be derived from milk powder and anhydrous milk fat, if the availability of fresh milk is limited. The technical name of this product is recombined SCM, which currently accounts for a significant part of the world production of SCM. Properties of this product need not be materially different from those of the product made from fresh milk. Recombined SCM is frequently made using vegetable fat instead of milk fat; this is usually labeled 'filled' SCM, but sometimes only by a trade name. Palm oil is most frequently used in filled SCM, because of its local abundance and low price. It gives a good-quality product. Coconut fat is less suitable, owing to its high content of lauric acid, which has a very low flavor threshold, so that even very low residual lipase activity may impart a soapy taste to the product.

The market share of the so-called 'sweetened creamer', a type of liquid sweet coffee or tea whitener, is increasing, especially for use in tea or coffee. The composition of this product varies among producers, but it always contains vegetable fat (7–15%), while the level of milk solids-not-fat is lower than in regular SCM. Sometimes, part of the milk solids-not-fat is replaced by whey solids or lactose.

Uses

The main consumer markets for SCM are the countries of the former Soviet Union, Southeast Asia, Africa, and Central America. The product is used for cooking, to enrich tea or coffee, and, after dilution, even as liquid milk although this yields a rather sweet product at a sugar content of 15–20%. SCM is also used in the food industry, for example, as an ingredient for the manufacture of sweets (candy) and chocolate bars. Fat, milk solids-not-fat, and sugar contents of products for these applications are mostly set according to the specification of the user. If the sugar content is too low to give sufficient microbiological stability, the product can be kept refrigerated for only a limited time.

Production Methods

Figure 1 shows processing schemes for SCM and **Figure 2** for recombined SCM. Essential processing steps are discussed in more detail in subsequent sections.

Addition of Sugar

Sugar can be dissolved in the cold milk before preheating. This is the least complicated process, and microorganisms present in the sugar are killed during preheating. The alternative method is adding sugar syrup after preheating or halfway during evaporation. Adding sugar to the milk is sometimes thought to result

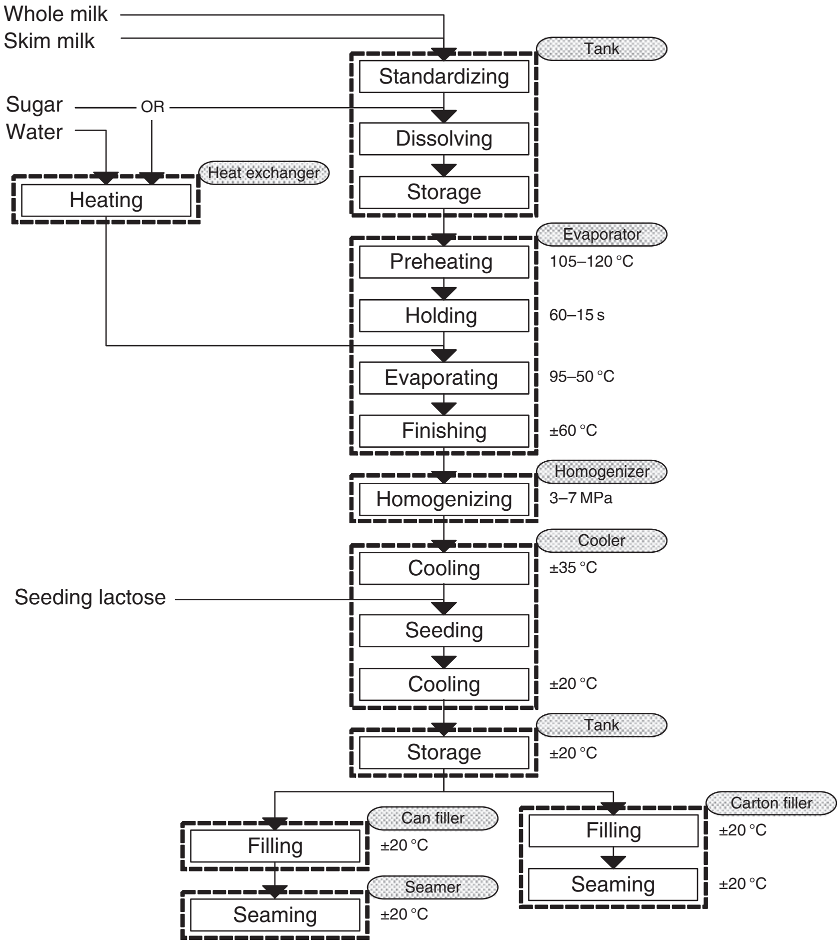
Whole milk
Skim milk
Tank
Standardizing
Sugar
OR
Water
Dissolving
Heat exchanger
Heating
Storage
Evaporator
Preheating
105–120 °C
Holding
60–15 s
Evaporating
95–50 °C
Finishing
±60 °C
Homogenizer
Homogenizing
3–7 MPa
Cooler
Cooling
±35 °C
Seeding lactose
Seeding
Cooling
±20 °C
Tank
Storage
±20 °C
Can filler
Filling
±20 °C
Seamer
Seaming
±20 °C
Carton filler
Filling
±20 °C
Seaming
±20 °C

Figure 1 Processing scheme for SCM from fresh milk.

in accelerated browning of the product due to the Maillard reaction, but as long as the sugar contains hardly any or no reducing sugars, this seems improbable. More important is the fact that this method gives faster age gelation. If sugar is added as a syrup, this must have been sufficiently heat-treated to kill osmophilic yeasts. It must be noted that to the author's knowledge, published results on this subject are for evaporation in a batch vacuum, and the effects may be different for evaporation in a continuous falling film evaporator, as is the industrial standard nowadays.

Preheating

Preheating is heat-treating the (sweetened) milk before it is concentrated. It is needed to kill all osmophilic yeasts, micrococci, and mold spores, to inactivate enzymes, and to regulate viscosity and age thickening.

Continuous-flow heating at 105–120 °C for about 15–60 s is generally considered to yield SCM with an appropriate viscosity and limited age gelation. If continuous-flow equipment is not available, preheating at about 82 °C for about 30 min may be used. As mentioned above, the appropriate time and temperature of preheating also depend on the method of sugar addition. For recombined SCM, preheating is done by the powder manufacturer, before concentration and drying; specifications for the skimmed milk powder are usually chosen for use in the recombined SCM. Heat treatment is in the same range as for medium-heat powder. However, using medium-heat skim milk powder without any further specification would result in recombined SCM with a widely fluctuating viscosity. Thus, a viscosity test (of the powder reconstituted to SCM, at laboratory scale) is usually done to adjust the preheating to such a time–temperature combination that the recombined SCM can be produced at a more or less constant viscosity.

Concentration

The milk, or the mixture of sugar and milk, is usually concentrated in a multistage falling film evaporator, designed with emphasis on high energy efficiency.

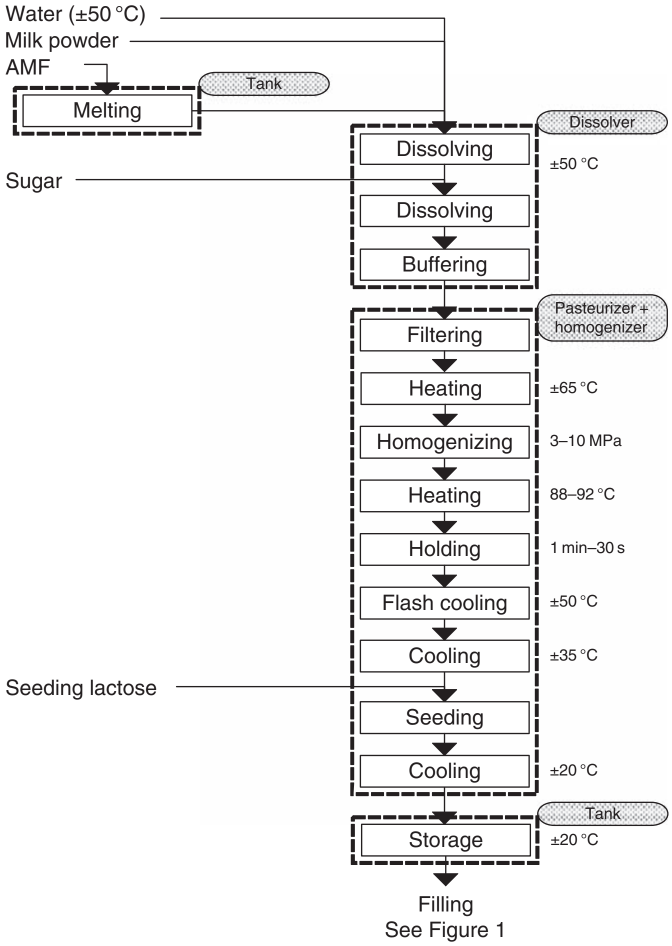
Water (±50 °C)
Milk powder
AMF
Tank
Melting
Dissolver
Dissolving
±50 °C
Sugar
Dissolving
Buffering
Pasteurizer + homogenizer
Filtering
Heating
±65 °C
Homogenizing
3–10 MPa
Heating
88–92 °C
Holding
1 min–30 s
Flash cooling
±50 °C
Cooling
±35 °C
Seeding lactose
Seeding
Cooling
±20 °C
Tank
Storage
±20 °C
Filling
See Figure 1

Figure 2 Processing scheme for recombined SCM. AMF, anhydrous milk fat.

Because of the high viscosity of the product, especially in the last effect, evaporators are usually equipped with a so-called finisher, in which the concentrate is reheated before concentrating to the final solids content. If this is not done, the flow of the product over the evaporator pipes may not be regular, resulting in fouling and low heat transfer.

Homogenization

Whether SCM is homogenized or not depends on the product type, market (shelf life), and preference of the manufacturer. Because of the high viscosity of the continuous phase, creaming of fat globules is very slow, even if they are not homogenized. However, especially if the product needs to be shelf-stable at tropical temperatures, homogenization at a pressure of about 5–10 MPa may be used. In addition, homogenization is done to regulate viscosity: it increases the viscosity of the fresh product, and reduces age gelation. Recombined SCM is, obviously, always homogenized.

Seeding and Cooling

To prevent the formation of large lactose crystals, seeding lactose, that is, lactose crystals that are not much larger than 1 μm, is added to the product. This can be done only at a temperature at which the SCM is supersaturated with respect to lactose (below 40–35 °C), because otherwise the seeding lactose crystals would dissolve before they could take an effect. After seeding, rapid cooling assures optimum crystallization of the lactose. This is usually achieved in a vacuum cooler, but a scraped-surface heat exchanger can also be applied.

Packing

After seeding, SCM is ready for packing. Product for retail sale is still mainly packed in tinned cans. Cans are flame-sterilized before use, and the filling machine should be placed in a closed room that is supplied with filtered air. These precautions are especially important to prevent recontamination with mold spores. The use of aseptic packaging systems is limited, although any system that offers sufficient mechanical resistance and permeability to water, gases, hydrophobic components, and light is suitable. Small (20–40 g) portion packs made of aluminum foil and plastic laminate, metal or plastic tubes, and aluminum-lined ‘milk’ cartons are commonly used. Bulk packages are metal drums or bag-in-box systems, holding up to 1000 kg of product.

Problems

Microbiology

SCM is preserved by its high sucrose content. The added sucrose is the principal component that gives the product its fairly low water activity of about 0.83–0.85, but milk salts and lactose also contribute. As a rule of thumb, the concentration of sugar in the aqueous phase, expressed as sugar/(sugar + water) ratio (also known as ‘sugar number’ or ‘sugar index’), should not be lower than 0.61 and not higher than 0.64, because this would result in crystallization of sugar. SCM is not sterile and may contain bacterial spores. These do not germinate and grow, but slowly decrease in number during shelf life.

Deterioration of the product occurs if mold spores, osmophilic yeasts, or micrococci are present after packaging. The product itself poses little risk of carrying contaminants if the usual preheating at a temperature above 100 °C is applied. Recontamination may occur if cleaning procedures in the production plant are inappropriate, or via the air during packaging if the filtration (or other method of sterilization of the air) is not functioning properly. Growth of molds and micrococci usually results in lumps and off-flavors in the product, while growth of yeasts causes bulging of cans (due to gas formation) and off-flavors.

Crystallization of Lactose

The lactose content of SCM is about 38 g 100 g^{-1} of water, which is about the saturation level for the product as it leaves the last section of the evaporator at 50–60 °C. At 25 °C, the solubility of lactose is about 11 g 100 g^{-1} of water (note that this is about half of the solubility in pure water, due to the presence of sucrose). Hence, lactose crystallizes during cooling of the product, but not at a high rate because the degree of supersaturation is very low. Thus, few nuclei are formed and these grow to large crystals that are perceivable during consumption as a gritty mouthfeel. Also, large crystals settle to the bottom of the package at an appreciably faster rate, which is unacceptable, especially for consumer products that require a long shelf life. Because crystallization cannot be prevented, the production process should yield a product that contains a large number of small crystals that are not perceivable and do not settle as sediment. The usual way to achieve this is to add seeding lactose, although an apparently acceptable product was made until some 50 years ago using rapid cooling only or the addition of SCM from the previous batch. About 0.02–0.05% of seeding lactose is added, that is, 0.25–0.6% of the amount to be crystallized. As a rule of thumb, lactose crystals smaller than 10 μm are not perceivable in the mouth; thus, the seeding lactose crystals are about 1–1.5 μm.

Seeding lactose is usually prepared by grinding with continuous air separation of the smaller crystals.

Color and Flavor

The appearance of SCM is somewhat glassy, with a yellowish white color. Because the refractive index of the water phase is about the same as that of the fat, due to the high sugar concentration, light scattering by fat globules and casein micelles is very limited. The main contributors to SCM’s whiteness are lactose crystals; this is in contrast to other milk products in which casein micelles and fat globules are responsible for whiteness. Due to this glassy appearance, even a limited formation of brown pigments by the Maillard reaction gives discoloration of the product. The production process has a relatively small effect on color, as it involves no sterilization. However, browning during storage can be considerable, especially at temperatures above 25 °C. In SCM for retail sale, replacing sucrose with glucose (syrup) or another reducing sugar results in much faster browning, which is undesirable. However, in products intended for the food industry, this may be acceptable, as storage times are usually short, or it may even be desirable, for example, for use in the production of fudge.

Products of the Maillard reaction also change the flavor of SCM during storage, but as long as this is not excessive, the flavor is considered normal for the product. Lipolysis incidentally results in a rancid off-flavor in products containing milk fat, and a soapy off-flavor if coconut or palm kernel oil is used to produce filled SCM.

Age Gelation

The viscosity of SCM increases during storage. As for evaporated milk, the main cause of age gelation appears to be an unknown physicochemical change in the casein micelles. Electron micrographs indicate that the protein particles in SCM become connected by threads of protein, similar to those in sterilized milk products that gel during storage. Reactions with reducing sugars seem to be involved, as SCM in which the sucrose is replaced by, for example, glucose syrup thickens faster. Proteolysis by heat-stable enzymes may play a part, but this does not seem to have been reported (this in contrast to lipolysis caused by bacterial enzymes).

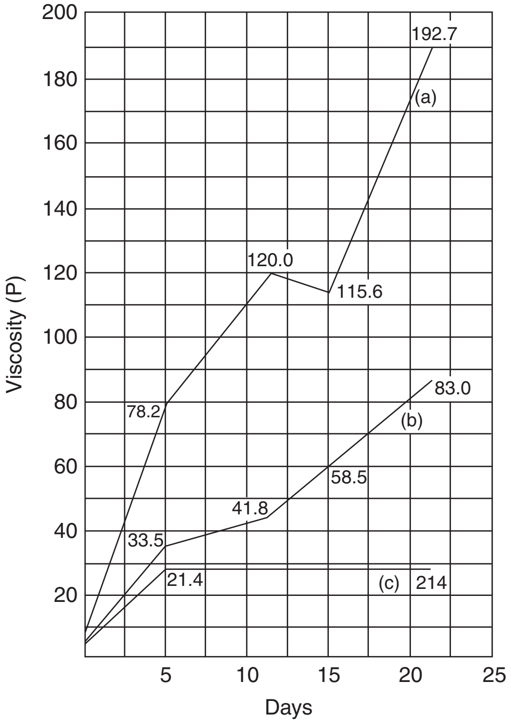

Figure 3 Effect of the point of sugar addition on the viscosity of SCM during storage. (a) Sugar and milk prewarmed together; (b) sugar syrup and milk prewarmed separately and mixed in the vacuum pan; (c) sugar syrup added near the end of the condensing period. Reproduced with permission from Hunziker OF (1947) *Condensed Milk and Milk Powder*, 7th edn. La Grange, IL: OF Hunziker.

Sucrose plays a pivotal role. The higher the sugar-in-water content, the slower the rate of age gelation, even though higher sugar-in-water also means a higher concentration factor of the milk solids-not-fat, which also hastens age gelation. The stage at which the sugar is added has a large effect. When added later in the concentration process, a slower rate of age gelation results. Adding the sugar syrup near the end of the evaporation results in a product that shows hardly any thickening (see **Figure 3**).

Preheating is important, but not (only) because it denatures serum proteins. The viscosity of SCM made from unpreheated milk hardly increases during storage; a preheating temperature between 80 and 100 °C gives rapid age gelation and a temperature above 100 °C results in a much lower rate. Finally, the effects of stabilizing salts are not clearly understood either. Added calcium appears to hasten age gelation if added after concentrating, but delays it if added between preheating and concentrating. Added orthophosphate and EDTA increase the rate of age gelation whether added before or after concentration, and added citrate has little effect if added before concentration, but slows age gelation if added after concentration. All in all, if additives are used in industrial practice, the type and method of addition should be carefully tested for the exact processing line.

See also: **Concentrated Dairy Products**: *Dulce de Leche*; Evaporated Milk. **Lactose and Oligosaccharides**: Lactose: Crystallization; Lactose: Chemistry, Properties; Maillard Reactions. **Plant and Equipment**: Evaporators. **Water in Dairy Products**: Water in Dairy Products: Significance.

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Dulce de Leche

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Introduction

Article 592 of the Argentinean Food Legislation, Código Alimentario Argentino (CAA), defines *dulce de leche* (DL) as "the product obtained by heat concentration, at normal or reduced pressure, of milk or reconstituted milk apt for human consumption, with or without addition of milk solids and/or cream, with the addition of white sugar and with or without the addition of other food ingredients".

This simple definition contains the essence of a concentrated milk product, which was homemade initially, but was undergoing a rapid industrial development. Its very pleasant sensory properties can be appreciated fully only by direct tasting.

It is widely consumed in Río de la Plata countries such as Argentina and Uruguay and at a lower level in other Latin American countries such as Chile, Paraguay, Brazil, and Bolivia. It is also consumed in Mexico, and in recent years in the United States, mainly as a consequence of the increase in Latin American population. DL is consumed as a spread, as a dessert, and at breakfast. It is widely used in bakery and confectionery in the preparation of desserts and cakes as a substitute for the creams used in other countries.

Most recently, DL has been marketed in Europe with some amount of success in Spain and France. In 2008, a factory began production of DL in France.

Milk–sugar mixture is concentrated by boiling to a solids content of 70%. Depending on the raw materials used and the processing conditions (i.e., time, temperature, and pH) in the boiling mixture, extensive nonenzymatic browning occurs, which gives a desirable color and texture, and a very pleasant flavor to the product.

Its low water activity (<0.85) is a consequence of its very high solids concentration. This low water activity is the principal factor responsible for the long shelf life of the product. In spite of its high stability against microbial spoilage, if it is stored for long periods at room temperature, some yeasts and molds can develop.

Production, Consumption, and Trade Statistics

Production of DL in Argentina represents only a small proportion of the total industrial milk utilization (2.2% in 2003, 1.9% in 2006, 2.2 in 2007, and 2% in 2008). Production has grown steadily to 124 982 tonnes in 2007, and per capita consumption is ~3.1 kg yr^{-1} (**Table 1**).

In 2003, DL represented 0.3% of the total of dairy products exported by Argentina. Exports of DL in 2003 were threefold higher than those in 1990. Major destinations of Argentinean exportations were Syria (456 tonnes), the European Union (260 tonnes), and Paraguay (154 tonnes).

Data of production, consumption, and exportations of DL in Argentina are presented in **Table 1**.

Uruguay is the second largest producer of DL in the world producing ~10 200 tonnes in 2006, with a per capita consumption of 2.9 kg yr^{-1}.

Types and Regulations

The industry produces several types of DL (**Table 2**). Details about the characteristics and regulations of each type were presented in the first edition of this encyclopedia and in the CAA.

Dulce de Leche familiar, tradicional, o clásico (family, traditional, or classic *dulce de leche*) is the type most widely known and consumed. Its main features are brightness and smoothness. The name 'familiar' is adopted because this product is mainly consumed by families at home.

Typical composition is moisture (30%, max), milk solids not fat (24%, min), milk fat (6–9%), protein (5%, min), and ash (2%, max).

Dulce de leche para respostería o pastelería (confectionery *dulce de leche*) is basically the same as *familiar* DL except that wetting, thickening, and stabilizing agents may be added. The maximum allowable thickening and stabilization agent content is 2%. These additives give the product the higher consistency necessary for confectionery applications.

Table 1 Statistics of *dulce de leche* in Argentina

Year	*Production (tonnes)*	*Export (tonnes)*	*Consumption per head (kg yr^{-1})*
1999	114 306	2200	3.0
2000	105 000	1850	2.8
2001	106 000	1950	2.9
2002	106 000	2147	2.6
2003	99 000	1237	2.5
2004	108 000	4608	2.7
2005	115 000	5605	2.8
2006	117 705	6228	2.9
2007	124 982	6365	3.1

Table 2 Industrial varieties of *dulce de leche*

Types	*Applications*
Familiar	Home consumption
Repostero o pastelero	Home consumption/bakery and confectionery
Heladero	Ice cream industry
Sólido	Candies and tablets
Con crema	Home consumption
Mixto	Home consumption

Dulce de leche heladero (*dulce de leche* for ice cream manufacture) is similar to *familiar* DL except that addition of some colorants is permitted.

Dulce de leche mixto (mixed *dulce de leche*) is the basic product with the addition of one or more of some ingredients such as chocolate, fruits, and dry fruits, with or without the addition of wetting, thickening, and/or stabilizing agents. In all cases, the DL content must be not less than 70%.

Dulce de leche sólido is the product with a moisture content of less than 20% (w/w).

Dulce de leche con crema (*dulce de leche* with cream) is obtained when cream is added to the raw milk to achieve a minimum fat content of 9% (w/w).

Technology of Production

Characteristics of Raw Materials and Additives

Milk

Although the manufacture of DL is performed at a high temperature over a long period of time, the milk used for manufacture must be of good microbial quality. High acidity, imbalance of milk components, and the presence of heat-stable enzymes in poor-quality milk result in an inferior DL.

It is also undesirable to use milk with a high somatic cell count or colostrum. Major variations in milk composition can cause defects in the final product.

An acidity higher than 0.20% (expressed as lactic acid) will require excessive addition of alkali for neutralization, which can cause saponification of free fatty acids with soap formation and, consequently, the production of undesirable flavors.

To reduce the duration of the manufacturing process, many producers increase the milk solids content by adding good-quality whole or skimmed milk powder (nonfat dry milk).

Milk fat

The fat content of each type of DL is established by regulations. To obtain the specified fat content, addition of cream to milk is common practice, especially when *dulce con crema* is produced.

Nutritive sweeteners

These products are added to milk to obtain a total solids content of 68–70% in the final product. Sucrose is often the sweetener of choice and generally accounts for 95–100% of the total sweeteners. Cane sugar is used in Argentina, but beet sugar may also be used.

Glucose is added only to *familiar* DL, because this product must be shining and smooth, but never to *dulce de leche repostero*, because it must be brittle and opaque. The content of added glucose is never higher than 2–5%, because this sugar gives a highly viscous texture, a glossier appearance, and a dark brown color (as a consequence of the Maillard reaction with proteins) to the final product. For the latter reason, glucose is added always at the end of the concentration process.

Neutralizing products

These products are used to reduce the lactic acidity of the raw milk and neutralize the acidity generated during the process of concentration. Calcium hydroxide and sodium bicarbonate are the most widely used alkalis. Sodium bicarbonate is used for *familiar* DL because it gives a nonbrittle, shiny, and smooth product, whereas calcium hydroxide is mainly used for *dulce repostero*, because it gives a product of brittle and opaque texture.

Aroma enhancers

For this purpose, natural vanilla in pod or artificial solutions of ethyl vanillin are used almost exclusively. Owing to the heat sensitivity of these products, they are added at the end of the heating process or when the DL is being cooled (preferably at about 65 °C).

Preservers

Legislation allows the use of sorbic acid or its potassium, sodium, or calcium salts for preventing the growth of mold in the product. The maximum quantity allowed is 600 mg kg^{-1} in DL for home consumption and 1000 mg kg^{-1} when DL is not directly consumed (bakery, confectionery, and ice creams). Both quantities are expressed as sorbic acid.

Preparation of the Starting Mixture

The first step in processing is the preparation of the starting mixture. This operation must be done very carefully, because the final composition and characteristics of the product are dependent on this step.

When the type and final composition of the DL have been established, a mass balance provides the quantities of raw milk and sucrose, and eventually of glucose for *familiar* type, to be used in the starting mixture. If milk powder (whole or skimmed) is a component of the starting mixture, it must be included in the mass balance.

Neutralizing products and aroma enhancers are not included in the mass balance because their quantities are very small in relation to the principal ingredients.

The following quantities of raw milk, sucrose, and glucose syrup will be used in the starting mixture for each 100 kg of *familiar* DL: milk, 218.8 kg; sucrose, 37.20 kg; glucose syrup (70%), 12.50 kg (8.75 kg of solid glucose).

After preparing the mixture of milk and sugar, it must be partially neutralized. The purpose of neutralization is to prevent destabilization of the casein micelles as a consequence of the decrease in pH during evaporation. The decrease in pH is caused by the increase in the amount of colloidal calcium phosphate, the formation of organic acids from partial degradation of lactose, and the hydrolysis of phosphoric esters from casein. This situation is known as 'corte' (acidic destabilization of casein), and its consequence is the separation of the mass into a heterogeneous mixture (solid protein and liquid 'whey').

The acidity of the raw milk is the basis for calculating the quantity of alkali to be added to achieve the desired reduction. The unit of Dornic degrees (°D), where 1 °D is equivalent to 0.01% of lactic acid, is widely used for acidity measurement during the manufacture of DL. The degree of neutralization depends on the type of DL being manufactured. For example, for *familiar* DL the mixture is neutralized to ~10 °D, whereas for *dulce repostero* the neutralization comes to 6 °D or may even be as low as 2 °D. The type of alkali used depends on the type of DL, and is added as a solid powder or, preferably, as an aqueous solution to improve its distribution in the milk–sugar mixture.

When the preparation of the mixture is completed, it is transferred to the evaporation kettle where the evaporation of water is accomplished. In this kettle, the reactions responsible for the characteristic texture, color, and aroma of the product take place.

Traditional Process in Open Kettles

This is the most widely used process in the industry. It is a classic batch process, and there is a general agreement that this process produces the best-quality DL.

The process is illustrated in **Figure 1**. The kettle is a hemispherical bottom tank with the upper part in the shape of an inverted bell. The upper part is closed, ending in a pipe for the evacuation of steam formed during concentration of the milk.

The most commonly used kettles have a capacity of 1000 l of mixture, equivalent to 500 kg of finished DL. The hemispherical bottom has a double wall to allow circulation of steam at a pressure of 4 kg cm^{-2}, for heating purposes.

To achieve a good mixing of the components, the starting mixture is prepared in a stirred tank separate from the kettle in which concentration will be performed. This working method also allows the gradual addition of the mixture to the evaporating kettle. If all the mixture was transferred to the kettle at the start, the boiling of the liquid would be very vigorous, and dangerous overflows could occur.

To avoid spills, only 20% of the mix is initially pumped or gravity fed into the kettle. This mix is heated to its boiling point and concentrated to 60% total solids. The remaining mixture (80%) is then pumped slowly into the kettle without stopping the boiling. As a consequence, the volume of liquid in the kettle remains at a constant level during the operation.

The kettle is fitted with a stirrer to avoid sticking of DL to the wall, and also to aid the release of vapors from the hot mass of product to the surface. To achieve an effective stirring, two contra-rotating stirrers are sometimes placed in the kettle. The blades of the stirrers are anchor-shaped and fitted with heat-resistant plastic scrapers to produce a scraping effect on the wall of the kettle.

As the solids content of the mixture increases, the Maillard and other nonenzymatic browning reactions involving sugars occur. Thus the viscosity increases, and the typical color and flavor characteristics of DL are achieved gradually.

If glucose is used, it should be added when the mixture reaches a solids content of 55–60%. The solids content is measured by refractometry.

The heating of the mixture is stopped when the solids content is 68%, 2% lower than that required by law, because during the discharge of the hot DL and during its cooling, a final concentration of 70% total solids is reached.

When the concentration process is over, the DL is discharged into a water-jacketed cooling tank.

Initially the mixture is cooled slowly so as to allow the crystallization of lactose in the β-anomeric form. This is

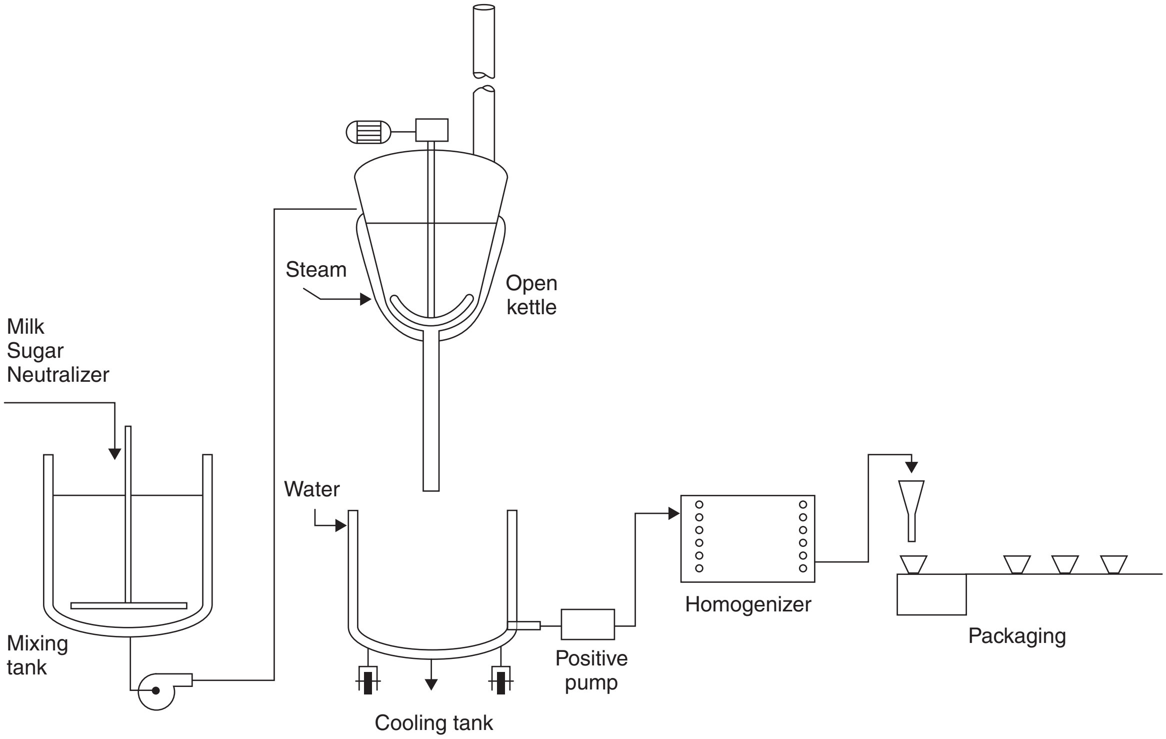

Figure 1 Batch production of *dulce de leche* in open kettles.

the more stable form of lactose above 93.5 °C, because at this temperature β-lactose is less soluble than the α-form. Furthermore, β-lactose crystals, which are in the form of uneven-sided diamond-shaped crystals, are smaller and softer than α-lactose monohydrate crystals, which give a gritty mouthfeel.

When the temperature of the mixture reaches below 93.5 °C, fast cooling is more convenient, to allow the formation of small crystals of α-lactose monohydrate. The cooling operation must be performed very carefully; otherwise, a gritty DL would be obtained as a consequence of the formation of large hard crystals of α-lactose monohydrate. However, it is not known how long it would take for β-lactose to crystallize above 93.5 °C, and maintaining a batch of DL for a long period at such a high temperature is highly inefficient.

After cooling, the product is gently transferred using a Moyno single-rotor screw pump with elastomeric lining into a homogenizer. It is homogenized at 10 MPa (100 bar) using a double-stage homogenizer. Homogenization is done to remove any clumps (due to the addition of milk powder) in the finished product. Homogenization also gives the DL a more brilliant and soft texture, and for these reasons the *dulce de leche repostero* is not homogenized.

DL is packaged while still hot (50–60 °C) to avoid microbiological contamination. It is packaged in plastic (0.25, 0.5, and 1.0 kg) or glass (primarily for export market) containers, or in paperboard (10 and 20 kg) for bakery and confectionery purposes.

Semicontinuous Process

To reduce steam consumption during processing, the mixture of sugar and milk can be concentrated as a first step in a multiple-effect evaporator. Afterward, the final solids content of the partially concentrated mixture is obtained by boiling in an open kettle. In this last step, the Maillard browning and sugar caramelization reactions occur leading to the development of color and flavor.

Continuous Process

The aim of this process is to achieve a more comprehensive reduction in steam consumption in addition to a continuous production of large volumes of DL. This process is suitable for large dairy plants. However, the utilization of this process for the production of DL is rather limited and has not increased in recent years, as the traditional batch process provides a finished product of better sensory characteristics.

Figure 2 shows the continuous method for the manufacture of DL. Preparation of the mix, homogenization, and packaging in the semicontinuous and the continuous processes are similar to those of the batch process. In the

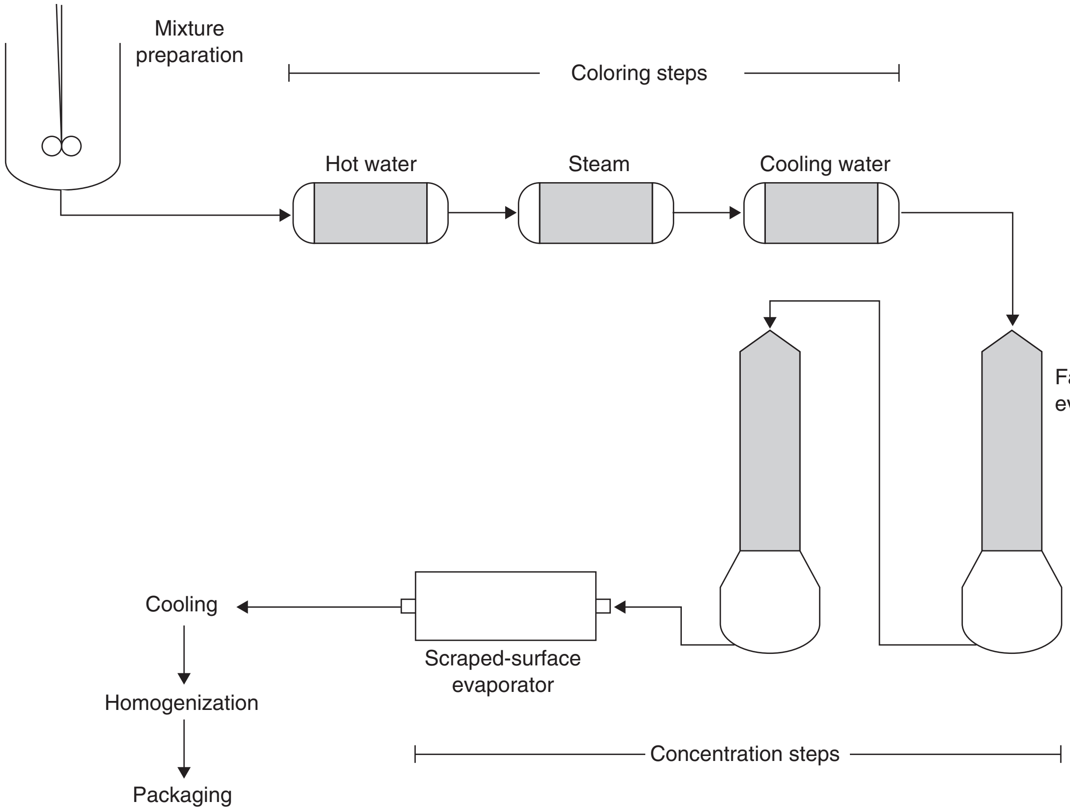

Figure 2 Continuous production of *dulce de leche*.

continuous process, the manufacturing steps are reversed. Basically, it involves a color development step followed by a concentration step. Color development is achieved in three heat interchange stages. The first and second stages are heating steps, whereas the last is a cooling step. The heat treatment steps result in the development of the desired final color, but the mix is still in liquid form.

The colored liquid mixture is then fed into a multiple-effect evaporator where it is concentrated to 55% total solids. Further concentration to 70% total solids is achieved in a scraped-surface evaporator. The hot product is then cooled to 60 °C in a tubular heat exchanger.

Nonenzymatic Browning Reactions

Unlike most food products, milk contains both proteinaceous material and a reducing sugar (lactose), which leads to nonenzymatic browning and changes in the nutritional value when it is subjected to heating. In some types of DL, sucrose is present in addition to lactose and glucose. Sucrose is a nonreducing sugar, whereas glucose and lactose, which are the main reducing sugars present in DL, readily react with the amino group of a protein by the Maillard reaction.

Perhaps DL is the only dairy product in which the Maillard reaction is desirable, because this reaction is mainly responsible for the color and flavor of the product. A negative consequence associated with the Maillard reaction is the loss of available lysine.

Nonenzymatic browning in DL also includes caramelization reactions; in this case, sucrose has been reported as the main sugar involved, although lactose may also contribute. Both reactions – caramelization and the Maillard reaction – continue during the storage of DL.

Defects and Problems in *Dulce de Leche*

Lactose Crystallization

DL is a concentrated dairy product, similar to sweetened condensed milk, in which crystallization of lactose occurs when the moisture in the product is supersaturated with lactose. Therefore, the presence of lactose crystals in DL is a common problem encountered during production and storage. Crystallization of lactose in DL can be prevented by careful control of the manufacturing process, especially the cooling step.

Typically, DL contains ~10% lactose and 45% added sugars (primarily sucrose and glucose) associated to 30% of moisture. Among the three sugars, lactose is the least soluble in water (~20% (w/w) in pure water at 20 °C).

However, due to the presence of sucrose and glucose in the mix, the solubility of lactose is ~10% (w/w). Consequently, lactose is highly supersaturated in DL; its concentration in the aqueous phase is equivalent to 36.3% (w/w). Due to the high lactose concentration in the aqueous phase of DL (i.e., intermediate equilibrium zone of the lactose solubility curves), lactose crystallization from the supersaturated solution may occur.

In DL, α- and β-lactose attain an equilibrium. When this happens, the rate of crystallization of lactose depends not only on the rate of crystal formation but also on the mutarotation rate. Therefore, the slowest reaction will limit the crystallization process. The problem of lactose crystallization in DL is very complex because of the high viscosity of the medium and the presence of salts, sucrose, glucose, citrate, and phosphates, products of the Maillard reaction. The available knowledge on lactose crystallization is mainly empirical, based on decades of observation and experience. In addition, lactose that precipitates under such conditions is in the form of α-lactose monohydrate crystals, which are large and hard. These crystals give a sandy texture and mouthfeel to the DL.

In good-quality DL, the formation of crystals larger than 10 µm, which are detectable in the mouth, does not occur before 120 days of storage at ambient temperature (always below 20 °C). Thus, the industry has adopted 120 days as the usual shelf life for DL, although the product is often consumed before 120 days because of its high demand.

There are some technological ways for avoiding lactose crystallization. One of the most commonly used approaches for preventing the crystallization of lactose is to hydrolyze lactose enzymatically to glucose and galactose before the manufacture of DL. Commercially available β-galactosidase (EC 3.2.1.23) is added to the milk and incubated for several hours to achieve hydrolysis. Because glucose and galactose are more soluble and sweeter than lactose, the amount of sugar to be added is reduced. If 30–35% lactose hydrolysis is achieved in the milk used for the manufacture of DL, lactose crystallization is avoided for a 180-day period. The disadvantage of this approach is the high cost of the enzyme, which is justifiable only if the product is to be exported. DL is not a recommended product for lactose-intolerant people.

A second approach is to seed with a certain quantity of finely ground α-lactose monohydrate crystals (not more than 10 µm) as nuclei for crystallization, to accelerate the process and produce a large number of very small, undetectable crystals. This practice is usual in sweetened concentrated milk (of ~0.5%) but not common in the production of DL.

Another technological approach is the slow cooling of DL up to 93.5 °C, to favor β-lactose formation, followed by fast cooling below this temperature.

Sandy Texture

The origin of this problem, as was mentioned previously, involves lactose crystallization. Occurrence of this defect can be aggravated by an inadequate mass balance of the ingredients or by the use of improperly sealed containers, resulting in a fast moisture loss from the finished product.

Formation of Clusters

This defect is commonly associated with a poor dissolution of milk powder, preservers, and stabilizers.

Rough Texture (Small Cluster-Like Flakes)

This problem can be associated with coagulation of casein caused either by the mishandling of acidity or by the use of milk with a high initial acidity.

Syneresis

It is caused by starting mixtures with a high acidity or by the use of milk with a high population of proteolytic bacteria.

Mold Formation

Mold may develop on the surface of DL under aerobic conditions. It is a consequence of using poorly sealed containers or packaging DL in containers at temperatures below 60 °C.

Dark Color

It is produced by an excess of neutralizing agent or by a prolonged processing time. This characteristic is, however, very much desirable in DL used in ice cream manufacture. In this case, a colorant is often added. Use of glucose instead of sucrose enhances the Maillard reaction and results in a darker-colored DL.

Weak *Dulce de Leche*

This problem can be a consequence of inadequate concentration of the mix, excessive stirring during cooling, or an inadequate mass balance of the initial mix (e.g., addition of an excess amount of glucose).

Future Trends in *Dulce de Leche*

Taking into account the actual trends of the markets toward 'premium products' in relation to the high quality of raw materials, technological processes, geographical origin, and final presentation, the Secretaría de Agricultura, Ganadería, Pesca y Alimentos of Argentina has approved the resolution N°798/2006 creating a Quality Protocol for DL. In this document, the quality considerations with regard to raw materials, processes, and containers that are to be followed to attain the classification of 'Argentinean Food: A Natural Selection' are detailed. The differentiated attributes in this protocol consist of physicochemical and biological parameters, beyond the requirements of CAA, related to the characteristics of milk, method for obtaining milk, nutritive sweeteners, manufacturing process, and the composition and characteristics of the final product, and transport and storage.

See also: **Concentrated Dairy Products**: Sweetened Condensed Milk. **Lactose and Oligosaccharides**: Lactose: Crystallization; Maillard Reaction. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose.

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Khoa

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Introduction

Khoa, also known as khoya, mawa, khava, and palghoa, is a traditional Indian dairy product. It is a partially dehydrated whole buffalo, cow, or mixed milk product. It is traditionally manufactured by boiling fresh milk in an open pan kept directly on a fire with continuous agitation and scraping to avoid burning and overheating. According to the Prevention of Food and Adulteration Act (PFA) of the Indian Government, khoa is a product obtained from the milk of cow, buffalo, goat, or sheep, or a combination thereof, by rapid drying, which contains not less than 20% milk fat (w/w) of in the finished product. It may contain citric acid, but not more than 0.1% (w/w). It should not contain any ingredient foreign to milk other than the added citric acid. The typical composition of khoa made from cow's and buffalo's milk is as shown in **Table 1**.

Khoa is an important base and/or filler material for the preparation of several indigenous Indian sweets such as peda, burfi, and kalakand. Based on the end use, khoa is classified into three categories: (1) Pindi khoa, which has a smooth and homogeneous body and texture (typically contains 67–69% total solids (TS) and 21–26% fat), and is used for manufacturing sweets like burfi and peda; (2) Dhap khoa, which has a loose, sticky body and smooth texture (typically contains 56–63% TS and 20–23% fat), and is used for making sweets like gulabjamun and pantua; and (3) Danedar khoa, which is characterized by a granular texture and uneven body (typically contains 60–65% TS and 20–25% fat), and is used for making sweets where granulation is desired, such as kalakand and milk cake. The Bureau of Indian Standards specifications for the three categories of khoa are as shown in **Table 2**.

Methods of Manufacture

Traditional Method

The traditional method of khoa manufacture is followed by unorganized traders for the production of khoa on a small scale. It involves boiling 3–4 kg of milk in a small, shallow, open, round, and thick-bottomed iron pan placed over a brisk nonsmoky fire. The milk is stirred vigorously and constantly with a circular motion using an iron stirrer. During this operation, all parts of the pan with which milk comes in contact are scraped lightly to prevent burning and overheating of milk. Constant evaporation of water takes place and the milk thickens progressively. At a certain stage of concentration (2.5 and 2.8 times for buffalo and cow milk, respectively), heat coagulation of milk proteins occurs resulting in a viscous mass, marked by an abrupt change in color. After this stage, the heat desiccation is continued with closer attention and increased speed of scraping until a semisolid consistency is obtained. The final product is ready when it shows signs of leaving the bottom and sides of the pan and starts sticking together. At this stage, the pan is removed from the fire, the product is worked up and down, and finally it is made into a pat before removing from the pan. Scraping in this process is so vital that the operator's skill is the single most important factor governing the quality of the product. In this process, possibilities of controlling and optimizing heat treatment are generally limited. The Pindi type khoa is prepared by heating for a longer period in the pan to achieve a lower moisture content than that of the Dhap type. If citric acid is added as soon as the milk starts to boil, Danedar type khoa is obtained.

The khoa produced in such small batches usually varies in quality owing to the varying temperature profile over the heat transfer surface of the pan and the uncontrolled heat input. Intensity and speed of agitation and scraping are also varied as evaporation progresses. If the scraping is not vigorous enough, the product is likely to caramelize or burn, leading to poor market acceptability and a low price.

Mechanized Methods

Several efforts have been made in the recent years to overcome the limitations of the traditional method of khoa manufacture. The following processes and equipment have been developed so that the khoa-making process can be adapted by the commercial dairy sector:

1. To increase the intensity of heating, a diesel-fueled burner is used in place of the wood-fueled open fire. To scale up production, more than one burner is used.
2. A double-jacketed, steam-heated stainless-steel kettle, with or without an in-built stirrer-cum-scraper, is used.
3. A hemispherical pan joined to a cylindrical water jacket by a safety valve to control steam pressure and a rotary stirring-cum-scraping mechanism is used.
4. Mechanized khoa making uses a scraped-surface heat exchanger (SSHE) in batch processing as well as continuous processing for commercial applications.
5. A roller dryer, with scraper blade(s), capable of manipulating process parameters such as concentration of milk, speed of rollers, steam pressure and flow rate is used.

Table 1 Typical composition of khoa

	Moisture	*Fat*	*Protein*	*Lactose*	*Ash*
Type of milk	*(%)*				
Cow milk	25	26	19	25	3.8
Buffalo milk	19	37	18	22	3.6

Table 2 Bureau of Indian standards specifications for khoa

	Minimum total solids	*Minimum fat (dry matter basis)*	*Maximum total ash (dry matter basis)*	*Maximum titratable acidity (as lactic acid)*	*Maximum coliform count*	*Maximum yeast and mold count*
Category	*% (w/w)*				*cfu g^{-1}*	
Pindi	65	37	6	0.8	90	50
Dhap	60	37	6	0.9	90	50
Danedar	55	37	6	0.6	90	50

6. Milk concentrated either in a vacuum condensing plant or by reverse osmosis (RO) has also been tried successfully as a base material for making khoa. With RO a concentration of ~30% TS is attained; this is followed by a final desiccation in open vats or a scraped-surface heat exchanger. This requires an additional step in the mechanized method; however, it also enables energy saving and speedy conversion of raw material to the final product.

In a comparative study of all the available systems for the commercial production of khoa, an inclined SSHE gave the best results. The sensory characteristics of khoa prepared by this system were similar to those of the traditional product. As there is no exposure of the product to the environment during manufacture, khoa made by using inclined SSHE is expected to have better microbial quality and shelf life, which can be further enhanced through packaging in a suitable container under hygienic conditions.

Physicochemical Changes during Khoa Making

The most apparent change during khoa making is the change of state of liquid milk to solid khoa. On heating, denaturation and coagulation of milk protein occur, as evidenced by a change in color and consistency of the product towards the end of the manufacturing process. The whey proteins are coagulated by the action of heat, whereas the destabilization of caseins may be attributed to the combined effect of progressively higher TS concentration, altered salt balance, decreased pH, and high temperature. The fat globules in milk undergo appreciable subdivision owing to the vigorous agitation at a high temperature during khoa making, and a considerable amount of free fat is present in the final product owing to rupturing of the fat globule membrane. From being a dilute solution in milk, lactose is present as a supersaturated solution in khoa. However, crystallization of lactose does not occur in khoa. A portion of the milk salts are precipitated by the action of heat. There is a significant reduction in the total, as well as soluble, content of calcium, magnesium, and phosphorus in khoa. During the traditional method of khoa making, the iron content of khoa increases to >100 mg kg^{-1} from 2–4 mg kg^{-1} in milk. This is due to the incorporation of iron from the surface of the pan caused by the vigorous scraping during manufacturing. Caramelization due to the interaction of proteins and carbohydrates imparts a typical flavor and other sensory characteristics to khoa.

Factors Influencing the Quality of Khoa

Type and Quality of Milk

Buffalo's milk is preferred for khoa making owing to its higher yield, light greenish color, soft loose body, smooth granular texture, rich nutty flavor, and slightly sweetish taste. Such khoa is more suitable for the preparation of most sweets than cow's milk khoa, which is slightly hard and has pale yellow color, moist surface, sticky body, sandy texture, rich nutty flavor, and slightly salty taste. Levels of fat and the ratio of nonfat solids to fat solids in milk influence both the yield and the moisture content of khoa. Buffalo's milk with a minimum of 5% fat or cow's milk with a minimum of 4% fat is necessary to obtain the desirable body and textural characteristics in khoa. Milk with a lower fat content results in a hard, rubbery, and coarse-textured khoa. Fresh milk is preferred for a better-quality khoa. Aged raw or heat-treated milk imparts a coarse texture, sour smell, and sour–bitter taste to khoa. Khoa made from goat's milk is characterized by a yellow color, slightly hard body, smooth texture, and pronounced salty taste. Unlike that from cow's or buffalo's milk, the product from goat's milk does not leave the sides of the heating surface easily during the final stages of manufacturing. The sticky nature of the resultant khoa is attributed to almost no release of free fat during its manufacture. Adulteration of milk with water lowers the yield without affecting the quality of khoa. The presence of added neutralizers and stabilizers gives an undesirable salty taste to the product. Adulteration of milk with starch yields puffed-up and sticky-bodied khoa with a starchy smell and taste.

Pretreatment of Milk

Homogenization of milk produces a softer product, improves the color of khoa, giving it a whiter appearance, and reduces the amount of visible free fat present. Hydrolysis of the lactose in milk prior to khoa manufacture gives a product with a higher peroxide value, sweeter taste, softer body, more uniform texture and brown color, and resistance to mold growth. Khoa made from unhydrolyzed milk exhibits higher proteolysis and lipolysis and is also susceptible to mold growth within 3–4 days of storage. Concentration of khoa milk by RO leads to a slightly higher moisture content, significantly higher fat content, and lower ash content on a dry matter basis in comparison with conventionally made khoa.

Heat Treatment

Heat treatment of milk in khoa making, in general, is aimed at meeting public health requirements, water removal, facilitating mixing and blending, and imparting desirable properties to the product. Removal of water to a desirable level provides a proper body and texture, and enhances the keeping quality of khoa by minimizing bacterial growth and slowing down some of the chemical and biochemical reactions. To obtain a better-quality khoa, the milk is kept under boiling conditions continuously until a semisolid/pasty consistency is achieved. Subsequently, the temperature is reduced to 80–85 °C until the final stage. A higher temperature during the later stages results in an undesirable coarse texture, a brownish color, and an intense cooked/burnt flavor.

Rate of Stirring

During khoa making, the milk is stirred and scraped continuously to (1) attain proper and uniform mixing, (2) facilitate rapid evaporation of water, (3) avoid localized action of heat, and (4) impart a desirable body and texture to khoa. A low speed of stirring (30–40 rpm) results in an undesirable appearance, texture, and flavor of khoa. A medium (100 rpm) to high (150 rpm) speed of stirring in a circular motion during desiccation is required for khoa making, which otherwise may undergo undesirable changes in its properties.

Amount of Milk per Batch

Generally, 3–4 kg of milk per batch is used in the traditional method of khoa making. This amount is ∼20–25% of the total capacity of the pan. A lesser quantity of milk may result in burning of some particles, whereas a larger quantity would be difficult to handle, particularly while boiling, and may cause yield losses.

Manufacturing Technique

Khoa made by a continuous method has a coarser texture and softer body when compared to the traditionally made khoa. Khoa manufactured from whole milk preconcentrated (31% TS) by RO has flavor and texture comparable to that of khoa made in a traditional open pan. The mineral content of RO khoa is slightly lower owing to permeation of these compounds through the RO membrane. RO khoa lacks graininess and shows a slightly higher free fat and a higher moisture content than the traditional product. The khoa obtained from reconstitution of khoa powder tends to have a pale yellow color with a brown tinge, moist surface, uniform texture, soft and smooth body, cooked smell, and slightly salty taste.

Yield of Khoa

The yield of khoa depends on several factors, including the type and quality of milk, the extent of dehydration, the type of khoa manufactured and its moisture content, and handling losses (e.g., overflow and sticking residues). Moisture content is the most important factor affecting the yield of khoa. The average yield from buffalo's milk is 21–23% and from cow's milk is 17–19%.

Packaging, Storage, and Shelf Life of Khoa

Although packaging is one of the major factors in determining the keeping quality of khoa, it has been observed for several years that it is the aspect most neglected by traditional manufacturers. This may be because of the fact that the product is usually sold at a nearby market soon after manufacture and hence does not need a protective container. However, even when the product needs relatively longer transportation, the traditional practice of wrapping the individual khoa pats in leaves and then packing in a bamboo basket is still followed. Now that the product has attracted the attention of the organized sector, it is packaged in vegetable parchment paper, mechanical parchment paper (glassine), or polyethylene pouches or bags. Many manufacturers have also started using aluminum-coated laminates or tinplate cans. As khoa is transported to the market soon after manufacture, its storage at the production place is not given much importance when made in small quantities. The hot climatic conditions prevailing in the Indian subcontinent are among the major factors responsible for the limited keeping quality of khoa, and hence refrigeration is preferred for bulk storage. Khoa contains about 20–30% moisture and thus falls under the category of intermediate-moisture food. This makes it highly perishable, and it remains acceptable for a maximum of 5 days at 30 °C (ambient temperature) or for ~25 days at 7 °C. Other factors that influence the shelf life of khoa are (1) the quality of raw milk, (2) the initial moisture content, (3) the sanitary conditions at the manufacture and storage places, and (4) the type and method of packaging. The use of vacuum packaging and gamma radiation (60 °C) to sterilize the polypacks before product packaging, and addition of potassium sorbate (0.3%) at the final stage of khoa making have been found to be beneficial in extending the shelf life of the product. However, addition of any preservatives to khoa is prohibited under the PFA rules.

Changes during the Storage of Khoa

Postmanufacture microbial contamination and the interaction of milk components during the storage of khoa lead to many physicochemical changes. Several studies have been undertaken on these aspects of khoa.

Moisture

It has been reported that khoa stored at room temperature without any preservative showed an increase in its moisture content for up to 4 weeks and thereafter the moisture content declined progressively throughout the storage period of 9 weeks. Studies have shown that the loss of moisture during the storage of khoa varies with the type of packaging used and the temperature of storage. Among the several packaging materials tested, khoa packaged in parchment paper and in a 5-ply laminate package showed the maximum and minimum loss of moisture during storage, respectively.

Lactose and Acidity

Significant breakdown of lactose occurs during the storage of khoa, which varies with storage temperature and the microbiological quality of khoa. It has been reported that the breakdown of lactose during storage at 22 °C for 9–11 days and at 37 °C for 5–7 days was about 19 to 60%, respectively. A progressive increase in the titratable acidity of khoa is observed during storage. The increase in acidity is positively correlated to flavor deterioration of khoa.

Lipolysis

The lipids in khoa undergo hydrolysis due to the action of microbial enzymes, which causes an increase in the free fatty acid (FFA) content. This influences the flavor characteristics of khoa. Lipolytic changes in khoa have been reported by several workers and usually are expressed either as the percentage of oleic acid or as the acid degree value. In a study of lipolysis in market samples of khoa, it was observed that the period of incubation required to reach different stages of deterioration, namely, slight change (grade I), perceptible change (grade II), and pronounced deterioration (grade III), depends on the temperature of storage and the microbiological quality of khoa. The liberation of FFAs was higher at 22 °C than at 37 °C. The higher concentration of FFAs at the lower temperature of storage is attributed to optimum growth of yeasts and molds. Besides the release of FFAs, the lipids of khoa undergo oxidative deterioration during storage and lead to the development of oxidative rancidity in the product. The extent of oxidation of lipids in khoa as a function of time is measured by estimating the peroxide value.

Proteolysis

Proteins in khoa undergo microbiological degradation during storage, which causes changes in flavor, body, and texture of khoa. The rate of proteolysis has been measured by various workers either by assessing the tyrosine level or by formal titration. It is observed that the rate of proteolysis in khoa is correlated with the bacteriological quality of khoa and the temperature of storage. In a market sample study, tyrosine levels in grade-I khoa stored at 22 and 37 °C were 94.71 and 71.03 mg $(100\,\mathrm{g})^{-1}$, respectively, whereas in grade-II khoa, the corresponding values were 217.33 and 133.37 mg $(100\,\mathrm{g})^{-1}$. The rate of proteolysis was higher at 22 °C than at 37 °C presumably because the former is the optimum temperature for the growth of yeast and mold.

Hydroxymethyl Furfural Content

It is observed that the initial concentration of 5-hydroxymethyl furfural (HMF) in khoa gradually increases on storage at 30 °C. In khoa made from lactose-hydrolyzed milk, this increase is very high at the end of 4 days of storage period and thereafter it declines.

Microbiological Growth

As milk is subjected to a very high heat treatment during khoa making, a low bacterial count, with very few survivors and sporeformers, is expected in the final product. Yet a high and varied microbiological count has been reported in market samples of khoa due to contamination during manufacture, handling, packaging, and storage. The general microflora of khoa (fresh or stored market khoa) has been reported to include acid producers, aerobic sporeformers, proteolytic, lipolytic, and pigmented organisms, coliforms, and yeasts, molds, and many other group of organisms, which on proliferation cause spoilage of khoa. The limited shelf life of khoa has been ascribed to microbial spoilage; particularly, the yeast and mold count has been shown to be closely related to this.

Khoa Powder

Manufacture of khoa powder has been attempted to reduce the bulk of indigenous product by moisture evaporation and to extend the shelf life of the product. Khoa powder can be produced in the flush season for marketing in the lean season. Various processing techniques have been tried to manufacture khoa powder.

In one of the processes, skim milk is preconcentrated to 28% TS by RO. The preconcentrate is then standardized to 6% fat using 20% fat cream and heated in an SSHE at 127–132 °C to impart the typical khoa color and flavor. After cooling the heat-treated concentrate to 65.5 °C, it is finally dried over a steam-heated roller dryer to obtain khoa powder. To produce tray-dried khoa powder, buffalo milk is heated to a suitable temperature to reduce the moisture content to about 20%. The partially desiccated khoa is pulverized to fine particles and uniformly spread in trays to a thickness of 1 cm. It is then dried in a drying chamber at 70 °C for 4 h or in a vacuum oven at 63.5 cm of mercury for 3 h. The preconcentrated khoa particles can also be dried in a fluidized bed dryer at an inlet air temperature of 92 °C and outlet air temperature of 80 °C.

To utilize roller dryers for making khoa powder, standardized buffalo milk is first heated to a predetermined temperature in a stainless-steel double-jacketed vessel to develop a cooked flavor. The heated milk is pumped to the milk distribution channel of the roller dryer at a predetermined flow rate and steam pressure of 4.5 ± 0.3 bar (448 ± 34 kPa). Khoa powder formed in thin sheets is then scraped, ground, sieved, and packaged. Spray drying is used in the large-scale manufacture of khoa powder. Buffalo milk is vacuum evaporated to 35% TS, heated to develop a cooked flavor, and spray dried at an inlet air temperature of 190 °C and outlet air temperature of 88 °C. Khoa powder can be preserved in tin containers for up to 7 and 9 months at 30 and 5 °C, respectively.

Defects in Khoa and Their Prevention

1. Flavor defects: The major flavor defects in khoa are sour/acid, rancid, and stale flavors caused by using poor-quality milk or storing khoa for excessively long periods. These defects can be prevented by using fresh milk, storing khoa at a low temperature, and early usage/marketing of khoa.
2. Body and texture defects: Low fat content of milk, low moisture content of khoa, and adulteration of milk with starch cause a hard body. A coarse texture is caused by high acidity and low fat content of milk, excessively high temperature, and low speed of stirring during khoa making. The presence of large lactose crystals due to incorrect method of manufacture causes grittiness in khoa. These texture defects in khoa can be prevented by using good-quality milk and proper manufacturing techniques.
3. Color and appearance defects: Poor-quality milk; dirty utensils; unhygienic surroundings during manufacture, transportation, and storage; and poor packaging of khoa lead to the presence of visible dirt or foreign particles in the product. Excessively high temperature, especially during the final stages of manufacture, and inadequate stirring and scraping during khoa making cause browning and/or presence of burnt particles in the final product. A very high moisture content of the khoa, high humidity of the storage room, and poor packaging lead to mold growth on the surface of khoa. A low fat content of milk cause dryness on the surface of khoa, whereas a high fat content and excessive stirring during manufacture causes free fat. These defects in appearance can be prevented by using good-quality milk, proper manufacturing techniques, and proper packaging and storage of the product.

Applications of Khoa

Almost all of the khoa manufactured in India is used as a base material for making various sweets. Some of the popular khoa-based sweets are peda, burfi, gulabjamun, kalakand, and milk cake. Some traditional manufacturers also use khoa in ice cream to replace all or a part of the fat and solids-not-fat sources, that is, milk powders, cream, or butter. Even though khoa-based ice cream has a granular body (as the mix is not homogenized), it is preferred by many consumers. Very little khoa is used for direct consumption.

See also: **Concentrated Dairy Products**: *Dulce de Leche*. **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture.

Further Reading

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CONTAMINANTS OF MILK AND DAIRY PRODUCTS

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Introduction

Improved analytical methods that allow detection of exquisitely low concentrations of chemicals have revealed that milk and dairy products may be contaminated with a wide range of potentially harmful chemicals. These compounds enter milk through various direct and/or indirect routes (**Figure 1**). For several reasons, their occurrence in milk is difficult to avoid and control, as is the case with the persistent environmental pollutants, for example, dioxins and heavy metals. Others result from agricultural, veterinary, and hygienic practices, which improve milk yield and quality, but may also leave trace levels of residues in the finished products.

As milk-based products contribute significantly to the overall human diet in many regions of the world, their contamination with potentially toxic chemicals may cause concern. However, it is important to realize that the detection of such chemicals alone does not contribute to the understanding of their significance to human health. Safety assessment should be applied to interpret occurrence data. It allows the establishment of limit values ensuring the safety of the products and provides tools to prioritize and focus resources and management measures on key issues (**Figure 2**).

Safety assessment combines toxicological and exposure information to evaluate the probability of adverse effects occurring in the human population (**Figure 2**). Most of the chemical contaminants found in milk show a threshold of toxicity. Their safety is addressed through the establishment of safety standards such as the acceptable or tolerable daily intake (ADI or TDI), or the provisional tolerable weekly intake (PTWI, for accumulating chemicals). They are usually derived from no observed adverse effect levels (NOAELs) obtained experimentally in toxicological studies. These safety standards correspond to an estimate of the amount of a chemical that can be ingested daily or weekly over a lifetime without appreciable health risk. The safety evaluation compares exposures with safety standards. Usually, average and high-exposure (e.g., 95th percentile, about 2–3 times the average) estimates are used in the evaluation. With respect to milk and dairy products, consumption may vary significantly according to the geographic region considered.

It is important to note that the safety standards cover exposure from all sources (e.g., food, air, water). Therefore, the safety significance of a contaminant should be evaluated in the overall context, allocating a proportion of the safety standard to all relevant sources of exposure.

Genotoxic carcinogens are considered to act through non-threshold mechanisms via direct DNA binding, resulting in mutations. For this class of toxicants, the ADI concept is not applicable and different approaches have been developed. Either contamination is minimized to

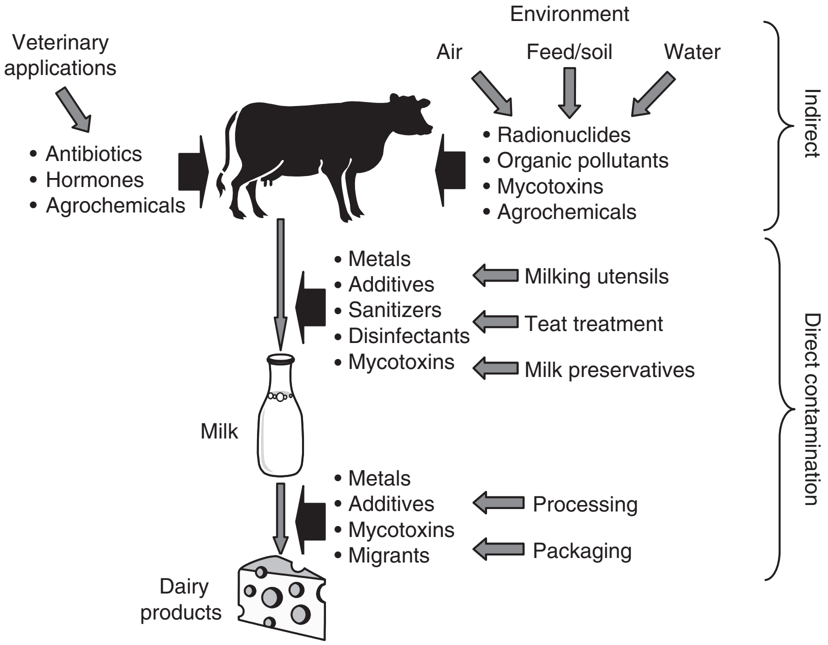
Environment
Air
Feed/soil
Water
Veterinary applications
• Antibiotics
• Hormones
• Agrochemicals
• Radionuclides
• Organic pollutants
• Mycotoxins
• Agrochemicals
Indirect
• Metals
• Additives
• Sanitizers
• Disinfectants
• Mycotoxins
Milking utensils
Teat treatment
Milk preservatives
Milk
• Metals
• Additives
• Mycotoxins
• Migrants
Processing
Packaging
Direct contamination
Dairy products

Figure 1 Contaminants of milk and dairy products and their respective sources. 'Migrants' refers to indirect food additives and polymerization residues.

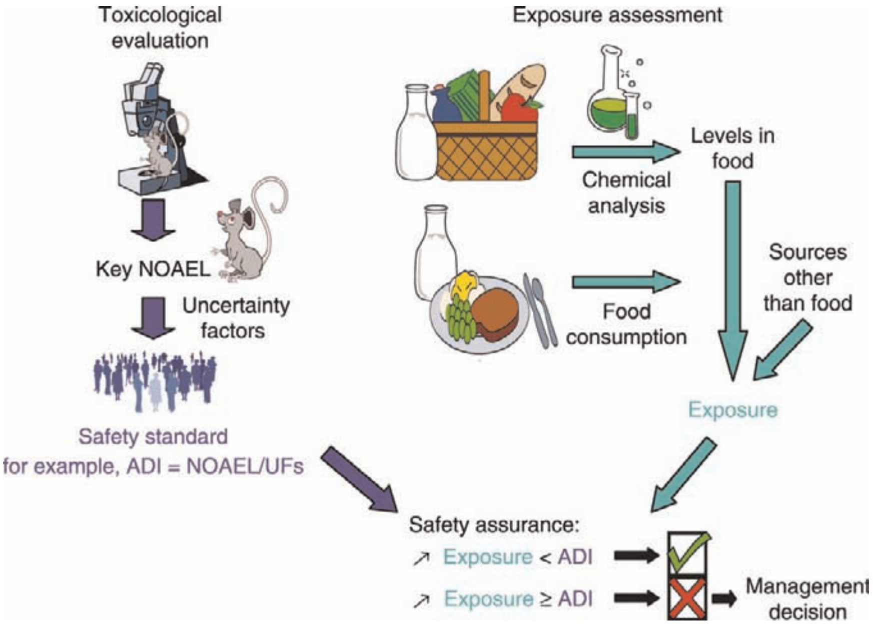
Toxicological evaluation
Key NOAEL
Uncertainty factors
Safety standard for example, ADI = NOAEL/UFs
Exposure assessment
Levels in food
Chemical analysis
Food consumption
Sources other than food
Exposure
Safety assurance:
↗ Exposure < ADI
↗ Exposure ≥ ADI
Management decision

Figure 2 The safety significance of contaminants in foodstuffs is addressed through a comparison of exposure with safety standards/tolerable levels such as the acceptable daily intake (ADI) value. Safety standards are derived by applying uncertainty factors (UFs) to a key no observed adverse effect level (NOAEL) experimentally obtained in animals. The NOAEL is the dose at which no adverse effect was detected in the most sensitive multiple-dose animal study. UFs take into account potential differences between humans and the test species, and the variability between human individuals. Factors of 10 have often been used for interspecies extrapolation and human variability. Contaminant occurrence data and food consumption models allow estimation of exposure. For specific contaminants, additional sources of exposure (e.g., water, air) have to be considered. There is no safety concern when the overall exposure is below the ADI, whereas if the ADI is exceeded, a refinement of the assessment is conducted. Usually, the easiest way is to refine the exposure component of the assessment through the use of more probable occurrence data and through the application of more realistic consumption scenarios. Refinement of the safety standard may also be possible. In the case of the ADI, it would involve replacement of the default UFs by real values based on scientific data. If despite the refinement the ADI is still exceeded, risk management measures to reduce exposure have to be planned. Safety-based guidance values for contaminants in milk and dairy products can be determined from the ADI. Information on representative concentrations in food and the environment is used to estimate the proportion of the ADI that should be allocated to milk and milk products. Safe levels in these products are then derived taking consumption data into consideration.

levels as low as technologically possible, or a quantitative risk assessment involving a dose–response relationship can be conducted.

In the process of evaluating the safety of milk as a source of contaminants, particular attention should be paid to infants and children. For this specific sector of the population, milk and dairy products play a particularly important role as a source of nutrients. On a body weight basis, milk consumption by infants and children is much higher than for adults. As a result, higher exposures to milk contaminants are expected. In addition, for some contaminants known to occur in milk, developing organisms appear more susceptible than adults to their toxic effects, for example, to lead and polychlorinated biphenyls (PCBs).

In this article, information on the concentrations of contaminants originating from agricultural, veterinary, and hygienic practices is provided. It provides a general overview of the types of chemical residues and the typical levels that may be encountered in milk and dairy products. High levels should be viewed in the context of safety and good manufacturing practices.

Environmental contaminants are covered in **Contaminants of Milk and Dairy Products**: Environmental Contaminants.

Pesticides

Sources and Occurrence

The employment of commercial man-made chemicals to combat insects as disease vectors and pests in the agricultural sector was particularly intensified on a global scale after World War II. These 'pioneer' chemicals were organochlorines (OCs) such as the insecticide 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), endrin (insecticide/rodenticide), and hexachlorobenzene (HCB) (fungicide), all showing persistence in the environment and thus prolonged efficacy. However, due to their lipophilicity and resistance to biodegradation, these compounds accumulate in the biosphere and detectable levels may be found essentially on a global scale in many foods including milk and dairy products.

Owing to this concern, more labile pesticides of the organophosphate (OP) class rapidly replaced the stable OC compounds (which were banned in many countries in Europe and in the United States already in the 1970s), which in turn has led to a steady decline of OC residues such as DDT (and its metabolites) and hexachlorocyclohexane (HCH) isomers in the environment and consequently also in dairy products. This tendency is reflected in the levels of major OCs in dairy products reported in national contaminant surveys conducted over the past decades. Such trends are evident in European countries. In Germany, for example, levels of OCs (total DDT, HCB, lindane, aldrin, dieldrin, heptachlor/heptachlor epoxide) in bovine milk declined considerably (>50%) from 1974 to 1981, mainly as a consequence of the OC ban. However, in some developing countries, recent surveys show regular elevation in the concentrations of certain OC residues as these may not be totally banned but restricted in their use, as a result of which some animals may still be exposed to the compounds (e.g., lindane, DDT). A seemingly contradictory picture is drawn when comparing the surveys of the United States Department of Agriculture (USDA) Pesticide Data Program (PDP) for the years 1998 and 2004 (or 2005). In the 2004 survey, the major isomer of 1,1-dichloro-2, 2-bis(4-chlorophenyl) ethylene (DDE p, p′) was detected in 96% of the milk samples tested versus close to 14% in 1998 (**Table 1**). Similarly, diphenylamine (DPA) was found in 98%, and the OC insecticide dieldrin in 41% of the samples. This large increase in the number and frequency of pesticides detected in the more recent surveys can be explained by much more sensitive analytical methods being employed. Essentially all the limits of detection (LOD) were significantly lower for the milk samples tested in 2004 and beyond. Importantly, all results were well below the established US Environmental Protection Agency (EPA) tolerances and Codex maximum residue limits/extraneous maximum residue limits (MRLs/EMRLs).

However, surveillance of OC pesticide residues remains important as they may still find their way into milk either through intensive insecticide use on field crops or from an earlier contamination of the environment. An example of the impact of previous intensive usage of DDT in the environment is New Zealand, where relatively high levels of DDT breakdown products have been found in butter, for example, DDE, albeit at levels below the Codex EMRL (0.02 mg kg^{-1} for the sum of DDT and metabolites). In pasture-based economies in particular, environmental contamination with OCs in animal products may be difficult to predict and control, particularly during drought or marginal malnutrition, where animal fat reserves are mobilized to maintain milk output, periodically leading to higher incorporation of OC residues into milk. Other potential routes of contamination are the application of sewage sludge to land, or dairy animal feedstuffs imported from certain countries where agricultural practices and/or malaria control still include the use of OCs such as lindane and DDT.

Notably, OCs and their metabolites (e.g., DDT/DDE) are also classified as environmental contaminants belonging to the so-called 'persistent organic pollutants' (POPs). In 2004, the United Nations Stockholm Convention on POPs came into force and became international law. About 100 nations agreed to sign the treaty to control the release of or to ban the 12 initial POPs (i.e., aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex, toxaphene, HCB, PCBs, dioxins, and furans).

Table 1 Excerpt of the pesticide residues found in milk in the United States Department of Agriculture 1998 and 2004 surveys

Pesticide	*No. of samples with detections*	*Samples (%) positive*	*Mean of the positives (ppb)*
1998 survey[a]			
Chlorpropham	1	0.2	2
DDE p,p′	82	13.8	3
Diphenylamine	1	0.2	17
Lindane	1	0.2	2
O-Phenylphenol	5	2.3	10
2004 survey[b]			
3-Hydroxycarbofuran	65	8.8	0.34
Bifenthrin	3	0.4	0.1
Cyfluthrin	11	1.5	1
Cyhalothrin, total	128	17.3	0.48
Cypermethrin	1	0.1	1
DDE p,p′	710	96.1	0.51
Dieldrin	307	41.5	0.2
Dimethoate	6	0.8	0.12
Diphenylamine	728	98.5	0.19
Endosulfan sulfate	134	18.1	0.22
Fluvalinate	3	0.4	1.82
Permethrin, total	33	4.5	1.07

[a] Average based on the maximum number of samples tested for a single pesticide ($n = 595$).
[b] Average based on 739 samples tested for all pesticides.

Pesticide residues in milk may have a number of potential sources, including environmental (water, soil, air) contamination of the animal feed (fodder), or treating dairy animals or their direct living environment to protect against disease vectors (mites, ticks, insects). Direct contamination of milk may ensue via uncontrolled sanitary measures of the immediate surroundings of the animal, or treatment of the dairy utensils that are employed during milking or storage. However, more important routes of contamination are indirect, one of which may be through the application and administration of pesticides to the milk-producing animal, orally, cutaneously, or via inhalation when animals are housed in a closed environment during treatment. In all cases, the active ingredient may be absorbed, subsequently metabolized, and eventually excreted into the milk of the lactating animal.

This potential route may be a consequence of treatment of the animal with ectoparasiticides and endoparasiticides, but only if this usage is uncontrolled and fails to respect the prescribed withholding time before milking. Carryover into milk – in both cases – may originate from absorption of the active ingredient passing the blood–milk barrier in the mammary gland, the efficacy of the process being dependent on the chemical/physical properties of the molecule (e.g., lipo- or hydrophilicity, molecular weight, and preference to carrier proteins).

Endoparasiticides, usually administered to animals orally, as feed additives, by injection or in the form of pour-on preparations, are employed mainly against helminths, which include the tapeworms, roundworms, and flukes. The most widely used compounds in the past were levamisole and the benzimidazoles (e.g., thiabendazole), which are largely replaced today by the highly effective fungal metabolite ivermectin. Studies on the excretion rate of anthelminthics show that prevalence of measurable residues in milk is – in most cases – shorter than 5 days after therapeutic treatment.

Cutaneous treatment of animals against ectoparasites (insecticides, acaricides) includes compounds mainly of the OP class, carbamates, pyrethroids, organotin compounds, and certain organonitrogen compounds such as the acaricide amitraz. Studies have shown that many residues are below the detection limit in the milk already 2 days after application (e.g., coumaphos, at a dose of 6 mg kg^{-1} body weight (bw)), but some may persist for up to 5 days at levels >0.01 mg kg^{-1} (fenchlorphos, permethrin). Thus, hygienic treatment of dairy animal presents no real risk of residues in milk if the treatment is carried out as directed. However, contamination of utensils or factory premises may result in the occasional presence of insecticides (e.g., chlorpyrifos, diazinon, malathion), used to control, for example, cockroaches.

Health Impact

The applications of modern pesticides in agriculture, on food and forage plants, practically bear no risk of significant residues in milk, a premise being strict adherence to good agricultural practice (GAP). Levels of OC pesticides, potentially contaminating milk via the environment, have been decreasing over the past decade(s) and international efforts are under way to further reduce environmental contamination. Current residue levels in milk fat do, however, reflect both past and current usage patterns. The results of surveys do not raise health concerns in developed countries.

Analytical Aspects

The range of pesticides and pesticide formulations registered for use in agriculture represent a large diversity of chemical structures characterized by different physicochemical properties. Today, approximately 800 compounds are used in pesticide formulations, and laboratory analysts developing detection methods also need to consider several metabolites, degradation products, and 'phased out' as well as 'old' persistent chemicals.

Residue analysis in food is probably one of the most demanding areas of analytical chemistry, requiring in many cases the determination of compounds at the microgram or nanogram per kilogram level. Without doubt, the most efficient approach to pesticide residue analysis involves the use of multiclass, multi-residue methods.

The basic steps of pesticide residue analysis can be defined as

- sampling
- extraction
- clean-up (e.g., gel permeation chromatography, silica gel, florisil columns)
- (separation) and identification
- quantification

The procedure chosen for the isolation, clean-up, and analysis of the food sample is dictated by the composition of the food matrix, especially the fat content. The polarity ranges of the different pesticide families in water are an important consideration in the development of a universal residue method, which should have the widest scope possible. A recently developed and much favored approach to extract residues is the QuEChERS method (quick, easy, cheap, effective, rugged, and safe), which entails extraction with acetonitrile and salting out with, for example, anhydrous magnesium sulfate and sodium acetate. The QuEChERS approach has been evaluated for pesticides in fatty foods including milk, coupled with a clean-up by dispersive solid-phase extraction to remove matrix coextractives. Good recovery was achieved with semipolar and polar pesticides. Today, more residue methods that are based on mass spectrometry as the choice of technology for detection are described in the literature, and are gradually replacing the classical capillary gas chromatography (GC) coupled to selective detectors.

Antimicrobial Drugs

Sources and Occurrence

Antimicrobial drugs are administered to treat bacterial infections or employed prophylactically to prevent spread of disease, or to augment growth and yield in animals and animal products. All antimicrobial drugs administered to dairy animals enter the milk to a certain degree, and each drug is given a certain withdrawal (waiting) period, during which time the concentration in the tissues declines and the drug is eliminated from the animal. The most frequently and commonly used antimicrobial drugs are antibiotics, used to combat mastitis-causing pathogens. Mastitis is a disease that inflicts significant economic losses on the industry on a global scale, estimated in the United States alone at approximately US$ 1.8–2 billion per annum. Other infectious diseases such as laminitis, respiratory diseases, and metritis are also treated with antimicrobial agents, but are of minor comparative importance.

Mastitis is an inflammation of the mammary gland, and is characterized by an increase in somatic cell count in the milk and by pathological change in the mammary tissue. Mastitis-causing pathogens include bacteria, mycoplasmas, and fungi, and can be broadly categorized into specific udder pathogens (contagious pathogens) and environmental pathogens. Numerous products are available for therapeutic and prophylactic treatment of the udder, and are usually administered by intramammary (intracisternal) injection of the infected quarter(s), and in severe cases systemically. One can distinguish between lactating-cow and dry-cow therapies, the latter characterized by slow-release preparations that are active over several weeks during a dry period, and which should be taken into account in the case of early parturition (*see also* **Mastitis Therapy and Control**: Management Control Options). Each registered antimicrobial preparation has a recommended withdrawal time, which must be adhered to in order to avoid illegal levels of residues in the milk. It is rare that antimicrobials will be retained for longer than the normal withdrawal times, but could be extended in certain cases of low milk yield, for example, due to milk fever.

The antimicrobials administered in mastitis treatment span a wide range of compounds, including the β-lactams (penicillins, cephalosporins), tetracyclines, macrolides, aminoglycosides, quinolones, and polymyxins. Of these, the β-lactams are the most widely used for lactating cows, and >170 formulations are currently available on the market (taking into account Switzerland, United Kingdom, France, United States of America, and Ireland). Of these, more than 50% contain either penicillin G or cloxacillin (semi-synthetic isoxazolyl penicillin), with roughly equal distribution of the two antibiotics in the preparations. Moreover, to improve the efficacy and to increase the spectrum of activity of the preparations, many of the compounds are used as formulations that contain two or even more different families of antimicrobials in the same preparation.

National surveys in developed countries show that contamination of bovine milk (residue-positive samples using a bacterial inhibition screening test) at tanker level is generally low (low single-digit percentage). Such findings may not necessarily be equated with the failure rate since it could also encompass the so-called 'false positives' (i.e., still within legislative limits but positive in the rapid test), thus depending on the sensitivity of the test toward certain individual antibiotics. In 2003, the US National Milk Drug Residue Database for example reported the results of over 4 million samples analyzed by using rapid test kits (milk from pickup tanks, pasteurized fluid milk, and milk products). Many of these tests were for β-lactam residues and the most violations, approximately 3000 (<0.1%), were found for these residues; 23 positive samples were found for sulfonamide residues (out of 66 000, 0.03%). Only a few non-compliant residues were found for tetracyclines and aminoglycosides in a limited number of samples. In the 2004 national residue monitoring plan by the European Union, 44 out of 32 000 (0.1%) samples were non-compliant for antimicrobials of which 5 non-compliant results for chloramphenicol were detected. In countries where no regular testing is done, this figure may lie between 1 and 10%. An additional concern is the usage of unapproved (non-compliant) compounds such as chloramphenicol, which may seem attractive considering the substantial economic losses inflicted by mastitis.

Technological Impact

The occurrence of residues of antimicrobials in milk besides being of interest in the context of consumer health and the development of antibiotic resistance has both economical and technological impact on the dairy industry. Antimicrobial residues at or below MRL levels can influence bacterial fermentation processes involved in the production of some dairy products, such as cheese or yogurt. In model trials with yogurt and mesophilic cultures, β-lactam antibiotics (penicillin G, cloxacillin), as well as oxytetracycline and some macrolides (spiramycin and tylosin), for example, significantly impaired lactic acid production by the bacterial cultures at EU-MRL levels. Differences are reported between cultures as well as between the various antibiotics tested. Yogurt cultures in most cases were shown to be more susceptible to antibiotic residues than mesophilic starter cultures. Within the tests employing yogurt cultures, L(+)-lactate formation was particularly inhibited by penicillin, whereas spiramycin impaired mostly D(−)-lactate formation.

Besides lactic acid formation, production of aroma compounds (e.g., diacetyl in butter), formation of carbon dioxide for eye formation in cheeses (e.g., Emmental cheese), and polysaccharide formation to thicken fermented milks (e.g., yogurt) are important characteristics of bacterial cultures used in the production of dairy products. Along with the inhibition of acid formation by lactic acid bacteria, antimicrobial residues have been reported to be able to result in inadequate ripening, off-flavors or lower flavor intensity, and uneven texture such as uneven eye development, as well as brown spot defects in propionic acid-fermented cheeses. Ultimately, residues of antimicrobials may lead to a deterioration of quality and to monetary losses in the dairy industry by inhibiting starter cultures in dairy technological processes.

Health Impact

A general concern linked to the widespread usage of antimicrobials at the farm level is the potential development of antibiotic-resistant pathogens, particularly if treatment is not diagnostically targeted. This may complicate human treatment and possibly cause selection of antibiotic-resistant strains in the gut.

Further concern was raised that sensitive individuals may exhibit allergic reactions to residues of antibiotics and/or their metabolites, mainly β-lactam antibiotics. However, the allergenic risk is very low. Only the individuals sensitized through previous therapeutic exposure can react with mild and transient symptoms around the tolerance levels (Codex MRL for penicillin G is $4\,\mu g\,l^{-1}$). National surveys on residues in milk in developed countries only very seldom reveal positive samples exceeding these levels.

Thus, regular monitoring is the only pragmatic approach to residue problems faced by the dairy industry in many countries, particularly in the manufacture of fermented milk products such as yogurt and cheese. The available data and global trends reflected by national surveys indicate that a direct health hazard for humans can be virtually excluded.

Other Drug Classes

Non-steroidal Anti-Inflammatory Agents

There is increasing evidence that non-steroidal anti-inflammatory agents such as phenylbutazone, flunixin meglumine, or dipyrone are used to treat bovine mastitis. These drugs are in most countries not approved for use on lactating dairy animals due to potential food safety issues. Scientific studies indicate that in the case of the lipophilic drug phenylbutazone, the elimination time of residues may be relatively extended in comparison to conventional antimicrobials, due to either milk protein bondage or active transport of the residue into milk.

β-Adrenergic Agonists

The β-agonists are hormonal-type growth promoters licensed in most EU member states for therapeutic use as bronchospasmolytics (horses, calves) and tocolytic agents (cows). The illegal usage of these compounds to improve the efficiency of feed utilization and/or to enhance carcass leanness in meat-producing animals has been reported in the literature. Thus, they are also referred to as repartitioning agents, because their effect on carcass composition is to increase the deposition of protein while reducing fat accretion. The administration of β-agonists, such as clenbuterol, in feedlots and consequently their presence in the edible tissues and milk of the animals can constitute a real health risk, with potential exposure of consumers to pharmacologically active levels that have in the past led to poisoning cases after ingestion of liver or meat.

Depending on their structures, β-agonists can have relatively long plasma half-lives, slow rates of elimination, and high oral potencies. For example, clenbuterol administered (10 mg kg^{-1} bw) to dairy cows is secreted into the milk resulting in levels directly related to those in the blood plasma, and range from 5.5 to 22.5 μg kg^{-1}. However, it is not known whether metabolites of the drugs are also excreted into milk.

Overall, the risk of physiologically active levels of residues in milk and thus a potential health impact on the consumer is minimal, because the illegal usage is in most cases limited to meat-producing animals.

Hormones

The employment of hormones in animal husbandry serves a number of purposes, which include increased food production, medical treatment, or improved reproductive performance. Hormones that have an impact on food production are classified as growth promoters, anabolics, or performance enhancers, with the prime goal of enhancing economic competitiveness. However, this use is acceptable only if no potential threats are known to the health of consumers and the animals involved.

The use of hormones as growth promoters is approved in some countries; for example, in the United States and Canada, the natural steroid hormones estradiol, testosterone, and progesterone and the (semi)-synthetic hormones melengestrol acetate, trenbolone acetate, and zeranol are approved for use only in meat-producing animals. Safety concerns have been raised by other countries, for example, EU member states, regarding hormone residues in meat. However, elevated levels of residues in milk are not expected if hormones are applied appropriately.

Sources and Occurrence

Natural hormones (steroids, peptide/protein)

The endogenous steroids 17β-estradiol, progesterone, and testosterone and their derivatives are the main sex hormones present in all mammals. They can be used for anabolic purposes, and the two female sex hormones 17β-estradiol and progesterone are also used to induce lactation, control/improve fertility, and synchronize estrous cycle.

The natural steroid hormone content of milk will fluctuate depending on the physiological and nutritional status of the animal and probably other factors. Hormone levels, mainly estrogens and progesterone, are also used for diagnostic purpose (estrus, pregnancy). Reported levels in the literature for whole milk are, for example, for total estrogens, 50–70 ng l^{-1} and for progesterone, 10–13 μg l^{-1}. Steroids are soluble in lipids; therefore, dairy products with lower fat content contain comparatively lower concentrations of steroids.

Steroid treatment, for most purposes at low levels, will not lead to a detectable increase of residues in milk. Only higher dose treatments, for example, for fertility treatment or other medical purposes, may lead to a short-term enhancement of steroids in milk.

Oxytocin is a naturally occurring peptide hormone (9 amino acids), excreted by all mammals for induction and maintenance of labor and promotion of milk ejection. It has an important pharmaceutical use in veterinary and human medicine.

Treatment is via injection, intravenously, intramuscularly, or subcutaneously. Since the peptide has a short half-life in animals, treatment can usually not be detected via increased oxytocin levels in milk. Oxytocin use has been reported in buffaloes to facilitate milking, as they seem to be more difficult to milk than cows. The use of oxytocin for the treatment of mastitis has also been reported in some countries, the mode of action being the stimulation of milk ejection, which is correlated with increased pathogen removal from the udder.

There is a paucity of data in the scientific literature on the levels of oxytocin in milk. For whole milk, approximately 50 microunits per milliliter has been reported, and skim milk apparently contains lower levels (15–20 microunits per milliliter). The legal status of the oxytocin applications may vary in different countries, and concerns have been raised with regard to animal welfare.

Bovine somatotropin (BST), also termed bovine growth hormone, is a polypeptide hormone (190–191 amino acids) produced by the pituitary gland in all cattle.

It promotes growth and regulates fat, protein, and carbohydrate metabolism. Many of the physiological effects of BST may be mediated via increased blood levels of insulin-like growth factor 1 (IGF-1), also called somatomedin, and produced mainly in the liver in response to somatotropin.

Recently, a virtually identical BST has been produced by recombinant DNA technology via genetically engineered bacteria (recombinant BST (rBST)). When injected into dairy cattle, rBST improves milk production efficiency by up to 20% under optimal management conditions.

This rBST is commercially available, and the first product was marketed in the United States under the trade name Posilac®, after approval by the Food and Drug Administration (FDA) in 1993. The use of rBST is considered safe and currently approved in many countries.

However, clinical trials with rBST have indicated an increase in the incidence and severity of mastitis, and other concerns have been raised with regard to animal welfare, which include increased food disorders, reproductive disorders, and localized swellings at injection sites (injections are repeated at fortnightly intervals).

Treatment of cows with rBST does not lead to an increase in BST in milk. Since rBST and natural BST are basically identical, analytical differentiation is not possible. BST levels in milk are generally less than 3 ng ml^{-1}, but may occasionally increase up to 10 ng ml^{-1}. The overall nutrient composition of milk is not altered by rBST treatment.

Natural BST and rBST are not biologically active in humans because they do not interact with the human somatotropin receptor. Moreover, orally ingested BST is readily digested. Bovine IGF-1, which may be elevated slightly in the milk of rBST-treated cows, is potentially bioactive in humans. However, reported increases remain within the normal range of variation and are orders of magnitude lower than the endogenous IGF-1 levels in the gut and body fluids of humans (*see* **Lactation**: Galactopoiesis, Seasonal Effects).

Prostaglandins are a group of potent hormone-like substances that are produced in various mammalian tissues and mediate a wide range of physiological functions, such as control of blood pressure, contraction of smooth muscle, and modulation of inflammation. Some prostaglandins, such as PGF2α, are employed for estrous cycle synchronization and multiple ovulations. Treatment usually does not induce a significant difference in residue concentrations compared to the natural physiological levels after a few hours of treatment.

Semi-synthetic and synthetic hormones

In mastitis treatment, synthetic corticosteroids, for example, dexamethasone, prednisolone, and derivatives thereof (flumethasone), are administered systemically or into the mammary gland to relieve inflammatory conditions.

The (semi)synthetic hormones, melengestrol acetate, trenbolone acetate, and zeranol, are approved in some countries as growth promoters in meat-producing animals.

rBST is virtually identical to the naturally occurring BST (see above).

Analytical Aspects – Hormones and Antimicrobial Drugs

For the analysis of antimicrobials, and hormones in milk and milk products, emphasis is placed on rapid tests that provide an accept/reject answer at the farm or slaughter house. These tests give qualitative or semiquantitative results, their aim being to check legislative compliance at an early stage in the food chain. Many of the tests are performance-validated and certified by the Association of Official Analytical Chemists (AOAC), and the majority are based on the inhibition of growth of microbial test organisms, ligand assays using biological receptors, or antibodies. Inhibition tests can detect a wide range of antimicrobial compounds, whereas receptor or immunological assays are specific for a family or limited range of compounds.

For some drugs, there are currently no reliable rapid tests to detect residues at the legislative levels, or, in certain cases, to detect traces of the drug if no MRL has been set. In such instances, liquid chromatography (LC) or GC methods must be employed; they also play a major role as quantitative and confirmatory techniques. These usually require sample preparation steps, which in the case of milk often involve defatting and protein precipitation with acetonitrile or trichloroacetic acid, prior to or concurrent with the extraction of residues. As in the case of pesticide residues, there is a growing trend in the development of liquid chromatography coupled to mass spectrometry (LC-MS)-based methods, which give a confirmation of the analyte, a high level of specificity, and usually a very low limit of detection. However, certain issues such as matrix effects and variable ionization that may have an impact on the performance of the method must be considered when developing determinative methods with MS detection. Matrix-based standards may be needed; deuterated or carbon-13-labeled internal standards are frequently used and highly recommended. Moreover, quantitative methods must be validated prior to widespread implementation for accuracy, precision, LOD, limits of quantification, and so on. Several guidelines have been published by enforcement bodies stipulating rigorous and necessary validation procedures.

Most methods, however, are focused on single drug classes and only a few that can detect multiple class chemicals have been reported. Recently, the

QuEChERS method has also been tested as an extraction method for veterinary drugs in milk, showing good recoveries of 18 analytes (70–110%) belonging to the quinolones, sulfonamides, macrolides, anthelmintics, and tetracyclines. Through coupling to ultra-performance liquid chromatography (UPLC)-MS/MS, a procedure that can simultaneously screen, quantify, and confirm multiple drug residues in one sample analysis is designed.

In the case of endogenous hormones, the natural variation in concentrations in tissues and milk of animals makes the detection of legal or illegal use particularly difficult. Furthermore, concentrations in healthy animals are in the same order of magnitude as those observed in animals that have received hormone implants. For this reason, sophisticated analytical techniques based on the detection of abnormal ratios of hormones to precursors or metabolites coupled with stable isotope dilution analysis have to be used to detect illegal use of these compounds.

Sanitizers/Disinfectants

Cleaning and disinfection are critical aspects of good manufacturing practice in the food production and dairy sector to ensure removal of bacteria and residual milk from the surfaces of equipment. Residues of detergents and disinfectants/sanitizers can be introduced into milk on the farm and at the dairy plant level, particularly if cleaning, disinfection, draining, and rinsing procedures of milking equipment and containers are improperly conducted. Sanitizer contaminants occur in milk and dairy products at very low concentrations and are present as indirect and incidental food contaminants.

Sources and Occurrence

Contamination of milk with disinfectants could potentially ensue via two principal routes, that is, application as teat and skin disinfectants, and treatment of the milk plants. Dipping or spraying of teats with bactericides after milking may help to control mastitis pathogens. Disinfection of the udder after milking is particularly useful against pathogens from the infected mammary gland, whereas a pre-milking treatment is more effective in controlling pathogens involved in environmental mastitis. Furthermore, contamination can occur during contact of milk with cleaned and disinfected surfaces in milking equipment and dairy plants.

The most commonly used disinfectants are iodine-liberating agents (iodophores) and chlorine-containing compounds, such as chlorhexidine and hypochlorite, as well as quaternary ammonium compounds and hydrogen peroxide (**Table 2**).

Table 2 Examples of commonly used sanitizers/disinfectants in the food industry

Halogens	Iodine-containing agents
	Chlorine-containing agents (e.g., sodium hypochlorite, trichloroisocyanurate)
Surface-active agents	Quaternary ammonium compounds (e.g., benzalkonium chloride)
	Amphoterics
	Acid anionics
Peroxy compounds	Hydrogen peroxide
	Peracetic acid

Iodophores are organic compounds containing iodine in a micellar cage of polyvinylpyrrolidone or nonoxynol complex. When diluted, iodine is liberated and can exert its bactericidal properties. Commercial preparations contain 0.3–1.75% iodine, of which 80–90% is released upon dilution. Chloramine T (*N*-chloro-*p*-toluenesulfonamide) usage as a teat disinfectant has been severely restricted over the past 20 years, due mainly to the risk of residues of *p*-toluenesulfonamide (*p*-TSA) and *p*-sulfamoylbenzoic acid, traces of which are undesirable in dairy products due to potentially toxic properties of these compounds.

Iodine ingested via feed is secreted in milk and presents an additional contribution to the total iodine in dairy products. Due to feeding practices, the iodine content in cow's milk increased by 300–500% from 1965 to 1980. High variations in mean residual iodine levels, reported in several countries, lie between approximately 10 and several hundred micrograms per liter. The mean iodine level in a German study was reported at 150 $\mu g\,kg^{-1}$.

Health Impact

Generally, disinfectants rarely pose serious residue problems. Many sanitizers have defined, specific antimicrobial activities and are consequently likely to have of low mammalian toxicity. Here, emphasis is on iodine, one of the most frequently used teat disinfectants and among the most effective antimicrobial agents, which at high doses can be of potential health concern. However, contamination of milk can largely be avoided by using formulae that do not contain more than 0.5% iodine, and by drying of the teats after dipping.

Iodine is an essential component of thyroid metabolism and the recommended dietary allowances (RDAs) are 150 $\mu g\,day^{-1}$ for adults and 90 $\mu g\,day^{-1}$ for children (1–3 years of age). High iodine intake can lead to disturbance of thyroid function. The tolerable upper intake level (UL) is defined as the highest level of intake that is likely to pose no risks of adverse effects in most individuals. The UL for iodine in adults is 1100 $\mu g\,day^{-1}$ and for children of 1–3 years of age 200 μg day^{-1}. Initial adverse effects observed in cases of excessive intake of iodine are characterized by elevated concentrations of thyroid-stimulating hormone. These effects were demonstrated at iodine intake in adults at or just above the UL.

At residual levels above 500 μg l^{-1}, exceeding of the UL for iodine by consumption of milk alone may be possible in children. In most people and at 'normal' residual iodine contamination, iodine intake from milk and other common foods is unlikely to exceed the UL and is therefore of no health concern.

The majority of sanitizers can be detected organoleptically. Despite occasional reports, however, taste and odor are poor indicators of contamination with respect to levels that may threaten human health. With the exception of phenolic compounds, some of which have extremely low perception thresholds, insufficient margins exist between perception thresholds (**Table 3**) and the contamination levels and that can pose potential human health risks.

Overall, only limited information is available on detergent and sanitizer toxicology and occurrence in food. Therefore, proper safety assessments are difficult. However, if applied within the frame of 'good hygienic practice', these products are unlikely to result in significant residue levels in milk and dairy products and thus are not expected to raise immediate health concerns.

Analytical Aspects

Due to the large variety of compounds that may be used as disinfectants, many analytical techniques have been described for their detection in food. These include simple colorimetric tests, potentiometry with ion-selective electrodes, thin-layer chromatography, and, for certain chemicals, more complex analytical techniques utilizing LC and GC.

Table 3 Organoleptic thresholds of selected sanitizers/disinfectants

Contaminant	*Taste threshold (ppm)*	*Odor threshold (ppm)*
Chlorine compounds	~100	50–200
Iodine	4–16	-
Quaternary ammonium compounds	8–50	-
Amphoterics	25–100	-
Phenolics	0.001–0.5	-

Adapted from IDF (1997) Monograph on residues and contaminants in milk and milk products. *IDF Special Issue 9701*. Brussels, Belgium: International Dairy Federation.

Melamine

Sources and Occurrence

Adulteration of milk

In 2008, deliberate contamination of milk with the synthetic chemical melamine (2,4,6-triamino-1,3,5-triazine) resulted in a major outbreak of renal disease and associated deaths in infants. In China, close to 52 000 infants and young children were hospitalized for urinary problems, related to the consumption of melamine-contaminated infant formulae and other dairy products. Melamine, a molecule high in nitrogen content, was illegally added to diluted milk to produce a false high reading of protein content in the standard measurement (the Kjeldahl method). Melamine has subsequently been detected in a variety of milk-containing products leading to regulatory measures in many countries, including bans of certain imports. Levels of melamine reported in dairy products (including infant formulae) ranged from 0.09 to 6200 mg kg^{-1}.

Other possible sources

Transfer of melamine from melamine-containing feed to cow's milk has been reported.

Melamine is used in the production of melamine resins, typically by reaction with formaldehyde. It has many industrial uses, including in the production of laminates, glues and adhesives, molding compounds, coatings, and flame retardants. Melamine is a minor metabolite of the pesticide cyromazine, and is used in some fertilizers.

Some approved uses of malamine in the United States are as an indirect food additive as a component of glues and adhesives, and in Europe as a monomer and as an additive in plastics.

Consequently, low levels of melamine can migrate into milk and dairy products from food contact material. These levels are typically below 1 mg kg^{-1}.

Health impact and Risk Assessment

The primary target for the toxic action of melamine is the kidney and urinary tract. Formation of bladder stones in rodents and kidney stones in humans has been observed as a result of high melamine exposure. In cats and dogs, co-exposure to melamine and its structural analogs, mainly cyanuric acid, as well as ammeline and ammelide, was found to induce the formation of crystals in the kidney and consequently acute renal failure.

Several authorities, as well as the WHO, have established preliminary risk assessments for melamine. In late 2008, WHO updated the risk assessment and established a TDI of 0.2 mg kg^{-1} body weight per day. This TDI is applicable to exposure to melamine alone.

Available data indicate that simultaneous exposure to melamine and cyanuric acid is more toxic than exposure to each compound individually, but available data are insufficient to derive a TDI for combined exposure.

Analytical Methods

Several methods have been published to date, from rapid screening (enzyme-linked immunosorbent assay (ELISA)) to selective quantitative methods, to determine melamine in dairy products and other foods (human and animal). Of the quantitative methods, liquid chromatography/tandem mass spectrometry (LC/MS/MS) meets all performance criteria in terms of sensitivity, specificity, and accuracy, and can be applied to many different matrices. Rapid methods to determine melamine in milk are also currently being evaluated.

See also: **Lactation**: Galactopoiesis, Seasonal Effects. **Mastitis Therapy and Control**: Management Control Options.

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Environmental Contaminants

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Introduction

Milk and dairy products are remarkably susceptible to environmental contamination. Dairy animals ingest environmental contaminants while grazing on the pasture and when fed on contaminated concentrate feeds. These contaminants may be

- naturally present in the soil and subsequently in fodder plants,
- inherent plant toxicants,
- mycotoxins from plants infected by fungi, and
- anthropogenic chemicals from industrial emissions, for example, dioxins, polychlorinated biphenyls (PCBs), or radionuclides from fallout.

Milk and dairy products are consumed in significant amounts in several regions of the world. This is even more the case for the sub-population of infants and small children, who, based on their body weight, consume greater amounts of milk than adults. For certain parts of the population, milk and dairy products may therefore represent the most important source of certain contaminants in their diet.

The objective of this article is to draw attention to the major sources of environmental contaminants in milk and dairy products and to highlight their occurrence and the resulting human exposure. Data are discussed in the perspective of potential human health impacts (*see* **Contaminants of Milk and Dairy Products**: Contamination Resulting from Farm and Dairy Practices).

Aflatoxins will not be covered here in detail (*see* **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds).

Dioxins, Polychlorinated Biphenyls, and Furans

Dioxins

'Dioxins' is a generic term for a series of related polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (**Figure 1**).

Of the 210 different congeners that can be encountered, only 17 are considered toxicologically relevant (2,3,7,8-chlorinated congeners). The most investigated and toxic representative is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), often simply referred to as 'dioxin' (**Figure 2**).

Dioxins are chemically and thermally stable and highly lipophilic. Due to their environmental persistence, they bioaccumulate via the food chain and can be found at low levels in food, mainly in animal fats. Hence, traces are found in milk and dairy products, fish, meat, eggs, and other fatty foods.

Sources and occurrence

Dioxins are formed as inadvertent by-products in many chemical processes involving chlorine and in any combustion process. The main sources of PCDDs/PCDFs are waste incinerators (municipal, hospital, industrial), metal sintering/recycling plants, cement kilns, and emissions by forest fires and volcanic eruptions. Dioxins, bound to particulate matter, are deposited via the atmosphere on any surface. Elevated contamination levels can be found in milk from farmland in the vicinity of latter industries.

Following surveys between the early 1990s and 2004, the mean background levels in dairy products expressed as toxic equivalents (TEQ) have decreased steadily and reached a plateau at 0.3–0.5 pg TEQ g^{-1} fat (10^{-12} g g^{-1} fat; ppt) in industrialized countries (with seasonal and regional variations). Less industrialized areas can reach lower plateaus of 0.1–0.3 pg TEQ g^{-1} fat without considering the dioxin-like PCBs (DL-PCBs; see below).

Health impact

Dioxins are very potent toxicants, and TCDD is one of the most potent animal carcinogens, recently classified as a human carcinogen. Apart from carcinogenicity, various effects have been demonstrated in animal models and suspected in humans, for example, on the immune system, reproduction and development, and neurobehavioral alterations.

The main sources of human exposure are foods of animal origin, and the estimated average daily intake of dioxins in industrialized countries is 1–3 pg TEQ kg^{-1} body weight (bw) day^{-1}. Dairy products contribute about one-fourth to one-half to the dietary intake of total dioxins, including DL-PCBs (see below). Dioxins occur as complex mixtures. They act through a common mechanism, but

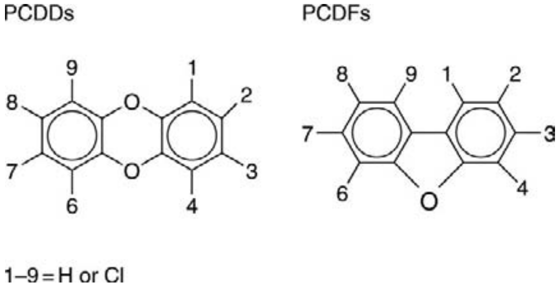

Figure 1 Structural formulae of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).

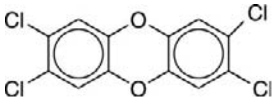

Figure 2 Structural formula of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

vary in their toxic potency. The compounds are assessed and regulated together as a group by the sum of the potency of the congeners relative to TCDD. The result is thus expressed as TEQ. DL-PCBs, which act in a similar fashion, are included in the TEQ concept.

A provisional tolerable monthly intake (PTMI) for dioxins, furans, and DL-PCBs of 70 pg TEQ kg^{-1} body weight $month^{-1}$ was set by WHO. The PTMI is a measure used by WHO for food contaminants with cumulative properties. Its value represents permissible human monthly exposure to contaminants unavoidably associated with otherwise wholesome and nutritious foods. The European Union has established a comprehensive set of maximum levels for dioxins, furans, and DL-PCBs in food and feed. For example, the maximum levels for the sum of dioxins, furans, and DL-PCBs have been set for raw milk and dairy products, including butterfat, at 6 ng WHO TEQ kg^{-1} fat or product. It appears that part of the population in some industrialized countries exceed the safe level of intake (PTMI). Therefore, dioxins in food are considered to be of health concern and efforts are undertaken to further reduce human exposure.

Analysis

Analysis of dioxins requires determination of extremely low levels (ppt, 10^{-12}, or even ppq, 10^{-15}) by gas chromatography/high-resolution mass spectrometry (GC/HRMS). The analysis is complicated and expensive and performed by only a few specialized laboratories. All relevant congeners are quantified, and the congener pattern may give indications of the source of the contamination.

However, there is also progress in the development of ultratrace methods, and improved chromatographic separation and better performance can be achieved with the help of fast-GC and GC × GC multidimensional chromatography. The latter coupled to a time-of-flight mass spectrometer (GC × GC-TOFMS) is a good alternative to conventional GC/HRMS particularly for challenging food matrices, and represents a robust and sensitive method with excellent peak resolution.

Cell-based assays have been developed to determine dioxins in food and feed. Some have been commercialized such as the CALUX (chemical-activated luciferase expression) bioassay. The CALUX assay is based on a genetically engineered cell line that contains the firefly luciferase gene under transactivational control of the aryl hydrocarbon (Ah) receptor. When these cells are exposed to dioxins, the dioxin enters the cell and binds to the Ah receptor. This complex is then translocated into the nucleus of the cell and binds to the dioxin-responsive element, inducing the expression of the luciferase gene, and subsequently the synthesis of the firefly luciferase protein, which can be measured. Several ring tests have shown that the CALUX assay is suitable for monitoring dioxin in food. However, positive results from the CALUX test are confirmed by GC/HRMS. Such alternative rapid tests allow large-scale screening due to faster total analysis time and comparatively lower costs per sample *versus* the confirmatory MS method.

Polychlorinated Biphenyls

PCBs are chlorinated hydrocarbons with the general structure shown in **Figure 3**.

In total, 209 different PCB congeners are theoretically possible, of which 36 are considered to be of environmental relevance and only about 100–140 are likely to be found in normal commercial mixtures. On the basis of structural characteristics and the associated toxicology, PCB congeners can be classified into ‘dioxin-like’ (DL) and ‘non-dioxin-like’ (NDL) PCBs.

Although the manufacture, processing, and distribution of PCBs have been prohibited in almost all industrial countries since the late 1980s, their entry into the environment may still occur, especially due to improper disposal practices or leaks in electrical equipment and hydraulic systems still in use.

The physicochemical properties of PCBs resemble those of dioxins, in that this group of compounds are chemically and thermally very stable and highly

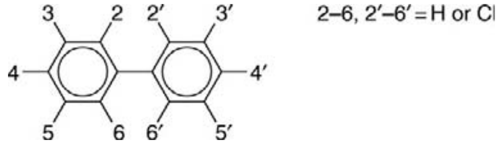

Figure 3 Structural formula of polychlorinated biphenyls.

lipophilic. They are also characterized by very low electrical conductivity, high boiling points, and fire resistance, which have led to their widespread use in various industries.

Sources and occurrence

It is estimated that more than one million metric tonnes of technical PCB mixtures were commercially produced worldwide since their first commercial use in the late 1920s. Commercial PCBs are mixtures of congeners; they always contain PCDFs as impurities in the range of 0.8–5 ppm. By the 1960s, PCBs became ubiquitous in the environment, and their toxicity came under closer scrutiny after several accidents and poisoning incidents. Although the manufacture and use of PCBs were phased out from the mid-1970s onward, low levels of persistent PCBs can still be detected in the environment and via bioaccumulation in certain fatty foods. Stricter environmental controls have led to decreased levels in foods and subsequently lower human exposure over the last decade, although to a lesser degree compared with PCDDs/PCDFs.

Following exposure of farm animals, NDL-PCB will accumulate in meat, liver, and particularly in fatty tissues. In addition, NDL-PCB will be transferred into milk and eggs, and levels in these products will reach a steady state following exposure over a period of several weeks. PCB 138 and 153, both with six chlorine atoms, show the highest carryover into milk, on the order of 50–60%. After cessation of exposure, levels in milk initially decrease rapidly to about 50%, followed by a slower elimination phase.

The amounts of NDL-PCBs in milk and animal fat are usually below 100 $\mu g\,kg^{-1}$ (ppb) on a fat basis. For example, the analysis of butter (138 samples from 15 non-European and 9 European countries) revealed the mean sum and median levels for the 7 indicator PCBs (including the DL-PCB 118) to be 8.2 and 7.1 $\mu g\,kg^{-1}$ fat, respectively. Variations in reported levels in foods as well as in human intake estimates are due to analytical differences (number of PCB congeners analyzed) and dietary habits.

Health impact

PCBs are of great health concern and can cause a variety of adverse effects. Accurate scientific assessment, especially of the potency, is difficult because PCBs occur only as complex mixtures and frequently together with other potent toxins such as dioxins and chlorinated pesticides.

PCBs have been classified as probable human carcinogens. In animal studies, PCBs exhibit reproductive, developmental, and immunotoxic effects.

The mean daily intake has been estimated as <0.1–1.9 μg person^{-1} day^{-1}. At current background exposure levels, there is no real evidence of adverse effects in humans. Of greatest concern in this context are the DL-PCB congeners (coplanar PCBs) because of their similar mode of action to dioxins. This group, consisting of 12 congeners, shows toxicological properties similar to dioxins. Furthermore, no health-based guidance values have been established for NDL-PCBs. In a 2005 evaluation by EFSA, the toxicological database was considered to be too limited to develop a guidance value for NDL-PCBs. However, DL-PCBs are included in the WHO PTMI for dioxins and dioxin-like compounds, set at 70 pg TEQ kg^{-1} bw month^{-1} (see above).

Many countries have set maximum residue limits for PCBs in dairy products, based mainly on the seven most abundant congeners. These are PCB 28, 52, 101, 138, 153, and 180 (all NDL-PCBs) and the DL-PCB 118. These congeners are considered as appropriate indicators of different PCB patterns in various matrices and represent about 50% of total NDL-PCB in food.

Analysis

PCBs are determined by GC. However, GC identification can be difficult since different commercial mixtures give different peak patterns. The traditional approach to identify and quantify these compounds was by 'peak pattern comparison' using GC with electron capture detection (ECD), and comparison to higher chlorinated technical mixtures, for example, Aroclor 1260. Because many countries have now set maximum residue limits for six or seven individual congeners, it is current practice to analyze only the seven above-mentioned 'indicator' congeners by capillary GC-ECD, with confirmation by GC-MS. This is an alternative to the costly and time-consuming analysis of the DL-PCBs (see above).

Other Persistent Halogenated Hydrocarbons

There are other halogenated hydrocarbons that persist in the environment and can be detected in milk, such as polybrominated flame retardants (e.g., polybrominated diphenylethers), toxaphene (mixture of chlorinated boranes), chlorinated paraffins, and polychlorinated naphthalenes. Analysis is difficult and in many cases not well developed, and only limited data are available. It is generally thought that these compounds are of less health concern compared to dioxins and PCBs. However, data on exposure and toxicity are scarce and efforts are under way to reduce environmental levels.

Organochlorine (OC) pesticides, which include compounds such as 1, 1, 1- trichloro- 2, 2- bis (4- chlorophenyl) ethane (DDT), hexachlorobenzen (HCB), lindane, and aldrin, are addressed in **Contaminants of Milk and Dairy Products**: Contamination Resulting from Farm and Dairy Practices.

Metals

Sources and occurrence

Metals are present in the environment either naturally or as a consequence of industrial and/or agricultural activities. They find their way to milk through several routes. Elements such as chromium and nickel from the stainless-steel dairy equipment or tin from soldered cans may enter milk through direct contact. Heavy metals such as cadmium, lead, mercury, and arsenic are not expected to have any direct contact with milk and milk products except in accidental cases. For these elements, the main pathway to milk is through the ingestion of contaminated feeds by milk-producing animals. However, in the cow, transfer of heavy metals from feed to milk is very low.

Typical analytical data for several metals in bovine milk are provided in **Table 1**. Data on buffalo and goat milk indicate that levels of heavy metals are likely to be similar to those encountered in bovine milk. With respect to milk products, contamination reflects the levels found in fresh milk, taking into account concentration factors. Metals may be associated with particular milk fractions. For example, lead and cadmium bind strongly to casein. The use of specific milk fractions may thus concentrate or remove metals.

Health significance

Of the metals to which humans are exposed, heavy metals have raised the highest safety concern since the margins between the actual overall exposures and the safe levels of exposure (PTWI, provisional tolerable weekly intake) are narrow. For adults, estimations of intake of heavy metals from milk and milk products indicate that, in general, this category of food is unlikely to contribute significantly to the overall exposure. The situation may be different for infants and children because of their higher milk consumption on a body weight basis. For this sector of the population, intake estimates suggest that milk is likely to significantly contribute to the overall exposure. The health significance of such exposures is difficult to assess since safety standards do not cover infants less than 12 weeks of age. It is important to note that for some heavy metals such as lead, bioavailability and susceptibility are likely to be higher in infants than in adults. In this context, it is advised to limit overall exposure, and in particular exposure through milk.

At the levels reported in milk, the other metals are unlikely to raise any safety issues. Some of them are thought to be essential trace elements and may be of nutritional relevance.

Table 1 Typical metal contents in bovine milk

Element	*Typical (μg kg^{-1})*	*Range (μg kg^{-1})*
Lead	<3	n.d.–20
Cadmium	<2	0.05–3
Mercury	<1	0.05–2
Arsenic	<1	n.d.–2
Chromium	<3	1–4
Nickel	<3	0.4–6

n.d., not detectable (limit of detection not specified).

Reproduced from Tahvonen R (1996) *Food Reviews International* 12: 1–70; Carl M (1991) Monograph on residues and contaminants in milk and milk products. *IDF Special Issue 9101*. Brussels, Belgium: International Dairy Federation; Blüthgen A, Burt R, and Heeschen WH (1997) Monograph on residues and contaminants in milk and milk products. *IDF Special Issue 9701*. Brussels, Belgium: International Dairy Federation.

Analysis

Methods based on atomic absorption spectrometry (AAS) are widely used to determine metals in foods. However, more sensitive multicomponent methods are available today for trace metal determination based on inductively coupled plasma (ICP) coupled to either atomic emission spectroscopy (AES) or MS. Because of the low absolute levels involved and the presence of metals in the laboratory environment, analysis may be challenging. Systematic errors and contaminations may explain some high levels reported in the older literature. It is well documented that metals such as lead, mercury, arsenic, or chromium may occur in various chemical species, which are characterized by specific toxic potentials. Presently, only little is known about the speciation of metals in milk and occurrence data are usually expressed as total metals.

Radionuclides in Dairy Products

Sources and occurrence

Humans have always been exposed to radioactivity. Radionuclides and their resulting ionizing radiation may stem from natural or anthropogenic sources. Contributions to natural radiation exposure comes from outer space, from radiation of soils and rocks as well as from inhaled and ingested natural radionuclides. The four most prominent and always present natural radionuclides in dairy products are potassium-40 (^{40}K), rubidium-87 (^{87}Rb), carbon-14 (^{14}C), and tritium (hydrogen-3, i.e., ^{3}H) (**Table 2**).

Radiocontamination in the environment and consequently in dairy products may also result from anthropogenic activities. Radionuclides from 'fallout', that is, from nuclear weapons testing and accidents in nuclear power plants, are by far the most predominant contributors to this environmental anthropogenic radiation. Anthropogenic radionuclides may find their way into dairy animals and milk either by inhalation during actual fallout, or after deposition directly through plants and later through the soil–roots–grass–animal–milk pathway. The most significant anthropogenic

Table 2 Commonly found radionuclides in milk

Radionuclide	*Half-life ($t_{1/2}$)*	*Major type of irradiation*	*Concentration in milk (Bq l^{-1})*	*Predominant origin*
Rubidium-87	49×10^9 years	β	0.5–3.5	Natural
Potassium-40	1.3×10^9 years	β, γ	40–60	Natural
Carbon-14	5730 years	β	14–16	Natural
Cesium-137	30.2 years	β, γ	<0.1[a]	Anthropogenic
Strontium-90	28.6 years	β	<0.04	Anthropogenic
Tritium	12.4 years	β	<6.3	Natural
Cesium-134	754.2 days	β, γ	<0.1[a]	Anthropogenic
Strontium-89	50.5 days	β	[b]	Anthropogenic
Iodine-131	8.1 days	β, γ	[b]	Anthropogenic

[a] Values for total cesium.

[b] Data not known.

Adapted from Paakkola O and Wiechen A (1990) Radionuclides in dairy products. *IDF Bulletin 247*, 3–15; Radioactivity in Food and the Environment (RIFE5) (1999) UK Food Standards Agency and Scottish Environment Protection Agency.

radiocontaminants in dairy products are strontium-89 (^{89}Sr) and strontium-90 (^{90}Sr), iodine-131 (^{131}I), as well as cesium-134 (^{134}Cs) and cesium-137 (^{137}Cs) (**Table 2**).

While all radionuclides will be dispersed after atmospheric nuclear explosions, in reactor accidents it is predominantly the volatile radionuclides, in particular iodine and cesium, that are liberated. This is also reflected in the dairy contamination pattern. After the Chernobyl accident in 1986, the ratio of contamination of ^{137}Cs to ^{90}Sr in milk was approximately 50:1, whereas during the period of heavy nuclear weapon testing and subsequent fallouts in the 1960s, the ratio was only about 10:1.

The strontium isotopes were of concern only after the 1960s due to nuclear weapons testings, and therefore both radioisotopes were ubiquitously detected in milk. Restrictions on nuclear testing have resulted in a significant drop in the amount of strontium isotopes, particularly ^{89}Sr, attributable also to its shorter half-life, and today the overall environmental radiostrontium activity in Western Europe is only slightly elevated compared to activities measured before the nuclear weapons testing era.

The fallout of volatile ^{131}I, which is characterized by a short half-life of 8.1 days, was very high after the Chernobyl accident. Notably, nuclear weapons testing had only little impact on ^{131}I activity, due to the fact that the radioisotope was lifted sufficiently high in the troposphere allowing ample time for decay before deposition. Therefore, levels were 100–1000 times lower as compared to the immediate post-Chernobyl period.

Radioactive disintegration can be in the form of particle emission (α- and β-radiation) as well as in the form of energy-rich photon emission (γ-radiation). The energy of the emitted α-, β-, and γ-radiation is characteristic of each specific radionuclide. The activity of any radionuclide is measured in disintegrations per second in units of becquerel (1 s^{-1}; Bq). Exposure to radiation, also referred to as the radiation dose, is measured in sievert (Sv). It is important in the estimation of human health effect of radiation dose, and is standardized for the sensitivity of various body tissues and the type of radiation.

Milk contributes considerably to the radioactive dose from foodstuffs. Mean radioactivity concentrations in milk in the United Kingdom in 1999 were reported to be <0.038 Bq l^{-1} for ^{90}Sr, <0.066 Bq l^{-1} for total cesium, <6.3 Bq l^{-1} for ^{3}H, and 16 Bq l^{-1} for ^{14}C.

The levels of radioactivity are considerably reduced through the food chain compared to direct intake from plant feed. Differential metabolic patterns in species of dairy animals introduce additional variation in the level of radioactivity in milk. Comparative biological trials were conducted to investigate the differential secretion into milk of radionuclides incorporated via feed. Highest ^{131}I contamination was found in sheep's milk, followed by goat, cow, and buffalo milk, which contained 21, 11, and 3%, respectively, of the radioactivity found in sheep milk. A similar trend – although less pronounced – could be observed for radiocesium. Similarly, while approximately 0.1% of the amount of ^{90}Sr given to cows daily is secreted per liter of milk, transfer to goat's milk may be more than 10 times greater.

Radionuclides partition differentially in aqueous and fatty milk fractions. These partition properties can be exploited to reduce radiocontamination of dairy products, especially in times of high contamination. Radiocesium, for example, predominantly concentrates in the aqueous fraction during physical separation procedures. In consequence, high-fat fractions, such as butter, cream, and high-fat cheeses, and high-protein fractions, such as cottage cheese curds, caseinates, and whey protein concentrates, will have comparatively low levels of radiocesium.

Health impact

Adverse health effects of radiocontaminants originate from the emission of ionizing radiation, which is a known carcinogen. An estimated 95% of human exposure from man-made sources originates from medicinal applications, such as X-rays. Exposure to natural sources of radiation contributes more than 98% of the radiation dose to the general population (excluding medical exposure). The global average human exposure from natural sources is on the order of 3 mSv yr^{-1}. In consequence, there is only a very small contribution from nuclear power generation and nuclear weapons testing to the overall human radiation exposure.

The relevance of specific radionuclides to milk and consequently to human health depends on various factors. The physical half-life (**Table 2**) is the chief characteristic in the relative significance of radioactive contaminants in foodstuffs. Whereas ^{131}I has a short half-life of 8.1 days, ^{137}Cs, for example, has a half-life of 30.2 years and, once incorporated, a much longer-lived effect in the body.

The distinct metabolism of specific radionuclides is another important factor that determines the relative health significance of radiocontaminants. Iodine-131 acts like stable iodine and is accumulated quickly in the thyroid, where it causes local effects, while cesium isotopes that behave like potassium in the body are consequently widely distributed in soft tissues. Radiostrontium acts like calcium and therefore accumulates in bone tissue.

The International Commission on Radiological Protection (ICRP) has set a maximum allowable annual dose above that from natural and medical sources at 5 mSv. The contribution of milk to the total radiation exposure is fairly small, being 0.02–0.3 mSv in the United Kingdom after the Chernobyl accident depending on the degree of deposition.

In conclusion, the amounts of hazardous radionuclides in dairy products are low today and do not present immediate health hazards.

Analysis

Standard methods have been published for determining gross α- and β-activity concentrations in food. However, this is useful only when quick results need to be obtained, for example, during catastrophe situations. Often, the intensity of naturally present radioactivity in milk is considerably higher than radioactivity resulting from fallout.

Radioactivity determination of specific radionuclides is therefore generally preferred. The radioactivity concentration of γ-emitting radionuclides can be determined using spectrometric analysis of γ-irradiation, generally without sample destruction.

Determination of the activity of β-radiation is comparatively more complicated, requires sample preparation, and is generally done indirectly by β-scintillation spectrometry.

Mycotoxins in Dairy Products

Mycotoxins are secondary metabolites of fungi, some of which possess high potential to cause adverse effects in animals and man.

Sources and Occurrence

Dairy contamination by mycotoxins can be via two different routes: indirect contamination and direct contamination.

Indirect contamination stems from fungus-infected feedstuffs consumed by dairy animals. Of highest importance and significance in this respect is aflatoxin M_1 (AFM_1), the major metabolite of aflatoxin B_1 (AFB_1). In fact, approximately 3–5% of AFB_1 initially present in the animal feedstuff appears as AFM_1 in milk. Its acute and chronic toxicity, including hepatocarcinogenicity in several species, is similar to that of AFB_1 (*see* **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds).

Carryover into milk of other mycotoxins (e.g., ochratoxin A, sterigmatocystin, deoxynivalenol, T-2 toxin, and zearalenone) has been reported. Their transmission rate, however, is significantly lower than that of aflatoxin and therefore does not cause immediate health concerns.

The cause of direct contamination is the colonization of dairy products, in particular of cheese, by mycotoxigenic fungi. Dairy products are susceptible to contamination by molds and once infested, mycotoxins can be formed. Their formation, apart from inadvertent fungal growth, may also be attributed to fungal starter cultures used for the production of specific dairy products.

Penicillium camemberti and *P. roqueforti* starter cultures are used in the production of white surface mold and blue-veined cheeses, respectively. While *P. camemberti* is a consistent producer of cyclopiazonic acid, *P. roqueforti* strains produce patulin, penicillic acid, isofumigaclavine A, mycophenolic acid, and roquefortine *in vitro*. Only the last three toxins produced by *P. roqueforti* could be found in commercial blue cheeses, sometimes at concentrations in the low ppm range. Cyclopiazonic acid originating from *P. camemberti* starter cultures could be detected in white surface mold cheese at similar concentrations.

Several mycotoxins result from unintentional fungal non-starter culture infestation of dairy products. AFB_1 can be formed in milk or processed dairy products infected with *Aspergillus* strains, although generally at lower levels than AFM_1. Sterigmatocystin originates from *Aspergillus versicolor* and was detected upon unintentional fungal infestation in the low ppm range, mainly in hard cheeses. It is structurally similar to AFB_1 and is believed to be a precursor in the biosynthesis of AFB_1. The toxic properties of sterigmatocystin are much the same as aflatoxin, although much less pronounced. The nephrotoxic and carcinogenic ochratoxin A has also been detected in cheese.

A large selection of dairy mycotoxins and causative mycotoxigenic molds is listed in **Table 3**, together with their typical sources relevant to human exposure.

Isolated incidences of further fungal toxins in several dairy products at low levels have been reported for penitrem A, citrinin, citreoviridin, β-nitropropionic acid, and PR toxin. Although *Fusarium* toxins, fumonisins B_1 and B_2, have been detected in dairy products, mycotoxins produced by species other than *Aspergillus* and *Penicillium* are of minor concern.

Health impact

The aforementioned toxins occur in milk and processed dairy products generally at low concentrations.

Indirect contamination of milk and dairy products with mycotoxins secreted by dairy animals is generally negligible and milk is not a major source of human exposure to these substances. The only exception is represented by AFM_1, which may be secreted into and occurring in milk in significant amounts, and which is relatively stable in milk and processed milk products such as cheese and yogurt. Like AFB_1, AFM_1 is of considerable health concern. Although the presence of moderately toxic metabolites, particularly of *Penicillium* species in cheese, cannot be excluded completely, it is unlikely that consumers are endangered by the consumption of milk and mold-ripened cheeses.

Milk and dairy products may be intermittently infested with mycotoxigenic fungi producing toxins such as AFB_1 or ochratoxin A. In such occasional cases, high levels of mycotoxins may be anticipated, which, owing to their high toxic potency, would represent a significant health concern. Such issues are difficult to identify and have to be dealt with through the strict application of good manufacturing practices.

Comparatively little knowledge exists on potentially harmful secondary metabolites of starter cultures. Only limited data are available on the adverse effects of a number of substances occasionally produced by certain strains used in the manufacture of dairy products. Therefore, it is relatively difficult to assess the safety of human exposure to such compounds.

By complying with good manufacturing practices, toxin-producing fungal infestation can be avoided or greatly reduced. Selection of starter strains with restricted capabilities for mycotoxin production can further reduce human exposure.

In conclusion, with the exception of AFM_1, transfer of mycotoxins from animal feed to milk is negligible. Direct contamination of dairy products with mycotoxin due to fungal infestation can occur sporadically at high levels.

By and large, dairy products do not contribute substantially to the overall mycotoxin intake in man and no overt concern exists in relation to human health.

Analysis

Various analytical methods have been developed for the identification and quantification of fungal toxins in milk and dairy products, ranging from simple techniques such as thin-layer chromatography (TLC) and immunochemical assays to confirmatory methods employing GC and high-performance liquid chromatography (HPLC), coupled to various detectors. For AFM_1, these methods must achieve very low levels of detection and quantitation in the sub-ppm range, which can be accomplished by HPLC with fluorescence detection, preceded by a sample cleanup step using an immunoaffinity

Table 3 Mycotoxins relevant to dairy products

Mycotoxin	*Main sources*	*Main sources of human exposure*
Aflatoxin B_1	*Aspergillus flavus*, *A. parasiticus*, *A. nomius*	Corn, peanuts, nuts, spices, wheat, oats, barley, rice, cheeses, etc.
Aflatoxin M_1	Dairy animal metabolism of aflatoxin B_1	Milk and dairy products
Cyclopiazonic acid	*Penicillium camemberti*, *P. commune*, *Penicillium* spp., *Aspergillus* spp.	Grain, legumes, meat, milk, cheese (Brie, Camembert), etc.
Fumonisin B_1	*Fusarium moniliforme*, *F. proliferatum*, *Fusarium* spp.	Corn and other cereal grains
Isofumigaclavine A	*Penicillium roqueforti*, *Penicillium* spp.	Blue cheeses
Mycophenolic acid	*Penicillium roqueforti*, *Penicillium* spp.	Blue cheeses, mainly Roquefort
Ochratoxin A	*Penicillium verrucosum*, *Penicillium*. spp., *Aspergillus ochraceus*, *Aspergillus* spp.	Corn, wheat, barley, beer, wine, oats, sorghum, coffee, etc.
Patulin	*Penicillium* spp., *Aspergillus* spp.	Apples, etc.
Penicillic acid	*Penicillium* spp., *Aspergillus* spp.	Cheese, etc.
PR toxin	*Penicillium roqueforti*	Blue cheeses
Roquefortine	*Penicillium roqueforti*, *Penicillium* spp.	Roquefort, blue cheeses
Sterigmatocystin	*Aspergillus versicolor*, *Aspergillus* spp., *Emericella* spp.	Cereals, cheese (Edam, Gouda), coffee, nuts, etc.

Adapted from Weidenbörner M (2001) *Encyclopedia of food mycotoxins*. Berlin, Germany: Springer; van Egmond HP (1989) *Mycotoxins in dairy products*. London: Elsevier.

column. Commercial rapid test methods are available for AFM_1, based, for example, on radioimmunoassay or solution fluorometry.

Inherent Plant Toxicants – Bracken Fern Toxin

Sources and occurrence

The ubiquitous bracken fern (*Pteridium* spp.) is one of the most abundant plants on Earth. Bracken fern, due to its several inherent toxins, is well known to cause a variety of diseases in livestock that ingest large amounts of bracken or are fed bracken-containing fodder. Bracken fern is used as an example to illustrate inherent plant toxicants that may be excreted into milk.

The major toxicant found to be responsible for the detrimental effects of bracken fern has been identified as the norsesquiterpene glucoside ptaquiloside (PT). The PT content of bracken fern is subject to significant regional and seasonal variations and can occasionally be extraordinarily high (up to $13\,\mathrm{g\,kg^{-1}}$ dry weight). It is well established that PT is excreted into milk in a dose-dependent manner. Excretion rates between 1 and 11% of ingested PT have been reported, corresponding to approximately 0.1–$22\,\mathrm{mg\,l^{-1}}$ in milk, respectively.

Health impact

Diseases reported in livestock upon ingestion of large amounts of bracken fern include thiamine deficiency in non-ruminants, acute hemorrhage, blindness due to retinal degradation, as well as bladder and intestinal cancer.

There is epidemiological evidence of elevated cancer incidence in humans who consume bracken crosiers (young non-unfolded fronds) and also in people who consume dairy products from animals exposed to bracken fern through feed. Furthermore, the milk of bracken-fed cattle has been demonstrated to be carcinogenic in rats.

The level of human exposure is difficult to quantify and probably inconsistent due to the seasonal variation of the PT content in bracken fern and sporadic ingestion of fern by dairy animals, which do not normally feed on bracken fern. However, during periods of drought when the ferns remain green and on overgrazed or heavily infested pastures, they may be forced to do so. Therefore, the principal strategy to minimize human risk appears to be careful management of dairy animal pastures, that is, strict adherence to good agricultural practices.

See also: **Contaminants of Milk and Dairy Products**: Contamination Resulting from Farm and Dairy Practices. **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds.

Further Reading

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Nitrates and Nitrites as Contaminants

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Occurrence of Nitrogen and Its Oxides

Nitrogen (atomic number 7) exists predominantly as isotope ^{14}N and makes up a substantial part of the known universe. On Earth, nitrogen occurs mainly as inert gaseous N_2, making 78% of the atmosphere and as such is not available for organic life unless converted to oxide form. This was achieved primordially by electrical storms and in the current biosphere by nitrogen-fixing bacteria. Thus, nitrogen exists commonly in the stable +5 oxidation state as NO_3^- as part of the well-studied 'nitrogen cycle', one of the most significant nutrient cycles in the terrestrial ecosystem (**Figure 1**).

Nitrate (NO_3^-) exists commonly throughout the geological world in sediments or dissolved in the oceans and waterways. Significant nitrate reserves are stored in soil, of which the majority is bound in dead biomass and is largely unavailable to plants. The remaining nitrogen pool is available for plant growth in the form of ammonium, both free and clay-bound. Within the soil environment, nitrogen is available for use by many life-forms for incorporation into nucleic acids, amino acids, and other essential compounds, and as such, about 16% of living organisms is nitrogen. Other inorganic compounds are also produced chemically and biochemically, containing nitrogen in a wide variety of oxidation states from −3 to +5, both gaseous and ionic (**Table 1**).

Nitrate is a requirement of modern industry and is made in significant quantities via ammonia by the Haber and Ostwald processes. This was instigated by its historic importance in gunpowder and explosives. More recently, the higher oxides are used as propellants in rockets. Nitrate and ammonium salts are most commonly used in modern agriculture as fertilizers to replenish soil nitrogen for pasture and crops and thus nitrogen salts enter the environment where the potential for environmental pollution is high. It should be noted that atmospheric sources of aquatic nitrate have also become an increasing concern in view of anthropogenic-induced emissions of nitrogen compounds.

The widespread use of nitrogen-based fertilizers, combined with domestic, agricultural, and industrial wastes, has indeed resulted in increases in the nitrate content of surface ground-water globally, since these nitrogen inputs have frequently exceeded the capacity of the biosphere. The extent of such nitrate leaching is dependent on the type of land use, and increases in the order forestry, agriculture, and livestock. In general, nitrate is largely unbound within the soil environment and has a much higher potential to leach compared to ammonium. The principal concern related to nitrate loss is its capacity to encourage excessive plant and algal growth in waterways and thereby interfere with air supplies to other flora and fauna. Although considered stable and not inherently toxic, nitrate is reduced to the reactive nitrite ion, NO_2^-. While agricultural use of nitrate is dominant in terms of volumes, nitrite in the form of its sodium salt is used as a food preservative, most commonly in cured meats, with nitrate salts also included in processed foods to provide a nitrite reservoir.

Physiological Role

While dietary (exogenous) intakes of nitrate and nitrite are commonly and justifiably regarded with concern, it must be noted that endogenous tissue generation of these ions is an alternative and normal consequence of the critical physiological functions of the highly transient nitric oxide. In humans, nitric oxide is endogenously produced from a range of sources, the most notable of which is L-arginine, under the action of a tissue-specific synthase system. Nitric oxide is involved in multiple metabolic functions, including vascular regulation, platelet aggregation, neurotransmission, inflammation, and immunity. Given its physiological significance, plasma nitrite levels are now considered a reliable diagnostic indicator of nitric oxide status in a range of human pathologies. Only recently is evidence available to suggest that, apart from the endogenous L-arginine route, dietary nitrate and nitrite may account for approximately half of systemic nitric oxide, a pathway that is dependent on endogenous mammalian nitrate reductases to provide the nitrite substrate required for nitric oxide production via several potential reductive mechanisms (**Figure 2**).

In fact, it is also becoming recognized that exogenous nitrite may have physiological properties that are independent of its role as a precursor of nitric oxide. It is likely that recent developments related to the *in vivo* conversion of dietary nitrate and nitrite to systemic nitric oxide will have novel implications beyond the traditional consensus that regards these nitrogen species solely as contaminants.

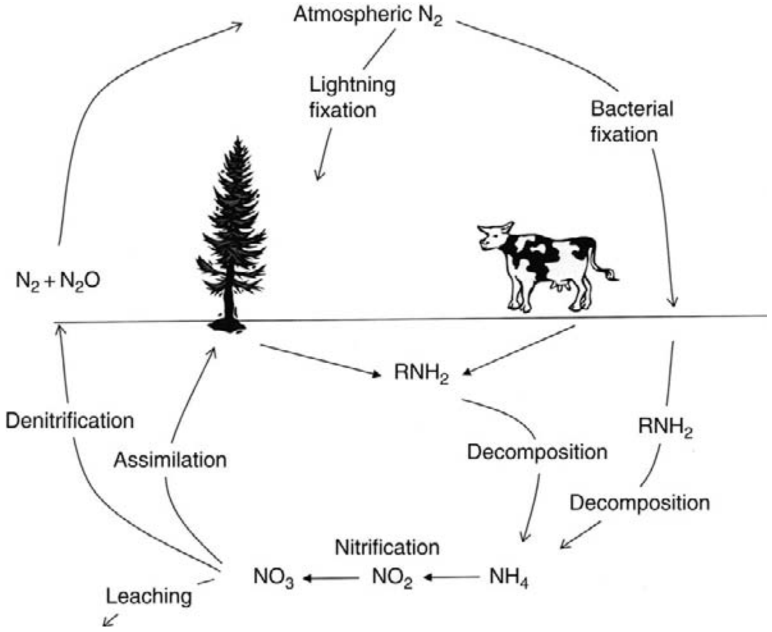
Atmospheric N_2
Lightning fixation
Bacterial fixation
$N_2 + N_2O$
RNH_2
Denitrification
Assimilation
RNH_2
Decomposition
Decomposition
Nitrification
NO_3
NO_2
NH_4
Leaching

Figure 1 Schematic illustration of the natural nitrogen cycle.

Table 1 Nitrogen oxidation states and representative compounds

Oxidation state	*Species*	*Name*
−3	NH_3, NH_4^+	Ammonia, ammonium ion
−2	N_2H_4	Hydrazine
−1	NH_2OH	Hydroxylamine
0	N_2	Nitrogen
+1	N_2O	Nitrous oxide
+2	NO	Nitric oxide
+3	HNO_2, NO_2^-	Nitrous acid, nitrite ion
+4	NO_2	Nitrogen dioxide
+5	HNO_3, NO_3^-	Nitric acid, nitrate ion

While dietary nitrates and nitrites have long been associated with increased risk of adverse health effects, recent clinical studies have indicated that exogenous nitrate and nitrite may increasingly be considered as part of normal nitrogen consumption, rather than a toxin to be avoided at all costs. Evidence that suggests that health benefits may be derived from consumption of dietary nitrate and nitrite, through maintenance of a sufficient pool of systemic nitrogen oxides for tissue defense and nitric oxide homeostasis during disease and stress, is accumulating. In addition, there have been recommendations to Codex that the acceptable daily intake (ADI) for nitrite is too low. This

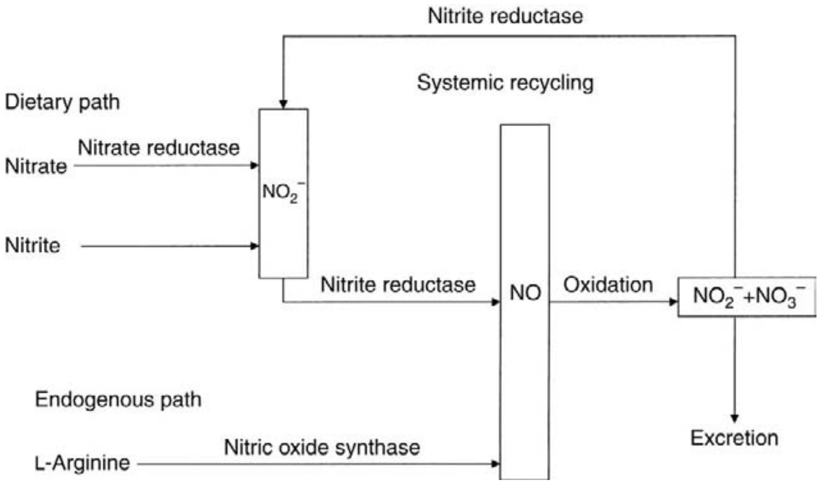
Nitrite reductase
Systemic recycling
Dietary path
Nitrate reductase
Nitrate
NO_2^-
Nitrite
Nitrite reductase
NO
Oxidation
$NO_2^- + NO_3^-$
Excretion
Endogenous path
Nitric oxide synthase
L-Arginine

Figure 2 Schematic of the sources and physiological cycling of NO, NO_2^-, and NO_3^-.

means that the significance of nitrate and nitrite in dairy products with respect to toxicological implications may diminish even further.

Toxicity of Nitrite and Nitrosamines

In view of poisoning events with humans and animals, particularly from contaminated drinking water, toxicological studies on nitrate and nitrite have been quite extensive. The current World Health Organization guideline for nitrate in drinking water is $50\,\mathrm{mg\,l^{-1}}$ and the provisional guideline for nitrite is $0.2\,\mathrm{mg\,l^{-1}}$ for long-term exposure, although individual water samples can contain up to $3\,\mathrm{mg\,l^{-1}}$ of nitrite. These drinking water levels are rarely exceeded in Australia, New Zealand, and other developed countries. However, concentrations well above these levels have been reported in countries noted for severe environmental pollution.

While nitrate itself is not reported as a significantly toxic compound, the principal toxicological problem related to dietary intake is with nitrite. This is largely attributed to oxidation of the ferrous ion in hemoglobin in a disease state known as methemoglobinemia. The oxygen-carrying capacity is thus reduced to a point that it can cause cyanosis (blue coloration) and minor or serious hypoxia (asphyxiation). Nitrite-forming bacteria in healthy adult humans are limited by the low pH of the stomach, so NO_3–NO_2 conversion *in vivo* is minimal. However, this is not the case with both the aged and neonates, who, in general, are characterized by reduced stomach acidity. Such individuals can also have inefficient enzyme systems to reverse the hemoglobin–methemoglobin conversion, notably diaphorase I (NADH methemoglobin reductase). Genetic disorders can also make certain individuals susceptible to clinical or subclinical methemoglobinemia through shortage of the enzyme or coenzyme.

A further commonly reported health issue related to dietary nitrite is its propensity to form nitrosamines in the acidic environment of the stomach. Nitrite forms nitrous acid with any mineral acid such as stomach HCl ($NO_2^- + H^+ \rightarrow HNO_2$). While unstable to dissociation ($2HNO_2 \rightarrow NO_2 + NO + H_2O$), it will also form the nitrosonium cation ($HNO_2 + H^+ \rightarrow H_2NO_2^+ \rightarrow H_2O + NO^+$). This strongly nucleophilic agent ($N{\equiv}O^+$) will react with secondary amines to form a potentially wide array of carcinogenic nitrosamines. The suspected pathway for carcinogenesis involves alkylation of DNA, primarily via formation of methylguanine, resulting in GC to AT point mutation. Modern analytical techniques have identified over 300 nitrosamines, most of which have been shown to have organ-specific carcinogenicity in animals. Epidemiological evidence in human populations remains inconclusive, but toxicity is likely to be similar to animals.

While the most frequent exogenous nitrosamines are associated with the use of tobacco and occupational exposure in chemical factories, dietary sources are also well established. Since the formation of nitrosamines is associated with cooking regimens, fried and smoked foods can contain significant concentrations, particularly when cured with sodium nitrite to reduce the potential for botulism poisoning. Fish contain high levels of amines, making them the first identified food type containing nitrosamine in the form of *N*-nitrosodimethylamine. All foods pickled in nitrate/nitrite (bacon, ham, etc.) will also yield high levels of nitrosamines, particularly dimethylnitrosamine, diisobutylnitrosamine, and nitrosopyrrolidine. Ascorbic acid (vitamin C) is known to limit nitrosamine formation, so it is added to modern meat-curing solutions. In the past, a significant source of dietary nitrosamines was barley used in beer production, although this problem has been largely remedied.

Avoiding dietary levels of nitrite is clearly a way of minimizing the risk of gastric and liver cancers associated with nitrosamines, and US and EU regulations are currently implemented to control the use of sodium (and potassium) nitrate and nitrites. This has helped reduce the ingestion of nitrosamines significantly in the past few decades from levels of approximately $1\,\mu\mathrm{g\,day^{-1}}$ to less than $0.1\,\mu\mathrm{g\,day^{-1}}$.

Daily Intake

The ADI for adults is 3.7 mg of nitrate per kg body weight and 0.07 mg of nitrite per kg body weight. Many official studies have been undertaken to trace the sources of nitrate and nitrite as part of regulatory decision making within Europe, the United States, Australia, and New Zealand. These generally report that selected vegetables, such as spinach and lettuce, can contain over $5000\,\mathrm{mg\,kg^{-1}}$ of nitrate, thus making them the principal contributor to the human diet. As importantly, nitrate and nitrite are used as food additives, particularly as microbial inhibitors and to impart flavor and color in processed meats. Thus, Western diets involving bacon and other cured meat products are responsible for nitrite regularly exceeding the ADI. The opportunity for large-scale poisoning is reduced by strict control of nitrate and nitrite salts used during food manufacturing.

Quite clearly, the impact of dietary nitrate and nitrite depends on three factors: (1) their concentration in food products; (2) the amount of such food in the diet; and (3) the body weight of the consumer. British studies in 1994 and 1997 concluded that the total intake of dietary nitrate from all sources can exceed $140\,\mathrm{mg\,day^{-1}}$, with vegetables such as spinach and lettuce contributing approximately 80% of the average daily dietary intake. This level is well short of the permissible ADI of $220\,\mathrm{mg\,day^{-1}}$ for a 60 kg

European adult. These studies also confirmed that dairy products are a measurable but not overwhelming source of these compounds.

In Asian countries, the average body weight of the population is considerably less (55 kg) and they consume larger portions of high-nitrate vegetables. The absence of widespread toxicity symptoms also indicates that nitrate is not a serious health risk to the general population. Individuals can, however, be at risk depending on unique circumstances, but these are not evident in epidemiological studies. In contrast, infants are high on the list of potential victims of nitrate/nitrite toxicity. While babies raised on mother's milk will not experience such problems, the reconstitution of milk powder or infant formula with contaminated water is a recognized causative factor of clinical manifestations, notably methemoglobinemia, leading to possible morbidity and mortality.

Sources in Dairy Products

As early as *c.* 7000 BC, man learned to domesticate selected animals, including the cow, buffalo, sheep, goat, and camel, for the provision of milk for human consumption, with milk from the dairy cow dominant by volume. The current availability of dairy products in the modern world combines the traditional with the application of modern science and technology. Total regional dairy consumption per person varies widely, although through globalization and migration, these trends are continuously evolving. Whereas the majority of nitrate and nitrite in the human diet is derived from potable water, vegetables, and cured meats, commodity dairy products, as staple food ingredients, are traded internationally. They are, therefore, well monitored for the presence of all contaminants, including nitrate and nitrite. The regular use of milk and processed dairy ingredients in infant nutriture is a major reason to minimize such contamination, particularly that of nitrite.

Within the dairy industry, cows ingest nitrate and nitrite during grazing and drinking, and milk can be contaminated by means of either secretory or post-secretory processes. It is, however, well established that the level of nitrate in the diet of dairy cows has little effect on secretory milk composition and does not lead to significant accumulation in the milk. Indeed, it has been estimated that the expression of nitrate in bovine milk is on the order of 10^{-3} of an orally administered dose. Thus, raw milk typically contains $1-5\ \mathrm{mg\,l^{-1}}$ of nitrate and $<0.1\ \mathrm{mg\,l^{-1}}$ of nitrite, giving rise to about 10 and $1\ \mathrm{mg\,kg^{-1}}$, respectively, in uncontaminated milk powders.

Post-secretory contamination with nitrate is, however, possible during milk collection and processing, either from intentional addition to bulk milk destined for cheese or from unintentional residues from plant-cleaning processes. Nitric acid has been used to sanitize dairy factory equipment, so inadvertent incorporation of NO_3^- is possible. The other significant source of contamination is incoming wash-water, and hence modern factories focus on purification by deionization. Furthermore, nitrate contamination of dried milk products is significantly more likely in directly heated spray dryers as compared to indirect steam-heated systems, as a consequence of fuel gas combustion products responsible for the formation of nitrogen oxides. The use of such technology to save energy costs has, therefore, been discouraged because of such contamination and also the presence of other combustion products, including polycyclic aromatic hydrocarbons.

With the reported nitrate content of only $3–27\ \mathrm{mg\,kg^{-1}}$ in typical dairy products, the amount of nitrate ingested from this food source is not likely to cause significant problems. In the case of nitrite, which is reported to typically range from 0 to $0.6\ \mathrm{mg\,kg^{-1}}$ in dairy products, the contribution is even less significant to the ADI of 4.2 mg for the average consumer. For most dairy products, nitrate and nitrite are restricted from use as intentional additives, so the majority of intake is from innate sources within milk itself and from the dairy production environment. Certain cheeses have nitrate added in small quantities (20–200 mg per kg bulk milk) to restrict 'late blowing' and defects associated with gas formation, but this practice is being reviewed within the European Union, which has, in the past, allowed a maximum of 50 mg NO_3^- per kg of finished cheese. However, nitrate in fresh cheese is very unstable and is rapidly reduced to nitrite by milk xanthine oxidase and various microbial nitrate reductases during cheese maturation. Nitrite so formed is itself rapidly reduced to other compounds such that its content in cheese is typically very low.

Ranges of nitrate, nitrite, and nitrosamines in dairy products collated from the reported literature are summarized in **Table 2**. It should be noted that reported levels in the surprisingly limited data available are of variable quality and consistency, perhaps reflecting the range of analytical methodologies utilized in the last few decades.

Analysis

The potential health and regulatory implications associated with consumption of nitrates and nitrites in the human diet necessitate the availability of accurate analytical methods. Current methods for the determination of nitrite and nitrate in milk and milk products predominantly rely on a wide range of manual, segmented flow or flow injection analysis variants of the traditional colorimetric Griess diazotization procedure. The kinetics, mechanism, and critical analytical variables have been studied comprehensively,

Table 2 Range of nitrate, nitrite, and nitrosamines in dairy products not known to be contaminated

	NO_3^-	NO_2^-	NOS
	(mg kg^{-1})	(mg kg^{-1})	(μg kg^{-1})
Raw milk	0.05–5.0	0.2–1.0	<0.1
Milk	tr–5.3	0–0.04	0.1–1.9
Pasteurized milk	<0.03–4.6	<0.02–0.15	
UHT milk	<0.03–1.7	<0.02–0.02	
Sour milk	<0.05–3.0	<0.04	
Condensed milk	0.13–1.00	<0.04–1.00	
Cream	<0.13–20.33	<0.01–0.05	
Yogurt	nd–13.77	<0.01–1.5	nd–0.01
Whey powder	1.9–50		
Milk powder	1–102	nd–8.6	nd–1
Buttermilk powder	30–35		
Cottage cheese	0.32–23.95	<0.10–15	
Soft cheese	<0.13–5.45	<0.10–0.49	0.01–0.5
Cheese	<0.13–133	nd–10	nd–2.7

NOS, nitrosamines; UHT, ultra-high temperature; tr, trace; nd, not detected.

and the Griess reaction is commonly regarded as specific for nitrite. The most frequently described assays utilize sulfanilamide and *N*-(1-naphthyl)ethylenediamine (NED) to form the azo chromophore, although related reagents are reported. These non-separative methods require nitrate to be determined by difference following its prior conversion to nitrite with cadmium metal. However, in view of the toxicity of cadmium, alternative reduction strategies utilizing vanadium, zinc, hydrazine, enzymatic (e.g., nitrate reductase from *Aspergillus niger*) or photolysis techniques have also been advocated. Griess-based methods are susceptible to numerous redox and other interferences potentially present in biological matrices such as milk, but nevertheless they form the basis for the many national and international regulatory reference standards for dairy products published by US and European organizations, including AOAC International, International Dairy Federation (IDF), European Committee for Standardization (CEN), Food Standards Agency (FSA), International Organization for Standardization (ISO).

In the case of commodity dairy products, the limitations of such assays with respect to potential interferences are generally minor in view of relatively high regulatory limits for both contaminants. However, and especially for nitrite, the implications of analytical uncertainty are more serious for highly regulated infant formulae, where acceptable threshold levels are very low (<0.5 mg kg^{-1}). In addition, protein hydrolysates and other highly manipulated milk-based foods challenge such assays due to the presence of significant levels of potentially interfering compounds.

Recently developed methods have been reported predominantly for environmental water monitoring and clinical applications. These include alternative spectrophotometric assays, UV detection, fluorimetry, electrochemical methods, detection based on ion-selective electrodes, chemiluminescence methods, and also separative methods involving gas chromatography, gas chromatography–mass spectrometry, liquid chromatography (LC), ion-exchange chromatography, and capillary electrophoresis, most of which have been reviewed comprehensively. In certain instances, these analytical strategies have also found application for specific foods, most commonly including processed meats, vegetables, fruits, and drinking water. However, application of these methods to milk and dairy products has been relatively limited due, in part, to the significantly lower contents of nitrate and nitrite compared to other dietary sources.

Quantitation of nitrite and nitrate by LC, based on ion-exchange or reversed-phase ion-interaction separation mechanisms, has been applied to several food matrices. With detection utilizing either the inherent low-wavelength UV absorption of these anions or conductivity, the techniques fail for the analysis of milk-based foods. UV detection is compromised due to low absorptivity and spectral interferences from closely eluting endogenous organic acids, while high levels of chloride significantly interfere with nitrite during conductivity detection. Alternative LC detection systems reported for biological samples include indirect UV, fluorimetry, electrochemical, chemiluminescence, and mass spectrometry as reviewed recently, although such strategies have not yet become established for the analysis of dairy products.

Two high-performance ion-exchange methods utilizing direct UV detection for reportedly successful application to dairy products include a collaborative study restricted to cheese and a procedure applied to yogurt and cheese. More recently, high-performance ion-exchange methods incorporating on-line post-column reduction with either cadmium or vanadium, coupled to derivatization with Griess reagent and detection at 540 nm, have been reported for dairy products and baby foods. The chromatographic separation of nitrate and nitrite, combined with specific post-column conversion to the chromophoric azo derivative, avoids the potential matrix interference limitations of conventional assays and the inherent disadvantages of other reported chromatographic detection modes.

Conclusion

It is well established that dairy products are relatively safe for consumers. Such products do not inherently incorporate high nitrate and nitrite concentrations and are monitored regularly by both the exporting and importing countries. They are also checked periodically for nitrosamine content. Analytical methods are available for fast detection of these anions at manufacturing plants, so the opportunity for inadvertent contamination is minimal. Dairy products are thus suitable as significant components of the diet and as ingredients for the production of infant formula with minimal risk of methemoglobinemia and other nitrate/nitrite-related maladies.

See also: **Additives in Dairy Foods**: Legislation. **Analytical Methods**: Chromatographic Methods. **Cheese**: Avoidance of Gas Blowing. **Dehydrated Dairy Products**: Infant Formulae. **Plant and Equipment:** In-Place Cleaning. **Utilities and Effluent Treatment**: Reducing the Negative Impact of the Dairy Industry on the Environment.

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CREAM

Contents
Manufacture
Products

Manufacture

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Introduction

Worldwide, about 550 million tonnes of cow's milk is produced per annum. Under the assumption that the average fat content is 4%, this yearly milk production generates about 22 million tonnes of milk fat. Milk fat is a valuable source of energy, and largely contributes to the taste and texture of many milk products and other foods. The properties of milk fat are commonly recognized as enhancing acceptability and turnover of these products. The word 'cream' has for a long time been associated with a premium product as milk fat develops a unique flavor and as its properties make it also a preferred ingredient in many other foods. However, there is no uniform definition of 'cream'. Generally, cream is considered the fluid milk product comparatively rich in fat, in the form of an emulsion of fat-in-skimmed milk, obtained by physical separation of raw milk. The special 'creaminess' results from the fine dispersion of the fat globules protected by a particular membrane against deemulsification. Although cream is regarded as a typical oil-in-water emulsion, a simple process such as whipping can change this physical state into a fat-stabilized stiff foam or into a completely coalesced, separated fat phase. Creams may differ in fat content, but also in the degree of fat dispersion, which is strongly affected by homogenization during processing. Both characteristics determine the functional, rheological, and sensory properties of the resulting product. Cream and cream products are sold in many forms, and traditional customary classes of cream products still exist in most countries (*see* **Cream**: Products). It should not be forgotten that cream is also the primary product in the manufacturing process of butter and butter oil. In the European Union, about 30% of the available milk fat is processed into butter (*see* **Butter and Other Milk Fat Products**: The Product and Its Manufacture).

The most typical of all cream products is whipping cream, which has a fat content of 30–40% and is processed without or with low-pressure homogenization. Originally, it requires no complicated preparation, just careful handling before whipping. A well-homogenized cream with the lowest legally permitted fat content (10–12%) is preferably used as coffee whitener. This coffee cream is added just before enjoying the coffee, whereas double cream (45–50% fat) is added already during the manufacture of another kind of beverage, cream liqueur. This product contains about 17% alcohol by volume and 16% milk fat that shows the finest distribution of all dairy-type products achieved by severe homogenization. Cultured or sour creams hold a special position within cream products. The homogenization before fermentation depends on the fat content, which ranges from 10% to more than 40%. The resulting fat globules directly participate in the following acid coagulation process and finally support the network structure of proteins.

Principles

Milk fat is a mixture of many different triacylglycerols with individual melting points and thus has an extensive melting range. Between –40 and +40 °C, milk fat usually consists of liquid as well as solid fat, that is, oil with various crystals. A substantial proportion of milk fat melts between 10 and 20 °C. In raw milk, nearly all of the fat is emulsified in small fat globules protected against coalescence by a natural interfacial layer, which originates from membranes of secretory cells of the mammary gland. This milk fat globule membrane maintains the integrity and particular stability of the globules and renders them compatible with their aqueous environment. Approximately 10^{10} fat globules are dispersed in 1 ml raw milk, with diameters mostly in the range between 1 and 8 μm (*see* **Milk Lipids**: Fat Globules in Milk; General Characteristics; and Milk Fat Globule Membrane).

The physical separation of a fat-rich portion (cream) from a nearly fat-free portion (skim milk) by centrifugation relies on the difference in the density between the fat globules ($\approx 0.9\,\mathrm{g\,ml^{-1}}$) and the aqueous phase ($\approx 1.0\,\mathrm{g\,ml^{-1}}$) in which the fat globules are dispersed. In theory, an increase in milk temperature leads to a decrease in viscosity and an increase in separation efficiency. In practice, the separating temperatures are in the range 50−60 °C. Temperatures above 60 °C give rise to deposition of denatured proteins on the separator discs, which reduces efficiency and leads to a higher fat content in the skimmed milk. On the other hand, shear stresses at temperatures lower than 35 °C affect the membrane of the fat globules and thereby reduce the degree of separation. Centrifugal cream separation considerably accelerates the natural creaming effect of raw milk in the gravitational field of the earth. A difference in densities is essential for creaming, but its rate mainly depends on the diameter of the fat globules and the viscosity of the serum phase (Stokes' Law). The centrifugal separation is additionally influenced by the bowl speed and radius. Normally, a cream with 30–40% fat and a skim milk with about 0.05% fat leave the separator. After separation and before further processing, the fat content of the cream is standardized by the addition of skim milk (*see* **Standardization of Fat and Protein Content**).

In order to avoid mechanical damage of the fat globules in milk and cream, some precautions have to be taken. The stress of stirring, pumping, and mixing should be minimized with increasing fat content of the cream. The flow velocity in pipelines should be limited by the critical shear rate, which can be calculated. Generally, summer fat with a less crystallized fat portion needs more careful cold transport than winter fat. Drawn air bubbles increase the risk of damaged fat globules or can act as centers for fat globule aggregation and subsequent coalescence. Disrupted fat globules can cause fat losses, sensory defects, butterfat floccules, or gel-like cream plugs.

Coffee Cream

Coffee cream products mostly have either 10 or 12% fat, rarely 15 or 18%. They are manufactured for a long shelf life either by in-bottle sterilization or by flow sterilization (**Figure 1**). The process of flow sterilization (<135 °C) in a ultra-high temperature (UHT) plant followed by aseptic filling in different packaging materials has largely replaced the traditional in-bottle sterilization (*see* **Heat Treatment of Milk**: Sterilization of Milk and Other Products, and Ultra-High Temperature Treatment (UHT): Aseptic Packaging).

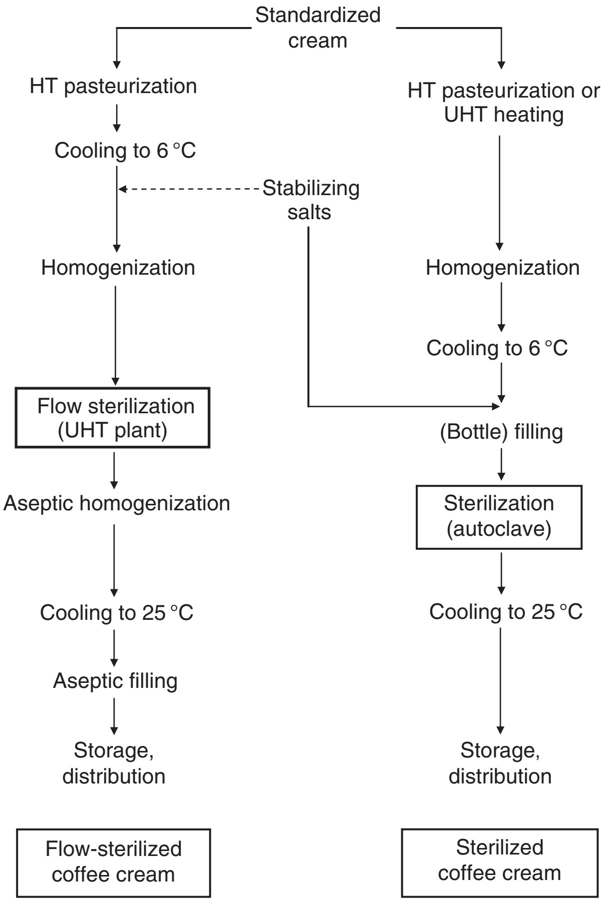

Figure 1 Manufacture of coffee cream. HT, high temperature; UHT, ultra-high temperature.

After separation of the raw milk and adjustment of the fat content, the cream is high-temperature pasteurized (90–95 °C) before cooling (6 °C). Subsequently, additives, that is, stabilizing salts, can be added as water-diluted suspensions. These salts have an effect on the pH value (buffer salt) and/or complex Ca^{2+} (ion exchanger) resulting in a decreased aggregation of casein micelles during the following heat treatment and in hot coffee beverages. The salts most often used are sodium phosphates and sodium citrates. Sodium monophosphates primarily stabilize the pH value, but also separate aggregated casein micelles. With a rising degree of phosphate condensation, the ability of ion exchange increases and the buffering capacity decreases. Trisodium citrate has both buffering and sequestering properties. Sodium phosphates and citrates are frequently added to sterilized coffee cream, but traditionally and predominantly to evaporated milk. Moreover, they are essential for the production of processed cheese where they are classified as 'emulsifying salts' (*see* **Cheese**: Pasteurized Processed Cheese Products and **Concentrated Dairy Products**: Evaporated Milk). However, flow-sterilized coffee cream (with at least 10 or 12% fat) may be produced without stabilizing salts provided the processing parameters are carefully adjusted.

Homogenization and flow sterilization are the decisive steps for producing a cream of high quality. Globules with a volume-moment average diameter of 0.4–0.6 μm, a narrow size distribution, and a minimum degree of fat globule clusters contribute to a relatively low viscosity of the cream, which is an indicator for high storage and coffee stability (resistance against feathering) and good whitening effect. In order to achieve these optimal conditions, single- or double-stage upstream homogenization (i.e., before flow sterilization) and double-stage downstream homogenization (i.e., after flow sterilization) with a total pressure of about 20 MPa are the method of choice. A heating temperature of below 130 °C results in reduced thermal aggregation of milk proteins compared with temperatures of 135–150 °C (UHT). While heating in the sub-UHT range, holding times of several minutes are essential for a sterile product, but this results in more advanced Maillard reactions.

The flow-sterilized coffee cream is then aseptically filled into glass bottles, into paper cartons with additional layers of aluminum foil and polyethylene (PE), or – preferably – into plastic packages. Usually, deep-drawn plastic cups contain 7.5–15 g, and preformed cups or cans 100–200 g of coffee cream. The small portion packs of white or brown polystyrene (PS) – seldom of multilayer plastic – are normally closed with lacquered aluminum foil by heat sealing. They are offered as single units or as rows with multiple units. Sterilization of the PS foil and the aluminum foil is achieved by a hot bath (>55 °C) of concentrated hydrogen peroxide (about 35% H_2O_2). After sterilizing, the plastic foil is immediately dried and deep-drawn before being filled with the cream. Although the water vapor permeability of PS is not negligible, commercial coffee cream with 10 or 12% fat has a minimum shelf life of about 4 months. At 20 °C and 50% relative air humidity, such portion packs lose about 10–15% of their mass by vapor permeation during this time. The water loss and the corresponding increase in cream viscosity can be reduced by using a multilayer plastic (e.g., PE and ethylene vinyl alcohol copolymer (EVOH)), or if a transparent polypropylene (PP) film encloses the row. Preformed plastic packages are sterilized by a finely sprayed hot hydrogen peroxide solution or by hot air and hydrogen peroxide vapor (at about 130 °C). They often consist of PP, exhibiting a considerably lower water vapor permeability and a prolonged shelf life of the cream. Also paper cartons with an aluminum layer or glass bottles are (nearly) gas proof. Uncolored glass affords no light barrier whereas brown PS or cartons with a layer of aluminum foil keep light transmission and induced oxidation at a low level (*see* **Packaging**).

Whipping Cream

Traditionally, whipping cream has a fat content between 30 and 40% and is pasteurized without homogenization. The demand for a prolonged shelf life has resulted in increased pasteurization temperatures and – at least in some countries – in UHT-heated (≥135 °C), aseptically homogenized and filled products with added stabilizers (**Figure 2**) (*see* **Heat Treatment of Milk**: Ultra-High Temperature Treatment (UHT): Heating Systems and **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects).

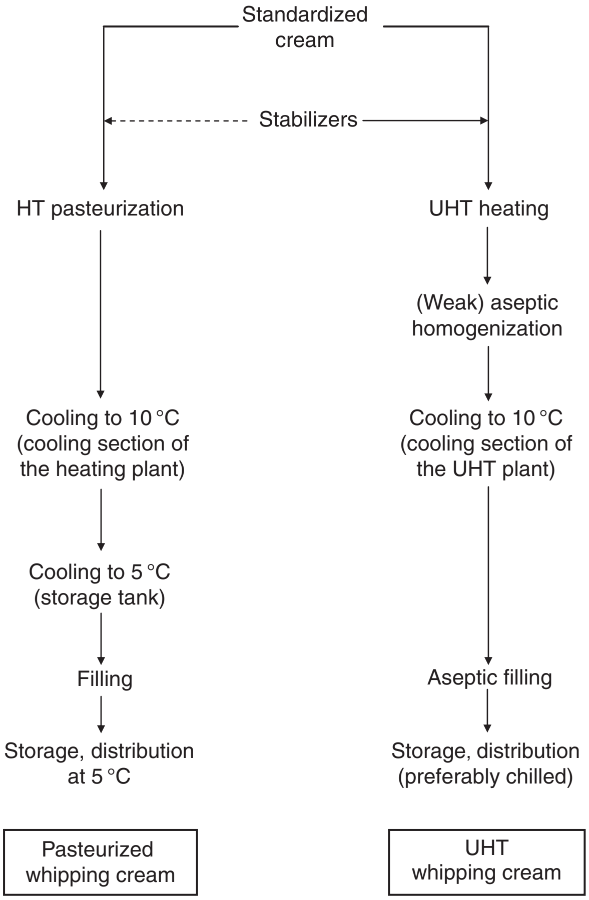
Standardized cream
Stabilizers
HT pasteurization
UHT heating
(Weak) aseptic homogenization
Cooling to 10 °C (cooling section of the heating plant)
Cooling to 10 °C (cooling section of the UHT plant)
Cooling to 5 °C (storage tank)
Filling
Aseptic filling
Storage, distribution at 5 °C
Storage, distribution (preferably chilled)
Pasteurized whipping cream
UHT whipping cream

Figure 2 Manufacture of whipping cream. HT, high temperature; UHT, ultra-high temperature.

Each manufacturing process for different milk and cream products has to consider the quality of raw milk. However, the demands can vary according to the resulting product and its purpose. The severe heat treatment of a flow- or in-bottle-sterilized coffee cream (see above) can destroy higher loads of enzymes and microorganisms than normal pasteurization or 'pure' UHT heating. In addition, original or heat-induced sensory deviations are partially masked after addition to the coffee beverage. Compared with this, the quality of raw milk for the production of pasteurized and UHT whipping cream is of particular importance since this cream is consumed for its pure flavor.

A prolonged refrigerated storage of raw milk will lead to proliferation of psychrotrophic bacteria, which are known to produce extracellular lipases and proteinases. These enzymes can survive pasteurization or even UHT heating and contribute to rancid and tallowy flavor, bitty cream, or even dramatic physical changes (*see* **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality).

After separation of raw milk at about 55 °C, the microbial load of cream is heavier than that of skim milk. The lower specific heat and heat transfer rate of cream compared to that of milk, the higher viscosity, and therefore the different residence time distribution in the holding section of a pasteurizer also have to be taken into account. A temperature of 72 °C for 15 s is a minimum regime for high-temperature short-time (HTST) pasteurized milk, whereas a temperature of 80 °C and higher is generally used for whipping cream. Pasteurization is usually carried out in a plate heat exchanger. A critical situation can occur in the cooling section because the fat globules are very sensitive to mechanical damage during crystallization. Therefore, a low pressure difference is desirable in the plate heat exchanger. In addition, the whipping cream should not be cooled to temperatures less than 8 °C. The final cooling has to take place in a storage tank filled from the bottom and with a gentle agitator. After 5–6 h of cold storage, crystallization is largely completed and the cream can be exposed to filling.

During processing of cream, the incorporation of air and air bubbles is another important factor in the mechanical destabilization of the fat globules and should be minimized. The plates of the pasteurizer should be arranged in such a manner that the inlets are at the bottom and the outlets at the top. The holding tube should have an upward slope.

The need to extend the shelf life of cream calls for temperatures higher than 80 or 85 °C. At least 110 °C for 10 s is essential for the inactivation of mesophilic spores and to achieve a shelf life of 3 weeks at 10 °C. Unfortunately, the free-fat content is highest in the temperature range between 115 and 130 °C resulting in an increased creaming and fat plug formation. A preheat treatment at 90 °C for 2–5 min denatures the whey proteins to a great extent and prevents negative physical effects but affects the sensory impression.

Homogenization (at more than 1.0 or 1.5 MPa) resulting in less satisfying whipping properties is not required even if a shelf life of 3 weeks is demanded. The addition of dairy or nondairy stabilizers – if legally allowed – can sufficiently slow down creaming during cold storage (see below).

The aim of UHT treatment is to produce sterile long-life products, which are aseptically packaged after heating and cooling. A whipping cream of good quality with 30–35% fat can reach a shelf life of up to 3 months at 20 °C. For UHT processing, mainly indirect heat exchange is applied. The heating temperatures range between 135 and 150 °C with holding times of a few seconds.

During the unchilled storage up to the best-before date, a sufficient emulsion stability of UHT whipping cream must be guaranteed. An unfavorable creaming and separation of serum can be delayed by a lower fat content (legally limited), additives (see below), or homogenization after UHT treatment. Upstream homogenization would cause an undesirable increase in the free-fat content. Whipping cream tolerates homogenization only at a relatively low pressure in order to keep still acceptable whipping properties. A compromise to achieve a long shelf life and adequate functional attributes is needed. Hence, a two-stage homogenization of cream (30% fat) with about 3.0 MPa in the first stage and 1.0 MPa in the second stage is recommended. The addition of carrageenan, special milk ingredients (protein and fat fractions), and emulsifiers (rarely legally permitted) can markedly improve the storage stability and also the whipping properties so that the difference from an unhomogenized product is very slight. Special fractions of the hydrocolloid carrageenan (kappa and iota) interact with the casein micelles at neutral pH values assisted by Ca ions forming a network. This results in delayed creaming during storage and reduced draining of the whipped cream, but whipping time and amount of incorporated air do not change. Some emulsifiers can substantially support the incorporation of air through their surface activity, whereas others will enhance fat globule interactions to decrease whipping times and form stiffer foams (*see* **Additives in Dairy Foods**: Legislation).

Pasteurized and UHT-treated whipping creams are packaged into a variety of retail containers made of different materials and enclosing different volumes. Most pasteurized cream is filled into preformed PS pots. Paper cartons supplied as flat sleeves with a coating of PE are also often used. UHT cream requires an aluminum foil laminated to the inner layer of the carton, which provides an efficient light and oxygen barrier. Plastic pots consist of PP or of different laminated plastics providing a suitable barrier against moisture, fat, and oxygen. Pots may be preformed or thermoformed just before filling and should be sufficiently colored against light transmission. A lacquered aluminum foil or a thin aluminated PP foil is recommended as a seal. All packaging materials should be carefully selected to avoid migration of monomers or softening agents into the cream (*see* **Packaging**).

For convenience, UHT-treated whipping cream is sometimes filled into aerosol cans. The lacquered aluminum or tinplate cans are sterilized by saturated steam, hot air, or a solution of hydrogen peroxide. Nitrous oxide (N_2O) or a mixture of both inert N_2O and carbon dioxide can be used as propellant gases.

Cultured Cream Products

Cultured or sour creams are manufactured in a number of countries. These products find various applications as additives in rounding off sauces or dressings.

The fat content of cultured creams ranges from 10% to more than 40%. The manufacturing process is largely equivalent to that of other fermented products (**Figure 3**). After standardization of fat content, dry matter can be enriched and stabilizers for improving texture and preventing syneresis (e.g., casein(ate) or hydrocolloids) may be added if they are legally allowed. A small amount of chymosin or other safe and suitable coagulating enzymes to improve texture without achieving enzymatic coagulation may also be added, preferably after the addition of the culture. The requirement for such steps decreases with increasing fat content. Addition of sodium chloride affects the flavor. Subsequently, the standardized cream is heat-treated and homogenized. Usual heating conditions are 85–95 °C for 15 s to 30 min or 120–130 °C for a few seconds. Homogenization after heating results in better texture properties compared to upstream treatment. Pressures applied vary according to the fat content of cream. A general guide is that the lower the fat percentage, the higher the required homogenization pressure. Homogenized fat globules directly participate in the

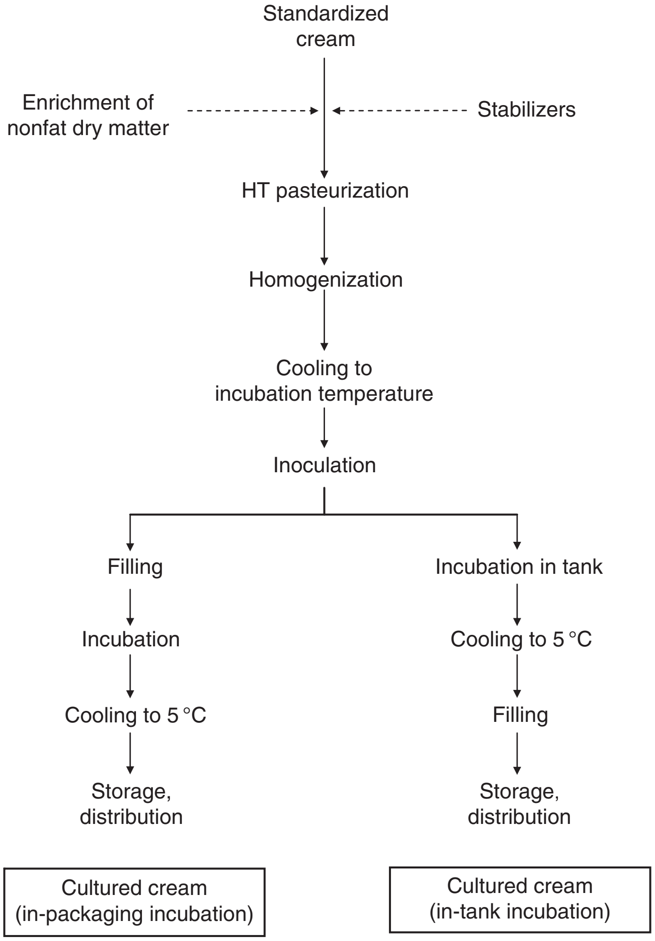
Standardized cream
Enrichment of nonfat dry matter
Stabilizers
HT pasteurization
Homogenization
Cooling to incubation temperature
Inoculation
Filling
Incubation
Cooling to 5 °C
Storage, distribution
Cultured cream (in-packaging incubation)
Incubation in tank
Cooling to 5 °C
Filling
Storage, distribution
Cultured cream (in-tank incubation)

Figure 3 Manufacture of cultured cream. HT, high temperature.

following acid coagulation process and finally become an integral part of the network structure.

Normally, souring is achieved by inoculation with mesophilic lactic acid bacteria. Less frequently produced, acidified sour cream is soured chemically, often by a blend of lactic and citric acid or by gluconic acid-δ-lactone. Lactic acid has bacteriostatic effects, while citric acid can be fermented by many organisms. The most common mesophilic cultures comprise *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive strains of lactococci, and *Leuconostoc mesenteroides* subsp. *cremoris*. *Lactobacillus acidophilus* plays an important role, owing to its perceived health benefits (*see* **Fermented Milks**: Starter Cultures). Incubation may take place after filling in the retail package, or in a fermentation tank before filling. It takes about 14–24 h at temperatures between 20 and 24 °C. This temperature range produces a good balance between organic acids and aromatic flavor compounds. Raising the temperature up to 30 °C shortens incubation time, but produces more acid flavor and less aromatic flavor. When the desired pH is almost reached, the microbiological process is interrupted by rapid cooling to 5 °C. A freshly produced cultured cream shall have a pH value of about 4.5 resulting in a slightly acidic, mild 'cheesy' or 'buttery' flavor. The texture of cultured cream should be uniform (without creaming), creamy, and viscous.

Cultured creams (possibly in a modified composition) may also obtain a nearly plastic consistency by appropriate processing conditions and may then be used as low-fat spreads.

Cream Liqueur

A cream liqueur combines the flavor of an alcoholic drink with the texture of cream in a product expected to have a shelf life of several years at ambient temperature. During that shelf life, the liqueur must be resistant to both bacteriological and physical changes. A sufficient alcohol strength (not less than 14%, i.e., 30° proof) together

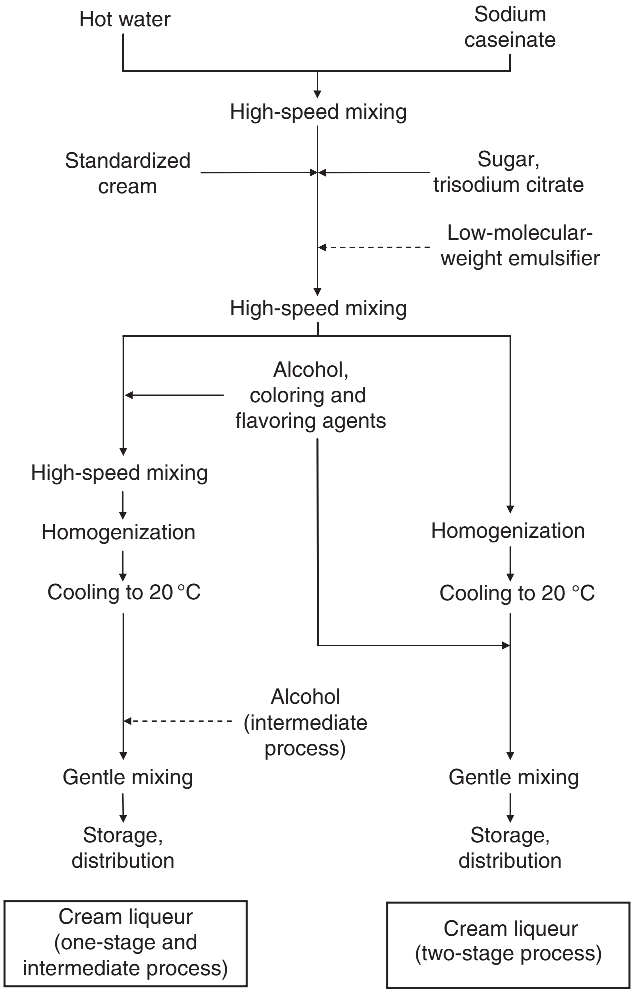
Hot water
Sodium caseinate
High-speed mixing
Standardized cream
Sugar, trisodium citrate
Low-molecular-weight emulsifier
High-speed mixing
Alcohol, coloring and flavoring agents
High-speed mixing
Homogenization
Cooling to 20 °C
Homogenization
Cooling to 20 °C
Alcohol (intermediate process)
Gentle mixing
Storage, distribution
Gentle mixing
Storage, distribution
Cream liqueur (one-stage and intermediate process)
Cream liqueur (two-stage process)

Figure 4 Manufacture of cream liqueur.

with a high sugar content (about 19%) makes the liqueur lethal to human pathogens and prevents germination of remaining viable spores. Attaining an acceptable level of physical stability places high demands on emulsion stability, formulation, and processing. Besides alcohol and sugar, the oil-in-water emulsion consists of milk fat (about 16%, contributed by cream with 48% fat), nonfat milk solids (*c.* 1.5%, contributed by cream), water (*c.* 46%), sodium caseinate (*c.* 3%), trisodium citrate (*c.* 0.2%), possibly low-molecular-weight emulsifiers like monoacylglycerols (*c.* 0.1%), and coloring and flavoring agents. Although deviations exist, many liqueurs on the market have compositions not too far removed from this standard.

The three general strategies available for producing cream liqueurs are the single-stage and the two-stage processes and an intermediate process for higher alcoholic contents (about 19%, i.e., 42.5° proof) (**Figure 4**). In all three, sodium caseinate is first dissolved in hot water with the aid of a high-speed mixer. Sodium caseinate has been found to be the only protein able to provide the required long-term stability of the emulsion. α_{S1}-Casein seems to be the fraction of sodium caseinate that is best soluble in ethanol. Therefore, the sole use of this fraction might be worth considering. The amount of necessary protein depends on the fat content and the globule size desired. Generally, the ratio of fat to total protein is approximately 5:1 (*see* **Milk Protein Products**: Functional Properties of Milk Proteins). Once casein is dissolved, pasteurized cream, sugar, trisodium citrate, and possibly a suitable emulsifier are added under continuous high-speed mixing at 55 °C. Trisodium citrate is used as a calcium sequestering agent, since the interaction between sodium caseinate and available calcium (mainly from cream) may lead to gelation and syneresis during storage. An increase in pH value up to 6.8–7.0 by citrate has a further stabilizing effect. Addition of different sugars affects mouthfeel, sweetness, and perception of alcoholic strength, but can also determine emulsion stability. The optimum content of cream results in adequate sensory properties.

In the two-stage process, the premix (cream base) is homogenized at 55 °C, the conditions being chosen such that more than 98% of the fat globules have a diameter of less than 0.8 μm. Cream liqueurs show the finest fat dispersion of all known dairy-type products (volume-moment average diameter about 0.2 μm). The small fat globule size is achieved by multiple high-pressure homogenization. After rapid cooling to 20 °C, the appropriate amount of alcohol, containing coloring and flavoring agents, is added under stirring. Most commercial liqueurs are based on a mixture of pure ethanol and another potable spirit (e.g., whiskey, brandy, rum). In the single-stage process, the alcohol is added prior to homogenization. Both methods are used, but the single-stage process is recommended. If a cream liqueur with an alcohol content of 19% is produced, a stable emulsion cannot be made directly. On account of the increased alcohol content, emulsion stability would decrease at constant total solids. Hence, an intermediate process comprises a second addition of alcohol after homogenization and cooling, which completes the product. Decreasing the total solids in the final liqueur results in a prolonged shelf life. The final step of all processes is filling into bottles of brown glass. Most cream liqueurs have a final alcohol content of 17% by volume.

See also: **Additives in Dairy Foods**: Legislation. **Butter and Other Milk Fat Products**: The Product and Its Manufacture. **Cheese**: Pasteurized Processed Cheese Products. **Concentrated Dairy Products**: Evaporated Milk. **Cream**: Products. **Fermented Milks**: Starter Cultures. **Heat Treatment of Milk**: Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality. **Milk Lipids**: Fat Globules in Milk; General Characteristics; Milk Fat Globule Membrane. **Milk Protein Products**: Functional Properties of Milk Proteins. **Packaging**. **Standardization of Fat and Protein Content**.

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Products

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Introduction

Cream is a comparatively rich emulsion of milk fat. Cream and cream products are sold in many varieties. In most countries, there exist traditional classes of cream products, mostly divided according to the fat content. However, no uniform legal definition and classification has gained worldwide acceptance. The fat content of the different liquid and cultured products ranges from 10 to 50%. Often, subclasses are used. Coffee, half, light, and single cream usually have a fat content of 10–30%, whipping cream has a fat content of 30–40%, and double cream 45–50%. Cream products are also classified according to their thermal treatment. Minimum pasteurization is the best way to preserve cream flavor; however, increasingly, in several countries, more severe heat treatments are practiced for the convenience of extended shelf life at ambient temperatures. Whereas in-bottle sterilization has become less common, ultra-high temperature (UHT) heating and pasteurization with temperatures well above 100 °C have gained ground. Nowadays, products such as coffee cream are predominantly flow-sterilized. Cultured or sour creams hold a special position within the range of cream products, because they belong to a diverse group of fermented products. Hence, regulations and manufacturing processes have been adopted from this group. Cream is also used as an essential ingredient in other dairy or nondairy products such as ice cream and cream liqueur (*see* **Cream**: Manufacture). In addition, cream is the primary product in the manufacturing process of butter and butter oil (*see* **Butter and Other Milk Fat Products**: The Product and Its Manufacture). Some cream products may even be directly used as spreads.

Types and Regulations

Cream and cream products have a variety of compositions and are normally defined according to fat content and, sometimes, also by function or heat treatment. Until now, the traditional classes of cream products in different countries have not been made uniform. In 1977, the Food and Agriculture Organization (FAO) and World Health Organization (WHO) suggested standards for nonfermented market cream combining fat content and function. Half cream should have a fat content ≥10 and <18%, cream ≥18%, whipping cream ≥28%, heavy whipping cream ≥35%, even though higher fat creams are less dense and thus lighter per unit volume, and double cream ≥45%. However, these suggestions did not prevail over national legislation. A new initiative was taken by the Codex Alimentarius Milk Commission and the International Dairy Federation (IDF). The revised 'Codex Standard for Cream and Cream Products' (2003) describes cream as "... the fluid milk product comparatively rich in fat, in the form of an emulsion of fat-in-skimmed milk, obtained by physical separation from milk. Prepared creams are the milk products obtained by subjecting cream... to suitable treatments and processes to obtain the characteristic properties as specified below." These prepared creams comprise prepackaged liquid cream, whipping and whipped cream, cream packed under pressure (aerosol whipping cream), fermented cream, and acidified cream. The minimum fat content of all these creams has to be 10%. National regulations make more distinctions regarding fat content. Products with a low fat content are German 'coffee cream' (≥10% fat), 'half-and-half cream' (≥10.5% fat) in the United States, and British 'half cream' (≥12% fat). Traditional (whipping) cream contains 30% fat in Germany and the Netherlands, whereas the same product contains 35% fat in the United Kingdom, Australia, and New Zealand. The US Food and Drug Administration makes a distinction between 'light whipping cream' (≥30% fat) and 'heavy whipping cream' (≥36% fat). Applied heat treatments for the different cream products include pasteurization, UHT heating (≥135 °C), flow sterilization in a UHT plant (<135 °C), and in-bottle sterilization (*see* **Heat Treatment of Milk**: Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Aseptic Packaging. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk).

Cultured or sour creams are regulated as are fermented products. Their fat content also ranges from 10 to more than 40%. In the United Kingdom and the United States, a minimum fat content of 18% is required. In countries such as France, Germany, Denmark, and Sweden, the term 'crème fraîche' has a different definition in each nation's legislation. 'Smetana' is a popular sour cream product in eastern Europe and Finland. In the

United States, the addition of 'acidified' to the name means that the cream is soured with safe and suitable acidifiers such as gluconic acid-δ-lactone, with or without the addition of lactic acid bacteria. The manufacturing processes of cultured creams are largely similar to those of other fermented products (*see* **Fermented Milks**: Yoghurt: Types and Manufacture).

'Clotted cream' ($\geq$55% fat) is a product that is virtually unique to South West England and is used for tea and dessert (with scones and jam). A thin layer of cream is heated at about 80 °C for 1 h (scalding), resulting in a cream crust and a rich, sweet flavor. Similar products are 'örom' (Mongolia), 'malai' (India), and 'kaymak' (Near and Middle East). Cream liqueurs, which contain an essential amount of cream, are normally regulated as are spirits. In a cream liqueur produced in the European Union, $\geq$15% of cream with $\geq$10% fat is required. Removal of water from cream by spray-drying yields cream powders with an extended shelf life if antioxidants are added and adequate storage conditions are guaranteed. To obtain functional products after reconstitution, incorporation of emulsifiers, such as monoacylglycerols, is necessary. Legally, cream powders normally belong to the group of dry milk products (*see* **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture). There are many more products with the word 'cream' in their designation. Well-known examples are ice cream, cream cheeses, and cream spreads (*see* **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat-Based Spreads. **Cream**: Manufacture. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Product Types).

During the manufacturing process of liquid cream products, certain substances can be added if legally permitted. Typically, coffee cream contains stabilizing salts such as phosphates and citrates, and the creaming of whipping cream within the package is delayed by carrageenan, which also reduces syneresis after whipping. Cream liqueurs are stabilized by citrate, whereas cultured cream products are usually processed without adding hydrocolloids.

In the European Union, the legal regulations concerning additives have been uniform for some years. The permitted substances in liquid cream comprise phosphoric acid and mono-, di-, tri-, and polyphosphates, lactic acid and lactates, citric acid and citrates, chlorides, alginic acid and alginates, agar-agar, carrageenan, xanthan gum, pectins, cellulose and cellulose derivates, starch derivates, emulsifiers (lecithin and mono- and diacylglycerols of edible fatty acids), and the propellants carbon dioxide, nitrogen, or dinitrogen monoxide in whipped creams. Not all of the additives on this extensive list are, however, really helpful or necessary for the production of high-quality cream products, and hence not all are used.

Quality Problems

Coffee Cream

In many countries, coffee cream is a popular product, which is manufactured for long storage either by in-bottle sterilization or by flow sterilization in a UHT plant. It competes with evaporated milk, liquid or foamed whole milk, and liquid or dried coffee whiteners, which often contain vegetable fats. After opening of a briefly shaken cream package, the consumer expects good sensory properties of the content and that the ingredients, that is, fat and protein, have remained in a homogeneously dispersed state. In a hot coffee beverage, a high whitening effect as well as a high coffee stability is demanded. Coffee stability, which is of outstanding importance for the quality of this product, means the degree of resistance against coagulation or 'feathering'.

Creaming or sedimentation phenomena during storage depend on the fat content of cream and on the conditions of heat treatment and homogenization during the manufacturing process (*see* **Cream**: Manufacture). It is obvious that creaming problems increase with the fat content. The fat content is limited to about 20% to obtain a desirable homogenization effect (volume-moment average diameter 0.4–0.6 μm, nearly identical size) because casein is partly bound to the fat as a major constituent of the newly formed fat globule membrane. The considerable enlargement of the total fat globule surface after homogenization as a result of the increased globule numbers and its reduced diameter requires a sufficient amount of casein, that is, a sufficiently high protein/fat ratio. The more the available casein is adsorbed on the fat globules, the more the aggregation of fat/protein complexes is favored (due to the higher sensitivity to changes in electrical charge). A thermally induced aggregation may occur during in–bottle or flow sterilization of the coffee cream. Its extent depends more on the heating temperature than on the heating time. That is why flow sterilization is carried out preferably with temperatures below 130 °C. In addition, the process of flow sterilization in a UHT plant allows a second two-stage homogenization after heating in order to split up aggregates (clusters) formed. A resulting coffee cream with predominantly nonaggregated fat globules shows a low viscosity, which is a desirable characteristic.

The aggregation of fat/protein complexes in hot coffee solutions is affected by the manufacturing process of the cream, but also by coffee brand, water ingredients, brewing conditions, actual temperature, and other factors. Most coffee solutions have pH values of about 5.0, which is near the isoelectric point of casein. In combination with high temperatures and low or very high water hardness, coagulation and aggregation can easily occur. When the temperature of a cold coffee solution with dispersed cream increases, the growing aggregates remain invisible to the naked eye until they have a sufficient size. At this temperature, feathering becomes perceptible and the whitening power of the cream decreases at the same time. After pouring the coffee cream from a small polystyrene (PS) package into a coffee solution, clearly defined large white floccules can float on the surface, and this is not a result of feathering. These floccules are dried particles from inside the package that were formed during storage because PS permits substantial water vapor permeability (*see* **Packaging**).

Whipping Cream

The whipping of a traditionally pasteurized, continuously and sufficiently cooled cream with a fat content of not less than 30% is unproblematic if a raw milk of good quality is used and the production of cream continues largely without mechanical stress. Each additional percent of fat up to 40% reduces whipping time and results in a firmer foam. Quality defects of raw milk, nonoptimal processing, a lower fat content, and, most of all, the demand for a prolongation of shelf life generally cause problems.

Raw milk contains enzymes with different activities and some of them are responsible for the development of specific flavor compounds and defects in milk and cream. The native milk lipase, for example, is lipoprotein lipase (LPL) that catalyzes the hydrolysis of triacylglycerols to free fatty acids. The activity of LPL is theoretically sufficient to cause rancidity in less than 1 min. However, liberation of free fatty acids is prevented by an intact milk fat globule membrane. Since the fat globule membrane protects milk fat against lipolysis, the milk must be carefully handled to minimize damage to the fat globule membrane. Homogenization of cream produces a greatly enlarged area of milk fat covered with a new membrane. This milk fat is vulnerable to the action of LPL, and subsequent rancidity occurs if no immediate pasteurization inactivates this heat-labile enzyme. Lipolytic rancidity is also induced by extracellular bacterial lipases of *Pseudomonas* spp. and other Gram-negative psychrotrophs. In many cases, these lipases are not inactivated by pasteurization and may even be present in UHT cream (e.g., lipases of *Pseudomonas fluorescens*). Extracellular proteinases of Gram-negative psychrotrophs may be also very heat-stable and can show activity even after in-bottle sterilization of cream. Phospholipases, proteinases, and glycosidasas from psychrotrophic *Pseudomonas*, *Citrobacter*, and *Enterobacter* may act synergistically in damaging the fat globule membrane. The aggregation of fat globules, which produces bitty cream, has been linked to the specific activity of phospholipase from *Bacillus cereus*. Psychrotrophic spore formers in raw milk such as *Bacillus* spp., which cause sweet coagulation, can survive pasteurization, and also heat-resistant spores may occur in UHT cream (*see* **Enzymes Indigenous to Milk**: Lipases and Esterases. **Psychrotrophic Bacteria**: *Pseudomonas* spp.).

The prolonged shelf life of UHT cream means that high demands are made on filling and packaging materials. In contrast to pasteurized cream, the UHT product must be aseptically filled and packed. Therefore, the filling equipment and packaging materials have to be tolerant to water, steam, hydrogen peroxide, heat, and UV light. Whether the whipping cream is pasteurized or UHT heated, there are some common important factors that must be taken into consideration. Light and/or oxygen may induce oxidation of unsaturated fatty acids, leading to flavor degradation. The appropriate filling conditions should be selected to minimize the oxygen content in the package (with small headspace volume) and in the cream (*see* **Packaging**). Homogenized cream is particularly susceptible to the action of light. The sensitivity of cream to light depends also on the heating conditions. UHT heating causes sulfhydryl groups and hydrogen sulfide to be released from β-lactoglobulin, initially creating an intense cooked flavor (*see* **Heat Treatment of Milk**: Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems). During storage, oxidation of these groups causes the cooked flavor to disappear and the strong reducing activity inhibits the development of off-flavor compared with those likely to occur in pasteurized cream. A balanced antioxidative/oxidative action of sulfhydryl groups and oxygen will probably help to ensure cream products of a good sensory quality. But even if well-balanced conditions can be maintained, light transmission must be kept at a low level.

UHT whipping cream is expected to have a shelf life of about 3 months. But particularly during the summer period, consumers complain about quality defects. A short-lived warming up of a cream package to a temperature of 30–35 °C, as may occur during transport without cooling, produces adverse effects after slow recooling. It not only supports creaming during subsequent storage at 20 °C, but may also lead to a distinct thickening after cooling before whipping. Although not all the fat is melted after the warming up, this ‘rebodying’ is caused by increased size of fat crystals during recooling. This results in partial coalescence without stirring provided that the fat content is so high that the fat globules are very close together. A continuous storage temperature of 5 °C delays creaming and the occurrence of sensory problems when compared to storage at 20 °C. The whipping time of the cream is extended as a result of cold storage, but it also results in an increased volume.

Whenever comparative studies or assessments of whipping properties are carried out, standardized temperature and whipping conditions must be ensured, for which special regulations exist in many countries. Usually, whipping time and overrun, and firmness and leakage of the foam are measured. Most devices used to test whipping are modifications of the one originally described by Mohr and Baur in 1937. It consists of two hexagonal, cylindrically arranged wire baskets rotating at a constant speed. The cream, which is stored for 24 h at 4 °C, is whipped in standardized cooled (5 °C) cups until there is no appreciable increase in the load required to turn the blades. Percent overrun is calculated as the volume difference after and before whipping, divided by the volume of unwhipped cream and multiplied by 100. An overrun of not less than 80% is desirable. Firmness can be equated with the length of time needed by a standardized plunger to penetrate the foam to a defined depth. When using a texture analyzer, the average force required to move the plunger at a constant speed within the foam yields a more sensitive indicator of firmness. For determining serum leakage, a formed square block (defined edge length) of the whipped cream is placed on a special sieve in a room of constant temperature and humidity. After 2 h, the quantity of dripped liquid is measured.

Development of the whipped cream structure depends on interactions between fat globules and between fat globules and air bubbles. This leads to the build up of a matrix in which bubbles are stabilized, and the majority of globules are clumped. A prerequisite for effective whipping of the cream is that part of the fat is solid and a space-filling network of mainly long and slender platelets is formed within the fat globules. Hence, deep cooling and a sufficient cooling time of the cream are indispensable.

The initial stage of whipping involves adsorption of soluble whey proteins and β-casein at the gas–liquid interface. This protein layer is not strong enough to stabilize a foam structure of large and still rather unstable air cells. However, it makes it easier to incorporate more air into the system until the maximum overrun is achieved. This first stage corresponds more or less to the formation of a protein foam. During the second stage of whipping, the bubbles are reduced in size, and the overrun remains approximately constant. In the end, the proteins are going to be replaced by strongly hydrophobic fat compounds of damaged fat globules. Induced by the mechanical stress, fat globules with slightly protruding fat crystals may collide with an unstable air bubble, and a bridging process occurs. The vigorous flow results also in a more frequent collision of fat globules with protruding crystals which have lost segments of the natural membrane, leading to conjunction of these globules via crystal bridging and released liquid fat as ‘viscous glue’. This partial coalescence results in an irreversible deposition of single fat globules or fat aggregates at the hydrophobic air/serum interface. The highly dynamic transformation of free fat globules into clumps finally leads to the third stage of whipping. This stage is initiated by a steep increase of power consumption of the whipping device. At the end point, probably only insufficient free fat globules and small clumps remain in the serum phase for the stabilization of newly formed bubble surfaces. The whole process of foam formation results in a partly coalesced fat globule network, which stabilizes the air cells, traps the serum phase, and forms the characteristic stiff texture (**Figure 1**). This applies to pasteurized or low-pressure-homogenized (max. 3 MPa) UHT whipping cream with a fat content of about 30% and with or without viscosity raising additives. Prolonged whipping would result in too large clumps of fat globules leading to rupture of bubble-enclosing lamella, initiation of bubble coalescence, and a reduction in overrun. The irreversible phase inversion into a greasy water-in-oil emulsion becomes visible as butter granules.

Homogenization creates smaller and more stable fat globules which are stabilized by an interface of adsorbed proteins covering an increased total surface area. Such fat globules that are in direct contact with air bubbles do not lose their globule membrane so that no partial coalescence occurs. The secondary membrane exposes the so-called calcium-sensitive regions of the micellar caseins, increasing the reactivity of fat globules. The resulting foam structure is stabilized by casein on the surface of fat globules via calcium bridges. Its development and stability are not comparable with that of nonhomogenized whipped cream (*see* **Cream**: Manufacture). Supporting the

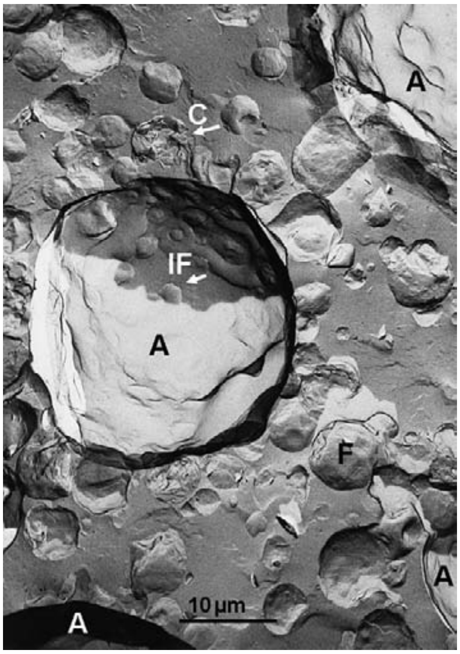

Figure 1 Transmission electron micrograph of whipped cream. A, air cell; C, cut fat globule membrane with crystallized fat; F, fat globule; IF, impression of fat globule.

surface layers with other surface-active substances (emulsifiers) decreases the formation of clusters and increases the tendency to clumping. Then homogenization at higher pressure may be applied.

Cream can also be aerated by means of suitable propellants resulting in an overrun of such aerosol products in the range of 300–500%. The resulting microstructure shows a clearly increased amount of fat globules, which adsorb at the interfaces of air bubbles. Concurrently, agglomeration of the fat globules and the corresponding network of different air bubbles is substantially reduced compared with regular whipped cream. The common structure is modified insofar as there is a reduction in the dimensions of the lamellae between air bubbles. The very low level of partial coalescence and the high solubility of the propellants decrease the stability of such foam.

Cultured Cream

During the manufacturing process of cultured cream (*see* **Cream**: Manufacture), incubation may take place in the retail package or in a fermentation tank. One disadvantage of in-tank souring is that the product will once more come into contact with the manufacturing equipment, which seriously raises the risk of reinfection. Apart from that, the viscosity of the cream after fermentation decreases during mechanical treatment till packaging. Therefore, the necessity of adding hydrocolloids increases. Set-style products have a markedly thicker consistency, but have a tendency to become slightly inhomogeneous during the long fermentation period at ambient temperature.

Direct acidified sour cream lacks the fine flavor of cultured cream in the first 2 weeks after production. However, during the following shelf life, enzymes from the culture can start producing an 'aged' flavor as a result of proteolysis.

Cream Liqueur

The cream liqueurs that are commercially available have typical shelf lives of several years when stored in sealed bottles under ambient conditions. Very occasionally, however, defects such as the formation of a cream or fat plug in the neck of the bottle can occur after prolonged storage. One explanation is that insufficiently severe homogenization conditions were used (*see* **Cream**: Manufacture). There is no analogy between the behavior of double cream in liqueur and pure double cream, for which even slight homogenization results in aggregation of the fat globules. By contrast, it is difficult to overhomogenize a cream liqueur because the fat to protein ratio is about 5, but about 25 in pure double cream. A neck-plug resulting from ordinary creaming only can be mostly redispersed by gentle shaking or even pouring, whereas a more solid kind of plug is not redispersible and thus unacceptable to the consumer. An essential aspect of this neck-plug is its fatty solid-like cohesive structure. So, while creaming remains the prerequisite, considerable variations in storage temperature, especially if accompanied by excessive mechanical agitation, may cause an appreciable destabilization. The formation of a solid neck-plug may be similar in origin to the churning of cream into butter or to the thickening of whipping cream after a short warming up at 30–35 °C and subsequent cooling to 5 °C (rebodying, see above). Finally, the oil-in-water emulsion is partially converted into a butter-like water-in-oil emulsion. Other physical and chemical factors may also contribute to neck-plugging.

At the bottom of the bottle, a slightly granular precipitate is occasionally observed. This deposit is composed of calcium and citrate, and is a direct result of the addition of trisodium citrate. High ambient temperatures during storage of cream liqueur accelerate the production of such crystalline material. The extent of deposition can be reduced by lowering the amount of citrate, but at the same time this increases the probability of subsequent gelation and separation of serum. Hence, it follows that the manufacturer has to assess carefully the composition of and production process for cream liqueurs (*see* **Cream**: Manufacture).

Finally, it must be taken into consideration that cream liqueur is basically unstable in the presence of lemonade or acidic mixers, because casein coagulates at its isoelectric point (around pH 4.6). The presence of traces of tannins and polyphenolic compounds in added spirits during the manufacture of cream liqueur may also destabilize sodium caseinate emulsions. When using white wine in the formulation of liqueur, the acidity can be neutralized by the addition of sodium hydrogen carbonate.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat-Based Spreads; The Product and Its Manufacture. **Cream**: Manufacture. **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture. **Enzymes Indigenous to Milk**: Lipases and Esterases. **Fermented Milks**: Yoghurt: Types and Manufacture. **Heat Treatment of Milk**: Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Product Types. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk. **Packaging**. **Psychrotrophic Bacteria**: *Pseudomonas* spp.

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John W. Fuquay, Professor Emeritus of Dairy Science at Mississippi State University, served on the faculty there from 1969 to 1999. His areas of emphasis in teaching and research were environmental physiology and reproductive physiology. He received his BS and MS degrees from North Carolina State University and his PhD degree from Pennsylvania State University, all in the area of dairy science. After completing the PhD degree in 1969, he accepted a teaching and research position at Mississippi State University, where he progressed through the ranks from assistant professor to professor before retiring in 1999. Professor Fuquay served as Coordinator for the Graduate Program in Animal Physiology from 1986 to 1999. He was a Visiting Professor in the Animal Sciences Department, University of California-Davis in 1979 and in 1985–86.

Professor Fuquay was active in his professional society, The American Dairy Science Association. He was a member of the editorial board of *Journal of Dairy Science* for seven years, an editor for four years, and served as the first Editor-in-Chief for six years (1997–2002). For his professional contributions and service to the Association, Professor Fuquay was recognized as a Fellow in the American Dairy Science Association in 2001 and received the Association's Award of Honor in 2002. Other recognitions include the World Association of Animal Production Jean Boyazoglu Award in 2003, the Distinguished Dairy Science Alumnus Award from Pennsylvania State University in 2003, and several teaching and research awards from his university.

Professor Fuquay has participated in a variety of international activities. He has presented short courses and lectures as well as provided consultations in a number of countries, primarily in Asia and Latin America. In addition to his research publications, he is the coauthor of a textbook, *Applied Animal Reproduction* (Prentice Hall), that has been widely used by universities in the United States and internationally. The first edition was published in 1980 and the last (sixth) edition in 2004. In 2010, he published a memoir, *Musings of a Depression-Era Southern Farm Boy* (Vantage Press), which reflects on how the experience of growing up on a farm in the southern United States during the great depression instills one with an understanding of the importance of strong family bonds and a sound work ethic in meeting the challenges of the adult world.

Patrick F. Fox was Professor and Head of the Department of Food Chemistry at University College, Cork (UCC), Ireland, from 1969 to 1997; he retired in December 1997 and is now Emeritus Professor of Food Chemistry at UCC. Prof. Fox received his BSc degree in Dairy Science from UCC in 1959 and PhD degree in Food Chemistry from Cornell University in 1964. After postdoctoral periods in Biochemistry at Michigan State University and in Food Biochemistry at the University of California, Davis, he returned to Ireland in 1967 to take up a research position at the Dairy Products Research Centre at Moorepark before moving to UCC in 1969.

Prof. Fox's research has focused on the biochemistry of cheese, the heat stability of milk, physicochemical properties of milk proteins, and food enzymology. He has authored or coauthored about 520 research and review papers, and authored or edited 25 text books on Dairy Chemistry. He was one of the founding editors of the *International Dairy Journal.*

In recognition of his work, Prof. Fox has received the Research & Innovation Award of the (Irish) National Board for Science and Technology (1983), the Miles-Marschall Award of the American Dairy Science Association (1987), Medal of Honour, University of Helsinki (1991), the DSc degree of the National University of Ireland (1993), the Senior Medal for Agricultural & Food Chemistry of the Royal Society for Chemistry (2000), the ISI Highly Cited Award in Agricultural Science (2002), the International Dairy Federation Award (2002), Gold Medal of the UK Society of Dairy Technology (2007), and an autobiography published in Annual Review of Food Science & Technology (2011).

Prof. Fox has been invited to lecture in various countries around the world. He has served in various capacities with the International Dairy Federation, including President of Commission F (Science, Nutrition and Education) from 1980 to 1983.

Paul McSweeney is Professor of Food Chemistry in the School of Food and Nutritional Sciences, University College, Cork, Ireland (UCC). He graduated with a BSc degree in Food Science and Technology in 1990 and a PhD degree in Food Chemistry from UCC in 1993 and also has an MA in Ancient Classics. He worked for a year in the University of Wisconsin (1991–92) as part of his PhD and as a postdoctoral research scientist in UCC (1993–94). He was appointed to the academic staff of UCC in 1995. The overall theme of his research is dairy biochemistry with particular reference to factors affecting cheese flavor and proteolysis during cheese maturation including the role of non-starter lactic acid bacteria and smear microorganisms, the ripening of hybrid and non-Cheddar varieties, the specificity of proteinases on the caseins, proteolysis and lipolysis in cheese during ripening, and characterization of enzymes important to cheese ripening (proteinases, peptidases, amino acid catabolic enzymes). He is the coauthor or coeditor of eight books, including the third edition of *Cheese: Chemistry, Physics and Microbiology* (Amsterdam, 2004) and the *Advanced Dairy Chemistry Series* (New York, 2003, 2006, 2009), and has published numerous research papers and reviews. Prof. McSweeney is an experienced lecturer and researcher and has successfully managed research projects funded through the Food Industry Research Measure and its predecessors administered by the Irish Department of Agriculture and Food, the EU Framework Programmes, the US–Ireland Co-operative Programme in Agriculture/Food Science and Technology, and Bioresearch Ireland and Industry. He was awarded the Marschall Danisco International Dairy Science Award of the American Dairy Science Association in 2004 and in 2009 a higher doctorate (DSc) on published work by the National University of Ireland.

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GUIDE TO USE OF THE ENCYCLOPEDIA

STRUCTURE OF THE ENCYCLOPEDIA

The material in the Encyclopedia is arranged as a series of entries in alphabetical order. Some entries comprise a single article, whilst entries on more diverse subjects consist of several articles that deal with various aspects of the topic. In the latter case the articles are arranged in a logical sequence within an entry.

To help you realize the full potential of the material in the Encyclopedia we have provided three features to help you find the topic of your choice.

1. CONTENTS LISTS

Your first point of reference will probably be the contents list. The complete contents list appearing in each volume will provide you with both the volume number and the page number of the entry. On the opening page of an entry a contents list is provided so that the full details of the articles within the entry are immediately available.

Alternatively you may choose to browse through a volume using the alphabetical order of the entries as your guide. To assist you in identifying your location within the Encyclopedia a running headline indicates the current entry and the current article within that entry.

2. CROSS REFERENCES

All of the articles in the Encyclopedia have been extensively cross referenced. The cross references, which appear at the end of an article, have been provided at three levels:

i. To indicate if a topic is discussed in greater detail elsewhere.
ii. To draw the reader's attention to parallel discussions in other articles.
iii. To indicate material that broadens the discussion.

Example

The following list of cross references appear at the end of the entry entitled **Bacteria, Beneficial** | Lactic Acid Bacteria: An Overview

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; *Streptococcus thermophilus*; Taxonomy and Biodiversity. **Pathogens in Milk**: Enterobacteriaceae.

3. INDEX

The index will provide you with the volume number and page number of where the material is to be located, and the index entries differentiate between material that is a whole article, is part of an article, or is data presented in a table or figure. Detailed notes are provided on the opening page of the index.

4. COLOR PLATES

The color figures for each volume have been grouped together in a plate section. The location of this section is cited in the contents list. Color versions of black and white figures are cited in figure captions within individual articles.

5. CONTRIBUTORS

A full list of contributors appears at the beginning of each volume.

6. GLOSSARY

A glossary of terms used within the work is provided in Volume Four before the Index.

PREFACE

We are pleased to present the second edition of the *Encyclopedia of Dairy Sciences*. The first edition was published in 2003 by the Major Reference Works Division of Academic Press, now part of Elsevier Sciences, and it comprised 427 articles. The objective was to satisfy the need for an authoritative source of information for people involved in the integrated system of production, manufacture, and distribution of dairy foods. It was realized from the beginning that a program of revision would be needed to keep the Encyclopedia up to date. This goal has been met in the second edition through 503 articles, of which 121 are new articles and 382 are revised articles. We express appreciation to the Editorial Advisory Board for its role in evaluating articles for needed revision, reviewing new and revised articles, and for help in identifying new topics to be included along with appropriate authors. Likewise, we are grateful for the contributions of the many authors who have either revised their articles or prepared new articles.

The main topics related to milk production and dairy technology are addressed in addition to providing information on nutrition, public health, and dairy industry economics including aspects of trade in milk and dairy products. All species that produce milk for human consumption have been included in this work. Some of these species are of regional significance only, but they have been included because of the essential role that their milk plays in the nutrition of people inhabiting various regions of the world. A significant addition to the second edition is four introductory articles addressing the history of Dairy Science and Technology. A synopsis has been prepared for each article in the second edition and will appear with the online listing of the articles in this publication.

The primary aim of the Encyclopedia is to provide a complete resource for researchers, students, and practitioners involved in all aspects of the dairy sciences as well as those involved with economic and nutritional policy and members of the media. We have tried to do this with a writing style that is easily comprehended by persons who are not highly trained in the technical aspects of the Dairy Sciences. Users should be able to access information on topics that are peripheral to their areas of expertise.

We express appreciation to the staff of the Major Reference Works Division, responsible for this Encyclopedia, for their timely responsiveness to the needs of the editors and their essential administrative role in keeping this major reference work on-track toward a satisfactory completion within the desired time schedule. We remember Nancy Maragioglio, Senior Life Sciences Editor, who initiated the work and was ever responsive to queries by the editors, as well as Sera Relton, Esmond Collins, Milo Perkins, and Claire Byrne, Development Editors, and Charlotte (Charlie) Kent, Publishing Administrator, who kept things moving through their communication with editors, authors, and reviewers and who exhibited almost flawless administrative skills. Sera Relton was particularly helpful as she assisted us in moving through the final submission and review stages. Laura Jackson is recognized for her contributions as Production Manager of the Encyclopedia.

Special recognition is due to Ms Anne Cahalane, Senior Executive Assistant, School of Food & Nutritional Sciences, University College, Cork, whose stylized representation of a cow, a milk can, and a wedge of cheese adorns the cover of the first and second editions of the Encyclopedia of Dairy Sciences.

John W. Fuquay
Patrick F. Fox and
Paul L. H. McSweeney

FOREWORD

The cow is the foster mother of the human race. From the days of the ancient Hindoo to this time have the thoughts of men turned to this kindly and beneficent creature as one of the chief sustaining forces of human life.

William Dempster Hoard (1836-1918)
Former governor, state of Wisconsin, USA (1889-1891)
Founder of **Hoard's Dairyman** (1885)

We must never forget that milk and milk products are and will always be important sources of basic food nutrients for humans both young and old. The more scientific facts we can discover, understand, and apply related to producing, processing, and marketing milk and milk products, the better we will serve the nutritional needs of humanity throughout the world.

More than 2000 years ago Aristotle noted, *Everyone honors the wise and excellent.* We are indebted to those *wise* enough to conceptualize and envision the favorable global impact that is certain to follow by bringing together this exhaustive, rich collection of 503 pertinent articles written and reviewed by more than 700 world-renowned disciplinary experts representing 50 countries – persons each of whom bears the mark of *excellence.* Happily these timely topics are now recorded in four informative, important, engaging volumes. We thank, commend, and salute the prodigious efforts of the *wise* and *excellent* authors who generated, compiled, and put the spotlight on the useful information and data, and who now share them through their well-written articles.

One noteworthy value and enduring virtue of these articles is bringing into clear perspective the context of both the state-of-the-art and the future of dairy sciences. When the history and contributions of scholarly publications related to the all-important global dairy industry are recorded, the second edition of the *Encyclopedia of Dairy Sciences* will be cited often and with great respect and appreciation.

Fundamental to continued progress and success in the dairy industry have been the signal service, cooperation, and collective contributions of dedicated scientists, teachers, agricultural advisors/extension workers, and representatives of governments and industries. Additional exciting breakthroughs in applying new findings and developments in research and technology to the production and processing of milk are sure to follow as we move surefootedly through the twenty-first century. This continued growth and success will be aided immensely by the vast and extraordinarily useful knowledge base made available by the idea-rich, insightful authors, editorial advisory board members, editors, and publisher of the second edition of the *Encyclopedia of Dairy Sciences.*

Indeed, by perusing the comprehensive and authoritative articles of this greatly needed and monumental encyclopedia, readers will be made even more aware of the tremendous progress that has occurred in the basic and applied sciences underpinning the global dairy industry.

Ours is an internationally competitive and incredibly technological world. And unless talented, creative scientists continue to work together in researching and applying the most effective and economical ways and means of providing

an abundant, safe supply of milk and milk products for an ever-increasing world population, we will never reach our noble goal of adequately feeding all the earth's people.

May we utilize the comprehensive scientific knowledge base made available through this second edition of the *Encyclopedia of Dairy Sciences* as we pledge to realize advances in the health and well-being of the undernourished millions – including many who need and deserve to be rescued from the ugly grip of hunger – by increasing the availability of nature's most nearly perfect food – milk!

Pure milk from healthy animals is a luxury of the rich, whereas it ought to be the common food of the poor.

Mohandas Gandhi (1869-1948)
Indian nationalist leader

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April 2010

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DAIRY EDUCATION

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Introduction

Educational and teaching programs in dairy education and dairy production have changed dramatically during the past 50 years. The progress and notable trends in dairy science education for the 50-year period from 1956 to 2006 in the United States include (1) an increase in the number of female students, (2) a decrease in the number of students from dairy farms, (3) an increase in the number of animal science departments that cover multiple animal species, and some loss of educational programs in dairy production and dairy foods/manufacturing areas as combined departments separated into individual departments, (4) a trend for faculty with more education and PhD degrees, (5) an increase in technology and computer use in courses, and (6) a small decrease in the number of student Dairy Science Clubs.

A summary of major advances and current trends in teaching dairy science was published as part of the 100th anniversary of the American Dairy Science Association in 2006. There are three structures of dairy production and education programs in the United States. These are land grant universities, the first of which was established in 1855, the non-land-grant programs, and 2-year technical programs. This article attempts to outline and discuss the status and changes in dairy education and curricula.

Current Trends in Dairy Education

Data were collected from dairy and animal science programs in North America representing all three educational structures including educational programs that are non-land-grant programs and 1- or 2-year degree programs. A survey instrument was sent to 38 universities that grant 4-year degrees and to 12 institutions that grant technical degrees. The survey instrument provided valuable information on the status of dairy education and revealed that degree programs in dairy production remain popular but have changed significantly during the last 40–50 years. Several universities did not respond to the survey because courses in dairy production were not offered. Several non-land-grant institutions also received the survey. A similar survey instrument was sent to 24 institutions that offer course work in dairy production that may lead to a 1- or 2-year technical degree, and data were collected from the 12 institutions that responded. These nonbaccalaureate degrees included educational programs that lead to 1- or 2-year technical or associate degrees. Some students elect to transfer to a land grant university for 2 years to complete a 4-year program.

The results and responses provided important information for an assessment of the state of dairy education in the dairy production area. Enrollment in 4-year baccalaureate degree programs is high with approximately 1200 students in dairy education programs in the United States. In nonbaccalaureate institutions that responded to the survey, there were 420 students enrolled in 12 dairy programs in the United States in 2004. The size and student enrollment in the dairy production programs in agriculture programs differ across the United States. The number of professors and faculty members is adequate in most universities. Most faculty members at universities in 2004 have split appointments and responsibilities among the three missions of the land grant universities: teaching, research, and extension/outreach. Faculty with split appointments often devote less time to teaching undergraduate students than required. The full-time equivalents (FTEs) devoted to teaching of dairy production was reduced 20% between 1994 and 2004.

The percentage of female students enrolled in 4-year programs has increased significantly. Computer and information technology has become a mainstream part of educational programs. A high percentage of undergraduate students elect to engage in an internship or work experience, and there is a high correlation between internships and career paths selected by students. The collaboration between universities and private industry is strong evidence that undergraduate programs are relevant to the dairy industry. Meaningful extracurricular activities including Dairy Science Clubs also remain popular and are perceived by faculty members to enhance personal development and leadership skills.

Dairy Industry Changes and The Impact on Education

Important changes in the dairy industry during the last 25 years have impacted dairy education and will continue to influence education programs in the future. A brief profile of the dairy industry illustrates some of these changes in the dairy industry (**Table 1**).

Between 1980 and 2008, the number of dairy cows and dairy farms continued to decrease at a modest rate, whereas cows per farm and milk yield per cow increased. The increasing farm input costs and stagnant milk prices emphasize the need for higher technical education and knowledge, use of specialized consultants, and increasing business knowledge. This has placed significant demands on educational programs in dairy production to remain relevant to the industry as well as to provide leadership for the future.

Table 1 Profile of the dairy industry since 1980

Measure	*1980*	*1994*	*2004*	*2008*
Dairy cows (million)	10.9	9.5	9.0	9.2
Dairy farms (no.)	320 160	148 140	81 440	78 000
Milk per cow (kg)	5414	7353	8614	9273

The merger of former dairy science departments into combined animal science departments increased the difficulty in identification of faculty and their distribution of time devoted to dairy education. Among the 38 universities that responded to the survey, 7 have the word ‘dairy’ in the department name. Many dairy manufacturing/technology programs have merged into food science departments. Four universities still have combined dairy production/dairy foods departments. Because of these changes, some dairy production programs are struggling to maintain identity and visibility on college campuses. Identifying students in some combined animal science departments who have a specific dairy interest is a challenge.

Ten of the land grant universities have more than 40 undergraduate students in 4-year dairy production programs. Several other university programs have fewer students per class and may not have adequate enrollment to offer courses. Overall, changes in total student numbers by enrollment group have not changed significantly but are primarily located in 10 land grant universities.

There has been a decline in the number of students from a dairy farm background (70% in 1994 vs. 64% in 2004), along with a significant increase in the number of female students (39% in 1994 vs. 54% in 2004). The percentage of female students enrolled in dairy production is higher than the 46% national average for all students in Colleges of Agriculture in 2003. A few questions on the survey related to the use of information technologies and the percentage of class time devoted to their use. In 2004, 87% of the courses in dairy production used computers and information technology compared with 30% of the courses in 1994. In 2004, 22% of course time in dairy education involved the use of computer programs for dairy farm management.

Important Skills and Experiences Required of Faculty/Professors

Included in the current survey was an assessment of the importance of various skills and experiences for students in dairy production. University faculty members at each institution were asked to rank each of the skills or experiences using a rating of 1 (not important), 2 (somewhat important), 3 (important), or 4 (extremely important). The authors ranked these skills after calculating the geometric means for all institutions (**Table 2**).

The top five skills as ranked by faculty members who teach undergraduate classes in dairy science were in the areas of critical thinking, oral and written communication skills, and computer skills. Also ranked very highly were

Table 2 Skills and experiences deemed important by faculty in dairy science programs

Skill/experience	*Mean response of importance (rank)*[a]
Interpersonal communication skills	3.75 (1)
Critical thinking skills	3.71 (2)
Oral presentation skills	3.58 (3)
Writing skills	3.53 (4)
Computer skills	3.50 (5)
Team problem-solving skills	3.42 (6)
Leadership development skills	3.22 (7)
Knowledge of general business management	3.19 (8)
Industry-related internships or work experience	3.17 (9)
Completed capstone course	3.14 (10 tie)
Knowledge of agribusiness marketplace	3.14 (10 tie)
Human resource management	2.94 (12)
International experience	2.20 (13)
Dairy cattle judging experience	2.00 (14)

[a] Based upon a scale from 1 (not important) to 4 (extremely important).

team problem-solving skills. A survey of 14 dairy and food industry executives was conducted and published by Hoard's Dairyman. This survey also placed critical thinking and interpersonal communication skills as the top priority. Industry executives placed business knowledge and marketing skills somewhat higher than did university faculty members.

Leadership development, general business management and knowledge of the market, and human resource management were ranked in the middle of the skill-sets rankings by faculty members. The scores received suggest that they are important skills to faculty, but are not as highly ranked as the top six skills and experiences. International experience, foreign language skills, and dairy cattle judging and evaluation were near the bottom of the list of scores (**Table 2**).

Initial Careers of Graduates, Internships, and Work Experiences

A major objective of an undergraduate program is to prepare students for future careers. In the survey, faculty members at the 38 universities that responded to the survey were asked to assess the number of graduates over the last 5 years who selected different career options upon graduation. Based on the information for 1700 graduates (**Table 3**), 31% of students chose dairy farming and production agriculture (**Table 3**). Approximately 27% of dairy production graduates elected to enter veterinary or graduate school, and 10% of the graduates entered the feed/nutrition field.

Table 3 Initial career paths of graduates from 4-year dairy production programs

Career path	*Percentage of graduates*
Dairy production	
Herdsperson or farm worker	7.6
Herd manager/assistant manager	7.4
Return to home farm	16.0
Veterinary school	16.3
Graduate school	10.9
Nutrition/feed sales and service	10.6
Education (includes cooperative extension)	5.3
Pharmaceutical sales and service	5.1
Animal breeding (service and sales)	4.8
Communications (advertising, promotion, and marketing)	4.2
Finance/business	4.0
Government/regulatory	3.8
Other	4.0

Data are from the results of authors' survey and represent 1700 graduates during the last 5 years.

Current and Future Perspectives of Dairy Production/Education Programs

Challenges exist in educational programs in dairy production. The curriculum in 1955–65 focused on chemistry, biochemistry, animal biology, mathematics, and more specialized courses including genetics and breeding, reproduction, lactation, nutrition, dairy farm management, and information technology. The curricula in the 1980s contained more science and more general agriculture courses. Most of the teaching and educational experience was typically performed in the classroom and/or laboratory, and is still taught in the classroom and/or laboratory to some extent. During the past 30 years, the outflow of scientific facts has placed a considerable burden on faculty to digest, evaluate, and incorporate the important facts and keep pace with new developments.

The dairy industry in the United States experienced major changes in structure and dairy farm management, and the profile of the industry changed (see **Table 1**). Fewer young people in dairy science were attending college with a practical background in the dairy industry, increasing the need for universities to train undergraduate students in practical aspects of dairy management. It became clear that undergraduate students with an interest in dairy science need more practical education in addition to key courses in science and management areas. During the period between 1975 and 2005, it was evident that the educational experiences are enhanced if students have more interactions and visits to progressive dairy farmers. Visits to the classroom by industry leaders and other people from the dairy industry enhanced the dairy educational experience.

North American Intercollegiate Dairy Challenge

The concept of the North American Intercollegiate Dairy Challenge (NAIDC) program was developed as a result of partnership between university faculty and industry representatives. The Dairy Challenge began at Michigan State University in 2000 as a practical on-farm evaluation experience for senior dairy science students. The contests in Michigan State in 2000 and 2001 were so successful that the National Dairy Challenge was established in 2002 as a dairy management activity or contest to incorporate all facets of a dairy farm business. The first National Dairy Challenge contest had 14 participating universities and 56 students with 4 students per team. In contrast, the 2009 national contest had 31 participating universities with 124 students participating. In 2003, the first regional Dairy Challenge was added in the northeastern United States. Subsequently, three other regional contests were added: the midwest, western, and southern regions. The regional events are structured much like the National Contest. However, the emphasis in regional contests is more on education than competition. As such, the universities are invited to bring more than one four-member team to the regional event allowing more students to participate in the regional Dairy Challenge program. Students are placed on aggregate or mixed teams with students from other universities with varying skills and backgrounds.

The mission statement developed by the 15-member board of directors is 'to create an intercollegiate dairy management competition based on the examination of all aspects of a dairy business'. This competition challenges students to recall basic dairy management principles and their practical application, while testing organizational, time management, data analysis, public speaking, leadership, and teamwork skills. Agribusiness and universities will cooperate in the conduct of this event to increase students' knowledge and comprehension of dairy business management and thereby promote a strong future for the dairy industry.

The Dairy Challenge program brings together the best and the brightest collegiate students who are studying for a career in the dairy industry. The Dairy Challenge is a unique 2-day competition among teams representing dairy science programs in North American universities. The competition challenges those institutions to inspire their students with a high-quality education – empowering them with the resources for real-world success. This challenge incorporates all facets of a working dairy business in a forum that is interactive, educational, and fun. The NAIDC is the competition for tomorrow's dairy leaders.

The Dairy Challenge allows dairy science students to apply theory and learning to a real-world dairy farm business while working as part of a team. Day one of the challenge begins with each four-person student team receiving information on a real-life dairy, including production and farm management data. Following an on-farm evaluation of the dairy farm business, and the strengths and weaknesses, each team develops a comprehensive program including recommendations for nutrition, reproduction, milking procedures, animal health, housing, and financial management for the dairy farm manager. The first day concludes with an informal dinner with NAIDC sponsors. Day two is a presentation day, where team members present their recommendations and evaluations to a panel of five judges. Competition can be intense, and team members must field questions from the judges. Presentations are evaluated based on the analysis of the dairy farm business and recommendations. The evening concludes with a reception and awards banquet. Year after year, the Dairy Challenge program benefits the students, universities, dairy producers, and agribusiness, that is, the dairy industry as a whole, as it generates prospective employees who are more experienced and knowledgeable and will be better prepared to serve the industry of the future.

Additional goals of the Dairy Challenge programs include the following:

- Encourage and develop networking – among students, faculty, and agribusiness professionals.
- Practice team building, teamwork, and team leadership.
- Provide the opportunity for application of classroom experience to real-world problems encountered in dairy operations.
- Provide potential job placement for many of the students through direct contact with potential employers.
- Provide practice for 'real-life' interpersonal relationships faced in business by forcing the students to work in mixed teams with new acquaintances and no prior knowledge.
- Provide an atmosphere that encourages participants to be more committed to the dairy industry following their participation.
- Receive feedback, both verbal and written, from participants and sponsors that allows the organizers to continually modify and improve their approach.
- Prepare students for the real world.

Coaches, Students, and Agribusiness

The Dairy Challenge has had a significant impact on the coaches and teaching programs of many colleges in North America by encouraging greater experiential learning and active participation in actual dairy business. Prior to the development of Dairy Challenge, few students were exposed to actual farm business evaluations and industry-related programs. This program, although focused on students and teaching, with team-building skills has had a similar impact on educational institutions and agribusinesses because it has forced them to reevaluate how they approach farms and farm businesses. At each Dairy Challenge (one national and four regional contests) during the year, the Board of Directors of the NAIDC have 1 h to visit with coaches (teachers). The following are the comments by coaches, which illustrate the changes in the dairy curricula and approach to teaching.

- The Dairy Challenge is where students work as a team like consultants who go to troubleshoot a farm – like the vet, nutritionist, the herd manager. They can be working together to evaluate a dairy.
- It is a systems-based approach and the students are looking at all aspects of the farm. It is key for training students who want to work in many different areas of the dairy industry. They have to understand a little bit about cow comfort. . .the financials of the dairy. . .and be a little broader based.
- The contests teach students how to manage times, be organized, and work as a team.
- The Dairy Challenge competitions also help the dairy industry thrive, building dairy leaders for the future. Many of the judges, moderators, and volunteers who help with the dairy challenges are industry representatives.
- Companies are looking at these students as potential employees because they know they are the best students and they are interested in working with dairy systems in the real world.
- Meeting more people in the industry will help in the future.
- Contestants leave this 3-day event with new skills not only in the technical aspects of dairy management, but also in teamwork, public speaking, time management, and other leadership competencies.

The following comments from students illustrate the value to the students and the dairy industry:

- There is not anything else like the NAIDC.
- Dairy Challenge is one of the most valuable things I did in college.
- By doing the Dairy Challenge, I was able to see things I learned in the classroom about nutrition, genetics, reproduction, and management.
- The value of a four-member team that have different experiences and areas of expertise is important.
- It amazes me of how interconnected every aspect of the dairy farm is.
- The Dairy Challenge becomes prioritizing recommendations for things that can get done immediately and changes that could improve profitability.
- There is no single educational event that is more relevant to the dairy industry that the NAIDC.
- The challenge prepared me for my current position as a nutritionist and consultant.

Today, Dairy Challenge strives to incorporate a higher-learning atmosphere with practical application to help prepare students for careers in the dairy industry. Since 2002, 2216 students have enhanced their dairy management, communication, and business and financial skills through participating in the 8 national contests and 20 regional events. The Dairy Challenge program in turn is generating highly qualified graduates to lead and further improve the dairy industry. To facilitate education, communication, and an exchange of ideas among students, agribusiness, dairy producers, and universities enhance the development of the dairy industry and its leaders. The program is funded through the generous support of 100–130 sponsors per year providing US$250 000–270 000 each year.

See also: **Business Management**: Management Records and Analysis; Roles and Responsibilities of the Manager. **Dairy Education**: Dairy Technology.

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Relevant Website

http://www.dairychallenge.org – North American Intercollegiate Dairy Challenge.

Dairy Technology

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Introduction

Specialized education and training programs in dairy science and technology are facing a number of issues in most developed countries. Industry demands that courses in dairy science and technology retain relevance to its needs, and that these courses equip graduates with the required skills to enhance the efficiency of production and the quality of the product. The education and training institutions that provide these courses are attempting to balance this requirement of industry with pressures from within the education sector; specialized dairy science and technology programs are seen by some within this sector as administratively and financially inefficient and not educationally essential. Both education and industry sectors purport to share a concern to attract high-caliber recruits to the industry but appear to differ when matters of implementation are concerned.

The provision of courses to service the needs of the dairy production or farm sector faces similar demands for relevance, for specialization, and for cost-efficient delivery. However, the situation for this sector has some significant differences in its approach compared to the processing sector.

There is debate as to appropriate strategies for balancing the demands of industry and the capacity of education to respond. This article attempts to outline the situation that exists in the provision of specialist dairy education, and analyze the prevailing influences that shape this situation. Models for the development of curricula are discussed as the basis for considering options available to industry and education in achieving a balance of interests in future dairy science and technology programs at all levels.

Current Trends in Dairy Education

Traditionally, specialist courses in dairy science emphasized the basic sciences that underpin dairy product manufacture and dairy production management. They included specific detail on the nature of dairy products, milk, cream, butter and related fat products, fermented milk products such as cheese and yogurt, dried and concentrated products, frozen products, and dairy desserts, and the industrial technologies in manufacturing dairy foods and in managing production.

Basic science courses in dairy production programs include chemistry, biochemistry, animal biology, plant biology, and microbiology, with specialist courses including dairy cattle evaluation, genetics and breeding, physiology of reproduction, physiology of lactation, ruminant nutrition, ration formulation, forages and pastures, and dairy farm management.

In an attempt to set a benchmark for dairy curricula, to be used by both educational and industry bodies, a group sponsored by the International Dairy Federation (IDF) in 2000 proposed typical training programs for dairy operators and dairy technologists. These programs are indicative of current practice in many countries, although variations can be expected as local industry and educational agendas are accommodated.

Modern dairy plants are large, automated, specialized production facilities. They exist as parts of complex businesses and organizations that frequently have interests in areas other than dairy. Within these plants and organizations, in common with many other professions, there is a call for graduates to develop generic skills such as communication, capacity to cope with and manage change, and an ability to lead and motivate staff. With the increasing rate of change within companies and the industry, employers are demanding greater flexibility in graduates. Adaptability is vital.

The technical demands on graduates are not diminished. Increasingly sophisticated technology requires greater depth and specialization along with greater breadth to achieve flexibility in matters relating to dairy science and technology as well as managing the workplace.

Dairy Studies at Degree Level

Of late, there has been a trend to incorporate specialist dairy education into more generic food science and animal science programs. Within universities and colleges, the extent to which the specific outcomes desired by the dairy industry have been reduced at the expense of the skills and knowledge seen to be relevant to the broader food industry or to the even broader disciplines of science or engineering causes concern. The pressures within the education sector originate from cost and resource efficiencies, not from specific industry requests. The IDF-sponsored seminar entitled 'The Future of Dairy Education' held in 1998 when addressing this issue concluded, "The professional competence required by the dairy industry is not adequately addressed within the general framework of food science education."

Specialist courses in dairy production have not existed separately in many countries in contrast to parallel programs in dairy technology. Candidates wishing to specialize in dairy production are normally able to take specific dairy electives within degrees or diplomas in agriculture, animal production, or animal science. Study areas such as animal breeding, nutrition and health, pasture management, and farm business management can be included as acceptable generic studies.

Various strategies have been adopted to compensate for this loss of specific dairy content in educational programs. In New Zealand, the Dairy Industry Graduate Training Programme provides an orientation course into the industry for graduates from science, technology, or engineering degrees. In other countries, similar pathways exist at Graduate Diploma or Vocational Masters level. It is the objective in each of these to provide the detailed and specific knowledge required by the dairy industry for graduates. Therefore, the move in undergraduate degrees toward generic studies is balanced by dairy-specific outcomes in a subsequent program of study. It should be noted that this approach leads to a formal and recognized qualification, generally within a university, and it constitutes a national industry approach to the problem.

At a company level, the lack of dairy-specific skills and knowledge of the food science graduate may be addressed through targeted in-house training courses designed to build on existing albeit generic expertise. Typically, such training has a narrow focus and generally does not lead to a formal qualification. However, such an approach has substantial appeal to a company because it equips the graduate with skills of particular significance to the company's needs.

The inclusion of dairy specialization within generic food science and animal science degrees is a strategy adopted by many educational institutions on the grounds of efficiency. A course in food chemistry or food analysis has a greater potential clientele than a course that focuses specifically on dairy chemistry or analysis of dairy foods. When universities and colleges are being required by funding bodies to do more with less, when minimum class sizes are prescribed, and when a greater diversity of customers and candidates has to be catered for, compromise is inevitable. Hence, programs that previously were referred to as dairy science are now hidden within food science and animal science programs with a consequential problem of a poor profile for prospective students at the same time as employers and educators ponder strategies to increase recruitment of graduates into the dairy industry.

Dairy or Food Processing Training for Operators

The tension between the needs of industry and the trend toward generic curricula is less significant in the area of skilled operator or technician training. Because the objective of training is more clearly focused on the skills specified by practices within the workplace, the needs of industry for competent operators are more readily defined and translated into training programs.

The strategies by which such training is provided are a function of the structure of the training system within a particular country. Variously, training of skilled operators may occur within training institutions or within the industry itself. Regardless of the delivery arrangements, there is a strong influence from industry as to the nature and content of training with a high level of skills assessment, often within the actual workplace.

While there does not exist the same tension between academic considerations of generic food science issues and the practical application of concepts and skills to production at skilled operator level, specific issues for the dairy industry may compete with issues that are seen to be generic to the food processing industry. In some countries, the extent to which the particular competencies prescribed by the dairy industry are compromised or diluted in the name of transferable skills for the food processing industry is a matter of concern.

What we see as structures for dairy education and training in different countries is therefore the result of various forces within industry, education and training, and government. These forces combine and interact as each of the vested interests attempts to influence the nature, the delivery, and the funding of courses in dairy science, production, and technology.

Processes in Curriculum Development

A major area of influence sought by industry and by education and training agencies is the development of curriculum for specific courses in dairy science, production, and technology. The problems outlined above arise from the conflict inherent in who has the major influence in constructing the curriculum and the premise upon which curriculum outcomes are based.

The process of curriculum development is best described by reference to two models that represent opposite and idealistic standpoints. In simplistic terms, these represent the industry-driven competency approach and the discipline-driven academic approach.

Industry-Driven Competency Approach

The emphasis in this approach is on assessment of industry-related outcomes, normally defined in terms of skills. Such outcomes may be referred to as competencies or performance-related outcomes that clearly specify what is required of the candidate as a functional employee in the workplace. A curriculum constructed on this approach has a substantial emphasis on assessment of practical skills with knowledge and understanding included as an attribute that underpins performance. This is distinct from a traditional academic curriculum where knowledge and understanding are prescribed, with the assumption that these lay the foundation for practical skills (**Table 1**).

This approach gained much favor in the competency-based movements that were part of many training reform programs in the late 1980s and early 1990s. A strong motivation in these reforms was articulated as a need to make training more relevant to the needs of industry. The associated rhetoric was critical of previous institution-based training as being irrelevant, theoretical, and out of date. Examples of this approach can be seen in the introduction of National Vocational Qualifications (NVQs) in the United Kingdom, National Dairy Industry Training Standards in Australia, and Company Competencies within Clover SA in South Africa. Each of these approaches used the term 'competency' as a key term to describe the nature of the skills and knowledge desired by industry as the training outcome.

The meaning of competence

The attractiveness of the term 'competence' has led to its adoption in other areas such as some higher education courses where it is applied to components of knowledge while ignoring the preeminent role of workplace performance. There is a need for caution when addressing courses that claim to be based on competence or competency standards. The term has become fashionable and hence as a result of wide usage has lost much of its precise meaning. Within the training sector, where it refers to artisans or craftsmen or skilled operators, the term normally refers to the minimum standard, measured in performance terms, for a task or operation that is a specified function for an employee at a given level of employment.

This approach, because of its close relationship to job functions, is well suited to on-the-job delivery and necessarily involves a significant amount of workplace assessment. Because the competency standards are derived through consultation with industry, the direction of the curriculum is set and controlled by industry and employers. Employee organizations such as unions have also endorsed this approach since it can be used as a relatively objective basis for employment conditions and for wage rates.

Support for the competence approach also grew from the claim that, as a consequence of defining a standard, recognition of a candidate's performance and ability was possible within a company, within a country, between companies, and globally.

The process of defining the competency standards is not without problem. Within a limited area of activity, it

Table 1 An example of competency-based criteria that can be used to build a University dairy technology curriculum (adapted from IDF Bulletin 358)

Level of demonstrated competency	*Background knowledge required*
Understanding of various milk processing technologies used to manufacture a wide range of dairy products	Structure and significance of the dairy industry
Proficiency in analyzing a wide range of dairy products and associated raw materials	Milk as a raw material; definitions of quality of dairy products
Operate, control, adjust, and maintain equipment used to produce, package and/or analyze dairy products	Principles and processes of unit operations and complex technologies used in the dairy industry
Implement and supervise appropriate cleaning and sanitizing procedures	Principles of equipment design, function, and maintenance
Oversee the maintenance of acceptable levels of personal hygiene by staff and of a safe workplace	Principles of procedures used for cleaning, sanitation, and hygiene
Organize, plan, and supervise the manufacturing program and its component tasks	Roles and responsibilities of a supervisor, the concepts of matching people to jobs
Develop, manage, and evaluate quality management programs and identify necessary action in quality control	The interaction of factors (technical, economic, environmental, etc.) in optimizing resources
Assess and advise on the use of resources, report and comment on successes and failures in production	Criteria of individual and corporate responsibility for producing safe and nutritious foods

is reasonably straightforward to define what skills and knowledge are required of an individual in the execution of a specific work function. Expressing this in terms that are acceptable across a number of workplaces within an industry requires that the standard becomes increasingly general. Terminology becomes necessarily vague and assessment of candidates becomes less objective. The applicability of the standard is lessened. This is demonstrated when food industry standards are applied to the dairy industry standards. It equally becomes problematic when industry standards are applied to both large and small plants where job functions are differently defined.

Implementation of competency-based programs

The implementation of competency standards has proven difficult because of the expense associated with individualized practical assessment. It has not alleviated the inevitable clash between training priorities and production deadlines, a factor that continues to limit a training culture within many companies and workplaces. It is also understandably hard within a company or workplace to develop supervisory staff with skills in an area of human development such as training when they have been employed for their skills in the technical and logistical issues of production.

On the positive side, the introduction of competency-based assessment within the workplace has made training more accessible to people who would otherwise be unable to participate because of the demands of day-to-day employment. It has also provided a mechanism by which management can integrate training and skill development with the enhancement of product quality and company culture.

The concept that competency standards could act as the foundation for recognition on a broad scale has been put forward. A number of European countries came to an agreement as to the skill base for employment at certain levels of employment, which led to the introduction of the Europel, the European Dairy Passport. Such a mechanism allows trainees to move between states with due recognition of their qualifications. In a similar initiative, the New Zealand dairy industry is in the process of adopting the Australian Dairy Industry Competency Standards, with minor amendment, as the basis of their industry qualifications for skilled operators. It should be noted that the success of such arrangements for cross-recognition depends on there being considerable similarities between the industries concerned. It should also be noted that there are a number of companies with interests in participating countries.

A similar attempt to develop global competencies for the dairy industry was undertaken by a former Group of Experts F14 within the IDF. The problems of defining specific workplace competencies were compounded by the diversity that exists between companies and countries and the various social, political, and educational contexts that influence the practice of industry training. The results of this work were published in the *IDF Bulletin 358* and provide recommendations to industry and to educational agencies as to the broad outcomes of training at the levels of dairy operator and dairy technician. While they are referred to as ‘Minimum Competency Standards’, they cannot be regarded as the precise statements that would be required by assessment schemes such as NVQs in the United Kingdom. They will, however, act as guidelines for the nature of skills required at a particular level as well as outlining the knowledge and understanding considered to be important underpinning the required skills.

Some educators are fearful of the competence approach lest it leads to the training of automatons. Some early versions of competency-based assessment took the view that if an operator could satisfactorily execute a task, this constituted competence. Concern was expressed that under this regime of assessment, a candidate could perform tasks by rote memory with neither understanding of the implications of their actions nor knowledge of the reason for certain actions, and hence they would have no capacity to adapt to contingencies. For this reason, underpinning knowledge and understanding have become essential parts of recent competency standards.

The competence approach can be understood as a response by industry to an academic preparation that lacks practical skill. It reflects the fact that the academic approach has in some situations failed to deliver the specific and immediately relevant outcomes desired by industry.

Discipline-Driven Academic Approach

It has been noted earlier that the industry requires greater technical depth in the graduates that it recruits. In addition, it is asking for skills in people and production management combined with the talents of adaptability and innovation. It is therefore understandable that those planning undergraduate programs seek to add more of the food and dairy science that is the basis of product innovation and manufacture. With increasing levels of sophistication in the equipment in production plants and laboratories, there is more for students to know if they are to be informed of the processes that they are being asked to manage.

Whereas the competency-based approach asks the question “What does the candidate need to be able to do to be regarded as a competent employee?”, the academic- or discipline-based approach is more likely to ask “What does a dairy science graduate need to know to cope with the demands of sophisticated technologies?”. This approach is exemplified in many articles attempting to define what is needed at operator, technician, or scientist level within the industry. It is described in terms of subjects and disciplines required rather than outcomes, behaviors, or competencies (**Table 2**).

Degrees and disciplines

It is pertinent at this point to observe that the discipline-driven approach is more prevalent at degree level whereas the competence approach appears to operate with some success at vocational (certificate and diploma) level. There is a concern expressed by industry that the academic discipline emphasis of degree programs risks producing graduates who have little appreciation of the practicalities of the workplace. Strategies such as industry placements during undergraduate courses are attempting to address this problem.

It can be argued that the academic discipline approach has contributed to the process by which specialist dairy courses have been subsumed into food science degrees. There is a substantial amount of common curriculum material that is relevant to generalist food degrees and specialist dairy degrees. Costs and administrative efficiencies make a compelling argument to combine courses even though the theoretical knowledge as well as the practical skills of such things as cheesemaking is not highlighted and hence the profile of dairy is diminished.

The changing nature of the industry exacerbates this problem. Many companies that previously manufactured only dairy products are now diversifying into other foods. An adaptable graduate trained in the scientific principles that apply to many foods and their technologies may be more attractive to such a company than a dairy specialist.

Many universities and colleges have attempted to include industry in a consultative process to determine the curriculum for undergraduate programs. Given the competitive nature of the industry, and the rate of change that is evident, such consultations often lead to frustration and bear little fruit. However, there are examples where productive interaction between the requirements of industry and the academic rigor of universities and colleges has proved successful. Companies have diverse policies with regard to employment of graduates. Their differing strategies have implications as to what companies are seeking in graduate recruits. Obtaining a coherent and cohesive mandate for the construction of a course from industrial parties who are clearly in competition with each other is therefore very difficult. The factors that lead to a successful outcome are complex and the achievement of an acceptable curriculum requires a significant effort on the part of both industry and academia.

Table 2 A model curriculum for a 4-year university degree in dairy science/technology (adapted from IDF Bulletin 318)

Subject areas, as percentages	*Study areas*
General subjects – science, economy, humanities, other prerequisites (40%)	**Chemistry** – general, organic, biochemistry **Physics** – mechanics (hydro-, thermo-) **Mathematics** – (calculus); statistics; computers **Biology** – general, introductory microbiology **Nutrition** – general, human **Economics** – micro- and/or macro- **Communications** – speaking and writing skills
Dairy science "core" subjects (40%)	**Dairy** (and/or food) **chemistry** incl. **laboratory analysis** **Dairy/Food microbiology** incl. **sanitation and dairy starter cultures** **Engineering principles of dairy processes** **Dairy technology** (fluid, concentrated, dried products, butter, and ice cream science) **Dairy fermentations** **Cheese and whey technology** **Dairy husbandry** **Dairy product quality evaluation**
Electives – dairy, food, other (15%)	**Emulsion science** **Process control** **Waste treatment/environmental engineering** **Food law** **Food toxicology** **Food preservation** **Biotechnology** **etc.**
Graduation project (5%)	**Integrated final project, product development, research, interdisciplinary activity, etc.**

Academic institutions such as universities that have strong research capacity and hence hold a significant amount of expertise in dairy science are well equipped to develop curricula that represent the current state of knowledge in the discipline. It is therefore reasonable to believe that they have the expertise to construct the academic syllabus that adequately prepares graduates for the dairy industry of the future. The challenge remains to connect with the industry in productive and practical ways to ensure relevance.

Postgraduate education and research

The strong research emphasis of universities has the potential to contribute to education of postgraduates in matters related to dairy science and to enhance the analytical and investigative skills of research workers. Collaboration between industry and academia in research is widespread and generally productive. The fact that such collaboration is productive is understandable because the matter under investigation is the prime concern of industry while the academic agenda for the university consists of issues of academic process. The curriculum for a research student is largely skill-based and set by the university, and the content is the problem that is of significance to the industry sponsor.

Issues of confidentiality, a concern for a commercial company sponsoring research, may limit the extent to which postgraduate students are free to discuss or publish their work. In addition, commercially sponsored research may be constrained by economic considerations of what a company believes is worth pursuing. However, the academic processes and requirements set down for examination of research candidates are seldom an issue between industry and universities. At this level, little if any tension as to who determines the curriculum is apparent.

Short courses

Many education and training organizations offer a multitude of short courses in dairy production and in dairy technology, primarily for people in full-time employment in industry although others often participate. These courses have a significant emphasis on content, often with minimal attention to practical skill development, because they arise from specific requests from industry for programs that bring employees up to date with specific technologies or innovations. The facilities and time available, which ranges from a couple of days to 2 or 3 weeks, limit the chance to develop and refine substantial levels of workplace skills.

There is little tension in these programs as to who sets the curriculum. Industry and short-course participants are recognized as customers with specific and clearly defined requirements. Education and training providers, having identified these requirements, customize the programs accordingly and the test of this tailoring is demonstrated by repeat business and customer approval. Because such courses constitute a mechanism for education and training providers to augment their income, they are a clear example of where the two parties have learned to work together productively in setting the curriculum.

A Future for Dairy Education?

Given the trends and pressures discussed, it is relevant to pose the question whether there is a future for specialized dairy education. The answer may not be a straightforward affirmative or negative, but a compromise strategy that permits specialization within a broader, more generic, food or animal science education framework. There is an interest and imperative for both industry and the education and training sector to discover a mutually satisfying approach. This mutual discovery requires certain things to happen.

First, the industry, at least at national level, needs to agree as to its needs and priorities for education and training of its existing and future personnel at the level of operators, technicians, scientists, and managers. This has been shown to be possible at operator level in the development of competency standards in a number of countries, and at higher levels in some specific companies.

Clearly, these training and education needs would best be developed in conjunction with education and training providers so that programs developed not only connect with industry but also enjoy recognition within academic circles. The linking of training and education at various levels is an essential tool for the ongoing professional development of people within the dairy industry. Pathways of progression are important to an individual's aspirations and can bring great benefits to companies who choose to invest in people.

Given the rapid rate of change in industry, the partnership between industry and education must be ongoing if courses at all levels are to remain relevant to industry needs. Such a partnership may inform both parties on possible improvements to course content, on innovations for more tailored delivery of courses, on addressing issues of resource requirements, and on shifts in the direction of training and education in light of industry changes.

The challenge remains to harmonize industry needs with intellectual rigor in the face of the inescapable economic imperatives for industry and the constraining policies in the educational sector. This challenge will continue regardless of the structures that are devised to provide the type of education and training that the dairy industry needs. The challenge will also be satisfactorily met only if both industry and education agree that they share an interest in determining the dairy curriculum.

See also: **Business Management**: Management Records and Analysis; Roles and Responsibilities of the Manager. **Dairy Education**: Dairy Production.

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DAIRY FARM LAYOUT AND DESIGN

Building and Yard Design, Warm Climates

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Introduction

There is a wide variety of building yard and other infrastructure designs in common use on dairy farms in warm climates. Several factors are important in determining the most suitable design. Some of these include:

1. The predominant forage source. Farms using grazed pastures require different facilities to those using conserved forages (silage, hay or by-products).
2. Heat stress in extreme climates demands facilities such as shade shelters, cooling barns, fans and water sprinkling, while grazing farms in more temperate regions require few such facilities.
3. Farms in extremely wet environments require facilities to avoid pasture damage and maintain animal health, particularly of young stock, in wet weather.
4. Labor considerations particularly including the cost of labor are another important factor. Substitution of capital for labor can be very economic where labor is expensive. Similarly family farms often have very different facilities to large farms owned corporately.
5. Smallholders commonly undertake dairy farming in developing countries, often with quite rudimentary facilities.

Milking Systems

With minor exceptions, machine milking systems tend to fall into three basic types commonly known as walkthroughs, herringbones and rotaries.

Walkthroughs

Walkthrough dairies (sometimes called abreast parlors or flat barns) were used extensively in the development of pasture dairy farms in Australia and New Zealand between 1940 and 1980. However they are labor intensive as performance is limited, usually to less than 50 cows per operator hour, by the amount of walking, bending and stooping. Walkthroughs are usually limited to herds under 80 cows (**Figure 1**).

Herringbones

Herringbone dairies are the most popular system, particularly for herds of from 50 to 250 cows. They come in many variations including swingover (one cluster per two cow positions), doubled-up (one cluster per cow position), conventional (30°–45° cow angle), close-spaced (60°–90° cow angle) while some of the more modern variations include stall gates and rapid exit mechanisms (**Figure 2**).

Various milking machine configurations are available in herringbones including high milk line (1.5–1.8 m above the cow platform), mid-line (1.2–1.5 m) and low-line (0.3 m below the cow platform). Low-line herringbones must of necessity be doubled-up systems.

Close-spaced herringbones offer reduced walking for the operator and better supervision of the milking process (**Figure 3**). However, this more compact design has reduced ability to hold cows in their correct bail positions sometimes leading to stressful milking and perhaps making the cows reluctant to enter the milking area.

Stall gate mechanisms are now commonly installed in close-spaced herringbones to improve cow flow and to reduce both operator and cow stress during milking, particularly when concentrate feeding during milking is practiced. In stall gate equipped sheds, cows exit via the end of the platform as in a conventional herringbone. Various mechanisms are used to either lift or lower the stall gates as the cows leave. Each stall is usually equipped with a small gate at the pit end, which prevents cows entering incorrect cow positions. Each stall gate is opened

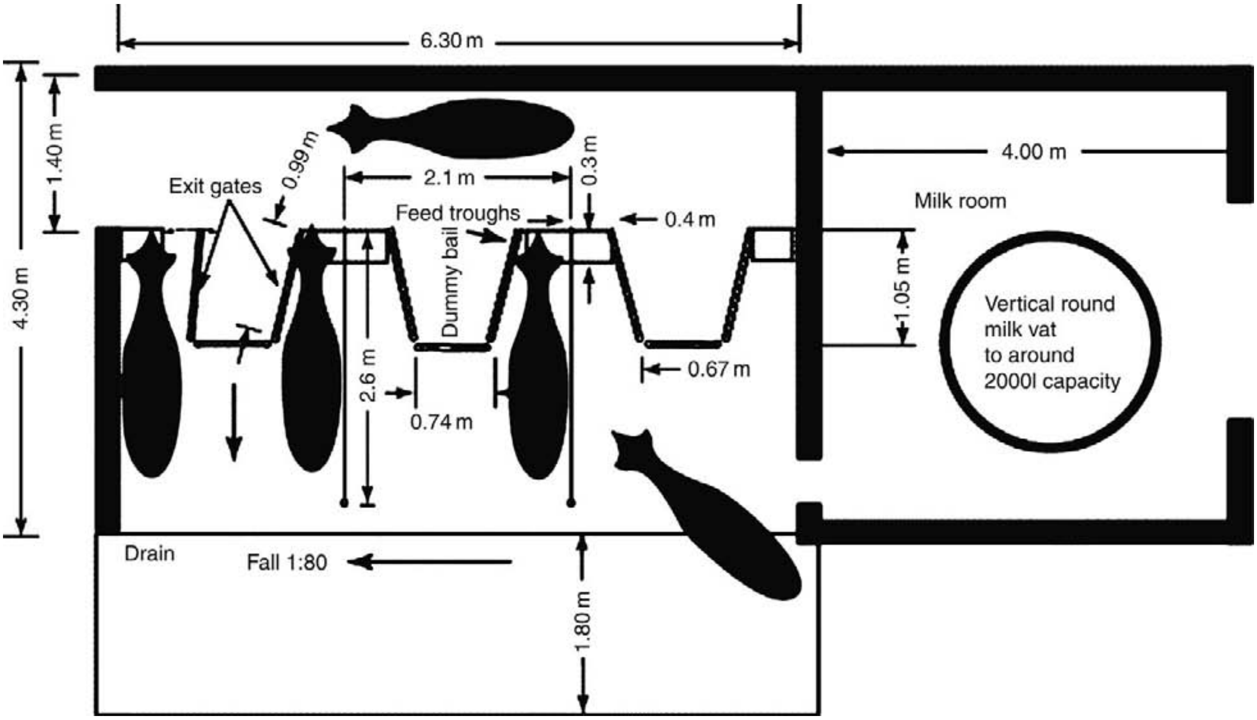
6.30 m
1.40 m
4.30 m
0.99 m
Exit gates
2.1 m
0.3 m
Feed troughs
Dummy bail
0.4 m
4.00 m
Milk room
1.05 m
Vertical round milk vat to around 2000l capacity
2.6 m
0.74 m
0.67 m
Drain
Fall 1:80
1.80 m

Figure 1 Three doubled-up walkthrough.

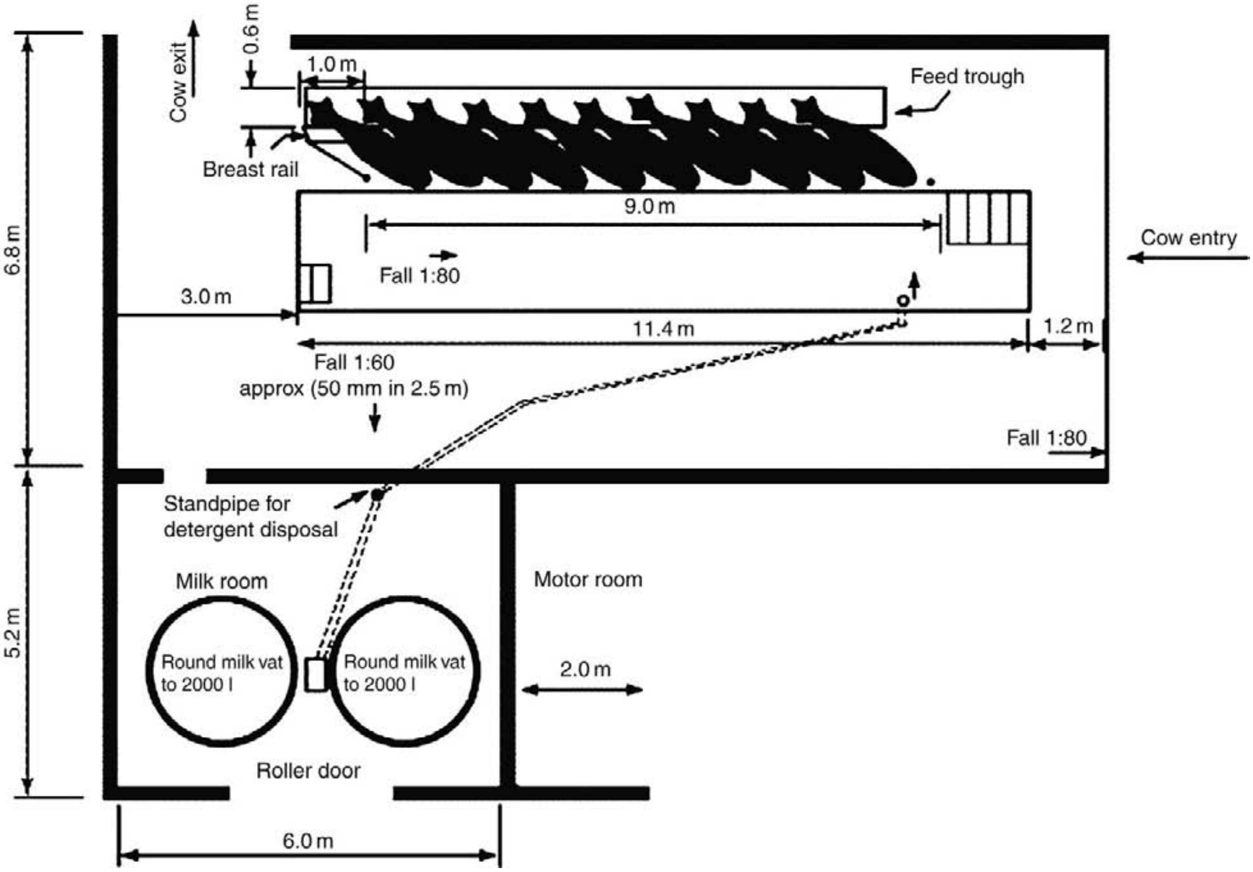
Cow exit
0.6 m
1.0 m
Feed trough
Breast rail
6.8 m
9.0 m
Cow entry
Fall 1:80
3.0 m
11.4 m
1.2 m
Fall 1:60
approx (50 mm in 2.5 m)
Fall 1:80
Standpipe for detergent disposal
Milk room
Motor room
5.2 m
Round milk vat to 2000 l
Round milk vat to 2000 l
2.0 m
Roller door
6.0 m

Figure 2 10-a-side swingover herringbone (900 mm spacing or 45°).

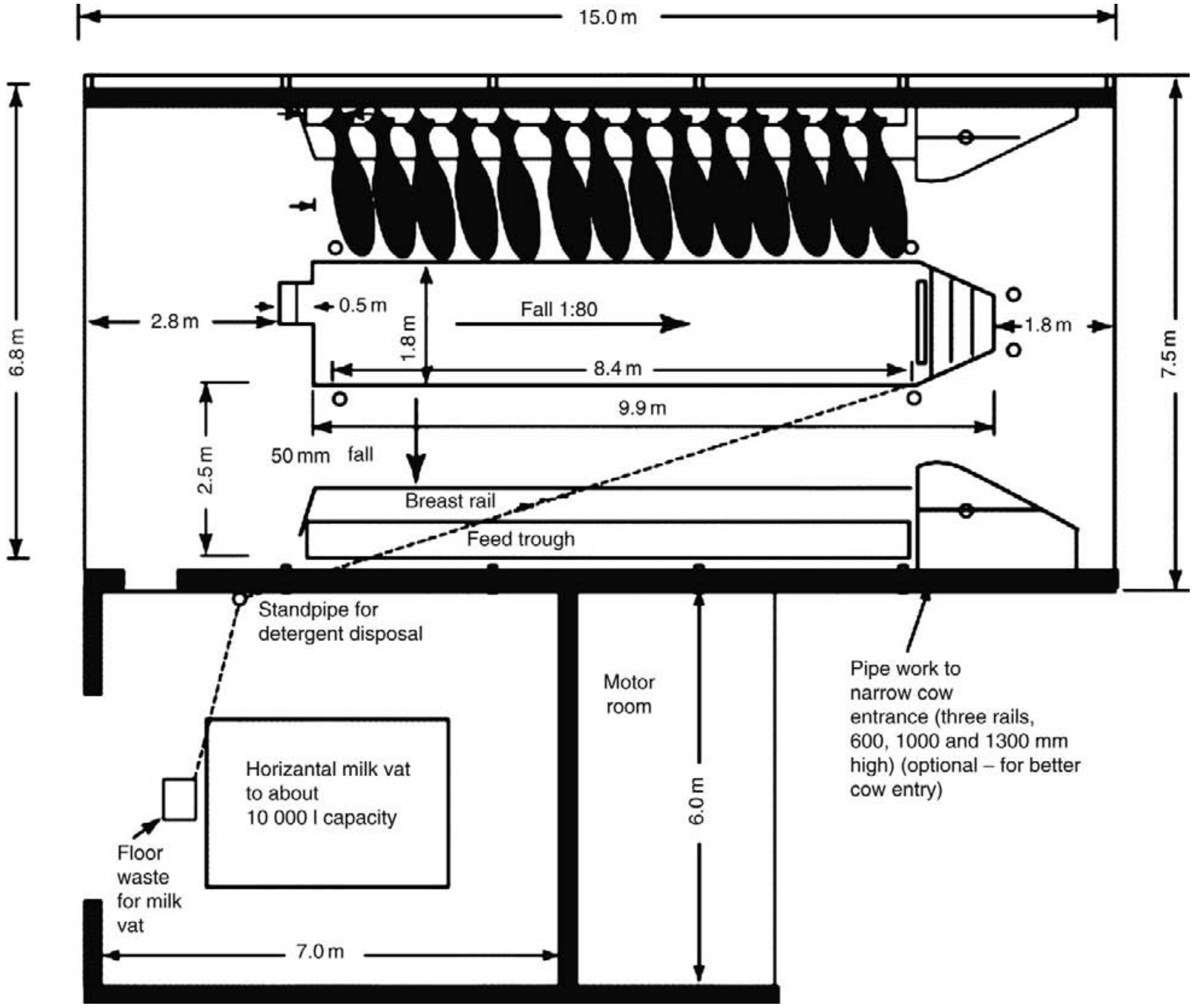
15.0 m
6.8 m
7.5 m
2.8 m
0.5 m
1.8 m
Fall 1:80
1.8 m
8.4 m
9.9 m
2.5 m
50 mm fall
Breast rail
Feed trough
Standpipe for detergent disposal
Motor room
Pipe work to narrow cow entrance (three rails, 600, 1000 and 1300 mm high) (optional – for better cow entry)
Horizantal milk vat to about 10 000 l capacity
6.0 m
Floor waste for milk vat
7.0 m

Figure 3 14-a-side close-spaced swingover herringbone (600 mm spacing or 80°).

sequentially by the action of the cow entering the previous bail position (**Figure 4**).

In recent years, rapid or side exit herringbones have become popular. Such sheds also often have stalls although the stalls are sometimes fixed permanently to the floor. Cows exit directly through the side of the herringbone as the breast rail and feed trough is lifted to about 1.8 m allowing the cows to pass underneath. Breast rails and feed troughs are sometimes lifted vertically, swung up like a pendulum while another design has duplicate breast rails and feed troughs which are rotated. Hydraulic, mechanical and pneumatic operating systems have been used. Sometimes the breast rail is lowered and cows step over. **Figure 5** shows two rapid exit systems.

Herringbones can milk up to 200 cows per operator per hour. Performance is maximized when each operator handles about 20 clusters, perhaps with the aid of automatic cup removers.

Rotaries

Rotary milking systems have been popular since 1970 in Australia and New Zealand particularly for herds of over 250 cows. Various types have been constructed including the carousel (or rotary tandem), rotary herringbone and, by far the most popular, the turnstyle (or rotary abreast).

The carousel has the cows standing nose to tail on a circular, moving platform while the rotary herringbone has the cows standing herringbone fashion in a similar manner. In both cases, the operator works inside the circle while the cows enter and exit outside the circle. While some operators with good stock handling skills achieve excellent performance in rotary herringbones, many sheds do not achieve their design capacity because of difficulty in encouraging cows to enter and leave the platform.

The operator working inside the circle is in a good position to handle the clusters but in a very difficult

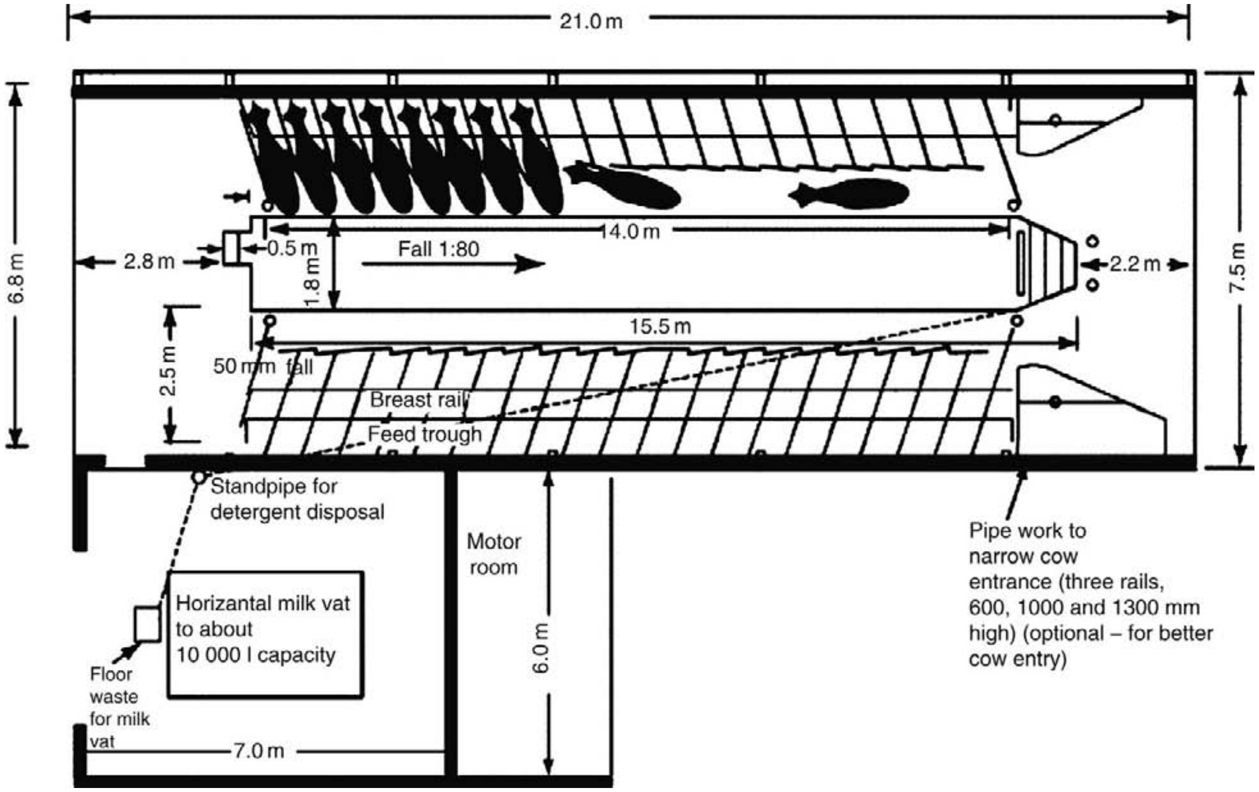
21.0 m
6.8 m
7.5 m
2.8 m
0.5 m
1.8 m
14.0 m
Fall 1:80
2.2 m
15.5 m
2.5 m
50 mm fall
Breast rail
Feed trough
Standpipe for detergent disposal
Horizantal milk vat to about 10 000 l capacity
Floor waste for milk vat
7.0 m
Motor room
6.0 m
Pipe work to narrow cow entrance (three rails, 600, 1000 and 1300 mm high) (optional – for better cow entry)

Figure 4 20-a-side herringbone with stall gates (700 mm spacing).

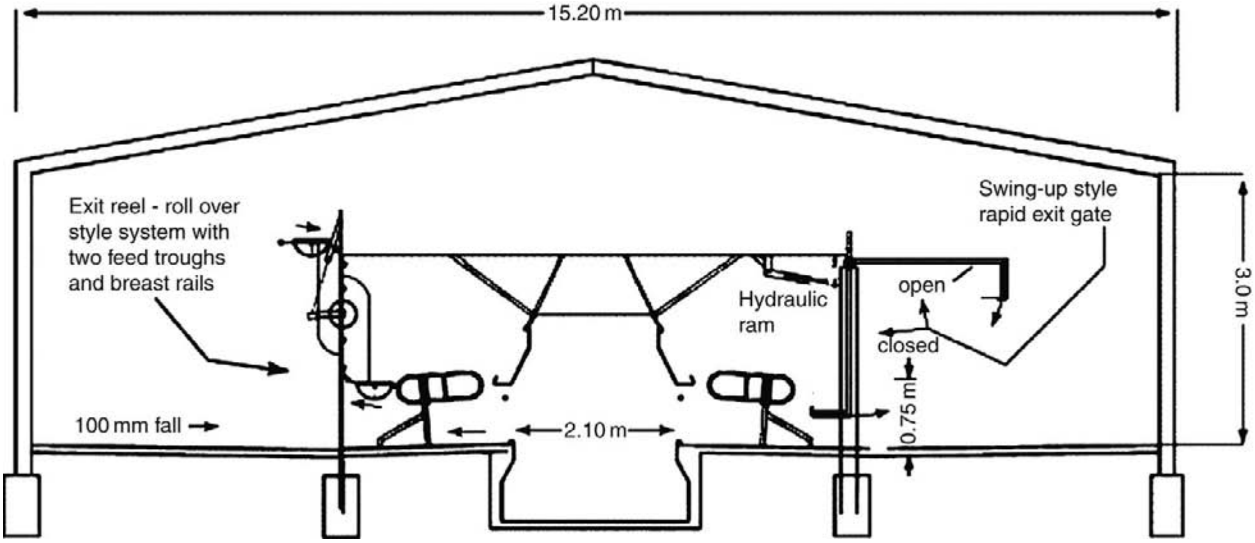
15.20 m
Exit reel - roll over style system with two feed troughs and breast rails
100 mm fall
2.10 m
Hydraulic ram
open
closed
0.75 m
Swing-up style rapid exit gate
3.0 m

Figure 5 Cross-section of a rapid exit herringbone.

location to supervise or assist cow entry and exit. The rotary herringbone is no longer popular in most warm climate countries.

Turnstile rotaries are very popular in Australia. Cows walk directly on to the platform, complete their rotation and then back off and turn to pass through an exit lane (**Figure 6**). The direction of rotation is not critical. The cups-on operator (or operators) stands near the cow entry point while the cups-off operator stands near the exit point and also handles teat disinfection. Turnstiles tend to need at least two operators and neither operator is in a good position to supervise the mid-phase of milking. Single-operator turnstiles are theoretically possible with automatic cup removers and

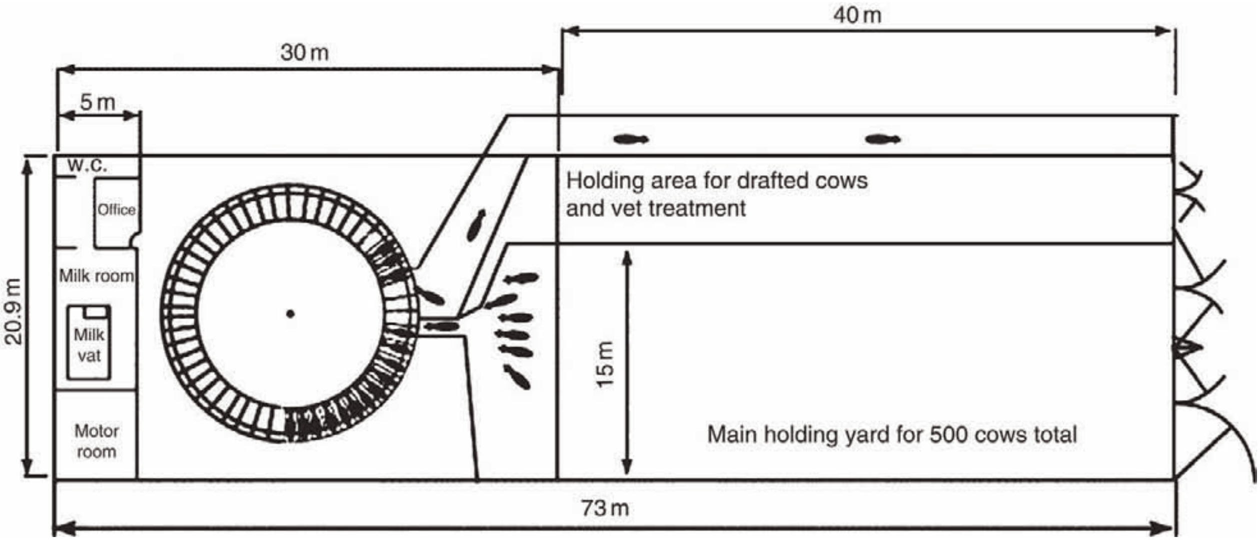
40 m
30 m
5 m
W.C.
Office
Milk room
Milk vat
Motor room
20.9 m
Holding area for drafted cows and vet treatment
15 m
Main holding yard for 500 cows total
73 m

Figure 6 50 bail turnstyle rotary milking system.

automatic teat disinfection but are not yet popular. Performance ranges from about 100 cows per operator hour in multi-operator sheds up to theoretically 300 cows per operator hour in a single-operator shed. All rotary platforms suffer from a lack of flexibility and many of the earlier installations proved too small to handle increasingly large herds. Few rotary platforms with fewer than 40 stalls are constructed today while very large turnstiles up to 120 stalls are available.

Corrosion was a problem with some of the earlier steel platforms. Since 1980, most rotaries have concrete platforms, usually turning on wheels or bearings although some concrete platforms float on water.

Comfortable Milking Environment

The milking system should provide a comfortable environment for both the cows and the operator. Milking machines in poor condition or poorly adjusted cause cow discomfort and teat damage. Cows enter a well-lit open milking facility more easily than a closed-in dark facility. Cows (and operators) are more at ease in milking systems which provide a constant and repeatable environment from day to day and which are arranged so that crowding, bullying and competition for in-bail feed are reduced. The turnstile rotary and herringbone systems with stall gates are good examples.

Factors important in operator comfort include pit depth (0.7–1.0 m, deeper in recent years) and ease of access to both milk room and holding yard. Lighting is obviously important and pit flooring which offers some flexibility (such as interlocking comfort tiles) reduce back aches and leg cramps. Reducing the reach to the cows will also reduce back strain. Operator safety is important. Adequate shielding with kick rails and belt guards is necessary. Some protection from dung and urine splash will reduce the chance of disease cross-infection, particularly leptospirosis, for the operators.

Stray Voltage

An unavoidable consequence of mains electricity distribution in many countries using the multiple-earthed-neutral (MEN) system is the possibility of small potentials on neutral wiring. Such potentials or stray voltages are readily transferred to the milking machine and bail pipework and can be felt by operators and cows during milking. Cows are reported to be able to feel potentials of 1.0 volts. Stray voltage can cause cows to be difficult to manage during milking and to baulk at shed entry. Although often blamed for milk production losses, mastitis and somatic cell count increases, stray voltage is not a proven factor under such circumstances.

Stray voltage can not be eliminated but a milking shed can be built so that neither cows nor operators can feel the electricity during milking. Such an equipotential plane can be created so that all the cow and operator contact surfaces are at the same potential. Usually this involves welding the reinforcing steel mesh in the cow platform and pit floor to the metal milking bail supports and consequently connecting to the milking machine. More complicated systems involve a steel or copper rim (30–50 mm × 1.6 mm) also connected to reinforcing, inserted vertically in the concrete at surface level around the cow platform to avoid surface electrical flow. A more gradual potential gradient or voltage ramp between cow platform and holding yard may also be necessary (see **Milking Parlors**).

Holding Yards

Yards for holding cows prior to milking may be rectangular or round in shape. Holding yard surface area needs to be from 1.0 to 1.5 m^2 per cow. Round yards are popular in New Zealand and can be half circle, three-quarter circle or full circle. Rectangular yards, usually 6–9 m wide, are more popular in Australia although yards for herds of 500 cows or more may need to be around 15 m wide. Both types of yard may be equipped with a backing gate. Solid pipe backing gates work best in round yards. Cables mounted over rectangular yards often carry light electrified-curtain-style backing gates.

Concrete surfaces in holding yards and any cow traffic areas must be finished with suitable rough pattern to avoid cows slipping. Holding yards should fall in one direction (usually lengthways in a rectangular yard) at 2.5–5% slope for easy cleaning.

Holding yards can be cleaned easily by hosing. High volume (minimum 230 l min^{-1} – measured at the end of the hose) systems are recommended. Flush or flood cleaning systems have become popular in recent years, particularly for rectangular yards. Such systems consist of a tank (capacity 500 l m^{-1} of yard width minimum) and a gravity delivery system, perhaps three or more (150 mm diameter) pipes and valves or a single (300–400 mm diameter) pipe with a suitable directional flow apparatus. Yard slope is particularly important for flush cleaning with the optimum at 3.0% slope in the lengthways direction and no crossways slope.

Holding yard fencing traditionally is constructed from galvanized iron pipe, commonly 50 mm nb posts and four to six rails (38 mm nb), although a combination of tensioned wire or cable and pipe (top rail only or top and mid rail) is more economical.

Machine Cleaning

Milking machines may be cleaned using several different systems. The simplest is the bucket or flush system using buckets carried manually with solutions flushed by vacuum through the machines. Surging, by periodically plunging and withdrawing clusters from the bucket, is necessary for good cleaning.

Larger machines are often cleaned with recirculation systems called jetter or third-line cleaning systems. Clusters are placed on jetters which are connected to an extra or third stainless steel line to carry cleaning fluids from a wash drum. This system has the capacity to recirculate cleaning solutions. Surging is necessary to create turbulent flow for good cleaning and to wash adequately the top of large milk lines. Surging is usually achieved in modern plants with an air injector (or flushing pulsator) which admits large quantities of air periodically into the machine during cleaning. It is possible substantially to automate jetter cleaning and such systems are regularly installed in modern dairies.

Reverse flow cleaning, where both hot and cold cleaning fluids are pumped through the milk chambers of the machine and allowed to run in the reverse direction through the teat cups, was popular in the 1970s and 1980s in Australia. Although the systems offer extremely fast and labor-efficient cleaning for large installations, popularity has waned in recent years because of large hot water and detergent usage and poor milk quality results, particularly when used with hard water supplies.

Effluent Management

Environmental considerations demand that wastewater from a milking shed is sustainably managed. Two rules of thumb often apply. Milking shed wastewater should remain on the farm at all times in any weather and the effluent should be spread evenly over an area of sufficient size so that the fertilizer nutrients in it are spread at rates consistent with good agricultural practice. Practical systems of managing effluent to satisfy these requirements include fresh effluent and ponded systems.

Fresh effluent systems consist of a manure sump, preferably preceded by a stone trap, which collects the washings. The sump is equipped with a solids-handling manure pump and, to ensure even distribution, a flexible delivery hose and manure sprinkler. For more labor-efficient operation, some systems have a low-pressure travelling irrigator while others have a trafficable solids trap that can be emptied with a tractor and front-end bucket. Fresh effluent systems optimize nutrient-return to pasture but are more labor intensive.

Ponded systems usually consist of one or two ponds. Ponds are best situated so that wastewater can easily enter by gravity. Single ponds must be sized carefully. Important factors include the volatile solids load (dependent on the number of cows involved and the time of manure collection), a sludge allowance (capacity to hold the end products of anaerobic digestion) and a water balance (based on the water used and the climatic conditions). Two-pond systems (often referred to as anaerobic and aerobic) offer a higher degree of effluent treatment, particularly useful when recycling washwater for flood cleaning. Ponds accumulate washwater for subsequent irrigation and need to be sized so that water can be held through sustained periods of wet weather. Irrigation of ponded water is less troublesome than fresh effluent as the solids level is greatly reduced. However, manure nutrients (particularly nitrogen) are lost during the ponding process.

Strategies to Minimize Heat Stress on Pasture Dairy Farms

The possible gains in feed intake, milk yield, milk composition and reproduction which can be obtained through minimizing heat stress have been accurately demonstrated and are available on CD-ROM in a format which allows easy calculation of the benefits of investment options to reduce heat loads.

Shade

The provision of shade is the most effective strategy to minimize heat stress. Galvanized iron shade structures for cows can be profitable but only if feed is available so that cows can feed when cool.

Shade can be provided by protected tree plantings on the northern side (in the southern hemisphere) of paddocks and deciduous trees along laneways. Trees may be planted on the western side of the holding yard to shade cows during the hot afternoon milking. Water troughs should be available in all paddocks preferably close to shade. It is particularly important that a water trough is available on the exit side of the milking system where cows may consume up to 40% of their daily water intake.

Water Sprinklers

Periodic wetting of the cow‘s coat with water sprinklers is also effective in reducing body temperature, particularly when combined with shade. Sprinklers should have large droplets and operate periodically to apply 1–2 mm water in 3 min in a 15 min cycle. Sprinklers may be installed in the holding >yard to cool cows for 30 min before milking and at other times during the day, when respirations are above 70 breaths min^{-1}, if the cows are close enough to the dairy. The holding yard concrete should be wet before the cows enter.

Ventilation

Natural and forced draft ventilation can also be effective in cooling cows particularly when combined with shade and sprinklers. Fans capable of moving 5–6 m^3 min^{-1} of air can be installed in holding yards and milking areas.

Herd Management

Herd management can also be varied in hot weather to alleviate heat stress. Mating programmes can be deferred from the summer months and heifers, rather than mature cows, should be calved during summer. Mustering and the afternoon milking should be delayed until the cooler part of the day. A diet with a higher energy concentration, less rumen-soluble nitrogen and higher sodium intake will benefit heat-stressed cows.

Feed Pads for Predominantly Pasture Farms

Many predominantly pasture farms have pushed production per cow to a level that can not easily be obtained from pasture alone or even from pasture plus concentrates fed in the dairy. Components of a ration of high nutrient value such as crops and by-products can be fed on a feed pad to increase nutrient intake and milk production.

Advantages of Feed Pads

Feed pads are constructed to reduce feed wastage, mastitis and lameness. Good feed pads also minimize heat stress and pasture damage and protect the environment.

Reduced feed wastage

Silage, hay, grain and by-products are all expensive materials and it common to waste up to 23% of such material when feeding along fence lines or straight into a pasture. A good feed pad can reduce the wastage to 5%, sufficient for up to 2 l day^{-1} additional milk production.

Cleaner udders and reduced mastitis and lameness

Concentrating cows together for extended periods on poorly setup feed pads usually leads to a buildup of manure mixed with mud. Udders get muddy leading to laborious udder washing and slow milking shed throughput unless additional labor is available. Such conditions are ideal for the growth of pathogenic bacteria and several mastitis pathogens (particularly *Streptococcus uberis*) can be expected in large numbers. During extended periods of dry weather, the risk of infection seems low, but during and after rain, the infection risk is higher particularly if the cows lounge in mud and manure.

Standing in mud softens cows' hoofs leading to lameness, particularly if stones are present to cause bruising. Good feed pad design is aimed at minimizing such problems.

Minimized heat stress

A prime reason for the construction of many covered feed pads in Northern Australia is to minimize heat stress.

Reduced pasture damage

A combination of high stocking rates and extended periods of wet weather can cause significant pasture damage from pugging and general traffic. A feed pad provides an alternative place for the cows to stand while also maintaining nutrition.

Environmental protection

Feed pads congregate large numbers of animals on to small areas, creating potential environmental problems. Earth or gravel areas around feed troughs always contain large amounts of manure and are high-risk areas for pollution of both surface and ground water. Good feed pad design will contain all potential pollution sources and present an attractive appearance to help maintain the clean, green image of dairy products.

Types of Feed Pads

Feed pads can be low cost (under A$10 per cow), medium cost (A$10–100 per cow) and high cost (over A$100 per cow).

Low cost

Feed pads in this category could include feeding out straight on the ground, along fence lines or roadways. Hollow logs, secondhand bath tubs, water troughs, round bale feeders and used conveyor belting from the mining industry can all be used. While these systems may be cheap, they usually have high wastage and thus hidden costs. Wet weather will turn feed pads on most natural surfaces into a quagmire. Although the cost may rise to the higher end of this category, a compacted gravel base for such a system is a sound investment. Gravel feed pads should fall away from the feed troughs at 2–4% to reduce mud accumulation and minimize possible odors.

Bath tubs, old tractor tires or round bale feeders are good for systems that use a tractor and loader bucket to feed out. Used conveyor belting on a compacted gravel base is probably the best of the low-cost systems for use with a feed-out wagon. A small depression can be left in the base to partially form the belt into a trough and the edges can be raised up further with star pickets or by tying the sides together.

Medium cost

Feed pads in this category could include precast concrete troughs as commonly used in beef feedlots. Such troughs are usually designed for one-sided feeding of high concentrate rations and often lack sufficient capacity for two-sided feeding of bulky forage rations. More elaborate conveyor belt systems with metal supports, a simple flat cement pad without any cover, and purpose-built precast concrete troughs for two-sided feeding all have been used.

Medium-cost feed pads often have a concrete floor and are often planned with view to covering with a shed later. Orientation must be considered when planning such a pad and if flush cleaning is ever planned, the pad should fall at 2–3% in the longitudinal direction.

High cost

High-cost feed pads are usually covered with a shed. The more economical pads have either one or two troughs while more expensive systems may have a central feeding alley, through which the tractor and forage wagon can be driven.

Troughs or a Central Feed Wagon Alley?

Trough design for dairy feed pads has been poorly researched. Initial investigations suggest a trough with about 1.5 m minimum internal width for two-sided feeding with sides at least 400 mm high. Cows may easily become cast or trapped in narrow feed troughs should they fall or be forced into them. Wider troughs or some system of a moveable trough, which can be tipped over with a front-end loader to get a cow out of such a situation, can be at least a partial solution. Some feed troughs can only be cleaned out manually which can be a real chore should cows reject significant quantities of feed.

Many experienced farmers consider that a central feeding alley through which the tractor and feed wagon can be driven is essential. Such a system allows very easy cleaning out of surplus or rejected feed allowing a completely fresh ration to be fed each day. However some system of pushing feed back to the cows is necessary. A simple blade attached to a quad motorcycle can be used. The central feed wagon alley also allows the installation of self-locking stanchion gates which can be a boon in capturing cows for artificial insemination or herd health purposes.

Loafing Areas

The primary purpose of most Australian feed pads is usually as a feeding system sometimes combined with a roof to minimize stress from heat or wet weather. As such the cows usually remain standing and loafing areas for the cows may not be necessary. Cows held for 3–4 h during the hot part of the day do not lie down much, particularly if fresh feed is kept in front of them. However cows held for 10–12 h day^{-1} will lie down. Concrete is a very poor surface for cows to lie down on. Some sort of loafing area becomes necessary.

A dirt yard or sacrifice paddock is the simplest form of loafing area. It can be satisfactory on some soils, particularly a hard hillside perhaps with shade trees. Unfortunately the cows often pick on a few of the best shady trees and transform the area underneath into a muddy slop. Usually the only solution is to electric fence off such areas until they dry out and force the cows to use other trees.

Compacted gravel is next cheapest alternative for loafing areas. The gravel should be carefully chosen avoiding sharp stones and sufficient clay or fine material should be present so the mixture will compact well and set hard hopefully minimizing the free stones. Gravel loafing pads should have fall at 2–4% to allow the water to drain away reducing mud.

Constructed loafing pads are becoming more common. The most common Australian example is rice hull pads usually used for calving pads in northern Victoria. These may consist of a compacted clay base often with slotted subsurface drainage pipes 1.5–2.0 m apart falling at 1.0% or more. The slotted pipes are covered by 20 mm gravel. This is in turn covered by an 80 mm layer of coarse sand and then 600 mm or more of the rice hulls. Manure is removed as much as possible from the top of the rice hulls and more hulls are added when the surface gets too contaminated. Once each year (or every 2 years) all the hulls must be removed as an impervious layer of manure and hulls is formed. These systems work well as calving pads for seasonal calving herds but not many have been used as loafing pads for milking cows near feed pads. Rice hulls are, of course, an organic form of bedding which will support bacterial growth particularly when wet. Environmental mastitis organisms could grow in such an environment. Pine bark, sawdust and even straight sand could be alternatives to the rice hulls. Some sawdusts are notorious for breeding mastitis pathogens (*Klebsiella* spp.).

Inert materials which will not support bacterial growth at least while they remain clean are the best bedding materials. Sand, usually river sand with not very much silt included, is clearly the material of choice.

The majority of loafing pads will have to be regularly cleaned mechanically and fresh bedding material added to maintain a clean loafing environment. Sterilants such as burnt lime, hydrated lime or formalin-based fluids have been used to reduce bacterial populations in bedding materials but the sterilizing treatment would need to be done very often (every second day) to have any real effect on pathogen populations. Some of these sterilizers could burn the cows.

Geotextile pads

Feed pads and cow tracks laid over boggy areas with little foundation can benefit from geotextile laid before gravel is installed. Geotextile will reduce the tendency of the gravel to sink through the mud. Geotextile fabrics are synthetically engineered materials often used in highway construction that were originally developed to provide additional soil stability and to distribute loads over a wider area. They are laid under the base material (usually gravel) of lanes and pads. The fabric is porous and allows water to pass through while holding the soil or rock in place. Often a two-layer decomposed rock base is laid over the geotextile. The base course (100–150 mm) is often coarse aggregate (up to 75 mm particles) with a cover layer (50 to 75 mm) of finer material (up to 25 mm particles) laid and compacted on top.

Feed Pad Cleaning

Mechanical scraping

Mechanical scraping with either a front or rear blade mounted on a tractor is a common pad cleaning system. Steel blades wear rapidly on concrete surfaces as does the concrete surface. A better alternative may be a rubber blade perhaps fashioned out of an old tractor or earthmover tire or a purpose-built box scraper.

Mechanical scraping has the advantage of being cheap and the manure remains dry (in dry weather), perhaps more suitable for spreading or for further processing. Mechanical scraping is the only option for pad surfaces other than concrete. Any manure stockpile formed by scraping must be in a controlled drainage area so that drainage is incorporated into the waste management system.

Hosing

Concrete surfaces can be hosed by a high-volume low-pressure system similar to that used for dairy holding yards. It is simple technology and a perfectly clean feed pad should result but the method is very labor intensive.

Flood or flush cleaning

Concrete feed pads with suitable fall can be flush cleaned. Prewetting of the manure with sprinklers (good for heat stress also) or soaker pipes will greatly assist manure removal and enable smaller pipes and flow rates. A 250 mm pipe cleaning a 6 m wide alley seemed to do a reasonable job although dry manure was often left behind.

Flood wash systems can use a flush pump which directly pumps the water from a pond to the pad. The flush pump system has the advantage that very long flush times can be used (60 min or more). A reasonable Australian compromise is a flush pump operating at the same time as mechanical scraping (mechanically assisted flood cleaning).

Liquid Waste Management

Management of waste water from feed pads flushed or hosed clean is little different to milking waste-water management. Flush-cleaned feed pads will usually need to use recycled water so it is likely that two-pond systems with their better water quality will be more suited. First pond size and sludge accumulation rates can be reduced by partial solids removal either with trafficable solids traps or with mechanical solids separation equipment. Unfortunately, such equipment comes at a high price either in labor or capital.

Orientation: East–West or North–South

The correct orientation is an often-discussed subject. For uncovered pads, orientation is probably not too important, unless it is planned to build a shed over the pad later.

An east–west orientation maximizes the shade but minimizes the entry of sun into the area reducing the drying and bactericidal effects of the sun.

A north–south orientation maximizes the sun penetration under the roof, promoting drying and reducing bacterial populations but minimizes the shade, particularly before mid-morning and after mid-afternoon.

The orientation decision is a definite compromise and depends on the individual farmer's concerns about minimizing disease, particularly mastitis, relative to his concern about heat stress. Some compromises suggest north–south for sheds with cow access to the sides of the shed and cows can move with the shade while east–west orientation is recommended when cows can access only from the ends.

North–south is probably a good orientation for sheds relying on a tractor cleaning of the pad. Some areas inside an east–west shed will never have direct sunlight leaving manure and surfaces always wet and a likely source of pathogens.

Other Environmental Considerations

Manure seal

Earth and gravel surfaces usually build up a seal in combination with manure after a period of use. It is important during cleaning or scraping that all the manure is not removed. A small amount should be left behind to retain a manure seal that will prevent infiltration of manure nutrients beneath the pad.

Controlled drainage area

The risk of surface water pollution during high rainfall periods can only be eliminated by the concept of a controlled drainage area. The area around the feed pad must be surrounded with banks or drains so that surface water from other areas does not run through the area and so that the runoff from the feed pad area is collected and does not enter watercourses.

Runoff collection ponds

Usually a pond must be constructed to hold the polluted stormwater for subsequent pumping and irrigation. When large numbers of cattle are held for long periods, a sedimentation basin or solids trap is often constructed before the pond to reduce the solids entering the pond. Such systems are common for large beef cattle feedlots but are probably not justified for most dairy farms. Runoff ponds should be designed to hold all the runoff from the controlled drainage area in a 90th percentile wet year. This size is easily calculated from rainfall records for the district, the area (m^2) and the runoff coefficient for the surface concerned. As the main variable in the size of the retention pond is the size of the controlled drainage area, it follows that this area should be no larger than necessary.

Dairy Housing Systems for Warm and Hot Climates

Hot arid environments demand different dairy housing systems from hot humid environments. A simple rule-of-thumb suggests an annual rainfall of approximately 375 mm as a suitable threshold between the two.

Hot Arid Dairy Environments

Although other systems are used, the dry lot dairy system performs well in dry environments. A dry lot dairy system holds the cattle in outdoor pens usually on a manure pack built up on the natural soil surface, often sand. In some cases, a well-graded and compacted gravel surface is installed similar to that used in beef feedlots. As rainfall is very low manure remains dry and mud is unlikely to be a problem. Cows normally present for milking with fairly clean udders although unseasonable heavy rain may require additional labor in the milking facility to maintain throughput while cleaning heavily soiled udders. Similarly, environmental mastitis is not a serious problem and somatic cell counts are low while the dry conditions are maintained.

Dry lots often have a concrete feeding alley along one side cleaned by flushing or scraping. Shade shelters are also often provided, usually in the middle of the lot and oriented north–south to promote drying underneath. Cow cooling is still maximized as the cows can move to remain shaded as the shade moves around the shelter during the day.

Surfaces under shade shelters need careful management. Earthen surfaces may be mounded up to promote drying. Wet material must be regularly removed and spread so that it dries and replaced with dry or fresh material. Areas of 2–5 m^2 shade per cow are necessary with a total pen area of 10–20 m^2 per cow. Dry lots must fall at 2–4% to a runoff collection pond to contain surface runoff while solid material is scraped into mounds for subsequent removal by loader and truck. Ideally solid material is spread back on to the land where the dairy cow feed was grown but sometimes it is sold to commercial manure recyclers.

Sometimes fan and high-pressure water mister cooling systems are provided in hot arid environments. Evaporative cooling systems (Korral Kool™) are used commercially in southern United States and the Middle East.

Hot Humid Dairy Environments

Hot humid dairy environments are more difficult to manage than hot dry environments. The most common facility for fully housed animals in such environments is the freestall barn, similar to that used in colder environments but modified to suit the warm climate. Serious mud problems caused by high rainfall in such humid environments render the dry lot system unworkable.

Freestall barns often have a central drive-through alley for the feed wagon while the cows feed on each side in the feed alleys. Bedding is provided in the freestalls that commonly measure about 1.2 × 2.4 m. Such has proved to be the best bedding material although sand can cause problems for effluent handling equipment. Rails are provided so that cows must lie in the beds in correct manner and a neck rail encourages cows to move backward when rising so the manure is dropped in the alleys rather than in the beds. The cow alleys in a freestall barn are often flush cleaned and effluent handled in ponding systems perhaps preceded by some type of solid–liquid separator. A cross-section of a freestall barn is shown in **Figure 7**.

Freestall barns are preferably oriented east–west in hot humid climates to promote the maximum shade under the roof. The cows normally remain within the building and are not free to move with the shade. Cow alleys are flush cleaned minimizing the disease risk from manure contamination.

Hot humid environments demand effective cow cooling systems to maximize milk production and reproduction. Cows are provided with shade but increasingly artificial cooling, normally fan and sprinkler evaporative cooling, is provided. Electric fans of at least 0.9 m diameter providing an air velocity of at least $3.0\,\mathrm{m\,s^{-1}}$ are placed every 9.1 m along the length of the shed above the cow alleys. Low pressure (70 kPa) large water droplet sprinklers which soak the cows' skin in about 1.5 min are placed above the feed bunk. Sometimes sprinklers are timer-controlled to operate for 1.5 min at 15 min intervals. Care must be taken with sprinkler systems to avoid wetting the feed.

Calf Rearing Facilities

Calf Housing

Calf paddocks

Calves can be successfully reared in open paddocks on grazing dairy farms provided environmental conditions are not too severe. Grazed pasture is usually the cheapest feed available, and should constitute the bulk of the diet for weaned heifers. Grassed paddocks need to be rotated and spelled regularly to minimize disease and intestinal parasites. Young calves may need protection from environmental extremes during the first 2 months of life and will benefit from some form of shelter in most dairying environments. Shelter should be provided from rain, cold winds and hot sun and should be draught-free, clean and hygienic. Care must be taken so that the shelter itself does not become a source of infection and illness. Wet, cold, soiled pens will spread disease and cause illness. Portable shelters, which can be moved regularly to clean areas, are preferred. Permanent shelters must be disinfected with hydrated lime or other bactericide and must be rested between batches of calves to break the disease cycle. Shelters constructed from metal are easier to disinfect than wooden shelters.

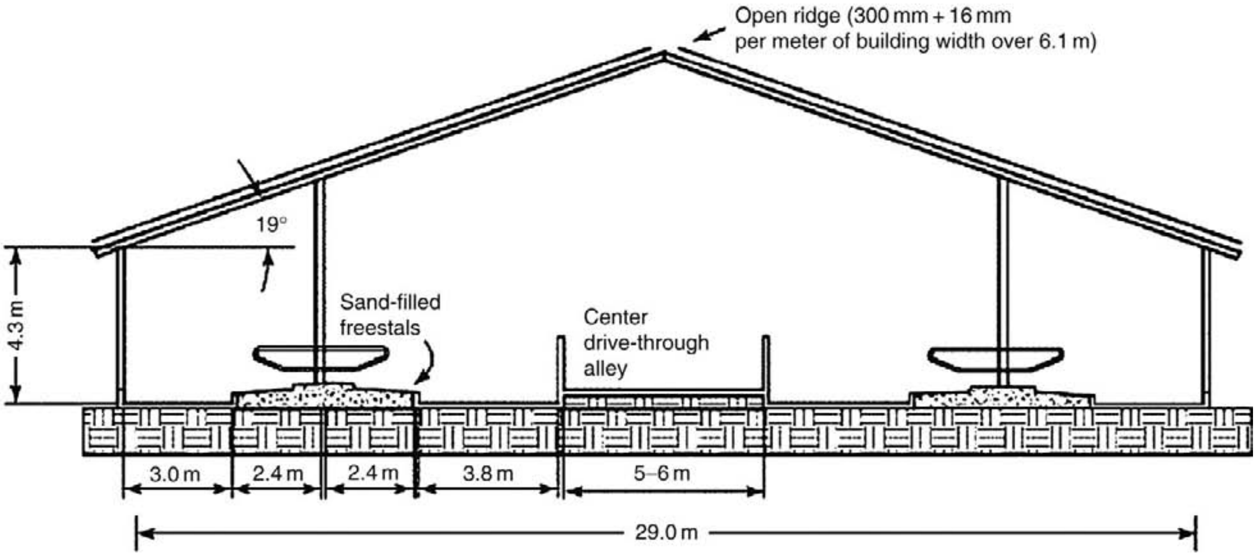

Figure 7 Cross-section of a free-stall barn.

Calf crates

Modern dairy farmers in tropical dairy regions often rear calves in elevated calf pens or crates installed inside an open well-ventilated building. Calf crates can provide a successful method of rearing calves in these areas of harsh weather and high disease and parasite risk.

Calf health is improved in the individual crates because access to other calves and the spread of disease is restricted. The calf is on a dry well-drained floor and the contact with fecal matter and other contaminants is limited. These benefits must be weighed against concern about animal welfare as calves are restrained in small pens and are not able to exercise or be exposed to direct sunlight.

Calf crates must be designed to maintain acceptable animal welfare standards. Social contact can be maintained with transparent flexible plastic or PVC partitions between calves so that the calves can see, hear and sense at least one other calf. Individual water troughs with protection from fecal contamination should be provided. Calves need around 2 m^2 space in the crate so they can move, turn, lie down, rise and groom themselves freely. Adequate total shed volume of at least 5.5 m^3 per calf will maintain air quality provided ventilation is satisfactory.

Some environmental enrichment is needed in the shed such as provision of dry teats to suck and perhaps playing a radio in the shed to accustom the calves to human voices and other noises. Because of the lack of exercise, calves should be kept in crates only as long as necessary. The disease risk is greatest in the first few days of life.

Elevated calf crates are suitable only for tropical and subtropical areas, not colder climates, as it is impossible to provide any bedding for the calves in the crates (**Figure 8**).

Calf hutches

Calf hutches are commonly used in cold environments and have also been successfully used in warm environments. The simplest are commonly constructed from plywood usually measuring about 1.2 × 2.4 × 1.2 m often with an open 1.2 × 1.8 m welded wire enclosure in front. Some bedding (straw, etc.) is normally provided. A wide variety of prefabricated fiberglass or plastic hutches are available.

Calf hutches are moved to a new site for each new calf thus breaking the disease cycle and the hutch itself should also be thoroughly washed and disinfected at the same time. Wooden hutches are difficult to disinfect properly. A base of compacted decomposed rock or sand is recommended to avoid mud and poorly drained sites should be avoided for calf hutches.

Heat stress can be a problem with calf hutches in warm climates. Translucent hutches (fiberglass or plastic) require additional shade in summer and sufficient shaded space must be available so the hutches can be moved. Earthen or gravel bases under shade do not dry well so the floor material may need to be removed and replaced or chemically disinfected between calves.

Deep litter systems

In less humid areas, deep litter systems for calf rearing are very successful. The calf loafing areas consist of a layer of rice hulls or wood chips 40–50 cm thick over a concrete floor. These systems have uncovered concrete in the feeding area and the deep litter area toward the back of the pen. The entire system is enclosed in a well-ventilated shed designed for easy waste management and removal and replacement of bedding material with tractor and loader.

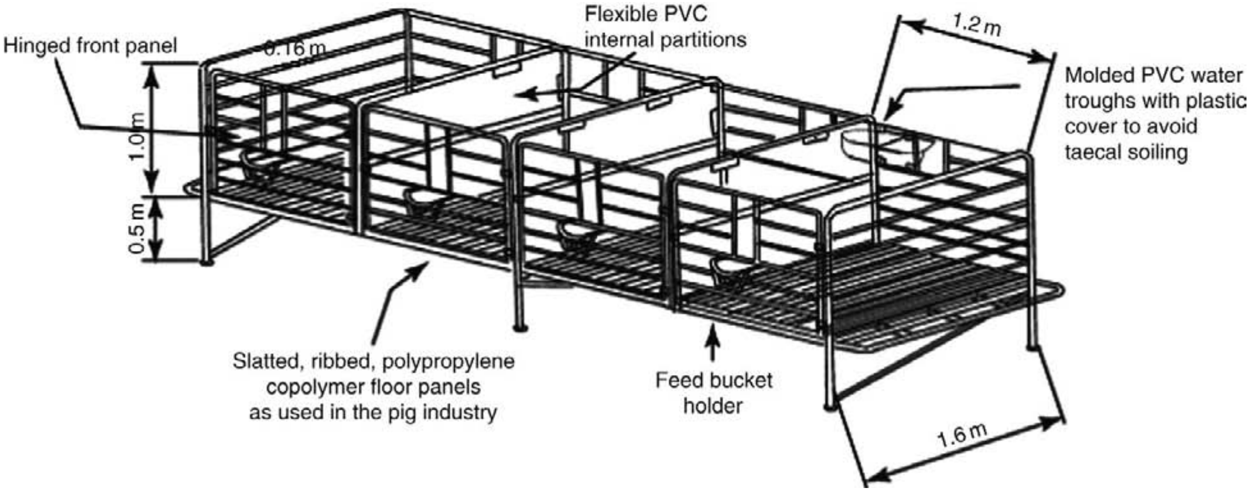
Hinged front panel
0.16 m
1.0 m
0.5 m
Flexible PVC internal partitions
1.2 m
Molded PVC water troughs with plastic cover to avoid taecal soiling
Slatted, ribbed, polypropylene copolymer floor panels as used in the pig industry
Feed bucket holder
1.6 m

Figure 8 A four-pen calf crate.

Milk Feeding Systems

Calf feeding can be labor intensive and modern dairy farmers have found a variety of ways to reduce labor input.

Manual systems

Simple systems include buckets and troughs with or without rubber teats. Suckle bars which consist of a series of self-closing teats attached to a 50 mm PVC milk line connected to a milk reservoir are sometimes used. Larger farms tend to use much larger containers usually transported or towed from the dairy to the calves. Such calfeterias or feeding drums can hold up to 300 l milk and a large number of rubber teats. Calves may have *ad libitum* (or restricted *ad libitum* – sufficient for a couple of hours) access to the milk. In some cases, milk may be preserved by acidification either through natural fermentation, the addition of various bacterial cultures or by addition of hydrogen peroxide or formaldehyde. Overacidification may restrict intake and regular stirring of the container is necessary. In temperate climates this system works well with through cleaning needed only once or twice a week but in tropical climates more attention to cleanliness is necessary. Very large specialized calf-rearing facilities using large numbers of calf hutches may use a mechanized calf bottle washing and filling machine.

Multiple suckling

Multiple suckling is a very labor-efficient system of feeding calves. Additional calves are either continuously fostered on to nurse cows or the nurse cows are restrained in a suckle race so the calves can have access at milking time. There is a risk of disease transfer from cow to calf and multiple suckling should not be used on farms where Johne‘s disease or other contagious diseases are present.

Automated calf feeding

Automated calf feeding systems are available which farmers claim save labor and veterinary bills and reduce deaths and ill thrift due to scours. Modern automated systems seem trouble-free in operation although such systems are relatively expensive, usually imported from Europe.

This system can use either milk replacer or fresh milk and comes equipped with an automated grain pellet dispenser. Each calf wears a transponder ear tag and when it approaches the feeder, the amount of milk or pellets is distributed depending on the computer program in the dispensing machine. Each calf ear tag is identifiable by the machine. Milk and pellets are available to each calf over a 24 h period, perhaps leading to better growth rate and healthier calves. The system comprises a dispensing machine, an identification and computer system, the transponder ear tags and the calf stalls.

Calves can be fed as much or as little milk or pellets as allocated depending on their stage of growth but once a calf has had its allotted feed of milk and/or grain for the day, no more is available from the machine until the next day. Electrolyte replacer, antibiotics, scour treatments or any other additives can also be incorporated into the feeding system.

Pasture Farm Subdivision

Selection of Site for Milking Shed

The following points should be considered before making a decision on the location for a new dairy.

Access

The site should be centrally located on the farm so that walking distances for the cows and mustering times are minimized. Production losses of 1 l per cow can result from cows walking long distances on hot afternoons and large herds need well-constructed cow tracks which can be expensive. Ideally, the site would also be close to the road for good tanker access.

Drainage

Elevated sites have an advantage over very flat areas because water can be drained easily from the shed and yards. Well-drained sites also reduce bogging around the dairy. The site should be chosen so that the yard is directly in front of the bails and at the same level as the entrance to the bails. Earthmoving is relatively cheap in relation to other costs in a dairy, so otherwise good sites should not be ignored because the slope is not ideal.

Effluent disposal

The shed and yards should be designed to allow for efficient effluent disposal. Elevated sites are preferred so that effluent can easily drain to a sump from which it may gravitate to an effluent pond. Flat sites are less satisfactory as effluent needs to be pumped. Sites very close to rivers, creeks, watercourses, public roads or neighboring properties need to be carefully planned in respect to effluent disposal.

Aspect

The bails should be located to give some protection from cold winds and driving rain and to minimize the entry of the afternoon sun into the milking area. In the southern hemisphere, the yard for holding cows before milking is usually best located on the northern or northeastern end of the building. Cows should enter the shed directly from the holding yard without turning. Most new dairies are very open buildings with few walls so an ideal aspect would have the yard on the exact northern end with the milk room, motor room, office, etc. on the western side of the milking area. Some additional wind protection may be necessary on the southern end of the milking area perhaps by extending the western wall well past the pit or by closing in the end wall forcing the cows to turn on the exit. Straight-through exits are preferable for cow flow.

Power and water supply

The availability and cost of reticulating electricity and water to the site must be considered. Most electricity authorities will not permit overhead power lines directly over a building. Three-phase power is preferable to single-phase for the significant electrical load required by a modern milking system.

Site approval

Many local authorities now require building permits for new dairies. The relevant dairy authority must also be informed.

Laneways

Laneways and farm tracks form an important part of pasture farm infrastructure. Well-designed and constructed farm tracks allow convenient movement of vehicles, promote easy and fast movement of cows to and from milking and keep udders cleaner. The track must remain as dry as possible, which is best achieved with a well-crowned and compacted soil base, a hard and impermeable surface and a functional track drainage system (**Figure 9**). On sloping land, track design should minimize nutrient runoff from the track into the surrounding environment by careful planning of the direction of the laneways, preferably across the gradient rather than up and down.

During construction grass and manure should first be removed and the base graded up. The base should be well compacted. Soils that do not compact well should not be used as a base. Some (but not all) clay soils can be stabilized with hydrated lime mixed *in situ* to depth of 200 mm.

The surface of the track must be impermeable to water and not cause damage to the cows' hoofs. A mixture of gravel, sand and clay laid to a depth of 100–150 mm after compaction makes the best surface for cow tracks. The clay will fill the voids between the larger particles, binding the material forming a hard-wearing and reasonably smooth surface. Large or sharp stones should be avoided as they may cause lameness and may be lost from the track surface leaving the track susceptible to water damage. Track material, particularly sand and sharp stones, carried on to concrete can be a problem damaging or wearing hooves and contributing to lameness. The choice of track surface material is important and local advice should be sought or alternatively a small section of track should be tried with the new material experimentally before committing to large track projects.

Junctions between gravel and concrete tracks or holding yards are best placed at a high position with both concrete and gravel sloped away from the junction or, if topography is not convenient, a kerb should be installed to prevent water running from concrete to gravel. Track width needs to be about 1 m for each 20 cows milked up to a maximum of 8 m. Fences should be placed on the track side of drains with lower wires high enough to allow a blade underneath. Track dryness should be promoted. In wet climates, trees near the actual track may prevent the track drying out and should be avoided although in drier areas trees near tracks are useful for shade. Tracks across boggy areas can benefit from geotextile stabilization (see 'Geotextile Pads', above). The track should have about 1 in 10 crossways gradient from crown to edge. Longitudinal gradients above 1 in 7 may lead to water scouring and track damage.

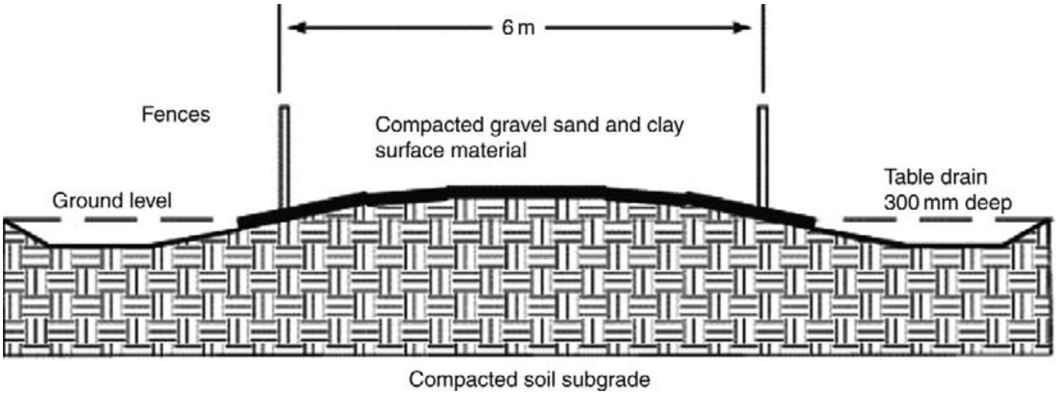

Figure 9 Cross-section of track.

Subdivision and Fencing

Whole farm plan

Modern dairy farms need to be comprehensively planned to protect the environment, make management easier and take advantage of any natural attributes offered by the farm location. Farmers are encouraged to prepare, often with specialized help, a whole farm plan.

Irrigation

The system of irrigation is an important consideration in subdivisional plans. Pastures are often divided into convenient paddocks to suit the irrigation system. Flood and traveling irrigators often have long narrow paddocks, while spray line, center-pivot or lateral-move irrigation systems may demand other subdivisional plans.

Permanent fencing

Permanent fences on pasture dairy farms are often limited to the boundary fence and fences delimiting laneways, roads and buildings. The type of fencing is usually determined by the economic availability of materials and varies enormously between regions.

Electric fencing

Electric fences are widely used on pasture dairy farms. There are a variety of labor-saving methods for convenient movement of electric fences.

Electric fences are often used in strip grazing systems. It is important for rapid pasture regrowth and optimum grazing intervals to provide a back fence so that cows do not have access to previously grazed areas in addition to their new strip.

Riparian Areas

Riparian land or the land immediately alongside small creeks and rivers includes the riverbank itself, areas surrounding lakes, and wetlands on river floodplains which interact with the river in times of floods. Pasture dairy farms often include riparian areas which are often highly productive. Riparian land is both an important agricultural resource and an important resource for native plants and animals. However, because of the enhanced environmental conditions, riparian land can serve as a repository for invasive exotic flora such as woody weeds and fauna such as feral pigs.

Important considerations in riparian land management include stream bank stability, weed populations, water quality and the general health of aquatic and terrestrial environments.

Stream bank stability

The vegetative cover of stream banks needs to be maintained. Stock crossing and stock watering points need to be placed and managed in appropriate locations. Good locations are on the inside of gently sloping creek bends or where erosion controls have been constructed. Off-stream watering facilities are preferred.

Weed populations

Weed invasion is a common in riparian areas. Weeds can be controlled with controlled grazing, physical removal and chemical control. Uncontrolled grazing should be avoided and grazing frequency should be monitored so that a good vegetative cover is maintained.

Water quality

Dairy farms often contribute a range of materials that can contaminate streams. Such materials include soil particles or sediment, nutrients from both manure and artificial fertilizer and biocides. Vegetative buffers between intensively used farming land and riparian vegetation will help filter pollutants before they enter streams. Direct contamination of streams with urine and feces must be avoided.

General environmental health

Riparian land provides shade, shelter and food for native plants and animals. Streamside vegetation is important and shading of streams lowers water temperatures, an important factor for the survival of native plants and animals. Increased light may promote algal populations, perhaps toxic blue-green algae. Organic matter accumulation such as leaf litter and wood debris should be promoted and may reduce soil erosion. Replanting native vegetation along stream banks is a positive step in improving riparian management but technical advice should be sort before undertaking such work.

Other Facilities

Calving Facilities

The ideal place for cows to calve is a shaded, comfortable dry area with low bacterial counts. Cows are very susceptible to mastitis at calving for many reasons. On pasture dairy farms, the ideal situation is a dry paddock, preferably not irrigated or contaminated with milking shed or feed pad effluent, with a good cover of grass on a elevated site without wet, boggy or poorly drained areas. Unfortunately such areas are rare on most dairy farms and cows are forced to use smaller calving paddocks where they can easily be supervised or calving pads, particularly on farms that experience high rainfall during the calving period.

Calving paddocks

Calving paddocks can work if they are large enough so that grass cover is maintained but a common problem occurs when small, sheltered areas are overused becoming boggy. The only practical solution is fencing such areas off until they dry and the grass recovers. Clean areas can be provided for new batches of calving cows by shifting electric fences across a paddock.

Calving pads

Calving pads can be a successful alternative for wet conditions. Drainage is probably the most important factor (see 'Loafing Areas', above).

Bedding materials

Some type of bedding material is often provided to make the cows more comfortable during calving but disease risk must be minimized. Nonorganic materials (washed sand, ground limestone) support lower bacterial populations than organic bedding materials (straw, rice hulls, shavings or sawdust). Finely chopped or ground organic materials have increased surface area for bacterial growth. Long straw and wood shavings are better bedding options than finely chopped straw or sawdust for this reason. Regular replacement of any bedding material is important because all bedding materials (organic and inorganic) will support high pathogen counts after becoming contaminated with manure.

Hospital Facilities

Pasture farms

Animal health is usually excellent on farms where cows spend most of their time grazing pasture so there is little need for dedicated hospital facilities. Such facilities are best placed and constructed similarly to calving paddocks or pads. There may be a need for shade and protection from the elements.

Intensive farms

Large farms with housing often provide a separate hospital barn that can be equipped with its own dedicated milking facility and sophisticated veterinary facilities including tilting crushes. The hospital milking facility is an aid to keeping milk unsuitable for sale away from the production facilities.

Conclusions

The information in this article presents a wide range of facilities in common use on dairy farms in warm climates. Of course, facility design has evolved over many years of practical use on commercial dairy farms. Actual experience in use is always the best evaluation. Farmers planning new facilities are encouraged to visit and talk to other farmers to gain valuable insight into the various design features exhibited. For milking sheds, it is best to plan visits at milking time and actually milk in the shed being inspected. Most farmers are only too willing to cooperate in such an activity. The state-of-the-art in farm facility design changes quickly, as new ideas are incorporated and older designs discarded. It is important that as many of the newer designs as possible are visited even if it requires travelling substantial distances to do so.

All facility design tends to be a compromise, often between many factors, and no single solution will be optimal for all concerned. However careful planning will alleviate many of the unforeseen management problems that often occur when new dairy farm facilities are put into service.

See also: **Dairy Farm Management Systems**: Dry Lot Dairy Cow Breeds; Non-Seasonal, Pasture-Based Milk Production Systems in Western Europe; Non-Seasonal, Pasture Optimized, Dairy Cow Breeds in the United States; Seasonal, Pasture-Based, Dairy Cow Breeds. **Manure/Effluent Management**: Nutrient Recycling; Systems Design and Government Regulations. **Milking and Handling of Raw Milk**: Milking Hygiene. **Milking Parlors**.

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DAIRY FARM MANAGEMENT SYSTEMS

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Seasonal, Pasture-Based, Dairy Cow Breeds

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Introduction

Pasture-based dairy production systems with seasonal calving have predominated in southern Australia and New Zealand (NZ) for many years. The NZ industry has grown substantially over the last 10 years, and is export oriented with a small domestic market. Milk production is still based on grazed pasture and seasonal calving. In Australia, about 80% of the milk is produced in the southeast (Victoria, Tasmania, and South Australia), and production peaked in 2001–02 and has declined since with a series of droughts and dry years. These conditions have contributed to increasing diversity and flexibility in feeding systems, and while grazed pasture remains the predominant feed-base, there has been increasing use of purchased feeds and a shift to more split and batch calving practices. Fifty percent of the milk produced in Australia is exported. Hence, in both countries, production systems must remain low-cost to be internationally competitive.

The seasonal patterns of growth and nutritive characteristics of pastures interact with prices received for milk and the availability and cost of supplementary feeds to determine the most cost-effective management systems. This means that milk production systems within and between regions are diverse and recognition of these differences has led to the differentiation of feed-base zones. Understanding the interactions between grazed pastures, conserved feed inputs, concentrate supplements, and cow genetics is essential to farm profitability and for managing the cost of milk production. The differences between feeding and management systems in southern Australia and NZ, and in the end uses of the milk produced, have led to differences in cow breed and type and considerable debate as to the 'best' cow for different systems.

Milk Production Patterns

In 2006–07, Australia and NZ produced 9.6 and 15.1 billion tons of milk, respectively, together accounting for less than 5% of world production. However, Australia accounted for 12% and NZ 32% of world exports in milk equivalents. Average herd size and milk production in Australia are 225 cows and about 5160 l per cow compared with 337 cows and 3790 l per cow in NZ. Dairy farm numbers have more than halved over the last 25 years in both countries.

Milk production in NZ is very seasonal, peaking in spring (September–November), and with less than 5% of annual milk supply delivered in the 'trough' quarter (May–July). Cows usually calve between late July and September. The industry appears to have accepted that capital investment in processing plant to handle this large variation in milk supply is unavoidable, since feeding concentrates to cows when pasture growth is slow is not an economic proposition because of the high cost of concentrate feeds. However, the use of maize silage and other supplements has increased in the last two decades.

The Australian dairy industry has changed quickly following deregulation in July 2000 followed by a series of droughts and dry years. For many years prior to deregulation in southern Australia, about 75% of cows calved in winter (June and July) to early spring (August and September) to match herd feed requirements with the seasonal pattern of pasture growth. In other regions, a much higher proportion of the milk produced was for domestic consumption, and year-round calving was common. While grazed pasture remains the predominant feed in southeastern Australia, there has been a shift to more split and batch calving across the country, with only 47% of herd-recorded cows calving in July–September in 2006–07. This change in calving pattern reflects the increasing use of supplements, failure to get cows in calf to maintain a seasonal system, and the payment of premiums in winter to encourage production of enough milk to satisfy the liquid milk sector and to improve utilization of manufacturing plant. Milk supplied in the 'trough' quarter (May–July) has increased from about 15 to 19% of annual production.

The average on-farm costs ($\mathrm{c\,l^{-1}}$) of milk production in NZ remain at about 80% of those in southeastern Australia. Importantly, average feed costs are now lower in NZ than in Victoria and Tasmania, which reflects the increased costs of supplements in Australia and their poor utilization on many farms.

Pasture Zones

The dairy industry in southern Australia is concentrated in three feed-base zones. The climatic diversity between these zones and the regions within them has a profound effect on the feeds used for milk production. The cool temperate zone (southern Victoria, Tasmania, and south coast of NSW) generally has annual rainfall in excess of 700 mm, with a relatively long and reliable pasture-growing season. The major pasture species sown are perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), but pastures vary in composition from being dominated by the sown species to heavily invaded by volunteer grasses and weeds (*see* **Forages and Pastures**: Perennial Forage and Pasture Crops – Species and Varieties).

The Mediterranean zone (WA, southeast SA, and parts of Victoria) has annual rainfall in excess of 600 mm, but this is winter dominant and the regions experience long hot summers, short growing seasons, and unreliable autumn breaks. In the truly Mediterranean regions, the long summer drought means that, in most situations, perennial ryegrass/white clover pastures cannot persist and pastures are predominantly annual ryegrass (*Lolium rigidum*) and subterranean clover (*Trifolium subterraneum*) (*see* **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties).

The inland irrigation zone (northern Victoria and southern NSW) experiences average annual rainfall of 350–550 mm. Pasture growth is dependent on irrigation. The percentage of the milking area on irrigated dairy farms sown to perennial pastures (perennial ryegrass/white clover) varies from less than 40 to 100%. These pastures are invariably invaded by summer growing species, in particular paspalum (*Paspalum dilatatum*). Irrigated annual pastures also form an important part of the feed-base, as do summer fodder crops, particularly maize.

In NZ, there is a 10-fold range in average annual rainfall (about 500–5000 mm), considerable variation in sea level temperature, which declines from north to south, and a wide spectrum of soil types. This leads to large variations in average annual and seasonal pasture production. Eighty percent of dairy farms are in the North Island, with the major milk production areas being South Auckland and Taranaki (temperate climate, dry summers, perennial ryegrass/white clover pastures) and Northland (warm humid climate, wet summers, perennial ryegrass/white clover pastures with subtropical grasses in summer). The South Auckland and Northland regions are used as examples here.

Pasture Growth

Of the rain-fed zones, pasture production is highest in Northland (about 17 t dry matter (DM) $\mathrm{ha^{-1}}$), followed by South Auckland and the cool temperate zone (about $11\,\mathrm{t\,DM\,ha^{-1}}$), and is lowest in the Mediterranean zone (about $6\,\mathrm{t\,DM\,ha^{-1}}$) (**Figure 1**). In the inland irrigation zone, irrigated perennial and annual pastures produce about 15 and $10\,\mathrm{t\,DM\,ha^{-1}}$, respectively. The variability in pasture growth and its distribution throughout the year (**Figure 1**) underpins the vast differences in the pasture-based systems that currently exist within and between regions. The seasonal pasture supply on farms is a key determinant of decisions in relation to time of calving, supplementary feeding, stocking rate, and targets for milk production per cow.

The key constraints to pasture-based dairy production systems are the amount of feed grown and the proportion of it that is utilized (consumed by cows or conserved). There is little doubt that removal of soil-based limitations (water, nutrients, and soil structure) and maintenance of sown species are essential in achieving the upper ends of the growth rates depicted in **Figure 1**. A key issue in both countries is whether plant systems that produce more digestible DM can be implemented on farms. Hence, there is increasingly more research on integration of pastures, fodder crops, or grain crops, and on the use of irrigation.

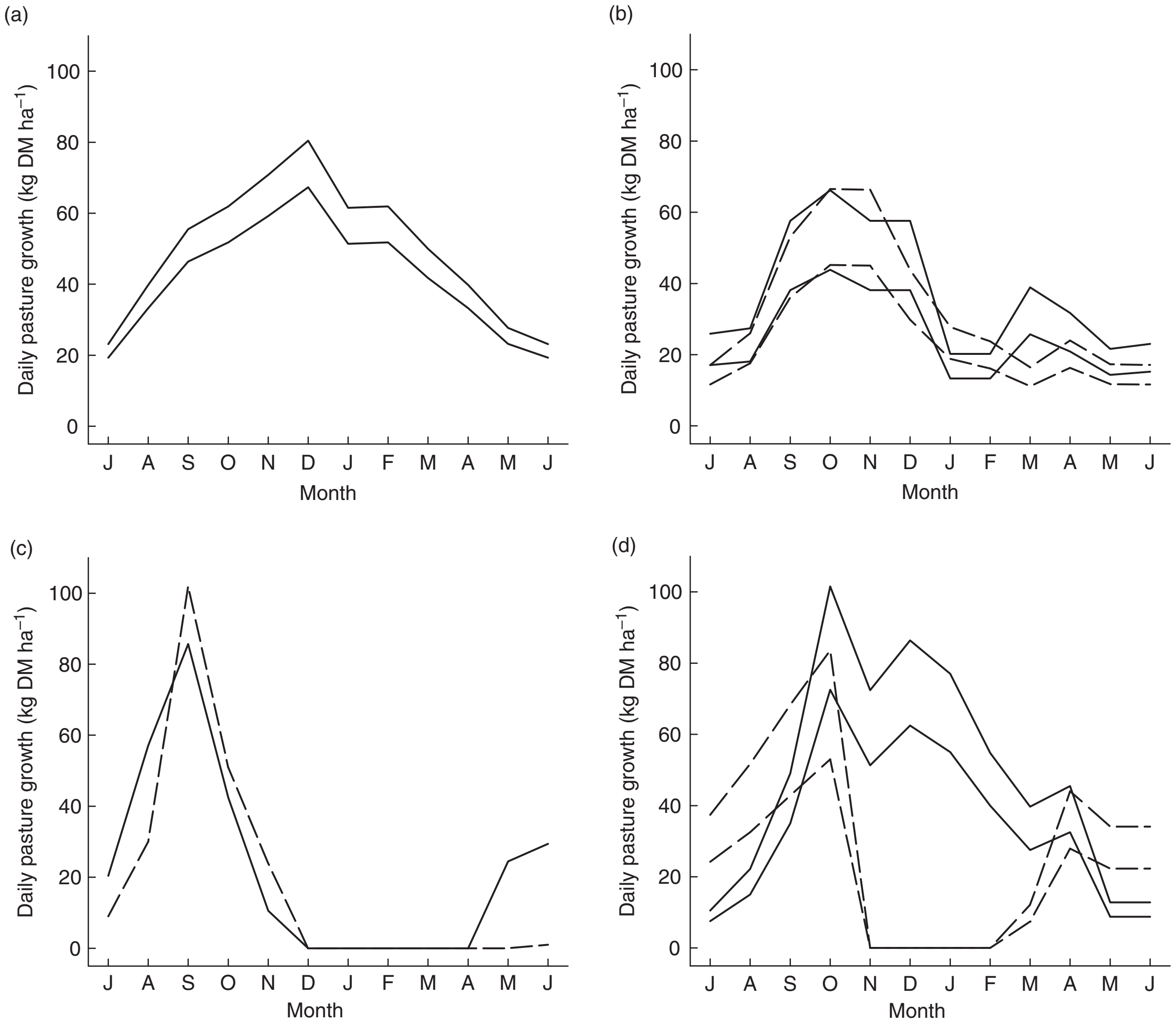

Figure 1 Pasture growth rates representative of the range occurring in dairy production systems in (a) Northland (15–18 t DM ha^{-1}), New Zealand, (b) South Auckland (8–12 t DM ha^{-1}; full line), New Zealand, and the cool temperate zone (8–12 t DM ha^{-1}; broken line) of southern Australia, (c) the Mediterranean zone (northeast Victoria (broken line) and Western Australia (full line)), and (d) the inland irrigation zone (12.5–17.5 t DM ha^{-1} for perennial pastures (full line); 7–11 t DM ha^{-1} for annual pastures (broken line)).

Grazing Management

In all feed-base zones, there is an emphasis on managing grazing to optimize pasture utilization without compromising growth or persistence of sown species. Strip grazing or rotational grazing of small paddocks is practiced. Rotation lengths vary throughout the year in accordance with pasture growth rates and are also modulated by the use of supplementary feeds. As an example, in northern Victoria, rotation lengths are 14–29 days in spring, 19–23 days in summer, and 17–35 days in autumn.

The principles of feed planning and understanding the supply of pasture in relation to demand at the cow and herd level are not new. However, they are becoming more important in containing feed costs in order to remain competitive in world markets. Hence, balancing seasonal feed demand with expected pasture supply, and feed budgeting to estimate how available feed may best be used to ensure optimal or target levels of milk production, are essential in making profitable decisions on pasture use. This information assumes greater importance where annual and seasonal variations in pasture growth are greatest and successful grazing plans include critical decisions on fodder conservation. For example, two key aspects to grazing management in the Mediterranean zone are the need to conserve the maximum amount of feed in spring to fill the summer feed gap and the rapid reestablishment of pastures after the autumn break.

Ideally, grazing management matches pasture consumption with pasture growth rate, such that plants are maintained in a productive state with minimum losses through death and decay. This is difficult to achieve in practice, but good management involves striking a balance between per cow and per hectare production, while minimizing trade-offs between pasture production, persistence, and nutritive value. In the cool temperate zone, the three-leaf stage of perennial ryegrass, just prior to senescence of the oldest leaf, is recommended as the time at which to graze. This ensures high growth rates and persistence, improves utilization, and should optimize the digestibility of the feed consumed. However, the pregrazing pasture mass at which ryegrass reaches this stage varies throughout the season, and has practical implications in terms of frequency of defoliation if adhering to these guidelines in winter and summer. It is also not possible to apply such guidelines across regions. For example, grazing of irrigated pastures offers particular challenges in summer when paspalum growth rates exceed those of perennial ryegrass and white clover. As a consequence, it is necessary to graze in relation to the stage of growth of paspalum through summer to limit ingress of this species.

Pasture Intake

Grazing dairy cows are unable to consume sufficient DM and metabolizable energy to achieve their potential milk production, because of constraints involved in harvesting grazed pasture and those imposed by managing the allocation of feed. While milk yields of grazing dairy cows are the direct result of the amount of pasture and supplements consumed, and the nutritive characteristics of these feeds, the regulation of intake of pasture by grazing dairy cows is complex and is undoubtedly affected by various factors:

- animal factors – cow size, milk yield, stage of lactation
- environmental factors – disease, climatic stress
- pasture factors – pasture mass, sward composition, digestibility/nutrient concentrations
- management factors – pasture allowance, amounts and types of supplementary feeds

We have a sound understanding of the relationships between pasture allowance and herbage intake by grazing cows (**Figure 2**). Although these relationships are influenced by pasture mass and species composition, enough knowledge exists to predict herbage intake for cows grazing green pastures with a degree of confidence. For example, pregrazing pasture mass affects the intake/pasture allowance relationship where (at a given pasture allowance) pasture intake will be lower at a low, compared with a high, pasture mass.

Grazing cows consume more pasture when grazing clover-dominant than grass-dominant swards. For example, cows grazing clover-dominant swards at pasture allowances of 15 and 30 kg DM cow^{-1} may consume about 11 and 19 kg DM day^{-1}, respectively. To achieve the same intakes on ryegrass-dominant swards, higher allowances of about 20 and 40 kg DM day^{-1} would be needed. Pasture intake is also positively related to digestibility, so intake and milk production generally increase as digestibility increases. Predicting pasture intake from pasture allowance and digestibility is more complex where dry pastures of low digestibility are grazed.

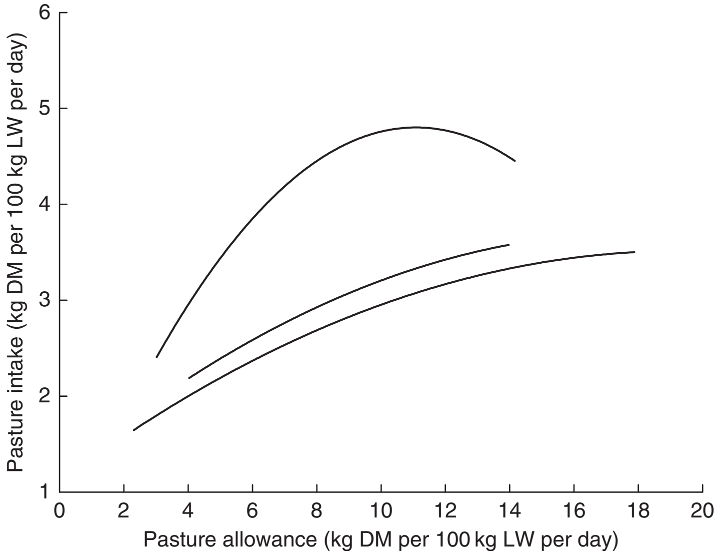

Figure 2 Relationships between pasture intake and pasture allowance for lactating dairy cows grazing pasture in the cool temperate zone in spring (middle curve; data supplied by D.E. Dalley) and annual (top curve) and perennial (bottom curve) pastures in the inland irrigation zone (data supplied by Richard Stockdale). DM, dry matter; LW, live weight.

Annual pasture consumption in the cool temperate zone varies from 3 to 9 t DM ha^{-1}, in the Mediterranean zone from 3 to 10 t DM ha^{-1}, and in the inland irrigation zone from less than 4 to more than 14 t DM ha^{-1}. This variation within regions may be due to differences in the amounts of pasture grown, in stocking rate, and in grazing management and feeding practices. The ranges illustrate that, on many farms, there is substantial room to improve conversion of pasture into milk.

Nutritive Characteristics of Pastures

Considerable effort has been invested in defining seasonal variation in digestibility, and crude protein (CP) and neutral detergent fiber (NDF) concentrations in pastures used for dairying. Digestibility and CP concentration are generally high during autumn to spring, but decline from late spring and are low in summer and into autumn (**Figure 3**). Regional differences in

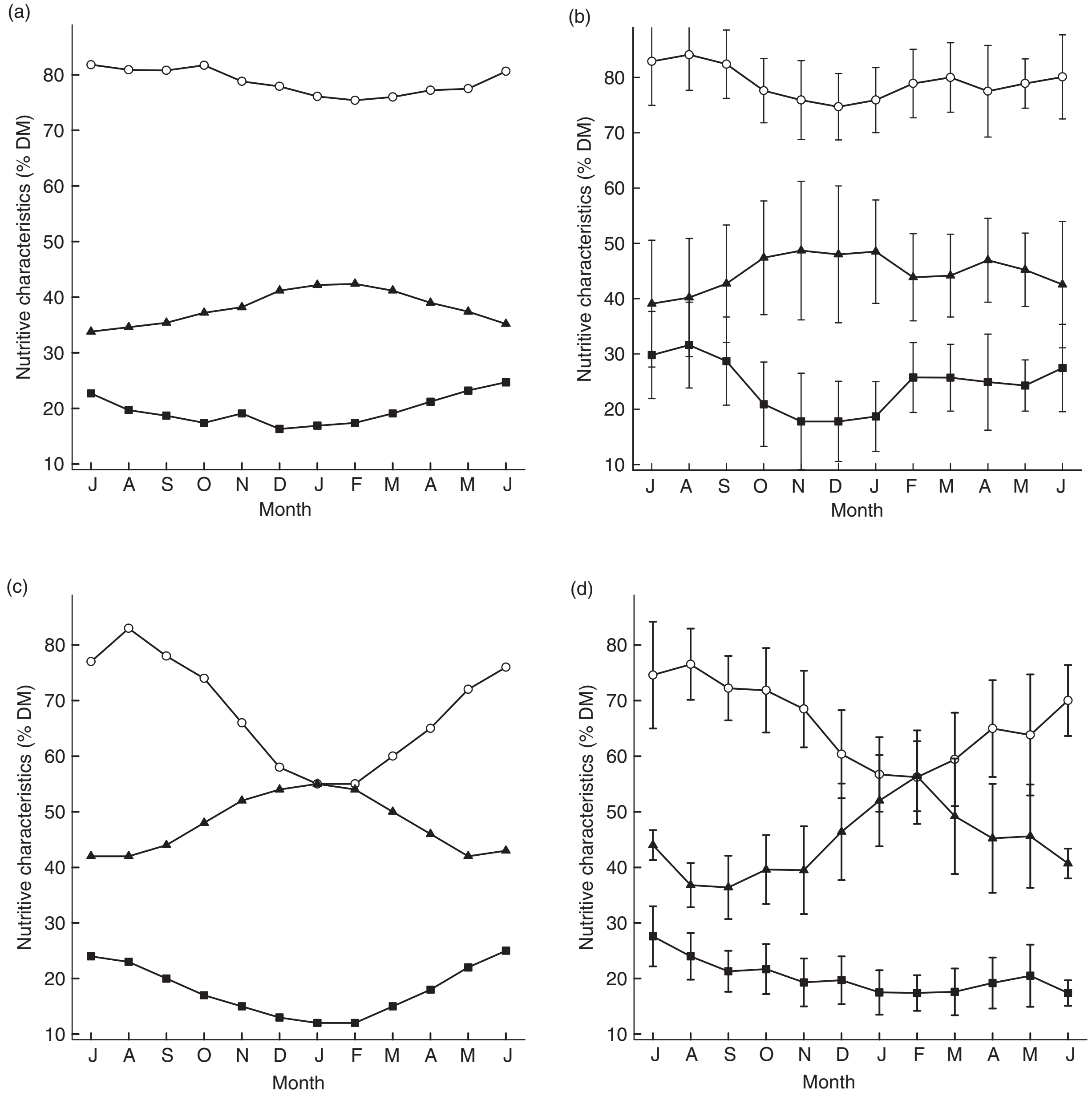

Figure 3 Nutritive characteristics (digestibility (○); crude protein concentration (■); neutral detergent fiber concentration (▲)) of herbage consumed in dairy production systems in (a) South Auckland, New Zealand, (b) the cool temperate (western Victoria) zone, (c) the Mediterranean zone, and (d) the inland irrigation zone. Data for (a) and (c) were supplied by John Penno and Martin van Houtert, respectively, while that for (b) and (d) were provided by Janna Heard from the Kyabram Dairy Centre pasture database. Bars are standard deviations about means.

digestibility exist because of differences in pasture species present, in the growth patterns of these species, and in climatic conditions. These variations are more extreme in regions or years with dry summers.

CP concentration in pastures follows a similar pattern to digestibility, but there is greater variation as the differences in protein concentration between leaf and stem components of pasture plants are greater than the corresponding differences in digestibility. The high CP concentrations in green pastures often exceed cow requirements, and there are energy costs incurred in excreting excess nitrogen as urea. In contrast, CP concentrations in summer are sometimes below 15% and protein supplements may be needed.

NDF concentrations are the inverse of digestibility and are lowest during winter and spring and highest in summer (**Figure 3**). While the concentrations in pasture on offer usually exceed those recommended for lactating cows, namely 30–40%, selection may create a problem in this regard on highly digestible pastures, particularly those with high clover contents.

To better understand the essential supply of nutrients to grazing dairy cows, it is important to be aware of their concentrations in the feed eaten compared with those in the pasture on offer. In the cool temperate and inland irrigation zones of southern Australia, cows grazing predominantly green pasture in winter and spring consume material that is higher in digestibility (1.05–1.15) and CP concentration (1.20–1.40) than the pasture on offer. The potentially high level of consumption of CP from pasture can have important implications in designing supplementary feeding practices that optimize the supply of energy and protein to the rumen microorganisms and to the cow.

At the same time, the pasture consumed is lower in NDF (0.75–0.95) than is the pasture on offer, principally because leaves have less NDF than stems. In cows grazing extremely high-digestibility pastures, rumen pH can be below 6.0 for a considerable portion of the day (**Figure 4**), presumably due to insufficient fiber in the diet. This predisposes the animals to an unstable rumen fermentation pattern and acidosis if they are fed cereal grain.

Supplement Use

In NZ, the feeding systems are predominantly year-round grazing supplemented with conserved forage. However, the use of maize silage and other supplements has increased over the last two decades.

Less intensive, lower stocked farms in the cool temperate and irrigation zones in southern Australia also have feeding systems that are predominantly year-round grazing supplemented with conserved forage and low amounts of concentrates. On more intensive, highly

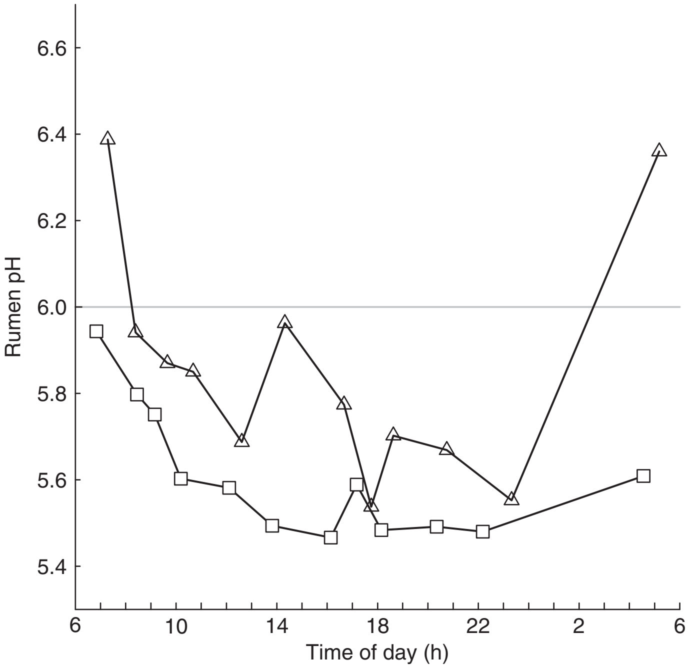

Figure 4 Rumen pH in lactating cows consuming about 19 kg DM day^{-1} while grazing either Persian clover (□) or perennial ryegrass (Δ) pastures during spring. Data provided by Yvette Williams.

stocked dairy farms, many different feeding systems exist, which can be broadly classified as follows:

- Twice-daily grazing with concentrates fed in the dairy and conserved fodder fed at pasture or on a feed pad.
- Twice-daily grazing with partial mixed rations fed on a feed pad twice daily.
- Once-daily grazing with partial mixed rations fed on a feed pad once daily.

In northern Victoria, energy consumption by milking herds varies from 25 to 100% from pasture, with the balance being brought in from off-farm areas. During periods of low rainfall or low availability of irrigation water, farmers may feed mixed rations of conserved fodder and concentrates with no grazing. Thus, almost all conceivable combinations of grazing and supplementation with various types and mixes of conserved fodder and concentrates can be found, which leads to considerable variation in feed conversion efficiency.

Since the early 1980s, the average amount of cereal grain-based concentrates fed to dairy cows in southern Australia has increased markedly from virtually 0 to about 1450 kg cow^{-1} in 2006–07. This is a fundamental difference between the pasture-based systems of southern Australia, where over 95% of farmers now feed concentrates, and those in NZ, where the use of grains and meals is much lower because of cost. In southern Australia, the amount of concentrates fed varies from 0 to 3000 kg cow^{-1}, while conserved fodder supplementation varies from 0 to over 1500 kg DM cow^{-1}.

Cereal grains (*see* **Feed Ingredients**: Feed Concentrates: Cereal Grains), by-products (*see* **Feed Ingredients**: Feed Concentrates: Co-Product Feeds), and formulated concentrates are used not only to alleviate pasture shortages, but also to overcome limitations in nutrients supplied by grazed pastures at key times of the year and to increase milk production per cow. Productivity increases on Australian dairy farms have been poor, and many farmers have not utilized opportunities afforded by access to relatively cheap concentrates. Thus, there is a need for improved understanding and matching of supplements and pasture to further increase productivity.

Interactions Between Pastures and Supplements

The efficiency of use of supplements is a critical issue in managing farm costs. In particular, substitution of supplement for pasture in the diet will affect pasture utilization and the milk response to additional supplement. Substitution will be less of an issue when pasture allowances are low, but is invariably an issue at moderate to high pasture allowances.

Many factors affect substitution of supplements for pasture, including pasture intake as affected by pasture allowance and mass, the nutritive characteristics of the sward, the amount and type of supplement fed, physiological state (stage of lactation) of the cows, and their body condition and size. An indication of the influence of pasture intake before supplementation on the level of substitution that occurs when supplements are fed is illustrated in **Figure 5**.

Over the past 10 years, a major research goal has been to understand the interactions between pasture, concentrate supplements fed in the dairy, and cows, to enable development of feeding systems that are profitable. This has illustrated the difficulties of feeding highly digestible concentrates twice daily in the dairy, to cows grazing high-digestibility pastures, without causing large fluctuations in rumen fermentation and instability in the rumen. However, adoption of the findings of this research has been slow. Similar information is not available for partial mixed ration systems even though this should lead to a more stable rumen fermentation. The diversity of feeding systems within and between regions and the differentials between costs and the different milk pricing systems, particularly across Australia, mean there will be no best system.

Cow Breeds

In NZ, 85% of herds participate in herd recording compared with about 50% in Australia. Hence, NZ data on herd composition are more reliable. There are 3.92 million dairy cows in NZ, with Holstein-Friesian (46% of the national herd) being the predominant breed, and Jersey (14%), Holstein-Friesian/Jersey cross (32%), and other breeds (8%) making up the balance. The percentage of Holstein-Friesian cows has declined by 10% over the past 7 years, with the number of Holstein-Friesian/Jersey cross cows increasing.

In Australia, there are 1.8 million dairy cows, and based on herd recording, Holstein-Friesian is the predominant dairy breed, comprising 78% of cows. Nearly all of these cows have some North American parentage. Jerseys comprise 13%, Holstein-Friesian/Jersey cross cows 5%, and other breeds (Australian Illawarra Shorthorn, Ayrshire, Australian Red Breed, Guernsey, and Brown Swiss) 4%. However, it is apparent that outside the herds in the Australian recording scheme, there has been a noticeable increase in crossbreeding.

Over the period 1980–2000, the shift to Holstein-Friesian animals in Australia led to a dramatic increase in the size and potential production of cows. For example, the average live weight of mature cows in southern Australia increased from around 400 to 550 kg and average milk production increased from

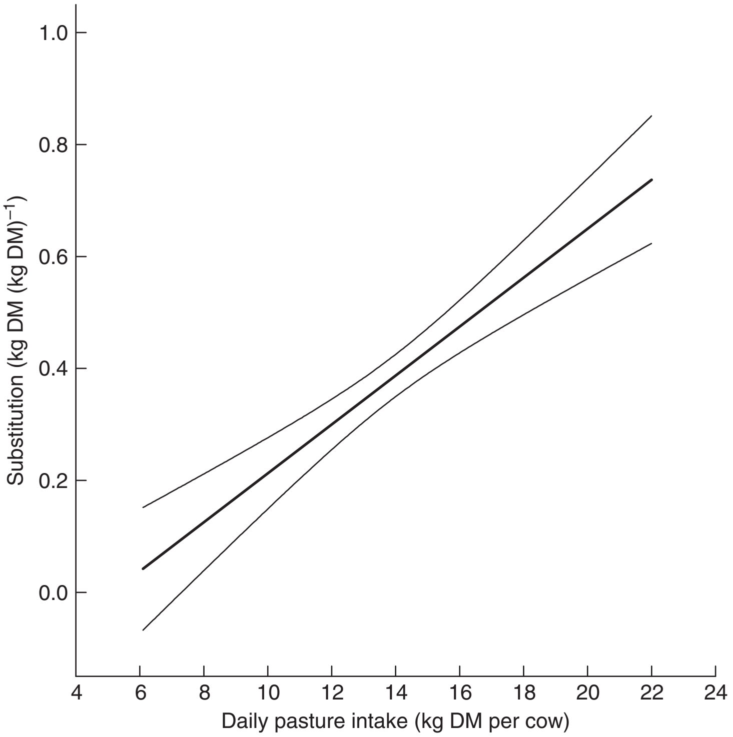

Figure 5 A relationship between level of substitution and unsupplemented pasture intake when concentrates are fed to lactating dairy cows in northern Victoria, with the 95% level of confidence about the curve indicated. Data supplied by Richard Stockdale.

3000 to 5000 l cow^{-1}. The increased milk production potential has contributed to the diversification of feeding systems. In NZ, these changes were not so dramatic and cow size (less than 500 kg for Holstein-Friesian cows) and milk production (about 3500 l cow^{-1}) remained relatively constant between 1990 and 2000. In both countries, cows in systems where grazed pasture is the majority of the diet are underfed due to the constraints on intake associated with grazing and constraints imposed by management to achieve high levels of pasture utilization. This has contributed to the shift toward crossbred cows in both countries.

The rationale for continued increases in genetic potential in dairy cows needs to be questioned for pasture-based systems. There is some evidence that the potential increases from improved genetic merit can only be achieved when feeding intensity is increased, and this invariably means increased unit costs of feed and other inputs. Where nutrient intake is inadequate, as is commonly the case with pasture feeding, increased genetic merit and the associated tendency for the cow to partition nutrients from feed and/or body reserves to milk can lead to metabolic disorders, reduced reproductive performance, and reduced longevity in the herd. Such effects have led to changes in the calculation of breeding values in many countries, with nonmilk production traits being given higher weightings.

Future Trends

Milk production per farm in southern Australia and NZ has continued to increase over the past decade. This has been associated with a general increase in intensification of farming, with increased farm and herd size, and with decreased farm numbers. Dependence of both countries on exports ensures that most production systems will remain pasture-based and low-cost. Further productivity gains will need to be achieved in an environment in which on-farm quality assurance systems for food safety and natural resource management are implemented by cooperatives and proprietary companies. Communities and consumers will require responsible management of water, nutrients, and effluent to limit off-farm impacts, and the industries will need to address greenhouse emission issues. It is likely that improvements in feeding systems on individual farms will be incremental, and to remain competitive, farmers will need to continually analyze their systems and apply the latest knowledge on matching pasture, conserved fodder, and concentrate supplement usage.

The need for research products/technologies to impact at industry level will require further development of predictive technologies and effective linkages with private agribusiness and government service providers. Increasing complexity and intensification of farming systems, particularly in Australia, will accelerate the shift to farming as a business, with consequent needs to improve management skills and workforce capability. Finally, increased differentiation in the prices received for milk, based on composition and end use, will mean farmers will look for cow genotypes that are best adapted to particular feeding management systems and end products.

See also: **Dairy Farm Management Systems**: Dry Lot Dairy Cow Breeds; Non-Seasonal, Pasture Optimized, Dairy Cow Breeds in the United States. **Feed Ingredients**: Feed Concentrates: Cereal Grains; Feed Concentrates: Co-Product Feeds. **Feeds, Prediction of Energy and Proteins**: Feed Energy. **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties; Perennial Forage and Pasture Crops – Species and Varieties.

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Non-Seasonal, Pasture Optimized, Dairy Cow Breeds in the United States

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Introduction

Pasture-based dairies account for a relatively small percentage of the approximately 71 000 dairies in operation in the United States. A 2000 US Department of Agriculture survey indicated that the average US dairy contained 133 hectares of land, but of that only 20 hectares or 14.9% of the total acreage was devoted to pasture production. Pasture production varies considerably throughout the United States. In the traditional dairying regions such as the upper midwestern states of Wisconsin and Minnesota, pasture use typically accounts for less than 10% of the farm area, but in the southeast and southern plain states where long growing seasons and low soil fertility are prevalent, more than half of the average dairy farm land mass may be in pasture.

Although most US dairies are confinement based, use of pasture in US dairy operations has grown steadily since the early 1990s, a time marked by relatively low milk prices and high production costs for housing, waste management, and supplemental feed. Recent surveys indicate that summer grazing of forages has been adopted by as many as 20% of northeastern and midwestern dairymen. Likewise, pasture use in Georgia, a state in the southeastern United States, grew from 34.5% in 1985 to 53.5% in 1995. Primary forages used for grazing during the spring and summer in the northeastern region include orchard grass, bluegrass, and perennial ryegrass often grown in combination with variable amounts (10–50%) of red or white clover. Major grazing crops grown for lactating dairy cows in the southern United States are annual ryegrass, wheat, oats, and cereal rye, which are normally grazed from November through April. Summer grazing crops used by many southern dairymen include perennials such as bermudagrass and bahiagrass and annuals such as millet, sorghum-sudan, crabgrass, and signalgrass.

In Missouri, a state located in the central United States, more than half of the state's dairy cattle graze pasture in some manner with the majority of cows being intensively grazed. Work at the University of Missouri indicated that the state's soils were well suited for intensive grazing. In order to generate local pasture system data, the Southwest Center at Mt. Vernon, MO, converted from a confinement feeding system to a 60-cow intensive grazing system in 1998. Major pasture systems have centered around perennial ryegrass and endophyte-free fescue. Although many of Missouri's own dairymen have returned to grazing, since 2003 there has also been an impressive influx of New Zealanders opening up grazing dairies in the state. Most of the increase in the number of cows (as many as 10 000 cows) attributed to the New Zealanders has been in the form of 500-cow pods utilizing Holstein–Jersey cows. Relatively cheap land, good grass production, and abundant water supplies appear to be the main attractants for these foreign investors. Most of these dairies are a hybrid of US and New Zealand management systems featuring minimal grain feeding, low labor requirements, low capital inputs, and intensive grazing on irrigated pastures. New Zealand investors have also shown interest in other southeastern states such as Georgia and Mississippi.

Increased interest in pasture dairying has prompted several studies comparing milk production and economics of cows receiving diets based on pasture to those fed corn or alfalfa silage-based total mixed rations (TMRs) in confinement (**Table 1**). Study or survey sites ranged geographically from the southern states of Mississippi and Georgia to the more northern states of New York and Minnesota. Generally, grazing seasons in northern states range from 120 to 160 days depending on first frost day, and in many southern states may extend from 200 to 300 days per year. In southern states, droughts and low forage quality often restrict the number of days pastures are suitable for grazing more than cold weather.

Based on university studies and farm surveys cited in **Table 1** (annualized data), pasture-based herds may be expected to produce 8.6%, or in this case 681 kg per lactation, less milk than confinement herds fed TMRs containing corn silage, alfalfa hay, ground corn, and protein supplements. Using the data from all eight locations, milk yield for pasture-based herds was 12.9% less than that for confinement herds, but several of the studies were not year-long comparisons. Milk from grazing herds contained less fat (3.62 vs. 3.77%), but similar amounts of protein (3.26 vs. 3.30%) compared to milk from confined herds. In spite of the lower milk production, several reports indicated $60–180 higher net return per cow due to savings in stored feed, facilities, bedding, equipment, and labor.

Several plant, animal, and environmental factors that may limit milk yield of pastured dairy cattle exist. Unlike stored forages, nutritional characteristics of pasture are dynamic, changing substantially with season, stage of

Table 1 Comparative performance characteristics of pasture-based and confinement dairy systems in the United States

					Milk yield (kg yr^{-1} or kg day^{-1})		***Milk fat (%)***		
Location (state)	***Dairy breed***	***Study duration***	***Pasture forages***	***Grain (kg day^{-1})***	***Past.***	***Conf.***	***Past.***	***Conf.***	***Source***
Virginia[a]	Holstein	150 days for 3 years	Orchard grass–clover	7.3	25.1	25.4	3.19	3.57	Polan *et al*. (1986)
Minnesota[a]	Guernsey	140 days for 2 years	Bluegrass, orchard grass, and white clover	7.0	22.1	23.7	4.47	4.64	Rust *et al*. (1995)
Pennsylvania[a]	Holstein	28 days	Perennial ryegrass, white clover, and orchard grass	0	28.3	40.5	3.72	3.48	Kolver and Muller (1998)
New York/ Pennsylvania[b]	NA	365 days	NA	NA	5843	6816	NA	NA	Hanson *et al*. (1998)
Mississippi[a]	Holstein	365 days for 2 years	Annual ryegrass, oat, and signal grass	11.2	8110	8332	NA	NA	Murphy and Thomlinson (1998)
Georgia[b]	Holstein	365 days	NA	NA	8005	8938	3.44	3.59	Smith and Ely (1997)
North Carolina[a]	Holstein	365 days for 3 years	Rye, orchard grass, bermuda grass, and clover	NA	NA	NA	7115	7910	White *et al*. (2002)
	Jersey			NA	NA	NA	5381	6147	
Florida[a]	Holstein	259 days	Annual ryegrass, rye, crimson/red clover, and bermuda grass	10.1	25.1	29.9	3.60	3.69	Fontaneli *et al*. (2005)

[a] Data obtained from university research.
[b] Data obtained from farm surveys.
Conf., confinement systems with corn silage–alfalfa hay-based total mixed rations; Past., pasture-based systems; NA, data not available.

maturity, and in some cases with time of day. A major pasture management goal is to maintain pasture mass and nutritional value at optimum levels in order to maximize dry matter (DM) intake and milk yield. Proper fertilization stimulates growth, but concomitant stem elongation often depresses feeding value. In order to maximize leaf production, and consumption by dairy cows, pastures should be grazed when leafy and vegetative, usually at plant heights ranging from 6 to 15 cm. Grazing small paddocks with many animals for a short duration (12–48 h) results in uniform defoliation and subsequent refoliation, which promotes high nutritional value and consumption. This management-intensive grazing (MIG) system, which relies on electric fencing to divide large pastures into small paddocks, is widely used in New Zealand, and is gaining acceptance in Europe and the United States. Higher fencing, water, and labor costs associated with herds employing intensive grazing are often offset by higher milk yield, greater carrying capacity (more animals per land unit), lowered stored forage needs, and improved milk quality.

The primary plant-related factors that affect lactation performance of dairy cattle are forage availability and quality (nutritive value). Adequate forage mass must be available to maximize bite size, minimize maintenance requirements, and maximize intake by grazing cows. Under intensive-grazing systems, efficiency of pasture utilization (percent of growth consumed) approaches 80%; however, pasture utilization may be 25% or less for continuous or other extensive-grazing programs. Low efficiency of pasture usage not only results in wastage of valuable forage, but may also decrease the nutritive value of pasture, increase weediness, and elevate per animal forage costs. Generally, a temperate pasture growth rate of 40–50 kg M ha^{-1} day^{-1} will meet the needs of Holstein cows stocked at 3 cows per hectare; however, such high growth rates normally occur only for 3–4 months in the spring. As a consequence, cows must be stocked at lower rates or supplemental forages must be provided to offset deficiencies that occur when plant growth rate is slowed or forage quality is unacceptable.

Pasture quality is affected by many factors including stage of maturity, plant species, fertilization, and environment. Since plant leaves are normally higher in protein and lower in fiber compared to stems, maintaining a high leaf-to-stem ratio is desirable. As the plant matures, stem elongation takes place followed by efflorescence and a decline in forage quality. For example, immature annual ryegrass growing in late February may exhibit a leaf-to-stem ratio of 8:1, a crude protein concentration of 30% or more, and a neutral detergent fiber concentration of 40% or less. But, at the mature seed stage in mid-May, the same forage may have a leaf-to-stem ratio of 1:3, a protein content less than 8%, and a fiber concentration above 70% of DM. This emphasizes the importance of pasture management strategies that delay plant maturation and promote availability of young leafy regrowth. As a rule, pasture quality in southern states is highest for winter annuals such as annual ryegrass, intermediate for summer annuals such as millet, and lowest for summer perennial grasses such as bermudagrass. In contrast, perennial temperate forages, such as orchard grass and bluegrass, often provide high-quality spring and summer grazing in the central and northeastern United States. Legumes, such as white and red clovers, are often grown with grasses in the central and northeastern United States and may account for as much as one-third of the total pasture used by grazing dairy cows. Clovers usually contain less fiber (lower cell wall content) than grasses, which improves the rate of digestion and increases forage DM intake by grazing cattle. Furthermore, the growth patterns of clovers promote higher green leaf consumption compared to grasses, which, in combination with improved digestibility, may increase milk yield 4–8% over pure grass swards.

Conserved Forage Supplementation

Year-round pasture production is an often-discussed, but seldom-achieved, goal of grazing dairies. Even states with subtropical climates, such as Louisiana and Florida, experience periods of cool weather and drought, which necessitates use of conserved forages. In these southern states, summer pastures are often grazed during the cool of the night and conserved forages are offered during the hot daytime hours to minimize the effects of heat stress on forage consumption. Conserved forages are also fed to compensate for the decline in the quality of grazed pastures, to increase effective fiber concentrations of lush pasture, and to increase the number of animals per unit land area (stocking rate).

Corn silage is a commonly fed conserved forage on pasture-based dairies in the United States. During winter, many central and northern US dairy farms feed TMRs; therefore, this stored forage is readily available to supplement pastures, particularly in summer and early fall, when pasture availability is marginal. Corn silage has generally proven superior to native hay and grass silage for supplementing pastures in both the United States and Australia. Growing conditions do not favor corn silage production in many European countries; therefore, grass silage conserved in either clamps or bales remains popular.

Research conducted during summer in Louisiana compared cows grazing bermudagrass with no supplemental forage to those grazing at night and offered fresh sorghum, 3 kg alfalfa hay plus *ad libitum* native hay, 7 kg alfalfa hay, or *ad libitum* corn silage. Sorghum and limited alfalfa hay increased milk yield about 9% above pasture-only controls, while the high level of alfalfa and corn silage supplementation increased milk yield 16.3 and 24.0% above controls, respectively. Although high levels of alfalfa increased milk production significantly, economics favored supplementation with locally grown corn silage.

Although corn silage is a superior supplemental forage, it is expensive to maintain equipment for growing, storing, and feeding silage. In many parts of the United States, graziers take advantage of custom producers to grow and harvest corn for silage. This does improve the economics associated with corn silage feeding. Still other pasture-based dairymen are turning to high-quality bale silage to meet stored forage needs. The long particle length of baleage promotes rumination and milk fat production, which is often an advantage for cows grazing lush pastures low in effective fiber. Because of its low wilting time requirement (an important consideration in high-rainfall areas), low equipment costs, low storage losses, and high nutritive value (relative to native hays), conservation of forage crops such as annual ryegrass, wheat, and millet as bale silage is on the increase, particularly among dairymen who graze their cattle for a significant portion of the year.

Feed Management

Concentrate supplementation of pasture is an accepted management strategy on most US pasture-based dairies. Economics, grain availability, and animal health are the major factors governing the amount and type of concentrate provided to grazing dairy cattle. Although there is considerable variation from year to year, price relationships between milk and grain normally average near 2:1 in the United States, while in other countries, such as New Zealand, price relationships between milk and grain are closer to 1:2 or lower. Relatively inexpensive grain costs have spurred considerable research aimed at defining the optimum level of grain feeding that maximizes milk yield while avoiding digestive upsets and milk fat depression.

Lactation responses to pasture supplements vary with the amount and quality of the supplement, pasture intake and quality, and stage of lactation of the cows. Pennsylvania researchers summarized research documenting the effect of supplementing pasture-fed dairy cows with concentrate on DM intake, lactation performance, and diet digestibility. Low pasture DM intake was identified as one of the primary factors limiting the production of high-producing dairy cows on pasture. Of the three major factors regulating pasture intake, that is, grazing time, biting rate, and bite mass, only grazing time was negatively affected by concentrate supplementation. For each kilogram increase in concentrate supplemented, daily grazing time on the average was reduced by 12 min. It was further noted that the substitution rate of supplement for pasture was negatively correlated to milk yield. High-quality pastures with a low substitution rate generated superior lactation performance compared to low-quality pastures in which cows substantially reduced pasture intake when provided significant amounts of concentrate. Milk production was found to increase linearly with increasing supplementation up to 10 kg concentrate DM per day. Overall response to grain was approximately 1 kg milk per kg grain independent of whether the supplement was high in starch or nonforage fiber. Average milk yield response to dietary fat supplementation was +6%; this increase occurs without affecting milk composition. Although energy supplementation reduced fiber digestibility and N intake, no overall effect on organic matter digestibility or microbial N availability was observed. The optimum ratio of concentrate to milk will likely vary with the availability and nutrient status of the pasture as well as the genetic merit, stage of lactation, energy status, and reproductive status of the cow.

Ideally, the chemical composition of the concentrate should complement the composition of the grazed pasture such that the combination meets the nutrient requirements of the grazing dairy cow. Immature pasture is often high in moisture, crude protein, and rumen-degradable protein, and is low in fiber. Therefore, most research indicates that pastured dairy cows rarely respond to concentrates that contain more than 16% crude protein. Although most fresh pasture contains low quantities of rumen-undegradable protein, addition of rumen-undegradable sources such as fishmeal, corn gluten meal, and expeller soybean meal has seldom improved milk yield. In most instances, protein needs for moderate levels of milk production ($<35\,\mathrm{kg\,day^{-1}}$) may be met by traditional protein supplements such as solvent soybean meal and cottonseed meal along with the protein in pasture and that synthesized through rumen microbial growth. Rumen-undegradable protein supplementation of pasture has proven beneficial with high-producing, early-lactation cows, but targeting supplements for specific groups of lactating cows, though common in confinement operations, is difficult on grazing farms. Nevertheless, varying the amount and type of concentrate with stage of lactation and pasture quality can be beneficial. Research at Pennsylvania State University demonstrated that weekly adjustments in concentrate composition and intake, based on changes in pasture availability and quality, reduced feed cost 12–15% without lowering milk yield.

Pastured dairy cattle typically experience greater postpartum condition loss than cows fed in confinement. This may be due to higher maintenance requirements, lower DM intake, low energy density, or poor dietary energy utilization. As a rule, grain mixtures that provide an abundant supply of soluble carbohydrates in combination with digestible fiber are most conducive to high milk yield while maintaining rumen health. In addition, supplementation with high-fat by-products such as whole cottonseed (at 10–12% of diet DM) has been shown to maintain milk yield similar to ground corn, while increasing milk fat content considerably. Addition of vegetable oils is often limited to 2–3% of concentrate DM since these rumen-soluble fat sources are known to inhibit fiber digestibility and lower milk fat concentration.

Health

Mastitis (intramammary infection) is the leading health problem of dairy cattle in the United States. In a Vermont study, milk quality and udder health of 15 dairy herds using traditional continuous grazing, MIG (rotational grazing), or confinement feeding were compared. It was found that mean standard plate bacterial counts were lower in milk from rotationally grazed herds than confined herds or continuous grazing herds. Milk from MIG herds with more than 60 cows contained more coliform bacteria than milk from MIG herds with less than 60 cows. These findings were confirmed in a 3-year North Carolina State University study in which the incidence of clinical mastitis among pasture-fed cows was 24% compared to 43% for cows fed in confinement. Jersey cows experienced a lower incidence of mastitis than Holstein cows in both pasture and confinement herds.

Although data are sparse, the incidence of other infectious diseases appears similar between grazing and confinement herds. Obviously, hygiene, vaccination practices, and overall animal care likely play a greater role in disease incidence among dairy herds than forage programs *per se*. Nevertheless, there are several diet-related disorders that are more likely to occur among pastured dairy cows than in cows receiving diets based on stored forages. Milk fever, a condition arising from the cows' inability to mobilize calcium reserves at parturition, may be elevated among grazing herds that allow prepartum cows to graze large quantities of lush pasture. Other disorders of concern for grazing dairy cows are bloat, chiefly associated with leguminous species, hypomagnesemia (grass tetany), and nitrate toxicity. Most of the above-mentioned disorders can be prevented with proper mineral supplementation and pasture management. There is some evidence that hoof damage is more prevalent in cows fed on concrete than in cows fed on pastures, but management factors such as level of dietary effective fiber, mineral nutrition, and overall cow comfort may have a greater effect on hoof disease than the specific housing system employed.

In summary, pasture-based herds tend to produce less milk (8–10%) of lower butterfat than confinement herds, but net returns may be higher due to lower labor, stored forage, and waste management costs. Dairies in more northern climes typically graze 4–5 months in the spring and summer, relying on stored forages and TMRs the remainder of the year. Many of these dairies use a fall calving program such that cows are being fed TMR in confinement at peak milk time, and at mid-to-late lactation, when diet nutrient requirements are lower, cows are turned onto spring pasture. Under the northern system, concentrate supplementation is minimal (2–6 kg day^{-1}). In contrast, in southern dairies in which cows are on pasture for much of the year, concentrate is usually supplemented at a higher rate to maximize peak milk production and assure adequate energy repletion for timely rebreeding. Forage supplementation is necessary on all dairies during periods of drought, cold, and low pasture quality. In high-humidity regions, many hay producers are adopting the bale silage technology to reduce field losses and improve forage quality. Pastured herds tend to experience less mastitis and foot problems than confinement herds, though caution must be exercised to avoid metabolic problems associated with low plant mineral concentrations and plant toxins. Pasture will continue to be an important forage component for many small- to moderate-sized (50–500 cows) US dairies.

See also: **Dairy Farm Management Systems**: Non-Seasonal, Pasture-Based Milk Production Systems in Western Europe. **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties; Grazing Management; Perennial Forage and Pasture Crops – Species and Varieties. **Mastitis Therapy and Control**: Management Control Options.

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Non-Seasonal, Pasture-Based Milk Production Systems in Western Europe

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Introduction

Milk production systems in Western Europe continue to be subject to significant change. For example, during the last 40 years the emphasis has changed from increased production per cow and per farm to quota restrictions on milk output at farm level (from 1984 to the present), but with the abolition of the European Union (EU) milk quota regime now planned for 2015. In addition, issues related to animal welfare, food safety, animal health, and the environmental impact of milk production have become increasingly important. Major structural changes have also taken place within the European dairy industry, with the number of dairy farms in steep decline, while average herd size and average milk yield per cow continue to increase. These changes are highlighted within the United Kingdom where the number of dairy farms decreased from approximately 41 000 to 19 000 between 1990 and 2006, while during the same period, the average herd size increased from approximately 67 to 95 cows, and the average milk yield per cow increased from approximately 5100 to 6800 l.

Management Systems

Within Western Europe, dairying systems can be classified into two major categories: seasonal spring-calving systems and non-seasonal production systems.

Seasonal Spring-Calving Systems

In this system, calving is targeted to coincide with the onset of grass growth in spring (February and March) and the primary objective is to achieve 7–9 months of pasture feeding. Supplementary concentrates are used in some instances to increase individual animal performance. During the winter period, when animals are housed indoors, grass silage is the predominant basal forage. This system is used widely throughout the Republic of Ireland and in western regions of the United Kingdom. The principles of this production system are very similar to those in other regions of the world with highly specialized pasture-based milk production systems (e.g., New Zealand, Australia, and South America).

Non-seasonal Pasture-Based Milk Production Systems

The majority of milk production systems in Western Europe can be classified as non-seasonal, with the degree of reliance on grazed pasture primarily dependent on grass growing conditions. In these systems, animals may calve through all 12 months of the year, but in the majority of the systems, calving is targeted during the autumn/early winter period. During the winter feeding period, which may last from 3 to 6 months depending on soil and climatic conditions, cows are housed indoors and have *ad libitum* access to either grass or maize silage supplemented with cereals, by-product feeds, or concentrates depending upon individual farm circumstances. However, in comparison with milk-producing regions in the southern hemisphere, the higher milk price in Western Europe (resulting from quota regulation of milk supply) enables profitable production of milk during the winter period. Animals are normally turned out to pasture for between 6 and 9 months of the year with supplementary concentrates fed to higher yielding cows during the grazing period. It is difficult to generalize on feed input–milk output relationships, given the wide range of feed inputs between herds, but in general these systems span the full range from 0.75 t concentrate and 5500 l per lactation to 3.5 t concentrate and 13 000 l per lactation. The primary objective of this article is to examine the major characteristics of production systems in Western Europe, to highlight some of the major challenges facing the systems, and to suggest how they might change in the future.

Dairy Cow Breeds

The Holstein Friesian breed is the predominant dairy breed throughout Western Europe, although other breeds (Simmental, Jersey, Montbeliarde, Normande, and Scandinavian Red) have a significant presence in localized regions. The predominance of the Holstein Friesian can be largely attributed to the substantial genetic progress in milk production that has been made with the breed, with research evidence highlighting improved efficiency of conversion of feed into milk with increasing

Table 1 Effect of dairy cow genetic merit (Holstein Friesian) on production efficiency for the first 150 days of lactation

Genetic merit	*Food intake (kg DM day^{-1})*	*Milk yield (kg day^{-1})*	*Gross energetic efficiency (ratio of milk energy output to feed energy input)*	*Condition score change*	*Partial energetic efficiency (kl)*
Low	19.0	29.0	0.25	0.54	0.59
Medium	19.4	30.6	0.27	0.52	0.58
High	20.2	37.2	0.30	–0.18	0.58

Reproduced with permission from Gordon FJ, Patterson DC, You T, Porter MG, Mayne CS, and Unsworth EF (1995) The influence of genetic index for milk production on the response to complete diet feeding and the utilization of energy and nitrogen. *Animal Science* 61: 199–210.

genetic merit for milk production (**Table 1**). In this study, cows of differing genetic merit were offered a total mixed ration *ad libitum* for 150 days in early lactation. While milk yields increased with increasing genetic merit, feed intakes were only marginally higher. However, over the experimental period, the high-merit cows exhibited a net loss of body condition, with approximately 50% of their increased milk yield being directly attributed to mobilization of body reserves. Further detailed studies using energy exchange measurements in respiration chambers suggest that genetic merit has little effect on the partial energetic efficiency for milk production, thus highlighting a major problem of increasing genetic merit in production systems with a high reliance on grazed or conserved grass. In such systems, the small increase in intake potential associated with increasing genetic merit is not sufficient to support the increased demands of lactation, and consequently, animals have to draw further on body energy reserves, particularly in early lactation. The higher metabolic turnover in these animals predisposes them to increased risk of a range of production diseases including mastitis, lameness, and infertility. Given the increasing consumer interest in animal welfare and food safety issues, and the negative economic impact of reduced longevity, breed selection goals within many European countries are increasingly focusing on 'secondary' traits such as fertility, longevity, and cow health.

Indoor/Winter Housing and Feeding

There is considerable variation in the duration of the winter housing period across Western Europe, ranging from a minimum of 1 month per year in southwest Ireland to 8 months per year in Scandinavia. This range primarily reflects differences in climate and soil type, which limit the duration of both the growing and the grazing seasons.

Housing Systems

During the indoor-housed period, the majority of dairy cows in Western Europe are loose housed in free-stall cubicle houses with *ad libitum* access to grass or maize silage. In recent years, there have been major improvements in the design of cubicles and cubicle floor coverings to improve cow comfort, with longer cubicles (up to 2.75 m) now being recommended for today's Holstein Friesian cow. More recently, in order to mitigate against possible negative effects of concrete floors on hoof health, a number of synthetic floor coverings have been developed with the aim of cushioning the hoof during housing. While some of these are commercially available at present, there appears to be limited evidence as to their effectiveness. Similarly, major advances have been made in slurry handling systems to minimize labor use, ranging from the use of slatted floors with underground slurry tanks to automated scraper systems, which scrape slurry from solid floors to external slurry stores. A key consideration on farms throughout many parts of Europe is the legislative requirement to ensure that there is sufficient slurry storage capacity to avoid the need for return of slurry/manure to the land during periods when it may be susceptible to nutrient loss.

Forage Conservation Systems

High-quality conserved forage is the basis for milk production during the indoor-housed period, with grass and maize silage the main conserved forages. In grass silage-based systems, grass is normally harvested from intensively fertilized perennial ryegrass-based swards on 2–3 occasions over the growing season. Key factors influencing the feed value of conserved grass include stage of growth or digestibility at the time of harvest, dry matter (DM) content, and fermentation characteristics. Under ideal conditions, the objective is normally to ensile grass with a D-value (digestible organic matter in the DM) of 720–750 g kg^{-1}, with this being achieved by harvesting the grass sward in early/mid-May, early July, and again in mid-August. The importance of harvesting a highly digestible forage is highlighted in that each 10 g kg^{-1} decrease in silage D-value will result in reductions in silage DM intake and milk yield of approximately 0.15 and 0.35–0.40 kg day^{-1}, respectively. Under typical Western European conditions, each week delay in harvest from early May onward results in a decline in digestibility (D-value) of approximately 30 g kg^{-1}.

When climatic conditions are suitable, field wilting of herbage is normally practiced, with the aim of achieving herbage DM content of between 250 and 300 g kg^{-1} fresh weight. In addition to the reduction in effluent loss, recent research has demonstrated substantial cow performance benefits when silage produced using rapid wilting techniques (24–36 h maximum) is offered. However, given the unstable climatic conditions in Western Europe, grass is often ensiled without a period of field wilting, or following a 'wilt' of only a few hours.

In many instances, especially on the most progressive farms, herbage is treated with an additive at the time of harvest. Additives used tend to be either 'acids' or bacterial inoculants, with country-to-country variation in the type of additive commonly employed. There is now a substantial volume of evidence to indicate that treatment of herbage at ensiling, especially low DM herbage, with either formic acid or an effective inoculant will improve food intake and animal performance. In most dairy systems in Western Europe, forage is normally ensiled in pit- or bunker-type silage clamps, although the use of big bale silage is reasonably common.

An important development in relation to grass silage has been the development of improved systems for the prediction of silage feeding value (intake potential and digestibility) based on near-infrared reflectance spectroscopy (NIRS). Given that NIRS is widely used to predict the chemical composition and digestibility of a wide range of feedstuffs, routine prediction of the intake characteristics and digestibility of grass silage is now commonplace, and can be undertaken by many laboratories at minimum cost. Future developments in this technology are likely to improve the accuracy with which dairy cows can be rationed.

While grass silage remains the predominant winter forage in the cooler wetter regions, the popularity of forage maize as a winter forage crop in these regions has increased dramatically in recent years. This change can be attributed to the higher DM yield potential of maize compared to grass silage, the fact that the crop can be harvested in one operation per year, and significant advances in plant breeding. Improved maize varieties can now be grown in areas previously considered climatically unsuitable and, in addition, recent developments allow the maize crop to be sown under plastic, thus extending its range even further and resulting in significant improvements in both DM yield and starch content.

Forage Feeding Systems and Supplementation Strategies

While self-feed forage systems (cows 'graze' silage behind a feed fence in the silo) were common in the past, these have now largely been replaced by easy feed and, more recently, total mixed ration systems. With easy feed systems, silage is removed from the silo and offered to the cows behind a feed barrier, with cows normally receiving supplementary feed during milking, or via electronic out-of-parlor feeders. However, as herd size has increased throughout Europe, there has been increased adoption of total mixed ration feeding systems in which silage and a range of other feeds including cereals (wheat, barley, and maize), by-product feeds (sugar beet pulp, citrus pulp, and maize gluten), and protein supplements (soya bean meal and rapeseed meal) are mixed prior to feeding using a mixer wagon, and offered behind a free-access feed manger.

When maize silage is offered, it is normally included as an additional forage in the diet, substituting for grass silage, with maize inclusion rates up to 60% of total forage within the ration. Recent evidence suggests that the effects of forage maize inclusion on food intake and animal performance are highly dependent on the DM and starch content of maize, with responses in animal performance increasing with starch content of up to 30% of DM.

The use of supplementary concentrate feeds within these systems is justified in that even with high-quality grass or maize, forage DM intake and animal performance are unlikely to exceed 16 kg DM and 20 kg milk day^{-1}, respectively. Thus the main objective of providing supplementary feed is to improve feed intake and enhance animal performance. Supplements are normally offered 'in-parlor' during milking, via electronic 'out-of-parlor' feeders, or as feed mixed with the forage component of the diet prior to feeding (complete mixed), or via a combination of these systems. At low supplement feed levels (less than 8–9 kg day^{-1}), all three systems are likely to be equally effective, while at higher supplement feed levels, twice-daily supplementation in-parlor becomes problematic. At supplement levels of greater than 13.0–14.0 kg day^{-1}, research data suggest that total mixed diets may result in increased animal performance compared to feeding the concentrate component of the diet separately. However, the provision of supplementary feed in addition to a basal forage normally results in a reduction in forage intake, with typical substitution rates ranging between 0.3 and 0.7 kg reduction in forage DM intake per kg increase in concentrate DM intake. Substitution rate is highly correlated with the intake of the forage as a sole feed, and consequently, with high forage intake, incremental increases in supplement feed level result in higher substitution rates and lower production responses compared to those obtained with low forage intake, as shown in **Figure 1**.

Pasture Management

Climatic and soil conditions in western and northern regions of Europe favor the use of grass as the primary

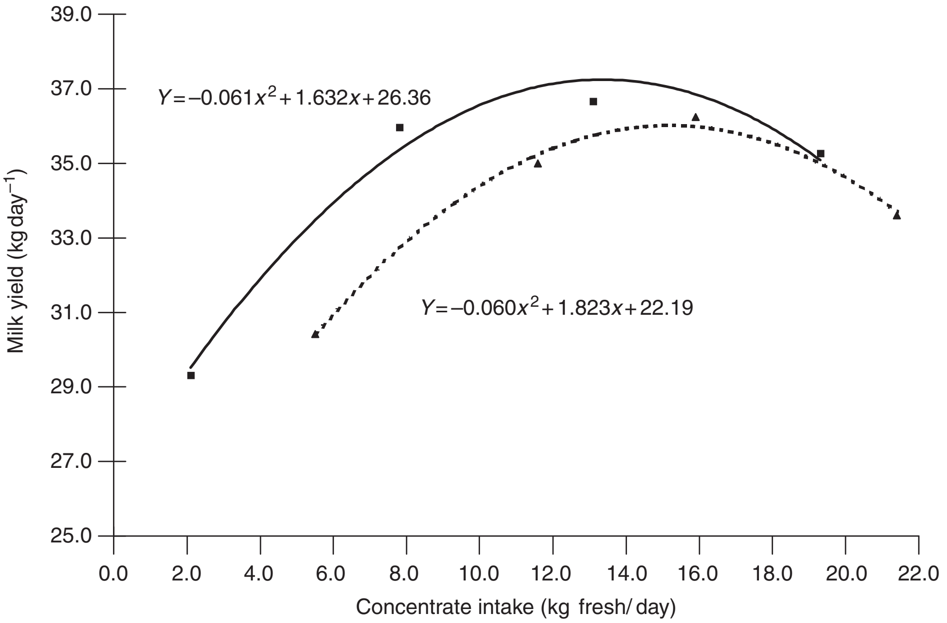

Figure 1 Effect of concentrate feed level on milk yield (adjusted to 32 g kg^{-1} protein and 40 g kg^{-1} fat) with high (——) and medium (- - - -) feed value silages. Reproduced with permission from Ferris CP, Gordon FJ, Patterson DC, Kilpatrick DJ, Mayne CS, and McCoy MA (2001) The response of dairy cows of high genetic merit to increasing proportion of concentrate in the diet with a high and medium feed value silage. *The Journal of Agricultural Science* 136(03): 319–329.

forage crop. Utilization of grass by grazing animals has long been recognized as the most economically efficient, but also most challenging, means of converting grass to animal product. It is also widely recognized that grazing requires substantially fewer resource inputs than other methods of pasture utilization, is more sensitive to the welfare needs of animals, and also helps to preserve the rural landscape. Given these advantages, it is clear why grazed grass continues to play a major role in non-seasonal milk production systems in Western Europe.

Grazing Systems

A wide range of grazing systems are operated on farms in Western Europe, ranging from controlled rotational grazing in Republic of Ireland, parts of the United Kingdom, France, and The Netherlands to more extensive set stocked systems with animals having access to the entire grazing area. Experimental evidence indicates little difference between grazing systems in terms of effects on individual animal performance or output per hectare, even with high-yielding cows. However, rotational grazing offers a number of management advantages compared with continuous grazing. For example, rotational grazing enables identification of grass shortages and surpluses and offers greater flexibility to adjust grass supply (through addition or removal of paddocks). Rotational grazing systems also facilitate a range of options for controlling sward quality in situations where the objective is to maximize grass intake and production per animal rather than utilization per hectare. For example, individual paddocks can be mechanically topped following grazing or a leader/follower grazing system can be used to utilize residual herbage with lower yielding cows or dry stock.

Intensively fertilized perennial ryegrass swards predominate within these systems, with levels of nitrogen (N) fertilizer up to 350 kg ha^{-1} yr^{-1} having been the norm on the most intensive farms until recently. While such swards are capable of producing up to 13.5 t DM ha^{-1} yr^{-1}, total inputs of N fertilizer over the growing season are now more likely to be between 200 and 300 kg N ha^{-1} yr^{-1}. Furthermore, tactical use of N through the growing season enables some manipulation of the seasonal pattern of grass growth to facilitate early and late grazing, and a more uniform pattern of grass growth through the summer period. For these reasons, grass/white clover swards are not widely used in the main dairying regions within Western Europe. Nevertheless, the spiraling cost of fertilizer, combined with increasing legislative restrictions on the use of inorganic N fertilizer, has resulted in renewed interest in the use of grass/white clover swards.

Grass Intake and Grazing Efficiency

The major challenge in grazing management is to balance the conflict between maximizing intake per cow and the need to ensure a high efficiency of grassland utilization. While maximum intake, and hence maximum production per animal, is achieved at low grazing intensity, a high grazing intensity is required to achieve efficient sward utilization. This conflict between yield per animal and efficiency of grassland use has resulted in the development of a range of grazing systems. For example, moderate genetic merit cows may be grazed relatively intensively so as to achieve a high efficiency of grass use, while high genetic merit cows may be grazed less intensively to achieve a high milk yield per cow, while accepting a lower level of grass utilization. Alternatively, high genetic merit cows may be grazed at moderate–high stocking rates to achieve high levels of grass utilization, but with supplementary feed offered so as to achieve a high milk yield per cow.

Supplementation at Pasture

The provision of supplementary feeds to dairy cows at pasture is normally undertaken either to improve animal performance over and above that which can be produced from pasture alone, or to maintain performance during periods of grass shortage. There is a wide variation in response to supplementation at pasture depending on grazing conditions, production potential of the cow, and the level and type of supplement offered. However, recent evidence suggests that high-yielding cows (>35 kg milk day^{-1}) respond better to supplements than lower yielding animals (<25 kg milk day^{-1}), a reflection of the inability of high-yielding cows to harvest sufficient additional herbage to meet their increased nutrient requirements. Recent recommendations, shown in **Table 2**, suggest target concentrate feed levels for cows of differing yield potential through the grazing season. Increased concentrate feed levels are required later in the season to compensate for the lower herbage intake characteristics of mid- and late season swards.

Animal Health Concerns

Detection of new and emerging diseases at the earliest possible opportunity is of increasing importance to minimize hazards to consumers, producers, and trade. Recent epidemics of bovine spongiform encephalopathy (BSE) and foot and mouth disease (FMD) in the United Kingdom, and more recently the spread of bluetongue virus into Northwest Europe, highlight the need to monitor the industry and to act promptly.

The major endemic disease problems in dairy cows are mastitis, infertility, and lameness. Concerns for animal welfare and consumer awareness of potential for transfer of antibiotic resistance to pathogenic bacteria have increased the need for prevention and nontherapeutic control of these conditions.

Mastitis

In a recent farm survey, mastitis accounted for a significant (10%) proportion of cows removed from the herd on an annual basis. Infections caused by *Escherichia coli* or *Streptococcus uberis* account for at least 50% of clinical mastitis cases. The former occurs most commonly in recently calved cows that are stressed, for example, through milk fever or prolonged labor. Controls include preventing such stress conditions, improving environmental conditions and careful hygiene particularly at calving, and reducing the severity of clinical signs of the disease by vaccination. *Streptococcus uberis* is an increasingly important cause of clinical mastitis in Britain and can persist following clinical recovery. Infections due to other streptococcal species, *Staphylococci* sp., and *Arcanobacterium pyogenes* occur less frequently but result in culling, particularly in older cows when there is residual pathology after clinical cure. Prevention by dry cow therapy is a common approach to minimize disease associated with these bacteria. *Mycoplasma* sp. infections are also increasing, and although there can be self-cure in younger cows, effective therapies are needed. Other infections that may compromise immunity have been linked to mastitis. For example, clinical mastitis increases in the year following the introduction of bovine diarrhea virus to naive herds. Fortunately, selection for low somatic cell counts in breeding programs has not

Table 2 Suggested concentrate feed levels for high-yielding dairy cows in early and late season offered a moderate herbage allowance

	Early season target milk yield (kg cow^{-1} day^{-1})			*Late season target milk yield (kg cow^{-1} day^{-1})*	
	25.0	***35.0***	***40.0***	***25.0***	***35.0***
Potential milk yield from grass (kg day^{-1})	27.0	29.4	30.9	20.0	24.5
Supplement feed level required (kg cow^{-1} day^{-1})	0	4.5	7.0	4.0	8.5

Reproduced with permission from Mayne CS, Wright IA, and Fisher GEJ (1999) Grassland management under grazing and animal response. In: Hopkins A (ed.) *Grass: Its Production and Utilization*, 3rd edn., pp. 247–286. Blackwell Science Ltd.: British Grassland Society.

increased susceptibility to mastitis and may reduce clinical cases when included as a breeding goal.

Early detection of mastitis is essential to improve cow welfare, reduce production loss, and meet consumer requirements for safe milk products. The installation of automated milking systems to increase milking frequency and reduce labor inputs offers fewer opportunities for observation by milkers. However, research into automated detection sensors that can detect clinical mastitis and a range of other metabolic parameters by monitoring changes in milk is progressing rapidly. As these systems become more accurate, they are likely to be routinely installed in commercial operations.

Lameness

Lameness in dairy cows, which is increasing in frequency, has a negative impact on animal welfare and is attracting increasing attention. Over 25% of dairy cows show signs of lameness at least once during lactation, and lameness is one of the major reasons for removal of cows from the herd (approximately 15% of removals). Claw lesions and interdigital conditions are the most common problems, accounting for over 50% of cases. Claw lesions, which are the most intractable, probably result from abnormal gaits and excessive exposure to hard or uneven surfaces or poor walkways. Other risk factors include prolonged housing, high concentrate inputs, and poorly designed cubicles. The effects may be cumulative throughout the productive lifetime; therefore, careful management begins during the rearing period of the heifer. Early recognition and treatment of the problems by the stockperson or specialist foot trimmer can reduce the incidence of lameness. Preventive measures include routine foot trimming, footbathing, improving surfaces, and avoidance of simultaneous challenges, for example, separation of calving and housing dates. The frequency of digital dermatitis is also increasing, particularly at or shortly after calving. Routine antibiotic footbathing can control but not eradicate the condition, and antibiotic footbathing is expensive and no longer acceptable to consumers. This form of lameness is associated with housing conditions, occurring least severely where cows are housed in straw-bedded yards. Minimizing exposure of cows' hooves to slurry is an essential element in control. There is evidence that a proportion of cows are resistant, but further research is needed to confirm breeding as a possible solution.

Infertility

Despite control of major infectious diseases, reproductive efficiency of dairy cows has fallen in many countries and the culling rate remains very high with approximately 25% of all removals being attributed to infertility. Increased herd size, high labor costs, and low profit margins have contributed to the decline. Management changes reduce time available for heat detection. Several recording systems estimate that only 55% of available heats are detected. Increased use of heat detection aids, including installation of automated heat detection systems, are being investigated to reduce loss resulting from this inefficiency. Estrus synchronization, by prostaglandin or progesterone treatments, to remove the need for heat detection is not sufficiently precise to be adopted as a herd routine. A sustained decline (estimated at 0.5–1% per annum) in dairy cow fertility has been experienced in several national dairy herds. Links between nutrition and fertility are under investigation and some Nordic breeds are benefiting from the earlier adoption of selection programs incorporating fertility indices. For example, in the Scandinavian red breeds, the total economic merit selection approach has counteracted the anticipated negative response in fertility expected from selection solely for increased production. Future national breeding research programs are likely to include early and objective measures of fertility, identification of genes that affect fertility, metabolic stressors, and length of productive life of cows.

National Eradication Schemes

Bovine tuberculosis continues to affect dairy herds across Western Europe. Infections are linked to increasing cow movements and to persistence in wildlife. Animal to animal transition remains the main route of infection and all movements are recorded in national registers. Trace back of movement assists in earlier eradication of infected and potentially infected animals. In the British Isles, the importance of the badger (*Meles meles*) is currently under investigation as a source of recurring infections in cattle. Environmental and conservation concerns oppose eradication of badgers and demand control by vaccination in these potential carriers.

Increasing attention has been focused on the prevalence and control of *Mycobacterium avium* ssp. *paratuberculosis* (Johne's disease in cattle), a suggested cofactor in the human intestinal disorder Crohn's disease, as this bacterium has been shown to survive the pasteurization process. National investigations have shown that the frequency of cow infection varies widely between EU member states, but an overall testing program leading to control and eradication has yet to be developed and agreed. Brucellosis (*Brucella abortus*) in cattle occurs in some localized areas and is controlled by test and slaughter programs.

The need for free trade throughout the European Community has also led to various programs to control major endemic diseases. Eradication of bovine diarrhea virus by the identification of persistently infected excreting cows is being introduced by local, regional, and national programs. Infectious bovine rhinotracheitis (IBR/IPV) is also being controlled by local programs. An increasing number of areas claim to be free of these infections.

Biosecurity

Improved biosecurity is a major new consideration for the dairy industry. In Europe, farming is an intensive industry. The dairy sector is becoming increasingly concentrated on larger farms, but these are confined into small land areas. Separation is thus often insufficient to prevent animal to animal contact and so there is enhanced opportunity to transmit infectious diseases. The newly introduced animal presents identifiable challenges that can be met by selective sourcing, pre-entry testing, and/or vaccination to ensure that health status is similar to that of the herd. However, many units also rely on contractors to conserve forage or to remove slurry, and their visits present additional risk. The combination of factors suggests that few farms will be able to follow full 'fortress farming' principles. However, risk management plans appropriate to individual farms or areas will become increasingly important aspects of dairy management.

Automation

The marked structural changes that have taken place in dairying in Western Europe in the last 20 years have resulted in major increases in farm size, with many smaller dairy farmers leaving the industry. This increase in farm size has created major difficulties, particularly in those countries with the largest herd size (Denmark, 86 cows; UK, 78 cows; The Netherlands, 61 cows). The particular problems relate to availability and cost of labor and this causes specific problems for those herds expanding from 1 to 2 person units (i.e., a considerable increase in scale is required to 'justify' the second employee). Alongside this underlying trend, major advances in automation, including robotic milking, automated heat detection, and automated feeding systems, offer considerable potential to reduce labor requirements, particularly for the more routine/mundane tasks. There is no doubt that automation offers considerable potential for the future, including integrated management systems in which information on milk yield, milk quality, and hormonal assays determined through the robotic milker can be combined with condition scoring via digital imaging and information on cow movements to 'manage' the feeding, health, and welfare of the individual cow. While considerable progress is required to develop a fully integrated management system, individual components of this approach are currently available and are already being incorporated into existing management systems.

Environmental Issues

European Union legislation, primarily in the form of the Nitrates Directive (91/676/EEC), has had a considerable impact on European milk production systems during the last 10 years. This legislation seeks to protect waters from nitrate pollution of agricultural origin, and necessitates the designation of 'vulnerable zones' in regions where waters are polluted or likely to become polluted by nitrates, or where eutrophication is a problem. When a region is designated as vulnerable (Nitrate Vulnerable Zone), an action program must be implemented to mitigate against further reduction in water quality, and to seek to improve water quality. These action programs will normally involve limits on stocking rates, restricted periods for manure, slurry, and chemical fertilizer applications, and a requirement for a minimum period for slurry (16–25 weeks). In addition, where eutrophication is a major issue, controls on the use of inorganic phosphorus fertilizer and farm phosphorus surpluses may be implemented. In the future, the contribution of dairy systems to climate change via greenhouse gas emissions will become increasingly important, and it is likely that future legislation will seek to control the production of methane from dairy systems.

Conclusions

The European dairy industry is the only major dairy industry (with the exception of Canada) with supply control measures in place for over 20 years. These supply control measures have maintained milk price above world market levels, but have also contributed to considerable restructuring within the industry, particularly in terms of business size. The dairy industry in Europe is also at the forefront of current consumer concern in relation to animal welfare, food safety, and potential environmental impact. There are resulting conflicts between operating production systems based on grazed and conserved forage with modest inputs of supplementary feeds, and the need to satisfy the requirements of the high genetic merit dairy cow. This reflects the fact that genetic selection of dairy cows for increased milk yield has resulted in cows with an enhanced ability to mobilize body reserves but which are predisposed to increased risk of a number of production diseases. While some of these problems may be resolved by increased use of automation, possibly including fully integrated management systems, it is likely that there will be increased diversity in milk production systems in Europe in the future. Farmers will increasingly have to adopt the most appropriate system for their individual farm circumstances, with dairy cows selected to perform within their particular system to meet changing consumer requirements.

See also: **Dairy Farm Management Systems**: Dry Lot Dairy Cow Breeds; Goats; Non-Seasonal, Pasture Optimized, Dairy Cow Breeds in the United States; Seasonal, Pasture-Based, Dairy Cow Breeds; Sheep. **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis; Infectious Diseases: Johne's Disease; Infectious Diseases: Tuberculosis.

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Dry Lot Dairy Cow Breeds

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Introduction

Confinement management systems vary greatly in the United States, Mexico, Canada, western European countries, and Middle East regions depending on herd size, environmental conditions, feed sources and availability, and management style. Drylot management systems occur in drier and warmer regions of the United States with larger herd sizes (over 500 cows). Building investment is lower compared to freestall confinement systems. **Table 1** summarizes herd sizes of dairy cattle in the United States in 1999 by number of herds and percent of cows.

Herd size in the United States has continued to increase annually by 4–5%, number of herds has dropped by 2–3% annually, the number of dairy cows has declined by 1% annually, and milk yield per cow has increased by 2–3% annually. In the United States, optimizing milk production (not always maximizing milk yield) is the goal on most farms. In some areas of the world, this goal will be at different levels of milk yield based on their economics and available resources. This article will emphasize feeding aspects and summarize current trends in the area of housing, milk systems, and farm management.

Feed Management Systems

A successful feeding system can be defined as one that delivers the needed nutrients to each cow to meet her requirements as economically as possible. Stage of lactation, gestation, and growth (young cows) will determine nutrient requirements and feeding system strategies. Four factors can impact the nutrient requirements of dairy cows. Each factor has a pattern as the cow progresses through the lactation and gestation cycle.

Factor 1: Milk Production Curve

Milk production drives nutrient needs of dairy cows. Peak milk sets the lactation curve for cows and should occur 40–60 days after calving. First lactation cows should reach 75% or greater peak milk levels compared to peak milk levels of mature cows in the herd. For example, if first lactation cows averaged 30 kg of peak milk while mature cows averaged 40 kg of peak milk, the ratio is 75% (30 kg divided by 40 kg times 100). If the ratio is less than 75%, first lactation cows are not peaking high enough compared to mature cows. If first lactation cows peak over 75% of mature cows, heifers are peaking higher due to improved genetics, health, and/or heifer rearing programs or mature cows are not peaking high enough due to health or management limitations.

Factor 2: Milk Fat and Milk Protein Curve

Milk fat and protein levels will vary by breed (**Table 2**). If milk fat test is below milk protein test by 0.4 of a percentage point or more (e.g., 2.7% milk fat and 3.2% milk protein), rumen acidosis can be occurring (reduce percent crude protein test by 0.2% (3.2% compared to 3.0%) if true protein test is used, as adopted in the United States in 2000). If the milk protein test is below breed average or drops during lactation, the following nutritional causes of lower milk protein could be occurring: (1) low levels of fermentable carbohydrate (lowers microbial protein synthesis); (2) low levels of dry matter intake (reduces the supply of nutrients available for the rumen microbes and cow); (3) protein shortage and/or imbalance of amino acids; or (4) use of fats and oils as energy sources (fat is not a source of rumen-fermentable energy).

In January 2000, the United States shifted to expressing milk protein as true protein instead of crude protein. This change was related to the shift of new milk marketing orders to component-based pricing (kilograms of milk protein and milk fat). True protein values are approximately 0.19% lower than crude or total protein. Values in **Table 2** reflect total or crude protein percentages based on 1999 data.

Factor 3: Dry Matter Intake Curve

Increasing dry matter intake (DMI) can minimize metabolic disorders, minimize weight loss, and improve reproductive performance. During late gestation, DMI can decline 2–4 kg per cow. Wisconsin workers concluded that DMI at calving impacted DMI 4 weeks postpartum. If dry matter is lower than predicted, the nutrient concentration must be increased to meet the cow's nutrient requirements. After calving, DMI slowly increases (**Table 3**).

Table 1 Distribution of herd sizes and cow numbers in the United States in 2008

Head (number of cows)	*Operations (number)*	*Cow number (percent of total)*	*Milk production (percent of total)*
1–29	20 400	1.8	1.2
30–49	11 500	4.9	3.8
50–99	17 300	13.0	11.4
100–200	8600	12.4	11.6
200–499	3850	12.3	12.5
500–999	1700	12.5	12.6
1000–1999	910	13.3	15.7
Over 2000	740	29.8	31.2

Table 2 US breed comparisons of milk fat, true milk protein test, and total or crude milk protein test

Breed	*Milk fat (%)*	*True milk protein (%)*	*Total milk protein (%)*
Ayrshire	3.85	3.15	3.34
Brown Swiss	3.97	3.30	3.49
Guernsey	4.46	3.31	3.50
Holstein	3.70	3.02	3.21
Jersey	4.59	3.58	3.77
Milking Shorthorn	3.78	3.10	3.29

Adapted from Hoard's Dairyman (2009) Hoard's focus on breed association activity. *Hoard's Dairyman Magazine* 154(13).

Table 3 Estimated dry matter intake (DMI) for first lactation (545 kg) and mature (636 kg) cows for the initial 5 weeks postpartum

	kg DMI day^{-1} per cow	
Week	*First lactation cows*	*Mature cows*
1	14.1	16.6
2	15.9	19.3
3	17.3	21.1
4	18.2	22.3
5	18.9	23.9

Adapted with permission from Kertz AF, Reutzel LF, and Thomson GM (1991) Dry matter intake form parturition to mid-lactation. *Journal of Dairy Science* 74: 2290–2295.

DMI for first lactation cows is less than that for mature cows, which must be considered when concentrate is fed independent of forages, especially in component-fed herds. Guidelines for DMI for various phases are listed in **Table 4**.

Factor 4: Body Weight Loss and Gain Curve

Monitoring weight changes provides valuable information on the energy status of cows. High-producing cows will lose weight to support high energy needed in early lactation. Body condition scoring (BCS) is a field method to monitor weight changes. The following guidelines can be used to evaluate weight changes:

1. One BCS point (using the 1–5 system) is equal to 55 kg of body weight.
2. Cows should not lose more than 1–1.5 BCS points (55–80 kg) in early lactation.
3. Weight loss should be limited to 1 kg day^{-1} in early lactation to avoid negative effects on reproduction and the risk of metabolic disorders.
4. The cow should be at the optimum BCS prior to drying off (3.0–3.5). If dry cows are thin, limit weight gain to 0.5 BCS point (e.g., shifting dry cows from 2.75 to 3.25), which represents 30 kg or 0.45 kg day^{-1} weight gain during the dry period.

Phase Feeding

By evaluating the four factors during the lactation and gestation cycles in dairy cows, six feeding phases or rations can be developed (**Table 4**). On some farms, fewer groups of cows may be needed to meet nutrient needs. Phase feeding approaches take into consideration the various phases of the lactation and gestation periods. The feeding system must provide the needed nutrients to complement the four factors or curves discussed above.

Table 4 Illinois nutrient recommendations for dairy cows in different stages of lactation and gestation

	Dry cows		*Milk cows*			
	Early	*Close-up*	*Fresh, 0–21 days*	*Early, 22–80 days*	*Middle, 80–200 days*	*Late, >200 days*
Major nutrients						
DMI (kg)	13	10	18	24	22	19
Metabolizable protein (%)	6.0	8.0	13.8	11.6	10.2	9.2
CP (%)	12	14	19	17.5	16	14
Rumen-degradable protein: percentage of CP (DM)	70 (8.4)	60 (8.4)	60 (11.4)	62 (9.7)	64 (10.2)	68 (9.5)
Rumen-undegradable protein: percentage of CP (DM)	30 (3.6)	40 (5.6)	40 (7.6)	38 (7.8)	36 (5.8)	32 (4.5)
Soluble protein: percentage of CP (DM)	35 (4.2)	30 (4.2)	30 (5.7)	31 (5.6)	32 (5.1)	34 (4.8)
Total digestible nutrients (%)	60	67	75	77	75	67
NE_L (Mcal kg^{-1})	1.39	1.52	1.72	1.78	1.72	1.52
(kcal kg^{-1})	1386	1518	1716	1782	1716	1518
(kJ kg^{-1})	5838	6376	7207	7484	7207	6376
Ether extract (%)	2	3	5	6	5	3
Acid detergent fiber (%)	30	24	21	19	21	24
Neutral detergent fiber (%)	40	35	33	30	33	36
Nonfiber carbohydrate (%)[a]	30	34	35	38	35	32
Major minerals in terms of percentage of DM[b,c]						
Calcium (Ca)	0.60	0.7 (1.0)[d]	1.10	0.90	0.80	0.60
Phosphorus (P)	0.26	0.30	0.40	0.38	0.36	0.34
Magnesium (Mg)	0.16	0.40	0.38	0.35	0.25	0.20
Potassium (K)	0.65	0.65	1.00	1.00	1.00	0.90
Sodium (Na)	0.10	0.05	0.33	0.30	0.20	0.20
Chloride (Cl)	0.20	0.15 (0.8)[d]	0.27	0.25	0.25	0.25
Sulfur (S)	0.16	0.2 (0.4)[d]	0.25	0.25	0.25	0.25
Vitamins in IU per day						
Vitamin A	100 000	100 000	110 000	100 000	50 000	50 000
Vitamin D	30 000	30 000	35 000	30 000	20 000	20 000
Vitamin E	1000	2000	2000	1000	600	400

[a] Ratio of nonfiber carbohydrate (NFC) to Rumen-degradable protein (percentage of DM) = 3.5:1.

[b] Trace minerals: iron (100 mg kg^{-1}), cobalt (0.1 mg kg^{-1}), copper (15 mg kg^{-1}), manganese (60 mg kg^{-1}), zinc (60 mg kg^{-1}), iodine (0.6 mg kg^{-1}), and selenium (0.3 mg kg^{-1}).

[c] Ratio of minerals in total ration: zinc to copper 4:1, iron to copper 40:1, potassium to magnesium 4:1, copper to molybdenum 6:1, potassium to sodium 3:1, and nitrogen to sulfur 11:1.

[d] When anionic salts are used: mineral/anionic salts (%).

CP, crude protein; DMI, dry matter intake; DM, dry matter; NE_L, net energy of lactation.

Adapted with permission from Hutjens MF (2008) *Feeding Guide*. Fort Atkinson, WI: Heard's Dairyman Publishers.

Phase 1: Far-Off Dry Cows

Phase 1 begins at drying-off time to 21 days before calving. This period is also referred to as the traditional dry-cow period. These cows must be in a separate group (not with the lactating herd). A balanced dry-cow program can increase milk production by 200–700 kg in the subsequent lactation. Thus, phase 1 actually initiates the next lactation. During this phase, the cow's mammary gland involutes (dries up), the calf increases in size, and body weight gain can occur. To avoid metabolic disorders, limit weight gain to 0.45 kg day^{-1} or 0.5 BCS point (from 3.0 to 3.5 for example). DMI can vary from 1.8 to 2.5% of the cow's body weight (**Table 5**). The amount of concentrate fed can vary from 1 to 3 kg day^{-1}. A minimum of 1 kg of concentrate serves as a carrier of minerals and vitamins. Do not depend on free-choice mineral consumption to meet the mineral and vitamin needs of the dry cow and developing calf. Higher levels of concentrate are needed if cows are thin, if young cows need to grow, if environmental stress (cold or hot weather) is occurring, and/or if low-quality forage is fed. Feeding a minimum of 7–10 kg of maize silage (as-fed or on a wet basis) or 2–4 kg (on a dry matter basis) can provide additional energy from forage, lower dietary calcium and potassium levels, and improve ration palatability. **Table 4** lists the recommended level of nutrients for phase 1 dry cows. Management strategies for the far-off dry cow (phase 1) ration are listed below:

Table 5 DMI based on body weight and milk yield (4% fat-corrected milk)

	Body weight (kg)			
	400	*500*	*600*	*700*
Milk yield (kg)	*(kg DMI per cow per day)*			
20	14.5	15.9	17.3	18.2
30	17.7	19.5	20.9	22.3
40	21.8	23.2	24.1	25.0
50	NA	26.8	28.2	28.6
60	NA	NA	32.3	33.6

DMI, dry matter intake; NA, not available

1. 12–13% crude protein.
2. 60–80 g of calcium (15% lower for Jersey and Guernsey cows).
3. 30–40 g of phosphorus (15% lower for Jersey and Guernsey cows).
4. Limit salt intake to 30 g.
5. Force feed trace minerals and vitamins.

Phase 2: Close-Up Dry Cows

Phase 2 (close-up dry-cow period) starts from 21 days prepartum (days before calving). If this period is less than 10 days, 24% of the dry cows will not receive the phase 2 ration for the minimum 5 days needed to achieve desired benefits. Iowa workers identified four physiological goals that the close-up dry-cow program must achieve:

1. Adapt the rumen for higher energy diets fed postpartum.
2. Maintain normal blood calcium levels.
3. Build and stimulate the immune system.
4. Maintain a positive energy balance to avoid fatty acid infiltration and subclinical ketosis.

Increasing the level of concentrate shifts rumen microbes that can ferment high-energy diets. Energy balance can be negative for several reasons: (1) DMI may be 15–30% below the phase 1 level; (2) the unborn calf is rapidly growing, requiring more nutrients; (3) cows with twin pregnancy have lower DMI, earlier decline in DMI, and a greater conceptus mass; and (4) formation of colostrum and mammary tissue regeneration.

Body weight loss can occur and ketosis risk increased due to fat mobilization. Nonesterified fatty acids (NEFAs) originate almost entirely from mobilized fatty acids. Dietary fatty acids are transported as triglycerides and phospholipids. The presence of NEFAs in plasma above normal (> 0.3 mEq l^{-1}) indicates that fat is being mobilized in response to negative energy balance. Plasma NEFAs begin to increase 3–10 days prepartum, reach peak level at calving (0.5–0.8 mEq l^{-1}), and decline postpartum (<0.6 mEq l^{-1}) in healthy cows. **Table 4** provides nutrient guidelines for phase 2. Heifers in the later stages of pregnancy (also called springing heifers) may need to be in phase 2 longer (30–60 days) due to added growth and pregnancy requirements. Management strategies during the close-up dry period (phase 2) are listed below:

1. Increase concentrate to 2–4 kg of dry matter.
2. Increase crude protein to 14–15% using rumen-undegraded protein (RUP) sources.
3. Limit added fat to 150 g day^{-1}.
4. Maintain 2–4 kg of long forage (hay, long hay silage, and/or straw).
5. Consider feeding 3–5 kg of dry matter from the high-group total mixed ration (TMR) containing RUP, fat, concentrates, and higher quality forages plus the phase 2 concentrate mix and long forage.
6. Reduce and restrict supplemental sodium.
7. Add anionic compounds to prevent low blood calcium.
8. Add yeast culture (10–120 g day^{-1} depending on the product selected).
9. Add niacin (6 g day^{-1}).
10. Drench with propylene glycol (225 g) starting 3–5 days before calving or feed calcium propionate (180 g) if subclinical ketosis is observed.

Phase 3: Fresh Cows

Phase 3 is the fresh cow phase beginning at calving to 2–3 weeks after calving. The key management factor is the ability to monitor and observe these cows to ensure they are healthy when moved to the high group and are challenged with higher nutrient-dense diets. Individual cow management occurs in this phase requiring headlock and palpation rail in freestall housing, or tie stalls (individual cow restraint is needed to evaluate the cow). The following evaluations should be recorded each day to assess the cow's status:

1. Monitor feed intake by evaluating how the cow consumes or 'attacks' fresh feed. Record the amount or develop a scoring system (1 = 0–33% consumed, 2 = 33–66% consumed, 3 = 66–99% consumed, and 4 = all consumed feed offered).
2. Record daily body temperatures until temperatures drop below 38 °C.
3. Listen for rumen movements with a stethoscope (cows should have one to two rumen movements per minute).
4. Observe uterine discharges for odors and characteristics.
5. Conduct a ketone test on the cow's urine or milk to assess energy status.

The fresh cow ration should be intermediate between the close-up ration and the high group ration. Wisconsin workers suggest that a change in a ration should not be greater than 10% increase in a nutrient (e.g., changing from 1.54 net energy (NE) lactation by 10% would be 0.15 unit shift in the next ration or a 1.69 Mcal kg^{-1} NE lactation). Maintain a 'healthy' level of fiber and avoid high starch levels leading to off-feed risks. **Table 4** lists the recommended nutrient levels for this phase. The following strategies can be considered for fresh cows (phase 3):

1. Feed 1–3 kg of high-quality long forage to maintain rumen function.
2. Consider a fresh cow top dress concentrate mixture that contains RUP and digestible fiber (such as soybean hulls or citrus pulp) as an energy source.
3. Increase the ration nutrient concentration to adjust for lower feed intakes.
4. Supplement yeast culture to stimulate fiber digesting bacteria.
5. Add a buffer pack to stabilize rumen pH.
6. Provide 12 g of niacin to minimize ketosis. Provide 12 g of rumen-unprotected niacin or 3 g of rumen-protected niacin and 3 g of unprotected niacin.
7. Add propylene glycol (225 g) or calcium propionate (180 g) to raise blood glucose.
8. Consider adding 15 g of rumen-protected choline in the diets of cows at risk for fatty liver.

Phase 4: Early Lactation Cows

Early lactation rations are fed to cows from 14 to 100 days after calving (some high-producing cows and first lactation heifers will remain on this ration for the entire lactation). During this phase, cows achieve peak milk production, undergo weight loss (providing an additional source of energy), and reduce DMI (**Table 4**). Protein type and level are critical to reach peak milk production. The amount of supplemental fat has to be reduced to maintain dry matter intake. **Table 4** lists nutrient guidelines. Feeding strategies for early lactation cows (phase 4) are listed below:

1. Feed high-quality forage to improve DMI.
2. Provide additional sources of lysine and methionine to maintain a 6.6% lysine to 2.2% methionine expressed as a percent of essential amino acids based on rumen modeling software.
3. Increase concentrate levels gradually (maximum of 0.45 kg day^{-1}).
4. Limit the amount of supplemental fat to 0.6 kg day^{-1}.
5. Allow for adequate feed trough space (60 cm per cow).

Phase 5: Midlactation Cows

Cows show a decline in milk production in phase 5. Peak DMI has been reached with associated weight gain. A sharp decline in milk production and/or components indicates that nutrient needs are not being met. The time period for phase 5 can range from 70 to 200 days postpartum or until the cow dries off. Injecting with bovine somato-tropin (BST) has been initiated in countries where this technology has been approved. The goal in this phase is optimizing DMI. **Table 4** lists nutrient guidelines for this phase. Feeding strategies for midlactation milk cows (phase 5) are listed below:

1. Optimize DMI to meet energy and nutrient needs.
2. Replace lost body condition at 0.45–0.70 kg day^{-1}.
3. Raise supplemental fat to desired levels (5–6% of the total ration dry matter).
4. Review the need for feed additives and remove those additives that are not cost-effective.

Phase 6: Late Lactation Cows

Some herds will not have cows that qualify for a phase 6 ration or group (also called tail-end lactating cows). These cows are pregnant and gaining weight, and their milk production is declining 6% per month (first lactation cows) to 9% per month (second and greater lactation cows). This phase can begin 200 days after calving and ends when the cow dries off. High-producing cows may not reach phase 6. Cows should be gaining body weight (0.45–0.7 kg day^{-1}) plus growth needed for young cows if they have not reached their mature size. **Table 4** provides guidelines for phase 6, with feeding strategies listed below.

1. Increase the proportion of forages in the ration to reduce feed costs while meeting nutrient requirements (assuming forage is less expensive than concentrate).
2. Supplemental sources of RUP can be reduced relying on microbial and ration sources for protein.
3. Remove supplemental fat sources unless forage quality and DMI are limiting energy intake.
4. Eliminate feed additives to save money unless they are economically favorable.
5. Replace lost body condition before cows are dried off.
6. Target BCS of 3.0–3.50 at dry-off time.
7. Reduce feed costs per cow per day.

Table 4 contains nutrient guidelines for a phase feeding system. Values have been adjusted to allow for modest nutrient increases (up 10%) and decreases (down 5%) between groups to avoid digestive disorders and large declines in milk production. All vitamins listed are supplemental amounts from commercial feed sources (unless cows are on pasture systems). Amounts of trace minerals include basal feed ingredients and added levels from commercial feed supplements. Nonfiber carbohydrate (NFC) values were calculated using the following formula (all values entered on a 100% dry matter basis):

$$\text{NFC} = 100 - (\%\ \text{crude protein} + \%\ \text{neutral detergent fiber} + \%\ \text{ash} + \%\ \text{ether extract})$$

Milking Management

Milking parlors are rapidly changing in size and design with the increase in herd size. Milking parlors on large farms operate for more than 20 h day^{-1} and cows are milked three times a day. While no one design is perfect, the following factors will impact milking parlor design and style:

- number of cows, group size, production level, and frequency of milking
- cow traffic pattern and number of groups
- initial investment, annual costs, and existing debt load
- labor cost, labor availability, and level of mechanization
- personal preference

Rapid-exit parallel parlors (both parabone and side-by-side styles) and rotary parlors are popular in larger dairy herds. Mechanization continues to invade the parlor, including automated cow identification, automatic take-offs, predipping and postdipping, electronic detection of mastitis by milk conductivity, and electronic tracking of treated or sick cows. Robotic milking is appearing in the United States, Canada, and western Europe, but may be more economic for smaller herd sizes, areas with high labor costs, and/or areas with family labor limitations.

Facility Management

Freestall (cubicle) housing is increasingly popular with larger milking herds compared to loose house with manure packs and conventional confinement housing. Other drylot dairy facilities will have a shade over a mounted dirt area, but excessive precipitation can lead to mud and manure sanitation problems. Cooling cows with water can be a limitation with dirt lots. Factors including initial investment, herd size, environment (cold and heat stress), operating expenses, labor availability and cost, and personal preference will determine the type and style of facility.

The advantages of freestall housing include environmental control, cleanliness, health advantage, and added cow comfort when environmental conditions are unfavorable. Stall size will be dictated by breed and size of cows, lunge room, cow configuration (cows facing in or facing out), and barn configuration (two-, three-, four-, or six-row barns). Stalls must be wide enough for ease of entering and to avoid mammary gland injury (stepped on teats), but narrow enough to prevent cows turning around in the stall. Cows need body space (from the rear of the cow to her knees), head space (allows for the cow's head to be in a comfortable position), and lunge space (area for the cow's head to move when the cow rocks forward to rise).

Compost Barn

Another approach for dairy cattle housing is a compost barn system especially in the midwest region of the United States. The compost barn (a type of loose housing system) allows for a deep-bedded area (80–100 ft^2 per cow) allowing cows to recline and rest in the deep area. The mulch area is aerated, 12–18 inches in depth, twice a day (while cows are being milked) to allow oxygen to continue the process of composting of the organic matter. A small amount of bedding (usually wood shaving) is used to keep the cows dry and to allow heat generation from compost. In spring, the compost is removed and applied to the land or sold. The compost depth during the cold periods from fall to spring can reach 4–5 ft. Cow comfort is excellent and older cows may remain in the herd for several additional lactations. Smaller herds find this approach more effective as bedding costs can be high.

Two stall surfaces are popular in the United States. Sand is recommended because no organic matter is available in it for mastitic organisms to grow, and it provides excellent cow comfort. Sand is inexpensive, bulky and heavy to handle, and provides footing for the cows. Sand is difficult to move (requires fast-moving water), leads to pump damage, and involves higher system maintenance costs. Fresh or recycled sand must be replaced every 1–3 weeks. Commercial sand-saving devices can reduce the amount of sand kicked out of the freestall (including burying used car tyres). Cows prefer sand to other stall surfaces if the sand is deep enough (20–25 cm deep). Mattresses are also popular with flushing manure and solid separation systems. Various types of mattresses and surfaces have been used (rubber, plastic, molded rubber and plastic particles with fabric or canvas covers).

Water Beds

Water mattresses can also be installed in freestalls adding cow comfort. The water bed is 4–6 inches thick and covered with a cover. A small amount of bedding can be added to reduce abrasions to hock and leg area. Mattress surfaces should have a small amount of litter or bedding on the surface to improve cow comfort, avoid abrasions to the hock and legs, and keep cows clean and dry. Bedding needs to be added once or twice a week and must work with the manure system (chopped straw, dry sawdust, and rice hulls have been used successfully). All stall surfaces should be inspected and maintained twice a day to remove cow droppings, level out sand or pockets, remove wet sand or bedding, and rack dry bedding or sand to the back of the stall keeping stalls dry. Daily maintenance lessens the risk of mastitis and keeps cows cleaner.

Ventilation in freestall barns is achieved by raising the roof higher (prevents heat from radiating down on cows from the roof), opening side walls (allows prevailing wind to cool the barn), and opening the ridge (allows warm air to move up and out). Check with successful building and agricultural engineers in your area for recommended heights, widths, and ridge openings. During hot weather, fans are added to move more air in large barns and water is used to wet cows to enhance cow cooling. Heat abatement must occur in the holding pen area, over the feeding area, and in the freestall resting area, in that order.

Cross Ventilate Barn

Crossventilated barns are a new concept in areas of high temperature and moderate humidity. These barns have a low profile, small footprint as barns may contain 10–12 rows of cows, and a series of fans that pull air (4–5 miles per hour) through water pad on the opposite end of the barn. Pulling air through the pad can reduce air temperature by 10 °F compared to the outside temperature. The crossventilated barn is built with a low ceiling, has baffles to force the air down on each row of cows, and is airtight to get proper airflow and velocity.

Water management is critical to encourage and maintain DMI. One watering space (with 60 cm of tank linear space) is required for every 15–20 cows. At least two waterers per group are needed to avoid boss cow and competition. Young cows need more waterers to allow and encourage water consumption. A source of water must be available when cows leave the milking parlor as cows consume large amounts of water after milking and prior to feed consumption. Providing water in milking parlors has been successful. Waterers must be clean and dumped/drained each day to avoid bacterial growth and feed contamination. Water depth should be 15–20 cm to keep water fresh, control changes in water temperature, and avoid contamination.

Floors must have a desirable texture for good footing, for reducing injuries from slipping and falling, and to encourage the cows to move to the feeding area and express heat. Grooving of fresh concrete can be done, but sharp edges must be avoided, as they lead to excessive hoof wear. Grooves can be cut in after the concrete has hardened, parallel to the direction of the scraping. In high-traffic areas or areas where cows turn, a diamond pattern (two-directional grooves) can improve cow traffic pattern. Midwest US agricultural engineers suggest grooves 12–18 mm wide, 18 mm deep, and 8.5–10 cm from center to center. A new application is to lay a rubber mat or belt next to the feeding manger and in a portion of the return alley from the milking parlor to provide more comfort when the cows are eating and walking. These rubber belt surfaces can be a limitation when scraping and handling manure.

Conclusion

Dairy managers must control feeding, milking, and housing systems to compete and succeed in the US dairy business. If the dairy manager manages the four factors that impact nutrient needs (milk yield, milk components, dry matter intake, and weight loss), several feeding phases can be developed. All dairy managers may not need six phases, but they must manage feed changes economically (considering milk yield vs. feed costs). The feeding system begins with phase 1 (dry cows), not the lactating cows. If the dairy manager can manage the transition feeding programs (phases 2 and 3), metabolic problems will be minimized and milk production optimized. The challenge of feeding systems is to deliver these identified nutrient needs.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction; Measurement Techniques and Data Processing. **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates. **Feeds, Ration Formulation**: Dry Period Rations in Cattle. **Milking Machines**: Robotic Milking. **Milking Parlors**.

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Goats

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Goat Husbandry Systems in the Europe

In the Greek and Roman civilizations, sheep and goats were the animals par excellence for income. Polyphemus milked sheep and made cheese, which is considered the ancestor of pecorino cheese; Columella exalts cheeses of both the species; and Virgil writes that goatskin was used for 'sailors' sails and tents'. In the Middle Ages, with economic recovery and demographic development, the demand for fibers and above all wool increased rapidly. The greatest part of lands was used to farm sheep in order to produce wool. Goats, which did not produce wool but continued to produce good milk and meat, were relegated to the margins of the main production system and ended up playing a role that was paradoxical in many aspects. For some people, in certain or most part of the world, the goat was the subject of strict laws – since it took wood and pastures away from sheep and the man himself – and this limited its farming. For others, on the contrary, it was charged with various symbolic values. Thus, on the one hand, it was ill treated and persecuted, and, on the other hand, it was invoked to cure diseases and human weaknesses. The situation started to change, and to worsen, in the 'century of lights (1700)'. When pestilences ended, economic recovery and demographic development increased pressure on farmers' land and, in the same period, physiocrats were theorizing for the privatization of public lands. Within a few decades, sheep farming had to come to terms with a society that did everything to hinder its development. In Great Britain enclosures underwent acceleration, and in Spain and Italy the 'Mesta' and the 'Dogana della Mena', the great organizations of transhumance, were terminated and public lands mostly fenced and privatized. Sheep still developed in Europe for centuries, until the forthcoming of fast-sailing ships made Australian wool cheaper. Since then, in Europe, sheep started their slow decline, while for goats the situation has not changed or better it has mainly improved after the movement of '1968, when this animal was taken as a symbol of the rebirth of a new way of living. On the contrary, the hostility against pastoral systems and pastoralism, which assumed a negative connotation with time, remains. Over and above this, the nonpastoral systems end up assuming a positive connotation, until reaching models that theorize an almost void relationship toward the land (zero grazing). Thus, pastoral systems have been considered from time to time as being responsible for desertification, fires, and land degradation and the concerned stakeholders and shepherds are relegated to the lowest level of society, whereas intensive and sedentary systems have benefited from incentives and laws supporting their development.

Why do motivations coming from afar continue to affect the two systems? Motivations must be essentially found in the unit of measurement that was adopted to assess farming efficiency and productivity. This is at least true as far as meat and milk are concerned, while wool followed an opposite course.

The Unit of Measurement

In the world, the quasitotality of goat farming is based on pastoral models that assume different connotations from area to area with relation to the orography and traditions of a territory. In many countries, the evolution has been slow and the changes almost nonexistent. The pastoral model, in its several forms (nomadic, transhumant, sedentary), is based on natural pastures, autochthonous breeds, and sufficiently balanced land–animal relations. In industrialized countries, where the pressure on land has been huge, they have settled on a type of intensive model that is similar to the one used for dairy cows: a few cosmopolitan breeds – Saanen, Alpine, Nubian – and a scarce relation with soil – zero grazing or high stocking rate, a feeding system based on corn silage and concentrates. Such a culture has also contaminated the majority of systems of many different countries originally based on pastoralism, where the attempts to introduce extremely productive breeds, above all Saanen, and intensive feeding systems have been frequent and often unsuccessful. The development of intensive models took place not as much because pastoral farming was inheriting a negative image but because in the accelerated economic development after World War II, the unit of measurement that was adopted to assess the efficiency of several systems was one that exalted quantity: kilogram or liter. On the basis of this trend, the development of intensive systems, favored by the availability of technology that industry and research put at their disposal, was overwhelming: above all in the cattle sector, and also, though to a lesser degree, in the goat sector. In France, Holland, Spain, and the United States and marginally in Italy, industrial models of goat milk and cheese production have been developed. Paradoxically, all this occurred and still occurs, while in the same countries we are rediscovering gastronomy, and the culture of quality is increasingly establishing itself. In fact, since about 10 years ago, dairy and meat sectors have perceived that quality was going to become something essential, and because of this we have identified new units of measurement able to describe and quantify milk and meat quality: fat, proteins, and meat–fat ratio. But these units of measurement, which represent the viewpoint of the intermediate ring of the chain (butcher, dairy), are still expressions of quantity: more fat, more protein, and more meat. On the contrary, when consumers think of and ask for milk, meat, or cheese quality, they refer to flavor, taste, and, more and more, nutritional value. Today we know that between fat, protein, and meat, and the flavor and nutritional value of milk, meat, and cheeses, the ratio is modest as other factors and parameters need to be taken into consideration. We will talk about this in the following section. Here we just want to highlight how the approach was addressed toward quality, not only in the overall agricultural and food sectors but also in the goat sector itself as far as fiber is concerned. In the case of wine, it is now accepted that to make a very good wine a reduction in grapes' production per hectare and per plant is needed. To the contrary, for animal productions we thought to improve upon quality by increasing productions per head and per hectare. In the world of fiber, the Loro Piana industry, one of the most renowned in the sector, boasts of the fact that it succeeds in winning the best batches of cashmere fiber in the world – of course the thinnest and not the most voluminous or the heaviest. Unfortunately, the selection of the unit of measurement still affects production systems and their development and potential.

The Parameters

Consumers choose cheese or meat because they like them, as they have that particular taste, a special flavor that brings familiar tastes to mind ("les madeleines"), which give the product an added value so that price becomes less of a determining factor in the choice. That is why, in the Mediterranean area, kid meat is one of the most expensive, while in Northern Europe this product has only room in foreign markets, because it does not belong to the gastronomic tradition of those countries. It is the same for cheeses, with hundreds of types, all with different organoleptic characteristics and prices. The aromatic notes of meat and milk are the expression of terpenic compounds, polyphenols, fatty acids, and amino acids, which in their turn mainly depend on the production system and feeding, and in some cases on breeds. Curiously, dietologists consider meat and cheese – which are food – as substances to be consumed carefully owing to their high content of cholesterol. Yet it is now known and accepted that the problem is not just cholesterol itself but cholesterol oxides. In fact there exists an extremely diversified nutritional quality: fatty acids, antioxidants, vitamins, and conjugated linoleic acids (CLAs) that are extremely variable and change with relation to the farming system. Finally, there is a quality that is not at all considered but has an important tools and health effect and concerns the environment and the parameters correlated to it: heavy metals, dioxins, benzenes, lead, and so on.

Production Systems and Management

What has been shown so far clarifies that the management and organization of production systems depend on the type of product and market sector we want to occupy. Until some years ago, when the objective was quantity and the units of measurement were fat and proteins, all the systems, even the pastoral ones, tended to intensify the production to select animals more, and increase intake rhythms. Nowadays the situation is changing: excess in milk and meat production, decrease in animal well-being, accumulation of nitrates in soil, and last but not least consumers who search more and more for products having a unique taste personality and nutritional value. It is no coincidence that the first production regulations on a goat cheese, the Banon, which is made in Provence (France), set a limit to milk production (850 l in 210 days).

The Feeding System

Now, let us talk about the two main types of production systems followed in the world: intensive – sedentary and confined – and extensive in their many different forms – nomadic, transhumant, roaming, on confined and not confined pastures. The former systems are much used in France, Spain, and above all Holland. They are usually zero-grazing and indoor, use extremely selected breeds, and have high milk production and feeding systems having a concentrate–fodder ratio that highly favors concentrates and, above all, using only one type of grass. The ration is made up so that the animal cannot select what to eat but is forced to eat everything, and all animals eat alike. In this way, not only the animal cannot express its own potential but the type of ration also causes a decrease in milk quality. Some years ago we tried to experiment a free-choice system, where each animal had the possibility to have *ad libitum* access to many and various foods different from each other as far as their content in energy, fiber, and proteins was concerned. Being able to manage their diet, goats increased their intake levels in an extremely balanced way by raising their milk production without unbalancing fat and proteins. If intensive systems can benefit from free choice, as they allow bearing high production levels while maintaining the animal's well-being, it is clear that these high production levels and rich contribution in concentrates must affect milk quality. We carried out a test by comparing three groups of Maltese breed goats: one always grazing without any concentrate supplements, another one always grazing but with concentrate supplements, and a third one, indoor, with both hay and concentrates. **Table 1** shows that terpenes, which significantly contribute to flavor formation, undergo a decrease due to the effect of concentrates – a decrease extremely significant, in molecules such as α- and β-pinene, limonene, *p*-cymene, all molecules that impart flavor to cheese. In this test, the hay administered to indoor goats was produced with the same pasture grass that was used by the other two groups' pasture, which was in average made up of more than 40 different species of plants. We know that each plant has a different composition of secondary metabolites, which differently affects milk and cheese flavor.

Table 2 shows how volatile organic compound (VOC) composition changes in cheese with relation to the types of plant ingested by animals. The integration with concentrates, apart from reducing the quota of grass intake, also affects animals' grazing behavior and, thus, negatively affects the aromatic fractions of milk.

In groups of goats kept on pasture but with three different levels of supplementation (**Table 3**), the levels of mono- and sesquiterpenes, which, as it is known, are much influenced by grass ingestion, decreased with relation to the season and the different type of concentrate.

The major reduction was observed in the groups that were supplied a slowly degradable concentrate and the least in those receiving a rapidly degradable concentrate. This phenomenon could not be ascribed to the effect of herbage intake but probably to the plants selected by the

Table 1 Terpene composition of goat milk

	Feeding system		
	G	*GS*	*ZG*
Total terpenes	246.8 ± 173.2	132.1 ± 107.8	55.4 ± 36.7
α-Pinene	65.3 ± 102.2	12.0 ± 9.8	6.6 ± 6.8
β-Pinene	3.3 ± 3.5	2.1 ± 2.8	2.7 ± 3.3
Sabinene	1.8 ± 3.1	1.2 ± 1.1	0.2 ± 0.4
Limonene	90.4 ± 85.8	54.7 ± 53.9	32.4 ± 23.2
A + γ-terpineol	24.4 ± 21.3	17.7 ± 6.6	6.6 ± 7.0
Tricyclene	7.0 ± 10.8	2.3 ± 2.3	0 ± 0
p-Cymene	15.2 ± 25.7	5.3 ± 5.9	4.3 ± 4.9
Camphene	7.4 ± 6.6	3.3 ± 2.8	2.1 ± 2.6
Δ-3-Carene	0 ± 0	5.0 ± 8.9	0 ± 0
Thujene	6.5 ± 11.2	1.6 ± 2.7	0 ± 0
Unidentified terpene	25.5 ± 44.2	26.9 ± 44.6	0.5 ± 0.8

Data are means ± s.d. expressed in ng l^{-1}.
G, grazing 8 h day^{-1}; GS, grazing supplemented (hay plus 500 g day^{-1} of commercial concentrate); ZG, zero grazing.
Data provided by Salvatore Claps.

Table 2 Volatile organic compound composition of goat cheeses from each diet treatment

	Dietary treatment				
	Avena sativa	*Lolium perenne*	*Medicago sativa*	*Trifolium incarnatum*	*Significance*
Aldehydes	9.06^b	8.62^b	24.07^a	8.87^b	**
Ketones	$9.70b^c$	14.97^b	39.62^a	3.69^c	***
Esters	3.97	3.32	9.68	5.55	NS
Hydrocarbons	60.42	47.66	78.1	46.78	NS
Alcohols	132.52^a	16.89^b	1.60^b	4.03^b	**
Terpenes	10.88^a	8.19^b	9.15^b	11.05^a	**
Total	226.55^a	99.65^b	$162.23^{a,b}$	79.96^b	*

^{a,b,c}Means with different superscripts differ for each item in a row ($p < 0.05$).
Data are mean values in arbitrary units. NS, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
Avena sativa, oats; *Lolium perenne*, perennial ryegrass; *Medicago sativa*, alfalfa; *Trifolium incarnatum*, crimson clover.
Data supplied by Salvatore Claps.

Table 3 Mono- and sesquiterpene composition of goat milk

	Season		
Group	*Winter*	*Spring*	*Summer*
Monoterpenes (ng l^{-1})			
G	1899 ± 190	243 ± 173	1730 ± 1771
GRD	1192 ± 175	201 ± 216	2077 ± 1645
GNRD	785 ± 138	130 ± 103	1429 ± 1512
Sesquiterpenes (ng l^{-1})			
G	2397 ± 2961	1609 ± 645	14 041 ± 3455
GRD	1124 ± 534	1130 ± 800	24 586 ± 23 000
GNRD	724 ± 80	1252 ± 158	20 698 ± 16 717

Data are means of three determinations. Data provided by Vincenzo De Feo.
G, grazing for 8 h day^{-1}; GRD, grazing plus 600 g day^{-1} of rapidly degradable concentrate, barley (*Hordeum vulgare*) and chickpeas (*Cicer arietinum*) – ratio 60:40; GNRD, grazing plus 600 g^{-1} day of slowly degradable concentrate, maize (*Zea mays*) and broad beans (*Vicia faba*) – ratio 80:20 (data provided by Salvatore Claps).

animal. The system affects not only aromatic complexity but also, and above all, the nutritional value of milk and cheese. In a test where two groups of goats were compared, one only grazing and the other kept in stable and fed with hay produced with the grass from the same pasture (thus with many herb species), the acidic composition of milk showed a lot of difference depending on the kind of feeding. Grazing goats produced milk with a higher content of omega-3, CLAs, docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), that is, milk rich in unsaturated acids; on the contrary, the content of omega-6 and saturated acids was lower (**Table 4**).

The effect of feeding involves not only fats but also vitamins, especially those that play an important role in blocking cholesterol oxidation. Pasture grazing causes an increase in α-tocopherol and a contemporaneous decrease in the content of cholesterol in milk (**Table 5**). This phenomenon permitted setting an index that kept in consideration the content of both cholesterol and

Table 4 Fatty acid composition of goat milk

	Season									
	Winter		*Spring*		*Summer*		*Effect*			
Feeding system	*G*	*ZG*	*G*	*ZG*	*G*	*ZG*	*MSE*	*Se*	*Fs*	*Se × Fs*
C18:1trans	2.271^{A}	0.711^{B}	1.988^{A}	0.910^{B}	1.141	0.872	0.129	***	***	**
C18:2ώ6	1.961^{B}	2.729^{A}	2.078	2.06	1.735^{b}	1.910^{a}	0.094	***	***	**
C18:3ώ6	0.104	0.1	0.105	0.102	0.119	0.114	0.006	*	NS	NS
C18:3ώ3	0.737	0.386	0.786	0.359	0.588	0.335	0.037	**	***	NS
C18:2$_{c}$9$_{t}$ 11 – CLA	1.130^{A}	0.462^{B}	0.738^{A}	0.503^{B}	0.703^{a}	0.510^{b}	0.033	***	***	***
C20:2ώ6	0.075	0.06	0.071	0.065	0.079	0.063	0.005	NS	**	NS
C20:3ώ6	0.021	0.018	0.016^{B}	0.022^{A}	0.018^{B}	0.024^{A}	0.001	NS	**	**
C20:3ώ3	0.172	0.203	0.147	0.179	0.158	0.195	0.006	**	***	NS
C20:4ώ6	0.075^{a}	0.022^{B}	0.057^{A}	0.040^{B}	0.055^{A}	0.036^{B}	0.003	NS	***	***
C20:5ώ3 – EPA	0.037	0.029	0.043	0.029	0.048	0.03	0.006	NS	*	NS
C22:4ώ6	0.019	0.023	0.017	0.016	0.012	0.022	0.003	NS	NS	NS
C22:5ώ3 – DPA	0.028^{a}	0.012^{B}	0.016	0.017	0.029	0.022	0.002	**	***	**
C22:6ώ – DHA	0.144	0.061	0.115	0.096	0.114^{A}	0.085	0.007	NS	***	***
SFA	69.063	73.301	70.51	73.031	69.44	73.286	0.709	NS	***	NS
MUFA	25.389	22.056	24.138	22.897	26.1	22.893	0.601	NS	***	NS
PUFA	5.547	4.643	5.352	4.072	4.455	3.82	0.161	***	***	NS
ώ3	1.118	0.691	1.108	0.68	0.931	0.668	0.042	*	***	NS
ώ6	2.399^{b}	3.014^{a}	2.459	2.403	2.134	2.255	0.097	***	**	**

Uppercase and lowercase superscripts indicate significant differences, $p < 0.05$ and $p < 0.01$, respectively; NS, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; MSE, mean square error; Se, season; FS, feeding system; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; G, grazing; ZG, zero grazing; EPA, eicosapentaenoic acid.
Data provided by Adriana Di Trana.

Table 5 Effect of two different pastures on α-tocopherol and cholesterol content and DAP values of cheese

Feeding treatment	*α-Tocopherol* (μg per 100 g)	*Cholesterol (mg per 100 g)*	*DAP ($\times 10^{-3}$)*
GVC	778.3 ± 57.6^{a}	84.5 ± 4.9^{a}	8.3 ± 0.7^{a}
GMC	842.0 ± 36.5^{a}	87.2 ± 0.7^{a}	8.7 ± 0.3^{a}
ZGC	646.9 ± 72.7^{b}	96.7 ± 6.1^{b}	6.0 ± 1.0^{b}

[a,b]Means with different superscripts differ for each item in a column ($p < 0.05$).
Values are mean data from three samples collected during lactation and analyzed in triplicate ±s.d.
GVC, grazing on valley pasture; GMC, grazing on mountain pasture; ZGC, zero grazing.
Data supplied by Laura Pizzoferrato.

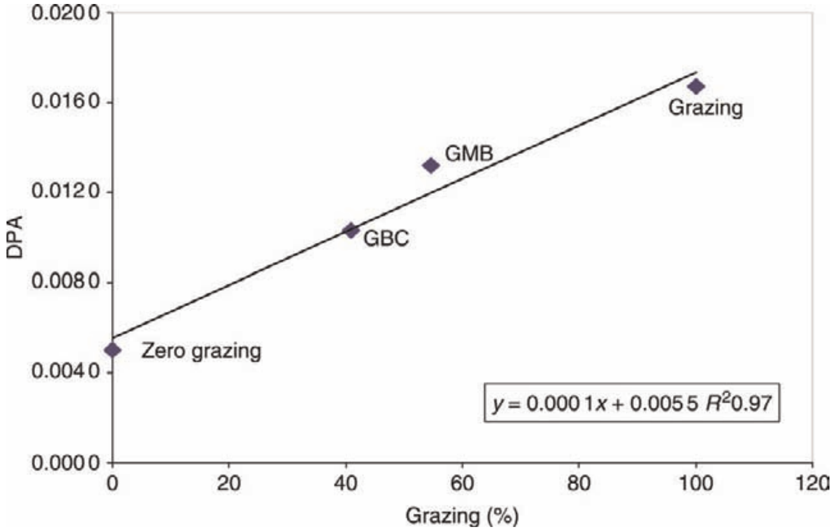

Figure 1 Goat milk: correlation DPA vs. weight of the pasture in the feeding system. DPA, degree of antioxidant protection; GBC, grazing + mixed barly (*Hordeum vulgare*) and chickpeas (*Cicer arietinum*); GMB, grazing + corn and board beans (*Vicia faba*). Data supplied by Laura Pizzzoferrato.

antioxidants, as the degree of antioxidant protection (DAP) is more important than cholesterol itself.

This index is not only higher in grazing animals but, as we can see in **Figure 1**, it also increases with the increase in herbage intake.

We should stress that all this research on indoor goats has been carried out basing the ration on permanent meadows' hay, rich in many herbage species. In general, instead, in intensive systems they use hay or silage based on only one type of grass, with clear major negative effects on secondary metabolites and nutritional value molecules. But overall, the majority of farming systems is based on free grazing where flora is represented by a huge variety of plants, each with its own characteristics, which animals are free to eat to their liking. We know that lots of these plants have molecules that are also exploited by indigenous populations for curative purposes and by pharmaceutical industry as the basic ingredient for many drugs. We wondered if the milk coming from goats that had eaten these herbs also contained such characteristic molecules. Thus we administered both borage (*Borago officinalis*) and hawthorn (*Crataegus oxyacantha*) to a group of goats.

As we can see in **Table 6**, flavonoids and quercitin contained in these herbs were found in milk. In conclusion, we can say that, if we want to produce in high quantities, we should use exceedingly high concentrates and supply animals with only one kind of herbage. On the contrary, if we want to have higher aromatic complexity and nutritional content in milk and cheeses, we need to act on the number of plant species and their ingested quantity.

More and more often, however, consumers are frightened by alarming news concerning food, above all milk and meat. Besides 'mad cow disease', we have often heard about meat and milk containing dioxin, a constituent coming from the environment and not from the breeding system. Therefore, it is clear that the environment can

Table 6 Plant metabolites found in plants and milk

	Plants			*Milk*	
Extract	*Borage*	*Hawthorn*	*Control*	*Borage fed*	*Hawthorn fed*
Chloroform	Quercetin $8.1 \pm 0.3 \times 10^{5}$ 5,7-dihydroxyflavone $2.7 \pm 0.3 \times 10^{5}$	Quercetin $8.4 \pm 0.6 \times 10^{-5}$ Vitexin-7-glucoside $0.8 \pm 0.1 \times 10^{-5}$	None	5,7,4′-trihydroxyflavonol $9.2 \pm 0.3 \times 10^{-6}$ 5,7-dihydroxyflavone $5.4 \pm 0.3 \times 10^{-6}$ Flavone	Quercetin $3.9 \pm 0.1 \times 10^{-6}$ 5,7-dihydroxyflavonol $8.3 \pm 0.3 \times 10^{-6}$ Flavone
Methanol	Rutin $1.5 \pm 0.3 \times 10^{-5}$ Kaempferol $2.0 \pm 0.2 \times 10^{-5}$ Kaempferol-3-*O*-β-D-glucopyranoside $0.9 \pm 0.2 \times 10^{-5}$	Rutin $2.9 \pm 0.2 \times 10^{-5}$ Apigenin $1.7 \pm 0.2 \times 10^{-5}$	None	Rutin $2.7 \pm 0.1 \times 10^{-6}$	None

Data are expressed as mol per 1000 g of dry plant or milk. Data are means of three determinations.
Data provided by Vincenzo De Feo.

pollute farms and products insofar as the system can pollute the environment, as it is in the case of nitrates and methane. A test carried out in two different areas, one in mountains and another near a motorway, highlighted that the content of C2 and C3 benzenes in the milk from goats grazing in the area near the motorway is much higher than that from goats grazing among the mountains (**Table 7**).

The Breed

One of the important components of the production system is breed, which is selected in order to make the system more efficient. It is clear that until the main objectives of farming were production level maximization and cost reduction, the main trend of all producers was that of introducing into their farms extremely productive animals, purebred or as reproducers to be used for crossbreeding. Intensive systems have used breeds that have soon become cosmopolitan, such as Saanen, Alpine, Nubian, and Granadina, while extensive ones have tried in every way to introduce these more productive breeds, sometimes successfully but often with many problems mainly due to the diffusion of diseases such as that caused by caprine arthritis–encephalitis virus (CAEV) and paratuberculosis. For quite a while, as we realized that endogenous resources have high potential if more pertinent units of measurement are used, and as consumers

Table 7 Contents and percent compositions of selected arenes detected in milk of goats grazing in a polluted and an unpolluted pasture

	Natural pasture		*Polluted pasture*		*Vehicular emission*
Compound	*ppbw*	*%*	*ppbw*	*%*	*%*
Ethylbenzene	0.04	16.0	0.4	10.4	17.1
(*m* + *p*)-Xylene	0.17	63.9	2.2	60.7	59.5
o-Xylene	0.05	20.1	1.1	29.0	23.4
Total C2-benzene	0.27		3.7		
Benzene, isopropyl	<0.01	0.2	0.3	1.0	8
Benzene, *n*-propyl-			0.7	2.9	1.2
Benzene, 1-ethyl-3-methyl-	0.02	3.4	2.7	11.0	21.2
Benzene, 1-ethyl-4-methyl-	0.01	2.2	1.1	4.3	9.7
Benzene, 1,3,5-trimethyl-	0.52	80.8	5.0	20.3	11.3
Benzene, 1-ethyl-2-methyl-	0.02	2.4	1.5	5.9	7.4
Benzene, 1,2,4-trimethyl-	0.06	9.2	8.9	35.8	33.9
Benzene, 1,2,3-trimethyl-	0.01	1.8	4.6	18.8	7.4
Total C3-benzene	0.65		24.8		

For the sake of comparison, the percent composition of vehicular emission is reported. The average uncertainty in these determinations was ±0.01 ppbw.
Data supplied by Paolo Ciccioli.

started to demand local products, not only this mode of cross-breeding stopped but genetic improvement also underwent a pause, allowing for reflection; in fact, in Europe, many production regulations with respect to Protected Designation of Origin (PDO) impose a productive limit to cows or goats. There are even regulations that bind the breed. For cheeses made from cows' milk such as Beaufort, Abondance, Salers, and Fontina, the regulations provide for the breeds Tarantaise, Abondance–Tarine, Salers, and Valdostana Pezzata Rossa only, respectively. For cheeses made from sheep milk such as Roquefort, Ossau-Iraty, and Idiazabal, the sheep breeds must be Lacaune, Basco-béarnaise, and Lacha–Carranzana, respectively. In the goat sector also, they are starting to define in a specific way the breed to use. For the Banon they provide for the Alpine and Rove, and for Robiola di Roccaverano the eponymous goat. But can the breed be a diversity factor? Namely, can we find different cheeses only because they are made from different breeds within the same food system? Comparing ricotta cheese, a type of cheese made from whey, produced from milk of different breeds (**Table 8**), the sensory analysis highlighted that there were differences not only for softness, greasiness, and granulometry but also for its goat flavor, an aromatic note that characterizes cheese and which is a distinction marker for consumers.

Why do breeds that eat in the same way give different products? It is true that each breed is the result of an adaptation to a special environment and, for this reason, has developed extremely specific characteristics that are different from those of animals living in other environments. But maybe this is not enough to explain these differences. **Table 9** shows that, in the same environment and breeds, VOCs are different from breed to breed.

Such diversity can be explained by the breeds' grazing behavior. In fact the breeds Maltese and Derivata di Siria are apparently very similar from a genetic point of view as they are for productive levels and area of origin; however, these two breeds used to choose their diet in a different way while grazing: the Maltese showed a greater selectivity and had a preference for grasses and, among them, for a small number of species, while the Derivata di Siria selected less and used a broader range of the available herbage.

Table 8 Effect of goat breeds on sensory properties of ricotta cheese

	Girgentana	*Siriana*	*Maltese*	*Local*	*Significance*
No. of samples	9	9	9	9	
Softness	7.5^{a} ± 0.25	6.5^{b} ± 0.25	7.5^{a} ± 0.25	7.5^{a} ± 0.25	***
Greasiness	5.5^{a,b} ± 0.54	4.5^{b} ± 0.54	4.0^{b} ± 0.54	7.0^{a} ± 0.54	**
Granulosity	3.0^{b} ± 0.54	7.0^{a} ± 0.54	2.0^{b} ± 0.54	2.0^{b} ± 0.54	***
Sweet	5.75 ± 0.88	5.75 ± 0.88	7.00 ± 0.88	7.00 ± 0.88	NS
Bitter	3.25 ± 0.28	3.00 ± 0.28	2.00 ± 0.28	2.00 ± 0.28	NS
Goat	2.0^{c} ± 0.20	5.0^{a} ± 0.20	4.0^{b} ± 0.20	2.0^{c} ± 0.20	***
Cooked	3.25 ± 0.28	3.0 ± 0.28	3.5 ± 0.28	4.0 ± 0.28	NS
Milk	5.50 ± 0.67	5.75 ± 0.67	6.00 ± 0.67	6.00 ± 0.67	NS

[a,b,c]Means with different superscripts differ for each item in a row ($p < 0.05$).
Data are means ± s.d.
NS, not significant; **$p < 0.01$; ***$p < 0.001$.
Each attribute was evaluated on a 0–9 point graduate scale.
Data provided by Michele Pizzillo.

Table 9 Volatile organic compounds in milk and cheese of goat breeds

	Milk						*Caciotta cheese*			
Parameters	*M*	*RS*	*I*	*G*	*SE*	*M*	*RS*	*I*	*G*	*SE*
Alcohols	2195.2^{a}	191.1^{b}	58.9^{c}	37.5^{d}	0.61	335.3^{a}	36.0^{d}	291.3^{b}	143.3^{c}	0.62
Aldehydes	373.3^{a}	272.1^{b}	188.5c	72.6d	16.1	171.1^{c}	178^{b}	201.1^{a}	129.1^{d}	0.59
Ketones	3.28^{d}	34.9^{b}	37.8^{a}	13.7^{c}	0.58	31.1^{b}	21.1^{d}	56.2^{a}	23.1^{c}	0.60
Terpenes	2243.0^{a}	285.1^{b}	953.6^{c}	72.7^{d}	0.51	551.0^{a}	160.2^{d}	455^{b}	319.3^{c}	0.60
Acids	14.9^{a}	11.6^{b}	11.7^{b}	8.7^{c}	0.58	1731.0^{a}	320.1^{b}	203.2^{c}	22.2^{d}	0.56
Aromatic hydrocarbons	505.9^{b}	584.1^{a}	385.2^{c}	54.7^{d}	0.55	165.2^{c}	81.1^{d}	440.1^{a}	214.0^{b}	0.59

[a,b,c,d]Means with different superscripts differ for each item in a row ($p < 0.05$).
Data are means ± s.d. in arbitrary units.
M, Maltese; RS, Red Syrian; I, Ionica; G, Girgentana breed.
Data provided by Salvatore Claps.

Conclusion

A world crisis of milk, not only goat's but also sheep's, cow's, and buffalo's, will oblige producers to either decrease production levels, a desirable but not credible point, or differentiate their production to enter new market niches. So far, the dairy sector has kept the problem at bay by relying on dairy techniques – many different cheeses from the same milk – but today we need to identify the difference of factors in the raw matter – the same cheese made from milks having different quality. Thus, we need to abandon quantity as the unit of measurement and start talking of quality classes with relation to farming system, food system, and breed and its productive levels. The goat system still has at its disposal a great part of its biodiversity; this is an important tool that can be valorized if we change the units of measurement used to assess efficiency.

See also: **Animals that Produce Dairy Foods:** Goat Breeds. **Husbandry of Dairy Animals**: Goat: Feeding Management; Goat: Health Management; Goat: Milking Management; Goat: Multipurpose Management; Goat: Replacement Management; Goat: Reproductive Management; Predator Control in Goats and Sheep.

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Sheep

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Introduction

The management of sheep has evolved through centuries of tradition based on religious rituals, cultural heritage, socioeconomic constraints, genetically diverse sheep populations, and agriculture in arduous terrains under diverse climatic conditions. Historically, sheep production in the mountainous pastures has played an integral role in maintaining rural customs, sustaining development in harmony with the environment, combating urban migration, preventing forest fires, and diversifying farming-based activities dedicated toward creating handicrafts, promoting tourism, marketing of specialty products, and conservation of biodiversity. The management of dairy sheep flocks that supply milk and milk products varies considerably from region to region. The influence of topography; climatic conditions; availability of pastures, stored feed for the winter and spring months, forage from irrigated pastures, concentrates, and human resources; access to roads and markets; degree of mechanization; and attack from predators ascertain the specific management pattern.

In the agroeconomically underprivileged semidesert of the Sahel region and the Steppe-like areas of North Africa, large numbers of nomadic sheep flocks remain. In this region, milk from sheep after a suckling period of 3 months is used primarily to supplement the diet of the human population. The Northern and Central European plains are not favorable for the development of modern agriculture because of irregularity of annual rainfall, sparse vegetation over rocky soil, severe climatic conditions in the coastal region, and scarcity of arable land. In Central Europe and in those countries with tremendous variations in topography and climatic conditions, ewes are milked only after the lambs suckle for 1–2 months. In the Balkan countries, Turkey, the Middle East, Iran, and Afghanistan, sheep milk is processed into popular milk products (yogurt and Feta-type cheese) for daily consumption. Ewes are milked after a suckling period of 1 month for fat lamb production, or later if lambs are utilized for replacement. In the countries of the northern Mediterranean basin, sheep milk collected from a large number of small flocks under a transhumance system is processed into high-quality cheese for the specialty market. These luxury products consist of Roquefort in France; Manchego in Spain; Fiore Sardo and Pecorino Romano in Italy; Feta, Kefalotiri, and Manouri in Greece; and Cachcaval and Feta in Bulgaria, Hungary, Macedonia, Romania, Serbia, and Bosnia–Herzegovina. An assortment of cheese is produced from mixed milk from cows and sheep in Spain, and from sheep and goats in Greece (Feta), Italy (Ricotta), and Corsica (Niollo). The flavors and tastes of individual cheese varieties are associated with specific regions.

In Canada, the newly developed Rideau breed has excelled in milk production, becoming the second highest registered sheep breed in the country; with the inherent potential for milking and raising more lambs, it has been popular as a dairy sheep. The technology for increasing efficiency and output of lamb production shows promise and the key components are being utilized by a number of sheep producers across the country. In North America, there is opportunity to increase efficiency of milk production at a much more rapid rate than is achieved through selection among non-dairy-type breeds. This is possible by complementary crossbreeding and development of composite breeds based on non-dairy-type sheep in North America and dairy-type breeds imported from Europe and the Middle East without jeopardizing the animal health status of the country.

In France, rural exodus resulted in constraints due to shortage of labor in dairy sheep farms, which had to be overcome by introducing mechanized milking. In the Mediterranean countries and in Central Europe, machine milking is an integral component of large flocks owned by industrial farms in Spain, cooperative farms in Bulgaria, and family farms in Italy. In this system, ewes are milked after lambs suckle for only 4–5 weeks, thus increasing the amount of milk produced. In Israel, during the first month of lactation, lambs are allowed to suckle simultaneously while ewes are milked, enabling a smoother transition from suckling to milking.

The development of modern dairy sheep industries in France and Spain can be attributed to higher financial returns from the sale of quality cheese; availability of forage from irrigated fields along with concentrates at a sufficiently low price; lower cost of labor for machine milking of flocks with 200 or more ewes; and government grants for fencing, housing, and the purchase of milking machines. Provision for technical assistance and organization of milk collection centers, milk and curd storage, and control of the manufacturing process including protection of specialty products are key factors in sustaining the enterprise.

Distribution and Production

The Food and Agriculture Organization of the United Nations has summarized statistics on the total number of sheep and their milk production in different continents (**Table 1**). In 1999 and 2007, the continents of Asia (44 and 47%), Europe (36 and 33%), and Africa (19%) accounted for most of the sheep milk produced in the world. The countries of Italy, Turkey, Greece, Sudan, Iran, Syria, Somalia, Spain, Romania, Afghanistan, France, Algeria, Iraq, Bulgaria, Portugal, Egypt, and Albania account for 61 and 66% of the sheep milk produced in the world. The remaining production comes from China (11 and 12%) and Central Asia where collection of data from remote areas can be challenging. Milk production is concentrated in the regions bordering southwest Asia, southern Europe, and northern Africa; the Mediterranean basin; and the Middle East. In 2003, ∼2–2.5 million kilograms of sheep milk was produced from about 11 000–12 000 ewes in Canada and the United States, even though sheep milk amounted to only 2% of the milk produced by cows. Besides, sheep numbers are significantly larger in developing countries than in developed countries. The sheep numbers and their milk production in the Mediterranean basin and the Middle East are presented in **Table 2**.

Traditional management of dairy sheep raised under transhumance and nomadism is associated with the availability of natural vegetation. In the transhumance system, flocks follow an age-old tradition of grazing in the mountain pasture during the summer and gradually descending to the plains in winter, making efficient use of vegetation in the forests and wasteland, and grain stubble in fields following harvesting. Flocks of 50–400 ewes are hand-milked, usually by 2–4 men, after lambs intended for slaughter are allowed to suckle for 3–6 weeks (or longer for replacement animals). There are no housing and milking facilities for sheep, and milk production per lactation is 40–100 l. Grazing on natural pastures of indigenous grasses, mountain vegetation, and shrubs is usually supplemented by agricultural by-products locally available in populated areas. Overgrazing the pastures has contributed to increased numbers of sedentary flocks relying on locally available feed and agricultural by-products. This is in contrast to the modern management of more productive breeds of dairy sheep under intensive production in flocks of more than 200 ewes, where ewes are fed balanced diets to meet their nutrient requirements based on nutrients derived from optimized grazing, fodder crops, and concentrates. Besides, ewes are milked after a suckling period of 4 weeks. Early-weaned lambs are provided milk replacer diets and have access to feed. Lambs intended for slaughter are fed high-energy diets to promote growth. The use of controlled reproduction based on induction and synchronization of estrus provides an opportunity for scheduling breeding, lambing, fattening, and marketing activities to avoid constraints due to season and labor shortage. In recent years, mechanized milking based on specialized equipment in milking parlors has gained popularity. The traditional system of management has been declining due to rural exodus, and following the end of World War I, many countries have closed their borders restricting the movement of sheep. At the same time, the high prices paid for sheep milk and milk products have contributed to an increase in the number of facilities that utilize modern management systems for milk production.

Advances in breeding, feeding, reproduction, and disease control have made it possible to more than double the productivity levels of milk and meat. The new

Table 1 Sheep numbers and milk production in different continents of the world

	Sheep numbers, 000 (%)		*Milk production, 000 mt (%)*		*Milk production per sheep (kg yr^{-1})*	
Country	*1999*	*2007*	*1999*	*2007*	*1999*	*2007*
World	1 056 056	1 086 882	8120	9044	7.7	8.3
Africa	242 256 (22.9)	279 722 (25.7)	1555 (19.2)	1753 (19.4)	6.4	6.3
North America	7954 (0.8)	7064 (0.7)	na	na	na	na
South America	75 617 (7.2)	72 830 (6.7)	35 (0.4)	36 (0.4)	0.5	0.5
Asia	408 270 (38.7)	455 740 (41.9)	3576 (44.0)	4239 (46.9)	8.8	9.3
Europe	152 048 (14.4)	136 475 (12.6)	2955 (36.4)	3016 (33.4)	19.4	22.1
Oceania	161 152 (15.3)	124 187 (11.4)	na	na	na	na

na, not available.

From FAOSTAT (2009) Food and Agriculture Organization of the United Nations, Rome, Italy. www.faostat.fao.org (accessed 2 August 2009).

Table 2 Sheep numbers and milk production in the Mediterranean basin and the Middle East

	Sheep numbers, 000 (%)		*Milk production, 000 mt (%)*		*Milk production per sheep (kg yr^{-1})*	
Country	*1999*	*2007*	*1999*	*2007*	*1999*	*2007*
Africa						
Algeria	17 988 (1.7)	19 851 (1.8)	180 (2.2)	205 (2.3)	10.0	10.3
Egypt	4391 (0.4)	5525[a] (0.5)	93 (1.1)	93 (1.0)	21.2	16.8
Libya	5150 (0.5)	4500 (0.4)	54 (0.7)	56 (0.6)	10.5	12.4
Morocco	16 576 (1.6)	16 894 (1.6)	27 (0.3)	27 (0.3)	1.6	1.6
Somalia	13 537 (1.3)	13 100 (1.2)	420 (5.2)	468 (5.2)	31.0	35.7
Sudan	44 802 (4.2)	50 944 (4.7)	461 (5.7)	498 (5.5)	10.3	9.8
Tunisia	6576 (0.6)	7618 (0.7)	17 (0.2)	18 (0.2)	2.6	2.4
Asia						
Afghanistan[a]	17 690 (1.7)	8105 (0.7)	264 (3.3)	122 (1.3)	14.9	15.1
China[a]	127 352 (12.1)	146 018 (13.4)	893 (11.1)	1072 (11.9)	7.0	7.3
Cyprus	240 (0.02)	259 (0.02)	17 (0.2)	16 (0.2)	70.8	61.8
Indonesia	7226 (0.7)	9860 (0.9)	87 (1.1)	118 (1.3)	12.0	12.0
Iran	53 900 (5.1)	53 800[a] (4.9)	549 (6.8)	534 (5.9)	10.2	9.9
Iraq	6000[a] (0.6)	6200 (0.6)	125 (1.5)	150 (1.7)	20.8	24.2
Israel	350 (0.03)	433 (0.04)	19 (0.2)	19 (0.2)	54.3	43.9
Jordan	1581 (0.1)	2496[a] (0.2)	20 (0.2)	90 (1.0)	12.7	36.1
Lebanon	378 (0.04)	340 (0.03)	23 (0.3)	22 (0.2)	60.9	64.7
Syria	13 998 (1.3)	22 865 (2.1)	446 (5.5)	874 (9.7)	31.9	38.2
Turkey	29 435 (2.8)	25 462 (2.3)	805 (9.9)	783 (8.7)	27.4	30.8
Europe						
Albania	1941 (0.2)	1853 (0.2)	73 (0.9)	75 (0.8)	37.6	40.5
Bosnia–Herzegovina	610[a] (0.06)	1033 (0.1)	17 (0.2)	21 (0.2)	27.9	20.3
Bulgaria	2774 (0.3)	1635 (0.2)	106 (1.3)	85 (0.9)	38.2	52.0
Croatia	489 (0.05)	680 (0.06)	8 (0.1)	7 (0.1)	16.4	10.3
France	10 240 (1.0)	9499 (0.9)	244 (3.0)	264 (2.9)	23.8	27.8
Greece	8930 (0.8)	8830 (0.8)	731 (9.0)	727[a] (8.0)	81.9	82.3
Italy	10 894 (1.0)	8227 (0.8)	844 (10.4)	560 (6.2)	77.5	68.1
Malta	16 (0.002)	12 (0.001)	3 (0.04)	2 (0.02)	187.5	166.7
Montenegro		249 (0.02)		9.4 (0.1)		37.8
Portugal	3590 (0.3)	3549 (0.3)	104 (1.3)	96 (1.1)	29.0	27.1
Romania	8409 (0.8)	7678 (0.7)	342 (4.2)	638 (7.1)	40.7	83.1
Serbia	1315 (0.1)[b]	1606 (0.2)	39 (0.5)[b]	14 (0.2)	29.7[b]	8.7
Slovenia	72 (0.01)	132 (0.01)	0.3 (0.004)	0.4 (0.004)	4.2	3.0
Spain	24 190 (2.3)	22 194 (2.0)	349 (4.3)	410 (4.5)	14.4	18.5
The Middle East[c]	120 964 (11.5)	129 319 (11.9)	2118 (26.1)	2620 (29.0)	17.5	20.3
Mediterranean basin[d]	152 970 (14.5)	157 809 (14.5)	3986 (49.1)	4257 (47.1)	26.1	27.0
Para-Mediterranean countries[e]	73 273 (6.9)	72 843 (6.7)	1159 (14.3)	1518 (16.8)	15.8	20.8
World	1 056 056	1 086 882	8120	9044	7.7	8.3

[a] Estimate.

[b] Former Yugoslavia.

[c] Saudi Arabia, Bahrain, Syria, Oman, Lebanon, Qatar, UAE, Israel, Cyprus, Yemen, Turkey, Kuwait, Iraq, Iran, and Jordan.

[d] Spain, France, Italy, Serbia, Montenegro, Slovenia, Croatia, Bosnia–Herzegovina, Albania, Greece, Turkey, Cyprus, Syria, Lebanon, Israel (and Palestine), Egypt, Libya, Malta, Tunisia, Algeria, and Morocco.

[e] Iran, Iraq, Jordan, Bosnia–Herzegovina, Bulgaria, and Romania.

From FAOSTAT (2009) Food and Agriculture Organization of the United Nations, Rome, Italy. www.faostat.fao.org (accessed 2 August 2009).

technology includes early weaning of lambs (at 1 month of age or earlier) to produce fat lambs for market and early mating of ewe lambs (at 8–9 months) raised on high-energy diets. Professor Hogue of Cornell University in the United States has achieved success in weaning ewes from their lambs as early as 1 week of age. Similarly, controlled reproduction based on induction and synchronization of estrus and the use of artificial insemination have enhanced the ability to manage breeding and lambing. The use of forage (green and stored) and concentrates along with vitamin and mineral supplements to supply the necessary dietary nutrients to lactating ewes, and the prevention of mastitis, abortions, and parasite infestation through flock health programs have contributed to increased production efficiency. Finally, there has been a significant improvement with the introduction of milk-recording programs and the application of quantitative genetic principles for the selection of breeding animals, including crossbreeding and the development of new sheep breeds.

Dairy sheep flocks in the Rocquefort region of France have undergone changes from traditional production to a highly specialized intensive type of production based on new husbandry procedures, highly productive sheep, large flocks, and modern equipment. In 1951, 10 200 farms produced 33 million liters of milk from 600 000 ewes. Later, in 1978, there were only 3784 farms remaining and producing 58 million liters of milk from 590 000 ewes. The decline in the number of dairy sheep flocks over 27 years has been almost threefold, while the average flock size has increased from 69 to 156 ewes and the individual production level has doubled. Furthermore, two-thirds of the total milk produced comes from more than half of the farms with milking machines.

Presently, there is a trend toward the conservation of indigenous sheep genetic resources derived from many generations of creative human activity and natural selection in harmony with the environment and available feed resources. This approach has sustained the traditional way of life, helping to maintain the evolution of society and the environment. Sheep can exploit land in rural areas with low production potential and land not suitable for larger ruminants. The low input requirements make these animals more effective in wealth generation for the poor people of the developing countries. The high prices paid for animal products and by-products from developing countries have transformed a cottage industry into an important instrument for sustainable development.

Nutrition

In developing feeding protocols to meet the nutrient requirements of dairy sheep, the quality of breeding stock, physiological status during growth and development, feeding behavior, and the source and amount of fodder available are important factors that need to be considered. Milk production depends to a large extent on the supply of energy and protein during gestation. When the energy supply is not a limiting factor, a lower level of crude protein could lead to a reduction in milk production. The dietary minerals and vitamins also have an important influence on milk production. Recently, there have been a number of studies to determine their exact role as described in *Nutrient Requirements of Small Ruminants: Sheep, Goat, Cervids, and New World Camelids*.

Diets containing higher levels of energy during late gestation and early lactation exert a direct and rapid influence on milk production. During early lactation, higher levels of dietary energy hasten the occurrence of peak milk yield; however, the persistency of lactation may be inadequate. In contrast, restricting the supply of dietary energy and protein during early and midlactation delays peak milk yield and reduces milk production. In the second half of lactation, dietary energy promotes building of reserves and persistency of lactation. During this period, it is important to provide an adequate supply of dietary protein essential for milk synthesis. The lactation pattern of dairy sheep can be modified by manipulating the dietary supply of energy and protein during pregnancy and lactation. The feeding protocols developed for dairy sheep are based on balancing the daily nutrient requirements to sustain milk yield, which may be driven by demand for milk and milk products.

Extensive System of Production

The essential components of farming consist of plowing, soil quality, seasonality and drought, and the availability of perennial or nonperennial cultivated species, industrial by-products, and concentrates. In the extensive system of production, nutrient requirements for dairy sheep can be satisfied by adjusting the diet for the amount of feed supplement according to the availability and source of forages on a year-round basis. Grazing of sheep and goats may be complementary on ranges because sheep are known to prefer herbaceous plants, while goats are known not to consume the same plant or same parts of the plant. Sheep raised on the fallow in the plains, mountain, and forest pastures, shrubs, and subdesert areas with little vegetative cover may be able to utilize all the ingested material. In many cases, nutrients derived from grazing may be comparable to that from hay of moderate quality. It may be possible to predict the nutrient composition of vegetation in the mountain pastures and the valleys despite tremendous variation in quality and quantity according to the season. In the Sahel region and the desert areas of the Middle East, water supply is a constraint. Rapid lignification of the plants reduces intake, adversely affecting the digestibility, and crude protein and mineral balance. Provision of mineral and protein (including nonprotein nitrogen) supplements can help promote intake, contributing toward a beneficial effect on health and reproduction.

Sheep raised on ranges are able to take advantage of the vegetation only for a limited number of days due to the prevailing climatic conditions that may vary in severity with the region. Dietary supplements from homegrown forage, farm by-products, and purchased feed may be used to sustain dairy sheep for an extended period. In Morocco, sheep under the pastoral systems receive little dietary supplement because cultivated crops are fed to cattle, and cereals are used for human consumption. Straw, weeds, and stubble associated with lower intake are fed to, or grazed by, small ruminants. Small amounts of cultivated crops are provided as a feed supplement to sheep during early lactation. During periods of feed shortage, decisive actions call for the purchase of forage or concentrates, leasing land with stubble to be used as feed, accepting weight loss, marketing surplus animals, and utilizing community pastures. The feeding of cultivated crops to sheep may vary with the possible benefit from marketing of milk and milk products.

In southwestern Europe, the climatic conditions are favorable for dairy sheep, and milk marketing is organized. Milk and milk products command high prices. Grazing in rangelands may not be able to meet the nutrient requirements of highly productive sheep. Under such conditions, intensification and provision of large amounts of dietary supplement from field crops or purchased forage, cereal, and mixed feed become essential. The potential for increased milk production at high prices under conditions of favorable rainfall, temperature, irrigation, and soil quality can justify permanent pasture or forage production. The complexity of the system becomes challenging when the permanent pasture provides a smaller proportion of the dietary requirements as compared to concentrate-based diets. Forage usage may be maximized by feeding sheep diets with a greater proportion of forage to extend lactation during the winter or dry period of the summer. Cash crops, tubers, beets, and other roots and various varieties of forage can be fed to sheep to substitute for green forage year-round or for stored hay or silage during periods of feed shortage.

Intensive System of Production

In France, higher costs associated with labor, fencing, wastage of forage on pasture, and movement of animals resulted in the development of the zero-grazing concept. In this system, intake may be increased by feeding leguminous plants, ryegrass, orchard grass, and indigenous grasses year-round. If forage needs to be harvested from a number of small fields scattered over a large area, feeding forage may not be cost-effective. Again, the risks associated with climatic conditions increase with the acreage because of inability to harvest crops at appropriate growth and maturity. Hay is the most common form of feed storage; however, the quality tends to vary with the climatic conditions and the stage at which the forage is harvested. Feeding a combination of silage and hay allows for more flexibility over forage production, mainly during the first growth cycles. Under conditions of finite land base, poor soil fertility, and high rainfall, dietary requirements may be satisfied by feeding concentrates in the form of cereal and oil meals together with purchased forage. To replace a greater proportion of concentrates in the diet of the sheep, an increasing number of producers are relying on pastures. Nevertheless, seasonal variation in the quality of feed from pasture can delay lambing from October to December in the northern hemisphere.

The feeding of concentrates and forage to dairy sheep is feasible in an enterprise with well-organized milk marketing commanding a high price for the product. Concentrate helps meet the nutrient requirements of the animal and provides an optimum efficiency for milk yield, thus reducing the costs associated with labor and equipment for forage production. This approach may be modified according to the demand for increased milk production and the amount of forage that needs to be produced. The proportion of concentrate in the diet may be reduced when cereals are to be used sparingly after parturition in order to improve the onset of lactation. In contrast, dry matter consumption may be increased to 70% under intensive systems of production. Under certain conditions, the production cost associated with growing forage may be higher than that of purchased concentrates, in terms of nutrients supplied to the sheep. Therefore, the feeding protocol cannot be generalized largely due to the influence of season and production factors.

Accelerated Lambings in a Controlled Environment

At the Animal Research Centre (later Centre for Food and Animal Research) in Ottawa, Canada, newly developed sheep breeds derived from imported and established breeds in Canada were housed indoors year-round on expanded metal mesh floors in windowless barns with light controlled by time clocks. The accelerated lambing program was based on separating sheep into two equal flocks and breeding for lambing at 4-month intervals under an 8-month breeding cycle. Specific pathogen-free lambs derived by hysterectomy were used to reestablish a disease-free flock. To minimize generation interval, rams were used for breeding at 11 and 15 months of age in both flocks, and were randomly mated to ewe lambs and mature ewes with the restriction that pen matings among half-sibs or more closely related animals were avoided. Ewes were mated in the ratio of 8–12 ewes per ram.

At parturition, each ewe and its lamb were placed in a temporary holding area (1.2 × 1.2 m) to ensure the lambs received colostrum. Lambs remained with their ewes for 8–30 h after birth. In cases where a ewe was deficient in colostrum or the lambs were weak, the lambs were moved to a special draft-free, bedded area. Such lambs were bottle-fed a minimum of 75 ml kg^{-1} body weight of frozen cow colostrum that had been thawed and warmed to body temperature. Each day, lambs were weaned from their dams and transferred to the liquid diet feeding barn. The newly born lambs were held in starter pens with wood shavings for bedding and taught to nurse from the nipple bar. The tail of all lambs was docked at 5 days of age and lambs were placed in starter pens equipped with two heat lamps (1 m above floor level), a nipple bar with 12 nipples, automatic waterers, and creep feed boxes. The animal density was 25 lambs per starter pen (2.4 × 6.1 m). Milk replacer at 5 °C containing 24% fat and 24% protein (from spray-dried milk products made from cow milk) in the dry powder was reconstituted (~17% solids) and circulated continuously to animal pens from refrigerated bulk tanks. Lambs had access to water and creep feed (~18% crude protein) composed of barley, oats, corn, wheat bran, linseed meal, and molasses plus minerals and vitamins at all times.

At 21 days of age, the lambs weighing 6 kg or more were provided solid feed. Lambs weighing less were kept on the milk replacer diets for an additional 1–2 weeks. Weaned lambs were transferred to growing barns and housed at a density of 25 lambs per grower pen (2.4 × 4.8 m). All lambs were fed high-energy diets containing ~90% grain and 8% hay plus vitamins and minerals to promote rapid growth. At weaning and at 49 days of age, all lambs were vaccinated against clostridial infections. Surplus lambs weighing about 50 kg were marketed for slaughter at ~100 days of age. As the older lambs moved out of the liquid-diet barn or growing barn, individual rooms that became empty were cleaned, disinfected, and fumigated before the next lamb crop entered the barn.

Lambs were maintained in light throughout the day (24 h) until about 35 days of age. Daylength was then adjusted to 16 h of light and 8 h of darkness until all animals in a given room reached 105 days of age. Subsequently, ewe lambs were exposed to 10 h of light and 14 h of darkness until breeding at 6.5–7.5 months of age. Lighting regimens were utilized to induce and synchronize estrus in ewes in each of the two flocks in asynchrony relative to photoperiod. Ewes were exposed to an alternating 4-month photoperiod of 16 h of light (long) per day followed by 9 h of light (short) per day as long as the animal remained in the flock. Consequently, in January, May, and September, one or the other of the flocks was bred at the end of a period of short days. Ram lambs remained at 9 h of light until used for breeding at 10.5–11.5 months of age.

Breeding

The East Friesian breed in Germany, the Manchega breed in Spain, the Zackel breed in Greece, the Chios breed of Cyprus, the Sarda breed of Sardinia, the Awassi breed in the Middle East, the Assaf breed in Israel, and the Lacaune breed in France are the most popular breeds among dairy sheep. Genetically diverse populations with considerable differences in milk composition, length of lactation, and milk yield may be classified on the basis of milk yield into superior-, high-, moderate-, and low-productivity categories. The East Friesian and improved Awassi breeds produce more than 300 kg of milk per lactation. Therefore, these breeds are considered to have superior productivity. The highly productive Awassi, Chios, Lacaune, and Sardinian breeds produce 200–300 kg of milk per lactation. The Basque-Bearn, Bergamasca, Bordaleiro, Churra, Comisana, Flemish milksheep, Garfagnana, Karaman, Kymi, Lacha or Manech, Langhe, Latxa, Manchega, Massese, Mehraban, Préalpes du Sud, Sfakia, Serra da Estrela, Stara Zagora, Skopelos, Tzigaja, and Zakinthos breeds produce 100–200 kg of milk per lactation and are considered to be of moderate productivity. In general, flocks of ewes raised on rugged terrain produce only 60–90 kg of milk per lactation. Similarly, milk production from Merino ewes on marginal farms is variable ranging from 10 to 20 l during late lactation of 180–210 days. Breeds producing less than 100 kg of milk per lactation, and therefore of low productivity, are Barbary, Corsican, Epirus, Florina, Karagouniko, Kivircick, Rouge de l’Ouest, Roumloukian, Serrai, Somassierra Blond, Tsigai, and Vlakhiko sheep. The newly developed sheep breeds that excel in milk production are Assaf (Friesian × Awassi), Bergschaf (Bergamo type), British milksheep (42% East Friesian, 16% Bluefaced Leicester, 15% ‘Prolific’, 13.5% Lleyn, 11.5% Polled Dorset, and others), FSL (East Friesian, Sarda, and Lacaune), Frisarta (75% East Friesian), Rideau (40% Finnish Landrace, 20% Suffolk, 14% East Friesian, 9% Shropshire, 8% Dorset Horn, and remaining from Leicester, North Country Cheviot, Romnelet, and Corriedale), Tahirova (75% East Friesian and Kivircik), and Synthetic milksheep derived from multiple breeds.

In the early twentieth century, the East Friesian breed of the long-wool type was milked in small flocks around mining areas. These ewes producing 600 kg of milk in a complete lactation of 240–270 days are the most productive dairy sheep in the world. The seasonal nature of reproduction is more prominent in the East Friesian and Nordic breeds compared to many other sheep breeds. In the Mediterranean countries, lactation terminates in the hot summer months of June and July. Advancing the month of lambing by controlled reproduction has been known to increase milk production by extending the duration of lactation. The potential for out-of-season breeding was found to increase annual milk production in the low-producing Massese and Churra ewes as compared to Sardinian ewes, and in Chios ewes as compared to East Friesian ewes. This phenomenon may be associated with increased availability of high-quality fodder.

In the Mediterranean countries and Central Europe, East Friesian sheep with potential for increased milk production have been crossed with indigenous sheep resulting in crossbred ewes with higher milk yield. Although crossbred ewes produce more milk, the wool quality necessary for weaving rugs in the mountain villages has often been compromised. These crossbred sheep can easily adapt to the environment, an important breed characteristic of indigenous ewes. In subsequent generations, however, the East Friesian crosses have failed to sustain the increase in milk yield. Furthermore, a higher incidence of neonatal mortality and health problems was reported. In many instances, attempts to introduce East Friesian sheep into Central Europe and the Mediterranean region failed because of the inability of the breed to adapt. This has been due to differences in climatic conditions, feeding, body size, and management, and incidence of diseases in the new areas.

As an alternative to crossbreeding, composite dairy sheep populations were derived from two or more breeds for specialized milk production systems. These include the Assaf breed in Israel, FSL breed in France, and the Frisarta breed in Greece. In the Awassi and Assaf breeds, milk production has been increased by implementing an accelerated lambing program. Normally, dairy sheep under such a breeding program are first mated as early as 1 year of age and begin producing milk before 2 years of age.

The potential for genetic improvement of morphological characteristics and production performance among the diverse sheep breeds associated with the wide range of environments and rearing systems is complex. Breeding programs for range-based production systems have been slow in improving the quality of sheep. A number of breeders have expressed growing concern over the improvement of dairy characteristics at the expense of adaptation to environment. The difficulty lies in exploiting the genetic potential of these breeds in the mountainous areas, Mediterranean ranges, irrigated areas, cereal-growing plains, and modern production systems. In the past, efforts were directed mainly toward improving the level of milk production and milking ability. Recently, there has been increased emphasis placed on adding value to milk products. This has been accomplished by considering fat and protein contents as selection criteria while maintaining protein-to-fat ratio in order to improve cheese production.

Udder morphology, particularly the basis of teat placement and cistern height traits, can influence the machine-milking aptitude of dairy sheep. Selection for uniform udder and teat conformation can be effective in overcoming one of the biggest faults of dairy sheep besides short lactation. It has been proposed that selection for udder morphology based on teat placement can be considered with little or no detrimental effect on milk yield when traditional hand milking is replaced by machine milking. Correspondingly, somatic cell count as an index of mastitis that can be easily measured is moderate to low in heritability and adequate to permit genetic progress from selection. Thus, selection for somatic cell counts may be useful in improving the health status of the sheep flock.

There appears to be adequate genetic variation necessary to select for milking ability under conditions of simplified milking and the practice of single daily milking. While attempting to minimize the high cost associated with milk recording, large numbers of flocks are employing simplified recording systems in conjunction with mass selection. In 1978, France initiated the official milk recording of 87 171 Lacaune ewes to promote progeny testing of 357 rams. The average size of the flocks that participated in progeny testing was 282 ewes with an average milk production of 145 l per lactation. Concurrently, a breeding program based on within-flock mass selection made use of simplified milk recording in 138 395 Lacaune ewes. The average size of flocks that participated in mass selection was 246 ewes with an average milk production of 111 l per lactation. The process of milk recording, progeny testing of young rams on the farms and test stations, and the use of semen from selected rams for artificial insemination across flocks have been implemented in the Lacaune breed in France, the Awassi breed in Israel, the Sarda breed in Italy, and some Spanish flocks with over 1000 ewes. This has resulted in 1.1–2.5% annual genetic response to selection for increased milk production. It has been suggested that allocation of greater resources for the selection of rams, particularly a large group of breeding rams under good management, may be more effective in accelerating genetic response to selection as compared to increasing the precision of carrying out progeny tests.

Management

In the Mediterranean region, lactation in sheep depends largely on the availability of roughage and the prevailing climatic condition. The peak lactation may be delayed in flocks that do not have access to stored forages during the winter and spring months. Dairy sheep, with their inherent capacity to utilize scrub and poor-quality fodder, have traditionally served a dual function by producing milk and meat from land not suitable for cultivation. Usually, milk collected from ewes weaned from their lambs is not consumed, but processed into fresh or ripened cheese and yogurt. Lambs surplus to the production requirements are slaughtered for meat, whereas wool and skins are exploited for local handicrafts. In the Corsican mountains of the Mediterranean region, nomadic flocks of indigenous sheep with minimal or no shelter can withstand conditions of drought. Thus investment in housing, fencing, and equipment does not exist. A small proportion of producers provide pasture and cultivated crops for the more productive animals such as Sarda sheep. This requires investment for equipment, fences, barns, and sheepfolds. In the coastal areas where pasture or crops from irrigated fields are common, milking is mechanized and intensive rearing systems are based on highly productive animals. Production of sheep milk is mainly a Mediterranean activity with a larger proportion produced in specialized flocks with improved breeds. In North America, despite the lower volume of sheep milk, its social, economic, and gastronomic importance needs to be considered.

In dairy sheep, milk production can be increased by modifying the management of ewes during the suckling period, without decreasing the amount of milk available for their offspring. In general, ewes are not milked until their lambs are weaned. The amount of milk produced increases with age, reaching a maximum at the third or fourth lactation. In the subsequent lactations, milk production tends to decrease, demonstrating a curvilinear response. Furthermore, prolific ewes are known to produce more milk, even in the absence of suckling. When there is a demand for the meat of suckling lambs, weaning occurs after 4 weeks of lactation. Only lambs intended for flock replacements are permitted to suckle for an extended period that could last 2–3 months.

In the Lacaune breed, artificial rearing of lambs on milk replacer diets has contributed to a 50% increase in milk production. In milking ewes, the amount of residual milk can be reduced more efficiently by encouraging lambs to suckle than by hand or machine milking. Consequently, the demand for labor, equipment, and milk replacer can be minimized.

Milking

The modern dairy sheep industry that produces luxury products is market-oriented, labor-intensive, and heavily mechanized, with requirements for substantial financial investments. In Germany, many flocks of dairy ewes are weaned from their offspring immediately or shortly after birth. Thus, the potential for increased milk production can be fully exploited for mechanized milking. The practice of abrupt weaning of ewes at lambing or following 1 month of lactation does not appear to have any adverse effect on milk production. This is because a few days after weaning, milk production tends to equalize in ewes whether the lambs were weaned abruptly or 1 month later. During milking, ewes may exhibit a single ejection corresponding to the immediate release of cisternal milk. This is due to the absence of a secondary ejection reflex or the simultaneous ejection of cisternal and alveolar milk, for example, in ewes of the Sarda breed. Milking ewes may also exhibit two successive ejections, the first corresponding to cisternal milk and the subsequent ejection reflex for milk derived from the myoepithelial cells ~30–40 s after the teat cups are applied. Milking is more complete with less residual milk in ewes that exhibit the ejection reflex. Manifestation of the reflex depends to a large extent on the environment. Transition from suckling to hand or machine milking could inhibit the reflex. The reflex is known to reappear after ewes are weaned from their lambs. In some ewes, the ejection reflex present during suckling does not reappear during milking, making the ewes unfit for milking.

Milk production may vary when ewes are milked individually or in groups depending on the length of milking, mean or maximum flow, and percentage of milk collected at different stages of milking. The time required for hand milking depends on the creative skills of the milker. The ewes of the Sarda breed appear to be more suitable for milking systems based on simplified methods or under conditions demanding a reduction in the frequency of milking as compared to those of the Lacaune breed. In Sarda ewes, the volume of milk lost in the absence of stripping is negligible. Furthermore, single milking reduces milk production by only 12–15% as compared to the usual two milkings. The residual milk arising from incomplete milking is similar to that in other sheep breeds. These breed characteristics determine hand milking, leading to differences in the number of sheep milked, which ranges from 20–25 Lacaune ewes to 80 Sarda ewes per hour. In mechanized milking, grouping of ewes and the organization of milking parlors can be regulated according to the length of milking. Currently, more attention is paid to the stages of milking because the length of individual milking is no longer an issue.

Mechanized milking is considered adequate when more milk can be collected without any intervention by the milker and a lower volume of milk is obtained from machine stripping and finally by manual stripping also known as 'milking out'. The time required for these activities is 70–75% for milking, 15–20% for machine stripping, and 10% for manual stripping. The ideal conformation, including shape and elasticity of the teat cup, contributes to the efficiency of machine milking. When the ejection reflex is not established, failure to perform stripping during machine and hand milking results in nonemptying of the udder. This amounts to a loss of 15–20% milk in the Lacaune breed, but a negligible amount in the Sarda breed. In dairy breeds (Lacaune and Préalpes du Sud) with moderate-to-low milk yield, there can be a 30% reduction in milk production during the transition from suckling to milking. It has been demonstrated in Préalpes du Sud ewes that increasing the frequency to seven milkings tends to compensate for lambs suckling 7 times a day, thus resulting in a similar volume of milk produced. The suppression of Sunday night milking has been advocated to minimize labor requirements. Ewes, however, appear to be more sensitive to the suppression, and losses in milk production may amount to 13% in Sarda ewes and 26% in Préalpes du Sud ewes.

Mechanized Milking

In the 1930s, the first milking machine was developed by Fleury in Roquefort, France. The two most important characteristics of the milking machine, establishing vacuum level and pulsation rate, have remained stable over the years. The performance of ewes during milking is influenced somewhat by changes in the machine characteristics. The incidence of cups or clusters falling off during milking may be lowered by adjusting the vacuum level and pulsation rate to foster rapid milking. The requirement for capital investment tends to vary considerably between milking strategies, with many ewes being milked continuously or in groups.

All milking parlors currently in use are based on either the abreast system or the rotating system. The most popular milking parlors until recently have been the Casse type developed in Roquefort. This is based on group milking and the abreast system according to the herringbone principle. The system does not require extensive investment in expensive equipment and facilities. However, efficiency depends largely on labor requirements to transport ewes into and out of the milking parlor. The management of dairy sheep in this system consists of weaning all ewes from their lambs at an early age. Offspring intended for fat-lamb production are fed high-energy diets from weaning to 120 days to promote rapid growth rate. Subsequently, these lambs are marketed for slaughter. The remaining lambs for replacement remain in the flock with their ewes and are allowed to suckle for an extended period of time.

In Israel, mechanized milking was introduced in dairy sheep farms to overcome the shortage of labor associated with managing flocks of several hundred ewes. The excessive labor requirements for moving ewes into and from the milking parlor do not occur in the more expensive Carrousel-type system. In this system, the flow of milking ewes is continuous because the ewes enter and leave the milking parlor while others are being milked. The management of dairy sheep in this system consists of gradually weaning ewes from their offspring with provision for simultaneous milking and suckling to promote the stimulation of the udder.

The design of milking parlors has been modified to minimize costs and labor requirements. The International Symposia on Mechanized Milking of Small Ruminants have been a platform for discussion of mechanized milking and specialized equipment. The French Rotostalle type of milking parlor is a constantly rotating carousel capable of handling 500–600 ewes per hour. The Leducteur type of milking parlor was developed in France by combining the continuous-milking design with the lower cost of the abreast system where the milking ewes enter and leave the parlor from the side. The various types of milking parlors described above are fixed, and therefore unsuitable for migratory sheep flocks on mountain pastures. In Sardinia, milking parlors based on simple and inexpensive structure were developed. These units are mobile and can be transported into remote areas without excessive energy costs.

Future Prospects

The sustainable development of dairy sheep farming for specialized and niche products based on natural ecosystems and complementary production systems may be more appropriate in the agroeconomically underprivileged areas. The lower investment cost and opportunity for higher returns by adding value through specialty products could facilitate wealth generation in these regions. A number of developing countries have failed to capitalize on the benefits of milk and milk products from dairy sheep. In recent years, genetic improvement programs have gained popularity in regions with a large concentration of dairy sheep. More recently, developing countries have been attempting to increase the revenue from sheep and goat production by promoting milking. Technologies developed for the production of cow milk are being applied to dairy sheep on farm, including the collection, processing, and commercialization of milk and milk products. Opportunities exist for improvement of morphological characteristics and production performance based on molecular and quantitative methodologies for the application of marker-assisted selection.

See also: **Dairy Farm Management Systems**: Dry Lot Dairy Cow Breeds; Goats; Non-Seasonal, Pasture-Based Milk Production Systems in Western Europe; Non-Seasonal, Pasture Optimized, Dairy Cow Breeds in the United States; Seasonal, Pasture-Based, Dairy Cow Breeds. **Husbandry of Dairy Animals**: Predator Control in Goats and Sheep; Sheep: Feeding Management; Sheep: Health Management; Sheep: Milking Management; Sheep: Multipurpose Management; Sheep: Replacement Management; Sheep: Reproductive Management.

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DAIRY PRODUCTION IN DIVERSE REGIONS

Contents
Africa
China
Latin America
Southern Asia

Africa

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Introduction

In Africa there are three different categories of dairy cow management. There is the large-scale commercial dairy farm sector with cow numbers ranging from 50 to 2000 head, the small-scale dairy sector with cow numbers ranging from 10 to 50 head and finally the communal area/subsistence farms with cow numbers ranging from one to 10 cows. Some authors define small-scale dairy farmers as those in communal areas who sell milk commercially, irrespective of the size of the herd.

Most large-scale farms utilize the 'exotic' breeds of cattle: Holstein–Friesian is the most common, as well as Jersey, Ayrshire, Guernsey, Red Dane and Montbéliard, to name but a few (**Figure 1**). The small-scale dairies utilize some exotic breeds, including Holstein–Friesians, Jerseys, Red Danes, Simmental and Hereford. A few use the indigenous breeds of cattle such as the Mashona, the Tuli and the Nguni. The communal area farmers mostly milk the indigenous breeds as they also use them for beef and a few farmers have one or two cows of an exotic breed.

Most large-scale farms use the purebred milking breeds or crosses of the pure breeds; they very rarely cross the exotics with the indigenous breeds for milk production, although this practice is common in beef production. Small-scale and communal area farms may use crosses of indigenous breeds and exotic breeds for milk production, but this is not as commonly done as it could be.

Research has been done on the milking capacity of these crossbreeds and their advantages as regards disease resistance. However, despite some favourable results, this information has not been widely distributed and most farmers prefer to stick to the known milking breeds.

Housing and Grouping

Most dairy cows in Africa are not housed as such. They are usually kept out in the open with some shelter provided by open sheds or trees (**Figure 2**). A small number of commercial farms use the freestall or tie-stall barn set-up, but even in these set-ups the postweaning calves, replacement heifers and dry cows are usually kept out in the open with minimal shelter provided.

Both types of management have their own problems, as can be expected. The open-style management leads to heat stress and excessive exposure to wet, muddy conditions during the short but intense rainy seasons that most African countries experience. The barn set-ups are very expensive to build and maintain and the low milk price and high input costs prohibit their use in most dairy set-ups. In fact, a large number of dairy farmers have stopped using their barns for cattle as they could not maintain them. In particular they could not afford the constant extensions required for the ever-increasing size of the dairy cow.

Preweaning calves in most commercial farms are taken away from their mothers at or just after birth and kept in

Figure 1 Zimbabwean Holstein–Friesian cow in typical pose.

Figure 2 Large-scale dairy farm with open paddocks.

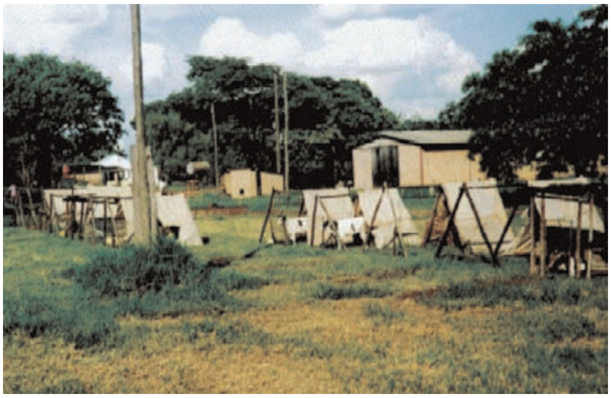

Figure 3 Home-made calf hutches.

home-made hutches (**Figure 3**), crates (**Figure 4**) or individual open brick pens. In the small-scale and communal farm set-up preweaning calves are usually kept with their mothers for short period of time so they can suckle, and then kept in kraals (corrals) together the rest of the time.

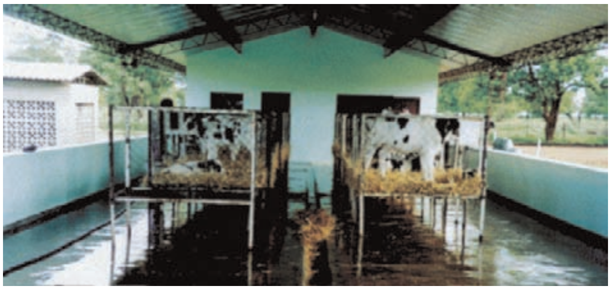

Figure 4 Large-scale calf-rearing facility.

Postweaning calves are usually kept together in small groups, which are age- and size-dependent, no matter what the set-up. However most small-scale and communal area farms have very few followers, so herds may be kept as one large group and sent out to graze. Replacement heifers are also kept in groups dependenton their age and management level or physiological status in the large-scale set-ups and occasionally in the small-scale set-up, depending on numbers.

Dry cows and pregnant heifers are kept separately from lactating cows in all large-scale set-ups and some small-scale set-ups, depending once again on cow numbers. As stated before, most communal area herds are kept as a single group no matter what their status.

Lactating cows in the large-scale set-up are kept in groups according to their production levels. In the small-scale set-up they are kept as one group no matter what their production level. In the communal set-up lactating cows are taken away from the main herd at milking time and given their extra feed then.

All management categories have some form of milking 'parlor' set-up, which will be discussed under 'Milking Management'.

Feeding Management and Feed Types

Feeding management systems in Africa range from zero grazing to minimal grazing with most of the feed provided, to extensive grazing with minimal supplementation. Zero grazing is mostly seen in the large-scale set-up and occasionally in the small-scale set-up. The other feeding management systems are seen in all three categories.

In the zero grazing system cows are kept in barns or paddocks and fed all their required nutrients in a total mixed ration (TMR) (**Figures 5** and **6**). Even if no mixer wagon is present on the farm, feed will be hand-mixed before being offered. Component feeding is no longer practised in these set-ups. The main types of feed sources used are maize silage, cottonseed (whole or as a meal), soya bean meal, maize (crushed or whole), molasses, maize or wheat bran, brewers' grains from clear and

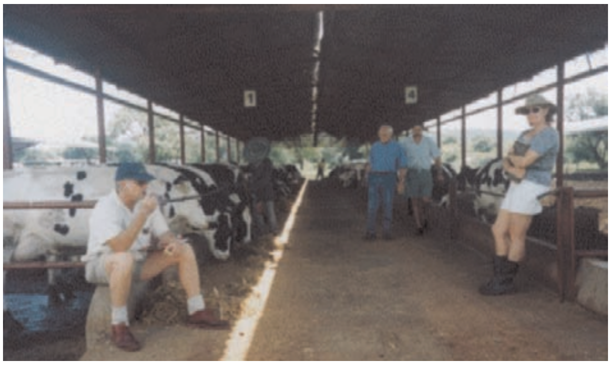

Figure 5 Large-scale dairy cow feeding shed.

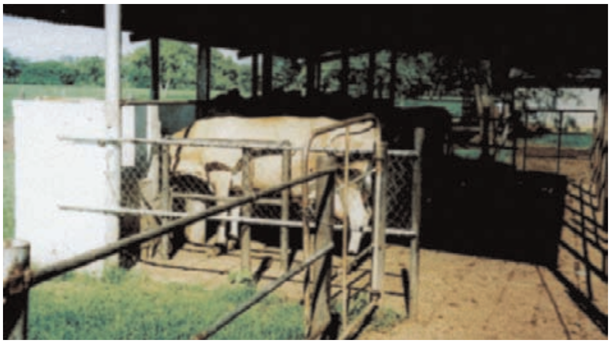

Figure 6 Small-scale dairy cattle feed bunk.

opaque beers and hay from various types of grass. Farmers may also use other byproducts such as citrus pulp, vegetables, bananas, sunflower seeds, water weeds, chicken manure, potato crisps and anything else they think will be of nutritive value to the cows. Home formulation of rations is very common, with occasional consultation with a nutritionist or veterinarian. Often there are shortages of or unavailability of certain products, so most rations are constantly changing. Feed companies also provide mixed-concentrate rations and then the farmer adds maize silage and/or hay for the roughage component. Feed companies mostly use the same concentrate sources as home-mixed rations and also suffer from the same shortages, which causes variable rations. The farms that utilize this system do so, in most cases, for all categories of animal. Some farms will use this system for their lactating cows and then one of the other two systems for all other categories of animal.

In the minimal grazing set-up, cows are sent out to graze during the day, usually on reinforced veld pastures, and then fed a TMR (with reduced amounts of roughage) when they are brought in for milking and kept in their paddocks at night. Once again, this results in variations in quality and quantity of nutrients received, as pasture quality varies significantly throughout the year depending on the season, as does the type of concentrate supplementation. Most farms using this system use the same system for all types of animal and some farms only use it for lactating cows.

In the extensive grazing system the cows are kept on reinforced pastures and only fed concentrates in the parlour at milking time. This system is commonly used in the small-scale and communal set-up and occasionally in the large-scale set-up, where it is referred to as dairy ranching. This system has the same problems as the other two systems, in addition to the fact that most exotic breeds of dairy cows cannot reach their full potential if left to obtain most of their nutrients from grazing. In this system it is usually only the lactating cows which are supplemented with concentrates.

As would be expected, the zero grazing systems have the highest average daily milk production, followed by the semi-intensive and the extensive. However, when it comes to profit, somatic cell counts (SCCs) total bacterial counts (TBCs), and milk components, the results are variable and depend on the farmer's viewpoint. Farmers will always argue that their system works just fine for them and can give you the most favorable economic viewpoint for their system, whilst still complaining about the milk price. As these systems are very different in terms of input, output, genetics, infrastructure and expectations, it is very hard to do a study that will solve the dispute. Basically, all farmers have to decide what works best for them.

The biggest feeding constraint in all systems is a good-quality roughage source. Due to the dry climate found in most African countries it is very hard to grow a good-quality grass and turn it into a good-quality hay. Water for irrigation is a major problem on most farms, even on large-scale farms, and in most cases is preferentially used for crops for human consumption, which give a better return than milk. In most communal areas the pastures are not irrigated and certainly not reinforced, so nutritive value in the dry season is very poor. There are even large-scale and small-scale farms which do not use irrigated or reinforced pastures. Farms which use the TMR system struggle to find good-quality hay that will not be sorted out by the cows. Ryegrass is very expensive to maintain and does not make good hay, so if farmers use it they feed it green. Most other grasses make hay that is usually of a poor quality because, even if it is irrigated, in the African climate it lignifies very quickly. Maize silage is a common feed even in the communal set-up; it is a reasonable roughage source if made correctly; however, as it is a wet feed some other form of roughage is also required. Much research is going into this area but no solution has been found yet. Despite the poor quality of most hays available in Africa it is still fed to dairy cows as it is an essential nutrient.

Good-quality water for the cows to drink is often another problem. Although all dairy cows will receive water, it is often of a poor quality and not provided in large enough quantities, especially in the communal areas where water for human consumption is scarce. As milk is 86–90% water, this leads to low milk production, especially if the cows are also subject to heat stress. Cows suffer from heat stress most commonly at the driest times of the year when water sources are low. Most water on dairy farms comes from boreholes, dams and occasionally rivers. Water in boreholes and dams can be contaminated with heavy metals, pesticides and dairy runoff. This causes problems with the amount of water cows will voluntarily take in, as well as problems in the milking parlour with mastitis, high TBCs and contaminants in the milk. However, until there is enough potable water for human consumption, water for dairy cows will take second place.

Milking Management

All dairy farms in Africa that provide milk for processing or human consumption outside the family are expected to have a milking parlor or shed (**Figure 7**). This applies to all three management systems. A number of communal farmers have cows which they milk for household consumption but they are not really regarded as dairy producers.

Most large-scale dairy farms have a fully operational parlor with a milking machine. Milking machines are of varying types depending on how old the farm is and what is available. No African country has a milking machine production plant so they rely on foreign importations, which makes replacement or upgrading of machines very expensive. There are a few large-scale farms that still use hand-milking, but these are usually the smaller herd sizes. Most large-scale farms have at least one bulk tank, although there is a minority that still use milk cans.

Some of the larger small-scale dairy farms will use portable bucket milking machines; however, most small-scale and communal area dairy farms use hand-milking. All small-scale and communal area farms use milk cans which they then deliver to a central depot which has a bulk tank.

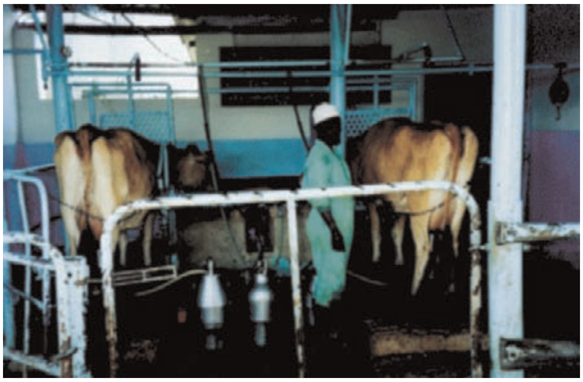

Figure 7 Small-scale milking parlor with bucket milking machine.

Milk Processing

All dairy producers will deliver their milk to a processor of some sort. In most African countries there are one or two large-scale processors which operate country-wide and then a few small-scale processors which distribute to local areas. Most large-scale farmers supply to a large-scale processor, although a few farmers have gone into medium-scale processing in cooperation with other producers in their area. Most small-scale and communal area farmers belong to a dairy cooperative, which has a central depot where milk is stored and processed. These dairy cooperatives may occasionally sell milk to the large-scale processors but it is mostly kept for local community consumption and any profit is shared with cooperative members.

Milk prices are set by the processors after lobbying from the producers. As in all other areas of the world, farmers are never happy with the milk price, consumers complain about the price of milk products and the processors claim to make a loss; however, the industry tends to tick along. There are always dairy farmers going out of business and there may be a downturn in levels of milk received by the processors, but then a new milk price is announced and supplies bounce back.

Although most African countries have dairy farms of some sort, a large number rely on imports of dairy products such as milk powder, cheese and yogurt as the farms only produce enough milk for raw milk consumption. There are even countries, for example, Zimbabwe and South Africa, which export some milk products to gain valuable foreign currency and import milk products to meet local demand.

Labor Management

Most dairy farms in Africa are owned by a family, no matter what scale of production. There are very few state-owned or cooperative dairy farms.

In the small-scale and communal set-up the dairy herd is managed entirely by the family. Each family member is assigned a duty on the farm and then profit from the farm is put back into the household.

In the large-scale set-up the owners are often not actively involved in the farm as they are concerned with the other sections of the farm. In some instances the owner is the manager of the dairy farm who gives instructions to the labor force. In many cases the owner will employ a dairy manager who gives instructions to the labor force but very rarely milks the cows or performs any other tasks. Most large-scale dairy farms rely on unskilled labor for all functions of the dairy farm, from milking, to feeding, and detection of oestrus and insemination. This causes a problem with dissemination of information as dairy owners or managers often do not relay information to their workers and do not send them on training courses. Most workers are told what they have to do but not why, as the owner/manager feels they will not understand the reasoning behind why things are done in certain ways.

Veterinarians in Zimbabwe can find their efforts frustrated when trying to deal with herd problems; the information often goes to the owner who is paying for veterinarians' services and often is not disseminated to the workers. Even if help is given to the workers they frequently revert to their old ways as advice may not be enforced by the owner or manager. Even when the workers are sent on a course, the owner or manager often does not let them implement what they have been taught. However, owners or managers can readily attribute problems to the workers' lack of knowledge and while ready to agree that they should be educated, rarely follow through. In the author's experience, in communication with colleagues in other African countries, the same difficulties with farm owners are evident.

Access to advice for small-scale and communal area dairy farmers is very scarce as very little research has been done on how to manage dairy farms on this scale. Most of the time the information is extrapolated from the large-scale set-up and ends up being not applicable. Research in these areas seems to be mostly aimed at diseases and trying to find cheap feed sources, but largely ignores other aspects of dairy management. More research needs to be done in the field, based around management in the field rather than on feeding trials in artificial set-ups and serology. The problem is lack of money for this type of research due to its extensive nature.

Constraints to Milk Production

The major problem is the lack of on-the-ground research that is applicable to the African situation, whether it be for the communal, small-scale or large-scale dairy farmer. A lot of research has gone into beef production in these sectors but very little into milk production. It is largely believed by most research funders that milk production is not very important in Africa and therefore more emphasis is placed on other farming activities. Milk and its products are regarded as luxury items in most African countries and not as they should be, i.e. as a good source of nutrition. In most African countries liquid milk is more expensive than some of the popular fizzy drinks sold around the world. Wherever you go in Africa you can find cold fizzy drinks but you can't always find milk. Surely if there are refrigeration systems available for fizzy drinks, there should be refrigeration available for milk and milk products. Milk powders are available but due to the fact that most available water is not potable, this product is not commonly used. Much could be done on the marketing of milk in Africa to make it more affordable for the whole population and not just for the economically elite. However, this requires motivation from all stakeholders and in most countries there are only a few processors and suppliers vying for a small market. Fear of competition and loss of market share are too great to allow better cooperation.

The other major constraint is disease. Most dairy farmers keep tick-borne diseases under control through the use of acaracides and vaccines against the specific diseases. These products are readily available as they are used on all forms of livestock and in communal areas the dip tanks are usually government-funded or subsidized.

The major disease seen in all dairy cattle in Africa is mastitis. Zimbabwe and South Africa are the only two African countries which have a milk recording set-up where individual cows are tested for somatic cell counts, milk production, and fat and protein percentages on a regular basis. Most countries have a set-up for testing milk delivered to the processor for SCCs, TBCs and fat and protein percentages as well as any contaminants. Zimbabwe and South Africa have a premium and penalty scheme for SCCs, TBCs and total solids. Despite this, due to the fact that all milk produced is required for processing, milk delivered to the processor is very rarely rejected, even if it is of very poor quality. This means that farmers will always receive some money for their milk, so even the less well-managed farms remain operational, although the dairy may be heavily subsidized by other farming activities. This means that the national average SCCs for most African countries are very high and are not available for publishing (even Zimbabwe and South Africa). This does not mean that all dairy farms have high SCCs; however there is only a small percentage of farms in any African country with herd SCCs below 200 000. Most fall into the 200 000–600 000 bracket and then there is a small percentage which are higher than this (according to bulletins of the various milk-producing organizations). It has been noticed that some farms have had cell counts above 6 million and their milk is still accepted by the processor, although they would never admit to this. Fortunately, only the best-quality milk is supposedly used for products earmarked for export. However, poor-quality milk is sold to local consumers who are then put off dairy products because of their poor shelf-lives.

Farmers will often claim that mastitis and high SCCs are insoluble problems on their farms due to the climate, tick-borne diseases and labor. Both South Africa and Zimbabwe have had major educational thrusts on mastitis over the last few years, in conjunction with a premium and penalty scheme being introduced by the processors. Before these schemes were started, no farm in either country had SCCs below 400 000. However, in the last 2 years Zimbabwe has seen about 10% of herds manage to drop their SCCs below this figure; in South Africa it is about 20%. The percentage of herds with SCCs over 1 million has not significantly changed and there are always dairy farmers at field days and workshops who claim that it is impossible to get SCCs below 400 000 despite the evidence before their eyes. Unfortunately, dairying is mostly a sideline business and until farm owners are prepared to place more emphasis on their dairy management and education of their workers, the situation is unlikely to change. Also, as can be expected, most of these farmers with high SCC problems do not attend workshops and field days.

Other diseases seen are the same as seen worldwide in dairying, including negative energy balance, ruminal acidosis and other metabolic diseases.

Conclusion

Dairy farming is common in African countries. However it is not a high priority in the eyes of the farmers or the market. This is despite the fact that studies done by the Dairy Development Project in Zimbabwe found that households which embarked on a small-scale or communal dairy production scheme benefited economically and nutritionally. This low priority means that little is done on research in these areas due to lack of funds. Specific machinery and inputs for dairying are mostly imported and therefore very expensive, making dairy a marginal profit business. Most stakeholders agree that generic marketing and better loan facilities for setting up dairy farms may improve the situation; however, due to the lack of willingness to cooperate on these schemes because of the small market available, these ideas have not taken off, except to a small degree in South Africa where the Milk Producing Organization has recently started a generic advertising campaign.

In summary, there is a lot of potential for dairying in Africa. However, some obstacles need to be overcome before it becomes more than just a sideline business.

See also: **Animals that Produce Dairy Foods:** Major *Bos taurus* Breeds; Minor and Dual-Purpose *Bos taurus* Breeds. **Dairy Farm Layout and Design:** Building and Yard Design, Warm Climates; Dry Lot Dairy Cow Breeds. **Dairy Production in Diverse Regions:** Latin America; Southern Asia. **Diseases of Dairy Animals:** Non-Infectious Diseases: Acidosis/Laminitis; Parasites, External: Tick Infestations. **Labor Management on Dairy Farms**.

Further Reading

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China

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Introduction

China is the third largest producer of milk in the world. By 2007, there were 13.87 million dairy cattle in total, providing 35.57 million tonnes of milk. China also produces 262 744 tonnes of goat milk, 2.90 million tonnes of buffalo milk, 1.07 million tonnes of sheep milk, and 14 600 tonnes of camel milk. The majority of dairy cattle are located in the northern part of China, with only a small number found in the south, mainly near large cities.

History and Development

History

Traditionally, Chinese people have not kept dairy cattle for milk, and Chinese native cattle were mainly used for crop cultivation. The first time a few dairy cows were introduced into China was by Western priests in the late Qing dynasty. Between 1842 and 1945, more and more dairy cows were brought in via port cities such as Shanghai, Guangzhou, Dalian, and Qingdao. The majority of these imported dairy cattle were Holstein, and only a small number of them were Friesian cows from the United Kingdom and Italy. Further into the twentieth century, a large number of Holstein cows were imported into China from European countries, Australia, and New Zealand. By 1939, the Holstein population reached 9430 head. By the time of the establishment of the People's Republic of China in 1949, the dairy cattle population had reached 120 000 head and many of them were crossbred cows.

Development

Between 1949 and 1979, the dairy industry in China grew at an annual rate of 1.18% to reach 475 000 head by 1979.

With China's rapid economic growth and rising incomes since the early 1980s, significant changes have taken place in the dairy industry. The total population of dairy cattle reached 12.34 million by 2008, 25 times that of 1978 giving an annual growth rate of 11.5% over the preceding 30 years (**Figure 1**). The second significant change has been the dramatic growth in the number of dairy processors and their capacities. The average daily processing capacity was over 100 tonnes in 2007, while it was only 8 tonnes in 1982. In 2008, 16.59 million tonnes of dairy products were produced, accounting for 4.6% of the total world output. The third change has been the high demand for fresh milk and dairy products by Chinese consumers during this period. By 2008, urban residents consumed about 22.7 kg per capita, while rural residents consumed 3.52 kg, which were, respectively, 145 and 203% higher than the corresponding figures in 1992. The income increase of urban residents is a major factor in the increased consumption of fresh milk and dairy products.

Dairy Production

Breeds and Breeding

Currently, most Chinese dairy cattle are derived from crossbreeding between the local yellow cattle (native breed) and Holsteins. A systematic dairy breeding program was started in 1974. The Chinese Black and White breed was formulated in 1981 and officially recognized in 1985, then renamed as the Chinese Holstein in 1992.

In general, Chinese Holsteins have an average milk yield per cow of about 5000 kg per lactation (305 days) with some individuals yielding up to 6400 kg. On major breeding farms, average annual milk production reaches 7000 kg per cow, and a high-yield herd in Beijing produced over 10 000 kg per cow per lactation in 1985. However, Chinese Holsteins do not always perform well on small private farms. Lower production in some herds is due to deficiencies in management, breeding program, roughage quality, stockman skills, and/or knowledge of dairying.

In addition, due to the increasing demand for milk, the crossbreeding of Holsteins with indigenous cattle is popular in some provinces. For example, Quinchuan cattle, a native breed of northwestern China, are crossbred with Holsteins and their offspring perform quite well in milking performance (**Table 1**).

Sanhe cattle were the first dual-purpose cattle bred in China. This breed originated from Inner Mongolia and was the offspring of Mongolian cattle crossbred with a few exotic breeds brought in by the Russians who built the railway in the Far East. Sanhe cows can produce 4000 kg milk annually with a fat content of 4%. They also have good carcass quality and lean meat. Breeding standards for the Sanhe breed were established in 1982.

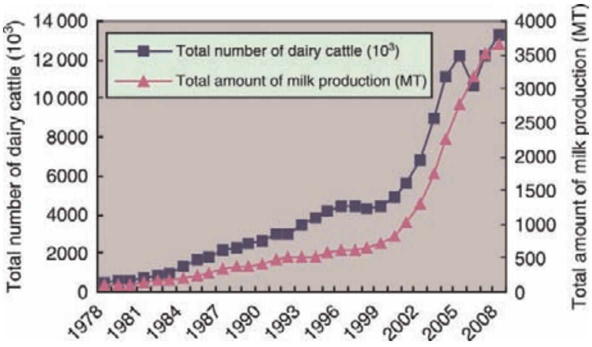

Figure 1 The increasing trends of milk production and the total number of dairy cattle in China.

Table 1 Milk production of Qinchuan × Holstein

Cross	*Milk yield (kg)*	*fat (%)*
Q × H	2076	3.7
QH_1	3718	3.3
QH_2	4307	3.3
QH_3	5110	2.3

There are a small number of Jersey cows in China, which are kept for the high fat content of their milk but make little contribution to the total milk production.

Artificial insemination (AI) was started in the 1950s and widely applied in the 1970s. By 2006, there were 26 Holstein breeding bull stations in China, containing 1319 bulls and providing 25.74 million straws of frozen semen per year.

Progeny testing was started in 1983, and by 2009, 219 bulls were selected using best linear unbiased prediction (BLUP) and test-day methods.

The dairy herd improvement (DHI) program was started in 1992, but up to now only 18 DHI centers have been built and only 250 000 cows, or about 4% of the total dairy cow population, have been registered.

Due to the shortage of quality breeding bulls and high-yielding cows, the importation of foreign semen has been encouraged by Chinese government in recent years. This has stimulated the inflow of frozen semen, and the quantity of the semen imported increased from 64 kg in 2000 to 1856 kg in 2006.

Feeding and Management

During the recent rapid development of the dairy industry in China, there were three main feeding management systems: state-owned farms, hotel farms, and small private farms.

State-owned farms

State-owned farms are very common in China and are usually located close to large cities. The number of cattle on such farms range from 1000 to 10 000 cows, with typically about 2000 cows on most of the farms. The total number of cows held by this farming system represents about 8% of the total dairy cattle population.

In this system, tethered feeding is the most popular feeding system, but housing with free stalls is also gaining in popularity. With either a free-stall or a tethered feeding system, the total mixed ration (TMR) technique, machinery feeding operation, DHI, and automatic milking systems are becoming popular although they vary from farm to farm.

In China, the majority of dairy cattle are reared in cropping regions; thus, a lack of high-quality grasses and legumes is a major problem for the dairy industry. Crop residues, with or without ammonization, are the main forages for dairy cattle. Corn and soybean meals are the main sources of energy and proteins. Other feedstuff such as by-products of grains, oilseed cakes, and sugar beets are also widely used depending on their availability.

In this system, the average milk yield per cow is 6500–7500 kg, with protein and fat contents of 3.8–4.0% and 3.0–3.2%, respectively. The cows are normally culled after 4–5 lactations.

Main problems

Usually, state-owned farms are relatively large and produce large quantities of waste. This waste has become a major agricultural pollutant to the nearby environments and is very expensive to correct.

The shortage of good-quality legumes and grasses results in a heavy reliance on concentrates, and it leads to excessive supplementation with concentrates or incorrect use of roughages. It has become a barrier to achieving higher milk yields.

Hotel farms

Hotel farms are a new type of dairy production system that emerged in the late 1990s. The hotel farms can involve investments by large dairies or the local governments, shareholders, and private investors, and subsidization by the government. The scale of the farm ranges from 500 to 2000 cows, commonly 1000 cows. The cows in the hotel farms are managed in either tethered stalls or free stalls. The advantage of hotel farms is their efficiency of management as compared with that of small private farms. The efficiency is based on standardization of what is known as the 'five standards procedures': (1) house design; (2) feed supply and management; (3) breeding and AI service; (4) disease prevention; and (5) milking system.

As with state-owned farms, the roughages fed to the cows consist of corn stalk silage, dried corn stalk, or dried native alkaline grass (*Aneurolepidium chinense*), so concentrates have to be fed to the cows. As the investors in hotel farms may provide the tenants with proper skills and training, the average milk yield has improved significantly and is 5000–6500 kg per cow per lactation, with protein a content of 3.4–3.6% and a fat content of 3.0%.

Main problems

Hotel farming is a new type of dairying system in China, so investors and policymakers are still lacking in personal experience and necessary knowledge. This has resulted in wrong farm site selection, application of inappropriate technique, poor feeding, and inappropriate feeding management. Also, managers and workers are not trained properly, so the lack of disease control and inefficiency of services are common. These problems, combined with a lack of facilities on some farms, means that the above-mentioned five standards are not being achieved.

Small private farms

Small privately owned farms are the traditional dairy production system in China, which has existed for almost a century. This type of dairy farming is commonly seen in the vast agricultural regions and represents more than 60% of the total dairy cattle population and thus is a main force in the Chinese dairy industry. The number of dairy cattle on an ordinary farm is less than 10 cows. Small private farms can be further divided into two subtypes: backyard farming and grazing-to-shed farming.

Backyard farming

The so-called backyard means rearing animals at the backyard belonging to a family. It is the traditional way of dairying in rural regions. The cows are either tied in a shed all day or let loose in the yard. They are normally fed twice a day and milked 2 or 3 times. The hygiene condition of the sheds or yards is poor: dirty, wet, and no bedding. The cows have little space to exercise. The farmers or owners have little knowledge of how to manage dairy cattle correctly. With such small herds, the farmers are not able to adopt any advanced technology. Furthermore, the poor economic status of the farmers makes it impossible for them to purchase the necessary facilities. In the aspect of feeding, the cows mainly rely on dried corn stalk and self-mixed concentrate feed. A feed such as silage is expensive for farmers and luxurious for the cows. Putting these factors together, many production problems and low yields are experienced in this type of farming.

Grazing-to-shed farming

In China, pastures and grasslands are scattered in many regions, particularly in areas close to Inner Mongolia. In these regions, the local farmers traditionally raise cows by grazing them in summer and feeding them in sheds during winter. During grazing, dairy cattle rely on natural grasses without any supplements and are brought back for milking twice a day. During winter they are offered dried corn stalk or dry grass with supplements of self-mixed feeds.

In this farming system, the average milk yield per cow ranges from 3500 to 4500, with a protein content of 2.8% and a fat content of 3.0–3.4%.

Main problems

Poor roughages and the excessive use of concentrates lead to serious disproportion between roughage and mixed feeds and can result in acidosis.

The self-mixed feeds are usually imbalanced in nutrition and lead to low feed conversion and low milk yield. The farmers sometimes offer extra concentrates in an effort to obtain the expected milk yield and by doing so worsen the milking capacity of the cows.

Generally speaking, the farmers are not trained properly and raise their cows through experience gained from neighbors and their own actions. Their lack of training makes it difficult to deal with daily problems, especially disease problems. The extent of problems usually varies from householders to householders and makes it difficult for the local governments to train them through systematic schemes. The problems on small private farms include the incidence of all kinds of diseases, poor management, high culling rates, metabolic disorders, abuse of antibiotics, and poor milk quality. This has prompted the Chinese government to encourage the new form of hotel farming.

Village Milking Centers

Chinese people have not been in the habit of drinking fresh milk, so much of the raw milk produced in China was made into dairy products. Historically, hand-milking was popular and 30 years ago it was rare to see automatic milking on any dairy farm, even the state-owned farms. With an increase in the consumption of fresh milk, the quality of raw milk has become a big concern for both the government and the major dairy processors.

In the past decade, village milking centers (VMCs) have been developed and their numbers boosted in order to ensure the quality of the raw milk. By 2007, the total number of VMCs was estimated to be about 20 000, of which about 10% were owned by large dairies, 11.7% by large dairy farms, 13.5% by hotel farms, and 38.5% by private investors, and the other 24.5% were the so-called movable milk collectors (a movable container, operating only in high-price seasons). Due to the lack of national standards for VMCs, not all are equipped with the same milking facilities, inspection equipment, or cooling systems.

Other Problems

Breeding problems

There is a need for a functional and effective mechanism to operate the dairy genetic improvement system and its components such as progeny testing, DHI, selection of cows, selection of bulls, culling of cows, and the purchase of breeding cows to enhance the genetic potential and milking capability of the whole dairy herd.

Metabolic disorders

Metabolic disorders are common in all dairy management systems in China but particularly serious for the managers of small private farms due to the excessive use of concentrated feeds, especially during the peak milking period. This results in low conversion of feeds to milk, low fat content, acidosis, ketosis, milk fever, and high culling rates.

Diseases

All kinds of diseases can be found in the Chinese dairy industry. The most common ones that seriously affect production and health of the cows are mastitis, endometritis, tuberculosis, digestive disorders, brucellosis, lameness, bovine viral diarrhea (BVD), and ketosis. The occurrence of these diseases differs from farming system to farming system and from farm to farm with an overall disease occurrence of about 73% in milking cows, 10% in heifers, and 17% in calves.

Milk quality

The high total bacterial count (TBC) and somatic cell count (SCC) in raw milk are the main contributors to poor-quality milk in China. These result from poor hygiene in housing conditions and subclinical mastitis contamination during milking (particularly hand-milking) or storage (containers without cooling system) in the milking parlor. Also residues of antibiotics are found in milk from time to time because of misuse of antibiotics. It has been estimated that the high TBC and SCC in milk lead to production losses of up to 20% in many herds.

Dairy Industry

Dairy Products and Processors

Dairy products and technology

The dairy processing industry is a new and evolving one, as compared with the rest of the food industry in China. Liquid dairy products and dried dairy products such as milk powder are the two major categories.

Liquid dairy products include pasteurized milk, UHT milk, fermented milks, and milk beverages. By 2007, the consumption of liquid dairy products accounted for 55–60% of the annual output of the dairy products. Urban residents consumed much more than did the people living in rural areas. At present, consumers prefer liquid milk products to milk powders. The annual per capita consumption of liquid milk by urban residents was 17.75 kg in 2007, while powdered milk consumption was only 3.15 kg.

Because of the similarity of the liquid dairy products manufactured by dairy processors, there is serious market competition among dairy processors. This has prompted large dairy processors to develop new products. Natural and organic milk are becoming popular, and flavored and fortified milks and lactose-free milk are suitable for the Chinese market. Some liquid milks with special functions such as immunity enhancement, imparted by adding probiotics, are being developed and introduced to the market.

In the past, the main type of milk powder was the whole milk powder, but this has changed in recent years. By 2006, the consumption of whole milk powder decreased to 20% (**Figure 2**).

Powdered milks are consumed mainly by rural residents, infants, and elderly people. Moreover, it is expected that consumption of milk powders by urban residents will continue to decline.

The proportion of infant formula and skim milk powder has increased. Low-lactose formulated powders have also been developed for the elderly. Infant formulae with oligosaccharides are available, and hypoallergenic infant formula products are being produced.

Fermented milk products such as yogurt and milk beverages, especially with functional ingredients, are becoming popular with the annual growth rate in consumption as high as 20% in some parts of the country. Starter cultures are mainly imported for fermented milk product manufacturing, but the government is encouraging companies and research institutes to develop their own starter cultures to support the industry.

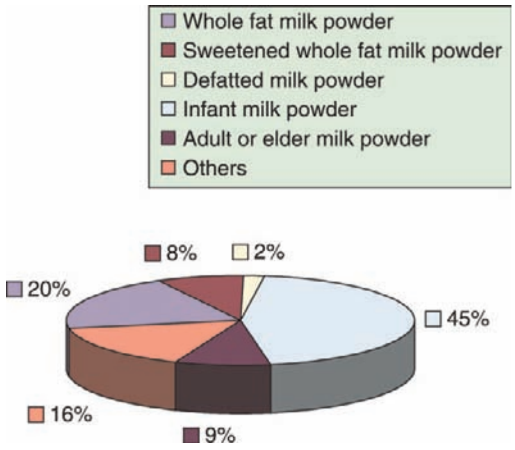

Figure 2 Consumption of milk powders in China.

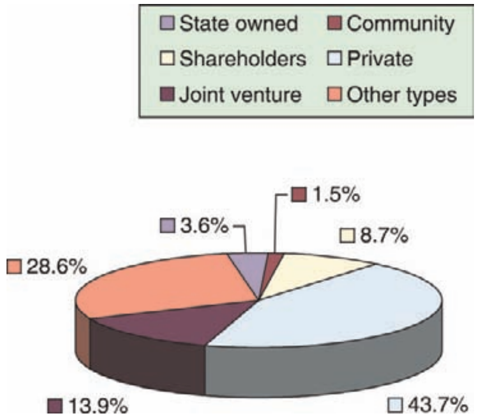

Figure 3 Disposition of ownership of dairy processing companies.

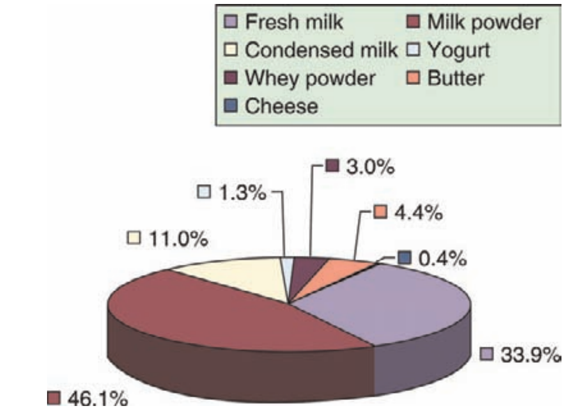

Figure 4 The proportion of imported dairy products in 2007.

Cheese is not a popular dairy food because its smell does not match the taste of Chinese people. Although cheese is considered as a typical Western food, it can be easily found on the market in China. Although little cheese is produced by Chinese dairy companies, research on cheese products is ongoing in many laboratories to create suitably flavored cheeses for the local market.

Dairy processors

The Chinese dairy industry is at a fast-growing stage with investment not only by state-owned processors, but also by privately owned and foreign dairy enterprises. In 2008, there were 807 dairy processors, of which 29 were state-owned companies, 12 community enterprises, 70 shareholder companies, 353 private firms, 112 joint-venture companies, and 231 other types. This indicates that the state-owned companies had declined significantly in number (**Figure 3**). One-third of the total sales value in 2008 was from the joint-venture companies, meaning that the joint-venture companies are more competitive. Their products are higher in price but better in quality than the products from others. On the other hand, larger native dairy processors are becoming the major players. *Meng Niu* Group and *Yili* Group (top two Chinese dairy companies) accounted for almost 50% of the total market share of liquid dairy products and this trend will probably continue.

Import and export

The overall processing capacity of Chinese dairy processors is greater than the total amount of milk produced. This means that some of the processors are unable to operate to their full capacity, so they turn to foreign dairy products as processing materials. Although 120 600 tonnes of Chinese dairy products were exported in 2008, 351 000 tonnes of foreign dairy products were imported. Among them, whey powder accounted for 46% of the total (**Figure 4**).

New Zealand, Australia, the United States, and France are the major countries supplying dairy products to the Chinese market. The major importers of dairy products from China are Hong Kong, Taiwan, and other Southeast Asian countries.

See also: **Dairy Production in Diverse Regions**: Africa; Latin America; Southern Asia.

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Latin America

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Introduction

Dairy cattle production systems in Latin America and the Caribbean (LAC) are extremely heterogeneous due to climatic, ecological, social and economic factors. This text refers to the tropical countries (i.e. LAC excluding Argentina, Chile and Uruguay) since the south is not appropriately classified as 'developing' and management systems are closely similar to those found in industrialized countries.

Production Systems

Production systems can be broadly classified into specialized dairying, which may be intensive or semi-intensive, and dual-purpose which are semi-intensive or extensive. Most intensive farms are located in the cool, high or coastal lands (e.g. surrounding Mexico City, San José, Bogotá, Cochabamba, Quito and Lima). They are also found to a limited extent in the hot lowlands. However, the great majority of lowland farms are dual purpose, with semi-intensive and extensive operations coexisting within geographical areas (e.g. Veracruz, Mexico; Atlantic Coast, Colombia; Zulia, Venezuela), although the extensive farms tend to be found more frequently in the regions with poorer soils and longer dry seasons. Some of the lowland semi-intensive farms do not rear the calves, so are more correctly classified as specialized dairying, as in Minas Gerais, Brazil, but in other respects they are similar to the dual-purpose operations. In general, livestock are quite poorly integrated with crop production, except on the smallest farms where production systems are typically mixed. Far closer integration would be beneficial and is to be expected in future.

Cool Regions: Intensive Systems

The cool region intensive systems are based on temperate zone forage species (grazed or cut) supplemented with concentrates, and pure European dairy breeds. Cows are machine milked two or three times daily. Male calves are generally sold at birth. Females are reared artificially, or purchased and even imported. Artificial insemination is used widely and embryo transplants are common in herds which sell breeding stock and specialize in livestock shows. Although preventive disease programs are established, brucellosis and tuberculosis are still important in localized areas (see **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis; Infectious Diseases: Tuberculosis). Otherwise, the main health problems are pneumonia and gastrointestinal diseases in calves and reproductive problems and mastitis in cows. Reproductive problems are typically the most important reason for cows leaving the herd. Production recording is generalized and while some countries process the data locally others, such as Mexico and Colombia, send them to the United States for processing. Management practices are very strongly influenced by technology in the United States, and a major proportion of inputs are imported (e.g. grain and feed additives, medicines, machinery, semen, embryos and even live animals).

Under these conditions, levels of production that are comparable with temperate zone countries are regularly reached: e.g. average herd milk yields in the order of 6000–9000 kg per lactation with calving intervals in the region of 420 days and mean ages at first calving of about 30 months.

Hot Lowlands: Intensive Systems

LAC has a long history of unsuccessful attempts to establish intensive farms in the hot lowlands, using imported or locally born pure or high-grade European breeds, high levels of concentrate feeding and temperate zone technologies. A major limiting factor is the innately poor quality of tropical forages, combined with the high cost of grain-based concentrates. Poor nutritional status and heat stress (see **Stress in Dairy Animals**: Heat Stress: Effects on Milk Production and Composition; Heat Stress: Effects on Reproduction) make cattle more prone to tropical diseases and parasites. In addition, there is a general shortage of the management skills required for high-yielding cows, and supplies of essential inputs are erratic due to poor communications. Intensive farms now make up a small minority of lowland dairy enterprises and, where they do persist, unusual economic circumstances are often to be found. The Cuban case provides an interesting example of how this kind of system could persist as long as imported grains were cheap. Since the breakup of the USSR, Cuba has changed its dairy production policy radically toward less intensive systems, using grazing, agroindustrial by-products and crossbred cows.

Milk yield is lower than in the cooler regions (2000–5000 kg per lactation), although still high by lowland tropical standards (see **Stress in Dairy Animals**: Heat Stress: Effects on Milk Production and Composition). Overall performance is usually severely limited by poor fertility and survival rates. Reproductive problems, lameness, tick-borne diseases and mastitis are the major health problems (see **Diseases of Dairy Animals**: Parasites, External: Tick Infestations. **Stress in Dairy Animals**: Heat Stress: Effects on Reproduction). A Venezuelan case study showed that losses from death and involuntary culling were so high in imported and local cows that they were generally unable to cover their own replacements. Similar problems have been widespread throughout the region but not thoroughly documented in the literature.

Table 1 Typical average values (range of mean values) for performance traits in lowland dual-purpose farms

Trait	*Extensive*	*Semi-intensive*
Age at 1st calving (months)	40–48	36–40
Milk yield		
kg per lactation	500–1000	2000–3500
days in milk	150–240	250–300
% lactations		
0 days	3–25	1–5
1–100 days	5–20	5–10
Calving interval (days)	390–450	420–450
Calf mortality (%)	3.0–10.0	5.0–15.0
Calf weight (kg)		
birth	28–34	30–38
4 months	58–85	
Growth (kg day^{-1} to 18 months)	0.3	0.3–0.45
Adult cow weight (kg)	370–420	450–520
Herd life (no. calvings per cow)	2.0–5.0	3.0–4.5

Hot Lowlands: Dual-Purpose Systems

Most lowland farms throughout the Latin American tropics use dual-purpose systems. It is estimated that about 70% of all milked cattle in the region are of this type and that they produce 40% of all milk sold. Sales from beef account for about 30% of income from cattle. The systems are very heterogeneous, but generally based on grazing, using crossbred (*Bos taurus* × *Bos indicus*) cows (see **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses), with restricted suckling of the calves which are raised for beef, although not necessarily on the same farm. Although performance recording is not generalized, accurate data are available from research projects carried out in representative samples of commercial farms over considerable periods of time in several countries. Typical ranges of herd means for different production traits are shown in **Table 1** for extensive and semi-intensive systems.

Extensive Systems

Under extensive conditions, cows are kept mainly on native pastures, sometimes supplemented with crop residues in the dry season. They receive little or no concentrate feed supplements. Salt and minerals are usually not supplied systematically, and may not be used at all. Long walking distances and scarce supplies of drinking water are severe limitations to production in the dry season. Milking is frequently seasonal and may be stopped at the most critical time of the dry season, and also in the peak of the rainy season. Cows are milked once daily, by hand, with the calf at foot. Typically, milking takes place on earth floors and udders are not washed beforehand. The cleaning of utensils is rudimentary and clean water supplies at the site of milking are rare. Milk letdown is stimulated by allowing the calf to suckle for about a minute before milking starts. Sometimes the milk from a whole quarter is reserved for the calf, but more commonly they suckle all quarters after the milker has extracted the proportion of the total yield considered appropriate, according to the calf's age and physical condition. Calf death leads to a reduction in lactation length, and hence milk yield, by up to about 50%. The cows are predominantly *Bos indicus* or low grade European × zebu crosses, often selected directly from beef herds on the basis of the size of their udder and amenable temperament. Apart from occasional epidemics (e.g. foot-and-mouth disease), the precarious nutritional status of the cattle at critical times of feed shortage is the main health problem in adults, especially when combined with tick-borne diseases. Internal parasites are probably the major cause of morbidity in calves, and despite natural rearing, the calves' nutritional status is frequently very poor due to overmilking of the cows. Very few farms identify their animals individually and performance recording is virtually nonexistent. Herd sires are usually purchased, and selected on the basis of external appearance and the reputation of the vendor. Continuous, uncontrolled mating is used and, in larger herds, several sires are in use simultaneously, so that sire identification of the calves is difficult or impossible. Most bulls are zebus or crossbreds of low to medium grades of *Bos taurus* breeding.

Semi-Intensive Systems

Semi-intensive farms coexist with the extensive system described above, but are usually located on the more fertile soils and where the distribution of rainfall is better. The most common modifications include the introduction of cultivated pasture species, sometimes with fertilization and irrigation, strategic supplementation of cows and calves, the use of higher-grade *Bos taurus* × *Bos indicus* crosses (see **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses), preventive disease control programs, including internal and external parasites, and twice-daily milking. The latter is one of the most important factors involved in the evolution of the extensive systems into the semi-intensive state, and rural electrification may trigger the change. Calf management varies considerably in the semi-intensive systems, but a common feature is that the calf suckles the residual milk until the end of lactation. They may not, however, always suckle at the start of milking to stimulate letdown, nor are they necessarily present during the milking process. Zebu crossbreds are reputedly difficult to milk without the calf, but their response is greatly conditioned by management. The major advantages of the system are the beneficial effects on total milk yield and on calf survival. Many commercial operations milk 1/2 *Bos taurus* × zebu crosses, and higher grades, with only restricted suckling after milking. On the other hand, once cows have been milked with the calf present to stimulate letdown, lactation length is curtailed if the calf is separated or dies. One study showed a 26% reduction in days in milk for 3/4 and higher grade European cows, due to calf death during the lactation under the traditional milking system. Comparative losses for 1/2 to 5/8 crosses and for zebu-type cows were 48% and 56%, respectively. The presence of the calf during milking does not prevent mechanization of the process, and suitably designed installations are commonly seen. When higher-grade crossbreds are introduced into the system, problems related to tick-borne diseases increase (see **Diseases of Dairy Animals**: Parasites, External: Tick Infestations). Internal parasites are a major problem in young stock. Reproduction and udder problems are usually the main causes of involuntary culling, but the proportion of all cows that leave the herd due to death and involuntary culling is about 50%, which is far lower than in the lowland intensive systems. Natural mating predominates and, even under these more intensive conditions, artificial insemination can lead to a notable decrease in pregnancy rates (10–15 percentage points). Considerable variation has traditionally been observed between and within farms with regard to the grade of crossbred cow which is used. More recently, there has been a clear tendency to consolidate herds at approximately the 1/2 to 5/8 level of European breed inheritance. Mating systems include rotational crossing and the use of crossbred bulls, but few sires are evaluated at all for performance traits. Very few farms have systematic performance-recording programs and even fewer make opportune use of records for management and selection. The low educational level of the farm workers is a major constraint in this regard. As **Table 1** shows, the higher levels of management are reflected in higher yields of milk per lactation and a lower age at first calving. However, calving intervals and calf mortality rates are often similar to, or even higher than, those found in the extensive systems, especially if the grade of crossbred cow exceeds the intermediate level. The slow growth rate of young stock remains one of the weak points of the system.

Feed Resources

The salient features of ruminant feed resources in tropical LAC are, first, the abundance of pastures, with approximately 590 million hectares in the lowlands. Second, locally produced energy-rich feeds (e.g. grains) are not usually available for ruminants: most countries import grain for human consumption and poultry feed, with importation having risen 250% between 1975 and 1996 for LAC as a whole. Third, the supply of agroindustrial by-products, suitable for ruminant feed, is large and generally increasing. They include milling wastes of wheat, maize and rice, brewers' grains, cotton seed, oil seed residues, fruit waste, poultry litter and sugarcane residues.

The cool zone intensive systems use a wide variety of temperate zone forage species (see **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties; Perennial Forage and Pasture Crops – Species and Varieties), including ryegrass, clovers, kikuyu, lucerne (alfalfa) and forage maize as the basis of production, usually heavily supplemented with concentrates. The latter have traditionally been composed of grain-based commercial concentrates, but the fuller incorporation of agroindustrial by-products into dairy rations is taking place, even though this usually leads to some decrease in the energy concentration of the ration. The use of probiotics, bovine somatotrophin and other additives is quite common.

In the lowlands, the extensive systems still depend heavily on native pastures, but cultivated species such as the brachiaria or signal grass (*Brachiaria* spp.), star grass and bermudagrass (*Cynodon* spp.), guinea grass (*Panicum maximum*), elephant grass (*Pennisetum purpureum*) and others are being introduced increasingly as the production system intensifies. The use of fertilizers is variable, but in general levels of application are low. Efforts over many years to incorporate legumes such as stylo (*Stylosanthes*) and centro (*Centrosema*) into grass pastures in the region have had very limited success. Tree legumes such as gliricidia (*Gliricidia sepium*) and leucaena (*Leucaena leucocephala*) have rapidly become popular, especially for use as protein banks. Despite the variety of species of grasses and legumes available, their energy content is characteristically low and this factor, combined with seasonal shortages in many parts of the region, is the main nutritional factor limiting production. Low protein and mineral contents are often an additional limitation, since mineral supplementation is generally restricted to common salt. Average milk yields of about 1700–2400 kg per lactation are obtained from tropical pastures without supplementation, and though higher levels have been reported, they are generally not sustained for any prolonged period of time under commercial conditions. The intensive systems are particularly affected by the poor quality of tropical forage species. Up to 70% of the diet may have to be derived from concentrate supplements, with negative effects on the utilization of the fibrous forage base. In the dual purpose systems, levels of concentrate supplementation are variable but generally do not exceed 3 kg daily. Problems of erratic supplies, quality control and transportation often limit opportunities for making wider use of agroindustrial by-products as dietary supplements, especially on small farms.

Cattle Breeds and Types

In most countries, the intensive systems are based predominantly on North American Holstein Friesians and, to a lesser extent, Brown Swiss and Jerseys (see **Animals that Produce Dairy Foods**: Major *Bos taurus* Breeds). Continuous importation of semen and embryos occurs in most countries, mainly from the United States, but there is relatively little exchange of germplasm between countries within the region itself. The regression of daughters' milk yield in the tropical countries on the sires' proofs for milk in the United States has been found to vary between about 0.3 and 0.6 kg kg^{-1}. Emphasis on type characteristics is generally high and in some countries has been the predominant criterion in the selection of imported semen. As pointed out above, the breeds have outstanding performance records in intensive systems in the cooler parts of the region, but have generally failed economically in the hot lowlands.

Criollo (*Bos taurus*) cattle now play an extremely limited role in commercial milk production systems, either pure or crossbred. There have been numerous efforts to conserve them, but numbers of most breeds remain very low. The Central American criollo, Harton del Valle (Colombia) and Criollo Limonero (Venezuela) are among those with most potential for milk production. The shortage of selected breeding stock and the poor growth rates of the criollos, when compared with European × zebu crosses, are probably the main factors limiting their current distribution.

Two specialized *Bos indicus* breeds, the Gir and Guzerat, have been selected for milk production in Brazil under high standards of management (see **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses). The average yield of officially recorded Gir cows is 3200 kg, with over 3500 cows in the program. Bull progeny testing was started in the Gir breed in 1985 and now both breeds have modern sire evaluation schemes. Semen and embryo sales are expanding at home and Gir bulls now account for 80% of total semen exports from Brazil. Although some Gir bulls are now performance tested for growth rate, the relatively small size and slow growth rate of the breed may limit its expansion for use more widely in dual purpose systems.

The region also has various composite breeds developed for lowland milk production. These include the Lucerna (Colombia), Carora (Venezuela), Girolando (Brazil), Siboney and Mambi (Cuba), all with recording and evaluation programs. Progeny testing of Carora sires is in progress. The potentially important Mambi and Siboney populations were formerly subject to rigorous selection but bull progeny test results have not been published from Cuba recently.

The vast majority of milked cows in the lowlands are indefinite crossbreds between *Bos taurus* breeds and zebus, mainly of Brahman type. The Holstein and Brown Swiss breeds predominate in the crosses. Available evidence is quite consistent in showing the Holstein crosses to produce more milk per lactation, but they may be poorer in fertility, calf growth and survival so not always more productive overall. There is generalized evidence that the performance of cows with 1/2 to 5/8 European inheritance is similar or superior to that of other grades when milk, growth, fertility and survival are taken into account. This holds true over a wide range of management levels for dual-purpose systems (**Table 2**). At the higher levels of management, higher grade crossbreds produce more milk per lactation, but are inferior in growth, survival and usually in reproduction. There is a strong movement in Brazil to generate F_1s in zebu herds specifically as replacements for dual-purpose systems. Pedigree-selected crossbred bulls (progeny of proven exotic *Bos taurus* sires and locally performance-evaluated dams) are recommended for use until progeny testing can be systematically established in these commercial populations.

Table 2 Relative production of different breeds groups according to management level (zebu cows at low level = 100% base)

	(Milk + calf) per 100 cows per year		
		European × zebu crossbreds	
Level of management[a]	*Zebu type*	*Medium*	*High grade*
Low	100	112	108
Medium	107	128	120
High	268	315	319

[a]Mean milk yields were 761 kg, 912 kg and 2563 kg per lactation at the low, medium and high management levels.

Adapted from Vaccaro *et al*. (1997).

Social and Economic Factors

Given the socioeconomic context of LAC, the region's intensive systems have the advantage of producing large quantities per farm of good quality milk, but their contribution to equity, rural development and to lowering milk price to consumers seems highly questionable. The capital per hectare required for their establishment is two to seven times higher than that necessary for dual-purpose systems. Interest rates on capital are frequently in the order of 25–40%. Thus, most intensive farms are in the hands of large producers with capital derived outside the dairy sector. Intensive systems make poor use of the region's most abundant feed resources and are highly dependent on imported inputs, which is a disadvantage given the heavy indebtedness of most LAC countries. They also require high management skills. Studies in the region have shown that milk produced in intensive herds has a 20–50% higher cost per liter and that they are usually less profitable than lowland dual-purpose systems. However, intensive system transportation costs may be considerably lower. In general, intensive systems are declining in importance throughout the region because of their high capital and operating costs. The land that they occupy is in increasing demand for vegetables, flowers, poultry and construction. In contrast, about 80% of the dual purpose cattle are in the hands of small producers. The dual-purpose systems are more easily integrated into local cropping systems, and also are more flexible and less risky than intensive systems in volatile economies. Thus, the dual-purpose systems have an important potential role in rural development but also appear to offer greater promise than the intensive systems for optimizing the use of local resources and producing milk at prices which are accessible to the majority of the population, which now includes over 200 million poor and 60 million people who are food insecure.

Marketing

Much of the milk produced on intensive farms is sold homogenized and pasteurized or converted into a variety of milk products of excellent quality. These are generally distributed through supermarkets in the major urban areas. However, the sale of raw milk to urban consumers persists, partly because it is usually not subject to price controls and consumers prefer the full fat product. Boiling in the household is standard practice. Milk from the tropical lowlands is usually of poorer hygienic quality, and several hours may elapse between milking and cooling. In most countries, the use of any additives is illegal, even though a strong case might be made for the use of harmless preservatives. Transport is costly and uncertain, given the typically poor condition of the roads, particularly in the rainy season. Much of the dual-purpose milk is made into cheese at the farm, to avoid the risk of spoilage and reduce transport costs. The remaining fluid milk is generally either sold raw in nearby urban centres, or converted into powder or cheese, since a relatively small proportion is of sufficient quality to be sold pasteurized. In general, price controls exist for raw milk, but not for inputs and in the unstable economies that characterize the region, there is a frequent imbalance between the two. Similarly, unpredictable seasonal price fluctuations and varying ratios between the prices of milk and beef are problems the dairy producer has to face. The lack of quality control of feeds, additives, vaccines, medicines, etc. and defective services (e.g. equipment repairs, spare parts) are also major concerns.

Potential for Improvement

The present average yield of cows in tropical LAC is about 1250 kg milk per cow per year, with eight countries below the 1000 kg level. There is wide consensus among local researchers in different disciplines that the current average level could be doubled, and that this could be achieved mainly by the better management of existing resources, rather than by major additional capital investment. Evidence in support of this view is available from numerous studies which demonstrate the wide differences in production levels between farms within similar geographical regions (**Table 3**), as well as considerable,

Table 3 Extreme differences in performance traits between farms in two geographical zones in Venezuela[a]

	Zone I		*Zone II*	
Trait	*Minimum*	*Maximum*	*Minimum*	*Maximum*
Milk yield				
kg per lactation	543	1076	894	2865
days in milk	154	253	172	284
Calving interval (days)	378	457	389	435
Calf weight at 4 months (kg)	61	88	64	76
Calf mortality (%)	2.4	9.3	6.4	22.3

[a] Least squares adjusted 6-year herd averages from seven farms in Zone I and five in Zone II.

though smaller, differences between breed groups within farms as shown in **Table 2**. A Venezuelan study showed increases in the annual production of milk and calf in the range of 30–80% over 6 years on farms which had made coordinated improvements in management and genetics. Closure of local milk plants due to limited demand, changes in national subsidy policies, absentee ownership and rural violence were associated with farms where improvements were not observed. It is concluded that well-tested technologies in the areas of feeding, health, genetics and management are in commercial use and that these, in proper combination, can lead to yields of 2000–2500 kg milk per year, with annual calving rates of at least 70%, using locally available resources. Coherent national policies for the cattle sector are now required to achieve these goals.

See also: **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses; Major *Bos taurus* Breeds. **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis; Infectious Diseases: Tuberculosis; Parasites, External: Tick Infestations. **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties; Perennial Forage and Pasture Crops – Species and Varieties. **Stress in Dairy Animals**: Heat Stress: Effects on Milk Production and Composition; Heat Stress: Effects on Reproduction.

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Southern Asia

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Nutrition Resources

In developing Asia, crop residues constitute the major component of feed for cattle in almost all small-scale crop livestock systems, for example, straw from rice, wheat, and barley, stovers from maize, sorghum, and millet, stalk from groundnuts and sweet potatoes, and molasses from sugarcane, to mention a few. In some areas, small amounts of cultivated forage are available. A limited amount of higher-quality crop residues is available such as wheat bran, rice polish, and crushed maize, in addition to leaves from trees and bushes and city by-products such as brewer's grains. A small amount of grazing along the roads and edges of fields and ponds is also available. In rain-fed areas, the fields can be grazed in the dry season.

Crop residues are fibrous by-products. They provide low-cost feed management of cattle. They are important adjuncts to natural pastures and planted forages and are used especially to fill in feed gaps during periods of acute shortage.

Crop residues are seasonally produced and become available only after the grain is harvested. Therefore, a good preservation of crop residues is essential to ensure their utilization year-round. Exposure to weather decreases the nutritive value of straws and stovers. Other problems include pest infestation, mold growth, and fire risks. In addition, crop residues alone are poor-nutrition feedstuff with low digestibility. This results in low intake by cattle. The digested products of crop residues are poorly balanced and hence result in poor performance with regard to reproduction and production in general. Supplementation of crop residues may improve the acceptability and nutritive values of the residues. Supplements enhance the rate of fermentation of fibrous carbohydrate for the production of microbial protein and reduce the rumen degradation of dietary protein and outflow of particulate matter from rumen. Complete feeds are now available, especially in some parts of India, that are composed of 50% of straws or stovers and 50% of milling by-products, vitamin and mineral mixtures, salts, and molasses. Weeds and grasses collected from cropland tending are used as fodder.

In India, Pakistan, and, to a limited extent, in Bangladesh, fodder cultivation is being practiced in some areas. Berseem (*Trifolium alexandrinum*), oats (*Avena sativa*), rape (*Brassica campestris*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), and millet (*Pennisetum typhoides*) are cultivated depending on the season. High-yielding fodders such as napier (*Pennisetum purpureum*), sadabahar (*Andropogon gayanus*), hybrid sorghum, and multicut oats are cultivated in considerable amounts.

Wheat bran and rice polish are the main milling by-products. Cottonseed (*Gossypium hirsutum*), rapeseed, maize, coconut (*Cocos nucifera*), mustard (*Brassica juncea*), and sesame (*Sesamum indicum*) oil cakes are the major protein supplements used to feed dairy animals. Many attempts to introduce molasses–urea blocks as a ruminant feed supplement were not successful in the farmers' community. Treatment of straw with urea was found technologically unsuitable for resource-poor farmers. The by-products of animal origin are of little significance as cattle feed.

In India, about 4% of the cultivable land (6.6 million hectares) is used for fodder cultivation. The average yield is about 40 tonnes per hectare. Over the past 30 years, the production of cereals, and hence straws and stovers, has increased markedly. The area devoted to green fodder production has increased from 3 to 4% of cropland, indicating a small increase.

In Pakistan, 13% of the cultivable land is devoted to fodder production. During the last 30 years, the ruminant population has increased, but the land devoted to fodder crops has decreased.

Concentrate feeds in Sri Lanka are coconut cake and rice bran. Farmers in the dairy zones feed their cattle with rice straw during the dry season. In the rainy season, animals are grazed on roadsides and noncropped areas. Sri Lanka estimated a requirement of 225 000 tonnes of concentrates in 1994 and it was projected to be 747 000 tonnes by 2000. The present demand must have changed although the growth rate in the cattle industry of Sri Lanka has not been promising over the last few years.

Cattle feed resources in Indonesia, the Philippines, and Thailand are of similar kind. Large amounts of legume by-products and forage grasses are produced in rotation with other crops. Legumes are also used as green fodder. Cassava peels and leaves have recently been introduced as cattle feed in many Southeast Asian countries.

In areas closer to the Indonesian mountains, green feeds are normally available during the dry season because of some precipitation and trees in nearby forests. In intensive milk-producing areas, rice straw is used to some extent; however, the production system is based largely on green feeds and concentrates. In urban dairy enterprises, concentrates are the main source of nutrients.

In Thailand, ipil-ipil (*Leucaena leucocephala*) and liricida (*Gliricidia sepium*) have long been used as cattle feed by farmers. Several pilot trials with ipil-ipil as cattle feed in Bangladesh found it to be useful; however, it has not been extended to the farm level. Thailand proved cattle feed supplementation worth by using low-cost concentrates, urea–molasses, and minerals (as blocks). Such systems are now common in large dairy farms and beef production operations.

In Indochina, the return from rice production tended to be higher than that from other industrial crops. About half of all rice land is double cropped. In Laos, replacing fallow vegetation with fodder crops seems to provide good potential for improving livestock production. Improved fallow systems, especially those in which leguminous plants and the animals themselves are used to increase soil fertility, can lead to increased fodder availability.

Potential Improvement in Cow Management

Asia is, by tradition, dominated by small-scale farmers, and cattle rearing is an important economic activity of indigenous farming system; however, both land and capital resources are limited. In recent decades, dairy farms of various sizes have been established. The establishment of dairy farmers' cooperatives and associations is an important milestone in the development of dairies in developing Asia. The success of Indian dairy farmers' cooperatives is well known worldwide. Bangladesh, Indonesia, and Thailand, among others, have also proved the benefit of cooperatives in milk production and marketing. The cooperatives have largely contributed to the development of dairy by (1) buying milk from farmers at a reasonable price, (2) running a breeding program for cattle development, (3) providing cattle healthcare support, and (4) ensuring farmers' participation in the whole system. The involvement of nongovernmental organizations (NGOs) has helped establishment of small dairy farms to some extent. The Bharatiya Agro Industries Foundation (BAIF) and Sabarmati Ashram Gaushala, in India, have been very successful in the dairy industry. These two NGOs are running good breeding programs with provision for testing sires for progeny performances. In Bangladesh, BRAC has introduced breeding programs in cattle and goats and has made remarkable success. Community-based veterinary service delivery through farmers' groups and associations and assurance of milk marketing proved to be instrumental for the rapid growth rate of the smallholder dairy industry in Bangladesh.

Developing Asia has made good progress in cattle breeding. Almost all countries practice artificial insemination (AI). AI programs use mostly frozen semen. Many countries have initiated performance testing of progeny for ranking potential sires. With the help of the International Atomic Energy Agency (IAEA), Bangladesh, China, India, Indonesia, Myanmar, Pakistan, the Philippines, Sri Lanka, Thailand, and Vietnam, for instance, adopted nuclear and related techniques (i.e., progesterone radioimmunoassay) that helped them develop improved feed supplementation strategies and reproductive management. Several pilot studies have shown clear economic advantages of feed supplementation and improved reproductive management. The measurement of progesterone in cows' milk has proved effective for identifying and rectifying constraints in AI and for improving AI services to farmers by diagnosing nonpregnant cows. Data on the use of milk progesterone enzyme-linked immunosorbent assays (ELISAs) in diagnosing nonpregnant cows are shown in **Table 1**. Milk progesterone data gave a clear interpretation in 94% of cows ($n = 390$) about their luteal functions when the data were compared with the data of pregnancy diagnoses made by per-rectal palpation at days 50–118. None of the 101 cows with a progesterone profile of low ($<1\ \mathrm{ng\,ml^{-1}}$), high ($\geq 1\ \mathrm{ng\,ml^{-1}}$), and low on days 0, 10–12, and 22–24, respectively, was found pregnant at rectal palpation. This indicates a high accuracy of nonpregnancy diagnosis by using milk progesterone ELISA. Ultrasonography and ovulation synchronizations (Ovsynch) have recently been introduced to study reproduction in cattle in developing Asia. The Artificial Insemination Database Application (AIDA), developed by IAEA, has proved useful in several countries as a tool for keeping and managing AI records. Thailand has developed a good database of their own for recording reproductive parameters and production management.

Methods have been developed for better preservation of paddy straws, particularly during the rainy seasons. As in other developing countries, Vietnam operates large-scale ensilage preservation of paddy straw as cattle feed. Furthermore, significant progress has been made in deworming, vaccination and prevention, and control and treatment of diseases, in general. One of the major improvements made in cattle management is the identification of the problems that significantly limit the calf crop productivity. More attention is given to enhance

Table 1 Milk progesterone profiles by ELISA of the artificially inseminated cows on the day of service and on days 10–12 and 22–24 with respect to pregnancy

Day 0	*Days 10–12*	*Days 22–24*	*Number of cases (%)*	*Rectal palpation results; interpretation*
	(days of AI)			
Low	High	High	239 (57.5)	Pregnant
Low	High	High	9 (2.2)	Nonpregnant, early embryonic death
Low	High	Low	101 (24.3)	Nonpregnant, fertilization failure, early embryonic death, post-AI anestrus
Low	Low	High	13 (3.1)	Nonpregnant, AI in nonestrous cows
Low	Low	Low	25 (6)	Nonpregnant, AI in anestrous cows
High	High	High	2 (0.5)	Pregnant, AI on pregnant cows
High	Low	High	1 (0.2)	Nonpregnant, AI at incorrect time/luteal phase
Clear interpretation			390 (94)	
Low	Low	High	1 (0.2)	Pregnant, assay problem
High	High	High	8 (1.9)	Nonpregnant, persistent corpus luteum, assay problem
High	High	Low	15 (3.5)	Nonpregnant, assay problem, short estrous cycle
High	Low	Low	2 (0.5)	Nonpregnant, assay problem, short estrous cycle, cows went to anestrus
Unclear interpretations			26 (6.3)	
Total number of observations			416	

Low = <1.0 ng progesterone ml^{-1} of milk and high = ≥1.0 ng progesterone ml^{-1} of milk.
From Khan AHMSI (2008) Development of Milk Progesterone ELISA and Its Application at AI Field Services in Cattle. PhD Thesis, Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh, Bangladesh, 102pp.

the growth and survival rates of replacement stocks (heifer calves and growing heifers) by improving calf health management. Many countries now operate udder health management programs to reduce the incidence of mastitis and ensure the production of wholesome milk.

Marketing

Milk, meat, and leather are the principal marketing products from cows in developing countries. Milk consumption is not widespread in the humid tropic of Southeast Asia, whereas it is widely consumed in South Asia. Milk in particular is a perishable good. Therefore, good preservation and market networks are essential prerequisites for dairy farming to run in the production line. Many countries have developed good networks for collecting milk from smallholding farmers through cooperatives. In India, as an impact of Operation Flood Project, over 100 million tonnes of milk was produced in 2008. Operation Flood, in collaboration with NGOs, has established 6000 women's dairy cooperatives and generated higher income for some women.

The average annual per capita milk consumption in Bangladesh has increased over the last decade (from 10.45 l in 1995–96 to 11.2 l in 2004–05), but it remains very low compared with regional consumption levels (e.g., 85 l per capita in India). According to data on household income and expenditure survey, total milk consumption grew by about 3% per year between 1995–96 and 2004–05. The average growth rate of national milk production, however, was only 1.2% per year from 1995 to 2005 (FAO) (**Figure 1**). Growth in milk production remains far behind that of other Asian countries (3.8% in India, 3.6% in Pakistan, and 13.2% in China from 1995 to 2005).

Many developing Asian countries have not yet been successful in developing proper marketing facilities and networks for milk and milk products. In many cases, farmers do not get proper margin due to the monopoly of intermediaries. Often, the farmers incur loss due to higher production costs than sale values. One of the important reasons for the loss incurred by dairy farmers is the low price of milk. The cheap supply of imported powder milk in large quantities in the local market has contributed significantly to the low price of milk. As a result, local producers and milk marketing organizations cannot compete with milk importers.

Marketing of livestock and their products is handled mainly by the private sector. Other than a few dairy processing enterprises, milk and milk products coming out from traditional small-scale dairies are marketed in an unorganized manner. Generally, three different systems of milk marketing exist in Bangladesh: (1) marketing of milk from farmers to consumers with or without the involvement of intermediaries, (2) organized collection of milk from farmers for processing and marketing by private enterprises, and (3) marketing systems for traditional dairy products such as sweetmeats and other fermented

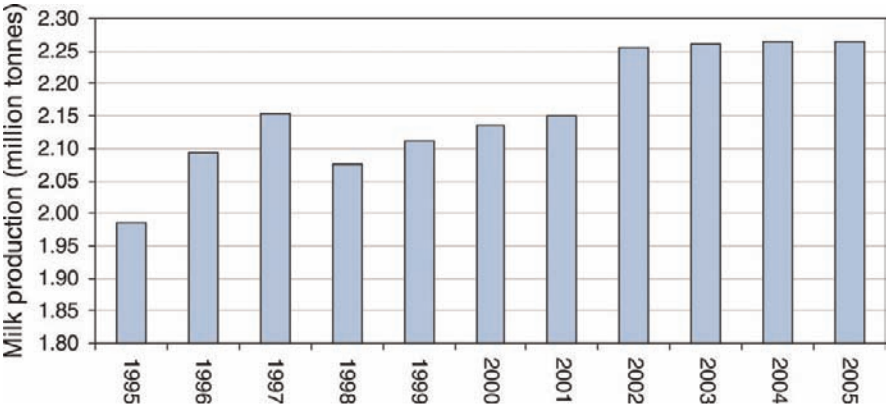

Figure 1 Milk production, 1995–2005. Source: FAOSTAT, 2005.

products. The imperfections in the village marketing systems resulting in high prices for input and low prices for output often discourage the development of dairies in a country.

In current years, cooperative and private milk processing and packaging enterprises have been established in developing countries. Organized collection, processing, and marketing of milk are accomplished by milk producers' cooperatives/societies. Some NGOs do collect milk from smallholding farmers, chill at the point of collection, carry to a central processing plant, and market the finished products mostly in the cities. Very recently, NGOs have also been involved in the collection of milk from contact farmers of urban and periurban areas. The private milk processing enterprises operate their activities in limited areas, so it is not possible to provide services to dairy farmers dispersed all over the country.

In Bangladesh, the Milk Producers' Cooperative Union Ltd. has made significant improvement in milk marketing. This cooperative collected and marketed 25 000 kg milk in 1990. In 2009, the average daily milk collection was 200 000 kg. In addition, NGOs and private entrepreneurs also collect and market about 200 000 kg milk per day.

In general, consumer prices of most commodities have increased worldwide. However, the price increase of animal products has been very low. This means that animal products have been available at relatively cheap prices.

Animal Use

Cattle provide agricultural power and animal products for human consumption and use in developing Asian countries. However, replacement of an animal by mechanical draft power has made good progress in recent years. In association with industrialization and modernization of most of developing Asia, the requirement for draft power is reducing gradually.

Specific cattle rearing for dairy or beef has to compete in the market with other sources of animal protein. Owing to the quick return, fish and poultry are the major competitors of dairy and beef industries. The benefit of cattle rearing is that the cattle can survive on crop residues and by-products. The hard line is that the crop residues are nutritionally imbalanced foodstuff for cattle, leading to poor reproduction and production in general. Poultry and fish farming are big competitors for utilizing crop-milling by-products. This means that specialized dairy, beef, or dual-purpose cattle rearing faces a hard economic reality in developing Asia.

In South Asia, there are tribes of people who traditionally rear cattle and market milk and milk products. These tribes are good candidates to work with for sustainable small-scale dairy development. This means that affordable rural-based technologies for preservation of milk and for processing of milk to other products will help dairy development.

Animal products for human consumption and use have been increasing gradually in developing countries with annual growth rates varying from 4.09 to 4.76%. There has also been an increase in the consumption of dairy products. Increased income has allowed people to choose animal products over grains and other plant products. Technological development has contributed to increased amount of produce and reduced production cost. This has increased the availability of animal products at low prices. Cows and their products provide direct cash income and are therefore considered as living banks for farmers. Cattle are critical to agricultural intensification because they provide manure,which can be used as fertilizer and fuel. Dairying has a direct impact on generating income, alleviating poverty, and making animal protein available. By quantifying livestock and poultry, it is seen that dairying is the predominant source of income generation.

Production Goals

In all developing countries, the dairy cattle population will increase by 1% per year up to the year 2010, 1.7% per year in 2010–25, and 0.8% per year in 2025–50. The beef cattle population in all developing countries including China will increase by 1.2% per year up to 2010 and 0.6% per year in 2010–25. In South Asia, the cattle population will decrease. **Table 2** projects the production goal for milk and meat with respect to the population increase in developing Asia up to the year 2100. This is due to a stable or decreasing per caput demand for beef combined with production increases. However, the FAO study in 2000 suggests that there will be a continuing strong rise in demand for dairy products in the developing countries at the beginning of the present century.

The smallholder individual dairy farmers in developing Asia set their goal to produce outputs that may be (1) more animals within the herd, (2) live animals for sale at times of necessity or opportunity, (3) meat and meat products, hides, or skins, (4) milk and milk products, and (5) manure. Therefore, the purpose of improving livestock system would be to meet more output or more diversified outputs for sale. In the widest sense, the goal of improvement is set both by farmers' attitude and by the policies and strategies of national or local authority, which may encourage or discourage changes.

In the areas where availability of feed and other resources vary depending on the seasons, farmers often arrange a strategic management. A yearly strategy involves matching the cycles of animal production to the changing availability of the sources of nutrients and market demand of the produces overtime. The results must be consistent with the objective of a cattle owner and the levels of nutritional support required to achieve the objective. The feed year strategic model is thus a tool for recognizing the best combination from available feeds to support a shift toward specified achievable animal production goals.

The prices of grains in developing countries have remained unchanged during the last few decades except a global price hike in 2007–08, although the costs of labor, fertilizer, and pesticides have been gradually increasing. This means, farmers are not getting their proper margin from cultivating crops. However, production of biofuel from grain is likely to change the price. There is a scope for using land for cultivating cattle feed and increasing the amount of animal protein for human consumption. This will be a big challenge and requires strong national political commitment and changes in consumers' attitudes toward consuming more food of animal origin. Political commitment will be required on the issue of importing or producing milk locally. Cattle rearing can be more environmental friendly and a good alternative to chemical agriculture.

Bangladesh, India, Pakistan, and Sri Lanka experienced a growth of 1.2, 3.8, 3.6, and 0.6%, respectively, in total national milk production from 1995 to 2005, and all developing Asian countries have been experiencing rapid growth in per capita income since 1980. Regionally, real GDP/capita rose from $1210 to $3355 from 1980 to 2001, leading to an overall annual growth rate of 5.0%. The regional population has also expanded at a rate of 1.7% per year. It seems clear that the region will need a large increase in milk production to keep pace with the projected increasing populations and per capita consumption of milk products.

Consumption of milk and dairy products has expanded dramatically with income growth, population growth, urbanization, and dietary changes. Income growth, population growth, and urbanization have contributed 60% or

Table 2 The projection of population (million inhabitants) and per caput consumption of milk and beef (kg per head per year) in developing Asia

		Year							
Regions	*Parameters*	*1989–91*	*2000*	*2010*	*2025*	*2050*	*2075*	*2100*	
East Asia	Population	360	419	472	540	625	659	696	
	Beef	3	3	4	5	7	9	11	
	Milk	12	13	14	21	30	40	48	
China and Central Asia	Population	1259	1413	1541	1706	1831	1863	1896	
		Beef	2	2	3	5	7	8	9
		Milk	5	6	7	12	15	18	20
Southeast Asia	Population	1159	1414	1676	2011	2436	2570	2712	
	Beef	3	3	3	2	2	2	1	
	Milk	54	58	61	85	123	137	137	
Near East Asia	Population	195	215	326	428	567	642	727	
	Beef	5	6	6	7	8	9	11	
	Milk	83	75	67	71	77	84	91	

From Bouwman AF (1997) Long-term scenarios of livestock–crop–land use interactions in developing countries. *FAO Land and Water Bulletin 6*. Rome, Italy: Food and Agriculture Organization of the United Nations.

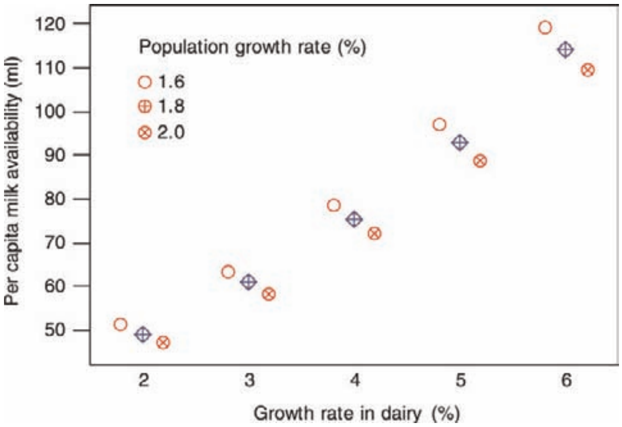

Figure 2 Estimated per capita milk availability in 2025 at different growth rates of milk production and population.

more to increased dairy consumption in Asia. It is expected that in 2030, meat, milk, and dairy product consumption in South Asian countries will be threefold the consumption in 1965. This trend will likely continue because most South Asian countries are projecting increases in national GDP. Taking a modest population growth rate of 1.6% per year and per capita daily milk consumption of 120 ml, 9.1 million tonnes of milk will be required in the year 2025 in Bangladesh (**Figure 2**). The total yearly requirement will be 19.0 million tonnes if per capita daily milk consumption is raised to 250 ml by 2025 in Bangladesh.

Indigenous versus Exotic Breeds

The native cattle of developing Asia are low producing and are poor converters of feedstuff to animal protein for human consumption. On the other hand, native cattle are more adaptable to the harsh environmental conditions, particularly they are tolerant to heat stress and high humidity. India is rich in native cattle, which is reflected in at least 26 recognized breeds of cattle. India has some of the world's best tropical dairy breeds and draft cattle. The world-famous dual-purpose Sahiwal cattle come from India and Pakistan.

Efforts have been made to improve the productivity and feed consumption ability of native cattle by crossbreeding with the European cattle. Such projects have not been successful in most cases because of poor heat tolerance and high susceptibility of crossbred cattle to diseases. Consequently, the targeted productivity has not been realized in most cases. An ideal cow for developing Asia would have been the one that produces more and also tolerates heat and resists diseases. Future development of animal biotechnology will perhaps lead to the solution of such problems. Nonetheless, individual productivity of cattle has increased, indicated by a greater increase in dairy products than in cattle number during the recent years.

Indigenous cattle in South Asia have low productivity with an average milk yield of only about 3 kg per day. Data show that milk production can be substantially increased by feeding indigenous dairy cattle well under the traditional system of husbandry with small quantities of feed concentrates and by providing better veterinary care. To obtain the desired level of production from crossbred cattle, a year-round supply of good-quality green fodder is essential, which is often difficult in many developing Asian countries.

In general, higher-yielding European dairy cattle (*Bos taurus*) are more sensitive to thermal stress than lower-producing *Bos indicus* cattle. This is perhaps due to increased body heat load associated with increased heat production in cattle with high milk production. Vietnamese data show that the milk yield per lactation of pure Holstein-Friesian (HF) cattle was 1900 kg at a temperature of 25 °C. However, HF cattle produced 3000–3200 kg milk when the average temperature was 18.2 °C. A different study showed that HF cows adapt well in areas where the average temperature is below 21 °C. With regard to beef cattle, under hot tropical conditions, the Zebu gained more weight than did the Hereford.

The most common cattle breeds of the Indian subcontinent are Sahiwal, Red Sindhi, Tharparkar, Gir, Ongole, Mewati, Hariana, and Siri. In most developing Asian countries, crossbreeding programs have long been used. The performance of crossbred cattle (*B. taurus* × *B. indicus*) in the tropical and subtropical conditions of developing Asia is not without questions. There are reports mentioning that, provided adequate nutrition and disease control programs are in place, crossbreeding offers the potential to improve dairy production significantly in tropical countries. F1 cows (native cows × HF bulls) produced 1329–2375 kg milk per lactation. A different study showed that milk yield per lactation period of 300 days of pure HF, F1 (50% HF), and F2 (75% HF) was 4100, 2640, and 3250 kg, respectively.

Numerous programs have been initiated to improve cattle production in developing countries of Asia through introduction of temperate genotypes. These programs were often not successful. Cattle production involves a complex interaction between genotype, environment, and management, with genotype dictating the potential to produce, the environment governing how much of this potential can be realized, and management influencing both these components. A long-term strategy for the continued development of production would be to select cattle that can adapt and be reasonably productive through a wide range of environmental conditions.

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DAIRY SCIENCE SOCIETIES, AND ASSOCIATIONS

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Introduction

Science, defined as the body of experimental, theoretical, and practical knowledge of the natural world, dates from prehistoric times and was well established in ancient Egypt, Greece, and Rome. However, the word scientist was not introduced until the nineteenth century (by William Whewell); previously, people who investigated Nature called themselves 'natural philosophers'. Learned societies established to promote natural philosophy date from the fifteenth century; examples of old societies include the Polish Sodalitas Litterarum Vistulana (1488), Italian Accademia dei Lincei (1603), Académie Française (1635), German Academy of Sciences Leopoldina (1652), and the Royal Society, London (formed in 1660 from one or two older, informal societies).

Like other areas of science and technology, dairy science and technology is served by several professional societies and associations, established to promote the advancement of all aspects of dairying, ranging from the supporting basic sciences to economics. The total network of structures is more complex than that for pure sciences because, unlike the latter, dairy science is the resultant of several more basic sciences, for example, chemistry, biochemistry, microbiology, nutrition, physiology, and genetics. Dairy science comprises two principal areas, dairy husbandry and dairy technology, which are clearly interdependent but, to a large extent, operate independently. Dairy husbandry involves the production of milk at the farm level, whereas dairy technology involves the conversion of milk to stable, wholesome, and sensorially desirable products. The scientific field supporting dairy technology is variously called 'dairy science', 'dairy technology', or 'dairy science and technology', the term which will be used in this article. Dairy science and technology involves the study of the chemistry of milk constituents, the control of microorganisms (desirable and undesirable) associated with milk and dairy products, all branches of engineering related to milk processing, and the economics of milk processing, distribution, and retailing.

The earliest studies in dairy science were by basic chemists who isolated and characterized the principal constituents of milk, for example, lactose (Bartolettus, 1663; Schelle, 1780), lactic acid (Schelle, 1780), casein (Berzelius, 1814; Hammersten, 1883), whey proteins (Seblein, 1885), and lipids (Chevreul, 1823), and published their results in chemical journals. An early driving force in food/dairy chemistry was the enactment of food laws, especially the (UK) Sale of Food and Drugs Acts of 1875 and 1879; implementation of these acts created a need for public analysts, who formed the Society of Public Analysts in 1874 and commenced publication of an official journal, *The Analyst*, in 1877. Bacteriology emerged as a discipline at the end of the nineteenth century, through the work of Louis Pasteur and Robert Koch.

Dairy scientists usually continue membership of the society serving their parent discipline; both the American Chemical Society and the Royal Society of Chemistry have an agricultural and food chemistry division and the American Society for Microbiology and the (British) Society for General Microbiology cater to such specialists in food/dairy microbiology. Considering the importance of dairy products in human nutrition in many regions of the world, dairy science and technology is closely linked with the science of nutrition. Clearly, dairy science and technology is a subset of food science and technology, and the institutes of food science and/or technology cater to dairy specialists, perhaps in a dedicated section. Owing to the importance of dairying in agricultural economy and human nutrition, dairy science and technology is linked with agencies of the UN (FAO, WHO) and other international bodies related to agriculture and nutrition.

Most countries with a substantial dairy industry have a national dairy science society/association, entrusted with the charge of promoting the various aspects of dairy science and technology. In this article, the principal societies, organizations, and associations serving dairy science and technology are described briefly. Many of these societies publish technical journals, which are the major conduits for dissemination of pertinent knowledge; in this article, the principal journals devoted to dairy science and technology are listed. For the most part, the societies/associations and the journals described are those with an international impact; in addition, there are numerous societies and journals important locally in their respective countries of origin.

American Dairy Science Association

The American Dairy Science Association (ADSA) is a nonprofit professional organization for the advancement of dairy science, with its headquarters at Savoy, Illinois.

The ADSA has ~4500 members, involved in research, education, and industry; it primarily serves the United States and Canada, but has members in most dairying countries and can be considered as the most senior society for dairy science and technology. The ADSA caters to both aspects of the dairy industry, namely, dairy husbandry and dairy technology. Its objectives include care and nutrition of dairy animals; management and economics of dairy farms and product manufacture; sanitation throughout the dairy industry; and processing of dairy-based foods.

Together with the American Society of Animal Science and the Poultry Science Association, the ADSA forms the Federation of Animal Science Societies. The principal activities of the ADSA are publication of the *Journal of Dairy Science*, organization of an annual meeting, scientific liaisons with other relevant organizations and agencies.

The ADSA emerged from a meeting at the Graduate School of Agriculture held at Ohio State University in the summer of 1905. Professor W.J. Fraser of the University of Illinois suggested the formation of a permanent Dairy Instructors and Investigators Association, and at an inaugural meeting on 17 July 1906, the National Association of Dairy Instructors and Investigators was established. At that time, dairy schools existed at Cornell, Iowa State, Wisconsin, Purdue, Pennsylvania State, Ohio State, Missouri, Minnesota, Illinois, and Guelph. At the second meeting on 11 October 1907, the name of the organization was changed to 'Official Dairy Instructors Association', and at the 10th annual meeting at Amherst, MA, on 17 October 1916, the name was changed to its current form.

The scientific journal of the ADSA is the *Journal of Dairy Science* (*JDS*); volume 1, issue No.1, appeared on 1 May 1917. Initially published bimonthly, *JDS* began monthly publication in 1934 and remains so till today. *JDS* is among the top five most-cited scientific journals in the agriculture category. By 1945, ADSA had 1407 members, which by 1985 had increased to ~3000 in 50 countries. One of the principal activities of the ADSA is its annual meeting, which from 1927 to 1997 was held on university campuses, but since 1998 has been held in convention centers, jointly with the American Society of Animal Science (ASAS).

To mark the 50th anniversary of the ADSA in 1956, volume 39, issue No. 6 of the *Journal of Dairy Science* was devoted to a set of articles describing the advances that had been made in dairy cattle husbandry, dairy chemistry, dairy bacteriology, and the technology of the principal dairy products during the previous 50 years; this is a very valuable source of historical information on dairy science and technology. Among the interesting articles is a compilation by Joseph Tobias of the journals and papers published in the United States at various times during the period 1880–1953; nearly 70 journals dealing with a wide range of topics of interest to dairy scientists, many of them commodity-specific, were published in 1953.

The centenary of the foundation of the ADSA in 2006 was also marked by a special issue of the *Journal of Dairy Science*, volume 89, issue No. 4 (2006).

Society of Dairy Technology (UK)

Founded in 1943, the Society of Dairy Technology (SDT) is dedicated to the advancement of dairy science and technology to the mutual benefit of milk producers, dairy processors, food retailers, and consumers. The SDT operates principally in the United Kingdom and the Republic of Ireland, but has members from other European countries and further afield. The history and highlights of the SDT are described in commemorative booklets published by the society to mark the 50th and 60th anniversaries in 1993 and 2003, respectively.

The SDT, in conjunction with Blackwell Publishing, publishes the *International Journal of Dairy Technology* (previously the *Journal of the Society of Dairy Technology*) and a series of technological monographs, which have been revised recently. It also organizes formal meetings, symposia, and conferences on scientific/technological matters related to the dairy industry and facilitates technological training and education in appropriate subjects. The SDT is an active member of the International Dairy Federation.

The society recognizes the work of its members in promoting the advancement of dairy technology and best practice in the industry by promoting prizes and awards to scientists and technologists working in the dairy field.

Genootschap ter Bevordering van Melkkunde (Netherlands Association for the Advancement of Dairy Science)

This society, which was established in 1908, has the objectives of improving knowledge of milk and dairy products and applications derived therefrom as well as facilitating contacts between people working in the dairy sector in Holland. At present, there are ~300 members, mainly in the Netherlands and Belgium.

This society published the *Netherlands Milk and Dairy Journal* (first published in 1947), which was merged with the *International Dairy Journal* in 1997. A special commemorative issue of the *Netherlands Milk and Dairy Journal* was published in 1996 to mark the 50th anniversary of its publication, and to mark the centenary of the Association in 2008, a special issue (volume 18, issue

No. 5) of the *International Dairy Journal*, in which all articles were contributed by Dutch or Belgian authors, was published.

Gesellschaft für Milchwissenschaft (Society of Milk Science)

This is the principal dairy science society in Germany; it is based at Kiel and coordinates dairy and milk science in Germany, and has members from Austria and Switzerland. This society organizes a scientific event called 'Milchkonferenz' every second year, which was most recently held at Vienna in September 2009. The current president is Prof. Wolfgang Kneifel from the Universität für Bodenkultur, Vienna.

The scientific journal *Milchwissenschaft* (Dairy Science International) is also based at Kiel, and its current editor is Prof. Heeschen. *Milchwissenschaft*, which is published by Volkwirtschaftlicher Verlag, is independent of the Gesellschaft für Milchwissenschaft, but has a certain historical relationship. The Gesellschaft uses *Milchwissenschaft* as its organ of communication.

Three other, more industry-orientated journals, are published in the German language and are intended to communicate science to the industry via short reports and technical articles. These are *Deutsche Milchwirtschaft*, *Deutsche Molkereizeitung*, and *Molkereiindustrie*, which appear biweekly, monthly, and bimonthly, respectively.

Association Laitiere Francaise

Although not a scientific society *sensu, stricto* the Association Laitiere Francaise (ALF) serves many important organizational functions on behalf of the French dairy industry. It is comprised of the Centre National Interprofessionnel de l'Economie Laitière, the Fédération Nationale des Coopératives Laitères, the Federation Nationale des Industries Laitères, and the Federation Nationale des Producteurs de Lait. The AFL participates in the activities of the Federation International de Laiterie (International Dairy Federation), for example, in working groups, publications, symposia, and congresses. It is a member of the Codex Alimentarius of the FAO.

One of the leading journals on the subject is *Dairy Science and Technology* (previously *Le Lait*), first published in 1921 and now at volume 89 (2009), which is edited by the staff of the Institut National de la Recherche Agrononique and is now published in English by EDP Sciences, SA.

Danmarks Mejeritekniske Selsab (Danish Society of Dairy Technology)

This society was founded in 1942 with the objective of promoting information and technical research in the dairy field and furthering the application of the results to benefit the Danish dairy industry. The society arranges meetings, lectures, and seminars, and cooperates with similar national and foreign organizations, associations, and experts. It also organizes field trips and study tours, both within Denmark and abroad.

Swiss Dairy Science Societies

In Switzerland, there are four associations involved in the dairy field:

1. Gesellschaft für Milchwissenschaft (The Society for Dairy Science in Germany, Switzerland, and Austria; see above).
2. Schweizerischer Milchwirtschaftlicher Verein, SMV-SSIL (The Swiss Dairy Association), which is mainly responsible for the organization and quality development of vocational dairy education.
3. Vereinigung Schweizer Milchindustrie, VMI (The Association of the Swiss Milk Industry), which consists of bigger dairy companies. This association compiles statistics on various aspects of the Swiss dairy industry, is an important contact with the government and the parliament, and commissions research.
4. Fromarte – Die Schweizer Kaesespezialisten (The Swiss Cheese Specialists). This is an association of about 600 village cheese factories in Switzerland. It is politically important, compiles statistics, and reports on the cheese market in French and German.

Indian Dairy Science Association

Scientists working at the Indian Dairy Research Institute (IDRI), Bangalore, under the leadership of the then director, Dr. K. C. Sen, decided in 1947 to establish the Indian Dairy Science Association (IDSA). The first general meeting of the Association was held on 15 May 1948 at IDRI, Bangalore. Membership of the Association is open to all persons engaged in teaching or research, or in advisory roles. It has undertaken the publication of a quarterly journal, *Indian Journal of Dairy Science*, devoted to the advancement of dairy science.

The headquarters of the IDRI were moved to Karnal in 1955, but the IDSA remained at Bangalore until 1968, when it was moved to Delhi. In 1976, the Association was renamed the Indian Dairy Association (IDA), with headquarters at Delhi.

Egyptian Society of Dairy Science

This society was established in 1972 with the objective of advancing dairy science and technology in Egypt. The society is open to national and international membership, which may be active or honorary; there were 270 members in 2009. The principal activities are (1) publication of the *Egyptian Journal of Dairy Science* (biannually), (2) organizing a triennial conference (the 11th to be held in 2010), (3) organizing seminars on selected topics of interest to the academia or industry, and (4) presentation of awards to young scientists.

South African Society of Dairy Technology

The interests of dairy scientists in South Africa are met by a number of societies, especially (1) The South African Society of Dairy Technology, which has four branches, which meet four times annually and organize an Annual National Congress; this society, which is affiliated with the International Dairy Federation, does not publish a journal but its activities are reported in the *Dairy Mail*; (2) The South African Society of Animal Science (which publishes the *South African Journal of Animal Science*); and (3) The South African Association of Food Science and Technology, which focuses on the food industry in general, with little specific interest in dairy science; it publishes the *Journal of Food Technology in Africa*. In addition, there are three industry-focused organizations: (1) The South African Milk Producers Organization; (2) Milk South Africa, the objective of which is to promote the image and consumption of dairy products and provide various services, including literature searches; and (3) Agritech South Africa, which offers various services to agriculturalists, including publication of *Dairy Mail*.

Dairy Industry Association of Australia

The Australian Society of Dairy Technology (ASDT), which was founded in 1946, merged with the Australian Dairy Institute Inc. in 1986 to form the Dairy Industry Association of Australia (DIAA). The ASDT started publication of the *Australian Journal of Dairy Technology*, first published in 1952 and now published by DIAA. The DIAA is not linked with the Australian Institute of Food Science and Technology (AIFST), which does not have a dairy division, but many Australian dairy scientists, including members of the DIAA, are also members of the AIFST.

Dairy Industry Association of New Zealand

The Dairy Industry Management Institute of New Zealand (DIMINZ) was formed in 1908 to promote the technical aspects of the dairy industry in NZ; it merged with the NZ Dairy Technology Society in 1969 to form the Dairy Industry Association of NZ (DIANZ). In 2004, the DIANZ merged with the NZ Institute of Food Science and Technology, which has a dairy division.

The DIANZ published the *NZ Journal of Dairy Science and Technology* during the period 1966–88. The NZIFST publishes a technical journal, *Food New Zealand*, which contains some material relevant to the dairy industry.

Dairy Science Societies and Associations in Japan

Dairy scientists in Japan are served by the Japanese Dairy Science Association, the aims of which are to contribute to dairy farming in Japan, to promote the science and technology of dairy products, and to develop the dairy industry. The Association, which has about 200 members, publishes the quarterly journal *Milk Science* (formerly the *Japanese Journal of Dairy and Food Science*) and organizes an annual symposium.

Many Japanese dairy scientists belong to the Japanese Society of Animal Science, which has a dairy science section. This Society, which has about 2000 members, publishes the *Animal Science Journal* in English (*Nihon Chikusan Gakkaiho* in Japanese). A national conference is held annually.

South America

In South America, most dairy societies consist of partnerships of milk producers and consortia of dairy industries.

In Argentina, Centro de la Industria Lechera (CIL), established in 1919, consists of dairy industries, dairy product traders, and milk producers. The objectives of the center are to obtain equal and fair laws for the promotion of the dairy sector, to improve scientific and technical skills of professionals and workers in the dairy sector, to disseminate knowledge related to the field, and to encourage the production and trade of dairy products. The Society publishes, since 1919, the journal *Industria Lechera*. Another scientific journal on dairy science and technology in Argentina is *Revista Argentina de Lactología*, published by the Instituto Argentino de Lactología (INLAIN). A third Argentinan dairy science journal, *Tecnología Láctea Latinoamericana*, is produced by a private publisher.

In Brazil, the Associação Brasileira dos Produtores de Leite, established in 1997, has the following objectives: to improve milk quality, to increase milk consumption, to carry out marketing of dairy products, to represent milk producers, and to protect the Brazilian market. It publishes the technical journal *Leite Brasil.*

In Uruguay, which has a strong dairying tradition, the Asociación Nacional de Productores de Leche (ANPL), composed mainly of farmers, is 70 years old. The ANPL publishes a journal, *El Tambo* (The Dairy Farm), related to primary milk production. CONAPROLE is a cooperative that has processed the maximum quantity of Uruguayan milk since 1936.

The Pan American Dairy Federation (FEPALE), formed in 1991, collates enterprises and institutions, both public and private, from the dairy sector in the Americas and the world. It works mainly on integration and coordination, dairy sector development, scientific events and business meetings, communication, education, increase in dairy consumption, and dairy policies and regulation.

European Dairy Association

The European Dairy Association (EDA) is the association of European dairy industries working toward their common interests in liaison with bodies such as the EU institutions (the Commission, Parliament, Council of Ministers, Economic and Social Committee, etc.) and international bodies (the Codex Alimentarius, the World Trade Organization, etc.). The EDA gathers together the dairy industry organizations of 22 EU member states. It is responsible for issues related to trade policy, legislation, and food safety, as well as matters of cooperation, and the supervision of interests in nutrition policy.

The EDA is the platform for the European dairy industry to work together on important issues like policy development on nutrition and health, food safety, sustainability, and market management as part of the EU Common Agricultural Policy.

Under the slogan 'Milk – Drink it up', the EU Commission has launched an EU school milk information campaign, which aims to increase awareness of the benefits of milk consumption and the availability of EU funds to schools. As part of this campaign, young people are invited to participate in a photography competition on the theme 'Milk Power'.

Federacion Panamericana de Lecheria

This Federation serves in Latin America a function similar to that of the EDA in Europe. It represents the interests of the dairy industry in Guatemala, Chile, Honduras, Mexico, Uruguay, Argentina, and Brazil, with headquarters in Montevideo, Uruguay. At least some of these countries also have national dairy associations.

Spain and Italy

These two countries have large sophisticated dairy industries, especially the cheese sector, and both have institutes active in dairy research. However, neither has a dedicated society for dairy science or technology. In Spain, a privately published journal, *Industrias Lácteas Españolas*, serves the dairy industry, aspects of which are also served by more general scientific societies, for example, Asociación Española de Licenciados y Doctores en Ciencia y Tecnología de los Alimentos (ALCYTA), Sociedad Española de Microbiología (which has a food microbiology group), and the Fundación Española de Ciencia y Tecnología.

In Italy, the National Dairy Research Centre (Centro Sperimentale de Latte, Lodi) publishes a dedicated journal *L'Industria del Latte.* The Italian Society of Food Science and Technology, based at the University of Perugia, serves some needs of the Italian dairy industry and publishes the *Italian Journal of Food Science.*

Canada

Canada is part of the home territory of the ADSA; in fact Guelph University was one of the founder members of the ADSA. The Canadian Institute of Food Science and Technology (CIFST), which is generally similar to the IFT, previously had a dairy technology interest group, which was quite active in the past, but is not so active now. The CIFST produced the *Journal of the Canadian Institute of Food Science and Technology* (JCIFST), which was metamorphosed into Elsevier's journal, *Food Research International*, which typically has a Canadian as the editor-in-chief. The *JDS* and other international journals also serve the needs of Canadian dairy scientists.

The Dairy Farmers of Canada (DFC) is devoted to lobbying for the dairy sector; it is the main nongovernmental organization representing the dairy industry. The DFC has two grant distribution programs for the support of research – one focused on production, the other on nutrition. The primary objective of these research grants is to promote the consumption of dairy products through scientifically supported positive facts. The Canadian National Committee of the IDF selects Canadian representatives for the various IDF action teams, committees, and dairy summits, and it is funded by the Government of Canada and the DFC.

International Dairy Federation

The International Dairy Federation (IDF) is an international organization established in 1903 to facilitate and promote the interchange of experience and ideas among experts on all aspects of dairy science and technology, nutrition, animal diseases, engineering, and economics. In 2008, the IDF had 53 member countries, which account for ~82% of the current total milk production worldwide.

The mission of the IDF is to represent the dairy sector worldwide by providing the best global sources of scientific expertise and knowledge in support of the development and promotion of quality milk and dairy products to deliver nutrition, health, and well-being to consumers. The IDF is a center for developing scientific knowledge, exchanging information, addressing global issues and developments, and facilitating networking within and outside the sector.

Each member country of the IDF is represented by a national committee, formed according to its own preferences. The national committees meet annually in a General Assembly, which is the supreme authority and which ratifies a board of directors, a science and coordination committee, and task forces to perform specific tasks. The chief executive is the Director General, who is based at the Federation's headquarters in Brussels.

The IDF provides a permanent source of authoritative scientific and technical information on a wide range of topics relevant to the dairy industry in the form of news briefs, newsletters, review papers, bulletins, special issues, best practices, guidelines, statistical digests, and standard methods for sampling and analysis. It also disseminates latest knowledge and information through various international events and via its public website. A network of more than 1200 experts covers a wide range of areas, including nutrition and health, food standards, methods of sampling and analysis, animal health and welfare, food safety and hygiene, farm management, environmental matters, dairy science and technology, dairy policies, and economics and marketing.

The IDF is a partner in many influential global organizations that influence the dairy sector, such as Food and Agriculture Organization of the United Nations (FAO); World Organization for Animal Health (OIE); World Health Organization (WHO); International Organization for Standardization (ISO); Association of Official Analytical Chemists (AOAC); and Codex Alimentarius Commission, in particular the Codex Committee on Milk and Milk Products.

The IDF promotes exchange of new ideas with fellow specialists across national borders, helps resolve issues, and spreads best practice. Its working groups provide experts with a platform to meet and jointly develop scientific knowledge and applications for industrial practice. It organizes an annual world dairy summit and a number of high-level international symposia, seminars, and workshops, which contribute to the progress and understanding of dairy issues worldwide and provide excellent opportunities for networking with peers, sharing experiences, and establishing contacts for future use. Commencing in 1906, the IDF has sponsored an international dairy congress every fourth year; these have served as opportunities to review the status of various areas of dairy science and technology.

Major contributions to various aspects of the dairy industry are recognized through a number of awards: the IDF award, the annual marketing award, and the Elie Metchnikoff prize.

Other Relevant Societies

Although not devoted primarily to the dairy industry, there are several societies that have a major impact on dairy science and technology, some via a dedicated section. The following are, perhaps, the principal among such societies.

The Institute of Food Technology (IFT), probably the oldest society for general food science and technology (established in 1939), is based in the United States and Canada but has members throughout the world (total membership, ~40 000); it has a strong role in dairy science and technology. The corresponding British institute is the Institute of Food Science and Technology, which has a lesser impact on dairy science and technology than the IFT. Many other countries have national institutes of food science and/or technology that have some impact on the local and perhaps the international dairy industry. As dairy science had evolved from chemistry, it is not surprising that chemical and biochemical societies play significant roles in the chemical aspects of dairy science, especially the American Chemical Society and the Royal Society for Chemistry. Microbiological societies generally are important in certain aspects of dairy science, especially the Society for General Microbiology, the Society for Applied Microbiology, and the societies related to public health. The British Association for Animal Science is active in dairy cattle husbandry and to a lesser extent in other aspects of dairy technology. Nutrition is an increasingly important aspect of dairy science, and the British Society for Nutrition and the American Society of Nutrition are particularly important.

Dairy Science Journals

For at least the past 200 years, scientists have disseminated the results of their studies through journals published initially by the scientists themselves and later by professional societies, which is the usual format today. Early scientists working on dairy subjects published their results in contemporary general journals. Agricultural experiment stations, usually associated with a state university, were established in the United States around 1870, and the US researchers in dairy science and technology published the results of their research in the bulletins (dating back to 1881) of the agricultural experiment stations in the principal dairying states (New York, Wisconsin, Vermont, Iowa, etc.). There were no similar publications in Europe, although Annales d'Institut Pasteur, established in 1887, served a similar function.

Today, there are a number of dedicated journals, of which the following are probably the most significant: *Journal of Dairy Science*, *International Dairy Journal*, *Journal of Dairy Research*, *International Journal of Dairy Technology*, *Dairy Science and Technology* (*formerly Le Lait*), *Milchwissenschaft*, *Lebensmittel Wissenschaft und Technologie*, *Australian Journal of Dairy Technology*, *New Zealand Journal of Dairy Technology* (1966–88), *Netherlands Milk and Dairy Journal* (1947–96; merged with the *International Dairy Journal* in 1997), and the *Egyptian Journal of Dairy Science.* There is a dedicated abstracting journal, *Dairy Science Abstracts*, as well as *Food Science & Technology Abstracts*, *Chemical Abstracts*, and *Biological Abstracts*, all of which are available online.

In addition to these dedicated journals, dairy scientists regularly publish in general food science journals, for example, *Journal of Food Science*, *Journal of Agricultural and Food Chemistry*, *Food Chemistry*, *Journal of Food Biochemistry, Hydrocolloids, Colloid and Interface Science*, *Journal of Nutrition*, *British Journal of Nutrition*, and *American Journal of Clinical Nutrition*, or in more basic journals, for example, *Biochimica et Biophysica Acta*, *Archives of Biochemistry and Biophysics*, *Analytical Biochemistry*, *Analytical Chemistry*, *Lipids*, *Journal of Chromatography*, *Applied and Environmental Microbiology*, *Applied Bacteriology*, *Journal of the Association of Analytical Chemists*, and *Process Biochemistry.*

Summary

This article was compiled to serve as a record of the principal societies and associations devoted to dairy science and technology. In addition to those recorded here, there are many others serving local scientific communities. It is hoped that the article will direct interested readers to potential contacts and sources of technical information.

DEHYDRATED DAIRY PRODUCTS

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Milk Powder: Types and Manufacture
Milk Powder: Physical and Functional Properties of Milk Powders
Dairy Ingredients in Non-Dairy Foods
Infant Formulae

Milk Powder: Types and Manufacture

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Introduction

The purpose of dehydration of milk is to stabilize milk constituents for their storage and later use. In the early 1970s, the industrial application of concentration and fractionation by membrane processes (e.g., microfiltration, ultrafiltration, nanofiltration, and reverse osmosis), electrodialysis or ion exchange, provided opportunities and versatility to drying of milk, milk components, and products. Dry milk products currently include milk powder, skim milk powder, whey powder, various whey protein powders, dry dairy-based beverages, casein, caseinates, coprecipitates, infant formula and cheese products, lactose, coffee whiteners, and dry ice cream mixes. The dairy industry has developed new technologies for isolating and purifying proteins (e.g., casein, caseinates, and whey proteins) such as milk protein concentrates (MPCs), milk protein isolates (MPIs), whey protein concentrates (WPCs), whey protein isolates (WPIs), micellar casein concentrates (MCCs), micellar casein isolates (MCIs), whey concentrates, and selectively demineralized whey concentrates. Availability of new membrane separation techniques and improvements in chromatographic resins now provide the dairy technologist with several options for the extraction and purification of almost all of the major milk proteins.

World production of dry dairy products has increased steadily in recent years, due to the main advantages of the powders, which

- retain high quality, without special storage conditions;
- reduce mass and volume compared to fluid products;
- provide balance between milk supply and consumption;
- provide an irreplaceable food component in hot climate regions;
- are a valuable food reserve for emergencies;
- are suitable for various tailor-made food products.

The most frequently used technique for the dehydration of dairy products is spray drying. It became popular in the dairy industry in the 1970s, but at that time there were few scientific or technical studies on spray drying and, in particular, none on the effects of spray drying parameters or on the effects of the physicochemical composition and microbiology of the concentrates on powder quality. Manufacturers acquired expertise in milk drying, and eventually in whey drying processes, through trial and error. Because of the variety and complexity of the mixes to be dried, a more rigorous method based on physicochemical and thermodynamic properties has become necessary. Greater understanding of the biochemical properties of milk products before drying, water transfer during spray drying, and the properties of powders and influencing factors is now essential for the production of milk powder. The lack of technical and economic information and the lack of understanding of scientific methods prevent the manufacturer from optimizing his plant in terms of energy costs and powder quality.

The aim of this article is to provide information on two areas, that is, the principles of spray drying and the manufacture of milk powders.

Principles of Spray Drying

Drying is defined as the removal of a liquid (usually water) from a product by evaporation, leaving the solids in an essentially dry state. A number of different drying processes are in use in the dairy, food, chemical, and pharmaceutical industries, such as spray drying, fluid bed drying, roller drying, freeze drying, microwave drying, and superheated steam drying.

In special circumstances, roller drying (**Figure 1**) is used for the production of milk powder for particular applications, for example, confectionery and animal feeds. Direct contact of concentrated milk with rotating steam-heated rollers adversely affects the components of milk, especially proteins and lactose. Some reactions such as protein denaturation, Maillard reactions, and lactose caramelization are irreversible.

Due to considerations related to drying economics and final product quality, the processes of greatest significance for milk and dairy powder manufacture are spray drying and fluid bed drying (most often in combination). Only these two combined drying processes will be discussed in this section.

The basic principle of spray drying is the exposure of a fine dispersion of droplets created by means of atomization of preconcentrated milk products over a hot airstream in a drying chamber. Spray drying as an industrial process for the dehydration of a liquid by transforming the liquid into a spray of small droplets and exposing these droplets to a flow of hot air (Pisecky, 1997). The very large surface area of the spray droplets causes water evaporation to take place very quickly, converting the droplets into dry powder particles. The small droplet size created, and hence the large total surface area, results in very rapid evaporation of water at a relatively low temperature, thus minimizing heat damage to the product.

When a wet droplet is exposed to hot dry gas, variations in the temperature and the partial pressure of the water vapor are spontaneously established between the droplet and the air:

- heat transfer from the air to the droplet occurs under the influence of the temperature gradient;
- water transfer occurs in the opposite direction, explained by variation in the partial pressure of water vapor between the air and the droplet surface.

The drying kinetics are related to three factors:

- the evaporation surface,
- the difference in the partial pressure of water vapor between the particle and the drying air,
- the rate of water migration from the center of the particle toward its surface.

The main components of a spray drying installation are as follows (**Figure 2**):

- Drying chamber (**Figure 2**, position 7).
- Air filtration (**Figure 2**, position 17).
- Air heating system. The drying air can be heated in different ways: either indirectly by steam, oil, or gas, or directly by gas or electricity (**Figure 2**, position 5).
- Air distribution. The most common system is where the air disperser is situated on top of the dryer ceiling and the atomizing device is placed in the middle of the air disperser, thus ensuring optimal mixing of the air and the atomized droplets.
- An atomizing device. The main functions of atomization are
  - – to produce a high surface-to-mass ratio, resulting in a high evaporation rate,
  - – to produce particles of the desired shape, size, and density.

There are three types of atomizing devices: nozzle atomizer (pressure, pneumatic, or sonic), rotary atomizer (wheel or disc), and combined atomizer (rotary and pneumatic) (**Figure 2**, position 3).

- Powder recovery system (**Figure 2**, positions 8 and 9). Separation of the dried product can be achieved by a primary discharge from the drying chamber, followed by a secondary discharge from a particle collector (using a cyclone, bag filter, or electrostatic precipitation), followed by total discharge from the particle collector and finishing with final cleaning of the exhaust air in a wet scrubber and dry filter.

The main advantages of spray drying over other drying techniques are

- the process is rapid, residence time in the chamber being less than 30 s;
- the product has a fine structure and excellent properties, with no adverse effects of heat (as for freeze

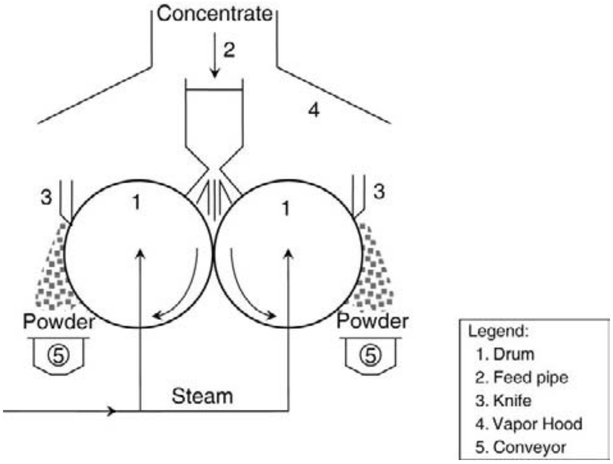

Figure 1 Roller dryer.

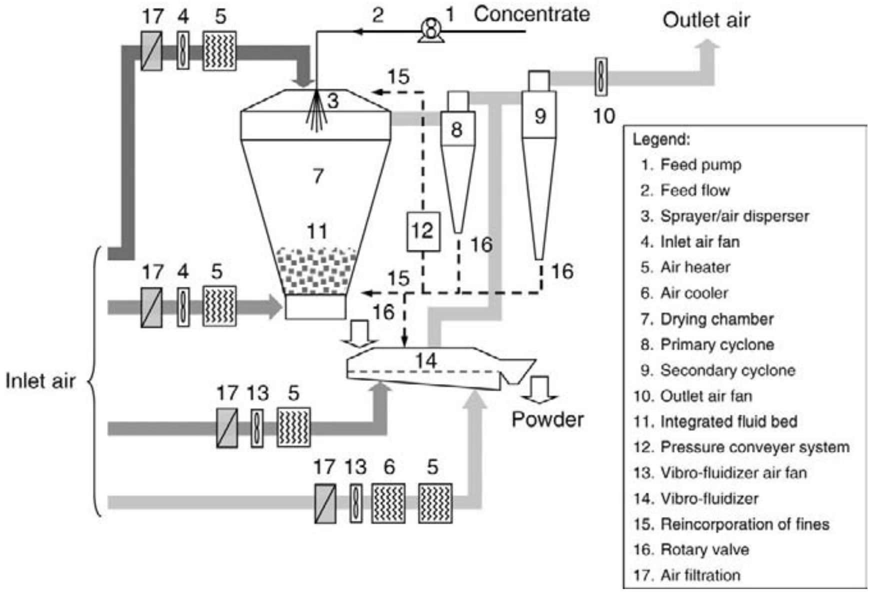
17 4 5
2
1 Concentrate
Outlet air
15
3
8
9
10
7
11
12
16
16
17 4 5
15
16
14
Inlet air
17 13 5
Powder
17 13 6 5
Legend:
1. Feed pump
2. Feed flow
3. Sprayer/air disperser
4. Inlet air fan
5. Air heater
6. Air cooler
7. Drying chamber
8. Primary cyclone
9. Secondary cyclone
10. Outlet air fan
11. Integrated fluid bed
12. Pressure conveyer system
13. Vibro-fluidizer air fan
14. Vibro-fluidizer
15. Reincorporation of fines
16. Rotary valve
17. Air filtration

Figure 2 Multiple effect spray dryer.

drying), as drying is accomplished in a very short time and at a low temperature;

- the process is fully automatic with complete control of drying parameters and minimal labor of any type;
- the product comes into contact with the drying chamber wall only in the powder form, so there is neither a problem of equipment maintenance nor a problem with the microbiological quality of the final product.

However, the investment cost for a spray drying plant is high, and is economically justified only for large quantities or for products with high added value.

Single-stage spray dryers are now considered obsolete although many are still in operation. The residence time is not long enough to obtain a real equilibrium between the relative humidity (RH) of the outlet air and water activity of the powder. The outlet temperature of the air must therefore be high, reducing the thermal efficiency of the single-stage spray dryer. The two-stage drying system consists of limiting the spray drying to a process with a longer residence time (several minutes) to provide a better thermodynamic balance. A second final drying stage is necessary to optimize the moisture content by using an integrated fluid bed (static) or an external fluid bed (vibrating) (**Figure 2**, positions 11 and 14). The integrated fluid bed can be either circular (e.g., Multi-Stage Dryer (MSD™) chamber) or annular (e.g., compact dryer (CD) chamber). The three-stage drying systems, with an internal fluid bed as a second stage in combination with an external vibrating fluid bed as a third-stage dryer, first appeared at the beginning of the 1980s and were called compact dryer instantization (CDI) or MSD™. Today, they dominate the dairy powder industry (**Figure 2**). Three-stage systems combine all the advantages of extended two-stage drying, using spray drying as the primary stage, fluid bed drying of a static fluid as the second drying stage, and drying on an external vibrating fluid bed as the third drying stage. Although two- and three-stage drying may produce both nonagglomerated and agglomerated powders, their principal products are instant milk powders, which are discussed later in this article.

Table 1 shows that energy consumption varies according to the drying processes. Energy consumption is 6677, 5362, 4602, and 4020 kJ for a one-stage spray dryer, two-stage spray dryer, and two-stage spray dryer with a vibro-fluidizer, with CD-type static fluid bed or MSD™ type with static fluid bed, respectively, to produce 1 kg of powder from skim milk concentrate containing 48% total solids. The reduction in energy consumption with increased number of drying stages is due to increased product residence time, allowing an increase in the inlet air temperature and increased concentrate flow rate.

Milk Powder

The flow chart of milk powder production, consisting of reception, clarification, cooling, standardization, heat treatment, evaporation, homogenization, drying, and packaging, is shown in **Figure 3**.

Raw full-fat milk used for powder production must be of high chemical, sensory, and bacteriological quality, which is regulated by government standards. After

Table 1 Comparison of one- and two-stage drying systems

		SD			
Drying system	*Unit*	*Single-stage SD*	*SD with VF*	*SD with SFB (Compact)*	*SD with SFB (MSD™)*
SD					
Inlet air temperature	°C	200	230	230	260
Drying air	kg h^{-1}	31 500	31 500	31 500	31 500
Skim milk with 8.5% solids	kg h^{-1}	12 950	19 800	24 000	31 300
Concentrate with 48% solids	kg h^{-1}	2290	3510	4250	5540
Evaporation in chamber	kg h^{-1}	1150	1720	2010	2620
Powder from chamber:					
3.5% moisture	kg h^{-1}	1140			
6.0% moisture	kg h^{-1}		1790		
9.0% moisture	kg h^{-1}			2240	2920
Fuel oil consumption	kg h^{-1}	175	205	205	230
Power consumption	kW	120	130	140	150
Energy consumption:					
Total spray drying	MJ	7612	8876	8918	9965
Energy per kg powder in chamber	kJ	6677	4959	3981	3413
FB			*VF*	*SFB*	*SFB*
Inlet air temperature	°C		100	115	120
Drying air	kg h^{-1}		4290	6750	11 500
Evaporation in VF/SFB	kg h^{-1}		45	125	165
Powder from FB, 3.5% moisture	kg h^{-1}		1745	2115	2755
Steam consumption	kg h^{-1}		167	290	400
Power consumption	kW		20	25	35
Total energy consumption in FB	MJ		481	816	1110
Total drying					
Total energy consumption	MJ	7612	9357	9734	11 075
Total energy per kg powder	kJ	6677	5362	4602	4020
Energy ratio	%	100	80	69	60
Dryer efficiency		0.54	0.66	0.69	0.79

FB, fluid bed; MSD, Multi-Stage-Dryer; SD, spray dryer; SFB, static fluid bed; VF, vibro-fluidizer.

reception, milk is clarified, usually by centrifugal separators, and cooled to 4 °C in plate heat exchangers, followed by storage at the same temperature. The next operation is standardization, which is used to adjust the ratio of milk fat to total solids as required in the final product. Heat treatment is commonly performed using the indirect method in a tubular or plate heat exchanger at 88–95 °C for 15–30 s, the aims being to destroy pathogenic bacteria and most of the saprophytic microorganisms, to inactivate enzymes, especially lipase, and to activate SH groups in β-lactoglobulin, which results in an antioxidative effect. Evaporation is used to concentrate milk prior to drying, and can be combined with reverse osmosis. Evaporation is performed in multiple effect vacuum evaporators with mechanical or thermal steam recompression, where energy consumption is about 10–30 times lower than in spray drying. The differences in the concentration of total solids are determined by the drying technique used: 30–35% total solids for roller drying and 45–50% total solids for spray drying. Concentrating milk prior to drying has a positive effect on milk powder quality: milk powder produced from concentrated milk consists of larger powder particles containing less occluded air, and therefore results in better storage stability. Homogenization is not an obligatory operation, but is usually applied with the aim of reducing the free-fat content in full-fat milk powder, which has a negative effect on powder solubility and its susceptibility to oxidation.

Skim Milk Powder

The procedure for the manufacture of skim milk powder (nonfat dry milk) differs in several features from the process for full-fat milk powder: fat standardization leads to very low fat content in skim milk, that is,

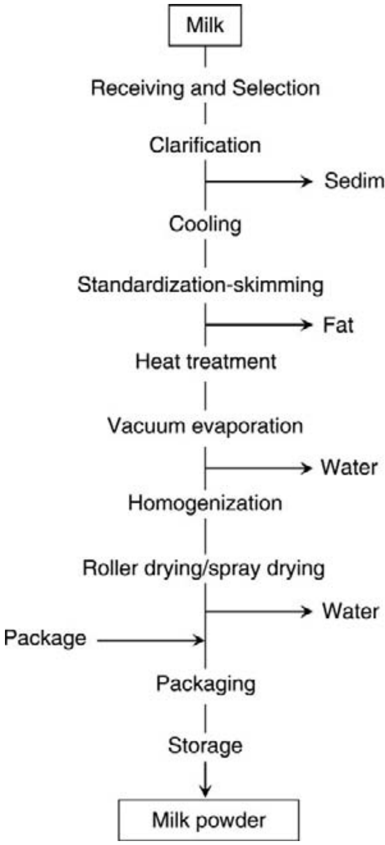
Milk
Receiving and Selection
Clarification
Sediment
Cooling
Standardization-skimming
Fat
Heat treatment
Vacuum evaporation
Water
Homogenization
Roller drying/spray drying
Water
Package
Packaging
Storage
Milk powder

Figure 3 Flow chart for milk powder production.

0.05–0.10%; heat treatment may be more intense compared to whole milk; and no homogenization is required.

The regime of skim milk heat treatment depends on the type of skim milk powder being produced. The essential classification of skim milk powder according to this criterion is shown in **Table 2**. Skim milk powder produced by a 'low-heat method' is only pasteurized, while the 'high-heat method' requires additional heat treatment, for example, 85–88 °C for 15–30 min. Such intensive heat treatment is necessary in the production of skim milk powders intended for use in the bakery industry, where a high degree of protein denaturation (low (undenatured) whey protein nitrogen index (WPNI)) is desired.

Table 2 Heat classification of skim milk powders

Class	*WPNI (mg N g^{-1} powder)*
Ultralow heat	≥9.00
Low heat	6.00–8.99
Medium heat	4.50–5.99
Medium high heat	1.51–4.49
High heat	≤1.50

WPNI, whey protein nitrogen index.

Pisecky J (1986) Standards, specifications and test methods for dry milk products. In: MacCarthy D (ed.) Concentration and Drying of Food, pp. 203–220. London: Elsevier; American Dairy Products Institute (1990) *Standards for Grades of Dry Milk Including Methods of Analysis*. Chicago, IL: American Dairy Products Institute; Schuck P, Piot M, Méjean S, Fauquant J, Brulé G, and Maubois JL (1994a) Déshydratation des laits enrichis en caséine micellaire par microfiltration; comparaison des propriétés des poudres obtenues avec celles d'une poudre de lait ultra-propre. Le Lait 74: 47–63.

Most of the problems in cheesemaking using medium- and high-heat milk powders are due to the cumulative effect of heat treatments applied for purposes of microbiological control and thermal efficiency during concentration and drying.

In 1992, a new process was proposed consisting of the partial removal of β-lactoglobulin from skim milk, followed by low- or medium-heat treatment, leading to production of a new milk powder (deseroproteinised skim milk powder) with improved cheesemaking potential compared to that of low-heat milk powders (Quiblier *et al.*, 1992). The process, patented by INRA, consists of four successive steps (**Figure 4**):

- partial or total removal of whey proteins from the milk by microfiltration performed on membranes with an average pore diameter of 0.1–0.2 μm;
- ultrafiltration of the microfiltration permeate with ultrafiltration membranes having a molecular weight cutoff threshold around 20 kg mol^{-1};
- blending of the microfiltration retentate with the ultrafiltration permeate;

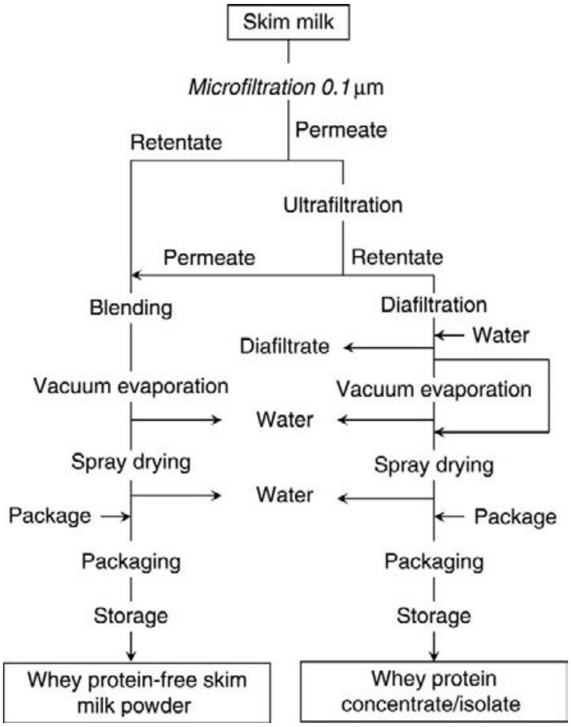
Skim milk
Microfiltration 0.1 μm
Retentate
Permeate
Ultrafiltration
Permeate
Retentate
Blending
Diafiltration
Water
Diafiltrate
Vacuum evaporation
Vacuum evaporation
Water
Spray drying
Spray drying
Water
Package
Package
Packaging
Packaging
Storage
Storage
Whey protein-free skim milk powder
Whey protein concentrate/isolate

Figure 4 Flow chart for whey protein-depleted skim milk powder production.

- vacuum evaporation and spray drying (medium-heat treatment) of the blend.

Mozzarella produced by recombination of the deseroproteinised skim milk powder (**Figure 4**) had the same composition and properties as control Mozzarella (produced with raw fresh milk), but when the deseroproteinised skim milk powder was used, the cheesemaking yields were 7.3 ± 1.8% higher in comparison with the control cheese (Garem *et al.*, 2000). The recovery of total solids, fat, and total nitrogen contents showed similar trends. Such a process may thus give rise to a new generation of milk powders, especially suitable for cheesemaking in countries where there is a shortage of milk.

Figure 5 shows an alternative to heat treatment. Treatment of raw skim milk by the Bactocatch® procedure (microfiltration 1.4 μm) before concentration by vacuum evaporation and spray drying leads to a high-quality milk powder. No heat treatment is required to obtain an ultralow-heat powder (**Table 2**) with a WPNI ≥ 9 mg N g^{-1} powder and with a maximum bacterial count of 3000 cfu g^{-1}. Such a powder has the same renneting time after water reconstitution as the original raw milk and can be used as a reference powder for either industrial or scientific purposes.

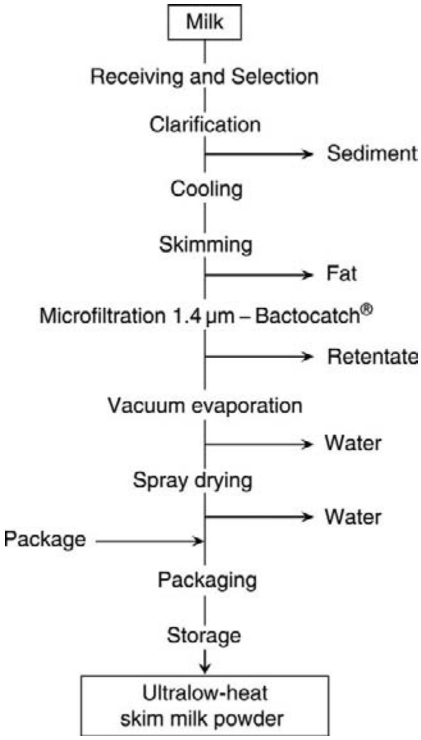
Milk
Receiving and Selection
Clarification
Sediment
Cooling
Skimming
Fat
Microfiltration 1.4 μm – Bactocatch®
Retentate
Vacuum evaporation
Water
Spray drying
Water
Package
Packaging
Storage
Ultralow-heat skim milk powder

Figure 5 Flow chart for ultralow-heat skim milk powder production.

Instantization

Instantization is a drying procedure that produces milk powder with better rehydration properties. By using two- or three-stage drying, this procedure significantly improves the quality and economics of the drying technology. The rehydration properties (e.g., wettability, sinkability, dispersibility, solubility, and rate of dissolution) are enhanced. Instantization is based on agglomeration, which enables a larger volume of air to be incorporated between the powder particles, resulting in a characteristic coarse, cluster-like, agglomerated structure (*see* **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders, **Figure 1(c)**). Air is replaced by water during rehydration and thus no viscous layer is formed around agglomerated particles, which regularly happens when nonagglomerated powders come into contact with water.

The three-stage drying procedure is shown in **Figure 2**, while a flow chart of both instantization procedures, 'rewetting' and 'straight through', is shown in **Figures 6** and 7. In the 'rewetting' procedure (**Figure 6**), instantization is performed after powder is obtained in the dry form, while in the 'straight through' process (**Figure 7**), instantization is performed during drying. Regardless of the techniques chosen, the main feature of instantization is agglomeration, that is, powder particles are wetted by steam, water, or skim milk

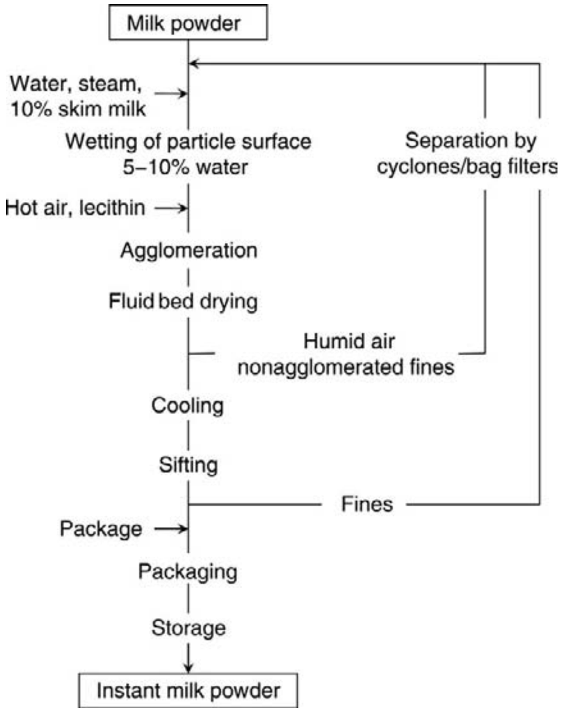
Milk powder
Water, steam, 10% skim milk
Wetting of particle surface 5–10% water
Separation by cyclones/bag filters
Hot air, lecithin
Agglomeration
Fluid bed drying
Humid air nonagglomerated fines
Cooling
Sifting
Fines
Package
Packaging
Storage
Instant milk powder

Figure 6 Flow chart for instantized milk powder production by 'rewetting' procedure.

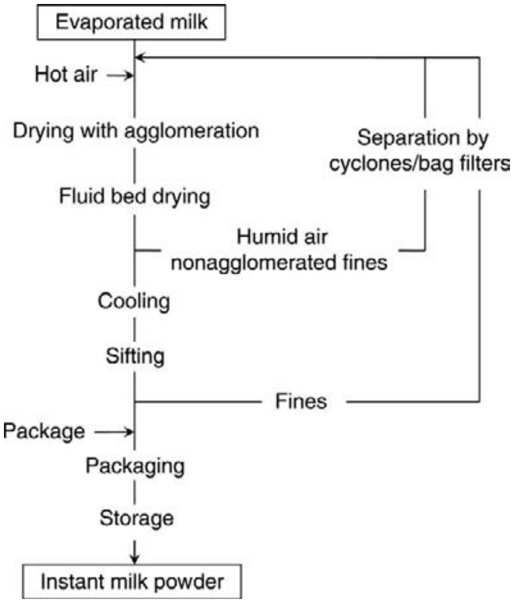

Figure 7 Flow chart for instantized milk powder production by 'straight through' procedure.

('rewetting'), or left wet during first-stage drying ('straight through'), having around 10% water and introduced into a fluid bed dryer (instantizer) (**Figure 2**, position 14) for final drying. The airstream in the instantizer blows the powder upward, thus 'fluidizing' it, through the vibrating, finely perforated plate, redrying the agglomerated product at 90–120 °C to a final moisture content of 3–4%. In addition to better reconstitution properties, improved heat utilization is achieved in multistage drying, as shown in **Table 2**.

A recent development in spray drying has been the three-stage drying technique (**Figure 2**), where even better product quality and thermal efficiency is obtained (specific heat consumption of 4070 kJ kg^{-1} evaporated water for a two-stage dryer compared to 3626 kJ kg^{-1} evaporated water for a three-stage dryer). In order to achieve better heat economy, it is necessary to perform the drying operation with large temperature differences, that is, a high inlet air temperature and a low outlet air temperature. In the two-stage drying system, it is impossible to reduce further the outlet air temperature (below 70–105 °C) in the first drying stage. A lower temperature of the outcoming air would result in a higher moisture content of the powder after the first drying stage, making it difficult to transport humid, sticky powder to the next drying stage, that is, the external fluid bed. The development of an integrated static fluid bed (**Figure 2**, position 11) built into the base of the spray dryer solved this problem. The wet powder (10–18% moisture) falls to the fluidized bed, coming into contact with fines of an already lower moisture content just above the fluid bed. The residence time is in the range of 10–20 min, after which the powder is pneumatically discharged either totally dry or to an external vibro-fluidizer for third-stage drying. The three-stage drying procedure means that the inlet air temperature can be 240 °C compared to an outlet air temperature of less than 70 °C, resulting in a marked reduction in energy consumption. Due to the very low outlet air temperature, the quality of the powder is significantly improved, especially its wettability, dispersibility, sinkability, and rate of rehydration. The fluid bed system makes possible simple online lecithination in the production of instant whole milk powder, or online blending in the production of various dairy-based blends.

Three-stage drying includes a classic spray dryer as the first stage, an integrated static fluid bed (**Figure 2**, position 11) as the second stage, and an external vibro-fluidizer (**Figure 2**, position 14) as the third stage.

In addition to the spray dryers shown, there are various other forms of drying equipment such as the 'tall form dryer', the 'Filtermat™ dryer', the 'Paraflash™ dryer', the 'Tixotherm™ dryer', and the Integrated Filter Dryer™ (IFD™). All these towers have characteristics related to the specific properties of the product being dried (e.g., high fat content, starch, maltodextrin, egg, and hygroscopic products).

Determination of Spray Drying Parameters

Due to the variety and complexity of the concentrates to be dried, a more rigorous understanding of spray drying based on physicochemical and thermodynamic properties has become necessary. However, the current technology and knowledge do not allow determination of the parameters of spray drying of dairy products. Studies have shown that drying by desorption is an excellent tool to determine and optimize the major spray drying parameters in relation to properties such as water availability and desorption behavior. Analysis of the desorption curve (RH vs. time), combined with knowledge of the temperature, total solids, density and specific heat capacity of the concentrate, air flow rates, water content in relation to water activity (a_w) and RH of the outlet air, the current weather conditions, cost per kWh, and the percentage of drying in the integrated fluid bed, allowed determination of enthalpy, temperature and RH for each inlet air, concentrate and powder flow rate, specific energy consumption, energy and mass balance, yield of the dryer, and cost (in € or $) to remove 1 kg of water or to produce 1 kg of powder. For reasons of calculation speed and reliability, this method has been computerized and it can already be used in the determination of parameters of spray drying for food products. The name of the new software is "Spray Drying Parameter Simulation and Determination Software"

($SD^2P^{®}$) registered under the following identification: IDDN.FR.001.480002.002.R.P.2005.000.30100.

Packaging and Storage

Milk powders are packed in suitable containers in order to protect the powder from moisture, air, light, insects, and other extraneous matter. The most important of these is moisture, particularly the water activity (a_w) and the glass transition temperature (T_g), because of the high levels of hygroscopicity of these powders. The most common packaging material for milk powder is a combination of multilayer 50 kg kraft paper and one layer of polyethylene lining (25–75 μm thickness). Metal barrels lined with polyethylene bags, or cans sealed with aluminum foil, are also used for packing powder. When the product (especially whole milk) must be stored for a long period, packing is carried out in an atmosphere of inert gas, or under a partial vacuum of 4.0–5.3 kPa, in order to avoid oxidative changes in fat and other milk components. When planning the quantity of wrapping material, it is necessary to take bulk density into account, as this is markedly affected by processing parameters and techniques. All milk powders can be stored at ambient temperature for a limited time, if properly produced and packed.

To summarize, spray drying, storage, and quality of dairy powders are significantly dependent on both the physical state of the lactose (one of the main components of dairy powders) and the other carbohydrates, which themselves are dependent on T_g and a_w. The maximum moisture content of a dairy powder (4% for skim milk powder) is defined in the product specification in relation to a_w, and this must be close to 0.2 at 25 °C for optimal preservation. In these conditions of water content and a_w, the T_g will be close to 50 °C.

Applications

Milk powder is a very valuable and nutritious food. It can be used in powder form in various dry food and beverage blends, as well as reconstituted to give liquid milk. Most milk powder is still utilized in confectionery (chocolate, caramel, soft sweets, coatings, biscuits, cakes) and animal feed. In these products, milk powders provide not only nutrients but they are also functional, improving the consistency, taste, or flavor of confectionery products.

Milk powders are also used in the dairy industry as an ingredient in products such as ice cream blends, yogurts, and processed cheeses. The whole dairy industry in non-dairy-producing countries is based on milk powders, which are reconstituted and processed.

Milk powders are used as supplements in other specific food products, such as coatings, creams, sauces, soups, ready-made dishes, breakfast blends, food blends for special categories (nutrition blends for geriatric subjects, sports people, or convalescents), blends for meal replacements, modified milk products, and imitation milk products.

Analysis

The quality of milk powders is particularly determined by the total heat treatment in the operations used, such as concentration by vacuum evaporation or drying. In addition, storage time and temperature affect the quality characteristics of powders.

The structure and physical properties of milk powders are most severely affected by the drying technique and parameters. Low bulk density is the main drawback for milk powder quality. However, modern spraying methods (e.g., using a steam swept wheel) increase bulk density. The dissolution rate is one of the most important characteristics. These and other physical characteristics are discussed in detail in **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders.

Chemical analysis of milk powders includes monitoring of moisture (free and total), total fat, free fat, oxidative changes, hydrolytic changes, and the intensity of the Maillard reaction.

Microbiological and sensory analysis of milk powders should also be performed, in order to confirm the high quality of the powder.

See also: **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders.

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Milk Powder: Physical and Functional Properties of Milk Powders

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Introduction

The quality of milk powders is influenced by their physical, functional, biochemical, microbiological, and sensory attributes – all of which are interrelated. The physical and functional properties of milk powders are especially important when the powders are intended for recombination and for use in the manufacture of various food products. When intended for use as a food ingredient, milk powders should be cream colored or white, free from off-flavors and easy to hydrate, disperse, and dissolve in water. The basic properties that determine the quality of milk powder, and where defects are most likely to be found, include powder structure, solubility, water content, scorched particles, flowability, floodability, oxidative changes, flavor, color, and contamination with microorganisms.

Thus, a dairy powder is characterized not only by its composition (proteins, carbohydrates, fats, minerals, and water) but also by its microbiological and physical properties (bulk and particle density, instant characteristics, flowability, floodability, hygroscopicity, degree of caking, whey protein nitrogen index, thermostability, insolubility index, dispersibility index, wettability index, sinkability index, rehydration time, free fat, occluded air, interstitial air, particle size, water activity (a_{w}), glass transition temperature (T_g), etc.), which form the basic elements of quality specifications, and there are well-defined test methods for their determination according to international standards. These characteristics depend on drying parameters (type of spray dryer, nozzles/wheels, pressure, agglomeration, and thermodynamic conditions of the air, such as temperature, relative humidity, and velocity), the characteristics of the concentrate before spraying (composition/physicochemical characteristics, viscosity, thermosensitivity, and availability of water), and storage conditions.

Powder Structure

The physical structure of a milk powder can be defined as the way in which its chemical components are distributed and connected between them. Powder structure is very strongly affected by the drying technique. Powder produced by roller drying has a compact structure of irregular shape with no occluded air. Roller dried powder particles have a low bulk density (300–500 kg m^{-3}) because of their irregular structure. The particles of spray dried powder are spherical, with diameters in the range of 10–250 μm. The particles contain occluded air and either large central vacuoles or smaller vacuoles that are distributed throughout the interior of the particles. The surface of spray dried skim milk powder particles is usually wrinkled, but it is smooth for high-protein powders. The high inlet air temperature and large temperature differential between the hot air and the powder particles are the main cause of wrinkle formation. The presence of particles of different morphology in the same sample is attributed to the different drying conditions to which individual particles are exposed in the spray drying chamber. The atomization method, whether centrifugal or nozzle, has no special effect on the structure of the particles.

There are three types of atomizing device: rotary atomizer (wheel or disk), nozzle atomizer (pressure, pneumatic, or sonic) and combined (rotary and pneumatic).

The basic function of pressure nozzles is to convert the pressure energy supplied by the high-pressure pump into kinetic energy in the form of a thin film, the stability of which is determined by the properties of the liquid, such as viscosity, surface tension, density, and quantity per unit of time, and by the medium into which the liquid is sprayed.

The energy available for atomization in two-fluid atomizers is independent of liquid flow and pressure. The necessary energy (kinetic) is supplied by compressed air. Two-fluid atomization is the only successful nozzle method for producing very small particles, especially from highly viscous liquids. It is not normally used in the drying of milk products.

In rotary atomizers, the liquid is accelerated continuously to the wheel edge by centrifugal forces produced by the rotation of the wheel. The liquid is distributed centrally; it then extends over the wheel surface in a thin sheet and is discharged at high speed at the periphery of the wheel. The degree of atomization depends on peripheral speed, properties of the liquid, and feed rate. To select an optimal atomizer wheel, the liquid feed rate, the rotation speed, and the viscosity of the liquid should be taken into consideration.

Powder produced by instantization has a completely different structure. The structure of milk powders obtained by various techniques is shown in **Figure 1**.

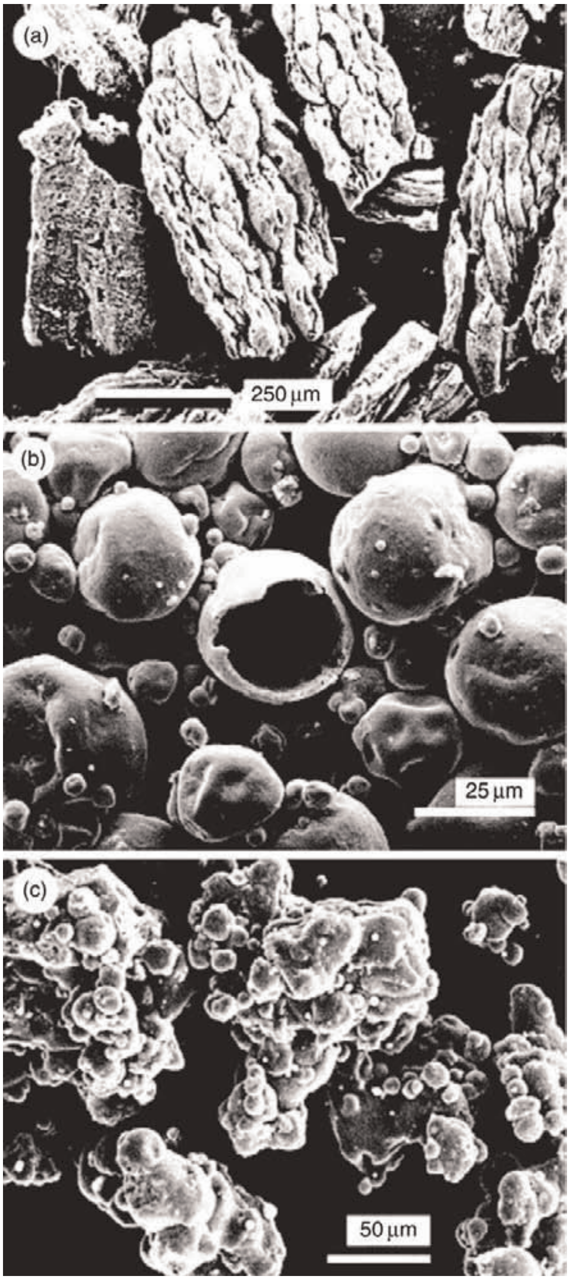

Figure 1 Microstructure of milk powder: (a) roller dried; (b) spray dried; (c) instant milk powder. Reproduced from Carić M and Milanović S (2003) Physical and functional properties of milk powders. In: Roginsky H (ed.) *Encyclopedia of Dairy Sciences*, pp. 1874–1880. London: Academic Press.

Particle Size Distribution

The particle size of a powder, which affects its appearance, reconstitution, and flow characteristics, depends on the atomization conditions and the viscosity of the concentrate prior to drying. A high atomizing pressure and a low concentrate viscosity result in reduced particle size.

Powder Density

Densities are classified into three groups: bulk (apparent) density, particle density, and the density of the dry milk solids; all three are very much interrelated.

Bulk Density

Bulk density is regarded as the weight per unit volume and is expressed as kg m^{-3}. It is a very important property, both from the point of view of cost and market requirements. Bulk density is currently determined by measuring the volume of 100 g of powder in a 250 ml^{-1} graduated glass cylinder. The bulk density of milk powders is a very complex property; it is a result of many other properties and is influenced by several factors such as feed concentration, feed temperature, feed foamability, milk preheating, age thickening, feed composition, type of atomizer, particle temperature history, and particle size distribution, as shown in **Figure 2**.

Nozzle atomization results in powder with a higher bulk density than centrifugal atomization. The manufacturing procedure and conditions greatly influence bulk density, primarily because of the effects of occluded air. Consequently, steps to reduce occluded air increase density. Minimizing the air content of the concentrate before drying, increasing the total solids of the concentrate (a higher concentration can be used with centrifugal atomization), reducing the spray pressure, or using a large orifice are examples of processing steps to reduce occluded air. Also, less uniformity in particle size distribution results in closer packing and a higher bulk density. The shape and the size of the particles also affect the bulk density of powder particles.

The relatively low density of milk powders has several practical implications. It is undesirable as it leads to higher packaging, storage, and transportation costs. By adding certain nondairy ingredients such as sucrose or additives to milk before drying, the bulk density of the dry product will be affected more and less depending on the true density of each ingredient.

Particle Density

Particle density corresponds to the mass (kg) of particles having a total volume of 1 m^3. Particle density is influenced mainly by the amount of entrapped air. The processing factors that contribute significantly to particle density are viscosity and incorporation of air into the concentrate before drying. The type of spray atomization affects air retention. Certain types of centrifugally spray-dried milk have more entrapped air than pressure spray products.

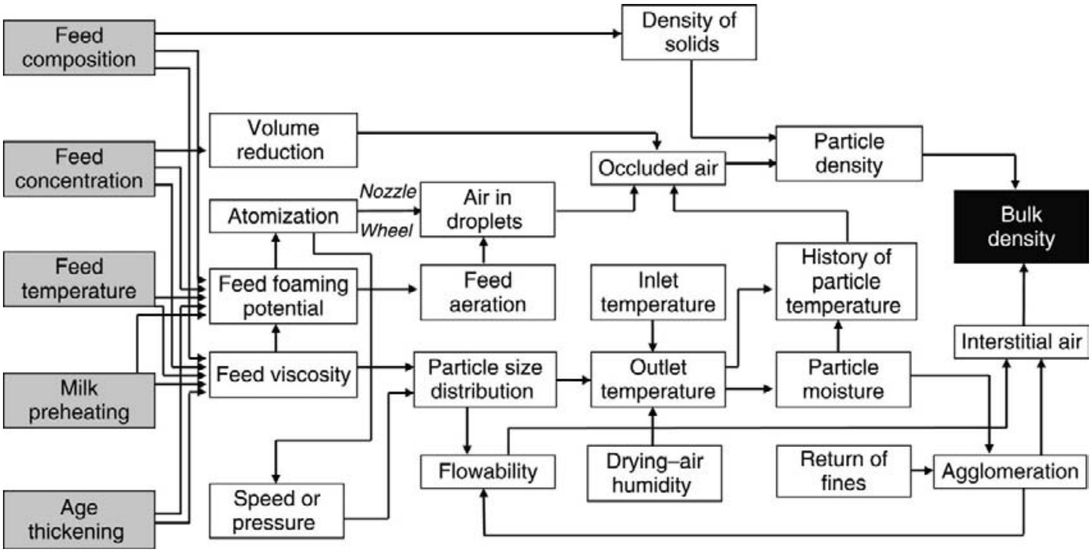
Feed composition
Feed concentration
Feed temperature
Milk preheating
Age thickening
Density of solids
Volume reduction
Atomization
Nozzle
Wheel
Air in droplets
Occluded air
Particle density
Bulk density
Feed foaming potential
Feed aeration
Inlet temperature
History of particle temperature
Interstitial air
Feed viscosity
Particle size distribution
Outlet temperature
Particle moisture
Flowability
Drying–air humidity
Return of fines
Agglomeration
Speed or pressure

Figure 2 Interrelationship of various drying parameters and physico chemical characteristics. Reproduced from Pisecky J (1997) *Handbook of Milk Powder Manufacture*. Copenhagen: Niro A/S.; Master K (2002) *Spray Drying in Practice*. Charlottenlund: SprayDryConsult International ApS.

Occluded Air

The occluded air content is defined as the difference between the volume of a given mass of particles and the volume of the same mass of air-free milk solids (determined by pycnometry). Milk powder normally contains between 10 and 200 ml of occluded air per 100 g depending on many factors such as incorporation of air into the feed, the system chosen for spray drying the concentrate, whipping action before and/or during atomization, the properties of the feed, and the ability of the feed to form a stable foam. The content and state of proteins can markedly affect stable foam formation, whereas fat has the opposite effect. High-fat concentrates are much less susceptible to foaming than is skim milk. Undenaturated whey proteins in skim milk have a greater tendency to foam. Denaturation of whey proteins by high-heat treatment reduces foaming. Concentrates with low total solids content foam more than do those with a high total solids.

Interstitial Air

Interstitial air is defined as the difference between the volume of a given mass of particles and the volume of the same mass of tapped powder. This property depends primarily on the particle size distribution and the degree of agglomeration.

Table 1 shows examples of bulk density and interstitial and occluded air content of skim milk and whole milk powders produced by spray drying.

Table 1 Bulk density and interstitial and occluded air contents of skim milk and whole milk powders produced by spray drying

	Units	*Skim milk powder*	*Whole milk powder*
Bulk density	kg m^{-3}	431	360
Interstitial air	cm^3 100 g^{-1}	41	120
Occluded air	cm^3 100 g^{-1}	119	63

Flowability

Flowability is the ability of a powder to flow freely, like dry sand, without forming lumps, clusters, or aggregates. Flowability is defined as the time (in seconds) necessary for a given volume of powder to leave a rotary drum through given slits (Carr, 1965). The evaluation of the flowability of a powder will involve the use of four properties: angle of repose, compressibility, angle of spatula, and either cohesion or coefficient of uniformity. The last are alternates. The evaluation of the potential floodabilty of a powder involves the use of the following four properties: flowability, angle of fall, angle of difference, and dispersibility. Lumps may form in some milk powders as a result of incompletely crystallized lactose. Under normal conditions, most of the lactose present in a powder is in the form of α-monohydrate crystals, and the rest is amorphous lactose. If the product is not hermetically packed, the amorphous lactose absorbs water from humid atmosphere and forms α-monohydrate crystals. This crystallization results in lumping and caking and leads to the loss of the free-flowing property of the milk powder. Flowability depends also on particle size and shape, density and electrical charge. Large particles flow more easily than fines (particles with diameter <90 μm). Consequently, agglomeration is beneficial, as is uniformity of size. Moreover, a wide variation in particle size permits fines to occupy spaces between the large particles, which results in closer packing. Flowability can be improved by adding various additives, free-flowing agents, moisture-absorbing compounds such as silicium oxide, silicates, calcium phosphate, calcium stearate, or modified starches. The main feature of these additives is that by covering the surface of powder particles, any adhesion between them is reduced, and this reduces the possibility of the formation of 'wet bridges'.

Scorched Particles

Scorched particles in dry milk, with colors ranging from light brown to black, are overheated or burnt particles. Scorched particles usually originate from milk solids with a longer-than-normal residence time in the evaporator and/or dryer, and which become overheated or burnt. The drying process also leads to the Maillard reaction. Particle color intensity depends on the processing and drying parameters applied and on storage conditions of the powder. Generally, these changes are much more prevalent in powder dried by roller drying methods and are sporadic when spray drying is used.

Rehydration

Most food additives prepared in powder form are hydrated before use. Water interactions in dehydrated products and dissolution are thus important factors in food development and formulation. Rehydration is an essential quality attribute of a dairy powder as a food ingredient. Rehydration properties indicate the total phenomenon of reconstituting milk powder with water. It consists of a combination of several properties, such as powder solubility, dispersibility, sinkability, and wettability. Lactose, undenaturated whey proteins, and some of the salts are soluble. Casein is dispersible. Sinkability refers to the particle's ability to penetrate the aqueous surface tension. Wettability is penetration of water into the particles. There are no distinct differences between these individual rehydration terms. They proceed simultaneously during rehydration and influence each other, thereby making it difficult to determine each individual property. Several methods have been proposed for measuring various rehydration properties of milk powders. The irreversible changes that have occurred during milk powder processing or storage remain in the rehydrated product. Rehydration properties are also affected by the processing parameters and techniques (i.e., type of dryer and spraying system, preheat treatment of milk and concentrate, total solids content of the concentrate, outlet air temperature, and storage conditions). Instant whole milk powder of high quality recombines rapidly with water without agitation, resulting in reconstituted milk with the same characteristics as regular milk. Ease of reconstitution is important for the use of powders in beverages, recombined milk, for milk solids fortification, and as ingredients in various other food products.

Many sensors and analytical methods can now be used to study water transfer in dairy protein concentrates during rehydration. Using combinations of various methods (insolubility, dispersibility, wettability indices, nuclear magnetic resonance (NMR) spectroscopy, turbidity, viscosity, and particle size distribution), it is easier to determine the different stages of the rehydration process (i.e., wettability, swellability, sinkability, dispersibility, and solubility).

Wettability

Wettability is defined as the time (in seconds, s) necessary for a given amount of powder to penetrate the still surface of water. It is the ability of a powder to absorb water on the surface and get wet. The wettability of powder particles depends on the surface activity of the particles, surface area, surface charge, particle size, density, porosity, and presence of moisture-absorbing substances.

Lack of wettability causes milk powder to form lumps after coming into contact with water. Small particle size and the symmetrical shape of spray dried milk powder enhance close packing of particles thereby inhibiting penetration of water. Large particles that are irregularly shaped (e.g., those produced by roller drying) have more space in the interstices for wetting. Instantization results in the formation of aggregates, with large spaces between individual particles as a result of agglomeration also favoring wetting. The amount and dispersion of fat or free fat in milk powder negatively affects wettability. Because fat is hydrophobic, it inhibits wetting of milk powder. To improve wettability of high-fat powders, the surface of powder particles is coated with a surface-active agent. Lecithin is one of the most commonly used surface-active agents for the instantization of whole milk powders.

Sinkability

Sinkability is the ability of powder particles to overcome the surface tension of water and sink into water after passing through the surface. Sinkability is expressed as milligrams of powder that sink per minute per square centimeter of surface area. This property of powder is influenced by the forces that tend to submerge a particle on the surface and depends on the density of the particles, that is, on the mass of the particles and the quantity of occluded air. Higher particle density and lower quantity of occluded air cause particles to sink.

Instantization of spray dried milk improves sinkability by increasing aggregate weight caused by agglomeration. The effect of agglomeration on sinkability is greater for skim milk powder than for whole milk powder. Foam spray dried milk has very low sinkability.

During initial stages of reconstitution, the density of powder particles decrease as the heavier components such as lactose and minerals dissolve faster than the other components. Simultaneously, the density of the solution increases, thus inhibiting farther sinking. Consequently, the difference in density between the particles and the medium decreases, so that particles start to rise again after the initial sinking.

Dispersibility

Dispersibility reflects the ability of the wetted aggregates of powder particles to become uniformly dispersed when in contact with water. This property measures whether or not the product is 'instant'. Increasing particle size by agglomeration improves dispersibility of whole milk powder, whereas there is a progressive decrease in dispersibility as the percentage of fines (particles with a diameter <90 μm) in the agglomerated powder increases. The effect of total heat treatment on casein–whey proteins during processing is important for good dispersibility. By increasing total solids, increased heat application causes more irreversible changes in protein, in particular in casein–whey protein interactions, which tends not to favor stable dispersion.

The dispersibility of milk powder can be improved by

- keeping the heat treatment during preheating to a minimum;
- minimizing the holding time and temperature of the concentrate;
- increasing the particle size of the powder;
- choosing the appropriate atomization technique and parameters; and
- agglomeration by instantizing the powder.

Solubility

The term 'solubility' is usually used to indicate the complete phenomenon of milk powder rehydration into liquid form, taking in account soluble (lactose, undenaturated whey protein, salts) and dispersible (casein) components. Thus, 'solubility' is not used in a strictly chemical sense, but more as the rate of dissolving, to describe the combination of all the properties connected with milk powder reconstitution. The solubility index (SI, in percentage) or insolubility index (ISI, in percentage) described by the International Dairy Federation (IDF) standard for skim milk is the volume of the undissolved residue after rehydration, mixing, and centrifugation.

Powder solubility is one of the properties required by quality standards. This means that reduced solubility is a serious defect, which can lead to rejection of the product. During rehydration, the insoluble portion of the powder settles and forms a sediment at the bottom. Because reduced powder solubility is mainly the consequence of milk protein denaturation, to a great extent, the degree of denaturation determines powder solubility. Preheat treatment of milk improves the stability of the final reconstituted product. The parameters of drying procedures have to be adjusted so that the last phase of drying is as short as possible when the concentration of total solids is high. Direct contact between milk and hot metal surfaces of roller dryers causes high degree of denaturation, which affects solubility. Ions (i.e., ion balance), including pH and possibly added salts, have a major influence on protein stability and powder solubility.

Insolubility index (IDI) as determined by the IDF method is only related to the decrease in water transfer needed for rehydration. This index cannot differentiate the true insoluble (related to thermal denaturation) from the false insoluble (related to biochemical composition). The difference between true insoluble and false insoluble material during powder reconstitution and the time required for reconstitution can be measured by NMR spectroscopy.

A rheometer, a turbidimeter, and a laser light diffraction apparatus can be used to measure viscosity, turbidity, and size distribution profiles.

By using a combination of all three methods, it is possible to follow water transfer during rehydration and the wetting time of the powder. Wetting time is determined from the viscosity or turbidimetry curves (kinetics) using the first peak of increased viscosity and turbidity, and the swelling time, determined using the second peak of viscosity in relation to the increase in particle size. The rehydration time is the time when viscosity, turbidity, and particle size values stabilize.

Hygroscopicity

Hygroscopicity is the ability of a powder to absorb moisture from its surroundings. It is measured in a desiccator using powder placed in a stainless steel dish on top of solutions at various degrees of saturation for various times at constant temperature (20 ± 2 °C). When equilibrium is obtained, the moisture absorbed is calculated by weighing the powder sample before and after absorption.

Absorption of water by milk powders is of practical importance in the functionality and use of powders. Therefore, it is important to select proper drying procedures, suitable packaging materials, and storage conditions for milk powders. The ability to absorb water is attributed primarily to milk proteins and lactose. Several important functional properties, such as swelling, viscosity, gelation, foaming, and emulsifying capacity, are affected by the protein–water interaction in most food systems. These interactions are usually expressed as water–sorption–desorption isotherms, which show hydration of protein as a function of water activity (a_w). At low a_w values (<0.3), the water is hydrogen bonded to charged and polar protein residues as a monolayer of bound water. At a_w values higher than 0.3, water associates loosely with the protein, thus forming a multilayer around the protein micelles. Furthermore, water is entrapped mechanically in the capillaries between protein particles. These types of water represent what is known as 'bound' water, which is desirable in various food systems. Water binding is usually affected by protein composition, pH, salt, ionic strength and temperature.

In addition to proteins, the form of lactose has the greatest effect on moisture equilibrium in dried milk. Exposure of milk powders to a humid atmosphere leads to the absorption of water by the amorphous α- and β-lactose isomers. α-Lactose crystallizes as α-lactose monohydrate at temperatures less than 93.5 °C. The formation of α-lactose monohydrate crystals after exposure to 85 and 100% humidity at 25 °C for 24 h is shown in **Figure 3**.

Heat Stability

The heat stability characteristics of reconstituted milk powders are important, especially when the powders are intended for use in hot beverages, custards, sauces, bakery products, recombined evaporated milk, and coffee whitener.

For example, skim milk powder, as the principal raw material in recombined evaporated milk production, must be sufficiently heat stable to withstand sterilization temperatures. Skim milk powder also has to be sufficiently heat stable to avoid 'feathering' in coffee whitener when it comes into contact with the hot coffee (though additives used in coffee whitener processing help to prevent this effect).

The principal processing factor influencing the heat stability of reconstituted milk powders is the level of heat treatment applied during the preheating stage of powder manufacture. In addition, milk constituents (proteins, salt balance) and pH can have a significant effect on the heat stability of powders.

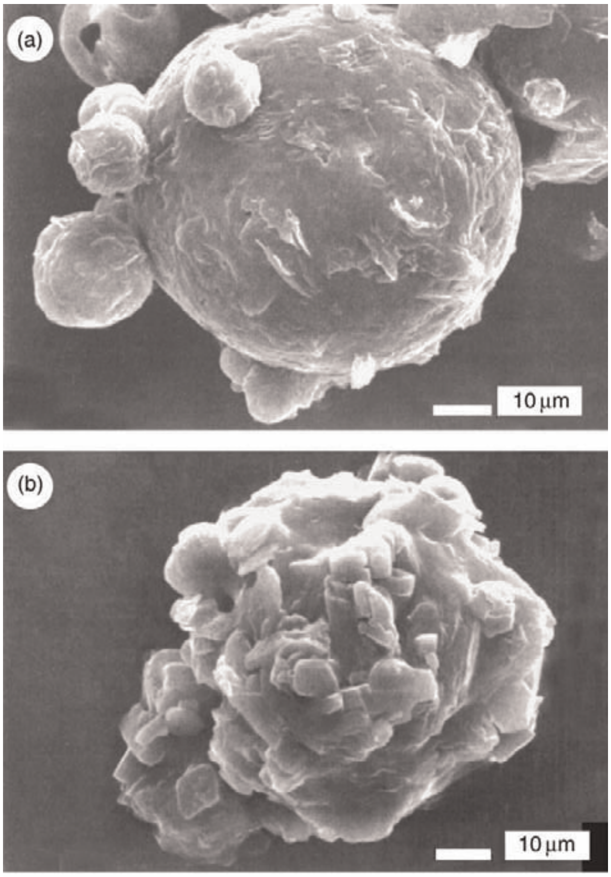

Figure 3 Whole milk powder exposed to 85% (a) or 100% (b) relative humidity for 24 h at 25 °C. Reproduced from Carić M and Milanović S (2003) Physical and functional properties of milk powders. In: Roginsky H (ed.) *Encyclopedia of Dairy Sciences*, pp. 1874–1880. London: Academic Press.

Emulsifying Properties

The emulsifying properties of milk powders are important in several food products such as soups, mayonnaise, coffee whiteners, comminuted meat products, composite milk products, and recombined milk products. Until recently, skim milk powders were the principal source of milk proteins used in the aforementioned products.

However, at present, the role of emulsifying agent is better performed by various milk protein products, such as caseinates, coprecipitates, or whey protein concentrates than by skim milk powders.

Glass Transition Temperature, Water Activity, Stickiness, and Cakiness

The physicochemical properties of free and bound water affect the physical state, transition temperatures, sticking temperature, reaction kinetics, and stability of milk products. The emphasis has been on the physical state of nonfatty solids and the effects of water and its physical state on chemical reaction rates, growth of microorganisms, and stability. Spray drying, storage, and quality of dairy powders are significantly dependent on the physical state of both lactose (one of the main components of dairy powders) and other carbohydrates, which themselves are dependent on T_g and a_w. The maximum moisture content of a dairy powder (4% for skim milk powder) is defined in the product specification in relation to a_w, which must be close to 0.2 at 25 °C for optimal preservation. Under those (4% moisture and a_w of ∼0.2), the T_g is be close to 50 °C.

Glass transition of dairy solids can be observed using differential scanning calorimetry (DSC). DSC measures any change in heat capacity that occurs over the glass transition temperature range. The glass transition of anhydrous lactose as observed using DSC has an onset temperature of 101 °C, which is one of the highest temperatures measured for 'anhydrous' disaccharides. The glass transitions observed in milk solids are very close to those of pure lactose.

Several techniques have been developed to characterize the stickiness behavior of food powders. All the tests have been empirical in nature, and techniques are still being developed because of inaccuracies and the difficulties in application to real processing and handling situations. Whatever the method used, the glass transition temperature of skim milk solids (which is related to stickiness and caking), is approximately 10 °C above T_g or higher measured by DSC.

The a_w of dried milk products is largely correlated with moisture content and temperature. The composition and state of individual components as influenced by various processing techniques also affect water activity. At low moisture content, characterized by a_w <0.2, the casein is the main water absorber. Within the intermediate range of >0.2 to <0.6, sorption is dominated by the transformation of the physical state of the lactose. Above this level, salts have a marked influence.

The water activity of whole milk powders is mainly controlled by the moisture content expressed in nonfat solids, because fat has no influence. Thus, differences in a_w of different kinds of dairy powders are mostly the result of the state of the proteins and the physical state of the lactose. The methods to determine a_w consist of putting the product in equilibrium with the surrounding atmosphere then measuring the thermohygrometric characteristics of the air in equilibrium with the product. The ideal moisture contents can be determined for the optimal stabilization (at 0.2 a_w and 25 °C) of some dairy powders by using practical or theoretical sorption isotherms. For example, the corresponding moisture contents must be close to 4, 2–3 and 6%, for skim milk, whey, and caseinate powders, respectively.

See also: **Dehydrated Dairy Products:** Milk Powder: Types and Manufacture.

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Dairy Ingredients in Non-Dairy Foods

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Introduction

In 2008, the global production of milk was about 700 million metric tonnes. It has been estimated that nearly half of this amount is converted to forms for use as food ingredients. Prior to 1950, the major products for ingredient use were dried whole milk, dried skim milk, and dried churned buttermilk. The major uses of these ingredients were for bakery products and confections.

New developments in concentration, fractionation, isolation, and preservation techniques in the dairy industry have allowed the manufacture of additional high-quality and safe dairy ingredients. Information on the composition and functionality of these ingredients increased their utilization in a large range of food products. Applications also appear in specific fields such as nutrition, pharmacy, and medicine, and as support for the functional properties of food products. In this article, a survey will be given of the current fractionation and isolation processes of milk and the composition of the recovered dairy ingredients. Subsequently, attention is focused on the utilization of these ingredients in a number of nondairy food products and pharmaceutical applications.

Recovery of Dairy Ingredients

Desired ingredients may be recovered systematically from milk according to a number of separation techniques (**Figure 1**).

Usually, whole milk is heated to 65–76 °C in a pasteurizer before centrifugal separation into cream (density 89 kg m^{-3}) and skim milk (density 1016 kg m^{-3}). Whole milk may also be separated cold. Well-known fat standards are 35–40% in cream and $\leq$0.05% in skim milk. Surplus cream is usually churned to butter containing 83% milk fat. The storage stability of butterfat may be increased by the production of anhydrous milk fat (AMF), containing at least 99.8% fat. AMF can be produced either directly from concentrated cream (75% fat) or from butter by melting at 60 °C and centrifugal separation of the aqueous phase. AMF products may be refined to tailor-made milk fat products for the bakery industry by fractionation into high-melting (40–41 °C), medium-melting (32–34 °C), and low-melting (24–28 °C) milk fat fractions. This is achieved by filtration or centrifugation of fat crystals of different size after a slow cooling process. The buttermilk produced is frequently spray dried for use as a food ingredient.

Skim milk may be subjected to different preheating processes before drying, depending on the intended application. The bakery industry usually requires a high-heat milk powder, obtained by a preheat treatment of at least 5 min at 95 °C. Skim milk (powder) used for the production of casein and caseinates is generally subjected to a medium-heat treatment (e.g., 0.5–1.0 min at 85 °C). Low-heat skim milk powder (15 s at 72 °C) is used for the standardization of milk and in applications where low-heat milk powder is legally required, for example, for the production of cheese.

Coprecipitate is recovered after heat treatment of skim milk at 90 °C, either with or without some added $CaCl_2$ and acid, followed by lowering the pH to 4.6 at this temperature and centrifugal separation. Coprecipitate was first made in the 1930s and was called 'lactalbumin'. Coprecipitate is used as a nutritional extender in a number of food products.

Ultrafiltration is commonly used for the production of milk protein concentrates (MPCs). A typical membrane for ultrafiltration of milk is permeable to any substance with a molecular weight of less than 10 000 Da. A standard ultrafiltration procedure results in a five- to sixfold concentration of the protein, that is, from 3.5% in skim milk to about 19% (70–80% of total solids) in the retentate. MPCs and milk protein isolates are preferentially used in dietetic and clinical formulations for nutrition. Milk protein isolates (>90% milk protein) can also be made by ion exchange processes. MPCs and milk protein isolates are gaining use in nonstandard cheese, processed cheese food, nutritional beverages, frozen desserts, and bakery.

Caseins and Caseinates

The general process for casein manufacture is to add a coagulant such as rennet or a culture of lactic acid bacteria or food-grade acids such as hydrochloric acid or sulfuric acid.

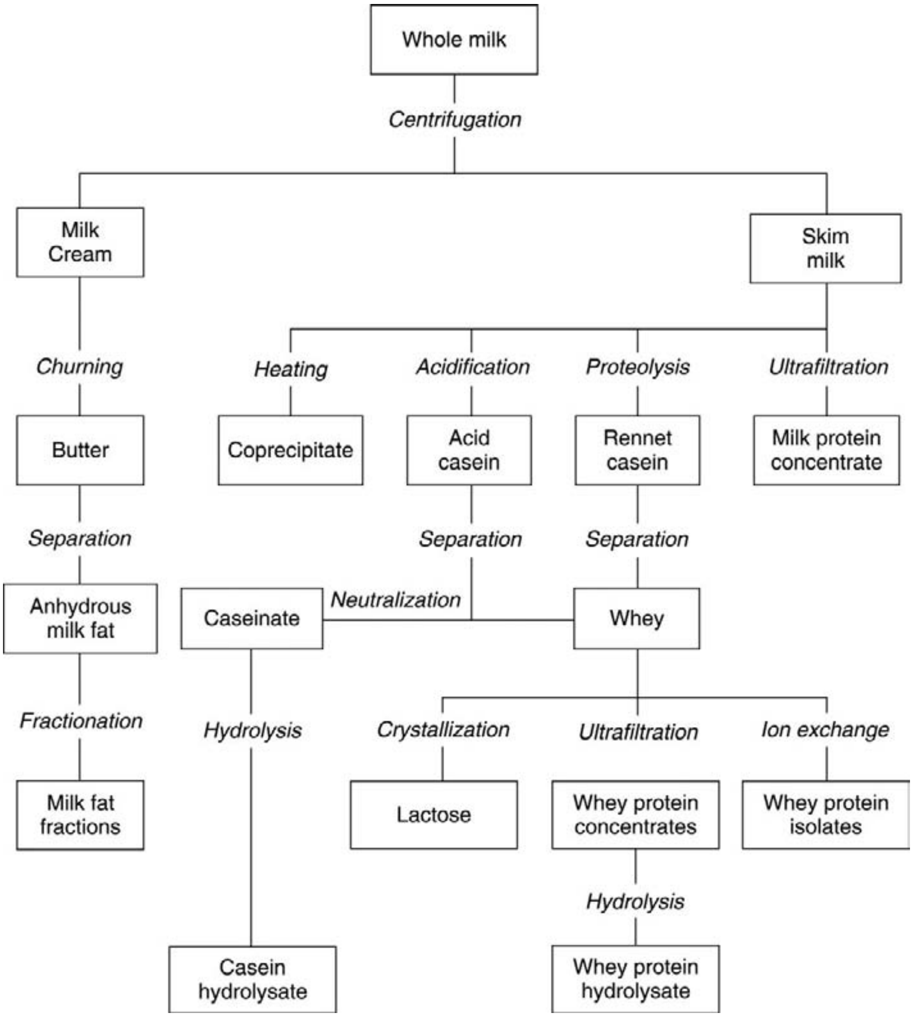
Whole milk
Centrifugation
Milk Cream
Skim milk
Churning
Heating
Acidification
Proteolysis
Ultrafiltration
Butter
Coprecipitate
Acid casein
Rennet casein
Milk protein concentrate
Separation
Separation
Separation
Anhydrous milk fat
Caseinate
Neutralization
Whey
Fractionation
Hydrolysis
Crystallization
Ultrafiltration
Ion exchange
Milk fat fractions
Lactose
Whey protein concentrates
Whey protein isolates
Hydrolysis
Casein hydrolysate
Whey protein hydrolysate

Figure 1 Flowchart for the manufacture of dairy ingredients by industrial fractionation processes of milk and milk products.

Rennet casein is produced by enzymatic coagulation from skim milk at pH 6.7. The enzymes traditionally used, referred to as rennet, are preparations of gastric proteinases of young calves. Current rennet extracts are produced by fermentation. In the traditional method of rennet casein manufacture, the skim milk is set with rennet in a manner similar to that used in cheesemaking. After coagulation, the gel is pumped to a cooking pipe where the curd is separated from the whey, washed, and dried. Rennet casein is a well-known ingredient in processed cheese and cheese analogues.

Acid casein is produced by acidification to pH 4.5. Then the casein is cooked, dewatered, washed, and dried with a ring dryer. Most casein is converted to caseinates by neutralization (to pH 7.0) with $Ca(OH)_2$ or NaOH, followed by drying. Caseinates may be sodium caseinate, calcium caseinate, or sodium/calcium caseinate, depending on the end product usage. Caseinates are heat stable, acid labile, and alcohol stable. They exist in different salt forms and can provide a range of functionalities, including viscosity, emulsification, aeration, opacity, and dispersibility.

Caseinates are used as functional ingredients in various food products. Different caseinates are used in different applications. Sodium caseinate is generally found in beverage powders, retorted or aseptic liquids, whipped toppings, coffee whitener, and nutrition bars. In addition to some of these uses, calcium caseinate is used in soups, bakery, confectionery coatings, and desserts.

Recovery Processes from Whey

Most of the whey is recovered as cheese whey, the liquid remaining during the production of cheese. Less than 10% of the whey is produced as a by-product from skimmed milk during the production of casein (**Figure 1**).

The principles of industrial recovery processes of ingredients from whey before evaporation and/or

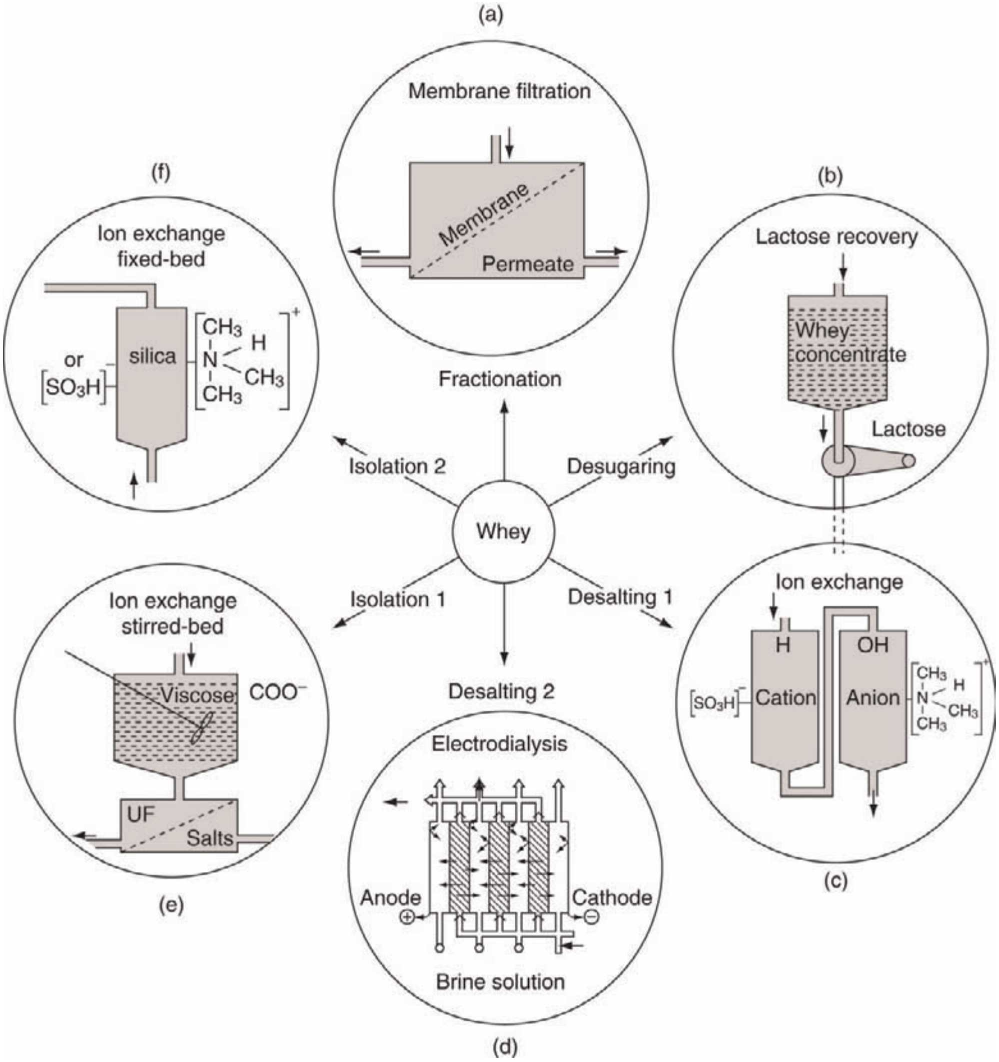
(a)
Membrane filtration
Membrane
Permeate
(b)
Lactose recovery
Whey concentrate
Lactose
Ion exchange
H
OH
$[SO_3H]^-$
Cation
Anion
CH_3
N
H
CH_3
CH_3
(c)
Desalting 2
Electrodialysis
Anode
Cathode
Brine solution
(d)
Ion exchange stirred-bed
Viscose
COO⁻
UF
Salts
(e)
(f)
Ion exchange fixed-bed
or
$[SO_3H]$
silica
CH_3
N
H
CH_3
CH_3
Fractionation
Isolation 2
Desugaring
Whey
Isolation 1
Desalting 1

Figure 2 Survey of fractionation processes for the recovery of ingredients from whey. UF, ultrafiltration.

spray drying are shown schematically in **Figure 2**. Membrane processes (**Figure 2(a)**) are used for the separation of whey ingredients having different sizes according to their molecular weight. Microfiltration is used for the removal of bacteria and fat globules by using membranes of fairly wide pores (>0.1 μm). Ultrafiltration is used for the separation of (whey) proteins, and these membranes are characterized by separation characteristics based on molecular weight. Nanofiltration is used for desalting and reversed osmosis for the separation of water.

Removal of lactose (**Figure 2(b)**) makes use of the poor solubility of lactose in whey, resulting in the crystallization and separation of lactose solids from concentrated whey. Pasteurized whey is concentrated by evaporation to 60–65% total solids. The concentrated whey is transferred to crystallization tanks at a temperature of about 50 °C and during gentle stirring cooled to 10 °C. The concentration of lactose in the concentrated whey is 40–45%, while its solubility at 10 °C is only 6%. After crystallization, the slurry proceeds to a decanter centrifuge for separation of the lactose mass.

Demineralization involves the removal of minerals and some organic acids by nanofiltration, ionic exchange, or electrodialysis. The most complete demineralization is achieved by using ionic exchange (**Figure 2(c)**). Whey (or desugared whey) first enters a strong cation exchanger, loaded with a resin in the H^+ form for exchanging cations from the whey. The resulting acid whey continues to a basic ion exchanger where its anions are exchanged for OH^-.

Electrodialysis (**Figure 2(d)**) is a more selective demineralization method, based on the transport of preferentially monovalent ions through semipermeable membranes, induced by a direct current as the driving force. Direct current electrodes are located along the end compartments and whey salts are discharged through a 5% brine solution.

Specific separation of whey proteins by ionic exchange may be achieved by mixing whey at pH 3.2 with porous, cross-linked, negatively charged, viscose particles in the so-called stirred-bed ionic exchange process (**Figure 2(e)**). The positively charged (at pH 3.2) whey proteins are bound during gentle stirring and thereafter released at pH >8. Separation of the whey proteins from the particles occurs through a sieve in the bottom of the tank. An additional ultrafiltration step is needed to remove excess salts, upon which a whey protein isolate is obtained.

Another ionic exchange method for the production of whey protein isolates is performed in columns according to a fixed-bed process (**Figure 2(f)**). Porous, positively charged silica particles bind the negatively charged (major) whey proteins at pH 6.5. An alternative is the use of negatively charged Sepharose columns for binding positively charged (specific) milk proteins, such as lactoferrin and lactoperoxidase, at pH 6.5.

By-products arising from the fractionation of milk and whey products are increasingly used as food ingredients. Examples are milk salts, which have the same salt sensation as table salt. Sources of milk salt production are delactosed whey and ultrafiltration permeates, and the minerals from electrodialysis. Hydrolysis of milk proteins is carried out to improve their nutritional characteristics, increase their solubility, and improve their foaming properties. Milk protein hydrolysis may be accomplished with preparations of enzymes that usually occur in the intestinal tract. After the desired degree of hydrolysis, the enzymes are inactivated by heat treatment or separated by membrane filtration. The resulting protein hydrolysates are used for specific nutritional applications.

Protein Fractionation

While limited in application at the present time, fractionation of both caseins and whey proteins has received much attention in recent years. These include α_s-, β-, and κ-casein, casein glycomacropeptide (GMP), β-lactoglobulin, α-lactalbumin, immunoglobulins, osteopontin, lactoferrin, and lactoperoxidase. Lactoferrin, lactoperoxidase, and GMP are commercially available. Growth in these products is expected in the future, especially with increased interest in functional foods.

Composition of Dairy Ingredients

The main components present in the dairy ingredients discussed so far are indicated in **Table 1**. All ingredients, except butter, are dried to less than 4% moisture before they are delivered to the food industry. Unsalted butter usually contains 16% moisture and 1% nonfat solids, and salted butter contains additional NaCl. Buttermilk, the by-product from the butter manufacture, has a composition similar to skim milk, although it has a slightly higher fat content (0.5%).

Whole milk or skim milk may be preserved by adding sugar (60%) to milk that has been pasteurized and concentrated (2.5:1) previously. This so-called sweetened condensed (skim) milk has an osmotic pressure high enough to inhibit the growth of bacteria. Sweetened condensed milk is a well-known ingredient in the confectionery industry.

Rennet casein has a slightly different protein composition compared to acid casein, caused by the absence of a peptide released from one of the casein molecules. This enzymatic modification results in (rennet) casein products with quite different properties than those of acid casein.

Table 1 Approximate composition (%) of milk ingredients recovered according to different processes, before concentration and drying

Ingredient	*Protein*	*Lactose*	*Milk fat*	*Minerals*	*Water*
Whole milk	3.6	4.6	4.1	0.7	87.0
Skim milk	3.7	4.7	0.05	0.7	91.0
Cream	2.8	2.8	40.0	0.4	54.0
Butter	0.4	0.5	83.0	0.1	16.0
MPC	17.0	2.5	1.5	0.5	78.5
Casein	43.5	0.1	0.4	1.0	55.0
Caseinate	21.0	0.05	0.2	1.0	77.0
Coprecipitate	35.0	0.4	0.6	4.0	60.0
Whey	0.9	4.8	0.05	0.5	93.0
WPC-35	3.3	4.8	0.2	0.7	91.0
WPC-60	11.5	5.2	1.0	0.8	71.5
WPC-80	20.0	1.0	2.0	1.0	76.0
WPI	19.0	0.2	0.2	0.6	80.0
Milk salts	4.0	20.0	0	16.0	60.0

MPC, milk protein concentrate; WPC, whey protein concentrate; WPI, whey protein isolate.

Whey still contains about 50% of the nutrients present in whole milk, comprising lactose, whey proteins, minerals, a small amount of fat, and most of the minor water-soluble nutrients from milk, such as vitamins and growth factors. The protein content is calculated from the total nitrogen content multiplied by a factor 6.38 (so-called Kjeldahl factor). This implies that both protein nitrogen and nonprotein nitrogen (NPN) are included, which should be taken into account, particularly for milk, skim milk, and whey. In cheese whey, 20% of total protein is NPN, which is nearly twice the amount present in (acid) casein whey. Electrodialysis of whey results in a partial desalting because multivalent ions such as calcium, magnesium, phosphates, and citrates are not or only slightly removed. The protein, lactose, fat, and mineral content of delactosed whey, demineralized by electrodialysis, is nearly identical to that of skim milk and is often indicated as 'skim milk equivalent'. This whey (by)product is a well-known extender for skim milk in ice cream and baby food.

Applications of Dairy Ingredients

Milk and dairy products are used by the food industry in many different types of food products on the basis of their excellent nutritional and functional properties. Dairy-based ingredients are manufactured globally, and their functionality in food products frequently varies for the same type of product from different manufacturers. Other factors that influence the final characteristics of a food product include

- type of food product being made – different foods require different functionalities as illustrated in **Table 2**,
- type of nondairy ingredients being used, causing interactions and modification of the characteristics of the food (protein/fat, protein/gum, etc.),
- difference in the processing methods for manufacturing the dairy ingredient, and
- differences in the processing methods for making the food product.

Table 2 Examples of multiple functionalities required of selected foods

Food type	*Multiple functionalities required*
Beverages	Solubility, heat stability, pH stability, color
Baked goods	Emulsification, foaming, gelation
Meat emulsions	Emulsification, foaming, gelation
Soups and sauces	Viscosity, emulsification, water binding
Frozen desserts	Foaming, gelation, emulsification

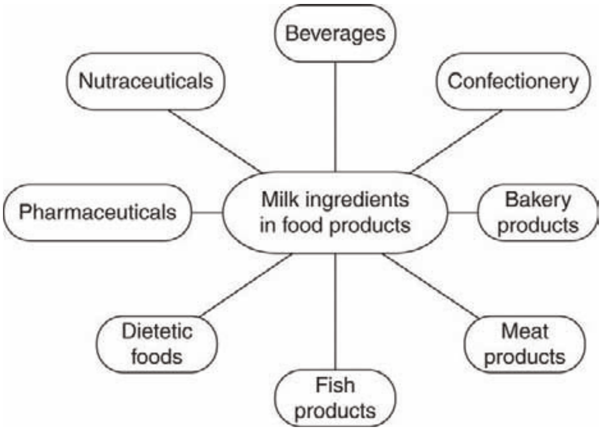

Figure 3 Summary of the principal applications of dairy ingredients in food products.

The main areas of application are summarized in **Figure 3**, and will be discussed successively in the next sections.

Beverages

The flavor of whey, especially that of acid whey, is most compatible with that of citrus fruits in beverages. However, utilization of whey as a refreshing drink is hampered by the presence of whey proteins and fatty components. After World War II, this problem has been solved by using deproteinated and defatted whey. A well-known example of such a refreshing drink is 'Rivella', produced in Switzerland since 1950 and nowadays still consumed in Canada and The Netherlands. Rivella is a carbonated, clear whey beverage with a bittersweet fruit flavor and a pH of 3.7.

Nutritional whey drinks based on pasteurized (desalted) whey mixed with fruit juices at pH 4.0 were developed in the 1970s. Whey or desalted whey was mixed with concentrated fruit juice and sugar. Pasteurization and aseptic packaging guaranteed a shelf life of 6 months without refrigeration.

The good solubility and bland taste of undenatured whey proteins over a broad pH range are important attributes for soft drinks. In the 1970s, the Coca-Cola Company selected whey protein concentrates (WPCs) as a nutrient to improve the nutritional quality of their drinks. The drinks could be fortified up to 1% whey proteins, without detectable changes in flavor and appearance. These WPC beverages retained their bland flavor and solubility after in-bottle pasteurization at around pH 3.0. However, the cost of these products appeared to be too high to continue their production on an industrial scale.

Confectionery Products

Dairy ingredients are well-known components in a large number of confectionery products because of their contribution to the characteristic texture, flavor, and color. Some of these products will be discussed with respect to their main functional demands. Candy-type products, such as toffees, caramels, and fudges, are cooked syrups; they were originally textured and flavored by using sweetened condensed milk. The palatability of confectionery products is often improved by the incorporation of air, supported by whipping proteins. Lactose contributes to the color and flavor of these products, in particular during cooking. Milk fat serves as a source of several significant flavor compounds. Moreover, milk fat improves the mouthfeel of candies due to its disposition in the candy product. Milk proteins enhance the miscibility of formula ingredients because of their emulsifying properties, and contribute to the lightness during whipping and the texture of the candy during cooking.

Milk ingredients are also valuable components in chocolate products, especially in milk chocolate, owing to their contributions to flavor, sweetness, and protein profile. According to the regulations of the European Community, milk chocolate should contain at least 14% dry milk solids and not less than 3.5% milk fat. A basic step in chocolate manufacture is ‘conching’, a heating process with aeration for some hours, which creates typical chocolate flavors. In order to maintain the chocolate flavor during an extended storage period, milk crumb has been introduced as an ingredient for the chocolate industry. Milk crumb is prepared from sweetened condensed milk, sugar, chocolate liquor, and cocoa mass. This mixture is usually drum-dried and subsequently crushed into grains, which may be stored for several months without loss of flavor when packed into closed sacks. Not all products that have the appearance of chocolate meet the official standards and by regulation these products may not be labeled as chocolate. Such products are generally referred to as confectionery coatings or compound coatings and are developed for specific uses, for example, as coatings on the centers of ice cream bars, enrobed candy bars, and baked goods. In these recipes, sweetened condensed milk whey, various sugars, and optimal additions of fats and emulsifiers may replace part or all of the sweetened condensed milk. Most aerated confectionery products are protein-type foams, which are highly sensitive to fatty components; examples are frappé and meringue. Frappé is a highly aerated sucrose–glucose solution, prepared with egg white, which is carefully folded into viscous candy fillings for confectionery products such as caramels, toffees, and nougats. Meringue is a whipped (egg white) protein/sugar preparation that is dried at 110–125 °C. In particular, the drying process puts high demands on foam stability and requires the absence of fat. Defatted WPC may replace egg white in meringue. Meringues made from normal (nondefatted) WPC collapse to flat cookies during drying.

Bakery Products

Whole milk powder is a well-known dairy ingredient in bread; it improves the flavor and color and retards staling of bread during storage. A requisite is that the milk should be heat-treated intensively before use, in order to prevent loaf depression of the bread during baking. High-heat milk powder meets this requirement through inactivation (denaturation) of a loaf-depressing protein. Whole milk powder may be replaced by a combination of high-heat skim milk powder and butter, depending on legal regulations. Butter and fractionated milk fat, prepared as described previously, are important ingredients in the bakery industry. Since butter is quite expensive relative to other fats, its use is restricted to those products in which flavor makes a significant contribution.

High-melting milk fat fractions are functional ingredients in the preparation of puff pastry, providing the crisp texture in dough coatings of almond bread, pies, sausage rolls, or apple dumplings. The low-melting milk fat fractions may be used in the production of recombined milk powder from skim milk and milk fat; it appears that the reconstitutability of milk powder is significantly improved.

Whey powder is a well-known ingredient in the bakery industry because of its flavor-enhancing and tenderizing qualities. Volume, texture, crust, and retention of freshness in wheat bread are improved by the incorporation of a combination of emulsifiers and whey powder in the flour. Usually, 1–2% of whey solids (on the basis of flour) are added, depending on the type and structure of the bread.

In the 1970s, there was an increasing interest in the production of milk protein-enriched biscuits as nutritional food for children in developing countries. A mixture of delactosed whey and buttermilk added to wheat flour resulted in tasty biscuits containing 20% milk proteins.

Hens’ eggs are widely used in the baking industry because of their unique properties. Whey proteins have a number of properties in common with egg (white) proteins, and many attempts have been made to substitute WPC for egg white proteins in bakery products. For economical reasons, there has been much interest in replacing whole eggs in Madeira-type cakes, consisting of whole eggs, sugar, flour, and (butter) fat. The main function of egg yolk in this cake is the emulsification of fat, a function that cannot easily be performed by other proteins. Preparing a pre-emulsion of fat and whey proteins has solved the lack of fat binding, allowing complete substitution of whole eggs by WPC-80 as shown in **Figure 4**. However, the typical egg yolk taste and the fatty mouthfeel are lacking in bland cakes. Fruit and chocolate cakes prepared from WPC according to this

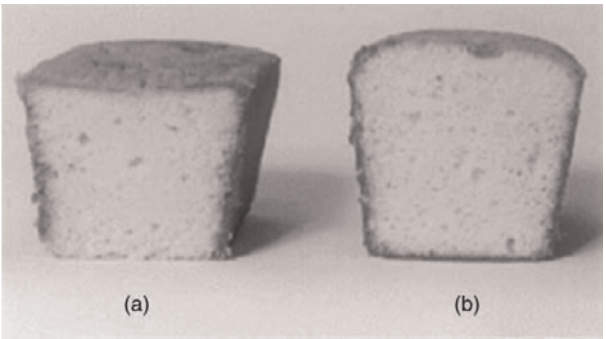

Figure 4 Cakes prepared from (a) equal amounts of wheat flour, sugar, fat, and whole eggs, or (b) whey protein concentrates as a complete replacer for whole eggs.

procedure have a structure and mouthfeel that resemble those of similar types of cakes prepared with whole eggs.

Meat Products

A number of milk products are used as functional ingredients or extenders in meat products. Two functional demands of comminuted meat products are of particular importance, that is, the water-holding capacity and the fat-binding ability. More than 70% of the water present in lean meat is 'free water', which is important for the tenderness and juiciness of whole meat products. After the animal is slaughtered, part of the free water is expelled and this can be restored with the help of salts and milk proteins such as caseinates and WPCs. Luncheon meat is a comminuted (fine-particle) meat product enriched with pork fat and flavoring additives. Fine-particle meat products are prepared by comminution of the muscle tissue in a grinder/mincer. Pork fat is usually dispersed as pre-emulsion stabilized by caseinates in a bowl chopper and then mixed with the minced meat slurry. WPCs may be used as an emulsifier for patés, which contain a greater amount of fatty tissues than luncheon meat.

Concomitant protein fortification of whole meat and recombined products (such as hams) is achieved by injecting a whey protein solution into the meat, using a multineedle system. The product is then tumbled to achieve mixing and binding. To achieve the desired elasticity and slicing characteristic of the final product, especially in recombined hams and other recombined meat products, a gelling whey protein is needed.

Fish Products

Numerous species of fish are caught annually throughout the world. The introduction of gel-type seafood products such as surimi on Western markets has opened a profitable outlet for deboned fish flesh. Surimi can be described as a myofibrillar protein concentrate, which is used primarily in the manufacture of various types of Japanese heat-gelled products, such as kamaboko. Egg white and WPCs are well-known texturizers for fish products. However, the gel-forming characteristics of pollock (*Pollachius virens*) deteriorate rapidly after the fish is caught at sea, which is caused by enzymatic degradation of its myofibrillar proteins. The addition of 3% WPC-80 appears to be very effective for the inhibition of this autolytic enzyme activity.

Finely comminuted fish products are usually prepared in bowl choppers and sometimes in a grinder. Examples of comminuted fish products are fish sticks, fish nuggets, and Japanese-style fish pastes such as kamaboko and tempura. Water binding capacity (and in turn juiciness) is improved in comminuted nuggets when both whey protein products and starch are present during chopping.

Dietetic Foods

Dietetic foods may be defined as food products designed for special dietary requirements. These include infant formulae, foods for the elderly, slimming foods, clinical or medical foods, and sports nutritional foods.

Infant formulae are mainly designed on the basis of cows' milk as a substitute for human milk. When in the early 1970s it became apparent that whey-based infant formulae can simulate human milk, attention was turned to the development of formulae containing whey or whey proteins. This was the start for formulae prepared by mixing equal amounts of skim milk and demineralized whey. Specific whey-predominant formulae, using WPC, have been developed for preterm low-weight infants, to support a more appropriate balance of amino acids for growth and metabolism.

As age advances, physical activity tends to decline and so less dietary energy is required. Food intake also declines with age, but information on the specific nutrient needs of elderly people is scarce. MPC with additional nutrients coming from fruits, vegetables, or cereal products meets the requirements of food for the elderly. Both high-quality proteins and bioavailable calcium present in MPC are important nutrients for (elderly) people.

Slimming foods are introduced to prevent or control obesity, the most prevalent nutritional disorder in prosperous communities. Obesity arises as a consequence of taking in more energy in food than is expended in the activities of daily life, leading to a positive energy balance, which is mainly stored as fat. Whey products fit well in slimming foods, owing to their excellent amino acid composition and low fat content.

Clinical or medical foods are designed to provide complete or supplemented nutritional support to persons who are unable to digest adequate amounts of food in a conventional form. Whey products have nutritional advances in medical diets because most whey nutrients are present in a bioavailable form. Some patients having defects in their (enzymatic) digestion system require a diet that contains previously (*in vitro*) hydrolyzed milk proteins or lactose. The degree of protein hydrolysis can vary from almost completely (65% of the peptide bonds broken) to partial (35% of the peptide bonds broken). The required degree of hydrolysis may vary according to the required use of the formula. Orally consumed enteral diets contain larger peptides, apt to further digestion in the gastrointestinal tract. A more rigorous hydrolysis is required for parenteral diets, which are injected directly into the bloodstream.

Sports nutritional foods developed to meet the needs of athletes have shown a growth of 5–9% in the past decade. These include supplements, beverages, and nutrition bars. In 2008, the sports nutrition market had grown to about US$25 billions.

Functional Foods and Pharmaceuticals

Lactose is quantitatively the most significant excipient (substance other than the active drug) in pharmaceutical applications. Tablets, capsules, and inhalers are the most widespread and convenient forms for administering drugs to patients. Refined lactose is well known as an inert carrier of drugs because of its purity and consistent chemical and physical stability. One of the most important physical properties in the manufacture of tablets is its capability for direct compression. A lubricant (e.g., magnesium stearate) is essential for almost all tablet formulations, which forms a thin film around the particles, necessary for the disintegration of the tablets in water.

Another category of medicines is the inhalers. The majority of inhalers contain the active drug bound to small homogeneously sized lactose particles. The drug particles must be in the size range 0.5–5 μm for optimal delivery to the deepest parts of the lungs. The small particles of the active drug are coated on the somewhat larger lactose particles, acting as carriers. These lactose particles are trapped in the respiratory tract, moved upward by the action of cilia, and then swallowed.

The versatility of lactose is demonstrated by the range of derivatives that can be obtained through chemical and biochemical reactions, as shown in **Figure 5**. Calcium salts of lactobionic acid (**Figure 5(a)**) are used as a carrier for antibiotics in pharmaceutical (functional) preparations. Lactulose (**Figure 5(b)**) has been identified as a bifidus factor (see section 'Functional Foods

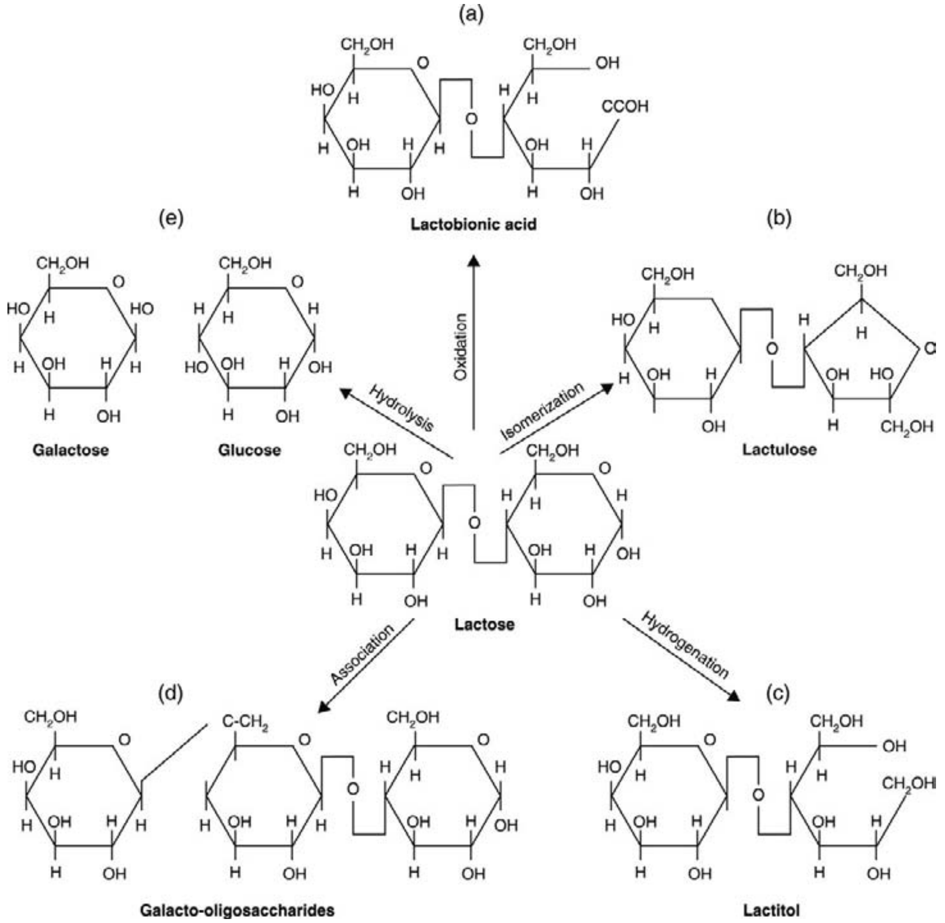

Figure 5 Lactose derivatives obtained by chemical and biochemical modifications.

(Nutraceuticals)'). Lactitol (**Figure 5(c)**) is a sugar alcohol prepared from lactose by catalytic hydrogenation of the glucose part of the molecule to an alcohol (sorbitol). Lactitol is not absorbed or hydrolyzed in the small intestine; instead, it is fermented by bacteria in the large intestine to biomass and short-chain fatty acids for absorption. Galacto-oligosaccharides (**Figure 5(d)**) are formed during the enzymatic hydrolysis of lactose under specific reaction conditions. Galacto-oligosaccharides cannot be digested by human intestinal enzymes and consequently arrive in the human colon where they are fermented by the colonic bacteria, preferably bifidobacteria (see below). The hydrolysis of lactose to glucose and galactose (**Figure 5(e)**) is catalyzed by the enzyme β-galactosidase, which is present in the intestines of mammals, including humans. Both galactose and glucose are absorbed from the small intestine and are used as an energy source in the body. Previous hydrolysis of lactose in milk products is important for lactose-intolerant people.

Recently, lactose has been fermented to produce polylactic acid, a biodegradable polymer that is finding use in a number of different applications, including implants and new packaging applications.

Functional Foods (Nutraceuticals)

Functional foods (or nutraceuticals) are food products or food ingredients that provide a physiological function other than nutrition and they have medical or health benefits, including the prevention and treatment of diseases. Examples of functional foods are bioactive proteins, bioactive peptides, probiotics, and prebiotics.

A group of specific bioactive milk proteins include lactoferrin, lactoperoxidase, lysozyme, and a number of growth factors present in milk and whey. Lactoferrin is credited with several beneficial health-promoting effects, including antibacterial activity in the intestinal tract, acting as a carrier for iron absorption, and regulation of the immune system. Lactoperoxidase and lysozyme are natural antimicrobial milk proteins, which have been described as prospective additives to protect milk and milk products against microbial deterioration.

Probiotics may be defined as, health-promoting live bacteria that beneficially affect the microbial intestinal balance of the host. *Bifidobacterium* spp. are predominant in the intestinal microflora of infants and are considered to play an important role in maintaining their health. These bacteria suppress harmful intestinal bacteria, which is also considered to be an important activity in human nutrition. Some *Lactobacillus* species and *Bifidobacterium* spp. are used in the production of (therapeutic) yogurts.

Prebiotics may be defined as nondigestible food ingredients of the diet that reach the colon intact and beneficially affect the host by selectively stimulating the growth and/or activity of probiotic bacteria in the colon. Lactulose (and oligosaccharides) are not absorbed in the small intestine, but migrate to the large intestine. In the large intestine, they appear to be utilized predominantly by all species of *Bifidobacterium* residing there. Moreover, the metabolism of these compounds stimulates the growth of a healthy intestinal flora.

Future Trends

In 2008, the global use of dairy ingredients reached 82 million metric tonnes, with an estimated growth of 3% per year. Growth is projected to continue into the future.

The interest in the use of dairy ingredients in nutritional and functional foods continues to grow. A greater knowledge on the relation between nutrition and health can be expected to stimulate the consumption of functional foods significantly. New product development will be important to the continued growth of dairy ingredients, since 50% of the growth of sales over the past decade has come from the development of new products.

Health and welfare concerns appear to be the driving force for future development of dairy ingredients. Market research suggests that the consumer wants appetizing products that will help control weight gain rather than just calorie restrictions.

Major growth areas will be in functional foods of all types. Most of the current knowledge of functionality has been gained through cellular and animal studies. There is a need for more and better clinical trials to confirm the value of these products in human health.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; Probiotics, Applications in Dairy Products. **Chocolate**: Milk Chocolate. **Dehydrated Dairy Products**: Infant Formulae; Milk Powder: Types and Manufacture. **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects. **Nutrition and Health**: Nutraceuticals from Milk. **Whey Processing**: Utilization and Products.

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Infant Formulae

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Introduction

Human milk from healthy and well-nourished mothers provides adequate nutrition for infants during the first few months of life and also significantly reduces the risk of acute and chronic diseases during this critical development period. When a mother cannot breast-feed or chooses not to breast-feed, the use of infant formula provides the next best option to meet the nutritional and health needs of the infant. Historically, the chemical composition of human milk served as a guide for the formulation of infant formula. In recent years, selected physiological outcomes have gained acceptance in documenting the functionality of novel ingredients, including some chemical entities not found in human milk. Technological sophistication may allow both composition and functional attributes of human milk to be achieved simultaneously.

This brief review describes many current nutritional and processing innovations in the infant formula industry.

Manufacturing and Packaging Technology

The manufacture of infant formula involves the blending of dairy or soy ingredients with vitamins, minerals, and vegetable oils to achieve a homogeneous solution, followed by sufficient heat treatment or dehydration to provide bacteriological safety. The final steps in the technology used for the manufacture of infant formulae have seen little change in the past 20 years, with powder manufacture, typically via spray drying, and heat sterilization (ultra-high heat or retort sterilization) of liquids being the processes of choice (**Figure 1**). The development of agglomeration (instantization) processes in the art of spray drying has contributed to improved reconstitution of infant formula powders. Dry blending of preprepared base powders provides flexibility for the manufacture of market-specific formulations, which may include, among others, heat-sensitive components, such as starches, flavors, probiotics, or bioactive proteins.

While the presentation of powders in nitrogen/carbon dioxide-flushed tinplate cans (400–2000 g) has many attributes such as robustness, tamper-proof, and rodent resistance, other packaging formats such as composite cans, aluminum foil packs, and single-serve foil sachets have also gained in popularity. Another recent novel packaging innovation is the use of tablets for commercial powder products.

Sterilized ready-to-feed (RTF) formulae conveniently presented in screw-capped glass jars (60–120 ml) or small plastic bottles continue to be the presentation of choice in hospitals and pediatric clinics. RTF formulae and liquid concentrates are also available in cans. Aseptically packaged ultra-high temperature (UHT)-sterilized RTF formulae are now available at retail outlets in several markets, while more expensive, UHT-sterilized RTF products, available typically in 200 ml to 1 l cartons, often with pouring and reclosure devices, or in plastic bottles with screw caps, are also popular with the consumers. These sterilized RTF formulae can be stored at room temperature prior to opening, so the inconvenience of bottle makeup is avoided. The development of new liquid-dosing technology has also allowed aseptic incorporation of heat-labile, bioactive components into UHT-treated products post-heat treatment and just prior to packaging.

Developments in Regulations and Authoritative Guidelines

Regulations governing the manufacture, composition, and labeling of infant nutritional products have evolved substantially over the last few decades. Today, despite some regional variations, there is substantial equivalency between the major regulatory groups, for example, the European Union Directives, Codex, and the USA Infant Formula Act (**Tables 1** and **2**). The European Union and Codex compositional standards have been reviewed and updated in the last 3–4 years. A generally accepted classification of infant nutritional products is outlined in **Table 3**.

Developments in Methods for Analysis of Human Milk and Infant Formulae

Many of the developments in formula composition have resulted from new analytical methods for the components in human milk, often from global human milk surveys,

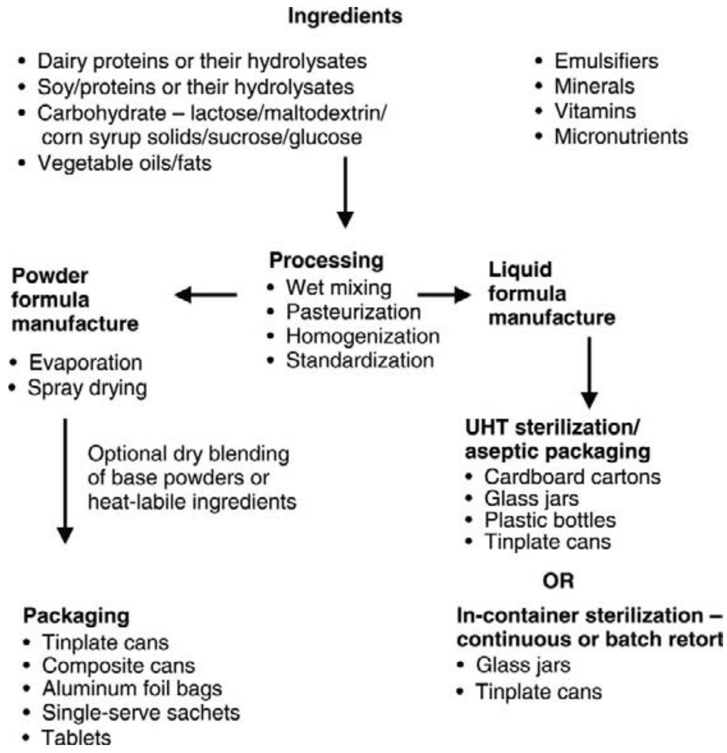
Ingredients
• Dairy proteins or their hydrolysates
• Soy/proteins or their hydrolysates
• Carbohydrate – lactose/maltodextrin/ corn syrup solids/sucrose/glucose
• Vegetable oils/fats
• Emulsifiers
• Minerals
• Vitamins
• Micronutrients
Processing
• Wet mixing
• Pasteurization
• Homogenization
• Standardization
Powder formula manufacture
• Evaporation
• Spray drying
Liquid formula manufacture
Optional dry blending of base powders or heat-labile ingredients
UHT sterilization/ aseptic packaging
• Cardboard cartons
• Glass jars
• Plastic bottles
• Tinplate cans
OR
In-container sterilization – continuous or batch retort
• Glass jars
• Tinplate cans
Packaging
• Tinplate cans
• Composite cans
• Aluminum foil bags
• Single-serve sachets
• Tablets

Figure 1 Commercial manufacture and packaging of infant formulae.

and from increased emphasis on the demonstration of functional benefits.

Methods for the determination of total protein have followed the lead of the dairy industry and measure total nitrogen using the Kjeldahl or the Dumas method. The new methods for the measurement of individual proteins in human milk and bovine milk-derived ingredients have enabled the development of infant formulae that match human milk in terms of the whey protein-to-casein ratio as well as the concentrations of specific individual proteins (e.g., α-lactalbumin (α-la)). The current techniques for protein identification and quantification include high-performance liquid chromatography (HPLC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunological methods such as enzyme-linked immunosorbent assays (ELISAs) and surface plasmon resonance (SPR). The methods for amino acid analysis ensure that the first-age infant formulae meet the minimum regulatory requirements for essential amino acids based on human milk levels. Legacy amino acid methods used ion exchange chromatography with post-column ninhydrin derivatization and had analytical run times of about 1 h. Currently, ultrahigh performance liquid chromatography (UPLC) uses pre-column derivatization, with chromatographic run times reduced to less than 10 min.

The analytical methods for lipids in human milk and infant formulae include the gravimetric procedure based on solvent extraction for total fat. In addition to matching the total fat composition range of human milk, measurement of the human milk fatty acid profile using gas chromatography with flame ionization detection (GC-FID) has provided another compositional target for infant formula manufacturers. Further development of these methods by incorporating the use of enzymes to simulate infant fat digestion can provide the positional distribution of individual fatty acids on the intact triglyceride.

The analytical methods for carbohydrates provide a relatively new area of opportunity. Through the development of carbohydrate analysis using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), individual human milk oligosaccharides can be measured in human milk and infant formulae.

Methods of analysis for vitamins have been developed and validated by both formula manufacturers and compendial sources for formula ingredients and finished products. HPLC assays for water-soluble vitamins have better precision and accuracy than the previous generation of microbiological assays. However, the molecular specificity of individual assays has sometimes resulted in misunderstandings between manufacturers and regulatory agencies. For example, folate that occurs naturally in milk is comprised of numerous polyglutamic acid derivatives of folic acid with known lesser bioavailability than that of folic acid itself. Selection of a test method therefore

Table 1 Regulatory limits for first-age (adapted) infant formulae

		CODEX A C[a]		*European Union*[b]		*USA IFA*[c]	
Nutrient	*Units per 100 kcal*	*Min*	*Max*	*Min*	*Max*	*Min*	*Max*
Protein	g	1.8	3.0	1.8	3.0	1.8	4.5
Fat	g	4.4	6.0	4.4	6.0	3.3	6.0
Linoleic acid	mg	300		300	1200	300	
Linolenic acid	mg	50		50			
Carbohydrate	g	9.0	14	9.0	14		
Vitamin A	IU	200	600	200	600	250	750
Vitamin D	IU	40.0	100	40.0	100	40.0	100
Vitamin E	IU	0.5		0.5	5	0.7	
Vitamin K	μg	4.0		4.0	25	4.0	
Vitamin B_1	μg	60		60	300	40	
Vitamin B_2	μg	80		80	400	60	
Vitamin B_6	μg	35		35	175	35	
Vitamin B_{12}	μg	0.1		0.1	0.5	0.15	
Niacin	μg	300		300	1500	250	
Folic acid	μg	10		10	50	4.0	
Pantothenic acid	μg	400		400	2000	300	
Biotin	μg	1.5		1.5	7.5	1.5	
Vitamin C	mg	10		10	30	8.0	
Choline	mg	7.0		7.0	50	7.0	
Inositol	mg	4.0		4.0	40	4.0	
Calcium	mg	50		50	140	50	
Phosphorus	mg	25		25	90	25	
Magnesium	mg	5.0		5.0	15	6.0	
Iron	mg	0.45		0.3	1.3	0.15	
Zinc	mg	0.5		0.5	1.5	0.5	
Manganese	μg	1.0		1.0	100	5.0	
Copper	μg	35		35	100	60	
Iodine	μg	10		10	50	5.0	
Sodium	mg	20	60	20	60	20	60
Potassium	mg	60	180	60	160	80	200
Chloride	mg	50	160	50	160	55	150
Selenium	μg	1.0		1.0	9.0		
L-Carnitine	mg	1.2		1.2			
Taurine	mg		12		12		
Nucleotides	mg				5.0		

[a] FAO/WHO Codex Alimentarius Commission Standard for infant formula: Stan 72-1981, including amendments and revisions.
[b] Commision of the European Communities Directive 2006/141/EC on infant formulae and follow-on formulae, Official Journal of the European Communities, 2006, amending Directive 1999/21/EC.
[c] Infant Formula Act of 2003, Code of Federal Regulations, Title 21: Food and Drugs.

defines the label claim analyte to either include or exclude particular naturally occurring forms. Similar examples exist in the case of fat-soluble vitamins where the biological activity may be contributed by various isomers or molecular forms. Since many infant formula manufacturers supply markets in various regulatory environments, a harmonization of analyte identity and modernization of reference methods could improve transparency between product composition and label claims.

Analysis of the mineral composition of infant formulae has evolved from atomic absorption to inductively coupled plasma with mass spectrometry (ICP-MS). The current methods are sufficiently rugged to ensure the finished-product levels of trace minerals as well as to guard against heavy metal contamination.

First-Age Infant Formulae

Proteins

In general, the formulation of dairy-based first-age infant nutritional products involves a combination of demineralized whey and skim milk solids to satisfy the regulatory requirements for total protein content and essential amino acid profile. The introduction of a whey protein-dominant (60:40, whey protein:casein ratio) formula in 1961 was a significant milestone in the humanization of infant formulae, as it mimicked the whey protein-to-casein ratio of human milk. However, the protein content of infant formulae, at 13–15 $g\,l^{-1}$, is higher than that of human milk (9–11 $g\,l^{-1}$). This is to compensate for the relatively low levels of certain essential amino acids such as tryptophan, tyrosine, and

Table 2 Regulatory limits for follow-on (second-age) formulae

		CODEX A C[a]		*European Union*[b]	
Nutrient	*Units per 100 kcal*	*Min*	*Max*	*Min*	*Max*
Protein	g	3.0	5.5	1.8	3.5
Fat	g	3.0	6.0	4.0	6.0
Linoleic acid	mg	300	300	1200	
Linolenic acid	mg		50		
Carbohydrate	g		9.0	14	
Vitamin A	IU	250	750	200	600
Vitamin D	IU	40	120	40	120
Vitamin E	IU	0.7		0.5	5.0
Vitamin K	μg	4.0		4.0	25
Vitamin B_1	μg	40		60	300
Vitamin B_2	μg	60		80	400
Vitamin B_6	μg	45		35	175
Vitamin B_{12}	μg	0.15		0.1	0.5
Niacin	μg	250		300	1500
Folic acid	μg	4.0		10	50
Pantothenic acid	μg	300		400	2000
Biotin	μg	1.5		1.5	7.5
Vitamin C	mg	8.0		10	30
Choline	mg				
Inositol	mg				
Calcium	mg	90		50	140
Phosphorus	mg	60		25	90
Magnesium	mg	6.0		5.0	15
Iron	mg	1.0	2.0	0.6	2.0
Zinc	mg	0.5		0.5	1.5
Manganese	μg			1.0	100
Copper	μg			35	100
Iodine	μg	5.0		10	50
Sodium	mg	20	85	20	60
Potassium	mg	80		60	160
Chloride	mg	55		50	160
Selenium	μg			1.0	9.0
L-Carnitine	mg				
Taurine	mg				12
Nucleotides	mg	-	-	-	5.0

[a] FAO/WHO Codex Alimentarius Commission Standard for Follow-Up Formula: Stan 156-1987, including amendments.

[b] Commision of the European Communities Directive 2006/141/EC on infant formulae and follow-on formulae, Official Journal of the European Communities, 2006, amending Directive 1999/21/EC.

cysteine in protein sources. Also, the protein profile of bovine milk and human milk differ significantly. Human milk does not contain β-lactoglobulin (β-lg) and α_{S2}-casein, but has much higher concentrations of β-casein, α-la, and lactoferrin (Lf) than bovine milk; thus, there is considerable commercial incentive for industrial-scale fractionation of bovine milk proteins for application in infant formula manufacture. The human and bovine fractions of these proteins have more than 50% homology and also have similar functionality. Fractionation processes, for example, selective precipitation, membrane separation, chromatography, or combinations thereof, have been used to isolate and enrich these protein fractions.

The recent development of α-la-enriched formulations has been shown clinically to promote gastrointestinal tolerance similar to that of human milk-fed infants. In addition, α-la is a rich source of essential amino acids such as tryptophan. Tryptophan and its metabolite serotonin are essential for brain maturation and the development of other neurobehavioral regulators of food intake, satiation, and sleep–wake rhythm.

Higher protein intake is proposed to be one of the causal factors that may lead to obesity in later life. It is also known that growth kinetics of breast-fed and formula-fed infants are different during the first year of life. Recently, novel enrichment technologies have led to ingredients that have a higher protein quality, enabling manufacturers of infant formulae to reduce the total protein content. Clinical studies have shown that these lower-protein formulae can bring some anthropometric

Table 3(a) General classification of formulae intended for infants and young children in good health

Product	*General description*	*General properties*	*Intended age bracket*	*Applications/comments*
First-age infant formulae	1. Whey dominant (adapted)	Whey:casein = 60:40	From birth to 6 months	Nutritionally complete Breast milk substitute for healthy infants
		Casein:whey = 80:20		
	2. Casein (curd) dominant			
	3. Hypoantigenic formulae (HA)	Hydrolyzed proteins 100-fold reduction in allergenicity	From birth to 6 months	Nutritionally complete Breast milk substitute for prevention of milk protein allergy
	4. Easy to digest formulae	Partially hydrolyzed proteins Reduced lactose	From birth to 6 months	Nutritionally complete Breast milk substitute for healthy term infants with general formula intolerance issues
	5. Soy-based	Milk protein-free and lactose-free	From birth to 6 months	Nutritionally complete For treatment of lactose or milk protein intolerance
Second-age or follow-on formulae	1. Casein-dominant formulae	Casein:whey = 80:20 High in protein, Ca, Fe, and vitamins	From 6 months to 1 year	To be used as substitute for cow's milk Not necessarily nutritionally complete
	2. Soy-based formulae	Milk protein-free and lactose-free	From 6 months to 1 year	For the treatment of lactose or milk protein intolerance Used as substitute for cow's milk

Table 3(b) General classification of formulae intended for infants with special nutritional needs – to be used under medical supervision

Product	*General description*	*General properties*	*Intended age bracket*	*Applications/comments*
Low-birthweight (LBW) formulae	Whey-dominant (adapted)	Whey:casein = 60:40	From birth as required until progression to post-discharge formulae or first-age formulae	Nutritionally complete
		High in protein, Ca, P		Breast milk substitute for LBW or premature infants
Post-discharge formulae	Whey-dominant (adapted)	Whey:casein = 60:40	From hospital discharge of LBW infants as required until progression to first-age formulae	Nutritionally complete
		High in protein, Ca, P		Breast milk substitute for LBW or premature infants
Foods for special medical purposes	1.Lactose-free (LF)	Lactose less than 0.2 g per 100 g powder	From birth to 6 months	Nutritionally complete
	Whey- or casein-dominant			For treatment of lactose intolerance
	2. Antiregurgitation (AR) formulae	Casein-dominant Contain starch or other thickeners	From birth to 6 months	Nutritionally complete
				Breast milk substitute for infants prone to gastroesophageal reflux
	3. High-caloric or nutrient-dense formulae	Generally whey-dominant	From birth to 12 months	Nutritionally complete
				Breast milk substitute for infants small for gestational age, in pre- or postoperative care
	4. Extensively hydrolyzed protein formula	Casein/whey protein hydrolysates	From birth to 12 months	Nutritionally complete
				Breast milk substitute for management of atopic CMA
	5. Low-phenylalanine formulae	Casein hydrolysates <0.08% Phe	From birth to 12 months	Nutritionally complete Breast milk substitute for the treatment of phenylketonuria
	6. Elemental diets	Free amino acids Chemically defined	From birth as required	Nutritionally complete Breast milk substitute for the treatment of IgE-mediated cow's milk allergy or atopic infants

and biochemical measures much closer to those of breast-fed infants.

Lactoferrin (Lf) is the second most abundant whey protein in human milk (~1.4 g l^{-1}) and has been shown to possess bacteriostatic and bactericidal activities. Relatively pure preparations of Lf from bovine milk and whey have been available for a number of years. However, Lf-enriched infant formulations have been commercialized to a limited extent, mainly due to the cost of the ingredients and the challenges in conserving the biofunctionality of Lf during the manufacture of infant formula. In clinical studies, infants receiving Lf-enriched infant formulae showed higher hematocrits and a significant reduction in the incidence of lower respiratory tract illnesses.

Casein-dominant formulae are generally perceived to be more satisfying for hungry infants than whey protein-dominant formulae. In contrast to bovine milk, β-casein is the principal casein in human milk, accounting for >70% of total casein. The recent industrial developments in the manufacture of high-purity β-casein may assist in furthering the humanization of infant formula; There are limited preclinical data and no clinical data available on β-casein-enriched formulae.

As potential ingredients for use in infant formulae, certain hydrolysis products of caseins have been shown to have antimicrobial activity toward pathogenic bacteria of significance in the infant formula industry (e.g., *Cronobacter sakazakei*). Other minor proteins of interest in the infant formula industry include milk basic protein, immunoglobulins, lactoperoxidase, osteopontin, glycomacropeptide, and milk fat globule membrane proteins. Enriched fractions of some of these components are commercially available, albeit to a limited extent.

Lipids

The production and supply of high-oleic safflower oil in the 1970s helped infant formula fat blends achieve a fatty acid profile and a ratio of saturated, monounsaturated, and polyunsaturated fatty acids closer to that of human milk. All-vegetable fat blends also enabled absorption of fat and calcium comparable to human milk. However, the progress in analytical techniques revealed that the positional distribution of fatty acids, especially palmitic acid (a major contributor of ~20% of total fatty acids in most infant formulae), was different from that of human milk. In vegetable oils, palmitic acid is located at *Sn*-1 and *S*n-3 positions of the triacylglycerol, whereas in human milk it is predominantly at the *Sn*-2 position. Due to cleavage preferences of digestive enzymes in humans, the palmitic acid at the *Sn*-1 and *Sn*-3 positions is cleaved from the triglyceride allowing free palmitic acid to form insoluble calcium soaps in the intestinal lumen. This may lead to differences in stool consistency between formula-fed and breast-fed infants and potentially decrease calcium bioavailability. Industrial transesterification of commercial vegetable oils to produce triacylglycerols enriched in *Sn*-2 palmitic acid, making them structurally similar to human milk triacylglycerols, enabled infant formula fat blends to be formulated with such oils. Alternatively, limiting the amount of palmitic acid in infant formula can improve net retention of minerals and increase bone mineral density, even in the absence of any detectable effect on anthropometric indices of growth.

Human milk contains both n-3 and n-6 long-chain polyunsaturated fatty acids (LCPUFAs), which are absent in infant formula, if not supplemented. Term infants need an exogenous supply of LCPUFAs to achieve similar accretion of fatty acids in plasma and red blood cells in comparison to breast-fed infants. The beneficial functions of LCPUFAs in infants include improvements in visual acuity and electrophysiologic (EEG) response, improved immune status, and better problem-solving skills. However, it is imperative that a balance of arachidonic acid (AA) and docosahexaenoic acid (DHA) (at least in 1:1 ratio) be supplemented, as decreased growth and impaired language development have resulted when DHA only was supplemented. Recommendations by authoritative bodies (WHO, Child Health Foundation) have stated that both DHA and AA should be supplemented simultaneously.

The advent of LCPUFAs in infant formula was closely linked with the development of a novel technology: production of DHA- and AA-rich oils from single-cell organisms grown in bioreactors. The use of DHA extracted from fish oil is limited by the presence of an LCPUFA not present in significant concentrations in human milk, namely, eicosapentaenoic acid, which is antagonistic to the functions of AA. An alternative food source of AA is egg phospholipids. Recombinant strains of canola have been created to produce DHA-rich food-grade oil, and transgenic approaches utilizing mammalian enzymes to synthesize both DHA and AA in plant sources have been developed, but these techniques have not been used in commercial infant formulae to date.

Another area of lipid research of interest is modification of the phospholipid and glycosphingolipid content of infant formulae. These components are present in human milk, and technological improvements have enabled commercial production of dairy-based fractions containing a mixture of these components. To a limited extent, the dairy industry has developed and commercialized milk fat globule membrane fractions, enriched in gangliosides (and nutritionally significant phospholipid fractions). Gangliosides have biological activities of importance in infant growth and development, for example, anti-infective, neural development, anti-inflammatory, and prebiotic. Functional benefits of these components (either individually or in combination) in infant formulae as compared to breast milk need to be explored through clinical trials.

Conjugated linoleic acid (CLA) is present in human milk at levels similar to that of LCPUFAs. Endogenously synthesized CLA from vaccenic acid or CLA mobilized from maternal adipose tissue stores can be secreted into milk. In addition, exogenous dietary CLA can also be secreted into human milk from the lactating mother's diet. Despite compositional similarity, the functional benefits of CLA addition to infant formula need further evaluation.

Carbohydrates

Surprisingly, the third most abundant component of human milk, behind lactose and fat, is not protein, but oligosaccharides, comprising about 1 g l^{-1} in mature milk. A large variety of human milk oligosaccharides (HMOs) have been identified, numbering in excess of some 130 distinct moieties. Two of the major acidic HMOs are 3′sialyllactose (3′SL) and 6′sialyllactose (6′SL). In bovine milk, 3′SL is secreted in higher amounts than 6′SL. Investigation into the biological functions of HMOs is still a rather young science, but some HMOs have structural similarity to glycoprotein sugar moieties projecting from intestinal epithelial cells into the lumen that act as receptors for bacterial pathogens. As such, HMOs may act as decoys to prevent the attachment of pathogens to the gastrointestinal (GI) tract. Alternatively, HMOs may bind to the cell surface carbohydrates, blocking the pathogen attachment on receptors. HMOs also have prebiotic effects, that is, HMOs are competitively metabolized by intestinal bacteria favorable to gastrointestinal health, such as bifidobacteria and lactobacilli.

Due to the interesting biological activities associated with HMOs, much work has been done on developing HMO-enriched fractions for incorporation into infant formulae. In the past, enzymatic synthesis of selected HMOs has been achieved and these have been tested with some success. Some exosaccharides produced by bacteria contain a core oligosaccharide unit structurally similar to HMOs. More recently, research has focused on the enrichment of selected HMOs from bovine milk-based sources using fractionation and enrichment techniques such as membrane filtration, ion exchange chromatography, demineralization, and crystallization. Chemical coupling of mono- or di-saccharides using specific linkages is also becoming an increasingly cost-effective approach to generate HMOs. In the future, each of these methods may provide a commercially viable source of HMOs for use in infant formulae.

In addition to HMOs, other oligomers of simple sugars, for example, oligofructose isolated from inulin, and galacto-oligosaccharides enzymatically synthesized from lactose have been shown to confer prebiotic activity. These molecules are chemically distinct from any naturally occurring HMOs, but have been added to commercial infant formula due to their clinically proven biological benefits, for example, prebiotic and immunostimulatory activity and improved gastrointestinal tolerance.

Vitamins and Other Micronutrients

Specific regulatory requirements for essential fat- and water-soluble vitamins enable infant formulae to provide adequate vitamin content to support infant growth and development. There has been little interest in mimicking the varied chemical forms of vitamins as they exist in human milk that may have unknown physiological effects. Vitamin E is present in human milk as various tocopherols, whereas the chemically pure, though racemic, active form of α-tocopherol is typically used in infant formulae. Folacin in human milk is comprised of a family of folates with only a fraction being present as free folic acid, while the latter form is added to the formula. In recent years, markers for assessing vitamin status have received interest. One such marker for vitamin D, 25-hydroxy D, has been measured in several population segments across the globe. Prevalence of vitamin D deficiency has been observed to be based on this marker status, and recently the American Academy of Pediatrics has increased the recommended intake of vitamin D for infants. Vitamin D deficiency may result in insufficient uptake of calcium and phosphorus leading to skeletal diseases.

Technological advances in the analysis of human milk have revealed the presence of both endogenous (e.g., nucleotides and choline) and exogenous nutrients (e.g., lutein and lycopene) derived from the mother's diet. Lutein is an example of one such component that confers important benefits on infants who are breast-fed. Lutein helps protect the infant's eyes from damage by filtering blue light and also acts as an antioxidant. Lactating mothers who ingest fruits and vegetables pass on lutein to their infants through breast-feeding. Lutein has recently been added to commercial infant formulae.

Minerals

Adequate calcium intake is vital to infant health, as calcium is the most abundant mineral in the body. Inadequate calcium uptake has long been associated with osteoporosis. Refined methods using stable isotopes have replaced classic balance studies and allowed a reassessment of the requirements for calcium during the first year of life. The use of dual X-ray absorptiometry (DEXA) facilitates the assessment of net calcium retention by measurement of bone mineral density. Measurement of bone mineral density has the advantage over short-term measurements of calcium bioavailability in that it integrates retention of calcium over the entire duration of the study.

Another mineral element critical to an infant's development is iron. Iron deficiency in the first year of life results in long-term cognitive decrements. Recent data support the adequacy of iron at 4 mg l^{-1} in milk fed to infants. This level accounts for the sequestration of iron from intestinal bacteria and is not believed to induce constipation.

Ultra-trace minerals (e.g., selenium) are added to most infant formulae. Since protein sources and lactose may also contribute innate minerals, including ultra-trace components, it is critical to manage the fortification of trace and other minerals to consistently meet label claim specifications in the finished product.

Follow-on Formulae and Growing-up Milks

Formulae developed for the second 6 months of life are designed as complementary foods for infants who have been introduced to some solid foods (**Table 3**). They are enriched in nutrients typically low in weaning foods, notably protein, calcium and iron, and n-3 fatty acids, though some second-age formulae may also serve as a complete source of nutrients. As the diet of young children diversifies, the task of supplying nutrients that otherwise are low in the total diet becomes increasingly difficult; then the objective is to provide a superior nutrient source as compared to cow's milk. Some suboptimal nutritional features of cow's milk include its high total fat content, high level of saturated fatty acids, presence of *trans* fatty acids albeit at low levels, a high solute load, excess electrolytes, paucity of iron, zinc, and other minerals, and a low content of some vitamins, for example, vitamin C and fat-soluble vitamins.

Foods for Special Medical Purposes

Some infants do not thrive on standard formulae. Formula intolerance is a catch-all term that includes any gastrointestinal distress, such as spitting up, constipation, or diarrhea. Each of the major energy sources in formulae has been modified in attempts to mitigate the discomfort of the affected infants.

Traditionally, soy-based formulae were used for their exclusion of both lactose and cow's milk protein, and they still account for ~20% of the formulae sold in the United States. Recently, milk protein isolates and whey protein concentrates from which lactose has been removed by membrane processing or enzymatic hydrolysis are used in the manufacture of lactose-free dairy-based infant formulae.

The enzymatic hydrolysis of milk proteins is a well-recognized process for reducing the allergenicity of the protein. Hydrolysates are typically prepared by partially or extensively hydrolyzing the protein using proteolytic enzymes, sometimes followed by ultrafiltration to remove the unhydrolyzed material and large polypeptides. Protein hydrolysates are generally bitter due to the exposure of hydrophobic amino acids.

The increasing incidence of allergic diseases in developed countries highlights the necessity and importance of hypoallergenic infant nutrition. Stringent regulations accompany claims for a hypoallergenic formula; for example, in Europe, a 99% reduction in immunoreactive protein must be demonstrated prior to claiming a reduction of risk to milk protein allergy. Extensively hydrolyzed whey proteins and free amino acids have been used in hypoallergenic, peptide-based formulae, as they have been proven clinically efficacious for management of highly allergic or atopic infants.

In comparison to intact protein-based formulae, partial hydrolysate-based formulae have shown improved absorption in an infant's digestive system. There is evidence that partial hydrolysis of proteins also results in more rapid and easier gastrointestinal digestion – something that underpins the recent development of easy-to-digest infant formulations. These formulae are targeted toward otherwise healthy term infants who have subclinical discomfort including fussiness, development of gas, and other gastrointestinal discomfort issues.

The occurrence of gastroesophageal reflux (GOR), that is, the involuntary passage of gastric contents into the esophagus, can be a distressing phenomenon. Food ingredients, such as locust bean gum or starches, which have the capacity to increase the viscosity of formulae after ingestion, are used in specially formulated anti-regurgitation (AR) formulae. The use of AR formulae has been shown to reduce the incidence of reflux episodes in GOR-prone infants.

Nutrient-dense formulae, typically 900 kcal l^{-1} (3800 kJ l^{-1}), are prescribed for infants with increased nutritional requirements, for example, infants categorized as small for gestational age (SGA) or those who fail to thrive. Collectively, these groups account for up to 5% of pediatric hospital admissions. Infants in pre- or post-operative care, or infants with conditions such as congenital heart disease or cystic fibrosis are also known to benefit from nutrient-dense formulae.

Besides classical nutrients, non-nutritive additions and processing methods are used in some formulae for special medical purposes. The area of probiotics in infant formulae has received much attention in recent years, and a wide range of probiotic-enriched infant formulae are now commercially available. Probiotics directly stimulate the GI immune system, with varying immunoglobulin responses, as a function of the bacterial strain and increase the populations of friendly bacteria in the GI tract.

Formulae for Premature and Low-Birthweight Infants

As medical technology improves, smaller and smaller low-birthweight (LBW) infants survive. Nutrition of very LBW infants is predicated on the concept of providing sufficient nutrient density to enable the same total body accretion of nutrients to occur as if the infant were still *in utero*. Therefore, higher concentrations of nutrients are required in LBW formulae than are present in term formulae. For example, the protein composition of human milk provides only about half the amount needed to maintain protein accretion at the intrauterine rate of tissue protein deposition. Similarly, increased calcium concentration in LBW formulae allows bone mineral accretion at greater levels than in infants fed human milk. Sodium is increased in LBW formulations to account for the reduced rate of sodium resorption in the immature kidneys. At the same time, a new constraint on the composition of early-life feeds has emerged with the advent of the concept of fetal origins of adult diseases. Suboptimal nutrition may result in organ systems with reduced metabolic capacity. If subsequent growth outpaces the trajectory of early-life growth, there may be excessive metabolic demand relative to capacity, resulting in the foundations of chronic diseases.

The milk of mothers having delivered preterm infants is nutrient-dense relative to the milk of mothers having delivered term infants, though not to the extent necessary to enable the infant to grow at the intrauterine rate. In many countries, there is an increased use of expressed milk from the preterm infant's mother, which may be supplemented by nutrient preparations, appropriately called human milk fortifiers (HMFs). HMFs are designed primarily to provide high-quality protein and calcium. Infants fed fortified mother's milk have improved growth rates as compared to infants fed nonfortified milk, though not always as great as infants fed premanufactured LBW formulae.

As nutrition science continues to progress, the composition of HMFs continues to improve. A new fortifier, comprised of bovine whey proteins, supported the same plasma amino acid levels as a special preparation of human milk protein.

A new product category has evolved in the form of an enriched formula for LBW infants, namely, post-discharge formula, for use after hospital discharge through the first year of life. At the time of discharge, LBW infants often have protein, energy and micronutrient deficits. Post-discharge formulae are designed to address these deficits and help with the catch-up growth for these infants.

Future Trends

Unlike other food manufacturers, the infant formula industry has a common gold standard in the form of human milk on which to model its product development. Therefore, it is reasonable to expect that the future trends will be substantially influenced by the developments in analytical science and the application of novel analytical tools in the study of human milk. Development of novel end-point measurements in human physiology will continue to demonstrate the biological role of milk components and other novel ingredients. The development of economically viable processes for the extraction and enrichment of such newly identified nutritional/functional components from other sources (primarily bovine milk) in quantities required by infant formula manufacturers will be key to realizing the benefits of such analytical advancements.

Much work has been done over recent decades in matching the chemical composition of human milk in infant formula. However, the development of next-generation infant formula is likely to expand to matching the biofunctionality of human milk-fed infants. This trend, coupled with the increasing regulatory challenges in substantiating health claims, will require significant investment in human clinical trials in the development and commercialization of scientifically advanced infant formulae.

Manufacturers face the challenge of devising robust processing technologies to facilitate the inclusion of bioactive molecules that maintain functionality and stability during the manufacture and shelf life of next-generation infant formulae.

Biotechnology and chemical synthesis will continue to be platforms for the manufacture of novel nutrients. The advent of methods to identify and quantify the minor components in human milk and new understanding into gene expression during human development and during infancy will identify new avenues for the industry.

Conclusions

Today, there is still much to learn about the composition and biology of human milk. This learning will trigger new technological approaches to formulate and produce infant formula that gets closer in composition and/or functionality to human milk. The research and development of infant formula will remain a lively area for some time to come.

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See also: **Cheese**: Public Health Aspects. **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders; Milk Powder: Types and Manufacture. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milk**: Human Milk. **Milk Lipids**: General Characteristics. **Vitamins**: General Introduction.

Further Reading

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DISEASES OF DAIRY ANIMALS

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Infectious Diseases: Bluetongue

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Introduction

Bluetongue virus (BTV) commonly infects cattle in wide tropical and subtropical zones over much of the world, typically between the latitudes 35 ° S and 40 ° N. Bluetongue (BT) is an infectious but noncontagious disease transmitted by biting midges of the genus *Culicoides*. However, since 1998, five BTV serotypes (BTV-1, BTV-2, BTV-4, BTV-9, and BTV-16) have been continuously present in the southern parts of Europe around the Mediterranean Sea. This extension of the territories where BTV is observed can be attributed mainly to climatic changes that allow the major vector (*C. imicola*) to survive and transmit the virus in those regions. Clinical disease rarely occurs in infected cattle; however, BT disease is important in sheep. More recently, in 2006, BTV-8 caused a severe epizootic of BT in northern Europe. This sudden emergence cannot be attributed to climatic changes. However, the spectacular spread of the infection can be explained by the favorable meteorological conditions that prevailed in Europe in the summer and fall of 2006, as well as in the spring of 2007. Since then, the epizootic has spread rapidly to many countries including Belgium, The Netherlands, Germany, France, Luxembourg, Switzerland, Denmark, Czech Republic, and the United Kingdom. This serotype is affecting cattle more often and more severely than what is usually seen with other serotypes in Europe. The epidemiological situation is further complicated in western Europe by the recent introduction of serotype 1 in Spain, which expanded to France in late 2007, and the outcomes of expected coinfection by serotypes BTV-1 and BTV-8 are unknown. BTV is a notifiable disease in the list of the World Organisation for Animal Health (OIE). It is also important to distinguish between potential BT in cattle and other diseases that are potentially economically devastating. Discovering the epidemiology of transmission and the role of BTV in producing disease in cattle has been an intense endeavor of many workers over several decades. Some of this interesting history and evolution of knowledge is summarized in a number of authoritative reviews. This article summarizes the current state of understanding of BT and BTV infection and control in cattle.

Causes

The BTV is the prototype virus of the *Orbivirus* genus (family Reoviridae), and species belonging to this genus have segmented, double-stranded RNA genomes. The genus *Rotavirus* is another well-known virus in the family Reoviridae that is relevant to dairy scientists. Other orbiviruses that cause animal disease are the African horse sickness virus in horses and the epizootic hemorrhagic disease virus (EHDV) in deer but also sporadically observed in cattle. These viruses are serotypically diverse, and this, combined with the use of varying laboratory assays over the years, each possessing differing sensitivity and specificity characteristics, has made it a challenging task to thoroughly describe the epidemiology and disease pathogenesis of bovine BT. Twenty-four serotypes of BTV are recognized, and there is substantial genetic diversity within serotypes. On the African continent, where BTV is largely endemic, serotypes BTV-1 to BTV-16, BTV-18, BTV-19, and BTV-24 have been isolated. Serotypes BTV-1 to BTV-4, BTV-7, BTV-9, BTV-10, BTV-12, BTV-15 to BTV-17, BTV-20, BTV-21, and BTV-23 have been isolated in Asia, and BTV-1, BTV-3, BTV-9, BTV-15, BTV-16, BTV-20, BTV-21, and BTV-23 are recognized in Australia. Since 1998, BTV has been considered endemic in southern parts of Europe (Spain, Portugal, Greece, Italy, Corsica, Sardinia, and the Balkans) and, since 2006, in northern parts also (France, Belgium, Germany, Luxembourg, and The Netherlands). Six BTV serotypes (BTV-1, BTV-2, BTV-4, BTV-8, BTV-9, and BTV-16) are present in those regions coming from three different epidemiological systems. The first one is located in the eastern part of the Mediterranean Sea and the serotypes involved (BTV-1, BTV-4, BTV-9, and BTV-16) seem to come from the Near, Middle, and Far East. The second system is located in the western part of Europe and the strains (BTV-1, BTV-2, BTV-4, and BTV-16) seem to come from North Africa. The third one (BTV-1 and BTV-8) is located in northern Europe; BTV-1 emerged in northern Spain and is spreading to the north. Strain BTV-8 seems to be related to a strain from South Africa. However, the manner in which these two serotypes spread to western Europe is unknown. Serotypes BTV-1, BTV-3, BTV-4, BTV-6, BTV-8, BTV-12, and BTV-17 have been isolated in Central and South America and the Caribbean, and BTV-2, BTV-10, BTV-11, BTV-13, and BTV-17 are recognized in North America.

Whether BTV has a role in causing bovine disease was uncertain for many years. Evidence observed in natural outbreaks of BT-like disease among cattle in several herds in South Africa in the 1930s first challenged the dogma that BTV is not pathogenic for cattle. A thorough investigation was instituted among these herds, which presented with several cases of ulcerative stomatitis and coronitis, in order to rule out foot and mouth disease. Blood taken from cattle before reaching a late stage of clinical disease induced BT symptoms in sheep that were not previously exposed to BTV. The sheep were subsequently resistant to challenge with known virulent BTV. Also, naive calves inoculated with blood from outbreak cases developed ulcerative stomatitis. Blood from these calves produced BT symptoms when inoculated into sheep. Sheep that were inoculated with blood from cattle in an advanced clinical state did not develop BT symptoms and remained susceptible to BTV experimental challenge. Although these investigations were subsequently challenged, the groundwork was laid for continued investigation of the bovine–BTV relationship. Investigations of some other outbreaks over the years failed to clearly establish BTV as the cause of BT-like symptoms in cattle. Part of the challenge has been in distinguishing BTV from EHDV. Indeed, on a few occasions, outbreaks of BT-like symptoms in cattle have been attributed to EHDV.

Experimental evidence for clinical BT in cattle has been variable too. Clinical symptoms have been successfully produced in some experiments, but not in others. Experimentation with the virus is complicated by the wide variety of serotypes and differential susceptibilities of bovids to individual serotypes. More recent experimentation has suggested that disease symptoms are a result of an inflammatory response to BTV and that clinical disease, including characteristic oral lesions, occurs when cattle are challenged after some previous exposure to BTV antigen. It is now accepted that BTV is capable of producing disease in cattle, but usually very rarely. However, the recent emergence of BTV-8 in northern Europe clinically affects up to 10% of the infected cattle. A persistent question though is why cattle are so differentially susceptible to pathological effects of BTV infection relative to ovids despite apparent similarities in the pathology. It has been hypothesized that bovine and ovine endothelial cells are differentially susceptible to BTV attachment and penetration.

Signs of Disease

Natural BTV infection of cattle is very rarely accompanied by clinical signs. There is some evidence that pathological changes are due to an IgE-mediated type I hypersensitivity reaction. First time infection of cattle with BTV would not be expected to produce clinical signs by this mechanism. When signs do occur, they are similar to many of those signs seen in clinical ovine cases, including initial superficial hyperemic lesions on dental pads, mucocutaneous junctions, and on the ventrum of the tongue, followed by erosions that range in size from very small to 10 mm in diameter (**Figure 1**). Inflammation of the coronary bands and even distal limb swelling may also be observed (**Figures 2** and **3**), sometimes accompanied by hyperemia and exudation, especially on the plantar surfaces, and ulcerations. Severe cases may also present with lesions on the external nares, buccal papillary areas, lips, tongue, and muzzle (**Figure 4**). Transient febrileness occurs, but may be resolved before clinical signs are evident. Similar lesions are sometimes observed on the teats, which become quite painful (**Figure 5**). Skin thickening and crusting lesions may appear later in the progression of clinical signs, especially on the back of the animals, and these can be

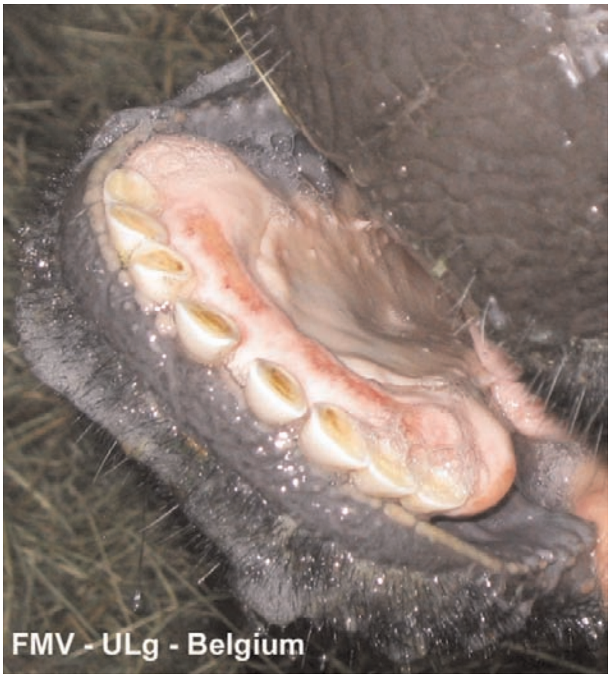

Figure 1 Ulcers on the tongue and on the gingiva, especially behind the incisors and on the incisor pad with hypersalivation.

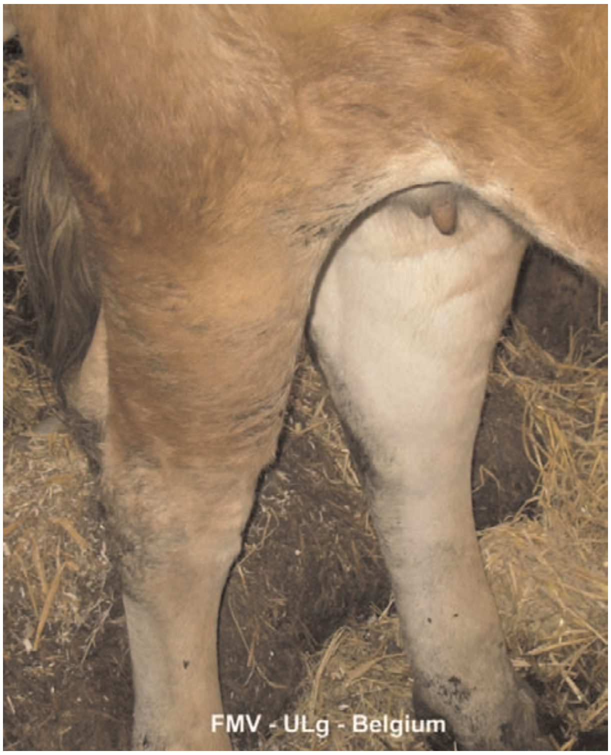

Figure 2 Edema of the limbs at various levels.

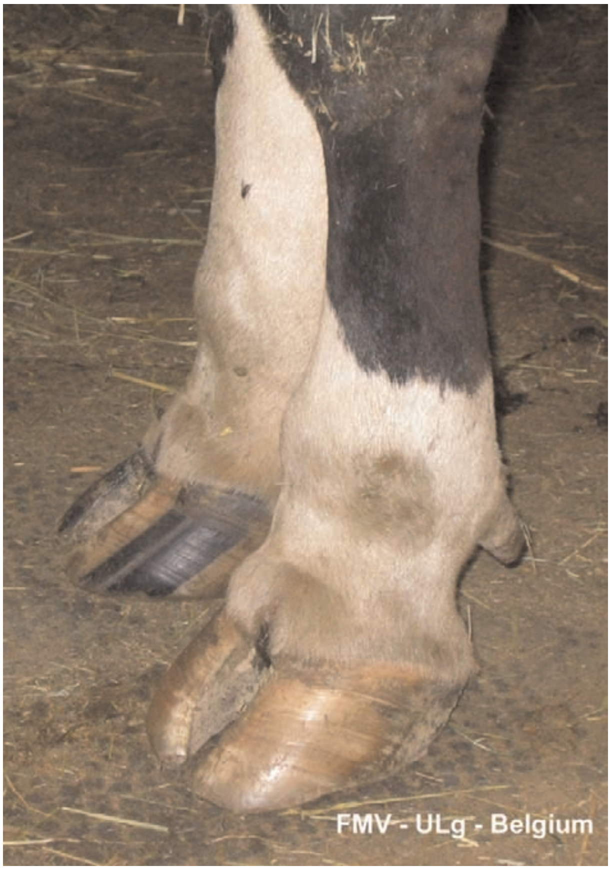

Figure 3 Edema of the distal limbs at various levels.

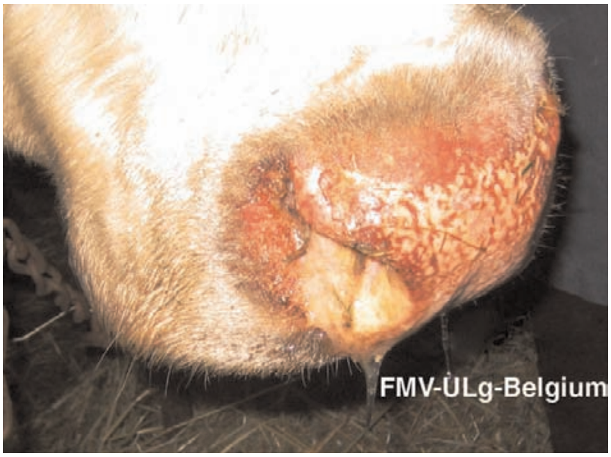

Figure 4 Ulcers inside the nostrils; mucous, mucopurulent to muco-hemorrhagic nasal discharge.

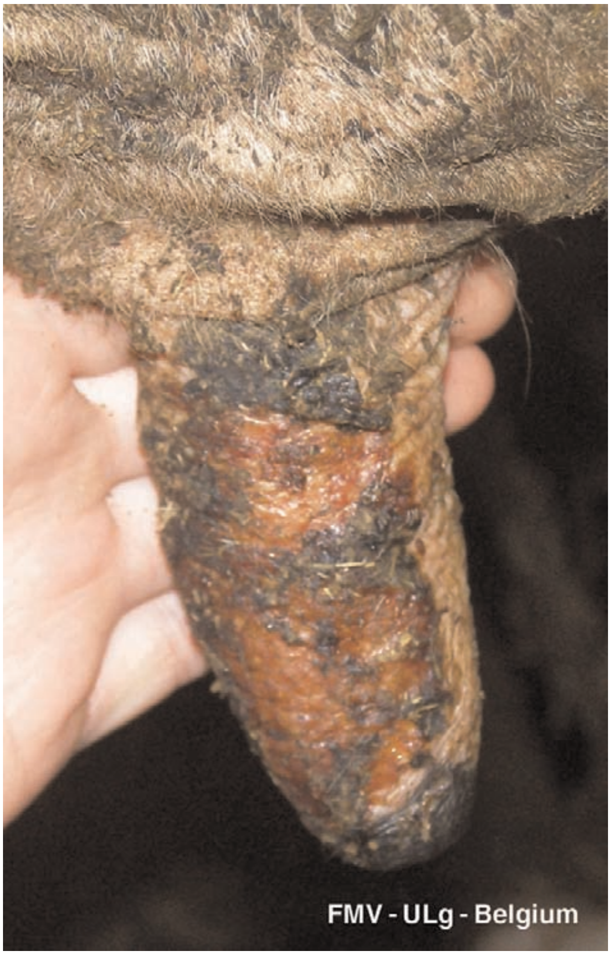

Figure 5 Ulcerative and necrotic lesions of the teats.

confused with photosensitization lesions in white areas of the skin (**Figure 6**). The first signs of clinical disease in a dairy herd may be a drop in milk production and stiffness of gait, or even more severe lameness in 10–15% of the animals. Care must be taken in the diagnostic workups of outbreaks of clinical signs to distinguish causation by BTV from EHDV and other viruses that cause erosive lesions or vesicular lesions of the oral mucosa or coronary band. Other important differential diagnoses include bovine viral diarrhea and mucosal disease, infectious bovine rhinotracheitis, malignant catarrhal fever, vesicular stomatitis, mycotic stomatitis, bovine papular stomatitis, rinderpest, and foot and mouth disease. Unfortunately, the minimum conditions necessary to stimulate pathological changes in infected cattle in a field environment are not understood.

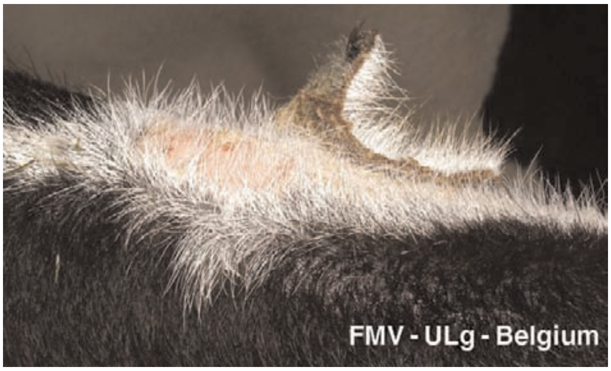

Figure 6 Various necrotic skin lesions can affect bluetongue-positive cattle, on the back and near the tail. In some cases, necrotic lesions are observed with skin flaps.

Reproductive health of BTV-infected dairy cattle may be compromised too. Abortions, early embryonic death, birth of full-term nonviable fetuses, and congenitally malformed fetuses have all been attributed to bovine BTV infection. Abortion caused by BTV infection has been experimentally induced. Some abortion outbreaks have been assumed to be attributable to BTV by virtue of failure to diagnose other known abortigenic infectious agents among cattle with rising BTV titers and are accompanied by isolation of BTV from aborted fetuses. In some outbreaks, abortions have been the first clinical sign recognized by producers. The hypothesis that BTV causes early embryonic death is based on descriptive investigation of herds with low first-service conception or calving percentages. Experimental efforts to confirm early embryonic death have failed and rigorous epidemiological studies under natural conditions have not been conducted. Hydranencephaly has been observed in calves that were experimentally infected *in utero* with serotypes BTV-10, BTV-13, and BTV-17 between 85 and 125 days of gestation. BTV has a predilection for undifferentiated neuronal tissue, ultimately leading to necrosis of the infected cells. The ability of BTV to establish congenital infection is not only serotype specific, but also varies by strains within serotypes as well. Neither persistent viremia nor immunotolerance to BTV results from fetal infection. Although it is clear that BTV can cause fetal anomalies and pregnancy wastage,

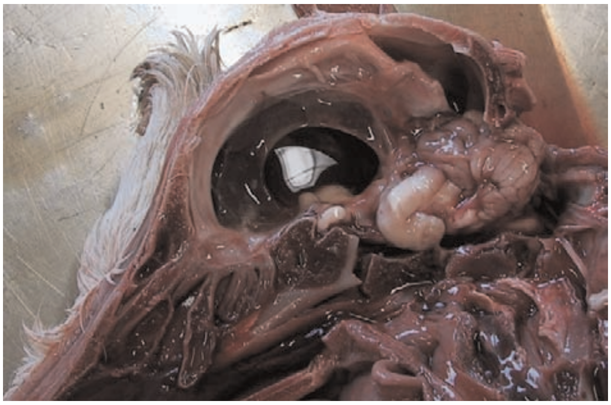

Figure 7 Hydranencephaly in a newborn calf born from a cow infected by BTV serotype 8 during pregnancy. Courtesy of Dr. M. Saulmont and J. Bughin, ARSIA, Belgium.

the importance of BTV in this regard relative to other infectious processes is not thoroughly described and, like nonreproductive clinical signs, is probably usually extremely low. However, many cases of reproductive failure at the herd level are reported with the serotype BTV-8 in northern Europe, mainly a low conception rate, increased rates of abortion, and congenital anomalies (hydranencephaly) (**Figure 7**).

Although male reproductive tract damage has been seen once in an experimental infection, the male bovine reproductive tract is not considered highly susceptible to pathological effects of BTV. The virus can, however, be secreted in the semen during periods of viremia, and is associated with contamination of semen with red blood cells or mononuclear cells carrying BTV. Transient infertility of viremic bulls has been reported, but is not well established. This has been especially observed in northern Europe during the BTV-8 outbreak. In contrast, infected rams are temporarily infertile in association with febrile signs. Just as is true with nonreproductive clinical symptoms, dogmatic statements of the effect of BTV on the male reproductive tract are limited by variation in experimental conditions and BTV challenge strains, and the degree to which alternative causes of natural disease have been ruled out. All things considered, clinical effects on male reproduction are infrequently recognized in cattle and are not of great importance, with the recent exception of the situation that occurred in the BTV-8 outbreak in northern Europe.

Diagnosis

The approach to diagnosis of BTV infection in cattle will depend on whether the goal is to clear animals for international import/export or to investigate on-farm clinical disease. The OIE prescribes two readily available serological assays, an agar gel immunodiffusion assay (AGID) and an indirect enzyme-linked immunosorbant assay (I-ELISA), for international trade purposes. However, a virus neutralization (VN) assay may also be employed if there is bilateral agreement between officials of the importing and exporting countries. All three of these assays detect antibodies as evidence of prior exposure to BTV antigen but do not indicate that infection is current.

Attempts to diagnose clinical infection are warranted when clinical signs are consistent with BTV-related disease and BTV infection is one of the conditions that should be ruled out. The AGID and VN assays and a competitive or blocking ELISA (C-ELISA) are readily available for detecting anti-BTV antibodies in serum. The AGID is not quantitative, but the VN and C-ELISA may be used to document rising antibody titers between sera obtained at a minimum of 2-week intervals. The C-ELISA and possibly the VN are considered to be more sensitive than AGID, although quantitative sensitivity and specificity estimates of the performance of these assays on cattle in the field have not been made. Cross-reacting EHDV-specific antibody may cause false-positive results in the AGID. The VN assay is used in a microtiter-plate format to identify antibodies against specific BTV serotypes.

Definitive diagnosis in clinical infection is done by polymerase chain reaction (PCR). Available PCR assays can detect BTV-specific nucleic acids with greater sensitivity and for a longer postinfection period than the virus isolation methods. Laboratory time for PCR is just a few days. New reverse transcription (RT) PCR test has been developed since 2006 and results are available within just a few hours. Quantitative real-time RT-PCR assays can estimate the quantity of viruses present in the sample. Virus isolation is an alternative method, but it is time consuming and is mainly used by reference laboratories to isolate and type the virus circulating in an outbreak. Common methods to isolate virus include inoculation in embryonated chicken eggs, susceptible sheep, or cell culture. These methods require 3–4 weeks. The difference between PCR and virus isolation methods lies in the fact that PCR detects both infectious and noninfectious viruses, while virus isolation detects only infectious viruses. Practically speaking, when viremia is looked for, its duration is much longer when investigated by PCR than by virus isolation. Whole blood in an anticoagulant (heparin, ethylenediamine tetraacetic acid (EDTA), or sodium citrate) may be obtained during febrile episodes for antemortem diagnosis. If storage in the absence of refrigeration is anticipated, blood should be collected in oxalate–phenol–glycerin (OPG). Whole blood collected in buffered lactose peptone may be frozen at −70 °C. BTV is not stable for long periods at −20 °C. Postmortem diagnosis is best performed on spleen or lymph nodes of postnatal to adult animals, whereas brain is the tissue of choice from aborted fetuses. Tissues should be stored and transported at 4 °C.

Control

Control of BTV infection and/or clinical disease may be based on immunization as well as on limiting exposure of livestock to the vector. Bovine BTV control recommendations are centered on fundamental knowledge of the descriptive and risk factor epidemiology of infection, modes of transmission, vector biology, and the epidemiology of clinical disease. Especially, costs and benefits of control must be carefully weighed.

The epidemiology of infection parallels the ecology of the vector. But in view of the high degree of exposure in endemic regions coupled with infrequency of clinical disease, efforts to control either infection or clinical disease are often economically unjustifiable. Seroprevalence within cattle herds is an indicator of the magnitude of exposure to BTV accumulated up to the point in time at which serum samples are drawn. Seroincidence measures the ongoing force of change in exposure of cattle to BTV, and as such is a more precise indicator of BTV infection. A number of seroprevalence studies indicate that there is a wide variation of within-herd seroprevalence from just a few percentage points up to more than 50% seropositive. Some variation is undoubtedly attributable to different sensitivity and specificity characteristics of the various assays that have been employed over the years. There is evidence of geographical clustering of within-herd seroprevalence as well, which is usually qualitatively associated with the distribution of specific *Culicoides* species known to be competent BTV vectors. Moreover, seroprevalence may be greater in increasing age categories of cattle on dairy farms. This could be the product of differential housing of age groups but may also result from cumulative exposure to BTV. Few descriptive studies document the incidence rate of new BTV seropositivities. One study conducted in Queensland, Australia, demonstrated a mean of 0.29 within-herd seroconversions per cow-year, but a very wide range among herds (0–3.45 seroconversions per cow-year). Seroconversions were temporally clustered from April to July in correspondence with temperate weather conducive to *Culicoides brevitarsis* breeding activity.

BTV is not transmitted directly between cattle, but by species of the *Culicoides* genus. In many locales, they are known as 'biting midges.' In Australia and the Caribbean, they are called 'sandflies' and carry the name 'no-see-ums' in the United States. (The *Culicoides* spp. 'sandflies' should be differentiated from the phlebotomous 'sandfly' (subfamily Phlebotominae, family Psychodidae), which are vectors for the genus of protozoal parasites that cause leishmaniases in humans and other species.) Experimentally infected bulls have been shown to infect naive heifers, but neither fetal infection nor maternal clinical disease is recognized to occur as a result. The natural incidence of BTV infection in cattle as a consequence of seminal shedding and sexual transmission is not known, but BTV shedding into the semen is inefficient. Past tests of commercial semen for BTV have shown that it is a very unlikely source of maternal BTV infection.

Although more than 1000 species of *Culicoides* (family Ceratopogonidae) are recognized, approximately 30 have been connected with BTV in some fashion and a mere 8 species have been shown before 1998 to serve as vectors for BTV: *C. actoni*, *C. brevitarsis*, *C. fulvus*, *C. imicola*, *C. insignis*, *C. variipennis*, *C. nubeculosus*, *and C. wadai*. A ninth species, *C. bolitinos*, may also be a competent vector in Africa. Since 1998, new species of *Culicoides* have been recognized as competent BTV vectors: *C. obsoletus*, *C. pulicaris*, *C. scoticus*, and *C. dewulfi*. They feed on livestock mostly outside and are typically most active at twilight. In northern Europe, the activity of several *Culicoides* species inside the buildings was recognized as an important epidemiological issue: when animals are kept indoors, potential vectors of BTV may be much more abundant in the stable than outdoors. Individual species of *Culicoides* exhibit preferences for certain hosts as the source of blood meals, but they are not absolutely host specific. Border areas between terrestrial and aquatic habitats that contain soft soil laden with biological material and suitable for burrowing are typical breeding grounds for these insects. In the United States, for example, the water–land interface of farm ponds or settling lagoons are a common breeding place for *C. variipennis*.

Fully elucidating the role of *Culicoides* spp. in BTV transmission, epidemics, and maintenance of endemicity has been a challenging task, unfolding over many decades. Competency of recognized *Culicoides* spp. that serve as BTV vectors varies by species and by BTV serotype. Although laboratory investigations have demonstrated that the susceptibility of specific *Culicoides* species to infection with individual serotypes of BTV can be modified by genetic selection, it is not known if natural selective pressure is strong enough to impact the epidemiology of BTV in specific locales. The *Culicoides* spp. are a biological vector, meaning that not only do they become infected with BTV upon taking a blood meal from a viremic animal, but BTV replicates in the insects as well. Efforts to demonstrate transovarial transmission of BTV in *C. variipennis* have failed and it is assumed that it does not occur among the other competent *Culicoides* spp. vectors. Other hypotheses that had been formulated to explain overwintering of BTV are persistence within surviving adult vectors themselves and prolonged or persistent infection in viremic hosts. Viremia is of longer duration in cattle (more than 100 days) than in sheep (14–45 days).

The morbidity risk of BT in cattle is extremely low, especially in endemic areas. Mortality risk is low to variable when clinical disease does occur. The recent European experience reinforces the fact that the introduction of BTV in a naive, high-density cattle population may be associated with a significant load of clinical cases. Clinical disease outbreaks, when they occur, are associated with coincident and irregular occurrence of climatic conditions that are unusually favorable to *Culicoides* spp. Outbreaks may also occur when infected *Culicoides* spp. are windborne from an endemic area to an incursive zone. Persistent infection of cattle does not lead to clinical manifestations.

Although it has been argued that regulatory, diagnostic, and vector control strategies are adequate to prevent major outbreaks of BTV disease in sheep in countries where BTV-associated disease has not been observed, BTV remains on the list of reportable diseases of the OIE. The last outbreak of BT in northern Europe in 2006 had proven that this decision was good, but it did not prevent the further spread of the infection. Windborne incursion of BTV-infected *Culicoides* into non-BTV endemic areas occurs and has caused clinical disease epidemics in the United Kingdom for example.

Vaccines for enhancing resistance of cattle and sheep to infection are available and transmission of BTV can be theoretically tempered by minimizing cattle exposure to *Culicoides* spp. Vaccines must be regionally tailored to include all of the relevant BTV serotypes and vaccination of cattle is not usually considered an efficient control method. Effective ongoing limitation of *Culicoides* spp. exposure is extremely difficult at best; consequently, BTV infection control in cattle herds is probably not economically justifiable from an individual farm perspective. BTV serotype 8 control in northern Europe could be more economically justifiable since more cattle are affected clinically and reproductive problems are more frequent. Inactivated BTV vaccines with adjuvant are proven efficient in a two-administration regime in cattle, enabling reduction or even elimination of viremia in short-term immunogenicity studies. The same vaccines are also used in one injection in sheep.

Treatment

There are no specific treatments for clinical BT. Therapy is purely supportive. Antibiotics may be strategically administered to aid prevention of secondary infections. Muzzle and teat lesions can be given topical care to alleviate discomfort and to minimize the risk of secondary infection. Animals with oral lesions should be separated and provided a diet designed to minimize mucosal trauma.

See also: **Diseases of Dairy Animals**: Infectious Diseases: Foot-and-Mouth Disease. **Office of International Epizooties**: Mission, Organization and Animal Health Code.

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Infectious Diseases: Brucellosis

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Introduction

Brucellosis (contagious abortion, infectious abortion, and Bang's disease) is an important disease of cattle, sheep, and many other mammalian livestock species. It is also a significant zoonosis, commonly via unpasteurized dairy products, and as a consequence of these two factors it is considered an important priority in many national animal health programs. It is widespread internationally, with relatively few countries not reporting the presence of the disease, at least prior to eradication programs, in the last 30 years. Brucellosis in humans (Malta fever or undulant fever) is recognized as an occupational hazard for those working with livestock or attending to derived tissue or fluid samples from them: for example, farmers, veterinarians, meat industry workers, and laboratory technicians. Both acute and chronic brucellosis is recognized, with the former characterized by undulant, severe fever, and the latter by a far wider range of clinical signs, with many nonspecific pathologies recognized.

Epidemiology

Brucellosis is a contagious disease caused by a facultative intracellular bacterium of the genus *Brucella*. The microorganism is a small Gram-negative coccobacillus, nonmotile, nonencapsulated, and non-spore-forming. There are numerous species that affect livestock, and there are also significant differences between biovars of each species in pathogenicity and specific pathologies arising from infection. *Brucella abortus* is the main cause of contagious abortion in cattle, and the majority of infections are due to a single biovar. *Brucella melitensis* is highly contagious to sheep and goats. *Brucella suis* infects pigs, and *Brucella ovis* infects mainly rams. Although brucellae have definite host preference, cross-infections do occur. Such infections are rare, significantly less severe, and are less likely to impact reproduction. Humans may contract infection with *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*. Whereas pasteurization, disinfectants, and direct sunlight kill the microorganism, it may survive for several months in tap water or damp soil. *Brucella* can survive for 30 days in urine, and 2–7 months in aborted fetuses and uterine exudate. The survival period of *Brucella* in liquid manure varies between 30 and 210 days, with viability extended in cooler temperatures. Domestic animals that excrete brucellae are the common sources of infection. Although horses or camels may carry *Brucella*, they do not play a significant role in the transmission of the organism. Numerous feral mammals are susceptible to brucellae, and feral pigs are known to transmit the disease to both domestic livestock and humans. In North America, wild bison and elk populations have been an important reservoir of *Brucella*, and have been implicated in transmission to brucellosis-free cattle populations. Dogs with naturally acquired brucellosis can become carriers and introduce brucellosis in *Brucella*-free herds. The single greatest source of infection in cattle is the contamination resulting from a *Brucella* abortion, with the placenta, amniotic fluid, and fetus containing very high concentrations of the organism. *Brucella abortus* continues to be excreted from the uterus in the discharges after abortion, but in significantly reduced numbers within several weeks.

The aborting dam is less likely to abort and excrete *Brucella* in subsequent pregnancies, but a carrier state is well-recognized postinfection and abortion. Carrier dams can transmit the organism, usually intermittently, in uterine fluids and fetal membranes, but also in milk, which has been suggested as a means of horizontal transfer within a herd. Infection with *Brucella* usually occurs by ingestion, through contact of the tongue or muzzle with aborted fetuses, fetal membranes, or uterine discharges of an aborting animal. Contamination of feed, water sources, and the general environment by direct contact with the aborted tissues may facilitate this, but is not considered a significant route of transmission. However, it is accepted that infection can also occur percutaneously, via the milk duct, mucous membranes, *in utero*, and infected semen produced in the acute phase of the disease in sires.

Age, sex, pregnancy, and stage of gestation are risk factors for brucellosis. Younger, sexually immature animals of both sexes are less likely to be clinically identified with brucellosis, reflecting a greater resistance or a shorter duration of natural infection compared with older stock, or both. Pregnant cattle are also more likely to become infected than sexually mature males, and the

[†] Deceased

risk of infection has been reported to increase with the stage of gestation. In addition, there would appear to be other significant risk factors associated with innate resistance, which are currently poorly understood, as show by the fact that individual infection or resistance in populations of broadly equal susceptibility and exposure could not be explained to date.

Clinical Findings

Brucellosis in Cattle

Brucellosis in cattle is nearly always due to infection with *B. abortus* although infection with *B. melitensis* is also recorded. The incubation period varies between 2 weeks and 2–3 months depending on the infective dose, stage of gestation, and previous exposure of the animal. After exposure, susceptible cattle become bacteremic for a short period without showing any symptoms. In pregnant cattle, the uterus is the predilection site for *Brucella*, whereas the udder, lymph nodes, testicles, seminal vesicles, and joints are also sites of aggregation in cattle generally. In susceptible cows, abortion beyond the fifth month of gestation is the most characteristic clinical sign. However, the birth of a premature weak calf or an infected full-term calf is also possible. In the majority of infected herds, the abortion rate will be 30% or lower, but in certain circumstances of high exposure with susceptible cows (abortion storms), the abortion rate may be far greater. Retained placenta and subsequent uterine infections are also commonly reported sequelae to abortion. In general, cows will abort due to *Brucella* and then carry calves to full term in subsequent pregnancies, but on occasion serial abortions do occur.

In bulls the seminal vesicles, testicles, and the epididymis are the sites of infection. Acute inflammatory changes can produce painful local swelling, and necrosis of these tissues can follow, with temporary or permanent infertility sometimes occurring. *Brucella* can be found in the semen of both acutely and chronically infected bulls, and when used for artificial insemination, such semen may introduce brucellosis in *Brucella*-free herds.

Brucellosis in Sheep and Goats

Brucellosis in sheep and goats can be considered two separate clinical entities on the basis of the species involved. Brucellosis from *B. melitensis*, and far less commonly from *B. abortus*, is the less significant clinical manifestation. In this infection, the source is an aborting animal. As in cattle, animals may contract brucellosis by oral or cutaneous routes as well as via an infected male. *Brucella* in aborting sheep is principally found in the tissues of the pregnant animal's uterus, udder, and the lymph nodes, similar to the case of cattle. Bacteremia occurs soon after infection and may last 6–8 weeks. Sheep usually do not show any signs until abortion occurs although changes in the milk may precede abortion. Abortion occurs at 3–4 months of pregnancy, and in a susceptible flock, it may reach epidemic proportions. Retention of the placenta and subsequent uterine infections may or may not occur. After abortion, brucellae are excreted in the uterine discharges, the milk, and the urine and feces for 6 months or longer.

Goats infected with a large dose of *Brucella* may become very ill, but rarely die. Mastitis and abortion at 3–4 months of pregnancy are common clinical signs. Retention of the placenta is not common. Brucellae are excreted in the uterine discharges for months and in the milk for years. Because urine and feces are often contaminated with *Brucella*, use of contaminated dung as a pasture fertilizer can lead to horizontal transmission in the flock. Both infected sheep and goats may have clinical signs of lameness, hygroma, respiratory compromise, and orchitis in males. However, with infection by *B. melitensis* and *B. abortus*, infertility is not a common sequela. Unlike cattle, sheep and goats are susceptible to brucellosis at all ages and males are more susceptible to infection than are females. Sheep and goats can recover from brucellosis but may also abort a second time due to a *Brucella* infection.

The second form of clinical manifestation of brucellosis in sheep and goats is due to *B. ovis*. This disease is characterized by significant epididymitis and orchitis, which impair fertility in rams in the acute phase of infection and commonly for long periods thereafter due to the destruction of the tissues. Infected rams shed *B. ovis* in semen, often intermittently, and as this can persist for several years after the initial infection, this is the principal route of transmission within the flock. A common clinical sign in affected rams is progressive fibrosis and atrophy of the testicles and particularly the epididymis. Infection in ewes, signaled by abortion and reduced viability lambs, is not common but may occur after mating with a naturally infected ram.

Diagnosis of Brucellosis

As the primary clinical sign of brucellosis in herd cows is abortion, diagnosis is achieved by a combination of methods including herd and clinical histories, examination of the dam and fetus, and laboratory analysis of samples from both. The laboratory techniques required for definitive diagnosis are complicated by the natural history and clinical pathology produced by the organism, so they include a combination of direct bacteriological assessment and serological assays.

History, Clinical Signs, and Fetal Necropsy

Brucellosis in a susceptible herd is characterized by relatively high rates of late-term abortions, so as in all abortion investigations a thorough herd history is very important. An abortion rate above 30% of the herd at a stage of gestation beyond 5 months in an unvaccinated or unexposed herd, with few additional clinical signs in the dams beyond retained placentas and metritis, is characteristic of brucellosis. However, several other abortifacient diseases can also present with similar clinical signs in a herd in some circumstances, so caution is required. Where *Brucella* is endemic in a herd, because resistance is generally exhibited after the first infection, abortions are usually confined to heifers and the recent arrivals.

There is a range of pathological changes associated with brucellosis in the placenta and fetus, and as there is considerable variation in which of these are present in any individual case, no single pathology can be considered characteristic. The placentitis observed in aborting cows is not characteristic, and all other clinical signs are nonspecific. Aborted fetuses can have subcutaneous edema and inflammatory foci in the muscles, lungs, and central nervous system.

Direct Bacteriological Assessment

As the organism is concentrated in the uterus, placenta, fetus, and associated fluids, direct bacteriological examination of these can sometimes identify *Brucella.* It is found in large numbers in the chorioallantoic epithelium and allantoic fluids of infected cows after abortion. It is also present in the cervical mucus, vaginal exudates, and colostral milk of the aborted cows, and all of these can be examined after abortion for the presence of *Brucella.* In the aborted fetus, *Brucella* may sometimes be identified from the abomasal contents, lymph nodes, meninges, and the lungs.

Bacteriological techniques used to identify *Brucella* include direct-staining (e.g., modified Ziehl–Neelson) microscopy, fluorescent antibody microscopy, culturing, and nucleic acid identification using polymerase chain reaction (PCR) methods. The microscopic techniques can be definitive if the organism is found in convincingly large numbers, but the range of pathologies associated with this disease means that a negative result from any single or few tissues does not automatically exclude *Brucella* from the diagnosis. *Brucella* is fastidious and slow growing, and the sites sampled are routinely highly contaminated with other bacteria, so it can be difficult to culture successfully. In addition, the zoonotic potential of the organism is sufficiently high to require stringent laboratory caution when handling, so this technique is rarely a first-order priority in diagnosis. There have been numerous PCR methods of identification developed in very recent times, and the rapidity, safety, high specificity and sensitivity, and relatively low cost of these will likely encourage increasing use.

Serological Assays

Antibodies to *Brucella* can be found in the serum, uterine and vaginal exudates, milk, or semen plasma of infected or exposed animals. There is a considerable range of known antibodies to *Brucella*: in particular IgM, IgG1, IgG2, and IgA. These are not produced in all *Brucella* infections, and some latent infections are not associated with any antibody titer. Also, the observed titers of the antibodies present appear to vary significantly with the species and biovars, and between infected individuals. These are challenges for the serological assay of *Brucella*-exposed animals to distinguish between the infected and noninfected animals, and as a consequence there have been a large number of methods developed to quantify all relevant isotypes (**Table 1**); there are advantages and limitations to each of them in practical use.

There are broadly two types of serological tests in current use: tests that measure directly the primary binding of antibodies with the antigen such as enzyme-linked immunosorbent assay (ELISA) and fluorescence polarization assay (FPA); and those that use the observed physiological consequences of that coupling, for example, the complement fixation test (CFT), the buffered antigen tests such as the rose bengal test (RBT), the card test, and the serum (plate or tube) agglutination tests (SATs, PATs, and TATs). The latter group comprises the original tests for exposure to and infection with *Brucella*, and the extensive use of these internationally for many years has critically defined both their weaknesses and optimal application. The former group has potential advantages in accuracy, but requires a greater level of technical expertise and sophisticated equipment to perform adequately, which initially slowed widespread adoption.

It is also possible to categorize the tests available on the basis of primary use, either as initial ‘screening’ tests or as ‘supplementary’ (follow-up) tests, although there is some overlap. The screening tests are necessarily rapid, simple, and inexpensive, while requiring a high sensitivity (e.g., RBT or the card test). A lower specificity can be allowed if the positively identified animals can then be reassessed with an independent supplementary test with greater specificity (e.g., ELISA or CFT).

A brief description of some of the key tests in current use in dairy animals and their practical limitations is valuable. The milk ring test (MRT) is a rapid and inexpensive screening test that detects IgM and IgA antibodies in milk, often used as a bulk milk tank test. The sensitivity of the MRT is reduced with a low prevalence in the contributing herd and due to certain

Table 1 The reactivity of the four major bovine immunoglobulins and the characteristics of selected historic and current serological tests

	Immunoglobulin type							
Test	*IgM*	*IgG$_1$*	*IgG$_2$*	*IgA*	*Sensitivity (%)*	*Specificity (%)*	*Test advantages*	*Test disadvantages*
MRT	+		+		56	99	No serology required, rapid, inexpensive, simple	Only in lactating animals, lower sensitivity and specificity
CFT	+	+	+	+	70	95	Detects acute infection, rapid, simple	Lower sensitivity and specificity, prozone phenomenon
SAT	+	+			81	98	Highly specific	Tests positive later than SAT, anticomplementary activity, inhibited by IgG$_2$
RBT	+	+	+		78	71	Suitable for screening, rapid, inexpensive, simple	Sometimes low sensitivity
Coombs			+				Confirms SAT, specific	Laborious, needs titers at least 2 times that needed for SAT
2-ME	+	+			56	97	Differentiates between infected and vaccinated cattle	Not suitable for acute infections
ELISA								
IgM	+				96	81	High specificity, moderate sensitivity, suitable for automation	Sensitivity may be too low for some applications
IgG		+			77	87		
SDTH	Cell-mediated immune response				81	99	Simple, detects latent carriers	Sensitivity may be low in certain applications

CFT, complement fixation test; ELISA, enzyme linked immunosorbent assay; 2-ME, 2 mercaptoethanol; MRT, milk ring test; RBT, rose bengal test; SAT, serum agglutination test; SDTH, skin delayed type hypersensitivity.

physical characteristics of some milks. Its specificity is also reduced with increasing amounts of colostrum, or late-lactation milk, in the sample, and by the presence of certain organisms. The MRT is not effective in detecting brucellosis in sheep's and goat's milk unless a small amount of *Brucella*-negative cattle milk is added to the this milk (at a ratio 1:3) before the test is performed. The milk ELISA is an improved test that overcomes the problems with low prevalence in larger herds, and has greater specificity.

The RBT is also a rapid and inexpensive screening test with a high sensitivity, performed on individual serum samples. The low pH (3.6) of the antigen alters the agglutination of IgG and thereby improves the specificity of the test. Oversensitivity is the principal limitation, particularly in vaccinated animals. However, the test may yield negative results for some infected cattle that test positive with the CFT, and so it has been used in combination with CFT in some national programs. It is less reliable in testing for brucellosis in sheep and goats.

The SAT has been the principal serological screening test used for the diagnosis of brucellosis in the past. The SAT measures the agglutinating antibodies IgM, IgG1, IgG2, and IgA. The test detects acute infections because antibodies of the IgM type are more reactive in the SAT than those of the IgG1 and IgG2 types. However, because the test has reduced specificity for IgM and IgG1 in cattle and reduced sensitivity in general, the test effectively detects brucellosis only on a herd basis, and its use has gradually given way to tests with superior sensitivity and specificity.

The 2-mercaptoethanol (2-ME) and 7-ethoxyacridine-3,9-diamine tests detect IgG by first disrupting or precipitating out IgM, and because of this specificity are often used as supplementary tests to differentiate between infected and vaccinated reactor cattle.

The CFT is highly specific and is very important for the diagnosis of brucellosis. Action of the CFT is based on the detection of specific antibodies of the IgM and IgG1 types that fix complements. Since antibodies of the IgG1 type usually appear after antibodies of the IgM type, many national eradication programs have used SAT and CFT together. To improve sensitivity and specificity in testing, the European Union had previously recommended that nonvaccinated cattle be considered infected only if they return $\geq$30 IU in the SAT and $\geq$20 international complement fixation test units in the CFT. Antibodies of the IgG2 type may hinder complement fixation, causing infected sheep or goats to test false negative with the CFT. When the combined use of the SAT and CFT cannot establish a clear-cut diagnosis, the Coombs (antiglobulin) test can be useful. This test detects IgG2, confirms SAT results, and is specific. This is particularly important when CFT results are negative or inconclusive. However, the Coombs test is laborious, and the results are indicative of infection only when its titers are at least 2 times those of the SAT. As not all infected cattle show this ratio, this is also the main limitation of the test.

The ELISA (and the competitive ELISA) can be used to detect *Brucella* antibodies in milk or serum. The specificity and sensitivity of the assay depend on the antigen, conjugate, substrate, and the cutoff point used to establish infection, but in general terms both are high. The ELISA is suitable for testing sera from sheep and goats for the presence of antibodies against *Brucella*.

Particle concentration fluorescence immunoassay (PCFIA) is a primary binding test measuring IgA, IgM, and IgG using a fluorescein molecule attached to a carrier molecule that binds to polystyrene particles. Changes in the light emitted result from binding to the target antibodies, and the method is highly automated. The specificity of this test is considered moderate to low.

FPA is a more recent method that uses the rotational properties of molecules to directly measure antigen–antibody binding in serum. The rate of rotation alters with binding, so the fluorescent dye attached to the carrier lipopolysaccharide antigen molecules displays a quantifiable difference with increasing antibody titer. The test has the valuable advantages of being very rapid, uncomplicated to perform, and suitable for multiple *Brucella* species, and is highly sensitive and specific. These qualities have led to a rapid adoption in contemporary laboratories internationally.

Infected animals that elude detection with serological tests complicate eradication programs. Use of the skin-delayed-type hypersensitivity (SDTH) test, which is independent of circulating serum antibodies, improves the diagnosis of brucellosis. The SDTH test is highly specific, confirms the serological test results, determines the infection status of cattle that had returned ambiguous serum test results, and detects infection in seronegative infected cattle. However, one limitation is that cattle with high serological titers may test negative with the SDTH test. Nevertheless, a combined use of screening tests such as CFT or ELISA with the SDTH test will detect a larger number of infected cattle than detected by either test alone. The test is also effective in sheep and goats when used in combination with ELISA or CFT.

Control of Brucellosis

There is no practical therapy for animal brucellosis, and therefore not a component of any control program. Hence, efforts are directed toward eradication of brucellosis from livestock. There are, however, many factors that influence the programs employed for this purpose. The principal determinants of the approach taken are the prevalence of infection and economics. Control of brucellosis is based on the identification and slaughter of infected animals, and the prevention of new infections. As there are no definitive pathognomonic clinical signs of brucellosis, identification is achieved by serological testing, with ancillary bacteriological testing for acute abortion cases. Prevention of new infections is achieved by restricting movement of infected animals into the herd, or vaccination where that is not possible, or by improved husbandry and management of high-risk cattle (e.g., pregnant and calving cows) where neither of the other options can be reliably instituted.

Preventing infected animals from entering the herd is usually accomplished by prescreening of the animals or the semen to be used in artificial insemination. Prevention against *Brucella* exposure by vaccination is usually done where eradication by elimination of infected animals cannot be achieved in a suitable time, or where movement restriction of infected animals is not possible.

Surveillance for brucellosis in brucellosis-free areas can be achieved by screening of milk of dairy herds and sampling cattle populations at slaughter, and tracking infected cattle back to the herd of origin for further serological testing.

In regions with *Brucella* infections, depopulating individual herds of demonstrated positive *Brucella* status is an effective method of regional control. As the economic impact of this approach is large, test and slaughter programs are routinely used. Although a large variety of specific programs have been used, the principles underlying them are very similar. Each animal in a herd is serologically tested (screening) and identified reactors are either slaughtered or retested with a supplementary test of greater specificity after which repeat reactors are slaughtered. The remaining herd animals are then retested at regular intervals until all animals test negative several times.

The test and slaughter approach is less suitable and more difficult to enact for sheep and goats, as the serological tests are less accurate, and individual identification is less common in some extensive production systems. The exception is *B. ovis* infection, which has been very successfully controlled in some countries (e.g., New Zealand) by a similar approach. In this national program, all rams were palpated for testicular and epididymal fibrosis, and culled on the basis of a presumptive positive status if any abnormalities were found. Annual and presale serological testing (CFT) was also used, and reactors were culled.

Vaccination of cattle with *Brucella* vaccines also prevents the spread of brucellosis. The early vaccine used most widely was the live *B. abortus* strain 19 culture. This vaccine was protective, but was associated with abortion in pregnant cows and various other health problems in other stock classes, and produced an antibody titer that would test positive on serological tests for 18 months after vaccination. Although vaccinated stock were generally permanently identified (e.g., ear tattoo) and specially tagged, the presence of an unidentifiable vaccine recipient in a herd was not an uncommon problem in *Brucella* surveillance. A reduced dose of the vaccine administered to adults partially ameliorated these issues, and when used in calves (4–9 months of age) minimized many of the reported health problems, significantly reduced the postvaccination period during which serological testing was unsuitable, and delivered broadly equivalent protection to *Brucella* challenge.

However, these issues led to the development of a new vaccine that was not associated with false positives on serological test and was more suitable for use in adult cattle. *Brucella abortus* 45/20 is composed of dead *Brucella* in an oil adjuvant, requiring two initial vaccinations and an annual booster. It is slightly less protective than *B. abortus* strain 19 and can cause large granulomas at the injection site. *Brucella abortus* RB51 was developed for use as a live vaccine by culturing the virulent *B. abortus* biovar 2308 in a medium containing rifampicin. It is a live vaccine, was broadly equivalent in conferred protection against *Brucella* challenge, has few associated health problems, and does not cause the serological difficulties associated with *B. abortus* strain 19. It was registered as an official vaccine by the US Department of Agriculture and has superseded the use of *B. abortus* strain 19.

The live, attenuated *B. melitensis* strain Rev 1 vaccine has been used in sheep and goats, and has formal registration in some countries, though not in the United States. The vaccine is suitable for young stock between 3 and 8 months of age, and use in adult stock, even at reduced doses, has been associated with health and serological problems similar to those reported for adult cattle with *B. abortus* strain 19. Immunogenicity and attenuation of this vaccine do differ between sites of production, and this has been associated with differences in efficacy and safety.

Other vaccines against brucellosis in sheep and goats were developed to minimize some of the problems associated with the Rev 1 vaccine, including a number of oil adjuvant-based vaccines. The most widely used one is the *B. melitensis* H38 vaccine, composed of dead *Brucella* in an oil adjuvant. The protection against *Brucella* challenge conferred by H38 vaccine is broadly equivalent to that of Rev 1, and it has a significantly greater safety index in all stock classes of sheep and goats. However, the vaccine is associated with serological changes that require careful interpretation when *Brucella* status surveillance testing is undertaken in vaccinated flocks, and the oil adjuvant can produce granulomatous injection site lesions similar to those seen with the *B. abortus* 45/20 vaccine in cattle.

Effective control of brucellosis has been achieved in many countries by the use of these programs of herd screening by serological testing, slaughter of identified *Brucella*-positive animals, and targeted vaccination.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Acidosis/Laminitis; Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Milk Fever.

Further Reading

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Infectious Diseases: Foot-and-Mouth Disease

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Introduction

Foot-and-mouth disease (FMD) is one of the most important infectious diseases that can affect cattle. This is due to the severe economic consequences of outbreaks, caused both by the direct losses in animal productivity and by the indirect losses associated with the high costs of control measures coupled to trade restrictions that usually result from outbreaks. Several characteristics of FMD contribute to this: the disease is highly contagious; it affects a wide range of artiodactyls; and the virus is relatively stable in the environment and can easily be transmitted, including via airborne spreads under specific conditions. In addition, seven serotypes of FMD virus (FMDV) exist, which confer little or no cross-protection, and many variants occur within each serotype. For this reason, vaccines containing specific strains are required to protect against antigenically different isolates of FMDV.

Since the cessation of vaccination against FMDV at the end of 1991 in the European Union, it was estimated from cost–benefit studies that mass vaccination campaigns were costlier than the estimated expenditure to control occasional outbreaks. Added to this, there is the longer waiting period necessary to regain the FMD-free status when using vaccines. Also, several FMD outbreaks have been associated with improperly inactivated vaccines, or by escape of the virus from the vaccine plants. The decision to cease all vaccination has led to a fully susceptible population of cloven hoofed animals where the disease can rapidly spread resulting in unprecedented economical and social damage. This was amply demonstrated when an outbreak of FMD occurred in 2001, starting in the United Kingdom, with limited spread to France and the Netherlands. More than 2000 FMD cases were confirmed in the United Kingdom, and more than 3.7 million animals were slaughtered. Vaccination was applied only in the Netherlands, followed by stamping out of vaccinated animals leading to 26 confirmed cases and 265 000 animals slaughtered. This has initiated an international debate on the role of vaccination in the FMD control policy in the European Union (EU).

FMDV is still endemic in many countries, which limits trade in live animals or animal products with FMDV-free countries. Illegal trade and the widespread occurrence of FMD in many parts of the world is a constant threat to free areas, justifying regular consideration, review, and update of surveillance programs and contingency plans, and creation of awareness among those working with susceptible animals in free countries. In endemic areas, it is essential to monitor the possible emergence of new viruses that may differ antigenically from existing vaccine strains with potential failure of protection against the disease in the face of an outbreak.

There is a trade incentive not to use vaccination and despite the developments in serological assays to distinguish between vaccinated and infected animals, countries free of the disease are often reluctant to accept live animals and their products from areas where vaccines are used. It is expected that the number of FMDV-free countries will gradually increase.

This article reviews the epidemiology, economy, and control of FMD, with special emphasis on ruminants.

Epidemiology

The Causative Virus and Its Characteristics

FMD is caused by a positive-sense, single-stranded RNA virus. The genome consists of approximately 8500 nucleotide bases, and encodes one large polyprotein. This polyprotein is posttranslationally cleaved by viral-encoded proteases into structural and nonstructural (NS) proteins. The virus has an icosahedral symmetry and is composed of 60 copies of each of the four structural proteins 1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1). Four structural proteins form a protomer, and five protomers form a pentamer (with a sedimentation coefficient of 12 S). Twelve pentamers assemble into one virion (with a sedimentation coefficient of 146 S). The NS proteins are L (leader protease), 2A, 2B, 2C, 3A, 3B (associated to the 5’-end of the viral RNA), 3C (protease), and 3D (RNA-dependent RNA polymerase). The function of each protein is not known, but the proteases are associated with the processing of the viral polyprotein into functional proteins and also mediate the host cell’s cap-dependent mRNA translation shutoff and interfere with the innate immune response.

FMDV has no envelope and is moderately resistant in the environment. A relatively high humidity of more than 60%, a pH of 7.2–7.6, and low temperatures are the environmental factors that favor long-term survival of the virus. The virus is unstable at pH below 6 or above 9, and is inactivated at temperatures above 56 °C. Consequently, FMDV is rapidly inactivated by citric acid and by bases such as caustic soda. FMDV isolates may differ in their resistance to heat inactivation, the type A strains being relatively more resistant. When the virus is associated with proteins, such as in milk or other dairy products where the virus is incorporated into the casein (CN) micelles and fat droplets, or present in organic material, such as slurry, the inactivation is greatly reduced, and more severe conditions or the use of detergents is necessary for complete inactivation. In skeletal muscles, reduction of pH to a value below 6 due to lactic acid formation leads to the inactivation of FMDV postmortem, but this requires that the meat be stored for about 48 h at 4 °C after slaughter. In lymph nodes and bone marrow, virus may survive for months.

Seven serotypes of FMDV have been identified (O, A, C, Asia-1, and the South African serotypes (SAT) 1, 2, and 3), of which serotype C is most likely extinct. These serotypes differ in their geographic distribution as summarized in **Table 1**. However, certain isolates appear from time to time and spread rapidly over vast areas. For example, the serotype O virus that caused the outbreak in Europe in 2001, the so-called Pan-Asia O virus, has replaced many other viruses in the Middle East and Asia. It was first identified in northern India in 1990 and spread westward into Saudi Arabia in 1994, throughout the Near East and into Europe (Turkish Thrace, Bulgaria, and Greece) in 1996. In addition, the A-Iran 05-strain has similarly become the predominant strain in western Asia, the Middle East, and Turkey. The SAT strains are restricted to sub-Saharan Africa, although incidental outbreaks have occurred in the Middle East (see **Table 1**).

Within each serotype, considerable genetic and antigenic variation occurs, notably within the three SAT serotypes and serotype A, which can explain the insufficient cross-protection by vaccines when the vaccine strain and the circulating viruses differ significantly from each other. This variation is due to the high rate of mutation, well known to occur with single-stranded RNA viruses. Within serotypes, differences in infectivity, virulence, and pathogenicity occur. Although generally one strain will infect ruminants as well as pigs, particular viruses belonging to serotype O have shown species adaptation, such as the O Taiwan isolate that infected pigs but not cattle in the 1997 outbreak in Taiwan. Molecular epidemiology, based on sequencing data from a genomic region encoding the VP1 protein, has contributed significantly to the classification of FMDVs. It has been shown that FMDV shows marked variation in time and between regions within serotypes. FMDVs are therefore also classified into separate genotypes or 'topotypes', reflecting the occurrence of a genotype within a given geographic region. The SAT serotypes show significantly more variation than the other serotypes, probably due to the involvement of the wildlife host and long-term carrier status. It has been shown that genetic and antigenic variants are generated during long-term persistence in African buffalo (*Syncerus caffer*). Serotype A also demonstrates marked variation, but Asia-1 seems to be the most conserved serotype (**Figure 1**). Such variations have an impact on vaccine strain development (see section 'Control Measures').

Hosts and Epidemiological Features

All cloven hoofed animals are susceptible to FMDV infection, and over 70 species have been found to be infected. Infected animals start excreting the virus in excreta and secreta during the incubation period before the onset of visible clinical signs. Milk may contain infectious virus up to 4 days before the onset of clinical signs. All excretions and secretions contain infectious virus and consequently may be a source of infection for other animals until the onset of neutralizing antibodies,

Table 1 Serotypes of foot-and-mouth disease and their geographic distribution

FMDV serotypes	*Geographic distribution*
A (Ardennes)	South America, southeastern Europe, Africa, Southeast Asia, the Middle East
O (Oise)	South America, southeastern Europe[a], Africa, Southeast Asia, the Middle East
C[b]	South America, Africa, Asia
Asia-1	The Middle East, the Far East, Southeast Asia
SAT-1 (South African Territories)	Sub-Saharan Africa
SAT-2	Sub-Saharan Africa
SAT-3	Sub-Saharan Africa

[a] 2001 outbreak in Europe caused by the Pan-Asia serotype O strain. The outbreak started in pigs, and later on was predominantly found in cattle, sheep, and goats.

[b] Possibly extinct.

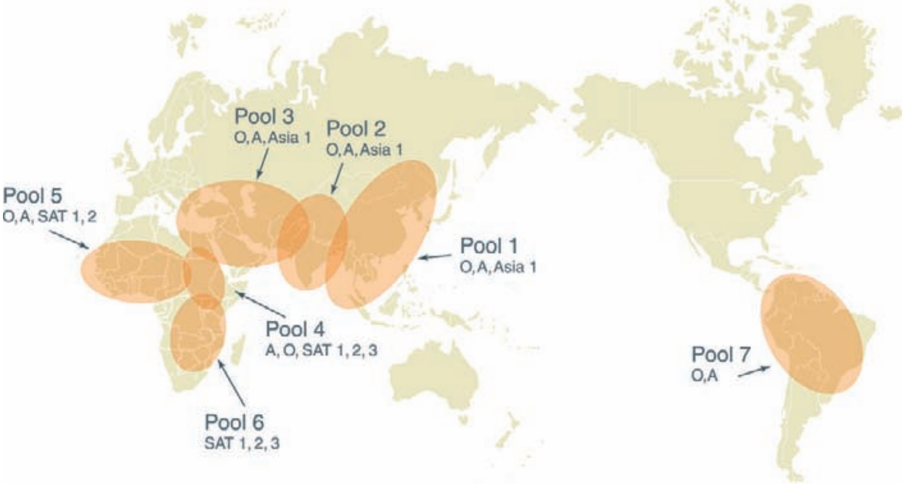

Figure 1 Several international organizations such as the OIE and Food and Agriculture Organisation (FAO) together with the WRL for FMD have divided FMD worldwide into virus pools as a first step in global control. Each pool contains similar serotypes and topotypes to assist with decisions regarding vaccines that could be used in controlling infection. The map represents a rough estimation of the geographic distribution and the serotypes included in each pool. Artwork by Frank Filippi.

usually between 3 and 5 days after infection, when a decrease in virus excretion relates to a concomitant rise in antibody titers.

The most common way of virus transmission is by direct animal contact. Other indirect sources of transmission are contaminated transport vehicles, humans, and, under very specific climatic conditions, air. Transmission by ingestion of infected milk or meat or even through abrasions on the skin is possible, but occurs less frequently. Cattle are highly susceptible to FMDV and can be infected by inhalation of the virus, by contact, by ingestion of the virus, and by contaminated semen. The incubation period is generally shorter when the infectious dose is higher, but also depends on the strain of the virus, the susceptibility and breed of the host, and the route of infection. For instance, after (artificial) intradermolingual inoculation of virus in the course of FMD vaccine control experiments, animals may show clinical signs as soon as 1 day after inoculation, whereas clinical signs may appear only after 10–14 days with aerosol infection. Consequently, the incubation period may vary, but generally lasts 2–14 days. Cattle are most susceptible to infection by the respiratory tract (~10–25 $TCID_{50}$ [tissue culture infectious doses]), whereas for infection by the oral route at least 10 000 times as much virus is required. Because cattle have a large inspiratory volume, they are highly susceptible to airborne infection. There are significant differences in virus excretion and susceptibility among domestic livestock. Cattle excrete a maximum of 120 000 $TCID_{50}$ a day, whereas pigs can excrete up to 400 million $TCID_{50}$ a day, and pigs need more viruses than needed by cattle to become clinically infected by the respiratory route.

Milk can be infectious for about 7–9 days, starting up to 4 days before the onset of clinical signs, and could be a source of transmission when infected milk is fed to piglets or calves. In one study, virus could be observed in milk 23 days postinfection while viremia lasted only 4–6 days. However, the infectious dose may not be sufficient to infect other animals via ingestion. Aerosolized infected milk generated during bulk handling procedures could also be a source of infection and, since the minimum infectious dose by the respiratory route is lower as compared to the oral route for both cattle and pigs, may even be a more important source. However, studies of recent outbreaks have shown that transmission of FMDV by milk or dairy products occurs relatively rarely.

The duration of virus excretion depends on the host and varies between different secreta and excreta. The highest amounts of virus occur in lesion material such as vesicular fluid and epithelium from the vesicles, and in saliva. In cattle milk, virus titers can reach up to $\log_{10} 10^{5-6}$ $TCID_{50}$. Importantly, pigs excrete up to 3000 times more infectious virus in the air than cattle.

Sheep and goats show less severe clinical signs, and this, often subclinical infection, makes them a threat for transmission of FMDV in many regions of the world. Cattle and pigs mostly show overt signs of infection, which are easy to identify.

A particular phenomenon is the occurrence of carrier animals post-FMDV infection in the absence of circulating virus and the presence of neutralizing antibodies. An animal is considered a carrier when the virus can be intermittently recovered from the esophago-pharyngeal fluid more than 28 days after infection. In a small proportion of animals, FMDV can be recovered from these throat scrapings up to 3.5 years in cattle and up to 9 months in sheep after infection. It is estimated that 50–70% of the cattle may become carriers soon after an outbreak with a sharp decrease over time. The African buffalo is the natural reservoir of FMDV in sub-Saharan Africa, and can probably harbor FMDV lifelong. There is no firm evidence yet that pigs can become carriers, although it has recently been described that viral RNA was detected in sera from infected pigs several months after infection; this finding still needs to be confirmed. Importantly, vaccinated animals can also become carriers. However, because the amount of virus in carrier animals is relatively low and seems to be confined to the light zone of the germinal centers in cattle, the risk of virus transmission by carriers is low as compared to that during the acute phase of infection. The occurrence of carrier animals is of special concern in eradication programs.

FMD is not a zoonosis and seroconversion has been detected only in a few individuals with high exposure to the virus.

Clinical Signs in Various Domestic Species

Clinical signs in dairy cattle usually start with fever, depression, a reduced appetite, and in lactating animals a sudden drop in milk yield that could be significant. Disease can range from a subclinical infection to overt clinical signs. Affected animals salivate, and vesicle formation can be observed in the mouth and on the dorsal surface of the tongue. Vesicles may also appear on the teats and the udder, but are usually smaller than the vesicles in the mouth, and could result in mastitis and become infected with bacteria (**Figures 2–4**). Cattle may become lame due to vesicles appearing in the interdigital space and coronary bands. Feet lesions usually take longer to heal, and bacterial infection often aggravates the symptoms. Based on the appearance of lesions in the mouth, experienced clinicians can estimate the age of the lesions to help determine the start of the infection and backward tracing during more widespread outbreaks. Young animals such as calves and piglets may suddenly die as a result of an acute myocarditis, called tiger heart disease based on the striped appearance of the heart muscle, and this may be the only clinical sign. Mortality in adult animals is usually low, but morbidity may reach 100%. The differential diagnosis is indicated in **Table 2**.

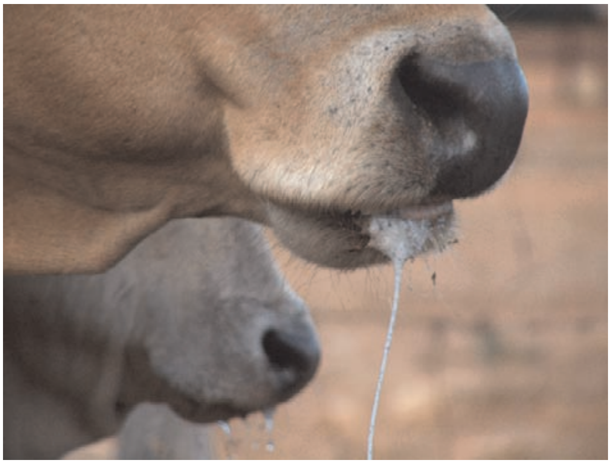

Figure 2 Infected cattle salivate due to the presence of lesions in the mouth. Salivation can be pronounced. Photo courtesy Peter Geertsma.

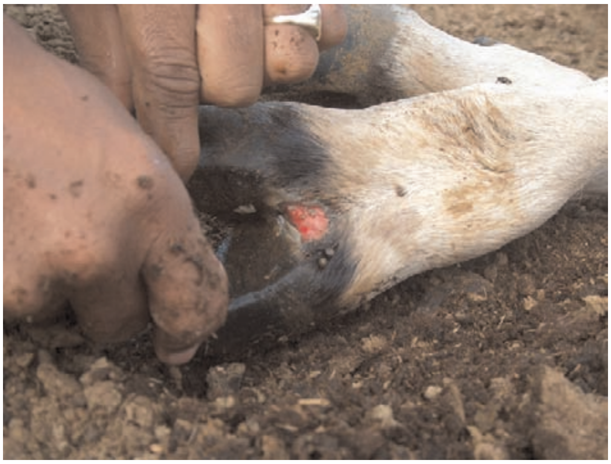

Figure 3 Feet lesions occur in the interdigital space and take longer to heal than lesions in the mouth. Photo courtesy Peter Geertsma.

Sheep and goats differ from cattle in that their clinical signs are much less apparent. Lameness is usually the most predominant clinical sign, but in a sheep flock only a small number of animals may show clinical signs. Lactating animals may show a sudden drop in milk yield, with pyrexia.

Pigs become recumbent, huddle together, and are reluctant to move. When forced to move, they may show lameness. In adult sows, FMD could go unnoticed, and in young piglets mortality may be the only sign of infection. Vesicles in the mouth and on the nose, and on the feet and the elbows may be pronounced when the animals are kept on hard surfaces, while vesicles may also form on the teats of lactating sows. Vesicles will easily rupture. The lesions in pigs are indistinguishable from swine vesicular disease, caused by the closely related swine vesicular disease virus.

Laboratory Diagnosis of Foot-and-Mouth Disease

The World Organisation for Animal Health (OIE) has issued a manual with standards for diagnostic tests and vaccines, which provides detailed descriptions of tests for

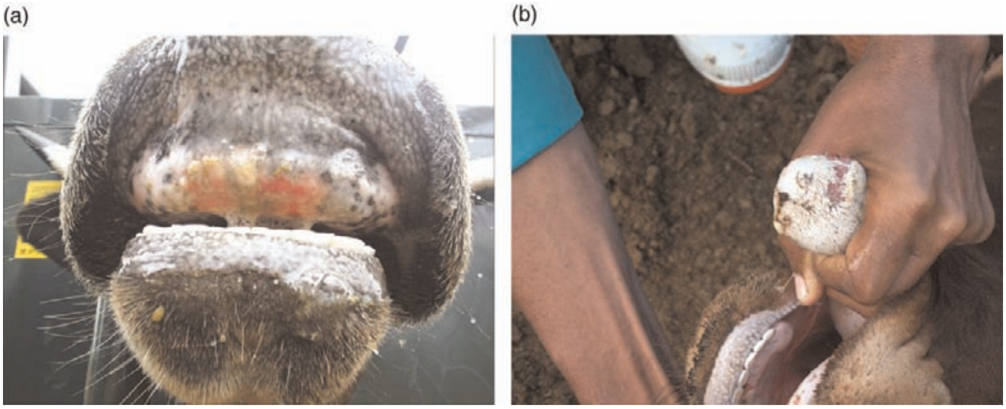

Figure 4 Lesions occur on the gums (a) and on the tongue (b) of infected animals. Photo courtesy Peter Geertsma.

Table 2 Differential diagnosis of foot-and-mouth disease

Cattle	*Sheep*	*Pigs*
Infectious bovine rhinotracheitis	Contagious ecthyma (orf)	Swine vesicular disease
Bovine viral diarrhea	Blue tongue	Vesicular exanthema of pigs
Malignant catarrhal fever	Foot rot	
Stomatitis papulosa		
Rinderpest		
Vesicular stomatitis		
Calf diphtheria		
Pseudocowpox		
Bovine herpes mammillitis		
Foul in the foot and foot rot		
Lumpy skin disease		
Orf (contagious pustular dermatitis)		

the purpose of international trade. In a number of countries where the disease is exotic, high-security FMDV laboratories maintained under biosecurity level 3 standards have been constructed, which are especially equipped to handle samples suspected of carrying FMDV without the risk of further disseminating the disease. In most countries where FMD does not occur, it is a notifiable disease included in the legislation and only government veterinarians or accredited personnel are allowed to collect and submit diagnostic samples. Great care should be taken to ensure that these people do not spread the infection between farms. If a high-security laboratory is not regionally available, samples can be submitted to the OIE World Reference Laboratory (WRL) for FMDV in Pirbright, UK.

The virus can be identified by virus isolation, an enzyme-linked immunosorbent assay (ELISA) that detects the viral antigen, or reverse transcription-polymerase chain reaction (RT-PCR). Virus isolation on specific cell lines or primary cells is very sensitive but requires a few days before the final result is available. This delay could be crucial when dealing with such a highly infectious disease. The antigen ELISA provides results much more rapidly (within a few hours) and can distinguish between the various serotypes. RT-PCR is a highly sensitive assay that similarly can deliver results within hours, and tests to distinguish between the serotypes are available. In addition, the RT-PCR products obtained from the structural gene regions can be sequenced and used to determine the molecular epidemiology of FMDV isolates and to establish the relationship with other viruses and possibly trace the origin of an outbreak. Should vaccination be a control option, knowledge about the serotype is essential.

The gold standard antibody test for detecting antibodies is the virus neutralization test, which relies on live cell culture and virus, but is not conducive for high-throughput testing. ELISAs such as the solid phase competition ELISA have been developed and are widely used due to their ease and the fact that no live cells or live virus is needed. All these assays measure antibodies to the structural proteins of the virus. Some NS proteins are sufficiently immunogenic to allow antibody detection after infection, but are not detectable in modern purified FMD vaccines and rarely induce antibody formation after one or more vaccinations. In addition, NS proteins are relatively conserved among the serotypes, and one test can therefore be used regardless of the serotype, in contrast to the other serological assays where reagents have to be serotype-specific. This allowed the development of differentiating ELISAs and immunoblot assays based on NS proteins, and especially 3ABC-specific antibody detection, to distinguish infected animals from vaccinated animals. The current tests are not sufficiently sensitive to detect single infected animals and can be used only on a herd basis. In addition, there is evidence that the NS proteins of the SAT-type viruses differ to such an extent from the other serotypes that commercial assays may not be sufficiently sensitive to use when those serotypes occur. It is expected that these tests will increasingly be used in eradication and control programs of FMDV.

Although assays for detecting antibodies to FMDV and viral genomic material detection using RT-PCR in cattle and sheep milk have been described, these are not widely used in surveillance and control programs. However, it has been indicated that especially the sensitive RT-PCR could detect one infected animal in a herd when testing pooled milk.

A particular problem in FMDV control is the occurrence of many antigenically different variants. This requires that reagents for both virus and antibody tests be regularly updated to ensure that they are suitable to detect antibodies against the circulating serotypes and that they are able to detect newly emerging FMDV isolates.

Diagnosis of carrier animals is challenging as these animals are seropositive to the structural proteins and virus, and viral RNA can only be intermittently detected in the esophago-pharyngeal fluid and cellular material, collected with a probang cup especially designed for this purpose. However, these animals may be negative for antibodies to the NS proteins and would therefore not be detected as having been infected. Newer assays looking for secretory IgA may in future assist to detect carrier animals.

Economy

Outbreaks of FMD in free areas are usually associated with significant economic losses. Direct losses are due to mortality and decreased production. Lactating animals could lose production in one or more quarters permanently. Indirect losses are due to trade restrictions on animals and animal products and the costs of control measures such as stamping out, compensation of farmers, vaccination, cleaning, and disinfection, as well as of movement control. When large numbers of animals are destroyed, loss of high-performing animals and difficulties in repopulation may also account for severe economic damage. In addition, reduced draft power may lead to food insecurity if fields cannot be plowed during the growth season in developing countries. Endemic FMD usually precludes countries from exporting animals and animal products and could further impact the already strained economies.

During FMD outbreaks that affect the dairy industry, milk from infected areas will be excluded from consumption or production of milk products, unless it has been treated appropriately to inactivate FMDV. In the European Union, directives have been adopted for milk and dairy products (85/511/EU and 92/46/EU) that contain prescribed treatments for these products. FMDV present in milk and dairy products is particularly resistant to inactivation, and even *in vitro* assessment of the absence of infectious FMDV does not exclude that cattle may become infected after inoculation. Despite this potential risk, the risk of transmission of FMDV by infected milk and dairy products under natural conditions and after treatments such as pasteurization may be considered low, because large amounts of FMDV-containing milk must be ingested by susceptible animals to establish an infection due to the high infectious dose needed to establish infection via the oral route. Production processes containing specific heat treatment, or pasteurization followed by acid treatment, decrease the risk of FMDV transmission to practically zero, provided the process is properly implemented. Thus, the highest risk of FMDV-containing milk will most likely be the direct feeding of the milk to susceptible animals, or spilling or aerosol formation during handling of the milk so that the disease can be transmitted by contact or by aerosolized virus.

The costs of outbreaks vary greatly depending on the species affected, density of the animal population, production systems, and trade implications, and no general estimate can be given. Widespread outbreaks in previously free regions or countries could easily lead to damages of several hundreds of millions of dollars. For instance, direct economic losses due to the 1997 outbreak in Taipei, Taiwan, were estimated at US$400 million, with indirect losses estimated at US$3650 million. During the 2001 FMD outbreaks in Europe, the European Commission authorized €400 million of advance payments to member states to reimburse compensation paid to farmers for animals slaughtered. Advances of €355 million were allocated to the United Kingdom, €39 million to the Netherlands, €3.3 million to France, and €2.7 million to Ireland.

Costs of controlling the disease in areas where vaccination is routinely used or where infected zones occur can also be significant. In southern Africa, a number of countries use game fences and limited vaccination of livestock at risk to prevent contact and disease transmission between infected buffaloes and domestic animals. Strict movement control of animals and products prevents the infection from reaching the disease-free zones. All these measures are costly, but a study performed in Zimbabwe indicated that the benefits from having access to export markets in the European Union outweighed the significant costs for control.

Control Measures

Control measures generally differ between endemic and free areas. Whereas free areas may take significant action to eradicate the disease should it be accidentally introduced, endemic countries may rely on limited vaccination and movement control to protect certain areas, zones, or industries. In many endemic countries, no actions are taken to control the disease as other priorities compete with the limited funding.

For effective control of FMDV, the number of farms or herds infected by one newly infected farm or herd (the reproduction ratio, *R*) must be significantly below 1 after implementation of control measures to assist with eradication. However, under natural conditions, the basic reproduction ratio (R_0) for FMDV is significantly higher than 1, based on observations that major outbreaks usually follow single introductions of FMDV in susceptible populations. The spread of infection depends on various parameters, such as population size and contacts between farms. In addition, little is known about the impact of herd size, contact structure, and control measures such as vaccination on the transmission of FMDV, and consequently on the *R* value. Evidently, control measures must be imposed aiming to reduce the *R* value, but these may vary for different regions where dissimilarities exist in animal population densities, production systems, and availability and suitability of vaccines and destruction plants.

To assist in defining control measures that are effective in reducing transmission, a thorough risk assessment for transmission of FMDV in the animal population at risk during an outbreak must be performed. The time between introduction of the virus and its identification, the so-called high-risk period (HRP), must be as short as possible. A long HRP allows the virus to spread and infect multiple farms, which significantly increases the magnitude of the outbreak and the necessary control measures. Therefore, in FMDV-free regions, an adequate surveillance system must be operational, aimed at reducing the HRP. This again requires risk assessments, aimed at identification, quantification, and subsequent reduction of risk factors. For the Netherlands, it has been estimated that 70% of the potential contagious animal disease introduction could be attributed to import of infected animals or contaminated transport vehicles, based on conjoint analysis of expert opinions. Studies of recent outbreaks in eastern European countries and Italy strongly suggest that animal movements – sometimes illegal – transport vehicles, and infected animal products have caused several outbreaks. Control measures must be targeted at mitigating these risk factors. Contingency plans are frequently legally required, but these must be regularly updated and practiced.

Important elements of control measures are stamping out of infected herds, movement control, and vaccination. The disease should be controlled in pigs first, because they excrete by far the maximum amounts of the virus, followed by cattle. Should the decision be to slaughter out, special emphasis must be given to the hygiene procedures involved in killing, removal, and destruction of animals, as the virus may easily be spread during these procedures. Therefore, disinfection must immediately follow killing of the animals to prevent virus escape by ventilation, people, or secretions. Movement control should prevent the spread of FMDV by infected animals, their products, transport vehicles, or people. In Europe, a protection zone directly around an outbreak and an outer surveillance zone are established after an FMDV outbreak, according to the EU directives (EU directive 85/511). Border inspection posts or buffer zones may also be useful, depending on the geographic situation. In some African countries, fences are used to separate FMDV-carrying buffaloes from disease-free cattle.

Vaccination is an important part of FMDV control. Vaccines against FMDV are still manufactured according to classical methods where large volumes are cultured on baby hamster kidney cells in suspension followed by inactivation using aziridines, purification, and concentration. For ruminants, both aluminum-adjuvanted vaccines and oil emulsion vaccines are available, while only oil emulsion vaccines are suitable for pigs. During emergencies in free zones and countries, high-potency vaccines are administered that should provide a rapid immune response. In contrast, during routine vaccination, payloads are generally lower and the duration of immunity is important. The duration of immunity is short-lived and the aluminum-based adjuvants need to be administered every 4–6 months while annual vaccination should suffice when using oil-adjuvanted vaccines. Vaccine strains should be chosen based on the serotype and its protective capacity against the virus causing the outbreak. The latter is determined in cross-neutralization assays, where the *R* value is an indication of the antigenic relationship between a virus and a vaccine strain. In general, it is accepted that *R* values of 0.4–1.0 indicate a sufficient relationship that would confer protection. The genetic relationships between viruses need to be used with care, as changes in critical regions of the gene encoding the structural proteins that make the virus capsid could lead to significant antigenic differences.

Although it is known that some highly potent vaccines are able to prevent transmission under experimental conditions, little is known of the rate by which transmission is

Table 3 Websites for further information on foot-and-mouth disease

Website	*URL*
OIE	www.oie.int
FAO	www.fao.org
World Reference Laboratory for FMDV	www.iah.bbsrc.ac.uk
European Union legislation	europa.eu.int/eur-lex
European commission for the control of FMDV	www.fao.org/ag/againinfo/commissions/en/enfmd/enfmd.html
Pan American Foot-and-Mouth Disease Center (PANAFTOSA)	www.panaftosa.org.br
Picornavirus home page	www.iah.bbsrc.ac.uk/virus/Picornaviridae/index.html

prevented by emergency vaccination in an outbreak in densely populated areas. Most vaccine studies are based on prevention of clinical disease, in agreement with the European Pharmacopoeia and OIE Manual of Diagnostic Tests and Vaccines, which prescribes PD50 challenge infection experiments in the natural host. Because the period before a country can obtain disease freedom from the OIE is extended if vaccination is used in previously free zones, the decision to start with vaccination may be postponed until other measures have failed to stop the outbreak.

Due to the inadequacies of the current vaccines, several attempts have been made to improve the vaccine using recombinant technologies. However, these are not yet commercially available and will most probably be too expensive for routine applications.

Surveillance of FMDV is aimed at reducing the HRP (high-risk period). Because FMDV has a short incubation period and normally causes overt disease, it will easily be recognized in cattle. Thus, if regular clinical inspection is guaranteed, serological surveillance for FMDV does not seem cost-effective in the face of an outbreak. However, most countries have their own emergency plans that describe the actions needed. Awareness among farmers and veterinary practitioners is critical for a quick identification of FDMV. If clinical inspection of animals cannot be performed regularly, serological surveys may be necessary to identify convalescent or carrier animals, such as in regions where little or no veterinary control is in place.

To aid in the decision process for control of FMDV, management support systems have been developed, and these may prove useful in the decision-making process in the emergency situation of an outbreak.

For online references, see **Table 3**, which contains a set of relevant websites.

See also: **Contaminants of Milk and Dairy Products**: Environmental Contaminants. **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health. **Husbandry of Dairy Animals**: Goat: Health Management; Sheep: Health Management. **Office of International Epizooties**: Mission, Organization and Animal Health Code. **Policy Schemes and Trade in Dairy Products**: Trade in Milk and Dairy Products, International Standards: World Trade Organization. **Risk Analysis**.

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Infectious Diseases: Hairy Heel Warts

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Introduction

Hairy heel warts, also known as digital dermatitis, interdigital dermatitis, interdigital papillomatosis, foot warts, heel warts, digital warts, strawberry foot rot, Mortellaro disease, verrucous dermatitis, and papillomatous digital dermatitis (PDD), is a contagious, painful disease characterized by a wart-like inflammatory and hyperkeratotic lesion, typically located on the plantar skin between the heel bulbs and adjacent to the coronary band of the bovine digit. Hereafter, the condition will be referred to as PDD. PDD cases were first described in Italy in 1974. Since that time the condition has been reported worldwide. In the United States, a 1996 survey of dairy operations indicated that PDD prevalence among herds was 47% with an overall prevalence of 17.2% in cows and 6.8% in bred heifers. A 1999 survey of 214 farms in Chile reported that 91% of the dairies surveyed had affected cows. A 2000 survey of the prevalence of PDD lesions in culled dairy cattle at a slaughterhouse in the southeast United States found that 29% of animals had gross lesions. In the northern parts of Germany, 59% of dairy farms had affected animals with an average prevalence of 12.5% per farm.

PDD results in significant economic loss on dairies due to associated lameness attributable to losses in milk production and quality, culling, decreased reproductive efficiency, and decreased feed intake with loss of body weight as well as treatment and control expenses. Regarding reduced reproductive efficiency, on a high-producing US dairy, lame cows took 36–50 days longer to conceive after calving than nonlame cows. Because of the high degree of discomfort in affected animals, PDD is also an animal welfare concern.

The etiology of PDD appears to be multifactorial with infectious agent, management, breed, individual animal, and environmental factors influencing disease pathogenesis. The specific cause of PDD has not been conclusively established but spirochetes have been implicated as playing a major role. The disorder is characterized by inflamed, painful lesions, which may be proliferative, hyperplastic, or ulcerative. Initial lesions appear as superficial inflammation of the skin. Mature lesions are circumscribed and are either erosive or proliferative. Lesions typically appear on the plantar aspect of the hind foot just proximal to the heel bulbs and often extend into the interdigital space. Occasionally, lesions may be observed on the cranial aspect of the foot between the toes, and lesions have been reported on the skin of mammary glands. Effective treatment and control options are available but eradication of the disease, even within an individual herd, is unlikely.

Etiology

The specific etiology of PDD has not been elucidated. Lesion resolution occurs with the application of antibiotics, indicating a bacterial etiology. A mixed population of Gram-negative bacteria, including anaerobes, microaerophilic organisms, and spirochetes, has been demonstrated within or isolated from PDD lesions.

Role of Bacteria

Even though the specific bacterial etiology of digital dermatitis remains equivocal, the lesions have been associated with the presence of Treponemes. Organisms found in PDD lesions of California dairy cows were identified as spirochetes that were most likely of the genus *Treponema*. Treponemes have been seen on sectioned lesion specimens and are found deep within the epidermis, invading the stratum spongiosum and dermal papillae (**Figure 1**). Treponemes have been consistently isolated and identified in lesions from the United States, Germany, Canada, and Japan. Presently, at least five phylotypes of *Treponema* have been detected in PDD lesions by analysis of PCR-amplified rRNA, of which three groups closely resemble cultivatable human spirochetes: *Treponema denticola*, *Treponema phagedenis*, and *Treponema vincentii/Treponema medium.* Microbiological examination of 51 cases of PDD demonstrated that all three Treponeme phylotypes were present in 74.5% of lesions, which suggests that, in many cases, the etiology is polytreponemal. *Treponema denticola* has been demonstrated in the surface debris and superficial layers of PDD lesions. This is in contrast to *T. phagedenis*, which has been found deep within the epidermis. Both genetic and antigenic diversity exist between strains of *T. phagedenis*-like organisms isolated from within active PDD lesions. The same study suggests, based on 16S RNA gene sequence analysis, growth characteristics, and flaB2 gene sequence analysis, that a unique and novel Treponeme named *Treponema pedis* sp. *nov.* represents a distinct cluster of phylotypes found in PDD lesions. As further evidence that Treponemes play an important role in the etiology of

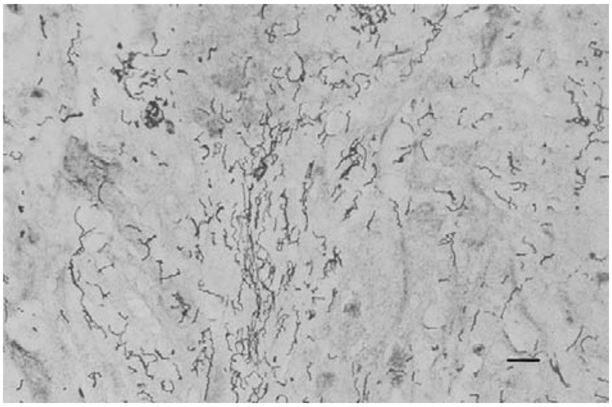

Figure 1 Photomicrograph of a section of tissue from the interdigital space of a cow with gross lesions of hairy heel warts. Spirochetes are abundantly present within the stratum spinosum. Silver stain; bar=100μm. (Reproduced with permission from Brown et al., 2000.)

PDD, affected cows show both a humeral and cellular immune response to PDD-associated spirochetes. Additionally, it has been suggested that the innate immune and wound repair functions of bovine macrophages exposed to *T. phagedenis*-like spirochete cellular constituents are impaired, thereby enabling bacteria to resist clearance and induce lesion formation. Spirochetes are strict anaerobes with fastidious nutritional requirements and are easily overgrown by more rapidly growing organisms commonly present in PDD lesions; few have been isolated in pure culture. Other bacteria including *Fusobacterium* spp., *Bacteroides* spp., *Campylobacter* spp., and *Peptococcus* spp. have been found in samples obtained from PDD lesions. It appears that the potential point of entry into the epidermis is via hair follicles and sebaceous glands. While PDD can be reproduced experimentally by inoculating the feet of fecal slurry-exposed cattle with lesion material, bacteria isolated from PDD lesions have not fulfilled Koch's postulates in experimental infections. Current belief is that together with the other bacteria, spirochetes are presumed to be causative agents. Although the lesion is commonly referred to as a wart, there is no evidence to support viral etiology.

Risk Factors

It appears that PDD is highly contagious based on the spread of disease regionally, high levels of disease within affected herds, within-herd spread after introduction of affected cattle, and higher prevalence in younger cows. Major risk factors for having a high herd prevalence of PDD include herd size (positively correlated with increasing herd size), muddy corrals, large dairy breeds, and the purchase of dairy replacement animals. In Chilean dairy cattle, loose-housed cows had a higher risk of developing PDD heel warts, followed by cows in freestalls or open corrals, compared with cows on pasture all year. Possibly, the disease is more common in freestall-confined herds because the feet are more likely to be constantly exposed to moisture and manure. The feet often become coated with a layer of dry manure, which may favor anaerobic conditions necessary for growth of the causal organisms. Additional factors associated with high (>5%) prevalence of PDD heel warts include region (southwest, northwest, and southeast regions in the United States), type of land accessed by lactating cows on a daily basis, flooring type where lactating cows walked (more common on slatted floors or grooved concrete, suggesting microtrauma), parity (more common in first-parity animals), use of a hoof trimmer who also trimmed hooves on other farms, and lack of washing hoof-trimming equipment between farms.

Clinical Signs

Animals affected by PDD are characterized by lameness with walking on the toes and clubbing of hooves. Cows are often affected in both hind claws and less commonly in the fore claws. Approximately 80% of PDD lesions occur on the plantar aspect of the rear foot. The predisposing factor for development of lesions on the hind feet may be that the hind feet are more exposed to manure slurry. The degree of lameness associated with PDD heel warts does not seem to correlate with the size of the lesion. Severely affected animals are reluctant to move and the affected limb may be held shaking in partial flexion due to intense pain. Less severely affected animals rest or bear weight on the toes to avoid contact with the heel area.

Lesion Description

Both clinical and gross pathological studies have been undertaken on lesions from affected cows on California dairies. Lesions associated with PDD are quite variable in appearance. They typically involve the skin adjacent to the heel or, less commonly, the proximal part of the interdigital space but can also be found on an interdigital overgrowth (fibroma), in a sole ulcer, or around the dewclaws (**Figure 2**). Initially, a circumscribed hyperemic area of 1–4 cm^2 appears on the skin just above the coronary band in the interdigital cleft of the plantar (or palmar) aspect of the claws and is associated with intense pain. Initial lesions appear as exudative, with superficial inflammation of the skin of the digit. At this stage, the exudate may have a distinctive pungent odor, likely due to secondary bacterial growth. In the early stages, the hairs of the diseased areas are often erect and later disappear. The pastern is occasionally diffusely swollen, but

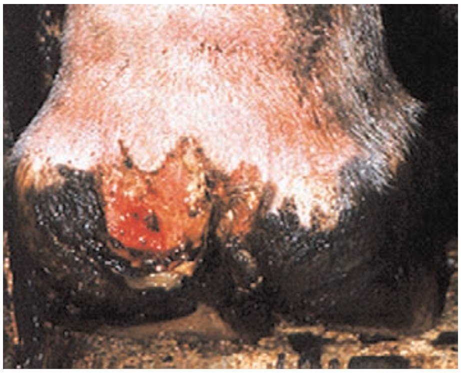

Figure 2 Plantar view of a cow's foot affected by hairy heel warts. This chronic lesion is circumscribed, erosive-granular and bleeds easily.

swelling is usually a secondary occurrence. As the lesion progresses, there is superficial loss of tissue from the skin of the coronary band of the claws and dewclaws. Many affected animals will have concurrent infection with *Dichelobacter nodosus*, leading to erosion of the horn in the heel area of the axial space. The lesion may have a grayish exudate covering the conical papillae such that the surface resembles terry cloth (**Figure 3**).

Cleansing exposes reddish granulation tissue (hence the name strawberry foot rot) with a concave profile. The area is circumscribed by a discrete line of raised hyperkeratotic skin often bearing erect hairs 2–3 times longer than normal. Lesions are prone to bleeding. In longer standing cases, there may be progression to undermining or erosion of contiguous horn resulting in permanent claw damage. Alternate forms of the lesions frequently have a distinctive proliferative reaction, which varies from a velvet-like appearance to clearly papilliform with hyperkeratotic papillae that may be 2 cm or more in length, hence the descriptive name hairy heel warts. Progression from the erosive to papillomatous form typically takes 2–3 weeks. Oftentimes, a single lesion will be mixed with areas of erosion (strawberry-like) and areas of proliferation (hence the name hairy). Epidermal erosion is the initial stage of the mature papilliform lesion, which evolves through the following phases: erosion, intermediate cutaneous plaque with peripheral papilliform reaction, and raised mature plaque with hyperkeratotic papillae. However, an Italian veterinarian reported that the lesions he typically observed did not progress through that evolutionary progress and erosive lesions remain apparently unchanged for an indefinite time unless treated. Regardless of lesion type, complete spontaneous recovery is seldom observed.

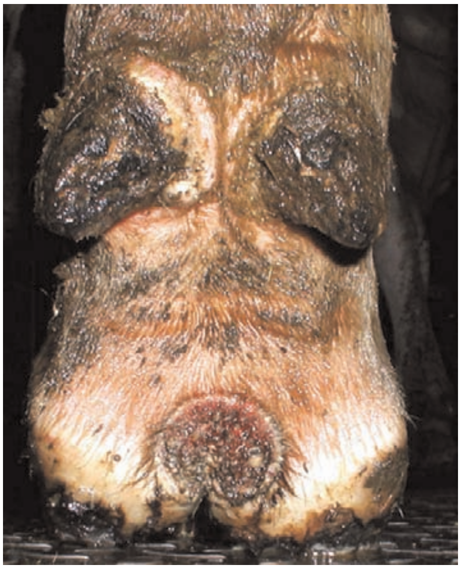

Figure 3 Hind foot of a cow with a hairy heel wart. This is the characteristic strawberry appearance.

Histological Description

Histological characteristics of PDD include extensive parakeratosis and focal hyperkeratosis with patchy papillomatous change; erosion of tips of papillary dermis; dense colonization of parakeratotic stratum corneum by spirochete-dominated bacterial flora; vacuolation of parakeratotic stratum corneum attended by neutrophils and erythrocytes; invasion of subadjacent stratum spinosum by spirochetes; loss of stratum granulosum; presence of multifocal to confluent acanthosis; lymphoplasmacytic perivascular dermatitis; and an absence of a fibromatous change. Lesions sometimes contain arteries affected by vasculitis in different stages. Some show intact neutrophils lying perivascularly and in vessel walls, and others demonstrate nuclear dust following leukocytoclasia.

Herd Epidemiology

Once a herd begins to experience PDD lesions, the spread is rapid among adult cows with the majority of animals becoming affected within a year. When the condition becomes established in a herd, most new cases of lameness are observed in first lactation animals as they enter the milking herd. Although lesions may be prevalent in older animals, the degree of lameness associated with lesions is often less than in lactating heifers. A seasonal increase in incidence seems to correlate with the presence of wet, muddy conditions. In California, the disease incidence is highest in the spring and summer months, while in the United Kingdom, PDD incidence is highest during the winter months.

Control

Without the continued use of appropriate control measures, up to 48% of affected cows experience a recurrence or development of new lesions within 7–12 weeks after an apparently complete therapeutic response. The reported recurrence rate in one herd during the 11 months following treatment where no subsequent control measures were practiced was roughly 88%. In addition to timely and effective treatment of lesions to reduce spread to nonaffected animals, control measures should also include appropriate housing so that exposure to moisture, mud, manure, and trauma-causing flooring is minimized. Development and implementation of a farm biosecurity plan to reduce the introduction of affected replacements into the herd and assurance that personnel and equipment do not serve as fomites are essential for effective long-term control.

Footbathing

Footbathing is commonly used in an attempt to treat and control PDD as well as other infections of the bovine foot. Footbaths typically contain solutions of antibiotics, copper sulfate, formaldehyde, or other nonantibiotic solutions. Despite several disadvantages, footbaths are likely to remain a preferred method of controlling PDD. However, unless carefully and conscientiously managed, footbaths are unlikely to be effective and may actually contribute to the spread of PDD within a herd.

Treatment

PDD is generally treated with one or a combination of three approaches: (1) systemic antibiotics; (2) individual topical treatment; and (3) mass topical treatment via footbathing. PDD lesions are usually responsive to either parenteral or topical antibiotic therapy.

Parenteral administration of antibiotics for PDD treatment has fallen into disfavor due to high cost, perceived lack of efficacy, and mandatory withholding of milk and meat from treated animals. However, a study involving a small number of affected cows (21) demonstrated that most cows with PDD heel warts treated with procaine penicillin G (18 000 U kg^{-1} i.m. b.i.d. for 3 days) or ceftiofur sodium (2 mg kg^{-1} daily for 3 days) responded favorably. Both parenteral therapy and topical therapy combined with radical trimming have been recommended when there is severe involvement of the coronary band with fissure development in the vertical hoof wall. A favorable response to parenteral cefquinome administered for 3 days has been noted in the United Kingdom and Argentina. Other reports suggest that systemic antibiotics (cefquinome or erythromycin) are ineffective in reducing lesion severity.

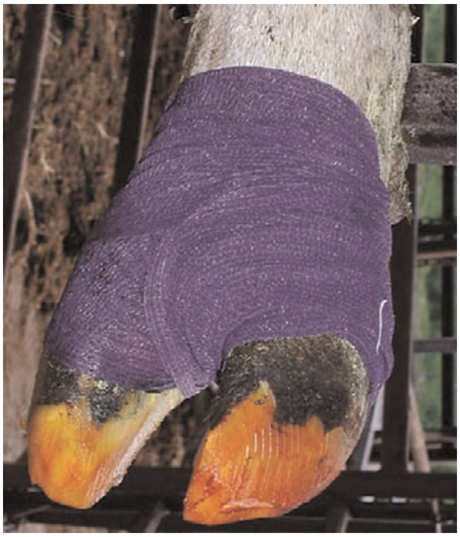

Figure 4 Lesions in this location are best treated by placing a bandage over an effective antimicrobial compound.

Following thorough cleaning of the lesion, topical antibiotic treatment, especially when applied under a thin bandage left in place for 3–5 days, is highly (>90%) effective (**Figure 4**). Cotton balls or gauze soaked in tetracycline, oxytetracycline, lincomycin, or a lincomycin/spectinomycin combination under a bandage is effective but labor-intensive. Either liquid or powdered forms of the preceding antibiotics are effective. The bandage should be applied in such a manner that it will fall off spontaneously after 3–5 days. Application of lincomycin-containing paste under a bandage has efficacy similar to that of oxytetracycline.

Topical spray with antibiotic solutions has become popular on many farms. A common protocol involves treatment with oxytetracycline (25–50 mg ml^{-1}) in a pump-up garden-type sprayer applied at a rate of 10–20 ml per foot. A reportedly successful protocol involves spraying all feet once a day for 5 consecutive days at monthly intervals and spraying all visible lesions daily. Similar results to those obtained with oxytetracycline can be expected with lincomycin (0.6 g l^{-1}) applied topically via a sprayer. A surfactant such as propylene glycol or dish soap (approximately 20 ml l^{-1}) will improve the ability of the antibiotic solution spray to adhere to and penetrate the lesions. The risk of violative antibiotic residues in milk has been evaluated when oxytetracycline was applied by spraying or under a bandage. With concentrations of oxytetracycline commonly used to treat PDD, the risk of violative residues is very low. However, circumstantial evidence suggests that milk contamination has occurred. Similar studies have not been reported with other antibiotics. In the United States, use of topical antibiotics for treatment of PDD heel warts is strictly extralabel and requires a veterinary prescription.

Anatomical location of the lesions results in variation in treatment response to topical oxytetracycline spray. Cows with lesions located in the interdigital cleft are less likely to respond than those with lesions on the heels or near the coronary band.

The increase in organic dairies has increased interest in nonantibiotic treatment alternatives. Many of these treatments have not been critically evaluated for efficacy. A topical product (PediCuRx Complete, WestfaliaSurge, Inc., Naperville, IL, USA) containing a mixture of soluble copper, a peroxide compound, and a cationic agent was reported to be equal or superior in efficacy to oxytetracycline, based on reduction of pain and lesion score. There are a number of other nonantibiotic products marketed for individual topical treatment of PDD, including antiseptics, acidified copper salts, organic acids with essential oils, and metallic enzymes (Hoof Care for Cattle, Probiotics International Ltd., Somerset, UK).

Walk-through footbaths are commonly recommended for treating as well as controlling herd outbreaks of PDD, particularly when within-herd prevalence is high. When used as the sole method of treatment or control, effectiveness varies considerably. Some of the variability is likely due to differences in footbath management. Antibiotics are widely used in footbaths for management of PDD. The major disadvantages to antibiotic use in footbaths are the rapid neutralization of activity in the presence of mud and manure, the expense of using large volumes of solution, and legislative restriction on the sale of milk from treated cows (even though violative antibiotic residues have not been demonstrated in milk from treated cows). The most frequently used antibiotics are tetracycline ($4\,\mathrm{g\,l^{-1}}$), oxytetracycline (2–$6\,\mathrm{g\,l^{-1}}$), lincomycin/spectinomycin ($1\,\mathrm{g\,l^{-1}}$), and lincomycin (0.1–$0.6\,\mathrm{g\,l^{-1}}$). Higher concentrations, such as oxytetracycline at 5–$10\,\mathrm{g\,l^{-1}}$ or lincomycin at 1–$3\,\mathrm{g\,l^{-1}}$, have been recommended when used in a once-per-month footbath program. A footbath containing erythromycin ($0.035\,\mathrm{g\,l^{-1}}$) has been critically evaluated and found to be effective when used twice within a 24 h period. The study also indicated that repeat treatment would be required in many cases that initially responded.

Formalin and copper sulfate are the most common nonantibiotic products used for footbathing even though there is significant safety (formalin) and environmental (copper sulfate) concern associated with the use of these compounds. Although widely used and supported by anecdotal reports of success, there is little experimental data to support the use of copper sulfate solutions in footbaths to control PDD. Formalin (37%) diluted to 2.5–5% concentration has been used extensively in footbaths. Concentrations greater than 5% are likely to cause skin irritation. The use of formalin in footbaths is complicated by concern for worker and environmental safety.

Because use of antibiotics for mass medication is extralabel drug use and has the potential to result in violative residues in milk, nonantibiotic treatments have been evaluated for efficacy. A commercial triplex solution (PediCuRx Complete, WestfaliaSurge, Inc., Naperville, IL, USA) containing soluble copper, peroxide compound, and a cationic agent was shown to be as effective when used in footbath solution. A 5% solution of a nonantibiotic product (Double-Action, West Agro, Kansas City, MO, USA) with undisclosed ingredients appears promising when used either as a topical spray or as a footbath. The manufacturer recommends use of the footbath twice daily for 3–7 consecutive days each week or as a topical spray twice daily for 14 days or as needed.

Two-stage footbaths, in which cows walk through a prerinse footbath prior to the treatment footbath, may extend the number of cow passages before the solution must be changed. One study recommended the guideline of 1 l per cow in a footbath. Therefore, a 200 l footbath should serve a single treatment of 200 cows. However, depending on the degree of organic material on the cow's feet, the footbath solution may be changed after 30–150 cow passages. Also, footbaths long enough to ensure all four feet become submerged in the solution are recommended. A footbath of length 3 m and volume 360 l has been advocated.

Response to treatment is characterized by complete transformation of moist, red, raw painful surfaces that were prone to bleeding to dry, dark-brown, nonpainful, tough rubbery keratinous surfaces. The keratinous layer is tightly adhered to underlying white-pink healthy-appearing skin. A reduction in lesion size and partial restoration of hair growth are observed during healing as well as a reduction in signs of lameness.

Regardless of the effectiveness of the treatment protocol used, recurrences are commonplace. Treatment failures may be due to inconsistent application of topical sprays, or failure to treat for a sufficient number of days. Use of overdiluted or improperly prepared solutions can result in lack of efficacy. When tetracycline or oxytetracycline solutions are made up using hard water, the antibiotic may be neutralized or precipitated; therefore, it is recommended that distilled water be used to mix these solutions and that fresh solutions be prepared daily just prior to use.

Nutrition

Since the cause of PDD heel warts is presumed to be multifactorial, it appears reasonable that adequate nutrition, especially nutrients associated with epidermal integrity, may play a role in disease control. One study indicated a reduction in the incidence of PDD in New York dairy herds when rations were supplemented with a combination of zinc methionine, copper lysine, manganese methionine, and cobalt glucoheptonate. The supplement supplied 360 mg Zn, 200 mg Mn, 125 mg Cu, and 25 mg Co per day per head.

Immunity

Even when successfully treated, the recurrence rate of PDD lesions is near 50%, suggesting that natural infection does not establish long-lasting immunity. Dairy cattle with PDD lesions develop an immune response primarily against the liposaccharide of spirochetes. Not surprisingly, attempts to develop a vaccine to control this problem have not been successful and will likely remain challenging.

Conclusion

PDD heel warts have emerged worldwide as a major cause of lameness in dairy cattle. Although the specific etiology has not been conclusively determined, current information supports a multifactorial etiology with spirochetes eventually causing development of lesions. At the present time, treatment and control strategies are effective but eradication on a herd basis is unlikely, especially in large herds or where replacement animals are purchased. Even when treatment is effective, lesions tend to recur. Although antibiotics are an effective treatment for PDD warts, the fact that they are used in an extralabel manner and may increase the risk of milk residue violation and development of antibiotic resistance, the use of nonantibiotic formulations is likely to increase. Improvement in dairy biosecurity should reduce the incidence of new lesions in low-prevalence herds. New additions should be visually screened for lesions and treated if lesions are observed or if there is a history of PDD on the farm of origin. Thirty-day isolation is recommended for purchased additions since PDD has an approximately 3-week incubation period. Changes in management and housing, including segregation of first lactation animals and minimization of exposure to wet muddy conditions, will reduce disease incidence. If it is conclusively demonstrated that lesion development is dependent on invasion of tissue by spirochetes, an effective vaccine may eventually be produced to aid in the reduction of disease incidence or severity or hasten recovery.

See also: **Dairy Farm Management Systems**: Dry Lot Dairy Cow Breeds. **Diseases of Dairy Animals**: Non-Infectious Diseases: Acidosis/Laminitis. **Feed Ingredients**: Feed Supplements: Microminerals. **Manure/Effluent Management**: Nutrient Recycling; Systems Design and Government Regulations.

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Infectious Diseases: Johne's Disease

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Introduction

Johne's disease (paratuberculosis) is a chronic contagious infectious disease affecting dairy cattle worldwide. The disease is economically important and continues to spread despite the implementation of control programs in many countries. The ability of the causative agent of Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis* (**Figure 1**), to infect and cause disease in humans is a controversial and unresolved scientific question.

Description of the Organism

Mycobacterium avium subsp. *paratuberculosis* was simply called *M. paratuberculosis* until relatively recently and many authors still favor the latter name. The proposal to reclassify it as a subspecies of *Mycobacterium avium* resulted from genetic studies that showed very high DNA homology (98–99%) with *M. avium* subsp. *avium*. However, recent sequencing of the *M. paratuberculosis* genome identified 35 unique coding sequences that distinguish *M. paratuberculosis* from *M. avium*. Both names, *M. a. paratuberculosis* and *M. paratuberculosis*, remain in common use in scientific literature and can be considered synonymous. In the remainder of this article, the organism will be referred to as *M. a. paratuberculosis*.

In spite of the close genetic similarity between *Mb. a.* subsp. *avium*, a cause of tuberculosis in birds, and *M. a. paratuberculosis*, there are several important phenotypic differences. *Mycobacterium a. paratuberculosis* grows much more slowly *in vitro*; it takes roughly 1 week for *Mb. a. avium* to form visible bacterial colonies on primary isolation media versus 12–16 weeks for *M. a. paratuberculosis*. *Mycobacterium a. paratuberculosis* is also dependent upon the addition of a mycobacterial siderophore called mycobactin to culture media for growth. It is virtually the only species of mycobacteria that exhibits mycobactin dependency *in vitro*. Colonies of *M. a. paratuberculosis* are very rough in appearance on most culture media, while those of *Mb. a. avium* are generally smooth.

Pathogenicity characterizes the most striking differences between *Mb. a. avium* and *M. a. paratuberculosis*. *Mycobacterium a. avium* is a primary pathogen in birds, where it causes avian tuberculosis, a chronic, debilitating disease of aged birds. It has very low virulence for mammals, being only sporadically involved in self-limited lymph node infections, particularly in pigs. The one exception is in humans in the terminal stages of HIV infection where, after severe suppression of cellular immunity occurs due to the viral infection, environmental strains of this agent commonly infect the human intestinal tract. In sharp contrast, *M. a. paratuberculosis* is pathogenic to dairy cattle and a wide variety of other ruminants, and has been reported as a sporadic cause of infection in several other mammals. It is not considered a pathogen of birds, although birds have been infected experimentally.

The ecology of *M. a. paratuberculosis* is directly related to its mycobactin dependency. Because of the inability to produce this siderophore, it cannot acquire iron from its environment as readily as *Mb. a. avium* can. The result is that *M. a. paratuberculosis* is essentially an obligate parasitic pathogen in that, except for laboratory culture conditions, virtually all replication of this organism occurs in the host, within macrophages. There it can 'steal' iron, essential for growth, from host iron-binding proteins such as transferrin. Once *M. a. paratuberculosis* leaves the host, it depends on its tenacious survival characteristics to persist in contaminated environments, such as soil, water, and animal feed, until it is ingested by another suitable host and establishes infection. *Mycobacterium a. avium* by contrast is commonly found in soil and water, particularly those with a lower pH, where it can multiply as a free-living organism. Hence, isolation of *M. a. paratuberculosis* from food, water, or soil indicates contamination of those substances with material, usually feces, from an infected animal.

Like other mycobacteria, *M. a. paratuberculosis* is more resistant to physical factors such as heat, cold, and drying than most vegetative bacteria. It shares the property of resistance to chemical disinfectants with other mycobacteria, phenolic disinfectants being the most efficacious against mycobacteria. Like *Mb. a. avium*, *M. a. paratuberculosis* is more resistant to antimicrobial agents than the causes of tuberculosis in humans and cattle, *Mycobacterium tuberculosis* and *M. bovis*, respectively.

Resistance to heat, particularly conditions of pasteurization, has become of paramount concern as medical scientists search for explanations as to why *M. a. paratuberculosis* is found in humans with Crohn's

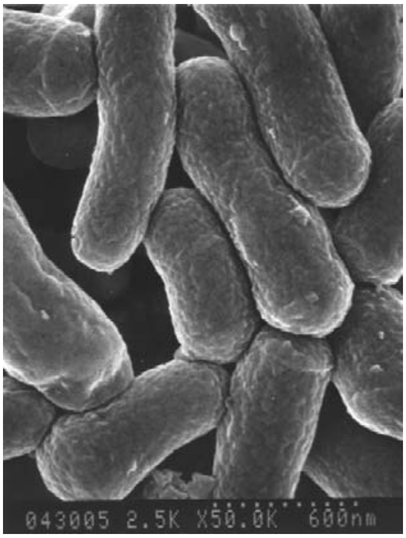

Figure 1 Scanning electron micrograph of a group of *Mycobacterium paratuberculosis* cells. Photograph courtesy of MT Collins.

disease (see section 'Zoonotic concerns'). Thermal tolerance studies and studies that mimic commercial pasteurizers on a laboratory scale provide conflicting results. In addition, studies reporting the recovery of viable *M. a. paratuberculosis* from retail-ready milk are also conflicting, adding more fuel to the debate about pasteurization efficacy and the association of *M. a. paratuberculosis* with human disease. A simulation model capturing published studies on the heat resistance of *M. a. paratuberculosis* concluded that the probability of detecting *M. a. paratuberculosis* in pasteurized milk is less than 1%. The reasons for discrepant figures in the literature include improper pasteurization and cross-contamination within the laboratory. More work is needed before these controversial issues can be resolved.

Prevalence of Infection in Dairy Cattle

Johne's disease has been reported in dairy cattle in virtually every country of the world. Only two locations, Sweden and Western Australia, can support claims of freedom from this infection in their dairy cattle populations. Published herd prevalence estimates in the remaining countries range from 7% (Austria) to 55% (The Netherlands). These estimates are only very approximate figures, being heavily dependent on the design and scope of each survey and the type of diagnostic test used. A large, statistically based 1996 survey of dairy cattle in the United States found that 22% of all dairy herds surveyed were infected with *M. a. paratuberculosis*. Infection rate was associated with herd size, being 19% for herds of less than 50 cows and 40% for herds of more than 300 cows. Within-herd prevalence for infected herds is typically 2–10% of cows. A more recent survey conducted in 2007 evaluated herd-level prevalence by culture of environmental samples. The study reported that 68% of US dairy operations were infected with *M. a. paratuberculosis*, demonstrating a significant increase in the incidence of Johne's disease in the last 10 years.

Pathogenesis

Nearly all infections of cattle with *M. a. paratuberculosis* begin in young calves. For as yet unknown reasons, calves are far more susceptible to infection than are adult cattle. Although *in utero* transmission from dam to fetus has been documented, the primary route of infection appears to be through ingestion of *M. a. paratuberculosis* in contaminated milk, water, feed, or anything else in the calves' environment. The ingested organism passes down the gastrointestinal tract eventually passing across the intestinal wall via M cells at the site of Peyer's patches. Peyer's patches are tonsil-like aggregates of lymphoid tissue and are common routes of entry for many intestinal pathogens. The target site of bovine Johne's disease is the ileum (terminal portion of the small intestine) (**Figure 2**). The ileum is targeted simply because of the abundance of Peyer's patches at this location rather than any complex biochemically based tissue tropism. After entry, host macrophages engulf and attempt to destroy this pathogen. Like other mycobacteria, *M. a. paratuberculosis* resists killing by macrophages and actually thrives in this intracellular location.

Over time, measured in months to years, the organism multiplies and infects other macrophages. In response to the infection, the host animal mounts a cell-mediated

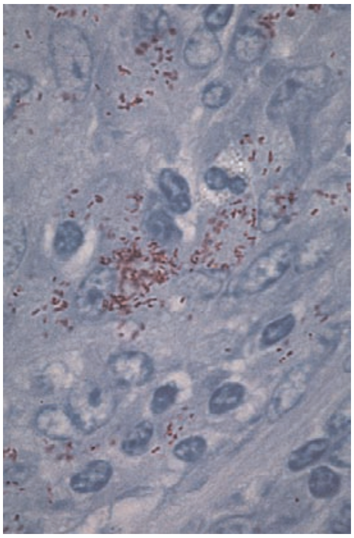

Figure 2 Bovine ileum containing *Mycobacterium paratuberculosis*. Photograph courtesy of MT Collins.

inflammatory response. Key cells in this response are macrophages and T lymphocytes, whose interactions are mediated by a complex interplay of cytokines and other chemical mediators. The net result is a steady accumulation of mononuclear inflammatory cells in the wall of the ileum. This inflammation becomes visible to the naked eye as a thickened intestinal wall with corrugated mucosal surface. Generally, the mucosal surface is intact, not ulcerated, and not hyperemic (reddened), pathological manifestations that help to distinguish Johne's disease from other causes of diarrhea and intestinal damage in adult dairy cattle. The lymph nodes adjacent to the infected intestinal tract trap *M. a. paratuberculosis* as they are carried away from the bowel inside macrophages. These lymph nodes become a secondary site of infection and also enlarge as the animal mounts a cell-mediated inflammatory response. If such affected lymph nodes are cut in cross section, this inflammation can be seen: normal lymph nodes are smaller and liver-colored, whereas those infected with *M. a. paratuberculosis* are larger and white colored.

Although *M. a. paratuberculosis* infection disseminates throughout the bloodstream in the later stages, host tissue reaction is not usually evident either grossly or microscopically, suggesting that this is a late event in the pathogenesis of the infection. However, this is conjecture and some investigators suggest that detection of *M. a. paratuberculosis* in blood, specifically circulating white blood cells, may be a good method for early diagnosis of the infection.

Damage to the intestine due to infection and inflammation leads to the development of protein-losing enteropathy: protein absorption is impaired and increasing amounts of protein are lost in the feces. This, coupled with the effects of cytokines such as tumor necrosis factor released by lymphocytes responding to the infection, leads to cachexia (wasting). The weight loss can be very pronounced, particularly when a cow is in the early part of the lactation cycle producing large quantities of milk, placing heavy demands on her ability to maintain body condition.

Protein deficiency induced by *M. a. paratuberculosis* can be detected in serum. Total protein levels may be depressed, but the serum albumin concentration is most markedly affected and is a better indicator that the animal may have Johne's disease. Hypoalbuminemia leads to an inability of the cow to retain fluid in the circulatory system leading to dependent edema, often in the submandibular space. This soft fluctuant mass under the jaw is called 'bottle jaw' and is considered very characteristic of Johne's disease although this is not the only condition that can cause bottle jaw.

Clinical Signs

Clinical signs result from the cascade of events described in response to *M. a. paratuberculosis* infection. In cows, they often, but not always, are initiated within 1 month of calving, most commonly after the second or third lactation. It is remarkable how cows that have harbored this infection in their intestine for 5 or 6 years, the preclinical or subclinical phase of infection, can suddenly develop clinical signs leading to death or culling from the herd. Although the 'stress of calving' is often incriminated as the event that triggers the onset of clinical signs, no specific mechanisms describing how this happens have been reported. The constellation of clinical signs considered most typical of Johne's disease is (1) diarrhea that does not respond to treatment, (2) rapid weight loss, (3) decreasing milk production, (4) low serum albumin levels with or without bottle jaw, in the face of (5) a good appetite and (6) no fever. Not every case of Johne's disease is typical or easy to diagnose and variations on this typical clinical presentation are common. General unthriftiness is often the primary complaint from owners (**Figure 3**). The signs of Johne's disease are sufficiently nonspecific that corroboration by laboratory tests is generally required.

At the herd level, nutritionists are often the first to detect that the herd may have *M. a. paratuberculosis*-infected cattle. When rations are well balanced and effectively delivered to cattle but herd production does not match expectations, the presence of Johne's disease in the herd should be suspected and tested for.

Economic Impact

Cattle infected with *M. a. paratuberculosis* produce less milk, have increased days open, a decreased carcass weight at slaughter, and a shortened herd life. The combined effect on herd profitability is a direct function of the prevalence of the infection in the herd. Most importantly, the economic impact increase geometrically as the infection spreads from the adult herd to heifer calves destined to join the milking herd, and then they further propagate the infection. It is vital that this cycle of infection be

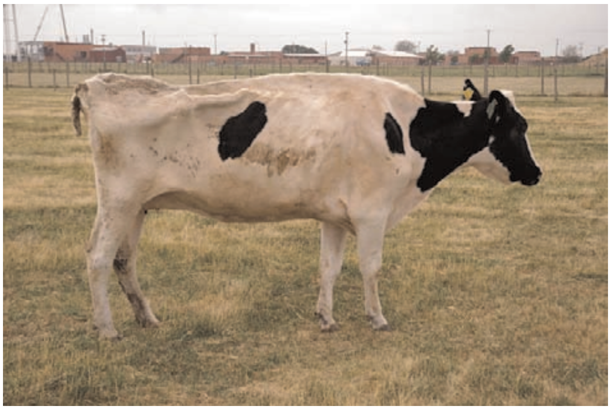

Figure 3 Emaciation associated with clinical Johne's disease. Photograph courtesy of JR Stabel.

broken if the economic damage to the herd caused by the infection is to be controlled or eliminated.

Diagnosis

Since the early 1990s, many new and/or improved diagnostic tests for Johne's disease have been developed and many of these have become commercial kits. Diagnostics for bovine Johne's disease fall into three general categories: (1) direct detection of *M. a. paratuberculosis*; (2) those that detect a serum antibody response to the organism; and (3) those that detect a cellular immune response to the organism.

Detection of *M. a. paratuberculosis* is considered definitive proof that an animal is infected (diagnostic specificity 100%) because the organism replicates only inside infected animals and numbers persisting in the environment are generally very low. However, it has been shown that *M. a. paratuberculosis* fed to adult cattle can pass through the intestinal tract and be detected in manure samples. Regardless, it is a reasonable assumption that, barring mistakes by the testing laboratory, detection of *M. a. paratuberculosis* in feces, milk, or tissue samples is proof that the animal is truly infected. Only under situations of very high environmental contamination, for example, herds with a very high infection rate, could 'pass through' be the cause of positive test for *M. a. paratuberculosis* in fecal samples.

Detection of *M. a. paratuberculosis* can be done by culture of the organism on bacteriological media or by genetic methods. Detection by chemical or immunological methods is also theoretically possible and has been tried to a limited extent, but as of this writing these are not functional diagnostic methods except in research laboratories. Genetic techniques generally target the IS900 sequence and use polymerase chain reaction (PCR) or equivalent technology to amplify target sequences. Highly specific gene sequences such as HspX, ISMav2, ISMap02, and F57 have successfully been incorporated into PCR tests for *M. a. paratuberculosis* as well. More recently, the use of standards (plasmids carrying *M. a. paratuberculosis* genes) has shifted the platform of PCR assays from a qualitative nature (positive or negative) to a more quantitative assessment of the amount of *M. a. paratuberculosis* DNA present within a sample. This information can be extrapolated back to the number of organisms present and can provide some information on the relative degree of animal infectivity. Genetic detection has the advantage of being much faster than culture, with many PCR assays achieving similar sensitivity to culture. However, genetic detection has the disadvantage of being more expensive than culture. Although culture remains the preferred diagnostic method on clinical samples, PCR detection is rapidly gaining a foothold in veterinary diagnostic laboratories.

Culture of samples for isolation of *M. a. paratuberculosis* has the disadvantage of being labor intensive, hence expensive, and slow. Culture on conventional, test tube culture media such as Herrold's egg yolk agar or modified Lowenstein–Jensen medium typically requires incubation for 12–16 weeks before definitive culture interpretation can be made. Using liquid culture media and automated detection systems like the BACTEC and Trek-ESP II systems can shorten the detection time to 8 weeks, save labor costs by automating culture reading, and increase the analytical sensitivity (allowing detection of fewer organisms per gram of clinical sample). The BACTEC MGIT 960 system is a novel liquid medium system utilizing an oxygen-quenching fluorochrome sensor to assess the presence of *M. a. paratuberculosis* in samples this system has recently been incorporated by some veterinary diagnostic laboratories.

Serological diagnosis of bovine Johne's disease can be accomplished by several techniques: the complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), or agar gel immunodiffusion (AGID). The CFT is the assay most often required by countries importing cattle, although this is slowly being replaced by the ELISA. The AGID is sold in the United States as a diagnostic kit. This serological technique is best applied only to cattle showing clinical signs of Johne's disease. IDEXX Laboratories Inc. in the United States (Herdchek), Prionics AG in Switzerland (Parachek), and Institut Pourquier in France, a subsidiary of IDEXX (Pourquier), all produce commercial ELISA kits for bovine Johne's disease. Most ELISAs are performed in a microtiter plate format enabling a rapid, inexpensive platform for diagnosis. Newer versions of ELISA tests for *M. a. paratuberculosis* utilizing improved methods of antigen preparation have been published but are currently being used only in research laboratories. The identification of novel antigens that are more specific for *M. a. paratuberculosis* and that do not cross-react with the closely related *Mb. a. avium* will further improve the specificity of the ELISA test. The detection of *M. a. paratuberculosis* antibodies in milk is another method of diagnosis that has been used extensively in Denmark. The milk ELISA is gaining consideration in the United States and other countries as a screening tool due to the ease of sampling and similar sensitivity to serum ELISA tests for Johne's disease.

Assays for cellular immunity include the delayed-type hypersensitivity (skin) test and the interferon-γ assay. Neither is used routinely as agreement on the sensitivity and specificity of these tests for Johne's disease has not been established to the satisfaction of experts. Disagreement as to the accuracy of sensitivity and specificity estimates reported in the literature stems from the difficulty in interpreting the actual infection status of the animals. In addition, both parameters can be markedly affected by the antigen preparations used in testing. Yet recent work demonstrates promise for these cellular immune-based tests to be used in the diagnosis of young stock and cattle that are in the early subclinical phase of disease.

The pattern of progression of *M. a. paratuberculosis* infection relative to diagnostic tests has not been well documented. In general, however, the following sequence of events is believed to occur: cellular immune responses precede fecal shedding of *M. a. paratuberculosis* and this precedes serum antibody production. The cellular immune response rises first, as early as 6 months of age, but may begin to wane around the time antibody production is rising and cattle are showing clinical signs of Johne's disease. Once fecal shedding begins, it usually steadily increases with time. Likewise, once serum antibody production can be detected, antibody levels tend to rise and reach the highest levels when cattle show clinical signs of Johne's disease. Deviation from this general pattern by infected cattle is common. Nevertheless, this pattern affects the time frame when use of different diagnostic tests is recommended: tests for cellular immunity are best used on young cattle, 6–24 months old; fecal detection methods such as culture and/or PCR are best applied to cattle more than 2 years of age; and serological tests are most effective on cattle more than 3 years old. Selection of specific tests under specific circumstances involves an understanding of the accuracy of the test, the cost of testing, and the consequences of actions taken on the tests, among other things, and is beyond the scope of this article.

In addition to the diagnosis of individual animals, the aforementioned tests can also be used to screen herds for the presence of *M. a. paratuberculosis* by performing the testing on a random subsampling of animals within the herd. This information is helpful to producers who may not be aware that their herd is infected. An additional method for screening herds is a pooling of fecal samples from 5 to 10 cows within the herd and processing the pooled sample for culture or PCR. Likewise, processing of environmental samples collected at specific sites on the dairy for culture or PCR is an excellent alternative for assessing *M. a. paratuberculosis* infection within a herd. These methods significantly reduce the cost of testing compared to a per-animal basis and provide important information concerning the infection status of the herd.

Treatment

Rarely is treatment of *M. a. paratuberculosis* infection in cattle attempted. Hence, there is a paucity of data on this subject. In general, multiple antimicrobial drugs like those used to treat tuberculosis **Diseases of Dairy Animals**: Infectious Diseases: Tuberculosis and leprosy in humans must be used in combination over a very extended period of time, for example, 1–2 years, to potentially achieve treatment success. All products from treated animals, meat and milk, can never be used for human consumption. The cost of drugs for such a treatment regimen would be in excess of US$10 000. For this reason, Johne's disease in cattle is considered untreatable, actually meaning impractical.

Control

Control of Johne's disease should be done at two levels: within herds and among herds at the state, regional, or national level.

Mycobacterium a. paratuberculosis infections are spread within herds by any means that exposes young calves to contaminated milk or manure from the adult herd. Multiple strategies are used to limit this exposure risk. Effective control begins with the birth of calves in a clean, uncontaminated maternity pen or outdoors. Calves should be promptly removed from cows. Colostrum to be fed to calves should be from cows that are test-negative for Johne's disease and hygienically collected to ensure no fecal contamination. After colostrum, milk fed to calves should be pasteurized. This can be accomplished by the use of on-farm pasteurization equipment or by simply purchasing powdered milk replacer since these products are pasteurized in the course of being manufactured. Water provided to calves should be free of contamination from manure of the adult herd. Likewise, solid feeds should be fed to young cattle in the most hygienic manner possible to avoid manure contamination. Practices such as using the same equipment for removing manure from barns and for moving feed to heifers must be avoided.

Mycobacterium a. paratuberculosis infections move among dairy herds when infected animals are mistakenly purchased. Good biosecurity procedures can avoid, or at least significantly limit, the risk that this will occur. The highest risk of introducing *M. a. paratuberculosis* infection into dairy herds occurs when untested animals are bought from untested herds. Given the prevalence of Johne's disease in most countries, each animal bought this way has a 5–10% chance of being infected. The larger the number of cattle bought as herd replacements each year, the greater the likelihood of introducing the infection to a herd. Strategies to limit this risk include (1) limiting the number of purchased animals, ideally keeping a totally closed herd, (2) buying replacement cattle from only test-negative herds, ideally from only certified-free herds, or (3) buying test-negative cattle. Given the accuracy of diagnostic tests for Johne's disease and that few tests are very sensitive on cattle less than 2 years of age, the age class most frequently purchased, buying cattle from test-negative herds is far better than buying cattle that are individually tested. Owners of noninfected herds should seriously consider using one or more of these strategies to keep their herd free of *M. a. paratuberculosis* infection. The costs incurred by effective prevention of infection far outweigh the costs of controlling or eradicating the infection after it becomes established within a herd. Herd testing programs organized by the breed organizations, milk producer cooperatives, or governmental agencies will greatly facilitate control of the spread of Johne's disease among dairy herds.

Principles of Control Programs at the State or National Level

There are two aspects to Johne's disease control at the state or national level. The first is to assist owners of infected herds with the implementation of on-farm control measures. This can be accomplished first and foremost by education of herd owners and managers about the biology of this infection. Financial assistance with the costs of testing and costs of culling test-positive cattle will further help expedite Johne's disease control on the infected farms.

The second form of Johne's disease control at the state or national level is aimed at preventing the spread of *M. a. paratuberculosis* infection among herds. The foundation of such programs is the classification of herds based on number of times each herd is annually tested and is found test-negative. National voluntary control programs have become established in countries such as Australia, Sweden, Denmark, Italy, Israel, the Czech Republic, Canada, and the United States. Other countries such as Japan, Thailand, and Austria have more stringent control programs in place. In time, harmonization of such programs among dairy producing countries will facilitate international trade of animals without the fear of introducing Johne's disease.

Vaccination

Vaccination of calves is one control option that can be utilized by producers to allay the spread of disease within their herd. Calves must be vaccinated prior to 30 days of age in order to achieve adequate protection. Vaccination does not prevent infection, but studies have demonstrated reduced incidence of clinical disease and fecal shedding. Several commercial vaccines are available for use in cattle, including Mycopar (USA), Strain ID-Lelystad (The Netherlands), Neoparasec (France), Paratuberkulose vaksine (Norway), and Silirum (Spain). Research is currently being conducted on the development of subunit vaccines for Johne's disease that may prove to be more efficacious and reduce cross-reactivity with other mycobacterial diseases such as bovine tuberculosis in the field. Vaccine candidates such as Ag85 complex, superoxide dismutase (SOD), and heat-shock protein 70 (Hsp70) show promise in reducing the bacterial burden in the host. Other protein, lipid, and DNA components of *M. a. paratuberculosis* are currently being evaluated as potential vaccines. It is likely that vaccination will become more acceptable as a method of control as the incidence of Johne's disease continues to increase in the field.

Zoonotic Concerns

Mycobacterial pathogens are notorious for their relatively broad host range and ability to infect both animals and humans. This is true in the case of tuberculosis in cattle (*M. bovis*), tuberculosis in birds (*Mb. a. avium*), and even leprosy (*M. leprae*). Pathogens capable of being transmitted from animals to humans are called zoonotic agents. Some evidence suggests that *M. a. paratuberculosis* too can infect humans.

The first reported isolation of *M. a. paratuberculosis* from humans was reported by Chiodini in 1984. In that report, he recovered the organism from three patients with Crohn's disease, a chronic inflammatory bowel disease with clinical and pathological similarity to Johne's disease. Subsequently, others have also isolated *M. a. paratuberculosis* from Crohn's disease patients but not very frequently, raising questions about the significance of such findings.

The use of IS900 PCR to detect *M. a. paratuberculosis* in human tissues has been employed by many investigators, but the results from such studies are conflicting. Key factors that contribute to these discrepant results are the differences between laboratories in test protocols, types and size of samples, and source populations used. A recent systematic review of published studies investigating the relationship between *M. a. paratuberculosis* and Crohn's disease was inconclusive, with 23 studies reporting significant positive associations, 23 studies reporting nonsignificant associations, and 14 studies not detecting *M. a. paratuberculosis* in any sample. The use of serological tests to provide evidence of a relationship between *M. a. paratuberculosis* and Crohn's disease has proven equally inconclusive. Association does not prove causation; therefore, it will take more time and careful investigations to determine if *M. a. paratuberculosis* is capable of infecting humans and/or causing Crohn's disease. If this is found to be true, it will have a profound effect on the nature and scope of Johne's disease control programs.

See also: **Office of International Epizooties**: Mission, Organization and Animal Health Code.

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Infectious Diseases: Leptospirosis

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Introduction

Leptospirosis has been diagnosed in cattle, horses, swine, dogs and humans and probably affects many other animal species, both wild and domestic. The causative organism belongs to the species *Leptospira interrogans*. The species contains a very large number (50 or more) of serovars that are widely distributed in nature. They seem to exist in a wide range of environmental conditions, but are more commonly found in poorly drained topography – pastures with areas where water stands for long periods, especially areas that are frequently flooded.

Serovars Involved

Leptospira serovars Pomona, Hardjo, Canicola, Grippotyphosa, Icterohaemorrhagiae and Sejroe have been associated with leptospirosis in cattle, while serovars Pomona, Hardjo, Icterohaemorrhagiae and Bratislava are associated with leptospirosis in horses and swine.

Leptospirosis does affect the reproductive processes in cattle, horses and swine; however it is not considered to be a venereal disease since the primary method of transmission is from contaminated environment. Also, the disease causes many symptoms totally unrelated to reproduction.

Symptoms

Leptospirosis causes a number of symptoms in dairy cattle, but the symptoms for an individual cow will vary in number and severity. This variation is probably related to the level of exposure to the antigen and to the level of the individual's immunity (from prior exposure or vaccination).The following is a list of symptoms that have been attributed to leptospirosis in cattle:

1. Sudden onset of loss of appetite: in severe cases cows will lose interest in both feed and water.
2. Decrease in milk production: the decrease in milk production will vary with the severity of the infection. Even in high-producing cows, the level of production may drop to near zero. In severe cases, the secretions from the udder will be abnormal, usually thick and dark yellow in color, and may contain some blood cells. Because of the abnormal udder secretion some have suggested that the *Leptospira* organism causes mastitis by invading the udder. However, it is more probable that the abnormal secretion is a result of abnormal body metabolism.
3. Rapid loss of body weight: body weight loss of up to 140 kg in 3 days has been reported in acutely infected mature dairy cows. Some of the weight loss is due to shrinkage related to decreased feed and water intake; however, much of it is true weight loss. The cows do not return to their former weight after they resume eating and drinking.
4. Blood-tinged urine: urine from acutely infected cows will often have a red tint. The color results from damage caused to kidney tissue by the invasive organisms and results in red blood cells undergoing a hemolytic reaction which releases hemoglobin from the blood cells. The hemoglobin will not settle out, thus the urine will remain red after standing.
5. Elevated body temperature: most infected animals will have elevated body temperatures ranging from 40.5 to 41.7 °C.
6. Abortions: the incidence of abortion in herds infected with leptospirosis will vary from a few to as high as 50%. This incidence is related to the stage of gestation of the herd as a whole. Most leptospirosis-induced abortions occur during the latter third of gestation, 7–10 days after the onset of the disease. As an example of the incidence of abortion when leptospirosis occurs during late pregnancy, a dairyman in western New York had 10 late-pregnancy heifers in a small pasture that was isolated from all other domestic animals. The heifers were checked daily, and the pasture provided adequate nutrients. In a 14-day period eight of the 10 heifers aborted. A diagnostic laboratory confirmed that the heifers were infected with *Lep.* Pomona. Early on it was thought that the high body temperature caused the death of the fetus. Later data have shown that the organisms have the ability to cross the placental barrier from the maternal bloodstream to invade the foetus. This passage apparently is made easier during advanced stages of gestation when some natural haemorrhage occurs at the hylus of the placentome. This concept is supported by the fact that aborted fetuses

are extensively autolysed (enzymatic degeneration of cells). Abortion without apparent illness may be the only symptom of the disease in some herd outbreaks. Some calves carried to full-term are stillborn or are weak and have low viability.

7. Reduced breeding efficiency: there are few research data to indicate that cows recovering from leptospirosis continue to experience reduced breeding efficiency. Many astute managers report that reproductive problems do continue for up to a year in the form of increased incidence of retained placentae and increased number of services per conception. In herds infected with *Lep.* Hardjo, these lingering problems seem to be more pronounced than with the other serovars. Cows recovering from severe leptospirosis frequently have slow and prolonged convalescence.

Leptospirosis has been reported to cause abortions and premature parturitions in mares. The clinical disease and abortions occur less frequently in horses than in cattle and swine. Leptospirosis in sows not only causes abortions but often infects the piglets carried to term so that they are weak and die in a few days. Along with the weak pigs, some mummified foetuses may also be seen. Extended periods of reduced breeding efficiency have been reported in both horses and swine following outbreaks of the diseases when the Bratislava serovar is involved.

Methods of Transmission

The most common mode of transmission is by ingesting and inhaling the organisms from a contaminated environment. The environment is contaminated by urine from infected animals, both domestic and wild. Once ingested or inhaled, the organism finds its way into the circulatory system and affects many parts of the body. The kidneys seem to act as a reservoir for collection and reproduction of the *Leptospira* organism, and serve as the principal means for eliminating organisms from the body along with urine. Each drop of urine can contain millions of organisms. The amount of damage done to the kidneys is related to the severity of the infection.

The high-density management systems usually practised with dairy cattle provide an ideal exposure situation. Infected cows urinate on concrete and produce tremendous numbers of water particles (aerosols), each of which contains many organisms for all of the cows to inhale. In addition, splashing urine along with the aerosols contaminates both the feed and water available to the cows. It is easy to see how one infected cow can expose a whole herd in a very short period of time. Cows recovering from the disease continue to shed the organism in their urine for 2–3 months or longer. There is evidence that swine and some species of wild animals may become permanent vectors.

The principal vector for introducing *Leptospira* organisms into a herd that is free of leptospirosis is wild animals. The list of animals is almost limitless when both active and passive contamination are considered. Visiting humans, domestic cats and even blackbirds at dairy facilities are examples of passive vectors. Wild animals ranging from skunks and opossums to white-tailed deer are examples of active vectors. Visits by neighbouring livestock also have to be considered as possible vectors.

The survival of the organisms left by vectors in the environment depends on several factors. Pastures with wet areas and pastures that are frequently flooded will retain viable organisms longer than well-drained pastures. Farm ponds and natural water-holes appear to be reservoirs for *Leptospira* organisms. Fencing these areas and providing a source of fresh water for dairy animals is recommended.

Leptospirosis is not classified as a venereal disease; however, it is possible for an infected bull to transmit the *Leptospira* organism to cows during natural mating. Commercial artificial insemination organizations do not process semen from bulls that are shedding *Leptospira* organisms in their semen.

It is impossible to prevent cattle from being exposed to *Leptospira* organisms. Therefore, control measures are of utmost importance.

Diagnostic Procedures

The serum agglutination test utilizing the tube or plate procedure is the most common diagnostic procedure used for leptospirosis. A positive titre (test) does not necessarily mean that the tested animal is currently infected with leptospirosis. It may mean that the animal has previously had the disease or that it has been vaccinated. To remove as much doubt as possible, a second test in 2–3 weeks and comparing the two is recommended. Animals that are negative to the first test and positive to the second test, or those that have a higher titre on the second test than they had on the first test probably have an active disease. Testing for all six of the serovars listed previously is recommended when leptospirosis is suspected.

Isolation and identification of the organism is a more positive diagnostic procedure. However, culturing the *Leptospira* organisms presents some serious problems, in that *Leptospira* do not grow well on most artificial culture media. Also, the samples, whether blood or urine, must be taken at a time when the disease is active. The aborted fetus is not usually a good source of material for culturing *Leptospira.* These organisms are very delicate and die readily when they are outside the host.

Control Measures

As mentioned earlier, it is virtually impossible to prevent animals from being exposed to *Leptospira* organisms. Since most species of animals, both wild and domestic, are susceptible to *Leptospira* organisms, the disease is a continuous threat to farm animals. Preventing access to farm and natural ponds and slow-moving streams may be helpful.

Vaccination is the best option for preventing the disease in cattle, horses and swine. The available vaccine is a bacterin (a preparation of killed organisms) which stimulates the animal's immune system but provides immunity for only a few months. A multivalent vaccine containing the first five serovars listed under 'Serovars Involved' (above) is available, but *Lep.* Sejroe has not yet been added. Under the lowest exposure conditions, cattle should be vaccinated every 6 months, but under high exposure condition, vaccination should be repeated every 3–4 months. All animals over 2 months of age should be vaccinated. *Leptospira* Bratislava bacterin should be added to the vaccination program for horses and swine. Horses should be vaccinated at 6-month intervals and swine should be vaccinated prior to each breeding season.

Until recently, leptospirosis has not been considered a serious disease of humans and the occasional case that did occur was not traced to farm animals. However, an outbreak of leptospirosis in three large dairy herds in Florida resulted in 11 of 14 milk-hands becoming infected. The disease causes many different symptoms in humans. The 11 infected individuals in the Florida outbreak showed the following symptoms, in order of frequency: fever, malaise, nausea and vomiting, myalgia and coryza, chills, enlarged lymph glands, pharyngitis, anorexia, stiff neck, photophobia, arthralgia, diarrhoea, conjunctival suffusion and skin rashes. Serological diagnosis incriminated *Lep.* Hardjo in nine and *Lep.* Pomona in two of the patients. Also, *Lep.* Sejroe is currently causing public health concerns.

In summary, leptospirosis is a serious disease in that the six serovars listed previously not only infect cattle, horses and swine, but can also infect humans. Since it is impossible to prevent livestock from being exposed to the *Leptospira* organisms, good management practices, including a vigilant vaccination program, are an absolute necessity. A good relationship between the herd manager and a practising veterinarian is highly recommended.

See also: **Replacement Management in Cattle:** Health Management.

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Infectious Diseases: Listeriosis

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Introduction

Listeria monocytogenes is a Gram-positive bacterial pathogen that can cause disease in humans and a variety of animal species. Although listeriosis is increasingly recognized as a severe disease in dairy animals, including sheep, goats and cattle, it is still regarded by some as a relatively uncommon disease, probably due to the fact that it may be underreported and underdiagnosed. *Listeria monocytogenes* is of particular concern not only because it may cause significant disease and financial tolls for the dairy livestock industry, but also due to the human health significance of this organism. Human listeriosis is primarily a foodborne disease, which is estimated to be responsible for about 2500 cases annually in the United States. Although the number of human listeriosis cases is low as compared to many other foodborne disease, listeriosis has a high case mortality rate of about 20%. Thus in the United States only, there are an estimated 500 human deaths attributable to listeriosis annually. Stringent control of *L. monocytogenes* in farm and food-processing environments and control and prevention of listeriosis in food-producing animals are important measures to assure human and animal health.

Causative Agent

The genus *Listeria* contains five species, namely *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri* and *L. welshimeri*. All members of this genus are Gram-positive nonsporeforming rods, closely related to the genera *Lactobacillus* and *Streptococcus*. The G + C% DNA content of *L. monocytogenes* lies around 40%. While *L. monocytogenes* causes both human and animal disease, *L. ivanovii* is predominantly associated with disease (specifically abortions) in sheep. The other *Listeria* species are considered nonpathogenic. The pathogenic species *L. monocytogenes* and *L. ivanovii* are both hemolytic, as is the nonpathogenic *L. seeligeri*, while *L. innocua* and *L. welshimeri* are nonhemolytic. *Listeria monocytogenes* can be separated into 13 different serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7), all of which appear to have been associated with animal disease, although some of them are less commonly isolated from affected animals than others. Generally serotypes 1/2a, 1/2b and 4b appear to be most common among animal isolates, although serotype 4c is also occasionally found. There are also some indications that the frequency of different serotypes and molecular subtypes among human and animal clinical cases differs. Thus, certain subtypes may show at least some level of host specificity for humans and animals.

All *Listeria* species are aerobic, microaerophilic, facultative anaerobic, catalase-positive, oxidase negative and esculin hydrolysis positive. *Listeria monocytogenes* is considered a ubiquitous organism, which can be isolated from many different environmental sources (surface water, soil, sewage, plant material, etc.). This organism has the ability to grow and survive under a variety of different conditions. *Listeria monocytogenes* grows from close to 0 °C to 44 °C and is thus considered a psychrotolerant organism. All *Listeria* species are heat sensitive and pasteurization effectively kills listeria. When cultured at 20–25 °C, *Listeria* is motile due to peritrichous flagella. In media, *Listeria* grows in a pH range from 4.4 to 9.6, with optimal growth at neutral pH. *Listeria monocytogenes* has a high level of osmotolerance; it can grow in media containing up to 10% (w/v) NaCl and it survives at even higher salt concentration.

All *Listeria* species are phenotypically very similar and their differentiation can sometimes be difficult. Commonly used tests to differentiate *Listeria* species include acid production from D-xylose, L-rhamnose, α-methyl-D-mannoside and mannitol as well as hemolysis patterns on blood agar plates. *Listeria monocytogenes* and *L. seeligeri* show narrow slight clearing zones (β-hemolysis), while most *L. ivanovii* strains show wide zones of hemolysis. Hemolysis is usually observed on sheep blood plates, but blood from other species can also be used (e.g. horses or cattle). The Christie–Atkins–Munch–Peterson (CAMP) test with *Rhodococcus equi* and *Staphylococcus aureus* is often recommended to clarify hemolysis patterns, but may be difficult to interpret, particularly for people without experience in the use of this test. While *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* show a positive CAMP test with *St. aureus* (i.e. enhanced hemolysis at the intersection with *St. aureus*), *L. ivanovii* shows a unique and typical CAMP test pattern with *Rc. equi* (shovel-shaped enhanced hemolysis zone). Sometimes hemolysis patterns and CAMP patterns are difficult to interpret, e.g. for weakly hemolytic or nonhemolytic *L. monocytogenes* isolates. While virulence tests in mice have historically been used for more definitive speciation, routine pathogenicity testing is generally unnecessary. Molecular and genetic methods generally provide reliable and more rapid means for speciation of *Listeria* isolates. For example, polymerase chain reaction (PCR)-based methods can be used to screen for the presence of the *L. monocytogenes* hemolysin gene (*hlyA*), which is unique to this species, or to screen for other *L. monocytogenes*-specific genes. While these genetic-based methods provide rapid means for detecting and defining *Listeria* isolates that carry genetic material unique to *L. monocytogenes*, they do not allow easy differentiation of virulent from avirulent *L. monocytogenes* strains. Multiple avirulent *L. monocytogenes* isolates have been described, including the ATCC *L. monocytogenes* type strain. The development of reliable genetic approaches to differentiate virulent from avirulent *L. monocytogenes* isolates still represents a challenge for researchers.

The pathogenesis and genetics of *L. monocytogenes* have been explored extensively. The genome sequences of *L. monocytogenes* and *L. innocua* have recently been completed and these data will provide new opportunities to better understand the pathogenesis and genetics of this organisms and hopefully to develop new therapeutic and preventive strategies. For many decades, *L. monocytogenes* has been recognized as a model system for a bacterial pathogen, which induces T cell mediated cellular immunity. Studies on the cellular pathogenesis of listeriosis showed that *L. monocytogenes* is a facultative intracellular pathogen, which has a unique ability to use host cell proteins to spread from cell to cell. The ability of *L. monocytogenes* to spread directly from cell to cell (without contact with the extracellular milieu) provides a morphological explanation of why cellular immunity plays a crucial role in the immune protection against listeriosis. Intracellular bacteria cannot be recognized by antibodies, while cytotoxic T cells will provide a mechanism for the killing of intracellular *Listeria*.

The cellular processes of *L. monocytogenes* infection have been well characterized at the morphological level. A key group of *L. monocytogenes* virulence genes and their specific functions in the intracellular infection process have been identified and characterized. In tissue culture models of infection, the following stages of infection can be defined: (1) internalization of *L. monocytogenes* within the host cell; (2) bacterial escape from the host vacuole; (3) multiplication of the bacterium within the host cell cytoplasm and movement through the cytoplasm by virtue of bacterially directed polymerization of host actin filaments; (4) bacterial movement to the host cell surface and extrusion of bacterial cells in pseudopod-like structures; and (5) phagocytosis of these pseudopod-like structures by neighboring cells, followed by escape of the bacterium from the resulting double-membrane vacuole, thus allowing the cycle to repeat.

Gene products essential for each step of the infection process have been identified. Six *L. monocytogenes* virulence genes (*prfA*, *plcA*, *hlyA*, *mpl*, *actA* and *plcB*) are located together in one virulence gene cluster. Additional *L. monocytogenes* virulence-associated genes (e.g. *iap*, *inlA*, *inlB*, *inlD*, *inlE*, *inlF*), which are not physically linked to this virulence gene cluster, have also been described.

Clinical Signs

Generally, the most common clinical signs of listeriosis in ruminants include encephalitis, septicemia and intrauterine infections, which may lead to abortion or to birth of weak and/or septicemic animals. Less common symptoms associated with *L. monocytogenes* infections are mastitis, iritis and keratoconjunctivitis. In addition to clinically affected animals, a significant number of animals may be asymptomatic carriers of *L. monocytogenes*, often shedding the organism in fecal material.

Listeria monocytogenes is predominantly transmitted by the oral route. Invasion generally occurs in the intestinal tract with subsequent hematogenous spread, leading to septicemia and possibly transuterine infection. The pathogenesis of listerial encephalitis is still somewhat controversial. There are some indications that *L. monocytogenes* may enter through the oral mucous membranes (possibly through preformed lesions) and subsequently migrate centripetally inside cranial nerves to the brain and particularly to the brainstem. For example, oral lesions in animals changing teeth may provide an entry port for *L. monocytogenes*.

Sheep

Widespread listeriosis in sheep was first recognized in 1931 in New Zealand, where it was known as 'circling disease'. This local name is a good descriptor of a common manifestation of ovine listeriosis: encephalitis. Encephalitic listeriosis is a result of brainstem lesions caused by *L. monocytogenes*. Common signs of encephalitis include fever, depression, lack of feed and water intake, dullness, turning or twisting of the head to one side, and movement in circles. In advanced cases unilateral facial nerve paralysis can develop, causing drooping of the eyelid and ear, and drooling. Encephalitis has been reported as the predominant symptom of listeriosis seen in sheep; it has been observed in lambs as young as 4–8 months. The incubation period can be up to 3 weeks and in most cases of listerial encephalitis in sheep, death occurs within 2 to 3 days of onset of clinical signs.

Abortion represents another common clinical manifestation of listeriosis in sheep. *Listeria monocytogenes* is transmitted to the fetus via the placenta, leading to a septic infection of the foetus. Clinical signs in the ewe are resolved following abortion of the fetus, which usually occurs as a stillbirth in the 3rd trimester of pregnancy. Septicemia is the most frequent infection type in neonates and lambs, and has been documented to develop in lambs as young as 5 weeks old. Often, when a dam has a subclinical listerial infection she will bear an apparently healthy lamb that will then become septicemic and die within a few days. This infection is thought to be a consequence of intrauterine infection and is often characterized by diarrhea. Other clinical signs associated with listerial septicemia include elevated body temperature and a loss of appetite. Septicemia is also often accompanied by hepatitis, splenitis and pneumonitis. Although the response to antibiotics is good in adult septicemic cases, pregnant animals may still abort following clinical recovery. Overall, the mortality rate is much lower for septicemic than for encephalitic cases.

It has been reported that up to 5–10% of sheep exposed to *L. monocytogenes* during an outbreak show clinical signs, and many more are likely to have subclinical infections, shedding the bacteria in their feces. Listeriosis is difficult to diagnose based solely upon clinical signs because other diseases manifest themselves in similar ways. For example, ketosis and enterotoxemia will produce clinical signs similar to listerial septicemia, and viral encephalitis produces the same clinical signs as listerial encephalitis.

Goats

The clinical manifestations of listeriosis in goats closely resemble those seen in sheep: encephalitis, septicemia and abortion. In cases of listerial encephalitis in goats, meningitis frequently develops secondary to encephalitic brainstem lesions. As in ovine cases, goats usually succumb to an encephalitic listerial infection within 2 to 3 days, with fatalities reaching 60%. Based on the extreme extent of brain damage in goats diagnosed with encephalitic listeriosis, goats may be more susceptible to *L. monocytogenes* than sheep.

Septicemia is also often seen in goats; initial clinical signs include general depression, loss of appetite, a decrease in milk yield, and an increase in body temperature. Affected goats may also have diarrhea. In pregnant does, *L. monocytogenes* may cross the placenta, enter the fetus and cause abortion, possibly by triggering a change in hormone levels that prompts myometrial contraction and abortion. Like sheep, goats may be asymptomatic carriers, shedding *L. monocytogenes* in the faeces and milk. This provides the potential for environmental contamination and infection of newborn kids housed with the does through the navel or through sucking on dirty teats.

Cattle

Some sources report that cattle account for about 80–90% of reported listeriosis cases in North America. This may, though, not necessarily reflect a higher true incidence of listeriosis in cattle, but could also reflect a lower reporting rate for listeriosis cases in small ruminants. Encephalitis, abortion, septicemia and mastitis due to *L. monocytogenes* have been documented in cattle. In an encephalitic infection, death usually occurs about 2 weeks after the appearance of the first clinical signs, in contrast to the very rapid death seen in sheep. Bovine encephalitic listeriosis is characterized by walking in circles, bending the head to one side, with the corresponding ear drooping, the tongue protruding from the mouth, and abundant nasal secretion and drooling. Finally, the animal is increasingly emaciated, irritable, and then comatose until death. Neural damage to cranial nerves may also cause general irritability of the affected animal and impaired locomotion. Some animals that survive the listerial central nervous system (CNS) infection show typical postencephalitic or postmyelitic symptoms like 'dumbness', difficulty locating feed, and occasionally falling down, all results of the neural damage caused by the bacteria. The clinical signs of encephalitic listeriosis are similar to those associated with rabies or lead poisoning in cattle. Thus, it is important to consider listeriosis as a differential diagnosis in suspect cases of rabies or lead poisoning.

Bovine listerial septicemia is characterized by the same clinical signs seen in sheep and goats; elevated body temperature, a loss of appetite and diarrhea. In cattle and particularly in newborn calves, septicemia is often accompanied by miliary abscesses, especially in the liver. Like in sheep, intrauterine infection may lead to the birth of septicemic calves; typical clinical signs in these calves include diarrhea. Listeriosis in cattle is also often associated with abortion, generally occurring in the final trimester of pregnancy.

Less common manifestations of listeriosis in cattle include mastitis, keratoconjunctivitis and iritis. Listerial mastitis is characterized by shedding of *L. monocytogenes* in the milk, often either accompanied by a general septicemia or without any other clinical signs. In mastitic cows, the condition may be chronic, with the animal shedding the bacteria in her milk intermittently for up to 12 months. An interesting note is that healthy calves can be born to chronic carriers who shed *L. monocytogenes* in their milk. Listerial keratoconjunctivitis and iritis are thought to be caused by direct contact between the eye and contaminated silage, e.g. when an animal reaches its head into the feed. These symptoms do not only occur in cattle, but also in small ruminants.

Diagnosis

Clinical diagnosis of listeriosis in dairy animals is generally difficult as a variety of inflammatory and infectious diseases can cause clinical signs similar to those of listerial encephalitis. Many diseases also cause clinical signs identical to those of listerial septicemia and abortion. Important differential diagnoses for listerial encephalitis include rabies, viral CNS infections, polyencephalomalacia, lead poisoning and other intoxications. In general, definitive diagnosis of listeriosis can be achieved by bacterial culture (i.e. isolation of the organism from generally sterile tissue or body fluid samples such as cerebrospinal fluid (CSF) or blood), by histopathology and sometimes by serology. Thus, in many cases definitive diagnosis of listeriosis cannot be accomplished in a live animal, but requires a *postmortem* pathological and histopathological examination and/or microbial culture on organs collected *postmortem.*

Bacterial Culture

In cases of encephalitic listeriosis, cultivation of *L. monocytogenes* from CSF is one of the most effective means for definitive diagnosis. CSF is normally a sterile fluid, which may be positive for *L. monocytogenes* in infected animals. Unfortunately, a significant number of animals with listerial encephalitis may show negative bacterial cultures for *L. monocytogenes*, particularly if an animal has been treated with antibiotics before sample collection. Increased white blood cell counts, elevated glucose levels and increased protein concentration in the CSF are also typical for listerial infections. A combination of bacteriological, cytological and chemical CSF examination in combination with clinical signs of cranial nerve dysfunction and depression may often allow a definitive diagnosis of listeriosis in a live animal. In cases of listerial septicemia and/or abortion, detection of *L. monocytogenes* from sterilely collected blood samples can establish definitive diagnosis, although blood samples may again be negative in some animals with listeriosis, particularly if antibiotic treatment has been initiated. Isolation of *L. monocytogenes* from nonsterile body fluids such as milk and feces, even in combination with typical clinical signs, is generally not diagnostic, as it has been shown that a significant number of healthy animals can shed *L. monocytogenes* in feces and milk.

Pathology and Histopathology

Definitive diagnosis of listeriosis is often only possible by *post-mortem* examination. Macroscopically visible lesions are rare, but microscopic examination of affected organs can often help in the diagnosis of listeriosis. Similar pathological changes are observed in different animal species with listeriosis.

In encephalitic cases generally no macroscopically apparent lesions are seen. Typical microscopic CNS lesions include foci of necrosis and inflammation with adjacent perivascular cuffs consisting mainly of lymphocytes with plasma cells, histocytes and occasional neutrophils. Bacterial cells can often also be visualized in these lesions. These histological lesions are usually unilateral and most severe in the pons and medulla oblongata.

Typical gross pathology findings in septicemic listeriosis cases include granulomatous lesions in different organs, including lesions typical for hepatitis, splenitis and pneumonitis. For example, small, round, white abscesses of the liver are often seen in cases of listerial septicemia, particularly in newborn animals with listerial septicemia.

Definitive *postmortem* diagnosis generally requires positive bacteriological cultures from the affected organs. Brain and particularly medulla oblongata specimens are appropriate for detection of *L. monocytogenes* in cases of listerial encephalitis. For abortions and septicemic cases, bacteriological culture from spleen, liver and lung is recommended.

Serology

Listerial septicemia and abortion have been shown to be reliably diagnosed by measurement of antibody responses to listeriolysin O, a hemolysin that is necessary for the pathogenicity of the organism. Nevertheless, healthy animals may have significant antilisteriolysin O antibodies, as most animals appear to be commonly exposed to *L. monocytogenes* without developing clinical signs. Thus, in most cases an increase an antibody titer, using two blood samples collected at least 10 days apart, would need to be shown to be diagnostic. This would rely on the availability of pre-exposure antiserum for the animal to be tested, but these samples are rarely if ever available for farm animals. Antilisteriolysin O titers have been reported to be negative or inconsistent in encephalitic listeriosis cases.

Treatment

Listeriosis is often a rapidly progressing disease; in encephalitic cases death usually occurs within 2 days to 2 weeks of the first onset of clinical signs. Therefore, rapid diagnosis and treatment initiation immediately after onset of clinical signs are necessary for successful treatment. Since early diagnosis prior to death is rarely achieved, appropriate treatment is often initiated late and unsuccessfully.

Listeria is a facultative intracellular pathogen, thus the organism may be protected from the action of some antibiotics. Surprisingly, some antibiotics, which do not penetrate intracellularly, still seem to be effective for treatment of listeriosis. Ampicillin and amoxicillin are often recommended as effective antibiotics for the treatment of listeriosis. Oxytetracycline and penicillin G also have been used successfully. All of these antibiotics, however, require administration of very high doses to be effective. For example, the recommended oxytetracycline treatment scheme requires doses as high as 10 mg kg^{-1} body weight per day for at least 5 days. Penicillin G should be given at 44 000 U kg^{-1} body weight daily for 1–2 weeks in order to be effective.

In addition to antibiotic therapy, supportive therapy may be necessary. This may include fluid and electrolyte replacement for animals having difficulty eating and drinking as a result of neurological damage. Acidosis caused by excessive salivation can be treated by intravenous replacement of bicarbonate ions.

Control and Prevention

As therapy of listeriosis in dairy animals is often difficult and unsuccessful, prevention and prophylaxis play a critical role in the control of this disease.

Feed Preparation

In most listeriosis outbreaks, particularly in cattle and sheep, silage appears to be the most likely source of infection. *Listeria monocytogenes* is a ubiquitous organism and is likely to naturally occur in plant materials used for silage preparation and/or in contaminating soil or other materials. Fecal contamination from birds and other animals, especially rodents, which can be asymptomatic carriers of *Listeria*, can also contribute to *Listeria* contamination of silage. While initial contamination of ensiled materials is probably common, multiplication and survival of *L. monocytogenes* depends on the quality of silage fermentation. Since listerial growth is inhibited by a pH lower than about 5.0–5.5, *Listeria* does not multiply in properly prepared silage. Improperly fermented silage or pockets of improper fermentation will have a high pH (>6.0), allowing multiplication of *Listeria*. Some poorly fermented silages have shown *Listeria* contamination in excess of 10^{8} cfu g^{-1} wet weight of silage. Careful preparation and use of silage is thus of the utmost importance in the prevention of listeriosis. Emphasis should be placed on reducing the likelihood of multiplication of *Listeria* within a bale or bunk of silage. This can be aided by cutting the grass or maize from which the silage is made early in the season and effectively compacting and sealing it into the bale. Early cutting will help prevent fecal contamination by wild animals, and effective compaction and sealing help prevent air pockets where *Listeria* could thrive. Fields with crops intended for silage preparation should be kept free of grazing animals before and during the time of cutting to decrease the incidence of fecal contamination.

When feeding silage, additional precautions can be taken to help prevent listeriosis. Mold growth on silage indicates conditions that also allow growth of *Listeria*. Thus, any obviously moldy material should not be fed to animals and should be discarded. In particularly susceptible animal populations and in flocks or herds with recent listeriosis cases extra care should to be taken to feed only high-quality silage and to discard improperly fermented silage. For example, ripped bales represent a particular risk and silage close to the top, sides and front in a bunker silo may often be improperly fermented. Also, it is very important to remove uneaten silage after feeding. Keeping pens and barns clean is also important for reducing the size of the rodent population and of other potential carriers of *L. monocytogenes*. In cases of listerial keratoconjunctivitis and iritis, measures to avoid direct eye contact with contaminated silage may minimize additional cases.

In order to help control listeriosis cases in goats there are additional measures that may be recommended. Grazing goats that are not being fed silage are known to develop listeriosis at times. It has been suggested that abrasion to the mouth by rough grazing materials provides an entry port for *L. monocytogenes*. Therefore, it is important to avoid grazing in locations that have been recently used by other species, or to reduce the amount of grazing time in order to avoid infection.

In addition to contaminated feed, there are several other factors that put a population of animals at risk of developing listeriosis. Stresses such as transport, a change of feed and the introduction of new animals to a herd all put animals at risk. The introduction of new animals not only introduces challenges to hierarchical position in the herd, but it also has the potential of introducing subclinical carriers of *Listeria* to the existing herd. Therefore it may be advisable to quarantine new animals for a period of time before introducing them to the herd.

The majority of listeriosis cases in dairy animals appear to occur from November to May, with a peak around February and March. This temporal distribution may be related to common feeding of silage rather than pasture during these times, with an increase in the likelihood of feeding poor-quality and older silage later in the season. Thus, prophylactic and preventive measures are particularly important during certain times of the year.

Vaccination and Immunoprophylaxis

Natural immunity to *Listeria* probably provides protection for most animals exposed to *L. monocytogenes*. Vaccination of susceptible populations appears to provide a means of minimizing the risk of significant health problems and subsequent economic losses caused by listeriosis outbreaks. For the present time, however, vaccination is not a valid option in the United States or the United Kingdom, because there is no licensed vaccine available. A live, attenuated vaccine is available in some European countries and is claimed to be effective for sheep and goats. Studies performed using this vaccine showed that it is effective in reducing the incidence of listerial encephalitis and abortions, speeding recovery and increasing response to treatment. As *L. monocytogenes* is an intracellular pathogen, only live vaccines are likely to induce protective cell-mediated immunity. Killed vaccines are unlikely to lead to a protective immune response.

See also: **Pathogens in Milk**: *Listeria monocytogenes*.

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Infectious Diseases: Salmonellosis

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Introduction

Salmonella has long been recognized as an important zoonotic pathogen of worldwide significance in man and animals. *Salmonella* are pathogenic bacteria that are isolated from man and a wide variety of animal species including cattle, pigs, poultry, horses, companion animals, wild birds, cold-blooded animals such as turtles and snakes, rodents, fish, flies, beetles, cockroaches and other insects. Salmonellosis, the disease caused by infection with *Salmonella*, is a common intestinal illness caused by numerous *Salmonella* serovars and manifested clinically in animals and humans as an acute or chronic enteritis, an acute septicaemic disease or as a subclinical infection. Animals with a subclinical infection or recovered from clinical salmonellosis may become carriers, shedding the organism in large numbers in the feces, and are a major source of infection of animals and humans and contamination of the environment. *Salmonella* are ubiquitous organisms that are dispersed widely in the environment, on pastures, and in rivers, streams and lakes. *Salmonella* bacteria contaminate many feed stuffs of animal origin such as bone meal, blood meal, feather meal, fish meal, and many foods including meat, raw milk, raw eggs, foods containing raw eggs, other foods of animal origin, fruits and fruit juices, and vegetables.

Like other members of the Enterobacteriaceae, *Salmonella* are gram-negative, facultatively anaerobic, non-sporeforming rods. *Salmonella* are fermentative, oxidase negative, catalase positive bacteria that are motile by means of peritrichous flagella.

Somatic or O antigens and flagellar or H antigens identify *Salmonella* serovars. The O antigen consists of repeat units of the same tri- or branched tetra- and penta-saccharides of the O-specific side chain attached via a core chain to lipid A embedded in the outer membrane of bacteria. The H antigen is the filamentous elongated part of the flagella consisting of a protein called flagellin. Expression of flagellar antigen alternates between two phases encoded by two genes, *H1* and *H2*, located at separate locations on the chromosome. When the promoter for the *H2* operon is in the correct orientation, the H2 flagellin is synthesized together with the repressor of the *H1* operon, the *rh1* gene product. When the promoter of the *H2* operon is in the opposite orientation, neither the H2 flagellin nor the *rh1* gene products are produced, thus the *H1* operon is no longer repressed and H1 flagellin is produced. Not all *Salmonella* express both phases of the flagellar antigens. The combinations of the O antigen and the two phases of flagellar antigens, identified by agglutination of the bacteria with group- and factor-specific antisera, determine the antigenic formula of the bacterial isolate. All *Salmonella* serovars are motile. The exceptions were thought to be *S.* Pullorum and *S.* Gallinarum. However, it has recently been shown that motility can be induced in *S.* Pullorum.

There are more than 2,500 *Salmonella* serovars or serotypes but less than 50 of these are frequently isolated from animals with salmonellosis. There are only two species of *Salmonella*, namely *S. enterica*, consisting of six subspecies, *enterica* (subsp. I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI), and *S. bongori*. The *Salmonella* serovars belonging to *S. enterica* subsp. *enterica* are the most numerous in that they total more than 1400 serovars, and only they have a name, e.g. *S.* Typhimurium, *S.* Dublin, *S.* Heidelberg and *S.* Enteritidis. *Salmonella* serovars belonging to subspecies other than subspecies I are denoted by the subspecies number (in Roman numerals) and the antigenic formula of the strain. The subspecies are identified by biochemical characterization and susceptibility to lysis by the Felix O1 bacteriophage. Phage typing, biotyping, resistance to antimicrobials, plasmid profile analysis and other, especially molecular methods, are used to identify isolates beyond the level of serovar and are useful for epidemiological studies.

Some of the *Salmonella* serovars are host-specific, infecting and causing illness in a specific animal species or in man (**Table 1**). An example is *Salmonella* Dublin that is isolated primarily from cattle, whereas *S.* Typhimurium has a much wider host-range than *S.* Dublin; it infects cattle, many other animal species, and man.

Causative organisms

Salmonella Typhimurium is the commonest serovar isolated from cattle in many countries worldwide, whereas *S.* Dublin has a more local distribution. The latter serovar

Table 1 Host-specific *Salmonella* serovars and the diseases, disease symptoms and pathological lesions they cause

Serovar	*Host*	*Disease, symptoms, pathological lesions*
S. typhi *S. paratyphi* A, B, C	Humans	Typhoid Fever, Paratyphoid Fever
S. dublin	Cattle, calves	Cattle: diarrhea, fever, necrotizing hemorrhagic enteritis, abortion Calves: diarrhea, fever, enteritis, septicaemia, pneumonia
S. choleraesuis	Pigs	Septicaemia, pneumonia, hepatitis, enterocolitis, and sometimes abortion
S. pullorum	Chickens, turkeys	Pullorum Disease
S. gallinarum	Chickens, turkeys	Fowl Typhoid
S. abortusequi	Horses	Abortion
S. abortusovis	Sheep	Abortion

is found in cattle in the UK, other European countries, and the US, but rarely in Canada. In the UK, *S.* Dublin was for most of the 1960s the commonest serovar isolated from cattle. Thereafter, the number of cases declined substantially and *S.* Typhimurium is now the most frequently isolated serovar from cattle. In Australia, *S.* Typhimurium is the commonest serovar isolated from cattle, followed by *S.* Bovismorbificans. Outbreaks of *S.* Dublin infections in dairy cattle have increased during the last two decades in Australia. *Salmonella* Dublin is also an important pathogen for cattle in South Africa and South America. In Canada, during the 1990s, *S.* Typhimurium has been the commonest serovar isolated from cattle, whereas much lower numbers of isolates were serotyped as *S.* Muenster, *S.* Mbandaka, *S.* Anatum, *S.* Heidelberg, *S.* Give and *S.* Cerro. In the US, during the early 1990s, *S.* Typhimurium was the commonest serovar isolated from cattle, followed by *S.* Dublin, *S.* Montevideo, *S.* Kentucky, *S.* Anatum, *S.* Cerro, *S.* Muenster, *S.* Newport, and *S.* Meleagridis. *Salmonella* Dublin used to be isolated primarily at dairy farms in California and other states west of the Rocky Mountains but more recently cases and outbreaks have occurred in cattle in Indiana, Ohio, New York and Pennsylvania. A considerable percentage of the *S.* Dublin strains (20-25%) were non-motile. These isolates were as virulent for calves and cattle as motile *S.* Dublin strains. Such strains may be further identified by considering the clinical and pathological characteristics of the infection in calves and cattle, by determining the plasmid profile and examining the strain for the presence of the *Salmonella* plasmid virulence (*spv*) genes on a virulence-associated plasmid of about 75 Kb, by pulsed field gel electrophoresis (PFGE), and by other means such as multilocus enzyme genotyping.

Epidemiology

The prevalence of disease and the incidence of infection in cattle vary between different geographical areas depending on climate, cattle density and farming practices. Infection rates of 10-15% in dairy cattle and of 4% in beef cattle have been recorded. In a survey of feedlot cattle in the US, *Salmonella* were isolated from fecal samples at 38% of feedlots, in 26% of feedlot pens and in 5% of the fecal samples. Surveys in North America showed that 2-3% of calves were infected and that calves on 14% to 22% of farms shed *Salmonella* bacteria.

Certain *Salmonella* serovars are more likely to persistently colonize the intestinal tract than others. Salmonellosis in cattle caused by *S.* Dublin is a serious disease that tends to persist in the herd. In contrast, disease caused by *S.* Typhimurium is sporadic, although sometimes fatal in individual animals; it tends to subside after initial exposure and to recur when the source of the infection reappears.

Cattle may not show any overt clinical symptoms but may carry and excrete as many as 10^5 *Salmonella* per gram of feces and transmit the pathogen to susceptible animals. Adult cattle with typical symptoms of salmonellosis are more likely to shed the organism in large numbers for an extended period of time, whereas in calves the excretion is more intermittent. Carrier animals include active carriers that excrete *Salmonella* continuously or intermittent for months or years, passive carriers that pass the bacteria through the intestinal tract and cease shedding shortly thereafter, and latent carriers that harbor the bacteria in tissues such as the mesenteric lymph nodes and tonsils but do not excrete the organism with the feces. Latent carriers may become active carriers or clinical cases when stressed, for example, at calving or during transportation.

The introduction of carrier cattle into a herd is a common cause of outbreaks of clinical salmonellosis in dairy herds. Infected animals excrete *Salmonella* bacteria and infect other animals directly or indirectly by contamination of the environment, the feed and the drinking water. Factors that may contribute to outbreaks of salmonellosis in calves include: infected dams that became active carriers during parturition and infected their calves, purchase in sales yards by dealers of calves that have received no or little colostrum and thus have minimal immunity to the infection, stress as a result of crowded and poor hygienic conditions in sales barns, and transport over long distances. Calves may become infected via the fecal-oral route and may infect one another when housed in groups and able to contact and lick one another or when drinking from a communal source.

Cattle may contract salmonellosis because of contamination of the pasture with *Salmonella* after the spreading of contaminated manure or slurry and when drinking water from contaminated troughs, sloughs, creeks and rivers. Infection of cattle also occurs by eating contaminated feed stuffs such as bone meal, meat meal, fishmeal and oilseed meals. Factors that predispose dairy cattle to salmonellosis include parturition, stress caused by transportation, crowding, poor weather conditions and parasitism.

In regularly fed cows the *Salmonella* tend to disappear from the rumen. A high concentration of volatile fatty acids results in a low rumen pH and inhibition of *Salmonella*. Anorexia results in low concentrations of volatile fatty acids and a high rumen pH and multiplication of *Salmonella*. Around parturition, immunological responses are depressed and dietary changes take place. Dry-matter intake may be depressed by up to 50% influencing the multiplication of *Salmonella* present in the rumen. Infection with concomitant pathogens such as bovine virus diarrhea (BVD) virus or fascioliasis due to *Fasciola hepatica* may exacerbate concurrent salmonellosis.

Pathogenesis

Infection of calves and cattle is usually by the oral route but it may also occur via other routes including the respiratory tract by inhalation of aerosols, and via the tonsils. Hematogenous spread from extraintestinal ports of entry may also result in intestinal infection. Oral infection of one-month-old calves with a dose of 10^6 *S.* Typhimurium bacteria induced an acute self-limiting enteritis and the development of diarrhea within 12 to 48 hours. Infection with 10^9-10^{10} *S.* Typhimurium bacteria resulted in diarrhea, dehydration, intestinal lesions and mortality of calves. Postmortem examination revealed a moderate to severe enteritis, particularly of the terminal ileum, and a fibrinopurulent necrotizing enteritis over the Peyer's patches and enlargement of the Peyer's patches and mesenteric lymph nodes. The contents of the small and large intestines were watery and sometimes bloody. The highest number of *Salmonella* bacteria were found in the ileal mucosa and the ileal lymph nodes, whereas low numbers were observed in liver and spleen.

Histopathological examination and scanning and transmission electromicroscopy of the intestinal lesions after a high-dose oral challenge with *S.* Typhimurium showed an initial invasion by macropinocytosis and later destruction of the M cells of the follicle associated epithelium of bovine Peyer's patches. The bacteria also penetrated the apical membrane of the enterocytes of the absorptive villi in the terminal ileum. This was followed by a shortening of the villi, extrusion of enterocytes, increased emptying of mucus from the goblet cells, and a neutrophilic reaction in the lamina propria accompanied by transepithelial migration of neutrophils into the lumen. Bacteria and an infiltration of neutrophils were found in the Peyer's patches and a depletion of lymphocytes in the germinal centres of the intestinal lymphoid follicles was noted. The tip of the villi became necrotic. The blood vessels in the lamina propria contained thrombi, and the wall of the vessels was damaged. Bacteria were found in degenerative phagocytic cells in the lamina propria. Most of *S.* Typhimurium infections in calves remained localized to the intestines and mesenteric lymph nodes. Bacteremia followed by retention of the bacteria in liver and spleen and phagocytosis and growth of the bacteria in the macrophages of the reticuloendothelial system of the liver and spleen is an uncommon occurrence in calves.

Salmonella Typhimurium strains possess about 200 genes that contribute to virulence. Genes contributing to virulence are divided in three groups: bona fide virulence genes, house keeping genes and regulatory genes. Earlier, five clusters of virulence genes called *Salmonella* pathogenicity island 1, 2, 3, 4 and 5 (SPI-1-5), located on the chromosome, were recognized. More recently, the number increased to as many as 14 SPIs. Other virulence genes are located on the chromosome outside the pathogenicity islands. Genes mediating virulence have also been found on plasmids of a limited number of *Salmonella* serovars including *S.* Choleraesuis, *S.* Dublin, *S.* Enteritidis, *S.* Gallinarum, *S.* Pullorum and *S.* Typhimurium. These are called *Salmonella* plasmid virulence (*spv*) genes. The *spv* genes are mainly found in serovars that are host specific and cause serious illness in their respective hosts, e.g. *S.* Dublin in cattle, *S.* Choleraesuis in pigs and *S.* Pullorum in poultry. Similarities observed between typhoid fever caused by *S.* Typhi in humans, and *S.* Typhimurium infection in susceptible mice, prompted the extensive use of an animal model called the murine typhoid model. Although many of the *S.* Typhimurium and *S.* Typhi genes mediating virulence have been identified with the murine typhoid model, a number of *S.* Typhimurium genes which encode virulence in mice play a less prominent role in virulence for calves. Virulence genes on the SPI-2 and the *spv* operon enable *S.* Typhimurium to cause systemic infection and to multiply in macrophages in the liver and spleen of susceptible mice. Mutation of the genes made the *S.* Typhimurium strain much less virulent for susceptible mice but had little effect on virulence of the bacteria for calves and did not reduce the severity of diarrhea in calves. In contrast, *S.* Typhimurium bacteria with mutations of SPI-1 genes are defective in colonizing the Peyer's patches and the ileal mucosa of calves. Such mutants cause less diarrhea and do not cause a fatal infection in calves when given a high oral dose of 10^{10} bacteria. The SPI-1 encodes a type III secretion system, associated regulatory genes, and the *Salmonella* invasion proteins (Sips). The SPI-1 proteins facilitate translocation of proteins from the *Salmonella* into eukaryotic cells. Some of the secreted *Salmonella* outer proteins (called Sops) facilitate the invasion of *Salmonella* into epithelial cells by stimulating the reorganization of the cell cytoskeleton. Mutation of the *sopD* (on SPI-1) or the *sopB* (on SPI-5) genes of *S.* Dublin reduced secretory and inflammatory responses in bovine ileal loops. Also, disruption of the SPI-1 genes *invH* and *sipB* genes of *S.* Typhimurium and *S.* Dublin abolished the ability of the mutants to elicit secretory and inflammatory responses in bovine ligated ileal loops.

Clinical Symptoms

Cattle and calves may be affected with an acute or a chronic form of salmonellosis. The disease manifestations depend on the virulence of different *Salmonella* serovars, the number of *Salmonella* ingested, and host immunity. Many *Salmonella* infections are opportunistic infections in compromised hosts. The morbidity rate of salmonellosis may be high and reach more than 50%, especially in young susceptible calves. Clinical signs are found mainly in calves of 2-6 weeks of age and are unusual in calves of less than 2 weeks of age. Symptoms in calves with septicaemic salmonellosis include depression, dullness, a high fever (41-42 °C), anorexia, dehydration, incoordination, and death within a few days. Respiratory distress, meningitis and polyarthritis may be seen in some calves with acute salmonellosis. Calves and cattle with acute enteritis may have a fever (40-41 °C), and fetid diarrhea with loose feces containing flecks of blood, mucus and casts of necrotic fibrinous mucosal debris. The animals may dehydrate and lose weight. The mortality in calves may be as high as 70%, but losses are usually 5-10%. Losses are often much higher among purchased calves than of calves that were born and raised at the same farm. Symptoms in cows with acute salmonellosis include fever, dullness, loss of appetite and depressed milk yield. Symptoms in cows with subacute salmonellosis are less severe. Salmonellosis in cows often occurs around parturition. Pregnant cows, especially when infected with *S.* Dublin, may abort without having displayed previously any obvious symptoms. Recovery from salmonellosis may take several weeks to months.

Diagnosis

To determine if a herd or an individual cow or calf is infected with *Salmonella* it is necessary to culture feces, blood, tissues and/or milk for the presence of *Salmonella.* Salmonellosis is particularly difficult to determine in clinically normal carrier animals. A plethora of media and methods exist to culture, isolate and identify *Salmonella.* Direct plating and direct selective enrichment procedures are often unsuccessful for the recovery of *Salmonella* bacteria. Optimal recovery of *Salmonella* requires pre-enrichment of the sample in a non selective medium, followed by culture in a selective enrichment medium and plating on selective or non selective agars. Pre-enrichment of fecal samples in buffered peptone water (BPW) results in higher isolation rates than pre-enrichment in lactose broth. Resuscitation and recovery of *Salmonella* does not depend on the nutritional value of the pre-enrichment medium. The fermentation of lactose by other members of the *Enterobacteriaceae* present in samples like feces would cause the pH of the medium to fall to levels that might be inhibitory to growth of *Salmonella.* In contrast, BPW has a high buffering capacity and does not contain lactose or any other sugars, and thus prevents acidification of the medium. Selective-enrichment broth selectively inhibits bacteria other than *Salmonella* allowing multiplication of *Salmonella* to levels detectable by plating. High *Salmonella* isolation rates have been obtained by inoculation of fecal and environmental samples at a 1:10 w/v ratio in BPW and overnight culture at 37 °C, followed by plating of 100 μL onto modified semisolid Rappaport-Vassiliadis (MSRV) agar. After culture of the MSRV plates at 41.5 °C for 24-48 hours, a loop full of the selective outgrowth of the motile putative *Salmonella* bacteria from the semisolid agar is streaked onto a non-selective agar such as Luria-Bertani agar or tryptic soy agar. After incubation of the agar overnight at 37 °C, the colonies are almost invariably identified as *Salmonella* colonies by a slide agglutination test with polyvalent *Salmonella* O antisera.

A number of serological tests such as the serum agglutination test and indirect enzyme-linked immunoabsorbent assays have been developed. Serological tests are useful for the identification of infected herds but are inadequate for the identification of persistently infected animals.

Since *S.* Typhimurium strains are important pathogens affecting humans and many animal species including cattle, strains of this serovar are often further characterized by phagetyping, examining antimicrobial resistance profiles, and determining the PFGE pattern of the strains. These procedures allow for an epidemiological trace-back to determine the source of the pathogen, are helpful when considering prognosis and treatment options and aid in prevention and control measures. During the 1990s, *S.* Typhimurium phage type (PT) or definitive type (DT) 104 has been isolated increasingly from cattle and calves, and from pigs, chickens and humans in the UK and other European countries, and in the US and Canada. DT104 strains are highly virulent for cattle and calves. The strains are resistant to a host of antimicrobials including ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline and may be additionally resistant to ciprofloxacin, trimethoprim, and the aminoglycosides kanamycin and neomycin.

Treatment

Oral treatment of clinical salmonellosis in preruminant calves and parenteral treatment of older animals with broad-spectrum antimicrobials within 1-2 days of the appearance of diarrhea is, except in cases of acute septicaemic salmonellosis, effective in reducing mortality and aids in recovery of the calves. The combination of trimethoprim and sulfonamide has been used successfully to treat calves with *S.* Dublin infection. However, treatment of salmonellosis with antimicrobial agents may induce the carrier state. Other concerns are the development of strains of *Salmonella* resistant to antimicrobials and the killing of normal intestinal tract flora, which may cause an increased susceptibility of the animal to salmonellosis and other gastrointestinal tract infections. Also, *Salmonella* resistant to antimicrobials may contaminate the food supply. The intravenous administration of fluids will increase the survival rates in calves with clinical salmonellosis and oral administration of fluids with electrolytes and nutrients will help calves to overcome a period of acute dehydration.

Prevention and Control

Salmonella infections in cattle may be prevented by purchasing replacement stock directly rather than via livestock dealers, by purchasing calves from farms free of salmonellosis, by purchasing calves of 6 weeks and older that have developed immunity, by purchasing from vaccinated herds, by maintaining a 4-week quarantine period of purchased cattle, by housing sick animals in dedicated isolation areas, and by preventing wild birds form having access to feed for cattle. Live attenuated vaccine strains (e.g. *aro* mutants) have been shown to be efficacious in preventing experimental salmonellosis, but few have been licensed. Vaccination with killed vaccines (bacterins) does not usually produce an effective immune response against *Salmonella.* However, in one study, a vaccine consisting of formalin-inactivated *S.* Typhimurium and *S.* Dublin strains led to the rapid cessation of excretion of *S.* Typhimurium DT104 in 7 out of 7 dairy herds. In contrast, 5 of 5 non-vaccinated herds were subclinically affected for at least 6 months and 2 of the herds experienced recurrence of subclinical infection after 2 years.

Public Health Aspects

Salmonellosis is a zoonotic illness in that infection of cattle or contamination of the milk with *Salmonella* spp. may by direct contact with infected animals or via the food chain, for example by drinking raw milk, cause illness in humans. These matters are discussed in detail in the article on *Salmonella* spp.

See also: **Feed Ingredients**: Feed Concentrates: Co-Product Feeds. **Pathogens in Milk**: *Salmonella* spp. **Replacement Management in Cattle**: Health Management.

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Infectious Diseases: Tuberculosis

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Introduction

Mycobacteria are common in the environment. Only a relatively small number of the 85 mycobacterial species cause disease in dairy animals. Sporadic infections causing mastitis, metritis, skin or other localized infections may be due to mycobacteria such as *Mycobacterium avium*, *Mb. scrofulaceum*, *Mb. kansasii* and *Mb. fortuitum*. The two major infectious diseases of cattle caused by mycobacteria are tuberculosis (TB), caused by *Mb. bovis*, and paratuberculosis, also called Johne's disease, caused by *Mb. paratuberculosis* (*see* **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease). This article will focus exclusively on bovine TB.

Bovine TB has plagued cattle since their domestication. Domestication and the increased animal density that results was, in fact, probably responsible for this disease entering the cattle population from humans by adaptation of the human cause of TB, *Mb. tuberculosis*. *Mycobacterium bovis*, while well adapted to cattle, is genetically so similar to *Mb. tuberculosis* that these two agents are technically members of the same species. Both are capable of infecting humans but have distinct host preferences.

The chronic debilitating nature of bovine TB results in impaired animal productivity. While this is a sufficient reason to control the infection, it is the zoonotic potential of this microbe that has prompted many countries to invest large sums of money to eradicate the disease. Bovine TB has largely been eradicated from North America, Australia and most of western Europe. It remains a common and difficult problem for developing countries and in those locations where *Mb. bovis* has become established in wildlife populations, making eradication virtually impossible.

Causative Organism

Mycobacterium bovis is a small, aerobic, nonmotile, nonsporeforming, Gram-positive and acid-fast- staining bacterium. It is closely related genetically to the cause of TB in humans, *Mb. tuberculosis*. Together with other similar but far less common mycobacteria, namely *Mb. microti* and *Mb. africanum*, these organisms form a group of mycobacteria called the TB complex. Members of the TB complex are zoonotic agents, meaning they are pathogenic for, and readily transmitted between, animals and humans.

Epidemiology/Pathogenesis

Bovine TB is prevalent in all countries that have significant cattle populations unless an organized campaign to eradicate the diseases has been undertaken. As a result of eradication efforts, cattle in Australia, most of Europe and North America are essentially free of bovine TB.

Bovine TB is primarily an airborne infection. Transmission rates rise are higher when animals are kept crowded and/or confined to stables or barns. Once inhaled, *Mb. bovis* is phagocytized by alveolar macrophages in which this facultative intracellular pathogen begins multiplying. Focal infection of lung tissue progresses with time and eventually the infection spreads to regional lymph nodes in the mediastinum of the lung or other lymphnodes in the head and neck region. The host animal continues to mount a cell-mediated immune response against *Mb. bovis*, resulting in steadily accumulation of macrophages and T lymphocytes at the site of infection. This focal mononuclear inflammatory response becomes organized into a lesion known as a granuloma. Such lesions are also called tubercles since they are so indicative of TB. Tuberculous granulomas are circumscribed by fibrous connective tissue and have a laminar structure resembling the cut surface of an onion. With time, cells in the centre of the granuloma die and the dead cellular debris takes the appearance and consistency of dry cottage cheese. The pathological term for this is caseous necrosis. The time from onset of infection to extensive pathology and clinical signs of TB is usually several years.

If *Mb. bovis* is ingested, it can infect the intestinal tract or become a disseminated infection involving multiple internal organs. This is a far less common form of bovine TB than is the respiratory form of the disease.

Clinical Signs

Clinical signs of TB in cattle are not dramatic and are nonspecific. Infected animals simply appear unthrifty and gradually decline in body condition until they are culled for being nonproductive.

The effects of *Mb. bovis* infection on the health and productivity of cattle are subtle and hard to quantify. The greatest economic impact of bovine TB comes from the costs of testing and its effect on animal trade among herds, states, regions or countries. Countries that have eradicated bovine TB are intent on maintaining their TB-free status. Consequently, there are strict import testing requirements placed on the cattle, and the herds and regions of the world that they originate from. In general, TB-free countries, or regions within countries, take the safest course of action to avoid importation of *Mb. bovis*-infected cattle. That is, they only accept cattle for importation that are TB skin test-negative and originate from a TB test-negative herd in a TB test-negative region. Veterinary certificates of inspection verifying that the animal has been TB-tested and found to be negative and that it originates from a TB-free herd must usually accompany imported animals.

Diagnosis

Essentially only one antemortem diagnostic test is used to detect *Mb. bovis*-infected cattle – the TB skin test. This test is performed, just as in humans, by inoculation of a small quantity of *Mb. bovis* antigens (called purified protein derivative, PPD) into the superficial layers of the skin (intradermal inoculation). The typical site for injection is the fold of skin directly under the tail (caudal fold). After 48–72 h the site of inoculation is examined visually and by feel for evidence of swelling. Significant swelling (as defined by government regulations, e.g. >3 mm) is caused by an accumulation of mononuclear inflammatory cells, called a delayed-type hypersensitivity reaction. This reaction to PPD, mediated by T lymphocytes, indicates past or present infection of the animal by *Mb. bovis*. Since spontaneous resolution of *Mb. bovis* infection is rare, the animal is considered infected and labelled a 'TB reactor'.

The sensitivity and specificity of the skin test are 86% and 96% respectively. This means that 86 of 100 actual cases of bovine TB will test positive but that four of 100 noninfected animals also may test positive: false-positive tests. This presumes that the skin test was performed and interpreted correctly. The precise measurement of antigen dose, careful injection into the superficial layers of the skin and careful examination of the injection site 2–3 days later are all steps critical to test accuracy.

TB skin testing is done differently in geographic areas where the disease is endemic than in areas with a very low *Mb. bovis* infection prevalence. If the infection prevalence is high, the predictive value of a positive test (the probability the test is correct when positive) is sufficiently high that caudal fold skin test-positive cattle are labelled 'reactors' and managed as infected animals. In countries or regions with a low prevalence of bovine TB the predictive value of a positive skin test is low, meaning a low probability that the positive test is correct. This results when the frequency of false-positive tests is as high as or higher than that of true-positive tests. A false-positive skin test is defined as a skin test-positive animal that has no evidence of bovine TB after necropsy and examination of tissues by histopathology and microbiology. False-positive TB skin tests occur when animals become sensitized to mycobacteria that occur in the environment.

To limit problems resulting from false-positive skin tests and improve the skin test specificity, the comparative cervical skin test was devised. In this test two intradermal injections are placed side by side in the neck skin (cervical region) of the animal. PPD antigens of *Mb. bovis* are inoculated in one site and PPD antigens of *Mb. avium* in the other. *Mycobacterium avium* is used because it is one of the more common mycobacteria found in the environment causing sensitization of cattle. The amount of swelling induced by each antigen is measured by comparing skin thickness measured before and 48–72 h after antigen injection. Using prescribed interpretation charts these two parameters can be used to determine if the animal is a true *Mb. bovis* reactor (larger amount of swelling at the site of *Mb. bovis* PPD inoculation) or is merely sensitized to some other mycobacterial agent.

Most countries have specific regulations governing the use and interpretation of tests for bovine TB. Many times it is only governmental veterinarians or specially trained and certified veterinarians who can perform these tests.

A laboratory test for interferon-γ (IFN-γ) has been shown to be a useful alternative to skin testing and in some countries is used as an alternative or supplemental test for bovine TB. The IFN-γ test is performed on peripheral blood leucocytes. To perform this test whole blood is divided into two portions. One is mixed with *Mb. bovis* PPD and the other serves as a control. After 24 h incubation at 37 °C, the plasma fraction of the blood is tested for evidence of IFN-γ release using an enzyme-linked immunosorbent assay (Bovigam, CSL, Melbourne, Australia). Significant levels of IFN-γ in the blood exposed to *Mb. bovis* PPD as compared to the control is considered a positive test. The IFN-γ assay shows good correlation with the skin test. The test has the advantage of only requiring handling of the cattle one time and of being both quantitative and objective. A disadvantage is the necessity of getting heparinized (heparin is a chemical that prevents clotting) blood to the laboratory within 24 h in good condition.

Diagnosis of bovine TB after death of the animal is accomplished by histopathology and culture of potentially infected tissues for *Mb. bovis*. These tests are done to confirm a suspected diagnosis.

Treatment

Rarely is treatment of *Mb. bovis* infection attempted. Rather, test-positive animals are slaughtered.

Prevention/Control

There are three principal reasons for controlling bovine tuberculosis: (1) risk of infection transmission to humans; (2) loss in productivity of infected animals and (3) risk that trade restrictions might be imposed by countries that are free of bovine TB.

Control of bovine TB within herds involves annual testing of the herd and removal of all reactors. Segregation of adult cattle from the young animals being raised as dairy replacements will help diminish the spread of infection. In situations of very high *Mb. bovis* infection rate, whole-herd depopulation may be warranted depending on the TB status of the region or country in which the herd is located.

At the regional or national level control programmes are aimed at limiting movement of animals from infected to noninfected herds. This begins with testing and classification of herds based on annual whole-herd skin tests. As TB programs expand, smaller geographic units, such as counties or states, will be declared free of bovine TB. The goal, successfully achieved by many countries, is to attain total freedom from bovine TB. The Office International des Epizooties (OIE) definition of a country being declared free of bovine TB is when: (1) bovine TB is compulsorily notifiable in the country and (2) 99.8% of the herds have been officially free from TB for at least the past 3 years; (3) cattle introduced to the country are required to be accompanied by a certificate from an official veterinarian attesting to their TB-free status and (4) the country has a veterinary administration system capable of tracing and testing the herd of origin for any TB test reactor.

Obstacles to successful eradication of bovine TB are several. The first, and for many countries, most significant, obstacle is political or financial. To launch a successful bovine TB control or eradication campaign requires that the majority of producers are strongly supportive of the program. It also requires that they can obtain sufficient support from the government to help finance what is a very expensive and long-term effort. Paradoxically, sustaining both dairy industry and government support for a bovine TB program is challenging, even if the program is successful. As the prevalence of the infection approaches zero it becomes increasingly hard to maintain enthusiasm for a program to eradicate a disease that is rarely seen. Decades of investment to obtain a TB-free status can be reversed in relatively short order if some level of surveillance is not sustained. The most common method of surveillance is by visual examination of carcasses at the slaughter plants. When granulomatous lesions are seen by inspectors, they are submitted for histopathology and culture by specialized reference laboratories.

The other main obstacle to bovine TB control or eradication is a biological one. In some countries the infection has spread from cattle to local wildlife populations. The best, but not the only, examples of endemically *Mb. bovis*-infected wildlife are Australian brushtail possums (*Trichosurus vulpecula*) in New Zealand and Eurasian badgers (*Meles meles*) in the United Kingdom. Epidemiological studies in both of these countries have clearly shown that *Mb. bovis* is not only resident in these wild populations but that the infection readily passes back to domestic animals in contact with those animals. This creates a situation of conflicting interests between animal agriculture and people who place a higher priority on protection of these wild animals. One strategy for finding compromise is to develop *Mb. bovis* vaccines for the wildlife and some research shows progress in this direction.

Public Health Concerns

Mycobacterium bovis has a broad host range and it includes humans, thus making it one of the zoonotic bacterial pathogens. The zoonotic potential of *Mb. bovis* and the fact that it is excreted in milk were responsible in large measure for the establishment of bovine TB eradication programmes in many countries and institution of pasteurization of milk for human consumption – a process that effectively kills the organism. In the early 1900s, *Mb. bovis* caused an estimated 5–30% of all cases of human TB in the United States and United Kingdom. Most human *Mb. bovis* infections were acquired by children through consumption of raw milk and resulted in an intestinal or generalized form of TB as opposed to the respiratory form of the disease typical of human TB due to *Mb. tuberculosis*.

TB remains one of the most common infectious diseases of humans, causing an estimated 8 million new cases and 3 million deaths worldwide each year. It must be recognized, however, that the vast majority of these human TB cases are caused by *Mb. tuberculosis*, not *Mb. bovis*, and that humans, not cattle, are the most common source of infection. It is possible for cattle to become infected with *Mb. tuberculosis* from humans and then transmit the infection back to other humans but such instances are very rare. Countries can virtually eliminate the public health problem of *Mb. bovis* by making pasteurization of milk and fresh cheese compulsory, eradicating bovine TB from cattle herds, or both. The positive impact these measures have on human health is clearly evident in the countries of North America and most of western Europe.

See also: **Diseases of Dairy Animals:** Infectious Diseases: Johne's Disease; Non-Infectious Diseases: Bloat. **Office of International Epizootics:** Mission, Organization and Animal Health Code.

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Non-Infectious Diseases: Acidosis/Laminitis

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Introduction

Ruminal acidosis refers to increased concentration of organic acids, particularly lactic acid, and lower ruminal pH that often occurs owing to rapid fermentation of soluble carbohydrates. Laminitis is the aseptic inflammation, ischemia, and subsequent degeneration of the dermal layers, particularly the sensitive laminae, between the keratinized hoof and the third phalanx (pedal bone). It may occur subsequent to ruminal acidosis or may occur independently. Acidosis most often occurs in the form of subacute ruminal acidosis (SARA) during the transition from a diet with higher fiber and lower energy to one with increased energy in the form of soluble carbohydrate. It is undesirable because of its direct effects on the rumen, including reduced fiber digestion, and the negative impacts on metabolism and health due to its systemic effects beyond the rumen. Prevention requires careful feed formulation, mixing, and delivery practices, and proper feed bunk management to avoid rapid shifts in dietary intake composition.

Laminitis in dairy cattle manifests as subtle, short-term changes in gait through to chronic and potentially debilitating lameness. The connection between rumen acidosis and laminitis includes alterations in blood flow and the systemic effects of endotoxins or other rumen bacterial products on cellular metabolism and on inflammation. Other causes include genetic developmental disorders, hormonal changes, season-related changes in metabolism and development of hoof structure, chronic or acute insult or injury related to prolonged standing time, the type of housing and hard-surface flooring, and particularly the cows' resting and feeding behaviors.

Acidosis

Basic Condition

Maximum milk production requires a stably functioning, healthy rumen, which in turn requires a consistent intake and balance of dietary fiber (plant cell walls: cellulose, hemicellulose, and lignin), protein, starch, and a variety of other carbohydrates. A significant portion of the fiber should be from a highly digestible source that is harvested, stored, mixed, and fed in a way that it retains sufficient fiber length to stimulate rumination with associated salivation and chewing of the regurgitated bolus, or cud. A healthy rumen supports a stable mixed population of bacteria and protozoa that grow on fiber, protein, or starch. These in turn provide a mix of chemical products and intermediates at a pH of 6.5–7. To prevent the accumulation of volatile fatty acids, the rumen mucosa must be able to metabolize them. The mucosal mass of the rumen of a cow consuming a ration with increased amounts of high-energy concentrate feeds increases about fourfold as compared to that of cows on rations high in fiber, but this mass requires up to 4–6 weeks of exposure to develop. If during any feeding episode the percentage of rapidly fermented carbohydrate is too high, if dietary fiber percentage is too low, if the fiber is chopped too short (usually <3–4 cm), if the feed mixing process reduces the effective fiber, or if selective intake occurs such that rumination (regurgitation and chewing of a bolus of matted fiber to decrease fiber length and stimulate saliva production) is reduced, then a cascade of events leads to increased lactic acid formation and decreased utilization with a decrease in rumen pH.

Ruminal acidosis (rumen overload, acute carbohydrate engorgement, ruminal lactic acidosis) refers to the condition of decreased ruminal pH that occurs due to increased production of lactic acid resulting from excessive ingestion of rapidly fermentable carbohydrates. Although acidosis can occur any time, it most often occurs as SARA during the transition from a prepartum diet with higher fiber to one with increased energy density in the form of soluble carbohydrate and lower fiber as fed during early lactation. The acute form occurs when an abrupt increase in rapidly fermentable carbohydrate enables lactic acid-producing bacteria to proliferate more rapidly than other rumen microflora. As a result, lactic acid and other metabolic intermediates accumulate and reduce the pH of the rumen.

Acute clinical acidosis, which is rare and occurs due to gross feed-mixing errors or due to accidental access to concentrates, occurs at a rumen pH below ~5.0; cases with rumen pH less than ~4.5 usually are fatal. Subacute acidosis occurs at a rumen pH between 5.0 and 5.5. SARA occurs when rumen pH is below 5.6 for more than 3 h day^{-1}, is the prevalent form, requires special procedures to detect, and often results in reduced performance that is difficult to detect. Because of the delicate balance of energy and fiber intake required for high milk production, prevention of acidosis requires careful feed formulation, proper mixing, and consistent delivery practices to avoid rapid shifts in dietary composition or the consumption of a diet leading to severe depression of rumen pH or repeated episodes of SARA.

With the ingestion of excess soluble carbohydrate, bacteria (such as *Streptococcus bovis* and many *Lactobacillis* sp.) that break down starch and ferment the glucose to D- and L-lactic acid proliferate more rapidly than those that use lactic acid as a precursor. When the production of lactic acid exceeds the ability of other bacterial species (such as *Megaspheria elsdenii* or *Selemonas ruminantium*) to convert lactic acid to acetate, propionate, or long-chain fatty acids, lactic acid concentration increases and rumen pH falls. To maintain a balance between lactic acid-producing and lactic acid-utilizing microflora, rumen pH must remain above ~5.5. Most lactic acid-utilizing bacteria are initially inhibited by the falling pH and then killed if the pH falls below 5.2, so the accumulation of excess lactic acid accelerates until the supply of fermentable carbohydrate substrate is exhausted or the pH falls so low that the rumen environment is toxic even to the lactic acid producers. As the pH decreases, more of the total volatile fatty acids (acetate, propionate, butyrate, and other intermediates) are found in the associated state (bound with a hydrogen ion). Because the associated fatty acids are not absorbed as well, the total acid concentration in the rumen increases more rapidly as absorption decreases.

If rumen pH falls below the clinical acidosis threshold, rumen motility is detectably decreased and stasis occurs if it drops low enough. A rumen pH below ~6.0 dramatically reduces the population of cellulolytic microflora, reducing cellulose digestion efficiency, and the production and absorption of the critical energy-yielding volatile fatty acids are altered, reducing milk production efficiency. Because cellulolytic microflora recover slowly relative to the proliferation of the lactic acid producers, bouts of excess soluble carbohydrate consumption, such as those resulting from feeding grain and fiber separately or other feeding errors, have prolonged adverse effects on milk production.

Serious secondary problems result from rumen acidosis. Severe acidosis causes ruminitis, starting with damage to the rumen wall, ulcer formation, and eventual scarring of the ruminal lining (**Figures 1–3**). This permanently reduces absorptive efficiency. During the acute phase, damage to the rumen wall allows penetration of fungus and bacteria, which can lead to systemic bacteremia and hepatic abscesses. In turn, damage to the caudal vena cava may then occur, along with pulmonary thromboembolism and systemic abscesses. Because mammalian enzyme systems do not metabolize D-lactic acid as efficiently as they metabolize L-lactic acid, this isomer has prolonged physiological effects.

Effects from subclinical acidosis include a reduction in absorptive efficiency due to hyperkeratosis and parakeratosis, a change in the proportion of fatty acids absorbed, which alters metabolic efficiency, and a reduction in milk fat synthesis. As bacterial populations change, those that make conjugated linoleic acids (specifically those containing a *trans* configuration double bond at C-10) increase. These intermediates are known to decrease fatty acid and triglyceride synthesis and may be most responsible for the reduction in milk fat content associated with high-grain diets. A reduction in rumen pH and the associated decreases in rumination and saliva production worsen the situation, as the buffers in saliva critical in acid neutralization and absorption are reduced. This eventually leads to a reduction in rumen activity, which in turn leads to depressed and inconsistent feed intake. Reduced feed intake in turn can lead to many other further problems, including lost production, subclinical or clinical ketosis, and displaced abomasum.

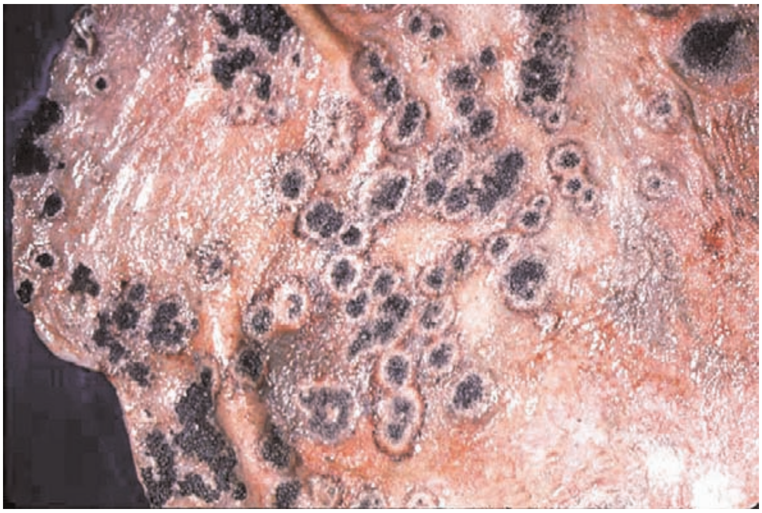

Figure 1 Early damage of acidosis – rumen mycotic plaques.

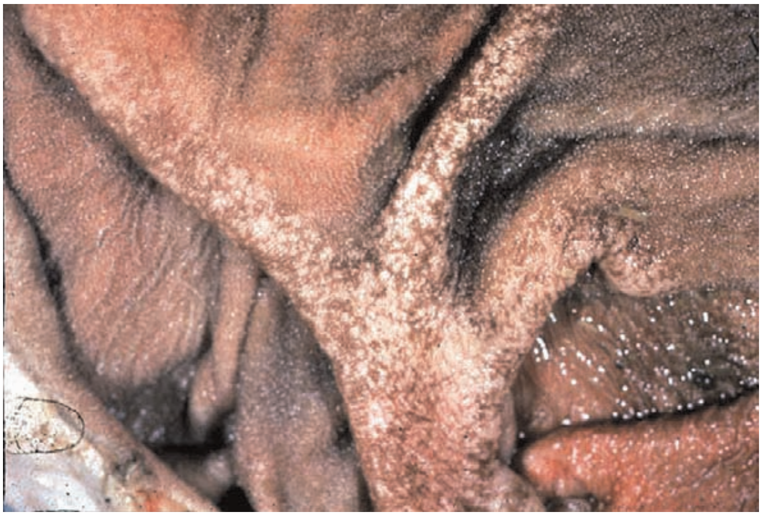

Figure 2 Further damage of acidosis – rumen ulcers.

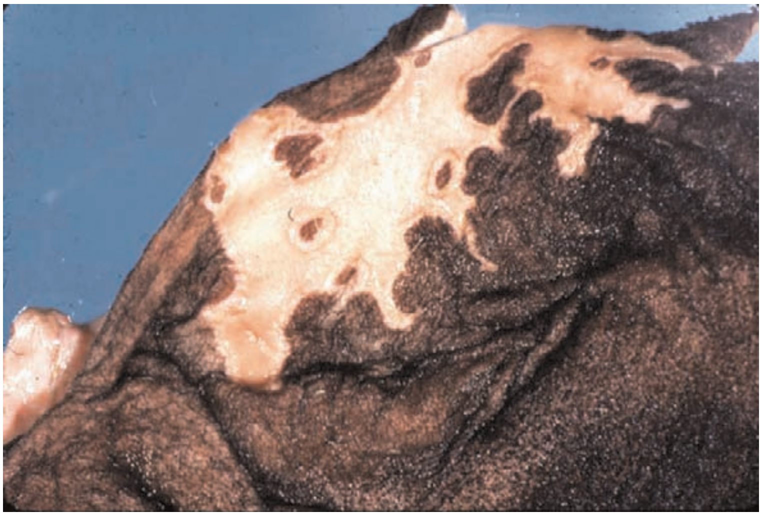

Figure 3 Permanent rumen scarring damage.

Prevention

In dairy cattle, SARA most often occurs during the transition from a diet with higher fiber for maintenance or pregnancy to one with increased soluble carbohydrate, as is usually fed during lactation. The development of total mixed rations (TMR) and abandoning parlor grain feeding are examples of industry innovations to prevent acidosis. Simple preventative measures include making stepwise increases in dietary concentrate percentage that increase net energy no more than 10% about every 5–7 days more frequent feeding during a day, minimizing feed bunk competition, reducing feed sorting, limiting the total amount of concentrate in the ration (usually in the range of 60%), and maintaining an adequate percentage of acid detergent fiber (ADF)-cellulose and lignin. Provision of sufficient fiber is critical to help prevent acidosis through salivary production and for maintaining the population of cellulolytic organisms. Attention must also be paid to providing plant material of sufficient length (such as 1–2 inches in a chopped diet, or long hay fed separately) to maintain a fiber mat in the rumen. This allows a rumen structure sufficient to help prevent displaced abomasums as well as long fiber sufficient to allow cud formation, which in turn stimulates saliva production and buffering capacity. Oligofructose, derived from fructans in pasture grasses, has recently been implicated as a cause of bovine and equine laminitis cases in pasture-fed animals.

Even with attention to all these factors, the energy density and feeding rates necessary to supply the production ability of very high-producing cows often exceed the ability of the rumen to maintain pH, leading to the aforementioned problems. In the 1970s, a simple preventative solution was found: incorporating buffering compounds into diets containing increased amounts of soluble carbohydrate. This effective solution saved biological resources and reduced food production costs. Using buffering salts has allowed increased feeding rates while preventing acidosis and subsequent production and health losses from feeding genetically inferior animals, reduced feed intake, ketosis, displaced abomasums, and lameness. Most cost analysis figures would use the prevention of disease and reduction of veterinary costs as the major savings from rumen buffers.

Without rumen buffers, the maintenance of the same level of total milk production would require feeding more feed to more cows having lower production per cow. If we aggregate all the cost savings of rumen buffers, which increase efficiency and reduce metabolic and clinical disease and death loss, we can see the benefits involved. We will use a number of 10 million cows in the United States (now this is a little high, 20 years ago it was low) and a starting point of 10% of the cows having rumen acidosis leading to problems (this is a realistic estimate from pre-buffer years). The loss of production, wasted feed, disease treatment, veterinary costs, and animal loss, all averaged together is conservatively estimated at US$500 per cow per year. Thus, prevention of even one-half of these losses (500 000 cases per year) is a cost savings of $250 million per year, or $5 billion saved in the United States alone in 20 years! On the basis of a farm containing 100 cows, this is a savings of at least (5 cows × $500 per case) $2500 per year. There are, frankly, few scientific discoveries or applications that have had such a positive impact on animal health, economy, and environmental resources as the use of rumen buffers. More recently, the feeding of monensin also has been suggested to reduce SARA episodes as well as improve productive efficiency. Readers should refer to the article on metabolic modifiers.

Prevention of acidosis and subsequent problems requires a thorough review of feed formulation, feeding practices, and animal management to avoid too rapid shifts in dietary composition or erratic consumption of a diet leading to repeated episodes of abnormally lowered rumen pH. For example, mixing smaller, timid first-calf heifers and dominant older cows or providing inadequate bunk space and inadequate feed access time may result in intermittent feed intake by the more timid animals. Prolonged or harsh feed mixing may significantly reduce the effective fiber component that would have otherwise been adequate. Combining of feeds with disparate physical properties in a TMR may enable cows to selectively consume the higher-energy components and discard the fiber components. Errors in ration sampling and analysis, failure to monitor ration particle size, failure to routinely monitor and adjust for changes in dry matter content of wet ingredients, poor management of wet forage feed out, and mistakes in combining ration ingredient amounts also contribute to SARA episodes. Mitigating intake declines due to heat stress has also been shown to reduce SARA.

Attention should be paid to changing a diet over a 2-week period at minimum. A standard practice on many farms would be to provide a slightly increased concentrate (perhaps 12% of dry matter) during the late dry period (2–3 weeks prior to calving) to help stimulate the proper bacterial population and increased rumen mucosal mass. Then, when starch content is increased postpartum, the bacteria are there to start utilizing it and the cow can absorb the fatty acids. After calving, a ration further increased in concentrate (20%) can be provided, followed by another week at about 30–40% and then allowing a ration concentrate percentage up to 60% or slightly more depending on production. Great care needs to be taken to observe any potential problems such as reduced intake or access to too much concentrate too fast, which can quickly escalate into further problems. Providing sufficient ADF (20–23%) with sufficient length (1.5–2 inches in chopped rations or some long hay) on very low-forage diets is also critical. Even with attention to all this, inclusion of a rumen buffering agent such as sodium bicarbonate and magnesium oxide is necessary. Usually, these are used at a few tenths of a percent in the ration, depending upon the percentage of grain in the ration, length of forage, and expected feeding and milk production rates. Following these simple guidelines (even though this can mean a lot of details in practice) has allowed support of high feed intake and high milk production rates with low incidence of acidosis and subsequent problems in well-managed dairy herds.

Treatment

Acute rumen acidosis is an emergency requiring aggressive treatment to remove the lactic acid-producing feed from the rumen, oftentimes by rumenotomy, and fluid therapy to correct the electrolyte imbalances. Treatment of SARA or chronic acidosis can be achieved through addition of buffering agents, providing access to more or longer lengths of fibrous material, or reducing the concentrate percentage of the ration. Quite often, the underlying acidosis problem is not recognized as a component of a more acute secondary problem, such as reduced feed intake, ketosis, or displaced abomasums (the reader should consult other articles for more information on these problems). But the underlying cause is usually in the makeup and management of the ration, and changing this will usually reduce the primary acidosis problem as well as the secondary problems.

Laminitis

Basic Condition

Laminitis (coriosis) is a microvascular disturbance resulting in aseptic inflammation and subsequent degeneration in the dermal structures of the hoof, particularly those that generate the hoof horn keratin, and the sensitive laminae and the associated corium that join the keratinized hoof to the third phalanx bone. The lameness associated with laminitis ranges from a mild soreness manifested by walking with the back arched upward to acute or chronic debilitating non-weight-bearing lameness. However, most often laminitis episodes occur without being detected. As this disruption affects the tissues generating the hard structures of the hoof beyond the period of acute insult, these hard structures are softer than normal as they grow out. The claw wall may have a groove ('hardship groove') from the episode; the sole may be waxy and tinged yellow and contain hemorrhages from serum and blood leaking from capillaries during the acute episode; and the claw may become flattened. As the hoof wall is normally produced at 5 mm per month and the sole somewhat slower, up to several months may be required for these lesions to appear. These defects lead to serious secondary conditions including sole ulcer, white-line separation, and heel horn erosion (**Figures 4–6**). Although often found in association with dietary and feeding management errors resulting in episodes of lowered rumen pH, laminitis has many causes including structural defects of the bone and hoof (genetic); normal physiological changes associated with impending calving (hormonal); developmental abnormalities (genetic and environmental); housing (time spent on concrete, uncomfortable stalls, likelihood of injury or falling); season of the year; nutritional management; and other disease conditions such as mastitis and metritis. Bovine laminitis is quite similar to laminitis and the associated lameness in horses, which is also recognized as having multiple potential causes.

Even though the details of the relationship between rumen acidosis and laminitis are not well understood, disruption of microvascular blood flow is the initial step in the pathology. Prevailing hypotheses focus on the connection between alterations in microvascular blood flow and cellular metabolism and the effects of increased acid and endotoxins or other bacterial products in the blood that may or may not originate from the rumen. It is thought that these directly or indirectly (through histamine or other circulating vasoactive substances) trigger an inflammatory cascade, altering blood flow in the sensitive structures. This in turn may alter the normal growth rate of the tissues, reducing their integrity and causing increased pressure and chances for damage. Alternatively, the increased blood pressure itself may lead to damage in this expansion-restricted area with tremendous pressures imposed during locomotion. An additional effect of acid-producing diets is the increased absorption of endotoxins and other microbial products into the bloodstream due to increased starch outflow from the rumen to the lower intestinal tract, which may also directly or indirectly affect tissue function in the hoof area.

Increased energy or protein intake that leads to rapid growth, and therefore increased stress on the hoof structures during development, is a risk factor for laminitis. In addition, the amount and proportion of dietary calcium and phosphorus play a part in mineralization of bony tissues (hoof is not bone; but surrounding bones and growth plates if affected can lead to lameness). Copper and zinc directly affect the enzymes involved in the production of collagen and keratin. The amino acid methionine may have direct or indirect (through

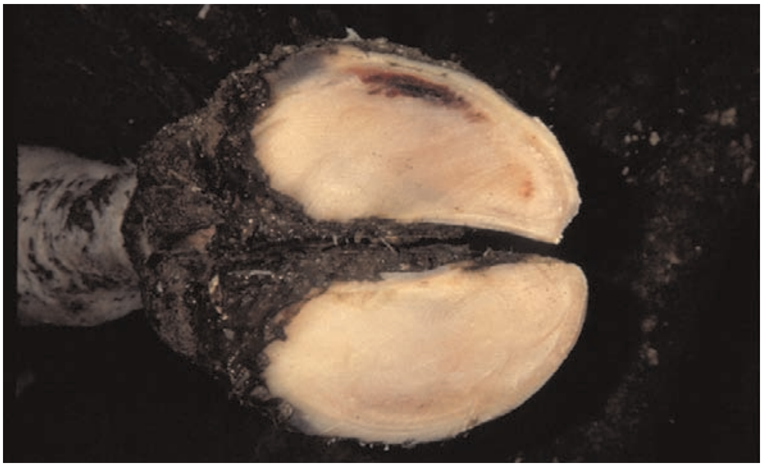

Figure 4 White-line separation disease in zone 2 of the lateral digit. Hemorrhagic area, which significantly reduces the integrity of the laminar region, can be seen at the top.

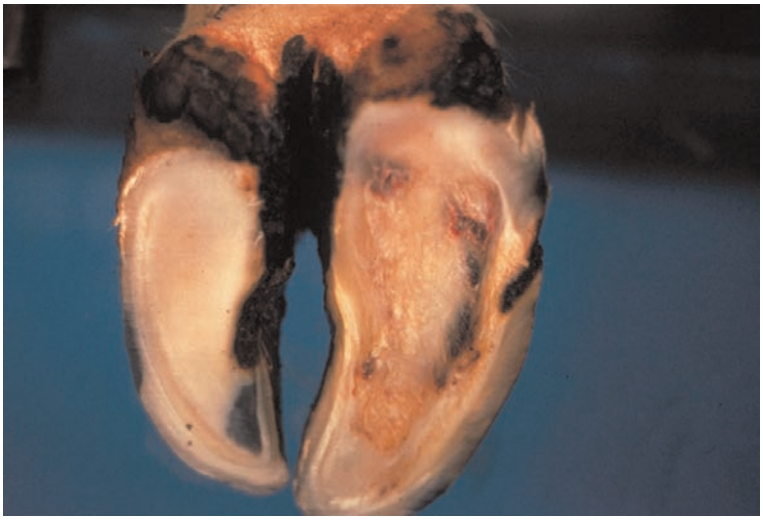

Figure 5 A variety of lesions including sole ulcers, solar bruises, yellow discoloration, and white-line separation. Apparent are the normal status of the medial digit and the extreme complexity of injuries and disease associated with clinical laminitis.

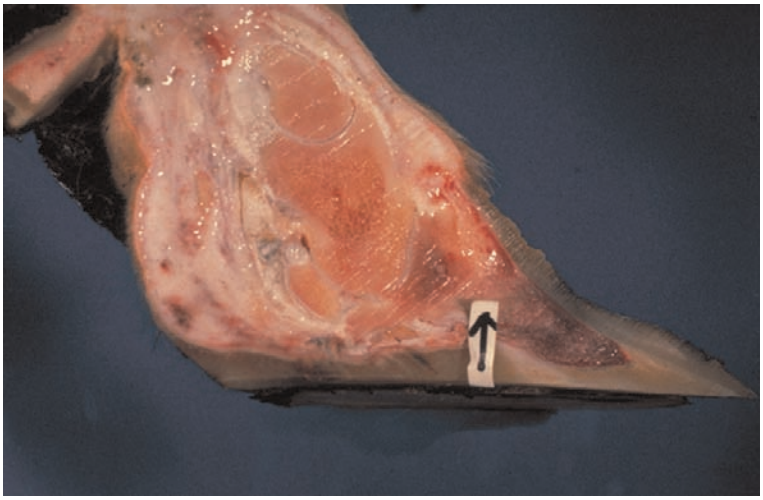

Figure 6 Extensive tissue scarring of the various dermal regions, along with solar hemorrhage and perforation. The arrow depicts the end of the pedal bone, which has been extremely degenerated in this case of chronic laminitis.

metabolic and vasoactive intermediates) influence on the process of hoof formation. Increasing biotin levels in the ration has been shown to improve hoof integrity.

In addition to the metabolic causes of laminitis, other causes may include developmental disorders, hormonal changes of late pregnancy, season-related changes in metabolism and development of hoof structure, and the interaction of all of these with chronic or acute insult or injury related to the type of housing and flooring in and on which the dairy cattle are kept. Hoof injury remains the major cause of hoof damage and lameness, with or without clinical laminitis. Normal hoof cell function is depressed in winter and enhanced in summer, and this may lead to seasonal changes in lameness, leading to a false conclusion that the diet was the causative factor. This theory is supported primarily by studies at the cellular level, and a clear causative relationship between season of the year, hoof function, and clinical laminitis has not been established. Based on the results of more frequent corrective trimming, others suggest that altered morphology from abnormal wearing of hoof structures on concrete surfaces may also be a cause.

Prevention

Normal growth and development of the hoof involves many steps that can be affected. The keratinization process involves formation of collagen matrix, basement membrane, basal cells, keratinocytes, keratinized cells, and horn squames, leading to horn or stratum corneum. Many things can affect each of these steps. These include rate of growth, which is of course a function of total energy, protein, vitamin, and mineral intake; pressure exerted by the animal's weight; the type of surface that the hoof must walk upon (wet, muddy, dry, slick, concrete, dirt, mats, etc.); and injury or insult. Laminitis in dairy cattle is clearly a complex, multifactorial disease. Rates of laminitis and lameness vary among herds from very low (5%) to very high (70%), and heifers are clearly at higher risk. Determination of the causes on a farm requires complete investigation of the environment, husbandry, and diet of the lactating cattle as well as of the replacement heifers.

Prevention must start with a thorough analysis of the environment, husbandry, and feeding management of both the lactating herd and the replacement heifers. Attention must be given to the flooring surface, minimizing wet and muddy conditions, maximizing stall comfort, and providing a surface less prone to high hoof impact, hoof slippage, and other potential injuries. Attention must be given to rate of growth and proper balance of energy, protein, calcium, phosphorus, zinc, and copper in the replacement heifers. In the lactating herd, a diet that optimizes consistent feed intake and milk production while minimizing other problems such as acidosis and displaced abomasums will also most likely minimize diet-related laminitis. Proper and regular trimming of hoofs also maintains normal hoof function and diminishes injury- and stress-related lameness.

Treatment

Treatment of acute individual cases of laminitis, although often not especially effective, involves treating the ruminal acidosis, metritis, or mastitis if these conditions are present, cooling the hoof area to diminish blood flow and inflammation, using approved antihistamines and anti-inflammatories, keeping the hoof dry, and making sure the animal has a safe surface upon which to walk, to minimize further injury. But as is the case for many other problems, unless all the items listed in the 'Prevention' section are examined and adjusted as needed, treatment will be a futile exercise in symptom management and will not improve the situation for the balance of the herd.

See also: **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems. **Nutrients, Digestion and Absorption**: Fermentation in the Rumen.

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Non-Infectious Diseases: Bloat

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Introduction

Bloat or tympanitis is a serious disorder of ruminants, chiefly affecting dairy and beef cattle, while its occurrence and severity are much less in dairy sheep and goats. The main symptom of bloat is an extreme distension or inflation of the animal's rumen. The etiology of bloat is still poorly understood. For over 50 years, researchers have been trying to answer many questions about bloat: What is bloat? Why does it occur mainly in spring? Why is its occurrence so intermittent and unpredictable? Why does it occur when animals graze in one field but not in an adjacent field? Why do some plant species cause bloat but not others? Why are cattle more susceptible to bloat than sheep or goats? In an outbreak of bloat, why are some animals affected but not others? Why does bloat occur repeatedly in some geographic areas but not at all in some other areas? Unfortunately, we still do not have certain answers to most of these questions, but we have the realization that bloat is a multifactorial syndrome(s) and a theory or hypothesis that can accommodate many of these questions.

There are two types of bloat: foamy bloat and free-gas bloat. Foamy bloat is caused by the formation of persistent foam in the cows' rumen, which inhibits the cow from eructating (burping) fermentation gases. There are two main types of foamy bloat: legume bloat and grain or feedlot bloat. Legume bloat principally occurs in spring when cattle graze temperate pastures containing legumes. In legume bloat, the foam is formed when fermentation gases become entrapped within the protein released into the rumen fluid from the leaves of consumed leguminous plants. The main leguminous pasture species associated with legume bloat (in decreasing order of bloat-inducing potency) are red clover *Trifolium pratense*, alfalfa *Medicago sativa*, and white clover *Trifolium repens*.

In grain or feedlot bloat, the dominant foaming agent is bacterial mucopolysaccharides (slime). In contrast to foamy bloat, in free-gas bloat, the rumen fluid is not foamy and there is little or no foam in the rumen headspace, and consequently the bloat must be caused by factor(s) other than foam that inhibit eructation. Research in Australia has shown that even in cases of subclinical bloat where there is no outward sign of bloat, pasture intake may be depressed, resulting in milk yields being reduced by about 1 l per cow per day. In severe bloat, accumulation of gas in the reticulo-rumen causes increased pressure on internal organs and death by cardiorespiratory failure. Thus, there is a continuum in degrees of bloat and researchers and veterinarians use a 0–5 scale to describe bloat severity: 0, no bloat; 1, mild bloat, left flank slightly distended; 2, medium bloat, left flank severely distended (see **Figure 1**), on palpation it is difficult to compress the rumen; 3, moderate bloat, left flank severely distended and right flank distended, cow showing signs of distress such as uneasiness and labored breathing; 4, severe bloat, both flanks severely distended, the animal showing more signs of distress and may cough-up rumen contents; 5, close to death.

The extent of the economic significance of bloat is unknown, but there have been estimates that the annual number of deaths from bloat in Australia and New Zealand combined may be as high as 50 000, while for Argentina there have been estimates of up to 500 000 (mainly beef animals). In the United States, where dairy cows are mostly fed on total mixed rations, deaths from bloat are infrequent. In beef feedlots, the death rate from bloat is between 0.1% and 0.2%, but if ionophores are not included in the diet, bloat deaths may be as high as 0.5–1%. On Australian and New Zealand dairy farms, annual deaths from bloat are only about 0.3%, but on individual farms bloat may occasionally kill 5–20% of animals in a single day. However, the main economic cost of bloat is not due to deaths but from the fear of bloat. Methods to control and treat bloat are expensive not only in terms of labor and material but also because the necessity to prevent, treat, and be alert to the onset of bloat may divert management from other pressing activities on the dairy farm. In addition, bloat may cause an opportunity cost since the fear of bloat may prevent farmers from growing or optimally utilizing legume-dominant pastures, which have been shown to produce more milk than grass-dominant pastures.

Foam Hypothesis of Bloat

The foam hypothesis as an explanation for the cause of bloat was first promulgated in the 1950s by researchers in New Zealand. They observed that, during spring, when hungry cows were milked and then allowed to graze on lush legume-dominant pasture, severe bloat could result, and can even culminate in death within 30 min of grazing the bloat-inducing pasture. In many of these cases, the rumen contents consisted of strong and persistent foam. It was realized that when receptors within the rumen near the esophageal sphincter could detect the presence of

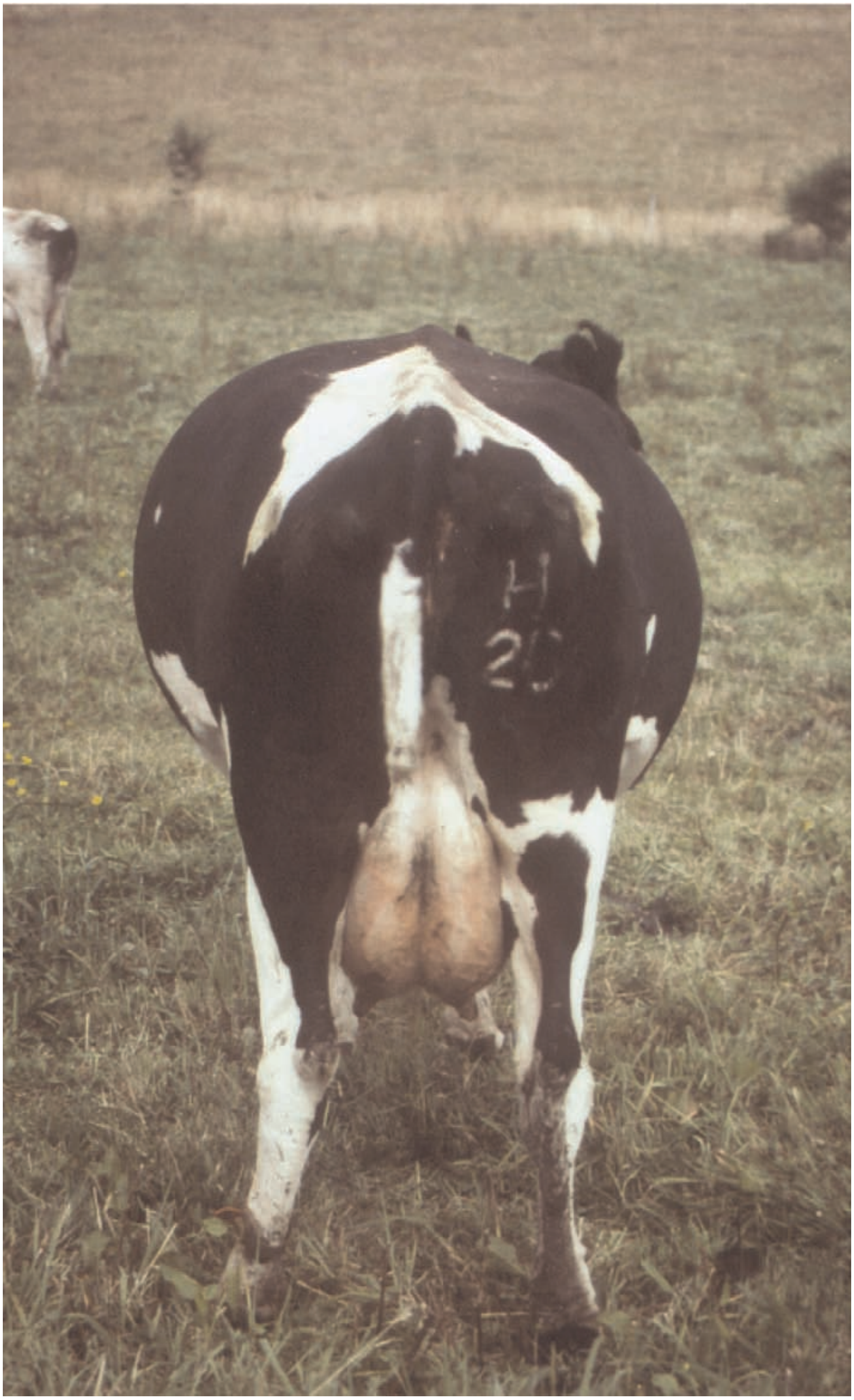

Figure 1 A Holstein cow with score 2 bloat.

foam, the esophageal sphincter remained closed and the animal was unable to eructate and hence gas accumulated within the rumen. Research in New Zealand in the 1960s implicated soluble leaf cytoplasmic protein in the foaming process. Although some research suggested the involvement of salivary proteins and even plant pectins, later research confirmed the central importance of the abundant leaf enzyme, ribulose bisphosphate carboxylase, as the dominant protein involved in foam formation in legume bloat. A number of other factors may be responsible for inhibiting or preventing the esophageal sphincter from opening and thus for causing free-gas bloat. These include obstruction of the esophagus by turnips, potatoes, or sugar beets, hardware disease (ingestion of foreign objects such as plastic), impaired rumen motility due to hypocalcemia, injury or damage to the vagus nerve (which controls the esophageal sphincter), and anesthetics. Free-gas bloat, from an as yet unknown cause, may also occur when cattle graze lush, young ryegrass pasture or green wheat crops.

There has long been consensus that the gas involved in the formation of foamy and free-gas bloat is fermentation gas. Moreover, recent research has shown that the gas in foamy bloat and free-gas bloat has similar proportions of carbon dioxide (CO_2), methane (CH_4), and nitrogen (N_2) as the rumen headspace gas in nonbloating animals. This finding was unexpected since *in vitro* studies had demonstrated that persistent protein foams could be generated with CH_4 and N_2, but not with CO_2, or when there was more than 30% CO_2 in a mixture of CO_2 and CH_4. A high rate of gas production in the rumen has long been considered to be a likely factor that triggers the bloat, onset but recent research indicates that bloat can occur even when the rate of production of fermentation gas is relatively low. Thus, the cause of bloat remains principally due to a failure of the animal to eructate.

A number of researchers have speculated that for foamy bloat to occur, powerful foam-stabilizing agents must be present within the rumen that can cause a persistent foam even in the presence of the normally high concentrations of CO_2 that occur in the rumen. In addition, since outbreaks of bloat are generally infrequent, it has been further hypothesized that the presence of foam-stabilizing substances in legumes must also be intermittent or that there must also be the presence of antifoaming agents in legumes. Thus the occurrence of bloat at any particular time may depend upon the balance between foam-stabilizing substances and antifoaming agents. For example, cattle grazing lush white clover in early spring may suffer from bloat, but in summer when white clover flowers, there is a much lower incidence of bloat. This observation is consistent with the fact that white clover flowers have recently been shown to contain condensed tannins or proanthocyanidins that have antifoaming properties.

Once the central importance of strong, stable, protein foams was understood, researchers in New Zealand and Australia conducted a long series of *in vitro* experiments to elucidate the factors involved in foam stability. Concentrations of protein, sodium, potassium, calcium, and magnesium and pH were all found to influence foam strength and persistence. Thus, a gradual understanding developed that management and environmental factors may influence fundamental factors within the plant and these could then determine under what circumstances bloat would occur. For example, the observation that bloat rarely occurred on many farms close to the sea could be explained in terms of an effect due to high concentrations of sodium in pasture.

An important factor influencing the formation of foam in the rumen is the concentration of leaf cytoplasmic protein in the rumen fluid. This depends upon many factors including the rate at which the pasture is consumed, the rate at which cytoplasmic protein is released from cells, and the rate at which cytoplasmic protein is digested by rumen bacteria. When alfalfa is young and lush, cows are able to very quickly eat great quantities and, not surprisingly, this coincides with a period when the grazing of alfalfa has a high risk of bloat. As the alfalfa plant ages, it becomes more fibrous and 'stemmy', the maximal rate of grazing tends to decline, and the incidence of bloat declines. As the alfalfa plant gets still older, cows are able to wrap their tongues around the alfalfa stem and quickly strip the leaves from the stem. Grazing alfalfa at this period or age is also accompanied by a high risk of bloat.

Not all legumes cause bloat. Bloat does not occur when cattle graze tropical legumes. Temperate legumes such as trefoil (*Lotus* spp.), sanfoin (*Onobrychis viciifolia*), and vetch (*Vicia* spp.), which all contain tannins (proanthocyanidins), generally do not cause bloat. Indeed, these legumes may partially protect against bloat if they occur in pastures containing red or white clover. Although many pasture weeds such as docks (*Rumex* spp.) also contain tannins, it has been the experience of the authors that when cows grazed mixed red clover/dock pastures, they still bloated severely (and died), because the cows avoided the dock and selectively grazed the clover.

Besides plant factors, there are many other factors that impact upon the bloat syndrome. In Argentina, Australia, New Zealand, and South Africa, there are many farmer anecdotes that suggest bloat is more frequent in the mornings when there is dew on the pasture or when the wind blows from a certain direction or at a particular prevailing atmospheric pressure or humidity. While the involvement of these factors in influencing the onset of bloat cannot be completely ruled out, extensive surveys in the above countries have generally failed to find strong correlations between weather parameters and bloat incidence. In contrast, there is good evidence that there are animal factors that influence the predisposition of an animal to bloat. It has been the observation of the authors that some animals will experience many episodes of bloat during a single year, while other animals grazing the same pasture will never be observed to bloat. Grazing dairy cattle tend to be more prone to bloat than grazing beef cattle, but, it has been argued that this may not be due to breed but due to twice-daily milking, which necessarily imparts on dairy cows an intermittent grazing pattern. However, even within dairy breeds, there are differences in bloat susceptibility. A survey in Australia showed that Jersey cows were 4 times more likely to suffer from bloat than their Friesian herdmates. In New Zealand, researchers, using a closed herd initially containing pure Jersey and Friesian dairy cows, sought in a long-term crossbreeding experiment to select and breed bloat-prone and bloat-resistant animals. This must have been a difficult undertaking since a survey in Australia has shown that of animals that die from bloat, 75% die on their first recorded case. Nevertheless, after approximately 30 years, the New Zealand researchers had indeed developed bloat-prone and bloat-resistant animals and had made some progress toward identifying genes that could serve as markers of bloat susceptibility. However, the bloat-susceptible animals were not as heavy and produced milk with higher fat test than the bloat-resistant animals. Thus it cannot be certain that the putative marker genes for bloat susceptibility were not in reality marker genes for the Jersey breed.

Treatment and Prevention

For the last 50 years, the most popular methods of bloat prevention and treatment have been based upon chemotherapeutics. Reid's seminal paper 'Bloat: The Foam Hypothesis' is the cornerstone on which, for the last 40 years, bloat research and antibloat prophylactic/therapeutic treatments have been based. The first antibloat medications were based upon the long-recognized antifoaming abilities of various oils. Vegetable oils such as peanut, sunflower, and olive oil, mineral oils such as paraffin oil, and other oils such as whale and fish oils have been used successfully to treat bloated animals.

Foams cannot persist without film or lamellar elasticity. Oils greatly reduce or eliminate film elasticity. The application of as little as 90 g of oil can prevent bloat in dairy cows for 2–4 h. Antibloat oils should be administered orally (drenched) to any cow with mild to moderate bloat. Usually, approximately 100–200 ml will be sufficient to successfully treat cows suffering from score 2–3 bloat. Sometimes, antibloat oil is applied as a flank spray with the intention that cattle will lick the bloat oil. This method of application is less effective than drenching since some cows may not lick the bloat oil. Antibloat oils may also be sprayed each morning on the area of pasture to be grazed that day. However, this technique is also less effective than drenching since rain may wash the oil off the pasture and cattle may manage to graze unsprayed pasture.

We generally think of detergents as foaming agents since they form foam when mixed with water. Paradoxically, with respect to bloat foam, detergents also have antifoaming effects. The explanation for this involves an interaction of detergent with the naturally occurring dietary fats and oils. Most pasture species including legumes contain 1–4% ether extract, and a substantial part of the ether extract is composed of long-chain fatty acids. These fatty acids and other plant lipids potentially have strong antifoaming abilities and ingested plant lipids are normally present in the rumen at quantities (0.3–1 kg) that theoretically should prevent the formation of protein foams. Unfortunately, when ruminants chew or ruminate on legumes, the lipid components are released from the plant cells into the rumen fluid and then they either bind to the surface of plant particles or form fatty micelles (small hydrophobic droplets of fat surrounded by a monolayer of protein). The antifoaming ability of these lipids is inactivated probably by their association with denatured protein. Detergents, by their wetting action, break down the weak protein/lipid associations and resuspend the liberated lipids, thus reactivating their antifoaming action. There are a number of different types of detergents that have antibloating abilities, but the most commonly used are the nonionic alcohol ethoxylates based on alcohols with chain lengths of 12–15 carbon atoms. Pluronic detergents including poloxalene are also very effective against bloat. The experience of farmers and researchers is that detergents are best used as a preventative of bloat. Routine twice-daily dosing (*per os*) with 5–10 g of these detergents usually prevents bloats in dairy cattle. This is a labor-intensive procedure, but it is perhaps the most effective method to reduce the incidence and severity of bloat. Detergents can also be added to drinking water or to supplementary feeds, but there can be no guarantee that every animal will consume these in sufficient amounts that can prevent bloat.

Monensin is an ionophorous antibiotic that has been used in many countries for over 50 years as a feed additive in beef feedlots since it improves feed conversion efficiency and reduces the incidence of feed-related bloat deaths. There is now incontrovertible evidence that monensin can reduce the incidence and severity of foamy bloat. In the United States, monensin has long been approved for use in beef feedlots but was approved for use in lactating cows only in 2004. In contrast, in Argentina, Australia, Canada, and New Zealand, monensin has been approved for many years for use in beef and lactating dairy cows. In Australia, the most popular means of bloat prevention and the main means of administering monensin to dairy and beef cows is by way of intraruminal controlled-release capsules (see **Figure 2**). These devices contain approximately 25 g of monensin and 35 g of excipient (hexaglycerol distearate) and are designed to constantly release monensin at a rate of 250 mg day^{-1} for 100 days. The devices are administered in early spring *per os* by a balling gun and are effective for approximately 100 days, covering the major risk period for bloat. The antibloat capsule resembles a syringe with a compressed

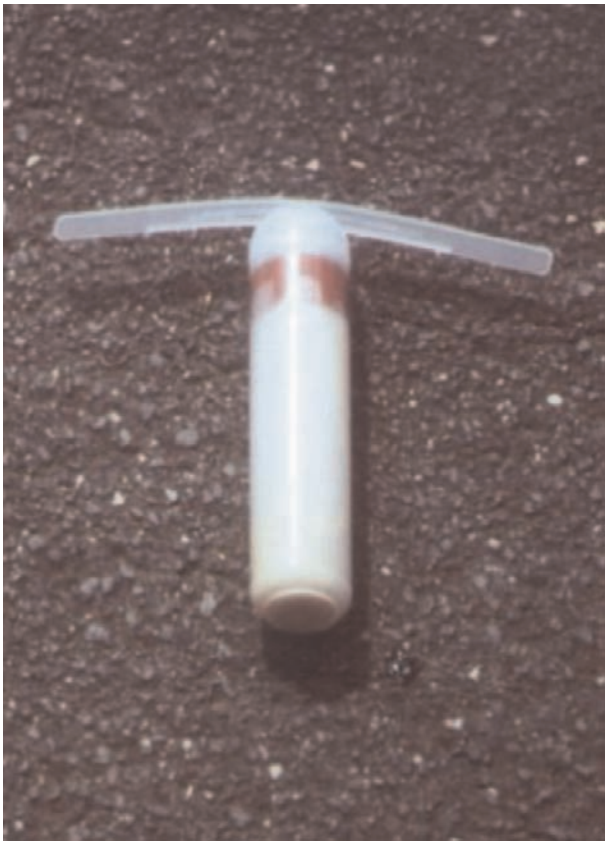

Figure 2 A monensin intraruminal controlled-release capsule.

steel spring pressing against a piston providing the motive force to expel the core contents through an orifice at the other end. In addition, the capsules are designed not to be regurgitated or to pass out of the rumen since they are fitted with flexible wings that extend once the capsules enter the rumen. Surveys have shown that these capsules reduce incidents of visible bloat and bloat deaths by approximately 80%. Monensin may also be included as an additive to supplementary feeds for grazing dairy cows, but care must be taken to ensure that other animals do not ingest the monensin since it can be extremely toxic and may cause death in horses. For many years, the antibloat activity of monensin was attributed to its ability to shift the pattern of rumen fermentation from acetic to propionic acid and thus to reduce the production of methane. However, recent research in Australia has shown that monensin antibloat capsules have no effect on the concentration of methane in the rumen and have little effect on the rate of gas production. Research has shown that monensin reduces the strength and persistence of protein foams, but the major mechanism of the action of monensin in preventing bloat is still uncertain.

Besides chemotherapeutics, the other main means to prevent bloat involves management. Many dairy farmers have found that the incidence and severity of bloat is greatly reduced if they offer their cows concentrate feed supplements before they allow them access to bloat-inducing pasture. Other farmers have found that the incidence and severity of bloat is greatly diminished if the pasture is mowed and wilted before offering it to their animals.

At present, there is no form of bloat prevention that can be guaranteed to be always 100% successful. Occasionally, the farmer may still have to treat some severely bloated animals. In the case of severe bloat, a cow may be only minutes from death and there is probably only one course of action that will save the animal. The rumen wall in the area of the paralumbar fossa must be punctured to allow the accumulated gas to escape. Sometimes, in the case of free-gas bloat, one or two large bore (10 gauge) needles inserted into the rumen will be sufficient to release the pent-up gas. In free-gas bloat, a large quantity of gas will issue forth for a considerable period of time (perhaps an hour or more). In the case of severe foamy bloat, the needle will almost instantly become blocked with foamy rumen contents. In these cases, as a last resort the condition will need to be relieved surgically by means of a sharp knife or trocar. Sometimes, the cut may become blocked, and a piece of tubing may have to be inserted to allow the foamy rumen contents to escape. These invasive procedures should be carried out only by experienced people, or by those instructed in the techniques by a veterinary surgeon. Following this procedure, it will be necessary for a veterinary surgeon to sew up the wound and administer antibiotics to the cow. Usually, cows that recover from this procedure are not nearly as productive as they were formerly.

Future Impact of Bloat on the Dairy Industry

As diets for dairy cows are increasingly comprised of total mixed rations (containing monensin or detergents) or of grazed pastures heavily supplemented with concentrates, the incidence of bloat should continue to decline in the major dairying countries of the world. Chemotherapeutics will probably remain the mainstay for preventing bloat in conventional dairy farming systems. However, if consumer concerns result in the withdrawal of these agents for bloat prophylaxis, then bloat may again cause a substantial impact on the grazing dairy industries of the world. Recent developments in the field of biotechnology and genetically modified organisms hold the potential for bloat-resistant animals and bloat-safe legumes. However, much basic research is still required to identify the appropriate genes for these attributes, and it is still uncertain if milk produced using genetically modified pastures or animals will be acceptable to consumers. In developed countries, including the

United States, there is a growing trend toward 'organic' dairy farming. On these farms, cows typically graze pastures instead of consuming total mixed rations fortified with monensin. Thus, bloat is likely to be an increasing problem on organic dairy farms.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever. **Forages and Pastures**: Annual Forage and Pasture Crops – Establishment and Management; Annual Forage and Pasture Crops – Species and Varieties; Perennial Forage and Pasture Crops – Species and Varieties. **Husbandry of Dairy Animals**: Goat: Feeding Management.

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Non-Infectious Diseases: Displaced Abomasum

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Introduction

Displacement of the abomasum within the bovine abdomen is a relatively common and economically important disease that affects dairy cattle worldwide. The disease is in fact a complex of three different types of displacements of the abomasum leading to decreased production, debility, and possibly even death. When the intensity of dairy farming increased and the genetic potential of cattle to produce milk increased rapidly, the nutritional management did not keep pace with these genetic improvements; the industry saw a dramatic increase in the incidence of displaced abomasums. Recently, with much better management of the chemical and physical properties of dairy cattle rations and their delivery through total mixed rations, the incidence of displaced abomasums has declined. Nevertheless, they can still be a problem in many situations. There is no single direct cause of abomasal displacements; instead it is believed that multiple risk factors come together to produce the disease. Factors involving pregnancy, nutrition, genetics, and management are all involved in the disease pathogenesis. Critical analysis of all of these areas is necessary to understand and lessen the importance of the disease complex within a herd.

Displaced Abomasum – Occurrence and Significance

Abomasal displacement is a disease complex of cattle that is of great significance to the dairy industry. Economic losses include decreased milk production by affected cows and increased treatment and/or replacement costs of affected cows. Abomasal displacement is one of the periparturient diseases that can reduce a cow's overall production for that lactation because it usually occurs at the beginning of the lactation period and can affect peak and persistency of the lactation. Cattle affected with displaced abomasums frequently have decreased fertility and subsequent increased days open.

As stated, the term 'displaced abomasum' is actually a disease complex wherein the abomasum becomes displaced from its normal position in the right ventral abdomen. The displacement can be either to the left side of the abdominal cavity, a left displaced abomasum (LDA), or dorsally to the right side, a right displaced abomasum (RDA). Further the RDA can become subsequently twisted leading to not only an obstruction to flow of ingesta through the abomasum but also interference with blood flow to the abomasum. This condition is often called a right abomasal volvulus (AV) and because of the interference with blood flow to the abomasum, serious, life-threatening consequences can result. Concerning the prevalence of the varying types, LDAs account for 85–95.8% of the displacements. It is generally believed that regardless of the abnormal position that the abomasum assumes, the reasons for the displacement are common.

Causes of Abomasal Displacement

Displacement of the abomasum occurs most commonly in middle-aged, high-producing dairy cattle but can be seen in other situations including heifers, bulls, and steers. The condition can occur sporadically in individual cows; however, clusters of cases often occur suggesting that common underlying risk factors are present in the herd. Eighty percent of displacements occur within 1 month of parturition. Less common presentations occur prepartum and during later lactation. The annual incidence on typical farms ranges up to 4.4%, although this is variable with clusters of displacements sometimes occurring. Mean decrease in milk production of affected cows is approximately 1600 pounds for that lactation. Subsequent lactations may also be affected by a displacement in a previous lactation.

A single, absolute cause of abomasal displacements is not likely. Instead, a number of managerial, environmental, and hereditary risk factors are thought to play a role in the disease process. These general factors along with the anatomy of the abomasum and its intra-abdominal attachments can combine to allow for displacement to occur. Although not totally proven, heredity can play a part in the risk for displacements. Selection for high milk production may play a part. In a study of 7416 Holstein cows, the heritability of abomasal displacement was 0.28. Conclusive judgment cannot be drawn from these studies; however, data suggest that long-term selection against metabolic disorders such as abomasal displacements may decrease the disease incidence, but milk production may also be adversely affected.

The largest predisposing factor for abomasal displacement is parturition and the changes that the cow must go through at the time of parturition. The majority of abomasal displacements occur from the time period 2 weeks prior to parturition to 2 weeks after calving. Obviously, calving is a risk factor that cannot be changed, as reproduction is an integral part of milk production. In late pregnancy, the uterus pushes the rumen cranially and dorsally whereas the abomasum may assume a position more to the left than normal. After parturition the abomasum may move further to the left to fill the void that was left by the emptying of the uterus. This situation may be a stronger factor in modern deep-bodied dairy cattle. In most dairy cattle, there is a decrease in feed intake during the periparturient period and this reduces rumen volume possibly allowing the abomasum to shift somewhat to the left. Many consider this reduced rumen volume during the periparturient period a major risk factor for development of an LDA as it signifies a decrease in dry mater intake which is often associated with a negative energy status in the cow. It is extremely difficult for the modern dry cow to meet all of her energy demands in late pregnancy and early lactation. Strong evidence would suggest that cows in a state of negative energy balance, as evidenced by increased serum nonesterified free fatty acids (NEFA) prepartum and increased serum beta-hydroxybutyrate (BHBA) postpartum, are at greater risk to develop many periparturient diseases including displaced abomasum. Further any other occurrence of periparturient diseases including hypocalcemia, mastitis, metritis, or retained placenta will further accentuate the issues of negative energy status.

Issues related to calcium status have often been considered to be significant in the development of displaced abomasums. Cattle suffering from clinical or even subclinical hypocalcemia in the transitional period and in later lactation are at greater risk for the development of a displacement. Equally cattle that have other risk factors for displacement development, such as retained placenta or mastitis, often have decreased serum calcium as a result of partial to complete anorexia secondary to these concurrent diseases. Controlling hypocalcemia in general as well as in the transitional period through dietary component manipulation including the use of anionic salts may be useful in the prevention of displaced abomasum.

Along with changes in the rumen size during this period, other dietary and metabolic factors may affect abomasal motility and increase abomasal gas production. Concurrent with the end of gestation are the nutritional changes that occur in this transition period. Typically, cattle that develop displacements are being fed a high-concentrate, low-roughage diet. The incorporation of these components in total mixed rations with short fiber lengths and silage-based diets seem to predispose cattle to displacements. High-concentrate, low-roughage diets are thought to be major risk factors in disease development. The increases in dietary concentrate and decreases in roughage cause increased volatile fatty acid (VFA) production in the rumen, leading to an increase in VFAs in the abomasum. This accumulation of VFAs may be a factor in decreasing abomasal motility. Further, gas production (carbon dioxide and methane) in the rumen increases in association with these dietary changes leading to an increased accumulation of gas in the abomasum. The decreases in abomasal motility along with an increase of gas accumulation in the abomasum are thought to be major factors in the final displacement. Whereas a postcalving void in the abdomen along with abomasal gas accumulation and decreased motility can combine to explain the left displacement; right displacements are due to gas accumulation and motility changes of the abomasum.

Along with the natural factors of pregnancy and parturition and the accompanying dietary changes, several other common conditions that occur in dairy cattle during this period have been linked to an increased risk to the development of a displacement. As emphasized previously, cattle affected with retained placenta, ketosis, dystocia, uterine infection, twinning, or hypocalcemia are more likely to develop a displacement than cows not affected with these conditions. Most likely these risk factors for displacement development relate to the motility of the abomasum during this critical postpartum period and are intimately associated with other risk factors such as changes in dry matter intake and the energy status of the cow.

Clinical Signs and Diagnosis of Abomasal Displacement

Clinically LDA and RDA will appear similar. Temperature, pulse, and respiratory rates will be normal unless there are concurrent diseases present such as retained placenta, metritis, or mastitis. The earliest clinical finding noticed will generally be a decrease (usually gradual) in milk production and a lessening in feed intake. It is not uncommon for the milk production of a cow with a displaced abomasum to fluctuate up and down on a daily basis. This often represents a waxing and waning of the functional obstruction caused by a displaced abomasum as it periodically fills and empties itself with gas and fluid. Correspondingly the cow may go through periods of apparent well-being followed by the reoccurrence of clinical signs of displacement. Eventually most of these cows become persistently obstructed leading to consistent signs. Cows with uncomplicated displacements have decreased appetites with generally complete refusal of concentrates and a lessening intake of roughage. Cattle consuming total mixed rations will be noted to have decreased intake of the ration. If a displacement becomes complicated, as is the case with an AV, the appetite for feeds abruptly ceases and anorexia is complete. Fecal output is usually decreased in amount and will appear pasty or occasionally mild diarrhea will be present in simple displacements. On occasion, one might notice excessive fiber length in the stool in cows with displacements resulting from interference of the omasal orifice function as a result of the displacement. Ketosis develops and increases in severity as the disease progresses over time. In the case of the LDA the cow may appear slab-sided on the left side due to the gas-filled abomasum distending the upper left side of the abdomen. In some cows, one can visualize the curvature of the abomasum extending caudal to the last rib into the paralumbar fossa in the case of an LDA. Occasionally a left displacement can be palpated rectally, whereas RDA and AV can usually be felt. More typically on rectal examination of a cow with an LDA, the rumen is noted to be displaced medially toward the right side of the abdomen. In most cases of simple LDA and RDA there is a slow but progressive debilitation of the cow. If the displacement is undetected the cow's condition will deteriorate characterized by dehydration and weight loss. These nonspecific signs and symptoms evidenced by the cow do not define an abomasal problem, just the developing debilitation. Concurrently, during this period of developing disability, the cow with an LDA or RDA will generally develop changes in her biochemical status, including blood pH, leading to a more systemic alkaline nature. This developing alkalosis is due to the sequestration of acid (hydrochloric acid) within the abomasum and rumen. Normally acid (chloride ion) produced in the abomasum is exchanged for bicarbonate in the small intestine. In the case of the LDA and RDA, this acid is prevented from transiting from the abomasum to the intestine by the displacement. These developing acid/base and electrolyte problems may account for some of the changes in the cow's demeanor along with developing ketosis.

If a right displacement progresses to an AV the clinical signs are significantly more striking. The cow will be markedly depressed and milk production significantly drops. She will evidence abdominal pain or discomfort. Feed intake ceases and dehydration will be readily evident. Abdominal distention may be present, and fecal output is scant and often dark in color. Signs of shock appear often rapidly related to abnormal vascular events occurring in the abomasum. As the abomasum twists, the blood supply to the organ is inhibited leading to, often, irreversible damage to the abomasum and rapid development of shock. As a result of the systemic events occurring in AV leading to shock, the alkalosis of a simple displacement is often overwhelmed by a most severe systemic accumulation of toxic products, and the body's pH often changes to a more acid state. Many other blood biochemical measurements will be found to be abnormal at this time.

On rare occasions, one might discover a chronic displacement of the abomasum. This is most commonly encountered as a chronic LDA. Through physical examination and other means it is found that the abomasum has been displaced for a considerable period of time, often with no or only minor clinical signs or debilitation. It is possible that some cows might pass through an entire lactation displaced and yet evidence only minimal or no signs of disease. Many chronically displaced abomasums develop ulceration and subsequent adhesions to the internal body wall and rumen. The adhesions when examined at surgery or autopsy appear to be long standing indicating a chronic clinical course. These patients may have exhibited nonspecific signs of abdominal sepsis.

In the case of each type of displacement a high-pitched musical sound or 'ping' can usually be elicited over the distended structure by simultaneous percussion and auscultation. A similar sound can be mimicked by placing one's ear next to a large metal storage drum or inflated child's rubber ball and gently tapping on the drum or ball to elicit a musical 'ping'. The examiner accomplishes simultaneous percussion and auscultation by placing the head of the stethoscope over the area of the suspected displacement or gas-filled structure and at the same time striking the side of the cow with a plexor (striking hammer or a sharp thump with one's finger) adjacent to the area of the stethoscope head to elicit a sound. The sound generated by this striking of the cow's side travels through the body wall and is reflected back to the stethoscope head by the structure underneath, which it encounters. The principle is very similar to sonar used by naval ships. Based on the character of the underlying structure (solid or gas-filled) a different sound is reflected back to the listener. Gas-filled structures that are tightly distended reflect back a high-pitched musical sound described as a 'ping' whereas more solid objects (non-gas-filled) such as the rumen or normal intestine yield a much duller sound. In the case of the LDA, the sound is best heard over the upper aspects of the 9th through 13th ribs on the left side of the cow. Similarly an RDA is heard in the same position on the right side of the cow. An AV is typically heard over an even larger area on the right side, both over the ribs and extending back over much of the paralumbar fossa. Not in all cases of abomasal displacement is a ping present, at times many pings are present and sometimes none is present. In the case of nonpinging other physical diagnosis techniques are often used by the examiner to define the presence or absence of displacement.

Other abnormal conditions within the abdomen can also be characterized by the high-pitched metallic sound or ping. Where the sound is heard will often lead the examiner to a tentative diagnosis as to the cause. On the left side of the cow, a gas cap within the rumen or within the peritoneal cavity may be represented by a ping. Also if the rumen becomes very small and collapses away from the left body wall, a ping may develop in the void created. On the right side, gas accumulations within the cecum, uterus, small intestine, and spiral colon can be associated with a ping. Generally these nonabomasal pings are found at different sites within the abdomen by auscultation and percussion leading the examiner to alternative diagnoses.

Another technique frequently utilized by the clinician to assess the potential presence of a gas-filled organ within the abdominal cavity is ballottement with simultaneous auscultation. This is a process where the abdomen is balloted rapidly in succession with one's fist in order to auscultate evidence of splashing sounds caused by fluid splashing within a gas-filled structure. As is the case in most conditions where gas is entrapped within an organ, fluid is also present. The ballottement causes the fluid to develop a wave-like movement and elicit a splashing sound. This procedure is performed on both sides of the cow. The hearing of splashing within the abdomen is not absolutely specific for a displaced abomasum; however, when considered with other findings it can help with a definitive diagnosis.

On rare occasions the examiner may elect to perform a diagnostic tap through the body wall, using a needle, to obtain fluid from a suspected displaced abomasum. This technique is most commonly described involving suspected left displaced abomasums that neither ping nor splash. Still, the examiner suspects that the abomasum is present between the rumen and the left body wall. In this case the examiner passes a sufficiently long, 14- to 16-gauge, needle through the body wall into the underlying structure, fluid is aspirated and the pH is determined. If the abomasum is present at the site of the tap, the pH will be very acidic, whereas if the rumen was tapped, the pH will be near neutral.

Further evaluation of these cases may involve laboratory testing to assess circulating acid/base status along with electrolyte determinations, as previously mentioned. Typically cows with displaced abomasums, either to the left or to the right, will develop low blood chloride and potassium along with a shift in pH toward the alkaline side of normal. In patients developing complications, such as RAV, blood changes reflecting shock and cardiovascular collapse typically develop. Other nondiagnostic laboratory findings that are usually present in cows with displaced abomasum include ketonemia, ketonuria, and elevation of liver enzymes. These findings reflect the negative energy status of the cow and fat mobilization in an attempt to offset the deficit.

The ultimate diagnostic technique for displaced abomasums is an exploratory celiotomy. This procedure would obviously not be limited to suspected, but unconfirmed displaced abomasums; instead it would give the surgeon the opportunity to explore the abdomen for abnormal conditions which could include a displacement. Although this procedure might seem extreme to diagnose what would seem to be a straightforward disease, there are times when all other diagnostic techniques are unsuccessful in definitively providing the final diagnosis. In these cases the exploratory celiotomy provides not only a diagnostic approach but also an opportunity for correction of the displacement by one of the described techniques.

Clinically, displacements must be differentiated from other conditions that present with similar signs. These can include simple ketosis, indigestion, intestinal gas accumulation, cecal dilation/torsion, twisted small intestine, intestinal obstruction, and peritonitis. A careful clinical examination should help in differentiating these conditions since many can and do occur in the periparturient period.

Treatment of Displaced Abomasum

Multiple methods of correction of simple displacements and the more complicated volvulus conditions of the abomasum are practiced. Several surgical and nonsurgical methods have been devised for the correction of displacement. Certain procedures are limited to one kind of displacement whereas another might be appropriate for all types. In some high-intensity dairy situations, many cattle with displaced abomasum are sold for slaughter rather than corrected.

Nonsurgical methods of correction of abomasal displacements are generally limited to correction of left displacements. These procedures, at minimum, involve casting the cow in left lateral recumbency, rolling her onto her back, and continuing the rolling until she is in right lateral recumbency from where she is allowed to rise. This rolling procedure is designed to reposition the abomasum from the left abdomen back to the right side. A serious complication of this procedure is that an LDA might subsequently become an RDA and even an AV. To prevent this from occurring, several methods of closed suture techniques have been designed to pexy or fix the abomasum in the correct position. One technique involves a large suture directed through the intact ventral body wall, purposely entering into the abomasal lumen and then backing out through the body wall again. The large suture is then tied to itself, to hold the abomasum in a normal position and allow an adhesion to form. This procedure is accomplished while the cow is on her back during the rolling procedure. A more current technique involves the use of a commercially available trocar and toggle specifically designed to perform the pexy. Again with the cow rolled on her back and the abomasum localized, a trocar is inserted through the ventral abdominal wall, entering the lumen of the abomasum. A small plastic toggle with an attached suture is fitted through the trocar and into the lumen of the abomasum. A second toggle is placed at a separate site and the sutures from the two are tied together, stabilizing the abomasum and allowing a permanent adhesion to form. Complications of these procedures can include peritonitis, localized infection, or redisplacement due to failure of the procedure. A recent report states that in the hands of qualified individuals the success rate of these blind techniques is similar to surgical correction techniques.

Three common surgical techniques are available for the surgeon. They are the right paramedian abomasopexy, right paralumbar fossa omentopexy, and left paralumbar fossa abomasopexy. The first two procedures are the most popular amongst veterinary surgeons. The purpose of each of these surgeries is to correct the displacement and then create a permanent adhesion of the abomasum or its attachments preventing further displacement and allowing the cow to return to normal function. The surgeries that are performed through the paralumbar fossas are accomplished with the cow in a standing position whereas the paramedian approach is performed with the cow cast on her back. Each surgical approach has its own advantages and disadvantages. It is up to the surgeons and their comfort level as to which technique they will utilize. Recently techniques involving laparoscopic approaches to the abdomen have been advocated for correction of LDA.

Regardless of the technique utilized to correct the displacement, postsurgical care is critical to insure adequate recovery. Underlying disease problems must be addressed. Infectious conditions such as metritis and mastitis must be aggressively treated. Primary or secondary metabolic condition such as hypocalcemia and ketosis must be resolved along with correction of dehydration and electrolyte deficits and reestablishing adequate rumen function. Each cow must be addressed separately as to its specific needs and complications.

Prevention of Displaced Abomasums

The prevention of all displaced abomasums is not practical or realistic. The goal should be to minimize the occurrence within the herd. Since there is no one specific cause of the displacements, one must evaluate a multitude of risk factors potentially involved. Since a high percentage of displacements occur in the transition period between late pregnancy and the early stages of lactation, nutritional and management procedures should be closely scrutinized. Risk factors such as twinning may be difficult to control. Occurrences of displacements at other stages of the production cycle may be difficult to rationalize. Prevention and early treatment of metabolic diseases such as ketosis and hypocalcemia are paramount. Proper ration formulation to provide balanced levels of nutrients in the proper form is critical. Cows must be encouraged to maintain a maximum dry matter intake during the later stages of gestation and through early lactation as lack of rumen fill has been suggested as an important risk factor for left displacement. One of the most important considerations involving nutrition related to displacements is to maintain an adequate intake of a high-fiber diet to dairy cows in late pregnancy and early lactation. Feeding a high-roughage diet and therefore a high-fiber diet with adequate fiber length is one of the most common ways recommended to decrease the incidence of displaced abomasums in the postpartum period. Recent advances in monitoring energy status of transition cows through the use of NEFA and BHBA measures can help address not only displaced abomasum but also other transition cow diseases.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Ketosis. **Nutrition and Health**: Diabetes Mellitus and Consumption of Milk and Dairy Products. **Reproduction, Events and Management**: Pregnancy: Periparturient Disorders.

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Non-Infectious Diseases: Fatty Liver

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Introduction

Fatty liver, or hepatic steatosis, is a pathological condition that is caused by chemical, nutritional, genetic, and endocrine perturbations. The condition is often termed a metabolic disease of dairy cattle in that the lipid content of liver deviates from the normal levels of <5% triacylglycerol (TG) (or triacylglyceride). During the late 1970s and early 1980s, fatty liver was recognized as part of a condition known as 'fat cow syndrome'. Clinical reports indicate that fatty liver occurs in high-yielding dairy cows immediately after calving, particularly when cows are overconditioned. The classic studies of Reid and coworkers provide some of the earliest characterization of this condition in lactating cows. More recently, fatty liver has been associated with the 'transition dairy cow' and the unique nutritional, management, and metabolic needs of the dairy cow during the last 3 weeks of gestation and first 3 weeks of lactation. At calving, liver TG can exceed 20% of the weight of liver, and a liver TG content of greater than 40% has been reported for individual cows. The consequences of severe hepatic lipidosis are apparent; however, the consequences of mild hepatic liposis are more difficult to define. Approximately 50% of transition cows develop moderate-to-severe fatty liver. Reducing the severity and incidence of this condition is a step toward improving the health and productivity of dairy cows. This article will outline the physiology and pathobiology of fatty liver in dairy cattle and describe the biochemistry underlying the development of this condition. Several excellent reviews that describe the classical nutrition and biochemistry associated with this disorder are available. Technologies and management strategies that reduce the incidence rate, severity, and duration of the occurrence of fatty liver in dairy cows continue to emerge. Current and emerging knowledge of this disorder and preventative strategies are discussed here.

How is Fatty Liver Diagnosed?

Accumulation of fat in the liver does not usually produce any easily identifiable outward symptoms because it is deposited over a period of several days to weeks. The most common method for diagnosis of fatty liver requires a cow-side surgery to obtain a sample of liver tissue. Cows do not react adversely to this minimally invasive procedure and the biopsy is usually obtained without a precise knowledge of the exact location within the liver but is assumed to be representative of the entire organ. The use of ultrasound to measure liver lipid content has been described recently by two independent groups and shows considerable promise as a noninvasive means of assessing fatty liver. Refinement of this technique will expand the possibilities for fatty liver diagnosis to larger groups of cows and broaden the assessment of nutritional and management strategies that influence fatty liver. Currently, studies to assess the impact of nutritional and management strategies on fatty liver in cow are limited due to the need for a biopsy sample in order to measure liver lipid content.

The fat content of liver can be expressed as total liver lipid or total liver TG and requires approximately 100 mg of fresh tissue for analysis. Total liver lipid is extracted with organic solvents and the lipid content of the extract is determined quantitatively. TG in the extract is determined following saponification to release glycerol, which is then quantified as a measure of TG content. Other estimates of liver lipid content have been obtained from liver samples that have been fixed, sectioned, and stained. The fractional volume of lipid relative to total volume of liver cells, determined by microscopy, is then used to calculate liver lipid content. Liver TG content accurately reflects fatty liver and is the most common method used to describe the degree of lipid accumulation in the liver. A cow-side test, based on the buoyancy of liver biopsy samples in aqueous solvents, has also been described but its use is restricted to gross classification of the condition. While biochemical analysis of liver lipid content is simple, it provides little information regarding the localization of fat within the liver or the relationship of fat to hepatocyte volume. As discussed below, the nature of TG depots within the hepatocytes and the location of TG within the liver acinus are used to classify hepatic steatosis in human disease states that include alcohol-induced fatty liver and nonalcoholic steatohepatitis.

At calving, TG accumulates in the liver and may be coupled with decreases in other nonfat cellular components such as glycogen and liver protein. Measures of liver TG content should reflect changes in lipid and nonlipid cell components. Liver lipid content in dairy cows has been expressed as TG per gram of liver dry matter, as liver TG per gram of wet tissue, or liver TG per unit of DNA.

Prevalence of Fatty Liver

Fatty liver in dairy cows was first classified as mild, moderate, and severe fatty liver corresponding to <5, 5–10, and >10 TG as a percentage of wet weight of the liver using a needle biopsy sample. The presence of 8–10% TG (% wet weight) in the liver sample relates to histological and functional differences. Ketosis is preceded by fatty liver and is the best recognized consequence of the condition. Healthy, mildly ketotic, and severely ketotic cows have a liver lipid content of 5, 8, and 17%, respectively, based on the analysis of fixed tissues using a light microscope and oil red O, a lipid-specific tissue stain. A liver lipid content of >17% TG of wet liver weight is classified as moderate-to-severe fatty liver based on 0.284 g liver dry weight per gram liver wet weight. Approximately 50% of multiparous cows in three trials at the University of Wisconsin had liver lipid contents >15% at calving and 30% had lipid contents >20%. Classification of liver TG as moderate to severe based on TG content per microgram DNA corresponds to values greater than 14 μg TG per μg liver DNA. Studies at Purdue University indicate that 26% of cows had greater than 14 μg TG per μg DNA in the liver at calving, but more than 70% of cows had liver TG above 14 μg per μg DNA by 28 days postcalving. Although differences may exist with regard to the timing of TG accumulation relative to calving using these methods, the data clearly indicate a high incidence of fatty liver in transition cows.

What Causes Fatty Liver?

Hormonal changes associated with parturition, feed intake depression at calving, and increased energy demands initiate the release of nonesterified fatty acids (NEFAs) from adipose tissue. Fatty liver results if the uptake and esterification of NEFAs to TG exceed the capacity of the liver to further metabolize NEFAs or to secrete them as very-low-density lipoprotein (VLDL). Intensive liver biopsy sampling around calving indicates that elevated plasma NEFA levels precede TG accumulation in the liver. Attenuating the prepartum rise in NEFA levels serves to decrease the severity of fatty liver. On the other hand, the clearance of lipid from the liver as VLDL is controlled by the rate of VLDL synthesis and the degree of loading of the VLDL particles with TG. The rate of VLDL release in ruminants is inherently compromised compared to other species. The biochemistry and molecular biology underlying the inability of ruminant liver to clear TG as VLDL is an area of active investigation.

The basic biology of lipid metabolism in ruminants is not completely understood. Advances in this regard are necessary to identify therapeutic and nutritional interventions that will reduce the incidence and severity of fatty liver in periparturient dairy cattle. Some of the recent information on the basic processes controlling lipid clearance from the liver is highlighted here.

The slow rate of TG export as VLDL is thought to be the main contributing factor leading to fatty liver in dairy cows. The VLDL particle is composed of four major lipid classes: phospholipids (mainly phosphatidylcholine), free cholesterol, TG, and cholesterol esters. The assembly and secretion of VLDL from the liver are dependent upon (1) the synthesis of apolipoprotein B (ApoB), the major structural protein associated with a stabilized VLDL particle; (2) ApoE, a component necessary for the VLDL assembly/secretion cascade; (3) the availability of lipids to form a surface coat (such as phosphatidylcholine and free cholesterol) and a neutral core (TG and cholesterol esters); and (4) the activity of microsomal triglyceride transfer protein (MTP) for VLDL filling.

The MTP is a dedicated endoplasmic reticulum-localized cofactor that is necessary for the assembly of ApoB with lipids. Within the endoplasmic reticulum, MTP functions with ApoB to form a small dense TG containing lipoprotein precursor particle. This initial phase of VLDL synthesis occurs coincident with the translation of the ApoB protein and its insertion into the endoplasmic reticulum and is referred to as the cotranslational phase of VLDL assembly. Within the endoplasmic reticulum, MTP also acts to transfer lipid to the VLDL precursor, known as the TG loading phase of VLDL synthesis, resulting in a mature VLDL particle for secretion. In the absence of MTP or sufficient TG, newly synthesized ApoB is delipidated and the ApoB protein associates with the inner leaf of the endoplasmic reticulum and is degraded. The relative abundance of MTP mRNA is elevated postcalving (154% of precalving values), but the mass of MTP protein is increased only 15% at calving. There is no correlation between hepatic MTP (mRNA and mass) and liver TG. Similarly, MTP activity and mass are not affected by nutritional status or insulin action and do not appear to be related to the rate of VLDL export. Likewise, a lack of coordinated change in ApoE with liver lipid concentrations during the periparturient period points to alternative limiting factors for triglyceride export from bovine liver.

Free fatty acids in the liver stimulate VLDL assembly and TG secretion, but their effects depend on the physiological state and the experimental model. In primary hepatocytes from rats, oleic acid increased secretion of VLDL by increasing the translocation of ApoB across the endoplasmic reticulum. Dietary conditions that favor *de novo* hepatic fatty acid synthesis in nonruminants act to increase VLDL synthesis, whereas increased supply of preformed fatty acids acts to reduce VLDL output. It has been proposed that a high rate of esterification of fatty acids in bovine liver at calving acts to reduce the capacity for VLDL secretion and promotes fatty liver. Recent measures of change in the gene expression of MTP, ApoE, and ApoB-100 in the liver demonstrate a decrease in ApoB-100 during the periparturient period, which is consistent with decreased synthesis and/or secretion of VLDL from the liver. Some of the most effective therapies for combating the development of fatty liver in dairy cows may act by increasing the rate of synthesis of ApoB-100.

Risks Associated with Fatty Liver

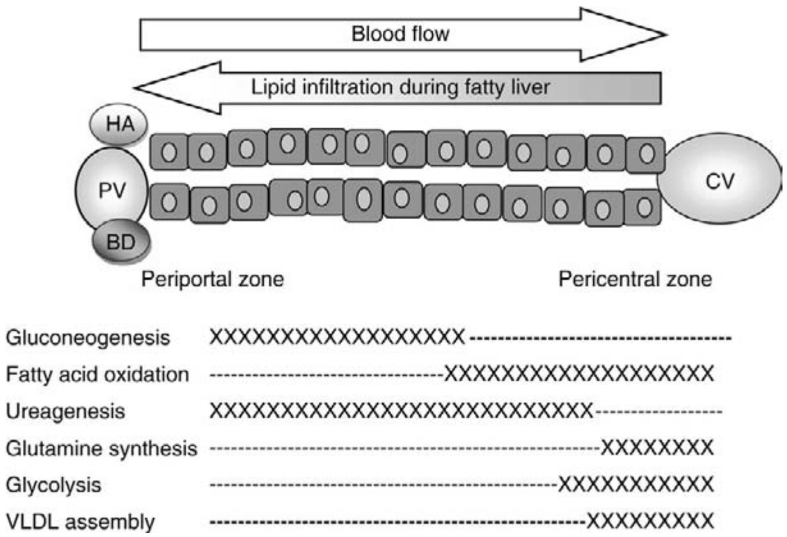
Fatty liver is implicated in the development of several metabolic disorders such as ketosis, impaired gluconeogenesis, impaired urea formation, increased incidence of mastitis, displaced abomasum, retained placenta, poor reproductive performance, and immune suppression. Fatty liver precedes the onset of clinical symptoms of ketosis. The prevalence of fatty liver can be reduced by common practices such as avoiding overconditioning at dryoff, maintaining body condition through the dry period, and managing the nutrition of cows during the transition to calving. Ideal condition scores, based on a 5-point scoring system, are in the range of 3.25–3.75 at dryoff and calving. While optimal management of body condition at dryoff may minimize the severity of fatty liver at calving, the prevalence of fatty liver in transition dairy cows is still a concern.

Genetic Component

There are several well-documented genetically linked disorders of hepatic lipid metabolism in humans and other species. Genetic analysis reveals a low heritability and repeatability of ketosis in dairy cows. These studies did not evaluate the genetic basis of fatty liver specifically, but due to the link between fatty liver and ketosis, one would conclude a lack of genetic basis for fatty liver in cows.

Consequences of Fatty Liver

The liver is geometrically organized into compartments or lobules, which are further configured to functional columns of 15–25 hepatocytes, which extend along sinusoids from the portal vein to the central vein called the liver acinus. Blood draining the rumen and intestines is collected in the portal vein, flows to the liver, and is dispersed through capillaries that flow across the cells of the liver acinus and emptied into the central vein of the liver lobule (**Figures 1** and **2**). The branches of the central vein coalesce to form the hepatic vein, which drains blood from the liver to the vena cava. Hepatocytes immediately adjacent to the branches of the portal vein (periportal hepatocytes) are therefore perfused with higher concentrations of oxygen and metabolites originating from the


Blood flow
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Ureagenesis XXXXXXXXXXXXXXXXXXXXXXXXXXX------------------
Glutamine synthesis ---XXXXXXXX
Glycolysis --XXXXXXXXXXX
VLDL assembly --XXXXXXXXX

Figure 1 Arrangement of hepatocytes along a plate of cells within a lobule of liver. Blood enters a liver lobule through the portal vein (PV) and hepatic artery and passes through hepatic sinusoids to exit the lobule through a central vein (CV). Bile flows in the opposite direction and is collected via the bile ductule. Cells in close proximity to the portal vein of the liver lobule are termed periportal cells, whereas cells surrounding the central vein are termed pericentral cells. Fat infiltration commonly observed in dairy cattle at calving is initiated in the periportal zone and, with the progress in severity, it extends to the pericentral zone. Zonation of primary metabolic functions across liver acinus is indicated by the symbol X. VLDL, very-low-density lipoprotein.

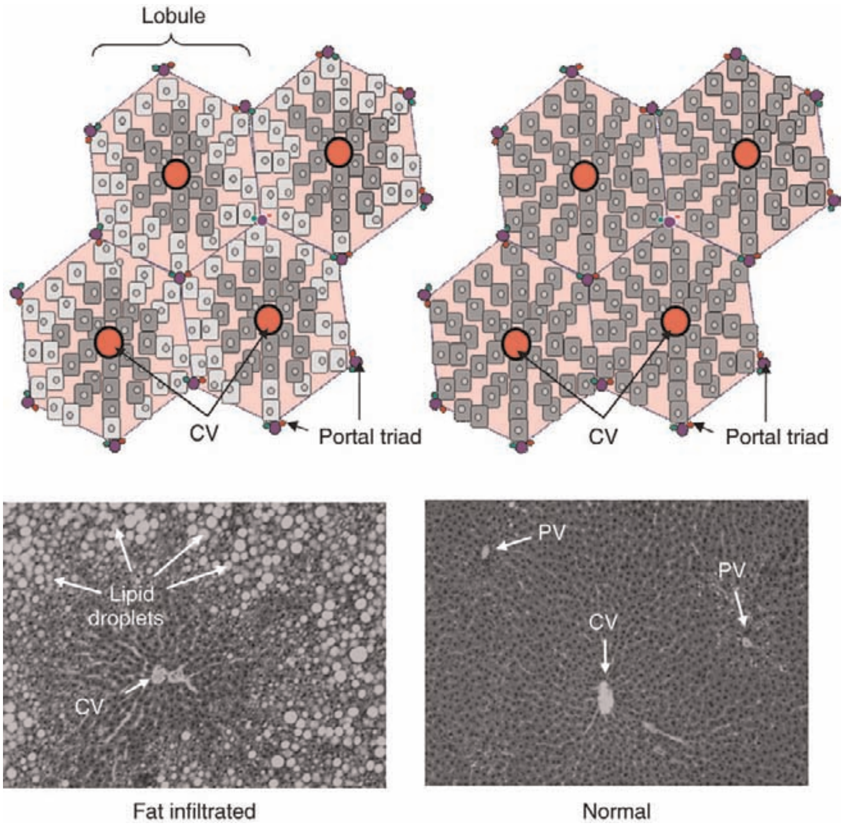

Figure 2 Arrangement of hepatocytes within the liver lobule and association of lobules. Plates of hepatocytes converge toward the central vein (CV) within lobules. The peripheral zone of lobules is demarcated by the portal triads, which consist of a branch of the hepatic artery, hepatic portal vein (PV), and a bile ductule. Blood flows from the portal triad along sinusoids between liver cells and is collected in the central vein (upper panel). Lipid infiltration is characterized by accumulation of lipid droplets first within cells in the periportal zone of the liver lobule and then extending toward the central vein (lower panel).

gastrointestinal tract. As a consequence, there is a marked functional and histological heterogeneity among cells that are located close to the portal vein (periportal hepatocytes) and cells located close to the central vein (pericentral hepatocytes) of the liver acinus. Within the liver acinus of nonruminants, betaoxidation of fatty acids, amino acid catabolism, ureagenesis, gluconeogenesis for the synthesis of both glucose and glycogen, cholesterol synthesis, and bile formation are predominantly located in the periportal zone, whereas glycolysis, glycogen synthesis, ketogenesis, glutamine formation, and metabolism of toxins are preferentially situated in the pericentral zone. Although the partitioning of biochemical functions within the microstructure of bovine liver has not been determined, it is reasonable to assume a heterogeneity in bovine that is similar to other species.

Hepatic steatosis (fatty liver) is accompanied by many changes in the hepatic structure including compression of the hepatic sinusoids, mitochondrial damage, and decreased volume of the rough endoplasmic reticulum. Microscopic examination of liver tissue indicates the presence of fat droplets within hepatocytes. The development of fatty liver associated with obesity, alcoholism, and diabetes in humans results in the accumulation of fat in cells in the periportal region of the liver, whereas the onset of acute fatty liver of pregnancy in humans results in fat deposition within pericentral hepatocytes. An accumulation of fat droplets proximal to the central vein has been noted during the development of fatty liver associated with calving in dairy cows.

Decreased gluconeogenic capacity is a notable change that accompanies the fatty liver *in vivo*. A reduction in gluconeogenesis from propionate is also observed in bovine hepatocytes that are induced to be lipid filled in culture by incubation with oleic acid. Ammonia is toxic to mammalian cells, and one of the main functions of the liver is to detoxify ammonia to urea for excretion by the kidney. Bovine hepatocytes loaded with TG have reduced capacity to detoxify ammonia, leading to reduced capacity for other cellular functions including gluconeogenesis. Under normal conditions, the synthesis of glutamate in the liver acts as a high-affinity low-capacity scavenger system to detoxify ammonia that escapes ureagenesis. Pericentral cells of the liver display the highest rates of glutamate synthesis and serve as a backup system to the removal of ammonia that is not sequestered as urea in periportal hepatocytes. There appears to be little adaptation in urea synthetic capacity in the liver of transition dairy cows and the positive relationship between liver TG content, blood ammonia, and glutamine concentrations in transition cows, suggesting that the capacity for ammonia detoxification is overwhelmed. These findings have important implications with regard to overfeeding protein during late gestation in cows that are at risk for fatty liver due to overconditioning. The heterogeneity of fat deposition and metabolic activities within the liver acinus coupled with the progression of fatty liver in the central regions of the liver leads to a sequence of events that have progressive adverse effects on liver function and animal health.

Prevention and Treatment of Fatty Liver

Several management practices are recognized that minimize the incidence and severity of fatty liver. Feeding practices that limit the rate and degree of fatty acid mobilization from adipose tissue, reduce the esterification of NEFAs as TG in liver, increase the export of TG from liver, and increase the oxidation of fatty acids all act to decrease fatty liver in dairy cows. It is recognized that strategies to reduce lipid accumulation in the liver must be implemented before calving in order to reduce the severity and incidence of lipid accumulation in the liver.

Reducing the severity of feed intake depression at calving decreases the severity of fatty liver and tends to increase milk production. There has been a concerted effort toward improved feeding management strategies for transition dairy cows. Supplying adequate energy by increasing the energy density of transition cow diet may act to counter the effects of feed intake depression at calving. Circulating NEFAs are increased during late pregnancy due to depressed intake at calving, a diminished responsiveness to insulin in adipose tissue, and the subsequent mobilization of adipose tissue. Cows that experience health problems after calving had 18–20% lower prepartum intakes. When intake was maintained during the transition period by applying a force feeding protocol to rumen-fistulated cows, the increase in NEFAs at calving was reduced in magnitude but was not eliminated.

Recent experiments indicate a lack of response to increased protein density or type in the prepartum diet of multiparous cows; however, heifers approaching first calving may require additional protein for mammary development. Feeding excessive protein to prepartum cows may be detrimental due to the impaired capacity to detoxify ammonia.

Increasing the energy density of the prepartum diets is beneficial in increasing liver glycogen content but not in reducing liver lipid. The ratio of TG to glycogen in the liver appears to be a predisposing factor for ketosis; therefore, increasing glycogen indirectly reduces the effects of fatty liver. Diets containing more energy may not reduce fatty liver but instead allow the cow to better cope with the condition. Feeding diets that have greater fermentative capacity in the rumen, such as steam-flaked corn, acts to decrease NEFA concentrations in the blood prior to calving and increase subsequent milk production. Greater energy intake will likely lead to physiological changes, such as increased insulin concentration, that reduce the mobilization of NEFAs from adipose tissue and promote glycogen storage in liver. Diets containing 1.62 Mcal of net energy of lactation (NEL) per kg are recommended, but energy content should be increased gradually beginning 2–3 weeks prior to calving to avoid adverse effects on rumen fermentation or predispose cows to other health disorders.

Several compounds have been investigated for their ability to diminish the incidence and severity of fatty liver. Niacin feeding decreases blood ketones but does not appear to reduce liver TG content. Increasing the supply of precursors for glucose synthesis in liver using propylene glycol reduces liver TG. Part of the response to propylene glycol may be a consequence of increased blood insulin, reduced circulating NEFA levels, and increased liver glycogen. Ionophores, which act to alter rumen metabolism in favor of propionate production, appear to reduce the plasma ketones and subclinical ketosis under experimental conditions.

Phosphatidylcholine, the major lipid component of the VLDL surface, may be limiting for VLDL assembly under some conditions in ruminants. Dietary choline deficiency accelerates ApoB degradation and decreases VLDL synthesis. Because choline is extensively degraded in the rumen and because choline and other methyl donors are critical to VLDL synthesis, the addition of rumen-protected choline has proved beneficial for transition dairy cows. Rumen-protected choline acts to reduce circulating NEFA concentrations and reduce the severity of liver lipid accumulation. Likewise, rumen-protected choline appears to enhance the rate of lipid clearance in cows that have been experimentally induced to express fatty liver. Choline is also necessary for carnitine synthesis, a necessary component of the translocation of fatty acids across the inner mitochondrial membrane mediated by carnitine palmitoyltransferase. Carnitine increases fatty acid oxidation in the bovine liver *in vitro*. Infusing carnitine into the abomasum of lactating dairy cows decreases plasma NEFA concentrations, leading to questions regarding the adequacy of carnitine and choline supply for fatty acid oxidation. Increasing the postruminal supply of L-carnitine reduces plasma NEFA concentrations and triglyceride accumulation in the liver by increasing hepatic oxidation of NEFAs. Feeding carnitine decreases total liver lipid in transition dairy cows, increases liver glycogen content in early lactation, and increases *in vitro* palmitate β-oxidation by liver slices, an indicator of hepatic capacity for fatty acid oxidation.

Hormonal interventions have been explored as a means of reducing liver lipids in dairy cows. Under normal conditions, plasma concentrations of somatotropin rise during late pregnancy, with a distinct peak at parturition and a decline to slightly elevated levels in early lactation. Somatotropin decreases the activity of key lipogenic enzymes in adipose tissue, apparently by opposing tissue response to insulin. Somatotropin also increases hepatic gluconeogenesis by activation of expression of the phosphoenolpyruvate gene, a pace-setting enzyme for gluconeogenesis in liver. Prepartum treatment with somatotropin increases plasma glucose and glucose disposal, and decreases plasma NEFAs prior to calving but does not alter liver TG content.

The use of exogenous glucagon also holds promise as a therapy for fatty liver in dairy cows. Glucagon acts directly on the liver to increase gluconeogenesis. Experimental glucagon infusions in dairy cows decreased liver TG, increased blood glucose, and decreased blood ketones. Because the clearance of glucagon from blood is relatively rapid, the use of glucagon as a practical therapy for fatty liver will require a sustained delivery method. Treatment of transition cows with 15 mg glucagon per day reduces liver lipid content and decreases some of the detrimental effects of fatty liver on health and reproduction of dairy cows. Some of these effects are due to the ability of glucagon to simulate hepatic capacity for ammonia detoxification and gluconeogenesis through changes in the expression of key genes in these metabolic pathways.

Although the balance of fatty acid metabolism and fatty acid storage as triglyceride is an essential component of fatty liver, it has been recognized recently that the profile of fatty acids presented to the liver may play a crucial role in determining the progression of this condition. The percentages of fatty acids within the liver are dramatically altered during adipose tissue lipolysis and these differences are more pronounced in cows that develop fatty liver. Likewise, the profile of fatty acids released from adipose tissue differs in cows that experience fatty liver. Recent evidence suggests a relationship between total liver lipid and fatty acid profile, in particular, an inverse relationship between long-chain unsaturated fatty acids and liver triglycerides has been demonstrated. Supporting data demonstrate that dry cows fed high levels of palmitic and oleic acid have increased hepatic fatty acid oxidation and reduced liver lipid in early lactation. The profile of fatty acids appears to be critical in this regard and has led to the introduction of feeding strategies that initiate metabolic priming in order to permit cows to cope with body fat mobilization in early lactation. Although the mechanism of action of fatty acids as regulators of metabolism in ruminants is not completely characterized, it is likely to involve effects of specific fatty acids as controllers of gene promoters for enzymes in liver that catalyze key metabolic processes related to fatty acid oxidation and hepatic energy metabolism.

New Directions and the Role of 'Omics' in Understanding Fatty Liver

Past approaches to understanding fatty liver have relied heavily on a reductionist approach, which is best exemplified by the focus on a causative dominant factor. In some cases, these have been linked to simple biomarkers such as reduced feed intake, increased mobilization of adipose tissue, and elevated blood NEFA levels. An implicit assumption in most studies is that fatty liver has a unique target, which would be most suitable for intervention. Although there are several examples where this reductionism has been tremendously helpful in understanding the etiology of fatty liver, there are other examples where this has not been the case. The advent of tools for genomics, proteomics, and metabolomics has provided an opportunity to simultaneously assess factors that at first glance effectively supersede the reductionist approach to understanding biology; however, when used effectively, these tools provide a greater opportunity to integrate the biology of the dairy cow on several levels with an ultimate goal of optimizing performance and minimizing the occurrence of metabolic diseases including fatty liver.

The application of 'omics' tools to understanding the biology of fatty liver has been limited, but shows potential in helping to unravel this complex condition. Temporal transcript profiling using genomics tools that permit simultaneous analysis of over 6000 gene products in the liver of dairy cows indicates that fatty liver is linked to 85 genes that code for key enzymes associated with hepatic fatty acid oxidation, gluconeogenesis, and cholesterol synthesis. Currently, there are only a handful of reports of proteomic analysis of fatty liver in dairy cows, but these data identify downregulation of several enzymes involved in β-oxidation, an increase in enzymes associated with ketogenesis, a reduction in enzymes associated with prevention of lipid peroxidation, and changes in lipid signaling molecules as well as key enzymes of gluconeogenesis, calcium homeostasis, and protein metabolism. Unfortunately, application of metabolomics to the study of fatty liver in cattle and other species has lagged behind genomics, but will be critical in validating the suspected control points in metabolism that are perturbed during the onset and progression of fatty liver. Regardless of the tools used to study this pathology, it is important to recognize that a complete cycle of hypothesis generation and testing is necessary to provide a complete understanding of the development, progression, and consequences of fatty liver in dairy cattle.

Conclusion

Fatty liver is characterized by the deposition of lipid to approximately 3 times the normal concentrations. The incidence of fatty liver is greatest in the periparturient dairy cow, and more than 50% of all cows experience mild to severe fatty liver. A major factor leading to fatty liver is the inherent lack of ability of the liver of ruminants to export TG as VLDL. As a consequence, the progression of lipid deposition within the microstructure of liver leads to a progressive impairment of specific hepatic functions. These impairments are manifested as ketosis, reduced health, reduced productivity, impaired immune function, and increased risk of health disorders. The incidence and severity of fatty liver may be managed best using strategies that act in combination to limit adipose tissue mobilization at calving, enhance lipid oxidation in liver, and enhance release of lipid from liver for use by other tissues.

See also: **Analytical Methods**: DNA-Based Assays. **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Diseases of Dairy Animals**: Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Pregnancy Toxemia.

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Non-Infectious Diseases: Grass Tetany

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Introduction

Magnesium is an essential mineral and is the fourth abundant cation in the body. Mg is required for the activation of enzymes such as ATPases and kinases, RNA, DNA, synthesis of proteins, regulation of membrane channels, and modulation of synaptic transmission. Hence, Mg deficiency causes a variety of symptoms including neurological disorders such as hypomagnesemic tetany in ruminants. This disease has been observed in goats, sheep, beef cattle, and, predominantly, high-producing dairy cows in all countries with intensive agriculture production since the 1920s. The decrease in blood Mg concentration is caused either by inadequate intake of Mg with diet (deficiency) or (mainly) by reduced absorption of Mg from the gastrointestinal tract (GIT) and high Mg output via milk.

This article describes the clinical symptoms, etiology, and pathogenesis of hypomagnesemia and grass tetany and possible measures of prophylaxis. Furthermore, information on the role of Mg in the pathogenesis of milk fever is also provided.

Clinical Symptoms and Occurrence

Hypomagnesemia causes a wide range of clinical symptoms such as reduced feed intake, uncertain gait, grinding of the teeth, salivation, ataxia, recumbency, convulsions, and finally tetanic muscle spasms. Recumbent animals with severe seizures and opisthotonus exhibit high mortality without treatment. The clinical symptoms, which are often induced by noise or stress, are triggered by a decrease in the Mg concentration in the cerebrospinal fluid ($<0.7\,\mathrm{mmol\,l^{-1}}$); the inhibitory effects of Mg on synaptic transmission are reduced in the central nervous system facilitating uncontrolled excitations. Animals with blood Mg concentration of $<0.7\,\mathrm{mmol\,l^{-1}}$ are hypomagnesemic and are at risk for tetany at blood Mg concentrations $<0.5\,\mathrm{m\,mol\,l^{-1}}$ because Mg in the cerebrospinal fluid cannot be maintained at $>0.7\,\mathrm{mmol\,l^{-1}}$ at this blood concentration ($<0.5\,\mathrm{mmol\,l^{-1}}$).

Reports concerning grass tetany have been published in many countries around the world. However, consistent epidemiological data on the actual frequency of clinical cases (tetany) or hypomagnesemia without clinical signs are still lacking. Most recent publications are case reports about sudden outbreaks of this disease in one herd, often with a high incidence of hypomagnesemia or dead cows. Nevertheless, some conclusions are possible. The high incidence reported in the 1950s and 1960s is historical. The actual risk is probably <<1%, with some exceptions under special conditions such as those in New Zealand or when lactating cows are kept on grass. Hypomagnesemia without clinical signs is much more frequent and, in many cases, is not detected because possible symptoms such as a decrease in milk yield or food intake are highly unspecific and rarely directly related to hypomagnesemia. Hence, hypomagnesemia without overt clinical signs is often detected only incidentally. However, in carefully directed studies, a decrease in blood Mg concentration of 10% or higher has been observed.

Etiology

Regulation of Mg Homeostasis

Magnesium homeostasis is not regulated by a hormonal feedback system like that for Ca but can be influenced by hormones such as insulin, noradrenaline, and adrenaline. These hormones induce a shift of Mg from the extracellular space (ECS) into the intracellular space (ICS); this can contribute to acute hypomagnesemia (transport tetany) or can exacerbate existing hypomagnesemia.

Since Mg homeostasis is not regulated by hormones, blood Mg (0.75–$1.10\,\mathrm{mmol\,l^{-1}}$) depends, under steady-state conditions, on the influx of Mg from the GIT into the ECS including blood (a) and on the efflux from ECS into the milk (most important; b), into the ICS including bones (c), into the intestine (endogenous losses; d) and, via the kidney, into urine (e). Hence, blood Mg concentration is constant according to this simplified scheme if $a = b + c + d + e$. Because c and d are relatively constant and low in adult animals, a constant blood Mg concentration primarily depends on absorption from the gut and demand for milk production and is finally regulated by the kidneys, which excrete surplus Mg (influx − efflux > requirement) with urine. The lability of the steady state of blood Mg concentration can easily be seen from the finding that the Mg pool in the ECS of a cow is 2–3 g. Because the concentration of Mg in milk is kept constant at 120–$150\,\mathrm{mg\,l^{-1}}$ independent of the blood Mg concentration, 4.8–6.0 g Mg or more than the amount of Mg in the ECS is solely required for milk production at

40 l day^{-1}. Because of the lack of hormonal regulation, the relatively large Mg pools in the ICS (150 g) or bones (~270–280 g; cow body weight 650 kg) cannot be acutely mobilized for maintaining physiological blood Mg concentrations. Therefore, the blood Mg concentration can be kept constant only when the daily Mg requirement, especially for milk production, is met by an adequate absorption of Mg from the GIT. A high Mg requirement for milk production may lead to hypomagnesemia at reduced Mg absorption from the GIT. Hence, a precise knowledge about the absorption of Mg from the GIT and its possible impairment appears to be a key factor in understanding hypomagnesemia.

Mg Absorption from the Gastrointestinal Tract

Digestible Mg

Digestible Mg has been estimated in a meta-analysis in lactating dairy cows (Holsteins) and was found to increase linearly with Mg intake within the range 40–80 g day^{-1}: digestible Mg (g day^{-1}) = 2.5 (±3.9) + 0.14 (±0.06) × g Mg intake. The values in parentheses are the standard errors of the coefficients. Within this range of intake (40–80 g day^{-1}), the apparent absorption of Mg is generally low at 20%, which can be further reduced to less than 10% by dietary components. Reduced apparent absorption is not caused by increased endogenous secretion of Mg into the GIT; this secretion is considered to be relatively constant and low (3–4 mg kg^{-1} body weight) and, hence, apparent absorption is a reliable estimate of true absorption. Most of the endogenously secreted Mg is probably recycled into the GIT via saliva with a Mg concentration of 0.30–0.40 mmol l^{-1}. The large volume of saliva in ruminants (>200 l day^{-1} in cows) causes a daily drain of Mg of approximately 50% in the ECS.

Reduced Mg absorption

An inadequate absorption of Mg from the GIT can be caused by (1) low Mg intake (Mg deficiency), (2) impairment of Mg absorption, or (3) a combination of these factors.

Mg deficiency

Mg deficiency is one cause of hypomagnesemia and, in many cases, the reason for the so-called winter tetany, which occurs when the animals are kept under harsh and poor conditions with low energy and low Mg intake. The low energy intake decreases the fermentation rate and hence the number and size of rumen papillae and absorptive surface, which depresses the absorption rate of Mg and exacerbates low Mg intake. Mg deficiency can be proposed when animals are kept on grass of poor quality (during the winter) or on grass heavily fertilized with K because K reduces Mg uptake by plants.

Impairment of Mg absorption

Mg absorption can be disturbed by dietary factors. Understanding of these effects has significantly improved because of our knowledge of (1) the site of Mg absorption, (2) the mechanism of Mg absorption, and (3) factors that change Mg absorption.

Site of Mg absorption: Studies of the flow rates of nutrients and minerals along the GIT of ruminants have led to the conclusion that Mg absorption occurs before the duodenum and is essential for maintaining normal blood Mg concentration as bypassing the rumen causes hypomagnesemia. The remaining GIT (the small and large intestine) does not or may only weakly contribute to total Mg absorption. Absorption of Mg from the hind gut is possible but requires high concentrations of Mg in the colon.

Mechanism of Mg absorption in the rumen: *In vivo* and *in vitro* experiments have demonstrated that Mg is transported across the rumen epithelium by an active mechanism. Paracellular and passive movements of Mg are of minor importance and can be neglected. The transcellular movement of Mg includes uptake across the luminal membrane and extrusion across the basolateral membrane (**Figure 1**).

Uptake across the luminal membrane is mediated by two mechanisms.

1. Mg^{2+} uptake (as ion) is driven by the potential difference of the apical membrane, PD_a. The high intracellular K concentration causes an efflux of K across channels in the luminal membrane thereby producing an intracellular negative PD_a of –40 to –50 mV. An increase of ruminal K concentration reduces K efflux and PD_a and, thus, the driving force for Mg^{2+} uptake. Consequently, this mechanism is called PD-dependent or K-sensitive Mg uptake, because PD_a is mainly modulated by the ruminal K concentration. The relationship between ruminal K and PD_a is described by the equation $PD_a = -82.8 + 21.3\lg[K]$. The logarithmic scale of the *x*-axis (K concentration) implies that an increase of the ruminal K concentration from 10 to 50 mmol l^{-1} causes a depolarization of PD_a by 15 mV (i.e., from –50 to –35 mV), whereas the same $\Delta[K]$ of 40 mmol l^{-1} from 50 to 90 mmol l^{-1} leads to a further decrease of PD_a of only 6 mV. This means that the PD-dependent or K-sensitive Mg uptake is impaired far more by an increase in K intake from low (1% K in dry matter of the diet) to medium (3% K) than from medium (3% K) to high (>4% K; see below).
2. Since the intracellular Mg concentration (0.8–1.2 mmol l^{-1}) is lower than in the rumen fluid (2–4 mmol l^{-1} or more), the chemical gradient of Mg contributes to the total driving force (electrical and chemical gradient) for the luminal uptake of Mg^{2+}.

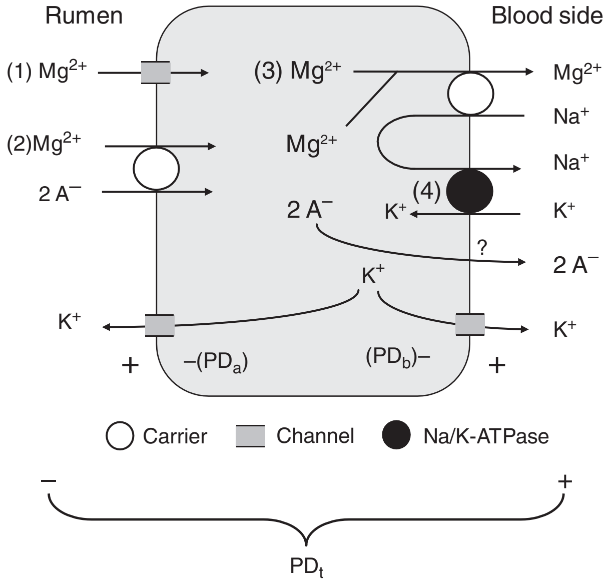

Figure 1 Scheme of ruminal Mg transport. (1) Mg^{2+} uptake (as ion) is driven by the potential difference of the apical membrane, PD_a, which depends on the K gradient across the luminal membrane. An increase of ruminal K concentration reduces K efflux and PD_a, and thus the driving force for Mg^{2+} uptake. (2) The second luminal uptake mechanism is a cotransport system, in which anions are transported together with Mg neutralizing the Mg^{2+} cations. (3) Basolateral extrusion of Mg is mediated by Na/Mg exchange. (4) Na that enters via this mechanism is pumped out of the cell via Na/K-ATPase. PD_a, potential difference of the luminal membrane; PD_b, potential difference of the basolateral membrane; PD_t, transepithelial or transmural potential difference (blood side positive). PD_t, is the difference of PD_a and PD_b: $PD_t = PD_a - PD_b$. A representative example of PD_t for sheep rumen epithelium is

$$PD_t = PD_a\ (-40\,\text{mV}) - PD_b\ (-55\,\text{mV})$$

$$PD_t = +15\,mV$$

Hence, a decrease of PD_a at high ruminal K concentration (see text) causes an increase of PD_t and a decrease of Mg uptake via (1), which leads to a reciprocal correlation between Mg absorption and PD_t.

Basolateral extrusion of Mg is mediated by Na/Mg exchange. Na influx is driven by Na gradient (extracellular >> intracellular) and is used for Mg extrusion. Na is pumped out of the cell across the basolateral membrane via Na/K-ATPase, which consequently energizes Mg transport indirectly as a secondary active transport mechanism (**Figure 1**). The stoichiometry of Na/Mg exchange is not known (probably 2 Na for 1 Mg). However, the Na concentration in the rumen is not linked to transcellular Mg transport.

This putative scheme of Mg is supported by many *in vitro* and *in vivo* observations. Nevertheless, convincing evidence has been presented for a second uptake mechanism that can be deduced from Mg balance data *in vivo*. Mg absorption is mainly reduced by an increase of K concentration in the diet from 1% in dry matter to 2 or 3%. A further increase causes only small effects or even levels off. The remaining Mg absorption clearly hints at a K-insensitive or PD-independent mechanism. Confirming evidence has been obtained from *in vitro* studies with isolated sheep rumen epithelia. A fraction of transcellular Mg transport is independent of alterations in PD_a and additional characterization of Mg transport has led to the proposal of a Mg cotransport system with anions as the second luminal uptake mechanism (**Figure 1**). Here, the anions are transported together with Mg, thereby neutralizing the Mg^{2+} cation. This cotransport solely uses the chemical gradients of the participating ions for luminal Mg uptake and not PD_a: PD-independent or K-insensitive Mg uptake.

The kinetic data (K_m and V_{max}) of the two parallel working uptake mechanisms are not known but an assessment of the driving forces shows that the PD_a-dependent mechanism has the capacity for Mg uptake even at ruminal Mg concentrations <1 mmol l^{-1}. A similar calculation for the PD-independent mechanism is not possible because of missing data with regard to the gradients of the anion; however, the ruminal [Mg] must be definitely higher. Hence, the two uptake mechanisms ensure Mg absorption over a wide range of ruminal Mg concentrations, at low and high Mg intake. This 'job sharing' of Mg uptake (high affinity/low capacity for low Mg concentrations, and low affinity/high capacity for high Mg concentrations) guarantees Mg absorption from the rumen at almost all physiological Mg concentrations as a prerequisite for the homeostasis of Mg metabolism.

Impairment of Mg absorption by dietary factors: The early study of Sjollema showed that grass can cause tetany despite a sufficient Mg content, when K and nitrogen concentrations are high and [Na] is low. K, N, and Na concentrations can clearly be related to disturbed Mg absorption.

The role of potassium: If the above-mentioned proposal with respect to 'job sharing' of the two Mg uptake mechanisms is correct, three consequences regarding possible effects of K can be predicted. (1) An increase of K at low Mg intake should reduce Mg absorption to a large extent, because Mg absorption at low ruminal Mg concentration primarily depends on PD-dependent or K-sensitive uptake. (2) An increase of K intake in addition to high basal K content in the diet would be probably without any effect or have only small effects on Mg absorption (see above: effect of K on PD_a). (3) An increase of Mg intake could compensate for the possible negative effects of high K intake, because the PD-independent or K-insensitive uptake is mainly active at high ruminal Mg concentrations. These assumptions are indeed confirmed by field and experimental observations.

Potassium is used as an artificial fertilizer and the growth rate of grass increases almost linearly with K content in the dry matter between 1 and 3%. K reduces Mg uptake by plants and an almost reciprocal correlation exists between K and Mg concentrations. The high content of K in grass and other crops leads to high oral intakes of K far above the daily requirement (1% K in dry matter). The rumen K concentration is linearly correlated with K intake and can increase to 100 mmol l^{-1}. Consequently, the increasing ruminal K concentrations depolarize PD_a and reduce PD-dependent or K-sensitive Mg uptake, which is pronounced at low rumen Mg concentration. Conversely, the negative effects of K on Mg absorption decrease proportionately with increasing Mg intake and Mg concentration in the rumen fluid. Hence, any approach for predicting digestible Mg must include the negative effect of K and the positive effect of increasing Mg intake. The equation is as follows:

Digestible Mg $= 4.5\ (\pm 4.0) + 0.24\ (\pm 0.07) \times$ Mg intake (g day^{-1})$-4.4\ (\pm 2.2) \times$ K (% dry matter). This means that, at 1% K, digestible Mg is 0.24, regardless of Mg intake, which confirms the proposed Mg transport via two mechanisms. At a low K intake (1%), the ruminal K concentration is low and hence the PD-dependent Mg transport is hardly disturbed and the PD-independent Mg transport is proportional to the Mg concentration. Furthermore, the equation predicts that, with an increase of K, for example, from 1 to 2%, the intake of Mg must be increased by 18.3 g day^{-1} (**Table 1**).

Na deficiency: Young spring grass often has a low Na content that does not cover Na requirement and hence Na deficiency has been reported in grazing cows and sheep. Na deficiency causes, via activation of aldosterone, a reciprocal exchange of Na with K in saliva, which leads to a high inflow of K into the rumen and K concentrations in the rumen fluid of >100 mmol l^{-1}. Daily saliva secretion of 200 l containing 100 mmol l^{-1} K amounts to 800 g K, which equals the K intake in 25 kg dry matter with 3.2% K. The negative consequences of Mg absorption are identical to an increase of K intake and, unfortunately, Na deficiency is still an overlooked factor in the pathogenesis of grass tetany, particularly in grazing animals such as beef cattle. Na intake far above requirement causes increased flow of urine and diuretic urinary Mg losses. Na deficiency as a risk factor for tetany has nothing to do with K intake. Although dietary sodium is important, the often discussed Na/K ratio is without any physiological basis.

Nitrogen: Grass prone to producing tetany frequently shows high N concentrations. Rumen ammonia has been suggested as a possible factor for impairing Mg absorption. However, classical balance studies with increasing N intake and ruminal ammonia concentrations have not led to any change in digestible Mg. On the other hand, a sudden increase of ruminal ammonia in acute experiments causes a concentration-dependent decrease of Mg absorption from the rumen. This contradiction has been solved by an experiment in which the urinary excretion of Mg has been determined after a sudden change in ruminal ammonia concentration from 5 to 48 mmol l^{-1}. The increase of ruminal ammonia concentration causes a transient decrease of urinary Mg excretion (probably caused by disturbed absorption) for only 2 days. At day 3 and later, urinary Mg is normalized and not different from the excretion before the ammonia load. Therefore, it is concluded that ammonia has an acute negative effect on Mg absorption. Obviously adaptation occurs. Neither the mechanism of the effect of ammonia on Mg absorption nor the mode of adaptation is known.

Fermentable carbohydrates: An increase of energy intake stimulates Mg digestibility. The underlying mechanisms

Table 1 K content of diet and its effect on required Mg intake for a cow with a body weight of 700 kg and with increasing milk production[a]

			Mg intake (g day^{-1}) depends on K content		
Milk yield (kg day^{-1})	*Mg in milk (g day^{-1})*	*Mg requirement (g day^{-1})*	*1% K*	*2% K*	*3% K*
0	0	2.8	11.3	29.6	47.9
20	3.0	2.8	23.8	42.1	60.4
40	6.0	2.8	36.3	54.6	72.9
60	9.0	2.8	48.8	67.1	85.4

[a] Mg requirement for maintenance and milk production is 2.8 g day^{-1} and 0.15 g Mg l^{-1} milk, respectively. The calculation was made using the following equation: Digestible Mg $= 4.5\ (\pm 4.0) + 0.24\ (\pm 0.07) \times$ Mg intake $- 4.4\ (\pm 2.2) \times$ K. Mg intake is in g day^{-1} and K content in % of DM. It must be emphasized that the equation was derived with K concentration in the range of 1–3% and is probably not correct for higher K concentrations. Please take into consideration the large standard errors of the coefficients.

Weiss WP (2004) Macromineral digestion by lactating cows: Factors affecting digestibility of magnesium. *Journal of Dairy Science* 24: 2167–2171.

are not clear because increased fermentation lowers the ruminal pH and the ammonia concentration, and increases the Mg solubility and short-chain fatty acid concentrations. Low energy intake is most probably involved in the pathogenesis of winter tetany, especially under other comparable harsh feeding conditions. Suggestions can be made with regard to the role of energy intake in classical tetany. The onset of classical tetany is characterized by reduced feed intake, uncertain gait, and other symptoms. Fermentation rates are probably decreased and accompanied by an increase of pH and a remarkable decrease of Mg solubility at $\mathrm{pH} > 7.0$. Combined with the reduced feed and Mg intake, decreased Mg absorption can be anticipated with negative effects on blood Mg concentration.

Role of kidneys in Mg homeostasis

The kidneys play a major role in Mg homeostasis, because any surplus of Mg between influx into and out of the ECS is excreted with the urine. Blood Mg concentrations above 1.1–1.3 $\mathrm{mmol\,l^{-1}}$ are accompanied by high concentrations of Mg in the urine and, conversely, a sharp decrease is observed at blood Mg $<1.00\,\mathrm{mmol\,l^{-1}}$ and a further decrease to $<1.0\,\mathrm{mmol\,l^{-1}}$ Mg in urine is noticed at hypomagnesemia ($<0.70\,\mathrm{mmol\,l^{-1}}$). Measurement of total Mg excretion is rarely possible, although Mg at $>2.5\,\mathrm{g\,day^{-1}}$ is considered as satisfactory and urinary Mg of $<1\,\mathrm{g\,day^{-1}}$ is closely related to hypomagnesemia. An important finding is that urinary Mg concentrations are significantly influenced by renal water excretion, which could be very large at high K intake.

Prevention and Treatment

A successful prevention of hypomagnesemia should include reduction or elimination of possible risk factors. For practical reasons, a reduction in K intake is hardly possible. Hence, an adequate increase of Mg intake is necessary according to K concentration (**Table 1**). Na deficiency can easily be avoided by mixing Na into the concentrate. The acute and negative effect of ammonia on Mg absorption can be prevented by a stepwise increase of N intake and/or by offering easily fermentable carbohydrates. These measurements are common under normal feeding conditions of dairy cows and the major reason nowadays for the low rate of clinical cases of tetany in cows. Conversely, the reduction of concentrate intake in dairy cows because of the political restriction of milk production by milk quota is the probable reason for the sudden increase of hypomagnesemia and tetany in cows in England.

Successful prevention of hypomagnesemia in grazing animals without regular supplementation of concentrates is much more difficult to achieve. Na lick stones combined with Mg are practicable as they are voluntarily accepted by cows, particularly at Na deficiency. Mg supplementation alone is refused even in animals with hypomagnesemia because of the lower palatability of Mg salts or MgO. Other measures such as pasture dusting with Mg salts or Mg supplementation with drinking water have been successfully used especially for grazing cattle. Mg bullets or drenches are a useful help for transient supplementation.

Clinical tetany is treated by intravenous or subcutaneous infusion of Mg (200–400 ml magnesium and calcium borogluconate) or, as ‘first aid’, by rectal application of Mg (60 g $\mathrm{MgCl_2 \cdot 6H_2O}$ in 200 ml water) by using the absorptive capacity of the colon for Mg.

Magnesium and Milk Fever

Hypomagnesemia impairs the regulation of Ca concentration in blood. The release and synthesis of parathyroid hormone (PTH) and the binding of PTH to its receptor require Mg. Hence, the signal cascade of Ca homeostasis is repeatedly disturbed by hypomagnesemia, which, in cows, reduces the capacity for Ca mobilization from the bone and increases the risk of milk fever. Assessment of Mg status from the blood Mg concentration is not satisfactory because surplus of Mg is excreted with urine and the blood Mg concentration can be kept within the normal range even under a brief shortage of Mg intake/absorption. However, parturition causes a decrease in blood Ca concentration, which stimulates PTH excretion. As PTH changes the renal threshold for Mg excretion, a decrease in blood Ca concentration is accompanied by an increase in blood Mg concentration. If this increase of blood Mg is not observed ($<0.8\,\mathrm{mmol\,l^{-1}}$ Mg), a shortage of Mg can be suggested. The results of a meta-analysis of milk fever are in agreement with these conclusions. A Mg content of 0.3–0.4% in dry matter reduces the incidence of milk fever. This concentration is above the recommended requirement of 0.2% Mg in dry matter, but cows reduce their voluntary feed intake before and especially on the day of parturition, and the high Mg content could compensate for this decrease of feed (and Mg) intake.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever.

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Non-Infectious Diseases: Ketosis

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Introduction

Clinical ketosis is the ultimate expression of the difference between milk production and the capacity of body tissue reserves and feed intake to provide the metabolic fuels for that production. The condition provides substantial insights into intermediary metabolism in the cow and is associated with many of the important disease conditions of dairy cattle. This article examines the epidemiology, biochemistry, prevention and treatment of ketosis.

Definitions

Clinical ketosis refers to the condition in which cattle typically have lower milk production, marked weight loss and low dry-matter intake. Cows with no other detectable or anteceding disorder are defined as having primary clinical ketosis, whereas cows with an anteceding or concurrent disorder are defined as having a secondary ketosis.

Cows with subclinical ketosis have similar biochemical changes to clinical cases, but maintain appetite.

Epidemiology

Careful studies report lactational incidence rates for clinical ketosis between 2% and 15% of adult cattle. Estimates of prevalence of elevated ketone concentrations in blood (ketonemia) or milk samplings (ketolactia) suggest a prevalence in the range of 7–20%, with some estimates as high as and exceeding 36%. These estimates vary with the threshold concentrations of ketones used to define the condition. Estimates of the heritability of ketosis and subclinical ketosis vary widely between a heritability of 0 and 0.31. Continued selection for milk production may increase the incidence of ketotic conditions.

The immediate periparturient period is that of greatest risk for ketosis; approximately 90% of all ketosis cases, clinical or subclinical or treatments occur within approximately 60 days postcalving. Incidence of ketosis increases with age and peak incidence may be in cattle in lactations 3–6. The occurrence of ketosis has been noted to vary markedly between herds. Higher-producing cows may be at more risk of ketosis and diets that are low in energy and higher in protein increase the risk of ketosis. The following increase the risk of ketosis: retained placenta, low milk yield in the last 120 days of the previous lactation and an extended dry period, parturient paresis, obesity at calving (body condition score >3.5/5), higher milk yields in the previous lactation, displaced abomasum, foot and leg conditions and hypomagnesemia. A large number of other conditions such as traumatic reticuloperitonitis have been associated with ketosis, but these are not part of the group of interacting periparturient disorders of the cow. Cows with ketosis have a greater risk of developing displaced abomasum, infections of the reproductive tract, mastitis, cystic ovarian disease, diseases of the digit and foot and leg problems and repeat breeding. Ketolactia has been associated with increased risk of metritis, clinical ketosis and cystic ovarian disease.

Blood ketone concentrations reflect the balance between hepatic production and peripheral utilization. Ketone concentrations decrease subsequent to feeding. The significance of elevated ketones is not clear as hyperketonemia is a normal physiological response to lactational demands. The values presented as normal do not, therefore, necessarily relate to increased risk for production loss via disease or milk loss but rather to a range of expected values. Recent studies from Canada suggest that an upper limit for normal β-hydroxybutyrate concentrations is $<1.4\,\mathrm{mmol\,l^{-1}}$ (approximately <15 mg $\mathrm{day^{-1}}$) for lactating cows and that concentrations exceeding this are associated with lower milk production.

Clinical Presentations of Ketosis

Cows with clinical ketosis typically have weight loss, decreased dry-matter intake and selective intake of forage, particularly hay, in preference to concentrates. Generally temperature, respiratory and heart rates are normal, but not uncommonly ruminal contraction rates are depressed, and ruminal contractions are weak and incomplete. A ruminal 'ping' may be detected on occasions. Dry, scant feces are often found on rectal examination. Milk production will be decreased in cows with clinical ketosis. Approximately 10% of cows may show signs of central nervous excitement. Cows that show excitement may repeatedly lick or suck on objects, and show incoordination, but cows are more commonly depressed or dull. The smell of acetone on the breath of cows may be detected. The relationship between ketosis and other disease conditions has been acknowledged in the clinical definition of ketosis into 'primary' and 'secondary' categories, with secondary ketosis being the result of an antecedent condition.

Biochemistry and Physiology

It has been contended that ketosis is a result of an imbalance of appropriate precursors for milk production. Three propositions were raised: (1) that a major determinant of the rate of milk secretion is the mammary uptake of glucose; (2) that a major determinant of lactational efficiency is the mammary uptake of long-chain fatty acids and (3) that a major determinant of the development of spontaneous ketosis is the ratio of glucogenic to lipogenic nutrients in the diet. These contentions are supportable, but fail to acknowledge adequately the integrated role of energy and amino acid metabolism in cattle and that ketone bodies are both metabolic modifiers and an efficient means to export energy to peripheral tissues, not merely as the 'dirty exhaust of an engine'.

The following are homeorhetic, that is, the orchestrated, longer-term adaptive changes to lactation: increased lipolysis, decreased lipogenesis, increased gluconeogenesis, increased glycogenolysis, increased use of lipids and decreased glucose use as an energy source, increased mobilization of protein reserves, increased absorption of minerals and mobilization of mineral reserves, increased food consumption and increased absorptive capacity for nutrients.

Changes in hormone concentrations around the time of calving are important extrahepatic influences for ketosis. These include a precipitous decrease in plasma progesterone concentrations that occurs at parturition and rapid increase in estrogens in the last week of gestation. Insulin concentrations are depressed both in animals developing ketosis and in animals with ketosis. Elevated insulin concentrations favor the production of malonyl-coenzyme A (CoA) and insulin administration *in vitro* has been shown to reduce oxidation of long-chain fatty acids in liver. Insulin increases reesterification of long-chain fatty acids in the liver and adipose tissue. Insulin can reduce lipolysis in cattle through its lipogenic action and insulin also increases the utilization of ketone bodies by peripheral tissues. The net effect of insulin is to reduce ketone concentrations through decreased synthesis and increased utilization.

Glucagon plays a gluconeogenic role in the bovine and injection of glucagon can reduce hepatic lipid accumulation and ketone concentrations in blood. Normal cows have increased blood glucagon concentrations following calving, while ketotic cows do not have increased blood glucagon concentrations. It is clear that insulin and glucagon concentrations are potentially critical to the development of ketosis.

Somatotrophin plays a key lactogenic role in the bovine, as evidenced by milk production responses to exogenous somatotrophin and positive relationships between production and somatotrophin concentrations in comparisons of high- and low-yielding cows. Injection of somatotrophin has increased blood ketone concentrations, and increased concentrations of β-hydroxybutyrate have been associated with elevated endogenous concentrations of somatotrophin. However, there is no evidence at present that either somatotrophin administration or somatotrophin concentrations are associated with clinical ketosis, and in many studies no increase in ketone concentrations was noted following somatotrophin treatment. Prolonged administration of somatotrophin to dairy cows has the potential to increase nitrogen retention and produce a leaner carcass. Reducing the amount of lipid available for mobilization and possibly increasing labile protein reserves can reduce the risk of ketotsis in subsequent lactations for animals treated with somatotrophin.

Plasma cortisol concentrations increase in the immediate periparturient period and are associated with a transient hyperglycemia at calving. Glucocorticoids have been used therapeutically in ketotic cows with good effect. The efficacy of glucocorticoids may be attributed to the following: a decrease in milk yield associated with decreased mammary uptake and utilization of glucose, increased availability of gluconeogenic amino acids from increased protein mobilization, increased blood glucose concentrations and increased hepatic glycogen concentrations.

Feed and Diet

Decreases in dry matter intake are present in cows with ketosis. However, it is not clear whether decreases precede ketosis or are subsequent to the onset of ketosis. It is apparent that the control of ketogenesis may be influenced by dry matter intake through provision of glucogenic precursors. The periparturient period is one of greatly increased risk of disease and these conditions are associated with decreased dry matter intake. Clinically ketotic cows had greater risk of other illness in the first 10 days postpartum than herdmates. However, ketonemic cows, with indistinguishable blood biochemistry to clinically ketotic cows, had significantly lower risk of other disease in the first 10 days postpartum than herdmates.

It is assumed that the secondary ketosis syndrome is a result of decreased feed intake. Cows in higher body condition scores have decreased dry matter intakes postpartum. Ketonemic cows were significantly heavier than nonketotic cows, but maintained higher dry matter intake than these cows – probably a key factor in reducing the risk of clinical ketosis (**Figures 1–4**)

Cobalt deficiency may predispose animals to ketosis. This is a result of impaired gluconeogenesis due to reduced activity of methylmalonyl-CoA mutase (a vitamin B_{12}-dependent enzyme), which converts methylmalonyl-CoA to succinyl-CoA. Low blood calcium concentrations have been found in ketotic animals; however, the significance of this finding is not clear. Magnesium intake can influence dry matter intake, somatotrophin concentrations and milk production and, therefore, may play a role in the pathogenesis of ketosis. Mineral inputs may be important in

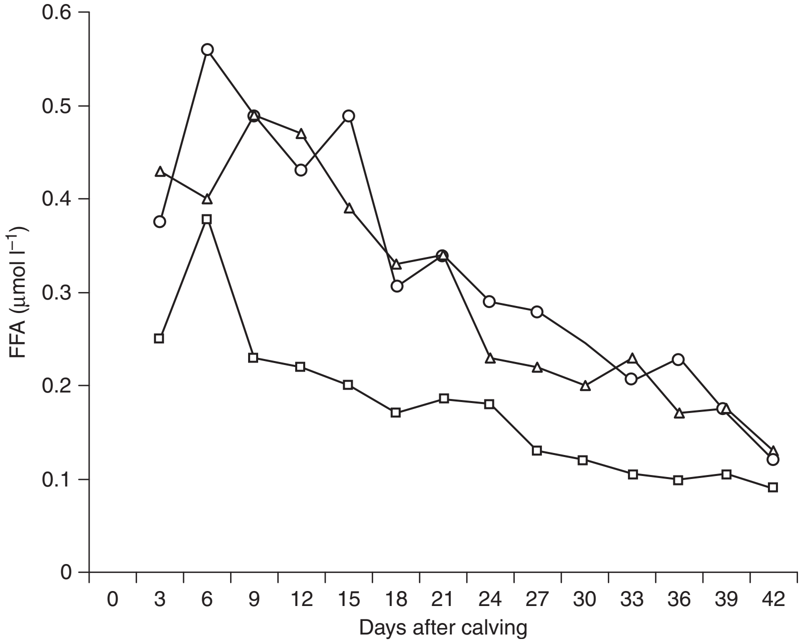

Figure 1 Free fatty acid concentrations for (□) nonketotic, (○) ketonemic and (△) clinically ketotic dairy cows after calving.

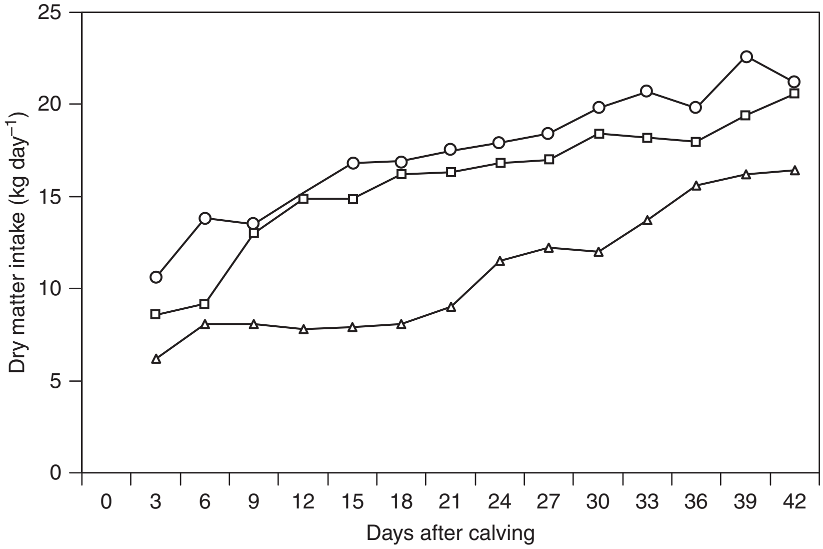

Figure 2 Dry matter intakes for (□) nonketotic, (○) ketonemic and (△) clinically ketotic dairy cows after calving.

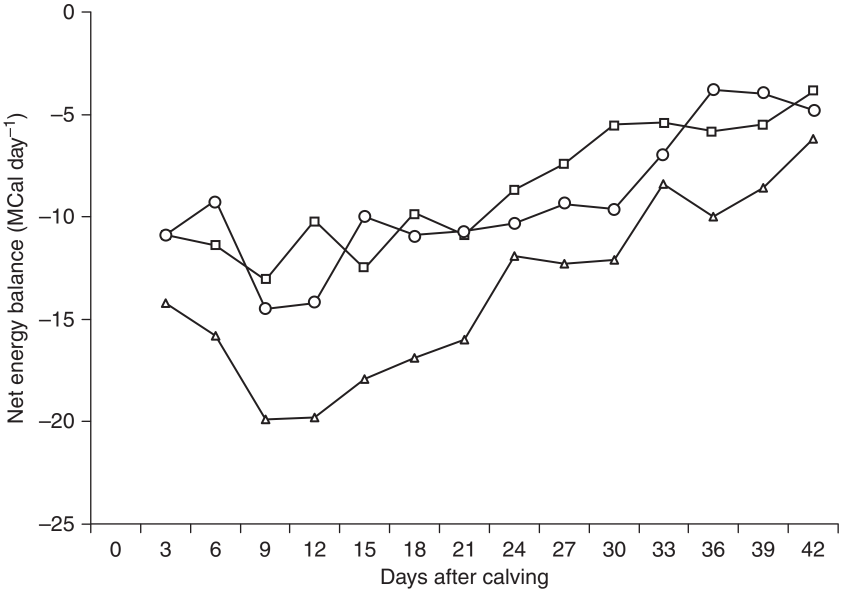

Figure 3 Estimated net energy balance for (□) nonketotic, (○) ketonemic and (△) clinically ketotic dairy cows after calving.

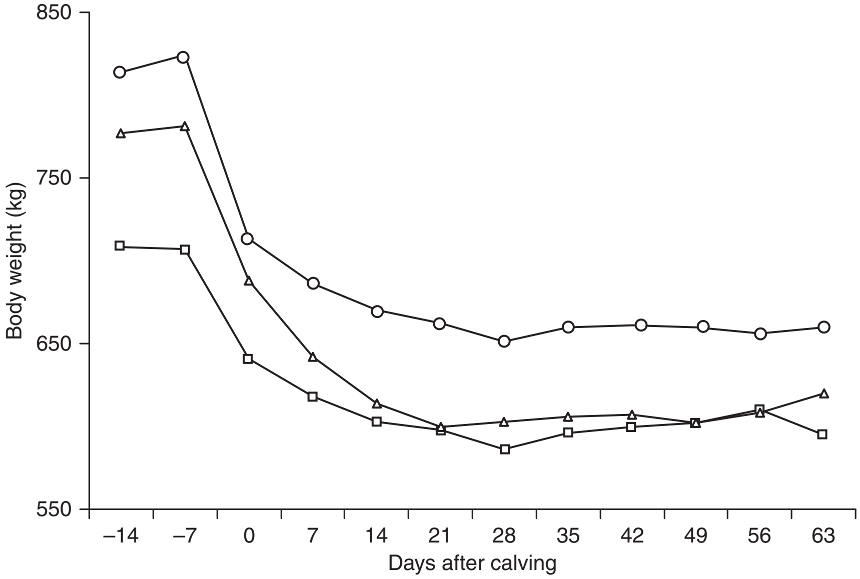

Figure 4 Body weights of (□) nonketotic, (○) ketonemic and (△) clinically ketotic dairy cows after calving.

circumstances where either deficiency or excess influences dry matter intake or influences the efficiency of energy metabolism.

Origin of Ketones

There are two different sources of ketones that are important in the pathogenesis of ketosis in cattle. The first is exogenous ketogenic precursors, not including acetate that is metabolized to long-chain fatty acids before acting as a precursor for ketone production, and which arise from the feeding of poor-quality silages containing butyrate or possibly from feeding fats containing medium-chain fatty acids. Short-chain fatty acids are absorbed from the diet into the portal circulation and are metabolized in the liver. Butyrate is a product of normal ruminal fermentation and this may be a key factor in the comparatively high circulating ketone concentrations of cattle. Approximately 50% or more of butyrate present in the rumen is taken up by the ruminal epithelium and converted to β-hydroxybutyrate in the rumen epithelium and the remaining butyrate is efficiently removed by the liver.

The second source of ketones is the production of ketones from endogenous precursors. The only endogenous precursors of significance are long-chain fatty acids, although ketones can derive from oxidation of essential or nonessential amino acids. The metabolism of cattle is directed toward the sparing of glucose. This is a consequence of the ruminant adaptation to diets which supplied very few glucose precursors and requirements for glucose are primarily met by intermediary metabolism because of the relatively small amount of glucose absorbed by the cow. Consequently lipid metabolism plays a key role in energy homeostasis in the bovine.

The pathways for hepatic utilization of acetate and synthesis of lipids, however, play an important role in the control of ketogenesis. The flux-generating steps for ketone production are the release of free fatty acids from triglycerides stored in adipose tissue and the release of ketogenic amino acids from labile protein pools. Free fatty acids are complexed with albumin for transport in the blood. Fat mobilization is increased in the immediate periparturient period and is associated with elevated blood concentrations of free fatty acids. Uptake by hepatocytes is a function of the extracellular concentration of free fatty acids. Once fatty acids are transported into the mitochondria by carnitine acyltransferase I and II, β-oxidation of the fats occurs, producing acetyl-CoA and NADH. Acetyl-CoA is either oxidized in the tricarboxylic acid (TCA) cycle or is metabolized to acetoacetyl-CoA and subsequently to acetoacetate and β-hydroxybutyrate. There is also endogenous production of acetate from acetyl-CoA by the liver.

Intrahepatic control over the fate of fatty acids results from inhibition of carnitine acyltransferase 1 (CAT1) by malonyl-CoA, an intermediate in the hepatic synthesis of fatty acids. Methylmalonyl-CoA, an intermediate in the gluconeogenic metabolism of propionate, also influences intrahepatic utilization of fatty acids through inhibition of CAT1. Malonyl-CoA inhibited carnitine palmitoyltransferase 1 in bovine liver and propionate, acetate and insulin also inhibited long-chain fatty oxidation *in vitro*. Long-chain fatty acids also act to inhibit acetyl-CoA carboxylase activity, which decreases malonyl-CoA concentrations. The dual control over fatty acid metabolism by malonyl-CoA and methylmalonyl-CoA may be a powerful adaptation in the ruminant, allowing a strict control over lipid metabolism despite the minor role of *de novo* fatty acid synthesis in the ruminant liver.

Role of Oxaloacetate and Gluconeogenic Precursors

Regulation of the utilization of acetyl-CoA for production of adenosine triphosphate or for ketogenesis may be effected through the availability of oxaloacetate in the mitochondria and by mitochondrial concentrations of acetoacetyl-CoA. Precursors of oxaloacetate are pyruvate or amino acids and portal uptake of dietary lactate and glycerol. Amino acids, including alanine, cysteine, glycine and serine, act as precursors for oxaloacetate, as do aspartate and malate, which may be derived from propionate. Other amino acids including glutamate may act as precursors for oxaloacetate.

The major precursors for gluconeogenesis are propionate and amino acids. Less important precursors are lactate and glycerol, which represent a recycling of glucose carbon through either the Cori cycle or lipolysis. However, appreciable amounts of lactate and glycerol can be absorbed on grain diets and silage diets. No glucose can be synthesized from acetate and butyrate.

Propionate is produced in the rumen from starch, fiber and proteins and propionate is the precursor for approximately 30–55% of glucose produced. Once propionate is absorbed it is potentially metabolized in the rumen wall and converted to lactate. Long-chain fatty acid oxidation in the bovine liver decreases as the concentrate-to-forage ratio of the ration increases, probably as a result of increased propionate precursors. Feeding propylene glycol, a propionate precursor, also significantly lowered correlations between blood free fatty acids and ketones. Propionate may act to reduce ketone production through increased methylmalonyl-CoA and oxaloacetate concentrations and may possibly reduce ketone concentrations through stimulation of insulin release by glucose.

While dietary protein is the most important quantitative source of amino acids for gluconeogenesis, cows are in negative nitrogen balance in early lactation and consequently the labile pool of body protein is also very important to gluconeogenesis. This pool is mobilized in response to energy demands, particularly in early lactation, and estimated labile protein reserves may be greater than 15–17 kg and in the order of 25% of body protein. Most amino acids are gluconeogenic, with lysine and leucine being exceptions. Tryptophane, isoleucine, phenylalanine and tyrosine can also be partly metabolized to acetyl-CoA and are, hence, partly ketogenic. Estimates of the gluconeogenic potential of protein have varied widely between researchers and have ranged from as little as 1–2% of glucose produced from amino acids to as much as 70%. The contribution of amino acids to gluconeogenesis is dependent on the physiological state of the animal; in periods of food deprivation, amino acids are mobilized and recent studies indicate that the contribution of amino acids to glucose synthesis is approximately 20% during the period from 10 to 30 days after calving. Variation in the ability of cows to mobilize amino acids may be an important predisposing factor in ketosis. Ketonemia reflects both the lack of glucogenic precursors and a relative oversupply of ketogenic precursors.

Overview of Hepatic Ketogenesis

Examination of the extrahepatic and intrahepatic controls for ketogenesis allows the generation of a hypothesis of subtly controlled energy regulation in the bovine. High concentrations of somatotrophin and catecholamines and low blood insulin concentrations increase the release of long-chain fatty acids from adipose tissue. Insulin concentrations are decreased by lowered blood glucose concentrations resulting from the mammary demand for glucose. Long-chain fatty acids exert control over ketogenesis both as substrate and through their potential to inhibit acetyl-CoA carboxylase and, hence, decrease malonyl-CoA synthesis. Propionate is potentially an important controlling factor through methyl malonyl-CoA inhibition of carnitine acyltransferase 1 and through regulation of insulin concentrations. If insulin concentrations are increased, fatty acid mobilization may be decreased, reesterification of long-chain fatty acids increased, malonyl-CoA synthesis stimulated through acetyl-CoA carboxylase and peripheral utilization of ketones enhanced. Animals that are able to mobilize more protein from labile reserves in this period may be able to increase gluconeogenic flux and, thereby, lower the risk of ketosis.

Uses of Ketones

Until relatively recently ketones were viewed as detrimental to the animal and as a signal of disease; however, ketones play an important part in the maintenance of energy homeostasis. Ketones supply acetyl-CoA for oxidation by the peripheral tissues and decrease the demand of peripheral tissues for glucose as a precursor for ATP generation under circumstances when glucose supply is limited. By moving succinyl-CoA to succinate, the oxaloacetate susequently produced may allow increased use of acetyl-CoA by the TCA cycle should greater production of ATP be required. One function of ketones is to supply a form of energy to peripheral tissues when glucose availability is limited. In contrast to long-chain fatty acids, ketones are more diffusible, do not require binding proteins for transport, and are not highly toxic if unbound. Exporting ketones from the liver as either β-hydroxybutyrate or acetoacetate allows the liver to regulate the mitochondrial redox rate – hence the benefit in having two major ketone bodies.

Ketone bodies can control the utilization and production of glucose and act as regulators of their own production by controlling long-chain fatty acid supply and utilization. Beta-hydroxybutyrate will act at physiological concentrations to reduce rates of lipolysis. Ketones may also influence the rate of muscle protein mobilization, apparently by indirectly inhibiting protein degradation. The metabolic actions of ketones are consistent with their role as part of a cycle of glucose, fatty acid and ketone metabolism that acts to ensure that protein resources are not rapidly depleted during periods of increased demand for endogenous energy precursors.

Prevention and Treatment

Herd Presentations of Ketosis

Ketosis is a multifactorial disorder of energy metabolism in the bovine. Alimentary ketosis is caused by excessive intake of butyrate in silages and is possibly due to inappetence associated with these silages. A second herd presentation of ketosis is that of cattle in poor body condition exposed to poor-quality feeds. While mobilizable reserves of adipose tissue may be limited, deficiency of propionate and possibly protein leads to limited gluconeogenesis from feed. There is very limited capacity for gluconeogenesis from mobilized tissue protein reserves. Low dietary protein concentrations are associated with decreased fermentation rates and decreased dry matter intake. Hence the observation that low protein rations are associated with ketosis is supportable.

A third herd presentation is for apparently well- fed cows in good to excessive body condition. High genetic merit, excessive body condition and high dietary protein intake lead to excessive lipid mobilization. Ketosis may result under circumstances where dry matter intake is inhibited, such as disease, acidosis due to ration adjustments or where excessive body condition impairs dry matter intake. The latter presentation is a function of production systems that encourage high yield per cow and requires careful ration formulation to manage. In particular, strategies that minimize the risk of inappetance are critical to success.

Within each of the three presentations, cows of high lactation potential are at greater risk than those of low lactation potential. Similarly, all depend on limited propionate precursors, early lactation demand for glucose and mobilized lipid or preformed ketones in the pathogenesis. A relative oversupply of ketogenic precursors to gluconeogenic precursors occurs as a result of exogenous ketogenic precursors, poor supply of propionate precursors and limited endogenous gluconeogenic precursors or excessive mobilization of lipids. The role of exogenous protein is important. Low levels of dietary protein reduce fermentation and limit dry matter intake, while excessive levels result in metabolic losses in urea synthesis and increased tissue mobilization and ketosis may result when bypass proteins are supplied to the small intestine. Endogenous protein may also play a key role in the pathogenesis of ketosis, as limited mobilizable reserves may predispose animals to ketosis.

General Principles of Herd Management to Avoid Ketosis

1. Avoid poor-quality silages that may contain large amounts of butyrate. If these feeds need to be fed, feeding should be directed toward cows in middle and late lactation, avoiding the immediate postpartum risk period.
2. Manage body condition carefully. Do not allow animals to become overconditioned or mobilize excessive amounts of body tissue. Body condition scores exceeding 3.5 on a 5-point scale are associated with greatly increased risk of ketosis. It has been demonstrated that there was no production benefit and substantial risk of ketosis in allowing cows to become overconditioned in the dry period.
3. Ensure that diets are designed to deliver propionate precursors to the maximum amount achievable given economic and health constraints. In intensive production systems, this will mean that concentrate-to-forage ratios should be in the order of 55–65:45–35 or higher in early lactation and nonstructural carbohydrates are approximately 36%. High-quality forages should be directed toward early-lactation cows and transition rations should be used in late gestation to adapt rumen flora to increased amounts of concentrates in the post-parturient period. Some consideration should be given to diet palatability and ensuring that sudden feed changes are not made. Similarly, complete diets or total mixed rations are preferable to feed management systems where concentrates are fed separately in large amounts (e.g. more than 4 kg a feeding).

   Protein concentrations should be closely monitored and consideration given to limiting protein intake in herds to 16–18% of the ration. Grain processing may be used to advantage for grains such as maize and sorghum to allow greater bypass to the small intestine, hence greater glucose absorption.

   In less intensively managed dairy systems, ensuring that cows calve on to high-quality pastures is important. Adjustments in calving pattern, pasture rotation and conservation management should be used to ensure high-quality forage availability.
4. Ensure that vitamins and minerals are supplied in the diet at National Research Council or Agricultural Research Council recommended levels (*see* **Feed Ingredients**: Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Vitamins).
5. Identify animals at risk to allow the instigation of appropriate preventive treatment. Workers were able to detect 69% of clinical ketosis cases prior to the onset of clinical signs using a weekly milk ketone test. Predictive tests with higher sensitivities and which are less labor-intensive would be valuable for detecting at-risk animals.

Fats and Feed Additives in the Management of Ketosis

Ionophores

Ionophores such as monensin and lasalocid act by altering ion transport across bacterial cell membranes and are selectively bactericidal in the rumen. Gram-positive bacteria from the rumen produce hydrogen, ammonia, lactate, acetate and methane and are more sensitive to ionophores, while the Gram-negative bacteria which produce propionate and succinate are less susceptible. Differences in cellular membrane structure between Gram-positive and Gram-negative bacteria are chiefly responsible for the different sensitivities of bacteria. Effects of monensin supplementation include increased ruminal propionate production, reduced *in vivo* and *in vitro* production of methane and increased dry matter and starch digestibility. Supplementation decreased the production of bacterial protein in the rumen, but increased nitrogen retention and significantly increased the flow to and digestion of amino acids in the duodenum. Treatment of lactating dairy cows with ionophores has resulted in increased plasma glucose concentrations, and decreased plasma ketone and free fatty acid concentrations. In dairy cattle the use of ionophores may lead to minor milk fat depression and increased milk production.

Ionophores reduce ketogenesis and clinical ketosis in grazing and housed dairy cattle. Timing of treatment may be important, as studies show that while plasma β-hydroxybutyrate concentrations are significantly lowered in cows treated both before and at calving, cows treated before calving tended to have lower levels than cows treated at calving. Relative risk analysis on monensin data indicated that monensin treatment administered after calving reduced the risk of ketonemia by 3.4 times over controls, but the risk for treated cows was 19 times less than controls when monensin was administered before calving.

Ionophores have a considerable advantage over other available propionic precursors such as 1,2-propanediol and sodium propionate, as they can be readily mixed into the ration, are inexpensive and do not significantly depress appetite. In pasture-based dairy systems the potential to control bloating is a significant advantage.

Fats

Feeding fats can help manage ketosis. Fats may be supplied in forms that are susceptible to hydrolysis, interconversion and hydrogenation by rumen flora, or in more rumen-inert forms such as the calcium soaps of long-chain fatty acids. Fats more available to metabolism in the rumen should be limited in order to provide not more than 5% of dry matter in the ration, as significant depression of rumen fermentation can occur. The use of more inert fats or protected lipids can allow a greater amount of metabolizable energy to be supplied as fat.

Supplying energy as fat will increase metabolic lactational efficiency. The use of protected lipids results in higher blood glucose concentrations and in lower blood ketone concentrations. Fat feeding can increase milk yields; however, prolonged fat feeding has resulted in shortened lactations and fat feeding can either increase or decrease milk fat content and decrease milk protein composition. In very high-producing herds fats should be carefully considered for routine inclusion in rations, and their potential to benefit herd management of ketosis should be a positive aspect of that consideration.

Niacin

Niacin has a number of actions that may be useful in the herd management of ketosis, and niacin has been successfully used at the rate of 3 or 6 g day^{-1} to reduce blood β-hydroxybutyrate concentrations. Higher concentrations of use are not associated with substantially greater reductions in ketone concentrations and are associated with a rebound effect in which ketone concentrations increase to normal or even higher concentrations after 24–48 h. Blood glucose concentrations may increase for 24 h following niacin supplementation, but also return to normal or below after 24–48 h.

Niacin acts to inhibit lipolysis and may also act to decrease protein mobilization. Niacin may also influence rumen metabolism and is associated with increased propionate concentrations, increased microbial protein production and increased microbial protein supply to the small intestine. Niacin supplementation at the rate of 3 g day^{-1} significantly increased milk production, but milk yield responses are not consistent. For herds with ketosis problems associated with excessive tissue mobilization, niacin at 3–6 g day^{-1} for early-lactation cows may be beneficial.

Somatotrophin

The capacity for somatotrophin to change body composition through increased mobilization of lipid and increased deposition of protein can reduce the risk of ketosis. It was found that cows previously exposed to bovine somatotrophin (bST) had substantially less risk of ketonemia and clinical ketosis than control cows. Some, but not all of the risk for clinical ketosis was attributable to lower body condition score of cows previously treated with bST.

Treatments

Many substances have been used to treat ketosis. It should be noted that the condition has the potential to remit spontaneously and consequently many older trials that lacked controls are unsound. On a herd basis, perhaps the only products other than those indicated above that merit attention are the gluconeogenic precursors, including sodium, magnesium or other salts of propionate, 1,2-propanediol (propylene glycol) and glycerol. These products have been extensively used in the treatment of ketosis and have had limited use as herd prophylactic agents. Sodium propionate was successfully used to lower the incidence of ketosis in a 3-year trial. Magnesium propionate acts to stimulate insulin release and may be a more potent antiketogenic agent than sodium propionate. However, sodium propionate may have the potential to depress feed intake and has not been widely used for ketosis prevention. Propylene glycol apparently does not induce insulin secretion, but is an effective prophylactic agent. Higher rates of administration may result in incoordination and scouring. The relatively large amounts of propionate salts and propylene glycol required and their relatively high cost have limited their adoption in preventive programs.

Corticosteroids are an effective treatment for ketosis, but are immunosuppressive. Given the already immunosuppressed state of the periparturient dairy cow, the use of corticosterids should be limited. Recent studies indicate the potential for glucagon to decrease both plasma ketone and free fatty acid concentrations, to increase plasma glucose concentrations and to decrease liver triacylglycerol content.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Diseases of Dairy Animals**: Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Grass Tetany; Non-Infectious Diseases: Milk Fever. **Feed Ingredients**: Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Ruminally Protected Amino Acids; Feed Supplements: Vitamins. **Feeds, Ration Formulation**: Dry Period Rations in Cattle. **Lactation**: Galactopoiesis, Effect of Treatment with Bovine Somatotropin; Galactopoiesis, Effects of Hormones and Growth Factors. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat. **Reproduction, Events and Management**: Pregnancy: Parturition; Pregnancy: Periparturient Disorders.

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Non-Infectious Diseases: Milk Fever

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Introduction

Milk fever (parturient paresis, hypocalcemia, paresis puerperalis, parturient apoplexy) is a nonfebrile disease of adult dairy cows and female goats caused by an acute calcium deficiency. In dairy cows, hypocalcemia causes progressive neuromuscular dysfunction with flaccid paralysis, circulatory collapse, and depression of consciousness. Hypocalcemia in goats causes varying combinations of tetany and flaccid paralysis. Although clinical cases of milk fever generally respond well to treatment, the costs of this disease are very high owing to clinical complications and milk production losses. The pathogenesis of milk fever is complex, and several prevention strategies have been proposed to counter the metabolic disturbances that lead to hypocalcemia.

Occurrence

Milk fever is one of the most common metabolic diseases of dairy cattle, affecting about 5–10% of cows around calving. The incidence of milk fever in high-producing female goats is similar. Annual incidence rates of clinical milk fever within herds vary from about 2 to 60%. Economic losses due to clinical cases of milk fever are substantial and include losses from deaths (~8% of affected cows), premature culling (~12% of affected cows), treatment costs, and decreased milk production in the subsequent lactation. In addition, each episode of clinical milk fever increases the risk for other parturient diseases such as retained placenta, ketosis, displaced abomasum, and environmental mastitis.

Subclinical hypocalcemia (depressed blood calcium concentrations without clinical signs) affects about 50% of all adult dairy cattle around the time of calving. Subclinical hypocalcemia may cause even greater economic loss than caused by clinical milk fever, because it affects many more animals. Subclinical hypocalcemia may lead to decreased dry matter intake after calving, increased risk of secondary disease conditions, decreased milk production, and decreased fertility later in lactation. Therefore, efforts to improve calcium metabolism in dairy animals may be financially rewarding even in herds without high clinical milk fever rates.

Breed, age, and milk production level are important risk factors for milk fever in dairy cattle. Jersey and Guernsey breeds are the most susceptible; Holstein and Brown Swiss breeds are moderately susceptible; and Ayrshire and Milking Shorthorn breeds are the least susceptible. Breed predilections may be explained in part by higher calcium content in the colostrum and by higher milk production per unit of body weight in the more susceptible breeds. Jersey cattle may also have fewer vitamin D receptors than Holstein cattle.

Regardless of the breed, the incidence of milk fever increases with parity (~9% increased risk for each successive lactation) and with higher levels of milk production. First-lactation dairy cattle rarely develop milk fever because they produce less colostrum and because they can rapidly mobilize calcium from bone owing to the high osteoclastic activity in their growing skeleton.

Etiology and Pathogenesis

Milk fever is caused by hypocalcemia that occurs as the dairy animal's complex mechanisms for maintaining calcium homeostasis fail during a sudden and severe calcium outflow. Any decrease in ionized blood calcium concentration causes the parathyroid glands to secrete parathyroid hormone (PTH). Within minutes, PTH increases renal reabsorption of calcium from the glomerular filtrate. If the perturbation in blood calcium is small ($<1\ \mathrm{g\ Ca\ day^{-1}}$), blood calcium returns to normal and PTH secretion returns to baseline levels. If the calcium drain from the extracellular pool is large, continued PTH secretion stimulates resorption of calcium stored in bone. This calcium comes from dissolved calcium in solution within the bones as well as from calcium released by osteoclastic activity on the organic bone collagen matrix.

Ultimately, calcium exiting the extracellular fluid must be replaced by dietary calcium. Calcium can be absorbed across the gastrointestinal tract by both vitamin D-dependent and vitamin D-independent means. Vitamin D-independent absorption of calcium is primarily by passive diffusion. Vitamin D-dependent absorption is by active transport and occurs when dietary calcium is low or when calcium demand is very high.

Vitamin D may be either ingested or produced during ultraviolet irradiation of the skin. It then enters the blood and is converted in the liver to 25-hydroxyvitamin D, and then to 1,25-dihydroxyvitamin D in the kidney. The activity of the renal enzyme responsible for converting 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D is stimulated and tightly regulated by PTH. The most important function of 1,25-dihydroxyvitamin D (a steroid hormone) is its ability to stimulate active transport of dietary calcium across the intestinal epithelium.

Calcium demands in dairy cattle and female goats are at their nadir just prior to parturition. Demands by the fetal skeleton are low relative to the amount of calcium needed to produce milk. Thus, bone calcium resorption mechanisms become quiescent and intestinal calcium absorption mechanisms are passive just before parturition. Diets typically fed to these animals at this time exceed their calcium requirements; therefore, the entire calcium demands for maintenance of body tissues and for the developing fetal skeleton can be met by vitamin D-independent absorption of calcium.

Sudden calcium outflow occurs most commonly at the time of the initiation of lactation, when the dairy animal's calcium requirement increases dramatically in a period of just a few hours. Calcium demand for dairy cattle prior to colostrum production is only about 15–20 g $\mathrm{Ca\,day^{-1}}$, which includes endogenous fecal loss of 6–10 g $\mathrm{Ca\,day^{-1}}$, urinary loss of 0.25–1.0 g $\mathrm{Ca\,day^{-1}}$, and fetal skeleton calcification needs of up to 10 g $\mathrm{Ca\,day^{-1}}$ by the end of gestation. Colostrum production requires an addition of 20–30 g $\mathrm{Ca\,day^{-1}}$ (2.0–2.3 g $\mathrm{Ca\,l^{-1}}$); this represents a sudden two- to threefold increase in calcium requirement.

During the critical first few days of lactation in dairy cows and female goats, calcium homeostasis is restored largely by release of PTH, which reduces urinary calcium losses, stimulates bone calcium resorption, and increases 1,25-dihydroxyvitamin D synthesis to enhance active intestinal transport of calcium. All three mechanisms must be operational to avoid hypocalcemia.

An animal's ability to adapt to hypocalcemia is influenced by a number of factors. An important determinant of milk fever risk is the acid–base status of the animal at the time of parturition. Metabolic alkalosis impairs the physiological activity of PTH so that bone resorption and production of 1,25-dihydroxyvitamin D are impaired. Metabolic alkalosis apparently induces conformational changes in the PTH receptor, which impairs PTH binding. Cows fed diets relatively high in potassium or sodium are in a state of metabolic alkalosis and thus have an increased risk for milk fever.

Hypomagnesemia is also an important risk factor for hypocalcemia. Low blood magnesium can reduce PTH secretion from the parathyroid glands. Hypomagnesemia also reduces tissue responsiveness to PTH by inducing conformational changes in the PTH receptor and G-stimulating protein complex. High dietary potassium increases the risk for milk fever because it both reduces ruminal magnesium absorption and causes metabolic alkalosis.

Excessive dietary phosphorus intake ($>80\,\mathrm{g\,day^{-1}}$) during late gestation can induce milk fever by raising blood phosphorus concentrations to the point that phosphorus directly inhibits renal synthesis of 1,25-dihydroxyvitamin D. This can occur when blood phosphorus levels at parturition exceed about 2.6 $\mathrm{mmol\,l^{-1}}$.

Hypocalcemia may also be influenced by estrogen, which is a potent inhibitor of osteoclast activity. Blood estrogen concentrations rise dramatically at the end of gestation and may blunt the effects of PTH on bone resorption.

Clinical Presentation of Dairy Animals with Milk Fever

The clinical effects of hypocalcemia in dairy animals are broad, as calcium serves many critical physiological functions. Calcium is required for the release of the neurotransmitter acetylcholine at the neuromuscular junction. Impaired acetylcholine release effectively blocks transmission of nerve impulses through the junction and on to muscle fibers, leading to flaccid paralysis. Hypocalcemia also hinders calcium-dependent actin–myosin interactions, which directly decreases muscle contractility and enhances the clinical presentation of flaccid paralysis. Finally, hypocalcemia inhibits contractility of smooth and cardiac muscle (including the muscle that closes the teat sphincter), causing a variety of additional clinical signs.

Some hypocalcemic animals show signs of hyperaesthesia and tetany, especially during the early phase of hypocalcemia. This occurs because calcium assists in stabilizing the membranes in peripheral nerves and muscle fibers. Thus, hypocalcemia may initially speed up impulse conduction or even allow spontaneous impulse production in peripheral nerves and muscle fibers. Animals initially affected with hyperesthesia and tetany will often later lapse into flaccid paralysis as the hypocalcemia worsens and neuromuscular junctions become completely blocked. Tetany is generally more pronounced in female goats than in dairy cows.

Whether tetany or flaccid paralysis occurs is also dependent on the relative concentrations of magnesium and calcium. Magnesium competitively inhibits calcium at the myoneural junction and prevents calcium from stimulating acetylcholine release. This promotes flaccid paralysis. Low magnesium at the junction removes calcium inhibition and promotes tetany, as long as hypocalcemia is not severe.

Approximately 75% of all cases of milk fever in dairy cattle occur within 24 h of calving. An additional 12% occur 24–48 h after calving. Some cases (~6%) occur at the time of delivery and cause dystocia because hypocalcemia inhibits uterine contractility. About 7% of all hypocalcemias in dairy cattle occur not around calving. Such cases are often termed 'nonparturient hypocalcemia' rather than milk fever.

Clinical signs of milk fever in dairy cattle may be divided into three stages, which are summarized in **Table 1**. Stage I milk fever is characterized by mild excitement and tetany without recumbency. This phase may go unnoticed because its signs are subtle and do not last very long (often <1 h).

Stage II milk fever in dairy cattle is characterized by sternal recumbency owing to flaccid paralysis. Instead of being hypersensitive and tetanic, affected cows are now depressed and paralyzed. Because of reduced cardiac muscle contractility, they have tachycardia, decreased intensity of heart sounds, and cold extremities. Affected cows usually have a lowered rectal temperature (35.6–37.8 °C). During hot weather, however, some cows in stage II milk fever may become hyperthermic, especially if they are dark colored and lying in direct sunlight. Impaired smooth muscle function owing to hypocalcemia may also lead to gastrointestinal atony, mild bloat, constipation, relaxed teat sphincter, and loss of the anal reflex. Pupils may be dilated and unresponsive to light owing to atony of the dilator pupillae muscle. Clinical signs of stage II generally last from 1 to 12 h.

Dairy cows in stage III milk fever are laterally recumbent and progressively lose consciousness to the point of coma. They are often severely bloated in this stage as a result of lateral recumbency combined with profound gastrointestinal atony. Cardiac output becomes severely compromised; heart sounds may be nearly inaudible; and heart rate increases to 120 beats or more per minute. Cows in stage III milk fever do not survive for more than a few hours without treatment.

Even seemingly uncomplicated cases of clinical milk fever or unobserved cases of subclinical hypocalcemia may be associated with important secondary problems. For example, milk production is reduced by about 14% in the subsequent lactation in cows affected with clinical milk fever. Milk fever is also associated with increased risk for fetal membrane retention, likely due to stress hormones and the subsequent immunosuppression associated with hypocalcemia. Cows affected with milk fever are also at a higher risk for ketosis, displaced abomasum, and clinical mastitis later in lactation.

Table 1 Stages of clinical milk fever in dairy cows

Stage	*Position*	*Possible clinical signs*	*Physical examination findings*	*Approximate total blood Ca (mmol l^{-1})*	*Preferred treatment*
Stage I	Standing	Excitability, nervousness Hypersensitivity Anorexia Weakness Weight shifting Shuffling of hind feet	Mild tachycardia Slight hyperthermia	1.40–1.90	Oral calcium supplement or subcutaneous calcium injection
Stage II	Sternal recumbency	Flaccid paralysis Depression Head turned or extended 'S' curve to neck Fine muscle tremors Cold extremities Mild bloat, constipation	Tachycardia Muffled heart sounds Hypothermia Loss of anal reflex Gastrointestinal atony Dilated pupils Pupils unresponsive to light	0.85–1.60	Intravenous calcium injection, followed by oral or subcutaneous calcium administration to prevent relapse
Stage III	Lateral recumbency	Same signs as stage II, except that they are more pronounced Loss of consciousness, coma Bloat, possibly severe	Severe tachycardia Heart sounds nearly inaudible	0.25–0.85	Same as for stage II

About 7% of all cases of milk fever in dairy cattle are nonparturient. In these cases, the sudden calcium outflow associated with the onset of lactation is not the stimulus for hypocalcemia. Instead, any severe stress (weather, transport, etc.) or period of feed deprivation may cause hypocalcemia owing to a sudden calcium outflow coupled with a lack of calcium uptake. Specific disease conditions that may trigger nonparturient hypocalcemia include hypomagnesemia, ruminal acidosis, alkaline digestive disturbances (such as urea toxicity or protein overload), oxalate toxicity, toxic infections, heat stroke (or any cause of severe alkalosis), and increased estrogen concentrations owing to estrus or ingestion of plant estrogens.

Milk fever may occur in female goats either pre- or postpartum because they have relatively large fetoplacental requirements associated with multiple births and the potential for high milk production. The parturient form of milk fever will dominate when goats are managed intensively for milk production. The prepartum form of milk fever will be more common when goats are managed extensively and not challenged for high milk production.

Clinical Pathology

Milk fever is confirmed by low blood calcium concentration. Clinical signs may begin as the total blood calcium value falls below 1.9 mmol l^{-1}; however, more than half of all mature dairy cows will have a total blood calcium concentration below 1.9 mmol l^{-1} following calving, and most of these cows will not display any clinical signs of hypocalcemia. Animals in stage I milk fever usually have mild hypocalcemia (1.4–1.9 mmol l^{-1} Ca). Some animals are able to remain standing with total calcium concentrations as low as 1.2 mmol l^{-1}, although most become recumbent before this level is reached. Animals in stage II milk fever typically have total calcium concentrations of 0.85–1.60 mmol l^{-1}, and calcium may be as low as 0.25 mmol l^{-1} in animals with stage III milk fever.

Blood ionized calcium concentration expresses the actual amount of metabolically active (ionized) calcium in the bloodstream and therefore presents the most accurate method of diagnosing hypocalcemia. Total blood calcium determinations include calcium that is protein-bound or complexed; these forms of calcium do not affect neuromuscular function. Unfortunately, ionized calcium determinations may be expensive and may not be available in commercial laboratories. In addition, determination of ionized calcium requires anaerobic collection of whole blood samples in heparinized syringes, refrigeration of the sample, and rapid analysis after sample collection. Fortunately, total blood calcium is highly correlated to ionized calcium when acid–base balance and protein metabolism are not disturbed (as is typically the case in uncomplicated milk fever).

Blood concentrations of phosphorus are typically lowered to 1 mmol l^{-1} or less in cases of milk fever. This is partly a result of the lactational drain of phosphorus and partly because PTH secretion causes renal tubular excretion of phosphorus.

Blood magnesium concentration generally increases slightly during milk fever because PTH stimulates renal tubular reabsorption of magnesium. Blood magnesium concentrations below 0.7 mmol l^{-1} at the time of clinical milk fever suggest that hypomagnesemia may have contributed to the development of milk fever.

Blood cortisol concentrations are typically elevated in animals with milk fever. Excessive cortisol causes immunosuppression in periparturient animals and may in part explain the increased susceptibility of cows with milk fever to placenta retention and mastitis. It may also account for the exaggerated stress leukogram (neutrophilia and relative lymphopenia) seen in the white blood cell count of animals with milk fever. Hyperglycemia is also evident during milk fever and is apparently caused by cortisol release combined with the inability of pancreatic β-cells to secrete insulin when extracellular calcium is low.

Laboratory confirmation of the diagnosis of milk fever is not always essential, as response to treatment is a useful and commonly used diagnostic method. Most cases of milk fever respond rapidly to a single parenteral treatment with calcium salts. However, it is good practice to collect a blood sample prior to initial treatment in all cases of milk fever. If the animal does not respond to initial treatment, then an accurate diagnosis can be made from the pretreatment blood sample. Posttreatment samples are of very limited value in diagnosing milk fever because they are temporarily influenced by the calcium administered.

It is important to rule out other possible causes of recumbency in parturient animals before initiating calcium treatment. Important differential diagnoses for clinical milk fever include toxemia from mastitis or metritis; physical injury such as pelvic fracture, obturator paralysis, leg bone fracture, ruptured gastrocnemius tendon, and ‘downer cow syndrome’ due to pressure necrosis; hypomagnesemia; fat-cow syndrome; and pregnancy toxemia (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Grass Tetany; Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Pregnancy Toxemia. **Mastitis Pathogens**: Contagious Pathogens. **Mastitis Therapy and Control**: Medical Therapy Options).

Milk fever must be diagnosed *ante mortem*, as there are no gross lesions or histological changes in affected animals at necropsy. Urine obtained from the bladder of cows that died from hypocalcemia will have very low calcium concentration, but this alone is not sufficient evidence to make a diagnosis. Postmortem blood samples cannot be used to assess calcium status.

Treatment

Cases of stage I milk fever may be treated by administering calcium via a slowly absorbed route (oral or subcutaneous). For example, subcutaneously administered calcium is gradually absorbed over a period of several hours. Rate of subcutaneous calcium absorption depends on the degree of peripheral perfusion and may therefore be ineffective in dehydrated animals. Solutions containing glucose should never be given subcutaneously, as they often cause tissue destruction, abscess formation, and/or sloughing at the site of injection.

Calcium provided by oral dosing is also gradually absorbed. A variety of oral calcium salt preparations are available. They typically contain between 25 and 100 g Ca in the form of calcium chloride or calcium propionate. They work by rapidly raising calcium in the intestine to such a high concentration that a small amount is passively absorbed. For example, about 4 g calcium will be absorbed and enter the bloodstream of a cow given an oral solution containing 50 g of calcium chloride. Calcium chloride also rapidly causes a compensated metabolic acidosis, which improves the animal's own calcium homeostatic mechanisms. However, high or repeated doses of calcium chloride can cause uncompensated metabolic acidosis. Calcium chloride is irritating and may cause transient ulcers in the mouth, esophagus, rumen, and abomasum of some cows. Calcium propionate is less irritating than calcium chloride and in high doses is nearly as effective in supporting blood calcium concentrations.

Care must be taken during administration of any oral calcium supplement to avoid laceration of the pharyngeal region or aspiration of the solution. Thinner liquid drenches, though absorbed faster, pose a greater risk for aspiration than do the thicker gels.

Animals in stage II or III milk fever require immediate treatment with intravenous calcium salts. Because these animals are recumbent, any delay in getting them to rise increases the risk for musculoskeletal damage. A variety of intravenous calcium solutions are available to dairy veterinarians and producers to treat milk fever. The standard dose is 500 ml of a 23% calcium gluconate solution. Intravenous treatments for milk fever should not contain glucose or magnesium, because animals with milk fever already have elevated blood concentrations of these metabolites. Intravenous treatments do not need to include phosphorus, which is usually provided as biologically ineffective hypophosphites.

It is important to administer intravenous calcium slowly, because hypocalcemic animals are at risk for cardiac arrest during calcium infusion. The smallest dose of intravenous calcium necessary to allow the animal to rise should be administered. This reduces the risk of cardiac arrest and later hypocalcemic relapse.

An unfortunate effect of intravenous calcium administration is that it raises blood calcium levels well beyond the physiological threshold. This shuts down PTH production and encourages calcitonin release to protect the animal against hypercalcemia. This leaves the animal vulnerable to a hypocalcemic relapse once the exogenous calcium is eliminated from her system. Not surprisingly, about 25–40% of dairy cows with milk fever that respond favorably to initial intravenous calcium therapy will relapse into hypocalcemia within 12–48 h. Animals with prepartum milk fever have an even greater relapse rate. Older cows are at greater risk for a hypocalcemic relapse because bone responsiveness diminishes with age and because older cows tend to produce more milk. A subgroup of cows experiencing hypocalcemic relapses may have an impairment in 1,25-dihydroxyvitamin D production that lasts for 2–3 days after calving.

The incidence of hypocalcemic relapses in dairy cattle may be reduced to only 5–10% of the total cases by administration of an additional 500 ml of 23% calcium gluconate subcutaneously at the time of initial treatment with intravenous calcium. Calcium is released apparently slowly from the subcutaneous depot and sustains the treated animal through the surge of calcitonin release that follows intravenous calcium administration.

Oral calcium supplements may also be used to prevent hypocalcemic relapses. Two doses of oral calcium are usually given for this purpose – one dose soon after successful intravenous treatment, and a second dose about 12 h later. Oral calcium supplements should be administered only when the animal is standing, alert, and able to swallow.

Udder inflation, the earliest treatment for milk fever, reduces the risk of hypocalcemic relapses because it temporarily slows calcium outflow through colostrum. However, udder inflation increases the risk of mastitis, which probably outweighs its benefits in preventing relapses. An alternative to udder inflation is to milk the cow less frequently following treatment for milk fever.

Prevention

Dietary Calcium Restriction

Feeding cows a calcium-deficient diet before calving reduces the risk for milk fever. Calcium-deficient diets stimulate PTH secretion prior to calving. This activates bone osteoclasts, stimulates bone calcium resorption, and activates renal tubules to resorb urinary calcium and begin producing 1,25-dihydroxyvitamin D. Thus, at the onset of lactation, all calcium homeostatic mechanisms are active and able to prevent hypocalcemia.

Calcium restriction prior to calving has limited practical application for milk fever prevention. When poor-quality pasture or hay is the only feed available to dry cows, it may be possible to restrict calcium intake sufficiently to stimulate PTH production prior to parturition. Low overall dry matter intake of these forages makes very low total calcium intake feasible.

There is also evidence that high-calcium diets fed before calving also reduce the risk for milk fever. However, research data available to support this practice are limited.

Acidification through Diet

The effect of diet on an animal's acid–base balance is the main dietary determinant of risk for milk fever. Prepartum diets that evoke an acidic response in the animal will tend to prevent milk fever, whereas diets that evoke an alkaline response will tend to cause milk fever (*see* **Feed Ingredients**: Feed Supplements: Anionic Salts). Dietary calcium should not be severely restricted when acidogenic diets are fed.

Calcium Binding with Zeolite A

An alternate application of the calcium deficiency approach to milk fever prevention is to bind calcium with synthetic zeolite A (sodium aluminosilicate). Feeding a daily dose of about 0.5–1.0 kg zeolite A creates a functional calcium deficiency and substantially reduces the risk for milk fever. However, this approach also may decrease prepartum dry matter intake, decrease blood phosphorus and magnesium concentrations at calving, and increase tissue aluminum concentrations. The magnitude of these side effects may be dose dependent, and their biological significance is unclear. The postcalving performance of cows fed zeolite A has generally been lower than what would be expected for cows experiencing a reduction in hypocalcemia around calving.

Dietary Magnesium Supplementation

Dietary magnesium supplementation prior to calving often reduces the risk for milk fever. This observation is supported by the known physiological role of magnesium in calcium metabolism. Optimum precalving dietary magnesium is between about 0.35 and 0.40% (dry matter basis).

Dietary Sulfur Supplementation

Dietary sulfur may have benefits in preventing milk fever independent of its acidifying effects. The mechanism for this possible effect is unknown. Optimum precalving dietary sulfur is between ~0.30 and 0.40% (dry matter basis); higher concentrations may cause toxicity.

Vitamin D Treatment

Earlier literature often recommended injecting up to 10 million units of vitamin D_3 intramuscularly 10 days to 2 weeks prior to calving to prevent milk fever. Unfortunately, the dose of vitamin D_3 that effectively prevents milk fever is very close to the dose that causes irreversible metastatic calcification of soft tissues. Active vitamin D metabolites are more effective in preventing milk fever and are safer than vitamin D_3. However, they share some of the same limitations of vitamin D_3, and few are approved for use in dairy animals.

Prophylactic Calcium Administration

Prophylactic treatment of cows with intravenous and/or subcutaneous calcium immediately after calving may reduce the risk of milk fever. Oral calcium supplements may also be used to prevent as well as treat milk fever. Treatment with four doses of an oral calcium supplement (given prior to calving, at calving, 12 h postcalving, and 24 h postcalving) reduces the risk of clinical and subclinical milk fever in dairy cows by about half. This protocol works best when at least one dose of oral calcium can be administered prior to calving.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Grass Tetany; Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Pregnancy Toxemia. **Feed Ingredients**: Feed Supplements: Anionic Salts. **Mastitis Pathogens**: Contagious Pathogens. **Mastitis Therapy and Control**: Medical Therapy Options.

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Non-Infectious Diseases: Pregnancy Toxemia

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Introduction

Pregnancy toxemia is a disorder of energy and protein metabolism that occurs in sheep, cattle, and goats. The condition reflects an imbalance between fetal demand for nutrients and the supply of nutrients by the dam from either exogenous (dietary) or endogenous (body tissue) reserves. Pregnancy toxemia has a greater gestational incidence in polytocous animals, and this is reflected in the synonyms for the disorder, which include twin disease, twin lamb disease, and pregnancy disease. The condition is very similar to ketosis in cattle (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Ketosis) and produces a profound hepatic lipidosis.

Clinical Signs

The clinical signs of the disease include separation from the herd or flock, followed by depression and recumbency. Neurological signs are common in sheep and include tremors, myoclonic twitching of the facial muscles, incoordination, circling, and apparent blindness. In some cases, clonic seizures may occur. Physical examination of the animals indicates a decrease in pupillary light reflexes and an absence of the eye preservation reflex. The animals can assume a 'star-gazing' posture both while walking and when recumbent. Even with treatment, the prognosis for recumbent animals is poor, and death commonly results 3–7 days after recumbency.

Epidemiology

The condition is most prevalent in late gestation, especially during the 4 weeks before parturition in cattle and sheep. Affected animals are almost invariably carrying multiple fetuses. Younger animals, which are growing, are at greater risk than older stock. A sudden onset of cold weather for animals on poor-quality feed and with inadequate shelter can precipitate a marked increase in the incidence of the condition. Movement of stock will exacerbate the condition. Older ewes with poor dentition or flocks with a high prevalence of lameness may be at greater risk of pregnancy toxemia.

Pathogenesis

The pathogenesis reflects increased demand for nutrients by the fetus and adnexa, including placenta and uterus, during late gestation. The fetus requires glucose and amino acids, in particular, for energy and protein deposition. These requirements are difficult to meet from exogenous sources when poor-quality forages are the basal diet or when multiple fetuses are present. Unlike lactational ketosis, when ruminants reduce milk production, there is little opportunity in pregnancy toxemia for the dam to reduce the irreversible transfer of nutrients to the fetus.

In both sheep and cattle, the primary sources of macronutrients for the fetus and placenta are glucose and amino acids (**Table 1**). There appears to be limited capacity for placental transport of ketone bodies or short- or long-chain fatty acids. Consequently, use of acetate and β-hydroxybutyrate by the fetus and adnexa is limited in well-fed dams and the use of ketones and nonesterified free fatty acids is limited in underfed dams. In the case of sheep in late gestation, the contribution of glucose to the fetus and adnexa on a daily basis exceeds that available in the blood of the dam by 4 times. This implies a requirement for substantial gluconeogenic activity in the dam to meet the glucose requirements of the fetus and adnexa. The dam has a decreased insulin sensitivity in late gestation, which reduces glucose use by peripheral tissues and increases dependence on lipid and ketone metabolism. There is some evidence that high concentrations of ketones, 5–7 $\text{mmol}\,\text{l}^{-1}$ in blood, decrease hepatic gluconeogenesis and that 3-hydroxybutyrate clearance decreases in late pregnancy as compared to dry nonpregnant lactating ewes. **Figure 1** shows the typical increase in plasma nonesterified free fatty acids and β-hydroxybutyrate, and the changes in glucose concentrations in well-fed cattle around calving. The fatty acids mobilized from the body stores are complexed with blood albumin for transport to the liver and extrahepatic tissues. Uptake of fatty acids by the liver is proportional to the concentration of fatty acids in the blood. Fatty acids within the liver pool are stored as triglycerides and may subsequently be converted to ketone bodies, be oxidized, or be incorporated into lipoproteins in the Golgi apparatus for release into general circulation. Failure to provide sufficient amount of substrate to allow oxidation of fatty acids results in the accumulation of triglycerides in the hepatic cytosol. These can be exported from the liver as lipoproteins; however, mechanisms that

Table 1 Fetal sources and requirements of energy and nitrogen in cows in late pregnancy

Nutrient sources and requirements	*Energy (MJ day^{-1})*	*Energy (kcal day^{-1})*	*Nitrogen (g day^{-1})*
Sources			
Glucose and lactate	184.5	775	
Amino acids	311	1306	38
Acetate	60.7	255	
Total	556.2	2336	38
Requirements			
Tissue deposition	144	605	12
Heat	382	1605	
Urea	29.8	125	23
Total	555.8	2335	35

Modified from Bell AW (1995) Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *Journal of Animal Science* 73: 2804–2819.

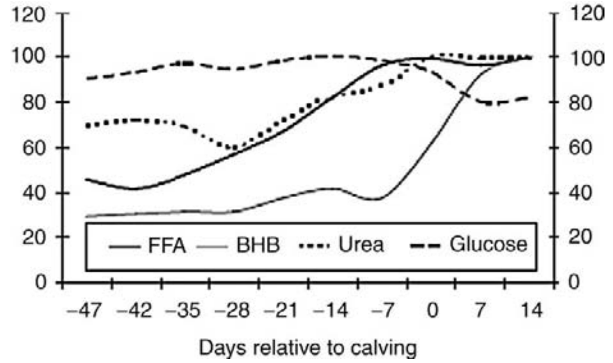

Figure 1 Relative changes in free fatty acids (FFA), β-hydroxybutyrate (BHB), urea, and glucose. Adapted from Stephenson KA, Lean IJ, Hyde ML, Curtis MA, Garvin JK, and Lowe LB (1997) Effects of monensin on the metabolism of periparturient dairy cows. *Journal of Dairy Science* 80: 830–837.

allow this are overwhelmed and hepatic lipidosis results in pregnancy toxemia.

In the relative absence of propionate influx from the rumen, there is a reliance on amino acid metabolism and lactate to provide carbon chains for gluconeogenesis. Sheep more susceptible to pregnancy toxemia have hepatocytes that produce less glucose from glucogenic precursors, including propionate, lactate, and alanine. The fetus has a strong demand for amino acids. Remarkably, these amino acids are used to supply energy to the fetus and adnexa (**Table 1**) and are used with a very low efficiency for fetal growth. Recent estimates suggest that up to 60 and 66% of the amino acids transported to the fetus and adnexa are catabolized in sheep and beef cattle, respectively. The demand for amino acids for gluconeogenesis is met from dietary amino acids and by mobilization of the labile protein reserves. Sheep on a higher plane of nutrition before being exposed to undernutrition are more capable of sustained gluconeogenesis than sheep previously fed on a low plane of nutrition. Once the labile protein reserves are depleted, there is little opportunity for the dam to produce glucose if the diet fails to provide sufficient protein or propionate. Because the ketones produced from the partial oxidation of fatty acids are metabolic acids, metabolic acidosis can occur in pregnancy toxemia.

Clinical Pathology

The clinical pathology associated with the condition includes hypoglycemia, increased nonesterified free fatty acids in plasma, hyperketonemia, and marked ketonuria. Blood glucose concentrations can fall below 2 mmol l^{-1}, and β-hydroxybutyrate concentrations can exceed 2.5 mmol l^{-1}. Plasma insulin and cortisol concentrations can be increased. Other changes in blood reflect electrolyte imbalances associated with acidosis, renal failure, and muscle damage, and include increased creatinine concentrations, increased serum aspartate aminotransferase, and dehydration. Plasma potassium concentrations are particularly sensitive to energy deficits in target tissues and increase with increasing deficit.

Necropsy

The most striking clinical finding is the characteristically enlarged, pale, fat-infiltrated liver. The edges of the organ are rounded and the liver is friable. Usually multiple fetuses are present, although a large single fetus may be found. There are signs of lipid mobilization from fat stores, and the kidneys, heart, and adrenal glands show signs of fat infiltration.

Diagnosis

Differential diagnoses usually include other conditions that cause sudden death in large numbers of sheep or goats within a flock. Usually, in sheep, an investigation of the epidemiology of the condition clearly indicates a strong probability of pregnancy toxemia. The diagnosis is confirmed following necropsy and the use of clinical pathology. In sheep, it may be necessary to differentiate the condition from hypocalcemia, which can also result after prolonged walking or transport, or with sudden feed change. In cattle, the condition is rarely as prevalent as in sheep and goats and the differential diagnoses include other causes of collapse and recumbency, including hypocalcemia and hypomagnesemia. The observation of body tissue loss in late gestation in cattle may indicate individuals and herds at risk of pregnancy toxemia.

Prevention

Prevention of the condition is achieved by ensuring that the nutrient requirements of heavily pregnant ruminants are met. The needs of the fetus for protein must be adequately recognized. This is particularly important under extensive pasture or range conditions, where the protein and energy content of forages may fall below requirements. Problems of feed quality can be exacerbated by a shortage of dry matter. Some of these forages may also be deficient in fat-soluble vitamins, which have a protective role in controlling free-radical damage.

Steps to prevent pregnancy toxemia can be outlined as follows:

1. Provide dry matter with sufficient energy, protein, mineral, and vitamin content to meet or slightly exceed the nutrient requirements of the stock. When the diet is forage-based, allocation of better pastures to stock in late pregnancy is a sensible strategy. When pasture is limited, use of fertilizers to increase pasture growth is advisable.
2. Identify stock with multiple pregnancies when feasible. Ultrasonic examination can achieve this, but good husbandry practices, such as identifying stock with greater abdominal fill and lower body condition, can also be effective in identifying stock at risk.
3. Provide additional supplements for stock carrying multiple fetuses and for stock on forages that do not meet nutrient requirements. Supplements should be grain-based and address protein needs in particular. Crude protein content of diets for ewes in late gestation should be 12–14% of dry matter.
4. Ensure that there is effective parasite control of the flock or herd.
5. Do not allow animals to become overly fat in pregnancy.
6. Provide shelter for animals likely to be exposed to marked adverse changes in weather conditions.
7. Do not allow animals to exercise excessively.
8. Ensure that ionophores are present at recommended rates (*see* **Feed Ingredients**: Feed Supplements: Organic-Chelated Minerals). Ionophores increase propionate flux and reduce ketone production.
9. Provide trace- and macroelements at recommended rates (*see* **Feed Ingredients**: Feed Supplements: Macrominerals; Feed Supplements: Microminerals). Cobalt and phosphorus have important roles as cofactors in energy metabolism, and transition metals such as copper, selenium, and zinc have roles in controlling free-radical damage.
10. Evaluate flocks for adequate dentition and control lameness in breeding ewes and does.

Treatment

The prognosis is poor for stock that are recumbent, whereas stock that are ambulatory with few clinical signs have a good prognosis. Treatments selected should reflect the severity of clinical signs. Costs of therapy are significant, and careful cost–benefit analysis is indicated before treatment can be recommended.

Hematology and blood chemistry can be useful in determining the most appropriate therapies and prognoses. Animals that have a poor prognosis show

- dehydration, indicated by increased packed cell volume
- acidosis, indicated by low blood bicarbonate
- evidence of renal failure, indicated by elevated blood urea nitrogen and creatinine
- markedly increased concentrations of β-hydroxybutyrate and free fatty acids in blood

Some animals may have evidence of sepsis associated with the death of the fetus. Treatments are essentially directed at correcting the changes in clinical chemistry or removing the irreversible loss of nutrients from the dam to the fetoplacental complex. They include the following:

1. Increasing the supply of exogenous glucogenic precursors by intravenous dextrose and glucose, and oral glucose preparations that are often combined with electrolytes to provide oral rehydration solutions, or oral propylene glycol (30–60 ml, 2 or 3 times daily, for ewes and does; 200–300 ml for cows).
2. Correcting electrolyte imbalances and dehydration by the use of calcium borogluconate solutions, intravenous fluids containing dextrose or glucose, and oral rehydration solutions.
3. Removing the fetus (emergency cesarean section can be indicated in valuable animals) and increasing endogenous glucogenic precursors by the use of glucocorticoids, including dexamethasone-21-isonicotinate and dexamethasone sodium phosphate.

Corticosteroids may also increase the availability of amino acids for gluconeogenesis, but corticosteroids are generally not recommended in cases with sepsis.

4. Hormonal treatments. Various hormones have been used to modify the metabolic changes that occur. These include anabolic steroids (e.g., trenbolone acetate; results are equivocal), insulin injections (e.g., 20–40 IU of protamine zinc insulin administered intramuscularly every 48 h for 4 days; results are equivocal), and recombinant bovine somatotropin (e.g., 0.15 mg per kg^{-1} of body weight daily; results appear promising).

On a herd or flock basis, the provision of larger quantities of better-quality feed is critical.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Diseases of Dairy Animals**: Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Ketosis. **Feed Ingredients**: Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Organic-Chelated Minerals. **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows.

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Parasites, External: Mange, Dermatitis and Dermatosis

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Introduction

Mange in cattle, sheep and goats is caused by any of several genera of host (species)-specific mites. Lesions result directly from the mechanical damage due to the presence of the mites. This damage occurs as the mites feed and move about, and from irritating secretions from the mites. Additionally, an immune-mediated hypersensitivity to foreign antigens from the mites produces inflammation. As the host responds to inflammation by rubbing or other types of self mutilation, the lesions increase in size and severity. Economic loss to the producer results from weight loss and decreased production. Diagnosis is provided by clinical signs and can be confirmed by microscopic identification of the mite. Treatment varies to some extent depending on the type of mite present.

Areas of concern with regard to the management and treatment of mange in cattle are transmission within the herd or to the handler and utilization of therapeutic modalities that do not have an impact on the wholesomeness of milk produced by treated animals. Also, infestation by some of the mange mite species should be reported to state regulatory agencies (United States) due to their communicable or contagious nature. Other skin diseases that may present similar lesions or clinical signs will also be discussed, so that a correct diagnosis can be determined and provide an appropriate treatment. Other skin diseases include ringworm, photosensitization, culicoides hypersensitivity and infestation with lice.

Epidemiology

There are five types of mange based on the genus of mite that infests the animal: psoroptic, sarcoptic, chorioptic, demodectic and psorergatic mange. Psoroptic mange is probably the most severe of the mange dermatoses and is caused by *Psoroptes* spp. *Psoroptes ovis* infects both cattle and sheep and *Psoroptes cuniculi* (the rabbit ear mite) affects goats. Psoroptic mange is spread by direct contact and fomites (brushes, blankets, etc.). It has been experimentally spread from cattle to sheep.

Sarcoptic mange is caused by *Sarcoptes scabiei* and, although considered to be relatively host-specific, can be transmitted to humans. Goats and sheep can also be affected. Sarcoptic mange is contagious and spread is typically by direct contact, although the mite survives off the host for a variable time, making spread by fomites possible.

Chorioptic mange is host-specific. The types affecting cattle, sheep and goats respectively are *Chorioptes bovis* (**Figure 1**), *Chorioptes ovis* and *Chorioptes capre.* Although the entire life cycle of the mite is spent on the host, transmission can occur by fomites in addition to direct spread. Because of the location of the resultant lesions (legs, perineum, etc.: **Figure 2**) and the fact that it is most evident in stalled animals during the winter, bedding is often incriminated as the source of transmission, but it is not the usual or most likely source.

Demodectic mange is host-specific. Cattle are affected by *Demodex bovis*, sheep by *Demodex ovis* and goats by *Demodex capre.* It is not considered to be contagious and in cattle at least is believed to be transmitted from dam to offspring very early in life.

Psorergatic mange is caused by *Psorergates bos* and is not considered to be of economic importance.

Clinical Signs and Diagnosis

The clinical presentations of the different types of mange are fairly similar. Most cause pruritus of varying degrees. Most result in loss of hair and damage to the hide. Clinical signs may be similar, to the point that a specific diagnosis can only be made by microscopic examination of the mite. Even then, slight morphological differences in the mites make referral to a parasitologist necessary if a specific diagnosis is needed.

Psoroptic mange causes a severe pruritus due to the severe inflammation resulting from the mites activity. Psoroptic mites live at the base of the hairs and actually pierce the skin. Their saliva is inflammatory. Exudate and scab formation follow. Affected animals rub and scratch continually and lose body condition. The lesions, which are most commonly found on the shoulders, withers and tailhead, are susceptible to secondary infections.

Severely affected animals may become very weak and die due to the secondary infections, inability to maintain body heat, fluid, protein and electrolyte loss, or a combination of these. It is most likely to be confused with

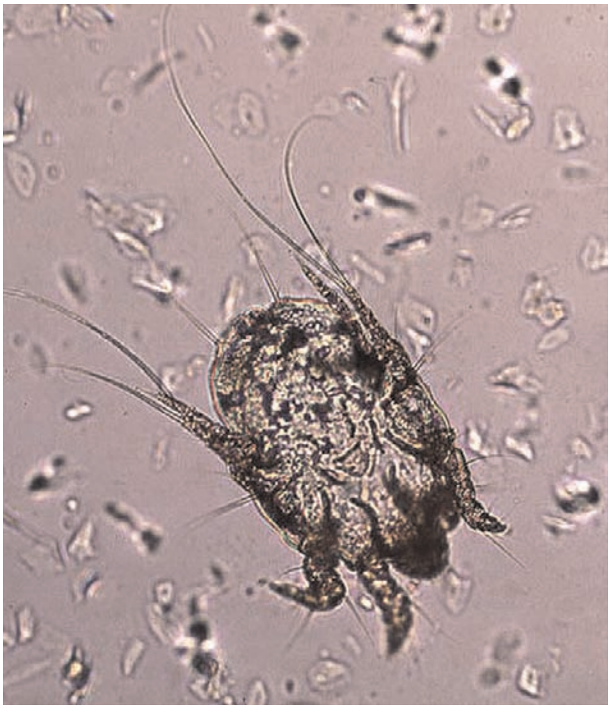

Figure 1 Chorioptic mange mite, *Chorioptes bovis*.

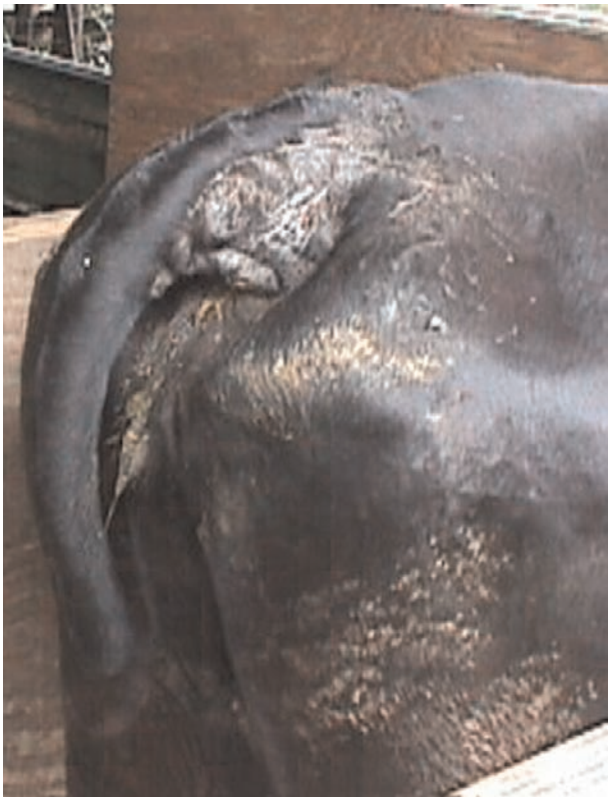

Figure 2 Holstein cow with chorioptic mange and excoriation.

chorioptic mange, although the intense itching and severity of lesions are more similar to the sarcoptic mange. Lesions in sheep and goats are similar to those found on cattle.

Sarcoptic mange, like psoroptic mange, produces severe itching. Because the female mite burrows into the skin, irritation and inflammation result. The pruritus can be so severe that animals will spend considerable time scratching and will not graze. Also, like psoroptic mange, lesions become worse with the excessive scratching and rubbing, leading to secondary infections. The lesions rarely form scabs, although there are crusts. The skin becomes thickened and folded due to the swelling and inflammation. Sarcoptic mange rarely affects sheep. Goats are affected about as much as cattle.

Chorioptic is probably the most common type of mange and causes a pruritus with lesions found on the perineum, legs, udder (or scrotum on a bull) and tail. When the legs are involved the lesions are often located on the pastern area and often the area near the hoofs (coronary band) will be affected. This usually results in lowered milk production. There is pruritus but it is not as severe as in either psoroptic or sarcoptic mange. Lesions begin as papules and progress to crusts and scabs.

In sheep and goats chorioptic mange lesions seem to be limited to the perineum, lower limbs and inguinal area. Young lambs with severe infestations in the scrotal area may later have lowered fertility. Some goats may be allergic to the mite, resulting in an exaggerated manifestation of the usual clinical signs. Others may be totally asymptomatic.

Demodectic mange seems to be of little clinical significance in cattle, goats or sheep. It does not cause pruritus and is usually manifested by nodules, crusts and small scabs. Because there is not as much inflammation as caused by other mange mites and since the animals do not itch and therefore do not scratch and rub, the skin and hair are not damaged as much. Animals severely affected may be immunocompromised.

Psorergatic mange is of little clinical significance. It causes hair loss and pruritus, but does not damage the deeper layers of the skin.

There are other skin diseases that cause clinical signs similar to mange. The two most likely to be confused with mange are ringworm (dermatophytosis) and lice infestation (pediculosis). Ringworm, a fungal infection, is more prevalent in young animals and, although secondary infection can occur, rarely causes the extensive lesions mange does. Lice infestation results in hair loss and pruritus, but lice can be visualized easily on close examination of the affected areas. Culicoides hypersensitivity, which is caused by an inflammatory reaction to the biting of *Culicoides* spp., causes a severe pruritus and because the animals will scratch and rub it may resemble mange

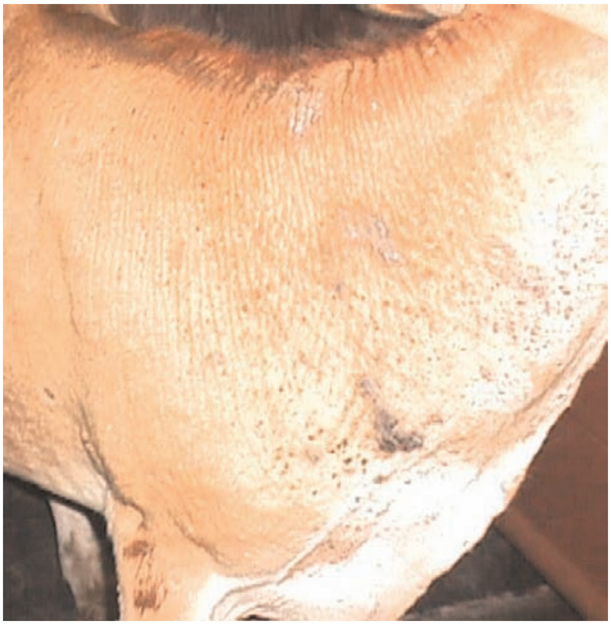

Figure 3 Jersey heifer with culicoides hypersensitivity.

(**Figure 3**). Photosensitization results from an increased response to sunlight or, more correctly, UV light. This is caused by increased levels of a photodynamic agent in the skin. The photodynamic agent can be toxin (plant or chemical) or phylloerythrin, which is a product of the liver. If the source is a plant or chemical toxin, the problem is termed primary photosensitization. If caused by the phylloerythrin, then it is referred to as hepatogenous photosensitization.

Treatment

Psoroptic, sarcoptic, chorioptic and psorergatic manges are all responsive to any of the antiparasitic drugs in the avermectin class. Specifically, ivermectin at $0.2\,\mathrm{mg\,kg^{-1}}$ administered subcutaneously is effective. However, this does not result in the immediate elimination of the mites, so infested animals should be isolated for at least 2 weeks following treatment. Although ivermectin is often utilized for demodectic mange and the author believes that its use is appropriate, its efficacy has not been established. Topical administration of amitraz at a concentration of 0.025% has been utilized for demodectic mange in cattle, goats and sheep. Various organophosphate compounds have also been utilized for psoroptic, sarcoptic and chorioptic manges. Caution should be exercised when treating lactating goats.

Lice can also be treated with ivermectin at the same dose. Ringworm and most other fungal infections can be treated with iodine-based shampoos or soaps. Culicoides hypersensitivity and photosensitization can both be treated with corticosteroids. The prognosis for photosensitization is poor if it is caused by liver dysfunction.

Prevention

Mange caused by the various mange mites can usually be prevented by the treatment of any herd additions with an avermectin product at the treatment dosage followed by a quarantine period of 2 weeks. Careful examination with close attention to any skin lesions prior to movement to a new area or herd is important whether one suspects mange or a fungal (ringworm) agent.

Conclusion

Although a specific diagnosis may require microscopic identification of the mite in question, the same treatment for the various manges and even lice infestation can be utilized. However, nonresponsive cases should be reevaluated and may require a definitive diagnosis. Also some of the manges are classified as reportable to the governmental livestock health agencies. Sarcoptic mange has the potential to be transmitted to humans. In any case, a veterinary surgeon should be consulted whenever a skin disease is nonresponsive or complicated by other infectious agents.

See also: **Diseases of Dairy Animals**: Parasites, External: Tick Infestations. **Husbandry of Dairy Animals**: Sheep: Health Management.

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Parasites, External: Tick Infestations

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Background

The ectoparasites that attack dairy cattle include insects, mites and ticks. There are at least 400 pest species that infest animals in the United States. The most important species of external parasites are shown in **Table 1**. These pest species are often more numerous in tropical regions due to longer summers, as in southern states of the United States. During winter months, heavy infestations of some tick species may also develop. Ticks are considered the most important external parasites of dairy cattle, mainly in tropical and subtropical areas of the world. They are responsible for considerable economic losses both through the direct effects of blood sucking and indirectly by maintaining and spreading vectors of pathogens, such as protozoan, rickettsial and viral diseases. Viral diseases cause major losses and are of great economic importance worldwide.

Ticks can survive in different ecological conditions, from ornamental shrubberies in suburban residential areas to pastures for cattle feeding (**Table 2**). Dairy cattle that graze in pastures are more susceptible to tick infestation than confined dairy cattle.

Figure 1 shows the adult stage of two different common ticks: an ear tick and the Rocky Mountain wood tick. It is not unusual for 1000 female ticks to be found on one cow at a time and together they can suck more than 0.5 l of blood in just 1 day.

Life Cycle of Ticks

In general, ticks have a three-stage life cycle with each stage depending on a new host on which it feeds. These stages are larvae (six-legged), nymphs (eight-legged) and adults (eight-legged). **Figure 2** depicts these stages in ticks. Ticks attach themselves to the host until feeding has completed and then drop back to the ground where they will look for shelter and develop to the next stage. This development may take several days or even months depending on weather conditions, but in general takes 2 months. The normal feeding time on the host is between 4 and 8 days; however, research on this topic has shown that it can last longer in extreme cases. All three stages feed on blood and after feeding, the tick falls from the feeding source and the larva will develop into a nymph; the nymph will moult to an adult and the female adult will lay eggs. Depending on the number of species they host, ticks can be one-, two- or three-host species.

Tick Families

There are about 840 tick species grouped into two major families, the Ixodidae or 'hard ticks' and the Argasidae or 'soft ticks'. The family Ixodidae includes approximately 80% of all tick species in tropical regions; the most important genera economically are *Hyalomma, Amblyomma, Boophilus* and *Rhipicephalus*. Most ticks found in the United States have a hard covering on the back and thus they are called hard ticks. Only one soft tick species affects dairy cattle and it gets its name because of the leathery texture of its body.

Hard Ticks

The bodies of hard ticks are more or less oval and pointed at the front. The hypostome anchors the tick to the host's skin, and the mouthparts are adapted for sucking blood. Flattened top to bottom, the abdomen can expand to several times its original size as a tick feeds on its host. This phenomenon is called engorgement, and is only seen in females. Male ticks are generally more colorful and ornate than females.

Soft Ticks

Soft ticks have a leathery outer skin rather than a hard cuticle, and both males and females engorge when feeding on the host. Their shapes vary between species. The spinose ear tick (*Otobius megnini*) is the only species from this family that affects dairy cattle. This tick lives in the ears of dairy cattle and the stage most frequently seen is the large engorging nymph. Adults are free-living and are found in hidden areas in the environment, such as within cracks in the wood of barns. These sites are preferred by females for laying eggs. Only larvae and nymphs of this species are parasitic.

Table 1 Important species of parasites of cattle in the United States

Common name	*Technical name*
House fly	*Musca domestica*
Stable fly	*Stomoxys calcitrans*
Face fly	*Musca autumnalis*
Horn fly	*Haematobia irritans*
Eye gnats	*Hippelates* spp.
Screwworm	*Cochliomyia macellaria, C. hominivorax*
Mosquitoes	*Aedes* spp., *Culex* spp., *Psorophora* spp., *Anopheles* spp.
Black flies	*Simulium* spp., *Prosimulium* spp.
Biting midges	*Culicoides* spp., *Leptoconops* spp.
Horse flies	*Tabanus* spp., *Hybomitra* spp.
Deer flies	*Chrysops* spp.
Biting louse	*Bovicola bovis*
Sucking lice	*Solenopotes capillatus, Linognathus vituli, Haematopinus* spp.
Mange mites	*Sarcoptes scabiei, Chorioptes bovis, Psoroptes* spp.
Cattle grubs	*Hypoderma bovis, H. lineatum*
Ticks	*Amblyomma* spp., *Dermacenter* spp., *Ixodes* spp., *Boophilus* spp., *Otobius megnini*

Adapted from Butler JF (1992) External parasite control. In: Van Horn HH and Wilcox CJ (eds.) *Large Dairy Herd Management*, p. 568. Champaign: American Dairy Science Association Press.

Table 2 Association between adult black-legged ticks (*Ixodes scapularis*) and vegetation ecotypes on dairy farms in Barron County, Wisconsin

Ecotype	*Number of ticks*[a]	*Ticks captured*[b]
Farmhouse garden		
Lawn	1/17	1/105
Ornamentals	0/19	0/120
Windbreak	0/11	0/95
Total	1/47	1/325
Pasture, lactating cows		
Open grass	0/14	0/75
Deciduous trees	0/2	0/20
Wooded or brushy	1/2	1/30
Total	1/18	1/125
Pasture, heifers and dry cows		
Deciduous trees	0/7	0/85
Wooded or brushy	9/30	17/265
Total	9/37	17/350
Ungrazed woodlands		
Total	27/53	70/505

[a] Number of positive samples out of total number of samples.
[b] Number of ticks captured for each minute of sampling.
Reproduced from Schmidtmann *et al*. (1998) *Journal of Dairy Science* 81: 718–721.

Worldwide Distribution of Ticks

Ixodidae Family

The genus *Hyalomma* is common in semi-arid regions such as China, Spain and Mauritania, where it is responsible for transmitting *Theileria annulata*, causing the disease known as tropical theileriosis. *Boophilus* species are one-host ticks and distribution includes all tropical and subtropical areas of the world. Most *Rhipicephalus* species are found in all

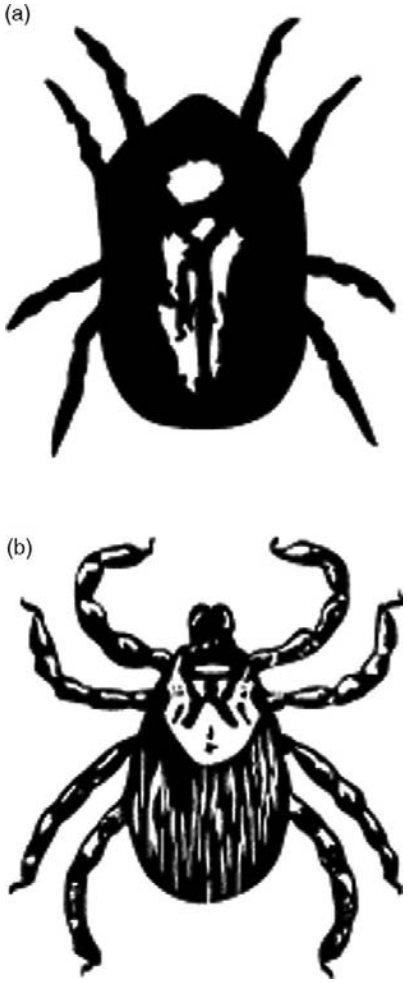

Figure 1 Examples of two ticks. (A) Ear tick, size 1–5 mm (courtesy of PG Koehler); and (B) Rocky Mountain wood tick, size 4–9 mm (courtesy of Ministry of Agriculture, Food and Fisheries, British Columbia Government).

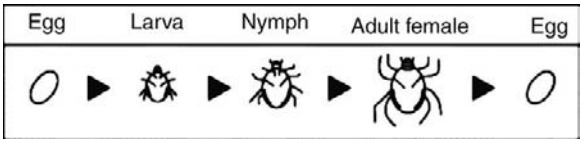

Figure 2 Common tick life cycle. (Courtesy of Frontline Products.)

countries of Africa. *Amblyomma* species are three-host ticks, which are distributed in tropical and subtropical areas of the world, especially in Africa. Some *Amblyomma* species, such as *Amblyomma variegatum*, have been introduced to Caribbean countries. *Amblyomma* ticks are vectors of cowdriosis or heartwater disease, which is considered to be one of the most important tick-borne disease in Africa.

Argasidae Family

Soft ticks of the family Argasidae, genus *Ornithodoros*, are vectors of endemic diseases caused by the pathogen *Borrelia* spp. Its distribution includes East, Central and South Africa, countries of the Mediterranean region (Portugal, Spain, North and West Africa) and it is extending eastwards through Cyprus, Israel, Iran, Central Asia and Kashmir to western China. In the American continent it can be found in central and western United States, and Central and South America southwards to northern Argentina.

Effects on Dairy Cattle

Tick infestation results in reduced milk production levels and reduced feed conversion efficiency. Ticks are also important because they expose dairy cattle to pathogenic microorganisms and cause blood loss and hide damage. Transmitted diseases include anaplasmosis, bovine piroplasmosis and tularaemia. Ticks also have the ability to transmit disease to the cows' offspring. They may also add stress on young replacement animals, delaying their entry into production and negatively affecting lifetime production performance. Heavy tick infestations, especially of sucking ticks, are very irritating to animals and rubbing and scratching behaviour may produce chronic sores and cause damage to fences, feed troughs or other equipment where the cattle are held. Other effects of ticks on the infected animal include inflammation, itching and swelling at the bite site, blood loss, production of wounds that may serve as sites of secondary invasion, obstruction of body openings and paralysis from the injection of toxic fluids.

Economic Impact

Losses to the livestock industry, in particular the production of cattle in tropical and subtropical areas, have been estimated up to be about US$109 billion annually. Each year US$150 million are lost in Australia's beef and dairy industries because of cattle tick infectations. Economic losses inflicted by cattle ticks in Latin America and the Caribbean, including the costs of acaricidal treatments, are calculated to range between US$6 to US$13 per head. This amount corresponds to an overall economic cost for this zone between US$2.5 and US$5.4 billion.

Control of Ticks

In order to obtain the maximum benefits from the use of tickicides it is important that the correct chemicals are chosen. Some factors to consider when choosing a tickicide are the cost, the extensive variety of species, variable life cycles and sometimes seasonal distribution.

Cost

Cost of tickicides is the most important factor for some dairy producers, especially in developing countries. A way to compare tickicides is by first estimating the cost per litre of made-up wash, both at the charging rate and the replenishment rate. The cost of treating an adult animal by plunge dipping or spray race is approximately the cost of 3 l of wash mixed at the replenishment rate. An adult animal treated by hand-spraying requires at least 10 l of fluid mixed at the charging rate.

Species

The dairy manager should know what kind of species is affecting the herd in order to be able to apply the most effective treatments at the proper time of the year. However, ticks spend most of their lives in the pasture because they seek humid conditions and therefore succeed in long grass and rushes. Areas with poor drainage can also harbour large tick numbers. Since most tick species bind to the external surfaces of cattle, the use of spray could be the most effective means of control.

Some chemicals used for control of cattle ticks are also effective against other external parasites. The correct identification of tick species is essential for a precise assessment of their disease vector potential, and in turn for decisions concerning appropriate treatment for pests and diseases of livestock.

Life cycle

The three stages of cattle tick – larvae, nymphs and adults – should be eliminated. Of these, moulting stages and engorged adults are most difficult to control. Dairy producers have to consider that there can be ticks at every stage of life cycle present on an animal at any one time. This situation makes implementing a method of control in one stage of the life cycle very difficult. Ticks have the capacity to survive for more than a year without feeding on a host, so leaving the pasture inactive for long periods of time is not a practical management control.

Treatments

There are several methods of controlling ticks but the most common and effective is chemical means. In general, tick control consists of treating pastured dairy cattle exhaustively with insecticides applied as dips or sprays. Other treatments include the use of liquid applications, dusts, insecticide ear tags and self-treatment devices, which frequently may not provide adequate control due to the fact that they are not exhaustive treatments. Experimental trials have shown that the persistence of liquid applications on dipping of dairy cattle is very poor. Some tickicides are appropriate for spraying but are not reliable in plunge dips, while others are not registered for spray races because of excess stripping. Recently, a tick development inhibitor (fluazuron) has been registered for cattle tick control. This product does not kill ticks but prevents their development. Improving drainage can control the number of ticks, as can keeping rushes and pasture trimmed through topping. One study reported that the use of neem oil diluted in water at a concentration of 1.0% has sufficient botanical insecticide in the form of azadirachtin to affect the life cycle and to control the cattle tick. It is recommended to spray the animals every 2 weeks in order to control ticks. **Table 3** shows some insecticides applied as sprays for tick control.

Control of tick-borne diseases (**Table 4**) depends on intensive control with acaricides. However, these products are toxic, expensive and generate ticks resistant to acaricides, which is the major concern to the dairy industry. Most acaricides have a residual effect, which gives protection against reinfestation for several days following dipping. This protection period varies between chemicals and can be affected by sunlight, heat, rainfall, dust and length of coat. Isolation of animals with a particular tick-borne disease is not recommended because it is possible that several tick-transmitted disease agents can occur

Table 3 Insecticides recommended as sprays for the control of ticks other than ear ticks on dairy cattle

Insecticide	*Tolerance (mg kg^{-1})*[a]	*Formulation and strength*[b]	*Amount per head*	*How to apply*
Crotoxyphos	0.02 in milk, meat and fat	EC, 0.15–0.3%	1–4 qt	Apply thoroughly; repeat after 1 week if needed; do not reapply more often than every 7 days
		EC, 0.5%	1–2 qt	Pen spraying with high-pressure device
		EC, 0.1–0.25%	1–2 gal	Apply second application 14 days later
Pyrethrins	0.5 in milk fat, 0.1 in meat, fat and meat byproducts	EC, 0.1%	Depending on size of animal and haircoat	Spray animals thoroughly as needed

[a] Legal tolerance for insecticide residues on food or feed products.

[b] EC, emulsifiable concentrate.

Reproduced from Harris RL (1971) Control of external parasites of dairy cattle. In: Esminger ME (ed.) *Dairy Science Handbook*, vol. 4, p. 90. Clovis: Agriservices Foundation Press.

Table 4 Tick-borne pathogen interactions

Disease	*Host*	*Geographical Area*
Babesiosis, anaplasmosis	Cattle	Australia, South Africa
Babesiosis, anaplasmosis Theileriosis, cowdriosis	Cattle	Sub-Saharan Africa
Babesiosis, anaplasmosis Tropical theileriosis	Cattle	Southern Europe Southern Asia, North Africa
Babesiosis, ehrlichiosis, louping-ill	Cattle	Northern Europe
Ehrlichiosis, louping-ill, tick pyaemia	Sheep	British Isles
Babesiosis, theileriosis	Sheep	Southern Asia, Southern Europe, North Africa

Adapted from Gray JS (1999) *Infectious Disease Review* 1: 117–119.

simultaneously when present in the same geographical region. This means that an effective tick-control programme will need to target several parasite species, including the vector tick, at the same time.

Future Trends in Tick Control

Epidemiological studies and socioeconomic evaluation of the impact of ticks and tick-transmitted diseases on survival or higher milk production systems are a priority, particularly for developing countries. At the present time, there are limited coordinated programs carried out in universities and research institutes, although on a country-by-country basis. Some interesting points to work towards in the near future are described below.

Vaccine Elaboration

The objective would be to replace infection and treatment procedures and prolonged vaccines using live parasites by inactivated or defined subunit vaccines, concentrating on tropical diseases such as theileriosis and cowdriosis. A re-evaluation of existing antitick vaccines would be another interesting topic. The possibility of developing subunit vaccines containing multiple components targeting different disease agents as well as tick components should be studied. In order to guarantee sustained antigen expression, different vaccine delivery systems, including DNA vaccination, should be examined.

Development of Improved Diagnostics

The advent of recombinant DNA technology should be exploited to produce standardized antigens for the diagnostic of tick-borne diseases. Diagnostics using enzyme-linked immunosorbent assays should be further developed and validated. Polymerase chain reaction technology for the identification of parasite DNA sequences in blood samples or tick extracts would be adapted to a format using simultaneous nonradioactive detection of different disease agents.

Epidemiology and Socioeconomic Surveys

Epidemiological and socioeconomic surveys of tick-borne diseases under different dairy production systems should be carried out in order to help in the design of optimal control strategies on a cost-recovery basis. The objective is to generate a database of existing information so that this can be assessed and the results used in the design of new surveys, aided by improved detection techniques. This is especially important in dairy herds located in tropical and subtropical areas as well as in developing countries.

See also: **Dairy Production in Diverse Regions:** Africa; Latin America; Southern Asia. **Diseases of Dairy Animals:** Parasites, External: Mange, Dermatitis and Dermatosis.

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Parasites, Internal: Gastrointestinal Nematodes

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Introduction

Traditionally, infections with gastrointestinal (GI) nematodes are considered to be important mainly in first-season grazing calves, and control measures are usually restricted to this age group. The effects of clinical disease in this age group have been clearly demonstrated and are well known to both farmers and practitioners. In contrast, infections with GI nematodes in older cattle were for a long time considered to be of no major importance, merely due to the absence of clinical symptoms and the (apparent) lower infection levels usually found in these animals. Data on the effect of GI nematodes on milk production have been controversial for a long time. However, two reviews of more than 75 clinical trials each showed that nematode infections in adult cows indeed have an effect on milk production. Due to the milk yield losses in adult animals, together with the continuing intensification of dairy production, the recognition of subclinical infections with a negative impact on production as disease, and the availability of highly efficacious anthelmintics with a zero-withdrawal time for milk, control of GI nematodes in adult cows is becoming established in an increasing number of dairy herds.

In this article, we will discuss (1) the importance of GI nematodes in dairy cattle, (2) the value of available diagnostic parameters to monitor infections in the adult dairy herd, and (3) the control of GI nematodes in dairy cattle.

Importance of GI Nematodes in Dairy Cattle

Parasites of Concern

Infections of domestic livestock with GI nematodes are an important cause of economic loss on farms throughout the world. Although infection levels generally decrease as animals age, several studies in the United States and Europe (United Kingdom, The Netherlands, Germany, and Belgium) have shown that GI nematodes are still widespread among pastured adult cows, with a prevalence of infection between 80 and 100%. This is not unexpected, since all pastures are likely to be contaminated, and all grazing animals are exposed to infection.

The common GI nematodes have a direct life cycle (**Figure 1**). The eggs, which are typical for the Strongylidae, are passed in the feces and develop in the fecal pat to the infective third stage (L3) within 2 weeks if temperature and humidity are optimal. When moist conditions prevail, the L3 migrate from the feces on to the herbage. After ingestion, the L3 exsheath in the rumen and further development takes place in the mucosa of the abomasum or the intestine. After two parasitic molts, adults emerge on the surface of the mucosa, around 3 weeks after infection. Under certain conditions, many of the ingested *Ostertagia ostertagi* L3 become arrested in their development at the early fourth stage (EL4), for a period of up to 6 months.

The important species of the GI nematodes of cattle in temperate climate areas are *O. ostertagi* and *Cooperia oncophora* and, to a lesser extent, *Nematodirus helvetianus*, *Trichostrongylus* spp., and *Oesophagostomum radiatum.* The relative importance of the genera differs with host age because of acquired immunity. A strong host resistance develops against *Nematodirus* and *Cooperia* within 1 year. *Ostertagia* and *Trichostrongylus* engender immunity more slowly and are therefore present even in older cattle. Burdens of *Ostertagia* in adult cattle vary from zero to over 200 000 worms. Within herds, the frequency distribution is such that about 75–80% of the animals harbor a low worm burden (0–10 000 worms) and about 15–20% harbor a high worm burden (>10 000 worms). The majority of these worms are present as inhibited (hypobiotic) fourth-stage larvae, which have a reduced pathology.

Subclinical Parasitism in Lactating Dairy Cows

Clinical symptoms due to GI nematode infections are rare in adult cattle and infections remain generally at the subclinical level. Subclinical parasitism is a term used to describe a degree of parasitism that interferes with production but is not evident by physical and visual examination of the animal. In practice, it is a state of parasitism usually diagnosed by a positive production response to the administration of an anthelmintic. For some parasites, such as liver fluke, it has been accepted for a long time that subclinical infections are responsible for a reduced milk production; however, it is only recently becoming generally accepted that the same effect occurs with subclinical GI nematode infections. Thus, the

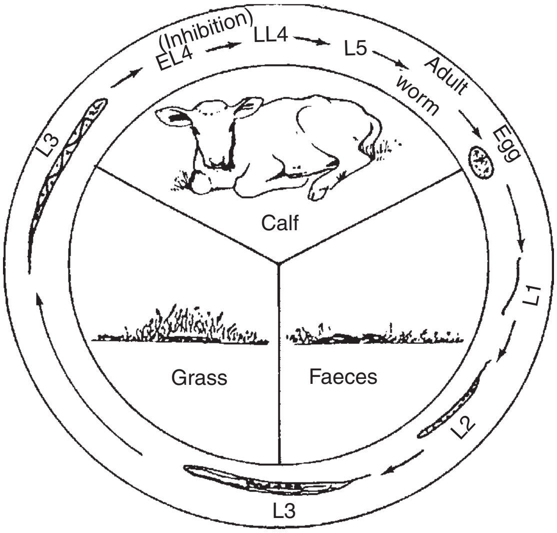

Figure 1 Life cycle of gastrointestinal nematodes in cattle. EL, early larval stage; L, larval stage; LL, late larval stage.

importance of subclinical GI nematode infection in adult cattle could also be referred to as 'economical' infection.

The economic importance of GI nematode infections in adult cattle, and especially lactating animals, has been debated for the last 50 years. Since the 1970s, reports from Europe and the United States have indicated that milk production of high-yielding cows increases following anthelmintic treatment around the time of parturition. This stimulated the worldwide interest in this subject, and since then many papers have been published on the effect of nematode infections on milk production. These studies can be divided into studies evaluating the effect of experimental infections, clinical trials evaluating the effect of anthelmintic treatment, and surveys investigating the relationship between *O. ostertagi*-specific antibody levels and milk yield. Most of these studies indicated that GI nematodes can cause significant milk yield losses. In an extensive review of 87 published clinical trials, it was found that a positive production response after anthelmintic treatment was observed in 80% of the trials with a median increase of 0.6 kg milk per cow per day. After taking into account small-study effects and publication bias, a meta-analysis of 75 published trials estimated the increase in milk production after anthelmintic treatment to be 0.35 kg per cow per day. In more recent trials ($n=6$), published after 2000, the average increase in milk yield after treatment in pastured dairy herds was around 1 kg per cow per day. This number agrees markedly with the results of studies that investigated the relationships of *O. ostertagi*-specific antibody levels in bulk tank milk and annual average milk yield, where an increase in the *O. ostertagi*-specific antibody level over the interquartile range was associated with a drop in milk yield of 0.9–1.2 kg per cow per day.

Next to the effect on milk production, a number of reports are available on the effects of GI parasitism on the reproductive performance of cows, suggesting that removal of the parasite burden in the dry-off or calving period may result in a reduced calving-to-conception interval.

From all this, we can conclude that in many cases anthelmintic treatment of adult cattle will be economically benefiting. However, whereas the above figures may reflect the average production response in a dairy population with access to pasture in a temperate climate zone, milk yield responses after anthelmintic treatment at the individual herd or cow level may vary from 0 to >4 kg per cow per day and it is a major challenge to identify key factors that determine this response. It has been shown that treatment responses vary depending on the study design and the anthelmintic drug used. Whole-herd treatment trials or trials that applied the treatments strategically throughout the year had higher responses than trials that applied the treatments at calving or in the dry period. Trials using endectocides had higher milk production responses than trials using older anthelmintics. In addition, a large part of the variation in the treatment response may be attributed to the fact that milk production is a highly variable trait, dependent on factors such as nutrition, breed, age, season of calving, and heritage. However, the single most overlooked fact is that unless sufficient numbers of parasites are present to inhibit the production performances, no response from anthelmintic treatment can be anticipated.

In addition, it is also important to distinguish between increased productivity and financial profit. There is a wide range of potentially hidden costs and benefits associated with parasitic disease and its control in production animals that have been largely ignored to date. Hidden costs may arise from increased labor, milk quota regulations, and selection pressure for anthelmintic resistance. Hidden benefits may accrue from increased resistance to other diseases. Incorporation of these factors could add much to the value and credibility of impact studies. Viewed broadly, we can conclude that the question is not whether anthelmintic treatment of cows is of any value, but rather when, under what conditions, and for which animals treatment is likely to be valuable.

Pathophysiology of GI Nematode Parasitism in Adult Cattle

Although many studies have shown an influence of subclinical GI nematode infection on production, little is known about the possible mechanisms for this interaction. It is known for a long time that parasitic nematodes, even in the absence of clinical disease, can cause a reduction in voluntary feed intake. The extent of metabolic impairment induced by a parasitic infection is influenced predominantly by the level of larval challenge and the number and species of worms that establish, but will be modified by host factors such as age, breed, nutritional status, and immune status. Inappetence is one of the main factors that can lead to reduced performance in subclinical infected animals. Recently, it was shown that lactating animals that were pastured under continuous stocking management and treated with an anthelmintic grazed on average 54 min per day longer than their untreated counterparts and this was associated with an increase in milk production. These effects occurred despite very low pasture infection levels. There are indications that the reduced appetite may be a result of the increased gastrin levels associated with the increased abomasal pH, which in turn is a result of damage to the parietal cells. However, the exact neuroendocrine mechanisms of parasite-induced inappetence are probably much more complex and are waiting to be explored by future research.

Besides reduced appetite, a second important mechanism related to the effect of subclinical GI nematode infection on productivity may be the energy requirements of the immune response. In sheep, it has been estimated that the maintenance of immunity to nematode parasites incurs a 15% loss of productivity due to the diversion of nutrients away from productive function to immunological tissues. Corticosteroid treatment of *Teladorsagia circumcincta*-infected lambs resulted in higher fecal egg counts and worm burdens, but improved energy utilization and performance as compared to nonimmunosuppressed infected control lambs. In cattle, it has been postulated that hypersensitivity reactions to even small numbers of developing larvae will produce changes in the gastric glands that are severe enough to result in mucosal permeability of the kind usually seen only in more heavy infections. The hypothesis on the economic cost of the immune response agrees also with the observation that cows with high *O. ostertagi*-specific IgG levels have on average a lower milk production than cows with low *O. ostertagi*-specific IgG levels.

Monitoring GI Nematode Infections in Adult Dairy Cattle: A Part of Herd Health Management

In adult cows in temperate regions, the only GI nematode of any significance is *O. ostertagi*. The other nematodes are of limited importance due to immunity development. Monitoring GI nematode infections in the dairy herd aims at determining whether the levels of infection or induced morbidity are high enough to induce significant production losses that could at least partially be recovered by implementing anthelmintic control measures. The determination of a threshold level for treatment of dairy herds is hindered by the rather low levels of infection in adult cows, the limited sensitivity of most diagnostic techniques, and the high variability of milk production, which is influenced by a number of factors other than GI nematode infection. Fecal egg counts and serum pepsinogen concentrations are considered poor indicators of the level of infection in adult cattle. In contrast, antibody levels against *O. ostertagi* are considered as a valuable parameter to monitor GI nematode infections in the adult herd. Especially the *O. ostertagi*-specific IgG level in milk has been evaluated, as milk samples enable noninvasive and automated measurements. Significant relationships were found between anti-*O. ostertagi* bulk tank milk antibody levels and several management practices known to be associated with GI nematode infection levels (e.g., level of exposure to pasture, anthelmintic treatment), suggesting that bulk tank milk antibody levels are a reasonable measure of the parasite infection level in dairy herds. In longitudinal studies in Belgium and Atlantic Canada, it was shown that milk antibody levels had a seasonal pattern that followed the expected intake of infective larvae from pasture. Other studies indicate that the *O. ostertagi*-specific antibody level can be used to evaluate whether GI nematode infections are impairing productivity. In a study of 863 dairy herds in Belgium, a negative relationship was observed between the *O. ostertagi*-specific antibody level in bulk tank milk and the herd's average annual milk production, after controlling for possible confounding factors. An increase in the anti-*O. ostertagi* antibody level over the interquartile range was associated with a drop in annual average milk yield of 0.9 kg per cow per day. A similar relationship was also observed in studies in Canada and France. In addition, three studies found that herds with a high bulk tank milk antibody level had a higher milk yield response to anthelmintic treatment than those with a low level, although two of these studies lacked statistical significance.

Based on all this, the anti-*O. ostertagi* antibody level in bulk tank milk is considered as a useful parameter to evaluate the level of infection with GI nematodes in a dairy herd and to estimate whether this infection is likely to cause production losses. Since recently, this parameter can be determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (Svanova, Uppsala) so that uniform test results can be obtained in different laboratories and monitoring GI nematode infections can become a part of herd health management in different countries (*see* Appendix; **Figure 2**). A drawback of the ELISA is that it is based on crude antigen from adult *O. ostertagi* worms and therefore cross-reactivity with other helminth infections such as lungworms and liver fluke occurs.

Current research focuses on the detection of anti-*O. ostertagi* antibody levels in individual milk samples to enable selective anthelmintic treatments within a herd. The investigations so far indicate that the precalving anti-*O. ostertagi* antibody level in individual milk samples can be used to identify the cows with the greatest milk

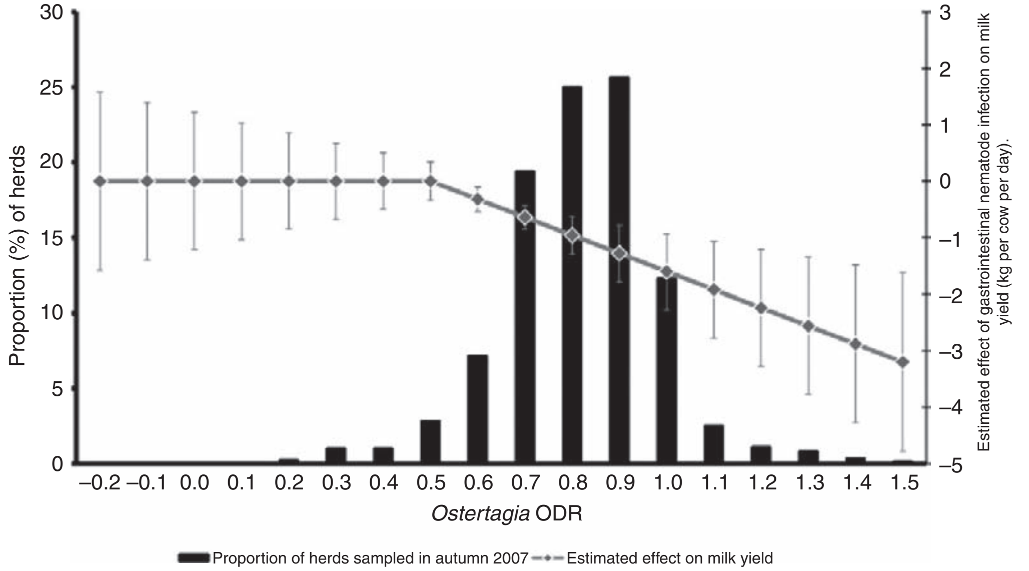

Figure 2 Interpretation diagram for the *Ostertagia ostertagi* ELISA applied on bulk tank milk in a monitoring program on 1053 dairy herds in 2007 in Belgium.

yield response after anthelmintic treatment, but further studies should confirm this.

Overall, since recently, it has become possible to include the measurement of *O. ostertagi*-specific antibody levels in herd health monitoring programs. However, rather than being the sole parameter to evaluate the GI nematode infection status, it should be interpreted in conjunction with information on pasture management, the likely presence of other helminth infections, and production measures. In this way, the herd owner can be assisted in deciding whether anthelmintic control measures are likely to result in an economic benefit. Further research should focus on the usefulness of individual monitoring, taking into account the costs of diagnosis and control measures and the likely benefits of increased production.

Control of GI Nematodes in Dairy Cattle

Many efficient anthelmintics are available for the treatment of GI nematode infections in cattle, for example, (pro-)benzimidazoles, levamisole, and macrocyclic lactones. Considering that in adult cattle the major part of the worm population are inhibited larvae, the use of macrocyclic lactones is preferred. The class of macrocyclic lactones, known as the avermectins/milbemycins (i.e., ivermectin, doramectin, moxidectin, and eprinomectin), is not only very potent against all parasitic life stages of nematodes, including inhibited fourth-stage larvae, but also has a persistent efficacy of several weeks. However, of all macrocyclic lactones, only pour-on formulations of eprinomectin and moxidectin are registered for use in lactating dairy cattle.

No recommendations for anthelmintic treatment strategies in adult dairy cows have been developed yet. To do so, there are three critical points that need to be considered. The first critical point is the unpredictability of the production response after treatment. Age and production level of the cows are two biomarkers that have been investigated to predict production responses, but with inconsistent results. The only identified useful parameter so far is the *O. ostertagi*-specific antibody level in serum or milk. It is important to notice however that even after using this parameter in conjunction with herd anamnesis, an important part of variation in production responses after anthelmintic treatment will remain unexplained, meaning that treatments may in some cases be given when little benefit will accrue and not be given when desirable in other cases.

The second critical point is the best timing of treatment. Generally, it is expected that when the production response over a whole lactation is considered, the largest benefit will be obtained by treatment in the dry-off or calving period because the energy demands of the cow are highest in the beginning of the lactation and it may modify the shape of the lactation curve. Treatment in the calving period will also allow to take advantage of possible beneficial effects on reproductive performance. On the other hand, a meta-analysis trial that applied the anthelmintic treatment in midlactation or strategically throughout the year estimated that milk yield responses increased by 0.4 kg per cow per day compared with trials treating at dry-off or at calving. In a study in the United Kingdom on 24 spring-calving naturally infected multiparous cows, significant treatment responses occurred following each of three treatments, which were administered at 7-week intervals during the grazing period and thus at different stages of lactation. Consequently, the timing of treatment seems less critical than previously thought and it seems that it can be chosen according to the farm-specific objectives and management system.

Since recently, a third critical point that should be taken into account when designing control programs is the consequence of treating adult cows on the development of anthelmintic-resistant nematodes. Although anthelmintic resistance is considered to be an important problem in nematodes of small ruminants, there are over the last few years reports on anthelmintic-resistant GI nematodes even in cattle. It is debated that important reasons why resistance has developed more slowly in bovine than ovine nematodes are the less intensive chemoprophylaxis generally applied in cattle and the fact that treatments are mostly restricted to the calves, leaving a greater proportion of the total parasite population in *refugia*. Based on this rationale, anthelmintic treatments in adult cows should be used rationally and concentrated on those herds with a high larval challenge and a reduced productivity. This makes monitoring GI nematode infections essential when treating adult animals is considered. In the same rationale, it would be desirable to target anthelmintic treatments to individual animals within a herd.

Besides anthelmintic treatment, control of GI nematode infections can also be achieved by grazing management. Control by grazing management aims at limiting the contact between host and parasite. In areas with a housing period, postponing turnout on pasture, mowing of pastures, and restricting the daily grazing time are considered as important protective measures.

Conclusion

There is substantial evidence that GI nematodes are of importance to the production and welfare not only in the replacement stock but also in the milking herd. However, a major challenge is to identify dairy herds or cows in which anthelmintic treatment is justified on economic grounds. An *O. ostertagi* ELISA has recently become available, which enables monitoring of the levels of exposure to GI nematodes and identification of herds that will most likely benefit from imposing an anthelmintic control strategy. However, further studies are necessary to define the optimal control strategy for different farm situations. Insight into the sustainability of different control options should be gained by modeling their effect on selection for anthelmintic resistance and by effectively monitoring drug efficacy in dairy herds. This should allow developing reliable and sustainable control recommendations of GI nematode infections in adult dairy cows.

Appendix: The Practical Use of the *Ostertagia ostertagi* ELISA

The *Ostertagia ostertagi* ELISA detects IgG antibodies against crude extracts of the adult worm in serum or milk. The test results are expressed as optical density ratios (ODRs). The recommended use in temperate climate zones where animals are housed is to apply the test on a bulk tank milk sample at the end of the grazing period. In this way, the level of exposure to GI nematodes over the past grazing season is evaluated. A high ODR points out that the animals have been exposed to a high larval challenge on pasture and that production losses have likely occurred. An interpretation diagram has been designed (**Figure 2**), where a line with error bars represents the estimated average loss in milk yield corresponding to a given ODR and vertical bars represent the frequency distribution of test results of tested herds in a specific region and time frame. In this way, the end user can judge whether the level of exposure on his farm can be considered high and whether the estimated production losses are important enough to implement new anthelmintic control measures. Such control measures could imply a (whole-herd or selective) anthelmintic treatment to prevent milk yield losses over the stabling period and control by pasture management to prevent the buildup of high larval challenges over the grazing period.

See also: **Diseases of Dairy Animals**: Parasites, Internal: Liver Flukes; Parasites, Internal: Lungworms. **Feeds, Ration Formulation**: Transition Cow Feeding and Management on Pasture Systems. **Forages and Pastures**: Grazing Management.

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Parasites, Internal: Liver Flukes

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Systematics

Liver flukes is the general name for those digenean trematodes (Phylum Platyhelminthes – Class Trematoda – Subclass Digenea) that live in the adult stage in the liver, the bile ducts, or the gallbladder of vertebrates. In veterinary science, two families are of importance: the Fasciolidae with the genera *Fasciola* and *Fascioloides* and the Dicrocoeliidae with the genus *Dicrocoelium*.

Particularly, the genus *Fasciola* with the species *F. hepatica*, the common liver fluke, and *F. gigantica*, the tropical liver fluke, is of utmost importance in cattle and sheep. The characteristics of infections with these species will be discussed in detail.

The genus *Fascioloides* with the species *F. magna* is primarily found in deer in North America and rarely in some parts of Europe. Cattle and sheep may occasionally become infected with this species. The infection is almost always without clinical signs in cattle, but in sheep the infection may have a pathogenic effect with sometimes lethal consequences. However, because clinical infections are rare, this parasite species will not be discussed further.

Dicrocoelium (*D. dendriticum* being the most important species) lives in the bile ducts and gallbladder. The genus has a worldwide distribution, but has a much lesser pathogenic effect than the *Fasciola* species. Further detailed information about *Dicrocoelium* can be found in the handbooks of veterinary parasitology. Here we will concentrate on *F. hepatica* and *F. gigantica* (**Figure 1**).

Geographical Distribution

Fasciola hepatica can be found on all continents, particularly in the temperate climatic zones. Within these zones, the presence of a suitable intermediate host determines the presence of *F. hepatica*.

Fasciola gigantica is the dominant species in tropical areas of Africa, South America, and Southeast Asia, but can also occur in more subtropical regions of the United States and southern Europe. There are areas in the world (e.g., India, Pakistan) where both *F. hepatica* and *F. gigantica* occur concurrently.

Life Cycle and Epidemiology

The life cycle of *F. hepatica* and *F. gigantica* involves a mollusk as first and only intermediate host. Many snail species of the genus *Lymnaea* (syn. *Galba*) can act as intermediate hosts. In Europe and many other parts of the world, *L. truncatula* is the most important host for *F. hepatica*, but in Australia and New Zealand *L. tomentosa* is the most important host. The favorite snail host for *F. gigantica* in southern Europe, southern United States, and the Middle East is *L. auricularia* and in Africa *L. natalensis* (**Figure 2**).

Part of the Life Cycle of *Fasciola hepatica* outside the Host

Fluke eggs produced by the adult worms reach the intestine with the bile and leave the host with the feces. Eggs have to be washed out of the feces, otherwise no further development is possible. The development must take place in a wet environment (**Figures 3** and **4**).

The developmental rate is dependent on the temperature. The minimum time required to develop to the first larval stage, the miracidium, is about 10 days at 26 °C and 6 weeks at 15 °C. Below 10 °C, no development is possible.

The miracidium leaves the egg after the 'lid' (operculum) of the egg is opened. The miracidium has to find a snail host within a few hours after hatching. On encountering a snail, it penetrates the snail with the help of proteolytic enzymes, and migrates as a young sporocyst to the hepatopancreas. The sporocyst develops into a redia and the redia may develop into daughter redia. Eventually, cercariae develop in the redia or daughter redia. The cercariae have many characteristics of the adult fluke, but they have a distinct tail. Cercariae leave the snail and swim around and settle on the vegetation. They lose their tail and form a cyst. This stage is called metacercaria. This is the infective stage for the mammalian final host. The development from miracidium to metacercaria requires a minimum of 5 weeks. Snails can shed cercariae for a couple of weeks. In dry periods, the release of cercariae can stop and can be continued when the conditions are wet again. Metacercariae can survive for long periods of up to almost 1 year. Survival depends strongly on humidity. Dry conditions are lethal. They can survive a few degrees below 0 °C, but severe frost is also lethal. So liver fluke can overwinter

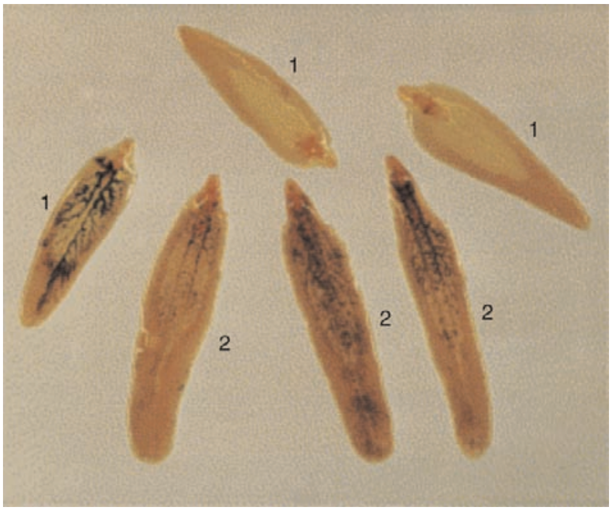

Figure 1 Adult *Fasciola hepatica* (1) and *Fasciola gigantica* (2).

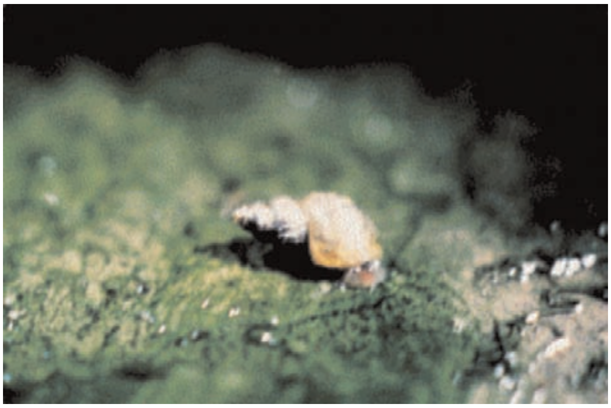

Figure 2 Adult *Lymnaea truncatula*.

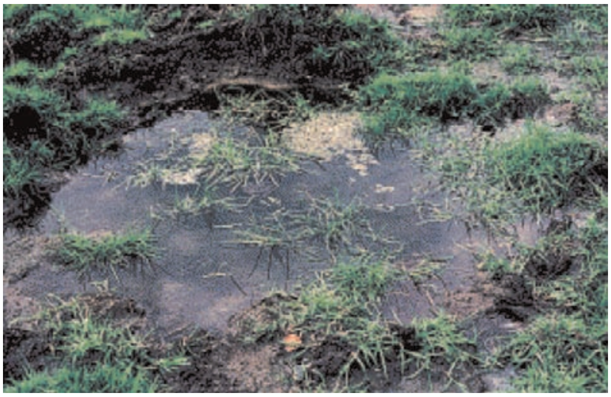

Figure 3 Typical liver fluke environment.

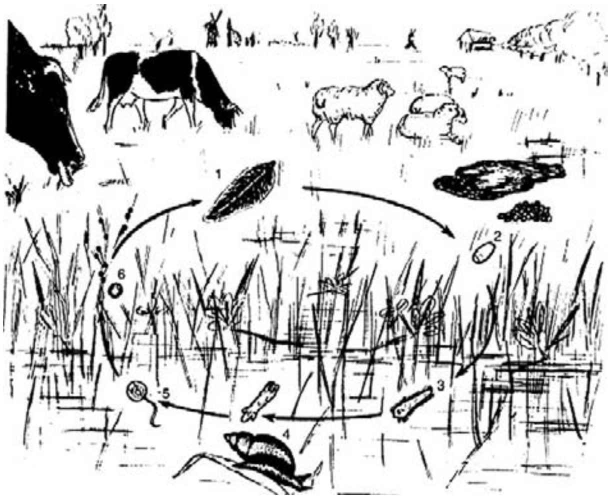

Figure 4 Schematic representation of the life cycle of *Fasciola hepatica*: 1, adult fluke; 2, egg; 3, miracidium; 4, redia; 5, cercaria; 6, metacercaria.

as egg, as sporocyst/redia in the snail, and as metacercaria on the vegetation. Within a year, two waves of infection of the vegetation are possible: the winter infection in the period April–July, caused by the shedding of cercariae by infected snails that have survived the winter; and the summer infection caused by snails that have been infected in the spring and summer due to overwintered eggs or eggs shed by cattle in the spring. This infection becomes available on the herbage from July onward with a peak in late autumn. It may be clear that in those places in the world where *F. hepatica* occurs the epidemiological pattern has been adapted to the local situation within the limits given above.

Part of the Life Cycle of *Fasciola hepatica* within the Host

After ingestion of the metacercariae, the outer cyst wall is removed under the influence of the digestive enzymes in abomasum and duodenum and the young fluke escapes actively from the cyst. It penetrates the wall of the small intestine with the help of proteolytic enzymes and reaches the peritoneal cavity. It can be found there within 1 day. The majority of the young flukes migrate in 3–4 days toward the liver, but occasionally flukes may reach other organs such as lungs, brains, or other organs (ectopic flukes). If young flukes are wandering in pregnant cows, they may reach the fetus and cause intrauterine infection, which may even result in abortion. However, most flukes penetrate the liver and wander through the parenchyma for a period of about 6 weeks to reach the bile ducts. In the larger bile ducts and gallbladder, they grow out in about 4 weeks to adult flukes of maximum length 5 cm and width 1.5 cm. Flukes are hermaphroditic (with male and female genital organs) and proterandric (male sexual maturity is reached first). The eggs that they produce are yellowish/brown gold in color with an operculum. The eggs have a maximum length of 0.15 mm and width of 0.09 mm (**Figure 5**).

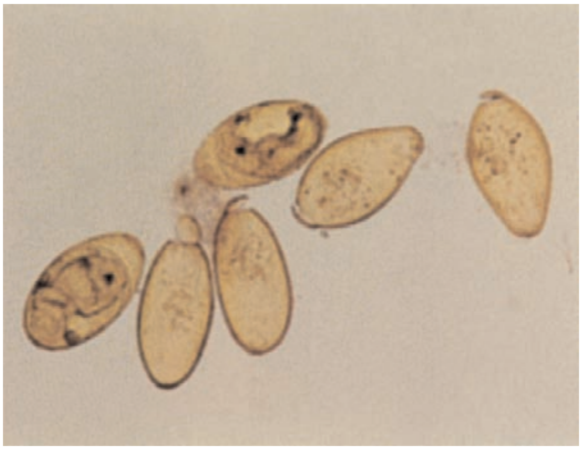

Figure 5 Eggs of *Fasciola hepatica*.

Egg production starts 10–11 weeks after ingestion of the metacercariae. This period may be delayed in secondary or later infections. In reinfections or heavy primary infections, flukes may become inhibited in their development due to a crowding effect, an immune response, or a combination of both. Without appropriate treatment flukes may live for years in their host.

Comparison of *Fasciola hepatica* and *Fasciola gigantica*

The life cycles of both species are almost identical. The intermediate hosts of *F. gigantica* are more aquatic than those of *F. hepatica.* The development of the parasitic stages of *F. gigantica* in the snail is slower. The same can be said for the migration process of *F. gigantica* inside the final host, resulting in a prepatent period of about 13–16 weeks. Adult flukes are larger (maximum 7.5 cm long and 1.2 cm wide) and of different shape compared to *F. hepatica.* Eggs are larger (maximum 0.19 mm long and 0.1 mm wide).

Pathogenesis

The pathogenic effect of a liver fluke infection is dose dependent. Low numbers do not cause harmful effects and thus infections are invisible to farmers and veterinarians.

We can distinguish between two phases of pathogenicity:

1. the effects caused by the migrating young stages and
2. the effects caused by the adult worms.

Young flukes escape from the metacercarial cysts in the small intestine and penetrate the intestinal wall. Massive penetration can cause peritonitis, but generally during the first weeks after infection no damage can be observed. Real damage occurs when the young flukes make their way through the liver parenchyma. They feed themselves with hepatocytes and erythrocytes, which are digested by the proteolytic enzymes that they excrete. These enzymes, in combination with excretion products of the flukes, also cause damage to the surrounding tissue, which becomes filled with blood and eosinophils. The end result is fibrosis of the liver tissue, which can be easily observed after slaughter. This first part of the migration phase is characterized by an increase in blood loss, a decrease in serum albumin level, and an increase in the level of hepatocyte enzymes in the serum such as glutamate dehydrogenase (GLDH). In cattle, the damage caused by young migrating flukes, though sometimes severe, rarely ends with the death of the animal. This may occur in sheep and is called acute fasciolosis, while in cattle the chronic disease is common. When the growing flukes reach the bile ducts, the liver parenchyma can be repaired by the formation of new liver tissue, which can be recognized by its lighter color. The level of the enzymes released due to the destruction of parenchymal cells decreases, but albumin loss and anemia persist.

In the bile ducts, the irritation of the wall by the migrating flukes causes hyperplasia and infiltration of fibrous tissue and an increase in the level of γ-glutamyl transferase (γ-GT) in the serum (**Figure 6**).

Secretory and excretory products of the flukes may increase the inflammatory processes. Calcium is deposited and the characteristic 'calcified liver fluke liver with pipes' is formed (**Figure 7**).

Immunity against Liver Fluke

It is generally accepted that cattle, in contrast to sheep, can build up immunity against liver fluke infections. This may be a combined effect of the immune system of the host and the changes in the liver caused by earlier infections, which may make the place unpleasant to live in for new incoming flukes. As a result, lower numbers of migrating flukes reach the bile ducts and gallbladder. These worms are generally of smaller size, produce fewer eggs, and have a shorter patent period. Immunity can be maintained only when herbage with new metacercariae has been ingested regularly. The best place to kill young flukes is when they try to penetrate the intestinal wall. It is possible to make rats 100% immune to new incoming infections, but in cattle this has not been accomplished so far.

Clinical Signs and Diagnosis

Light infections are without clinical signs. Fasciolosis in most cattle is not acute, but chronic. In temperate regions, disease will be mostly observed in late autumn

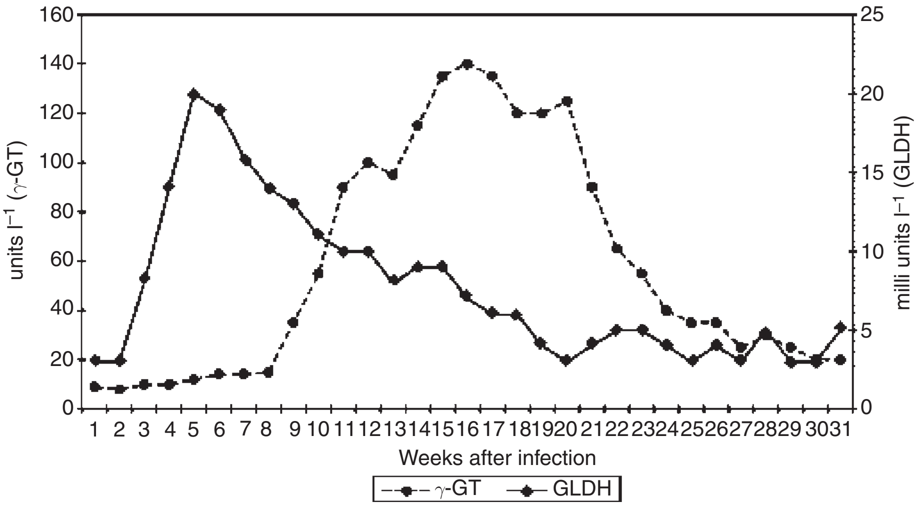

Figure 6 Pattern of serum enzymes after primary infection with 1000 metacercariae of *Fasciola hepatica*. GLDH, glutamate dehydrogenase; γ-GT, γ-glutamyl transferase.

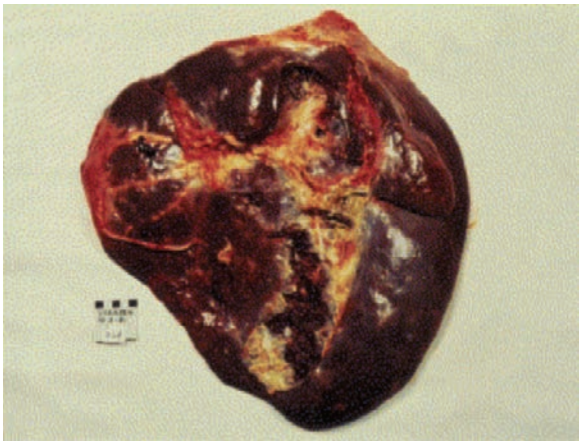

Figure 7 Liver of a calf heavily infected with *Fasciola hepatica*.

and winter. Calves and yearlings may show anorexia, loss of weight, and signs of anemia, including sometimes submandibular edema. In dairy cattle, there is a lower milk yield; the fertility might be influenced, resulting in a longer interval between pregnancies and, rarely, abortion. Food conversion in infected animals is far from optimal and at slaughter the carcass quality is lower.

The presence of adult flukes can be demonstrated by the presence of eggs in the feces. Various techniques are available, and most of them are based on sedimentation, because fluke eggs are rather heavy compared with the eggs of gastrointestinal nematodes, which easily float in flotation media such as saturated NaCl. Fluke eggs can be recognized by their color and shape. People who are not specialized may possibly confuse them with the eggs of paramphistomes, although these eggs are generally larger and of different color.

Diagnosis can be confirmed by serum parameters such as increase in the level of γ-GT and GLDH and the number of eosinophilic leukocytes. However, these parameters may vary according to the stage of the infection and are not always specific.

Serological techniques have also been developed. The ‘old’ indirect hemagglutination test has been replaced by modern enzyme-linked immunosorbent assays (ELISAs) with very high sensitivity and specificity. Both antibody- and antigen-based ELISAs are available. Very high-sensitive antigen-based ELISAs are able to demonstrate the presence of even a few flukes.

The ‘earliest’ ELISAs can be used from 2 weeks after primary infection.

Diagnosis of fasciolosis after slaughter is easy. The lesions caused by the worms are typical (fibrosis and cholangitis) and cannot be confused with other infections of the liver (e.g., larval tapeworms).

Heavily affected livers will be condemned and are excluded for human consumption. Apart from the already mentioned weight loss and reduced milk yield, this forms another important source of economic loss.

Control of Liver Fluke Infections

Liver fluke infections can be controlled in several ways. First of all, in some countries, a forecast system has been developed to indicate the risk of infection. Control itself can be divided into control measures related to the intermediate host and control measures directed toward the parasitic stages in the host. Finally, in the future, development of vaccines can be expected.

Forecast of Liver Fluke Infections

Since the second half of the twentieth century, forecast systems have been developed in the United Kingdom and other countries. In most systems, the ingredients for the forecast are a combination of rainfall or days with rain in 'important' months, evapotranspiration, condemnation figures of lamb livers in the second half of the year, and infection percentages of snail populations. Systems may be adapted to the local situation. In a small country such as The Netherlands, the forecast is given per district, because local differences in meteorological data may vary reasonably. In September, a preliminary forecast is published, and in November, a definitive forecast is distributed by television, radio, and appropriate journals. Today, modern satellite sensor techniques and GIS (global information systems) are also available in some countries as new tools.

Measures to Prevent Uptake of Metacercariae

On 'fluky' farms or in 'fluky' areas, it is possible to make a map with places where snails are present and infection of herbage with metacercariae may occur. Pasture management may be directed toward avoiding these places. This may vary from fencing of dangerous spots to grazing on pastures only in safe periods. Another possibility in some countries is to lower the ground water level, thereby influencing the snail habitats. All these measures fit very well in an integrated management system for sustainable agriculture with the aim to reduce the application of chemicals.

The use of molluscicides has been practiced in the past. Today, this is no longer an option in many countries due to the costs, the sometimes disappointing results, and for obvious ecological reasons.

Control of the Flukes in the Host

A number of anthelmintics to control flukes in the host are available. These flukicides are listed in **Table 1**. The availability of these drugs may vary from country to country depending on the local registration. Today, triclabendazole is the first-choice flukicide, although not registered in all countries with liver fluke problems. Triclabendazole is the only drug with excellent activities against all stages of the infection. That means that it can be used in the prepatent period, thus preventing the damage caused by adult worms and preventing egg excretion and pasture contamination.

Apart from their costs, a big disadvantage of all flukicides is that, if applied in dairy cows, there is a withdrawal period for the milk. The use in the dry period does not always coincide with the optimal time of treatment. Another drawback is the emergence of anthelmintic resistance.

Vaccination

Following the successful introduction in the late 1950s of a vaccine against lungworm in cattle by dosing them with irradiated infective larvae, studies have been undertaken to achieve the same with irradiated (γ- or X-ray) metacercariae. These studies have been carried out for both *F. hepatica* and *F. gigantica* and have shown some spectacular results. Protection percentages of up to 70–80% and sometimes even more have been reached in cattle, but this technique has never been commercialized.

Table 1 Fasciolicides (drugs against liver flukes) for usage in cattle

			Active against flukes			*Withdrawal time (days) for*	
Generic name	*Route of administration*	*Dose (mg kg^{-1} BW)*	*0–6 weeks old*	*6–12 weeks old*	*>12 weeks old*	*Milk*	*Meat*
Albendazole	*Per os*	10	−	−	+	5	8
Bromphenophos	*Per os*	12	−	−	++	5	21
Closantel	*Per os*/injection	2.5	−	+	++	*a*	30
Clorsulon	*Per os*/injection	8	−	+	++	*a* (injection) 3 (*per os*)	3 (injection and *per os*)
Netobimin	*Per os*/injection	20	−	−	+	5	14
Niclofolan	*Per os*	3	−	−	++	5	7
Nitroxynil	Injection	10	−	+	++	5	30
Oxyclozanide	*Per os*	10	−	−	++	4	14
Rafoxanide	*Per os*	7.5	−	+	++	*a*	28
Triclabendazole	*Per os*	12	++	++	++	28	42

[a] Not registered for milk-producing animals.

−, not active; +, 50–90%; ++, >90%.

Other vaccination studies have been carried out with crude somatic antigens, antigens derived from secretory and excretory products, and with well-defined antigens. Although there is not yet a commercial vaccine available, modern work concentrates on the possibility to use fatty acid binding proteins (FABPs), glutathione *S*-transferase (GST), hemoglobin, and particularly proteolytic enzymes (e.g., cathepsins) alone or in combination as major vaccine candidates. Production of these candidates by recombinant techniques is on its way. It is expected that more vaccine candidates will become available, with DNA vaccination being another option to be worked out in the future. With the growing threat of resistance to anthelmintics, development of vaccines is necessary to remain in the race against liver fluke.

Genetic Resistance

It has been demonstrated that there is a difference in susceptibility among breeds of sheep against liver fluke. Although comparable studies in cattle have not yet been carried out, it is reasonable to think that these differences can also occur among cattle breeds. This may give us a clue for identifying resistant genes and their use in selection studies.

Drug Resistance

Although laboratory selection studies carried out in the last decade of the twentieth century had already shown that *F. hepatica* in sheep was able to develop resistance against anthelmintics such as rafoxanide, closantel, benzimidazoles, and triclabendazole, the first reliable field observation of triclabendazole resistance was reported in sheep in Australia in 1995. Later, in Ireland, the United Kingdom, and The Netherlands, field cases in sheep had been observed as well; in The Netherlands, resistance was also found in flukes in cattle. The number of reported cases is still increasing and it poses a threat to the future of anthelmintic usage. Another worrying factor is that, similar to the situation in benzimidazole-resistant nematodes, there is no reversion to susceptibility when triclabendazole treatment is stopped and then replaced by another flukicide. Therefore, sustainable agriculture with minimal use of flukicides is necessary to keep the existing drugs effective.

No cases of anthelmintic resistance against the above-listed drugs in *F. gigantica* have been described in the literature. A possible explanation might be the lesser frequency of treatment of *F. gigantica* and thus the lower selection pressure on the worm populations.

Recently, in 2007, the European Union has started a special liver fluke project (DELIVER) in which many aspects of liver fluke and liver fluke disease, such as epidemiology, drug resistance, and vaccine development, will be studied by expert scientists. Information can be found on the website.

See also: **Diseases of Dairy Animals**: Parasites, Internal: Gastrointestinal Nematodes; Parasites, Internal: Lungworms.

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Relevant Websites

www.deliver-project.eu

Parasites, Internal: Lungworms

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Introduction

Dictyocaulosis or lungworm disease in cattle is still common, particularly in dairy cattle in western Europe. In the tropics, lungworm disease is rare and only in Cuba it is considered a problem. In North America dictyocaulosis is less important than in Europe. Usually the disease is seen in calves at the end of the grazing season. However, throughout western Europe the incidence of the disease in lactating dairy cows has increased considerably since the early 1990s. The population dynamics of lungworm infections is characterized by a rapid dispersal of larvae to pasture, a high mortality rate on pasture, and a rapid development of immunity. This implies a 'race' between the buildup of infections and resistance against these infections in susceptible cattle. The outcome decides whether disease will occur.

For the control of lungworm a good understanding of the epidemiology of infections is required, and to determine epidemiology of infections, proper diagnostic methods need to be available.

Life Cycle

The adults live in the bronchi and bronchioles. Eggs containing first-stage larvae (L1) are coughed up and swallowed, and they hatch during passage through the digestive tract. At mean temperatures above 10 °C the infective third-stage larvae (L3) develop within 1 week. Immediately after development almost all the L3 that are dispersed to the herbage do so in the sporangia of the coprophagic fungus *Pilobolus*. Thus, in western Europe lungworm larvae reach the herbage within a week throughout the grazing season. Other means of dispersal such as diarrhea, rain, earthworms, insects, and mechanical means (hoofs, boots, machines, etc.) are less important, although there is some evidence that torrential rain and subsequent flooding may be important in southern United States. In spring and summer, most larvae die within 2–3 weeks in western Europe. In autumn and winter, survival is longer. This rapid appearance of lungworm larvae on pasture by *Pilobolus* and the high mortality rate of these larvae differ completely from the situation with gastrointestinal nematodes.

After ingestion the L3 penetrate the intestinal wall and reach the lungs where they mature after two additional molts via lymph, mesenteric lymph nodes, and blood. The prepatent period is 3–4 weeks. Because immunity develops rapidly, the patent period is only 6–8 weeks when primary infections are high. When initial infections are low patent infections last 1 or 2 months longer. Later on, larvae may reappear in low numbers in a small proportion of reinfected animals. These are often nonsymptomatic 'carriers' and are very important in the epidemiology of lungworm infections. The occurrence of carriers in spring is usually associated with the maturation of larvae that overwintered as inhibited early fifth-stage larvae in the host. As in the case of *Ostertagia ostertagi*, inhibited development is primarily a seasonal phenomenon.

Clinical Signs

In the course of a heavy primary infection the following four phases can be distinguished:

1. Penetration phase (Day 1–7): penetration of the larvae into the body and migration to the lungs
2. Prepatent phase (Day 7–25): development of the larvae in the lungs
3. Patent phase (Day 25–55): production of eggs by mature worms
4. Postpatent phase (Day 55–90): occasional flare-up of severe respiratory signs at the end of the disease

The first clinical signs appear at the end of the prepatent phase when blockage of the bronchi and small bronchioles by eosinophilic exudate produced in response to migrating larvae occurs. Clinical signs are most severe during the patent phase. This phase is associated with adults in the bronchi producing bronchitis. Most of the major clinical signs are caused by primary parasitic pneumonia, in which macrophages and giant cells, engulfing aspirated eggs and larvae, consolidate the lung lobules. In the postpatent phase, patients gradually recover. However, in severely affected animals a sudden exacerbation of dyspnoea may occur during week 7 or 8 as a result of widespread alveolar epithelialization.

When immune cattle are reinfected, larvae may reach the bronchioles before being destroyed by the immune response. When many larvae have been ingested, this can lead to disease, which is called the reinfection syndrome. Diagnosis of this type of lungworm disease is often difficult because there will be no or very few adult worms. Consequently, fecal or serological examination is usually negative. The reinfection syndrome is probably the commonest form of lungworm disease in adult cattle.

The gradual development of bronchitis and pneumonia results in coughing and tachypnea accompanied by varying degrees of anorexia, decreased weight gain, and dyspnea. Pyrexia may occur when secondary bacterial infections are involved. In severe infections coughing becomes frequent in weeks 2 and 3. The respiration rate may exceed 70 breaths per minute in week 3. During the patent phase, respiration rate exceeds 70 breaths per minute and animals cough frequently. Dyspneic cattle stretch their necks in a characteristic air-hunger position and stick out their tongues each time they try to cough. On auscultation of the lungs, the inspiratory and expiratory sounds are harsh, and rhonchi are present. Varying degrees of emphysematous crackling occurs.

Epidemiology

Development of Immunity

Cattle are capable of rapidly acquiring a strong immunity against *Dictyocaulus viviparus*. However, there appears to be two expressions of immunity, acting against either the larvae before they reach the lungs (pre-lung phase of infection) or the juvenile and adult stages in the lungs (lung-phase of infection). The major difference between the two expressions of immunity is the apparent absence or presence of an 'immune memory' toward the migrating larvae, or the developing juveniles and adult worms, respectively.

Between 8 and 11 days after a high primary infection, protection against establishment of reinfection develops rapidly. In the presence of developing larvae and adult worms, protection is slowly reinforced during patency. However, in the postpatent period, protection against establishment wanes gradually in the absence of reinfection, and after 6–12 months cattle are virtually fully susceptible again.

Immunity also develops against the maturation of juvenile larvae in the lungs, between 10 and 30 days after primary infection. Patent infections appear to be necessary for the development of this type of acquired immunity. In contrast to the protection against establishment, this type of acquired immunity does not seem to wane. This likely explains why high infections in adult animals may result in clinical disease without development of patent infections. Protection against establishment has then virtually been lost in the absence of reinfection, whereas protection against maturation of the worms still exists.

Against lungworm a live-attenuated larval vaccine exists. Using this vaccine by itself will not result in patent infections and consequently will not generate a significant level of immunity against juveniles and adult worms in the lungs. The vaccine mainly triggers protection against establishment of larvae, which gradually wanes in the absence of reinfection. Booster infections are therefore necessary for animals to fully acquire and maintain both types of protection. The level of protection is exposure-dependent, and low primary infections will not lead to a sufficient immunity level to offer protection from disease after immediate reinfection. However, low natural exposure levels, even when cut by chemoprophylaxis, can result in sufficient immunity levels later on.

Prevalence of Infections

Very few studies are available on the proportion of cattle farms with lungworm infections. High-prevalence regions, where infections occur on the majority of farms, are found in some countries in western Europe, such as the United Kingdom, Ireland, and the Netherlands. Studies in the latter suggest that prevalence in dairy replacement heifers may have decreased during the last two to three decades. This coincides with an increase in outbreaks in dairy cows, not only in the Netherlands but throughout western Europe. Possibly this is related to the increase in suppressive anthelmintic treatments in dairy replacement heifers. On the one hand, decreased lungworm transmission may then have resulted in elimination of lungworm infections on some farms, leaving a fully susceptible adult herd that will be affected after reintroduction of lungworm. On the other hand, many adult dairy herds may contain some carriers. Introduction of heifers with no previous exposure to lungworm to the dairy herd containing such carriers may result in low primary infections in those heifers, this subsequently may be followed by heavy infections and disease in the whole herd. In the latter case, adult cows will show the 'reinfection syndrome'. In the Netherlands, this is the most likely cause of outbreaks in dairy cows.

In other regions in Europe, such as Scandinavia, prevalence rates have always been lower. Outbreaks then occur in any age class of cattle and not mainly in the young stock, unlike in high-prevalence areas.

Sources for Primary Infections

The onset of primary infections is very important for build up of infections and disease. The following situations may occur.

Overwintering on pasture

Overwintering of larvae on pasture is not a reliable phenomenon in Europe, except in the very humid Atlantic regions of the United Kingdom and Ireland. The size of the herd and the moment of turnout are important because whenever larvae are present on pasture in spring, numbers decrease rapidly. Thus, it is more likely that at least one animal develops a patent infection when a large herd is turned out early.

Carriers

Carriers may be yearlings, other groups of calves, or cows. The latter appear to be particularly important in the Netherlands. Many outbreaks in dairy replacement heifers can be linked to carrier cows having contaminated calf pastures. It has also been shown that patent infections occur in some cows in almost any herd in spring. This also implies that most Dutch dairy cows are exposed to low lungworm infections annually, thus allowing maintenance of immunity. The carrier situation in other countries in western Europe is somewhat less clear, but it may be similar to the situation in the Netherlands.

A separate category of carriers may be deer. Nowadays, it is recognized that *D. eckerti* from deer and *D. capreolus* from roe deer and moose are closely related, though separate species. Experimental cross-infections with *D. eckerti* from deer to cattle have been demonstrated. Patent *D. viviparus* infections have been demonstrated in red deer in New Zealand. Nevertheless, it is difficult to quantify the possible role of deer in the transmission of *D. viviparus.* The most common deer species in Europe, the roe deer, does not seem to be susceptible to *D. viviparus* and molecular studies in Sweden have demonstrated that *D. viviparus* is host specific for cattle. Infections of calves with *D. filaria* from sheep and goats and *D. arnfieldi* from donkeys do not result in patency.

Stable infections

As a rule stable infections are not important. However, they may occur and they may even occasionally result in disease.

Possible Causes for Outbreaks

The rapid and massive dispersal of larvae from the feces through *Pilobolus* results in a rapid sequence of worm generations. Each generation takes approximately 1 month (**Figure 1**). The prepatent period is 3–4 weeks, and it takes 1 week for larvae to develop to L3 and become dispersed onto the pasture. The occurrence of disease depends on the primary infection level and the weather conditions. Disease in the first generation only occurs after heavy primary infections, for instance, when calves are grazed on a pasture contaminated by other calves, or occasionally, yearlings. When primary infections result from overwintered larvae on pasture or from carriers such as cows or yearlings, they will be low or moderate. The subsequent infection patterns are then highly predictable. Reinfection results in an increase in fecal larval counts, and occasionally in disease, 4–5 weeks after the beginning of patency (**Figure 1**). Usually, fecal larval counts decrease again after a further 4–8 weeks as a result of developing immunity. However, when infections are still low during the second lungworm generation a further increase of fecal larval counts and disease may be observed during the third generation of lungworm (**Figure 1**). When the third generation does not result in disease it is unlikely that it will ever occur, because immunity will develop during the long period of low infections. Thus, disease will occur approximately 1, 2, or 3 months after primary infection. The reason that disease is less common in beef cattle is probably the transmission of low infections from the dam herd to the calves. Suckling calves therefore will not be exposed to high infections and can easily develop immunity.

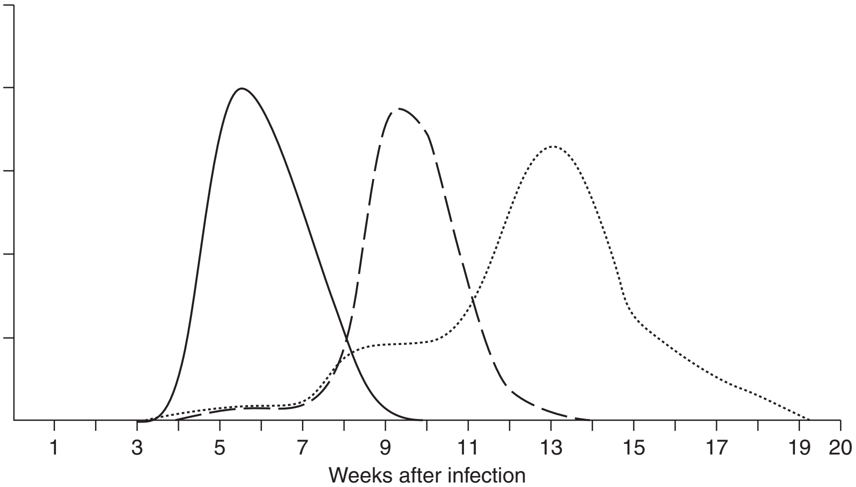

Figure 1 Fecal larval output patterns after primary lungworm infections. Disease may occur after approximately 1, 2, or 3 months following high (solid line), low (broken line), or very low (dotted line) primary infections, respectively. As a result of partial immunity mean peak fecal larval counts decrease in each worm generation. Reproduced from Eysker M (2002) *Lungworms, Encyclopedia of Dairy Sciences*, 1st edn., pp. 2220–2225. Amsterdam: Elsevier with permission from Elsevier.

Weather conditions also influence the occurrence of lungworm disease. Although transmission of lungworm infections also occurs under hot and dry summer conditions, outbreaks predominantly occur when summer and autumn are wet. This probably explains why dictyocaulosis is a lesser problem in the dairy regions of northern United States and Canada than in Europe. The hot summer and the cold winter imply that transmission of lungworm infections is mainly restricted to relatively short periods in spring and autumn.

Diagnosis

Diagnosis is based on the clinical signs and grazing history. Experienced practitioners in endemic regions have no difficulty in recognizing the typical signs in young animals. The diagnosis may be confirmed by parasitological and immunological means. Diagnosis is often more difficult in adult animals because infections may not reach patency.

The most convenient parasitological diagnosis is through detection of L1 with the Baermann technique. When performed properly, it is highly sensitive in young animals with primary infections; it enables detection of one adult female worm when 30 g of feces is examined. Proper performance of the Baermann method implies the following:

1. Fecal samples are taken rectally to prevent contamination with soil nematodes.
2. Feces is processed the same day or stored at 4–8 °C until examination.
3. Glassware with steeply sloping sides is used.
4. Fine-meshed (25–30 μm) screens (cheesecloth or metal gauze) are used to avoid leaking of fecal material.
5. Baermann samples are left for 24 h (or at least overnight) before being examined; recovery is much lower when samples are examined the same day.
6. Samples are preferably examined with a dissecting microscope (magnifications ×16–25).
7. Lungworm larvae are differentiated from free-living nematodes and from first-stage larvae of *Strongyloides papillosus* and trichostrongylids. *D. viviparus* larvae are 450 × 25 μm, have a pointed tail and intestinal food granules, and show a characteristic sluggish movement.

Flotation techniques should not be used because they are far less sensitive than the Baermann method. Sputum examination for eggs and larvae is obviously more rapid than the Baermann method because detection can be done on the day of sampling. Moreover, egg-producing lungworms can be detected 1 or 2 days earlier than with fecal examination. When fecal examination is negative immature worms may be recovered through lung washings in severe cases.

Diagnosis can also be performed through serology. Crude worm antigens in an ELISA were used for seroepidemiological surveys in the 1980s in the Netherlands. The disadvantage of these tests was a lack of specificity. Subsequently, during the 1990s a highly lungworm-specific test based on a purified adult worm protein was developed (both in the Netherlands and in Hannover, Germany). This test allows detection of positive animals individually. The adult worm protein used as antigen is a major sperm protein and only allows detection of a patent infection, similar to fecal examination for L1. Seroconversion is not obtained before patency, and in fact, larvae can be found in the feces before seroconversion. Vaccinated cattle remain negative with this test, and in the postpatent period antibody levels gradually decrease to negative values. Therefore, this test often fails when used to confirm lungworm disease in nonpatent, older cattle showing the reinfection syndrome.

The major advantage of the lungworm-specific ELISA over fecal examination for L1 is the fact that lungworm antibodies gradually decrease over a period of some months during the postpatent period. Therefore, the ELISA can be easily used for large-scale prevalence studies. A recent development is to use the ELISA for detection of antibodies in (bulk) milk samples to screen for lungworm infections on a herd level.

Eosinophilia is a characteristic phenomenon in cattle with respiratory signs due to dictyocaulosis. Nonetheless, it provides inconclusive support for the diagnosis of lungworm disease and can only be used in adjunction to clinical signs and grazing history.

Treatment

Treatment implies application of an anthelmintic, and when necessary, further supportive treatment. When clinical signs are severe, they may aggravate after anthelmintic treatment, and farmers have to be warned for that. Anthelmintics that can be used are the benzimidazoles, levamisole, and the avermectins/milbemycins. Compounds of the latter group all have a residual effect against lungworm of at least 3–4 weeks. Only two compounds, topical formulations of eprinomectin and moxidectin, are licensed in many countries for lactating animals without a withdrawal period for the milk.

Recently, the first case of *D. viviparus* showing reduced susceptibility to an anthelmintic has been reported in Brazil. However, so far anthelmintic resistance is not considered important and has not been reported anywhere else.

Prevention

General

The aim of control measures in high-prevalence regions is to suppress lungworm infections to the extent that no disease occurs. However, sufficient exposure to infection is necessary for the buildup and maintenance of immunity. The latter is of vital importance because infections will be introduced inevitably on lungworm-free farms. In that case disease may occur in any age class. Outbreaks in lactating animals can cause serious economic losses. This means that in high-prevalence regions the aim is to seek a balance between acquisition of infection and acquisition of immunity.

The aim of control measures in low-prevalence regions is less easy to define. Acquisition of immunity may be less relevant than attempts to maintain a lungworm-free status on the majority of farms. However, it is not easy to guarantee a lungworm-free status for a particular farm, and it is virtually impossible to guarantee maintenance of this situation.

Control may be achieved by vaccination, suppressive anthelmintic treatment, grazing management, and vigilance and treatment.

Vaccination

Vaccination using an irradiated vaccine has been a highly effective control measure in high-prevalence regions in Europe since the late 1950s. Two doses of approximately 1000 irradiated larvae are given 4 weeks apart, and calves are turned out 2 weeks after receiving the last dose. On-pasture reinfection is necessary for a further buildup and maintenance of immunity. However, during the last decades the use of the vaccine in dairy replacement heifers has decreased. On farms where lungworm outbreaks occur in adult cows that can be linked to introduction of lungworm-naïve heifers, vaccinating heifers shortly before they enter the adult herd can help to prevent new outbreaks. Vaccination is not justified in low-prevalence regions.

The irradiated larval vaccine is not available in all countries. The production of this vaccine is also laborious and requires donor calves. Therefore, some groups are working on novel recombinant vaccines for dictyocaulosis.

Suppressive Anthelmintic Treatment

These include suppressive systems for the control of gastrointestinal nematodes (**Table 1**). The principle implies suppression of pasture contamination with nematode eggs in the first half of the grazing season through application of a long-acting device or through a treatment at turnout and another one 6–10 weeks later with an avermectin/milbemycin. Such pastures can safely be used until the end of the grazing season. Although not designed for this purpose, most of these systems will suppress lungworm infections. **Table 1** shows that systems vary in the length of suppression of lungworm. The suppression of lungworm by these early season systems implies a low risk for lungworm disease in the first grazing season. However, development of immunity may also be impaired. Thus, outbreaks may be delayed to later grazing seasons.

Grazing Management

Considering the rapid dispersal of lungworm larvae to pasture, dangerous reinfection can only be prevented by frequent moves to clean pasture. On the other hand, survival of larvae is short and pasture infectivity will decrease rapidly. Therefore, control schemes involving rotational grazing for 4- or 3-day periods in the former

Table 1 Early season treatment systems

Compound	*Name*	*Approximate length of activity*
Levamisole	Chronomintic bolus	3 months
Morantel	Paratect flex bolus	3 months (not effective against lungworm)
Oxfendazole	Repidose 5 bolus	4 months
Oxfendazole	Repidose forte bolus	4 months
Fenbendazole	Panacur-SR bolus	5 months
Ivermectin	Ivomec-SR bolus	5 months
Ivermectin[a]	0–6 or 0–8 w ivomec	2.5–3 months
Eprinomectin[a]	0–6 or 0–8 w ivomec/eprinex	2.5–3 months
Abamectin[a]	0–6 or 0–8 w enzec	2.5–3 months
Doramectin	0–8 w dectomax	3 months
Moxidectin	0–10 w cydectin	3.5 months

[a]These systems are not recommended as such by the producer, but may be considered.
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Democratic Republic of Germany or the former East-Germany (DDR), Belgium, and Cuba, gave good results. In all schemes calves did not return to the same pasture within 32–40 days. Experiments in the Netherlands confirmed that such rapid rotational grazing systems prevent lungworm disease. However, they did not prevent parasitic gastroenteritis and consequently cannot be recommended.

Vigilance and Treatment

This implies anthelmintic treatment of susceptible animals at first appearance of respiratory signs. This is often practiced, although rarely as a deliberate control system. In the Netherlands lungworm disease was mentioned as the most common reason to apply anthelmintics in the first grazing season. Relapses of disease after treatment are rare, because immunity will have developed. This may be the control method of choice in low-prevalence regions. However, it implies regular examination of cattle at risk and swift action upon the first signs of disease.

See also: **Diseases of Dairy Animals**: Parasites, External: Mange, Dermatitis and Dermatosis; Parasites, External: Tick Infestations; Parasites, Internal: Gastrointestinal Nematodes; Parasites, Internal: Liver Flukes.

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E

ENZYMES EXOGENOUS TO MILK IN DAIRY TECHNOLOGY

Contents

β-D-Galactosidase
Lipases
Proteinases
Transglutaminase
Catalase, Glucose Oxidase, Glucose Isomerase and Hexose Oxidase

β-D-Galactosidase

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Introduction

β-Galactosidases have been extensively described in the scientific literature. A major part of this research has been focused on the β-galactosidase of the intestinal Gram-negative bacterium *Escherichia coli.* Not only did this enzyme play a key role in developing the understanding of operon structure and control of gene expression, but also continues to play an important role as a cell biology probe. This β-galactosidase has been used in biochemical and molecular biology studies as a convenient marker for gene expression studies, as a tool to detect plasmid transformation of bacteria, and to study protein–protein interactions in the yeast two-hybrid system. A major advantage of the use of β-galactosidase as research tool is the relative ease of detection of its activity by convenient color-based enzyme assays using substrates like X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and ONPG (*O*-nitrophenyl-β-D-galactopyranoside). An important disadvantage of the *E. coli*-derived enzyme is that products from this production host are not considered as GRAS (generally regarded as safe), which is a major obstacle for the application of such an enzyme in the food industry.

This article solely focuses on β-galactosidases with industrial relevance for the dairy industry. The industrially relevant β-galactosidases belong to the subgroup of lactases (EC 3.1.2.23). Lactose is a specific substrate and while all lactases, regardless of the source, can be classified as β-galactosidases, the converse is not true. Many β-galactosidases in plant cells and mammalian organs (except for intestinal β-galactosidase) act on cell wall polysaccharides, galactolipids, or glycoproteins and have little or no activity on lactose.

By far, the most important reason for the use of lactases in the dairy industry is to allow people who are lactose intolerant to consume dairy products that are high in lactose. Several different lactases and different enzyme formulations are on the market for both producers of dairy products and consumers. The main applications are the treatment of liquid milk and the use of the enzyme as a nutritional supplement. Another industrial application of lactases is the production of the prebiotic galacto-oligosaccharides (GOSs) using the transglycosylase activity of lactases. These major applications will be discussed later in this article. Further advantages of lactose hydrolysis like increased sweetness (chocolate milk, yogurt), prevention of crystallization (dulce de leche, ice cream), and decreased freezing point (ice cream) have been noted in the literature and may be the reason for the use of lactases in specific applications. These topics are only briefly discussed here.

Lactose Intolerance

Milk is the major food of newborn mammals, but most human adults cannot digest it because they lack sufficient quantities of lactase. With the exception of the population of northern and central Europe and its offspring in the Americas and Australasia, about 75% of the world population consists of lactose nonpersistent subjects, of which about 30% are without any symptoms. This means that 50% of the world adult population suffers from lactose intolerance. The prevalence of maldigestion is above 50% in South America and Africa, and reaching almost 100% in some Asian countries. In the United States, the prevalence varies from 15 (in Caucasians) to 80% (in African–American populations). In Europe, it varies from around 2% in Scandinavia to 17% in Finland and 2–70% in Italy. Therefore, milk and many other dairy products are not part of the regular diet of a large part of the world population. Since milk is a major source of essential nutrients like protein, riboflavin, calcium, vitamin D, niacin, vitamin B_{12}, phosphorus, magnesium, vitamin A, zinc, and iodine, a lack of dairy intake may lead to nutrient deficiencies if adequate substitutes are not provided.

In juveniles and people who are lactose tolerant, lactose is hydrolyzed into galactose and glucose by the enzyme ‘lactase-phlorizin hydrolase’ (LPH) in the small intestinal brush border of the jejunum. The LPH is encoded by a single lactase gene *LCT*, and it is found that the ability to digest lactose in adulthood is due to *cis*-acting mutations in gene expression, which are inherited in a dominant manner. Lactose, in contrast to monosaccharides (i.e., glucose and galactose), is poorly absorbed in the small intestine. People who do not carry mutations in the *LCT* gene will not digest and absorb lactose, and malabsorbed lactose will osmotically attract fluid into the bowel lumen, which will lead to a loose stool. Additionally, lactose is a substrate for intestinal bacteria in the colon, which metabolize it, producing volatile fatty acids and gases such as carbon dioxide, hydrogen, and methane, leading to flatulence and cramping.

The degree of lactose malabsorption varies greatly between humans, but most can ingest up to 14 g lactose daily without any symptoms. The severity of the symptoms very much depends also on the rate of gastric emptying, the intestinal transit time, and the colonic microflora. The constitution of the meal ingested together with lactose is therefore important. In general, lactose ingestion via a higher viscosity product like yogurt will give fewer problems than a liquid milk product.

Nowadays, more and more low-lactose dairy products are commercially available. The use of low-lactose or lactose-free products alleviates the problems associated with the digestion of dairy products by a large part of the world population, which then can benefit from the essential nutrients present in dairy products.

Lactases

β-Galactosidase is very widely distributed in nature because of its multiple functions, which include digestion, lysosomal degradation, and catabolism. Lactases, however, are found only in the mammalian intestine and in microorganisms. In the intestine, lactase is located in the microvilli of the mucosal cells of the brush border membrane. In bacteria and yeasts, the enzyme is usually intracellular but in fungi it can be intra- or extracellular. For commercial use, microorganisms are the only practical source and the enzyme has been identified in a large number of species. Several of the enzymes have been purified and characterized; a few have been cloned and expressed in other microbial hosts.

Sources of Industrial Lactase

Industrial lactases can be subdivided into two groups: neutral and acid lactases. The applications of both types of lactases are completely different and depend very much on their pH optima and stability. Properties and the production processes for both types of lactases are also distinctly different. Neutral lactases are generally used in neutral food products, such as milk, while acid lactases are used as nutritional supplements.

Currently, a number of neutral lactase products are sold by companies like DSM Food-Specialties, Godo, Chr. Hansen, Kerry, Novozymes, and others, under different brand names. All neutral lactases are produced by one of the two closely related yeast species *Kluyveromyces lactis* or *Kluyveromyces marxianus* (previously named *Kluyveromyces fragilis*). Although the biochemical properties of these enzymes are fairly similar, they are sold in different qualities, which can have a different impact on the final product (*see also* ‘New Developments’).

Acid lactases are produced by the filamentous fungi *Aspergillus oryzae* and *Aspergillus niger*. The main supplier is the Japanese firm Amano Enzyme, Inc., which produces the *A. oryzae* enzyme under the brand name Lactase F. DSM Food-Specialties has recently launched an *A. oryzae* acid lactase named Tolerase®. The acid lactase of *A. niger* has been on the market in the past but is currently not in production.

A third, bacterial, β-galactosidase from *Bacillus circulans* fermentation is produced by Amano Enzyme, Inc. This lactase is used in the industry for its transglycosylase activity and not as a hydrolase like the other commercial lactases.

Enzyme Structure

The most widely studied and best understood β-galactosidase is that from *E. coli* encoded by the *lacZ* gene. It is easy to purify and has served as a model for understanding the mechanism of catalysis. The enzyme has a molecular weight of ~450 kDa and is made up of four identical subunits each containing 1023 amino acid residues. Each subunit contains 16 cysteine residues and a binding site for magnesium, a cofactor of the enzyme. β-Galactosidases from other bacteria and yeasts are of approximately similar subunit size and can occur in dimeric or tetrameric form, in contrast to the fungal β-galactosidases, which are active as monomers.

Primary amino acid sequences have been established for β-galactosidases from several bacteria, and comparison with the enzyme from *E. coli* shows extensive sequence homologies and highly conserved regions. Also the enzymes from *Kluyveromyces* are >30% identical to the *E. coli* enzyme, suggesting an evolutionary relationship. Glycosyl hydrolases have been grouped in to different families (*see* 'Relevant Websites' section), based on sequence and structure similarity, and the yeast lactases belong to family 2 (see **Figure 1**). The primary amino acid sequence of the fungal enzymes is much less related (<15%) to the *E. coli* prototype, explaining the annotation of these enzymes to a different glycosyl hydrolase family. The tertiary structures of the β-galactosidases of both *E. coli* (family 2) and *Penicillium* sp. (family 35) are known. Despite their importance for the alleviation of lactose intolerance, the tertiary structure of industrial *Kluyveromyces* and *Aspergillus* lactases has not been determined.

Mechanism of Hydrolysis

β-Galactosidases hydrolyze the disaccharide β-D-galactopyranosyl-(1 → 4)-D-glucose (lactose) to β-D-galactose and D-glucose. The mechanism of the action of the β-galactosidase from *E. coli* has been studied extensively and clarified. β-Galactosidase is a retaining glycosidase. It hydrolyzes its substrates in a double-displacement reaction where the product retains the same stereochemistry as the starting state. A generalized outline of the mechanism of action is shown in **Figure 2**.

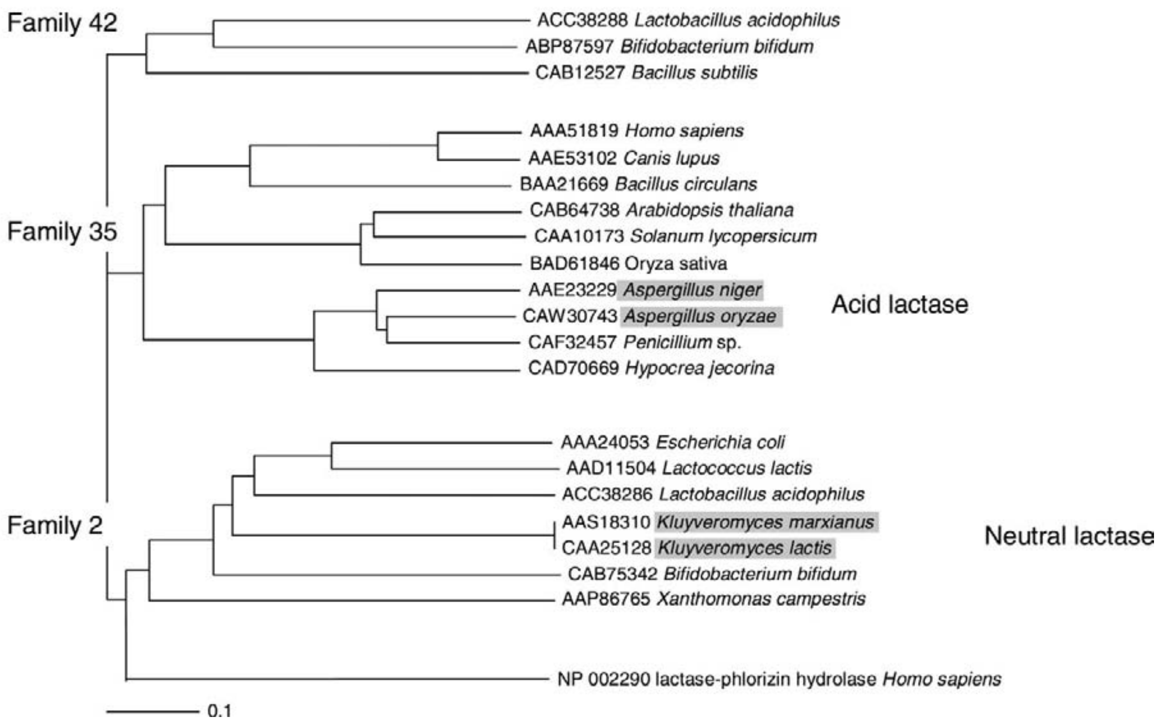
Family 42
ACC38288 Lactobacillus acidophilus
ABP87597 Bifidobacterium bifidum
CAB12527 Bacillus subtilis
AAA51819 Homo sapiens
AAE53102 Canis lupus
BAA21669 Bacillus circulans
CAB64738 Arabidopsis thaliana
CAA10173 Solanum lycopersicum
BAD61846 Oryza sativa
Family 35
AAE23229 Aspergillus niger
CAW30743 Aspergillus oryzae
CAF32457 Penicillium sp.
CAD70669 Hypocrea jecorina
Acid lactase
AAA24053 Escherichia coli
AAD11504 Lactococcus lactis
ACC38286 Lactobacillus acidophilus
Family 2
AAS18310 Kluyveromyces marxianus
CAA25128 Kluyveromyces lactis
Neutral lactase
CAB75342 Bifidobacterium bifidum
AAP86765 Xanthomonas campestris
NP 002290 lactase-phlorizin hydrolase Homo sapiens
0.1

Figure 1 Phylogenetic tree of a small selection of lactase amino acid sequences present in public databases, made using the program Phylip tree (bootstrap neighbor-joining tree, 500 seeds, 1000 trials). Neutral yeast lactases belong to family 2 and differ from the fungal acid lactases of family 35.

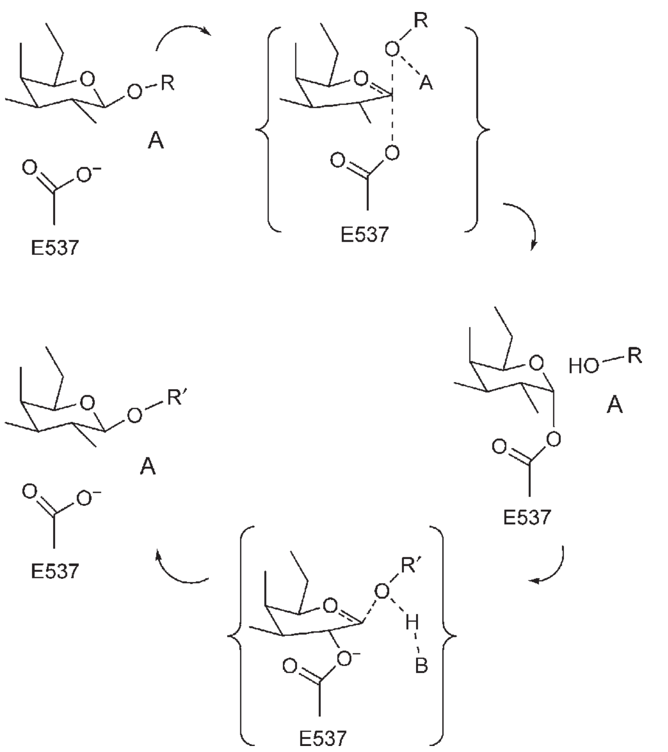

Figure 2 Generalized outline of a double-displacement reaction catalyzed by β-galactosidase. In the first step (top), the substrate, a β-D-galactopyranoside with OR as the aglycone, forms a covalent α-D-galactosyl-enzyme intermediate with the nucleophile Glu537 and with assistance from an acid, A (either Glu461 or a magnesium ion). In the second step (bottom), release of the intermediate is facilitated by a base, B (probably Glu461), which abstracts a proton from the acceptor molecule. Adapted from Juers D, Heightman TD, Vasella A, *et al*. (2001) A structural view of the action of *Escherichia coli* (lacZ) β-galactosidase. *Biochemistry* 40: 14781–14794.

One amino acid acts as a general acid and donates a proton to the glycosidic oxygen, while a negatively charged group stabilizes the positively charged galactosyl-enzyme carbonium intermediate, which then reacts with water in the hydrolytic reaction. The nucleophile, Glu537 in the *E. coli* enzyme, binds covalently to the galactosyl moiety. Of the two potential acids, Glu461 is in the best position to assist directly in the departure of the leaving group, suggesting that magnesium acts in a secondary role. For the *K. lactis* enzyme, the active site residues are thought to be represented by Glu551 and Glu482, based on homology to the *E. coli* enzyme.

If the enzyme-galactose$^+$ intermediate reacts with another sugar (e.g., lactose, glucose, or galactose) instead of water, synthesis of oligosaccharides occurs by a transglycosylation reaction. This reaction can become quantitatively significant at high lactose concentrations. There is a significant difference in the efficiency of the transglycosylation reaction between lactases from different microorganisms.

Biochemical Properties

The commercial lactases currently available differ widely in their properties, particularly with respect to their pH and temperature optima. The neutral lactases from the *Kluyveromyces* yeasts have a neutral pH optimum and are therefore well suited to hydrolysis of lactose in milk (**Figure 3**(**a**)). Acidification leads, however, to a rapid loss of activity, which limits the use of the yeast enzyme. However, depending on the fermentation temperature and enzyme dosage, a fast and even complete hydrolysis can be achieved with a neutral lactase during the acidification process of yogurt manufacture.

Fungal acid lactases are activated by acidification but perform poorly at the pH of milk. The *A. oryzae* acid lactase has a pH optimum at 4.5 and the pH optimum of the *A. niger* enzyme is at 3. The main application of acid lactases is as a dietary supplement where lactose hydrolysis takes place in the stomach.

Temperature is another important factor to consider when choosing a commercial source of β-galactosidase. Fungal lactases have a high temperature optimum for both activity and enzyme stability, and can be used for hydrolysis of lactose at 50–55 °C (**Figure 3**(**b**)). In contrast, yeast neutral lactases are rapidly denatured above 40 °C, although the milk matrix may have some stabilizing effect on the enzyme. The inactivation of yeast lactase at pasteurization temperature prevents the presence of active enzyme in the final product. In some countries, legislation prohibits the use of enzymes that are not inactivated in dairy products. Another advantage of the yeast lactases is that they still show reasonable activity at

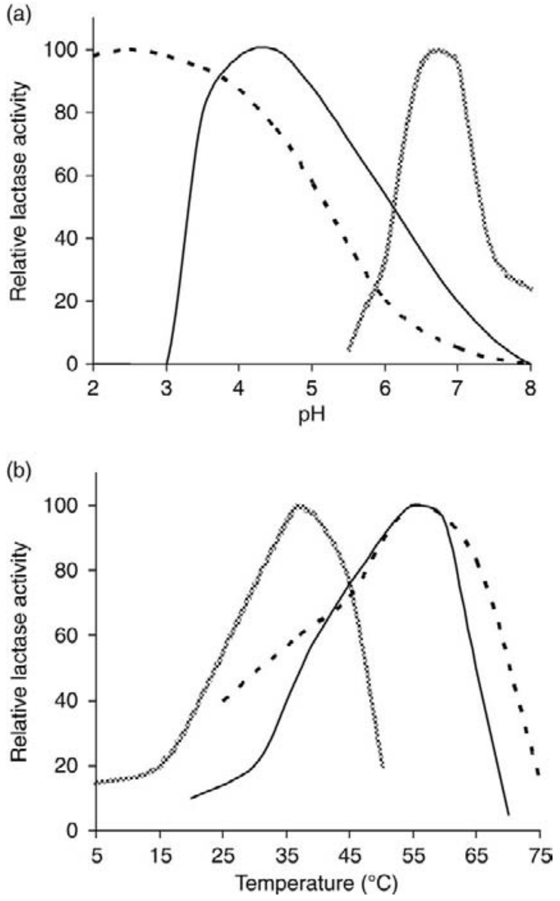

Figure 3 Activity of lactases of *Aspergillus niger* (dashed line), *Aspergillus oryzae* (black line), and *Kluyveromyces* (gray line) dependent on pH (a) and temperature (b). Activities are expressed relative to the optimum of each enzyme.

low temperatures, which allows hydrolysis to take place during refrigerated storage of milk. At low temperature, spoilage of milk due to microbial contamination is diminished.

The effect of metal ions and other compounds on the activity of neutral lactases has been investigated extensively. Most of these studies have been performed in buffers instead of milk, and ONPG has been used as the substrate instead of lactose. There are some clear inconsistencies in the literature regarding the possible activating or inhibiting effect of different metal ions when individual publications are compared. Nevertheless, some general conclusions can be drawn from these studies: (1) it has been found that galactose is a competitive inhibitor of all lactases studied. For instance, for the yeast lactase, the K_i for galactose was found to be 42 mmol l^{-1}, which is not much higher than the K_M for lactose (12–37 mmol l^{-1}) under the same conditions. It is therefore expected that galactose may play an inhibitory role during batchwise hydrolysis of milk. The effect of glucose as a noncompetitive inhibitor seems to be much less severe. (2) It has become clear that heavy metals are inhibitory, whereas potassium, magnesium, and manganese stimulate the activity of the enzymes from yeast. However, the type of buffer used in the assay can have an influence on the effect of metal ions. Additionally, the magnitude of the effect of these ions also differs depending on whether the enzyme is used in whey, milk, or buffered lactose. For instance, conflicting results have been reported for zinc and calcium, once activating and in the next experiment inhibitory. The fungal β-galactosidases do not appear to be dependent on activating ions. (3) Finally, it has been found that milk proteins help to activate and stabilize the yeast lactases. Interestingly, preheating of milk was shown to activate the enzyme, and this correlated with the liberation of free SH groups. Activation can be reversed again by oxidation of the reactive SH groups, suggesting that a reducing environment may be beneficial for the activity of the yeast enzyme.

Applications of Lactases

Low-Lactose Milk

For the application of lactases in liquid dairy products, several properties of the enzyme, besides the evident prerequisite that it should be obtained from a microorganism that is GRAS, are important: (1) the enzyme should have a relatively high pH optimum (pH 6–7) to allow efficient hydrolysis of lactose at milk pH. (2) It should be possible to inactivate the enzyme by pasteurization and/or ultra-high temperature (UHT) treatment to prevent its activity in the final product. (3) The enzyme should ideally be active at low temperatures (6–8 °C) since many processors prefer the treatment of the milk during overnight storage. This prevents the introduction of an additional processing step in the dairy plant and the low temperature prevents microbial growth. (4) The enzyme should be sufficiently pure to prevent off-flavor formation upon prolonged storage of UHT milk.

Neutral lactases obtained from the dairy yeast *Kluyveromyces* have the first three properties mentioned above and are therefore ideally suitable for application in milk and milk products. Additional purification and strain selection are needed to satisfy the requirement for purity and prevention of off-flavor formation, and help to extend the shelf life of the end product (*see also* 'New Developments').

In principle, there are three ways to obtain lactose-free milk:

1. Pretreatment of milk with yeast lactase after which the product is heat treated, packed, and sold. To avoid microbial growth during hydrolysis, in most

applications temperatures between 6 and 8 °C are used. As this is not the optimum temperature of the enzyme, the hydrolysis time is quite long, between 24 and 30 h, depending on the enzyme dosage. Only when good quality Grade A raw milk is used, hydrolysis at high temperature (38–40 °C) may be applied. For food safety reasons, the milk should always be pasteurized or at least thermized prior to hydrolysis.

2. Posttreatment of milk after heat treatment. In this application, a sterile lactase is added in-line immediately after UHT treatment of milk, followed by packing. The lactose hydrolysis takes place in the final package at ambient temperature. Since active enzyme is present in the milk during storage, this method requires a lactase preparation of high quality, lacking side activities.
3. Treatment of regular milk with yeast lactase by the consumer. Consumers can buy small packages of neutral lactase. A few drops of lactase are added 1 day prior to consumption of the milk. Depending on the dosage, the lactose can be hydrolyzed within 12–24 h.

Other Lactose-Free Products

In the past 40 years, the main applications of neutral lactase were in milk and dulce de leche to make low-lactose products. Presently, a lot more commercial lactase-treated dairy products are available, for example, yogurt, ice cream, fresh cheeses (cottage/quark), milk shakes, condensed milk, milk powder, cat milk, and many other applications.

It is generally thought that fermented milk products should not pose a problem to lactose-intolerant consumers because the lactose is used by the acidifying bacteria. However, yogurt cultures remove only part of the lactose, and lactose concentrations of 3–5% have been measured in yogurts depending on the product formulation. When skim milk powder is added in a yogurt formulation, the extra lactose from the skim milk powder results in a high lactose content in the end product. Treatment of such yogurt with lactase still leads to a reduced-lactose or lactose-free product. In addition, lactases may be used in yogurt to increase sweetness without the addition of extra sugar because hydrolysis of lactose into glucose and galactose leads to a doubling of the sweetness perception.

Also in ice cream applications, neutral lactase is primarily used for lactose reduction. Besides this, functional advantages also have been identified. Lactose hydrolysis improves the scoopability of the ice cream due to the decrease of the freezing point. Also sandiness due to the crystallization of lactose can be prevented by hydrolysis with lactase.

There is a worldwide trend to produce lactose-free products instead of lactose-reduced products. Market growth rates for lactose-free products for the period 2003–08 (Euromonitor 2009) are +11% compared to +6% for standard dairy products, and +11% for lactose-free ice cream compared to +9% for standard ice cream.

Dietary Supplements

An enzyme that is supplied as dietary supplement should have a number of properties: (1) the enzyme should be active at the low pH of the stomach (pH 2.5–4); (2) the enzyme should be resistant to drying since it is formulated in pills; and (3) it should not be vulnerable to proteolytic degradation to extend its life span in the stomach.

Currently, the most commonly used acid lactase in this application is obtained from *A. oryzae*. There is a growing demand for the use of acid lactase as a dietary supplement in areas or situations where lactose-free dairy products are not available. The dosage can be adjusted to the personal need by varying the number of pills.

Galacto-Oligosaccharides

GOSs consist of saccharides (glucose and galactose) with different chain lengths. All commercial lactases form different types of GOSs (with $1 \rightarrow 3$, $1 \rightarrow 4$, or $1 \rightarrow 6$ linkages), but often at low efficiencies. Historically, the primary reason for investigation of the transgalactosylation reaction of lactases has been nutritional concerns regarding the indigestibility of these saccharides. Recently, long-chain GOSs have been recognized to stimulate the growth of healthy bacteria in the colon and are applied as prebiotics mainly in infant foods and also in other dairy products and beverages.

For the efficient production of GOSs, the lactase should have a number of properties: (1) it should be functional at high lactose concentration because transgalactosylation is most efficient at high lactose concentration; (2) it should be able to function at high temperatures to increase lactose solubility; (3) it should be efficient in long-chain GOS formation; and (4) it should have poor GOS hydrolysis activity. A successful product with the brand name Vivinal GOS is produced by Royal FrieslandCampina – Domo (The Netherlands) using a bacterial β-galactosidase from *B. circulans*.

New Developments

Use of Immobilized Enzyme

Immobilization of lactase has been studied extensively and many reports on this subject are present in the scientific literature. Immobilization has the potential to decrease enzyme costs since the enzyme can be used several times or is used in a continuous process. Many researchers have studied possibilities to immobilize yeast or fungal lactases to hydrolyze lactose in milk or whey. Development of a suitable reactor for milk or whey has not been easy, and the percentage of hydrolysis is often not sufficient. Despite all the efforts, to our knowledge, immobilized lactases have not been used successfully in the dairy industry today. One reason that immobilized processes have not been implemented widely in the dairy industry may be the increased chance of fouling and bacterial contamination by the long process times at high temperature. Additionally, the cost advantage of using immobilized enzyme may not be as high as generally thought in the scientific community.

Sweetness Reduction

One obvious difference between lactase-treated milk and normal milk is the increased sweetness of the former product. The relative sweetness of lactose is 0.25 compared to sucrose, while galactose has a sweetness of 0.30 and glucose a sweetness of 0.70. Complete hydrolysis of all lactose in milk will therefore lead to twice the sweetness of regular milk. This difference can be clearly perceived by consumers. While in some countries the increased sweetness is regarded as an advantage, in countries that have a tradition of drinking plain milk the increased sweetness can be regarded as a defect.

The Finnish dairy company Valio has therefore developed a process to reduce the amount of carbohydrate in milk. In the patent-protected technology, approximately half of the lactose is removed from the milk by a chromatographic separation. The remaining part of the lactose is hydrolyzed with lactase. The claimed advantages of the resulting milk drink are a taste similar to the original milk (no extra sweetness) and reduced proteolysis and browning during storage.

Prevention of Off-Flavor Development

The majority of the lactose-free milk is currently produced as UHT milk and stored at ambient temperature in supermarkets up to 3 months. Additionally, some UHT milk is treated with sterile enzyme in the final package. For both processes, it is essential that the lactase used is free from side activities that could alter the flavor of milk during prolonged storage. Indeed, a recent study in the United States has revealed that the sensory quality of lactose-free UHT milk products was different from regular UHT milk. Besides the obvious effect on sweetness, more negative attributes like ‘cooked’, ‘chalky’, and ‘animal (sulfur)’ have also been associated with some batches of lactose-free milk. For the producers, it is therefore imperative that the raw milk and the final enzyme-treated product are as clean as possible, and lack other enzymatic activities that could have a negative influence on milk flavor. The production of neutral lactase free of side activities is especially difficult due to the recovery process during production of this intracellular yeast enzyme.

Several developments in recent years have addressed the problem of quality loss and have led to lactase enzyme preparations that have higher purity than earlier lactase preparations. DSM Food-Specialties currently supplies a neutral lactase preparation under the brand name Maxilact® LG, which has been chromatographically purified. The production process has been patented and the final product is superior to nonpurified products with respect to enzyme stability and side activities.

Another recent discovery by DSM Food-Specialties was that the development of off-flavor in lactase-treated UHT milk is related to the accumulation of *p*-cresol – which when present in minute quantities leads to a severe ‘medical’ or ‘animal’ off-flavor. It was found that the accumulation of *p*-cresol in UHT milk was due to hydrolysis of sulfonated cresol, which is naturally present in milk, by the enzyme arylsulfatase. Arylsulfatase was introduced in the milk as a side activity in the lactase enzyme preparation, and was found to be present in all commercial neutral lactases. DSM Food-Specialties has recently selected a *K. lactis* strain that is devoid of arylsulfatase activity. This strain was used to produce a new neutral lactase with superior qualities (Maxilact® LGX), lacking off-flavor formation in UHT milk, even after prolonged storage.

Implementation of new neutral lactases like Maxilact® LGX in the production of lactose-free milk may lead to a better acceptance of these products by the consumer, and thereby the more general use of lactose-free dairy products.

See also: **Lactose and Oligosaccharides**: Lactose Intolerance.

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www.cazy.org – Carbohydrate-Active enZYmes Database (CAZy).

Lipases

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Introduction

Enzymes inherently present in microorganisms and food substrates can be termed endogenous and indigenous enzymes, respectively. In contrast to these enzymes, biocatalysts may also be added to substrates, and these are termed exogenous enzymes. This article deals specifically with a type of exogenous enzyme that acts on lipids (lipase) and its use in dairy technology.

Lipids in milk and dairy products are mainly triglycerides with a variety of other fatty constituents. The uniqueness of ruminant milk fat is the presence of 15–20 mol% of short-chain fatty acid residues with 4–10 carbon atoms. These fatty acids are also mainly responsible for the unique flavor of ruminant milk fat. If liberated in milk, these acids in small amounts cause profound and undesirable flavor changes, which are characterized as soapy and rancid. These same acids when liberated by controlled lipolysis result in flavor concentrates.

Free fatty acids themselves are potent contributors to flavors; however, they also serve an important role as a precursor for other flavor compounds. For example, *n*-methyl ketones in Blue cheese are produced from free fatty acids. Free fatty acids also serve as precursors to the formation of fatty lactones and 4-*cis*-heptenal (creamy aroma). There is also the possibility that free fatty acids serve as precursors to esters found in cheeses via the action of ketone monooxygenases. Finally, free fatty acids may modulate the activity of proteases and thus affect the flavor attributes of fermented dairy foods.

The enzymes that result in the degradation of lipids are generally called lipases, but a more scientific classification reveals that lipases are glycerol ester hydrolases (EC 3.1.1.3) that hydrolyze tri-, di-, and monoacylglycerols at the oil–water interface liberating free fatty acids. Substrates requisitely have to be water insoluble, and therefore emulsified, for the lipases to act.

In this article, the characteristics of lipases and the uses of lipases in the generation of flavors in dairy foods will be discussed.

Sources and Properties of Lipases

Plants, animals, and microorganisms produce lipases. Castor bean and wheat germ contain lipases; a wide variety of microorganisms are sources of these enzymes; and animals contain pregastric esterases (lingual lipases) and pancreatic lipase. Plant lipases have not been used as commercial sources of enzymes for producing modified flavors. Lipases derived from microorganisms and animals have been exploited for flavor generation in the dairy industry.

As early as 1940, it was recognized that calves secreted a salivary lipase, which was named pregastric esterase. This enzyme is a true lipase in spite of the name. The commercially important pregastric lipases are derived from kids and lambs. Pancreatic lipase and microbial lipases have also been studied extensively.

The temperature optima for pregastric esterases range from 28–30 °C for calf enzyme to 32–34 °C for kid, goat, and lamb enzymes. The pH optima for calf, kid, goat, and lamb pregastric esterases are 5.3, 6.2, 7.5, and 8.5, respectively. Commercial preparations of pregastric esterases contain sodium chloride that has an effect of increasing the activity of the enzyme. Enzyme activity is inhibited by sodium taurocholate, milk salts, and mono- and dibutyrins, while the presence of lecithin, nonfat dry milk, casein, lactose, calcium, and egg albumin increase the activity of pregastric esterases. Both inhibitory and stimulatory activities of these additives are attributable to interfacial and emulsification activities.

Most lipases of animal and microbial origin exhibit alkaline pH optima (pH 8–9), but depending upon the substrate used, presence of salts, and type of emulsifier used, the optimum may shift to the acidic range. Several microbial lipases exhibit pH optima between 5.6 and 8.5. With respect to temperature optima, most lipases are active between 30 and 40 °C. Salts affect lipases in different ways. Porcine pancreatic lipase activity increased in the presence of up to 7 $\text{mmol}\,\text{l}^{-1}$ sodium chloride, while activities of bovine and milk lipase were unaffected. Calcium and bile salts stimulate activities of most lipases by counteracting the inhibitory effects of soaps formed during hydrolysis.

Microbial lipases are divided into two groups depending upon their positional specificity of hydrolysis on a triacylglycerol molecule. Lipases from *Geotrichum candidum*, *Corynebacterium acnes*, *Chromobacterium viscosum*, *Penicillium cyclopium*, and *Staphylococcus aureus* are nonspecific and can cause complete hydrolysis of triacylglycerol to glycerol and free fatty acids. The second group of lipases based on positional specificities hydrolyzes fatty acids esterified in the *sn*1 and *sn*3 positions only, resulting in di- and monoacylglycerols.

Lipases derived from *Aspergillus niger*, *Mucor javanicus*, *Rhizopus arrhizus*, *Rhizopus delmar*, and *Pseudomonas fragi* are 1,3-specific. Microbial lipases do not exhibit any specificity toward the chain length of the fatty acids in triacylglycerol substrates.

With the advent of biotechnology and the identification of appropriate lipases and esterases from microbial sources, alternatives to animal pregastric esterases have been discovered. Microbial enzymes offer the advantages of being less expensive and free of protease contamination, no risk of animal virus contamination, suitability for kosher products, and suitability for manufacture of vegetarian products.

The time–temperature combinations reported for the destruction of enzyme activity are provided in **Table 1**. The temperature of inactivation of lipase is influenced by the composition of the medium in which the inactivation is being determined. For example, destruction in milk requires higher temperatures and longer times than in an aqueous buffer medium. Water activity is another critical parameter influencing the thermal destruction of lipase activity. In most dairy foods, except powders, the water activity is high enough that this variable becomes less important than in grains and cereals.

The relative rate of release of free fatty acids from butteroil by various lipases has been reported (**Table 2**). The enzymes from kid, lamb, calf, and *Penicillium roqueforti* produce a proportionately larger amount of lower-chain fatty acids that have an impact on flavor development.

If the release of the lower-chain fatty acids is investigated in detail, an interesting picture emerges. The liberation of free fatty acids from cream by various lipases has been studied (**Table 3**). Of the enzymes studied, pregastric esterase from lamb released the maximum amount of butyric acid, followed by pregastric esterase from kid and lipase of *A. niger*. Butyric acid has a characteristic flavor with a low odor threshold value. Caproic, caprylic, and capric acids (the so-called goat acids) when summed up showed that *A. niger* lipase produced the maximum amount of these acids followed by pregastric esterases of kid and lamb, and kid rennet paste. The important point to be inferred from this is that the type and abundance of fatty acids influence the flavor of the lipolyzed substrates. When choosing enzymes, attention must be paid to the activity of the enzyme, nature of the products resulting from the reaction, and the processing of the hydrolyzed substrates.

Table 1 Conditions for the thermal destruction of some microbial lipases

	Thermal inactivation at	
Source of lipase	*Time (min)*	*Temperature (°C)*
Pseudomonas fragi	15	72
Rhizopus delmar	15	50
Aspergillus niger	15	45
Penicillium roqueforti	10	50
Staphylococcus aureus	30	70
Geotrichum candidum	15	60
Achromobacter lipolyticum	40	99

Table 2 Relative rate and proportion of lower-chain fatty acids released from butteroil by various lipases

	Total free fatty acid	*Proportion of lower chain fatty acids*
Lipase source	*(μ eq.)*	*(%)*
Kid	171	42
Kid–lamb	142	40
Calf	108	31
Milk	80	19
Bovine pancreas	140	17
Penicillium roqueforti	110	38
Achromobacter	96	22

Production of Modified Ingredients and Their Uses

The general process for the manufacture of lipolyzed products involves the following steps:

1. preparation of the substrate (generally condensed milk, butteroil, or cheese curd);
2. preparation and standardization of the enzyme solution;
3. placing the enzyme in contact with the substrate (enzyme addition);
4. homogenization to facilitate emulsion formation and to enhance activity;
5. incubation to achieve the desired conversion;
6. enzyme inactivation with minimum loss of the generated flavor volatiles; and
7. final product standardization, formulation, and packaging.

There are two main types of products produced by this process, namely, lipolyzed cream and enzyme-modified cheese (EMC) flavors.

Lipolyzed Cream Products

Several patents have been issued on enzyme-modified milk fat composition for use in imparting or intensifying butter-like flavors in baked products and sauces (**Table 4**). Lipolyzed creams have been evaluated in bread along with commercial shortening, 3% butteroil,

Table 3 Liberation of free fatty acids from cream by a variety of lipases

	Relative abundance (mol%)				
Source of enzyme	*Butyric ($C_{4:0}$)*	*Caproic ($C_{6:0}$)*	*Caprylic ($C_{8:0}$)*	*Capric ($C_{10:0}$)*	*Lauric and higher ($>C_{12:0}$)*
Kid rennet paste	32.8	11.3	7.1	11.8	33.6
Kid pregastric esterase	44.4	15.2	7.6	12.3	21.5
Lamb pregastric esterase	48.1	8.6	14.2	9.3	19.8
Calf pregastric esterase	36.7	8.9	4.8	10.7	39.0
Calf rennet	10.7	3.1	Trace	Trace	86.5
Milk lipase	13.5	8.2	10.2	8.7	60.0
Porcine pancreatic lipase	8.4	2.1	Trace	Trace	89.1
Aspergillus niger lipase	43.1	18.9	20.2	17.5	Trace

Table 4 Examples of patents pertaining to the use of lipases in modifying butterfat in milk and cream

Application	*Patent reference*	*Year of issue*
Modification of fat in milk	US 1,966,460	1939
	US 2,638,418	1953
	US 3,469,933	1969
Enzyme-modified milk powder	US 2,531,329	1950
	US 2,794,743	1957
Lipolyzed milk fat buttery flavor	JP 3187/70	1970
	UK 1,251,272	1971
	JP 72-45108	1972
	CD 912,905	1971
Lipolyzed cultured cream flavors	US 3,469,993	1969
Lipolyzed Blue cheese flavors	US 2,965,492	1960
	US 3,072,488	1963
	US 3,100,153	1963
Lipolyzed cheese-like flavors	UK 1,326,516	1971
	US 3,780,182	1973
Yogurt flavor	JP 3107/71	1971

2% butteroil + 1% modified butteroil, 1% butteroil + 2% modified butteroil, and 2% commercial shortening + 1% modified butteroil. The resulting breads were evaluated for flavor, color, softness, appearance, and internal structure by a panel of experienced judges. The experimental breads were slightly tenderer than the controls, and the flavor of the breads containing modified butteroil was judged to be superior. After 24 h of storage, the control breads developed a stale flavor while the experimental breads did not. It has been recommended that 35–40% replacement of shortening with modified butteroil is optimal. Lipolysis of butteroil for such purposes should not use the enzymes from *Achromobacter lipolyticum*, *P. roqueforti*, and *G. candidum* to avoid soapy, musty flavors in bread. Kid and lamb pregastric esterases also are avoided because they impart a rancid flavor to bread.

Lipolyzed cream products are natural dairy flavors produced by treating fresh cream with lipases. Hydrolysis of milk fat liberates free fatty acids, and the four short-chain fatty acids contribute to flavor volatiles. To control flavor development in the finished product, the lipolyzed cream is heat processed to inactivate the added enzymes. The process of heating can generate secondary flavor compounds such as lactones. Sometimes lipolyzed cream products are made by inoculating cream with *Lactobacillus delbrueckii* subsp. *bulgaricus* to develop acidity in the cream prior to lipolysis. Instead of cream, pure butteroil can also be used as a substrate. Butteroil is solid at 25 °C (room temperature), and the lipolyzed butteroil returns to this state when the reaction is terminated and the mixture is cooled.

Lipolyzed cream and lipolyzed cultured cream products enhance flavors in candies, cheesecakes, sauces, dips, salad dressings, sweet doughs, soups, and baked goods. For subtle flavor effects, the use levels can be as low as 0.05–0.1% (w/w), and for a more pronounced effect 0.1–0.5% (w/w) levels may be used. Partially lipolyzed butteroils are used in oils, fats, cereals, snacks, and baked goods. For example, the oils used to popcorn or the oil used to cover the popped corn may contain 0.05–1.0% of lipolyzed butteroil.

Cheese Flavors

Natural cheese is expensive and is replaced with EMC (concentrated cheese flavor) in many food formulations. Cheese that has been enzymatically treated to enhance its flavor was first introduced in the late 1960s. EMCs have a flavor markedly different from the natural cheese flavor. An EMC used in food recipes can be the sole source of flavor or impart a specific taste characteristic to a more bland-tasting cheese (*see* **Cheese**: Enzyme-Modified Cheese). EMC flavors include Parmesan, Romano, Provolone, Gouda, Cheddar, Blue, and Swiss.

One popular cheese flavor line is the Blue cheese flavor. The flavor of Blue cheese is largely derived from lipids and involves four major enzymatic processes wherein (1) free fatty acids are liberated from milk fat by lipases, (2) the free fatty acids are oxidized to β-keto acids, (3) the β-keto acids undergo decarboxylation to generate methyl ketones, and (4) the methyl ketones are reduced to yield secondary alcohols.

The levels of lipolysis in Provolone, Romano, and Blue chesses are higher than in Cheddar, Swiss, Edam, and Gouda. It has been claimed that addition of rennet paste, pregastric esterase, or gastric lipase improves the flavor of Cheddar cheese, and several patents have been awarded for such applications. Acceleration of Cheddar cheese ripening by the addition of proteases and lipases has been reported. Also, it has been reported that the use of lipases in enzyme-modified Cheddar cheese production could eliminate the need for extensive proteolysis. Generation of intense flavors by lipolysis requires less proteolysis by proteases, thereby minimizing the chances of producing the bitter peptides and other undesirable flavor components resulting from extensive proteolysis.

Several lipases derived from *A. niger*, *Pseudomonas fluorescens*, and calf and porcine pancreas were added to curd slurries. The free fatty acid profile of the slurries containing *P. fluorescens* lipase was similar to that of the slurries containing porcine pancreatic lipase; however, the ratio of free fatty acids was similar to that of the control slurry. These slurries developed good Cheddar flavor initially, but upon continued incubation, developed lipolysis defects. From this study it appears that the ratios of free fatty acids as well as the concentrations of individual components of these acids determine the occurrence of lipolyzed flavor defects in Cheddar cheese.

It has been reported that Novo Nordisk used medium-aged Cheddar emulsified in water and added *Rhizomucor miehei* lipase (Palatase). The mixture was incubated at 40 °C for periods of 12–96 h in the presence of proteases and esterases. Heating at 66–72 °C for 4–8 min terminated the reaction, and the resulting paste was spray dried. The EMC produced in this manner had 5–20 times the flavor intensity of mild Cheddar cheese.

The flavor of processed Cheddar cheese was not improved by the addition of lipolyzed Edam cheese to the formulation. However, blending of small quantities of lipolyzed cheese with a bland cheese might result in a pronounced cheese flavor. In such experiments, cheeses with acid degree values of 2, 6, and 16 meq KOH $100\,\mathrm{g}^{-1}$ fat were formulated, and it was speculated that an acid degree value of 3 meq KOH $100\,\mathrm{g}^{-1}$ fat might have resulted in a better cheese flavor.

A Gouda-type EMC was developed by chopping Gouda cheese adding water, salt, cultures, and enzymes. The enzymes tested were kid–lamb pregastric esterase, kid pregastric esterase, lamb pregastric esterase, and calf pregastric esterase. A proteinase from *Penicillium camemberti* was also used for the proteolysis of the casein. The pH of this reaction was reported to be 5.5, and the temperature was 30 °C for 10 days. To stop the reaction, the mixture was heated to 80 °C. Two EMC products with different moisture contents were successfully produced by this process.

Conclusions

This article has briefly overviewed the use of lipases in dairy foods. Characteristics of the enzymes used and the nature of the resulting enzyme-modified products are also presented. A number of key factors are involved in the production of enzyme-modified lipid ingredients. Among these are the type and specificities of the enzymes used, nature of the substrates that the enzymes act on, and the reaction conditions such as time duration, temperature, pH, moisture content, presence of reaction aids (e.g., emulsifiers, salts, and precursors), the enzyme-to-substrate ratio, and the processing steps used to prepare the substrates and to inactivate the enzyme.

As the demand for convenience, nutrition, and flavor in consumer foods increases, enzyme-modified ingredients will continue to find greater use in such products. In nature, almost all flavors have their origin in enzyme reactions. Some of the flavor-impact compounds are primary end products, and some others are secondary or tertiary reaction products of chemical and enzymatic changes accorded to the primary end products. Therefore, flavor is a dynamic system and in a state of flux. Enzymatic modification of milk fat accelerates the development of 'aged' flavor in chronologically young cheeses and shows greater potential as the demand by consumers for fast, convenient, nutritious foods increases.

See also: **Cheese:** Enzyme-Modified Cheese.

Further Reading

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Proteinases

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Introduction

Proteinase is the generic term given to enzymes that catalyze the breakdown of proteins into large peptide fragments. In dairy processing, proteinases work in tandem with peptidase activities, which hydrolyze large protein fragments into smaller peptides or individual amino acids. Proteinases can be classified according to different parameters such as (**Table 1**)

- pH optimum (alkaline, neutral, and acid proteinases) and temperature range
- specific amino acids that are central for hydrolytic activity (aspartic, cysteine, serine) and/or the requirement for metal ions (metalloproteinases)

Different proteinases have traditionally been used in fermented dairy products (cheese and fermented milks) to improve nutritional value, sensory properties, and shelf life. Among these, rennet, a crude gastrointestinal proteinase preparation, has been reported as being used in cheese making as early as 6000 BC. Interestingly, rennet is still the most extensively used exogenous enzyme in the dairy industry. The aim of this article is to review the main sources of exogenous proteinases used in the dairy industry. The discussion will focus only on exogenous proteinases that are intentionally added into dairy products. However, milk naturally contains indigenous proteinases (e.g., plasmin and cathepsin D) that may remain active after pasteurization (*see* **Enzymes Indigenous to Milk**: Plasmin System in Milk and **Heat Treatment of Milk**: Sterilization of Milk and Other Products) along with other exogenous proteinases (derived from psychrotrophs) that may contribute to proteolysis of milk proteins and dairy product spoilage. The application of exogenous proteinases will be discussed in relation to specific dairy products, techno-functional additives, foods for specific populations, and nutraceutical/functional food ingredients.

Proteinase Sources

Exogenous proteinases used in dairy processing are sourced from plants, animals, and microorganisms (**Table 1**). These are generally commercialized as food-grade preparations in liquid, paste, and powder forms. Some of these enzyme preparations can also be encapsulated for greater stability during storage and food processing, and for targeted/controlled release during processing. Most of the food-grade enzyme preparations have a relatively low purity as the process used in their extraction must comply with food-grade procedures which generally do not allow multistep extractions. Therefore, other enzymatic side activities, notably peptidase activities, are quite often associated with the main proteinase activity.

Animal Sources

Animal rennet is a blend of enzymes principally containing chymosin. It also contains other side activities such as pepsin and lipases (*see* **Cheese**: Rennets and Coagulants; Rennet-Induced Coagulation of Milk). The main source of animal rennet is the fourth stomach of newborn calves; however, other sources include lamb, buffalo, sheep, goat, and camel. Chymosin preferentially cleaves the Phe_{105}–Met_{106} bond of κ-casein and releases a fragment called (glyco)macropeptide (negatively charged fragment of κ-casein (f106–169)) and para κ-casein. In native casein, (glyco)macropeptide contributes to electrostatic repulsion between casein micelles, maintaining them in a colloidal suspension. The removal of this fragment decreases electrostatic repulsion and steric factors, causing aggregation of casein micelles, followed by gel formation.

Mammalian gastrointestinal proteinase preparations mainly from bovine and porcine sources are used extensively in dairy processing. Pepsin, which is found in the mammalian stomach, has its optimum pH between 1.0 and 4.0 and can be used to generate milk protein hydrolysates. Pepsin preferentially hydrolyzes peptide bonds involving aromatic and hydrophobic amino acids. Trypsin, a gastrointestinal proteinase released from mammalian pancreas, preferentially hydrolyzes peptide bonds involving basic amino acids (Arg and Lys) and has an optimum pH between 6.0 and 9.0. Chymotrypsin, another pancreatic proteinase, cleaves at the C-terminus of Phe, Tyr, Lys, and Trp aminoacyl residues. Chymotrypsin is often associated with trypsin as both enzymes have similar properties (size and isoelectric point) and are therefore relatively difficult to separate using conventional food-grade extraction

Table 1 Characteristics of some animal, microbial, and plant proteinases

Enzyme	*Specificity*	*Type*	*pH*	*Main sources*
Animal				
Pepsin	Phe-, Leu-	Aspartic	1–4	Bovine and porcine gastric mucosa
Chymosin	Phe–Met	Aspartic	3–6	Calf, goat, and lamb stomach
Trypsin	Arg-, Lys-	Serine	6–9	Bovine and porcine pancreas
Chymotrypsin	Tyr-, Trp-, Phe-, Lys-	Serine	6–9	Bovine and porcine pancreas
Elastase	Leu-, Ala-, Val-, Ile-	Serine	6–8	Bovine and porcine pancreas
Microbial				
Bacterial neutral	-Phe, -Leu, -Val	Metallo	6–8	*Bacillus subtilis*, *Bacillus amyloliquefaciens*
Bacterial neutral	Broad	Serine	6–9	*Bacillus subtilis*
Bacterial neutral	Broad	Serine	7–10	*Bacillus licheniformis*
Bacterial alkaline	Ile–Phe, Leu–Val	Metallo	7–9	*Bacillus thermoproteolyticus*
Bacterial alkaline	Broad	Serine	7–11	*Bacillus* spp.
Fungal acid	Pepsin-like	Aspartic	2–5	*Aspergillus niger*
Fungal acid	His–Leu, Phe–Phe	Aspartic	2–5	*Aspergillus niger*
Fungal acid	Pepsin-like	Aspartic	4–6	*Rhizopus* spp.
Fungal acid	Chymosin-like	Aspartic	3–7	*Cryphonectria parasitica*
Fungal acid	Chymosin-like	Aspartic	3–7	*Rhizomucor miehei*
Fungal alkaline	Broad	Aspartic	6–9	*Aspergillus oryzae*
Fungal alkaline	Phe–Tyr	Aspartic	6–9	*Aspergillus oryzae*
Fungal alkaline	Leu–Tyr	Metallo	6–9	*Aspergillus oryzae*
Plant				
Ficin	Broad, Lys-, Ala-, Tyr-, Gly-	Cysteine	5–8	Figs
Papain	Broad, Arg-, Lys-, Phe-	Cysteine	5–9	Papaya fruit
Bromelain	Lys-, Tyr-, Gly-	Cysteine	5–8	Pineapple fruit and stem

Godfrey T (1996) Protein modification. In: Godfrey T and West S (eds.) *Industrial Enzymology*, 2nd edn., pp. 303–325. London: Macmillan Press.

procedures. Elastase, a proteinase present in many mammalian preparations, preferentially hydrolyzes at the C-terminus of Ala, Leu, Val, and Ile residues.

Microbial Sources

Proteinases from microbial sources can be used in two modes in the dairy industry: either by direct inoculation of microorganisms or as a proteinase extract (cell-free) from a microbial culture. Various peptidase activities are also found within (starter) bacteria and therefore these activities will be present in commercial preparations from these bacterial sources. The proteolytic activities of lactic acid bacteria (LAB) aid in their ability to grow in milk: protein breakdown allows the bacteria to utilize small peptides and amino acids in their metabolism to promote growth and acid production (*see* **Lactic Acid Bacteria**: Proteolytic Systems). Bacterial proteinases can be located inside the cell or can be associated with the cell wall (cell envelope proteinase (CEP); *see* **Lactic Acid Bacteria**: Proteolytic Systems). While CEPs are involved in the degradation of intact caseins, the intracellular proteinases are potentially not available for protein breakdown due to their intracellular location. The intracellular proteinases may however be released into the dairy product following bacterial autolysis, and once released, these proteinases are readily available for proteolytic action.

In addition to their contribution to protein digestion when added in the form of starter bacteria, microbial proteinases from different sources (see **Table 1**) are used extensively for the generation of milk protein hydrolysates. A specialist application of microbial proteinases is as rennet substitutes. The major microbial rennet sources are from *Rhizomucor miehei*, *Cryphonectria parasitica*, or *Rhizomucor pusillus* Lindt. Additionally, genetically engineered microorganisms have been developed using recombinants for calf prochymosin cloned in *Escherichia coli*, *Kluyveromyces marxianus* var. *lactis*, *Aspergillus niger*, and *Aspergillus oryzae* to yield the so-called fermentation-produced chymosins. These genetically engineered microbial rennets have received regulatory approval and are being used by the major cheese companies (*see* **Cheese**: Rennets and Coagulants; Rennet-Induced Coagulation of Milk).

Plant Sources

Plant-derived proteinases are primarily used for their milk-clotting activity in cheese making (*see* **Cheese**: Rennets and Coagulants; Rennet-Induced Coagulation of Milk). Interest in the utilization of vegetal renneting activities has developed in response to the growing demand for vegetarian cheeses. Different plants, including fig, melon, wild thistle, and safflower, naturally contain proteinases that can coagulate milk proteins. For instance, plant rennets extracted from *Cynara cardunculus* flowers have been used to manufacture a wide variety of Spanish and Portuguese cheeses. A crude aqueous proteinase extract from *C. cardunculus* has been obtained according to a patented method. The mechanism(s) of coagulation greatly depend on the milk source as the bonds preferentially cleaved on κ-, α_{s1}-, α_{s2}-, and β-casein differ from ovine to caprine milk proteins. The primary cleavage site associated with milk clotting is the Phe_{105}–Met_{106} peptide bond in bovine κ-casein and the Lys_{116}–Thr_{117} bond in caprine κ-casein. Compared to animal rennet (chymosin activity), vegetal rennet is reported to have a relatively stronger proteolytic activity. This makes these enzymes potential candidate activities for accelerated ripening of cheese. Other vegetal proteinases such as enzyme extract from germinated barley may also have potential in accelerated Cheddar cheese ripening. Papain from papaya leaves, bromelain from pineapple, and ficin from figs are examples of commercially available proteinase preparations with potential in the generation of milk protein hydrolysates. Papain, active between pH 5.0 and 9.0, specifically hydrolyzes Arg, Lys, and Phe bonds, whereas bromelain hydrolyzes Lys, Tyr, and Gly bonds at pH between 5.0 and 8.0 (**Table 1**).

Application of Proteinases in the Dairy Industry

Cheese Processing

Two different sources of exogenous proteinases are generally required during cheese processing: chymosin, from rennet, which coagulates milk and which hydrolyzes the caseins during primary proteolysis releasing large and intermediate-sized peptides (*see* **Cheese**: Rennet-Induced Coagulation of Milk), and bacterial proteinases and peptidases, which hydrolyze peptides during secondary proteolysis leading to the formation of smaller peptides and free amino acids.

The release of bacterial proteinases and peptidases is a complex process and needs to be carefully balanced during cheese ripening. Following growth in cheese a certain proportion of dead starter bacterial cells lyse subsequently releasing their enzymes into the cheese matrix. These activities significantly contribute to the organoleptic (flavor, taste, and texture) properties of the cheese (*see* **Cheese**: Cheese Flavor). The other population of dead cells, also called spheroplasts or protoplasts, remain intact in cheese retaining their proteinase/peptidase activities and may be subject to autolysis at later stages of ripening (*see* **Lactic Acid Bacteria**: Physiology and Stress Resistance). Extensive autolysis of the starter is generally not desired as a balance between autolyzed/intact live/dead LAB is needed for other biochemical reactions in cheese.

Acceleration of Cheese Ripening and Enzyme-Modified Cheese

Different biochemical reactions are involved in cheese ripening: glycolysis and lipolysis occur relatively quickly within the cheese environment, whereas proteolysis can be relatively slow due to delays in LAB autolysis (up to several months). Proteolysis is the rate-limiting step in the ripening process of most cheeses and it therefore governs flavor, taste, and texture development (*see* **Cheese**: Accelerated Cheese Ripening). Acceleration of cheese ripening has been proposed as a means of reducing costs associated with cheese ripening and as an approach to the manufacture of a fast-ripened curd for processed cheese (*see* **Cheese**: Pasteurized Processed Cheese Products). The use of exogenous enzyme preparations can accelerate ripening time by up to 75%. Different ways of accelerating cheese ripening have been proposed and these are mostly based on the utilization of proteinases and peptidases via

- microbial inoculation using adjunct cultures as a means of increasing starter biomass and/or attenuated bacterial cell numbers
- incubation with bacterial cell-free extracts
- incubation of the cheese mass with various exogenous enzyme activities including proteinases and peptidases from plant, animal, and bacterial sources, which is the most common technique for acceleration of cheese ripening. This includes increasing the rennet dose rates along with the use of plant rennet from *C. cardunculus*. These exogenous enzymes may also be presented as liposome-entrapped activities; however, although this approach is successful, it is currently restricted due to associated costs.

Combinations of the aforementioned methods may also be utilized to produce a more balanced product. This is particularly the case in relation to reducing bitterness development following extensive proteolysis.

Enzyme-modified cheeses (EMCs) find application in processed foods (soups, sauces, dips, spreads, biscuits, and analogue cheese) where they are mainly used as low-cost flavoring ingredients with a relatively strong cheese flavor (20–50 times more intense than cheese). The main ingredients for EMCs are medium-aged cheeses, cheese curd, and a blend of microorganisms and/or exogenous enzymes (proteinases, peptidases, lipases, and esterases) (*see* **Cheese**: Enzyme-Modified Cheese). Specific combinations of enzymes can be exploited to develop particular flavors such as that of Cheddar, Parmesan, Swiss, Gouda, Blue, and Mozzarella cheese. The enzyme preparations used to generate EMCs are mainly from microbial sources (*Bacillus* and *Aspergillus* spp.), with acidic and neutral proteinase activities. Some plant-derived preparations such as bromelain have also been used for this purpose.

Milk Protein Hydrolysates

Milk proteins are subjected to enzymatic hydrolysis using various proteinase activities to generate a range of hydrolysate products. These hydrolysates find application as technofunctional and biofunctional ingredients in various formulated food products ranging from beverages, infant formulas to foods for specific population needs.

Technofunctionality of milk peptides

The functional properties of proteins and peptides govern their behavior in food matrices, and these are directly linked to their physicochemical properties. Milk protein hydrolysates are employed as ingredients in various formulated foods due to for example, their enhanced gelling, solubility, foaming, or emulsifying properties in comparison to the intact/unhydrolyzed protein (**Table 2**).

Enzyme-induced gels

In general, hydrolysis results in the formation of peptides, which are less hydrophobic and more charged than the parent protein. These changes result in a decrease in the interactions between peptide fragments and are

Table 2 Some characteristics of the technofunctional properties of milk protein hydrolysates

Technofunction	*Substrate*	*Enzyme*	*Functional peptide characteristics*
Solubility	Casein	Corolase PP, bromelain	-
	β-Casein A1	Trypsin	-
	κ-Casein	Chymosin	Caseinomacropeptide
	Lactalbumin	Commercial proteases	-
	Whey protein isolate	Trypsin, chymotrypsin, neutrase, Alcalase	-
Emulsification	Casein	Trypsin, chymosin, Alcalase, neutrase, pronase, pepsin, acid fungal protease, prozyme 5	β-Casein (f101–145), (f107–145), and (f107–135)
		Bacillus subtilis protease	DH: 0.5–1%, fragments active at pH 8–10
		Plasmin	γ-Casein
		Trypsin	α_{s1}-Casein (f167–208), β-casein (f48–63) and (f129–184), fragments active at pH 4–5
	Acid casein	Trypsin, chymotrypsin, Rhozyme 4 (immobilized enzymes)	DH: 2–5%
	Casein κ-Casein	Chymosin	Caseinomacropeptide, active at pH >5.5
	α_{s1}-Casein	Pepsin	1–23 N-terminal residue of α_{s1}-casein
	β-Casein	Trypsin	Fragments active at pH 1.5–3.5
		Plasmin	β-Casein (f29–105) and (f29–107), active at pH 6.7
		Chymosin	-
	Whey protein concentrate	Trypsin	Fragments >5 kDa
		Trypsin, chymosin	Molecular weight >1 kDa
	Whey protein isolate	Trypsin Alcalase	10 kDa retentate 10 kDa permeate
	β-Lactoglobulin	Trypsin (immobilized)	-
Gelation	Casein, κ-casein	Chymosin	-
	Whey proteins	Alcalase 2.4L (*Bacillus licheniformis*)	Hydrolysates with DH >18%
		Bacillus licheniformis protease	Hydrolysates with DH 2.2 and 1.8–6.8%
	Whey protein isolate	*Bacillus licheniformis* protease, Alcalase, esperase, multifact P300, protease M	-
	β-Lactoglobulin	Trypsin	Low DH
		Alcalase 2.4L (*Bacillus licheniformis*)	Extensive DH
		Bacillus licheniformis protease	Low DH
	α-Lactalbumin	*Bacillus licheniformis* protease	Low DH

DH, degree of hydrolysis.

Creusot N and Gruppen H (2007) Enzyme induced aggregation and gelation of proteins. *Biotechnology Advances* 25: 597–601; Panyam D and Kilara A (2003) Peptides from milk proteins and their properties. *Critical Reviews in Food Science and Nutrition* 43: 607–633.

therefore detrimental to gel formation. However, under specific enzymatic hydrolysis conditions, milk proteins can acquire improved gel-forming properties. This phenomenon is the basis of cheese making, which involves gelation of casein micelles following the removal of κ-casein fragments with rennet (*see* **Cheese**: Rennet and Coagulants; Rennet-Induced Coagulation of Milk).

Furthermore, it is also known that under certain conditions, whey proteins form gels following enzymatic hydrolysis. The gelation of whey protein hydrolysates generated with Alcalase 2.4L (a serine alkaline protease from *Bacillus licheniformis*) has been attributed to the presence of glutamyl endopeptidase (GE) in Alcalase. While GE hydrolyzes hydrophilic bonds, relatively large hydrophobic peptides are also obtained, which can form self-aggregates. Gelation has been attributed to the establishment of hydrophobic interactions between these aggregates, with hydrogen bonds and electrostatic interactions playing a minor role. The central role of GE in the formation of aggregates in whey protein hydrolysates has been demonstrated in studies involving incubation with subtilisin (the main proteinase activity in Alcalase) along with combinations of subtilisin and GE activity.

Other types of gels can be obtained via the so-called plastein reaction, which has been defined as the enzymatic formation of peptide bonds between peptides. This reaction is observed at relatively high protein concentrations (20–40%) in the presence of a proteinase catalyst. Generally, gels are formed with peptides within the 1 kDa size range at pH values between 4 and 7. Hydrophobic bonding between plasteins results in the formation of aggregates, which is subsequently followed by gelation. Besides being of interest for the production of gels, it has been proposed that this proteinase-induced reaction can be used for other purposes such as the enrichment of peptides or proteins with essential amino acids, and the generation of new peptides with improved solubility, reduced bitterness, or decreased allergenicity. Plasteins have been obtained following the action of enzymes such as pepsin, chymotrypsin, and papain on sodium caseinate.

Solubility and water-holding capacity

From a technofunctional perspective, good protein solubility is central for the efficient incorporation of protein ingredients into formulated foods. Partial hydrolysis of proteins is generally associated with an increase in their aqueous solubility. The increased solubility is linked to the smaller size of peptides and to the increased number of ionized groups (-NH_3^+ and -COO^-). The extent of enhancement of solubility is enzyme specific. There appears to be no direct correlation between the degree of hydrolysis (DH: percentage of peptide bonds cleaved) and solubility. Milk protein hydrolysates demonstrate increased solubility when various enzyme preparations from microbial, plant, and animal sources are used to hydrolyze caseins and whey proteins (**Table 2**).

Water-holding capacity refers to water bound to proteins and peptides. Water uptake increases with the increased number of ionic groups. Therefore, protein hydrolysates generally exhibit a greater water-holding capacity as compared to the parent protein. Water-holding capacity directly influences water activity of the food and therefore affects the rheological behavior and shelf life. The impact of hydrolysis on water-holding capacity depends on the proteolytic activity and the reaction conditions during hydrolysis (milk preheat treatment, pH, and temperature).

Emulsification and foaming

Hydrolysis of milk proteins alters their physicochemical characteristics and therefore modifies their foaming and emulsifying properties. Peptides with a molecular mass greater than 5 kDa are reported as being highly efficient at improving emulsifying properties. Hydrolysis of whey proteins results in the release of hydrophobic peptides that are masked within the native protein structure. These hydrophobic peptides are likely to readily interact with the interface of fat globules, thus improving emulsion stability. Nevertheless, there appears to be no direct correlation between peptide hydrophobicity and improvement in emulsifying properties. It is considered more likely that it is the amphiphilic character of the peptides (distribution of hydrophobic and hydrophilic groups) that governs adsorption at the interface. For sodium caseinate hydrolysates, the emulsifying properties were highest around the isoelectric point. Regarding foaming capacity, low molecular weight peptides appear to exhibit higher foaming properties than larger peptides or native proteins. Emulsification of milk proteins is improved by using different enzyme preparations for protein hydrolysis. Digestion of casein with gastrointestinal activities appears to be the most common approach to improving emulsification properties (**Table 2**).

Biofunctionality of milk peptides

Numerous biologically active peptides are encrypted within the primary structures of the milk proteins. These include peptides with opioid, immunomodulatory, antimicrobial, blood pressure-reducing, and antithrombotic activities. Release of these peptides requires a hydrolytic step, which can be catalyzed via proteinase and peptidase activities from endogenous and exogenous sources. Three main routes exist for the release of bioactive peptides: during gastrointestinal digestion, during bacterial fermentation, or during hydrolysate manufacture following addition of exogenous food-grade proteinase/peptidase activities.

Bioactive peptides

The main bioactive properties attributed to milk peptides and the proteinases capable of releasing these peptides from different milk proteins are summarized in **Table 3**. Clearly, enzymatic specificity is central to the release of bioactive peptides. Nevertheless, the presence of the enzyme is not sufficient as the target sequence in the protein must be accessible to hydrolysis. This access can be hindered by the spatial arrangement of the protein, which is directly correlated to its tertiary and quaternary structure. Therefore, a denaturation step may sometimes be beneficial in the hydrolysis process. Optimization of different parameters during hydrolysis is crucial to the efficient release of bioactive peptides. In certain instances, for example, extensive hydrolysis may result in a loss in bioactivity. To date, a limited number of studies have addressed process optimization to release bioactive peptides during hydrolysis. Central composite rotatable designs including various parameters (i.e., pretreatment temperature, pH, temperature, hydrolysis time, enzyme to substrate ratio) may be used to optimize

Table 3 Examples of bioactive peptides released by the action of exogenous proteinases on milk protein substrates

Bioactive property	*Substrate*	*Enzyme*	*Bioactive peptide characteristics*
Angiotensin-I-converting enzyme inhibitory	β-Lactoglobulin and α-lactalbumin	Trypsin	-
	Whey proteins	Proteinase K	-
	Casein	Trypsin, protease from *Aspergillus oryzae*	-
	α-Lactalbumin	Pepsin Pepsin + trypsin + chymotrypsin Trypsin	(f50–52), (f99–108), (f104–108)
	β-Lactoglobulin	Pepsin Pepsin + trypsin + chymotrypsin Trypsin	(f22–25), (f32–40), (f81–83), (f94–100), (f106–111), (f142–146)
Antithrombic	κ-Casein	Trypsin	Glycomacropeptide
Microbial growth inhibition	Lactoferrin	Pepsin	Lactoferricin
	α_{s2}-Casein	Chymosin	Casocidin
	β-Lactoglobulin and α-lactalbumin	Gastrointestinal proteinases	-
	Lactoferrin	Trypsin	(f345–689), (f1–280), (f222–230), and (f264–269)
Antimicrobial, antiviral, and antipathogen adhesion	Casein	Papain	Iracidin
Regulation of the gastrointestinal system	Casein	Alcalase, thermolysin, subtilin, pepsin, trypsin	Caseinophosphopeptides, β-casein (f1–25)
Opioid	Casein	Gastrointestinal enzymes (pepsin and trypsin)	β-Casomorphins, β-casein (f60–70), α_{s1}-casein (f91–100)
	β-Lactoglobulin	Pepsin + trypsin	β-Lactorphin
	β-Casein	Pepsin + trypsin	(f59–68)
	κ-Casein	Pepsin	(f33–38)
		Pepsin + trypsin	κ-Casoxins
	α_{s1}-Casein	Pepsin	(f90–96)
	α-Lactalbumin	Pepsin	α-Lactorphin
Reduced allergenicity	β-Lactoglobulin and α-lactalbumin	Pepsin, chymotrypsin	Hydrolysates with 1–20% DH
	α_{s1}-Casein	Pepsin + chymotrypsin + trypsin	5 kDa permeate
	Casein	Porcine pancreatin	-
	Whey proteins (bovine serum albumin and IgG)	Pepsin, Corolase PP	-
Immunomodulatory	Casein	Trypsin	Caseinophosphopeptides
	α_{s1}-Casein	Trypsin	-
	κ-Casein	Trypsin	Glycomacropeptide

DH, degree of hydrolysis.

FitzGerald RJ and Meisel H (2003) Milk protein hydrolysates and bioactive peptides. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 1: Proteins. Part B*, 3rd edn., pp. 675–698. New York: Kluwer Academic; Plenum Publishers; Korhonen H (2009) Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods* 1: 177–187; Panyam D and Kilara A (2003) Peptides from milk proteins and their properties. *Critical Reviews in Food Science and Nutrition* 43: 607–633.

the enzymatic hydrolysis process. The resultant response surface model is a useful tool in conjunction with liquid chromatography mass spectrometry (LC–MS) for peptide identification/quantification for estimating the release of bioactive peptides during hydrolysis. The combined utilization of bioinformatic and artificial neural network approaches along with a detailed knowledge of enzymatic specificity has significant potential in the identification and targeted release of specific bioactive peptide sequences from food protein sources. However, the challenge still remains in that while to date many studies have demonstrated bioactivity of specific peptides *in vitro*, there is still a lack of data on the impact of these peptides *in vivo*.

Development of foods for specific populations

Milk protein hydrolysates have been used for populations with specific nutritional requirements linked to athletic performance, infant development, food protein allergies, and phenylketonuria (PKU). The DH depends on the final application: hypoallergenic formulas require extensive DH, while nutritional supplements are generally moderately hydrolyzed. Different enzymes are used to generate hypoallergenic formulas; however, this process mainly involves gastrointestinal enzymes. Subjects who suffer from PKU require a diet low in phenylalanine, and specific formulas consisting of hydrolyzed milk proteins have been developed for this purpose. A certain proportion of Phe residues are released from the protein, using enzyme preparations such as papain or Pancreatin™. These Phe residues can be removed from the hydrolysate using adsorbents (activated carbon and/or a polymeric resin XAD-4). Up to 98% removal of Phe can be achieved. Another peptide fragment of interest for PKU sufferers is (glyco)macropeptide, which is released from caseins by the action of chymosin. (Glyco)macropeptide does not contain Phe residues and is therefore of interest as a nitrogen source for PKU diets.

Future developments in milk protein hydrolysates

Different issues arise during the production of enzymatic hydrolysates of milk protein. In many instances, hydrolytic processes that beneficially modulate technofunctional properties such as enhanced surface active properties (generally hydrolysis to low DH values) may not be suitable for hydrolysates having significantly reduced allergenic potential (generally hydrolysis to high DH values). Furthermore, it is generally accepted that extensive proteinase-catalyzed hydrolysis of milk/food proteins results in the development of undesirable bitterness. Bitterness defects associated with extensive proteolysis have been reported in cheese products and in milk protein hydrolysates. The peptides implicated in bitterness development are low molecular weight structures with hydrophobic amino acid residues. These hydrophobic fragments are generally hidden within the folded structure of the protein molecule and are made available to interact with the taste buds following hydrolysis. Therefore, hydrolysates at high DH generally exhibit relatively strong bitter sensations. The occurrence of exopeptidase activities in the enzyme preparation is generally responsible for a lower bitterness in the resultant hydrolysate as compared to hydrolysates generated with proteinase activities alone. Furthermore, peptides containing internally located Pro and indeed hydrophobic amino acid residues are associated with bitterness perception. This observation is being exploited in the debittering of cheeses, EMCs, and milk hydrolysates by utilizing peptidases such as PepX, which releases X-Pro dipeptides from the N-terminus of peptides. This specific peptidase activity is found in a number of commercial enzyme preparations including Debritase® (Danisco) obtained from *Lactococcus lactis* and *A. oryzae* strains. Another strategy to avoid development of bitterness is the use of specific non bittering proteinase preparations such as Corolase PP and leucine aminopeptidase or masking strategies such as the addition of mature cheese or flavor enhancers (monosodium glutamate). Other sapid compounds can appear following amino acid release during protein hydrolysis. These compounds generally have sweet, sulfurous, sour, salty, or umami attributes and can contribute to the overall acceptability/unacceptability of different protein hydrolysates targeted for oral ingestion.

Conclusion and Perspective

The utilization of exogenous proteinases is central in the production of traditional and in the development of new dairy products. This includes various fermented milks and cheeses along with milk protein hydrolysates. A more detailed understanding of the role of proteinases in milk protein hydrolysis and the interactive effects, specifically those of environmental conditions (pH, temperature, substrate matrix effects, etc.) that contribute to hydrolysis is necessary. Such an understanding will greatly increase the ability to produce hydrolyzed dairy foods/food ingredients with defined technofunctional as well as biofunctional properties.

See also: **Cheese**: Accelerated Cheese Ripening; Enzyme-Modified Cheese; Flavor; Pasteurized Processed Cheese Products; Rennets and Coagulants; Rennet-Induced Coagulation of Milk. **Enzymes Indigenous to Milk**: Plasmin System in Milk. **Heat Treatment of Milk**: Sterilization of Milk and Other Products. **Lactic Acid Bacteria**: Physiology and Stress Resistance; Proteolytic Systems.

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Transglutaminase

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Introduction

There is a strong interest for tailoring the technofunctional and sensory properties of various foodstuffs by the targeted alteration of already present, natural food constituents rather than by using additional auxiliary substances, such as thickeners and stabilizers. Microbial transglutaminase (mTGase) is an enzyme that, for example, creates isopeptide bonds by cross-linking proteins. This cross-linking results in the modification of several physicochemical properties such as solubility, heat stability, water-binding or emulsifying capacity, foaming, viscosity, elasticity, and gelation behavior of proteins intended for human consumption, where modification using chemical reagents is not acceptable. Numerous patents exist concerning the application of mTGase in the production of, for example, meat and fish products, bakery products and other foods containing vegetable proteins, and dairy products.

Transglutaminases (TGases) are naturally present in various living organisms and contribute to a great variety of biological functions, such as blood clotting and wound healing. All TGases are protein-glutamine γ-glutamyltransferases and belong to the enzyme class EC 2.3.2.13. Research on industrial applications of these enzymes started with the isolation of TGases from mammalian tissues and body fluids; the first commercially available TGase isolated from guinea pig liver was, however, not very attractive for industrial use because of high production costs owing to limited supply and the requirement of Ca^{2+} for activation. Factor XIII, another TGase isolated from blood plasma, had the disadvantages of red pigmentation and the need of thrombin for activation. A subsequent screening of microorganisms in the late 1980s with respect to their TGase activity resulted in the detection of mTGase from *Streptomyces mobaraensis*. mTGase is Ca^{2+}-independent, has a low substrate specificity, and is, up to now, the only TGase commercially available for the food industry. It is produced by conventional fermentation of the wild-type *S. mobaraensis* strain (GRAS status) with subsequent isolation of the secreted protein. Several attempts are ongoing to apply genetic engineering to reduce production costs, but the activation of the secreted pre-proenzyme, which occurs simultaneously with secretion in the case of *S. mobaraensis* cultivation, is not fully understood and thus not sufficiently controllable yet. Screening of microorganisms, especially of *Streptomyces* sp., for new microbial TGases and improvement of the purification step are still topics of research interest.

Mechanism and Characteristics of mTGase

Generally, mTGase catalyzes the formation of covalent cross-links in protein-containing systems as acyl transfer reactions occur between the γ-carboxyamide group of peptide- or protein-bound glutamine, serving as acyl donor, and primary amines, serving as acyl acceptor. This reaction might be useful for binding amino acids or peptides to a protein, for example, to improve its nutritional value. When the ϵ-amino group of lysine acts as the acyl acceptor, intramolecular and/or intermolecular isopeptide bonds form between the protein molecules, which result in their polymerization and an accompanying modification of their technofunctional properties (**Figure 1**). When primary amines are not accessible, water may act as the acyl acceptor, which then leads to the deamidation of the glutamine residue and the formation of glutamic acid and ammonia. Deamidation may also lead to improved functionality, but as proteins possess a large amount of glutamine and only a few lysine residues, the latter have to be blocked to prevent cross-linking. As no suitable blocking reagents exist, the deamidation reaction is not of significance in the food industry.

mTGase is a monomeric protein with a sequence of 331 amino acids and an isoelectric point of 8.9. As regards its secondary structure, its single polypeptide chain consists of eight β-strands, which are surrounded by 11 α-helices (**Figure 2**). The enzyme adopts a disk-like shape with a deep cleft at the edge, where a single cysteine residue (Cys64) is located. Molar mass as assessed by mass spectrometry and amino acid sequencing is 37.86 kDa. Generally, mTGase is active at pH values between ~4 and 9, with the optimum pH being in the range 6–7. Optimum temperature is 50 °C; the enzyme is inactivated at 70 °C within minutes and, on the other hand, retains some residual activity at near-freezing temperatures. mTGase is sufficiently stable under high hydrostatic pressure, which offers the possibility of modification of proteins even in cases where they are not accessible for mTGase under atmospheric pressure.

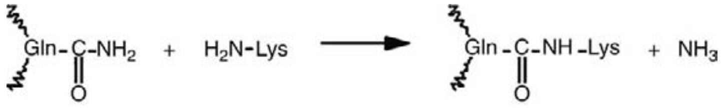

Figure 1 Principle of the cross-linking reaction catalyzed by transglutaminase.

Figure 2 Three-dimensional (3D) structure of microbial transglutaminase. The 3D structure of the coordinate file (1iu4.pdb), which is supported by the Research Collaboratory for Structural Bioinformatics's Protein Data Bank, was displayed by molecular visualization program RasWin Molecular Graphics version 2.7.2.1.1.

Essential for the catalytic activity of TGase is the Cys64 residue, which can be blocked by dicarboxyethylating the thiol group with *N*-ethylmaleimide. Other inhibitors, which also do react with the thiol moiety, include cystamine, monoiodoacetate, parachloromercuribenzoic acid, and a number of heavy metals (Cu^{2+}, Zn^{2+}, and Pb^{2+}). Natural inhibitors of TGase are scarce; there are reports of an antimicrobial agent of fungal origin that inhibits the activity of plasma TGase, and of a small (~250 Da), temperature-sensitive mTGase inhibitor present in milk serum.

Analytical Aspects

The most frequently used method to determine the activity of mTGase is the hydroxamate method. This assay is based on the coupling of hydroxylamine to a synthetic peptide, which forms a colored component in the presence of ferric ions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to determine semiquantitatively the degree of cross-linking of the mTGase-treated proteins. Before protein separation by SDS-PAGE, a reducing agent such as dithiothreitol (DTT), which cleaves the disulfide bonds, is added to the sample. A more quantitative determination of mTGase-induced cross-linking of proteins is possible by gel permeation chromatography, which separates the proteins into monomers, dimers, trimers, and polymers. Cation-exchange chromatography or reversed-phase (RP) HPLC analysis allows determination of the amount of isopeptide bonds. In this method, the protein samples are subjected to exhaustive proteolytic digestion with several enzymes such as pronase, leucine aminopeptidase, prolidase, and carboxypeptidase, resulting in amino acids and isopeptides. Prior to chromatographic separation, the mixture is derivatized with *o*-phthaldialdehyde for fluorometric detection or with phenyl isothiocyanate for UV detection. Most recently, HPLC/mass spectroscopy has been used for the detection and quantification of isopeptides.

Substrate Specificity

The susceptibility of a particular protein to mTGase-induced cross-linking is determined by its macromolecular structure. In milk, the caseins are excellent substrates for mTGase because they do not possess a pronounced tertiary structure. The association of casein molecules into micelles affects the cross-linking rate of particular casein monomers. The extent of cross-linking of κ-casein by mTGase is higher than that of β-casein and of α_{s1}- and α_{s2}-casein because of the location of κ-casein on the surface of the casein micelle, with the glutamine and lysine residues being more accessible to the enzyme. Particle size measurements by dynamic light scattering clearly indicate that cross-linking is exclusively intramicellar rather than between the micelles. In sodium caseinate – an ingredient important for the production of a variety of foodstuffs – the casein molecules are readily accessible to the enzyme owing to their open structure, and this results in fast reaction rates and high degrees of cross-linking. In sodium caseinate systems, the order of cross-linking is β-caseins followed by α_{s1}- and α_{s2}-caseins, and then κ-caseins.

Owing to their ordered tertiary structure, whey proteins have to be unfolded before cross-linking to overcome steric inaccessibility. Unfolding of whey proteins can be induced by adding DTT, increasing the pH to ~9, heating, or applying high hydrostatic pressure.

Applications in the Dairy Industry

Until 2008, the use of enzymes as processing aids was regulated in the European Union on a national basis and, frequently, the declaration of enzymes was not necessary as long as the enzyme was inactive in the product (which is the case when mTGase is used in the production of fermented milk products). Beginning 20 January 2009, Regulation 1332/2008 of the European Parliament and of the Council took effect to generalize the rules for the scientific evaluation of enzymes in food processing. According to the regulation, with a few exceptions, food enzymes should be used only if they are safe, if there is a technological need for their use, and if their use does not mislead the consumer. A food enzyme permitted within a community will appear on a community list describing the enzyme and the specific conditions governing its use. The procedure for authorization depends on the origin of the enzyme and includes tests for genotoxicity in the case of enzymes of microbial origin.

Milk Proteins in Emulsions

Cross-linking by mTGase also affects technofunctional characteristics of milk proteins such as emulsifying and foaming properties. In the case of caseins, pH, enzyme-to-substrate ratio, and cross-linking time significantly affect their behavior in multiphase systems. Although the emulsifying capability of mTGase-treated caseins is hardly affected, their emulsion stability improves. Excessive cross-linking results in increased surface load at the oil–water (O–W) interface, reduced coalescence, more pronounced viscoelasticity at the surface, and improved mechanical resistance of O–W emulsions against shearing. Cross-linked caseins have high heat stability, making them important valuable functional ingredients in foods requiring severe heat treatment.

When β-lactoglobulin is used as the emulsifier in O–W systems, destabilization of the system occurs on treatment with TGase. The protein film on the surface of the oil droplets is highly susceptible to cross-linking, and the isopeptide bonds between different droplets are responsible for flocculation and viscosity increase. However, when β-lactoglobulin is cross-linked in bulk before adding to the disperse phase, it shows a reduced affinity to the O–W interface, and the lower surface coverage is responsible for a reduced stability of the emulsion.

Fermented Milk

The most significant application of mTGase in the dairy industry is in fermented milks. Most of the research using mTGase has been on improving the textural properties (e.g., improved firmness and reduced syneresis) of set-style yogurt (**Figure 3**). More recent research on stirred yogurts shows that mTGase treatment results in increased viscosity. The texture-enhancing effects resulting from mTGase treatment allow a reduction of dry matter enrichment of the yogurt base mix still giving product characteristics similar to those of regular products. In yogurt manufacture, enzymatic modification may be performed either prior to acidification or concomitantly with fermentation by lactic acid bacteria. When cross-linking is done before fermentation, it is necessary to perform additional heat treatment to inactivate mTGase for better control of the degree of cross-linking. The time × temperature requirement for sufficient mTGase action in yogurt is similar to that used for fermentation during yogurt manufacture. Therefore, it may be advantageous to add mTGase and starter culture at the same time to the preheated yogurt base mix. However, there is no additional heat treatment to inactivate the enzyme. Its activity is reduced by the low pH in yogurt and the possible mobility restrictions owing to gelation. In model systems, mTGase is inactivated at a pH below 4.0; above this pH value, some residual activity remains. For both set-style and stirred yogurts, addition of mTGase simultaneously with the starter may cause textural defects such as coarseness or grittiness and viscosity increase during cold storage.

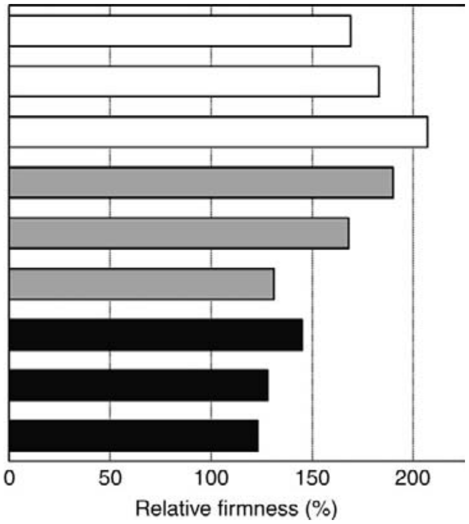

Figure 3 Firmness of set-style yogurt from mTGase-treated skim milk (open bars), medium-fat milk (gray bars), and full-fat milk (black bars). For each study, the firmness of control yogurt made from untreated milk was 100%. Data are compiled from various sources.

Cheese

Suitability of mTGase-treated milk for cheesemaking is restricted by the degree of cross-linking. Formation of the rennet gel network is significantly delayed by cross-linking of the caseins. Because the κ-casein is located mainly on the surface of the micelles and is the preferred target for mTGase, cross-linking hinders the Phe105–Met106 bond of κ-casein, limiting its accessibility to milk-clotting enzymes. In high-heat-treated milk in which β-lactoglobulin interacts with κ-casein, cross-linking of casein micelles results in sealing of the micelle surface.

Nondairy Products

Fish and meat

In the fish and meat industries, mTGase is used alone or in combination with, for example, caseinate as a cold binder to produce restructured products at low temperature. The mechanical and viscoelastic properties of the product depend on the source of protein, the amount of mTGase and additives, and the time and temperature conditions during enzyme treatment.

Plant protein-based foods

Application of mTGase in plant-based foods is mainly on wheat proteins, which, besides being suitable as substrates for mTGase, are easily available on the world market. mTGase cross-linked flours exhibit higher water-holding capacity and better thermal properties than exhibited by regular flours. Doughs from mTGase cross-linked flours show higher tensile strength and higher resistance to extension when compared to dough from regular flours, and the volume and crumb characteristics of breads are improved. Also, mTGase treatment enhances the baking quality of dough containing weak protein and improves the texture and cooking stability of noodles. Proteins of other vegetable origin, such as pea or lupine, are in the focus of interest to produce innovative new plant-based foods.

Applications of TGase in Nonfood Products

There is an increasing research interest in nonfood-industry applications of mTGase. For example, in the leather industry cross-linked casein may be used as a filler to smoothen irregularities on the leather surface. In wool textiles, treatment with mTGase may reduce loss in tensile strength that occurs after proteolytic treatment of wool. In the field of tissue engineering, mTGase cross-linked scaffolds may replace scaffolds derived from collagen or gelatin.

See also: **Fermented Milks**: Yogurt: Types and Manufacture. **Milk Proteins**: Casein Nomenclature, Structure, and Association; Caseins, Micellar Structure. **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications and Regulatory Aspects; Functional Properties of Milk Proteins; Milk Protein Concentrate.

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Catalase, Glucose Oxidase, Glucose Isomerase and Hexose Oxidase

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Introduction

Exogenous enzymes have two principal functions in foods: (1) they may be added to foods to effect some change and (2) they may be used as components in enzyme assay kits. Adding enzymes to foods to create new and unique products is an age-old process. While many indigenous oxidoreductases are of significance in dairy foods, with some exceptions, these enzymes and isomerases are rarely used as exogenous enzymes.

In comparison to rennets, other exogenous enzymes have limited applications in the dairy sector; principal among these enzymes are lipases and β-galactosidase (*see* **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase; Lipases). Other enzymes with limited applications in dairy processing include superoxide dismutase, sulfhydryl oxidase, lysozyme, lactoperoxidase (LP), catalase, glucose oxidase (GO), hexose oxidase, and glucose isomerase (GI). GO and catalase are often used together in selected foods for preservation. This article will focus on the applications of catalase, GO, and GI in the dairy sector.

Catalase

Catalase (EC 1.11.1.6) is a tetrameric heme-containing enzyme and is present in the cells of all aerobic organisms. It catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen:

$$H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$$

Commercially, catalase is produced mainly by extraction from bovine liver and, in recent years, from *Aspergillus niger* and *Micrococcus luteus*. Sweet potatoes are also a good source of catalase.

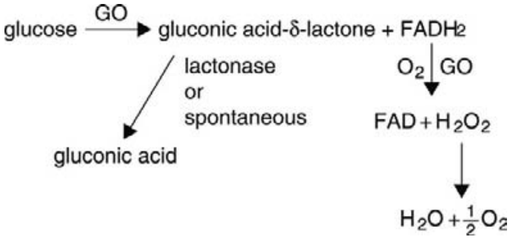
Catalase has potential uses in the food, dairy, textile, wood pulp, and paper industries. In regions lacking refrigeration, H_2O_2 is used for the cold-sterilization of milk. The US Food and Drug Administration (FDA) permits the use of H_2O_2 in products such as cheese, milk, eggs, and whey, but stipulates that residual H_2O_2 must be removed from the product by appropriate physical or chemical means. While the FDA does not actually define a method for the removal of residual H_2O_2, many of the regulations dealing with specific products suggest the use of catalase. The use of catalase is more cost-effective and environmentally friendly than chemical methods for reducing H_2O_2. H_2O_2 may be decomposed slowly by the indigenous catalase of raw milk. However, the amount of catalase in milk is not sufficiently high to ensure complete destruction of added H_2O_2 in an adequate time period. Catalase is added to avoid the high oxidation/reduction potential caused by the presence of residual H_2O_2, which also interferes with the active growth of starter organisms in fermented dairy products. In developing regions, milk is treated with H_2O_2 at ambient temperature and excess H_2O_2 is reduced by catalase or by chemical interaction with milk proteins to which it causes some physicochemical changes, principally by the oxidation of methionine, with adverse effects on cheese quality. These side effects can be reduced by short-term exposure of H_2O_2 to ~65 °C, after which the residual H_2O_2 can be reduced by the addition of commercially available catalase.

Another use of catalase in the preservation of milk is its use as a component of the LP–H_2O_2–thiocyanate system in which a low level of H_2O_2 is required to enhance the antibacterial activity of LP in milk. The addition of small amounts of thiocyanate ($15\ \text{mg kg}^{-1}$) and H_2O_2 ($8.5\ \text{mg kg}^{-1}$) to milk activates the natural LPs in milk. With good sanitary practice, the LP system is applicable in providing a simple and cheap method of preventing spoilage of raw milk during collection and storage in tropical regions. Catalase can be used to degrade excess H_2O_2.

There has also been interest in the use of immobilized catalase for the sterilization of milk using H_2O_2. Although catalase may be immobilized readily, a major disadvantage of this system is that catalase is inactivated rapidly on exposure to H_2O_2. Catalase is frequently used together with GO in many applications of the latter (see below).

Glucose Oxidase

GO (EC 1.1.3.4) is a flavoenzyme that catalyzes the oxidation of β-D-glucose to gluconic acid (via gluconic acid-δ-lactone) according to the following reactions:



The H_2O_2 produced is normally reduced by catalase present as a contaminant in commercial preparations of GO or by deliberately added catalase. Since its discovery as an 'antibiotic' (its antimicrobial activity was shown subsequently to be due to the formation of H_2O_2), there has been an interest in GO, chiefly because of its use in an enzyme assay for glucose. However, exogenous GO has not lived up to its promise in the food industry. GO has a number of potential uses in food processing.

Removal of Trace Levels of Glucose

Egg white is used as a foaming agent in the manufacture of confectionery and cakes. Dehydrated egg white contains trace amounts of glucose, which can cause the food to turn brown due to the Maillard reaction when stored for significant periods of time. The addition of GO to degrade trace levels of glucose prior to dehydration can greatly improve the quality of egg whites, although there are alternatives to the use of this enzyme (e.g., using yeast fermentation).

Removal of Trace Levels of Oxygen

Trace amounts of oxygen can cause loss of flavor, discoloration, and/or oxidation of ascorbic acid. GO has been used to remove trace levels of O_2 from products including lemon juice, beer, soybeans and soy milk, and potato chips and to remove O_2 from the headspace above canned or bottled products. The addition of GO and xylanase during bread processing may greatly improve the quality and structure as well as the appearance of the bread. The oxidation of oil or fat is inhibited in meat if the O_2 is removed by treatment with GO and superoxide dismutase. Chemical reducing agents may be used as oxygen scavengers but the use of enzymes such as GO is the preferred treatment. GO has been proposed as an antioxidant in high-fat foods such as mayonnaise, butter, and whole-milk powder, but it does not appear to be used widely, possibly due to the low cost of chemical antioxidants (if permitted).

Generation of H_2O_2 *In Situ*

The H_2O_2 generated by GO has bactericidal properties and these properties can be effectively exploited as a component of the LP–H_2O_2–thiocyanate system.

Production of Acid *In Situ*

Direct acidification of dairy products is performed by the addition of preformed acid or an acidogen (usually gluconic acid-δ-lactone) or by a combination of acid and acidogen. The action of GO on glucose may also be used to produce acid *in situ*. One suggested application of GO is in the replacement of slow-growing *Streptococcus thermophilus* in yogurt manufacture. GO can be used in conjunction with β-galactosidase and H_2O_2 to substitute *Sc. thermophilus* and to reduce the initial pH of milk at which point *Lactobacillus delbrueckii* subsp. *bulgaricus* is added.

Other Uses

Fructose syrup without glucose can be produced from invert sugar using GO. Packaging materials impregnated with GO and glucose are capable of consuming oxygen and thus prevent the oxidation and decay of lipid-containing foods and the growth of microorganisms.

Hexose Oxidase

Danisco A/S (Copenhagen) has commercialized hexose oxidase, an enzyme related to GO but also with activity on lactose as substrate. The hexose oxidase gene was isolated from the red seaweed *Chondrus crispus*, cloned into a host microorganism, and the enzyme was produced by fermentation. In addition to being used as an antibacterial agent, an antioxidant, and probably in some of the potential applications of GO mentioned above, hexose oxidase could have applications in controlling Maillard browning of Mozzarella cheese used as pizza topping.

Glucose Isomerase

GI (D-glucose ketoisomerase) catalyzes the isomerization of glucose to fructose:

$$\text{glucose} \xrightarrow{\text{glucose isomerase}} \text{fructose}$$

The major application of GI in the food industry is in the production of high-fructose syrups (e.g., high-fructose corn syrup (HFCS)) for use as a sweetener. The production of HFCS is possibly the largest industrial use of immobilized enzymes. Immobilized GI used in the production of HFCS is isolated from various bacterial sources. A potential use of GI is in the production of high-fructose syrups from whey following hydrolysis of lactose by β-galactosidase, but we are not aware of any commercial application of this process.

See also: **Additives in Dairy Foods**: Legislation. **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase; Lipases; Proteinases.

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ENZYMES INDIGENOUS TO MILK

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Lipases and Esterases

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Introduction

Lipases and esterases are hydrolases (EC 3.1.1.–). Esterases prefer soluble over emulsified substrates, a property that is used to distinguish esterases from lipases. Lipases hydrolyze ester bonds in emulsified esters at a water–oil interface, although some have limited activity on soluble esters. Three types of esterases have been reported in milk, namely A-type carboxylic ester hydrolase (arylesterase; EC 3.1.1.2), B-type esterase (glycerol tricarboxyl esterase, aliphatic esterase, lipase; EC 3.1.1.3), and C-type esterase (cholinesterase; EC 3.1.1.7; EC 3.1.1.8). A-type carboxylic esterases hydrolyze aromatic esters, for example, phenylacetate, but do not hydrolyze tributyrin. B-type esterases hydrolyze aliphatic esters rapidly and aromatic esters very slowly, and are inhibited by organophosphates. C-type esterases are active on choline esters. They also hydrolyze some aromatic and aliphatic esters slowly and are also inhibited by organophosphates. The ratio of A:B:C esterase activities in normal milk is 3:10:1. Milk from cows with mastistis contains a higher level of A-type esterase activity than B- and C-type esterases. A- and C-type esterases seem to have no significant technological importance in milk. Human milk contains a bile salt-stimulated lipase, which plays a role in the digestion of milk fat in neonates. It has been shown to be very closely related to pancreatic lipase.

Milk lipase is a lipoprotein lipase (LPL). It occurs at the endothelial surfaces of blood vessels where it hydrolyzes triglycerides of blood lipoproteins to liberate fatty acids. Milk LPL belongs to the family of lipases that catalyze steps in the digestion and transport of triglycerides. Mammalian milk contains varying concentrations of LPL: bovine milk contains $\sim 2\,\mathrm{mg\,l^{-1}}$, buffalo milk contains a similar concentration of LPL as bovine milk, but human milk contains less than 50% of the LPL concentration in bovine milk. Porcine milk contains 4 times the concentration of LPL present in bovine milk, while guinea pig milk contains 20–50 times as much, which represents 0.1% of total protein. The concentration of LPL in ewes' milk decreases during lactation but it increases in bovine, goat, and guinea pig milk. LPL has an important role in milk production in the mammary gland. LPL activity in the mammary gland is low before and during pregnancy; it increases shortly before parturition and remains high throughout lactation. The reverse occurs in adipose tissue. Changes in the activity of LPL in the mammary gland and adipose tissue result in the transfer of blood triglycerides to the mammary gland for the synthesis of milk lipids. Fatty acids generated in human milk by the action of LPL are reported to have a powerful antiparasitic function.

Origin, Isolation, and Characterization

The importance of LPL in milk is well known because of its role in the development of hydrolytic rancidity in milk and milk products. LPL is synthesized in mammary gland secretory cells. Most of it is transported to capillary endothelium where it hydrolyzes the circulating triglycerides of lipoprotein complexes to free fatty acids (FFAs) and monoglycerides. These FFAs and monoglycerides are absorbed by the mammary gland and used for milk fat synthesis. Milk LPL appears to be identical with the LPL in mammary gland, and to be transferred to milk from the mammary gland. In bovine and guinea pig milks, most of the LPL is associated with the casein micelles by electrostatic bonding between negatively charged phosphates on the caseins and positively charged amino acid residues in the 'heparin'-binding sites of the LPL. Hydrophobic bonding may also be involved as the lipase can be dissociated from the casein micelle by dimethylformamide.

The distribution of LPL between the skim milk (casein) and the cream (fat) phases of milk is variable between species. Most of the bovine LPL is in the skim milk fraction, some is in the soluble form, and a small amount is associated with the milk fat globule membrane (MFGM). More becomes associated with the MFGM after some physical manipulations and also in spontaneously lipolytic milk (see below). In human milk, most of the LPL is associated with the milk fat globules. In buffalo milk, about 23% of LPL is in the cream phase and the rest is attached to casein micelles. In goats' milk, LPL is distributed equally between the cream and serum phase and about 10% is attached to caseins. The differences between goat and bovine milk in the distributions of LPL are probably due to a smaller proportion of α_S-casein in goats' milk compared to bovine milk.

Cooling or freezing of bovine milk results in the transfer of LPL from the skim milk fraction to the milk fat globules. LPL can be dissociated from the casein micelles by treatment with dimethylformamide, 1 mol l^{-1} NaCl, or heparin. The lipase present in bovine colostrum is not bound to casein and is not activated by blood serum. Hence, colostral lipase can be classified as a triacylglycerol lipase, but it has no apparent homology to the bile salt-stimulated lipase in human milk. It has also been shown to have carboxyl (B-type) esterase activity. The lipolytic activity of colostrum measured on a tributyrin emulsion at pH 8.5 and 37 °C is about one-third of that of skim milk; however, the esterase activity measured against the carboxylesterase substrate heptanoyl 4-methylumbelliferone is 10 times higher in colostrum than in skim milk.

The first step in the isolation of LPL from milk involves absorption onto heparin-agarose. The complex is then washed with 0.7–0.9 mol l^{-1} NaCl, and LPL is eluted from the complex by 1.5–2 mol l^{-1} NaCl. An alternative method reported for the purification of LPL involves addition of a lipid emulsion to skim milk and collection of the lipid–LPL complex by centrifugation. Further purification is achieved by the adsorption of the crude LPL complex to heparin-agarose, followed by gradient elution with NaCl.

LPL is a glycoprotein (containing 8.3% carbohydrate). It is optimally active at pH 9.2 and 37 °C, and has a native molecular mass of 100 kDa. Blood serum albumin and Ca^{2+} are activators of LPL because they bind FFAs which inhibit LPL. Blood serum also contains a low-molecular-weight apoprotein cofactor that stimulates the activity of LPL by bringing the enzyme into contact with its substrate; the apoprotein cofactor helps LPL to act at an oil–water interface. Organophosphates are potent-specific inhibitors of LPL. The K_{cat} for milk LPL under optimum conditions (pH 9.2 and 37 °C) is $\sim 3000\,s^{-1}$. LPL is strongly inhibited by its products, for example, long-chain fatty acids, which probably prevents milk from becoming rancid rapidly. LPL is also inhibited by 1 mol l^{-1} NaCl, pyrophosphate, protamine sulfate, apolipoproteins (CI and CIII), and polyanions. LPL is an unstable enzyme. It is inactivated by ultraviolet light, heat, acid, and oxidizing agents including ozone; hence it is unstable in heated milk and when milk pH is reduced. It is partially inactivated by high pressure (200 MPa) at low temperature (−4 °C) and by pulsed electric field treatment (35 kV cm^{-1}, 35 °C, 163 kJ l^{-1}) but activated by high-pressure homogenization. In the mammary gland (at body temperature), LPL is inactivated slowly probably due to proteolysis, to give active and inactive LPL in milk. The inactive lipase has a lower affinity for heparin-Sephadex and is therefore removed during purification.

Ultracentrifugation and gel filtration studies show that active LPL from bovine milk is an elongated noncovalent dimer of two identical subunits. Studies using target analysis (radiation inactivation) also show that the enzyme remains dimeric when it is complexed with heparin or when it is bound to lipid without apolipoprotein. Each subunit contains 450 amino acid residues, 5 disulfide bridges, and 2 oligosaccharide chains. Guinea pig LPL has three N-linked oligosaccharide chains. Physical studies show that the dimer is in a rapid reversible equilibrium with its monomers, but the monomers undergo irreversible changes of conformation, resulting in loss of catalytic activity.

Reactions Catalyzed by LPL

LPL, being a nonspecific esterase/lipase, liberates fatty acids from the 1,3-positions in tri-, di-, and monoglycerides, and from the 1-position in glycerophospholipids. In milk triglycerides, long-chain fatty acids are attached to glycerol at the *sn*-1 and *sn*-2 positions, while shorter chain fatty acids are attached at the *sn*-3 position (**Figure 1**). LPL does not hydrolyze cholesterol esters or sphingolipids at a significant rate.

The action of LPL involves two distinct steps. First, the enzyme adsorbs at the lipid–water interface; then, in the second step, the enzyme seeks out a single substrate molecule at the interface and aligns it at the active site and hydrolyzes it.

Lipolysis in milk leads to the preferential release of short- and medium-chain fatty acids. Triglycerides probably orient at the lipid–water interface, thereby positioning short-chain fatty acids for hydrolysis. LPL also catalyzes the formation of ester bonds. Therefore, it is a transacylase. In the formation of ester bonds, fatty acids are substrates for LPL and are incorporated into di- and triglycerides.

The binding of LPL to the milk fat globules as substrate is pH dependent, occurring optimally at pH ~8. LPL also binds to a variety of lipid structures such as liposomes and lipoproteins. Binding is rapid and reversible and is mediated by a lipid-binding site, resulting in stabilization of the enzyme.

Technological Significance

The LPL in bovine milk has the capability of producing about 2 μmol min^{-1} of FFAs under optimum conditions (pH 9.2 and 37 °C). The activity of LPL in bovine milk is theoretically sufficient to cause rancidity in 10 s; however, liberation of FFAs by LPL does not happen in practical situations because of low storage temperature (~5°C), the pH of the milk (6.7), the intact fat globule membrane, and the presence of indigenous inhibitors.

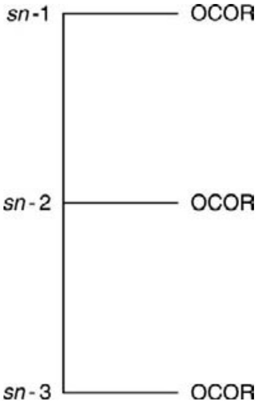

Figure 1 The basic structure of a triacylglycerol, showing positions *sn*-1, *sn*-2, and *sn*-3.

Lipolysis increases the levels of short-chain, volatile fatty acids, which results in rancid, butyric, bitter, unclean, soapy, and astringent flavors that render market milk and most dairy products unacceptable to the consumer. LPL probably causes significant lipolysis in cheese made from raw milk, but contributes little to lipolysis in cheese made from pasteurized milk, as virtually no milk LPL remains active when milk is pasteurized.

Lipolysis in milk is initiated at the farm due to the damage caused to MFGM by machine milking, agitation by pumps, agitators in bulk tanks, or turbulence in milk pipelines. The level of FFAs in freshly drawn milk from healthy cows is ≤0.5 μmol ml^{-1}. Good management practices at the farm result in only small increases in the level of FFAs in milk.

Considerable increases in the concentration of FFAs in raw milk occur as a result of induced or spontaneous lipolysis. Induced lipolysis in raw milk results from damage of the MFGM by vigorous agitation. The temperature of milk during agitation has a major influence on the activity of LPL, which is greatest at 37–40 °C and slight at <5 °C. Homogenization of raw milk or cream also damages the MFGM and produces a large surface area of milk fat, making it vulnerable to the action of LPL. The highest rate of lipolysis occurs in raw milk immediately after homogenization and later remains constant due to the accumulation of FFAs, which inhibit LPL activity. Mixing of raw milk with homogenized pasteurized milk results in rapid lipolysis. This phenomenon has practical significance in dairy factories as any recirculation of inadequately pasteurized and homogenized milk back to the raw milk tank during a breakdown can cause a high level of lipolysis in the finished product.

The milk from some cows may become rancid spontaneously without agitation, probably due to enhanced activity of LPL by a high level of lipoprotein activator leakage from blood into the milk. In spontaneous lipolysis, the LPL complexes to the MFGM. Spontaneous lipolysis can be prevented if the formation of the LPL–MFGM complex is prevented by delayed cooling of milk or addition of NaCl. Normal milk will become spontaneously rancid if blood serum is added to it, because of the high level of lipoprotein activator found in blood serum. Dilution of spontaneously rancid milk with normal milk may reduce rancidity.

Quantification of Lipase Activity

Determination of LPL activity is usually based on measurement of the concentration of the products of lipolysis by titrimetric, colorimetric, fluorimetric, turbidometric, chromatographic, radiometric, enzymatic, physical, or immunological methods. The simplest but tedious method for assaying the activity of LPL is by incubating the sample with an emulsified lipid substrate, for example, milk fat or tributyrin, followed by extraction of the liberated fatty acids with a mixture of diethyl ether and petroleum ether. The extracted fatty acids are then titrated with ethanolic KOH to determine their concentration in the sample. The titrimetric method using triacylglycerols, such as milk fat or triolein, as substrates gives reliable results but lacks sensitivity and is time consuming. Colorimetric methods that use substrates such as β-naphthyl esters, fluorimetric methods that use 4-methylumbelliferyl esters, and turbidity clearing methods based on tributyrin are rapid and suitable for screening purposes. However, differences between the specificity of LPL toward synthetic substrates and milk fat, and interference from fat and protein in the enzyme source, limit the usefulness of the various methods for predicting lipolytic changes in milk and milk products. Assays involving triacylglycerols, such as milk fat, as substrates and determination of the reaction products by chromatographic (gas chromatography (GC) or high-performance liquid chromatography) methods are most reliable for predictive purposes. A method based on triheptanoin with detection of the released heptanoic acid by GC–mass spectrometry (GC–MS) has been used for measuring low levels of lipase in milk powders.

See also: **Analytical Methods**: Chromatographic Methods. **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee. **Cheese**: Biochemistry of Cheese Ripening. **Homogenization of Milk:** Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Milk Lipids**: Fatty Acids; Lipolysis and Hydrolytic Rancidity; Milk Fat Globule Membrane.

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Plasmin System in Milk

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Introduction

Proteolysis in milk is an important quality criterion that can have either beneficial or detrimental effects, depending on the purpose of processing and processing conditions. Proteolysis of milk proteins can be attributed to both indigenous proteases and proteases produced by psychrotrophic bacteria during cold storage of milk. This article focuses on proteolysis caused by plasmin (PL; EC 3.4.21.7), the predominant and most thoroughly studied of the indigenous proteases in bovine milk. Proteolysis induced by PL is sometimes essential and desirable for flavor development and texture changes during ripening of cheese, thus enhancing product quality. The loss of PL from the casein micelle may slow down the cheese-ripening process, and consequently increase the processing expenses. Conversely, in other products such as pasteurized milk, ultra-high temperature (UHT)-heated milk, and nonfat dry milk (NFDM), proteolysis by PL causes undesirable precipitation or gelation. Additionally, uncontrolled proteolysis can result in poor curd formation and in degradation of stored milk proteins intended to be used as functional ingredients in food. The molecular properties of PL and related proteases, and their activators and inhibitors, and isolation and activity of these during processing and storage (both refrigerated and room temperature storage) of milk and milk products are discussed in subsequent sections.

The Plasmin System

PL is part of a complex protease–protease inhibitor system in milk, commonly referred to as the PL system. PL exists in milk primarily in its zymogen form, plasminogen (PG), which can be converted into active PL by plasminogen activators (PAs). The conversion of PG to PL is mediated by at least two types of PAs, tissue-type PA (t-PA) and urokinase-type PA (u-PA). The PL system also includes plasminogen activator inhibitors (PAIs) and plasmin inhibitors (PIs), whose effects on PA and PL, respectively, are greatly dependent on the processing conditions. The components of the PL system (**Figure 1**) interact together and with other components of milk, such as whey and casein proteins, and promote or inhibit proteolysis depending on the processing and storage conditions of milk.

Plasmin and Plasminogen

The PL and PG found in bovine milk are essentially identical to those found in bovine blood, as indicated by their heat and pH stabilities, pH optimum, specificity for casein hydrolysis, inhibition patterns, and amino acid sequences. In blood, PL breaks down blood clots, and its activity is controlled by enzyme activators and inhibitors. PL, mostly in its zymogen form, PG, enters milk from the blood via the mammary cell wall lining and associates mainly with the casein fraction of the milk. In fresh milk, PG is the predominant form, where its concentration is 2–30 times that of PL (reports mention 0.8–2.8 $\mu g\,ml^{-1}$ PG compared to 0.1–0.7 $\mu g\,ml^{-1}$ PL). Therefore, any potential activation of PG could contribute significantly to PL activity in milk. The conversion of PG into PL by PAs can occur while the milk is in the mammary lumen prior to milking and during milk storage. The levels of both PL and PG can vary significantly with the stage of lactation, lactation number, and mastitis. The level of PL and PG in milk is higher at the end of lactation, in older cows, and in mastitic milk.

PL (48 kDa), an alkaline serine proteinase, is similar to trypsin in its activity and characteristics. Like trypsin, PL is inhibited by diisopropylfluorophosphate (DFP), soybean trypsin inhibitor, and tosyllysine chloromethylketone (TLCK). While both PL and trypsin hydrolyze Lys-X and Arg-X bonds, PL preferentially hydrolyzes Lys-X bonds. PL hydrolyzes α_{S1}-, α_{S2}-, and β-caseins, but the rate of hydrolysis of α_{S1}-casein is slower than that of α_{S2}- and β-casein. Much of the work on PL action on casein has focused on β-casein, which has three PL-sensitive bonds. **Figure 2** is a schematic of β-casein breakdown by PL. PL has little or no effect on the whey proteins α-lactalbumin and β-lactoglobulin.

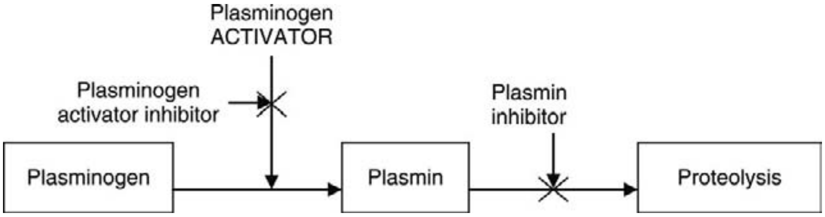
Plasminogen
ACTIVATOR
Plasminogen
activator inhibitor
Plasmin
inhibitor
Plasminogen
Plasmin
Proteolysis

Figure 1 Plasmin system in bovine milk.

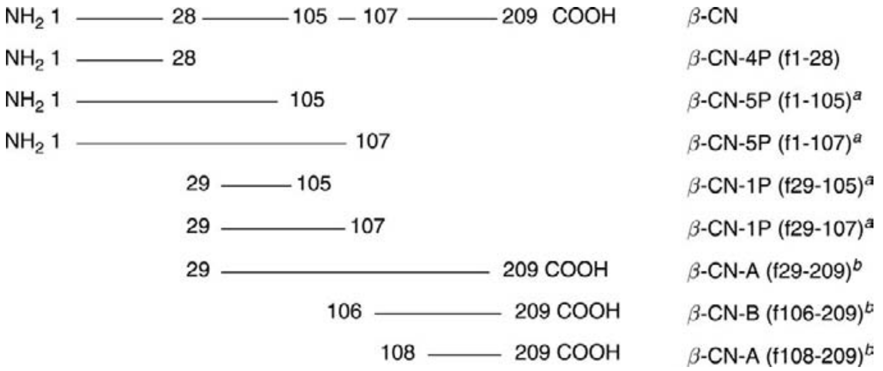
NH2 1 28 105 107 209 COOH
β-CN
NH2 1 28
β-CN-4P (f1-28)
NH2 1 105
β-CN-5P (f1-105)a
NH2 1 107
β-CN-5P (f1-107)a
29 105
β-CN-1P (f29-105)a
29 107
β-CN-1P (f29-107)a
29 209 COOH
β-CN-A (f29-209)b
106 209 COOH
β-CN-B (f106-209)b
108 209 COOH
β-CN-A (f108-209)b

Figure 2 Hydrolysis of β-casein (CN) by plasmin to form γ-caseins and proteose peptone components. The superscript *a* denotes hydrolysis of β-casein that does not take into account amino acid differences between variants A1, A2, A3, and C, which occur between residues 29 and 107. Superscript *b* denotes forms that are called B (vs. A) or A (vs. B).

Plasminogen Activators

u-PA and t-PA also are serine proteinases that activate PG to PL by cleaving the Arg557-Ile558 bond in PG (**Figure 3**). PL autocatalytically cleaves a Lys77-Arg78 bond to produce Arg-plasminogen. The cleavage of PG (88 kDa) by PL at the Lys-Arg bond causes the release of the preactivation peptide and a marked conformational change in PG. The resultant PG form can be easily activated to PL by PAs. Researchers have found that t-PA activity is significantly enhanced by fibrin, and both t-PA and u-PA activities are stimulated by casein. Additionally, PL can lead to the conversion of single-chain u-PA and t-PA into two-chain proteins with significantly enhanced activities. On the other hand, amiloride inhibits u-PA activity but has no effect on t-PA. It is commonly believed that t-PA is associated with casein and u-PA is associated with somatic cells. u-PA can dissociate from the somatic cells and be picked up by casein and associate with it via lysine or electrostatic binding. Therefore, the somatic cell count in milk, the time the somatic cells stay in milk, and the heat treatment of milk could be factors affecting the concentration of u-PA and its dissociation from the somatic cells and the association with casein micelle instead.

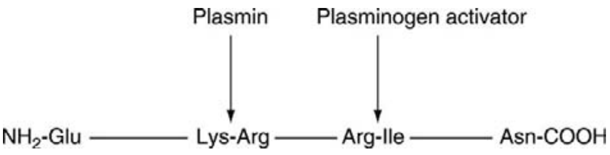
Plasmin
Plasminogen activator
NH2-Glu
Lys-Arg
Arg-Ile
Asn-COOH

Figure 3 Activation of bovine plasminogen.

Inhibitors of the Plasmin System

The conversion of PG to PL can be slowed down by the action of PAI. Similarly, the proteolysis of casein induced by PL can be slowed down by the action of PI. PI and PAI are present mainly in milk serum (whey), and their activity is affected mainly by pH fluctuation and heat treatment. Inhibitors of PL and PAs have been studied as one potential means of controlling PL-related activities. Numerous researchers have reported the presence of protease inhibitors in colostrum and bovine milk. The protease inhibitor in regular milk differs from the colostral inhibitor, in that the former is larger and presumably not acid- or heat-stable. A putative α_1-antitrypsin has been isolated and partially characterized, and α_2-antiplasmin and PA inhibitor-1 have been identified in milk and partially purified.

Association of Plasmin-Related Activities with Casein Micelle

Lysine binding and, to a lesser extent, electrostatic forces are involved in the binding of PL, PG, and PA with the casein micelle. Occurrence of PL in the whey fraction is the result of its dissociation from the casein micelle due to several factors such as milk storage temperature, NaCl concentration, pH, and hydrolysis of casein by PL. While evidence on the effect of temperature is conflicting, pH clearly influences PL dissociation from casein micelles, with most or all of the PL activity dissociated at pH 4.6–4.7. This effect of pH explains, at least in part, the higher levels of PL in acid whey (44 $\mu g\,g^{-1}$ protein) than in sweet whey (4 $\mu g\,g^{-1}$ protein). Also, PL is dissociated from casein micelles upon long exposure to ethanol and when 1 $mol\,l^{-1}$ sodium chloride is added to milk. Any shift of PL from the casein to the whey fraction, by various mechanisms, may negatively affect the quality of food products containing whey protein as a functional ingredient.

Factors that Affect Plasmin-Induced Proteolysis

Many factors such as heat treatment and storage of milk can affect the PL system. The interaction between components of the PL system can be very complex due to interference of other milk components (such as whey proteins), under various conditions (pH, heat, and storage conditions). Furthermore, each of the components of the PL system can be influenced by various conditions, and in turn can affect the kinetics of PL-induced hydrolysis of caseins. Kinetics in this case is related to enzyme (PL or PA) properties, namely the efficiency and speed of their activity during hydrolysis of caseins by the action of PL or conversion of PG to PL by the action of PA.

Heat Treatment of Milk

The heat treatment of milk varies depending on the end usage of milk. In general, milk can be pasteurized at various temperatures (65–75 °C) and for different time durations (15–30 s), or commercially sterilized following UHT processing (135–150 °C for few seconds). Also, prior to processing into products such as NFDM (skim milk powder), a preheating treatment is applied, which includes low heat (75 °C for 15 s), medium heat (75 °C for 1–3 min), high heat (80 °C for 30 min), or ultra-high heat (120 °C for 1 min). Having such a wide range of heat treatments applied, various effects on the components of the PL system are expected. Reported results show that the components of the PL system vary considerably in their thermal stability, highlighting the complexity of the PL system activity.

PL and PG can fully survive pasteurization conditions (72 °C for 15 s), and are somewhat resistant (the remaining activity is 20–40%) to certain high-temperature, short-time (HTST) and UHT heat treatments. Reported *D*-values of PL are 35.7 and 12.4 min at 72.5 °C, and 7 and 10 s at 142.5 °C. However, there is more to that than just the direct effect of heat on PL and PG. Like any protein, PL and PG are prone to denaturation and structural modification upon heating. The temperature range for denaturation of PG is between 50.1 and 61.6 °C. In this temperature range, PG loses its naturally occurring tertiary structure. PG becomes more accessible to the action of PA, by unfolding of its kringles. Both PL activity and PG activation increase upon pasteurization of milk, seemingly due to inactivation of the heat-labile PAI and enhanced activation of unfolded PG. Almost complete inactivation of PAI occurs upon pasteurization (75 °C for 15 s); however, PI retains two-thirds of its activity. The inhibition of PAI in milk enhances activation of PG by PA, while the remaining PI may inhibit part of the PL generated. However, PA is even more heat stable than PL and PG, being quite stable during pasteurization and UHT processing, with a *D*-value of 109 min at 70 °C and 32 s at 140 °C. Therefore, in spite of the remaining active PI, PL activity may continue to increase during storage of processed milk, due to the enhanced activation of the unfolded PG by heat-stable PAs. However, considering the complexity of the PL system, it is also important to draw attention here to the complexity of the milk system as a whole. Whey proteins, namely β-lactoglobulin (β-LG), interfere, upon heating, with PL activity and PG activation. PL activity is significantly affected by interaction with whey proteins. Upon severe heat treatment, such as UHT processing, β-LG, which contains one free sulfhydryl group (SH), can cause irreversible denaturation of PL by S-S/-SH interaction. Unfolding of PG also can promote interaction with β-LG, more so under elevated temperatures, and thus hinder PG activation. However, the PL system is not completely shut down upon heating, even at elevated temperatures. Although intense heat treatments can irreversibly inactivate most of the PL in the presence of β-LG, any residual PG and PA in the system, coupled with inactivation of the system's inhibitors, will result in active PL during storage of milk.

The pH of Milk

PL has an optimum pH of 7.4–7.5. The pH of milk can range between 6 and 7, with an average of 6.8. Lowering the pH, as in the case of cheese and yogurt production, leads to precipitation of the caseins, thus forming a curd. Low pH causes dissociation of the casein micelle, and with it dissociation of PL and PG from the micelles into the whey fraction of milk. The effect of pH on PG activation has not yet been fully researched.

Cold Storage of Milk

In the United States, extended refrigerated storage of milk on the farm, in transport, at the dairy plant, and in supermarkets has led to a total age of the milk before consumption of 20–21 days. Refrigerated storage of raw and pasteurized milk for long periods has resulted in quality problems for the dairy industry. Although the optimal temperature for PL activity is 37 °C, PL can still be active during cold storage. Similarly, PA can be active during refrigeration, and thus mediate the conversion of PG to PL. On the other hand, during refrigerated storage, changes occur to the casein micelles. β-Casein dissociates from the micelle at lower temperatures. Because of the change in the location of β-casein, PL may have more access to caseins, resulting in more PL-mediated proteolysis during refrigerated storage of milk.

Room Temperature Storage of Milk

UHT milk is normally stored at room temperature. PL can undergo autolysis with prolonged storage, thus losing some of its activity. However, the chances of PG activation are considerably high, since the temperature (22–25 °C) is close to optimum (37 °C) and PAIs are already inhibited by the heat treatment applied to the UHT milk. Therefore, more casein hydrolysis is likely to take place, leading ultimately to the gelation of UHT milk.

Microbial Proteases

Microbial proteases have activity on α_{S1}- and β-caseins, and can also affect the PL system, which in turn will affect the quality of dairy products. PL activity decreases with microbial growth and storage time, due to hydrolysis of PL by the bacterial proteases. Also, these proteases can affect PL location by disrupting the casein micelle to release enzymes of the PL system into the whey fraction. On the other hand, certain bacterial proteases can enhance activation of PG either by acting as PA or by enhancing the activity of the native PA in milk. Therefore, when the intent is to study the activity of the PL system in milk, it is crucial to minimize or eliminate the presence of bacterial proteases, ideally by starting with very fresh milk. Also, many psychrotrophic proteases are metalloproteases and can be inhibited by the addition of a metal-binding agent, for example, ethylenediaminetetraacetic acid (EDTA).

Isolation

Plasmin and Plasminogen

The first attempts to isolate PL from bovine milk involved fractionation by ammonium sulfate and pH adjustment using commercial casein as the starting material. In later studies, further purification from casein micelles was achieved using anion-exchange and cation-exchange chromatography columns, followed by size-exclusion chromatography. More recent isolation of PL and PG from milk is by affinity chromatography on a column such as lysine-Sepharose 4B. Milk has been subjected, in sequence, to ultracentrifugation, acid treatment, ammonium sulfate fractionation, and affinity chromatography to obtain PL and PG.

Plasminogen Activators

In early efforts to isolate PAs, the procedures developed to isolate PAs from human milk applied to bovine milk. This was unsuccessful, presumably due to differences in the protein content of bovine and human milk. PAs can be purified from milk by treatment with sulfuric acid, ammonium sulfate, and dimethylformamide, followed by zinc chelate chromatography. Further purification can be achieved with PA inhibitor affinity chromatography, but the PAs are not stable to this treatment. Similar to procedures for separating other milk proteases from casein, some researchers have isolated PAs using cation-exchange chromatography and then lysine-Sepharose chromatography.

Inhibitors of Plasmin System

Many early studies on protease inhibitors in bovine milk focused on trypsin-inhibitory activity in colostrum, which was reported to contain isoinhibitors. Subsequent reports revealed the presence of protease inhibitor activity in mature bovine milk, without complete purification and characterization. Many attempts to isolate protease inhibitors from bovine milk concentrated on inhibitors of trypsin or PL, and included an acid treatment to extract the interfering proteins such as casein and indigenous milk serine proteinases. These attempts were not successful, presumably because the acid treatment inactivated the inhibitors. A putative α_1-antitrypsin was isolated from bovine milk, using ammonium sulfate fractionation, followed by metal chelate interaction, hydrophobic interaction, and size-exclusion chromatography. Further efforts followed in an attempt to isolate α_2-antiplasmin and PA inhibitor-1, both of which had been isolated from human blood, and which are most likely the principal protease inhibitors present in milk, important in controlling the PL system. Using ammonium sulfate fractionation and affinity chromatography on concanavalin A, these protease inhibitors were identified in milk and were partially purified. Other chromatographic procedures, including anion- and cation-exchange chromatography, hydrophobic interaction chromatography, immunoaffinity chromatography (using commercial antibodies to these inhibitors), and anhydrotrypsin affinity chromatography, have been assessed in an effort to obtain pure protease inhibitors. However, cross-reactivity problems with commercial antibodies and unique characteristics of contaminating milk proteins have made it difficult to isolate these protease inhibitors to homogeneity.

Significance and Applications

Cheeses and UHT milk are the principal dairy products for which the advantages and disadvantages of PL activity have been studied. However, PL activity may influence the quality of other dairy products such as casein and whey protein products, and NFDM, hence the need to control the activation of PG and the activity of PL to optimize the quality of dairy products. Understanding the function of the components of the PL system and their interactions with other milk constituents is crucial for efficient control of PL activity. Currently, dairy industries are trying to find the best conditions for the processing of the aforementioned products to enhance the quality. Although attempts are being made, a complete control of the PL system has not yet been achieved.

UHT Milk Products

There is conflicting evidence on the role of PL in the age gelation of UHT milk. Results obtained and conclusions reached seemingly depend on factors such as processing conditions, storage conditions, level of PL, and concentration of milk and other ingredients used in the product. However, it seems clear that when PG or a low level of PL is added to UHT milk, the milk gels faster than milk with no added enzyme. Heat can cause whey proteins to bind to κ-casein located on the surface of the casein micelles, causing aggregation. Increasing the heating time and temperature of milk results in increased whey protein association with casein micelles. Therefore, in the production of UHT milk, for instance, raw milk is preheated (80–95 °C for 30–60 s) prior to high-temperature heating (135–150 °C for a few seconds) to avoid the formation of complexes between whey proteins and casein, and thereby delay gelation. However, this attempt does not shut down the PL system completely.

Cheeses

While research suggests that hydrolysis of casein by PL does not significantly influence rennet clotting time in cheesemaking, PL has been shown to affect the ripening of certain cheeses. Proteolysis during cheese ripening results in the production of flavor compounds, modification of texture, and an increase in pH due to the formation of ammonia. The importance of PL in contributing to proteolysis during cheese ripening is still debated. However, increased PL activity in cheese, as a result of either PG activation or addition of PL, affects the flavor and overall quality of certain cheeses. The importance of PL in cheese ripening depends on the variety of cheese, due to differences in cooking temperature during cheesemaking and in pH during ripening.

PL activity is lowest in Cheddar and Cheshire cheeses, higher in Gouda, and highest in Emmental, Blarney, and Romano-type cheeses. For example, Swiss cheese has 1.5–3.25 times higher PL activity than Cheddar cheese. In Swiss-type cheese, the high cooking temperature inactivates chymosin and other rennets, making PL the predominant protease, and thus very important and even essential in casein hydrolysis during ripening. Even in Cheddar cheese, for which PL is considered less important, adding PL inhibitor during the cheesemaking process resulted in significantly less proteolysis of β-casein during ripening compared to the control cheese. PL is more active in high-pH cheese (e.g., Camembert) than in low-pH cheese (e.g., Cheshire). However, there is evidence that PL contributes to the ripening process even in a fairly low-pH cheese such as Mozzarella. As is typical of PL activity, β-casein is degraded to γ-caseins during the aging of Mozzarella cheese.

Cheese ripening is a time-consuming and expensive process. Therefore, considerable benefit is gained from accelerating ripening. In cheeses for which PL is important for ripening, reduced cheese quality and/or increased costs of production due to delayed ripening could result from reduced levels of PL in the casein micelles. An excessive shift of PL from the casein to the whey fraction may reduce the rate of cheese ripening, affecting the quality of cheese and the cost of production.

Milk Protein Products

Although PL activity is probably important in various milk protein products, little is known about the amount of PL present and its effect on the properties of such products. Caseinates and whey protein concentrates and isolates are produced in large quantities. These are important as functional ingredients for formulated foods such as bakery and dairy products, beverages, desserts, pasta, confectionery, and meat products. In addition, both casein and whey protein products are used in animal feeds, and caseinates have numerous industrial applications. Casein and whey protein products are used in foods and other products because of their gelation, coagulation, hydration, emulsifying, and foaming properties. These functional properties are largely controlled by the chemical composition and physicochemical properties of the protein products. These in turn are determined by the composition of the milk and the processing conditions used for their isolation. One factor that can affect the functional properties of milk protein products is the PL content. For example, the functional properties of β-LG are reportedly improved by a 4% degree of hydrolysis caused by PL. However, thinning has been reported in industrial products to which caseinates are added, possibly due to PL activity. For both casein and whey protein products, it may be desirable to have little or no PL present, which would hydrolyze other proteins in a system to which they are added as functional ingredients.

Nonfat Dry Milk

NFDM is yet another common protein-derived ingredient used to enhance functional properties such as viscosity, emulsion stability, and foam stability in many food products. Most yogurts, pasteurized processed cheeses, bread machine mixes, and ice cream products contain significant amounts of powdered milk ingredients. The PL, PG, and PA, which are associated with casein micelles, are retained in the NFDM. PL activity in powdered milk can cause unwanted proteolysis, thus affecting the functional properties of milk powders and the quality of the food products thereafter. The processing of NFDM includes defatting, heat treating, concentrating, and then spray drying. Predrying heat treatment conditions typically employed during the production of NFDM have not been researched with regard to PL system activity.

Future Trends

Identifying and characterizing the components of the PL system have improved our understanding of this system and its effects on the quality of dairy products. However, we must further improve our understanding of the PL system to be able to control it. Improved control of the PL system will be possible only by understanding better the conversion of PG to PL, and thus the factors that influence the PA kinetics, such as Ca content, pH, storage temperature, and whey proteins. The onset of the interference, the amount of β-LG required, and the mechanism of the interaction between β-LG and PG have to be further studied. Additionally, determining the factors that enhance or inhibit PL activity and the factors that influence the shift of PL from the casein to the whey fraction can lead to better control of the PL system. Manipulating the heating and storage (refrigerated and room temperature) conditions might result in better control and possible shutdown of the PL system. Research in this regard is a pressing need, with specific emphasis on UHT milk, NFDM, yogurt (viscosity), and cheese (ripening) applications. Utilizing this knowledge has the potential for improving the quality and reducing the production costs of these dairy products.

See also: **Cheese**: Biochemistry of Cheese Ripening; Cheese Flavor; Cheese Rheology. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk. **Milk Proteins**: Casein Nomenclature, Structure, and Association. **Milk Protein Products**: Whey Protein Products.

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Phosphatases

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Milk Alkaline Phosphatase

Alkaline phosphatase (ALP) occurs in the milk of all mammals, and its level varies considerably between species. In bovine milk, inter-cow and seasonal variations in ALP activity have been reported. The level of ALP is very high in colostrum, and it falls to a minimum within 1–2 weeks after parturition and reaches a constant level after about 25 weeks. ALP is a glycoprotein containing sialic acid. Its activity is used as an index of the efficiency of adequate pasteurization of milk, because ALP is slightly more resistant to heat than *Mycobacterium tuberculosis*, the most heat-stable pathogen in milk known at the time of introduction of pasteurization in dairy industry. The ALP test is of great significance to public health as a means of checking the thoroughness of pasteurization of milk or the addition of raw milk to pasteurized milk or milk products.

Origin, Isolation, and Characterization of Milk Alkaline Phosphatase

ALP is found in varying amounts in many tissues and secretions. The best characterized mammalian ALPs are of intestinal or placental origin because these organs are rich sources of the enzyme. However, the indigenous ALP in milk does not belong to the intestinal or placental phosphatase group but is similar to the enzyme present in the mammary gland. ALP is concentrated in the milk fat globule membrane (MFGM), where it is intimately associated with the phospholipid particles referred to as microsomes. Hence, cream is a rich source of ALP. About 30–50% of ALP is found in the cream phase, and the remainder is dispersed throughout the skim milk, probably in the lipoprotein particles. ALP is released from the phospholipids by treatment of milk with phosphatidyl inositol-specific phospholipase C, indicating that ALP is bound to microsomal membranes of the mammary gland and MFGM through linkage to phosphatidyl inositol. Electrophoresis of the isolated enzyme revealed that the ALP of microsomal membranes exists as several isozymes, compared to only one enzyme form in MFGM. ALP activity has been reported in myoepithelial cells of the bovine mammary gland.

Bovine milk ALP occurs in three forms, namely, α, β, and γ. The α form predominates in skim milk, β form is found mostly in MFGM, while the γ form is present in both skim milk and MFGM. The MFGM is released into buttermilk on phase inversion; consequently, buttermilk is most frequently used as the starting material for the purification of ALP. Buttermilk is treated with *n*-butanol (29%, v/v) to break the lipoprotein complex of the milk microsomes in order to release ALP. The solubilized enzyme is purified by pH manipulation and repeated precipitation with acetone or ammonium sulfate, and low-molecular-weight impurities are then removed by treatment with activated charcoal. A combination of gel filtration on Sephadex G-200 and chromatography on DEAE-cellulose is then used to further purify ALP. A 7440-fold purification can be obtained with a yield of 28%. The combination of gel filtration and ion-exchange chromatography has been used most frequently to purify ALP after butanol extraction, but affinity chromatography on concanavalin A-Sepharose 4B has also been used. A single-step extraction using 8% (v/v) *n*-butanol at room temperature has been used to extract >90% ALP activity from bovine MFGM. The K_M values and substrate specificity of ALP do not change following extraction with 8% (v/v) *n*-butanol. Crude ALP has been purified from skim milk by electroelution from native polyacrylamide gels or by sequential chromatography on Macro-Prep High Q, Sephacryl S-200, and concanavalin A-agarose. Electroelution is faster and results in a higher yield (23.4 vs. 1.6%) than column purification.

ALP is a phosphomonoesterase with optimum activity in the pH range 9–10.5, depending on the type of buffer used in the assay mixture. It catalyzes the reaction

$$\text{orthophosphoric monoester} + H_2O \\ \rightarrow \text{alcohol} + \text{orthophosphate}$$

ALP is thought to be similar to inorganic pyrophosphatase (EC 3.6.1.1), which dephosphorylates pyrophosphate (pyrophosphate $+ H_2O \rightarrow 2$ orthophosphates). The characteristics of milk ALP are summarized in **Table 1**. The *D*-values for ALP at 60 and 63 °C are 27.2 and 8.3 min, respectively. The enzyme is a dimer of two identical 85 kDa subunits and contains four Zn atoms. It is activated by the addition of 1 mmol l^{-1} Mg^{2+} to the assay mixture. The enzyme is strongly but reversibly inhibited by metal chelators; the apoenzyme is reactivated by Zn^{2+}, Mg^{2+},

Table 1 Characteristics of milk alkaline phosphatase

Characteristic	*Conditions*	
pH optimum	Casein	6.8
	p-Nitrophenylphosphate	9.65, 10.5
Temperature optimum	37 °C	
K_M	0.69 mmol l^{-1} on *p*-nitrophenylphosphate	
Activators	Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}	
Inhibitors	Metal chelators (EDTA, EGTA, etc.), orthophosphates	
Native molecular weight	170 kDa	
Quaternary structure	Two subunits (each of 85 kDa)	
Zn content	4 mol mol^{-1}	
Thermal stability		
D-value at 60 °C, pH 9	27.2 min	
63 °C, pH 9	8.3 min	

and other metal ions. Activation of the apoenzyme may, in fact, be used as a sensitive assay for available Zn in foods. Inorganic orthophosphates are strong competitive inhibitors of the hydrolysis of *p*-nitrophenylphosphate. ALP can dephosphorylate phosphoproteins, including casein, at a pH optimum of 6.5–7.0; however, it does not appear to do so in milk, probably owing to inhibition by high level of orthophosphate in milk. Homoarginine is a potential inhibitor of ALP. β-Lactoglobulin has been reported to have an inhibitory effect on ALP, but the effect is not large and probably of no real technological importance.

Significance of Alkaline Phosphatase in Dairy Products

ALP is an important indigenous milk enzyme because of its technological significance. Fluid milk and its products with ALP activity below the legal limit (i.e., <1 μg phenol per ml in pasteurized milk by the Schaar rapid method, <350 mU of ALP per liter for fluid milk products, and <500 mU of ALP per liter for other products by the fluorometric or equivalent method) are considered to be adequately pasteurized and safe for consumption. The US Food and Drug Administration guidelines for screening cheese for residual phosphatase activity stipulate a negative test to ensure that the milk used for cheese manufacture was properly pasteurized. However, cheeses manufactured with starter cultures that produce ALP may show a positive ALP test.

Microfiltration of skim milk at 55 °C does not affect ALP activity. However, ALP loses >99.9% activity when skim milk is heated at 70 °C for 15 s, and shows a decimal reduction when heated at 65 °C for 70 s. ALP activity in spray-dried milk powder decreases with increasing outlet temperature in the spray dryer, but significant reactivation of the enzyme can occur in the critical region of 70–80% total solids.

Analyses of ALP activity in commercial butters showed that the presence of salt influences ALP activity. There are reports that most of the unsalted butters made from pasteurized cream have little or no ALP activity but salted butters made from pasteurized cream always possess some activity and often yield a positive test for ALP activity (as determined by the AOAC method).

Although ALP is used universally as an index of high-temperature short-time (HTST) pasteurization of milk, it may not be the most appropriate enzyme for this purpose because

1. reactivation of ALP under certain conditions complicates interpretation of the test results,
2. the enzyme appears to be fully inactivated by subpasteurization conditions (70 °C × 16 s), and
3. the relationship between $\log_{10}$ % initial activity and pasteurization equivalent (PE) is less linear than the relationship between PE and lactoperoxidase or γ-glutamyl transpeptidase activity.

The removal of lactose during ultrafiltration (UF) reduces the heat resistance of ALP and therefore the phosphatase test cannot be used to determine the efficiency of pasteurization of milks that have been concentrated by UF. ALP is only partially inactivated by ultra-high pressure treatment of milk (200–400 Pa for 15–120 min); therefore, the ALP test is not a suitable indicator of the effectiveness of the ultra-high pressure treatment process (a substitute method for pasteurization of milk).

Although ALP can dephosphorylate casein under suitable conditions, the technological significance of casein dephosphorylation by ALP is unknown. Proteolysis is a major contributor to the development of flavor and texture in cheese during ripening. Most of the small water-soluble peptides in cheese are from the N-terminal half of α_{s1}- or β-casein; many of these peptides are phosphorylated and show evidence that they have been acted on by phosphatase, that is, they are partially dephosphorylated. In cheese made from pasteurized milk, indigenous acid phosphatase (ACP) or bacterial phosphatase is probably responsible for dephosphorylation, but in raw milk cheese, such as Parmigiano Reggiano or Grana Padano, ALP appears to be more important. It has been suggested that indigenous ALP in milk may play a role in cheese ripening, possibly by dephosphorylating peptides, as shown by different peptide profiles in miniature Cheddar-type cheese made from pasteurized milk containing different levels of exogenous ALP.

Methods Used to Study the Activity of Alkaline Phosphatase

The inorganic phosphate content of raw milk increases during storage due to enzymatic hydrolysis of the phosphoric esters by phosphatase. Therefore, most of the tests for phosphatase in milk are based on the principle that ALP hydrolyzes monophosphate esters at an appropriate temperature and pH, thereby liberating compounds that can be detected, often by color development. The intensity of color developed is proportional to the activity of the enzyme. The usual substrates are phenyl phosphate, *p*-nitrophenylphosphate, and phenolphthalein phosphate, which are hydrolyzed to inorganic phosphate and phenol, *p*-nitrophenol, and phenolphthalein, respectively. The release of inorganic phosphate may be assayed but the second product is usually determined. Phenol is colorless but forms a colored complex on reaction with one of several reagents, for example, 2,6-dibromo- or 2,6-dichloroquinonechloroimide, with which it forms a blue complex. *p*-Nitrophenol is yellow and phenolphthalein is pink at the alkaline pH of the assay (~10) and hence are easily quantified.

The development of the Technicon AutoAnalyzer in 1966 (Technicon Controls Inc., Chauncey, New York) was the first attempt to automate the ALP testing procedure. This automated test for ALP is based on the principle of manual tests, that is, hydrolysis of disodium phenyl phosphate under alkaline conditions to free phenol, which reacts with 4-aminoantipyrine. A colorless complex results from the reaction between 4-aminoantipyrine and phenol at pH 10.0, but upon oxidation with potassium ferricyanide, the complex yields a red-colored dye, which is quantified colorimetrically.

The 'Technicon AutoAnalyzer' method has been modified by using *p*-nitrophenylphosphate as substrate in 2-amino-2-methyl-1-propanol buffer at pH 10. The modification enables the analysis of 40 milk samples per hour, with no decrease in sensitivity or reproducibility compared to the original method.

A quantitative assay for measuring residual ALP activity in dairy products is the Fluoro-Test-system FML 200, developed for the rapid and precise determination of low levels of ALP activity to monitor adequate pasteurization of milk and milk products on a large scale. The test is based on mixing a sample of the milk product with an aromatic monophosphoric ester and measuring the resulting fluorescence (excitation at 440 nm, emission at >505 nm).

The International Dairy Federation (IDF) has established three standards for determining ALP activity. Standard 63 (IDF, 1971) uses sodium phenyl phosphate as substrate and a quantitative determination by the 'indophenol reaction'. Standard 82A (IDF, 1987), defined as a screening method, uses *p*-nitrophenylphosphate as substrate and a qualitative evaluation of a positive or negative enzyme reaction. Standard 155 (IDF, 1992) specifies a fluorometric method for the determination of ALP activity in whole, part skim, and flavored milk. This method is quite rapid and about 10 times more sensitive than the other two methods.

AOAC specifies two methods for the determination of ALP activity. In the AOAC method 972.17 for residual phosphatase in milk, the milk is incubated with phenolphthalein monophosphate in carbonate buffer. Free phenolphthalein liberated by residual ALP activity is measured directly using a spectrophotometer. In the AOAC method 1991.24, ALP activity in fluid dairy products (whole milk, skim milk, and chocolate milk) is measured by continuous fluorometric direct kinetic assay. A nonfluorescent aromatic monophosphoric ester substrate is hydrolyzed by ALP to give a highly fluorescent product. ALP activity is expressed in mU l^{-1}, where 1 mU of ALP is the amount of enzyme that catalyzes the transformation of 1 μmol of substrate per minute per liter of sample.

The chemical principles involved in the detection and measurement of ALP activity are the same for all dairy products, but different dairy products require modifications of methods because of their different physical properties, compositions, and especially buffering capacity.

Reactivation of Alkaline Phosphatase

The reactivation of ALP has been studied widely due to the observation that ultra-high temperature (UHT)-treated milk is phosphatase-negative immediately after processing but becomes phosphatase-positive on standing. Bulk HTST milk does not show reactivation and HTST pasteurization after UHT treatment usually prevents reactivation. The reactivation phenomenon of ALP does not occur in in-container-sterilized milk.

The ALP test was originally designed to determine effectively the adequacy of low-temperature long-time (LTLT) (63 °C for 30 min) pasteurization of milk.

However, with the advent of HTST and UHT treatment of milk, in which exposure to a high temperature is for a brief period (only a few seconds), it became apparent that milk heated to a temperature ranging from 82 to 180 °C or cream heated to a temperature ranging from 74 to 180 °C for a short time acquired ALP activity during storage at 4–40 °C, indicating partial reactivation of the enzyme. In general, reactivation of ALP increases with an increase in pasteurization temperature and a decrease in holding time. In addition, the presence of Mg^{2+}, Zn^{2+}, and NaCl increases reactivation of ALP. It has been proposed that Mg^{2+} or Zn^{2+} causes a conformational change in the denatured enzyme that is necessary for renaturation.

Sulfhydryl (SH) groups appear to be essential for the reactivation of ALP: perhaps that is why phosphatase becomes reactivated in UHT milk but not in HTST milk. The role of –SH groups, supplied by denatured whey proteins, is considered to be the chelation of heavy metals, which would otherwise bind to –SH groups of the enzyme (also activated on denaturation), thus preventing renaturation. Reports suggest that Hg and Cd inhibit reactivation.

The maximum reactivation of ALP occurs when skim milk, cream, or buttermilk is heated to 104 °C and then incubated at 34 °C. The level of reactivation is highest at pH 6.5. The mean activation energy (E_a) for reactivation of ALP in milk that had been heated to 87.8 or 104.4 °C for 6 s and incubated in the presence of Mg^{2+} (27.4 mmol l^{-1}) is 24.108 kJ mol^{-1}, while samples incubated without Mg^{2+} had a mean E_a of 22.646 kJ mol^{-1}. The reactivation of ALP is spontaneous and is inversely proportional to homogenization pressure, but is independent of fat content.

Because the reactivation of ALP results in a false-positive phosphatase test, it raises doubts about the reliability of the phosphatase test for determining the adequacy of milk pasteurization. Methods for distinguishing between renatured and residual native ALP are based on the increase in phosphatase activity resulting from the addition of Mg^{2+} to the reaction mixture.

Acid Phosphatase

The activity of ACP in milk is much lower (~20%) than ALP activity. The activity of ACP in normal bovine milk ranges from 2.6×10^{-4} to 2.6×10^{-3} IU ml^{-1}. The concentration of ACP reaches a maximum 5–6 days postpartum, then decreases and remains low till the end of lactation. The activity of ACP in ewes' milk increases fourfold (17 mU ml^{-1}) in early lactation and then remains constant up to the end of lactation. It has been reported that milk from healthy cows contains one ACP isozyme while mastitis milk may contain two additional ACP isozymes. The activity of ACP is about 4–10 times higher in milk from cows with mastitis than in normal bovine milk. The enzyme is very heat stable. Complete inactivation of ACP in milk requires heating at 88 °C for 30 min. About 10–20% of the activity of ACP is lost during LTLT pasteurization and the activity is not affected by normal HTST pasteurization. No activity of ACP is lost during heating of milk at pH 6.7 for 5 s at 100 °C, while 90% activity is lost on heating at 100 °C for 20 s. In-container sterilization or UHT treatment completely inactivates ACP. Thermal denaturation of ACP follows first-order kinetics and is unaffected by casein substrate. Activity of ACP is rapidly lost in visible light. The *D*-value of ACP ranges from 4.8 s at 100 °C to 36 744.4 s at 65 °C. The *Z*-value of ACP ranges from 6.6 at 75 °C to 27.6 at 85 °C.

Origin, Isolation, and Characterization of Acid Phosphatase

ACP is found mostly in the skim milk phase and also in MFGM (cream phase).

The first step in the isolation of ACP from skim milk or cream is by using an acidic ion-exchange resin, which gives about 300-fold increase in the specific activity (IU mg^{-1}) of the enzyme. The second step is usually gel filtration (Sephadex), which gives a 30-fold increase in the specific activity of the crude ACP isolated in the first step. A homogeneous ACP, with a specific activity of over 30 IU mg^{-1}, can be obtained by subjecting the ACP isolated in the first two steps to sequential separations on ion-exchange, cellulose phosphate, and affinity chromatography.

ACP is a phosphomonoesterase. It hydrolyzes aromatic phosphomonoesters, pyrophosphates (ADP/ATP), polyphosphate, and the phosphoserine residues of phosphoproteins. The optimum pH of ACP is 4.0 and is activated by Mn^{2+} and by reducing agents. It is strongly inhibited by fluoride and also inhibited to some extent by iodoacetate, oxidizing agents, and heavy metals. Bovine caseins are competitive inhibitors of ACP in the order of α-caseins ($\alpha_{s1} + \alpha_{s2}$) > β-casein > κ-casein when assayed on *p*-nitrophenylphosphate.

ACP consists of a single polypeptide chain with a molecular weight of about 4200 Da. The purified enzyme is a glycoprotein, containing 2 mol mol^{-1} of galactose, 2 mol mol^{-1} of mannose, and 4 mol mol^{-1} of *N*-acetyl glucosamine. The enzyme contains low levels of proline and histidine and relatively large amounts of lysine and arginine. An Arrhenius plot (based on 50% inactivation) for ACP in the presence of 1% casein at pH 6.7, 5.2, or 4.9 shows activation energy (E_a) values of 245, 270, or 297 kJ mol^{-1}, respectively, suggesting that the stability of ACP to thermal denaturation increases with decreasing pH.

Significance of Acid Phosphatase

The higher heat stability and lower pH optimum of ACP compared to ALP suggest that ACP is of significance in dairy processing. The dephosphorylation of caseins by ACP activity may reduce the heat stability of dairy products. Milk caseins are phosphoproteins, and therefore are excellent substrates for the action of ACP. The micellar integrity of caseins is lost due to cleavage of phosphate groups from the serine residue of casein by ACP. The activity of indigenous milk and/or microbial ACP has been found in Cheddar cheese, suggesting that ACP activity may influence cheese flavor via its effect on proteolysis. Several phosphopeptides, probably due to phosphatase activity, have been isolated from Cheddar and Parmesan cheese. ACP from ewe's milk has been shown to retain 16% of its activity when assayed at cheese ripening temperatures compared to its activity at 37 °C. The activity of ACP has been reported to increase twofold during the 180 days of ripening of ewes' milk cheese made in summer months as compared to cheese made in winter and spring.

See also: **Cheese**: Starter Cultures: Specific Properties. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milk Lipids**: Milk Fat Globule Membrane.

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Lactoperoxidase

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Introduction

Lactoperoxidase (LPO) is an important indigenous milk enzyme because

- it exhibits antimicrobial activity,
- it is utilized for the preservation of milk quality,
- it is used as an index of the thermal history of milk – it is used as an index of super-high-temperature short-time (HTST) pasteurization,
- it is used as a commercial source of enzyme,
- it plays an important biological role in protecting the lactating mammary gland, and
- it causes nonenzymatic oxidation of unsaturated lipids, acting through its heme group.

LPO (EC 1.11.1.7) is a member of the peroxidase family, which includes a group of enzymes that are widely distributed in nature, having been found in plants and animals, including man. The peroxidase enzymes catalyze the oxidation of numerous organic and inorganic substrates by hydrogen peroxide:

$$2HA + H_2O_2 \rightarrow 2A + 2H_2O$$

where HA is an oxidizable substrate or a hydrogen donor, for example, aromatic amines, phenol, aromatic acids, or leuco dye.

LPO together with myeloperoxidase (MP), eosinophil peroxidase (EP), and thyroid peroxidase (TP) constitutes the mammalian peroxidase superfamily II, which is distinguished from the peroxidase superfamily I (includes enzymes from plants, fungi, and bacteria) in that the prosthetic heme group is covalently attached to the protein matrix. Most peroxidases, including LPO, contain ferriprotoporphyrin IX as a prosthetic group. A characteristic feature of hemoprotein peroxidases is their ability to exist in different oxidation states. There are five known enzyme intermediates. The major intermediates of LPO are ferric peroxidase (the native enzyme), compound I, compound II, compound III, and ferrous peroxidase.

LPO is an oxidoreductase secreted into milk; its primary role is to protect the mammary gland and the gut of infants against bacterial infections. LPO is found in the mammary, salivary, and lachrymal glands of all mammals tested so far and in their respective secretions, that is, milk, saliva, and tears. The peroxidases of the above-mentioned glands are chemically and immunologically similar. The LPO system acts as an antioxidant, thereby protecting mammalian cells against the highly reactive and damaging oxygen-derived species. Mammalian cells are not affected by the oxidation products of thiocyanate (SCN^-); the LPO system is not only atoxic to human cells but also protects these cells against the toxic effects of H_2O_2. Two principal forms occur, A and B, each of which exhibits microheterogeneity with regard to amide groups, glutamine and/or asparagine, and carbohydrate content, giving a total of 10 variants. There is no significant difference in the enzymatic activity of the various LPO fractions.

Physicochemical Properties of Lactoperoxidase

LPO was first recognized as early as 1881 by Arnold. Enriched preparations of LPO were obtained for the first time by fractional precipitation with $(NH_4)_2SO_4$ and the enzyme was subsequently isolated and crystallized by Theorell and Akeson in 1943. Improved isolation and characterization of the enzyme from rennet whey by salting out, displacement chromatography, and crystallization was reported in 1953 by Polis and Shmukler, which indicated that the enzyme was green in color and that it was contaminated with a red protein, now known as lactoferrin.

Since LPO is cationic at the pH of milk, it has been isolated and purified from milk or concentrated sweet (rennet) whey by cation-exchange chromatography using carboxymethyl cellulose at pH 5.1 or 5.7 and also using 0.05 mol l^{-1} phosphate buffer (pH 7.7) and a linear gradient of NaCl from 0 to 0.55 mol l^{-1}. LPO consists of a single polypeptide chain of 612 amino acid residues and shows homology with human MP (55%), EP (54%), and TP (45%). LPO is a basic protein and is highly structured, with 65% β-structure, 23% α-helical structure, and 12% unordered structure; it has a high isoelectric point of 9.6. The LPO enzyme is a 78 kDa glycoprotein with a heme group (protoheme 9) at its active site. The heme group in the catalytic center of the LPO molecule is a protoporphyrin IX; the heme is covalently bound to the polypeptide chain through two ester bonds, formed between the heme 1- and 5-position hydroxymethyl side chains and glutamate 375 and aspartate 275, respectively. LPO also binds Ca^{2+}, which stabilizes the molecular conformation of the enzyme and thus maintains its structural integrity. There is one calcium per iron atom and the protein has a high affinity for calcium. The iron content of LPO is 0.07%, corresponding to one iron atom per LPO molecule; iron is part of the heme group of the enzyme and is also associated with the carbohydrate chains of the enzyme. LPO has four or five carbohydrate chains, which constitute 10% of the 78 kDa mass. LPO has an absorbance maximum at 412 nm; its purity ratio is measured at A_{412}/A_{280} and it is approximately 0.95. The three-dimensional structure of LPO is in essence similar to that of MP found in the leukocytes of mastitis milk. An important difference is the more constraint heme pocket of LPO, being primarily responsible for the difference in the halide specificity of LPO and MP. At neutral pH, LPO oxidizes only iodide and thiocyanate; it can oxidize bromide only slightly but is unable to oxidize chloride.

Maximum LPO activity in milk is obtained at pH 6.0 using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate. Since many of the reducing agents are chromogenic, a recommended method for measuring the LPO activity in milk is by using ABTS as a chromophore and measuring the absorbance at 412 nm. In milk, LPO is the second most abundant enzyme after xanthine oxidase, constituting ~0.5% of the total whey proteins and ~0.1% of total protein (30 mg l^{-1}).

LPO has high thermal stability in milk, whey, permeate, and buffer. Its destruction has been used as an index of the pasteurization efficiency of milk. LPO maintains its activity at low pasteurization temperatures for extended periods of time (63 °C, 30 min) and during HTST pasteurization (72 °C, 15 s), leaving sufficient activity (~70%) to catalyze the reactions between thiocyanate and hydrogen peroxide. Complete inactivation of LPO occurs by heating at 78 °C for 15 s. However, LPO loses its activity slowly at temperatures below 70 °C with a sharp decrease in its activity at 72 °C. LPO is denatured when heated at 80 °C for 2.5 s and it is possible that when LPO-activated milk is pasteurized, the LPO system can be reactivated to extend the shelf life of milk.

At low pH (5.3), LPO is less heat stable. The loss of calcium, which is responsible for the structural integrity of LPO enzyme, is the likely reason for the lower denaturation temperature of LPO at low pH. The greatest decrease in LPO activity at low concentrations (0.5 ppm) in the pH range 4.4–6.7 was 15% per 15 min at pH 5.4. At higher concentrations, >25 ppm, LPO did not lose activity. LPO is deactivated by storage at pH 3.

LPO is relatively stable against a number of proteolytic enzymes, for example, trypsin and thermolysin. LPO appears to be very sensitive to light in the presence of riboflavin and is inactivated by 55% in milk at 6000 lux after exposure for 4 h. Photochemical inactivation is irreversible but can be prevented by the addition of cysteine. LPO has a high tendency to adhere to surfaces, which leads to a decrease in activity. Structural prerequisites for the aggregation and adsorption are still unknown, but indications are that the LPO molecule is equipped for both ionic and hydrophobic interactions.

Variations in enzyme level are influenced by the sexual cycle of the cow, season of the year, feeding practices, and breed type. Its concentration in bovine milk is around 30 mg l^{-1}, constituting about 1% of the whey protein. The LPO concentration is low in bovine colostrum, unlike other antibacterial proteins; however, it increases rapidly to reach a maximum at 3–5 days postpartum. Varying levels of LPO activity have been reported in bovine, caprine, ewe, and buffalo milk. Bovine milk contains 1.2–19.4 U ml^{-1} LPO and is about 20 times richer in peroxidase activity than human milk. Human colostrum has a high level of MP and a low level of LPO. However, LPO is the principal peroxidase in mature human milk. Mean LPO activities in cow and caprine milk are 2.3 and 0.1 U ml^{-1}, respectively. LPO activities of 0.79 and 4.5 U ml^{-1} have been reported for Saanen goat milk and Creole goat milk, respectively. The mean LPO activity for buffalo and ewe milk has been reported as 0.9 and 4.0 U ml^{-1}, respectively.

Lactoperoxidase System

The LPO system is an antimicrobial system and has been recommended as an alternative to chilling for the preservation of raw milk, especially where lack of capital, unreliable energy supply, and high ambient temperatures make chilling of raw milk practically unattainable. The LPO system has many applications as a natural preservative in the food industry; however, its use has been generally recommended for the dairy industry. Unlike pasteurization and fermentation, the LPO system does not render raw milk safer for consumption; it preserves the initial quality of the product. Since LPO retains its activity at pasteurization temperatures applied to milk, it can be used in combination with heat treatment for the preservation of milk and milk products.

LPO catalyzes the oxidation of thiocyanate (SCN^{-}) by hydrogen peroxide to yield thiocyanogen $(SCN)_2$, which is then hydrolyzed to hypocyanous acid (HOSCN) or hypothiocyanate (OSCN). OSCN is the major intermediate oxidation product of the LPO-catalyzed oxidation of SCN. Other short-lived intermediates that can be found in varying amounts are thiocyanogen $(SCN)_2$, cyanogen thiocyanate (NC-SCN), cyanosulfurous acid (HO_2SCN), and cyanosulfuric acid (HO_3SCN). Hypocyanous acid and hypothiocyanate are highly reactive oxidizing agents. They react with the sulfhydryl groups and reduced nicotinamide nucleotides of microbial cells. The oxidation of these cellular components, cytoplasmic membranes, carbohydrate and amino acid transport systems, and glycolytic pathways are impaired.

$$SCN^- + H_2O_2 + 2H \xrightarrow{\text{Lactoperoxidase}} (SCN)_2 + 2H_2O$$

$$(SCN)_2 + H_2O \longrightarrow OSCN + SCN^- + H$$

$$OSCN \rightleftharpoons H + OSCN$$

Thiocyanate can also be directly oxidized to hypothiocyanate:

$$SCN^- + H_2O_2 \xrightarrow{\text{Lactoperoxidase}} OSCN^- + H_2O$$

Apart from the LPO enzyme, exogenous thiocyanate and hydrogen peroxide are needed for a complete and functional LPO system. Thiocyanate (SCN^-) is widely distributed in animal tissues and secretions. It is present in the mammary, salivary, and thyroid glands and their secretions, in organs such as the stomach and kidney, and in fluids such as synovial, cerebral, cervical and spinal fluids, lymph, and plasma. Thiocyanate is found naturally in milk at levels of $\sim$24 mg kg^{-1} in bovine milk, $\sim$18 mg kg^{-1} in ewe's milk, and $\sim$10 mg kg^{-1} in goat milk. The thiocyanate level found in milk varies with breed type, species, feed, and udder health of the animal and also varies with the season of milking. SCN^- is usually present in milk at sufficient concentrations to serve as the principal electron donor in the enzymatic reaction. The thiocyanate level required for activation of the LPO system is 15 mg kg^{-1}. The addition of an exogenous source of thiocyanate, at a concentration of 100–800 mg kg^{-1}, to activate fully the LPO system will enhance the antibacterial effect of the system. Hydrogen peroxide is one of the inorganic peroxide compounds and a strong oxidizing agent and exhibits varying degrees of antimicrobial activity. Unlike thiocyanate, hydrogen peroxide is not detected in milk under normal conditions. However, it can be generated endogenously by polymorphonuclear leukocytes during phagocytosis or by catalase-negative lactic acid bacteria (LAB) such as lactococci, lactobacilli, and streptococci during growth under aerobic conditions. A hydrogen peroxide generating system, for example, sodium percarbonate, is said to be more effective than hydrogen peroxide as a component of the LPO antimicrobial system. This system is 50–100 times more effective than H_2O_2 alone. Hydrogen peroxide has been found to be highly toxic for mammalian cells; however, at low levels of hydrogen peroxide (100 μmol l^{-1}) and in the presence of LPO and SCN^-, mammalian cells are protected from the toxicity of hydrogen peroxide.

Antimicrobial Action of Lactoperoxidase System

The antimicrobial effect of the LPO system stems from the reaction of unstable hypothiocyanite with sulfhydryl groups in cell membrane proteins and with low-molecular-weight components of cytoplasmic thiols forming disulfides, sulfenyl thiocyanates, or sulfenic acids. The oxidation of sulfhydryl (SH) groups in microbial enzymes and other proteins is considered to be the key to the antimicrobial action of the LPO system.

$$\text{protein-SH} + OSCN^- \rightarrow \text{protein-S-SCN} + OH^-$$

$$\text{protein-SH} + (SCN)_2 \rightarrow \text{protein-S-SCN} + SCN^- + H^+$$

$$\text{protein-S-SCN} + H_2O \leftrightarrows \text{protein-S-OH} + SCN^- + H^+$$

The products HOSCN and $OSCN^-$ react rapidly with protein sulfhydryl groups to yield sulfenyl thiocyanates (R-S-SCN). At low concentrations of sulfhydryls (R′-SH), the R-S-SCN may react to form mixed disulfides (R-S-S-R′). At higher concentrations of R′-SH, the R-S-SCN may be reduced back to R-SH. The R-S-SCN may also be hydrolyzed to sulfenic acids (R-S-OH). LPO catalyzes the incorporation of SCN^- into protein substrates. The reaction of $(SCN)_2$ or $OSCN^-$ with proteins oxidizes the protein sulfhydryls to sulfenyl thiocyanate derivatives. Sulfenyl thiocyanate derivatives can undergo further modifications, including reversible hydrolysis, to yield sulfenic acids.

These reactions inhibit bacterial enzymes responsible for respiration and metabolism, notably enzymes having cysteine residues in their active sites. At pH <5, HOSCN inhibits microorganisms by entering the cells as undissociated acid. In the cytoplasm of the microbial cell, the equilibrium favors the undissociated acid; analogous to the antibacterial organic acid mechanism, the generation of protons inside the cells is responsible for the antibacterial activity of LPO at low pH. LPO in milk exhibits an activity maximum at pH 5 and combined with elevated HOSCN concentrations, LPO system has optimal antimicrobial activity. The cytoplasmic membrane or the cytoplasm is the major target of the LPO system's antimicrobial products. The structural damage of microbial cytoplasmic membranes by the oxidation of SH groups results in leakage of potassium ions, amino acids, and polypeptides. Subsequently, uptake of glucose, amino acids, purines, and pyrimidines in the cell and the synthesis of proteins, DNA, and RNA are also inhibited. Cessation of essential cell functions, mainly respiration and metabolism, results in eventual cell death. The cell wall and the membrane may partially limit accessibility of the LPO system's products into the cell but do not exclude it completely.

The stability of hypothiocyanite, $OSCN^-$, is affected by many factors, such as pH, light, metals (Fe, Ni, Cu, Mn), glycerol, and ammonium sulfate as well as by the presence and removal of LPO; however, it is very heat stable.

The antimicrobial activity can be inhibited by reducing agents containing sulfhydryl (SH) groups, such as cysteine, glutathione, mercaptoethanol, dithiothreitol, and sodium hydrosulfite, either by direct binding to the heme group of the enzyme or by scavenging thiocyanate ions. Neither HOSCN nor $OSCN^-$ appears to oxidize SH groups of milk proteins, such as β-lactoglobulin.

Lactoperoxidase Activity in Milk

The LPO system can inhibit the growth and metabolism of different species of microorganisms. It is capable of inhibiting viruses, Gram-positive bacteria, Gram-negative bacteria, fungi, mycoplasms, and parasites, and can be applied at ambient temperatures ranging from 15 to 30 °C for 6 h for the preservation of raw milk. LPO may have a bactericidal or bacteriostatic effect against a range of spoilage and pathogenic bacteria that occur in raw milk. Compared to lysozyme, LPO has a much wider antibacterial spectrum because of the lower specificity of its antibacterial mechanism. The susceptibility of microorganisms to the LPO system depends on the state of their growth. Cells in the stationary phase are more susceptible to inhibition or killing than metabolically active cells; the LPO system is also more effective at low cell densities than at high densities. The lower the cell density, the more lethal the effect of the LPO system and the permeability of the cell wall, with rough mutants showing higher susceptibility. The effect of the LPO system on bacteria can be reversible or irreversible, due to stress response of cells, as well as cross-protection. The capacity of cells to recover from inhibition depends on environmental conditions, for example, temperature and pH, and is also strain specific. Bacteria that survive the initial bactericidal activity of the LPO system exhibit an extended lag phase or recovery period. Cross-protection occurs because some stress response systems share the same/overlapping regulatory pathways. Strains may exhibit cross-protection when subjected to combination treatments during processing. Acid-adapted *Escherichia coli* elicits cross-protection against combined low pH (pH 4.0 and 5.0) and activated LPO. Changes in the outer membrane porins (*omp*C and *omp*F) and outer membrane fatty acids contribute to cross-protection. Porin-mediated outer membrane permeability for small hydrophilic molecules leads to increased tolerance to the LPO enzyme system because of the reduced uptake of $OSCN^-$.

The bacteriostatic effect of the LPO system on *Staphylococcus aureus* and its bactericidal effect on enteric pathogens including multiple antibiotic-resistant *E. coli* strains, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Brucella melitensis* in goat milk have been demonstrated. Inhibition of other pathogens such as *Campylobacter jejuni* and vegetative cells of *Bacillus cereus* has been reported. Other bacteria that were found to be inhibited by the LPO system include *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Salmonella* Typhi, *Vibrio cholerae*, *Helicobacter pylori*, *Streptococcus uberis*, and *Staph. aureus*. Acid production, oxygen uptake, and consequently H_2O_2 excretion were inhibited in *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus mitis*, and *Streptococcus thermophilus*. *Streptococcus sanguis* and *Sc. mitis* showed more resistance to the LPO system than *Sc. mutans* and *Sc. thermophilus*. This was attributed to the higher activity of NADH-OSCN oxidoreductase in the former strains. The primary target of $OSCN^-$ in the glycolytic pathway was glyceraldehyde 3-phosphate dehydrogenase.

The LPO system can also be applied in combination with other treatments used to preserve milk. The LPO system has been shown to delay proliferation of psychrotrophic spoilage bacteria, being bactericidal against *Pseudomonas fluorescens*, and thus prolong the keeping quality of raw milk stored under chilled conditions for several days. LPO extends the shelf life of milk at 4–8 °C for 3–6 days. The use of the LPO system in combination with heat treatment has also proved effective in eliminating vegetative microorganisms in milk. The LPO system in combination with low pH also reduced *E. coli* and *Shigella* spp. cell numbers to undetectable levels after 24 h exposure in fruit and vegetable juices. Activated LPO may enhance resistance to sublethal heat treatments; therefore, the sequence of application of heat and LPO treatments is important. High-pressure homogenization together with the activated LPO system and lysozyme at 37 °C reduced both *L. monocytogenes* and *E. coli* to undetectable levels. A synergistic effect of LPO and nisin in ultra-high temperature (UHT) skim milk resulted in a decrease in *L. monocytogenes*, and activation of the LPO system greatly enhanced the thermal destruction of *L. monocytogenes* in milk during pasteurization. Whey protein films incorporating the LPO system inhibited *Salmonella enterica* and *E. coli*.

The LPO system plays a role in protecting the lactating mammary gland from infection with *Sc. uberis* and LPO may be used is an index of mastitic infection. Any disease condition that causes an increased concentration of leukocytes in milk will increase the activities of certain enzymes in milk. LPO is synthesized mainly by polymorphonuclear leukocytes and colostrum peroxidase activity increases with an increase in the somatic cell count (SCC) of milk. Although high SCC increases the level of LPO in bovine, goat, and human milk, the correlation between SCC and LPO level is variable.

The biochemical effect that the LPO system has on individual lactic cultures varies with the inherent resistance of LAB cultures and the type of milk used. Exposure to the LPO system causes a general decrease in lactic acid production by thermophilic LAB cultures used for milk fermentation and delays the coagulation of milk. Mesophilic starter cultures have also been shown to exhibit resistance to the LPO system. Phage-resistant mutants can be more susceptible to the LPO system than their respective parent strains.

Single-strain cultures of *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, and *Leuconostoc mesenteriodes* subsp. *cremoris*, and probiotic *Bifidobacterium longum* have also been shown to be unaffected by the LPO system. LPO activity depends on the severity of the heat treatment of milk, due to the effect of heat on LPO. Acid production by LAB can be affected by HTST, but can be restored by the addition of LPO. Inhibition of LAB strains in yogurt, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Sc. thermophilus*, is variable.

The quality of various cheese types, Gouda, Cottage, and French soft cheese, made with LPO-activated milk has been affected by the LPO system. Aerobic organisms, LAB, coliforms, and coagulase-positive staphylococci decreased during ripening in LPO-activated Gouda cheese. Activation of LPO effectively reduced the numbers of *Pseudomonas*, *E. coli*, and *S. typhimurium* in Cottage cheese and eliminated *L. monocytogenes* on the surface of French soft cheese.

The LPO–thiocyanate–H_2O_2 system was found to inhibit the growth and proliferation of many fungal and yeast species, such as *Trichoderma* spp., *Corynespora cassiicola*, *Phytophthora meadii*, *Alternaria* spp., *Penicillium chrysogenum*, *Claviceps* spp., and *Corticium salmonicolor*. *Candida albicans* and *Pythium* spp. were not affected by the LPO system. Different species were inhibited at different concentrations of goat milk LPO, for example, *Aspergillus niger* required a minimum of 475 μg ml^{-1}, whereas *Claviceps* spp. required 62 μg ml^{-1} for total inhibition. LPO has the ability to degrade aflatoxin in the presence of sodium chloride (225 mmol l^{-1}) and H_2O_2 (50 mmol l^{-1}). Comparable amounts of LPO degraded aflatoxin G1 ~1.5 times faster than aflatoxin B1. The rate of degradation of aflatoxin B1 increased from 3.6 to 5.1% per 24 h as the amount of LPO in the reaction mixture increased from 50 to 500 U ml^{-1}.

LPO has been shown to affect both poliovirus and vaccinia virus with halides (I^-, Br^-) as electron donors. LPO and glucose oxidase are virucidal to HIV-1 in the presence of sodium iodide, as assessed by the loss of viral replication in a syncytium-forming assay or by the inhibition of cytopathic effects in infected cells.

See also: **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview. **Cheese**: Public Health Aspects; Raw Milk Cheeses. **Fermented Milks**: Starter Cultures. **Heat Treatment of Milk**: Non-Thermal Technologies: Introduction. **Mastitis Therapy and Control**: Management Control Options. **Pathogens in Milk**: *Brucella* spp.; *Escherichia coli*; *Listeria monocytogenes*; *Salmonella* spp.; *Staphylococcus aureus* – Molecular. **Psychrotrophic Bacteria**: *Pseudomonas* spp. **Yeasts and Molds**: Yeasts in Milk and Dairy Products.

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Xanthine Oxidoreductase

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Introduction

Xanthine oxidoreductase (XOR) has long been known to be present in cows' milk, from which it can be readily purified, even in gram quantities. Indeed, XOR was first purified from this source over 60 years ago and is consequently one of the best studied of all enzymes. It is not confined to milk, having been detected in all species examined to date, including bacteria. In mammalian tissues, the enzyme is widely distributed, particularly high levels being found in the liver and intestine. It is conventionally seen as a late enzyme of purine catabolism, catalyzing the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. However, XOR is capable of much more than this.

In milk, XOR occurs in the milk fat globule membrane (MFGM). Fat droplets originate in the endoplasmic reticulum of the mammary secretory cell. In the secretion process, they migrate to the luminal surface and bud off from the cell, enveloped by the apical cell membrane. This becomes the MFGM (**Figure 1**). Immediately after leaving the cell, therefore, the fat droplet is surrounded by a true biological membrane. Churning disrupts the MFGM, allowing the fat droplets to coalesce, forming butter and leaving buttermilk, the latter of which contains MFGM.

The MFGM contains some eight major proteins including XOR, and procedures for its purification are well established. XOR is a complex homodimer, each 147 kDa subunit of which contains three redox centers, comprising one Mo, two Fe_2-S_2 groups (regarded here as one center), and one FAD. Apart from hypoxanthine and xanthine, XOR has a wide range of reducing substrates, including many nitrogen heterocycles and also relatively simple aldehydes. Once reduced, XOR passes electrons to NAD^+, yielding NADH, or to molecular oxygen. Reduction of oxygen yields the reactive oxygen species (ROS), hydrogen peroxide, and superoxide anion.

The enzyme occurs naturally in two inactive forms. These are demolybdo-XOR, which lacks Mo, and desulfo-XOR, in which Mo=S, essential for catalytic activity, is replaced by Mo=O. Active enzyme can exist in one of two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). Both forms will reduce O_2, although XO is more efficient in this respect. Only XDH can reduce NAD^+, which is its preferred substrate.

The terminology is potentially confusing. All forms of the enzyme, including XDH and XO, are widely referred to as 'xanthine oxidase'. However, the enzyme, in general, is more correctly referred to as 'xanthine oxidoreductase'. In this chapter, 'xanthine oxidase' is reserved for the form of XOR that cannot reduce NAD^+ (i.e., XO).

XOR as a Source of ROS and Reactive Nitrogen Species in the Vasculature and the Digestive Tract

As noted above, XOR catalyzes the reduction of molecular oxygen to yield superoxide anion and hydrogen peroxide. These ROS can interact, particularly in the presence of iron, to generate a range of cytotoxic agents, including hydroxyl radicals. In the early 1980s, such XOR-derived agents were implicated in the pathogenesis of ischemia–reperfusion injury and this hypothesis focused unprecedented attention on the enzyme. Following much debate, the involvement of XOR in such injury is generally accepted, although the precise pathogenic mechanisms remain uncertain. It has been suggested that ischemia–reperfusion injury represents an aberrant response to a normal physiological process in the vasculature, namely inflammation.

In the context of the inflammatory response, XOR is believed to combat infection by generation of ROS and can be seen as an agent of innate immunity. Such a role can be played by XOR not only in the vasculature, but also in the gastrointestinal tract, where the enzyme is concentrated in surface epithelial cells in the frontline of antimicrobial defense.

XOR has recently been shown to catalyze the anaerobic reduction of inorganic nitrite to nitric oxide (NO). In the presence of oxygen, this also is reduced, to superoxide, which reacts rapidly with NO to give peroxynitrite. NO and, especially, peroxynitrite have bactericidal properties and these reactive nitrogen species (RNS) might be expected to complement the antimicrobial activities of XOR-derived ROS in the gut, provided that nitrite is available in sufficient concentrations. Nitrite is, indeed, present in the gastrointestinal tract, originating largely from swallowed saliva although the levels are likely to be in the micromolar range, rather than in the millimolar range that would be required for maximal rates of XOR-catalyzed NO production. There is, however, a further

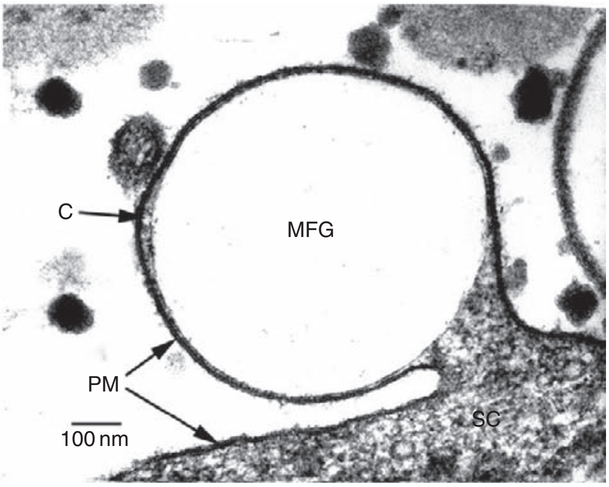

Figure 1 Electron micrograph of a section of goat mammary tissue showing fat globules (MFG) in the process of secretion. The globules approach the plasma membrane (PM) of the secretory cell (SC) and bulge through it. The globule forms a protuberance, which pinches off forming a free milk fat globule. It can be clearly seen that the trilamellar plasma membrane of the secretory cell is continuous with the globule membrane. Small crescents of cytoplasm (C) are sometimes trapped in the globules. The micrograph was kindly provided by Dr. FBP Wooding, University of Cambridge, and was originally published in Harrison R (2006) Milk xanthine oxidase: Properties and physiological roles. *International Dairy Journal* 16: 546–554.

factor to be considered. Nitrite concentrations are expected to be particularly high in the immediate neighborhood of enteric bacteria, which have been shown to excrete millimolar levels of nitrite, originating from dissimilatory nitrate reductase. It is accordingly possible that such bacteria create a microenvironment that makes them especially vulnerable to XOR-catalyzed attack.

Antimicrobial Activity of Milk XOR

Milk XOR has long been known to show antibacterial activity, and may well fulfill this function in the neonatal gut, at a time when other immune mechanisms are not fully developed.

While this antibacterial activity is commonly attributed to XOR-derived hydrogen peroxide, the recently demonstrated ability of XOR to generate RNS (see above) suggests that these cytotoxic species also might contribute to the antibacterial activity of milk.

It was noted above that enteric bacteria are likely to be particularly susceptible to XOR-mediated attack by RNS by virtue of their secretion of nitrite and the potentially high levels of nitrite concentrations in their immediate microenvironment. This applies to XOR in both gut epithelial cells and milk fat globules. A further consideration applies in the case of milk. XOR is located on the surface of the MFGM, and pathogenic bacteria that target epithelial membranes of the digestive tract will be attracted to similar antigens on the MFGM, itself of epithelial cell origin. This will not only divert bacteria from their primary target but will bring them into contact with XOR. Such proximity will also be further induced by the known affinity of XOR for acidic polysaccharides, such as occur in many bacterial capsules. All these factors combine to juxtapose XOR and the bacteria in a microenvironment favorable to the destruction of the latter.

It might be expected that XOR of the MFGM performs a similar antimicrobial function in the mammary gland. Indeed, there is recent evidence that implicates XOR in the mammary innate immune system. From a practical viewpoint, it is of interest that addition of hypoxanthine to fresh milk prevents bacterial growth for up to 7 days.

In developed countries, bottle-fed infants show a higher incidence of gastrointestinal infections than breast-fed. Xanthine oxidase activity is absent from infant formula preparations and, in view of the likely contribution of XOR to antimicrobial defense in the neonatal gut (see above), this absence may partially explain the greater susceptibility of bottle-fed infants to infection. There is, accordingly, a case to be made for introducing active enzyme into infant formula.

Similar considerations apply to cows. Calves are commonly weaned after 2–3 days and given milk substitute. They are susceptible to scours, which is similar to human infant enteritis. It has been shown that animals fed supplements containing active XOR gained weight faster than controls and showed a 50% reduction in the incidence of infective scours.

While arguments in the breast feeding versus bottle feeding debate are complex, supplementation of infant formula with active XOR is worthy of consideration.

Role of XOR Protein in Milk Secretion

In the lactating alveolar cell, XOR represents a significant component of the soluble protein. It has been known for some years that expression of XOR mRNA in such epithelial cells increases during pregnancy and that the enzyme is present in the apical plasma membrane at the time of lactation. On the basis of this and other information, the involvement of XOR in the secretion of milk fat globules has been proposed, and recent studies of mice have provided convincing evidence in support of this idea. Thus, on lactation, XOR was shown to move from the cytoplasm to the apical membrane of the cell, where it colocalized with two other proteins, adipophilin (ADPH) and butyrophilin (BTN). Within the cell, ADPH is associated with the lipid globule, while BTN is an integral protein of the plasma membrane. In the secreted milk fat globules, these same three proteins (XOR, ADPH, and BTN) are known to form part of the proteinaceous layer that separates the lipid core from the MFGM, and the clear implication of the latest study is that XOR mediates coupling between the lipid globule and the apical cell membrane in the secretion process. Chromatographic analysis of solubilized MFGM proteins showed that XOR, ADPH, and BTN occurred in the form of a sulfhydryl bond-dependent complex.

In other recent works, mice with a targeted disruption of the XOR gene were studied. While homozygous (−/−) mice lived for a maximum of only 6 weeks, heterozygous (+/−) individuals survived to maturity, with normal fertility and litter size. Their lactation, however, was defective and all their pups died of starvation. Ablation of the BTN gene has also been shown to severely compromise secretion of lipid droplets in mice.

The above and similar studies strongly suggest that the role of XOR in milk secretion depends solely on its protein content and is independent of enzymatic activity.

Variation of Milk XOR between Species

XOR preparations from milk of different species differ greatly in their Mo content. Thus, while bovine milk XOR contains 0.61 atoms Mo per subunit, the enzyme purified from humans contains only 0.04 atoms Mo, while that from goats and sheep contains 0.09 and 0.18 atoms Mo, respectively. Because xanthine and most reducing substrates donate electrons directly to the Mo redox center, the Mo contents are reflected in the relative specific xanthine oxidase activities of XOR from the various species. Xanthine oxidase activity of human milk XOR, for example, is some 15-fold less than that of the bovine milk enzyme.

A further complication is that the specific xanthine oxidase activity of human milk has been shown to vary with time postpartum. In the first weeks, activity rises to a peak before falling back to basal levels (when milk is collected for purification). It has been proposed that inactive enzyme is produced during the early stages of milk secretion, for which XOR protein alone is required; the metabolically expensive process of Mo incorporation is spared until active enzyme becomes essential for antimicrobial defense. However, although specific xanthine oxidase activity certainly varies, there is currently no comparable information on Mo content, and other factors could be involved.

The question arises as to why this apparent evolutionary advantage is manifested in humans, sheep, and goats but not in cows. It may be that the decades-long selection of cows for high milk yield has led to loss of some relevant controlling factors.

Milk XOR, anti-XOR Antibodies, and Heart Disease

During the 1970s and early 1980s, cows' milk XOR was linked to ischemic heart disease. It was claimed that XOR ingested from bovine milk, particularly homogenized milk, could be absorbed, in enzymatically active form, from the intestine and deposited in arterial intima, where it could cause lesions and initiate atherosclerosis. These ideas provoked considerable controversy but were generally refuted.

At about the same time, other workers reported elevated levels of anti-cows' milk antibodies in the serum of patients who had suffered a myocardial infarction (MI). Again, interest was aroused and, again, the findings were largely refuted. Nevertheless, the same workers went on to show that anti-cows' milk antibodies were directed to the bovine MFGM. They proposed that anti-bovine MFGM antibodies might cross-react with platelet membrane antigens, inducing platelet aggregation and hence atherogenesis. Subsequent studies by others have confirmed that levels of anti-MFGM antibodies are raised in MI patients compared to controls and, moreover, that the relevant antigen in MFGM is XOR. Circulating anti-XOR antibodies need not, of course, arise in response to ingested milk; they could be autoantibodies, and convincing evidence for this has been provided. Human XOR occurs at particularly high levels in the liver and intestine and is present in endothelial and epithelial cells of many tissues. Low concentrations of the enzyme are also found in the blood and levels can rise dramatically in various disease states, particularly those involving the liver.

If circulating anti-XOR antibodies are indeed autoantibodies, the question arises as to their role and as to how raised levels relate to heart disease. It may be that their role is protective, serving to remove endogenous XOR arising from various pathological states. Elevation in their levels in MI patients could be in response to the enzyme released from vascular endothelial cell lesions, possibly chronically repeated prior to MI.

See also: **Milk Lipids**: Lipid Oxidation; Milk Fat Globule Membrane.

Further Reading

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Other Enzymes

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Catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) is a heme protein that catalyzes the decomposition of hydrogen peroxide as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase also catalyzes peroxide-dependent oxidation of acids (e.g., hydrazoic, formic, and nitrous acids), lower aliphatic alcohols, and hydroxylamine. The enzyme is present in various living tissues and cells. It enters milk via somatic cells, in which it is associated with the membranes. Therefore, catalase activity in raw milk is directly proportional ($r = 0.7$) to the somatic cell count. As such, catalase activity may be an indicator of mastitis as the mammary gland cell wall becomes increasingly permeable to blood constituents, such as somatic cells, during mastitis.

Catalase activity in milk varies with the stage of lactation: it is high in colostrum, decreases during lactation, and then increases again in late lactation. In bovine milk and caprine milk, about 22 and 73% of the catalase activity is found in the cream and skim milk fractions, respectively, and the remaining 5% sediments with the separator sludge. Approximately 56% of the activity in cream is firmly bound to insoluble lipoproteins of the milk fat globule membrane (MFGM). Catalase activity can be determined by measuring the evolution of O_2 or by titrimetrically measuring the reduction of H_2O_2.

Catalase has been purified (23 000-fold) and crystallized from bovine milk, yielding 0.1 mg kg^{-1} milk. Purification of catalase from milk, using buttermilk as starting material, involves extraction with *n*-butanol, $(NH_4)_2SO_4$ fractionation (35–55% saturation), chloroform:methanol extraction, followed by chromatography of the extract on diethylaminoethyl (DEAE)-Sephacel and Sephacryl S-300.

Bovine milk catalase is an oligomer with a molecular mass of 225 kDa, consisting of subunits associated by hydrophobic interactions. Catalase dissociates into five subunits when treated with 1% SDS for 48 h; the subunits have a molecular mass of 55, 40, 34, 24, and 11 kDa. All the subunits are inactive except for the 55-kDa subunit. Milk contains at least two catalase isozymes that are immunochemically similar but have different pH optima of 7 and 8. Catalase is inhibited by Cu^{2+}, Fe^{2+}, Hg^{2+}, Sn^{2+}, CN^{-}, and N_3^{-}.

Crystalline catalase from milk is stable in salt solutions, retaining >90% of its activity in 10% NaCl. The enzyme is inactivated below its optimum temperature of 20 °C or when heated at 70 °C for 30 min in a buffer at pH 8.0. When milk is heated to 77–83 °C for 20 s, 88–90 °C for 3 min, or 91–95 °C without holding, it loses approximately 63, 74, and 84%, respectively, of its catalase activity. Heating milk for 16 s at 60, 65, or 72 °C results in the loss of 26, 84, and 92% of catalase activity, respectively, during Cheddar cheese manufacture, and a corresponding loss of 55.6, 90.3, and 94.4%, respectively, of catalase activity in the finished cheese. Some reactivation of catalase occurs during refrigerated storage of heated milk, presumably due to the release of microbial catalase from microorganisms that survive pasteurization or postpasteurization contaminants. Therefore, catalase activity in pasteurized milk may be a useful indicator of bacterial growth within 25 h of processing. High catalase activity in raw milk may be an indicator of high microbial load and, hence, poor quality.

It has been suggested that catalase plays a role in lipid oxidation because the enzyme contains a prosthetic ferric group; however, this role has not been demonstrated experimentally. Technologically, catalase is used to remove excess hydrogen peroxide during bleaching of cheese whey.

Lactate Dehydrogenase

Indigenous lactate dehydrogenase (LDH; EC 1.1.1.27; also called lactic acid dehydrogenase, (*S*)-lactate:NAD$^+$ oxidoreductase) in milk originates from somatic cells, leukocytes, and invading microorganisms. It plays an important role in the Embden–Meyerhof pathway by catalyzing the reversible oxidation of lactate to pyruvate, as follows:

$$\text{L-lactate} + \beta\text{-NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \beta\text{-NADH}$$

The conversion of lactate to pyruvate occurs under aerobic conditions, whereas the reverse reaction is anaerobic. The change in the concentration of β-NADH is measured spectrophotometrically to quantify the LDH content of milk. Alternatively, LDH can be quantified in milk by a fluorometric assay in which nonfluorescent resazurin is quantitatively reduced by NADH + H$^+$ into a highly fluorescent substance, resorufin.

LDH is a tetramer with a molecular mass of 140 kDa. Each LDH molecule consists of two polypeptide chains, designated H and M, so named according to their origins. Type H originates from aerobic tissues, such as cardiac muscle, whereas type M is from anaerobic tissues, such as skeletal muscle. Milk contains five LDH isozymes, namely LDH-1 to LDH-5, that result from combinations of the two polypeptides as follows: H_4, H_3M, H_2M_2, HM_3, and M_4. LDH-4 and LDH-5 are associated with glycolysis, and LDH-1 is associated with oxidative metabolism. The distribution of LDH differs in the milk of various species (**Table 1**).

The LDH activity in mastitic milk or colostrum is higher than that in normal milk because the enzyme enters milk from blood. The concentration of LDH isozymes decreases from LDH-5 to LDH-l in colostrum, but the order reverses during lactation, such that 95% of the LDH activity in mature bovine milk is due to LDH-l.

LDH activity in milk has been suggested as a useful indicator of mastitis. The significance of LDH in dairy processing has not been demonstrated, although it is conceivable that it may play a role in dairy fermentations. When buffalo milk is heated to 70 °C, LDH loses 50% and more than 90% of its activity after 3 and 10 min, respectively. At 80 °C, 90% of LDH is inactivated within 3 min. LDH in milk also loses its activity rapidly during high-pressure processing (HPP) at $\geq$200 MPa due to denaturation and aggregation. Hence it is considered as a possible indicator of adequate pasteurization by HPP.

Superoxide Dismutase

Superoxide dismutase (SOD; superoxide:superoxide oxidoreductase; EC 1.15.1.1) catalyzes the dismutation of the superoxide anion, as follows:

$$2O_2^{\bar{}} + 2H^+ \rightarrow H_2O_2 + O_2$$

The H_2O_2 formed in the reaction may serve as a substrate for catalase, peroxidase, or other suitable reducing agents. The biological function of SOD is to protect tissue against free radicals in anaerobic systems. The radical, which is potentially dangerous to living matter, is a product of the oxidation of xanthine by xanthine oxidase at high pH, high O_2, and low xanthine concentrations. The superoxide ion is also formed during autooxidation of hemoglobin.

There are three distinct types of SOD isoenzymes, Mn-SOD, Fe-SOD, and CuZn-SOD, which are found in prokaryotes, microorganisms and plants, and eukaryotes, respectively. Also, there is an extracellular SOD (EC-SOD) that has been isolated and characterized from human extracellular fluid. This article is limited to CuZn-SOD, the type found in milk.

CuZn-SOD is a dimer consisting of two 16-kDa subunits with a total molecular mass of 31–33 kDa. The subunits are chemically identical and are noncovalently associated but stabilized by intrachain disulfide bonds. Each subunit contains approximately 153 amino acids, one disulfide bridge, one Cu^{2+}, and one Zn^{2+}. The Cu^{2+} is located in the active site and is partially exposed to the solvents, whereas the Zn^{2+} is completely buried

Table 1 Activity and distribution of lactate dehydrogenase (LDH) in milk of some species

		Percentage of total activity				
Species and milk type	*Activity (U ml^{-1})*[a]	*LDH-1*	*LDH-2*	*LDH-3*	*LDH-4*	*LDH-5*
Bovine						
Normal	0.28 ± 0.15	93.5	6.5			
Mastitic	4.54 ± 1.21	34.8	22.3	16.7	15.4	7.3
Human						
Colostrum	0.90 ± 0.41	Low				High
Normal	0.39 ± 0.17	High				Low
Porcine						
Normal	1.31 ± 0.28					
Goat						
Colostrum	0.50 ± 0.14					
Buffalo						
Colostrum	0.73 ± 0.30					
Normal	0.39 ± 0.18					
Sheep						
Colostrum	0.83 ± 0.12					

[a] One unit of LDH activity oxidizes 1 μmol of β-NADH per minute.

within the protein structure. Due to the presence of Cu, isolated SOD has a blue-green color. The native enzyme is resistant to thiol-modifying reagents. Complete removal of the Zn exposes the SH groups, suggesting that a Zn–S complex is essential for stability while Cu^{2+} is responsible for electron transfer during the action of the enzyme. Removal of Cu by ethylenediaminetetraacetic acid (EDTA) results in the loss of enzymatic activity.

SOD is present only in milk serum and is absent from the cream. Homogenization does not affect the distribution of SOD in milk. Its concentration in bovine milk ranges from 0.15 to 2.5 mg l^{-1}. Milk from Jersey cows contains about 38% more SOD activity than Holstein milk, and SOD activity in human milk is 2.0–2.3 times higher than that in bovine milk. The level of SOD in milk parallels that of xanthine oxidase, suggesting that SOD may be produced in an attempt to offset the prooxidant effect of xanthine oxidase.

The electrophoretic and chromatographic properties of bovine and human milk SOD are identical to SOD purified from their corresponding erythrocytes, suggesting that milk SOD originates from blood plasma. The activity of SOD in bovine milk is 100–150 times lower than that in bovine blood. However, the activity of SOD in milk is independent of the stage of lactation, age of cow, or mastitis, all of which affect the permeability of the mammary gland cell membrane. There is a controversy regarding the effect of stage of lactation on SOD concentration in human milk. Some studies suggest that the SOD concentration is higher in colostrum than in mature milk, whereas others suggest that it is higher in milk of the third week of lactation than in colostrum.

Purification of SOD from acid whey is by a sequence of chloroform:methanol fractionation, gel filtration (Sephadex G-25) of the methanolic phase (which contains SOD activity), followed by ion-exchange chromatography on DE32 (grade of DEAE cellulose). Alternatively, SOD may be isolated from skim milk by ultrafiltration (using 100 and 10 kDa molecular mass cutoff membranes), followed by gel filtration on Sephadex G-l00.

Although CuZn-SOD predominates in milk, it has been suggested that some Mn-SOD may also be present. However, this is questionable because bovine milk and human milk lose their SOD activity in the presence of cyanide, which binds to copper strongly, thus inhibiting CuZn-SOD. The occurrence of Mn-SOD in milk may be due to contaminating bacteria.

Normal pasteurization (72 °C for 15 s) has little effect on the SOD activity of milk, but only 25% of its activity remains in milk heated at 75 °C for 20 min. The activity of CuZn-SOD remains unaffected by the pH in the range 5–10. As high SOD activity results in low levels of oxidative rancidity in milk, SOD may be important in maintaining the oxidative stability of milk by scavenging the superoxide ion produced by xanthine oxidase during oxidation of its substrate. As SOD loses its activity rapidly at even slightly higher temperatures than those used for normal pasteurization, slight variations in the pasteurization temperature may contribute to variations in the stability of milk to oxidative rancidity.

There are two assays that are commonly used for measuring SOD activity, one using cytochrome *c* and the other using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) as the indicating scavenger of superoxide. However, the assay methods for SOD are complicated.

Galactosyltransferase

β-1,4-Galactosyltransferase 1 (Gal-T1; EC 2.4.1.38) is a member of the family of glycosyltransferases that comprises at least seven members (Gal-T1 to Gal-T7) that are involved in the synthesis of complex carbohydrates present on glycoproteins and glycolipids. Gal-T1 to Gal-T7 all catalyze the transfer of galactose (Gal) from uridine diphosphate (UDP)-Gal to different sugar acceptors. The milk galactosyl-transferase enzyme is Gal-T1.

Gal-T1 (EC 2.4.1.38) or UDP-galactose: *N*-acetylglucosamine galactosyltransferase catalyzes the transfer of galactosyl groups from UDP-galactose to *N*-acetylglucosamine (NAcGlc) of oligosaccharides attached to glycoproteins (e.g., glycan) to form β-1,4-linked galactosylated glycan according to the following equation:

$$\text{UDP-galactose} + N\text{-acetyl-}\textsc{d}\text{-glucosaminyl-glycopeptide} \leftrightarrow \text{UDP} + 4\text{-}\beta\text{-}\textsc{d}\text{-galactosyl-}N\text{-acetyl-}\textsc{d}\text{-glucosaminylglycopeptide}$$

Gal-T1 also catalyzes the transfer of Gal to free NAcGlc to form *N*-acetyllactosamine. Gal-T1 does not have an absolute requirement for UDP-Gal as a sugar donor. It can also transfer glucose (Glc), 2-deoxy-Glc, arabinose, and NAcGal at reduced efficiencies. For example, catalytic transfer of Glc is 0.3–0.5% that of Gal. Catalytic transfer of Glc from UDP-Glc to NAcGlc constitutes glycosyltransferase activity (Glc-T), which is stimulated 30-fold by α-lactalbumin.

Gal-T1 is part of the enzyme lactose synthetase (EC 2.4.1.22) along with α-lactalbumin (α-lac) and is designated the A protein, whereas α-lac is designated the B protein. The specific function of α-lac is to modify the catalytic site of Gal-T1 and promote the binding of glucose to the enzyme for lactose synthesis as shown below:

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}$$

α-Lac reduces the K_M of the enzyme for glucose, leading to lactose synthesis at physiological glucose concentrations. The enzyme requires Mn^{2+} for activity at an optimum concentration of 13.3 mmol l^{-1} at pH 7.5.

Other ions (e.g., Mg^{2+}, Zn^{2+}, or Ca^{2+}) can replace Mn^{2+}, but are less effective. In the absence of α-lac, Zn^{2+} competes with Mn^{2+} for the same binding site. Purified Gal-T1 from bovine skim milk has a specific activity of 14.1 μmol lactose min^{-1} mg^{-1} at 37 °C (pH 5.8 at 25 °C).

The concentration of Gal-T1 in bovine milk is 3–4 mg l^{-1}. The enzyme is found in the membranes of Golgi vesicles; therefore, it enters milk with Golgi membrane fragments. The enzyme contains 12–13% carbohydrates (of which 8% are neutral sugars, like galactose, mannose, and glucose, 1% glucosamine, 1% galactosamine, and 2% sialic acid), and it has a molecular mass of 42 kDa. Some research, however, has shown that there are two enzymatically active forms of bovine Gal-T, one with a molecular mass of 45 kDa and the other with a molecular mass of ~58 kDa. The molecular mass of human Gal-T is 53 kDa. Gal-T activity in bovine milk is independent of the breed of the animal and is highly correlated with the stage of lactation, with higher concentration during late lactation.

Sulfhydryl Oxidase

Sulfhydryl oxidase (SOx) functions in the formation of the three-dimensional structure of proteins by catalyzing the oxidation of cysteine and SH-containing peptides and proteins, using molecular O_2 as an electron acceptor, and yielding H_2O_2 and their corresponding disulfides. The reaction equation is summarized as follows:

$$2RSH + O_2 \rightarrow RSSR + H_2O_2$$

At present, no EC number has been assigned to SOx found in milk, so it is often confused with thiol oxidase (EC 1.8.3.2). However, the molecular and biochemical properties of bovine milk SOx are distinctly different from those of thiol oxidase. The mammalian enzyme also differs from the SOx of *Aspergillus niger*. Unlike bovine milk SOx, the microbial enzyme is a soluble flavoprotein with a subunit molecular mass of 53 kDa; it is acid stable and survives at pH 3.

SOx is present in colostrum and milk from various species. Bovine milk contains about 10 mg l^{-1} SOx, most of which is bound to skim milk membranes. Similarly, 95% of human milk SOx is present in the skim milk.

SOx has been purified (to different degrees) from skim milk or sweet whey (pH >6) by different methods. However, purification by transient covalent affinity chromatography on cysteinylsuccinamidopropyl-glass yields a relatively pure enzyme. SOx is a highly aggregated FeCu-containing glycoprotein with a blocked N-terminus. The enzyme has a molecular mass of 80–90 kDa and contains 11% carbohydrate by weight and 0.5 atom of Fe per subunit. SOx contains two chemically reactive SH groups. However, activity is due to one of the SH groups plus Fe. The carbohydrate moiety increases the solubility of the enzyme.

Bovine milk SOx is acid labile. However, the human milk enzyme partially survives passage through the gastrointestinal tract, losing about 50% of its activity after 1 h at pH 2–5. SOx is optimally active at pH 7.0 and 37 °C; it is inhibited by 1 $mmol^{-1}$ EDTA.

Commercially processed milk contains about 40% of the SOx activity of raw milk, suggesting that the enzyme partially survives pasteurization. Storage of human milk at a low temperature (4 or −20 °C) has no effect on its SOx activity. SOx activity retained in microwave-processed skim milk is significantly higher compared with ultra-high temperature (UHT) -sterilized samples. SOx activity is usually assayed using glutathione as a substrate. Disappearance of sulfhydryl groups is measured by the reaction with 5,5′-dithio-bis-(2-nitrobenzoic acid), with which glutathione forms a yellow product. This product is quantified by measuring absorbance at 412 nm.

The H_2O_2 generated from SOx activity may be involved in the lactoperoxidase system of milk. During high-temperature treatment of milk, the SH groups, which are responsible for cooked flavor, are exposed and activated. Hence, addition of purified enzyme to UHT milk reduces the cooked flavor defect and enhances flavor stability due to reduced lipid oxidation.

Recent studies suggest that milk might contain a flavin-dependent SOx (QSOx1) in addition to the iron-dependent SOx described above. QSOx1 belongs to a newly recognized family of flavoenzymes called quiescin-sulfhydryl oxidases (QSOx). Both iron- and flavin-dependent SOx catalyze disulfide bond formation. The QSOx1 in milk is shown to have a molecular mass of 62 kDa, contains only small levels of Fe, and is not tightly associated with skim milk membrane. Also, 1 mmol l^{-1} EDTA did not significantly affect the QSOx1 activity. It is also suggested that QSOx1 might constitute the dominant disulfide bond-generating activity in skim milk. Further research is warranted in this area to confirm these new findings.

Lysozyme

Lysozyme, also called muramidase (peptidoglycan *N*-acetylmuramoyl hydrolase; EC 3.2.1.17), catalyzes the hydrolysis of β1→4-linkages between *N*-acetylmuramic acid and *N*-acetyl-β-glucosamine residues in peptidoglycan, a constituent of bacterial cell walls. Lysozyme acts mostly on Gram-positive bacteria and a few Gram-negative bacteria. Gram-positive bacteria are more susceptible to the action of lysozyme because their cell wall contains up to 90% peptidoglycan, whereas Gram-negative bacteria are more resistant because of the smaller amount of peptidoglycan in their cell wall. Heat denaturation, slight modification with hydrophobic ligands, or high-pressure homogenization can increase the activity of lysozyme against Gram-negative bacteria. Lysozyme has been shown to be effective against Gram-positive bacteria such as *Micrococcus*, *Sarcina*, *Lactobacillus*, and *Bacillus* and Gram-negative bacteria such as *Salmonella*, *Pseudomonas*, *Aeromonas*, *Escherichia coli*, *Clostridium botulinum*, and *Listeria monocytogenes*. Lysozyme also inhibits viruses, parasites, and fungi. There are two types of lysozymes: those found in hen egg whites (hen egg whites lysozyme; HEWL), also known as chick-type (*c*) lysozyme; and those found in Embden goose egg whites, or goose-type (*g*) lysozyme. Lysozymes *c* and *g* differ in their amino acid sequence, molecular mass, and extinction coefficient ($E_{280\,\mathrm{nm}}$) (**Table 2**).

Lysozyme *g* is heat labile and contains half as many cysteine (3) and tryptophan (4) residues as lysozyme *c*. The concentration of lysozyme in mammalian milk varies from <0.3 mg 100 ml^{-1} in bovine milk to about 100 mg 100 ml^{-1} in donkey milk. Generally, lysozyme activities in precolostrum and colostrum are higher than in corresponding human milk or bovine milk. In bovine milk, lysozyme activity increases with somatic cell count and mastitis.

Human milk lysozyme (HML), camel milk lysozyme (CML), equine milk lysozyme (EML), and donkey milk lysozyme (DML) are considered to be type *c* lysozymes because the molecular mass of each is identical to HEWL. However, it is unclear whether bovine milk lysozyme (BML) is a type *c* or *g* lysozyme. Both *c*- and *g*-type lysozymes are present in bovine stomach tissues and fluids, raising the possibility that both types of lysozyme are present in bovine milk. BML consists of two components that differ by seven amino acids in the first 39 residues. Although HML and HEWL are structurally homologous, they differ in their amino acid composition. HEWL and EML have 129 amino acid residues compared with 130 for HML, which has an extra glycine residue. HML and BML are antigenically and serologically different. DML has two variants, LYS A and LYS B, which differ in three amino acids at positions 48, 52, and 61. The molecular weight of buffalo milk lysozyme (BfML) is 16 kDa. The lysozyme activity in buffalo milk is twice as much as that in bovine milk. BfML has 115 amino acids.

Purification of lysozyme from milk or acid whey involves affinity chromatography on heparin-Sepharose, followed by gel filtration on Sepharose 4B or Sephadex-G50. Chromatography on hydroxyapatite has also been used for purification. Lysozyme activity is normally assayed by the lysis of *Micrococcus lysodeikticus* but can also be measured by reversed-phase high-performance liquid chromatography.

The three-dimensional structures of milk lysozyme and α-lac and the intron–exon organization of their genes are similar, suggesting that both proteins have similar genetic lineage. BML and HML show optimum activity at pH 7.5 and 6.35, respectively. Both enzymes are most stable in the pH range 3–4 and retain >75% of their activity in milk heated to 75 °C for 15 min or 80 °C for 15 s. HML retains >95% of its activity after HPP at 400 MPa for 120 min. BfML has maximum activity at pH 7.4 and 37 °C and is inhibited by 0.1 mmol l^{-1} heavy metals, such as Ni^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, and Fe^{3+}. In buffer, BfML retains 90% of its activity when heated at 74 °C for 15 min and is completely inactivated after 30 min at 100 °C.

Lysozyme loses its enzymatic activity when its intrachain disulfide bonds are reduced. Oxidation of the reduced enzyme renatures the enzyme by restoring its native conformation. The different lysozymes have different renaturation rates. For example, HEWL renatures more rapidly than HML because of the greater hydrophobicity of the latter. Also, oxidation of reduced HML and BML results in regeneration of 84 and 328% of their original activities, respectively.

In association with lactoferrin, lysozyme functions as a bactericidal agent in milk. BML, HML, and CML have been shown to have lytic action toward *E. coli*. Thus, premature infants fed precolostrum containing a high level of lysozyme may have a lower incidence of gastrointestinal invasion by pathogenic bacteria.

Table 2 Comparison of lysozymes from milk of different species

Source	*Concentration (mg 100 ml^{-1})*	*Molecular mass (kDa)*	$E_{280\,nm}$
Bovine (BML)	>0.3	18	7.1
Human (HML)	10–12	15	
Equine (EML)	79	14	
Camel (CML)	0.5	15	
Donkey (DML)	100	15	
Lysozyme *c*		15	27
Lysozyme *g*		20	15

Gamma Glutamyltranspeptidase

Gamma-glutamyltransferase, or γ-glutamyltranspeptidase (GGT; (5-glutamyl)-peptide:amino acid 5-glutamyltransferase; EC 2.3.2.2), catalyzes the transfer of γ-glutamyl residues from γ-glutamyl–containing peptides to an acceptor α-amino acid (except proline) or peptides according to the following reaction:

$$\gamma\text{-glutamyl-peptide} + \text{amino acid} \xrightarrow{\text{GGT}} \text{peptide} \\ + \gamma\text{-glutamyl-amino acid}$$

GGT is a secretory enzyme present in mammalian tissues and fluids and in plants and microorganisms. The enzyme is located in the Golgi apparatus where it is involved in the endocytotic and/or exocytotic transport of proteins. GGT in milk is associated with the membrane fractions of skim milk and fat globules. About 25% of GGT activity is located within the cream fraction and the rest (75%) within the skim milk. The fat content does not have any significant effect on the inactivation of GGT during heating of milk. Also, the level of GGT varies very little with seasonal variations. Its activity in both human milk and bovine milk is influenced by the stage of lactation, with 3 times higher activity in colostrum than in normal milk.

GGT has been purified from bovine milk (11 000-fold) by solubilization with Lubrol WX, treatment with acetone, deoxylcholate and bromelain, and then separation on ion-exchange and molecular-sieving resins. Active GGT is a glycoprotein with a molecular mass of 80 kDa. It consists of two subunits with molecular masses of 57 and 25.5 kDa. GGT has a pI of 3.85 and, depending on the acceptor amino acid or peptide, its optimum pH for activity is in the range 8.0–9.0. The enzyme is inhibited by iodoacetamide, diisopropyl fluorophosphate, and metal ions such as Cu^{2+} and Fe^{3+}.

Bovine milk GGT is stable at 20 °C. During storage at 4 or −20 °C, milk loses 30% of its GGT activity. Milk retains <20% of its GGT activity after pasteurization (72–75 °C for 15 s). Thus, GGT activity may be useful for detecting milk heated to 70–80 °C. GGT in milk is more abundant and more heat resistant than alkaline phosphatase (ALP). Also, there is a good correlation between reduction in GGT activity and destruction of streptococci. Hence, GGT may be a useful indicator of proper pasteurization. In camel milk, inactivation of ALP does not serve as an indicator of adequate pasteurization; GGT may be a better indicator. GGT heat-inactivation kinetics follow a first-order reaction, and it can be easily quantified by using commercially available kits. In the assay, GGT acts on L-γ-glutamyl-*p*-nitroanilide and releases *p*-nitroanilide, which is then quantified spectrometrically. During HPP, the kinetics of inactivation of GGT at 20 °C and pressures above 500 MPa are sufficiently close to the inactivation of *Leuconostoc monocytogenes* and *E. coli*. Therefore, it may prove a useful process marker for the destruction of these organisms during HPP.

GGT plays a role in the transport of amino acids from blood into the mammary gland for use in milk protein synthesis. GGT is readily absorbed in the gastrointestinal tract, resulting in high levels of GGT in the serum of newborns fed colostrum or early breast milk. Because heat treatment of infant formula destroys its GGT activity, the activity of this enzyme in the sera of newborns can be used to distinguish between infants fed breast milk and those fed a milk-based formula. There is a significant correlation between GGT and the concentration of gammaglobulins in the colostrum. Hence, this enzyme can be used as a marker for the evaluation of colostrum quality. Low concentrations of GGT, and hence antibodies, in colostrum will result in poor immunity in newborns.

Amylases

Three types of amylases (α (EC 3.2.1.1), β (EC 3.2.1.2), and γ (EC 3.2.1.3)) occur in nature. Milk contains both α-amylase (1,4-α-D-glucan glucanohydrolase) and β-amylase (1,4-α-D-glucan maltohydrolase), but the former predominates. Amylases generally function by digesting polysaccharides not present in milk (e.g., starch and glycogen). α-Amylase hydrolyzes 1,4-α-D-glucosidic linkages in polysaccharides containing at least three $\alpha 1 \rightarrow 4$-linked glucose units. Bovine milk and human milk α-amylases are identical to the salivary isozyme.

The α-amylase activity in human milk varies (1000–8000 $\mathrm{U\,l^{-1}}$) but is generally about 25-fold higher than that in bovine milk. Human milk contains 10–25 times more α-amylase than does blood, suggesting that the enzyme does not enter milk from blood but is produced ectopically in the mammary gland in response to a hormonal stimulus. In humans, amylase activity varies among women; activity is present even after a prolonged lactation of up to 27 months and maintains a plateau at 6–27 months.

α-Amylase has been purified from milk by chromatography on Sephadex G-100 or G-150. The purified enzyme is stable at pH ≥ 3 and shows optimum activity in the pH range 6.5–7.5 and at 44 °C. It is resistant to proteolysis by pepsin, suggesting that it can survive passage through the gastrointestinal tract after ingestion of milk. α-Amylase activity increases in the presence of Ca^{2+}, Cl^{-}, and bovine serum albumin, but activity is reduced in the presence of I^{-}. The enzyme is stable in human milk frozen at −20 or −70 °C. Milk retains its amylase activity when stored at 15, 25, or 38 °C for 24 h. Pasteurization (75 °C for 15 s) of milk results in the loss of <45% of its amylase activity.

Camel milk contains 59.9 U mg^{-1} β-amylase. The molecular mass of β-amylase isolated from camel milk is 61 kDa. The purified enzyme shows optimum activity at pH 7.0 and in the temperature range 30–40 °C. The enzyme is thermally stable up to 40 °C, followed by a rapid decline in its activity at 50 °C. While monovalent and divalent ions, such as Mg^{2+}, Ca^{2+}, Na^{+}, and Cl^{-}, activated the enzyme, EDTA, ethylene glycol tetraacetic acid (EGTA), urea, sodium dodecyle sulfate (SDS), dithiothreitol (DTT), and 5-5-dithiolius (2-nitrobenzoic acid) (DTNB) significantly inhibited its activity.

The presence of α-amylase in milk is, perhaps, surprising because milk is thought to lack a suitable substrate for the enzyme. However, recent high-performance liquid chromatography (HPLC) analysis shows that human milk and colostrum contain about 120–150 mg l^{-1} of oligosaccharides (mostly penta- to tetradecasaccharides), suggesting that milk may contain substrates for the enzyme. As little pancreatic α-amylase activity occurs at birth, breast milk appears to be the immediate source of the enzyme for premature infants who may require high-calorie starch-containing supplements in their diets.

Ribonucleases

Milk ribonuclease (RNase; EC 3.1.27.5) is identical to pancreatic RNase, which catalyzes the endonucleolytic cleavage of 3′-phosphomononucleotides and 3′-phosphooligonucleotides ending in polycytidylic (Cp) or polyuridylic (Up) acids with 2′,3′-cyclic phosphate intermediates. It is suggested that the RNase in milk originates in the pancreas and is absorbed through the intestinal wall into the blood and enters milk through the blood serum; this would require active transport as the level of RNase in milk is considerably higher than in blood serum.

RNase is present in bovine, caprine, human, porcine, and buffalo milk. Bovine milk contains 11–25 mg l^{-1}, most of which is in the milk serum. RNase activity in bovine milk is more than 3 times higher than in human milk, which contains 3 mg l^{-1}. Generally, RNase activity is higher in colostrum (3–5 times) and mastitic milk (2 times) than in normal milk. RNase may protect milk from viruses as it is reported to inhibit the activity of RNA-dependent DNA polymerase, an enzyme involved in the replication of viruses. A bactericidal and growth promotion role of RNase has also been suggested.

The purification of RNase from milk (acid whey) involves chromatography on the carboxylic acid resin IRC-50, Sephadex G-50, or phosphocellulose. Three RNase isoenzymes (A, B, and II-1) occur in bovine milk in the ratio 72:27:1. The major difference between the three isoenzymes is that RNase B is a glycoprotein containing 3.0% glucosamine, 1.2% galactosamine, and 5.17% mannose, RNase A contains no carbohydrate, and RNase II-1 is unable to hydrolyze phosphonucleotides ending in Cp.

The molecular mass of pancreatic RNase is in the range 12.7–15.5 kDa. The molecular mass of bovine milk and caprine milk RNase is 13.6 and 29 kDa, respectively. Bovine milk RNase exhibits optimum activity at 37 °C and pH 7.5 compared with 50 °C and pH 9.0, respectively, for caprine milk RNase. Inhibitors of RNase activity include Cu^{2+}, Ag^{+}, Fe^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, and Mn^{2+}.

Less than 0.3% of bovine milk RNase survives sterilization (121 °C for 10 s) of milk, whereas about 60% survives in milk heated to 80 °C for 15 s or 72 °C for 2 min. However, the enzyme retains its activity when it is heated in buffer at 90 °C for 20 °C per min at pH 3.5, suggesting that RNase is stable at low pH.

The RNase purified from human milk (HmRNase) has a much higher molecular weight (80 kDa) compared with any other source. HmRNase has a broad pH optimum in the range 7.5–8.0, with an isoelectric point at pH 6.8. It is more thermal labile compared with bovine RNase. HmRNase loses 60 and 85% of its activity when heated at 60 and 90 °C, respectively, for 5 min; it is completely inactivated by heating at 90 °C for 10 min. The effect of cations on HmRNase activity is similar to that on bovine RNase; it is inhibited by 1–2 mmol l^{-1} Mn^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Co^{2+} and by 10 mmol l^{-1} Mg^{2+}. Due to its physical, chemical, and antigenic similarities, HmRNase was previously considered as an isoform of lactoferrin. However, their functional properties are very different. It is speculated that HmRNase may be synthesized in the mammary gland. There may be a correlation between the underexpression of RNase in women and the risk of breast cancer.

See also: **Lactose and Oligosaccharides**: Lactose: Galacto-Oligosaccharides. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk). **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose; Secretion of Milk Constituents. **Milk Lipids**: Lipid Oxidation; Milk Fat Globule Membrane. **Milk Proteins**: Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins.

Further Reading

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FEED INGREDIENTS

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Feed Concentrates: Cereal Grains

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Introduction

Livestock and poultry animal production requires a large amount of food energy. The primary energy source for livestock production is cereal grains. Carbohydrates constitute about 70% of the diet for livestock, with proportion of fibrous and nonfibrous carbohydrate (NFC) varying in the diet, depending on type of digestive system of the species. The NFC portion of nonruminant diets may be as high as 55%, whereas it is usually less than 42% in ruminant diets to avoid ruminal acidosis and related disorders and to utilize high-fiber feedstuffs that would otherwise be underutilized, including by-products from the processing of cereal grains for human consumption. The NFC portion of diets for nonruminants and ruminants is provided primarily by the cereal grains, with the primary cereal grain used in a geographical area based on availability and cost of grain commodities. Some cereal grains are used very little for livestock because of their use for human food; however, those cereal grains with little value for human food use and whose production far exceeds the demand for human food, are fed in large quantities to the livestock. Although some people hold the view that livestock compete with humans for cereal grains for food, without the use of cereal grains by livestock, there would be an enormous surplus of cereal grains in the world. World hunger is caused more by problems with distribution of food rather than the availability of food, such as cereal grains, within the world market.

The composition of cereal grains is somewhat similar, but the grains possess some unique characteristics. The digestibility of the cereal grains can be affected by processing, for example, mechanical processing to break the seed coat and reduce particle size and by application of heat and moisture. The utilization of cereal grains by ruminants will be affected by type and amount of grain fed, processing of the grains, and the species, quality, and amount of forage in the diet. This article will review the characteristics of major cereal grains and their utilization by ruminants, with primary emphasis on their feeding to dairy cattle.

Availability

Corn (maize), wheat, and rice are the major cereal grains produced in the world (**Table 1**). However, rice is almost exclusively used for human food. Using the US market as an indicator of cereal grain usage, about 53% of the corn produced is used for feed but only 7% of the wheat is used for feed. Although barley ranks fourth in the world production of cereal grain, 87% of it is used for human food in the United States. The world production of sorghum is about 41% that of barley. This is in direct contrast to the United States, but their utilization for feed is somewhat similar in the United States. World production of millet, oats, rye, and triticale in total ranges from 11 to 32 million Mt per year, but except for oats, others are produced in negligible amounts in the United States. Oats are used for both animal feed and human food.

Given the amount of production and utilization for feed, the cereal grain used in the greatest quantity in the United States is by far corn, followed by wheat and sorghum, and then followed by oats and barley. Much of the wheat is fed to nonruminants, whereas much of the oats is fed to young ruminants and horses. Most of the discussion in this article will address the utilization of corn, barley, sorghum, and wheat.

Composition

Carbohydrates are the major nutrient provided by cereal grains, constituting 75–85% of the grain (**Table 1**). Of the carbohydrates present, 60–90% are NFCs. Therefore, cereal grains are somewhat low in protein (8–17% of dry matter (DM)) and fat (1.5–5%). Most of the fat and protein are located in the germ portion of the seed. The fiber is primarily in the pericarp and seed coat, and the NFC is in the endosperm areas of the seed. The neutral detergent fiber (NDF) in the cereal grains is low in its effectiveness in stimulating rumination but does serve the role of diluting NFC in high-grain diets, especially when the cereal grain by-products that are high in NDF are included in diets. The starch consists of amylose (linear starch polymer) and amylopectin (branched chain polymer). The relative contribution of amylose for barley, oats, wheat, sorghum, and dent corn is 22, 27, 26, 25, and 28% of the starch. Some specialty hybrids (e.g., waxy hybrids) for high amylopectin have been developed for corn and sorghum. In contrast, amylopectin in high-amylose, dent, flint, and waxy corn is 50, 72, 99, and 100% of the starch. The grain with a higher proportion of amylopectin is expected to be more susceptible to enzymatic digestion, and this has been demonstrated by the higher *in vitro* digestibility of waxy corn compared to dent corn. Average daily gain by lambs and steers was observed to be higher with waxy corn compared to dent corn when the corn was fed at 90% of the diet, but these gains were also affected by nitrogen source (soybean meal vs soybean meal plus urea). A diet with waxy corn grain (27.9%) and waxy corn silage (32.8%) increased milk yield, apparent total tract digestibility of starch, and ruminal concentration of propionate compared to a similar diet with dent corn grain and silage.

Besides altering the nature of the starch, other specialty hybrids of cereal grains have been developed. For example, corn hybrids have been developed with increased concentration of protein, lysine, sugar, and/or oil. Thus, these efforts from the livestock feeding perspective are aimed at improving protein quality or energy availability from the cereal grains. Antinutritional factors affecting starch utilization include enzyme inhibitors, phytates, lectin, and tannins. For example, high tannin in the bird-resistant varieties of sorghum reduces the digestibility of protein and starch.

Many factors must be considered when evaluating the utility of the different grain hybrids, including yield, cost of production, feasibility to market and distribute the grain separately, and the portion of the plant that changed by the genetic selection (i.e., seed vs stem or leaves). The species of animals most likely to benefit from the change in composition based on the digestive system and nutritional requirements must also be evaluated. Several trials have been conducted with dairy cattle to determine the value of grain and forage from high-oil corn. The seed has an increased concentration of oil; high-oil corn grain has about 2.4 times higher oil concentration than dent corn. Based on the availability of other fat sources and the agronomic factors of high-oil grain production, high-oil corn is of limited economic value for feeding to dairy cattle except for possibly high-producing cows.

As discussed earlier, the primary cereal grains (nonspecialty hybrids) are similar in the concentration of amylose and amylopectin; however, the digestion of starch differs among these grains. Therefore, the nature of the starch has an impact on digestion. Even though the starch granules in corn and sorghum are similar in size, shape, and composition, the starch in sorghum is generally less digestible than the starch in corn. The endosperm of corn and sorghum is made up of both corneous and floury areas, but sorghum contains proportionally more corneous endosperm than corn. The corneous endosperm is dense, hard, and somewhat resistant to water penetration, caused by smaller starch granules and a continuous (vs incomplete) protein matrix with cross-linking. Floury endosperm tends to have larger starch granules

Table 1 Production, usage, and composition of major cereal grains

		United States				*Chemical composition (% of DM)*[c]			
			Usage (%)[b]				*Carbohydrates*		
Cereal grain	*World production*[a] *(Million Mt)*	*Production (Million Mt)*	*Feed*	*Food*	*Usage for feed (Mt)*	*CP*	*NDF*	*NFC*[e]	*Fat*
Maize	707	268	53.1	13.0	142	9.4	9.5	75.4	4.2
Wheat	592	49.3	6.7	51.8	3.29	14.2	13.4	68.1	2.3
Rice	421	8.79	NA	NA	NA	8.4	26.4	57.4	1.8
Barley	139	3.92[d]	31.1	86.7	1.22	12.4	20.8	61.7	2.2
Sorghum	56.5	7.06	39.2	16.2	2.77	11.6	10.9	72.4	3.1
Millet	31.8	0.30	NA	NA	NA	13.5	19.5	59.4	4.5
Oats	23.1	1.36[d]	133	78.7	1.81	13.2	30.0	48.4	5.1
Rye	13.3	0.18	NA	NA	NA	13.8	11.4	71.2	1.7
Triticale	11.3	NA	NA	NA	NA	17.3	13.3	65.7	1.7

[a] Taken from US Department of Agriculture (2008) *World Agricultural Supply and Demand Estimates WASDE-458*. 9 May. Washington, DC: World Agricultural Outlook Board; Food and Agriculture Organization (2006) Rome: FAO. http://faostat.fao.org (accessed 9 June 2008); based on production from 2006 to 2007, 67% of corn produced was used for feed and 18% of wheat produced was used for feed.

[b] Usage as a percentage of production; the food category includes food, seed, and industrial use with the exception of ethanol production for fuel use with maize.

[c] Taken from National Research Council (NRC) (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy of Sciences; Ensminger ME, Oldfield JE, and Heinemann WW (1990) *Feeds and Nutrition*, 2nd edn. Clovis, CA: The Ensminger Publishing Co.

[d] The amount used for feed and food exceeds 100% because of imports and beginning stocks.

[e] NFC is calculated by 100% DM – % CP – % NDF – % fat – % ash.

CP, crude protein; DM, dry matter; NA, not available; NDF, neutral detergent fiber; NFC, nonfiber carbohydrate.

surrounded by a discontinuous protein matrix. Wheat proportionally has more flourly endosperm than corn, thus explaining its faster ruminal degradation compared to corn. With respect to rate of starch degradation, cereal grains can be ranked from highest to lowest as follows: wheat, barley, oats, corn, and sorghum.

Utilization by Ruminants

The primary role of dietary carbohydrates in ruminant animals is the provision of energy and fiber for good ruminal function. Because cereal grains are low in the concentration of fiber and the fiber present is low in its effectiveness in stimulating rumination, cereal grains primarily provide energy in the form of starch. About two-thirds of the starch is degraded in the rumen by microorganisms (**Figure 1**). This fermentation provides substrates for microbial protein synthesis and for the production of propionate, which serves as a precursor of glucose for the animal. About 90–95% of the starch will be digested in the total tract of the animal, with the digestibility being reflected by the digestible energy (DE) at 1× maintenance. The starch digestibilities would be expected to decrease with incremental increases in intake above maintenance (e.g., 3× to 4× expected for lactating cows) because higher DM intake results in faster rate of particulate passage in the digestive tract.

There are differences in total tract digestibilities among cereal grains due to the compositional differences discussed earlier. However, these digestibilities can be increased by grain processing. Rolling or grinding grain will reduce particle size and thus increase surface area for attack by digestive enzymes, leading to increased digestibility in the rumen and total tract and thus increased energy availability. Steaming, through rolling or flaking, adds moisture and heat, because of which the starch granules swell (gelatinize), resulting in increased surface area for enzymatic attack and disruption of the protein matrix (allows easier penetration of the starch granules by the enzymes). These effects on availability of energy from processing of cereal grains are described using 'processing adjustment factor' (PAF) in the dairy industry. These factors are used to adjust the NFC digestibility, assuming that true total tract digestibility of NFC is 0.98 at 1× maintenance and 0.90 at 3× maintenance. Dry, ground corn is set at a PAF of 1.00 at 3× maintenance, with other cereal grains adjusted relative to the ground corn. For example, the NFC in steam-flaked sorghum is 4 percentage units more digestible than the NFC in ground corn but 12 percentage units more digestible than the NFC in dry, rolled sorghum (**Figure 1**). The PAF does not correspond directly to DE because DE is affected by the total composition of the cereal grain and PAF is used directly on the NFC fraction. For example, the DE is higher for ground corn than for rolled barley even though the PAF for barley is 1.04, which is caused by corn being higher in NFC and lower in NDF than barley.

Although cereal grains are not good sources of effective fiber in the diet for dairy cattle, the amount and source of cereal grain must be considered in diet formulation to optimize ruminal function. It is now recognized that the minimum amount of forage NDF (provides most of the effective fiber in diets) depends on the maximum amount of NFC in the diet: 19% forage NDF and 44% NFC, 18 and 42, 17 and 40, 16 and 38, and 15 and 36, respectively. The minimum amount of forage NDF in the diet should be 14–16% and the maximum NFC should not exceed

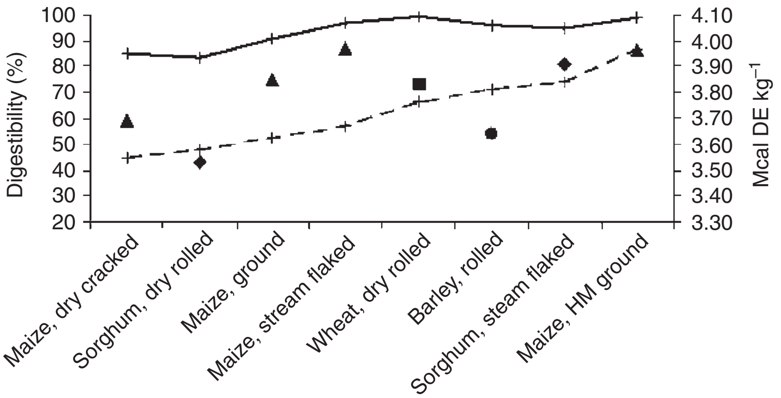

Figure 1 Ruminal (dashed line) and total tract (solid line) digestibilities of starch and digestible energy concentrations (solid symbols) in various cereal grains that have undergone different methods of harvesting and processing. HM, high moisture. Data taken from Firkins JL, Eastridge ML, St-Pierre NR, and Noftsger SM (2001) Effects of grain variability and processing on starch utilization by lactating dairy cattle. *Journal of Animal Science* 79(electronic supplement): E218–E238; National Research Council (NRC) (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy of Sciences; Espindola MS, DePeters EJ, Fadel JG, Zinn RA, and Perez-Monti H (1997) Effects of nutrient digestion of wheat processing and method of tallow addition to the diets of lactating cows. *Journal of Dairy Science* 80: 1160–1171.

Table 2 Ruminal and total tract digestibilities, digestible energy, processing adjustment factors, chewing, and microbial *N* flows to the duodenum when feeding various cereal grains

Cereal grain	*Ruminal starch digestibility*[a] *(% of intake)*	*Total tract starch digestibility*[a] *(% of intake)*	*Digestible energy at 1× maintenance*[b] *(Mcal kg^{-1})*	*Processing adjustment factor*[b]	*Chewing (min day^{-1})*[a]	*Microbial* N[a] *(g day^{-1})*
Corn						
Dry, cracked	44.6	85.0	3.69	0.95	-	276
Ground	52.3	90.7	3.85	1.00	649	257
High moisture, ground	86.8	98.8	3.96	1.04	760	236
Steam flaked	56.9	96.6	3.97	1.04	-	296
Barley						
Rolled	71.2	95.8	3.64	1.04	671	299
Sorghum						
Dry rolled	48.1	83.5	3.53	0.92	-	278
Steam flaked	74.0	94.9	3.91	1.04	-	357
Wheat						
Dry rolled	66.4[c]	99.3[c]	3.83	1.04	546[d]	-

[a] Taken from Firkins JL, Eastridge ML, St-Pierre NR, and Noftsger SM (2001) Effects of grain variability and processing on starch utilization by lactating dairy cattle. *Journal of Animal Science* 79(electronic supplement): E218–E238.

[b] Taken from National Research Council (NRC) (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy of Sciences.

[c] Taken from a single experiment; Espindola MS, DePeters EJ, Fadel JG, Zinn RA, and Perez-Monti H (1997) Effects of nutrient digestion of wheat processing and method of tallow addition to the diets of lactating cows. *Journal of Dairy Science* 80: 1160–1171.

[d] Diets included corn and wheat in combination.

1.0 Mcal = 4.184 MJ.

42%. The lower dietary levels of forage NDF are more apt to be used if whole cottonseed is the diet. The NDF from cottonseeds is more similar to forage NDF at simulating chewing than the fiber from other grain by-products. Another approach that can be used to monitor the interaction between dietary forage NDF and NFC is by keeping their ratio between 0.45 and 0.50. However, this index does not take into account either the particle size of the forage or the source and processing of the grain. The extent (**Figure 1**) and rate of ruminal digestibility of starch vary by cereal grain. Thus, the source of cereal grain and the processing that occurs can affect ruminal pH. The amount of cereal grain that can be safely fed depends on the source of the grain, processing of the grain, and level of effective fiber in the diet. For example, wheat is more prone to decrease chewing than high-moisture ground corn, even though the ruminal starch digestibility may be higher with the corn. Wheat should not be fed as the sole source of cereal grain in a diet for dairy cattle and should be limited to about 25% of DM in a high-forage diet and 15% of DM in a low-forage diet. Corn, barley, and sorghum can be fed as the sole sources of grain in a ration, but the maximum inclusion will depend on the dietary concentration of forage NDF and the nature of the grain processing.

Among cereal grains, digestibility of starch is 4 times more variable in the rumen than in the total tract. In other words, the amount, source, and processing of grain are more likely to have a greater impact on the site of digestion (ruminal vs. postruminal) than on digestibility in the total tract. Thus, the primary strategies should be to feed adequate grain to supply energy to the high-producing cow and to process the grain to increase its ruminal digestibility to the extent that ruminal fermentation will not be impaired. At this threshold point, grain will need to be decreased in the diet or the grain should be less rigorously processed when at the same dietary inclusion rate, which should result in more starch digested postruminally.

The cereal grain fed and the amount of processing can affect microbial protein synthesis. Dry, rolled barley resulted in more microbial N flowing to the duodenum than mechanically processed corn or sorghum. However, steam flaking of the corn and sorghum resulted in similar flows of microbial N as when feeding dry, rolled barley. Increasing the ruminal digestibility of starch would be expected to increase microbial N flow to the duodenum as long as DM intake is not affected. When ruminal starch digestibility is increased, rumen degradable protein may need to be increased in the diet.

Besides the availability within a market area and the utilization aspects discussed above, usage of cereal grains in diets for dairy cattle is also affected by their cost. Cost comparisons can be made within given geographical areas using local commodity prices and typical concentrations of DE (**Table 2**) to calculate cost per megacalorie of DE. Given typical prices, value for human food (**Table 1**), and the rapid rate of starch digestion in the rumen, very little wheat is fed to lactating dairy cows. Based on agronomic production, ease of distribution, price, and digestibility, corn is the dominant cereal grain in diets for dairy cattle.

Conclusions

Corn, barley, sorghum, wheat, and oats are the most common cereal grains fed to livestock. These cereal grains primarily provide energy to animals by their concentration of starch. Although the starch in the typical hybrids of these cereal grains contains about 25% amylose and 75% amylopectin, the site and extent of their digestion vary because of the differences in the structural nature of the endosperm. Reduction of particle size and processing with heat and moisture increase the digestibility of the cereal grains in the rumen and total tract, thus resulting in increased energy availability. The amount of cereal grains used in diets should depend on cost, production level of animals, dietary concentration of forage NDF, and the nature of the grain processing. Generally speaking, the ratio of the percentage of forage NDF and NFC in diets for lactating dairy cows should be between 0.45 and 0.50. Adequate forage NDF is needed to stimulate ruminal fermentation, a minimum amount of NFC is needed to support microbial protein synthesis, and maximum NFC is necessary to reduce the risk of ruminal acidosis. Because of the agronomic conditions for their production, ease of distribution, and the comparative low costs of energy provided by grains, the cereal grains and their corresponding by-products will remain as staple ingredients in diets for dairy cattle.

See also: **Feed Ingredients**: Feed Concentrates: Co-Product Feeds; Feed Concentrates: Oilseed and Oilseed Meals. **Feeds, Prediction of Energy and Proteins**: Feed Energy; Feed Proteins. **Feeds, Ration Formulation**: Dry Period Rations in Cattle; Lactation Rations for Dairy Cattle on Dry Lot Systems; Models in Nutritional Research; Systems Describing Nutritional Requirements of Dairy Cows.

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Feed Concentrates: Co-Product Feeds

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Introduction

Co-product feeds are generated from the production of food, fiber, and bioenergy products for human consumption. They include plant feedstuffs such as hulls, stalks, peels, and oil seed meals, and animal co-products such as blood meal, fats, bone meal, or processed organ meats. Some feed co-products are a result of the production of food that may not be of the quality or composition appropriate for human use; however, they provide economical feeds for dairy cattle and other ruminants. The composition and quality of co-product feeds have the potential to vary greatly, depending upon the specific starting product and particular process. To optimize their use, special attention must be paid to monitoring nutrient composition and absence of factors that could impair animal performance or affect human health. The use of co-product feeds has been and will continue to be important for the efficient use of our natural resources in food production.

Benefits of Using Co-products

Co-product feeds are often utilized for livestock because their composition (high fiber) and quality (taste, texture, contaminants) are such that they are not considered suitable for human use. The feeding of co-product feeds to cattle represents one of the oldest forms of recycling; it reduces the waste mass that must be disposed of by human society by diverting these 'wastes' from landfills or land application and into the production of animal products (**Figure 1**). The use of these co-products as feeds also increases the economic return of producing the parent product by providing additional income from the sale of the co-product, rather than incurring additional costs for its disposal.

Use of co-products replaces more expensive feeds provided to food- and fiber-producing animals. This substitution for traditional feeds may be even more important than the efficiency gained directly from using these co-products. The more efficiently that cattle utilize these feeds, the less the amount of nutrients that have potential to pass into the environment. In properly formulated diets, co-products can provide excellent economical sources of protein, energy, and fiber to fill nutrient requirements.

General Classifications

Co-product feeds are generally classified as high-fiber, high-protein, and high-energy feeds. However, these general classifications do not provide needed information about composition and quality factors essential to proper formulation of rations. For instance, fat content of feeds may restrict the amount that is feasible to feed in order to avoid potential reductions in ruminal fiber digestibility and microbial yield. In another case, inclusion of feeds high in starch may be limited by restrictions placed on total dietary starch in order to avoid ruminal acidosis and digestive upset. Palatability issues including dustiness, fineness of particle size, and general acceptability to the animals are largely evaluated on the basis of experiences in a particular feeding system with the specific feeds.

Co-product Analyses

Compositional analyses for a number of co-product feeds are provided in **Table 1**. The reader should recognize that these values may vary appreciably among processors and regions. The 'sugars' referred to are mono- and oligosaccharides extractable by 80% ethanol and these sugars are typically rapidly fermented in the rumen. Sucrose, glucose, and fructose are the predominant sugars for most feeds, with the possible exception of leguminous seeds such as soybean meal, which contains stachyose and raffinose. Soluble fiber consists of nonstarch polysaccharides such as pectic substances, galactans, fructans, and noncellulosic beta-glucans that are not included in neutral detergent fiber (NDF). Soluble fiber is digestible only by microbes. All values are presented as a percentage of feed dry matter.

Fibrous Feeds

Cottonseed hulls. The seed coat of cottonseeds removed before oil extraction of the seed. They contain variable amounts of cellulosic lint. Although the hulls are highly lignified (~20%) and have low dry matter digestibility (~34%), they are very palatable and can increase dry matter intake, possibly through increasing the rate of digesta passage from the rumen.

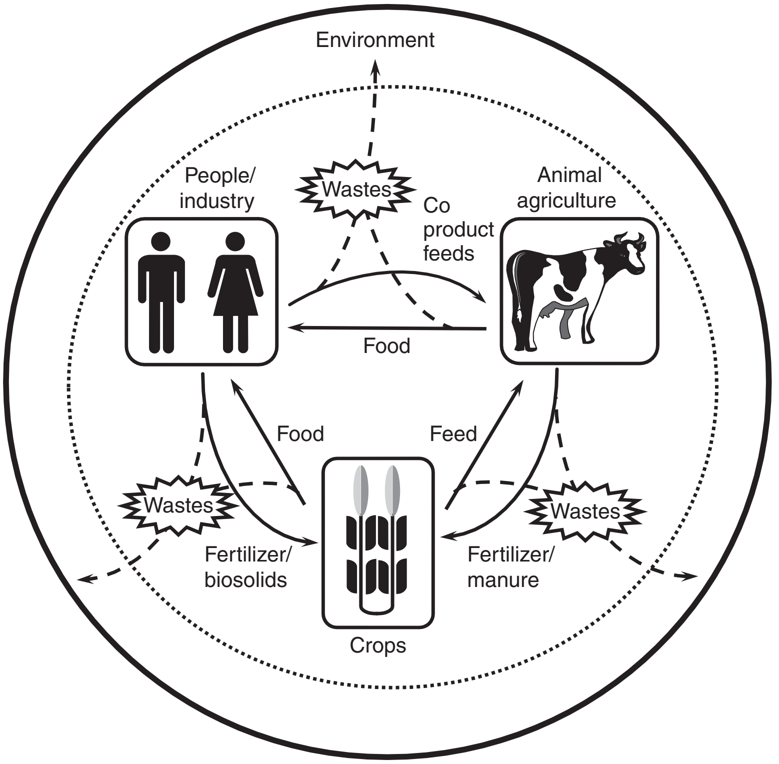

Figure 1 Relationships among human society and animal and crop agriculture. Efficiency in the use of products or wastes generated dictates the quantity of material that enters the environment. (Adapted with permission from Van Horn HH and Hall MB (1997) Agricultural and environmental issues in the management of cattle manure. In: *Agricultural Uses of By-Products and Wastes*, Rechcigl JE and MacKinnon HC (eds.) ACS Symposium Series 668. Copyright 1997 American Chemical Society.)

Rice hulls. The outer covering of rice grains. They are very high in ash and fiber, and low in crude protein (CP). They are not recommended as a desirable fiber source.

Soybean hulls. The seed coat of the soybean. Both the soluble fiber and NDF are extensively but slowly fermented in the rumen. They contain approximately 20% soluble fiber.

Sugarcane bagasse. The fibrous material remaining after extraction of sugars from sugarcane. Digestibility of its fiber can be increased through steam pressure or sodium hydroxide treatments.

Wheat bran. Consists primarily of the outer seed covering and some portion of the germ removed from wheat during the production of flour.

Wheat middlings. A co-product of the flour milling industry that contains varying portions of bran, aleurone layer, germ, and flour. It can contain significant quantities of starch (~20%).

Energy Feeds

Almond hulls. The hulls and associated twigs and discarded nuts from the production of almonds. Almond hulls tend to contain substantial quantities of sugars (~30%) and soluble fiber (~17%).

Animal fats. These are extracted/rendered tissue fats obtained from animals and include tallow (from ruminants) and lard (from swine). Animal fats tend to be high in glyceride esters of predominantly saturated fatty acids. Inclusion of these fats into ruminant diets may be limited to approximately 3% of diet dry matter, depending upon palatability and inclusion of other fat sources within the ration.

Apple pomace. A co-product of apple juice industry. It contains peels, cores, and flesh remaining after pressing apples for juice or other products.

Bakery waste. A highly variable product that may contain dough, stale product, and the entire range of types of baked products (bread, muffins, cookies, cakes, etc.). Bakery waste tends to be high in fat and starch content, which may limit amounts that may be included in rations. Much of the starch is likely to be rapidly fermented due to gelatinization during cooking.

Citrus pulp, dried. Peel, internal membranes, ruptured juice vesicles, and seed remaining after squeezing citrus fruits for juice. A calcium alkali added to the pulp to allow removal of water for effective drying also increases the calcium content of the pulp. Composition can vary with season, fruit type, and amounts of citrus molasses and calcium base that are added to the pulp. It is a source of sugars (~20%), soluble fiber (pectic substances ~35%),

Table 1 Composition and characteristics of selected co-product feedstuffs

Feed	*DM*	*Ash*	*CP*	*NDF*	*Fat*	*Ca*	*P*	*SQ*
Almond hulls	90	6.5	5.7	34	2.4	0.27	0.12	Su, SF
Apple pomace, dehydrated	89	2.2	4.9	40	5.1	0.13	0.11	
Bakery waste	92	4.4	10.7	18	12.7	0.14	0.26	Su, St
Blood meal	90	2.5	95.5		1.2	0.30	0.30	BP
Brewers' grains								
Dry	92	4.8	25.4	46	6.5	0.20	0.60	BP
Wet	21	4.8	25.4	42	6.5	0.20	0.60	BP
Citrus products								
Pulp, dried	91	6.2	6.8	22	3.7	1.84	0.12	Su, SF
Molasses	68	7.9	8.2		0.3	1.72	0.13	
Corn products								
Distillers grain and solubles, dried	92	4.8	25.0	44	13	0.15	0.71	BP
Gluten feed	90	7.0	24	40	3.4	0.03	0.84	St
Gluten meal	90	1.8	67.2	14	2.4	0.08	0.54	
Hominy feed	90	3.0	11.5	22	7.0	0.05	0.57	St
Cottonseed products								
Whole, linted	92	4.8	23.0	45	22	0.21	0.64	
Whole, no lint	90	4.5	25.0	37	23.8	0.12	0.54	
Hulls	91	2.8	4.1	90	1.7	0.15	0.09	
Solvent-extracted meal	91	6.7	48.9	28	1.7	0.17	1.00	BP
Feather meal, hydrolyzed	90	3.5	85.8		7.2	1.19	0.68	BP
Fish meal, menhaden	92	20.8	66.7		10.5	5.65	3.16	BP
Meat and bone meal	94	30.4	54.2		10.4	10.6	4.73	BP
Molasses								
Beet	78	11.3	8.5		0.2	0.17	0.03	Su
Cane	75	13.1	5.8		0.1	1.00	0.11	Su
Poultry litter	80	25	25	34		2.3	1.6	NPN
Rapeseed meal, solvent extracted	91	7.5	40	27	4.5	0.67	1.04	
Rice products								
Bran	91	12.8	14.1	33	15.1	0.08	1.70	
Hulls	92	20.6	3.3	82	0.8	0.10	0.08	
Soybean								
44% meal	89	7.3	49.9	15	1.0	0.29	0.68	Su, SF
48% meal	90	6.5	55.1	8	1.5	0.30	0.68	Su, SF
Hulls	91	5.1	12	65	2.1	0.49	0.21	SF
Sugar beet pulp, dried	92	6.1	10.1	44	0.6	0.60	0.10	Su, SF
Sugarcane bagasse	91	5.5	1.5	49	0.4	0.51	0.29	
Wheat products								
Bran	89	6.9	17.1	51	4.4	0.13	1.38	
Middlings	89	5.2	18.4	37	4.9	0.13	0.99	St

CP, crude protein; DM, dry matter; NDF, neutral detergent fiber; SQ, special qualities.
Nonprotein nitrogen (NPN), starch (St), sugars (Su), rumen bypass protein (BP), soluble protein (SP), and soluble fiber (SF) are found in appreciable quantities in some co-product feedstuffs.

and very digestible NDF, which all tend to ferment rapidly.

Corn hominy feed. A variable co-product of the production of hominy grits, corn table meal, or flour. It contains variable proportions of corn bran, germ, and starch (~55 to 65%). The germ may have some portion of the fat extracted. The final product is often very finely ground. Inclusion in rations may be limited based upon permissible levels of dietary starch.

Cottonseed, whole. The seed remaining after ginning to remove the long cotton fibers. It consists of kernel, which contains the majority of protein and fat, and hull, which consists of the lignified seed coat and the attached cellulosic lint. Linted cottonseed does not have to be cracked prior to feeding. However, delinted cottonseed may be better utilized if cracked before feeding. Limits on fat levels in the diet (e.g., 3% of diet dry matter as added fat that is not inert in the rumen) may restrict the amount that can be fed.

Molasses. Generally, a liquid co-product high in sugar content. It is made from sugarcane, sugar beets, or citrus, and is often used for reducing dustiness or enhancing palatability. Citrus molasses may have a bitter flavor. Sugar content of molasses is expressed as 'invert sugars', which is the amount of monosaccharides present after hydrolysis of sucrose by invertase (sucrase).

Rice bran. Consists of the seed coat and germ removed during the polishing of rice.

Sugar beet pulp. Pulp remaining after roots are pressed to remove sugar. It is a source of soluble fiber (~30%) and rapidly fermentable NDF. Sugar content will likely vary depending upon the efficacy of the extraction process and addition of sugar beet molasses back to the pulp.

Protein Feeds

Animal Protein Sources

Blood meal. A dried feed produced from blood. It is a source of rumen escape or bypass protein, and is low in calcium and phosphorus.

Feather meal, hydrolyzed. Consists of feathers that have been subject to steam and pressure treatments to increase digestibility.

Fish meal. Produced from fish and fish co-products that have been dried and ground. Composition and degradability of the protein may vary greatly with source. Palatability problems may be encountered with high inclusion rates. The unsaturated fats present in fish meal have been associated with decreases in ruminal fiber digestion.

Meat and bone meal. A dry, ground, rendered product containing both bone and animal tissues, not including hair, hooves, hide, and horn. Meat and bone meal is typically identified by the class of animal (poultry, swine, ruminant) from which it was produced. Feeding of ruminant-derived meat and bone meal to ruminants is widely prohibited due to concerns about the transmission of bovine spongiform encephalopathy.

Poultry manure/litter. Poultry waste with bedding litter. It is typically fed to nonlactating animals, and it can be high in nonprotein nitrogen. The composition can be quite variable, depending upon the proportion of manure to litter and the type of litter material used. Proper composting or other treatment of the material is necessary to control microbiological contamination or toxins.

Milling Co-products

Dry milling and wet milling of cereal grains are used to produce ethanol and to partition the grains into an array of feed components (**Figure 2**). Wet milling is a more complex process and requires high-quality grains to produce high-value products suitable for human use. Some of the co-products, such as maize gluten meal, may be marketed in higher value markets such as the pet food or poultry industry.

Maize gluten feed. Produced from the wet milling process of maize, and sold as a wet or dried product. It contains varying amounts of steep liquor, which increases its content of lactic acid. This feed usually consists of corn bran and steep, with germ meal added if the processing plant possesses the capabilities. It contains 16–25% CP, with a rumen-undegradable protein (RUP) value of approximately 24–30% CP.

Maize gluten meal. Produced from the wet milling of corn to remove starch and the corn germ. Maize gluten meal contains more protein and less of the corn bran than does maize gluten feed.

Distillers grains and solubles, dried. Co-products from the production of ethanol for distilled beverages or for fuel through the dry milling process (**Figure 2**). Maize or possibly another starch source such as sorghum or wheat is ground, fermented, and the remaining sugars are converted to ethanol and CO_2. Approximately one-third of the dry matter remains as the feed product following starch fermentation. As a result, all the nutrients are concentrated threefold because most grains contain approximately two-thirds

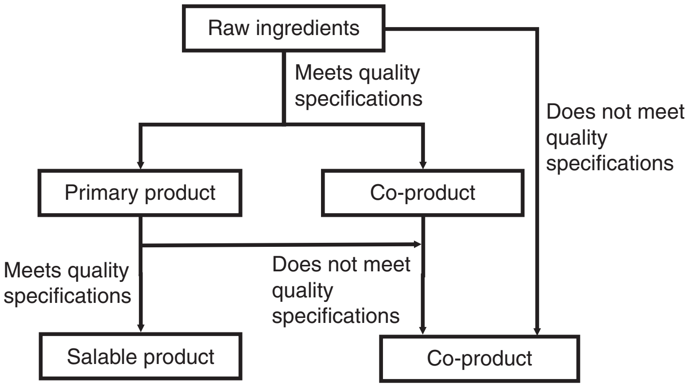
Raw ingredients
Meets quality specifications
Does not meet quality specifications
Primary product
Co-product
Meets quality specifications
Does not meet quality specifications
Salable product
Co-product

Figure 2 Production of co-product feeds.

starch. In this process, distillers grains and distillers solubles are produced and may be sold individually or combined and sold as feed co-products. These co-products may be combined and sold in a high-moisture form or dried and sold as dry distillers grains and solubles (DDGS).

Other Plant Protein Sources

Cottonseed meal. A co-product from the production of cottonseed oil. The dehulled seed is pressed and solvent extracted to remove oil. Varying amounts of ground hulls are added to the extracted seeds to modify the protein content. Cottonseed meal contains gossypol, a toxin that affects nonruminants to a greater extent than ruminants. Accordingly, the feeding of cottonseed meal to calves should be limited.

Maltage/brewers' grains. Co-products from the production of beer. The type of grain used in the brewing process varies. The feed is often fed as a high-moisture feed, and hence it is prone to 'souring', which necessitates its feeding in a relatively short period (3–7 days). The time frame for feeding decreases with elevated ambient temperatures. The high-moisture product may be ensiled.

Rapeseed/canola meal. Produced from oil extraction of rapeseed or canola. Compared to rapeseed meal, canola meal contains lower levels of glucosinolates, which may be toxic to livestock.

Soybean meal. Produced from oil extraction of soybeans. Meals with lower protein contents contain greater quantities of soybean hulls. The non-NDF carbohydrates consist of sugars (mono- and oligosaccharides) and soluble fiber, which account for approximately 11 and 14% of the dry matter, respectively, in 49% CP meals.

Quality and Variability of Co-product Feeds

The quality and composition of many co-product feeds tend to be variable (**Table 2**) and different from the raw material that entered the process. With the possible exception of the soybean meals, co-products are not the main salable materials from a process, and their composition often is not tightly monitored. Co-product feed composition will change depending on the proportions of the co-product, and of raw ingredients and primary products that did not meet specifications for quality (**Figure 3**). Excessively heat-dried or improperly stored co-products may show heat damage, as evidenced by increased contents of acid detergent-insoluble nitrogen or charring. Acid detergent-insoluble nitrogen is used as an indicator of 'bound' CP that is unavailable for digestion. Knowledge of the raw ingredients, the processing

Table 2 Variation in the composition of co-product feeds (percentage of sample dry matter)

Feed	*CP*	*Ash*	*NDF*	*Crude fat*
Whole cottonseed (number of samples = 15)				
Mean	22.5	4.14	53.2	16.7
Standard deviation	1.44	0.49	3.28	1.69
Minimum	19.9	3.63	46.7	14.5
Maximum	24.7	5.35	58.4	21.2
Corn hominy feed (number of samples = 16)				
Mean	11.8	3.49	22.2	3.95
Standard deviation	1.76	1.37	3.06	1.46
Minimum	9.1	1.58	14.7	1.90
Maximum	13.7	6.04	26.7	6.10
48% soybean meal (number of samples = 17)				
Mean	53.9	7.05	6.71	1.68
Standard deviation	1.26	0.38	1.86	0.37
Minimum	51.6	6.45	4.20	1.10
Maximum	55.9	8.09	9.40	2.70
Dried citrus pulp (number of samples = 79)				
Mean	7.20	6.77	22.1	26.5
Standard deviation	0.90	0.91	2.24	5.21
Minimum	4.14	4.37	17.8	12.5
Maximum	9.42	8.68	29.4	40.2

CP, crude protein; NDF, neutral detergent fiber.
Whole cottonseed, corn hominy feed, and soybean meal were sampled over a 4-month period from loads of feed delivered to a single dairy farm. Citrus samples were collected from five processing plants in Florida over an 8-month period.

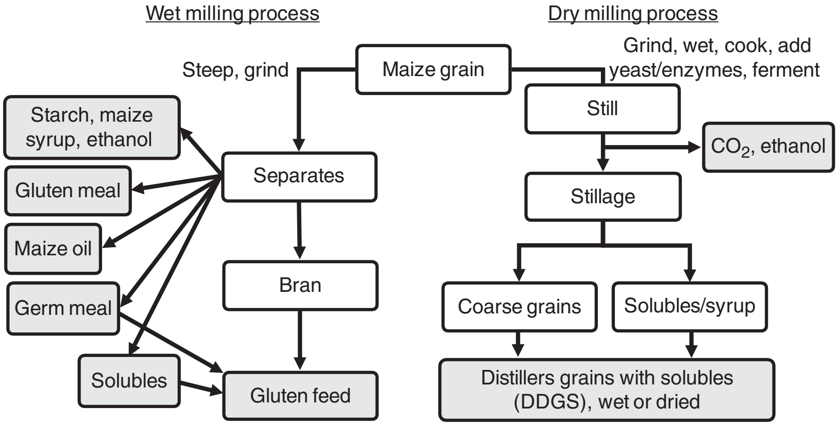

Figure 3 Outline of wet and dry milling processes for grains.

methods, and subsequent storage conditions can offer useful information on a co-products' likely composition and final quality.

Digestibility and nutritional quality of feed fractions affect their value in rations. Although crude fat or ether extract has been most commonly used to describe fat content of feeds, this value includes cutins and other indigestible ether-soluble materials. Fatty acid content and profile more accurately describe nutritional value to the animal. Digestibility of both fiber and starch can differ substantially among feedstuffs. Laboratory assays for digestibility can provide relative comparisons among feeds, but the values achieved in cattle will be affected by rate of passage, other dietary components, and ruminal conditions.

When appraising co-product feeds for inclusion in rations, consideration must be given to the amount of product available, variability in nutrient content, feasibility for setting criteria on composition and quality to define the acceptable variation, and other factors that may alter composition, including on-farm handling and storage. Acceptable variation in the feed will depend in part upon price and level of inclusion; more variation can be acceptable in an inexpensive feed that forms a small portion ($\leq 5\%$) of the ration, than in a higher priced commodity or one that makes up a substantial portion of the diet. High-moisture feeds may have a specific 'shelf life' that requires them to be fed in a time frame that limits the extent of molding or 'souring' of the feed. Additionally, high-moisture feeds such as wet brewers' grains (22–35% dry matter) may undergo large changes in moisture content during on-farm storage due to loss of water. In some cases, storing of wet feeds in plastic bags may be feasible.

The potential for factors that negatively impact animal performance or human health must be considered, particularly relative to the amount that will be fed and the class of animals (young calves, older heifers, lactating cows, dry cows, bulls) that will receive it. Tannins (coffee hulls, peanut skins), mycotoxins (any moldy feed; may be a particular problem with cotton, maize, and peanut products, and inadequately dried or improperly stored materials), naturally occurring toxins (e.g., gossypol in cottonseed products), microbiological contamination (improperly processed poultry litter), pesticide residues (cotton gin trash), and several other contaminants may make certain co-products unsafe or undesirable to feed if they have not been properly processed and handled. Careful monitoring of the feed, knowledge of the supplier, and understanding of the normal composition of the feeds are essential elements for assuring a safe and nutritionally effective feed supply.

See also: **Additives in Dairy Foods:** Safety. **Feed Ingredients**: Feed Supplements: Anionic Salts; Feed Supplements: Fats and Protected Fats; Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Organic-Chelated Minerals; Feed Supplements: Ruminally Protected Amino Acids; Feed Supplements: Vitamins. **Nutrition and Health**: Diabetes Mellitus and Consumption of Milk and Dairy Products; Effects of Processing on Protein Quality of Milk and Milk Products; Galactosaemia; Milk Allergy; Nutraceuticals from Milk; Nutrigenomics and Nutrigenetics; Nutritional and Health-Promoting Properties of Dairy Products: Bone Health; Nutritional and Oral Health-Promoting Properties of Dairy Products: Caries Prevention and Oral Health; Nutritional and Health-Promoting Properties of Dairy Products: Colon Cancer Prevention; Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake; Nutritional and Health-Promoting Properties of Dairy Products: Fatty Acids of Milk and Cardiovascular Disease.

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Feed Concentrates: Oilseed and Oilseed Meals

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Glossary

Canola A variety of rapeseed that contains low concentrations of eruic acid and glucinolates, which have been shown to reduce animal performance. Produced primarily for oil, considered very healthy, but can be fed to dairy cattle as well.

Canola meal Protein supplement produced as a by-product of crushing canola seed to extract oil. Contains moderate concentrations of protein and low concentrations of eruic acid and glucinolates.

Cottonseed Oilseed separated from cotton lint during ginning. Contains moderate concentrations of oil and protein and high concentration of fiber from lint remaining on hull.

Cottonseed meal Protein supplement with high fiber concentrations that is a by-product from crushing cottonseed to extract oil.

Extrusion Process used to reduce degradability of protein in a feed. Involves forcing feed through a die using pressure. Heat generated during the process coupled with pressure alters protein structure and susceptibility to degradation by ruminal microorganisms.

Gossypol A polyphenolic compound concentrated in the pigment glands of cottonseed. Toxic to animals without a functioning rumen, or when cottonseed and cottonseed meal are fed in large quantities.

Peanut meal Protein supplement produced as a by-product of crushing peanuts for oil. Limited amounts are available for feeding dairy cattle as it is primarily used for human consumption.

Roasting Process used to reduce degradability of protein in a feed. The process consists of exposing a feed to heat using a direct or indirect heat source for a fixed time. After heating, the feed is typically steeped for an additional time to achieve the desired reduction in protein degradability.

Soybean meal Protein supplement produced as a by-product of crushing soybeans for oil. The primary oilseed meal used in diets for all animals and serves as the standard against which all other protein supplements are compared.

Soybeans Oilseed containing moderate concentrations of oil and protein. The by-product produced from the extraction of soybean oil is used in diets for animals. Protein concentration is standardized through the addition of hulls.

Sunflower meal Protein supplement produced as a by-product of crushing sunflower seeds for oil.

Sunflower seed Oilseed containing high concentrations of oil, which is primarily linoleic acid (C18:2). Grown primarily for extracting oil for human consumption, it can also be fed to dairy cattle.

Introduction

Whole oilseeds contain high concentrations of energy and moderate concentrations of protein and fiber (**Table 1**). Because of this, oilseeds are frequently used as an energy source in the diets of lactating dairy cows. Processing whole oilseeds alters the site and extent of nutrient digestion and the production response when fed to lactating dairy cows. Meals from oilseeds are used as protein supplements for all classes of dairy cattle. The type of processing used to extract the oil from the oilseed influences the resulting nutrient content of the meal (**Table 1**). Because the protein from whole oilseeds and oilseed meals is highly degraded by the ruminal microorganisms, it is desirable to treat these ingredients to reduce their degradability before feeding in certain situations.

Oilseeds

Whole cottonseed (WCS), soybeans (SB), and sunflower seed (SF) are three oilseeds that are frequently included in dairy rations. Because of the value of the oil for human consumption, SF is not used as commonly in dairy rations as SB and WCS. Whole SB contain more protein but less fiber than the other oilseeds. The protein in SB has the most favorable balance of digestible amino acids (AA), but without additional heat treatment the protein in all oilseeds is highly degraded by the ruminal microorganisms. Oil concentrations are higher in SF than in WCS or SB, which limits the total amount that can be fed. The oil in whole oilseed is released slowly and is biohydrogenated in the rumen without negatively affecting fiber digestion when added at rates to provide no more than 2–3%

Table 1 Chemical composition of oilseeds and oilseed meals (DM basis)

Feed stuffs	*DM*	*CP*	*RUP*	*EE*	*NDF*	*ADF*
Canola seed	89.9	20.5	21.3	40.5	17.8	11.6
Canola meal, mechanical	90.3	37.8	35.7	5.4	29.8	20.5
Cottonseed, fuzzy	91.6	22.5	22.9	17.8	47.2	38.8
Cottonseed, delinted	90.0	25.0	22.9	23.8	37.0	26.0
Cottonseed meal						
Expander solvent	90.6	48.4	47.9	1.2	26.0	16.7
Mechanical	93.9	45.8	ND	5.8	28.0	17.1
Expander-expeller	89.7	31.7	ND	8.6	ND	34.2
Peanut meal, solvent	92.3	51.8	13.2	1.4	21.4	13.5
Soybeans, whole	90.0	39.2	30.4	19.2	19.5	13.1
Soybeans, roasted	91.0	43.0	39.4	19.0	22.1	14.7
Soybean meal						
Expeller, 45%	89.6	46.3	69.0	8.1	21.7	10.4
Nonenzymatically browned	89.0	50.0	79.4	2.3	29.7	9.5
Solvent, 44%	89.1	49.9	34.6	1.6	14.9	10.0
Solvent, 48%	89.5	53.8	42.6	1.1	9.8	6.2
Sunflower	91.8	19.2	11.2	41.9	24.0	16.7
Sunflower meal, solvent	92.2	28.4	15.9	1.4	40.3	30.0

From NRC (1989) *Nutrient Requirements of Dairy Cattle*. Washington, DC: National Academy Press; NRC (2001) *Nutrient Requirements of Dairy Cattle*. Washington, DC: National Academy Press; Calhoun M, *et al.* (1995) *Proceedings of the Second National Alternative Feeds Symposium*. Columbia, MO: University of Missouri; Noftsger SM, Hopkins BA, Diaz DE, Brownie C, and Whitlow LW (2000) Effect of whole and expanded-expelled cottonseed on milk yield and blood gossypol. *Journal of Dairy Science* 83: 2539–2547. ADF, acid detergent fiber; CP, crude protein; DM, dry matter; EE, ether extract; ND, data not available; NDF, neutral detergent fiber; RUP, ruminally undegraded protein.

added fat. However, if oilseeds are cracked or ground, the oil may reduce fiber digestion unless the amount fed is limited to no more than 1.5–2.0% added fat.

Cottonseed

WCS provides a unique blend of digestible energy, protein, and fiber compared with other feed ingredients. The high concentration of energy from oil and fiber from the lint and hull in WCS are especially useful when formulating rations for high-producing dairy cows. With the introduction of new varieties that have greater yield of lint, the average seed size and concentrations of oil and protein in WCS have decreased slightly. The energy and protein content of mechanically or naturally delinted WCS is approximately 10% higher due to the removal of lint from the hull, which represents approximately 10% of the weight of the seed. The fiber in WCS has been shown to be an effective source of fiber for maintaining rumen function. The physical effectiveness of WCS neutral detergent fiber (NDF) is considered to be 50% of long-cut and 127% of short-cut alfalfa silage NDF.

WCS is limited to no more than 15% of the ration dry matter (DM) in most circumstances. In an analysis of 18 research trials in which WCS was included at rates up to 25% of the diet DM, no differences in DM intake were noted, but milk fat percentage increased in most trials and milk protein percentage decreased in approximately half of the studies. Milk yield typically remains the same when WCS is fed, but the yield of fat-corrected milk may increase because of the increased milk fat yield. Milk protein percentage may be slightly lower when WCS is fed, which is most likely related to protein quality and lower amounts of fermentable carbohydrate to support microbial protein synthesis. Supplemental niacin has prevented the reduction in milk protein percentage in some cases when oilseeds were fed but had no effect in other trials. The performance of lactating dairy cows fed delinted WCS (natural or acid delinted) is similar to that of cows fed fuzzy WCS, but 11–15% of delinted WCS consumed passes through the digestive tract undigested compared with less than 1–5% for fuzzy WCS. Grinding or cracking delinted WCS reduces the passage of undigested delinted cottonseed and improves nutrient digestion. When ground or cracked WCS is fed fiber digestibility may be reduced due to the negative effects of the free oil on microbial populations in the rumen and physical coating of fiber when large amounts of WCS are fed and dietary fiber levels are marginal.

Different processing methods have been applied to WCS to improve handling characteristics or ruminal-undegradable protein concentrations. Mechanical delinting, pelleting, extrusion, and coating processes are used to improve the handling characteristics of WCS. Ruminal protein degradation is reduced by pelleting, extruding, or roasting WCS. Mechanically delinted WCS has approximately 3% lint remaining on the seed hull, which is presumed to be adequate to prevent passage of whole seed through the digestive tract. Nutrient digestibility and performance of lactating cows fed mechanically delinted WCS are similar to those of cows fed fuzzy WCS. Roasting WCS has been shown to reduce ruminal N solubility from 79 to 37% of total N; however, fiber digestion is reduced slightly because of increased formation of Malliard reaction compounds. The optimum conditions for roasting WCS involve roasting at 146 °C followed by steeping for 30 min before cooling. Lactating dairy cows fed WCS roasted according to these guidelines show an increase in milk yield of approximately 0.9 kg day^{-1}. Extruding WCS reduces N solubility and improves the flow of AA to the small intestine. One study reported 17% greater flow of AA to the small intestine and 25% greater disappearance of AA from the small intestine for extruded WCS compared with unprocessed WCS. Milk yield, and concentrations of protein and conjugated linoleic acid are greater for cows fed extruded WCS compared to those from diets without oilseed.

Coating WCS with gelatinized corn starch improves handling characteristics significantly. Lactating dairy cows fed WCS coated with 2.5%, by weight, gelatinized corn starch produce similar amounts of milk containing similar percentages of fat and protein as cows fed uncoated WCS. Feeding WCS coated with higher concentrations of gelatinized corn starch, or a combination of gelatinized corn starch and maltodextrin sugar reduced fiber digestion and depressed milk fat percentage. The reduction in fiber digestion appears to be because of a reduction in the activity of cellulolytic microorganisms in the presence of the gelatinized corn starch. A recent *in vitro* trial using WCS coated with different combinations of gelatinized corn starch and feed grade urea suggests that including urea in the coating with gelatinized corn starch at a 1:10 ratio of urea to corn starch may prevent the negative effects on fiber digestion and maintain fiber digestion.

Cottonseed and cottonseed meal (CSM) contain gossypol, a polyphenolic compound that is concentrated in the pigment glands of the seed. Gossypol is produced by the cotton plant as a natural defense mechanism against insect pests, but may be toxic when consumed by animals. Gossypol is detoxified by the microorganisms in the rumen presumably by binding the ε-amino group of lysine, but toxicity can occur in dairy cows fed large amounts of gossypol for extended periods. Total plasma gossypol concentrations below 4 μg ml^{-1} are considered safe for dairy cattle. Gossypol disrupts spermatogenesis, reducing fertility in the male. Recent research indicates that high levels of gossypol (greater than 35 mg free gossypol per kg body weight) reduce embryonic fertility, but lower concentrations of gossypol do not appear to negatively affect embryonic fertility. For these reasons, cottonseed and cotton by-products are not fed or are fed in limited quantities to young calves until their rumen is fully functional or to bulls because of the negative impact on spermatogenesis.

Total and free gossypol concentrations are typically reported for WCS and CSM. Free gossypol is considered to be toxic, but free gossypol intake is not always correlated with toxicity. Gossypol exists naturally as a mixture of two stereoisomers, (+)- and (−)-gossypol. The negative isomer has the greatest biological activity and is responsible for many of the toxic effects. Pima cottonseed, a naturally delinted cottonseed, has higher concentrations of gossypol and a greater proportion of the negative isomer than upland or short staple varieties. Grinding, cracking, or roasting WCS increases gossypol availability, resulting in plasma concentrations that are 30–40% higher than those of cows fed unprocessed WCS. Roasting WCS appears to increase gossypol availability based on plasma concentrations, but the heat used to dry the gelatinized corn starch coating on WCS does not alter gossypol concentrations or availability. The effects of extruding or pelleting WCS on gossypol content and form have not been examined.

Soybeans

Whole raw SB is used as a source of energy and protein for lactation cows; however, SB contains urease, which hydrolyzes urea to ammonia, and trypsin inhibitors, which reduce protein digestion in the small intestine. Including raw SB in diets containing urea is not recommended, especially if the SB has been ground or cracked. Raw SB also contains lipase, lipoxidase, and peroxidase enzymes that alter fatty acid quality over time. Proper heat treatment inactivates these enzymes improving nutrient utilization and flow of AA to the small intestine. Raw SB should not be fed to young calves and should be limited to 10% or less of the ration DM in diets fed to lactating cows to reduce the potential negative effects of the antinutritional enzymes.

When whole SB is fed, 8–12% of the SB passes through the digestive tract without being digested. Grinding or rolling SB reduces the proportion of whole SB passing through the digestive tract undigested and increases DM digestibility and ruminal protein degradability. Ruminal fiber digestibility declines when ground or rolled SB is fed because the polyunsaturated fatty acids are toxic to the ruminal microbes and the fat may physically coat the dietary fiber preventing the microbes from attaching to fiber. Rolling SB to half and quarter sizes does not alter ruminal fiber digestion and results in higher milk yield than that achieved with SB rolled to quarter or smaller sizes or ground. Grinding, cracking, or rolling

SB increases the rate of fatty acid oxidation; so processed SB should be fed quickly.

Roasting and extruding processes are used to reduce ruminal protein degradability of SB and inactivate enzymes that interfere with digestion. The milk yield of lactating dairy cows fed diets containing either roasted or extruded SB has been shown to differ that from when compared to feeding raw SB. Some trials have not demonstrated any difference in production, whereas others show a positive response to feeding roasted or extruded SB. The reason for the lack of production response to roasted or extruded SB is often due to ineffective heat treatment. To optimize ruminal-undegradable protein content and lysine availability, SB should achieve an exit temperature from the roaster of approximately 146 °C before steeping for 30 min. The effectiveness of heat treatment from roasting can be measured using the protein dispersibility index (PDI). The PDI of optimally roasted SB is 9–11%, whereas the PDI of marginally roasted SB is 11–14% and the PDI of underheated SB is greater than 14%. Estimated ruminal-undegradable protein content of optimally roasted and steeped SB is approximately 60% compared to 27% for raw SB. Extruded SB should exit the extruder at 132–149 °C and will have a ruminal-undegradable protein content similar to that of roasted SB. Roasted SB may be fed at up to 18% of the dietary DM, but extruded SB should be limited to less than 15% of the dietary DM because of the free oil that may interfere with ruminal fiber digestion.

Based on a review of 26 trials, milk yield was on average 1.5 kg day^{-1} greater for cows fed roasted SB and 1.3 kg day^{-1} greater for cows fed extruded SB compared with milk yield for cows fed diets supplemented with soybean meal (SBM) or raw SB. The heat treatment applied to SB in these trials was not always optimal. Milk fat percentage was 0.06% higher with roasted SB and 0.17% lower with extruded SB compared with SBM or raw SB. The decline in milk fat percentage observed with extruded SB is due to incomplete ruminal biohydration of fatty acids and altered ruminal fiber digestion in the presence of polyunsaturated fatty acids. Milk protein percentages were 0.07 and 0.06% lower for roasted and extruded SB, respectively, compared with SBM or raw SB. When optimally roasted SB was fed at 13% of the dietary DM, milk yield was 4.5 kg day^{-1} greater compared with feeding either SBM or raw SB, illustrating the importance of applying proper heat treatment and the production potential of optimally roasted SB.

Two additional methods of treating whole SB to reduce ruminal protein degradation are nonenzymatic browning and micronizing, which have been used on SBM. However, only limited information is available on the effects of these methods on nutritional value. A recent study reported similar DM intake, milk yield, and milk composition for cows fed cracked nonenzymatically browned SB or calcium salts of long-chain fatty acids. Compared with SB oil, feeding nonenzymatically browned SB does not negatively affect ruminal fiber digestion. Micronization uses infrared radiation to reduce ruminal protein degradation. In a recent report, raw SB processed at 115 °C and rolled prior to feeding supported similar intake, milk yield, and milk composition as extruded or roasted SB. These limited data suggest that these processes have the potential to adequately protect soy protein from ruminal protein degradation.

Sunflower Seed

SF contains higher concentrations of oil, which is primarily linoleic acid (C18:2). The amount of SF fed is limited to no more than 10% of the dietary DM. When fed at 10% or less of the dietary DM, SF will maintain normal milk yield, but milk fat percentage typically decreases. The decrease in milk fat percentage is caused by reduced synthesis of C12:0 and C14:0 by the mammary gland although concentrations of C18:0, C18:1, and C18:2, are higher which is consistent with incomplete biohydrogenation of the fatty acids by the ruminal microorganisms. Compared with extruded SB, rolled SF supports similar milk yield and tends to maintain normal milk protein percentage when both were included in diets to provide 3.7% supplemental fat.

High oleic acid (C18:1) varieties of SF (HOSF) that have reduced concentrations of linoleic acid (C18:2) and maintain milk fat percentage compared with regular SF have been developed. Milk fatty acid concentrations show a trend that is similar to that observed with regular SF but intermediate in degree when individual fatty acids are considered.

Oilseed Meals

Canola meal (CM), CSM, peanut meal (PM), SBM, and sunflower meal (SFM) are some of the more commonly used oilseed meals. The protein content of these meals is higher than that of the original oilseed. The type of processing used to extract the oil influences the concentration of oil remaining in the meal. In general, mechanical processing and expeller processing are less effective in removing oil, resulting in higher energy values for these meals compared with solvent extraction processes. The protein content of oilseed meals is typically standardized through the addition of hulls, and it may be less than the protein content of whole oilseed in some situations. The degree to which protein is degraded in the rumen varies according to the amount of heat applied to process the meal. Commercially other processes and treatments are used to reduce ruminal protein degradation of these meals and improve animal performance.

Canola Meal

Canola is a cultivar of rapeseed that has low concentrations of erucic acid and glucosinolates, which have been shown to reduce animal performance. CM is a by-product of crushing canola seed to extract oil and has low concentrations of these antinutritional compounds. Protein in CM is readily degraded (approximately 78.7%) by the ruminal microorganisms to a degree similar to that with SBM. When included in a balanced diet, CM supports the efficiency of microbial protein synthesis and flow of non-ammonia nitrogen to the omasum similar to SBM or CSM. When fed to lactating cows, CM supports similar levels of DM intake, milk yield, and composition as SBM or CSM although some trials have shown improved milk protein percentage for diets supplemented with CM compared with CSM, which is related to the more desirable AA content of CM. Treating CM with either heat or lignosulfonate improves DM intake and milk yield, and decreases ruminal protein degradation compared with CM. When fed to weaned dairy calves, CM supports similar intake and body weight gain as SBM or CSM.

Cottonseed Meal

The majority of CSM available in the United States is produced using the expander solvent extraction process. Limited quantities of mechanical and prepress solvent CSM are available, but these meals could become more common if increased amounts of cottonseed oil are used for biodiesel production. Protein concentrations of CSM vary with the type of processing and are lowest for prepress solvent-extracted CSM (41.6% of DM), intermediate for mechanically extracted CSM (45.8% of DM), and highest for direct (49.0% of DM) or expander solvent-extracted CSM (48.4% of DM). Ruminal protein undegradability is highest for mechanically processed CSM (52.4% of crude protein (CP)) and similar for prepress, direct, and expander solvent-extracted CSM (36–37% of CP). Ruminal protein degradability is lower for CSM than for SBM and may not support adequate ammonia concentrations to maintain ruminal organic matter digestibility in diets containing less than 13.5% CP. The AA quality of CSM is less than that of SBM because of lower concentrations of lysine (4.13 vs. 6.29% of CP, respectively). The detoxification process of gossypol involves binding of some of the lysine in CSM, thereby reducing available lysine further.

Dry matter intake, milk yield, and milk composition are similar for lactating cows fed either CSM or SBM when isocaloric and isonitrogenous diets are fed. Including a high-quality source of ruminal-undegradable protein in diets containing CSM to provide additional essential AA improves the efficiency of converting DM to milk, but does not improve milk yield or alter milk composition.

Partially delinted cottonseed processed through an expander-expeller contains more fat and less protein than CSM, but has a higher proportion of rumen-undegradable protein (approximately 55% of CP). Dry matter intake and milk yield of cows fed diets containing expander-expeller cottonseed were similar to those of cows fed WCS in one trial; however, multiparous cows had a greater yield of milk and milk components when fed expander-expeller cottonseed than when fed WCS in a second trial.

Peanut Meal

PM is a by-product of crushing peanuts to produce peanut oil. The protein in PM contains lower concentrations of several essential AA than proteins SBM in and is very degradable. Research is limited regarding the feeding value of PM to cattle, as most of the available meal is used for human consumption. The available research indicates that PM can be used to replace SBM or other oilseed meals in most diets; however, PM is susceptible to contamination with aflatoxin and should be tested before feeding. Aflatoxin is a naturally occurring toxin produced by *Aspergillus flavus* and occurs when peanuts are grown under stressful conditions such as a drought or when stored improperly after harvest. If PM is found to contain aflatoxin, it should not be fed to lactating cows as the aflatoxin will be absorbed and excreted in milk, nor should it be fed to young calves as they are more susceptible to aflatoxin. PM can also be contaminated with *Salmonella*, which could also pose health problems when fed to animals, especially young or animals under stress.

Soybean Meal

SBM is the most commonly used protein supplement in dairy rations and is the standard used for determining the value of other protein supplements. The most common forms of SBM are solvent extracted (44% CP, as-fed basis) and dehulled, solvent extracted (48% CP, as-fed basis). SBM is very palatable and contains large quantities of essential AA (approximately 45% of total AA), but a large proportion of the protein is degraded by the ruminal microorganisms. In terms of essential AA balance, methionine is more limiting in SBM than lysine.

SBM is commonly heat or chemically treated to reduce ruminal protein degradability and increase the flow of dietary AA to the small intestine. Commercial processes used to produce heat-treated SBM include cooker-expeller processing of SB, extruder-expeller processing of SB, nonenzymatically browning of dehulled, solvent-extracted SBM, and cooker processing of dehulled, solvent-extracted SBM. Controlled research studies are lacking for SBM produced by extruder-expeller or expeller-extracted processing of SB and cooker processing of dehulled, solvent-extracted SBM. Heat-treated SBM increases the total flow of essential AA to the small intestine and supports higher milk yields. Chemically treating SBM with sulfate liquor and xylase, sodium hydroxide, or formaldehyde increases the flow of dietary AA to the small intestine but does not increase the total flow of essential AA to the small intestine because of reduced microbial protein flow.

Production response to feeding treated SBM has resulted in similar or increased milk yield when compared with solvent-extracted SBM. Positive milk yield responses to treated SBM are more common when diets contain alfalfa silage as the primary forage compared with diets based on corn silage. Heat-treated SBM products usually support greater levels of milk yield compared with solvent-extracted SBM (35.9 vs. 34.7 kg day^{-1}, respectively). Cows fed nonenzymatically browned SBM showed an increase in milk yield compared with those fed solvent-extracted SBM. In another trial, milk yields were similar for cows fed either solvent-extracted SBM or nonenzymatically browned SBM fed at half the amount of solvent-extracted SBM in the control diet. Few trials have reported increased performance or efficiency when chemically treated SBM replaced solvent-extracted SBM. Replacing solvent-extracted SBM with cooker-expeller SBM increased milk yield 3% and efficiency of DM utilization 4%.

Sunflower Meal

SFM is a by-product of crushing SF for oil. The nutrient content of SFM varies with the type of processing used for extracting the oil. Solvent-extracted SFM has higher concentrations of protein and lower concentrations of oil and fiber compared with mechanically extracted SFM. Dehulled SFM has slightly higher protein concentrations and less fiber. The protein in SFM is more soluble than SBM protein (34 vs. 17% of N, respectively), but processes such as extrusion decrease solubility. Without processing (heat, enzymatic, or chemical) to reduce solubility, greater proportions of the dietary nitrogen provided by SFM are degraded by the ruminal microorganisms. Microbial protein synthesis is improved with SFM compared with blood meal or corn gluten meal so that total flow of nitrogen to the duodenum is similar. The lysine content of SFM is less than that provided by SBM, CSM, or CM, which could limit the potential milk yield response in very high-producing lactating cows.

Lactating dairy cows when fed diets supplemented with either SFM or SBM consumed similar amounts of DM and produced equal amounts of milk with similar concentrations of fat and protein. Body weight gain and DM intake are similar for replacement dairy heifers fed diets supplemented with either SBM or SFM. Because the mechanically extracted SFM has higher oil concentrations, the amount fed should be limited to no more than 20% of the total dietary DM to avoid any negative impact from the residual oil.

Conclusions

Oilseeds and oilseed meals may be used in dairy rations to provide nutrients in support of growth and milk synthesis. When using heat-treated oilseeds or oilseed meals, it is important to ensure that proper heat treatment is applied to provide optimal protection of protein from ruminal protein degradation and inactivation of naturally occurring enzymes that interfere with digestion. Optimally treated oilseeds and oilseed meals have positive effects on milk yield, especially in diets based on ensiled high-protein forages.

See also: **Feed Ingredients**: Feed Concentrates: Co-Product Feeds; Feed Supplements: Fats and Protected Fats. **Feeds, Prediction of Energy and Proteins**: Feed Energy; Feed Proteins. **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems.

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Feed Supplements: Anionic Salts

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Introduction

For years it was assumed that the primary nutritional cause of milk fever in dairy animals was high calcium intake prior to parturition. However, strict feeding of low-calcium diets is often difficult or impractical in practice (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever). An alternative and often more effective means of milk fever prevention in dairy animals is acidification through diet.

It is very challenging to evaluate the effect of diet on the risk for milk fever because of its relatively low incidence and multifactorial etiology. It is necessary to pool data from large numbers of previously published studies in a meta-analysis before making conclusions. In two recently published meta-analyses, reducing dietary cation–anion difference (DCAD) by a reasonable increment of 300 meq kg^{-1} resulted in a two- to fivefold reduction in the relative risk for clinical milk fever. This effect was essentially linear through the range of practical diets fed to dairy cattle. Therefore, any reduction in DCAD can be expected to reduce the risk for clinical milk fever. In contrast, the effect of dietary calcium on risk for milk fever was quadratic, with the highest risk at 1.35% dietary calcium (dry matter basis). Reducing dietary calcium by a practical increment of 0.50% (starting and ending with calcium content below the peak risk of 1.35%) resulted in a twofold reduction in the relative risk for clinical milk fever.

Diets may be made acidogenic (lowered DCAD) by selecting feed ingredients that are naturally acidogenic, by feeding anionic salts, or by adding mineral acids to the diet. Dietary acidification has important practical limitations and cannot be fully applied in all feeding management situations.

Effect of Metabolic Acidosis on Calcium Metabolism

Diets fed prior to parturition that evoke an acidic response in the animal reduce the risk for milk fever, while diets that evoke an alkaline response will tend to cause milk fever. Normal, healthy dairy animals are typically in a state of either mild alkalosis or acidosis. Metabolic alkalosis is common in herbivores, because they eat forage-based diets that are often high in potassium. Metabolic alkalosis impairs tissue responsiveness to parathyroid hormone (PTH) and increases the risk for milk fever. Metabolic acidosis, in contrast, enhances tissue responsiveness to PTH. Metabolic acidosis is defined as the accumulation of acid and/or depletion of bicarbonate content in blood and body tissues. The metabolic acidosis associated with feeding acidogenic diets is compensated; blood pH does not decrease below the normal range of about 7.35–7.45. Metabolic acidosis makes more calcium available to meet the sudden outflow of calcium that occurs around parturition. The source of this calcium is likely from a combination of increased bone resorption, increased intestinal absorption, and decreased renal excretion.

Calculating the Effect of Diet on Acid–Base Balance

The potential of a diet to cause either metabolic alkalosis or acidosis can be estimated by calculating its DCAD. This calculation takes into account dietary minerals that strongly influence systemic acid–base balance. For purposes of DCAD calculation, dietary minerals are divided into either cations (positively charged) or anions (negatively charged). The most important dietary cations are sodium and potassium. The most important dietary anions are chloride and sulfur.

Ions must be absorbed into the bloodstream in order to participate in acid–base balance. The monovalent ions sodium, potassium, and chloride are highly bioavailable and are not metabolized within the body. Therefore, they exert the strongest effects on acid–base balance. Other macrominerals (mostly divalent) may have modest effects on acid–base balance. They are excluded from DCAD calculations because of their weak acid–base effects and because they have independent effects on the risk for milk fever. These include calcium (which is cationic but has a quadratic effect on milk fever incidence), magnesium (which is cationic but reduces the risk for milk fever), and phosphorus (which is anionic but increases the risk for milk fever). Microminerals may participate in acid–base balance as well, but they constitute a very small portion of the total diet compared to the macrominerals and thus are not included in DCAD calculations.

The equations used to calculate DCAD and the units used to express it vary among dairy publications

Table 1 Conversion of dietary mineral concentrations from a percentage basis to a milliequivalents per kg basis

Element	*Molecular weight (g)*	*Valence*	*Equivalent weight (g)*	*To convert from percentage to meq kg^{-1}, divide by*
Cations				
Sodium	23.00	1	23.00	0.002300
Potassium	39.10	1	39.10	0.003910
Anions				
Chlorine	35.45	1	35.45	0.003545
Sulfur	32.06	2	16.03	0.001603

and ration evaluation software programs. Recent meta-analyses support the use of the original and simplest DCAD equation, where DCAD (meq) = (Na + K)−(Cl + S).

Calculation of the DCAD of a diet requires using the equivalent weights of the strong ions. This is necessary because acid–base balance is affected by electrical charge rather than mass. **Table 1** lists the factors used to convert dietary mineral concentrations from percentage to milliequivalents per kg.

Creating Acidogenic Diets for Milk Fever Prevention

Reducing Dietary Cation–Anion Difference

The DCAD of a diet can be lowered by reducing cations, increasing anions, or a combination of both. Typical diets (without supplemental anions) fed to dry dairy cows in confinement dairies in the United States have a DCAD of about +250 to +350 meq kg^{-1} of diet dry matter. Pasture diets typically have a higher DCAD (about +400 to +750 meq kg^{-1} of diet dry matter). Any reduction in DCAD prior to calving will lessen metabolic alkalosis and reduce the risk for milk fever. Reducing the DCAD below about 50 meq kg^{-1} creates a consistently measurable metabolic acidosis, which is marked by a reduction in urinary pH. A DCAD of about 0 to −100 meq kg^{-1} of diet dry matter appears to be optimal for milk fever prevention. This DCAD corresponds to a urinary pH of about 7.0.

Lowering DCAD linearly reduces the risk for milk fever; however, lowering DCAD does not linearly reduce urinary pH. Reducing DCAD from high concentrations down to about 50 meq kg^{-1} has little impact on urinary pH. But reducing DCAD from about 50 meq kg^{-1} to about −150 meq kg^{-1} dramatically lowers urinary pH (see **Figure 1**). Note that a reduction in the risk for milk fever may occur without a measurable response in acid–base status. Altering urinary pH is a marker for optimum acidification but is not the goal *per se* of feeding an acidogenic diet.

Blood pH remains in the normal range and is slightly decreased when acidogenic diets are fed. A 300 meq kg^{-1}

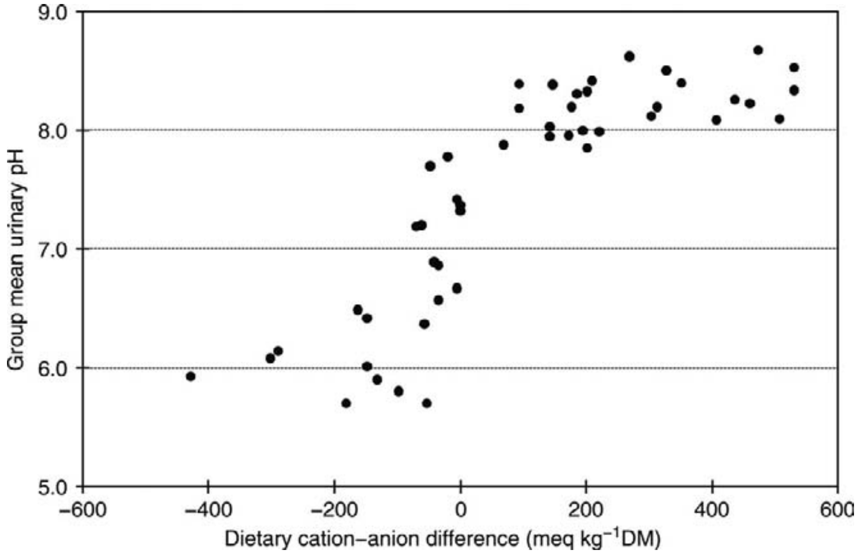

Figure 1 Effect of dietary cation–anion difference (calculated as (Na + K) − (Cl + S)) from 75 treatment groups in 22 published studies on group mean urinary pH. Data adapted from Charbonneau E, Pellerin D, and Oetzel GR (2006) Impact of lowering dietary cation–anion difference in nonlactating dairy cows: A meta-analysis. *Journal of Dairy Science* 89: 537–548.

reduction in DCAD results in a blood pH drop of only 0.018 pH units (−0.25%), despite a substantial drop (−2.2 mmol l^{-1} or −8.6%) in blood bicarbonate concentration. Respiratory mechanisms are apparently invoked to compensate for the dietary acid load (pressure of carbon dioxide dissolved in the blood is reduced by 1.9 mmHg or 4.5%).

Feeding acidogenic diets slightly increases the proportion of blood calcium that is ionized. The increase is minor (0.06 mmol l^{-1} or 4.8%). The slight shift of blood calcium to the ionized fraction probably plays only a minor role in the improved calcium metabolism observed when acidogenic diets are fed. Total blood calcium is not affected by feeding acidogenic diets, except around the day of calving, when it is substantially improved (0.20 mmol l^{-1} or 10.7%).

Reducing Dietary Cation–Anion Difference by Feed Ingredient Selection

Potassium is the most variable of the strong ions found in feed ingredients and is the most important determinant of the DCAD of a diet. A good first step in formulating a low-DCAD prepartum diet is to reduce dietary potassium to less than 1.5% of diet dry matter. However, limiting potassium in a diet can be difficult because forages are often relatively high in potassium compared to other fixed ions. All plants must have access to a certain amount of potassium for maximal growth. However, when soil potassium is high, many plants will accumulate potassium within their tissues beyond the required concentrations (luxury consumption). High soil potassium is caused by heavy soil fertilization and by heavy application of manure to soils. Increasingly high soil potassium is often associated with intensive crop production and expanding animal production units with inadequate land space for manure disposal.

Warm season grasses tend to have the lowest potassium concentrations among forages. For example, maize silage contains only about 1.1–1.5% potassium. Some other warm season grasses, such as switchgrass, big bluestem, and indiangrass, also tend to be low in potassium but have the disadvantage of being poorly digestible and low in protein. Cool season grasses such as bluegrass, orchardgrass, and brome have a fibrous root system, which makes them very efficient utilizers of soil potassium. Thus, these grasses will be high in potassium if they were grown on high-potassium soil.

Forages produced on some soil types are naturally higher in chlorine than others. Chlorine is a strong anion that lowers DCAD and helps prevent milk fever. Some forage growers in parts of the United States are able to produce forages that are naturally high in chlorine and low in potassium. Some of these forages have a DCAD near 0 meq kg^{-1}, which makes them highly desirable for prepartum dairy rations. Fertilization with calcium chloride can also assist in producing forages that are inherently lower in DCAD.

Examples of book values for several common dairy forages are listed in **Table 2**. Remember that book values for mineral content are not sufficiently accurate to formulate diets that will prevent milk fever. Individual forages must be sampled in a representative fashion and then tested for their mineral content by wet chemistry procedures. Testing by near-infrared reflectance spectroscopy (NIRS) is not a reliable method for determining the mineral content and DCAD of a feed sample.

If a producer has access only to forages with high DCAD content, then a forage low in DCAD may need to be purchased to feed to the prepartum animals. If this is not practical, then a high-fiber concentrate feed that is low in potassium may be used to replace some of the high-potassium forage. Examples of concentrate feeds useful for this purpose include brewer's grains, corn gluten feed, and beet

Table 2 Mineral and dietary cation–anion difference concentration (dry matter basis) of dairy feed ingredients

Feed ingredient	*International feed no.*	*Na (%)*	*K (%)*	*Cl (%)*	*S (%)*	*DCAD (meq kg^{-1})*
Legume silage, immature	3-07-795	0.03	3.03	0.55	0.33	427
Grass hay, cool season, midmaturity	1-02-243	0.08	2.13	0.92	0.24	170
Corn silage, normal	3-28-248	0.01	1.20	0.29	0.14	142
Corn grain, ground, dry	4-02-854	0.02	0.42	0.08	0.10	31
Soybean meal, solvent, 44% crude protein	5-20-637	0.04	2.22	0.13	0.46	262
Brewer's grains, dried	5-12-024	0.04	0.50	0.07	0.38	–112
Corn gluten feed, dried	5-28-243	0.13	1.46	0.20	0.44	99
Beet pulp, sugar, dried	4-00-669	0.31	0.96	0.18	0.30	142
Soybean hulls	1-04-560	0.01	1.51	0.05	0.12	302

These values are estimates based on previously published data; actual mineral composition of feed ingredients varies considerably. The mineral content of all feed ingredients should be determined by wet chemistry analysis before formulating diets for milk fever prevention. DCAD, dietary cation–anion difference, calculated as meq kg^{-1} of (Na + K) – (Cl + S).

Feed mineral composition data from National Research Council (2001) *Nutrient Requirements of Dairy Cattle,* 7th edn., pp. 304–310. Washington, DC: National Academy Press.

pulp. These concentrate feeds have relatively low DCAD values compared to a high-potassium forage (see **Table 2**). Not all high-fiber by-product feeds have a low DCAD, however (e.g., soybean hulls).

Addition of a cationic salt (such as sodium bicarbonate, sodium carbonate, or potassium carbonate) to the prepartum diet increases DCAD and the risk for milk fever. Such salts should be avoided in the diet of animals that are near parturition.

Anion Supplementation

Once the diet has been made as low in DCAD as possible by forage selection and forage replacement with high-fiber concentrates, the nutritionist must carefully decide whether or not the feeding management of the herd allows for supplementation with anions. Before attempting to supplement additional anions to the prepartum diet, the herd should ideally be feeding a total mixed ration (TMR) and should have palatable forages. Additionally, the nutritionist should be able to accurately monitor dry matter intake of the prepartum diet. Addition of anions to a grain or concentrate mix usually results in markedly decreased intake of the mix, unless the amount of concentrate used to carry the supplemental anions is large (about 3.5 kg or more). A better option is to mix the anion source with a wet forage when TMR feeding cannot be accomplished. Even when a TMR is fed, supplementation with anions will likely reduce dry matter intake. A recent meta-analysis indicated that a 300 meq kg^{-1} reduction in DCAD resulted in a 1.3 kg (11.3%) reduction in daily dry matter intake.

Because of the expected reduction in dry matter intake when anions are supplemented, feeding management is of the utmost importance. It is particularly critical to maintain dry matter intake and energy balance just prior to parturition. It is not acceptable to reduce the risk for milk fever by feeding supplemental anions but at the same time increasing the risk for fatty liver and ketosis by creating a prepartum energy deficiency. Both milk fever and ketosis can be held to low rates when feeding management and diet formulation are excellent.

If the herd's feeding management is adequate, then anions can be added to the prepartum diet to further reduce DCAD to the desired end point. Anion sources include anionic salts (any mineral salt high in Cl and S relative to Na and K) and mineral acids (hydrochloric or sulfuric acids). Optimal acidification generally occurs when anions are added to achieve a final DCAD between about 0 and $-100\ \text{meq kg}^{-1}$ dry matter. However, any reduction in DCAD lowers the risk for milk fever.

The total dose of anions is expressed as the number of equivalents supplemented per day. Equivalent weights of different anion sources are presented in **Table 3**. A typical daily dose of anions is 3 equivalents per cow per day. Larger doses than this may cause unacceptable depression in dry matter intake.

It is sufficient to add anions for about the last 3 weeks prior to calving, which corresponds to the time period when dry animals are commonly switched to a 'close-up' or 'prefresh' diet that is higher in concentrates than the 'far-off' dry cow diet. A few studies indicate that feeding supplemental anions for more than about 3 weeks results in higher urinary pH values, perhaps because bone needs several weeks to fully adapt to buffering the acidity generated by the diet.

Anionic salts have been added to prepartum diets either by using standard doses or by systematic calculation of mineral content and DCAD. An example of a standard daily dose is 114 g $MgSO_4{\cdot}7H_2O$ plus 114 g NH_4Cl (3.0 equivalents of anions). While standard doses of anionic salts may perform satisfactorily in many herds, this dosing strategy may not properly adjust for the existing mineral and protein content of the prepartum diet. A better approach is to analyze the available forages and

Table 3 Molecular weights, equivalent weights, and anion content of mineral supplements commonly used as anion sources

Mineral supplement	*Molecular weight (g)*	*Equivalent weight (g)*	*Cl (%)*	*S (%)*
Chlorine sources				
Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$)	147.0	73.5	48.23	-
Magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$)	203.3	101.7	34.88	-
Ammonium chloride (NH_4Cl)	53.5	53.5	66.28	-
Hydrochloric acid (HCl)	36.5	36.5	97.23	-
Sulfur sources				
Calcium sulfate dihydrate ($CaSO_4{\cdot}2H_2O$)	172.2	86.1	-	18.62
Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$)	246.5	123.3	-	13.31
Ammonium sulfate ($(NH_4)_2SO_4$)	132.1	66.1	-	24.10
Sulfuric acid (H_2SO_4)	98.1	49.0	-	32.69

Molecular and equivalent weight data in part from Oetzel GR (1993) Use of anionic salts for prevention of milk fever in dairy cattle. *Compendium on Continuing Education for the Practicing Veterinarian* 15: 1138–1146. Feed mineral composition data in part from National Research Council (2001) *Nutrient Requirements of Dairy Cattle,* 7th edn., p. 312. Washington, DC: National Academy Press.

then formulate a specific concentrate and mineral mixture to create the desired final diet.

Mixtures of chloride and sulfate anionic salts are often used for milk fever prevention. Chlorides are more potent systemic acidifiers, but they apparently depress dry matter intake more than sulfates. Examples of chloride anionic salts are $MgCl_2$, $CaCl_2$, and NH_4Cl. Examples of sulfate anionic salts are $MgSO_4$, $CaSO_4$, and $(NH_4)_2SO_4$. Sulfur may be beneficial in reducing milk fever independent of its effect on acid–base balance. Anionic salts are often mixed to avoid potential toxicities due to excessive magnesium from magnesium salts, excessive sulfur from sulfates, or excessive nonprotein nitrogen from ammonium salts. Nonprotein nitrogen contributed by the ammonium salts may be useful in diets needing additional protein, but could be harmful if the diet already contains large amounts of soluble protein.

Mineral acids were used as anion source in the original research studies with low-DCAD diets. Mineral acids are less expensive than anionic salts per equivalent of anion supplemented. They may also be less likely to depress intake than anionic salts, perhaps because they have an acidic rather than salty taste. However, the extremely corrosive properties of mineral acids make them difficult to feed safely. Liquid mineral acids are too dangerous for dairy producers to handle on-farm. Commercial feed companies have developed methods of preblending mineral acids with concentrate feed ingredients such as fermentation by-products, oilseed meals, and beet pulp. These mixtures are safe to handle and may not inhibit dry matter intake as much as anionic salts. They are typically formulated to provide about 3 equivalents of anions per kg of as-fed product.

Calcium Supplementation with Acidogenic Diets

Once the DCAD and anion sources for the prepartum diet are determined, the calcium content of the diet should be evaluated. Limited evidence suggests that anionic salts work best in dairy diets that provide about 120 g of daily calcium. Clinical experience and limited research data indicate that anions should not be supplemented when the diet contains less than about 60 g Ca per day.

Simultaneous Magnesium and Anion Supplementation

Inclusion of some supplemental magnesium in the prepartum diet (to about 0.35–0.40% of diet dry matter) is recommended for all prepartum dairy diets, whether anions are supplemented or not. Magnesium appears to play an important role in calcium metabolism and milk fever prevention (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever). Magnesium is supplemented to ensure that adequate magnesium will be absorbed despite high potassium concentrations and/or reduced feed intake.

Magnesium sulfate can supply magnesium in a readily soluble form and is also an anion source. Magnesium chloride, where available, is also a good source of soluble magnesium and is more acidogenic than magnesium sulfate. These two salts may be particularly useful in pasture systems, where dry cows are routinely drenched with supplemental magnesium. Magnesium sulfate or magnesium chloride can be used in the drench instead of magnesium oxide, which is not acidogenic.

Acidogenic Diet Example

An example of a prepartum dairy diet, before and after anion supplementation, is presented in **Table 4**. Feed ingredients used in these diets are typical of those fed in the United States. In the acidogenic example diet, some of the high-DCAD legume silage was replaced with dried brewer's grains. Three equivalents of anions were then supplemented – one equivalent each from calcium chloride, ammonium chloride, and magnesium sulfate. The calcium and magnesium concentrations of the acidogenic diet were increased by supplementing limestone and magnesium oxide.

Monitoring the Response to Dietary Acidification

Monitoring urinary pH after feeding supplemental anions is a direct approach to establishing the optimal dose of anions within a dairy herd. An advantage of this approach (overrelying on calculated DCAD alone) is that it accounts for inaccuracies in mineral analyses and for unexpected changes in forage mineral content. Mean urinary pH can be evaluated by obtaining urine from a group of at least eight animals near parturition. The animals chosen should have been on the anionic diet for at least 2 days prior to testing. When acidification is optimal, mean urinary pH for the group of cows tested will be about 7.0. Mean urinary pH values below about 6.0 indicate overacidification and suggest that the dose of anions could be reduced. Impaired dry matter intake is the main risk associated with dietary overacidification. Conversely, urinary pH values above about 7.5 reflect inadequate acidification and suggest that more anions are needed. Only a small addition of anions may be necessary to reduce urinary pH to the optimal range, because urinary pH decreases rapidly as DCAD decreases from about 50 meq kg^{-1}.

Because there may be significant variations in urinary pH related to time after feeding, most accurate results will be obtained by collecting urine samples at a standard

Table 4 Examples of prepartum dairy diets, formulated to be nonacidogenic or acidogenic

Item	*Units*	*Nonacidogenic*	*Acidogenic*
Feed ingredients[a]			
Grass hay, cool season, midmaturity	kg day^{-1}	3.70	3.70
Corn silage, normal	kg day^{-1}	3.70	3.70
Legume silage, immature	kg day^{-1}	3.70	1.85
Brewer's grains, dried	kg day^{-1}	0.00	1.50
Corn grain, ground, dry	kg day^{-1}	2.80	2.80
Salt	kg day^{-1}	0.03	0.03
Magnesium oxide (ore)	kg day^{-1}	0.04	0.03
Calcium carbonate	kg day^{-1}	0.00	0.10
Trace mineral–vitamin premix	kg day^{-1}	0.04	0.04
Calcium chloride ($2H_2O$, 77–80% $CaCl_2$)	kg day^{-1}	0.00	0.07
Magnesium sulfate ($7H_2O$, Epsom salts)	kg day^{-1}	0.00	0.12
Ammonium chloride	kg day^{-1}	0.00	0.05
Nutrient analysis			
Dry matter	kg day^{-1}	14.0	14.0
Net energy for lactation	Mcal kg^{-1}	1.56	1.57
Crude protein	%	13.9	14.5
Neutral detergent fiber	%	38.7	39.0
Nonfiber carbohydrates	%	39.7	37.7
Calcium	%	0.62	0.90
Phosphorus	%	0.30	0.33
Magnesium	%	0.35	0.40
Sodium	%	0.12	0.12
Potassium	%	1.76	1.42
Chlorine	%	0.61	1.06
Sulfur	%	0.20	0.31
DCAD	meq kg^{-1}	207	−78
Anions supplemented	eq day^{-1}	0.00	3.00

[a] The mineral composition (including calculated DCAD) of the nonmineral feed ingredients used in these diets is presented in Table 2. All feed amounts and nutrient analyses are on a dry matter basis.
Diets were formulated to meet the nutrient requirements of a Holstein dry cow, 725 kg body weight, 260 days pregnant, and a body condition score of 3.0 using the Computer Model Program for Predicting Nutrient Requirements described in National Research Council (2001) *Nutrient Requirements of Dairy Cattle*, 7th edn. Washington, DC: National Academy Press.
DCAD, dietary cation–anion difference, calculated as meq kg^{-1} of (Na + K) – (Cl + S).

time, preferably within a few hours of feeding. Urinary pH may be measured on-farm by dairy producers or veterinarians using pH paper, urine dipsticks, or a calibrated pH meter.

Oral Dosing with Acidogenic Compounds

Anionic salts can be provided as boluses, packed into gelatin capsules, or mixed with water and then given orally to dairy animals. Good results were obtained when anionic salts were administered twice daily in a bolus to cows in pastured herds with high-DCAD diets. However, oral dosing of anion sources for cows in confinement herds already consuming a low-DCAD diet could cause severe, acute systemic acidosis. It is better to provide supplemental anions as part of the diet in this situation. When anion supplements are consumed along with the rest of the diet, the consumption of anions is spread out through the day. Additionally, including the anions with the rest of the diet provides a margin of safety in case an excessive amount of anions is accidentally mixed with the diet (animals will simply refuse to eat the diet). But if an excessive dose of anions is administered orally, the animal has no means of refusal.

Anions can be orally dosed in the form of calcium chloride in commercially prepared products. In this situation, the calcium chloride is both an anion source (chlorine) and a source of readily absorbable calcium. Both effects result in a rapid rise in blood calcium concentrations.

Oral calcium chloride can be used both to prevent cases of milk fever and to treat clinical cases of milk fever in their earliest stage. It is important to follow label directions when using such products and to avoid overdosing (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever).

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever.

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Feed Supplements: Fats and Protected Fats

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Introduction

Adding fat to dairy rations increases energy for high milk production without sacrificing fiber content in the ration. When used properly, fat supplements enhance milk yield significantly without adverse effects on milk composition or feed digestibility. However, fat supplements must be limited to relatively small amounts (generally <5%) in dairy rations to avoid negative effects on fiber digestion, feed intake, or fatty acid digestibility. In response to the need for fat supplements with fewer limitations and better handling, several commercial 'rumen-inert' fats were introduced that prevented negative effects on feed digestibility and were solid, dry powders that could be transported and mixed easily on the farm. Fat supplements are also being utilized in dairy rations to enhance the absorption of specific unsaturated fatty acids, which have been linked to improved reproductive performance of the cow or may be beneficial to human health when enriched in milk. Protected fat supplements are most suitable for increasing the absorption of unsaturated fatty acids, as they are designed to resist biohydrogenation by ruminal microbes. The type and amount of fat to include in dairy rations depend on a multitude of factors such as identifying the desired outcome (e.g., increased milk yield or modified fatty acid profile in milk), the interactions between dietary fat and microbial population and transformations of the fat supplement in the rumen, and understanding the influences of animal and feed factors on fat utilization.

Fat Definitions

The value of fats and oils as animal feed ingredients is based on their fatty acid content and fatty acid composition. Content refers to the total concentration ($\mathrm{g\,kg^{-1}}$) of fatty acids in a lipid supplement and composition (g per 100 g) refers to the mixture of individual fatty acids that make up the lipid supplement. Fatty acids are chains of carbons that end in an acid group, or carboxyl group as is referred to in biochemistry. An example of a common fatty acid is stearic acid with 18 carbons and no double bonds. Fatty acids such as stearic acid are referred to as saturated because all the carbons are holding the maximum number of hydrogens possible, or the fatty acid is 'saturated' with hydrogen. Stearic acid is low in plant oils, but present in higher amounts in animal fats, particularly in fats obtained from ruminant species such as beef tallow (**Table 1**). Oleic acid, linoleic acid, and linolenic acid are examples of unsaturated fatty acids containing one, two, and three double bonds, respectively (**Figure 1**). Oleic acid is the predominant fatty acid in animal fats and some plant oils such as canola oil (**Table 1**). Linoleic acid is the predominant fatty acid in many plant oils, including cottonseed oil, soybean oil, and corn oil. Linolenic acid is the predominant fatty acid in linseed oil extracted from flaxseed.

Fat content has traditionally been determined as the ether-extractable component of the feed. The ether extract of grains and forages has the lowest fat content. The fatty acid content of forages typically ranges from 10 to 30 $\mathrm{g\,kg^{-1}}$ dry matter (DM), with the majority of the fatty acids classified as unsaturated (predominately oleic, linoleic, and linolenic acids). In addition to extracting fat, ether also extracts some carbohydrates, vitamins, and pigments. Therefore, fatty acids in cereal grains, forages, and the total mixed ration often comprise less than 60% of the ether extract. Because of the problems inherent with ether extract, many laboratories have moved to determining fatty acid content of feeds instead of ether extract.

Most plant oils contain 100% ether extract with a high percentage of fatty acids. The impurities extracted, such as water and pigments, are removed during refining leaving the commercial plant oils (soybean oil, canola oil, corn oil, etc.) and animal fats (tallow, grease, etc.) with mainly triglycerides consisting of 90–93% fatty acids. The remaining 7–10% is mainly glycerol. Glycerol is readily utilized as an energy source, but only contains the energy of carbohydrates. Caution is advised when obtaining fats from unknown vendors to ensure that considerable impurities that lower the fatty acid and energy content do not remain in the product. Rather than guessing, it pays to have a sample of the fat analyzed for fatty acid content and profile.

According to Nutrient Requirements for Dairy Cattle published by the National Research Council, the ether extract values exceed 99% for all fat categories (vegetable oil, tallow, hydrolyzed tallow fatty acids, and partially hydrogenated tallow) except for calcium soaps. Ether extract for the vegetable oil, tallow, and partially hydrogenated tallow is assumed to contain 90% fatty acids and 10% glycerol. Ether extract for the hydrolyzed tallow fatty acids is assumed to contain 100% fatty acids. Ether extract for calcium soaps is listed as 84.5%, which is assumed to be all fatty acid.

Table 1 Fatty acid composition of several animal fats (tallow and poultry) and vegetable oils (cottonseed and canola)

Abbreviation[a]	*Common name*	*Tallow*	*Cottonseed*	*Canola*	*Poultry*
$C_{14:0}$	Myristic	3.0	1.0		1.0
$C_{16:0}$	Palmitic	25.0	23.0	4.0	21.0
$C_{18:0}$	Stearic	21.5	3.0	2.0	8.0
$C_{18:1}$	Oleic	42.0	18.5	60.0	41.0
$C_{18:2}$	Linoleic	3.0	52.5	20.0	19.0
$C_{18:3}$	Linolenic			10.0	1.0

[a] Number of carbons:number of double bonds.

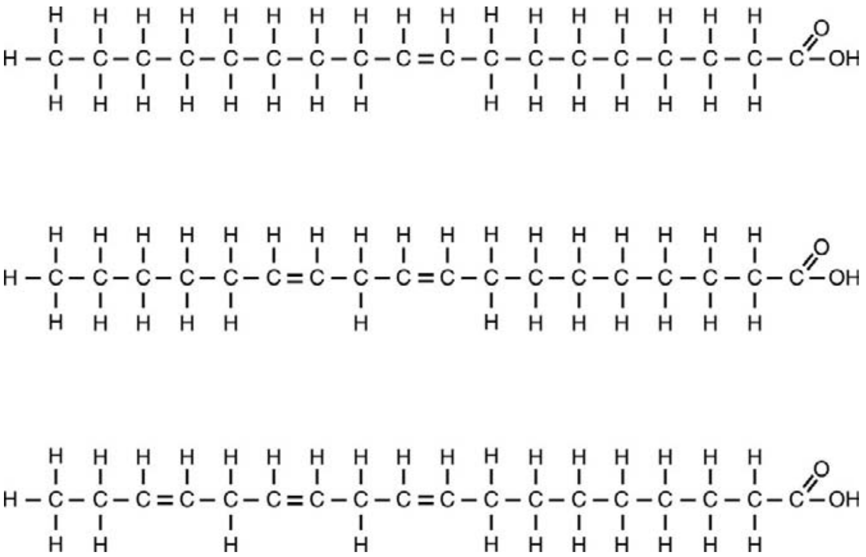

Figure 1 Structures of the three primary unsaturated fatty acids consumed by cattle: oleic acid (top), linoleic acid (middle), and linolenic acid (bottom).

Benefits of Fat Supplements

Adding fat to dairy rations can affect the productive efficiency of dairy cows through a combination of caloric and noncaloric effects. Caloric effects are attributable to greater energy content and energetic efficiency of lipid compared to those of carbohydrate or protein, and the overall benefit is increased milk production. Noncaloric effects are caused by benefits from added fat that are not directly attributable to its energy content or increased milk production. Examples of the proposed noncaloric effects include improved reproductive performance and altered fatty acid profile of milk.

Caloric Benefits

Caloric effects are the main reason for the increased usage of fat supplements in dairy rations over the past several decades. Replacing grains with fat has provided a means to increase energy available for milk production while still maintaining adequate fiber intake based on gross energy (GE) values for fat averaging 9.45 kcal g^{-1} compared with 4.10 kcal g^{-1} for carbohydrates. This translates into fat supplements providing more than twice the digestible energy (DE) of cereal seeds when compared on an equal weight basis. The energy advantage of fat supplements over corn is further accentuated by fatty acids having lower energy losses (per 100 kJ GE) in methane (because of negligible fermentation of fatty acids by ruminal microbes), urine, and heat (because energy from fatty acids is utilized more efficiently by body tissues) compared to carbohydrates.

Energy value of the fat supplement only partially explains reported variation in animal performance. Production is improved only if the added fat increases DE concentration of the whole diet. All fat sources are grouped together in Nutrient Requirements for Beef Cattle published by the National Research Council with an assigned DE value of 7.30 Mcal kg^{-1} (**Table 2**). The Nutrient Requirements for Dairy Cattle divide fat sources into five categories, which range in DE value from 7.70 Mcal kg^{-1} for vegetable oils to 4.05 Mcal kg^{-1}

Table 2 Energy values (TDN and DE) for fat sources listed in Nutrient Requirements for Beef and Dairy Cattle published by the National Research Council

	TDN (%)	*DE (Mcal kg^{-1})*
Beef	177	7.30
Dairy		
Calcium soaps	163.5	6.83
Hydrolyzed tallow	176.3	7.37
Partially hydrogenated tallow	96.6	4.05
Tallow	147.4	6.17
Vegetable oil	184.0	7.70

DE, digestible energy; TDN, total digestible nutrients.

for partially hydrogenated tallow. Low fatty acid digestibilities have been reported when dairy cows were fed hydrogenated fats, especially when fat particle size was large. Because of variation in the digestibility of all fat sources, it is difficult to suggest a single digestibility value that applies to each fat source in all instances. Even the hydrogenated fats have a range of digestibilities and their addition to dairy rations has resulted in normal fatty acid digestibilities in many instances.

More important than the fat DE value is the increase in total ration DE resulting from the addition of a fat source. Even if fatty acid digestibilities are high, fat supplements may fail to elevate total ration DE if the fat reduces feed intake or interferes with feed digestion.

Noncaloric Benefits

The interest in adding fat to dairy rations for reasons other than increased milk production is rapidly growing. Fat continues to be added to animal feeds for dust control, to improve feed intake by dairy cows as well as working conditions for feed mill employees. Much of the recent interest in noncaloric benefits of fat supplements stems from the reported role of fatty acids as regulators of cell function or for their ability to alter the fatty acid composition of milk. In both cases, the focus is on enhancing the absorption and uptake of selected unsaturated fatty acids by body tissues or the mammary gland.

The mechanism by which fat supplements alter reproductive performance is not clear, but three possible means receive the most attention: (1) fats as a source of energy; (2) fats as a source of essential fatty acids; and (3) fats as hormones. Fats may function by providing additional energy during early lactation to support improved productive functions, including reproduction. Negative energy balance delays ovulation and the initiation of the first normal luteal phase. However, early studies suggested that the mechanism involves an energy-independent response to fat. When glucose, saturated animal fat (tallow), or unsaturated fat (yellow grease) on an equal energy basis was infused into lactating dairy cows via the abomasum, the fat but not carbohydrate decreased plasma estradiol and increased progesterone. These studies also demonstrated the potential to decrease $PGF_{2\alpha}$ synthesis by supplying elevated concentrations of polyunsaturated fatty acids (PUFAs).

Fats serve as a source of essential fatty acids. Linoleic acid is an ω-6 fatty acid (**Table 3**) that is converted by tissue desaturases and elongases to arachidonic acid, which in turn is converted to $PGF_{2\alpha.}$ Omega is a system for identifying the location of the terminal double bond in a fatty acyl chain. The omega carbon is the last carbon on the fatty acyl chain, or the one farthest from the carboxyl group. Omega fatty acids contain one or more double bonds, but only the position of the one closest to the omega carbon is given. For an ω-3 fatty acid, there are three carbons between the omega carbon and the closest double bond.

The parent ω-3 fatty acid is linolenic acid, which is converted to eicosapentaenoic acid (EPA) and eventually to the series-3 prostaglandins. Supplemental ω-3 fatty acids have been proposed to enhance reproduction by providing parent omega fatty acids for the synthesis of adequate prostaglandins involved in reproduction.

Despite strong evidence of a link between reproduction and the ω-3 and ω-6 fatty acids, questions remain regarding the proper amounts, types, and timing of feeding additional fatty acids for maximum reproductive response. On average, duodenal flow of linoleic and linolenic acids is 38 and 5 g day^{-1}, respectively, in dairy cattle fed control diets with no added fat. This background flow of unsaturated fatty acids is not enough in some situations based on reports that feeding additional fat often improves reproductive performance. Additional intestinal supply of unsaturated fatty acids can be met by increasing the dietary concentration of unprotected fat sources. Based on a summary of duodenal flow studies, each

Table 3 Parent omega fatty acids in the feed of dairy cattle and their major metabolites resulting from the action of tissue desaturases and elongases

Family	*Parent fatty acid*	*Major metabolite*
ω-9	Oleic acid ($C_{18:1}$ ω-9)	Eisosatrienoic acid ($C_{20:3}$ ω-9)
ω-6	Linoleic acid ($C_{18:2}$ ω-6)	Arachidonic acid ($C_{20:4}$ ω-6)
ω-3	Linolenic acid ($C_{18:3}$ ω-3)	Eicosapentaenoic acid ($C_{20:5}$ ω-3) Docosahexaenoic acid ($C_{22:6}$ ω-3)

100 g of additional linoleic acid intake from unprotected fats will increase intestinal flow of linoleic acid by 18 g. Using soybean oil as the unprotected fat source would require adding about 200 g soybean oil to the diet to increase duodenal linoleic acid flow by 18 g. Eventually, the risks of high levels of unprotected fats will outweigh the benefits, with negative effects seen on feed intake, ruminal fermentation and digestion, and depressed milk fat percentage. In some cases, rumen-protected fat sources (oilseeds, calcium salts) can increase duodenal flow of essential fatty acids beyond that seen for unprotected fats, but their response is often inconsistent.

The third mechanism by which fat supplements improve reproductive performance is related to their hormonal action, including their effects on progesterone and prostaglandins. The progesterone explanation centers on reports that fat feeding increases follicular growth and diameter. Progesterone is secreted by the corpus luteum and is responsible for preparing the body for pregnancy and, if pregnancy occurs, maintaining it until birth. On average, the diameter of the dominant follicle increased from 14.1 mm on the control diets to 17.3 mm for the fat-supplemented diets ($p < 0.10$) across several major published studies. Because there is a linear relationship between corpus luteum volume and serum progesterone, and fat increases corpus luteum and follicular size, then fat may logically lead to increased progesterone production. An alternative hormonal explanation is that fat feeding reduces uterine secretion of $PGF_{2\alpha}$. Prostaglandins are like hormones in that they act as chemical messengers, but do not move to other sites, but work right within the cells where they are synthesized. A developing embryo produces interferon-τ, which inhibits production of $PGF_{2\alpha}$ so that pregnancy is maintained. Fat supplements may assist interferon-τ by helping to suppress the synthesis of $PGF_{2\alpha}$.

The possibilities of feeding fat supplements to dairy cows to alter milk fatty acid profile are either being investigated or already in practice. Applications include increasing unsaturated fatty acids in milk to reduce hardness and improve spreadability of butter, offer consumers more choices in the content of saturated fatty acids in milk and other dairy products, and enhance milk concentration of fatty acids with positive health benefits to humans. An example of potential health benefits to humans includes the conjugated linoleic acids (CLAs) that have been identified as potent anticarcinogens, antioxidants, and modulators of the immune system.

Fat Interactions in the Rumen

The benefits derived from feeding additional fat to dairy cattle are greatly influenced by two interactions between dietary fats and the microbial population in the rumen: (1) the extent that the fat source inhibits growth and function of the microbial population and (2) the extent that the lipid is transformed by the microbial population.

Antimicrobial Effects of Fatty Acids

As fatty acid concentration increases in ruminal contents, eventually a point is reached where growth and metabolism of the microbial population are suppressed. This antimicrobial effect of fatty acids causes several changes in ruminal fermentation, including decreased methane production, decreased acetate-to-propionate ratio, and decreased protozoal numbers. Reduced digestibility of fiber in the rumen is another major consequence of fatty acids disrupting the microbial fermentation process. If hindgut fermentation fails to compensate for the additional flow of fiber to the intestines, the end result is increased fecal fiber excretion. In this instance, DE intake and milk production are not increased to the extent expected.

Disruptions of ruminal fermentation and fiber digestibility are more severe when the fat supplement is rich in unsaturated fatty acids or in free fatty acids. Some reports have indicated improved ruminal fermentation from added fat depending on the composition of the basal diet, especially when fiber content of the ration is high.

Fatty Acid Transformations in the Rumen

Many of the noncaloric benefits of fat supplements, including improved reproductive performance and modifying milk fatty acid profile, are contingent on the supplement increasing intestinal absorption of unsaturated fatty acids. However, in dairy cattle and other ruminant species, it is difficult to control the type of fatty acid absorbed simply by modifying fatty acid composition of the diet. This difficulty arises because unsaturated fatty acids in the diet are converted by ruminal microbes to saturated fatty acids prior to the site of lipid absorption in the small intestine.

Feed consumed by ruminants first passes through the largest of the four stomach compartments or rumen, which acts like a fermentation vat. Countless numbers of bacteria, protozoa, and fungi in the rumen ferment the feed releasing end products that are utilized by the host animal for maintenance and growth of body tissues. The microbial population in the rumen is also responsible for extensive transformation of dietary lipid. Lipid transformations include lipolysis to release free fatty acids from complex plant lipids, and biohydrogenation to convert unsaturated fatty acids in plant matter to more saturated lipid end products.

The main types of lipids entering the rumen are triglycerides, phospholipids, and galactolipids from

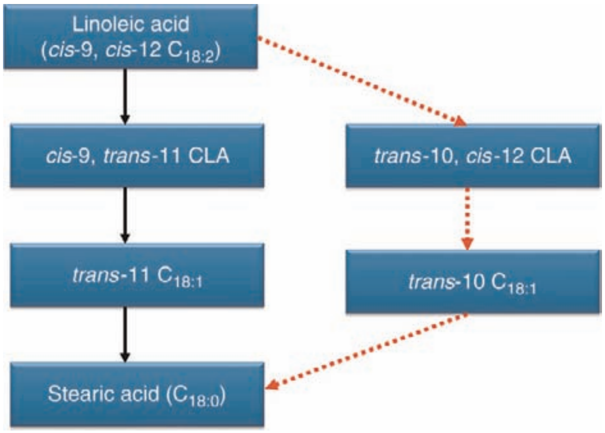

Figure 2 Major intermediates and end products in the biohydrogenation of linoleic acid by ruminal microorganisms. Black arrows show major intermediates during a normal ruminal fermentation. Red arrows show major intermediates accumulating in the rumen when fermentation is altered by excessive grain or fat content in the diet.

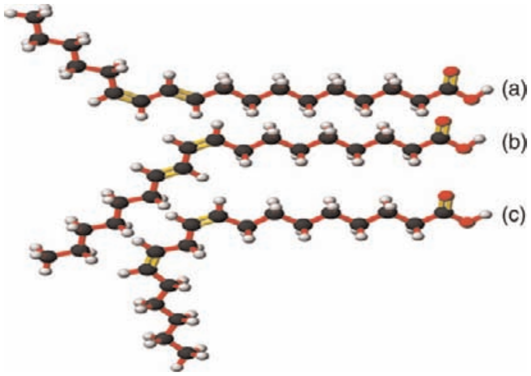

Figure 3 Structures of conjugated linoleic acid (CLA) isomers that are formed as intermediates during biohydrogenation of linoleic acid by microorganisms in the rumen. (a) *trans*-10, *cis*-12 CLA (b) *cis*-9, *trans*−11 CLA; and (c) linoleic acid.

forages and concentrates in the diet. Rapid hydrolysis of triglycerides occurs by microbial enzymes, resulting in greater than 75% of the total lipid recovered in the small intestine as free fatty acids. Phospholipids and galactolipids also undergo rapid and extensive breakdown in the rumen as a result of the lipase activity of ruminal microorganisms.

Following lipolysis, unsaturated fatty acids accumulating in ruminal contents undergo biohydrogenation causing a reduction in the number of double bonds. The biohydrogenation of linoleic acid in the rumen (**Figure 2**) begins with its conversion to CLA. In this initial step, the number of double bonds remains the same but one of the double bonds is shifted to a new position by microbial enzymes. Normally, the double bonds in linoleic acid are separated by two single bonds, but in CLA, the double bonds are separated by only one single bond. Many types of CLA are produced in the rumen of dairy cows, but a common CLA produced from biohydrogenation of linoleic acid is *cis*-9, *trans*-11 $C_{18:2}$ (**Figure 3**). Diet manipulation can alter fermentation patterns in the rumen and enhance the production of other CLA isomers. One of these is the *trans*-10, *cis*-12 $C_{18:2}$ isomer that accumulates in ruminal contents from feeding excessive grain or fat and has been associated with milk fat depression.

As biohydrogenation progresses, double bonds in the CLA intermediates are hydrogenated to *trans* fatty acids having only one double bond. *Trans* double bonds differ from *cis* double bonds only in the placement of the hydrogens around the double bonds. The hydrogens are located on opposite sides of the double bond for *trans* fatty acids, but on the same side of the double bond for *cis* fatty acids. Although the difference in the structure between *trans* and *cis* fatty acids appears small, it causes significant differences in their physical and metabolic properties. The major *trans* $C_{18:1}$ normally present in ruminal contents is *trans*-11 $C_{18:1}$, but the remaining isomers have double bonds distributed among carbons 12 through 16. A final hydrogenation step by the ruminal microbes eliminates the last double bond yielding stearic acid as the final end product. As a result of biohydrogenation, there is an extensive loss of unsaturated fatty acids as food passes from the mouth to the duodenum of the animal.

Types of Fat Supplements

Fat sources used as supplements for dairy rations are generally grouped based on structural differences or based on known nutritional effects, with considerable overlap between the two systems. Classification by structure is generally based on differences in fatty acid composition (g fatty acid per 100 g total fatty acids). The majority of the fat sources fed to dairy cattle are comprised of mixtures of the same 5–8 major fatty acids that differ in carbon length and number of double bonds.

Terminology for classifying fat sources according to nutritional effect varies widely, but most groupings consider the extent that a fat source depresses digestibility of the basal feed ingredients and the extent that the fat source resists biohydrogenation. On this basis, fats are often referred to as rumen-active, rumen-inert, or protected.

Rumen-Inert Fats

The term ‘rumen-inert’ has been assigned to fats that were specifically designed to have little, if any, negative effect on feed digestibility when fed to dairy cattle.

Rumen-inert fats often have the added advantage of being dry fats that are easily transported and can be mixed into the diet without the need for specialized equipment. Rumen-inert fats are often high in calcium salts of fatty acids, saturated fatty acids, or hydrogenated fats. Fats in this category have also been referred to as ‘bypass’ fats.

Rumen-Active Fats

The ‘rumen-active’ fats have the potential to interfere with microbial fermentation in the rumen and reduce feed digestibility to varying degrees. Digestibility of the fibrous carbohydrate fraction is especially susceptible to antimicrobial effects of rumen-active fats. Generally, unsaturated fatty acids depress ruminal fermentation and digestibility more than saturated fatty acids. Rumen-active fats include fats of animal origin (tallow, grease, etc.), plant oils (soybean oil, canola oil, etc.), oilseeds (cottonseeds, soybeans, etc.), and fat by-products such as distillers grains and residues from food processing plants. Rumen-active fats undergo biohydrogenation by ruminal microbes and generally have little impact on modifying milk fatty acid profile.

Protected Fats

The term ‘protected fat’ is most applicable to fat sources specifically designed to resist biohydrogenation by ruminal microbes and modify fatty acid profile of body tissues and milk. Many protected fat technologies were based on surrounding unsaturated fatty acids by a protective capsule to shield internal fatty acids from biohydrogenation.

Feeding whole oilseeds (i.e., whole soybeans, whole cottonseeds, whole sunflower seeds, etc.) to cows increases tissue and milk unsaturation according to some reports. Increasing cottonseed or other oilseeds in the diet of dairy cows steadily increases milk oleic acid, but increases in milk linoleic or linolenic acids were inconsistent. Processing of the seed can affect the degree of protection from ruminal biohydrogenation and the extent that milk fatty acids are altered. Whole seeds provide some protection from biohydrogenation because of the nature of their hard outer seed coat. Disruption of the seed coat exposes the oil to the microbial population and increases the potential for fermentation problems and biohydrogenation. The seed coat can be sufficiently broken by chewing and rumination, or through a variety of processing techniques such as extrusion or grinding.

A single fat source may overlap two or even all three fat groups to some extent. For example, at normal levels of supplementation, some rumen-active fats, such as tallow, are fed to dairy cows without evidence of consistent problems with fiber digestion. Even whole oilseeds help to lessen the severity of digestion problems by encapsulation of antimicrobial fatty acids within their hard outer seed coat. However, classification according to ruminal digestion is better defined at high levels of supplementation, where the frequency of digestibility problems for tallow and oilseeds is much greater than for the rumen-inert fats. The oilseeds may also overlap as protected fats in instances where their hard outer seed coat provides protection from biohydrogenation. However, disruption of the outer seed coat by chewing and rumination often leads to oilseeds having little ability to enhance unsaturated fatty acids in milk.

Feeding Recommendations for Fat Supplements

Over the years, numerous recommendations have been cited for feeding fat supplements to dairy cattle, some of which have been proven repeatedly and others that have been accepted with little evidence. Most of the recommendations relate to the caloric benefits of fat and are aimed at maximizing the milk response to added fat. Examples of some common (but not necessarily proven) recommendations include the following:

- Feed fat to cows milking more than 75 lb of 4% fat-corrected milk (FCM). This recommendation is based on the presumption that cows with lower milk yield will utilize the additional energy for excessive body weight gain rather than increased milk yield.
- Wait until weeks 5–7 postpartum to begin feeding fat. This is one of the most controversial recommendations associated with feeding fat. Some trials have indicated little benefit from feeding fat supplements prior to week 5–7 postpartum. However, one-group total mixed ration (TMR) and adapting cows to fat prior to calving often encourage feeding at least limited amounts of fat at calving or prepartum.
- Limit total fat in dairy rations to 7–9% of the ration DM, with one-third from basal feed ingredients, one-third from oilseeds and rumen-active fats, and one-third from rumen-inert fats. Recommendations for levels of added fat are diverse, but most recognize that total fat must be limited and that combining fat sources is the best strategy for minimizing both feed digestibility problems and fat costs.
- Maximize forage intake when feeding fat. Studies continue to show that fat supplements enhance milk production more when fiber content of the ration is high. Proposed mechanisms for the beneficial effect of fiber include the following: provision of greater surface area for adsorption of fatty acids to lessen antimicrobial effects; maintenance of saliva production for normal rumen function and pH; and maximization of

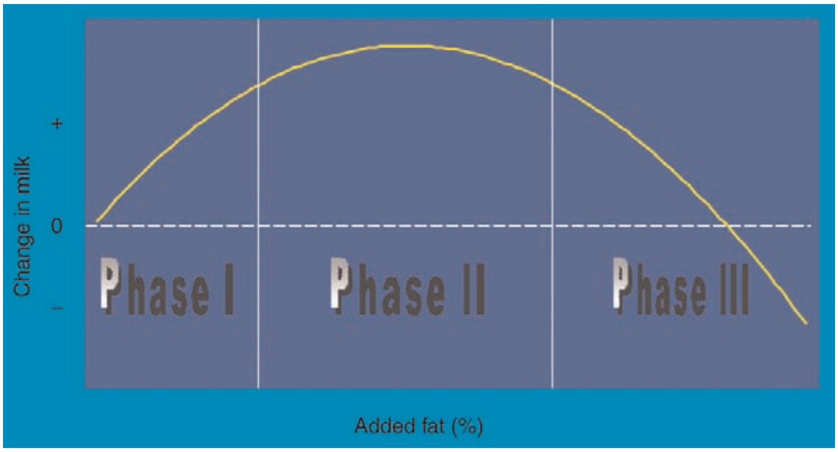

Figure 4 A hypothetical model describing changes in milk yield as fat content is increased in the diets of lactating dairy cows. Compared to a control diet, the added fat can cause milk yield to increase (+), decrease (–), or remain the same (dotted line). It is proposed that milk initially increases with dietary fat (phase I) as energy density of the diet increases, then levels off and remains stable (phase II) as the negative effects of the fat offset increased energy, and finally declines (phase III) as these negative effects exceed the increased energy. Adapted with permission from Jenkins TC (1998) *Proceedings of the 1998 Mid-South Ruminant Nutrition Conference*. Dallas, TX, USA.

biohydrogenation potential. The source of fiber was also important in some studies, as the milk response to added fat was higher when the source of fiber was alfalfa hay compared to corn silage.

- Maintain adequate rumen-undegradable protein when fats are added to dairy rations. It is proposed by some nutritionists that the lack of rumen-undegradable protein is responsible for the reduced milk protein percentage, reduced feed intake, and reduced milk yield that sometimes accompanies the addition of fat to dairy rations.

With all the recommendations that have been written on feeding fat to dairy cows, perhaps the most important, yet most elusive of these, might be the proper amount to feed. To effectively utilize the vast array of fat products available, it is essential that practical guidelines be developed for matching sources of fat with proper levels of supplementation. Proper feeding rates for fat may be the single most important management tool affecting the success of using fat supplements.

Figure 4 presents a model describing milk yield changes as fat percentage is increased in the diet of dairy cows. Three distinct changes or phases in milk yield can be identified.

- *Phase I* – milk yield increases as fat percentage increases. Fat supplies additional DE in this region because the supplement has minimal negative effects on feed intake, fatty acid digestibility, or feed digestibility.
- *Phase II* – further increases in fat percentage of the diet yield no additional increases in milk yield. Depressions in feed intake, fatty acid digestibility, or feed digestion caused by the fat supplement counterbalance the additional fat energy supplied resulting in no change in DE.
- *Phase III* – continuing to increase fat percentage in the diet eventually causes milk yield to decline. In phase III, the negative effects of the fat supplement on feed intake, fatty acid digestibility, or feed digestion exceed the additional fat energy supplied resulting in a decline in DE. Eventually, a point is reached where milk yield of the fat-supplemented diet is lower than the control diet.

All fat sources conform to this model, but may differ in the actual percentages marking the start of each phase. For instance, fats with greater unsaturation would be expected to have a smaller phase I and phase II compared with saturated fats. The 'ideal' fat percentage from **Figure 4** corresponds to the point at the junction of phases I and II where milk response to the added fat is maximized. Increasing fat beyond this point will yield little additional milk or perhaps even reduce it compared to a 0% added fat diet. Unfortunately, this 'ideal' fat percentage is not the same for all fat sources.

See also: **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows.

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Feed Supplements: Macrominerals

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Introduction

Macrominerals, or major minerals, are those elements that are required daily in gram amounts, and include calcium, chlorine, magnesium, phosphorus, potassium, sodium, and sulfur. These mineral elements are important structural components of bone and other tissues. The percentages of these elements present in the body of steers of varying age (excluding gastrointestinal contents) average 1.33, 0.11, 0.04, 0.74, 0.19, 0.16, and 0.15%, respectively. The requirements for each macromineral for lactating cows will be presented. These are taken from the National Research Council publication *Nutrient Requirements of Dairy Cattle*, seventh edition, the Agricultural Research Council publication *The Nutrient Requirements of Ruminant Livestock*, and Underwood and Suttle's *The Mineral Nutrition of Livestock*, third edition. These are the most recent and comprehensive summaries of nutrient requirements of dairy cattle, and are used widely throughout the world. The minerals are presented in alphabetical order.

Calcium

Although about 99% of the body's calcium is present in bones and teeth, extracellular calcium is also essential for the transmission of nerve impulses, blood clotting, and the normal contraction of skeletal and cardiac muscle. Furthermore, intracellular calcium is involved in the activity of enzymes and serves as an important secondary messenger conveying information from the surface of the cell to the interior.

Approximately 50% of plasma calcium is bound to plasma proteins, other organic blood components, and inorganic elements, with the other 50% free in ionic form. The real challenge of all mammals is to maintain calcium concentrations in plasma and the extracellular fluids (ECFs) at around 2.5 mmol l^{-1} (100 mg l^{-1}) and, more importantly, blood ionized (free) calcium concentrations at 1.0–1.25 mmol l^{-1}, despite large fluctuations in demand and supply. The primary challenge in the lactation of the dairy cow is around calving, a 24-h period during which a cow with only 3 g of available ionized calcium in her bloodstream can maintain her normal physiological functions and secrete 21–34 g of calcium in 10–15 kg of colostrum. A further complication is that this occurs when a cow may not have eaten for several hours. A failure of calcium homeostasis to sufficiently increase calcium entry into the ECF when losses increase at calving can lead to recumbency and the clinical condition known as milk fever.

Calcium Homeostasis

Due to the requirement for eucalcemia in the regulation of vital cellular and tissue functions, the concentration of ionized calcium is closely regulated by an efficient feedback control mechanism. Homeostasis in adult ruminants is controlled largely by hormonal regulation of absorption and, to a lesser extent, hormonal regulation of excretion. Absorption is primarily under the control of parathyroid hormone (PTH) and the physiologically active form of vitamin D_3 (1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$)). The parathyroid gland is acutely sensitive to changes in ionized calcium concentrations in carotid blood, such that PTH concentration rises quickly when ionized calcium concentration falls. The precipitous increase in calcium requirements during the second stage of lactogenesis can cause a 10-fold increase in PTH concentrations. PTH immediately increases renal reabsorption of calcium and facilitates the activation of vitamin D_3 to 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ acts on the intestinal mucosa to allow calcium uptake; however, this process is slow, with peak concentrations occurring up to 3 days postcalving. When dietary calcium is insufficient to meet cow requirements, calcium will be withdrawn from bone.

Calcium Requirements

The requirement for calcium represents the sum of the requirements for maintenance, growth, pregnancy, and lactation. The maintenance requirements of nonlactating cattle for absorbed calcium is 0.0154 g kg^{-1} body weight (BW), and lactating cows require approximately twice as

[†] Revised posthumously.

much (0.031 g kg^{-1} BW). The increased requirements of lactating cows reflect the impact of increased dry matter intake (DMI) on intestinal secretion of calcium during digestion.

Actively growing cattle require more calcium than older animals:

$$\text{calcium}\ \left(\text{g day}^{-1}\right) = \left(9.83 \times \left(\text{MW}^{0.22}\right)\right) \times \left(\text{BW}^{-0.22}\right) \times (\text{WG}/0.96)$$

where MW is the expected mature live BW (kg), BW is the current BW (kg), and WG is the daily weight gain (kg day^{-1}).

The requirement for absorbed calcium for pregnancy is negligible until the last trimester. At 190 and 280 days of gestation, accretion rates of calcium are 2.3 and 10.3 g day^{-1}, respectively. These amounts equal the requirements for pregnancy. The amount of absorbed calcium required per kg of milk is dependent on the protein content of milk and is therefore breed dependent; it is approximately 1.22 g kg^{-1} milk for Holstein-Friesian cows, 1.45 g kg^{-1} milk for Jersey cows, and 1.37 g kg^{-1} milk for other breeds. Cows require about 2.1 g of absorbed Ca per kg of colostrum produced.

Calcium Absorption

The requirements outlined above refer to calcium absorbed from the intestine into blood. Therefore, the amount of dietary calcium that must be fed to meet the requirement for absorbed calcium is dependent on the amount and availability of calcium from the feedstuffs and inorganic supplements being offered and the efficiency of intestinal calcium absorption in the animal being fed.

The estimates for calcium absorption and availability vary between 25 and 70% of total calcium in feed. Forages tend to be reasonable sources of calcium, with temperate and tropical legumes containing >1.0% Ca (as percent dry matter (DM)) and temperate and tropical grasses containing around 0.4% calcium. In comparison, most concentrates are very low in calcium.

Determining the availability of dietary calcium is not as simple as estimating total calcium content. The proportion of dietary calcium absorbed will decrease as dietary calcium increases above requirements. To truly test the efficiency of absorption of calcium in a feedstuff, the animals being tested should be fed less total dietary calcium than the amount of absorbed calcium required to meet their needs.

The Agriculture and Food Research Council plotted all absorption coefficients for calcium in sheep and reported no evidence to indicate that the absorption coefficient of calcium for any feed could not be raised when necessary; they therefore suggested an average absorbability of calcium of 0.68 for all feeds, including forages. However, subsequent research has indicated much lower absorption of calcium from forage diets, particularly in animals with a large calcium demand (e.g., lactating cow). The National Research Council disagreed with the appropriateness of a single coefficient of calcium absorption, and instead recommended separating the efficiency of calcium absorption into forages (0.30), concentrates (0.60), and mineral compounds ($CaCO_3$ – 0.75; $CaCl_2$ – 0.95). However, their figure for efficiency of absorption of calcium from forages may unduly penalize temperate grasses, as their forage of choice was alfalfa, in which 30–40% of calcium is bound with oxalate.

Physiological State

The capacity to absorb calcium varies with physiological state. Young animals absorb calcium efficiently, and this efficiency declines with age. In early lactation, all cows tend to be in negative calcium balance for 6–8 weeks, after which increased intestinal absorption and increased DMI result in cows entering a positive calcium balance. Intestinal absorption of calcium increases by 50–60% from the week before to the week after calving.

Age and Breed

Periparturient hypocalcemia and its clinical manifestation, milk fever, are more prevalent in older cows and cows of certain breeds (Channel Island Breeds, Scandinavian Red) (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever). The reasons for this increased susceptibility remain unclear. Some of the difference may be a result of differences in calcium output in colostrum; older cows produce more colostrum than younger cows, and cows from breeds susceptible to milk fever tend to produce milk with greater protein concentrations, and therefore greater calcium concentrations, than other less susceptible breeds. There is also some evidence that intestinal receptors for vitamin D decline with age, and that Jersey cows have fewer vitamin D receptors than Holstein-Friesian cows, making older cows and Jersey cows less physiologically able to adapt quickly to a severe calcium shortage (as happens around calving). It is also known that the ability to resorb bone calcium diminishes with age.

Dietary Influence on Absorption

Dietary calcium concentration adversely affects the proportion, but not the amount, of calcium absorbed. This is because the parathyroid-dependent active calcium absorption process is regulated by blood calcium, the animal only absorbing sufficient calcium to maintain eucalcemia. A deficiency of dietary magnesium can also result in hypocalcemia. Magnesium is an essential cofactor in the production of PTH, and increases tissue sensitivity to PTH. A cow that is hypomagnesemic will, therefore, have both a blunted PTH response to a hypocalcemic challenge and a reduced effect of PTH at target tissues. Dietary calcium to phosphorus ratio was once regarded as an important variable in determining calcium absorption. Recent data indicate that the ratio is not critical, unless calcium to phosphorus ratio is >7:1 or <1:1. Excess dietary phosphorus is probably more important than the calcium to phosphorus ratio, as it interferes with the renal activation of vitamin D_3.

Dietary concentrations of metabolically strong cations (sodium and potassium) and anions (chlorine and sulfur) also affect calcium homeostasis, primarily through their effect on acid–base balance. Cations increase blood pH and interfere with the integrity of the PTH receptor. A diet with a greater preponderance of anions to cations (a negative dietary cation–anion difference (DCAD)) reduces blood pH and improves the hypercalcemic response to PTH. There is also some evidence that anions improve calcium homeostasis through a non-DCAD route.

Although all ions possess these acidifying and alkalizing properties, differences in the actual effect on pH as well as differences in the absorbability of the ions have led to debate about the correct equation to use when determining the DCAD. Recent meta-analyses have concluded that the equation

$$\begin{aligned}\text{DCAD} = (&\text{sodium} + \text{potassium}) - (\text{chlorine} + \text{sulfur}),\\ &\text{meq per } 100\,\text{g DM}\end{aligned}$$

is the most appropriate equation for determining a diet's effectiveness in preventing milk fever. This is therefore the most accepted equation for calculating DCAD.

There has been also some concern that the addition of fat or oil to the dairy diet can reduce calcium availability through formation of calcium soaps of fatty acids. This is unlikely as the calcium has to be dissociated from the fatty acid in order for the fatty acid to be absorbed. Since absorption of fatty acids is extensive, it is obvious that calcium is not irretrievably bound to fatty acids in the intestine. Further evidence of this is the very high digestibility (80–90%) of the fat in palm oil products, despite the fact that the calcium soap can be 7–9% calcium.

Ration Requirements

Most lactation diets should contain between 0.6 and 0.7% calcium (on a DM basis). The requirement, expressed as a percentage of the diet DM, can reach as high as 1.0% in the first weeks of lactation when DMI is still relatively low. This is particularly important during the colostrum period, when the animal is at greatest risk. Calcium mobilized from bone can provide a significant amount of the calcium requirement through the negative calcium balance period, subsequent to the colostrum challenge, and it should not be necessary to formulate diets especially high in calcium during early lactation. Concentrations of 0.45–0.5% are sufficient for dry cows.

Chlorine

Chlorine is one of the least studied of the macrominerals. Chlorine will likely be in adequate supply when sodium chloride salt is supplemented in amounts sufficient to meet sodium needs. If no salt is supplemented, it is possible that both sodium and chlorine could be deficient. Forages contain 0.2–3.0% chlorine kg^{-1} DM, but most grains contain only 0.1–0.15% chlorine kg^{-1} DM. Typical concentrations of chloride in blood plasma, rumen fluid, and milk are 100, 20, and 25 mmol l^{-1}, respectively.

Chlorine Requirements

The maintenance requirement for absorbable chlorine is 2.25 g per 100 kg BW, with 1.0 g chlorine kg^{-1} daily BW gain required for growth. The requirement for pregnancy is negligible until 190 days of gestation, and it increases to 1.0 g chlorine day^{-1} from 190 days to parturition. The concentration of chlorine in milk is approximately 1.15 g kg^{-1} and is independent of diet (except in severe deficiency), although the concentration can vary with the stage of lactation (greater in colostrum and late lactation) and environmental temperature (increasing with environmental temperature). The coefficient of absorption is estimated to be 90% for all feedstuffs and supplemental sources.

Ration Requirements

Diets containing 0.3–0.4% DM chlorine will meet the requirements of lactating cows during the first 6 weeks of lactation, and thereafter 0.25–0.3% DM chlorine should suffice.

Magnesium

Magnesium is abundant in most common feedstuffs. About 70% of the body's magnesium is in the skeleton, with the remainder contained in cells (29%) and ECFs (1%). Magnesium is a major intracellular cation and an essential cofactor in enzymatic reactions vital to every metabolic pathway. It is also important in normal nerve and muscle function, and is part of bone mineral formation. Normal blood plasma and milk concentrations range from 0.75 to 1.0 and 5 to 6.25 mmol l^{-1}, respectively.

Magnesium Absorption

Magnesium is absorbed primarily from the small intestine in calves and from the rumen and reticulum in adult cattle. Absorption from The rumen is dependent on the concentration of magnesium in ruminal fluid and the integrity of the active transport mechanisms.

The concentration of magnesium in ruminal fluid is dependent on dietary magnesium content, magnesium solubility, and possible interacting compounds. The solubility of magnesium declines dramatically with increasing pH. *In vitro* studies have reported a reduction in magnesium solubility from 80 to 20% with an increase in rumen fluid pH from 5 to 7. Similarly, as rumen pH increased from 5.6 to 7.2 in dairy cows, magnesium concentrations in rumen fluid declined from 6 to 0.5 mmol l^{-1}.

Magnesium is absorbed from the rumen by two active transport mechanisms across an electrochemical gradient. These mechanisms are sodium dependent and are inhibited by potassium. Potassium can increase the potential difference across the rumen and infusions of potassium in the rumen reduce magnesium absorption. In comparison, abomasal or ileal infusions of potassium do not reduce magnesium absorption. However, the effect of both sodium and potassium will be dependent on the accompanying anion. For example, the alkalizing effect of bicarbonate contributes to the negative effect of potassium supplements on magnesium absorption when compared with the acidifying effect of chloride.

There is postruminal absorption of magnesium, but it was traditionally believed that this process was inefficient, hard to saturate, and was unable to compensate for compromised ruminal absorption. Considerable research over the last 20 years has brought that conclusion into question, with several studies reporting significant postruminal absorption of magnesium. These studies have also highlighted that the potassium-induced reduction in magnesium absorption was independent of magnesium intake, implying that the negative effect of a high-potassium diet could be offset by increasing dietary magnesium concentration. The most recent edition of National Research Council's, *Nutrient Requirements of Dairy Cattle* reports that previous recommendations of maximum tolerable dietary magnesium concentrations were probably conservative, and so increased dietary magnesium may be a viable mechanism to overcome surplus potassium in predominantly forage rations.

Genetics

There is considerable evidence of a genetic effect on magnesium metabolism, with beef and dairy breeds showing marked differences in plasma magnesium concentrations, when supplied with similar concentrations of dietary magnesium. This is consistent with species differences in the ability to absorb magnesium (sheep are 1.75 times more efficient at absorbing magnesium than cattle) and consistent with an interaction between dietary potassium concentration and species on plasma magnesium concentration. Among dairy breeds and strains, North American Holstein-Friesian cows have consistently lower plasma magnesium concentrations than Holstein-Friesian cows of New Zealand origin with similar milk-producing ability.

Magnesium Availability

In reviewing the literature on a wide variety of natural feedstuffs, the Agricultural Research Council determined that the absorption coefficient for magnesium was 29.4 ± 13.5% (mean ± standard deviation). Because of the danger associated with overestimating magnesium absorption, the coefficient of absorption of magnesium from natural feedstuffs was assigned a value one standard deviation below the mean (i.e., 16%). In comparison, the coefficient of absorption from inorganic sources is set at 50%. The particle size of magnesium oxide, however, can have major effects on magnesium solubility; magnesium oxide particles should be $<250\,\mu$m in diameter to ensure 50% availability of magnesium, but solubility declines with increasing ruminal pH.

Magnesium Requirements

There is general agreement that fecal loss of magnesium is 0.3 g per 100 kg BW and obligate urinary loss of magnesium is negligible (although surplus magnesium is excreted via this route). Magnesium content in the tissue of growing cattle is 0.45 g kg^{-1} BW gain, and milk contains 0.12 g magnesium kg^{-1}. It should be noted, however, that although the magnesium content of milk is low, it is maintained during dietary depletion, and is a continuous drain on maternal plasma magnesium. As with other minerals, the requirement for fetal development (pregnancy) is negligible until day 190 of gestation and increases to 0.33 g day^{-1} subsequently.

Ration Requirements

Based on these requirements and previously mentioned absorption coefficients, recommendations for dietary magnesium are 0.18–0.20% DM.

Pasture-Based Cows

At the recommended levels of magnesium, a dietary magnesium deficiency is rarely a problem, with the exception of cows grazing temperate pastures. The term 'grass tetany' or 'grass staggers' has been applied to the convulsive condition resulting from subnormal serum magnesium concentrations occasionally experienced in cows grazing pastures during spring (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Grass Tetany).

Intensification of grassland production through increased use of nitrogen and potassic fertilizers, as well as excessive applications of farm yard manure, provides pastures with very high levels of nitrogen and potassium. The resultant ingestion of such pasture results in increased concentrations of ammonia and potassium in the rumen fluid, raising rumen pH and reducing magnesium solubility, and reducing the functionality of the active magnesium transport mechanism. Additionally, lush high-moisture forages increase the rate of passage of material from the rumen, preventing magnesium concentrations within rumen fluid from reaching critical values required to fully saturate absorption sites (about 11 mmol l^{-1}).

However, despite all these complications that grazing cows endure, by far the greatest factor influencing this metabolic condition is inclement weather conditions. Periods of high rainfall tend to reduce grazing time and, ultimately, DMI. Dairy cows have access to very little stored magnesium, and as magnesium continues to be secreted in milk, in an almost obligatory fashion to nurture the neonate, plasma concentrations of magnesium decline rapidly and hypomagnesemia ensues. For these reasons, it is recommended that grazing cows are supplemented daily with 20–25 g magnesium per cow.

Phosphorus

Approximately 80% of phosphorus is found in bones and teeth, with the remaining 20% involved in numerous functions throughout the body. Phosphorus concentrations in blood plasma are usually 1.3–2.6 mmol l^{-1}. Phosphorus is also required by the rumen microorganisms; diet digestibility and microbial synthesis can be reduced at low concentrations of dietary phosphorus. Estimates of minimal dietary phosphorus for normal rumen function vary, but probably fall within the range of 0.25–0.35% DM.

Phosphorus Absorption

Passive absorption from the small intestine is the predominant mechanism for phosphorus absorption, although there is a vitamin D-dependent mechanism to increase absorption when low-phosphorus diets are fed. Synthesis of 1,25-$(\mathrm{OH})_2\mathrm{D}_3$ can be stimulated by low blood phosphorus, enhancing intestinal absorption. Excess phosphorus is predominantly excreted in feces, with only 2–5% of consumed phosphorus excreted in urine.

Phosphorus Requirements

Net phosphorus retention is <1 g day^{-1}; therefore, maintenance requirements for phosphorus are approximately equal to fecal phosphorus output. Consequently, maintenance requirements for phosphorus can be related to DMI, as about 50% of fecal phosphorus is derived from microbial mass, which is directly related to fermentable energy intake. The maintenance requirement for phosphorus is about 0.1% DMI.

An allometric equation developed by the Agriculture and Food Research Council for growing cattle recommends that the amount of absorbed phosphorus required daily is

$$\mathrm{P}\left(\mathrm{g\,day}^{-1}\right) = \left(1.2 + \left(4.635 \times \mathrm{MW}^{0.22}\right) \times \left(\mathrm{BW}^{-0.22}\right)\right) \\ \times (\mathrm{WG}/0.96)$$

where MW is the expected mature live BW (kg), BW is the current BW (kg), and WG is the daily weight gain (kg day^{-1}).

As for most minerals, the phosphorus requirement for pregnancy is very low until the last trimester. The requirement for phosphorus then increases through the final trimester, and is described by the model

$$\text{absorbed phosphorus } \left(\mathrm{g\ day}^{-1}\right) \\ = 0.027\,43\mathrm{e}^{(0.055\,27 - 0.000\,075\,t)t} \\ - 0.027\,43\mathrm{e}^{(0.055\,27 - 0.000\,075\,(t-1)(t-1))}$$

where t is the day of gestation (≥190). This equates to an increasing absorbed phosphorus requirement for pregnancy of 1.8 g day^{-1} at 190 days of gestation to 5.4 g day^{-1} at 280 days. The requirement for milk production is equal to the amount of phosphorus in milk and has been set at 0.9 g phosphorus kg^{-1} milk, although concentrations will be modified slightly by the fat and protein content of milk.

Phosphorus Availability

To relate the absorbed phosphorus requirement to dietary phosphorus, phosphorus availability must be known. As with calcium and magnesium, the estimates of availability vary. The National Research Council recommends use of the following phosphorus absorption coefficients: forages – 64%; concentrates – 70%; dicalcium phosphate – 75%; bone meal – 80%; and monosodium phosphate – 90%. Phytate phosphorus, the main form of phosphorus in most grains, is almost totally available to ruminants because of phytase production by rumen microbes.

Ration Requirements

Based on the requirements and absorption coefficients, recommendations for dietary phosphorus are 0.32–0.42% DM. The National Research Council recently reviewed the published literature to assess the effects of dietary phosphorus on reproductive performance of cattle. Although a number of studies have demonstrated improved reproductive performance in cattle when diets were fortified with phosphorus, typically dietary phosphorus concentrations were very low (<0.2% DM), the deficient diet was fed for an extended length of time (1–4 years), and, where measured, DMI was also depressed. There was no evidence of a direct effect of phosphorus on reproductive performance. They concluded that reproduction will not be improved by surpassing recommended dietary inclusion rates for phosphorus.

Environmental Consideration

Dietary phosphorus concentrations must also be considered from an environmental standpoint. Surveys in many parts of the United States indicate that dairy producers typically formulate dairy diets to contain 0.45–0.50% phosphorus (i.e., 20–25% greater than the recommended requirements). There is no evidence to support this inclusion level of phosphorus in the diet for milk production or DMI, and the hypothesis that high dietary phosphorus will improve reproductive performance has also been discounted. It is therefore incumbent upon all dairy producers to remove excess phosphorus from diet formulations to minimize the risk of environmental damage.

Potassium

Potassium is the third most abundant mineral element in the body, and must be supplied on a daily basis because there is little storage in the body. In comparison to sodium and chloride, which are the major extracellular electrolytes in the body, potassium is the major intracellular electrolyte. It is involved in the regulation of osmotic pressure and acid–base balance, water balance, nerve impulse transmission, and muscle contraction, and is an activator or cofactor in many enzymatic reactions. Potassium consumed in excess is primarily excreted in urine.

Potassium Requirements

The maintenance requirement for absorbed potassium, like all minerals, is the sum of the endogenous losses in feces and urine when animals are fed very near the true requirement. The maintenance requirements for growing animals and nonlactating pregnant cows are estimated to be 3.8 g per 100 kg BW (endogenous urinary losses) and 2.6 g kg^{-1} DMI (endogenous fecal loss). For lactating cows, values of 3.8 g per 100 kg BW (endogenous urinary losses) and 6.1 g kg^{-1} DMI (endogenous fecal loss) are used, the greater requirement justified on the basis of potassium's role in ruminal function and acid–base balance. Furthermore, an additional 0.04 g potassium per 100 kg BW is considered part of maintenance requirements at environmental temperatures between 25 and 30 °C, with an additional 0.36 g potassium per 100 kg BW (i.e., a total of 0.40 g of additional potassium per 100 kg BW) suggested when environmental temperatures exceed 30 °C. The potassium requirements for growth, pregnancy, and lactation are 1.6 g kg^{-1} BW gain, 1.027 g day^{-1} (>190 days of gestation), and 1.5 g kg^{-1} milk, respectively.

Ration Requirements

Potassium is absorbed in the duodenum by simple diffusion, and has an absorption coefficient of about 90%. Expressing these requirements as a percentage of expected DMI, dry cow diets should contain about 0.5% DM potassium and lactating cows about 1.2% DM. There is some evidence of an increase in DMI and milk yield up to dietary potassium concentrations of 1.5% DM in cool seasons and 2% DM in the warm season.

Considerations for Grazing Animals

Almost all rations will inherently contain the minimum 1.5% DM potassium, and in general, forages contain more potassium than concentrates. In grazing systems however, dietary potassium concentrations are often 2–3 times greater than the recommended concentrations. This has implications for magnesium absorption and the risk of hypomagnesemia, and in manipulating cation–anion differences during the transition period.

A further complication is the significant variability in pasture potassium concentrations across a grazing area, with nutrient gradients from differences in stocking intensity and grazing behavior, inaccuracies in fertilizer distribution, and concentrated potassium in urine patches, all contributing to differences in pasture potassium concentration. Any attempt to 'balance' potassium, as in the DCAD concept, is rendered almost impossible because of this inter-grazing area variability.

Farmers and their advisers must be considerate of these two significant risks when deciding on the timing and rate of application of potassium fertilizers.

Sodium

Sodium is the primary extracellular cation, but as much as 30–50% of total body sodium is in a nonexchangeable fraction in the crystalline structure of bone. Typical concentrations in blood plasma and milk are 150 and 27 mmol l^{-1}, respectively, and 160–180 mmol l^{-1} in saliva. As dairy cattle evolved under conditions of low sodium intake, they have developed very efficient mechanisms to absorb, utilize, and recycle sodium. Sodium is absorbed throughout the digestive tract and is generally regarded to be 90% available from common feedstuffs and 100% available from inorganic compounds. Consequently, there is little sodium excreted in feces, with the primary route of excretion, as for potassium, being urine.

Sodium Requirements

The maintenance requirement for absorbed sodium is 1.5 g per 100 kg BW for growing cattle and nonlactating pregnant cows, and 3.8 g per 100 kg BW for lactating cows. At environmental temperatures between 25 and 30 °C, maintenance requirements for sodium increase by 0.1 g per 100 kg BW and by a further 0.4 g per 100 kg BW when environmental temperatures exceed 30 °C (i.e., 0.5 g sodium per 100 kg BW in total).

The requirement for growth is 1.4 g sodium kg^{-1} daily BW gain (between 150 and 600 kg BW). As with other mineral elements, requirements for pregnancy prior to day 190 of gestation are negligible. Requirements for absorbed sodium for pregnancy after day 190 of gestation are 1.39 g day^{-1}. Milk production makes up the largest component of total sodium requirement with 0.63 g sodium kg^{-1} milk.

Ration Requirements

Although there is evidence that DMI and milk yield are improved by dietary concentrations of sodium well above those needed to meet requirements, this effect is confounded by the concentration of potassium, chloride, calcium, and phosphorus in the diet. Sodium content of most feeds is low, with grains typically containing 0.01–0.05% DM sodium and forages containing 3–5 times this amount. Therefore, a high-forage ration (e.g., grazing cows) should provide sufficient sodium to meet requirements. If concentrates or other low-sodium feeds constitute more than 20–30% of the ration, most growing cattle and certainly lactating cows will be deficient in sodium if not supplemented. Removal of salt supplement from such lactating cow diets can result in sodium deficiency and intense craving for salt within 2–3 weeks. The recommendation in balanced rations is for dietary sodium concentrations of 0.30–0.35% for the first several weeks of lactation. As lactation progresses, the dietary requirement is 0.22% DM.

Special Consideration

The maximum tolerable dietary concentration of sodium is believed to be 1.6% DM, and this is dependent on a supply of clean drinking water. Drinking water that contained 12 000–25 000 $\mathrm{mg\,l}^{-1}$ sodium chloride was toxic to growing cattle, but a concentration of 10 000 $\mathrm{mg\,l}^{-1}$ was tolerable.

Sulfur

Sulfur is an essential element only for plants and microbes. They synthesize sulfur amino acids, and hence proteins, from inorganic sulfur sources. The requirement for sulfur for ruminants, therefore, is actually a ruminal microbe requirement. Elemental sulfur is not well utilized by rumen microbes, but cysteine, sulfides, and sulfates are readily used. The nitrogen to sulfur ratio found in rumen microbes is about 15–20, and close to 15 for animal tissues and products.

The situation where sulfur is most likely to be limiting is when low-protein dairy diets are supplemented with nonprotein nitrogen, such as urea. Urea or other nonprotein nitrogen sources do not contain sulfur, as most all protein sources do. In general, the sulfur content of feedstuffs is directly related to protein content.

Sulfur Requirements

The results of animal feeding trials have indicated that a dietary nitrogen to sulfur ratio of 1–12 will support maximum microbial growth in the rumen. The National Research Council has set the requirement for sulfur at 0.2% of dietary DM. Sulfur is seldom limiting for ruminal microbes with modern dairy diets. An upper level of 0.5% of DM has also been set, to avoid the risk of polioencephalomalacia. However, dietary concentrations greater than 0.5% DM sulfur has been fed to nonlactating dairy cows with no ill effect. Nevertheless, sulfur is a mineral that must be viewed with caution, as a narrow range exists between deficiency and toxicity. All feed sources (including water) should be evaluated for sulfur content, before supplementary sulfur is offered.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Grass Tetany; Non-Infectious Diseases: Milk Fever.

Further Reading

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Feed Supplements: Microminerals

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Introduction

At least eight trace minerals can be classified as essential for cattle, based on identification of one or more metabolic functions. Many trace minerals function as essential cofactors for enzymes involved in biochemical processes in the body. Other trace elements are involved in normal functioning of hormones or vitamins. A severe deficiency of each essential mineral results in abnormalities that can be corrected by only supplementation of the deficient mineral. Even marginal trace mineral deficiencies can reduce growth, milk production, reproduction, or health in cattle showing few, if any, clinical signs of deficiency. Trace mineral deficiencies can be primary or secondary in nature. A primary deficiency results from a low concentration of a given trace mineral in the diet. In contrast, a secondary deficiency is not caused necessarily by a low dietary concentration of a mineral but by other factors, such as other nutritional deficiencies or parasitic or infectious diseases. When present in diets, either naturally or via supplementation, at high concentrations some essential trace minerals can cause toxicity problems and possibly induce a deficiency of another trace mineral.

Trace Mineral Requirements

Dietary trace mineral requirements for early lactating cows, dry dairy cows and growing heifers are shown in **Table 1** The trace mineral levels indicated in **Table 1** should not be considered concrete values because mineral requirements can be affected by a number of factors such as breed and bioavailability of the mineral from the diet. Requirements for certain trace minerals are greatly affected by bioavailability of the mineral from the diet. Low bioavailability of a mineral from the diet may relate to the chemical form of the mineral or to the presence of high concentrations of other minerals and/or other dietary components that reduce absorption or utilization. Factors that may affect trace mineral requirements will be discussed in more detail in the sections on individual minerals.

Cobalt

Cobalt functions as an essential component of vitamin B_{12} (cobalamin). Vitamin B_{12} is required for two enzymes found in ruminant tissues. Methylmalonyl CoA mutase is involved in the metabolism of propionate to glucose. Methionine synthase is important in the recycling of methionine following transfer of its methyl group. In cattle, rumen microorganisms synthesize sufficient vitamin B_{12} from cobalt to meet animal requirements provided that adequate cobalt is supplied by the diet. Adequate cobalt is also necessary for optimal ruminal fermentation. Vitamin B_{12} is a growth factor for a number of ruminal microorganisms. Low dietary cobalt impairs ruminal fermentation by reducing ruminal production of vitamin B_{12}. In addition to active vitamin B_{12}, ruminal bacteria also produce a number of B_{12} analogs that are not active in animal tissues.

Depressed appetite and failure to grow or slight weight loss are early signs of cobalt deficiency. If the deficiency becomes severe, cattle exhibit (1) rapid weight loss and unthriftiness, (2) pale skin and mucous membranes resulting from pernicious anemia, (3) fatty degeneration of the liver, and (4) reduced disease resistance. Cobalt deficiency also alters ruminal fermentation resulting in impaired ability of microorganisms to convert succinate to propionate.

Dietary cobalt concentrations below $0.11\ \mathrm{mg\,kg^{-1}}$ diet for an extended period of time suggest cobalt deficiency. Vitamin B_{12} concentrations in the liver of $0.10\ \mu\mathrm{g\,g^{-1}}$ wet weight or less are indicative of cobalt deficiency. Serum or plasma vitamin B_{12} concentrations may be of limited value in assessing cobalt status because of the presence of B_{12} analogs in bovine blood. Cattle fed adequate cobalt have sufficient vitamin B_{12} stored in their liver to last for several months even if they are abruptly switched to a cobalt-deficient diet.

Cobalt sulfate and cobalt carbonate are the major cobalt sources supplemented to cattle diets and the two sources appear to be similar in bioavailability. Cobalt toxicity is unlikely to occur unless a major error is made in formulating a mineral supplement.

Table 1 Trace mineral requirements of dairy cattle

	Requirement ($mg\ kg^{-1}$ dry diet)		
Mineral	*Growing heifer*[a]	*Dry cow*	*Lactating cow*[b]
Cobalt	0.11	0.11	0.11
Copper	10	13	11
Iodine	0.27	0.40	0.50
Iron	43	18	15
Manganese	22	18	14
Selenium	0.30	0.30	0.30
Zinc	32	22	48

[a]Six-month-old heifer. [b]Holstein cow producing 35 kg milk day^{-1}.
From National Research Council (2001) *Nutrient Requirements of Dairy Cattle*, 7th edn. Washington, DC: National Academy Press.

Copper

Copper is an essential component of a number of enzymes including cytochrome oxidase, superoxide dismutase, lysyl oxidase, tyrosinase, and ceruloplasmin. These enzymes function in energy metabolism, detoxification of superoxide radicals, structural integrity of collagen and elastin, pigmentation, and iron transport.

In young ruminants, copper is well absorbed prior to rumen development. However, copper is poorly absorbed in ruminants with a developed rumen. Considerable amounts of copper can be stored in the liver and liver copper can be mobilized and used by other tissues if absorbed copper is insufficient.

Copper deficiency in cattle is a problem in many areas of the world. In most instances, copper deficiency results from the presence of other minerals (sulfur, molybdenum, and/or iron) in the diet that interfere with copper utilization rather than a simple lack of copper in the diet. Depigmentation or bleaching of hair is usually the earliest visual sign of copper deficiency. Loss of hair pigmentation in copper-deficient cattle is also associated with a rough hair coat. Other signs of deficiency that may be seen in copper-deficient cattle include (1) poor growth, (2) fragile bones, (3) anemia, (4) sudden death owing to heart failure, and (5) increased incidence of disease owing to reduced immunity. Diarrhea and low reproduction have been attributed to copper deficiency, but these appear to be more related to high intakes of molybdenum rather than copper deficiency *per se*. However, supplying additional supplemental copper will generally prevent or correct the adverse effects of high molybdenum.

Copper requirements vary greatly depending on other dietary components, especially molybdenum and sulfur. The copper requirements listed in **Table 1** should meet requirements if molybdenum is below 2 mg kg^{-1} diet and sulfur is less than 0.30%. If molybdenum is present in diets at levels of only 4–6 mg kg^{-1} diet, the amount of copper required by cattle may be increased by 50–100%. The antagonistic action of molybdenum on copper metabolism is greater when dietary sulfur is also high. Molybdate interacts with sulfide (produced from sulfate and sulfur-containing amino acids) in the rumen to form thiomolybdates. Copper can react in the gut with thiomolybdates to form insoluble complexes that are not absorbed or at least very poorly absorbed. Some thiomolybdates formed can be absorbed and affect systemic metabolism of copper. Thiomolybdates can result in copper being tightly bound to plasma albumin and not available for biochemical functions and they may directly inhibit certain copper-dependent enzymes by removing copper from the enzyme.

Sulfur reduces copper absorption, perhaps through the formation of copper sulfide in the gut, independent of its role in the molybdenum–sulfur–copper interaction. Copper present in copper sulfide is insoluble and unavailable for absorption by the animal. Studies have indicated that increasing dietary sulfur from 0.1 to 0.3 or 0.4% reduced copper absorption by 30–40%. High iron concentrations in feedstuffs may also lead to copper deficiency in cattle. A number of studies have shown that feeding levels of iron (250–1200 mg kg^{-1} diet) typical of those sometimes found naturally in feeds reduces copper status in cattle.

Concentrations of copper in feeds are of limited value in assessing copper adequacy unless concentrations of copper antagonists such as molybdenum, sulfur, and iron are also considered. Liver copper levels less than 20 mg copper kg^{-1} dry weight or plasma concentrations less than 0.5 μg copper ml^{-1} are indicative of copper deficiency. However, copper in liver and plasma may not accurately reflect copper status if dietary molybdenum and sulfur are high, because the copper can exist in tightly bound forms unavailable for biochemical functions.

Available sources of copper that may be supplemented to ruminant diets include copper sulfate, copper carbonate, tribasic copper chloride, and various organic copper compounds. Tribasic copper chloride and certain organic forms of copper appear to be more bioavailable than copper sulfate when dietary molybdenum and sulfur are high. Because of its low solubility, even under acid conditions in the abomasum, copper oxide is very poorly available in cattle and should be avoided in mineral supplements. Copper oxide needles, which remain in the reticulo-rumen and slowly release copper over a period of months, have been used successfully to provide long-term copper supplementation in grazing cattle.

Copper toxicity can be a problem in dairy cattle. Diets containing as little as 30–40 mg copper kg^{-1} diet may result in toxicity signs in adult animals if fed for a prolonged period of time. Young calves are even more susceptible to copper toxicity because of their greater ability to absorb dietary copper. When cattle consume diets high in copper, the liver accumulates high concentrations of copper before toxicosis becomes evident. The sudden release of copper from the liver into the blood results in signs of toxicity characterized by hemolysis, jaundice, methemoglobinemia, hemoglobinuria, widespread necrosis, and frequently death.

Iodine

Iodine functions as a component of the thyroid hormones thyroxine and triiodothyronine. The thyroid hormones regulate the rate of energy metabolism in the body and are required for growth and development in young animals.

Dietary iodine is well absorbed in the form of iodide from the rumen and small and large intestine. Absorbed iodide is primarily taken up by the thyroid gland for thyroid hormone synthesis or excreted in urine and milk. When iodine intake is low or marginal with regard to requirements, a higher percentage of absorbed iodide is taken up by the thyroid gland. Milk generally contains between 30 and 300 μg iodine l^{-1} and the iodine content of milk increases as iodine intake increases.

The first sign of iodine deficiency is usually enlargement of the thyroid gland (goiter) in the newborn calf. Iodine deficiency can also result in calves being born hairless, weak, or dead. Adult cattle are less likely to show clinical signs of iodine deficiency. However, iodine deficiency may cause reduced reproduction in cows characterized by irregular cycling, low conception rate, and retained placenta, and decreased libido and semen quality in males. Protein-bound iodine in serum, thyroid gland weight in newborns, and milk iodine have been used to assess iodine status.

Iodine requirements vary from 0.27 mg kg^{-1} diet in nonlactating cattle to 0.50 mg kg^{-1} diet in lactating dairy cows. However, goitrogenic substances (substances that induce goiter) in certain feeds may increase iodine requirements by two- to fourfold depending on the amount and type of goitrogens present. Cyanogenetic goitrogens can be found in white clover, millet, raw soybeans, sweet potatoes, and maize. They impair iodine uptake by the thyroid gland and their effect can be overcome by increasing dietary iodine. The thiouracil goitrogens found in turnips, rape, kale, and cabbage inhibit iodination of tyrosine residues in the formation of thyroid hormones. The action of thiouracil goitrogens is more difficult to reverse through iodine supplementation and the responsible feedstuffs may need to be removed from the diet.

The iodine content of feeds varies greatly depending on the iodine content of the soil. Most supplemental sources of iodine are readily available for absorption by the animal. Iodine is usually supplemented in diets or in free-choice minerals as ethylenediamine dihydroiodide (EDDI) or calcium iodate. Iodide forms such as potassium and sodium iodide are less stable in mineral supplements and considerable losses can occur as a result of heat, moisture, light, and exposure to other minerals. At concentrations well in excess of dietary requirements, EDDI has been used in cattle to prevent foot rot. In the United States, currently 10 mg of iodine from EDDI is the maximum amount that can be fed per head per day. Feeding higher levels of EDDI can result in iodine concentrations in milk and meat that are unsafe for human consumption.

Iron

Iron functions in oxygen transport in the blood as a component of hemoglobin and in oxygen storage and transport in muscle as a component of myoglobin. Iron is also an integral component of a number of cytochromes and iron–sulfur proteins involved in the electron transport chain, which is responsible for oxidative production of energy in cells. Several enzymes also either contain iron or are activated by iron. Over 50% of the iron in the body is present in hemoglobin, with smaller amounts found in other iron-containing proteins and enzymes, and in protein-bound stored iron.

A deficiency of iron results in anemia, listlessness, reduced feed intake and weight gain, atrophy of the papillae of the tongue, and pale-colored muscle and mucous membranes. Iron-deficient animals are also more susceptible to infectious diseases. Milk is low in iron and young calves housed in confinement and fed exclusively milk for an extended period can develop iron deficiency. Most practical feedstuffs are more than adequate in iron, and iron deficiency is unlikely in cattle consuming dry feeds unless parasite infestations or diseases that cause chronic blood loss exist. Only small amounts of absorbed iron are lost in urine or feces in the absence of blood loss. When adequate iron is present in the diet, considerable storage of iron also occurs in the liver.

In young calves fed milk diets, 50 mg iron kg^{-1} dry diet is adequate to support growth and prevent anemia. The iron requirements of older cattle are much lower than in young calves because considerable recycling of iron occurs when red blood cells turn over, and in older animals blood volume is not increasing or at least not to the extent that it is in young animals.

Cattle grazing on pastures or being fed harvested silage or hay may be exposed to excessive levels of iron through forage, water, or soil ingestion. The iron content of forages is highly variable but most forages contain from 70 to 500 mg iron kg^{-1}. Water and soil ingestion can also be significant sources of iron for cattle. Iron from soil is probably of poor availability; however, research has indicated that a significant amount of iron from various soil types was soluble in rumen fluid. Iron toxicity signs include diarrhea, reduced gain and feed intake, reduced milk production, metabolic acidosis, and hypothermia. The maximum tolerable concentration of iron for cattle has been estimated at 500 mg iron kg^{-1} diet by the National Research Council. As discussed earlier, dietary iron concentrations as low as 250–500 mg kg^{-1} diet have caused copper depletion in cattle.

Manganese

Biochemically, manganese functions as an integral component of the enzymes pyruvate carboxylase, arginase, and superoxide dismutase. In addition, several enzymes, including a number of kinases, transferases, hydrolyases, and decarboxylases, can be activated by manganese. Of the enzymes that can be activated by manganese, only the glycosyltransferases are known to specifically require manganese. Most of the other enzymes activated by manganese can also be activated by other metals especially magnesium. Glycosyltransferases are involved in the synthesis of mucopolysaccharides and, thus, are required for formation of skeletal cartilage.

Calves born to cows deficient in manganese exhibit skeletal abnormalities characterized by stiffness, twisted legs, enlarged joints, and short leg bones with reduced breaking strength. Dwarfism, unsteadiness, and a shortening of the nasomaxillary bones, causing the lower jaw to appear extended, may also be seen in young calves deficient in manganese. Cows deficient in manganese have impaired reproductive performance characterized by depressed or irregular estrus, low conception rate, and increased incidence of abortion and embryonic death.

Manganese is poorly absorbed. In lactating dairy cows, less than 1% of the manganese supplied from radiolabeled manganese chloride was absorbed. High dietary calcium and phosphorus increase excretion of manganese in feces. Manganese absorbed in excess of tissue is excreted largely via the bile with only small losses via urine.

Manganese requirements of dairy cattle vary from 14 to 22 mg kg^{-1} diet depending on age and physiological stage of productivity. Dietary factors that may affect manganese requirements are not well defined. Forages generally contain adequate manganese to meet animal requirements, assuming that the manganese is available for absorption. Maize silage can be low, or at least marginal, in manganese. While cereal grains contain 5–40 mg manganese kg^{-1}, and that in maize is especially low. Manganese is normally supplemented to ruminant diets as manganese sulfate or manganese oxide. The availability of manganese from manganese oxide is approximately 60% of that available from manganese sulfate.

Toxicity of manganese is not likely to occur in cattle. Young calves supplemented with 1000 mg manganese kg^{-1} diet show reduced weight gain and feed efficiency. However, older cattle have been shown to tolerate a level of 1000 mg manganese kg^{-1} diet for 100 days without any adverse effects.

Molybdenum

Molybdenum functions as a component of the enzymes xanthine oxidase, sulfite oxidase, and aldehyde oxidase. Cattle requirements for molybdenum are not established, but are obviously very low. There is no evidence that molybdenum deficiency occurs in cattle under practical conditions. However, molybdenum supplementation of ruminant diets has enhanced ruminal digestion in some studies, apparently by altering ruminal microorganisms.

Toxicity of molybdenum can be a problem in cattle. Acute toxicity signs, including severe diarrhea, loss of weight, anorexia, stiffness, and changes in hair color, may be observed in cattle fed diets containing molybdenum at concentrations of 20 mg or greater per kilogram. Molybdenosis can generally be overcome by providing large amounts of copper. Concentrations of molybdenum much lower than those needed to cause acute toxicity signs can result in copper deficiency, depending on the length of time the cattle are exposed and the concentration of dietary copper.

Molybdenum levels in forages vary greatly depending on soil type and soil pH. Neutral or alkaline soils with high moisture and organic matter favor molybdenum uptake by forages. Cereal grains and protein supplements are less variable in molybdenum than forages.

Selenium

Selenium first received attention because of problems associated with severe selenium toxicity. In 1957, selenium was shown to be an essential trace mineral and since that time it has become evident that selenium deficiency is a much greater problem than selenium toxicity.

In the early 1970s, selenium was identified as an essential component of glutathione peroxidase. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide and lipid hydroperoxides, thus preventing oxidative damage to body tissues. More recently, a second selenometalloenzyme, iodothyronine 5′-deiodinase, was identified. This enzyme catalyzes the deiodination of thyroxine (T_4) to the more metabolically active triiodothyronine (T_3) in tissues.

In young calves, selenium deficiency results in degeneration and necrosis in both skeletal and cardiac muscle. This condition is referred to as white muscle disease and affected animals may show stiffness, lameness, and even cardiac failure. Selenium deficiency in young calves has also been associated with unthriftiness, weight loss, and diarrhea. Selenium supplementation of diets low in selenium has decreased the incidence of retained placenta, metritis, cystic ovaries, and udder edema in dairy cows. The immune response is also reduced by selenium deficiency and a number of studies have indicated that low selenium status is related to greater prevalence and severity of mastitis in dairy cows.

A level of 0.3 mg selenium kg^{-1} diet is generally considered adequate to meet animal requirements. The functions of vitamin E and selenium are interrelated, and a diet low in vitamin E may increase the quantity of selenium needed to prevent certain abnormalities. High dietary sulfur and substances present in some legumes may also increase selenium requirements. In addition to dietary supplementation, alternative methods of supplementing selenium include injecting selenium at critical production stages or using boluses retained in the rumen that release selenium over a period of months.

Selenium concentrations in plasma, serum, and whole blood, and glutathione peroxidase activities in plasma, whole blood, or erythrocytes may be used to assess selenium status. Glutathione peroxidase activities, which are consistent with selenium deficiency, vary from one laboratory to another depending on storage of samples and assay conditions. Plasma or serum selenium concentrations between 0.07 and 0.10 $\mu\mathrm{g\,ml}^{-1}$ are considered adequate in dairy cows. Selenium that is naturally present in feeds affects selenium status in a way different from that with supplemental selenium, which is usually provided as sodium selenite. Selenomethionine, the major chemical form of selenium found in feeds, can be incorporated into nonspecific body proteins in place of methionine. Some natural selenium absorbed may end up in proteins other than enzymes that specifically require selenium. However, selenomethionine appears to be absorbed more efficiently than selenite, because it is believed that selenite is reduced to insoluble forms of selenium in the rumen that are poorly absorbed from the small intestine. When fed to ruminants deficient in selenium, selenomethionine is at least 40% more effective than selenite in increasing glutathione peroxidase activity.

Feedstuffs grown in many areas of the world are deficient or at least marginally deficient in selenium. The selenium content of forages and other feedstuffs varies greatly depending on plant species and the selenium content of the soil.

Selenium toxicity can occur as a result of excessive selenium supplementation or consumption of plants naturally high in selenium. Clinical signs of toxicity include lameness, sloughing of hooves, loss of hair, and emaciation. The maximum tolerable concentration of selenium has been estimated to be 5.0 mg selenium kg^{-1} diet.

Zinc

Zinc is a component of over 70 metalloenzymes found in mammalian tissues. Carbonic anhydrase, RNA polymerase, DNA polymerase, alcohol dehydrogenase, copper–zinc superoxide dismutase, and alkaline phosphatase are examples of enzymes that require zinc. Enzymes that require zinc are involved in nucleic acid, protein, carbohydrate, and lipid metabolism. Zinc is also important for normal development and functioning of the immune system and in cell membrane stability. Recent research has indicated that zinc plays a role in gene expression, and a number of transcriptional regulators have been shown to contain zinc.

Zinc absorption occurs primarily from the abomasum and small intestine in cattle. Absorption of zinc is homeostatically controlled and animals adjust the percentage of dietary zinc absorbed based on their need. If a diet is low or marginal in zinc, cattle will absorb a high percentage of dietary zinc assuming that dietary zinc is present in an available form.

Reduced feed intake is the earliest sign observed when cattle are fed a diet severely deficient in zinc. Other signs that can develop with prolonged zinc deficiency include reduced growth and feed efficiency, listlessness, excessive salivation, reduced testicular growth in males, swollen feet with scaly lesions, parakeratotic lesions that are most severe on the neck, legs, head, and around the nostrils, failure of wounds to heal, and loss of hair. Thymus atrophy and impaired immune response have been observed in Dutch-Friesian calves with a genetic disorder that results in an impaired ability to absorb zinc and consequently severe zinc deficiency. Plasma or liver zinc concentrations may be useful in diagnosing severe zinc deficiency, but plasma zinc is of little value in detecting marginal deficiencies. Stress or disease causes a redistribution of zinc in the body that can temporarily result in low plasma zinc concentrations characteristic of severe deficiency.

Zinc requirements vary depending on factors such as age, level of milk production, and bioavailability of zinc in the diet. Milk contains 3–5 mg zinc l^{-1}, but the increased demand for milk production is probably met by increased absorption, provided that dietary zinc is in a form that can be absorbed. Dietary factors that may affect zinc requirements in cattle are not well defined. High dietary calcium may reduce zinc absorption.

Supplemental forms of zinc used in diets include zinc sulfate, zinc oxide, and a number of organic zinc sources such as zinc methionine and zinc proteinate. Zinc in the sulfate and oxide form appears to be of similar bioavailability in ruminants.

The amount of zinc necessary to cause toxicity is much greater than requirements. The National Research Council recommended a maximum tolerable concentration of zinc of 500 mg kg^{-1} diet. Young calves fed milk replacer tolerated 500 mg zinc kg^{-1} for 5 weeks without adverse effects, but 700 mg zinc kg^{-1} reduced weight gain, feed intake, and feed efficiency. Feeding lactating dairy cows 1000 mg zinc kg^{-1} diet for 16 weeks did not affect feed intake or milk production. Zinc is supplemented at concentrations well above the recommended maximum tolerable level in some areas to prevent facial eczema. Facial eczema occurs in cattle grazing pastures infected with the fungus *Pithomyces chartarum* and is characterized by liver damage and photosensitization.

See also: **Feed Ingredients**: Feed Supplements: Macrominerals. **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows. **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins.

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Feed Supplements: Organic-Chelated Minerals

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Introduction

Organic trace minerals include a variety of different types of compounds. In broadest terms, they consist of a trace mineral ion bound to an organic molecule (typically an amino acid or carbohydrate). Trace minerals in plant material are present as inorganic (i.e., salts and ions) and organic (i.e., amino acid complexes and chelates) molecules; however, currently, very little is known about the bioavailability of these different 'natural' forms. A number of different classes of organic trace minerals are commercially available as trace mineral supplements for use in ruminant diets. In the United States, definitions of different supplements are used based on the manufacturing process (**Table 1**).

Bioavailability of Trace Minerals

It is known that inorganic trace mineral sources vary in bioavailability. It would be expected that bioavailabilities of different classes of organic supplements would also vary. However, attempting to correlate a laboratory analysis with *in vivo* results has been problematic. Bioavailability of a mineral is defined as the proportion of the ingested mineral that is absorbed, transported to its site of action, and converted to a physiologically active form. To be absorbed, a source must be soluble in the gastrointestinal tract; however, solubility in the rumen may be associated with formation of insoluble complexes that render the trace mineral unavailable in the small intestine. Research is continuing to attempt to characterize the stability of these organic trace minerals in the digestive tract. It is accepted that after absorption trace minerals are present in the body almost entirely as organic complexes or chelates and not as free inorganic ions; therefore, it has been suggested that dietary organic trace minerals could be more efficiently utilized.

Zinc

Zinc methionine has been studied to the greatest extent of any commercially available organic trace mineral in dairy cattle. This is due to the fact that zinc is an important component of many enzymes affecting the metabolism of proteins, carbohydrates, lipids, and nucleic acids. Therefore, zinc has important roles in milk synthesis, in tissue repair, and in the mammalian immune system. In addition, calcium, which is high in most legume forages, may interfere with the absorption of zinc. Copper and cadmium are also antagonists to zinc but are much less likely to pose a practical problem in dairy rations. Peptides and amino acids have improved intestinal absorption in some species by forming organic complexes with zinc. Zinc is typically much lower in forages (24 mg kg^{-1} in legume hay) than is required in the diet (63 mg kg $^{-1}$ diet) of high-producing dairy cows, so a dietary supplement is usually required. There is a wide variation in the zinc content of plant species, and flowering heads and leaves usually contain much more zinc than stems.

Zinc oxide and zinc sulfate have been the traditional supplements, but zinc lysine and zinc methionine are at least equally available, and there are many indications that zinc from the organic complexes is metabolized differently. Zinc methionine has provided the most rapid zinc repletion in deficient calves compared to zinc lysine and zinc sulfate. Stressed cattle have responded to supplemental zinc methionine compared to inorganic zinc, whereas in nonstressed cattle the source may have minimal effects. Levels of zinc in plasma are dynamic and fall dramatically during stress due to internal shifts to the liver.

In some experiments, dietary zinc methionine at concentrations of 4.5–9.0 g per cow per day has been shown to result in higher milk yield in cows. This has also reduced somatic cell counts in milk, improved hoof health of cows, improved recovery from viral infections (if evidence gathered from beef steers can be extrapolated to dairy cows), and perhaps improved reproductive efficiency too. From the summary of results from 10 experiments, the average increase in milk yield was 4.2% with zinc methionine in the ration compared with control cows fed equal amounts of inorganic zinc (**Table 2**). Improved hoof health apparently results from the ability of zinc to aid the repair of tissues. Zinc supplementation with equal amounts of zinc methionine and zinc sulfate has improved some reproductive traits (fewer days to first estrus, fewer days to first service, and a tendency for fewer retained placentas) in dairy cows, although it is possible that some benefits on reproductive performance occurred through indirect effects of improved protein metabolism, reduced lameness, and improved health of the cow.

Table 1 Brief description of different categories of organic trace minerals

Metal amino acid complex	A product resulting from complexing of a soluble metal salt with an amino acid(s)
Metal (specific amino acid) complex	A product resulting from complexing of a soluble metal salt with a specific amino acid
Metal amino acid chelate	A product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acids to form coordinate covalent bonds
Metal polysaccharide complex	A product resulting from complexing of a soluble salt with a polysaccharide solution
Metal proteinate	A product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein
Metal propionate	A product resulting from the reaction of a metal salt with propionic acid

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Table 2 Summary of results from 10 experiments comparing supplementation of zinc methionine with supplementation of zinc oxide in dairy cow diets

Treatment	*Milk yield (kg)*	*Milk protein (%)*	*Milk fat (%)*	*Milk somatic cell count (cells ml^{-1})*
Control	30.5^{a}	3.14	3.47	294 000^{b}
Zinc methionine	31.8^{b}	3.11	3.48	196 000^{a}

[a,b]Means within a column without a common superscript are different ($P \leq 0.001$).
Adapted with permission from Kellogg DW, Tomlinson DJ, Socha MT, and Johnson AB (2004) Review: Effects of zinc methionine complex on milk production and somatic cell count of dairy cows: Twelve-trial summary. *The Professional Animal Scientist* 20: 295–301.

There have been fewer experiments reported comparing zinc proteinate with inorganic zinc supplements for dairy cows. In one study, milk production and somatic cell count did not vary significantly between zinc proteinate and zinc oxide, but there were fewer new mammary infections with zinc proteinate. However, in another study, no effects of zinc proteinate versus inorganic zinc were observed with respect to milk production, somatic cell count, or rates of new mammary infections. A study with growing bulls found some improvement in claw quality with feeding organic zinc sources (proteinate or polysaccharide) versus zinc oxide, but no influence of zinc source on growth rate, feed intake, or efficiency.

Copper, Manganese, and Cobalt

In addition to zinc, most feeds for dairy cattle contain less copper, manganese, and cobalt than are required in the diets of high-producing cows.

Copper is needed for the activity of enzymes involved in the metabolism of bone tissue, in the regulation of reactive oxygen metabolites generated by cellular metabolism, in the utilization of iron, and in the pigmentation of hair. High molybdenum is especially antagonistic to copper, because insoluble copper thiomolybdates are formed in the rumen; therefore, copper will be needed in greater quantities than usual if even small amounts of molybdenum are present in the diet of cattle. Additionally, supplementation of zinc lowers blood copper concentration, probably because of competition for intestinal absorption. In a study with beef calves, copper lysine was as equally available as cupric sulfate. Two studies have been published in refereed journals that compared copper proteinate and copper sulfate in the diets of dairy cattle. In one of these studies, there were no detectable differences in the availability of the sources for Holstein or Jersey cows; however, in the other study using calves fed hay with high concentrations of molybdenum, copper proteinate supplementation resulted in greater liver copper concentrations than copper sulfate supplementation. In Holstein heifers that had been fed diets high in sulfur and molybdenum to deplete their liver copper, supplementing copper as an amino acid complex or as sulfate did not affect the rate of liver copper repletion when the repletion diets did not have additional sulfur or molybdenum.

Rumen microorganisms need cobalt to synthesize vitamin B_{12}. Carbonate, sulfate, and glucoheptonate forms of cobalt are supplemental sources that have been compared in the diets of sheep; however, to the best of authors' knowledge, there is no published research comparing cobalt sources in dairy cattle. In one study with growing and finishing beef steers, vitamin B_{12} status was positively correlated with dietary cobalt level, but there was no difference between two cobalt sources (cobalt carbonate and cobalt propionate) with regard to vitamin B_{12} status; however, some other metabolites were affected by the source of cobalt.

Manganese metalloenzymes are critical for energy metabolism and bone growth. Manganese is also involved in reproductive efficiency of cows, and conception is reduced when diets, usually involving high maize silage, are low in manganese. When manganese is deficient, puberty may be delayed, estrus is suppressed, follicular development is poor, conception rates are reduced, there is an increased incidence of abortions, and birth weights are small. In dairy cows, the requirement for manganese is 17–25 mg kg^{-1} diet. Forages often provide that amount of manganese, but there is a wide variation in plants. The maximum tolerable level of dietary manganese is 2000 mg kg^{-1}, so there is latitude for adding additional manganese to rations.

In sheep fed extremely high levels of manganese for short periods of time, manganese methionine was more available than manganese sulfate. Most experiments with manganese methionine in dairy cattle have fed the mineral in combination with zinc methionine, copper lysine, and cobalt glucoheptonate. This combination of organic trace minerals improved reproductive efficiency as evident from the reduction in days to first observed estrus and in the time from calving to conception in field trials with dairy cows (**Table 3**).

In an experiment in which trace mineral-specific amino acid complexes of zinc, copper, and manganese, and cobalt glucoheptonate were added to a control diet, days to first observed estrus were fewer for supplemented cows (46.9 days) compared to control cows (67.6 days). In that experiment, overall means were not significantly different between treatments for days to first service, days open, days from first service to conception, or services per conception. A major contributing factor to inefficient reproduction is failure to remove placental tissues after parturition, and supplemented cows that had experienced retained placenta had fewer days to first estrus (44.0 vs. 81.0 days), fewer days to first luteal activity (30.0 vs. 41.8 days), and fewer days to first corpus luteum (34.8 vs. 40.2 days) than control cows with retained placenta. In more recent research with isolevels of trace mineral supplementation, replacing a portion of the inorganic trace mineral sources with amino acid complexes of zinc, copper, and manganese, and cobalt glucoheptonate increased milk production (41.8 vs. 40.6 kg) and reduced days open (147 vs. 169).

Limited research indicated that a mixture of amino acid chelates (copper, zinc, manganese, magnesium, and potassium) may have benefited reproductive efficiency of young dairy cows. In other research, there was no change or increase in milk production, and the content of fat and protein in milk did not vary between supplemental treatments consisting of inorganic forms and proteinates of zinc, copper, and manganese; however, somatic cell counts were lower with the proteinates in the diet. A chelated product containing zinc, copper, and manganese did not change milk yield or milk components in another experiment. Therefore, the animal response to different forms of organic trace mineral products may be an important consideration. Again, limited research information is available for some products.

Selenium

Selenium deficiency of cows is associated with reproductive disorders including erratic or silent estrus, delayed conception, abortions, and birth of weak or dead calves. Retained placenta at parturition is a common occurrence during selenium deficiency, and some forms of mastitis are affected by selenium and/or vitamin E status. In dairy cows and their newborn calves, serum selenium concentrations were greater when selenium yeast was fed compared with sodium selenate; however, there were no differences in the cows' neutrophil function or clinical responses following an intramammary challenge with endotoxin.

Selenium has a narrow range between the amount required and the amount that is toxic, so amounts and forms that may be supplemented are tightly regulated by some governments. Sources of selenomethionine, including yeast, may be more than twice as bioavailable for cattle as sodium selenite or selenate. Supplementation with organic selenium (high-selenium yeast) is more

Table 3 Summary of results from eight experiments comparing a combined trace mineral supplement of 360 mg day^{-1} of zinc from zinc methionine, 125 mg day^{-1} of copper from copper lysine, 200 mg day^{-1} of manganese from manganese methionine, and 25 mg day^{-1} cobalt from cobalt glucoheptonate with inorganic supplements in dairy cow diets

Treatment	*Milk yield (kg)*	*Milk protein (%)*	*Milk fat (%)*	*Days to first service*	*Days open*
Inorganic trace mineral	35.7^{a}	3.2	3.67	81^{b}	131^{b}
Organic trace mineral	36.8^{b}	3.2	3.68	74^{a}	115^{a}

^{a,b}Means within a column without a common superscript are different ($P \leq 0.02$).

Adapted with permission from Kellogg DW, Socha MT, Tomlinson DJ, and Johnson AB (2003) Review: Effects of feeding cobalt glucoheptonate and metal specific amino acid complexes of zinc, manganese, and copper on lactation and reproductive performance of dairy cattle. *The Professional Animal Scientist* 19: 1–9.

effective than inorganic selenium in increasing milk selenium concentration. An experiment comparing sodium selenite, a chelated selenium product, and a selenium yeast observed that with all three sources, increasing inclusion levels increased milk selenium concentration; however, selenium yeast gave a much greater response than the other two selenium sources.

Iodine

Iodine is necessary for the production of the thyroid hormones, thyroxine and triiodothyronine, that regulate body metabolism. Commercial sources of supplemental iodine include potassium or sodium iodide, calcium iodate, and ethylenediamine dihydriodide (EDDI), an organic source. All are thought to be highly available. In two studies with cattle, EDDI was equivalent to potassium iodide and slightly more available than sodium iodide.

Chromium

Chromium is a required ultratrace mineral that potentiates the action of insulin and also has effects on protein and nucleic acid metabolism. In monogastrics, organic forms of chromium including chromium picolinate, chromium methionine, chromium nicotinate, and high-chromium yeast are more available than inorganic forms of chromium such as chromic oxide and chromium chloride. However, limited studies have been conducted thus far with ruminants. In an experiment with dairy heifers, supplementing chromium chloride or a chromium–yeast complex similarly increased body weight gain compared to an unsupplemented control. Supplemental chromium as chelated chromium improved the immune function of early-lactation cows. In addition, two studies indicate that supplementation with chelated chromium enhances milk yield of primiparous cows in early lactation, with no effect on multiparous cows. Chelated chromium supplementation may also be more beneficial in heat-stressed cattle. It has been suggested that chromium supplementation alters ketone body metabolism during the transition period, and reduces serum nonesterified fatty acid concentrations.

Conclusion

The sites of potential improvement of the effects of organic forms of trace minerals over inorganic forms are in the rumen where minerals are subjected to formation of insoluble complexes, in the intestines where absorption occurs, or in the body where the mineral is needed for metabolism. Certain trace minerals may be protected from forming insoluble complexes in the rumen by their association with an organic compound, but the supplemented form must remain stable in the rumen environment and be delivered to the small intestine intact. While bioavailability of selenomethionine is dramatically greater than inorganic supplements of selenium, evidence thus far indicates that availability of organic forms of other trace minerals is only slightly higher than, or equal to, inorganic supplements. Data indicate that absorption is not dramatically improved; however, the amount absorbed may be less important than the form absorbed. Organic complexes or chelates may interact with cellular processes differently and thus impact animal responses. For example, zinc from zinc chloride that had been incorporated into maize during plant growth was metabolized differently than zinc from zinc chloride fed as a dietary supplement to calves. Several organs contained more zinc from the organic source than from zinc chloride in the diet. In some experiments, high-producing dairy cattle have responded with increased milk yield, improved mammary health, more efficient reproduction, and enhanced immunity when offered supplements of organic trace minerals compared to inorganic forms of the minerals.

It should be noted that most commercially available organic trace mineral supplements are typically more expensive than inorganic trace mineral supplements. In some cases, similar responses may be achieved by feeding even greater levels of the inorganic form of the trace mineral(s) if the greater amounts do not interfere with other nutrients. However, it should also be considered that there is increasing concern about the accumulation of some trace minerals on land where dairy manure is routinely applied, and feeding increasing amounts of cheaper but less bioavailable sources of trace minerals may not be a viable long-term solution. An intriguing recent article found similar production responses through two lactations in dairy cows fed 75% of the recommended levels of zinc, manganese, copper, and cobalt as organic trace minerals versus 100% of the recommended levels of these same trace minerals from inorganic sources.

See also: **Feed Ingredients**: Feed Supplements: Microminerals; **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins.

Further Reading

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Feed Supplements: Ruminally Protected Amino Acids

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Amino Acid Nutrition of Dairy Cattle

Sources of Absorbed Amino Acids

Absorbed amino acids are provided by ruminally synthesized microbial protein, rumen-undegradable feed protein (RUP), and, to a lesser extent, endogenous protein. Microbial protein refers to the constituent proteins of the bacteria, protozoa, and fungi that grow and multiply in the rumen. RUP is that portion of feed protein that escapes or resists ruminal degradation. Endogenous protein refers to protein originating in the body. Sources of endogenous protein include mucoproteins in saliva, sloughed epithelial cells (from the respiratory tract, mouth, esophagus, rumen, omasum, and abomasum), and enzyme secretions into the abomasum. The endogenous protein contribution to the duodenum includes free endogenous secretions and endogenous proteins incorporated into the microbial protein flow.

Microbial protein typically supplies a majority of the amino acids required by the ruminant animal. However, RUP may supply more than 50% of the absorbed amino acids in high-producing dairy cows fed a high-concentrate diet that is balanced to meet requirements for rumen-degradable protein and RUP. The quantity of amino acids provided by free endogenous protein secretions is small and appears to be correlated closely to intake of indigestible organic matter. It appears that about 10% of the total supply of absorbed amino acids is provided by endogenous protein.

Factors Affecting the Profile of Absorbed Amino Acids

Two factors account for most of the variation in amino acid profiles of duodenal protein. These are the proportional contribution that RUP makes to total protein passage and the amino acid composition of that portion of total dietary protein predicted to be RUP. This would be expected because feed proteins vary in amino acid composition and usually differ from ruminally synthesized microbial protein (**Table 1**).

Limiting Amino Acids

Limiting amino acids are essential amino acids in digested protein that are in shortest supply relative to body requirements for absorbed amino acids. Methionine, lysine, and histidine have been identified most often as the most limiting amino acids for dairy cattle. The extent and sequence of their limitation is affected primarily by the amount of RUP in the diet and its amino acid composition.

Methionine has been shown to be first limiting for growth and milk protein production when dairy cattle were fed high forage or soybean hull-based diets and intake of RUP was low. Methionine has also been identified as first limiting for growing cattle and lactating cows that were fed a variety of diets in which most of the supplemental RUP was provided by soybean protein, animal-derived proteins, or a combination of the two. In contrast, lysine has been identified as first limiting for growth and milk protein synthesis when maize and feeds of maize origin provided most or all of the RUP in the diet. Relative to concentrations in microbial protein, feeds of maize origin are low in lysine content and similar in methionine content, whereas soybean products and most animal-derived proteins are similar in lysine content and low in methionine content (**Table 1**).

Methionine and lysine have been identified as colimiting amino acids for milk protein synthesis when cows were fed maize silage-based diets with little or no protein supplementation. Histidine has been identified as first limiting for milk protein production when dairy cows were fed grass silage/cereal (barley and oats)-based diets, with or without feather meal as the sole source of RUP supplementation.

It should not be too surprising that these amino acids have all been shown to be first limiting. First, all have been identified as being among the most limiting amino acids in microbial protein. Methionine has been identified as first limiting and lysine as second limiting in microbial protein for nitrogen retention of both growing cattle and growing lambs. Histidine has been identified as possibly third limiting for sheep.

Second, concentrations of methionine and lysine in most feed proteins are lower than those in microbial protein (**Table 1**). Thus, most feed proteins are not complementary to microbial protein and instead, when they are fed, will exacerbate rather than eliminate deficiencies of methionine and lysine in metabolizable protein. This also appears to be why methionine and lysine become more limiting (relative to the other essential amino acids) with increasing intakes of complementary sources of RUP.

Table 1 A comparison of the essential amino acid (EAA) profiles of body lean tissue and milk with that of ruminal bacteria and protozoa and some common feeds

	Arg	*His*	*Ile*	*Leu*	*Lys*	*Met*	*Phe*	*Thr*	*Trp*	*Val*	*EAA*
Item	*(% of total EAA)*										*(% of CP)*
Animal products											
Lean tissue[a]	16.8	6.3	7.1	17.0	16.3	5.1	8.9	9.9	2.5	10.1	–
Milk[b]	7.2	5.5	11.4	19.5	16.0	5.5	10.0	8.9	3.0	13.0	–
Rumen microbes											
Bacteria[c]	10.4	4.1	11.5	15.9	16.5	5.1	10.1	11.3	2.7	12.4	–
Protozoa[d]	9.3	3.6	12.7	15.8	20.6	4.2	10.7	10.5	2.8	9.7	–
Forages[e,f]											
Alfalfa hay	12.5	4.7	10.3	17.9	12.4	3.8	11.6	10.6	3.6	12.7	41.2
Alfalfa silage	10.9	4.7	11.1	17.9	12.1	3.8	11.7	10.7	2.7	14.1	35.6
Corn silage	6.2	5.7	10.6	27.2	7.9	4.8	12.1	10.1	1.4	14.1	31.6
Grass hay	11.7	4.9	10.0	18.8	10.5	3.9	11.8	10.9	3.7	13.6	33.1
Grass silage	9.4	5.1	10.9	18.8	10.1	3.7	13.4	10.2	3.3	15.0	32.6
Grains[e]											
Barley	13.4	6.1	9.2	18.5	9.6	4.5	13.5	9.1	3.1	13.0	37.7
Corn	11.5	7.8	8.2	27.9	7.1	5.3	11.5	8.8	1.8	10.0	40.1
Oats	16.6	5.9	9.1	17.7	10.1	4.2	12.5	8.4	2.9	12.6	41.2
Sorghum	9.4	5.7	9.3	31.9	5.4	4.2	12.3	7.8	2.5	11.6	42.8
Wheat	13.6	7.1	9.6	19.3	8.1	4.6	13.3	8.4	3.5	12.3	34.4
Plant proteins[e]											
Brewers' grains	14.7	5.1	9.8	20.0	10.4	4.3	11.7	9.1	2.5	12.1	39.2
Canola meal	16.5	6.6	9.0	15.9	13.2	4.4	9.5	10.4	3.4	11.1	42.6
Corn DDG w/sol.	10.7	6.6	9.8	25.4	5.9	4.8	12.9	9.1	2.3	12.4	37.8
Corn gluten meal	7.1	4.7	9.1	37.2	3.7	5.2	14.1	7.5	1.2	10.3	45.2
Cottonseed meal	26.0	6.6	7.3	13.8	9.7	3.7	12.5	7.6	2.8	10.0	42.6
Linseed meal	20.9	4.8	11.0	14.5	8.7	4.2	11.1	8.9	3.7	12.3	42.2
Peanut meal	27.6	6.0	8.1	15.9	8.3	2.9	12.1	6.7	2.4	9.8	40.1
Soybean meal	16.2	6.1	10.1	17.2	13.9	3.2	11.6	8.7	2.8	10.2	45.3
Sunflower meal	20.8	6.2	9.9	15.2	8.0	5.6	11.0	8.7	2.9	11.7	42.2
Animal proteins[e]											
Blood meal	7.8	1.3	2.2	22.7	15.9	2.1	12.1	7.7	2.8	15.4	56.4
Feather meal	16.2	2.7	11.4	19.9	6.0	1.8	11.6	11.1	1.7	17.6	42.7
Fish meal	13.1	6.4	9.2	16.2	17.2	6.3	9.0	9.4	2.4	10.8	44.5
Meat and bone meal	19.5	5.3	7.7	17.2	14.5	3.9	9.4	9.1	1.6	11.8	35.7
Whey, dry	5.0	4.5	12.1	21.2	17.6	3.3	7.0	14.1	3.5	11.7	42.2

[a] Average values of empty, whole body carcasses as reported in three studies.

[b] Average values as reported in three studies.

[c] The mean of average values from over 100 dietary treatments.

[d] Average values from 15 literature reports.

[e] Calculated from values presented in National Research Council (2001) *Nutrient Requirements of Dairy Cattle,* 7th revised edn. Washington, DC: National Academy Press.

[f] Legume and grass hays and silages are mid-maturity.

Third, lysine is more vulnerable to heat processing than the other essential amino acids. Overheating feed proteins can decrease lysine concentrations as well as decrease the digestibility of the remaining lysine more than that of total protein.

Finally, concentrations of histidine are lower in grasses and legumes, oats, barley, and particularly feather meal, as compared to most other feeds (**Table 1**). This is probably why diets consisting solely of these feeds have been shown to be more limiting in histidine than in methionine or lysine.

Ruminally Protected Amino Acids

History of Development

Interest in protecting free amino acids from ruminal degradation dates back to the 1960s and early 1970s, when it became apparent from abomasal, intestinal, and intravenous infusion trials that the profile of absorbed amino acids was not always optimum in ruminants. These trials indicated that the sulfur amino acids were clearly first limiting for wool growth and body weight gains of sheep and that methionine was a limiting amino acid for growing cattle and lactating dairy cows. Thus, several laboratories began to devise procedures to protect methionine from ruminal degradation. Subsequent interest developed in protecting lysine when it was discovered to be a second limiting for growing lambs and either first or second limiting for growing cattle and lactating dairy cows.

Many approaches have been evaluated to physically protect methionine and lysine from ruminal degradation. Initial attempts focused on protecting methionine with lipids, often in combination with inorganic materials and carbohydrates as stabilizers, softening agents, and fillers. For example, in the 1960s, Delmar Chemicals of Canada developed a product in which a core of DL-methionine, colloidal kaolin, and tristearin was wrapped in a continuous film of tristearin. The product contained 20% methionine. A few years later, Rumen Kjemi A/S of Oslo, Norway, introduced a somewhat more efficacious product (Ketionin®) that had a higher intestinal release of the encapsulated methionine. The product contained 30% DL-methionine, 2% glucose, 4% stabilizer, antioxidant and flavoring agents, 6% $CaCO_3$, and 58% tristearin and oleic acid.

Several other lipid-protected methionine products have also been evaluated. The greatest challenge with using lipids as the primary encapsulating material is to identify a combination of materials and process that has both a high ruminal escape and intestinal release of the amino acid.

The most effective approach has been to surface-coat amino acids with enzyme-resistant, pH-sensitive synthetic polymers that are insoluble in the more neutral pH environment of ruminal digesta but highly soluble in the acidic abomasum. This approach provides for a postruminal delivery system that is independent of enzyme function and, instead, relies on the pH differences between the rumen and the abomasum for ruminal protection and intestinal release. Polymer-protected amino acids have higher ruminal protection and intestinal release coefficients than other products. The patent rights for the use of pH-sensitive polymers for protecting nutrients from ruminal degradation is currently held by Adisseo Animal Nutrition, Antony, France.

Another method that has been explored for increasing supplies of methionine and lysine to ruminants is the use of derivatives and analogues of these amino acids. Most of the derivatives and analogues that have been investigated for level of rumen protection are those of methionine.

Amino acid derivatives are free amino acids to which a chemical blocking group has been added to the α-amino group, or in which the acyl group has been modified. Some examples of derivatives that have been shown to have some resistance to ruminal degradation are isopropyl-DL-methionine, *t*-butyl-DL-methionine, *N*-stearoyl-DL-methionine, *N*-oleoyl-DL-methionine, capryl-caproylic-DL-methionine, and di-hydroxymethyl-L-lysine-Ca. There is evidence that the extent of ruminal escape is greater with shorter-chain alkyl esters than with longer-chain alkyl esters. Although these and other derivatives show promise, most have not been investigated adequately to determine the extent to which they will increase postruminal supplies of absorbable methionine.

An amino acid analogue results from the substitution of the α-amino group of an amino acid with a non-nitrogenous group. The most studied amino acid analogue is methionine hydroxy analogue (MHA; DL-α-hydroxy-γ-mercaptobutyrate), more appropriately called 2-hydroxy-4-(methylthio)butanoic acid (HMB). Studies indicate that free HMB (1) is more resistant to ruminal degradation than free methionine, (2) is more absorbable from the rumen and omasum than methionine, and (3) is converted to methionine in most mammalian tissues. However, because of no measurable effects on blood methionine concentrations when fed to cows on methionine-adequate diets, or on the content of milk protein when fed to methionine-deficient cows, it appears that feeding HMB, in either the acid or salt form, has little or no replacement value for ruminally protected methionine.

Research has shown that several esters of HMB enhance ruminal escape of HMB, at least in part, because of their apparent ability to be absorbed across the rumen wall. The isopropyl ester of HMB (HMBi) has been shown to have an excellent replacement value for absorbed methionine.

Examples of Commercial Protected Methionine Products

Mepron® M85 (Evonik-Degussa Corporation, Germany): This is an example of a surface-coated, carbohydrate-protected product. The small pellets have a diameter of 1.8 mm, a length of 3–4 mm, and an approximate density of 1.2 g cm^{-3}. The pellets consist of a core of methionine and starch coated with several thin layers of ethylcellulose and stearic acid. The final product contains a minimum of 85% DL-methionine, and approximately 8.5% nonstructural carbohydrates, 3.5% neutral detergent fiber, 1.5% ash, 1.0% moisture, and 0.5% crude fat. The technology is a combination of coating materials and application that allows for a large payload of methionine. Because enzymatic digestion of the ethylcellulose is minimal, degradation of the product occurs primarily through physical action and abrasion. The result is a product that is slowly degraded in the rumen and slowly releases methionine into the intestine.

METHIOPLUS™ (Kemin Industries, Des Moines, IA, USA): Methioplus is produced by encapsulating DL-methionine with hydrogenated vegetable oils. The preparation method is described as microencapsulation by spray freezing using the patented MICROPEARLS® process. The coating material and method of application are designed to provide a coating matrix that is able to resist rumen breakdown and release the ingredient in the intestine. The yellow microcapsules contain 55% DL-methionine.

Met-Plus™ (Novus International, Inc., St. Louis, MO, USA): This is an example of a lipid-protected product. It is a matrix compound that contains 65% DL-methionine embedded in a mixture of calcium salts of long-chain fatty acids, lauric acid, and butylated hydroxytoluene (BHT); BHT is a preservative for the fatty acids. However, like other lipid-coated products, the technology relies on achieving a balance between ruminal protection and intestinal release so as to maximize the amount of methionine available for intestinal absorption while minimizing losses in the rumen and in feces.

Smartamine™ M (Adisseo, Antony, France): This is an example of a lipid/pH-sensitive polymer-protected product. It is a surface-coated product that contains a minimum of 75% DL-methionine. The small 2-mm pellets consist of a core of DL-methionine and ethyl cellulose that is covered with a coat of stearic acid containing small droplets of poly(2-vinylpyridine-*co*-styrene). The copolymer contributes 3% by weight of the final product. The presence of the copolymer appears to alter the stereochemistry of the stearic acid such that the surface coating becomes enhanced in its resistant to ruminal degradation. The presence of the copolymer, because of its solubilization at low pH, also allows for rapid release of the methionine in the abomasum.

MetaSmart® (Adisseo, Antony, France): This is the HMBi. While technically an amino acid analogue, it is usually referred to as a protected methionine product because of its ability to provide significant amounts of absorbable HMB to the cow. The compound is formed by reacting Rhodimet™ AT88 (HMB) with isopropanol. The resulting HMBi provides for a limited degree of protection from ruminal degradation, but it provides a vehicle for a more rapid absorption across the ruminal and omasal walls.

Commercial Sources of Methionine Analogues

There are three commercial sources of free methionine analogues. These are Alimet® (Novus International, Inc., St. Louis, MO, USA), Rhodimet™ AT88 (Adisseo, Antony, France), and MFP™ (Novus International, Inc., St. Louis, MO, USA). Alimet and Rhodimet AT88 are both liquid sources of HMB. Chemically, the compounds are the same. Both are used extensively as a substitute for methionine in the poultry and swine industry. However, Novus International also has Alimet® patented for use in dairy cows and recommends its use as a source of ruminally protected methionine. Adisseo considers the ruminal escape and ruminal absorption of Rhodimet™ AT88 to be too low to recommend its use as a source of ruminally protected methionine. Novus International also recommends the use of MFP™, which is the Ca salt of HMB, and thus the dry source of HMB, as a source of ruminally protected methionine. Both companies recognize the potential benefits of feeding HMB on rumen function (e.g., increased fiber and protein digestion and increased bacterial protein synthesis and efficiency of synthesis) and animal performance (e.g., increased milk yield and butterfat content).

Commercial Sources of Rumen-Protected Lysine

AminoShure™-L (Balchem Corporation, New Hampton, NY, USA): Launched in 2008, AminoShure™-L was the first commercial source of rumen-protected lysine to become available in the United States. The product is formed by encapsulating L-lysine-HCl with multiple layers of food-grade lipids. The spherical shaped tan to light brown granules contain 38% crude protein, a minimum of 36% lysine, 53% ether extract, and 9.0% chloride.

MEGAMINE-L® (Church & Dwight Co., Princeton, NJ, USA): Released in 2009, the final product contains 80% Megalac and 20% lysine-HCl. The product is formed by adding lysine-HCl to the Megalac production process. The resulting 0.32 cm pellets have a density of 1.05 and contain 68% fat, 16% lysine, 7% calcium, and 5% lipid-soluble materials. Ethoxyquin is added as a preservative.

LYS-50™ (Kemin Industries, Des Moines, IA, USA): LYS-50™ is produced by the same MICROPEARLS® process as described for Methioplus. The product typically contains 50% L-lysine-HCl.

Efficacy of Products

Responsible use of ruminally protected methionine and lysine products requires estimates of their ability to provide methionine and lysine for protein synthesis. Insofar as possible, estimates of 'methionine and lysine bioavailability' must be accurate and reliable under the conditions in which they are fed in production systems.

Approaches

Unfortunately, there is no universally accepted, standardized procedure(s) for obtaining estimates of methionine and lysine bioavailability. These are needed to bring uniformity to estimates of amino acid bioavailability and to more accurately compare the efficacy of different products. Current approaches can be categorized as factorial approaches, blood response approaches, and production response approaches.

The factorial approach involves independent measurements of ruminal escape, intestinal disappearance (digestibility), and, in the case of free or protected forms of HMB, metabolic conversion to methionine. Animals with cannulae in the rumen, duodenum, and preferably also in the ileum are required. Estimates of ruminal escape and intestinal disappearance of amino acid from protected methionine and lysine products have been obtained using both the *in situ* nylon-bag procedure and the cannulated cow *in vivo* procedure. Use of the *in situ* procedure requires measurements of rate of passage of protected amino acid products from the rumen.

Factorial approaches, particularly those involving *in situ* nylon-bag techniques, have been questioned as to their appropriateness for measuring the intestinal release of amino acids from a rumen-protected product that relies on abrasion and physical forces for its degradation. Moreover, the procedures do not account for the physiological aspects of digestion such as mastication and digesta passage. The *in situ* procedure is also not suitable for soluble or liquid products such as HMB and HMBi.

Blood response approaches are an attractive alternative to factorial approaches because they are easier to conduct and they allow liquid and pulverulent products to be evaluated. Studies with cattle have shown that a linear relationship exists between increasing supplies of absorbable methionine and lysine and plasma methionine and lysine concentrations. Two variations of the approach have been used. The first is the dose–response approach. This approach involves determination of differences in the slope of measured blood amino acid concentrations between graded dietary doses of the product and graded intestinal doses of the infused amino acid. The second is the 'area under the curve' (AUC) approach. This approach involves the ruminal administration of a pulsed dose of equimolar amounts of different methionine or lysine sources and comparing the AUC of the plasma methionine and lysine response curves that result. To obtain estimates of methionine bioavailability for methionine products, Smartamine™ M is often used as the positive control treatment with the assumption that it has a methionine bioavailability value of 80%.

A milk protein content response approach has also been used to obtain estimates of amino acid bioavailability. This approach involves determination of differences in slope of measured milk protein concentrations between graded dietary inclusion levels of the product and graded intestinal infusions of the free amino acid when cows are fed a basal diet deficient in the amino acid in question. As with the blood response approach, some of the studies that focused on obtaining estimates of methionine bioavailability for methionine products replaced intestinally infused methionine with Smartamine™ M as the positive control treatment with the assumption that Smartamine™ M has a methionine bioavailability value of 80%. Numerous studies with lactating cows indicate that content of milk protein is the most responsive criterion to small changes in concentrations of methionine and lysine in metabolizable protein.

Using production responses such as milk yield, milk fat concentration, and milk component yields as criteria for comparing or evaluating products as sources of rumen-protected amino acids is not valid because of the effect that changes in rumen function can have on them. For example, several experiments have indicated increases in forage and fiber digestion, protein digestion, acetate-to-propionate ratios, bacterial protein synthesis, efficiency of bacterial protein synthesis, and protozoan numbers as a result of feeding methionine hydoxy analogue. When these benefits are realized and one or more of the effects are limiting animal performance, it should not be surprising that increases in feed intake, milk yield, and milk fat concentration are observed.

A Comparison of Some Available Products

Relying on the blood and milk protein content response approaches as being the most appropriate techniques for arriving at estimates of 'methionine and lysine bioavailability', a summary of available research indicates that Smartamine™ M clearly has the greatest efficacy as a source of absorbable methionine. The industry use of a methionine bioavailability value of 80% appears reasonable when the manufacturers recommendations for mixing with other supplements and rations are followed. The data are not as consistent for Mepron™ M85 and MetaSmart™ as they are for Smartamine™ M but it appears that the methionine bioavailability value for the two products is somewhere between 40 and 50%. It is the opinion of the author that there is not enough data to make similar claims of efficacy.

Research indicates no measurable effects of Alimet® or Rhodimet™ AT88 on blood methionine concentrations when fed to cows on methionine-adequate diets, or on content of milk protein when fed to methionine-deficient cows. Therefore, it appears that feeding HMB, in either the acid or salt form, has little or no replacement value for ruminally protected methionine. It appears quite certain that the methionine bioavailability of these two products is less than 5%. Pulse-dosing large amounts into the rumen has yielded apparent rumen escape values of 40–50%. However, adding incremental amounts of these products to methionine-deficient diets has not increased milk protein concentrations or blood methionine concentrations.

The rumen-protected lysine products have been introduced only recently and have not had the research scrutiny of most of the methionine supplements. However, because they are all lipid-encapsulated or lipid-protected products, and depend on both the mechanical and enzymatic aspects of digestion throughout the gastrointestinal tract for lysine release, it is unlikely in most cases that more than half of the protected lysine is available for absorption in the small intestine.

The Benefits of Using Rumen-Protected Methionine and Lysine Products

The use of these products is of significant help in achieving a more desirable profile of the most limiting amino acids in metabolizable protein. Clearly, their use allows the alleviation of methionine and lysine deficiencies without increasing the intake of RUP or changing the profile of the other essential amino acids in metabolizable protein. However, their use also allows for more selective use of protein supplements, without regard to their content of methionine and lysine, to more adequately meet desired levels of other limiting amino acids in metabolizable protein. The result is more efficient use of absorbed amino acids for protein synthesis, increased weight gains and higher levels of milk protein production, and reduced need for RUP in the diet.

The use of rumen-protected methionine and lysine products has been limited. Reasons for this include (1) inadequate knowledge of amino acid requirements and the required concentrations of limiting amino acids in metabolizable protein, (2) lack of easy-to-use computer models that predict accurately their need, (3) issues of product efficacy and cost relative to anticipated benefits, and (4) lack of ease of incorporating desired concentrations into the diet. However, their use continues to increase as some of these issues have been resolved. The greatest increase in use has occurred where there has been a genuine desire to increase milk protein production while maintaining or decreasing the use of dietary protein.

Conclusions

Identifying technologies for manufacturing high-quality ruminally protected amino acids has proven to be a challenging task. Currently, commercial products are limited to protected methionine and lysine. It is anticipated that derivatives and analogues of methionine, and modifications thereof, will be evaluated further as effective alternatives to protected methionine. The availability of an efficacious technology for increasing lysine supplies to dairy cattle would be desirable where high corn diets are fed and high-lysine protein supplements such as blood meal and meat meal are not an option. The use of protected amino acids with established efficacy values, along with selective use of protein supplements, gives dairy producers the opportunity to balance diets for amino acids more adequately. The recent introduction of rumen-protected lysine products is welcomed because they allow for achieving higher concentrations of lysine in metabolizable protein than otherwise possible, particularly when high corn diets are fed and high-lysine protein supplements such as blood meal and meat meal are not an option.

See also: **Nutrients, Digestion and Absorption**: Fermentation in the Rumen.

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Feed Supplements: Vitamins

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Introduction

Vitamins are organic compounds needed in minute amounts and are essential for life. A vitamin must be in the diet or be synthesized by microorganisms in the digestive system and then absorbed by the host animal. Currently, there are 10 water-soluble (**Table 1**) and 4 fat-soluble vitamins (**Table 2**), but not all animals require all 14 vitamins. Although choline does not perfectly fit the definition of vitamins, many consider it a vitamin and it is included in **Table 1**. Dairy cattle are known to require 12 vitamins; ascorbic acid (vitamin C) and choline are synthesized by bovine cells.

Vitamins differ greatly in chemical structure and have diverse biological functions. Many of the water-soluble vitamins are cofactors of enzymes involved in numerous metabolic pathways. Fat-soluble vitamins play a role in a variety of functions such as gene regulation, hormone synthesis, mineral homeostasis, antioxidant defense, and vision. When an animal absorbs an inadequate quantity of a particular vitamin, various responses are observed depending on the vitamin, and the degree and duration of deficiency. The most severe situation (seldom observed in US dairy cows) is a clinical deficiency resulting in a disease such as rickets. Marginal deficiencies of vitamins usually have more subtle and less defined signs, but can include reduced growth and milk production, poor reproductive performance, and increased prevalence of infectious diseases.

Most vitamins are available as feed supplements but dietary supplementation of water-soluble vitamins and vitamin K is rarely needed to prevent clinical deficiencies. Many common feedstuffs contain appreciable concentrations of water-soluble vitamins, and bacteria in the rumen and colon can synthesize most water-soluble vitamins and vitamin K. Conversely, the vast majority of diets fed to dairy cows contain supplemental vitamins A, D, and E because these vitamins are often low in concentration in feeds and also because bacteria in the rumen and colon cannot synthesize these vitamins.

Water-Soluble Vitamins

An abbreviated list of known functions of some water-soluble vitamins is given in **Table 1**. Essentially no research has been published regarding the response by dairy cows to supplemental pantothenic acid, riboflavin, and vitamin B_6, and these vitamins are not included in the list. With the exception of niacin, biotin, and choline, dairy cows are not commonly supplemented with water-soluble vitamins. Microorganisms within the rumen and, to a much lesser degree, those in the large intestine can synthesize all known B vitamins; therefore, clinical deficiencies are extremely rare and usually occur only when an inhibitory substance is present (e.g., thiaminase) or when ruminal fermentation is greatly reduced (e.g., extensive antibiotic treatment). Because cobalt is a component of vitamin B_{12}, cobalt deficiency can lead to vitamin B_{12} deficiency.

Ruminal microorganisms not only synthesize water-soluble vitamins, but also catabolize them. As much as 98% of supplemental niacin and folic acid disappear before reaching the duodenum, and ruminal disappearance of supplemental B_{12} and biotin average 60 and 40%, respectively. The extensive degradation of niacin brings into question the value of typical supplementation rates (6–12 g day^{-1}) because most of the supplementation will be destroyed before it is absorbed by the cow. It has been shown that if niacin is used in large enough doses, sufficient amounts can possibly become available to alter lipid metabolism. Some ruminal bacteria require certain B vitamins, so ruminal effects of supplementation cannot be ruled out. Once the B vitamins reach the small intestine, most are absorbed fairly efficiently (>75%), but biotin and riboflavin appear to be absorbed with only about 35% efficiency and for B_{12} absorption efficiency is <20%.

Biotin

Biotin is found in most plant-based feedstuffs, but due to the great difficulty in assaying biotin accurately, the reported concentrations of biotin in typical diets fed to dairy cows are extremely variable. The majority of the studies report concentrations between 0.2 and 0.4 mg of biotin per kg dry matter; however, a few studies (using a different analytical method) have reported concentrations between 6 and 7 mg kg^{-1}. Assuming the actual dietary concentration is approximately 0.3 mg kg^{-1}, a typical lactating dairy cow (with an assumed dry matter intake of 20 kg day^{-1}) will consume about 6 mg biotin day^{-1}. Biotin is produced by ruminal bacteria, but again because of analytical difficulties synthesis estimates vary widely.

Table 1 Sources and functions of some water-soluble vitamins (not a complete list)

Vitamin	*Sources*	*Functions*
Biotin	Oilseed meals, brewers' grains, forages, microbial fermentation, supplements	Gluconeogenesis, fatty acid synthesis, amino acid metabolism, hoof integrity
Choline	*In vivo* synthesis from methionine, rumen-protected supplements	Neural transmission, lipid metabolism, component of cell membranes
Folic acid	Soybean products, fresh forage, brewers' grains, alfalfa meal, microbial fermentation, supplements	Nucleotide synthesis, protein synthesis, choline synthesis
Niacin	*In vivo* synthesis from tryptophan, distillers' and brewers' grains, microbial fermentation, supplements	Energy metabolism, urea synthesis, ketone metabolism
Thiamine	Grains, forages, supplements	Energy metabolism, fatty acid synthesis, branched-chain amino acid metabolism
Vitamin B_{12}	Animal protein meals, microbial fermentation (requires cobalt), supplements	Nucleic acid and protein synthesis, gluconeogenesis, propionate metabolism
Vitamin C	*In vivo* synthesis, rumen-protected supplements	Water-soluble antioxidant activity, collagen synthesis

Table 2 Sources and functions of fat-soluble vitamins

Vitamin	*Sources*	*Functions*
Vitamin A	Fresh forages (β-carotene), silages, supplements	Vision, maintenance of epithelial and skeletal cells, immunity, gene regulation
Vitamin D	Exposure to sunlight, sun-cured hay, animal protein products, supplements	Calcium and phosphorus homeostasis, immune function
Vitamin E	Fresh forages, silage, oilseeds, supplements	Lipid-soluble antioxidant activity
Vitamin K	Fresh forages, microbial synthesis, supplements supplements	Blood coagulation

For a lactating cow consuming a typical diet, net ruminal synthesis is probably around 4 or 5 mg day^{-1}, but one study reported no net synthesis and some studies using *in vitro* rumen systems found synthesis rates exceeding 15 mg day^{-1}. Based on a typical dry matter intake and on the majority of data generated from lactating cows (i.e., excluding *in vitro* data), the amount of biotin reaching the small intestine for absorption is probably about 10 mg day^{-1} when cows are not fed supplemental biotin. Research conducted with swine and horses found that supplemental biotin improved hoof integrity. The high prevalence of hoof disorders and lameness on many dairy farms led to research to determine the value of supplemental biotin in dairy cow diets. Several large clinical studies have been conducted and all the studies reported that supplemental biotin had a positive effect on some aspect of hoof health such as sole hemorrhages, white line separation, and general lameness. The exact mode of action is not yet clear but may involve hoof cell differentiation, keratinization, and production of a cementing substance necessary for hoof integrity. For hoof health, 10–20 mg of supplemental biotin is needed per day and it must be supplemented for several months.

Many of the clinical studies found that supplemental biotin (10–20 mg day^{-1}) increased milk production. Initially, this was thought to occur as an indirect effect of improved hoof health, but subsequent production research disproved this. The majority of production studies using cows with milk yields >35 kg day^{-1} reported increased milk production (average increase was about 1 kg day^{-1}) with supplemental biotin. The response occurs within a few days; therefore, the milk response cannot be caused by improved hoof health and must be caused by some change in metabolism. Biotin is a cofactor of four carboxylating enzymes three of which are involved in the synthesis of lactose and milk fat and supplementation of biotin increases the activity of at least one of these enzymes (propionyl carboxylase).

Choline

Choline does not fit the classical definition of a vitamin because it can be synthesized *in vivo* and is required in gram quantities per day. However, it is often considered a vitamin probably because it does not fit into any other nutrient classification. Choline is an essential component of cell membranes, is needed to transport fat out of the liver, and is a precursor of an important neurotransmitter. *In vivo*, methionine is a precursor of choline; therefore, supplemental choline may spare methionine for other purposes such as synthesis of milk protein. Essentially all the choline consumed by a cow is destroyed in the rumen so cows must either synthesize all their choline or be fed rumen-protected sources of choline. Milk production by cows in early lactation is often increased when choline is infused postruminally or when added to diets in a rumen-protected form. When cows in early lactation (usually first 60 days following calving) are supplemented with about 50 g day^{-1} of protected choline (actual choline is 15 g), milk yield increases by an average of 2.2 kg day^{-1}. In early lactation, milk yields are high but feed intake is low, which means less methionine is available for choline synthesis; milk yield responses may be substantially less in later lactation when intake is high. Because of choline's role in lipid transport, supplemental rumen-protected choline may have value in reducing the prevalence of fatty liver and ketosis during the peripartum period. At this time, data on positive responses to choline are limited, and additional research is needed.

Folates

Folic acid is the most biologically active form of the folates, and many feedstuffs contain appreciable concentrations of folic acid. Based on limited research, average flow of folic acid to the small intestine is about 25 mg day^{-1} for a lactating cow with about half the amount coming from the diet and half from ruminal synthesis. The main function of folic acid is to transfer single carbon units among different compounds, which makes it important for the synthesis of major milk components, but milk yield responses to supplemental folic acid (usually 1–3 g day^{-1}) have not been consistent. The reasons for the inconsistent response include extensive ruminal degradation of supplemental folic acid, variation in the supply of methionine (major source of single carbon units), and variation in vitamin B_{12} status. Vitamin B_{12} is needed for folate to transfer single carbon units and if vitamin B_{12} status is marginal, responses to supplemental folic acid will be attenuated. Feeding supplemental folic acid consistently increases its concentration in milk, which may have value to consumers of the milk.

Niacin

Niacin is found in cereal grains, grain and seed by-products, and forages. Intake of niacin by a typical lactating cow fed a diet without supplemental niacin is about 1 g day^{-1} and net ruminal synthesis adds another 1–1.5 g to the daily flow of niacin to the small intestine. Niacin is involved in many oxidation–reduction pathways via NADP and NAD. Energy is often considered the most limiting factor for milk production, and niacin is essential for cellular energy pathways. This linkage has led to a plethora of studies evaluating responses to supplemental niacin in dairy cows. Numerous meta-analyses have been conducted to quantify milk yield responses to supplemental niacin; the average milk yield response across studies is 0.3–0.6 kg day^{-1}, but most individual studies report no statistically significant effect of supplemental niacin. A milk yield response is more likely when supplementation rate is 12 g day^{-1} rather than 6 g day^{-1} and when cows are supplemented in early lactation rather than mid- and late lactation. One of the reasons for the modest response to supplemental niacin may be that almost all the supplemental niacin fed disappears in the rumen. Because of the extensive ruminal metabolism of niacin, rumen-protected forms of niacin have been developed, but, at present, data evaluating responses in cows have been published only in abstract form. Supplementing rumen-protected niacin (10–15 g of actual niacin per day) can reduce core body temperature and increase milk production by cows under heat stress conditions. The antilipolytic activity of niacin stimulated research into evaluating the effectiveness of niacin as a treatment and preventative of ketosis. However, the preponderance of data shows that niacin supplementation, especially at rates <12 g day^{-1}, has little effect on the prevalence of ketosis. Very high supplementation rates of niacin (approximately 50 g day^{-1}) have been found to reduce plasma concentrations of free fatty acids in some studies.

Thiamine

Thiamine is found in all plant-derived feedstuffs, especially grains. Based on limited data, an average lactating dairy cow fed a typical diet probably consumes about 40 mg of thiamine each day, and net ruminal synthesis is 20–30 mg day^{-1}, resulting in a flow of about 65 mg day^{-1} to the small intestine. Thiamine is a cofactor of enzymes involved in pyruvate metabolism, branched-chain amino acid metabolism, and fatty acid synthesis. Most of the research involving supplemental thiamine has been with feedlot cattle fed high-concentrate diets. Under certain conditions, rumen bacteria can produce thiaminase, the enzyme that breaks down thiamine. In these situations, a clinical condition called polioencephalomalacia occurs, and afflicted animals display severely abnormal behavior and convulsions, and usually will die unless thiamine is administered orally or parenterally. This condition has not been reported in dairy cattle. In one research trial, supplemental thiamine (150 mg day^{-1}) increased milk yield, but additional research is needed before conclusions can be drawn regarding the value of supplemental thiamine.

Vitamin B_{12}

Vitamin B_{12} is a large molecule that contains an atom of cobalt. Vitamin B_{12} is not found in plants, but ruminal and intestinal bacteria can synthesize adequate amounts if the diet contains sufficient cobalt (approximately 0.1–0.2 mg cobalt per kg diet dry matter). Vitamin B_{12} is involved in folate activation pathways and in propionate metabolism. In general, supplemental vitamin B_{12} has had negligible effects on milk yields and milk composition. One possible reason for the lack of response is that vitamin B_{12} and folic acid are closely linked, and some studies have shown that production responses to vitamin B_{12} can occur only when folic acid is adequate.

Vitamin C (Ascorbic Acid)

Vitamin C is not a required dietary vitamin for dairy cattle because it can be synthesized *in vivo*. Because of its effect on immune function in other species, research has been conducted on the use of supplemental vitamin C in cattle. Concentrations of vitamin C in plasma and milk are reduced when cows have mastitis, and the decrease is correlated with the severity of the disease. Some studies have shown that injected vitamin C lessens the severity and duration of clinical mastitis. Because ascorbic acid is extensively metabolized in the rumen, rates of oral supplementation have to be very high ($>10\,\mathrm{g\,day}^{-1}$) before plasma concentrations of vitamin C are increased. Current data do not show any benefits of oral supplementation and data on the effects of feeding rumen-protected sources of vitamin C are lacking.

Fat-Soluble Vitamins

An abbreviated list of known functions of fat-soluble vitamins is shown in **Table 2**. Unlike water-soluble vitamins, clinical deficiencies of fat-soluble vitamins can occur, and supplementation is often necessary.

Vitamin A

Plants do not make vitamin A (retinol) but some feedstuffs contain β-carotene, which animals can convert to vitamin A. Fresh green forage is an excellent source of β-carotene but concentrations are markedly reduced in silage and especially hay. Concentrate feeds are generally poor sources of β-carotene. Vitamin A is stored in the liver, and a deficiency of vitamin A may require months to be expressed. The two most common sources of supplemental vitamin A are all-*trans* retinyl palmitate and all-*trans* retinyl acetate. The activity of vitamin A supplements decreases during storage, and the loss of activity is exacerbated by pelleting and on exposure to light, moisture, and certain trace minerals. Destruction of vitamin A occurs in the rumen and is greater when animals are fed high-concentrate diets than when fed high-forage diets.

Vitamin A requirements and dietary concentrations are expressed in international units (IU). One IU of vitamin A is defined as 0.3 μg of retinol or 2.5 μg of β-carotene. Vitamin A is involved in diverse functions such as vision, maintenance of epithelial cells, and gene regulation. For dairy cows fed hay and silage (i.e., no fresh forage), supplemental vitam, in A is usually recommended. Poor reproductive efficiency (especially abortions) is common, and increased prevalence of mastitis and retained fetal membranes can occur when cows are fed inadequate vitamin A.

Vitamin D

Vitamin D is another compound that does not perfectly fit the definition of a vitamin because it can be synthesized *in vivo* by exposure of the skin to UV radiation (sunshine). Vitamin D requirements and dietary concentrations are expressed in IU with 1 IU equal to 0.025 μg of cholecalciferol. Most feeds contain insignificant concentrations of vitamin D with the exception of sun-cured hay, and as much as 50% of the vitamin D consumed is broken down in the rumen. Vitamin D exists in two forms: vitamin D_2 (ergocalciferol) derived from plants and vitamin D_3 (cholecalciferol) derived from animals. Vitamin D_3 is the most common type of supplemental vitamin D. Vitamin D is involved in the regulation of calcium and phosphorus absorption from the gut and calcium resorption by the kidney and bone. Limited but increasing amounts of research show that vitamin D is also involved in immune function and gene regulation. Rickets can occur in dairy cows when they are not fed hay and supplemental vitamin D and are housed inside without access to sunlight.

Vitamin E

Fresh, green plants and some oilseeds contain substantial concentrations of α-tocopherol (vitamin E), all of which is in the *RRR* isomer form. The most common form of supplemental vitamin E is all-rac α-tocopherol acetate, which is an equimixture of all eight isomers. Vitamin E concentrations and requirements are expressed in IU with 1 IU equal to 1 mg of all-rac α-tocopheryl acetate. Because the *RRR* isomer is more active biologically (probably because other isomers are discriminated against during absorption and liver metabolism), 1 mg of *RRR* α-tocopherol equals 1.5 IU of vitamin E. Recent data suggest that *RRR* α-tocopherol may have even greater biological activity relative to all-rac vitamin E than implied by the current conversion factor. Vitamin E does not appear to be extensively metabolized in the rumen.

Vitamin E is primarily a lipid-soluble antioxidant. Clinical deficiencies of vitamin E are difficult to produce in ruminants, but several studies have shown positive effects when vitamin E intake by dairy cows is increased. Several measures of immune function are improved and the prevalence of mastitis and retained fetal membranes is reduced by vitamin E supplementation. Cows in poor selenium status have higher vitamin E requirements because selenium is a component of enzymes that are important cellular antioxidants. Cows in good selenium status require less vitamin E, but adequate selenium does not eliminate the need for vitamin E and vice versa.

Vitamin K

Fresh forage is a good source of vitamin K, but most concentrate feeds contain only negligible amounts of the vitamin. The predominant source of vitamin K, however, is not diet, but bacterial synthesis that occurs in the rumen and large intestine. The most common source of supplemental vitamin K is menadione salts. Vitamin K is involved in several steps of the blood coagulation cascade, and deficiencies of vitamin K result in prolonged clotting times and uncontrolled hemorrhaging. Because of gut synthesis, vitamin K deficiencies are extremely rare and usually occur only when cattle consume dicoumarol, an antagonist of vitamin K that is found in moldy sweet clover hay. Ideally, animals should not be fed moldy sweet clover hay, but, if they are fed, supplemental vitamin K should be included in the diet.

Supplementation Strategies

Quantifying precise vitamin requirements for dairy cattle is extremely difficult because of the following reasons:

1. The concentrations of vitamins in the feeds are highly variable and often difficult to measure
2. Substantial synthesis and degradation of vitamins occur in the rumen
3. Certain fat-soluble vitamins are stored in the body and experiments may have to last months before body stores are depleted
4. Responses to vitamins are often subtle and involve health and reproduction measures. Obtaining statistically valid results requires large experiments that last several months or years and are quite expensive to conduct. Because of the cost, most experiments have only two treatments (diets with and without supplemental vitamin). The limited number of treatments precludes accurate titration of requirements

Therefore, presenting vitamin recommendations, rather than requirements, is more appropriate.

A recommendation should be based on expected concentrations of the vitamin, the presence of any antagonists, the cost of supplementation, the cost of a deficiency, and the amount needed to prevent an overt or clinical deficiency. Toxicity of the vitamin could be an issue, but, under practical conditions, toxicity is extremely rare. Vitamins A and D are the most toxic known vitamin and its toxic effects are observed only when it is fed in amounts that are 4–10 times the commonly recommended amount for several months.

Water-Soluble Vitamins

Normal feedstuffs and ruminal synthesis almost always provide enough water-soluble vitamins that deficiency is unlikely, and nationally recognized publications on nutrient requirements of dairy cows (e.g., National Research Council (NRC)) have not established requirements for any water-soluble vitamin. However, as milk yields continue to increase, supplemental water-soluble vitamins may become more beneficial. Based on current research, supplementation of only four water-soluble vitamins should be considered. If the diet is inadequate in cobalt, supplemental vitamin B_{12} will be needed; however, cobalt is usually less expensive than vitamin B_{12}. All diets fed to dairy cows should have at least 0.11 mg cobalt per kg diet dry matter, and some data suggest that for maximal vitamin B_{12} status, 0.2–0.3 mg k g^{-1} is needed. Providing approximately 50 g day^{-1} of rumen-protected choline (15 g of actual choline) during the peripartum and early lactation periods usually increases milk production (about 2 kg day^{-1}) and may help with fatty liver. The milk price must justify the cost of the rumen-protected choline (which is substantial) before the product is used. Feeding 12 g day^{-1} of niacin is expected to increase daily milk production by approximately 0.5 kg. Niacin is relatively inexpensive, but the expected milk response is quite modest and routine supplementation may not be profitable. Early lactation cows are more likely to respond. Feeding 6 g day^{-1} (a common supplementation rate) generally does not increase milk yield enough to justify supplementation. Using niacin to prevent or treat ketosis is not justified based on current data. Supplemental biotin (approximately 20 mg day^{-1}) has consistently improved hoof health in dairy cows and should be used if hoof

Table 3 Fat-soluble vitamin requirements and recommendations for dairy cattle (assumed body weight of lactating cow, 600 kg; assumed body weight of pregnant and periparturient cow, 690 kg)

Vitamin–animal class	*NRC (2001) requirement (IU day^{-1})*
Vitamin A	
Lactating cow	66 000
Dry pregnant cow	71 500
Periparturient cow	71 500
Vitamin D	
Lactating cow	18 000
Dry pregnant cow	19 500
Periparturient cow	19 500
Vitamin E	
Lactating cow	480
Dry pregnant cow	1040
Periparturient cow	1040

Requirements for vitamins A, D, and E are for supplemental, not total, vitamins.

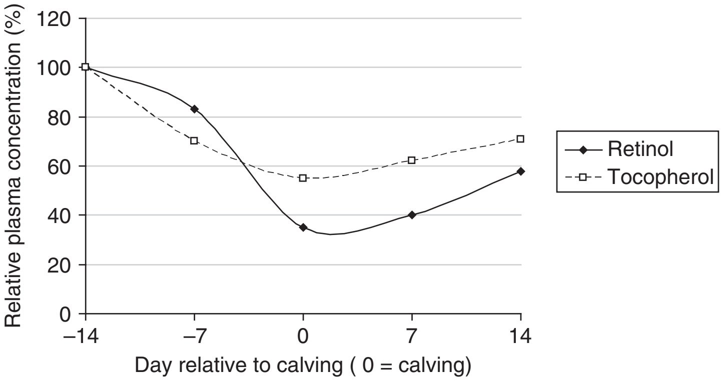

Figure 1 Changes in plasma concentrations of retinol (vitamin A) and tocopherol (vitamin E) in dairy cows during the periparturient period. Concentrations at 14 days before calving set to 100% and supplementation rates of the vitamins did not change over time. Data were compiled from several published studies.

integrity and lameness are a concern. Supplemental biotin often, but not always, increases milk production.

Fat-Soluble Vitamins

Requirements (or recommendations) for dairy cattle have been established for vitamins A, D, and E (**Table 3**). Because assaying feeds for vitamins is difficult and expensive, the actual dietary concentrations of these vitamins are usually unknown, and requirements are often expressed as the amount of supplemental vitamins needed. Vitamin A requirements are usually based on the amount needed for good reproductive performance. Vitamin D requirements are based on blood calcium, phosphorus, and 25-hydroxyvitamin D concentration. Historically, vitamin E requirements were based on reproductive performance, but more recent national standards have used health measures to define the vitamin E requirement. The response measures used to establish requirements for vitamins A and D and, to a lesser extent, E ignore possible effects on immune function and prevention of diseases other than those directly related to vitamin deficiency. Because of variability in vitamin supply, decreased activity of supplemental vitamins during storage, relatively low supplementation costs, and potential benefits on immunity and general health, vitamins A, D, and E are often fed at rates above national standards. Research has also shown that requirements for vitamins A and E are dependent on the stage of gestation and lactation. Plasma concentrations of retinol (vitamin A) and tocopherol (vitamin E) drop precipitously shortly before calving and remain low for a few weeks (**Figure 1**), suggesting that additional supplementation of these vitamins is warranted during this period. Cows are immunosuppressed during this period and have increased susceptibility to disease; increasing vitamin A and E intake during this period has improved cow health.

Conclusions

A rational program of vitamin supplementation is cost-effective, but excessive supplementation increases feed costs and can reduce profitability. Current data do not support routine supplementation of most water-soluble vitamins, but supplemental choline to increase milk yield in early lactation and supplemental biotin throughout lactation to improve hoof health may be profitable. Supplemental vitamins A, D, and E are usually necessary when cows are fed silage or hay, but because fresh forage contains substantial amounts of β-carotene and vitamin E, grazing cattle should require less supplemental vitamins A and E.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Acidosis/Laminitis. **Feed Ingredients**: Feed Supplements: Microminerals. **Feeds, Ration Formulation**: Dry Period Rations in Cattle; Lactation Rations for Dairy Cattle on Dry Lot Systems; Lactation Rations in Cows on Grazing Systems; Systems Describing Nutritional Requirements of Dairy Cows; Transition Cow Feeding and Management on Pasture Systems. **Mastitis Therapy and Control**: Management Control Options. **Nutrients, Digestion and Absorption**: Absorpotion of Minerals and Vitamins. **Reproduction, Events and Management**: Pregnancy: Periparturient Disorders.

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FEEDS, PREDICTION OF ENERGY AND PROTEINS

Contents

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Feed Energy

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Introduction

Formulating diets so that adequate energy is consumed by cows is a challenge because factors governing the availability of energy from diets are complex and energy availability cannot be measured directly using laboratory techniques. Knowing the energy content of feeds is important for diet formulation and comparing the economic value of different feeds. A variety of methods are available to estimate the energy content of feeds ranging from the use of reference values found in feed composition tables to extremely complex mathematical models.

Basic Energy Concepts

Energy is defined by physicists as the amount of work a system is capable of doing. In dairy cattle nutrition, a feed energy system should accurately estimate the amount of work (maintenance of the cow, milk production, growth, etc.) a diet can support. To estimate the available energy content of feeds, the potential losses of energy must be estimated. Intake energy is partitioned into digestible energy (DE), metabolizable energy (ME) and net energy (NE) (**Figure 1**). In theory, the more energetic losses that are considered, the more accurate the estimate of feed energy. However, in practice some of these losses are extremely difficult to measure which reduces the overall accuracy and precision of the estimated energy value. Even with this problem, the consensus among nutrition scientists is that NE more accurately describes feed energy than ME which is more accurate than DE. Because of this, a good energy estimation method should estimate NE. Although describing feed energy in terms of digestibility (e.g. DE) is not as accurate as ME or NE, digestibility must be accurately estimated if accurate estimates of ME or NE are to be obtained. The efficiency of converting gross energy of a feed or diet to DE (i.e. digestibility) is more variable and therefore more difficult to estimate accurately than the efficiency of converting DE to ME or to converting ME to NE.

Digestibility

In the late nineteenth and early twentieth centuries, measuring energy was extremely difficult and systems based on digestible mass, rather than energy, were developed to describe the energetic value of diets. Digestibility of organic matter (OMD), dry matter (DMD), and total digestible nutrients (TDN) were (and still are) used as proxies for DE. This approach was acceptable when diets for cows were very simple and consisted mostly of forages; however, dairy cows are now fed complex diets with several different ingredients. Because of the diverse nature of feeds and diets fed to dairy cows, DMD, OMD and, to a lesser extent, TDN, do not accurately describe the energy content of feeds. Ash, fat, carbohydrate and protein contain different amounts of energy per unit of mass. Two feeds that have the same DMD can have

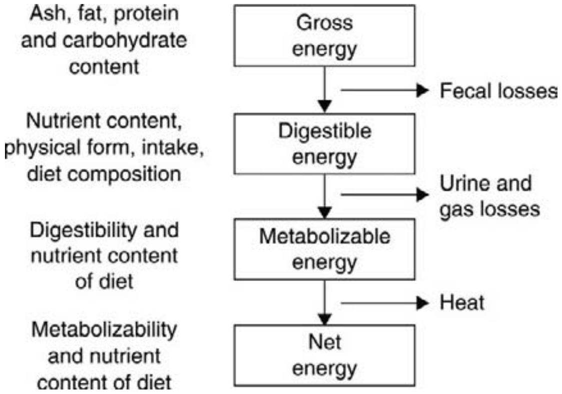
Ash, fat, protein and carbohydrate content
Gross energy
Fecal losses
Nutrient content, physical form, intake, diet composition
Digestible energy
Urine and gas losses
Digestibility and nutrient content of diet
Metabolizable energy
Heat
Metabolizability and nutrient content of diet
Net energy

Figure 1 The classical energy partitioning scheme for feeds (center column). Gross energy represents the total energy content of a feed or diet, net energy represents the amount of energy in a diet that is available for productive purposes. The left-hand column includes factors known to effect each respective energy expression. The right-hand column illustrates energetic losses associated with each energy expression.

different DE contents because of differences in concentrations of ash, fat, protein and carbohydrate. Organic matter digestibility accounts for variation in ash content but not for variation in protein, carbohydrate and fat concentrations. The TDN content is based on mass but incorporates some factors (ash and fat) that partially adjust for differences in digestible nutrient content. Because digestible protein is assumed to have the same energy content as digestible carbohydrate, TDN actually reflects both digestive and urinary losses of energy. TDN, DMD and OMD do not include energy lost as methane and heat. These losses are not constant and are greater for forages than concentrates. Therefore, measures of digestible mass overestimates the true energy value of forages relative to concentrates. Using TDN, DMD and OMD as measures of feed energy for dairy cows should be abandoned because they are not biologically correct, they are biased, and energy content of feeds and feces can now be measured easily and accurately.

Estimating DE at Maintenance Intake

Measuring DE requires feeding a test diet to several cows for at least 2 weeks and then measuring total feed intake and total fecal output or estimating fecal output using a digestibility marker. This technique is time and labor consuming, extremely expensive, and impractical for routine feed analysis. Therefore, several different methods have been developed to estimate DE. Almost all the methods currently used involve measuring DE *in vivo*, analysing certain characteristics of the feed, and then deriving equations that use feed characteristics to estimate *in vivo* DE. A major limitation of this approach is that the energy content of a feed depends not only on its characteristics but also on the composition of the entire diet and on feed intake of the cow. To reduce intake effects, many digestion trials are conducted with cows (or sheep) that are fed only enough energy to maintain themselves (maintenance intake). Another factor affecting digestibility of a feed is the composition of the diet in which the feed is fed. The digestibility of a specific feed may differ when fed in different diets. This response is referred to as an associative effect. An example of a positive associative effect is that when protein is added to a low-protein diet digestibility of a fibrous feed often increases. Conversely, feeding excessive amounts of starch can reduce the digestibility of a fibrous feed, i.e. a negative associative effect. To avoid associative effects many digestion trials are conducted using very simple diets with only one or two ingredients. Restricting intake and using simple diets reduces experimental variation but also reduces the accuracy of the measurement because dairy cows usually are fed mixed diets at high intakes. Equations then are derived using the *in vivo* data to estimate DE at maintenance intake and other equations are used to convert those values into energy values when cows are fed at productive levels of intake.

Equations Based on Nutrient Composition

Numerous equations relating feed composition to different measures of available energy have been published during the last 50 years. Most of the equations were derived by regressing the energy measure on the concentration of a single nutrient. Because the digestibility of fiber (usually measured as acid detergent or neutral detergent fiber (ADF, NDF)) is usually less digestible than nonfiber, the concentration of fiber is negatively correlated with DE and is most commonly used to estimate energy content. Some example equations are shown in **Table 1**. Several problems are associated with the use of single component regression equations: (1) they lack sensitivity because nutrients other than fiber can affect digestibility and energy content, (2) they are population-specific therefore different equations are needed for different feeds and an equation developed from feeds grown in one year under specific conditions may not be accurate for feeds grown under different conditions, and (3) they lack precision and tend to have high prediction errors.

Multiple regression has been used to overcome some of the problems associated with single component equations. The prediction error for multiple regression equations is usually, but not always, less than for simple linear regression equations. Two approaches can be followed when using multiple regression. The ‘shotgun’ approach is when all measured nutrients are entered into a stepwise regression and an equation that includes

Table 1 Example equations based on acid detergent fiber concentrations (g kg^{-1} of dry matter) to estimate some measure of digestibility

Equation		*Residual standard deviation*
Legume forages		
	$DE = 4.308 - 0.00505 \times ADF^a$	0.15
	$OMD = 92.3 - 0.091 \times ADF^b$	2.2
Grass forages		
	$DE = 3.313 - 0.00225 \times ADF^a$	0.11
	$OMD = 118.0 - 0.1399 \times ADF^a$	4.9
Maize silage		
	$DE = 4.103 - 0.00446 \times ADF^a$	0.15
	$OMD = 86.25 - 0.060 \times ADF^c$	2.1

[a] Harlan DW, Holter JB and Hayes HH (1991) Detergent fibre traits to predict productive energy of forages fed free choice to nonlactating dairy cattle. *Journal of Dairy Science* 74: 1337–1353.
[b] Minson (1982).
[c] Aufrere J, Gravious D, Demarquilly C *et al*. (1992) Estimation of organic matter digestibility of whole maize plants by laboratory methods. *Animal Feed Science and Technology* 36: 187–204.
DE, digestible energy (Mcal kg^{-1}); OMD, organic matter digestibility (%).

all significant variables is produced. This approach is statistically flawed because of collinearity among independent variables and can generate equations that are extremely specific to the samples used to generate the equation. A better approach is to include only composition variables that are thought to directly influence the energy content of feeds and are independent of each other. These equations usually include fiber plus components such as ash, lignin and fat.

A mechanistic approach should be a more robust method of estimating DE from feed constituents, and mechanistic models should be population independent (i.e. they should work for most or all feeds). These models partition feed components into energetically uniform fractions. To be a uniform fraction, digestibility and the gross energy per unit of digestible mass must be relatively constant among sources. Ash and lignin (no gross energy), and protein, nonfiber carbohydrates and fatty acids (relatively constant digestibility and gross energy), are commonly measured uniform fractions. Neutral detergent fiber (or any measure of fiber) is not a uniform fraction because digestibility is extremely variable among sources. To make the mechanistic approach feasible, NDF digestibility must be estimable. Such a model can be written as (nutrients as g kg^{-1} of dry matter):

$$\begin{aligned} \text{DE (Mcal Kg}^{-1}) = {} & (0.93 \times 0.0056 \times \text{CP}) \\ & +(0.98 \times 0.0042 \times \text{NFC}) \\ & +(0.98 \times 0.0094 \times \text{FA}) \\ & +(\text{dNDF} \times 0.0042 \times \text{NDF}) - \text{M} \end{aligned}$$

where 0.93, 0.98 and 0.98 = true digestibility of crude protein (CP), nonfiber carbohydrates (NFC) and fatty acids (FA), respectively; 0.0056, 0.0042 and 0.0094 = heat of combustion (Mcal g^{-1}) for CP, carbohydrates (NFC and NDF) and FA; NFC = (1000−NDF−CP−FA−ash); dNDF = digestibility coefficient for NDF; and M = metabolic fecal losses. The energetic value of the M fraction has not been determined experimentally but estimates based on metabolic fecal dry matter have been made (approximately 0.3 Mcal kg^{-1} of feed dry matter).

Equations or *in vitro* techniques (discussed below) can be used to estimate NDF digestibility. Two mechanistically based equations that include terms to estimate NDF digestibility are shown in **Table 2**.

Table 2 Theoretically based equations for estimating digestible energy (DE, Mcal kg^{-1}) or digestible dry matter (DMD, %) content of feeds at maintenance intake

Equation[a]	*Source*
$DE = (0.98 \times 0.0042 \times NFC) + (0.93 \times 0.0056 \times CP) + (0.0094 \times FA) + \{0.75 \times 0.0042 \times (NDF - L) \times [1 - L/NDF)^{0.67}]\} - 0.3$	National Research Council (2001)
$DMD = 0.098 \times (1000 - NDF) + \{147.3 - 78.9 \times \log[(L/ADF) \times 100]\} \times (NDF/1000) - 12.9$	Goering and Van Soest (1970)

[a] Nutrients entered as g kg^{-1} of dry matter.
NFC, nonfiber carbohydrates; CP, crude protein; FA, fatty acids; NDF, neutral detergent fiber; L, lignin; ADF, acid detergent fiber.

In Vitro Methods

In vitro disappearance consists of incubating a feed in either buffered rumen fluid or in an enzyme solution and then measuring disappearance of some fraction. For this discussion, *in vitro* disappearance (IVD) will refer to the use of rumen fluid and enzymatic digestion will refer to techniques using only enzymes. In 1963, the Tilley and Terry method was published and became the standard for IVD measurements of hay crop forages. The method consisted of a 48-h incubation in buffered rumen fluid followed by an acid-pepsin digestion. This method accurately estimated *in vivo* dry matter digestibility for sheep fed all forage diets at low intakes. This procedure has been modified several times but the most significant modification was the replacement of the acid-pepsin step with a neutral detergent extraction. With mathematical transformation of the data, the IVD with neutral detergent method can be used to estimate *in vivo* DM digestibility. The IVD methods are generally more accurate at estimating *in vivo* digestibility for hay crop forages fed at maintenance than are equations based on nutrient composition, but IVD is more laborious and requires a source of rumen fluid.

The IVD method is less accurate for feeds other than hay crop forages and its accuracy for estimating digestibility of a feed as part of a mixed diet is generally unknown. Digestibility of fiber *in vivo* and *in vitro* is usually reduced when starchy concentrates are fed or included with the substrate but most IVD methods do not include the addition of exogenous starch. Therefore, IVD may overestimate fiber digestibility by cows fed typical lactation diets at high intakes. Furthermore, for IVD, substrates are ground finely, and IVD of starch (for example that provided by maize silage) is almost 100%. Starch-containing feeds are usually not ground as finely when fed to cows as for IVD measurements, and when fed at high rates of intake (as for high-producing cows) *in vivo* starch digestibility is usually lower than 100%.

Enzymatic digestion involves incubating feeds with various mixtures of enzymes for various amounts of time. The main benefits of enzymatic digestion are that it can be standardized and enzymes are available from commercial sources (a cannulated cow is not required). The main disadvantage is that fiber digestion by enzymatic digestion is often considerably lower than *in vivo* digestibility. Significant improvements in methodology has reduced the difference between enzymatic and *in vivo* values. One of the better methods involves incubating feeds in an acid-pepsin solution for 24 h, followed by a high temperature (80 °C) hydrolysis of starch, followed by incubation in cellulase. Enzymatic digestion does not directly estimate *in vivo* digestibility; the values must be entered into an equation to convert the enzyme values into *in vivo* values. Most of the equations (*in vivo* digestibility $= a + b \times$ enzyme digestibility) have slopes significantly less than 1 and an intercept significantly greater than 0.

Both IVD and enzymatic methods estimate apparent digestibility, not DE. The concentrations of ash, fat and protein must be measured and then equations are used to convert digestibility into DE. As with the equations discussed above, *in vitro* methods estimate digestibility in cows fed simple diets (generally low starch) at maintenance intake. They do not estimate digestibility of high concentrate diets fed at high intakes.

Estimating DE for Typical Diets at Productive Levels of Intake

Mathematical models have been developed to convert DE estimated at maintenance to DE values in high concentrate diets fed at high intakes. It is beyond the scope of this article to discuss these methods in detail but these equations generally discount (i.e. reduce) digestibility as intake and the amount of concentrate in the diet increase. Some models are based on estimated rate of passage and rate of digestion (at high rates of passage, feeds with a rapid rate of digestion are discounted less than feeds with slower rates of digestion). Other models are based on intake but the principles are the same. The effects of high concentrate diets are handled in various ways. Some models estimate the effect high concentrate diets have on rumen pH and then estimate digestibility discounts based on estimated rumen pH. Other models use regression techniques to estimate discounts when concentrate is increased. Discounts for individual feeds are calculated in some models whereas other models calculate a discount factor for the entire diet and that discount is applied to each feed within the diet. The discounts range from essentially 0 (low intakes, high forage diets) to more than 15% (high intakes, high concentrate diets). Once the discount is calculated, discounted DE (Mcal kg^{-1}) is calculated as DE$\times$(1$-$discount) where discount is as a proportion of 1. For example, if DE at maintenance $= 3.0$ Mcal kg^{-1} and the discount is 12%, discounted DE $= \Sigma\times(1-0.12) = 2.64$ Mcal kg^{-1}.

Estimating ME

In addition to fecal losses, ME includes urinary and gaseous losses. Most of the energy lost via urine is in nitrogen-containing compounds and most of the gaseous losses are as methane. Feeds (or more correctly nutrients) that increase urinary and methane losses have a lower efficiency of DE to ME conversion. Following the classical energy partitioning scheme (**Figure 1**), ME should be calculated from DE, or more accurately, from the DE provided by each nutrient. Because of urinary losses, the DE from crude protein will have a lower efficiency going to ME than carbohydrates and fats. The DE to ME efficiency of digestible fiber will be less than that for digestible NFC because of methane losses. The DE to ME efficiency for digestible fat is greater than that for carbohydrates and protein because fat does not contribute greatly to urinary or methane losses. If the efficiency values are known, ME can be calculated directly from discounted DE values:

$$\mathrm{ME}\left(\mathrm{Mcal\ Kg}^{-1}\right) = 3(k_i \times \mathrm{DE}_i)$$

where k_i = efficiency of converting DE from nutrient i to ME, and DE_i = discounted DE from nutrient i. Currently, the efficiencies of each nutrient are not known with great precision. Based on limited data, DE to ME efficiencies are approximately 1.0 for DE from fat, 0.7 to 0.75 for DE from protein, 0.76 to 0.81 for DE from NDF, and 0.83 to 0.88 for DE from NFC. If the DE provided by each nutrient can be calculated and if these efficiency are correct, ME can then be calculated directly from DE in a rational manner. Because of the inaccuracies associated with the efficiency values, some systems estimate ME directly from DE. This approach works reasonably well for balanced diets but for diets with high concentrations of protein or fat, ME can be over- or underestimated, respectively.

Several regression equations that estimate ME directly from nutrient composition or *in vitro* digestibility measurements have been derived (**Table 3**). For limited sample populations (e.g. grass hay) these equations are about as accurate as estimating digestibility. Estimating ME directly from nutrient composition may have limited application in dairy nutrition because of the diverse nature of feeds and diets. The more scientifically rational approach of first estimating DE and then estimating ME should be more robust.

Estimating NE

The NE value of feeds represent the amount of energy that is actually used for productive work. Measuring NE content of diets is quite difficult since it requires that heat production be measured. Few measured NE values are available and few equations have been developed to directly estimate NE. For several years, the US energy system calculated NE-lactation (NEL) from TDN. This system was flawed, but because DE and ME data were scarce when the system was developed, this approach was a good compromise. The National Research Council system developed in 2001 calculates NEL from ME rather than TDN. Although the equations differ, many European energy systems have calculated NE from ME for several years. In general all the equations account for the increased efficiency of converting ME to NE as the ME content of the diet increases.

Equations have been developed that estimate NEL directly from feed characteristics. This approach is usually flawed because in most experiments, NEL is not actually measured. Equations are developed to use feed characteristics to estimate 'estimated NEL'. The regression statistics that are reported generally ignore the errors associated with

Table 3 Example equations for estimating metabolizable energy (ME, Mcal kg^{-1}) content of feeds from nutrient composition

Equation[a]	*Residual standard deviation*
Legume forages[b]	
ME = 4.039 − 0.00535 × MADF	nd
Grass forages[c]	
ME = 1.47 + 0.00225 × PC	0.57
ME = 3.98 − 0.0040 × ADF	0.80
Maize silage[d]	
ME = 0.942 + 0.00229 × PC	0.28
ME = 13.31 − 0.0098 × ADF	0.33

[a] Values entered as g kg^{-1} of dry matter.
[b] Agricultural Research Council (1993) *Energy and Protein Requirements of Ruminants*. Wallingford: CAB International.
[c] Givens DI, Moss AR and Adamson AH (1993) Influence of growth stage and season on the energy value of fresh herbage. 2. Relationships between digestibility and metabolizable energy content and various laboratory measurements. *Grass Forage Science* 48: 175–180.
[d] De Boever JL, Cottyn BG, De Brabander DL, Vanacker JM and Boucque CV (1996) Prediction of the feeding value of maize silages by chemical parameters, *in vitro* digestibility and NIRS. *Animal Feed Science and Technology* 66: 211–222.
MADF, modified ADF; PC, pepsin-cellulase digestibility; ADF, acid detergent fibre.
nd, not determined.

the 'estimated NE' and only reflect the errors in the regression equation used to estimate the estimated NE. If all prediction errors were considered, equations that produce an NEL value from feed composition are usually inaccurate. Rather than use feed characteristics to directly estimate NEL, following the classical energy scheme is preferred.

The efficiencies of converting ME to NEL for the different nutrients are not known with any degree of accuracy. For total diets, the average efficiency of converting ME to NEL is between 0.60 and 0.64. Diets with high fat will be at the high end and diets with high fiber will tend to be at the low end of the range. The narrow range in efficiencies for converting ME to NEL for diets means that knowing the efficiencies for each nutrient is not necessary. However, the range in efficiencies would probably be much greater if individual feeds, rather than diets, were used. Since most lactating dairy cows are fed diets with a blend of forages and concentrates, the use of ME to directly estimate NEL does not introduce large errors.

Limitations and Needed Improvements

In addition to the statistical and analytical problems discussed above, all the current equations to estimate feed energy have limitations. Perhaps the greatest limitation is the data available to derive the equations. Very often, the dependent variable is determined in sheep or cattle fed relatively high forage diets at low intakes. The dependent variable is usually DE or less often ME. Experiments that measure NE are still extremely limited. The DE or ME values obtained with this approach may have little semblance to values that would be obtained when the feed was fed as part of a typical lactation diet fed at high intakes. Computer models to estimate intake and associative effects are continually being improved but much more work is needed to increase accuracy. Most equations are based on chemical components, but physical characteristics of feed (e.g. particle size) can have a substantial impact on digestibility and metabolizability. Most energy prediction systems are still based on aggregate expressions of energy (e.g. NE) rather than substrate supply (e.g. glucose, acetate, amino acids). Truly accurate and robust systems for evaluating the energy value of feeds and for diet formulation must eventually be based on the energy yielding substrates provided by feeds when fed in a specific diet at a specific level of intake. Research should be directed to meet that goal.

See also: **Feeds, Prediction of Energy and Proteins**: Feed Proteins. **Feeds, Ration Formulation**: Models in Nutritional Management; Models in Nutritional Research; Systems Describing Nutritional Requirements of Dairy Cows.

Further Reading

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Feed Proteins

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Introduction

Most milk-producing animals are ruminants. Interestingly, ruminants are the only mammals with the unique ability to survive, grow, reproduce and lactate without a source of dietary true protein.

Forestomach fermentation of feedstuffs supplies substrates for synthesis of microbial protein. These include amino acids, peptides, ammonia-nitrogen (N), and carbon skeletons. Rumen microbes are composed of 55% to 65% crude protein (CP) based on their total N content. The amino acid profile of microbial protein is very similar to that of milk protein. In addition to microbial protein, ruminants utilize amino acids and peptides from the dietary protein that escapes rumen degradation. Based upon intrinsic characteristics of the protein source, as well as animal factors such as dry matter intake, proteins can either be degraded within the forestomachs or remain intact and escape rumen degradation. Research conducted in the 1960s demonstrated that lactating cows were able to produce up to 4500 kg of milk per lactation when fed diets containing no true protein. This demonstrates the importance of microbial protein in supplying essential and nonessential amino acids to lactating dairy cows. However, ruminal microbes do not provide enough protein for maximum milk production in most contemporary dairy animals, and sufficient dietary protein must be fed to escape ruminal degradation and supply a sufficient amount of amino acids.

Generally, dairy cattle are not efficient animals in converting dietary N into milk protein. Because extensive degradation of proteins within the rumen can increase N wastage and decrease efficiency of N utilization, strategies to formulate diets with protein sources that are more resistant to microbial degradation have been proposed. However, a balance between optimum microbial protein synthesis and supply of ruminally undegraded protein (RUP) with a balanced amino acid profile and of high intestinal digestibility is important for optimizing animal performance.

Rumen Fermentation

The rumen is a unique microbial ecosystem composed of three major types of microorganisms: bacteria, protozoa and fungi. The total number of microbes found in the rumen contents ranges from 10^{10} to 10^{12} cells g^{-1}. Because microbial protein can represent 40% to 70% of the total metabolizable protein available, optimizing rumen fermentation and microbial growth usually improves animal performance.

Microbial growth in the rumen–reticulum requires energy in the form of ATP, N in the form of NH_3 N, amino acids and peptides, branched chain fatty acids, and minerals such as potassium, sulfur and cobalt. In addition to nutrient supply, rumen pH, osmolarity, and outflow rate influence the growth of microbes and synthesis of microbial protein.

Energy in the form of fermentable organic matter is the key component dictating microbial protein synthesis. The duodenal flow of microbial protein can be predicted based upon the intake of net energy for lactation as follows:

$$\text{microbial protein} = 6.25(-30.93 + 11.45 \times \text{intake of net energy for lactation})$$

The most recent National Research Council report for dairy cattle observed that microbial N synthesis was related linearly to total tract dry matter digestibility and it can be calculated according to total tract apparently digestible organic matter (microbial N, g $\text{day}^{-1} = 21.03 \times$ kg of total tract digestible organic matter) or according to the intake of total digestible nutrients (microbial N, g $\text{day}^{-1} = 0.13 \times$ kg total digestible nutrients).

During the process of feed digestion, microbes must attach to feed particle, gain access to the least resistant areas (inner portion of the feed particle) and digest the material in a process called 'the inside out concept'. Therefore, proteins and carbohydrates are digested from the inside to the outside. This is one of the reasons why feed processing such as mechanical and physical processing of cereal grains improves nutrient digestion within the rumen. When sorghum and maize grain were processed by steam-flaking, the flow of microbial protein to the duodenum of lactating dairy cows was increased by 15% to 20% compared with dry-rolling. This effect of processing of cereal grain increasing microbial protein synthesis is caused by the increase in rumen starch digestibility, and subsequent increase in energy availability (ATP) within the rumen.

Because of the interrelationships between energy and protein in ruminants, it is extremely difficult, if not impossible, to predict availability of metabolizable protein, flow of amino acids to the small intestine and animal performance when only the dietary protein sources are considered.

Fractionation of Dietary Protein from Feedstuffs

Crude protein is determined by quantifying the total N content and multiplying that value by a specific factor. Because it is generally assumed that N represents 16% of the total weight of an amino acid, determination of CP in feeds have been based on their total N content multiplied by 6.25 (6.25 = 100/16). However, proteins vary in N content, and not all N is of true protein origin.

Until recently, protein nutrition of ruminants was based upon their requirements for CP. However, because of the increased level of productivity of these animals and increased knowledge of feed analysis and nutrient requirements, proteins have been fractionated into different categories such as CP, soluble protein, ruminally degraded protein (RDP), RUP and unavailable protein. In addition to those protein measurements, the amino acid profile of protein supplements can also be determined.

The Cornell Net Carbohydrate and Protein System (CNCPS) fractionates dietary protein into three major fractions: nonprotein N (fraction A), true protein (fraction B) and bound true protein (fraction C). True protein is further divided into three subfractions, B_1, B_2 and B_3, based on their rates of ruminal degradation. Determination of protein fractions is based upon protein solubility in borate phosphate buffer, neutral detergent and acid detergent solutions (**Table 1**). Fraction A is constituted by ammonia N, nitrate, soluble amino acids and proteins, and it represents the soluble protein fraction. Fraction B_1 is the rapid degrading protein composed by globulins. Fraction B_2 is the medium degrading protein, and it is composed by albumins and glutelins. Fraction B_3 is the slowly degrading protein, and it is composed by prolamins. Fraction C is the protein fraction associated with fiber, which is mostly unavailable and composed by bound protein and Maillard products. Fractions A, B_1, and most of B_2 represent the RDP. Part of B_2 and fractions B_3 and C represent the RUP.

Table 1 Determination of protein fractions according to the Cornell Net Carbohydrate and Protein System

	Solubility	
Solution	*Soluble*	*Insoluble*
Borate–phosphate buffer	A, B_1	B_2, B_3, C
Neutral detergent solution	A, B_1, B_2	B_3, C
Acid detergent solution	A, B_1, B_2, B_3	C

Because ration formulation systems require partition of dietary CP into fractions degraded in the rumen (RDP) and resistant to ruminal degradation (RUP), fractionation of proteins in feedstuffs becomes an important aspect of feed analyses. Protein sources highly degradable in the rumen, such as soybean meal and canola meal, are rich in fractions A and B_1, but those that are resistant to rumen degradation, such as fish meal and blood meal, have fractions B_2, B_3 and C as their major components.

Crude Protein

Ruminants like any other mammal have no requirements for CP, but for specific amino acids. However, because ruminant animals have an active pregastric fermentation, dietary CP becomes an important source of N to rumen microbes. For optimum rumen fermentation, the diet has to provide N in the form of NH_3 N, peptides and amino acids. When a minimum amount of CP is not supplied in the diet, rumen fermentation is compromised and feed digestibility and dry matter intake are reduced. For sheep and goats, a minimum of 7–8% CP in the diet is required for maintenance of rumen fermentation. In dairy cattle, based on *in vitro* studies, it has been estimated that a minimum concentration of $2.5\,\text{mg}\,\text{dl}^{-1}$ of NH_3 N is required for optimum microbial growth. However, several studies with ruminally cannulated high yielding dairy cows have demonstrated that the optimum NH_3 N concentration in the rumen fluid is between 10 and $20\,\text{mg}\,\text{dl}^{-1}$ to maximize dry matter intake and milk yield.

Although NH_3 N concentration in the rumen is not solely dependent on the amount of CP and RDP offered in the diet, dietary protein often has a large effect on rumen environment and animal performance. In dairy cattle diets, a minimum of 10–12% CP is often required to maintain adequate rumen function and dry matter digestion. However, lactating cows will require more CP in their diets according to body weight, pregnancy status, level of milk production and protein content in milk.

Diets for lactating dairy cows usually contain 16–19% CP. Early lactation cows appear to positively respond to CP percentages between 18% and 19%, probably to compensate for the lower dry matter intake and decreased microbial protein synthesis during the first weeks postpartum. Dry cows do not require more than 12% of the diet as CP, but as parturition approaches, dry matter

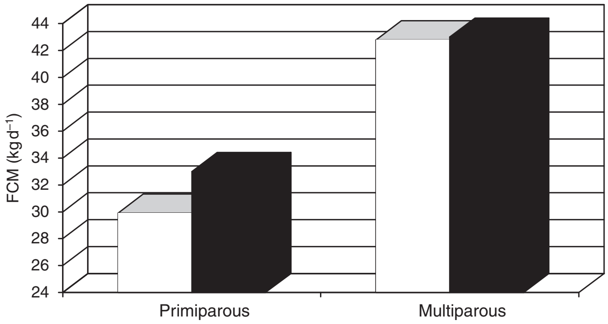

Figure 1 Effect of prepartum dietary CP/RUP content on 3.5% fat-corrected milk (FCM) yield during the first 120 days in lactation. Moderate CP/RUP (12.7% CP and 36% RUP; white solid bar); High CP/RUP (14.7% CP and 40% RUP; black solid bar). Significant interaction between treatment and parity ($p < 0.01$). (Adapted from Santos *et al*., 2001.)

intake decreases while protein requirements increase for fetal growth and colostrum synthesis. During the last 3 weeks of gestation, multiparous cows can still maintain a positive N balance when fed diets with 12% CP, but recent studies suggest that primigravid cows may benefit from diets with 14–15% CP during the last weeks of gestation (**Figure 1**).

Because metabolism of nitrogenous compounds uses energy, excessive CP feeding can be detrimental to animal performance. Ammonia passively diffuses through the rumen wall, and it is converted to urea by the liver. For every mol of NH_3 that enters the urea cycle, 4 moles of high-energy phosphate are consumed in each turn of the cycle. Because energy is consumed both fixing N and excreting it, dietary intake of protein beyond its need for biosynthesis of nitrogenous metabolites is energetically wasteful. Some have estimated that the energy cost for formation and excretion of extra urinary N is 7.3 kcal of metabolizable energy (ME) per g of N in dairy cattle and 3.8 kcal of ME per g N in sheep. When the energy cost of excreting excess N in the urine was calculated, 4.21 MJ of net energy for lactation were consumed for every 100 g of excess of N or 625 g excess CP excreted in the urine of dairy cows. This is the energy equivalent of almost 1.3 kg of 4% fat-corrected milk.

Rumen Protein Degradability

Different methods have been proposed to determine the rate and extent of protein degradation within the forestomachs of ruminants. These methods use direct determination of the flow of N fractions to the small intestine of animals cannulated in the abomasum or proximal duodenum. In addition, determination of protein degradation in feedstuffs can also be estimated by disappearance of protein from nylon bags incubated into the rumen, and from estimates of rumen passage rates of dietary protein. Because such techniques are costly and not readily adaptable to routine commercial analyses, less invasive procedures have been utilized to estimate protein degradation by *in vitro* methods. Incubation of feeds enclosed within nylon bags in rumen fluid *in vitro*, and solubility of proteins in borate–phosphate buffers, as well as neutral detergent and acid detergent solutions, have been utilized to determine protein solubility and to estimate protein degradability in the rumen. Although the latter systems provide information on rate of protein disappearance, no information on rumen outflow rate is obtained and effective protein degradability has to be estimated.

When the different protein fractions are analysed (**Table 1**) and the degradation rate of fraction B is known, the RDP of CP can be estimated by the following formula:

$$\mathrm{RDP} = \mathrm{A} + \mathrm{B}[\mathrm{Kd}/(\mathrm{Kd} + \mathrm{Kp})]$$

where Kd is the degradation rate of fraction B (% h^{-1}) and Kp is the rumen outflow rate (% h^{-1}). Fractions A and B are entered as percentage of CP.

Protein degradation within the rumen–reticulum involves two major steps: hydrolysis of peptide bonds, also called proteolysis, and deamination of amino acids. Enzymes present in the solid-associated phase of the rumen content are responsible for these two processes. Although it is not completely clear which of the two processes is the rate-limiting step, available data are consistent with the concept that proteolysis is usually the controlling reaction during protein degradation within the rumen.

The energy contribution from protein degradation within the rumen–reticulum varies with the amount and type of protein, and rate of intake. However, when casein is degraded in the rumen and all its amino acids deaminated, followed by decarboxylation of the respective α-keto acids, the stoichiometry of proteolysis and amino acid oxidation yields 1 mole of ATP per amino acid fermented, in addition of 1 mole of ATP per mole of propionate produced.

Protein solubility and other intrinsic characteristics of the feed material influence the rate and extent of protein degradation in the rumen. The composition of protein determines its solubility in an aqueous solution. Generally, globulins and albumins are more soluble than glutelins and prolamins. In addition, protein sources that contain high amounts of NH_3 N, urea, nitrates and free amino acids have a great degree of solubility in the rumen fluid. An example of protein sources that differ in their solubility and rumen degradability are represented in **Figure 2**.

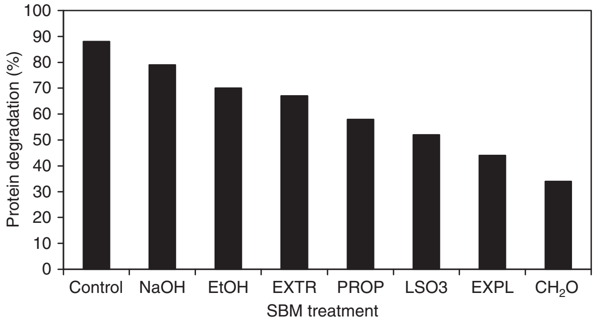

Figure 2 Extent of ruminal protein degradation of solvent-extracted soybean meal (SBM – control), soybean meal treated with sodium hydroxide (NaOH), ethanol (EtOH), extrusion (EXTR), propionic acid (PROP), calcium lignosulfonate (LSO3), expeller processed (EXPL) and formaldehyde (CH_2O) determined *in situ*. (Data from Stern *et al*., 1994.)

Silage-making increases protein solubility and rumen degradability because silage fermentation activates proteolysis and increases the amount of NH_3 N in the feed material. On the other hand, heat treatment increases the formation of Maillard products, which bind to the fiber and tend to be unavailable for digestion. Protein sources rich in keratin, such as feather meal, and proteins that have been heated, such as fish meal and blood meal, can have large amounts of disulfide bridges crosslinking from sulfydryl oxidation, which makes them poorly soluble in rumen fluid and decreases rumen degradability (**Table 2**). These disulfide bridges present in proteins of animal origin prevent bacterial colonization and enzymatic hydrolysis of peptide bonds.

Chemical and physical treatment of protein sources can also alter their protein composition and the degree to what they can be degraded in the rumen. Soybean meal, which is normally highly degradable in the rumen, when treated with different chemical substances, or extracted by extrusion or expeller processed can dramatically increase its undegradable fraction (**Figure 2**).

Animal factors can also affect protein degradability. The most important of them is rumen retention time. In order for feed materials to be digested, they need to remain in the rumen for a period of time that allows the microbes to colonize, have access to the inner portion of the material and complete digestion. When rumen retention time is decreased, protein degradation also decreases. Increasing dry matter intake increases rumen outflow rate, which decreases rumen retention time and protein degradability. Furthermore, rumen pH, feed particle size, defaunation and use of feed additives such as ionophores can all have an effect on protein degradation within the rumen.

Intestinal Degradation of Proteins

With increased productive capacity of dairy animals during the last decades, more attention has been given to the intestinal supply of proteins and specific amino acids. As the level of milk production increases, the contribution of microbial protein to the total metabolizable protein pool decreases. Because high-yielding dairy cows depend more upon the true protein that escapes rumen degradation than lower-yielding dairy cows, intestinal degradation of RUP and amino acid absorption become important aspects of protein nutrition for high-yielding animals.

Protein quality is dictated by its amino acid profile and intestinal digestibility. In general, protein sources considered to be of superior quality are those with an amino acid profile similar to that of milk protein and of high intestinal digestibility. Protein digestion in the small intestine of ruminants is similar to that in nonruminants. Proteins are hydrolyzed by proteolytic enzymes secreted by the

Table 2 Ruminal degradability and intestinal digestibility values of protein sources

Protein source	*Crude protein (%)*	*Rumen Crude protein degradability*[a] *(%)*	*Intestinal Crude protein digestibility*[b] *(%)*	*Essential amino acid index*[c]
Rumen microbes	60	NA	80	82
Soybean meal	48–54	65	93–96	71
Alfalfa hay	18–25	70	65.7–75	65
Maize gluten meal	67	32	92–97.4	52
Distillers' grains	30	48	80–84	54
Brewers' grains	29	38	80–85	67
Blood meal	93	22	56.3–80	60
Feather meal	86–92	20	65–70	34
Fish meal	68–71	30	90–96	68
Meat and bone meal	45–54	48	60–78	51

[a] Rumen protein degradation based on *in situ* incubation of protein samples.
[b] Intestinal digestibility based on mobile nylon bag technique. Expressed as percentage of the protein reaching the small intestine.
[c] Essential amino acid index from Chandler PT (1989) *Feedstuffs* 61(26): 14, 25.
Adapted from National Research Council (2001), O'Mara *et al*. (1997) and Erasmus *et al*. (1994).

pancreas and single amino acids are absorbed by specific transport systems across the intestinal cells. A summary of ruminal degradability and intestinal digestibility of different protein sources is given in **Table 2**.

Absorption of Amino Acids and Peptides

Amino acid and peptide absorption is unequally distributed throughout the digestive tract. In general, the distal duodenum and ileum are the major sites for free amino acid absorption. Also, evidence exists that the forestomachs of ruminants, specially the omasum, are capable of absorbing dipeptides. The expression of H^+-peptide cotransporters and the presence of H^+/Na^+ exchanger and Na^+/K^+ ATPase proteins in the epithelium of the omasum supplies evidence supporting that peptide absorption takes place in tissues other than the small intestine of ruminants.

Experiments with sheep have demonstrated that a smaller quantity of amino acids appears in the portal-drained viscera compared with that disappearing from the lumen of the digestive system. This suggests that amino acids absorbed by the gastrointestinal tissues can be utilized before they appear in the portal vein, which is the case of glutamine and glutamate used as energy source by intestinal cells.

In sheep, lactating dairy cows and beef steers the amino acid and peptide flux across the portal-drained viscera is responsive to dietary changes. Generally, increasing the intake of ruminally fermentable organic matter in the diet increases the flow of microbial protein and the net absorption of amino acids by the portal-drained viscera. However, protein intake has a poor relationship with portal-drained viscera α-amino acid N flux. In reality, intake of metabolizable energy is a better predictor of net flux of α-amino acid N by the portal-drained viscera than total protein intake.

Metabolizable Protein

Metabolizable protein (MP) is the true protein available and absorbed by the gastrointestinal tissues supplied by microbial protein and RUP. Because microbial protein can represent 40% to 70% of the total MP available for a lactating cow, ewe or goat, it is impossible to predict the MP content of a feedstuff based only upon characteristics of the dietary protein sources. Associative effects between the protein source, fermentable energy and rumen environment that might influence microbial yield are extremely important when predicting the MP of a diet. When protein degradation is synchronized with availability of fermentable organic matter in the rumen, microbial protein synthesis is favored, and this associative effect enhances the MP content of the diet.

Since rumen digestion rates and outflow of protein from the rumen can affect the amount of RDP and RUP of a diet, ruminal digestion and passage rates of protein should be integrated to predict the MP value of an ingredient or diet. Because the rumen is a dynamic compartment, any factor that influences microbial protein synthesis and alter the flow of microbial protein from the rumen can impact the MP content of feedstuffs and diets.

Determination of MP requirements of dairy animals is, in many cases, based on a factorial method. Factors such as milk yield, fetal growth, scurf losses and metabolic fecal losses are utilized for those determinations. Studies utilizing N balance data have estimated that the MP requirements for dairy cows to be between 3.5 to 3.9 g MP kg^{-1} of metabolic body weight ($BW^{0.75}$). Others have suggested a more detailed information on MP requirements for maintenance of dairy cows: MP g day^{-1} = scurf protein + urinary protein + metabolic fecal protein, or $MP = (2.75\ BW^{0.5}/0.67) + (0.20\ BW^{0.6}/0.67) + (0.09 \times \text{indigestible dry matter})$. Furthermore, the MP requirement for milk production is dependent upon the milk yield and the true protein content of milk. The efficiency of MP utilization for milk protein yield has been measured repeatedly and it is approximately 65%.

Amino Acids

Amino acid supply to the small intestine is provided by microbial protein and RUP. Ruminant tissues can synthesize nonessential amino acids but not the essential amino acids. However, rumen microbes can synthesize all 20 essential and nonessential amino acids. Between 40% and 70% of the amino acids entering the small intestine of dairy cows, goats and sheep are of microbial origin. As a result, the amino acid profile of the protein flowing to the small intestine can be considerably different from that in the ingested feeds, and more similar to that of microbial protein.

In many studies conducted to determine amino acid requirements for milk production, lysine appears to be the first limiting amino acid when dairy cows are fed maize-based diets. Methionine is likely to be the first limiting amino acid in lactating diets based on legume or animal proteins. In a series of duodenal infusion studies conducted by New Hampshire researchers, the need for supplemental lysine to optimize synthesis of milk protein was relatively more important than methionine in early and peak lactation.

French researchers observed that maximum milk protein output in lactating dairy cows is achieved when lysine and methionine represent 7.3% and 2.5% of the total metabolizable amino acids. Utilizing a different approach, New Hampshire researchers observed that maximum milk protein output was obtained when lysine and methionine represented 15% and 5% of the duodenal flow of essential amino acids.

Characterization of Protein Sources

Protein sources commonly utilized in dairy rations can be divided into different groups, based on their ruminal degradability and origin:

1. High in RDP: soybean meal, canola meal, cottonseed meal, maize gluten feed
2. High in RUP:
   - Animal origin: fish meal, blood meal, meat and bone meal, and feather meal
   - Vegetable origin: maize gluten meal, distiller's dried grains, distiller's dried grains with solubles, brewer's dried grains, and brewer's wet grains.

Table 3 calculates the essential amino acid index, taking into account an utilization factor for each amino acid and the chemical scores of protein sources in relationship to milk protein. It also lists the three most limiting essential amino acids in each protein source. Microbial protein is most often the best available source of protein for milk synthesis. Soybean meal received the second highest score and is considered to have a fair amino acid profile. However, soybean meal is extensively degraded in the rumen (65–75%), and its essential amino acid profile may not be as important as that of feedstuffs high in RUP. Nevertheless, processed soybean supplements (**Figure 2**) of low rumen degradability might supply a balanced amount of essential amino acids to the small intestine that might complement the amino acid profile of microbial protein.

Of the high RUP protein supplements, only fish meal possesses a good balance of essential amino acids such as lysine and methionine. In general, high RUP supplements of animal origin are adequate in lysine, but marginal or low in methionine. On the other hand, high RUP supplements of vegetable origin tend to be adequate in methionine, but low in lysine. The exception is feather meal, which is a poor source of both lysine and methionine.

It is clear the importance of both the amount and balance of essential amino acids in duodenal digesta. Therefore, protein sources should be compared based on their content and profile of essential amino acids, especially lysine and methionine (**Table 4**).

Protein Sources and Flow of Protein Fractions to the Small Intestine

Soybean meal is the most common protein supplement fed in dairy rations in the United States, and possibly in many other parts of the world. In 27 comparisons from 14 metabolism studies, replacing soybean meal with a high RUP source decreased microbial N flow in 10 comparisons. Cows fed soybean meal had a numerically higher flow of microbial N in 25 of the 27 comparisons, which suggests that a shortage of RDP limited microbial protein synthesis in the diets higher in RUP (**Table 5**).

Feeding a high RUP supplement increased feed protein escaping ruminal degradation in 9 of the 24 comparisons. However, the duodenal flow of feed protein and microbial N was increased in only 5 comparisons, decreased in 1 and did not change in 18 when RUP supplements replaced soybean meal. Because some of the RUP supplements did not have a well-balanced

Table 3 Essential amino acid (EAA) index and limiting amino acids as estimated by chemical score when compared with milk protein[a]

Protein source	*EAA index*	*Limiting amino acids*		
Blood meal	60	Ile (10)	Arg (33)	Met (45)
Fish meal	68	Ile (47)	Leu (58)	Val (59)
Feather meal	34	His (11)	Lys (13)	Met (23)
Meat meal	53	Ile (36)	Trp (39)	Leu (46)
Meat and bone meal	51	Trp (32)	Ile (36)	Leu (46)
Maize gluten meal	52	Lys (18)	Trp (30)	Arg (36)
Lucerne (alfalfa) meal, dehydrated	65	Lys (46)	Arg (50)	Ile (51)
Brewers' grains	67	Lys (34)	Arg (53)	His (56)
Distillers' grains with solubles	54	Lys (24)	Ile (38)	Arg (42)
Soybean meal	71	Ile (55)	Leu (56)	Met (56)
Microbes	82	Leu (84)	Ile (61)	Val (66)

[a] Calculated as E = [(log of amino acids in feed protein)/(log of % amino acids in milk protein)] × 100, where E = the 10 essential amino acids.

Adapted from Chandler (1989).

Table 4 The lysine and methionine contents of microbial protein and protein supplements compared with milk

	Lysine	*Methionine*	
Item	*(% of total essential amino acids)*		*Essential amino acids*[a] *(% of CP)*
Milk	16.1	5.7	38.4
Microbes	15.9	5.2	33.1
Protein supplement			
Blood meal	17.5	2.5	49.4
Brewers' dried grains	6.7	4.5	46.3
Maize gluten meal	3.8	7.2	44.2
Maize distillers' grains with solubles	5.9	5.9	37.7
Distillers' dried grains with solubles	6.5	3.7	43.3
Feather meal	3.9	2.1	31.4
Fish meal	16.9	6.5	44.8
Meat and bone meal			
45% CP	12.4	3.0	39.4
50% CP	14.2	3.7	36.6
Soybean meal (solvent)	13.8	3.1	47.6
Expeller soybean meal	13.0	2.9	49.6

From Santos *et al*. (1998).

Table 5 Summary of N flow to the duodenum of cows fed soybean meal or a high RUP supplement (14 studies with 27 comparisons)

	Treatment[a]		*Difference*		
Item	*SBM*	*RUP*	*g day^{-1}*	*%*	*p<*
N intake, g day^{-1}	469.1	463.6	−6.5	−1.4	0.67
Flow to the duodenum, g day^{-1}					
Microbial N	275.6	240.2	−35.4	−12.9	0.001
Feed N[b]	201.1	248.9	47.8	23.8	0.002
Microbial N and feed N[b]	474.3	486.7	12.4	2.6	0.31
Essential amino acids	1102.0	1159.0	57.0	5.2	0.11
Lysine	230.5	138.7	−91.8	−39.8	0.14
Methionine	45.1	46.5	1.4	3.2	0.42

[a] SBM, soybean meal; RUP, ruminally undegradable protein supplement.
[b] Feed N, Feed protein N escaping rumen degradation.
Adapted from Santos *et al*. (1998).

Table 6 Intestinal absorption of total essential amino acids, lysine and methionine of cows fed soybean meal or a high RUP supplement (4 studies with 11 comparisons)

	Treatment[a]		*Difference*		
Item	*SBM*	*RUP*	*g day^{-1}*	*%*	*p<*
Intestinal absorption, g day^{-1}					
Essential amino acids	762.3	802.0	39.7	5.2	0.17
Lysine	116.0	129.9	13.9	12.0	0.21
Methionine	36.0	31.7	−4.3	−11.9	0.21
Absorption, % of intestinal flow					
Essential amino acids	77.2	77.3	—	0.1	0.96
Lysine	80.8	80.9	—	0.1	0.97
Methionine	77.7	73.8	—	−3.9	0.14

[a] SBM, soybean meal; RUP, ruminally undegraded protein supplement.
Adapted from Santos *et al*. (1998).

amino acid profile and no effective gain in the flow of total N in protein was observed, the intestinal flow of methionine was decreased in 3 comparisons, increased in only 1 and did not change in 21. In addition, lysine flow decreased in 1 comparison, increased in 2 and did not change in 22. Furthermore, intestinal absorption of total essential amino acids, lysine, and methionine were not altered by feeding a high RUP supplement (**Table 6**).

In summary, replacing a RDP source with a high RUP supplement usually decreases the flow of microbial N, which negatively impacts intestinal flow of essential amino acids, including lysine and methionine.

Protein Sources and Lactation Performance

Optimizing protein utilization requires the ability to formulate diets that provide sufficient N for an adequate rumen metabolism, and absorbable amino acids in an amount and balance as required by the lactating animal. Because feed proteins differ in their degree of ruminal degradability and in amino acid composition, it should be no surprise that different protein sources may alter the pattern of amino acids flowing to the small intestine, which influences yields of milk and milk protein.

When protein sources are fed, the goal is to formulate a diet that maximizes the flow of microbial protein at the same time that the protein fraction that escapes rumen degradation supplies absorbable amino acids that complement the amino acid profile of the microbes. Unfortunately, not all protein supplements have an adequate amino profile when compared with that of microbes and milk (**Tables 3** and **4**). This becomes important when such protein sources are highly undegradable, which increases the contribution of their amino acids to the MP content of the diet. When protein sources with an unbalanced amino acid profile or of low intestinal digestibility are fed, absorption of limiting amino acids may be compromised and yields of milk and milk protein might suffer.

Because soybean meal is the most common protein supplement fed in dairy rations, and higher-yielding dairy animals have greater requirements for RUP, studies have compared the effects of replacing soybean meal with protein sources that are high in RUP. In many studies, simply replacing soybean meal with a high RUP supplement reduced yields of milk and milk protein. Many factors can contribute to the lack of response to RUP supplements. Some of these factors are:

1. Decreased microbial protein synthesis in the rumen.
2. Poor amino acid profile of the RUP source.
3. Low digestibility of the undegradable fraction of the protein supplement.
4. Diets with no RUP supplement may already supply enough MP for synthesis of milk and milk protein.

Although response to RUP is dependent upon several factors, generally, the sources high in RUP that most consistently benefit lactation performance of dairy cows are those with a balanced amino acid profile and a high essential amino acid index, such as fish meal and treated soybean meal.

Conclusions

Protein sources differ in their degree of rumen degradability, amino acid profile and intestinal digestibility. In ruminants, as milk production increases, the contribution of the rumen undegraded protein to the total metabolizable protein increases. Formulating diets with protein sources that maximize microbial protein synthesis and increase the supply of highly digestible, well-balanced undegraded feed protein improves yields of milk and milk protein.

See also: **Feed Ingredients**: Feed Concentrates: Co-Product Feeds; Feed Concentrates: Oilseed and Oilseed Meals. **Feeds, Prediction of Energy and Proteins**: Feed Energy. **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows; **Nutrients, Digestion and Absorption**: Fermentation in the Rumen.

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FEEDS, RATION FORMULATION

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Systems Describing Nutritional Requirements of Dairy Cows

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Introduction and a Rationale for Developing Systems for Describing Nutritional Requirements

Systems used for describing nutritional requirements of dairy cattle are a means of understanding nutrition and an essential step toward implementing more sophisticated nutritional strategies to control production from cattle. Part of the challenge for those involved in developing recommendations on nutrition is to distill the current knowledge of nutrition into usable systems to influence nutritional practice. The methods used to describe nutrition can be classified into two broad categories: those based on mechanistic approaches, which describe responses to nutrients in chemical, biochemical, and physiological terms; and those based on empirical approaches, which use relationships observed in carefully designed experiments to describe responses to nutrients. Many systems describing nutritional requirements extend beyond nutrition and evaluate the effects of environment and other influences on production.

There are three key components common to currently used systems:

- estimates of the energy, protein, mineral, vitamin, and water needs for maintenance and productive outcomes (milk, meat, fetal growth, and power)
- estimates of the value of feeds to cattle
- predictive equations that provide estimates of responses of cattle to feeds

There are four criteria for a successful feed system. It must be

- based on measurements of feed chemistry, physical form or biological degradation that can be adopted by a feed compounder
- deterministic and sufficiently descriptive of ruminant physiology to be able to incorporate the essentials of present and future knowledge
- able to predict the yield of the major truly absorbed substrates for energy and protein metabolism
- demonstrably better than existing empirical systems of prediction when tested in production trials

The challenge in the development of systems is to predict precisely the responses to nutrients in terms of productive outcomes, specifically milk production, milk composition, weight gain and the composition of weight gain, and weight of conceptus.

This article briefly examines the historical aspects of the development of feeding systems, and addresses differences and limitations of some commonly used energy and protein systems.

Historical Aspects

Our desire to predict precisely the productive responses of cattle to feed has developed rapidly since the nineteenth century. Early systems of prediction were based on the value of a single feed and simplified units were used, such as hay equivalents or the starch equivalent system. The latter system ranked a feed based on a value relative to feeding starch. The use of chemical feed analysis, such as the Wende system, to describe the composition of feeds provided additional impetus to develop systems of nutritional evaluation. There was interest in the 1900s in producing estimates of animal response based on energy, and these systems were more fully developed in the United Kingdom and United States. The ability to conduct calorimetry on cattle, either by direct calorimetry to measure heat production or by indirect means by measurement of respiratory exchange, was an important breakthrough in the development of the new systems. There was widespread adoption of the indirect calorimetric procedures in Europe and North America from the late 1940s. Commonly used systems for describing nutrient requirements have been, of necessity, gross simplifications of complex processes. These provide, however, valuable and useful tools for the management of nutrition.

Subsequent developments were based on metabolizable energy (ME) or net energy (NE) systems and refinements that more carefully examine the interactions of the animal with feed and the environment to predict performance. New systems will ultimately predict the generation of substrates used in metabolism from feed inputs. Recent improvements in computing power are of great significance to the development of better systems for assessing nutritional requirements.

Feed Intake

Prediction of the feed intake of cattle is an essential first step for determining probable responses to diet. Feed intake is influenced by stage of lactation (intake being lower in early lactation), weight of the animal, milk production, weight gain, and feed characteristics, particularly fiber estimates that reflect rumen fill and digestibility. Interestingly, in 1993, the Agricultural and Food Research Council (AFRC) produced estimates of intake for cattle eating silage-based diets that reflect lower feed intakes of these diets. A major review of feeding standards for ruminants was undertaken by the Feed Into Milk (FIM) consortium in the United Kingdom in 2004, which adopted a series of interrelated empirical equations that accurately predicted dry matter intake (DMI). These equations included concentrate feed level, milk energy output, live weight, silage intake potential, week of lactation, condition score, concentrate crude protein intake, forage intake potential and forage starch content, and an early lactation adjustment. The equations provided a model that predicted intake across the range of grass silage, whole crop, wheat, forage maize, and concentrate diets commonly used in the United Kingdom.

The Institut National de la Recherche Agronomique (INRA) in 1989 issued a document describing feed intake in terms of forage fill values and fill units per kilogram of dry matter, in which it is assumed that the feed intake is determined by a fixed amount of fill units. Also in 1989, a National Research Council (NRC) publication provided a table estimating the DMI of cattle weighing between 400 and 800 kg and producing between 10 and 45 kg of milk per day. In 2001, the NRC produced tables describing daily nutrient requirements, including estimated DMI, for cattle of live weight 454–680 kg at varying stages of lactation and with milk production varying from 15 to 55 kg^{-1} day and of, generated these tables from an underlying computer model. The Cornell Net Carbohydrate and Protein System (CNCPS) and the closely related CPM Dairy computer model estimate DMI from shrunk current live body weight (96% of full body weight), milk yield, and milk fat content for lactating cattle. DMI during the first 100 days of lactation is discounted at a decreasing rate. For heifers, shrunk current live body weight (96% of full body weight) and ration's NE content are the primary determinants of DMI. DMI is adjusted for effects of body fat (based upon maturity), temperature, night cooling, and mud.

Energy Use in Cattle

Figure 1 depicts the flow of energy through cattle. Energy is measured in joules, although the United States, among other countries, continues to use calories as a measure of energy (1 calorie = 4.182 joules (J)). Ultimately, at a biochemical level, work is performed in the release of energy from the reduction of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADP). A number of important concepts are inherent in **Figure 1**. Intake energy (IE) of a feed is a measure of the energy potentially available in the feed and can be estimated by the measurement of the energy released when the feed is burnt in a bomb calorimeter. At each lower level, the energy available for productive outcomes, recovered energy (RE), is better described. ME comprises heat, wasted energy in urine, and RE. NE is calculated after accounting for losses of energy created by excretion of urine. The substrates used for energetic work include carbohydrates, long-chain fatty acids, organic acids including the volatile fatty acids, and proteins. These are provided as carbohydrates, proteins, lipids, and organic acids in feed. Nucleic acids are absorbed in the small intestine, but are almost entirely excreted in the urine and provide little nutritive value.

The strength of the ME/NE systems is that these are integrated estimates of a number of underlying metabolic processes reflecting the use of nutrients in tissue maintenance and production functions. The energy requirements of cattle are described generally using

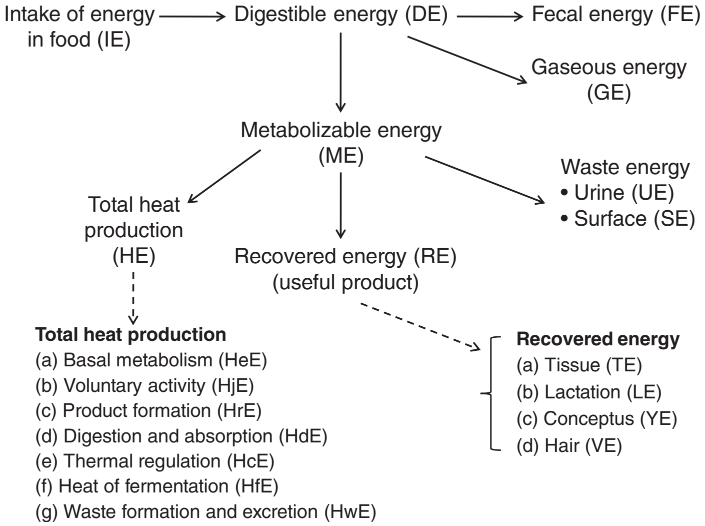
Intake of energy in food (IE)
Digestible energy (DE)
Fecal energy (FE)
Gaseous energy (GE)
Metabolizable energy (ME)
Waste energy
• Urine (UE)
• Surface (SE)
Total heat production (HE)
Recovered energy (RE) (useful product)
Total heat production
(a) Basal metabolism (HeE)
(b) Voluntary activity (HjE)
(c) Product formation (HrE)
(d) Digestion and absorption (HdE)
(e) Thermal regulation (HcE)
(f) Heat of fermentation (HfE)
(g) Waste formation and excretion (HwE)
Recovered energy
(a) Tissue (TE)
(b) Lactation (LE)
(c) Conceptus (YE)
(d) Hair (VE)

Figure 1 Energy flow in animals and suggested terms (NRC, 1981). Modified with permission from Reynolds CK (2000) Measurement of energy metabolism. In: Theodorou MK and France J (eds.) *Feeding Systems and Feed Evaluation Models*, pp. 109–128. New York: CABI Publishing.

factorial methods that account for costs of maintenance, milk production, body tissue gain or loss, growth, exercise, environmental impacts, and costs of gestation. The weaknesses in these approaches include

- an assumption of additivity that may not be correct
- a failure to distinguish between fermentable and nonfermentable substrates
- a failure to distinguish patterns of fermentation and their possible effects on the efficiency of utilization of ME, or composition of milk or body tissue
- a failure to permit a logical interpretation of the effects of plane of nutrition on fermentation, yield of ME, or microbial protein production

Simply, a measured ME provides no information on the profile of nutrients that are available to tissues for production and maintenance. These weaknesses in approach have been addressed in the CNCPS, by NRC in 2001, and FIM. Whereas feeds were described in terms of 'fermentable metabolizable' energy by AFRC in 1993, or NE value by NRC in 1989, more mechanistic approaches that reflect the potential for feeds and diets to stimulate the production of microbial protein were developed in the CNCPS and adopted by the NRC in 2001 and FIM in 2004.

Protein and Amino Acid Use in Cattle

Ruminants provide special benefits to humans through the conversion of feeds unsuitable to monogastric livestock or humans to products containing substantial amounts of high-quality protein. The symbiosis between ruminal microbes and cattle provides the opportunity for cattle to improve the nutritional quality of ingested nutrients. Systems that assess nutritional requirements need to reflect

- ruminal degradation of dietary protein
- the capacity of the rumen to utilize nonprotein nitrogen sources for the synthesis of microbial protein
- the variation in value to the animal of different proteins that escape ruminal degradation
- endogenous protein losses

Older systems for describing nutritional requirements of dairy cattle were based on digestible crude protein and they calculated apparently digested crude protein and used this as a measure of amino acid availability to the cattle. The more recent systems described below are semimechanistic and consider to some degree the interactions between carbohydrates and protein in the rumen, but often fail to consider amino acid metabolism in detail or interactions with other nutrients. **Figure 2** provides a conceptual framework for understanding protein metabolism. There are a large number of elements, in principle, that are similar in the protein systems, as identified by Jones and others in 1996. **Tables 1** and **2** provide evidence for the similarity in approach and estimated requirements derived using the different systems.

Mineral and Vitamin Requirements

Mineral and vitamin requirements have been calculated using factorial methods and generally include a margin for 'safety'. The NRC in 2001 estimated mineral needs in

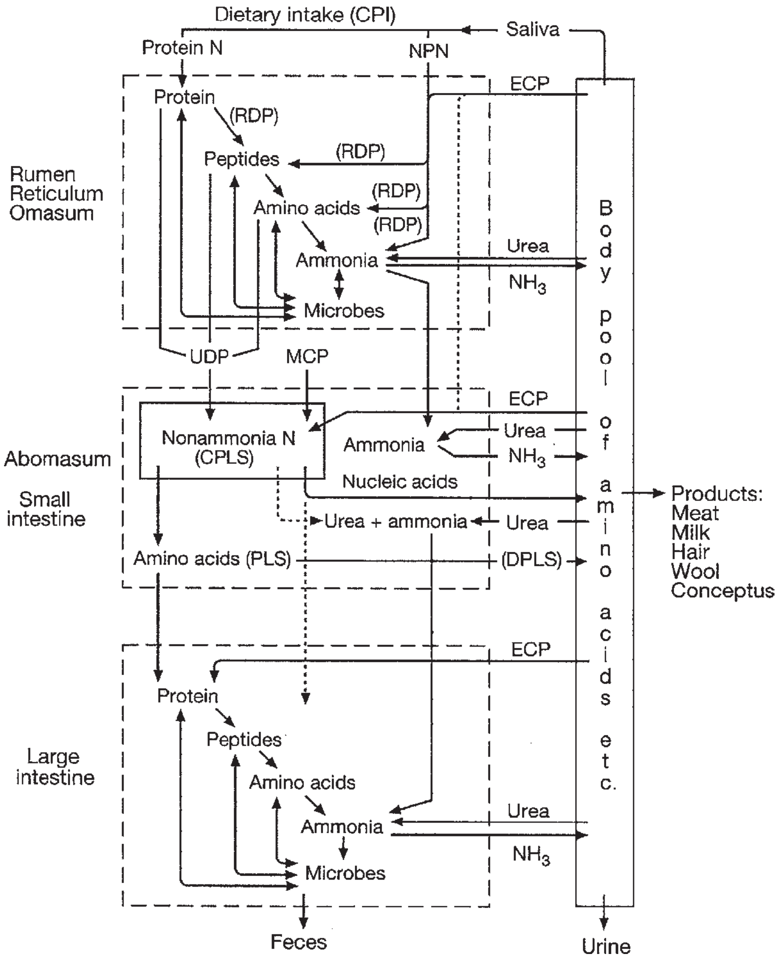
Dietary intake (CPI)
Saliva
Protein N
NPN
ECP
Protein
(RDP)
Peptides
(RDP)
(RDP)
Amino acids
(RDP)
Rumen
Reticulum
Omasum
Ammonia
Urea
NH3
Microbes
UDP
MCP
ECP
Nonammonia N
(CPLS)
Ammonia
Urea
NH3
Abomasum
Small
intestine
Nucleic acids
Urea + ammonia
Urea
Amino acids (PLS)
(DPLS)
Body pool of amino acids etc.
Products:
Meat
Milk
Hair
Wool
Conceptus
ECP
Protein
Peptides
Large
intestine
Amino acids
Ammonia
Urea
NH3
Microbes
Feces
Urine

Figure 2 The protein nutrition of ruminants. CPI, crude protein intake; NPN, nonprotein nitrogen; RDP, rumen-degraded protein; UDP, undegraded dietary protein; MCP, microbial crude protein; CPLS, crude protein leaving stomach (UDP + MCP); PLS, true protein leaving stomach = CPLS − (nucleic acid N × 6.25); DPLS, digestible true protein leaving stomach; ECP, endogenous crude protein. Reproduced from CSIRO (2007) *Nutrient Requirements of Domesticated Ruminants*. Collingwood, VIC: CSIRO; Figure 2.1.

terms of absorbed grams per day. These were incorporated into CPM Dairy, the computer-based nutritional model developed by Cornell University, the University of Pennsylvania, and the Miner Institute (*see* Models in Nutritional Management). **Table 2** provides estimates of requirements for the macrominerals as percentage of diet for the NRC and as absorbed grams for CPM Dairy.

Systems for Describing Nutritional Requirements

Some of the major systems used for nutritional evaluation are those of the Agricultural Research Council (ARC), FIM based on ARC, NRC, and INRA, as well as computer-based systems including Cornell and CPM Dairy, PC Dairy, and CamDairy. There are a number of outstanding attempts to provide more mechanistic systems of nutritional evaluation; however, these should be considered primarily experimental at this time. All systems have limitations that influence the capacity to predict responses of cattle to nutrients. The historical development of these systems provides an important context for understanding recent developments in the nutrition of cattle and advances in this field reflect the rapid increase in milk production of dairy cattle in technologically advanced regions of the world. A comparison of the estimated requirements for metabolizable protein (MP) produced using different systems is provided in **Figures 3** and **4**. There is much greater consistency of estimates of MP and energy produced from these systems than from those developed earlier. Differences in estimating nutrient requirements between current systems are arguably less important to the prediction of milk yield responses than problems with the accuracy and timeliness of methods for evaluating the value of feeds for cattle.

Table 1 Factors in the calculation of protein requirements

Factor	*Symbol*	*FIM 2004*	*NRC 2001*	*CamDairy*	*CNCPS*	*INRA 1989*
Efficiency of utilization of absorbed amino acids	effAA	na	na	0.70	na	na
Metabolic fecal protein	MFP	0.03DMI	0.03DMI	0.02DMI	0.09IDMI	ns
Efficiency of use of fecal metabolic protein	effFMP	ns	ns	ns	ns	ns
Endogenous urinary protein	EUP	$4.1LW^{0.5}$	$4.1LW^{0.5}$	$0.2LW^{0.75}$	$0.2LW^{0.67}$	ns
Basal endogenous protein	BEP	ns	ns	ns	ns	$3.25LW^{0.75}$
Efficiency of use of endogenous urinary protein	effEUN	1.0	1.0	ns	0.67	ns
Scurf protein	SP	$0.3LW^{0.6}$	$0.3LW^{0.6}$	$0.1125LW^{0.75}$	$2.75LW^{0.5}$	ns
Efficiency of use of scurf protein	effSP	1.0	1.0	ns	0.67	ns
Protein requirement for lactation	LP	13.57 x CP%	Linear	Curvilinear	Predicted	48 g kg^{-1}
Efficiency of use of lactation protein	effLP	0.68	0.67	ns	0.65	0.64
Protein requirement for live weight change	LWG	138 g kg^{-1}	134 g kg^{-1}	Linear	Predicted	250–150 g kg^{-1}
Efficiency of use of protein for live weight gain	effLWG	0.59	0.58	ns	Predicted	0.68–0.40
Efficiency of use of protein for live weight loss	effLWL	1.0	1.0	ns	Predicted	0.68–0.40
Protein requirement for pregnancy	PregP	Curvilinear	Curvilinear between days 190 and 279 of gestation	Curvilinear	Predicted	Tabulated
Efficiency of use of protein for pregnancy	effPregP	0.85	0.33	ns	0.5	0.60

CP, crude protein; DMI, dry matter intake; LW, live weight; na, not applicable; ns, not specified. Linear denotes that the equation used is a linear model, whereas curvilinear denotes that the equation used is a curvilinear model.

Table 2 Comparison of requirements based on CamDairy in 2000, NRC in 2001, FIM in 2004, INRA in 1989, and CPM Dairy, Version 3.09, in 2006, for a lactating cow weighing 600 kg producing 35 kg of milk, with 4% fat per liter, with no weight change

	NRC (2001)	*FIM (2004)*	*CPM Dairy*	*INRA*	*CamDairy (2000)*
Energy intake					
UFL				20.4	
NE intake (MJ (Mcal) day^{-1})	156 (37.1)	156 (37.1)	145 (34.5)	145 (34.4)	
ME intake (MJ (Mcal) day^{-1})	255 (60.8)	255 (60.8)	237.5 (56.4)	237 (56.3)	269 (64)[a]
Protein					
CP (% in diet DM)	-	-			16.5
UIP (% in diet DM)	-	-			6.48
DIP (% in diet DM)	-	-			10.02
DgP (% of protein)	-	-			60.7
MP (g day^{-1})	2450	2208	2496		-
Protein digestible in the small intestine (g day^{-1})				2075	
Fiber		Rumen stability factor 67			-
NDF (% in diet DM)	25	-	-		30 (20% eNDF minimum)
Nonstructural carbohydrate (g kg^{-1} minimum)	-	-			200 (minimum)
Ether extract (% in diet DM)	-	-	-		5.5 (maximum)
Ca (% in diet DM)	0.56	na	62[b]	0.7–.73	0.496
P (% in diet DM)	0.27	na	56[b]	0.37–0.39	0.35
Mg (% in diet DM)	0.13	na	7[b]	0.15–0.2	0.25
K (% in diet DM)	0.35	na	219[b]	70 mg k g^{-1} LW day^{-1}	0.9
Na (% in diet DM)	0.213	na	45[b]	0.17	0.18
Cl (% in diet DM)	0.26	na	54[b]		0.25
S (% in diet DM)	0.205	na	47[b]	0.2	0.2
Fe (ppm (mg kg^{-1}) in diet DM)		na	15[b]		50
Co (ppm (mg kg^{-1}) in diet DM)	0.11	na	0.11	0.1	0.1
Cu (ppm (mg kg^{-1}) in diet DM)	11	na	11	10	10
Mn (ppm (mg kg^{-1}) in diet DM)	14	na	14	50	40
Zn (ppm (mg kg^{-1}) in diet DM)	48	na	48	50	40
I (ppm (mg kg^{-1}) in diet DM)	0.6	na	0.6	0.2	0.6
Se (ppm (mg kg^{-1}) in diet DM)	0.3	na	0.3	0.1	0.3
Vitamin A (IU kg^{-1} in diet DM)	3169	na	3169	3200	3190
Vitamin D (IU kg^{-1} in diet DM)	864	na	864	1000	990
Vitamin E (IU kg^{-1} in diet DM)	23	na	23	15	15.4

[a] Allows a 10% margin above production energy for increased metabolic activity associated with high levels of production.

[b] Absorbed amounts (g day^{-1}).

CP, crude protein; DgP, degradability of protein; DIP, rumen degradable intake protein; DM, dry matter; eNDF, effective neutral detergent fiber; ME, metabolizable energy; MP, metabolizable protein; NDF, neutral detergent fiber; NE, net energy; UFL, unit of fill volume; UIP, undegraded intake protein.

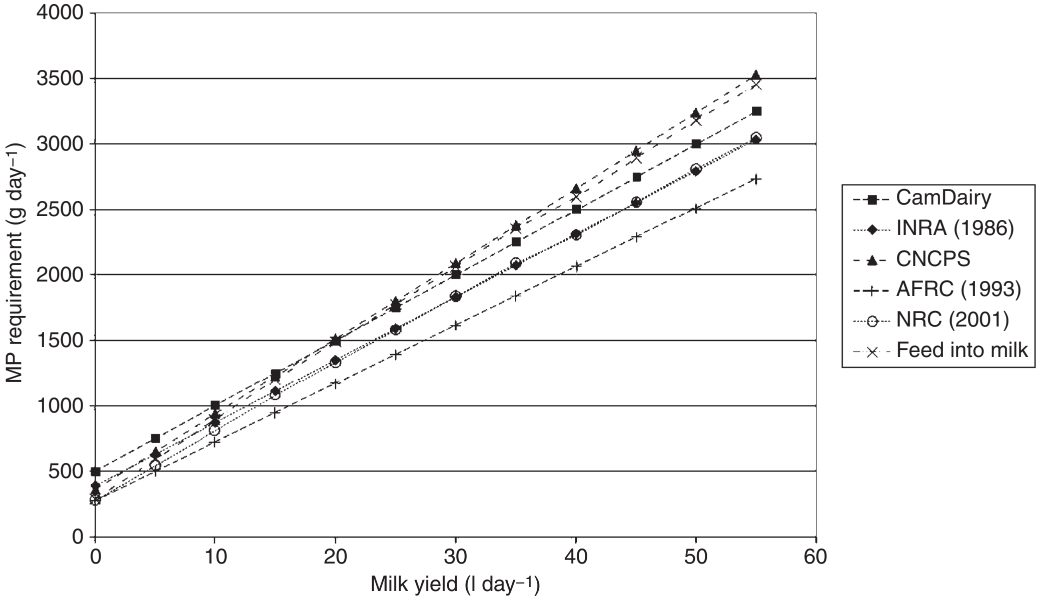

Figure 3 Requirements for metabolizable protein (MP) for milk production, produced using CamDairy, INRA (1989), CNCPS, AFRC (1993), NRC (2001), and FIM.

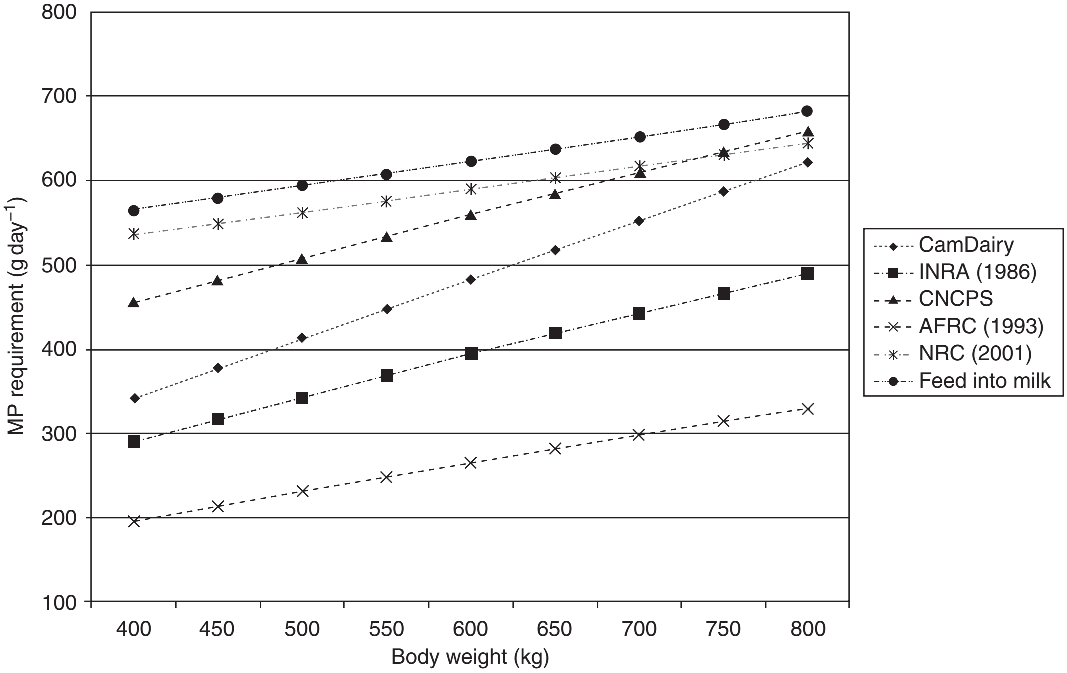

Figure 4 Requirements for metabolizable protein (MP) for maintenance, produced using CamDairy, INRA (1989), CNCPS, AFRC (1993), NRC (2001), and FIM.

ARC Systems

In 1965, the ARC produced a text that provided an evaluation of the ME, protein, mineral, and vitamin needs of cattle. Using this as a model, in 1975 the Ministry of Agriculture, Fisheries and Foods (MAFF) produced a simple model for evaluating the energy requirements of dairy cattle and despite subsequent estimates this remains the most practical means for evaluating energy requirements and predicting production responses in the field.

In 1993, the AFRC linked the processes of fermentation and microbial protein production to predict ruminal microbial protein output and developed an MP system. MP was defined as the total digestible true protein available for the cow for metabolism after digestion and absorption of feed. Digestible microbial protein, the result of microbial protein synthesis, was assumed to be 85% digestible and 75% of that digested is available for absorption from the small intestine. Digestibility of undegraded protein was estimated from the acid detergent-insoluble fiber or modified acid detergent fiber content of feeds. This MP system used information derived from *in sacco* digestion to estimate the rates of degradation of proteins to estimate the soluble fractions, those proteins slowly degraded in the rumen and those available to the small intestine. This system was a substantial development that addressed some of the limitations of earlier systems. There were, however, a number of failings in the model and estimates of protein requirements were substantially lower than those determined by NRC, Cornell, and CamDairy models.

The FIM review in 2004 adopted many of the equations defined by AFRC, but conducted a comprehensive review of nutritional requirements. New equations for estimating DMI were developed. The factorial method to calculate energy requirements was retained. New equations were developed to describe requirements for maintenance based on recently collected calorimetric data from 642 cows. The new equations corrected for feeding level and the efficiency of use of energy for lactation.

The FIM review contains considerable changes to the MP system developed by AFRC in 1993; however, requirements for MP for pregnancy and dermal losses and efficiency of utilization of MP for milk protein synthesis and live weight change proposed by AFRC in 1992 were not changed in the FIM model. The FIM model also notably incorporated an allowance for metabolic fecal N losses based on DMI and the estimates of MP required for maintenance are consistent with those of NRC published in 2001. The efficiency of use of MP for milk protein synthesis is, however, modified when MP supply exceeds requirements.

A new model of rumen function was developed for FIM. The model predicts the amount of rumen microbial protein and digestible rumen-undegradable protein that a diet will supply. The estimate of microbial protein produced is based on energy, or estimated ATP available to ruminal microorganisms, and availability of degraded nitrogen for the microorganisms. The model is dynamic, accounting for rumen outflow rate and degradability of the feed. The yield of ATP varies according to the protein, fiber, and starch content of the diet. The model has two subcomponents: one of the subcomponents predicts whether a diet is likely to result in problems with rumen acidosis and the other estimates supply of digestible amino acids to the cow and predicts whether either lysine or methionine is likely to be limiting. Fiber requirements and the potential to generate acid from the diet are used to determine a factor called potential acid load, which is used in conjunction with milk production, milk fat concentration, parity, and number of times that cows are fed per day to highlight diets that pose a risk of acidosis. Amino acid supply is estimated from the rumen microbial protein and rumen-undegraded protein (RUP) supply and the amino acid profiles of these. Uniquely among the systems reviewed, FIM uses a series of empirical equations to predict milk composition from diet composition.

NRC Systems

The NRC produced a series of publications from 1935 providing guidelines for energy nutrition of livestock. The most recent on dairy cattle, published in 2001, provides a useful method for predicting responses to nutrients. The review of dairy nutrition associated with the document provides a sound precis of dairy nutrition. The NRC energy system is suggested to be an NE system, but standard regressions are used to predict urinary losses and the system is in reality an ME system for lactating cows. The growth estimates for NRC, however, are based on NE estimates derived from slaughter experiments and are a true NE system. The protein system used is a factorial method based on ruminally degraded and ruminally undegraded intake proteins.

The NRC requirements published in 2001 represent a major change from the NRC requirements developed previously. The requirements are no longer based on tabulated values but on a dynamic computer model. Predicted DMI is based on body weight, fat-corrected milk production, and week of lactation. Estimates of maintenance are not changed from those used in 1989, and include a component for exercise. The energy costs of milk production include consideration of milk protein content. The energy costs of pregnancy reflect the increase in energy demands from days 190 to 279 of gestation, and partitioning of energy allows for growth according to age and mature body weight. Body condition score is also considered in partitioning of nutrients.

The energy content of feeds is calculated from the composition and accounts for the true digestible crude protein content of a forage or concentrate, the true digestible nonfiber carbohydrate (NFC) content, true digestible fatty acid content or ether extract, and true digestible neutral detergent fiber (NDF). A processing adjustment factor is applied to account for the availability of true digestible NFC. The level of feed intake above maintenance determines the rate of passage of feed and results in a discount of the energy value supplied compared to that provided at maintenance. Regression equations are then used to predict ME and NE of feeds and diets.

Protein requirements are now described as MP, being composed of rumen-degradable protein (RDP) and RUP. Estimates are based on total tract digestible organic matter and account for digesta flow rates.

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The INRA system is based on NE; however, this system also uses a standard regression on ME to estimate the NE value of feeds used for lactation. A modification of the starch unit is used to describe feeds, with feeds being described in terms of the NE content of a kilogram of standard barley. The protein truly digestible in the small intestine (PDI) system of protein evaluation is based on the efficiency of utilization of truly absorbed amino acids and provides responses consistent with those of NRC published in 2001. The PDI system, however, does not have an explicit requirement for losses of endogenous fecal nitrogen. Importantly, the PDI system is based on protein requirements determined from feed trials. One of the most impressive aspects of the PDI system is the extensive feed library that provides detailed information on the protein values of feeds.

Computer Models

Systems that use computer models such as Cornell, CPM Dairy, CamDairy, and PCDairy provide a powerful, relatively user-friendly means of predicting production responses to nutrients and evaluating dietary manipulations. These models allow greater opportunity for semimechanistic algorithms, as hand computations are not required. However, an increased knowledge of nutrition is probably a prerequisite to ensure that the solutions provided are feasible.

CNCPS and CPM Dairy

The CNCPS model provides many theoretical advantages in the evaluation and formulation of diets. It provides accurate estimates of the responses of dairy cattle to total mixed rations or pasture, and addresses rumen fermentation and microbial protein production using a semimechanistic model. There is a detailed consideration of the carbohydrate content of the ration and the effect of this is considered in the algorithms that predict microbial protein formation. The model represents a significant development in systems of nutritional evaluation. The CNCPS model provides a strong framework for assessing responses to diet. The rumen submodel provides substantial benefits in predicting yields of protein and amino acid supply to the small intestine and ME from the submodels of metabolism. This detailed estimation of protein yields and amino acid metabolism breaks new ground in practical ration evaluation. In CNCPS, predicted DMI is adjusted for environment and discounted during the early stages of the lactation cycle.

The lipid submodel developed for CPM Dairy and incorporated into CNCPS is a semimechanistic model that provides details on the ruminal metabolism of lipids and provides estimates of the amounts of individual fatty acids absorbed from the small intestine. Additions to CNCPS provided in Version 6.1 address the availability of free amino acids and peptides in the soluble protein fraction on the basis that these differ substantially from ammonia and other more basic nitrogenous compounds. Ruminal outflow rates of the soluble protein fractions have been changed to link these more closely with liquid flow rates than to solids passage rates. These changes are theoretically sound and pertinent to pasture-based diets. The carbohydrate pools have been expanded to include the organic acids. Organic acids, such as malate, can be quite high in forages and are used less efficiently for microbial growth compared to sugars.

CamDairy

The algorithms used in CamDairy are empirically derived equations that result in similar predictive responses to other models. Advantages of this computer model include ease of use and the ability to formulate least-cost and maximum profit rations using linear programming. The energy and protein estimates used are derived from independent analyses of responses to incremental increases in energy. The protein system used gives similar responses to Standing Committee on Agriculture in 1990 and NRC in 2001. One limitation is the lack of inclusions to account for the metabolic costs of detoxification of excess rumen-degradable nitrogen.

Limitations to Systems of Nutritional Evaluation

Biochemical Limitations to Predictions of Energy Use

The energetic efficiency of milk production varies with the mix of precursors available. **Tables 3** and **4**, derived from the work of Baldwin, demonstrate differing efficiencies of use of metabolites for milk production, and show that the theoretical efficiency of milk synthesis may vary from 0.75 to 0.92, depending on the precursors. Use of fatty acids for milk fat formation is more efficient than use of acetate, and use of glucose is more efficient than use of propionate. Observed efficiencies of production also vary with factors such as processing of grains, use of rumen modifiers, and amino acid composition of the ration. Estimates of ME used in feed systems do not indicate whether the energy is in the form of lipids, starches, sugars, or structural carbohydrates, and crude or even real protein and amino acid estimates do not indicate which amino acids will be available to the liver and mammary gland. Many of these limitations are addressed in the CNCPS, CPM Dairy, FIM, and NRC models. Differences between systems in the estimation of

Table 3 Energetic efficiency of milk synthesis per kilogram from efficient precursors

Milk component (precursor)	*Percentage in milk*	*Output (MJ)*	*Input (MJ)*	*Efficiency (%)*
Protein (amino acids)	3.1	0.743	0.907	82
Fat (fatty acids)	3.7	1.478	1.607	97
Lactose (glucose)	5.0	0.882	0.928	95
Total		3.104	3.373	92

Table 4 Energetic efficiency of milk synthesis per kilogram from less-efficient precursors

Milk component (precursor)	*Percentage in milk*	*Output (MJ)*	*Input (MJ)*	*Efficiency (%)*
Protein (amino acids)	3.1	0.743	0.907	82
Fat (acetate)	3.7	1.478	2.111	70
Lactose (propionate)	5.0	0.882	1.116	79
Total		3.104	4.139	75

nutrient requirements are understandable, as the flux of different precursors into the body is not easily estimated from data normally presented on the composition of ruminant diets. A key component of future developments will be the more effective description of the value of feeds to ruminants. Importantly, none of the current systems described here explicitly consider the issue of inconsistent feed supply within a day. While rumen bacteria grow rapidly with increased availability of substrate, these also die rapidly. Also, the effects of rapid changes in diet and particle size are not included in most of the requirement systems and there is limited consideration of the effects of the following on nutritional responses:

- disease states
- antinutritional factors such as endophytes, mycotoxins, and other plant toxins
- production modifiers such as ionophores, antimicrobial agents, and other rumen modifiers
- partitioning of nutrients to different pools, including body tissue

Homeorhesis – Homeostasis

A limitation to systems of nutritional evaluation is a failure to address the homeorhetic adaptations to lactation. Homeorhetic changes are the long-term adaptive changes that occur when an animal changes from being nonlactating to lactating or from being a nonruminant to a ruminant. Current feeding systems evaluate nutrient needs on a given day and do not consider the impact of diet formulation on longer-term adaptive changes. Furthermore, current systems of feed evaluation do not consider carryover effects of altered plane of nutrition on growth, body condition, mammary gland development, and appetite. These responses may not be simply linear, but follow a recursive pattern. That is, increasing plane of nutrition now may allow increased production later, but this production may require further or even an increasing supply of nutrients. This concept is exemplified by the strong association between a 1-l increase at peak milk production and a 200-l milk response over the whole lactation.

Protein or Amino Acids

Problems with estimating responses to protein or amino acid supplementation also indicate limitations with current systems. Limiting amino acids may substantively influence milk or total milk protein output; however, responses have not always been highly predictable. This problem is specifically referred to in the 1993 report of AFRC, in which the failure of the model to adequately predict responses to fish meal is acknowledged. The AFRC document also noted that the biological value of different proteins varies for different productive purposes, because of differences in amino acid composition. The CNCPS, CPM Dairy, FIM, and NRC models all have addressed amino acid nutrition. The CNCPS and CPM Dairy provide detailed estimates of the amounts of essential amino acids in MP and provide estimates of adequacy. The NRC also provides estimates of essential amino acids in MP, and FIM has a decision support module to evaluate the potential for milk production to be increased by the addition of protected methionine or lysine. The potential to accurately and repeatedly manipulate milk production or composition through use of amino acids remains a goal of dairy nutrition.

Conclusion

The substantial gains made in physiology, biochemistry, nutrition, and feed chemistry during the past 100 years are reflected in the sophisticated nutritional models that are now available. While there is substantial agreement among systems on the energy and protein requirements of adult cattle, there is still potential to improve given the differences in systems highlighted in this article and given the limitations also detailed. There is also evidence that milk production is driven rather than requirements met. Specifically, additional protein supplementation and injection of bovine somatotropin may initially cause cows to mobilize body tissue to meet additional demands for milk protein, fat, and lactose synthesis. Therefore, rather than feeding being considered a passive action of meeting nutrient requirements, it should be considered as an active process that determines the level of milk production by strategies that determine appetite. Given the potential to incorporate more mechanistic approaches to nutrition in dynamic models, the opportunity will develop to improve the sophistication of prediction of response incorporating the longer-term responses to nutrition.

See also: **Replacement Management in Cattle**: Growth Standards and Nutrient Requirements.

Further Reading

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Models in Nutritional Research

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Introduction

Models of nutrient utilization have long been used to estimate the nutrient requirements of animals. The earliest models were static, factorial, algebraic representations devised at the turn of the twentieth century by a number of workers, including Von Voit, Rubner, Atwater, Armsby, and Kellner. Subsequent and most current models of the nutrient requirements of domestic livestock have incorporated improvements in our understanding of digestion and energy metabolism but, largely, retain the static, factorial, algebraic characteristic of the original models. Over the past 30 years or so, many investigators have adopted the views that mechanistic models, which explicitly capture knowledge of cause-and-effect relationships, offer more promise than the statistically deduced empirical relationships, which comprise the bases of most current models, and that previous as well as current nutrition influences subsequent animal performance and that these variables are best accommodated by dynamic as opposed to static models.

We share these views and, with a view to the future, will emphasize progress toward the development of dynamic, mechanistic models of digestion and metabolism in dairy cattle that enhance understanding of the processes (**Figure 1**). The major research questions in this type of modeling include the problem of parameterization of dynamic, mechanistic models: what types of data are adequate and satisfactory? Is our understanding of digestion and metabolism in dairy cows adequate? What experiments and measurements are required in areas where current knowledge is found to be inadequate for the formulation of dynamic, mechanistic equations? Recognition of these issues led to the definition of models used in support of experimental research programs as research models. These models have been utilized to evaluate current knowledge, understanding, and data for adequacy in the quantitative and dynamic domains and to deduce parameter values not directly measurable using current technology.

Mechanistic Modeling: Hierarchy and Calculus

Modeling is a central and integral part of the scientific method. In particular, the importance of mathematics in dairy cattle research programs is in connecting quantitative observations to hypotheses related to cow physiology. A mathematical equation or model can be viewed as an idea, hypothesis, or relation expressed in mathematics. To appreciate fully the role of mathematical modeling in nutritional research in dairy cattle, it is necessary to consider the nature and implications of organizational hierarchy. A typical scheme for the hierarchy of organizational levels proposed by France and Thornley is shown in **Figure 2**. Any level of the scheme can be viewed as a system, composed of subsystems lying at a lower level, or as a subsystem of higher level systems. Such a hierarchical system has some important properties. First, each level has its own concepts and language. For example, the terms of animal production such as milk production and body weight gain have little meaning at the cellular level. Second, each level is an integration of items from lower levels. The response of the system at level i can be related to the response at lower levels by a reductionist scheme. For example, a description of amino acid (AA) utilization in the liver (level $i-1$) can provide a mechanism for responses in protein efficiency (amount of milk protein per unit dietary protein) of the cow (level i). Finally, successful operation of a given level requires lower levels to function properly, but not vice versa. For example, a microorganism can be extracted from the rumen and grown in culture in a laboratory, so that it is independent of the integrity of the rumen and the cow, but the rumen (and hence the cow) relies on the proper functioning of its microorganisms to function fully itself.

Mechanistic models in dairy cattle research programs consider processes at a level (level i) in relation to those at lower levels (level $i-1$). Other types of models include empirical models that examine a single level, and teleonomic models that usually look upward to higher levels. Mechanistic modeling follows the traditional philosophy and reductionist method of the physical and chemical sciences. This type of modeling gives rise to differential

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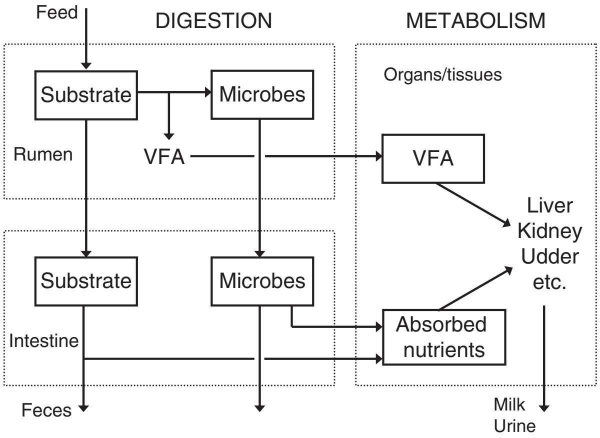

Figure 1 A simplified diagram of the major digestion and metabolism processes to be represented in an integrated whole dairy cow model. VFA, volatile fatty acids.

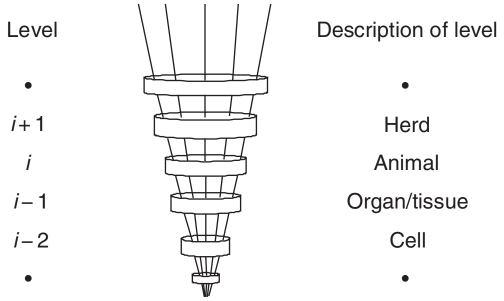

Figure 2 Levels of organization in animal science. The different levels are labeled . . ., $i+1$, i, $i-1$, ...

equations, and the standard way of representing such models is called the rate:state formalism. The system under investigation is defined at time t by q state variables: X_1, X_2, . . ., X_q. These variables represent properties or attributes of the system, such as quantity of substrate and organ mass. The model then comprises q first-order differential equations, which describe how the state variables change with time:

$$\frac{\mathrm{d}X_i}{\mathrm{d}t} = f_i(X_1, X_2, \ldots, X_q; S); \quad i = 1, 2, \ldots, q$$

where S denotes a set of parameters and the function f_i gives the rate of change of the state variable X_i. The function f_i comprises terms that represent the rates of processes, and these rates can be calculated from the values of the state variables together with the values of any constants and parameters. If the system under investigation is in steady state, solution to the differential equations is obtained by setting the differential terms to zero and manipulating to give an expression for each of the components and processes of interest. Stable isotope data generated using constant infusion, for example, are usually resolved in this way. However, to generate the dynamic behavior of a model, the rate:state equations must be integrated. For the simple cases, analytical solutions are usually obtained. An example is their use to interpret kinetic data from marker experiments, where the functional form of the solution is fitted to the data using a curve-fitting procedure. However, only numerical solutions to the rate:state equations can be obtained for the more complex cases. Such models are used to simulate dairy cattle digestive and metabolic systems. They are often used as tactical research tools to evaluate current understanding for adequacy and, when current understanding is inadequate, help identify critical experiments. Thus, they play a useful role in hypothesis evaluation and in the identification of areas where knowledge is lacking, leading to less *ad hoc* experimentation.

Dairy Cow Digestion

The first dynamic, mechanistic model of ruminant digestion, within our knowledge, was reported by Baldwin *et al.* in 1970. The objectives were to illustrate the utility and flexibility of using mathematical models in support of research and to summarize a number of ruminant digestive and metabolic energetic relationships. Model evaluation indicated an inadequate understanding of the utilization of substrates by microbes, in particular the utilization of nitrogen (N) sources, and microbial growth efficiency. This stimulated research into utilization of ammonia, AA, and small peptides by microbes in relation to microbial growth rate and growth efficiency. Part of this experimental research was performed using isotope-labeled substrates that require kinetic models to analyze and interpret the results. Using ^{14}C-labeled peptides and free AA, it was shown that during short-term incubations mixed rumen bacteria assimilated both peptides and AA, and their intracellular metabolism was also rapid. Studies using ^{15}N-labeled ammonia have greatly helped to elucidate the relative importance of various N sources for microbes. Depending on the diet, from 40 to 95% of the N in bacteria is derived from ammonia. Studies with labeled substrates also indicated extensive crossfeeding between microbial species. Although experiments using isotopically labeled substrates are expensive and laborious, their approach allows better identification (unique definition) of key microbial parameters required in rumen models.

Since the early 1970 model of Baldwin *et al.*, a number of other models were developed and new knowledge on substrate degradation and microbial metabolism integrated in those models. A significantly modified version of the 1970 model was employed to test additional hypotheses when evaluation of model behavior indicated inadequate representation. The number of microbial groups represented was reduced from a cellulolytic group, an amylolytic group, and a saccharolytic group to microbes free in rumen fluid fermenting soluble nutrients and microbes bound to particles fermenting insoluble nutrients. The reasoning in doing this was that microbial interactions known then and availabilities of individual substrates were not adequate to describe the complex competitions among microbes in the rumen. Furthermore, it was proposed that the rumen microflora were determined by the physical and chemical characteristics of the diet and, hence, that the products of rumen fermentation are best defined by diet components and not competitions among individual rumen microbes. Implementation of this concept required improved descriptions of the chemical composition of diets. However, further reduction in the number of microbial groups represented in rumen models appeared to be inadequate. For example, when both structural carbohydrates (SCs) and nonstructural insoluble carbohydrates (NSCs) are degraded by the single microbial pool represented, an increase in the availability of NSCs would increase the size of the microbial pool and therefore increase the degradation of SCs as well, whereas in actual experiments, SC degradation is often reduced upon an increase in NSC availability.

The large amount of new experimental results on microbial metabolism allowed Dijkstra and coworkers to improve significantly representation of rumen microbial metabolism, in particular protozoal metabolism. The need for reliable estimates of protozoal biomass, turnover, and outflow on a range of diets was indicated by model simulations, as well as the lack of quantitative data on bacterial engulfment and protozoal maintenance requirement and death rate. This in turn stimulated experimental work on protozoal metabolism to address these issues and shows again the important interplay between modeling and experimentation, and the positive effect of such a symbiosis on the improvement of our understanding of dairy cow nutrition. No direct *in vivo* measurements are available on the contribution of protozoa and bacteria to degradation of fiber in the rumen. The integration of concepts and data on various microbial groups in the rumen in a mechanistic model allowed quantification of the contribution of protozoa and bacteria to fiber degradation. The predicted protozoal contribution, as influenced by the diet, provided possible explanations for the differences in rumen fiber degradation observed when animals are defaunated.

Volatile fatty acids (VFA) deliver most of the metabolizable energy (ME) in a lactating cow. The need to maintain redox balance through reduction and reoxidation of pyridine nucleotides controls fermentation reactions in the rumen. A number of methods have been used to estimate the rates of individual and total VFA production in the rumen. Since the most accurate method employing isotopes to estimate VFA production is difficult and expensive, only VFA concentrations are measured routinely in rumen fermentation trials. Hence efforts were made to predict the stoichiometry of rumen fermentation using various modeling approaches. Murphy compiled two large data sets from the literature – one for largely concentrate-based diets and one for high-forage diets. Using these data sets, an algebraic rumen balance model was solved iteratively to deduce improved estimates of stoichiometric parameter values for rumen fermentation. In a number of subsequent evaluation studies, prediction of VFA molar proportions in rumen fluid was still shown to be inaccurate. Bannink and coworkers evaluated the various sources of error in predicting VFA molar proportions. Detailed simulation studies demonstrated that this inaccuracy was most likely caused by inadequate representation of the stoichiometry of VFA production or the rate of VFA absorption. This led to more experimental work to establish the factors influencing rate of VFA absorption. Bannink adapted the model of Murphy assuming a fixed incorporation of each substrate into microbial biomass. This model was fitted to data from experiments with dairy cattle obtained from the literature in which truly degraded substrate and VFA molar proportions were presented. The coefficients derived by Murphy and by Bannink are presented in **Table 1**. Since studies with rumen microorganisms have indicated a shift in VFA profile upon changes in pH of fluid, even upon incubation of single substrates, new stoichiometric values were recently derived related to rumen fluid pH. These values have been shown to be of great importance in predicting the production of methane per unit of degraded feed in the rumen, and triggered research into devising diets that reduce methane emissions. Excess reducing power generated during the conversion of hexose to acetate or butyrate is utilized in part during the formation of propionate or valerate and by conversion to methane. Thus adequate knowledge of the type of VFA formed contributes to proper prediction of methane formation in the rumen as well. Enteric methane production is a major item of interest since methane is a potent greenhouse gas, contributing to climatic change and global warming. The amount of methane formed per unit of feed is highly variable and may vary between 2 and 12% of gross energy intake. A sound prediction of the type of VFA formed is therefore of great interest to correctly predict enteric methane emissions and evaluate mitigation options.

In 1987, Baldwin *et al.* developed a model of metabolism in a lactating dairy cow (see the next section). In order to provide nutrients to the animal model at appropriate rates and patterns over time, a model of ruminant digestive function was devised. Despite the limited initial objective set in the development of this digestion model, it has continued to evolve, partially in response to

Table 1 Stoichiometric yield parameters (mol VFA mol^{-1} fermented substrate) for production of acetic acid (Ac), propionic acid (Pr), butyric acid (Bu), and branched-chain plus valeric acid (Vl) from fermentation of substrates on roughage and concentrate diets in the approach of Murphy and coworkers (M) and of Bannink and coworkers (B)

	Ac		*Pr*		*Bu*		*Vl*	
Substrate	*M*	*B*	*M*	*B*	*M*	*B*	*M*	*B*
Roughage diets								
Soluble sugars	1.38	1.29	0.41	0.16	0.10	0.24	0.00	0.04
Starch	1.19	0.98	0.28	0.43	0.20	0.21	0.06	0.08
Cellulose	1.32	1.12	0.17	0.41	0.23	0.17	0.03	0.07
Hemicellulose	1.13	0.88	0.36	0.35	0.21	0.32	0.05	0.06
Protein	0.40	0.62	0.13	0.32	0.08	0.09	0.33	0.07
Concentrate diets								
Soluble sugars	0.90	1.06	0.42	0.31	0.30	0.26	0.04	0.06
Starch	0.80	0.97	0.60	0.62	0.20	0.15	0.10	0.05
Cellulose	1.58	1.37	0.12	0.23	0.06	0.20	0.09	0.00
Hemicellulose	1.12	1.02	0.51	0.24	0.11	0.32	0.07	0.05
Protein	0.36	0.49	0.16	0.20	0.08	0.19	0.33	0.23

numerous challenges by us and others, and must now be considered a research model. Because of the increasing interest in protein supplements and rumen-protected AA to enhance the supply of critical AA to lactating cows, provisions for estimating the supply of individual AA to the animal have been included. It was found that provisions in the model for accommodating the effects of pH upon rates of fiber fermentation were inadequate. Based upon subsequent *in vitro* experiments that relate fiber degradation to pH, Dijkstra and coworkers developed a nonlinear relationship between rumen fluid pH and fiber degradation, through which interactions between availability of NSC and SC degradation were represented. Also, model predictions of starch digestion in the rumen in the Baldwin model are considerably higher than observed values at the high intakes characteristic of high-producing cows. Accurate estimates of starch fermentation in the rumen are essential for estimating rumen microbial growth (protein) yields. Dijkstra and coworkers further improved representation of starch dynamics in the rumen by allowing the polysaccharide content of microbial mass, which is assayed along with starch in chemical analysis, to vary.

Another problem that became apparent from the Baldwin and Dijkstra models that must be addressed is better definition and parameterization of equations for the computation of fractional rates of passage. Fractional passage rate not only has a major impact upon outflow of carbohydrates and proteins in the rumen, but it is also a major determinant of microbial efficiency (amount of microbial protein synthesized per unit of degraded organic matter), as established already in the 1960s by Pirt. Such research model evaluations also indicate which concepts or factors need to be addressed in practical feed evaluation systems to improve prediction of feed value in practice. At present, the digestive element appears to be adequate to meet the purpose for which it was originally designed, but advances from ongoing research will undoubtedly yield improvements.

Dairy Cow Metabolism

In the late 1960s, a dynamic, mechanistic model of the metabolism of a lactating cow was developed by Smith, based on biochemical pathways recently discovered. Several major benefits were realized from this early modeling effort. The first was the collection and summarization of a great many data from the literature and this has proven very useful in subsequent attempts to model dairy cow metabolism. A problem arose during the development of the data summaries, which illustrates the benefit to research arising from the modeling process. Data on organ and tissue weights in lactating dairy cattle were lacking in the literature. This required that the data be collected. The issue of relative organ weights and energy expenditures in animals in differing physiological states has since become a major focus in nutritional energetics. This helps emphasize our often-stated view that the modeling process aids in the identification of critical experiments and that persons conducting modeling must have the ability and resources required to collect critical data. A second benefit was the identification of significant gaps in our understanding of metabolism in adipose, liver, and mammary tissues of lactating dairy cows. This led to extensive experimental research and the development of models of metabolism in these tissues by Baldwin, Hanigan, and others. Another benefit was the initiation of efforts directed to the development of strategies for the simplification and formulation of more stable models, resulting in less expensive computer solutions.

The most notable failure of the early models was that milk fat percentage increased when the simulated cow was fed diets known to depress milk fat percentage. This failure clearly indicated that our knowledge and representations of adipose tissue metabolism were inadequate. This led to the conduct of a series of studies of adipose tissue metabolism to define and parameterize improved equations to represent metabolite interactions, the regulation of lipogenesis, energy storage, and lipolysis in research models of lactating cow metabolism. Tests of the modified research model against full lactation data still resulted in pronounced cumulative errors. Milk composition predictions appeared to be inversely related to observed composition as lactation progresses. Furthermore, a simulated net accumulation of body fat occurred during lactation on several but not all diets. The errors are relatively small on a daily basis (3–6 MJ day^{-1}) but the cumulative error is large: up to 100 kg empty body weight. These errors may be corrected by minor adjustments of energy expenditures in protein turnover and ion transport associated with the Na^+–K^+ ATPase. Such adjustments fall within the confidence limits of experimental measurements. However, experimental variance in the measurements is high, so it is difficult to ascertain whether these adjustments are the correct ones, as opposed to other possible model errors. These (model) errors become especially relevant in view of recent publications indicating that base estimates of apparent maintenance requirements of lactating dairy cows published by various governmental bodies of ~0.56 MJ of ME per kg metabolic body weight per day used indirectly in model development may be low.

However, adjusting energy expenditure in protein turnover and ion transport does not solve the problem of incorrect milk composition predictions during lactation noted before. Therefore, Hanigan and coworkers changed the representation of mammary cells and enzyme activity in the mammary gland, and active and quiescent cells with cell growth during gestation and early lactation periods, and first-order cell death, were represented, with enzyme capacity for fat and protein synthesis assumed to be proportional to cell numbers. Milk composition predictions clearly improved upon this modification, but body fat stores were still not predicted accurately.

The initial modeling analyses and subsequent modeling work indicated problems with parameterization of the liver and mammary elements of the model. This led to the conduct of a series of experimental studies of ruminant liver and mammary metabolism and evaluations of the data obtained using detailed models of liver and mammary function by Freetly, Hanigan, and Baldwin. Many of the concepts and data in those detailed models arose from compartmental analysis of experimental data using tracers to study VFA, glucose, long-chain fatty acid, and AA metabolism. Compartmental systems consist of a finite number of well-mixed, lumped subsystems (the compartments), which exchange with each other and with the environment so that the quantity or concentration of material within each compartment can be described by a first-order differential equation and expressed using the rate-state formalism. Such compartmental models are constructed to resolve data generated by *in vivo* experiments of organs and tissues. The use of isotopes allows various unidirectional fluxes to be quantified and thus provides far more of the insight required to develop organ or tissue metabolism models than data based on arteriovenous (A-V) techniques alone.

An example scheme for the uptake and partition of an AA (e.g., leucine (LEU)) by the mammary gland is provided in **Figure 3**. This scheme has been applied for various individual AAs. For example, this analysis was applied to experimental data with three lactating dairy cows, in which LEU exchange across the mammary gland was measured as well as the enrichment of [1-^{13}C]LEU in blood and secreted milk, to investigate the partitioning of LEU between milk protein output and other metabolic activities. The LEU A-V difference was on average 101% of LEU excreted in milk protein. LEU oxidation appeared to be minor. However, there was considerable synthesis and degradation of constitutive mammary gland protein, as evidenced by estimated LEU fluxes to and from this protein pool. LEU from degraded mammary gland protein contributed some 40% to net A-V flux of LEU, and the amount of LEU used to synthesize constitutive mammary gland protein was some 35% of LEU in milk protein. When applied to various AAs, such models provide a means of ranking the nutritional importance of individual AAs and identifying the most limiting in the gland, which is essential knowledge to further improve research models and also of high value in feeding practice. It may indicate aspects of metabolic regulation that could be manipulated to direct more AAs toward muscle (growth) or milk protein synthesis.

The studies described above also emphasized the importance of the interplay between modeling and experimental research – modeling analyses lead to the conduct of experimental research required to advance the overall research process, followed, in turn, by additional modeling studies. Several additional benefits derive from the use of modeling in support of a research program. One of these is that the modeling process aids in the design of required experiments. As the approaches to modeling dairy cow metabolism evolved, many mass action equations were replaced with Michaelis–Menten type equations in recognition of the fact that most metabolic systems exhibit saturation kinetics. While parameterization of a mass action equation simply requires an estimate

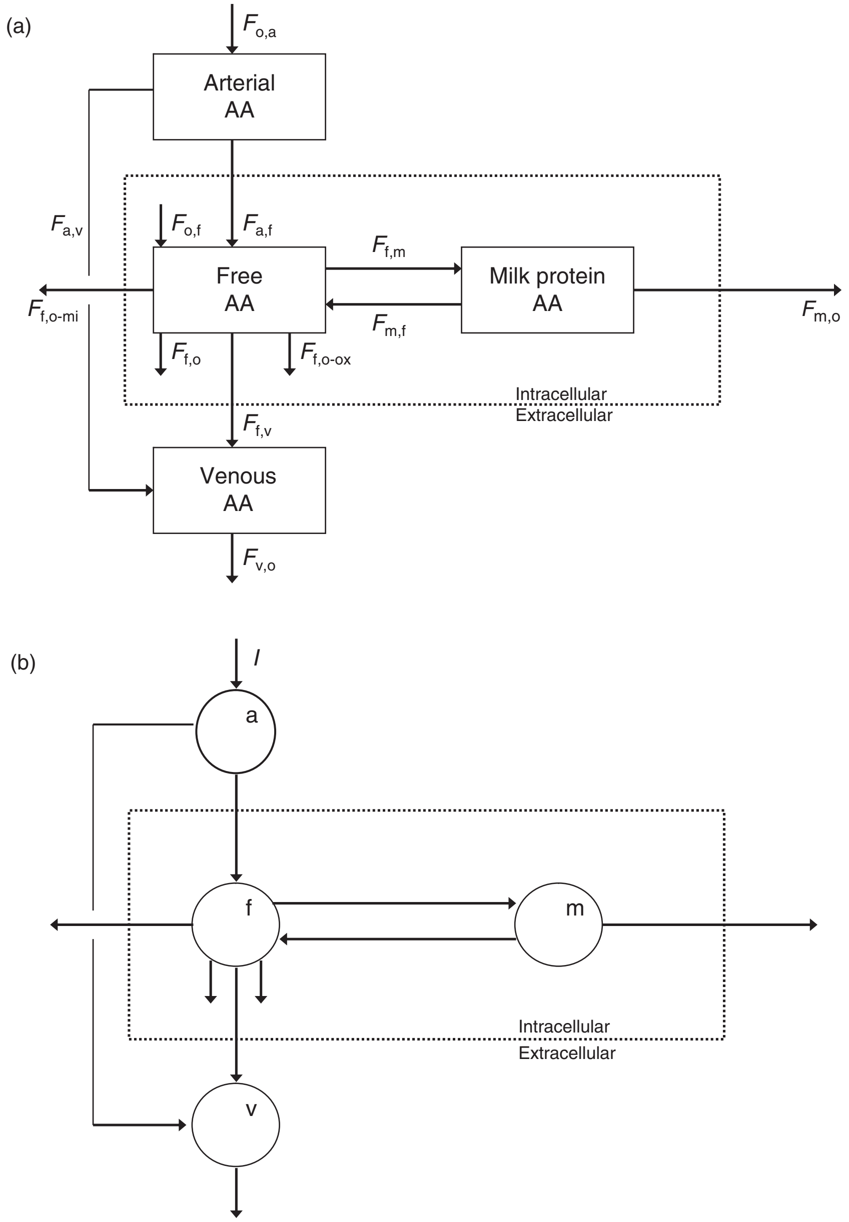

Figure 3 Scheme for uptake and partition of amino acids (AA) by the mammary gland: (a) total AA; (b) labeled AA. Fluxes in (a) are as follows: $F_{a,f}$, influx into intracellular free AA pool from arterial AA pool; $F_{a,v}$, influx into venous AA pool from arterial AA pool; $F_{f,m}$, efflux from intracellular free AA pool to incorporation into milk protein; $F_{f,o}$, efflux from intracellular free AA to synthesis of constitutive mammary gland protein; $F_{f,o\text{-}mi}$, efflux of intracellular free AA pool to secretion of free AA in milk; $F_{f,o\text{-}ox}$, efflux of intracellular free AA pool to AA oxidation; $F_{f,v}$, influx into venous AA pool from intracellular free AA pool; $F_{m,f}$, influx into intracellular free AA pool from degradation of milk protein; $F_{m,o}$, secretion of protein in milk; $F_{o,a}$, influx into arterial AA pool; $F_{o,f}$, influx into intracellular free AA pool from constitutive mammary gland protein degradation; $F_{v,o}$, efflux from venous AA pool. Pools and fluxes in (b) are as follows: a, arterial AA; f, intracellular free AA; *I,* infusion rate of labeled AA in arterial AA; m, milk protein; v, venous AA.

of metabolite flow through a reaction or pathway at a given substrate concentration, the full relationship between concentrations of substrates and reaction rates is essential in parameterizing equations representing saturation kinetics. Another advantage in modeling arising from the use of Michaelis–Menten type equations is that these yield more stable computer solutions.

Conclusion

We have attempted to present a more or less chronological overview of the evolution in the practice of using modeling approaches, methods, and results in support of an overall research program. Research models are, and will remain for some time, constructed to satisfy a different set of objectives than those intended for application in direct support of dairy cow feeding and management. The primary objective in applied modeling is accurate prediction of nutrient requirements and utilization for production functions under field conditions. As research models and thus our understanding of physiological and metabolic functions improve, there is a tendency to view and evaluate research models as competitors of models used in practical animal agriculture. However, research models are designed to be used by research scientists to advance their research endeavors. Such users may find specific elements of research models overly simplified and, as a result, add complexity in order to help advance the field and integrate and evaluate their experimental work directed at advancing our understanding of dairy cow systems. In this regard, research models are always a work in progress. This does not detract from our view, stated in 'Introduction', that future applied models should be dynamic and mechanistic in nature. In addition, it is clear that incorporation of specific elements from research models into applied models often improves the utility of such models. Thus, we do not expect that research models *per se* will be used directly in applied animal agriculture, but do expect that mechanistic equations validated using research models will continue to be simplified and incorporated into applied models to increase their utility and correct systematic errors of prediction observed during the evaluation and use of applied models.

See also: **Feeds, Prediction of Energy and Proteins**: Feed Energy; Feed Proteins. **Feeds, Ration Formulation**: Dry Period Rations in Cattle; Lactation Rations for Dairy Cattle on Dry Lot Systems; Lactation Rations in Cows on Grazing Systems; Models in Nutritional Management; Systems Describing Nutritional Requirements of Dairy Cows; Transition Cow Feeding and Management on Pasture Systems. **Milk**: Bovine Milk; **Nutrients, Digestion and Absorption**: Fermentation in the Rumen; Fiber Digestion in Pasture-Based Cows; Small Intestine of Lactating Ruminants.

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Models in Nutritional Management

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Introduction

For some years now, it has been evident that dairy cow nutrition and nutrient management models are vital to the continued success of the dairy industry. This is especially true as we recognize the importance, for example, of ruminal microbes and metabolism in body tissues to nutrient requirements. In addition, our production emphasis has shifted from only milk volume and fat to include milk protein percentage and yield. Mathematical models of nutrition have been in use for over three decades and have stimulated improvement in feeding cattle. However, more complete data sets available in recent years combined with more precise mathematical approaches have now allowed us to improve models of nutrient use tremendously. They will be used more frequently in the future for support of decisions not only on the nutrition of cattle, but also on other aspects including farm economics and environmental impact.

Dairy Nutrition Models: Their Form and Role

Nutritional models vary in complexity according to the objectives. A typical scheme of model levels needed to represent a system is presented in **Table 1**. Information about a system must be at least one level below the system explored with the model. Thus, models describing herds operate at the animal level or below, those describing animals require details at the organ level and lower, and so on.

In practice, models need only those details that have significant bearing on the consequences of changes arising from inputs to the system (production model) or as much detail as is necessary to explore the system in new and different ways (scientific model). Salient properties of production and scientific models are presented in **Table 2**.

Scientific Models

Scientific models are usually developed upward from basic experimental data pertaining to metabolic processes. Scientific models assume that a living system can be described in terms of a set of 'critical' metabolic transactions encapsulated in organs. The goal is to translate *in vitro* experimental data into chemical reactions representing the essential metabolic processes. Differential equations of the mass balance and Michaelis–Menten forms are used to describe substrate-level changes as the system equilibrates to a (new) steady state because of nutritional and digestive inputs. Implicit to these models are two basic assumptions: first, that *in vivo* metabolic pathways can be represented using the critical transactions modeled from *in vitro* experimental data, and second, that cellular-level metabolic processes can be aggregated to the organ level to effectively model whole-animal function. Baldwin at the University of California and his colleagues have produced a comprehensive integrated model that describes digestion and metabolism of the dairy cow with dynamic, mechanistic equations of physiological processes.

Production Models

Production models are primarily used to portray animal responses to different inputs. They are usually created from collections of response surface models that are developed from animal- or herd-level experiments. Thus, these models are developed downward. They are valid within the domain of data underpinning the individual response surfaces and are as accurate as the response models themselves.

A theme for the development, refinement, and deployment of empirical production models is seen in the development and implementation of the US National Research Council (NRC) dairy cow models. In 1978, response equations were used to predict crude protein (CP) and energy needs of the dairy cow. The 1989 model used a system of protein utilization that partitioned dietary protein into rumen-degradable (DIP) and rumen-undegradable (UIP) fractions. Growth of microorganisms in the rumen was driven by energy intake (total digestible nutrients (TDNs), net energy of lactation (NEL)). Other empirical production models include the VEM-DVE/OEB (Dutch), AFRC (British), CSIRO (Australian), and INRA (French) systems. These early production models stimulated more precise thinking and experimentation. Better data were incorporated into newer versions of the models. Largely because of the concepts in these increasingly precise models, rations for dairy cows usually now contain feed ingredients that are resistant to ruminal degradation. This increases the overall efficiency of dairy cow feeding.

Table 1 Model levels

Level	*Description of level*
$i+1$	Collection of organisms (herd, flock, crop)
i	Organism (animal, plant)
$i-1$	Organs
$i-2$	Tissues
	Cells
	Organelles

The need for more accurate models to define rumen bacterial and whole-animal requirements, to assess feed utilization, and to predict production responses led to the development of the Cornell Net Carbohydrate and Protein System (CNCPS). CNCPS integrates empirical and mechanistic approaches to describe feed intake, ruminal fermentation of protein and carbohydrate, intestinal digestion and absorption, excretion, heat production, and utilization of nutrients for maintenance, growth, lactation, and pregnancy. The system can be applied at the farm level because diets are characterized according to fractions that are measured in most feed analysis laboratories. We have found the system to be valuable in estimating ruminal degradability of dietary protein and in determining whether ruminal microbes are provided with proper types and amounts of carbohydrates and nitrogenous nutrients (i.e., ammonia, peptides). The system is also useful in balancing rations based on amino acids.

In 2001, the NRC released a new dairy cow model that contains some of the mechanistic approaches in the CNCPS.

Components of Dairy Cow Nutrition Models

Dairy cow nutrition models used to evaluate and formulate rations are production rather than scientific models. They consist of integrated submodels that are classified as input or calculation. In the following discussion, the 2001 NRC dairy cow model (NRC-2001) and the CNCPS are used as examples of models that combine mechanistic and empirical approaches. The discussion is limited to organic nutrients and does not consider mineral and vitamin components of the models.

Input Submodels

The user provides information to input submodels. These inputs are used in calculation submodels to estimate dry matter intake (DMI), requirements for maintenance, growth, production, and pregnancy, and supplies of nutrients. Factors such as physical condition of the surroundings (bunk space, cow comfort, etc.), eating patterns, water availability, and milking time are important but are not currently included in the models.

Animal

In addition to yield and composition of milk, animal descriptors usually include age, body weight, body condition score, days in milk, and days pregnant.

Environment

The environment that surrounds the animal has a large effect on maintenance requirements and DMI. Models usually do not contain an environmental submodel. CNCPS has a complex submodel that has many defining inputs. These include temperature, humidity, wind speed, hair depth, heat stress, night cooling, and mud. The CNCPS environmental submodel is applied to growing heifers but not to lactating cows in the NRC-2001.

Ration

The ration submodel describes feed ingredients and their composition and amounts in diets. Most nutrition models have active feed sections that allow entry of feed nutrient composition directly or allow use of default values in feed dictionaries.

Productivity of dairy cattle is sensitive to supplies of absorbed nutrients. Absorbed nutrients are provided by fermentation of dietary nutrients to yield bacteria and volatile fatty acids and by dietary nutrients that escape fermentation. Nutrition models contain equations to

Table 2 Properties of production and scientific models

Feature	*Production model*	*Scientific model*
Purpose	Predict response	Understand process
Form	Response surface equations	Differential equations (state equations)
Parameters	Polynomial coefficients derived from data fitting	Biochemical reaction properties
Aggregation step	None; model derived from aggregated experiments	Chemical processes aggregated to organ- and animal-level functions
Solution process	Simple explicit solution of equations	Complex systems of differential equations requiring special software
Outputs	Computed indicators of adequacy of inputs and production cost measures	Steady-state solutions to transactions in terms of scientifically significant indicators
Character	Empirical and static	Dynamic and mechanistic

predict supplies of nutrients. Predictions are very sensitive to input of feed ingredient nutrient profiles. Our understanding of digestive processes is now fairly adequate, and thus, when nutrition models fail to predict animal performance, an inaccurate description of ingredients is a likely cause. Improvements in predicting supplies of absorbed nutrients have now allowed scientists to focus efforts on metabolism of nutrients in important organs such as the mammary gland, viscera, muscle, and adipose tissue, all of which contribute to variation of responses to nutritional inputs.

A key component of the CNCPS is the recognition that feed ingredients have unique chemical and physical properties that affect the amount and pattern of absorbed nutrients. The model identifies feed fractions (A, B, and C) that have uniform rates of digestion in the rumen.

In the CNCPS, the detergent system for analyses of carbohydrates in conjunction with extraction with borate–phosphate buffer is used to describe protein fractions (A, B_1, B_2, B_3, and C) (**Figure 1**). Fractions A and B_1 are soluble in borate–phosphate buffer. These can be partitioned further by extraction with trichloroacetic acid (TCA). Fractions B_2, B_3, and C are insoluble in borate–phosphate buffer. Extraction with neutral detergent prepared without sodium sulfite isolates fractions A, B_1, and B_2 (soluble in neutral detergent) from fractions B_3 and C (insoluble in neutral detergent). Acid detergent partitions proteins into fraction C (insoluble in acid detergent) and fractions A, B_1, B_2, and B_3 (soluble in acid detergent). Fraction B_2 is calculated as the difference between borate–phosphate buffer-insoluble protein and neutral detergent-insoluble protein (NDFIP). Fraction B_3 is calculated as the difference between NDFIP and acid detergent-insoluble protein. Proteins insoluble in acid detergent are fraction C. Degradation rates of protein fractions were determined by plotting the natural logarithm of residual protein following enzymatic incubation or *in situ* digestion versus time and applying curve peeling. The composition and ruminal degradation of protein fractions are shown in **Table 3**.

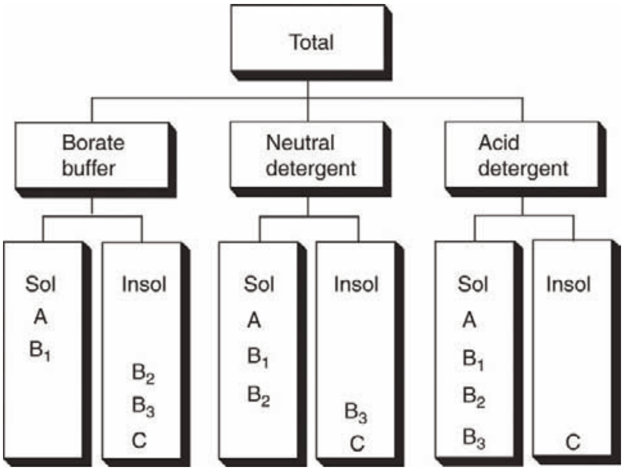

Figure 1 Analysis of protein fractions in the Cornell Net Carbohydrate and Protein System. Sol, soluble; Insol, insoluble.

Table 3 Composition and digestion of protein fractions in the Cornell Net Carbohydrate and Protein System

		Digestion	
		Rumen	*Intestine*[a]
Fraction	*Composition*	*(% per hour)*	*(%)*
A	NH_3, NO_3, amino acids, peptides	Instantaneous	100
B_1	Globulins Some albumins	200–300	100
B_2	Most albumins Glutelins	5–15	100
B_3	Prolamins Extensin proteins Denatured proteins	0.1–1.5	80
C	Maillard products, N bound to lignin	0	0

[a] Digestibility of the rumen escape fraction.

In the NRC-2001, proteins are partitioned into fractions A, B, and C. There are no subfractions of protein B. Fractions A, B, and C in the NRC-2001 are not the same as fraction A, the sum of fractions B_1, $B_{2,}$ and B_3, and fraction C in the CNCPS, respectively. No laboratory methods are offered in the NRC-2001 for determining the protein fractions. Instead, the protein fractions are calculated from *in situ* digestion at defined times. Fraction A is assumed to be completely fermented in the rumen. Fraction C is assumed to completely resist fermentation in the rumen. Rates of degradation for fraction B were estimated by applying the curve peeling technique to protein residues after *in situ* digestion.

Carbohydrates are partitioned using the detergent system in both the CNCPS and NRC-2001. The fibrous carbohydrates (neutral detergent fiber (NDF)) are hemicellulose, cellulose, and lignin that are insoluble in neutral detergent. The CNCPS further partitions fiber into available (B_2) and unavailable (C) fractions. Unavailable fiber is lignin × 2.4. Lignin does not impede digestion by simply encrusting or covering nutrients. Instead, carbohydrate associated with lignin is not digested. The nonfibrous carbohydrates (NFCs) are silage acids, organic acids, sugars, starch, pectins, and β-glucans. NFC is calculated by difference. Because protein in NDF is accounted for in NDF and in CP, NFC calculations should be corrected for NDFIP. The equation for calculating NFC is

$$\mathrm{NFC} = 100 - (\%\mathrm{CP} + \%\mathrm{fat} + \%\mathrm{ash} + \%\mathrm{NDF} - \%\mathrm{NDFIP}) \tag{1}$$

The CNCPS but not NRC-2001 uses degradation rates to calculate ruminal fermentability of carbohydrate fractions. The composition and ruminal degradation of carbohydrate fractions are shown in **Table 4**.

Calculation Submodels

Calculation submodels use information from input submodels to predict DMI, nutrient supplies, and nutrient requirements. Ration formulation is simply the process of selecting types and amounts of feed ingredients within a specified DMI so that nutrient supplies equal nutrient requirements.

Dry matter intake

DMI is predicted based on animal size, milk yield and composition, growth, and pregnancy. In both the CNCPS and NRC-2001, predicted DMI is discounted during the early stages of the lactation cycle. In the CNCPS, predicted DMI is also adjusted for environment.

Rumen

Fermentability (degradation)

Feeds that enter the rumen can disappear from the rumen only by the competing processes of digestion in the rumen and passage from the rumen. Rates of passage and degradation of feed ingredients can be used to calculate fermentability of nutrient fractions using the following equation:

$$\text{fermentation of nutrient fractions (\%)} = \left(\frac{k_\mathrm{d}}{(k_\mathrm{d} + k_\mathrm{p})}\right) \times 100 \tag{2}$$

where k_d is the rate of degradation (% per hour) and k_p is the rate of passage (% per hour).

In both the CNCPS and NRC-2001, eqn [2] is used to calculate fermentability of protein fractions. In both the models, rumen-degraded protein is fermented B

Table 4 Composition and digestion of carbohydrate fractions in the Cornell Net Carbohydrate and Protein System

		Digestion	
		Rumen	*Intestine*[a]
Fraction		*(% per hour)*	*(%)*
A	Sugars	100–300	100
	Fermentation acids	1–2	100
B_1	Starch	10–40	75
	Soluble available fiber	40–60	75
	Pectins		
	β-Glucans		
B_2	Insoluble available fiber	2–15	20
	Cellulose		
	Hemicellulose		
C	Unavailable fiber	0	0
	Lignin		
	Fiber associated with lignin		

[a] Digestibility of the rumen escape fraction.

fraction(s) plus fraction A. Rumen-undegraded (escape) protein is unfermented B fraction(s) plus fraction C.

In the CNCPS, ruminal fermentability of carbohydrates is calculated by applying the above degradation:passage equation to the carbohydrate fractions in **Table 4**. Carbohydrate fermentability is not calculated in the NRC-2001.

Bacterial growth

Only carbohydrates or products of carbohydrate fermentation provide energy at rates sufficient for the growth of most ruminal bacteria. Thus, the amount of metabolizable protein and amino acids derived from bacteria depends primarily on the amount and ruminal fermentability of feed carbohydrate.

Bacterial growth in the CNCPS is driven primarily by fermentability of carbohydrates in the rumen. The following equation is used to calculate the yield of bacteria that ferment sugars, starch, and soluble fiber (pectins and β-glucans), and available fiber (NDF − (lignin × 2.4)):

$$\frac{1}{Y} = \left(\frac{k_m}{k_d}\right) + \left(\frac{1}{Y_g}\right) \tag{3}$$

where Y is the yield efficiency (gram of bacteria per gram of carbohydrate fermented), k_m is the maintenance rate (gram of carbohydrate fermented per gram of bacteria per hour), k_d is the growth rate of bacteria (=degradation rate of carbohydrate) (% per hour), and Y_g is the theoretical maximum yield of bacteria (gram of bacteria per gram of carbohydrate fermented); total bacteria (g day^{-1}) is estimated as the product of Y and gram of carbohydrate fermented.

The dynamic approach taken in the CNCPS recognizes that bacteria have a maintenance energy requirement. In addition, bacterial yield is adjusted for ruminal pH and the amount and type of ruminal available nitrogen.

Bacterial yield is affected by low ruminal pH in two ways. First, the degradation rate of available fiber is discounted. This affects bacterial yield by decreasing fermentable carbohydrate. Second, maximum growth yield (Y_g) of both fiber- and nonfiber-digesting bacteria is decreased.

Bacterial yield is discounted if there is insufficient ruminal available nitrogen. In addition, the form of available nitrogen is important. Bacteria that ferment fibrous carbohydrates can grow on ammonia alone. Bacteria that ferment NFCs also take up peptides. Whether peptides are used for bacterial protein synthesis or are degraded to ammonia depends on the amount of fermentable NFCs. When fermentable NFCs allow growth, 34% of the bacterial protein comes from ammonia and 66% comes from peptides. If there is insufficient carbohydrate, peptides are converted to ammonia. In addition, as the ratio of peptides to peptides plus NFCs increases from 0 to 14%, the yield of that ferment NFCs fermenting bacteria can increase by as much as 18.7%.

Because carbohydrate fermentability is not calculated in the NRC-2001, a surrogate must be used to drive growth of ruminal bacteria. The approach is empirical and uses a static efficiency. Bacterial CP yield is calculated as 130 g kg^{-1} of TDNs discounted to account for reduced digestibility with increasing DMI. Using TDNs to drive growth of ruminal bacteria presents a problem because TDNs contain digested fat, and bacteria theoretically do not grow on fat. However, publications reviewed in the NRC-2001 showed that while added fat reduced fermentability of organic matter in the rumen, total production of bacterial protein did not decrease. This occurred because the efficiency of bacterial growth increased. Bacterial growth is discounted if there is insufficient rumen available nitrogen.

Intestine

In the NRC-2001, only bacterial protein and rumen-undegraded protein are assigned values for intestinal digestibility. In CNCPS, absorption of protein, amino acids, carbohydrates, fats, and ash in rumen escape feed fractions and in bacteria is calculated by multiplying each fraction by its respective digestibility.

Protein

Protein flowing to the small intestine includes bacterial protein, rumen-undegraded dietary protein, and endogenous protein.

Bacteria contain nonprotein nucleic acids and true protein in cell walls and in cell contents. Bacterial nucleic acids are completely digested in the small intestine, but they are excreted in urine and do not contribute to the animal's protein nutrition. Cell wall protein is indigestible. In the NRC-2001, bacterial protein is 20% nucleic acids and 80% true protein (cell wall plus cell contents). Bacterial true protein is assigned a digestibility of 80%. Thus, 64% ((0.80 × 0.80) × 100) of intestinal bacterial CP is converted to metabolizable protein. In the CNCPS, bacterial protein is 15% nucleic acids, 25% cell walls protein, and 60% protein present in cell contents. Cell wall bacterial protein is not digested. Bacterial true protein is completely digested. Thus, 60% of intestinal bacterial protein is converted to metabolizable protein.

In both the NRC-2001 and CNCPS, intestinal digestion of rumen-undegraded protein is variable. Most of the values in the NRC-2001 were derived from experiments using the mobile bag technique. Feeds placed in bags are subjected to ruminal fermentation, and later the bags are placed into the duodenum and recovered in feces. Where data were not available, values from the French Institut National de la Recherche Agronomique were used. The CNCPS uses intestinal true digestibilities of

100, 100, 100, and 80%, respectively, for feed peptide, B_1, B_2, and B_3 protein fractions (**Table 3**). Fraction C is completely unavailable for digestion and does not contribute to metabolizable protein.

Endogenous protein passing to the small intestine consists of mucoproteins in saliva, epithelial cells from the respiratory tract, cellular debris from sloughing and abrasion of epithelial tissue of the rumen, omasum, and abomasum, and enzyme secretions into the abomasum. Much of the endogenous protein from saliva and the mouth, respiratory tract, and rumen is probably degraded by ruminal microorganisms. The NRC-2001 estimate is 1.9 g of endogenous nitrogen per kg of dry matter consumed. Intestinal digestibility is 40%. The CNCPS does not consider endogenous protein flowing to the intestine.

Carbohydrate

In the CNCPS, intestinal digestibilities are applied to rumen escape carbohydrate fractions as shown in **Table 4**. Little sugar (fraction A) reaches the intestine, but any that escapes ruminal fermentation is completely digested. Starch digestibility is 75%. However, starch digestion can vary from 60 to 100%. For example, digestion of starch in maize silage can vary considerably depending on plant maturity, silage dry matter, and kernel processing. If considerable proportion of maize grain appears in feces, intestinal starch digestibility should be adjusted downward. Enzyme systems for digestion of cellulose and hemicellulose are not present in the small intestine. Available fiber can be fermented by bacteria in the large intestine, but the CNCPS does not have a hindgut submodel. Postruminal digestibility of 20% for available fiber is an attempt to account for fermentation in the large intestine. Bacterial carbohydrate has a postruminal digestibility of 95%.

Because NRC-2001 does not have a carbohydrate submodel, digestibility values used represent ruminal and intestinal digestion. Ninety-eight percent of NFCs are digested. The base digestion of fibrous carbohydrates is 75% with discounts for the amount of lignin in NDF.

Fat

Rumen bacteria hydrogenate fat, but they do not ferment it. CNCPS assumes that feed fat and bacterial fat have intestinal digestibilities of 95%. This ignores the fact that fatty acid composition can affect intestinal digestibility of fat. For fats with high concentrations of stearic acid, especially if in the form of triglycerides, the user should adjust intestinal digestibility of fat downward. In the NRC-2001, feed fats have variable digestibilities (43–86%), which depend mainly on the fatty acid profile and whether the fat is in the form of free fatty acids, calcium salts, or triglycerides.

Metabolizable (absorbed) protein value of feed ingredients

Metabolizable (absorbed) protein is the sum of intestinally digested rumen-undegraded protein, bacterial protein, and endogenous protein. Metabolizable protein values are not constant but vary as a function of the amount of feed consumed. As intake increases, the residence time of feeds in the rumen decreases so that fermentability of nutrients is decreased. Ruminal escape of feed proteins is greater, but production of bacterial protein per unit of feed consumed is less.

In the CNCPS, undegraded protein and bacterial protein are calculated for each feed ingredient. Thus, each feed ingredient has a metabolizable protein value that is summed to give the ration metabolizable protein. As noted before, the CNCPS does not include an endogenous supply of protein to metabolizable protein.

In the NRC-2001, metabolizable protein derived from rumen-undegraded protein is calculated for each feed ingredient. However, metabolizable protein from bacteria and from endogenous sources is calculated on a total ration basis. Thus, metabolizable protein values of individual feed ingredients cannot be calculated.

Energy value of feed ingredients

Energy values (metabolizable energy (ME), NEL, net energy of maintenance (NEM), net energy of gain (NEG)) have usually been estimated from TDNs. In CNCPS, ruminal digestion rates and passage rates of nutrient fractions, bacterial yield, and intestinal digestibility are used to predict TDNs. Because of the dynamic rumen submodel, TDN values have been discounted for the reduced digestibility that occurs when feed intake increases. Digestible energy and ME are then calculated from TDNs.

In the NRC-2001, digestibilities for proteins, carbohydrates, and fats are applied to compositional data to obtain digested nutrients. Caloric values (protein 5.6 Mcal kg^{-1}, carbohydrate 4.2 Mcal kg^{-1}, and fat 9.4 Mcal kg^{-1}) are applied to the digested nutrients to obtain digestible energy values. Equations to calculate ME and net energy from digestible energy vary, depending upon whether the feed ingredient is a forage, concentrate, animal protein, or fat supplement. Adjustments are made to account for decreases in digestibility that occur when feed intake increases and for the impacts of grain processing methods that affect ruminal fermentability of starch. Net energy values in the NRC-2001 are lower than values in previous NRC dairy cow nutrition models.

Metabolic requirements

The NRC-2001 and CNCPS use the factorial system to calculate energy (metabolizable and net) and protein requirements for maintenance, growth, pregnancy, and milk production. Both nutrition models contain amino acid submodels. Two approaches are used. The factorial method used in the CNCPS and the CNCPS option of DPM-Dairy calculates supply and requirements in grams per day. The ideal protein method used in the CNCPS option of DPM-Dairy and in the NRC-2001 expresses amino acids as percentages of metabolizable protein.

The factorial system first estimates net requirements and then relies on efficiency factors (transfer coefficients) to transform net requirements into metabolizable (absorbable) amounts. Net requirements are based on the amount of product and nutrient composition of the product. These can be measured reliably. Estimating the efficiency of nutrient use is difficult. Transfer coefficients used in factorial systems are usually constant. This dictates that production responses are linear regardless of the amount of nutrient supplied. Thus, the factorial method may describe production responses correctly when nutrients are limiting but will overestimate production responses when nutrients are in excess. Consequently, dairy nutrition models that use factorial methods to estimate nutrient requirements will overpredict production when nutrient supply exceeds the animal's productive ability.

Optimization and Ration Formulation

Ration formulation involves the selection of feed ingredients within a specified DMI so that nutrient supplies equal nutrient requirements at the best cost. Autobalancing is a key computer tool in ration formulation. The reasons are clear when we reflect on the magnitude of the task of balancing the nutritional intake of the cow with regard to her health and her production, and the costs of meeting these needs. Whereas a skilled nutritionist using a 'hunt and peck approach' can produce a ration that meets a few of simple nutritional needs of the cow, balancing for multiple nutrients moves the effort of cost containment of the balanced ration beyond the domain of 'manual' specification.

Autobalancing Rations

The usual objective of autobalancing is to produce an 'optimal ration' at the lowest cost. Constraints (minimum and maximum amounts) are set for both nutrients and feed ingredients. Nutritional constraints are based upon application of the factorial approach to describe the requirements of cows to perform specific or multiple functions (maintenance, growth, lactation, pregnancy). Nutritional constraints include DMI, energy (metabolizable and net), protein (crude, soluble, bypass, absorbed, or metabolizable), carbohydrates (fiber and nonfiber), fat, minerals, and, in the case of newer models like CNCPS, amino acids and rumen available nitrogen (peptides and ammonia). Feed ingredients are selected on the basis of the major nutrients that they provide (i.e., fiber from forages, nonfiber carbohydrates from grains, protein from oilseed meals). Feed constraints are set based on the availability of purchased ingredients and inventory of ingredients on the farm or contracted for purchase. The amount of an ingredient specified is often adjusted by the formulator to take into account a minimum amount that the formulator feels rations should contain or the maximum amount that the formulator feels can be tolerated by the animal. The amount of a feed ingredient should not be limited by high cost because optimization programs will control the inclusion of expensive feeds. Thus, the autobalancing (optimization) task is to find the least-cost combination of feed ingredients within their minimum and maximum constraints that provide nutrients that are within the specified minimum and maximum ranges. When the foregoing is achieved, the autobalancing process has provided a solution to the specifications defined by the formulator.

Ration formulators often are discouraged when the optimization process does not give a solution as defined above. This simply means that a combination of feed ingredients in amounts within the minimum and maximum ranges cannot provide nutrients within the specified ranges. To find a solution, the formulator should either expand (relax) the feed ingredient and nutrient constraints or include additional ingredients that are good sources of limiting nutrients. Older optimization methods simply indicated that there was 'no feasible' solution. This provided no direction to obtaining a solution. Newer optimization methods, by using 'dummy variables', provide direction by listing nutrient constraints that are not met.

Linear programming

Linear programming is used for autobalancing in most empirical dairy cow nutrition models. In fact, ration formulation was one of the first applications of linear programming. Not only could solutions be found in seconds, but building on Danzig's contributions to operational research, we were also able to derive an array of other very helpful economic properties (shadow prices) relating to our optimal solution. For example, we could discover over what cost ranges feeds within the optimal ration remained there, as well as which among the feeds not selected in the optimal ration were candidates for inclusion if cost decreased.

The suitability of linear programming for optimization depends on the linearity of the problem. That is, the objective and nutrient supply must be expressible as linear functions. Clearly, the objective function, feed cost, is a linear function of feed amounts. In both empirical models and CNCPS, nutrients like CP, fat, carbohydrates (fiber and nonfiber), and minerals are constant proportions of the ingredient regardless of the amount of feed consumed. Thus, supply of these nutrients is a linear function of intake. In empirical dairy cow models, absorbable (metabolizable) protein and energy (metabolizable and net) values are also not affected by intake and thus are constant.

Nonlinear programming

The CNCPS has a dynamic rumen submodel wherein the passage rate of feeds (determined mainly by feed intake but also adjusted by ration forage content and particle size) determines the outflow of nutrients from the rumen system. Thus, nutrients like metabolizable protein, ME, amino acid content of metabolizable protein, and rumen available protein (peptides and ammonia) are not constant but vary according to feed consumption and ration ingredients. These features of the dynamic digestion models, together with the fact that novel nonlinear mathematical techniques are needed to bridge nonoverlapping digestive model components from different dairy research centers, mean that the problem of dairy cow ration optimization is no longer the province of the linear programming package. A group at the University of Pennsylvania has now demonstrated that by using the 'forward sequential quadratic programming' approach, the problems alluded to above can be effectively resolved without serious loss of computational efficiency.

Implementing constrained, nonlinear optimization is not without problems. If the nutrition model contains discontinuous (breakpoint) functions, continuous mathematical models must be developed to describe the discontinuous functions. Whereas a linear programming problem can be solved from any starting point, a nonlinear programming problem requires a 'good' feasible starting point to 'effectively' start the solution process. In the CNCPS option of DPM-Dairy, the linear solution to an NRC-based ration is used as the starting point for the nonlinear CNCPS optimization. Finally, a linear programming optimization problem has just one solution. This is not so for nonlinear optimization. The entire field of biomathematics is still under investigation. Nevertheless, a number of points are clear:

1. The era of the linear program as the exclusive tool for dairy cow ration optimization has ended.
2. The use of nonlinear optimization techniques has been shown to be not only feasible but also practicable in the field.
3. Starting the nonlinear optimization problem calls for an approach that yields good initial estimates for the final solution. Using the same starting point ensures that, when presented with the same problem twice, nonlinear optimization will provide the same solution. This area represents an opportunity for the continued use of linear programming in conjunction with nonlinear optimization. Work needs to be continued to explore the array of properties of the final nonlinear ration optimization solution, which may provide similar information to the optimizer as did opportunity prices and shadow prices in the linear programming context. Clearly, marginal patterns will emerge to be major players here.
4. Feed cost is a sensible objective for ration formulation. However, with legislative imposition on dairymen to be mindful of the environment, and with health and fitness promoters seeking milk with special nutritional qualities, broadening the focus of optimization to nutrient excretion and milk components may be desirable.

Linear and nonlinear optimization methods are powerful tools for assisting the formulator in obtaining least-cost combinations of feed ingredients to meet nutrients required for maintenance and productive functions like growth, lactation, and pregnancy. Solutions obtained are based on constraints for feed ingredients and nutrients selected by the formulator. Rations obtained by optimization should always be assessed by a trained nutritionist before they are implemented.

Dairy Nutrition Software

Dairy nutrition models often do not contain tools for computer-assisted ration formulation. Software included with the 1989 and 2001 NRC dairy nutrition models allowed calculation of nutrient requirements but did not provide for formulation of rations.

Bath and colleagues at the University of California were among the earliest to employ linear programming to formulate rations for maximum income over feed costs. Galligan and coworkers at the University of Pennsylvania programmed the 1978 NRC dairy nutrition model into Lotus 1-2-3 with autobalancing of rations provided by Einfin. Spartan represented an excellent effort in software development by the group at Michigan State University that was based on NRC models and included autobalancing.

Table 5 contains a list of commercially available software for formulation of dairy cattle rations. NRC, INRA, and CNCPS dairy nutrition models are used in some of the software packages, whereas proprietary or user-defined models are used in others. Linear programming is used for autobalancing in empirical models. In CNCPS, biological values for ME, metabolizable protein, passage rate, bacterial yield efficiencies, and degradation rate of available fiber, which depend upon feed intake and the ingredients selected, are first estimated and then rations are balanced using linear programming. In DPM-Dairy, a nonlinear optimizer is used to autobalance rations according to CNCPS.

Table 5 Some commercially available dairy nutrition software

Software	*Developers*	*Dairy nutrition model*[a]
CamDairy	Cam Software	Proprietary model
The Consulting Nutritionist	Dalex Computer Systems, Inc.	User-defined nutrient requirements, CNCPS
DPM-Dairy	Cornell University, University of Pennsylvania, Miner Institute	NRC with modifications, CNCPS
CNCPS	Cornell University	CNCPS
Dairy Ration System	ACS Computer Services	NRC
Formulate2	Central Valley Nutritional Associates	NRC
INRAtion-PrevAlim	INRA	INRA
Mixit-Win	Agricultural Software Consultants, Inc.	User-defined minimum and maximum nutrient amounts
Molly	University of California, Davis	MOLLY
Ohio Dairy Ration Program	Ohio State University	NRC
PCDairy-2	University of California, Davis	NRC
Perfo-Lact Feeding Tool, Individual Performance Feeding	Shur-Gain (Maple Leaf Foods, Inc.)	Proprietary model similar to CNCPS
RationPro	ProfitSource	NRC, user-defined
RumNut	A.T. Chamberlain	AFRC, PDI
Shield	University of California, Davis	NRC
SigaDairy	Siga Farm Software	NRC, user-defined
Spartan	Michigan State University	NRC with modifications
Trilogic	Trilogic Systems	NRC, user-defined requirements, proprietary amino acid/carbohydrate model

[a] AFRC, Agricultural and Food Research Council; CNCPS; Cornell Net Carbohydrate and Protein System; INRA, Institut National de la Recherche Agronomique; MOLLY, a dynamic, mechanistic computer model of a dairy cow; NRC, National Research Council; PDI (INRA), Proteines vraies réellement digestibles dans l'intestin grêle.

Whole-Farm Nutrient Management

Nutrient Flows on Dairy Farms

Dairies need to operate in ways that maintain efficiency as well as minimize impact on the environment. Models can be used to explore the effects of feeding decisions on the overall efficiency of key elements to help us reduce potentially negative effects on the environment (*see* **Manure/Effluent Management**: Nutrient Recycling). Nitrogen and phosphorus are the central concerns to the environment due to their impact on water quality. Despite their essential roles in both animal and crop production, excess amounts of these two nutrients increase the potential for water pollution. Groundwater nitrate exceeding the drinking water standard and surface water eutrophication stimulated by excessive N and P are the most widespread problems.

Nitrogen and phosphorus enter a dairy farm through the purchase of feeds, fertilizers, and bedding materials, and through symbiotic fixation of atmospheric N by legumes. Nutrients leave the farm through sold animal products and occasionally sold crops. On a typical dairy, purchased feeds account for over 50% of the nutrient inputs. Only 20–30% of the nutrient intake is captured in animal products. A similar percentage applies to whole-farm nutrient efficiency, that is, 20–30% of the nutrients entering the farm are exported as production output. The remainder is cycled on the farm in a managed pathway of feeds → animals → manure → fields and back to feeds. A schematic illustration of dairy farm nutrient flow is presented in **Figure 2**.

Nutrient Management Approaches

Nutrient losses from a dairy farm to the environment occur throughout the cycling process, especially during the handling and application of manure from barnyard to crops in the fields. For over a decade, nutrient management for environmental protection has been focused on manure management (*see* **Manure/Effluent Management**: Nutrient Recycling). This approach is inadequate for resolving the problem because, as dairy operations intensify, the reliance on imported feeds increases, and nutrient imbalance worsens. Responsible nutrient management must include animal feeding programs. By eliminating excess consumption of nutrients through ration balancing, not only is nutrient utilization maximized but also nutrient excretion in manure is minimized, benefiting the environment without impairing animal productivity.

Nutrient Management Models

Various computer programs are available for farm nutrient management planning. Most of these programs estimate the amounts of manure and manure nutrients

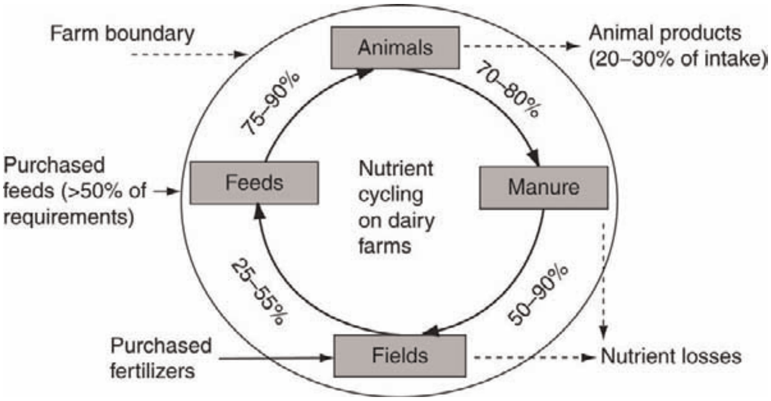
Farm boundary
Animals
Animal products (20–30% of intake)
75–90%
70–80%
Purchased feeds (>50% of requirements)
Feeds
Nutrient cycling on dairy farms
Manure
25–55%
50–90%
Purchased fertilizers
Fields
Nutrient losses

Figure 2 Nutrient (nitrogen and phosphorus) flow on a typical dairy farm, which features large inputs as feed purchases and small output as animal products. Nutrients remaining and cycling within the farm are subject to potential losses, especially during the handling and application of manure from farmyard to crops in the fields.

by multiplying fixed book values for excretion of N and P per unit of animal body weight by total animal units in the herd, and then allocating the manure to fields based on soil fertilization information and crop requirements. Programs of this type do not have an explicit animal component and thus are unable to handle the impact of animal feeding on nutrient excretion.

Accurate nutrient management requires the consideration of all key components, that is, animal and feeding, manure handling and storage, and field application and cropping. Modeling of whole-farm nutrient management is still in its early stages. Nevertheless, the integrated systems approach is a critical step for addressing nutrient imbalances in dairy farming and other livestock industries. Only a few models fit this criterion. Their basic characteristics and contact information are given in **Table 6**.

Dairy forage system model

The dairy forage system model (DAFOSYM) is a comprehensive program developed by the US Department of Agriculture. This model consists of several components with a mix of dynamic (mechanistic) and static (empirical) functions simulating the growth, harvest, and feeding of crops, production and handling of manure, and economic returns to dairy farms.

DAFOSYM deals with animals and feeding by splitting the herd into six groups (dry cows, two heifer groups, and three lactating cow groups), allocating homegrown feeds to these groups and supplementing with purchased feeds if necessary. Rations are determined with a linear programming algorithm and follow certain established rules and commonly used criteria regarding energy consumption, rumen bacterial growth, digestibility of NDF, and protein requirements. The manure component determines the amounts of N, P, and K in fresh manure by subtracting the nutrient output in milk and that used for animal growth from nutrient intake. Nutrient losses are then calculated and subtracted to determine the amounts that are available for plant growth. Nitrogen losses during manure collection, storage, and field application are predicted as functions of temperature, storage period, and application timing and methods, while P and K losses are

Table 6 Whole-farm nutrient management models

	DAFOSYM	*DNP*	*CuNMPS*
Developers	USDA Dairy Forage Research Center; USDA Pasture Systems and Watershed Management Research Unit	University of Pennsylvania	Cornell University
Components	Crop growth	Ration formulation	Herd nutrition (CNCPS v.4.0)
	Harvest	Whole-farm cycling	Crop rotation
	Feed storage	Herd	Manure
	Feed allocation and animal performance	Manure Fields	
	Manure production and use	Feeds	
	Tillage and planting		
	Economics		
Nutrients handled	N, P, K	N	N, P, K

set at 5% of fresh excretion. Crop growth and nutrient uptake, and changes in the quality and quantity of forages during harvest and storage are simulated.

As a research and teaching tool, DAFOSYM has the unique strength of predicting the long-term impact of various technological and management alternatives on the economic costs and returns as well as nutrient and environmental implications.

Dairy nitrogen planner

Dairy nitrogen planner (DNP), developed at the University of Pennsylvania, deals with dairy farm N flow by linking a ration formulation model (CNCPS) with a whole-farm model. The whole-farm model has a module-type structure to reflect the managed pathways of N flow as illustrated in **Figure 2**. Feed intake and N excretion data are aggregated for the herd and routed to the relevant modules. Calculations of N and material (feeds, manure, etc.) flows across the farm and farm components employ tabulated, empirical parameters routinely used by agronomists when planning manure utilization for farmers.

Cornell University nutrient management planning system

CuNMPS is a multicomponent system program under development at Cornell University. The CNCPS is used to predict feed requirements and manure nutrient excretion for the herd on an annual basis. A crop rotation program is used to predict homegrown feeds available with alternative crop rotations. A crop and manure management program is used to develop nutrient management plans and record keeping. A unique attribute is the capability of CNCPS for whole-herd optimization. Homegrown feeds are allocated with a linear programming procedure to satisfy set requirements of each animal group while optimizing return over feed costs and reducing manure nutrient excretion.

See also: **Feeds, Prediction of Energy and Proteins**: Feed Energy; Feed Proteins. **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems; Models in Nutritional Research. **Manure/ Effluent Management**: Nutrient Recycling. **Nutrients, Digestion and Absorption**: Fermentation in the Rumen.

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Dry Period Rations in Cattle

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The Dry Period

At the end of a lactation cycle, high-producing dairy cows require a dry period, where milk production is halted, to prepare the mammary gland and rumen for the next lactation. Traditionally, nonlactating (dry) cows were considered inactive; they were largely unmanaged and their nutritional requirements were poorly characterized. However, it is now clear that preparations for a successful lactation begin during, or even before, the preceding dry period. This article will focus on recent advances in the understanding of dry cow nutrition and management techniques used to maximize milk production. For the purposes of this article, dry cows will be considered in two groups: cows recently dried off (far-off dry cows) and those within 2–3 weeks of calving (close-up or transition cows).

The decision of when to dry off cows can represent a management dilemma. At best the decision is based on the marginal cost of production: a cow should be dried off when the value of milk produced drops below the added costs of feed and labour to keep her in the milking herd. However, the length of the dry period must also be considered. The dry period must be long enough to permit mammary gland involution and recovery of the rumen before the next lactation begins. Numerous studies have shown that milk yield is dependent on the length of the preceding dry period (**Figure 1**), and that the optimal dry period length is 50–60 days. After 60 days dry, the production response begins to decline and the added cost of maintaining nonlactating cows argues against extending the dry period any longer. The decision of when to dry off cows is complicated further by the fact that cows with good persistence may be producing 20 kg day^{-1} or more at the end of their lactation (60 days before their expected calving date). However, the consequences of decreasing the dry period to less than 45 days are generally greater than the benefits of extending the preceding lactation.

At the end of a lactation cycle, milk production is stopped in cows producing less than 15 kg day^{-1} by using the 'cold turkey' method, i.e. simply stopping milk removal. Once milk removal is ceased, the increase in intramammary pressure rapidly stops milk synthesis and the process of involution begins. For cows producing more than 15 kg day^{-1}, the process can be more stressful and can increase the incidence of mastitis if steps are not taken to reduce the volume of milk produced before drying off. To decrease production and ease the drying-off process in high-producing cows, feed can be reduced for a period of 10–14 days before drying off. In addition, switching to a high-forage diet will decrease the nutrient supply to the udder and rapidly decrease milk synthesis. Other techniques used to decrease milk production before dry-off include the use of once-a-day milking or altering the normal routine for cows entering the parlour to avoid any cues that stimulates milk let-down. Reducing water intake for 1–3 days has also been used to decrease milk production rapidly, but at best this should be considered as an extreme measure and should never be attempted during hot weather. In any case, if growth hormone (somatotrophin; bST) is being used, injections should be stopped 30 days prior to dry-off.

Milk production is ceased during the dry period, yet it is not a period of inactivity for dairy cows. During the dry period, energy, protein and mineral metabolism must be precisely coordinated to support the demands of pregnancy and prepare for the subsequent lactation. Cows that are unable to adapt to the changing demands are susceptible to a complex of metabolic and digestive disorders associated with the transition into lactation (fatty liver and ketosis, milk fever, fat cow syndrome and displaced abomasum). The use of established management practices can, however, help dry cows meet this challenge.

Far-Off Dry Cows

The far-off stage of the dry period begins at dry-off and continues until 14–21 days prepartum. During this stage, milk production is stopped and the mammary gland undergoes involution, wherein the mammary secretory tissue degenerates. This stage normally corresponds to a period of rapid foetal growth, but the estimated 3–4 Mcal net energy of lactation (NE_L) day^{-1} required for pregnancy is substantially less than that required for lactation. The goals of management and feeding programs for the far-off dry period are to provide nutrients for the developing calf, to prepare the mammary gland and rumen for

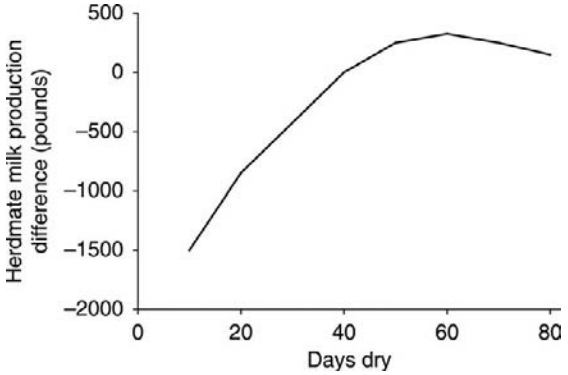

Figure 1 The effect of dry period length on subsequent milk yield in comparison with herdmates.

the following lactation and to minimize the occurrence of metabolic, digestive and infectious diseases.

An additional goal of dry-period management programmes is to maintain the cow's body condition. The body condition of dry cows represents the energy reserves that will be utilized to support milk production in early lactation, when high-producing cows are expected to be in a negative energy balance. Too little body condition can limit milk production, but excessive body condition can increase the cow's susceptibility to metabolic diseases, including fatty liver, ketosis and fat cow syndrome. On a 5-point scale, a body condition score of 3.5–4 at calving is considered ideal. Furthermore, it is recommended that this condition be attained before dry-off and then maintained during the dry period. This is because: (1) adding body condition is more efficient while cows are lactating; (2) adding body condition during the dry period increases the likelihood of metabolic and digestive disorders (fatty liver, ketosis, retained placenta and displaced abomasum); and (3) decreasing body condition during the dry period requires feeding below requirements at the time of maximal foetal growth. Thus, adjustments in body condition are generally made before the end of the preceding lactation and cows are fed to maintain body condition throughout the dry period.

The nutrient requirements of far-off dry cows are summarized in **Table 1** and corresponding sample diets are shown in **Table 2**. Maximizing feed intake after calving is a critical factor in determining milk production. Maintaining rumen fill (rumen distension) during the dry period will not only help maximize feed

Table 1 Prepartum nutrient recommendations for dry cows[a]

	Far-off	*Close-up regular*[b]	*Close-up anionic*[c]
Maximum DMI (% of body weight)	2.0	1.8	–
Energy			
NE_L (Mcal/d)	15.7	16.8	–
Protein			
CP (%)[d]	13.1	13.8	–
MP (%)	762	823	
RDP (%)	1062	–	–
RUP (%)	169	248	–
Fibre and carbohydrates			
Minimum NDF (% DM)	33	–	–
Minimum ADF (% DM)	21	–	–
Maximum NFC (% DM)	43	–	–
Macro-minerals (Total Supplied)			
Calcium (g/d)	48.0	–	74–186
Phosphorus (g/d)	28.5	37–50	–
Magnesium (g/d)	14.9	43.5–50	–
Chlorine (g/d)	18.6	–	100–150[e]
Potassium (g/d)	64.5	–	–
Sodium (g/d)	12.4	–	–
Sulphur (g/d)	24.8	–	37–50[e]
DCAD (mEq/100 g)	–	–	−7.5–0
Vitamins (Total Supplied)			
Vitamin A (1000 IU/d)	74.8	90.5	–
Vitamin D (1000 IU/d)	20.4	22.5	–
Vitamin E (IU/d)	1088	1623	–

[a] Requirements for a second-lactation Holstein with a body weight of 629 kg (without conceptus), a body condition score of 3.5 and gaining 0.79 kg/d (including conceptus) at 40 days prepartum.

[b] Recommendations for close-up diets that differ from far-off diets.

[c] Recommendations for close-up 'Anionic' diets that differ from regular close-up diets.

[d] The recommended crude protein content may be decreased if rumen degradable and rumen undegradable protein are carefully balanced in the ration.

[e] Dietary anions concentrations increased to adjust DCAD.

Table 2 Sample dry cow rations[a]

	Far-off	*Close-up Regular*	*Close-up Anionic*
Ingredient	Percent of ration dry matter		
Bermudagrass hay	48.0	29.3	25.9
Corn Silage	34.8	48	43.7
Corn Grain	10.6	12.5	14.3
Soyabean Meal, 48%	4.2	8.2	4.1
Corn Distillers Grains with solubles	1.0	1.0	6.7
Anionic Salts	–	–	2.1
Vitamin/Mineral premix	1.4	1.0	3.2
Nutrient	Nutrient densities (dry matter basis)		
NE_L (Mcal/kg)	1.4	1.5	1.47
Crude protein (%)	13.3	14.2	14.0
Rumen undegradable protein (% of crude protein)	30.0	42.3	39.8
ADF (%)	25.5	25.2	23.6
NDF (%)	49.6	46.6	44.2
NFC (%)	32.8	30.5	30.0
Calcium (%)	0.39	0.40	0.90
Phosphorus (%)	0.27	0.35	0.38
Magnesium (g/d)	0.15	0.39	0.39
Chlorine (g/d)	0.35	0.31	1.13
Potassium (g/d)	1.18	1.23	1.06
Sodium (g/d)	0.10	0.12	0.14
Sulphur (g/d)	0.25	0.24	0.31
DCAD (mEq/100 g)	9.6	27.7	−4.1

[a] Formulated for a second-lactation Holstein with a body weight of 620 kg (without conceptus), a body condition score of 3.5 and gaining 0.79 kg/d (including conceptus) at 40 d prepartum.

intake after calving, but will also decrease the incidence of displaced abomasum, fatty liver and ketosis during early lactation. Thus, far-off dry cows are fed high-forage diets to promote and maintain rumen distention. With most grass hay diets the cows will require supplementation with 2–3 kg day^{-1} of concentrate (grain) during the dry period. Medium protein levels found in dry hay and wet forages such as oat silage work well for this phase. However, *ad libitum* maize silage should be avoided since this can overcondition cows and it does not promote adequate rumen distention. In addition, *ad libitum* hay should be avoided as overconsumption may prevent cows from consuming required energy and minerals.

Maintaining dry cows on a low calcium diet (<50 g day^{-1}) has been used for years to reduce the incidence of milk fever (pariparturient pariesis), which results from a calcium imbalance when cows begin lactating; the sudden drain of calcium used in milk synthesis outpaces the cows' ability to replace it from bone reserves and intestinal absorption. By formulating dry-cow diets to put cows in a slightly negative calcium balance, their metabolic machinery is primed to mobilize calcium at the onset of lactation. Recent studies suggest that feeding excessive potassium during the dry period also plays a major role in predisposing cows to milk fever; maintaining dietary potassium levels below 1.5% of the total ration dry matter can be used to decrease its occurrence. While the incidence of milk fever can be decreased by maintaining the proper dietary calcium, phosphorus and potassium concentrations during the dry period, recent research has also demonstrated that milk fever can be prevented by carefully adjusting the balance of cations and ions in the diet of transition cows (see below).

It is important to note that about half of all new cases of mastitis occur during the dry period and cows are most susceptible to mammary infections in the 7–10 days following dry-off (*see* **Mastitis Therapy And Control:** Medical Therapy Options). As a result, cows should be observed closely while drying off to ensure their udders are involuting properly and do not appear swollen or inflamed. In addition, most dairy cows are given an antibiotic infusion 'dry cow treatment' into each quarter of the udder at the time of dry-off. The US Food and Drug Administration (FDA)-approved antibiotic products, formulated specifically for single-dose intramammary infusions, are available commercially for use in dry cows. Dry-cow treatment can dramatically reduce the incidence of new infections during the far-off dry period and is also used to treat existing infections. If extreme care is not observed, however, the infusion procedure can introduce new mastitis-causing pathogens into the udder. Product label directions must be followed exactly to avoid antibiotic residues in milk after freshening, particularly in cows with shorter than normal dry periods.

Transition Cows

The transition (or close-up dry) period is generally considered to be the last 2–3 weeks before calving. During the transition period, dairy cows undergo a dramatic adaptation in metabolism, to prepare for parturition and lactation. The transition period is also used to gradually adapt cows to the high-grain diets that typify production rations. These physiological and nutritional changes may cause stress and predispose cows to metabolic disorders, which can persist well beyond calving and have a dramatic impact on milk production. Thus, a major goal of transition cow management programs is to avoid stress and other disorders associated with calving and the onset of lactation.

As with the far-off dry period, another goal of the transition period is to minimize the incidence of mastitis. Dry-cow therapy can greatly reduce the incidence of mastitis in far-off dry cows, but as antibiotic concentrations decline, the udder becomes vulnerable to new infections during the transition period. In addition, the immune status of cows is generally suppressed during the transition period and these factors lead to a significant risk of developing new cases of mastitis around the time of calving. Increasing dietary concentrations of vitamins A and E, as well as copper, zinc and selenium, can aid stimulation of immune function during the transition period. Keeping pregnant cows and heifers in a clean environment, such as a grassy lot or a dry-bedded stall, can also decrease exposure to mastitis-causing pathogens. Recent studies with teat barriers show that their application from 10–14 days prepartum may also reduce the number of new mastitis cases at calving.

Transition rations are used to gradually introduce cows to high-grain 'production rations'. Without a gradual adaptation to high-grain diets, there is a risk of upsetting rumen fermentation and causing the cows to go off-feed. In addition, the added concentrates in transition diets (termed 'lead-feeding') has many beneficial effects, including:

- increasing the dry matter intake
- adapting the rumen microbe population to a production ration
- stimulating the growth of rumen papillae
- increasing absorption of volatile fatty acids from the rumen
- increasing blood insulin concentration postpartum
- decreasing nonesterified fatty acid (NEFA) mobilization from adipose tissue postpartum.

Maximizing intake during the close-up dry period will not only ease the transition into lactation, but will also decrease the incidence of metabolic disturbances and improve milk production. Consequently during this period, producers frequently use techniques to maximize intake, such as frequent feedings and the use of palatable feeds. Transition diets are generally introduced 2–3 weeks prior to calving. Intake of concentrates (grains) should be 4.5–7 kg per cow prior to calving (0.5–1% of body weight). Less grain is needed on high maize-silage diets and grains should be increased gradually, approximately 0.5 kg day^{-1} per head, to avoid digestive upset. Despite the increased concentrates in the diet, there remains a need for bulky forages to promote rumen distention, which is critical during this stage to maximize milk production for the entire lactation and to prevent the problem of displaced abomasum.

One of the more significant changes in the 2001 National Research Council's Dairy recommendations is the allowance for feed intake depression in transition cows. Dry matter intake decreases by approximately 30% during the last 2 weeks of gestation (**Figure 2**) and, while the cause of this intake depression is not well characterized, it corresponds with dramatic shifts in the cow's endocrine status. The magnitude of intake depression is related to the development of fatty liver and other peripartal disorders. Therefore, dietary nutrient density is increased in the transition diet (**Table 1**) and highly palatable feeds are used to minimize the intake depression and maintain the desired nutritional balance. However, feeding rumen buffers, added fat or excess protein is not recommended during the transition period.

The inclusion of anionic salts in transition diets is an emerging technique to prevent milk fever. Dietary cations are positively charged molecules (sodium and potassium) while anions are negatively charged molecules (chlorine and sulphur). The dietary cation–anion difference (DCAD) is calculated using the following equation and is expressed in milliequivalents (mEq) 100 g^{-1} of dry matter.

$$\text{DCAD (mEq100g}^{-1}) = (\%\text{Na} \times 43.5) + (\%\text{K} \times 25.6) \\ -(\%\text{C1} \times 28.2) + (\%\text{S} \times 62.4)$$

Balancing transition diets for a negative DCAD (−7.5 to 0 mEq 100 g^{-1}) results in a mild metabolic acidosis and decreases the incidence of milk fever by increasing calcium

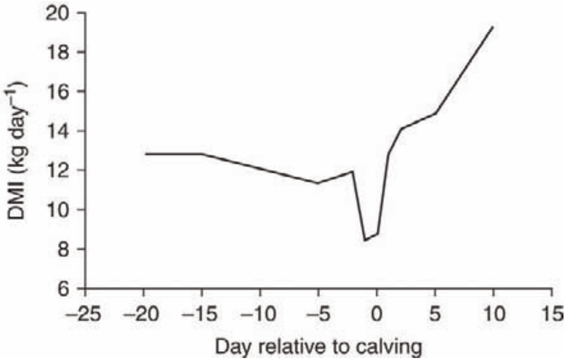

Figure 2 Peripartal dry matter intake in dairy cows. (Adapted from Bertics *et al*. (1992) *Journal of Dairy Science* 75: 1914.)

Table 3 Relationship of DCAD in rations to urine pH and calcium status at freshening in dairy cows

	Close-up Dry Cow		
Ration DCAD	*Urine pH*	*Acid-Base status*	*Calcium Status at Freshening*
Positive (>0 mEq/100 g)	7–8	Alkalosis	Low blood Ca
Negative (<0 mEq/100 g)	5.5–6.5	Mild metabolic acidosis	Normal blood Ca
A	<5.5	Kidney overload crisis	–

availability in bone and absorption from the intestine. The easiest way to obtain a negative DCAD in the transition diets is to start by feeding nonlegume forages and forages with a low potassium concentration. Urine acidity is also affected by DCAD and urine pH should be used to monitor the effectiveness of rations containing anionic salts (**Table 3**). In transition diets with a negative DCAD, urine pH should be between 5.5 and 6.5. Anionic salts are generally not palatable and should be included as part of a mixed ration to improve their intake. Anionic salts should also be fed only during the transition period and calcium is not restricted in diets when feeding anionic salts (**Table 1**).

Summary

In dairy cows, the dry period is an essential part of the lactation cycle and preparations for a successful lactation begin during, and even before, the preceding dry period. The management goals of the dry period are to minimize stress and the occurrence of infectious diseases, and to feed in order to maximize intake, maintain body condition, nourish the calf and minimize the occurrence of metabolic and digestive disorders. Each cow's body condition must be monitored regularly and cows should be fed to attain the proper conditioning (3.5–4.0) before dry-off. Maintaining rumen fill throughout the dry period is important in maximizing intake and production after calving. In addition, minimizing intake depression around parturition is key to maximizing production and avoiding metabolic and digestive problems. With proper management, cows will calve in good health and body condition, which will enable them to cope with the physical and metabolic challenges of the next lactation cycle.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction; Measurement Techniques and Data Processing. **Diseases of Dairy Animals:** Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Fatty Liver; Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Milk Fever. **Feed Ingredients**: Feed Supplements: Anionic Salts. **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems. **Mastitis Pathogens:** Environmental Pathogens. **Mastitis Therapy and Control**: Management Control Options; Medical Therapy Options. **Nutrients, Digestion and Absorption**: Fermentation in the Rumen. **Reproduction, Events and Management**: Pregnancy: Periparturient Disorders.

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Lactation Rations in Cows on Grazing Systems

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Background

The profitable production of milk and milk components in pasture-based systems is dependent on high utilization of the pasture grown. This is achieved by targeting appropriate stocking rates and calving dates to match the herd's feed demand with pasture supply. Even with the most ideal of climates, however, these supply and demand curves rarely, if ever, match completely, and pasture surplus to requirements must be harvested for silage/hay, or supplementary feeds provided during periods of pasture deficit (**Figure 1**).

Grazed pasture is the primary feed ingredient in the dairy cow's ration. But in many countries, there is the opportunity to supply the cow with a rich array of supplementary and possibly complementary feed ingredients in an effort to improve animal efficiency and increase milk production. The optimal combination of these ingredients will be discussed.

Nutrient Supply

A summary of the nutrient supply from pasture in a temperate climate throughout the year is presented in **Figure 2**. The repeatability of herbage quality traits within fortnights across years varies from moderate (22% for ether extract) to high (54% for metabolizable energy (ME)), indicating that although weather influences, at least in part, some of the quality factors, there is reasonable predictability of these nutritional parameters across years. All nutrients follow a cyclical pattern, with ME and nonstructural carbohydrate content high in spring and low in summer, and fiber content (neutral detergent fiber and acid detergent fiber) low in spring and high in summer. Crude protein and ether extract content have two peaks and troughs, peaking in spring and autumn and declining in both summer and winter.

From an examination of these data, it is evident that fresh pasture can be a very nutritious feed, with adequate to high crude protein concentrations (22.3 ± 2.35% dry matter (DM)), a reasonable digestible fiber (42.5 ± 2.44% DM) and nonstructural carbohydrate content (11.3 ± 2.06% DM), a desirable and relatively consistent ether extract (4.21 ± 0.23% DM) concentration, and a moderate to high ME content (11.7 ± 1.60 MJ ME kg^{-1} DM).

Although fresh pasture can be a high-quality feed for ruminant production when well managed, there are some specific nutritional factors to consider. The fiber in high-quality pasture is very digestible (~70%); however, the data presented in **Figure 2** indicate that there are instances when the pasture may have a reduced fiber digestibility because of a greater indigestible component (~55–60%). This could result in a reduced rate of fiber digestion (e.g., from 13–15% per hour in spring to 8–10% per hour in summer). In addition, the rumen-degradable protein content of pasture is generally in excess of the requirements of the cow.

Nutrient Requirements

The nutrient requirements recommended by NRC 2001 model are largely suitable for cows grazing high-quality pasture (**Table 1**), and computer simulation exercises using these guidelines are consistent with *in vivo* measurements. Comparisons of cows fed total mixed rations (TMRs) or fresh pasture indicate that differences in dry matter intake (DMI) explain more than 60% of the difference in milk production between the systems, and almost 90% of the difference is nonnutritional in origin. Kolver modeled the requirements of a 550 or 650 kg cow grazing high-quality fresh pasture (**Table 2**) and showed that there was sufficient metabolizable protein and amino acids for milk yields of 35–40 kg per cow per day, but actual production was limited to between 80 and 90% of this potential yield by ME intake. By his estimates, a dairy cow would have to consume between 3.0 and 3.5% live weight to produce 25–35 kg milk day^{-1}. This is in line with the published estimates of maximum achievable DMI for unrestricted high genetic merit cows, suggesting that this is the upper range of milk production for a cow fed solely on pasture.

Kolver's simulations (**Table 2**) suggest that the primary limiting nutrient in most intensive grazing scenarios is energy and this is consistent with farm systems' experiments undertaken internationally. Recognizing that energy is the dietary component most limiting milk production in grazing dairy cows, and that fresh pasture will be the primary dietary ingredient in lactating cow's rations in these systems, the main factors to be considered are

1. the energy density of pasture,
2. the total amount of energy produced per hectare, and
3. the most appropriate supplement to fill feed deficits.

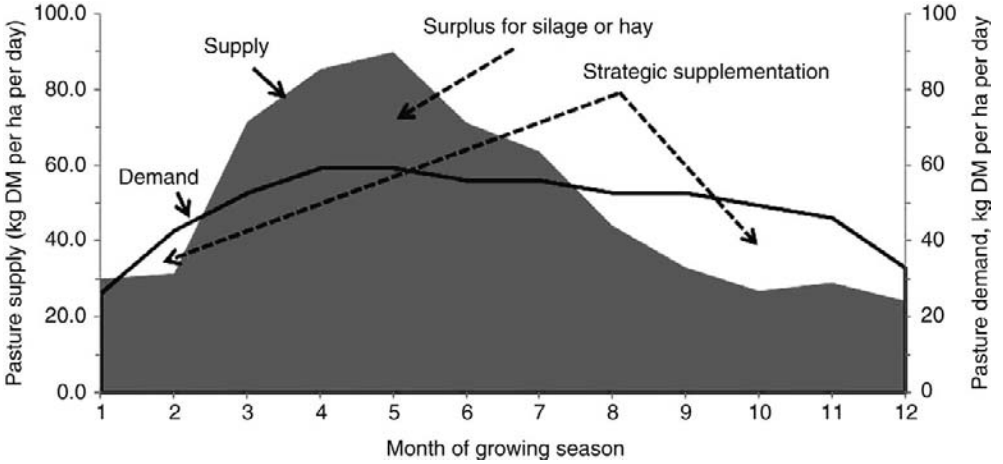

Figure 1 A stylized relationship between pasture supply (ha^{-1}) and dairy cow demand (ha^{-1}). The shaded area represents pasture supply, and the solid line represents cow demand assuming a stocking rate of 3.3 cows per hectare.

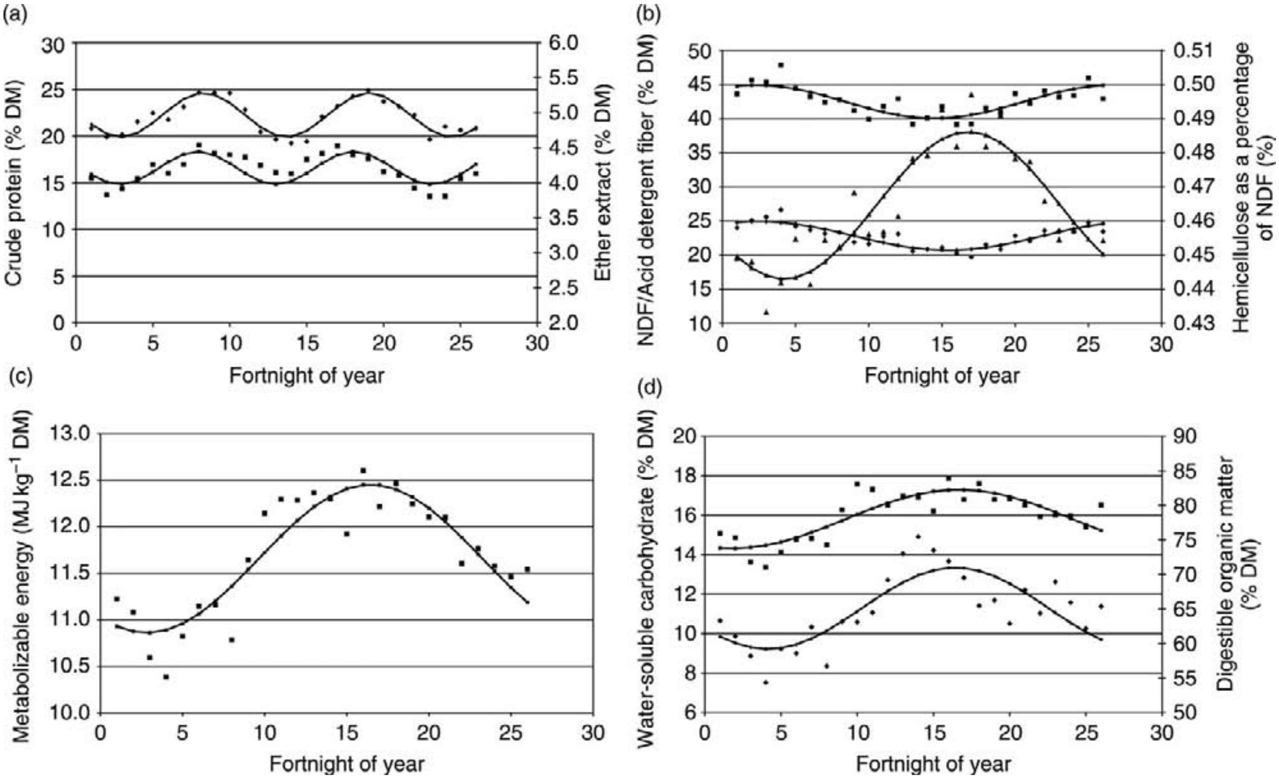

Figure 2 Temporal change in (a) crude protein (◆) and ether extract (■) concentrations, (b) acid detergent fiber (◆) and neutral detergent fiber (NDF; ■) concentrations, and hemicellulose as a percentage of NDF (▲), (c) metabolizable energy content (■), and (d) water-soluble carbohydrate (◆) and digestible organic matter (■) concentrations. Least square means for each variable are depicted without connecting lines, while the cosine functions are included in the figure within connecting lines among data points.

The Energy Density of Pasture

Table 3 indicates the potential milk production from cows grazing fresh forage of differing energy densities. Assuming the same DMI, a 13% increase in pasture ME content could result in 19% greater milk yield. These data highlight the positive effect of pasture energy density on potential milk production, and emphasize the need to manage pasture effectively to maximize quality.

The primary management factors influencing pasture energy density are

- pregrazing pasture mass and
- previous grazing's postgrazing mass (i.e., pasture residual).

Table 1 Nutritional guidelines for high genetic merit dairy cows grazing fresh pasture

	% DM
Crude protein	23
Nonstructural carbohydrates	<35
Neutral detergent fiber	>30
Acid detergent fiber	>12
Fat	<5–6
Calcium	1
Phosphorus	0.35
Magnesium	0.25–0.30
Potassium	1.0
Sulfur	0.30
Sodium	0.20
Chlorine	0.25

DM, dry matter.

Table 2 Nutrient requirements and supply for cows of 550 or 650 kg live weight

Live weight (kg)	*550*		*650*	
Milk yield (kg)	*25*	*35*	*25*	*35*
Nutrient requirements				
Metabolizable energy (MJ day^{-1})	207	229	216	260
Metabolizable protein (g day^{-1})	1704	1947	1730	2222
Methionine (g day^{-1})	31	35	31	40
Lysine (g day^{-1})	103	118	105	134
Calcium (g day^{-1})	136	155	145	184
Phosphorus (g day^{-1})	67	76	72	90
Nutrient supply				
Dry matter intake				
kg day^{-1}	17.3	19.1	18.0	21.7
g per kg live weight	31	35	30	33

Reproduced from Kolver ES (2003) Nutritional limitations to increased production on pasture-based systems. *Proceedings of the Nutrition Society* 62: 291–300.

Pregrazing pasture mass

Pregrazing pasture mass can have a significant effect on pasture quality. Most temperate grass species support between three and four live leaves per tiller, with leaf emergence and senescence tightly coordinated. Limited data on the nutrient content of the plant with advancing physiological maturity indicate little deterioration in quality until either senescence of the primary leaf or canopy closure (i.e., when light cannot penetrate the whole sward because of high pasture mass).

Previous grazing's postgrazing mass

The previous postgrazing mass can also have a significant effect on the quality of the pasture regrowth. Digestibility and energy density can be 3–5% less in pasture swards that have residuals of 1600–1800 kg DM ha^{-1} compared with those that have 1300–1500 kg DM ha^{-1} residuals. In addition, DMI has been reported to decrease by 15% in swards that were previously managed to leave high pasture residuals postgrazing, compounding the negative effect of reduced energy density and reducing milk yield by 10–20%.

Therefore, to maximize the energy density of the pasture consumed by the cow grazing management should be such that pasture swards are grazed close to full emergence of the last leaf, but before canopy closure, and should be grazed to a postgrazing residual height of around 35–40 mm.

The Total Amount of Energy Produced per Hectare

Although energy density is an important component of a lactating dairy cow's ration, the total quantity of energy that is available is more important for total milk production per hectare. Pasture should, therefore, be managed to maximize the production of ME per hectare, without unduly affecting energy density per kg DM.

Table 3 ME available for milk production in cows grazing fresh pasture containing either 11.5 or 13.0 MJ ME kg^{-1} DM

Live weight (kg)	*450*		*550*		*650*	
DMI (% live weight)	*3.0*	*3.5*	*3.0*	*3.5*	*3.0*	*3.5*
11.5 MJ ME kg^{-1} DM						
ME intake (MJ day^{-1})	155	181	181	221	224	262
ME available for milk production (MJ day^{-1})	101	127	127	159	153	191
Potential milk production (kg day^{-1})	18.5	23.0	23.0	29.0	28.0	34.5
13.0 MJ ME kg^{-1} DM						
ME intake (MJ day^{-1})	176	205	215	250	254	296
ME available for milk production (MJ day^{-1})	122	151	152	188	183	225
Potential milk production (kg day^{-1})	22.0	27.5	27.5	34.0	33.0	41.0

DM, dry matter; DMI, dry matter intake; ME, metabolizable energy.

The most digestible fraction of the pasture plant is the leaf. Temperate pasture follows a sigmoidal growth pattern after grazing, with each successive leaf considerably larger than the previous leaf. It is estimated that 8, 26, and 66% of potential pasture production result from the emergence of the first, second, and third leaf, respectively. Production of green leafy material is, therefore, maximized when the grass plant is allowed to reach its maximum leaf number (i.e., just before senescence of the first leaf). In addition, leaf digestibility does not decline from the first to the third leaf. However, care must be taken not to pass canopy closure, or a large proportion of additional growth will be lower digestibility stem, sheath, or senescent leaf, reducing the ME energy density of the pasture.

The Most Appropriate Supplement to Fill Feed Deficits

Computer-simulated feeding scenarios generally agree that the primary limiting nutrient in cows grazing fresh pasture is energy. However, supplementary feeding is not as simple as merely providing cows with additional energy. When a grazing cow is supplemented, she reduces her intake of pasture (substitution). The greater her pasture intake before supplementation, the greater the reduction in pasture eaten with supplementation, and substitution rates of 50–80% (i.e., for every kg DM of supplement offered, the cow decreases pasture intake by 0.5–0.8 kg DM) are commonly quoted, particularly when pasture quality is very high.

Ration formulation in grazing scenarios is, therefore, a balance between achieving desired postgrazing residuals to ensure maximum pasture quality and achieve high DMI per cow. If postgrazing residuals are greater than 35–40 mm, grazing dairy cows should not be provided with a supplement. In these situations, substitution rates are very high (>60%) and energy intake is not greatly improved by supplementation. Pasture, with consideration for some mineral supplementation, can be the sole feed ingredient in the ration.

Type of energy supplement

If postgrazing residuals are less than 35–40 mm, the dairy ration can be expanded to incorporate supplements and, in general, the supplement of choice should be a relatively energy-dense feed. However, assuming a common price per unit of energy, choice of supplement used may depend on milk payment structure.

Starch- and sugar-based supplements increase milk volume to a greater extent than similar intakes of fermentable fiber-based supplements, which is an important consideration in systems where producers are paid primarily for fluid milk. This is probably because the former feeds result in greater yields of precursors for hepatic gluconeogenesis (i.e., propionate and possibly microbial protein) and greater concentrations of glucose circulating in blood. As uptake of glucose by the mammary gland is primarily dependent on blood glucose concentration, the increase in glucose supply should increase mammary lactose production, with a consequent increase in milk volume to maintain osmolarity. In general, where pasture postgrazing residuals are approximately 35–40 mm, responses to starch- and sugar-based feed ingredients are between 0.08 and 0.10 kg milk MJ^{-1} ME.

In systems where producers are paid primarily for milk components, protein tends to be worth approximately twice as much as fat. However, milk protein is resistant to nutritional manipulation, whereas milk fat can be easily altered by diet change. In general, supplementation with starch- or sugar-based feed ingredients tends to increase milk protein percent, but decrease milk fat percent, and the decline in milk fat is often close to twice the increase in milk protein. In comparison, supplementation with forage or fiber-based concentrates may not alter milk composition greatly, but component yield increases are similar to those from nonstructural carbohydrate supplements. In these situations, whether the energy is from nonstructural carbohydrates or fermentable fiber supplements is largely irrelevant.

There have been some suggestions that starch- or sugar-based supplements result in less milk production per MJ of ME than fermentable fiber-based supplements; however, there is little agreement in the published literature on this. If real, the effect may be a result of greater pasture substitution with the use of starch- and sugar-based supplements, because of the greater ruminal propionate production and associated effects on the neuroendocrine satiety factors, or may be dose related (i.e., how much supplement is being fed). Further research is required in this area.

Protein

In general, pasture-based systems rarely require protein supplementation. The feed protein is of high quality (good amino acid profile), passage rate is rapid, ensuring that a high proportion of potentially degradable protein reaches the small intestine, and the efficiency of microbial protein synthesis is equal to that reported in cows fed TMRs. Although modeling exercises have identified methionine, lysine, and histidine as primary limiting amino acids, in studies where rumen-protected forms of these amino acids have been provided to dairy cows grazing fresh pasture, milk production has not increased. However, milk production may not have been sufficiently high for amino acids to become the primary limiting nutrient in those studies. Certainly, in herds producing 40+ kg milk day^{-1}, adequacy of metabolizable protein and specific amino acids should be considered.

Minerals and vitamins

Cows fed fresh pasture tend to consume sufficient vitamins to maximize milk production. However, their ration must contain supplementary magnesium during risk periods for grass staggers (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Grass Tetany) because of the high potassium and nitrogen content of pasture. Furthermore, intensively managed pastures often contain inadequate amounts of copper, cobalt, selenium, iodine, and zinc for optimal animal health and production; therefore, dietary adequacy of each of these minerals should be evaluated with a feed test.

Conclusion

Fresh pasture is a very high-quality feed ingredient and can constitute the entire ration for a lactating cow. DMI and energy intake can be maximized through careful attention to pasture management, but maximum milk production from pasture alone is probably between 30 and 35 kg per cow per day.

Nutrient requirements recommended by NRC 2001 model are largely appropriate for pasture-based dairy cows, but there must be recognition that there is limited benefit to providing a cow that has sufficient pasture with an additional supplement because she will refuse pasture. Adequacy of pasture can be defined by postgrazing residual; if residuals are >35–40 mm, pasture refusal will be large if supplements are offered.

If postgrazing residuals are below 35–40 mm, supplementing cows with an energy supplement will increase milk production. Starch- or sugar-based supplements would be expected to increase milk yield and milk protein to a greater extent than fermentable fiber-based supplements, but they would also be expected to reduce milk fat content. Decisions on the choice of supplement should primarily reflect the cost of energy in the different supplements; however, components of the milk payment structure may also be important.

See also: **Dairy Farm Management Systems**: Seasonal, Pasture-Based, Dairy Cow Breeds. **Nutrients, Digestion and Absorption**: Fiber Digestion in Pasture-Based Cows. **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems.

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Lactation Rations for Dairy Cattle on Dry Lot Systems

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The continuing change in animal productivity is another factor that influences the formulation process. The average lactation yield in 1960 for dairy cows enrolled in the Dairy Herd Improvement (DHI) program was 4790 kg. This level of production increased to 9281 kg per lactation in 1999. This is an average milk production increase of 115 kg year^{-1}. Individual cows have produced >30 000 kg milk in a lactation. There are a number of Holstein herds in the United States with herd averages in excess of 13 000 kg per cow. A number of Jersey herds have herd averages exceeding 9000 kg year^{-1}.

There are a number of considerations that must be kept in mind when developing nutrition programs for dairy farms. These can be thought of as goals and may include the following factors:

1. To provide an adequate level of DMI and nutrient intake to permit dairy cows to express their genetic potential.
2. To provide rations and feeding management programs that maintain a healthy rumen environment and maximize microbial protein production.
3. To maintain herd health and reproductive performance in the herd.
4. To optimize economic returns from the feeding program.
5. To provide rations that minimize that nutrient excretion to the environment.

The above list presents an imposing challenge for both the dairy producer and the agriservice professionals who are part of the farm advisory team. This list should be modified depending on the goals and objectives of the specific farm unit. It is important to have a defined set of goals and objectives so that progress can be monitored.

As a base for developing dairy rations, it is essential to understand the relationships between DMI, milk production, nutrient use and profitability. **Table 1** contains the relationships between milk production and the partition of energy and protein intake for milk production. As milk production increases, a larger portion of the total nutrient intake is used for milk synthesis. Conversely, a lower percentage of the total nutrient intake is required to meet the maintenance requirement of the cow.

Table 2 contains the relationships between milk production, feed cost and income over feed cost (IOFC). The key points are:

- feed cost per cow per day increases as milk production goes up
- feed cost per 45 kg of milk production decreases with higher levels of milk production
- IOFC increases as milk production increases
- Dry-matter efficiency (DME) increases with increased rates of milk production DME is a measure of kg milk produced kg^{-1} DMI.

The overall interpretation is that higher levels of milk production are related to higher levels of profitability. It is important to remember that this is a generalized chart based on biological principles. These results are related only to the nutrition program on the farm. They may not always translate to a higher level of total farm profitability. Other factors, such as total production costs, labor efficiency and debt load, will influence total farm profitability.

This table also contains information on DME. DME is a term that can be used to monitor the efficiency of the nutrition program in converting feed nutrients into milk. Note that the DME factor increases as milk production increases. This term is beginning to be used by dairy managers as a monitoring tool for the feeding program. The goal for DME will vary with the milk production average of the herd. DME values should be in the range of 1.3–1.5 for dairy herds producing 25–35 kg milk day^{-1} per cow. The DME value in a specific dairy herd or group will be meaningless unless a measured DMI value is available.

It is important to remember that the actual ration formulation process accounts for only a small portion of the total nutrition program on a dairy farm. Some consultants estimate that the formulation of the ration accounts for <20% of the total nutrition program. Other key components of the nutrition program include forage quality, feed mixing, ration delivery, cow behavior, feed sorting and feedbunk management. The goal is to ensure that the 'formulated' ration is delivered to and consumed by the cow. This can be a difficult task in many farm situations.

Table 1 Percentage of total nutrient intake used for milk production

Milk (kg day^{-1})	*Net energy (%)*	*Metabolizable protein (%)*	*Crude protein (%)*
15	51.2	53.0	75.0
25	63.7	61.8	83.6
35	71.0	66.0	87.7
45	75.9	69.4	90.1
55	79.4	71.4	91.8

Other articles provide specific information on nutrient requirements, computer models and nutrient use in dairy cattle (*see* **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows; Models in Nutritional Research; Models in Nutritional Management). This article will focus on some of the current concepts that nutritionists must consider when developing or evaluating dairy rations. These may not be totally quantified but must still be incorporated into the thought process. The practical application of nutrition principles on dairy farms requires the recognition of the ability to work with variations.

Dry Matter Intake

One of the most important variables influencing milk production is DMI. Surveys in both the United Kingdom and the United States have indicated that DMI is one of the key management attributes defined in high-producing herds. Accurate measures of DMI are needed for ration formulation. When involved in investigating a 'suspected' nutritional problem in a dairy herd, a measured DMI is essential. A large number of equations have been developed by various groups to predict DMI. Body weight and milk production are two variables that consistently appear in these equations. A number of other parameters are also used in some prediction equations. Milk production has been estimated to account for about 45% of the predicted DMI. Other factors that influence DMI include body weight (17%), environment (10%), body condition (6%) and herd feeding and management practices (22%). The equation used to predict DMI in the 2001 nutrient requirements of dairy cattle publication by the National Research Council is:

$$\mathrm{DME}\left(\mathrm{kg\ day}^{-1}\right) = \left(0.372 \times \mathrm{FCM} + 0.0968 \times \mathrm{BW}^{0.75}\right) \times \left\{1 - e^{[-0.192\times(\mathrm{WOL}+3.67)]}\right\}$$

where FCM = kg of 4% fat-corrected milk, BW = body weight and WOL = week of lactation.

This equation was based on about 17 000 cow week records on Holstein cows. This equation may not work for other dairy breeds. The WOL adjustment factor is used to calculate the lower intake in early-lactation cows. Maximum DMI will be attained at about 10–14 weeks of lactation in most cows. It should be emphasized that any prediction equation may not fit well for animal groups on individual dairy farms. The preferred approach is to have actual DMI determinations made on the dairy farm. Feed quality and feeding practices can have a significant impact on DMI.

Forage Quality

Forages provide the base upon which dairy rations are built. High-quality forages have a high intake potential, along with high digestibility. They are characterized by a high rate of fiber digestion in the rumen. The quality of the

Table 2 Feed cost and income over feed cost at varying levels of milk production[a],[b]

Milk (kg)	*DMI (kg)*	*Feed cost per cow day^{-1} (US$)*	*Feed cost 45 kg^{-1} milk (US$)*	*IOFC[c] (US$)*	*DME[d]*
15	16.8	1.70	5.10	3.30	0.9
25	19.9	2.27	4.09	6.06	1.25
35	23.3	2.88	3.70	8.78	1.5
45	26.3	3.46	3.46	11.54	1.7
55	28.9	4.20	3.43	14.13	1.9

[a] 640 kg cow, milk fat test = 3.7%.
[b] Feed and milk prices used:
[c] Income over feed cost = total milk income–total feed cost.
[d] Dry matter efficiency (DME) = kg milk kg^{-1} dry matter intake (DMI).
Milk = 33 cents kg^{-1}
Maize silage = \$27.5 t^{-1}
Alfalfa silage = \$38.5 t^{-1}
Maize meal = \$110 t^{-1}
Soya bean meal = \$231 t^{-1}
Soya bean meal = \$253 t^{-1} (heat-treated)
Animal fat = \$440 t^{-1}
Mineral mix = \$495 t^{-1}
Note: Feed prices are on an as-fed basis.

Table 3 Alfalfa (lucerne) hay quality and predicted milk production

	Sample A	*Sample B*	*Sample C*
Crude protein (% of DM)	23	20	18
Neutral detergent fiber (% of DM)	36	43	51
Net energy lactation (Mcal kg^{-1})	1.4 (5.85)[b]	1.3 (5.44)	1.1 (4.6)
F-NDF intake (quantity of NDF contributed by forages) (kg)[a]	6.4	6.4	6.4
Forage-DMI intake (kg)	17.8	14.9	12.5
Crude protein intake (kg)	4.1	3.0	2.25
Net energy lactation (Mcal)	24.9 (104)[c]	19.4 (81)	13.75 (57.5)
Crude protein–milk (kg)	43.7	30.7	21.7
Net energy lactation–milk (kg)	21.3	13.3	5.2

[a] F-NDF intake = 1% of body weight for a 640-kg cow.
[b] MJ kg^{-1}.
[c] MJ day^{-1}.

forage available dictates the maximum milk production that can be attained while still maintaining rumen health.

Table 3 contains an example of potential milk production that could be attained using three different qualities of alfalfa (lucerne) hay. These calculations are based on crude protein (CP) and net energy lactation (NE$_l$) content of the forages. Forage DMI is calculated using a forage neutral detergent fiber (NDF) intake of 1% of body weight. Projected milk production is based only on the nutrients contained in the forage after accounting for the animal maintenance requirements. Predicted milk production drops rapidly as forage quality declines. The predicted milk from CP is always higher than the milk that could be produced from energy. This information emphasizes the importance of forage quality and forage testing in determining potential herd milk production while maintaining rumen health.

There are at least three factors which can be used to assess forage quality. One is to calculate the forage lignin content as a percentage of NDF. Since lignin is basically unavailable to the cow, forage digestibility declines as the lignin-to-NDF ratio increases. One use of this factor is to select the groups of animals to which specific forages should be allocated. Forages with a lower lignin-to-NDF ration should be allocated to cows in the early part of the lactation cycle.

A second tool that can be used is determining forage digestibility. This can be done by *in vitro*, *in situ*, enzymatic or near-infrared spectroscopy (NIRS) techniques. The variation in NDF digestibility from forages submitted for analysis by dairy producers can be 20–30%. Research workers at Michigan State University summarized data from 23 studies with measured NDF digestibility values. They reported that an increase of 1 unit of NDF digestibility was related to an increase of 0.17 kg of DMI and 0.25 kg of 4% FCM. A number of strategies exist to utilize forage digestibility results on dairy farms. The majority of the adjustments will be made when a low NDF digestibility forage is found on a farm. One would be to stop feeding this forage to cows in the first part of the lactation cycle. A second alternative would be to feed less of this forage and more of a higher-digestibility forage. A third approach would be to substitute a highly digestible byproduct feed (beet pulp, soya hulls) for some of the low-digestibility forage. These approaches have improved daily herd milk production by 0–5 kg. If NDF digestibility is higher than normal, then the quantity of forage fed in the ration can be increased. If the quantity of forage fed is not increased in this situation, there is a greater risk of ruminal acidosis. This analysis is currently provided by a number of forage testing laboratories.

A third factor is forage particle size. Forage particles must be of an adequate particle size to stimulate chewing, rumination and saliva flow. If forage particle size is too small, then both the rate of digestion and passage of the forage increase. Rumen pH will decline and rumen fermentation will be altered. The physically effective NDF (peNDF) system measures this by the percentage of the dried particles which stay on top of a 1.18-mm screen. This system still requires additional validation and testing. However, minimum ration peNDF levels of 20–22% seem to be a reasonable guideline at this time. Adjustments to this guideline will need to be made for factors such as feeding practices, feedbunk management, cow behavior and feed sorting.

Feed Energy Values

The energy value of a specific feed or ingredient is not constant. The energy value of a feed varies with rate of passage and DMI. As DMI increases, rate of passage increases and the feed energy value decreases. This concept was quantified in research trials many years ago but until recently has not been incorporated in ration formulation programs. Information on this approach is contained in both the Agricultural and Feed Research Council (AFRC) and National Research Council (NRC) nutrient requirement publications. The metabolizable energy (ME) value for maize silage in the ration for a dairy cow producing 25 kg day^{-1} milk is listed as 2.35 Mcal of ME kg^{-1} DM (9.83 MJ) by the NRC ration program. The same maize silage has a value of 2.18 Mcal of ME kg^{-1} DM (9.1 MJ) for a cow producing 55 kg of milk. The depression in digestibility with increasing levels of DMI is not constant across feeds but varies for each individual feed. Ration formulation programs are available which incorporate these concepts.

Carbohydrates

The carbohydrate nutrition of high-producing cows is critical for optimizing milk production, rumen health, microbial protein synthesis and profitability. At the same time, balancing ration carbohydrates is the most difficult and uncertain component of the formulation process. One reason is that many of the requirements for the various carbohydrate fractions have not been clearly defined. Examples include ration sugar, starch, pectin and effective fiber levels. A second reason is that there are such a large number of carbohydrate fractions contained in various feeds. A third reason is that routine analysis for some carbohydrate components is not available. Typically, forage laboratories can analyze for acid detergent fiber (ADF), NDF, sugars and starches. The rate of digestion of various carbohydrate fractions is also variable between feeds but is not a routinely available laboratory analysis. Ration formulation programs are available which incorporate these concepts.

The feed carbohydrates can be divided into two basic groups. These are the structural and nonstructural fractions. The total carbohydrates in a typical dairy ration may comprise 60–75% of the total ration DM. The structural and nonstructural fractions are composed of a number of different components. The structural fraction contains primarily cellulose, hemicellulose and pectic substances. The nonstructural fraction contains sugars, starches and fructans. Organic acids from silage fermentation may also be included in this fraction if a difference calculation is used.

Structural carbohydrates are commonly defined as ADF and NDF. The ADF fraction includes the cellulose fraction while NDF includes both cellulose and hemicellulose. Typically, the lignin fraction is also included in both ADF and NDF. However, lignin is not a carbohydrate by a strict chemical definition.

There has been some confusion between the terms NSC (nonstructural carbohydrates) and NFC (nonfiber carbohydrates) in the last few years. The definition of these terms is now standardized as follows:

- NSC: this term is used when this fraction is determined by enzymatic analysis
- NFC: this term is used when this fraction is determined by a difference calculation rather than by actual analysis. The following calculation should be used:

$$\text{NFC }\% = 100 - (\text{NDF} + \text{CP} + \text{fat} + \text{ash} - \text{NDFIP})$$

The NDFIP term (the crude protein contained in the residue from the NDF analysis) corrects for the CP contained in the NDF fraction. The NFC calculated by this equation would include the organic acids in silages. The NFC calculated by this method could overestimate the quantity of rumen fermentable carbohydrate that is available to support microbial function in the rumen.

An additional challenge is the effects of both particle size and processing methods on the rate and extent of carbohydrate degradation in the rumen. Smaller particles tend to have faster rates of degradation. Processing methods, such as steam-flaking, may also increase both the rate and extent of carbohydrate degradation in the rumen.

Protein

Ration formulation programs have used a number of protein terms to describe feeds and determine requirements. The term crude protein (CP) has been replaced in most nutrient requirement systems and formulation programs. Metabolizable protein (MP) is the term used by the AFRC (United Kingdom), NRC (United States) and a number of ration formulation models. MP includes microbial crude protein (MCP), feed protein undegraded in the rumen (RUP) and a small amount of endogenous protein. Both the AFRC and NRC systems are based on MP, even though the requirement calculation equations are different.

A primary source of the MP in the small intestine should be MCP. The quantity of MCP produced per day depends primarily on the quantity of carbohydrate available in the rumen and the quantity of rumen-degraded protein (RDP). A number of equations are available to predict daily MCP production. The 2001 Dairy NRC predicts MCP as 130 g kg^{-1} of total digestible nutrients (TDN) intake.

The crude protein in the feed is divided into a number of fractions in both the AFRC and NRC publications. Typically, these fractions are defined as A, B and C. The definition of these fractions is not always consistent. One approach to defining these fractions is:

- A: that portion of the protein that is rapidly degraded in the rumen. This fraction may be primarily nonprotein nitrogen compounds.
- B: this is the remainder of the CP and should be available for degradation in the rumen.
- C: this is the portion of the CP remaining undigested in the bag after an *in situ* digestion.

Amino acid nutrition of the dairy cow has received a lot of research attention in the last 20 years. However, the absolute requirement for specific amino acids is still not elucidated. There is adequate information on methionine and lysine to use these, with some caution, in both formulation and evaluation programs. The most common approach is to set guidelines for methionine and lysine as a percentage of the MP. The evaluation or formulation of dairy rations for amino acids requires the use of specialized computer models (*see* **Feeds, Ration Formulation**: Models in Nutritional Management).

Environmental Considerations

Many countries are imposing nutrient management constraints on dairy farms. These are designed to minimize excess nutrient transfer to the environment and to protect water and air quality. There is a large diversity in both the structure and degree of implementation of these requirements in various countries. Currently, the majority of these regulations are focused on nitrogen (N) and phosphorus (P). Other nutrients may be added to this list in the future.

It is common that ration formulations provide both N and P in excess of animal requirements. There is logic to this situation. Animal nutrient requirements are determined in very tightly controlled and regulated research settings. Typically, there is much more variation in an actual dairy farm setting. Variations in forage quality, silage fermentation characteristics, feed mixing, ration delivery, cow behavior, feed sorting by cows, feedbunk management, housing systems and environmental conditions exist on farms. Feed formulators commonly formulate above requirements in an attempt to account for these factors and minimize variations in milk production. The size of this 'safety' factor can vary greatly between farms. Assume that one farm does not use routine forage testing, feeds silage and concentrate in the barn and has free-choice access to hay in an outside lot. A second farm uses routine forage analysis, feeds a total mixed ration (TMR), has a 4% feed refusal factor, adjusts rations for variations in forage DM content and has minimal feed sorting by the cows. The ration balanced for the second farm can be balanced with a much smaller 'safety' factor than the first farm if the same level of milk production is to be attained.

The challenge is to reduce overfeeding of nutrients while maintaining production and profitability. Surveys in the United States have indicated that P is often overfed by 20–50% in excess of requirements. Recent studies in the United States, Germany and The Netherlands have indicated no milk production or reproduction benefits to feeding P in excess of requirements. Decreasing P overfeeding has been estimated to lower yearly purchased feed costs by $10–15 per cow. The decrease in purchased feed costs will depend on the degree of P overfeeding in the original ration. The excretion of P by the cow can also be reduced by 20–35% in this situation.

A number of research trials have been conducted examining ration protein levels and feeding strategies. One recent trial used cows averaging about 11 000 kg of milk per lactation with four protein feeding options. Yearly N excretion could be reduced by about 20 kg per cow while maintaining milk production. It appears that decreases of 10–30% in N excretion per cow are attainable without decreasing milk production. Recent papers have indicated that milk urea nitrogen may be a practical tool that can be used as an index of N excretion.

Feedbunk Management

The goal is to provide a fresh, consistent and palatable ration to dairy cows. Ideally, this mix would be available 24 h day^{-1}. However, this is difficult to achieve on many dairy farms. There are frequently variations in feed mixing, feed delivery, feed availability and sorting of feed by cows. A key concern in mixing is the tendency to overmix and this can reduce the particle size of the feed delivered to the cow. This is more of a concern with auger-type mixers. The consistency of the feed delivered to the cows may also vary from different parts of the same load. In some situations, feedbunks are empty for a number of hours per day and this can both decrease DMI and change eating patterns. The other variable in the feeding system is the potential for the cows to sort the feed and discriminate against the coarse particles. This can be significant on some farms and can decrease fiber intake. The result can be less chewing activity, less saliva flow and an increased risk of acidosis. The addition of water or a liquid molasses-type supplement may be helpful to minimize sorting.

A practical tool that can be used to monitor some of these feedbunk items is a forage particle separator. There are a number of these that could be used on farms. In the United States, the Penn State separator is the most commonly used. This device can assess the particle length of either forages or TMRs. This tool has two screens and a solid pan. The sizes of the circular openings are 19 and 8 mm. A TMR should have a minimum of 10–12% of the material on the coarse screen and <50% that collects in the pan.

How Does This All Fit Together?

The challenge for a nutritionist is to integrate these concepts into a practical ration. A number of key decisions need to be made to accomplish this task. The first is to determine which nutrient requirement standards will be used as a base for formulation. A number of options exist in various countries that provide nutrient requirements. The second decision is to determine the computer software that will be used in the formulation process. Again, there are a large number available for consideration. Increasingly, nutritionists are beginning to utilize computer models that incorporate the concepts described above (*see* **Feeds, Ration Formulation**: Models in Nutritional Management). Once these decisions are made, then the move can be made to the formulation process. The following comments and guidelines may be helpful in this overall process. These are guidelines based on both nutrient requirement standards and field experience. They should be appropriate for Holstein cows producing 35–45 kg of milk day^{-1}:

1. DMI: this can be predicted with equations or a system established at the dairy farm to determine actual intakes. Equations may vary by 1–3 kg of predicted DMI for the cows at the same body weight and level of milk production. The actual intake has a significant impact on the ration nutrient density required to meet requirements.
2. Quantity of forage in the ration: a simple way of calculating this is as F-NDF (the quantity of the total NDF contributed by forages) as percentage of body weight. Typically, I use about 1% of body weight. This range can be adjusted down or up as forage quality varies. Cows grazing lush, low-fiber pastures may have F-NDF intakes up to 1.3–1.5% of body weight.
3. Ration carbohydrate levels:
   - ADF: 19–21% of ration DM
   - NDF: 27–32% of ration DM (forage NDF should be 70–80% of this total)
   - NFC: 37–42 % of ration DM.
4. Ration protein levels:
   - MP: this value will be determined by the specific nutrient requirements used
   - RDP: this should be about 65% of the total MP
   - RUP: this should be about 35% of the total MP
   - MP from microbial protein: this should be greater than the MP provided by RUP.
5. Ration fat levels: normally, total ration fat should not exceed 5–6% of total ration DM. The maximum quantity of fat provided by vegetable fats and other rumen active fats would be about 1.5–2% of total ration DM. If additional fat is needed, bypass fat sources can be used.
6. Ration energy levels: a ration ME content of about 1.6–1.7 Mcal kg^{-1} (6.7–7.1 MJ kg^{-1}) is needed at 'normal' DMI to meet the ME requirements. This will vary slightly with the specific ME system used.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction; Measurement Techniques and Data Processing. **Feeds, Ration Formulation**: Dry Period Rations in Cattle; Models in Nutritional Management; Models in Nutritional Research; Systems Describing Nutritional Requirements of Dairy Cows. **Manure/Effluent Management**: Nutrient Recycling; Systems Design and Government Regulations.

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Transition Cow Feeding and Management on Pasture Systems

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Background

Traditionally, the nonlactating cow was managed by neglect. The suggestion, however, that marginal quality feeds and lack of proper care of dry cow were responsible for lower milk production, an increased incidence of periparturient health disorders, and lower reproductive performance has led to a recharacterization of the dry period as an important period in the cow's life with regard to productivity, and no longer just a resting time between lactations.

The period that most encompasses the physiologically coordinated (homeorhetic) changes that the cow undergoes between late pregnancy and lactation is referred to as the transition period and is loosely regarded as the interval between 3 weeks precalving and 3 weeks postcalving. It is a period where the dairy cow must alter her metabolic priorities for available nutrients from fetal growth and net tissue deposition (mid- to late gestation) to milk production and the mobilization of tissue reserves (early lactation). If the dairy cow cannot adapt quickly to these challenges, the substantial cost of conceptus growth during late pregnancy and milk synthesis during early lactation places the transition dairy cow in a state of severe negative energy balance (NEBAL), where energy output exceeds energy intake. To avoid this, the cow must substantially increase dry matter intake (DMI) and alter the size and metabolic rate of many organs and tissues in a very short time period.

Although there is much interest in this important time in the cow's life, much of the accepted dogma around nutrient requirements of cows and the failure to achieve those requirements is not supported by research results. Furthermore, much of the cited research has been undertaken in indoor-fed dairying systems where the dietary ingredients are very different from those fed to nonlactating cows in pasture-based systems. Many of these findings are being introduced liberally into pasture-based dairying systems with little thought for the relevance or applicability of this advice. This article addresses some of these nutritional concerns.

Dry Matter Intake

Managing the transition cow involves digestive system adaptation to the lactation ration and the avoidance of metabolic disorders, with the aim of increasing DMI, reducing body condition score (BCS) loss, and improving milk production, health, and reproduction. Even though the energy requirements for pregnancy are small compared with the requirements during lactation, inadequate nutrition of dry cow will result in a substantial drain of maternal nutrient reserves to sustain the developing fetus and to meet the increasing energy demands of the mammary gland.

A severe NEBAL during the transition period has been suggested to predispose cows to metabolic disorders. Milk fever, ketosis, retained fetal membranes, metritis and endometritis, and left displaced abomasum primarily affect cows during the transition period, and they have all been associated with a periparturient NEBAL. Most associations, however, have been epidemiological, and the causality of periparturient NEBAL on these metabolic problems is not necessarily supported by experimental evidence. For example, a precalving NEBAL has been reported in many studies to reduce the risk of milk fever and ketosis, and there is conflicting evidence regarding the association between periparturient NEBAL and metritis. The importance of DMI in the weeks preceding calving on subsequent milk production is, therefore, unclear.

DMI Requirements Precalving

As fresh pasture provides metabolizable protein in excess of the requirements of the nonlactating cow, even during the last month of gestation, a cow's requirement for energy is what dictates her DMI requirements. The energy requirements of pasture-fed dairy cows during the month preceding calving were recently defined as 1.05 MJ of metabolizable energy (ME) per $kg^{0.75}$ body weight (BW) or a DMI requirement of approximately 2% BW (assuming 11 MJ ME per kg pasture/forage dry matter (DM)). This was the amount of energy required daily to maintain BCS through the month preceding parturition and is approximately 20% greater than the energy requirements calculated from previously published estimates.

Maintaining DMI Precalving

One of the primary concerns of nutritionists during the precalving period is preventing the decline in DMI with approaching parturition. It has generally been accepted that DMI declines 20–30% in the weeks preceding calving in systems where cows consume considerable amounts of nonfiber carbohydrates. This decline has been associated

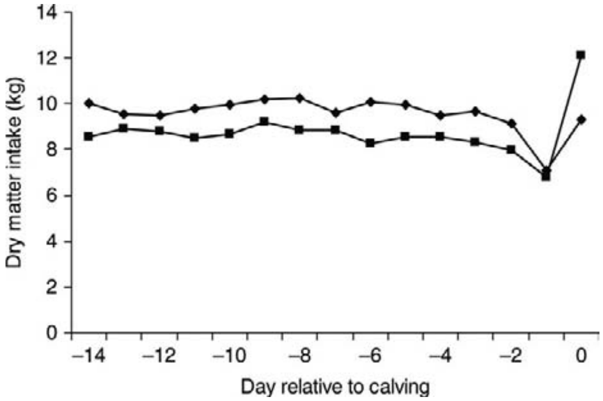

Figure 1 Dry matter intake (kg per cow per day) in cows consuming approximately 75% (■) or 95% (◆) of predicted precalving energy requirements as fresh and conserved pasture. From Roche JR (2006) Dry matter intake precalving in cows offered fresh and conserved pasture. *Journal of Dairy Research* 73: 273–276.

with greater circulating concentrations of free fatty acids in blood and up to a 300% increase in liver fat infiltration precalving. Seminal research undertaken at Purdue University highlighted that the reported depression in DMI precalving occurred only on diets with greater than 25% concentrate feeds in the complete ration. This is consistent with behavioral preference research in sheep, where ewes reduced their DMI of a complete ration containing forages and concentrates when injected with a pharmacologically relevant dose of estrogen, but maintained their DMI of hay under a similar physiological challenge. The observation that no DMI reduction occurs in dairy cows fed fresh pasture and grass hay was subsequently confirmed (**Figure 1**) and implies that cows fed primarily forage (fresh pasture/grass silage or hay) during the prepartum phase of the transition period do not consume less in the weeks preceding parturition as those in systems where they are fed concentrate feeds.

Effect of a Precalving NEBAL

Because of the induced hypotheses from association analyses that prepartum DMI influenced postcalving health and milk production and the assumption that DMI declines prepartum, there has been considerable research effort during the last decade in determining the postcalving effect of precalving level of feeding. Irrespective of the dairy system, there is general agreement that failing to provide the required amount of energy (or DM in a pasture-fed cows) has only minor effects on milk production (<75 kg fat-corrected milk for a 50% reduction in energy intake in the month precalving). In fact, the effect can almost completely be explained by the effect of the prepartum diet on calving BCS.

In many of these recent studies, postcalving DMI was greater and predictors of energy balance indicated a more positive energy balance in cows on a restricted diet precalving. Recent microarray studies confirm the advantage of 'preconditioning' cows to a NEBAL prepartum, with results indicating a more balanced metabolic profile postpartum, a reduced risk of fatty liver, and, perhaps, enhanced overall liver health during the periparturient period.

Conclusion

There is general consensus among published studies that providing a cow has not got an underlying malaise, precalving DMI is not positively associated with postcalving DMI. Consistent with such a premise, precalving DMI has only minor positive effects on milk production, commensurate with differences in calving BCS.

Furthermore, there is a growing body of physiological and molecular evidence that providing a cow with energy surplus to requirements precalving has detrimental effects on periparturient metabolic health, and it may even be sensible to impose a NEBAL precalving. If this is to happen, the system must ensure that cows are at sufficient BCS before the NEBAL imposition to still achieve a calving BCS of 3.0–3.25 (5-point scale).

Importance of Carbohydrate Type

The drain of nutrients by the mammary gland and the fetus places considerable nutrient demand, above maintenance, on the cow. Compared with monogastric animals, very little glucose is absorbed from the digestive tract in ruminants, but is instead produced by the liver through gluconeogenesis from propionate, amino acids, glycerol, and lactate. The observation that very little fetal metabolic energy is derived from acetate (the primary volatile fatty acid produced in rumen fermentation in grazing dairy cows) and reports of glucose oxidation accounting for approximately 30% of fetal energy demands has led to recommendations for inclusion of gluconeogenic precursors (i.e., starch and sugar) in the diet of grazing dairy cows. The assumed benefits achieved through feeding such ingredients precalving are believed to be threefold:

1. acclimatization of the rumen microorganisms to the highly fermentable carbohydrate ration offered postcalving;
2. increased rumen papillae development; and
3. a more readily available supply of nutrients, such as glucose, for the fetus to prevent maternal loss of energy reserves.

Until recently, however, there was very little research undertaken to test these hypotheses, which have been induced from an increased knowledge of fetal requirements and metabolism. Therefore, little was known about the benefits of altering structural carbohydrate (SC): nonstructural carbohydrate (NSC) ratio in the prepartum ration, and recommendations on the basis of the induced hypotheses presented have become accepted dogma. These recommendations require revision on the basis of recent research results.

Acclimatization of the Microorganisms to Diets High in Carbohydrates

When highly fermentable carbohydrates are introduced into the diet, the production of volatile fatty acids increases, reducing rumen pH. This change in pH exerts selective pressure on the microbial population and needs to be undertaken gradually to allow a stable fermentation pattern to continue. Of the reasons cited earlier for the feeding of a total mixed ration (TMR) precalving, in lieu of 100% forage, this reason is probably the most plausible, especially when cows are supplemented with such feeds postcalving. However, cows in pasture-based systems will rarely receive more than 30–40% of their diet postcalving from concentrate feeds high in rapidly fermentable carbohydrates. Therefore, although this may be a valid reason for provision of NSC in the cow diet prepartum, the decision to feed on this basis must be made with each farm individually.

Rumen Papillae Development

Feeding concentrates precalving is often recommended to enhance rumen papillae development, because research results indicated greater papillae development on a high-concentrate diet compared with a high-forage diet. However, the forage evaluated in the study most often cited was low-digestibility straw.

The development of papillae is induced and maintained by chemical stimuli, primarily volatile fatty acids. Therefore, comparing a poorly digestible forage, such as straw, with a highly digestible concentrate does not provide a true reflection of the benefits of altering dietary SC: NSC on papillae development. Rumen papillae development is most influenced by ruminal butyrate production and not the production of acetate or propionate. Pasture in winter and spring is highly digestible, producing similar quantities of ruminal butyrate compared with cows consuming pasture and large amounts of concentrates. Therefore no improvement in papillae development should be expected from offering concentrates to cows consuming highly digestible forages. Although there has been no research undertaken to validate this hypothesis, it is consistent with the lack of a milk production response or evident metabolic effect postcalving, from altering dietary SC: NSC ratio in the precalving diet.

Provision of More Readily Available Nutrients for the Fetus – Sparing Maternal Nutrient Reserves for Milk Production

The feeding of concentrate feeds precalving is by no means a new phenomenon, with its origins dating back to the recommendations of Boutflour in 1943. However, with the increased understanding of fetal and lactogenic requirements for glucose during the final weeks prepartum, and the increased interest, in general, in the transition dairy cow, there has been a renewal of the recommendations for increased NSC in the prepartum diet.

A 400% increase in mammary uptake of glucose in the week preceding parturition, as well as the continued rapid development of the fetus, has been reported. It, therefore, appears logical that there would be an advantage to replacing SC with NSC during late gestation. However, this hypothesis is not necessarily supported by experimental results. Although there are some studies in which altering SC: NSC ratio in the precalving diet affected postcalving health and production, the effect has been small and the experimental design was generally confounded by energy intake and, therefore, calving condition. A positive effect of supplementary feeding precalving on subsequent milk yield has been reported; however, it is acknowledged that the effect was greater with low-energy basal rations. Consistent with this premise, a study comparing isoenergetic diets differing in their SC to NSC content reported no metabolic or production effects of precalving dietary carbohydrate type (**Figure 2**); postcalving SC: NSC ratio also did not have any effect. These results are consistent with those reported in cows offered a TMR in confinement. In addition to the lack of effect on production

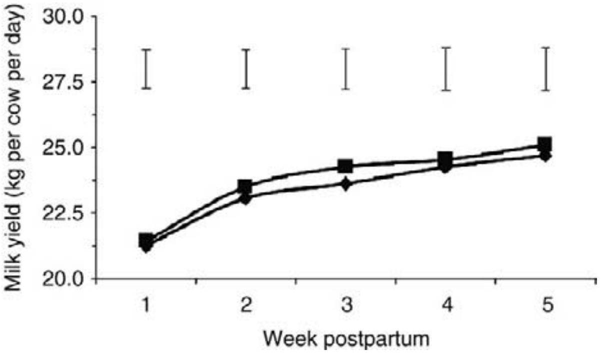

Figure 2 Milk yield (kg per cow per day) from cows consuming isoenergetic diets with either a high (■) or a low (◆) dietary structural carbohydrate: nonstructural carbohydrate ratio. From Roche JR, Lee JM, Aspin PW, *et al*. (2006) Supplementation with concentrates either pre- or post-partum does not affect milk production when diets are iso-energetic. *New Zealand Society Animal Production* 66: 416–422.

parameters, there was also no evident metabolic difference between cows receiving pasture and pasture silage precalving and those receiving a TMR.

Conclusion

It can be inferred from the majority of published research studies that there is no advantage to manipulating SC:NSC ratio in the prepartum ration, and that any reported advantage is a result of confounding effects of energy intake.

Feed Additives, Minerals, and Micronutrients

It is probably in the area of feed additives, minerals, and micronutrients that there is most confusion around the requirements of the transition cow. This confusion largely stems from a lack of interventionist research (as opposed to epidemiological profiling), but also the use of inductive reasoning to justify a position about a particular product – for example, many micronutrients and feed additives are purported to enhance gluconeogenesis and that this will benefit the cow through reduced risk of ketosis and maintenance of maternal body reserves for subsequent milk production. However, there is little or no evidence to support these claims. For example, prepartum supplementation with ionophores has been reported to reduce the circulating concentrations of ketone bodies peripartum, but it has not been reported to improve dairy cow health or production postcalving. Until there is published evidence in reputable scientific journals that such benefits are consistently attainable, such products should be avoided.

Macrominerals

The primary consideration in manipulating precalving dietary macromineral concentrations is for the maintenance of peripartum eucalcemia and the prevention of milk fever. The most important macromineral for the pasture-based transition cow is magnesium, although calcium, sulfur, and phosphorus must also be considered.

Magnesium is essential in the production of parathyroid hormone and the renal activation of vitamin D, both essential hormones in maintaining eucalcemia. In support of this critical role of magnesium in preventing periparturient hypocalcemia, hypomagnesemic cows are less able to reabsorb calcium renally and are slower to initiate bone calcium resorption. Magnesium supplementation is even more important in pasture-based systems because of the hypomagnesemic challenges of surplus dietary potassium and nitrogen (**Disease of Diary Animals**: Non-Infectious Diseases: Grass Tetany). This was confirmed by a study that reported a consistent drop in the incidence of milk fever (from approximately 10% per year to 5% per year) when precalving magnesium supplementation was introduced.

In addition to supplying important magnesium, magnesium sulfate has been reported to reduce the risk of peripartum hypocalcemia more effectively than other forms of magnesium, highlighting the importance of dietary sulfur in the precalving diet (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever). This effect of sulfur is, at least in part, independent of sulfur's importance in manipulating the dietary cation–anion difference (DCAD). This is consistent with previously published meta-analyses.

Precalving dietary potassium content is also an important factor in maintaining peripartum eucalcemia. Potassium acts locally in the rumen, to reduce magnesium absorption (**Disease of Diary Animals**: Non-Infectious Diseases: Grass Tetany), and systemically, to increase DCAD and blood alkalosis (**Feed Ingredients**: Feed Supplements: Anionic Salts), and in both ways reduces the effectiveness of peripartum eucalcemic mechanisms. The effect of potassium on plasma magnesium concentrations can be overcome through greater magnesium supplementation (link to Magnesium Chapter), increasing the amount of magnesium absorbed passively. However, in situations where this does not rectify the milk fever problem, high-potassium feeds can be replaced with low-potassium alternatives and/or magnesium chloride and magnesium sulfate can be used as primary magnesium sources to counter the alkalizing effect of dietary potassium.

Pasture tends to have reasonable concentrations of dietary calcium and phosphorus (ratio of 1:1 to 1.5:1). Although there is some evidence of a lowering of the milk fever risk at very high dietary calcium concentrations, this should only be attempted as a last resort. However, supplementing pasture-based dairy cows with calcium (ground limestone) during the first four milkings postcalving has been reported to reduce the risk of clinical and subclinical hypocalcemia significantly.

Dietary Cation–Anion Difference

A comprehensive review of DCAD is given in. However, the farm system implications for the concept are worthy of discussion here.

Grazing dairy cows are offered feeds varying substantially in their mineral concentrations daily, and within the day they select pastures that also differ significantly in their mineral concentration. It is, therefore, impossible to predict the DCAD consumed by grazing dairy cows – whether day to day or hour to hour. This makes manipulating DCAD for milk fever prevention a very difficult task.

Recent meta-analyses acknowledge the quadratic nature of the blood and urine pH response to alterations in the concentration of metabolically strong ions in the diet (link to DCAD Chapter), with urine and blood pH declining significantly only when DCAD is less than 15 meq per 100 g DM. However, they claim a linear effect on milk fever. This may be an artifact of treating DCAD as a continuous variable, as no effect of DCAD on urinary excretion of calcium (a proxy measure for calcium absorption) has been reported until DCAD was also less than 15 meq per 100 g DM. There may be a non-DCAD sulfur-related effect from supplementing certain anionic salts above this DCAD, but this is not an effect of reducing DCAD.

In general, it is very difficult to reduce DCAD below 15 meq per 100 g DM in pasture-based systems because of the very high potassium concentration in the base feed ingredient. In addition, as mentioned previously, it is impossible to verify if the diet consistently below this threshold mark. Therefore, in general, this is not a technique of much practical use for pasture-based dairy systems, but may have applicability in situations where low-potassium feeds are employed to reduce DCAD consistently, such that anionic salts can be supplied in practically relevant amounts.

Micronutrients

Although cows grazing fresh forages tend to receive all of the required vitamins, dietary copper, cobalt, iodine, selenium, and zinc often tend to be insufficient for optimal health. These need to be supplemented for at least 2 weeks precalving and preferably throughout early lactation (*see* **Feed Ingredients**: Feed Supplements: Microminerals).

Conclusion

Pasture-based cows require magnesium supplementation during the transition period to minimize the risk of hypocalcemia and associated disorders. In addition to this, calcium must be supplemented, at least during the 4–8 milkings immediately postcalving. This will reduce the risk of milk fever. If milk fever remains a problem, energy intake should be restricted to between 50 and 75% of estimated requirements during the 2 weeks preceding calving and attempts made to reduce the potassium content of the diet. As a last resort, precalving calcium supplementation may be considered.

Pasture-based cows will generally have a sufficient intake of vitamins, but will sometimes be deficient in cobalt, copper, iodine, selenium, and zinc, although the degree of deficiency (and necessary supplementation) will vary across farms. Other feed additives should generally be disregarded, unless they are consistently proven to work.

Conclusion

Although it should be acknowledged that the transition period across calving is important, there is little need to complicate it unduly in pasture-based systems, where the prepartum and postpartum diets are not dramatically different from each other.

Cows require 1.05 MJ ME per $kg^{0.75}$ BW per day in the month precalving to maintain BCS, but there is evidence that DMI and liver metabolic function may be enhanced by subjecting a cow to a NEBAL during the 2–3 weeks prior to calving.

Cows' feed should be supplemented with magnesium, cobalt, copper, iodine, selenium, and zinc prepartum, assuming that the diet is lacking in these trace elements, and with calcium during at least the first 4–8 milkings after calving. Other micronutrients and feed additives are unlikely to be beneficial or profitable.

See also: **Disease of Diary Animals**: Non-Infectious Diseases: Grass Tetany; Non-Infectious Diseases: Milk Fever. **Feed Ingredients**: Feed Supplements: Anionic Salts; Feed Supplements: Microminerals. **Milk Salts**: Trace Elements, Nutritional Significance.

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FERMENTED MILKS

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Types and Standards of Identity

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Introduction

Originally, the primary function of fermenting milk was to extend its shelf life. With this came numerous advantages, such as an improved taste and enhanced digestibility of the milk, as well as the manufacture of a wide variety of products. Historically the fermentation of milk can be traced back to around 10 000 BC. It is likely that fermentation initially arose spontaneously from indigenous microflora found in milk.

Fermented milks have been an important component of nutrition and diet. In addition to yogurt, there are numerous types of fermented milks manufactured in different parts of the world. Throughout the world, around 400 names are applied to traditional and industrially made fermented milk products. In general, the different types of fermented milks tend to be classified according to the methods of fermentation and/or processing, which are related to the microorganisms involved. Each type of fermented milk involves specific microorganisms, based primarily on the optimum growth requirements of the starter cultures (i.e., mesophilic and thermophilic microflora); however, there are strong similarities between manufacturing technologies used.

General Features

Milk fermentations generally involve the metabolism of lactose to lactic acid, a characteristic common to all fermented milks, by lactic acid bacteria (LAB), mostly lactococci and lactobacilli.

Fermented milk is obtained by fermentation by suitable microorganisms resulting in reduction of pH with or without coagulation. These starter microorganisms should be viable, active, and abundant in the product to the date of minimum durability. If the product is heat-treated after fermentation, the requirement for viable microorganisms does not apply.

As long as the starters are handled correctly, and added to the milk of good quality, and the manufacturing technology is adhered to, fermentation will produce a stable distinctive fermented milk product. Standard processes are now available for most types of fermented milks.

The basic identity of each type of fermented milk results from both the specific microorganisms and the process conditions providing suitable environment for the microorganisms concerned. Basic metabolism of the species or groups of species plays an important role in supplying metabolites that ensure a complex range of chemical interactions, producing numerous functional properties such as preserving ability, flavor as well as texture development, and thus contributing to the sensory quality of fermented milk, which in turn improves the nutritional value.

Careful selection of species is important in maintaining the identity of each fermented milk product, with temperature as an essential factor. **Table 1** shows examples of established types of fermented milk, with the microorganisms used as starter cultures.

Types of Fermented Milks

Fermented milk products are classified into three different types: (1) products of lactic fermentation, where strains of mesophilic or thermophilic LAB are used; (2) products obtained through alcohol–lactic fermentation, involving yeast and LAB; and (3) products where, in addition to fermentation of type (1) or (2), there is mold growth. All products are the result of fermentation of lactose into mainly lactic acid.

Lactic Fermentation of Milk

There are two types of lactic fermentation based on the incubation temperature, namely, mesophilic fermentation, employing mesophilic starters, and thermophilic fermentation, involving thermophilic starters, composed solely of LAB.

Cultured buttermilk, cultured cream, 'lactofil', 'filmjölk', nordic ropy milks, 'ymer', 'shrikhand', and 'chakka'

Table 1 Some established types of fermented milk and their starter cultures

Product/origin	*Starter organisms*
Acidophilus milk	*Lactobacillus acidophilus*
Biogarde^{R}	*Streptococcus thermophilus* *Lb. acidophilus* *Bifidobacterium bifidum*
Bioghurt^{R}	*Sc. thermophilus* *Lb. acidophilus*
Bifighurt^{R}	*Bif. bifidum* *Sc. thermophilus*
Buttermilk (Bulgarian)	*Lb. delbrueckii subsp. bulgaricus*
Buttermilk (cultured)	Commercial butter starter or a mixture of: *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive *Lc. lactis*, and *Leuconostos mesenteroides* subsp. *cremoris*
Cultura	*Lb. acidophilus* *Bif. bifidum*
Cultured cream	As for cultured buttermilk, but usually without *Leuconostoc* spp.
Dahi	*Sc. thermophilus* *Lb. delbrueckii* subsp. *bulgaricus* or *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive *Lc. lactis* (the final choice depends on the country of manufacture)
Dadih	*Lb. casei* subsp. *casei*, *Ln. paramesenteroides*, *Lb. plantarum*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive *Lc. lactis Enterococcus faecium* (depends on the place of manufacture)
Filmjölk	*Lc. lactis* subsp. *lactis* citrate-positive *Lc. lactis* *Ln. mesenteroides* subsp. *cremoris*
Kefir	*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive *Lc. lactis*, *Ln. mesenteroides* subsp. *cremoris*, *Ln. mesenteroides* subsp. *dextranicum*, *Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, *Lb. helveticus*, *Lb. kefir*, *Lb. kefiranofaciens*, *Kluyveromyces marxianus*, *Saccharomyces* spp.
German kefir	*Lc. lactis* subsp. *lactis*, citrate-positive *Lc. lactis* subsp. *cremoris*, *Sc. thermophilus*, *Lb. acidophilus*, *Lb. lactis*, *Lb. brevis*, *Candida kefyr*
Kumys	*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Saccharomyces lactis*, *Torula koumiss*
Tätmjölk	As for Filmjölk
Viili	As for Filmjölk, plus *Geotrichum candidum*
Yakult	*Lb. casei* subsp. *casei*
Yogurt (including labneh and related products)	*Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*

Adapted from Kurmann JA and Rasic JLj (1988) Technology of fermented special products. In: *Fermented Milks Science and Technology*. Bulletin of the IDF No. 227; Lee YK and Wong SF (1993) Stability of lactic acid bacteria in fermented milk. In: Salminen S and Wright A von (eds.) *Lactic Acid Bacteria*, pp. 103–114. New York: Marcel Dekker, Inc.; Tamime AY and Robinson RK (1999) *Yoghurt. Science and Technology*, 2nd edn. Cambridge, OK. Woodhead Publishing Limifed.

are the types of products obtained through mesophilic fermentation of milk, while yogurt, 'laban', 'zabady', 'labneh', 'skyr', 'yakult', acidophilus milk, and Bulgarian buttermilk represent products obtained as a result of thermophilic fermentation of milk.

Mesophilic fermentations

Cultured buttermilk

Cultured buttermilk, a low-acid fermented milk, is a pasteurized skim milk fermented by a lactic culture and by aroma-producing bacteria. High-quality cultured buttermilk has mild acid flavor with an aromatic diacetyl overtone and a smooth viscous body and texture. Its appearance is soft white, without gas holes or whey separation, and the product remains fresh for at least 10 days at 5 °C. *Lactococcus lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* are responsible for acid production, while citrate-positive strains of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris* are the primary sources of flavor and aroma, because of their ability to produce diacetyl, an important volatile compound that is critical for cultured buttermilk quality, giving the product its characteristic buttery flavor and aroma.

Most cultured buttermilks are made with mixed cultures as shown in **Table 1**. Milk is fermented at 22 °C until a titratable acidity of 0.9% is reached. Incubation at 22 °C is necessary to enable both species of starter culture to produce the desirable characteristics of cultured buttermilk. Incubation at a higher temperature would favor the growth of *Lc. lactis* subsp. *lactis*, resulting in excess acid production, and diminish aroma production by *Ln. mesenteroides* subsp. *cremoris*. Buttermilks are most popular in Germany and Scandinavian countries. See **Fermented Milks**: Buttermilk.

Cultured cream

Cultured cream or sour cream, a low-acid fermented milk, is an extremely viscous product with the flavor and aroma of buttermilk. The final pH value of a freshly produced sour cream is about 4.5. It normally contains not less than 18% butterfat. The functions of the starter cultures are the same as in cultured buttermilk, dominated by *Lc. lactis*. The incubation temperature is 21–24 °C.

Nordic (Scandinavian) fermented milks

Characteristic types of Nordic fermented milks are noted for their high viscosity and ropiness. They owe these properties to the vigorous growth of capsule-forming lactococci, mainly *Lc. lactis* subsp. *cremoris*. If one applies a needle or a spoon to the milk surface, long strings will appear when the needle or spoon is lifted. The ropy character of these fermented milks is derived from the formation of exopolysaccharides formed by the lactococci involved in fermentation. An example of a traditional product of this type is 'långfil', also referred to as 'tätmjölk', produced in Sweden. It has a mild, sour taste and a ropy consistency. A similar fermented milk traditionally made in Norway is called 'tettemelk'.

'Filmjölk', developed in Sweden in the early 1930s, is a sour milk used as a drink, often consumed with meals. It contains 3.0% fat and it has a characteristic flavor and aroma, and a relatively high viscosity.

'Ymer' (Denmark) and 'lactofil' (Sweden) are concentrated after fermentation by removal of a fixed percentage of whey. Both these products are made with nonropy culture. (see **Fermented Milks**: Nordic Fermented Milks).

Thermophilic fermentations

Yogurt

Yogurt, a medium-acid fermented milk, is fermented by symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* characterized by a smooth, viscous gel with a delicate walnutty flavor. The two yogurt bacteria reside either naturally in the milk (in the regions where yogurt is traditionally made) or are added as starter cultures at 2–5% inoculum, preferably at a 1:1 ratio. Incubation is at 42–45 °C for 3–6 h until pH 4.4 and 0.9–1.2% titratable acidity is reached.

The delicate flavor of plain yogurt is achieved through a protocooperation relationship between rods and cocci, which is influenced by factors such as incubation temperature and acid concentration. *Lactobacillus delbrueckii* subsp. *bulgaricus* produces amino acids and peptides required by *Sc. thermophilus* as growth factors, while folate is produced by *Sc. thermophilus* to support the growth of *Lb. delbrueckii* subsp. *bulgaricus*.

Volatile compounds include small amounts of acetic acid, diacetyl, and acetaldehyde produced by *Lb. delbrueckii* subsp. *bulgaricus*, which contributes much to the unique flavor of yogurt. Heat treatment of yogurt after fermentation is permitted in most countries. Alternative nutritive sweeteners such as aspartame are also permitted. Based on the method of production, there are two types of yogurt, namely, set and stirred yogurt.

Yogurt and yogurt-like products are made widely in the Mediterranean area, Asia, Africa, and central Europe. Synonyms for yogurt or related fermented milks throughout various countries are shown in **Table 2**. 'Zabady' or Egyptian yogurt is traditionally made from sheep's milk. 'Dahi' and 'dadih' are Indian and Indonesian yogurts, respectively. Buffalo milk is often used in the manufacture of these products, sometimes in combination with bovine milk. See **Fermented Milks**: Asian Fermented Milks; Middle Eastern Fermented Milks; Yogurt: Role of Starter Culture; and Yogurt: Types and Manufacture.

Bulgarian buttermilk

Bulgarian buttermilk, a high-acid fermented milk, is made by inoculating *Lb. delbrueckii* subsp. *bulgaricus* alone (at 2%

Table 2 Yogurt-related fermented milks in various countries

Synonym	*Country*
Dahi	India
Dadih	Indonesia
Katyk	Kazakhstan
Laben, leben	Iraq, Lebanon, Egypt
Laben, rayeb	Saudi Arabia
Mast	Iran, Iraq, Afghanistan
Matzoon, madzoon	Armenia
Roba, rob	Egypt, Sudan, Iraq
Tarho	Hungary
Tiaourti	Greece
Yaourt	Russia, Bulgaria
Zabady, zabade	Egypt, Sudan

inoculum) into pasteurized whole milk and incubating at 38–42 °C for 10–12 h, until a curd forms with about 1.4% titratable acidity, which gives it a sharp acidic flavor. This product is popular only in Bulgaria.

Acidophilus milk

Acidophilus milk is cultured with *Lactobacillus acidophilus*, whose primary function is to produce lactic acid from lactose. Moreover, *Lb. acidophilus* is considered to be a probiotic bacterium, and has been claimed to confer various nutritional and health benefits on consumers. An ability to grow in the presence of acid and bile acids enables it to survive in the intestinal tract.

Lactobacillus acidophilus grows only slowly in milk and therefore it is essential to maintain the inoculum's activity by daily transfers of mother culture; this will help to ensure consistent results. Acidophilus milk is fermented at 38 °C with the inoculum of 2–5% active culture, until a curd forms (which usually happens after 18–24 h). The final product contains 1.5–2.0% lactic acid but no alcohol. It is cooled to 10 °C before agitation and pumped to a filler where it is filled into bottles or cartons. In some countries, doctors recommend acidophilus milk to patients with various gastrointestinal tract disorders including constipation, nonulcerative colitis, and diarrhea. See **Fermented Milks**: Health Effects of Fermented Milks.

Yakult

Yakult is renowned in Japan mainly on account of claims of its health-promoting properties. It is made with *Lactobacillus casei* subsp. *casei* strain Shirota, which appears to remain viable in the intestinal tract of humans and is also a probiotic bacterium.

The milk solids content in yakult is 3.6%, but it has 16% added sucrose. Yakult is a Drink based on Fermented Milk, consisting of 40% fermented milk, which is fermented by a single-strain culture. The final product contains 10^8 cfu ml^{-1} viable *Lb. casei* subsp. *casei* strain Shirota, and the protein content is 1.08%. See **Fermented Milks**: Asian Fermented Milks and Health Effects of Fermented Milks.

Probiotic fermented milks

Probiotic fermented milks are made with specific strains of various probiotic bacteria, including bifidobacteria. Some strains of *Lb. acidophilus*, *Lb. casei*, and *Bifidobacterium* spp. are the most commonly used probiotic bacteria in the manufacture of fermented milks. These and some other microorganisms are thought to confer health and nutritional benefits, through their activity in the intestinal tract. The traditional yogurt starter cultures *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* do not grow in the intestinal tract. However, they do improve the utilization of lactose in persons who are lactose maldigestors, and some probiotic fermented milks are processed in a similar manner to yogurt, fermented by yogurt starter cultures enriched with probiotic bacteria.

The number of fermented milks made with probiotic microorganisms has increased markedly over the past few decades. Such fermented milks are usually made with more than one organism although products made with single-strain cultures are also known.

In Germany for example, fermented milks made with probiotic bacteria include 'bifighurt', 'bioghurt', and 'biogarde', while a product called 'cultura', made with *Bifidobacterium bifidum* and *Lb. acidophilus*, originated in Denmark. Often, fermented milks presently manufactured in various regions and countries reflect local traditions of homemade fermented milks.

The success of fermented milk products relates to nutrition and health, versatility, and marketing. Scientific and clinical evidence is also mounting to support the consumer perception of health from fermented milks. See **Bacteria, Beneficial:** Probiotics, Applications in Dairy Products and **Fermented Milks:** Health Effects of Fermented Milks.

Yeast–Lactic Fermentations

Kefir

Kefir, an acidic and alcoholic fermented milk, has a sour cream-like consistency and a distinct flavor. This foamy, effervescent drink containing 0.9–1.1% lactic acid and 0.3–1.0% alcohol has achieved great popularity in eastern Europe, but its consumption is limited elsewhere, which is in part due to problems with packaging and distribution (if low temperature is not maintained, yeasts present in kefir will continue to produce alcohol and carbon dioxide during storage).

Kefir is usually made using whole milk by subjecting it to severe heat treatment (95 °C for 5 min) to denature the whey proteins and therefore improve the viscosity of the end product. The so-called kefir grains, a mixture of bacteria and yeasts, constitute the inoculum. Starter culture is prepared from kefir grains, *Lactobacillus kefiri*, and species of the genera *Leuconostoc*, *Lactococcus*, and *Acetobacter* growing in a strong specific relationship. Kefir grains constitute both lactose-fermenting yeasts (*Kluyveromyces marxianus*) and non-lactose-fermenting yeasts (*Saccharomyces unisporus*, *Saccharomyces cerevisiae*, and *Saccharomyces exiguus*).

Incubation temperature for the kefir fermentation is 18–22 °C. After approximately 20 h, the product will contain up to 0.8% ethanol and 1.0% carbon dioxide. Fresh kefir is often held for several hours to ripen, which improves viscosity and stability of the coagulum. See **Fermented Milks**: Kefir.

Kumys

Kumys, another fermented milk containing acid and alcohol, is traditionally made from mares' milk. This product is a result of the fermentation of milk by *Lb. delbrueckii* subsp. *bulgaricus* and lactose-fermenting yeasts, for example, *Saccharomyces lactis* and *K. marxianus*. Carbon dioxide imparts frothy appearance to the product and contributes to flavor.

Starter culture for kumys, which may include *Lb. acidophilus*, is added at a rate of 10–30% at 26–28 °C, to give an initial acidity of 0.45% lactic acid, and is incubated further. Depending on the duration of fermentation, the final product may have (1) 0.6% lactic acid and 0.7% alcohol, (2) 0.8% lactic acid and 1.1–1.7% alcohol, or (3) 1.0% lactic acid and 1.7–2.5% alcohol. See **Fermented Milks**: Asian Fermented Milks.

Acidophilus-yeast milk

Acidophilus-yeast milk is produced only in countries of the former USSR. Whole or skimmed milk is heated at 90–95 °C for 10–15 min, cooled to 35 °C, and inoculated with 3–5% mixed starter culture of *Lb. acidophilus* and *Sacch. lactis* at 35 °C, until 0.8% lactic acid and approx. 0.5% ethanol are produced. The product is described as viscous, with slightly acidic and yeasty taste.

Mold in Lactic Fermentations

Viili

Generally, fermented milks are not made with a mold as a component of the starter culture. An exception to this rule is the Finnish product viili. The starter culture includes citrate-positive *Lc. lactis* and *Ln. mesenteroides* subsp. *cremoris*, together with a mold, *Geotrichum candidum.*

The milk, which contains 2.5–3.9% fat, is inoculated with starter cultures at 18–21 °C, and incubated to reach a final acidity of 0.9% lactic acid. The ropy character of viili is derived from the formation of capsules by lactococci. (*see* **Fermented Milks**: Nordic Fermented Milks).

Standards of Identity

The International Dairy Federation (IDF) published general standards of identity for fermented milks, which could be briefly described as follows: Fermented milks are prepared from milk and/or milk products (e.g., any one or combinations of whole, partially or fully skimmed, concentrated or powdered milk, buttermilk powder, concentrated or powdered whey, milk protein (such as whey proteins, whey protein concentrates, soluble milk proteins, edible casein and caseinates), cream, butter or milk fat – all of which have been manufactured from raw materials that have been at least pasteurized) by the action of specific microorganisms, which results in a reduction of the pH with and without coagulation.

Codex Alimentarius Commission (CAC) and its committees, together with the IDF, have established regulations of international standards for fermented milks, Codex Standard for Fermented Milks (Codex Stan 243-2003), describing fermented milk, concentrated fermented milk, and flavored fermented milks. Since drinkable fermented milk products such as yakult that existed in the market were not covered by the current standards of identity, proposed draft amendment to the Codex Standard for Fermented Milks pertaining to drinks based on fermented milk is currently being developed, proposed to have a minimum content of 40% fermented milk, with a minimum protein content of 1.08% and a minimum titratable acidity of 0.1% (titratable acidity expressed as percent lactic acid (% w/w)).

Fermented milks with a minimum protein content of 2.7% (**Table 3**) are usually classified into set yogurt, stirred yogurt, alternative culture yogurt, cultured buttermilk, cultured cream products, kefir, kumys, and fermented special products. Each country has its own standards for milk as raw material, starter culture, manufacturing procedures, quality requirements, and legal requirements.

In the United States, the Code of Federal Regulations (CFR) of the Food and Drug Administration sets the standards of identity for yogurt. Three categories are listed, namely, yogurt, low-fat yogurt, and nonfat yogurt (**Table 4**).

Heat treatment after fermentation of yogurt results in an extension of shelf life, but kills the yogurt cultures. IDF Standard 47 defines fermented milks as products with no heat treatment after the fermentation. The current Codex Alimentarius trend is to differentiate between live product and heat-treated product by clear labeling, for example, 'contains no active cultures', for the consumer information. IDF provides a recommended method for the enumeration of total viable count in yogurt. The total viable count is proposed to be 10^8 cfu g^{-1} yogurt at the time of manufacture, and Codex Standard for Fermented Milks (Codex Stan

Table 3 Minimum composition types and standard of identity of fermented milks

	Fermented milk	*Yogurt, alternate culture yogurt, and Acidophilus milk*	*Kefir*	*Kumys*
Milk protein[a]–(% w/w)	Min. 2.7%	Min. 2.7%	Min. 2.7%	
Milk fat (% w/w)	Less than 10%	Less than 15%	Less than 10%	Less than 10%
Titrable acidity, expressed as percent lactic acid (% w/w)	Min. 0.3%	Min. 0.6%	Min. 0.6%	Min. 0.7%
Ethanol (% v/w)				Min. 0.5%
Sum of microorganisms constituting the starter culture defined in section 2.1 ($cfu^{-1}g$ in total)	Min. 10^7	Min. 10^7	Min. 10^7	Min. 10^7
Labeled microorganisms[b] ($cfu^{-1}g$ total)	Min. 10^6	Min. 10^6		
Yeasts ($cfu^{-1}g$)			Min. 10^4	Min. 10^4

[a] Protein content is 6.38 multiplied by the total Kjeldahl nitrogen determined.

[b] Applies where a content claim is made in the labeling that refers to the presence of a specific microorganism (other than those specified in section 2.1 of Codex Standard for Fermented Milks CODEX STAN 243-2003 for the product concerned) that has been added as a supplement to the specific starter culture.

Source: Codex Standard for Fermented Milks CODEX STAN 243-2003.

Table 4 Composition standards for yogurt in the United States

Composition (%)	*Yogurt*	*Low fat yogurt*	*Nonfat yogurt*
Fat	>3.25	>0.5 to <2.0	<0.5
Protein	4.4	5.7	5.2
Carbohydrate	7.5	7.5	6.9
Milk solids nonfat	>8.25	>8.25	>8.25
Titratable acidity	>0.9	>0.9	>0.9

243-2003) defines the viable count of yogurt starter after the product has been stored under the storage conditions specified in the labeling at the end of expired date as a minimum $10^6\,cfu\,g^{-1}$ viable yogurt starter organisms (**Table 3**).

Many countries have their own standards of identity for yogurt and other fermented milk, normally based on fat and solids nonfat content and acidity. The FAO/WHO CAC has drafted standards for yogurt as partly skimmed and skimmed yogurt. Four yogurt types are included, namely, yogurt, flavored yogurt, sweetened yogurt, and product pasteurized after fermentation.

Concentrated fermented milk is a fermented milk in which the protein content has been increased prior to or after fermentation to a minimum of 5.6%. Concentrated fermented milk includes traditional products such as stragisto (strained yogurt), labneh, ymer, and ylette.

Flavored fermented milks are composite milk products, which contain a maximum of 50% (w/w) of nondairy ingredients (such as nutritive and nonnutritive sweeteners, fruits, and vegetables as well as juices, purees, pulps, preparations and preserves derived there from cereals, honey, chocolate, nuts, coffee, spices, and other harmless natural flavoring foods) and/or flavors. The nondairy ingredients can be mixed in prior to or after fermentation.

The current standards of identity for fermented milks concern milk fat, milk protein content, amount of starter cultures, a minimum live and active culture content of labeled microorganisms added as supplement to the specific starter cultures as well as titratable acidity expressed as lactic acid.

See also: **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products. **Fermented Milks**: Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Kefir; Middle Eastern Fermented Milks; Nordic Fermented Milks; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture.

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Starter Cultures

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Introduction

Starter cultures were unknown until 1878, when Lister isolated pure cultures of the lactic acid bacteria responsible for milk acidification. In the 1880s, improvements to standardization and ripening of butter were made by introducing pure starter cultures (Storch in Denmark, Conn in the United States, and Weigmann in Germany). During the period 1910–20, Orla-Jensen, von Freudenreich, Sherman, Hammer, and other dairy microbiologists prepared pure cultures for fermented milks. Commercial production and use of starter cultures grew rapidly and was widespread at the beginning of the twentieth century.

Dairy starters are the ‘heart’ of fermented milk products, the most crucial component in the manufacture of high-quality fermented milks. The cultures are harmless food-grade microorganisms, such as active bacteria, that are intentionally grown in milk or whey or other formulated media to impart desirable and predictable flavor and texture to fermented milk products.

The microorganisms employed in milk fermentation are single-strain or multiple-strain cultures of lactic acid bacteria, producing different types of fermented milk products. Usually, one or two strains dominate the milk environment. Individual strains can be selected in advance for their resistance to both bacteriophages (phage) and antibiotics. Mixed starter cultures are used to ensure that fermentation will continue after a bacteriophage attack. Bacteriophages are highly strain specific, and if the dominant strain in a mixed-strain starter culture succumbs to an attack, the phage-resistant mutant or the next dominant culture maintains a satisfactory rate of lactic acid production.

Performance indicators of starter cultures include (1) adaptation to various manufacturing conditions, (2) rapid acid production in the vat, (3) minimal acid production during distribution and storage, (4) maintenance of viability during the shelf life of fermented milk, and (5) typical flavor, body, and texture formation.

Strains are selected for the rate of growth and lactic acid production, aroma and/or carbon dioxide production, resistance to phage, ability to produce viscous or ropy fermented milk, ability to maintain desirable ratios of component organisms, and viability during preparation of starter culture, preservation steps, storage, and distribution.

Starter cultures should contain the highest possible number of viable microorganisms, be highly active under production conditions, and be free from contaminants. The fermentation process of any cultured dairy product relies entirely on the purity and activity of the starter culture. In addition, milk or growth medium should not contain inhibitory agents, such as antibiotics and bacteriophage.

Types of Starter Cultures

Diverse lactic starter cultures are used in the manufacture of commercial fermented milk products in the world (**Table 1**). They can be classified into mesophilic cultures, which grow best at 25–30 °C, and thermophilic cultures, which grow at higher temperatures (37–45 °C).

Mesophilic Starter Cultures

Mesophilic cultures are widely used in the fermented milk industry (**Table 2**) in the manufacture of products such as ‘filmjölk’ and ‘lactofil’ (in Sweden) and ‘ymer’ (in Denmark). Mesophilic starters will almost certainly contain *Lactococcus lactis* subsp. *cremoris*, but rarely will this species be used alone. Buttermilk, ‘långfil’, and ‘viili’, which are popular in Norway, Sweden, and Finland, combine *Lc. lactis* subsp. *cremoris* with *Leuconostoc* species. Other fermented milk products made with mesophilic starters include sour cream, cultured buttermilk, and kefir (*see* **Lactic Acid Bacteria**: *Lactococcus lactis*; *Leuconostoc* spp.).

Thermophilic Starter Cultures

Thermophilic starter cultures (**Table 3**) are used for the manufacture of yogurt, Bulgarian buttermilk, and the whole range of products made with intestinal bacteria, primarily lactobacilli and bifidobacteria. Thermophilic cultures are classified into two main types: artisanal

Table 1 Primary starter cultures used for milk fermentations

Mesophilic	*Thermophilic*
Lactococcus lactis subsp. *lactis*	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Lc. lactis subsp. *cremoris*	*Streptococcus thermophilus*
Lc. lactis subsp. *lactis* biovar *diacetylactis*	*Lb. helveticus*
Lactobacillus kefir	*Lb. acidophilus*
Lb. casei	*Lb. paracasei* subsp. *paracasei*
Leuconostoc spp.	*Bifidobacterium* spp.

Table 2 Microbiological and biochemical attributes of typical mesophilic lactic acid bacteria used in fermented milks

Characteristic	Lactococcus lactis *subsp.* lactis	Lactococcus lactis *subsp.* cremoris	Lactococcus lactis *subsp.* lactis *biovar* diacetylactis	Leuconostoc mesenteroides *subsp.* cremoris	Leuconostoc mesenteroides *subsp.* dextranicum
Cell shape and configuration	Cocci, pairs, short chains	Cocci, pairs, short/long chains	Cocci, pairs, short chains	Cocci, pairs, short/long chains	Cocci, pairs, chains
Catalase reaction					
Growth temperature (°C)					
Optimum	28–31	22	28	20–25	20–25
Minimum	8–10	8–10	8–10	4–10	4–10
Maximum	40	37–39	40	37	37
Incubation temperature (°C)	21–30	22–30	22–28	22	22
Heat tolerance (60 °C for 30 min)	±	±	±		
Lactic acid isomers	L(+)	L(+)	L(+)	D(−)	D(−)
Lactic acid produced in milk (%)	0.5–0.7	0.5–0.7	0.5–0.7	0.1–0.2	0.1–0.2
Acetic acid production (%)				0.2–0.4	0.2–0.4
Gas (CO_2) production			+	±	±
Proteolytic activity	+	+	+	±	±
Lipolytic activity	±	±	±	±	±
Citrate fermentation			+	+	+
Flavor/aroma compounds	+	+	+++	+++	+++
Mucopolysaccharide production	±	±	±	No dextran from sucrose	Dextran from sucrose
Hydrogen peroxide production	+	+	+	±	±
Alcohol production	±	±	±	±	±
Salt tolerance (% max.)	4–6.5	4.0	4–6.5	6.5	6.5

Adapted from Heller KJ (2001) Probiotic bacteria in fermented foods: Product characteristics and starter organisms. *American Journal of Clinical Nutrition* 73(supplement): 374S–379S; Pettersson HE (1988) Starters for fermented milks. 2. Mesophilic starter cultures. *International Dairy Federation Bulletin* 20–28.

starters consisting of undefined strains, and the defined starters. Examples of the defined thermophilic starter culture systems are (1) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, where growth and acid production are enhanced owing to the protocooperative relationship between these two species, (2) *Lb. acidophilus* used for the production of acidophilus milk, and (3) *Lb. paracasei* subsp. *paracasei* for the production of yakult (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Streptococcus thermophilus*).

Manufacture of Starter Cultures

The traditional method for the production of bulk starter commences with stock culture, liquid, freeze-dried, or frozen at −196 °C, which is inoculated and prepared in a small volume (approximately 100 ml of

Table 3 Characteristics of thermophilic starters

Characteristic	Streptococcus thermophilus	Lactobacillus delbrueckii *subsp.* bulgaricus	Lactobacillus acidophilus	Lactobacillus casei *subsp.* casei	Bifidobacterium bifidum
Cell shape and configuration	Spherical to ovoid, pairs to long chains	Rods with round ends, single, short chains, metachromatic granules	Rods with round ends, single, pairs, short chains, no metachromatic granules	Rods with square ends, short/ long chains	Curved rods with bifurcated ends (Y shaped); can also be multibranched
Catalase reaction					
Growth temperature (°C)					
Optimum	40–45	40–45	37	37	37
Minimum	20	22	20–22	15–20	22
Maximum	50	52	45–48	40–45	48
Incubation temperature (°C)	40–45	42	37	37	37
Heat tolerance (60 °C for 30 min)	++	+			
Lactic acid isomers	L(+)	L(−)	DL	L(+)	L(+)
Lactic acid produced in milk (%)	0.6–0.8	1.7–1.8	0.3–2.0	1.2–1.5	0.1–1.4
Acetic acid production (%)	Trace	Trace	+	+	+++
Gas (CO_2) production					±
Proteolytic activity	±	+	±	±	+
Lipolytic activity	±	±	±	±	±
Citrate fermentation					
Flavor/aroma compounds	++	++	+	±	++
Mucopolysaccharide production	±	++	−	±	++
Hydrogen peroxide production	±	+	+	+	+
Alcohol production		Trace	Trace	Trace	Trace
Salt tolerance (% max.)	2.0	2.0	6.5	2.0	2.0

Adapted from Heller KJ (2001) Probiotic bacteria in fermented foods: Product characteristics and starter organisms. *American Journal of Clinical Nutrition* 73(supplement): 374S–379S; Dellaglio F (1988) Starters for fermented milks. 3. Thermophilic starters. *International Dairy Federation Bulletin* 227: 27–34.

starter growth medium) as 'mother culture', which in turn is subcultured daily into three or more bottles, with the best bottle selected for bulk starter production. This is followed by inoculation, using 1% inoculum, into larger volumes of the growth medium, that is, into 'feeder' or intermediate culture, and finally into the bulk starter unit. This method is still widely used, even though the propagation procedure is time consuming, requiring skilled operators, and may expose the culture to bacteriophage infection, which is one of the major hazards in the industry.

Milk is the usual growth medium for bulk starters, but other media may also be used. These may contain nonfat milk powder, phosphate salts, sodium citrate, dextrose, dextrin, pancreatin, dried autolyzed yeast, lactose, and sucrose.

It is essential that starter cultures are preserved so that stock cultures are always available in case of starter failure. In addition, successive subculturing can lead to the emergence of mutant strains, which may alter the overall behavior and general characteristics of the starter. Too many transfers may result in the loss of certain functional plasmids such as Lac^- or Prt^-.

Starter cultures for fermented milk manufacture are also available in freeze-dried or frozen concentrated form, and either as direct-vat-set (DVS) type or as cultures for bulk starter production. The popularity of DVS type cultures is increasing. Application of DVS cultures eliminates the risk of phage contamination during starter preparation in the plant and ensures appropriate strain balance.

In the mid-1960s, vacuum-dried cultures were replaced by frozen concentrated cultures. Frozen concentrated cultures contain 10^{10}–10^{11} cfu g^{-1}, a sufficient concentration to allow 70 ml to inoculate 1000 l medium for the bulk culture preparation. Preparation of frozen concentrated cultures involves growing cultures under optimal pH conditions, harvesting cells by centrifugation or ultrafiltration, standardizing the cell suspension to a specified activity, adding a cryoprotectant (glycerol, monosodium glutamate, sucrose, lactose, or skim milk), packaging, rapid freezing in liquid nitrogen (−196 °C) or in a dry ice–ethanol mixture, and storing in a deep freeze. The pH of the cell concentrate should be 6.6 for lactococci and 5.4–5.8 for lactobacilli.

Frozen concentrated starters can be used daily or as stand-by or emergency starter stock. Bacteria in frozen starter cultures are immediately active on thawing. They do not go through the lag phase when added to the vat.

In 1982, a French culture company, Eurozyme, introduced a new type of DVS culture, concentrated and freeze-dried. It is available in aluminum pouches under nitrogen. Each package may contain a single strain or a blend of defined strains, which are individually propagated, concentrated, freeze-dried (to less than 3% moisture), and then blended to attain specified activity. Each pouch has a starter population of approximately 10^{11} cells g^{-1}, does not require deep freeze storage temperatures, and can be stored at 4 °C for 1 year without loss of activity.

The major disadvantage of using freeze-dried concentrate cultures is the longer lag phase in the vat, adding an additional 30–60 min to the time required to ferment milk.

It has been demonstrated that most lactic acid bacteria can be successfully preserved by freeze-drying, with the exception of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*. The optimum rehydration temperature for both mesophilic and thermophilic lactic acid bacteria is 20 °C.

Inhibition of Starter Cultures

A number of factors may adversely affect the activity of starters, leading to poor quality of fermented milks and financial losses to the manufacturer. When lactic acid is not produced by a starter culture at the desired rate, the culture is called 'slow'. The slowness can be due to either the genetic makeup of the strains or extrinsic factors. The latter include, among others, (1) bacteriophage, (2) residues of antibiotics and sanitizing agents, (3) inhibitory compounds naturally found in milk, (4) variations in milk composition due to mastitis or seasonal factors, and (5) metabolites of spoilage bacteria. A lower rate of acid production can also be caused by irregular culture transfers, by fluctuations in incubation temperature, and by overacidification.

Viability of a starter culture can be determined by the simple direct microscopic count on a methylene blue-stained slide. This method can also be used to assess the physiological state of the bacterial cells. The shape of old cells, cells exposed to excessive acidity and inhibitors, and those grown on solid media will be changed.

Various simple tests can be used to quantify the activity of starter cultures. The activity of yogurt starter bacteria can be measured by a decrease in pH or rise in titratable acidity of sterile 12% reconstituted skim milk incubated at 40 °C for 8 h. A pH of 4.2 and titratable acidity of 1.05% under the above conditions are expected.

Inhibiting Factors

Bacteriophages in the dairy plant come from lysogenic bacteria in the starter culture and may also originate on farms. It is not possible to eliminate the entry of phage into the dairy plant because raw milk continuously enters the facility. However, growth of the phage population can be controlled by effective sanitation.

In general, thermophilic starters are not affected as much as mesophilic starters. In recent times, phages of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* have been reported, with *S. thermophilus* being relatively more susceptible than *Lb. delbrueckii* subsp. *bulgaricus*. In yogurt production, fermentation is relatively fast – it may take only 3–4 h. It is unlikely that all components of the culture would be simultaneously attacked by phages. In case of a phage attack on one strain, acid production by the unaffected strain/s will continue, causing few or no problems in production. Measures employed to minimize the risk of phage attack include (1) the use of mixed and phage-unrelated cultures, (2) strict adherence to aseptic techniques, and (3) proper heat treatment of the starter growth medium (e.g., at 85 °C for 30 min).

Residues of antibiotics and sanitizers such as quaternary ammonium compounds, iodophors, hypochlorites, and hydrogen peroxide inhibit the growth of starter cultures. They may contaminate milk as a result of human error. Antibiotics are used in the treatment of mastitis, while detergents and disinfectants are used for cleaning and sanitation purposes. In practice, it is necessary to ensure that the rinsing cycle is long enough to wash down these chemicals from the bulk starter tank. Starters differ in their susceptibility to antibiotics and sanitizers.

Natural inhibitors present in raw milk, namely, lactenins, the lactoperoxidase/hydrogen peroxide/thiocyanate system (LPS), agglutinins, and lysozyme, are generally destroyed by proper heat treatment prior to manufacture. However, mesophilic starter cultures are inhibited by the LPS system if the heat treatment of milk is milder, for example, after high-temperature short-time (HTST) pasteurization.

Starter growth and acid production are slower in abnormal milk, for example, in milk from mastitic cows or in milk with high hydrolytic rancidity. Seasonal variations in milk composition resulting in lower micronutrients (trace elements, nonprotein nitrogenous compounds) may also affect starter performance.

Progressive inhibition of acid production and a decline in the rate of acid production by the culture have been observed in yogurts produced with the addition of sucrose, which raises osmotic pressure in the system. The acid-producing ability of yogurt culture is fairly normal in mixes containing 4–7% sucrose. Commercial strains that are relatively osmotolerant may allow higher usage levels without delays in acid production during yogurt manufacture (*see* **Bacteriophage**: Biological Aspects; Technological Importance in the Diary Industry).

Exopolysaccharide Formation by Bacteria

In the early 1950s, the first mucoid variants of bifidobacteria were reported, followed by the characterization of 'bifidan', an exocellular polysaccharide (EPS) from a mucoid nonencapsulated strain of *Bifidobacterium bifidum*.

Thermophilic starters of yogurt, both *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, can produce as much as 0.2% (w/w) of mucopolysaccharides after 10 days of fermentation. Likewise, the mesophilic lactic starter *Lc. lactis* subsp. *cremoris* is able to produce a capsular polysaccharide in the Swedish 'långfil', containing rhamnose, glucose, galactose, and glycerol.

Most of the polysaccharides produced in yogurt contain glucose and galactose along with minor quantities of fructose, mannose, arabinose, rhamnose, xylose, and *N*-acetylgalactosamine, individually or in combination. The polysaccharides form a network of filaments visible under scanning electron microscope. The bacterial cells are partly covered by polysaccharide and the filaments link the cells and milk proteins. The texture of yogurt results from a complex interaction between milk proteins, acid, and EPS produced, which may influence important physical properties such as firmness, smoothness, and viscosity, and gel stability or susceptibility to syneresis.

Ropy strains of yogurt starters are commercially available, and are particularly suitable for drinking yogurt and stirred yogurt production, contributing to smooth texture, higher viscosity, lower syneresis, and better tolerance of mechanical handling. The capsule also slows diffusion of lactic acid away from the cell, causing the cells to stop acid production sooner, which helps prevent overacidification of fermented milk. It is conceivable that some of the exopolysaccharides play a physiological role in the human digestive system because of their chemical structure resembling the fiber of grains and vegetables.

Bacteria that are known to produce EPS include *S. thermophilus, Lb. kefiranofaciens, Lb. helveticus, Lb. sake, Lb. delbrueckii* subsp. *bulgaricus, Lc. lactis* subsp. *cremoris, B. longum*, and *B. infantis*. Recent studies consider EPSs as having probiotic properties since they may contribute to human health either as a nondigestible food fraction or because of their claimed antitumoral activity. The industrial value of the EPS depends on their ability to modify the rheological properties of aqueous solutions. They have been traditionally used in the food industry as thickening, gelling, or stabilizing agents.

A number of important functions have been ascribed to EPS:

1. Promotion of adhesion to oral surfaces and biofilm formation
2. Improvement of viability after adhesion
3. Promotion of resistance to nonspecific host immunity

EPSs play an important role in the rheology, texture, and mouthfeel of fermented milks, and are found in yogurt, kefir, viili, and some other products.

Concluding Remarks

Starters are carefully selected microorganisms that are added to milk to initiate and carry out the desired fermentation. Precaution should be taken in starter culture preparation, especially while 'mother culture' is prepared in a traditional way, to hinder bacteriophage infection by effective aseptic and sanitation practices, as well as thorough attention to detail in all manufacturing steps.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks. **Bacteriophage**: Biological Aspects; Technological Importance in the Diary Industry. **Biofilm Formation**. **Fermented Milks**: Nordic Fermented Milks; Types and Standards of Identity. **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactococcus lactis*; *Leuconostoc* spp.; *Streptococcus thermophilus*.

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Health Effects of Fermented Milks

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Introduction

In many parts of the world, from the distant past, fermented milks have been consumed as valuable food sources. They have also been recognized as foods that have health effects. The modern-day interest in the health effects of fermented milks is said to have been stimulated by the theory of longevity formulated by Metchnikoff in the early twentieth century. This hypothesis proposed that people consuming fermented milk regularly live longer, as lactic acid bacteria (LAB) ingested in the fermented milk colonize the intestine and inhibit putrefaction caused by harmful bacteria, thus slowing the ageing process. The longevity of peasants from the Balkans who regularly consumed fermented milk was cited as the evidence. However, the information such as birth certificates was not accurate then, and we still lack scientifically reliable epidemiological data regarding the prolongation of life by consuming fermented milks. In animal experiments, expansion of life span was observed by giving mice a traditional type of fermented milk, produced with a starter containing *Lactobacillus helveticus*, for their entire life (**Figure 1**).

The studies that followed Metchnikoff's claims proved that the LAB from yogurt does not colonize the intestinal tract. Based on the assumption that LAB of the intestinal origin may colonize the host's intestines, many trials have been done, beginning with *Lb. acidophilus* or bifidobacteria, as advocated in the 1920s. These days, the development of fermented milk products is aimed at the ability to colonize or to enhance health functions. The microorganisms from such fermented milks are generally referred to as probiotics.

The starters of traditional fermented milks vary depending on the method of production in each locality. Normally, they contain many bacteria, predominantly LAB such as lactobacilli and lactococci, but often including other bacteria and yeasts, etc.

Health effects are defined as those that help the organism to grow soundly, to live healthily, to produce offspring and to live long. When there is not enough food, it is important to find an adequate supply of basic nutrition. Conversely, in the developed countries diseases caused by overeating are common. Following the demands of the times, investigations have been undertaken to determine the effects of fermented milks on humans and animals. Several principal health effects so far studied over the world are summarized below. They are usually divided into two groups. One group refers to 'nutritional function', which is expressed as the function of supplying more nutrition efficiently. The other is 'physiological function', which includes the prophylactic and therapeutic functions beyond nutritional effects.

Nutritional function

Milk is a food of almost complete nutrition. Many changes occur to the components of milk during fermentation. Although there is no significant difference between the composition of fermented milk and unfermented milk, the following investigations show that these changes affect the nutritional value and bioavailability.

Improvement of Protein Digestibility

LAB require several amino acids, but there are not enough free amino acid molecules in milk to support their growth. They produce proteases to break down milk proteins and utilize the degradation products. The unused protein degradation products remain in the fermented milk as amino acids and peptides. As the pH decreases, caused by lactic acid formation from lactose, nondegraded or partially degraded proteins become insoluble and form a gel. With these changes accompanying fermentation, the following observations have been reported regarding digestion and absorption of milk proteins. First, when yogurt is digested with artificial gastric juice, the size of the protein particles decreases and there are increases in nonprotein nitrogen and amino acids compared to the levels in nonfermented milk, suggesting that the digestibility will be increased by fermentation. In an animal test, when *Lb. casei*-fermented milk was given to rats, there were more nonprotein nitrogen and free amino acids in the stomach compared to when nonfermented milk was given. Gastric emptying was faster, and amino acid concentration in the portal vein reached the peak sooner. These observations show that the digestion and absorption is enhanced.

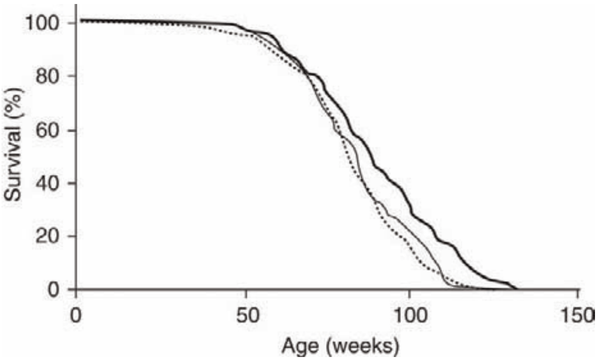

Figure 1 Change of survival ratio of ICR mice fed a fermented milk diet throughout their life span. —, calpis sour milk; ....., whole milk; —, control. Female ICR mice ($n = 30$ in each group) were fed with either the diet containing 14% sour milk fermented with a starter containing *Lb. helveticus*, 1.6% whole milk powder, or control diet from 6 weeks of age to death. (Reproduced with permission from Arai K, Murota I, Hayakawa K, Kataoka M and Mitsuoka T (1980) Effects of administration of pasteurized fermented milk to mice on the life-span and intestinal flora. *Journal of Japanese Society of Food and Nutrition* 33: 219–223.)

Some animal studies showed increase in weight and efficiency of food conversion, thus indicating the higher protein value of fermented compared to unfermented milk, although the results from several reports are not always consistent. However, in a human trial using ^{15}N-labelled milk protein, there was no difference in the digestion rate between fermented and unfermented milk, and gastric emptying was slowed down. At present, no definitive evidence exists about the superiority of fermented milks over unfermented milk with regard to protein nutrition.

Alleviation of Lactose Intolerance

Lactose is the main carbohydrate of milk. Lactose serves as a source of energy during infancy but after weaning, the activity of lactose-degrading enzymes in the brush border of the small intestine decreases, particularly in some ethnic groups. When ingested lactose is not digested in the small intestine, it reaches the large intestine and causes symptoms of lactose intolerance. These include bloating from the osmotic pressure of the lactose itself and gas production by the resident microorganisms in the large intestine, which also cause diarrhoea and flatulence induced by their metabolites. Because of these properties of lactose, it is nutritionally beneficial to remove lactose; for example, converting it to lactic acid when fermenting milk, and removing the fraction containing lactose when making cheese and butter.

In fact, if fermented milk is given to a person who suffers from lactose intolerance, the symptoms are alleviated compared to those caused by lactose consumed in unfermented milk. The main reason for such results could be a decrease in the amount of lactose during the fermentation. Lactose is present in milk at a concentration of 4–5%, but it is reduced to about 3% in yogurt. Secondly, the lactose degrading enzymes of LAB help break down lactose in the intestine. When yogurt with live bacteria was given to rats, a greater amount of free galactose was found in the blood than if they were given an unfermented milk or pasteurized yogurt. In human studies, when a water solution of lactose, or milk containing lactose at the same amount as in a yogurt was ingested, gastrointestinal discomfort such as diarrhoea was greater than when lactose was consumed in yogurt. Also, it was observed that only part of the ingested lactose from the yogurt reached the colon, as estimated from the amount of hydrogen gas produced by intestinal bacteria and released with exhaled air. The bacterial strains that are most effective in reducing the symptoms of lactose intolerance have been reported to be the strains showing higher resistance to bile acid, rather than the strains showing higher lactose-degrading enzyme activity, or higher lactose active transport capability, suggesting that adaptation to the environment of the intestine may be more important.

Enhancement of Mineral Absorption

Not only does milk contain more calcium than other foods, but also its absorption from milk has been considered to be superior to when the same amount of calcium is given to humans in other forms. Several possible explanations have been proposed for this, including the hypothesis that lactose or phosphopeptides released by the hydrolysis of casein act as absorption accelerators. However, as calcium concentration in the blood is controlled strictly by homeostasis, it has been difficult to obtain exact data. Recent animal studies on the amount of calcium in bone, as well as bone weight and strength, showed that lactic acid is involved in the utilization of calcium. Also, increases in bone density and bone strength of femur were observed when administering *Bifidobacterium longum* bacterial powder to the ovariectomized osteoporotic model rat. These observations suggest that fermented milk can enhance calcium absorption.

However, it is not possible to state unequivocally that fermented milk is definitely superior to unfermented milk. When ^{45}Ca was given in yogurt or milk to humans and assessed for absorption into the blood, there was no observable difference. Also, animal balance studies estimating the utilization of each mineral by measuring the amount of calcium excreted in the urine and the amount retained in the bone demonstrated that fermented milk performed better than the standard feed, but not better than milk.

Physiological Effects

Control of Intestinal Health

The digestive tract has a large surface and an abundance of indigenous microorganisms, which are collectively called intestinal microflora, and also transient microorganisms. It may be affected by many types of illness and disturbances of its function. These include diarrhoea caused by infection by pathogenic bacteria or viruses, and the opposite symptom, constipation. Although 'control of intestinal health' mainly means treating these direct symptoms, it is also widely understood to include improvement in the composition and metabolic activity of intestinal microflora.

The effects of fermented milks on intestinal health have been known since ancient times. For 'probiotic' products, made with selected intestinal bacteria, whose main target is the intestinal tract, many studies have been done to evaluate their various effects, and the mechanism of interaction with the intestinal tract. Some traditional fermented milks show beneficial effects on intestinal health (although the number of reports is smaller than for probiotic milks). Alleviation of gastric achlorhydria, infant diarrhoea, diarrhoea caused by malnutrition, diarrhoea caused by the use of antibiotics, chronic constipation, etc. have been reported. Also, an increase in the count of bifidobacteria in the faeces and a decrease in putrefactive compounds in the faeces have been shown after taking yogurt in human clinical studies. Studies have been conducted to identify a mechanism of action of fermented milk, which is prepared with lactic acid bacteria that do not possess the ability to colonize the intestinal tract. The hypotheses tested included enhancement of intestinal immune function by LAB in the fermented milk, effect of oligosaccharides ('prebiotics'; *see* **Prebiotics**: Types; Functions; **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products) and antimicrobial substances generated during fermentation on the improvement of intestinal microflora, and acceleration of the cell growth in the upper intestine by fermented milk.

Lowering Serum Cholesterol

High serum cholesterol is a risk factor in heart disease, which is one of the main causes of death in developed countries. It is believed that the arterial sclerosis that proceeds to cardiac infarction is caused by cholesterol accumulation in the blood vessel wall. However, according to recent study results, cholesterol does not accumulate as it is. Macrophages have been shown to retain oxidized cholesterol to become foam cells and accumulate on the blood vessel wall. Lowering serum cholesterol may help decrease oxidized cholesterol and hence the risk of heart disease.

Investigations of the cholesterol-reducing potential of fermented milk were triggered by the observation that the Masai people of East Africa have low blood cholesterol levels although they consume much milk and meat. Fermented milk with surfactant, which accelerates fat absorption, was given to them. Although their weight increased from consuming fermented milk, their blood serum cholesterol decreased. A decrease in cholesterol level was reported in a US study in which 720 ml of either yogurt with live bacteria or pasteurized yogurt was administered daily to normal subjects. However, results of subsequent human studies were not always as positive as results of these early studies. For example, the cholesterol level did not decrease when 2 l day^{-1}of yogurt was given to 10 high-school students for 3 weeks. In addition, the pretrial results from administering LAB capsules showed decreases in blood cholesterol level, but the results from the subsequent large-scale trial did not confirm this observation. It is difficult to compare results of different trials, owing to differences in the type and volume of fermented milk, the types of LAB, as well as the age, gender and food habits of the subjects. It seems now necessary to investigate the mechanism(s) of action and screen available strains.

Cholesterol is an essential component of cell membranes, and is used to produce some hormones and bile acids. It is synthesized in the liver and skeletal muscle and also supplied by absorption through the alimentary tract from ingested foods. Blood cholesterol level is controlled by a complex mechanism. Cholesterol is supplied to each organ through the bloodstream as lipoprotein, and also transported to the liver from each organ, to be finally excreted as bile acids. As a possible mechanism for lowering serum cholesterol level by fermented milks, a substance which inhibits hydroxymethyl-glutaryl CoA reductase, an important enzyme in cholesterol synthesis in the body, has been suggested, but experimental results were inconclusive. It has also been reported that in rats serum cholesterol was lowered and cholesterol synthesis was inhibited by a methanol extract of milk fermented by *Streptococcus thermophilus*. However, the active substance has not yet been identified. In addition, inhibition of cholesterol absorption from the intestine has been studied. Because conjugated bile acids enhance the absorption of cholesterol, attempts were made to select a strain with an ability to deconjugate bile acid or a strain that directly decreases absorption of cholesterol in the intestine, by assimilating or adsorbing cholesterol to the bacterial cells. In addition, viscous exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in fermented milks have been suggested to interfere with absorption in a similar manner to dietary fibre.

Antihypertensive Effects

High blood pressure is another risk factor in cardiovascular diseases. Blood pressure is controlled by complicated neural and humoral factors, which are interrelated and form an auto-control system. Among them, the renin–angiotensin system plays a particularly important role. Renin, a proteolytic enzyme secreted from the kidney, acts on angiotensinogen to generate angiotensin I. The two C-terminal amino acid residues of angiotensin I are cleaved off by the angiotensin converting enzyme (ACE) to generate an octapeptide, angiotensin II, which raises blood pressure. ACE inhibitors are widely used clinically and have been proven to work effectively in hypertensive patients. Therapeutic effects on cardiovascular diseases have been attributed to fermented milks since ancient times. They were used for high blood pressure, diabetes and heart disease. Yogurt is included in the group of foods that exhibit relatively strong ACE inhibitory activity. Also, some cheese or peptides obtained by proteolytic breakdown of milk proteins have been reported to show ACE inhibitory activity.

When hypotensive effects of several fermented milks made with various LAB were compared by feeding these milks to spontaneously hypertensive rats (SHR), only milk fermented by *Lb. helveticus* was observed to decrease blood pressure, along with a strong ACE inhibitory activity. The stronger proteolytic activity of *Lb. helveticus*, in comparison to other LAB, was thought to be related to the hypotensive effect of milk fermented by *Lb. helveticus*.

When a starter culture with *Lb. helveticus* was added to skim milk, ACE inhibition increased during the process of fermentation, suggesting that an ACE inhibitor(s) was/were generated. The active compounds were purified by using high performance liquid chromatography and identified as two tripeptides; Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). Most of the ACE inhibitory activity in the fermented milk has been attributed to these two tripeptides, which were generated from β-casein by the action of proteinase and several peptidases of *Lb. helveticus*.

The blood pressure of SHR rats given the chemically synthesized tripeptides decreased in a dose-dependent manner. Furthermore, there was lowered ACE activity in the aorta of SHR rats that were orally given fermented milk, and these tripeptides were detected in the aorta. It has thus been proved that peptides taken orally are absorbed in the intestine and produce hypotensive effects by inhibiting ACE activity in the body. No change in blood pressure was observed when fermented milk or these peptides were given to WKY rats which have normal blood pressure.

In the clinical studies, ingesting 95 ml of fermented milk daily for 8 weeks significantly lowered the systolic and diastolic blood pressure of hypertensive patients (**Figure 2**). Based on this evidence, a beverage using fermented milk as its main ingredient has been approved as a ‘Food for specified health use’ (FOSHU) in Japan.

Anticancer Effects

Anticarcinogenesis

Cancer is one of the main causes of death in developed countries. According to epidemiological studies, the incidence of cancer is greatly affected by environmental factors, especially diet. Research in The Netherlands comparing breast cancer patients and healthy people showed that patients with breast cancer consume less fermented milk, suggesting that fermented milk may prevent cancer. On the other hand, some results show that there is no relationship between fermented milk consumption and colon cancer, so this effect is yet to be unequivocally proved.

As an experimental animal model of human cancer, rats were given dimethylhydrazine (DMH) to induce colon cancer and fed with fermented milk or selected LAB. It has been reported that feeding *Lb. acidophilus* bacteria delayed the onset of cancer, and that milk soured by *Lb. helveticus* or yogurt decreased cancer incidence. The following possible mechanisms for the inhibitory effect on cancer onset have been proposed:

Decrease of mutagenic activity

The first step in cancer is the mutation of genes, and many carcinogens have mutagenic activity. By using the Ames test in an *in vitro* experiment, the antimutagenic effects of milk fermented with *Lb. bulgaricus*, *Sc. thermophilus* and *Enterococcus faecalis* have been reported. The active element was either casein, which is a major component of milk, or the cell wall of *Ec. faecalis*, and further research is needed to elucidate the mechanisms of action. Furthermore, oral administration of mutagens such as DMH trigger DNA damage in intestinal cells of rats, but feeding yogurt or LAB prevents the DNA damage.

Modification of intestinal microflora

Activity of intestinal microflora is related to carcinogenesis. The incidence of liver tumour of pathogen-free C3H/He mice was 30%, which is low compared to that of conventional mice, 75%. Addition of *Escherichia coli, Ec. faecalis* and *Clostridium paraputrificum* to pathogen-free mice increased the liver cancer rate to 100% and the further addition of *Bif. longum* or *Lb. acidophilus* reduced the rate to 46% and 60%, respectively. In rats and humans who are on a predominantly meat diet, considered to be a high-risk diet for colon cancer, high activities of such enzymes as β-glucuronidase, nitroreductase, azoreductase and steroid-7-dehydroxylase, that are all linked to the generation of carcinogenic compounds, have been observed. However, the activity of these enzymes was lowered by supplementing *Lb. acidophilus* or acidophilous

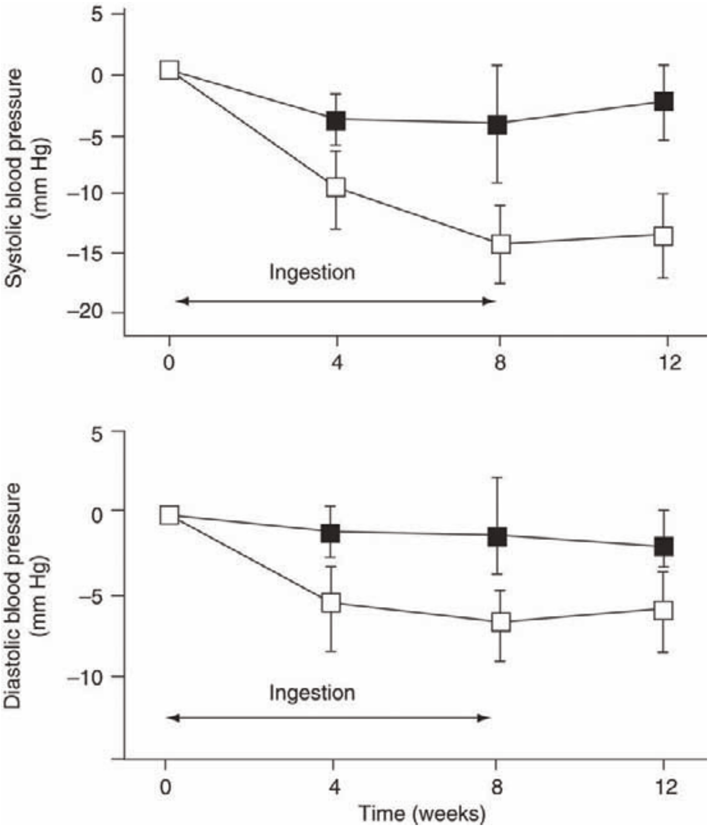

Figure 2 Effect of fermented milk ingestion on blood pressure of hypertensive patients. ■, Sour milk group; □, artificially acidified milk as placebo. Subjects were daily given 95 ml of the fermented milk ($n = 17$) or placebo ($n = 13$) for 8 weeks. Each point indicates a mean value and vertical bars represent standard errors. (Reproduced with permission from Hata Y, Yamamoto M, Ohni M *et al*. (1996) A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *American Journal of Clinical Nutrition* 64: 767–771.)

milk. Together with results showing inhibitory effects on chemical carcinogenesis in rats, administration of live *Lb. acidophilus* is suggested to be effective in inhibiting colon cancer.

Suppression of cancer cell growth

The method based on transplanting cancer cells into mice and studying the survival time or the growth of cancer cells is used as a primary screening for anticancer agents and in separation and identification studies of effective elements, because it is possible to obtain results within a short period of time. In an early anticancer study with use of LAB, the inhibitory effect of parenterally administered *Lb. bulgaricus* cell wall glycopeptide was reported. Later, in oral administration studies, several types of fermented milks, such as yogurt, cow colostrum fermented by *Lb. acidophilus* or *Lb. bulgaricus* plus *Sc. thermophilus*, milk fermented with *Lb. helveticus*, etc., have been found to have an inhibitory effect on cancer cell growth.

Several factors have been reported as effective in suppressing the growth of cancer cells. These include the anion fraction of yogurt dialysate separated by ion exchange chromatography, a fraction separated by ion chromatography of the supernatant of milk fermented by *Lb. bulgaricus*, LAB cells in yogurt such as *Lb. delbrueckii* subsp. *bulgaricus*, *Sc. thermophilus* and *Lb. helveticus* subsp. *jugurti*, polysaccharides made by *Lb. helveticus* subsp. *jugurti*, and kefir, as well as heated skim milk. These fermented milks or effective compounds in them are thought to work primarily on the immune system. Cancer cells can be considered foreign to the organism, and their growth is usually suppressed by the immune surveillance system. Those that escape this system proliferate and develop cancer. Results showing that the inhibitory effect of *Lb. casei* on cancer cell growth decreases after treatment with the carrageenan which is known to be an anti-macrophage agent, and that there is an effect on T cell function-deficient nude mice, suggest that this effect depends on macrophages of the host's immune system. On the other hand, yogurt and milk fermented with *Lb. helveticus* have been proven to inhibit the growth or induce differentiation of cell line derived from human colon cancer. Thus, direct action on cancer can also be possible.

Effect on Immunological function

The immune system not only defends the body against bacterial and viral infection, but also plays a role in many diseases such as cancer, allergies and autoimmune diseases. The intestinal tract has been shown to be a large immune organ which works not just as a peripheral organ to protect against intestinal infection but also affects systemic immunological function. Its function is affected by the intestinal microflora and diet.

In addition to the effect on cancer discussed above, consumption of fermented milks has been reported to inhibit infection. Studies on mice administered salmonellae to develop intestinal infections or on mice infected in the nasal cavity with *Klebsiella pneumoniae* to cause pneumonia demonstrate that mice given fermented milk lived longer. The translocation of *Candida albicans* to the liver in immunosuppressed mice was also suppressed. Alleviation of allergies by fermented milks has also been reported.

LAB can affect functions of immune cells; for example, activation of macrophages and 'natural killer' (NK) cells has been observed. Activation of these cells was reported in relation to anticancer properties of parenterally administered *Lb. casei*. Effect of oral administration was shown by administering *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. casei* and observing the increase in phagocytosis activity and lysozome release by peritoneal macrophages. In the study in which an increase in the numbers of surviving mice by administering fermented milk after nasal infection with *Kl. pneumoniae* was demonstrated, a concomitant increase in phagocytosis activity by pulmonary macrophages was observed.

The effects of lactic acid bacteria and fermented milk on cytokines, which regulate immune responses by mediating information between cells, have also been reported. Parenteral administration of *Lb. casei* to mice increased the serum levels of colony growth stimulating factor (CSA) which takes part in macrophage differentiation as well as those of γ-interferon which activates NK cells and macrophages. An increase in interferon production *in vitro* by human peripheral lymphocytes has been observed by the addition of yogurt. In a human clinical study, an increase in the serum level of γ-interferon as well as in the NK cell count was observed after the ingestion of yogurt or LAB used in yogurt, compared to unfermented skim milk.

See also: **Bacteria, Beneficial:** Probiotics, Applications in Dairy Products. **Fermented Milks:** Types and Standards of Identity; Yogurt: Types and Manufacture. **Milk Protein Products:** Bioactive Peptides.

Further Reading

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Buttermilk

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Introduction

The definition of buttermilk is very flexible. Depending on the country, it is associated or confused with sour milk, natural (conventional) buttermilk, cultured buttermilk, cultured skim milk, cultured milk, some types of Scandinavian fermented milks and sometimes even with Bulgarian fermented milk.

Buttermilk obtained by churning cultured or soured cream into butter is natural (conventional) buttermilk and a fermented product. Fresh (sweet) cream may be also churned into butter and yields sweet buttermilk which can be used further as a substrate for mesophilic fermentation to yield conventional buttermilk. Cultured or fermented buttermilk as it is widely produced by lactic acid fermentation of skim milk or milk containing less fat than whole milk should semantically be called cultured milk. However, the product, when of proper quality, is not simply sour milk since it possesses distinctive characteristics of a product obtained by fermentation with a typical butter starter culture. The term ‘cultured buttermilk’ will be, therefore, further referred to the product obtained by fermentation of milk. Some manufactures add fat granules or flakes to the product to associate its name stronger with true or conventional farm buttermilk.

According to the Standard of Identity (IDF Standard 163:1992), the requirements for buttermilk are: acidity not less than 0.60% (w/w), expressed as lactic acid, fermented by *Lactococcus lactis* or its subspecies and biovariants and/or *Leuconostoc mesenteroides* and its subspecies. Minimum counts of specific culture microorganisms should be 10^7 cfu g^{-1} at the time of sale.

Natural (Conventional) Buttermilk

Semantically, buttermilk is the aqueous phase released during the manufacture of butter. Traditionally, buttermilk was the fresh serum which was separated during buttermaking on farms after churning cream ripened with naturally occurring lactic acid bacteria. Sometimes, it contained small flakes of butter. Under industrial conditions, buttermilk is a byproduct of buttermaking. Depending on the processing conditions, either sour cream or sweet cream buttermilk is obtained. Its characteristics depend on the nature of the cream used for butter production. Sweet cream buttermilk can be processed further to fermented buttermilk by mesophilic lactic acid bacteria. **Figure 1** is a flow diagram of the protocol for the production of conventional buttermilk.

The chemical composition of buttermilk depends to a large extent on the buttermaking technology and season of the year. Traditionally, fermented buttermilk contains 7–10% total solids, including 3.5–4.9% lactose, about 0.5% lactic acid, 2.7–3.8% nitrogenous compounds and 0.6–0.75% ash. Fat content ranges from 0.3% to 1.0%. Besides skim milk proteins and lactose, it contains also proteins and phospholipids derived from milk fat globule membrane (**Table 1**).

In some countries (Russia, Poland, Czech Republic, Finland, Germany), naturally fermented buttermilk is on sale as a beverage for human consumption or sometimes is used for animal feed. Problems with increasing the consumption of traditional buttermilk is its short shelf-life (about 1 week at 4–7 °C), the difficulty in obtaining a uniform quality and the lack of promotion as a beverage. Therefore, without a special effort to commercialize natural buttermilk, consumption will decrease and it will be treated only as a regional drink.

In most countries, sweet buttermilk is concentrated by evaporation, then spray-dried and used in the food industry, mainly as an ingredient for breads, biscuits, pancakes, waffles or cakes. In the United States, for example, it is generally used as an ingredient in ice cream and processed cheese foods. Buttermilk contributes unique flavour characteristics to chocolate. Conventional sweet buttermilk may also provide additional emulsifier functionality in products like chocolate, reconstituted butter, cheese analogues and cheese sauces.

Sour cream buttermilk is difficult to dry due to the high content of lactic acid; therefore, it is usually evaporated to 26–28% of solids and can then be added to bread or cakes.

World production of natural buttermilk is quite stable and is about 6.3 Mt year^{-1}, corresponding to world butter production of 4.2 Mt.

[†] Deceased

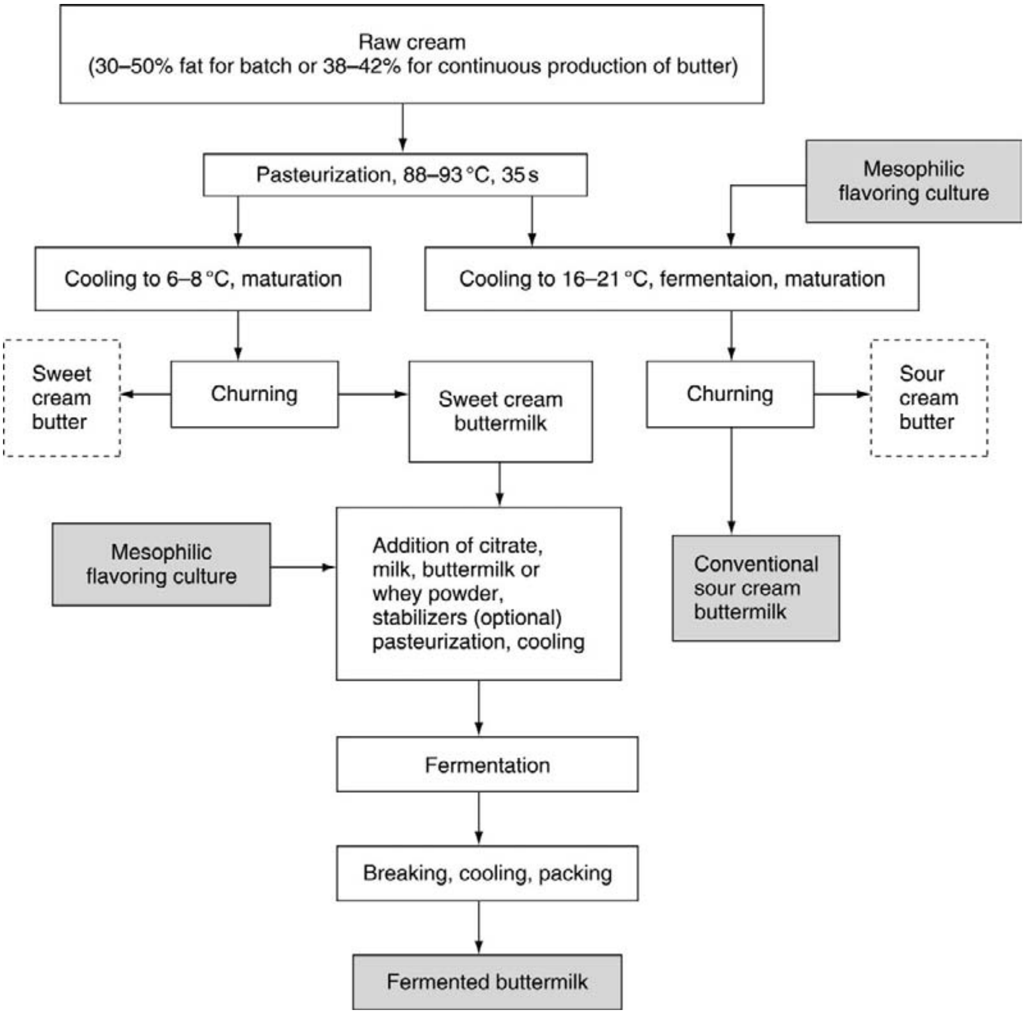
Raw cream
(30–50% fat for batch or 38–42% for continuous production of butter)
Pasteurization, 88–93 °C, 35 s
Mesophilic flavoring culture
Cooling to 6–8 °C, maturation
Cooling to 16–21 °C, fermentaion, maturation
Sweet cream butter
Churning
Sweet cream buttermilk
Churning
Sour cream butter
Mesophilic flavoring culture
Addition of citrate, milk, buttermilk or whey powder, stabilizers (optional) pasteurization, cooling
Conventional sour cream buttermilk
Fermentation
Breaking, cooling, packing
Fermented buttermilk

Figure 1 Flow diagram for production of conventional buttermilk and butter.

Table 1 Chemical components of conventional and cultured buttermilk

Component	*Conventional buttermilk*	*Cultured buttermilk*
Total solids (%)	7–10	8–10
Fat (%)	0.3–1.0	0–2.5
Lactose (%)	3.5–4.9	4.8–5.8
Protein (%)	2.66–3.75	3.4–3.8
Phospholipids (%)	0.075–0.25	nd
Conjugated linoleic acid (mg 100 g^{-1})	nd	0.6 (0.1 in yogurt)
Minerals (%)	0.6–0.75	0.6–0.9

nd, no data

Adapted from Kosikowski F (1984) *International Dairy Federation Bulletin* 179: 116–119; Heiler C and Schieberle P (1997) *International Dairy Journal* 7: 659–666; Dajnowiec Z, Jaroń E, Szczepański S and Korzeniowski W (1999) *Przeglad Mleczarski* 2: 51–53; Lin H, Boylston TD, Chang MJ, Luedecke LO and Shultz TD (1995) *Journal of Dairy Science* 78: 2358–2365.

Cultured Buttermilk

Cultured buttermilk is made from fresh pasteurized skimmed milk or homogenized, pasteurized low-fat milk, usually containing less than 1.0% fat (**Table 2**).

After pasteurization, the milk is cooled to 22 °C and inoculated with about 1–3% mesophilic starter or a direct set culture at the level recommended by the manufacturer. The milk is fermented at 19–22 °C to ensure the balanced growth of acid- and aroma-producing strains. At

Table 2 Preparation of milk for the production of buttermilk compared to some other fermented milks

	Cultured buttermilk	*Yogurt*	*Kefir*
Fat			
Range (%)	0–2.5	0–10	0–3.5
Average (%)	<1	0–4	>3
Solids-not-fat			
Range (%)	7–9	9–16	8–10
Average (%)	8–8.5	9–11	8–8.5
Heat treatment			
Range (°C)	80–95	90–100	85–95
Holding time (min)	2–30	5–20	5–30

Adapted from Puhan Z (1988) *International Dairy Federation Bulletin* 227: 138–164 and Puhan Z and Zambrini AV (1992) *International Dairy Federation Bulletin* 277: 22–27.

a temperature higher than 24 °C, acid producers (see next section) multiply at a much faster rate than aroma bacteria and, usually, the product lacks the specific diacetyl flavour. The milk used for buttermilk production should be of the highest quality from the compositional and microbiological points of view. Because the amount of citric acid in milk is not constant and varies during the year (see next section), supplementation of milk with 0.1–0.2% of citric acid or sodium citrate is recommended to obtain a sufficiently high level of diacetyl (2–3 mg kg^{-1}) by aroma-producing bacteria. Owing to the wide latitude which the legal provisions in general allow, the composition of buttermilk can vary over a wide range (**Table 1**).

Optional supplements, apart from citric acid or sodium citrate, are stabilizers (0.01–0.02%), nutritive carbohydrate sweeteners, flavouring ingredients, skim milk-, buttermilk- or whey powders (1.5–2.0%), NaCl (0.1%), freeze-dried butter flakes or granules at a level of 0.002%. After incubation for 15–20 h, the coagulum at pH 4.6–4.7 (0.75–0.85% titratable acidity) is broken by gentle agitation. Overly vigorous agitation, improper cooling and the use of unsuitable pumps to feed filling machines can lead to a loss of texture. Cooling must be coordinated with the breaking of buttermilk. Usually, cooling starts 15 min before breaking the buttermilk coagulum. Thereafter, the buttermilk is packed, cooled further and stored (**Figure 2**).

Disruption of the gel can occur due to the production of CO_2 by citric acid-fermenting bacteria, giving rise to the phenomenon of 'curd floating'. Fermentation parameters for the production of buttermilk are compared to those for some other fermented milks in **Table 3**.

The important sensory characteristics of buttermilk resulting from mesophilic fermentations are the typical consistency, which is due to the coagulation of milk proteins by lactic acid, and the aroma and flavour produced by the fermentation of citric acid and lactose. Cultured buttermilk should have a thick, smooth, fairly viscous body. The texture also depends on whether or not polysaccharide-producing strains are included in the starter culture and on the concentration of total solids. There should be no or very little separation of whey. The buttermilk should properly be held at temperatures not exceeding 4–5 °C. The keeping quality of buttermilk is approximately 2–3 weeks.

Starter Cultures

For the production of ripened butter and cultured buttermilk, mesophilic lactic acid bacteria are used as a starter. The mesophilic cultures are those which contain strains of *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris*. The first two species produce mainly lactic acid and are often referred to as acid producers, in contrast to *Leuconostoc* spp. which also ferment citric acid and produce important metabolites, such as CO_2, acetaldehyde and diacetyl. These are referred to as aroma and flavour compounds. The balance between aroma- and acid-producing strains is very important and not more than 20% of aroma-producing bacteria is recommended. Diacetyl, at a concentration of 2–5 mg kg^{-1}, is responsible for the characteristic 'buttery' flavour and aroma. In a culture of lactic acid bacteria, the formation of diacetyl is low when lactose is the only carbon source but it is increased if an additional source is available, e.g. citrate. Milk contains an average of 0.17–0.2% citrate but shows great fluctuation, with a range of 0.07% to 0.4%; lower levels of citrate occur in winter milk. Moreover, the citrate content in milk is highly dependent on its microbiological quality; for example, in summer, it is closely correlated with the level of contaminating bacteria, particularly with *Enterobacter* spp. and pseudomonads. Therefore, supplementation of milk with citrate or citric acid is often recommended. It is also possible to introduce citr^{+} *Lc. lactis* subsp. *lactis* (*Lc. lactis* subsp. *lactis* biovar. *diacetylactis*) strains but with low acetaldehyde-producing ability. Citr^{+} *Lc. lactis* subsp. *lactis* strains usually show high diacetyl reductase activity and after an extended incubation period, the concentration of diacetyl quickly decreases.

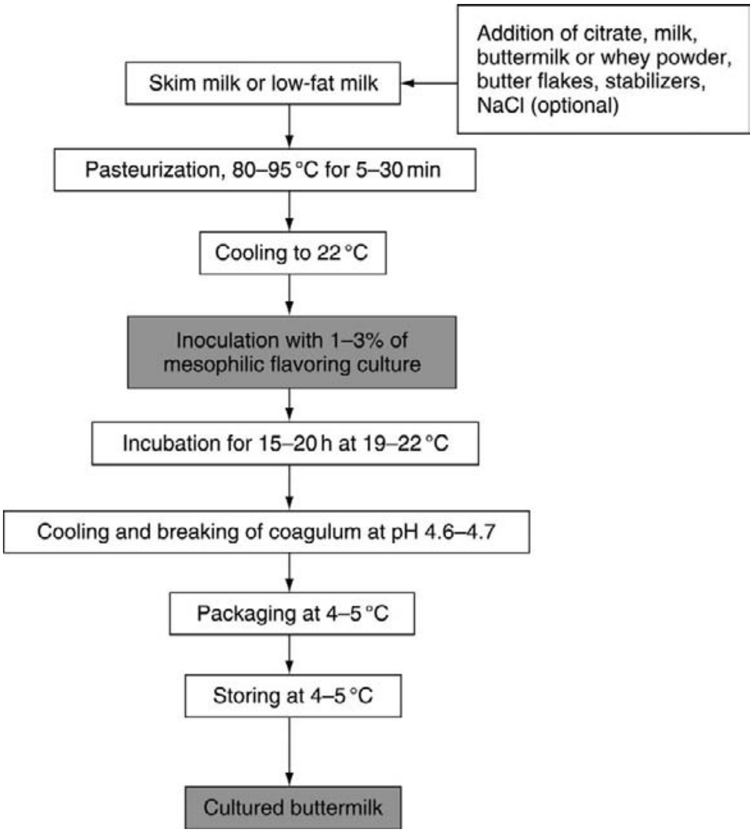
Addition of citrate, milk, buttermilk or whey powder, butter flakes, stabilizers, NaCl (optional)
Skim milk or low-fat milk
Pasteurization, 80–95 °C for 5–30 min
Cooling to 22 °C
Inoculation with 1–3% of mesophilic flavoring culture
Incubation for 15–20 h at 19–22 °C
Cooling and breaking of coagulum at pH 4.6–4.7
Packaging at 4–5 °C
Storing at 4–5 °C
Cultured buttermilk

Figure 2 Flow diagram for production of cultured buttermilk.

Table 3 Fermentation parameters for the production of buttermilk compared to some other fermented milks

	Cultured buttermilk	*Yogurt*	*Kefir*
Starter culture (%)			
Range	0.3–4.0	0.02–6	1–10
Average	1.0–3.0	0.5–4	1–4
Incubation Temperature (°C)			
Range	15–32	30–45	18–32
Average	19–22	37–45	20–23
Time (h)			
Range	5–24	2–18	8–24
Average	15–20	2.5–6	16–18
Final pH			
Range	4.7–4.4	4.8–4.0	4.6–4.2
Average	4.7–4.6	4.6–4.2	4.4–4.3
Lactic acid (%)			
Range	0.68–0.86	0.63–1.36	0.68–0.9
Average	0.75–0.85	0.8–1.0	

Adapted from Puhan Z (1988) *International Dairy Federation Bulletin* 227: 138–164 and Puhan Z and Zambrini AV (1992) *International Dairy Federation Bulletin* 277: 22–27.

Another very important carbonyl flavouring compound produced by lactic acid bacteria in buttermilk is acetaldehyde. It is undesirable and if present in excess is responsible for the flavour defect described as ‘green’ or ‘yogurt’-like; only relatively small amounts, less than 1 mg kg^{-1}, are required for the development of a balanced flavour in the product. The concentration of acetaldehyde in milk fermented by *Leuconostoc* strains is usually very low, less than 1 mg kg^{-1}. It is reduced to ethanol by leuconostocs as a major mechanism for the re-oxidation of pyrimidine nucleotides. In starters for the production of buttermilk, in which acetaldehyde is undesirable, it is advantageous to use *Ln. mesenteroides* subsp. *cremoris* rather than citr^{+} *Lc. lactis* subsp. *lactis* for flavour production. To obtain buttermilk with desirable organoleptic properties the optimum ratio of diacetyl and acetaldehyde is 4:1. However, diacetyl-to-acetaldehyde ratios of 8:1 or greater were noticed in commercial buttermilk receiving the high flavour scores.

The strains used in buttermilk fermentation are selected on the basis of the rapid acid production at 21–24 °C, production of clean lactic acid flavour with no trace of maltiness or ‘green’ acetaldehyde flavour, compatibility with the other bacterial strains included in the starter, and resistance to bacteriophage. The culture should be properly balanced for acid and flavour production, and low in diacetyl reductase activity. From a nutritional point of view, it is very desirable that *Lactococcus* spp. ferment milk lactose with the production of mainly L(+)-lactic acid, at about 93–100%. Therefore, the consumption of buttermilk by babies need not be restricted, as is the case with yogurt.

Ropy or slime variants of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, which are capable of producing polysaccharides in milk, are often used.

A variety of other neutral and acidic compounds may also contribute to the flavour and aroma of fermented milks. Important acids are formic, acetic, propionic, caproic, caprylic, capric, butyric and isovaleric. They are products of lactose fermentation and/or enzymatic transformation of amino acids. Carbon dioxide, formed mainly in lactose and citrate fermentations, causes carbonation. All of the above compounds are necessary for the total flavour of the fermented product. Even the minor metabolic products, at trace concentrations, may have an important role in balancing the desirable flavour in buttermilk.

Much of the cultured buttermilk is produced using frozen concentrated starters. The traditional scale-up system (stock culture–mother culture–bulk culture) for propagating starter cultures is also used in some countries. Bulk starter culture is fermented to final acidity of 0.8–0.9% lactic acid and final pH of 4.5–4.3, depending on the country, incubation temperature is 18–32 °C, usually 20–23 °C. Commercial cultures for the production of butter or buttermilk display wide variations in growth and flavour-forming properties (**Table 4**) (*see* **Fermented Milks:** Starter Cultures).

Defects of Buttermilks

Flavour defects are more frequent than the texture defects. The flavour criticisms most frequently associated with cultured buttermilks are ‘coarse’, ‘coarse-acid’ and ‘lacking in flavour’; less frequent criticisms are ‘rancid’, ‘stale’, ‘unclean’ or ‘bitter’ flavours. Overacidification during storage is an occasional problem, but the frequently occurring ‘lack of flavour’ in cold-stored buttermilk is associated with reduction of diacetyl to acetoin. *Pseudomonas, Enterobacter* and yeasts are major contaminants of commercial buttermilk. These organisms can all produce diacetyl reductase.

Metallic flavour develops in conventional cold-stored buttermilk. This defect develops markedly faster when the conventional buttermilk is obtained after churning of sour cream than it is produced by the fermentation of sweet buttermilk. The major compound responsible for the metallic flavour is *trans, cis*-2,6-nonadienol.

Table 4 Biochemical, microbial and flavour characteristics of cultured buttermilk (BM) containing 1% fat or cultured milks (CM) containing 2.5% fat obtained using 16 different commercial buttermilk cultures, cultures were ‘direct set’ type, freeze-dried or deep frozen

Product/property	*Concentration*
BM Diacetyl (D), range (mg kg^{-1})	0.2–25
Acetaldehyde (A), range (mg kg^{-1})	0.1–10
Ratio A/D, range	0.1–114
CM Diacetyl (D), range (mg kg^{-1})	0.2–25
Acetaldehyde (A), range (mg kg^{-1})	0.1–10
Ratio (A/D), range	<0.1–48
BM Proteolysis (% increase in alanine equivalent)	104–291
CM	94.1–265
BM Number of cultures with tendency to overacidification during storage	6
BM Number of cultures with high CO_2 production	3
CM Number of cultures giving buttery flavour	7
BM Number of cultures that increase folic acid content	3
% Increase	30–130
CM Number of cultures increasing folic acid content	9
% Increase	25–200
BM Number of cultures that increase thiamin content	3
% Increase	55–170
CM Number of cultures that increase thiamin content	8
% Increase	10–150
BM Number of lactic acid bacteria (cfu ml^{-1})	
Fresh product	3.6×10^7–2.1×10^9
After 2 weeks at 6 °C	5.5×10^4–8.3×10^8
CM Number of aroma-producing bacteria (cfu ml^{-1})	
Fresh product	8.6×10^6–1.7×10^9
After 2 weeks at 6 °C	5.1×10^4–2.0×10^8

Adapted from Kneifel W, Kaufmann M, Fleischer A and Ulberth F (1992) *Journal of Dairy Science* 75: 3158–3166.

Modified Technologies and Further Processing of Cultured Buttermilk

To improve texture and to increase the viscosity of buttermilk, concentration of the milk by a factor of 1.2 by ultrafiltration at 54 °C may be recommended. The main objective, besides improved quality, is to avoid adding milk powder and to reduce the level of lactose.

Technologies for fermentation in a membrane dialysis fermenter or direct acidification of milk, following citrate fermentation by *Ln. mesenteroides* subsp. *cremoris* have also been developed for the production of cultured buttermilk with a high and stable level of diacetyl. The membrane dialysis fermenter contains a 'flavour compartment' where milk is incubated with *Leuconostoc* spp. or citr^{+} *Lc. lactis* subsp. *lactis* and a 'product compartment' where the milk is incubated with citrate-nonfermenting *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. Diacetyl, which passes through membrane to the product compartment, is stable during storage of the product since citrate-nonfermenting lactococci do not possess diacetyl reductase. Treatment with oxygen further stabilized the diacetyl content.

In the direct acidification technology, which uses only citrate-fermenting *Ln. mesenteroides* subsp. *cremoris* strains, milk is first partially acidified to pH 5.3 with a mixture of lactic and acetic acids and, after incubation with *Leuconostoc*, is acidified further by the addition of glucono-δ-lactone to a final pH of 4.5–4.6 and treated with oxygen. However during 1 week storage, the concentration of diacetyl decreases drastically; glucono-δ-lactone may somehow enable diacetyl reductase remain active even at pH 4.5.

Nutritional Value

The concentration of lactose in buttermilk is rather high and varies from 3.5% to 4.9% in traditional and from 4.8% to 5.2% in cultured product. The level of galactose is much lower than in other fermented milks; the mean value is about 0.02%.

The biological activities of mesophilic lactic acid bacteria also result in changes in the levels of vitamins in fermented milks. The level of vitamins in buttermilk depends on the culture used and may be lower or higher than in noncultured milk (**Table 5**).

The level of vitamins is also influenced by seasonal variations. During storage at 5 °C, there are considerable losses of riboflavin, pyridoxine, folic acid and vitamin B_{12}, while biotin, thiamin and pantothenic acid are stable. The content of conjugated linoleic acid is somewhat higher in cultured buttermilk than in yogurt (**Table 1**). There is no nutritionally significant change in micronutrients during buttermilk fermentation.

Probiotic properties are not associated with the mesophilic lactic acid microflora of buttermilks. Probiotic buttermilk has been produced experimentally by incubating bifidobacteria together with the typical lactococcal microflora of buttermilk. Some inhibition of *Escherichia coli* O157:H7 in such fermented milks and more complete hydrolysis of lactose in the gastrointestinal tract of lactose-intolerant individuals were reported.

Concluding Remarks

Cultured buttermilk is still popular in certain European countries and North America, both as a refreshing beverage and as an ingredient for bread and bakery products. However, the consumption of cultured buttermilk has declined steadily, probably owing to competition from a variety of different styles of yogurt and the growing acceptance of new fermented milks containing probiotic bacteria.

Table 5 Vitamin content in conventional and cultured buttermilk (μg 100 g^{-1})

Vitamin	*Conventional buttermilk*	*Cultured buttermilk*	*Kefir*	*Yogurt*	*Milk*
Thiamin	12–34	23–40	18–22	25–65	20–43
Riboflavin (vitamin B_2)	48–202	120–170	70–351	163–480	106–200
Pantothenic acid	44–68	280–300	No data	280–381	330–460
Vitamin B_6 (pyridoxine)	32–96	27–35	43–49	35–68	17–70
Niacin	44–68	60–110	55–87	90–163	71–100
Biotin	No data	No data	No data	1.2–5.1	1.5–4.9
Vitamin B_{12} (cobalamins)	No data	0.0007–0.2	No data	0–0.52	0.3–0.57
Folic acid	No data	0.017	No data	0–4	0.13–7.5

Adapted from Shahani KM and Chandan RC (1979) *Journal of Dairy Science* 62: 1685–1694; Blanc B (1984) *International Dairy Federation Bulletin* 179: 33–53; Secomska B and Nadolna I (1987) *PrzemysNot-found SpoNot-foundywczy* 41(11): 320–322; Wituszyńska B (1989) *PrzemysNot-found SpoNot-foundywczy* 43(9): 242–243; Kneifel W, Kaufmann M, Fleischer A and Ulberth F (1992) *Journal of Dairy Science* 75: 3158–3166.

See also: **Bacteria, Beneficial:** Probiotics, Applications in Dairy Products. **Butter and Other Milk Fat Products:** The Product and Its Manufacture. **Fermented Milks:** Health Effects of Fermented Milks; Nordic Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Types and Manufacture.

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Nordic Fermented Milks

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Introduction

Fermented milks have traditionally played an important role in the nutrition of people inhabiting northern Europe. In Scandinavia in particular, consumption of fermented milks has a long tradition. Historically, fermented milks, often referred to as sour milk, were made and consumed at home. Most of these products have a characteristic viscous consistency and ropiness, and can be stored for weeks or even months in a cool room.

Home production of fermented milks is now much less common in Scandinavia, but many types of commercially manufactured products have the same attributes as their home-made precursors. Consumption statistics provide ample evidence of the continuing demand for Nordic fermented milks, although the growing popularity of yogurt has led to some erosion of the traditional market in recent decades.

Interest in Nordic fermented milks has recently been stimulated by reports of prophylactic and therapeutic effects associated with starter microorganisms and their metabolites, observed in experiments on animals.

General Features

Nordic fermented milks constitute a group of products distinctly different from fermented milks made elsewhere – primarily owing to their unique physical properties, characterized by high viscosity and ropiness. In the cool climate of northern Europe, home-made fermented milks are the result of the spontaneous growth of mesophilic microorganisms present in raw milk. Low temperatures seem to be critical for the domination of the milk microflora by encapsulated strains of lactococci. Lactobacilli are only rarely isolated from spontaneously fermented milk in Scandinavia. Molds and lactose-fermenting yeasts grow well in acidified milk and are therefore found in some types of Nordic fermented milks, influencing their properties.

Other traditional fermented products popular in Scandinavia include cultured buttermilks and cultured creams, which have a characteristic aroma and flavour, derived primarily from diacetyl and related volatile compounds. Also popular are concentrated milks, made by the removal of whey from the fresh coagulum or, more recently, using membrane- concentrated milk.

Microorganisms

Diverse cultures are used commercially in Scandinavia for various types of fermented milk, and have been derived from traditional products. In many of these cultures, the bacteria responsible for ropiness are indispensable. However, the presence of nonropy organisms is also essential in many cultures to ensure the formation of metabolites that impart typical organoleptic properties to the product.

Overall, the following groups of microorganisms have been isolated from various types of traditional Nordic fermented milks:

1. Mesophilic bacteria. *Lactococcus lactis* subspp. *cremoris, lactis* and *lactis* biovar *diacetylactis*; *Leuconostoc mesenteroides* subspp. *cremoris* and *dextranicum.*
2. *Lactobacillus helveticus*. It is significant that the optimum growth temperature of a strain isolated from the Norwegian and Finnish versions of 'tätmjölk' was 10 °C lower than that reported for other strains of this species. The thermophilic *Lb. helveticus* has been isolated from the Icelandic fermented milk 'skyr'.
3. *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, found in skyr.
4. The yeasts *Candida kefir* and its teleomorph *Kluyveromyces marxianus*, as well as *Torulopsis holmii* and its teleomorph *Saccharomyces exiguus*. These have been isolated from various types of Nordic fermented milks. The long shelf-life of ropy milk has been attributed in part to the presence of yeasts, which are inhibitory against a number of potential mold contaminants.
5. The white fungus *Geotrichum candidum.* This is found in 'viili', 'tette' and other products. *Geotrichum candidum* isolated from a sample of ropy milk and added to a ropy culture (*Lc. lactis* subsp. *cremoris*), which was previously not associated with this organism, stimulated the growth of the culture in milk. A cell-free filtrate of the organism's growth medium (sterile whey) had a similar effect.

Forms of lactococci classified as 'intermediate' between *Lc. lactis* subspp. *cremoris* and *lactis* (on the basis of their physiological characteristics) have also been found in some ropy milks.

The total number of viable bacteria in these products can be very high. For example, up to 10^{10} cfu g^{-1} have been reported in the Finnish ropy milk 'pitkäpiimä'. This is a higher number of live bacteria than that in some starter cultures used in the commercial production of fermented milks.

Traditional Nordic fermented milks owe their characteristic high viscosity and ropiness to the vigorous growth of capsule-forming lactococci, mainly *Lc. lactis* subsp. *cremoris.*

Glycocalyx

Terms like 'slime', 'capsule' and 'mucoidal (Muc^+) phenotype' are used interchangeably in the literature, usually without attempting to ascertain whether the material in question is of capsule type or slime type. 'Capsule' is defined as a compact layer of polysaccharide, and 'slime' as a diffuse layer of polysaccharide, both exterior to the cell wall. As a rule, the capsule of lactococci is tightly bound to the cell that produced it. In cases where the physical nature of the material has not been studied, it is more convenient to refer to 'glycocalyx', a general term that describes the polysaccharide-containing material lying outside the cell wall, although this term is seldom used. **Figure 1** shows an encapsulated strain of *Lc. lactis* subsp. *cremoris.*

Normally, both encapsulated and nonencapsulated forms of the same species can be isolated from a product. As an example, the types and numbers of bacteria isolated from pitkäpiimä are shown in **Table 1**. *Lactococcus lactis* subsp. *cremoris* was the organism most often encountered in this product. It is worth noting that encapsulated *Lc. lactis* subsp. *lactis* biovar *diacetylactis* was found only in samples of ropy milk that did not contain slime-forming cells of *Lc. lactis* subsp. *cremoris.*

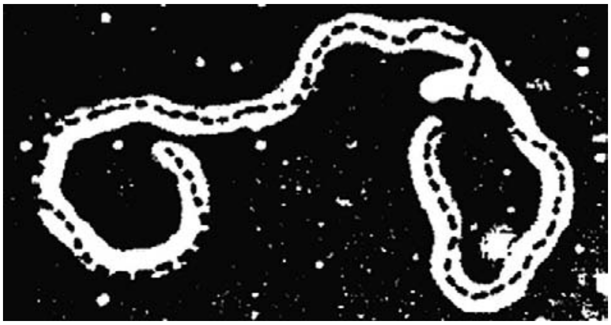

Figure 1 *Lactococcus lactis* subsp. *cremoris*, strain Va, after 6 h of growth. Preparation stained with methylene blue. (Reproduced with permission from Forsén (1966).)

The growth of slime-forming lactococci isolated from pitkäpiimä depended on the presence of nicotinic acid and riboflavin in the growth medium. The O_2 demand and CO_2 production of slime-forming cultures of *Lc. lactis* subspp. *cremoris* and *lactis* from pitkäpiimä has been observed, at 19 °C, to be two to three times higher than in strains that do not produce slime.

Polysaccharide slime material seems to be produced in particular abundance during the late exponential and early stationary phases of growth, and remains associated with the cells that produced it, while also attached to casein micelle clusters, forming a network responsible for the ropy consistency of the product. Other milk proteins may also be involved. In addition, proteins of bacterial origin may be present in slime.

In one study, the slime produced by a strain of *Lc. lactis* subsp. *cremoris* isolated from a Swedish ropy milk was found to contain 47% protein, 20% methyl pentoses, approximately 10% hexose-like sugars and almost 3% sialic acid. The composition of a phosphorus-containing polysaccharide produced by *Lc. lactis* subsp. *cremoris* isolated from a sample of the Finnish fermented milk viili is shown in **Table 2**. Rhamnose, glucose and galactose were present in this phosphopolysaccharide at the molar ratio 1:1.45:1.75. The purification procedure (preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)) removed protein, glycerol, hexosamine, sialic acid and uronic acids from the crude slime material. The phosphate group was attached to the β-D-galactopyranosyl residue. The molecular weight of this phosphopolysaccharide was estimated by gel permeation chromatography to be 17 MDa. The phosphopolysaccharide has a negative charge; it is, therefore, possible that it could form complexes with basic proteins found on the cell surface of lactococci, owing to electrostatic interactions.

Determinants of Glycocalyx Formation

Plasmid DNA

Observations of the loss of the mucoidal (Muc^+) phenotype at higher incubation temperatures led to studies that have demonstrated the involvement of plasmid DNA in the synthesis of glycocalyx by lactococci. The ropy phenotype of strains of *Lc. lactis* subsp. *cremoris* was found to be associated with a 7-MDa plasmid in Swedish strains and with a 30-MDa plasmid in Finnish strains. This indicates that distinctly different plasmids coding for the ability to produce slime are present in separate populations of starter bacteria. Plasmids on which slime synthesis is encoded can be transferred to non-slime-forming strains by a number of currently available techniques, including electroporation or cotransfer

Table 1 Types of bacteria identified in pitkäpiimä (the number of slime-forming colonies are shown in parentheses)

Ropy milk culture	*Number of characterized colonies*	*Leuconostoc spp.*	*Lactococcus lactis subsp. lactis*	*Lactococcus lactis subsp. lactis biovar diacetylactis*	*Lactococcus lactis subsp. cremoris*	*Streptococcus viridans group*[a]
H_1	29(4)	9	2(2)	–	18(2)	–
H_4	16(5)	3	6(1)	2	5(4)	–
H_5	20(2)	6	3	4(2)	7	–
VA	42(10)	5(5)	7(4)	30(1)	–	–
Jo	13(3)	–	7(1)	–	2(2)	4
In	17(4)	2	3(1)	4	8(3)	–
Total	137(28)	(5)	(9)	(3)	(11)	–
Percentage of slime-forming colonies	100	18	32	11	39	–

[a] *Streptococcus viridans* physiological group includes *Sc. thermophilus*, but the result reported above does not imply the presence of *Sc. thermophilus* in pitkäpiimä. Reproduced with permission from Forsén (1966).

Table 2 Composition of the slime material and phosphopolysaccharide excreted by *Lactococcus lactis* subsp. *cremoris* SBT 0495

	Content (%)	
	Crude slime material	*Purified polysaccharide*
Total carbohydrate	42.3	78.9 (90.5)[a]
Rhamnose	–	21.7
Glucose	–	31.4
Galactose	–	38.0
Methylpentose	20.6	21.4
Hexosamine	4.8	nd
Sialic acid	0.8	nd
Uronic acid	1.9	nd
Glycerol	–	nd
Phosphorus	1.8	3.1
Protein	21.2	nd

[a] Values after hydrofluoric acid cleavage.nd, Not detected. Reproduced with permission from Nakajima *et al*. (1990).

involving a bigger plasmid on which an ability to ferment lactose is encoded.

Proteins of the cell surface

Two cell-surface proteins, of molecular weight 26 kDa and 42 kDa, have been shown to be associated with slime formation in lactococci. The synthesis of these proteins is probably plasmid-encoded.

Lipoteichoic acid

The presence of lipoteichoic acid (LTA) on the cell surface of lactococci isolated from viili has been demonstrated by immunochemical techniques. LTA is present on the entire cell surface and may form associations with polysaccharides of the slime material and proteins of the bacterial surface.

Effect of limiting factors

Even in the presence of an energy source, bacterial growth may be affected by some limiting factor(s), such as an unfavourable physical or chemical environment or the exhaustion of an essential nutrient. It has been suggested that the continuing catabolism of energy substrates can then provide an impetus for the production of polymers, such as the slime material, as either energy reserves or waste products.

Instability of slime production

The variability of the rate at which lactococci produce slime is a well-known phenomenon, especially during incubation at higher temperatues, e.g. 30 °C. In addition, the serial transfer of a ropy strain, even at lower temperatures that normally favour the production of slime material, often leads to the loss of this trait. Sometimes, this unpredictability of slime formation causes difficulties in the commercial manufacture of ropy fermented milks.

Although the loss of the ropiness trait in starter strains has been observed in the commercial production of ropy milks, a similar problem has not been reported in the traditional manufacture of these products, in which a mixture of undefined organisms is used. A mixed population of lactococci and leuconostocs is likely to have effective mechanisms for retention in the culture of the plasmid(s) on which the ability to produce capsular material is encoded, especially if other properties important for the survival of the bacterial population are encoded on the same plasmid(s). The data reported in **Table 1** lend support to this concept.

Bacteriophages

Attack by bacteriophages can cause problems in the manufacture of ropy fermented milks. Thirteen phages of different morphology have been found in an extensive study of 90 viili samples from 20 dairies in Finland. The main morphological type was characterized by an isometric head with a long, noncontractile tail. The host organisms for these phages included strains of *Lc. lactis* subspp. *cremoris* and *lactis* biovar *diacetylactis* and *Ln. mesenteroides* subsp. *cremoris*. Some phages differed only in their host specificity.

A phage of nonencapsulated *Lc. lactis* subsp. *cremoris* isolated from viili was shown to degrade the capsules of some strains, including a strain of *Lc. lactis* subsp. *lactis*. The capsules disappeared soon after infection, and maturing phages were seen inside the cells 2 h after infection, appearing as tightly packed bundles. The phage titer declined rapidly after heating for 15 min at 50–70 °C, but particles capable of plaque formation were detected even after heating for 5 min at 100 °C.

Products and Processes

In the production of fermented milks in the home, no heat treatment was applied to milk prior to fermentation, and the milk was inoculated with the residue remaining in the fermentation vessel from the previous batch. In this way, a characteristic flora dominated by lactococci became established in the vessel. Nowadays, in the commercial production of Nordic fermented milks, defined strains of starter microorganisms are used. The type of starter flora has a distinct influence on the properties and shelf-life of these products.

Scandinavian fermented milks fall into six major classes, as shown in **Table 3**. Not all products in each row are identical to each other; the table is provided only as a guide.

Långfil

'Långfil' or tätmjölk has a mild, sour taste and a ropy, doughlike consistency produced by encapsulated variants of lactococci. Occasionally, lactobacilli are also found in this product. Traditionally, the leaves of plants such as butterwort (*Pinguicula vulgaris*) and sundew (*Drosera* spp., especially *Drosera rotundifolia*) were added to milk before leaving it to sour; these seem to exert a rennet-like action. No relationship has been established between the bacterial flora of butterwort or other plants and the bacterial composition of långfil.

Långfil cultures were preserved for later use by simple techniques, such as dipping a piece of cloth (in Sweden) or birch twigs ('tettemelk', in Norway) in the finished product and then allowing them to dry. A new batch was started by immersing the twigs or placing the dried cloth in fresh milk.

Viili contains various lactose-fermenting yeasts and *G. candidum*, in addition to lactococci and leuconostocs. The cream layer is usually covered with the mold. Viili is made from nonhomogenized milk and is eaten with a spoon.

Pitkäpiimä, which is always ropy, is used as a drink. In domestic production, the cream layer, removed from the pitkäpiimä after fermentaion, was used to make butter. Viilipiimä, from which the cream is not removed, also serves as a drink.

The following steps are included in the commercial production of viili:

- standardization of the fat content at the minimum level of 3.9%
- heat treatment at 89–90 °C for 15–30 min or 92–96 °C for 4–5 min
- cooling to 18–20 °C
- addition of 4–8% starter culture
- incubation for 18–20 h to reach the pH of 4.6
- cooling to the storage temperature of 8–10 °C.

The final pH, after cooling, is 4.3–4.4 and the shelf-life is between 10 and 15 days.

Table 3 The major classes of Scandinavian fermented milks[ab]

Sweden	*Norway*	*Denmark*	*Finland*
Långfil	Tettemelk	–	Viili
Filmjölk	Kulturmelk	Tykmælk	Talouspiimä
Lättfil	Skummet kulturmelk	–	Rasvatonpiimä
Kärnmjölk	Kjernemelk	Kaernemælk	Kirnupiimä
Gräddfil	Rømme	Crème fraîche	Kermapiimä
Lactofil	–	Ymer	Kokkeli

[a] Each row represents a separate class of products.

[b] There are also many special product names used by commercial manufacturers of fermented milks.

Adapted from Bertelsen (1983).

Cultured Milk

'Filmjölk', sometimes also known as 'kulturmjölk' (cultured milk), is a popular Swedish fermented milk characterized by a typical flavour and aroma (derived primarily from diacetyl) and a fairly high viscosity, with a fat content of 3.0%. Filmjölk, which is used as a drink, was developed in the early 1930s. The starter culture contains the acid-producer *Lc. lactis* subsp. *lactis* and the flavour and aroma producers *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Ln. mesenteroides* subsp. *cremoris*. All these strains are propagated together.

In the production of filmjölk:

- standardized milk is often deaerated at 78 °C, to prevent or to alleviate defects such as syneresis of the coagulum, granulation, lumpiness and low viscosity
- the deaerated milk is homogenized at 10–20 MPa and 70 °C, and then heated at 95 °C for 2–6 min
- the milk is cooled to 17–24 °C and inoculated with 1% starter culture
- after 17–24 h, a coagulum forms at pH 4.6
- the product is cooled to 8 °C and packaged into retail containers.

Normally, a shelf-life of 10 days at 8 °C is achieved.

Filmjölk with 0.5% fat is called 'lättfil' the same product is known as 'skummet kulturmelk' in Norway.

'Tykmælk', a characteristic Danish fermented milk, is produced commercially in a similar manner to the Swedish filmjölk; its fat content is 3.5%.

Buttermilk

Buttermilk ('kärnmjölk', 'kjernemelk', 'kærnemælk', 'kirnupiimä') of traditional type is made by churning cultured cream that has a relatively low fat content. In this manner, a small amount of cultured butter and a relatively large volume of buttermilk is obtained. A product known as 'cultured buttermilk' is made by the fermentation of skim milk or low-fat milk.

Starter cultures contain, in addition to the bacteria used for filmjölk manufacture, *Lc. lactis* subsp. *cremoris*. Slime-productung strains are included in the starter cultures for some types of cultured buttermilk made in Finland. The production process involves:

- heat treatment at 85–90 °C for 20–30 min or 92–96 °C for 4–5 min
- cooling to 20–23 °C
- incubation with 1–4% starter culture
- fermentation for 15–20 h
- cooling to 5–10 °C, during which the pH drops to 4.4–4.45.

The flavour and aroma of cultured buttermilk are very similar to those of filmjölk. The shelf-life varies from 7 to 12 days.

Cultured Creams

'Gräddfil', 'rømme', 'crème fraîche' and 'kermapiimä' are cultured creams made by the fermentation of heat-treated cream with the same cultures that are used in the manufacture of cultured buttermilk. The legal requirements regarding the fat content of cultured creams and the production practices vary between Scandinavian countries. In Denmark, for example, production involves:

- standardization of the cream at 9%, 18%, 38% or 50% fat, then homogenization
- heat treatment at 90 °C for 5 min
- cooling to the incubation temperature, 20–27 °C
- inoculation with 2% starter culture
- incubation in the tank for 16–20 h
- cooling to 5 °C, during which the pH of the final product falls to 4.4.

Concentrated Fermented Milks

These include 'ymer', 'lactofil', 'kokkeli' and skyr.

Ymer is a Danish fermented milk that contains at least 11% non-fat milk solids (including 5–6% protein) and 3.5% fat. It is usually produced from the ultrafiltered retentate, which contains about 15% total solids. Production involves the following:

- blending with cream and homogenization
- heat treatment and cooling to 20–27 °C
- inoculation with 4% starter culture containing *Lc. lactis* subspp. *lactis* and *cremoris* and *Ln. mesenteroides* subsp. *cremoris*
- fermentation until the pH reaches 4.5 (after 16–20 h)
- stirring, cooling to 5 °C and storage for 24 h
- stirring again and packaging.

The cooling is often carried out in two stages, first to 14 °C and then to 5 °C.

In the traditional process, fermentation of the skim milk is followed by cutting of the coagulum and drainage of the whey at 40 °C. Alternatively, a quark separator can be used to remove the whey. Pasteurized cream is then blended with the skim product, and this is followed by homogenization, cooling to 12–14 °C and packaging. The shelf-life of ymer is 20 days at 5 °C.

Lactofil, produced in Sweden, is similar to ymer. The fat content in lactofil is 5% and the starter culture is similar to those used in the manufacture of filmjölk and karnmjölk.

Kokkeli, traditionally made in eastern Finland, is prepared by warming spontaneously soured milk in an oven and removing the separated whey.

Skyr, produced in Iceland, is manufactured from skim milk. Unlike all other traditional Scandinavian fermented milks, it is fermented by a thermophilic flora. Starter cultures for the commercial manufacture of skyr consist of *Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. casei*. The protein coagulum is heated to facilitate the syneresis. Whey separation is achieved either by straining through linen bags or using a quark separator. Ultrafiltration is sometimes used to recover whey proteins, which are added to skyr before packaging. Microorganisms isolated from home-made skyr include thermophilic lactic acid bacteria (*Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*) and lactose-fermenting yeasts.

Product Composition and Shelf-Life

Composition

The composition of Nordic fermented milks in terms of total solids and fat content is determined by law. Often, the minimum number of viable microorganisms is prescribed by the food standards.

Starter activity in milk leads to production of lactic acid, as well as flavour and aroma compounds. For example, *Lc. lactis* subsp. *lactis* biovar *diacetylactis* metabolizes the citrate present in milk to diacetyl, acetoin, 2,3-butylene glycol and CO_2. Citrate is also metabolized by leuconostocs, mainly to diacetyl and acetoin (at $pH \leq 5.5$), and acetate (at $pH > 5.5$).

Differences in vitamin content have been observed between fermented milks and raw milk. For example, viili and cultured buttermilk (containing 1.9% fat) were found to contain more folate than raw milk, by 48% and 65%, respectively. However, their vitamin C content was lower than that of milk, by 58% and 24%, respectively, and their riboflavin was lower by 12% and 11%, respectively. The concentration of vitamin B_{12} in both products was also lower than in milk, by >20%.

Shelf-Life

Very high numbers of viable bacteria have been observed in some spontaneously fermented milks. This may be due to the reduced rate of cell autolysis, which has been attributed to the lipoteichoic acid present on the cell surface.

The shelf-life of traditional ropy fermented milks is exceptionally long. The glycocalyx synthesized by bacteria improves the rheological behaviour of these products. Features such as the cream layer and the presence of the mold *G. candidum* on the surface of some products provide added protection from spoilage organisms, thus further extending the shelf-life.

Commercially made products are also noted for their long shelf-life. Even if some pre-existing defect reduces the water-holding capacity of the protein matrix, for example, if proteolysis is allowed to proceed unhindered in raw milk, the presence of glycocalyx disguises the syneresis of the protein coagulum.

Health-Related Effects

Considerable progress has been made in demonstrating certain beneficial effects of Nordic fermented milks in animals. However, unequivocal experimental and/or epidemiological evidence still needs to be gathered to substantiate claims of similar effects in humans.

Effect on Immunity

It has been suggested that fermented milks may play an immunomodulating role; for example, they stimulate the functions of gut-associated lymphoreticular tissue, and this effect has been attributed mainly to antigenic structures of the surface of lactococci. In particular, *Lc. lactis* subsp. *cremoris* isolated from viili has been shown, in studies with human lymphocyte cultures, to stimulate the secretion of immunoglobulins, primarily those of the IgM class. In addition, T lymphocytes showed considerable proliferation in response to the same strain.

A significant induction of the cytotoxicity of peritoneal murine macrophages against sarcoma cells, by *Lc. lactis* subsp. *cremoris* isolated from viili, has been demonstrated *in vivo*. An intraperitoneal injection of the freeze-dried cells retarded the growth of ascitic and solid sarcomas in mice. However, the same preparation showed no direct cytotoxic activity against the same sarcoma cells *in vitro*, which suggests that the antitumour effect of this organism is through the enhancement of the cytotoxicity of the host's macrophages. The exact mechanism of this enhancement is not known, but the slime has been observed to increase glucose consumption *in vitro* by intraperitoneal macrophages.

Freeze-dried preparations of viili, långfil and ropy yogurt, used as intraperitoneal injections daily for 9 days after tumour inoculation, also significantly retarded the growth of murine solid sarcomas *in vivo*. The maximum antitumour effect was induced by the dose of $10\,\mathrm{mg\,kg}^{-1}$ of the villi preparation, $50\,\mathrm{mg\,kg}^{-1}$ of the långfil preparation and $100\,\mathrm{mg\,kg}^{-1}$ of the ropy yogurt preparation. Thus, the effect of both viili and långfil was clearly stronger than that of ropy yogurt. All three preparations significantly enhanced the delayed cutaneous hypersensitivity response to oxazolone, which was depressed in tumour-bearing mice. The antitumour effect of these ropy milks is thought to be mediated by the immune responses associated with host's macrophages and/or T cells.

Antimutagenic Activity

Strains of *Lc. lactis* subsp. *cremoris* isolated from viili, both ropy and nonropy, reduced the mutagenicity of nitrosated beef extract by 40%, as determined by the Ames test, using *Salmonella typhimurium* as the test organism.

Lowering of Serum Cholesterol

In experiments on rats fed diets containing viili, nonropy fermented milk or acidified skim milk, the serum cholesterol level of rats on a viili-containing diet was lowest of the three groups. Their ratio of high-density lipoprotein cholesterol to total cholesterol was the highest of the three groups. The mechanism of this cholesterol-lowering effect is unknown.

Antibacterial Effects

Antagonistic effects of lactic acid bacteria against common pathogens and spoilage bacteria have been well demonstrated *in vitro*. For example, lactococci and their capsular material have been shown to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli* and some clostridia.

Future Trends

The future position of Nordic fermented milks in their traditional markets seems assured, despite the popularity that yogurt and 'probiotic' products have gained in the past few decades.

Many types of fermented milks are nowadays produced in Scandinavia and some of them do not clearly fit into the categories outlined above; for example, products that are mixtures of ropy milks with acidophilous milk. With the growing interest in various 'probiotic' cultures, this trend is expected to continue, with more combinations of this kind being offered to consumers.

See also: **Fermented Milks**: Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Kefir; Middle Eastern Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture.

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Middle Eastern Fermented Milks

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Introduction

The evolution of fermented milks in the Middle East can be traced to the prehistoric era, although there is no tangible evidence for how people at that time knew how to ferment milk. However, there is a concrete evidence of milk fermentation by later communities apparent from the archaeological remains found in Egypt, Iraq, and Syria. Fermented milks became an integral part of the diet of these people.

In order to understand the situation of traditional milk processing and utilization in this part of the world, one should recall the following points:

1. Milking animals are not limited to cows: sheep, goat, camel, and buffalo milks are available in significant quantities.
2. Milk production has an obvious seasonality related to climatic conditions.
3. A significant part of the inhabitants are nomads, dessert dwellers, and people living in the rural areas who tend to preserve surplus sour milk in different products with extended shelf-life.
4. Most of these products are homemade following neither standardized conditions nor proper hygiene standards.

However, this picture has changed dramatically in the second half of the twentieth century. The modern dairy industry and its products have begun to replace the indigenous fermented milks. Several attempts have been made to develop and standardize the indigenous fermented products and some of these developments have been applied on industrial scale.

Type of Fermented Milks

The fermented milk products of the Middle East can be classified in different ways. However, the following classification has been adopted, which is based on the total solids (TS) content of the product.

1. Fermented milks with normal milk composition (12–18% TS), which can be either liquid or set gel. This group includes 'zabady', 'laban rayeb', 'laban kad', and 'gariss'.
2. Concentrated fermented milks (20–40% TS). This group includes 'labneh' and 'laban zeer'.
3. Dried fermented milks (>85% TS), which include 'kishk' and related products.

Fermented Milks with Normal Milk Composition (12–18% TS)

Zabady (Laban Zabady)

Zabady is the traditional plain set-type yogurt in Egypt and several Arabic countries. The main differences between zabady and the classical yogurt are summarized in **Table 1**.

Zabady is usually made from buffalo milk (partially skimmed). The milk is boiled for few minutes, left to cool to 37–45 °C, and then inoculated with part of the previous batch as starter culture. The milk is distributed into the marketing packages (traditional porcelain pots have been replaced nowadays with shallow plastic containers with separate lids). The containers are transferred to an incubator and left uncovered. This allows the formation of the surface skin. Also fat globules rise during the incubation giving a fat-rich top layer. After the zabady has set, the containers are removed, covered, and stored in the refrigerator. Normally, zabady is made by small producers (milk shops) to be distributed and consumed within 24 h of its manufacture.

Chemical composition

The chemical composition of zabady is close to that of yogurt. Its gross composition falls within the following range: fat, 2.60–3.67%; protein, 2.91–4.96%; lactose, 2.92–3.98%; ash, 0.7–0.82%; and titratable acidity, 0.93–1.20%. Zabady is characterized by high acetaldehyde (1.67–5.9 mg kg^{-1}) and low diacetyl content (0.0–0.15 mg kg^{-1}).

Microbiology

The count of lactic acid bacteria in zabady falls in the range of 1×10^8–7×10^8, which matches the standard of yogurt. However, it contains large numbers of yeasts and molds (5.0×10^4–6.9×10^5), which can be attributed to postcontamination as zabady surfaces are kept uncovered during incubation.

Streptococcus thermophilus and *Lactobacillus delbrueckii* subsp. *bulgaricus* predominate in zabady. *Bacillus subtilis, Microbacterium lacticum, Alcaligenes tolerans*, some strains of *Micrococcus* spp., yeasts, and also coliform bacteria have

Table 1 Differences between zabady and yogurt

	Zabady	*Yogurt*
Type of milk	Mainly buffalo milk	Cows' milk
Fortification/additives	Nothing used	Skim milk powder, stabilizers, flavors
Heat treatment	Boiling	High-temperature pasteurization
Homogenization	Not homogenized	Homogenized
Starters	From previous batch	Defined starters
Product characteristics	Surface skin with high-fat top layer Cooked flavor	Smooth surface, homogeneous composition
Packages/containers	Shallow porcelain pots/plastic containers with separate lids	Several types of glass and plastic containers with sealed lids

been found as contaminating microorganisms in some market zabady samples.

Zababy has antibacterial properties as it markedly inhibits the growth of *Staphylococcus aureus, B. subtilis, Shigella shigae*, and *Escherichia coli.*

Laban Rayeb

Laban rayeb is an indigenous product in Egypt. Traditionally housewives milk their animals into a shallow earthenware pot ('shalia'/'matred') that has been washed and sterilized by drying in an oven. The milk containers are left undisturbed until the next day in a warm place where milk is fermented by the natural milk flora. The top layer (sour cream) is removed for butter making, while the coagulated bottom layer (laban rayeb) is used as it is. Laban rayeb is also used as a base for the manufacture of karish cheese or in salad.

Laban Kad (Rob)

Small quantities of milk are collected successively in goatskin bags ('kerbah') and left to sour naturally for a period determined by experience and the season. Air is blown into the kerbah, closed tightly, and then shaken until the fat globules coalesce. Laban kad (buttermilk) is the product left after the removal of the butter grain. Laban kad is either made into karish cheese in the cold season or concentrated as laban zeer in the hot season. The containers used for the fermentation and churning are not washed and traces from the previous batch serve as a starter for the new one. The freshly prepared product has the characteristic buttery flavor.

Differences can be found in the flora of laban kad produced in different seasons. Streptococci dominate in the cold season, while lactobacilli dominate in the hot season. The lactic acid bacteria in laban kad include homo- and heterofermentative streptococci and lactobacilli. *Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *dextranicum, Ln. mesenteroides* subsp. *cremoris, Lactobacillus casei, Lb. plantarum*, and *Lb. brevis* are found in laban kad.

In Sudan, rob is diluted with 2–3 volumes of water to give a product known as 'ghubasha', a thirst quencher. Pastoralists in Sudan make salad from a mixture of rob and grated fruit of a wild plant called 'feggous' (*Cucumis melo*).

Gariss

Gariss is made from camel's milk in Sudan. Traditionally, the milk is fermented in two large leather bags lashed to the saddle of a camel. Whenever part of the product is taken for consumption, an equal volume of camels' fresh milk is added. The microorganisms for the fermentation of gariss have been identified as lactobacilli (*Lb. helveticus, Lb. delbrueckii* subsp. *lactis*) and yeast that belong to the genera *Candida* and *Kluveromyces*. Gariss has the following composition: lactose, 1.35–1.40%; fat, 2.15–2.40%; protein, 3.40–3.85%; ash, 0.75–0.80%; ethanol, 1.3–1.4%; volatile fatty acids, 0.13–0.20%; titratable acidity, 1.0–1.8%; pH, 3.25–3.40.

Concentrated Fermented Milks

Labneh

Labneh (22–26% TS) is a popular product in Lebanon and Syria. It has a soft, smooth, and spreadable texture with clean acid flavor. Labneh is made from cow, sheep, goat, or buffalo milks. Consequently, differences are expected in the composition and properties of labneh according to the different milks used.

Manufacture

Labneh is usually made from full-cream yogurt or zabady. The method of making labneh differs slightly in the different producing countries. In Egypt, yogurt is stored overnight in a cooled room. The next day, salt is added to the yogurt and mixed well to ensure uniform distribution of salt and to break the gel structure. The stirred yogurt is then transferred to cheesecloth bags, hung on racks, and left to drain for about 12–24 h. Labneh is then packaged in suitable containers and stored under refrigeration. In Saudi Arabia, a similar method is followed but the straining of the yogurt is enhanced by a light pressing of bags filled with yogurt. In Lebanon, labneh is made by straining stirred yogurt without the addition of salt. Labneh is garnished before consumption with dried herbs, salt, and olive oil and served with bread.

The traditional methods for making labneh have been subjected to modifications to overcome the substantial time needed for whey removal and to offer proper hygienic conditions during processing. These modifications include (1) removal of whey by mechanical separation, (2) culturing ultrafiltered milk retentate, (3) ultrafiltration of fermented milk, and (4) the use of low-lactose high-heat whole milk powder, which can be reconstituted to a TS similar to that in labneh to avoid the need for a whey drainage step.

Labneh is also used for the manufacture of another product, 'labneh anbaris' (about 40% TS), in Lebanon. This product is made by shaping labneh into balls and partial sun drying of these balls, which are then packaged into glass jars and covered with olive oil. However, labneh anbaris is properly classified under cheeses rather than fermented milks.

Characteristics

Labneh has a soft, smooth, spreadable, and creamy texture. Fresh labneh has no sign of wheying off, with clean acid taste and milky white color. The method of making labneh has a determining role in the texture and structure of the final product. Homogenization has a marked effect on the firmness of labneh made from goat and sheep milk and to a lesser extent on that made from cows' milk. A homogenized labneh is less firm than an unhomogenized one. Labneh made by ultrafiltration of coagulum has rheological properties closer to the traditional labneh than that made by the fermentation of ultrafiltered milk retentate. The microstructure of labneh is greatly influenced by the temperature of concentration: a firmer product is produced when the yogurt is ultrafiltered at 50–55 °C. This effect is associated with the formation of complex micellar chains, which are not evident in traditional labneh and in products made by ultrafiltration at 35 °C.

Chemical composition

The composition of labneh falls within the following ranges: fat, 9–11%; protein, 8.5–9.0%; lactose, 3.5–4.0%; TS, 22–26%; titratable acidity, 1.5–2.5%; and sodium chloride, about 1%.

Microbiology

Labneh is normally made from naturally fermented milk or plain yogurt. However, several other starters can be used in its manufacture. A mixture of *Lc. lactis* subspp. *lactis* and *cremoris* has been used at the rate of 2% as starter for the manufacture of labneh. Also, different combinations of some strains of *Enterococcus faecalis* isolated from laban rayeb and *Lb. delbrueckii* subsp. *bulgaricus* have been used to produce labneh.

Laban Zeer

Laban zeer is a concentrated fermented product made in some parts of Egypt. The fermented buttermilk ('laban khad') obtained from the churning of naturally fermented cream is stored in an earthenware jar ('zeer'). The walls of the zeer are porous, which allows the filtration of water and subsequent evaporation from the outer surface of the zeer. This gives rise to an increase in the TS of the zeer's contents. Laban zeer contains 11.5–17.4% total protein and about 0.2% soluble nitrogen. The finished product has a pH value of 3.5–3.8. The product has a high total bacterial count (10^8–10^9 cfu g^{-1}) and yeast count (10^6–10^7 cfu g^{-1}). The genus *Bacillus* is the most prominent type of bacteria found in laban zeer followed by *Lactobacillus* spp. The yeasts belong to the genera *Actinomyces* and *Saccharomyces*. Laban zeer serves as the base for the manufacture of kishk (see below).

Biruni

Biruni is a typical product of the Nuba Mountains in Sudan. Cows' milk is stored in large gourds in which milk undergoes fermentation and concentration through filtration and evaporation from the outer surface of the gourds. Fresh milk is added continuously to the old fermented and concentrated milk. Biruni is allowed to age for at least 1 year and up to 10 years before its consumption. It turns brown with a rancid flavor. The product falls in consistency between fermented milk and soft cheese. However, no report is available on its composition or microbiology.

Dried Fermented Milk Products (Kishk and Related Products)

Dried fermented milk products are traditionally produced to secure nutritive supplies during seasons of limited milk availability. The products can be eaten as biscuits with or without tea or after reconstitution in different ways, for example, as a porridge-like product, in soup preparation, or as a beverage.

Kishk has several names and varying composition throughout the Middle Eastern countries. The products in this group can be subdivided into the following:

1. Dried fermented milks without additives: ‘kurut’ (Turkey) and ‘madeer’ and ‘oggt’ (Saudi Arabia)
2. Products based on mixtures of fermented milks and cereals: kishk (kushuk, kichk) (Lebanon, Syria, Egypt)
3. Flavored products containing cereals and other additives (vegetables, spices, herbs, or fruits): ‘kashk’ and ‘kekg’ (Iran) and ‘zhum’ (Yemen).

Of the dried fermented milk products, kishk is the major and most important one. It has received much attention and developmental work.

Manufacture

The cereal additive (‘burghol’) used in kishk is prepared from soft wheat. The grains are cleaned of stalks and dirt, then sized, and the large grains are steeped in boiling water for 1 h until soft. The grains are then dried in the sun for 24 h. The following day, the dried grains are moistened with water (about 20%), cracked and dehusked by winnowing, and then sized as coarse and fine. It is recommended that coarse burghol be used for kishk making.

The fermented milk (skim milk or low-fat yogurt; laban zeer) is mixed with burghol at a ratio of 2:1 to 4:1 and NaCl (about 6%) is added. The mixture is left for several days with continuous kneading to complete fermentation and to achieve proper hydration of the burghol. The mixture is then shaped into small balls or nuggets and dried for 7–8 days in the hot shade or sun. The dried kishk is either milled to a powder or packed as dried balls or nuggets. The final product is not hygroscopic and can be stored for 2–3 years without deterioration. Kishk with a high fat content does not store well since it is prone to oxidative rancidity.

Chemical Composition and Nutritive Value

The chemical composition of kishk varies widely as follows: moisture, 3.0–16.0%; protein, 8.9–54.5%; fat, 1.6–19.9%; carbohydrate, 31.0–65.7%; dietary fiber, 0.5–2.5%; and ash, 3.8–9.5%. These differences are due to several factors, namely differences in the methods of manufacture, efficiency of fat separation of the fermented milk or buttermilk, and the ratio of fermented milk to cereal additive.

Kishk contains substantial amounts of starch (digestible and nondigestible fractions) and fiber that originate from burghol. In addition, an appreciable amount of galactose is present in kishk as the microflora responsible for the fermentation of kishk is not able to metabolize galactose.

The milk proteins undergo extensive proteolysis in kishk. The major casein fractions can be detected with difficulty in kishk samples. Kishk can be considered as a good source of iron and manganese, which originate from burghol.

Kishk has a characteristic pattern of organic acids, which reflect the fermentation processes during its preparation. Lactic, propionic, acetic, orotic, uric/formic, and citric acids have been identified in kishk samples. Compared to yogurt, kishk contains 2–3 times the amount of lactic acid, and has higher propionic and lower orotic, citric, and uric/formic acid contents.

Kishk can be considered as a functional healthy product. Fermenting milk makes it easier to digest and the presence of fiber is in accordance with the current nutritional trends.

Microbiological Quality

The acidic nature of kishk, its low moisture content, and the high salt content explain the good microbiological quality of traditional kishk. Coliforms and *S. aureus* are generally absent in market samples of traditional kishk.

See also: **Fermented Milks**: Health Effects of Fermented Milks; Types and Standards of Identity. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactococcus lactis*. **Yeasts and Molds**: *Kluyveromyces* spp.; Yeasts in Milk and Dairy Products.

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Asian Fermented Milks

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Introduction

The origins of fermented milk are unclear. However, when the goat was first domesticated in Mesopotamia in about 5000 BC, the nomadic communities stored the milk warm in animal skin bags or in crude earthenware pots in a hot climate, which led to spontaneous curd formation. Fermented milks probably originated in the Middle East before the Phoenician era. The Old Testament mentions that fermented cream existed in Mesopotamia in *c.* 1300 BC and laban rayed and laban khad were manufactured perhaps as early as 5000 BC. Kumys was probably known in the Ukraine around 2000 BC and dahi was fermented in India as early as 1500 BC. The skills of making fermented milk were introduced to Russia and other European areas by the Tartars, and to central Asia by the Huns and Mongols. The 'Silk Road' contributed to the early development and spread of fermented milks. A common characteristic of all fermented milks is the presence of lactic acid.

Various methods have been used for the manufacture of traditional fermented milks. The bacteria essential for fermentation originate from fermentation vessels, raw milk, the alimentary tract of animals, and plants. Warm raw milk from the cows, sheep, goats, water buffaloes, camels, or horses of the nomads was turned into clabber or curd by indigenous bacteria, by means of traditional fermentation.

Dahi (Dadhi)

Yogurt-like products are widely produced in the Mediterranean area, Asia, Africa, and central Europe. In India, dahi, which resembles yogurt, is still made traditionally at home using the milk of various mammals, for example, water buffaloes, cows, and goats. Dahi is a semisolid product obtained from pasteurized or boiled milk by souring naturally, or otherwise using a lactic acid culture. A commercially available mixture of buffaloes' and cows' milk is used. The milk is boiled, cooled, inoculated with dahi starter left over from the previous day, and incubated at ambient temperature for 4–6 h until it turns to a thick consistency. Dahi made from buffaloes' milk is thick because of the higher solids content of buffaloes' milk. The composition of the milk of various dairy species is shown in **Table 1**. Industrial production of dahi is now being attempted in metropolitan areas.

Mild dahi is made with mesophilic lactococci. *Leuconostoc* strains can be used as adjunct organisms for added buttery odor and flavor. Sour dahi contains additional cultures of thermophilic microorganisms, which are generally employed in the manufacture of yogurt. It takes less than 4 h to produce dahi. Dahi makes an important part of Indian meals. It is an essential material to make Indian cold soup 'raita' and curry 'kadhi'. Additionally, dahi may be consumed as a sweet or savory 'lassi' drink or as a dessert containing sugar and fresh-diced banana, orange slices, mango, and other seasonal fruits. The microorganisms involved in the production of dahi and other fermented milks of Asia, as well as the major functions of starter cultures, are listed in **Table 2**.

Kumys

Kumys (koumiss, kumis) (*see* **Fermented Milks**: Koumiss) is a unique lactic acid–alcohol fermented milk drink that originated in central Asia. The name is derived from the Kumanese, who survived until 1235 as a Kumane River tribe on the central Asian steppes. The proteins of horses' milk are somewhat different from those of the milk of other species because when the milk is fully renneted, it forms no visible curd, owing to higher whey protein and lower casein content. The fat content of horses' milk is lower than that of cows' milk. Traditionally, kumys was made from horses' milk, but now that horses' milk is scarce, cows' milk is used for the preparation. To overcome the difference in the casein and whey protein ratio between horses' and cows' milk, a membrane ultrafiltration method is used. By this method, the whey protein content could be adjusted, so that whey protein is concentrated, but not lactose.

Lactose-fermenting yeasts, such as *Candida* spp., *Kluyveromyces lactis*, and *Torula* spp., and the thermophilic starter *Lactobacillus delbrueckii* subsp. *bulgaricus* are involved in the fermentation process. The lactic acid bacteria

Table 1 Composition of milks used in the preparation of fermented milks

Species	*Fat (%)*	*Casein (%)*	*Whey protein (%)*	*Lactose (%)*	*Ash (%)*	*Total solids (%)*
Cow	3.7	2.8	0.6	4.8	0.7	12.6
Buffalo	7.5	3.2	0.6	4.9	0.8	16.9
Goat	4.5	2.5	0.8	4.4	0.8	13.0
Sheep	7.5	4.2	1.4	4.4	0.9	18.4
Horse	1.6	1.0	1.1	6.0	0.4	10.1
Camel	4.2	3.7[a]		4.1	0.8	12.8
Yak	6.5	5.5[a]		5.0		17.0

[a] Total protein content.

Adapted from Ling ER, Kon SK, and Porter JWG (1961) The composition of milk and the nutritive value of its components. In: *Milk*, Vol. 2, pp. 195–263. London: Academic Press; Fox PF and McSweeney PLH (1998) Production and utilization of milk. In: *Dairy Chemistry and Biochemistry*, pp. 1–2. London: Blackie Academic and Professional; Degan AA (2007) Sheep and goat milk in pastral societies. *Small Ruminant Research* 68: 7–19.

(LAB) are responsible for acid production, and the yeasts are responsible for the production of ethanol and carbon dioxide. Some starter cultures may include *Lactobacillus acidophilus* and *Lactococcus lactis*, as shown in **Table 2**.

In the commercial manufacture of kumys from horses' milk, the bulk starter is prepared first. A yeast culture of *Torula* spp. and the LAB culture are inoculated separately into pasteurized skim milk from cow and incubated at 30 °C for 15 h and at 37 °C for 7 h, respectively. To prepare the bulk starter, the incubated cultures are added to horses' milk and incubated at 28 °C for about 4 days, after which the titratable acidity usually reaches 1.4%. Thirty percent of the bulk starter is added to the fresh horses' milk at 28 °C and the milk is agitated vigorously to introduce air essential for good yeast growth. The fermenting milk is dispensed into bottles with crown-capped closures and incubated for a further 2 h at 20 °C. The product is stored at below 6 °C until sold.

Large-scale production of kumys from cows' milk is carried out by adding 2.5% sugar and 10% starter (*Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, and *Saccharomyces lactis*). The inoculated milk is incubated at 26–28 °C until a firm curd forms at the titratable acidity of approximately 0.8%. It is held at approximately 17 °C with aeration and intermittent stirring for the optimum yeast growth and, after several hours, the titratable acidity reaches 0.9%. The acidified milk is then bottled. Accumulation of alcohol and carbon dioxide occurs during ripening and the product is subsequently stored at or below 6 °C until sold.

Kumys is a milky white liquid with a grayish cast. It is not watery but possesses a uniform consistency without any tendency to flake or whey off. A starter for kumys patented in 1990 in the former USSR consisted of *Lc. lactis* subsp. *lactis*, *Lb. delbrueckii* subsp. *Bulgaricus*, and the yeast *Torula* spp. The milk is first aerated by stirring for 20–25 min and is then kept at room temperature for 2–3 h. Lactic acid, ethanol, and carbon dioxide are the end products. Hence, kumys has a sourish flavor and bubbles on shaking.

Kumys is produced with varying contents of lactic acid and alcohol. It is classified into three categories depending on the extent of fermentation. Mild-flavored kumys has an acidity of 0.6–0.8% with an alcohol content of 0.7–1.0%; medium-flavored kumys contains 0.8–1.0% lactic acid and 1.1–1.8% alcohol; and strong-flavored kumys has an acidity of 1.0–1.2% with 1.8–2.5% alcohol. Viable counts of 4.97×10^7 and 1.43×10^7 cfu ml^{-1} for bacteria and yeast, respectively, have been reported in kumys.

Yakult

Today, yakult is the best-known fermented milk product in the world on account of various claims of its health-promoting properties. After the Meiji Restoration (1868) in Japan, dairy products gained general acceptance and were appreciated as a special food for the sick, because this was the period when people were suffering from chronic malnutrition and infections, particularly intestinal infections such as dysentery and colitis. At that time, M. Shirota was aware that certain indigenous intestinal bacteria played a role in the body's defense mechanisms, including by competing with invading pathogenic bacteria. In 1929, he succeeded in isolating and cultivating *Lactobacillus casei* strain Shirota (now reclassified as *Lactobacillus paracasei* subsp. *paracasei*), an indigenous human intestinal bacterium, and in 1935 he developed a fermented milk drink named yakult. Commercially produced yakult is made by fermenting skim milk with this organism. The total milk solids are rather low, about 3.7%, and yakult has 14% added sugar. The fermentation time is around 16–18 h at 37 °C. The viable count at the end of the fermentation exceeds 10^8 cfu ml^{-1}. LAB are widely distributed in nature, but not all of them can survive in the human intestine. *Lactobacillus paracasei* subsp. *paracasei* is occasionally found in the intestine and is capable of reaching the intestine in a viable state. The strain used in yakult manufacture is resistant to gastric juice and bile.

Table 2 Starter cultures involved in the manufacture of commercial and traditional fermented milks of Asia

Product	*Primary microorganism(s)*	*Secondary/optional microorganism(s)*	*Incubation temperature and time*	*Major function of culture*
Calpis	*Lactobacillus helveticus* *Lactobacillus fermentum* *Saccharomyces cerevisiae*		37 °C, 18–20 h	Acidity, aroma, probiotic
Dahi, lassi, cultured buttermilk, and sour cream	*Lactococcus lactis* subsp. *lactis* *Lactococcus lactis* subsp. *cremoris* Citrate-positive strains of *Lactococcus lactis* *Lactobacillus delbrueckii* subsp. *bulgaricus*	*Leuconostoc lactis* *Leuconostoc mesenteroides* subsp. *cremoris*	22–25 °C, 16–18 h	Acidity, flavor, aroma
Dadhi	*Leuconostoc paramesenteroides* *Lactobacillus casei* subsp. *casei* Citrate-positive strains of *Lactococcus lactis*		28–30 °C, 24 h	Acidity, texture, aroma, flavor, probiotic
Kefir	*Lactobacillus brevis* *Lactobacillus kefir* *Lactobacillus acidophilus* *Leuconostoc mesenteroides* *Leuconostoc cremoris* *Streptococcus thermophilus* *Torulopsis kefir* *Torulopsis holmii* *Saccharomyces cerevisiae* *Candida pseudotropicalis*		15–22 °C, 24–36 h	Acidity, aroma, flavor, gas (CO_2), alcohol, probiotic
Kumys	*Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus kefiranofaciens* *Lactobacillus lactis* *Lactobacillus acidophilus* *Lactococcus lactis* subsp. *lactis* *Saccharomyces lactis* *Kluyveromyces lactis*		20–25 °C, 12–24 h 30–37 °C, 7–15 h	Acidity, aroma, flavor, gas (CO_2), alcohol, probiotic
Yakult	*Lactobacillus paracasei* subsp. *paracasei*		30–37 °C, 16–18 h	Acidity, probiotic

From Kosikowski FV (1982) *Cheese and Fermented Milk Foods*, 2nd edn., pp. 37–57. New York: FV Kosikowski and Associates; International Dairy Federation (1988) Fermented milks: Science and technology. *International Dairy Federation Bulletin no. 227*. Brussels, Belgium: IDF; Sansawa H (1999) Mass production of *Lactobacillus casei* strain Shirota. In: Yakult Central Institute for Micro-biological Research (ed.) Lactobacillus casei *Strain Shirota*, pp. 97–102. Tokyo, Japan: Yakult Honsha; Watanabe J, Ikeda N, Mizutani J, *et al*. (1998) Comparison of microbiological and chemical characteristics among types of traditionally fermented milk in Inner Mongolia in China and Calpis sour milk (sannyuu). *Milk Science* 47: 1–8; Lang F and Lang A (1970) A study of kumys manufacture as a potential new outlet for milk. *Milk Industry* 67: 22–25; Sukumar (1980) *Outlines of Dairy Technology*, p. 404. Delhi, India: Oxford University Press.

Many culture methods have been studied to alleviate the inhibition of growth medium. These included batch culture, continuous culture, fed-batch culture, dialysis culture, and extractive fermentation. The best results were obtained in fed-batch culture, by supplying fresh medium to the culture broth continuously or intermittently to maintain a desirable growth environment. In the fed-batch system, culture broth containing lactic acid is separated by filtration and an equal amount of fresh medium is added. This method achieves a very high final cell concentration. A final cell mass of 40 g l^{-1} was obtained using this technique. Batch culture is the simplest method, but both final cell concentration and metabolites are generally low, while continuous culture keeps the culture environment constant and cell productivity has been reported to be 1.05 g cell h^{-1} l^{-1}, over 3 times higher than that reported for batch culture (0.33 g cell h^{-1} l^{-1}).

The characteristic light coffee color of yakult is the result of the Maillard reaction. Glucose, added to skim milk before the heat treatment, plays a major role in this reaction.

Other Traditional Fermented Milks

Products of Mongolia

The fermented milks of the state of Mongolia and the province of Inner Mongolia in China are obtained using LAB and yeast fermentation.

Although fermented milk products are regarded as predominantly lactic fermentations, the frequent interaction of yeast and LAB has led to specific product characteristics and quality. Types of fermented milks from this region include 'edosensuu', 'airag', and 'tarag'. Edosensuu is made from cows' milk and it resembles soft yogurt in appearance. It is fermented by *Lc. lactis*, *Leuconostoc lactis*, and yeast at 17–20 °C, which results in the production of small quantities of lactic acid (0.8%) and alcohol (0.2%). The cream layer is removed after fermentation.

Airag (also called tsege in Inner Mongolia) is made from the milk of various mammals, and especially horse milk and camel milk are considered most valuable. In the traditional Mongolian procedure, the milk is poured into a large sack made of cows' or sheep's leather, or alternatively, a vat made of wood can be used. Within these containers, the milk is stirred with a wooden paddle over 1 or 2 days. This suggests that milk must be supplied with air. The oxygen in the air helps the fermentation process by a combination of lactic acid bacteria and yeast. *Lactobacillus helveticus*, *Lc. lactis*, and yeasts are the dominant microflora of this product, which contains a small amount of carbon dioxide and up to 1–3% of alcohol.

Tarag (also called airag in Inner Mongolia) is like a hard-type yogurt made from cows' milk or sometimes from the skim milk of sheep or goats. *Lactobacillus kefiranofaciens* dominates the microflora of this product. A bacterial culture is added when the boiled milk has cooled to the touch. It takes a few hours until curd is formed. Tarag contains relatively high amounts of lactic acid (pH 3.6–3.9). Tarag is usually consumed just by itself with some fruit added. Additionally, these alcoholic milk products will often be distilled to Mongolian milk liquor (arkhi) with a homemade distiller.

These Mongolian fermented milk products are considered to be a rich source of vitamins and minerals for nomads.

Tsege is often classified as airag, but it is a unique product basically made from horse milk. Traditional tsege contains *Lb. helveticus*, *Lactobacillus fermentum*, and *Saccharomyces cerevisiae*. A similar culture has been used in Japan in the commercial manufacture of a pasteurized fermented milk drink called Calpis.

Calpis was developed in Japan and first manufactured in 1919 based on the fermented milk of Mongolian nomads. This pasteurized fermented milk drink was manufactured from two fermentation processes using *Lb. helveticus* and *S. cerevisiae*. First, a starter culture of yeast and *Lactobacillus* is added to skim milk, thereby turning it into the lactobacillus-cultured milk. In the second fermentation process, sugar is added. Together with the yeast, this gives Calpis its sweet and tangy taste with the hint of an aroma and yogurt flavors.

Calpis contains several peptides derived from milk proteins, which provide a physiological effect such as lowering of blood pressure in spontaneously hypertensive rats and also in hypertensive human subjects. The starter organisms are responsible for the release of a range of potent bioactive peptides.

The well-studied milk peptides are the tripeptides isoleucine–proline–proline (IPP) and valine–proline–proline (VPP), derived from β- and κ-casein, and they inhibit angiotensin-I-converting enzyme (ACE). For more than 90 years, Calpis has continued to be in favor far and wide.

Dadih, which is similar to Indian dahi, has been produced in Indonesia for hundreds of years. The root word seems to be common. The Minangkabaus of West Sumatra traditionally ferment raw milk from buffaloes in fresh bamboo tubes capped with banana leaves to produce dadih. Interestingly, no heat treatment is applied to milk, which is fermented spontaneously by indigenous LAB present in the raw milk from buffaloes. The milk curdles after 24 h fermentation at 28–30 °C. At the end of fermentation, the total count of LAB is typically 10^8 cfu g^{-1}.

The major bacterial species found in this product are *Lb. casei* subsp. *casei*, *Leuconostoc paramesenteroides*, and citrate-positive strains of *Lc. lactis*. Some strains are acid and bile tolerant and their cell wall shows an ability to bind mutagenic 3-amino-1,4-dimethyl-5*H*-pyrrido-[4,3-*b*]-

indole. The composition of LAB involved in spontaneous fermentation may vary depending on the conditions of fermentation.

It is believed in West Sumatra that dadih has a beneficial effect on health. Claims of prophylactic and therapeutic properties of indigenous LAB isolated from dadih warrant further investigation. These bacteria may be a useful addition to the probiotic organisms currently used in the commercial manufacture of fermented milks around the world.

See also: **Fermented Milks**: Health Effects of Fermented Milks; Kefir; Koumiss; Middle Eastern Fermented Milks; Starter Cultures; Types and Standards of Identity; **Yeasts and Molds**: Yeasts in Milk and Dairy Products.

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Koumiss

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Introduction

Koumiss (kumys or kumiss), fermented equine milk, is widely consumed in western and central Asia (e.g., Mongolia, Kazakhstan, and Kyrgyzstan) and Russia for its nutritive and therapeutic properties. Koumiss is milky-gray in color and effervescent, with a sharp alcohol and acidic taste. Russians, in particular, have long advocated the use of koumiss for the treatment of a wide variety of illnesses, but the variable microbiology of this product has made it difficult to confirm any theoretical basis for the claims. The name koumiss is derived from the Kumanese tribe, who survived until ~1237 along the Kumane river in the central Asian steppes. Koumiss has been produced since ~2000 BC and was drunk by the earliest herdsmen, the Aryans, of central Asia. The Scythians, who existed long before the Christian era, are said to have consumed fermented milk; various artifacts have been excavated in central Asia, Russia, and Ukraine that detail the preparation of koumiss by these nomadic Scythian tribes.

In Mongolia, koumiss has high social, ritual, and religious value and is the national drink (airag or chigee); a high-alcohol drink made by distilling koumiss, called arkhi, is also produced. In the fourteenth century, the Mongols sprinkled koumiss on banners to ensure victory in battle, a custom that was witnessed and recorded by Marco Polo in his 'Memoirs of the Orient' where he referred to the Tartars (Mongols) liking for koumiss.

The first documented account of koumiss is thought to be a reference to it in the Ipatof Chronicles in the twelfth century and later during the thirteenth century, a French missionary, William de Rubruquis, wrote about his liking for koumiss, a drink he found on his travels through Tartary. The first account of the therapeutic properties of koumiss is thought to have been written in 1784 by a surgeon in the Russian army, Dr. John Grieve, who advocated its use in the treatment of tuberculosis. Later, the first organized use of koumiss for medical treatments began with the establishment of a hospital producing and specializing in the therapeutic use of koumiss in 1858 near Samara, Russia, by Dr. N.V. Postnikov. In 1866, he wrote a book on the therapeutic value of koumiss for the treatment of digestive, urogenital, and cardiovascular diseases. Doses of up to $1.5\,\mathrm{l\,day^{-1}}$ were frequently used for patients but were determined based on an individual's needs. At present, there are ~23 hospitals using koumiss therapy, combined with antibiotic and other treatments, in the former USSR. Sanatoriums in Kazakhstan use ~2 million liters of koumiss a year, while those of Kyrgyzstan use ~100 000 l and the Crimean region ~140 000 l. Kööřrzik, koumiss-based brandy, diluted with fresh equine milk, is used today in some parts of Russia for infant nutrition, while Bässrik, a fermented mixture of equine and bovine milk, is consumed elsewhere by adults. The principal breed of horse used for milk production is the relatively new Kirghiz breed, which produces over 2500 l of milk per lactation, with a daily average of ~16 l. Mongolia has ~0.5 million milking mares and produces ~13.5 million liters of koumiss annually (~30% of its raw equine milk resource). Per caput consumption of koumiss in Mongolia is estimated at about 50 l per year.

Because equine milk resembles human milk in many respects, and is claimed to have special therapeutic properties, it is becoming increasingly important in western Europe, especially in France, Italy, Hungary, and the Netherlands. Equine milk and koumiss are often used in these countries for the treatment of a myriad of ailments including anemia, nephritis, diarrhea, gastritis, and cardiovascular disease and in postoperative care, as well as for stimulation of the immune system. Koumiss is still manufactured in remote areas of Mongolia by traditional methods, but with increased demand elsewhere, especially in western Europe, it is now also produced under more controlled and regulated conditions.

Fermentation of Equine Milk

Milk has always soured spontaneously but at some point in human history artisans deliberately caused milk to sour or ferment. Fermentation is one of the oldest methods for preserving milk and probably dates back ~10 000 years to the Middle East where the first evidence of organized food cultivation and production is known to have occurred. Traditional fermented milk products have been developed independently worldwide and were, and continue to be, especially important in areas where transportation, pasteurization, and refrigeration facilities are inadequate. There are ~400 named traditional and industrially fermented milk products, which can be divided into three broad categories based on their metabolic products, that is, lactic fermentation, yeast–lactic

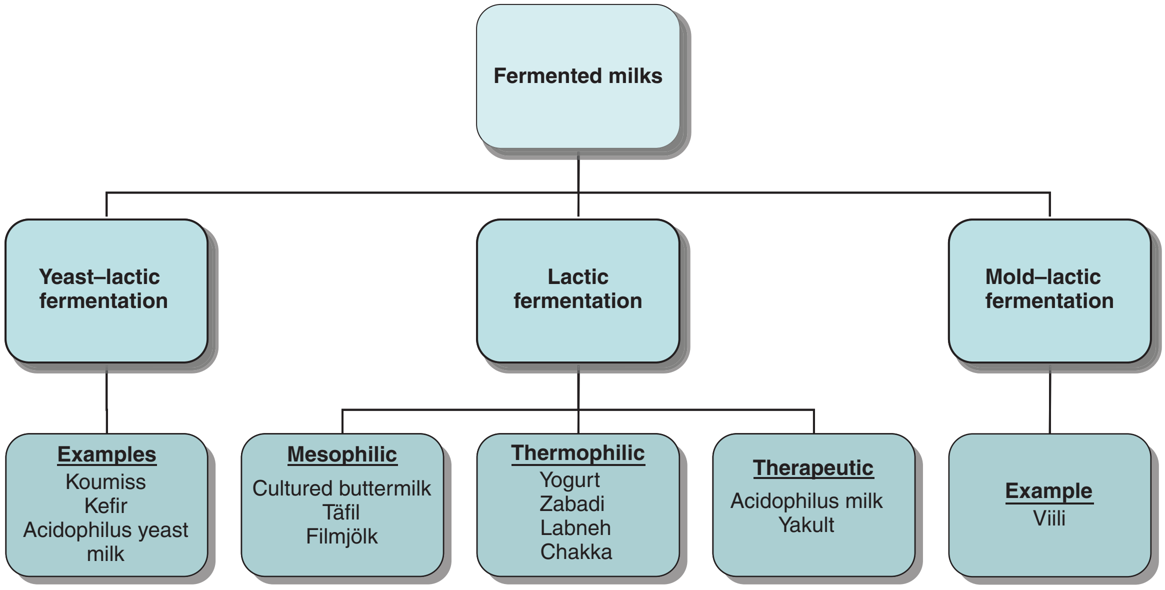

Figure 1 Classification of fermented milks.

fermentation, and mold–lactic fermentation (**Figure 1**). Koumiss and kefir belong to the yeast–lactic fermentation group where alcoholic fermentation by yeasts is used in combination with a lactic acid bacterial fermentation.

Biochemical Products of Fermentation

The conversion of milk to a fermented product has several important advantages; besides being a means of preservation, it also improves the taste and digestibility, and metabolites of lactic acid bacteria may have therapeutic value. The lactic–yeast fermentation of equine milk produces lactic acid, ethanol, carbon dioxide, and other by-products such as volatile acids, fusel alcohols, and other compounds with important characteristic aroma and taste. The production of flavor compounds by starter microorganisms during fermentation is important in fermented milks as the manufacturing process does not include a ripening stage, like that of most types of cheese. Limited hydrolysis of casein (~10% of total casein) occurs, depending on the length of time the product is stored. Lactic acid, diacetyl, and acetaldehyde are the major flavor compounds produced during the fermentation of equine milk, and the amount of lactic acid produced determines the acceptability of the end product. The content of some, but not all, water-soluble vitamins in equine milk increases on fermentation of the milk (**Table 1**); vitamins of the original equine milk are metabolized initially by microorganisms but are synthesized subsequently. The lactose content is reduced during fermentation (by ~20–30% of the level in the original milk), while the concentration of some free amino acids increases, for example, proline, serine, alanine, valine, leucine, and histidine.

Therapeutic Benefits of Koumiss and Fermented Milks

Modern-day interest in the health benefits of fermented milks was started by the theory of longevity proposed by the Russian microbiologist Professor Elie Metchnikoff (1845–1916), who proposed that people who consumed fermented milks live longer, as lactic acid bacteria from the fermented product colonized the intestine and inhibited 'putrefaction' caused by harmful bacteria, thereby

Table 1 Concentration of some vitamins in raw equine milk and koumiss

	Ascorbic acid (vitamin C)	*Retinol (vitamin A)*	*Thiamine (vitamin B_1)*	*Riboflavin (vitamin B_2)*	*Nicotinic acid (niacin or B_3)*	*Cobalamin (vitamin B_{12})*
Equine milk	250–333	125	291	261	299	3.3
Koumiss	200–250	78	194	275	1060	2.25

Concentrations of all vitamins are expressed in μg l^{-1} except vitamin C, which is expressed in mg l^{-1}.

slowing down the aging process. Metchnikoff drank sour milk daily and his theory on longevity led Dr. Minoru Shirota (1899–1982), a Japanese scientist, to isolate a unique strain of lactic acid bacterium, *Lactobacillus casei* Shirota, which could pass through, and survive, the acidic environment of the stomach and colonize the intestine and prevent the growth of harmful bacteria. The end result was a product called Yakult, a fermented milk, which was first marketed in 1935 and is now sold in over 31 countries (*see* **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products). In 1953, the term 'probiotics' was introduced to define microorganisms that stimulate the growth of other microorganisms and, in 1989, was redefined to include reference to positive health effects, that is, 'live microbial food supplements which benefit the host by improving its intestinal microbial balance'. A summary of the benefits attributed to probiotics is presented in **Figure 2**.

For some medical conditions, fermented milks are considered preferable to nonfermented milks as it is believed that they do not propagate infectious diseases due to their low pH, which prevents the growth of many pathogenic organisms. Furthermore, the low pH reduces buffering action in the gastrointestinal tract and is believed to enhance the absorption of calcium. The low lactose content of koumiss compared to raw equine milk is favorable for those suffering from lactose intolerance; ~88% of Mongolians are lactose intolerant but consume koumiss without ill effects, probably due to intraintestinal hydrolysis of lactose by microbial β-galactosidase in koumiss, an enzyme that is not denatured in the acidic environment of the stomach. Furthermore, koumiss is thought to be more effective than raw equine milk in treatment of disease due to the presence of additional bioactive peptides and bactericidal substances produced during microbial metabolism while retaining the high levels of lysozyme and lactoferrin of the original milk, which have proven antibacterial activity.

Traditional and Nontraditional Koumiss

Fermented milk products can be divided into traditional and nontraditional types. As outlined above, traditional fermented milk products, including koumiss, have a long history although it was not until the work of Louis Pasteur that the microbiology underlying fermentation was revealed. Nontraditional fermented milks have been developed more recently and their manufacture is based on known scientific principles using pure microbial cultures.

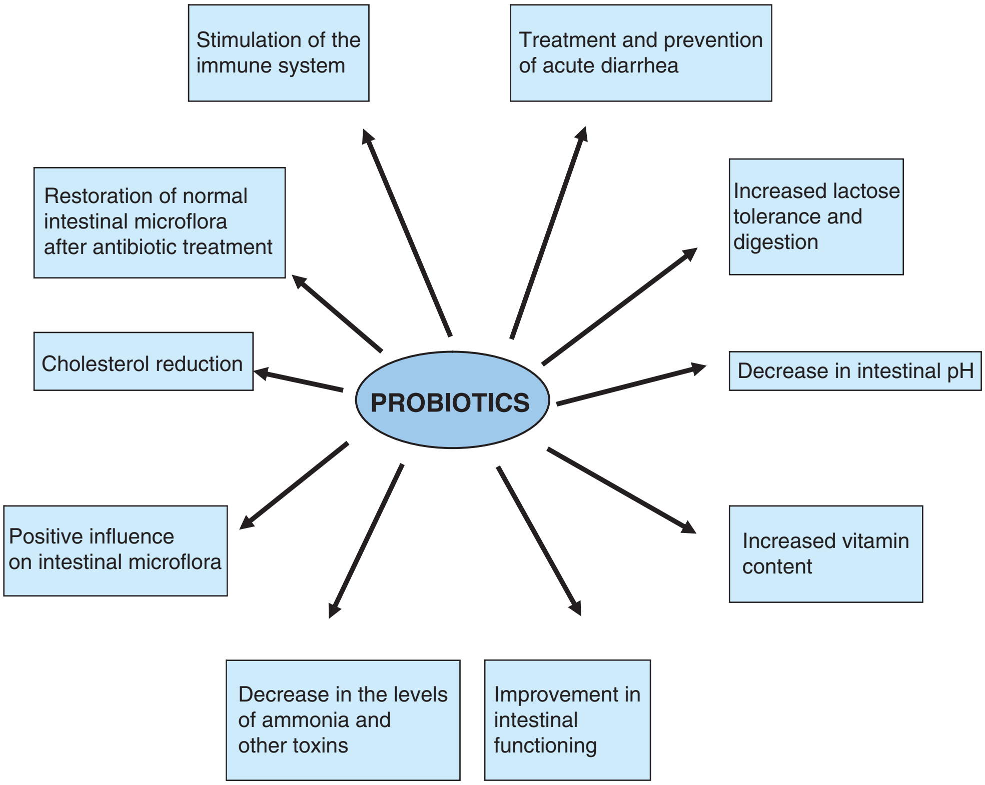
Stimulation of the immune system
Treatment and prevention of acute diarrhea
Restoration of normal intestinal microflora after antibiotic treatment
Increased lactose tolerance and digestion
Cholesterol reduction
PROBIOTICS
Decrease in intestinal pH
Positive influence on intestinal microflora
Increased vitamin content
Decrease in the levels of ammonia and other toxins
Improvement in intestinal functioning

Figure 2 Benefits of probiotics on human health. Adapted from Prado FC, Parada JL, Pandey A, and Soccol CR (2008) Trends in non-dairy probiotic beverages. *Food Research International* 41: 111–123.

Traditional Koumiss

The oldest method for the production of koumiss from equine milk in the central Asian steppes was by fermentation due to adventitious bacteria of lactose to lactic acid and ethanol. Horses were hand-milked with the foal in proximity (**Figure 3**).

Traditional koumiss (from fresh raw milk) was usually prepared by seeding milk with a mixture of bacteria and yeasts using part of the previous day's product as an inoculum (slop-back culture). The milk was held in a leather sack called a 'turdusk' (also called a 'saba' or 'burduk'), that was made from smoked horsehide taken from the thigh area of the horse, that is, it has a broad bottom and long narrow sleeve, with a capacity of 25–30 l. Fermentation took from 3 to 8 h with a mixed microbial population that were identified in the 1960s, and consisted mainly of *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lactococcus lactis* subsp. *lactis*, *Kluyveromyces fragilis*, and *Saccharomyces unisporus*. During the agitation and maturation stages of production, more equine milk is frequently added to control the acidity and alcohol level. The whole process was poorly controlled and often resulted in a product with an unpleasant taste, due to the presence of too much yeast or excess acidification. Turdusks, often containing caprine milk from the previous season, were stored in a cool place over winter and the starter culture was reactivated in spring by gradually filling the turdusk with equine milk over about 5 days.

Nontraditional Koumiss

Nowadays, the production of koumiss and other fermented milk products is carried out using a more standardized protocol for manufacture and is of considerable interest for increasing the market and consumption of equine milk products in countries where it has not normally been consumed. In addition to using pasteurized equine milk, pure cultures of lactobacilli, such as *Lb. delbrueckii* subsp. *bulgaricus,* and yeasts are used for koumiss manufacture. The use of *Saccharomyces lactis* is considered best for production of ethanol and *S. cartilaginosus* is sometimes used for its antibiotic activity against *Mycobacterium tuberculosis.* Other microorganisms such as *Candida* spp., *Torula* spp., *Lb. acidophilus,* and *Lb. lactis* may also be used in koumiss production. A schematic diagram of the manufacture of commercial koumiss is presented in **Figure 4**, which outlines the three stages of production: mother culture preparation, bulk starter preparation, and koumiss manufacture. The inoculation rate of equine milk with bulk starter at 30% is probably the highest inoculate rate used in the manufacture of any fermented milk. The agitation stages indicated in **Figure 4** are crucial for aeration of the mix, which promotes the growth of the yeast. The characteristics of good koumiss are optimal when lactic and alcoholic fermentations proceed simultaneously so that the products of fermentation occur in definite proportions. In addition to lactic acid, ethanol, and CO_2, volatile acids and other compounds are formed, which are important for aroma and taste, and ~10% of the milk proteins are hydrolyzed. Products with varying amounts of lactic acid and ethanol are produced and generally three categories of koumiss are recognized: mild, medium, and strong (**Table 2**).

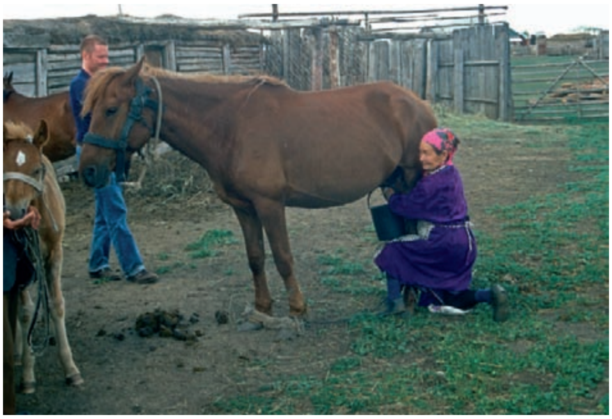

Figure 3 Hand-milking a mare in northern Kazakhstan. Photo courtesy of Dr. Alan Outram.

Apart from the end products of microbial fermentation, koumiss contains about 90% water, 2–2.5% protein (1.2% casein and 0.9% whey proteins), 4.5–5.5% lactose, 1–1.3% fat, and 0.4–0.7% ash. Viable bacterial and yeast counts of $\sim 4.97 \times 10^7$ and $\sim 1.43 \times 10^7\,\mathrm{cfu\,ml^{-1}}$, respectively, have been reported in koumiss.

Lactic acid in koumiss may occur as either the L(+) or D(−) isomer, depending on the type of lactic acid bacteria used (**Table 3**). Both L(+) and D(−) isomers are absorbed from the gastrointestinal tract but differ in the proportions converted to glucose or glycogen in the body. The L(+) isomer is rapidly and completely converted to glycogen, whereas the D(−) isomer is converted more slowly and a significant quantity is excreted in urine. The presence of unmetabolized lactic acid results in metabolic acidosis in infants. Since 1973, fermented milks manufactured commercially use cultures that produce high amounts of the L(+) isomer and very low amounts of the D(−) isomer.

Technological Developments

Some technological advancements have been made recently in the manufacture of koumiss, such as the development of blends of microorganisms in starter cultures that enhance flavor development and extend the shelf life up to 14 days. The presence of a high level of

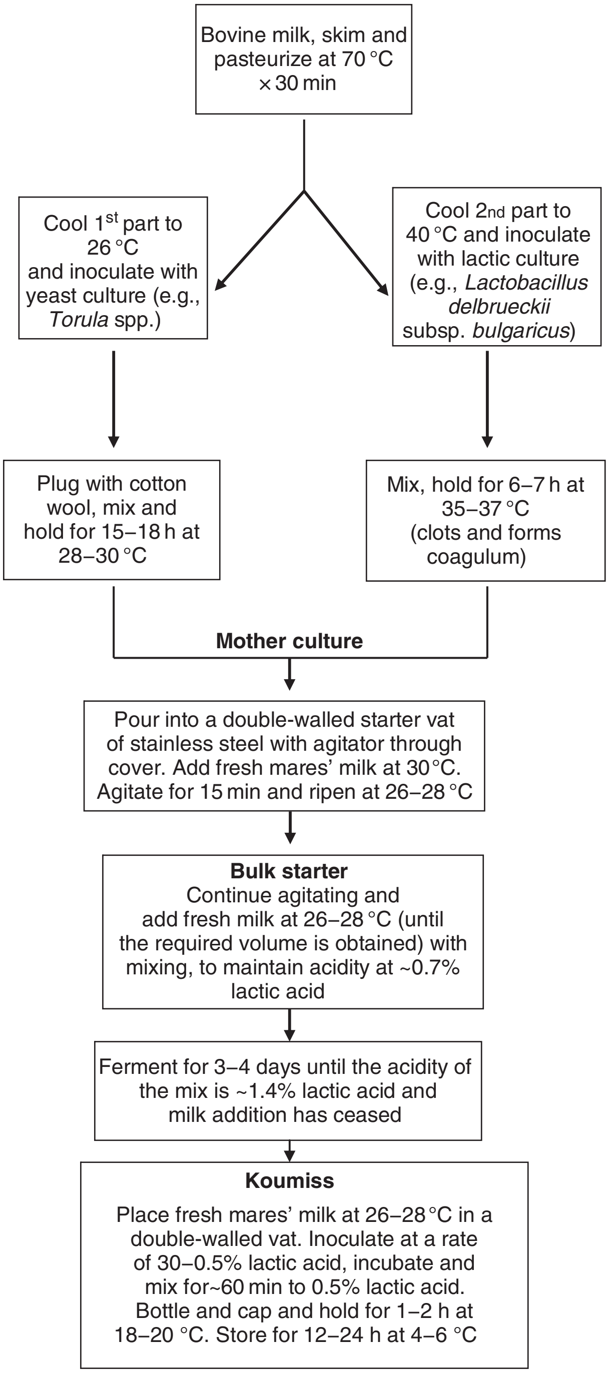
Bovine milk, skim and pasteurize at 70 °C × 30 min
Cool 1st part to 26 °C and inoculate with yeast culture (e.g., *Torula* spp.)
Cool 2nd part to 40 °C and inoculate with lactic culture (e.g., *Lactobacillus delbrueckii* subsp. *bulgaricus*)
Plug with cotton wool, mix and hold for 15–18 h at 28–30 °C
Mix, hold for 6–7 h at 35–37 °C (clots and forms coagulum)
Mother culture
Pour into a double-walled starter vat of stainless steel with agitator through cover. Add fresh mares' milk at 30 °C. Agitate for 15 min and ripen at 26–28 °C
Bulk starter
Continue agitating and add fresh milk at 26–28 °C (until the required volume is obtained) with mixing, to maintain acidity at ~0.7% lactic acid
Ferment for 3–4 days until the acidity of the mix is ~1.4% lactic acid and milk addition has ceased
Koumiss
Place fresh mares' milk at 26–28 °C in a double-walled vat. Inoculate at a rate of 30–0.5% lactic acid, incubate and mix for~60 min to 0.5% lactic acid. Bottle and cap and hold for 1–2 h at 18–20 °C. Store for 12–24 h at 4–6 °C

Figure 4 Schematic diagram for the production of koumiss. Adapted from Berlin PJ (1962) Koumiss. *International Dairy Federation Bulletin IV*, pp. 4–16.

Table 2 Categories of koumiss

	Acidity	*Ethanol*
Flavor category	*(%)*	*(%)*
Mild	0.6–0.8	0.7–1.0
Medium	0.8–1.0	1.1–1.8
Strong	1.0–1.2	1.8–2.5

Table 3 Optical isomers of lactic acid produced by some species of lactic acid bacteria used in koumiss production

L(+) lactic acid (≥95%)	All *Lactococcus* strains *Lactobacillus casei*
D(−) lactic acid (100%)	*Lactobacillus debrueckii* subsp. *bulgaricus* *Lactobacillus debrueckii* subsp. *lactis*
Racemic lactic acid mixture L(+)/D(−)	*Lactobacillus helveticus* *Lactobacillus acidophilus* *Lactobacillus plantarum* *Lactobacillus brevis*

thermostable lysozyme in equine milk may interfere with the activity of some starter cultures in the production of fermented products and thus cause technological problems in the processing of equine milk. Equine milk heated to 90 °C for 3 min to inactivate lysozyme has been reported to produce an acceptable fermented milk. In sensory tests in western countries, fermented unmodified equine milk has an unacceptable viscosity and scores very low in comparison to fortified products for appearance, consistency, and taste. In an attempt to improve the rheological and sensory properties, fortification with sodium caseinate ($1.5\,g\,100^{-1}\,g$), pectin ($0.25\,g\,100^{-1}\,g$), and threonine ($0.08\,g\,100^{-1}\,g$) has been investigated; the resultant products are reported to have good microbiological, rheological, and sensory characteristics even after 45 days at 4 °C. Addition of sucrose and sodium caseinate has a positive effect on the rheological properties of the product due to strengthening of the protein network.

Koumiss-Like Products from Non-Equine Milk

The physicochemical and microbiological properties of asinine milk, such as low microbiological load and high lysozyme content, make it a good substrate for the production of fermented products with probiotic *Lactobacillus* strains. Asinine milk has been fermented with the probiotic bacteria *Lb. rhamnosus* (AT 194, GTI/1, GT 1/3), which was unaffected by the high lysozyme content of the milk and was viable after 15 days at 4 °C and pH 3.7–3.8. *Lactobacillus rhamnosus* is known to inhibit the growth of most harmful bacteria in the intestine and acts as a natural preservative in yogurt-type products, considerably extending shelf life. Fermented asinine milk produced using a mixed culture of *Lb. rhamnosus* (AT 194, CLT 2.2) and *Lb. casei* (LC 88) had a high viable bacterial count after storage for 30 days. Some sensory differences have been reported for fermented asinine drinks, and those made with the *Lb. casei* strain developed a more favorable and balanced aroma than the boiled vegetable/acidic taste and aroma of the product made with *Lb. rhamnosus* alone.

Koumiss-like products are produced in several areas, for example, Mongolia, the former USSR, southern Europe, and North Africa, from camel milk (shubat), donkey milk (koumiss), goat milk (tarag), ewes milk (arak or arsa), and buffalo milk (katyk).

Due to shortages of mares' milk and the cost, when it is available, research has now turned to producing koumiss-like products from bovine milk, which must be modified to make it suitable for koumiss production. Methods have been developed, with varying degrees of success, where a single constituent of bovine milk has been altered to resemble that of equine milk, that is, the carbohydrate content has been increased or the protein content reduced but both had not been altered simultaneously, until recently. Koumiss of a reasonable quality has been produced successfully from whole or skimmed bovine milk containing added sucrose using a mixture of *Lb. acidophilus*, *Lb. delbrueckii* ssp. *bulgaricus,* and *Kluyveromyces marxianus* var. *marxianus* or var. *lactis* as starter culture. Koumiss has also been made from diluted bovine milk with added lactose and, more successfully, from bovine milk mixed with concentrated whey using a starter culture of *Kluyveromyces lactis* (ATCC 56498), *Lb. delbrueckii* subsp. *bulgaricus,* and *Lb. acidophilus*. Starter cultures for koumiss manufacture from bovine milk may also include *S. lactis* (high antimicrobial activity against *M. tuberculosis*) in order to retain the 'antituberculosis image' of equine milk.

More recently, bovine milk has been modified to approximate the composition of mares' milk using a series of membrane filtration steps and a starter culture (*K. lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. acidophilus*) used that ensures consistent fermentation; the resulting product was found to be very similar to koumiss with respect to pH, titratable acidity, ethanol content, proteolytic activity, apparent viscosity, and microbial composition, both when fresh and after storage (15 days at 4 °C).

Conclusion

Unlike other milks, equine milk cannot be used for cheese production (it is not coagulable by rennet) but forms a weak coagulum under acidic conditions, which is exploited in the production of koumiss in central and western Asia and more recently in the Netherlands and Belgium, where fermented equine milk is flavored with concentrated fruit extract. Standardized protocols for manufacture of koumiss are still lacking and current demand for the product indicates that research is warranted.

Fermenting equine milk combines the nutritional advantages of the raw milk with those of probiotic lactic acid bacteria. Bioactive peptides released by limited hydrolysis of casein during the fermentation of equine milk have important physiological roles, for example, opioid-like properties, immunostimulating and antihypertensive activities, and the ability to enhance calcium absorption.

Worldwide, the consumption of fermented milk has grown faster than the consumption of fresh milk and the demand for koumiss made from equine milk is reported to exceed current production.

Acknowledgment

The author would like to thank Dr. S. Ishii for assistance with this article and for providing the photograph from Mongolia of horse milking.

See also: **Animals that Produce Dairy Foods**: Donkey; Horse. **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products. **Feed Ingredients**: Feed Supplements: Vitamins. **Fermented Milks:** Health Effects of Fermented Milks; Types and Standards of Identity. **Milk**: Equid Milk. **Milk Proteins**: Lactoferrin. **Milk Protein Products**: Bioactive Peptides.

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Kefir

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Introduction

Kefir is a traditional fermented milk product, believed to originate from the Caucasus region of Europe. It is also known by the names of kefyr, kephir, kefer, kiaphur, knapon, kepi, and kippi. Kefir is produced by the inoculation of kefir grains into milk, followed by a fermentation period of about 1 day at room temperature. The product is unique in the sense that the inoculant, that is, the kefir grains, are recovered after the fermentation process by sieving or filtration of the milk. Traditionally, the fermentation of milk was carried out in goat skins, clay pots, or wooden buckets in which milk from cows, goats, sheep, camels, or buffalos was used as the fermentation substrate. Other substrates for kefir production include soy milk, fruit juices, sugar, or molasses. The fermented milk product is characterized by a distinctive yeast-like flavor and a fizzy or sparkling mouthfeel. In fact, kefir has a highly complex flavor because the kefir grains used in its manufacture have a highly diverse and complex microbiota. The principal fermentation products in kefir are lactate, ethanol, and carbon dioxide, while other minor fermentation products include diacetyl, acetaldehyde, free amino acids, and, in some cases, acetate. Analysis of kefir grains has revealed that its flora is comprised of various lactic acid bacteria and yeasts, which coexist in a symbiotic relationship within the kefir grain. Kefir grains are remarkable in their physical resemblance to cauliflower florets, measure about 1–3 cm in length, and have an off-white or cream color (**Figure 1**). The grains are irregularly shaped and have a slimy texture, but are structurally strong enough for physical separation and recovery from the milk following fermentation. In addition to the microflora, exopolysaccharide, milk protein, and lipids constitute the other principal components of the kefir grain.

Microbial Aspects of Kefir Grains and Kefir

In kefir, lactic acid bacteria are mainly responsible for the conversion of lactose to lactic acid, resulting in a pH drop and thus aiding preservation of the milk. In addition to the fermentation of lactose by lactic acid bacteria, lactose-fermenting yeasts convert lactose to ethanol and CO_2. Other microbial constituents of kefir include non-lactose-fermenting yeasts and in some cases acetic acid bacteria. Following fermentation, the kefir grain increases in biomass by about 5–7%, with new florets forming in the process. The microfloral composition of the kefir grain is about 65–80% lactobacilli, 10–15% yeasts, and 5–25% *Lactococci* and *Leuconostoc* spp. In contrast, kefir, into which the kefir grain sheds a proportion of its flora, is about 80% lactococci and *Leuconostoc* spp., 10–15% yeasts, and 5–10% lactobacilli. The total microbial counts within the kefir grain are estimated to be lactobacilli, 10^8–10^9 cfu g^{-1}; lactococci and *Leuconostoc* spp., 10^8–10^9 cfu g^{-1}; and yeasts, 10^6–10^8 cfu g^{-1}, while in the fermented kefir product the total microbial counts are in general somewhat lower, and are estimated to be lactobacilli, 10^7–10^8 cfu g^{-1}; lactococci and *Leuconostoc* spp., 10^8–10^9 cfu g^{-1}; and yeasts, 10^5–10^6 cfu g^{-1}. Not surprisingly, the exact ratio of the various species of lactic acid bacteria, yeasts, and other flora within the kefir grain and kefir is highly variable, and depend on factors such as fermentation time, temperature, degree of agitation, and type of milk. The effect of kefir grain-to-milk ratio on the microbial composition of kefir has demonstrated that at a kefir grain-to-milk ratio of 1:10, the pH of kefir is about 3.7, while at a ratio of 1:100 the pH is considerably higher at around 5.4. As the kefir grain-to-milk ratio increases from 1:10 to 1:100, the lactococci count in the resulting kefir produced increases from about 10^7 to 10^9 cfu ml^{-1}. A ratio of 1:10 gives a product with high acidity, low viscosity, and highly effervescent taste, while a ratio of 1:100 gives a product of high viscosity and low acidity. For optimum fermentation and product consistency, a kefir grain-to-milk ratio of about 1:30 to 1:50 is normally recommended. Agitation during fermentation has also been shown to affect the microbial composition of kefir, with agitation favoring the development of homofermentative lactococci and yeasts.

Extensive research efforts have been directed toward investigating the highly complex microbiota of the kefir grain. Numerous species of bacteria and yeasts have been isolated from kefir grains and from the fermented kefir product (**Table 1**). Traditionally, classical microbiological methods such as the measurement of various biochemical activities, fermentation profiles, and morphological characteristics have been used to study the microbiota of kefir grains. While these methods are useful, in some cases they

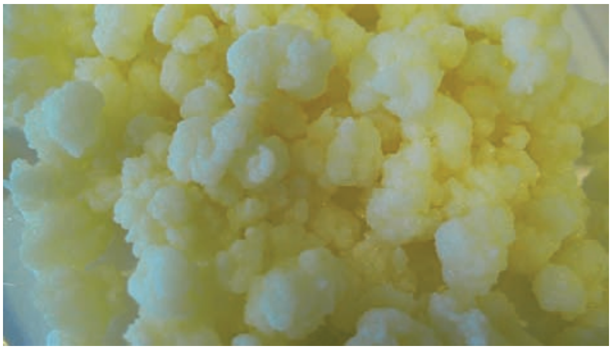

Figure 1 Freshly washed kefir grains harvested from milk.

are not discriminating enough to identify closely related species or indeed completely new species. Therefore, in recent years, culture-independent methods, using various genetic techniques, have been used to supplement the classical microbiological methods. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been shown to be useful for analyzing highly complex microbial consortia, while partial 16S rRNA sequencing has been used for the identification of yeast species.

Kefir Bacteria

Homofermentative lactic acid bacteria in kefir primarily produce lactic acid from the fermentation of lactose, and consist of lactobacilli such as *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, and *Lactobacillus acidophilus*; lactococci such as *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*; and *Streptococcus thermophilus* (**Table 1**). The heterofermentative lactic acid bacteria produce lactic acid and CO_2 from the fermentation of lactose, and include lactobacilli such as *Lactobacillus kefir*, *Lactobacillus parakefir*, *Lactobacillus fermentum*, and *Lactobacillus brevis*. The heterofermentative lactococci and the citrate-fermenting species such as the citrate-positive strains of *Lc. lactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, and *Leuconostoc mesenteroides* ssp. *mesenteroides* are of special interest. Utilization of citrate by the citrate-positive strains of *Lc. lactis* results in the

Table 1 Microflora identified in kefir grains

Lactobacilli	*Lactococci and Leuconostoc spp.*	*Yeasts*	*Others*
Homofermentative	***Homofermentative***	***Lactose-fermenting***	
Lb. acidophilus	*Lc. lactis* ssp. *lactis*	*Kluyveromyces marxianus* (t)/ *Candida kefyr* (a)	*Streptococcus thermophilus*
Lb. delbrueckii ssp. *bulgaricus*	*Lc. lactis* ssp. *cremoris*	*Kluyveromyces lactis* var. *lactis*	*Acetobacter aceti*
Lb. helveticus		*Debaryomyces hansenii* (t)/ *Candida famata* (a)	*Acetobacter rasens*
Lb. kefiranofaciens		*Dekkera anomala* (t)/ *Brettanomyces anomalus* (a)	*Enterococcus durans*
Lb. kefirgranum			*Galactomyces geotrichum* (t)/ *Geotricum candium* (a)
Heterofermentative	***Heterofermentative***	***Non-lactose-fermenting***	
Lb. kefir	*Lc. lactis* (citrate-positive)	*Saccharomyces unisporus*	
Lb. parakefir	*Leu. mesenteroides* ssp. *cremoris*	*Saccharomyces turicensis*	
Lb. brevis	*Leu. mesenteroides* ssp. *mesenteroides*	*Saccharomyces cerevisiae*	
Lb. plantarum	*Leu. mesenteroides* ssp. *dextranicum*	*Saccharomyces exiguus*	
Lb. casei ssp. *casei*		*Saccharomyces pastorianus*	
Lb. paracasei ssp. *paracasei*		*Pichia fermentans* (t) (a)/*Candida firmetaria*	
Lb. fermentum		*Torulaspora delbrueckii*	
Lb. rhamnosus		*Candida friedrichii*	
Lb. fructivorans		*Candida humilis*	
Lb. hilgardi		*Issatchenkia orientalis* (t)/ *Candida krusei* (a)	
		Candida maris	
		Debaryomyces occidentalis	
		Yarrowia lipolytica (t)/*Candida lipolytica* (a)	

(t): teleomorph (sexual reproductive stage); (a): anamorph (asexual reproductive stage).

production of diacetyl, acetaldehyde, ethanol, and acetate, while utilization of citrate by *Leu. mesenteroides* ssp. *cremoris* or *Leu. mesenteroides* ssp. *mesenteroides* produces diacetyl, ethanol, and acetate, all of which are key flavor compounds in kefir. The acetic acid bacteria *Acetobacter aceti* and *Acetobacter rasens* have also been isolated from kefir grains; however, in some countries, the presence of these species is considered undesirable.

Lactobacillus kefiranofaciens, which owes its name to the product from which it is isolated, is of particular interest due to its production of a polysaccharide known as kefiran. Other lactobacilli, including *Lb. kefir*, cannot produce this unique polysaccharide. Kefiran is a water-soluble glucogalactan composed of a repeating unit of 3 glucose and 3 galactose residues. Production of kefiran by *Lb. kefiranofaciens* is stimulated when it is grown together with *Saccharomyces cerevisiae*. Electron microscopy of kefir grains has revealed that kefiran together with protein and lipids constitutes the kefir grain matrix, around and within which the microbes are associated. Additional analysis has shown that, within the grain, cocci are observed on the surfaces of the yeasts, while rod-shaped bacteria are found between the yeast cells. Immunofluorescence staining has demonstrated that *Lb. kefiranofaciens* is located throughout the kefir grain, especially in the interior, while *Lb. kefir* is located only in small regions at the surface layers.

Kefir Yeasts

The yeasts isolated from kefir can be classified as either lactose-fermenting or non-lactose-fermenting; the non-lactose-fermenting yeasts dominate. The non-lactose-fermenting yeasts are found in the deep layers of the kefir grains, while the lactose-fermenting yeasts are located at the surface layers. The primary lactose-fermenting yeasts are *Kluyveromyces marxianus*, *Kluyveromyces lactis* var. *lactis*, *Debaryomyces hansenii*, and *Dekkera anomala*, while the main non-lactose-fermenting yeasts include *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Torulaspora delbrueckii*, *Pichia fermentans*, *Saccharomyces turicensis*, *Issatchenkia orientalis*, and *Debaryomyces occidentalis* (**Table 1**). The yeasts are primarily responsible for the production of ethanol and CO_2 in kefir. Nevertheless, some heterofermentative lactobacilli, such as *Lb. kefir*, are also capable of producing moderate amounts of ethanol (up to 0.25%) and CO_2.

It is highly probable, due to currently unknown interactions and inadequate selective media, that a number of species of lactic acid bacteria and yeasts still remain to be isolated and identified from kefir grains. Some of the species isolated from kefir grains and kefir may not necessarily be key microorganisms, but may be more appropriately considered as contaminants. Recently, using internal transcribed sequencing of rRNA genes, the fungi *Dipodascus capitatus* and *Trichosporon coremiiforme* have been identified in kefir grains. These fungi are known pathogens, and thus underlie the need for careful control of the microbial quality of kefir grains.

Bacteria–Yeast Interactions in Kefir

The complex interactions between the yeasts and bacteria in the kefir grain and their interdependence are not fully elucidated. Indeed it has been shown that when the kefir bacteria are separated from the kefir yeasts, the bacteria do not grow as efficiently as in the presence of yeasts. For example, it is believed that vitamin production by the yeasts and *Acetobacter* spp. may stimulate the growth of lactic acid bacteria. Also, some yeast species such as *Debaryomyces hansenii* and *Yarrowia lipolytica* assimilate the lactic acid formed by the lactic acid bacteria, and thus raise the pH and stimulate further growth of the lactic acid bacteria. Regarding the free amino acid level in kefir, there is a significant increase following fermentation of the milk, with high levels of threonine, lysine, valine, isoleucine, methionine, phenylalanine, and tryptophan reported. It is not clear whether this increase is due mainly to the activity of the yeasts or lactic acid bacteria, but it is likely, given the fact that some lactic acid bacteria are auxotrophic for certain amino acids, that a bacteria–yeast interaction in the metabolism of amino acids also occurs within the kefir grain, and during the fermentation of the milk.

Production of Kefir

The origins and production of kefir are lost to memory, and, traditionally, the method of producing kefir and kefir grains was a closely guarded secret. Today, kefir or kefir-like beverages are made across the globe, with reports of kefir-making in Ireland, Spain, Turkey, Malaysia, Indonesia, Tibet, and North and South America. However, kefir remains most popular in Eastern Europe, where it is generally made from cow's milk.

Production of Kefir Grains

Kefir grains are made from existing grains, which are incubated in milk until they increase in mass, thus generating more kefir grains. If carefully maintained, these grains can retain their activity for several years.

However, there is a lack of information on how kefir grains can be produced from first principles. One report demonstrated the production of kefir-like grains by inoculating a goatskin bag with the intestinal flora of a sheep, and then filling the bag with pasteurized milk. The bag was incubated at 24–26 °C for 48 h, with hourly agitation of the contents. When the milk coagulated, approximately 75% was removed and replaced with fresh milk. This process was repeated for 12 weeks, whereupon a polysaccharide layer was found to have developed on the inner surface of the bag. When this layer was removed and added to milk, kefir-like grains formed.

Commercial Kefir Production

There are two primary ways of making kefir: (1) kefir produced by fermenting milk with kefir grains and subculturing the resultant fermentate (**Figure 2**); and (2) kefir produced through the application of commercial starter cultures that are directly inoculated into the milk (**Figure 3**).

Kefir produced with kefir grains can vary substantially due to the diverse microbiological profile of the kefir grains. Also, variation in the fermentation process can result in significant changes in flavor and texture. Incubation at higher temperatures (>30°C) favors growth of thermophilic lactic acid bacteria, at the expense of yeast and mesophilic lactic acid bacteria; similarly, agitation during fermentation increases the numbers of homolactic lactic acid bacteria, whilst leaving the numbers of acetic acid and heterofermentative lactic acid bacteria unchanged. All of these factors impact the organoleptic characteristics of the final product.

Kefir production using kefir grains includes a stage called ripening, which occurs in the retail pack, where the kefir is held at 8–10 °C for up to 12 h, whereupon it is

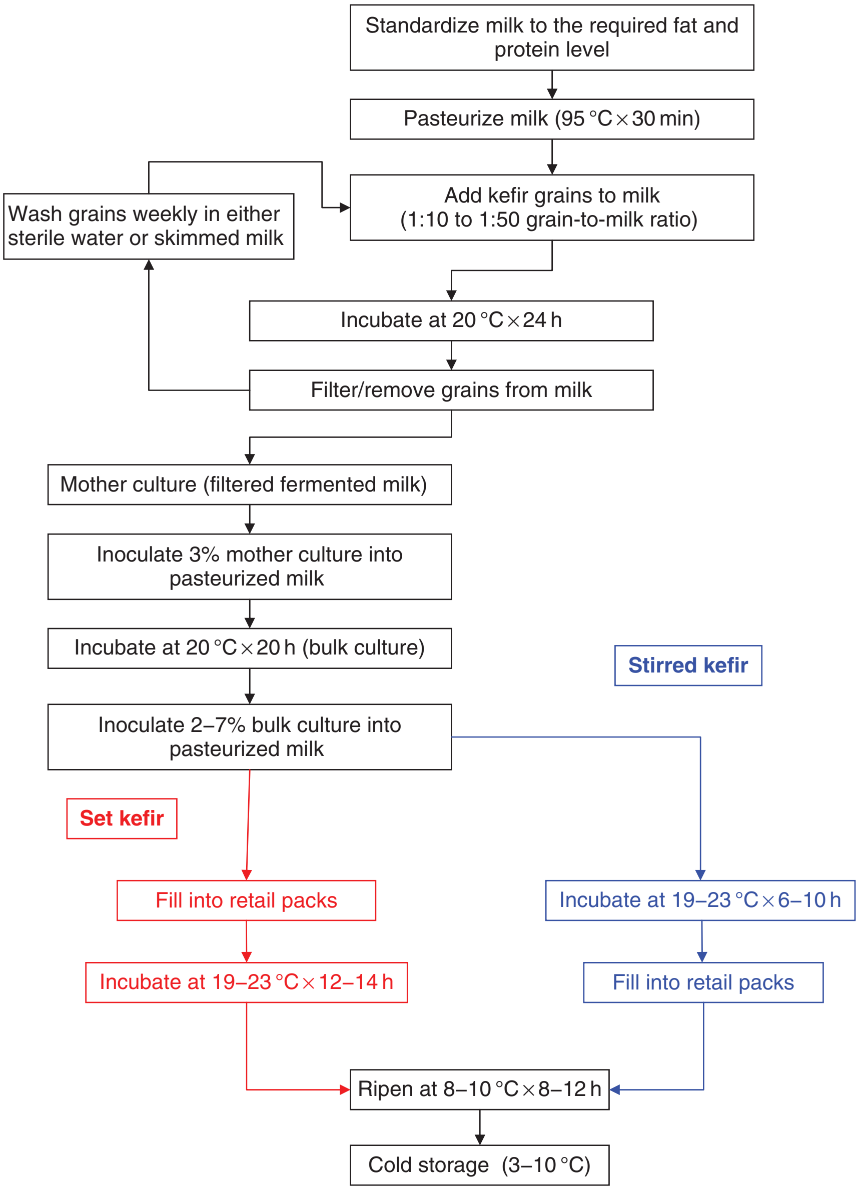
Standardize milk to the required fat and protein level
Pasteurize milk (95 °C × 30 min)
Add kefir grains to milk (1:10 to 1:50 grain-to-milk ratio)
Wash grains weekly in either sterile water or skimmed milk
Incubate at 20 °C × 24 h
Filter/remove grains from milk
Mother culture (filtered fermented milk)
Inoculate 3% mother culture into pasteurized milk
Incubate at 20 °C × 20 h (bulk culture)
Stirred kefir
Inoculate 2–7% bulk culture into pasteurized milk
Set kefir
Fill into retail packs
Incubate at 19–23 °C × 6–10 h
Incubate at 19–23 °C × 12–14 h
Fill into retail packs
Ripen at 8–10 °C × 8–12 h
Cold storage (3–10 °C)

Figure 2 Schematic of kefir production using kefir grains.

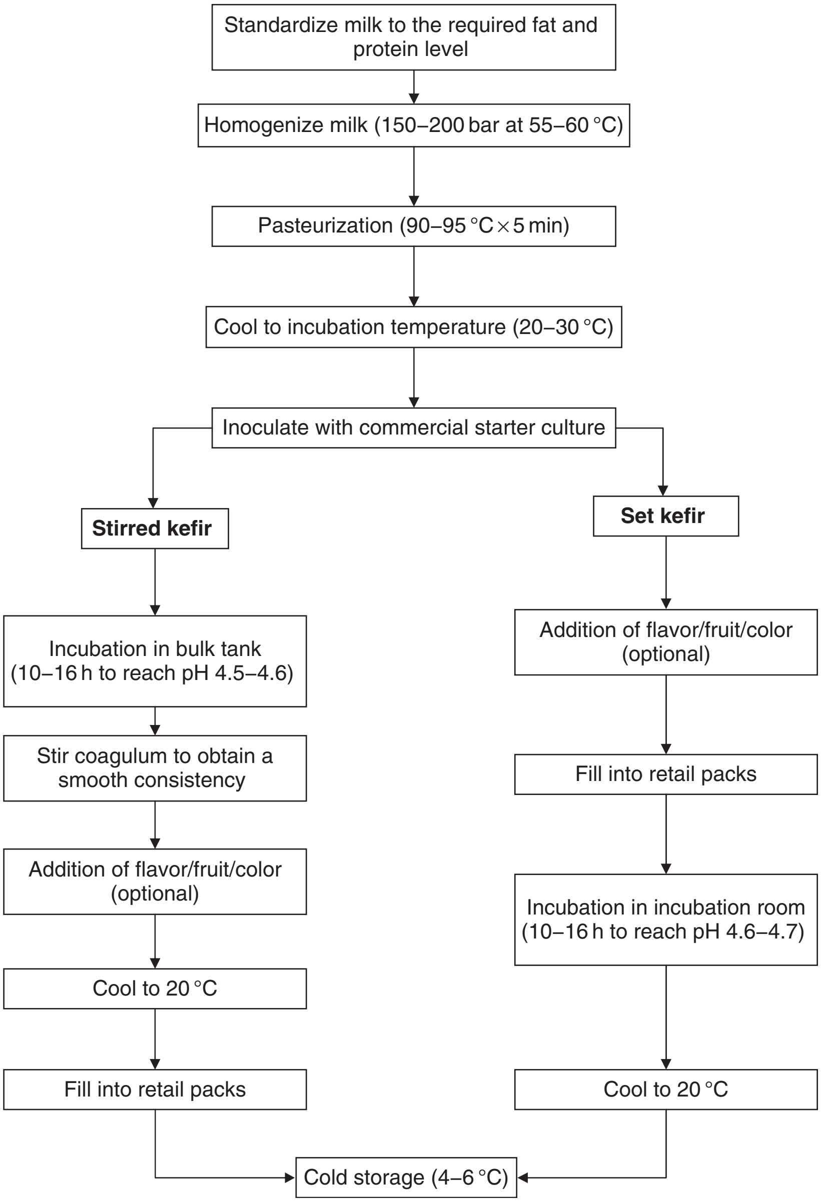
Standardize milk to the required fat and protein level
Homogenize milk (150–200 bar at 55–60 °C)
Pasteurization (90–95 °C × 5 min)
Cool to incubation temperature (20–30 °C)
Inoculate with commercial starter culture
Stirred kefir
Set kefir
Incubation in bulk tank (10–16 h to reach pH 4.5–4.6)
Stir coagulum to obtain a smooth consistency
Addition of flavor/fruit/color (optional)
Cool to 20 °C
Fill into retail packs
Addition of flavor/fruit/color (optional)
Fill into retail packs
Incubation in incubation room (10–16 h to reach pH 4.6–4.7)
Cool to 20 °C
Cold storage (4–6 °C)

Figure 3 Schematic of kefir production using commercial direct-to-vat cultures.

chilled to refrigeration temperatures and is then ready for consumption. The purpose of this ripening stage is to allow for the correct growth of yeast and bacteria within the kefir. Omission of this step is linked to atypical flavor development.

Use of commercial direct-to-vat cultures simplifies the commercial production of kefir, and with careful selection of yeasts and bacterial species, it is possible to produce a product that has an acceptable kefir flavor and very good keeping qualities. Kefir made this way can have a shelf life of up to 28 days, as compared to kefir produced with kefir grains, which has a shelf life of 3–12 days. However, this type of kefir lacks the microbial diversity of kefir made with grains and thus may not have the same therapeutic and probiotic characteristics.

Probiotic Effects of Kefir

Kefir has traditionally been regarded as a beverage with added health benefits and, consequently, perhaps one of the oldest probiotic food stuffs. It is possible that kefir was part of the sour milk preparations discussed by Metchnikoff in his early work describing probiotics and their benefits on the increased longevity of some groups of Bulgarian people. The name kefir, derived from the Turkish word *kefi*, translates as 'good feeling'. Historically, kefir has been recommended in the treatment of conditions such as gastrointestinal problems, hypertension, allergy, and ischemic heart disease.

The reported scientific benefits of kefir include antipathogenic activity, antitumor and anticarcinogenic activity, aid in lactose maldigestion, enhanced synthesis of certain B vitamins, anti-inflammatory/immune modulation effects, and hypocholesterolemic effects. However, there are several mitigating factors to be considered with regard to the probiotic benefits of kefir consumption.

There is considerable variation in kefir preparations used in the reported studies. The inherent microbial variability of kefir grains and the differences in production conditions used in kefir manufacture result in final fermentates with different microbial profiles and bioactive components released/produced during fermentation. Also, the kefir grain-to-milk inoculation ratio used in the fermentation of kefir appears to influence its probiotic efficacy, as higher inoculation levels tend to yield better results. In addition, fermentation time and fermentation conditions (e.g., fermentable carbohydrate source, fermentation temperature) will alter the microbial and chemical composition of the final kefir preparation. This inherent variability in the base material used in trials makes comparison between different scientific reports difficult and should be borne in mind when considering the probiotic attributes of kefir, as they are not universal to all kefir preparations.

Fermentations of animal milk, soy milk, and fruit and sugar juices with kefir grains have been examined, and a wide variety of different bioactive compounds have been found, such as organic acids (lactic, acetic, butyric, and propionic), CO_2, H_2O_2, ethanol, bioactive peptides, exopolysaccharides (kefiran), and bacteriocins. These compounds may act independently or in concert to produce the health benefits long attributed to kefir consumption.

Impact on Gut Microflora

A key focus of probiotic effects is the ability to modulate the gut flora of the host/consumer, where there is a reduction in undesirable microorganisms and an increase in the numbers of positive bacteria, such as bifidobacteria or lactobacilli. This effect on the composition of the gut flora is likely to derive from a combination of various effects: direct inhibition of pathogens through acid and bacteriocin production; competitive exclusion of pathogens at the gut mucosa; various effects, such as: direct inhibition of pathogens through acid and bacteriocin production and competitive exclusion of pathogens at the gut mucosa. Bifidokefir has been shown to reduce the numbers of *Clostridia* spp. in the gut and to increase the numbers of bifidobacteria and lactobacilli.

Antibacterial Effect

Kefir preparations have been reported to have bacteriocidal and bacteriostatic effects on Gram-positive and Gram-negative bacterial pathogens, respectively. Pathogens inhibited by kefir, kefir extracts, or bacterial isolates from kefir include *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enteriditis*, *Shigella sonnei*, *Yersinia enterocolitica*, *Micrococcus luteus*, *Micrococcus flavans*, *Escherichia coli*, *Bacillus cereus*, *Klebsiella pneumoniae*, and *Candida albicans*. Consumption of kefir has also reduced the duration and severity of *Salmonella* and *Shigella* infection in young infants. There is also a commercial interest in this antipathogenic effect, as experiments have shown that feeding kefir can prevent *Salmonella* colonization of the gastrointestinal tract in young chicks and Enterobacteriaceae in domestic geese, thus showing the potential to improve the hygiene of poultry carcasses.

A reported primary mechanism of pathogen inhibition is the antibacterial/bacteriostatic action of the undissociated organic acids present in kefir. The primary organic acids present in kefir are lactic acid and acetic acid, which have been shown to inhibit pathogenic *E. coli* strains in an acidic environment, but not at neutral pH. However, the bacteriostatic effect of acidic kefir has been found to slightly exceed that of the same organic acids at an identical concentration. This suggests the presence of an inhibitory compound(s) in kefir, which is(are) activated in an acidic environment.

Antitumor and Anticarcinogenic Effects

There is an interest in the antitumor and anticarcinogenic benefits of kefir consumption. Work has been conducted using animal models indicating some success in increasing apoptosis and reducing tumor size through dietary supplementation with kefir, and in preventing lipid peroxidation and increasing glutathione in rats with colonic abnormal crypt formation. Similarly, kefir extracts have been shown to suppress the growth of breast cancer cells *in vitro*.

Some antitumorigenic abilities of kefir have been associated with the exopolysaccharide kefiran, which was shown to inhibit Ehrlich carcinoma and Sarcoma 180 in a mouse study, where it was proposed that the polysaccharide stimulated the host immune system via T-cell activity, rather than acting against the cancerous cells directly. Other suggested mechanisms of kefir-mediated immune modulation are increased natural killer cell and macrophage activity. Several studies have shown kefir extracts and kefir bacterial isolates to have the potential to reduce the risk or arrest the development of cancerous growths *in vitro* or in animal models.

Lactose Maldigestion

About 75% of the world's population suffer from lactose maldigestion; however, many people can tolerate some lactose in the diet in the form of fermented milks, such as kefir (*see* **Lactose and Oligosaccharides**: Lactose intolerance). In kefir, the diverse microbial population invariably has some degree of β-galactosidase activity, which converts lactose into its, monomeric components glucose and galactose, which can be easily digested. At the point of consumption, typically 20–40% of the lactose present in kefir will have been hydrolyzed, and this decrease appears sufficient for many lactose-maldigesting individuals to tolerate fermented milk in the diet. Therefore, kefir can be used to provide dairy nutrition in people with lactose maldigestion, and kefir is often well tolerated by lactose-intolerant subjects when compared with nonfermented milk drinks (reduction of breath hydrogen scores and increased blood glucose levels).

Cholesterol Reduction

The evidence that kefir consumption will reduce serum cholesterol is limited, with some research indicating a decrease in total serum cholesterol and phospolipids in rats fed a high cholesterol diet supplemented with kefir, although other biomarkers such as high density lipoprotein (HDL) and serum triglycerides were unaltered by kefir consumption. Other effects of kefir fermentation refer to the ability of some kefirs to assimilate cholesterol in milk, from which it was inferred that kefir had a cholesterol-reducing enzyme system. However, human trials have not yet demonstrated that kefir consumption has cholesterol-lowering effects in humans.

The traditional view of kefir as a fermented dairy beverage with wide-ranging health benefits appears quite promising, with many positive scientific studies indicating that kefir, microbial isolates from kefir, and kefir extracts merit closer study. However, this, and characterization of kefir in general, will continue to prove difficult due to the inherent variation in kefir grains and production methods.

See also: **Fermented Milks:** Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Koumiss; Middle Eastern Fermented Milks; Nordic Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactococcus lactis*. **Lactose and Oligosaccharides**: Lactose Intolerance. **Milk Protein Products**: Bioactive Peptides. **Yeasts and Molds**: *Kluyveromyces* spp.

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Yogurt: Types and Manufacture

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Introduction

Yogurt and similar fermented milks have been produced and consumed throughout the Middle East and India for centuries, but the precise origins are a matter for debate. It is generally accepted, however, that the continual storage of raw milk in vessels of earthenware or animal skin leads to the selection of a fermentative microflora appropriate for the location so that, in the Middle East for example, a thermophilic microflora emerged that gave rise to a fermented milk that was quite unique.

Partial concentration of the raw milk over an open fire prior to transfer to the storage vessel was found to improve markedly the consistency of this yogurt-like product and, in this way, a standardized procedure for making this popular food gradually emerged. Although yogurt retained a place in the diet of communities with a long history of consumption, it was not until the middle of the twentieth century that yogurt emerged as a formidable presence in the European or North American markets. In the event, it was the addition of fruit that proved the turning point, and fruit yogurts began to find favor as pleasant 'snacks' for picnics or similar occasions. Control over the fermentation also increased, so that a consumer could purchase products and know, with some degree of confidence, that the flavor and consistency would match his/her expectations of quality.

Today, the nature of the products is, of course, changing to meet new consumer demands and/or expand into new markets, but the essential characteristics of the products have not altered. Thus, yogurt is still defined as a product manufactured from milk – with or without the addition of some natural derivative of milk, such as skim milk powder, whey concentrate(s), caseinates or cream, – and with the gel structure being the result of coagulation of the milk proteins by lactic acid secreted by defined species of bacteria. Furthermore, these same bacteria must be 'viable and abundant' at the time of consumption, and a definition along these lines is enshrined in the food laws of many countries. Elsewhere, common practice ensures retention of the essential characteristics of yogurt, and this protection of the name is essential to prevent blatant exploitation of the traditional 'concept' of the product.

Types of Yogurt

Both historically and commercially, yogurt is the most popular product made with thermophilic cultures, and a typical retail sample will contain millions of viable cells of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Indeed, retail cartons should only be labeled with the word 'yogurt' if the product contains *L. delbrueckii* subsp. *bulgaricus*; labels like 'mild yogurt' and 'bioyogurt' are permitted in some countries to describe a product that is 'yogurt-like' in texture, but the prefix implies that the culture may be different from normal yogurt.

At present, the retail markets of Europe, North America, Australasia, and other places are dominated by two types of yogurt. One variant has a firm, gel-like structure together with a clean, mildly acidic and slightly aromatic flavor – 'natural set yogurt' – while the other has the consistency of 'double cream' and the background flavor of yogurt is usually modified by the addition of fruit/flavors and sugar (sucrose) – 'stirred yogurt.' More recently, a yogurt with a high level of milk solids (∼20% total solids with ∼10% fat) has appeared on the commercial market, and this so-called 'Greek-style' yogurt has proved popular as a base for 'party dips' like 'tzatziki.' However, a similar product has a much longer history of usage in the Middle East where it goes under the name of 'labneh' or concentrated/strained yogurt.

While each of the above products has very different sensory properties, they are readily identifiable as 'yogurt' and, despite their apparently contrasted natures, the manufacturing procedures for the three variants have much in common.

Method of Manufacture

The raw material for yogurt production is usually cows' milk, although milk from other mammals, such as sheep, goat, camel, or buffalo, is equally suitable for fermentation. Milk fat can be present or absent according to taste and/or market demand and, in Northern Europe for example, 'luxury' yogurts with 4.5% fat are to be found on the same supermarket shelf as yogurts for the diet-conscious with <1.0% fat. However, if the retail product is to have a consistency and mouthfeel that is acceptable to consumers, the critical aspect of the chemical composition of the milk is the level of milk solids-not-fat (MSNF).

In cows' milk, the MSNF level is 8.5–9.0%, of which around 4.5% is lactose, 3.3% protein (2.6% casein and 0.7% whey proteins), and 0.7% mineral salts; each of these components is vital for the production of a satisfactory yogurt. In particular, lactose is the principal energy source for the bacteria in the starter culture, while casein, together with calcium and phosphorus, gives rise to the basic structure of the gel. However, the level of casein in normal cows' milk is not sufficient to produce a gel that is strong enough to give a satisfactory end product, and hence the following first steps in manufacture are carried out:

1. Filtration of the milk to remove leaves or other debris.
2. Removal of a sample(s) to check for the presence of antibiotics or other chemical compounds that might interfere with the activity of the bacteria during the fermentation stage.
3. Separation of the milk fat and adjustment of the fat content of the skim milk stream to the level required in the finished yogurt. In general, while 0.05% fat may be attractive for the diet-conscious, a minimum of around 1.0–1.5% tends to give the retail yogurt a more attractive mouthfeel.
4. Elevation of the MSNF to a level that will give a gel that is strong enough to have consumer appeal as a set yogurt, or can withstand – with or without additional ingredients – stirring to allow incorporation of fruit or flavors.

Fortification of the Milk

Milk solids

Traditionally, the solids content of milk was raised by gently heating the milk in an open pan over a fire, and evaporation of the water was sufficient to achieve the desired effect. Nowadays, three alternative systems are available: (1) addition of skim milk powder to liquid milk; (2) evaporation of water from liquid milk under vacuum; and (3) removal of water from liquid milk by ultrafiltration.

Irrespective of whether the base milk is skim, semi-skim, or full-cream milk, the method of fortification tends to depend upon the process plant available. For example, as long as the material meets internationally recognized standards with respect to its physical, chemical, and microbiological properties, skim milk powder is a versatile ingredient that can be stored at ambient temperature and used as required. Consequently, small factories with a low throughput of product tend to rely on the addition of powder, while large factories may find that the financial investment in process plant is more than offset by the economies offered by vacuum evaporation. Ultrafiltration provides an alternative method for the removal of water, and both membrane processing and evaporation under vacuum give process milks suitable for yogurt-making. It may also be relevant that the ultrafiltration of standard yogurt provides an excellent route for the manufacture of Greek-style/concentrated yogurt, so that dual-purpose plants could become popular in the future.

However, as long as the process milk has around 4.0–5.0% protein (equivalent to a total solids content of 13–14%), it will provide a suitable base for most commercial fermented milks. For some niche markets, total solids levels of 16–18% may be used, but the additional expense is rarely justified for routine production.

Other ingredients

Although natural yogurt is based entirely on milk, some countries permit the use of stabilizers to achieve an acceptable consistency with stirred yogurts. Many of these stabilizers are complex carbohydrates, and the incorporation of starch/modified starch or one of the plant gums like guar and locust bean at a level of 0.5–0.75% can give a smooth texture to a yogurt without the need for high levels of milk solids. Thus, starches, and especially materials like guar gum, locust bean gum, and carrageenan, are long-chain polysaccharides composed of regular arrangements of monosaccharide units, and it is this structure that gives them the ability to bind water. It is also significant that the more complex molecules cannot be attacked by digestive enzymes in the human body, and hence they may contribute to human nutrition by providing a 'bulking agent' for the contents of the intestine and, by stimulating intestinal peristalsis, help to prevent some types of colonic malfunction.

Sweetening agents like sucrose and high-fructose syrups tend to be added to the milk at this stage as well, because the subsequent heat treatment of the milk will ensure that any contaminant yeasts or molds associated with the sugars are destroyed.

Heat Treatment of the Milk

Once the desired level of MSNF has been achieved, the milk may be homogenized to ensure full incorporation of any dry ingredients and to break down the fat globules to a uniform size of around 1 μm. This size reduction is essential to prevent the separation of cream during the production of full-fat set yogurts, and it also improves the consistency of stirred yogurts.

The next stage involves passing the milk through a plate heat exchanger in order to raise the temperature to 90–95 °C with a residence time in the holding tube of 5–10 min. Alternatively, the milk may be heated in the main process vessel to 80–85 °C and then held for 30 min. The choice of treatment depends on the sophistication of the available plant but, either way, the heating step is essential to achieve the following:

1. Give a yogurt with the desired textural properties. Thus, the heating/holding regime both alters the physicochemical properties of the caseins and denatures the whey proteins, so that β-lactoglobulin, in particular, may become attached to the casein micelles; this linkage improves the texture (set yogurt) or viscosity (stirred yogurt) of the final product.
2. Cause some breakdown of the whey proteins to liberate free amino acids that stimulate the activity of the starter culture.
3. Expel oxygen from the process milk for, as the starter bacteria are microaerophilic, deaeration provides the correct environment for rapid growth.
4. Kill any nonsporing pathogens that may be present, to ensure that yogurt retains its image as a 'safe' product.

In order to achieve these aims, the critical feature is the holding time, for high temperatures alone, for example, ultra-high temperature (UHT) treatments at 140 °C for around 2 s, do not give rise to organoleptically attractive yogurts.

Microbiology of the Process

Once the heat treatment has been completed, the milk is cooled to just above the desired incubation temperature to allow for heat loss during inoculation and then dosed with a culture composed of equal numbers of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The technology for handling these cultures can vary but, currently, the most popular routes are (1) production of a liquid 'bulk starter' using 'sterile milk' made from reconstituted skim milk powder and a freeze-dried or frozen culture purchased from a supplier; the process milk will receive a $20\,\mathrm{g\,l^{-1}}$ inoculation of prefermented bulk starter (pH around 5.0), and (2) the purchase of a concentrated frozen or freeze-dried culture that can be added direct to the process milk. For sheer convenience, the latter route is becoming more popular, but either path is acceptable as long as both species are present (*see* **Fermented Milks**: Yogurt: Role of Starter Culture).

Fermentation

Once the milk has been inoculated, it will follow one of two routes to give either set or stirred yogurt. In the case of set yogurt, the inoculated milk is dispensed directly into the retail cartons, and the most popular size of carton, as with the stirred fruit yogurts, is the individual portion (125–150 g). The necessary cartons can either be (1) purchased preformed, filled, and then heat-sealed with an aluminum foil lid or (2) made from polystyrene or similar material, which is supplied as a roll and can be formed into cartons on the filling machine – the form/fill/seal process. Family packs (500 g) are available as well, usually with press-on lids to allow for consumption over several days. For stirred yogurt, the same milk will be pumped into insulated tanks capable of holding several thousand liters and with walls of sufficient thickness to hold the temperature of the milk at 42–43 °C for the duration of the fermentation.

After 3–4 h in cartons or tanks, the fermentation will be complete, that is, the acidity of the milk will have risen to 1.2–1.4% lactic acid (around pH 4.2–4.3), and the total bacterial population of starter origin may well exceed $20 \times 10^{8}\,\mathrm{cells\,ml^{-1}}$. At this acidity, which is probably the level preferred by most consumers, the milk proteins will have coagulated to form a firm gel, and the product must be cooled to avoid overacidification. If this control is not exercised, then (1) the product may develop an excessively sharp sour taste and (2) the protein gel may begin to shrink and cause whey to separate as a discrete layer on the surface of the yogurt. This free whey can, of course, be stirred back into the body of the product, but in set yogurts, at least, its presence must be regarded as a fault.

Final Processing

For set yogurt coagulated in the retail cartons, cooling can be achieved by blowing cold air through the incubation room or by carefully transferring groups of cartons in their retail trays to a chill room at 2–4 °C. In-tank cooling of the base is often practiced for stirred yogurt, and this stage requires the circulation of chilled water (2 °C) through the jacket/in-tank cooling system of the vessel. Alternatively, the yogurt may be stirred and pumped through a plate or tubular cooler, but this process can lead to a loss in viscosity unless the starter culture is one that produces gel-forming polysaccharides; in the latter case, the product will regain a degree of viscosity during cooling and storage. As most stirred yogurts are further processed, the initial cooling is usually to around 15 °C. In-tank mixing of the fruit or other flavoring mixture ($100\text{–}150\,\mathrm{g\,l^{-1}}$ of yogurt base) can be used, but large factories tend to feed the yogurt base and the fruit through a blending tube and then directly into cartons.

Concentrated Yogurt

As mentioned earlier, the total MSNF content of normal yogurt will be around $140\,\mathrm{g\,l^{-1}}$ but, for concentrated (labneh) or Greek-style products, this level has to be raised to $210\text{–}230\,\mathrm{g\,l^{-1}}$. On a small scale, this concentration can be achieved by pouring 5–10 l of stirred natural yogurt (no fruit, sugar, or stabilizer) into a bag made from a double layer of cheesecloth or some similar material, and then hanging the bag in a cold room overnight. As the whey drains out of the bag, the total solids level of the contents increases and, by the following morning, the bags of concentrated yogurt can be tipped into a bulk tank for blending and packaging.

The sensory properties of the product made with this traditional system are excellent and, while the acidity may have increased to 18–20 g lactic acid kg^{-1}, any sharp taste will be masked by the fat content which will have risen to ~100 g k g^{-1}. The viscosity/mouthfeel of the product should be excellent as well, but the actual process suffers from two major drawbacks: (1) it is labor intensive and (2) the various manipulations associated with emptying the bags and blending/packaging the end product render it liable to contamination with yeasts or molds.

Consequently, most commercial operations have switched to removing the whey by ultrafiltration, which, as with gravity drainage, removes the desired quantity of water along with lactose and mineral salts from the natural yogurt to give a smooth, concentrated product that can be packaged with a minimum risk of spoilage.

Quality Considerations

As long as the fermentation stage has been completed satisfactorily, yogurt should have a shelf life at 4–5 °C of 2–3 weeks, while for concentrated yogurt, storage for 4–6 weeks under refrigeration should be feasible.

This confidence stems from the fact that the severe heat treatment received by the process milk, together with the low pH of the final product, makes yogurt extremely safe in respect to public health, for none of the recognized pathogens can survive or grow below pH 4.3. The spores of *Bacillus cereus*, for example, will not germinate at low pH, while organisms of concern in soft cheeses, for example, *Listeria monocytogenes*, will be inactivated long before the yogurt reaches the consumer. In addition, there is good evidence that metabolites such as hydrogen peroxide or antibiotics released by the yogurt organisms can actively depress the viability of many enteric pathogens like *Campylobacter*, *Escherichia*, and *Salmonella* spp.

Spoilage, however, can occur through the activities of acid-tolerant yeasts, or occasionally molds, and widely distributed yeasts, like *Candida* and *Saccharomyces* spp., can be associated with gas formation and/or carton 'doming' of fruit yogurts. In natural yogurt, lactose is the principal sugar available and, as few yeasts can ferment lactose, the major concern is species like *Kluyveromyces marxianus* var. *lactis* and *K. marxianus* var. *marxianus*; both of these lactose-utilizing species grow readily on poorly cleaned surfaces in a dairy. Molds from genera like *Mucor*, *Rhizopus*, *Penicillium*, and *Aspergillus* can grow readily at the yogurt/air interface in cartons, and hence protection of a filling line from airborne contamination is essential.

Excessive acidity, as a result of continued starter activity during prolonged storage above 5 °C, can also be a problem, because the acid-tolerant *L. delbrueckii* subsp. *bulgaricus* has the ability to generate lactic acid to levels of 1.7% or above. Such a level is too harsh for the palates of most consumers, and it is this postproduction acidification that, in general, tends to determine the shelf life of commercial yogurt.

See also: **Fermented Milks:** Health Effects of Fermented Milks; Types and Standards of Identity; Yogurt: Role of Starter Culture. **Lactic Acid Bacteria:** *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Streptococcus thermophilus*.

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Yogurt: Role of Starter Culture

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Introduction

For thousands of years, yogurt has been a popular fermented milk in the Middle East and, for the most part, the product was made in individual households or on a limited communal scale. No knowledge of microbiology existed but, because the same utensils and vessels were used day after day, a typical microflora began to evolve. Sometimes 'old' yogurt would have been used to start the fermentation of a fresh batch of milk and, given the high ambient temperatures in the Middle East, the natural selection of a thermophilic microflora became inevitable.

The advantages to the householder were: (1) the avoidance of spoilage of any surplus the raw milk – raw milk will spoil within hours at the ambient temperatures found in the Middle East (20–25 °C); and (2) the derivation of a nutritious and organoleptically pleasing supplement to the diet, and one with a nonrefrigerated storage life of several days. In some communities, this latter advantage was reinforced by concentration of the yogurt to give 'labneh' (concentrated yogurt with a more extended storage life), or by mixing the yogurt with crushed burghol wheat and drying to 'kishk' (ambient storage life of ~2 years).

However, the production of a basic yogurt-like material was the essential first stage and, even today, the traditional properties of yogurt have not altered. What has improved is control of the fermentation process to give end products of consistent quality, a consistency that is achieved by employing starter cultures with defined characteristics. Thus, features like the rate and extent of lactic acid production, the level of flavour compounds generated and/or the nature of any extracellular polysaccharides produced by the bacteria can all be controlled, so that with reliability of starter culture performance guaranteed in advance, there is no reason why retail yogurts should vary from batch to batch. However, achievement of this aim has been possible only through a thorough understanding of the microbiology of the fermentation and, in particular how the microorganisms concerned can be manipulated to give end products that suit a wide range of different consumer tastes.

Starter Cultures for Yogurt

When the microflora of traditional yogurt from the Middle East was examined, it was found that Gram-positive rods and cocci were the dominant types and, although the nomenclature has changed over the years, it is now agreed that the bacteria essential for the production of yogurt are *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. In some countries, there is a legal requirement for *Lb. delbrueckii* subsp. *bulgaricus* to be included in the starter culture of any diary product labelled as 'yogurt', and this stipulation is based upon the grounds that: (1) there is an historical association between the two species and 'yogurt'; and (2) the typical flavour of yogurt tends to depend on the presence of *Lb. delbrueckii* subsp. *bulgaricus*. In other countries, the same requirement is achieved by a 'code of practice' to which most manufacturers adhere voluntarily. Terms like 'mild yogurt' or 'bioyogurt' are permitted in some countries to describe a product that is merely 'yogurt-like' in texture, and it is assumed that the prefix indicates to the consumer that the culture may be different from normal yogurt.

However, it seems likely that the current position will have to reviewed in the near future and, in particular, two points appear to be in need of clarification:

1. It is generally agreed that yogurt must contain an "abundant and viable" microflora of starter origin, but it has yet to be decided if there must be a minimum cell count of *Lb. delbrueckii* subsp. *bulgaricus* for the product to be labelled as 'yogurt'.
2. Most commercial manufacturers use cultures that have been identified as *Lb. delbrueckii* subsp. *bulgaricus* by classical biochemical and other tests, and yet the application of DNA analysis has suggested that a number of isolates should be reclassified as different subspecies of *Lb. delbrueckii*. Whether or not current regulations and/or codes of practice will have to be changed to include additional subspecies of *Lb. delbrueckii* remains to be seen, but adherence to the current definition could create difficulties for yogurt manufacturers and culture suppliers alike.

Types of Starter Culture

One of the reasons why any decisions about the taxonomy of starter cultures is so important is that there are numerous strains designated as *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* in commercial use. These strains have been isolated and retained by yogurt manufacturers or culture suppliers because each culture has one or more characteristics that are qualitatively or quantitatively unique, and which provide the finished yogurt with a property that is helpful to the manufacturer or attractive to the consumer.

For example, whereas most natural yogurts are acidic enough for the average consumer at ~10 g lactic acid kg^{-1} some strains of *Lb. delbrueckii* subsp. *bulgaricus* can generate levels of lactic acid >18 g kg^{-1} of yogurt. Consequently, the selection of a strain of *Lb. delbrueckii* subsp. *bulgaricus* that ceases to release lactic acid at ~10 g kg^{-1} would benefit a manufacturer wishing to avoid the risk of product overacidification during transport or storage. Similarly, some strains of *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* produce copious amounts of extracellular polysaccharides during fermentation and, equally important, the chemical composition and functional properties of such polysaccharides may differ from strain to strain.

The advantage of this availability of strains is that culture suppliers can blend a number of strains together to give an 'industrial culture' with a specific and well-defined set of characteristics and, if necessary, can fashion a culture to meet the demands of a particular yogurt manufacturer. Indeed, now that the genetic modification of strains is legal in some countries, the extent of strain and/or culture manipulation could become extensive – perhaps cultures for fruit yogurts that can synthesize strawberry or raspberry flavour notes during fermentation might even emerge.

However, whatever the problems over the taxonomy of starter bacteria for yogurt, it is widely accepted at present that the product should be manufactured with cultures currently designated as *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* and, as indicated earlier, there are sound reasons for employing these two species for the fermentation.

Microbiology of the Fermentation

Once the initial processing of the milk has been completed, the milk is cooled to 42 °C prior to inoculation with a culture composed of equal numbers of *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. The technology for handling the cultures can vary, but irrespective of whether the culture added to the process milk is a concentrated frozen/freeze-dried culture or a liquid bulk starter grown in the factory, the ratio between *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* should be around 1 : 1. For frozen or freeze-dried commercial cultures, it is normal to assume the existence of this ratio, but verification for liquid bulk cultures is most easily obtained by a direct microscopic count with the requirement that one 'chain/clump' of streptococci should be visible for every 'chain/clump' of lactobacilli.

If necessary, confirmation can be obtained by selective enumeration on appropriate media but, as the long chains of *Sc. thermophilus* may consist of 15–20 individual cells compared with chains of 3–4 cells for *Lb. delbrueckii* subsp. *bulgaricus*, the break-up of the chains during dilution and plating alters the anticipated 1 : 1 ratio quite dramatically. Indeed, a differential colony count may well indicate that *Sc. thermophilus* accounts for some 75–85% of the viable cells present in a yogurt culture.

The Individual Species

Streptococcus thermophilus

Streptococcus thermophilus is a Gram-positive bacterium with spherical/ovoid cells of 0.7–0.9 μm diameter, and it is a natural inhabitant of raw milk in many parts of the world (*see* **Lactic Acid Bacteria:** *Streptococcus thermophilus*). It occurs in milk in long chains of 10–20 cells, and ferments lactose homofermentatively to give L(+) lactic acid as the principal product. Above ~10 g of lactic acid kg^{-1} of yogurt (around pH 4.3–4.5), the growth and metabolism of *Sc. thermophilus* is normally inhibited and cell numbers, which may have reached $10 \times 10^{7-8}$ cfu ml^{-1} of yogurt, tend to stabilize. Glucose, fructose and mannose can also be metabolized, but the fermentation of galactose, maltose and sucrose is strain specific; the loss or gain of alleles for specific aspects of metabolic performance is not uncommon.

The principal sugar in the yogurt base, lactose, is actively transported across the cell membrane of *Sc. thermophilus* through the mediation of a membrane-located enzyme, galactoside permease, and once inside the cell, another enzyme, *β*-galactosidase, hydrolyses the sugar to glucose and galactose. The glucose is then metabolized to pyruvate via the Embden–Meyerhof pathway, and lactic dehydrogenase converts the pyruvate to lactic acid. The galactose and lactic acid usually leave the cell and accumulate in the medium, but some strains of *Sc. thermophilus* possess a galactokinase which converts the galactose to galactose-1-P. This phosphorylated form of galactose can then be transformed into either glucose-1-P or galactose-6-P, depending on the strain, and further metabolized into lactic acid.

Despite its protein-rich habitat, *Sc. thermophilus* displays limited proteolytic ability, and hence its source of nitrogen is, at least initially, free amino acids occurring naturally in the milk or released during the heat treatment. However, some amino acids, such as glutamic acid, histidine, cysteine, methionine, valine or leucine, are not present in milk at levels sufficient to support the essential growth of *Sc. thermophilus*. Consequently, the increase in cell numbers depends upon the absorption of short-chain peptides released by the breakdown of milk proteins by *Lactobacillus delbrueckii* subsp. *bulgaricus*, and the hydrolysis of these peptides to their constituent amino acids.

The optimum growth temperature for *Sc. thermophilus* is 37 °C, but it is sufficiently thermophilic in nature to grow alongside *Lb. delbrueckii* subsp. *bulgaricus* during the commercial production of yogurt at 42 °C. The growth of *Sc. thermophilus* ceases at ~10 °C.

Lactobacillus delbrueckii subsp. *Bulgaricus*

Lactobacillus delbrueckii subsp. *bulgaricus* is also Gram-positive, and it occurs in milk as chains of 3–4 short rods (0.5–0.8 × 2.0–9.0 μm) with rounded ends (*see* **Lactic Acid Bacteria:** *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group). Its basic metabolism is again homofermentative but, in this case, the end product is D(−) lactic acid at levels of around 18 g kg^{-1} of yogurt; this form of lactic acid is less readily metabolized by humans than the L(+) isomer. The tolerance of *Lb. delbrueckii* subsp. *bulgaricus* to acidity also contrasts dramatically with that of *Sc. thermophilus*. Lactose, fructose, glucose and, in some strains, galactose can all be utilized by *Lb. delbrueckii* subsp. *bulgaricus* but, unlike *Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* can hydrolyse casein – especially β-casein – by means of a wall-bound proteinase to release peptides. However, the peptidase activity of *Lb. delbrueckii* subsp. *bulgaricus* is limited, and it is likely that it relies on the metabolism of peptides by *Sc. thermophilus* for a source of free amino acids.

The optimum growth temperature for *Lb. delbrueckii* subsp. *bulgaricus* is 45 °C, and hence the value of 42 °C selected for the commercial production of yogurt is an effective compromise between the growth optima of the two essential species.

Growth in Milk for the Production of Yogurt

Although the use of these two organisms is historical in origin, there are good reasons for continuing with the tradition, for when growing in milk, the two organisms interact synergistically. This interaction, or protocooperation as it is sometimes called, is based upon the fact that there is remarkable metabolic compatibility between the two species. This point can be easily demonstrated by isolating strains of *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* from a commercial starter culture, and then inoculating the individual species into milk (20 ml of liquid culture l^{-1}). If the rate of acid development in the separate milks is then compared with the level of lactic acid liberated in a 'control' (10 ml of *Sc. thermophilus* and 10 ml of *Lb. delbrueckii* subsp. *bulgaricus* liquid cultures l^{-1}), the contrast is most dramatic. Thus while the combined culture may well generate an acidity of >10 g l^{-1} in 4 h, the values in the individual cultures could be around 4 g l^{-1} for *Sc. thermophilus* and 2 g l^{-1} for *Lb. delbrueckii* subsp. *bulgaricus*.

The implications of this pattern for the dairy industry are self-evident, and it suggests also that *Sc. thermophilus* grows more rapidly than *Lb. delbrueckii* subsp. *bulgaricus* in milk and, at least initially, releases more lactic acid. This developing acidity provides an environment that is conducive to the growth and metabolism of *Lb. delbrueckii* subsp. *bulgaricus* so that, at the end of 4 h, the latter component of a combined culture will be releasing more lactic acid than *Sc. thermophilus*. The total colony count of *Lb. delbrueckii* subsp. *bulgaricus* also increases so that, although the growth patterns of the two species are markedly different over a typical 4-h fermentation, 'abundant and viable' populations of both species should be present at the end and in the retail product.

The contrast in cell counts observed during the initial stages of the fermentation provides additional evidence of protocooperation for, in addition to producing lactic acid, a rapidly growing culture of *Sc. thermophilus* releases CO_2 from the breakdown of urea in the milk and, usually, formic acid (up to 40 μg ml^{-1}); both of these latter metabolites act as further stimulants to the growth of *Lb. delbrueckii* subsp. *bulgaricus*.

As a result of this cooperation, both species actively metabolize lactose to lactic acid and, in addition, other metabolites liberated by the two species give yogurt an aroma and flavour that is distinctly different from any other fermented milk. Acetaldehyde at levels up to 40 mg kg^{-1} is the major component of the flavour profile, and the major pathway for production by *Lb. delbrueckii* subsp. *bulgaricus* – and to a lesser extent, *Sc. thermophilus* – is the conversion of threonine to glycine by threonine aldolase:

$$\mathrm{CH_3{-}{-}{-}CHOH{-}{-}{-}NH_2\ \ {-}{-}{-}COOH \rightarrow CH{-}{-}{-}NH_2\ \ {-}{-}{-}COOH + CH_3{-}{-}{-}CH{=}O}$$

Threonine Glycine Acetaldehyde

In some lactic acid bacteria, for example *Lb. acidophilus*, the enzyme alcohol dehydrogenase reduces the acetaldehyde to alcohol, but as neither *Sc. thermophilus* nor *Lb. delbrueckii* subsp. *bulgaricus* possesses this enzyme, the acetaldehyde accumulates to a level dependent upon the strains involved. It may be relevant also that the activity of the threonine aldolase produced by *Sc. thermophilus* decreases significantly as the temperature of incubation is raised above 30 °C, while the comparable enzyme in *Lb. delbrueckii* subsp. *bulgaricus* is unaffected; this latter organism is, therefore, likely to be the main source of acetaldehyde in commercial yogurt. Other metabolic pathways, such as the transformation of pyruvate by α-carboxylase, may also be involved with a build-up of acetaldehyde, but the relative importance of such pathways is probably strain dependent.

The contribution of other compounds of starter origin, such as free fatty acids, amino acids, acetone, diacetyl and keto/hydroxy acids, to the perceived flavour and aroma of yogurt is poorly understood, but it is widely agreed that it is the high level of acetaldehyde that makes the flavour of yogurt unique.

With natural, set yogurts, the properties of major importance for the consumer are consistency of the gel and the perceived acidity/flavour. However, with stirred, fruit products, the major flavour components may well come from the fruit, and hence it is the texture/viscosity and mouthfeel of the basic yogurt that will be of especial note. The milk solids nonfat content of the base milk will make an important contribution to viscosity, as may added stabilizers like guar gum, locust bean gum or modified starch. Equally relevant, however, may be the contribution of the starter culture, for strains of both *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* can produce appreciable levels of extracellular polysaccharide materials. Glucans or polymers involving glucose, galactose and rhamnose as the constituent sugars have been detected, and the presence of these metabolites enhances considerably the viscosity/consumer appeal of the end product.

Commercial starter manufacturers have available a range of cultures that differ with respect to both the type and quantity of polysaccharide(s) synthesized. Consequently, a yogurt manufacturer can select a culture that gives a 'gum-like' or 'spoonable' product, or one that generates a 'glucan-like' polymer and gives rise to a more fluid yogurt. However, in either case, the mouthfeel of the yogurt will be improved, as will the stability of the product during transport and storage. Thus, whilst some of the polysaccharide will form a layer over the cell wall of the bacterium, the remainder will form a network that binds the cells and the casein micelles together as a viscous mass. As a result, the whey remains firmly trapped within the coagulum, and does not separate as the yogurt is moved from the factory to the retail outlet.

Inhibitors of Starter Activity

It is evident that that the growth and metabolism of both *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* are essential for the production of a satisfactory yogurt, and hence that a defective starter culture will produce a substandard product. Failures of starter cultures in a yogurt factory can occur, and hence it is worth highlighting the principal causes of these potential microbiological problems:

1. If the factory relies on the in-house production of a liquid bulk starter culture, contamination by adventitious microorganisms like coliforms or yeasts can have dire economic consequences in terms of customer complaints/product recalls.
2. Strains of both *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* are susceptible to infection by host-specific viruses (bacteriophages) and, although infections by phages are not usually as serious as with some mesophilic fermentations, the risk does exist, and yogurt producers need to maintain high standards of hygiene to avoid the build-up of bacteriophages in pools of 'stagnant' whey.
3. Changes in the activity of a culture, e.g. rate of acid production or level of aroma/flavour compounds produced, can arise as a consequence of routine subculturing and the gradual emergence of a numerical imbalance between *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*.
4. The presence of antibiotics or other inhibitory substances in the milk is a major cause of poor fermentations in some countries, and *Sc. thermophilus* is especially sensitive to antibiotics like penicillin, streptomycin, neomycin and ampicillin which are widely used to control mastitis; levels of contamination as low as 0.004 International Units (IU) of penicillin can inhibit cell wall development of *Sc. thermophilus*. Strains of *Lb. delbrueckii* subsp. *bulgaricus* tend to be more tolerant (0.02 IU of penicillin), as are cultures of both species growing together. However, even when the two organisms are present and under optimum conditions, as little as 0.01 IU of penicillin can delay fermentation. Sanitizing agents employed to clean a plant, such as chlorine ($100\,\mathrm{mg\,l^{-1}}$) or iodophors ($60\,\mathrm{mg\,l^{-1}}$), can also cause inhibition of the mixed cultures, and hence the screening of bulk milk for microbiocidal agents is essential.
5. Although commercial cultures are selected for their performance under industrial conditions, incompatibility between strains of *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* can result in an almost complete absence of protocooperation between the species. Hence any attempt by a yogurt-maker to create his/her own cultures by isolating and blending strains of *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* from different sources could lead to disappointment.

See also: **Fermented Milks:** Health Effects of Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Types and Manufacture. **Lactic Acid Bacteria**: *Lactobacillus* Spp.: *Lactobacillus delbrueckii* Group; *Streptococcus thermophilus*.

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FLAVORS AND OFF-FLAVORS IN DAIRY FOODS

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Desirable Flavor Compounds in Milk

Milk is such a bland-tasting food that consumers have a difficult time describing its taste attributes. Indeed, dairy researchers have devoted far more time and effort studying chemicals responsible for off-flavors (OF) in milk than in understanding what chemicals are most important to good flavor quality.

Although processed milk of good flavor quality has a bland taste, it does, nonetheless, have a characteristic flavor, described by some as a slight salty-sweet taste. To some degree, this flavor quality can probably be attributed to milk salts and lactose, but the reason for milk's characteristic taste is far more complicated than this.

The sensory perception of milk is significantly impacted by its pleasant mouthfeel, which is due primarily to the emulsion of fat globules in an aqueous colloidal protein phase, but the volatile constituents in milk make the strongest contribution to its aroma and flavor. Dimethyl sulfide was one of the first important aroma-contributing chemicals identified in milk (in 1956). Significant improvements in analytical techniques and instrumentation have occurred since then, and dozens of other odoriferous chemicals have been identified in milk, including aldehydes, ketones, alcohols, fatty acids, lactones, esters, sulfur compounds, nitrogen compounds, and aromatic hydrocarbons. Complicating the understanding of what odorants are most important to good milk flavor are three factors: (1) the large number of volatiles present in milk, (2) their low concentration levels, and (3) in some cases, their low odor-detection threshold levels.

Recently, researchers identified 80 neutral volatiles in raw milks from different species using vacuum distillation and liquid–liquid extraction followed by high-resolution gas chromatography (HRGC). In studying the flavor constituents of bovine, ovine, caprine, and water buffalo fresh raw milks, these researchers found similar volatiles in milk from the four species. Different levels of odorants from species to species were probably responsible for the different odors and taste of the milk. Dimethylsulfone alone comprised approximately 25% of the volatile components in bovine, caprine, and ovine milk, but only 4% in buffalo milk. Pentanal and nonanal were the prevalent aldehydes, while 3-methylbutanal was found only in buffalo milk, and phenylacetaldehyde and benzaldehyde were present in large quantities in caprine milk but only in trace amounts in the other milks. The concentrations of ketones, predominantly 2-methyl ketones, were higher in buffalo milk than in the other three types. Phenylethanol, a component with a floral-rose odor, was not found in ewes' and goats' milk; its concentration in buffalo milk was 100 times higher than in bovine milk. The level of 1-octene-3-ol, a compound with a metallic note, was highest in buffalo milk, followed in order by ewe, goat, and cow milks.

Although a significant number of volatiles were observed, results of this work did not identify which compounds were actually the most important to good milk flavor. As an important follow-up, the researchers added an olfactometry technique to their HRGC–MS (mass spectrometry) method. To determine which of the 80 volatiles observed in milk samples contributed odor notes, individual chromatographic peaks were sniffed as they eluted from the GC column. With this approach, 14 different odorants were identified. To determine which of these were 'odor-impact' compounds, the researchers used the CharmAnalysis technique. In this olfactometry method, stepwise dilutions (typically 1:2 or 1:3) of the prepared extract with a solvent are made, followed by evaluation of each dilution by chromatographing and sniffing column effluents. Using CharmAnalysis, the odor-impact volatiles in the four types of milk were determined. For raw bovine milk, the two most important odor-impact chemicals were ethyl butanoate and ethyl hexanoate.

It is interesting to note that significantly higher levels of ethyl butanoate and ethyl hexanoate are detected consistently in raw milk than in pasteurized milk. In fact, these esters are frequently not detected at all in pasteurized milk when analyzed by purge-and-trap gas chromatography or other GC-based techniques capable of $\mu g\,kg^{-1}$ detection levels. The majority of these esters, which apparently are the primary odor-impact chemicals in raw bovine milk, are lost or destroyed during pasteurization and processing.

Other popular gas chromatograpy–olfactometry (GC–O) techniques include aroma extraction–dilution analysis (a dilution technique) and Osme, a cross-modal matching technique. GC–O techniques are invaluable for identification of potent odorants in dairy products, including desirable odorants as well as those that contribute OF. (Further discussion of GC–O is beyond the scope of this article.)

Table 1 lists some of the volatiles most commonly present in normal-tasting raw and pasteurized milk, and describes their possible origin. **Figure 1** is a GC–MS chromatogram of fresh, pasteurized milk (2% milk fat content) stored in a plastic (high-density polyethylene) bottle. This particular sample had a normal flavor profile, free of OF. Some of the components, such as hexanal and styrene, have been known to cause OF in milk, but in this particular sample these compounds were present at concentrations below their flavor-odor threshold detection level.

Flavor Development in Cultured Dairy Products

Modification of milk components by starter culture enzymes is responsible for the flavor of cultured dairy

Table 1 Volatiles most commonly present in raw and processed milks of normal, good flavor quality

Volatile[a]	*Possible origin*	*Comments*
Acetone	Feed; bovine ketosis	Volatile present in highest concentration
2-Butanone	Feed	Volatile usually present at second highest concentration
Ethyl butanoate, ethyl hexanoate	Microbial metabolites (ethanol + fatty acid → ester)	Significantly more in raw milk than in pasteurized milk; most potent odorants in raw milk
Dimethyl sulfide	Thermal decomposition of methionine and cystine; microbial metabolite	Present in trace amounts; higher levels in pasteurized milk compared with raw milk
Toluene	Degradation of β-carotene	Trace amounts; packaging materials can also contribute toluene
Limonene	Forages	Trace amounts
Methyl ketones (C_5–C_{12})	Microbial metabolites; thermal degradation of β-keto acids naturally found in milk	Trace amounts
3-Methylbutanal	In raw milk, because of microbial growth (*Streptococcus lactis* var. *maltigenes*); in heated milk as a result of nonenzymatic browning reaction involving leucine (Strecker degradation)	Trace amounts; highest levels typically found in raw milk
Chloroform	Unknown (perhaps from chlorinated sanitizers used to wash teats before milking)	Trace amounts; highest levels typically found in raw milk, less in pasteurized milk
Fatty acids (C_4–C_{10})	Incomplete triacylglycerol synthesis in the mammary gland; possibly also lipase activity	Trace amounts
Xylene isomers, styrene	Contaminants from packaging materials	Trace amounts in processed milk; not normally present in raw milk
Hexanal	Autoxidation of linoleic acid	Trace amounts

[a] Except for ethyl butanoate and ethyl hexanoate in raw milk, all volatiles are at concentrations below the taste–odor detection threshold level.

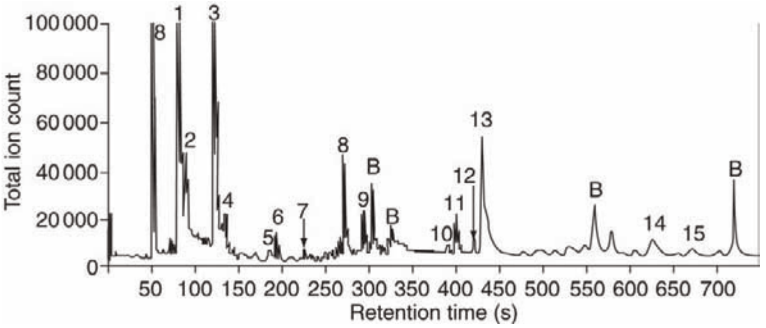

Figure 1 A GC–MS chromatogram of fresh, pasteurized milk (2% milk fat) of normal, good-tasting flavor quality stored in a plastic (high-density polyethylene) bottle. Extraction of volatiles performed by SPME using a 75 μm Carboxen-PDMS fiber. Peak identities are as follows: (1) acetone; (2) dimethyl sulfide; (3) 2-butanone; (4) chloroform; (5) 3-methyl butanal; (6) pentanal; (7) ethyl butyrate; (8) toluene; (9) hexanal; (10) and (11) xylene isomers; (12) 2-heptanone; (13) styrene; (14) limonene; (15) 2-nonanone; (B) blank, chemical from GC septum.

products. Lactic acid fermentation is critical to the characteristic flavor development of cultured dairy products, including buttermilk, sour cream, and yogurt.

Buttermilk and Sour Cream

Sour cream and buttermilk depend for their flavor on the combined action of strains of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* to produce lactic acid and on *Leuconostoc mesenteroides* subsp. *cremoris* and citrate-positive (Cit^+) strains of *Lc. lactis* for production of aroma chemicals (especially diacetyl). In recent years, some dairies have stopped using Cit^+ *Lc. lactis* subsp. *lactis* biovar *diacetylactis* because it generates a rather harsh flavor profile (*see* **Fermented Milks**: Buttermilk).

Lactic acid imparts a pleasant acid flavor. Excessive lactic acid from over-ripening or improper incubation temperature (or delayed cooling/under-cooling) makes the finished product taste acrid and gives it a coarse mouthfeel. Furthermore, excessive acidity masks the delicate flavor notes contributed by other important aroma chemicals. Only about 18% of the naturally available lactose in milk is fermented to lactic acid. The desired lactic acid level in good-flavored buttermilk is 0.75–0.85%.

Trace amounts of acetic, formic, propionic, and/or pyruvic acids also are formed in secondary metabolic reactions in cultured buttermilk and sour cream; these relatively volatile acids impart a pleasant acid flavor. The critical 'buttery' nutlike flavor characteristic of most cultured dairy products is attributed to diacetyl, a potent fragrance compound.

Diacetyl is derived from the fermentation of citric acid by *Ln. mesenteroides* subsp. *cremoris* and Cit^+ *Lc. lactis*. The pathway for the metabolism of citrate has been elucidated (**Figure 2**). High-performance liquid chromatography (HPLC) has been used to monitor changes in organic acids during buttermilk fermentation (**Figure 3**), while static headspace gas chromatography has been used to monitor volatile organics (**Figure 4**).

Chemical changes that occur during storage of buttermilk at refrigerated temperatures are shown in **Table 2**. The increase in acetaldehyde and the decrease in diacetyl are perhaps the most noteworthy changes. Loss of diacetyl is a result of its conversion (enzymatic reduction) to acetoin by diacetyl reductase (from *Ln. mesenteroides* subsp. *cremoris*). The increase in acetaldehyde and the decrease in diacetyl can result in an OF that is commonly described as the 'green flavor defect'.

Yogurt

Most commercial yogurt is produced by the action of two dissimilar organisms, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which grow simultaneously to give the desired flavor, mouthfeel, and body to the yogurt. It is generally agreed that lactic acid, acetic acid, and acetaldehyde are among the most important contributors to yogurt flavor and that the symbiotic action of *Sc. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* is necessary to obtain the desired flavor profile and acid balance. However, other secondary metabolites contribute significantly to the delicate flavor nuances of natural yogurt (*see* **Fermented Milks**: Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture).

Dozens of flavor compounds have been identified in yogurt. Headspace and simultaneous distillation–extraction have been commonly used for the analysis of yogurt flavor volatiles. Because of the low intensity of yogurt odor, much of the research performed in the past involved heating samples to increase the volatility of flavor components and improve the sensitivity of the

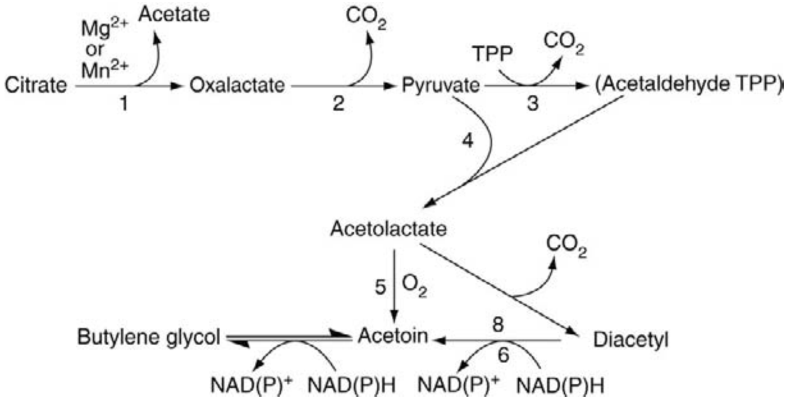

Figure 2 Pathway for the conversion of citrate to diacetyl and other compounds. The reactions are catalyzed by the following enzymes: (1) citrate lyase; (2) oxaloacetate synthase; (3) pyruvate decarboxylase; (4) acetolactate synthase; (5) acetolactate decarboxylase; (6) diacetyl reductase; (7) acetoin reductase. Reaction 8 is the oxidative conversion of α-acetolactate to diacetyl. Adapted from Jenness R and Patton S (1959) *Principles of Dairy Chemistry*. New York: John Wiley.

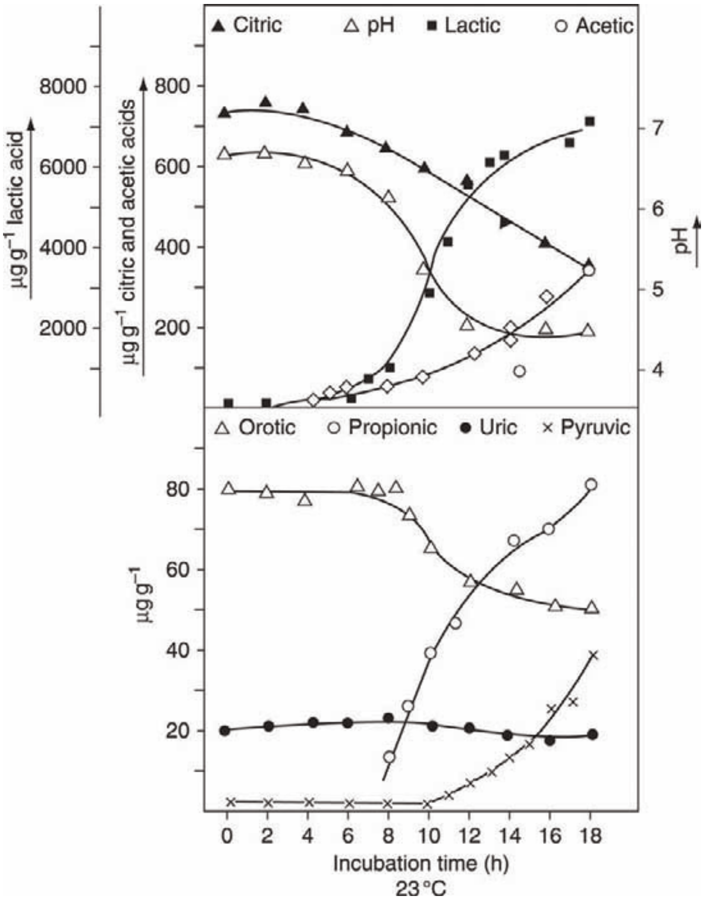

Figure 3 Changes in the concentration of organic acids in cultured buttermilk during a typical fermentation, as determined by HPLC. Reproduced with permission from Marsili RT (1981) Monitoring bacterial metabolites in cultured buttermilk by high-performance liquid chromatography and gas chromatography. *Journal of Chromatographic Science* 19: 451–456.

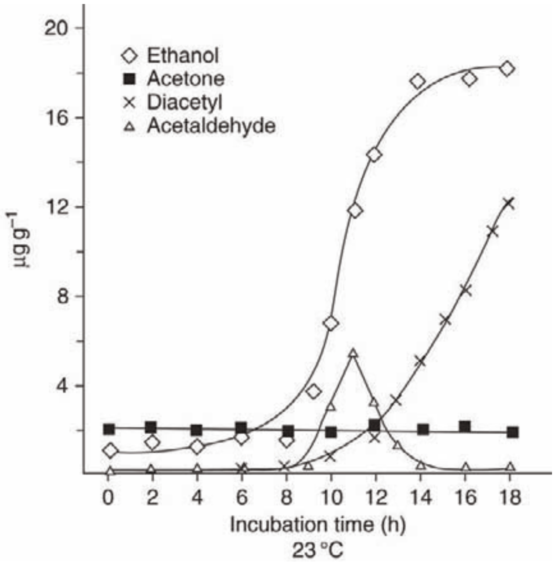

Figure 4 Changes in the concentration of volatile organic compounds in cultured buttermilk during a typical fermentation as determined by headspace GC. Reproduced with permission from Marsili RT (1981) Monitoring bacterial metabolites in cultured buttermilk by high-performance liquid chromatography and gas chromatography. *Journal of Chromatographic Science* 19: 451–456.

Table 2 Changes in concentration of organic acids (high-performance liquid chromatography) and volatile organic compounds (headspace gas chromatography) in buttermilk made from skim milk, at end of fermentation, and after an additional week of refrigerated storage

	Concentration ($\mu g\,g^{-1}$)		
Chemical	*0 h at 23 °C*	*18 h at 23 °C*	*168 h at 3 °C*
Orotic acid	79.1	42.2	34.7
Citric acid	730	176	20
Pyruvic acid	1.3	33	<0.2
Lactic acid	45	7410	5820
Uric acid	20.4	18.8	19.7
Acetic acid	<5	610	730
Propionic acid	<10	90	40
Acetaldehyde	0.02	0.77	1.46
Acetone	2.10	1.98	2.14
Ethanol	1.00	24.2	37.3
Diacetyl	<0.05	11.5	1.90

Reproduced with permission from Marsili RT (1981) Monitoring bacterial metabolites in cultured buttermilk by high-performance liquid chromatography and gas chromatography. *Journal of Chromatographic Science* 19: 451–456.

analytical test. Heating samples, however, can alter the composition of sensitive aroma compounds and lead to artifact peaks that can be misinterpreted as being potent odor-flavor contributors that occur naturally in the product. Therefore, much of this work may be invalid.

Recently, a variety of low-temperature headspace procedures have been used to extract volatiles from yogurt. In one low-temperature headspace technique, aroma volatiles were allowed to equilibrate between the matrix and the sample headspace at a controlled temperature (30 °C) before being swept from the headspace into a Tenax trap. The volatiles were then thermally desorbed from the Tenax, cryrofocused, and finally injected into a GC–MS equipped with an olfactometry detector.

One unique aspect of this work was the olfactometry method used to detect odor-impact chemicals. This GC–O technique is referred to as the detection frequency method and is neither a dilution nor a cross-modal matching technique. Only one dilution level was used, but GC–O experiments are repeated several times (e.g., 8–10). Aromagrams of individuals were then 'averaged' to eliminate reproducibility problems. Resulting aromagrams permitted odor profile comparisons as peak intensities were related to the frequencies of odor detection.

Using this technique, researchers detected some 91 different volatiles in the yogurt headspace, with 21 of these exhibiting significant odor impact. These odor-impact chemicals are shown in **Table 3**. Five compounds – 1-nonen-3-one, methional, 2-methyltetrahydrothiophen-3-one, 2(E)-nonenal, and guaiacol – with intense odor were reported for the first time in yogurt flavor. Identification of 1-nonen-3-one is especially significant. This component appears to be one of the most potent flavorings identified to date. Its low odor threshold justifies its impact role in yogurt despite its low concentration.

Common Causes of Off-Flavors in Milk and Dairy Products

Light-Induced Off-Flavor

Light-induced OF in milk is common and of major concern to the dairy industry. It has been estimated that exposure of milk in high-density polyethylene bottles to fluorescent lights in supermarket dairy cases is responsible for the development of light-induced OF in some 80% of samples sold in US supermarkets.

Light-induced OFs have two distinct components. Initially a burnt, activated sunlight flavor develops and predominates for approximately 2 or 3 days. Degradation of sulfur-containing amino acids of the serum (whey) proteins is probably responsible for this reaction. The exact reaction products responsible for this so-called light-activated flavor (LAF) have not been elucidated clearly. Methional (3-(methylthio)propanal), however, has been implicated as a possible contributor. One mechanism that has been postulated involves the light-induced reaction of methionine and riboflavin, producing methional.

Understanding the true impact that methional has on LAF is difficult, because it is relatively unstable and breaks down into more stable components, including mercaptans, sulfides and disulfides. Some researchers have suggested that methanethiol, dimethyl disulfide, and dimethyl sulfide also contribute to LAF. A recent

Table 3 Compounds contributing to the aroma of yogurt

Retention index[a]	*Compound*	*Odor descriptors*
716	Acetaldehyde	Fresh, green, pungent
757	Dimethyl sulfide	Milk, lactone-like, sulfury, warm
995	2,3-Butanedione	Butter, diacetyl, vanilla
1082	2,3-Pentanedione	Butter, vanilla, milk
1120	2-Methylthiophene	Gasoline, plastic, styrene
1221	3-Methyl-2-butenal	Metallic, aldehydic, herbaceous
1322	1-Octen-3-one	Mushroom, earthy
1406	Dimethyl trisulfide	Sulfury, hydrogen sulfide, fecal
1424	1-Nonen-3-one	Mushroom, earthy
1462	Acetic acid	Pungent, acidic, vinegar
1479	Methional	Soup, cooked vegetables, pungent, sulfury
1551	(*cis*, *cis*)-Nonenal + 2-methyltetrahydrothiophen-3-one	Green, leather, sulfury
1680	2-Phenylacetaldehyde	Flowery
1684	3-Methylbutyric acid	Sweaty, cheese, soy sauce, flowery
1715	Unidentified	Flowery, warm, caramel
1750	Unidentified	Metallic
1882	Caproic acid	Rancid, flowery
1896	Guaiacol	Bacon, phenolic, smoked, spicy
2002	Benzothiazole	Burnt, rubbery
2043	Unidentified	Hydrocarbon, chemical, burnt rubber

[a] Retention index on DB-Wax phase, using headspace injection method. Reproduced with permission from Ott A, Fay LB, and Chaintreau A (1997) Determination and origin of the aroma impact compounds of yoghurt flavor. *Journal of Agricultural and Food Chemistry* 45: 850–858.

dynamic headspace multivariate analysis study of OF in milk showed a correlation between increasing levels of dimethyl disulfide with increasing exposure time of milk to fluorescent light. In two recent studies, when light-exposed samples with LAF were analyzed by dynamic headspace GC–MS, methional was not detected at measurable levels. This has inspired researchers to propose an alternative mechanism, one that does not involve methional, to explain the formation of dimethyl disulfide. According to this mechanism (**Figure 5**), dimethyl disulfide is formed by singlet oxygen oxidation of methionine.

The second component of light-induced OF in milk is attributed to lipid oxidation (*see* **Milk Lipids**: Lipid Oxidation). This OF, often characterized as metallic or cardboardy, usually develops after 2 days and does not dissipate. Aldehydes (especially pentanal and hexanal) and, to a lesser degree, ketones (e.g., 1-hexen-3-one and 1-nonen-3-one), alcohols, and hydrocarbons have been observed to form in milk as a result of light-induced lipid oxidation reactions. The unsaturated aldehydes and ketones have the lowest sensory thresholds and are usually considered the primary sources of oxidized OF. When milk is exposed to light, various carbonyl compounds form from the reaction of light and oxygen with unsaturated fatty acids in the milk fat triacylglycerols and other milk fat components. Autooxidation of unsaturated fatty acids involves a free radical reaction, forming fatty acid hydroperoxides that degrade to various malodorous compounds, for example, hexanal, the predominant lipid reaction byproduct in light-exposed milk, in the case of linoleic acid.

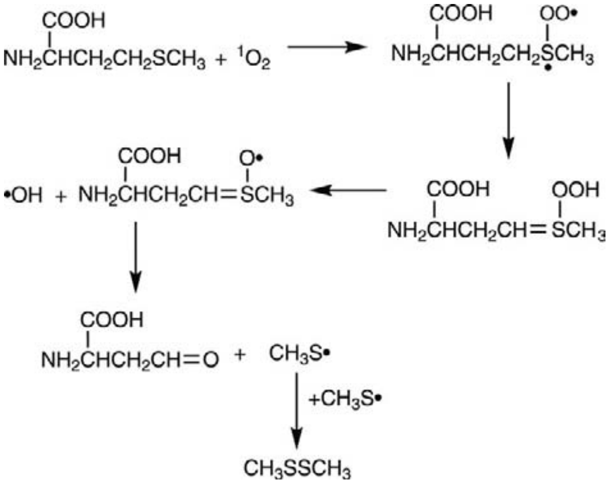

Figure 5 Postulated mechanism for the formation of dimethyl disulfide by singlet oxygen oxidation of methionine. Adapted from Jung MY, Yoon SH, Lee HO, and Min DB (1998) Singlet oxygen and ascorbic acid effects on dimethyl disulfide and off-flavor in skim milk exposed to light. *Journal of Food Science* 63: 408–412.

Other dairy products besides milk have been known to develop significant LAF OF. One example is a vanilla ice cream sample that developed a strong putrid flavor defect (**Figure 6**). Several samples developed the flavor defect, which was concentrated on the surface of the ice cream nearest the lid; several different production runs developed the defect. The ice cream was packaged in a round 1.5 gallon paperboard carton fitted with a clear plastic lid. Analysis of complaint samples by purge-and-trap

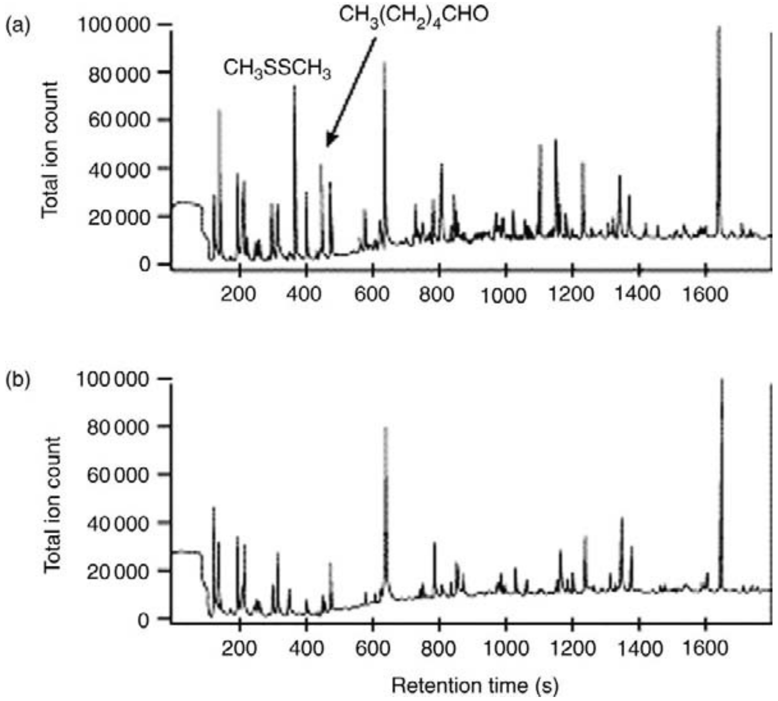

Figure 6 Chromatograms (obtained by purge-and-trap GC–MS) of (a) vanilla ice cream with severe putrid flavor defect caused by light-induced production of dimethyl disulfide, and (b) control vanilla ice cream with no off-flavor.

GC–MS revealed that, compared with control samples, the complaint samples had significant concentrations of dimethyl disulfide and hexanal. Records showed that the flavor defect first became noticeable when samples were stored in a new warehouse. Careful inspection of the freezer warehouse storage conditions revealed that samples were stored in proximity to high-intensity light. Lighting adjustments were made, and the problem was easily and quickly remedied.

Oxidized Off-Flavors from Contact with Prooxidant Metals

The presence of pro-oxidant metals like copper, iron, and nickel can significantly accelerate the rate of lipid oxidation OF development in milk and processed dairy products. This problem has been observed, for example, in sour cream (**Figure 7**). (Note that the size of the aldehyde peaks C_5–C_8 in normal-tasting sour cream would be similar to the size of these peaks shown in the 2% milk chromatogram in **Figure 1**.) In this example, a copper valve was used in the processing equipment. The high level of hexanal is an excellent indicator of lipid oxidation catalyzed by pro-oxidant metal contamination. One interesting aspect of this problem was that dairy technologists were unable to recognize the OF as an oxidation OF. Although the dairy technologists were experts at distinguishing oxidation OF in milk, the strong culture flavor background of the sample altered the perception of the oxidized flavor so dramatically that oxidation was not even considered as a potential cause of the OF. This example shows how valuable good analytical support can be in resolving OF issues with dairy products.

Microbial Off-Flavors

Milk is an excellent medium for the growth of microorganisms. Malodorous chemicals produced as metabolites during the bacterial growth phase are a common cause of acid, malty, fruity, bitter, putrid, and unclean OF in dairy products.

Post-pasteurization contamination of milk with psychrotrophic bacteria can cause milk OF. Some characteristic OF notes in milk are clearly associated with specific types of psychrotrophs. For example, contamination by *Pseudomonas fragi*, a psychrotrophic Gram-negative organism, often causes ‘fruity’ off-notes in milk. *Pseudomonas fragi* lipase and esterase hydrolyze short-chain fatty acids from milk fat and convert the acids to ethyl esters by reaction with ethanol. Strains of *Bacillus* spp. have also been observed to produce fruity OF in milk.

Another common psychrotroph associated with a particular flavor defect is *Lc. lactis* var. *maltigenes*. The malty aroma of milk cultures of *Lc. lactis* var. *maltigenes* is caused by the production of 3-methylbutanal, which at concentrations as low as 0.5 mg l^{-1} in milk generates the characteristic malty flavor defect. The action of bacterial enzymes on leucine produces 3-methylbutanal. Another

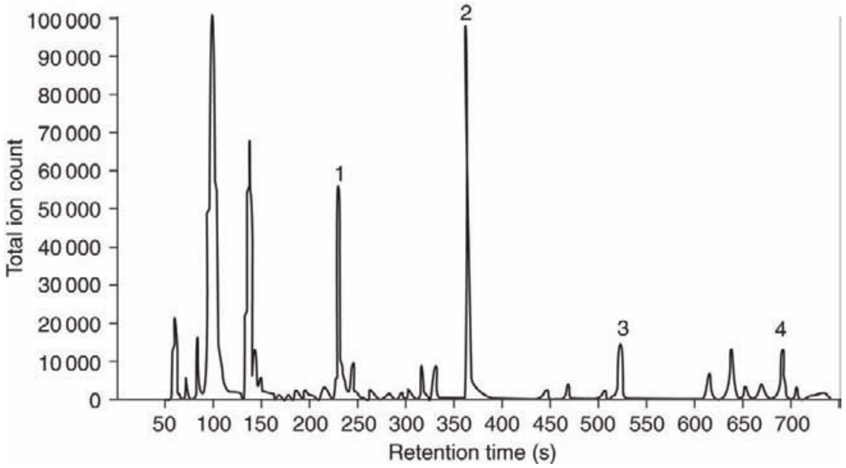

Figure 7 Chromatogram (obtained by solid-phase microextraction GC–MS using a 75 μm Carboxen–PDMS fiber) of a sour cream sample severely oxidized by contact with processing equipment containing a copper valve. Oxidation of unsaturated fatty acids in milk fat generates numerous malodorous aldehydes. Peak identities are as follows: (1) pentanal; (2) hexanal; (3) heptanal; (4) octanal.

organism capable of producing 3-methylbutanal is *Lb. maltaromicus*.

Other common metabolites include ethyl acetate, dimethyl sulfide, dimethyl disulfide, ethanol and other alcohols, methyl ketones, C_4–C_{10} free fatty acids, lactic acid pyruvic acid, and bitter peptides.

One problem that complicates understanding of the causes of OF in milk is that many of these chemicals can be produced by two or more mechanisms. For example, the production of methyl ketones and secondary alcohols (**Figure 8**) and lactones (**Figure 9**) in milk can result from microbial reaction pathways as well as from high-heat treatment. Dimethyl sulfide is generated (but dissipates after a few hours) by pasteurization and by microbial spoilage. Dimethyl disulfide is produced when milk is irraditated with fluorescent light, as well as by enzyme-induced reactions on milk proteins.

Lactones can impart an undesirable burnt, fruity, stale, and coconut-like OF to dairy products. Elevated levels of hydroxy acids sometimes occur in milk when cows are fed stored forages rather than pasture. Hydroxyacids may be incorporated into the triacylglycerols. These esters of hydroxy acids are relatively unstable and are readily hydrolyzed from the triacylglycerol in the presence of water and heat. They then cyclize to form lactones, which can cause a stale flavor in milk.

As long as they are bound to the triacylglycerol, the hydroxyacids do not impart OF to milk. However, thermal processing frees the acid, leading to the formation of undesirable-tasting lactones. Various microbial reactions can also generate lactones. For example, yeasts such as

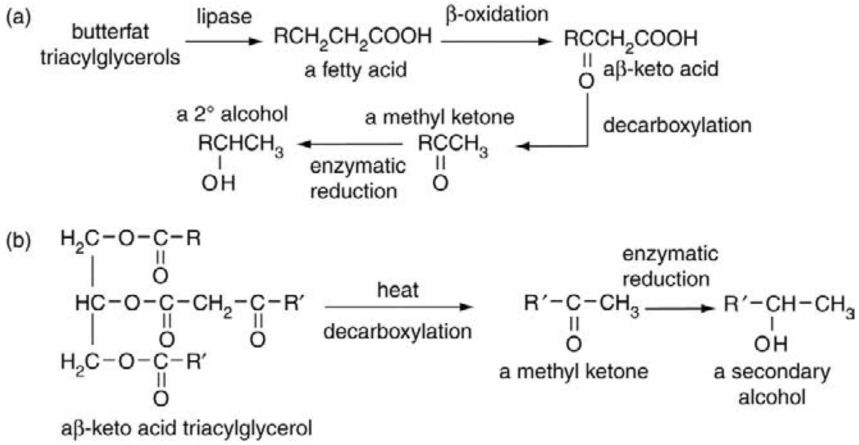
(a)
butterfat triacylglycerols
lipase
RCH2CH2COOH
a fetty acid
β-oxidation
RCCH2COOH
aβ-keto acid
decarboxylation
a methyl ketone
RCCH3
enzymatic reduction
a 2° alcohol
RCHCH3
OH
(b)
H2C–O–C–R
HC–O–C–CH2–C–R′
H2C–O–C–R′
aβ-keto acid triacylglycerol
heat
decarboxylation
R′–C–CH3
a methyl ketone
enzymatic reduction
R′–CH–CH3
OH
a secondary alcohol

Figure 8 Formation of methyl ketones and secondary alcohols in milk by microbial and thermally induced reaction mechanisms. (a) By action of enzymes from fungi, molds and some types of bacteria on butterfat triacylglycerols; (b) by thermal degradation of β-keto acids in triacylglycerols. Adapted from Jenness R and Patton S (1959) *Principles of Dairy Chemistry*. New York: John Wiley.

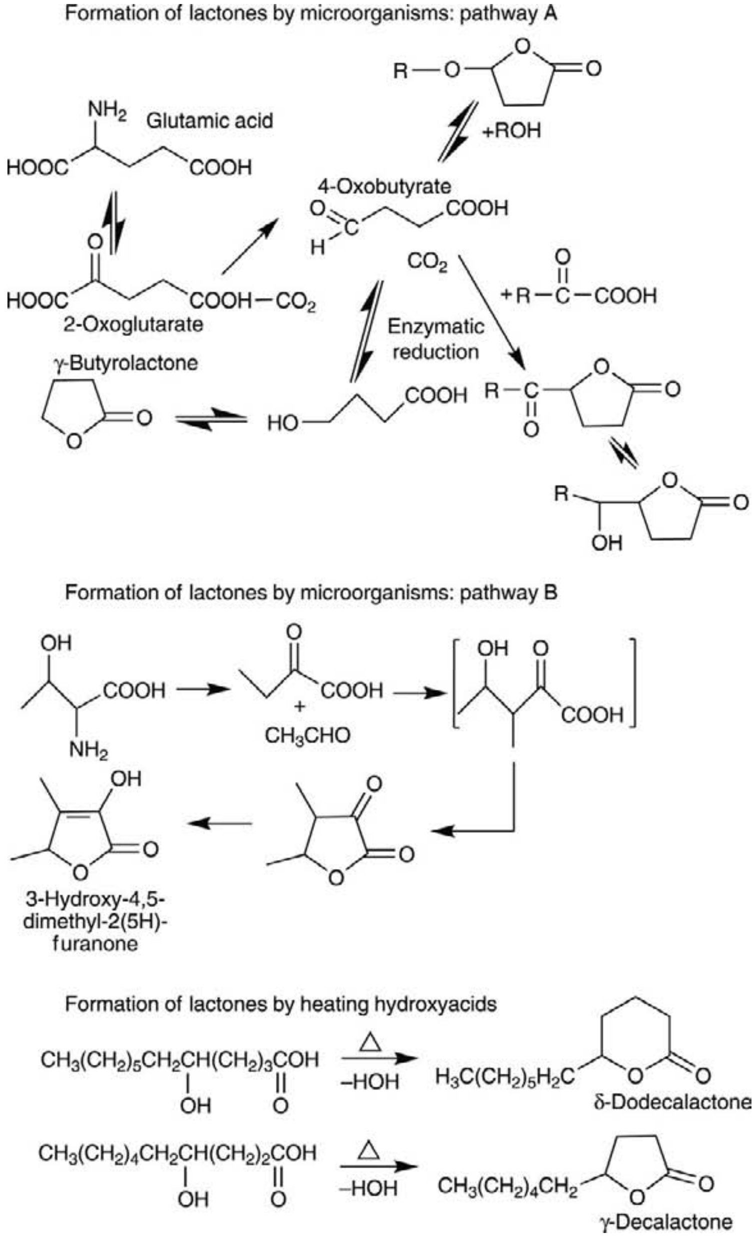
Formation of lactones by microorganisms: pathway A
Glutamic acid
HOOC
COOH
NH_2
HOOC
COOH−CO_2
2-Oxoglutarate
4-Oxobutyrate
COOH
+ROH
R—O
CO_2
+R—C—COOH
Enzymatic reduction
γ-Butyrolactone
HO
COOH
R—C
OH
Formation of lactones by microorganisms: pathway B
OH
COOH
NH_2
COOH
+
CH_3CHO
OH
COOH
3-Hydroxy-4,5-dimethyl-2(5H)-furanone
Formation of lactones by heating hydroxyacids
$CH_3(CH_2)_5CH_2CH(CH_2)_3COH$
–HOH
$H_3C(CH_2)_5H_2C$
δ-Dodecalactone
$CH_3(CH_2)_4CH_2CH(CH_2)_2COH$
–HOH
$CH_3(CH_2)_4CH_2$
γ-Decalactone

Figure 9 Formation of lactones in milk by microbial pathways and by thermally induced reactions. Adapted from Jenness R and Patton S (1959) *Principles of Dairy Chemistry*. New York: John Wiley.

Candida spp. and *Saccharomyces cerevisiae* and molds such as *Penicillium notatum* and *Cladosporium butyri* are known sources.

Raw milk contaminated with psychrotrophic bacteria before pasteurization can develop OF and malodor problems, even though most of the bacteria are killed by pasteurization. Although pasteurization destroys the bacteria, their heat-stable lipases, proteases, and other enzymes sometimes survive, and the action of these extracellular enzymes on milk components can also generate OF. These OF chemicals can be monitored by GC–MS (**Figure 10**). (See **Figure 1** for comparison with chromatogram of normal-tasting 2% processed milk.) To minimize the chances of this problem, raw milk should never be held for longer than 48 h in refrigerated silos before pasteurization.

Microorganisms can generate OF in unexpected and interesting ways. Potassium sorbate is often used as a fungistatic agent for cultured dairy products and cheeses. It has been shown that a large number of molds in the

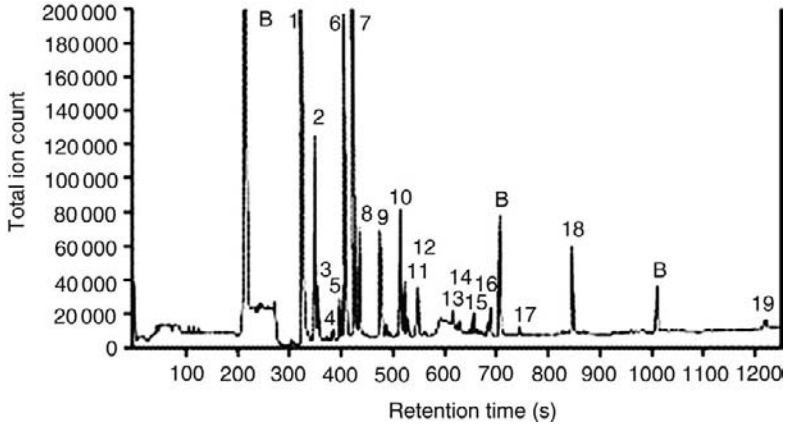

Figure 10 Off-flavor chemicals formed in processed milk by exogenous enzymes. Raw milk held for too long before pasteurization. Processed milk made from this raw form developed elevated levels of numerous bacterial metabolites 4 days after processing, even though microbiological plate counts were normal. Peak identities are as follows (* indicates chemical is a metabolite): (1) acetone; (2) methyl acetate*; (3) extraneous chemical from SPME fiber; (4) 2-methyl-2-propenal*; (5) diacetyl*; (6) 2-butanone; (7) ethyl acetate*; (8) chloroform; (9) 3-methyl butanal*; (10) 2-pentanone*; (11) 3-methyl pentane; (12) propyl acetate*; (13) 3-methyl-2-pentanone*; (14) isoamyl alcohol*; (15) propanoic acid*; (16) hexanal; (17) 4,4-dimethyl heptane; (18) 2-heptanone*; (19) 2-nonanone*; (B) blank, chemical from GC septum.

genus *Penicillium* can grow in the presence of up to 7100 mg l^{-1} potassium sorbate. The occurrence of a plastic, paint, or kerosene OF in Feta cheese, cottage cheese, and other dairy products has been attributed to the production of *trans*-1,3-pentadiene, a strong odorant produced from the enzymatic decarboxylation reaction of sorbic acid (*trans*, *trans*-2,4-hexadienoic acid) (**Figure 11**).

Feed-Related Off-Flavors

What cows eat can profoundly impact the flavor of the milk they produce. Feeds that are known to cause OF in milk include fermented musty silage (maize, legumes, and grass), alfalfa (green or hay), clover hay, brewers' grain, and green barley. The actual chemicals responsible for the OF flavor have been identified in some cases. Freshly cut alfalfa hay contains high levels of *trans*-2-hexenal, *trans*-3-hexenals, and *trans*-3-hexenols, which impart a green, grassy flavor to milk. Many types of weeds – especially wild onion, garlic, and related plants – impart serious OF to milk when ingested by dairy cows (predominantly because of various sulfur-containing organic compounds). (Note that there is another good reason to avoid feeding dairy cows musty, fermented silage: aflatoxin contamination. Mold-contaminated feed runs the risk of transmitting aflatoxins B_1, B_2, G_1, and G_2 to the cow, which are converted to aflatoxin M_1 in the cow's liver and transmitted in that form to the milk. Aflatoxins are among the most potent carcinogens known.)

The cow's diet can affect flavor in unexpected, subtle ways. In one study, for example, dozens of milk samples from one geographical region of the United States were analyzed by purge-and-trap GC–MS to determine the source of the severe OF characterized as 'oxidized' by dairy technologists. Testing revealed unusually high concentrations of hexanal, suggesting autoxidation of linoleic acid in milk fat. The OF problem had occurred during the late winter months for the previous 4 years but was getting progressively worse each successive year. Because the level of hexanal was so high, copper contamination from processing lines or from diet supplements was suspected initially as the source of activation catalysis. Previous studies on milk samples intentionally abused by exposure to fluorescent light and contact with copper revealed that hexanal levels tend to be significantly

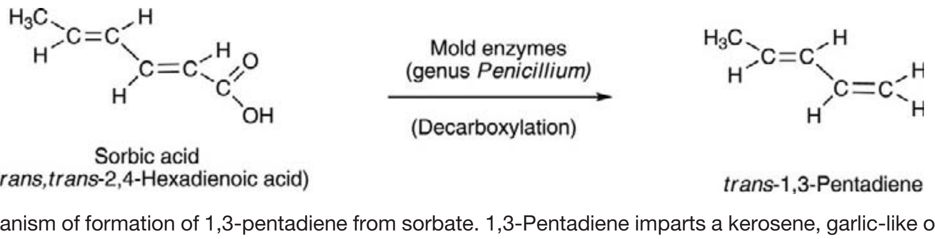

Figure 11 Mechanism of formation of 1,3-pentadiene from sorbate. 1,3-Pentadiene imparts a kerosene, garlic-like off-flavor to dairy products at low concentrations.

higher when pro-oxidant metals are involved than when milk is light-abused without added prooxidant metal.

Further testing of 'bad' milk samples by atomic absorption spectrometry ruled out contamination by copper, nickel, or iron. Another suspected contributing factor was lack of naturally occurring antioxidants (e.g., α-tocopherol) as a result of changes in winter feed regimens. However, HPLC analysis showed that samples contained normal levels of α-tocopherol.

When fatty acid profiles of complaint and normal samples were compared, samples with intense oxidized OF consistently contained 200–300% more linoleic acid than normal-tasting milk from different geographical areas. Linoleic acid ($C_{18:2}$) is highly susceptible to photoxidation.

The high linoleic acid content was traced to the cows' diet. Dairy farmers in this region had been feeding cows increasingly high levels of soya beans each year. Feeding high levels of soya beans has been shown to increase the linoleic acid content of milk fat and make milk unusually susceptible to oxidation. This problem was corrected by regulating levels of unsaturated lipids in feed to decrease the proportion of unsaturated fat in milk fat and/or by supplementing feed with an antioxidant like α-tocopherol.

Packaging-Related Off-Flavors

Ironically, packaging materials, which are designed to preserve the freshness and flavor of foods and beverages, can actually be directly responsible for causing OF defects. Although plastic packaging material consists primarily of nonvolatile high-molecular-weight polymers, volatile low-molecular-weight compounds are often added to improve the functional properties of the materials: plasticizers to improve flexibility, antioxidants to prevent oxidation of the plastic polymers or the food inside the packaging and UV blockers to prevent 'yellowing' of polymeric material when it is exposed to light. Additional additives include polymerization accelerators, cross-linking agents, antistatic chemicals, and lubricants.

Occasionally packaging materials are not cured before they are used, and a small amount of solvent associated with the manufacturing of the packaging materials remains. Residual solvent in packaging materials can migrate into the dairy product, imparting malodors and OF. Residual styrene monomer from polystyrene packaging is a common example.

One example of a dairy product that developed a severe OF problem because of packaging occurred with half-and-half packaged in 13-ml polystyrene cups. (Note that half-and-half is a mixture of milk and cream with a milk fat content ranging from 10.5 to 18.5%; it is commonly used in the United States to flavor coffee and tea beverages.) Normal-tasting control samples and complaint samples with a 'chemical, solvent-like' OF were analyzed by purge-and-trap GC–MS. The most significant differences in the chromatograms of control and tainted samples were an increase in the acetone levels and the presence of a significant proplyacetate peak in the tainted samples (**Figure 12**). The odor of the propylacetate peak was characterized by GC–O experiments (using a sniff port at the end of the GC column) and judged to be similar to the sample's odor defect.

The packaging lid-stock consisted of three layers: an outer paper layer impregnated with water-based inks, a middle metal foil layer, and an inner plastic film used for heat sealing. The inner heat-seal film, which is actuated by heat and pressure to seal the lid to the cup, is applied to the foil as a slurry and dried. The solvent used by the packaging supplier to form the slurry was propylacetate. After application of the plastic film to the foil, sheets of the lid-stock material pass through drying ovens to remove all residual propylacetate solvent. According to the packaging supplier's specifications, the finished lid-stock should contain less than 1000 mg propylacetate per 280 m^2 of material. After the problem was reported to the packaging supplier, quality control specifications were monitored more closely and no further OF occurred.

Techniques for Analyzing Flavors and Off-Flavors

Gas chromatography with mass spectrometry detection is the method of choice for analyzing volatile and semivolatile odor-active compounds in dairy products. The volatile and semivolatile compounds in the headspace are of interest because they can travel to the nose during eating and stimulate the olfactory receptors in the nasal cavity.

Perhaps the most critical and challenging step in the analytical process is the sample preparation technique used to isolate and concentrate the flavor compounds from the dairy matrix. Because it is not uncommon for the chemicals responsible for food malodors to be present at $\mu g\,kg^{-1}$ and even $ng\,kg^{-1}$ levels, the extraction technique must collect as many molecules of OF chemicals as possible. It is important that the extraction technique does not introduce or create volatiles that are not in the dairy product being tested. For example, sample preparation techniques that involve heating the sample to a high temperature (e.g., steam distillation) can generate artifact peaks in sample chromatograms, and these odoriferous artifacts may be misinterpreted as the cause of the malodor OF problem.

Although some excellent flavor work has been done with HPLC, supercritical fluid extraction, supercritical fluid chromatography, and other analytical methods, GC techniques have been the most useful. Dairy chemists now have a wide variety of sample preparation methods that they can use to isolate and concentrate odor-active compounds before GC analysis. The most frequently used

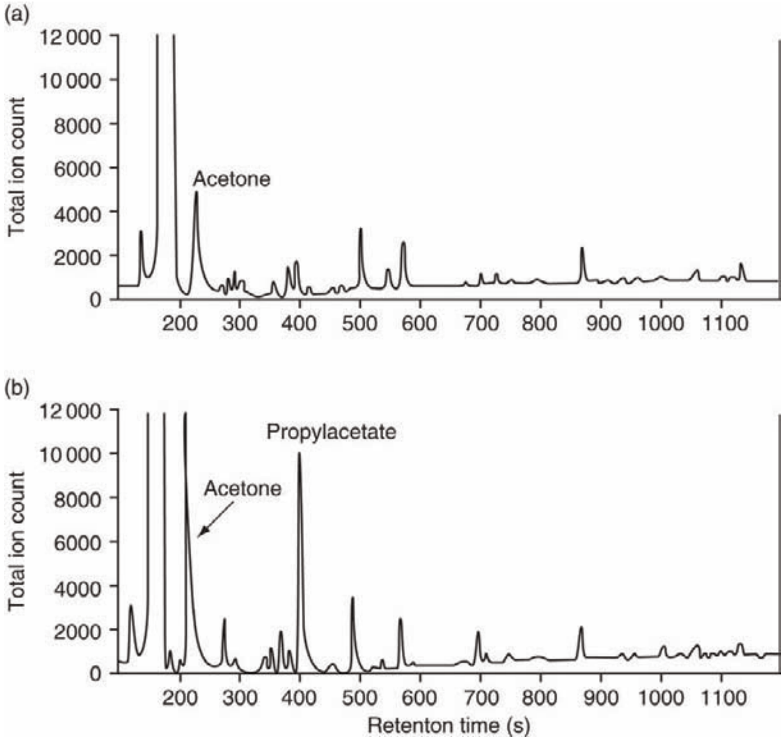

Figure 12 Chromatograms (obtained by purge-and-trap GC–MS) of (a) control (normal tasting) half-and-half and (b) half-and-half with chemical off-flavor because of contamination by propyl acetate solvent from packaging film.

techniques include vacuum distillation, simultaneous steam distillation–extraction (also referred to as the Likens and Nickerson extraction procedure), static headspace, dynamic headspace purge-and-trap, direct thermal desorption (for solid and semisolid samples), and solid-phase microextraction (SPME).

Each sample preparation method has its own advantages, disadvantages, and biases. Selection of the most appropriate method for a specific application depends on the number of samples to be tested, the type of sample, the specific analytes of interest, the desired sensitivity, the nature of the information desired, cost of instrumentation and equipment, and so on.

One increasingly popular analytical extraction technique used before GC–MS analysis is SPME. SPME utilizes a short, thin, solid rod of fused silica (typically 1 cm in length with an outer diameter of 0.11 mm) coated with an absorbsent/adsorbent polymer. The coated fused silica (the SPME fiber) is attached to a metal rod, and both are protected by a metal sheath that covers the fiber when not in use. The assembly is placed in a fiber holder. The system resembles a modified syringe (**Figure 13**).

Two sampling methods can be used with SPME depending on the placement of the fiber relative to the sample – immersion or headspace sampling. For dairy products, which contain high levels of fat, carbohydrates, and protein, the headspace technique is preferred. In SPME headspace analysis, a fiber is placed in the headspace above an equilibrated sample. For example, when analyzing volatiles in a milk sample, 3 ml of milk can be placed in a 9-ml glass GC vial containing a small stirring bar and sealed with a septum closure. The sample is then heated (e.g., to 50 °C). After allowing approximately 5 min for the sample to reach thermal equilibrium, the needle assembly is inserted through the septum and into the headspace above the milk sample while the sample is stirred with the stirring bar. The fiber is then exposed to the headspace gases. After an additional 10 min, the fiber is retracted into the needle assembly and removed. The extracted volatiles are thermally desorbed from the fiber in the heated GC injector port and into the capillary GC column.

Several types of fiber with varying affinities for specific classes of compounds are now available. As a result, the type of SPME fiber can be selected to optimize results for a particular analyte class. If lipid oxidation is being studied, for example, the analyst could select a Carboxen™–PDMS (polydimethylsiloxane) fiber to measure aldehydes in the 1–500 μg kg^{-1} range. If concentrations of aldehydes higher than 500 μg kg^{-1} are present, the Carboxen–PDMS fiber will become saturated, and a 100 μm PDMS fiber would be a better choice. Two fibers that work particularly well for the analysis of volatiles in dairy products are 75 μm

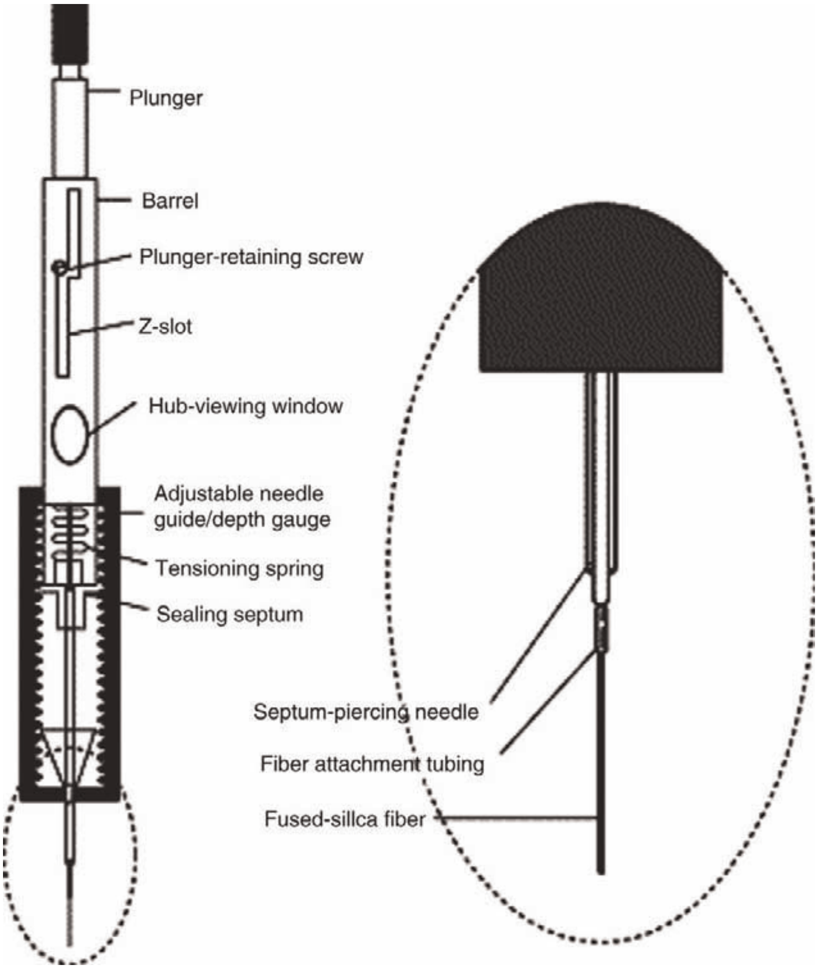

Figure 13 Solid-phase microextraction (SPME) fiber with holder. Courtesy of Supelco, Bellefonte, PA, USA.

Carboxen–PDMS and 65 μm PDMS–divinylbenzene. The fibers are relatively inexpensive, and they are simple and easy to use. The technique is relatively rapid, easy to perform, sensitive, reproducible, quantitative, and automatable.

SPME is particularly well suited to the analysis of dairy products. The technique is more capable of extracting a broader range of analytes than most other sample preparation methods. For example, SPME is capable of $\mu g\,kg^{-1}$ detection levels of both low-molecular-weight, highly volatile compounds like acetaldehyde, acetone, and 1,3-pentadiene, as well as high-molecular-weight, high-boiling-point compounds like vanillin, lactones, and dodecyl aldehyde. Furthermore, it can be used to quantify free fatty acids (C_4–C_{14}) in dairy products. This important class of flavor compounds can be particularly challenging and time consuming to extract by other techniques.

As an example of the capabilities of SPME, consider the analysis of milk contaminated with sanitizer. One popular sanitizer used by some dairies is Matrixx™ (Ecolab, St Paul, MN, USA). Matrixx has the following composition (approximate): 4.4% peroxyacetic (PAA), 6.9% hydrogen peroxide, and 3.4% octanoic acid. Initial samples of milk off the bottling line may be contaminated with Matrixx if the processing lines are not flushed thoroughly (**Figure 14**). Comparison of chromatograms of a control milk sample (no OF) and a sample with a severe OF that was believed to be caused by contamination with Matrixx shows that peaks for acetic acid, octanoic acid, and heptanal are good indicators that the sample was contaminated with Matrixx sanitizer. (Note that acetic acid is a decomposition product of PAA.) Heptanal is a lipid oxidation product. The following conditions were used for the analysis: Sample: 2 ml of low fat milk +1 ml 0.033M phosphoric acid +1 g salt in a 9-ml vial; SPME fiber: 75 μm Carboxen–PDMS; extraction method: headspace (with stirring) for 12 min at 40 °C; desorption: 2 min at 250 °C. The analytical capillary column was FFAP (30 cm × 0.25 mm with a 1.0 μm film thickness). **Figure 1** shows a

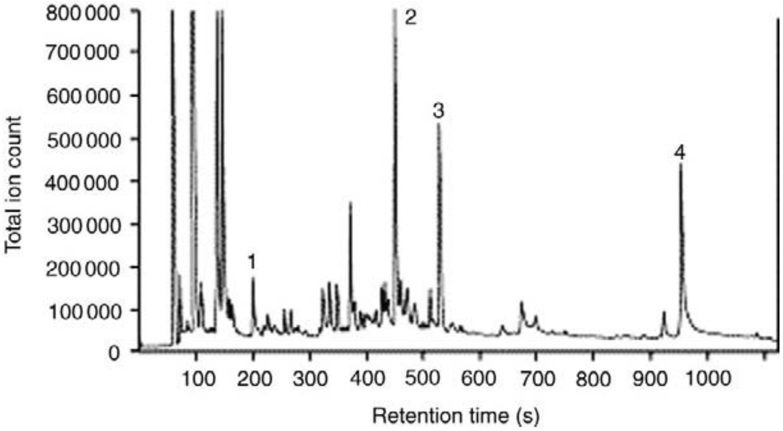

Figure 14 Chromatogram (obtained by SPME GC–MS using a 75 µm Carboxen–PDMS fiber) of a milk sample (2% milk fat) contaminated with 1300 ppm Matrixx sanitizer. Peak identities are as follows: (1) acetic acid; (2) chlorobenzene (internal standard); (3) heptanal; (4) octanoic acid. Acetic acid, heptanal, and octanoic acid were not detected in control milk (normal tasting) 2% milk before spiking with sanitizer.

chromatogram of a typical non-contaminated 2% processed milk sample.

Electronic Nose Applications for Measuring Off-Flavors in Milk

A potentially significant development for quality control monitoring of flavors and OFs in dairy products is the recent introduction of so-called 'electronic nose' (e-nose) instruments that employ an array of solid-state chemical sensors based on conducting polymers, metal oxides, surface acoustic wave devices, quartz crystal microbalances, or combinations of these devices. E-noses have been used to monitor the quality of edible oils and to distinguish different heat treatments of milk.

While e-nose instruments are tools for visually comparing the aromas of samples, they do not provide the same type of specific detailed chemical information that is possible with GC–MS methods, and the technique is probably not sensitive enough for some analytes and some types of matrices. The primary advantage of e-noses as a quality assurance tool for the dairy industry is speed of analysis, in terms of both data generation as well as data interpretation. Rapid, meaningful data interpretation is possible with various chemometric (multivariate analysis) techniques. As another alternative, data systems offered by some instrument manufacturers incorporate artificial neural networks for interpretation of data. Neural networks are data-processing algorithms based loosely on the structure of the human brain. Quality control models can be developed by 'training' the e-nose, and then routine samples can be tested against the model, providing a goodness-of-fit approximation or a sample accept/reject answer.

To date, commercial e-nose instruments, in general, have not lived up to expectations. Memory problems (carryover), irreversible poisoning of the chemical sensors and the need for frequent sensor recalibration have limited their usefulness.

Recently, e-noses instruments that use mass spectrometers as the chemical sensor array seem to have overcome many of the deficiencies of previous solid state-based sensor instruments. For example, one analytical system, consisting of SPME to isolate and concentrate volatiles from the dairy product, MS as a chemical sensor, and multivariate analysis to decipher meaningful trends in the MS output, has been applied to the study of OFs in milk. Using the multivariate analysis method of principal component analysis, this technique has been used successfully to differentiate control, good-tasting 2% milk, and 2% milk samples with various types of flavor defects, including samples contaminated with sanitizer, samples subjected to light abuse, samples that have been oxidized because of contact with prooxidant metals, and samples that have spoiled because of microbial growth. Preliminary studies have shown that the SPME–MS multivariate analysis system can even be used to accurately predict the shelf life of processed milk. The multivariate analysis method used in this study was partial least squares regression.

Off-Flavors Generated in Dry Dairy Ingredients

Dairy-based ingredients are widely used in various food and beverage segments because of their high nutritional, functional, and taste appeal. Therefore, monitoring dairy-based ingredients for OFs and malodors is important to avoid expensive product recalls.

Problem with 2,4,5-Trimethyloxazole in Spray-Dried Dairy Products

Reactions arising from carbonyl compounds can generate OF chemicals even at low temperatures. Several interesting malodorous compounds can be generated from sulfur amino acids, in particular cysteine, such as pyrazines, alkylpyrazines, methylthiazoles, acetylthiazole, acetylthiazolidine, trimethyloxazole, and dimethylethyloxazoles. These various compounds produce flavor notes of sulfur, popcorn, hazelnut, toasted, roasted, and ripe fruits. Because of their OF character and low odor and taste thresholds, these compounds can lead to serious taste and odor defects.

One OF problem that has occurred in spray-dried dairy products through this mechanism is the formation of 2,4,5-trimethyloxazole in spray-dried buttermilk, sour cream, and yogurt products. For example, **Figure 15** is a chromatogram of a spray-dried sour cream powder sample, showing a 2,4,5-trimethyloxazole peak at 43 μg kg^{-1}. This contaminant was responsible for the rejection of the entire production lot of the sour cream powder by sensory paneling. Analysis was performed by solid-phase microextraction (SPME) GC–MS using the PDMS–divinylbenzene fiber. One gram of sour cream powder and 5 ml of NaH_2PO_4 (25% w/v) solution were added to a 20 ml GC vial, vortexed for 1 min, preincubated for 4 min at 50 °C with agitation and extracted with the SPME fiber for 20 min at 50 °C with agitation before GC–MS analysis.

L. Pripis-Nicolau and colleagues have shown that the release of acetaldehyde by cysteine in the presence of α-diketones can generate alkyloxazoles. With diacetyl, the authors identified the formation of trimethyloxazole and with pentane-2,3-dione the formation of two oxazole isomers (2,5-dimethyl-4-ethyloxazole and 2,4-dimethyl-5-ethyloxazole) in model system studies. A possible mechanism for the formation of 2,4,5-trimethyloxazole from diacetyl, acetaldehyde, and ammonia is shown in **Figure 16**. 2,4,5-Trimethyloxazole has a taste threshold in water of 5 μg kg^{-1}. It has been detected in wine and dairy products and has an OF characterized as melonlike with pungent ripe kiwi notes.

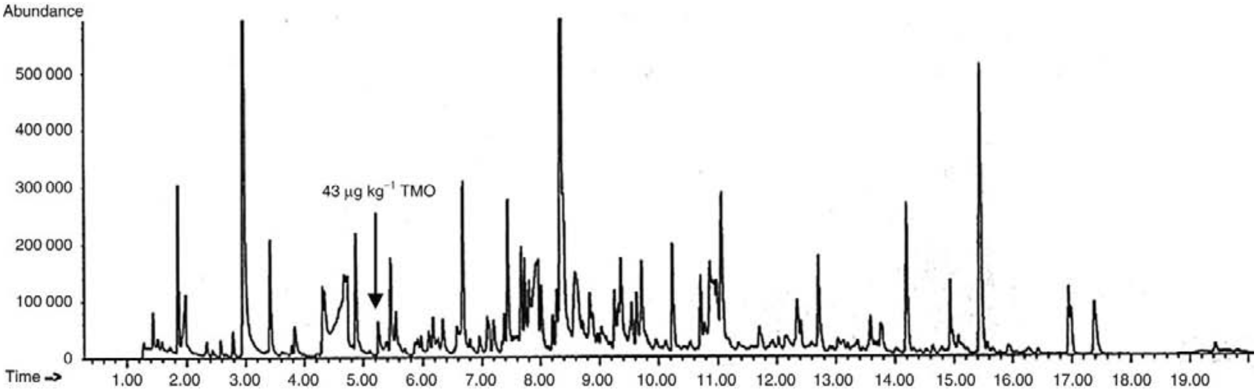

Figure 15 2,4,5-Trimethyloxazole (43 μg kg $^{-1}$) in spray-dried sour cream powder.

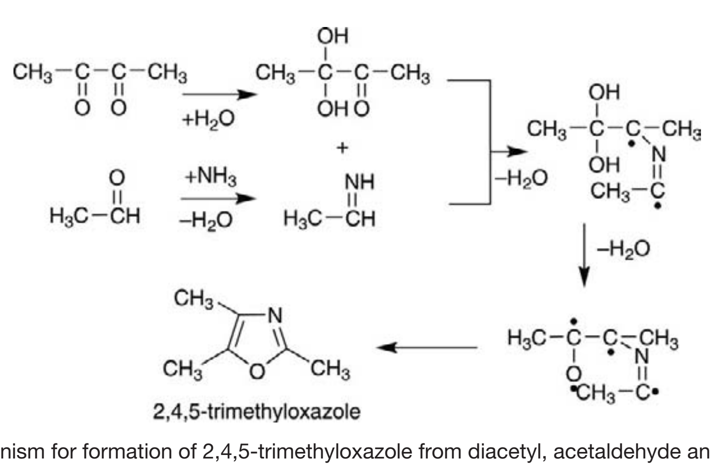

Figure 16 Possible mechanism for formation of 2,4,5-trimethyloxazole from diacetyl, acetaldehyde and ammonia.

Problem with Musty Trihaloanisole Malodors in Casein Powders

Another dried dairy powder that can develop OFs and malodors is casein. Rennet casein, produced by enzymatic (rennet) coagulation from pasteurized milk, is used in both industrial and food applications. Other types of caseins are produced by isoelectric precipitation by addition of acid or fermentation. The resulting range of acid and rennet casein products is used for nutritional and medical foods. Because of their flavor stability and functional properties, casein ingredients have wide appeal in cheese analogs, bakery, meat, confectionary products, desserts, nutritional beverages, and energy bars.

Casein powders have a characteristic unpleasant stale flavor. E. H. Ramshaw and E. A. Dunstone described the flavor as stale, gluelike, and burnt feathers. Others have described the typical odor of rennet casein as ‘wet-dog.’ *o*-Aminoacetophenone has been identified as an important odor-active component in stored skim milk powder. It is important to understand the nature of the flavor defects of casein powders because of their wide application in the food industry; the flavor of casein is a critical quality parameter.

Y. Karagul-Yuceer and colleagues have identified the primary odorants responsible for the typical odor of rennet casein powder. Results of aroma extraction–dilution analysis (AEDA) indicated that *o*-aminoacetophenone is a potent odorant. However, sensory descriptive analysis of model aroma systems revealed that the typical odor of rennet casein was caused principally by hexanoic acid, indole, guaiacol, and p-cresol.

To isolate volatiles prior to GC–MS analysis, Y. Karagul-Yuceer and colleagues used direct solvent extraction followed by high-vacuum distillation. This analytical technique to isolate volatiles is often referred to as solvent-assisted flavor evaporation (SAFE). Although it is an excellent technique for isolating volatiles from complex sample matrixes before GC–MS, SAFE analysis is time consuming and requires the use of large amounts of organic solvent (typically diethylether).

Recent advances in analytical extraction technologies have significantly simplified the extraction procedure. One example is stir bar sorptive extraction (SBSE) developed by E. Baltussen and colleagues and marketed by GERSTEL Inc.

SBSE is a simple, solvent-free technique that permits the extraction and concentration of analytes in a single step. SBSE is an excellent technique for studying odor-active compounds in casein powders. It was applied recently to the study of samples of rennet casein that had developed an unusual strong musty odor. SBSE is based on mixing a sample solution with a GERSTEL Twister™, a magnetic stir bar sealed in glass and coated with PDMS. The advantages of PDMS phase as an extraction medium have been described previously (M. A. Baltussen). Once the odor-active chemicals are extracted by SBSE, they are desorbed from the Twister and onto a capillary GC column using a thermal desorption unit (GERSTEL TDU) equipped with an MPS-2 auto-sampler and a CIS 4 programmed temperature vaporization (PTV) inlet (Gerstel, Mulheim an der Ruhr, Germany) installed on an Agilent 6890 gas chromatograph with a 5973 mass-selective detector (Agilent Technologies, CA, USA).

The sample preparation technique involved mixing 1 g of casein and 25 ml distilled water with a Twister stir bar (2 cm × 0.5 mm) for 3 h at 900 rpm. The Twister is removed from the sample, rinsed with distilled water, dried with a lint-free paper towel, and thermally desorbed in a Gerstel TDU. Musty casein samples from one supplier showed that the samples contained 2,4-dichroloranisole; 2,4,6-trichloroanisole (7.2 μg kg^{-1}); 2,3,6-trichloroanisole; and 2,4,6-trichlorophenol. Another set of musty casein samples was found to contain 2,4,6-tribromophenol and 2,4,6-tribromoanisole (215 μg kg^{-1}). Three major international food companies had produced nutritional beverages and nutritional bars using these trihaloanisole-contaminated caseins, resulting in expensive product recalls. Microbial (fungal enzymatic) o-methylation of the trihalophenols formed the strong musty trihaloanisoles which were responsible for the musty OFs. Trichlorophenol is used as a pesticide and wood preservative, and tribromophenol is used as a flame retardant.

In addition to measuring low concentration levels of trihalophenols and trihaloanisoles, the technique is useful for studying odor-active chemicals in casein and could provide a better understanding of the cause of the common wet-dog malodor of rennet casein. To get high recovery of both hydrophilic and hydrophoboic malodorous chemicals from casein, a technique called sequential SBSE was used. The technique was developed by N. Ochiaia and colleagues (GERSTEL KK, Tokyo, Japan). One gram of casein is vortexed with 10 ml water for 3 min in a 20 ml GC vial. A GERSTEL Twister® stir bar (1 cm × 0.5 mm) is added, and the vial is sealed with a screw cap. The sample is extracted for 1h by stirring with the Twister at 1500 rpm. The Twister is removed, dried with a lint-free paper towel and added to a thermal desorption tube. A second Twister (1 cm × 0.5 mm) and 3.0 g NaCl are added to the same casein/water sample. The vial is sealed with a screw cap, and the sample is re-extracted an additional hour while stirring at 1500 rpm. This second stir bar is removed, rinsed in distilled water, dried with a paper towel, and added to the first stir bar in the same desorption tube. Both Twisters are then simultaneously desorbed in the Gerstel TDU. The experimental setup is shown in **Figure 17**.

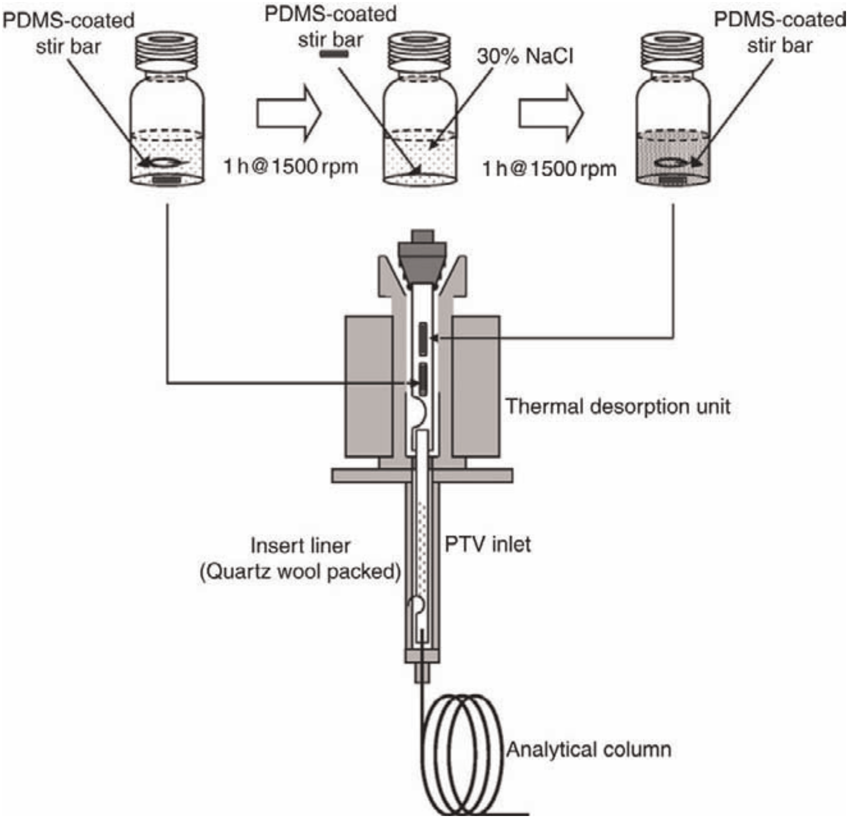
PDMS-coated stir bar
1 h@1500 rpm
PDMS-coated stir bar
30% NaCl
1 h@1500 rpm
PDMS-coated stir bar
Thermal desorption unit
Insert liner (Quartz wool packed)
PTV inlet
Analytical column

Figure 17 Experimental setup for sequential SBSE. Sample is 1 g of casein in 10 ml of distilled water.

Table 4 Levels (μg kg^{-1}) of selected odorants in various caseinate samples by sequential SBSE

Name	*Ca cas control source A (μg kg^{-1})*	*Ca cas musty source A (μg kg^{-1})*	*Acid casein source A (μg kg^{-1})*	*Rennet casein source A (μg kg^{-1})*	*Na casein source A (μg kg^{-1})*	*Ca cas musty source B (μg kg^{-1})*
Hexanal	549	480	2578	538	1835	719
2-Heptanone	0.0	0.0	52.5	17.6	29.0	3.4
Heptanal	1392	514	539	271	588	55
Hexanoic acid, ethyl ester	6.4	0.0	2.3	0.0	0.0	0.0
Octanal	319	150	236	103	231	131
Phenol	1589	2292	0.0	0.0	0.0	0.0
2-Nonanone	46.4	67.0	30.7	8.6	24.0	15.8
Nonanal	1658	1138	1474	552	1023	7447
Maltol	0.0	296	0.0	0.0	0.0	0.0
Phenylethyl alcohol	1282	0.0	0.0	0.0	0.0	0.0
2-Nonenal, (E)-	0.0	42.1	146	31.1	71.5	0.0
γ-Nonalactone	4.5	0.0	0.0	0.0	0.0	0.0
Decanal	132	179	417	123	183	1997
Benzothiazole	0.0	0.0	0.0	0.0	0.0	28.7
o-Aminoacetophenone	0.0	464	430	177	310	0.0
Indole	284	0.28	3.10	0.03	0.00	0.03
2,4,6-Trichloroanisole	0.0	7.2	0.0	0.0	0.0	0.0
δ-Nonalactone	125	0.0	815	0.0	654	0.0
2,4,6-Tribromoanisole	0.0	0.0	0.0	0.0	0.0	215
2,4-di-t-butylphenol	0.0	0.0	202	384	431	0.0
γ-dodecalactone	211	1624	2168	135	0.0	0.0

Table 5 Some additional odorants detected in caseinate by sequential SBSE but not quantitated.

Name	*Retention time (s)*	*CAS registry number*
Butanoic acid	407	107-92-6
3-Heptanone	434	106-35-4
Isovaleric acid	457	503-74-2
Pyrazine, 2,5-dimethyl-	475	123-32-0
Pyrazine, 2,3-dimethyl-	485	5910-89-4
2-Heptenal, (E)-	524	18829-55-5
Benzaldehyde	538	100-52-7
Dimethyl trisulfide	544	3658-80-8
2,4-Pentanedione, 3-methyl-	556	815-57-6
Furan, 2-pentyl-	560	3777-69-3
Pyrazine, trimethyl-	584	14667-55-1
3-Octen-2-one	622	1669-44-9
Hexanoic acid	626	142-62-1
1-Hexanol, 2-ethyl-	625	104-76-7
3,5-Octadien-2-one	663	38284-27-4
Acetophenone	667	98-86-2
Pyrazine, tetramethyl-	685	1124-11-4
3,5-Octadien-2-one, (E,E)-	692	30086-02-3
Acetic acid, phenylmethyl ester	774	140-11-4
Octanoic acid, ethyl ester	796	106-32-1
2,4-Dichloroanisole	816	120-83-2
Octanoic Acid	831	124-07-2
2,4-nonadienal, (E,E)-	830	5910-87-2
Acetic acid, 2-phenylethyl ester	875	103-45-7
Decanoic acid, ethyl ester	995	110-38-3
Thiazole, 2-ethyl	999	15679-09-1
n-Decanoic acid	1022	334-48-5
2,4,6-Trichlorophenol	1025	88-06-2
2,3,6-Trichloroanisole	1026	50375-10-5
2,4,6-Tribromophenol	1121	118-79-6
Dodecanoic acid	1158	143-07-7
γ-dodecalactone	1201	2305-05-7

With the sequential SBSE, hydrophobic analytes are extracted with maximum recovery in the initial aqueous extraction, and hydrophilic analytes are extracted with maximum recovery with the salting-out procedure used in the second extraction.

Thirty-two odor-active compounds were analyzed as standards. Five levels of each standard were prepared in the 20–6000 μg kg^{-1} range (spiked in control casein sample) and analyzed by sequential SBSE. The average linear least squares correlation coefficient for these standards was 0.9955. A mid-range standard was analyzed in triplicate for each of the 32 standards; the average standard deviation of triplicate determinations for the 32 samples was 4.3%.

Quantitative results for 21 analytes in various types of caseins are reported in **Table 4**. **Table 5** lists other odor-active analytes that were detected in the caseins by sequential SBSE but were not quanitated.

Conclusion

The advent of new sensitive and rapid analytical methods in conjunction with olfactometry techniques and traditional sensory taste paneling approaches has greatly improved our understanding of flavor-impact chemicals in dairy products. Application of new analytical technologies, as well as emerging analytical technologies such as electronic nose instruments, will allow dairy processors to better understand why OF development occurs in dairy products and how to minimize it. In the near future, enhanced analytical tools will likely move beyond dairy research laboratories and into production plant quality control laboratories as instrumentation becomes less expensive, easier to use, and automated.

See also: **Analytical Methods**: Biosensors; Sensory Evaluation; Chromatographic Methods. **Cheese**: Cheese Flavor. **Enzymes Exogenous to Milk in Dairy Technology**: Proteinases. **Fermented Milks**: Buttermilk; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture. **Milk Lipids**: Lipid Oxidation; Lipolysis and Hydrolytic Rancidity. **Psychrotrophic Bacteria**: *Pseudomonas* spp.

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FORAGES AND PASTURES

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Annual Forage and Pasture Crops – Species and Varieties

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Introduction

Annual forages are important components of feed supply for dairy cows worldwide. They include crops with very high forage yield potential such as maize. Many annual forages can be grazed but with conservation as hay or silage they are also important in spreading feed supply across the seasons.

Annual forages are available with summer–autumn production or winter–spring production. There are legumes and grasses that produce in either period. Brassicas also make a useful addition to summer–autumn and winter feed supply.

The annual forages are discussed in five groups: (1) warm season grasses, (2) warm season legumes, (3) cool season grasses, (4) cool season legumes, and (5) Brassicas. Within each group, there is a range of suitable species and within these species a much larger number of varieties are available worldwide. Suitable species within each of the annual forage groups have been presented elsewhere but here the varieties within each species are listed. Many varieties have been evaluated in different regions and the most suitable varieties for the local environment are well understood. Suitable varieties have been bred for local conditions or have been imported, tested, and shown to be effective. The best varieties are usually tolerant of local diseases and insects, and are adapted to the local climate and management systems. They are also bred or selected for feed quality, including digestibility, energy and protein content, and also the time taken to reach maturity.

High yields of annual forages should be sought to cover the high costs of production. Management must be effective at all stages of crop development to produce high yields. Land preparation, sowing, growing, grazing, or conserving the crop should all be managed to maximize yield. High dry matter (DM) yields may be obtained but feed quality, particularly energy and protein content, declines rapidly as maturity approaches, particularly in annual forage grasses. Care should be taken to harvest hay and silage crops from annual forages at a stage of development where the feed quality level will be effective in the milk production system.

There is a wide range of water use efficiencies (WUEs), that is, DM produced relative to water from irrigation or rainfall inputs. With the annual forages useful for milk production, WUE should be an important criterion when selecting the best forage, for example, maize growing in the summer–autumn has a very high WUE.

Annual Forages and Pastures

Annual forages and fodder crops are used worldwide as key feed sources for dairy systems. They produce all their feed in 1 year or season and usually are reestablished from fresh seed in the following year. Annual forages include

- true annuals, which set seed and die within 1 year,
- self-regenerating annuals, which reestablish from seed set and returned to the soil in previous years, and
- biennials and weak perennials, which are replaced with fresh seed in the second year because of reduced plant populations and hence low production potential.

Grasses, legumes, and brassicas either sown alone or in mixtures are used as annual forages.

Annual forage crops are fed fresh by grazing or green-chopping or conserved as hay, silage, or grain. Specialist annual forage crops are grown for conservation of feed reserves. Maize silage is an important component of dairy cattle feeding worldwide.

Feed costs from annual forage include land preparation, sowing, fertilizing, and harvesting, and these have to be recovered in a single year, whereas perennial pasture costs are spread over several years. High yield of utilized feed is essential in realizing maximum profit from annual forages. Adequate feed quality to sustain milk production should be sought but lower quality feed can be used for dry and young stock. Cost-effectiveness should be analyzed when choosing between forages. Some annual forages can be more cost-effective than perennials. The most profitable forage crop can also differ between farms.

Strategies for use of annual forages include

1. high-intensity production systems in which high-yielding forages are grown in combination to produce DM yields in excess of 40 tonnes ha^{-1} (e.g., maize followed by brassica and annual clover),
2. filling seasonal feed gaps in perennial pasture systems,
3. developing productive rotations,
4. building fodder reserves, to use in seasons when feed supply is restricted, or establishing drought reserves,
5. specifically augmenting protein or energy available to the dairy herd,
6. oversowing perennial pasture to increase production across seasons or to extend production for a further year,
7. supplying feed when establishing and replacing perennial pastures,
8. emergency feed supply after drought, flood, or winter-kill, and
9. cover crops to restrict erosion when establishing perennials.

The useful annual forages are discussed below.

Warm Season Grasses

Maize

Maize has the potential to yield more feed energy per hectare than other forages. Maize forage is high in energy but low in protein content. Attempts to improve the protein content of maize include breeding or intersowing with a high-protein legume crop that can be ensiled with the maize. The usual effect is to reduce total yield. Maize should be regarded as a specialist energy source with additional protein supplied from a protein-rich feed.

The high yield potential of maize is valuable in

- reducing the area of farm required for conservation,
- allowing sufficient stored silage to meet forage shortages in a dry year,
- using manure and effluent to reduce fertilizer imports to the farm, and
- taking advantage of the high WUE of the maize crop.

Variety selection

Maize breeding emphasizes on increasing grain yield and quality, and not on total forage production. Varieties bred for grain production can be used for forage. The forage potential of the local grain varieties is not always clear.

Varietal characteristics that count in maize forage production are as follows:

- *Potential for high total DM yield*: The highest forage yielding varieties should be identified from the grain varieties used locally.
- *High grain yield*: Grain is highly digestible (80% of DM) and high grain yield per hectare in the forage enhances its energy content.
- *High grain to stover ratio*: The grain content of maize forage is in the range of 30–50%. Stover, the nongrain component of the plant, has a high fiber content and is less digestible than grain; thus, a high proportion of grain increases digestibility and available energy in the feed.
- *High stover digestibility*: Increased stover quality is linked to decreases in lignin or fiber. Brown midrib (BMR bm$_3$ gene) maize varieties have lower lignin and increased stover digestibility but they yield less forage than current hybrids because of lower yields and lodging. Acidosis can be induced by the low fiber in diets containing more than 50% BMR maize. Breeding continues to improve the yield potential of BMR varieties. Improvement in feed quality has to be balanced with higher seed costs and potential yield loss.
- *Appropriate maturity*: Maize silage maturity is controlled by the accumulation of heat units measured as growing degree-days (GDD). Varieties require different GDD to stimulate flowering: varying from 85 to 150 days. The days to maturity guides the choice of sowing and harvest times. Harvest date should avoid frosts. If sowing is delayed, the maturity of the sown variety can be changed.
- *Harvest window*: Varieties that stay green until harvest provide an extended harvest window at the correct moisture content for ensiling; however, they can be too moist for ensiling at the best grain development stage. When the maize is harvested at less than 28% DM, feed quality is reduced and high levels of effluent produced during the ensiling process. When the stay-green crop is dry, the grain may have matured and forage digestibility will be reduced. A balance is required.

- *Disease resistance*: Locally bred grain varieties usually have acceptable resistance to major corn leaf diseases, and stalk and ear rots.
- *Lodging resistance with changing population*: Lodging can cause yield losses through restricted growth and harvesting difficulties. Standability is influenced by stalk rot resistance, stalk strength, lignin content, plant height, and ear placement. High plant populations can produce smaller stalks and increase lodging risk.
- *Nutrition profile*: The nutritional profile of maize has been changed by breeding varieties with high sugar, high oil, high lysine, more amylopectin, high amylose, less fiber, increased nutritional density, and greater leafiness. Often, the nutritional composition is improved but yield potential is lost. The benefits from breeding specialist forage varieties have yet to be realized. Effective grain varieties with high forage yield potential and a high grain to stover ratio remain useful forage varieties.

Sorghum

Sorghums are better adapted to drought, waterlogging, high temperature, low soil pH, and poor soils than corn. Sorghums are drought resistant and become dormant in extended dry periods. They have moderate tolerance to salinity and are less expensive to establish.

Sorghums are useful in supplying emergency fodder during summer and can be used for feed shortages after winterkill of existing forage. Sorghums can be conserved as chopped pit silage or wrapped bale silage, utilized fresh as green chop, or grazed. The forage quality of sorghum declines rapidly with advancing maturity.

Sorghums can be divided into two groups:

- *Single cut*: Grain sorghums and sweet sorghums (forage sorghum) are usually harvested for silage in a single cut. They provide sufficient quality for milking cows when harvested early.
- *Multicut*: Sudan grass and hybrid forage sorghum (sorghum × Sudan grass hybrids) can be cut 2–3 times per season at about 1 m. Later cuts produce higher yields but lower quality feed.

Single cut: Grain sorghum. Under favorable conditions, sorghum will yield less than maize but under less favorable conditions it could produce higher yields than maize. Sorghum silage is harvested between the milk and dough grain stage. The grain content of the forage is near 50%, which is higher than maize.

Special silage varieties have been developed that are taller than the dwarf grain varieties. They can yield close to 20 tonnes DM ha^{-1} and are nearly equal in quality to corn silage.

Factors influencing feed quality are

- *Tannin content*: Varieties with low levels of tannin in both grain and plant may be more digestible.
- *Low apparent digestibility of grain*: Nearly 20% of whole sorghum grain can pass into the dung undigested. Rolling the silage so that 95% of the grain is cracked improves grain digestion. Specialist forage varieties with high grain digestibility are required.
- *Protein digestibility*: The crude protein content of sorghum forage is usually higher than that of maize but the protein is less digestible and as with maize protein supplementation is required.
- *Other improvements*: Grain sorghum forage could be improved by increasing some or all of stem digestibility, stem sugar content, DM yield, stay-green attributes, and drought resistance.

Forage sorghum (sweet sorghum) (*Sorghum vulgare*). Sweet sorghum or sorgo has tall sweet juicy stems with small grain heads and is used mostly for silage. Quality and yield are stable near harvest with a balance between stem sugar content and grain yield. DM yields can be as high as those of maize but the yield of total digestible nutrients is much lower. Protein content is similar to that of maize but energy content is usually less than that of maize. BMR varieties have been developed with similar digestibility to maize but they will lodge.

Multiple cut: Sudan grass (*Sorghum bicolor* L. Moench var. *sudanense*). Sudan grass tillers extensively and regrows rapidly. Stems and tillers are finer and more suitable for grazing, and dry down for haymaking more readily than other sorghum types. Flowering is day length sensitive, which restricts running to head early in the season. Sudan grass has lower yield than sorghum × Sudan hybrids but can be managed to produce higher quality feed.

Sudan grass hybrids have been developed from crosses of Sudan grass strains. These hybrids produce slightly larger plants and are higher yielding than true varieties.

Sorghum × Sudan grass hybrids. Hybrid forage sorghums are usually developed from a forage sorghum female and a Sudan grass male plant. Hybrids have more propensity to run to head than Sudan grass and generally produce higher yields of lower quality feed. More than 50% of the yield is in the stems.

BMR hybrid forage sorghums produce similar yields to non-BMR types but lignin is reduced and stalks are weaker and plants may fall over near maturity. BMR varieties should be harvested early. BMR hybrid forages offer real improvements so that production and quality are similar to that of maize when managed efficiently.

Important antinutritional factors of sorghum include

- HCN poisoning,
- sulfur deficiency,
- low sodium, and
- nitrate poisoning.

Millets

Millets are useful forages for emergency sowings to fill feed gaps both early and late in the summer–autumn period. Pearl millet managed efficiently is a useful high-yielding and high-quality summer–autumn forage. Short-season millets can be sown much later than other summer forages and still give reasonable DM yields. One benefit is that they can use nutrients from a failed crop. Millets tend to be lower yielding than other summer crops and forages, particularly in wet and cool years; however, they can withstand dry and relatively low-fertility conditions.

Some millets can be grazed effectively when plants are 15–30 cm tall, as with more mature plants the nutritive value declines and regrowth is restricted. Hay can be made at the boot stage but thick culms make drying difficult. Millets ensile readily.

Pennisetum *millets*

The *Pennisetum* millets have various names worldwide including pearl, bulrush, candle, or cattail millet. Synonyms for the species name include *Pennisetum glaucum* (L.) R. Br., *Pennisetum typhoides* (Burm.) Stapf & Hubb., and *Pennisetum americanum* (L.) Leeke. Pearl millet has the highest yield potential of all millets. It tillers profusely and has a high leaf to stem ratio and strong regrowth potential. Flowering is induced with increasing day length and late-maturing varieties give leafy feed late in the season. Pearl millets mainly grow in the summer and early autumn, producing forage with about 15% protein at 1.5 m, which is higher than other millets, sorghums, and maize. Pearl millet is useful for hay, silage, and grazing. Pearl millet crops are more susceptible to establishment failure than other summer annual forage grasses if they are not sown carefully.

Japanese millet (Echinochloa crus-galli)

Japanese millet has superior cold tolerance to all summer forages and will establish when soil temperature reaches 14 °C. It is the most useful forage to fill early summer feed gaps. Grazing is available 4–6 weeks after planting and grain ripens 45 days from seeding. DM yield is lower than other summer forages. Japanese millet is adapted to wet soils and tolerant to low pH and salinity.

There are four millets that can be used as late-sown rescue crops or sown under more extreme conditions than other summer forages:

- foxtail (German, Italian, Siberian, or Hungarian) millet (*Setaria italica* L. Beauv),
- Siberian millet (*Echinochloa colonum* var. *frumentacea* Ridley),
- proso (white French) millet (*Panicum miliaceum* L.), and
- browntop millet (*Panicum ramosum* L.).

Sugarcane

Sugarcane is used in Brazil and other tropical and subtropical areas as a high-energy roughage. Mineral content is low, and phosphorus, sulfur, zinc, and manganese supplements could be required. Protein levels are low and urea is mixed with the forage as a nonprotein nitrogen source and added sulfur may also assist in rumen protein synthesis.

Cane varieties with high sugar content should be used. The forage should be chopped just before feeding and the additives mixed in carefully. The harvested material should not be stored for more than 2 days. Sugarcane can fill feed gaps. Preparing and feeding sugarcane is labor intensive.

Cool Season Grasses

Annual ryegrass

Annual ryegrass produces high-quality feed and is used worldwide for grazing and hay and silage production. It prefers mild to warm climates with production suppressed by low temperatures in winter and by high temperatures in summer. Annual ryegrasses grow further into the summer than many other cool season grasses, with peak growth in the temperature range of 20–25 °C. Rapid establishment and a long growing season mean that the first grazing of annual ryegrass is 2–3 weeks earlier than for perennial ryegrass and lasts 2–3 months longer than for oats.

Effective systems incorporating annual ryegrass include the following:

- Overseeding into existing summer grass pastures (e.g., kikuyu, couch, paspalum) to extend the growing season. Oversowing in autumn at a high seed rate will provide autumn, winter, and spring feed. Annual legumes, red clover, or white clover can also be combined in mixtures for oversowing to enhance feed quality.
- Oversowing of small-grain and annual ryegrass mixtures to provide a good spread of production. Small grains (oats, barley) produce their highest production from autumn to midwinter and then ryegrass takes over in late winter and spring.
- Oversowing into perennial ryegrass pastures to increase late autumn, winter, and early spring production; however, the perennial ryegrass may be weakened.
- Oversowing into brassicas (e.g., turnips) to extend summer–autumn production into winter–spring, or

autumn–winter production into spring (kale, rape, or pasja).

- Cover crop for perennials (fescue, cocksfoot).

The following annual ryegrass types are useful for annual forage production:

Italian ryegrass (Lolium multiflorum Lam.)

Italian ryegrasses are biennials to short-term perennials, which can grow in the second year but produce much less forage than from a fresh sowing. Best results are obtained by resowing each year.

Doubling the chromosomes in normal diploid varieties breeds tetraploid varieties. Tetraploids have higher sugars, higher digestibility, larger leaf and seed size, and fewer but larger tillers than diploid varieties. The tillers are more open and less competitive with legumes. They are also grazed preferentially. Nevertheless, diploids often grow better, are more grazing tolerant, and regrow more rapidly, providing more grazings.

Westerwolds ryegrass (Lolium multiflorum var. westerwoldicum)

Westerwolds ryegrass is a true annual, which will mature, set seed, and die in the year of sowing. It has less heat tolerance than other annual ryegrasses.

Short-rotation ryegrass (Lolium hybridum Hausskn (Lolium perenne × Lolium multiflorum))

Short-rotation ryegrasses or hybrid ryegrasses are variable; some are like Italian ryegrass, some closer to perennial, and some intermediate. They usually grow more aggressively than perennial ryegrass and often have the flowering characteristics of Italian ryegrass.

Many cultivars have been developed within each of the three types. The main differences are in maturity and resistance to disease, particularly leaf, crown, and stem rusts. Annual ryegrass can usually flower when day length is greater than 10 h. There are varietal differences in day length requirement. Stress speeds up flowering and favorable conditions hold it back. Snow cover will assist winter survival in cold climates. Annual ryegrass has a deep root system, prefers fertile soils, responds significantly to N fertilizer, and competes successfully with weeds and other crops.

Self-regenerating annual ryegrass

Annual ryegrasses are available that will regenerate from seed set in the previous year. These ryegrasses are valuable in Mediterranean climates characterized by cool and wet winters and springs, and hot and dry summers.

Wimmera ryegrass (Lolium rigidum Guad): Wimmera is early maturing, and has heavy seed set, which will not germinate during hot and dry summers. It is not useful in high-rainfall environments. Subterranean clover (sub-clover) is often sown in combination with Wimmera ryegrass. Wimmera ryegrass has a high weed potential in cropping areas.

Small-grain cereals

The small-grain cereals, wheat, oats, barley, triticale, and rye, are useful as annual forages. Potential uses of small-grain cereals as forage for dairy production include the following:

1. Whole-crop silage or hay harvested at the optimum time for accumulation of digestible nutrients per hectare. Legumes can also be sown with cereals to increase protein.
2. Double-cropping with maize to produce high per-hectare production.
3. Grazing followed by growing out for hay, silage, or grain production.
4. Grazing only.
   - ◆ Specialist crop planted for grazing may be mixed with legumes and sown on clean seedbed or direct drilled.
   - ◆ Oversown into existing pastures either alone or in mixtures with annual ryegrass or legumes to extend the season of growth of the pasture into the autumn and winter.
5. Emergency forage when stands or new sowings of perennial forage fail.

Winter and spring cereals

The flowering stimulus in different cereal varieties can be managed to produce forage from cereal in most seasons. Winter cereals need a period of low temperature before they are vernalized and will proceed to flowering. Spring cereals have no cold requirement and will flower in response to increasing day length. Some winter cereals, once vernalized, also require increasing day length to flower. In cold climates, winter hardiness is important in the seasonal distribution of cereal forage.

Cereals with adequate cold tolerance are effective in very cold climates. Rye has the best winter hardiness potential (−25 to −33 °C) followed by wheat, triticale, barley, and oats (−15 °C); however, there can be large differences between cultivars in winter hardiness. Acclimatization is needed to harden off plants. Planting early allows tolerance to increase from(?) −3 °C at the beginning of autumn to −19 °C by early winter. Crowns should be well developed before freezing conditions commence (2–3 leaves sufficient for survival). Cold hardiness is maintained if crown temperatures remain below freezing, but hardiness declines rapidly with warm winter temperatures. Snow cover helps crowns survive when temperatures fall below minimal survival temperatures.

Varying combinations of variety, vernalization requirement, day length response for flowering, and sowing date provide a range of opportunities for forage production from cereals.

- Spring sowings of winter cereals provide forage through late spring, summer, and autumn as the cereal will not flower until vernalized. Spring cereals sown at the same time will flower and set grain as day length increases.
- Intercropping of spring-sown spring and winter wheat provides earlier forage production from the spring wheat and later production from the winter wheat.
- Silage and grazing can be obtained by spring sowing mixtures of winter and spring cereals. Silage is made from the spring cereal, and the winter cereal is grazed after the silage is removed.
- Sowing winter wheat in autumn allows grazing in late autumn and early spring, but care must be taken not to graze when stems start to ascend. Rye is the best cereal to do this at high latitudes. Sow early to get grazing and also to increase winter hardiness.

Specialist forage varieties of cereals are available but grain varieties predominate as forage varieties mainly for their high level of disease resistance. Specialist forage varieties also need high levels of disease resistance to leaf rusts, stem rusts, and viruses. Tall varieties usually produce more forage than dwarf varieties, and late-maturing varieties also increase forage yield potential.

Forage quality in cereals can fall rapidly with advancing maturity. Stage of harvest for hay and silage is critical in ensuring forage of sufficient feed quality is obtained. At the preboot stage, cereals usually contain about 20% protein and have an IVDM digestibility of about 80%. By the milk stage, protein falls to about 12% and In vitro dry matter (IVDM) digestibility to about 62%. The stage of harvest for different cereal types to produce forage of sufficient quality for milking cows is as follows:

- *Rye*: Should be harvested no later than the boot stage. Preboot is best as chemicals in the head reduce feed intake through unpalatability, and feed quality also falls rapidly.
- *Triticale*: Should be harvested in the boot stage with less than 25% of heads with visible seed heads; any later, feed becomes unpalatable and intake is reduced.
- *Oats, wheat, and barley*: Should be harvested in the boot to milk stage.

Insect pest attack should be avoided with appropriate sowing dates or controlled if infestations occur (e.g., Hessian fly in North America).

The most suitable cereal will vary between environments and farmers should use the cereal that best meets local climatic conditions. Choices are between the following cereals:

Wheat: Wheat varieties are separated into winter- and spring-flowering stimulus, soft or hard grain, and red or white grain. Red grain varieties resist sprouting in the head, and hard and soft grain varieties are suitable for forage. Many of the grain varieties are dwarfs, which restricts forage yields.

Oats: Oats is the best cereal for oversowing into existing pasture, alone and in association with annual ryegrass, to fill feed gaps. Careful grazing management, controlling the interval and height of defoliation, is required to ensure that the growing points are not removed. Oats grown as a pure stand can give three grazing plus a hay crop. Oats tolerates acid and poorly drained soils but requires more moisture than other cereals, and is prone to heat damage and lodging. Days to maturity vary among oat varieties.

Barley: Barley matures earliest of all the small grains. There are 2- and 6-row varieties. Some have rough awns and should not be used for hay, but ensiling adequately softens the awns. Barley uses moisture efficiently, is tolerant to salinity but is sensitive to acid soils, and is responsive to soil fertility and good management. Winter barley is less winter hardy than winter wheat.

Rye: Rye will grow at cooler temperatures and provide later autumn and earlier spring pasture than other winter grains. It is the earliest of winter cereals. Early plantings in North America avoid Hessian fly damage and it is the best cereal for autumn and spring pasture. Rye is the most winter hardy of all grains and is resistant to winterkill. Quality is maintained when rye is used as pasture. Rye matures rapidly with declining feed quality and can be lower in quality than other small grains when taken for hay or silage. It is highly unpalatable if matured past the boot stage. Lighter and poorer soils that are not suitable for other cereals can be used for rye production.

Triticale: Triticale varieties are developed from wheat × rye or durum wheat × rye hybrids. Spring and winter varieties are available that have potential for high forage yield. Spring types need a long growing season and will produce more silage than oats or barley.

The forage yield of winter triticale is usually higher than that of wheat. It takes longer to develop winter hardiness than wheat, so it must be sown earlier than wheat. Once developed, the hardiness of triticale is similar to that of wheat. Tall varieties are susceptible to lodging but shorter varieties are available. Triticale is drought tolerant and produces adequate forage in areas with restricted water supply. In some countries, triticale grain is not harvested as there are no markets, but in Australia the grain is used in the dairy industry as a stock food.

Annual Legumes

Warm Season Legumes

Summer annual legumes produce high-quality feed in late summer and autumn in tropical and subtropical environments at a time when quality is declining in summer grasses and forages. Frequently, half their forage is leaf and half is stem. Leaf digestibility is 60–75% and stem digestibility is 50–55%. Leaf crude protein is about 20% and that of stem about 10%. Grazing stock should be removed when the leaves are eaten to allow regrowth. Severe cutting will restrict regrowth. Bloat can occur but the risk is low. At the first grazing stock will avoid the legume and eat grasses first. These legumes can be used in mixtures with millets and sorghums. They fix nitrogen and supply protein to summer diets. Important summer legumes include the following:

Cowpeas (Vigna unguiculata): DM yield potential is 2–3000 kg DM ha^{-1} from dryland and 8000 kg DM ha^{-1} from irrigation. Regrowth is obtained if grazing is delayed to flowering and 2–3 grazings are possible. Silage taken at midflowering provides the best quality. Cowpeas can also produce grain.

Soybeans (Glycine max): Yield potential in irrigated crops is up to 10 000 kg DM ha^{-1}. Grazing is available only once when green pods are present. Stock reject stems when fed as hay but will eat more as silage. Late-maturing varieties will maximize yield potential.

Lablab (Lablab purpureus): Dryland yields are variable (500–5000 kg DM ha^{-1}) and irrigated yields up to 14 000 kg DM ha^{-1} can be obtained. Lablab can be grazed up to 3 times. Lablab flowers later than other summer legumes (12–14 weeks compared to 10–12 weeks) with a higher growth rate in autumn. Trampling tolerance is better than cowpeas. Silage can be cut after 12 weeks growth.

To widen the range of potentially useful summer legumes, the following can be useful in specific situations:

Phasey bean and *Aeschynomene* (*Aeschynomene americana*) can be useful in wet areas prone to flooding.

Alyce clover (*Alysicarpus vaginalis* (L.) DC.) can be useful for late sowing for hay production. Quality is retained through a 4- to 6-week harvest window.

Annual lespedezas (common *Kummerowia striata* and Korean *Kummerowia stipulacea*) tolerate soil acidity and low soil phosphorus. They are adapted to infertile sites and produce high nutritive value feed from low-input systems.

There is a huge potential for further worldwide development of legumes as many legume genera have not been effectively exploited. Domestication of wild legumes for specific purposes could enhance dairy productivity, particularly in tropical and subtropical environments.

Cool Season Legumes

Annual cool season legumes are important in supplementing protein and providing a highly digestible feed source. They fix nitrogen, which stimulates grass growth and builds nitrogen fertility, but can cause bloat.

Strategies for use of these legumes include

1. as pure stands to be grazed or made into hay or silage,
2. planting with other legumes and grasses such as annual ryegrass or cereals for grazing and conservation, and
3. as break crop in dryland cropping rotations.

Different legumes have seasonal differences in growth. Legume seed is more expensive than that of grasses and although seeding rates are lower than for grasses cost can influence sowing of some legumes. The erect habit of some varieties is valuable in competing with grass species. Deep-rooted species like arrowleaf and crimson clover extend green feed supply.

Legume protein is highly degradable in the rumen. Degradability is reduced with increasing tannin content and advancing maturity. When crude protein in the diet exceeds 25%, energy is required to excrete the excess and essential amino acids may be used inefficiently.

Annual legumes can be divided into two groups:

1. legumes resown annually and
2. legumes self-regenerating from hard seed.

Resown annuals

Egyptian or berseem clover (Trifolium alexandrinum): Berseem clover falls into two groups:

1. *Single cut* (var. *alexandrinum* Boiss.): Unbranched or slightly branched Fahl group of cultivars, which have later maturity.
2. *Multicut* (var. *serotinum* Zoh and Lern): Branches from the base. This includes the Mescawi group of varieties such as Bigbee and Multicut.

Berseem is adapted to neutral to alkaline soils and has tolerance to salinity. Winter growth rate is better than that of other annual legumes, and berseem lasts longer into the spring, but it will not tolerate severe winters and requires irrigation.

High yields of up to 22 tonnes ha^{-1} are possible. When making hay, conditioning helps to dry forage cut with a high moisture content. Several cuts are possible after autumn sowing. High growing points restrict grazing potential. A quick grazing rotation is required with resting periods for regrowth, rather than set stocking or prolonged grazing.

Trifoliums

Trifoliums make up the largest group of cool season annuals. Varieties with the best resistance to local diseases and insects should be used.

Crimson (**Trifolium incarnatum**) Crimson clover can be grazed in winter and cut for silage or hay in the spring. The Caprera variety of crimson clover is characterized by high levels of soft seed, which restricts regeneration potential. It is quick to establish, and grows erectly with some autumn and early winter growth, but its highest production is in early spring. Deep rooting extends the spring growing period. Sow early with wheat or oats for grazing. Seed production is cheaper than for sub-clover.

Persian (shaftal) (Trifolium resupinatum) Persian clover has rapid regrowth after grazing, high tolerance to waterlogging, and moderate tolerance to salinity. It is sometimes called shaftal or giant shaftal, but shaftal is really *T. clusii* (annual strawberry clover).

There are two subspecies of Persian clover:

1. *Trifolium resupinatum* var. *majus* has an erect habit, thick hollow stems, large leaflets, low hard seededness (1–2%), and late flowering and maturity. Varieties include Maral, Leeton, Laser, and Lightning.
2. *Trifolium resupinatum* var. *resupinatum* has a more prostrate habit, thinner stems, and smaller leaflets. It flowers earlier than *majus* with more hard seededness and higher seed yields. Varieties include Kyambro and Nitro Prolific. Persian clover is palatable and nutritious providing up to five grazings and a hay cut, yielding up to 16 tonnes DM per year. Regrowth is rapid after grazing. The plant's erect habit allows it to grow and compete effectively with annual ryegrass and small-grain cereals. Mixtures with sub-clover can extend the growing season after sub-clover seed is set.

Arrowleaf clover (Trifolium vesiculosum) Arrowleaf originated in the Mediterranean region and is characterized by rapid spring growth and is one of the latest maturing of the annual clovers. Establishment is slow due to delicate and drought-susceptible seedlings and grazing must be delayed to allow establishment. Arrowleaf will regenerate from seed, but is best used as a one-season annual. Arrowleaf responds well to rotational grazing (every 2–3 weeks), but when not grazed efficiently, it will become rank and be rejected by stock and associated grasses will be shaded. High level of hard seed affects regeneration and seed should be scarified before sowing new stands.

Rose clover (Trifolium hirtum) Rose clover is useful when a balance between cold tolerance and winter production is required. It will reseed naturally but does not tolerate poor drainage and is sensitive to heavy grazing. Rose clover is used in mixtures with medics and sub-clover.

Self-regenerating annuals

Self-regenerating annuals perform best if the seed is buried and the appropriate level of hard seed is available for regeneration by autumn rains. Sub-clover stands out as a regenerating annual.

Subterranean clover

Sub-clover is useful in Mediterranean climates with hot and dry summers, cool and wet winters, and rainfall in the range of 350–1200 mm.

There are three subspecies:

1. The subspecies *subterraneum* is the most common and requires well-drained, slightly acid soils.
2. The subspecies *brachycalycinum* is adapted to neutral to slightly acid soils. On alkaline soils, varieties require self-mulching cracking soils to enable seed burial.
3. The subspecies *yanninicum* tolerates poorly drained soils. Plants are not hairy and the seed color is cream to light brown. Plants are not suited to sandy soils but will produce well in acid soils of pH 5–6 ($CaCl_2$) and are tolerant to soil aluminum (<15% of cation exchange capacity (CEC)).

Sub-clover can be sown in mixtures with annual and perennial grasses. The main annual is Wimmera ryegrass, and perennials include fescue, cocksfoot, and phalaris. Sub-clover is useful in areas marginal for dairy production and is productive for seasonal dairying in Mediterranean climates. Maturity in sub-clover varieties ranges from 77 to 150 days.

High levels of seed reserves are required in the soil for persistence of sub-clover. An adequate proportion of the seed should be hard to resist early germination. Seed set is increased by not grazing close to flowering. Seed reserves are depleted by false seasonal breaks, which reduce the viable seed in the soil.

Sub-clover can be highly persistent as a regenerating annual if managed correctly. Leaves of sub-clover plants are less digestible than the rest of the plant. Lenient grazing increases the proportion of leaves and reduces quality.

Balansa clover (Trifolium michelianum Savi)

Balansa has low estrogen and is resistant to clover scorch (*Kabatiella* spp.). It is also suitable for waterlogged or salty soils and as a companion species to salt-tolerant grasses in salty areas. Balansa can regenerate from seed if managed for heavy seed set by restricting grazing close to flowering. Up to 80% of seed is hard seed and 30–40% of the seed eaten passes through stock, which compares to 5% passing with sub-clover. Varieties are available with a range in maturity. Small seed size leads to slow establishment. Hay production leads to low seed set and no regeneration.

Medics (Medicago spp.)

Medics can produce high-quality feed that will remain green longer than sub-clover. Some varieties are more drought tolerant than sub-clover. Medics may not tolerate extended waterlogging. They can produce high levels of hard seed, which may restrict regeneration in the second year. Medics can be used effectively as break crops for disease control in cereal rotations and at the same time be a high-yielding forage crop that can be conserved or grazed

Other annual legumes

There is a wide range of cool season legume species that are used in limited situations. Many of these have not been effectively exploited for intensive dairy production.

Examples of such annual legume species include serradella (*Ornithopus* spp.), *Biserrula* spp., sweet clover (*Melilotus* spp.), and gland clover (*Trifolium glanduliferum*).

The twining legumes, vetch (*Vicia* spp.) and peas (*Pisum* spp.), can be usefully sown in combination with small-grain cereals to increase the protein content of harvested hay or silage, and to widen the harvest window. Pea seed can be expensive and seeding rates may need to be restricted to contain sowing costs.

Brassicas

Plants from the family Cruciferae are useful as annual forages, with the genus *Brassica* the largest source. Brassicas supply highly digestible and high-quality feed with a high moisture content. They are usually grazed but can be green chopped, and ensiling techniques have been developed for kale.

Brassicas are useful in extending summer feed supply (e.g., turnips sown in the spring) or for sowing in late summer to produce autumn and winter feed. Some brassicas are biennials but are usually managed as annuals. They are relatively drought tolerant.

Brassicas usually supply adequate protein, with leafy types such as kale or rape having 20–25% protein in their tops but only about 10% in the stems. Root types such as turnips have about 15% protein in tops and 8% in the roots. They are all highly digestible with a DM digestibility of about 90%. Moisture content is also high, with DM content down to 7–8%. The fiber content of brassicas is low (15%) and adequate roughage must be supplied to assist digestion. The amount of brassicas in the diet should not be more than two-thirds of the total diet on a DM basis. Care must be taken in introducing cows to brassicas because rumen flora need 2–3 days to adjust.

Brassicas can be grown alone or in mixtures with grasses.

- Mixtures allow ensiling with kale.
- Oversowing with annual ryegrass will extend growth period into spring in mild climates.

Early autumn growth of brassicas is not always better than from the existing pasture if it is adequately fertilized and watered. The potential production from existing pasture should be evaluated before replacing it with a brassica crop.

Brassica species and varieties have major differences in root to leaf and stem proportion, yield potential, cold tolerance, and capacity to regenerate after grazing. Some brassicas are suitable for only one grazing, while others will regenerate for 2–3 grazings. DM yields are in the range of 5–15 tonnes ha^{-1}.

Brassica species suitable for annual forage for dairy cattle include the following:

Turnip (Brassica rapa var. rapa Barkant)

Turnips have rapid growth, reaching maximum yield within 80–90 days. A range of maturity is available between varieties. The varietal tops to root ratio ranges from 90/10 to 15/85. Top growth predominates in early growth (first 45 days), then there is a change to root development. The protein content of roots is about half that of the tops and thus the root to top ratio influences protein content. Turnips can be green chopped or grazed more than once if grazing is commenced at or near 30 cm of growth and grazed down so that more than 12.5 cm of residue remains. With adequate residue, growing points are not damaged and regrowth potential is preserved. One or more grazings are for top growth followed by once for root growth. DM yields are not as high as for rape or kale.

Swede (Rutabagas) (Brassica napus L. var. napobrassica)

Swedes have a large edible root (swollen stem) and are slow to mature (150–180 days). They are grazed once and do not regrow after harvest. Cold-tolerant varieties of swedes can be the best brassica in terms of DM yield and protein content. Stems will elongate at the expense of root development when sown with tall crops. Swedes are susceptible to waterlogging.

Rape (Brassica napus var. napus Bonar)

There are two rape types: giant and dwarf. The giant type is more suitable for cattle as it is more palatable and provides higher yields. Stems vary in thickness and length between varieties. The most suitable grazing interval varies depending on the variety. Rangi is best grazed at 90-day intervals, while the variety Winfred is best when allowed to accumulate yield for a single 180-day period. Some rape hybrids can be first grazed after 60 days, regrazed 30 days later, and then the regrowth utilized after a further 30 days.

Kale (Chou Moellier) (Brassica oleracea Kestral)

Leafy kale can be divided into two groups based on stem development: stemless and marrow-stemmed. Stemless kale establishes rapidly and reaches maturity in 90 days at 60 cm height. In contrast, marrow-stemmed kale is slower to establish and matures in 150–180 days at 150 cm height.

Generally, kale can be grazed only once, but there is one stemless kale that will regrow if not grazed too heavily. There is the opportunity to plant a second crop of stemless kale in the same season. Kale is the most cold-tolerant brassica and can survive severe cold. Feed quality is intermediate between turnip and rape.

Other crucifers used as forage for dairy cattle include

1. Hybrids Hybrids between Chinese cabbage and other brassicas give useful cultivars:
   (a) Chinese cabbage × rape *Brassica campestris sensulato* × *B. napus*: varieties Pasja and Perko.
   (b) Chinese cabbage × turnip *B. campestris sensulato* × *B. rapa* L.: variety Tyfon.
   (c) Chinese cabbage × swede *B. campestris sensulato* × *B. napus* L. var. *napobrassica*: variety Wairangi.
2. Fodder radish (*Rhaphanus sativus*)
3. Mustard (*Sinapis alba*)
4. Fodder beet and sugar beet (*Beta vulgaris*) are fed to dairy cows in Europe as a high-energy supplement and as a concentrate substitute. Fodder beet tubers are stored and fed back, and sugar beet pulp and sugar beet mash silage are also used.

Genetically Modified Annual Forages

Genetically modified (GM) plants are being developed in many counties with a range of aims. The major aims are

1. herbicide resistance (e.g., glyphosate tolerance),
2. disease resistance, particularly for virus diseases, and
3. insect pest resistance; reducing requirement for insecticides (e.g., Bt (*Bacillus thuringiensis*) toxin incorporated through genetic engineering).

GM crops include maize, sugar beet, canola, and soybean. These modified crops could be used for annual forage production for dairy cows. There are concerns, however, that genetic modification could be harmful through the generation of harmful toxins in the food chain or may have severe ecological consequences due to GM varieties outcrossing with other varieties and native or naturalized plants.

GM crop plants have been widely adopted in many countries particularly in North and South America. In other countries, a more conservative approach has been adopted and in many countries the sowing of GM crop plants has been controlled. In Europe, Bt maize was cultivated on 21 000 ha under regulatory approval in 2007. GM rapeseed was sown for the first time in Australia in 2008.

Studies have been conducted for evaluating the impact of GM plants on dairy animals. Up to 2008, no harmful effects had been detected. The impact of GM plants on the food chain does however remain an open question.

See also: **Forages and Pastures:** Annual Forage and Pasture Crops – Establishment and Management; Grazing Management; Perennial Forage and Pasture Crops – Establishment and Maintenance; Perennial Forage and Pasture Crops – Species and Varieties.

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Annual Forage and Pasture Crops – Establishment and Management

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Introduction

Annual forages can be used to meet specific feed supply goals. There is a high potential for crop failure if the forages are not managed carefully and attention is not paid to details in the total management of the crop.

Useful annual forages can be divided into five major groups:

- Warm-season grasses including maize
- Cool-season grasses
- Warm-season legumes
- Cool-season legumes
- Brassicas

The annual forages can be used in the following ways:

1. Grown as a single crop to supply feed at a specific time or for specific purposes. The forage may be grazed when required or conserved as hay and silage for feeding later.
2. As mixtures sown as a bi-crop with other annuals or perennials to increase feed production per hectare and extend seasonal feed availability.
3. Oversown onto perennials or other annuals to increase yield, manipulate quality, or spread seasonal production.
4. As high-producing systems, where annual crops are planted in succession on the same area. Maize followed by brassicas and Persian clover or pea can produce more than 40 t DM ha^{-1}.
5. Integrated into perennial pasture systems to optimize pasture production and total feed supply to the milking herd. The usual pastures are maintained, and the annual forages are grown separately. Annual forages and perennial pastures can be rotated to assist in the renovation of pastures.
6. As conserved feed – hay or silage – to transfer feed from one season to another or provide a feed reserve for emergencies. Conservation is capital-intensive with special sowing, harvesting, and storing equipment and facilities required. In this case, losses through grazing damage and limited utilization are reduced.
7. Used in rotation to provide a break crop to reduce pathogens and weed competition and to improve the reestablishment of perennial pastures.

Integration of Annual Forages into Farm Production

Selection and adoption of annual forages into the farm feed supply for the dairy herd should follow careful planning based on the feed required onfarm to adequately feed the dairy herd and the capacity of a forage to provide the feed. The forage system chosen must be reliable in meeting productivity expectations. The potential feed production from each annual forage and its impact on milk production should be carefully costed, with the growing, harvesting, and feeding costs carefully analyzed.

Detailed planning and analysis will lead to the development of a potentially profitable feed supply system.

The factors to take into account include the following:

- Potential total production from the annual forage in terms of both quantity and feed quality.
- Efficiency of utilization by the stock and the potential milk production from the feed provided.
- Stocking rate required to utilize the feed efficiently and the area required of each feed source to meet production goals.
- Seasonal distribution of the feed supply. Will the forage effectively fill feed gaps in the existing pasture system and provide a balanced feed supply, or, alternatively, will it be possible to set up an effective feed supply to meet seasonal needs with annual forage combinations?
- Matching the forage crop or pasture to suitable paddocks on the farm and not planting particular forages in unsuitable paddocks. For example, some forages are better adapted to wet or poorly drained areas than others.
- Optimum long-term rotation of annual forage crops and perennial pastures required throughout the farm to maximize feed production and prevent the buildup of disease and insects. For example, planting brassicas for more than 2 years in the same paddock can lead to severe production losses through the club root disease.
- Cost of reseeding each year, including land preparation and all other crop establishment and maintenance costs, as compared to perennial pasture systems.
- Additional supplementary feeds that will be required to meet diet deficiencies in energy, protein, and minerals from feeds produced on farm.
- High water use efficiency can maximize production with available moisture from rain or irrigation. Maize is a

highly water-efficient summer grower, while annual ryegrass is a highly efficient winter–spring producer.

- Fertilizer requirement and cost, particularly fertilizer use efficiency; for example, capacity to respond to N fertilizer, that is, kilograms of dry matter (DM) produced per kilogram of nitrogen.
- Suitability for grazing including palatability and ability to meet grazing management requirements.
- Costing the feed obtained from grazed forage. Grazing is usually the least expensive feeding system. Grazing management needs to maximize percentage utilization and minimize trampling damage.
- Suitability for conservation as hay or silage. The feed quality of the conserved forage can be highly variable and requires assessment. Many forages decline in quality as they mature, and supplements may be required to balance the herd's diet. The hay or silage should be harvested at the optimum stage for yield and feed quality.
- Capital and continuing cost of conservation. Conservation as hay or silage requires special equipment for sowing, harvesting, storage, and feeding out of the conserved feed and is capital-intensive. However, annual forages stored as hay or silage can transfer feed to periods when feed from pasture and other sources is not available seasonally and can also provide feed in periods of drought and severe weather conditions. Feed quality losses in storage of hay and silage should be evaluated. Conserved feed costs should also be compared with the cost of purchased feed alternatives. The cost of contracting out the operations in the silage-making process should be compared with containing them on farm.
- Timing of sowing and the crop development stage for grazing or conserving.
- Balance between perennial pasture and annual forages based on the criteria of the cost of meeting the herd's feed requirement.
- Risks such as frost damage, drought, and crop failure.
- Antinutritional factors and their management.
- Seed cost for the best seeding rate.
- The cost of land preparation and planting, including the capital cost of required machinery. Cost should be compared with the cost of contracting the operations.
- Disease and insect problems affecting the production of individual forages and the cost of effective control.
- Potential competition from weeds. Cost of the management required include herbicides for successful weed control.

Role of Different Forage Types

In this section, some of the characteristics of the annual forages and where they can fit into feed provision system for the dairy are discussed for the major groups. More details are provided in the article **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties.

Warm-Season Grasses

Maize

Maize has the highest productivity potential among the annual forages (up to 25 t DM ha^{-1}). The crop is mainly cut and stored as silage, but it can be grazed as a pure stand or in a bi-crop. It can also be green chopped and fed directly. Maize can also be conserved as earlage, by utilizing silage-making principles on corn ears and a small amount of stalk; yield is reduced, but grain content and digestibility are enhanced. Maize silage production requires specialized equipment for land preparation, planting, harvesting, storing, and feeding out. When harvested at the best stage, maize has a high grain content and energy content (metabolizable energy (ME) 9.5–10.5 MJ kg^{-1}) and a low protein content (7–9%); hence protein supplementation is required. The forage is highly efficient in water use during crop growth.

Sorghums

Sorghums can be divided into three basic groups.

Forage types

The sorghum forage types include Sudan grass, Sudan grass hybrids, forage sorghum, and hybrids between forage sorghum and Sudan grass. These forages can be grazed or conserved as hay or silage. Grazing at around 1 m height restricts the potential for prussic acid poisoning and provides a fair-quality feed (ME 9.5–10 MJ kg^{-1}; protein 12–18%). Hay or silage can be produced by letting the crop grow on after several grazings. Feed quality reduces rapidly with increased lignification as the crop approaches maturity. Potential DM yield, however, increases sharply with advancing maturity. Lower-lignin varieties developed though the brown mid-rib (BMR) gene produce higher-quality feed as the plant matures.

Sweet sorghum

Sweet sorghum is usually conserved as silage or grazed or green chopped near maturity as stand-over feed. It can provide dry matter yields similar to that of maize, but is prone to lodging and more difficult to harvest. Sweet sorghum can stand over for a 6-week harvest period with stable yield and feed quality. Grain content is variable, and water-soluble carbohydrate (WSC) content is high. There is substitution between grain and WSC content. The high WSC content allows ready fermentation of silage.

Grain sorghum

Grain sorghum can be conserved as silage. It will produce higher yields than produced by maize in areas where maize growth is restricted under unfavorable conditions, including poorer soils and less rain. Harvesting at the dough stage of grain development usually provides the maximum yield and the optimum dry matter content for harvest. Grain sorghum at harvest has a low protein content. High tannin content inhibits protein digestion, and the waxy or yellow endosperm types have higher whole-plant digestibility than the normal endosperm type. Digestibility is also reduced by whole grains remaining after harvest and chopping for silage; there is more grain breakdown in maize during silage making.

Millets

Millets suitable for forage production include Japanese, pearl, foxtail, Siberian, poroso, and brown millet. They do not contain prussic acid, and grazing management is easier for millets than for sorghums. Also the forage quality of millets is usually higher than that of sorghums, but the DM yields may be lower. Millets can be grazed only, or grazed and allowed to grow on for hay or silage.

Japanese millet can be planted early and will germinate and establish in the early spring when soil temperatures reach 14 °C. Early germination allows the provision of feed in 6–7 weeks in early summer and quick feed after floods or dry spells.

Pearl millet requires a higher temperature for germination and establishment, and will provide feed later as compared to Japanese millet. Pearl millet has a high feed value when grazed at 45–90 cm, but quality declines rapidly with advancing maturity. Grazing pearl millet down to less than 20 cm will damage growing points and restrict further regrowth. Millet crops often fail to establish effectively. The small seed requires adequate moisture and effective seed–soil contact for germination.

Cool-Season Grasses

Annual ryegrass

Forage ryegrasses

Annual forage ryegrass, which includes Italian and Westerwold types, can be sown into clean seedbeds or directly drilled into existing perennial pastures after suppression of pasture growth through slashing or chemical herbicides. The ryegrass is grazed regularly, and excess production can be made into hay or silage. Feed quality deteriorates late in season as seed heads are developing.

High seeding rates in excess of 20 kg ha^{-1} can be used to increase the rapidity of establishment and feed availability. Forage ryegrass responds to frequent N fertilizer application. Careful grazing management with grazing at the three-leaf stage and controlling residue level will provide high utilization.

Wimmera ryegrass

Wimmera ryegrass is a reseeding annual usually grown in association with subterranean clover as a self-regenerating pasture. The pasture is grazed, and the excess production is harvested for hay or silage. Loss of seed removed from the paddock during conservation process can restrict pasture regeneration.

Cereals

The cereals wheat, oats, triticale, barley, and rye can all contribute to forage production. They can be

- grazed as an early feed in autumn as a whole crop or in combination with annual ryegrass;
- grazed as a crop, and then grown out to produce hay, silage, or grain;
- grown as a crop for whole-crop cereal silage or hay production only; or
- grazed and grown out for grain production.

High feed quality is available when the cereals are grazed at vegetative stage, but quality declines with heading and grain formation. The stage of harvest is critical in determining forage yield and quality. Around the heading stage, the yield and quality of the forage change rapidly. Protein content is ∼20% in the vegetative stage and declines to ∼6% at late milk stage of grain formation. Cereals are suitable for dryland farming.

Oats are effective in producing autumn feed for grazing when grown alone or in association with annual ryegrass, but are not as effective as other cereals if grown out for grain, hay, or silage production.

Winter wheat and rye require a cold period to stimulate flowering; this allows early autumn sowings for forage production.

Barley is suitable for later sowings and is useful in meeting emergencies such as crop failure from earlier plantings of other crops and pastures. It is less tolerant to acid soils than other cereals.

Triticale is more water-logging tolerant and frost susceptible than other cereals. It can supply autumn and winter feed similar in quality to ryegrass.

Warm-Season Legumes

Cowpea and Dolichos lablab

Cowpea and lablab are usually grazed. They can grow for a second grazing. Feed quality is reduced with leaf loss. The leaf-to-stem ratio is ∼50:50 at 6 weeks, but shading leads to leaf drop and the leaf-to-stem ratio becomes ∼20:80. Lablab has heavy stems, which lignify and become indigestible as the plant matures, affecting feed quality. Cows reject the stems if fed in hay, but will eat them in precision-chopped silage.

Soybean

Soybean is difficult to graze, but can be grown and harvested for a high yield of quality silage (8–10 t DM ha^{-1}). There is usually less leaf loss from soybean than from cowpeas and lablab.

Cool-Season Legumes

The main cool-season annual legumes include Persian clover, berseem clover, crimson clover, arrowleaf clover, and subterranean clover. They can be grown alone or along with grasses. Annual legumes produce high-quality feed with a high digestibility and protein content. Clovers can be used to balance the protein deficiencies in maize, sorghum, cereal, and other forages. They can be grazed and conserved as hay or silage. Bloat can occur during grazing and should be controlled. Careful drying is required for silage production as the legumes have a low WSC content. They need to be dried efficiently to concentrate the WSC and ensure suitable silage fermentation. The legumes fix nitrogen, which contributes to the production of associated grasses following crops or pasture.

Brassicas

Brassicas useful for annual forage production are turnips, swedes, rape, kale, and hybrids. They have a high yield potential of quality feed with adequate energy and protein. Turnips and swedes provide feed in the form of leaves, stems, and bulbs, whereas rape, kale, and hybrids provide feed as leaves and stems. Selection of varieties within the brassica group can produce the following:

- summer and autumn feed when pastures are declining in quality as they mature
- autumn and winter feed with frost-tolerant varieties

Brassicas can be sown as a break crop for pasture development allowing the opportunity for further weed control, land preparation, and fertilizer application before establishing a perennial pasture. Brassicas produce glucosinolates, which assist in the biofumigation of endophytes, nematodes, and insects. The root diseases such as take-all, crown rot, and root rot in cereals are also restricted.

Brassicas can be used

- alone to fill feed gaps in late-yielding and late-sowing varieties of pastures;
- oversown with ryegrass or legume to extend production;
- sown in combination with maize as a bi-crop, providing increased DM yield. The maize will be grazed out at first grazing; or
- planted following a maize crop, and then grazed and oversown with annual clover or peas to produce very high annual DM yields.

Management of Annual Forages

General Principles

The challenge is to realize the forage's potential for producing high yields of high-quality feed and to utilize a high proportion of the feed produced. Careful management and attention to detail are required at each management stage to meet this challenge.

The general principles for the establishment and management of each group are similar, with some variation in detail within and between groups.

Failure of annual forages to produce adequate yields is very costly. Attention to detail in the establishment and management of the forage will assist in realizing the full yield potential. The major areas where careful attention must be paid in the management of annual forages are as follows:

- establishment
- maintenance of effective growth
- efficient harvesting by grazing, green chopping, or conserving as hay or silagemanaging antinutritional factors

Establishing Annual Forage Crops

Management issues

Effective establishment of annual forages depends upon resolving the following management issues:

Time of sowing. Sowing times should be chosen to ensure that the objectives of feed provisioning for the herd with the forage are met. The objectives could include obtaining the highest possible yield of acceptable-quality feed, filling specific feed gaps, or supplying feed when required in a feed supply plan. The correct sowing time should also enable rapid establishment and reduce the risk of damage through low or high temperatures near maturity.

Optimum population. A population of the forage plants sufficient to sustain optimum production under the local conditions should be established. The spatial distribution of the population is also important in terms of row spacing and plant density. When the population is too dense, problems with moisture availability and forage development result. High populations may restrict root development in brassica forage and lead to choking and reduced grain content in maize. Low populations lead to lower yields and greater risk of weed invasion.

Rapid establishment. The population should establish quickly through rapid germination and early growth. The temperature should be high enough for germination, and sufficient moisture and nutrients should be available for the establishing seedling. The main aims of rapid establishment are to beat competition from weeds and associated species, and to achieve high production levels.

Restrict competition. Competition from weeds or the associated species for light and moisture at establishment and during early growth of the forage crop should be restricted. Weeds and undesirable species should be controlled. Also there should be minimum competition from the existing pasture when oversown.

Planning well ahead. Early planning will assist in ensuring that the production goals are set and efficiently met. Some of the operations have to be done well in advance of the year or season of growth. Crop rotation also should be planned carefully so that potential yield is not affected. For example, brassicas should not be planted in the same area 2 years in a row, as club root disease can build up and be very damaging.

Applying effective management. Species and varieties within species vary in potential yield, but poor management can be the largest constraint on production. Effective establishment is the foundation of forage crop production. Follow-up management will help to realize the potential.

Ensuring effective establishment

Four important components of delivering effective establishment are time of sowing, land preparation, sowing, and fertilizer at establishment. These components are discussed in more detail.

Time of sowing

Correct sowing time will vary among the important annual forge crops and pastures. There will be differences between species within the five groups and also between varieties within species. Temperature for germination and early growth is the main driver for the earliest sowings. Adequate moisture should also be available at or soon after planting to allow germination. Varietal differences in maturity allow for the spread of sowing dates with quicker-maturing varieties used with later sowing dates. Damage before the end of crop cycle should be avoided by selecting a combination of sowing date and maturity, so that plants that can be damaged by frost or cold are not exposed to excessive risk. Species or varieties that are frost- or cold-tolerant can be chosen for later sowings or when the feed is required further into the late autumn–winter period. The same applies to heat effects on cool-season plants. Sowing of a particular crop may be staggered so that the quality of the feed supply can be maintained through not having it all maturing at the same time. The staggered crop can then be grazed or harvested quickly enough, without having unacceptable decline in quality with advancing maturity.

Land preparation

The main goal of land preparation is to allow successful germination and rapid establishment through ensuring the following:

- Effective seed–soil contact to obtain moisture for germination.
- Sufficient moisture available for germination as stored moisture, rainfall, or irrigation.
- Effective weed control so that there is no competition from weeds for light and moisture at germination and early growth stages. Weed control could be preplanting, preemergent, or postemergent.
- Competition reduction to boost seasonal production when sown onto an existing pasture. The growth of existing species needs to be controlled so that they do not compete with and smother out the establishing seedlings.
- Maintenance of surface structure of the soil. Surface crusting, induced by excessive cultivation, will interfere with germination and establishment. Loose and fluffy surface soil should be rolled or compressed to obtain soil–seed contact.

Methods of land preparation include the following:

- *Clean cultivation*, where a fine and smooth seedbed is prepared through plowing and cultivation. The existing species are removed and germinating weeds controlled through cultivation. Care must be taken not to destroy soil structure, particularly at the soil surface.
- *Reduced tillage or minimum tillage*, where the soil is disturbed less often than under clean seedbed preparation. A firm seedbed is not obtained, but there is less damage to the soil structure. Plowing and turning over the soil is usually omitted. With less cultivation, more chemical weed and pest control is required.
- *Conservation tillage* also includes stubble retention where the stubble of the previous crop is maintained to protect the soil surface and to help retain moisture. Conservation tillage aims to control erosion. Tillage is usually markedly reduced with stubble retention and may be down to no tilling at all.
- *No-till* method, where the existing species are killed out with herbicides and planting is done directly into the previously undisturbed soil. A chemical seedbed is prepared by killing existing plants with glyphosate or knocking them down with paraquat, which burns off green tissue but does not kill the plants. No-till method can be difficult on clay soils, and poor surface drainage may develop. No-till method provides a better base for grazing, as the surface has not been disturbed.

Weed control: An essential component of land preparation In cultivated systems weeds are controlled by initial plowing and with additional cultivation if weeds germinate before the crop is ready to plant. In no-till and minimum-till systems, weeds are suppressed with herbicides glyphosate or paraquat. Weeds can regenerate after paraquat treatment, but usually after the sown species have successfully established. Paraquat can kill small weeds and winter annuals.

Preemergent herbicides, such as Trifluralin (Treflan®), incorporated into soil in a clean seedbed can kill grass and broadleaf weeds in establishing some crops. Preemergent herbicides are sprayed on soil surface after the maize crop is planted to protect the crop from germinating weeds, which are killed by the herbicide. Preemergent herbicides can also control weeds in sorghum crops. The germinating sorghum must be protected from the herbicide with safeners applied to the seeds before sowing.

When sowing into an existing pasture, the objective is not to kill the pasture, but to restrict competition with the establishing annual forage. The existing pasture can be suppressed by hard grazing, mechanically by mowing, slashing, or mulching the existing vegetation back to near the ground, or chemically with paraquat. Light cultivation may also be required to provide seed–soil contact. Harrowing forces the seeds closer to the soil surface and rolling improves soil–seed contact. Grazing after sowing can have a rolling effect by trampling the seeds into the soil.

Weed control and suppression at seeding is important because many forages can compete with weeds once established. Chemical recommendations and regulations on weed control vary in dairying regions worldwide. Locally approved and recommended chemicals should be used and applied carefully according to the directions on the label. Any withholding periods should be strictly applied.

Sowing

The objective of the sowing operation is to place the seed and fertilizer in the correct place and under the best conditions to obtain an effectively established population, which will allow the maximum potential yield. The plants should be distributed to provide the best arrangement for them to establish and produce.

The arrangements include

- *Broadcast*, where seeds are spread evenly on the surface. This method is useful for sward-forming and shorter plants.
- *Row planting*, where plants are planted in rows. There are differences in the spacing between rows and the spacing of plants within rows among different crops and pasture types. Maize is sown in rows 80 cm apart, whereas small-grain cereals are sown in 15–18 cm rows.

Plant density should be such that competition between plants does not restrict yield and the potential plant population is available to produce the maximum yield for the conditions under which the crop is being grown.

Crops planted in rows are easier to harvest and lodging is reduced. Rows can be made wider under more difficult conditions. Row planting allows better animal access with less trampling damage. Wider rows also allow mixed intersowings with legumes and cereals. With narrow rows, there is more competition between plants and less herbicide control is required. Broad rows allow management options such as weed control, fertilizer application, and spraying for disease- and insect control to be applied more effectively while the crop is growing.

Production is not highly influenced by moderate changes in row spacing. When maize is planted in rows 76 cm apart, there may be a 6–7% increase in yield over maize planted in rows 92 cm apart.

Components of establishing an appropriate population

Effective seeding rate Seeding rates recommended for annual forages vary widely throughout the world. The rates recommended locally for the local varieties should be used. The effective seeding rate is based on the final established population required and is influenced by seed size, seed quality, and germinability. The seed should be free of weed seeds and disease. Seed treatment should be applied, if required, to control fungal disease and insects.

The population required after establishment is affected by the probability of losing young plants under the prevailing conditions. Land preparation, seasonal conditions, and disease and insect attack can reduce the number of established seedlings. The seeding rate chosen should be increased to cover these risks. The seeding rate chosen should also be cost-effective. Normally, seeding rates and the subsequent populations are higher for grazing and conservation crops of small-grain cereals than for grain crops. Cereal stem size will reduce with higher populations. The seeding rate can be increased by 25% for hay or silage cereal crops.

Mixtures of seeds of annual forages are frequently sown. Mixtures commonly sown include legume/cereals, brassicas/ryegrass, different maturity cereals, ryegrass/legumes, and ryegrass/cereals. When sowing mixtures of different forages, the seeding rate for a variety in a mixture is lower compared to the rate used when sown alone.

Seed treatment to ensure healthy stands includes insect and disease control, inoculation of legumes with the appropriate strain of nitrogen-fixing rhizobia, and the scarification of hard seeds of annual legumes to allow immediate germination. Molybdenum can also be applied to coat the seed when it is required to meet deficiencies in plant growth.

Sowing and seed placement Seed is usually planted with sowing machinery. The seed should be placed at the correct depth. Too deep and the seedlings may not emerge, and too shallow, the surface may dry out and the moisture will be insufficient to stimulate germination. Also there should be effective seed–soil contact. Covering the seed with soil through harrowing or light cultivation can help to increase soil–seed contact. Rolling to compress the soil and bring it in closer contact with the seed is also an important component of effective sowing techniques.

Sowing methods include the following:

- Broadcasting, where the seed is spread uniformly on the soil surface.
- Drilling in rows with special machinery developed for different crop types. The row spacing can vary from 90 cm apart for corn to 15–17.5 cm apart for cereals.
- Direct drilling, where specific direct-drilling equipment is used to sow into an uncultivated seedbed. The machinery opens a slot, plants the seed, and covers and rolls the seed in the slot, all in one pass.
- Mulch sowing, where seed is broadcast onto an existing pasture and then the area is mulched to get the seed closer to the soil surface in the mulch. Annual ryegrass can be effectively mulch sown.
- Drilling into stubble after harvest needs machinery that can sow into the stubble and not be blocked through dragging the stubble.

Specialist sowing machinery can be a high-cost item in the use of annual forage crops in dairy farming systems and should be costed carefully into the system when comparing the economic efficiency of different annual forages.

Fertilizing Effective early forage crop growth will depend on an adequate supply of readily available nutrients for the establishing seedling. Nutrient availability in soil should be evaluated with soil tests on samples collected correctly from the area to be sown. Deficiencies are also identified with soil tests. Soil acidity may need to be ameliorated through liming.

Timing of fertilizer application

Presowing. Fertilizer can be spread and incorporated into the seedbed before sowing; nitrogen and potassium can be applied presowing. Lime to ameliorate acidic soils is most effective when incorporated into the soil before sowing. It can take up to 6 months for the lime to have full effect on changing soil acidity.

At sowing. Fertilizer can be drilled in the row with the seed or banded near the seed but not in contact. When the fertilizer is applied in the row with the seed, care must be taken to ensure that the germinating seedling is not damaged through osmotic effects and burning effect of soluble fertilizers such as potassium chloride and nitrogen fertilizers. Restricting the rate of application will reduce the potential for damage.

Direct drilling into no-till systems bands the phosphorus and potassium fertilizers. This has little effect on the availability of phosphorus to the crop, but potassium availability is reduced through accumulation in bands near the surface. Low potassium availability may have to be amended through the incorporation of phosphorus and potassium fertilizers into soil as part of crop rotation.

Sufficient amounts of nitrogen, phosphorus, and potassium should be supplied at sowing to ensure establishment and rapid early growth. It is also important to be alert to other potential deficiencies and remedy them when required. Sulfur deficiency can restrict growth and protein synthesis. Brassicas have a high requirement for sulfur and can also be boron-deficient. Molybdenum is important for legumes for nitrogen fixation and should be supplied when deficiency is detected. Zinc deficiency restricts maize production on some alkaline soils. A combination of soil and plant tissue tests can identify deficiencies that should be remedied with adequate fertilizer application.

Further, fertilizer may be required postsowing to obtain maximum production, which is discussed in the section 'Maintenance'.

Replanting Decisions Severe loss of seedlings through frost, moisture stress, flooding, or insect attack may lead to the need for replanting.

Replanting decisions are critical for forage crops like maize where high-cost inputs of fertilizer, weed control, or insect control may have been applied to the damaged crop area. The best decision on whether or not to replant can provide an effective crop that will help to minimize expenditure losses while still adding to the annual feed supply.

Before deciding to replant, the following factors should be carefully considered:

– The population of plants that are likely to survive should be carefully estimated. After a short recovery period, the surviving plants should be counted and the yield potential estimated. The most profitable alternative may be to continue with the remaining plants.
– With a new planting date whether there will be sufficient time to produce an adequate crop or whether the crop meets seasonal difficulties like frost in late growth should be estimated. Potential problems may be resolved by changing to an earlier maturing variety.
– Plants left from the failed crop can compete strongly with the replanted crop and reduce yield potential or interfere with crop management. These plants should be killed with herbicide or cultivation.
– The cost of replacement seed and further insect and weed control if required is an important factor in replant decisions and should be accurately estimated. The seeding rate may have to be changed for later planting after crop failure.

Maintenance of Forage Growth

After the annual forage crop has been effectively established, careful management is required to maintain and stimulate growth to attain the targeted yields of quality feed. The production has to be utilized through effective grazing management or fodder conservation. The aim is to maximize growth and utilization of the annual forage crop.

Factors in optimizing fodder growth

Competition control

The best method for restricting competition from in-crop weeds is to be diligent in presowing and preemergent weed control prior to crop establishment. Further weed control may be needed if weeds establish postgermination of the forage.

Postemergent weed control is more difficult. Inter-row cultivation can control weeds in row crops. Selective herbicides are available to control broadleaf weeds in grass crops or grasses in legume or brassica crops. But controlling grass weeds in grass crops and broadleaf weeds in legumes and brassicas is more difficult. Weed control may also be necessary to remove weeds at seedling or young stage and reduce competition with establishing seedlings close to sowing time to restrict competition later as the forage crop grows.

When many weeds establish within the crop despite weed control and weed seed reduction prior to planting, it is often best to plow out the crop and start again. Some weeds may be edible, particularly grassy weeds, and can be used as feed.

Early competition management When annual forages are sown into any associated species, early management may be required to reduce competition from the associated species so that seedlings can establish effectively. Slashing, mowing, or light grazing can be used to reduce competition from the associated species.

Competition management in mixed species sowing Often in mixed species sowing, one component may be more vigorous in establishing and early growth than the other, for example, annual ryegrass or cereals and small-seeded cool-season legumes or ryegrass oversown onto brassicas. The vigorous species will tend to compete out the less vigorous species. The vigorous species may have to be controlled by grazing, slashing, or mowing early in the growth cycle to restrict competition and to allow the less vigorous species to establish.

Fertilizer management

Fertilizer is applied postsowing to maintain optimum growth. Adequate phosphorus and potassium are usually applied at or prior to sowing. The main fertilizer applied after sowing is nitrogen (N) to stimulate production. In grazed annual forages, N is applied immediately after grazing usually at a rate of ~50 kg N ha^{-1}. In cereals, sufficient amount of fertilizers can be applied at establishment for grain crops, but further application of N during crop growth can stimulate higher forage yields and higher protein content in the forage. When the cereal is grazed, an ideal time to apply N is after the last grazing and before the crop is allowed to grow out for silage or hay production. In cropping systems, N may be required at key stages in crop development and can be applied in row crops as a side-dressing during crop growth. For example N can be side-dressed onto maize with specialized equipment, when maize is near 1 m tall. It can also be applied dissolved in irrigation water. Pure annual legume crops are usually not N fertilised but N is often used on legume/grass mixtures.

Manure can be a useful nutrient source for high-yielding crops. Nutrient use should be carefully budgeted to ensure that sufficient amounts are available to grow the forage and there is no excess that will create environmental damage. A 20 t DM maize silage crop will consume nearly 200 kg N ha^{-1}, 50 kg P ha^{-1}, and 300 kg K ha^{-1}. At least these amounts should be applied to sustain soil fertility. N rates should be adjusted by the N available from the previous legume or N-fertilized crops.

Care should be taken to avoid the use of excess fertilizers. Excess N can lead to high nitrate levels in the forage and toxicity for the grazing cows. Excess N and K can lead to low levels of magnesium in the forage plants on low magnesium soils, and the consequent hypomagnesia leads to grass tetany in the stock.

Harvesting the Forage

Grazing Management

The main principles involved in effectively grazing annual forages include the following:

- A high proportion of the available feed grown should be eaten. Waste should be minimized and damage to the forage by overgrazing or through trampling restricted.
- Nutritive value and feed quality of the forage at grazing should be sufficient to sustain effective milk production.
- Maturity of the forage at grazing has an impact on quality. Feed quality can decline rapidly in fast-growing crops such as hybrid forage sorghums. Protein and energy content can decline to levels where milk production is reduced. Rotational grazing should be adopted to ensure sufficient feed quality.
- Grazing should be controlled so that forage is not wasted and sufficient residue remains to ensure rapid regrowth. Undergrazing restricts utilization and overgrazing restricts potential production by delaying

recovery and regrowth. Growing points of the plants should be preserved, and the plants should be grazed at a stage when they have sufficient carbohydrate reserves for rapid regeneration. For lablab, stems are required for regeneration and should not be grazed too heavily. Annual ryegrass provides optimum production when grazed at the three-leaf stage.
- Do not allow regrazing of the new leaves as they emerge in the first 1–2 days after grazing.
- Growing points of cereals should not be grazed. They can be protected by leaving sufficient residue and also by timing grazing in relation to height of growing point.
- Control grazing to ensure that optimum management requirements are met. Strip grazing with back fencing will give a high level of control over grazing and ensure high utilization. Rotational grazing will usually allow effective utilization and regrowth. Grazing interval should be set based on the significant physiological characteristics of the plant.
- Stimulate tillering and maintain vegetative growth. Quality can be retained when grazing ryegrass if seed heads are removed to stimulate vegetative tillering. Heavy grazing may remove the seed heads, but 'topping' through slashing or mowing may be required. Care must be taken not to damage the growing points of the existing vegetative tillers.
- Stocks take time to adjust to some forages. It usually takes 3–4 days to introduce cows to brassicas. Cows also take time to start grazing lablab. They will eat out associated grasses before acquiring a taste for lablab.
- Brassicas should comprise no more than three-fourths of the diet. When brassicas are being grazed because of their high digestibility, they should be supplemented with a high-fiber roughage or the cows should be allowed access to grass pastures.
- Manage bloat when grazing legumes.

Mechanical Harvesting Including Hay and Silage Production

Some annual forages are grown only for conservation. Maize and grain sorghum are usually conserved as silage. Small-grain cereals can also be grown solely for whole-crop silage. Many of the annual forages can be grazed and also produce hay or silage, for example, small-grain cereals and excess spring production of annual ryegrass. Small-grain cereals can also be grazed and then grown on for grain production.

Mechanical harvesting includes green chopping and hay and silage production.

Green chopping

Forage can be mechanically chopped, removed from paddock, and fed to the cows green. Green chopping usually starts at a younger stage than for hay and silage production. Most forages, including brassicas, can be green chopped. The forage crop being green chopped will advance in maturity during the chopping program. Rapid-growing crops, which advance in maturity quickly, such as hybrid forage sorghums are difficult to manage for green chopping. Staggered sowings can assist in balancing maturity at harvest and yield. Field losses are usually lower for green chopping than for grazing or hay production.

Gazing is usually cheaper than machinery-based systems, which are capital-intensive. Harvesting by green chopping also takes more time and labor than grazing.

Green chopping can be difficult under wet conditions, and crops proposed for green chopping should be sown on well-drained fields.

Green chopped forage may deteriorate if not fed out quickly. Forage containing nitrate can become more risky because heating after harvest will increase the rate at which nitrate turns into poisonous nitrite.

Hay

Hay production is most effective when quality forage can be cut and dried down quickly and baled with minimum damage through leaf loss, mold, or deterioration. Forage crops can be harvested as hay in difficult conditions if the crop had been sown for another purpose.

The stage of harvesting is determined by a balance between the total dry matter available and the quality of the crop. The energy and protein content of the crop are dictated by the maturity of the crop at harvest. Delaying harvest may increase yield, but quality could also decline. The harvest stage should provide a balance between forage yield and quality. When the forage has been cut for hay, it needs to dry down quickly to become dry enough to bale without deterioration through mold or heating. Drying rate is strongly influenced by seasonal conditions and will be slowed down by wet, cold, or humid conditions.

The physical characteristics of plants such as stem thickness, the bulk of the material available, and its moisture content also influence drying rate. Physical treatments such as crushing or conditioning at cutting, rolling, and tedding, or turning the cut material can speed up moisture loss and allow it to dry down quickly. High-quality hay can be obtained if the cut crop can be dried down in 2 days. Cows will reject lablab and soybean stems even when the crop has been made into quality hay. They will eat a greater proportion of stems when conserved as silage.

Machinery is available to produce a range of bale sizes and shapes. Bale sizes range from small bales with ~25 kg DM bale^{-1} to large bales that can contain 300 kg of DM. Balers that produce round bales or square bales are also available. The baler can be an expensive item in the management system, and the return on the capital invested should be carefully analyzed.

Table 1 Effective stage for harvesting annual forage crops when conserved as hay or silage

Annual forage	*Optimum stage for harvest for conservation*
Warm-season grasses	
Maize	Milk line on maize grain nearly half way up the grain
Grain sorghum	Dough-stage grain formation
Sudan grass	At ~1 m or at boot to early heading
Hybrid forage sorghum	At ~1 m or at boot to early heading
Sweet (forage sorghum)	Soft to medium dough stage
Millet Japanese	Early flowering
Millet pearl	Nearly 1 m or early flowering
Cool-season grasses	
Annual ryegrass	Excess spring growth that cannot be grazed
Wheat	Dough-stage (short varieties); heading to flowering (tall varieties)
Oats	Boot or early heading
Barley	Dough stage
Rye	Harvest early at boot stage
Triticale	Boot or early flowering
Warm-season legumes	
Lablab	Early flowering
Soybeans	Half pod fill
Cowpeas	Early flowering
Cool-season legumes	
Beseem clover	Multiple cuts at an adequate height (45 cm) to early flowering
Persian clover	Early flowering

The most effective stage provides a good balance between yield and quality

Cutting height should be managed to allow regeneration of the crop after haymaking. Growing points can be removed if the forage is cut too low, and the forage will not regenerate for further cuts, for example, berseem clover (**Table 1**).

Silage

Effective silage making is contingent on two major components:

- Growing a high-yielding crop with an appropriate quality for dairy production.
- Harvesting the crop at the correct stage and preparing and storing it so that it is preserved effectively and fed with minimum wastage.

The correct harvesting stage for silage is similar to that for hay, chosen with the aim of balancing quality with yield. The decline in quality with advancing maturity must be managed carefully to conserve adequate-quality feed. Maize silage under ideal conditions offers the opportunity for the highest yield of forage among all the annual forages. Maize and grain sorghum forage are usually conserved only as silage.

Silage may be chopped and stored in a pit, bunker, or tower, or compressed into round or square bales and wrapped in plastic to exclude air and allow fermentation to proceed.

The plastic wrapping on baled silage can deteriorate, and the silage will not keep as long as bunker or pit silage.

Major considerations in the preparation of forage for ensiling are as follows:

- *Fineness of chopping*. This influences the capacity to compress silage, exclude air, and stabilize the anaerobic conditions required for effective silage fermentation. Particle size also affects the rate of digestion and passage of silage through the rumen when it is fed back to the cows.
- *Moisture content*. When the forage is too wet, the silage will produce excessive amounts of effluents. The dilution effect of the excess water will reduce concentration of the fermentable substrate and restrict the fermentation process. Dry forage is difficult to compress in the stack to expel air, and fermentation is also restricted. Water may need to be added to dry forage.

The silage should be covered so that air is excluded and potential for aerobic spoilage is prevented. Aerobic spoilage of silage occurs when it is exposed to air. The breakdown process is similar to composting.

Brassicas are difficult to ensile, as they are difficult to dry because of their high moisture content. Kale can be conserved as round bale silage. Brassicas can also be ensiled when sown in mixtures with grasses.

Pest and Disease Control

Insect pests and diseases can reduce production and in the worst case decimate the crop. Pest and disease control like weed control can be managed prior to sowing, at sowing, and during crop growth. Pests and diseases that affect annual forages vary worldwide. Local recommendations are usually available for their control. Toxic chemicals are often used for control, and extreme care is required in their use. The chemicals have withholding periods when the animals should not be allowed to graze the sprayed crop. These periods should be adhered to carefully to reduce risks.

Pesticides can be applied to the seed prior to sowing or sprayed directly onto the soil or the crop.

Antinutritional Factors that Affect Annual Forage Use

Annual forages contain a range of compounds that if eaten can restrict production, and even kill livestock compounds. The content of these antinutritional in the forage and their intake must be managed to restrict damage. Some of the main antinutritional factors are outlined for each annual forage group.

Warm-Season Grasses

Sorghums

1. *HCN poisoning.* All sorghum types can cause hydrogen cyanide (HCN, prussic acid, hydrocyanic acid) poisoning. The glucoside dhurrin is converted to HCN by an enzyme released when the plant cells are damaged. The glucoside concentration (HCNp) is higher in young plants and gets diluted with age. Stress from frost or drought increases HCNp. Haymaking reduces the enzyme content but not HCNp. There is sufficient enzyme in the rumen to allow poisoning. Ensiling reduces HCNp, because gaseous HCN is lost during the ensiling process. Fatalities from HCN have not been recorded after properly ensiling sorghums. Fermentation for 3–4 weeks should be allowed for breakdown.
2. *Sulfur deficiency.* The desired nitrogen-to-sulfur ratio in feeds is ~14:1. Usually sorghums are not severely deficient in sulfur; however, the quantity of S gets reduced as it is used to detoxify the HCN ingested from the sorghum plants and thus they can become S-deficient. S fertilizers are ineffective in supplying S. The most effective technique is to supplement the animal's diet with S in a 5% S salt block or lick.
3. *Low sodium.* Sorghum forage is usually low in sodium, which may be deficient for milk production. Fertilizing with sodium often does not increase sodium content of the forage to adequate levels. However, the animals respond to supplying sodium in feed supplements and in salt blocks or licks.
4. *Nitrate poisoning.* Nitrate problems can occur when sorghums are
   - grazed at <90 cm, after a killing frost, or in regrowth after killing by an early frost;
   - harvested during or immediately after severe stress including drought, shade, frost, or temperature extremes; or
   - exposed to high soil N levels from heavy manure application or a high rate of N fertilization particularly late in the growing season.

Conservation and storage methods can alter the concentration and composition of nitrates in the conserved fodder.

- Ensiling reduces nitrate content up to 50% after 21 days of fermentation.
- Green chop, which heats up before feeding, changes nitrate to nitrite, which is more dangerous.
- Nitrate levels stay high in hay. If the hay gets wet in storage, nitrate can be changed to nitrite, which can accumulate at the bottom of the bales.
- Analyze for nitrate before feeding. If the nitrate content is more than 6000 ppm, the feed is potentially toxic.
- Feed with proper precautions. Do not feed to hungry, sick, stressed, or pregnant animals.
- Dilute high-nitrate feeds with low-nitrate feeds, including grain supplements. Gradually adapt the cows to increasing nitrate levels. Leave 25–30 cm of stalk in the field, as nitrate accumulates in the stem bases. Molybdenum is an important component in nitrate reductase, which breaks down nitrates. Nitrate reduction is inhibited in Mo-deficient plants, and nitrate accumulates.

Millets

Millets do not accumulate prussic acid, but nitrate accumulation can be a problem. Stressed growth of young plants or regrowing plants can take up high levels of nitrate. Grazing may need to be delayed until nitrate concentration dilutes in the accumulated dry matter. Cows grazing pearl millet often show a depression in fat concentration in milk. This effect may be due to the high oxalic acid content in the forage.

Maize

Maize can also accumulate nitrate. High levels of nitrate can result when a long period of drought is followed by rain, N fertilizer or manure are applied to the crop at heavy rates, or leaves are frosted and killed and the roots and stems remain active.

Cool-Season Grasses

Italian ryegrass

Ryegrass staggers occurs in cattle grazing perennial ryegrass and is caused by the alkaloids Lolitrem B and ergovaline from the endophyte *Neotyphodium lolii*. Other alkaloids from the endophyte protect the ryegrass from insect attack and aid in its persistence (e.g., argentine stem weevil in New Zealand, Black beetle in Australia). The endophyte usually found in Italian ryegrass (*Neotyphodium occultans*) is not as vigorous as *N. lolii*. It appears to provide some protection to young Italian ryegrass seedlings from insect attack, but has no effect on insects in established ryegrass. Infected Italian ryegrass does not appear to cause ryegrass staggers. Perennial ryegrass will hybridize with Italian ryegrass, and *N. lollii* infection may get established in hybrid plants, which look similar to Italian ryegrass. Ryegrass staggers can occur when these hybrid plants are grazed.

Grass tetany (hypomagnesia) results from low accumulation of magnesium in the forage. Uptake of magnesium is restricted when high rates of N and K fertilizers are applied, particularly in the spring period of rapid growth, to crops grown on soils with marginal levels of available magnesium. Direct supplementation of the cows with magnesium in concentrate feeds is the most reliable method of grass tetany control.

Annual ryegrass

Lolium rigidum (or Wimmera ryegrass) develops a toxicosis through an association of the nematode *Anguina agrostis* and the bacterium *Clavibacter toxicus*. The bacterium is also infected with a bacteriophage. Infection occurs in the seed head and shows as a yellow slime. In cows, the corynetoxins produced develop neurological symptoms, including incoordination, brain damage, and death.

Small-grain cereals

Small-grain cereals can also accumulate high levels of nitrate when excess nitrogen is available and when conditions are stressful. Grass tetany can also develop from induced low magnesium levels in grazed cereals.

Cereals, especially rye, are susceptible to ergot infection. Ergot infects during grain formation and can be important in late-harvested whole crops. Toxins cause vasoconstriction and psychoactive effects in grazing animals. Some of the ergot toxins are similar to LSD.

Legumes

All legumes

Bloat can be a difficult problem associated with grazing legumes. Stable foams are developed by associated bacteria when highly digestible legumes are eaten. These foams trigger off the bloating process, which stresses the grazing cow and can result in death. Bloat is also generated from other highly digestible feeds including brassicas and young cereal forage. The effects of bloat can be reduced by

- introducing cows onto legume pastures slowly;
- sustaining a grass component in legume pastures;
- feeding hay before commencing grazing to ensure that the cows are not hungry;
- using antibloat drenches and bloat oils; and
- supervising grazing carefully, watching out for bloating animals.

Soybeans

Soybean hay damaged by wet weather can contain toxins from the fungus *Phomopsis* spp., which develops on the immature grain in the hay. The toxin causes liver damage and produces symptoms similar to those of lupinosis.

Brassicas

Brassicas contain a range of antinutritional compounds that can be detrimental to production. Nevertheless, the forage is useful and effective management can contain the effects. The antinutritional factors include the following:

1. *Glucosinolates*, which are broken down by enzymes in the plant and in the rumen to metabolites that can be toxic; these include
   - *Thiocyanates* – interfere with thyroid uptake of iodine; can be supplemented by iodine
   - *Goitrin* – interferes with iodine metabolism and can lead to goiter (enlargement of the thyroid gland)
   - *Nitriles* – can increase kidney and liver size and restrict growth rate
2. *S-Methylcysteine sulfoxide (SMCO)* is an amino acid that breaks down in the rumen to form dimethyl disulfide, an oxidant that attacks red blood cells and leads to hemolytic anemia. Symptoms are reduced appetite and red color in the urine. The syndrome is known as kale anemia as it is seen most often when grazing kale. SMCO increases as the plant matures, and care should be taken in late-winter grazings. Nitrogen fertilizer also tends to increase SMCO content.
3. *Milk tainting* will occur if cows are fed brassicas less than 2 h before milking.
4. *Nitrate* poisoning can also occur after stress periods.
5. *Bloat* can be initiated by rapid intake of the highly digestible brassica forage.
6. *Choking* can occur when the bulbs of turnips and swedes are small as a consequence of high plant populations.

Brassicas can also contain potentially dangerous antinutritional compounds. The impact of most antinutritional compounds can be controlled if

- the cows are introduced slowly to grazing the brassica crop;
- brassicas do not make up more than three-fourths of the diet; and
- roughage as pasture hay or silage is fed with the highly digestible brassica.

The major antinutritional factors in annual forages have been covered in the above discussion. There are, however, additional factors that were not discussed; some of these are not fully understood and others will be discovered as we improve our comprehension of the composition of plants and their interaction with ruminants.

See also: **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties.

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Perennial Forage and Pasture Crops – Species and Varieties

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Pasture Species Used for Dairying

Most dairy cows are grazed on temperate C_3 pastures although a proportion is also grazed on naturalized C_4 tropical grasses in the subtropical regions of Australia, South Africa, South America, and the southern areas of the United States. Plants are designated C_3 or C_4 based on their photosynthetic pathway: C_3 plants have a Benson–Calvin pathway, whereas C_4 plants have a PEP photosynthetic pathway.

Temperate Pasture Species

Perennial ryegrass (Lolium perenne)

By far, the most common dairy pasture is based on perennial ryegrass, although this species is restricted to cool temperate regions as it lacks persistence in subtropical (hot and humid) and Mediterranean (hot and dry) environments, even when irrigated.

Ryegrass has high forage quality with an average metabolizable energy density of 11.5 MJ kg^{-1} dry matter (DM) and crude protein (CP) levels of 18–30%. Perennial ryegrass does require high-fertility soils. Although often grown with white clover to improve quality and to reduce nitrogen fertilizer needs, ryegrass yields are higher when grown as a monoculture with 350–500 kg N ha^{-1} yr^{-1}.

Cocksfoot or orchardgrass (Dactylis glomerata)

Cocksfoot is also commonly used as a dairy pasture in areas where perennial ryegrass is not suitable, as cocksfoot's drought and pest resistance are better. It is slow to establish and its low water-soluble carbohydrate (WSC) content makes it difficult to conserve as silage.

Tall fescue (Festuca arundinacea)

Tall fescue is a hardier temperate grass than ryegrass with a far better root system and greater tolerance to heat and cold. When sown as a mixture with white clover, it has notably higher clover contents than ryegrass-based pastures. Drawbacks include its slow establishment, lack of palatability, and reputedly poorer quality. Particular attention is therefore needed to grazing management to maintain palatability. Breeding is improving the quality and animal acceptability of tall fescue, but generally persistence and yield are sacrificed for better animal acceptance.

Prairie grass (Bromus willdenowii)

Prairie grass is a temperate grass species that, because of its greater drought tolerance, is suitable for areas marginal for perennial ryegrass. In such situations, its ability to promote milk production is superior to that of perennial ryegrass. Prairie grass is rather intolerant of pugging and therefore grows best on well-drained soils. It has the advantage over ryegrass in prolific seed set even in regions such as the subtropics where perennial ryegrass becomes nonreproductive. It is generally short-lived, and management needs to be adjusted to let it seed in spring and reestablish in autumn.

Timothy (Phleum pratense)

Timothy is a species commonly grown in Europe for hay or silage. Timothy is tolerant of cold and wet conditions.

Phalaris (Phalaris aquatica)

Phalaris is of minor importance in dairying. Its forage quality is believed to be inadequate for milking cows, and, in addition, high alkaloid levels can cause toxicity problems. It has a deep root system that makes it more drought-resistant than most other temperate grasses.

White clover (Trifolium repens)

Globally, white clover is the most useful dairy legume because of its high quality and its ability to tolerate high grazing pressures, fix substantial quantities of atmospheric N, and tolerate a range of soil and climatic conditions. The actual amount of N fixed by white clover can be extremely variable from <10 kg N ha^{-1} yr^{-1} in infertile soils to over 380 kg N ha^{-1} yr^{-1} in fertile soils in temperate regions. In its establishment year, white clover is a taprooted plant, but in early spring (in summer in the subtropics) the plant 'breaks' into stolon sections and at each node a new fibrous-rooted seedling is initiated.

Milk production increased by up to 33% by increasing the component of white clover in the diet to ~50%. However, cows grazing clover-dominant pastures are prone to bloat. Although there are management practices that will prevent bloat, the best long-term solution would be to breed bloat-free cultivars and this will probably involve increasing tannin content. The larger-leafed cultivars are most suitable for intermittent grazing, and there is evidence that Ladino types are more heat-tolerant, with maximum production obtained up to 30 °C compared to 25–26 °C being optimal for other cultivars.

Red clover (Trifolium pratense)

Red clover grows from late spring to early summer but usually persists only for 2 years. The high estrogen concentrations in older cultivars have been selected out of most commercially available cultivars. Red clover can also cause bloat. It may be more tolerant of some insect pests than white clover.

Lucerne or alfalfa (Medicago sativa)

Lucerne is an extremely useful legume for grazing or for conservation as hay or silage. Its deep taproot allows it to utilize soil moisture not available to most other plants. It requires well-drained soil with a high pH (>6).

Selections have been made against fungal and insect attack and for winter activity. The winter-active varieties have been reported to yield up to 40 kg DM ha^{-1} day^{-1} in warm subtropical winter conditions. Yields in these areas have been up to 21 tonne DM ha^{-1} yr^{-1} (ranging from 26 tonne DM ha^{-1} in the sowing year to 17 tonne DM ha^{-1} in the third or fourth year).

Lotus major (Lotus pedunculatus)

Lotus major is a spreading, rhizomatous legume that provides good summer–autumn growth. It is more tolerant of acidic soil conditions and waterlogging than white clover, and hence it thrives in coastal swamp regions. It is slow to establish but is unique in that it does not cause bloat due to its high tannin content (2–4% DM) and is tolerant of low soil phosphorus status.

Strawberry clover (Trifolium fragiferum)

Strawberry clover can tolerate wet conditions and is also one of the most salt-tolerant clovers. It prefers a neutral to alkaline soil.

Tropical Pasture Species

When moisture or fertility is nonlimiting, DM yields from tropical C_4 grasses are more than double the yield obtainable from temperate grasses, but only a small proportion of this potential can be utilized by the dairy cow. The low utilization rate for this type of pasture is due to the extremely seasonal growth peaking in summer and the animal's selection for higher-quality leaf.

Globally, kikuyu (*Pennisetum clandestinum*) and paspalum (*Paspalum dilitatum*) would be the two most important tropical grasses used for dairy cows, with other species such as Rhodes grass (*Chloris gayana*), setaria (*Setaria sphacelata*), the panics (e.g., *Panicum maximum*), pangola grass (*Digitaria eriantha*), and Bermuda grass (*Cynodon dactylon*) being of lesser importance.

Kikuyu

Kikuyu is a stoloniferous plant and is regarded as a 'cool' tropical grass. It differs from other C_4 grasses in that it continues to produce stem without setting seed and sets seed only when grazed hard. The protein content of, and milk production from, well-managed kikuyu is higher than that from other tropical grasses. Under management systems promoting the availability of high-quality leaf, a maximum of 12–14 tonne DM ha^{-1} of utilized pasture can be expected.

Paspalum (or dallis grass)

Paspalum is a tufted grass, which is very much a summer-growing plant. It runs to seed in late summer–early autumn with consequent reduced forage quality unless it is grazed hard or the stems are removed by slashing or mulching. Ergot (*Claviceps paspali*) can produce a toxin in its seed and this exacerbates the poor animal performance in autumn.

Setaria (or golden timothy)

Setaria is a true tufted tropical grass. Cultivars have been selected for tolerance of drought, waterlogging, salinity, and frost. It probably tolerates hard grazing and slashing better than most other tufted tropical grasses. Setaria declines in palatability with leaf maturity and seeding. It contains oxalates that bind calcium and make it unavailable to the stock; this can cause acute hypocalcemia and death.

Guinea grass

Guinea grass is a tufted perennial suited to humid tropical areas. Cultivars need medium to high soil fertility and a rainfall above 1000 mm. It is not tolerant of severe drought, waterlogging, or frost.

Green panic (or slender guinea grass)

Green panic is similar to guinea grass but has smaller stems and leaves. It has a longer growing season and is more tolerant of drought, but more susceptible to frost than Rhodes grass. Green panic is best grown on medium- to high-fertility soils, and is one of the most palatable of the tropical grasses.

Bermuda grass (or couch grass, or kabuta, or dhoub grass)

Bermuda grass is a stoloniferous tropical grass, which is drought-resistant and has good tolerance to salt. Bermuda grass withstands close grazing or cutting. It is palatable if kept short and is highly responsive to nitrogen.

Pangola grass

Pangola grass is a stoloniferous tropical grass that will grow on poorer soils than kikuyu or paspalum. It tolerates waterlogging and drought, but its foliage is susceptible to frost. Pangola grass is responsive to high soil fertility and irrigation. It is highly palatable if kept short, leafy, and not allowed to seed. Seeds, if produced, are sterile so pangola can only be established vegetatively.

Rhodes grass

Rhodes grass is a stoloniferous tropical grass that has good tolerance of drought and salinity. It is responsive to high soil fertility and has yielded up to 58 tonne DM ha^{-1} yr^{-1} in Zambia. There are large differences in the seasonality of production, foliage quality, and palatability of its cultivars. Tetraploid cultivars are more palatable than diploid types.

Tropical legumes

The use of twining tropical legumes such as glycine (*Neonotonia wightii*) and siratro (*Macroptilium atropurpureum*) in dairy pastures has not been very successful, as most did not persist at stocking rates over 0.5 cows ha^{-1}. The attraction of tropical, like temperate, legumes in a pasture is the improvement in forage quality and reduced need for N fertilizer. More recently, tropical legumes that can withstand heavier grazing have been introduced, including Kenya white clover (*Trifolium semipilosum*), creeping vigna (*Vigna parkeri*), and the grazing peanuts (*Arachis pintoi* and *Arachis glabrata*).

Leucaena (*Leucaena leucocephaIa*) and gliricidia (*Gliricidia sepium*) are two browse legumes that may provide standing protein-rich fodder in marginal dairy lands. However, it is doubtful if leucaena can be used as a major component of the diet of milking cows, as it has been implicated in tainting milk.

Herbaceous Species

While grasses and legumes form the major forages grazed by dairy cattle, there are many herbs and forbs that are consumed as components of mixed pastures. The nutritive value of these has rarely been evaluated, but many have adequate levels for dairy cow nutrition and some contain high levels of minerals. Unfortunately, some contain high levels of tannins that affect digestibility and restrict intake.

Chicory (*Cichorium intybus*) and plantain (*Plantago lanceolata*) are two herbaceous species used in Australia and New Zealand as components of mixed pastures. The milk-tainting properties of chicory mean that its proportion in the diet must be restricted although newer cultivars have been bred to reduce this risk. Chicory is more active in winter, whereas plantain produces more summer growth and is more affected by leaf-sucking insects. The nutrient quality of herbs, like that of grasses, is determined by soil fertility and fertilizer application. Protein content and organic matter digestibility of chicory forage, 22.6 and 84.8% DM, respectively, are as high as those of perennial ryegrass/white clover. Potassium, calcium, magnesium, zinc, sulfur, boron, and manganese concentrations are considerably higher in chicory than in most other pasture species.

Forage Quality

The quality of the forage ingested by dairy cows grazing a pasture is determined by the quality of the pasture and the ability of the animal to select its diet from the material on offer. The nutrients actually available to the animal will depend on digestion and absorption in the rumen and intestine. A natural pasture is generally a complex mixture of many species, each of which has its own nutrient profile. Sown pastures, on the other hand, are generally simple mixtures with components that have been selected as having better nutrient profiles than those found in naturalized or native pasture species. Single-species forages provide even less variability and choice, particularly in situations where soil nutrients are limiting or environmental pressures restrict optimum growth. A plant community will have plants at different stages of maturity, and these plants have different proportions of leaves, stems, and seedheads, which will also vary in their nutrient profile.

Comparison of Grasses and Legumes

Grasses generally contain less CP than legumes (**Figure 1(a)**), but this is influenced by fertility of the soils in which they grow, by fertilizer inputs (particularly N), and by environmental conditions. CP levels in grasses generally range from 60 to 300 g kg^{-1} DM, whereas the range for legumes is 30–280 g kg^{-1} DM. Well-nodulated legume swards will be higher in CP because of the supply of N for protein synthesis through the symbiotic nature of the nodulation process. On the other hand, grasses need to extract N from the soil, and this is influenced by soil fertility, environment, fertilizer application, and nitrogen transfer from legumes. Levels are also influenced by plant maturity. In the vegetative stage, grasses have relatively higher levels of CP, but these fall rapidly as they mature. In legumes, the decline

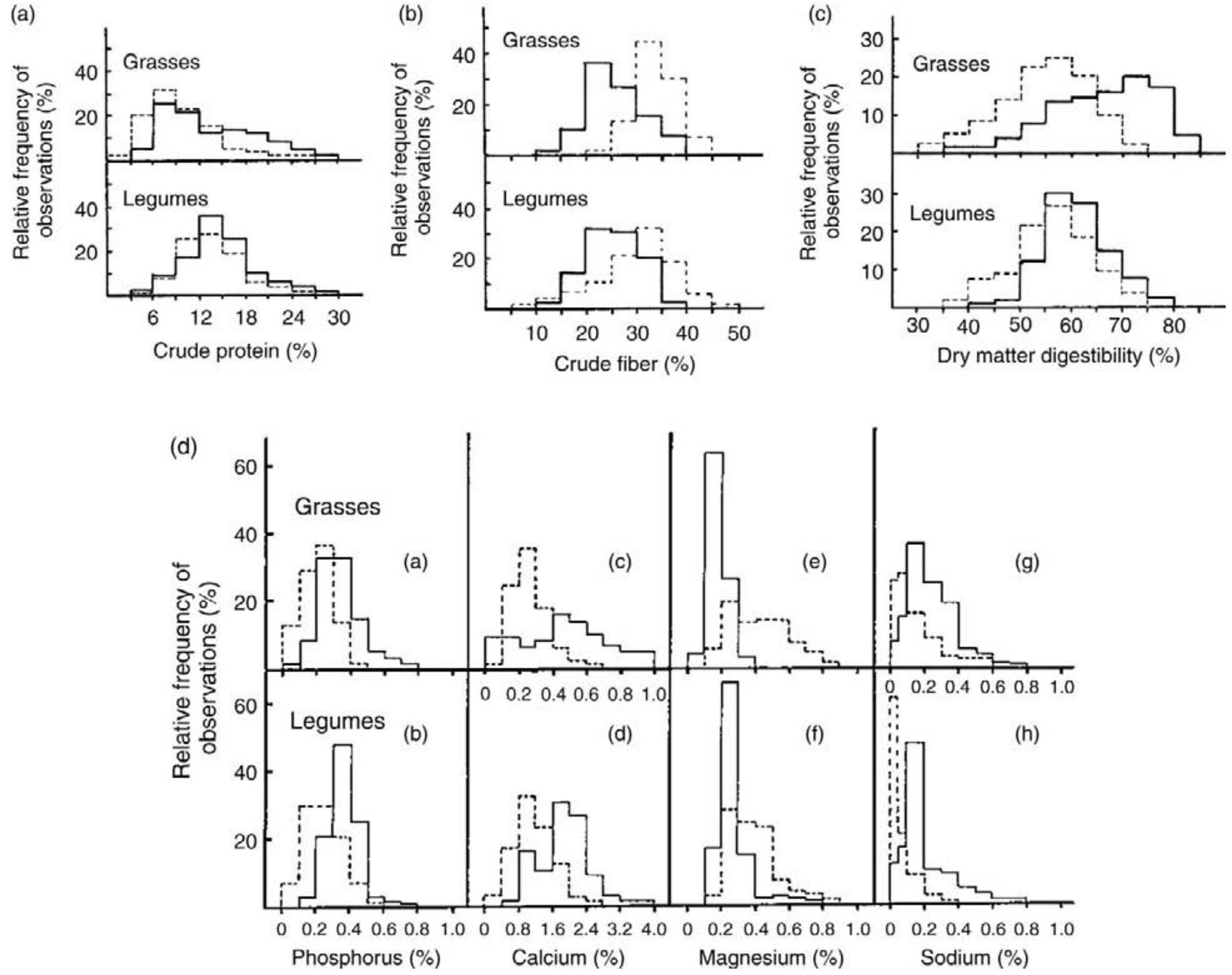

Figure 1 The range of values for (a) crude protein, (b) crude fiber, (c) digestibility, and (d) mineral content of temperate (—) and tropical (. . .) grasses and legumes. Adapted from Norton BW (1982) Differences between species in forage quality. In: Hacker JB (ed.) *Nutritional Limits to Animal Production from Pastures*, pp. 89–110. Farnham: Commonwealth Agricultural Bureaux.

in CP content with maturity is lower because of the continued supply of nitrogenous products through rhizobial fixation. In both grasses and legumes, the CP content of older leaves falls more quickly than in young leaves. For this reason, management systems that maintain a high proportion of new growth will optimize the CP intake by dairy cattle.

Nonstructural carbohydrates (NSC) are the readily fermentable source of energy for herbivores. What is presented as NSC in the literature is influenced by the method used for extraction; WSC extract the short-molecule sugars, whereas alcohol-soluble carbohydrates also contain longer-chain polysaccharides such as starch. Legumes and tropical grasses store starch and sucrose in their leaves, whereas temperate grasses store glucose and fructosans in the base of their tillers. Legumes such as lucerne also store substantial amounts of NSC in their taproots. The NSC content of grasses usually increases with maturity, but there does not appear to be the same consistent trend with legumes. Application of N fertilizer to grasses generally decreases their WSC content as reserves are utilized in new growth. Legumes generally have higher levels of NSC in their tissue than grasses, but this may be because many of the studies have measured only the WSC content ignoring the starch component, especially in tropical grasses. Perennial ryegrasses have been selected in Europe and South Africa for higher WSC content, but expression of these traits is weaker and more variable in Australia and New Zealand.

As structural carbohydrates form the major component of plant tissue or cell wall, they have a major influence on forage quality. Cell wall content of both grasses and legumes increases as plants age. Leaves have lower levels of structural carbohydrates than stems. The proportion of cell wall components (i.e., celluloses, hemicelluloses, tannins, proteins, minerals, and lignin) varies in grasses and legumes, and this influences quality differences between grasses and legumes and between species and cultivars. With maturity, grasses normally show higher cell wall concentrations and a more rapid accumulation of lignin than legumes. Crude fiber levels are similar for temperate grasses and legumes, but tropical grasses have higher levels than tropical legumes (**Figure 1(b)**).

Digestibility of temperate grasses is higher than that of temperate legumes, but the differences between tropical grasses and tropical legumes, and temperate legumes, are smaller (**Figure 1(c)**). Digestibility in legumes is determined primarily by the degree of leafiness. Generally, grass digestibility is more maturity-dependent than legume digestibility, whereas environment is more likely to affect legume digestibility (i.e., leaf loss from stress).

Mineral content is influenced by soil fertility, maturity, fertilizer application, and species. Forage quality will be affected only when nutrient concentrations in forage are above or below the nutrient requirements of the dairy cow. There appear to be few differences in the levels of P and Na between the tissues of grasses and legumes, but legumes are inherently higher in the levels of Ca, Mg (**Figure 1(d)**), and Zn. Potassium is required in greater amounts for plants than for animals, and therefore concentrations of K in the forage are unlikely to restrict milk production. Potassium levels in grasses are usually lower than in legumes. However, in well-fertilized pastures the differences are not large. Information is inconclusive differences in the levels of other trace elements.

Temperate legumes are acknowledged as being capable of supporting higher milk production than well-fertilized temperate grasses. However, herbage production per hectare is likely to be lower as legumes generally cannot sustain stocking rates as high as that of grasses. **Figure 2** illustrates the difference in milk production between white clover-dominant and perennial ryegrass-dominant pastures. Milk production from tropical grasses has been shown to be higher than that from the twining tropical legumes in a number of studies in subtropical Australia despite voluntary intakes being higher from the legumes. Milk production in short-term studies using Jersey cows averaged 9 l milk day^{-1} cow^{-1} from pangola grass, compared with 7.7 l milk day^{-1} cow^{-1} from siratro or Greenleaf desmodium (*Desmodium intortum*) swards. In longer-term studies, tropical grass/legume mixtures produced around 3500 l milk cow^{-1} lactation^{-1} compared with 4000 l milk cow^{-1} lactation^{-1} from grass/N pastures. This may have been a forage density effect, however, as milk production from Amarillo grazing peanut (*A. pintoi*), a tropical legume with a dense canopy and high digestibility, was around 1 l more than that from N-fertilized pangola grass: 14.6 and 13.8 l milk day^{-1} cow^{-1}, respectively.

Comparison of Tropical and Temperate Species

The nutritional limits of unsupplemented tropical pastures appear to be restricted to the levels quoted above when liveweight remains unchanged, compared to around 21 l milk day^{-1} cow^{-1} from unsupplemented temperate species. It has been suggested that the difference between the potential of tropical and temperate pastures to produce milk may, in part, be related to morphological differences, with tropical species generally having a more open growth habit than temperate species. Low leaf density affects the bite size of cattle, and this means that they have to expend more energy and time in harvesting material. Tropical legumes are more likely to have low leaf densities, particularly those with twining growth habits such as siratro or glycine. Leaf and stem

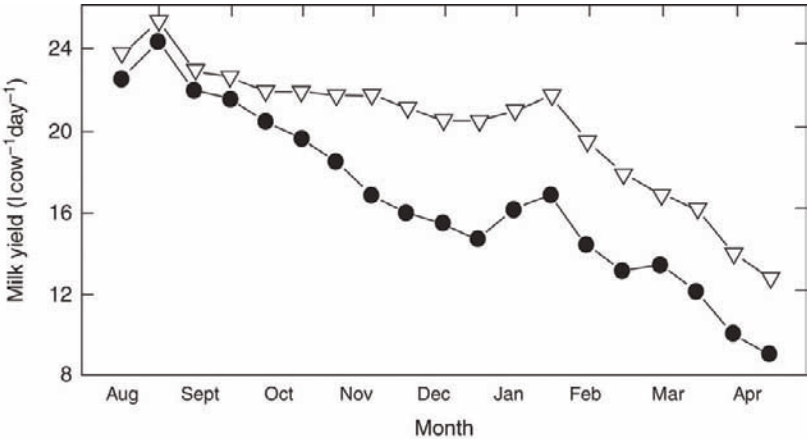

Figure 2 Milk production by cows grazing white clover (▽) or perennial ryegrass (●) to appetite throughout lactation. Reproduced with permission from Rogers GL, Robinson I, and Moate PJ (1986) Milk production of dairy cows grazing white clover and perennial ryegrass. *Proceedings of the Australian Society of Animal Production* 16: 427.

anatomy also differs between tropical and temperate species; tropical grasses have a higher proportion of bundle sheath and vascular tissue and a lower proportion of thin-walled mesophyll cells as compared with temperate grasses. The bundle sheaths in tropical grasses have thicker walls that are resistant to mechanical breakdown and may be responsible for increased rumen retention time. Temperate grasses contain more easily digested mesophyll tissue and it is less densely packed, making it easier for rumen microflora to access. These structural differences can result in leaves with different tensile strengths, which affect the ability of the animals to bite material and may lead to greater retention and breakdown time in the rumen.

Temperate and tropical legumes appear to have a similar range of CP values, but under similar fertilizer regimes, tropical grasses have a substantially lower concentration of protein than temperate grasses (**Figure 1(a)**). There are many instances where the concentration of CP in tropical grasses falls below the 15% minimum required by lactating animals.

In cool environments, temperate grasses and legumes have higher WSC concentrations than tropical species, but in warm environments WSC content is low in all types. One reason for this is the higher respiration rate at higher temperatures, with respiration expenditure being related to temperature. The WSC content of tropical grasses ranges from 50 to 90 g kg^{-1} DM, while that of temperate grasses ranges from 60 to 500 g kg^{-1} DM.

Studies indicate a twofold difference in the cell wall content between tropical and temperate species grown at the same temperature. However, when temperatures increased, the cell wall content of tropical grasses fell and that of temperate grasses increased. Tropical grasses and legumes have higher crude fiber (cellulose, hemicellulose, and lignin) content and hence lower digestibilities than temperate species (**Figures 1(b)** and **1(c)**). A survey of published results showed that the mean digestibility of temperate legumes and grasses was 61 and 68% DM, whereas that of tropical legumes and grasses was 57 and 55% DM, respectively. On average, this makes the tropical grasses 13% units less digestible than temperate grasses, and tropical legumes about 4% units less digestible than temperate legumes. Crude fiber content of tropical species also increased with age, although kikuyu, coastal Bermuda grass, and pangola grass showed less seasonal variation.

Figure 1 shows that there is a considerable range in these attributes with a substantial overlap between the values recorded for tropical and temperature grasses and legumes. In temperate species, digestibility and WSC content decline as ambient temperature increases. However, in tropical species, hotter environmental conditions have less effect.

Mineral concentrations are generally lower in tropical grasses and legumes than in temperate species (**Figure 1(d)**). Few temperate species, and only a small proportion of tropical grasses and legumes, contain less than the minimum requirement of P for growing stock, but for high-producing dairy cattle a greater proportion of tropical species fail to supply animal needs. Calcium concentration is higher in temperate than in tropical species. On the other hand, temperate species are more likely to create Mg deficiencies in lactating dairy cows than tropical species. Tropical grasses and legumes are likely to be deficient in sodium, affecting the K:Na ratio, which can result in reproductive problems, and high-producing dairy cows grazing tropical grasses are usually supplemented with Na. However, within both types of grasses, there are species that can be considered Na accumulators and those that will be deficient in Na. Cobalt also appears to be an element deficient in both tropical grasses and legumes, and supplementation has increased animal production even in growing stock. Mineral contents of some temperate and tropical species grown under subtropical conditions, along with National Research Council standard requirements for milking cows, are presented in **Table 1**.

Kikuyu was found to have lower levels of WSC, Na, Ca, Zn, and Mn, but higher levels of S and Fe than biennial ryegrass (*Lolium multiflorum*). Na and Zn in kikuyu were below the requirement levels for high-producing dairy cattle, meaning that cows grazing only kikuyu may need Na and Zn supplementation to reduce the problems related to milk production, reproduction, and disease.

Species and Cultivar Differences

Although the largest differences in quality are between the different groups of pasture plants (e.g., grasses and legumes, tropical and temperate species), there are still substantial differences between species and between cultivars within species. As dairy nutrition demands optimization of forage quality, the efficiency of forage utilization will depend on the choice of species and cultivars. Although the initial decision about which species to select is most probably made on how well the species is adapted to the farm environment, the right balance between quantity and quality will determine the efficiency of a dairy forage system. An example of this can be found in the choice of grass for dairy pastures in New Zealand. Though perennial ryegrass is accepted as the most productive grass for milk production, tall fescue has replaced it in harsher environments in the northern half of the North Island. Farmers then make choices on which of the fescue cultivars are best suited, based on quality and animal acceptance criteria. Similarly, in the subtropical

Table 1 Mean concentration of nutrients and minerals in three pastures grown under subtropical conditions, together with the requirement of a 600 kg Friesian cow producing 20 l milk day^{-1}

N (% DM)	*WSC (% DM)*	*Na (% DM)*	*Ca (% DM)*	*P (% DM)*	*K (% DM)*	*Mg (% DM)*	*S (% DM)*	*Cl (% DM)*	*Cu (mg kg^{-1})*	*Zn (mg kg^{-1})*	*Mn (mg kg^{-1})*	*Fe (mg kg^{-1})*
Requirement for a 600 kg cow												
2.3	NA	0.18	0.51	0.33	0.9	0.20	0.20	NA	10.0	40	40	50
Biennial ryegrass												
4.0	12.0	0.37	0.59	0.31	3.4	0.27	0.10	0.43	11.0	38	123	188
Perennial ryegrass–white clover												
3.8	8.9	0.47	0.70	0.30	3.0	0.31	0.06	1.23	13.6	37	119	386
Kikuyu												
3.2	5.9	0.10	0.42[a]	0.28	2.9	0.29	0.14	1.31	14.5	29	88	210

[a] High proportion bound to oxalate and therefore not available to the animal.

WSC, water-soluble carbohydrates; NA, not available.

Reproduced with permission from Fulkerson WJ, Slack K, Hennessy DW, and Hough GM (1998) Nutrients in ryegrass (*Lolium* spp.), white clover (*Trifolium repens*) and kikuyu (*Pennisetum clandestinum*) pastures in relation to season and stage of regrowth in a subtropical environment. *Australian Journal of Experimental Agriculture* 38: 227–240.

National Research Council (NRC) (2001) *Nutrient Requirements of Dairy Cattle*, 7th edn. Washington, DC: National Academy Press.

dairying areas of Australia, South Africa, and the Americas, the use of a combination of tropical species to provide warm-season forage and temperate species for cool-season production has optimized the forage base. Substantial gains have been made by selecting cultivars of perennial ryegrass, tall fescue, cocksfoot (orchardgrass), and *Bromus* species, which are adapted to such harsh (for temperate species) environments.

Many temperate species are grown in a marginal environment to provide cool-season forage for dairy cows. Differences in quality between temperate grass species are significant and can be increased by environmental conditions; for example, in the subtropics, grasses react differently to stress. Thus, though the quality of tall fescue is lower than that of perennial ryegrass, the differences become less significant in summer (**Table 2**).

Within species, cultivar differences can reflect differences in the origins of experimental material on which the cultivar is based. In tall fescue, for example, cultivars derived from Mediterranean-sourced material show superior winter vigor as compared to those derived from European-sourced germplasm. Selection for quality attributes has been successful and has made significant gains. The infusion of the brown midrib (*bmr*) gene has improved maize quality by reducing the lignin content, thereby improving digestibility. Animal production gains of up to 60% have been demonstrated between different cultivars of Bermuda grass and setaria by selecting for improved digestibility and leafiness. Research in Japan has demonstrated substantial increases in voluntary intake of cattle grazing crosses between tall fescue and biennial ryegrass (*Festulolium* spp.). Maturity date is another

Table 2 Quality of three species of temperate grass grown in a subtropical environment of Australia

	Lolium perenne cv. Yatsyn	*Festuca arundinacea cv. AU Triumph*	*Bromus willdenowii cv. Grasslands Matua*
Nitrogen content (%)			
Summer	3.10	3.10	2.30
Winter	3.88	3.49	3.84
In vitro *digestibility (%)*			
Summer	56.4	60.7	56.7
Winter	78.7	67.1	78.7
Acidic detergent fiber (%)			
Summer	32.1	32.3	41.6
Winter	24.9	27.8	26.8
Neutral detergent fiber (%)			
Summer	56.0	53.0	65.5
Winter	41.4	49.5	42.6

Adapted from Lowe KF, Bowdler TM, Casey ND, and Moss RJ (1999) Performance of temperate perennial pastures in the Australian subtropics 1. Yield, persistence and pasture quality. *Australian Journal of Experimental Agriculture* 39: 663–676.

characteristic that can influence the performance of cultivars. The digestibility of late-maturing cultivars of perennial ryegrass declines more slowly than that of early-maturing types, and this can be exploited in milk production systems. However, maturity characteristics must also be assessed in the context of forage production (i.e., whether the cultivars have their most active growing period in winter or spring) and persistence.

Figure 3 presents CP and digestibility data collected on a range of tall fescue cultivars in a subtropical environment. Though cultivar differences in the two attributes are substantial and there is a rapid falloff with the age of regrowth, there is no cultivar by age of foliage interaction. Differences in palatability and intake between tall fescue cultivars cannot easily be correlated with 'normal' quality measurements such as digestibility, neutral detergent fiber, acid detergent fiber, and metabolizable energy. Other physical attributes, such as leaf shear strength, number and size of silica denticles on the edges and surfaces of leaves, and concentrations and types of alkaloids present, may be important.

Cows grazing tetraploid perennial ryegrasses had 4.5% higher milk production than cows grazing diploid cultivars. Although the tetraploid cultivars did produce higher DM yields, the increased milk production was mainly attributed to differences in intake, a higher concentration of volatile fatty acids, lower rumen pH, and reduced leaf shear strength. Studies on annual ryegrasses have not shown the same conclusive result, and this may in part be due to the high moisture content of the tetraploid cultivars.

The presence and the type of fungal endophytes in tall fescue and perennial ryegrass plants have been implicated in differences in the animal production attributed to different cultivars. Although the alkaloids produced as a result of endophyte-infection appear to extend the range of adaptation of grasses and provide protection against insect pests, removal of detrimental alkaloids has

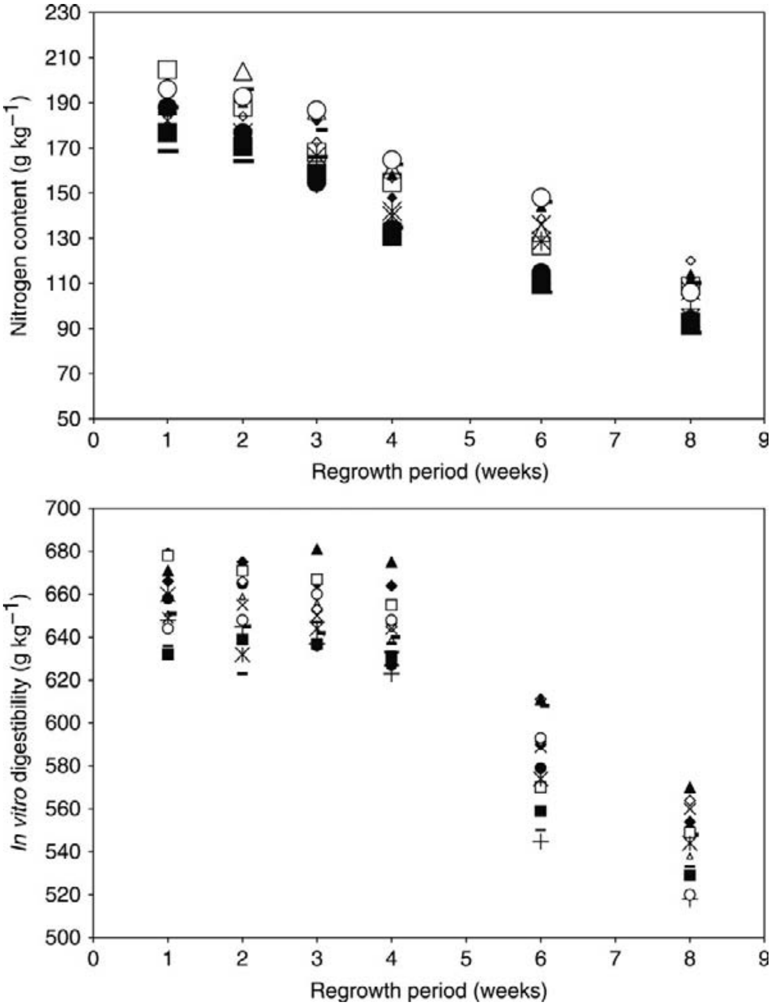

Figure 3 Nitrogen content and *in vitro* digestibility of 12 cultivars of *Festuca arundinacea* (Advance ◆, AU Triumph +, Bombina △, Cajan □, Dovey ●, Midwin -, Quantum ⁻, Torpedo ▲, Vulcan □, Georgia 5. ×, Jesup *, PWF 29 ○) in response to age of regrowth. From M. Callow and K. F. Lowe, unpublished data.

improved animal health, production, and heat tolerance. The presence of wild-type endophytes in a perennial ryegrass cultivar significantly reduced milk yield of dairy cows by 5 and 14% in spring and autumn, respectively. The loss of milk solids resulting from wild-type endophyte-infected perennial ryegrass as compared with that from the novel endophyte (AR1) was 9% in New Zealand.

It has been shown that cell, organic cell, high- and low-digestibility cell wall, organic cell wall, ash content, and digestible organic matter content varied between cocksfoot (orchardgrass), perennial ryegrass, and tall fescue, and between a range of cocksfoot cultivars. Within the temperate grasses, perennial ryegrass recorded the highest levels of WSC, cocksfoot the lowest, and tall fescue the intermediate. Perennial ryegrass cultivars have been shown to vary in WSC content, and this appears to be associated with palatability. The absolute value of WSC content is strongly influenced by the environment in which the swards are grown. WSC levels in a selection of perennial ryegrass cultivars grown in the subtropics were lower than those for the same cultivars grown in temperate environments although there was no cultivar by environment interaction. Tropical grasses showed less seasonal variation in NSC (**Figure 4**). Panicum had lower levels of alcohol-soluble carbohydrates than setaria, particularly over winter when the frost-resistant setaria retained more green leaf. On the other hand, hybrid ryegrass (*Lolium* × *boucheanum*) and prairie grass showed higher levels and a greater seasonal range.

Digestibility differences between cultivars have been demonstrated in many environments and in many species. For example, average digestibility ranged from 51.3 to 56.6% for 11 *Panicum maximum* cultivars and from 68.8 to 73.5% for 15 tall fescue cultivars. There was also a substantial digestibility range for sources of lucerne germplasm; it varied from 92.4 to 66.7% for leaf and stem of *Medicago falcata*, 92.8 and 72.1%, respectively, for *Medicago varia*, and 94.8–95.5% and 70.1–71.8%, respectively, for cultivars of *Medicago sativa.*

Cultivars can differ in their response to environmental conditions. Selection in *Setaria anceps* has provided increased frost resistance in the cultivar Narok as compared with the cultivars Nandi and Kazungula, which were selected for DM yields *per se.* As the importance to dairy production of available green leaf in tropical species cannot be overemphasized, the superiority of such a selection in early winter is obvious.

Breeding Aims for Species and Cultivars for Improved Nutrition

Improvement in the nutritional profile of temperate species is likely to be achieved by increasing the digestibility of the cell wall, changing the composition of the fiber content (e.g., reducing lignin), increasing the energy

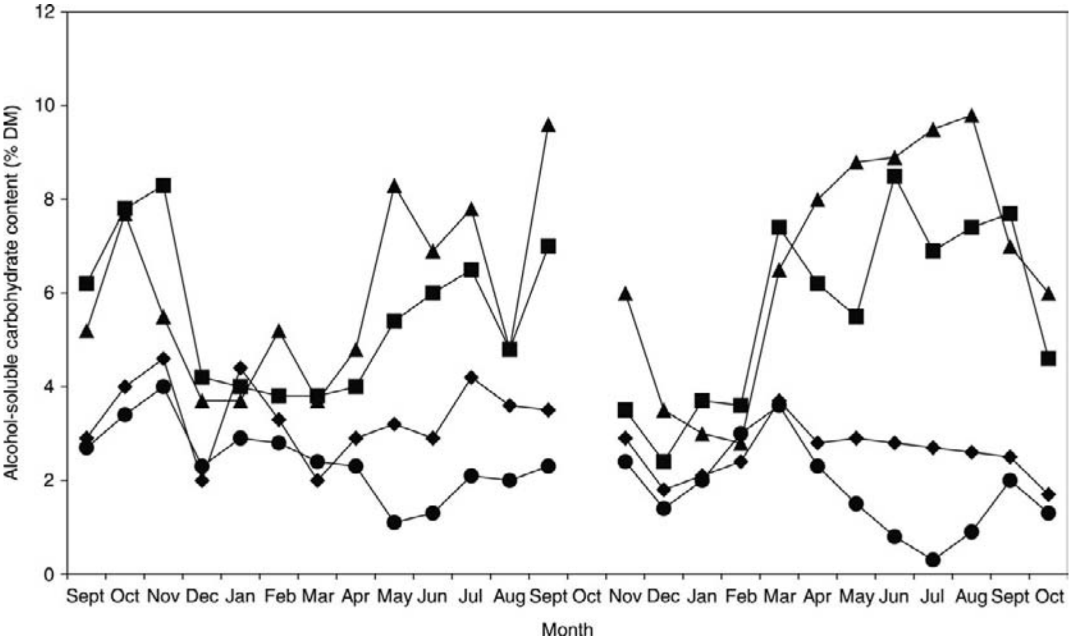

Figure 4 Levels of alcohol-soluble carbohydrates in foliage of *Setaria anceps* (●), *Panicum maximum* (◆), *Lolium* × *boucheanum* (▲), and *Bromus willdenowii* (■) defoliated monthly and grown under irrigation in the subtropics. Reproduced with permission from Noble A and Lowe KF (1974) Alcohol-soluble carbohydrates in various tropical and temperate pasture species. *Tropical Grasslands* 8: 179–187.

content (e.g., increased WSC, starch, and lipid contents), and improving the digestive characteristics in the rumen (e.g., protection of CP). Even though CP is usually not a limiting factor for temperate grass species, an improvement in CP content of tropical grasses should benefit animal nutrition. Selection of grasses for increased tannin levels may improve protein nutrition of the animal, while increases in tannin levels may also reduce bloat from clovers and lucerne. Continued search for cultivars with increased mineral content, for example, the development of high-Mg cultivars of *Lolium* spp., will assist in solving nutritional disorders. Improvement in legumes could best be achieved by selecting against levels of 'antiquality factors' rather than selecting for improved digestibility. Attention to such antiquality factors will benefit all forages (e.g., alkaloids and bloat in legumes, alkaloids, tannins, or oxalates in grasses, and tannins in herbs such as chicory and dock (*Rumex* spp.)).

Improving quality at the expense of agronomic adaptation can be counterproductive. Selection using nylon bag digestibility procedures resulted in the development of the Coastcross-1 hybrid, which successfully increased digestibility by 12% and increased dairy heifer growth rates. However, while selection for increased digestibility in finger grass (*Digitaria milanjiana*) and tall fescue developed cultivars capable of supporting higher milk production, these selections failed to persist under grazing. The improved milk production from finger grass was attributed to a higher leaf yield rather than increased leaf digestibility under grazing. It is in the area of increased leaf bulk density that the greatest gains in nutrition can be expected in breeding tropical grasses. Introduction of the *bmr* gene into annual grass crops such as maize, sorghum (*Sorghum bicolor*), sudangrass, and millet has successfully lowered the lignin content. Attempts to introduce this gene into kikuyu are in progress, but any large shift in leaf digestibility is yet to be achieved.

Improved CP content in lucerne proved to be a poorly heritable trait but, as in tropical grasses, selection for physiological characters that improved leaf content (i.e., leaf retention) could produce lucerne cultivars with higher CP levels.

Cultivar differences in leaf and stem mass and in total and reducing sugar content have been established in both chicory and plantain, and this suggests that breeding can further improve the potential of these herbaceous species.

See also: **Forages and Pastures:** Annual Forage and Pasture Crops – Species and Varieties; Grazing Management; Perennial Forage and Pasture Crops – Establishment and Maintenance.

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Perennial Forage and Pasture Crops – Establishment and Maintenance

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Introduction

In this article, the major components of pasture management are covered, including establishment of pastures, irrigation, fertilizer requirements and nutrient recycling, and mechanical removal of unwanted herbage. Other articles cover the issues of the pasture and forage species used for grazing dairy cows and the influence of management (and species and varieties) on forage quality (*see* **Forages and Pastures**: Perennial Forage and Pasture Crops – Species and Varieties) and the important aspect of grazing management (*see* **Forages and Pastures**: Grazing Management).

Pasture Establishment

The aim of preparing a seedbed for establishing a new pasture is to provide the best possible conditions for the seed to germinate and grow quickly into a productive sward with high plant density capable of suppressing weed growth. This can be achieved to varying levels through three intensities of seedbed preparation:

1. Full seedbed preparation includes plowing to bury the old sward, sometimes preceded by spraying with glyphosate. The seedbed should be fertile, fine, firm, weed-free, moist, and warm with the seed drilled at the optimal depth of 10–15 mm. Achieving this as part of a cropping phase increases the success rate and reduces overall costs. It is common to drill grass but broadcast clover, which, because of its small size, must be sown shallow. On most lighter soils, rolling to compact the seedbed is essential.
2. Direct drilling or broadcasting seed after killing the existing pasture with herbicide has the advantage of lower cost and does not leave the seedbed prone to erosion. However, whereas a complete plowing increases the mineralization of organic nitrogen (N), with direct drilling extra N must be applied to accommodate the decaying plant material. A triple-disc seeder is an excellent machine that cuts a groove, plants the seed, and then covers it. Broadcasting has the advantage of more even distribution of plants but is more affected by adverse climatic conditions immediately after sowing. In both cases, extra attention will need to be paid to control of pests (e.g., slugs), and slower establishing species risk establishment failure if conditions are not close to optimum.
3. Direct drilling or broadcasting seed into existing pasture without herbicide pretreatment is usually undertaken at the change of season (autumn or spring) and timing is critical. For example, annual ryegrass or white clover can be oversown into tropical grass pasture at about 5–6 weeks before the first frost is expected. The summer-growing grasses (kikuyu, paspalum) are set back long enough to allow establishment, by severe grazing, by mechanically slashing, or by taking a silage cut of the resident pasture. In this situation, the key to successful pasture establishment is to allow light to the establishing seedlings. The seed has a finite reserve of energy (carbohydrate), which is used to grow the initial shoot, and when this is exhausted the plant will die unless it can begin to photosynthesize. To prevent shading of the sown species by the resident species, the sward can be mob-grazed every 6–10 days to remove the foliage until the ryegrass dominates. Rapid grazing removes leaf (shade); once shade is removed, summer grass is set back at a time when it is still very palatable – far more plants are lost by shading than by treading by cows.

When establishing pasture, the seed rate varies but in general increases from colder to warmer climates (e.g., from 6–8 to 20–30 kg ha^{-1}, respectively, for pure ryegrass stands). The higher seed requirement in the warmer regions is to counter the higher plant loss and lower potential to tiller. Higher seeding rates are also used to obtain earlier feed and for better suppression of weeds. Seeding rate should be adjusted for seed quality (% germination) and seed size. For example, tetraploid grasses have larger seeds and need to be sown at somewhat higher rates than diploid cultivars.

The season of sowing is often governed by weed potential. For example, sowing temperate pastures in spring in the subtropics is not feasible in view of invasion of summer grasses in an open establishing sward at that time of the year.

Tillering of the sown grasses should be promoted after establishment as it determines the productive potential of the pasture. The critical factors that promote tillering are N and light. As a consequence, a newly established pasture should be grazed as soon as the young plants cannot be pulled out by stock to ensure exposure to sunlight at the base of the plant to stimulate tillering. N should be available from first tiller formation. Frequent but a moderate grazing intensity during establishment is required not only to promote the developing grass plants but also to ensure that any sown clovers are not shaded out by the faster establishing grasses, in particular ryegrass.

Seedling vigor varies considerably between species; generally, temperate grasses have vigorous seedlings and can handle oversowing well but the seed of tropical grasses is usually smaller and needs a full seedbed for reliable establishment. Within the temperate grasses, tall fescue has a less vigorous seedling than perennial ryegrass and can tolerate little competition during establishment; thus, the choice of seedbed preparation and postemergence grazing and weed control become more critical.

Grasses are generally less likely to be successfully sown into existing grassland than legumes because of problems with competition for space, light, and nutrients. Ryegrasses can be oversown into tropical swards but existing grass needs to be suppressed; seeding rates need to be high and N fertilizer must be applied. Tropical grasses are rarely successfully established in this manner.

Tropical legumes generally have larger seeds and more vigorous seedlings than temperate legumes such as white clover. They tolerate being oversown better than clovers, although reliability of establishment will be increased by a short-term reduction in the vigor of the existing vegetation (i.e., by herbicide or mechanical renovation).

Lucerne (alfalfa) can be sown in pure swards or in mixtures with tropical, temperate, or annual grasses or fodder crops. Sowing rates will be determined by the accompanying species or whether lucerne is being grown under irrigated or rain-grown conditions. Pre- and postemergent herbicides or cultural practices may be required to control weeds in establishing pure swards as contaminants reduce the quality if the stand is used for hay.

Fertilizer Application and Nutrient Recycling

Quantities of nutrients to apply to pastures as fertilizer are best predicted through assessment of current nutrient status of the soil, in combination with a calculation of total inputs and losses using a nutrient budget (**Table 1**). The meaningfulness of the results depends on the way the soil is sampled and how the results are used. Generally, 20–25 samples are taken per paddock or soil type with a corer to the required soil depth, which varies with country and species.

Table 1 Nutrient budgeting

Inputs	*Losses*
Supplements brought in	In products as milk or cull cows or calves
Fertilizer required	Transferred to laneways, cattle camps, dairy
	Runoff in heavy rain
	Volatilization
	Leaching

Nitrogen Fertilizer

The most important soil nutrient for plant growth is N. In clover and other leguminous plants, atmospheric N is fixed by the rhizobia within nodules on the roots. N fixation may account for 75–280 kg N ha^{-1} yr^{-1}. In grasses, the N must come from applied N fertilizer or mineralization of N from soil organic matter by soil microbes. Mineralization of N may account for 20–120 kg N ha^{-1} yr^{-1} in temperate regions. Providing aerobic conditions such as plowing or otherwise opening up the soil increases mineralization. On the other hand, there is denitrification under waterlogged soil conditions but little N mineralization, and this is reflected in the response to N applied. Thus, in a study carried out in a warm subtropical region of Australia, the response was 10 kg dry matter (DM) kg^{-1} N when ryegrass was sown after an extremely wet summer in 1993 (indicating low native soil N status) but the response was only 3.8 kg after a dry summer in 1994 (**Figure 1**).

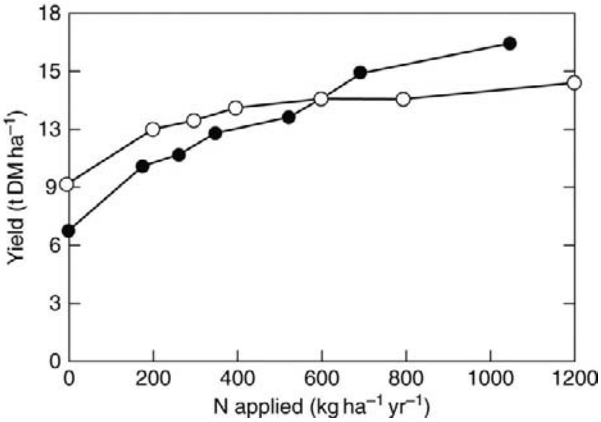

Figure 1 The annual yield (t DM ha^{-1}) at rates of 0–1200 kg N ha^{-1} yr^{-1} for annual ryegrass pastures sown in autumn following wet (○) and dry (●) summer conditions. From KF Lowe *et al.*, unpublished data.

In contrast to plowing, herbicide application and direct drilling lead to a N-deficient situation, as N is tied up by microbes decomposing the abundant organic matter.

Most pasture crops absorb N primarily in the nitrate (NO_3) form from the soil but can also take up nitrite (NO_2), ammonium (NH_4^+), and even N in organic form, such as in amino acids. The N taken up combines with carbohydrates to form protein:

$$\begin{array}{l} \{\quad NO_3 \quad\} \\ \{\quad NO_2 \quad\} + \text{carbohydrates} \rightarrow \text{proteins} \\ \{\quad NO_4^+ \quad\} \\ \{\text{amino acids}\} \end{array}$$

Nitrate is the most common form of N in the soil and its levels are higher and more variable than the ammonia ion.

Grasses generally show a linear response to N at low to moderate rates of N fertilizer application but the response curve flattens at higher rates as other factors (moisture, temperature, the plant itself) become limiting (**Figure 1**). For example, soil moisture and the availability of other elements (e.g., phosphorous (P) and potassium (K)) influence the response of grasses to N. Water is required to carry the NO_3 to the roots of the plant; moisture stress is often seen as N deficiency. A response rate of 15–30 kg DM kg^{-1} N applied appears to be common with temperate or tropical grasses when few other inputs are limiting.

The application of N fertilizer can markedly change pasture composition by favoring the most N-responsive species. The most obvious example is in a grass–clover pasture. Clover responds well to applied N fertilizer and shuts down its nodulation system as it is energetically more efficient to use applied N. However, the grass responds better to N and the clover cannot compete, unless grazing is frequent and hard, in which case total yields are likely to be reduced. A balance between these two species can be kept by applying N strategically, that is, only under conditions of very low clover growth (low winter temperatures) or when the grass component shows signs of N deficiency.

For tropical grass species, kikuyu is more responsive to high rates of N than paspalum. Milk production is more influenced by N applied than by species. In a subtropical environment, there were no differences in annual milk production between Callide rhodes, Pioneers rhodes, and a naturalized pasture, all fertilized with the same rate of N. However, seasonal differences were evident.

The interaction between N application and grazing interval is important. The imposition of grazing stops root growth and reduces demand for N by the plant, providing the conditions for greater loss of N to the environment by leaching and volatilization. The appropriate application interval depends on pasture growth and may be as frequently as 4 weeks in spring (or every two grazings) for temperate grasses or in summer for tropical grasses, but it could be at 6- to 8-week intervals in the colder season. Application of N at the beginning or in a period of declining or low grass growth can lead to excess accumulation of N in the plant and high losses from the soil – this is because N is taken up in luxury amounts by the plant – in situations where availability is high and/or requirements are low. Excess N in the plant also leads to metabolic problems in stock grazing such pasture when levels of NO_3 accumulate above 500 mg kg^{-1}. Although the obvious symptoms in stock of excess N in pasture are nitrate poisoning and death, excess NO_3 is converted to NO_2 in the rumen and this is toxic to the microbes and can lead to reduced breakdown of the forage component of the ration. In Australia, studies have shown that application of N, as urea, at a rate of 50 kg ha^{-1} per month is the most efficient rate in terms of N usage (44% recovery in pasture utilized) and yield response for annual ryegrass.

The most common N fertilizers used are ammonium nitrate (32–35% N), calcium ammonium nitrate (21–26% N), and urea (45% N). The loss of ammonia through volatilization when applying urea has been found to be only 14% under the worst conditions experienced in temperate regions (short pasture, heavy dew, wind in summer), although it could be higher in warmer subtropical regions. In Europe, there are strict controls on the use of N fertilizer in order to reduce N contamination of groundwater and a maximum of 150–200 kg N ha^{-1} yr^{-1} as mineral fertilizer has been recommended under grazing conditions. In New Zealand, the emphasis is on limits on the allowable amount of N leached from the land and not on the inputs of fertilizer N, stocking rate, stock type, or farm system/enterprise.

Gaseous ammonia dissolved in water can also be injected into the soil as a N fertilizer to reduce N losses but there are logistic difficulties in injecting liquid ammonia into established pastures.

P, K, S, and Mg Fertilizers

Phosphorus

Although measured available soil P status is a useful indicator of potential availability, it is more meaningful to look at trends in soil nutrient status with time and in relation to a fertilizer program based on replacing nutrients removed.

Removal of P and K can be calculated from nutrient budgeting, as shown in **Table 1**. Thus, the use of trends in soil P (and K) status and calculation of nutrients removed from a given area seem to be the most meaningful way to decide the fertilizer application rate. When the initial soil nutrient status is low, it may be necessary to add high levels of P rapidly (capital dressing).

When the soil is deficient in P, plant growth rates are low and leaves are small and tend to be bluish-green to purplish in the younger leaves. Although P concentrations in the plant reflect soil status, it is seldom economic to provide P to stock by increasing P fertilizer application above plant requirements.

In general, tropical grass pastures are less responsive to P than temperate pastures and this may be because they have a greater root volume. The clover component of pasture is usually more responsive to P than the grass and the response of the grass is often a consequence of the increase in N available from increased clover growth.

There are a number of P fertilizer sources available:

1. Single superphosphate (9% P, 11% sulfur (S), 20% calcium (Ca)).
2. Triple superphosphate (21% P; also contains ≈15% Ca and <1% S).
3. Rock phosphate (12% P, 1% S, 30% Ca), but the P is not water soluble and is released more slowly and as a result is more appropriate for acid, rapidly leaching soils in high-rainfall areas.
4. Diammonium phosphate is also a slow-releasing source of both N and P (18% N, 20% P, 1.6% S), but there is no Ca.

Potassium

The foliage of grass should contain over 2.5% K if this element is not limiting pasture growth. Unfortunately, the K requirements of dairy cattle are well below this (at <1% DM) and excess levels can cause metabolic problems.

Pastures deficient in K lack vigor and older leaves turn light in color early (at 2–2½ leaves/tiller stage of regrowth), as available K is used for the growth of new leaves when K is transferred from older leaves. Root growth is also restricted and a lowered leaf transpiration rate brings on early signs of wilting. K deficiency is most likely to be seen in spring, when all other inputs are optimal, as browning or burning along the leaf margins of clover.

Potassium fertilizers are usually available as

1. Muriate of potash (46% K).
2. Potassium sulfate (41% K, 18% S).

Sulfur

The N:S ratio in grass is a good indicator of S adequacy in the plant and the ratio should be below 13:1. Sulfur deficiency is seen as reduced growth with yellow and small leaves.

Sulfur fertilizers are available as

1. Ammonium sulfate (24% S) – the most appropriate form of S for clay soils but it does acidify the soil and more lime is needed to ameliorate the effects.
2. Single superphosphate (11% S).
3. Gypsum (15–18% S) – more appropriate for light-textured soils and sands.

Magnesium

In Mg deficiency, the older leaves are yellow, as scarce Mg is used for the growth of young leaves. There is competition between K and Mg for uptake by plants and this is important in problems with grass tetany in cattle. Most cases of grass tetany (Mg deficiency) are seen in stock grazing young succulent grass in early spring.

Magnesium limestone (or dolomite; 12% Mg) is the favored source of Mg but another source in New Zealand is products with serpentine, for example, potash serpentine super (4.7% Mg).

Nutrient Recycling – Impact of Management

Fertilizer input is only one, and in some ways a small part, of the soil nutrients made available to pasture plants.

Other sources include (1) mineralization of N from organic matter in the soil, (2) fixing of N_2 in the atmosphere by leguminous plants, and (3) weathering of parent material. However, probably the most important influence on soil nutrient status is nutrient recycling through dung and urine. It is estimated that 70–80% of N, P, and Ca and 80–90% of K, Mg, and other minerals ingested by cows is returned to the soil through dung and urine. The concentration of N is extremely high in a urine patch ($800\,\mathrm{kg\,N\,ha^{-1}}$) and as a consequence there is considerable loss (>50%) through leaching and volatilization. Management in general, and grazing management in particular, can have a marked effect on the evenness of nutrient distribution.

Night paddocks: In general, the ratio of eating to defecation activity is higher during the day if daytime temperatures remain below 27–28 °C. In temperate regions, the daytime intake (between a.m. and p.m. milking) is about 60% of the total daily intake. This means that night paddocks become relatively more fertile and day paddocks become less fertile unless countered by differential fertilizer input, by alternating day and night paddocks between seasons, or by cropping night paddocks.

Paddock size: The larger the paddock, the more the nutrients concentrated at gateways and water points, and fewer and larger the cattle camps; this is another argument for rotational grazing.

Milking time: The nutrients transferred off the paddocks to laneways and yards are directly related to time spent away from the paddock. Nutrients from the yards can be returned to paddocks by spray irrigation or as slurry, if effluent is captured and stored for this purpose.

Strip grazing: If cows are intensively strip-grazed for 1–2 h and then transferred to a standoff area, there can be nearly 100% nutrient transfer off the stripped area as dung and urine.

Removal in silage or hay: The nutrients removed by hay and silage can be substantial. As an example, consider $2 \times 4\,\text{t DM ha}^{-1}$ cuts $= 8\,\text{t DM ha}^{-1}$.

Potassium (K)

$$\text{K concentration in ryegrass} = 3.5\%\ \text{DM}$$

$$\therefore\ 0.035 \times 8000\,\text{kg} = 280\,\text{kg K or}$$

$$\frac{100}{46\text{–}50\%\ \text{K}} \times 280 = 609\,\text{kg muriate of potash ha}^{-1}$$

Phosphorus (P)

$$\text{P concentration in ryegrass} = 0.30\%\ \text{DM}$$

$$\therefore\ 0.003 \times 8000\,\text{kg} = 24\,\text{kg P or}$$

$$\frac{100}{9\%\ \text{P}} \times 24 = 267\,\text{kg single superphosphate ha}^{-1}$$

Nitrogen (N)

$$\text{N concentration in ryegrass} = 3\%\ \text{DM}$$

$$\therefore\ 0.03 \times 8000\,\text{kg} = 240\,\text{kg N or}$$

$$\frac{100}{46\text{–}48\%\ \text{N}} \times 240 = 500\,\text{kg urea ha}^{-1}$$

Mechanical Removal of Unwanted Herbage

Mechanical removal of unwanted pasture growth is variously named and relates to function.

Topping

Topping, as the name implies, is removing the ‘tops’ of temperate pastures to about 10–12 cm stubble height to remove seed heads in late spring and encourage initiation of vegetative tillers and improve forage quality. This is taken a step further in New Zealand where pastures are topped before seed set. The reproductive tillers of ryegrass accumulate substantial amounts of water-soluble carbohydrates (WSCs) in their bases to ‘export’ to the forming seed. Removing the developing seed head makes the WSC reserves available for initiation and growth of new vegetative tillers. In tropical grasses, topping is done in late summer to increase leaf production and delay seeding. Although the benefits of this have been shown experimentally, they have not been proven to be consistent under farm conditions.

Bottoming

Bottoming is generally undertaken in autumn to remove dead and senescing material so that light can penetrate to the bottom of the canopy and stimulate tillering. Bottoming is usually severe: to 4–6 cm stubble height.

Mulching or Slashing

Mulchers are similar in design to rotary hoes but the ‘shoes’ are sharp and they cut and pulverize the sward down to ground level if required. The advantage of mulchers over slashers is that they do not leave windrows, which often shade and kill plants. On the other hand, mulchers require substantial tractor power (>80 hp for a 1.6 m cut). Mulchers are commonly used to control excess growth of C_4 summer grasses such as kikuyu before oversowing with ryegrass.

Mechanical removal of excess growth of temperate grasses should be minimized as it is costly in terms of labor, machinery, and fuel and probably indicates inappropriate grazing management or inadequate stocking rate. If mowing or slashing is contemplated, it needs to be completed no later than 48 h after the commencement of grazing; otherwise, the regrown shoots and young tillers will be cut and more harm than good will be done. Slashing of tropical grasses rarely improves milk production as it reduces feed on offer and the animals’ ability to select a high-quality diet.

Mowing Pregrazing

Mowing pregrazing is sometimes undertaken to encourage animals to eat more pasture when pasture is in excess and it is also used to reduce bloat. This will increase intake, as the animals do not need to graze and the DM content may have increased to over 30% if left overnight. However, it also removes the ability of the animals to select against inferior species and against lower quality stem and therefore should only be undertaken with good leafy pasture.

Irrigation

Although irrigated pastures are important for grazing dairy cows in many parts of the world, irrigation practices lag behind the more sophisticated methods used, for example, in the horticultural industries, to schedule irrigation and ensure high water-use efficiency. This is surprising, in view of the relatively low potential water-use efficiency of pastures compared to horticulture and various cash and/or fodder crops.

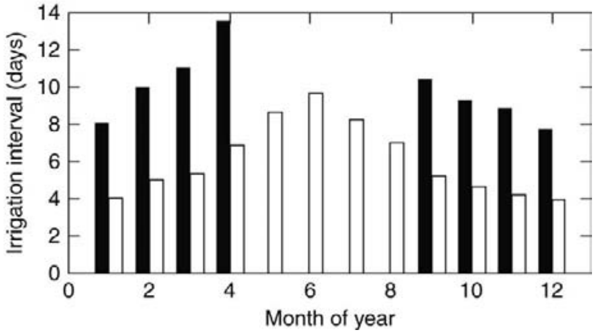

Figure 2 The recommended irrigation intervals (days) for perennial ryegrass (□) and kikuyu (■) growing on a clay soil in a subtropical climate. Reproduced with permission from Fulkerson WJ, Lowe KF, and Launders T (1993) Northern dairy feedbase 2001. 3. Winter pastures and crops. *Tropical Grasslands* 27: 162–179.

Irrigation interval and rate are dependent on the effective root depth or available soil water volume of the pasture, and the evapotranspiration rate. In this way, shallow-rooted plants, such as ryegrass (**Figure 2**), require short irrigation intervals with low application rates while deep-rooted plants, such as lucerne, tropical grasses, and many weeds, can tolerate longer watering intervals at higher rates. In fact, the composition of a pasture can be changed by watering frequency. For example, in a ryegrass–lucerne sward, 3-week watering intervals favor lucerne, whereas weekly watering would shift the pasture to grass dominance.

The recommended watering rates for various pasture species are shown in **Table 2**.

Thus, with the knowledge of the effective rooting depth of a particular species, and average evapotranspiration rates, an irrigation schedule can be developed for a given situation. For example, for ryegrass in a subtropical environment, the optimum irrigation interval in winter would be 22 days, whereas in late spring it would be closer to 4 days.

Note that the differences in irrigation interval between kikuyu, a deep-rooted C_4 grass, and ryegrass, a shallow-rooted grass, in the same environment can be dramatic for the same soil type. These are predicted requirements or a 'water budget', but there is large day-to-day variation in evapotranspiration rate and various devices (**Table 3**) are used to assist in monitoring soil moisture.

Table 2 Recommended irrigation application rates (mm) for various pasture species on three soil types

	Maximum water applied/irrigation (mm)		
Species	*Sandy loam*	*Loam*	*Heavy clay loam*
Clover	10–15	10–25	10–20
Ryegrass	15–25	20–40	20–35
Kikuyu	25–40	25–40	25–45
Oats	15–30	25–50	25–40
Lucerne (alfalfa)	15–30	25–40	25–40[a]

[a] Recommended for moderately heavy clays (<75% clay content) only.

The use of weather data would be the most common tool used on dairy farms to schedule irrigation – it is simple, cheap, and relatively accurate. On larger operations, irrigation is in relation to available soil moisture measured by capacitance meters such as the Gopher or Enviroscan.

There are many irrigation systems used on dairy farms – all have advantages and drawbacks and they are outlined below.

Hand Move

The whole sprayline is disconnected in sections and shifted to a new location (pressure: 200–300 kPa; labor: 1.6 h ha^{-1}, maximum 25 ha person^{-1}; capital cost: A\$1800 ha^{-1}; operating cost: A\$600 ha^{-1}).

Side Role

The sprayline is shifted by rolling along intact wheels. These lines can be up to 200 m long. This system is best suited for flat rectangular paddocks, free of obstacles (pressure: 200–300 kPa; labor: 0.5 h ha^{-1}, maximum 25 ha person^{-1}; capital cost: A\$3200 ha^{-1}; operating cost: A\$750 ha^{-1}).

Bike Shift or Long Lateral

A portable sprinkler on a low-density polypipe is shifted to 9 or 12 locations around a central turf valve covering an area of 0.4 ha. Benefits include minimum effect of wind, low pump costs, suitability for fertigation (liquid fertilizer application), and the ability to water odd-shaped and hilly paddocks (pressure: 200–300 kPa; labor: 0.2 h ha^{-1}, maximum 40 ha person^{-1}; capital cost: A\$1800 ha^{-1}; operating cost: A\$450 ha^{-1}).

Traveling Irrigators

Travelers are propelled by the water moving through the irrigators. They are suitable for irrigating crops but are not suitable for hill country (pressure: 400–600 kPa; labor: 0.5 h ha^{-1}, maximum 25 ha person^{-1}; capital cost: A\$3000 ha^{-1}; operating cost: A\$800 ha^{-1}).

Center Pivot

The center pivot consists of a single sprinkler lateral that rotates around a central water inflow pivot point. The speed of rotation can be varied – maximum lateral length is 600 m (113 ha) (pressure: 200–300 kPa; labor: low,

Table 3 Commonly used devices to determine the soil moisture status

Device	*Approximate cost*[a] *(A$)*	*Measure*	*Labor input*[b]	*Accuracy*[b]	*Continuous log*	*Comment*
Thin rod, shovel, or auger	0–30	Hand feel	7	1	No	Simple, penetrates to depth of wetting
Daily evaporation	Nil	Weather	7	2	No	Simple, lots of estimation?
Tensiometer	100	Soil tension	7	4	No	High maintenance
Gypsum block	750	Soil capacitance	2–7	4	No	Needs frequent reading
Gopher	2200	Soil capacitance	6	5	No	Simple to use; problem with cracking clays
Soil moisture probe	1500	Soil capacitance	4	5	Yes	Three depths; easy to install
Enviroscan	12 500	Soil capacitance	1	7	Yes	Multisite and multidepth. Problem with cracking clays
Neutron probe	12 500	Soil neutrons	7	7	No	Need license to use
Intelligent irrigation	12 500	Heat probe	2	4	Yes	Use with automatic irrigation systems

[a] These costs are in Australian dollars at the time of publishing and are only meant to be indicative of relative costs.

[b] Score 1, very low; 7, very high.

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maximum 60 ha person^{-1}; capital cost: A$3100 ha^{-1}; operating cost: A$680 ha^{-1}).

Linear Moves

This is as per center pivot but the laterals are propelled forward allowing watering of a rectangular area. Spraylines are generally 800 m long and draw water from a flexible hose or a water channel (pressure: low; labor: 0.01 h ha^{-1}, maximum 200 ha person^{-1}; capital cost: A$1650 ha^{-1}; operating cost: A$420 ha^{-1}).

Set Sprinklers

Set sprinklers are permanently set to water a fixed area (pressure: low – any area; labor: near zero; capital cost: A$3100 ha^{-1}; operating cost: A$680 ha^{-1}).

Subterranean Lines

Flexible drip lines are buried just below the root and plowing depth at 1–3 m spacings, depending on the soil and crops to be grown. The advantages are as for set sprinklers but in addition there is a reduction in evaporative loss (pressure: low; labor: near zero; capital cost: A$3100 ha^{-1}; operating cost: A$680 ha^{-1}).

General

Of the above systems, the most commonly used system on smaller properties is travelers, with bike shift and center pivot being increasingly used for larger operations.

Management of Pure Legume Swards

Lucerne is the most likely perennial legume to be sown in a pure sward and required to stay that way for best results. It is one of the most versatile pastures available to the dairy farmer; it can be grazed or cut for hay or a combination of both can be used.

The best way to maintain a weed-free sward of lucerne is to start with a clean seedbed and to reduce the potential weed seed crop by adequate cultural treatment before sowing. Preemergent herbicides are available to assist this process. A well-drained soil with a deep profile and the right choice of cultivar will assist in maintaining a good, dense stand. Disease-resistant and grazing-tolerant cultivars will minimize stand losses but management will be the most important factor in maintaining stand longevity.

Highly winter-active cultivars suit the dairy situation best by providing more winter grazing, particularly in warmer climates. However, their high crowns make them more susceptible to damage from grazing. If long stand survival is desired (i.e., more than 3 years), then semidormant cultivars are the most appropriate. Postemergent grass control will ensure that stands remain clean and productive but it is unlikely to be justified on dairy farms. Leaving a stubble of over 5 cm and the crown shoots ungrazed will achieve the best persistence, irrespective of the activity level of the cultivar. Lucerne requires rotational grazing, although a rigid fixed schedule is not essential.

See also: **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties; Grazing Management; Perennial Forage and Pasture Crops – Species and Varieties. **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows.

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Grazing Management

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The Use of Pasture and Forage Crops Worldwide

There is great variation worldwide in the extent to which dairy cows are grazed on pasture or forage crops. The two extremes are New Zealand and North America.

New Zealand is blessed with an even and reliable rainfall in a relatively mild environment, ideal for the growth of high-quality temperate pastures such as perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). In order to maximize pasture utilization, average stocking rates are high (>2.4 cows ha^{-1}), and consequently, production per cow is low (~4000 l milk per cow). Thus the axiom is: 'In New Zealand, production criteria are measured in terms of milk solids per hectare'.

In North America, cows are invariably confined to feedlots and fed total mixed rations (sometimes called zero grazing). Consequently, production based on maize silage and lucerne (alfalfa) hay is high (>7000 l milk per cow). However, there is some interest in grazing cows in order to reduce unit costs of production, primarily in the south where winters are milder. Nonetheless, the axiom is: 'In North America, production criteria are measured in terms of milk per cow'.

In Australia, South Africa, and South America, and to a lesser extent in Europe, where the climate is harsher and less reliable than in New Zealand, supplements are brought in to fill feed gaps at times of low pasture growth. As a consequence, the production criteria are a compromise between production per cow and production per hectare.

Grazing Management Should Be a Win–Win Situation for Both Pasture and Animals

Many dairy farmers simply look at pastures as a feed source for high-producing cows. However, in their natural state, pasture plants provide vegetative growth for grazing herbivores only to entice them to proliferate the species – seeds are spread on the coats of grazing animals or end up in dung pats after ingestion and excretion by stock. As a consequence, native grasses have short vegetative periods and seed prolifically. Man has selected in favor of vegetative top growth over root and reproductive growth. To exploit the high potential production of modern pasture cultivars, the environment often has to be modified by management of soil fertility, irrigation, and grazing.

Grazing is one management factor that can have a major impact on growth and survival of the pasture plant, as well as its utilization by stock, with the impact being dependent on the following:

- intensity of grazing (reflected in stubble height post-grazing) (stock per unit area)
- grazing interval (or rotation interval – period between one grazing and the next) (days)
- grazing duration (or period of occupation – period of grazing continuously on an area of pasture) (days)

There are many systems of grazing with set stocking (or continuous stocking or grazing) at one extreme and various degrees of controlled (or intermittent) grazing, commonly known as 'rotational grazing', at the other.

Grazing Management for C_3 Temperate Pastures

Set or Continuous Stocking

Set stocking is, in fact, not continuous grazing but intermittent grazing at the plant level, with grazing interval and intensity being determined by the grazing animal through stocking rate. In this instance, duration of grazing is not relevant. For example, studies in the United Kingdom have found that the interval between individual ryegrass tillers being grazed was 19–36 days at a stocking rate of 1000 kg liveweight ha^{-1}, but this shortened to 6 days when the stocking rate was increased to 3000 kg liveweight ha^{-1}.

At the plant level, bite size has been found to be fairly constant at 40% of profile height – until the animal reaches the maximum capacity of a bite (which probably varies with animal size). As there is only a small variation in grazing time (h day^{-1}), there must be a sward height at which pasture intake is maximized on a pasture with a given tiller density. This has been found to be the case, and a sward height of 5–8 cm is recommended for dairy cows grazing ryegrass pastures in temperate dairy regions. This is also the range within which net dry matter (DM) accumulation is maximized, coinciding with the maximum average growth rates.

The use of a logical criterion for determining optimum stocking rate, such as sward height, overcomes a major drawback of set stocking by giving the capacity to increase (take out supplements, reduce area, close for silage) or decrease (put in supplements) pasture availability or change effective stocking rate in a calculated way. In practice, very few dairy farmers set stock in New Zealand, Australia, or South Africa, but a proportion do in the United Kingdom.

There are some good practical reasons why set stocking may not be attractive to dairy farmers:

1. Selection for the most palatable, desirable species in a pasture can be extreme with set stocking, leading to the opportunity for undesirable grasses and weeds to proliferate and dominate.
2. The inconvenience of finding and moving, say, 200 cows in a 140 ha paddock is obvious.
3. The larger the paddock the more uneven is the distribution of nutrients in dung and urine, concentrating nutrients at gateways, water points, and cattle camps.
4. Damage to soil structure occurs if stock graze areas being irrigated or that have recently been irrigated.
5. Health problems can arise if cows graze pasture recently fertilized with N. However, in recent studies beef cattle set-stocked on pasture fertilized with up to 990 kg N ha^{-1} have exhibited no health problems. There may be a preference against pasture recently fertilized with N where NO_3 levels would be expected to be high, and this has been shown in recent studies.
6. Animals select the youngest leaf, which invariably has the highest concentrations of K and N, with both normally well in excess of cow's requirements, and this can cause metabolic problems (milk fever, grass tetany, nitrate poisoning).
7. Pasture cannot be rationed accurately and intake can fluctuate to extremes as growth rates change due to factors such as frost or moisture deficit, unless the pasture available can be altered by changing stock numbers or pasture area available. This cannot always be achieved in practice.

Controlled or Intermittent Grazing

Controlled grazing involves moving cows to new pasture at intermittent intervals – the intervals, intensity, and duration of grazing are controlled by man rather than by the animal, as is the case with set stocking. Controlled or intermittent grazing is commonly known as rotational grazing but goes by many other names, including cell, block, or short-duration grazing or mob stocking. However, the criteria on which movement of stock and stock numbers are based vary considerably, and this means that comparisons between different rotational grazing systems, and between rotational and set-stocking systems are often not valid.

A definition of intermittent grazing has been given in the following terms: "Rotational grazing rations pasture to meet the needs of the animal (as feed is rationed in a *confinement* system) while protecting the plant from overgrazing." By inference, with controlled grazing, there is an assumption that the outcome will lead to better pasture growth and persistence and higher pasture utilization by stock, and that forage will be of higher quality than if the animal determines its own grazing options as in a set-stocked situation.

The criteria used to determine timing of grazing within a controlled grazing system are various:

1. *Set days.* This is probably the most common criterion used, with the farm permanently subdivided into a number of paddocks – usually 20–35. This criterion does not consider the needs of the pasture or the animals in terms of changes in growth rate, or rate of leaf maturation and hence forage quality.
2. *Leaf area index (LAI).* Leaf area index is the ratio of leaf area to the corresponding ground area, and it has been shown, for example, that the LAI at the maximum growth of a perennial ryegrass/white clover pasture is between 2 and 4. Below this there is inadequate leaf area to capture all the incoming solar radiation, and above this, growth slows and net accumulation tends toward zero. The two issues with LAI are (1) it is not practical to measure LAI on-farm, although it is a useful research tool; and (2) an LAI of 2–3 may maximize pasture growth, but this may be beyond the period when average growth rates are maximized and after the oldest leaves have begun to senesce and decline in forage quality.
3. *Pasture height.* Sward height can be estimated visually or with the aid of a sward stick. It is more appropriate than set days, as it is related to the DM-on-offer (animal factor) and pasture growth (plant factor), but does not consider plant (tiller) density.
4. *DM-on-offer.* This attribute is usually estimated using a rising plate meter (measures the height to which a plate of known weight per unit area (4 kg m^{-2}) is held up by the pasture) or a pasture probe (measures the capacitance as related to leaf DM), or visually. The value of visual estimation of nominated fixed areas from which DM yield is later determined is that it considers plant density and reflects pasture growth and feed availability. Recommendations are often based on pasture mass with a maximum pregrazing mass of <2600 kg DM ha^{-1} (above which cows tend to waste ryegrass pasture) and a minimum postgrazing mass of 1500 kg DM ha^{-1} (which falls to 1000 kg DM ha^{-1} as ryegrass pasture density declines in warmer climates (discussed later)).

5. *Leaf growth and maturity.* With this criterion, leaf number per tiller is used to indicate leaf maturity, with the knowledge that the lifespan of an individual leaf is related in a constant way within species to leaf number. In ryegrass this is equivalent to the time taken to grow 3 new leaves per tiller, and for prairie grass (*Bromus willdenowii*) and cocksfoot or orchard grass (*Dactylis glomerata*) it is about 5 leaves per tiller. The use of this criterion is based on two key considerations: (1) *minimum grazing interval* – the time required to replenish water-soluble carbohydrate (WSC) reserves (which coincides with the growth of more than 2 leaves per tiller); and (2) *maximum grazing interval* – onset of senescence of the oldest leaf after 3 new leaves per tiller have grown, with a commensurate fall in forage quality and wastage as leaves die.

Water-Soluble Carbohydrates

WSCs or nonstructural carbohydrates are the first products of photosynthesis in plants, and these are the labile energy source used for the processes associated with growth and maintenance, with any amount not immediately used being stored as plant reserves. As a consequence, the balance between photosynthesis (source), and growth and respiration (sinks) determines the concentration of WSC reserves, mainly in the lower stems of the plant (discussed later). Similarly, in lucerne the timing of defoliation is determined by the initiation of the crown buds. Grazing should occur before the buds grow above grazing height (or cutting height in the case of hay production) and this coincides with less than 10% flowering. At this time, carbohydrate reserves have been replenished in the taproot of the lucerne plant rather than in the lower stem as in grass. Research has shown that this optimizes both yield and plant persistence. Like the leaf number method in ryegrass, the use of bud initiation and flowering reflects the level of replenishment of WSC reserves in the plant, and is an in-field practical criterion to flag the most appropriate time to graze or harvest lucerne.

The stored WSC reserves in the tiller bases of grasses are used for plant recovery after grazing as shown in **Figure 1**.

Stage 1: 1–3 days after commencement of grazing

In a well-grazed ryegrass pasture, most or all of the leaves are removed and only the tiller bases (stubble), containing carbohydrate (WSC) reserves, remain. Immediately after grazing, the roots stop growing. The youngest leaf, which was expanding prior to grazing, must continue to extend in order to catch sunlight and produce its own WSCs through photosynthesis. This is the highest priority; otherwise, respiration will use all the reserves and the plant will die. In the meantime, the plant relies on WSC reserves in the stubble for growth and respiration. If this new shoot is removed by stock, as a result of a long grazing duration (or occupation time), regrowth is substantially reduced because there is little WSC left to initiate more regrowth. In practice, if animals are able to graze regrowth shoots, duration of grazing is too long – as a rule of thumb, duration of grazing should be restricted to 2 days.

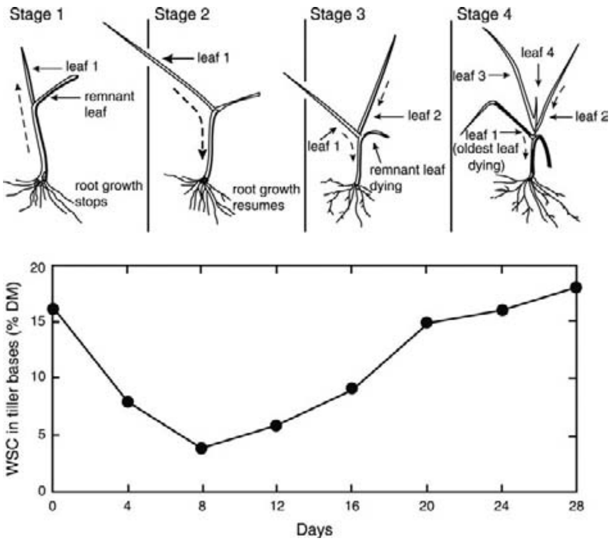

Figure 1 Changes in water-soluble carbohydrates (WSCs) (%DM) with regrowth time (days and number of leaves per tiller) in relation to initiation and expansion of new leaves. Movement of WSCs is shown as –→. Adapted from Fulkerson WJ and Donaghy DJ (2001) Plant soluble carbohydrate reserves and senescence: Key criteria for developing an effective grazing management system for ryegrass-based pasture: A review. *Australian Journal of Experimental Agriculture* 41: 261–275.

Stage 2: Plants are most vulnerable to grazing

When the ryegrass tiller has about three-fourths of a new leaf, it is producing enough WSCs to meet the requirements for growth and respiration, and replenishment of WSC reserves begins. As a consequence, the roots also begin to grow again. At this stage, the plant is most vulnerable to regrazing as it has very low levels of WSC reserves to again regrow.

Stage 3: Minimum grazing interval

At 2 new leaves per tiller, the WSC reserves are adequately replenished for the plant to cope with being grazed again, and subsequent regrowth will not be affected.

In the glasshouse experiment shown in **Figure 2**, regrowth was not affected if plants were previously defoliated at a 2 leaves-per-tiller interval, provided the subsequent defoliation interval was long enough, that is, at least at the 2-leaf stage. On the other hand, plants

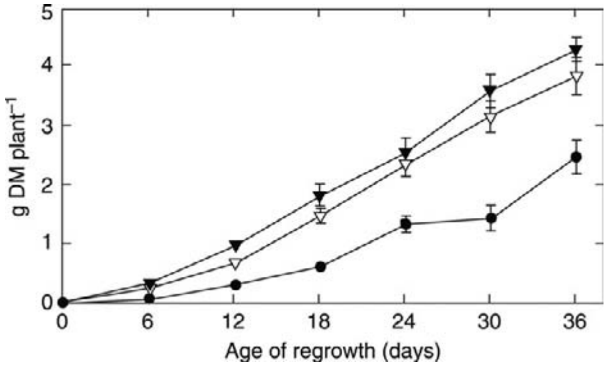

Figure 2 Yield (g DM plant^{-1}) of perennial ryegrass with regrowth time for plants previously defoliated once at 3 leaves per tiller (▼), once at 1 leaf per tiller, and once at 2 leaves per tiller (▽), or 3 times at 1 leaf per tiller (•). After Donaghy DJ and Fulkerson WJ (1997) The importance of water-soluble carbohydrate reserves on regrowth and root growth of *Lolium perenne* (L.). *Grassland Forage Science* 52: 401–407.

defoliated at 1 leaf per tiller had significantly lower regrowth rates over extended periods. In this study, regrowth at 12 days (1-leaf stage) was related to WSC reserves according to the equation:

$$\text{leaf DM}\left(\text{g plant}^{-1}\right) = 0.28 + 0.04$$
$$\text{WSC}_{\text{tiller bases}}\left(\text{g kg}^{-1}\ \text{DM}\right)\left(r^2 = 0.97\right)$$

More frequent defoliation will reduce the survival of perennial ryegrass plants over the stressful summer period (**Table 1**).

Stage 4: Maximum grazing interval

After 3 leaves per tiller have regrown, the oldest leaf (leaf 1) begins to die as a new one (leaf 4) appears. As a consequence, pasture quality begins to decline and pasture is wasted, through either rejection by stock or decay of dead leaves. Plant WSCs provide an important source of readily available energy for grazing ruminants. Thus, as expected from the pattern of change of WSCs in tiller bases, WSCs in the leaves accumulate with leaf maturity until the commencement of senescence at 3 leaves per tiller (**Figure 3**), after which the concentration of WSCs falls as they are exported to other parts of the plant.

Crude protein (CP) levels in ryegrass pastures at a vegetative stage of growth are normally too high for dairy cows. In contrast to WSCs, CP is particularly high in early regrowth with a high proportion of N present as nitrates (NO_3). High NO_3 intake can inhibit digestion of pasture in the rumen, as NO_3 are converted to nitrites, and in the extreme case can cause death from nitrate poisoning. Grazing at the 2½–3 leaf-per-tiller stage of growth provides a very different, and improved, ratio of WSC to CP in the feed for dairy cows. A ratio >2 of WSC to digestible protein intake (DIP) should optimize microbial protein synthesis (MPS) in the rumen (**Figure 3**). Assuming a 0.5% unit h^{-1} increase in WSC from 10 a.m., we can estimate that the WSC:DPI ratio would be 3 in ryegrass pasture grazed in late afternoon in midwinter, at the 3-leaf stage of growth, a ratio that maximizes MPS. On the other hand, the ratio would be only 0.3 when grazed in the morning at the 1 leaf-per-tiller stage of regrowth.

The change in WSC content in **Figures 3(a)** and **3(b)** is very different. In **Figure 3(a)**, the skies are clear in this environment, so solar radiation and hence photosynthesis are high; temperature is low in winter; hence, growth is slow and respiration is low – conditions for maximum accumulation of simple sugars from photosynthesis are prevalent.

In **Figure 3(b)**, although solar radiation and hence photosynthesis are at a maximum, the high temperatures lead to a high growth potential and very high levels of respiration – thus, the amount of simple sugars that can accumulate is small. Even though the data are from a subtropical region, it is also typical of the situation on frosty clear days in early spring in temperate regions.

Potassium is another nutrient, like N, taken up by the plant to luxury levels (>4%), which is normally well in excess of cow's requirements (0.8% DM), and has been implicated in metabolic problems in dairy cattle (grass tetany, milk fever). The reason for this is that high levels of K can interfere with the uptake of Mg and Ca, which are marginal in most pasture grasses. Again, grazing at 2½–3 leaves per tiller improves the balance

Table 1 Yield of perennial ryegrass in the establishment year (1993) and plant density (m^{-2}) in the following autumn (1994) in pastures defoliated at 1 or 3 leaves per tiller in 1993

Defoliation interval	*Annual yield (1993)*	*Ryegrass plants (autumn 1994)*
(leaves per tiller)	*(kg DM ha^{-1})*	*(m^{-2} ± SD)*
3	10 905	12 ± 2.2
1	7673	4.7 ± 0.4

Data from Fulkerson WJ and Donaghy DJ (2001) Plant soluble carbohydrate reserves and senescence: Key criteria for developing an effective grazing management system for ryegrass-based pasture: A review. *Australian Journal of Experimental Agriculture* 41: 261–275.

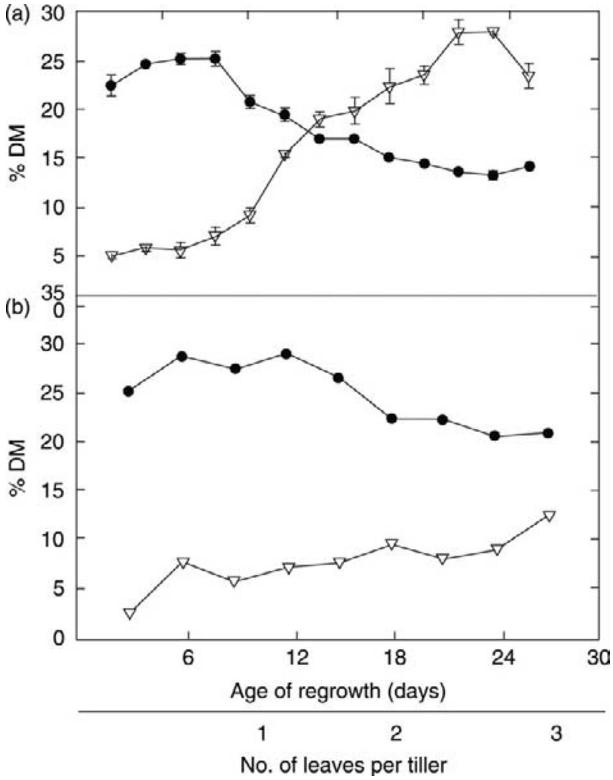

Figure 3 Concentration (%DM) of WSCs (▽) and crude protein (•) with regrowth time (days and number of leaves per tiller) in tiller bases of perennial ryegrass in a mixed pasture with white clover, in (a) July and (b) September. After Fulkerson WJ, Slack K, Hennessy DW, and Hough GM (1998) Nutrients in ryegrass (*Lolium* spp.), white clover (*Trifolium repens*) and kikuyu (*Pennisetum clandestinum*) pastures in relation to season and stage of regrowth in a subtropical environment. *Australian Journal of Experimental Agriculture* 38: 227–240.

of these minerals when compared to younger grass (**Figure 4**). Only phosphorus declines with regrowth, in contrast to cow's requirements. In fact the ratio of K:(Ca + Mg) (in milliequivalents) in forage indicates the potential to lead to metabolic problems in dairy cows (milk fever and grass tetany). It has been suggested that the ratio should not exceed 2.2, and only perennial ryegrass at the 3-leaf stage of growth has a ratio below 2.2.

The rate of leaf appearance is almost entirely related to ambient temperature (up to a maximum temperature of about 25 °C for ryegrass) such that the 3-leaf appearance interval in spring may be as short as 12 days, but over 100 days in winter in the colder dairy regions. Leaf appearance interval can be estimated from ambient temperatures from the following relationship:

$$\text{leaf appearance interval (days)} = 20 - 0.55\left(\frac{\max T\ (^\circ\text{C}) + \min T\ (^\circ\text{C})}{2}\right)$$

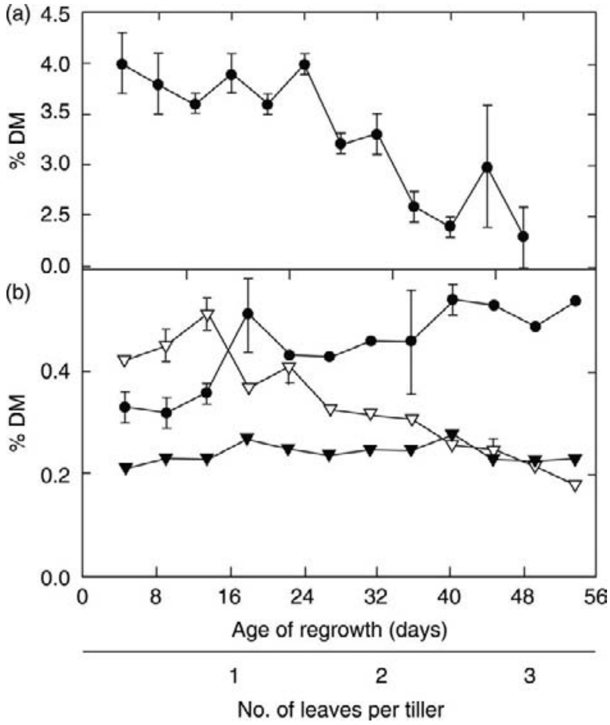

Figure 4 Concentration (%DM) of (a) potassium (•) and (b) calcium (•), magnesium (▼), and phosphorus (▽) with regrowth time (days and number of leaves per tiller) in tiller bases of perennial ryegrass.

Leaf number per tiller is a logical, convenient, and practical in-field indicator of replenishment of WSC reserves and leaf maturity, or the readiness of pasture to be grazed – the knowledge of its pattern of change can be used as a basis for farm layout for a controlled grazing program. For example, an appropriate farm layout on a farm with a maximum temperature of 22 °C and a minimum temperature of 6 °C in the coldest month of the year would need 18 × 2-day grazing blocks. This would be based on

$$\text{3-leaf interval (days)} = 3\left[20 - 0.55\left(\frac{22+6}{2}\right)\right] = 37 \text{ days}$$

Such an arrangement would optimize pasture growth and quality on each paddock, and any feed deficit for stock would need to be made up from supplements. Under this system, because the total intake by stock is the same, response to supplements should remain high and constant.

Another goal of grazing management should be to optimize tiller density in recognition that this has a major influence on the productivity of grass pastures. The management criteria here are to ensure that conditions are optimal for (1) tiller initiation (usually in late spring and autumn) by allowing light to reach the tiller bud at the base of the plant and ensuring an adequate availability of N; and (2) minimizing tiller death (usually in summer) by not overgrazing.

Using Both DM-on-Offer and Leaf Number-per-Tiller to Control Grazing

In practice, to flag the time of grazing, it makes sense to use both DM-on-offer (difficult to measure), an animal-related criterion, and leaf number (easy to monitor), primarily a plant-related criterion, although consideration of forage quality also makes it animal-related.

In this context, the use of either criterion alone to flag the time of grazing can mislead. For example, pasture 1 is growing on a relatively infertile soil and has 1600 kg DM ha^{-1} on offer but is at the 3½-leaf stage of regrowth; pasture 2 is on fertile soil and has 2000 kg DM ha^{-1}, but is only at the 1½-leaf stage, while paddock 3 has 2500 kg DM ha^{-1} and is at the 2½-leaf stage and is lodging. The choice: based on DM-on-offer, paddock 2 would be chosen over paddock 1, whereas paddock 1 has stopped growing but paddock 2 has potential for more growth – as indicated by leaf number. Paddock 3 is the one that should be grazed based on DM-on-offer but not leaf number – its growth will fall due to shading and its palatability will also fall. Experience indicates that it is difficult to utilize pastures with >2600 kg DM ha^{-1} on offer and still achieve reasonable levels of milk production per cow.

Likewise, DM-on-offer is, or could be, used as the criterion for determining grazing intensity, in terms of pasture intake (calculated as pregrazing minus postgrazing DM) by stock. However, as far as the plant is concerned, stubble height (4–6 cm), rather than DM, is the more important criterion. The actual pasture on offer corresponding to the 3–5 cm stubble will depend on (tiller) pasture density, and this in turn is also influenced by climate. For example, in cool temperate regions where tillering is prolific, tiller density may be 8000–30 000 tillers m^{-2}, whereas in warmer subtropical regions tiller density may be only 2500–4000 tillers m^{-2}.

Comparison between Set Stocking and Controlled Grazing

In general, comparisons between set stocking and controlled grazing have shown results ranging from no difference to a moderate increase in milk production at higher stocking rates for controlled grazing. Some caution is needed in interpreting these results for the following reasons:

1. Nearly all comparisons have been made at the same stocking rate. Thus at an equal feed requirement, the pasture actually utilized (measured as growth) would be the same. In other words, the potential to improve utilization needs to be created by increased stocking rate to show any benefits.
2. There are various definitions of controlled grazing. For example, having a set rotation of 1 week grazing and 3 weeks spell would be expected to be far worse than set stocking in view of the previous discussions on replenishment of plant WSC reserves, leaf maturity, and senescence.
3. There are practical problems associated with continuous or set stocking as outlined previously. In this regard, 22 dairy farms in the United Kingdom were surveyed to compare production under rotational and set-stocking systems of farming, and a significantly higher level of milk production was observed in the rotationally grazed properties.
4. There is a very real difference between block grazing with time of grazing based on leaf maturity and a system where rotations are kept at a constant value irrespective of pasture growth rate.

Grazing Management for C_4 Tropical Pastures

Whereas the vegetative growth of temperate pastures is generally of adequate quality for reasonable levels of milk production (~21 l milk per cow day^{-1} at no liveweight loss or gain), and therefore grazing management goals are to maximize growth, utilization, and persistence, management goals for C_4 tropical grass are very clearly to optimize forage quality. For example, yields of over 50 tonne DM ha^{-1} from tropical grass pastures have been achieved with optimal N fertilizer and irrigation but in practice only 6–10 tonne DM ha^{-1} of high-quality leaf can be utilized. The aim of management of C_4 grasses is to maximize the availability of leaf, as the metabolizable energy (ME) of leaf can be in excess of 9 MJ kg^{-1} DM, while the ME of stem and stolons can be as low as 7.5 MJ kg^{-1} DM. It is generally considered that 12 l milk per cow day^{-1} is all that can be derived from C_4 tropical grass pastures although cows grazing well-managed pangola grass (*Digitaria eriantha)* and kikuyu (*Pennisetum clandestinum*) have been found to produce up to 15 l milk per cow day^{-1}. Kikuyu differs from other C_4 grasses in a number of ways: (1) it tends to be a cooler or subtropical C_4 grass, (2) it seeds from stolons when grazed or cut short, and (3) it tends to be of better quality if managed properly.

Controlled Grazing of Tropical Grass Pastures

Studies in Australia have related changes in the quality of kikuyu to number of new leaves per tiller, as in ryegrass, making recommendations for grazing more applicable over regions and seasons (**Figure 5**).

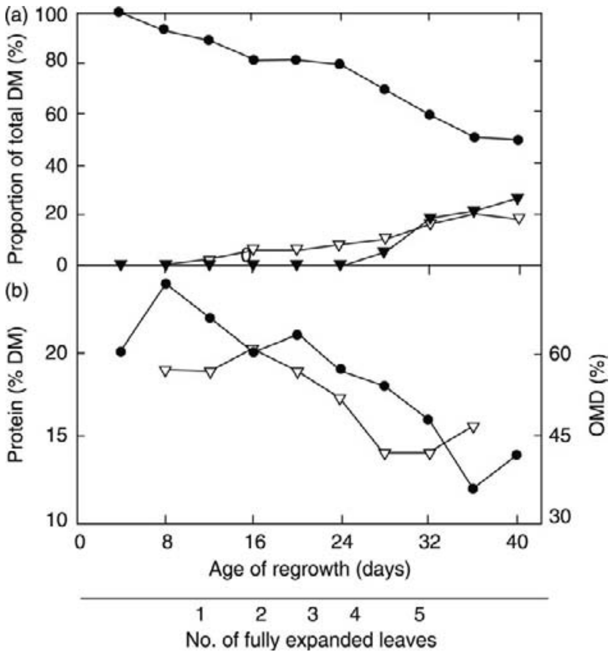

Figure 5 The changes (%DM) in (a) components: leaf (•), stem (▽), and dead material (▼), and (b) organic matter digestibility (OMD) (•) and crude protein (▽) in kikuyu grass in summer with regrowth time (days and number of leaves per tiller). After Reeves M, Fulkerson WJ, and Kellaway RC (1996) Forage quality of kikuyu (*Pennisetum clandestinum*): The effect of time of defoliation and nitrogen fertiliser application and in comparison with perennial ryegrass (*Lolium perenne*). *Australian Journal of Agricultural Research* 47: 1349–1359.

The proportion of stem and dead material increased substantially after 4 leaves per tiller had regrown and this was reflected in a decline in organic matter digestibility. Thus, grazing management is geared to grazing before 4 new leaves per tiller have regrown, and this could be as frequently as every 10–12 days in summer extending to 35 days in late autumn. The aim is to graze two-thirds of the available pasture and then remove the inedible (to milking cows) stem as it accumulates every 2–3 grazings using cows having lower feed requirements (dry stock) or mechanically (slashing or mulching).

With many tufted tropical grasses such as Rhodes grass (*Chloris gayana*), green panic (*Panicum maximum*), and to a lesser extent setaria (*Setaria anceps*), the practice has been to allow uninhibited growth with the cows selecting the leaf within a high canopy. A common recommendation has been to provide 1 tonnes leaf DM ha^{-1} to satisfy milking cows, and this variously equates to 2.4–4 tonnes total DM ha^{-1} on offer. Intensification of management by controlled or intermittent grazing management of tuft-forming tropical grasses has been ineffectual in increasing production per cow. A study with Rhodes grass found that despite changes in DM-on-offer within 2-, 4-, or 6-weekly grazed swards, pasture quality did not differ significantly, and this was reflected in similar levels of milk production per animal because of the ability of the animals to select their own diet. On the other hand, intensification did substantially increase milk production per hectare.

The incorporation of tropical legumes (e.g., glycine (*Neonotonia wightii*) and siratro (*Macroptilium atropurpureum*)) into grass pastures for dairy cows in order to reduce N fertilizer use and improve forage quality has been largely unsuccessful in view of their cost, unreliability of establishment, and extreme sensitivity to overgrazing (*see* **Forages and Pastures**: Perennial Forage and Pasture Crops – Establishment and Maintenance). Recent attempts to select tropical legumes with more tolerance to grazing (e.g., *Arachis* and *Vigna* spp.) may lead to the use of mixed pastures of C_4 species in dairy pastures.

Key Decisions

These are the three basic decisions every pasture-based dairy farmer needs to make every day of the year:

1. *grazing intensity* – how many animals (feed requirement) and/or what area of pasture (feed availability);
2. *grazing interval* – how long is each pasture area spelled; and
3. *grazing duration* – the period of grazing on a particular area of pasture.

The key considerations in choosing a grazing system should be to clearly identify desirable goals. These goals will be achieved only if the set of grazing criteria are relevant. For temperate species, these criteria include replenishment of WSC reserves, monitoring of leaf maturity to optimize quality and palatability, and considering critical periods when tiller initiation and tiller death peak, in the recognition that tiller density is a key determinant in the potential productivity of a pasture. Adherence to such criteria will ensure attainment of the goals of optimizing pasture growth and utilization, persistence, and forage quality. For C_4 tropical grasses, management clearly aims to optimize forage quality by maximizing leaf density at a young leaf age.

See also: **Forages and Pastures**: Annual Forage and Pasture Crops – Species and Varieties; Perennial Forage and Pasture Crops – Establishment and Maintenance; Perennial Forage and Pasture Crops – Species and Varieties.

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GAMETE AND EMBRYO TECHNOLOGY

Contents

Artificial Insemination
Cloning
***In Vitro* Fertilization**
Multiple Ovulation and Embryo Transfer
Sexed Offspring
Transgenic Animals

Artificial Insemination

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Historical Outline

Today we know that artificial insemination (AI) is the first animal reproductive biotechnology that has been applied to bring about (1) a major reduction in the incidence of venereal diseases, (2) an enormous improvement in practical type traits and the genetic capacity of dairy cattle to produce milk, and (3) a reduction of lethal and semilethal genes in the dairy cattle population. The development and general acceptance of initial AI procedures provided the foundation upon which newer animal reproductive biotechnologies have been built. These technologies include freezing semen, sexing sperm, and insemination of superovulated cows, followed by embryo collection and transfer. By ensuring superior genetics, eliminating the cost of secure housing and the danger of having mature bulls on the farm, and eliminating the farm bull(s) as a vector for spreading venereal diseases, AI has changed the practice of natural mating in commercial dairying.

Leeuwenhoek and his pupil Hamm in 1677 opened the door to investigations on the role of sperm in fertilization, when they peered through their simple microscope and discovered these small motile cells they called ‘animalcules’. News of their discovery was sent to the Royal Society of London, where the king was fascinated, and the paper was published. Almost 100 years later, in 1780, Spallanzani recorded the first successful AI, when a bitch that he inseminated had puppies. In 1899, Ivanoff organized studies on AI of horses and other domestic animals in Russia, followed shortly thereafter by Ishikawa in Japan.

In the 1930s, AI was further developed by Danish veterinarians who established the rectovaginal technique of insemination. This procedure required fewer sperm per insemination because the semen was deposited past

[†] Deceased.

the cervical barrier. This procedure was brought to the United States in 1938, where several investigators were exploring the use of AI. The AI cooperative movement spread rapidly in the United States, and much research was done, which contributed to the remarkable development of AI with liquid semen. Later, the AI industry in most countries changed to frozen semen, following successful freezing of bull sperm in England (*see* **Reproduction, Events and Management**: Mating Management: Artificial Insemination, Utilization).

Many steps are involved in the production of an excellent package of semen containing sperm that can be conveniently transferred to the cow to initiate pregnancy. These include proper techniques of handling bulls and collecting semen, critical evaluation of the quantity and quality of the sperm collected, and expeditious gentle handling and preservation of the sperm until they are used for insemination. The shift from liquid to frozen semen required new methods of packaging, cryopreservation, storage, and thawing of sperm, as a part of the current advanced technology of AI.

Semen Collection and Evaluation

Classical studies at Pennsylvania State University on sexual behavior of bulls were put into practice to prepare the bulls and the semen collection area so that it was ‘sexy’ for the bulls, sanitary for semen collection, and safe for the handlers of bulls and bull mount animals. The development of an artificial vagina for dogs by Amantea in Italy in 1914 prepared the way for multiple designs of artificial vaginas for the sanitary and effective collection of semen from many domestic animals.

Research at Cornell University demonstrated that bulls could be ejaculated daily for months without loss of libido, semen quality, or fertility. The common practice is to harvest sperm twice per morning, 2 or 3 days per week, from the sires with high semen sales. After collection, semen should be evaluated promptly, and not exposed to possible photooxidation by bright light. The most important characteristics of high-quality semen are samples (1) free from any contamination, (2) containing a high sperm concentration, (3) with a high proportion of sperm swimming rapidly forward, and (4) with a minimal proportion of abnormal sperm. Dairy bulls have been selected for high semen quality and genetic merit in AI for many generations.

The number (quantity) of sperm harvested per bull is of great importance because it is the major factor determining the number of potential progeny per sire. The number of sperm per ejaculate depends on the volume (milliliters) of semen collected and the sperm concentration per milliliter. These traits are measured with precision, the volume by weighing each semen collection and the concentration by determining sample optical density or counting sperm with particle counters.

Bulls produce an enormous number of sperm. These can be harvested with frequent semen collection, and preserved with good semen extenders. Holstein bulls on a typical schedule of four semen collections per week might average 7 ml of semen containing 1 billion sperm per ml. This represents 28 billion sperm collected per week. Because only a few million sperm are required per insemination, one sire’s superior genetics can be passed on to thousands of progeny (**Table 1**). In the 1950s and 1960s, conception rates of 60% or higher were common with liquid semen. As milk production

Table 1 Interrelationship of factors affecting the possible number of progeny per dairy bull per year

Sperm per bull per year[a]	*÷Sperm used per cow*	*×Collected sperm used*	*×Cows calving after one insemination*	
Number	*Number*	*%*	*%*	*=Number of progeny per year*
1.0 trillion (10^{12})	20 000 000	80	40	16 000
1.0 trillion (10^{12})	20 000 000	80	50	20 000
1.0 trillion (10^{12})	20 000 000	80	60	24 000
1.0 trillion (10^{12})	10 000 000	80	40	32 000
1.0 trillion (10^{12})	10 000 000	80	50	40 000
1.0 trillion (10^{12})	10 000 000	80	60	48 000
1.5 trillion (10^{12})	20 000 000	80	40	24 000
1.5 trillion (10^{12})	20 000 000	80	50	30 000
1.5 trillion (10^{12})	20 000 000	80	60	36 000
1.5 trillion (10^{12})	10 000 000	80	40	48 000
1.5 trillion (10^{12})	10 000 000	80	50	60 000
1.5 trillion (10^{12})	10 000 000	80	60	72 000

[a] Based on a conservative estimate of collecting 20–30 billion sperm per week during 50 weeks each year. Some bulls ejaculate more sperm than shown, and fewer than 10 million sperm can be inseminated.

per cow increased, and frozen semen was used, the pregnancy rates have declined to around 40% (**Table 1**).

The quality of semen depends primarily on the proportion of progressively motile sperm. This can be estimated with surprising accuracy by experienced technicians, provided the sperm are sufficiently diluted and magnified to observe them individually in a microscopic field containing many sperm cells. The observations should be made on a microscope stage at 37 °C using sperm diluted with a compatible diluent and several fields viewed with a high-quality binocular microscope at a magnification of about ×400.

With equipment for computer-assisted sperm analysis (CASA), the proportion of motile cells, the rate of progressive motion, and the types of swimming patterns of thousands of sperm can be monitored rapidly. The information can also be stored on video tapes for later reference when desired.

A supravital stain can be useful to determine the proportion of live versus motile sperm. The commonly used stain is 1% eosin, for staining dead cells, with 5% nigrosin included as a background stain. Alternatively, thousands of sperm stained with a fluorescent dye can be evaluated by flow cytometry, but the equipment is generally not available in an AI laboratory. The nigrosin–eosin stain can also be used to determine gross abnormalities, which is important when a bull with fertility problems has more than about 20% abnormal sperm. Also, abnormal forms of sperm can be detected in wet preparations with a differential interference contrast microscope.

The acrosome, on the apical portion of the sperm head, is of major importance for sperm to bind to the egg with resultant fertilization. The acrosome can be evaluated following Giemsa staining, or in wet preparations with the aid of a special microscope, or with the new CASA technology. Acrosome evaluation is especially valuable with frozen–thawed semen because of potential damage to the acrosome during cryopreservation.

Many other tests of semen quality have been studied experimentally, but are not widely applied commercially because either they are complicated and time consuming, or they measure the same characteristic monitored by simpler procedures. These include metabolic tests of glucose utilization and/or lactic acid production, including color changes in methylene blue or resazurin dye, as sperm utilize oxygen in closed tubes. Other tests measure the ability of sperm to penetrate synthetic gels, or cervical mucus, or to fuse with matured bovine oocytes recovered from the ovaries of slaughtered cattle.

A test that accurately ranks fertility of males is competitive fertilization. In this test, sperm from two males are mixed in equal numbers to compete for fertilization. Sperm from each male must be identifiable after mixing. Research at Cornell showed that sperm from a variety of pairs of bulls could be stained individually with a red fluorescent dye, tetramethyl rhodamine isothiocyanate, versus fluorescein isothiocyanate, which fluoresces green. The competitive fertilization results of oocytes penetrated *in vitro* were highly correlated ($\geq$0.89) with the fertility of bulls extensively tested in AI. Heterospermic insemination of cows is also efficient in ranking bulls on fertility, but it is not practical to use mixed semen commercially, where pedigree information is important.

Preserving Liquid Semen

The development and expansion of AI to a large segment of the dairy cattle population occurred with the use of liquid semen in the 1940s and 1950s (**Figure 1**). The characteristics of a good extender to preserve sperm viability at 5 °C include (1) a source of energy, (2) a proper concentration and balance of ionic components, (3) a near-neutral pH and good buffering capacity, (4)

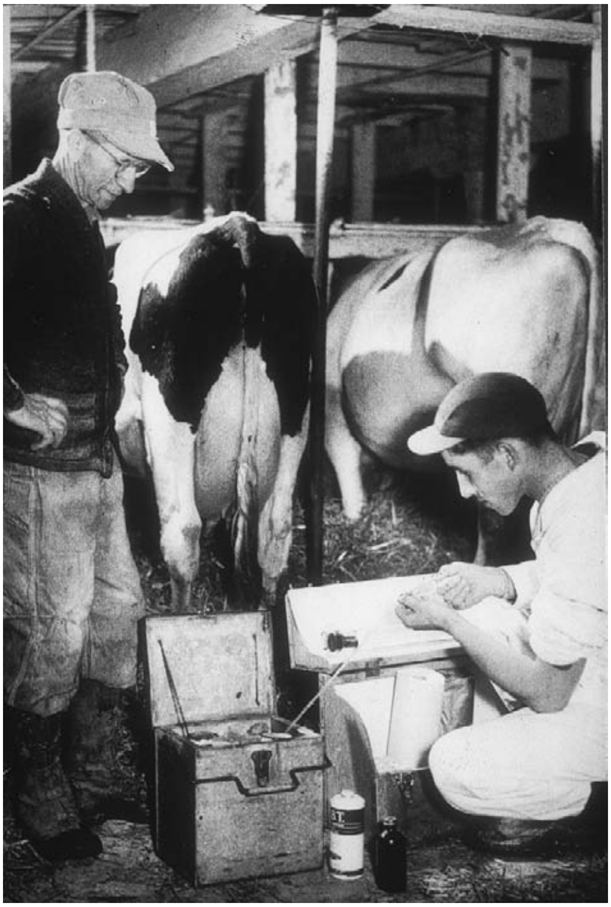

Figure 1 A dairyman watches an inseminator in 1941 drawing liquid semen into a glass catheter in preparation for insemination. Note the insulated case keeping semen at +5 °C.

macromolecules to protect the sperm during cooling, and (5) antibacterial agents to control bacterial growth.

The first practical semen extender was the egg yolk–phosphate medium tested in Wisconsin by Phillips and Lardy in 1940. In 1941, Salisbury and coworkers, at Cornell, developed a widely used extender for bull semen by combining egg yolk equally with a 2.9% solution of sodium citrate dihydrate. Later at Cornell, an extender known as CUE, with 20% egg yolk by volume, combined with a balanced ionic buffer, or 20% egg yolk with trihydroxy(methylamino)methane (Tris), was widely used. The CUE extender was effective in prolonging sperm livability at 5 °C and at ambient temperature. Researchers in New Zealand modified CUE; they reduced egg yolk to 5% by volume and added caproic acid and improved sperm survival, particularly at the New Zealand ambient temperature of 18–21 °C. This extender has been used successfully in New Zealand with liquid semen since the 1960s.

Membrane phospholipids of sperm cells, similar to many other types of cells, are damaged when cooled below about 15 °C. Membrane damage results in disruption of key metabolic processes of the sperm cells. The macromolecules in egg yolk, particularly the lipoprotein, protect the sperm cells during cooling. Cooling sperm from body temperature to 5 °C during a period of 2 h is recommended. The survival and fertility of bull sperm are preserved for a few days at this temperature, allowing the semen to be packed with ice and shipped rapidly to inseminators.

Whole milk, skim milk, and reconstituted skim milk powder have also been used effectively for preserving bull sperm. The milk must be heated to at least 90 °C for 10 min to destroy a spermicidal enzyme, lactenin. If water is lost, sterile water should be added to maintain correct osmotic pressure. A disadvantage of whole milk is that fat globules obscure sperm, making motility estimation more difficult. The problem is overcome in CASA equipment with a fluorescent light source and sperm stained with Hoechst 33342 DNA stain.

Another major problem that seriously affected the efficiency of dairying for years was the embryonic and fetal losses due to transmission of venereal diseases through semen from infected bulls. When AI first started, many sexually mature bulls selected for use in AI were infected with what was then called *Vibrio fetus* (now *Campylobacter fetus*). There was no successful treatment of bulls at that time. An important discovery at Cornell University and Pennsylvania State University was that semen treated with a combination of penicillin and streptomycin could increase the fertility of semen from infected bulls by 10–20%. The Cornell studies included polymyxin B. This antibiotic was added later to streptomycin and penicillin to become the standard prophylactic treatment of bull semen for 40 years. Fertility in herds using AI was increased markedly, thus saving the dairy industry billions of dollars by reducing pregnancy loss due to venereal diseases. Today, bulls in most AI organizations are free from organisms that cause known diseases. However, as a precaution to control possible pathogens that might be present in the environment, currently bull semen is treated with a combination of gentamycin, tylosin, and linco-spectin.

Background of Freezing Sperm

The preservation of bull sperm by freezing was a change with enormous impact. The technique did not improve the quality of semen used for insemination. In fact, even with the best procedures for sperm cryopreservation, many sperm cells are killed, and fertility is slightly lower than fertility obtained with liquid semen. However, the technique allowed AI personnel to collect and process semen on a convenient schedule, and to distribute the sperm from genetically superior bulls worldwide. The semen can be used at any time and any place, provided that equipment for proper storage of frozen semen is available (**Figure 2**).

Successful sperm cryopreservation has had a great impact upon all cryobiological research, as the procedure of incorporating glycerol and related cryoprotectants into the freezing medium stimulated the field of cryobiology. Today, embryos and blood and embryonic stem cell lines, used in cloning, are preserved by methods based upon the principle discovered with sperm cells by Polge, Parkes, and Smith in England. In addition, efficient containers for extended storage of living material in liquid nitrogen were developed initially to store bull sperm. Mr. Rockefeller Prentice, owner of American Breeders Service, provided the Linde Company with a major grant to develop much more efficient insulation in their storage

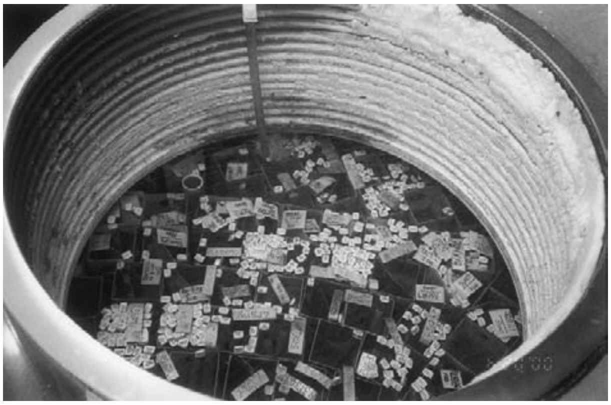

Figure 2 A tank of liquid nitrogen capable of holding 100 000 straws of frozen semen at −196 °C until they are distributed for use worldwide.

units, which extended the time between required liquid nitrogen refills from a few weeks to several months.

Principles of Cryopreservation and Extenders for Successful Cryopreservation of Sperm

The initial collection and processing of liquid and frozen semen are similar. However, for freezing, a cryoprotectant must be included in the egg yolk or milk semen extender. Glycerol is usually added after cooling semen to 5 °C because antibiotics may be more effective in its absence, and glycerol depresses sperm motility in some extenders at room temperature. The optimal freezing rate and thawing rate should be determined for each extender used, as the osmotic and thermal effects induced by freezing may interact with both the extender and the thawing rate.

The most widely used extender for freezing bull sperm is the egg yolk–Tris medium consisting of 3.028 g of Tris, 1.7 g of citric acid monohydrate, and 1.25 g of glucose in 92 ml of distilled water plus 8 ml of glycerol. To this mixture is added 20% egg yolk by volume. A surfactant mixture of 0.5% sodium and triethanolamine lauryl sulfate has been used experimentally to improve sperm survival under variable methods of cryopreservation. High fertility was achieved.

Glycerol can be included in the yolk–Tris extender at room temperature, but when egg yolk–citrate or heated milk extenders are used, glycerol is omitted from the initial extender. After being cooled to 5 °C the extended semen is diluted to contain the desired number of sperm cells per milliliter and glycerol is added, usually slowly over a period of 30 min. The final concentration of glycerol is 7–11%, depending upon the extender. The final number of bull sperm for insemination is usually about 10 million total sperm (**Table 1**).

The extended semen is then packaged and frozen after a few hours at 5 °C. Originally, it was thought that sperm should be held at 5 °C until glycerol equilibration had occurred. However, glycerol penetrates bull sperm membranes rapidly. Any beneficial effect of this equilibration period is more likely due to a restabilization of sperm cell membranes after undergoing a phase change during cooling than due to glycerol equilibration.

Freezing Sperm in Pellets

In the development of frozen semen for cattle AI, liquid nitrogen equipment was not generally available. Semen was processed in vials for freezing to −79 °C, and stored at that temperature with solid CO_2. A popular method was developed to freeze sperm rapidly on solid CO_2. Extenders containing high concentrations of raffinose or lactose and a low concentration of glycerol, combined with 20% egg yolk (v/v), were successfully tested by Nagase in Japan, and Graham in Minnesota, for pelleting semen. The pelleting procedure involved preparation of concentrated extended sperm. Then, 0.1–0.2 ml of semen was pipetted into small hemispheric holes on a block of solid CO_2. These small droplets froze rapidly. The sperm were fertile. It was an inexpensive method of freezing and storing semen, but identification and contamination of pellets by microorganisms, or with sperm from other pellets, were the potential problems. Separate tubes of extender for thawing the sperm were required. Pelleted semen is seldom used today, except in some Third World countries.

Freezing Sperm in Ampoules or Straws

Glass ampoules were available commercially for freezing sperm before the Cassou family in France produced plastic straws on a commercial scale in 1970. The 1.0 ml glass ampoule used first was relatively bulky, and 0.8 and 0.5 ml capacity ampoules were produced. The reduction in size also increased the ratio of surface area to volume of extended semen, a possible advantage in freezing. Fertility trials, comparing many methods of processing sperm in glass ampoules of different sizes, gave similar results. A problem with glass was that some ampoules broke when placed in warm water to thaw. Also, liquid nitrogen could leak into incompletely sealed ampoules, causing an explosion upon thawing.

The development of straws holding about 0.25 or 0.5 ml of semen for insemination of each cow overcame these problems, and these became the standard containers worldwide for freezing bull semen. The large surface area to semen volume permitted a more uniform cooling, freezing, and thawing of the sperm. This generally resulted in more sperm surviving freezing. Many more straws, and consequently semen from more bulls, could be stored in the liquid nitrogen tanks centrally (**Figure 2**), or in the smaller units (1200 0.5-ml straws per unit) used by inseminators. Equipment was developed to label, fill, and seal straws effectively at a high rate.

A common procedure for freezing straws is to lower the temperature of the straw from +5 to −100 °C over a period of 7–8 min. This cooling rate is about $-15\,°\mathrm{C\,min}^{-1}$. The sperm supercool to about −14 °C before crystallization starts, bringing the temperature to −4 °C. This supercooling should be minimized, but damage due to supercooling appears to be more important to avoid with larger groups of cells, such as embryos. Thawing sperm can be done rapidly, usually by thawing in water at 35 °C. Special equipment for thawing straws and for inseminating the sperm is described in the article **Bull Management**: Artificial Insemination Centers.

Some types of cells can be combined with a high concentration of cryoprotectant and frozen extremely rapidly. This causes a glassy state called vitrification.

This system has not been as successful as the conventional slower freezing procedure for bull semen.

Sexing Sperm

Methods for producing offspring of the desired sex have perfused the writings throughout recorded history. Finally, in the past 20 years, a repeatable procedure has become a reality, not only in theory but in commercial and clinical application. The basis for this technology is conceptually quite simple. The extra 'arm' of the X compared to the Y chromosome in haploid sperm confers a small percentage increase in total nuclear DNA (3.8% in the bull and comparable differences in many mammalian species). Using the DNA intercalating dye Hoechst 33342 that can permeate living sperm and specially designed flow cytometers that orient sperm to reduce variation in fluorescence emission, analyzed sperm exhibit a bimodal distribution of fluorescence corresponding to the DNA difference between X- and Y-chromosome bearing sperm. This difference in DNA content can be exploited not only to detect X and Y sperm, but also to sort them into populations with ≥90% accuracy.

While effective, the procedure has limitations and inefficiencies, which have been only partially overcome. These include the relatively slow throughput of sperm and thus low output of sorted cells compared to conventional semen processing, negative effects of the additional processing procedures carried out at ambient temperature on sperm viability and fertility, and the wastage of unresolved sperm. These difficulties have been surmounted to a significant extent by removing nonviable sperm during the sorting process and reducing the number of sperm per insemination dose after titrating sperm numbers to ensure acceptable fertility. Fertility of sorted, frozen–thawed bull sperm is consistently higher when used in heifers compared to cows.

Increasingly, major AI companies in the United States and abroad are adopting this technology and offering sexed semen from selected bulls commercially. Throughput and cost remain significant limitations that will require simpler, more efficient cell-sorting approaches. A recently developed strategy to reduce cost while satisfactorily skewing sex ratio is to change the conditions for sorting to increase speed while achieving a lower probability of the desired sex, for example 75 rather than 90% heifer calves. While commercial sorting has been applied most extensively with dairy cattle, resulting in millions of sex-selected offspring per year, the technology has also been applied in other domestic animals, companion animals, wildlife, and humans, primarily in attempts to prevent heritable, sex-linked birth defects.

Frozen-Dried Sperm

Early works to store sperm at a low cost for long periods by freeze-drying them were unsuccessful. Sperm were killed by the process, but the DNA remained intact. Killed sperm can fertilize eggs following microinjection of the sperm, but it is not a useful procedure for practical AI.

The Nonreturn Rate to Measure Fertility

The success of AI has been fuelled by the genetic superiority of the bulls used to produce large numbers of progeny, and the good conception rates (*see* **Bull Management**: Artificial Insemination Centers). Much of the reproductive success was a result of research based upon the availability of an extensive and inexpensive system of measuring fertility to test experimental hypotheses, to evaluate different methods of processing and preserving semen, and to improve the skills of inseminators and dairy farm managers. Pregnancy testing of cows was not a workable solution. Research workers at Cornell and the local AI unit developed the system of recording all cows not returning for service, adopted officially by the American Dairy Science Association in 1947. This nonreturn system facilitated conducting large field trials. It was vital for establishing a tremendous bank of knowledge concerning the biology of fertilization, and useful for managing successful AI organizations. The nonreturn rate parallels actual pregnancy rate, but is somewhat higher (**Figure 3**).

Predicting Male Fertility

The ability to predict male fertility based on laboratory evaluation of sperm has long been a goal in animal breeding and for diagnosing male factor infertility in

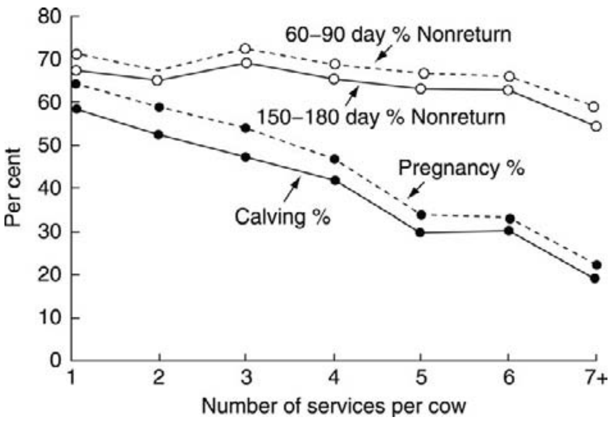

Figure 3 The relationship between nonreturn rates and pregnancy and calving rates for liquid semen used for cows inseminated one or more times. With frozen semen and high milk production, fertility is about 20% lower than is shown here.

humans. Since the refinement of microscope optics by Antonie van Leeuwenhoek that revealed novel ‘animalcules’ (sperm) in semen, numerous morphological, biochemical, and functional sperm attributes have been assessed for their correlation with fertilizing ability. While subjective assessment of the percentage of motile sperm remains the simplest and most widely used method for evaluating sperm function, numerous other sperm characteristics have been considered, including viability based on plasma membrane permeability or integrity, mitochondrial function, nuclear or head shape and chromatin stability, acrosomal integrity and capacitation state, and egg (zona) binding and penetration, among others. Unfortunately, the correlation of these assays with male fertility is generally low or at best inconsistent. Promising results have been presented for the relationship between sperm head shape and fertility based on Fourier harmonic amplitude analysis for identifying bulls with low fertility.

Both intrinsic and extrinsic sperm proteins, originating during spermatogenesis or from excurrent duct and accessory gland fluids (seminal plasma), have received much attention as potential markers for male fertility (high and low) and potential semen additives for enhancing sperm fertility. Similarly, various proteins contributed by seminal plasma have been identified and purified but strong evidence for their correlation with fertility or infertility is often variable and based on insufficient breedings to be compelling.

Factors that are an absolute requirement for fertility are self-limiting and are recognized relatively quickly. However, male fertility is multifactorial, affected by many intrinsic and extrinsic male and female factors. While modeling a number of sperm and semen quality parameters may improve fertility assessment, to date this approach has not been implemented because technical requirements and expense outweigh the benefit.

Aging Gametes and Optimal Time of Insemination

The importance of proper timing of insemination to obtain maximal fertilization rates and minimal embryonic mortality stimulated many *in vitro* and *in vivo* studies on gamete aging before fertilization. The times that each cow was first reported in estrus and inseminated were recorded on breeding receipts for later study relative to nonreturn rates. These studies, and research at several agricultural experiment stations, particularly at Nebraska in the early 1940s, led to the AM–PM rule. Professional inseminators should schedule their calls so that cows first seen in estrus in the morning (AM) should be inseminated the afternoon (PM) of the same day. Cows first observed in estrus in the afternoon (PM) should be inseminated the next morning (AM), or insemination will be too late for the best conception rates. With the variation in times of checking cows for estrus and variation in ovulation time with respect to the onset of estrus, this rule still is a good one to follow. When the insemination is performed by farm personnel, cows can be inseminated when detected in estrus. If timing of the insemination is mismanaged, either the sperm or egg may die, or aged sperm or eggs may result in fertilization but produce an embryo unable to survive.

Several systems of synchronizing estrus and controlling the time of insemination have been extensively researched. The objective is to be able to inseminate a group of cows at a fixed time with acceptable conception rates. These systems are described in the articles **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination.

Species other than Cattle

Equipment used for semen collection and insemination varies with species (for more details, *see* **Bull Management**: Artificial Insemination Centers). However, the principles of semen evaluation are the same for all species. Goat sperm freeze well in Tris–yolk or Tris–yolk–skim milk extenders, with 100×10^6 to 200×10^6 sperm used for intracervical insemination. Ram sperm are processed similar to goat sperm. However, intracervical insemination with frozen ram sperm gives poor results, and 200×10^6 fresh sperm intracervically or 20×10^6 sperm deposited surgically into the uterus is commonly used. Mares can be inseminated with centrifuged semen, containing about 500×10^6 fresh sperm, resuspended in a yolk–milk extender. Fresh sperm gives substantially higher fertility than frozen sperm.

Conclusion

AI has been the most powerful biotechnology applied for the widespread genetic improvement of dairy cattle and the reduction of venereal diseases. AI has resulted in an enormous increase in the knowledge of sperm physiology, fertilization, and the changes (including DNA) in aging gametes. Modified techniques of AI are useful in other species. It has paved the way for many other reproductive biotechnologies, from embryo manipulation to cloning, to first proceed in the laboratory and then to be accepted by the agricultural sector.

See also: **Bull Management**: Artificial Insemination Centers. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus;

Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Mating Management: Artificial Insemination, Utilization.

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Cloning

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Introduction

Since the first successful nuclear transfer (NT) of adult somatic cells in mammals in 1997, animal cloning has been in the spotlight, not only in the field of animal science, but also in xenotransplantation in the medical field. In this article, we discuss the history of animal cloning, how to produce cloned animals, how cloning of the animals is important and useful for humans, and the future aspects of animal cloning.

Overview of Nuclear Cloning

The word 'clone', defined as 'the descendant of a single plant or animal nonsexually produced from any one cell and with exactly the same form as the parent', was first used by Webber in 1903. Now, the term is broadly used to include also cell or gene replication (e.g., cell cloning or gene cloning). Animal clones are produced by embryo separation, splitting, and NT. As described later, animals cloned by embryo splitting are identical, not only in genetic components, but also in cytoplasmic components such as organelles. The cytoplasmic components of NT clones are hybrids, because the donor nucleus is introduced into host eggs, which includes maternal RNAs, proteins, and organelles that differ from donor cells.

Animal cloning can be traced back to the eighteenth and nineteenth centuries when the developmental potential of blastomeres and cells was first examined. Experiments to examine disaggregated blastomeres in sea urchins and amphibians were conducted, and similar experiments were later performed in mammals. Humans have improved farm animals by selective breeding. Many techniques have been developed to increase the number of animals that are superior in terms of producing high quantities of milk or high-quality meat, such as artificial insemination (AI), *in vitro* fertilization, and embryo transfer. These techniques have been extended to the cloning of farm animals to effectively produce superior animals or, more precisely, to produce copies of superior animals. Transgenic animals have also been produced using this technique. For example, NT of gene-manipulated somatic cells can give rise to a cow that produces milk containing growth factors.

First, we briefly discuss the history of genetic improvement in bovines.

History of Breed Improvement in Bovines

Artificial Insemination, Superovulation, and Embryo Transfer

AI has dramatically contributed much to breed improvement via male bovines. Sperm from males with genetically superior characteristics can be broadly distributed and used to inseminate a large number of females. However, to breed improvement through males has a limited potential because female bovines produce only one offspring a year, and this result in the production of only a few offspring during the female's reproductive life span. Female animals have many immature oocytes (50 000–300 000). However, most of the oocytes are not ovulated and therefore degenerate. Thus, less than 1/10 000 of the available oocytes in the ovary develop into calves in one life cycle. These immature oocytes have been considered for use in breed improvement through female animals. The superovulation technique, in which females are treated with appropriate gonadotropins to increase oocyte maturation and ovulation, can be used to increase breed improvement. via females (*see* **Gamete and Embryo Technology**: Multiple Ovulation and Embryo Transfer). When female bovines are superovulated and artificially inseminated with sperm from superior males, multiple fertilized embryos are obtained, which are expected to develop into superior offspring because both male and female genetic characteristics are superior. By transferring such fertilized embryos into surrogate mothers, which need not be superior, more offspring can be obtained. By cryopreservation of the embryos, fertilized embryos can be transferred to recipient females when convenient. This method improves both male and female sides of the breed. At present, the advantage of the 'nonsurgical embryo transfer' technique is that it dramatically contributes to breed improvement (*see* **Gamete and Embryo Technology**: Multiple Ovulation and Embryo Transfer). Before the successful development of this nonsurgical embryo transfer technique, embryos were surgically transferred to surrogate mothers, which is a high-risk and labor-intensive procedure.

Individual Steps in Cloning

Separation

The next step in animal breeding improvement was the production of identical multiplications in farm animals. For development of the cloning technique, blastomeres of

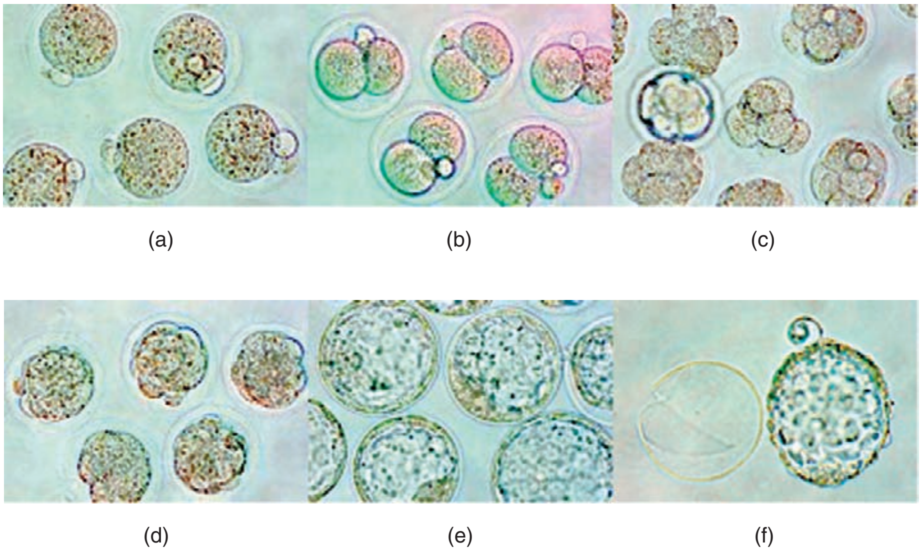

Figure 1 Preimplantation mouse embryos: (a) zygote; (b) 2-cell stage embryos; (c) 4- to 8-cell stage embryos; (d) compacted morula; (e) blastocysts; (f) hatched blastocyst.

preimplantation stage embryos (**Figure 1**) are separated into two or more groups (separation). Early embryos at the precompacted stages (**Figures 1(b)** and **1(c)**) are used for the separation method. Zonae pellucidae of precompacted embryos are removed, and blastomeres are rearranged into two or more groups. Separated blastomeres are occasionally placed back into empty zonae pellucidae to avoid disaggregation. **Figure 2** (left) shows a separation method at the 2-cell stage. Then, the separated blastomeres are cultured *in vitro* or *in vivo* until embryo transfer. By this method, identical twins have been obtained from the 2-cell stage in goat, sheep, bovine, and mouse. Because all separations are derived from a single embryo, the resulting embryos are all clones. Beyond 4-cell stage embryos, however, the separated blastomeres seldom develop to term, because the cell number at the blastocyst stage is insufficient.

Splitting

Another multiplication method is splitting of the embryo at the morula (**Figure 1(d)**) to blastocyst (**Figures 1(e)** and **1(f)**) stage. Embryos are split into two or more fragments to produce identical embryos (**Figure 2**, right) using a micromanipulator. The embryos at the compacted morula to blastocyst stage are recovered from the uterus and split into two or more fragments (**Figure 2**, right) using a fine knife or a glass needle mounted on a micromanipulator. After splitting, each fragment is briefly incubated *in vitro* and then transferred to the surrogate mother. This approach to obtain identical animals has been successful in cattle, sheep, goats, and pigs. This procedure, however, produces only a limited number of clones, mostly twins. The embryos generated from separated or split embryos can be called cloned embryos, but the number of cloned embryos produced is limited to only a few.

The next step in the evolution of cloning utilizes the NT technique. Compared with separation and splitting, NT can generate multiple clones.

Nuclear Transfer

As discussed above, both separation and splitting methods generate only a few cloned embryos and do not allow for large multiplications. For NT, recipient cytoplasm and donor cells must be prepared. Fertilized eggs at the pronuclear stage were first used as recipient cytoplasm for NT in mice. It was soon clear, however, that zygote cytoplasm has limited potential to support development after NT. In 1986, unfertilized oocytes at the metaphase II (MII) stage were successfully used as recipient cytoplasm for NT in sheep. In amphibian NT, maturing oocytes such as those in the germinal vesicle (GV) phase and metaphase I (MI) have been examined. At present, MII oocytes have the most supportive recipient cytoplasm for NT in mammals. The advantage of NT over separation and splitting is the possibility of producing a large number of cloned animals. In 1996, an embryonic cell line was established from an embryo disc in sheep and developed to a lamb after NT. In 1997, the first successful adult somatic cell nuclear transfer (SCNT) was reported in sheep. The production of animals from cultured cells indicates a high potential for cloning and, moreover, SCNT allows for the production of not only cloned animals but also copied animals. This technique would be a breakthrough, if the problems associated with this technique, which are discussed below, can be reasonably solved in the future. **Figure 3** shows the procedure of NT with blastomeres or somatic cells in bovines.

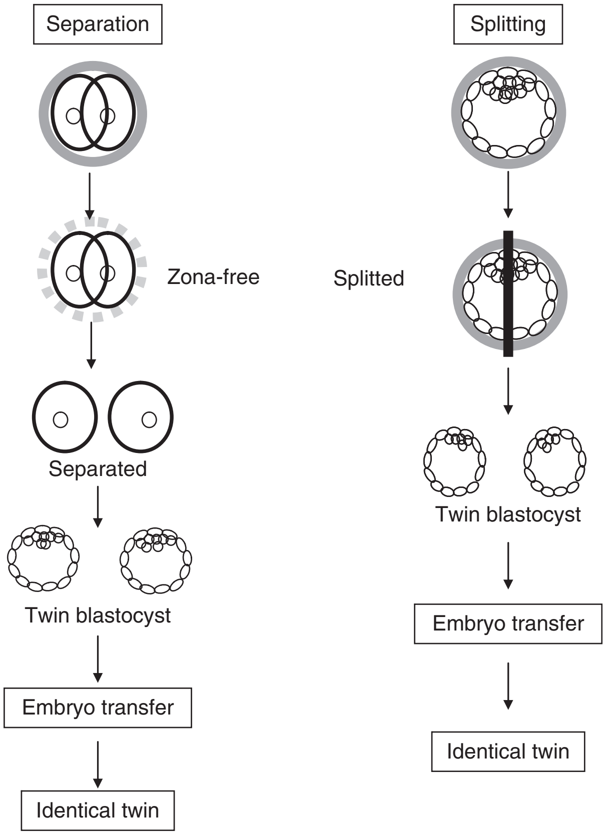

Figure 2 Separation and splitting for the production of identical twin. Left: A separation method at the 2-cell stage. See the text for detail. Right: Splitting method at the compacted morula to blastocyst stage. See the text for details.

Recipient oocytes and enucleation

Cumulus–oocyte complexes (COCs) are isolated from 1–10 mm follicles of matured bovine ovaries recovered from the local slaughterhouse and are cultured *in vitro* for 22–24 h in culture medium such as TCM-199 supplemented with 10% fetal bovine serum (FBS). At the end of the culture, oocytes with a polar body are selected as mature and used as NT recipients. For nuclear recipients, maternal chromosomes in MII oocytes must be removed to avoid mixing the chromosomes with the donor nucleus after NT. MII oocytes are mechanically enucleated using a micromanipulator. For experimental convenience, oocytes are usually pretreated with cytoskeleton inhibitors, such as cytochalasin, during mechanical enucleation. This treatment facilitates manipulation of the cytoplasm of mammalian eggs.

Donor cells

Preimplantation stage embryos as donors

Single blastomeres at the 8-cell stage to morula stage are used for NT (**Figure 3**). Zonae pellucidae of donor embryos are removed, and the blastomeres are disaggregated into single blastomeres to be used as nuclear donors. With this approach, the number of cloned embryos is the same as the number of blastomeres. If NT is repeated several times by using cloned donor embryos, more cloned embryos could be produced. This number is greater than that produced by separation and splitting, but the total number of cloned embryos is still limited.

Culturing cells: Embryonic and somatic cells

As shown in **Figure 3**, isolated tissue is disaggregated and cultured *in vitro*. Cells can be subcultured several times before use. Before NT, cells should be synchronized to the G0/G1 phase or M phase. Serum starvation (0.5% FBS) or contact inhibition is effective for G0/G1-phase synchronization, and drugs such as nocodazole, which depolymerize tubulin, are effective for M-phase synchronization. For SCNT, activated oocytes do not support development. Only oocytes that are rich in maturation-promoting factors can be used for SCNT at present. As discussed below, there might be reprogramming factors in MII oocytes.

Incorporation of donor cells using the fusion method or the direct injection method

Single donor cells are introduced into the perivitelline space of the enucleated oocytes. Electric pulses are useful to produce membrane fusion between recipient oocytes

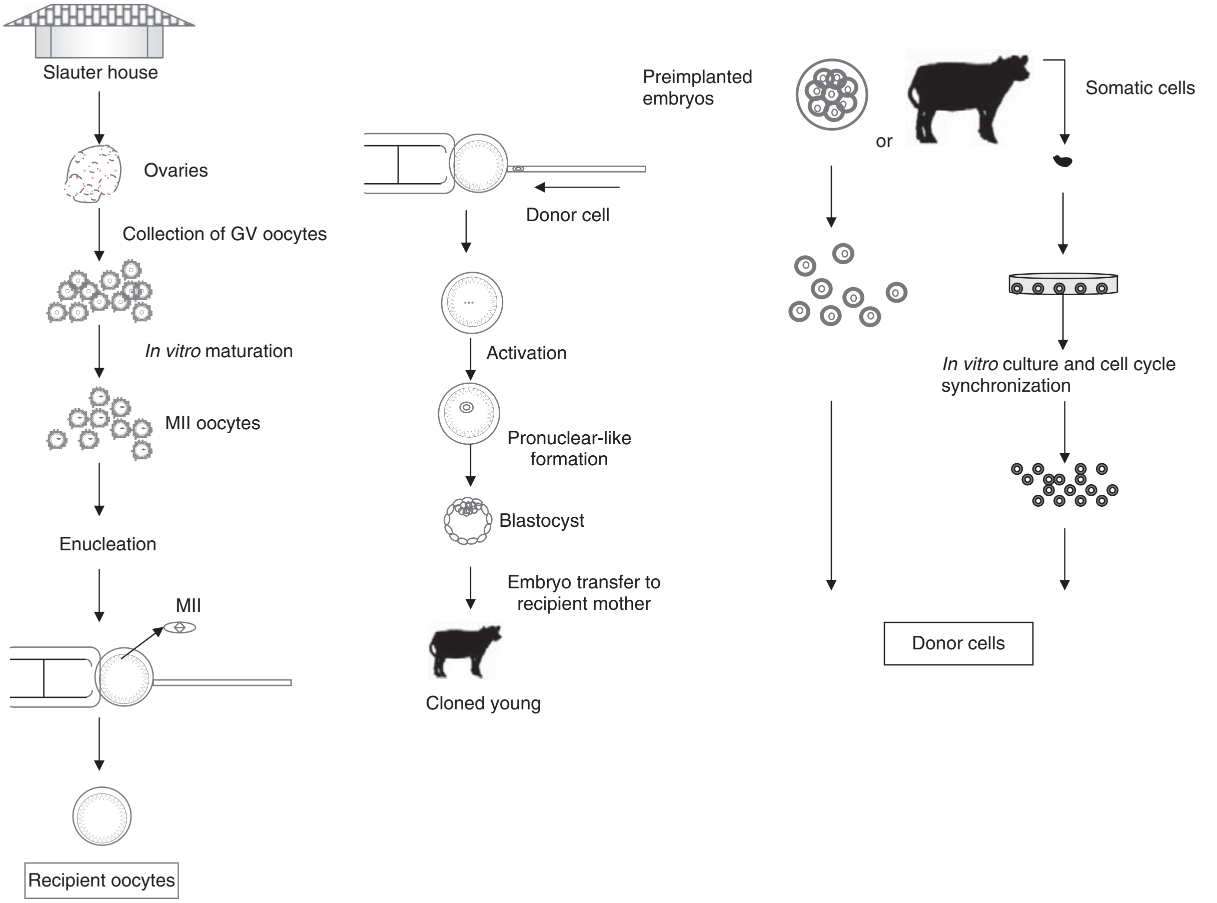
Slauter house
Ovaries
Collection of GV oocytes
In vitro maturation
MII oocytes
Enucleation
MII
Recipient oocytes
Donor cell
Activation
Pronuclear-like formation
Blastocyst
Embryo transfer to recipient mother
Cloned young
Preimplanted embryos
or
Somatic cells
In vitro culture and cell cycle synchronization
Donor cells

Figure 3 Nuclear transfer method for the production of cloned animals. Left: How to prepare the enucleated recipient oocytes. Middle: Nuclear transfer using enucleated recipient oocytes with donor cells. Right: Donor cell preparation from preimplantation stage embryos and somatic cells. See the text for details.

and donor cells. When appropriate direct current pulses are administered to the reconstituted oocyte, which is placed adjacent to the donor cell, the membranes of both the recipient oocyte and the donor cell will fuse. We usually use two pulses of 150 V mm^{-1} of DC for 20 μs with a 0.1 s interval, and 70–90% fusion rate is obtained. In another method, the donor nucleus is directly introduced inside the recipient cytoplasm, a technique often used for the cloning of mouse.

Artificial activation

Fused or injected oocytes stop developing unless they are artificially activated to simulate sperm penetration at fertilization. For artificial activation, electric pulses and/or other chemical reagents such as cycloheximide are effective. The conditions vary depending on the laboratory. In our laboratory, two DC pulses (20 V mm^{-1} for 20 μs) are used to obtain a good result. For preimplantation donor cells, previously activated oocytes can be used as recipients.

In vitro *culture and embryo transfer*

Activated oocytes are cultured *in vitro* until they reach the compacted morula to blastocyst stages for embryo transfer. For *in vitro* culture of activated oocytes, chemically defined media or media supplemented with 10% FBS are often used. We usually culture NT oocytes in 5% CO_2, 5% O_2, and 90% N_2, and under these conditions, 30–50% of NT oocytes develop to the morula or blastocyst stage, which is similar to the results from *in vitro* fertilization.

Conclusion and Speculation

The developmental totipotency of a blastomere of the preimplantation stage embryos and somatic cells from various tissues is shown in **Table 1**. The first successful somatic NT was reported in 1997. Various somatic cells such as fibroblasts, follicle epithelial cells (cumulus cells), oviductal cells, uterus cells, mammary gland cells, muscle cells, ear epithelial cells, Sertoli cells, tail tip cells, and

Table 1 Mammalian cloned animals from adults, fetuses, and embryonic cells

	Somatic		*Embryonic*	
	Adult	*Fetus*	*Blastomere*	*Cultured cell*
Sheep	Mammary gland	Fibroblast	2-cell 4-cell 8- to 16-cell Morula Inner cell mass (blastocyst)	Embryonic disc
Cattle	Cumulus Oviduct Granulosa Muscle Leukocyte Mammary Skin (ear, body)	Fibroblast	8-cell Morula Inner cell mass (blastocyst)	Embryonic stem-like cell
Goat		Fibroblast	2-cell 4-cell Morula Blastocyst	
Pig	Granulosa Kidney		4-cell	
Rabbit	Cumulus		8-cell Morula Inner cell mass (blastocyst)	
Mouse	Cumulus Tail tip Sertoli[a] B cell[b] T cell[b]		2-cell 4-cell 8-cell Morula Inner cell mass (blastocyst) Trophectoderm (blastocyst)	Embryonic stem cell
Feline	Cumulus			

[a] Isolated from newborn.
[b] Injection of inner cell mass from cloned blastocyst into tetraploid embryos.

skin cells have been developed to offspring in various species, for example, sheep, bovine, goat, pig, rabbit, and mouse. Moreover, transgenic animals are also obtained from somatic cells that are gene transferred or gene targeted before NT. This is also a breakthrough because in farm animals, embryonic stem (ES) cells, which are obtained from the inner cell mass of blastocysts having pluripotency and totipotency, are very difficult to obtain.

Cloned embryos produced by separation and splitting methods are identical. The genetic composition of NT embryos is a little more complicated, however, because the donor nucleus is reprogrammed and rearranged within the oocyte cytoplasm after NT, and the cytoplasm is heterogeneous, having both donor nucleus and recipient cytoplasmic components. The most abundant organelle present in the cytoplasm is the mitochondria. In normal fertilized eggs, sperm mitochondria degenerate during the first several rounds of cleavage, whereas mitochondria from the donors in NT embryos often remain. The role of such mitochondria in cloned animals is unclear, and the mitochondrial distribution in NT embryos is not uniform.

NT embryos often have developmental abnormalities, including a high rate of abortion, heavy body weight, peri- or postnatal death, and morphologic abnormalities of offspring. Although the reasons are unclear, the frequency of abnormalities increases when somatic cells are used for donor cells. In particular, NT embryos from somatic cells often abort at a later stage of pregnancy, injuring the surrogate females. Irreversible damage of donor DNA and insufficient reprogramming of donor nuclei after NT might be responsible for such abnormalities. In any case, some expression of imprinted genes affects development.

When donor cells are incorporated into oocytes, the donor nuclei are reprogrammed in the oocyte cytoplasm. Although the reprogramming mechanism of nuclei in the ooplasm is not clear, it is suggested that several proteins are specifically lost from donor nuclei, and other proteins are taken from the oocyte cytoplasm. When somatic cells are used for the donor nucleus, nonactivated MII oocytes support the development of reconstituted oocytes, but not activated oocytes. The potential of mouse SCNT embryos to develop into blastocysts and full-term development are enhanced by treatment with a histone deacetylase (HDAC) inhibitor, trichostatin A (TSA). It was clarified recently that epigenetic factors are important for improving not only the blastocyst formation rate, but also the more complex process of full-term development of SCNT embryos.

Cloning techniques using somatic cells cultured *in vitro* should be very useful for breed improvement in bovines in the future. Studies on NT of cultured cells began in 1996 with embryonic disc cells and in 1997 with fetal and adult somatic cells. Further studies are necessary, however, to resolve the problems in somatic NT discussed above, such as the high frequency of abnormalities of conceptuses or the mechanism of reprogramming of the somatic nuclei after fusion with oocytes and the identity of cloned animals. This technique has potential for other fields of research as well. For example, transgenic animals can be produced via somatic cells in which the DNA has been modified or partially removed *in vitro* (*see* **Gamete and Embryo Technology**: Transgenic Animals).

See also: **Gamete and Embryo Technology:** Multiple Ovulation and Embryo Transfer; Transgenic Animals.

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In Vitro Fertilization

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Introduction

After artificial insemination and multiple ovulation and embryo transfer (MOET), *in vitro* production (IVP) of embryos represents the third generation of techniques aimed at a better control of animal reproduction. This technique involves four major steps (**Figure 1**): oocyte collection, oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* development (IVD) of the resulting embryos. These different steps are now well established in domestic ruminant species (cattle, sheep, and goat), although the variability of the number and quality of the oocytes collected and the low viability of frozen–thawed, *in vitro*-produced embryos still limit the large-scale use of this promising technology. Nevertheless, more than 250 000 IVP embryos were transferred to recipients worldwide in 2005, compared to 600 000 *in vivo*-produced embryos. The number of transferred IVP embryos is increasing regularly in some regions (South America and Asia), as well as the proportion of frozen IVP embryos transferred, now reaching 50%. Beyond the potential use of IVP in breeding schemes or to overcome some female subfertility problems, this technique is also required for the establishment of new biotechnologies such as the use of sexed semen, cloning, and transgenesis. In addition, the knowledge of oocyte and embryo physiology acquired through IVP techniques may stimulate further development of other techniques such as MOET.

Techniques and Facilities

Oocyte Collection

For the setting up of the techniques as well as for the large-scale production of average genetic merit embryos, large quantities of material can be obtained at low cost by collecting ovaries at slaughterhouses. These ovaries are shipped to the laboratory (dry or in warmed saline) where the content of the follicles is aspirated. The needle used and the aspiration vacuum are important factors in determining the number and quality of the oocytes collected. These parameters have to be established for each species. In the cow, good results can be obtained with an 18-gauge needle connected to a 3 cm Hg vacuum. An average of 5–10 oocytes can be collected from each bovine ovary (1–2 in sheep and goat during the breeding season). Alternatively, the ovaries may be sliced with a razor blade and washed with phosphate-buffered saline to collect the oocytes. After aspiration or slicing, the collected fluid is screened under a stereomicroscope to select the oocytes. The oocytes are surrounded by several layers of somatic cells (cumulus oophorus, **Figure 2**). These cells establish contacts with one another, as well as with the oocyte, to allow cell communication through gap junctions, which is necessary for the efficient maturation of the oocyte. Only cumulus–oocyte complexes (COCs) with complete and compact cumulus investment should be selected. Cumulus expansion observed at oocyte collection may reflect follicular atresia or premature meiotic resumption in the oocyte.

To use this technique in a breeding scheme, the COC could be obtained from females of high genetic merit. This can be done after accidental culling of a female (in the same way as described for slaughterhouse oocytes) or from a living female through the ovum pickup (OPU) technique. In the bovine, OPU is done by transvaginal aspiration of follicle content under ultrasonographic control. In this case, OPU could be performed as frequently as twice a week, without any hormonal stimulation of follicular growth. Alternatively, OPU could be performed every week after a weak follicle-stimulating hormone (FSH) priming with a similar yield of total good-quality oocytes. Oocyte collection twice a week seems to provide better results as compared with once a week, probably because the appearance of a dominant follicle may induce some atresia in the latter case. An average of 10 oocytes may be collected per donor at each OPU session without stimulation, resulting in an average of 2.5 transferable embryos. In zebu cattle (*Bos indicus*), the high basal follicular activity of the ovaries results in a high number of aspirated oocytes in unstimulated OPU cycles (more than 20 COCs per session) and in a high embryo yield (up to 10 per session), making the IVP technique more efficient as compared to conventional MOET scheme.

Few data are available with regard to small ruminants. However, OPU may be done in these species by endoscopic aspiration of ovarian follicles. Good results can be obtained in this way after an ovarian stimulation by FSH or eCG.

Oocyte Maturation

Nuclear maturation

In the ovary, all oocytes are blocked at the prophase stage of the meiotic cycle. The block of meiotic progression during follicular growth is controlled by unknown

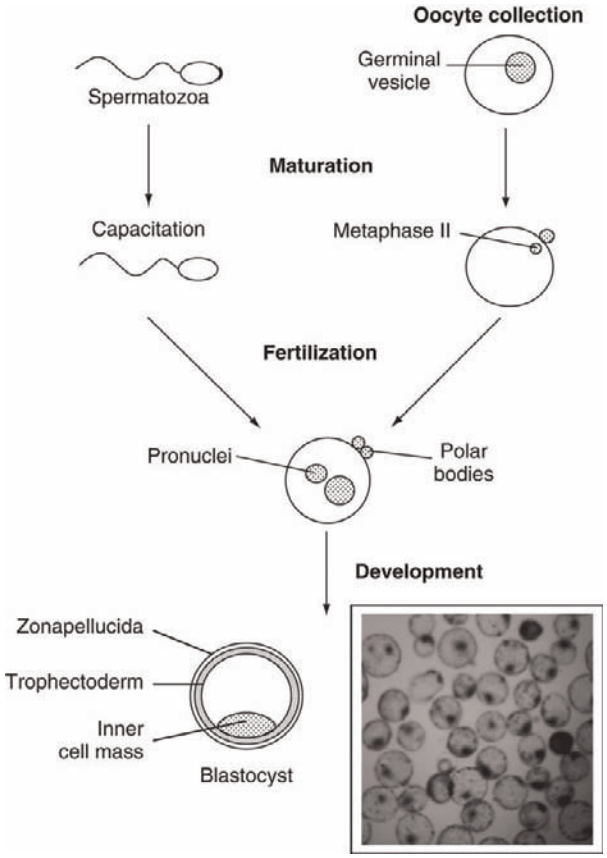

Figure 1 Schematic representation of *in vitro* production of embryos in domestic mammals. Four major steps are represented (bold). Oocytes are collected at the germinal vesicle stage of meiosis and reach the metaphase II stage after maturation. They are fertilized by capacitated sperms and cultured for 7–9 days to reach the expanded blastocyst stage. A batch of *in vitro*-produced bovine blastocysts is represented.

factors produced by the surrounding somatic cells. At this stage, the round-shaped nucleus, called the germinal vesicle (GV), contains diffuse chromatin. During early folliculogenesis, the oocyte grows, undergoes strong morphological modifications, and stores the molecules (proteins, RNAs) that will be determinant for the success of fertilization and early embryo development. The zona pellucida, a glycoprotein envelope surrounding the oocyte membrane, is synthesized by the oocyte during this period of growth. The zona pellucida has an important function in regulating fertilization and protection of the embryo until hatching. The oocytes resume meiosis and progress to the metaphase II (M II) stage only if they encounter a gonadotropin surge in the preovulatory follicle. They stop their progression at this M II stage until fertilization in the oviduct, after ovulation.

Oocytes collected from growing follicles for IVP are blocked at the prophase stage of meiosis. As soon as they are removed from the follicular inhibitory environment, meiotic resumption occurs spontaneously and progresses to M II. This spontaneous meiotic resumption of the oocyte outside of the follicle is the basis of IVM.

Cytoplasmic maturation

Cytoplasmic maturation covers all morphological and molecular events accompanying nuclear maturation after the luteinizing hormone (LH) surge in preovulatory follicles and preparing the oocyte cytoplasm for successful fertilization and embryo development. Cytoplasmic maturation includes the well-known morphological modifications such as the migration of cortical granules in the cortical region of the ooplasm. These granules are stored during oocyte growth and release their enzymatic content

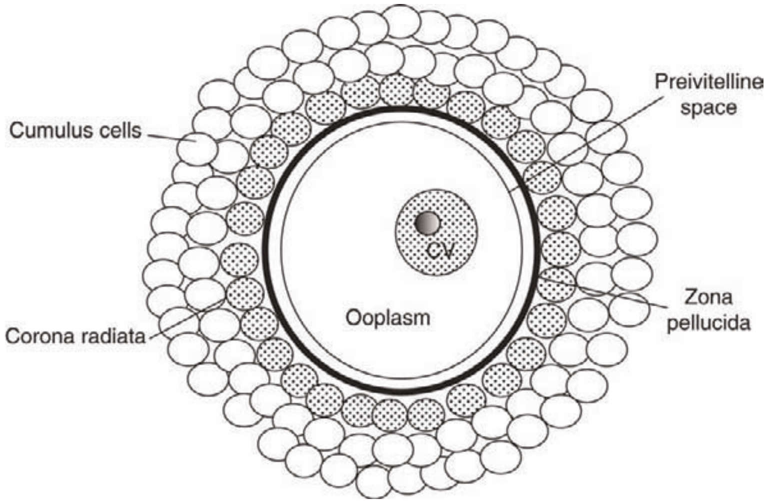

Figure 2 The cumulus–oocyte complex (COC). The germinal vesicle stage oocyte is surrounded by cumulus cells. The cells closer to the zona pellucida form the corona radiata. These cells maintain contact with the oocyte plasma membrane by foot processes crossing the zona pellucida and by forming gap junctions. The ooplasm is opaque due to its high lipid droplets load. The perivitelline space is virtual in immature oocytes and becomes visible in the mature one, after emission of the first polar body.

in the perivitelline space after fertilization. These enzymes modify the structure of the zona pellucida, preventing the penetration of additional spermatozoa. Meiotic competence is acquired during early folliculogenesis, soon after the apparition of the antral cavity in the follicle. However, after this acquisition, the oocyte requires a further differentiation period during late follicular growth to reach the full competence for cytoplasmic maturation. This late differentiation occurs under the meiosis-inhibiting signal sent by somatic follicular cells that maintain the meiotically competent oocyte at the prophase stage.

During IVP, more than 90% of the oocytes collected from follicles larger than 3 mm in cattle are able to complete nuclear maturation. However, only a few of them are competent for cytoplasmic maturation. Consequently, only 30–40% of the oocytes reach the blastocyst stage after IVF and IVD (**Figure 3**). When oocytes harvested from larger follicles or *in vivo*-matured oocytes are processed under the same IVF–IVD techniques, the success rate increases. This contrast highlights the functional importance of cytoplasmic competence.

Ovarian stimulation by gonadotropins increases the number of follicles growing to large size classes by protecting them against involution through the atresia process. Consequently, ovarian stimulation increases the number of competent oocytes collected through the OPU process.

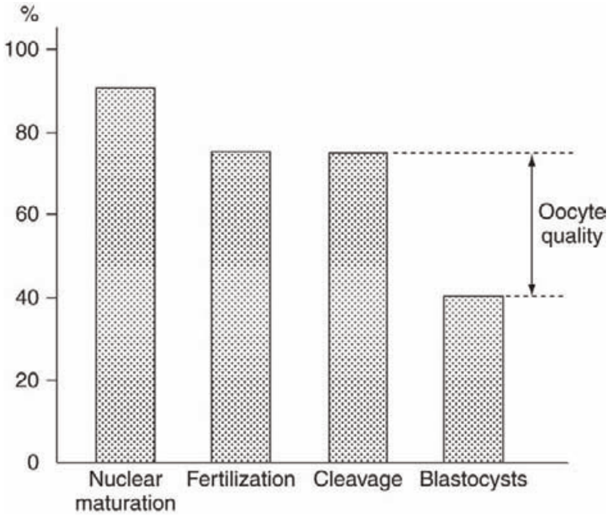

Figure 3 Success rate of *in vitro* embryo production. The maturation step is very efficient in terms of nuclear maturation (90%). The fertilization and cleavage steps are also efficient when appropriate conditions are used. However, the rate of development to the blastocyst stage is reduced (40%). After numerous works on *in vitro* development techniques, it is now clear that oocyte quality and oocyte maturation are limiting the whole process. The contrast between the high success of nuclear maturation and the low ability of these oocytes to develop after fertilization highlights the importance of cytoplasmic maturation of the oocytes responsible for their developmental potential.

In vitro *maturation*

In vitro maturation is probably the most critical part of the whole process of *in vitro* embryo production. Ruminant oocytes are usually matured at 39 °C in a 5% CO_2-containing humidified atmosphere. The optimal maturation time (more than 90% of the oocytes at the M II stage) is 22–24 h. Due to the high lipid content of oocyte cytoplasm, it is not possible to follow the progression of their nuclear status during culture. In addition, the presence of cumulus cells is required during IVM for an efficient cytoplasmic maturation and these cells mask the oocyte (**Figure 4**). Consequently, the only visible sign of oocyte maturation during IVM is the expansion of the cumulus cells. These cells produce hyaluronic acid, which is secreted and polymerized in the extracellular matrix, leading to the increase of intercellular space (**Figure 4**).

Several culture media have been proposed for IVM (MEM, Waymouth, Ham-F12). However, the most efficient one seems to be the TCM199 medium, bicarbonate buffered and containing minerals, carbon, and energy sources (glucose, glutamine), as well as vitamins and amino acids. The medium is generally supplemented with high-molecular-weight molecules that exert a surfactant effect (bovine serum albumin (BSA)) and provide hormones and growth factors (fetal calf serum, serum of estrous female, follicular fluid). These complex additives from animal sources may raise sanitary questions due to the possible presence of pathogen agents, and decrease the reproducibility of experiments. They may be successfully replaced by high-molecular-weight polymers for the surfactant effect (such as polyvinyl alcohol) and by cocktails of purified or recombinant hormones and growth factors.

Hormones are usually added to the maturation medium (FSH, LH, estradiol), in addition to the biological fluids, although their exact function is not clearly established. Growth hormone (GH) stimulates some aspects of cytoplasmic maturation (cortical granules migration) as well as nuclear maturation, resulting in a higher blastocyst yield. GH action seems to be mediated by the cumulus cells. Epidermal growth factor (EGF) has been shown to stimulate nuclear and cytoplasmic maturation in a wide variety of species including human, pig, cattle, and sheep. High blastocyst yields could be obtained after maturation of cattle oocytes in TCM199 supplemented only by EGF. EGF action is exerted through cumulus cells as well as directly on the oocyte. Insulin-like growth factor-I (IGF-I) also has a positive effect on cytoplasmic maturation of cattle oocytes. Oocyte-secreted factors such as growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) have recently been

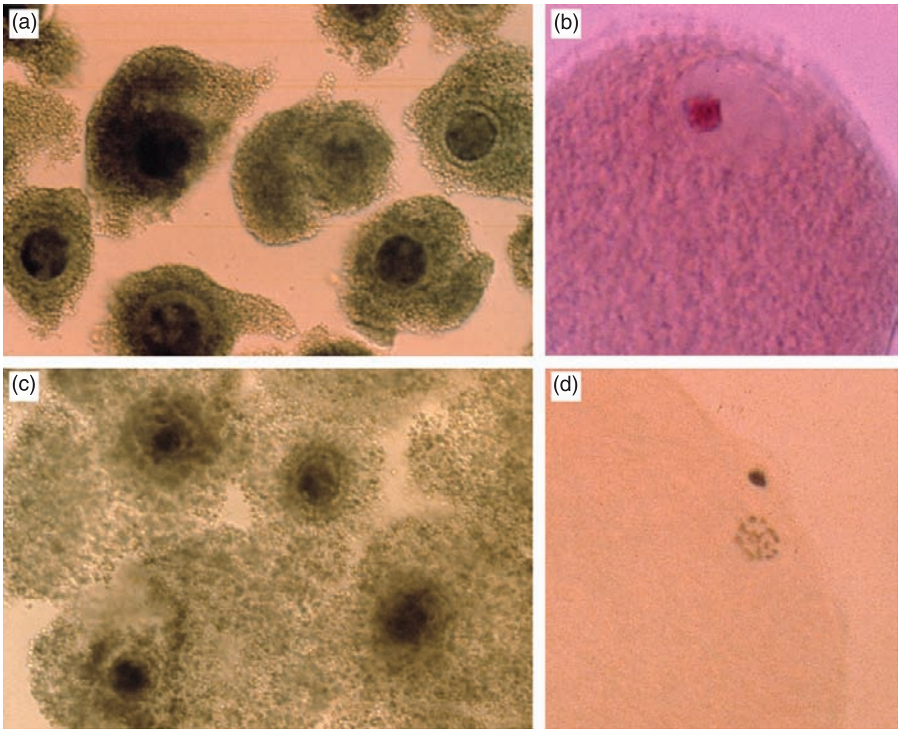

Figure 4 (a) Bovine cumulus–oocyte complexes (COCs) at the time of collection. The cumulus investment makes it difficult to see the oocytes. The corona radiata and zona pellucida are visible in some of the COC ($g = 120\times$). (b) Orcein-stained immature oocyte. The cumulus cells have been removed to allow visibility of the germinal vesicle with a large nucleolus ($g = 600\times$). (c) Bovine COC after *in vitro* maturation. Note the expansion of the cumulus cells ($g = 120\times$). (d) Orcein-stained bovine oocyte after *in vitro* maturation. Note the metaphase II plate and the extruded polar body ($g = 1000\times$).

shown to stimulate oocyte maturation, highlighting the interest in respecting a fixed COC density during IVM.

In Vitro Fertilization

The techniques used for IVF are well established in cattle as well as in small ruminants. The medium used for IVF is Tyrode-derived saline supplemented with BSA, lactate, and pyruvate, and with a capacitating agent. Sperm capacitation occurs physiologically in the female genital tract. This phenomenon involves modification of spermatozoa membrane as well as changes in its mobility properties that lead to spermatozoa fertilization ability. Many protocols support sperm capacitation *in vitro*. In cattle, heparin is widely used for this purpose, usually without pretreatment. In small ruminants, spermatozoa are usually capacitated by preliminary treatment with serum collected from females in estrus. Frozen semen is generally used, and a selection step is required to enrich the sperm suspension in living and motile spermatozoa. In the swim-up method, spermatozoa are deposited at the bottom of a culture medium-containing tube and allowed to swim in the medium. The top fraction, containing motile sperms, is then collected after a given incubation time. Alternatively, motile sperms may be purified by centrifugation in a discontinuous density gradient (Percoll). The motile fraction is collected at the bottom of the tube after centrifugation.

The sperm concentration used for IVF ranges between 0.5×10^6 and 2×10^6 spermatozoa ml^{-1} in the fertilization medium. This concentration, as well as the concentration of the capacitating agent, should be optimized for each male and even for each ejaculate from a given male. Fertilization rate increases with sperm concentration. However, after a given value, an increasing proportion of the oocytes become polyspermic. A compromise has to be established for a high fertilization rate with a low polyspermy rate. For better convenience, sperm and oocytes are usually coincubated for 18–24 h, although a 6-h incubation time seems to be sufficient to reach the higher level of normal fertilization.

A strong male effect has been reported on the success rate of IVF. Different males may provide different cleavage. In addition, males providing similar fertilization rates may also differ in their ability to promote the development to the blastocyst stage. Therefore, each male to be used in IVF has to be tested for optimized fertilization conditions and for its ability to promote embryo development. In the case of OPU–IVP with high-genetic merit females, the bull's ejaculates to be used should previously be tested on slaughterhouse oocytes. The use of sexed semen for IVP requires special attention due to lower fertilization potential, reduced numbers of available sperm cells, and high dilution.

Embryo Development

In standard culture conditions, ruminant embryo development blocks at the 8–16 cell stage. This block was first overcome through the use of coculture systems involving oviduct epithelial cells or other cell lineage in standard culture media (TCM199). The effect of cells has not been clearly established. They may produce some growth factors positively regulating embryo development or remove toxic media components. In a second step, culture media have been designed to more exactly fit embryo requirements without the need for coculture. One of the first media designed for this purpose was synthetic oviduct fluid (SOF). The composition of SOF was established from the composition of bovine oviduct fluid. Nevertheless, SOF or SOF modifications have also been successfully used in small ruminant species. More recently, sequential media have been proposed to fit the evolution of embryo requirements during early development. For example, glucose seems to be toxic to early cleaving embryos, whereas morulae need some glucose in the medium for further development.

The development takes place at 39 °C in droplets of culture medium overlaid with oil for 7–9 days. A high density of embryos per unit volume (1 embryo μl^{-1}) seems beneficial to development. When embryos are cultured without cell support, the oxygen concentration in the atmosphere should be reduced to 5% to avoid induction of oxidative stress on the embryos. Serum is usually added to the culture medium from the beginning or after the first cleavages (48 h postinsemination). The rate of cleavage is usually high (over 80% of the total oocytes), when an appropriate bull and appropriate fertilization conditions are used. However, the rate of development to the blastocyst stage plateaus at around 40% of the total oocytes. This limitation seems to be related more to the oocyte quality than to the conditions used for embryo development.

The pregnancy rates obtained after the transfer of fresh IVP embryos are usually good (40–50% of cows receiving an embryo produce a calf). Some problems of calf abnormality and perinatal mortality have been reported. However, these problems are less frequent than those observed with cloned embryos and seem to be linked to the presence of serum in the culture medium.

The main limitation to a wider use of OPU–IVP technique in breeding schemes is the low cryoresistance of the resulting embryos. Although well-established techniques of embryo freezing are described in cattle, sheep, and goat, and provide good results of pregnancy rate after transfer of the thawed embryos, these techniques appeared suboptimal for the cryopreservation of IVP embryos. Several alternative methods of embryo cryopreservation have been proposed to overcome the low viability of these embryos. Some vitrification procedures (high-speed cooling with a high concentration of cryoprotectants leading to amorphous solidification without the formation of ice crystals) appeared to partially solve the problem. The quality of the embryo, which is often determined by the culture conditions, appears to be one of the critical factors. However, recent advances in culture systems have allowed an increase in embryo quality and cryoresistance of IVP embryos through careful design of embryo development techniques using efficient sequential media or coculture of the embryos with somatic cells (oviduct epithelial cells) to mimic the *in vivo* environment. It has been clearly demonstrated that the maternal environment provides better results than *in vitro* conditions, especially regarding the viability and cryoresistance of the resulting embryos. A better knowledge of the precise embryo requirements at the different steps of early development and of the interactions between the developing embryo and maternal tissues (oviduct) will allow the design of more efficient culture systems.

Facilities

In vitro production of embryos requires two different places and sets of equipment: an oocyte collection facility and an IVP laboratory. These places may be distant since IVM could be performed during transport of the COC under controlled conditions.

Oocyte collection

All equipment required for oocyte collection may be transportable. Therefore, oocyte could be collected on-farm and subsequently transported to a distant IVP laboratory. Maturation can occur during transportation, in sealed tubes shipped in thermoregulated cases. Transportation may take up to 24 h, which is the optimal maturation time for oocytes from ruminant species. Alternatively, oocyte donors could be maintained in an oocyte collection station closer to IVP laboratory during the time of treatment. In cattle, OPU is done under the control of an ultrasound machine with a 5–7.5 MHz sectorial probe. This probe is fitted in an intravaginal device including an aspiration needle connected to a vacuum pump (**Figure 5**).

For small ruminants, the conventional endoscopic device should be used. Atraumatic forceps are used to maintain the ovary and a needle connected to a vacuum device is used to aspirate the follicles. A lower aspiration pressure should be used for small ruminants (1 cm Hg) as compared with cattle (3 cm Hg).

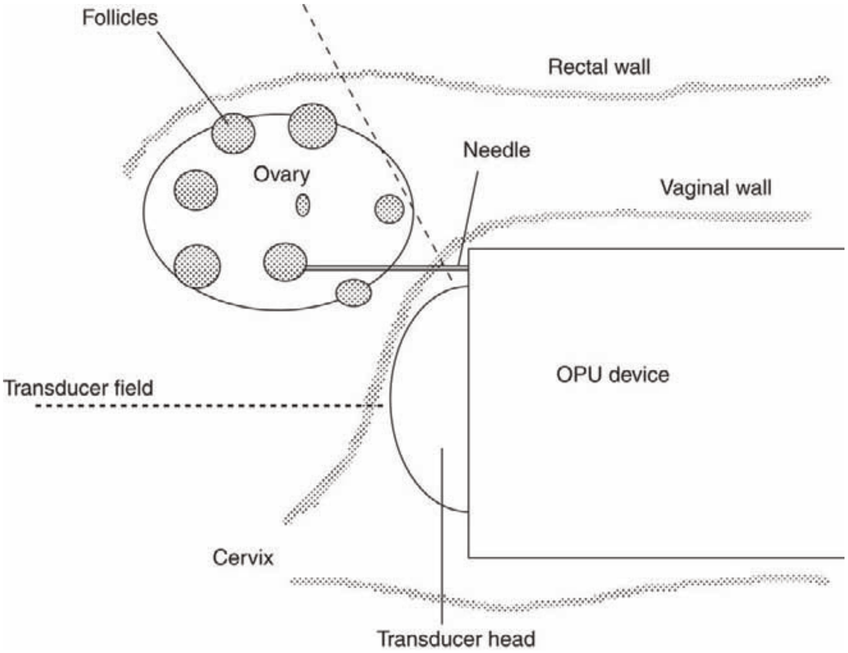

Figure 5 Schematic representation of ovum pickup. The technician maintains the ovary in close contact with the transducer probe. The ovarian follicles are visible on an echograph screen, as well as a line fitting the course of the aspiration needle. The needle is pushed through the vaginal wall to reach a follicle through the ovarian stroma. The aspiration system is turned on at that time, and follicular fluid, as well as cumulus–oocyte complexes, is collected in a tube connected to the needle.

IVP laboratory

The IVP laboratory should contain all equipment necessary for cell culture (laminar flow hoods, incubators) as well as the equipment for oocyte/embryo observation and manipulation (microscope, stereomicroscope). The room should be clean and preferably maintained under a high atmospheric pressure of filtered air. One should pay strict attention to potential toxic effects of the environment and the water used for media preparation, and the quality of disposable material. Oocytes, sperms, and early embryos appear to be very sensitive to toxic residues.

Impact and Potential

In vitro production of embryos presents several advantages over the more classical *in vivo* production through ovarian stimulation and artificial insemination. Nowadays, over 500 000 cattle embryos are produced worldwide each year by this technique. Although embryos could be produced from oocytes collected from slaughtered females in specific cases (culled genetically valuable females, mass production of embryos from specific breeds, etc.) the main genetic potential of IVP results from the possibility to collect oocytes through OPU and produce embryos from genetically interesting living females. The number of embryos produced through OPU–IVP technique from a given female over a given period of time is higher than the number that may be collected *in vivo*. Indeed, OPU could be performed twice a week for a long period of time without altering the reproductive potential of the females, whereas several weeks or months of recovery are required between two treatments of ovarian stimulation. OPU can be performed in young females (from 3 months of age) as well as in old cows. In addition, OPU could be continued during early pregnancy (4 months) without risk for the fetus, and it could also be performed on animals with some pathologies of the oviduct or uterus that prevent *in vivo* embryo production. OPU–IVP also allows the use of different males for producing the embryos (different males could be used for the oocytes from one aspiration session or from different OPU sessions). Furthermore, OPU–IVP could be beneficial in the case of females not responding to ovarian stimulation.

However, there are still some limitations to the use of OPU–IVP. The embryos produced by this technique have a low viability following cryopreservation and should be transferred fresh to get an optimum pregnancy rate. This limitation implies the disposition of large recipient herds and the synchronization of recipients at each oocyte collection. Consequently, the possibility of embryo transportation is limited and embryo storage is not recommended. In addition, some females appear to be poor oocyte donors in terms of both quantity and quality (developmental potential). Finally, some abnormalities have been reported during pregnancies after transfer of IVP embryos (large-calf syndrome). This translates into abnormally large offspring after a longer gestation time and a higher rate of perinatal mortality. In sheep, this syndrome has been clearly related to the presence of serum in the embryo culture medium.

In conclusion, OPU–IVP appears to be a promising tool for increasing the reproductive potential of high-genetic value females in ruminant species. However, research toward a better knowledge of oocyte differentiation leading to developmental competence may open the way to improvement of the efficiency of this technology and in turn to its increasing use in ruminant selection and breeding.

See also: **Gamete and Embryo Technology:** Artificial Insemination; Multiple Ovulation and Embryo Transfer. **Reproduction, Events and Management:** Pregnancy: Characteristics; Pregnancy: Physiology.

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Multiple Ovulation and Embryo Transfer

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Introduction

Artificial insemination (AI) allows high selection intensity on the male side such that superior males can sire in excess of 50 000 offspring. In contrast, the contribution of genetically superior cows is limited by the fact that they are monovulatory (i.e., only one oocyte is ovulated during each estrous cycle) and have a gestation period that lasts 9 months following which a period of uterine involution is required before the subsequent pregnancy. In addition, there has been an overall decline in fertility in high-yielding cows during the past 50 years. Thus, most cows contribute fewer than 5–10 calves in their lifetime. Superovulation and embryo transfer (ET) provide an opportunity to substantially increase the impact of superior females on a breeding program by allowing the gestation to occur in a surrogate recipient.

The first successful ET in cattle was recorded more than half a century ago. In the intervening period, this technology has played an important role in cattle breeding in dairy and beef cattle. When ET use was being extended in the 1970s, it involved the use of surgical procedures, which were not suited to lactating dairy cows. However, the development of simple nonsurgical recovery and transfer procedures ensured that this technology can be used widely in the dairy herd. Data collated annually by the International Embryo Transfer Society (*see* 'Relevant Websites' section) indicate that just under 1 million cattle embryos were transferred worldwide in 2007, the latest year for which figures are available. Approximately two-thirds of these embryos were *in vivo*-derived embryos by superovulation, while the remaining one-third were produced *in vitro* (**Table 1**).

Despite the fact that much research has primarily focused on methods to increase the number of ovulations and fertilized ova from the donor female, the total yield of transferable embryos has not changed markedly during the last 30 years. Variability in superovulatory response and the effort required for treatment and estrus detection have been among the primary limiting factors affecting the success of ET technology in genetic improvement programs. The variability in the superovulatory response continues to be one of the most frustrating problems with ET in cattle; as much as 20% of donors may not yield a viable embryo at any superovulatory attempt.

Multiple Ovulation

The term multiple ovulation and embryo transfer (MOET) was coined by Nicholas and Smith to consider ET and related technology in the context of optimizing genetic improvement of cattle. The main objective of MOET is to select on the basis of performance tests and pedigree analysis in order to reduce the generation interval, in comparison to progeny testing. The initial suggestion was that MOET would be confined to replacement heifers whereby these animals would be superovulated before entering the herd; however, it was not long until cows that had completed one lactation were included in the MOET program. The MOET proposals required replacements to be chosen on the basis of the performance of sisters (sibs) rather than on the performance of progeny. This produced a reduction in the generation interval, but a decreased accuracy of selection compared with progeny testing. Current MOET programs are characterized by the formation of a central nucleus herd with repeated superovulation of donors selected from cows and heifers. These selected breeding programs are more likely to be successful from a selection aspect due to the higher selection intensity among females and a shorter generation interval. In beef cattle, genetic gains can be increased by 30–65% by using MOET on yearling females to produce bulls and dams. In dairy cattle, the rate of genetic gain is 10–25% faster than using a national progeny testing system. Although the essential procedures associated with ET are now well established, there is considerable scope for improvement in various areas such as superovulation, embryo storage, and transfer. While MOET schemes have demonstrated that genetic gains are possible, the number of embryos produced per donor is variable and hence the numbers of offspring per donor have been lower and more variable than predicted. It has also taken longer than predicted to produce the given number of offspring.

Methods of Superovulation

Superovulation, as the name suggests, is a method of increasing the numbers of oocytes that are released at ovulation and that are available for fertilization.

Table 1 Overall bovine embryo transfer activity in 2007

		Number of transferred embryos		
Type of embryo	*Number of transferable embryos produced*	*Fresh*	*Frozen*	*Total*
In vivo derived	763 467	281 740	296 137	577 877
In vitro produced	434 581	215 512	29 745	245 257
Total				823 134

Adapted from Thibier M (2008) Data Retrieval Committee Report 2007. *Embryo Transfer Newsletter* 26(4): 4–9.

It is well established from endocrinological and ultrasonographical studies that follicular growth occurs in cattle in a wave-like fashion with two or three waves of growth occurring during each cycle (*see* **Reproduction, Events and Management**: Estrous Cycles: Characteristics). Emergence of a follicular wave is associated with a transient rise in follicle-stimulating hormone (FSH), which reaches its peak when the largest follicle of the wave is approximately 4 mm. By the time the follicles reach about 5 mm, they develop the ability to suppress FSH. The largest (dominant) follicle of a wave emerges at 3 or 4 mm and about 6 h before the second-largest follicle. The dominant follicle grows and very soon suppresses the growth of all subordinate follicles. The dominant follicle will only persist for 7–10 days and will undergo atresia unless its growth occurs in association with low progesterone concentrations and luteinizing hormone (LH) pulses, which are occurring about every hour and are sufficient for final maturation and ultimately ovulation. If it grows in the correct endocrine environment, it will ovulate; if not, it will undergo atresia like all other follicles of the wave and a new follicle wave will emerge.

Early research on superovulation indicated that the optimum response was obtained when gonadotropin was administered about 10 days after the previous estrus. This timing normally coincides with regression of the first dominant follicle and before the second follicle becomes dominant. However, superovulatory response is higher when gonadotropin treatment is initiated at the precise time of follicle wave emergence. Thus, the ability to control precisely follicle wave emergence would permit initiation of superovulation treatment at any time and eliminate the need for estrus detection or for waiting 8–12 days to initiate treatment. It has been demonstrated that all follicles are capable of suppressing FSH concentrations, but the dominant follicle may have more potential to do this than all others. Although there are many methods used for superovulation, it is generally accepted that the use of FSH gives the best response in terms of embryo production. Equine chorionic gonadotropin (eCG) can also be used; its main advantage is that a single injection will induce a superovulatory response because of its long half-life, but a major disadvantage is that it may stimulate the growth of follicles after ovulation, which may have adverse effects on embryo development. It is necessary to repeatedly inject FSH to induce a good superovulatory response because of its short half-life; animals generally receive 6–8 injections at 12-h intervals over a 3- or 4-day period. This is normally associated with a luteolytic dose of prostaglandin F2α (PGF2α) or an appropriate analogue administered with the fifth or sixth injection of FSH. When PGF2α is administered at 48 h after the start of gonadotropin, donors should show signs of estrous behavior about 2 days later, thus allowing 4 days from start of gonadotropin to onset of estrus. Both constant and descending doses of FSH have been used to induce superovulation. There is a substantial body of evidence to suggest that the descending dose method is superior in inducing higher numbers of follicles to grow and ovulate. However, animal-to-animal variability is as important as the particular hormone used in determining the ovulatory response (**Figure 1**).

Response to Superovulation

Not all animals respond to the superovulatory treatment. Normally, in excess of 90% of donors show signs of behavioral estrus, but about 80% are recorded as having three or more ovulations. The mean ovulation rate will be on the order of 15 with about 10 ova/embryos recovered of which 6–7 are transferable. If embryos are to be cryopreserved, only the top quality can be considered; the normal expectation is about four per donor. If embryo yield is considered as the response criterion, then 60–80% of donors will respond. A considerable variation in embryo production is found with a range of 0–40 embryos per donor. In general, heifers are more likely to respond to superovulation but they may produce fewer embryos than mature cows. Aging is associated with fewer 2–5 mm follicles at follicular wave emergence and a lesser follicular and ovulatory response after superstimulatory treatment. New data are emerging indicating that while the number of follicles in a wave is highly variable between animals, it is highly repeatable within an animal; thus, animals can be characterized as having a high or low number of follicles per wave and this can be used to accurately predict superovulatory response (**Figure 2**).

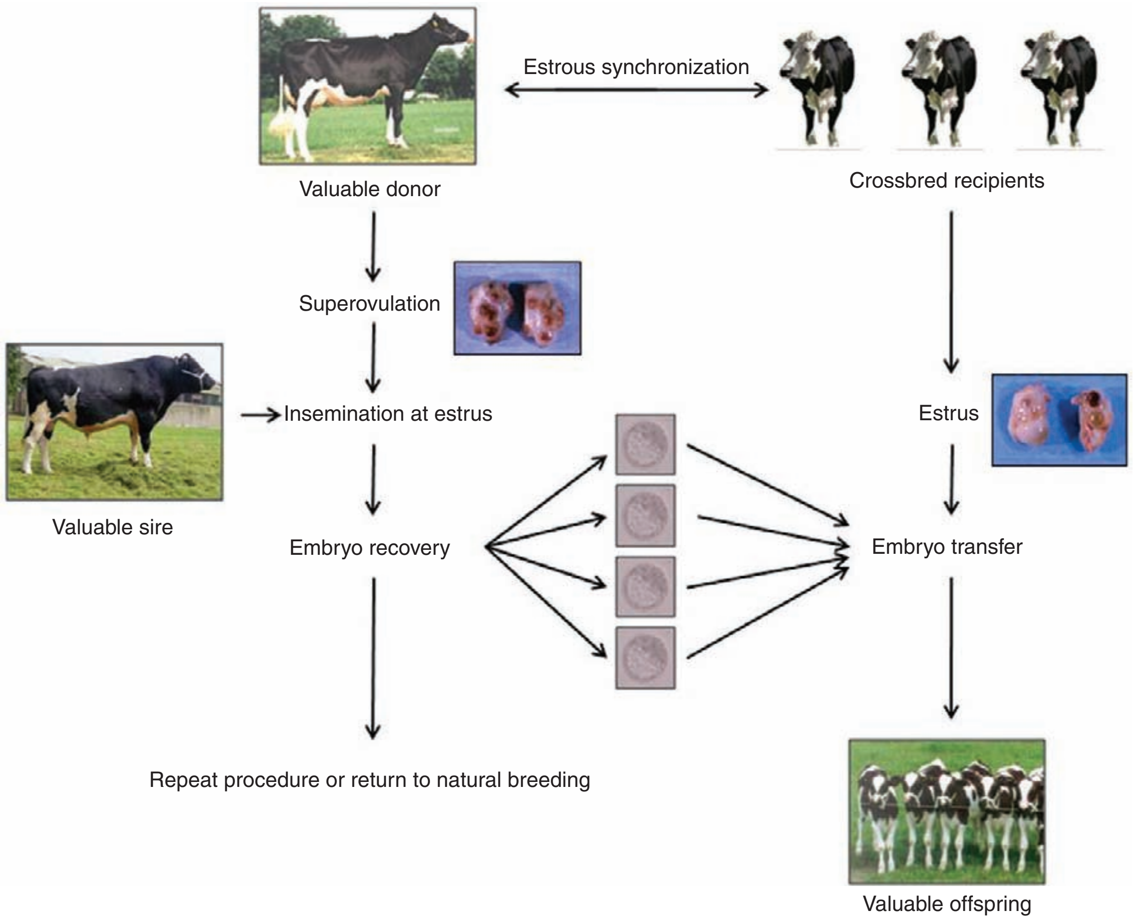

Figure 1 Overview of superovulation and embryo transfer. Following superovulation treatment, the valuable donor is inseminated with semen from a high genetic merit sire. Seven days later, embryos are recovered by nonsurgical uterine flushing. Grade 1 embryos are transferred to synchronized recipient animals, which carry the pregnancy to term. Meanwhile, the valuable donor can be resynchronized and superovulated to produce more embryos, or she can be returned to natural breeding.

Factors Affecting the Superovulatory Response

A reference estrus is necessary before initiation of gonadotropin treatment if a good response is to be expected. This may be related to the stage of follicular development, as most of the treatments are imposed within the interphase between the first and second dominant follicles of the cycle. Where donors are stimulated in the absence of a prior reference estrus, the response is normally extremely poor. In such cases, a beneficial effect of a progestagen device can be observed and, if such animals are superovulated with an exogenous progestagen device in place, the response will be intermediate between that observed after a reference estrus and that with no observed estrus.

Protocols controlling follicular wave emergence and ovulation have had a great impact on the application of on-farm ET as they allow the initiation of superovulatory treatment at a self-appointed time. Until recently, the most commonly used approach for synchronization of follicle wave emergence involved estradiol, which is no longer allowed in many countries. Possible alternative treatments include the following: (1) mechanical removal or ablation of the dominant follicle by ultrasound-guided aspiration; (2) use of exogenous gonadotropin-releasing hormone (GnRH) or LH to induce ovulation of the dominant follicle, thus synchronizing new wave emergence (however, efficacy is dependent on the stage of development of the follicle at treatment); and (3) use of FSH or eCG to initiate a new wave, irrespective of the presence of a dominant follicle, followed by superovulation at a predetermined time (this protocol has still to be thoroughly tested but shows some promise). Cows with low endogenous progesterone before superovulation have been recorded as yielding fewer embryos compared with those with normal or high progesterone values. The interval postpartum when gonadotropin is administered has a significant effect, as there are indications that treatment between 90 and about 110 days postpartum will result in lower embryo production, and this may be related to the population of medium follicles at this time. Treatment between 50 and 70 days or after 110 days results in normal

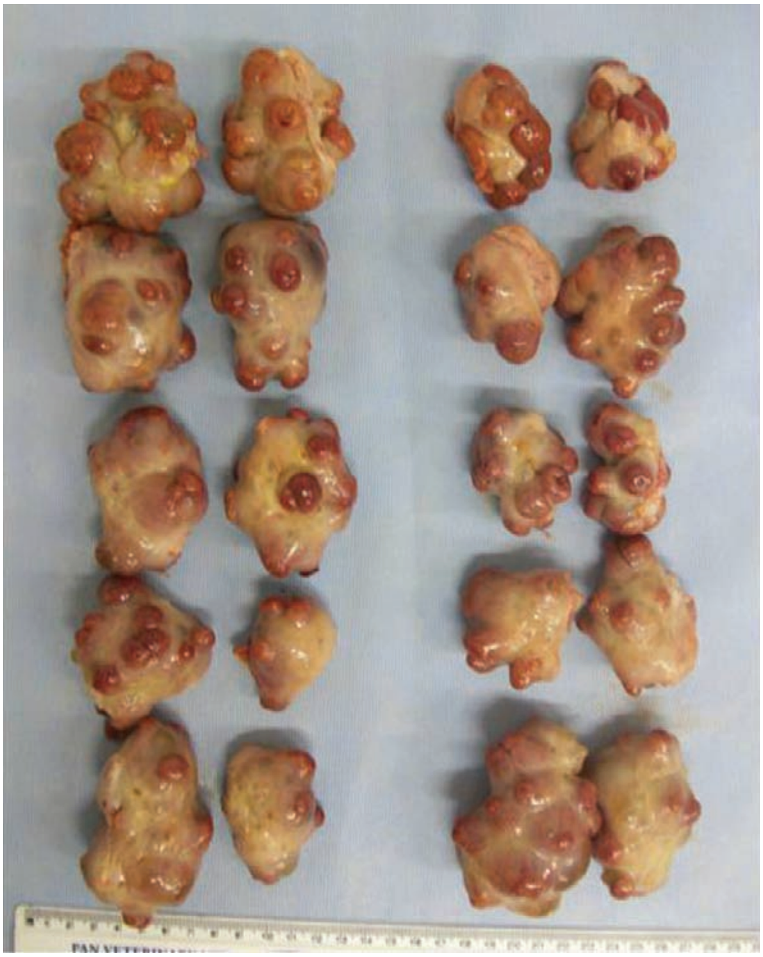

Figure 2 Pairs of superovulated ovaries collected from cows slaughtered 7 days after insemination. Note the presence of multiple corpora lutea on each ovary, each indicating a previous ovulation, and also the variation in individual response to treatment.

embryo production rates. If cows are known to have fertility problems, they are not good donors or recipients. Donor age is not regarded as a major limiting factor to superovulation. Indeed with the current trends in dairy production, very few cows will be in the herd when follicle populations are depleted through atresia to the extent that they will limit superovulation.

Nutrition has a major effect on certain aspects of reproduction in ruminants. Nutrition of the donor cow can influence oocyte and embryo quality, and thus affect the success of ET. Severe undernutrition compromises follicular development with implications for superovulatory response. In postpartum lactating cows, undernutrition or severe negative energy balance can delay resumption of ovulation, reduce the number of follicles, and compromise oocyte quality. Similarly, overfeeding energy and protein to cows is detrimental and should be avoided. The type of concentrate will influence the response in that slowly digested material such as beet pulp will give a better response than rapidly digested material such as barley. Feeding a high-fat diet to heifers has been shown to increase the numbers of follicles, but did not enhance the ovulatory response or yield of good-quality embryos. Feeding organic minerals especially selenium, zinc, and copper has been shown to increase the yield of good-quality embryos without having a major effect on ovulation rate. This will be particularly helpful if mineral deficiencies occur. Other factors such as season, particularly if associated with high environmental temperatures, will decrease the response to superovulation.

Mating the Donor Animal

The onset of estrus should be used to time inseminations. PGF2α-treated donors show estrous behavior sooner than nontreated animals (*see* **Reproduction, Events and Management**: Mating management: Detection of Estrus). The intensity of estrus may affect the ovulatory response; thus animals with no or weak signs of estrus may have a poor superovulatory response and low embryo yields. It is generally best to inseminate donors twice at 12-h

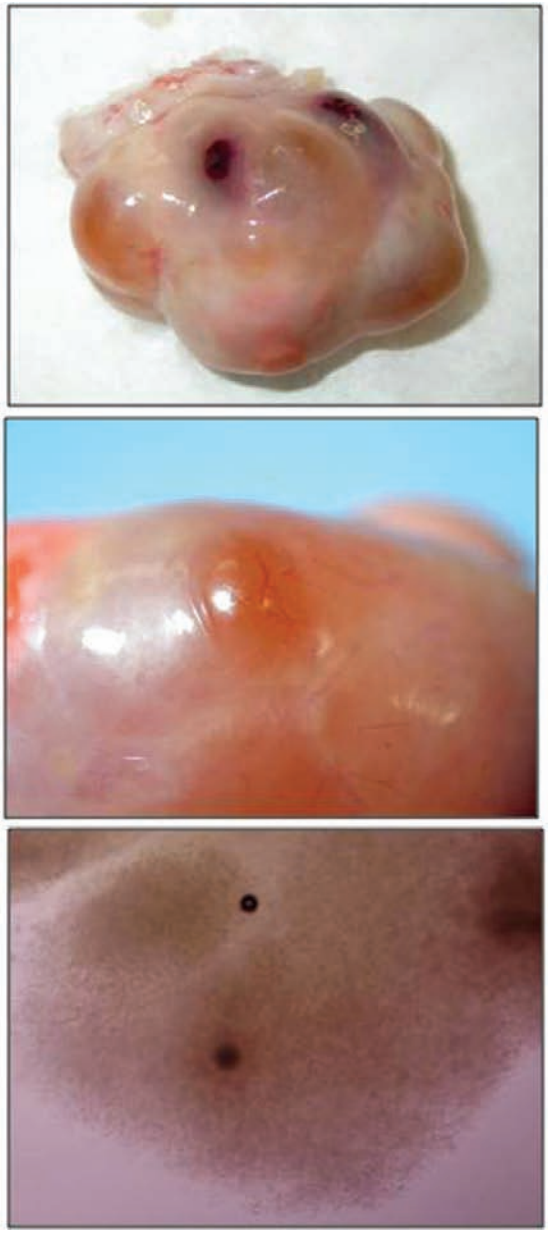

Figure 3 Top: Superovulated ovary around the time of ovulation. Ovulations occur over a period of hours; note the presence of two fresh corpora lutea indicating that two follicles have ovulated. Middle: Close up of preovulatory follicle on the point of rupture. Bottom: *In vivo*-matured oocyte recovered from a preovulatory follicle. Note the expanded cumulus cells surrounding the oocyte.

intervals during the superovulatory estrus. Inseminating donors late after the end of estrus will adversely affect embryo recovery. The use of high-quality semen is essential to ensure a supply of good-quality embryos. The choice of bull will affect the pregnancy rate to AI. With superovulated donors, this effect may be more accentuated and it has been suggested that AI bulls with above average fertility could be selected on the basis of the results obtained with superovulated cattle (**Figure 3**).

Embryo Recovery and Evaluation

Although the initial work on cattle ET involved surgical techniques, currently simple nonsurgical procedures are used except in experimental situations. Most embryo recoveries are timed to occur about 7 days after estrus, by which time the embryos have entered the uterus and are still contained within the zona pellucida making them more easily identified and more suitable for cryopreservation if required.

Using simple nonsurgical procedures it is possible to perform repeated recovery on the same animal without detrimental effects on either ovulatory response or embryo recovery rates. Animals normally receive a caudal epidural anesthetic, taking care to avoid the risk of overdosing in which case the cow will lose control of the hind limbs. When the epidural block is effective, the rectum is emptied and the vulva and perineal area are washed, scrubbed, treated with an antiseptic skin wash, and dried. The catheter (either a two-way or three-way) is inserted through the cervix and passed about two-thirds the way up the uterine horn. The cuff is inflated and irrigation of the uterine horn commences. Generally, about 400 ml of phosphate-buffered saline (PBS) supplemented with bovine serum albumin or serum is used to flush each horn. When the first horn is flushed, the procedure is repeated on the second side. Normally, in excess of 90% of the fluid administered is recovered but only about 60–70% of ovulated oocytes are recovered as ova/embryos. Even using surgical procedures at this time, the recovery rate will not be much higher.

To achieve optimum pregnancy rates following transfer, it is necessary that clearly defined criteria are available to assess the quality of the recovered embryos. There are now defined chronological and morphological criteria, as outlined by the International Embryo Transfer Society, for evaluating the normality and developmental potential of cattle embryos. Many of these center on characteristics such as cell size, shape of the embryo, degree of blastomere extrusion, and color and overall dimension of the embryonic mass. Essentially, embryos are classified as excellent, very good, good, fair, or degenerate, although individual groups may use numbers to indicate stage of development and morphological grade. There is a clear relationship between morphological grade and subsequent pregnancy rate following transfer. Attempts to relate embryo quality and more objective measurements like oxygen consumption are not yet developed to the stage of commercial application.

Two main factors are taken into account when evaluating an embryo; these include general appearance and stage of development in relation to its estimated age (**Figure 4**). The diameter of the embryo remains essentially unchanged from the zygotic stage until the

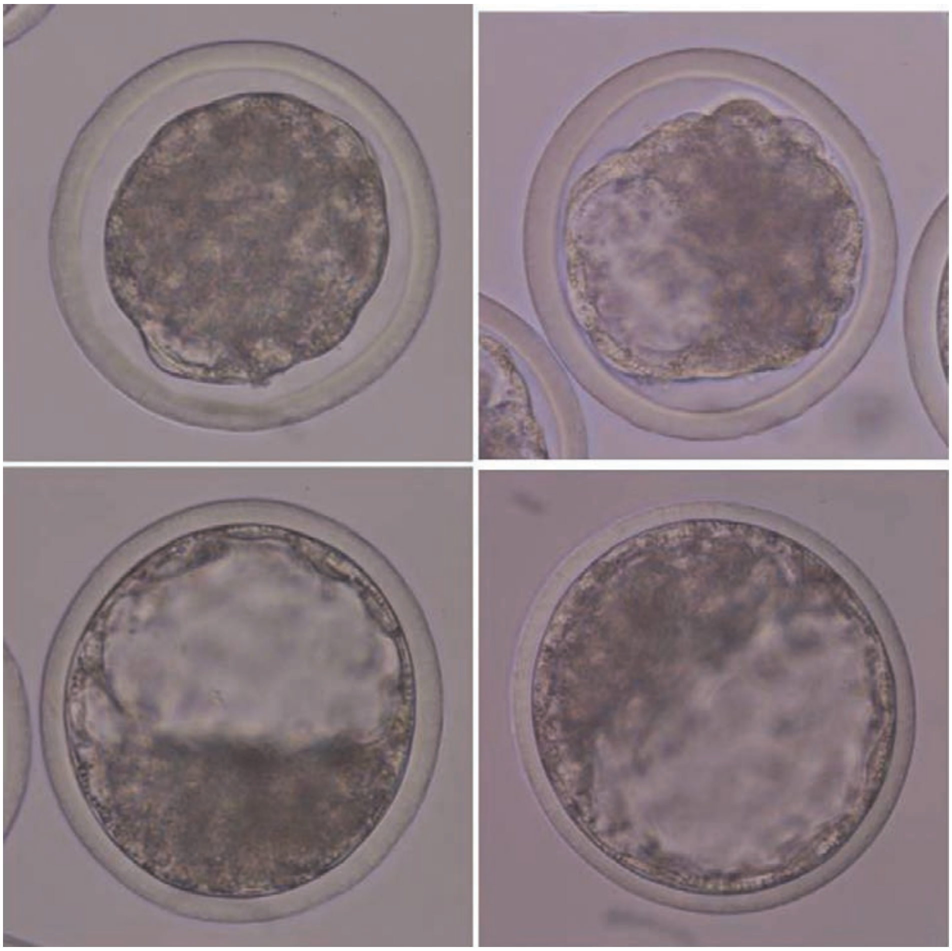

Figure 4 Representative photographs of good-quality transferable embryos recovered 7 days after insemination from a superovulated donor (top row: morulae prior to blastocoel cavity formation; bottom row: blastocysts with fully formed blastocoel cavity). Following nonsurgical flushing on day 7, a variety of structures can be obtained ranging from nonfertilized oocytes and arrested or degenerating embryos to good-quality transferable embryos as shown here.

commencement of blastocyst expansion. Recovery of embryos at day 7 would be expected to yield mainly morulae or blastocysts. However, there are many recorded instances of embryos at much earlier stages of development. It is reasonable to believe that these embryos are less likely to be competent to establish and maintain a pregnancy, as efforts to culture some of these resulted in relatively poor development. There is some evidence that the incidence of abnormalities may be higher in superovulated cattle than in unstimulated animals. This is not too surprising since superovulation may well rescue some atretic follicles and result in ovulation of oocytes that are less competent. Since it has been demonstrated that age of the dominant follicle at ovulation will influence pregnancy rates, it is safe to assume that some oocytes from superovulated follicles may not have sufficient developmental competence to develop to the transferable stage and, ultimately, to establish a pregnancy.

Embryo Storage

To achieve optimum pregnancy rates, it is essential that embryos are transferred to synchronized recipients as soon as possible after recovery or else are cryopreserved for future transfer. However, it is possible to store embryos *in vitro* for several hours, and as part of the normal ET practice, there is an obvious need to store embryos, on a temporary basis, before transfer. However, embryo viability will start to decline after about 12 h storage *in vitro*. It has been shown that pregnancy rates after the transfer of frozen/thawed cattle embryos may be inversely proportional to the time from embryo recovery to the start of freezing.

The choice of media for embryo recovery and storage has ranged from complex media such as M-199 and Ham's F-10 to simple formulations such as PBS supplemented with serum. For on-farm conditions, the use of PBS with a low (2–3%) serum content is quite adequate for flushing, and for storage the serum content should be increased to at least 10%. For short-term storage, room temperature will suffice, but high viability has been recorded after low-temperature (4–5 °C) storage for 24–48 h. Like spermatozoa, embryos experience a reduction in metabolism when maintained at refrigeration or lower temperatures. This will facilitate movement to a different geographical location without losing the synchronization effects necessary for the establishment of pregnancy.

It is widely held that frozen embryos will remain viable indefinitely, provided they are not damaged during the freezing or thawing process. In practice, it is normal to store the frozen embryos for a limited period of time, but cryopreservation facilitates international transport of embryos, which has helped to increase milk production in many parts of the world. While there is a clear relationship between embryo quality and pregnancy rates using fresh embryos, there is also a definite relationship between embryo quality and viability postthawing. Only top-quality embryos will survive the cryopreservation process, as up to 20% or more of blastomeres are damaged during freezing or thawing. The principles of cryopreservation are similar for many cell types, with the most important step being dehydration through the use of cryoprotectants before the cells are frozen in order to avoid intracellular ice crystal formation. Most of the work on cryopreservation has included about 10% of a cryoprotectant (e.g., glycerol or ethylene glycol) in the medium and embryos are cooled at a rate of 0.3–0.5 °C $\min^{-1}$ to -30 to -35 °C using controlled conditions before being plunged directly into liquid nitrogen. Thawing techniques will depend on the methods used for freezing, but they generally involve brief exposure to air before transfer to water; exposure to air warms them slowly prior to rapid warming in water at 37 °C. Initially, thawing involved stepwise dilution of the cryoprotectant, but with the use of ethylene glycol it is now possible to remove this in the straw using sucrose at low concentrations (0.25 mol l^{-1}). This has major practical applications, as an embryologist is not required on the farm at the time of transfer provided that the technician applies strict criteria to thawing the embryos. More recently, inclusion of high concentrations of cryoprotectants has enabled the process of vitrification to be employed. This procedure requires a very strict exposure time of the embryo to cryoprotectant, as the high cryoprotectant concentrations are detrimental to embryo survival. This technique requires further development before it becomes widely applied in the field.

Embryo Transfer

Initially, most embryos were transferred surgically using either midventral or flank incisions. This resulted in high pregnancy rates but was not suited to most on-farm applications. The development of simple nonsurgical transfer procedures facilitated the widespread application of this technology on farms. To achieve high pregnancy rates, the estrous cycles of the donor and recipient animals should be closely synchronized. If synchrony is out by more than 1 day, there will be a drop in pregnancy rate. The embryo should be deposited as far up the ipsilateral uterine horn as possible without causing undue trauma to the cow or the uterus. The loaded ET gun is inserted into an aseptic sheath, which is passed into the vagina as far as the cervix; the plastic sheath is then ruptured and the gun passed through the cervix and as far up the ipsilateral horn as possible. Transfers can be carried out under the influence of epidural anesthesia or without anesthesia, particularly if recipients are cows. When using maiden heifers, it may not be possible to pass the gun through the cervix in about 10% of animals at day 7. Embryo quality has the most significant effect on whether the recipient becomes pregnant, while the operator skill is also very important.

Selection of Donors and Recipients

Donors are normally selected on the basis of genetic merit, but once selected they must have a reference estrus so that a good superovulatory response and yield of embryos may be obtained. Selection of the recipient may be more problematic as the breed of embryo transferred needs to be considered carefully. Maiden heifers are considered optimum in terms of establishing and maintaining a pregnancy. However, care must be taken to ensure that the breed of calf will not lead to undue calving difficulties or that cesarean sections are required unless unavoidable. It is not well established why heifer recipients exhibit higher pregnancy rates regardless of whether they receive fresh or frozen embryos; the transfer of embryos to the parous cow is easier because of the anatomy of the cervix. Much discussion and thought has gone into methods of selecting recipients for transfer. This is based on the fact that if recipients have low progesterone concentrations, pregnancy rates will be lower. It is difficult to be accurate in selecting recipients on the basis of corpus luteum size and texture per rectum; ultrasonography will have a role to play in this selection. Monitoring progesterone concentration is probably the best method. However, a single sample taken just before transfer may not be ideal as recent data suggest that increases in progesterone concentrations around day 4 or 5 may be critical to the establishment of pregnancy in lactating dairy cows. Further work is required to establish a similar role in recipients.

Techniques and Facilities

The facilities required will be related to the degree of sophistication of the operation. For simple embryo recovery and transfer, a small mobile unit will produce very good results provided that attention to detail is good. The farm requires good animal handling facilities, which must be covered in inclement weather.

Impact and Potential

ET technology has facilitated the international transport of genetic material at low cost and with high efficiency. This has had major influences on breeding programs in certain parts of the world. Embryos can be cryopreserved and stored for hundreds of years, which has applications for the maintenance of genetic diversity and endangered breeds. In addition, it is possible to accurately determine the sex of the embryo prior to transfer by taking a small biopsy and subjecting it to PCR. Further developments in the sex-sorting of sperm will have positive effects on both *in vivo*- and *in vitro*-produced embryos (*see* **Gamete and Embryo Technology**: Sexed Offspring). ET is an essential technique for the exploitation of the numbers of selected and sexed embryos that will be available for the dairy industry. It is also possible that ET could alleviate some of the low pregnancy rates observed in summer heat stress conditions (see **Stress in Dairy Animals**: Heat Stress: Effects on Reproduction), by providing embryos that were harvested under good environmental conditions and frozen.

See also: **Gamete and Embryo Technology**: Artificial Insemination; Cloning; *In Vitro* Fertilization; Sexed Offspring. **Reproduction, Events and Management**: Estrous Cycles: Characteristics; Mating Management: Detection of Estrus. **Stress in Dairy Animals**: Heat Stress: Effects on Reproduction.

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Relevant Websites

http://www.iets.org – International Embryo Transfer Society

Sexed Offspring

J F Hasler, Bonner Creek Ranch, Laporte, CO, USA

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Introduction

Beginning with the earliest domestication of livestock, man has desired to predetermine the sex of offspring. Ineffective folklore approaches have been described for millennia. The first success, approximately 30 years ago, involved karyotyping of cells obtained via biopsy of preimplantation bovine embryos. Much faster and accurate sexing of embryo biopsies became possible within the last 10 years with the development of specific protocols using the polymerase chain reaction (PCR). Determination of embryo sex results in the inefficiency of discarding the embryos of undesired sex. In addition, it is necessary to employ embryo transfer procedures to use embryo sexing as a tool. Sexing semen prior to fertilization eliminates these drawbacks. Thus, recent advances in sperm sexing technology have provided an accurate, efficient, and less wasteful means of predetermining the sex of offspring.

Sexing Embryos with PCR

A number of different techniques have been employed to determine the sex of preimplantation bovine embryos. Elongated 14- to 15-day-old bovine embryos can be biopsied and sexed by karyotyping in approximately 3.5 h. However, only approximately 60% of embryos provide usable karyotypes, and the necessity of utilizing older, hatched, elongated blastocysts is a major drawback in commercial embryo transfer programs. Biopsies taken from younger embryos recovered prior to hatching provide few cells and very low rates of successful karyotyping. A noninvasive approach to sexing involving the detection of H-Y antigen received a good deal of attention in the early 1980s. However, this technology has never been commercialized, partly due to its subjective nature and partly due to the difficulty of generating large amounts of suitable antibodies.

Another method of sex determination that appeared to hold promise was the quantification of X-linked enzymes. In mice, this technique, which required approximately 12 h to run, proved to be over 90% accurate when applied to only one blastomere. It has not, however, been successfully commercialized for use in cattle.

The field of molecular biology has provided extremely sensitive and accurate tools for embryo sexing. Repetitive, male-specific DNA sequences on the bovine Y-chromosome provide a basis for distinguishing between X- and Y-chromosomes by utilizing PCR. This procedure permits amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA.

Embryo Biopsy

PCR protocols designed for determining the sex of preimplantation bovine embryos vary in sensitivity, requiring DNA from as few as 1 to as many as 6–10 cells. Biopsies are obtained either manually with a handheld blade or with the use of a micromanipulator (**Figure 1**). Manual biopsy is an art requiring a very steady hand. The ability to obtain suitable manual biopsies varies greatly among individuals. The two most common techniques involving the use of micromanipulators are biopsy removal by cutting with either a knife or a fine needle and biopsy removal by aspirating cells from the embryo with a pipette. Neither method is particularly suitable for the removal of only one blastomere in that biopsies ranging in size from 6 to 15 cells are often reported. Bovine *in vivo*- and *in vitro*-derived embryos are normally biopsied at 7–7.5 days, coinciding with the age at which most embryo transfer or freezing is performed. While biopsies can be taken from any portion on the periphery of morulae, blastocysts should be oriented such that the biopsy is obtained from the trophectoderm, leaving the inner cell mass intact. Extruded cells that are sometimes found in the perivitelline space should not constitute the entire biopsy sample since there is no guarantee that they contain amplifiable DNA. During the biopsy procedure, embryos must be maintained in separate droplets of medium so that there is no chance of contamination among biopsies.

Biopsies taken with a blade are normally done in protein-free medium so that the embryos adhere to the bottom of the Petri dish. The blade can be used to roll the embryo to achieve the desired orientation. Biopsy removal involves lowering the blade over the edge of the embryo until it contacts the bottom of the Petri dish and then applying a gentle sawing action to the blade. A finely drawn glass needle can be used in a very similar fashion.

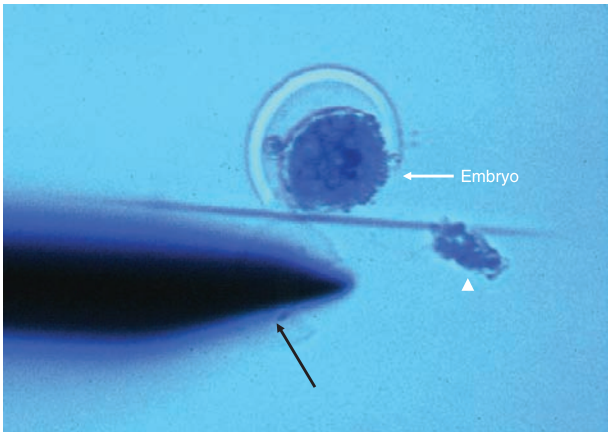

Figure 1 Biopsy of tissue from an early blastocyst. A portion of the embryo (arrowhead) was excised for subsequent PCR analysis using an ophthalmic knife (black arrow). The faint line below the embryo resulted from scoring the plastic dish during the process.

Biopsies obtained by aspiration involve the use of a holding pipette to which a slight suction is applied to the zona pellucida (ZP) in order to stabilize the embryo. A micropipette with a sharpened beveled tip is introduced through the ZP and the biopsy is aspirated into the pipette. The biopsy is then expelled into the drop holding the embryo so that it can be picked up with a handheld pipette and transferred to a PCR tube.

Biopsy Handling

To facilitate the release of the embryo and biopsy from the bottom of the Petri dish, a surfactant such as polyvinyl alcohol is used. The use of serum or bovine serum albumen increases the risk of introducing DNA into the system. Biopsies are individually picked up with a disposable pipette tip and placed into a reaction tube. DNA is usually extracted from the cells by exposure to proteinase K or by a freeze–thaw cycle.

Polymerase Chain Reaction

PCR protocols for detection of the sex of embryos are based on the amplification of Y-chromosome-specific DNA. There are a number of species-specific sequences on the Y-chromosome that are repeated hundreds and, in some cases, thousands of times in mammalian species, including cattle. Primers to identify an autosomal chromosome or the Y-chromosome are often included in PCR trials as controls. The number of amplification cycles required varies with different PCR protocols. Typically, 30–50 cycles of denaturation, annealing, and primer extension are conducted. The specific length of these steps and the recycling speed of the particular thermocycler result in total amplification times ranging from 1.5 to 3 h.

Analysis of PCR

Following amplification, most sexing protocols for sexing embryos require a step in which the PCR products are supported by agarose gel electrophoresis. The resulting bands contain amplified DNA stained with the DNA intercalating dye ethidium bromide. The bands are then visualized on an ultraviolet transilluminator (**Figure 2**). A nonelectrophoretic PCR method of sexing is based on visualizing the presence of ethidium bromide-stained Y-chromosome DNA at the end of the amplification cycles directly in the reaction tubes (**Figure 3**). This approach shortens the time for completion of the assay and decreases the chances of contamination in the laboratory that could result from the release of amplified DNA when opening the reaction tubes. The nonelectrophoretic PCR method has recently been shortened to a total run time of about 35 min by incorporating an extremely fast DNA polymerase and amplification in a new generation of thermal cyclers with ultrafast ramp times.

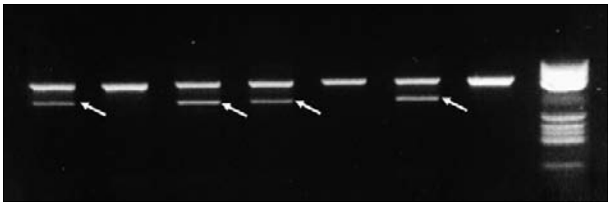

Figure 2 Electrophoretic characterization of PCR products separated through an agarose gel. The resulting bands of amplified DNA were stained with ethidium bromide for fluorescence visualization. Y-chromosome-specific bands from male embryos are indicated (arrows).

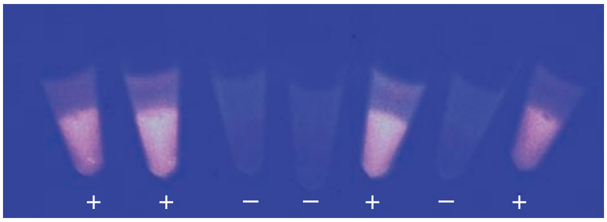

Figure 3 PCR determination of the presence of Y-chromosome in biopsied tissues from seven embryos using ethidium bromide-labeled Y-chromosome-specific PCR primers. Fluorescent illumination is used to identify the male embryos by the pinkish-red stain (+) while female embryos do not fluoresce (–).

Non-PCR Methods of Sexing Bovine Embryos

A non-PCR method of sexing embryos based on loop-mediated isothermal amplification (LAMP) was recently developed in Japan. The LAMP technology is based on the amplification of a target DNA using a DNA polymerase, resulting in the accumulation of increased amounts of amplification products. The end result is a precipitate of magnesium pyrophosphate, which is detected photometrically. This technique has been shown to work with a small embryo biopsy and is currently available in a kit form.

Another recently developed non-PCR approach to embryo sexing is based on the incubation of an embryo biopsy with a peptide nucleic acid probe that targets a unique Y-chromosome-specific sequence. The probe is conjugated to a fluorescent dye and appears as bright spots under fluorescent microscopy.

Freezing and Transfer of Biopsied Embryos

Following biopsy, bovine embryos can be maintained in culture while the PCR process is being conducted. When PCR results are available, embryos can be transferred, frozen, or discarded. Alternatively, embryos can be either transferred or frozen immediately after biopsy. Then the frozen embryos of undesired sex can be discarded at a later date. Similarly, recipient cattle carrying embryos of undesired sex can be recycled with prostaglandin, although this is not an optimally efficient management of recipients. For embryos held in culture after biopsy, there are no conclusive data available indicating whether there is an advantage in embryo viability based on either short-term culture in a CO_2 incubator at 39 °C or culture on the benchtop at ambient temperature.

Pregnancy rates in recipients receiving either fresh *in vivo-* or *in vitro-*derived embryos are slightly to moderately less than what is observed for intact control embryos. Pregnancy rates following transfer of frozen–thawed biopsied embryos are substantially lower in some cases than those for recipients receiving intact embryos. The quality of embryos at the time of biopsy has a substantial effect on the pregnancy rate of biopsied embryos whether they are transferred fresh or after being frozen and thawed. Whether or not the ZP is intact on biopsied embryos prior to freezing is not clearly proven. PCR of biopsies obtained after thawing of embryos previously frozen has been successful, with reports of moderate pregnancy rates.

PCR for sexing bovine embryos has not been widely applied in the embryo transfer industry. This is partly due to the fact that the equipment for conducting PCR is relatively expensive, as is the micromanipulatory setup required if manual biopsies are not conducted. Performing biopsies requires a rather high level of skill even with the use of a micromanipulator. Most embryo transfer procedures are conducted on-farm and inclusion of embryo biopsy and PCR may add substantially to the time spent on site on any given day. Also, most PCR procedures are rather sensitive to contamination, and thus it is not practical to conduct them in a confined mobile laboratory. Consequently, most embryo sexing is conducted at permanent embryo transfer facilities. In addition, there are some situations where embryos are biopsied on-farm and then frozen or transferred, with the PCR conducted in the permanent base facility at a later date. This, of course, is less efficient. Another factor limiting the spread of embryo sexing is the concern regarding the potential loss of income among practitioners. This is because embryo transfer services are often charged on the basis of the number of embryos handled, be they frozen or transferred. Therefore, discarded embryos of unwanted sex may represent lost income.

Gametes: Sex Preselection by Flow Sorting Bull Sperm

Sex-Sorting Sperm

Sex-sorting sperm by DNA content provides a means for predetermining the sex of calves with 85–95% accuracy. This sexing process, which is now commercialized at numerous sites worldwide, uses a flow cytometer/cell sorter to separate sperm according to their DNA content. X-chromosome-bearing sperm, which produce heifers, contain nearly 4% more DNA than Y-chromosome-containing sperm, which produce bull calves. DNA content is measured by staining freshly collected semen with a specific bisbenzimidazole DNA-binding dye, Hoechst 33342. After incubating the sperm for 1 h with the dye, the stained sperm fluoresce bright blue when exposed to a laser beam of short wavelength light (**Figure 4**). Bisbenzimidazole-stained X-sperm emit proportionally brighter fluorescence than Y-sperm because of the greater DNA content. The fluorescence signals emitted from the stained X- and Y-sperm can be accurately measured with a photomultiplier tube (PMT). Sperm must be oriented properly within the nozzle for their DNA content to be measured accurately. The signals emitting from the sperm are quantified and processed by a computer as they pass single file in front of the PMT and thereby allow accurate measurement of DNA content for most, but not all, sperm. This measurement occurs just as the fluid stream containing the sperm exits the nozzle of the sorter. The nozzle itself is vibrated at a high frequency causing individual droplets to form at a rate of about 80 000 s^{-1}. Droplets that contain sperm are given a positive or a negative charge, depending on the DNA

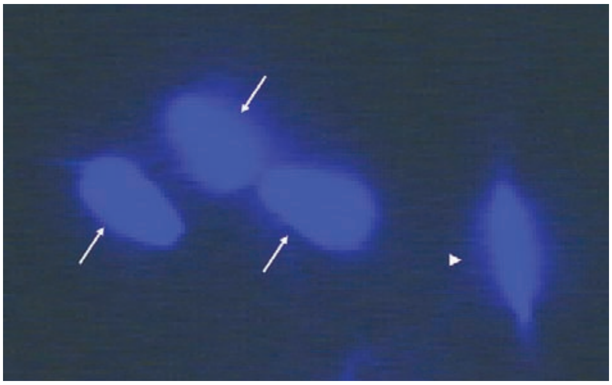

Figure 4 Bovine sperm fluorescently stained with Hoechst 33342 for measurement of the 4% DNA difference between X- and Y-chromosome-bearing sperm. Orientation is necessary because DNA content can be measured accurately only from the flat surface of precisely oriented sperm heads (arrows) rather than from the narrow edge (arrowhead) or from misaligned sperm nuclei.

content information from the detector. No charge is applied to droplets without sperm, droplets with more than one sperm, or those sperm whose DNA content cannot be measured precisely. Dead sperm are differentiated from the live sperm by uptake of a membrane-impermeant second dye and are simply disposed of as waste. The flow cytometer/cell sorter system that is used to sex sperm is a MoFlo® SX (Dako, Fort Collins, CO, USA) (**Figure 5**).

Droplets containing sperm, which had been negatively or positively charged according to sperm DNA content, are deflected by a brass plate charged opposite to that of the sperm. The streams of droplets flowing from the nozzle, which contain X-sperm, Y-sperm, or more than one sperm or no sperm, are collected into three separate vessels. This sorting process allows sexing and collection of up to 40% of the sperm that transit the sorter at a speed of approximately 90 km h^{-1} at an event rate of 30 000 total sperm per second. From 3000 to 5000 live sperm per second of each sex can be sorted simultaneously, thereby producing nearly 12–18 × 10^{6} live sperm per hour of each sex at an accuracy of 85–95%. Separation of the X- and Y-sperm peaks is shown in **Figure 6**. This sperm-sorting system is capable of producing more than 200 straws of sexed sperm per machine per 24 h. However, the process loses considerable numbers of sorted sperm due to post-sorting steps such as centrifugation required to concentrate the sperm after sorting. Some sperm-sorting facilities have more than a dozen machines simultaneously operating 24 h, 7 days a week. Thus, sexed semen from reasonably high-quality sires is now available worldwide. Current recommendations are that sexed semen be used for heifers only due to their inherent greater fertility.

Packaging Sperm for Low-Dose Insemination

The first sex-selected calves were from embryos derived from *in vitro* fertilization (IVF) with sex-sorted sperm. Most recent sex to selection work, however, has used artificial insemination (AI) with low numbers of sex-sorted, cryopreserved sperm. The sorting process dilutes sperm, so they must be reconcentrated by centrifugation prior to packaging for insemination. The reconcentrated sperm,

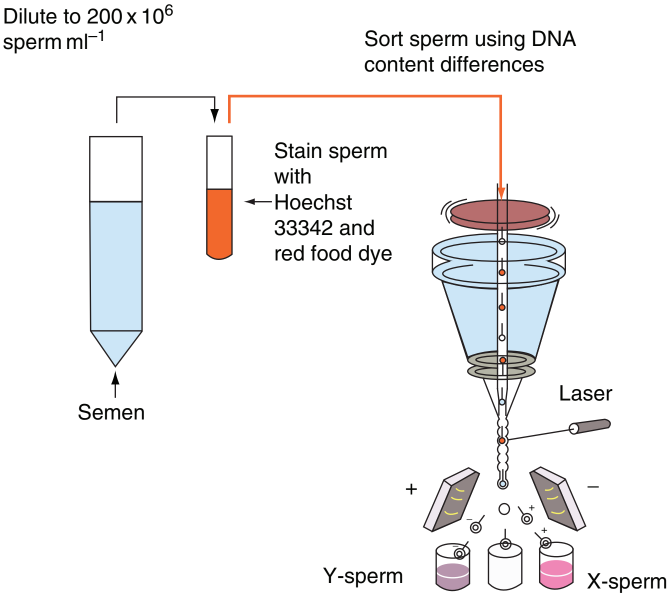
Dilute to 200 x 10^{6} sperm ml^{-1}
Sort sperm using DNA content differences
Stain sperm with Hoechst 33342 and red food dye
Semen
Laser
+
−
Y-sperm
X-sperm

Figure 5 Semen processing, staining, and flow sorting with a MoFlo® SX to produce sexed sperm for use with artificial insemination or *in vitro* fertilization.

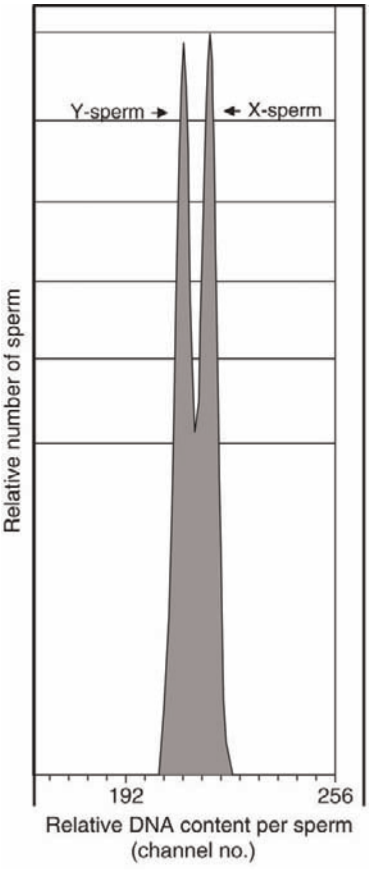

Figure 6 Histogram showing the Hoechst 33342-stained X- and Y-chromosome-bearing bovine sperm populations as revealed by laser illumination and quantified with a flow cytometer/cell sorter (MoFlo® SX).

200×10^6 sperm ml^{-1}, are packaged at about 2×10^6 sperm per dose in 0.25 ml^{-1} French straws. This dose is about 1/10 of the sperm normally used for conventional AI. When semen from highly fertile bulls is used, pregnancy rates of 80% of that achieved in heifers with only 2×10^6 sperm per dose can be achieved. The semen from most, but not all, bulls can be used for production of sexed sperm. As expected, low doses of sexed sperm magnify the variation among bulls in pregnancy rates achieved when sperm are sexed and used for insemination.

Successful cryopreservation of sexed sperm makes AI of estrus-synchronized heifers much more efficient. Thus, the sorting effort can take place at the site of semen collection rather than where insemination is to occur.

Estrus Synchronization

Successful commercial application of low doses of sexed sperm can be achieved only if properly managed heifers are used for insemination. Careful estrus detection and specific synchronization regimens have been successful with low doses of sexed sperm. Appropriate synchronization regimens for sexed sperm include (1) 500 mg of melengestrol acetate (MGA) fed daily in grain for 14 days followed by an intramuscular (i.m.) injection of 25 mg prostaglandin $F_2\alpha$ 17, 18, or 19 days after the last day of feeding MGA; (2) a single i.m. injection of 25 mg prostaglandin $F_2\alpha$; (3) 20 or 25 mg prostaglandin $F_2\alpha$ injected i.m. at 12-day intervals; and (4) 50 or 100 μg gonadotropin-releasing hormone (GnRH) injected i.m. followed by 25 mg prostaglandin $F_2\alpha$ 7 days later.

Insemination

Even though heifers have been synchronized, it is prudent to use only those animals that exhibit estrus when using sexed sperm. The current practice is to inseminate only once a day at 4.00 p.m., which is approximately 1/2 or 1 day following the onset of estrus. This insemination regimen with sexed sperm has not revealed any difference in pregnancy rates between heifers inseminated after 1/2 day (45%) and those inseminated after 1 day (49%).

Cryopreserved straws of sex-sorted sperm are normally thawed for 20–30 s in a 34–37 °C water bath before being used immediately. Two deposition sites have been examined: (1) placement into the lumen of the uterine body, as is done for conventional AI, or (2) deep in the uterine horn. Deposition of sperm at the end of the uterine horn has not resulted in consistently higher pregnancy rates than body insemination.

Pregnancy Rates

Nearly a million bovine pregnancies have been produced from sexed sperm using 2×10^6 sexed, cryopreserved sperm per dose. The pregnancy rates for the sexed sperm have been about 80% of that achieved in unsexed control samples with 7–20 times more sperm per insemination dose. For most inseminations, sperm are placed in the uterine body as is done for conventional AI.

Some fertility differences among bulls have been noted. Thus, only highly fertile bulls should be used to maximize the likelihood of achieving acceptable pregnancy rates.

Calving Results

The percentage of heifer calves born to sex-sorted sperm has been about 89%, which is consistent with that estimated from the proportion of X-sperm in sex-sorted inseminates. Most sex-predetermined calves have been produced with the use of sexed sperm in heifers. While not recommended, marginal success has also been achieved with the use of sexed sperm in cows. Although rigorous epidemiological studies have not been done, no apparent increase in embryonic deaths is observed between 1 and 2 months of gestation, with very few abortions occurring between 2 months and term.

Angus heifers that were themselves produced from sexed sperm calved as a result of being inseminated with sexed sperm. This second generation of calves demonstrates the feasibility of using an all-heifer production system, whereby heifers are bred with X-sperm to produce their own replacements.

In Vitro Fertilization

Although most sex-sorted sperm have been used in AI, efforts toward optimizing them for IVF continue. Further work is needed because embryos derived from sex-sorted sperm tend to develop somewhat slower than those derived from unsexed, conventional semen sources. Interest in predetermining sex by use of sex-sorted sperm in IVF continues and demand will likely increase in the future.

Conclusion

Sex preselection in mammals can be achieved either by sexing embryos and discarding those of the unwanted sex, and is likely to cease to be or by sexing sperm prior to fertilization. The goal of predetermining sex has been achieved after a millennium of efforts beginning when livestock were first domesticated. Advances in embryo and semen sexing technologies for cattle now provide accurate and efficient methods for predetermining the sex of offspring on a commercial basis. Embryo sexing will likely be replaced by semen sexing. It is likely to be relegated to the status of a quality control procedure and is likely to cease to be a production entity.

See also: **Gamete and Embryo Technology**: *In Vitro* Fertilization; Multiple Ovulation and Embryo Transfer. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Mating Management: Artificial Insemination, Utilization.

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Transgenic Animals

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Introduction

Over millennia, humans have shaped the genetic composition of today's livestock. Traditional breeding schemes and, more recently, marker-assisted selection strategies have been successfully used for incremental genetic improvement of livestock. However, the approach is relatively slow and nontargeted and also affects many nonselected traits associated, and hence has the potential for the concomitant introduction of undesirable characteristics. In principle, transgenic technology, which creates the potential to enhance existing characteristics at unprecedented magnitude and speed, can be seen as the logical progression from these more traditional efforts to modify livestock genomes. Unlike traditional breeding and selection, the technology is not restricted by the species barrier and can utilize the gene pool of other species to introduce entirely novel and unique characteristics. This can be achieved not only by additive strategies to introduce a new gene function (gain of function) but also by deleting gene functions (knockout, loss of function), replacing a gene function with a different one (knockin, exchange of function) or specifying when and where the genetic alteration is applied (conditional knockout).

Some of the initial transgenic livestock studies were hampered by the inability to appropriately control the activity of the newly introduced transgene. However, continual technological advances (**Table 1**), in particular the ability for precisely targeting modifications to defined genomic loci and advances in identifying the function of genes and their relationship with phenotypic traits, provide new opportunities for the purposeful modification of livestock animals to enhance their welfare, produce superior quality food and biomedical products, and reduce the environmental impact of farming.

With its wide range of applications, transgenic livestock technology could revolutionize animal agriculture to a similar or even greater extent than transgenic crops, which represent one of the most rapid introductions of a new technology in the history of agriculture. The first approval of a biomedical product from a transgenic goat has seen transgenic animals making the final transition from the research to commercial arena. However, several scientific, regulatory, and ethical issues that need to be resolved before transgenic livestock are found on a large number of farms and accepted in the marketplace remain.

Methodology for the Generation of Transgenic Animals

Although most transgenic livestock animals produced in the last 25 years have been generated by microinjection, this technology is becoming quickly superseded by new technological developments. In particular, cell-mediated transgenesis in combination with somatic cell nuclear transfer (SCNT) and transgenesis with vectors derived from lentiviruses have taken the production of transgenic livestock to a new level. The following is a brief description of today's most commonly used methods for the generation of transgenic animals.

Pronuclear Microinjection

Microinjection has been until recently the main method for the production of transgenic animals. The method involves the direct injection of many copies of a gene construct into one of the two pronuclei of a recently fertilized egg. The technology is limited by the lack of control over when and where the exogenous gene construct integrates into the genome. Only when the gene construct integrates will all daughter cells contain the new gene construct. In many cases, integration happens after the embryo has undergone cell division, which results in a mosaic animal in which some cells contain the gene constructs while others do not. Additional uncertainty is introduced by the integration of the transgene into a more or less random site of the genome. The transgene can be affected by the surrounding genomic DNA and result in animals that fail to express the transgene at adequate levels.

While the method has been very successful for the production of transgenic mice, transfer of the technology to other animals has been challenging. Injection is more technically difficult than for mice. For livestock, in addition, the efficiency of producing transgenic animals has proven very low with only 0.1–1% of injected zygotes typically resulting in transgenic offspring. Despite these hurdles, microinjection was used to produce the first transgenic livestock in 1985 and is the technology responsible for production of recombinant form of human antithrombin, ATryn, in the milk of transgenic goats, which represents the first transgenic product to gain market approval in Europe and the United States.

Table 1 Milestones in the development of transgenic animal technology

Year	*Milestone*
1980	First transgenic mice
1985	Transgenic sheep and pigs by microinjection
1989	Mice with site-specific genome modifications
1997	Transgenic sheep produced by SCNT
2000	Sheep with a site-specific genome modification
2002	Transchromosomal cattle with a human minichromosome
2003	Homozygous disruption of an endogenous gene in pigs
2004	Sequential targeting of multiple genomic loci in cattle
2006	Approval of first transgenic animal-produced protein as human drug
2009	Cattle with homozygous disruptions of multiple genes

SCNT, somatic cell nuclear transfer.

Viral-Mediated Transgenesis

Viruses are natural delivery vehicles for exogenous genetic material and are capable of introducing RNA or DNA into cells with very high efficiencies. Their often broad host range makes viral-mediated transgenesis a strategy with general applicability to many different species. To use them as carriers for exogenous genetic material, large parts of the viral genome are replaced with the transgene construct intended for introduction into cells and assembled into a viral particle. This results in a defective, replication-deficient virus that can efficiently infect and introduce the transgene construct into cells. Most importantly, it can do so only once. After the infection has taken place and the transgene construct has been introduced into the cells, the defective virus has no program to replicate so that formation of new infectious virus particles is prevented and its existence is terminated.

Viral-mediated transgenesis approaches initially used vectors based on retroviruses, which have the ability to stably integrate into the genome of dividing cells. In recent years, retroviruses have been superseded by the development of more sophisticated vector systems based on lentiviruses. Lentiviruses are more efficient at transfection than retroviral vectors because they infect dividing and nondividing cells. To generate transgenic animals, oocytes or embryos are simply injected or infected with lentiviral vectors. Following such a treatment, up to 80% of the animals born may be transgenic, making it one of the most simple and efficient methods for transgenesis.

The main disadvantages are the random nature of the insertion, frequently into multiple loci, and the strict limitation for the size of the exogenous genetic material that can be introduced due to the physical size constraints of the viral particle. In the case of lentiviral vectors, the viral particles can accommodate genetic material of up to 10 kb in size and thus are not suitable for transgenesis requiring large constructs.

Sperm-Mediated Transgenesis

Sperm are natural vectors for transmitting DNA in the sexual propagation of all mammalian species. The sperm-mediated transgenesis approach essentially tries to harness this natural ability to introduce foreign DNA into oocytes by 'loading' sperm with exogenous DNA prior to using it for fertilization or intracytoplasmic sperm injection. For 'loading', sperm are treated to achieve binding or internalization of foreign DNA by incubation of sperm with naked DNA or active transfection of sperm cells. Although conceptually elegant and technically very simple, sperm-mediated transgenic methods are often inconsistent and show high variability from species to species. Furthermore, in common with some of the other technologies, sperm-mediated transgenesis offers no control over the time of integration, and thus commonly results in mosaic animals.

Spermatogonial Stem Cell Transplantation

An alternative approach targets the precursor cells, the so-called spermatogonial stem cells (SSCs), which develop into sperm. SSCs are isolated from the testis and can be cultured *in vitro* for long periods. Routine transfection methods to introduce exogenous DNA into cultured cells, such as lipofection or electroporation, are used to genetically modify SSCs. Furthermore, the ability to culture SSCs provides the opportunity to fully characterize the cells and validate the stable integration of the exogenous DNA. To generate transgenic offspring, the modified SSCs are transplanted into the testes of a recipient male whose endogenous sperm-producing cells have been depleted. Following colonization of the host testes with the transgenic SSCs, functional sperm of the new genotype will be produced by the recipient male and routine breeding practices can be used to produce transgenic offspring.

Cell-Mediated Transgenesis Using Nuclear Transfer

SCNT, which is often referred to as cloning, describes a technology that essentially enables the generation of a whole animal from a single cell. For animal transgenesis, the SCNT process uses an individual transgenic cell from which an animal is generated. The nucleus from a suitable transgenic donor cell is transferred into an oocyte following removal of its own maternal chromosomes. This reconstructed one-cell embryo is then artificially stimulated to initiate embryonic development. After transfer into surrogate females for *in vivo* development to term, a proportion of embryos develop to term to result in transgenic offspring with the same genomic makeup as the original transgenic donor cell.

The main limitation of SCNT-mediated transgenesis is the poor efficiency of production of live transgenic offspring; only between 1% and 10% of transferred transgenic embryos result in live births. This inefficiency is assumed to be predominately caused by an inadequate transition of the gene activity program of the specialized somatic donor cell to the requirements of a developing embryo, which can result in a range of complications including embryonic and fetal losses, abnormal placentation, higher birth weights, and greater postnatal mortality. However, even with these limitations and particularly in comparison to other technologies, the SCNT cell-mediated approach provides an attractive technological platform for the production of transgenic animals with a number of distinct advantages, including (1) compatibility with readily available cells from a variety of sources and the ability to choose a specific genetic background and sex, (2) the potential to perform a wide repertoire of genetic modification in the cultured cell population, including not only the introduction of new DNA sequences from the same or a different species and even artificial chromosomes, but also functional deletion or repression of endogenous genes, (3) the ability to screen and validate cells for the specific genetic modification before producing the transgenic animals, (4) transgenesis in all of the embryos/offspring produced without any being mosaic, and (5) the generation of small herds from each cell line in the first generation, rather than individual founder animals that need to be subsequently bred. Although possible, SCNT faces limitations for the introduction of multiple or precisely controlled (site-specific) changes into the genome due to the limited proliferative capacity of somatic cells in culture and generally low efficiencies in producing live animals.

Cell-Mediated Transgenesis Based on Pluripotent Stem Cells

The use of pluripotent stem cells presents a great opportunity to improve the presently low gene targeting efficiencies for livestock animals. Such cells can divide indefinitely in culture, provide higher cloning efficiencies, and have a greater ability to recombine introduced exogenous DNA with homologous chromosomal sequences, which facilitates the introduction of more complex and controlled genome alterations. Thus, such cells would provide a superior type of donor cell that could be readily integrated into existing cloning systems. Today's exceptional ability for site-specific alteration of the mouse genome is intimately linked with the unique characteristics of pluripotent stem cells derived from early embryos called embryonic stem cells (ESCs). Here, the genetic modification is first introduced into mouse ESCs. Subsequently, these transgenic ESCs are injected into or aggregated with a 'normal', early mouse embryo where they can contribute to all cell types of the developing animal, including the germ cells because of their pluripotent nature.

This methodology generally results in the so-called chimeric animals, which contain cells of two different origins: normal cells derived from the early embryo and the modified ESCs. Transgenic mice containing the genetic modification in all cells are routinely produced by breeding mice with a chimeric germ line. For larger animals with longer generation intervals, the requirement of an additional breeding step for the production of a fully transgenic animal presents a major limitation for this particular ESC technology for livestock applications. In the mouse, there are alternative routes available that allow for the direct production of a completely transgenic animal from ESCs. This includes the use of a tetraploid embryo instead of a normal embryo that can contribute cells only to the placenta but not to the developing animal or the *in vitro* differentiation of transgenic ESCs into oocytes or sperm from which transgenic animals can be produced through *in vitro* fertilization methodology.

For many years, ESCs were the only pluripotent stem cells available. Recently, however, it has been demonstrated that it is possible to reprogram somatic cells into pluripotent stem cells by transiently expressing a specific set of four transcription factors. The resulting induced pluripotent stem cells (iPSCs) bear all the hallmarks of true ESCs, including morphology, gene expression, and germline competence. Unfortunately, ESCs and iPSCs are presently available from only rodents and humans. The immense progress over the last few years in the pluripotent stem cell field would strongly suggest that such cells will become available for livestock species in the near future. The versatility of pluripotent stem cells is highlighted in **Figure 1**, which summarizes the different strategies to produce a transgenic animal from pluripotent stem cells.

Pluripotent stem cells can be grown indefinitely in culture, which allows for the introduction of complex genetic modifications. In addition, their unique characteristics provide a variety of options to produce transgenic livestock carrying these defined genetic modifications.

Nuclear transfer technology allows for the reconstruction of an embryo from a single transgenic pluripotent stem cell and an oocyte that had its own chromosomes removed. A transgenic animal with the genetic makeup of the transgenic pluripotent stem cell can be produced following the transfer of the reconstructed embryo into a recipient animal for development to term.

Transgenic pluripotent stem cells can also be used directly and aggregated with a conventional embryo. Due to their pluripotent nature, these transgenic cells can contribute to all cell types of the developing

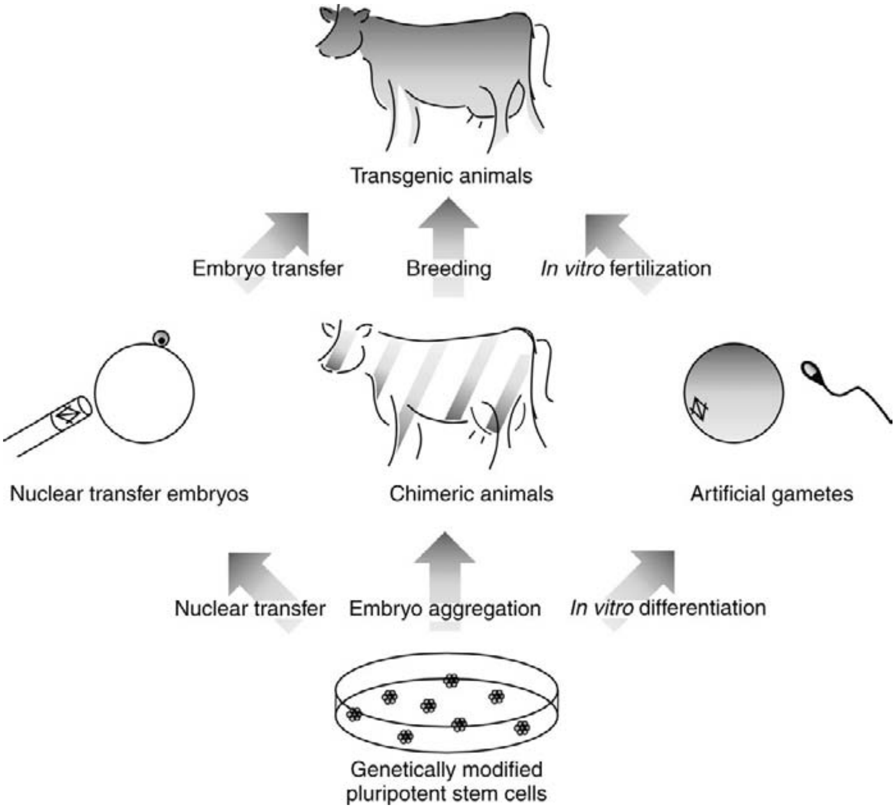

Figure 1 Pluripotent stem cells – a versatile tool to generate transgenic animals.

animal. Initially, this will result in a chimeric animal that contains cells of two different origins: cells derived from the conventional embryo and from the transgenic pluripotent stem cells. Depending on the pluripotent stem cells' contribution to the germ cell population of the chimeric animal, breeding enables the production of a transgenic animal containing the genetic modification in all cells.

Under the right conditions, pluripotent stem cells can be directed *in vitro* to differentiate into various cell types, including oocytes and sperm. These so-called artificial gametes can then be used for *in vitro* fertilization procedures to produce transgenic embryos and subsequently transgenic animals.

Transgenic Animal Applications

The continual advances in genomics toward identification of genes and their function(s) are steadily increasing our ability and versatility to redesign livestock with specific and purposeful genome alterations. Animal transgenesis is a platform technology that is relevant for a broad range of applications, which can be divided into those for biomedical and agricultural purposes.

Biomedical Applications

Pharming

The term pharming refers to the concept of producing rare human pharmaceutical proteins in animals or plants following the introduction of a gene construct that directs their production. While both manufacturing systems are very cost effective and provide excellent scalability compared to other production systems, livestock have the advantage that they are more closely related to humans than plants and are thus able to produce complex human proteins not achievable with other systems. Production in livestock can be targeted to various bodily fluids to allow for easy recovery of the pharmaceutical protein. The mammary gland of dairy species has been favored because of its ability to produce high amounts of protein that can be easily harvested in the milk. Since this approach was first demonstrated two decades ago, numerous recombinant human proteins have been produced by transgenic animals where the transgene is under the control of mammary-specific promoters and the protein is secreted into the milk. Yet, the final milestone of full regulatory approval was reached only recently with the approval of the first human pharmaceutical protein produced in transgenic animals for the European and the US market in 2006 and 2009, respectively. ATryn, a recombinant form of human antithrombin III (ATIII) produced in the milk of

Table 2 A selection of the most advanced transgenic animals for (a) human biopharmaceutical protein production and (b) agricultural applications

(a) Biomedical protein production

Protein	*Application*	*Animal species*	*Stage of development*
Human antithrombin alpha (ATryn)	Prevention of blood clots	Goat	Approved for EU and US market
Human C1 inhibitor	Treatment of hereditary angioedema	Rabbit	Phase 3
Human alpha-fetoprotein	Treatment of autoimmune diseases	Goat	Phase 2
Human alpha-glucosidase	Treatment of Pompe disease	Rabbit	Phase 2, on hold
Human lactoferrin	Beneficial food supplement	Cattle	Phase 1
Human butyrylcholinesterase	Treatment of nerve agent toxicity	Goat	Phase 1
Human growth hormone	Treatment of growth hormone deficiency	Cattle	Preclinical
Collagen	Use for implants and hemostatic products	Cattle	Preclinical
Fibrinogen	Treatment of fibrinogen deficiency	Cattle	Preclinical
Human serum albumin	Blood volume expander	Cattle	Preclinical
CD137 monoclonal antibody	Enhancing immune responses	Goat	Preclinical
MSP-1(42) malaria antigen	Malaria vaccine	Goat	Preclinical
Human polyclonal antibodies	Therapeutic antibodies	Rabbit, pig, cattle	Research
Human alpha-1-antitrypsin	Treatment of cystic fibrosis and chronic obstructive pulmonary diseases	Goat	Research
Factors VIIa, VIII, and IX	Treatment of hemophilia	Rabbit, pig, sheep, goat	Research

(b) Agricultural applications

Introduced modification	*Application*	*Animal species*	*Stage of development*
Growth hormone	Increased meat production	Pig, sheep	Research
Caenorhabditis elegans n−3 fatty acid desaturase	Omega-3-containing meat	Pig	Research
Casein	Improved milk composition	Pig, cattle	Research
Human lysozyme	Milk with added health benefits	Goat	Research
Human lactoferrin	Milk with added health benefits	Cattle	Research
Rat stearoyl-CoA desaturase	Healthier milk fat	Goat	Research
Disruption of the prion protein gene	BSE free production animals	Sheep, goat, cattle	Research
Bacterial lysostaphin	Prevention of mastitis	Cattle	Research
Bacterial phytase	Reduced environmental impact	Pig	Research

BSE, bovine spongiform encephalopathy.

transgenic dairy goats, is an anticoagulant that is indicated for the treatment of patients with hereditary antithrombin deficiency to prevent the formation of blood clots during surgical procedures. With many other proteins in development or in preclinical stages for the treatment of various human conditions (**Table 2(a)**), it can be expected that additional biopharmaceuticals derived from transgenic animals will receive market approval in the coming years.

Xenotransplantation

Pig xenografts are considered as a promising source of tissues to overcome the growing shortage of human donor organs to treat various degenerative tissue diseases. Pigs are favored because of their similarities in organ size and physiology to humans. Transgenesis offers the opportunity to overcome major immunological hurdles that limit successful xenotransplantation. The first hurdle is the hyperacute rejection (HAR) of pig xenografts immediately after transplantation. HAR is triggered by a specific carbohydrate epitope, the so-called α-1,3-galactose (Gal) epitope, which is found on the surface of cells in pigs, but not humans. Recently, pigs have been engineered that completely lack the enzyme responsible for synthesizing the Gal epitope. Pig-to-baboon xenografts with tissues from these pigs demonstrated the extension of the survival period to several months following the transplantation of hearts or kidneys. Subsequent immunosuppressive drug therapy or additional genetic modifications could be used to manage the human body's other rejection processes to prolong the functionality of the transplanted organ.

Animal models of human diseases

The ease of engineering the mouse genome with mutations found in human genetic disorders makes the mouse the most accessible model for human diseases. Numerous genetic mouse models exist and are routinely used to decipher the molecular mechanisms of human disease and identify potential therapeutic targets. However, the marked differences between mouse and human in their anatomy and life span restrict mouse models as some do not accurately reflect the human disease pathology or do not permit the evaluation of novel therapeutic strategies. Thus, large animals with organs that are structurally and functionally similar to human organs could provide more appropriate models for certain human genetic disorders.

Human degenerative photoreceptor diseases are an example of how a transgenic model in a large animal such as the pig, with eyes more closely related to the human eye than the murine eye, can provide a more precise histopathological model than mice. A transgenic pig model has been developed for the rare human eye disease, retinitis pigmentosa, and the model has proven valuable to study the disease and to test novel therapeutic treatments. Another example is the life-threatening human disease cystic fibrosis (CF), which is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Although many mouse models that carry the relevant CFTR mutations have been generated, all mouse models were unable to replicate the devastating lung infections of CF patients. To provide access to an appropriate CF model, pigs, which have lungs of much greater anatomical and functional similarity to the human lung, were engineered with nonfunctional CFTR genes. Newborn transgenic pigs with this CFTR mutation have phenotypes that replicate that observed in newborn humans with CF. Further technological advancements in the ability to introduce specific mutations into livestock genomes are expected to increase interest in the production of large animal models to study human diseases.

Agricultural Applications

There are a wide variety of agricultural applications for livestock transgenesis aimed at improving animal productivity; quality of meat, milk, and fiber components; animal welfare; and environmental sustainability. Improvements in any of these areas are expected to result in economic benefits for farmers and processors. In addition, consumers could benefit from food with additional health-promoting or disease-preventing attributes, for example, through functional foods, livestock from better health, and the environment through a reduction of the impact from livestock farming. Despite this potential, few studies have focused on agricultural applications, mainly because of the greater demands on cost, efficiency, consumer acceptance, relative value of agricultural products, and lack of an established regulatory pathway for transgenic animals intended as production animals. **Table 2(b)** provides a summary of studies targeting agricultural applications.

Production traits

The improvement of production traits holds great promise to produce more food with fewer animals and land resources. Early work attempted to increase growth rates and improve meat production in pigs and sheep by introducing genes for growth factors such as growth hormone. These early studies were hampered with poor control over the transcriptional regulation of the gene constructs, and this resulted in a number of unwanted side effects and small a few-to-moderate improvements in growth characteristics. While rather disappointing in pigs and sheep, subsequent studies with the same approach produced spectacular results in transgenic fish with massively increased growth rates. For large animals, alternative strategies with greater control that directly interfere with regulators of skeletal muscle development are investigated. The functional loss of myostatin, a negative regulator of muscle growth, due to a natural mutation in some existing beef cattle breeds, results in an approximately 20% increase in muscle mass. Whereas these known double-muscling breeds are associated with major calving difficulties and resulting welfare concerns, transgenic technology could provide the opportunity to limit the myostatin-related effects to only postnatal muscle growth, thereby eliminating the adverse health effects of an otherwise attractive production phenotype. Using a conditional myostatin knockout strategy, the feasibility of this concept has been validated in the mouse.

An entirely different concept for improving meat is based on the introduction of novel beneficial characteristics. Enzymatic activities normally not found in mammals can provide livestock with the ability to endogenously synthesize polyunsaturated omega-3 fatty acids (FAs) implicated in the prevention of coronary heart disease. Transgenic pigs that constitutively express the fat-1 FA desaturase gene of a nematode were shown to produce meat highly enriched in omega-3 FAs, in particular two of the most potent varieties mainly found in fish. This may prove to be a more economical, safe, and sustainable strategy to fortify meat compared to the current practice of feeding animals with fish meal in order to satisfy the growing demand for omega-3 FAs in human nutrition.

Livestock have been selected for milk production characteristics for millennia, but milk composition, in particular, proved to be relatively refractory to change by conventional means. Transgenic technology allows for a much more direct approach with the potential to introduce desired milk production characteristics in livestock. Although possible transgenic strategies to improve milk production were already discussed two decades ago, most of the concepts have only been tested in mouse models. Only a few studies have been extended to target species, including pigs and cattle. In pigs, milk production capacity is a limiting factor for piglet growth and survival, and thus pig production. The whey protein α-lactalbumin, which is implicated in lactose synthesis and the regulation of milk volume, has been proposed as a target to boost lactational performance of sows. Indeed, overexpression of α-lactalbumin in milk resulted in higher milk carbohydrate content, up to 50% increased milk yield, and greatly improved piglet growth and survival.

To improve the nutritional value and functional properties of cows milk, transgenic cows were engineered with additional β- and κ-casein genes. Their milk showed marked changes in composition and physicochemical characteristics, in particular a threefold increase in κ-casein, more calcium, and smaller casein micelles, which are the main protein particles in milk. Although these attributes have been generally associated with increased heat stability and improved cheese manufacture, this has not yet been demonstrated for this novel milk, which still remains to be fully evaluated. Additional approaches have focused on enhancing the levels of beneficial proteins found in milk and, with human applications in mind, used the corresponding human version of the proteins. The antimicrobial proteins lysozyme and lactoferrin were overexpressed in the milk of goats and cattle, respectively. Dairy milk with human lysozyme or lactoferrin, at concentrations much higher than the natural-milk levels of the bovine equivalent, constitutes a functional food that might offer new health benefits such as increased protection against infections and improved gastrointestinal health. A second major target for improving milk characteristics is milk fat, which is very rich in ‘unhealthy’ saturated fatty acids (SFAs) that have been associated with cardiovascular and coronary heart disease. The feasibility of engineering animals for the production of healthier milk fat was demonstrated in transgenic goats. The mammary gland-specific expression of a key enzyme (stearoyl-CoA desaturase) involved in converting SFAs into unsaturated forms resulted in goats producing milk with less SFAs and more unsaturated fats.

Livestock serve as not only the source of food products but also provide humans with valuable fibers. Initially, introduction of growth factor genes was evaluated for the ability to boost wool production. There was only a small gain, which could not be sustained long term and was associated with a decrease in fiber quality. A different approach explored the potential to alter the fiber composition to improve its processing and wearing qualities. The overexpression of a specific wool fiber protein in the wool follicle of transgenic sheep substantially altered the fiber structure, demonstrating that novel fiber types with unique characteristics can be produced with transgenic technology.

Animal health and welfare

The application of transgenic technology to protect animals from infectious diseases could have a multitude of positive effects. It would improve the welfare of the animal itself, result in more efficient production, increase food safety, and reduce risk for zoonotic diseases transmitted from infected animals to humans. For these reasons, this is a very active research area with studies in a number of different species at advanced stages. The first animals engineered for disease resistance have already been described for cattle, addressing mad cow disease (bovine spongiform encephalopathy (BSE)), one of the most serious health risks for humans, and mastitis, one of the most costly diseases for the livestock industry. The development of BSE is dependent on the presence of the so-called prion protein (PrP). Using a gene knockout strategy, transgenic cattle that are devoid of PrP have been produced. Results so far indicate that these animals are resistant to BSE but otherwise entirely normal. In a different approach to provide protection against mastitis caused by bacterial infection of the mammary gland, cattle were engineered for the expression of the bacterial antimicrobial protein lysostaphin. Lysostaphin is an enzyme that has been shown to be very effective in killing *Staphylococcus aureus*, one of the main mastitis-causing bacteria. Expression of lysostaphin in the mammary gland conferred close to complete protection when the transgenic cattle were challenged with the mastitis-causing bacteria. While antimicrobial proteins can provide beneficial health attributes, they also have the potential to compromise the microorganisms involved in the processing of milk into dairy products such as yogurt or cheese. Although this might be highly dependent on the amount and specificity of the antimicrobial produced and present in the milk, for the existing models with lysostaphin and human lysozyme, cheese manufacture revealed no signs of any detrimental effects.

The recent discovery of the RNA interference (RNAi) mechanisms to disrupt the activity of specific genes provides an attractive new disease prevention strategy, particularly to combat viral diseases. It is based on the expression of small double-stranded RNA molecules that mediate sequence-specific inhibition of a selected target gene. Depending on the degree of interference, increased or complete resistance could be achieved by engineering livestock that express an interfering RNA targeted at a crucial viral gene, essentially disrupting the life cycle of the virus. The potential of this concept to provide resistance to viral infections has been validated *in vitro* and in mouse models. Several studies are now evaluating the potential of RNAi-mediated approach to generate chickens and livestock that are resistant to viral diseases such as influenza and foot-and-mouth disease.

Environmental impact

Farm animals have an impact on the environment by requiring land, water, and energy, and through the excretions they produce. Improving the efficiency of production would lessen the environmental impact of animal production. In addition, there are specific strategies to use transgenic technology to directly reduce deleterious consequences of animal production on the environment. For example, pigs are unable to access most of the phosphate in their feed, which is present mainly in the form of phytate. About 60% of the phosphate from ingested feed is excreted in the manure, which constitutes a major form of environmental pollution for pig farming operations. Using a transgenic approach, a bacterial gene for phytase was introduced into pigs, which now enables them to digest the dietary phytate and more efficiently use the phosphate therein. These animals have lower dietary requirements for phosphate and also have up to 75% lower excretion of phosphate.

Regulatory and Ethical Issues

Transgenic technology provides a very versatile new tool to genetically enhance existing production and health traits beyond the normal biological boundaries. However, many important phenotypic livestock traits are complex and controlled by multiple genes, and major advances in our understanding of the functional relationship between genotype and phenotype are still required to realize the potential of transgenic animal technology. Aside from technical limitations, low public acceptance, associated with the belief that the technology is 'unnatural' and poses incalculable risks for the safety of food and the environment, is a major factor restricting progress and use of transgenic animals. In addition, the technology raises animal welfare concerns, which become more prevalent with the use of domesticated animals. Health and fitness of animals can be compromised in case of poor control over the activity of a newly introduced gene or the unintended disruption of an endogenous gene function through the integration of the transgene at a random genomic locus. Improved technologies allowing for more precise, site-specific genome modifications and the ability to conditionally express transgenes are under development and have the potential to eliminate possible adverse effects for the animals. Presently, the views of proponents and opponents are nearly antipodal considering that food safety, animal health, and the environment are by many suspected to be at risk while at the same time they coincide with the declared specific targets of transgenic animal applications for improved and safer food, greater animal health, and reduced environmental impact. Rigorous scientific evaluation of the risks and benefits that are sufficiently valued by the consumers and a robust regulatory framework will be necessary to change consumer perceptions. Although scientific advancements have provided many opportunities, the lack of established regulatory systems eroded consumer and investor confidence and has so far prevented progression from research to commercialization. After years of delays, the US Food and Drug Administration has finally issued a guidance document for the regulation of transgenic animals, which proposes to evaluate transgenic animals irrespective of their intended application for biomedicine or food, under the federal Food, Drug and Cosmetics Act. Importantly, there will be alignment with the Guidelines for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Animals recently developed by the Codex Alimentarius Commission. A clear regulatory pathway and the assurance for rigorous safety testing should lift consumer confidence. Whether consumers will perceive the benefits offered by the transgenic animal applications to be sufficient for accepting the residual risk of the unknowns of a relatively new technology is likely to be revealed in the coming years.

See also: **Gamete and Embryo Technology**: Cloning; *In Vitro* Fertilization; Multiple Ovulation and Embryo Transfer; Sexed Offspring. **Genetics**: Selection: Economic Indices for Genetic Evaluation. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat; Milk Protein. **Welfare of Animals, Political and Management Issues**.

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GENETICS

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Selection: Concepts

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Introduction

Genetics is the source of all permanent change or improvement in value of dairy cows. Changes in environment, management or nutrition can affect their productivity or appearance, sometimes by large amounts, but such effects are usually of a fleeting nature. Certainly they are not transmitted to offspring.

In the past most selection was within breed. Most breeds adopted a hair color or pattern as an identifier. Many selected for a specific trait as a specialty for the breed. For instance the black-and-white spotted breed (Friesian or Holstein) of the Rhine valley was widely known for its high milk volume in Roman times. The Jersey has been known for high milk fat content for centuries.

Historically, sons of the 'best' cows have been mated to other top cows to produce bulls to sire the next generation. This type of breeding was called 'best-to-best'. Such a breeding system was relatively inaccurate for identifying the genetic merit of individual animals because of inability to separate genetic and nongenetic causes of differences, both within and between herds. It was effective in identifying above-average animals. Yet, it was the main selection procedure for centuries.

Predicting Response to Selection

The equation that predicts genetic change per generation (usually called ΔG) is:

$$\Delta G = (\text{accuracy of selection}) \times (\text{intensity of selection}) \times (\text{genetic standard deviation}).$$

Accuracy simply means how well we identify the true genetic merit of animals chosen as parents relative to all possible parents. Intensity is a measure of what proportion of possible parents of the next generation are needed and used. The genetic standard deviation is the square root of the observed phenotypic variation times the heritability of the trait. To put ΔG on a per-year basis, it needs to be divided by the average age of the parents when their offspring are born (generation interval).

Accuracy of Selection

Accuracy varies among traits. Those that are most closely related to survival and reproduction are much lower in accuracy than traits such as body size, shape, milk yield and hair color or pattern.

Because mature bulls of dairy breeds are very aggressive toward humans and dangerous, most were not kept for more than a year or two. This reduced the generation interval and also maintained a high degree of genetic variation within and between herds.

The low accuracy of the traditional selection procedure for identifying the truly genetically best individual cows and bulls was exposed by the development of artificial insemination (AI) and its wide adoption in the middle of the twentieth century. At the start bulls from highly productive herds were used. When the daughters of these AI bulls produced little more if any than those of bulls used in ordinary herds previously, improved methods of genetic evaluation were developed. Some call the most accurate method currently in wide use the 'animal model' or 'individual animal model' (*see* **Genetics**: Selection: Evaluation and Methods and articles in Further Reading for more details).

Basic Discoveries in Genetics that Affected Dairy Cattle

Mendel first documented the concept of dominant and recessive genes. It is an easily recognized form of inheritance in dairy cattle as it is in most other species. Most dairy animals have horns at birth, which is recessive to the dominant 'polled' condition (absence of horns at birth). Another common dominant gene is black hair color compared to the recessive red hair.

Only one of each pair of genes has to be present for the animal to show the dominant trait but both alleles of a pair must be recessive for the trait to be expressed. Defects due to alleles of a single gene pair are usually recessive.

Most Traits Are Affected by Many Genes

Many genes, each with small effects, control the size and shape of horns in contrast to their presence or absence. This type of inheritance is called additive and is the kind that affects most traits of economic importance in dairy cattle. It is normally expressed as a decimal fraction of 1.0 or as a percentage. For instance, the estimates of genetic variation in yields of milk, fat and protein range from 25% to 40% of the total variance within lactation, herd, month, year and breed. The range is given because estimates differamong breeds, time and how well environmental effects are removed. Genetic variation in fraction of hair that is colored in a spotted breed such as Holsteins or Friesians is 90% or higher. The decimal fraction of additive genetic variation in a trait is defined as 'heritability', or 0.90 in the case of degree of spotting in Holsteins. Heritability of longevity, reproduction and disease resistance or tolerance is much lower, 0.02 to 0.10, but these are important economically.

Inbreeding and Crossbreeding

Inbreeding generally has deleterious effects because it causes more recessive genes or alleles to become homozygous (have two identical alleles because of descent from the same ancestor). Experience shows typical inbred mammals to contain four or more loci homozygous for deleterious alleles. This contributes to more loci being homozygous for undesirable as well as desirable ones. This contributes to the widespread observation that inbreds are weaker or 'less fit'. Certainly, inbred dairy cattle have a higher percentage of deaths particularly and especially as calves. Theoretically, inbreds have less genetic variation among members of the same family and are more uniform.

The idea of breeding best-to-best has increased the frequency of both desirable and undesirable genes. This has been one of the consequences of the accurate identification and extensive use of the 'best' bulls and cows as parents of future cattle through AI. Intensive selection of the best bulls and cows to be parents of the next generation has increased the rate of inbreeding in all major dairy breeds.

Crossbreeding is the mating of a bull of one breed with a cow of a different breed. Because loci in the cross will have one allele from each parent breed, the effects will be opposite from that of inbreeding. The phenomenon that this causes is called 'heterosis'. Experience shows that crossbreds are more 'fit' than those with alleles from only one breed. Particularly crossbreds show higher calf survival and reproductive efficiency. Yields of milk, protein and fat are higher than the average of the parent breeds in the same environment but only rarely above that of the higher parent. Changes in relative market values of milk components and increases in inbreeding in major dairy breeds has led to new interest in crossbreeding in the United States.

Goals of Dairy Cattle Breeding

The objective of dairy cattle improvement is to increase the future value of outputs per unit time of descendants within herds similar to the ones that parents were in. These should be adjusted to change in market values of all outputs and for all costs. Values for milk and its components will be most of the output. Inherited effects of disease resistance and fertility seem to be becoming of more importance as genetic gains in milk and its components increase, at least in some breeds (*see* **Genetics**: Selection: Economic Indices for Genetic Evaluation).

Computing Genetic Changes over Time

An effective method of determining possible genetic change for any selection program in dairy cattle is to compute the contributions of gene flow from sires of a

Table 1 Percentages of genetic gain from each of the four paths of selection

Sire to son	40–70
Dam to son	30–50
Sire to daughter	20–40
Dam to daughter	10–20

new generation of bulls, dams of new bulls, sires of new cows and dams of new cows. Ranges of the relative changes from each path for typical breeding programs for a breed are given in **Table 1**.

The sire-to-son path has the most potential gain because few sires of sons are needed, at least in the short run. It also has the potential for being highly accurate. The dam-to-son path has a high potential with superovulation and modern embryo technology but probably will be less important than the sire-to-son path. The sire-to-daughter path can be highly important depending on the availability of semen from the top bulls and affordable prices. Genetic gain from this path is variable depending on the accuracy of the genetic evaluations on sires and the willingness of breeders to pay the premiums for semen from top bulls, especially to breed cows that may be culled before they have the resulting calves.

Gain from the dam-to-daughter path is limited by the percentage of heifer calves that must be retained as replacements for culled cows. With longer productive lives, fewer replacement cows are needed and genetic change can increase. It is limited by relatively low accuracy of identifying true genetic merit of individual cows.

Summary

Analyses indicate that genetic changes have been responsible for at least 75% of the increased yearly production of dairy cows in the developed world over the past few decades. This trend has been predicted to remain similar for the next decade.

See also: **Genetic Defects in Cattle**. **Genetics**: Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. **Reproduction, Events and Management**: Mating Management: Artificial Insemination, Utilization.

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Selection: Evaluation and Methods

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Introduction

The ultimate goal of animal selection is to create a new generation of animals that are superior to the current population. Superior is interpreted broadly to include functionality of animals, cost reduction of production, consumer perception, and quality of products. These factors contribute to overall sustainability and long-term economic profitability of animal production. An essential element of selection is a genetic evaluation system for the detection of superior animals to be used to produce future generations. Current genetic evaluations use phenotypic records and advanced statistical methods to separate genetic and environmental effects. These traditional methods are complemented by DNA-based technologies that provide genetic information at a molecular level.

Genetic evaluation systems are highly complex and involve collection of data from thousands of farms, determination of milk characteristics in laboratories, processing and storage of data in regional computing centers, and application of advanced statistical procedures to estimate genetic merit. Genetic evaluations are widely distributed and are the primary determiner of the value of semen and embryos. Internationally, bull evaluations are combined across countries so that each country has a single national ranking of all bulls worldwide. Selection decisions on farms and by artificial insemination organizations are highly dependent on that genetic information.

This article covers aspects of genetic selection that stretch from basic data collection (including identification systems), traits recorded and evaluated, and characteristics of current and future evaluation systems to new DNA-based technologies.

Identification Systems

Accurate genetic evaluation systems require knowledge and use of relationships among animals. An identification system is needed to record performance and pedigree information. Breed associations (also called herdbooks) initially performed recording of pedigrees. Later, milk-recording agencies also recorded pedigree information. In some countries, these functions have been assumed by a single organization. Standards of identification are defined by the International Committee for Animal Recording (Rome, Italy). Historically, ear tags, neck chains, tattoos, leg bands, and brands have been used to identify animals. For cattle recorded by breed associations, sketches or pictures have also been used. More recently, electronic identification has been introduced. Some work has been done on implanting identification devices, but ear tag and neck chain mounting are the most common methods for attaching identification to animals. Some of the interest in improving identification systems has been for food safety to allow tracing animals back to the farm of origin for meat production.

Identification systems may be implemented at a farm, national, or international level. Recently, an international system of identification has been adopted. A 3-character country code is included with a 12-digit number. With the acceptance of the country code as part of the identification number, countries no longer need to assign a local identification number to a foreign bull when his semen is used in that country. Breed and sex codes also may be part of an animal's identification and, in some cases, are required for uniqueness, particularly for identification numbers from older systems.

Recently, radiofrequency identification devices have been adopted for countries with a numeric code (840 for the United States). These devices are expected to aid animal identification at low cost and be the basis for lifetime tracking of animal location. Large herds have found radiofrequency identification to be extremely useful for management purposes. For example, an identification reader connected to a handheld computer can provide information on which individual treatments are needed for a line of cows in head locks.

The international identification system implemented in most countries assigns a number to an animal at birth. This number stays with the animal and is used in milk recording and breed association herdbooks. In some countries, this number is centrally managed and stored in a national database. In the European Union, a unified identification system exists for bovines, and birth identification numbers are retained even if animals are moved across European Union countries. However, some of these countries did not use the international identification numbers as their herdbook numbers; therefore, some animals continue to have multiple identification numbers.

Genetic markers have been used for parentage verification for many years. Recent advances in genotyping single nucleotide polymorphisms (SNPs) indicate that using a panel of 3,072 markers will allow initial screening for genetic merit as well as determination of the parentage of an animal at a cost similar to that of the current parentage verification if both parents have been genotyped.

Milk Recording

Production records are the foundation of evaluation systems for dairy cattle. International standards of milk recording are defined by the International Committee for Animal Recording. The historical standard has been recording the milk production of each cow once per month. These daily yields are used to estimate lactation yields. The standard lactation measure has been yield for the 10-month (305-day) period following calving. Most milk-recording (dairy herd improvement) agencies provide a supervisor to conduct the on-farm recording. The supervisor contributes to the authenticity of the data. Milk samples may be collected and analyzed in a laboratory to obtain fat and protein percentages, somatic cell counts, and, in some cases, additional traits such as urea and lactose. Currently, research is being conducted to add new milk quality and animal robustness traits such as milk fatty acids, lactoferrin, or acetone.

Because of the recent increases in herd size, reduction in government support, and economic pressure on dairy producers, alternatives to traditional milk-recording plans have been developed to reduce the cost of milk recording. One popular approach has been to record only one milking on test day. The interval between milkings is recorded to determine what portion of the daily yield is represented by the measured milking. This reduced recording is called an AM–PM plan because the recorded milking is typically at alternating times (AM or PM). Another approach has been to allow longer intervals between recordings. In some countries, a supervisor is not employed; the owner or an employee records all the information. Greater flexibility has also been allowed in scheduling the frequency and type of tests (supervised, or not) and frequency of laboratory analysis of milk samples. Electronic milk meters can record the milk yield at every milking. A common method of integrating these data into the current milk-recording system is to report the average yield for a period of 5–10 days as the yield on test day. Future developments in data management may allow collection of yields for every milking.

Laptop computers are widely used by milk-recording supervisors for on-farm collection of data. These computers can perform preliminary validation to identify errors for immediate correction. Some management information such as which cows to breed or which cows to dry off may be available for the owner on test day. On farms with resident computer systems, production records can be calculated and maintained there. Data are then uploaded to regional computing centers. The computing center provides assistance to dairy producers and acts as a backup site for herd data. Milk-recording agencies are increasingly involved in providing extended services to farmers in support of the management of the dairy herd. Regional computing centers assemble yield and laboratory data and compute lactation information.

Traits

Milk Production and Quality Traits

Milk, fat, and protein yields are the major determinants of income to dairy producers and are included in nearly all evaluation systems. Milk and fat yields have been part of milk recording since it began in the early 1900s. Widespread collection of protein yield began in most countries during the late 1970s. If milk is used primarily for fluid consumption, component information may not be collected. Interest in fat and protein content has been increasing because of their potential use in determining individual cow feed requirements.

Type

Visual appraisals of cows for conformation (type) traits have been carried out for many years. A program to collect information on Holstein appraisals on a categorical scale began in 1925 in Canada and in 1929 in the United States. Conformation traits are now scored on a linear scale and include udder, locomotion, and other body traits in most countries. An overall (final) score is usually assigned by an appraiser and generally is not an index of the scores for individual traits. Typically, daughters of young bulls and some randomly selected herdmates are scored once during first lactation. However, some type systems make use of multiple scores. Scoring is usually done by breed association personnel or classifiers. Recent research efforts have focused on using type traits to select for increased profitability through increased herdlife and greater disease resistance, or as an indicator of maintenance cost. Body condition score has been increasingly used as a management tool.

Functional

Longevity (or herdlife) is an important trait from an economic viewpoint. This trait is usually determined from milk-recording data. Somatic cell score is a logarithmic representation of the somatic cell count and is used as an indicator of udder health. Other fitness traits that are measured and evaluated in various countries include health and disease resistance, fertility, calving ease, stillbirth, body weight, feed intake, milking speed, and temperament.

Genetic Evaluation System

The goal of a genetic evaluation system is to calculate the genetic merit of animals and to rank them as a tool for selection decisions for genetic improvement. Many developments have been made in both national and international genetic evaluation systems over the last 60 years. Evaluation systems have evolved in complexity and have become more accurate in estimating genetic differences as statistical techniques and computing power have improved.

Genetic evaluation systems generally differ by country. These differences reflect differences in the structure of data and the dairy industry. Some of the ways in which systems differ include calculation of lactation records (if still required), parameter estimation, accounting for age, definition of environmental groups, definition of unknown-parent groups, accounting for inbreeding and heterosis due to crossbreeding, and reporting scale of evaluations.

Evolution of Production Evaluation Models

An early method for genetic evaluation of dairy bulls was daughter–dam comparison. With this method, the difference in yield between a bull's daughter and her dam was assumed to have resulted from the genetics of the bull; no effect of differences in the environment was considered. The daughter–dam comparison was followed by the herdmate comparison, which accounted for the effect of the environment by comparing animals in the same herd that calved during the same season. These animals were called a contemporary group or herdmates; therefore, the method was often referred to as contemporary comparison. Selection index theory was used to obtain genetic evaluations of sires by regressing the herdmate deviations toward the expected value of the sire. The herdmate comparison did not account for genetic differences between herds or the genetic contribution from the parents of the contemporaries. This limitation was overcome by the best linear unbiased prediction procedure or an equivalent (modified contemporary comparison), which enabled the joint estimation of phenotypic deviations and genetic values. A sire model also accounted for relationships among cows through their sires (and later maternal grandsires in some countries), thereby joining the genetic accounting of the daughter–dam comparison and the environmental accounting of the herdmate comparison.

Currently, nearly all major dairy countries use an animal model. An animal model accounts for all relationships among animals and results in simultaneous evaluation of cows and bulls. An animal's evaluation is a function of the evaluations of its parents and its offspring (progeny) as well as its own records. Other relatives affect its evaluation through either parents or progeny. Because of the simultaneous nature of the animal model system, information from one animal can affect the evaluation of others.

In recent years, many countries have switched from analyzing lactation yields to analyzing individual test-day yields. A test-day model allows more accurate accounting of the environment because the effects of specific test days are estimated. A recent advance in test-day models is to allow for genetic differences by test day. In most countries, a lactation curve is fit for each lactation of each cow through random regression effects. This approach provides genetic evaluations of lactation persistency. Some countries have added a random regression effect for a common herd environment. By 2008, most countries had adopted a test-day model, and nearly all are working on one. An approximate test-day model used within the herd can also provide estimates of test-day environmental effects for use in management decisions.

Breeds are often still evaluated separately. However, with the increased presence of crossbreds, some countries have adopted multibreed models.

Some countries have also created joint genetic evaluation systems. Examples are the Nordic (Denmark–Sweden–Finland), Flemish–Dutch, and German–Austrian–Luxembourg collaborations. Such systems need careful consideration for data acquisition, modeling, and reporting of estimated breeding values to meet the needs of producers in the collaborating countries.

Evaluation Model

An evaluation model is a representation of the factors that affect a given trait. It includes the genetic merit of the animal, the factor of primary interest. A typical single-trait linear model is

$$y = h + f + a + p + e$$

where y is the observed value, h is the effect of the contemporary group or herdmates, f is the effect of other known environmental factors (e.g., age), a is the effect of the animal's genetics, p is the effect of the animal's permanent environment, and e is any unexplained residual effect (error). A contemporary group is composed of animals subjected to the same environmental influences. For milk production, it would include cows that calved or produced milk during the same period in the same herd. The permanent environmental effect is an estimate of the similarity among repeated records of an animal that is not the result of genetics.

The effects are classified into fixed and random effects. Random effects include residual, genetic, and permanent environmental effects. These effects are assumed to have some distribution of values. Solutions for random effects have the desirable property that they are regressed toward their expected values. The expected values of the permanent environmental effect and the residual effect are typically 0; for the animal's genetic effect, the expected value is the average of the evaluations of the animal's parents. The expected value of the animal's genetic effect means that only as information accumulates for an animal can its evaluation deviate substantially from the parental average. Without such a model characteristic, the most extreme evaluations would usually be for animals with the fewest data.

The classification of an effect as fixed or random is not always clear. The contemporary group is assumed to be a random effect in some systems with contemporary groups that are extremely small. Although this assumption allows small groups to be included, it may result in biases in the genetic evaluations. For random effects, the relationships among observations (covariances) can be specified. For instance, observations for the same animal or related animals are obviously not independent. These relationships are specified by adding the inverse of the covariance matrix to the systems of equations for each random effect. Typically, the residuals of all observations are considered to be uncorrelated, and nongenetic covariances are assumed not to exist among animals. For the genetic effect, the relationship matrix specifies the covariances among animals. The inverse of this matrix can be constructed, using remarkably simple rules, from a list of animals and their parents. This matrix connects animals with their parents and progeny so that information on one animal affects evaluations of all relatives.

Ancestors are traced to the point where parents of an animal are unknown or do not provide connections to observations of other animals. The unknown animals are replaced by unknown-parent groups that typically are defined to reflect known reasons for genetic differences: birth year, sex, and country or breed of origin. Unknown-parent groups improve the accuracy of evaluations by accounting for genetic differences in the origins of current animals.

The equations for most evaluation systems are solved through iteration. In iteration, the solution for one effect is updated using earlier solutions for all other effects. All the solutions are updated during one round of iteration. In some cases, hundreds of rounds of iteration are required for solutions to reach convergence. Convergence occurs when the changes in solutions from one round of iteration to the next are no longer significant (convergence criterion). Many convergence criteria could be used. One common criterion is based on mean squared relative differences, where squared differences are collected during a round of iteration and summed. When the ratio of this sum and the sum of squared solutions falls below a specified threshold, convergence is declared. The threshold selected is affected by the numerical accuracy possible in the computer. A reasonable threshold is 1×10^{-8}.

Multitrait Analysis

An advance in evaluation systems is multitrait analysis. Multitrait systems provide evaluations of several traits simultaneously, which allows the information from one trait to contribute to the accuracy of the evaluations of other traits. The covariances among the traits determine the degree of the influence of individual traits. In a multitrait analysis, an animal may receive an evaluation of a trait that was not recorded for that animal. For example, if a herd does not have milk component concentrations measured, a multitrait analysis would allow cows in that herd to be evaluated for fat and protein yields based on the correlations of these traits with milk yield.

Multitrait analysis can account for selection. When traits are positively correlated, selection for one trait results in genetic progress for the other trait even if the second trait was not recorded. To avoid bias, an evaluation system must include the data that were used in making selection decisions.

Multitrait analysis is commonly applied to linear type traits. If all traits are observed for all animals, a canonical transformation is used to create uncorrelated traits, which are then evaluated separately. The reverse transformation is then applied to the solutions to achieve the same result as would have been achieved with the much more computationally demanding multitrait analysis. A technique has been developed that allows a canonical transformation to be applied even when values are missing.

Categorical Analysis

Although a linear model is a powerful tool for analyzing data, some data are not linear. If a trait is recorded as an assignment to a category, a linear model is not appropriate. Instead, a categorical analysis is used. The results are interpreted as the probability of being a member of a particular class or category. Conceptually, an underlying linear scale exists for the observed classes. An ordering of the classifications is assumed, but the difference between consecutive classes is not specified. In the United States, an ordered categorical analysis is applied to calving difficulty scores (also known as calving ease or dystocia). The evaluation is reported as the expected percentage of difficult calvings. Calving difficulty is reported on a scale of 1 (no difficulty) to 5 (extreme difficulty).

Longevity and Survival Analyses

Longevity data can also be problematic. In fact, longevity data are of two types: realized (animal's observed longevity) and censored (animal is still alive). Therefore, a large number of different models have been proposed for longevity. Advanced statistical techniques based on survival analysis can be used. With these techniques, the survival of an animal is modeled using a proportional hazard function.

Heterogeneous Variance Adjustment

The accuracy of an evaluation is determined by how well the model represents the data. The random effects in the model represent the variability remaining after the fixed effects have been removed. If the actual genetic variance is different in various subsets of the data and the model does not account for this, the resulting evaluations will not be optimal. If a bull's daughters were primarily in herds with low genetic variance, probably he would not receive a high evaluation even if he really had the best genetics. To account for differences in variance, the data can be adjusted, or the model can be designed to better reflect the data. To adjust the data, an estimate of the variance within each contemporary group is needed. This variance may be approximated from the phenotypic variance. Other factors such as herd size, year, and region may also contribute to the estimate. By multiplying the record or deviation from an appropriate average by a factor, the genetic variance is adjusted to a common value. Adjustment can be made before evaluations or as part of the evaluation system.

Genetic Base

Genetic evaluation systems allow selection candidates to be compared. They do not provide absolute estimates of genetic ability. To simplify presentation, a group of animals is selected as the reference group (base) and their evaluations are forced to average a constant, usually 0 or, in some cases, 100. The standard deviation may also be set. The evaluations of all animals are expressed relative to this group. The current base in the United States is the average evaluation of cows born in 2005, and this base group is changed every 5 years to reflect genetic improvement (stepwise base). Some countries change their genetic base every year. This practice is called a rolling base. With an animal model, cows and bulls are evaluated simultaneously; therefore, their evaluations are comparable. The selection pressure for bulls used in artificial insemination is much greater than for cows, and bulls in widespread use generally have higher evaluations. In a few countries, cow evaluations are presented relative to a different base.

International Evaluation

The extensive marketing of semen and embryos worldwide has led to great interest in international comparison of bulls. The Interbull Centre in Uppsala, Sweden, combines national bull evaluations from participating countries to generate rankings that include the bulls from all countries. These international rankings are reported on each country's national evaluation scale. Such a multitrait across-country evaluation allows correlations to be less than 1 between bull performances in different countries; therefore, the bull rankings for different countries may not be the same. The multitrait across-country evaluation was first used in 1994.

Previous to 1994, conversion equations were used to compare bulls through ties between countries. The most obvious tie is when the same bull is used in two countries or more. Sons also can help support evaluations across countries. The Interbull Centre also conducts research to improve international evaluation systems, to promote harmonization, and to find ways to extend international evaluation procedures to additional traits. **Figure 1** illustrates the flow of information from farm through evaluation systems and on to the Interbull Centre for production traits in two hypothetical countries.

Control of Inbreeding and Use of Crossbred Animals

An important consideration in genetic selection is the control of inbreeding, which causes greater homozygosity of recessive genes that can have harmful effects. Even dairy cattle breeds with large populations have in reality very small effective population sizes (below 100). Holsteins, for example, are experiencing a loss of genetic variation and show annual increases in the percentage of inbreeding of about 0.2.

Inbreeding is a consequence of selection. Its rate of increase can be minimized by accounting for potential inbreeding when making mating decisions. Inbreeding depression may have its greatest economic effect on functional traits, such as fertility. Reduced fertility associated with increased inbreeding may result from an increase in matings that generate homozygous lethal or sublethal genotypes.

Crossbreeding is one way to avoid inbreeding and related problems. Crossbred animals will express heterosis (hybrid vigor). However, until recently, crossbreeding of dairy cattle was rather uncommon except in New Zealand. Because of the interest in high milk yields, purebred Holsteins were generally selected. In the last few years, interest in the use of crossbred dairy cows has

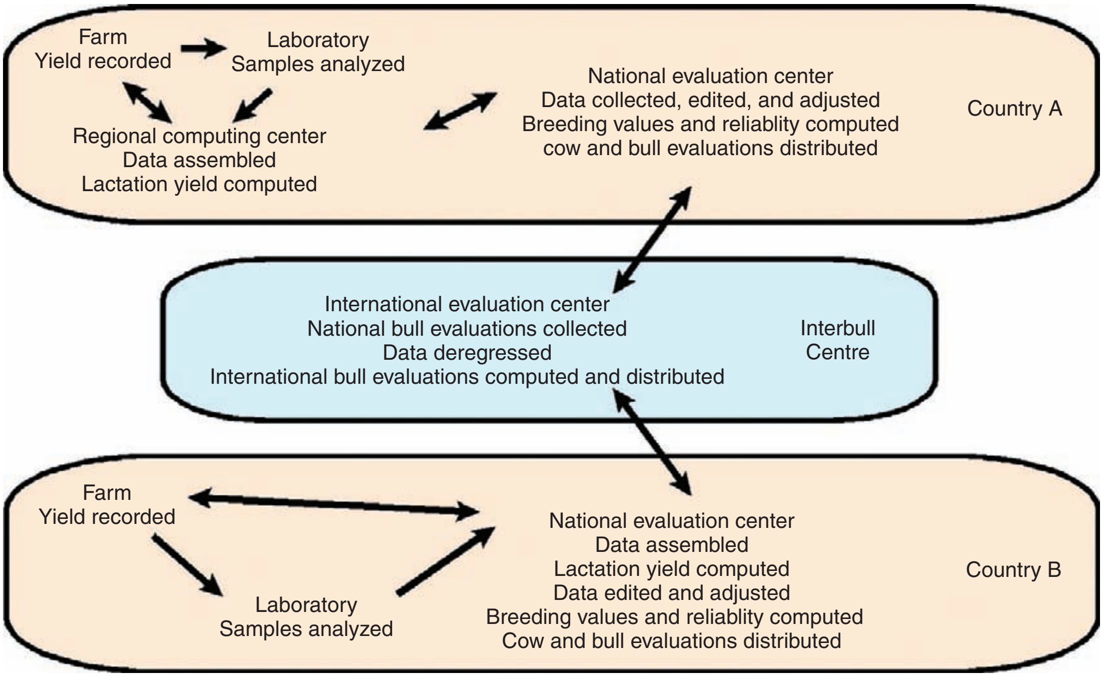
Farm
Yield recorded
Laboratory
Samples analyzed
Regional computing center
Data assembled
Lactation yield computed
National evaluation center
Data collected, edited, and adjusted
Breeding values and reliablity computed
cow and bull evaluations distributed
Country A
International evaluation center
National bull evaluations collected
Data deregressed
International bull evaluations computed and distributed
Interbull
Centre
Farm
Yield recorded
Laboratory
Samples analyzed
National evaluation center
Data assembled
Lactation yield computed
Data edited and adjusted
Breeding values and reliablity computed
Cow and bull evaluations distributed
Country B

Figure 1 Example of flow of data for production traits in two hypothetical countries through a genetic evaluation system (nationally and internationally).

increased because of changes in selection objectives and increased inbreeding in purebred populations.

Selection Based on DNA Technology

Currently, DNA tests are used to detect carriers of some genetic diseases such as deficiency of uridine monophosphate synthase (DUMPS) and bovine leukocyte adhesion deficiency (BLAD).

The yield of milk and its components and most other economically important traits are affected by many genes as well as by environmental factors. With the advances in DNA technology, some of the genes that affect these traits are being discovered. Such genes are called quantitative/economic trait loci (QTLs/ETLs). Evaluation systems can be adapted to estimate the effect of the various alleles of these genes and to improve the accuracy of evaluations by using this information. Some QTLs have been identified or mapped by linkage to genetic markers. DNA tests for genotype at these loci provide additional information on the genetic value of bulls and cows and could lead to more accurate selection as long as the detected QTL is large enough.

Genotypic information is most useful early in an animal's life. Genotypic information can also be valuable for traits that are not easily measurable or that have low heritability and for nonadditive genes, because mass selection techniques are not very effective for such traits and genes. However, QTLs with large effects are rare and often show opposite effects on economically important traits, such as the relationship between milk yield and diacylglycerol *o*-acyltransferase 1 (DGAT1) for fat percentage. A limited fraction of the genetic variation is explained by the identified QTL because stringent tests for statistical significance must be performed to identify a QTL. These tests must be stringent because many positions are tested for the presence of a QTL.

A new approach called genomic selection is based on dense panels of SNP markers. Genomic prediction does not rely on significance testing. Instead, the effects associated with all the SNP or chromosomal segments are estimated simultaneously. These estimates of SNP effects are combined with traditional evaluations (or parent averages for young animals) to calculate genomic evaluations. Some countries have begun including genomic information in their official evaluations. However, because genomic information has only recently become available, appropriate procedures for its international use have not yet been developed. Genomic evaluations, which are available early in life, are expected to increase the rate of genetic improvement by making a large reduction in generation interval with only a small loss in accuracy.

See also: **Genetic Defects in Cattle. Genetics**: Cattle Genomics; International Flow of Genes; Selection: Concepts; Selection: Economic Indices for Genetic Evaluation. **Mastitis Therapy and Control:** Management Control Options. **Reproduction, Events and Management:** Mating Management: Artificial Insemination, Utilization; Mating Management: Fertility.

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Selection: Economic Indices for Genetic Evaluation

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Introduction

This article is written on the premise that selection decisions in dairy cattle breeding should improve net returns across the lifetime of the dairy cow. Selection to improve a single trait will not accomplish this objective, although some traits will advance the effort more than others. At a minimum, production, longevity, fertility, and health traits contribute to the lifetime economic merit of commercial dairy cows. A properly weighted combination of these and other traits of economic importance that maximizes economic gain should guide selection decisions. Dr. LN Hazel of Iowa State University, in a landmark paper published in 1943, proposed a method to accomplish this task. His approach required that breeders define a selection objective or 'aggregate genotype' that was to be improved through selection on traits for which performance information was available.

The aggregate genotype or breeding objective, then, is the target gene complex to be improved through selection. As traits in that complex can be expected to have different relative values, the Hazel approach combines important traits using the relative economic values of each trait. The selection index used to improve the aggregate genotype consists of phenotypic or genetic information on traits that are part of or genetically correlated with traits in the aggregate genotype. Choices of traits to include in the index are based on availability of phenotypes, costs of collecting data, genetic correlations between traits, age of expression, and so on. Genetic correlations between traits in the index and the aggregate genotype are combined with economic values to determine a final set of selection index weights. These weights are applied to genetic evaluations of the different traits on individual animals to create a single number or 'selection index' that is used to rank animals for selection. A straightforward explanation of this process is provided in Chapter 14 of *Understanding Animal Breeding* (see Further Reading).

Dr. Hazel's approach has guided the development of many useful selection indexes, but implementation of the procedure involves some challenges. One of the major difficulties is to define the appropriate aggregate genotype. That genotype will not be the same for all breeders, particularly when marketing conditions change. One aggregate genotype may not be appropriate for all environments under which animals perform. Another problem is determining the economic values of traits in that genotype at the time when offspring produced would generate income. Genetic correlations between various traits are required. Genetic correlations have been challenging to estimate and tend to change between pairs of traits as gene frequencies change. Finally, the economic values need to be linear – perhaps 'constant' is the more descriptive term – across the range of genetic values for traits in the breeding objective. Despite limitations, selection indexes created with reasonably strict adherence to theoretical requirements have been very successful in improving the economic utility of many livestock species including dairy cattle.

Defining the Breeding Objective

Traits in the aggregate should be those that affect income and costs of production. The dairy cow must produce, reproduce, and avoid debilitating diseases in order to compete effectively for stall space on a modern dairy. She must also be able to accomplish these objectives without becoming an exception to normal management routines. This general description of ideal genetic makeup of the dairy cow and a comprehensive list of economically important traits are separated by a wide range of opinions. Economic conditions affect the choice of traits in the aggregate and are not constant across the global dairy industry.

Table 1 shows the traits included and emphasis placed on each trait in some of the selection indexes used in different countries with important Holstein populations. The data shown were summarized in the June 2009 issue of *Holstein International*. These indexes have evolved over time, with increasing emphasis on nonproduction traits as new genetic evaluations have become available. An example of such change is given in **Table 2**, which compares the last three versions of the Net Merit index used in the United States. The indexes shown in **Table 1** are not necessarily the only ones used within each country, but they are available on a national basis. The trait groupings ('trait' requires a very specific definition) included in **Table 1** are those on which selection is practiced. The aggregate genotype to be changed is not shown but can be inferred from the combination of traits listed in **Table 1**. All indexes show positive emphasis on fat and protein, justifying the conclusion that production of milk

Table 1 Weights (%) applied in selection indexes used worldwide in 2009 ordered by emphasis on production traits

Country	*Protein*	*Fat*	*Milk*	*Conformation*	*Productive life*	*Udder health*	*Fertility*	*Other health traits*
Japan	54.7	20.3		25				
Australia	36.8	14	−18.5		8.2	7.1	5.1	10.3
New Zealand	40	12	−14		6	−7	8	−13
Italy	45	14		23	8	10		
Spain	35	12	12	35	3	3		
Israel	42	14.6			8.2	12.8	15.5	6.9
Hungary	40	15		35		10		
Switzerland	38.7	14.3		24	7	10	6	
South Africa	26	26		45		3		
Canada	30.6	20.4		27.2	6.8	3	10	2
France	37	13		12.5	12.5	12.5	12.5	
Czech Republic	33.6	15.4		25	7	7	12	
USA, Net Merit	16	19		17	22	10	11	5
UK	21.9	12.4	−10.9	5.6	21.1	5.5	18.5	4.1
Germany	36	9		15	20	7	10	3
USA, TPI	26	16		25	14	5	10	4
Ireland	25	5	−12	11			23	24
The Netherlands	21.7	4.8	−6.5	22	20	6	19	
Scandinavia	20	5	−5	13	4	14	13	26

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Table 2 Relative emphasis on traits included in the Net Merit index used in the United States[a]

Trait	*US 2003 NM$*	*US 2006 NM$*	*US 2010 NM$*
Protein	33	23	16
Fat	22	23	19
Milk	0	0	0
Productive life	11	17	22
Somatic cell score	9	9	10
Daughter pregnancy rate	7	9	11
Udder composite	7	6	7
Feet and legs composite	4	3	4
Size composite	−3	−4	−6
Calving ability[b]	4	6	5

[a] VanRadan, 2003, 2006 and Cole 2010.

[b] Includes sire and daughter calving difficulty plus sire and daughter stillbirth evalutions.

NM$, Net Merit Dollars.

VanRaden P (2005) An example for the dairy industry: The Net Merit index. *Proceedings of the 37th Beef Improvement Federation Research Symposium and Annual Meeting*, pp. 96–100. Billings, MT, 6–9 July 2005; VanRaden P (2006) *Net Merit as a Measure of Lifetime Profit: 2006 Revision*. Beltsville, MD: Agriculture Research Service, USDA. http://aipl.arsusda.gov/ (accessed 2 March 2009).

components is a primary function of genetic improvement of dairy cattle around the world. Almost all indexes listed include significant emphasis on longevity, udder health or somatic cell score (SCS), and fertility. Udder conformation and feet and legs are included in many of the indexes under the category 'conformation'. Traits are defined differently in different countries. For instance, 'productive life' is defined variously as months in milk by specified ages with different maximum lengths of lactation, total months of life in the herd, and survival from lactation to lactation.

Production has dominated selection programs for at least 30 years in the United States, but emphasis on improved production has declined markedly in recent years with increased interest among producers in fitness traits. Producers in almost all dairy-producing countries have abandoned dual-purpose meat/milk breeding programs, if they existed, for selection to improve dairy-related traits during that period. The standard measure of production has been the 305-day, twice daily milking, mature-equivalent record. Production is calculated from 24 h milk weights recorded periodically during a 305-day lactation interval. Recently, development of computing power and statistical methodology has enabled genetic evaluation of individual daily milk weights instead of lactation totals. Some countries use 'test day models' where daily milk weights recorded at various intervals are the measure of performance on which selection is based. Almost all genetic evaluation systems include multiple lactation records across the lifetime of the cow. The effect of this practice is to reward persistent production within lactation and a reasonable rate of maturity in addition to an acceptable level of production in young cows.

Weights applied to milk, fat, and protein vary considerably from country to country in **Table 1**. Those differences reflect variation in selection pressure to improve health, longevity, and fertility traits in addition to production. Only Spain practices direct selection to increase milk volume, but milk volume will increase in response to heavy selection pressure to increase protein yield unless negative pressure is applied to volume. Such selection is practiced in six of the countries listed in **Table 1**. Reasons include the expense of hauling and processing of carrier (a declining problem with the growing importance of whey in food manufacture) and limitations on milk volume imposed by quota systems. Positive selection pressure is applied to volume of fat and protein in all countries.

Traits related to udder health and fertility are included in the selection indexes for many of the countries in **Table 1**. Several years ago, Sweden and Denmark were unique in placing heavy emphasis on improved fertility and udder health in their national selection goals. Today, most countries have followed their lead, as selection for higher yields has led to increased health costs and reduced fertility. SCS information is included in several of the indexes, but is coded as 'udder health' to reflect differences between countries in how selection is practiced. SCS serves as an indicator trait for incidence of mastitis and as a measure of milk quality. In the latter role, SCS has direct economic value. Many countries include type components in their selection index, generally to reduce production costs for maintenance (size), diseases or injury of the mammary system (udder), or foot health and care costs. There is a large variation in emphasis on conformation traits. Some countries place no emphasis at all on conformation traits, while three countries place over one-third of all selection pressure on such traits. Change in the final score is difficult to justify on the basis of reduced costs of production, but several countries still incorporate 'appearance' traits due to producer interest.

Economic Value of Traits in the Aggregate Genotype

One of the most difficult, yet important, steps in designing an appropriate economic index is to determine the value of traits in the aggregate genotype. Dr. Hazel's method required that economic values be fixed across the range of merit for each trait. The first kilogram of milk produced must have the same value as the last one added through selection. The value of milk in the market is constant across the range of genotypes evaluated, but the cost of producing the last unit is not likely the same as the cost of the first unit. Ideally, economic values would reflect the value of an additional unit of each trait when all other traits are constrained to average merit. Economic values so defined are partial regressions of each trait in the aggregate on overall economic merit. Such measures of economic merit are difficult to calculate, and are hard to explain to producers whose acceptance of an index really determines the impact on genetic change. Consequently, marginal economic values have been used infrequently.

Some indexes, for instance the Net Merit index used in the United States (**Table 2**), apply a net economic value to milk, fat, and protein by subtracting from market value of each component the expected costs of production. The net value of products such as protein yield is assumed to be linear throughout the range of genotypes, although nonfeed costs of production likely increase with higher yields. For some traits, net values may add complexity to an index to reflect true economic conditions, but the approach leads to a more realistic estimate of the real value of changing a trait. For example, reduced dystocia may be such a trait, as daughters of bulls born more easily tend to give birth themselves with greater difficulty. Bigger cows tend to incur increased health and maintenance costs, implying a negative economic value for body size. However, large body size increases salvage value for cull cows.

Selection index theory can accommodate such situations by adjusting the weights for several traits with favorable and unfavorable genetic correlations with each other or perhaps through creation of subindexes that accomplish the same task. The Net Merit index used in the United States was changed from the 2003 to the 2006 and 2010 versions shown in **Table 2** to include a 'composite' called calving ability. This composite combines genetic evaluations for calving ease as a service sire and sire of females giving birth, and stillbirth evaluations as service sire and sire of the cow giving birth. Expressing the traits related to difficulties during parturition allows a single number to be used in Net Merit. This makes the index easier to explain to producers while applying appropriate selection pressure to all four of the calving traits. Such flexibility can produce highly effective and easily used guides to selection for complex genetic and economic conditions.

One set of economic weights may not be applicable for all producers in a country. For instance, New Zealand producers supplying fresh milk for consumption in local towns face quite different economic conditions compared to producers whose milk is manufactured into products for sale on the world market. Canadian producers operating under quota systems have different income structures compared to their neighbors in the United States who operate without production restrictions. Such differences affect the economic values of traits included in the aggregate genotype. In the United States, for instance, market conditions vary considerably from one region of the country to another. Higher milk prices in markets where most milk is used for fluid sales reduce the value of longevity compared to milk markets dominated by butter and cheese production where prices are somewhat lower.

Fertility of individual cows is critical in grazing management conditions where it is essential that cows be clustered in peak production when grass is most plentiful. Fertility is relatively less valuable in confinement systems where forage availability is closely controlled.

Data Availability

Traits in the aggregate genotype and traits on which selection is based need not be the same, as long as genetic correlations between the traits are not zero and are known. Data collection is expensive. The most widely used traits in **Table 1**, the production traits, are those for which data collection is either less expensive or useful enough for herd management to justify investment in data collection by public or private sources. 'Other health traits' from **Table 1** are less widely used, in part because of data availability. Some traits with high economic impact are not considered because of cost or difficulty of measurement. Feed efficiency, estimated well only if intakes are measured, has major economic importance in almost all economies, but does not appear in **Table 1**. Such traits will be a basis for selection only when data can be collected at a reasonable cost on many individuals. The definition of 'reasonable cost' can change as needs for the information increase or decrease. For instance, genetic change for milk yield in dairy cows proceeded for years without regard to the role of mobilization of body tissue in early lactation when intake cannot fully support energy requirements for production in high-producing dairy cows. Health and fertility of dairy cows declined in a number of Holstein populations in recent years. One response has been to practice direct selection for improved fertility and to give additional weight to longevity or survival as protection against loss of cow health. The value of fertility data from herds on production recording increased from 'no genetic value' to high utility. It is possible that the cost of measuring energy balance during periods of peak yield may become more 'reasonable' in the future.

An additional issue affecting traits included in selection indexes is the age of the animal when the trait is expressed. Productive life is a classic example of a trait expressed too late in life to allow the superior animals to leave progeny for the next generation. Such traits should be included in the aggregate genotype if they have sufficient economic value. They can be improved through selection on correlated traits expressed earlier in life. Certain of the udder traits and feet and leg structure meet such standards for improving productive life as do censored herd life measures and even milk production itself.

Changing the Aggregate Genotype

The traits in the aggregate genotype need not be the traits on which selection is actually practiced, as use of correlated traits may reduce costs of genetic improvement programs or allow decisions to be made in a more timely fashion. The weights applied to traits in the selection index are assigned to maximize the correlation between the index and total economic merit. To maximize progress in the aggregate genotype, all traits related to traits in the aggregate should be included in the index. Practical considerations, however, dictate formation of effective – as opposed to maximally effective – indexes.

Selection should be practiced on traits for which genetic evaluations are available on many individuals. This standard improves the accuracy of evaluations and increases opportunities for high selection intensity. Mastitis is the most expensive single disease of dairy cattle, yet it is not included for direct selection in the Net Merit index used in the United States and detailed in **Table 2**. Selection is practiced on SCS because genetic evaluations are widely available and the trait is genetically correlated with incidence of mastitis. Direct selection on incidence of mastitis to reduce its incidence makes sense where incidence is recorded on many animals, as in Scandinavian countries. The recent development and application of genomic evaluations of dairy cattle add a new layer to selection processes, as reasonably accurate estimates of genetic merit are now available on much younger animals than in the past. The ability of genomic sequences to identify major genes for traits not previously available for selection holds out promise that the number of traits included in selection indexes may soon increase. Examples would be degrees of immunity to specific diseases or to categories of diseases such as metabolic diseases of early lactation.

Challenges to Creating Useful Selection Indexes

The most limiting information in selection index theory is the economic value of traits in the aggregate genotype. The value of traits is frequently nonlinear over the range of expression of a trait. Economic value can change over time and from one set of management conditions to another. Economic parameters are not the only challenge, however. When the 1943 paper on selection indexes was first published, estimates of genetic correlations between traits were almost completely unknown. Estimates of genetic correlations can still be a limiting factor in dairy cattle breeding decisions, despite improvements in computing power, data, and methodology. Phenotypic information of traits such as feed intake or efficiency of digestion is very limited, thus preventing an accurate estimation of genetic correlations necessary to utilize indicator traits as a proxy in selection indexes.

An optimum selection index includes those traits correlated with traits in the aggregate genotype, provided they are evaluated on relatively large numbers of animals in a population. Neither low heritability nor low economic value should eliminate a trait from consideration as correlations with traits of high economic value in the aggregate genotype may make them useful. Additionally, traits expressed several times over the life of a dairy cow should first be studied as separate traits before simplifying genetic evaluations with a 'repeatability' model. A single measure of production that ignores shape of the lactation curve or differences in first, second, or later parities eliminates potentially useful genetic information. To this point in dairy cattle breeding, computational challenges of evaluating national data sets have prevented multitrait genetic evaluations for individual parities. Advances in computer technology and methods of genetic evaluation will reduce such compromises in the future, provided treatment of repeated expressions as separate traits improves the utility of an index.

Theory and Reality: Creation of a Useful Index

The weights and traits included in selection indexes for many countries in **Table 1** reflect the intent to improve the lifetime economic merit of dairy animals. The discussion that follows is skewed toward the Net Merit selection index used in the United States, not because it is 'superior' to any other index but because the author is most familiar with it, how it has evolved, and what it is intended to do. Net Merit began in the mid-1990s as a way to include the then new genetic evaluations for productive life and SCS in a selection index. It replaced an index called Predicted Difference Dollars, which contained only production traits. It has evolved through several iterations since, the last three of which appear in **Table 2**. Through the years, emphasis on yield traits has been reduced to increase selection emphasis on longevity, fertility, health, and calving ability traits. In the 2010 version, only 35% of total emphasis is on production, compared with 55% emphasis in the 2003 version. It is difficult to compare emphasis on yield in the United States to other countries in **Table 1**, because six countries place negative emphasis on 'milk' while giving positive weights to fat and protein yield. However, a widespread concern for fitness traits at the expense of intensive selection for higher yields is evident. **Table 2** shows that productive life, daughter pregnancy rate, and calving ability all received higher emphasis in more recent versions of Net Merit than in the 2003 version.

How did the weights shown in **Table 2** come about? For Net Merit, the process described by Dr. Hazel in 1943 and outlined in the previous discussion was not followed in any formal manner. The traits included in Net Merit were traits for which data were widely available and for which genetic evaluations could be routinely calculated. The traits in the aggregate genotype were not formally defined, but a conceptual target genotype certainly affected the final form of the index. Researchers at Animal Improvement Programs Laboratory (AIPL), in cooperation with a regional dairy cattle breeding research group in the United States, designed the merit indexes (three versions exist to accommodate fluid, manufacturing, and cheese market conditions) to reflect lifetime economic merit. The choice of aggregate genotype, even when not formally stated through trait definition and estimation of economic values, affects the weights applied to traits in the resulting index. Several linear type traits, for instance, were included in Net Merit because of their impact on costs of production, but with relatively low weights. Some producers question the low emphasis on udder conformation, for example. The value of type traits in Net Merit is reduced because of the predictive role of type traits in productive life, which receives more emphasis than any trait other than production. Genetic evaluations for productive life are based on a multitrait approach that relies heavily on conformation data for herd life prediction, particularly when animals are young. Thus, weights for type traits in Net Merit are for their additional impact on lifetime economic merit beyond their role in extending productive life.

The value of a selection index in changing the economic utility of populations of animals depends primarily on acceptance of the index by the industry that manages those animals. To be effective, the index must be successful in identifying those animals with superior genes for the aggregate genotype to be changed. One of the most controversial steps in following Dr. Hazel's procedure is to declare some combination of traits to be the correct aggregate genotype. One possible definition of the correct breeding goal would be the net economic returns generated by a dairy cow across her lifetime. A lifetime profit function for dairy cows called Relative Net Income (RNI) was used for a number of years at the author's home institution and this function credits cows for value for milk produced, net value of calves born, and salvage value. Expenses considered are rearing costs until first calving, followed by feed costs for maintenance, reproduction, and production during her lifetime. It would be presumptuous to consider RNI to be the appropriate aggregate genotype for all dairy producers, but the function did allow a test of the utility of the Net Merit index used at the time of the study.

RNI estimates were used on over 2 million cows and Net Merit 2000 on sires of those cows in a regression analysis. Daughter pregnancy rate and calving ability were not part of the Net Merit 2000 equation. We used Net Merit 2000 to predict RNI and then used the eight different traits that were part of Net Merit at that time in a least squares prediction of RNI. The latter method would be equivalent to finding the marginal economic values for Dr. Hazel's procedure if our definition of RNI was the economic value of the aggregate genotype. What we learned was that the accuracy of predicting RNI of cows using Net Merit 2000 was nearly as large (over 97%) as the accuracy from least squares solutions for the eight different traits fitted simultaneously. The weights used in Net Merit 2000 differed from the least squares solutions, but the accuracy of prediction of cow performance was very similar. While Net Merit 2000 was not based on weights that maximize the correlation between the index and a measure of total economic worth, the weights used functioned quite well in predicting our measure of lifetime economic merit.

Two other indexes are used widely in the United States, each developed by a breed society. Holstein Association calculates and publishes an index called Type Production Index (TPI), which is included in **Table 1**. This index does not include genetic evaluations for milk or a composite evaluation of body size. It does include an evaluation for overall conformation score. The American Jersey Cattle Association publishes a measure called Jersey Performance Index (JPI), which features a composite type rating called the functional trait index. TPI and JPI have evolved with Net Merit, incorporating new trait evaluations and adjusting economic weights as the breed societies deemed appropriate. Both TPI and JPI perform functions similar to Net Merit, but reflect breed preferences for some traits. Long-term use of either Net Merit or TPI by Holstein breeders or Net Merit or JPI by Jersey breeders would achieve very similar results.

Mating Systems

Selection indexes are tools for selection rather than mating. Traditionally, mating systems for dairy cattle in the United States have avoided 'worst fault' matings for specific type traits where both sire and dam expressed a weakness in the same type trait(s). Applied judiciously, this process, called negative-assortative mating, reduces phenotypic variance in the next generation. No change in gene frequency occurs unless the mating program also involves selection of mates in addition to mate assignment.

Mating programs that emphasize assignment of mates to correct physical faults without selection for an economically justifiable breeding objective can harm genetic progress within a herd. Mating programs that select superior sires using a well-constructed selection index first, followed by mate assignment to avoid similar physical weaknesses, will be at least as successful as random use of the same bulls. If mating programs are to serve a long-term useful purpose in a breeding program, there must be emphasis on total economic merit of the next generation. That estimate of total merit needs to include traits with important economic impact. For instance, mating programs designed to improve longevity with no emphasis on productivity in the process may not improve lifetime economic merit. Carefully constructed selection indexes avoid this pitfall.

Recent concern for the buildup of relationships between proven artificial insemination bulls and females in dairy herds has opened an important new function for mating programs. Management of inbreeding through mate assignment improves the lifetime economic merit of the resulting progeny compared to random use of the same bulls. Estimates of the lifetime economic merit of cows produced by mate assignment considering inbreeding compared to cows produced through random matings show an economic advantage of as much as $37 per cow resulting from mate assignment, through avoidance of inbreeding depression. The investment necessary to achieve this improvement is good pedigree data on cows in the herd and a computer program to assign mates. The objective function maximized in the simulation study that produced this estimate was a comprehensive estimate of lifetime economic merit. Many commercially available mating programs now incorporate inbreeding considerations in mate assignment decisions. Development of genomic tools to avoid inbreeding in the coming years may further increase the advantages of controlled inbreeding in mate assignment programs.

Conclusion

Selection indexes are used globally to combine information on several traits into a single estimate of genetic merit. The process to create indexes in an optimum manner is well known. Economic conditions affecting dairy production differ considerably from country to country to affect the traits and weights used in selection indexes. Only one country places positive emphasis on the volume of milk produced but all countries apply positive weight to protein and fat. Most countries include some measure of longevity in their index. The most recent change in widely applied national indexes has been the incorporation of genetic evaluations for fertility. Increasingly complex and comprehensive indexes have been adopted as additional traits with known economic merit or closely correlated traits of known economic merit have become available.

The Net Merit index used in the United States was constructed using recent research results, but without strict application of Dr. Hazel's procedure. However, the resulting index was almost as effective in predicting lifetime net income in a large group of cows as a multiple regression equation with optimum weights based on the same traits. The empirical approach to creation of the Net Merit index created a useful tool without the controversy of defining and weighting traits in the aggregate genotype.

Selection indexes provide a systematic method to make optimal or near-optimal use of the variety of genetic evaluations available to dairy producers. Selection indexes, combined with mating efforts to control undesirable effects of inbreeding, offer promise of even more effective breeding programs for individual dairy producers in the years ahead.

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Cattle Genomics

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Introduction

Large gains in milk production from dairy cattle have been made over the past 50 years through the use of national genetic evaluation programs and artificial insemination (AI), especially for dairy cattle. These genetic gains have been made by combining pedigree and herd performance recording information to calculate breeding values for dairy bulls and cows for important economic traits. Following the sequencing of the bovine genome and subsequent targeted discovery of very large numbers of DNA markers, genomic information is available to improve the accuracy of these breeding values early in the animal's lives.

The Bovine Genome Sequence

The first draft of the bovine genome sequence was publicly released on 6 October 2004. The animal chosen for sequencing was an inbred Hereford cow 'Dominette'. Choosing an inbred animal was a deliberate strategy, because with an inbred animal fewer differences in sequence between the animal's paternal and maternal chromosomes are expected, and this simplifies the sequence assembly. The strategy used to sequence the bovine genome was a hybrid of two shotgun approaches: clone-by-clone sequencing and whole-genome shotgun sequencing.

The first step in clone-by-clone shotgun sequencing is to cut the genomic DNA randomly into pieces between 100 000 and 200 000 base pairs (bp) long. This can be done by a restriction enzyme that cuts the DNA whenever a particular sequence appears. For example, the *Hin*dIII enzyme cuts the DNA whenever the sequence ANAGCTT occurs. In mammalian genomes, this sequence occurs approximately every 110 000 bases. The second step of the clone-by-clone shotgun sequencing approach is to clone these short DNA fragments into the genome of bacteria, that is, to create bacterial artificial chromosomes (BACs). The BAC library then contains the whole bovine genome in many short fragments. The next step is to choose the minimum number of BACs that represent the entire bovine genome. First, each BAC fragment is cut with a single enzyme (e.g., *Hin*dIII) and the fragments are mapped to chromosomes using common sequence landmarks. Then the minimum set of BACs that span the genome, the minimum tiling path, is selected. Finally, the DNA within the clones selected from the minimum tiling path is randomly fragmented into stretches of approximately 800 bp. These fragments are sequenced. Assembly of the sequence involves finding sequences that have the same DNA for some of their length and, therefore, represent overlapping parts of the genome. In this way, the sequence of the fragment of bovine genome in each BAC can be reconstructed.

Whole-genome shotgun sequencing omits the BAC library step; therefore, the entire genome is fragmented into short fragments of approximately 800 bp. Many more reads are required with this approach to ensure a full coverage of the genome because the fragments are generated randomly. To ensure that all locations in the genome are sequenced, some locations are sequenced many times. For example, the bovine genome was sequenced to provide sixfold coverage; that is, each base was sequenced 6 times on average. However, because of the random nature of fragmentation, 0.25% of the genome was still unsequenced. Approximately 22.5 million reads were required to achieve that depth of coverage.

The current version of the bovine genome includes sequencing from both approaches. In fact, the most recent version incorporates information from a wide variety of sources including the original shotgun sequencing, linkage maps of genetic markers, and radiation hybrid maps comparing the order of sequence of the bovine genome with that of other mammals. The aim of integrating all this information was to achieve the most accurate assembly possible.

Comparing Bovine and Human Genome Sequences

Comparison of the bovine genome sequence with the human genome sequence and sequences of other mammalian species reveals the changes in DNA sequence that make cattle unique. Some interesting examples are gene families that have been altered or expanded (more bovine than human genes of that type) because of a possible role in rumen function. For example, a gene family called bactericidal/permeability increasing-like (BPI-like) proteins, which are expressed in saliva, has been investigated.

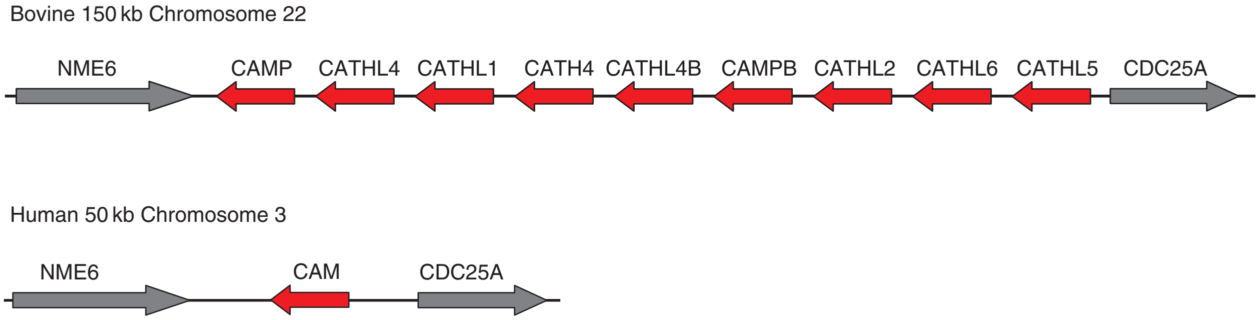

Figure 1 Human/bovine antimicrobial locus comparison: a schematic representation of the gene structures of cathelicidin loci on bovine chromosome 22 and human chromosome 3.

In humans, BPI-like proteins play a role in immune function. However, these genes were 4 times more frequent in cattle than in humans. As the rumen contains many thousands of species of bacteria, and these bacteria are the key to a ruminant's ability to survive on low-quality feed, the additional BPI-like proteins may play a role in modulating rumen function. Another gene family that is expanded in the bovine, compared to human and mouse, is the cathelicidins (**Figure 1**). Cathelicidins are potent antimicrobial peptides that rapidly inactivate microbes by destroying their membranes. These genes are expressed in many tissues, including the salivary gland. As for the BPI-like proteins, extra cathelicidins in the bovine could play some role in controlling the composition of rumen microflora.

Sequence Variation within and between Bovine Populations

Concurrent with efforts to refine the bovine genome sequence, much effort has been devoted to characterizing differences between and within cattle breeds at the genome level. Much of this variation occurs in the form of single nucleotide polymorphisms (SNPs, pronounced 'snips'), which are base-pair differences when the genome sequence is compared between two animals or two chromosomes at the same location in the genome (**Figure 2**). Some of the SNPs that have been characterized in cattle are a result of a low-coverage shotgun sequencing of additional animals, all of different breeds, followed by comparison of these sequences with the genome sequence from Dominette. Others are the results of a targeted SNP discovery effort through resequencing. In humans, at least 10 million sites in the genome have differences between individuals in the form of SNPs. The number of SNPs within and across cattle populations may be similar or even greater.

Genomic variation among cattle also exists in the form of insertions or deletions of small numbers of base pairs (indels) and, for some genes, in the number of copies of the gene an animal has (copy number variants (CNVs); **Figure 2**). In the human genome, many genes vary in copy number among individuals. Examples have been reported for immune function genes. Currently, little is known about CNVs in livestock, particularly their contribution to variation among animals for economically important traits. For the gene for agouti signaling protein, CNVs have recently been implicated as a cause of black fleece color in Australian merino sheep.

The SNPs are proving to be extremely useful tools for both population genetics and dairy cattle breeding. New genotyping technologies, such as high-density SNP arrays, allow tens of thousands of SNPs to be genotyped on a single chip at a cost of a fraction of a cent per SNP. For example, there is a commercially available SNP array with approximately 51 386 SNPs (the BovineSNP50 chip from Illumina®). The low cost of acquiring such a large volume of genetic information has led to increased use of the technology in the bovine research community and, more recently, by dairy cattle breeding companies.

By genotyping a large number of animals for SNPs across the genome, inferences can be drawn about the history of that population. For example, the genotype information has been used to infer the effective population size of dairy cattle breeds at different times in the recent and distant past. This analysis revealed the effect of domestication, breed formation, and the recent widespread use of AI in the dairy industry on genetic diversity among dairy breeds. The SNPs can also be used to locate areas of the genome that have been under strong selection (e.g., a region of the genome that contains a mutation that increases milk yield in dairy cattle).

Whole-Genome Association Studies

The SNP information can be used to locate genetic mutations that contribute to variation in economically important traits. Regions of the DNA called quantitative trait loci (QTLs) are associated with economically important traits in cattle. Knowledge of QTL location is useful

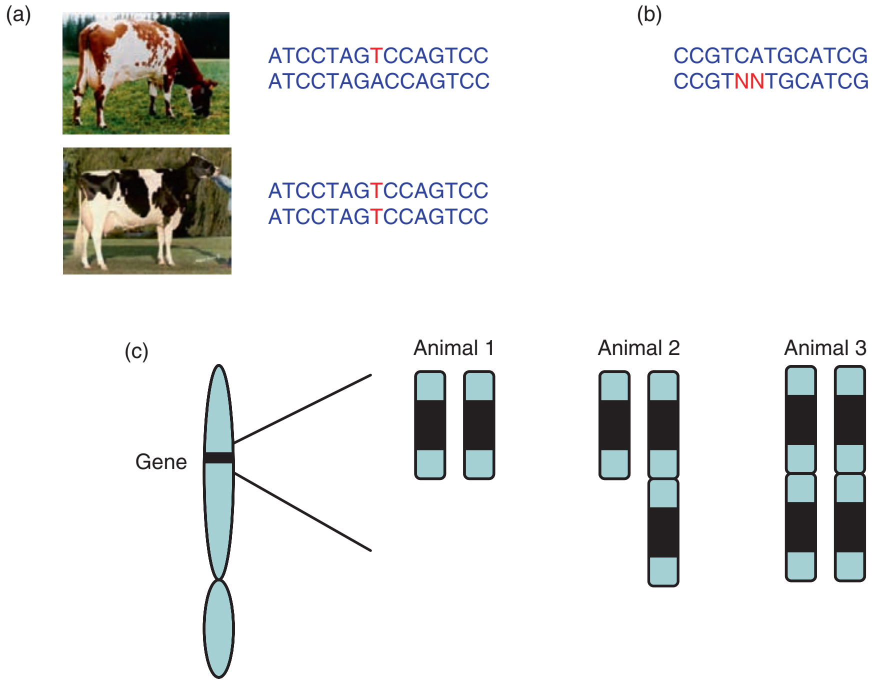

Figure 2 Types of genomic structural variation: (a) a single nucleotide polymorphism (SNP) is a base-pair difference between two chromosomes from the population at the same genomic position (the first cow is heterozygous at the SNP and the second cow is homozygous for the T allele); (b) a deletion is the absence of 1 bp or more on a chromosome from the population relative to a reference or other genome sequence; (c) a copy number variant is an increase or decrease in the number of copies of a particular gene among chromosomes from the population (the first animal is homozygous for a single copy of the gene, the second is heterozygous, and the third is homozygous for two copies).

for understanding the biology of different traits, and QTL information can also be used in marker-assisted selection (MAS).

In whole-genome association (WGA) experiments, a group of animals are genotyped for a number of SNPs. Then, each SNP is tested in turn for an effect on the trait of interest, such as protein content of milk. For example, for a SNP with alleles A and T, do cows with the A allele produce significantly more protein over their lactations than cows with the T allele?

For WGA experiments, a phenomenon called linkage disequilibrium is exploited. Linkage disequilibrium arises in a population because of small chromosome segments in the current population that are from the same common ancestor. These chromosome segments, which trace back to the same common ancestor without intervening recombination, have identical marker alleles and, if a QTL is located somewhere within the chromosome segment, identical QTL alleles (**Figure 3**). Linkage disequilibrium results in a higher correlation than expected between the allele carried at a QTL and the allele at a marker on the same chromosome. Consequently, animals that differ at the DNA marker will also tend to carry different QTL alleles and, therefore, tend to differ in phenotype.

The results of a WGA experiment conducted for an Australian Holstein–Friesian population are shown in **Figure 4**. The experiment was carried out by genotyping 730 bulls for approximately 54 000 SNPs. The phenotypic trait for each bull was the average protein yield of his daughters. Each point on the graph represents a SNP on a particular bovine chromosome. The *y*-axis is an *F*-value (a measure of significance) reported for each SNP. The graph shows a cluster of highly significant SNPs toward the end of the chromosome (~140 million bp), which indicates the presence of a QTL affecting protein yield in that region.

Mapping QTLs with linkage disequilibrium has led to the identification of mutations affecting dairy production traits. A mutation in a gene involved in fatty acid synthesis (*DGAT1*) explains up to 30% of the genetic variation in fat percentage in milk in some dairy breeds. A mutation in the growth hormone receptor (*GHR*) gene explains a significant proportion of the genetic variation in protein percentage in milk in Holstein–Friesian dairy cattle.

WGA experiments have also been used to locate mutations that lead to rare genetic defects in cattle. A WGA experiment was used to locate the mutation causing congenital muscular dystonia type 1 (CDM1). Calves affected with this disease have episodes of generalized muscle contractions, difficulty in swallowing, fatigue after stimulation and exercise, and muscle myotonia that results in an inability to flex their limbs. Calves with CMD1 usually die within a few weeks as a result of respiratory complications. The disease has been observed in Belgian Blue

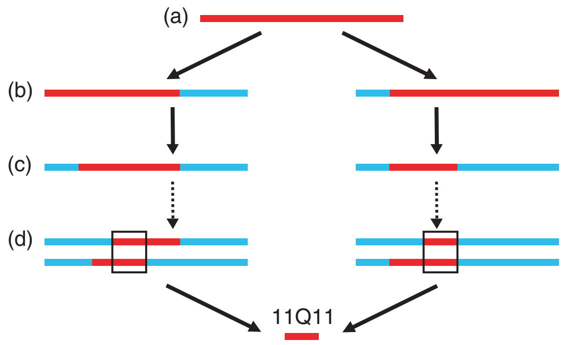

Figure 3 An ancestor many generations ago (a) has offspring (b). Each subsequent generation (c), the ancestor's chromosome is broken down by recombination until all that remain in the current generation (d) are small conserved segments of the ancestor's chromosome. Animals with these segments have identical marker and quantitative trait loci (QTLs) alleles for all loci within the segment. In this case, the chromosome segment contains four single nucleotide polymorphism loci with the 1 allele and one QTL with the Q allele.

cattle at a low frequency in the population (0.1%). The WGA experiment involved genotyping 2081 Belgian Blue cattle, 26 of which were affected by the disease, with approximately 60 000 SNP markers. The mutation was mapped to a 2.12 million bp region on chromosome 25. Out of the 95 genes in this region, 6 were plausible candidates for carrying the disease-causing mutation based on their function. Sequencing of these six genes in a sample of the affected animals revealed an amino-acid changing mutation in the gene *ATP2A1*. The gene encodes a protein that transports calcium ions into fast-twitch skeletal muscle fibers. This process results in muscle relaxation. Further supporting evidence for the hunch that the *ATP2A1* mutation was causing the disease was the observation that mutations in the same gene in humans caused a rare genetic disorder with symptoms of exercise-induced muscle cramps and impaired muscle relaxation. Now that the mutation causing CMD1 has been identified, bulls can be screened for the mutation and culled if they are a carrier, which will aid in reducing the frequency of the disorder in the population.

Marker-Assisted Selection and Genomic Selection

MAS can also be used to increase the frequency of QTL alleles with favorable effects. Such selection integrates pedigree, phenotype, and marker information to increase the accuracy of estimated breeding values (EBVs), and thus to increase the rate of genetic gain. The idea of using DNA markers to improve the rate of genetic gain in dairy cattle has been around for many years. However, the adoption of MAS by the dairy industry generally has been limited until recently. Unlike genetic diseases caused by a single mutation, quantitative traits (such as production and health) are typically affected in dairy cattle by a large number of loci. Any one locus then explains a limited proportion of the total genetic variance. As a result, only small additional gains are possible if a limited number of QTLs are included in the estimation of breeding values. Furthermore, until high-throughput SNP genotyping became available, the limited number of genetic markers available meant that the number of QTLs characterized for all of the economically important dairy traits was small.

A major advance was the demonstration that accurate selection decisions are possible when breeding values are predicted from dense marker data alone using an approach called genomic selection. Genomic selection

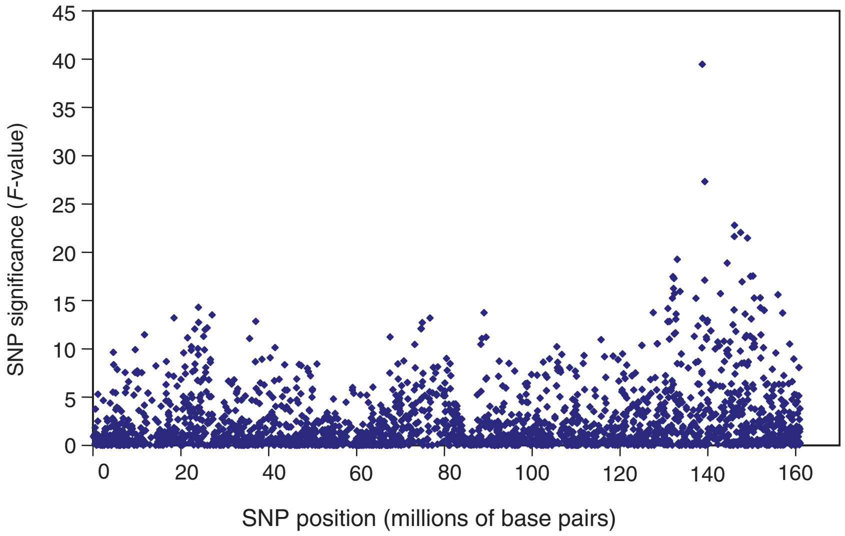

Figure 4 Results of a whole-genome association experiment for protein yield in dairy cattle for an individual chromosome.

refers to selection decisions based on genomic EBVs (GEBVs). The GEBVs are calculated by deriving a prediction equation based on the SNPs. The entire genome is divided into small segments defined by the location of the SNPs. The hypothesis is that each of the SNPs is in linkage disequilibrium with a QTL. The effects of all the QTLs are estimated in a reference population in which animals are both phenotyped and genotyped. In this way, the effects of all loci that contribute to genetic variation are captured, even if the effects of the individual loci are extremely small. In the next generation, animals need only be genotyped for the markers to determine which chromosome segments they carry to calculate their GEBVs. The estimated effects of the segments that the animal carries are summed across the whole genome.

The accuracy of GEBVs predicted in this way has already been evaluated in experiments in the United States, New Zealand, Australia, and The Netherlands. Accuracy is the correlation of the GEBVs with the true breeding values of the bulls. These experiments used reference populations of size between 650 and 4500 progeny-tested bulls that had been genotyped for approximately 50 000 genome-wide markers. The accuracy of GEBVs for young bulls that were not in the reference population ranged from 0.4 to 0.82. The accuracy achieved depended on the heritability of the trait evaluated, the number of bulls that had records used to calculate the SNP effects, and the statistical method used to estimate these SNP effects.

The implications of achieving such GEBV accuracies for animals at birth are profound. The results suggest that the accuracy of the GEBV for a bull calf can be less than but approaching the accuracy of an EBV following a progeny test, depending on the trait in question. If such an increase in accuracy is achieved, genomic selection could lead to a doubling of the rate of genetic gain through selection and breeding from bulls at 2 years of age based on their GEBVs rather than at 5 years of age or later based on their progeny test results (**Table 1**). A further attractive feature of genomic selection is that by avoiding progeny testing, bull-breeding companies can save some costs allocated to progeny testing.

Further genetic gains could be made by genotyping elite bull dams and selecting a smaller number for mating specific sires and by screening large numbers of bull calves with the markers to increase selection intensity. Bulls potentially are able to breed from 1 year of age; therefore, the generation interval could be reduced even further. Already 1-year-old bulls are being mated to a small number of cows to check for genetic diseases before these bulls receive widespread use at 2 years of age.

A benefiting feature of genomic selection may be more appropriate balance in genetic gain among traits. At present, large gains are made for production traits in the dairy industry. However, gains for traits like fertility are relatively small or negative, partly because of the lower accuracy of fertility EBVs and because production and fertility are unfavorably correlated. Genomic selection should improve the accuracy of fertility EBVs if records are sufficient to estimate SNP effects accurately. Then fertility would have a greater contribution to the total breeding objective.

The impact of genomic selection on inbreeding from a dairy breeding program should be carefully considered. If the generation interval is not altered, genomic selection will actually result in a lower rate of inbreeding than that from a breeding scheme based on nongenomic selection, especially for traits with low heritability. To understand this, consider the selection of young bull calves to become part of a progeny test team. If no genomic information is available, the EBV for a young bull calf is the average EBV of his sire and dam. Two full brothers would have the same EBV. If this EBV is high enough, both would be selected to be part of the progeny test team. However, if genomic information is available, the Mendelian sampling term (the result of the random sampling of paternal and maternal chromosomes during gamete formation) is captured. Then the two full brothers would have different GEBVs, and selecting both for progeny test would be less likely. Avoiding selection of close relatives decreases the rate of inbreeding.

Table 1 Timelines for possible dairy breeding programs based on traditional progeny testing or genomic selection

	Year				
Breeding program	*1*	*2*	*3*	*4*	*5*
Progeny testing	∼100 bull calves selected	Daughters born	Daughters mated	Daughters milking	∼20 bulls chosen for industry-wide semen sales
Genomic selection	∼20 elite bull calves selected based on genomic estimated breeding values	Semen from elite bulls available for industry-wide sale	***3 years advance in genetic gain***		

However, if the generation interval in the breeding program is halved, to take advantage of accurate GEBVs available at birth, the resulting increase in inbreeding per year may outweigh the decrease in inbreeding from capturing Mendelian sampling. If the generation interval is reduced, inbreeding could potentially be managed by screening a much larger number of selection candidates for bull teams than has been done previously. Then the contribution of any individual sire family to the selected bulls could be restricted such that inbreeding remains at an acceptable level.

Management of inbreeding is just one of the challenges remaining with genomic selection and its implementation. Other challenges include increasing the accuracy of GEBVs, integration of genomic information into national and international genetic evaluations, and management of long-term genetic gain.

Conclusion

Largely as a result of the bovine genome sequencing project, extremely large numbers of SNP markers are now available for cattle. In the dairy industry, the SNP markers are being used in genomic selection to increase the rate of genetic gain. By associating the SNP information with variation in the traits that affect the profitability of dairy production, genomic selection leads to accurate prediction of breeding values for young bulls and heifers. The GEBVs enable early selection and use of elite dairy bulls, which greatly accelerates the rate of genetic gain. Genomic selection is under way in at least four dairy breeding programs around the world.

The next development in genomics that is likely to have a large impact on the dairy industry is the new generation of sequencing technologies. Technologies such as Illumina Solexa® and Roche 454® have dramatically reduced the cost of resequencing specific genes, chromosomes, or even entire genomes. Within a few years, many elite dairy bulls are likely to have had their genomes sequenced, with the aim of uncovering their mutations that will have favorable effects on milk production and other important traits.

One area where this resequencing technology is being applied is microbiomes, which coexist with different species. Rumen microflora, the supplementary bovine genome, has already been partially sequenced. Analyses of this sequence suggested that less than 0.1% of the sequences from the dairy cattle rumen match published sequences, revealing a unique and complex microbial community. Just as the sequencing of the bovine genome has led to tools for selection to increase the profitability of dairy farming, tools and genes discovered from sequencing the rumen could enable the development of strategies for reducing methane emissions and improving feed conversion efficiency.

See also: **Genetic Defects in Cattle**. **Genetics**: Cattle Genomics; International Flow of Genes; Selection: Concepts; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods.

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International Flow of Genes

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Introduction

Milk producing cattle have accompanied mankind as they explored the earth. Despite their large mass relative to some other livestock, cattle were included in early ocean voyages and land excursions, attesting to their perceived importance. For hundreds of years, movement of cattle genetics required transport of the whole animal. In the mid-twentieth century, artificial insemination (AI) technology created the opportunity for genetic progress because the very best bulls could be used to breed large numbers of cows. This movement was facilitated and amplified by the development of frozen semen. In addition to removing the dangers from live bulls and disease transmission through natural mating, AI made semen available on a reliable basis and promoted the transport of genetic material nationally and internationally. Frozen embryos have made possible importation of fully foreign genetics. Due to the extent of impact, the use of AI has become a key factor in the genetic improvement process. However, full realization requires the ability to identify which are the truly superior bulls for extensive use.

Historical and present methods of assessing the genetic merit of animals, particularly bulls, from other countries will be described. Information will be presented on the direction of gene flow in connection with changing relative national genetic levels. Guidance will be given on the ethics of presenting marketing data.

Selecting Source of Genes

FAO Trial

Selection of genetics from other countries was initially based on intuition and phenotype. Local evaluation systems, although crude by today's standards, generally sorted out the North American genetics as superior for yield for Holsteins, Brown Swiss, and Jerseys. Perhaps the pivotal examination for black-and-white cattle (Friesians and Holsteins) was from the Food and Agricultural Organization (FAO) trial that started in the early 1970s. Ten countries provided frozen semen for this designed trial from a random sample of progeny-tested young bulls. Semen was used on cows in Poland and extensive recordings were made on the resulting daughters. Bulls from the United States ranked highest for milk and fat yield and longevity. Although the level of environment was low in that trial, a subsequent trial at higher levels in Poland and Israel confirmed the genetic superiority of North American Holsteins in a range of environments. These useful demonstrations are less pertinent with the passage of time – continuous international comparisons are needed.

Interbull Formed

The need for better methods for comparing genetic material across countries was evident decades ago. Trials such as those conducted by FAO, including the one comparing red-and-white cattle in Bulgaria, could give general information about national strains, but did very little to facilitate translation of evaluation results for individual bulls across borders. Working groups of the International Dairy Federation (IDF) and the European Association for Animal Production (EAAP) were leaders in this effort. In 1982, a decision was made to establish a single international forum for these discussions and the International Bull Evaluation Service (Interbull) was formed in 1983. Efforts were directed toward exchange of information and harmonizing trait definitions and national evaluation methodology. While this served (and continues to serve) the most useful purpose, comparison of bulls across countries was by procedures established by importing countries and therefore was subject to criticism and suspicion.

In the early years of 1980s through the mid-1990s, genetic information from one country was translated to that of another through conversion equations. These equations were of the simple linear regression form, $y = a + bx$, where y is the estimated evaluation in the importing country, x is the known evaluation in the exporting country, a is the intercept (value of y when $x = 0$), and b is the slope (regression of y on x). In the Goddard or Wilmink approaches to developing conversion equations, the y- or x-values were modified to account for the imperfect estimate of merit in the importing country. These conversions were very time consuming as they produced equations for only a pair of countries at a time. They depended on bulls evaluated in both countries and such bulls might be rare or nonexistent. Even if there were a suitable number of bulls evaluated in both countries, there could be problems associated with the direction of gene flow and preferential management of daughters produced by imported semen.

This procedure does little to provide information on bulls with evaluations in multiple countries. An unfortunate characteristic of regression procedures is that prediction is least accurate for the animals of most interest, those extremely positive and beyond the range of the data used for developing the conversion equations.

Interbull Centre

Selection decisions were relatively easier in the times when the North American cattle, particularly Holsteins, were recognized as clearly superior for yield. With the closing of the genetic gap, additional countries have competitive bulls and it is increasingly important to have accurate comparisons of bulls from many countries on the scales of all countries. Formation of the Interbull Centre in Uppsala, Sweden, in 1991 led to international evaluations of combined data at a single, unbiased location. In August 1994, routine production trait evaluations were provided for four Scandinavian countries and two breeds. Coverage was extended to 10 countries and 5 breeds in February 1995 and as of August 2008, there were 27 countries and 6 breeds. Traits have been added over time: conformation in August 1999, udder health in May 2001, longevity in November 2004, calving traits in February 2005, female fertility in February 2007, and milking speed and temperament in January 2009. Not all breeds or countries were included from the beginning, and participation for nonyield traits ranges from 3 breeds and 14 countries for calving traits to 6 breeds and 23 countries for conformation. Data from participating countries are now processed 3 times per year (following periods of 2 times, then 4 times per year) and results are presented on each country's scale. An important feature of the multiple-trait across-country evaluation (MACE) procedure of combining national evaluations is that it uses the estimated genetic correlations between countries and the products are customized to each country's scale. It is the responsibility of each country to determine the status and availability of results on their scale. Generally, the Interbull results are deemed official unless there is a local evaluation of a relatively high accuracy. Interbull evaluations have facilitated and promoted international movement of dairy semen.

Database Offers Research Possibilities

Sharing of national evaluations and pedigree data are a requirement for the Interbull evaluation process. Availability of the Interbull results and the necessary pedigree files facilitates research as well as selection decisions. Comparisons of trends in genetic merit, source of genetics, and other characteristics have become readily available through the international database. Much of the data in this article was obtained from the Interbull file, specifically that available at the USDA Animal Improvement Programs Laboratory website. For example, one can track the frequency of bulls that have daughters from more than one country and see that nearly 10% of Holstein bulls included in the Interbull evaluation and born in the 1990s had daughters in more than one country. More recent years show some decline due to insufficient time for bulls found to be outstanding in the original country to be used and have qualifying genetic evaluations elsewhere. One bull, Maizefield Bellwood, has daughters included in the Interbull evaluation from 22 countries. Public files contain evaluations only on the scale of the providing country, but all of the rest of the data are the same regardless of source: bull ID, pedigree information, amount of data for each trait (daughters and herds), number of countries with daughters and country with the most daughters, and so on.

Changes in Gene Flow

Direction of Genetic Flow

Most of today's familiar dairy breeds were developed in Europe. Breeders in North America exploited the dairy characteristics instead of dual-purpose characteristics and thus developed cattle with superior milk producing ability. The attractiveness of high-yield phenotypes, trials like those of FAO, experience from North American semen, and now the Interbull results have spurred transport of semen from North America to Europe and all parts of the world. The August 2008 Interbull results showed that North American Holstein bulls were almost exclusively by North American sires and dams until birth year 1999 when 5% of sires were from elsewhere. Since then, the percentage has varied and was 9% in the most recent birth year for evaluated bulls, 2002. Dams have continued to be essentially all North American. The pattern for non-North American bulls is given in **Table 1**. The percentage of North American sires for bulls from other countries was about 90% for 1987 through 1992. For dams and maternal grandsires, percentages reached 30 and 87%, with most being from the United States, especially for dams. The situation varied by country. Most French and Dutch bulls born in 1990 and now in Interbull evaluations were sired by US bulls (98 and 97%, respectively), while the average for other countries was 87% and lower. In recent years, the situation has changed considerably regarding the direct impact of North American animals. Increasingly, parents are from elsewhere. However, it may still be largely North American genes, just now carrying the identification of another country. Examination of this situation would be a very interesting

Table 1 Percentage of ancestors from North America and specifically the United States for bulls from outside North America (August 2008 Interbull)

	Percentage North American			*Percentage US*		
Year of bull birth	*Sire*	*Dam*	*MGS*	*Sire*	*Dam*	*MGS*
1986	87	13	74	53	11	56
1987	89	17	79	62	15	62
1988	90	25	82	65	22	64
1989	89	30	83	74	28	66
1990	88	30	83	79	28	69
1991	91	29	84	77	28	69
1992	88	27	85	72	25	71
1993	85	20	85	61	18	71
1994	84	17	86	65	16	72
1995	86	13	87	79	12	72
1996	79	17	87	73	15	72
1997	74	14	87	61	12	74
1998	65	13	84	57	10	73
1999	47	12	80	43	10	70
2000	48	11	75	44	9	67
2001	41	9	66	36	8	55
2002	44	7	57	36	6	47
2003	51	4	46	33	3	37

MGS, maternal grandsire.

project, but was not attempted for this article. The decline in North American maternal grandsires occurs later than for sires because of the additional generation.

The use of the same sires and maternal grandsires in much of the world contributes to increases in inbreeding on a global scale. Another way of describing the situation is that the effective international population size and global genetic diversity are diminishing. Years ago, concerns about increases in inbreeding could be answered by suggesting that if serious problems arise, relatively remote genetics from another country could be introduced. This is no longer an option, so inbreeding issues must be faced more directly.

The upgrading of various Holstein populations with North American genetics has reduced genetic differences considerably. This has brought about some reversal in the gene flow. Data in **Table 2** are for Holstein bulls being sampled in AI centers in the United States. Numbers of bulls in the sampling programs could be underreported as some bulls in progeny testing for the most recent years

Table 2 Percentage of foreign sires of Holstein bulls progeny tested in the United States

Year of bull birth	*Bulls sampled*	*Percentage of all foreign sires*	*Percentage of Canadian sires*	*Percentage of other sires*
1990	1224	1.9	1.9	0.0
1991	1206	6.9	6.9	0.0
1992	1412	5.2	5.2	0.0
1993	1294	12.9	12.9	0.0
1994	1212	6.6	6.2	0.4
1995	1249	6.2	6.0	0.2
1996	1312	4.0	3.5	0.5
1997	1334	18.8	14.9	3.9
1998	1405	9.4	3.6	5.8
1999	1258	17.3	2.0	15.3
2000	1253	32.7	3.0	29.7
2001	1214	28.6	5.6	23.0
2002	1328	23.3	8.7	14.6
2003	1297	31.2	24.3	6.9
2004	1262	26.9	11.4	15.5
2005	1255	40.0	10.3	29.7
2006	1274	38.8	13.5	25.3
2007	1199	29.9	3.1	26.8

may not have been reported. Numbers of bulls might have also declined slightly in recent years due to mergers, increasing numbers of daughters per sampled bull, decreasing US cow population, and perhaps the marketing of foreign semen. The impact of foreign sires of bulls sampled in the United States is clear. In the early years, foreign sires were all from Canada. The percentage of non-US sires is generally increasing, but whether it is from Canada or elsewhere is less consistent. For bulls born in 2000 or 2005, nearly 30% were from outside North America while the figure for 2003 was only 7% while Canadian sires of sons were nearly 25%. This strong Canadian representation for the sires of sons was largely due to 250 sons (19%) of just 3 bulls. These 3 bulls contributed only 39 sons (3%) the following year. Sourcing of sires of sons is quite dynamic, driven by the 'hot' prospects at a given time. While the foreign sires of sampled sons have reached as high as 40%, to what extent are these genes of North American, or specifically US, origin?

Crediting a country as the source of genes or even animals is becoming increasingly difficult. International AI organizations, multicountry sampling, and reidentification of bulls contribute to the uncertainty. However, it is the repackaging of North American genetics, now owned by others, that begs the question, 'Are we interested in where the genes came from, or where the parents came from, or where we can buy the product?' For example, in the 1997 crop, the two European bulls siring the most US sons were Etazon Lord Lily (the Netherlands) and Fatal (France) with 80 and 58 sons, respectively. Lord Lily has a solid US pedigree. Fatal has a US sire and the dam is 93% North American. The Holstein bull siring the most US progeny-tested sons born in 2005 and 2006 (148 and 197, respectively) was Picston Shottle. Shottle is from Britain but his sire is Italian (Mtoto). However, examination of Mtoto's pedigree shows that all eight grandparents are US. Shottle's dam is Canadian and five grandparents are US. Thus, although Shottle is at first glance British and his sire Italian, his genes are all North American and at least 81% US. The goal should be to use the best genetics available anywhere. Customers expecting exotic pedigrees and heterosis from use of foreign bulls are likely to be disappointed as the same sires and maternal grandsires appear in pedigrees in many countries. In this presentation, bull origin is the country possessing the bull and marketing the semen.

Shift in Merit Globally

Average predicted transmitting abilities (PTAs) on the US scale for milk and protein are given in **Table 3** for countries with the largest Holstein sampling programs among the Interbull participants. These data from the August 2008 Interbull run need to be viewed with some caution. They are on the US scale and genetic correlations less than unity with other countries would tend to reduce the apparent improvement for other countries versus presentation on their own scales. Countries may differ in the consistency in sampling programs over time. If programs are expanding (or the coding of bulls as AI sampled changes over time), trends within countries will be affected. Heavy use of a few sires of sons that turn out particularly good or bad, coupled with relatively few bulls sampled, can impact these statistics considerably. With these caveats, and without dwelling on specific country data, it is clear that differences among leading countries are diminishing and that countries with a low average merit before tend to have the largest gains. Correlations between mean PTA in 1993 and annual change were −0.17 for milk and −0.76 for protein. Obviously, countries starting at a low genetic level have the opportunity to make more rapid progress than genetically superior countries even though they do not overtake the leaders in actual level of merit. This 'catching up' means that differences among countries in genetic merit are generally lessening. Interbull data from the May 2005 routine run for Holsteins showed that in 18 countries with more than 25 multicountry full-brother families, there were 9920 bulls in 3600 families. In other words, there were 3600 sets of full brothers that spanned national boundaries. Who is to say which country selected the better brother?

Managing Information Flow

Currently, there are 26 countries contributing data to the Interbull evaluations for Holsteins and 6–14 countries for other breeds. Then there are scores of nonparticipating customer countries. There is a huge opportunity for confusion regarding relative merit for sources of genetics and individual bulls. For participating countries, genetic evaluations for most bulls of interest are available on their national scale. For other bulls and for cows, Interbull provides conversion equations. Nonparticipating countries may decide to have evaluations expressed on a familiar scale or on the scale of a country that seems to be most similar to them, although that may be difficult to define. As a minimum, selection needs to be made among bulls whose data are presented on the same national scale, regardless of how that scale is chosen.

Interbull has developed guidelines for the advertising of genetic merit of dairy animals, available at their website. Key points in the guidelines are that advertisers must identify the date, source, and units of the evaluations and not manipulate them, such as changing original breeding values in kilograms to transmitting abilities in pounds or vice versa. However, it is difficult, if not impossible, to control information in its many forms, particularly when marketing advantages are involved.

Table 3 Genetic merit means and average changes over a 10-year period of birth years on the scale of US Holsteins for pounds of PTA for milk or protein

	Mean PTA for milk (lb) for bulls born in		
Country	*1993*	*2003*	*Annual increase (lb)*
Australia	−417	7	42
Canada	−628	715	134
Denmark	−448	564	101
France	−331	913	124
Germany	−751	−402	35
Great Britain	−610	554	116
Italy	−496	791	129
Netherlands	−343	593	94
New Zealand	−827	−444	38
Poland	−1296	356	165
United States	−219	842	106
	Mean PTA protein (lb) for bulls born in		
	1993	*2003*	*Annual increase (lb)*
Australia	−4.8	13.8	1.9
Canada	−15.6	19.7	3.5
Denmark	−8.5	26.2	3.5
France	−2.2	29.6	3.2
Germany	−15.6	18.0	3.4
Great Britain	−10.1	22.2	3.2
Italy	−8.8	23.4	3.2
Netherlands	0.8	28.6	2.8
New Zealand	−12.0	12.7	2.5
Poland	−29.3	18.5	4.8
United States	−4.1	23.4	2.8

Conclusion

Advances in physiology made it possible to transport genetic material over great distances economically in the form of semen and embryos. However, this opportunity is of only academic interest without a means to assess the genetic value of animals across borders (genetic evaluation systems and scales). The ability to make these comparisons has improved rapidly in the past 15 years. Procedures to combine data and estimate evaluations across countries are available for yield, udder health, type traits, calving ease, longevity, female fertility, milking speed, and temperament. The system had many positive effects but is recognized as imperfect and refinements will continue. As of this writing, efforts are under way to incorporate genomic estimates of merit into the Interbull evaluations.

The ability to identify and use the best genetics globally will continue to result in reduced genetic differences among countries and increases in genetic levels. More countries will be represented among exporters. Consideration of inbreeding will become more important in mating decisions as national breed populations become joined and increasingly related.

See also: **Gamete and Embryo Technology**: Artificial Insemination. **Genetics**: Selection: Evaluation and Methods.

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Relevant Websites

http://www.aipl.arsusda.gov – Animal Improvement Programs Laboratory

http://www.interbull.org – INTERBULL

GENETIC DEFECTS IN CATTLE

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Introduction

Cattle are known to possess various genetic defects. A genetic defect usually refers to any abnormality that has a genetic origin. Most genetic defects identified have a rather simple mode of inheritance, often controlled by alleles at a single gene site. However, as scientists learn more about the complexity of the mammalian genome, they are learning that the inheritance of some genetic defects may involve multiple gene sites. The widespread use of artificial insemination in cattle and the extensive use of semen from a few elite bulls have led to elevated levels of inbreeding in some dairy breeds. As inbreeding levels increase, the likelihood of finding more genetic defects becomes greater. Genetic tests are available for some genetic defects so that parents can be tested to determine if they might transmit the defect to their offspring.

Inheritance

Gene mutations are believed to be the origin of most genetic defects. Different kinds of mutations are possible. For example, the gene mutation may have originated as a simple change in a DNA base pair, resulting in coding for a different amino acid. An alternative gene mutation may have originated from a deletion of DNA, resulting in a lack of coding for a particular protein. Mutations that alter the normal coding for certain amino acids or proteins can lead to genetic defects.

Inheritance is usually straightforward for genetic defects controlled by a single gene site. The more common defects observed in cattle are caused by recessive alleles. With recessive alleles, the genetic defect is not expressed unless both alleles at the gene site are recessive alleles. Individuals that have both a normal allele and a recessive allele generally do not exhibit the symptoms of the defect and are commonly called 'carriers' of the recessive. Because carrier individuals appear normal, the recessive allele can be passed on from generation to generation undetected. Only when the individual receives the recessive allele from both parents is the genetic defect observed. **Figure 1** demonstrates various modes of inheritance of recessive alleles.

The total number of genetic defects in cattle is not known, but is likely to be in hundreds. Most are quite rare, as breeders generally try to avoid using parents that are known carriers of a genetic defect. The frequency of a defective allele can increase rapidly in a population if an unknown carrier is used extensively as a parent.

In cattle, with the advent of artificial insemination, a sire may have thousands of offspring. If this sire is a carrier of a recessive allele, 50% of the sire's offspring will receive the recessive allele from the sire. Even in this situation, the genetic defect may go unnoticed in his progeny if the gene frequency for the recessive allele is very low in the cow population, as the defect is expressed only when both the sire and the dam transmit the recessive allele. If the sire is popular, many of his progeny and grand progeny may be used extensively in breeding programs. The frequency of the recessive allele can increase dramatically, and the incidence of genetic defects will rise when the original sire begins appearing as an ancestor on both sides of an individual's pedigree.

Inbreeding

Inbreeding can lead to higher frequencies of genetic defects. Inbreeding occurs when the alleles at a gene site are identical by descent. This can occur when a common ancestor appears on both the maternal and paternal side of the pedigree. In recent years, dairy bulls with high genetic merit for performance traits have been used extensively in artificial insemination programs. As a result, inbreeding levels have been increasing in some dairy breeds due to the extensive use of a few individual bulls and their offspring. This has been especially true for the Jersey and Holstein breeds. **Table 1** lists the inbreeding levels for the Holstein breed in recent years and the projected levels to the year 2020.

As inbreeding levels increase, there is a greater chance for recessive genes that trace back to a common ancestor to be expressed. Recessive genes that may have been rare at one time are now more widely spread through the population due to extensive use of an elite sire in breeding programs.

Examples of Genetic Defects

The following are some of the more common genetic defects identified in cattle. The defects are believed to be inherited as simple recessives, meaning individuals

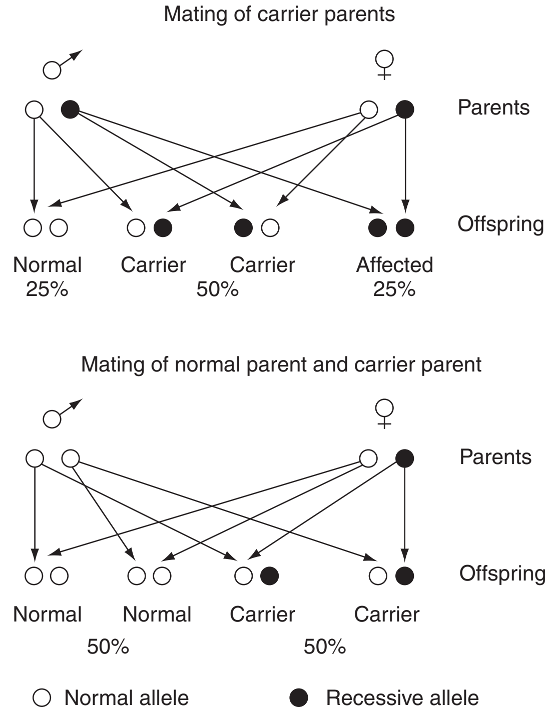

Figure 1 Modes of inheritance of recessive alles.

Table 1 Estimated and projected average inbreeding percentage of US Holstein population

Year	*Average inbreeding (%)*
1970	2.7
1980	4.6
1990	5.4
2000	6.8
2010	8.2
2020	9.7

Adapted from Young CW and Sekora AJ (1996) Estimates of inbreeding and relationship among registered Holstein females in the United States. *Journal of Dairy Science* 79: 502–505; Hansen LB (2000) Consequences of selection for milk yield from a geneticist's viewpoint. *Journal of Dairy Science* 83: 1145–1150.

express the symptoms when the recessive allele is found on both chromosomes.

Mulefoot: Mulefoot (syndactylism) is a condition characterized by a single toe on one or more feet (**Figure 2**). The foot looks more like the foot of a mule than a cow. Mulefoot has been identified in the Holstein and Angus breeds. Although not a lethal recessive, mulefoot animals have reduced mobility, especially if more than one foot is affected. As a result, mulefoot animals are often unthrifty.

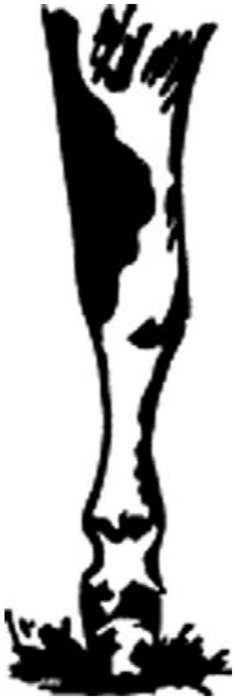

Figure 2 Mulefoot.

Bulldog: Bulldog (achondroplasia) is a defect found in the Holstein breed. The Holstein Association USA recognizes three different defects: bulldog 1, bulldog 2, and bulldog 3. All three are considered lethal. Bulldog 1 calves (**Figure 3**) are usually aborted prior to the normal gestation period. The aborted calves will have very short limbs, a bulging forehead, a shortened under jaw, and a depressed nose. Bulldog 2 calves may be born alive, but usually die shortly after birth. Although bulldog 2 symptoms are similar to bulldog 1 symptoms, the symptoms are usually not as pronounced. Bulldog 3 calves may have more normal-length limbs compared to the other two types of bulldog, but calves will still have a characteristically wide skull.

Weaver: The weaver condition (bovine progressive degenerative myeloencephalopathy) is found in the Brown Swiss breed. Weaver animals appear completely normal for 6–18 months of age. Symptoms include a staggering gait, lack of coordination of the rear limbs, a wide stance to maintain balance, and stumbling or falling,

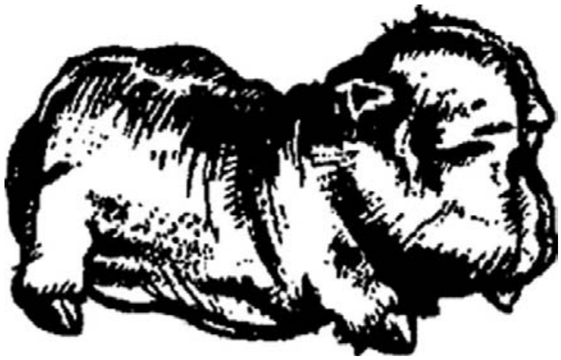

Figure 3 Bulldog.

especially if excited. Eventually the animals lose control of their rear limbs. The disorder is caused by degeneration of the nerves that control the muscles in the rear legs.

Limber leg: Limber leg is found in the Jersey breed. Symptoms include grossly rotated shoulder and hip joints. The calf will attempt to stand but will be unable to do so because of deformed joints and muscles. The defect is considered a lethal, as limber leg calves are unable to stand on their legs.

Spinal muscular atrophy (SMA): SMA is found in the Brown Swiss breed. The defect does not have symptoms that are as obvious as the previously discussed defects. SMA affects the nervous system and results in a deterioration of muscle tone and reflexes. Symptoms are usually observed in calves when they are 3–6 weeks of age. Calves begin to have difficulty standing, the skeletal muscles begin to deteriorate, and the calf often will have difficulty breathing.

Rectovaginal constriction (RVC): RVC is a defect found in the Jersey breed. Jerseys with RVC appear completely normal. Females with RVC have a constriction around the posterior portion of the reproductive tract. Both males and females have a constriction of the anal sphincter area. The defect is commonly observed at the time when heifers are bred by artificial insemination, as passage of the human arm into the rectum may be either difficult or impossible due to the anal constriction. The defect may not be noticed at breeding time if the heifers are bred by natural service. However, if the heifer becomes pregnant, the constriction around the reproductive tract prohibits passage of the calf through the birth canal.

Bovine leukocyte adhesion deficiency (BLAD): BLAD is a defect observed in Holstein cattle. The defect results in a lack of coding for a surface protein on white blood cells that is necessary for passage of the white blood cells out of the bloodstream to attack infections. Defective calves die within a few months of age when they acquire normal calf-hood infections and the white blood cells cannot reach the infection sites. There are no symptoms specific to BLAD except for an extreme elevation of the white blood cell count of the sick calves. The calves die of whatever infection they may have acquired due to the inability of the white blood cells to reach the infection.

Deficiency of uridine monophosphate synthase (DUMPS): DUMPS is an enzyme defect found in Holsteins. Uridine monophosphate synthase is an enzyme necessary for normal development. Defective fetuses do not produce the synthase and, as a result, death of the fetus occurs between 45 and 60 days. The fetus is either aborted or reabsorbed. Symptoms are not obvious. Cows that have been confirmed pregnant prior to 45 days are later found to be not pregnant.

Complex vertebral malformation (CVM): CVM is a lethal defect most commonly found in Holsteins. Affected calves have a malformation of the vertebral column with an abnormal curvature of the spine and a shortened neck. Legs are also malformed with flexed and rigid pasterns. Most of the affected calves are reabsorbed as embryos or aborted as fetuses prior to the 260th day of gestation. Remaining pregnancies result in stillborn calves.

Detecting Carriers of Genetic Defects

The ability to determine if a parent is a carrier of a genetic defect has become easier as a result of recent advances with genomics. The typical process involves two steps: (1) gathering DNA and phenotypes from afflicted individuals and (2) analyzing the DNA for genetic results. A pathological assessment of the afflicted individual is needed to verify that the individual has phenotypes consistent with the defect. A DNA sample from the afflicted individual is then sent to a high-throughput genotyping laboratory to determine the genetic makeup of the individual. The individual is genotyped for a large number of single-nucleotide polymorphisms (SNPs). The SNPs are single base-pair sites in DNA that can differ among individuals. The SNPs are used as genetic markers in an attempt to find the defective genetic location on the chromosome.

In most cases, individuals are genotyped using a commercially available chip that provides SNP results for approximately 50 000 SNPs across the entire bovine genome.

The SNP results from afflicted individuals are compared with the SNP results for individuals that do not have the affliction. If the SNP is either located within the causative gene or tightly linked to the causative gene, all afflicted individuals will be uniquely homozygous at a particular SNP location, whereas nonafflicted individuals will be either heterozygous or homozygous for the alternative SNP base pair.

With small sample sizes, the afflicted individuals may be uniquely homozygous at multiple SNP sites by chance. Therefore, it is desirable to have DNA available from several afflicted individuals to more quickly identify the homozygous SNP location that is unique to the afflicted individuals. Once a unique homozygous SNP location can be identified, all heterozygous individuals at this SNP location would be carriers for the genetic defect, and the individuals that are homozygous for the alternative SNP would be homozygous normal for the defect and would not transmit the defective allele to offspring.

Comparative mapping: Mammals have many genes in common. As a result, comparative mapping has been useful when attempting to identify the specific gene that may be responsible for a genetic defect. Because SNPs are markers that may or may not be located within the gene complex causing the defect, it is always desirable to find the specific gene causing a defect as opposed to relying entirely on an SNP result for a location that may only be linked to the causative gene. BLAD is a classic example of how comparative mapping has been used successfully. Humans can also inherit leukocyte adhesion deficiency, and the gene responsible for the defect had already been identified and characterized before the defect was even known to exist in cattle. When BLAD was identified in cattle in the early 1990s, the researchers noticed that the symptoms appeared to be identical to leukocyte adhesion deficiency in humans. Because the gene responsible in humans had already been identified, a comparative mapping search in cattle successfully found the same defective gene in cattle. A rather simple DNA test can determine whether a parent is homozygous normal with two normal alleles, or is a carrier with one normal and one defective allele.

Conclusion

A phrase often used in animal breeding is 'Select the best and cull the rest.' Intense selection pressure has been practiced in dairy cattle, especially in the last 40 years. The widespread use of frozen semen in dairy cattle, combined with selection intensity, has resulted in a rise in average inbreeding levels in the major dairy breeds. As inbreeding levels rise, we will find even more genetic defects in dairy cattle.

Although the symptoms associated with some genetic defects are obvious, such as mulefoot, the symptoms associated with others are less obvious, such as BLAD. Genetic defects that are lethal but do not have obvious symptoms will be the most difficult defects to characterize and manage in breeding programs.

The ability to identify carriers of genetic defects has improved greatly as a result of advances in high-throughput genotyping. The DNA from individuals afflicted with a particular genetic defect can be genotyped for thousands of SNPs, and if these afflicted individuals are all uniquely homozygous at a particular SNP location, then the chromosomal region causing the defect has been identified. Additionally, comparative mapping may identify the specific genes responsible for genetic defects. Genomic research has been especially successful at finding the specific genes that cause defects in humans and mice, and these findings are proving helpful in finding genes responsible for defects across other species as well, including cattle.

See also: **Genetics:** Cattle Genomics; International Flow of Genes; Selection: Concepts; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. **Lactic Acid Bacteria:** Genomics, Genetic Engineering. **Nutrition and Health**: Nutrigenomics and Nutrigenetics.

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H

HAZARD ANALYSIS AND CRITICAL CONTROL POINTS

Contents
HACCP Total Quality Management and Dairy Herd Health
Processing Plants

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Introduction

Animal health management and product quality control have evolved from a solely curative veterinary practice, where disease or quality problems were addressed once the problem had arisen, to the application of veterinary herd health and production management programs, where herd performance was monitored in an operational setting in order to better prevent disorders. Herd health and production management programs much more focused on early disease detection, herd monitoring and risk assessment of disorders in health, reproduction and productivity.

More recently, more emphasis is put on the issue of quality of the product (e.g. milk and meat) and of the production process. The latter includes aspects like animal health status and animal welfare, and environmental issues. There is an increasing tendency that livestock production is integrated into food production chains, from the farm level up to the consumer level. An important element in such production chains is that each link should provide the next link with safeguards regarding the product delivered. Certification of products and production processes is hereby introduced. This article addresses the different quality control concepts as applied at dairy farm level. Moreover, it is discussed how quality management at farm level can be integrated with herd health management.

Quality and Quality Control on the Dairy Farm

During the last decades, consumers have increasingly paid attention to the livestock industry. This was partly caused by the foodborne disasters such as mortality due to hamburger consumption, outbreaks of salmonellosis or cryptosporidiosis, and the epidemics of BSE in Europe. Food safety now plays a paramount role in the consumer attitudes and concerns about the livestock industry and the products of animal origin.

On the other hand, the general public puts increasing demands on the way that farmers produce. In this situation it is not food product safety that is referred to, but rather husbandry methods with regard to animal health and welfare (*see* **Welfare of Animals, Political and Management Issues**), to productivity and to environmental issues. Although the consumer perception of the livestock production sector appears to be highly biased by a lack of knowledge and although there is a large psychological gap between the farmers' community and the urban population in their mutual understanding, it has become clear to the farmers that the quality demands set by the general public have to be met. Livestock production has become market-driven and quality control is an important part of meeting the demands of the society. Certification and quality assurance programs have been introduced.

Table 1 A comparison between different quality control concepts with regard to animal health as a quality feature

Feature	*International Standardization Organization (ISO) system*	*Good Manufacturing Practice (GMP) codes*	*Hazard Analysis Critical Control Points (HACCP)*
Bottom–up approach?	No	No	Yes
Orientation?	Process	Product	Process/product
Health demonstrable?	Yes	No	Yes
Fit for true certification?	Yes	No	Yes
Documentation needed?	Very much	Much	Little
Self-management?	No	No	Yes
Farm-specific?	No	No	Yes
Labor-intensive?	Yes	No	No
Many people involved?	Yes	No	No

Quality control can be conducted according to different concepts: Good X Practice codes (GXP), International Standardization Organization systems (ISO-9000), Hazard Analysis Critical Control Points (HACCP) and Total Quality Management (TQM). These concepts are extensively addressed in textbooks on quality control. Differences between GXP, ISO and HACCP with regard to on-farm application have been categorized as given in **Table 1**. The starting issue was the question to what extent a quality control concept could be applied to animal health hypothesized as a feature of the quality of the production process. It is clear that HACCP can be regarded as the best choice for safeguarding and certifying animal health in dairy practice. However, before any action is to be taken to design and implement a quality control program, the appropriate attitude and mentality toward quality is necessary. This is the area of GXP codes.

These GXP codes, where the ‘X’ can be replaced by any term like veterinary (V), hygiene (H), clinical (C), manufacturing (M) or farming (F), comprise general statements of attitude regarding the production process. Take for example the statement in the GHP code that the calving pen should be cleaned and disinfected after every calving. Such statements are not specific and do not refer to specified locations or steps in the production process. An example of a GFP-like approach is given below.

The ISO concept refers to a whole systems approach, where all respective elements, suppliers and advisors are included in and elaborated by the system. This concept is far too elaborated to be practically feasible at farm level; it can function nicely in regular industries. Most quality control systems, like ISO, emphasize the need for creating a quality team before starting the build-up of a quality control system. On a dairy farm of considerable size, say 500 cattle or more, this seems feasible because of the number of workers and the labor organization. On smaller, family-based farms, say 80–100 cattle, however, such a quality team would basically mean the farmer himself full time, possibly assisted by his wife or a coworker. ISO is commonly addressed in textbooks on quality control, but is not further addressed in this article.

The HACCP concept has been proposed as an alternative quality control system for dairy farms, because it addresses hazards and risks (exactly as is being done in disease control and disease management), and focuses on preventive risk management in a farm-specific setting. Its orientation is on the product (e.g. milk) by addressing the production process steps (e.g. risks). HACCP is discussed in this article in more detail.

TQM can be considered as a mixture of both GXP codes and HACCP, and therefore could represent the best choice in the end, especially in situations where for reasons of efficiency an integration of quality management and herd health management is warranted. This option is also presented in this article.

Good Farming Practice

The first response of the dairy sector to society’s demand for quality control was the development of Quality Assurance Programs (QAP) in the areas of microbiological hazards and residue control, which are still product-oriented. QAP are aiming at monitoring and controlling these hazards and risks by product testing and by implementing codes of practice and standard operational procedures throughout the whole production chain. Recently, issues of animal welfare and environmental pollution and their control have also been addressed through more integrated programs, like Chain Quality Milk (CQM).

CQM was formulated in The Netherlands in 1998 by a joint action of the Dutch Farmers’ Association and the national milk industry, and is compulsory for dairy farmers. The Dutch CQM comprises six modules:

1. Animal health and welfare.
2. Handling and storage of antimicrobial products (including withdrawal periods).
3. Milk harvesting and storage.
4. Hygiene and disinfection procedures.
5. Feed and feedstuffs.
6. Environment, water and waste management.

Quality is now defined in a much broader sense: not only the product is involved but also the production method and the production unit surroundings.

CQM could – up to the present time – be regarded as GFP codes aiming at the improvement of attitude and mentality. For each module in CQM, the farmer conducts a written self-evaluation of GFP. These forms are checked for compliance with CQM regulations and the farms are audited by an independent organization.

Each year the demands in each module are becoming stricter. A veterinarian who wants to serve such a CQM-acknowledged farm needs to act according to a Good Veterinary Practice (GVP) code to obtain access to that particular farm. The veterinarian has to sign that GVP code. Feed producers, e.g. of concentrates, have to demonstrate that they have operated according to a Good Manufacturing Practice (GMP) protocol. Such a GMP is primarily meant to reduce the risk of delivered feed stuffs containing salmonella, undesired antibiotics or mycotoxins.

These good practices codes basically regard codes of behavior, attitudes and mentality in general. GFP is not a control program in itself. It may point, however, to certain critical issues on a farm, like the extent to which manure-borne pathogens such as salmonella and *Mycobacterium paratuberculosis* are introduced into and spread within the farm. The critical spots in that respect are for example the entrance of the farm where both farm workers and extension people cross, crossing-lines between cows leaving the milking parlor on their way to the pasture and farm machinery bringing feed to the barn, a worker passing from the lactating cow group to the dry cow group or to the young stock barn without crossing hygiene barriers.

Drawbacks of GFP are that it is not farm-specific and that such attitude or mentality can not be truly demonstrated to third parties which is required for certification. On the other hand, it appears that quality control programs can only be successful if the proper foundation for adoption is present; GFP helps in building that foundation by creating awareness in the farmer.

Hazard Analysis and Critical Control Points (HACCP)

The HACCP concept appears to be the best choice so far for the farmer who wants to act according to quality control principles, not in the least where animal health is considered a quality feature. Key issues in the HACCP concept are the design of a production process decomposition diagram, the identification of hazards, risk assessment, definition of critical control points and critical management points, the monitoring of the production process, planning of corrective measures, and the risk management procedures. An example of a general production process decomposition diagram for a dairy farm is presented in **Figure 1**. This shows where the animals on the farm pass from one step to the other, including the pathways and pasture plots. Further detailing can be done on each particular farm (*see* **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates). Hazard identification means that major disorders and potential threats are identified as well as the risk factors contributing to their occurrence. Examples of such hazards are introduction to the farm of pathogens, like that of Johne's disease or bovine virus diarrhea through purchased animals; spread of infections within the farm over units and between herds through poor hygiene or transport trucks; contamination of milk due to insufficient milking technique of the milker. Within herds, infections have to be eliminated before they can cause dramatic economic losses. Infections between herds have to be eliminated if they involve highly contagious diseases or diseases that threaten larger regions such as infectious bovine rhinotracheitis or brucellosis.

Risk factors are obtained through either literature analysis, observational–analytic field surveys, or adaptive conjoint analysis procedures, as has been reported in literature. These risk factors are assigned to the respective sites in the production process where they have their impact. Critical control points (CCPs) and critical management points (CMPs) then need to be defined on the basis of these risk factors.

The CCP is a measuring point, a condition or a sequence in the production process which is related to the hazard, for which the control is crucial and where deviations can be detected by comparison with accepted target and tolerance levels, for which corrective measures are available which after loss of control in their turn lead to full restoration of control.

Examples of CCP are bacteria counts per milliliter of milk; temperature of the cleaning water of the milking machine at the end of the cleaning process; air velocity in the barn; animal (blood antigen) check before delivery. There are not many CCPs defined for dairy production. One reason could be that the number of physical entities (e.g. water temperature for cleaning the milking machine) on a farm is relatively small as compared to other industrial sectors. Another reason could be that it is hard to define true CCPs for biological entities such as cows due to the wide intra- and interanimal variation of many variables.

The CMP is a point, a condition or a sequence in the process which is related to the hazard but where control cannot be fully guaranteed because there are no true target levels or tolerances defined, or which is hard to measure, but which is still crucial in the control process. CMP focuses on the best possible practice to reduce a risk

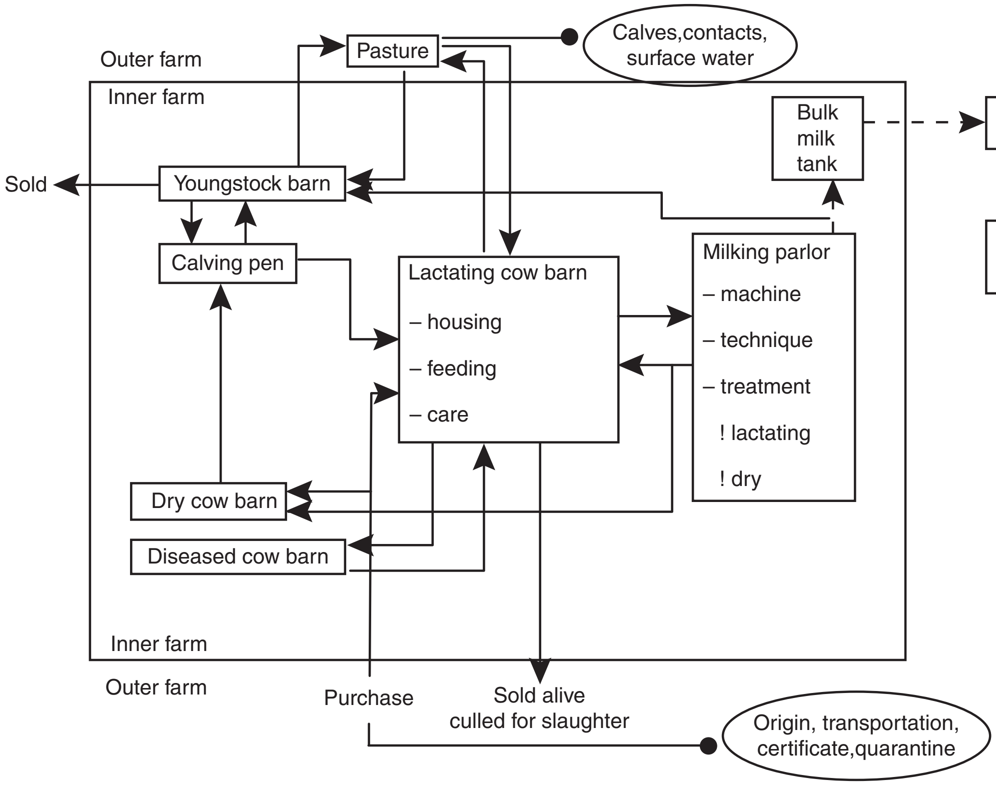

Outer farm
Inner farm
Pasture
Calves,contacts, surface water
Bulk milk tank
Milk truck
Dairy factory
Sold
Youngstock barn
Calving pen
Lactating cow barn
– housing
– feeding
– care
Milking parlor
– machine
– technique
– treatment
! lactating
! dry
Dry cow barn
Diseased cow barn
Inner farm
Outer farm
Purchase
Sold alive culled for slaughter
Origin, transportation, certificate,quarantine

Figure 1 Example of a production process decomposition diagram of a dairy farm. Boxes represent major production process steps, ovals represent risk factors.

or its impact. Examples of CMP are sero-titres; managerial actions to deny access of calves to pastures where manure has recently been spread which potentially carries salmonella bacteria; level of maintenance of the slatted floor with regard to traumatic hoof disorders. It can be stated that CMPs are the most critical issues in a biosecurity program which have a scientifically sound basis.

An example related to the determination of CMPs is presented below.

- Hazard: milk quality deviations.
- Hazard specification: milk quality deviations due to mastitis, teat lesions and poor teat-cup liners.
- Risk conditions related to the hazard (CMPs in a qualitative sense are given between brackets) are for example:
  - microbiological quality of the milk (expressed in somatic cell counts; coliform bacteria count; udder pathogens; clinical and subclinical mastitis)
  - teat hygiene (expressed in coliform and total bacteria count of the teat skin)
  - teat condition and health (expressed in coliform and total bacteria count of the teat skin; teat end condition as expressed in teat end callosity scores)
  - milking technique and milking hygiene
  - milking machine function and maintenance (e.g. poor teat-cup liners)
  - general hygiene scores of cows and surroundings
  - body condition scores of the cows
  - nutrition, especially in fresh cows, related to the effects of severe negative energy balance and hence (subclinical) ketosis
  - the situation regarding lameness in the herd.

The risk conditions are translated into CMPs. CMPs for microbiological quality of milk are the acceptable percentage of lactating cows with somatic cell counts above 200 000 ml^{-1} (e.g. 15% or less); the percentage of clean udders in the herd (at least 80% of lactating cows); the acceptable percentage of cows with clinical mastitis (20% or less); a proper herd mastitis treatment plan (antibiotic sensitivity determination; known profile of mastitis-causing pathogens); replacement of teat-cup liners after 1200 milkings; optimal milking hygiene and milking technique (e.g. udder preparation time 30 s or less per cow); milking machine evaluation at least twice yearly; general hygiene conditions according to Good Farming Practice codes; the percentage of ketotic cows in early lactation (less than 10% of the fresh cows up to 70 days postpartum: ketosis is associated with an increased mastitis risk); patterns of body condition scores throughout the lactation according to the standards set for milking cows; the percentage of lactating cows with deviating teat end callosity scores in the worst class (less than 15%).

With regard to the corrective measures in this case, we must design an udder health control program (UHCP) for mastitis as cause of contamination (too high somatic cell count and or bacteria) of the milk. This UHCP should focus on the prevention of new infections and the elimination of existing infections, e.g. by addressing milking machine function, milking technique, control of clinical as well as subclinical mastitis, culling of cows with an udder health problem. In such a UHCP cows and farm conditions are regularly monitored. This monitoring may also be part of a regularly executed veterinary herd health and production management program (HHPM).

All CCPs and CMPs together on a farm represent the on-farm monitoring network.

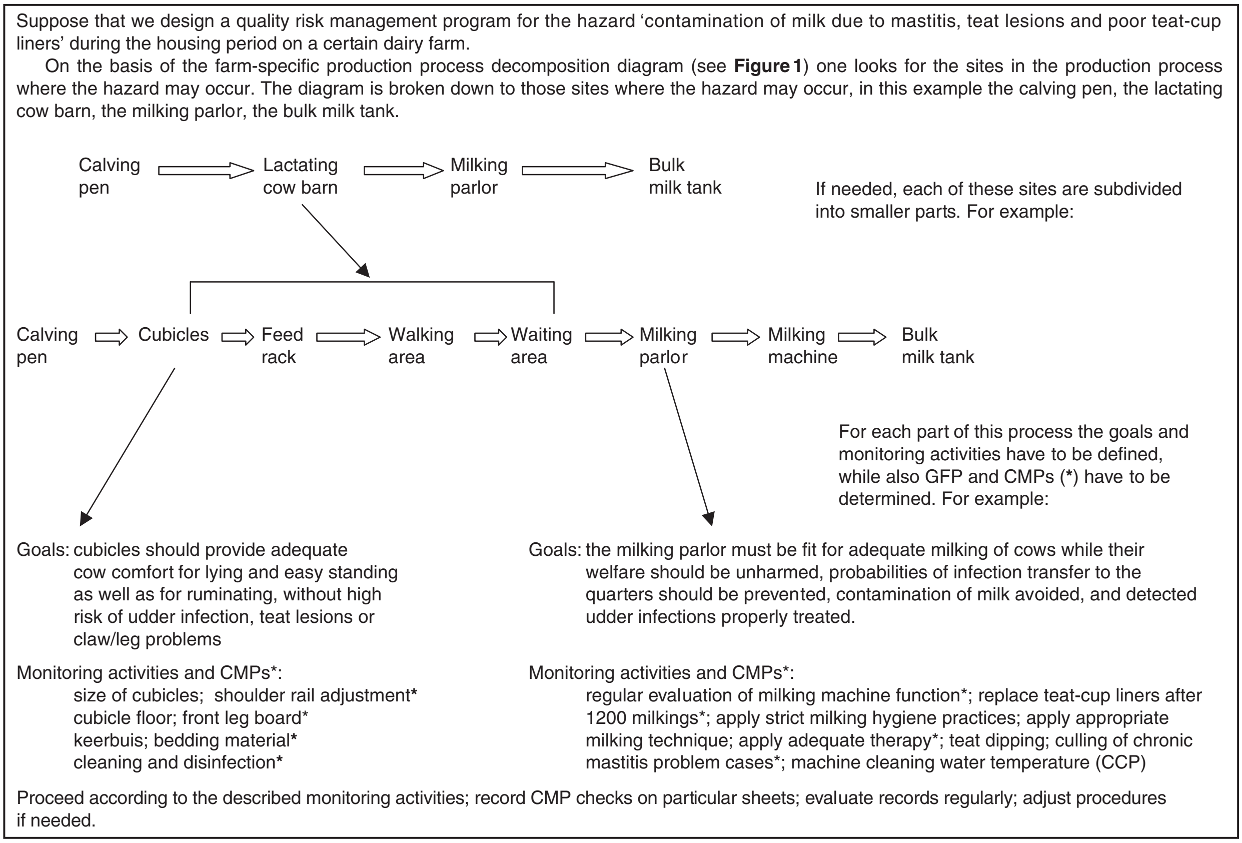
Integrating the HACCP Concept and Total Quality Management on a Dairy Farm

True CCPs are hardly defined for a dairy farm and therefore more focus is on the CMPs. CMPs are critical points for management attention and often part of a biosecurity plan. Many CMPs are known on a dairy farm because – as was stated previously – they are derived from risk factors related to the occurrence of certain disorders on a farm. Paramount activity in a quality control program is to determine these CMPs in the different production process steps. These production process steps can be defined by designing a process decomposition diagram of the farm (see **Figure 1**). Next, the respective activities and goals in the different steps in that diagram are logically and chronologically organized. Then, the CMPs are assigned to the different activities within each step and a monitoring procedure is developed, implemented and evaluated. Parts of this concept are elaborated in **Figure 2**.

We then have a mixture of Good Farming Practice codes and the HACCP concept, comprising CMPs and CCPs. This mixture can be regarded as Total Quality Management, especially if – in addition to quality control of the product itself and to process control with regard to animal health – emphasis is also put on animal welfare, public health and food safety, and environmental issues.

Herd Health and Production Management Programs and their Integration with Quality Control

In more business-like conditions, TQM can be integrated with veterinary herd health and production management programs (HHPM). The latter are focused on operational



Suppose that we design a quality risk management program for the hazard ‘contamination of milk due to mastitis, teat lesions and poor teat-cup liners’ during the housing period on a certain dairy farm.

On the basis of the farm-specific production process decomposition diagram (see **Figure 1**) one looks for the sites in the production process where the hazard may occur. The diagram is broken down to those sites where the hazard may occur, in this example the calving pen, the lactating cow barn, the milking parlor, the bulk milk tank.

If needed, each of these sites are subdivided into smaller parts. For example:

For each part of this process the goals and monitoring activities have to be defined, while also GFP and CMPs (*) have to be determined. For example:

Goals: cubicles should provide adequate cow comfort for lying and easy standing as well as for ruminating, without high risk of udder infection, teat lesions or claw/leg problems

Monitoring activities and CMPs*:
size of cubicles; shoulder rail adjustment*
cubicle floor; front leg board*
keerbuis; bedding material*
cleaning and disinfection*

Goals: the milking parlor must be fit for adequate milking of cows while their welfare should be unharmed, probabilities of infection transfer to the quarters should be prevented, contamination of milk avoided, and detected udder infections properly treated.

Monitoring activities and CMPs*:
regular evaluation of milking machine function*; replace teat-cup liners after 1200 milkings*; apply strict milking hygiene practices; apply appropriate milking technique; apply adequate therapy*; teat dipping; culling of chronic mastitis problem cases*; machine cleaning water temperature (CCP)

Proceed according to the described monitoring activities; record CMP checks on particular sheets; evaluate records regularly; adjust procedures if needed.

Figure 2 Integrating TQM, HACCP and HHPM: a simplified example.

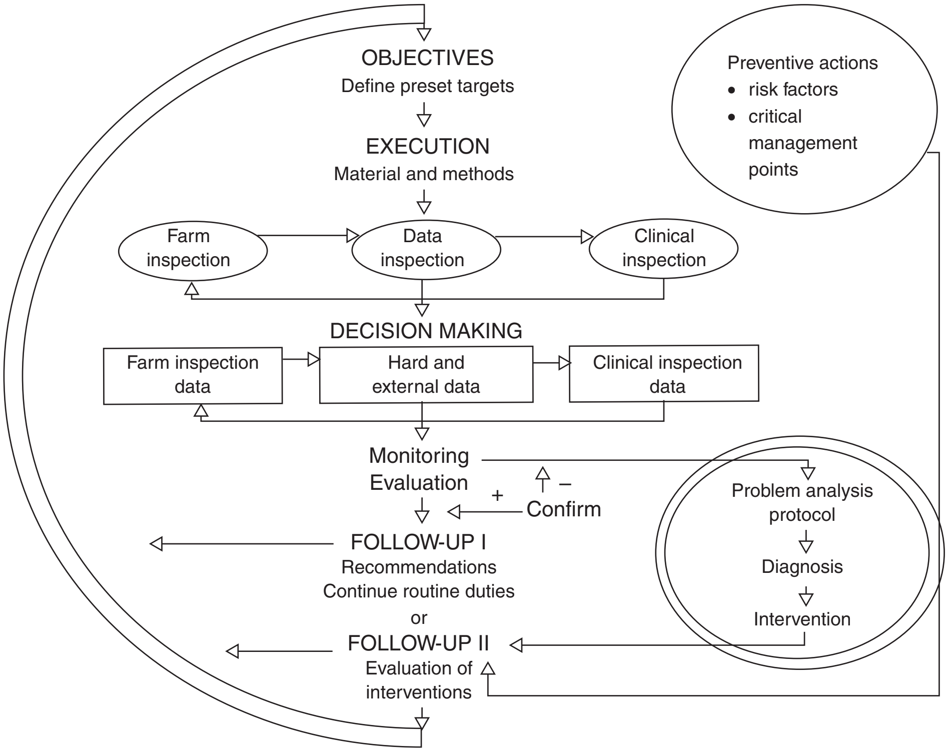

OBJECTIVES
Define preset targets
EXECUTION
Material and methods
Farm inspection
Data inspection
Clinical inspection
DECISION MAKING
Farm inspection data
Hard and external data
Clinical inspection data
Monitoring
Evaluation
+
–
Confirm
FOLLOW-UP I
Recommendations
Continue routine duties
or
FOLLOW-UP II
Evaluation of interventions
Preventive actions
• risk factors
• critical management points
Problem analysis protocol
Diagnosis
Intervention

Figure 3 A standard protocol for veterinary herd health and production management with three components: routine monitoring, problem analysis, and prevention. (From Brand *et al*., 1996.)

and tactical management support with regard to decision-making on health, reproduction and productivity of the herd according to standard protocols. Such a protocol is presented in **Figure 3**.

The ultimate objective of HHPM is to reduce operational costs and/or to increase farm income. These programs have three major characteristics: (1) the basis is routine monitoring and surveillance of animals, farm conditions and information during regularly planned farm visits, (2) the analysis of herd problems and related risk conditions in order to solve those problems, and (3) the prevention of disorders by monitoring, eliminating or reducing risk conditions. During biweekly or monthly farm visits the monitoring leads to early signals of possible deviations, on the basis of which farm-specific plans of action are designed, implemented and followed up by the veterinary surgeon at subsequent farm visits. Examples of routine monitoring are presented in **Table 2**.

Table 2 Examples of routine monitoring activities in a Herd Health and Production Management (HHPM) program

Cow/herd level	*Farm environment*	*Farm information*
Body condition score	Milking technique	Performance figures
Rumen fill score	Hygiene procedures	Milk recording data
Feces score	Nutrition and pasturing	Milk quality data
Claw score	Housing conditions	Roughage analysis
Teat end score	Climatic factors	Soil analysis
Reproductive examinations	Boiler temperature	Surface water quality
Growth measurements	Rodent/insect control	Quality audit reports
Clinical cow examination	Other managerial activities	

This farm monitoring can be regarded as an overall farm inspection, the prestage of problem analysis or an epidemiological survey wherein risk factors for disorders are searched for in order to provide means for elimination or control of that disorder. Such a survey yields a list of risk factors for one or more disorders, as well as a quantification of their contribution to the occurrence of that disorder, the parameter being the relative risk or the odds ratio. The strength of such epidemiological surveys is that contributing risk factors can be ranked according to the impact they have on disease occurrence at population level. On the individual farm it is checked with this list which risk factors are prevalent there and which are not. In this way it is easier to set priorities in addressing risk factors through managerial action, especially when cost–benefit calculations have been done. This is the area of risk management.

From the previous paragraphs on quality management, HACCP and TQM it is clear that TQM including

HACCP principles and HHPM can be easily integrated. They show many similarities in approach and have the same basis in monitoring and surveillance. In **Figure 2** an example is given of the integration of the TQM concept including GXP and HACCP principles with HHPM on the dairy farm, following the sequences outlined in the previous paragraphs.

Record-Keeping

In every organization it is necessary that the manager is able to assess the current situation and has insight into the past herd performance. Therefore, it is necessary that some relevant documentation is available for scrutiny and evaluation (*see* **Business Management**: Management Records and Analysis). On-farm information in that respect regard are the milk production data (test day results), the herd fertility–health overview, insemination data, results from laboratory testing on e.g. serology or milk samples, feed analysis, culling, farm economics. Veterinary herd health programs usually show a more extended database. This includes for example data on clinical mastitis, cell counts and pathogens, data on lameness and other diseases, data of monitoring rumen fill, body condition, feces consistency, teat end callosity. Moreover, a formal herd health status certificate can be acquired after testing of the herd (e.g. on infectious bovine rhinotracheitis, bovine virus diarrhea, leptospirosis). HHPM uses these data to better support the farmer in his decision-making about health, reproduction and productivity of animals.

In the case of quality control on dairy farms, usually the dairy industry carries out the control of the delivered product before acceptance (e.g. antibiotics residues, somatic cell counts, pathogens). If the dairy production process is to be addressed, as was described in this article, monitoring of hazards and risks in this production process on the farm needs to be done. Furthermore, if one has to demonstrate to third parties what activities have been executed to achieve a certain animal health status or quality level, documentation is paramount.

Important in this respect may be the specific listing of the CCPs and CMPs which have been monitored over time, with dates, observers, outcome, and incidental corrective measures applied and their effect. This may support both the HHPM and the QAP activities.

Concluding Remarks

Many developments in the dairy sector have taken place over the last decades. The currently most important one regards the quality assurance related to food safety and public health. Furthermore, due to ongoing urbanization, city-dwellers live closer to the farming communities in several regions and countries. As a consequence and even more enhanced by problems like BSE, general public opinion has a greater impact on livestock production than before. Farmers need to demonstrate to third parties how they proceed with regard to animal health, animal welfare, food safety and environmental issues.

Quality control, certification and animal health status are all part of a so-called licence to produce. If a farmer does not comply with the rules set, he will lose his licence. This is the reason that dairy farmers want management support in their operational, tactical and strategic decision-making on issues including quality. They expect this support from different experts among whom is the veterinary surgeon. Given the different sociological farming styles, it is crucial that advice on quality risk management is given tailor-made. It could be worthwhile to have a 'quality team' functioning closely around the farm. The members of that team are, for example, the veterinary surgeon, the milking-machine advisor, the extension officer and the nutritionist. The team members are responsible for their own discipline and should address the monitoring, interpretation and advice in that discipline. The team approach should lead to the best possible advice to the farmer and avoid contradictory advice being given.

One of the main advantages of applying quality control would be that emphasis will be on disease risk management instead of disease control on the farm. This means that focus is on prevention rather than on curative action. Prevention will be more cost-effective and represents investment, while curative action refers to economic losses.

This has also been the incentive of the Bovine Alliance on Management and Nutrition in the United States for providing the documentation on biosecurity for dairy farms in 2000 and 2001. The effects of these efforts should be twofold: on the one hand the farmer responds to the public health and food safety demands from society and on the other hand it refers to optimal cost–benefit dairy farming.

Quality control goes beyond the common issues of residues and use of antibiotics, or even animal health. Also animal welfare is to be included, as well as environmental issues. The first steps regarding environmental issues have been made in, for example, The Netherlands and Denmark. Currently, practical welfare scoring indices are being developed and tested in the field, for example, in The Netherlands and the United Kingdom. With regard to environmental issues, surface water quality indicators have been developed, as well as farm mineral-status bookkeeping. Such indicators need to be translated into CMPs in an on-farm network for monitoring. It can be foreseen that, particularly in Europe, compulsory farm and herd inspections will be implemented on a regular basis for the sake of consumer protection. The quality of dairy products and dairy production in its broadest sense will hence continue to play a paramount role in our society.

See also: **Business Management**: Management Records and Analysis. **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates. **Risk Analysis**. **Welfare of Animals, Political and Management Issues**.

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Processing Plants

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Introduction

The Hazard Analysis and Critical Control Points (HACCP) technique for the identification, evaluation, and control of food safety hazards was developed in the mid-1960s and publicly presented in the early 1970s. Since then, it has been adopted progressively by the food industry and enforced by regulators. It is the technique preferred by the Codex Alimentarius Commission for the enhancement of food safety.

The technique was developed originally as a system for the control of microbiological hazards of public health significance, due to the limitations of microbiological testing for the control of food processes. HACCP has now been adopted widely internationally by the dairy processing industry for the development of process-control procedures within the framework of documented quality management systems. Dairy processors use the technique for the identification and control of hazards to the safety and quality of a product as well as conformance to legal requirements.

HACCP is one of a number of worksheet-based hazard identification techniques but is unique in that it was developed specifically for the food processing industry. There are many variations of the HACCP worksheet and an example is given in **Table 1**.

HACCP is very effective when used in conjunction with process design verification tools, risk assessment techniques, and cause and effect analysis to develop process control procedures within documented quality management systems. HACCP-based process control procedures also need to be supported by well-developed and documented Good Manufacturing Practices (GMPs).

Applying HACCP to Dairy Processing

There are 12 generally accepted steps to the application of the HACCP technique. They are the following:

1. Assemble the HACCP team.
2. Describe the product.
3. Identify the product's intended use.
4. Construct a process flowchart (PEC).
5. Verify the PFC.
6. List the potential hazards associated with each process step and identify control measures.
7. Determine Critical Control Points (CCPs).
8. Establish critical limits for each CCP.
9. Establish a monitoring system for each CCP.
10. Establish corrective actions.
11. Establish verification procedures.
12. Establish documentation and record keeping.

Steps 6 to 12 are referred to as the seven principles of HACCP.

When applying HACCP to dairy processing operations, it is important to be aware of the complexity of these processes compared to other sectors of the food processing industry. Much of the information on applying HACCP has been written with labor intensive batch processing of solid food in mind. The highly automated continuous processing of liquid milk into a large variety of dairy products involves complex, capital-intensive plant.

The first task undertaken by the HACCP team after it has been given the scope of the HACCP plan is to develop system description documents. The dairy process is too complex to attempt hazard identification by observation of the process alone and must be simplified and described on paper. The two main system description documents used are the product specification and the process flowchart (PFC).

The product specification is a full description of the product including measurable limits that address all hazards included in the scope. While another team may develop this document, it is the responsibility of the HACCP team to review its adequacy. Those compiling this document need to ensure that they are up to date with the epidemiology of illnesses associated with the product in question.

HACCP uses the PFC to describe the process. While a PFC listing the process steps may be adequate in less complex food processes, other system description documents such as Piping and Instrumentation Diagrams (P&ID) should be consulted when analyzing a dairy process.

A typical dairy process PFC is shown in **Figure 1**. While it is not a strict requirement, many dairy processors use the symbols shown in **Figure 1**. These symbols are also widely used throughout the broader food processing industry. A key to the use of the symbols is given in **Figure 2**. A typical P&ID appears in **Figure 3**.

Table 1 HACCP worksheet

Process step	*Hazard*	*Control measure*	*Assessed risk*	*CCP*	*Critical limits*	*Monitoring procedure*	*Corrective action*

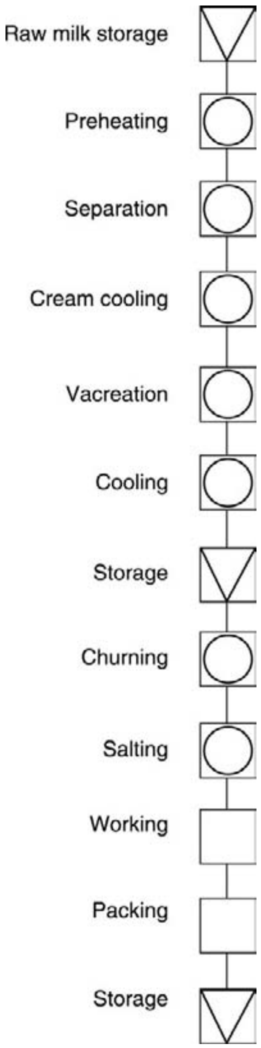


Figure 1 Process flowchart (PFC).

The HACCP team should ensure that the design of the process has been verified before conducting a HACCP on a dairy process. Process design verification will normally be conducted by engineers using a technique such as Hazards and Operability studies (HAZOP) that uses the more detailed P&ID as the main system description document. This verification should have been conducted as a part of plant commissioning as well as after any subsequent changes to the plant design.

In dairy processing there are certain control measures that are relevant to every process step in every process undertaken in the factory. These control measures are typically documented in the GMP section of the quality manual and should include

- equipment and premises cleaning and sanitation,
- personnel hygiene,
- pest control,
- equipment maintenance and calibration, and
- staff training.

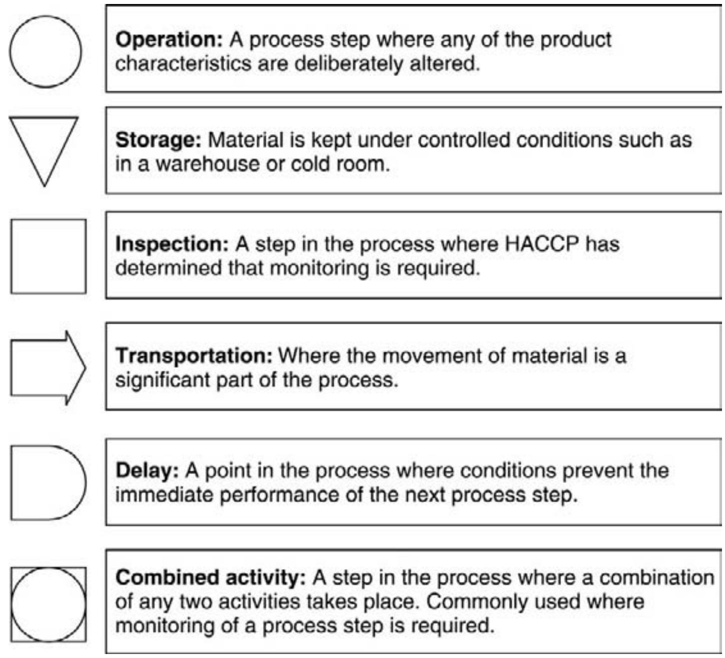
Identifying Hazards

The most important aspect of HACCP is the identification of potential hazards, which may occur at each step in the process. HACCP is used as a predictive tool in that the team is not only assessing hazards that have occurred in the past but also those that have the potential to occur.

The rigor of a hazard identification technique is judged on its thoroughness in uncovering potential hazards. Each technique includes a mechanism for enhancing this rigor. HAZOP, for example, utilizes a series of guide words, which are used to create questions with which the team challenges the process. HACCP relies heavily on the experience of the team; therefore, the selection of an appropriate team is of utmost importance. HACCP teams should include both technically qualified people and experienced process operators. The team must include someone with the knowledge of the microbiology of the product.

Well-written product specifications are essential for the identification of all potential hazards. The specifications serve as a checklist to be referred to as each step in the process is analyzed for hazards to determine which, if any, of the specifications are at risk. It is essential that the latest epidemiological data have been consulted in the writing of the product specifications. In the absence of the latest data, it is conceivable, for example, that in the mid-1980s HACCP plans for soft cheeses might have been developed with no consideration given to the pathogenic organism *Listeria monocytogenes*. This might have occurred during the early days of the organism's importance being recognized, with the epidemiological data being in existence but perhaps not widely available.

The intended use of the dairy product should always be identified in the product specifications as an aid to the identification of hazards, for example, the differences between the intended uses of high-heat skim milk powders



Operation: A process step where any of the product characteristics are deliberately altered.

Storage: Material is kept under controlled conditions such as in a warehouse or cold room.

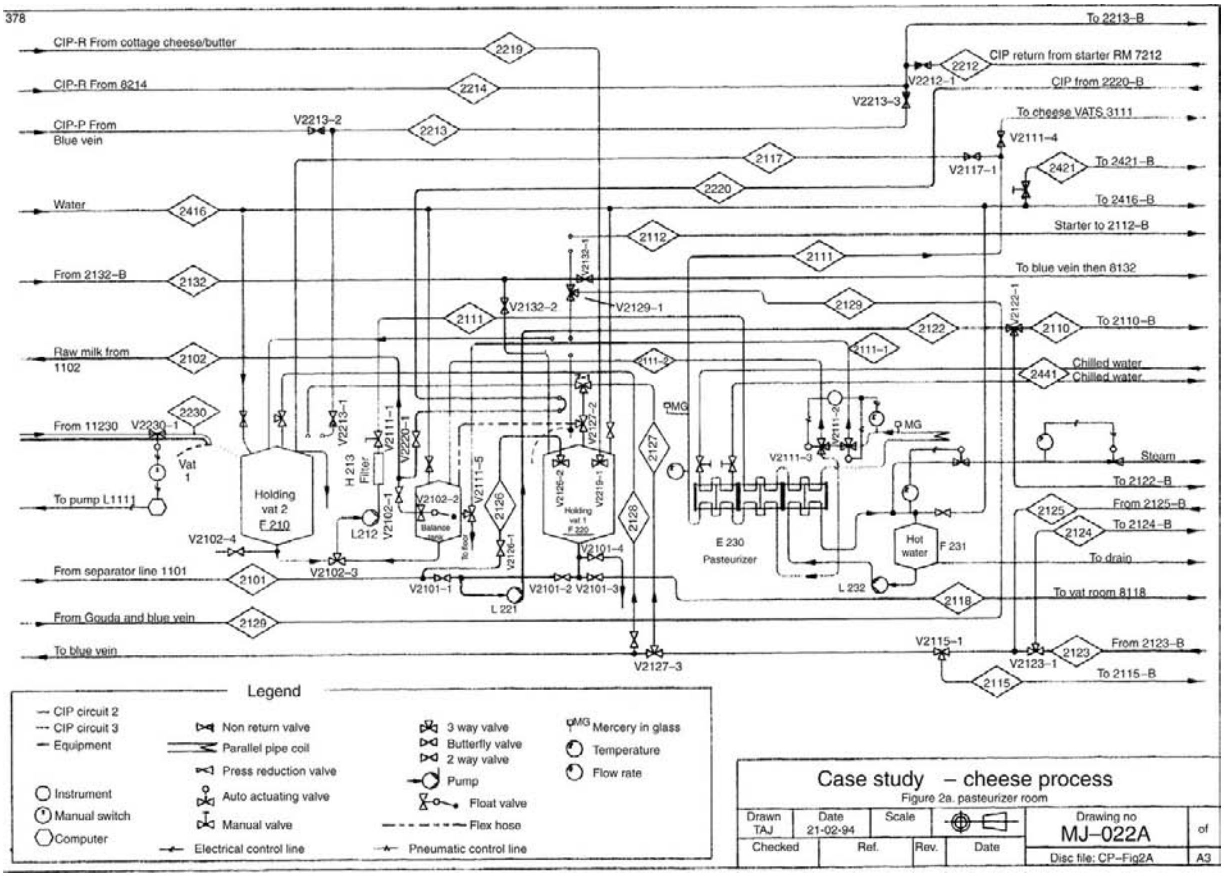
Inspection: A step in the process where HACCP has determined that monitoring is required.

Transportation: Where the movement of material is a significant part of the process.

Delay: A point in the process where conditions prevent the immediate performance of the next process step.

Combined activity: A step in the process where a combination of any two activities takes place. Commonly used where monitoring of a process step is required.

Figure 2 Key to typical process flowchart symbols.


378
CIP-R From cottage cheese/butter
2219
CIP-R From 8214
2214
CIP-P From Blue vein
V2213-2
2213
Water
2416
From 2132-B
2132
Raw milk from 1102
2102
From 11230
V2230-1
2230
Vat 1
To pump L1111
Holding vat 2 F 210
V2213-1
H 213 Filter
V2111-1
V2220-1
V2102-1
L212
V2102-2
Balance tank
To floor
V2111-5
2126
V2126-1
V2132-2
2111
V2132-1
V2129-1
V2127-2
V2125-2
V2219-1
Holding vat 1 F 220
2128
2127
V2102-4
V2102-3
From separator line 1101
2101
V2101-1
L 221
V2101-2
V2101-3
V2101-4
From Gouda and blue vein
2129
To blue vein
V2127-3
To 2213-B
2212
CIP return from starter RM 7212
V2212-1
CIP from 2220-B
V2213-3
To cheese VATS 3111
V2111-4
2117
V2117-1
2421
To 2421-B
2220
To 2416-B
2112
Starter to 2112-B
2111
To blue vein then 8132
2129
V2122-1
2122
2110
To 2110-B
2111-1
2111-2
2441
Chilled water
Chilled water
MG
V2111-2
MG
V2111-3
Steam
E 230 Pasteurizer
To 2122-B
2125
From 2125-B
2124
To 2124-B
Hot water
F 231
To drain
L 232
2118
To vat room 8118
V2115-1
V2123-1
2123
From 2123-B
2115
To 2115-B
Legend
CIP circuit 2
CIP circuit 3
Equipment
Instrument
Manual switch
Computer
Non return valve
Parallel pipe coil
Press reduction valve
Auto actuating valve
Manual valve
Electrical control line
3 way valve
Butterfly valve
2 way valve
Pump
Float valve
Flex hose
Pneumatic control line
MG Mercery in glass
Temperature
Flow rate

Case study – cheese process

Figure 2a. pasteurizer room

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Figure 3 Piping and instrumentation diagram (P&ID).

and infant dietetic powders. One is to be used as an ingredient in a product that undergoes further heat processing, while the other is obviously intended for a sector of the population with a high risk of fatality from food poisoning.

On a more process-specific level, the company's customer complaints register can offer an insight into the HACCP team.

It is also important when identifying hazards to consider the inputs to the process. Dairy processing includes the use of raw materials and ingredients other than milk, such as flavors, thickeners, and stabilizers. These other ingredients need to be considered in terms of the hazards they may introduce into the process.

Assessing Risk

The team approach to hazard identification works best if a brain-storming process is used. Using this approach requires trivial hazards to be eliminated and the critical hazards highlighted. Over recent years, the importance of risk assessment in a HACCP plan has been recognized increasingly.

Other hazard-identification techniques such as Failure Mode Effect and Criticality Analysis (FMECA) utilize a risk assessment method. There are as many risk assessment techniques available as there are hazard identification methods. Regardless of the risk assessment method selected, it must consider both the likelihood of the hazard occurring and the severity of the consequences. Either a quantitative or a qualitative measure of both needs to be undertaken. The total assessed risk is

$$\text{total assessed risk} = \text{frequency of occurrence} \times \text{severity of consequence}$$

The simple scoring system shown in **Tables 2** and **3** has been used effectively in the dairy processing industry in Australia. A score of 1–5 (from **Tables 2** and **3**) is given to both the likelihood of the hazard occurring and the severity of the consequence should it occur and the total assessed risk calculated using **Table 4**. The risk of a hazard is determined assuming that no process control procedures (monitoring and corrective action) are in place. If the risk is unacceptable, process control procedures that will reduce the risk to an acceptable level need to be put in place.

Table 2 Scores for frequency of occurrence

Frequency score	*Frequency of event*
5	Frequent
4	Probable
3	Occasional
2	Remote possibility
1	Improbable

Table 3 Scores for severity of consequence

Severity score	*Severity of consequence*
5	Critical
4	Major
3	Minor
2	Marginal
1	Negligible

The aim in this system is to reduce all hazards so that the total assessed risk score is 5 or less ('acceptable risk' category in **Table 4**) where all critical hazards are improbable. Other systems used simply assign a high, medium, or low rating to the frequency and severity.

Determining Critical Control Points

The intention of HACCP is to ensure that the focus of process control is at the CCPs. A CCP is a point in the process where control can be applied to prevent (or eliminate) a food safety hazard or reduce the total assessed risk of that hazard to an acceptable level.

There are decision trees available to help identify a CCP; however, a simple and logical approach that works well in the analysis of a dairy process is found in the first five columns of the HACCP worksheet in **Table 1**.

First, determine if there is a potential hazard at the process step being analysed. If a potential hazard exists, determine whether a control measure exists at that process step for the hazard. If a control measure exists, determine the significance of the hazard (i.e., the acceptability of the risk). If the hazard is significant then the process step is a control point. In summary, if a significant hazard exists at a process step for which there is a control measure available then the process step is a control point.

With the application of HACCP no longer restricted to microbiological hazards of public health significance, a distinction needs to be drawn between critical public health hazards and less serious quality issues. This is generally being achieved in the dairy processing industry by restricting the label CCP to process steps with public health hazards and describing other control points as QCP.

The main point of confusion in determining control points in a dairy process is at the beginning of the process, usually the raw milk storage in silos. It is at this point that the team may nominate hazards that can only be prevented on farm, such as the risk of antibiotic contamination of the milk. The result of conducting HACCP should be a process control procedure based on

Table 4 Total assessed risk matrix

	Severity of consequence score				
Frequency score	*1*	*2*	*3*	*4*	*5*
1	1	2	3	4	5
2	2	4	6	8	10
3	3	6	9	12	15
4	4	8	12	16	20
5	5	10	15	20	25
	Acceptable risk		Unacceptable risk		

prevention. In order to achieve this, the control point for this hazard must be on the farm. If the scope of the HACCP were always "from paddock to plate", this confusion would not occur.

Developing Effective Process Control

An important aspect of process control is that it is preventative. In order for a process control system to be preventative, it must concentrate on monitoring the control measure and not the hazard. For example, in order to assure the desired low level of bacteria in the process, the time and temperature of storage should be monitored and held within desired limits rather than testing milk for bacterial levels. Checking bacterial levels is, however, an important means of verifying the adequacy of an HACCP-based process control system.

Effective process control relies on the correct control measures being monitored. This often requires cause and effect analysis, as control measures focus on the cause of the hazard. For example, in the production of vacuum-packed cheese, leaking packages are likely to constitute a significant hazard. For this hazard, the possible causes could include: incorrect vacuum, incorrect temperature of the heating bars, inadequate time allowed for sealing, product on the sealing surface, or holes in the packaging material. One of these five possible causes is likely to be responsible for 80% of the leaking packages.

Critical limits need to be set at each control point for each control measure. Setting critical limits makes the process measurable and therefore controllable. By setting upper and lower limits, a truly preventative process control procedure is developed. Trends in the process can be observed and the process adjusted before the hazard has occurred. This principle has been long understood in the canning of 'sterile' milk. In this process, can-seam measurements are taken and graphed so that trends can be monitored and can-closers adjusted before any leaking cans are produced.

It is also useful to set an aim or target level usually in the middle between the upper and lower limits. To set only an upper limit or only a lower limit should be seen as an exception. For example in milk or product storage, it may seem logical to set the critical limit as a maximum of 4 °C. A lower limit of 0 °C should, however, be considered, as freezing milk is also undesirable (milk of normal composition would freeze below −0.5 °C).

As a general principle, the critical limits should be validated. In other words, evidence should be obtained indicating that the hazard will be controlled by maintaining the control measure within those limits. In many cases in the dairy processing industry, the critical limits are industry standards. For example, each processor need not validate the pasteurization critical limits (minimum of 72 °C for a minimum of 15 s). However, when *Listeria monocytogenes* became widely recognized as a hazard in dairy products, pasteurization had to be validated again for this organism.

When the frequency and rigor of monitoring are being set, the assessed risk should be considered. For example, there will be similarities between HACCP plans for various milk powders, but process control for a skim powder for further processing will have less rigorous monitoring for *Salmonella* control than an infant dietetic powder.

With the aim of developing practical process control procedures, it needs to be recognized that there will be times when monitoring indicates that the process has not remained in control and within critical limits. It is therefore necessary to include a plan of action to correct the process. Corrective actions should include actions to bring the process back to within critical limits; isolate, and determine the disposition of affected product; determine the cause of the loss of control; and maintain a record of actions taken.

While every effort is maintained to ensure the HACCP plan is effectively developed by a diverse and experienced team and, where necessary, by validation that critical limits are appropriate, until the resultant process control procedures are tried in practice, there is no absolute guarantee of the efficacy of the plan. It is therefore essential that verification procedures also are developed. The principle of modern quality management is to ensure the quality and safety of product by controlling the process and not by testing for and rejecting defective finished product. However, product testing is still an essential component in verifying the system. No matter how rigorous the monitoring of a process producing infant dietetic powder it would never be justifiable to stop testing the final product for *Salmonella*.

Finally, the HACCP plan must be documented. This involves both the documentation of HACCP procedures and the development of a record-keeping system. Ideally, this should be as part of a documented quality system as this will ensure that the HACCP plan is not only verified and kept up-to-date but also continuously improved.

See also: **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health. **Risk Analysis**.

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HEAT TREATMENT OF MILK

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Thermization of Milk

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Introduction

Thermization is the generic description for a range of sub-pasteurization heat treatments of milk. Thermization markedly reduces the number of spoilage bacteria with minimum collateral heat damage to milk components and it does not cause changes in flavor. Originally, thermization was developed as a heat treatment of milk before cheesemaking. Thermization at 57–68 °C for 10–20 s was aimed at destroying the adventitious bacterial flora and providing a suitable environment for the multiplication of lactic acid bacteria introduced in starter cultures. In the 1970s, when the cold storage of raw milk became a widespread practice, a concept was developed to extend the keeping quality of raw milk by its thermization on delivery to dairy plants or between collections on farms. The thermized and promptly cooled milk could be cold-stored for an additional 3 days because thermization markedly reduces the heat-labile psychrotrophic microflora responsible for spoilage at low temperatures (spoilage without souring). Those microorganisms produce at low temperatures heat-stable proteinases, lipases, and phospholipases. Extension of the keeping quality allowed dairies to be closed over weekends. Thermization at 62–68 °C for 15 s is practiced widely. The treatment can be conveniently performed in a plate heat exchanger designed for the pasteurization of milk using a standard 15 s holding time.

Heat treatment at sub-pasteurization temperatures (55–65 °C for 20 s–10 min) has been used also to prolong the shelf life of cultured milk products such as yogurt and quark.

Extracellular proteinases and lipases produced by pseudomonads during cold storage of milk survive ultra-high temperature (UHT) sterilization but are less heat stable at 55–65 °C than at temperatures >70–80 °C. Low-temperature inactivation (60 °C for 5 min or longer) has been proposed to inactivate these enzymes before or after UHT sterilization of milk.

Effect of Thermization on Milk Components, Bacteria, and Enzymes

The residual activity of some indigenous enzymes, the amount of denatured whey proteins, or the concentration of compounds generated by heat are used as indices to detect or quantify the degree of heat treatment applied to milk.

[†] Deceased

An appropriate enzyme for the detection of milk thermization at 50–60 °C is α-L-fucosidase. The enzyme originates from somatic cells and at 62 °C, 90% of its activity is lost within 15 s. The somewhat more stable phosphohexose isomerase may be an appropriate enzyme to detect thermization at a higher (60–70 °C) temperature range (**Table 1**). Except for the colostral phase, the concentration of ribonucleosides, adenosine, cytidine, guanosine, and inosine in bovine milk varies only slightly throughout lactation. During thermization at 62 °C, the concentration of inosine increases with holding time. Because inosine is produced from adenosine by adenosine deaminase during holding at 62 °C or during the come-up time for pasteurization at 72 °C, the fold increase of the inosine content of milk treated at 62 °C for 20 s or at 72 °C for 20 s over that in raw milk is quite similar (**Table 1**). Therefore, measurement of the inosine content may be used to distinguish between raw and freshly thermized or pasteurized milks but not between thermized and pasteurized milks. A rapid and sensitive fluorogenic method for pH 4.6-soluble compounds in milk allows distinguishing a wide range of heat treatments.

Residual catalase activity in Cheddar cheese is not reliable for distinguishing between cheeses produced

Table 1 Effect of thermization and pasteurization on bacteria, enzymes, whey proteins, and inosine in milk

	Temperature (°C)	*D value (s)*
Bacteria in milk		
Psychrotrophic *Pseudomonas* spp.	60	1.19
	70	0.063
Listeria monocytogenes	61.8	38.5
	67.5	3.5
	67.5	3.5
Psychrotrophic sporeformers	85	990–1230
Enzymes, indigenous or from somatic cells		
Alkaline phosphatase	66	180
	70	15
α-L-Fucosidase	62	15–16
Phosphohexose isomerase	66	25
Catalase	65	2400
Lactoperoxidase	75	47
Enzymes, bacterial		
Extracellular from *Pseudomonas* spp.		
Proteinase	55	36–66
	70	14 000
Lipase	55	1700–8000
	70	450–2100
		% inactivation
Aminopeptidase from *Lactobacillus*	68	55% after 16 s
β-Galactosidase from *Streptococcus thermophilus*	60	<50% after 30 min
Penicillinase from *Bacillus cereus*	65	50% after 30 min
Whey proteins		*% denaturation*
β-Lactoglobulin	65	12% after 15 s
	85	60.5% after 30 s
Bovine serum albumin	65	31% after 15 s
Ribonucleosides		*% increase*
Inosine	62	411 after 20 s
	72	529 after 20 s

Adapted from Griffiths MW, Phillips JD, and Muir DD (1986) The effect of sub-pasteurization heat treatments on the shelf-life of milk. *Dairy Industries International* 51(5): 31–35; Suhren G (1989) Producer microorganisms. In: McKellar RC (ed.) *Enzymes of Psychrotrophs in Raw Food*, pp. 3–34. Boca Raton, FL: CRC Press; Fairchild TM and Foegeding PM (1993) A proposed nonpathogenic biological indicator for thermal inactivation of Listeria-monocytogenes. *Applied and Environmental Microbiology* 59: 1247–1250; Stepaniak L and Sørhaug T (1995) Thermal denaturation of bacterial enzymes in milk. In: Fox PF (ed.) *Heat Induced Changes in Milk*, 2nd edn., pp. 349–364. Brussels: IDF; Zehetner G, Bareuther C, Henle T, and Klostermeyer H (1996) Endogenous milk enzymes as indicators for low treatment of milk. *Proceedings of the International Dairy Federation Symposium*, pp. 403–408. Vienna, Austria, to 6–8 September 1995. *Heat Treatments and Alternative Methods*. Brussels: IDF; Martin D, Kiesner C, and Schlimme E (1997) Ribonucleosides: Chemical parameters for controlling the heat treatment of milk. *Nahrung* 41: 258–267; McKellar RC and Piyasena P (2000) Predictive modelling of inactivation of bovine milk α-L-fucosidase in a high-temperature short-time pasteurizer. *International Dairy Journal* 10: 1–6; Morales FJ, Romero C, and Jiménez-Pérez S (2000) Characterization of industrial processed milk by analysis of heat induced changes. *International Journal of Food Science and Technology* 35: 193–200.

from thermized and raw milks. Although fresh cheeses made from raw milk have several times higher catalase activity than in those made from thermized milk, catalase activity varies markedly in matured commercial cheese samples. This is due to the fact that the enzyme is produced during maturation by some nonstarter bacteria and yeasts.

A positive or negative result in the test for alkaline phosphatase activity is reliable to discriminate between raw milk and milk pasteurized at 72 °C for 15 s but not between pasteurized milk and thermized milk. Data in **Table 1** show that thermization denatures very little of the whey proteins. Raw and thermized milks could not be reliably distinguished by applying multiple analysis of variance to study statistically significant changes in the concentrations of hydroxymethylfurfural, lactulose, or soluble whey proteins.

Inactivation of Heat-Stable Proteinases and Lipases

Heat treatment at 50–70 °C inactivates, to various degrees, proteinases and lipases from pseudomonads (**Table 1**) and several other species of psychrotrophic microorganisms. The most efficient low-temperature inactivation was found for *Pseudomonas* proteinases in the temperature range ~50–60 °C and for *Pseudomonas* lipase at ~60–70 °C.

UHT sterilized milk produced from raw milk containing $>10^6$ bacteria per milliliter by heating at 140 °C for 5s had a shelf life, due to defects caused by lipolysis or proteolysis, 3–8 times shorter than that of milk which was first UHT sterilized and then held at 60 °C for 5 min.

Effect of Thermization on Microbial Quality of Cold-Stored Milk

Psychrotrophic pseudomonads account for ~10% of microflora of freshly drawn milk, but they become the dominant microorganisms in raw milk stored in farm tanks at 4–5 °C for 60–72 h; the most frequent isolate after that period is *Pseudomonas fluorescens*. A rapid increase in the bacterial population is noted between the second and third days of storage. *Pseudomonas* species are markedly more heat-labile than *Listeria* spp. or bacterial and indigenous enzymes (**Table 1**). Decimal reduction times (*D* values) for *Pseudomonas* spp. indicate that thermization should reduce these microorganisms by several log. Treatment at a temperature of 60–63 °C gives at least a 4-log reduction of *Salmonella* spp. and a 2-log reduction of *Listeria monocytogenes*. Thermoduric or sporeforming psychrotrophs grow markedly slower at <7 °C than *Pseudomonas* spp. Provided that recontamination after thermization is avoided, thermized milk has markedly better microbial quality after 3 days of cold storage than milk which has not been thermized (**Tables 2** and **3**). The effect of thermization on keeping quality during subsequent cold storage clearly depends on the temperature of thermization and the microbial quality of raw milk before thermization (**Table 3**). Alternatives to thermization may be cold storage at a very low temperature (not higher than 2 °C) which is expensive and/or purging the milk with nitrogen or CO_2.

Thermization reduces lactoperoxidase activity only slightly (**Table 1**) and apparently has little effect on the natural lactoperoxidase–thiocyanate–hydrogen peroxide antibacterial system in milk. This system is most effective in promptly cooled milk during a short period of storage.

Activation of Spores

Thermization at 65 °C for 10 s may be sufficient to stimulate the germination of *B. cereus* spores; activated spores may germinate after 6 h at 10 °C. If post-thermization contamination is avoided, the vegetative cells which have developed from germinating spores during cold storage

Table 2 Effect of thermization at dairy plants on the bacteriological quality of milk cold-stored for 3 days

	Dairy I[a]		*Dairy II*[b]	
Plate count ($cfu\,ml^{-1}$)	*Control*	*Thermized*	*Control*	*Thermized*
Total count	2.1×10^6	2.2×10^4	3.2×10^5	8.2×10^3
Coliforms	3.5×10^2	1.8×10^1	6.3×10^2	0.5×10^1
Psychrotrophs	-	-	2.1×10^5	1.5×10^2

[a] Unthermized (control) milk and milk thermized at 66–68 °C for 15 s upon delivery to dairy plant and cold-stored at 2–4 °C for 3 days. Averages from 23 deliveries of 4000 l each. Adapted from Rukke EO (1985) Råstofftiltak ved lagring av melk til osteproduksjon. *Meieriposten* 74: 62–65.

[b] Unthermized (control) milk and milk thermized at 67 °C for 15 s upon delivery to dairy plant and stored at 4–5 °C for 3 days. Averages from 18 deliveries of 4000 l each. Adapted from Heskestad R (1985) Termisering av melk til ystingsformål. *Meieriposten* 74: 66–68.

Table 3 Effect of thermization temperature and quality of raw milk on microbial quality of milk after thermization and storage

			Count (cfu ml^{-1})	
Initial count (cfu ml^{-1})	*Thermization temperature (°C)*	*Holding time (s)*	*After thermization*	*After thermization and storage for 3 days °C*
				At 7 °C
80×10^3	59.5	15	14×10^3	20×10^3
	64	15	8×10^3	6×10^3
1500×10^3	60	12.5	560×10^3	1400×10^3
	64	12.5	14×10^3	13×10^3
				At 4–5 °C
170×10^3	55	60	17×10^3	1100×10^3
	65	15	1.7×10^3	3.4×10^3
				At 6 °C
199×10^3	65	15	No data	63×10^3
18×10^3	65	15	No data	1.6×10^3

Adapted from van der Berg MG (1984) The thermization of milk. In: *The Thermization of Milk: On-Farm Use of Membrane Systems*, pp. 3–11. *International Dairy Federation Bulletin No. 182*. Brussels: IDF; Banks JM, Griffiths MW, Phillips JD, and Muir DD (1986) The yield and quality of Cheddar cheese produced from thermized milk. *Dairy Industries International* 51(7): 31–35; Griffiths MW (1986) Use of milk enzymes as indices of milk treatment. *Journal of Food Protection* 49: 696–705.

between thermization and pasteurization may be destroyed effectively by pasteurization and the resulting quality of the pasteurized milk may be improved. Thermization also reduces the formation of flocks of fat in laboratory-pasteurized milk; these flocks are caused by the phospholipase of *B. cereus* which damages the fat globule membrane.

Effect of Thermization on Cheese Yield and Quality

Thermization of milk has very little effect on the renneting properties of milk. Thermization of milk on farms or in dairies increases cheese yield primarily because the treatment reduces and even reverses, at least partially, low-temperature dissociation of β-casein and calcium from casein micelles. A reduction of cheese yield of 3–5% due to bacterial proteolysis is observed for milk of very poor microbial quality, containing $\sim 10^7$ psychrotrophs per milliliter.

The frequency of unclean and rancid flavor in Norvegia (Gouda-type) or Jarlsberg cheeses produced from pasteurized milk which had been thermized and cold-stored as specified in **Table 2** was lower than in cheeses from unthermized, cold-stored, and pasteurized control milk. Cheddar cheese produced from milk thermized at 65 °C for 15 s and stored for 3 days at 4–5 °C (**Table 3**) had better quality than cheese produced from the same milk thermized at 55 °C for 60 s or from control unthermized milk that had been cold-stored for 3 days and then pasteurized. Addition of selected lactic acid bacteria, which do not grow below 7 °C, to thermized milk before cold storage and pasteurization resulted in improved renneting properties of the milk. In cheeses the treatment improved the quality, provided that the milk before thermization was of reasonably good microbial quality.

Long cold storage of cheese made from unpasteurized milk is mandatory in some countries. During storage of some cheese varieties at 2 °C for not less than 60 days, many pathogens die off. The combination of thermization of cheese milk and cold storage of some cheeses is considered to be a safety precaution equivalent to the pasteurization of cheese milk.

It has been reported that unsatisfactory quality of fresh, ripened, and semihard cheeses made from thermized milk may be due to levels of *Staphylococcus aureus*, *Escherichia coli*, and *L. monocytogenes*. These results emphasize the need for applying and maintaining good hygiene practices throughout the food chain to prevent contamination and/or bacterial growth. Thermization to safe levels of enterobacteria, coagulase-positive *Staphylococcus*, and *Listeria* was accomplished by heating cheese milk (90:10 of ewe's and goat's milk) at 60 °C for 30 s. Nisin-producing lactococci in raw milk were not detected in thermized milk.

Regarding semihard cheese quality and anticlostridial effects of *Lactobacillus*, it is reported that all of nine isolated strains of *Lactobacillus paracasei* withstand low-temperature treatment at 60 °C for 5 min.

It has been observed that thermization (65 °C for 15 s) reduces the levels of individual free amino acids (FAA)

Table 4 Survival of microoganisms in thermized yogurt and quark

		Thermization			*Survival (%)*	
Product	*Microorganism*	*Temperature (°C)*	*Time (s)*	*Initial count (cfu ml^{-1} or g^{-1})*	*At pH ~4.5*	*At pH ~4.0*
Yogurt	Thermophilic lactic acid bacteria	60	22	6×10^9	100	77
		65	22	6×10^9	97	6
	Escherichia coli	55	22	5×10^5	40	0
		60	22	5×10^5	0.8	0
	Geotrichum candidum	55	22	1×10^5	8.2	0.1
		60	22	1×10^5	0	0
	Kluyveromyces fragilis	60	22	1×10^5	0.06	0
Quark	Mesophilic lactic acid bacteria	55	60	3×10^7	0.2	0.02
		60	60	3×10^7	0.005	0.01
	Escherichia coli	55	60	5×10^5	0.08	0
		60	60	5×10^5	0	0

Adapted from Puhan Z (1979) Heat treatment of cultured dairy products. *Journal of Food Protection* 42: 890–894.

during ripening of Cheddar cheese due to changes in the raw milk microflora that thus influence the FAA production.

Biogenic amines are undesirable in cheese. Decarboxylation of amino acids by microbial enzymes produces biogenic amines. Thermization (67 °C, 20 s) of cheese milk was not sufficient to inactivate amino acid decarboxylase-positive bacteria.

Thermization of Cultured Dairy Products

The main spoilage microorganisms in cultured products are molds and yeasts, and their usual sources are fruits added to yogurt or other fermented milks. Either the activity of rennet used in the production of quark or the activity of heat-resistant proteinases from psychrotrophic microflora may also contribute to the development of a bitter taste in quark. Spoilage of quark occurs due to 'post-acidification' or 'over-acidification' by starter microflora during storage.

Thermization of cultured milk products may be performed before or after the fermentation process. To avoid recontamination before packing, heat treatment is combined with aseptic or hot filling. Thermization of fermented products requires scraped-surface heat exchangers. Hydrocolloids are usually added to thermized cultured milk to improve the consistency of the final product.

Data in **Table 4** show that thermization of yogurt and quark reduces the number of both lactic acid bacteria and contaminants. Although lactic acid bacteria, especially in yogurt, are more resistant than yeasts, molds, and coliforms, their viable counts after thermization are reduced substantially. Measurable post-acidification did not occur in thermized yogurt or quark after storage of these products for 2 months at 20 °C. Quark that contained added proteinase from *P. fluorescens* and thermized at 55 °C for 6 min had a markedly better taste and less proteolysis than the unthermized control after storage at 7 °C for 5 weeks. Proteinase from *Pseudomonas* was inactivated due to self-hydrolysis or aggregation with casein at both pH 6.6 and pH 4.5. Thermization also reduced proteolysis in quark without added *Pseudomonas* proteinase.

Although thermization achieves its goal, that is, extension of the shelf life of cultured dairy products, its rationale is often questioned. The importance of viable bacteria is emphasized in standards and regulations for yogurt and other fermented milk products. The viable microflora assures maintenance of all probiotic properties of these products. Some properties, like the enhancement of lactose hydrolysis in the digestive tract, are similar for viable and nonviable cultured milk, but stimulation of the immune system by probiotic cultures has been observed with viable bacteria only.

See also: **Heat Treatment of Milk**: Sterilization of Milk and Other Products. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Plant and Equipment**: Pasteurizers, Design and Operation.

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Ultra-High Temperature Treatment (UHT): Heating Systems

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Introduction

Ultra high temperature (UHT) treatment of milk is a continuous process aimed at producing a 'commercially sterile' product, that is, a product in which bacteria will not grow under the normal conditions of storage when packaged aseptically. It involves heating at temperatures higher than 130 °C (usually 138–145 °C) for a holding time of 1–10 s (usually 3–5 s).

The basic principle of UHT processing of milk is that, for the same bactericidal effect, high-temperature, short-time heating causes less chemical change than low-temperature, long-time heating. Thus, sterilization by UHT heating at ~140 °C for a few seconds causes much less chemical change than batch sterilization in retorts at ~120 °C for several minutes.

The minimum time–temperature combinations for UHT heating are dictated by the need to destroy bacterial spores while the maximum time–temperature combinations are those causing the maximum acceptable amount of chemical damage, usually set at 3% destruction of the B vitamin, thiamine. In practice, the lowest temperature used is that at which the spores are destroyed in a reasonable time (~135 °C) while the upper limit is determined by the capability of the equipment (~150 °C); very short holding times (fractions of a second) at elevated temperatures are practically impossible to achieve with most equipment. However, a recent Dutch invention, innovative steam injection (ISI), is capable of heating milk at temperatures up to 180 °C for a very short time, ~0.2 s.

Nominal holding times are normally based on the average velocity of the particles in the product. Depending on the product and the dimensions of the holding tube, the velocity of the fastest particles can be several times that of the slowest particles. As a consequence, at time–temperature combinations at the lower limit of UHT processing, the fastest particles may receive insufficient heat while at time–temperature combinations at the upper limit, some particles may be overheated. The residence time distribution in the holding tube is particularly significant for destruction of heat-resistant bacterial spores as the time taken for the fastest moving spores to traverse the holding section can be much less than half that taken by the average particle, that is, the nominal holding time.

In addition to heat treatment and aseptic packaging, UHT processing of whole milk also involves homogenization. When down-stream of the sterilization step, homogenization must be performed aseptically. Homogenization reduces the average size of the fat globules in milk from ~3 to ~0.5 μm and coats the new small globule with a membrane consisting largely of casein. This markedly reduces the rate at which the fat rises in the milk during storage. Ideally, UHT milk should not develop a layer of fat on the top of the milk even after storage at ambient temperature for up to 9 months. In practice, many UHT milks do develop a fat layer after storage.

The heating step in UHT processing usually involves superheated steam as the heating medium. As the temperatures used for UHT processing are higher than the boiling point of water (and milk), processing has to be performed under pressure (~0.4 MPa) to prevent boiling. The steam can be either mixed directly with the product (direct heating) or used indirectly via metal tubes or metal plates (indirect heating). In indirect systems, the steam can be used alone or to superheat water which is then used as the heating medium. Combinations of both indirect and direct modes of heating have been introduced to take advantage of the benefits of both types.

There are, however, other ways of performing the high-temperature heating. These are mostly electrically based. They include microwave, radiofrequency, inductive, ohmic, and electrical tube heating (ETH). The first four have found little application in the dairy industry to date and are not discussed in this article. Electric tube heating, on the other hand, has been used for some time for UHT processing of milk and milk products in some countries and is discussed below.

Viscous products and products containing particulate matter have additional requirements over liquids such as milk in UHT processing. While some of these products can be processed by the systems mentioned earlier, particularly ohmic heating, another form of heat exchanger, the scraped-surface heat exchanger, is used for this purpose. These are mostly indirectly heated although a variation utilizing direct steam injection heating is commercially available.

Steam-Based Heating Systems

UHT heating systems utilizing steam are shown in **Figure 1**.

Indirect Heating Systems

The most common type of UHT heating system utilizes tubular heat exchangers. Different configurations of the heat exchanger are available with the most common being the shell-in-tube and the concentric tube designs. In some systems, the heated tube does not contact the product directly but heats a closed water jacket which in turn heats the product. This double-jacket arrangement eliminates the risk of the product becoming contaminated if the heated tube develops a leak.

In plate heat exchangers, the heat transfer rates are high, due to the large contact surface area, and high turbulence levels can be achieved. However, they are more easily fouled than tubular systems due to the narrow channels through which the product flows.

All UHT plants employ some heat regeneration, that is, hot milk heats incoming cold milk. In this way, indirect heating systems can achieve 80–95% energy recovery, with plate systems achieving higher regenerative rates than tubular systems.

In indirect systems, the milk to be heated flows in the opposite direction to the heating medium (hot milk or steam). This minimizes the temperature differential between the two flows which in turn reduces the amount of fouling on the heat exchanger surface and allows longer operating times and better product quality. The temperature differential should be not more than 3 °C.

A flow diagram for an indirect (tubular) UHT system is shown in **Figure 2**. A system using plate heat exchangers is similar, with the heat exchangers 1, 4–6 being plate instead of tubular. The homogenizer is shown upstream of the high-heat section but it can also be downstream in indirect systems. Upstream homogenization has the advantage of not requiring the homogenizer to be aseptic.

A heating profile for indirect systems is shown in **Figure 3(a)**. The initial heating is achieved in a regeneration section where the previously heated milk heats and is cooled by the incoming cold milk. Many indirect systems include a preheat holding section (shown in **Figure 2**) where the milk is held at about 90 °C for 30–60 s, before being heated in the high-heat section. It is commonly known as a protein stabilization section as it causes denaturation of much of the whey protein β-lactoglobulin and so prevents it from denaturing and depositing on the walls of the subsequent heating tubes or plates.

Fouling or deposit formation is a major issue with indirect heating systems. The buildup of deposit restricts the flow of product through the tubes or plates and causes a decrease in pressure and a decreased transfer of heat from the heating medium to the product. This is more significant in plate than in tubular heat exchangers. Typically, this limits the run time in plate systems to 8–12 h and in tubular systems to 16–20 h before partial or complete cleaning is required to remove the deposit.

Direct Heating Systems

Two types of direct systems, which differ in the manner in which steam and product are mixed, are used. In one system, steam injection, steam is mixed with a stream of product (steam-into-product) via a steam injector nozzle (**Figure 4**) and in the other, steam infusion, the product is passed through a steam infusion chamber (item 2 in **Figure 5**) filled with steam under pressure at the sterilization temperature (product-into-steam). The flow diagram of a direct heating system using steam infusion is shown in **Figure 5**. A system using steam injection is similar to that shown in **Figure 5**, but has a steam injector rather than the stream infusion chamber. As shown in

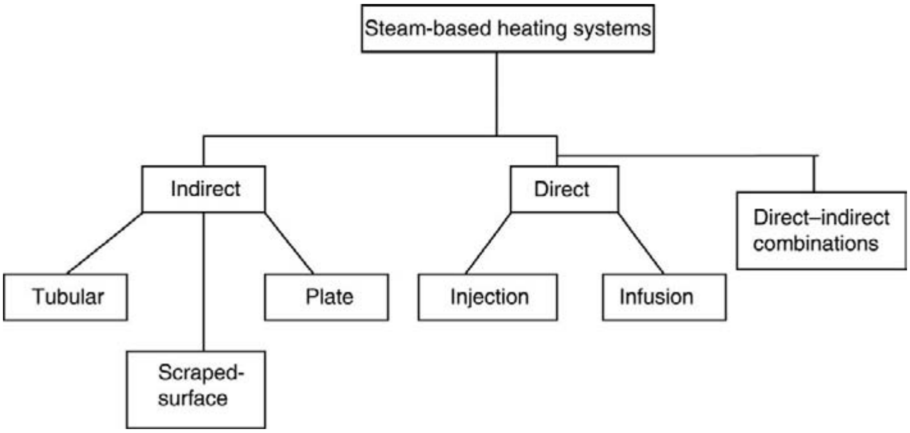
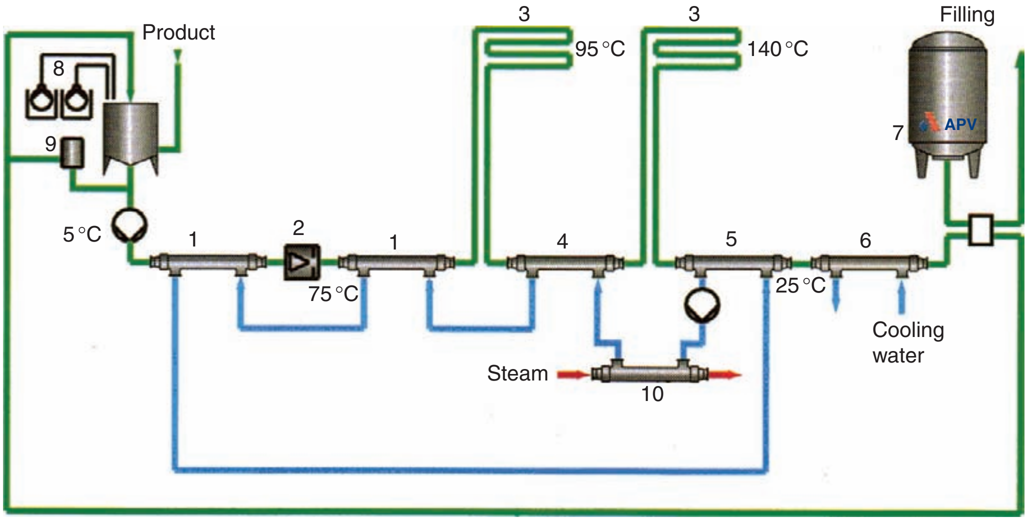


Figure 1 UHT systems based on steam heating.



1. Tubular regenerative preheaters
2. Homogenizer
3. Holding tubes
4. Tubular final heater
5. Tubular regenerative cooler
6. Final cooler
7. Sterile tank
8. CIP unit
9. Sterilizing loop
10. Water heater

Figure 2 Flow diagram for an indirect (tubular) UHT system. Reproduced with permission from APV.

Figure 5, direct heating is used only for the high-heat section; other heating sections utilize indirect heating.

The major feature of direct heating is its high heating rate compared with indirect systems. Typical rates are 60–70 °C in less than 1 s, with some reports as low as 0.1 s. This is achieved by utilizing the latent heat of the steam and as a consequence some water condenses into the milk. For a 60 °C rise, the amount of water condensed is about 11% of the product volume.

Following direct heating to a high temperature and holding for a few seconds, the product enters a vacuum chamber which fulfils the dual role of removing the water taken up in the heating step and rapidly cooling the product to approximately the same temperature as that of the product immediately before the heating step (typically about 70–80 °C).

Therefore, the high-temperature heating and subsequent cooling steps are very rapid, as illustrated in **Figure 3(b)**. This is quite different from the situation for indirect heating (**Figure 3(a)**), where heating and cooling are much slower. For the same bactericidal effect, the faster heating and cooling in direct heating means that a higher sterilization temperature can be achieved without excessive chemical change in the product. For UHT milk the most notable effect is a lower cooked flavor intensity in directly heated milk.

Figure 3(b) also shows the much slower heating prior to the high-temperature (direct) heating step and slower cooling after the vacuum cooling; these sections are heated/cooled by indirect means, largely via heat regeneration. Total heat regeneration in systems where direct heating is used for the high-temperature section is limited (to about 40%) as very little of the heat removed in the vacuum chamber can be utilized. The exact percentage of heat regeneration is determined by the relative amounts of direct and indirect heating used in the whole system.

A preheat holding section is usually not included in direct systems as fouling at heating surfaces is much less than in indirect systems. In infusion systems, the high-temperature heating occurs in the steam chamber before the product touches the conical base of the chamber. At this point the wall is usually 'cooled' to about 120 °C which prevents burn-on. In steam injection systems, some product may contact the hot metal surface of the injector and cause some fouling. However, injectors are designed to minimize this.

As indicated in **Figure 5**, homogenization is performed after the high-heat section (downstream homogenization) in direct heating systems to break up large casein aggregates which may form in the process. These aggregates can impart a 'chalky' and 'astringent' mouthfeel to the UHT milk. Hence, aseptic homogenizers must be used. Some homogenization occurs in steam injectors due to the violent mixing of the steam and milk with resultant cavitation and shear forces. No homogenization occurs in the gentler steam infusion systems.

Combination Direct–Indirect Systems

Major equipment manufacturers have developed systems incorporating both direct and indirect heating (and cooling) in the high-heat section of UHT plants. The reason

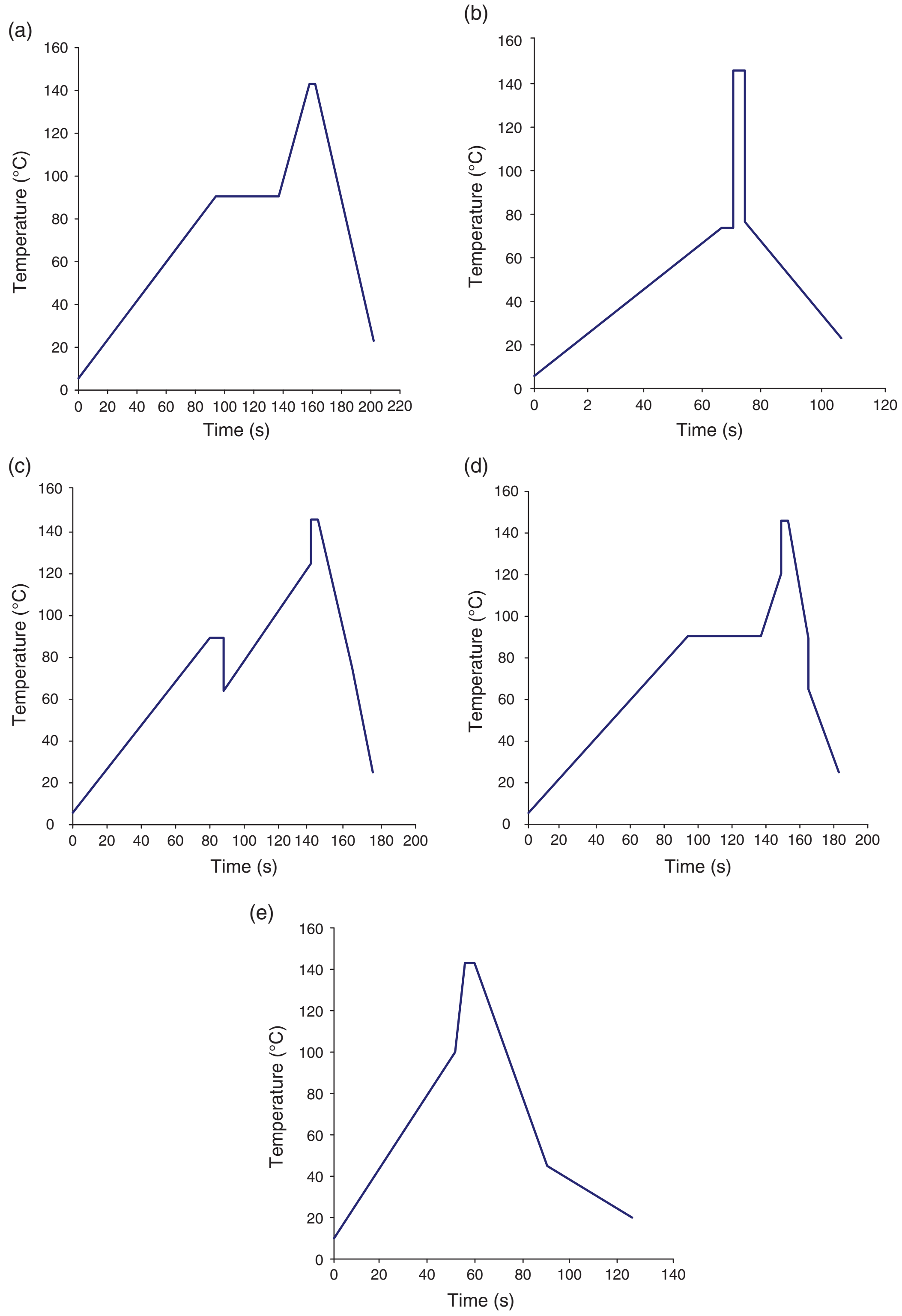


Figure 3 Representative temperature–time profiles for UHT systems: (a) indirect; (b) direct; (c) indirect–direct combination (APV's *High Heat infusion*); (d) indirect–direct combination (Tetra Pak's *Tetra Therm*® *Aseptic Plus Two*); (e) electrical tube heating.

for this is to exploit the advantages of each system, in particular, the better flavor and superior bacterial spore destruction capability of direct heating (higher temperatures can be attained without excessive chemical damage to the milk) and the higher heat regeneration capacity of indirect systems.

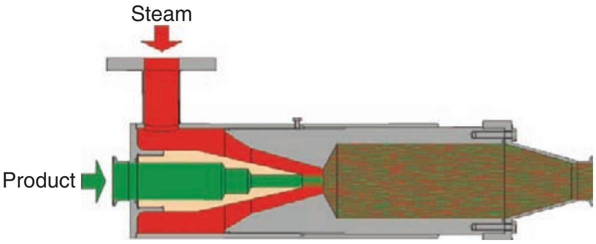


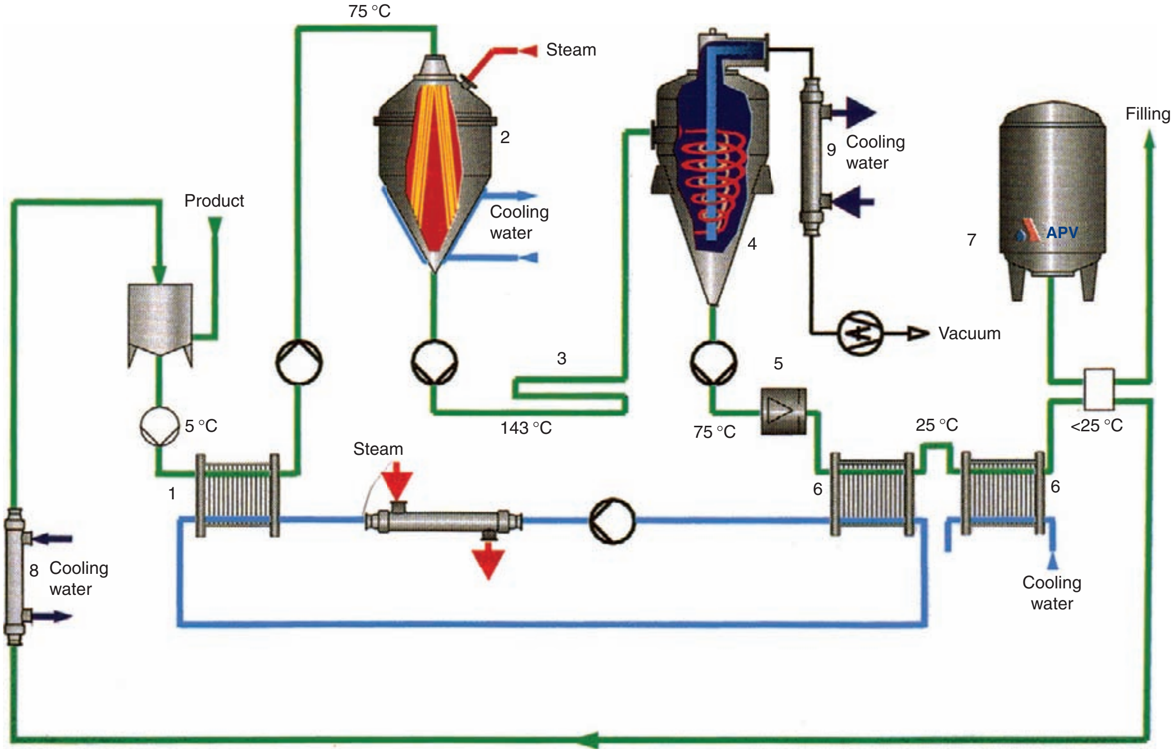
Figure 4 A steam injector nozzle used for direct heating. Reproduced with permission from APV.

The temperature profiles of the systems marketed by APV (High Heat Infusion) and Tetra Pak (Tetra Therm® Aseptic Plus 2) are illustrated in **Figures 3(c)** and **3(d)**, respectively. In both systems, the temperature rise achieved by direct heating is 20–30 °C, compared with 60–70 °C for the direct heating systems. They differ in the sequence of heating and cooling steps. In the High Heat Infusion system, the vacuum chamber is placed after preheating and before the high-heat sections, while in the Tetra Therm® Aseptic Plus 2 system, it is placed after an initial cooling step following the high-heat (steam injection) section. Heat regeneration and the extent of chemical 'damage' to the milk in these combination systems are intermediate between the conventional direct and indirect systems. Up to 75% regeneration is claimed for the High Heat Infusion system.

The discovery of very heat-resistant bacterial spores in UHT milk in Europe was a major trigger for the development of these combination systems. The major organisms of concern are *Bacillus sporothermodurans* and *Paenibacillus lactis* which have been isolated from UHT milk after heating the milk at 100 °C for 30 min. As well as their spores showing high heat resistance (heating at ~150 °C for 6 s is required for a satisfactory kill of *B. sporothermodurans* spores) are also capable of growth in the temperature range for mesophilic bacteria. The high temperatures (up to 150 °C) which can be achieved in direct heating are more likely to destroy the spores of these organisms than are the highest practical temperatures possible with indirect heating.

Scraped-Surface Heat Exchanger Systems

Scraped-surface heat exchangers are used for high-viscosity products for which alternative heat exchangers are impracticable. They use rotating mechanical scraper blades to continuously remove product from the heat transfer surface and create turbulence to improve heat transfer. They operate with high-temperature differentials



1. Plate preheaters
2. Steam infusion chamber
3. Holding tube
4. Flash vessel
5. Aseptic homogenizer
6. Plate coolers
7. Aspetic tank
8. Non-aspetic cooler
9. Condenser

Figure 5 A flow diagram for a direct heating system using steam infusion. Reproduced with permission from APV.

between the product and the heating surface and therefore have a high potential for burn-on on the heating surface. They have a relatively low energy efficiency compared with plate and tubular systems used for low-viscosity products, as regeneration of heat is not possible with this system.

A variation of the scraped-surface system, the *RotoTherm*™, utilizes a series of steam injectors along the scraped surface. In this system, the steam condenses and transfers its latent heat to the product. It also forms a film of water on the inside of the barrel which reduces burn-on and lubricates the surface. Processed cheese products are typical dairy products heated by this system.

Electrical Tube Heating

In this process, a stainless steel tube carrying a pumpable product acts as an electrical resistor and is heated by the passage of an electrical current by what is known as the Joule effect. The heat is then transferred to the circulating product from the surface of the tube by conduction and convection.

The technology has no generally recognized generic name but is known by several names such as 'Current Passage Tube', 'Tube with Direct Current Flow', 'Constant Wall Flux Tubular Heat Exchanger', 'Heating Electric Tube', and '*Actijoule*®', based on the brand name for the equipment marketed by the company, Actini. Other manufacturers include Rossi & Catelli in Italy and Elecster in Finland. In this article, the generic term, ETH, is used. The technology has not been widely adopted by the dairy industry of most countries but several plants are in use in Europe for pasteurization and sterilization of foods including milk, sweetened milk, cream, and other dairy products.

An ETH system typically consists of a power supply (a transformer converting high-voltage, three-phase power to low voltage (18–60 V)); three sets of stainless steel heating tubes of matched resistances, each connected to one of the three phases of the transformed power supply; heat-regeneration heat exchange tubes for preheating the cold incoming raw material with heated product; and a control unit from which the final temperature of the product can be set. Commercial plants operate at high amperage (~500–600 amps) and power ratings up to 500 kW.

A major feature of this technology is the constant flux density along the length of the heating tube, producing a linear rise in temperature. There is also a small and constant temperature differential between the tube and the product. As a fouling layer develops, this differential increases to ensure constant product exit temperature.

ETH is suitable for free-flowing liquids, viscous products, and products containing particulates up to ~4 mm. High flux densities (up to 350 W cm^{-2}) and high product velocities (7–9 m s^{-1}) are used for free-flowing liquids while low flux densities (~0.1 W cm^{-2}) and correspondingly low rates of heating (e.g., 0.8 °C s^{-1}) are used for viscous and heat-sensitive products such as dairy desserts. These different heating modes enable heated liquid products to retain good organoleptic quality and minimize overheating of viscous products.

The system has low thermal inertia, enabling quick start-up and shutdown. This and a control system which acts directly on the heating medium, the electrical power, allow a quick response time to changes in conditions and lead to only small volumes of product being lost when the plant is stopped.

ETH has high energy efficiencies (~95%). These are comparable to plate heat exchangers and much higher than for direct UHT heating systems because most of the heat can be regenerated.

A major attraction of the technology is that it requires no steam generation and reticulation facilities. ETH systems are simpler than conventional steam-based heating systems and require less maintenance.

While this technology is being used commercially for milk and other dairy products, there is limited available information on its application to these products. For UHT milk, a typical heating–cooling regime is shown in **Figure 3(e)**.

Compared with conventional indirectly heated UHT milk, ETH-processed milk is reported to be less chemically changed. This is evidenced by less browning, shorter rennet coagulation time, and a fresher taste and odor after 3 days. The difference is largely attributable to the faster heating rate to attain the sterilization temperature.

Comparison of Heating Systems

Several criteria can be used to compare the different heating systems. **Table 1** summarizes several of these for the steam-based and ETH systems discussed earlier. A considerable amount of data exist for indirect and direct steam heating systems but less information is available on the ETH system.

In general, the slower the high-temperature heating and cooling to achieve a given bactericidal effect, the greater the chemical change. The major chemical changes of practical significance are whey protein denaturation, protein–protein interactions (particularly the interaction of β-lactoglobulin and κ-casein but also cross-linking via dehydroalanine forming lysinoalanine), Maillard browning, formation of sulfhydryl compounds (largely by degradation of β-lactoglobulin and milk fat globule membrane material), production of carbonyl compounds, destruction of vitamins, formation of insoluble sedimentable material, and fouling of hot surfaces. These changes

Table 1 Comparison of UHT heating systems

Criteria	*Direct heating*	*Indirect heating*	*Direct–indirect combinations*	*Electrical tube heating*
Relative chemical changes (denaturation of whey protein, cooked flavor production, Maillard browning, lactulose production, water-soluble vitamin loss)	Low	High	Intermediate	*Intermediate*[a]
Susceptibility of product to age gelation	High	Low	Intermediate	*Intermediate*
Heat regeneration capability	Low	High	Intermediate	High
Fouling	Low	High	Intermediate	Relatively low
Run times	Long	Short	Intermediate	*Intermediate*
Ability to reach very high temperatures (i.e., >145 °C)	Capable	Limited	Capable	Capable
Effectiveness for destroying heat-resistant spore-forming bacteria without excessive chemical damage to milk	High	Low	High	*Reasonably high*
Ability to process viscous products	Reasonable, especially with infusion	Some capability with tubular, little with plate	Depends on direct and indirect components	Capable
Homogenizer placement	After high-heat treatment	Before or after high-heat treatment	After high-heat treatment	*Before or after high-heat treatment*
Aseptic homogenizer	Not required	Required	Required	Not required
Options available	Injection and infusion	Plate and tubular	Placement of vacuum chamber before or after high heat section	Low flux, low flow rate and high flux, high flow rate
Oxygen level in product	Low	High	Low	*High*
Loss of nutritive value	Very low	Low	Very low	*Low*
Requirement for culinary steam	Yes	No	Yes	No steam used
Ease of control	Careful control of amount of water removal required	Need to control pressure increase and temperature differential between product and heating tube or plate as fouling layer builds up	Careful control of amount of water removal required product and heating tube or plate as fouling layer builds up	Need to control pressure increase and temperature differential between product and heating tube or plate as fouling layer builds up
Possibility of contamination from heating medium through pinholes	Nil for high-heat section. Possible in regeneration and other indirect heating and cooling sections	More significant than for direct, especially with plate heat exchangers	Intermediate between direct and indirect systems	Nil for high-heat section. Possible in regeneration and other indirect heating and cooling sections
Commercial use	Common	Common	Some plants in operation	Some plants in operation
Other features	Infusion heating ‘gentler’ than injection; injection causes some homogenization	Tubular is most common UHT heating type	Capital cost high because both indirect and direct components required	Easy start-up and shutdown due to low capacity of heating system; electricity cost a consideration

[a] Entries in italics are based on limited data or theoretical considerations.

occur more in indirectly heated milk than in directly heated milk. A beneficial effect of the heating severity of indirect heating systems is that age gelation is retarded.

A useful measure of the chemical effect of heat processes is the C^* value which has a value of 1 for a process in which 3% of thiamine (vitamin B_1) is destroyed.

Mathematically it can be calculated from the formula

$$C^* = 10^{(T-135)/31.4} \frac{t}{30.5}$$

where T is the temperature in degrees Celsius and t the time in seconds.

The corresponding bacteriological index is B^* which has a value of 1 for a 9-log reduction of a mixture of thermophilic spores. It is given by the formula

$$B^* = 10^{(T-135)/10.5} \frac{t}{10.1}$$

A UHT process is deemed satisfactory if it has a C^* of <1 and a B^* of >1. **Table 2** gives a comparison of the C^* and B^* values for some commercial direct and indirect UHT plants.

During treatment of milk in direct heating systems, the vacuum chamber, which removes water and rapidly cools the product, also removes a large proportion of oxygen present. As a consequence, UHT milk processed in direct systems contains about 1 mg kg^{-1} of oxygen while milk heated in indirect systems contains up to 10 mg kg^{-1}. The major consequences of this difference are that, in indirectly heated milks, the initial sulfury, cabbagey flavor dissipates rapidly; staler, oxidized flavors develop; and oxidation-sensitive vitamins such as vitamin C are destroyed during storage. However, the amount of headspace in the package has a major effect on the oxygen content of the UHT milk as a large headspace can saturate the milk with oxygen, and hence negate the advantage of low oxygen content resulting from direct heating processes. TetraBrik cartons have a very small headspace volume, a few milliliters, while plastic bottles have much higher volumes; an average of 58 ml has been reported.

Several chemical indices of heat treatment are recognized. These include lactulose, furosine, and undenatured β-lactoglobulin (an indirect measure of denatured β-lactoglobulin). Furosine is an indirect measure as it is formed during acid treatment of the milk before analysis. Lactulose and furosine increase and undenatured β-lactoglobulin decreases with increased severity of heat treatment. **Table 3** illustrates the levels of these indices in some commercial UHT milk samples compared with high-temperature, short-time (HTST) pasteurized milk.

Table 2 Comparison of C^* and B^* for the overall process of some commercial direct and indirect UHT plants

Type of UHT plant	*Holding conditions (°C s^{-1})*	*C^**	*B^*(adj)^a*
Direct heating	143/2	0.4	1.7
	143/4	0.5	2.7
	143/10	0.8	5.6
	145/13	1.5	11.8
	146/6	0.5	4.1
Indirect heating	137/9	0.9	1.6
	138/10	1.8	3.1
	139/6	1.3	2.6
	140/4	1.7	3.7
	142/4	1.5	4.8
	144/5	1.9	8.7
	145/2.5	0.9	4.6

[a] Adjusted to account for residence time distribution assuming holding time for fastest particle is 1.2 times average holding time, that is, B^* (adj) = B^*/1.2.
Adapted from Tran H, Datta N, Lewis MJ, and Deeth HC (2008) Processing parameters and predicted product properties of industrial UHT milk processing plants in Australia. *International Dairy Journal* 18: 939–944.

Table 3 Chemical indices of some commercial UHT milks

Milk type	*Undenatured β-lactoglobulin (mg l^{-1})*	*Furosine (mg per 100 g protein)*	*Lactulose (mg l^{-1})*
UHT (direct) ($n = 9$)	639	55.1	139
UHT (indirect) ($n = 47$)	79	180	548
HTST-pasteurized ($n = 6$)	3512	7.0	Not detected

Source: Birlouez-Aragon, *et al.* (1998) *International Dairy Journal* 8: 771–777.

They indicate much greater chemical change in the UHT milks than in the pasteurized milks and more change in indirectly than directly heated UHT milks, in line with the reported C^* values shown in **Table 2**. Hence, they are useful for distinguishing between milks subjected to these different heat treatments. However, there is some overlap as conditions in commercial plants of both direct and indirect types vary considerably. In addition, some of these indices change during storage which makes comparison of UHT milks of different ages difficult. Since the kinetics for each of these changes has been determined, computer programs based on the kinetic data can be used to predict the changes that will occur in a plant with a certain temperature–time profile.

See also: **Heat Treatment of Milk**: Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Aseptic Packaging. **Plant and Equipment**: Heat Exchangers.

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Ultra-High Temperature Treatment (UHT): Aseptic Packaging

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Introduction

Aseptic packaging is the filling of a commercially sterile product into sterile containers under sterile conditions and sealing the containers so that reinfection is prevented. The first aseptic packaging of food (specifically milk in metal cans) was carried out in Denmark by Nielsen prior to 1913, and a patent for this process (termed 'aseptic conservation') was granted in 1921. In 1923, aseptically packaged milk from South Africa reached a trade fair in London, England, in perfect condition.

At the end of the 1940s, a dairy enterprise (Alpura AG, Bern) and a machinery manufacturer (Sulzer AG, Winterthur) in Switzerland combined their knowledge to develop ultra-high temperature (UHT)-sterilized, aseptically canned milk, which was marketed in Switzerland in 1953. However, this system was not economic, mainly because of the cost of the cans, and Alpura, in collaboration with Tetra Pak AB of Sweden, developed an aseptic system based on paperboard laminate cartons. The first milk with a long shelf life to be packaged in this manner was sold in Switzerland in October 1961.

The size of the aseptic market for milk products has since grown rapidly and is now estimated to be in excess of 30 billion liters of product annually.

Sterilization of Packaging Material

Three main sterilization processes for packaging material are in common use, either individually or in combination: irradiation, heat, and chemical treatments.

Irradiation

Ionizing radiation

Particle irradiation techniques using gamma rays from cobalt-60 or cesium-137 have been used to sterilize the interior of sealed but empty containers, especially those made of materials that cannot withstand the temperatures needed for thermal sterilization, or that, because of their shape, could not be sterilized conveniently by other means. A radiation dose of 25 kGy or more is sufficient to ensure sterility. The packages are sealed into microbial-proof containers prior to the irradiation treatment. It is also possible to use low-energy (80–150 keV), large-area electron beams for the surface sterilization of packaging materials and containers, including pre-forms, bottles, and caps.

Pulsed light

By storing electrical energy in a capacitor and releasing it in short pulses, high peak power levels can be generated. The use of intense and short-duration pulses of broad-spectrum 'white' light (200–1000 nm) to sterilize aseptic packaging material underwent considerable research and development efforts in the 1990s. The duration of the pulses ranges from 1 μs to 0.1 s, and the flashes are typically applied at a rate of 1–20 flashes per second. Approximately 25% of the emitted light, which has an intensity of about 20 000 times that of sunlight at the Earth's surface, is UV; 45% visible; and 30% infrared. Generally, a few flashes applied in a fraction of a second provide high levels of microbial inactivation. Despite promising trials, this technology has not been commercialized.

UV lasers

UV lasers have been evaluated to achieve chemical-free sterilization of pre-formed carton packaging. Excimer gas lasers using gases of halides mixed with an inert gas such as krypton fluoride (emitting at 248 nm) have been commercialized recently. Illumination of the carton interior by laser light is problematic: the beam has to be projected physically to different areas of the carton interior by moving the carton into the laser beam.

Plasma

A low-pressure microwave plasma reactor has been evaluated for sterilization of polyethylene terephthalate (PET) bottles. A mixture of nitrogen, oxygen, and hydrogen gases is ignited inside a bottle; the resultant plasma consists of both UV photons and short-lived antimicrobial-free radicals. A treatment time of 5 s provides a reduction of 10^5 cfu. Commercialization of this cold sterilization process is presently under way.

Heat

Heat sterilization processes can involve either steam (moist heat) or dry heat. Steam is pure gaseous water with no air or other gases present. Dry heat is hot air in the absence of water molecules. The sterilization effect depends on time and temperature, and steam is much more efficient than dry heat. Steam sterilization at 121 °C for 20 min is equivalent in effectiveness to dry heat at 170 °C for 60 min.

The most reliable sterilant is, without doubt, steam. However, in order to reach temperatures sufficiently high to achieve sterilization in a few seconds, the steam (and thus the packaging material with which it comes into contact) must be under pressure, necessitating the use of a pressure chamber. Also, any air that enters the pressure chamber with the packaging material must be removed; otherwise, it would interfere with the transfer of heat from the steam to the package surface. Finally, condensation of steam during heating of the packaging material surface produces condensate, which may remain in the container and dilute the product.

Despite these problems, saturated steam under pressure is used to sterilize plastic containers. To limit the heating effect to the internal surface of the cups, the exterior of the cups is simultaneously cooled.

Chemical Treatments

Hydrogen peroxide

The lethal effect of hydrogen peroxide (H_2O_2) on microorganisms (including heat-resistant spores) has been known for many years. However, it has been found to induce higher heat resistance in *Bacillus sporothermodurans* strains, indicating that sub-lethal stress conditions may affect heat resistance.

The most successful commercial aseptic filling system was devised in 1961 and used a combination of H_2O_2 and heat to sterilize the surface of the carton packaging material. For rapid sterilization, both a high concentration of H_2O_2 and a high temperature are needed. Recognized minimum parameters are 30% at 70 °C for 6 s.

The US Food and Drug Administration has ruled that when H_2O_2 has been used to sterilize food packaging material, no more than 0.5 ppm H_2O_2 should be detected in distilled water packaged under production conditions, the assay to be performed immediately after filling.

A number of systems have evolved to achieve faster peroxide sterilization processes by combining heat and radiation. UV radiation has a wavelength of 100–400 nm, and the lower part up to 280 nm is named UV-C radiation. In terms of microbial destruction, it has a peak at 240–280 nm. Mercury vapor lamps emit UV-C at 253.7 nm. UV irradiation and H_2O_2 act synergistically when used together, the UV irradiation promoting the breakdown of the peroxide into hydroxyl radicals. The advantage of such a combination is that much lower concentrations of peroxide can be used (0.5–5% compared with 30–35% for peroxide and heat together), and the problems of atmospheric contamination by peroxide and residual peroxide in the filled product are reduced.

Peroxyacetic acid

Peroxyacetic acid (PAA) is a liquid sterilant that is particularly effective against spores. It is produced by the oxidation of acetic acid by H_2O_2, and the solution containing PAA and H_2O_2 is effective against resistant bacterial spores even at 20 °C. It is used for sterilizing filling machine surfaces as well as packaging materials such as PET bottles prior to aseptic filling. The PET bottles are rinsed with sterile water rather than with hot air.

Aseptic Packaging Systems

The aseptic packaging system must be capable of filling the sterile product in an aseptic manner and sealing the container hermetically so that sterility is maintained throughout the handling and distribution processes. Commercial requirements of less than one faulty package out of 10 000 produced are common.

The type of packaging material used is influenced by the nature of the product, the cost of both the product and the package, and the preferences of the consumer. The most widespread consumer package for aseptic dairy products is the paperboard carton. Five major categories of aseptic packaging equipment are available; their major features and characteristics are described below.

Carton Systems

The carton material consists of layers of paperboard coated internally and externally with polyethylene, resulting in a carton that is impermeable to liquids and in which the internal and external surfaces may be heat-sealed. There is also a thin (6.3 μm) layer of aluminum foil that acts as an oxygen, flavor, and light barrier. The structure of a typical paperboard laminate carton is shown in **Figure 1**.

Form-fill-seal cartons

The packaging material is supplied in rolls that have been printed and creased, the latter being necessary to ease the forming process. A plastic strip is sealed to one edge (the

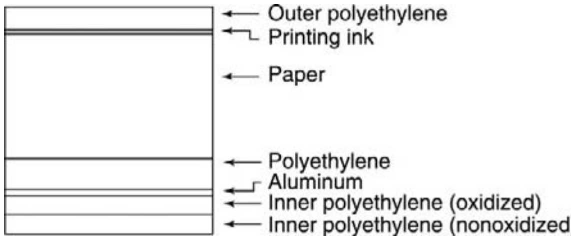
Outer polyethylene
Printing ink
Paper
Polyethylene
Aluminum
Inner polyethylene (oxidized)
Inner polyethylene (nonoxidized)

Figure 1 Structure of a paperboard laminate carton for aseptic filling.

reason for this is described later), and the packaging material sterilized using one of two procedures: a wetting system or a deep bath system. In the wetting system, a thin H_2O_2 film (15–35% concentration) containing a wetting agent (Tween-20 or polyoxyethylene sorbitan monolaurate at 0.2–0.3%) to improve the formation of a liquid film is applied on the inner surface of the packaging material. The material then passes through a pair of rollers for removal of excess liquid and is formed and sealed into a cylinder. It then passes under a tubular electric heater, which heats the inside surface to about 120 °C and evaporates the H_2O_2. In the deep bath system, the packaging material is fed through a deep bath containing H_2O_2 (35% concentration) at a minimum of 70 °C, the residence time being 6 s. After squeezer rollers have removed much of the peroxide, air at 125 °C is directed through nozzles onto both sides of the material to heat it to increase the sterilizing effect and to evaporate the peroxide.

The sterilized packaging material is fed into a machine where it is formed into a tube and closed at the longitudinal seal by a heat-sealing element. In the process, the strip that was added prior to sterilization is heat-sealed across the inner surface of the longitudinal seal to provide protection of the aluminum and paperboard layers from the product, which could corrode or swell the layers if such a strip were absent. Product is then filled into the tube, and a transverse seal made below the level of the product, thus ensuring that the package is completely filled. Alternatively, the packages may be produced with a headspace of up to 30% of the total filling volume by the injection of either sterile air or other sterile inert gases. The sterilization, filling, and sealing processes are all performed inside a chamber maintained at an overpressure of 0.5 atm with sterile air. The sealed packages are then pressed by molds into rectangular blocks, after which the top and bottom flaps or wings are folded down and heat-sealed to the body of the package using electrically heated air.

The most common method of forming and filling cartons from a continuous web is shown in **Figure 2**, which also includes a cross-section of the longitudinal seal.

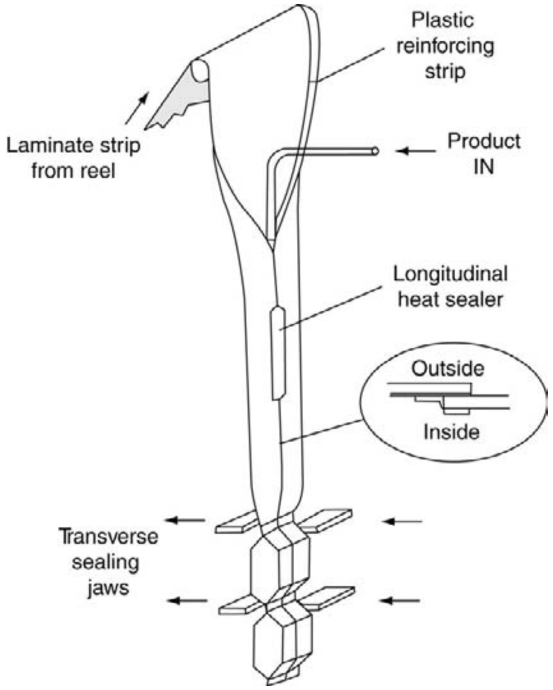

Figure 2 Forming cartons from a continuous web. The cross-section of the longitudinal seal is enlarged to show the plastic strip that protects the internal edge of the carton.

Prefabricated cartons

In systems of this type, prefabricated carton blanks are used, the cartons being die-cut, creased, and the longitudinal seam completed at the factory of origin by skiving the inner layer of board and folding it back. The cartons are delivered to the processor in lay-flat form, ready to be finally shaped in the filler and the top and bottom seams formed and bonded.

The aseptic area of the filling machine consists of several separate functional zones where operations are carried out in sequence. Sterility is maintained in each zone by a slight overpressure of sterile air. The inside surface of the carton is sterilized with a 35% solution of H_2O_2, delivered as either a fine spray or peroxide vapor in hot air so that the vapor condenses as liquid peroxide on the carton surface. The peroxide is removed by a jet of hot air at 170–200 °C. Alternatively, the inside of the carton can be sprayed uniformly with a 1–2% solution of H_2O_2 and then irradiated for about 10 s with high-intensity UV radiation. The peroxide is then heated and removed by hot-air jets. Because the total quantity of peroxide used in this latter process is 20–30 times less than that in the former, the problems of residual peroxide in the carton and peroxide contamination in the surrounding atmosphere are dealt with more easily.

After filling, the carton top is folded and closed, the seal being made by either heating or ultrasonic welding. The protruding flaps or ‘ears’ on each side are folded down and sealed to the package with hot air. The finished cartons are then discharged from the pocket chain onto a conveyor belt.

Plastics

For many years, blow-molded plastic bottles made from high-density polyethylene (HDPE) or polypropylene (PP) have been used, sometimes with pigments added to give greater protection to the contents against light. It is also possible to mold bottles from multilayered material, resulting in much improved barrier properties and thus longer shelf lives for the products packaged in them.

More recently, aseptic PET systems capable of packaging dairy products have been commercialized. UHT low-fat milk stored under light (700 lux) in clear PET bottles for 12 weeks at 23 °C showed 100% loss of vitamin B_2, 93% loss of vitamin A, and 66% loss of vitamin D_3. In pigmented PET bottles, the vitamin retention was only slightly higher: between 70 and 90% for vitamin A, 63 and 95% for vitamin B_2, and 35 and 65% for vitamin D_3 depending on the pigmentation level. Significant color changes in UHT milk packed in PET bottles have also been reported, 1.6% fat milk showing less change compared with 3.2% fat milk.

Four different types of systems are in use for bottles: non-sterile bottles; sterile blown bottles; sterilization of preformed containers; and single station blowing, filling, and sealing.

Non-sterile bottles

After blowing (either immediately prior to the following steps or on a separate site), the plastic bottles are conveyed into a sterile chamber that is kept at a slight overpressure with sterile air. The bottles are inverted and sprayed inside and outside with a solution of H_2O_2 or PAA. The H_2O_2 is evaporated by passing the bottles through a hot-air tunnel prior to filling. Bottles sterilized with PAA are rinsed with sterile water and then filled. A chemically sterilized, heat-sealable closure such as a plastic film or cap is then applied.

Sterile blown bottles

Bottles are extruded, blown with sterile air, and sealed under conditions that ensure internal sterility. The sealed bottles are then introduced into a sterile chamber (maintained under a slight positive pressure) where the outside surfaces are sterilized with H_2O_2 sprays. The closed top of the bottle is cut away, the neck trimmed, and the bottle filled. A foil cap or heat-sealable closure that has been sterilized outside the chamber is applied.

Sterilization of preformed containers

Recently, a dry decontamination system that uses 5–15 mg of H_2O_2 per preform (40 times less than ones involving bottle rinsing) was commercialized; no water is needed for the process. The method works by first transferring preforms by the neck on a wheel from the in-feed to the oven entrance. Nozzles, calibrated to between 120 and 140 °C, apply the H_2O_2 vapor, which condenses evenly on the internal walls of the performs; these are then heated in the oven to 100 °C.

Single station blowing, filling, and sealing

In this mechanically complex system, the separate operations of parison (the extrusion or injected molded tube from which the molded containers are blown) extrusion, blow molding, bottle filling, and sealing all take place in sequence in a single mold. Sterility of the inside surface of the containers is ensured by the high temperature of the plastic material during extrusion of the parison, and the use of sterile air for blowing. The thermoplastics used for this type of container include HDPE and PP. After filling, the tube projecting from the bottle mold is vacuum-formed or sealed with jaws into a cap, which closes the bottle. No special arrangements to ensure sterility are required since the filling and sealing are carried out within the closed mold.

Sachet and Pouch Systems

Lay-flat tubing

This system uses a blown film polymer in the form of lay-flat tubing so that only a transverse seal is required to form the bag. The assumption is made that the inside of the tubing is sterile due to the temperature achieved during the extrusion process. Either a single film or a coextrusion can be used. The tubing is fed from the reel into a sterile chamber in which an overpressure of air is maintained. The sachets are sealed at the bottom, cut, and moved to a filling section. After filling, they are sealed at the top and leave the chamber through a water seal.

Form-fill-seal pouches

In this system, a vertical form-fill-seal machine operates in a sterile chamber. The packaging material is passed through 35% H_2O_2 and then drained, dried, and irradiated with UV light. The film used is typically a laminate of linear low-density polyethylene (LLDPE) with a center layer of ethylene vinyl alcohol copolymer (EVOH) and carbon black to give the pouch the required shelf life. Typically, the pouch has fin seals on all four sides.

Recently, a new chalk-filled, multilayer plastic pouch material was commercialized. From outside inward, it consists of a PP layer; a 40% w/w calcium carbonate-filled PP layer to provide stiffness and integrity; EVOH to protect against oxygen; a carbon black layer to protect against light; and LLDPE as a sealing and food contact layer (**Figure 3**). The material is presterilized using electron beams. Compared with traditional aseptic packaging concepts, this system is claimed to offer lower environmental impact in terms of energy consumption, waste generation, and emissions to air and water.

Bag-in-box system

An aseptic bag-in-box system for packaging UHT dairy products consists of a filler that provides a sterile, enclosed product transfer path from process to package utilizing a unique steam-sterilized double-membrane technology. The inner membrane, heat-sealed under

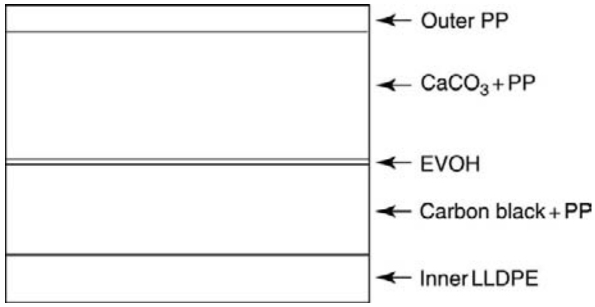

Figure 3 Structure of chalk-filled plastic material for aseptic pouch.

completely secure conditions during the fill process, remains sealed until the pack is opened by the consumer, thus eliminating any risk of product contamination during storage and transport. Bags range in size from 1.5 to 1400 l and contain EVOH and metalized PET (mPET) as barrier layers (Figure 4). Bags are manufactured fully sealed with minimum air content and then sterilized using gamma radiation. During filling, the transfer area is fully sterilized before the first membrane is pierced to ensure a completely aseptic transfer of product. Sterilization is accomplished using only steam, eliminating the need for chemical sterilants, and the steam inlet is specially designed to safeguard the quality of the product. The double membrane on the bags also ensures complete tamper evidence both before and after filling.

Cup Systems

Pre-formed plastic cups

The cups are usually made from high-impact polystyrene (HIPS), PP, or coextruded, multilayered material if improved barrier properties are required. The cups are fed onto a conveyor that is inside a sterile tunnel supplied with sterile air. The cups are sprayed inside with 35% H_2O_2, and after about 3 s, the solution is removed with compressed hot air at a maximum temperature of 400 °C, depending on the material from which the cups are made. The inside surface of the cups reaches a temperature of about 70 °C, which completes surface sterilization and reduces the peroxide residue to an acceptable level.

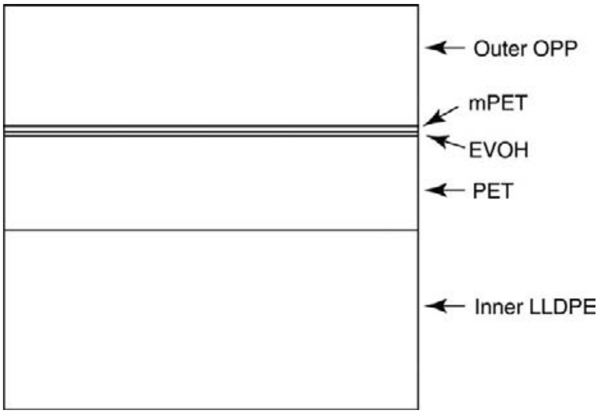

Figure 4 Structure of bag used for aseptic bag-in-box system for packaging ultra-high temperature (UHT) dairy products.

Cups can also be sterilized by carrying them through a 35% H_2O_2 bath at 85–90 °C before heating and passing through a water bath. The cups then enter a sterile chamber where sterilization is completed by spraying with sterile water and drying with hot air.

The sealing material (usually aluminum foil with a thin coating of a thermoplastic material to provide sealability) is typically sterilized with 35% H_2O_2, which is then removed by either radiant heat or hot sterile air or by passing the material over a heated roller. In some systems, UV radiation is used, either alone or in conjunction with peroxide.

Form-fill-seal cups

The plastic material (commonly PS because it is easily thermoformed) in the form of a web is fed from a roll into a thermoformer to give multiple containers, still in web form. More complex coextruded multilayer materials incorporating a barrier layer can also be treated in this way. If an aluminum foil layer is incorporated into the laminate, mechanical forming, rather than thermoforming, is used.

Sterilization of the web is carried out prior to forming by passing it through a 35% H_2O_2 bath at room temperature, typical residence times being in the order of 15 s. Air knives remove surplus liquid prior to the web passing through a sterile tunnel where it is heated to 130–150 °C to prepare it for thermoforming. Alternatively, radiant heat can be used to heat the web after it has left the peroxide bath. The containers are then formed (usually by a combination of mechanical forming and compressed air) into a water-cooled mold below the web. Sterilization of the lidding material is achieved in a similar manner to the container web. An alternative type of form-fill-seal system sterilizes the containers after they have been formed, using saturated steam under pressure at 3–6 atm (135–165 °C) for about 1.5 s; the lidding material is sterilized in a similar manner.

Integrity Testing of Aseptic Packages

Assessment of package integrity is one of the most critical issues in the aseptic packaging of foods. Several performance tests are in use to assess the likelihood of an aseptic package maintaining its integrity during distribution and handling. Traditionally, package and seal integrity has been verified using destructive methods such as a biotest, electrolytic test, dye penetration, or bubble test. However, destructive test methods are often laborious and time-consuming to perform, and it is clearly not possible to test and reject all defective packages.

There is growing interest in non-destructive (or non-invasive) package integrity testing that allows the online testing of every package produced while leaving both product and package intact. Such tests need to meet three criteria: non-specificity, high sensitivity, and rapidity. Most non-destructive leak inspection systems are based on a stimulus–response technique, stimuli to the package including pressure, a trace gas such as carbon dioxide or helium, or ultrasound. The package response can be package movement, pressure change, trace gas detection, and sound attenuation. Recently, a cyclic voltametric method was developed to identify pinholes arising from cracks in the inner plastic layers of cartons. An online pressure differential leak detector has also been shown to be effective in assessing the quality of seals in cartons.

Other non-destructive tests involving computer-aided video inspection or automatic profiling of the packages have been developed and are being improved all the time. The problem with profile scanners and aseptic packages is that even if the package leaks and air enters, the package profile does not change immediately, making profile scanning ineffective.

Despite considerable research in recent years, the availability of commercially viable non-destructive package integrity testing equipment is still very limited. Research is in progress, and new possibilities may be commercialized in future years.

See also: **Heat Treatment of Milk:** Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Heating Systems. **Liquid Milk Products**: Liquid Milk Products: UHT Sterilized Milks. **Packaging**.

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Sterilization of Milk and Other Products

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Introduction

Continuous sterilization, in-container sterilization in autoclaves, and ultra-high temperature treatment (UHT) are used to sterilize milk and milk products to prevent microbial spoilage during storage. In addition, a more or less complete inactivation of enzymes, as well as physicochemical changes of the constituents, occurs during sterilization, depending on temperature and treatment time. To retain as many of the sensory and nutritive properties of the raw material as possible, heating conditions can be selected by using kinetic data. Process conditions for continuous and in-container sterilization can be optimized by applying mathematical formulae that quantify the phenomena occurring during sterilization.

Principle of Sterilization

The objective of sterilization is to produce a long-life product by destruction of microorganisms capable of causing spoilage and also to destroy microorganisms detrimental to public health. At the same time, one has to realize that the term 'sterilization' does not ensure that the product is sterile in the strict microbiological sense because absolute sterility cannot be obtained by common heat treatment if statistics are taken into consideration. Concerning the health aspects, the current policy of the US Food and Drug Administration (FDA) requires temperature–time combinations that ensure a reduction of *Clostridium botulinum* spores by 12 log (12 D_ϑ reduction). However, to obtain commercial long-life, stable milk and milk products, the thermal treatment conditions required to deliver a 12 log reduction of *C. botulinum* have to be extended depending on the product; for example, for sterile milk the conditions are based on the reduction of thermophilic spores by 9 log.

The methods for the sterilization of milk products include continuous sterilization with aseptic filling and in-container sterilization in a temperature range from 105 to 120 °C, with an effective treatment time of 10–40 min, higher temperatures being associated with shorter times. Sterile products are also obtained by applying UHT at 135–150 °C with corresponding times from about 2 to 20 s (**Figure 2**). Application of extreme high-temperature treatment with very short treatment times using a newly developed innovative steam injection (ISI) system, a direct heating system enabling fast heating (<0.2 s holding time) at high temperatures (150–180 °C), has been reported recently.

Under sterilization conditions, most of the enzymes in raw milk are inactivated. If, however, the raw milk has undergone a prolonged storage at low temperatures that allow psychrotrophic bacteria to proliferate, then there might be problems in maintaining quality. Although psychrotrophic bacteria themselves are destroyed at a relatively low temperature, they may produce extremely heat-resistant lipases and proteinases that endure the heating process. For reasons that will be discussed later, higher or lower losses of vitamins and chemical changes occur depending on the sterilization conditions applied.

Kinetics

Sterilization processes, characterized by the heating temperature and the holding time, are responsible for reactions taking place in milk and milk products. To evaluate the effectiveness of a sterilization process regarding destruction of microorganisms and to quantify the chemical changes, the time, temperature, and concentration of the target components need to be considered. However, it is only rarely possible to follow the chemical events at a molecular level. Empirically obtained data are used to describe the kinetics of changes occurring during the process, but this is not molecular kinetics (which would give a better understanding of the mechanism of reactions).

The decomposition of a substance after a certain reaction time at a constant temperature is described by

$$\frac{\mathrm{d}C}{\mathrm{d}t} = -k_T \cdot C^n \qquad [1]$$

which has been found generally valid for decomposition reactions of the *n*th order. The rate of reaction k_T multiplied by the present concentration C to the power of the order of the reaction is proportional to the change of the concentration dC during the time dt. k_T depends mainly on the temperature T, the environmental conditions, and, occasionally, on any catalysts present. The order of reaction is an empirically determined value and may be a noninteger, or even zero, because it does not depend on the effect of intermediate products, nor on parallel or subsequent reactions.

Integration of eqn [1] leads, for a decomposition reaction with an order of $n=1$, to

$$C_t = C_0 \cdot \exp(-k_T \cdot t) \qquad [2]$$

and for $n \neq 1$, to

$$C_t = C_0 \left[1 + (n-1) \cdot k_T \cdot t \cdot C_0^{(n-1)}\right]^{1/(1-n)} \qquad [3]$$

Chemical reactions may also lead to the formation of new substances, C_F, which may be attributed to the decomposition of the substance C_0. It follows an order of reaction of $n=0$, which is often found for chemical reactions in milk and milk products shown in **Table 3** according to eqn [4]:

$$C_{Ft} = C_0 \cdot k_T \cdot t + C_{F0} \qquad [4]$$

The rate constant k_T of a reaction strongly depends on temperature. The effect of the absolute temperature T on the rate constant, k_T, is given by the Arrhenius equation:

$$k_T = k_{\mathrm{ref}} \cdot \exp\left[-\frac{E_A}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{\mathrm{ref}}}\right)\right] \qquad [5]$$

where k_{ref} represents the rate constant at the reference temperature T_{ref} and E_A is the activation energy (J mol^{-1}). $R = 8.314$ J, mol^{-1} K^{-1} is the universal gas constant. For a comparison or for the calculation of thermally induced changes like inactivation of microorganisms and enzymes or chemical changes, the relationship given in eqn [5] is inserted in eqns [1]–[4] depending on the kind and order of reaction. In principle, no other parameters than those given in **Tables 1–3** and the equations are needed, but in practice additional parameters like D_ϑ value, z_ϑ value, and Q_{10} are often used, which are related to the kinetics presented above.

The D_ϑ value is defined as the time necessary at a specific temperature ϑ to reduce the population of microorganisms to one-tenth of the original value. Only for an order of $n=1$ the D_ϑ value can be calculated from the reaction rate constant at the same temperature

$$D_\vartheta = \frac{2.303}{k_T} \quad \text{with} \quad T(\mathrm{K}) = 273\mathrm{K} + \vartheta\,(^\circ\mathrm{C}) \qquad [6]$$

where ϑ is the temperature in degree Celsius.

The F value is based on the D_ϑ value and is used for comparing heat sterilization procedures. The F value represents the time in minutes at a specific temperature needed to reduce the numbers of a specific microorganism in the product by a multiple of the D_ϑ value

$$F = y \cdot D_\vartheta \qquad [7]$$

The reference value F_0 is used to describe the destruction of microorganisms at a temperature of 121 °C. For example, F_0 value is 2.5 min for a log 12 $D_{121\,^\circ\mathrm{C}}$ reduction of *C. botulinum*, that is, the time of 2.5 min is required for a 12 log inactivation of *C. botulinum* with a $D_{121\,^\circ\mathrm{C}}$ value in milk of 0.21 min (**Table 1**). In a narrow temperature range, the z_ϑ value represents the increase in temperature necessary to obtain the same effect of destruction or reaction in one-tenth of the time

$$z_\vartheta \cong \frac{2.303 \cdot R \cdot T^2}{E_A} \quad \text{with} \quad T(\mathrm{K}) = 273\mathrm{K} + \vartheta\,(^\circ\mathrm{C}) \qquad [8]$$

Finally, the Q_{10} value is sometimes mentioned in the literature, and it also describes a dependence of the reaction on temperature. It expresses how much faster a reaction runs when the temperature is increased by 10 °C.

$$Q_{10} \cong \exp\left(\frac{10 \cdot E_A}{R \cdot T^2}\right) \cong \exp\left(\frac{23.03}{z_\vartheta}\right) \qquad [9]$$

The classical concept of D and z values, developed by Bigelow, Ball, and Stumbo, is widely accepted and practiced. However, it is unable to deal with the nonlinear behavior of survival curves during heat treatment. Most microbial survival curves do not obey first-order kinetics and exhibit a nonlinear behavior. Survial curves having a downward concavity (presence of a shoulder) and an upward concavity (presence of a tail) are frequently observed. For the observed phenomena, the physical interaction of the population to be inactivated, such as aggregation, adsorption to the walls of the vessel, and protection against inactivation processes (mechanistic approach) have been reported as possible explanations. Others suggest (vitalistic approach) a heterogeneous heat stability of the population. Commonly observed types of survival curves (linear, linear with tailing, sigmoidal-like, linear with a preceding shoulder, biphasic, concave, biphasic with a shoulder, and convex) are shown in **Figure 1**. To model these linear and nonlinear survival curves several approaches have been proposed. A detailed analysis of different modeling approachs using a range of experimental data for different species was conducted, and the limitations of each approach were discussed by Geeraerd coworkers.

Optimization of Processing

The heat treatment conditions, in terms of temperature and time, can be optimized, ensuring a sufficient destruction of pathogenic and spoilage microorganisms and, at the same time, limiting the heat load on the product, thus avoiding undesirable chemical changes. In addition, the following procedure makes it possible to compare heating processes or plants by their temperature–time profile as

Table 1 Summary of reported temperature-dependent kinetic data for inactivation of highly thermal resistant spore-forming bacteria

Bacteria	*Medium*	*Temperature, ϑ_{ref} (°C)*	D_{ref} *value (min)*	z_{ref} *value (K)*	*Absolute temperature,* T_{ref^*} *(K)*	*Order* (n)	k_{ref^*} *(s^{-1})*	E_A *(kJ mol^{-1})*
Single strains								
Bacillus anthracis	Milk	112	0.027–0.056	8.7–11.0	385	1	0.69–1.42	227–291
Bacillus cereus	Milk	121	0.04	9.4–9.7	394	1	9.6e−1	306–316
Bacillus cereus	Buffer	103	0.12	8.5	376	1	0.32	335
Bacillus licheniformis	Skim milk	111	0.48	8	394	1	7.90e−2	350
Bacillus licheniformis	Evaporated skim milk 40% TS[a]	111	0.84	7.88	384	1	4.56e−2	355
Bacillus licheniformis	Coffee cream (15% fat)	111	0.46	7.31	384	1	8.18e−2	379
Bacillus subtilis	Milk	111	0.5	10.7	384	1	7.7e−2	264
Bacillus subtilis	Buffer	112	0.14	9.0	385	1	0.27	
Bacillus coagulans	Skim milk	121	1.58	8.52	394	1	2.42e−2	339
Bacillus coagulans	Evaporated skim milk 40% TS	118	1.85	9.32	391	1	2.07e−2	313
Bacillus flexus	Buffer	121	0.07	16	394	1	0.55	180
Bacillus pumilus	Milk	118	1.4	9.7	391	1	2.7e−2	302
Geobacillus stearothermophilus, spore	Buffer	135	0.07	8.1	408	1	0.55	377
Geobacillus stearothermophilus, spore	Milk	120	4.1	7.54	393	1	9.37e−3	390
Geobacillus stearothermophilus, spore	Evaporated skim milk 40% TS	120	2.6	7.89	393	1	1.49e−2	372
Geobacillus stearothermophilus, spore	Coffee cream (15% fat)	120	4.25	7.78	393	1	9.02e−3	374
Bacillus sporothermodurans J16	Skim milk	121	2.68	13.1	394	1	1.40e−2	242
Bacillus sporothermodurans B93-20-12	Skim milk	121	3.47	14.2	394	1	1.10e−2	224
Bacillus sporothermodurans MB921	Skim milk	121	2.03	13.2	394	1	1.19e−2	240
Clostridium sporogenes	Milk (pH 7.0)	121	1.7	-	-	1	2.3e−2	-
Clostridium botulinum	Milk (pH 7.0)	121	0.21	10	394	1	0.18	297
Clostridium tyrobutyricum	Skim milk	105	0.483	9.4	378	1	7.90e−2	291

Natural mixed flora including spores								
Mesophilic mixed flora	Milk	121	0.18	10.4	394	1	0.21	286
Mesophilic mixed flora	Skim milk	121	0.35	12.0	394	1	0.11	248
Mesophilic mixed flora	Evaporated skim milk 40% TS	121	0.44	12.0	394	1	8.7e−2	248
Thermophilic mixed flora	Milk	121	0.42	10.4	394	1	9.1e−2	286
Thermophilic mixed flora	Skim milk	121	0.14–0.35	12.0	394	1	0.11	248
Thermophilic mixed flora	Evaporated skim milk 40% TS	121	0.12–0.19	12.0	394	1	0.20	248

[a] TS, total solids. Data calculated and adapted from Horak FP (1980) Reaction Kinetics of Spore Inactivation and Chemical Change during Thermal Treatment of Milk for Optimisation of Heating Processes (Über die Reaktionskinetik der Sporenabtötung und chemischer Veränderungen bei der thermischen Haltbarmachung von Milch zur Optimierung von Erhitzungsverfahren). PhD Thesis, Technische Universität München, Germany (in German); Behringer R and Kessler HG (1991) Heat resistance of spores in skim milk and skim milk concentrates and determination of reaction kinetic parameters. *Milchwissenschaft* 46: 488–492; Condon S, Bayarte M, and Sala FJ (1992) Influence of the sporulation temperature upon the heat resistance of *Bacillus subtilis*. *The Journal of Applied Bacteriology* 73: 251–256; Wescott GG, Fairchild TM, and Foegeding PM (1995) *Bacillus cereus* and *Bacillus stearothermophilus* spore inactivation in batch and continuous flow systems. *Journal of Food Science* 60: 446–450; Kessler HG (1996) *Lebensmittel- und Bioverfahrenstechnik Molkereitechnologie*. Munich, Germany: Verlag A. Kessler; Huemer IA, Klijn N, Vogelsang HWJ, and Langeveld PLM (1998) Thermal death kinetics of spores of *Bacillus sporothermodurans* isolated from UHT milk. *International Dairy Journal* 8: 851–855; Walstra P, Geurts TJ, Noomen A, Jellema A, and van Boekel MAJS (1999) Dairy Technology. New York: Marcel Dekker; Schreiber R (2000) Functional Properties and Rennet Gel Formation of High Heated Casein Micelle Suspensions – Modelling of Process Parameter for Safe Cheese Production (Funktionalität und Labgelbildung hocherhitzter Caseinmicellsuspensionen – Modellierung von Prozessparametern zur sicheren Käseherstellung). PhD Thesis, Technische Universität München, Germany (in German, with English abstract); Xu S, Labuza TP, and Diez-Gonzalez F (2006) Thermal inactivation of *Bacillus anthracis* spores in cow's milk. *Applied and Environmental Microbiology* 72: 4479–4483; Dogan Z, Weidendorfer K, Müller-Merbach M, Lembke F, and Hinrichs J (2009) Inactivation kinetics of *Bacillus* spores in batch- and continuous-heating systems. *LWT – Food Science and Technology* 42: 81–86.

Table 2 Summary of the reported temperature-dependent kinetic data for the inactivation of indigenous and bacterial enzymes

Enzymes (EC number)	*Medium (C_0)*	*Temperature, ϑ_{ref} (°C)*	D_{ref} *value (min)*	z_{ref} *value (K)*	*Absolute temperature, T_{ref^*} (K)*	*Order (n)*	k_{ref^*} $(C_0^{(1-n)}s^{-1})$	E_A *(kJ mol^{-1})*
Indigenous enzymes								
Alkaline phosphatase (3.1.3.1)	Skim milk (100%)				∞	1.3	2.33e + 67	449
Alkaline phosphatase (3.1.3.1)	Milk	70	0.55	5.6	343	1	6.9e−2	402
Lactoperoxidase (1.11.1.7)	Skim milk				∞	1.6	8.79e + 101	691
Lactoperoxidase (1.11.1.7)	Milk	70	0.33	4.2	343	1	0.12	536
Plasmin (70–91.8 °C)	Skim milk	80	7.99e − 22		353	1	1.72e + 23	169
Plasmin (91.8–140 °C)	Skim milk	120	0.0001		393	1	9.85e + 5	48
Acid phosphatase (3.1.3.2)	Milk	100	0.75	9.8	373	1	5.1e−2	272
Bacterial enzymes[a]								
Lipase, *Pseudomonas fluorescens*	Milk	130	8.33	(88)	403	1	4.6e−3	(35)
Lipase, *Pseudomonas fluorescens* B52	Buffer, pH 7.2	130	1.52	36	403	1	2.5e−2	81.7
Lipase, *Pseudomonas* spp.	Milk	130	11.7	26	403	1	3.3e−3	120
Protease, *Pseudomonas fluorescens*	Milk	130	10.5	31	403	1	3.6e−3	100
Protease, *Pseudomonas fluorescens* NCDO 2085	Milk whey, pH 7.2	130	1.79	31	403	1	2.1e−2	93.5
Protease, *Achromobacter*	Milk	130	8.5	31	403	1	4.5e−3	100

[a] The values vary widely among the strains and depending on the conditions during growth.

Data calculated and adapted from Horak FP (1980) Reaction Kinetics of Spore Inactivation and Chemical Change during Thermal Treatment of Milk for Optimisation of Heating Processes (Über die Reaktionskinetik der Sporenabtötung und chemischer Veränderungen bei der thermischen Haltbarmachung von Milch zur Optimierung von Erhitzungsverfahren). PhD Thesis, Technische Universität München, Germany (in German); Meyer MJ (1996) Comparative Study of the Heat Stability of Milk Enzymes, ß-Lactoglobulin, and Vitamins in Milk and Ultrafiltration Retentates (Vergleichende Untersuchung zur Hitzestabilität von originären Enzymen, ß-Lactoglobulin und Vitaminen in Milch und daraus hergestellten Ultrafiltrationsretentaten). PhD Thesis, Swiss Federal Institute of Technology Zurich, Switzerland (in German, with English abstract); Walstra P, Geurts TJ, Noomen A, Jellema A, and van Boekel MAJS (1999) *Dairy Technology*. New York: Marcel Dekker; Van Asselt AJ, Sweere APJ, Rollema HS, and De Jong P (2008) Extreme high-temperature treatment of milk with respect to plasmin inactivation. *International Dairy Journal* 18: 531–538. Vercet A, Burgos J, and Lopez-Buesa P (2002) Manothermosonication of heat-resistant lipase and protease from *Pseudomonas fluorescens*: Effect of pH and sonication parameters. *Journal of Dairy Research* 69: 243–254.

Table 3 Summary of reported temperature-dependent kinetic data for chemical reactions induced during high-temperature treatment

Other milk constituents	*Medium (C_0)*	*Absolute temperature, T_{ref^*} (K)*	*Order (n)*	*k_{ref^*} ($C_0^{(1-n)}s^{-1}$)*	*E_A (kJ mol^{-1})*
Protein denaturation					
α-Lactalbumin (70–80 °C)	Skim milk ($\sim$1 g l^{-1})	∞	1	7.59e + 36	269
α-Lactalbumin (85–150 °C)	Skim milk ($\sim$1 g l^{-1})	∞	1	4.62e + 8	69
β-Lactoglobulin A (70–90 °C)	Skim milk ($\sim$3 g l^{-1})	∞	1.5	8.79e + 101	691
β-Lactoglobulin A (95–150 °C)	Skim milk ($\sim$3 g l^{-1})	-	1.5	1.81e + 6	54
β-Lactoglobulin B (70–90 °C)	Skim milk ($\sim$1.6 g l^{-1})	∞	1.5	1.84e + 28	207
β-Lactoglobulin B (95–150 °C)	Skim milk ($\sim$1.6 g l^{-1})	-	1.5	3.15e + 5	48
Other chemical reactions					
Loss of thiamine (B_1)	Milk ($\sim$0.4 mg l^{-1})	∞	2	8.57e + 9	101
Loss of lysine	Milk	∞	2	1.95e + 10	109
Formation of hydroxymethylfurfural	Milk (μmol l^{-1})	∞	0	1.62e + 17	139
Formation of lactulose	Milk (mol l^{-1})	∞	0	1.06e + 8	100
Browning	Milk	-	-	-	107

Data calculated and adopted from Horak FP (1980) Reaction Kinetics of Spore Inactivation and Chemical Change during Thermal Treatment of Milk for Optimisation of Heating Processes (Über die Reaktionskinetik der Sporenabtötung und chemischer Veränderungen bei der thermischen Haltbarmachung von Milch zur Optimierung von Erhitzungsverfahren). PhD Thesis, Technische Universität München, Germany (in German); Fink R (1984) Storage Depending Changes of UHT Milk and Reaction Kinetics (Über lagerungsbedingte Veränderung von UHT-Vollmilch und deren reaktionskinetische Beschreibung). PhD Thesis, Technische Universität München, Germany (in German); Dannenberg F and Kessler HG (1988) Reaction kinetics of the denaturation of whey proteins in milk. *Journal of Food Science* 53: 258–263; IDF (1996) *Heat Treatments and Alternative Methods*. Brussels: IDF; Meyer MJ (1996) Comparative Study of the Heat Stability of Milk Enzymes, ß-Lactoglobulin, and Vitamins in Milk and Ultrafiltration Retentates (Vergleichende Untersuchung zur Hitzestabilität von originären Enzymen, ß-Lactoglobulin und Vitaminen in Milch und daraus hergestellten Ultrafiltrationsretentaten). PhD Thesis, Swiss Federal Institute of Technology Zurich, Switzerland (in German, with English abstract); De Rafael D, Villamiel M, and Olano A (1997) Formation of lactose and furosine during heat treatment of milk at temperatures of 100–120 °C. *Milchwissenschaft* 52: 76–78.

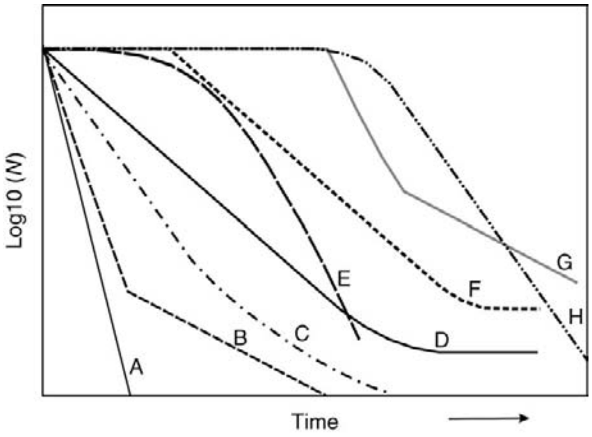

Figure 1 Commonly observed types of survival curves: linear (A), linear with tailing (D), sigmoidal-like (F), linear with a preceding shoulder (H), biphasic (B), concave (C), biphasic with a shoulder (G), and convex (E).

applied to milk or milk products during treatment. Depending on the microorganisms responsible for limiting the shelf life and important chemical (e.g., loss of vitamins) or textural changes (e.g., in a milk product like sterilized milk rice), and other special demands or standards, lines of equal effects can be plotted using the kinetic parameters (**Tables 1–3**). In milk, pathogenic bacteria (spores and vegetative cells) are already inactivated at about 100 °C. To obtain commercially sterile milk and milk products, the thermal treatment conditions have to be extended so that spoilage bacteria and their spores can be destroyed; for example, for sterile milk, the conditions are based on the reduction of the thermophilic mixed flora by 9 log. To obtain this effect of inactivation, which is set as a dimensionless value of $B^* = 1$, the necessary treatment time at a certain temperature T can be calculated by inserting eqn [5] in eqn [2]

$$t = \frac{\ln\left(\frac{C_0}{C_t}\right)}{k_{\text{ref}} \cdot \exp\left[-\frac{E_A}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}}\right)\right]} \qquad [10]$$

and using the parameters given in **Table 1** for the thermophilic mixed flora in milk.

Furthermore, other changes such as the loss of thiamine (3% loss is defined as $C^* = 1$), inactivation of pathogens (e.g., *Bacillus cereus*, 3 log), and inactivation of heat-resistant lipase and protease from *Pseudomonas*

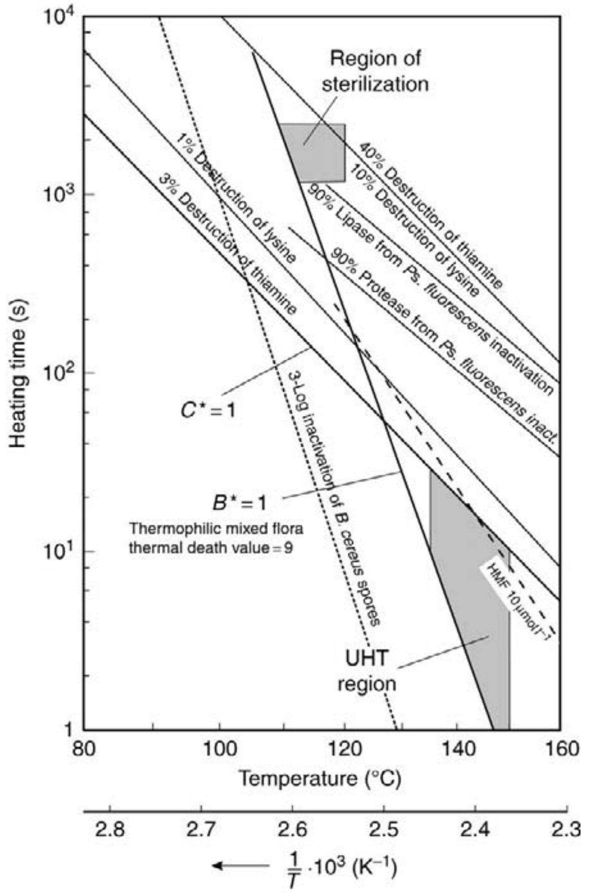

Figure 2 Lines of equal effects for the inactivation of the thermophilic mixed flora and chemical reaction in milk, and regions for sterilization and UHT treatment (enzymes from *Pseudomonas*). HMF, hydroxymethylfurfural. Adapted with permission from Kessler HG (1996) *Lebensmittel- und Bioverfahrenstechnik Molkereitechnologie*. Munich, Germany: Verlag A. Kessler.

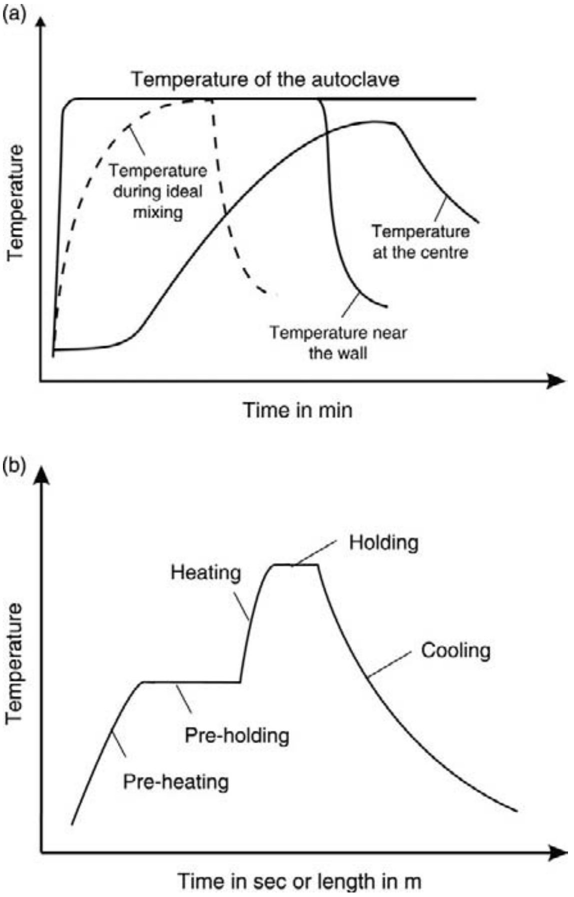
(a)
Temperature
Temperature of the autoclave
Temperature during ideal mixing
Temperature at the centre
Temperature near the wall
Time in min
(b)
Temperature
Holding
Heating
Cooling
Pre-holding
Pre-heating
Time in sec or length in m

Figure 3 Temperature–time pattern for (a) in-container sterilization of a mixable (dotted line) and a nonmixable product (solid line) and (b) continuous sterilization in heat exchangers.

fluorescens (1 log or 90%) in the system can be estimated by the kinetic parameters given in **Tables 1–3** and the related kinetic equations depending on the order of the reaction. **Figure 2** shows several lines of equal effects in a temperature–time graph. Temperature–time combinations above the B^* line ensure commercially sterile milk. However, the same antibacterial effect B^* attained by treatment in the UHT region causes smaller losses of thiamine (vitamin B_1), lysine, and other valuable constituents than the losses caused by treatment in the sterilization region. For heat-resistant spores (HRS), for example, *B. sporothermodurans B93-20-12* (see **Table 1**), the temperature–time combination given by the $B^* = 1$ line has to be extended according to the desired log reduction of spores.

In summary, heating methods for milk and milk products are characterized by a certain holding time at a defined heating temperature, as described above. However, the temperature pattern of the heating stage and that of the cooling stage can be quite different (**Figure 3**).

The influence of the heating method and type of equipment on the antimicrobial effect and the loss of nutrients can be estimated by integration of eqn [1]:

$$C_t = \int_0^t -k_T\langle t\rangle \cdot C^n\langle t\rangle \cdot \mathrm{d}t \qquad [11]$$

In most cases, an analytical solution of the integral is not possible because the reaction rate and the present concentration are related to the temperature–time pattern. However, by dividing the pattern into m small intervals Δt_i at a constant temperature T_i, eqn [11] can be integrated numerically and the inactivation effect of the heating and the cooling stages can be taken into account:

$$C_t = C_m = C_0 - \sum_{i=1}^{m} k_{T,i} \cdot C_i^n \cdot \Delta t_i \qquad [12]$$

Utilizing the Arrhenius equation in eqn [12] leads to

$$C_m = C_0 - k_{\text{ref}}\cdot\sum_{i=0}^{m}\exp\left(-\frac{E_{\text{A}}}{R}\left(\frac{1}{T_i}-\frac{1}{T_{\text{ref}}}\right)\right)\cdot C_i^n\cdot\Delta t_i \qquad [13]$$

Using the measured parameters C_m and C_0, and the recorded temperature–time profile in eqn [13], the solution of the corresponding inactivation experiment gives numerous possible combinations of k_{ref} and E_{A} on a curve. Calculated curves obtained from several inactivation experiments intersect in a small region, which includes the solution. The parameters (k_{ref}, E_{A}) for the best fit for all inactivation experiments are calculated by iteration, minimizing the sum of the squares of errors in the logarithmic concentration of each curve.

For in-container sterilization (**Figure 3a**) of liquid milk products and for continuous-flow sterilization in a heat exchanger (**Figure 3b**), assuming ideal mixing of the contents, eqn [12] allows the determination of both the reduction in the number of microorganisms and the chemical changes from the temperature–time pattern. If the content is not, or hardly, mixable during in-container treatment, the temperature at the center has to be used for the estimation of the reduction of spoilage bacteria population (**Figure 3a**). In addition, it is anticipated that rather than the temperature at the center, it is the temperature near the wall that induces the maximum amount of chemical changes in the product.

Processes and Equipment

For more than 100 years, in-container sterilization of milk and condensed milk, according to the early work of Appert, has been applied to produce long-life stable products. Meanwhile, the range of sterilized milk products has been extended, for example, to infant formulae, coffee cream, milk mix drinks, ice cream mix, milk desserts, and also milk rice or soft cheese in metal cans. In addition, developments in aseptic filling and packaging combined with continuous-flow heating to high temperatures have enabled manufacturers to produce sterile milk and milk products. **Figure 4** demonstrates two variations of condensed milk production. Up to the second step of standardization of the product, that is, adjusting it to the desired final composition, the steps are comparable. Then, in the continuous-flow sterilization process, the evaporated milk is homogenized before and after sterilization. There is an advantage in homogenizing after heating; it prevents or reverses protein–protein and fat globule–protein aggregation. This is not possible for in-container-sterilized products because the product is filled into separate containers before sterilization. A further advantage of the continuous process is that the product can be filled into flexible packages, which are not suitable for in-container sterilization. In the latter, different types of packaging like metal cans, glass jars or bottles, and plastic bottles are used.

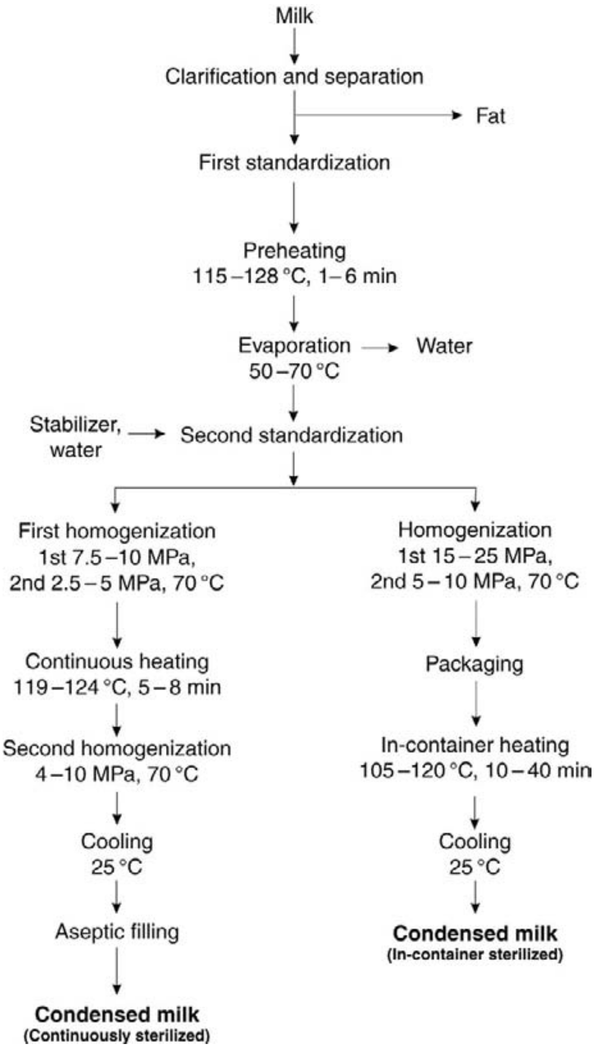
Milk
Clarification and separation
Fat
First standardization
Preheating
115–128 °C, 1–6 min
Evaporation
Water
50–70 °C
Stabilizer, water
Second standardization
First homogenization
1st 7.5–10 MPa,
2nd 2.5–5 MPa, 70 °C
Homogenization
1st 15–25 MPa,
2nd 5–10 MPa, 70 °C
Continuous heating
119–124 °C, 5–8 min
Packaging
Second homogenization
4–10 MPa, 70 °C
In-container heating
105–120 °C, 10–40 min
Cooling
25 °C
Cooling
25 °C
Aseptic filling
Condensed milk
(In-container sterilized)
Condensed milk
(Continuously sterilized)

Figure 4 Flowchart of continuously sterilized and in-container sterilized unsweetened condensed milk.

Continuous-Flow Sterilization

Two systems are used for continuous-flow sterilization: indirect heating in tubular or plate heat exchangers and direct heating by steam injection or infusion. Plants for continuous-flow sterilization consist of four main sections. In the heating-up section, the product is preheated and often held for a certain time to denature the whey proteins so as to reduce fouling in the heating section and to prolong the operating time of the heating plant. In the heating section, the product is brought up to sterilization temperature via indirect heating or direct heating. The holding time depends on public health aspects and on the requirement to reach commercial sterility (see **Figure 2**). The product is held at the required temperature in a tubular holding section. Heat regeneration with the incoming product or via an auxiliary water circulation in the cooling section allows a heat recovery of about 90%, and, if necessary, final cooling of the product with water. The temperature–time pattern along the heating plant is shown in **Figure 3a**. Homogenization is performed before and after sterilization (**Figure 4**). The first homogenization is applied to reduce the fat globule size to achieve the physical stability of the emulsified fat phase (according to Stokes's law). The second homogenization step separates heat-induced protein–protein and fat globule–protein aggregates, which may also lead to physical instability during storage.

In-Container Sterilization in Batch Autoclaves

Milk or milk products are pre-processed to modify the constituents according to physical and biochemical long-life stability and to special product demands, such as color, flavor, and rheology (**Figure 4**). After filling into containers, the required microbiological effect for long-life stability is obtained by heating in batch autoclaves or continuously operating autoclaves. The heat is transferred to the food by means of hot water or condensing saturated steam via the container wall. Sterilization temperatures above 100 °C are attained by pressures above atmospheric pressure in pressure vessels: the so-called autoclaves. Usually, upright or horizontally mounted cylindrical pressure vessels are used, into which the containers to be sterilized are placed on racks, baskets, or carriages. In batch autoclaves, short heating-up and cooling times are achieved by agitating the content by means of rotation or swinging of the inserts with the containers from side to side (**Figure 5**). After raising the temperature and holding it for the required time, the cooling-down period starts and the contents of the autoclave are cooled by the circulation of water. Typical temperature–time patterns for mixable and nonmixable milk products are shown in **Figure 3**. To save energy in hot water-operated autoclaves, the hot water is pumped into a storage tank situated above the autoclave and reused for the next batch. In practice, the containers are removed from the batch autoclave at a temperature of about 35°C, to enable the moisture on the surface to evaporate so that they are dry for labeling. Batch autoclaves are mainly used for small quantities and in processes where the type of product, container size, and therefore the sterilization procedure have to be changed frequently.

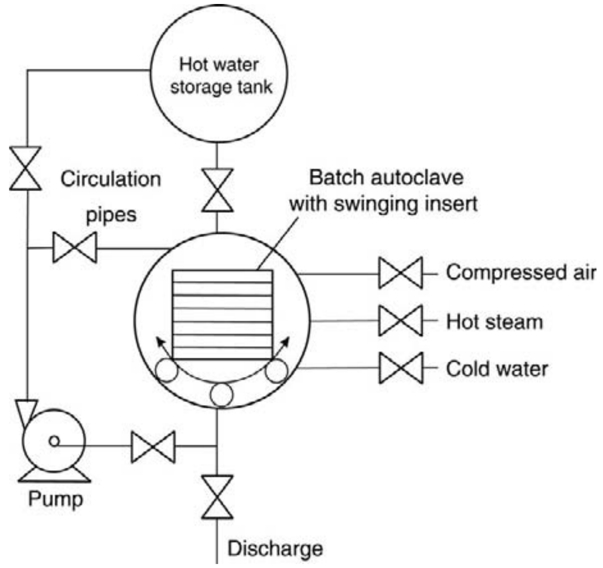

Figure 5 Schematic representation of a batch autoclave with swinging insert. Adapted with permission from Kessler HG (1996) *Lebensmittel- und Bioverfahrenstechnik Molkereitechnologie*. Munich, Germany: Verlag A. Kessler.

In-Container Sterilization in Continuously Operating Autoclaves

For large throughputs, as in the production of sterile milk or condensed milk in bottles or metal cans, continuous systems are used. One type of apparatus is the hydrostatic sterilizer, which uses columns of water to counterbalance the pressure gradient between the inside and the outside of the pressure-sensitive packages during heating up, sterilization, and cooling down. The columns of water act as transit pressure valves for cans and bottles transported in pockets or recesses on continuous conveyor chains through the hydrostatic sterilizer (**Figure 6**).

Another continuously operating autoclave is only suitable for sterilizing products filled in cans (**Figure 7**). In this rotating autoclave, the cans are fed by means of a bucket wheel into a horizontal cylindrical pressure vessel. They are fed in at the circumference at one end and discharged at the other end. Within the vessel, a rotor rotates with continuous radial flanges at its circumference, between which the cans lie in rows. By means of guide bars on the stator, that is, on the inner wall of the vessel, the cans are transported spirally through the vessel and a thorough mixing of the contents of the cans is achieved depending on the consistency of the product. In addition to the system shown in **Figure 7**, there are rotating sterilizers that may also consist of three sections such as a preheater, a pressure vessel for sterilization, and a final cooler section, in which the water in the cooling

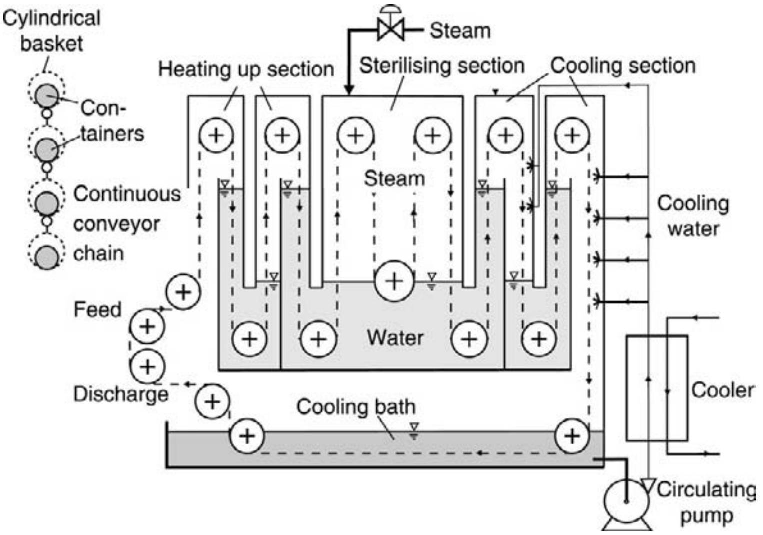
Cylindrical basket
Con-tainers
Continuous conveyor chain
Feed
Discharge
Steam
Heating up section
Sterilising section
Cooling section
Steam
Water
Cooling water
Cooling bath
Cooler
Circulating pump

Figure 6 Schematic representation of a continuously operating hydrostatic sterilizer. Adapted with permission from Kessler HG (1996) *Lebensmittel- und Bioverfahrenstechnik Molkereitechnologie*. Munich, Germany: Verlag A. Kessler.

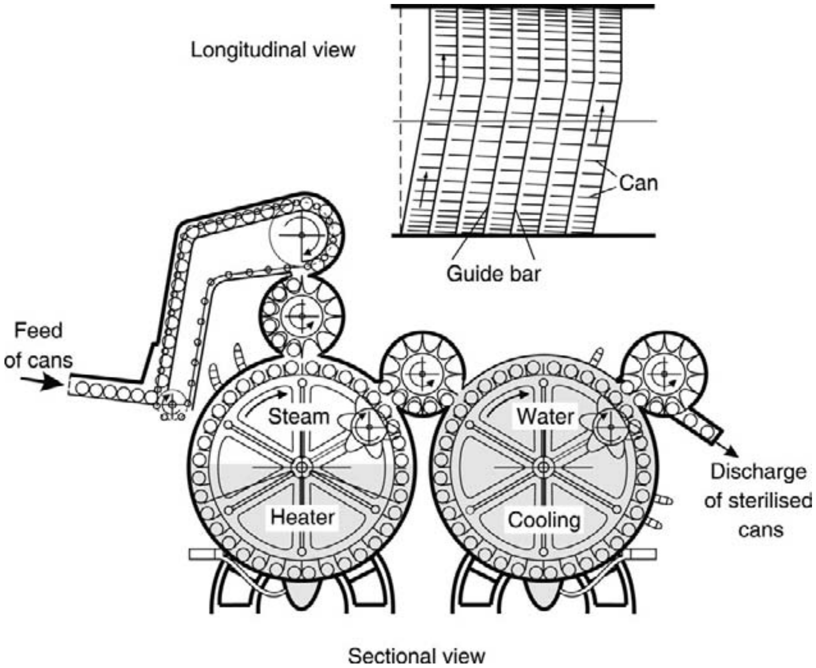
Longitudinal view
Can
Guide bar
Feed of cans
Steam
Heater
Water
Cooling
Discharge of sterilised cans
Sectional view

Figure 7 Schematic presentation of a rotating autoclave. Adapted with permission from Kessler HG (1996) *Lebensmittel- und Bioverfahrenstechnik Molkereitechnologie*. Munich, Germany: Verlag A. Kessler.

section is reused for preheating so as to recover energy. The discharge temperature of the cans is about 35 °C to enable labeling.

See also: **Heat Treatment of Milk**: Heat Stability of Milk; Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems.

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Non-Thermal Technologies: Introduction

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Introduction

Heat treatment has been and continues to be the major method for destroying microorganisms, both pathogenic and spoilage-producing, in milk and dairy products. It has proved to be a reliable method and its introduction for the pasteurization of milk has achieved a high level of safety of milk and derived products. In addition, it has enabled the shelf life of these products to be extended by several days in the case of pasteurization and up to several months in the case of sterilization procedures.

Despite the reliability and proven benefits of heat treatment in destroying microorganisms, it has the disadvantage of causing chemical modifications to milk constituents, which can result in adverse changes to the flavor, color, and nutritive value of milk. With the increasing consumer trend toward 'natural' and minimally processed foods, alternative technologies have been investigated for use in the food industry instead of, or in conjunction with, thermal technologies.

The International Dairy Federation recognized this trend in the 1990s and commissioned the 1996 report 'Alternatives to heat treatment of milk and dairy products'. In that report several technologies of potential application in the dairy industry were identified. Since that time there have been major developments in several of these alternative technologies and other technologies have emerged with potential application in the dairy industry.

Bacterial endospores, particularly those of *Bacillus* and *Bacillus*-like species, are very resistant to heat and only the most severe thermal treatments used in the dairy industry, ultra-high-temperature (UHT) and in-container sterilization, are capable of destroying them. However, even these heat treatments are incapable of inactivating the most heat-resistant spores, those of *Geobacillus thermophilus* and *Bacillus sporothermodurans*, without causing unacceptable chemical damage to the product. Consequently, there has been much interest in exploring the potential of nonthermal technologies to remove or destroy the spores in milk. Unfortunately, at this time, no nonthermal technology, when used alone, is capable of reducing the spore counts in milk sufficiently to produce a shelf-stable product. Microfiltration and centrifugation (Bactofugation$^{®}$) remove a large proportion, up to 6 and 3 logs, respectively, but this falls short of the requirement of a 9-log reduction for a sterile, and hence shelf stable, product. Most of the nonthermal technologies have little or no effect on bacterial spores when used at temperatures around the ambient temperature. However, in most cases, when combined with a heat treatment, before, during, or after the nonthermal treatment, they can inflict some damage on spores. In some cases, this damage is sublethal but renders the spore susceptible to a subsequent heat treatment, enabling a lower temperature to be used to produce a product of desired shelf stability than would be possible with a thermal treatment alone. This approach is expected to find application in dairy processing in the future.

Barriers to Adoption by the Dairy Industry

Although some nonthermal technologies have been in use in food industries for some time, the adoption of others by the dairy industry has been relatively slow. This is despite the huge research effort in the last 20 years. Microfiltration and centrifugation have been widely adopted by the dairy industry to remove bacteria from milk, although they have generally been used as adjuncts to (thermal) pasteurization rather than as stand-alone 'pasteurization' technologies. One advantage of these two technologies is that they remove the bacterial cells from milk, unlike other technologies, including heat treatment, which leave the cells dead, injured, or alive in the milk. This may have quality implications owing to the enzymes associated with the bacterial cells.

There are several reasons why nonthermal technologies have not been widely adopted by the dairy industry:

- Thermal processing is well established and has served the industry very well. Any new technology to replace a thermal process must offer clear advantages in terms of costs and/or product quality over thermal processing before it can be introduced. Alternatively, it must be able to perform additional functions that cannot be performed by heat alone.
- Suitable tests of efficacy equivalent to the alkaline phosphatase test for thermal processing of milk have not been developed. This has led to a reluctance on the part of regulatory authorities to approve the technologies for 'pasteurization' purposes. A related issue is that some potential pathogenic organisms have been shown

to be resistant to certain technologies; for example, some strains of *Escherichia coli* are resistant to high pressure and some *Listeria* strains are resistant to high-intensity pulsed electric fields.

- The difficulty in precisely defining the operating conditions of treatment. A good example is pulsed electric field technology, the efficacy of which in destroying bacteria depends on several factors, including the design of the treatment cell, which are difficult to prescribe in regulations. It also makes it difficult to utilize the results from different research laboratories for scale-up purposes.
- The unavailability of equipment of sufficient capacity for treatment of bulk products. Such technologies include high-pressure processing and high-pressure homogenization.
- The unsuitability of some milk products for certain treatments due to their physical nature. For example, milk is difficult to treat with high-intensity light technologies because of its opacity.
- The production of undesirable flavors. For example, production of oxidized flavors by ionizing radiation and UV light at energy inputs necessary to achieve the desired reduction of pathogens.
- Inability of most technologies to destroy spores, as discussed above. Ironically, those that are able to destroy spores, for example, UV light, high-intensity pulsed light, and cold plasma, are effective on surfaces and in water but are less effective or ineffective in liquid foods such as milk.
- Adverse consumer perceptions, for example, of ionizing irradiation.

Nonthermal Technologies and Their Applications in the Dairy Industry

There are several nonthermal technologies that may have application in the dairy industry. They vary from those that are well established, such as microfiltration, to others that are still in the research phase, such as cold plasma. A list of nonthermal technologies, together with an outline of their effects on milk and their possible uses in the dairy industry, is given in **Table 1**. This list is not exhaustive, but the other technologies are either in the early stages of assessment or unlikely to be applied in the dairy industry in the foreseeable future; bead milling, which successfully disrupts bacterial cells, is an example of the former category, whereas ionizing irradiation is an example of the latter. Another method of milk preservation is the use of chemical additives such as nisin or other bacteriocins, and hydrogen peroxide, which can be added with thiocyanate to activate the natural milk lactoperoxidase system. These are covered in other articles (*see* **Bacteriocins**. **Enzymes Indigenous to Milk**: Lactoperoxidase).

The first three entries in **Table 1**, high-pressure processing, ultrasonication, and pulsed electric field technology, have been chosen for detailed consideration in two separate articles. Short descriptions of the other technologies listed in the table appear below.

As different technologies have different capabilities, combinations of technologies have been proposed to increase their effectiveness. However, with the exception of nonthermal–thermal combinations, for example, microfiltration followed by HTST pasteurization, combinations have found little commercial application, presumably due to the additional cost involved.

High-Pressure Homogenization

High-pressure homogenization (HPH), also called ultra-high-pressure homogenization (UHPH) or dynamic high-pressure processing, differs considerably from conventional homogenization, as it operates at pressures up to 20 times higher (400 MPa). It achieves very efficient homogenization and emulsification, but it also has a destructive effect on bacteria and viruses, causes some inactivation of some enzymes, and induces changes to casein micelles and whey proteins. However, despite being increasingly used in the chemical, pharmaceutical, and biochemical industries, and showing potential for use in the dairy industry, HPH has found little application in dairy processing to date. One reason for this is the relatively small capacity of the equipment available.

Though the name ‘dynamic high-pressure processing’ suggests that it is similar to (static) high-pressure processing, it varies in many respects. The pressures used are lower and, because it is a continuous process, the time during which the product is subjected to high pressure is much less, $\sim 10^{-4}$ s. Besides, the physical effects of HPH are due to shear, cavitation, turbulence, and impact, whereas the effects of static high-pressure processing are largely due to compression.

There are two major types of high-pressure homogenizers: a high-pressure version of the conventional valve homogenizer and the microfluidizer, which operates on completely different principles. Whereas the valve homogenizers are based on the same design principle as that of conventional homogenizers, microfluidizers involve splitting the liquid feed into two streams that are forced to collide with each other at a high velocity (up to $50\,\mathrm{m\,s^{-1}}$) at 180° in an interaction chamber. Pressures commonly achieved in microfluidizers are in the range 100–200 MPa. In both types, the temperature of the treated liquid rises by ~17–23 °C per 100 MPa. This almost instantaneous rise in temperature can be exploited in using HPH for pasteurizing milk and, in some cases, superpasteurizing milk for extended shelf life (ESL). In general, HPH at a

Table 1 Nonthermal technologies and their applications in dairy processing

Technology	*Major effect(s) in milk*[a]	*Application(s) in dairy processing*[b]
High-pressure processing	Disintegration of casein micelles resulting in clarification of skim milk Denaturation of whey proteins Inactivation of some enzymes Solubilization of calcium and phosphate Destruction of spoilage and pathogenic bacteria Improvement in water-holding capacity of milk proteins Enhanced crystallization of milk fat	'Pasteurization' of low-volume, high-value products, for example, functional foods containing heat-sensitive bioactives and/or probiotics 'Pasteurization' of cheese spread Pretreatment of milk for manufacture of, for example, cheese, yogurt, ice cream Acceleration of cheese ripening
Ultrasonication	Homogenization Emulsification Inactivation of enzymes Destruction of spoilage and pathogenic bacteria Modification of functional properties of proteins Improvement of yogurt texture after milk treatment Particle size reduction, for example, in dairy waste treatment Collapsing of milk foams	Homogenization of milk Reduction of fouling Defoaming Tailoring functional properties of milk powders, for example, for improved solubility, heat stability, foaming Reducing heat stability of spores Waste treatment
Pulsed electric field technology	Destruction of spoilage and pathogenic bacteria Inactivation of some enzymes Improvement of cheese curd properties after milk treatment	Liquid milk processing (with heat) to produce 'pasteurized', ESL, long-life milk Pretreatment of milk for cheese manufacture 'Pasteurization' of products containing bioactive components
High-pressure homogenization	Homogenization Emulsification Thermal effects from heat generated Inactivation or activation of enzymes Destruction of spoilage and pathogenic bacteria Modification of functional properties of proteins	Homogenization, with heating, of liquid milk – pasteurized or ESL milk production Pretreatment of milk for manufacture of cheese, yogurt, ice cream Tailoring functional properties of whey proteins, for example, for improved foaming
Microfiltration	Separation of particulate matter from milk – casein micelles, bacteria (including spores), somatic cells, membrane material	Removal of bacteria from milk to extend shelf life, with or without final thermal pasteurization Preparation of micellar casein (native phosphocasein) Removal of lipid material from whey Pretreatment of milk to reduce spore levels in cheese (to prevent 'late blowing') and powders
Centrifugation/ bactofugation	Removal of bacteria, including spores and somatic cells	Pretreatment of milk to reduce spore levels in cheese (to prevent 'late blowing') and powders

(*Continued*)

Table 1 (Continued)

Technology	*Major effect(s) in milk*[a]	*Application(s) in dairy processing*[b]
High-intensity pulsed light	Destruction of bacteria, including spores, on surfaces and in water and low-opacity dairy liquids	Bacterial reduction on packaging material and in whey, brine, and water
Continuous UV light	Destruction of bacteria on surfaces and in low-opacity liquids	Bacterial reduction on packaging material and in whey, brine, and water
Carbon dioxide	Inhibition of growth of bacteria Reduction of rennet use in cheesemaking	Extending the shelf life of raw and pasteurized milks and fresh cheese varieties
	Precipitation of casein at pH ∼5.6	Preparation of casein
Cold plasma	Destruction of bacteria, including spores, on surfaces	Sterilization of packaging material and equipment surfaces Reduction of bacteria in powders

[a] The extent of the effects listed depends on the severity of the treatment and the nature of the target, for example, type of enzyme or bacterium.

[b] Includes commercialized and potential use.

final temperature of $\leq 50\,°C$ inactivates vegetative cells of bacteria nonthermally by up to 5 logs, whereas HPH combined with higher temperatures is capable of producing milk with an ESL. HPH causes little inactivation of bacterial spores (usually ≤ 1 log); however, HPH treatment may render spores more susceptible to subsequent heat treatments and enable sterilized products to be produced at a lower temperature than possible by conventional thermal processing.

HPH efficiently homogenizes the fat in milk by markedly reducing the size of the milk fat globules to $\leq 0.5\,\mu m$. However, this effect is most pronounced at ≤ 200 MPa as treatment at higher pressures tends to cause aggregation of the fat globules to larger particles.

The effect of HPH on the proteins in milk, particularly the whey proteins, is of particular interest as it can markedly affect their functional properties. For example, it improves the solubility and foaming properties of whey protein powders largely through reducing the size of particles. It also increases the water-binding capacity of milk proteins, which can also have favorable effects on yogurt properties.

More comprehensive coverage of high-pressure homogenization and microfluidization appears in the article **Homogenization of Milk**: High-Pressure Homogenizers. Alternative Homogenization Devices.

Microfiltration

Microfiltration, which separates particles from milk using a semipermeable membrane with a pore size of 0.3–1.4 μm, is used commercially to remove bacteria from milk. The treated milk is either marketed as drinking milk or used for cheesemaking.

As milk fat globules are about 1–10 μm, the cream must be removed from milk before it is microfiltered. For production of a whole-milk product, the cream is heat-treated separately at about 120 °C for 4 s to destroy bacteria, including sporeformers, and added back to the microfiltered skim milk. The microfiltration retentate, which contains most of the bacteria and somatic cells, and some large casein micelles, is treated similarly. In some systems, the retentate is fed back into the inlet of the cream separator and the sludge is discharged by the normal desludging procedure. This obviates the need for high-heat treatment of the retentate. To meet the current regulatory requirements, drinking milk produced using the microfiltration process usually undergoes a final HTST pasteurization step. Pasteurized milk produced in this manner has a refrigerated shelf life of 20–32 days, compared with 6–18 days for normal pasteurized milk.

Microfiltration of skim milk using ceramic membranes with a pore size of 1.4 μm reduces the bacterial load by 3–4 logs. However, spores may be reduced by up to 5 logs. Higher reductions of 5–7 logs can be achieved by the use of membranes with an effective pore size of 0.8 μm.

Microfiltration is applied to cheese milk to remove somatic cells and spore-forming bacteria, particularly *Clostridium* species such as *Clostridium tyrobutyricum* and *C. butyricum*. This reduces quality defects associated with somatic cells and the problem of gas production and ‘late blowing’ in cheese. It is also used in the production of micellar casein or native phosphocasein. In this process, skim milk is first microfiltered and then diafiltered to remove the soluble whey proteins, lactose, and minerals.

A more detailed coverage of microfiltration appears in the article **Liquid Milk Products**: Liquid Milk Products: Membrane-Processed Liquid Milk.

Centrifugation (Bactofugation)

Centrifugation, sometimes called ‘bactofugation’ because the commercial equipment manufactured by Tetra Pak is marketed under the trade name *Bactofuge*™, uses centrifugal force of ~9000 ***g*** to separate bacteria (and somatic cells) from milk. Separation is based on differences in the specific gravity (SG) of milk and bacterial cells. Milk has an SG of 1.028–1.038 g ml^{-1}; bacterial spores 1.30–1.32 g ml^{-1}; and vegetative bacterial cells 1.07–1.12 g ml^{-1}. Thus, centrifugation is more efficient in removing spores than in removing vegetative cells from milk.

Typically, when operating in the optimum temperature range of 55–60 °C, centrifugation reduces the total bacterial count of milk by 80–90% (~1-log cycle). However, it can remove 98–99.5% of anaerobic spore-forming microorganisms such as *Clostridium* and ~95% of aerobic sporeformers such as *Bacillus*. A slightly higher reduction can be achieved by a second centrifugation. The gain in shelf life of refrigerated milk by centrifugation is about 4–5 days.

A major application of centrifugation, as for microfiltration discussed above, is in cheese manufacture where it is used to remove bacterial spores such as *C. tyrobutyricum* and *C. butyricum*, which can cause the ‘late blowing’ defect in cheese. Centrifugation can also be combined with lysozyme addition to reduce the level of nitrate added to inhibit *Clostridium* growth and prevent late blowing of cheese.

The use of centrifugation is also beneficial for removing spores in the production of UHT milk, whey protein concentrates, infant formulae, and milk powders. In these cases, application of sufficient heat to destroy spore-forming bacteria is not possible because of its effect on heat-labile milk components, especially whey proteins.

A more detailed coverage appears in the article **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry.

Pulsed High-Intensity Light Technology

Broad-spectrum white light in intense, short-duration pulses is capable of reducing the microbial load on surfaces and in transparent fluids. A typical spectrum of the light is 25% UV, 45% visible, and 30% IR. Significantly, it includes the UV wavelengths 200–300 nm, which are filtered out by the atmosphere; these are important for killing microorganisms. The intensity of the pulses is 0.01–50 J cm^{-2}, about 20 000 times the intensity of sunlight at the surface of the Earth. The duration of pulses ranges from 1 μs to 0.1 s, and the frequency of the flashes applied is in the range 1–20 s^{-1}. A high level of microbial inactivation requires only a few flashes, so the treatment time is typically less than 1 s. The normal light energy required in a treatment is 6 J cm^{-2}, for example, 2 pulses of 3 J cm^{-2} per pulse; an excessive treatment uses 10 pulses. Use of light pulses with a relatively high UV content minimizes the total fluence necessary to achieve the desired microcidal effect.

High-intensity pulsed light is more bactericidal than continuous UV light, but the UV content of the pulses is important. It has a broad-spectrum microcidal effect and destroys bacteria, including spores, resistant to other agents such as heat, H_2O_2, and gamma radiation.

The most effective application of this technology is in the destruction of bacteria on surfaces. On smooth, nonporous surfaces, light pulses can cause a 9-log reduction of vegetative organisms. This is the situation for sterilization of the surfaces of packaging material used in packages for ESL and UHT dairy products. A process for the sterilization of the continuous roll paperboard material used for aseptic cartons has been described and patented. By contrast, on porous and complex surfaces, such as cheese wrapped in plastic, reductions of ≤3-log cycles are observed. For example, a 1.5-log reduction was observed when curds of commercial dry cottage cheese inoculated with *Pseudomonas* were treated with light pulses of a total energy of 16 J cm^{-2}; no effect on the taste of the cheese was noted.

Microorganisms are inactivated by a combination of photochemical and photothermal mechanisms. The biological effect of the UV wavelengths contained in the pulse light spectrum is through absorption by conjugated double bonds in proteins and nucleic acids. Some enzymes are affected by high-intensity light pulses. For example, alkaline phosphatase is reduced by 60–70% with a single full-spectrum flash of light of fluence 1 J cm^{-2}. Inactivation of enzymes due to photochemical effects may also contribute to the microcidal effect of pulsed light.

Continuous Ultraviolet Light Irradiation

The use of ultraviolet (UV) light for the destruction of microorganisms has a long history. It has been used for decontamination of air, surfaces of packaging materials and equipment, and water since the 1960s. However, in 2000, UV treatment of fruit juices as an alternative to thermal pasteurization was approved by the FDA, as it was demonstrated to achieve a 5-log reduction of the most resistant pathogens.

UV light processing for germicidal purposes involves the application of UV-C radiation for a short time. Typically, the wavelength for UV-C processing is in the range 200–280 nm, the so-called germicidal range, as it effectively inactivates bacteria, viruses, yeasts, molds, and protozoa. The maximum germicidal effect occurs between 250 and 270 nm, with 254 nm being considered the most germicidal.

However, UV-C light shows poor penetration into opaque liquids such as milk. Its efficacy in such liquids can be maximized by irradiating a very thin film of the liquid or generating turbulent flow in the liquid to ensure that as much of the liquid as possible is exposed to the UV light at the surface during the treatment. The second approach has been employed for UV irradiation of milk and dairy liquids. Using this approach, the total aerobic bacterial count in raw bovine milk can be reduced by ~3 logs before light-generated off-flavors become noticeable. The threshold energy input is ~1 kJ l^{-1}. These levels of UV also reduce bacterial spores, with the very heat-resistant *B. sporothermodurans* spores being inactivated by 1–2 logs. Larger reductions in all bacteria, including spores, up to 4–5 logs, can be achieved at higher UV dosages but flavor impairment in milk becomes excessive. However, 7- and 8-log reductions have been accomplished in whey and cheese brine solutions, respectively, without excessive off-flavor production, indicating the usefulness of the technology in the cheese industry.

Carbon Dioxide

Addition of carbon dioxide to milk up to ~30 mmol l^{-1} inhibits the growth of bacteria and extends the shelf life under refrigeration of both raw and pasteurized milk. Gram-negative bacteria are inhibited more than Gram-positives. Higher concentrations of CO_2 enhance inhibition but also decrease the stability of the milk. At 30 mmol l^{-1}, the pH is reversibly reduced to about 6.0; removal of the CO_2 can be achieved under vacuum, or the CO_2 can be left to dissipate naturally. It is removed for sensory purposes, as it has a reported flavor threshold of 11.9 mmol l^{-1}, and before heat treatment of the milk when it can promote fouling of heat exchangers.

When CO_2 is added to milk until the pH is ~5.6, it causes precipitation of the casein. Curiously, this pH is 1 pH unit higher than the isoelectric point of casein. The exact mechanism of the precipitation of casein by CO_2 is not known.

Carbon dioxide is now being used commercially for extending the shelf life of raw milk, particularly when it is necessary to transport milk in bulk over long distances. In such situations, extensions of shelf life of up to 14 days are possible if the milk is chilled to ~2 °C before being transported in insulated but nonrefrigerated containers. It is also used, at ~10 mmol l^{-1}, for the shelf-life extension of fresh cheese such as cottage cheese and quarg.

Cold Plasma

Plasma, sometimes referred to as the fourth state of matter, is produced when a gas is excited by electricity or electromagnetic waves in the radio frequency or microwave range. An everyday example is a fluorescent light bulb, which contains a plasma that acts on the phosphor coating of the bulb to produce white light. Plasmas consist of ions of the gas molecules, excited molecules, free radicals, UV light, and, if oxygen is present, ozone, and have the ability to inactivate bacteria, including spores.

Cold (or low-temperature) plasma, produced at atmospheric pressure or below and at close-to-ambient temperature, is useful for decontaminating surfaces and, because it has the ability to inactivate spores as well as vegetative cells, has attracted interest for sterilizing food packaging material as well as processing and packaging equipment. Light technologies such as continuous UV and high-intensity pulsed light are also effective on surfaces; however, cold plasma has the advantage of being multidirectional and hence able to penetrate all presented surfaces, whereas the light technologies are directional and ineffective on surfaces in shadow. When used on food surfaces, cold plasma does not affect the vitamin content, structure, appearance, or color of the food, and leaves no residue. Though the technology is promising, more research is required before it can be industrially applied.

See also: **Bacteriocins**. **Enzymes Indigenous to Milk**: Lactoperoxidase. **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification). **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Liquid Milk Products: Membrane-Processed Liquid Milk; Liquid Milk Products: UHT Sterilized Milks; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milk Protein Products**: Functional Properties of Milk Proteins; Membrane-Based Fractionation. **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry.

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Non-Thermal Technologies: High Pressure Processing

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Introduction

The application of high pressure, rather than heat, to food enables microorganisms to be destroyed without causing significant changes to the color, flavor, and nutritional attributes of the food. In this way, the food can be preserved in a safe state but still have most of the attributes of a fresh product. In addition, high pressure can cause rheologic changes in food that result in beneficial sensory and structural effects.

The potential of high-pressure treatment of food was first recognized by Hite in 1899 who investigated it as an alternative method for pasteurization of milk. However, it attracted little attention until it was 'rediscovered' in the 1980s. The first foods produced commercially by this technology appeared on the market in Japan in 1990. They included yogurts, fruit jellies, salad dressings, and fruit sauces. Several other pressure-treated foods are now manufactured, including juices, seafood (especially oysters), ready-to-eat meals, and cooked meats. With the exception of some cheese-based spreads, it has not been adopted for milk or dairy foods. Some applications that have been patented look promising and may soon be commercialized. According to the company NC Hyperbaric, Burgos, Spain, 130 high-pressure plants for processing food were installed in 61 establishments throughout the world in 2009.

The Process

Typically, high-pressure processing is performed at 300–600 MPa at room temperature for 2–30 min. During pressurization, an increase in temperature (3–9 °C per 100 MPa, depending on the pressure-transmitting fluid) occurs due to adiabatic heating; a corresponding decrease occurs during depressurization. These changes can be minimized by temperature control of the high-pressure equipment by circulating water. Conversely, this temperature rise can be used to achieve desired effects in the treated foods.

During high-pressure processing, the pressure is instantaneously and uniformly transmitted in all directions, regardless of shape or volume (based on Pascal's principle). Consequently, the food is treated evenly throughout, and no particles escape the treatment. This contrasts with processes involving heating where different parts of the food can be heated at very different rates and hence to different extents.

High pressure disrupts noncovalent bonds but has little effect on covalent bonds. Thus, the large biomolecules such as proteins, nucleic acids, and polysaccharides that depend on noncovalent bonding such as hydrophobic interactions and hydrogen bonds to maintain structure and function are most affected. On the other hand, smaller organic molecules such as those responsible for colors, flavors, and nutrients (e.g., vitamins) in which covalent bonding is the dominant or only type of bonding are not affected or are affected very little by the pressure treatment. The low temperature at which high-pressure treatments are usually performed ensures little or no heat-induced changes in these components. However, recent developments in the use of pressure-assisted thermal processing (PATP) and pressure-assisted thermal sterilization (PATS) utilize both heat and pressure and hence cause some heat-induced changes.

The kinetics of pressure-induced reactions can be described by the transition state theory in a similar manner to heat-induced reactions. According to this theory, the rate constant of a reaction is proportional to the equilibrium constant for the formation of an activated complex. In heating reactions, a certain energy, known as the activation energy (E_A), is required for the reaction to proceed. In pressure-driven reactions, a certain volume reduction, known as the activation volume (ΔV^*), must be achieved before a reaction can proceed. At constant temperature, the pressure-dependent reaction rate constant (k) is described by the equation

$$\ln k = \ln k_0 - \frac{\Delta V^*}{R \cdot T} \cdot p,$$

where R = the gas constant (8.314 $\text{cm}^3\,\text{MPa}^{-1}\,\text{K}^{-1}\,\text{mol}^{-1}$), T = temperature (K), and p = operating pressure.

Thus, when $\Delta V^* < 0$, the reaction rate increases with pressure; when $\Delta V^* = 0$, the reaction rate is unaffected by pressure; and when $\Delta V^* > 0$, the reaction rate decreases with pressure. This effect can also be described by the Le Chatelier-Braun principle, which states that whenever stress is applied on a system in a state of equilibrium, the system will always react in a direction that will counteract the applied stress. Accordingly, reactions that result in a volume decrease are promoted by the application of high pressure. Thus, rates of hydrolysis reactions, such as proteolysis, are increased by high pressure.

Equipment and Operation

A high-pressure system usually consists of four main parts: (1) a high-pressure vessel and its closure; (2) a pressure-generation system; (3) a temperature-control device; and (4) a materials-handling system. The pressure vessel in which the processing occurs is generally constructed of forged steel or low-alloy steel of high tensile strength, or reinforced with tensioned wire windings. Products are usually sealed in flexible packages and placed into the pressure vessel filled with a pressure-transmitting medium, which is often water mixed with a small percentage of soluble oil for lubrication and anticorrosion purposes or organic liquids such as alcohols and glycols, with or without added water and/or oil. The type of transmission fluid influences the adiabatic temperature rise during pressurization; the rise is much greater with oils than with water. Liquid food can be compressed directly in the pressure vessel, eliminating use of a pressure-transmitting medium. The success of such systems greatly depends on maintaining the integrity of the seal between the piston and the pressure vessel, which is a challenge at such high pressures and, in some cases, over a range of temperatures.

Pressure treatment can be performed in batch or semicontinuous operations. In batch operations, a pressure chamber containing the liquid or solid food is pressurized for a given holding time and then decompressed. In 2009, the highest-capacity pressure chamber was 420 l. A variation of the batch method is the pulsed high-pressure process where the pressure is raised and lowered repeatedly at intervals of several minutes.

In semicontinuous processing, several vessels are connected in series; while some are under constant pressure, others are being pressurized, unloaded, or loaded. This minimizes the operation time and allows a portion of the energy contained in the vessel under pressure to be used to pressurize another vessel, thus reducing operating costs. A continuous system suitable for liquid foods such as milk and fruit juice has been developed, but only on a research scale. If continuous systems can be built on an industrial scale, high-pressure processing is likely to find widespread application in high-volume liquid foods such as milk.

Microbiological Effects

Vegetative Organisms

A major function of high-pressure processing of food is destruction of microorganisms. When a microbial cell is subjected to high pressure, the following detrimental changes take place:

- cell membranes are destroyed via irreversible changes to the structure of the membrane macromolecules, particularly proteins;
- the homogeneity of the intermediate layer between the cell wall and the cytoplasmic membrane is disrupted;
- membrane ATPase is inactivated; and
- the nucleic acids and ribosomes involved in the synthesis of proteins are disrupted.

The result is permeabilization of the membranes and concomitant leakage of the contents of the cells and organelles, with eventual death of the bacterial cell.

In general, Gram-negative bacteria are inactivated at a lower pressure than Gram-positive bacteria, and rod-shaped bacteria are more sensitive to pressure than cocci. The pressure sensitivities of yeast are reported to be intermediate between these two bacterial groups. The lower resistance of Gram-negative bacteria compared with Gram-positive bacteria is attributable to their lack of teichoic acid, which strengthens the cell wall of Gram-positive bacteria. Bacteria in the log phase of growth are more sensitive than those in the stationary phase.

The bactericidal efficiency of high pressure can be increased by combining it with heat and other ‘hurdles’ as well as other energy forms such as ultrasound, shear, electromagnetic fields, or high-voltage pulses. Concomitant treatment of foods with chemical agents such as ethanol, lysozyme, chitosan, and sorbic and benzoic acids enhances the effect of pressure on microorganisms and allows use of lower pressures, lower temperatures, or shorter application times to achieve inactivation. The medium containing the bacteria can also affect the bactericidal efficiency. For example, *Listeria monocytogenes* is more susceptible in buffer solutions than in milk as proteins, carbohydrates, and fats seem to protect the bacterial cells.

Bacterial Spores

High-pressure processing at around ambient temperature is limited in its inability to destroy bacterial endospores. Because of this limitation, it cannot be used for producing sterile products, and all pressure-treated foods have to be kept refrigerated. However, high pressure can stimulate germination of bacterial spores, which enables the resulting vegetative form to be destroyed. Some variations of the standard single-pressure treatment, such as repeated cycling between high and low pressures, 'pulsed' or 'oscillatory' pressurization, and combined high pressure and high temperature, enhance the sporicidal effect of high pressure.

PATS, aimed at producing sterile, shelf-stable food at a lower temperature than is possible by thermal processing only, has been an active area of research in recent years. It utilizes the temperature rise caused by adiabatic heating during pressure treatment, which can vary with the temperature and chemical composition of the food material and the pressure transmitting liquid. However, in contrast with pressure, which is transmitted instantaneously and uniformly throughout the pressure vessel, the heat generated in this manner may not be transferred uniformly throughout all parts of the vessel if the temperature of the vessel wall is lower than that of the pressure-transmitting liquid; heat transfer to the colder vessel wall will occur, and the product near the wall will be colder than that in the middle of the vessel.

Clostridium botulinum, the target organism for sterilization of food products, is used as the marker pathogen for PATS treatment. This is due to its higher resistance to PATP than *Geobacillus stearothermophilus*, one of the most heat-resistant spores of relevance to dairy processing. Numbers of *C. botulinum* spores in phosphate buffer are reduced by 5 and 6 log cycles when treated at 827 MPa for 10 min at 40 °C and 827 MPa for 20–30 min at 75 °C, respectively.

The flavor of PATS-treated milk differs from that of thermally sterilized (UHT) milk. Straight-chain aldehydes are produced at higher levels, but methyl ketones and some sulfur compounds are produced at lower levels. Therefore, PATP has the potential to produce shelf-stable milk with a better flavor profile than that of UHT milk.

Effect on Bacterial Flora and Keeping Quality of Milk

Hite in 1899 observed a 5–6 log cycle reduction in the number of microorganisms when milk was treated at 680 MPa for 10 min at room temperature. Subsequent reports have supported these observations, all finding a small proportion of the bacterial population that could not be inactivated under normal high-pressure operating conditions. These resistant organisms are mostly spore formers. It has been concluded that to achieve the shelf life of thermally pasteurized milk of 10 days at 10 °C, a pressure treatment of at least 400 MPa for 15 min or 500 MPa for 3 min is required. However, a PATP treatment, 586 MPa at 55 °C for 5 min, can extend the shelf life of milk beyond 45 days, similar to that achieved in thermally produced extended shelf-life milk. The volatile flavor compounds formed in pressure-treated milk at moderate temperatures are similar to those of thermally pasteurized milk.

While a reasonable shelf life of milk may be obtained with pressure treatments of 400 or 500 MPa, some strains of the pathogenic bacteria *L. monocytogenes* and *Staphylococcus aureus* are quite pressure resistant and may not be sufficiently inactivated. Some mutant strains of *Escherichia coli* (LMM 1010, LMM 1020, and LMM 1030 developed from *E. coli* MG 1655) are particularly barotolerant being only reduced 2 log cycles at 600 MPa for 30 min. It has also been suggested that high-pressure treatment might sublethally injure a proportion of cells that could then grow slowly at refrigeration temperatures.

The risk of the presence of barotolerant pathogenic bacteria in milk must therefore be considered before this technology is adopted for producing 'pasteurized' milk. The safety aspects of high pressure and other nonthermal processes have been addressed by many bodies, including the International Dairy Federation Task Force on Alternatives to Traditional Heat Treatments.

Chemical Effects

High-pressure processing of food differs from heat processing in the chemical effects produced. The pressure-induced effects are mostly concerned with secondary and tertiary structural changes in large molecules. Consequently, proteins, including enzymes, and polysaccharides in an aqueous environment undergo reversible or irreversible conformational changes resulting in denaturation, dissociation, aggregation, or gelation. By contrast, the heat-induced breaking of covalent bonds in both small and large molecules causes changes to color, flavor, and other sensory attributes that are not observed with pressure treatment.

Effects on Water

Changes to the water component by high-pressure processing have significant direct and indirect relevance to foods. The changes include the following:

- Reduction of volume, which at high pressure can be substantial (e.g., 15% at 600 MPa).
- Reduction of the freezing point of water. At 50 MPa, the freezing point is reduced to −4 °C, at 100 MPa to −8 °C, and at 210 MPa to −22 °C. Thus, water at 210 MPa remains liquid down to −22 °C. This phenomenon gives rise to interesting effects including subzero food storage without ice crystal formation, rapid thawing of conventionally frozen foods, 'pressure-shift' crystallization, and pressure processing of

foods containing a high proportion of water at temperatures less than 0 °C while still liquid.

- Decrease in pH. Increasing the pressure on water from 100 to 1000 MPa causes a decrease of about 1 pH unit, due to dissociation of water molecules. This can have a significant effect on the stability and characteristics of some food products and also contribute to the effect of high-pressure treatment on microorganisms.

Effect on Milk Components

Proteins

Casein

Three types of change occur in the casein micelle when pressure is applied at around ambient temperature. Little or no change occurs at 100–200 MPa; at ~250 MPa the casein micelle size is increased; and at >300 MPa the casein micelle gradually disintegrates until at ~500 MPa the particles remaining are about 50% of the size of the original micelle. Thus, skim milk that has been pressure treated at ~500 MPa has a clear, almost transparent appearance and a turbidity of about one-third of that of untreated skim milk. At the same time, the viscosity is increased by about 20%. Formation of these smaller casein particles, which have a similar structure to the original micelles by electron microscopy, results from solubilization of micellar calcium phosphate and disruption of intramicellar hydrophobic bonds and electrostatic interactions. As a consequence, there is an increase in soluble calcium and phosphate; however, the ionic calcium shows no change. Concomitantly, individual caseins are dissociated from the micelle with the order of dissociation being β-casein > κ-casein > α_{s1}-casein > α_{s2}-casein. The caseins remain dissociated when the milk is stored at 5 °C but reassociate when the temperature is raised. Retort heating at 115 °C for 15 min restores the appearance and the casein micelle size of pressure-treated (600 MPa for 10 min at 20 °C) milk to those of raw skim milk.

Whey proteins

The sensitivity of whey proteins to denaturation by pressure is in the following order: lactoferrin > β-lactoglobulin > immunoglobulin > bovine serum albumin > α-lactalbumin. Denaturation of β-lactoglobulin commences at ~150 MPa and increases with increasing pressure and temperature. Almost complete denaturation occurs at 750 MPa at 30 °C for 30 min or 450 MPa at 60 °C for 15 min. The denatured β-lactoglobulin mostly becomes associated with the casein micelle; some of them attach milk fat globule membrane in whole milk. The attachments are via disulfide bonds resulting from sulfhydryl-disulfide interactions. Among whey proteins, α-lactalbumin is the most resistant to denaturation by pressure due to its intramolecular disulfide bonds and absence of free sulfhydryl groups. It is denatured only at pressures >400 MPa, becoming 70% denatured at 800 MPa.

Immunoglobulins have increased resistance to denaturation by high pressure at low pH. This phenomenon is utilized in a patented process for production of a colostrum beverage.

High-pressure treatment alters the functional properties of whey proteins. When whey protein concentrates are treated at 300–600 MPa, they show increased foaming ability and foam stability. High-pressure-treated whey protein concentrates improve the foaming properties of low-fat whipping cream formulations and ice cream mixes. Whey protein solubility at pH 7.0 is also enhanced by high-pressure processing.

Solutions containing whey proteins increase in viscosity and turbidity when subjected to pressure, and at high concentrations (>10%), whey protein concentrate forms a gel (at >400 MPa). These changes are due to denaturation of the β-lactoglobulin, which undergoes polymerization reactions through the formation of disulfide linkages to form protein aggregates with increased water-binding capacity.

Enzymes

Milk enzymes vary in their sensitivity to high pressure. Lipase, xanthine oxidase, and lactoperoxidase are resistant to pressures up to 400 MPa. Phosphohexose isomerase, γ-glutamyl transferase, and alkaline phosphatase in milk are partially inactivated at pressures >350, 400, and 600 MPa, respectively, and almost completely inactivated at ~550, 630, and 800 MPa, respectively. The effect on alkaline phosphatase is of interest in milk processing because of its universal use as an internal indicator of effective pasteurization. As its complete inactivation would occur only at very high pressures, alkaline phosphatase is not an appropriate indicator of effective 'pasteurization' by high-pressure treatment. The z_p value for this enzyme is 368 MPa compared with 168 MPa for microorganisms. In contrast with alkaline phosphatase, acid phosphatase is substantially inactivated at pressures $\geq$200 MPa.

Plasmin, the natural milk alkaline protease that causes protein degradation during storage leading to bitter flavors and age gelation in UHT milks, is not inactivated by treatment at 400 MPa for 15 min at room temperature. Plasminogen, the inactive precursor of plasmin in milk, is partially inactivated under these conditions. However, treatment at the same pressure at higher temperatures causes inactivation of plasmin, reaching 86.5% at 60 °C; this reduces proteolysis and improves the organoleptic quality of long-shelf-life milk compared with the same treatments at 25 °C.

Fat

Crystallization of fat can be accelerated, enforced, or initiated because of the shift in the phase transition temperature caused by application of high pressure. As a consequence, high-pressure treatment reduces the aging time of ice cream mixes and enhances the physical ripening of cream for making butter.

The fat globule size distribution and flow behavior of pasteurized liquid cream are not significantly modified by high-pressure processing at 450 MPa and 25 °C for 15–30 min or 10 °C for 30 min. At 40 °C, some fat globule aggregation occurs, but physical stability remains unchanged. With UHT-sterilized cream, treatment at 450 MPa induces fat globule aggregation and increases viscosity, whether carried out at 25 °C for 15 or 30 min or at 10 or 40 °C for 30 min. This aggregation, which may be initiated by the high heat treatment on the milk fat globule membrane, is partly reversed during chilled storage.

Effects on Dairy Products and Processes

Yogurt

Pressure treatment at 200–300 MPa for 10 min at 10–20 °C can be used to control after-acidification of yogurt without decreasing the number of viable lactic acid bacteria or modifying the yogurt texture. Treatment at higher pressures destroys lactic acid bacteria. Above 400 MPa for 15 min, *Lactobacillus delbrueckii* subsp. *bulgaricus* is inactivated, whereas *Streptococcus thermophilus* is more resistant but loses its acidifying capacity.

An extended shelf-life probiotic yogurt has been developed using pressure treatments of 350–650 MPa at 10–15 °C. The process inactivates spoilage microorganisms such as yeasts, molds, and lactobacilli but not specially selected pressure-resistant probiotics, thus extends the shelf life of the yogurt up to 90 days.

Yogurt made from milk treated at 600 MPa for 15 min shows different rheologic properties and gel microstructure from conventional yogurts. The pressure-treated (PT) yogurt gels are less continuous with larger interstitial spaces, which may cause the gel to flow or break at lower deformation. Although β-lactoglobulin is associated with the protein network in the PT gels, the long-range β-lactoglobulin bridges found in the corresponding heat-treated yogurt gels are not present. This makes the PT yogurt less firm and cohesive, which significantly affects its mouthfeel.

Acid-Set Gels

Acid-set gels made from milk treated at high pressure (600 MPa for 15 min) have improved mechanical properties (gel rigidity and gel breaking strength). An eight- to ninefold increase in gel rigidity with increased resistance to syneresis has been reported. This is attributable to a pressure-induced increase in cross-linking sites and improved gel structure due to increased numbers of network strands.

Cheese

High-pressure treatment of milk at pressures $\leq$300 MPa decreases the rennet coagulation time and increases the curd firming rate, curd firmness, and curd yield during cheese manufacture. At these pressures, the casein micelle is largely intact or slightly increased in size, and the extent of denaturation of β-lactoglobulin is modest. At higher pressures, the rennet coagulation time increases to be equal to or greater than that of untreated milk. The effect of high pressure appears to be the result of two mechanisms with opposite effects: disintegration of the casein micelle and denaturation of β-lactoglobulin with association of the denatured protein with the casein micelle. The former increases the surface area of the micelles and facilitates aggregation of para-casein micelles, and the latter hinders rennet action on κ-casein and subsequent aggregation of the caseins to form the coagulum. Interestingly, the rennet coagulation time of heat-treated milk can be decreased by high-pressure treatment, and high-heat-treated milk (90 °C for 10 min), which is uncoagulable by rennet, is rendered coagulable.

The quality of Cheddar cheese manufactured from high-pressure-treated (31-min cycles at 586 MPa) milk is not significantly different in sensory quality to that made from high-temperature, short-time (HTST) - pasteurized milk. However, the pressurized-milk cheese has a higher moisture content, which causes pasty and weak texture defects. This is attributable to the increased water-holding capacity of the milk proteins. The higher moisture content of the pressurized-milk cheese can be exploited for improving the texture of reduced-fat cheese, which is usually very hard.

Several authors have reported increased yield of cheese from pressure-treated milk. Though some of the increased yield may be due to higher moisture contents, some denatured whey protein is incorporated into the curd. This is also reflected in decreased protein losses in the whey.

Generally, pressure treatment of low-fat milk prior to cheesemaking improves cheese texture and overall acceptability. Cheese made from HTST-pasteurized, pressurized milk has been reported to be significantly more tasty than that made from either pressurized or HTST-pasteurized milk. Protein breakdown is faster in cheese made from pressurized milk, which leads to a softer texture; this has been attributed to a higher level of residual rennet.

Whereas a small number of coliforms can survive pressure treatment of milk, much lower numbers, or none at all, can be isolated from the pressurized-milk cheese. This may be explained by the recovery of sublethally injured cells in the pressurized milk during the enumeration procedure and the subsequent death of the injured cells in the cheese during storage.

High-pressure treatment of cheese curd rather than cheese milk has beneficial effects. For example, treatment of Cheddar cheese curd (345 MPa for 3 min) produces a microstructure that is similar to that of ripened cheese and produces shreds that are equivalent to those obtained from unpressurized 27-day-old Cheddar cheese; treatment of fresh lactic curd cheese ($\geq$300 MPa for 5 min at ambient temperature) extends its shelf life up to 8 weeks, compared with 3 weeks for an untreated control, through inactivation of spoilage yeasts; and treatment of 1-day-old Mozzarella cheese (200 MPa for 60 min at 20 °C) improves its functional properties such as melt time, flowability, and stretchability.

Proteolysis of Whey Proteins

Pepsin, which is unable to hydrolyze β-lactoglobulin at atmospheric pressure, completely hydrolyzes it in less than 40 min at 300 MPa due to denaturation of β-lactoglobulin. This has been explained in terms of a decrease in reaction volume ($\Delta V = -44.8\ \mathrm{ml\ mol^{-1}}$) during the hydrolysis, an example of the application of the Le Chatelier-Braun principle.

In making modified milk for infants, β-lactoglobulin can be selectively eliminated from whey protein concentrates by hydrolysis with thermolysin at elevated hydrostatic pressure. α-Lactalbumin is resistant to hydrolysis due to the presence of its four disulfide bonds. When the thermolysin digestion is performed under high pressure, β-lactoglobulin is hydrolyzed faster and more completely than at atmospheric pressure, but there is no effect on α-lactalbumin. Thus human milk, which contains α-lactalbumin but no β-lactoglobulin, can be simulated by the use of pressurized thermolysin treatment of cows' milk.

See also: **Heat Treatment of Milk**: Non-Thermal Technologies: Introduction; Non-Thermal Technologies: Pulsed Electric Field Technology and Ultrasonication.

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Non-Thermal Technologies: Pulsed Electric Field Technology and Ultrasonication

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Pulsed Electric Field Technology

Pulsed-energy technologies for treatment of foods utilize high energy levels in short bursts or pulses (nanoseconds to milliseconds) with average power consumption. The energy is generated initially as electrical energy and accumulated in a storage capacitor. The accumulated electrical power (many megawatts) is discharged almost instantaneously in the form of high-energy electric field pulses (pulsed electric field technology) or, with the appropriate hardware, high-intensity light pulses (pulsed light technology) or high-intensity magnetic field pulses (oscillating magnetic field technology). The electronics that drive the dose-generating devices for each of the technologies are similar. This article concerns pulsed electric field (PEF) technology only.

PEF technology has the potential to replace, or at least complement, the traditional thermal processes for pasteurization and even sterilization of liquid milk products. As most PEF operations are performed under mild conditions, often at ambient temperature, the technology offers considerable consumer benefits in terms of better sensory quality (flavor, color) and safety comparable to, or better than, that of the corresponding thermal processes. It may also offer the manufacturers benefits in terms of lower operational costs as compared to thermal processes.

Equipment

The essential components of a PEF processing system are (a) a high-voltage power supply (~30 kV); (b) an energy storage capacitor; (c) a pulse generator and switching system; (d) a treatment chamber, containing two electrodes, between which liquid food is pumped; (e) a cooling system; (f) a unit for control and monitoring of voltage, current, and electric field strength; and (g) a product delivery and packaging system.

Energy from the high-voltage power supply is stored in the capacitor and discharged almost instantaneously at high levels of power. The discharge is extremely fast and hence the pulse width is very short, typically 1–5 μs. The frequency of pulses is 200–400 pulses per second. Total treatment times are generally much less than 1 s. The strength of the electric field that passes through the food is directly proportional to the voltage supplied across the electrodes, and inversely proportional to the gap or distance between the electrodes. PEF technology utilizes electric field strengths of 10–50 kV cm^{-1}. When applied as pulses, the disadvantages of continuous high-voltage current treatments, electrolysis, and ohmic heating are minimized.

The design of the treatment chamber is critical to the efficacy of this technology. It is essential that a uniform electric field exists throughout the cell so that there are no ‘cold spots’ and no areas that promote electrical breakdown of the food product. The electrodes are typically 3–5 mm apart. Several different treatment chamber designs have been developed and patented. Common configurations are parallel-plate, cofield, colinear, and coaxial. Some have specific characteristics such as conical-shaped electrodes and insulators to prevent gas depositing within the treatment volume, which can lead to electrical breakdown of the food. Some systems operate under pressure to prevent such gas development. Multiple treatment chambers in series can be used to minimize the effect of nonuniformity of electrical field strength within individual chambers.

Bactericidal Effects

The most important aspect of PEF technology in the treatment of liquid milk products is its effect on bacteria. Most of the vegetative cells can be destroyed by the technology, but bacterial spores are much more resistant.

Several hypotheses have been advanced to explain the inactivation of bacteria by PEF, but a generally accepted one is the electroporation theory. When a high voltage is applied to a liquid containing microbial cells, a transmembrane potential is induced across the membrane of the cell. When this exceeds a critical value, ~1 V, electroporation occurs, that is, pores form in the cell membrane. As a consequence, the contents of the cell are released and the cell dies. The phenomenon can be explained by electromechanical compression caused by the induced separation of charge between the inside and the outside of the cell. This tends to compress the membrane through the attractive forces between the opposite charges. In addition, PEF-induced bud scars on the surface of some cells and extensive disruption of cellular organelles have been observed.

Pores, which are either predominantly hydrophobic or predominantly hydrophilic, are formed by the electrical breakdown of lipid bilayers. Hydrophilic pores conduct current, resulting in localized Joule heating, which causes changes in the liquid crystalline structure of the lipid layer in the cell membrane. This impairs the semipermeability of the membrane and increases its permeability to ions. If the pore radius exceeds a certain limit, the pores cause mechanical breakdown of the membrane.

Factors influencing the effect of PEF on vegetative microorganisms

The damage inflicted on a microorganism, and hence the cell's viability after PEF treatment, depend on several factors. These include the following:

- The nature of the cell. This determines the critical field strength (E_c) above which electroporation, and hence cell death, can occur. Large cells have a lower E_c than smaller ones. Typical yeast cells have an E_c of $\sim$5 kV cm^{-1}, whereas the E_c values for Gram-negative bacilli such as *Pseudomonas fluorescens* and *Escherichia coli* are $\sim$10–15 kV cm^{-1}. Cells also have a critical treatment time (T_c) below which no damage occurs at a particular electric field strength. Typical T_c values are <5 μs for yeasts and 46 μs for *E. coli*.
- The electric field strength, number of pulses, and total treatment time. For any organism, at electric field strengths higher than its critical value, E_c, viability decreases with increased field strength, increased treatment time, and increased number of pulses. For example, *E. coli* in UHT milk was reduced by 4 log cycles at $\sim$22 kV cm^{-1} after 20 pulses of $\sim$5 μs duration, 2 log cycles at $\sim$22 kV cm^{-1} after 10 pulses of $\sim$5 μs duration, and 2 log cycles at $\sim$18 kV cm^{-1} after 20 pulses of $\sim$5 μs duration.
- The shape of the pulse waveform. The most common forms of the pulse are of constant polarity with either an exponential or a square wave shape. In an exponential pulse, the voltage increases to a selected peak value and decreases exponentially after a very short period of time at the peak voltage. Voltage levels lower than the peak may not have a bactericidal effect. Square wave pulses deliver electrical energy at the maximum voltage during most of the pulse width. Where peak voltages are required for inactivation of bacteria, this waveform is more effective than the exponential waveform and requires a smaller number of pulses for the same bactericidal effect. The energy efficiency of square wave and exponential decay pulses have been reported to be 91 and 64%, respectively.
- Polarity of the pulses. Pulses of alternating polarity can also be used. The bipolar pulse is generated as a sinusoidal wave and uses very short duration pulses of alternating polarity. These alternating polarity pulses, which reduce the risk of undesirable electrochemical reactions and deposit formation on the electrode, have been reported to have a more lethal effect on bacteria than pulses of constant polarity. A variation of the bipolar pulse is the instant charge reversal pulse where a sharp spike is formed in the waveform when the polarity is reversed, resulting in an oscillating field. This waveform has been reported to have superior bacteria killing power than waveforms without the instant charge reversal spike.
- The stage of growth of the microorganism. Microorganisms are more sensitive to PEF in the log phase of growth than in the stationary phase.
- The composition of the medium. Studies on the effect of the composition of the medium have shown that fat protects microorganisms against electric pulses. For example, destruction of bacteria in milk containing 3.5% fat is less than that in milk with 1.5% fat. Similarly, higher electric field strengths are required to destroy bacteria in a xanthan gum solution than in a saline solution. This is attributed to the protective effect of the gum afforded by interaction between the polymer chains of the gum, forming a network of molecular aggregates around the microorganism. Another important factor is the presence of air in the medium being treated, which may result in arcing. The pH of the medium may also affect the outcome of PEF treatment, but reports on this have not been consistent.
- The electrical characteristics of the food. Many liquid food materials conduct electricity because they contain ions, which act as electrical charge carriers. Under a given set of conditions, the degree of microbial inactivation achievable by PEF decreases with increasing conductivity (or decreasing resistivity) of the food, which varies according to composition. Resistivity (the inverse of conductivity) ranges from 0.4 Ω m for foods with high ionic and water content to >100 Ω m for pure fats and oils, which are electrical insulators. Reported resistivities for milk and yogurt range from 1.3 to 3.1 Ω m.
- Presence of antimicrobials. PEF can have a synergistic effect with antimicrobials. For example, PEF treatment with nisin resulted in >6 log reduction of *Staphylococcus aureus* in skim milk; no reduction in *S. aureus* occurred after treatment with nisin alone.

Effect on bacterial endospores

Bacterial endospores, such as those formed by *Bacillus* and *Clostridium* species, are more resistant than vegetative cells to PEF. There are several reports of PEF being ineffective against endospores. The reason for the greater resistance of spores to PEF is that electrical conductivity is considerably less in endospores than in vegetative cells, owing to the low water content and the relative immobility of ions within the spore core. However, it may be possible to inactivate spores by PEF if used in conjunction with heat.

It has been suggested that spores are more susceptible to PEF at the time of germination and outgrowth. However, germination is not initiated by PEF. Therefore, processes capable of inducing germination (e.g., treatment with high hydrostatic pressure, heat, or ultrasound) may enhance the effectiveness of PEF in inactivation of spores. A 95% reduction in *Bacillus subtilis* spores treated at 30 kV cm^{-1} for 500–3000 μs at 36 °C in the presence of L-alanine, which enhances germination, has been reported.

Effect on enzymes

PEF has a variable effect on enzymes, with some being completely or partially inactivated (e.g., milk plasmin), some being stimulated (e.g., lysozyme and pepsin), and some remaining unaffected (e.g., pectinesterase). The effect also varies with electric field strength, pulse width, and treatment time. Significant enzyme inactivation generally requires a higher electric field strength than that required for the destruction of vegetative cells. A summary of the effects of PEF on some endogenous milk enzymes and bacterial lipases and proteases of significance in the dairy industry is given in **Table 1**. This not only shows a distinct variation between enzymes but also demonstrates the variation between reports for the same enzyme. This highlights the difficulty of comparing the results between laboratories because of the large number of variables involved, including, for enzymes, assay methodology.

In dairy processing it is of interest that milk plasmin (alkaline protease), which is resistant to heat treatments, even UHT, is partially inactivated by PEF. However, alkaline phosphatase and lipoprotein lipase, which are completely inactivated by high-temperature, short–time (HTST) pasteurization, are only partially inactivated by PEF when only the nonthermal effects of the technology are taken into account.

Mechanisms proposed for the inactivation of enzymes by PEF include alteration of secondary and tertiary structures of the protein and oxidation of key components of the enzyme. In the case of enzymes present in microorganisms, localized Joule heating of cell membrane components due to PEF may cause thermal denaturation of membrane-bound enzymes. Stimulation of some enzymes may be caused by creation of more active sites or increase in size of the existing sites.

Applications in dairy processing

Milk has been subjected to PEF by several researchers and the results are reasonably consistent. Most report 4–5 log reductions of non-spore-forming bacteria. Typical conditions used are 50–55 °C at 20 kV cm^{-1}; lower reductions were observed at lower temperatures (20–40 °C). PEF treatment, without additional heat treatment, can achieve a safe product (e.g., the bacterial count of raw milk inoculated with 10^8 cfu ml^{-1} *Listeria* was reduced to ~10 cfu ml^{-1}, the 'floor' level of spores, by treatment at ~60 °C; the corresponding thermal effect was 1–2 log reduction) and up to 14 days extension of shelf life. However, when combined with heat treatment, a further extension of shelf life is achievable. When an HTST treatment follows PEF treatment of milk, a product with

Table 1 Effect of PEF on some endogenous milk enzymes and *Pseudomonas fluorescens* lipase and protease

Enzyme	*Medium/temperature (°C)*	*Electric field strength (kV cm^{-1})*	*Number of pulses/pulse width (μs) or (total treatment time in μs) or [energy input in kJ l^{-1}]*	*%Inactivation*
Lipoprotein lipase	Raw milk/NR	21.5	20/NR	60
	Raw milk/35, 55	35	[163]	33, 21[a]
Alkaline phosphatase	Raw milk/NR	21.5	[400]	0
	Raw milk/30	37	(19.2)	42
	Raw milk/22	18.8	70/400	60
	Raw milk/45, 50	21.5	(780)	5
	Raw milk/35, 55	35	[163]	24, 21[a]
Lactoperoxidase	Raw milk/NR	21.5	[400]	0
Lipase (*P. fluorescens)*	SMUF/34; 35	27.4 (batch)	(314.5)	62.1
		37.4 (continuous)	(136)	13
Protease (*P. fluorescens)*	Skim milk/50	15	98/2	60
Plasmin	SMUF/15	45	50/NR	90
	Raw milk/35, 55	35	[163]	17, 36[a]
Xanthine oxidase	Raw milk/35, 55	35	[163]	23, 27[a]

[a] Nonthermal effect only.
NR, not reported.

extended (refrigerated) shelf life (≥60 days) results, whereas a thermal treatment at 112 °C for 31.5 s following PEF has been reported to produce a product (chocolate milk) that was shelf-stable for 3 months.

PEF is a promising technology for 'pasteurizing' liquid products containing heat-sensitive bioactive components. For example, successful treatment of products fortified with water-soluble vitamins and with bovine IgG has been reported.

Little or no change in flavor, color, lipid oxidation, whey proteins, fat globule membrane, and casein micelles has been observed. A reduction in vitamin C in milk treated with PEF has been observed. PEF-treated yogurt containing yeast (10 cfu ml^{-1}), which can reduce its shelf life, had no detectable yeast after storage for 30 days at 7–9 °C. Cheese made from PEF-treated milk is reported to have better curd properties than traditionally made cheese, and this has led some researchers to conclude that the use of milk 'pasteurized' by PEF may be a feasible option to improve the quality of cheese.

Ultrasonication

Ultrasonication refers to the use of sound waves of frequencies higher than those audible by the human ear (i.e., >18 kHz). Ultrasound can travel through gas, liquid, and solid materials and is therefore suitable for use with milk and milk products. It has applications in the dairy industry in both its high- and low-power forms. The high-power form (10–1000 W cm^{-2}) at relatively low frequencies (20–1000 kHz) causes changes in bacteria and the physical structure of food and food components, whereas the low-power form (1 W cm^{-2}) at high frequencies (0.1–20 MHz) is nondestructive and is used for evaluation of the physical state of products and for diagnostic purposes. This article is concerned only with the high-power form.

When ultrasound is applied to a food, the sound waves create small bubbles, which increase in size and eventually implode violently. This phenomenon, known as cavitation, causes high sheer and turbulence in the food, which can result in significant physical changes near the imploding bubbles. Some of the changes are brought about by the generation of high local temperatures (up to 5000 K) and pressures (up to 100 MPa). Cavitation is considered to be the major mechanism responsible for many of the effects observed with ultrasonication, including disruption of bacterial and other cells. However, the rapid longitudinal compressions and rarefactions that occur during ultrasonication also cause strong eddies in the liquid around the sonotrode, and these can result in what is known as microstreaming or acoustic streaming when they spread throughout the liquid. The considerable amount of energy associated with this streaming can impact surfaces (e.g., of bacteria) and cause physical damage.

The physical phenomena of microstreaming and cavitation are most important in the lower ultrasound frequency range (<100 kHz). At higher frequencies, 100 kHz to 1 MHz, chemical phenomena associated with the production of free radicals are more significant. The free radicals can lead to chemical reactions such as oxidation and hydroxylation, which can be either detrimental or beneficial. Most often ultrasonication is performed at 20–100 kHz to avoid these chemical changes.

The effects of an ultrasonic treatment depend on several factors, including the frequency of the ultrasound, the intensity of the energy input (which is also related to the amplitude of the sound wave), the duration of treatment, the temperature of treatment, and the amount of product being treated. Increasing the pressure in the treatment system also has a significant effect by increasing the actual energy input into the system; it also enables ultrasonication to be used at elevated temperatures where cavitation caused by the ultrasound is reduced by the elevated vapor pressure exerting a cushioning effect on the imploding bubble.

During ultrasonic treatment of foods, much of the sound energy is converted to heat, which raises the temperature of the food. The extent of the temperature increase is proportional to the time of treatment and the energy input. This heating effect can be used to advantage in some applications. The extent of heating can also be used to estimate the total energy input of the ultrasonic treatment.

Equipment

Ultrasonication equipment consists of a frequency generator and power supply, a transducer, and a horn or sonotrode. The vibrational energy produced by the frequency generator is transferred by the transducer to the sonotrode, which vibrates and transmits its vibrations to the target material. This may be a liquid, semisolid, or solid food; the air; or other solid material such as food processing equipment. This generates sound waves in the material. One of the most common ultrasonic treatment arrangements is a flow-through cell through which a pumpable liquid is passed. However, products can be treated in batch mode or in some other arrangement depending on the application. For example, the ultrasound waves can be transmitted through air for defoaming applications or the ultrasonic probes can be placed in contact with equipment to reduce fouling during membrane separations and heat treatments.

The power of laboratory ultrasonic equipment is typically 100–1000 W. However, large industrial ultrasonic units are now available with power outputs of several kilowatts. Such units can also be used in parallel or series for greater treatment capacity.

Effect on Bacteria

Ultrasonication can inactivate bacteria, but the extent of inactivation depends on many factors, including the type of bacterial cell and the conditions of treatment. Larger cells are more sensitive than smaller ones; rod-shaped are more sensitive than cocci; Gram-negatives are more sensitive than Gram-positives; anaerobics are more sensitive than aerobics; young cells are more sensitive than older cells; and vegetative cells are much more sensitive than spores. Inactivation increases with the time and energy intensity of the ultrasound treatment, and also the temperature of the treatment medium and pressure in the system. In general, ultrasound has minimal effects on bacteria at temperatures < approximately 50 °C, where the effects are almost completely nonthermal, but at higher temperatures it has a synergistic effect with heat. In fact, the synergistic action with heat is the major effect of ultrasound on bacteria.

Bacterial cells become more sensitive to heat treatment after ultrasonication. Sequentially or simultaneously applied sonication and heat treatment (thermosonication) achieve destruction of bacteria at much lower temperatures than would be required for heat treatment alone. Simultaneous application of mild pressure (~0.4 MPa) and ultrasound further enhances the bactericidal effect. Applying pressure with heat and ultrasound (manothermosonication) overcomes the loss of cavitation at high temperatures.

The destructive effect of ultrasonic waves on microorganisms has been attributed to a combination of phenomena, namely, cavitation and associated shear forces, localized heating, and free radical formation. Hence the greater the intensity of these phenomena, the greater the effect on the microorganisms. For example, the intensity of cavitation is affected by the frequency and amplitude of the ultrasound wave. At low frequencies (e.g., 20 kHz), large bubbles are produced, and high-energy forces result when they collapse. At higher frequencies, bubble formation becomes increasingly difficult, and at frequencies higher than 2.5 MHz, cavitation does not occur. The intensity of cavitation increases as the amplitude of the ultrasound wave increases.

Application of ultrasound in an aqueous environment can lead to the formation of the free radicals $OH^{\bullet}$, $H^{+\bullet}$, and $H_2O_2^{\bullet}$ species, which have bactericidal effects. The prime target of free radicals in cells is the DNA. Hydroxy radicals are very reactive and can initiate the formation of peroxy radicals on amino acid residues, causing substantial loss of tryptophan, tyrosine, and other amino acids. Subsequently, a chain reaction is initiated, which in the presence of oxygen usually leads to protein fragmentation and under anaerobic conditions to the formation of higher-molecular-weight aggregates through cross-linking.

The medium in which the microorganism is treated also influences the effect of ultrasound treatment. The effectiveness of ultrasound decreases as the level of solids (fat, protein, lactose, gums) and the viscosity increase. For example, thermosonication at 63 °C caused a 4.9 log reduction of *Listeria* in UHT skim milk, but only a 2.5 log reduction occurred in UHT whole milk. The effectiveness of ultrasonication in viscous liquids is maximized by the use of high-intensity, low-frequency ultrasound, and by increasing, within limits, the temperature of the medium.

Effect on Enzymes

The effect of ultrasonication on enzymes depends on their molecular structure and the nature of the medium, especially the presence of dissolved gases. Enzymes vary widely in their response. For example, polyphenoloxidase and lysozyme are sensitive, whereas catalase and pepsin are not. Ultrasound has no effect on the endogenous milk enzymes, alkaline phosphatase, lactoperoxidase, and γ-glutamyltranspepsidase at room temperature, but has a synergistic effect with heat in inactivating these enzymes at higher temperature. Similarly, the heat-resistant proteases and lipases of *Pseudomonas* are much more sensitive to inactivation by heat when applied with ultrasound, under mild pressure to maintain cavitation at elevated temperatures.

Inactivation of enzymes generally requires long treatment times and the presence of oxygen. The effect of sonication has been attributed to splitting off of prosthetic groups (such as heme in heme proteins and the lipid portion in lipoproteins); fragmentation of the polypeptide chain; and oxidation or degradation of amino acid residues via a free radical mechanism. The loss of activity of pepsin and trypsin after sonication has been attributed to the oxidation of cyclic amino acids and sulfhydryl groups, as inactivation is significantly reduced when hydrogen is substituted for oxygen in the sonicating medium or when antioxidants are present.

Applications in Dairy Processing

Ultrasonication reduces the size of the fat globules in milk to <1 μm and forms a stable emulsion. Both the average size and the size range of the fat globules are smaller after ultrasonication than after conventional homogenization. Thermosonication at 63 °C was reported to result in whiter and better homogenized milk than conventionally pasteurized and homogenized milk.

Whey proteins are denatured to some extent by ultrasound treatment and this effect is synergistic with heat. However, the effect of ultrasonication on the physical properties of whey protein powders has recently attracted considerable attention. In general, ultrasonication increases their solubility, largely through disrupting protein aggregates, and improves their foaming and emulsification properties. The viscosity of whey protein solutions may also be altered; both decreases and increases have been reported. Combined with a heat treatment, ultrasonication has also been shown to increase the heat stability of whey protein products and increase the strength of heat-set gels made from the treated whey proteins.

Sonication of milk at 40–45 °C before inoculation for yogurt production significantly improves the texture and rheological properties of yogurt. The improvement in yogurt from skim milk is substantial, and it has been suggested that sonication may reduce the need for addition of texture-modifying agents to enhance the rheological properties of low-fat yogurt. The improvements in textural characteristics caused by the ultrasound have been attributed to increased water-holding capacity, whey protein denaturation, and, in fat-containing yogurt, homogenization of the fat globules to very small particles.

There have been some reports of improvement in cheese properties by ultrasonication. In particular, ultrasonicated brined cheese exhibits greater firmness and more intense aroma, odor, and flavor than exhibited by conventionally brined cheese. It also undergoes more proteolysis and lipolysis during ripening.

See also: **Heat Treatment of Milk**: Non-Thermal Technologies: High Pressure Processing; Non-Thermal Technologies: Introduction. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Liquid Milk Products: UHT Sterilized Milks; **Milk Protein Products**: Functional Properties of Milk Proteins.

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Heat Stability of Milk

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Introduction

The first attempts to preserve milk by heat treatment were made by Nicolas Appert in 1810. These were unsuccessful because the milk coagulated during heat treatment. About 1860, Louis Pasteur applied a low-heat treatment (a process now known as pasteurization) to preserve wine and beer. This process was first applied in the dairy industry in 1885 by N. Fjord to improve the microbiological quality of cream for butter making. In 1886, Franz Soxhlet suggested that milk fed to children should be heat-treated and in 1895 it became a legal requirement in Denmark to pasteurize milk at creameries to prevent the spread of tuberculosis among farm animals through skimmed milk returned to farmers. In 1906, the philanthropist Nathan Straus established depots in New York City for the distribution of pasteurized milk for children. In 1908, legislation enacted in Chicago required that liquid milk offered for sale in that city should be pasteurized. In all of the above cases, unconcentrated milk was pasteurized and this almost always was sufficiently heat stable to withstand the process. In 1856, Gail Borden attempted to produce heat-sterilized concentrated milk but coagulation during heat treatment made the process unpredictable and Borden used sucrose to preserve the product instead. In 1884, John B. Meyenberg developed a process for the production of heat-sterilized concentrated (evaporated) milk; since then, evaporated milk has been a substantial dairy product but precautions are required to avoid heat-induced coagulation.

Today, heat treatment of milk is an integral step in dairy processing, and almost all milk, regardless of its ultimate use, is subjected to at least one heat treatment. The objective for heat treatment of milk varies considerably, from being an essential part of the manufacturing process for specific products, for example, protein coprecipitates and yogurt, to enabling the production of stable and safe products through the reduction or elimination of spoilage and pathogenic microbes. Accordingly, heat treatments vary considerably from mild (thermization at 65 °C for 15 s) to very severe (in-container sterilization at 110–115 °C for 10–15 min). Milk is extremely heat stable, owing principally to the ill-defined tertiary structure of the caseins, and can tolerate most processing conditions to which it is normally subjected; for example, it can be heated at 140 °C for up to 20 min before it coagulates. However, problems may be encountered with thermal processing and subsequent storage or processing of concentrated milk, homogenized milk products, and cheese milk.

Compared to other food systems, milk is very heat stable but when concentrated may not be sufficiently stable to withstand heat sterilization. Since the early twentieth century, the heat stability of milk has been the subject of considerable research at the basic and practical levels; this article presents a brief overview of the principal compositional and processing factors that affect the heat stability of milk.

Assessment of the Heat Stability of Milk

In the context of this article, 'heat stability' may be defined as the ability of a sample of milk product to withstand heating at a particular temperature without the onset of visible aggregation/coagulation. The denaturation of proteins, including enzymes, or other 'invisible' heat-induced changes are not included, even though it is these changes that ultimately cause coagulation of proteins.

Several experimental procedures are used to assess the heat stability of milk: (1) It may be expressed as the time elapsed between placing a sample in a sealed tube in an oil bath, usually at 140 °C for unconcentrated milk or 120 °C for concentrated milk, and the onset of coagulation or flocculation of milk proteins, that is, heat coagulation time (HCT; referred to as the subjective heat stability assay; **Figure 1(a)**). (2) It may be recorded as the temperature at which milk coagulates instantaneously, that is, heat coagulation temperature; this procedure is essentially a measure of the inherent stability of milk proteins as it is not affected by heat-induced reactions such as thermal oxidation of lactose, dephosphorylation and proteolysis of caseins, and the Maillard reaction. In practice, the heat coagulation temperature is the temperature at which a sample coagulates in 2 min, which is the approximate time for the sample in a sealed tube to reach the temperature of the oil bath, and is determined by intrapolation. (3) It may be determined objectively by measuring the percentage of total protein sedimentable by a low gravitational force (400 ***g***) as a function of heating at a constant temperature, with a sudden increase in the amount of sedimentable protein denoting coagulation

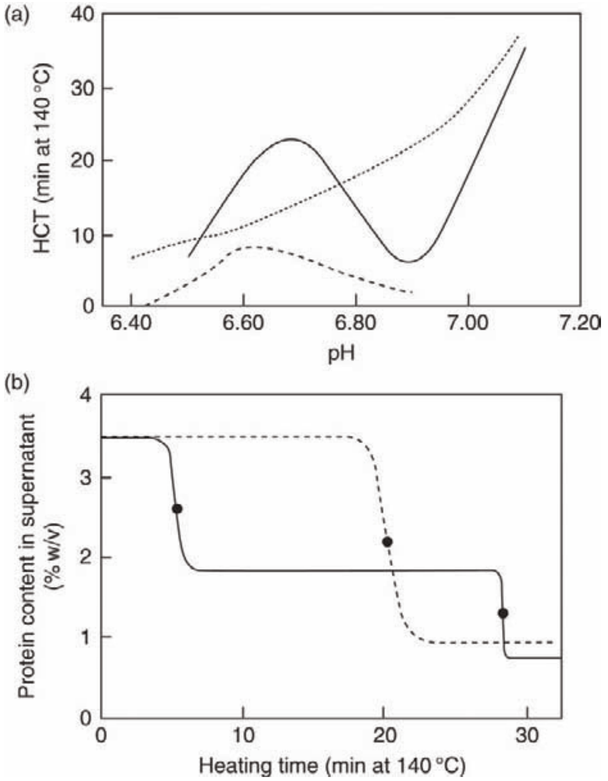

Figure 1 (a) HCT–pH profile of unconcentrated type A (—) and B (. . .) milk and concentrated milk (- - -); (b) protein depletion curve of type A milk at pH 6.7 (- - -) or 6.9 (—). HCT, heat coagulation time.

(**Figure 1(b)**). (4) It may be determined based on viscosity measurements. As milk coagulates, its viscosity increases and may be monitored using a viscometer, for example, a falling ball viscometer. (5) It may also be determined by using an ultrasonic device.

Unfortunately, the heat stability of milk determined by any of the above methods does not correlate well with that of concentrated milk during actual processing and this is best determined by pilot-scale retorting, for example, in McCartney bottles, and may be detected as the grain point.

Heat-induced coagulation of milk has a Q_{10} value of $\sim$2–3 and an apparent activation energy (E_a) of 140 kJ mol^{-1}. There is a slight inverse curvilinear relationship between temperature and the log of HCT.

Effect of Compositional Factors

The heat stability of milk is affected by a myriad of factors, of which pH is the most significant. The majority of unconcentrated milk ($\sim$80–90% of milk from individual cows and $\sim$100% of bulk milk) exhibits a type A HCT–pH profile; stability at 140 °C is low at pH values <6.3 but increases progressively to a maximum at pH 6.7 (HCT $\sim$20 min); stability then decreases to a minimum at pH 6.9 (HCT $\sim$5 min), with stability increasing on the alkaline side of the minimum (**Figure 1(a)**). For most of the year, the natural pH coincides with the pH of maximum stability. In type B milk, stability increases as a function of pH, being less stable in the region of the maximum and more stable in the region of the minimum than type A milk (**Figure 1(a)**). Concentrated milk (20% solids-not-fat) is relatively unstable and has maximum stability at pH 6.4–6.6 (HCT $\sim$20 min at 120 °C), with stability on either side of the maximum being very low. Coagulation of unconcentrated type B milk throughout the pH range 6.4–7.4 and of type A milk in the region of the maximum and on the alkaline side of the minimum is a single-stage process as determined by the objective heat stability assay. However, concentrated milk at its pH of maximum stability and type A milk in the region of the minimum coagulate by a two-stage process (**Figure 1(b)**).

The heat stability of milk is affected by milk salts, although natural variations in the heat stability of milk do not correlate well with those in the concentration of individual milk salts. A reduction in the total mineral content of milk eliminates the minimum in the HCT–pH profile, whereas an increase in the total serum ion concentration destabilizes milk in the region of the minimum, with little effect on maximum stability. A relatively small (15%) reduction in the concentration of divalent cations, Ca^{2+} or Mg^{2+}, in milk enhances stability throughout the pH range 6.5–7.5. Addition of phosphates to milk increases stability, presumably owing to their capacity to chelate calcium and also perhaps to their buffering ability, while the removal of indigenous phosphate shifts the HCT–pH profile to a more alkaline value. Citrate, which is present in milk at 1800 mg kg^{-1}, is likely important for the heat stability of milk because of its ability to chelate calcium and its high solubility at elevated temperatures. The addition of 10 mmol l^{-1} citrate markedly enhances HCT$_{max}$ and shifts it to more acid values; stability does not recover on the alkaline side of the minimum, which may be related to the disruption of colloidal calcium phosphate links in the casein micelles. Surprisingly, oxalate, which is a very strong calcium chelator, has a very strong destabilizing effect. The addition of NaCl or KCl to milk shifts HCT$_{max}$ to a more acid value and stability on the alkaline side of the minimum is reduced.

The macro-components of milk also affect heat stability. Stability is unaffected by lipids *per se*, but reducing the size of the fat globules by homogenization has a destabilizing effect (see below). Removal of lactose from type A milk results in a decrease in HCT$_{max}$ but an increase in HCT$_{min}$, while enzymatic hydrolysis of lactose to glucose and galactose enhances stability throughout the pH range. Increasing the concentration of lactose to 150% of its normal level destabilizes type A milk throughout the HCT–pH profile, while doubling

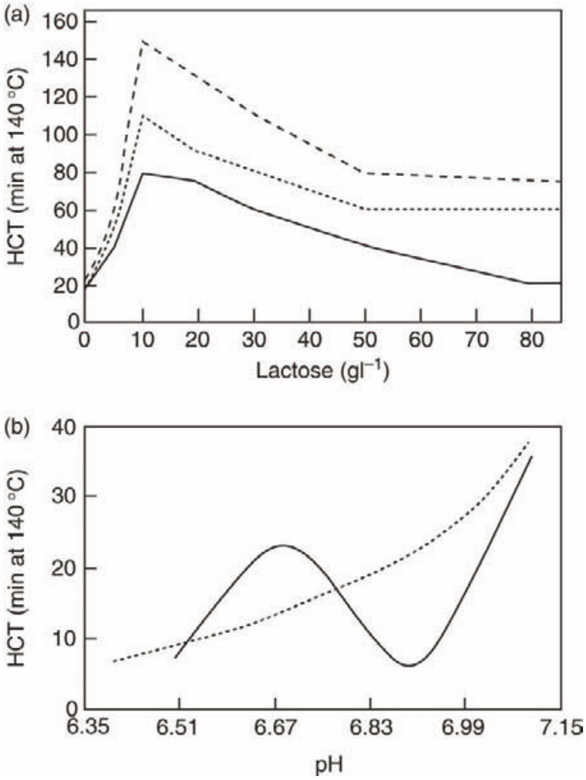

Figure 2 (a) Effect of lactose on the HCT$_{max}$ of lactose-free milk containing 5 (—), 10 (. . .), or 15 (- - -) mmol l^{-1} urea; (b) HCT–pH profile of serum protein-free casein micelle dispersion with (—) and without (. . .) added β-lactoglobulin. HCT, heat cogulation time.

the lactose content of type B milk converts it to type A milk. The addition of lactose (up to 1% (w/v)) to lactose-free milk increases stability, but higher levels reduce stability (**Figure 2(a)**).

Of the milk proteins, β-lactoglobulin and κ-casein have the greatest impact on the heat stability of milk. Addition of β-lactoglobulin to a serum protein-free casein micelle (SPFCM) dispersion or to type B milk results in the development of a type A HCT–pH profile, increasing and reducing the HCT at pH values 7 and 6.9, respectively (**Figure 2(b)**). Addition of κ-casein to type A milk eliminates the minimum in the HCT–pH profile. The addition of α_{S2}-casein to milk reduces stability, possibly because of its high sensitivity to calcium. The ratio of α_{S2}-casein to β-casein also appears to affect stability, a high ratio resulting in high stability. γ-Caseins, the products of the action of plasmin on β-casein, destabilize milk. The addition of α-lactalbumin to an SPFCM dispersion has an effect similar to that obtained by adding β-lactoglobulin, while the addition of bovine serum albumin destabilizes milk throughout the pH range.

Urea, the major nonprotein nitrogenous constituent in milk at 6 mmol l^{-1}, stabilizes milk at HCT$_{max}$ and at pH values on the alkaline side of the minimum and is thought to be responsible for up to 90% of the natural variability in the heat stability of milk (the natural range for the concentration of urea in milk is ~190–450 mg l^{-1}). The heat stability of unconcentrated type A milk in the region of the minimum is unaffected by urea and the stability of concentrated milk is unaffected by urea at levels up to 15 mmol l^{-1}. The heat-induced degradation of urea in milk has a Q_{10} value of ~2 and an E_a of ~84 kJ mol^{-1}, follows (pseudo-) first-order reaction kinetics, and the principal degradation products appear to be cyanate and ammonia. The stabilizing mechanism of urea has not been elucidated fully, although its ability to buffer against heat-induced acidification (through the formation of ammonia) appears to be important. Other mechanisms through which urea may affect heat stability include the interaction of cyanate with the nucleophilic amino acid residues lysine and cysteine, an increase in micellar steric stabilization and net negative charge, and the inhibition of the Maillard reaction. It is noteworthy that urea stabilizes milk only in the presence of lactose.

Other factors that may contribute to the variations in the heat stability of milk include the following:

- Health of the cow – stability decreases during mastitic infection.
- Stage of lactation – late-lactation milk is unstable.
- Season – stability is lowest during winter/spring.
- Genetic polymorphism of milk proteins – milk containing β-lactoglobulin AA has a high HCT$_{max}$.
- Diet – generally, milk produced by cattle on pasture is more stable than that from cattle fed silage and dried fodder crops.

However, the significance of any one of the above factors is obscured by the fact that they are interrelated; for example, the effect of season cannot be considered in isolation, as diet, stage of lactation, and protein content also vary as a function of season.

Effect of Processing Factors and Additives

Variations in the heat stability of milk are caused by compositional changes; several processing factors may also affect stability. Preheating under ultrahigh-temperature conditions (e.g., 140 °C for 2 min) increases the heat stability of type A milk and concentrates prepared therefrom, while the heat stability of type B milk is unaffected by preheating. It has been proposed that the stabilizing effect of preheat treatment is due to the denaturation of whey proteins and precipitation of calcium phosphate under relatively mild conditions. Homogenization of milk prior to heat treatment markedly reduces the heat stability of whole milk. The destabilizing effect of homogenization is related to the adsorption of casein submicelles onto the interface of newly formed fat globules, which then behave like κ-casein-depleted casein micelles. The deleterious effect of homogenization on the heat stability of whole milk may be offset by preheat treatment, two-stage homogenization (rather than single-stage), or the addition of stabilizers, for example, lecithin or sodium caseinate. Homogenization of skimmed milk has no effect on HCT that is increased by high-pressure (200–400 MPa) homogenization, which shatters casein micelles. Stability is unaffected by storage under refrigeration for up to 1 week but is reduced if the milk is stored at ambient temperature under controlled bacteriological conditions. In an attempt to enhance the heat stability of milk proteins or to determine the cause of pH dependence and the coagulation mechanism of milk, the effect of numerous additives on the heat stability has been studied:

1. The effect of hydrocolloids varies considerably; starches destabilize milk while κ-carrageenan, at low levels, has a stabilizing effect.
2. Some polyphenols increase heat stability very strongly.
3. Reagents that modify sulfhydryl groups also affect stability. Oxidizing agents such as Cu^{2+}, H_2O_2, BrO_4, KIO_3, and iodobenzoate convert a type A profile to a type B profile, while reducing agents such as β-mercaptoethanol markedly reduce stability.
4. Alcohols such as butanol, propanol, ethanol, and methanol reduce the heat stability of milk in proportion to their ability to reduce the dielectric constant. It is thought that by reducing the dielectric constant, these solvents collapse the protruding C-terminal segment of κ-casein located on the surface of the micelles (hairy chains), making the micelles susceptible to calcium-induced precipitation.
5. Reagents that react selectively with nucleophilic amino acid residues, for example, formaldehyde with lysine and diketones with arginine residues, increase the heat stability of milk. The stabilizing mechanism appears to be related to their ability to crosslink proteins (thereby maintaining micellar integrity) or increase the net negative charge on proteins (thereby protecting against calcium-induced precipitation).

Heat-Induced Changes

Up to ~90 °C, heat-induced changes are relatively slow and largely reversible (with the exception of whey protein denaturation), while at temperatures >100 °C, reactions occur rapidly and are irreversible. The main heat-induced changes in milk are

- aggregation of micelles,
- dissociation of κ-casein from the micelles,
- acidification,
- dephosphorylation of caseins,
- proteolysis of caseins,
- hydrolysis of the caseins,
- Maillard reaction,
- covalent polymerization of protein, and
- changes in micellar hydration and zeta potential.

The true significance of any of these changes has not yet been elucidated; for example, it is unclear which reactions cause coagulation, predispose the milk system to coagulation, or are merely a consequence of coagulation. On heating at the assay temperature ($\geq$120 °C), the casein micelles do not disintegrate but appear distorted; at pH values <6.7, the whey proteins denature and complex with the casein micelles, presumably through sulfhydryl–disulfide interchange reactions between κ-casein and the whey proteins, particularly β-lactoglobulin. At pH values >6.7, κ-casein, which plays an essential role in protecting the other caseins against calcium-induced coagulation, dissociates into the serum phase. It appears that on heating, dissociation occurs owing to electrostatic repulsion; at elevated temperatures, colloidal calcium phosphate links and hydrophobic bonds are weakened and are no longer strong enough to prevent the dissociation of κ-casein due to electrostatic repulsion. Dissociation of micellar κ-casein is affected by a number of factors:

- Calcium – an increase in calcium reduces dissociation.
- Concentration – dissociation increases with the concentration of milk.
- Protein profile – the addition of β-lactoglobulin to an SPFCM dispersion reduces heat-induced dissociation at pH values <6.7 and has the opposite effect at pH values >6.7.

Acidification, which occurs on severe heating, has a Q_{10} value of ~2 and starts initially at a rate of 5 mmol $H^+ l^{-1} min^{-1}$ but decreases to 1 mmol $H^+ l^{-1} min^{-1}$ after 10 min at 140 °C; the pH on cooling at coagulation approaches pH 5.5, although it is thought that the pH at the assay temperature is ~4.9 at coagulation. The three principal reactions that cause acidification are

1. thermal oxidation of lactose to organic acids (mainly formic acid, which is formed at 0.4 mol $m^{-3} min^{-1}$; this accounts for 80% of total acids formed and a pH decrease of 0.03 units min^{-1}),
2. the precipitation of primary and secondary phosphates as tertiary phosphate with a concomitant release of H^+, and
3. the dephosphorylation of casein and subsequent precipitation of the liberated phosphate.

The first two reactions are thought to contribute to ~50 and 30% of total acid formation, respectively, while the significance of dephosphorylation of casein with respect to heat-induced acidification is unclear. Heat-induced acidification likely plays an important role in the coagulation of milk proteins, as shown by the fact that milk may be heated for at least 3 h at 140 °C without coagulation if the pH is readjusted periodically to its original value. Acidification is likely to directly affect the stability of milk by reducing the micellar zeta potential and hydration, and consequently should promote protein–protein interactions. However, heat-induced coagulation of milk proteins should not be seen as an indirect acid coagulation process, as the Q_{10} values for the two processes differ and, unlike isoelectric casein, coagula produced on heat coagulation are not redispersible if the pH is increased. Also, the fact that the pH at the point of thermal coagulation is not constant would preclude the possibility of heat-induced coagulation being an indirect form of acid-induced coagulation. Other heat-induced changes that may result in an increase in protein–protein interactions include thermal dephosphorylation, deamidation of glutamine and asparagine residues, proteolysis, and the formation of covalent crosslinks. Thermal dephosphorylation of caseins has an E_a of 120 kJ mol^{-1} and a Q_{10} of ~2.5, follows first-order reaction kinetics, and appears to occur either by alkali fission of the phosphate esters at the –C–O– linkage or by β-elimination of the -O-P- linkage. The significance of dephosphorylation has not been elucidated fully, but, in addition to increasing protein–protein interactions by reducing the net negative charge on proteins, it may result in the disruption of colloidal calcium phosphate linkages and the formation of dehydroalanine, which would be expected to affect stability.

Heat-induced proteolysis of milk proteins has an E_a of 70 kJ mol^{-1} and a Q_{10} of ~2 and follows zero-order reaction kinetics. The hydrolysis of κ-casein, which is linear with time and has a Q_{10} value of ~3 from 110 to 150 °C, is likely to be significant in that 30% of total κ-casein is hydrolyzed at the point of coagulation, regardless of assay temperature. Deamidation of glutamine and asparagine residues is a zero-order reaction and has an E_a of 90 kJ mol^{-1}. Deamidation may affect stability by altering net protein charge and also possibly by enhancing the Maillard reaction.

Many forms of covalent crosslinks may be formed on heating, such as sulfhydryl–disulfide reactions, by isopeptide bond formation, by Maillard reaction, and by the reaction of dehydroalanine (which is formed by the β-elimination of proteins containing cysteine or phosphoserine residues or carbohydrate moieties) and nucleophilic amino acid residues, for example, lysine, cysteine, arginine, ornithine, and histidine. The Maillard reaction, which involves a condensation reaction between the ϵ-amino group of lysine (and to a lesser extent the guanidino group of arginine, indolyl group of tryptophan, or imidazole group of histidine) and the carbonyl group of lactose, is extensive on prolonged heating. The Schiff base formed by the condensation reaction then undergoes Amadori rearrangement to form *N*-substituted 1-amino-1-deoxy-2-ketose, which may then enolize to form 1-amino-1,2-enol or 1-amino-2,3-enodiol, which are then broken down to form hydroxymethylfurfural and an α-dicarbonyl, respectively, or alternatively engage in a Strecker degradation process to form aldehydes, CO_2, and pyrazines. Compounds formed by both pathways then interact and polymerize to form melanoidins (*see* **Lactose and Oligosaccharides**: Maillard Reaction). The role of the Maillard reaction in the heat-induced coagulation process is unclear. Some researchers have suggested that it destabilizes the milk system by polymerizing proteins, while others have proposed that products of the Maillard reaction stabilize milk proteins against heat-induced coagulation through their capacity to chelate calcium.

pH Dependence and Mechanism of Heat-Induced Coagulation

It would be expected that the heat stability of all milk samples would increase as a function of pH (type B HCT–pH profile) owing to the increase in protein charge, hydration, and zeta potential with pH. An attempt to explain the pH dependence of type A milk has been made. At pH values <6.3, owing to the high Ca^{2+} activity and low zeta potential and micellar hydration, milk has very low heat stability and coagulates rapidly at 140 °C. In the region of the maximum, it is thought that the formation of β-lactoglobulin–κ-casein complexes stabilizes milk by reducing the dissociation of micellar κ-casein and by increasing steric stabilization, zeta potential, and hydration. Within the region of the minimum, κ-casein (independently or in a complex with β-lactoglobulin) dissociates from the micelles, leaving κ-casein-depleted micelles, which are inherently unstable and coagulate rapidly through a calcium-induced precipitation mechanism. On the alkaline side of the minimum, stability increases, possibly because of a reduction in Ca^{2+} activity and higher protein hydration and zeta potential. In concentrated milk, dissociation of κ-casein is extensive throughout the pH range 6.3–7.4 and, consequently, the coagulation mechanism is likely to be similar to that of unconcentrated milk within the region of the minimum, that is, it is calcium-mediated. It appears that the mechanism for heat-induced coagulation of concentrated milk throughout the pH range 6.3–7.3 and of unconcentrated milk at pH <6.3 and within the region of the minimum entails some form of calcium-induced precipitation; the mechanism at HCT_{max} and on the alkaline side of the minimum in unconcentrated type A milk is unclear, although covalent polymerization and/or the collapse of the ill-defined secondary and tertiary structure of the caseins has been suggested. The heat-induced decrease in pH is likely to be very significant in creating an environment that favors coagulation.

Interspecies Comparison of the Heat Stability of Milk

This article has focused on the heat stability of bovine milk, which, because of its commercial importance, has been studied thoroughly. However, buffalo, caprine, ovine, camel, and equine milks are also heat-treated under certain circumstances. Like bovine milk, buffalo milk shows a type A HCT–pH profile but is less heat stable than bovine milk; its stability is affected by the same factors as those for bovine milk. Caprine and ovine milks are also less stable than bovine milk and their HCT–pH profiles are different – they show a maximum at pH ~6.8 above which stability decreases and does not recover at pH >7.2, that is, like concentrated bovine milk, there is a maximum but no minimum.

Equine milk is very unstable; HCT at pH 6.6–6.7 is <10 min at 100 °C but it increases in a sigmoidal manner as the pH is increased and is quite stable at pH >7.0. Camel milk is very unstable at 140 °C, with an HCT <1 min at pH 6.5–7.2; when assayed at 100 °C, it shows a type B HCT–pH profile. At its natural pH, porcine milk is relatively unstable to heat and stability increases with increasing pH. Human milk is reported to be quite stable and shows type B behavior.

See also: **Heat Treatment of Milk**: Sterilization of Milk and Other Products; Thermization of Milk; Ultra-High Temperature Treatment (UHT): Heating Systems. **Lactose and Oligosaccharides**: Lactose: Production, Applications; Maillard Reaction. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milk Proteins**: Casein Nomenclature, Structure, and Association; α-Lactalbumin. **Plant and Equipment**: Pasteurizers, Design and Operation.

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HOMOGENIZATION OF MILK

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Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers

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Introduction

Bovine milk contains approximately 4% fat, dispersed as milk fat globules enclosed by the milk fat globule membrane (MFGM). These globules vary in size from less than 1 μm to more than 10 μm, with a mean of approximately 3–4 μm, with some variation between breeds. Since the milk fat is less dense than milk serum, the fat globules will tend to rise and form a cream layer on top of the milk. This flotation follows Stokes' Law, where the rising velocity, *v*, of the globule may be expressed as

$$v = \frac{d^2 g(\rho_s - \rho_f)}{18\eta}$$

where *d* is the diameter of the fat globule, p_s the density of the milk serum, p_f the density of milk fat, and *h* the viscosity of milk serum.

Those fat globules less than 1 μm will tend to stay in suspension as Brownian motion has a significant effect in disturbing flotation. However, the presence of cryoglobulins in raw bovine milk will bring about agglomeration of fat globules and hasten creaming. At temperature–time combinations in excess of minimum high-temperature short-time (HTST) pasteurization (72 °C for 15 s), the cryoglobulins are denatured and the creaming rate in the resulting product is reduced.

The effects of shear forces on milk include disruption of fat globules. The MFGM may not be able to reform immediately with the result that some fat may be lost as free fat, which will promote aggregation of fat globules and accelerate creaming. In some instances, the damage to fat globules may be so severe that a plug of agglomerated fat globules will form in containers of pasteurized milk. If the milk is subjected to high shear at a temperature above the melting point of the fat, then the fat globules may be broken into smaller particles, stabilized by a new interfacial membrane, to give homogenized milk that does not exhibit creaming, that is, the milk remains homogeneous.

Principle of Homogenization

The basic principle of homogenization is to subject the milk fat globule to sufficiently severe conditions to disrupt it and then maintain the new globules in dispersion while a replacement MFGM is formed at the fat–serum interface. These conditions are attributed to a combination of factors:

1. Shear results from the flow of the milk over the surfaces in the homogenizing device. Velocities of 200–300 m s^{-1} may be achieved through a 100 μm gap. The shear between the fat globule and the surface will be complemented by the wire-drawing effect as the fat globule is accelerated, the globule becoming progressively less stable as it elongates.
2. Turbulence results from the high velocity of the milk serum, the eddy currents adding to the shear effects. Impacts between milk fat globules are increased, causing disruption.
3. High-frequency vibrations (>10 kHz) may be generated mechanically or induced as a result of the flow pattern or be a result of cavitation. These shock waves,

which may be >100 MPa in intensity, will disrupt fat globules.

4. Impact may provide an additional shock force in some equipment, where a high-velocity jet of milk leaving the homogenizing valve at $\geq 200\,\mathrm{m\,s^{-1}}$ strikes a perpendicular surface.

The extent to which each of these factors contributes to the homogenizing process will depend on the particular equipment being used.

Homogenization should normally be carried out at a temperature above the melting point of the fat to ensure that the fat has the mobility to form new globules. High temperatures, for example, >65 °C as in the case of ice cream mixes, will increase the likelihood of cavitation contributing to the homogenization process.

Design of Homogenizers

Most milk homogenization is carried out using high-pressure homogenizers, the invention being attributed to Auguste Gaulin, whose first patent was granted in 1899. Modern high-pressure homogenizers have been developed based on the principles introduced by Gaulin, to give better hygiene and higher efficiency. The equipment consists of a piston pump to generate the high pressure that is used as the driving force to direct the milk through a homogenizing valve or valves. For homogenization of milk and most dairy products, the pressure is typically in the range of 15–30 MPa, although some machines are now being built to operate at much higher pressures, leading to some confusion in the terminology since the term 'high-pressure homogenizer' is used across the range of machines while those operating in the normal range of pressures may also be referred to as 'valve homogenizers' and those working at very high pressures as 'ultra-high-pressure homogenizers' (**Figure 1**).

Homogenizer Pump

The pump is usually of a triplex design, sometimes with five or seven pistons, operating consecutively to ensure the generation of a steady driving pressure. Single piston pumps are avoided in all but the smallest machines, as they generate a pulsating output with fluctuating pressure that is difficult to damp. The pump block should be of stainless steel construction, possibly with some components made of ceramic materials.

The milk should be fed to the homogenizer from a preheater at a low pressure. This milk enters the inlet manifold, from which it may be drawn up into each of the pumping chambers. A tubular sieve is often placed in the inlet manifold to prevent foreign bodies from entering the pumping chamber. The valves used for milk have been mainly of a poppet design with relatively large contact surfaces; this ensures that a close-fitting seal can be made under optimal conditions. Sometimes, the homogenizer may be fitted with ball valves, which can exert a greater pressure on the much smaller seal area and are particularly appropriate for very high-pressure applications, suspensions with small particles, or where higher viscosity media are to be processed. A mushroom valve has been introduced by TetraPak, seeking to combine the advantages of the poppet and ball valves. On the pumping strokes, the liquid is forced past a second set of valves, usually spring loaded to assist rapid closure, into a high-pressure manifold, as illustrated in **Figure 2**.

The pump pistons have been a source of problems since they need to move through seals that must be capable of resisting both high pressure and microbial colonization. Pressures of up to 20 MPa are common for milk homogenization but may be up to 30 MPa for other dairy applications. In most homogenizers, chevron seals made from relatively soft composite materials are used, held in place by a threaded sleeve so that wear can be taken up. Piston movement must be sufficient to ensure that the seals are wetted by cleaning and disinfecting

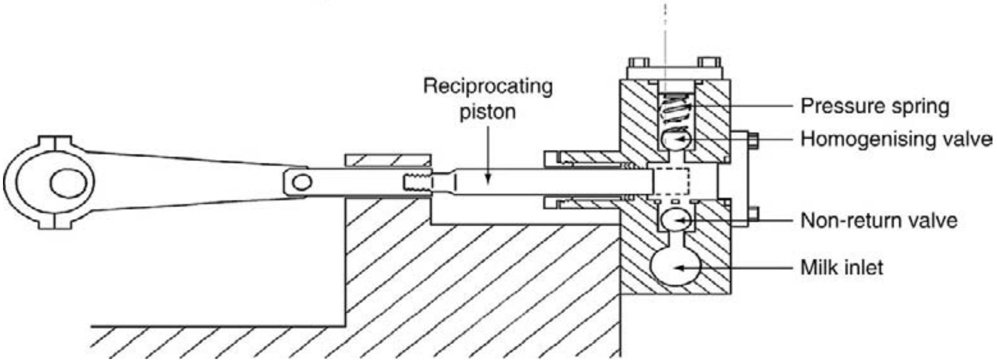

Figure 1 General arrangement of a high-pressure homogenizer. Courtesy of the Society of Dairy Technology.

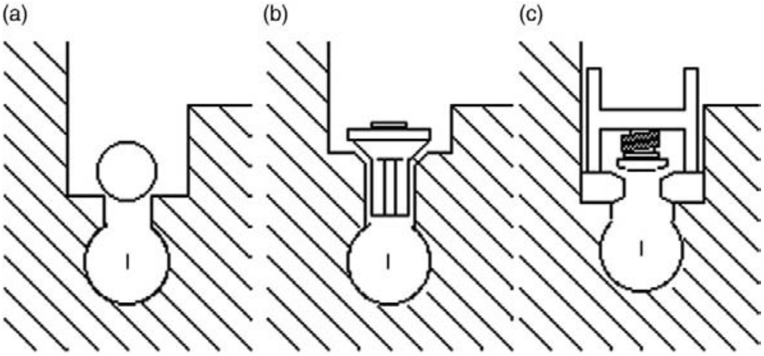

Figure 2 Inlet valve types used in high-pressure homogenizers: (a) ball, (b) poppet, and (c) mushroom. I, milk inlet. In each case, flow of milk is upward through the value.

agents during cleaning-in-place, without there being significant leakage. Each piston is lubricated by a fine water jet to reduce wear, but in hard water areas, care must be taken to avoid a buildup of water scale. Where aseptic processing is required, the water lubrication must be replaced by a steam box or an equivalent disinfection system.

Homogenizing Valve Assembly

Whereas the various pump designs are essentially similar, there are a great variety of homogenizing valves and many conflicting claims for their efficiency. Of these, the Gaulin type of valve (**Figures 3** and **4(a)**) is still widely used and provides a good model.

Well-mixed liquid at a high pressure enters the center of the valve seat and is accelerated as it passes into the constriction between the fixed and adjustable faces of the valve. The gap is maintained against the pressure of the feed by a counterforce from an adjustable heavy-duty spring, torsion bar, hydraulic actuator, or a simple screw mechanism, the last mechanism being the least favored as it will not compensate for changes in feed or temperature. Liquid passing across the valve at up to 200–300 m s^{-1} will drop in pressure and the pressure drop may be so low as to drop below the saturation vapor pressure and permit microscopic steam bubbles to form for a few microseconds before collapsing and setting up highly disruptive shock waves. These cavitation conditions are more likely to occur if homogenizing at an elevated temperature (>65 °C). The high-velocity jet then impinges

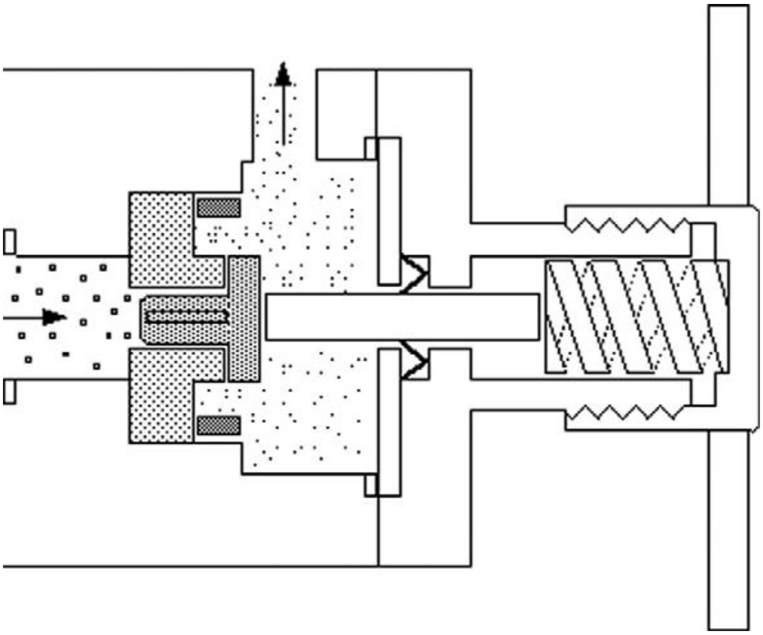

Figure 3 Section through single-stage Gaulin-type homogenizing valve assembly.

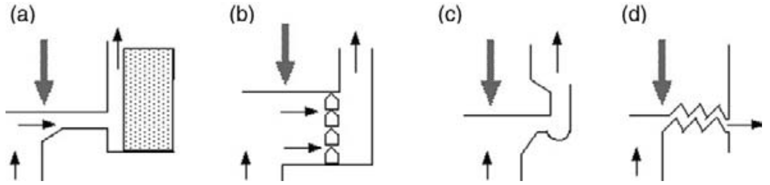

Figure 4 Part sections of homogenizing valves: (a) Gaulin, (b) Gaulin Micro-Gap, (c) Tetra Alex, and (d) Rannie Liquid Whirling.

on a perpendicular impact ring to inflict a further mechanical shock on the fat globules. The severity of the process conditions can be demonstrated by inspection; worn valves exhibit one or more concentric rings eroded by the turbulence and possibly by cavitation, while the impact rings may exhibit an eroded ring. This wear occurs despite their construction from extremely tough and corrosion-resistant materials such as stellite, tungsten carbide, or ceramics. Any valve parts exhibiting these features should be replaced as their efficiency will have declined.

The original MFGM is not sufficient to cover the greater surface area of the newly homogenized fat globules. Proteins, predominantly caseins, migrate from the milk serum to form a new composite membrane with the existing MFGM. Some aggregation may occur as the new membrane is formed, attributable to some hydrophobic interaction and the sharing of casein micelles between fat globules. These agglomerated globules would have a larger equivalent diameter and cream more rapidly, thus frustrating the objective of homogenization. The introduction of a second homogenizing step at a lower pressure drop, about 10% of the primary homogenization pressure or up to 3.5 MPa, will overcome the problem by disrupting these agglomerates and provide some extra time for a stable, fine dispersion to be achieved. In addition to reducing the mean particle size, the particle size range should also be reduced.

The quest for reduced energy expenditure has led to a wide range of homogenizing valve developments and claims for improved efficiency. The Rannie Liquid Whirling valve (**Figure 4(d)**) uses a series of concentric rings within the valve to create a multistage effect within a single valve, while the Gaulin Micro-Gap homogenizing valve assembly (**Figure 4(b)**) splits the flow between a series of rings with a knife-edge across the flow to achieve up to 40% claimed reduction in pressure for a given particle size in the product. The Tetra Alex valve employs a different configuration (**Figure 4(c)**) plus larger diameter to achieve up to 30% claimed reduction in energy consumption at pressures up to 20 MPa.

Applications and Significance

High-pressure homogenization is the main method used for the commercial homogenization of milks. With pasteurized milks, the homogenization step may be regarded as an option, increasingly desirable as longer shelf lives are sought.

In the case of semiskimmed milk containing 1.5–1.8% milk fat, the advantages of homogenization are as follows:

1. a slight increase in the perceived creaminess compared to the unhomogenized product;
2. absence of creaming, which the consumer is likely to view as detrimental in a product chosen for its lower fat content; and
3. stabilization of an emulsion that might have been damaged during the general handling, separation, and standardization processes.

With whole milk, the advantages of homogenization are not so clear-cut, since there is already sufficient creaminess from the natural fat content, typically about 4% but this may exceed 5% in the case of Channel Island milk. In one milk product, ‘Breakfast Milk’, high-fat milk is homogenized so that the richness may be appreciated without the fat content being so apparent.

With the continuing concentration of milk processing into fewer much larger units, there is greater potential for damage to the MFGM from excessive shear during milk handling. One manifestation of this problem is the formation of cream plug in whole milk, which is most evident in bottled milks, both glass and plastic. While the problem could be avoided by correct sizing of pumps, pipes, and valves, the symptoms can be alleviated by homogenization.

Sometimes, fluctuation will be noticed in the pressure gauge reading. This is seldom due to a fault in the pressure gauge but primarily results from leakage across one or more valves in the homogenizer pump. This leakage may be due to poor seating, particularly with worn poppet valves, but may be the result of a buildup of particles, for example, incompletely dispersed stabilizer in the case of ice cream mixes, on the face of the valve.

Raw milk contains lipoprotein lipase, which will attack damaged milk fat globules, liberating free fatty acids and thus generating off-flavors. Homogenization, by both modifying and increasing the surface area of fat globules, will increase the susceptibility of homogenized milk to lipolysis. Fortunately, lipoprotein lipase is heat labile and its effects on the homogenized fat globule can be avoided by heat treatment before or immediately after homogenization.

Upstream homogenization immediately followed by heat treatment avoids microbial hazards associated with seal problems in homogenizers. Occasionally, downstream homogenization is desired, either to minimize shear damage to the product or to avoid thermal stability problems associated with homogenized fat globules. The latter may be a problem with some ultra-high-temperature (UHT)-treated products and in this case it is essential that the machine be built for aseptic use (*see* **Heat Treatment of Milk**: Heat Stability of Milk).

Whereas one homogenization process, whether single or two stage, is normally sufficient to produce a stable dispersion, a yet finer dispersion with a narrower size distribution range may be achieved by running two high-pressure homogenizers in series. This approach may be used in the production of cream liqueurs where the product must remain stable at room temperature.

Homogenization Efficiency

The efficiency of the homogenization process may be viewed in terms of the energy requirement of the process or the properties of the product, the latter being important in monitoring the continuing effectiveness of the equipment. Product properties may be described by resistance to separation or by obtaining a measure of particle size.

One of the simplest methods (that can be carried out in any dairy laboratory) is to store a 100 ml sample of the fluid in a measuring cylinder in a refrigerator for 24 h and then measure the fat content of the top and bottom portions of the liquid. The storage time can be reduced by centrifuging the fluid for a standard period of time instead of holding in the refrigerator. Homogenization efficiency is then expressed as a homogenization index:

$$\text{index} = \frac{\text{difference in fat contents}}{\text{fat value in the upper layer}} \times 100$$

Particle size can be estimated by various light scattering techniques. The simplest techniques using turbidity or backscatter give mean values related to particle size and concentration and when calibrated for a given product can indicate when a homogenization process is drifting. More sophisticated laser-based systems such as the Malvern Mastersizer can provide a detailed particle size analysis from less than 0.1 μm to more than 100 μm in diameter.

Direct observation of samples by light microscopy using an oil immersion objective with a total magnification of about ×1000 can indicate whether a sample has been finely emulsified or not. Contrast between the fat globules and the aqueous phase may be enhanced by using fat-soluble dyes such as Sudan Black B, although resolution below 1 μm is difficult as the smaller globules are subject to Brownian motion. Ultramicroscopes, phase-contrast microscopes, and laser scanning confocal microscopes have also been used. Where resources permit, electron microscopy and image analysis techniques will give reliable quantitative data.

See also: **Heat Treatment of Milk**: Heat Stability of Milk. **Milk Lipids**: Milk Fat Globule Membrane.

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High-Pressure Homogenizers

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Introduction

Ever since the invention of the valve homogenizer at the end of the nineteenth century, homogenization has taken a central place in dairy processing. For liquid milk products, for example, pasteurized, sterilized, and ultra-high temperature (UHT)-treated varieties, it is crucial to homogenize the milk to prevent fat separation during storage. Likewise, homogenization is crucial in the preparation of ice cream mixes and recombined milk, and it significantly improves the texture of yogurt. Homogenization is traditionally carried out at a pressure in the range 10–30 MPa (100–300 bar). However, in the past two decades, a new generation of homogenizers that are capable of reaching far higher pressures, that is, up to 400 MPa in some cases has been developed. Such homogenizers are generally referred to as high-pressure homogenizers or ultra-high-pressure homogenizers; the former term will be used here. High-pressure homogenizers are already employed in the cosmetic and pharmaceutical industries. The use of much higher pressures for homogenization of milk and dairy products has also been studied extensively in the past decade and will be summarized here. Other homogenizing devices that can operate at comparably high pressures, for example, microfluidizers, will be dealt with elsewhere.

Principles of High-Pressure Homogenization

A typical design for the homogenizing valve of a high-pressure homogenizer is shown in **Figure 1**. The valve seat and piston for high-pressure homogenizers are often made of ceramics, to be able to withstand the high pressures and stresses encountered in the high-pressure homogenization process. In the homogenizing valve, the pressurized fluid flows axially past the mobile part of the homogenizing valve, that is, the piston. Subsequently, the fluid flows at an extremely high velocity through the valve gap, that is, the radial gap formed between the piston and the valve seat. Subsequently, the fluid leaves the valve seat at atmospheric pressure. The pressure and velocity that the liquid encounters in the homogenizing valve are determined by the size of the valve gap, which can be adjusted accordingly to regulate the homogenization pressure and intensity.

High-Pressure Homogenization of Milk

Milk or comparable products that pass through a high-pressure homogenizer encounter a number of simultaneous and interlinked physical phenomena, including high velocity, shear, collision of particles, turbulence, rapid increases and decreases in pressure, and accompanying cavitations. In high-pressure homogenizers, fluid velocity increases very rapidly, from $<0.5\,\mathrm{m\,s^{-1}}$ up to as high as $100\,\mathrm{m\,s^{-1}}$, on approach to the valve gap in the homogenizing valve. This high acceleration results in elongational flow, which is likely to be a main mechanism for disruption of emulsion droplets. Velocity is further increased as pressure drops when the fluid accelerates into the gap between the valve and the valve seat and may, at pressures of 300 MPa, reach values in the region of $200\,\mathrm{m\,s^{-1}}$. These high velocities make the residence time in the homogenizing valve extremely short. During passage through the valve, much turbulence and cavitation can occur. Along with these phenomena, large increases in temperature are observed when milk is homogenized at high pressure. Temperature increases up to the point where the milk has passed through the first homogenizing valve. Recently, temperatures of ~65, 84, or 103 °C have been reported after the first homogenizing valve when milk of an initial temperature of 40 °C is homogenized at 100, 200, or 300 MPa, respectively. The temperature of milk has generally been found to increase in a near-linear fashion with homogenization pressure, at a rate of $\sim0.15-0.20\,^{\circ}\mathrm{C\,MPa^{-1}}$ on high-pressure homogenization; temperature increases observed during high-pressure homogenization are largely independent of the temperature of the milk prior to homogenization. However, increase in temperature during high-pressure homogenization depends linearly on the fat content of the milk, and the increase in temperature encountered on high-pressure homogenization of milk at 150 MPa occurs at a rate of ~0.5 °C per 1% fat in milk.

Effects of High-Pressure Homogenization on Fat Globules in Milk

Like any other emulsification technique, the primary reason for the application of high-pressure homogenization in dairy technology is to reduce the size of emulsion

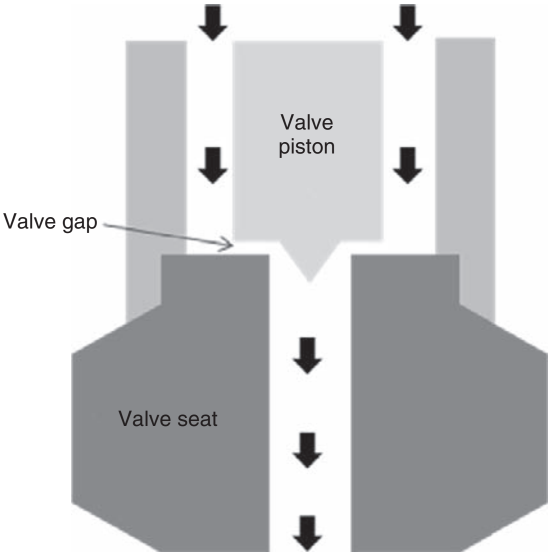

Figure 1 Schematic diagram of a homogenizing valve in a high-pressure homogenizer. The black arrows indicate the direction of flow of the product.

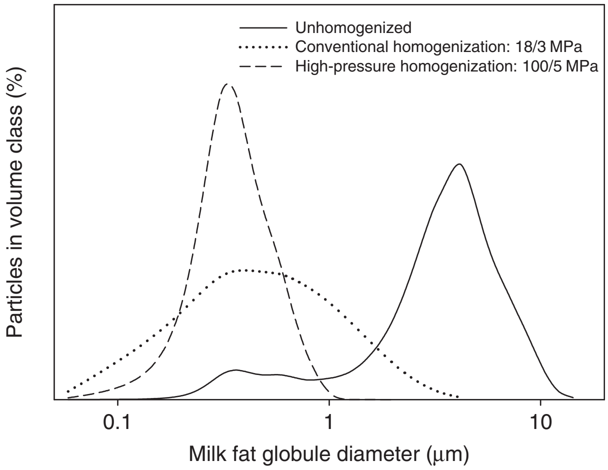

Figure 2 Volume-based particle size distributions of unhomogenized, conventionally homogenized, and high-pressure homogenized bovine milk.

droplets, that is, the milk fat globules in milk or the emulsion droplets in the pre-emulsions of ice cream mix, recombined milk, or infant formula. Not surprisingly, a major body of research has focused on this aspect. On passing through the homogenizing valve, emulsion droplets simultaneously encounter several processes: deformation and disruption of droplets, adsorption of surfactant at the newly formed interface, and collision and possible recoalescence of droplets. The balance between breakup and recoalescence of droplets determines droplet size. In general, fat globule size in milk decreases on high-pressure homogenization and the decreases in particle size that can be achieved by high-pressure homogenization are considerably greater than those achievable by conventional homogenization. The two main determinants of fat globule size in homogenized milk are homogenization pressure and the temperature at which homogenization is carried out.

As with conventional homogenization, milk fat globule size decreases with increasing homogenization pressure. At comparatively low homogenization pressures, for example, <50 MPa, conventional homogenizers are actually more effective than high-pressure homogenizers in reducing fat globule size, which is probably related to differences in the design of the homogenizing valve. However, at higher pressures, considerable further reductions in fat globule size can be achieved by high-pressure homogenization. An example thereof is given in **Figure 2**, which shows particle size distributions of unhomogenized raw whole bovine milk, of raw milk homogenized using a conventional homogenizer at (first/second stage) 18/3 MPa, and of raw milk homogenized with a high-pressure homogenizer at 100/5 MPa. Conventional homogenization reduces the median and width of the particle size distribution considerably, but is unable to achieve the narrow particle size distribution achieved by high-pressure homogenization. Typical particle size parameters for the size distribution of high-pressure homogenized milk shown in **Figure 2** are a volume-surface-weighted mean diameter (*D*[3,2]) of ~0.2 μmm and a volume-weighted mean diameter (*D*[4,3]) of ~0.5 μmm. These parameters commonly have values of ~1.0 and 4.5 μmm, respectively, in unhomogenized milk and ~0.5 and ~1.0, respectively, in conventionally homogenized milk. Further increases in homogenization pressure, up to ~200 MPa, can decrease milk fat globule size further, but the magnitude of such decreases is considerably smaller than those achieved in the 0–100 MPa region. At even greater homogenization pressure, that is, >250 MPa, milk fat globule size may actually begin to increase again. This is probably due to the fact that the newly created fat globule surface is too large to be covered immediately by surface-active material, and coalescence of the fat globules occurs.

With respect to temperature, several studies have shown that prewarming milk to >30 °C prior to high-pressure homogenization results in a considerably smaller average particle size and a narrower particle size distribution than from prewarming milk at temperatures <30 °C. This is, as for conventional homogenization, due to the fact that the milk fat should be in the liquid phase when homogenization is performed. Commonly, a minimum temperature of 45 °C is required to have milk fat completely devoid of fat crystals. As outlined previously, the rapid increase in pressure during high-pressure homogenization increases temperature considerably, in some case even far above the melting point of milk fat. However, such increases appear insufficient to ensure that the milk fat is in the liquid state when cold milk (e.g., <20 °C) is forced through the homogenizing valve, probably due to the short timescale involved in the process.

Effects of High-Pressure Homogenization on Milk Proteins

Compared to effects on the milk fat globules, effects of high-pressure homogenization on casein micelles are considerably less. High-pressure homogenization decreases the average particle size in skim milk, which is indicative of some disruption of casein micelles. However, decreases in particle size after homogenization at high pressure (e.g., ~200 MPa), even with the application of up to six repeated passes through the homogenizer, are only on the order of ~10–15%. Reductions in micelle size on high-pressure homogenization are probably due to the physical disruption of casein micelles or aggregates thereof, as the pressures and timescale involved in the process are unlikely to be sufficient to induce significant solubilization of micellar calcium phosphate, the major contributor to disruption of casein micelles on treatment of milk at high hydrostatic pressure. Homogenization of milk at pressures >250 MPa can cause increases in casein micelle size. In the presence of calcium, considerable aggregation of casein micelles can occur during high-pressure homogenization at a pressure >200 MPa. Such aggregation is probably induced by the combination of elevated calcium concentration and high temperature during homogenization.

Whey proteins are also susceptible to denaturation during high-pressure homogenization of milk. In skim milk, whey protein denaturation increases with increasing homogenization pressure and the application of a second homogenization stage. Homogenization of skim milk at a milk inlet temperature of 30 °C and a primary and secondary stage pressures of 300 and 30 MPa, respectively, can induce denaturation of β-lactoglobulin up to ~45% and denaturation of α-lactalbumin up to ~30%. The degree of denaturation achieved is probably largely due to the heat load encountered during high-pressure homogenization. For whole milk, the degree of whey protein denaturation following high-pressure homogenization is more difficult to determine due to the fact that whey proteins may, in addition to denaturing and associating with casein micelles or other whey proteins, also associate with the milk fat globule membrane during homogenization, either in their native or unfolded state. This makes interpretation of data on the denaturation of whey proteins resulting from high-pressure homogenization more difficult. In general, however, values for denaturation appear to be of the same order as those reported for skim milk.

Effects of High-Pressure Homogenization on Enzymes

In relation to homogenization of dairy products, lipases are probably the most crucial category of dairy enzymes, as it is the homogenization-induced changes in the milk fat globule membrane that facilitate access for lipases to the fatty acid core. Residual active lipases in homogenized milk can thus result in considerable lipolysis, with concomitant decreases in pH and the development of rancid flavor. As such, inactivation of lipase is crucial to ensure a good shelf life of high-pressure homogenized milk or products prepared therefrom. High-pressure homogenization at a pressure of 100 or 200 MPa and a milk inlet temperature of <40 °C does not yield complete inactivation of lipoprotein lipase in milk; in fact, treatment at this pressure at an inlet temperature of 10–30 °C appears to greatly aid the lipolysis process in milk, probably as a result of incomplete inactivation of lipase combined with greatly facilitated access of the enzyme to the milk fat. Homogenization of milk at 200 MPa at a milk inlet temperature of >40 °C appears to result in complete inactivation of lipase in milk. Such inactivation is probably primarily the result of considerable thermal load during processing.

The effect of high-pressure homogenization on the indigenous milk proteinase plasmin has also been studied in considerable detail. Studies have highlighted that plasmin is stable to inactivation on high-pressure homogenization of skim milk, but with increasing fat content of the milk, residual plasmin activity in milk appears to decrease. Likewise, reductions in plasmin activity induced by high-pressure homogenization appear to be more extensive at higher homogenization pressure and increasing inlet temperature of the milk. However, care should be taken when interpreting such data, as the methods commonly used for determining plasmin activity in milk do not account for plasmin associated with the milk fat globule membrane, which is centrifugally removed in most plasmin assays. Recent studies have shown that previously reported decreases in plasmin activity on high-pressure homogenization are most likely largely due to the association of casein micelles, where plasmin is naturally found in milk, onto the milk fat globules during homogenization. As a result, part of the plasmin is removed centrifugally prior to assay, rather than actually inactivated. Such findings are in agreement with the fact that the degree of proteolysis in high-pressure homogenized milk differs only slightly from that in untreated milk. Only at combinations of a high homogenization pressure and a high inlet temperature, for example, 200 MPa and 50 °C, does some limited (<20%) inactivation of plasmin occur. The aforementioned should be taken into account not only for plasmin, but also for other enzymes associated with the casein micelles.

Alkaline phosphatase is quite readily inactivated by high-pressure homogenization and the degree of inactivation appears to be correlated quite closely with the heat load the product encounters during processing. When milk is homogenized at an inlet temperature of 4 °C, alkaline phosphatase is not inactivated at pressures <250 MPa but homogenization at 200 MPa at inlet temperatures >20 °C induced considerable inactivation of alkaline phosphatase. Inactivation of lactoperoxidase in milk occurs on high-pressure homogenization of milk at 200 MPa at milk inlet temperatures >10 °C.

Microbiology of High-Pressure Homogenized Milk

The combination of shear, cavitation, temperature, and pressure that occurs during high-pressure homogenization not only affects the milk fat globules, but also the bacterial cells present in milk. High-pressure homogenization induces inactivation of a wide range of bacteria, with bacterial inactivation generally increasing with increases in homogenization pressure, the number of passes through the homogenizer, and the temperature at which homogenization is carried out. As shown in **Figure 3**, the inactivation of bacteria by high-pressure homogenization appears to be related to the actual physical destruction of bacterial cells. In line with this, Gram-positive bacteria have been shown to be more resistant to high-pressure homogenization than Gram-negative bacteria, due to the fact that their cell wall provides more protection to disruption on high-pressure homogenization. The viscosity, rather than the composition, of the medium has been highlighted as one of the crucial factors in determining the degree of bacterial inactivation by high-pressure homogenization. In low-viscosity media, deformation and resultant disruption of bacterial cells are readily achieved under the forces encountered during high-pressure homogenization. However, if viscosity is increased, the susceptibility of bacterial cells to disruption decreases. The influence of viscosity on bacterial disruption also, at least partially, contributes to increasing bacterial inactivation with higher inlet temperature because viscosity typically decreases with increasing temperature.

Ever since the first observations that high-pressure homogenization of milk can induce reductions in the microbial load of raw milk, the process has been suggested as a potential one-step alternative for the pasteurization and homogenization of milk. Several studies have shown that combinations of sufficiently high pressure and inlet temperature, for example, >150 MPa and >40 °C, can indeed result in degrees of microbial inactivation that are comparable to those achieved during high-temperature short-time (HTST) pasteurization processes commonly applied for the production of consumption milk. Shelf-life studies have shown that the refrigerated shelf life of such high-pressure homogenized milk is comparable to that of traditionally pasteurized milk and homogenized milk. However, incomplete inactivation of lipoprotein lipase by high-pressure homogenization can lead to significant lipolysis in high-pressure homogenized milk, as observed by increased levels of free fatty acids and reductions in pH. Milk homogenized at 300 MPa has also been shown to be more susceptible to lipid oxidation, possibly due to the fact that the extremely large fat globule surface area created at this pressure cannot be covered adequately by the amount of surface-active material present in the milk. Despite the presumed high-pressure homogenization-induced decreases in plasmin activity, levels of proteolysis in high-pressure homogenized milk are comparable to those in pasteurized milk. So from a microbiological perspective, high-pressure homogenization could indeed form a

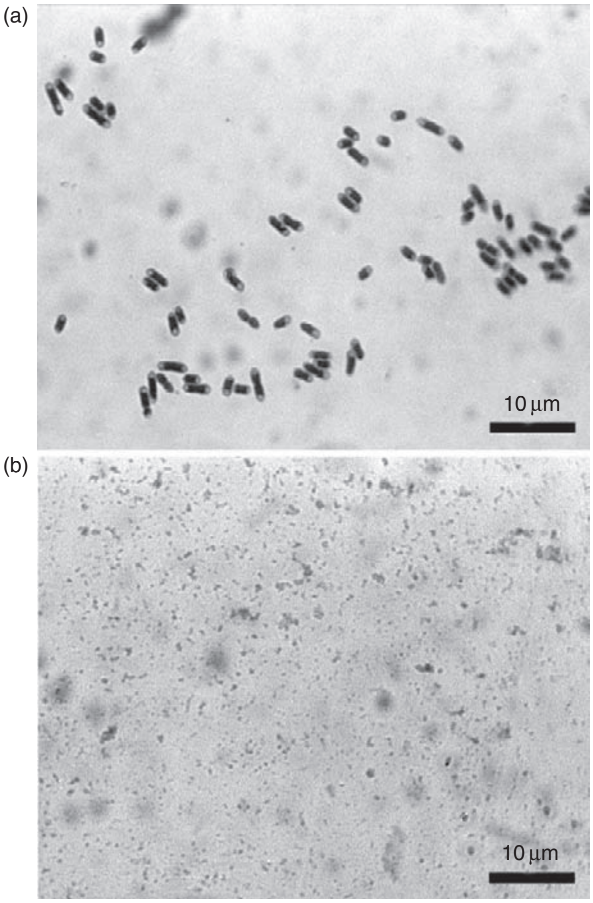

Figure 3 Light micrographs of a pseudomonad isolated from milk and suspended in a quarter-strength Ringer's solution (a) before or (b) after high-pressure homogenization at 250 MPa at 70 °C.

one-step alternative for pasteurization and homogenization of milk. However, increased levels of lipid oxidation and lipolysis in high-pressure homogenized milk are likely to create an undesirable flavor profile. Furthermore, the limited scale, high investment cost, and substantial energy use of the technology combined with the comparatively low economic margins on the final product make the application of high-pressure homogenization of milk for consumption milk unlikely in the near future.

High-Pressure Homogenization in the Manufacture of Yogurt

Homogenization is a common step in the manufacture of yogurt. The primary function of homogenization in yogurt manufacture is to improve the texture of the product. This is achieved by increasing the exposed casein surface area through the creation of a large number of casein-covered fat globules, which can then participate in the acid-coagulated milk gel. High-pressure homogenization has been shown to improve the texture of set yogurt. The firmness of set yogurt prepared from milk (3.2% protein, 3.5% fat) homogenized at 200–300 MPa at an inlet temperature of 30 or 40 °C was comparable to or greater than that of milk fortified with 3% skim milk powder, homogenized conventionally (15 MPa), and heat treated at 90 °C for 10 min. The improved textural properties of yogurt from high-pressure homogenized milk are probably largely attributable to the considerably lower milk fat globule size, and hence considerably increased casein surface area in high-pressure homogenized milk. Since whey protein denaturation during high-pressure homogenization is not complete, it is actually likely that the combination of high-pressure homogenization and the conventional high-heat treatment applied in yogurt manufacture is capable of improving gel firmness to even greater extents. Along with improvements in texture, reductions in susceptibility to syneresis have also been observed for yogurt produced from high-pressure homogenized milk, whereas fermentation times remain largely unaffected. For stirred yogurts, high-pressure homogenization of milk (3.5% fat, 3.2% protein) at 200 or 300 MPa also yields a product with improved textural properties and water-holding capacity compared to stirred yogurt prepared from the same milk fortified with 3% skim milk treated by conventional homogenization and heat treatments. In contrast, set yogurt produced from high-pressure homogenized skim milk had a lower firmness and higher susceptibility to syneresis than its counterpart produced from heated skim milk. The reduced textural properties of set yogurt from high-pressure homogenized skim milk are probably due to the considerably lower degree of whey protein denaturation compared to heated milk. These results indicate interesting opportunities for high-pressure homogenization in the manufacture of yogurt products, particularly since the added cost as a result of the process may be balanced by saving in the reduced amount of protein required to prepare a product of desirable properties.

High-Pressure Homogenization in the Manufacture of Cheese

Rennet coagulation properties of high-pressure homogenized milk have been studied by several research groups. For unheated skim milk, high-pressure homogenization causes small decreases in the time required for rennet-induced coagulation of milk to commence. For whole milk, high-pressure homogenization-induced decreases in the rennet coagulation time and increases in the rate of gel formation have been reported. However, in such studies, the pH of high-pressure homogenized milk was in many cases considerably lower, in some cases >0.3 pH units, than that of the control milk due to incomplete inactivation of lipase and resultant lipolysis. The lower pH strongly contributes to a reduced rennet coagulation time and an increased rate of gel formation. As a result, it is not possible to attribute directly the aforementioned changes in the rennet coagulation properties of milk to the actual homogenization process itself.

Cheeses made from high-pressure homogenized milk have been characterized as having a considerably higher yield and moisture content than their counterparts prepared from unhomogenized milk. Considerable lipolysis has also been reported in cheese made from high-pressure homogenized milk, probably due to the incomplete inactivation of lipase combined with the facilitated access to the triglycerides in the protein-covered fat globules in the high-pressure homogenized milk. Overall though, it is unlikely that high-pressure homogenization will find great application in cheese manufacture as the cheese curd produced from homogenized milk is generally weak and brittle due to a lack of fusion of the casein network.

See also: **Enzymes Exogenous to Milk in Dairy Technology**: Proteinases. **Homogenization of Milk**: Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Milk Lipids**: Fat Globules in Milk; Lipolysis and Hydrolytic Rancidity.

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Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification)

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Introduction

Since its invention in the late nineteenth century, homogenization has become a crucial process in the preparation of a variety of dairy products. While most homogenization processes are currently performed using so-called valve homogenizers, a number of alternative homogenization techniques are of use or potential use for the dairy industry. High-pressure valve homogenizers are covered in the article **Homogenization of Milk**: High-Pressure Homogenizers. This article focuses on alternative techniques for the preparation of either primary or secondary emulsions, that is, high-speed blending and mixing, colloid mills, microfluidization, ultrasonic homogenization, and membrane emulsification.

High-Speed Blending and Mixing Devices

High-speed blending and mixing devices are the most commonly used types of equipment for emulsifying fats or oils into an aqueous phase in the food and dairy industries. Typical applications for this purpose are, for instance, in the preparation of clinical and infant foods, recombined milk, and some ice cream mixes where a mixture of fats and oils is commonly emulsified into an aqueous phase containing proteins, carbohydrates, minerals, and other components. For high-speed blending and mixing, several types of stirrers are available, for example, blades, propellers, and turbine mixers. The design of the stirrer determines to a large extent the efficiency of the emulsification process. The rapid rotation of the stirrer generates velocity gradients in liquids, which result in the disruption of the oil–water interface, resulting in the emulsification of oil and the disruption of larger droplets into smaller ones. High-speed blending and mixing is most efficient when both horizontal and vertical flow profiles are obtained, which result in the even distribution of liquids throughout the mixing vessel. High-speed blenders and mixers typically yield a coarse pre-emulsion with emulsion droplets ranging in size from ∼2 to 20 μm. The size of emulsion droplets is subsequently further reduced to the desired values using a conventional valve homogenizer or another type of homogenizing device. Droplet size during emulsification with high-speed blending and mixing devices generally decreases with increasing time and speed of rotation, which may vary from several tens to several thousands of revolutions per minute (RPM).

The efficiency of high-speed blending and mixing devices can be improved further by using so-called rotor–stator systems, wherein the stirrer (rotor) is surrounded by a perforated stator. The high-speed rotation of the rotor blades creates a powerful suction, drawing materials upward from the bottom of the vessel and into the center of the workhead. The material is then driven toward the periphery of the workhead by centrifugal forces. Here, the material is subjected to a milling action in the clearance between the ends of the rotor blades and the inner wall of the stator. This is followed by subsequent intense hydraulic shear as the materials are forced, at high velocity, through the perforations in the stator and circulated into the main body of the mix. Rotor–stator high-shear blenders are typically employed in the production of recombined milk and ice cream mixes, as well as in the preparation of clinical and infant foods.

Colloid Mills

Colloid mills are also rotor–stator homogenizing devices that find wide application in the homogenization of medium- to high-viscosity liquid food and dairy products. Colloid mills are effective in reducing the size of emulsion droplets of coarse pre-emulsions but are not effective in emulsifying a fat phase directly into an aqueous phase. The physical principle of operation, as outlined in **Figure 1**, is based on the feeding of a liquid pre-emulsion into the colloid mill, where it flows through a narrow gap between a rotating disk (rotor) and a stationary disk (stator). Due to the shear stress in the gap between the rotor and the stator, caused by the rotation of the rotor, disruption of emulsion droplets occurs. Shear stress can be increased by increasing rotation speed, by narrowing the gap between the rotor and the stator, or by using disks that have roughened surfaces. Typically, rotation speed can vary from several hundreds to tens of thousands of RPM, whereas the thickness of the gap can vary from several tens of micrometers up to a millimeter. Reducing flow rate also increases the homogenizing effect of a colloid mill. Flow rates can typically vary between several liters per hour to tens of thousands liters per hour. Since the minimum droplet size that is achievable with a colloid mill is ∼1–5 μm, it does not find application in the

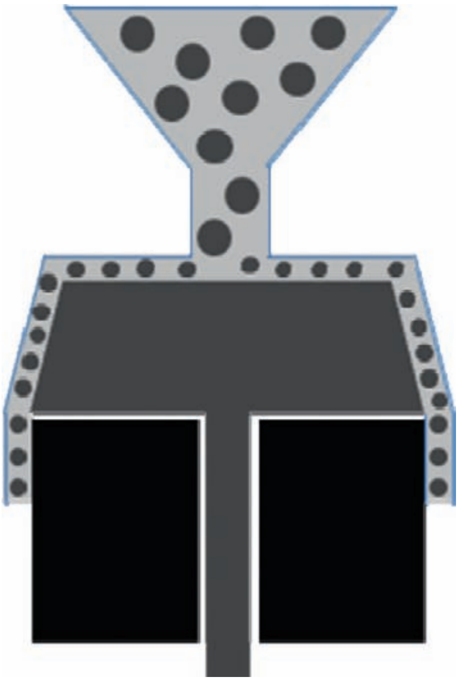

Figure 1 Schematic illustration of a colloid mill.

homogenization of milk or dairy products with the aim of reducing fat globule size. However, colloid mills are widely used in the manufacture of caseins or caseinates, where the casein curd, following milling, washing, and mixing with water, is passed through a colloid mill to prepare a homogeneous suspension prior to drying (in the case of acid or rennet casein) or reneutralization (in the case of sodium, potassium, or calcium caseinate). The use of the colloid mill for this purpose converts the casein–water mixture from a particulate suspension into a thick paste, which can subsequently be dried or neutralized.

Microfluidization

Microfluidization can be used to create emulsions with a very narrow particle size distribution, either from individual oil and aqueous phases or from an existing emulsion, for example, milk. The technology of microfluidization is based on a double-acting intensifier pump and an interaction chamber. The intensifier pump, which may be either air-driven or electric-hydraulic-driven, creates the pressure required to force the liquid into the interaction chamber. The geometry of the interaction chamber is such that the liquid is split into two streams, which are forced to collide at an angle of 180° in a reaction chamber. Pressures up to 300 MPa can be achieved using this technology. As the two streams collide in the interaction chamber, there is a rapid drop in pressure and emulsification occurs as a result of cavitation, turbulence, and shear effects that occur on impact.

Microfluidization has been successfully shown to decrease fat globule size in milk and cream and to yield narrow particle size distributions with fat globules as small as 30 μm. Particle size decreases with increasing homogenization pressure up to ~100 MPa. It should be noted that the dependence of particle size on microfluidization pressure for microfluidized milk is less than that for milk homogenized using a conventional valve homogenizer; at comparatively low pressures, microfluidization appears more effective than valve homogenization in reducing particle size but this difference becomes smaller at higher pressures. At pressures $\geq$100 MPa, little additional effect of increasing microfluidization pressure is observed on particle size in milk. In fact, particle size may even increase due to the formation of homogenization clusters. Further decreases in particle size, and particularly in the width of the particle size distribution, can be achieved by employing multiple passes through the homogenizer. Microscopic analysis of microfluidized milk has indicated that the fat globules in microfluidized milk have fewer intact casein micelles or micellar fragments at their interface than their counterparts in milk homogenized with a valve homogenizer. However, surface protein load of fat globules in microfluidized milk appears higher than in milk homogenized with a valve homogenizer, with the fat globule surface in microfluidized milk consisting primarily of casein. Fat globules that appear to be embedded in casein micelles have also been observed in microfluidized milk.

Ultrasonic Homogenization

Ultrasonic waves are defined as waves with a frequency >20 kHz, which are capable of traveling through liquids, solids, and gases. High-intensity ultrasound, that is, at a power level in the range of 10–1000 W cm^{-2} and commonly with a frequency <100 kHz, can be used for homogenization purposes. Ultrasonic homogenization uses the intense shear and pressure gradients caused by high-intensity ultrasonic waves to disrupt emulsion droplets. Such disruption is primarily due to cavitational effects. As such, ultrasonic homogenization is suitable for reducing the size of existing emulsions but not for the formation of a primary emulsion. Ultrasonic homogenization is commonly carried out at frequencies ranging from ~20 to 50 kHz. At frequencies <16 kHz, the ultrasonic waves become audible, whereas at frequencies >50 kHz, homogenization efficiency tends to decrease with increasing frequency.

Ultrasonic homogenizers are available on both laboratory and industrial scale. In laboratory-scale ultrasonic homogenizers, high-intensity ultrasonic waves are generated by either piezoelectric transducers or liquid jet generators. An ultrasonic transducer consists of a piezoelectric crystal contained in a protective metal casing.

A high-intensity electric wave is applied to the transducer, which causes the piezoelectric crystal to oscillate rapidly and generate an ultrasonic wave. This wave is directed to the tip of the transducer, where it generates intense pressure and shear gradients, primarily due to cavitational effects. These pressure and shear gradients cause liquids to be broken up into smaller fragments, which are forced to intermingle. Because the ultrasonic energy is focused on only a small volume of the sample, good mixing in the sample container is required. This is achievable with small volumes by the flow induced by the ultrasonic field, but in larger systems agitation is required. Continuous application of ultrasound causes considerable heating of the product, so intermittent cooling may be required to prevent overheating.

Laboratory-scale ultrasonic treatment of milk has indeed been shown to reduce the size of fat globules in unhomogenized milk. The degree of homogenization that can be achieved by ultrasonic homogenization of milk does not offer significant advantages over traditional valve homogenizers. In fact, to achieve results comparable to those achieved by traditional homogenizers, ultrasonic treatment for several minutes or several cycles of ultrasonic treatment are required. Treatment of milk with an ultrasonic homogenizer may also result in inactivation of enzymes and denaturation of whey proteins, but these effects appear largely related to the heat generated by the ultrasonic treatment. Ultrasonic treatment of milk has also been shown to be effective in reducing the microbial count of pathogenic and spoilage bacteria in milk, leading to suggestions of the use of ultrasonic treatment of milk as a nonthermal alternative to pasteurization of milk. The true extent of its potential for this purpose, however, remains to be elucidated in terms of both commercial relevance and product properties. With respect to product properties, the limited inactivation of indigenous milk enzymes, particularly lipoprotein lipase, presents a limitation, which needs to be dealt with.

On an industrial scale, ultrasonic jet homogenizers are available for the continuous preparation of emulsions at a scale of up to several thousand liters per minute. In ultrasonic jet homogenizers, a stream of the pre-emulsion, is forced through an orifice and made to impinge on a sharp-edged blade. This causes rapid vibration of the blade as a result of which an ultrasonic field is generated. Droplets in the immediate vicinity of this ultrasonic field are broken up as a result of the combined cavitation, shear, and turbulence.

Membrane Emulsification

Membrane emulsification involves forcing one immiscible liquid, the potential dispersed phase, into another through a membrane, usually glass, which contains a uniform pore size. As outlined in **Figure 2**, droplets are formed on the membrane surface and become emulsified in the continuous phase surrounding the membrane. Membrane emulsification can be operated in both batch mode and continuous mode. In the batch process, emulsion droplets are created by forcing the to-be-dispersed phase through a membrane that is held in a vessel containing the continuous phase. In the continuous process, the continuous phase flows through a cylindrical membrane that is located in a tube that contains the potential dispersed phase. The to-be-dispersed phase is pressurized so that it is forced through the membrane. The particle size obtained using membrane emulsification depends on the diameter of the pores in the membrane and the interfacial tensions of the two product phases. Membranes with different pore sizes are available and allow the production of emulsions with very narrow and tailored particle size distributions. The main advantages of membrane emulsification are the high energy efficiency of the system and the very narrow particle size distributions that can be achieved with this technique. However, the technique has not found application beyond the laboratory scale at present. The limited flux that can be achieved with membrane emulsification limits its applicability for dairy processing.

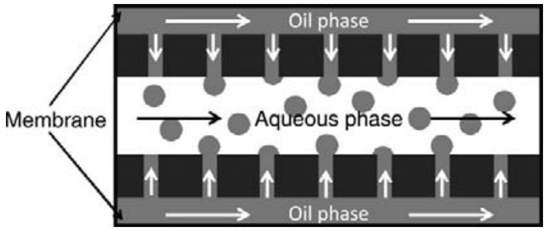

Figure 2 Schematic outline of the principle of membrane filtration.

See also: **Dehydrated Dairy Products**: Infant Formulae. **Homogenization of Milk**: High-Pressure Homogenizers; Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Liquid Milk Products**: Recombined and Reconstituted Products. **Milk Lipids**: Fat Globules in Milk.

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HORMONES IN MILK

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Introduction

Milk and honey are the only two natural animal substances with the sole purpose of being a consumed food. Milk remains the only food that is solely capable of sustaining a newborn mammal. In addition to the major components (proteins, carbohydrates, lipids, water), low or trace levels of other factors may contribute to the neonate's capacity to thrive. Among these trace components are an ever-expanding list of hormones, growth factors, and cytokines. Although the first reports of hormones in milk appeared over 75 years ago, it was not until the development of sensitive radioimmunoassays or enzyme-linked immunosorbent assays (ELISAs) that the capacity to measure hormones became common and the identification of hormones in milk expanded. Since those historical developments, many reviews have appeared on the presence of hormones and growth factors in mammary secretions, focusing mainly on rodent and bovine milks. A recent report has reviewed the impact of cow's milk on human growth and includes milk hormones in the discussion. Other reports have demonstrated that dairy products are the major source of steroid hormones in human diets. These and other reports have motivated studies on the relationship between dairy consumption and disease, with both increased risks and decreased risks being reported. A recent meeting in Boston considered this topic, but failed to arrive at a clear result. This article assesses the presence of hormones and growth factors in milk and considers some possible physiological roles in the mammary gland, the neonate, and the consuming public.

Why Are Hormones Found in Milk?

The list of hormones, growth factors, and cytokines continues to grow as more sensitive assays are devised and new 'factors' are discovered. A case in point is leptin, which was discovered 15 years ago and was first shown to occur in milk; a current search of PubMed indicates >184 articles when leptin and milk are searched. We expect the list of milk-borne regulatory factors to continue to expand. However, coming back to the question at hand, why are hormones found in milk? There are five potential reasons for the appearance of milk hormonal factors: (1) they are a means of mammary disposal; (2) hormones are autocrine/paracrine factors that have important roles in the local regulation of mammary cells; (3) the factors affect the function of the gastrointestinal tract (GIT) of the newborn; (4) the factors are absorbed by the GIT and have peripheral functions in the newborn; and (5) they do not have any function, they just result from normal biological processes involved in the secretion of milk. In addition, the potential reasons are not mutually exclusive. For example, a hormone may be functional in the mammary gland and also be effective in the GIT. Although there have been a number of scientific conferences that have approached this topic, no clear evidential concept has emerged. Perhaps the most promising evidence for the physiological function of milk hormones is that relating to colostrum-delivered cytokines and the role in immunocapacity in the neonate and effects on the GIT. Although the National Women's Health Information Center claims that breast-feeding is advantageous to the neonate, the descriptions are broadly described by the words 'may' and 'do better' and 'have slightly higher IQ tests'. However, it is difficult to establish that endocrine factors in milk are the cause of clear consequential physiological functions in the neonate or in the mammary gland. The key word here is 'consequential' because short-term effects of endocrine factors in milk may be observed to be different in some measurement functions when compared to control animals, but the latter have not been shown to be disadvantaged over time. For example, neonates who do not receive mother's milk and are fed an manufactured milk substitute formula grow and thrive without any detectable deficiencies. In addition, formula-fed infants generally gain weight faster than breast-fed neonates do, which is currently considered a health risk. In defense of an altered physiological function stemming from milk consumption, perhaps such physiological impacts are subtle and require much more time (years) to emerge. Generally, in science, subtle effects are difficult to detect or require extended time to produce notable effects and are very difficult to establish as a cause due to the complexity of milk components.

To that end, epidemiologists have taken up the challenge (subtle effects and long-term studies) and have utilized survey data (Nurse's Health Study) to evaluate the effect of milk or dairy consumption on the expression of disease. Most of the links between dietary milk and dairy products and disease (cancer, etc.) are derived from correlation studies utilizing rating scales. These studies rely on survey data obtained from individuals who list their diets (mostly from memory). Recent surveys are enhanced by the fact that they are obtained from 'professional nurses', who are better at recording or remembering their diets, but such information is less than 100% reliable. However, the accumulation of thousands of survey results over many years (starting in 1976) from numerous countries provides statistical power to diet and correlations. Nevertheless, statistical correlation works for data in which numbers are quantities of some type and it cannot be utilized for categorical data with rating scales such as glasses of milk consumed per day (0–1; 2–4; 5–7, etc.). Although such scales represent quantity, their meaning is not precise. Most statisticians avoid correlations with rating scales because the technique assumes that the differences between numbers are exactly equal.

Mechanism of Appearance of Milk Hormones

The appearance of endocrine factors in mammary secretions occurs by one or more of the eight different pathways shown in **Figure 1**. Secretion of the major milk components (proteins and fats) occurs by mechanisms 1 (apocrine; caseins and lactose) and 2 (merocrine; fat). Other proteins that are synthesized in the mammary secretory epithelial cells and have a signal sequence could also be secreted by mechanism 1. For example, both insulin-like growth factor II (IGF-II) and insulin-like growth factor-binding proteins (IGFBPs) are synthesized and secreted by mammary cells, apparently by pathway 1. Endocrine components that are fat soluble, such as steroid hormones, are known to pass biological membranes (enter cells) and have cellular binding proteins that provide retention capacity in aqueous solutions. Because of this membrane-passing capacity, the steroids found in milk are most likely of blood origin; however, mammary synthesis and processing of steroids from preformed testosterone or dehydroepiandrosterone (DHEA) has been reported. In the mammary cell, steroid hormones have signaling functions, but they are also concentrated in the fat globule and are secreted by pathway 2 into milk. Pathway 3 is associated with membrane-bound transporters that appear on the apical side of the plasma membrane and that account for the movement of ions and small molecules from the cytoplasm to the alveolar lumen. It is unlikely that this pathway contributes to endocrine factors found in mammary secretions. Pathway 4 is involved in endocytosis/transcytosis, which has a clear role in the massive movement of immunoglobulins from the circulation through the mammary cell during the formation of colostrum. Whether this is integrated into endosome packaging is not known. Clearly, this also provides a means to deliver blood-borne proteins such as transferrin and serum albumin to the colostrum or milk pool. Pathways 5 and 6 show that mammary cells form an epithelium with junctional

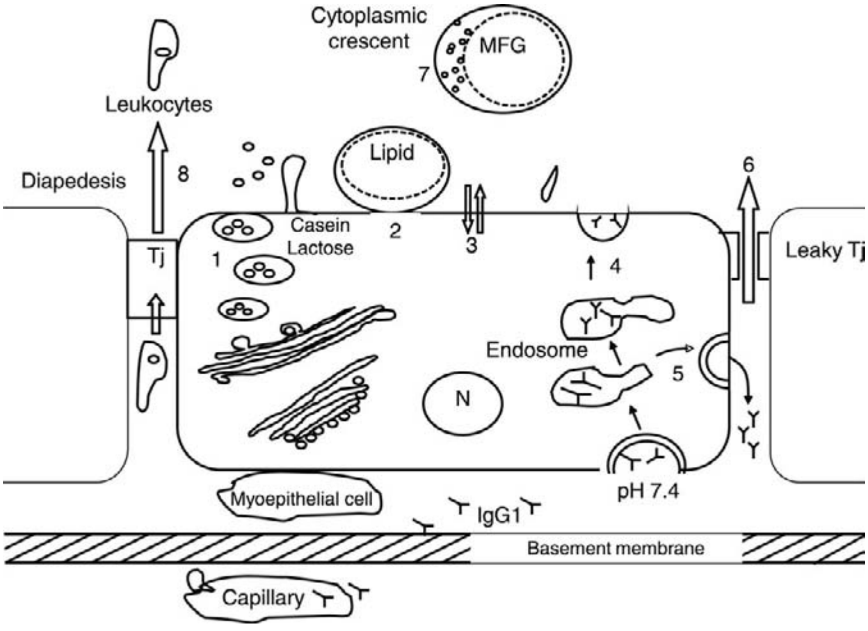

Figure 1 Model of bovine mammary cell mechanism to transfer components to the mammary gland lumen (colostrum or milk). Tj, tight junction; MFG, milk fat globule; N, nucleus. Figure drawn by Y. Wang (M.S. Physiology, Penn State University).

complexes that restrict intracellular passage of components; however, it has been long known that the junctional complexes can be leaky (as occurs in the pre-partum stage) and with infections (mastitis), thereby providing a means for blood-borne endocrine factors to penetrate the epithelial layer of cells. In addition, polymorphonucleocytes (PMNs) may move through intact tight junctions by means of diapedesis, producing a pool of PMNs (pathway 8) that are known to synthesize and secrete cytokines. Finally, the formation of cytoplasmic crescents may serve as a rather limited means of delivering components to milk (pathway 7). This contribution accounts for the appearance of components of the cytoplasm, including membranes (endoplasmic reticulum (ER)), mitochondria, and cytoplasmic enzymes, as well as the discovery that RNA is also shed into this compartment along with ribosomes and may be extracted for molecular evaluation.

Which Peptide Regulatory Factors Are Present in Milk?

Major bovine colostral and milk peptide endocrine factors are shown in **Table 1**. Although there have been multiple reports of different concentrations of some of these factors, the concentrations listed appear to represent the consensus of the reports. In bovine colostrum, many non-nutrient components appear to be derived from blood, as is the case for IgG$_1$, growth hormone (GH), prolactin (PRL), IGF-I, insulin, and glucagon. Nevertheless, these blood-originating factors may be of higher concentration than the concentration in blood, which suggests a specific transport mechanism. Because mammary epithelial cells have leaking tight junctions until parturition, and the endocrine factors are rather small (MW <24 000 daltons), it is likely that at least some factors (e.g., IGFs) leak back into the extracellular fluid and blood compartments. This is clearly the case for lactose that is synthesized pre-partum and leaks into the blood and appears in cow urine. Unfortunately, the molecular passage range (size) of leaky tight junctions is not clearly defined for bovine mammary epithelial cells.

Whatever the capacity of leaky tight junctions to allow passage of colostral or milk components through the epithelial barrier separating the extracellular fluid/blood compartment from the mammary alveolar lumen, the concentration effect observed for many hormonal factors, especially in colostrum, would require either a specific transport mechanism or some means of trapping the endocrine component, to prevent continued passage and the attainment of equilibrium. Specific transport mechanisms are clearly evident in mammary cells and are depicted in **Figure 1**. As for the trapping, many hormonal factors are known to bind to specific proteins. The best example of this may be the IGF/IGFBPs. IGF-I is not synthesized by bovine mammary cells, but a number of IGFBPs are synthesized and secreted. The transported

Table 1 Reported endocrine factors found in bovine colostrum and milk

Endocrine ligand	*Colostrum*	*Milk*
IGF-I	312–1850 ng ml^{-1}	2–10 ng ml^{-1}
IGF-II	394–1500 ng ml^{-1}	1–20 ng ml^{-1}
IGFBPs	$\sim$3000 ng ml^{-1}	$\sim$11 ng ml^{-1}
IGFBP-2	3503 od	15 od
IGFBP-3	17 097 od	51 od
IGFBP-4	135 od	7 od
IGFBP-5	114 od	3 od
Epidermal growth factor (likely betacellulin)	3 ng ml^{-1}	1.5 ng ml^{-1}
Betacellulin	2.6 ng ml^{-1}	1.93 ng ml^{-1}
Relaxin	9–19 ng ml^{-1} (pig)	
Platelet-derived growth factor	1–2 ng ml^{-1}	?
TGF-α	2.2–7.2 μg ml^{-1}	0–8.4 μg ml^{-1}
TGF-β2	74–143 ng ml^{-1}	12–21 ng ml^{-1}
TGF-β1	6.3–42.6 ng ml^{-1}	1.5–3.49 ng ml^{-1}
FGF (acidic)	?	$\sim$6 ng ml–^{1} (cheese whey)
FGF (basic)	?	$\sim$20 ng ml^{-1} (cheese whey)
Insulin	6–327 ng ml^{-1}	4–47 ng ml^{-1}
T3, T4	2 ng ml^{-1}	0.5 ng ml^{-1}
Growth hormone		
Oxytocin	?	10 ng ml^{-1}
Prolactin	500–800 ng ml^{-1}	6–8 ng ml^{-1}
Leptin	13.9 ng ml^{-1}	6.1 ng ml^{-1}

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; TGF, transforming growth factor; FGF, fibroblast growth factor; od, optical density units; ?, unknown.

IGF-I would bind to the IGFBPs, which are larger proteins, and become trapped in the colostrum mix. Furthermore, IGF-I would have little biological activity as long as it remained bound.

The greatest mass of bioactive proteins, peptides, and hormones is present in the first colostrum. A reason for this specific phenomenon may be the occurrence of leaky tight junctions (blood origin), an enhancement of transcytosis mechanisms, the transfer of other protein hormones along with the established IgG_1 transfer mechanism, and a prolonged period to concentrate other factors in the colostrum (weeks). There are species differences with respect to their colostrum contents. For example, in bovine colostrum, the concentrations of IGFs are high but the concentrations of members of the epidermal growth factor (EGF) family are low when compared to human and rat colostrum. These hormonal and growth factors are distributed among the casein fraction (minor), the whey fraction (major for protein regulatory factors), and the fat fraction (steroids). However, steroid hormones are also partitioned into the aqueous phase, as many are sulfonated (estrone sulfate) and become less metabolically active and more water soluble. Although the mechanism of appearance in milk of bioactive components synthesized by mammary epithelial cells would be expected to follow milk secretory pathways, the transfer mechanism of components originating from blood or other non-epithelial mammary cells is not well understood.

The IGF system is of significance among the various bioactive components of milk. It consists of four ligands (insulin, IGFs, and relaxin) and the corresponding high-affinity receptors, six IGFBPs that associate with the IGFs with high affinity, and five IGFBP-related proteins that bind to IGFs with approximately 10-fold lower affinity when compared with IGFBPs. Within the bovine species, IGFs and IGFBPs are highly concentrated in the prepartum secretion and in the first-milked colostrum and then decrease rapidly to low levels in mature milk. The detectable level and rank of specific IGFBPs in bovine mammary secretions are IGFBP-3 > IGFBP-2 > IGFBP-4 > IGFBP-5 (**Table 1**); the pattern of change during the lactation cycle is different from that of the circulation. Initially, the IGFBPs were thought to act principally by modulating the action of IGF by inhibiting or enhancing its binding to IGF receptors (IGFRs). More recent concepts have indicated an independent role for IGFBPs in cells. Although circulating IGFBP-3 primarily originates from hepatic cells, this protein is also produced locally in many tissues. In bovine mammary tissue, IGFBP-3 is synthesized in lactating mammary epithelial cells, and there is especially a high rate of synthesis during the involution and pre-partum periods.

IGF survives stomach acid and appears in the small intestine, especially in neonates. This survivability of IGFs is the reason why they are perhaps the most studied endocrine factors found in bovine milk. The impetus for this interest came from the finding that GH stimulated milk production without or with a low level of growth hormone receptors (GHRs) on mammary epithelial cells. However, GH increased the level of circulating IGFs in cows and there is evidence for the presence of IGFRs on mammary epithelial cells; thus the action of GH in inducing greater milk production was initially linked to increased IGFs. Current information suggests that the GH action involves multiple mechanisms, and new evidence suggests that the GHR may play a role in increased milk production via interaction with other receptors. Increases in milk IGFs were also detected for the same animal after GH administration and this suggested that this protein could be absorbed by the GIT and then appear in the circulation. Experiments with calves indicated that perhaps some dietarily supplemented IGF would be absorbed by the GIT in the pre-closure neonate, but there is little evidence for uptake after GIT closure. While the epidemiological evaluations of the Nurse's Health Study indicate changed risk for disease linked to bovine milk consumption (both increased risks and decreased risks), the consideration of mass action provides some insight into the potential systemic effects of IGFs. Human blood contains approximately 50–800 ng ml^{-1} of IGF-I, and most is bound to IGFBPs. Because there is a turnover of IGF-I in the human body, the body synthesizes and secretes (multiple sites) ~350 000 ng of IGF-I per day. Cow's milk contains 2–10 ng ml^{-1} of IGF-I (**Table 1**) and if milk is consumed at normal volumes, approximately 2000–10 000 ng of IGF-I could be consumed per day. This figure would represent less than 3% of daily body production and secretion and it does not include some destruction in the GIT and experimental evidence of no GIT absorption.

Steroid Hormones

There is growing concern among the general public about steroid hormones in milk. Dairy products have been implicated in the development of breast, uterine, and ovarian cancers; male reproductive disorders; adolescent obesity; and teenage acne. Steroid hormones were cited as the likely cause of these adverse health effects. Most of these reports were based on epidemiological studies where cause and effect cannot be proven. Until recently, most of the data on milk steroid hormones present in the literature were based on the analysis of skim milk. However, because steroid hormones are lipophilic, it is likely that they usually associate with the fat fraction of milk. Therefore, to get a true measurement of steroid concentrations in milk, it is vital to analyze whole milk as well as its fractions.

Steroid hormones appear in blood and milk in both free and conjugated forms. Only the free forms are capable of binding to receptors and eliciting biological responses. The steroids are conjugated to sulfate or glucuronide groups in the liver, which increases their solubility in water and therefore aids in disposal from the body. Nonconjugated steroids can diffuse through cell membranes, so it is not surprising that they appear in milk. However, it is not clear how the water-soluble conjugated steroids are transported into milk. It is possible that they are conjugated within the mammary epithelial cell and secreted into milk along with other milk components. **Table 2** lists individual studies that reported the concentrations of various steroids in whole milk. The number of samples analyzed in each study is included so that the reader can judge the accuracy of the reported results.

Estrogens

Estrone (E_1), 17β-estradiol (E_2), and estriol (E_3) are the primary estrogens in the bovine. By far, E_2 is the most potent in biological activity, being approximately 10 times more active than E_1. In turn, E_1 is more potent than E_3. Reported concentrations of E_2 range from nondetectable to 85 pg ml^{-1} (**Table 2**). However, most studies showed mean concentrations less than 20 pg ml^{-1}, with most mean values falling into the 1.0–6.6 pg ml^{-1} range. It is important to note the small number of samples analyzed in the majority of studies and this brings to question their accuracy. Studies with high sample numbers are impressive and their results showed that milk contains a very low concentration of E_2 (<2 pg ml^{-1}). The concentration of E_1 in milk is higher than that of E_2 and ranges from 2.1 to 130 pg ml^{-1} in the studies listed in **Table 2**. The majority of studies report E_1 concentrations less than ~30 pg ml^{-1}, but they have analyzed a small number of samples. In contrast, studies in which over 200 samples were analyzed showed mean E_1 concentrations in whole milk at <10 pg ml^{-1}; other studies have estimated that 85–96% of E_1 in milk was in the conjugated form. Conjugated E_1, primarily in the form of estrone sulfate, appears to range from 30 to 193 pg ml^{-1}; however, the small number of samples question the validity of these values. Studies with greater sample numbers showed mean estrone sulfate concentrations to be <85 pg ml^{-1}. Very little is reported on estriol concentrations in milk, with a reported mean of 9 pg ml^{-1} in 2–4 milk samples.

Concentrations of E_1, E_2, and estrone sulfate in milk begin to increase at around day 110 of gestation and continue to increase throughout pregnancy. Milk E_1 and E_2 are highly correlated with plasma E_1 and E_2, respectively, suggesting that these estrogens may diffuse passively from blood through the mammary epithelial cells and into milk. At approximately 180 days of pregnancy, milk concentration of E_2 surpasses plasma level, which suggests that there may be a specific transport mechanism, that cells within the mammary gland may begin to produce E_2, or that cells may convert other estrogens or steroids to E_2. Mammary venous blood contains greater concentrations of E_2 than arterial blood, indicating that E_2 was somehow produced in the mammary gland. Mammary tissue from late-pregnant cows converted androstenedione to E_2. This indicates that mammary tissue contains the aromatase enzyme necessary for this conversion. Also, 17β-hydroxysteroid dehydrogenase transcripts were detected in milk somatic cells, but it is not known which cell type(s) contains the transcript. This enzyme catalyzes the conversion of E_1 to E_2. The presence of aromatase and 17β-hydroxysteroid dehydrogenase may at least partially explain the higher concentration of E_2 in milk than in plasma during late pregnancy.

The GIT and the liver play an important role in steroid metabolism. Conjugation as well as 17β-hydroxysteroid

Table 2 Means and ranges for concentrations of steroid hormones in whole milk grouped by sample number

Sample n	*Estrone (E_1)* *pg ml^{-1}*	*Estradiol (E_2)* *pg ml^{-1}*	*Estrone sulfate* *pg ml^{-1}*	*Progesterone* *ng ml^{-1}*	*Testosterone* *pg ml^{-1}*	*Glucocorticoid* *ng ml^{-1}*
206		1.4				
13	11.0	1.9				
2–12	33.7–97	6.4–85	30–193			
7–8	14.1	3.0	173		4.1	
1	130	<20		9.8	<10	
54					20–120	
3					45–71	
334	4.97–6.63			12.0–13.9		
24				9.5–14.1		
44						3.0
6–30						0.25–0.7
468						0.4

dehydrogenase-mediated conversion of E_2 to a less active form of estrogen drastically reduces the amount of intestinally absorbed bioactive steroids in the first pass through the GIT and the liver. Modeling of metabolic clearance of 17β-estradiol based upon the amount of E_2 ingested by consuming a 237 ml (8 oz) glass of milk (0.33 ng) indicates much lower plasma concentrations, which reflects significant metabolism of E_2 before it reaches systemic circulation. This well-known mechanism protects against potential systemic effects of orally administered or ingested steroids.

Progesterone

Milk progesterone concentrations are in the nanogram rather than picogram range observed for most steroid hormones. Values range from 0.7 ng ml^{-1} in early lactation to 26.2 ng ml^{-1} at 90 days of pregnancy. Milk progesterone increases from 0.8 ng ml^{-1} on the day of breeding to 3.6 ng ml^{-1} on day 4 of pregnancy, 16.8 ng ml^{-1} on day 10, and 20–25 ng ml^{-1} by day 30. Therefore, milk progesterone has been used to determine pregnancy status in lactating cows. Levels remain relatively stable throughout the 210 days of pregnancy.

Progesterone concentrations are 2–5 times higher in milk than in blood and are greater in higher fat products. One reason for progesterone concentrating in milk is that it is more hydrophobic than estrogens or cortisol, and therefore it is relatively more soluble in lipids. Progesterone may simply diffuse from blood to milk and be concentrated by absorption into milk fat. It is known that progesterone concentrates in fat stores in the body. When radiolabeled progesterone was infused into cows, approximately 80% of the portion that entered milk associated with the fat fraction. It is not known if there are transport mechanisms for transferring progesterone from blood to milk or if there is mammary synthesis of progesterone.

Time of sampling has a significant effect on milk progesterone concentration. Samples collected after milking had threefold higher progesterone concentrations compared with those collected before milking. This was highly correlated ($r = 0.98$) with fat concentration. It has been reported that the place of storage within the mammary gland affects progesterone concentration and this was not independent of fat content. Alveolar milk contains more progesterone than does cisternal milk. Therefore, the time of sample collection and timing and method of udder preparation can significantly affect the concentration of milk progesterone. This reinforces the need for consistency in collecting samples.

Few studies have measured progesterone in commercial milk. As mentioned earlier, progesterone concentration is greater in higher fat dairy products. Unprocessed whole milk, skim milk, and cream contained 11.3, 4.6, and 58.7 ng ml^{-1} progesterone, respectively. Values reported for pasteurized/homogenized dairy products were similar in magnitude with whole milk, skim milk, and cream containing 12.5, 1.4, and 43 ng ml^{-1} progesterone, respectively. More recently, analysis of 334 commercial whole milk samples showed mean progesterone concentrations ranging from 12.0 to 13.9 ng ml^{-1}.

Androgens

There is very little information in the literature about androgens in milk. Reported values for testosterone ranged from <0.03 to approximately 120 pg ml^{-1}. Androstenedione has also been measured, but the studies are limited in number, and there is little agreement in reported values. Testosterone concentrations are higher in plasma than in milk, whereas androstenedione levels are higher in milk. It appears that values of both hormones may increase over the course of pregnancy.

Glucocorticoids

Glucocorticoid concentration in milk ranges from 0.25 to 3.5 ng ml^{-1} and is lower than levels observed in serum. Some studies indicate that corticosterone is the predominant glucocorticoid in milk, whereas others report that cortisol is the primary glucocorticoid. Most studies report concentrations of cortisol. Whole and skim milk contain similar concentrations, indicating that this steroid does not preferentially associate with the fat fraction. Concentrations of milk glucocorticoids increase around the time of parturition but return to baseline within 1 week. Most studies report data from relatively few animals. Of note, however, are the experiments that measured milk cortisol in 468 cows in modern milking parlors and reported a mean concentration of 0.4 ng ml^{-1}. This study concluded that the milking parlor is not a stressful environment for cows accustomed to it. Other studies attempted to use milk cortisol to evaluate transportation stress in cows and reported that milk and plasma concentrations were correlated ($r = 0.91$) but that concentrations varied widely between animals, limiting its use as a diagnostic tool. The high correlation between milk and plasma concentrations indicates that cortisol enters milk via a simple diffusion mechanism. Various time and temperature combinations of pasteurization and homogenization had no effect on the concentration of milk cortisol.

See also: **Lactation**: Galactopoiesis, Effects of Hormones and Growth Factors; Galactopoiesis, Effect of Treatment with Bovine Somatotropin; Lactogenesis. **Mammary**

Gland, Milk Biosynthesis and Secretion: Milk Fat; Secretion of Milk Constituents. **Milk Lipids**: Fat Globules in Milk. **Milk Protein Products**: Bioactive Peptides. **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Colon Cancer Prevention. **Reproduction, Events and Management**: Pregnancy: Parturition.

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HUSBANDRY OF DAIRY ANIMALS

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Buffalo: Asia

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Introduction

Tropical Asia is the homeland for slightly more than 169.4 million of the total world population of 178.2 million domestic buffaloes (*Bubalus bubalis*) estimated in 2006. Domestic buffaloes in Asia are of two types: the swamp type for draught in the eastern half of Asia and the river type for milk in the western half of Asia. The river-type buffaloes, the once-neglected farm species, currently produce about 73.2 million tonnes of milk annually from some of the world's best buffalo breeds in India and Pakistan. They breed throughout the year, conceive at 250–275 kg body weight, calve for the first time at 3–5 years of age following a gestation period of 305–320 days, and produce two calves every 3 years. Lactating animals are fed mainly on straw, crop residues, and mineral supplements such as urea–molasses–mineral block (UMMB). In most rural areas, animals are hand-milked twice daily and the calf is used to stimulate milk letdown. The lactation period is 200–300 days with first lactation milk yields of 1500–1800 kg. Breeding females are retained in the herd until about the ninth lactation (16 years of age) with reasonable economic returns. River buffaloes are vulnerable to most infectious and metabolic diseases affecting cattle. In India and Pakistan, milk is marketed through a network of milk cooperatives, which guarantee a stable price throughout the year for the farmer. Buffalo milk contains twice as much butterfat as cow milk. Besides ghee, several other products are manufactured from buffalo milk, such as butter, cheese, full cream milk powder, skim milk powder, and infant milk powder. Thus, the domestic buffalo is emerging as an alternative source for the manufacture of dairy products worldwide.

Buffalo Species, Types, Population

The term 'buffalo' refers to three species in the family Bovidae. The African buffalo (*Syncerus caffer*) and the North American buffalo (*Bison bison*) have yet to be domesticated. On the contrary, the Asian buffalo (*B. bubalis*) was domesticated around the same time in history as cattle for draft power, milk, and meat (**Figure 1**). The domestic

Figure 1 Buffaloes plowing in a paddy field – Myanmar.

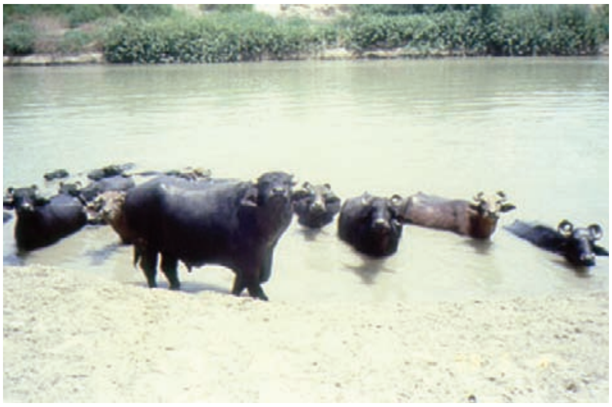

Figure 2 Wallowing behavior of the river and swamp buffaloes.

buffalo is also known as the 'water buffalo' because of its fondness to cool itself in water (**Figure 2**).

The two types of domesticated water buffaloes found in Asia, that is, the river and the swamp type, differ in their wallowing habits, chromosome numbers, and physical features (**Table 1**). The river buffalo makes up nearly 75% of the buffalo population in Asia. The dairy breeds of river buffaloes are the Murrah, Nili-Ravi, and Surti in India and the Nili-Ravi and Kundi in Pakistan.

Breeding Management

Buffaloes are polyestrus, breeding throughout the year, but the calving pattern is influenced by rainfall, feed supply, ambient temperature, and photoperiod. In India and Pakistan, most buffaloes calve between November and March. Although natural mating is the most common method of breeding, artificial insemination (AI) is also practiced.

Puberty

The buffalo attains puberty between 1.5 and 3.0 years of age. On recommended levels of nutrition, most conceptions occur when the female weighs 250–275 kg, which is usually achieved at 24–36 months of age. In the male, viable spermatozoa appear at about 24 months of age.

Estrous Cycle

The estrous cycle length is about 21 days with estrus lasting 12–30 h and ovulation occurring spontaneously after the end of estrus (**Table 2**). However, factors such as climate, temperature, photoperiod, and nutrition have been shown to influence the length of estrous cycle and also the extent of estrus manifestation.

Unlike cattle, overt signs of estrus are not pronounced. In most smallholder farms, a male buffalo may not be available for estrus detection. Homosexual behavior or standing to be mounted by another female is observed only occasionally in the buffalo. As a result, most inseminations are based on less-reliable signs such as clear vulval discharge, restlessness, frequent urination, vocalization,

Table 1 Some characteristics of swamp and river buffaloes

Characteristics	*Swamp buffalo*	*River buffalo*
Location	Eastern half of Asia	Western half of Asia
Countries	Burma, China, Indonesia, Malaysia, the Philippines, Thailand, and Vietnam	India, Pakistan, Iran, Iraq, Nepal, Sri Lanka, and Bangladesh
Horns	Grow outward and curve in a semicircle but remain on the plane of the forehead	Grow downward and backward
Breed(s)	Single breed	Seven recognized breeds, for example, Murrah, Nili-Ravi, and Surti
Wallowing habits	Swamps	Clean running water, for example, rivers or streams
Chromosome number (2*n*)	48	50
Purposes	Draft and meat	Milk and meat

Table 2 Reproductive parameters of buffalo and cattle

Parameter	*River buffalo*	*Cattle*
Sexual season	Polyestrus	Polyestrus
Age at puberty (months)	15–36	10–24
Estrous cycle		
Length (days)	18–22	14–29
Estrus (h)	12–30	17–24
Gestation length (days)	305–320	278–293
Age at first calving (months)	36–56	24–36
Calving intervals (months)	15–21	12–14
Ejaculate volume (ml)	3–6	4–10
Sperm concentration (10^6 ml^{-1})	300–1500	800–2000
First-service conception rate (frozen semen) (%)	10–50	45–75

Reproduced from Jainudeen MR and Hafez ESE (2000) Cattle and buffalo. In: Hafez B and Hafez ESE (eds.) *Reproduction in Farm Animals*, 7th edn, pp. 159–171. Baltimore: Lippincott Williams & Wilkins.

and reduction in milk. Estrus commences toward late evening with peak sexual activity at night.

Artificial Insemination

Since the early 1950s, AI has been practiced in the river buffalo in the Indian subcontinent, but its progress has been very slow because of the difficulty in detecting estrus and low conception rates in smallholder farms.

Buffalo semen is routinely collected in AI centers using an artificial vagina (AV), similar in design to that of cattle. Ejaculate volume and concentration of semen are lower in buffalo than in cattle. Techniques of semen evaluation, processing, and cryopreservation are as in cattle with minor modifications.

AI centers in India and Pakistan provide an AI service with either chilled or frozen semen. In Pakistan, an AI network consisting of more than 140 main centers and about 400 subcenters provides more than three million inseminations annually.

Most inseminations are usually performed between 12 and 24 h from the onset of estrus. At this time, the cervix is sufficiently dilated for the deposition of semen in the uterine body with the same insemination equipment as for cattle.

Both India and Pakistan export frozen semen to upgrade or crossbreed indigenous buffaloes in Thailand, China, and the Philippines.

Embryo Transfer Technology

Several countries are engaged in developing embryo transfer (ET) technology in the buffalo. The basic principles of ET technology in cattle are applicable to buffalo except that embryos are collected from the uterus on day 5 of the cycle instead of on day 7 or 8 adopted in cattle. Also the pregnancy rates have been less than 10% in Bulgaria and India, as compared with 50–70% in dairy cattle. Previous studies have shown that the superovulatory response to gonadotropins is comparable to that of cattle. However, low embryo recovery rates were not necessarily due to poor superovulatory responses, but instead due to failure of oocytes to enter the oviduct and/or impairment of embryo transport in the reproductive tract.

In vitro fertilization (IVF) of buffalo oocytes is an alternative to superovulation. Several laboratories have produced buffalo embryos by IVF. In 1997, the first IVF buffalo calf was born in India. Since oocytes can be collected at slaughter from high-producing buffaloes at the end of their lactation (see section 'Feeding the Lactating Buffalo'), IVF has potential applications in Pakistan and India.

Gestation

Gestation is longer in buffalo than in cattle, varying from 305 to 320 days for the river buffalo and from 320 to 340 days for the swamp buffalo. Pregnancy is routinely diagnosed by rectal palpation of the uterus from about 40 to 45 days following insemination. Transrectal real-time ultrasound scanning to determine early-stage pregnancy and fetometry have also been used.

Parturition

The birth process is similar to that of cattle. The fetus is delivered in anterior presentation with fully extended limbs, and fetal membranes are expelled 4–5 h later. Twinning is rare, and the incidence is less than 1 per 1000 births. Birth weights range from 26 to 35 kg, with male calves weighing 2–3 kg heavier than female calves.

Postpartum Period

After calving, the first estrus and ovulation occur at about 60 and 90 days, respectively, in well-managed herds. Postpartum anestrus or failure to resume estrous cycles after calving remains a major problem contributing to long calving intervals.

Fertility

Conception rates based on the nonreturn rates to AI are inaccurate, because of the inherent difficulty in detecting estrus (see section 'Artificial Insemination'). Pregnancy rates, based on rectal palpation, usually range from 50 to 60% with chilled semen, 25 to 45% with frozen semen, and more than 60% for hand matings.

A buffalo usually produces, on average, two calves every 3 years. However, in well-managed herds, calving intervals of 14–15 months have been achieved.

Several southeast Asian countries have embarked upon crossbreeding the indigenous swamp buffalo with the river buffalo. The F1 crossbreds (river × swamp) and F2 offspring possess an intermediate karyotype of $2n = 49$. Unlike other mammalian hybrids possessing chromosome complements differing from their parents, both male and female hybrids are fertile. As a matter of fact, the F1 crossbreds had improved body growth, larger body size, and better draft power and milk yield (4.0 kg day^{-1}) than the local buffaloes.

Reproductive Management

As mentioned previously, seasonal calving patterns in buffaloes have been attributed to ambient temperature, photoperiod, and feed supply. In India and Pakistan, buffaloes calving in summer or fall resume ovarian cyclicity earlier than those calving in winter or spring. Perhaps decreasing day length and cooler ambient temperatures favor cyclicity.

In the past, 'silent estrus' – ovulation not preceded by estrus – was believed to be a major problem in buffalo breeding, but recent hormonal studies have revealed that it is due to the farmer's inability to detect estrus.

Improvements in nutrition could increase growth rates and hasten the onset of puberty. Similarly, early weaning, induction of estrus with prostaglandin (PG)- or progesterone-releasing intravaginal devices, and better nutrition have hastened the resumption of early postpartum ovarian activity and reduced the calving-to-conception intervals. Induction of estrus with synthetic analogues of PG_{2a} and fixed-time insemination with frozen semen may prove useful in restricting mating seasons so that calving occurs when water and green feed are abundant.

Male buffaloes show marked seasonal fluctuations in libido and semen quality, which may be overcome by providing cooling facilities during the hot season. In addition, females could be inseminated with semen collected and cryopreserved during the cooler months.

Most reproductive management programs adopted for cattle can be effectively applied for the buffalo, but the commercial and smallholder farmers have not realized the benefits of such programs.

Feeding Management

Many Asian countries have limited feed resources for feeding their buffaloes. The available resources are essentially tropical pastures (both green and mature), straw, and crop residues, which are generally low in protein.

Feeding the Calf

Two systems are practiced for rearing buffalo calves. In smallholder farms, calves are allowed to suckle their dams to stimulate milk letdown and then allowed to suck 1–2 l of milk. As they grow older, suckling time is gradually reduced and replaced by grass and small quantities of concentrate. Beyond 4–6 weeks of age, the calf is used only for milk letdown. In commercial farms, calves are weaned at birth and managed as for dairy calves. Often male calves are neglected and die of starvation.

Feeding the Lactating Buffalo

Feeding systems of buffaloes for milk can be broadly classified as (1) extensive, (2) semi-intensive, and (3) the intensive system. The second system is most common, with animals tethered in the farmer's backyard and fed mainly on cut fodder and crop residues. Lactating animals receive 0.5 kg concentrate mixture per liter of milk produced.

Large herds of high-producing buffaloes are located near big cities in India and Pakistan. These animals, purchased from the villages immediately after calving, are transported to cities where they are confined in large holding areas and fed with dry fodder and large quantities of discarded bread and other preparations made of flour. 'Dry' animals are sent to the abattoir since it is uneconomical to transport them back to their original villages.

Nutritional Requirements

The energy and protein requirements have been established for maintenance and milk production for the river buffalo (**Table 3**). There is no physiological need for concentrate feed to maintain butterfat content that is about twice as much as cow milk. Feeding concentrates increases milk fat content as high as 15%, since the

Table 3 Metabolizable energy and digestible crude protein (DCP) requirements for maintenance and milk production of the river buffalo

Parameter	*Measured values*
Metabolizable energy	
Dry and lactating buffalo (kcal kg $W^{-0.75}$)	97.8–188.8
Milk production (kcal kg $W^{-0.75}$ 4% fat-corrected milk (FCM))	1171–1863
Digestible crude protein (DCP)	
Dry and lactating animals (g kg $W^{-0.75}$)	1.28–3.48
Milk production (g per100 g of protein in milk)	126.6–166.34

Reproduced from Mudgal VD (1988) Energy and protein requirements for dairy buffaloes. In: Nagarcenkar R (ed.) A Compendium of Latest Research Based on Indian Studies, pp. 130–141. New Delhi: Indian Council of Agriculture Research and Ranjhan SK (1998) Text Book on Buffalo Production, 4th edn, 397pp. New Delhi: Vikas Publishing House.

buffalo releases unwanted fat into the milk and stores only a minimum in body tissues.

Utilization of Crop Residues

Several physiological and physical factors contribute to the buffalo's ability to utilize poor quality roughage and crop residues. Among these factors are the large rumen volume, high rate of salivation, slower rate of passage of digesta through the reticulorumen, slow rumen motility, and higher cellular activity.

The dry matter intake and digestibility of roughage can be improved by supplementing with a mixture of urea–molasses. The mixture is available as a block lick – UMMB. This block supplies fermentable energy, bypass protein, and macro- and microminerals to make the rumen microflora and microfauna more efficient in digesting roughages. Buffaloes fed these supplements show better body condition, shorter calving intervals, and higher milk yields.

Milk Harvesting and Storage

The annual production of buffalo milk in the Asia–Pacific region exceeds 55 million tonnes (see **Table 4**), with India and Pakistan contributing more than 50 million tonnes (**Figure 3**). Almost all the milk is produced in smallholder farms.

Milking Technique

Milk letdown is slower in buffalo than in cattle. The presence of the calf initiates the milk letdown reflex. In most smallholder farms, animals are hand-milked and the calf is used to stimulate milk letdown, whereas in big herds in India and Pakistan, they are machine-milked as for cattle. Normally, buffaloes are milked twice a day.

Table 4 The domestic buffalo's contribution to milk and meat production in Asia

Country	*Nos. (10^6)*	*Milk (10^6 MT)*	*Meat (10^6 MT)*
River type			
Bangladesh	0.854	0.022	0.004
India	92.090	35.340	1.403
Iran	0.465	0.169	0.011
Iraq	0.065	0.190	0.001
Nepal	3.419	0.729	0.117
Pakistan	21.213	16.456	0.603
Sri Lanka	0.721	0.067	0.005
Swamp type			
Cambodia	0.694		0.013
China[a]	20.818	2.300	0.242
Indonesia	3.145		0.053
Laos	1.286		0.016
Malaysia	0.150	0.010	0.004
Myanmar	2.379	0.104	0.020
Philippines	3.006	0.018	0.051
Thailand	4.200		0.061
Vietnam	3.000	0.031	0.105
Asia	158.032	55.356	2.713
World	163.134	57.353	2.933

[a] China has a population of about 150 000 crossbred buffaloes (river × swamp buffalo).

Reproduced from FAOSTAT (1999) Food and Agricultural Commodities Production. Countries by Commodities.http://faostat.fao.org/site/339/default.aspx.

Milk Yield

The lactation length is about 300 days in the Murrah breed and about 320 days in the Nili-Ravi breed. Milk yields range from 1500 to 1800 kg for the first lactation with a steady increase to a peak in the fourth lactation, and are then maintained at peak levels until the ninth lactation. Thus, a buffalo could be retained in the herd up to about the ninth lactation (16 years of age) with reasonable economic returns.

With selective breeding, improved management, and the establishment of more dairy herds, milk yields are

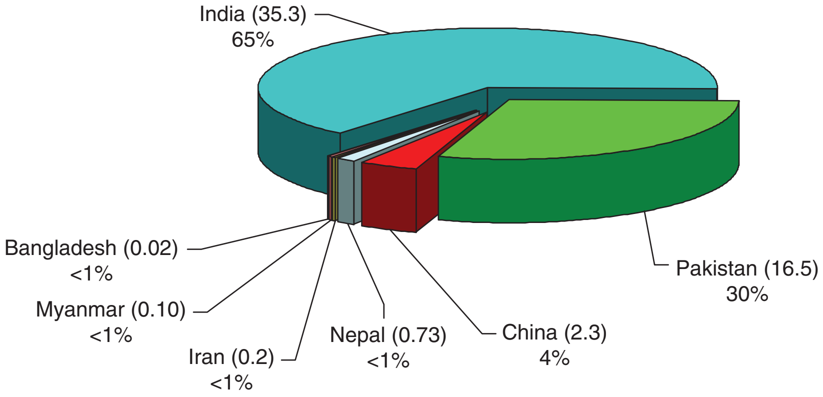

Figure 3 Major producers of buffalo milk in Asia (in million MT). Reproduced from FAOSTAT (1999) Food and Agricultural Commodities Production. Countries by Commodities.http://faostat.fao.org/site/339/default.aspx.

increasing. The individual 3000-l-per-lactation female, considered a record 30 years ago, is now common. There are many that yield 4000 l in a lactation period of 300 days – some have even attained 5000 l.

Most Asians consume buffalo milk in liquid form. Surplus milk is processed into butter, ghee, condensed milk, curd, and cheese. Dairy products, made from cow's milk, are also produced from buffalo milk in modern dairy plants.

The dairy industry has grown from small creameries to large dairy plants supported by thousands of small farmers who supply between 5 and 10 l of milk per day.

Milk Marketing

The rapid expansion of the buffalo dairy industry in the past two decades can be attributed to the Cooperative Milk Marketing model, first developed in Gujarat, India (**Table 5**), and then adopted by other states in India and Pakistan. In this model, the smallholder farmer is guaranteed a stable price for milk throughout the year, eliminating the middleman from the profits. In addition, these cooperatives provide loans to farmers to purchase superior animals, sell animal feed, and provide a routine veterinary and AI service. Their extension programs help producers to increase production and reduce costs.

Table 5 Some statistics of the buffalo dairy industry in Gujarat, India (1999–2000)

Members: District Cooperative Milk Producers' Union	12
No. of producer members	211 755
No. of village societies	10 411
Daily milk handling capacity (10^6 l)	6.7
Total milk collection (1999–2000) (10^6 l)	1586
Average daily milk collection (10^6 l)	43.46
Milk drying capacity (tonnes per day)	450
Feed manufacturing capacity (tonnes per day)	1450
Sales turnover (US$ million)	500

Reproduced from Gujarat Cooperative Milk Marketing Federation, Anand, India.

Composition and Nutritive Value

Few differences exist between buffalo and cattle in the nutritive value of milk and milk products. The milk of the buffalo is healthy as it is richer in saturated fatty acids. The lower water and higher fat contents make buffalo milk better suited for the manufacture of fat-based and solid non-fat (SNF)-based milk products, such as cheese, butterfat, many kinds of traditional pastries, candies as well as ice cream, ghee, and milk powder (**Table 6**). In fact, swamp buffalo milk has higher fat (9–15%), protein (7.1%), lactose (4.9%), and ash (0.89%) contents. In general, calcium, iron, and phosphorus contents are higher in buffalo milk than in cow milk. The lower cholesterol content in buffalo milk should make it more popular than cow milk in the health-conscious public.

Table 6 Composition of milk (g l^{-1}) of river buffalo and cow

Constituent	*Buffalo milk*	*Cow milk*
Water	820	870
Total solids	172	125
Lactose	55	46
Proteins	44	33
Fat	75	36
Cholesterol (mg g^{-1})	0.65	3.14

Reproduced from Rajorhia GS (1988) Dairy technology applied to buffalo milk. In: Bhatt PN (ed.) Invited Papers and Special Lectures Proceedings, Vol. II, Part II, pp. 624–640. New Delhi: Indian Council of Agriculture Research and Ganguli NC (1992) Milk processing and marketing. In: Tulloh NM and Holmes JHG (eds.) Buffalo Production in Subseries: Production – System Approach World Animal Science C6, pp. 393–411. London: Elsevier.

Unlike the cow, the buffalo converts the yellow pigment beta-carotene into vitamin A, which is colorless, and is passed on to milk. Therefore, buffalo milk is distinctively whiter than cow milk; not only cow milk is pale creamish-yellow but also the milk fat is golden yellow.

Proteins of buffalo milk, particularly the whey proteins, are more resistant to heat denaturation than those of cow milk. Dried milk products prepared from buffalo milk exhibit higher levels of undenatured proteins when processed under similar conditions.

Ultra-heat-treated (UHT)-processed buffalo milk and cream are intrinsically whiter and more viscous than their cow milk counterparts, because greater levels of calcium and phosphorus are converted into the colloidal form.

Milk Products

Ghee accounts for about 45% of the total milk produced in India. Ghee is clarified butterfat and contains about 99% of milk fat. Ghee from buffalo milk has no color, unlike ghee from cattle, which is golden yellow due to the presence of carotenoids as stated earlier. Ghee is the only source of animal fat in the vegetarian diet of the human population in India.

Cheese made from buffalo milk displays typical body and textural characteristics. For the manufacture of Mozzarella cheese, buffalo milk is preferred to cow milk. Certain traditional cheese varieties, such as paneer in India or pickled cheeses from the Middle East countries, are best made from buffalo milk.

Amul is a cooperative factory in Gujarat that produces a range of milk products exclusively manufactured from buffalo milk. The products include butter, full cream milk powder, skim milk powder, ghee, infant milk powder, cheese, chocolates, ice cream, and nutramul. Amul products are exported to the United States, New Zealand, and the Gulf states. The sale figures for Amul's butter have increased from 1000 tonnes a year in 1966 to more than 25 000 tonnes a year in 1997.

Health Management

Contrary to the popular belief that domestic buffaloes thrive in the harsh, humid conditions in the tropics, they are susceptible to thermal stress, infectious diseases, and disorders similar to cattle.

Thermal Stress

With less than one-tenth the density of sweat glands of cattle, the domestic buffalo's ability to sweat and lose heat through evaporative cooling is significantly diminished. In addition, their dark body coat promotes heat absorption from the direct rays of the sun, whereas the thick epidermal layer prevents heat dissipation through conduction and radiation. Thus, the domestic buffalo is more sensitive than cattle to direct solar radiation and high ambient temperatures during the summer months.

Thermal stress may lead to higher calf mortality, lower milk yields, slow growth, and depressed signs of estrus. Thermal stress can be reduced by providing cooling facilities such as shade and wallows and sprinkling water on to the skin during the hotter part of the day, and feeding roughage during the night.

Table 7 Common diseases/disorders of the domestic buffalo in Asia

Etiology	*Diseases/disorders*
Viral	Rinderpest, foot-and-mouth disease, malignant catarrhal fever
Bacterial	Hemorrhagic septicemia (*Pasteurella multocida*), Johne's disease (*Mycobacterium paratuberculosis*), tuberculosis (*Mycobacterium bovis*), mastitis (*Staphylococcus* and *Streptococcus* spp., *Escherichia coli*, *Corynebacterium pyogenes*)
Parasitic	Hemoprotozoan: *Anaplasma*, *Babesia*, *Theileria*, *Trypanosoma*, and *Schistosoma* species
	Gastrointestinal nematodes: *Haemonchus contortus*, *Toxocara vitulorum*, liver fluke *Fasciola gigantica*, *F. hepatica*
	Ectoparasites: Tick infestation (*Boophilus microplus*, *B. annulata*), mange (*Sarcoptes scabiei*, *Psoroptes* sp.)
Metabolic disorders	Hypocalcemia (milk fever), hypoglycemia (ketosis), hypomagnesemia, hypophosphatemia, selenium toxicity, bracken fern poisoning
Abortion, retention of fetal membranes, repeat breeding	Brucellosis (*Brucella abortus*), vibriosis (*Campylobacter fetus*), trichomoniasis (*Trichomonas fetus*), leptospirosis (*Leptospirosis pomona* and *L. hardjo*)
Vaginal, uterine, and ovarian disorders	Prepartum vaginal prolapse, postpartum uterine prolapse, puerperal metritis, endometritis, cystic ovaries, delayed resumption of ovarian cycles

Reproduced from Adlakha SC and Sharma SN (1992) Infectious diseases. In: Tulloh NM and Holmes JHG (eds.) Buffalo Production in Subseries: Production – System Approach World Animal Science C6, pp. 271–303. London: Elsevier.

Infectious Diseases

River buffaloes are susceptible to most diseases affecting cattle (**Table 7**). Compared with cattle, buffaloes show greater resistance to foot-and-mouth disease (FMD) and brucellosis but have a higher incidence of parasitic diseases because of their wallowing habits.

The dairy buffalo is as susceptible to mastitis as the dairy cow. Bacteria causing mastitis and their treatment and control are similar to those for cattle.

There is a high incidence of calf mortality caused by *Toxocara vitulorum*, virulent strains of *Escherichia coli*, and rota and corona viruses. Larvae of *T. vitulorum* are transmitted from the dam to the calf through the milk during the first month of life.

Puerperal metritis and retained fetal membranes occur in the buffalo. The high incidence of metritis and other genital infections has been partly attributed to the unhygienic practice of dilating the vagina either by inserting objects or by blowing air for stimulating milk letdown.

Metabolic Disorders

High-milk-producing river buffaloes are as susceptible to metabolic disorders as dairy cows. Apparently, the etiology is similar because affected buffaloes respond to therapy and control the same way as dairy cows.

See also: **Animals that Produce Dairy Foods**: Water Buffalo. **Gamete and Embryo Technology**: Artificial Insemination; *In Vitro* Fertilization; Multiple Ovulation and Embryo Transfer. **Husbandry of Dairy Animals**: Buffalo: Mediterranean Region. **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens.

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Buffalo: Mediterranean Region

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Introduction

The buffaloes reared in the Mediterranean region are the Asian buffalo or water buffalo, i.e. *Bubalus bubalis*. This species includes two types: (1) the river type, with 50 chromosomes, with an adult male weight ranging between 450 and 1000 kg, and annual milk production of 1000–3000 kg; and (2) the swamp type, with 48 chromosomes, with an adult male weight of 325–450 kg, and annual milk production up to 600 kg. While the major purpose of the river buffalo is milk, the swamp buffalo is reared mainly for draught.

Only 3% of the world buffalo population is reared in the Mediterranean region, which includes a few countries of Europe and the Near East; these buffaloes are all of the river type. The number of buffaloes in the Mediterranean region declined during the twentieth century for three reasons: Holsteinization of dairy cows, mechanization and the poor market demand of buffalo products.

Significant numbers of buffaloes (over 100 000) are at present found only in Italy, Romania, Egypt, Turkey, Azerbaijan, Iraq and Iran. In Bulgaria the number of buffaloes has decreased to fewer than 20 000. In all these countries, buffaloes represent only a very small portion of total livestock, except in Egypt, where buffaloes are more numerous than cattle. In the last 15 years, because of the strong market demand for buffalo cheese and as a consequence of the 'milk quotas' restriction imposed by the European Union, the number of buffaloes has increased in Italy. In Egypt, Iran and Azerbaijan also, there is a preference for buffalo dairy products compared to cows' milk products. During the 1990s, buffaloes have been imported to Germany, the United Kingdom and the Netherlands, where a few milk-producing herds can be found.

Buffaloes of the Mediterranean region differ phenotypically from country to country because of the different environment and management practices and because no exchange of genetic material between countries has occurred, except in Bulgaria, where crossbreeding with the Murrah breed was carried out by importing in 1962 a significant number of animals from India. Major morphological differences between the buffalo populations of different countries include: (1) the variable size, ranging between a minimum of 280 and 300 kg liveweight for adult females and males, respectively, in Egypt to a maximum of 900 and 1000 kg in Iraq, the most frequent weights being 600 and 800 kg; (2) the shape of the horns; and (3) the coat color, from dark-gray and dark-brown to black, showing white spots in some cases.

Breeding Management

The main objective of breeding buffaloes in Europe and the Near East is milk production. Everywhere in this region, their milk is sold at a higher price than cows' milk.

Average herd size (number of females of breeding age) is below eight in the whole region, except Italy (90), Syria (35) and Iran (34). The proportion of breeding females to total buffaloes is 50% in all countries, with a minimum of 33% in Azerbaijan, where a dual-purpose line of buffaloes is maintained, and a maximum of 62% in Italy, where males have no market. In countries where the majority of buffaloes are reared in very small herds there are also a few bigger private, cooperative or state herds (Bulgaria, Egypt and Turkey) (**Figure 1**).

The number of calves produced per cow per year varies from 0.5 in Egypt to 0.9 in Azerbaijan and Syria, with average of 0.7. The age at first calving averages 36 months. In Italy a good proportion of buffaloes calve at 28 months of age, whereas in Egypt and Syria a high number are 40 months or more at first calving.

In Mediterranean countries, all herds have their own bull except in the areas with very small herds (2–3 breedable buffaloes) in Romania, Bulgaria, Egypt and Turkey, where there are groups of bulls for breeding at village level.

Official milk recording for the productivity of buffaloes is performed in Egypt, Italy, Bulgaria, Romania, the United Kingdom, Azerbaijan and Iran. Genetic evaluation of buffaloes is done in Italy, Bulgaria, Romania, Egypt, Iran and Azerbaijan.

Choice of breeding bulls/heifers is made on the basis of:

1. Lactation yield (Bulgaria, Greece, Azerbaijan).
2. Lactation certificate (Italy, Egypt, Iran).
3. Cow indexes (Italy, Iran).
4. Bull indexes (Italy, Iran).

Where milk recording is not practiced, a better bull is judged on his appearance, size and strength. Natural breeding stations exist in Egypt and Bulgaria. Bulls in the breeding stations are provided by development programs of the government or other agencies. Due to

Figure 1 Egyptian buffalo, Nile delta. (Owner: Sami El Tahir.)

difficulties in estrus detection and lack of organization, artificial insemination (AI) is still practiced only to a very limited extent for buffaloes in the Mediterranean region: in Italy for 2.5% of the buffaloes, in Egypt and Iran for 0.5% and in Romania for 0.1%. In the large cooperative and state farms in Bulgaria, AI is used on 80% of the buffaloes. In the other countries it is not used at all. The low use of AI has slowed down the implementation of national selection schemes for genetic improvement of milk productivity. Research trials conducted in Italy have indicated that artificial insemination is feasible and successful in buffaloes using the following technique to synchronize estrus and boost fertility. A progesterone-releasing intravaginal device (PRID) of silicon coils is inserted and left for 10 days; on the 7th day injections of 1000 IU of eCG (equine chorionic gonadotropin) and 15 mg of luprostiol (prostaglandin $F_{2\alpha}$) are given; artificial insemination is performed at 48, 72 and 96 h after removal of the PRID on day 10.

Housing

The most common housing system is that referred to as 'traditional', consisting of keeping buffaloes indoors at night and confined in fenced areas during the day (Egypt, Turkey, Iraq, Syria); in the favorable season they are allowed to graze during the day (Romania, Turkey and on some farms in Italy). In the marshes in the southwest of Iran, buffaloes are kept outdoors on pasture throughout the year, whereas in the northern areas, around the Caspian Sea, they are kept in barns in winter. Lactating buffaloes are kept tied throughout the year in Bulgaria, Romania and Azerbaijan. In Italy, they are housed loose in paddocks all year, with the same modern systems used for dairy cows; one-third of Italian buffaloes are also put on pasture in spring. One-third of Iraqi buffaloes wallow in marshes all year, the water reaching a level halfway up their bodies. They swim far and wide to find food and when the water is high, they stand on platforms made of papyrus, reeds and mud; on these platforms the farmers sometimes build huts to house the buffaloes; these platforms can be pushed to different places in the marshes.

Lactation

The length of lactation varies from 200 to 290 days, the most frequent being 260. Average lactation milk yield is 1600 kg. Italian buffaloes give the highest average yield (almost 2000 kg) (**Figure 2**), while in Turkey, average yield is below 1000 kg. In the Iraqi marshes buffaloes produce no more than 700 kg in 180 days. The fat content of the milk throughout the lactation is over 8% in Italy, Turkey, Azerbaijan and Iraq, around 7% in Bulgaria, Romania and Egypt, and less than 7% in Iran.

The average daily milk yield of river buffaloes shows very wide variability, depending on the breed, the country and especially the management and feeding system. It can range from 3 to 4 kg day^{-1} for poorly fed animals (grazing and fed byproducts) to 15 kg day^{-1} in intensive management systems.

In the large commercial herds in Italy and Bulgaria, buffaloes are machine-milked, twice a day. In the smaller herds elsewhere in the Mediterranean, buffaloes are usually hand-milked, and often the calf is allowed to stand with its dam to assist letdown which otherwise may be slow.

Feeding Management

Extensive management systems, as practiced in Europe and the Near East, include grazing in the favorable seasons. In all cases, green forage 'cut-and-carry' – composed of legumes varying from country to country – concentrates and by-products are the basic foodstuff. Green forage and hay are made mainly of lucerne (alfalfa) in

Figure 2 Italian buffalo, in her fifth lactation; she produced 2 730 kg milk in 270 days, at 8.7% fat, 4.75% protein. (Tor Mancina farm, Rome.)

Italy, Bulgaria, Romania and Turkey and *Trifoliulm alexandrinum* in Egypt. The most common by-products fed to buffaloes are brewers' grains in Italy and Bulgaria, sugar-beet pulp in Italy, Bulgaria and Iran, cotton waste in Egypt and Azerbaijan, tomato peel in Italy, apple-juice wastes in Iran, sugar cane wastes in Egypt and Iran, maize stalk and cobs in Iran, Egypt and Romania and straw everywhere.

In the Iraqi marshes, when the buffaloes return at night to the floating islands where they live, they are fed green forage cut by the farmer during the day; this forage is composed of reeds, papyrus, various water plants, and rice hulls when available. In Italy, dairy buffaloes are managed in the same intensive way as dairy cows, maintained in loose housing paddocks throughout the year. Maize silage and grass silage are the main feeds. Average yearly milk production for buffaloes in Italy is 2000 kg although 5% of recorded buffaloes yield more than 3000 kg. An example of feeding schedules for high-yielding buffaloes is given in **Table 1**.

Similar high-energy diets (0.80–0.85 milk feed units (MFU) kg^{-1} dry matter) are used in Italy for particular selected genotypes where highest milk yield is desired because of the high prices for buffalo milk and because such diets increase milk protein (4.5–5.0%) and milk fat (8–9%) resulting in higher cheese yields.

Health Management

Buffalo pathologies are similar to those described for cattle. Few diseases are peculiar to buffaloes. They tend to show more resistance to diseases than cattle, perhaps because of their adaptation to hot–humid climates.

Parasitic Infections

Parasitic infections, particularly in developing countries, are very common in buffaloes. They include: gastrointestinal helminths (*Strongyloides*, *Toxocara*, *Moniezia*, *Mammomonogamus*) and coccidia (*Eimeria*, *Giardia*, *Cryptosporidium*), liver parasites (*Fasciola*), tick parasites (*Hyalomma*, *Sarcoptes*) blood parasites (*Theileria*), all of which produce important economic losses in buffalo breeding.

Bacterial Infections

Escherichia coli can cause gastroenteric pathologies in buffaloes, particularly in calves, associated with other bacteria (*Enterobacter*, *Pseudomonas*, *Klebsiella*) or with coccidia or verminosis or virosis. Respiratory diseases are caused by *Pasteurella*, *Staphylococcus*, *Streptococcus*, *Escherichia coli*, and can result in high mortality, if the animals are not treated with antibiotics.

Pasteurella multicida is responsible for hemorragic septicemia, the most serious disease in buffaloes because of the high mortality particularly in tropical Asian countries; however, it can be controlled with antibiotics and vaccines. Tuberculosis, produced by *Mycobacterium*, is a serious zoonosis, which could be eradicated after tuberculin diagnosis. Brucellosis, caused by *Brucella*, is another zoonosis that needs to be eradicated after serological diagnosis. It can cause serious disease in the human population and reproductive disorders and infertility in buffaloes. Vaccination can be applied only in developing countries.

Leptospirosis is another zoonosis, produced by infection with *Leptospira* from water sources contaminated by rodents. Listeriosis, caused by *Listeria*, produces meningoencephalitis, abortion and septicemia: the source of the infection may be silage in the buffaloes' diet. *Chlamydia*, *Rickettsia* and Johne's disease (caused by *Mycobacterium tuberculosis*) are present even in developed countries also, and the evidence of mastitis incidence links them to dairy buffaloes.

Viral Infections

Prophylaxis is very important to control some viral infections that could cause neonatal diarrhea (Rotavirus, Coronavirus), particularly in intensive systems. Bovine rhinotracheitis (IBR), bovine diarrhea (BVD) and bovine herpesvirus (BHV) have all been diffused by animals in intensive buffalo farms. Buffaloes are also susceptible to foot-and-mouth disease.

Other Pathologies

Buffaloes are affected by fungal infections, tumors and reproductive disorders including prolapse of the uterus.

Table 1 Example of daily feeding schedule for a buffalo producing 10 kg day^{-1} in Italy

Component	*kg*	*kg dry matter*	*Milk feed units*	*Crude protein (g)*	*Fiber (g)*
Lucerne (alfalfa) hay	7.5	6.45	3.87	650	2220
Maize silage	16.0	5.12	4.56	385	950
Concentrate (38% protein)	3.0	2.64	2.90	1000	320
Maize grains	1.3	1.14	1.45	115	25
Total	27.8	15.35	12.78	2150	3515

Milk and Milk Products

Compared to that of cattle, buffalo milk is richer in fat (6–9.5%) and protein (4–5%); it has a lower cholesterol content and higher tocopherol content. It is richer in calcium and phosphorus and has less sodium and potassium. Peroxidase activity is 2–4 times higher than in cows' milk so that it can be preserved longer; β-carotene is more completely transformed to retinol and therefore the milk looks whiter than cows' milk. The milk can be consumed in liquid form or processed into a wide range of products, either alone or mixed with milk from other livestock: fermented milks, butter, ghee, condensed and powdered milk, and cheeses are produced.

Cheese and By-products from Cheese-Processing Plants

Many farms produce their own cheese and cream which they sell directly. Classifying the types of cheese according to water content, the following are typical:

1. Soft cheese (water content >45%): Karish, Mish and Domiati in Egypt; Madhfor in Iraq; Mozzarella in Italy; Alghab in Syria; Vladeasa in Romania.
2. Semi-hard cheese (water content 40–45%): Beyaz peyneri in Turkey.
3. Hard cheese (water content <40%): Braila in Romania; Rahss in Egypt; white brine in Bulgaria; Akkawi in Syria.

The more common classification of cheese is based on the type of coagulation: either enzymatic coagulation (by rennet), or acid coagulation (after natural acidification or by the action of lactic bacteria). Most of the cheeses produced in the Mediterranean area, including Mozzarella, belong to the acid-enzymatic category, meaning that acid coagulation prevails. The demand for high-quality Mozzarella in Italy and the world has stimulated an increase in buffalo farming in Italy and improved animal management techniques. Mozzarella production is higher than all other cheeses and is forecast to increase still further.

Fermented Milks

Yogurt is produced in Bulgaria, Romania and Albania from either buffaloes', cows' or sheep's milk. In Egypt, rayat is produced from natural acidification of raw milk, after the removal of cream, with no addition of bacteria, and zabadi is produced industrially. Raha is the fermented milk of Iraq, produced either from whole or skim milk, and laban or khather are the fermented milks of Syria, all of which are often produced from buffaloes' milk.

Creams

Creams also show variable techniques of production. In Egypt, queshta mosakhana is the floating cream removed after boiling milk. Gaymar in Iraq is obtained both from spontaneaous floating or from spinning, in which case it is then pasteurized. In Italy, cream from buffalo milk is obtained after spinning; it is then pasteurized. After thermoacid coagulation (citric acid) of cream, another dairy product is obtained, called mascarpone. Quishada (made in Syria) is obtained from raw or boiled milk; sometimes, this cream is heated to make it more concentrated.

Butter and Ghee

Industrial butter is produced by churning of cream, often after pasteurization. The home made product is obtained simply by churning acidified milk. A peculiarity of buffalo butter is the color, which is much whiter than cows' milk butter, due to the lack of carotenoids. Ghee is obtained by boiling butter. It was very popular in Egypt and was found very useful in the baking industry. In Azerbaijan ghee is the only product obtained from buffalo milk.

It is evident that the dairy products made from buffalo milk in the Mediterranean area need to be more deeply studied; the variability of their technologies is an important part of global biodiversity.

See also: **Animals that Produce Dairy Foods**: Water Buffalo. **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Milk Fat-Based Spreads. **Cheese**: Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese. **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates. **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis; Infectious Diseases: Johne's Disease; Infectious Diseases: Listeriosis; Infectious Diseases: Tuberculosis; Parasites, External: Tick Infestations; Parasites, Internal: Gastrointestinal Nematodes. **Feed Ingredients**: Feed Concentrates: Co-Product Feeds. **Fermented Milks**: Middle Eastern Fermented Milks. **Husbandry of Dairy Animals**: Buffalo: Asia. **Milk**: Introduction. **Milking Machines**: Principles and Design.

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Goat: Feeding Management

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Introduction

Goats are very popular animals the world over for meat and milk production. With there being 830 million goats in the world, the number of goats is exceeded only by the number of cattle, pigs, sheep, and chickens in the world. Goats are especially important in countries with scarce animal food resources because of their ability to scavenge from the limited available resources and produce meat and milk. Asia has 59% of the world's goats and Africa 34%, accounting for the majority of the world's goats. Asia accounts for 58% of the world production of fresh goat milk, while Africa accounts for 20%. Although Europe has only 2% of the world's goats, it produces 17% of the world's fresh goat milk. In more developed countries, goats are appreciated for the exotic flavored cheeses that can be produced from goat milk. The goat cheese market has expanded, and strong cheese prices have resulted in an increase in dairy goat milk and cheese production in North America as well as in many other developed countries. As much as 500 tonnes of goat cheese is produced annually in the United States and an additional 500 tonnes is imported for consumption, predominantly from Europe. In addition, goat milk is used for feeding infants and as a health food throughout the world. There is a high demand for goat meat in many developed countries because of the increase in ethnic populations that traditionally consume goat meat. Because goat population and goat milk production are important in developed as well as developing countries, there is a need for scientific information on how to provide them with proper nutrition.

Nutrients

Nutrition is the study of nutrients, the quantity of nutrients needed for specific animals, and how to supply those nutrients, usually in a cost-effective manner. Therefore, it is important to understand nutrients and factors that affect nutrient requirements. A nutrient is a substance that aids in the support of life. The six nutrient classes are carbohydrates, proteins, fat, water, minerals, and vitamins. Energy, although required, is not a nutrient because it is not a substance, but we often speak of energy requirements when addressing the nutrient requirements of an animal. Energy, however, is derived from nutrients, notably, carbohydrates and, to a lesser extent, proteins and fat. Nutrients are required to provide energy, precursors, and cofactors for the body's metabolic machinery. Nutrients are necessary for maintaining basic animal functions and locomotion as well as providing for productive functions, be it gestation, lactation, or tissue gain. Also, the functioning of the immune system, vitally important to the health of the animals, is impaired when nutrition is inadequate.

Water is a very essential nutrient without which an animal cannot survive. Goats can use water very efficiently, which enables them to thrive in a desert environment. Water in the body is a solvent for many chemical reactions and a component of intracellular fluid, extracellular fluid, and blood plasma. In addition, it is the liquid in the reticulorumen in which fibrous feed components are digested and microbial protein and vitamins are synthesized. The major sources of water for the animal are free water, which they may consume often from a trough, and water that may be a component of their feed, especially fresh grass and silage, which contain several times more water than dry matter. Water may be lost from the body as evaporated water in the breath or as liquid in saliva, urine, and feces. In addition, considerable water is lost in the production of milk. The calculation for water requirements in lactating goats ranges from $150\,\text{ml}\,\text{kg}^{-1}\,\text{BW}^{0.75}$ for animals under a thermoneutral environment to $360\,\text{ml}\,\text{kg}^{-1}\,\text{BW}^{0.75}$ for animals under thermal stress plus the water that is contained in the milk.

Water quality is important because poor water quality can limit water intake by the animal, leading to reduced dry matter intake and lower animal production. In addition, water may have contaminants that supplement or interact with dietary nutrients (e.g., sulfur, iron, or phosphorus) or may contain toxins or pathogens with subclinical or clinical toxic effects. Calcium, phosphorus, magnesium, and sulfur can affect water intake as can total dissolved solids (a measure of total dissolved salts in the water) and chlorides. Sulfates at low concentrations may be beneficial to ruminal microbial protein synthesis, but at higher levels may contribute to polioencephalomalacia. Trace amounts of minerals such as copper, cobalt, fluoride, and zinc may support animal production.

Since water is critical to animal production, quality drinking water should be available at all times. In addition, it is important for water to be clean and kept clean because it is a good media for transmission of pathogens, from bacteria to coccidia. Therefore, water must be presented in a way that minimizes fecal contamination not only by goats but also by other animals and birds. In addition, water intake can be increased by keeping it warm in the winter and cool in the summer, such as by shading.

Proteins are composed of amino acids and are the major structural component of the body. These may also function as enzymes, expediting many chemical reactions in the body, making life possible. Goats are ruminants and can use nonprotein nitrogen for synthesizing protein by the ruminal microbes. In general, little nonprotein nitrogen is used in goat diets because goats seem to be more susceptible to urea toxicity than are cattle. Dairy goats do not seem to respond to ruminal escape protein as do dairy cattle. Because goats can consume as much as 7% of their body weight as dry matter, they must have a rapid rate of digesta passage. This rapid rate of passage would be expected to increase the amount of protein escaping ruminal degradation as well as increasing the efficiency and quantity of microbial protein synthesized in the rumen.

Carbohydrates are the main source of energy in dairy goat diets. Most grains contain large quantities of starch that are highly digestible and provide high levels of energy. Some starches are fermented more rapidly than others and are more likely to cause problems with acidosis. The fibrous carbohydrate components of forages augment buffering by increasing rumination and the resultant production of saliva that aids in buffering the rumen, preventing acidosis. The fibrous carbohydrates are often measured as acid detergent fiber (ADF) or neutral detergent fiber (NDF). Goats are less tolerant of high-starch diets compared with sheep or cattle and, therefore, require higher fiber levels. Typically 28% NDF, 21% ADF, and 17% crude fiber are considered the minimum levels, and a significant amount of the fiber must be long fiber (>2.5 cm in length), requiring the animal to chew. Generally, if half of the animal's diet is hay or forage, the level of fiber in the diet will usually be adequate.

Fats are generally present at low levels in ruminant diets and are generally poorly digested because much of the material comprising fat is plant waxes such as on the surface of plant leaves. Fats are an important energy storage mechanism for the body, with 2.25 times greater energy content than carbohydrates. Fats are also important in the absorption of fat-soluble vitamins. Salts of long-chain fatty acids may be fed to dairy goats to increase energy intake. This is especially important in early lactation when the level of milk production exceeds energy intake, and the energy deficit must be made up from body stores that diminish with lactation. Feeding salts of fatty acids can increase peak milk production as well as milk production throughout lactation.The cost of the salts of fatty acids is, however, often a limitation to their use, and these salts are more likely to be economically beneficial in early lactation compared with later lactation.

Energy sources are digested, with varying amounts being lost in the feces because of the innate characteristics of various feedstuffs. The digested energy is subject to losses in the urine and by gaseous products of digestion, chiefly methane before it becomes metabolizable energy. Metabolizable energy has losses in heat by fermentation by the rumen, cecum, and large intestine, and nutrient metabolism resulting in net energy. Net energy is often used with a greater efficiency to support lactation, with a little less efficiency to support maintenance, and with even less efficiency to support bodyweight gains. There are many different ways of expressing energy requirements because energy can be measured at various points in the energy metabolism scheme as well as in different units, generally calories in the United States and joules in Europe.

Vitamins are cofactors used in many enzymatic reactions of the body. Vitamins are typically divided into fat soluble (vitamins A, D, E, and K), which are stored in the liver, and water soluble (B vitamins), which have little storage in the body. The rumen synthesizes sufficient B vitamins for the animal under most conditions. However, anything that disturbs ruminal fermentation can impact the production of B vitamins. Vitamin K is synthesized by microflora in the intestine and absorbed in the lower intestine. Vitamin D is the sunshine vitamin and is usually produced in adequate quantities if animals are exposed to the sun for a significant portion of the day. Vitamin A is present in green forages as carotene, which the body converts to vitamin A. The liver may store up to a 90-day supply of vitamin A and a 60-day supply of vitamin E. Vitamin E is present in green forage material and is very important where selenium levels are marginal to deficient because of its interaction with selenium. Vitamin E decreases faster in stored forages than does vitamin A. Vitamins A, D, and E are often added to dairy goat diets. Nutrient requirements are listed in **Table 1** for vitamin A and E only because requirements for vitamin D are assumed to be met by exposure to the sun. Vitamin levels in the concentrate part of the feed that would meet the new NRC requirements are vitamin A, 3300 RE kg^{-1} (11 000 IU kg^{-1}), vitamin D, 4400 IU kg^{-1}, and vitamin E, 200 IU kg of feed. The new NRC (2007) presents vitamin A requirements as retinol equivalents, 1 IU = 0.3 RE of all *trans* retinol.

Mineral requirements include macrominerals of calcium, phosphorus, sodium, chloride, potassium, magnesium, and sulfur. Rations should be balanced to meet the requirements for calcium and phosphorus. Sometimes, feed by-products have very high levels of phosphorus or sulfur, which limit their level of inclusion in feed. Salt at 0.5% in the total ration will meet the requirements for sodium and chloride. Potassium and magnesium are seldom deficient in normal feedstuffs, but animals grazing lush pastures may have a magnesium deficiency known as grass tetany. Sulfur is not deficient in diets if natural protein sources are being fed, but

Table 1 Nutrient requirements for growing replacement kids and dry, breeding, and gestating does and bucks

BW	*Gain*	*DMI*	*ME*[a]	*CP*[b]	*Ca*	*P*	*Vit A*[c]	*it E*
kg	*g day^{-1}*	*kg day^{-1}*	*MJ day^{-1}*	*g day^{-1}*	*g day^{-1}*	*g day^{-1}*	*RE day^{-1}*	*IU day^{-1}*
Growing dairy doe kids								
20	100	0.73	7.4	82	4.0	2.0	2000	200
	150	0.65	8.6	103	5.1	2.4	2000	200
	200	0.73	9.7	124	6.4	3.0	2000	200
30	100	0.91	9.2	97	4.2	2.3	3000	300
	150	1.02	10.3	118	5.6	2.9	3000	300
	200	1.14	11.5	138	7.0	3.5	3000	300
40	100	1.47	10.8	111	5.0	3.0	4000	400
	150	1.19	12.0	131	5.8	3.1	4000	400
	200	1.30	13.1	152	7.2	3.7	4000	400
Growing dairy buck kids								
10	100	0.44	5.9	66	3.6	1.6	1000	100
	200	0.60	8.2	107	6.2	2.8	1000	100
20	100	0.63	8.2	82	3.8	1.9	2000	200
	200	0.79	10.6	124	6.5	3.0	2000	200
30	100	1.02	10.3	97	4.4	2.4	3000	300
	200	0.96	12.7	138	6.7	3.3	3000	300
40	100	1.21	12.2	111	4.6	2.7	4000	400
	200	1.44	14.5	152	7.4	3.9	4000	400
Mature dairy does, maintenance only								
40	0	1.00	8.0	64	1.9	1.5	1256	212
50	0	1.18	9.4	75	2.1	1.7	1570	265
60	0	1.35	10.8	86	2.4	2.0	1884	318
70	0	1.52	12.1	97	2.6	2.2	2200	371
80	0	1.68	13.4	107	2.8	2.4	2512	424
Mature dairy doe, breeding								
40	0	1.10	8.8	70	2.0	1.6	1.256	212
50	0	1.30	10.4	83	2.3	1.9	1570	265
60	0	1.49	11.9	95	2.6	2.1	1884	318
70	0	1.67	13.4	106	2.8	2.4	2200	371
80	0	1.84	14.8	117	3.1	2.6	2512	424
Mature dairy doe, twins early gestation								
40	26	1.23	9.83	108	5.7	3.1	1256	212
50	31	1.44	11.51	125	6.0	3.4	1570	265
60	35	1.64	13.14	142	6.3	3.7	1884	318
70	40	1.83	14.60	156	6.6	3.9	2198	371
80	44	2.02	16.15	172	6.8	4.2	2512	424

(*Continued*)

Table 1 (Continued)

BW	*Gain*	*DMI*	*ME*[a]	*CP*[b]	*Ca*	*P*	*Vit A*[c]	*it E*
kg	*g day*$^{-1}$	*kg day*$^{-1}$	*MJ day*$^{-1}$	*g day*$^{-1}$	*g day*$^{-1}$	*g day*$^{-1}$	*RE day*$^{-1}$	*IU day*$^{-1}$
Mature dairy does single late gestation								
40	63	1.15	11.5	128	4.1	2.4	1820	224
50	75	1.67	13.5	152	4.8	3.1	2275	280
60	86	1.89	15.1	178	5.1	3.4	2730	336
70	97	2.11	16.9	197	5.4	3.7	3185	392
80	107	2.31	18.4	214	5.7	4.0	3640	448
Mature dairy doe, twins late gestation								
40	106	1.28	12.84	157	5.8	3.2	1820	224
50	125	1.49	14.94	181	6.1	3.5	2275	280
60	143	1.69	16.95	203	6.4	3.7	2730	336
70	161	1.87	18.74	222	6.6	4.0	3185	392
80	178	2.06	20.7	245	6.9	4.3	3640	448
Mature dairy doe, triplets or quadruplets late gestation								
40	109	1.14	13.72	168	7.0	3.5	1820	224
50	137	1.32	15.9	191	7.2	3.8	2275	280
60	163	1.78	17.82	224	7.8	4.4	2730	336
70	186	1.98	19.79	247	8.1	4.7	3185	392
80	209	2.17	21.71	269	8.4	4.9	3640	448
Mature dairy bucks, maintenance								
50		1.36	10.9	82	2.4	2.0	1570	265
75		1.84	14.7	111	3.0	2.6	2355	398
100		2.28	18.3	137	3.7	3.2	3140	530
125		2.69	21.6	162	4.2	3.8	3925	663
Mature dairy bucks, prebreeding								
50		1.49	12.0	90	2.6	2.1	2275	280
75		2.02	16.2	122	3.3	2.9	3413	420
100		2.51	2.00	151	4.0	3.5	4550	560
20.1								
125		2.96	2.37	178	4.6	4.1	5688	700
23.7								

[a] Additional nutrients may be required for animal activity if other than stall feeding.
[b] Assumes 40% undegraded intake protein.
[c] Vitamin A requirements expressed as retinol equivalents.

is important when nonprotein nitrogen sources are present at significant levels.

Microminerals required include iron, copper, cobalt, selenium, molybdenum, manganese, zinc, and iodine. Iron is generally not deficient due to soil contamination of feedstuffs, although some people feel that iron injections may be helpful when large quantities of blood have been lost, such as in severe parasitism by *Haemonchus contortus*. Cobalt and molybdenum are seldom deficient in the United States, but cobalt is deficient in many areas of Australia and New Zealand. Feedstuffs produced in some regions of northern United States are often deficient in iodine. Most salt in the United States contains iodine to prevent deficiency in humans and animals. Selenium is deficient in a number of regions in the United States and across the world, where it needs to be supplemented along with ensuring that diets contain adequate levels of vitamin E. Feedstuffs such as hay or grain produced in selenium-deficient areas would be expected to be selenium deficient, and feedstuffs imported from these regions need to be supplemented with selenium and vitamin E. Copper may be deficient or poorly absorbed due to interaction with dietary levels of molybdenum and sulfur. Zinc is important for skin integrity, immune response, and reproduction. Mineral considerations in various geographic areas are usually known from production experience with other animal species, especially cattle. Such knowledge should be utilized when formulating goat diets to ensure that mineral requirements are met.

Factors Affecting Nutrient Requirements

Nutrient requirements are increased over the maintenance level by any requirement for bodily function beyond maintenance. This includes the functions of milk production, pregnancy, weight gain, and locomotion. As production levels increase, so do the nutrient levels required to support production. Production is often limited by nutrition, which may be due to a diet deficient in required nutrients, or the ability to consume a sufficient quantity of the diet to support a higher level of production. Intake is affected by many variables, including diet composition. Nutrient requirements for reproduction are generally small until the last 6 weeks of pregnancy. Also, since goats usually have twins and triplets, and quadruplets are not unusual, these nutrient demands can be considerable, which is exacerbated by the multiple fetuses taking up abdominal space and reducing the ability to consume feed. When the doe cannot consume sufficient nutrients at this time, it may lead to pregnancy toxemia, which is discussed later. Nutrient deficiencies in late pregnancy may cause abortion, low birth weight, weakness of the newborn, and low milk (and colostrum) production at birth. Growth of the fetuses requires minerals such as calcium, phosphorus, iron, zinc, and manganese. Functions such as milk production and tissue gain require additional protein and energy, as well as minerals that are excreted in the milk (Ca and P) or comprise new tissue, whereas other functions such locomotion generally require little additional vitamins and minerals.

Feedstuffs

Feed analysis is often necessary for balancing a diet, especially for forages. Many forages can be rapidly analyzed for protein and energy by near infrared spectroscopy at minimal cost. Mineral contents of feedstuffs are more costly to analyze, particularly those minerals requiring special analytical techniques such as selenium, iodine, and sulfur. In the absence of analysis, the composition of feedstuffs may be estimated from feed tables that are based on the average of a number of samples obtained from commercial sources. This average nutritional content is less variable for concentrate feedstuffs as compared to forages, again emphasizing the importance of forage analysis. Feedstuffs vary with production system and locale. Goats may be produced under a confinement system where feedstuffs are all transported to the goat or at the other extreme where goats obtain all their nutrients from pasture or browse. Feedstuffs may include haylage, silage, tree trimmings, and by-product feeds. The range of feedstuffs is limited only by availability and imagination.

Assessing Nutritional Adequacy

Nutrient adequacy for protein and energy can be assessed by body condition score (BCS) and milk production, although other nutrients may also be limiting. Milk production responds very rapidly to nutrient allowance. Other functions, such as poor reproduction or health issues, may also indicate nutritional deficiencies, but determination of which nutritional deficiencies are responsible is often difficult. Occasionally, there may be classic symptoms of a deficiency, making diagnosis straightforward. Often, symptoms are only reduced levels of production from marginal deficiencies of nutrients, making diagnosis difficult. It is better to determine the requirements for all nutrients (energy, protein, especially minerals most likely to be deficient in your region) and then using feed tables and feedstuff analysis to eliminate those nutrients for consideration that can be demonstrated to be adequate in the diet. Specific analysis may be required for some of the remaining nutrients. It is better to plan for good nutrition by determining nutrient requirements and balancing the ration to meet nutrient requirements than to try to diagnose bad nutrition. A source of information on assessing mineral adequacy is included in Kincaid (2000).

Body condition tells us how much flesh (muscle and fat) an animal is carrying. Several websites provide training materials for body condition scoring for goats. BCSs have been found to be a better measure of body stores than body weight because body weight can be affected by differing amounts of digesta in the digestive tract. In addition, BCS can be quickly assessed by an experienced eye and hand without requiring a scale, but it also has the disadvantage of some degree of subjectivity. Nonetheless, body condition is a good measure of the protein and energy adequacy of a feeding program and can be used to fine-tune the nutritional program. A doe should be in a BCS of 3.0–3.5 (on a scale of 1–5) when she is dried off, and that body condition should be maintained throughout the dry period. Does should not gain above a 4.0 BCS because they will be more prone to pregnancy toxemia. Early in lactation, the doe will lose weight as nutrient requirements for milk production exceed her ability to consume nutrients. The doe makes up for these deficiencies by drawing nutrients from her body stores. Heavy milking does will often lose body condition down to a BCS of 2.0 during this time. If the nutrient deficit is so great that she goes much below this body condition, the doe will often reduce milk production to minimize the nutrient deficit. The doe should be able to regain body condition in late lactation when milk production is lower.

Feeding Replacement Does

An article in this series covers raising replacement kids in detail, but nutrition for the replacement doe is briefly summarized here. It is critically important to keep young replacement kids growing at the proper rate to meet the desired breeding weight at the target breeding date. This generally requires gaining 150 g day^{-1} from birth through breeding age at 7–8 months. For weaned kids through breeding, this requirement can be met by feeding 0.36 kg of concentrate day^{-1} (12.0 MJ kg^{-1} ME and 17% CP) and high-quality grass or mixed hay (9.5 MJ kg^{-1} ME and 12% CP) *ad libitum*. With lower quality hay, higher levels of concentrate will have to be fed to compensate, but one must be careful to ensure that there is sufficient fiber in the diet to prevent ruminal acidosis and that the kids are vaccinated against enterotoxemia. After the doeling is bred, she can be fed to gain 100 g day^{-1} to reach 85% of her mature body weight by kidding. This can be achieved by feeding 0.27 kg of the aforementioned concentrate and allowing doelings *ad libitum* access to high-quality hay as mentioned earlier. A summary of nutrient requirements for nonlactating dairy animals is provided in **Table 1** (for more detailed requirements, see NRC (2007)).

Feeding the Doe through Her Life Cycle

The goals of the dry period are to provide sufficient nutrition to maintain the body condition of the doe, to provide nutrients for her growing fetus, and to prepare her digestive tract and metabolic machinery for the changes that happen in the periparturient period. Does replenish body fat more efficiently while lactating than during the dry period. It is more efficient for does to gain weight while still lactating than during the dry period. In addition, it is difficult to change body condition more than half a score during the 2-month dry period. The dry doe should be fed a quality mixed grass legume hay and approximately 0.25–0.5 kg of concentrate during the dry period. The dry period diet should provide 11.5% CP. Extra nutrition is required in the last 6 weeks of gestation for does that are carrying triplets or greater. However, one should be aware that overfeeding animals can cause health problems. In addition, it is important that the doe be provided with sufficient levels of minerals and vitamins, especially those known to be limiting in your area. These may include selenium, vitamin E, iodine, and copper.

The transition period is from a month before parturition to a month after parturition. Work with dairy cows has shown that the level of milk production at the end of this period is a major determinant of milk production during the whole lactation. During this time, the doe's energy requirements will increase from 130% of maintenance to 350% of maintenance and metabolism will change from anabolism to catabolism. Therefore, it is important for extra care and management to be given to animals during this time to prevent periparturient diseases and digestive upsets, and reduce stress to gradually transition the doe through great changes in intake and metabolism so that the animal can achieve a high level of production. This is best accomplished with gradual changes in nutrition and by providing preventive care. Transition period care starts with having the doe in appropriate body condition when she is dried off.

The nutrient requirements of the doe increase exponentially during the last 6 weeks of gestation because of the rapid growth of the fetuses, because they gain over 80% of their weight in this short period of time. The doe needs to be fed 0.25 kg concentrate at the beginning of the transition period and gradually increased so that the doe in consuming 0.8–1.0 kg of concentrate at kidding. The doe should be gradually adjusted to the forage that the lactating herd is consuming during the last 1–2 weeks before parturition. Every effort should be made to make changes gradually and reduce stress. It may be useful to evaluate social stress since animals are usually moved between groups, and it is important for animals to establish their social hierarchy, which is an additional stress with each change in herdmates.

Following parturition, the doe's concentrate should be increased by 0.10 kg every 3 days until she is consuming 1.5 kg of concentrate. Thereafter, concentrate levels can be adjusted for milk production with allowance for lead feeding (0.20 kg day^{-1}, discussed later). **Table 2** summarizes doe nutrient requirements for various levels of milk production (for more detailed nutrient requirements, see NRC (2007)). In a study at Langston University, does were fed either 0.33 kg of concentrate (**Table 3**) for every kg of milk over 1.5 kg or 0.66 kg of concentrate for every kg of milk over 1.5 kg. Does were grazing high-quality forages, and average milk production peaked over 4.5 kg day^{-1}. There was no difference in milk production between the two grain levels. However, the higher grain level would be beneficial if forage quality were not superior. With knowledge of forage and concentrate analysis and using intake estimates, one can approximate whether a doe's nutrient intake is meeting her requirements.

The biggest stress that the doe faces at this time is that milk production increases faster than her ability to consume nutrients, which results in a negative energy balance. Milk production peaks 6–9 weeks after kidding (approx 1 week later in doelings), whereas intake does not peak until 12–16 weeks after kidding. Consequently, the doe will lose body condition during this time, often to a BCS of 2.0. When body condition gets much below 2.0 BCS, the doe stops increasing milk production at that time, reducing milk production for the remaining lactation. This is why it is important that the doe have sufficient body condition before parturition and be encouraged to increase energy intake after parturition,

Table 2 Nutrient requirements for lactating does

BW *kg*	*DMI* *kg day^{-1}*	*ME*[a] *MJ day^{-1}*	*CP* *g day^{-1}*	*Ca* *g day^{-1}*	*Phos* *g day^{-1}*	*Vit A*[b] *RE day^{-1}*	*Vit E* *IU day^{-1}*
Does producing 1.0 kg of milk, 3.5% fat, 3.1% protein							
40	1.31	14.0	143	4.9	3.5	2140	224
50	1.46	15.7	164	5.5	3.9	2675	280
60	1.60	17.4	185	6.1	4.3	3210	336
70	1.73	18.8	204	6.7	4.7	3745	392
Does producing 2.0 kg of milk, 3.3% fat, 2.9% protein							
40	1.70	18.7	220	6.9	4.9	2140	224
50	1.85	20.4	241	7.5	5.3	2675	280
60	1.99	21.9	262	8.1	5.7	3210	336
70	2.12	23.4	281	8.7	6.1	3745	392
Does producing 3.0 kg of milk, 3.1% fat, 2.8% protein							
40	2.06	23.0	291	8.9	6.3	2140	224
50	2.23	24.8	312	9.5	6.7	2675	280
60	2.35	26.3	333	10.1	7.1	3210	336
70	2.48	27.8	352	10.7	7.5	3745	392
Does producing 4.0 kg of milk, 3.0% fat, 2.7% protein							
40	2.42	27.4	358	10.9	7.7	2140	224
50	2.57	29.1	379	11.5	8.1	2675	280
60	2.70	30.7	400	12.1	8.5	3210	336
70	2.84	32.2	419	12.7	8.9	3745	392
Does producing 5.0 kg of milk, 3.0% fat, 2.7% protein							
50	2.98	34.0	450	13.5	9.5	2675	280
60	3.09	35.3	471	14.1	9.9	3210	336
70	3.22	36.8	490	14.6	10.3	3745	392
Does producing 6.0 kg of milk, 2.9% fat, 2.6% protein							
50	3.29	37.8	509	15.5	10.9	2675	280
60	3.43	39.4	530	16.1	11.6	3210	336
70	3.60	40.9	548	16.6	11.6	3745	392
Does producing 7.0 kg of milk, 2.9% fat, 2.6% protein							
50	3.66	42.3	577	17.5	12.3	2675	280
60	3.80	43.9	598	18.1	12.6	3210	336
70	3.94	45.4	617	18.6	13.0	3745	392

[a] Activity allowance of 25% of maintenance was used in this table.

[b] Vitamin A requirements expressed as retinol equivalents.

Table 3 Sample lactating concentrate diet

Ingredient[a]	*% in diet*
Rolled corn grain	67.2
Cane molasses	5.0
Soybean meal	21.0
Limestone	1.4
Sodium bicarbonate	1.5
Magnesium oxide	0.8
Monoammonium phosphate	0.15
Trace mineral premix	0.5
Vitamin premix	0.5
Yeast	1.0
Salt	1.0
Analysis	
12.2 MJ ME kg^{-1}	
16.6	
0.6% Ca	
0.39%	

[a]Many other ingredients could be used depending on their availability in any locale. Barley could be used in place of rolled corn. There are many types of oil meals and by-product feeds that could be used as the protein source. This diet represents only an example in one locale.

without causing it to have a digestive upset. Research has demonstrated the usefulness of bypass fat products for increasing postpartum energy intake and increasing milk production throughout the lactation. Research with a tallow-based product resulted in milk production increasing for more days to a higher peak milk production level, which was sustained throughout lactation. These products tend to be expensive but may be cost effective during early lactation (for further information, see Brown-Crowder (2001)).

Early in lactation, the doe is fed additional 0.2 kg of concentrate (lead feed) in addition to the amount necessary to support milk production. After 8 weeks, the lead feed can be tapered off over a 1-week period. Care needs to be taken that there is adequate fiber in the diet (approx 50% forage will usually provide sufficient fiber). Also, does cannot tolerate consuming over 1.0 kg of concentrate per feeding without risking acidosis. If more than 2.0 kg of concentrate is needed per day, the does should be given a third feeding late at night to reduce the potential for acidosis.

The lactating concentrate diet should have a high energy level (10.5 MJ ME kg^{-1}) but only needs to have 14% CP if high-quality legume hay is being fed. If high-quality grass hay is being fed, 16 or 18% CP may be required in the diet. A sample diet composition is shown in **Table 3**. The calcium level should be 0.5% minimum, and the phosphorus level should be a minimum of 0.35%. Most sweet feeds (so called because they are ground mixtures of grains and molasses) are very low in protein and have only a moderate energy level and are poorly suited for feeding lactating does. Commercial goat milking diets have limited availability and tend to be expensive. Nonmedicated calf creep diets are generally suitable substitutes in that they are fairly high in energy and usually have adequate levels of protein, calcium, and phosphorus. They should be examined to see whether they supply the necessary trace minerals or whether a mineral supplement needs to be provided.

Young bucks must reach sufficient size by breeding season if they are going to be used to breed many does. Nutrient requirements for growing bucks are shown in **Tables 1** and **4**. Bucks are often neglected until shortly before breeding. Although bucks usually maintain themselves adequately if given sufficient forage of a medium quality, attention also needs to be paid to mineral and vitamin nutrition (see **Tables 1** and **4**) because both have a major effect on fertility. Bucks need to be evaluated 2–3 months before body season for body condition and feet problems. The buck needs sufficient body stores before breeding season, because they often lose significant amounts of weight during the breeding season due to breeding activity reducing feeding activity. The buck should be in BCS of 3.5 prior to breeding season and may lose BCS down to 2.0. After breeding season, the buck will often be able to gain weight if given sufficient forage of medium quality is provided. Sometimes concentrate feed is fed at 0.1–0.25% of bodyweight. This level of concentrate will not promote urinary calculi.

A nutrient calculator is available to calculate protein, energy, calcium, and phosphorus requirements for goats. Feed ingredients are selected and added to the diet in various levels until the doe's nutrient requirements are met. One can select the forage and the grains used to formulate the diets. Often corn is used as the energy source, although there are often regional feeds such as barley or by-product feeds such as wheat middlings or distillers dried grain that may be used. Generally, molasses is used to reduce dustiness. The corn is coarsely ground or rolled, as this will reduce problems with ruminal acidosis. Soybean meal is a common protein source although other by-products such as whole cottonseed or wheat middlings may be used. If a forage or feed analysis is available, it can be entered into the calculator. Because the calculator does not calculate requirements for other minerals and vitamins, the user must know which nutrients are limiting and provide for them. Vitamin A, D, and E are often added to diet as a supplement and should be added to the level that meets the requirements for these vitamins.

To provide macromineral requirements, calcium carbonate (limestone) can be used to provide for calcium, whereas several sources of phosphorus may be used (dicalcium phosphate, monocalcium phosphate). Sodium chloride (salt) is generally included at 1.0% of the concentrate, which more than meets the sodium and chloride requirements. Potassium, sulfur, and magnesium are usually present at adequate levels unless some by-product feeds are used that may be deficient in these minerals.

Table 4 Trace mineral requirements for growing replacement kids and dry, breeding, gestating, and lactating does

BW kg	*Prod*[a] *day*$^{-1}$	*DMI kg day*$^{-1}$	*Co*[b] μ*g kg*$^{-1}$	*Cu* μ*g kg*$^{-1}$	*Fe* μ*g kg*$^{-1}$	*I* μ*g kg*$^{-1}$	*Mn* μ*g kg*$^{-1}$	*Se* μ*g kg*$^{-1}$	*Zn* μ*g kg*$^{-1}$
Growing dairy doe and buck kids									
20	100 g	0.67	0.11	25	51.0	0.50	18.0	0.72	16.4
	150 g	0.71	0.11	25.5	70.5	0.50	21.3	0.79	22.5
30	100 g	1.03	0.11	25.2	35.0	0.50	13.6	0.48	12.6
	200 g	1.05	0.11	24.8	63.8	0.50	20.0	0.63	20.0
40	100 g	1.34	0.11	25.4	27.6	0.50	12.7	0.38	10.5
	200 g	1.34	0.11	24.6	51.5	0.50	17.2	0.51	17.2
Mature dairy does breeding									
40		1.01	0.11	19.8	5.9	0.50	10.9	0.16	11.9
60		1.37	0.11	19.7	5.8	0.50	11.7	0.12	13.1
80		1.50	0.11	22.7	7.3	0.50	14.0	0.12	16.0
Mature dairy doe, twins early gestation									
40		1.24	0.11	15.3	34.7	0.50	13.7	0.16	16.9
60		1.66	0.11	15.1	33.7	0.50	15.1	0.13	18.1
80		2.04	0.11	15.2	32.8	0.50	15.6	0.11	19.1
Mature dairy doe, twins late gestation									
40		1.52	0.11	15.1	43.4	0.50	24.3	0.19	32.9
60		2.02	0.11	14.9	41.6	0.50	24.8	0.16	33.2
80		2.76	0.11	14.9	36.6	0.50	22.5	0.14	30.4
Mature dairy doe, triplets or quadruplets late gestation									
40		1.42	0.11	14.8	54.9	0.50	29.6	0.21	35.9
60		2.23	0.11	14.8	44.8	0.50	25.6	0.17	35.0
80		2.73	0.11	15.0	44.3	0.50	26.4	0.16	35.5
Lactating does									
50	2.0	2.48	0.11	14.9	19.4	0.80	12.9	0.24	39.9
70	2.0	3.09	0.11	14.9	19.1	0.80	13.3	0.22	39.5
50	4.0	2.43	0.11	15.1	27.1	0.80	16.7	0.31	56.2
70	4.0	3.87	0.11	15.0	21.7	0.80	13.7	0.24	44.7
50	6.0	2.81	0.11	14.9	19.2	0.80	14.9	0.32	60.9
70	6.0	4.14	0.11	15.0	15.9	0.80	12.8	0.26	50.9
Mature bucks									
50		1.31	0.11	19.8	5.3	0.50	9.9	0.13	11.5
75		1.78	0.11	20.2	6.2	0.50	11.2	0.10	12.9
100		2.21	0.11	19.9	6.3	0.50	12.2	0.09	13.6
125		2.61	0.11	19.9	6.9	0.50	12.6	0.08	14.6

[a] Production level, gain in g day^{-1} or milk production in kg day^{-1}.

[b] Required levels in diet.

Some by-product feeds contain excesses of sulfur or phosphorus, and this limits their level of inclusion in the diet or may require an adjustment in the diet. To determine needs of trace minerals requires knowledge of which minerals are limiting in an area and the required level in the diet (**Table 4**). Trace mineralized salt can vary widely in concentration of trace minerals, and one that provides sufficient levels of minerals known to be deficient in the diet, such as selenium, copper, iodine, manganese, and zinc, should be selected. It should also be noted that trace mineral supplements vary greatly in their content of trace minerals, and one that provides an adequate amount of the limiting trace minerals should be used.

Several ingredients may be added to the goat's diet depending on the producers' needs. Niacin may be added at 1.0 g day^{-1} to help reduce the incidence of pregnancy toxemia based on research with dairy cows. Sodium bicarbonate (~1.5% of total diet) and magnesium oxide (~0.8% of total diet) may be added to the diet to reduce ruminal acidosis, reduce fluctuation in intake and may potentially increase milk fat production. Various probiotics and/or yeast may be added to the diet with potential benefits of preventing acidosis.

Nutrition-Related Diseases

Acidosis/founder is caused by too rapid an increase in grain consumption, excessive grain, or a lack of sufficient fiber in the diet. Because it takes time for rumen microbes to adapt to diet changes, all diet changes should be gradual. Also, fiber, especially long fiber, stimulates animals to ruminate and produce saliva, which buffers the rumen. A deficiency of fiber, including long fiber (such as hay), can contribute to acidosis. All ruminant diets should contain adequate fiber levels and some long fiber.

Dairy does are predisposed to pregnancy toxemia by being either excessively thin or excessively fat in late pregnancy. In fat does, the abdominal fat and fetuses (usually multiple) depress rumen size and ability to consume feed, and fat infiltrates the liver, which reduces the liver's ability to process metabolites such as ketones. In response to low intake, the doe metabolizes body fat, and the liver cannot adequately process ketones, the metabolite from fat catabolism. This results in an accumulation of ketones in the blood, causing metabolic acidosis. Ketosis may also be precipitated by any event that disrupts feed intake. Thin does are subject to ketosis because the nutritional demand of the fetuses causes the doe to draw heavily on her meager body reserves, resulting in a very high level of ketones in the circulation. Ketosis may also occur in early lactation, but this is less common. For prevention, does should be in a body condition of 3–3.5 (on a 5-point scale) when they are dried off and that body condition should be maintained during the dry period. In addition, encouraging animals to exercise, such as providing hay, feed, and water in different corners of the lot, can be helpful. Does should be fed sufficient nutrients to provide for their gestational needs. It is important to maintain a consistent feeding routine during late gestation. Supplemental niacin (1.0 g day^{-1}) has been used for the prevention of ketosis. Treatment of ketosis includes stimulating appetite and increasing energy intake, often with supplements such as polyethylene glycol, commercially available energy drenches, or yogurt. Electrolytes are also useful to restore hydration and treat metabolic acidosis. Late pregnant does can become very sick and die from pregnancy toxemia. Sometimes, it is necessary to induce labor or do a cesarean section to remove the fetuses and save the doe's life.

Enterotoxemia is caused by too rapid a change in diet, which results in an increase in starch or other fermentable carbohydrate in the small intestine and causes overgrowth of *Clostridium perfringens*, which produces endotoxins. These endotoxins (types C and D are most common, but there are other types) cause intestinal necrosis and vascular damage. Enterotoxemia can be prevented by vaccination, and it appears that some vaccines are more effective than others. Gradual dietary changes that allow ruminal microflora time to adapt reduce the incidence of enterotoxemia. Limiting concentrate to 1.0 kg/feeding can aid in preventing enterotoxemia. Sometimes enterotoxemia can be caused by very lush pastures or by young animals consuming large quantities of milk. Animals can be treated with oral penicillin, antitoxin, fluids including electrolytes, and nonsteroidal anti-inflammatories.

High-producing dairy goats may develop milk fever (parturient paresis; hypocalcemia) 1–3 weeks after kidding, but this is not as common as in dairy cattle. Does may also become hypocalcemic during parturition. It is being recognized that low calcium levels may impair labor during the birthing process because calcium is an important mineral for muscle contraction. Hypocalcemia can be effectively treated by intravaneous infusion of calcium solutions. There are oral calcium gels for treatment or prevention, but some of these are caustic, and the mouth needs to be thoroughly flushed with water after administration.

Polioencephalomalacia is caused by a deficiency of thiamine, which is normally produced in adequate quantities by ruminal bacteria. Thiamine deficiency causes an animal to have various neurological symptoms, including stargazing, circling, stiffness, muscle tremors, and possibly progress to blindness. Conditions of nutritional stress may affect ruminal microbes that result in inadequate thiamine, including high-concentrate diets or being off feed. High-concentrate diets have also been implicated in feedlot cattle, especially those that have high levels of sulfur. However, a significant portion of polioencephalomalacia cases in goats cannot be ascribed to these classical causes. In addition, a number of poisonous weeds have been shown to cause polioencephalomalacia. Thiamine has been included in feedlot diets for goats at 0.10 kg/800 kg of feed (target dose 100 mg hd^{-1}). The disease may be treated by giving large doses of thiamine several times a day for several days and correcting any ruminal dysfunction.

White muscle disease is caused by vitamin E or selenium deficiency. Kids may be too weak to suckle or may have sudden death or inhalation pneumonia relating to muscle pathology. White streaks are often seen in the heart muscle on postmortem examination. This disease is more likely in regions that have an endemic soil with selenium deficiency. The disease can be very effectively prevented by feeding feedstuffs or mineral supplements containing selenium and vitamin E. Although injections comprising selenium and vitamin E may be given in the short term to overcome a deficiency, correcting the feeding program to meet selenium and vitamin E requirements will effectively prevent the disease.

Bloat occurs when ruminal gasses become entrapped by foam and are unable to be released by belching. As a result, the rumen is inflated as can be seen in the left paralumbar fossa and the whole abdomen may be distended. The problem is acute and needs to be treated promptly such as by introduction of one of many bloat remedies into the rumen by stomach tube or direct injection into the rumen. These substances break down the foam and allow separation and elimination of the gas by belching. Goats are more resistant to bloat than sheep or cattle. Some bloat-disposing feeds include clover or alfalfa in new hay or pastures, often lush grass pasture, or sudden introduction of grain to the diet, especially finely ground grains. Most bloat can be prevented by management, gradually increasing the level of grain in the diet, or by filling animals up on hay before allowing them to graze new lush pastures for limited amount of time, which is gradually increased.

Grass tetany is a disease caused by the grazing animal's failure to absorb sufficient magnesium from the forage. It is more prevalent in aged late pregnant or early lactating animals that are grazing lush, rapidly growing pastures usually in the spring, with cool, wet weather. The pasture may have been recently fertilized with nitrogen and sometimes potassium. Affected animals often graze away from the herd, are irritable, and progressively develop other symptoms such as muscular twitching in the flank, wide-eyed and staring, muscular incoordination, staggering, paddling convulsions, thrashing, head thrown back, or coma. Sometimes the main symptom is sudden death. The primary cause of the disease is a low blood magnesium level that can be treated by careful intravaneous administration of a magnesium solution for that purpose. The disease can be prevented by feeding a palatable source of MgO, often available from many commercial sources. A similar supplement can be made by mixing equal parts (by weight) of MgO, trace mineralized salt, and ground grain.

Urinary calculi or urolithiasis occurs predominantly in male (both intact and castrated) animals when stones precipitate from the urine and obstruct the flow of urine in the penis, which can result in a ruptured bladder. Treatment of the condition has been described in Smith and Sherman (1994). The condition is usually associated with feeding of high levels of concentrate for long periods of time, although some pasture plants such as subterranean clover (very high in calcium) or forages high in oxylate may also cause calculi. Often the disease can be prevented by limiting grain fed to less than 0.5% of BW. If higher levels of concentrate must be fed, it should be balanced to have minimum levels of phosphorus necessary to meet nutrient requirements, over twice as much calcium as phosphorus, and inclusion or a urinary acidifier such as ammonium chloride in the feed at 0.5–1.0%. Salt level in the diet is often doubled to encourage water consumption. Water consumption should be encouraged by shading water in summer and warming water in winter. Castrating goats after 6 months of age may increase urethral diameter, reducing the incidence of urinary calculi. Vitamin A supplementation may possibly be helpful for prevention. Animals should be removed from pastures if the disease is caused by pasture plants.

Posthitis or pizzle rot is a disease of both intact and castrated males. It is caused by consumption of excess protein in the diet and the urea in residual urine around the preputial area is hydrolyzed to ammonia, which results in irritation and swelling and inflammation in the preputial area. The disease may be caused by grazing pastures containing high levels of legumes resulting in a high-protein diet. The disease is easily controlled by reducing protein level in the diet to match the animal's requirement and clipping the hair in the preputial area.

Nutritional Effects on Milk Composition

Milk produced by animals consuming all forage diets is very high in conjugated linoleic acid and other desirable omega-3 fatty acids. These products have been found beneficial in human health by preventing cancer and atherosclerosis. However, by feeding as little as 25% concentrate feed to animals on forage-based diets will dramatically reduce the levels of CLA in the milk. Supplementing goats with canola oil or canola seed has been shown to increase CLA in the milk.

The rumen and milk synthesis mechanism modulate most of the variation in the goat's diet on milk composition although diet may still have some effect on milk composition. Diet affects milk fat more than milk protein. Milk protein is generally in proportion to fat concentration except when protected fats are fed, or when milk fat depression occurs. Milk fat depression is often due to insufficient fiber in the diet, which may be associated with ruminal acidosis. In milk fat depression, the milk fat concentration often decreases below the milk protein concentration. To correct this problem, additional forage fiber needs to be fed, preferably long fiber, which promotes chewing and salivation. Ruminal buffers can also help alleviate milk fat depression. In addition, the feeding of slower fermenting starches or coarser ground grains can also help alleviate milk fat depression. Feeding rumen-protected lipids can increase milk fat concentration by up to 1.5% in a low milk fat situation. Feeding unsaturated oils can depress milk fat percentage.

Milk protein can be affected by energy intake. Restricting energy intake can decrease milk protein by as much as 0.3%. Feeding a higher digestible forage that may also increase feed consumption has been shown to have a slight effect on milk protein (0.1%). Feeding rumen-protected lipids or whole cottonseed may decrease milk protein concentration (0.05–0.15%), which can be overcome by supplementation with niacin. Feeding additional protein has minimal effect on protein content if protein nutrition is already adequate.

Nutritional Causes of Milk Off-Flavors

Properly handled goat milk should smell sweet and have virtually no flavor, similar to cow milk. The majority of off-flavors in goat milk are due to feed. Consumption of plants such as onions, garlic, mustard, chamomile, fennel, sneezeweed, and ragweed will cause various milk off-flavors. Removing animals from these feedstuffs will correct off-flavor problems. Some feedstuffs such as alfalfa, silage, cool-season small grains, and brassicas can cause off-flavors if consumed within several hours of milking. This problem can be corrected by feeding hay only for several hours prior to milking. A vitamin B-12 deficiency will cause an off-flavor described as sweet sickly smell. The deficiency may be due to a cobalt deficiency, caused by gastrointestinal nematodes infestation, and can usually be corrected by administration of an efficacious anthelmintic. Rapid cooling of milk is important to prevent lipolysis in the milk, which causes off-flavor. Milk sanitation is also important in preventing off-flavors.

See also: **Husbandry of Dairy Animals**: Goat: Replacement Management.

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Goat: Health Management

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Introduction

Herd health programs developed by veterinarians in cooperation with producers strive to maximize health and production while decreasing the incidence of a variety of economically important diseases. Development of a comprehensive biosecurity program recognizes the interaction between the animal and its ability to resist disease, a variety of infectious agents, and the environment. Changes in management practices can be very cost effective in reducing the incidence of preventable diseases and decreasing the need for pharmaceuticals. Insidious diseases such as caprine arthritis-encephalitis (CAE), mycoplasma, caseous lymphadenitis, and Johne's disease do not respond to treatment regimens and are best prevented through adoption of specific pathogen prevention programs and culling of affected animals. Lastly, cleanliness and good nutrition cannot be overstressed to prevent disease and increase productivity.

Routine Health Practices

Good herd health programs assume that certain general management practices are performed on a routine basis. Vaccination of young stock, bucks, and pregnant does against *Clostridium perfringens* types C and D and tetanus decreases the incidence of these common diseases. The herd veterinarian and producer should consider the use of other available vaccines based on the incidence of specific diseases or infections such as contagious ecthyma, caseous lymphadenitis, *Chlamydophila* infections, or campylobacteriosis. Feeding newborns 1 ounce of heat-treated colostrum per pound of body weight 3 times in the first 24 h of life provides antibodies against those pathogens present in their specific environment. Dipping navels of newborn kids in 7% iodine solution prevents navel infections. Elevating feed off the ground and keeping water sources clean help prevent parasitism and bacterial gastroenteritis. Separating newborns from the adult population at birth prevents transfer of microbes and parasites from infected adults to immunologically naive young. Addition of coccidiostats to milk, milk replacer, water, or creep feeds markedly decreases the incidence of coccidiosis. Hooves should be trimmed at least 4 times yearly and medicated footbaths may be used in some environments where foot rot is prevalent.

No vaccine or drug can replace keeping the environment clean. Producers are encouraged to test the cleanliness of the environment by kneeling on the bedding to check for wetness and breathing the air at goat level to detect high levels of ammonia or other odors. Removing dirty bedding and opening a closed barn prevent parasitism, pneumonia, and paratuberculosis. Adequate space and shelter should be provided for the number and size of goats housed, as overcrowded goats are more stressed, are less healthy, and have a higher rate of disease. The incidence of scours in kids is markedly reduced when their pens and feeding devices are cleaned frequently. In certain environments, the use of raised floors or dry lots markedly decreases the incidence of parasitism. Encouraging exercise by housing animals in large paddocks or pastures reduces the incidence ketosis, hypocalcemia, and obesity. Adequate amounts of nutritious feeds appropriate for the type and number of animals present prevent nutritional deficiencies and allow livestock to maximize productivity. Consultation with a competent nutritionist aids in development of a complete balanced ration based on grazing or locally available forage in combination with locally available concentrates. All mineral nutrition is local and selection of trace mineral supplements should be based on analysis of the complete ration. Well-fed goats with adequate trace minerals appropriate to the local feeds are more likely to mount an effective immune response when challenged.

Diseases with High Economic Impact

Enterotoxemia due to toxins produced by the digestive tract bacterium *C. perfringens* is one of the most common causes of sudden death in goats. This bacterium is a normal inhabitant of the digestive tract and it grows rapidly and elaborates several different toxins quickly when the rumen environment changes suddenly. Common inciting factors include, but are not limited to, changes in feed, roughage, water source, or weather and failure of passive transfer of antibodies from dam to offspring. Young kids may exhibit a peracute form characterized by depression, colic, fluid diarrhea, paddling, recumbency, convulsions, and death within 24 h in spite of treatment. An acute form with less severe symptoms of dehydration, anorexia, acidosis, and diarrhea occurs in older animals, while postpartum lactating animals may exhibit a chronic form characterized by intermittent episodes of mild anorexia, listlessness, soft feces, and depressed milk production. Body temperature is quite variable and may reflect the severity of disease at the time recorded. Diagnosis is often based on history, clinical symptoms, and response to treatment with *C. perfringens* types C and D antitoxin, nonsteroidal anti-inflammatory drugs, and appropriate antibiotics. Animals found suddenly dead should be necropsied to determine the cause, and during outbreaks of enterotoxemia, other goats may benefit from administration of antitoxin and vaccination in addition to correcting the cause of the outbreak.

Eradication and control programs prevent major disease problems such as CAE, mycoplasma, caseous lymphadenitis, and Johne's disease. These four diseases cause significant economic loss through decreased longevity, growth rate, milk production, and animal sales. Direct contact can transmit these diseases between adults over time, but they can be transferred quickly from adults to young stock through colostrum, milk, and direct contact. All four diseases have insidious onset and may be acquired through the introduction of asymptomatic carriers.

CAE often presents as chronic progressive arthritis affecting multiple joints in adult goats, and some goats may also develop chronic progressive interstitial pneumonia or weight loss associated with chronic disease. Two less common presentations include ascending paralysis in otherwise healthy afebrile kids and an unusual form of postpartum udder edema that is nonresponsive to diuretic therapy. The percentage of infected animals that exhibit clinical disease is low, but stress and poor management increase the appearance of clinical symptoms. There is no difference in prevalence between breeds or sex, but the incidence of infection increases with age in those herds with virus-positive animals. Tests for both antibody (agargel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA)) and for virus (PCR) are commercially available. CAE does not kill goats but decreases productivity, life span, and quality of life.

Mycoplasma mycoides mycoides large colony type (Mmm) may present a complex sequence of symptoms that can have devastating impact on different age groups at different times. Young kids develop high fever, pneumonia, inflamed joints, and meningitis, while adult does may demonstrate fever, abortion, chronic degenerative arthritis, chronic weight loss, septicemia, and severe mastitis. Morbidity and mortality are very high, and surviving animals become carriers that shed mycoplasma in their colostrum and milk. Special transport media are required for culturing mycoplasma, and not all diagnostic laboratories are capable of isolating these difficult bacteria. Clinically normal carriers may be identified by culturing ear canal swabs or first-milking colostrum. Other species of mycoplasma may cause conjunctivitis or mastitis, but those infections are usually self-limiting and have less economic impact than Mmm large colony type.

Corynebacterium pseudotuberculosis is the causative agent of contagious abscesses or caseous lymphadenitis in both sheep and goats. This organism is found in the thick purulent discharge from ruptured abscesses and survives for many years in contaminated soil or barns and on equipment or instruments. The bacteria are capable of penetrating intact skin but presence of skin wounds facilitates infection. The bacterial toxin phospholipase D allows the organism to spread from lymph node to lymph node throughout the body even though the immune system attempts to encapsulate it with fibrous connective tissue. Most lesions begin in the lymph nodes about the head and neck before spreading to internal nodes around the lung, heart, liver, kidneys, intestine, and mammary glands. Lactating does with abscesses in the mammary glands or mammary lymph nodes transmit large numbers of bacteria to their offspring in milk and colostrum. Abscesses caused by *Corynebacterium* frequently increase in size with age and interfere with body function. Goats infected with *C. pseudotuberculosis* are permanently infected and shed the organism in body fluids, abscess contents, and coughed aerosol droplets. The disease is most commonly confirmed by bacterial culture. The use of commercially available vaccines or autogenous bacterins combined with good management practices to reduce environmental contamination leads to a decreased incidence of abscesses in infected herds.

Johne's disease or paratuberculosis affects both wild and domestic ruminants. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) can survive for extended periods (for over 1 year) in contaminated environments or in manure spread on pasture. Contaminated soil and bacteria-laden feces coat the teats and udder of the dam and are nursed by newborns, while infected animals shed mycobacteria in both milk and colostrum. *In utero* transmission of MAP has been documented in both sheep and cattle, but the percentage of goats born with infection has not been determined. The incubation period is very long and clinical symptoms are more common in animals between 2 and 4 years of age. Severe stress associated with pregnancy, parasitism, or environmental change may trigger clinical disease characterized by severe progressive weight loss leading to emaciation in spite of good nutrition, anemia, lethargy, rough hair coat, flaky skin, and occasionally bottle jaw or dependent edema. Diarrhea is uncommon in small ruminants but may appear terminally. Fecal culture and AGID or ELISA testing of blood samples may be used for antemortem diagnosis, but most cases are confirmed by histopathology at necropsy.

Specific Pathogen Prevention Program

The four diseases mentioned above can be economically devastating to the commercial producer and the incidence of all four can be decreased through the use of a specific pathogen prevention program. The infectious agents of these diseases are readily passed from infected adult populations to newborns through direct contact, colostrums, and milk. Newborn kids should be removed from the adult population at birth and should be fed heat-treated colostrum (heated to 135 °F and held at that temperature for 1 h), and pasteurized milk or milk replacer. Raw cow colostrum or cow milk should not be substituted due to the high incidence of mycoplasma and paratuberculosis in dairy cattle. If cow colostrum or milk must be used, then it should be heat-treated as described previously. The new clean population must not share feeding devices, water sources, or housing with infected goats, and there should be at least 10-foot-wide alleys separating status groups. Clean animals should be milked, fed, and cared for first, and equipment must be cleaned after each use in the infected population. Raising all kids on the specific pathogen prevention program increases the sale value of extra replacements and provides an extra source of income for the herd.

While many producers see the benefit of developing herds free of these insidious diseases, not all are convinced that the effort involved in developing a clean herd will provide an increased economic return. Does with chronic wasting diseases do not live as long and do not produce as much milk on a yearly basis as does under the same management and without chronic disease. Animals infected with these chronic diseases are permanently infected and serve as a source of contagion to the rest of the herd. Many progressive commercial herds enjoy greater productivity and sales of healthy stock after adopting a specific pathogen prevention program and eliminating these chronic diseases (**Table 1**).

Prepurchase Procedures and Quarantine

In order to prevent introduction of new diseases into the commercial herd, goats should be tested for brucellosis, CAE, mycoplasma, caseous lymphadenitis, paratuberculosis, parasites, and tuberculosis prior to purchase. Vaccination status and nutritional history should be determined. If possible, young goats that have not previously been bred should be purchased to decrease exposure to pathogens transmitted through breeding. Milk from lactating does should be cultured prior to purchase to prevent introduction of new mastitis pathogens into the herd. Upon arrival, herd additions should be treated for internal and external parasites and vaccinated for the same diseases as the rest of the home herd. When transporting incoming stock, a clean vehicle should be used and it should be cleaned again before being used for another purpose.

Many producers cannot elude the appeal of the show ring. Producers should arrive early and pick pens as far away from other herds as possible. Tack pens, tarps, and walls or fences should be used as barriers between herds, and producers should avoid penning back to back with other herds. Herds should be grouped by health status or prevention programs. Pens should be cleaned on arrival and bedded heavily to prevent transmission of soil-borne organisms. Pens should be cleaned frequently and the goats should not be overcrowded. Gastrointestinal upsets can be minimized by maintaining a steady diet and feeding the same roughage and concentrates as the home herd. Upon return home, the exhibited goats should be treated as if they were new additions and quarantined separately from the home herd.

New arrivals and returning herd mates should be housed separately for a minimum of 30 days to prevent introduction of new diseases into the main herd. There should be no contact between the new stock and the main herd through feeders, fences, housing, or water sources. If possible, different people should care for the main herd and the new acquisitions using separate equipment. If labor is in short supply, the main herd should be cared for first before working with the new or returning stock. Any equipment used on the new stock should be cleaned

Table 1 Specific pathogen prevention program

Step 1: Remove newborn kids from the adult population at birth
Step 2: Feed newborn kids 1 ounce of heat-treated colostrum per pound of body weight 3 times in the first 24 h of life
Step 3: Transition kids to pasteurized milk or milk replacer until weaning
Step 4: House, feed, and water kids separately from the adult herd permanently
Step 5: Provide 10-foot-wide alleys between affected adults and replacements
Step 6: Use serology and culture annually or semiannually in heavily infected herds to identify affected animals
Step 7: Separate affected adults from the adult population and milk them last
Step 8: Clean equipment used in the affected population before using it in the clean herd
Step 9: Cull affected animals to slaughter when their production decreases below a profitable level

before using it with the main herd. If the new additions have not been vaccinated or treated for internal and external parasites on the same program as the main herd, then they should receive this treatment while in quarantine. If lactating animals are entering the herd, they should be milked last, separate towels should be used for each animal, and the equipment should be cleaned thoroughly after milking the new stock. Producers should observe new animals at least twice daily, examine promptly any that appear abnormal, and seek veterinary advice early in the course of a problem.

Biosecurity

People, equipment, and animals can transmit disease, and infectious agents can be carried on hands, clothing, boots, and pets. Producers should not wear their barn clothes to another goat producer's facility, and they should leave their dogs at home. If a visitor arrives with a dog in tow, it should be kept in the vehicle away from the herd. Visitors should wear disposable plastic boot covers or washable boots and should wash their hands before and after visiting the livestock. Herds should not exchange equipment unless absolutely necessary, and then it should be thoroughly cleaned before using it and again before returning it. It is recommended that producers selling stock have the buyer park on the road and then walk the animals off the property to the vehicle. Likewise, dead animals should be placed at the edge of the property so that the renderer will not enter the pens or come in contact with the livestock.

Health Problems of Specific Populations

Dietary Imbalances

Good nutritional management can reduce the incidence of dietary diseases such as mineral imbalances, urolithiasis, pregnancy toxemia, and hypocalcemia. Numerous mineral supplements are actively marketed to goat producers without regard to dietary need, so producers should be cautioned to have the entire diet including water sources analyzed prior to selection of specific trace mineral supplements. All mineral nutrition is local, so it is important to base supplementation on the diet actually fed to the goats and not on what might be recommended over the Internet or from a distant location. The absorption of several trace minerals such as copper, zinc, iron, iodine, and molybdenum is interrelated and excess of one element may lead to poor absorption of another. Proper feed analysis and assistance of a competent nutritionist help prevent copper toxicity and deficiencies of zinc, iron, iodine, selenium, and copper.

Mineral imbalances, excessive grain intake, decreased water availability, poor water quality, and early castration are all associated with development of urolithiasis or inability to urinate. This condition is most common in castrated males on high-grain diets but may occur in bucks on imbalanced diets or those who do not have adequate access to clean water. Diets high in phosphorus and magnesium are often implicated, and the calcium-to-phosphorus ratio in the complete ration should be 2:1 to help prevent calculi formation. Affected males may dribble blood-tinged urine initially before the condition progresses to full obstruction and inability to urinate. Clinical symptoms of urolithiasis include anorexia, depression, vocalization, tenesmus, tail twitching, treading of the rear legs, and pulsation of the urethra ventral to the anal sphincter. Treatment of urolithiais requires establishment of urine outflow through amputation of the urethral process, identification of the blockage, and a variety of surgical procedures. Addition of ammonium chloride to the ration to acidify the urine and sodium chloride to increase water consumption may help reduce the incidence of urinary calculi. Adequate provision of clean water, reduced access to excessive minerals, and feeding correct levels of appropriate concentrates decrease the incidence of urinary calculi. In general, goats that are not producing a product such as meat, milk, or fiber do not need grain.

One of the more common health problems on a commercial dairy is ketosis or pregnancy toxemia. Does that are underfed do not have enough energy or protein in the diet to provide for fetal and mammary development in addition to their own maintenance, while overfed does deposit fat in the liver, which interferes with protein and energy metabolism. Many dairymen do not separate their pregnant dry stock from the milking herd. Pregnant dry goats housed with the milking strings have access to a higher nutritional plane than is required for their nonlactating state and become obese. Early symptoms of ketosis include anorexia and depression that progress to weakness, staggering gait, apparent blindness, recumbency, coma, and death. Some toxemic does grind their teeth, breathe rapidly, and exhibit swollen lower legs. Laminitis may develop a few days after the initial onset, and some does recover spontaneously following delivery of their kids. Diagnosis is based on demonstration of elevated ketones in urine, serum, or milk and may be confirmed by response to treatment. Pregnant does in the early stages of ketosis may recover if the protein, energy, and calcium contents of the diet are supplemented. More severely affected does may respond to administration of oral and intravenous glucose, fluids, electrolytes, and vitamins. Induction of labor or Cesarean section may be necessary to salvage recumbent does. Pregnant dry stock should be separated from the milking does, sorted into groups by body condition and stage of pregnancy, and fed appropriately. Increasing exercise for pregnant does improves energy metabolism, and this can be accomplished by moving the feed, water, and shelter as far apart as possible. Addition of niacin to the grain ration during the last 2 months of gestation and the first 3 months of lactation may reduce the incidence of ketosis.

Hypocalcemia or low blood calcium is related to the level of calcium in the diet, calcification of developing fetuses in the pregnant doe, maintenance and growth of the adult doe, and production of colostrum. Stressed pregnant does in poor body condition, goats that have recently traveled, and those fed low-calcium diets are more prone to hypocalcemia. Symptoms of hypocalcemia or milk fever appear when the demand for calcium exceeds immediately available blood calcium. Clinical symptoms are often progressive and begin with anorexia and mild bloat and then progress to disorientation, staggering gait, inability to rise, recumbency, subnormal body temperature, coma, and death. Two common presentations of hypocalcemia are the pregnant doe with weak contractions during labor that fails to progress and the postpartum doe with subnormal body temperature and no milk. Diagnosis of hypocalcemia may be based on clinical symptoms, laboratory confirmation of low blood calcium, and response to treatment. Treatment includes administration of supplemental calcium, glucose, magnesium, phosphorus, and potassium. Prevention of hypocalcemia includes minimizing stress in the pregnant does, provision of adequate nutrients appropriate for each stage of pregnancy and lactation, and maintenance of a calcium-to-phosphorus ratio in the total diet of 2:1.

Kid Health Management

Digestive and respiratory diseases in kids are often reported as major health issues for commercial producers. Removing kids from the adult population to clean, dry housing at birth helps prevent transmission of pathogens from the adult herd to the naive young. Dipping navels of newborns in 7% iodine prevents navel infections and septicemia. Feeding adequate levels of good quality colostrum helps prevent enteric disease from a variety of pathogens. Providing coarse concentrates and long-stem forage at an early age encourages rumen development and promotes bacterial fermentation. Providing adequate pen space and reducing overcrowding increase growth rate and decrease the spread of disease if a pathogen enters the herd. Segregation of kids by age, maturity, and body size improves access to feed and increases growth rate while decreasing the impact of parasites such as coccidia and cryptosporidia. Kids raised in humid climates may benefit from housing on slats or grates to decrease fecal contamination and spread of coccidia, while appropriately timed use of coccidiostats in milk, milk replacer, or feed dramatically increases growth rate and survival.

Reproductive Manipulation

Changing management practices so that does kid in the fall and winter to maintain high milk production through the winter can make a tremendous difference in how much money the dairy earns for the same amount of annual milk production. Many milk cooperatives pay a premium price for winter milk and most quotas are established by how much winter milk a producer can generate. Regulations regarding the use of drugs that alter reproduction vary by country, but treatment with prostaglandins and progestagins has been used to stimulate estrus outside the normal breeding season. The 1994 Animal Medicinal Drug Use Clarification Act prohibits the use of drugs to alter reproduction in goats in the United States. Some herds meet this challenge by using fluorescent lights to artificially increase day length both for out of season breeding and for maintaining higher milk production in the winter. Provision of 20 h of daylight for 2 months in deep winter followed by return to normal day length will bring goats into estrus several weeks later. Sudden introduction of an odoriferous buck into a pen of does that have not been housed within the sight or smell of a buck will stimulate cycling. Both are effective techniques that require simple changes in management. Increased use of ultrasound also improves reproductive efficiency by allowing accurate diagnosis of false pregnancy as a cause of reproductive failure.

Buck Health Management

Often the most neglected animals on the goat dairy are the bucks or intact male goats. All preventative management practices performed on does such as vaccination, dehorning, foot trimming, body condition scoring, delousing, deworming, and disease control programs should be part of the routine care of bucks. Due to the early onset of puberty in dairy breed bucks, young males should be separated from the doelings by 4 weeks of age and they should be grouped and housed in well-constructed facilities appropriate to their age and size. Male goats grow very rapidly and should receive a rising plane of balanced nutrition from birth through first service. Provision of clean water and appropriate roughage, concentrate, and trace minerals should be monitored to prevent development of urolithiasis. Regular body condition scoring of adult bucks is important to maintain proper weight year-round so that they will be in correct condition and ready for service prior to onset of the breeding season. Breeding soundness examination should be conducted prior to semen collection or natural service each year and should be repeated if the does served fail to conceive.

Mastitis

Commercial goat producers should be aware of the many management practices pioneered in cattle dairies that decrease the incidence of mastitis. To maximize production and minimize mastitis caused by nursing kids, newborns should be removed from the doe immediately after parturition and should be fed and housed separately from adults. Does should be milked in a clean area with properly maintained equipment on a regular schedule. Employee use of disposable gloves during milking improves cleanliness and helps prevent transmission of mastitis from one goat to another. Use of a strip cup to observe the first milk from each teat for abnormalities before every milking can help identify problem animals early for diagnosis and treatment. Goat-side use of the California mastitis test on does with abnormal milk or those with changes in volume aids in early detection of mastitis. Dipping teats in a 0.5% iodine solution followed by drying of the teats with a single-service paper or cloth towel prior to milking helps prevent introduction of pathogens into the teat canal. Milk removal should begin within 60 s of teat preparation in order to maximize the effect of oxytocin release and should stop when full streams cease. An effective postmilking dip should be thoroughly applied to the bottom two-thirds of the teat after milking to prevent bacteria from entering the open teat orifice. Separation of affected animals to the last milking string can help prevent spread of mastitis. Herds with a history of mastitis caused by *Staphylococcus* species or Gram-negative bacteria may benefit from the use of specific bacterins and toxoids. Cleanliness and sanitation of the housing and bedding area for the milking does help prevent mastitis due to environmental pathogens. Providing adequate levels of trace minerals such as copper, zinc, and selenium appropriate to the rest of the diet can improve immune function and decrease the incidence of mastitis.

Scrapie

Scrapie is an infectious disease of sheep and goats characterized by intense pruiritis, varying neurological symptoms, and long incubation period. In sheep, this transmissible spongiform encephalopathy is thought to be caused by an abnormal prion protein transmitted in the placenta and uterine fluids during the first 4–6 weeks after parturition. The method of transmission in goats has yet to be determined. The abnormal prion protein has been found in the brain, spinal cord, lymph nodes, and spleen of infected sheep and goats. The incubation period is very long and infected animals may genetically control the length of the incubation period. The onset of symptoms may be insidious, occur over a long period of time, and may be associated with stress such as change in herd, parturition, or sale. Affected animals may isolate themselves or lag behind the herd and startle easily. Hair loss from pruiritis, nibbling, lip smacking, ataxia, and weight loss in spite of good nutrition are common symptoms. All affected animals die. Diagnosis is based on histological examination of characteristic brain lesions, and the disease is federally reportable. An immunohistochemical test of rectal biopsy was approved for live animal testing in 2009. Regulatory programs vary between countries, but most affected herds are quarantined and depopulated.

Zoonotic Diseases

Many goat producers are unaware of zoonotic diseases that can be passed from animals to man. Some zoonoses such as pinkeye or ringworm cause symptoms in the host animal so that the producer knows the animal is ill, but the producer may not realize that the disease could be transmitted to himself or his family. Other zoonotic diseases such as enterohemorrhagic *Escherichia coli* infections and toxoplasmosis may not cause symptoms in the affected animal, and the producer may not take adequate precautions to prevent transmission of these diseases to humans. Some zoonoses are transmitted directly from the host to humans, while others are passed indirectly through vectors such as milk, meat, or fiber. Healthy, vigorous dairymen may not demonstrate symptoms of many of the zoonotic diseases, whereas immunosuppressed individuals, infants, and elderly debilitated adults may be more susceptible to disease. Those individuals taking immunosuppressive drugs or affected with immunosuppressing diseases such as leukemia or HIV may die of zoonotic disease. Infectious agents such as *Campylobacter*, *Chlamydophila*, or *Toxoplasma* that cause abortion in goats can also cause abortion in pregnant women, so pregnant women should not handle fetal membranes or aborted fetuses. Cleanliness, sanitation, vaccination, use of protective gloves and face masks, and proper cooking and handling of food prevent transmission of zoonotic diseases from goats to man (**Table 2**).

Drug Residue Prevention

The production of safe and wholesome dairy products for human consumption is the primary goal of commercial goat milk producers. Veterinarians should work with producers to develop effective management programs combined with stress reduction, sound nutrition, and use of vaccines and pharmaceuticals to reduce the incidence of disease. Prudent use of pharmaceuticals is a necessary tool to treat livestock, reduce suffering, and decrease

Table 2 Zoonotic diseases of dairy goats

Anthrax
Brucellosis
Campylobacteriosis
Chlamydophila infection
Corynebacterium pseudotuberculosis infection
Cryptosporidiosis
Echinococcus granulosus infection
Escherichia coli 0157:H7 infection
Fungal skin disease (ringworm)
Johne's disease
Leptospirosis
Listeriosis
Parapox (contagious ecthyma)
Q fever (*Coxiella burnetii)*
Rabies
Salmonella infection
Staphylococcal dermatitis
Staphylococcal enteritis
Toxoplasmosis
Tuberculosis

shedding of zoonotic agents into the environment and food chain.

Veterinarians and producers should carefully select antimicrobial drugs and other pharmaceuticals used in goats producing milk for human consumption. Before choosing a specific antibiotic, there should be strong evidence to identify the cause of the disease based on clinical symptoms, history, examination, laboratory data, and past experience. The antibiotic selected should be appropriate for the suspected pathogen and it should be administered in adequate dosage and duration to effectively treat the identified pathogen. If a labeled product is available for that disease, that product should be chosen unless there is reason to believe that it would be ineffective. Antibiotics should be used with a specific clinical outcome in mind such as fever reduction, return to function, decreased recurrence of disease, or decreased environmental contamination. When possible, antibiotics with a narrow spectrum of activity should be chosen over broad-spectrum drugs. Antibiotics should be administered for as short a period of time as is reasonable to achieve the desired result. Antibiotics that are of lesser importance in human therapy should be chosen whenever possible. Compounding of medications and use of prophylactic antibiotics should be avoided whenever possible. Producers should be educated in the proper administration, handling, and storage of all pharmaceuticals. Prolonged treatment of chronically ill animals should be discouraged and consideration should be given to their removal from the herd. The selection of appropriate drug withdrawal times to prevent drug residues in dairy products offered for human consumption cannot be overstressed. Treated animals should be clearly identified and accurate treatment records should be maintained to prevent accidental contamination of dairy products for human consumption. The veterinarian should stay in contact with the producer to monitor whether the elected treatment has been effective. It is imperative that goat producers and veterinarians work together to prevent drug residues in dairy products for human consumption.

See also: **Analytical Methods**: Microbiological. **Diseases of Dairy Animals**: Infectious Diseases: Foot-and-Mouth Disease; Infectious Diseases: Johne's Disease; Non-Infectious Diseases: Pregnancy Toxemia; Parasites, External: Mange, Dermatitis, and Dermatosis; Parasites, External: Tick Infestations. **Husbandry of Dairy Animals**: Goat: Feeding Management; Goat: Milking Management; Goat: Replacement Management; Goat: Reproductive Management. **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality.

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Goat: Milking Management

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Introduction

Goat milk production is of significant importance to the economy and survival of large populations of many countries in the world. Goat milk production is spread throughout the world: in developing countries such as Asia, Africa, the Middle East and Mediterranean countries and South America, as well as in developed countries such as Europe (France, Greece, Italy, Spain, Norway, The Netherlands), North America (United States, Canada) and Oceania.

A global survey carried out by the International Dairy Federation (IDF) in 1998 showed that most goats bred in the world are generally hand-milked except in some developed countries (France, Italy, Spain, Norway, The Netherlands, Israel, for example), where the number of heads per herd is increasing while staffing levels are decreasing. These countries have established new technologies in order to milk goats with milking machines.

Unfortunately, in the past milking machines for goats were merely adaptations of machines used for dairy cattle and this led to milk quality and udder health problems.

Nowadays, a new and modern technology of special milking machines for goats being developed. Most world-famous manufacturers of milking machines for dairy cattle, as well as smaller local manufacturers, have made a great deal of effort over the last 10 years to offer machines suited to the special anatomy and physiology of dairy goats. At the same time research was carried out in many countries to understand better animals and their needs.

Although more research is needed, especially on the adjustment of milking machines (vacuum level, pulsation characteristics, cluster and liners), in relation to animals (breed, milking ability, milk production, milking conditions), the first international guidelines were published by the IDF in 2002.

Types and Size of Milking Installations

When goats are milked with a milking machine, three types of installations are traditionally used: buckets and milk pipelines in sheds, mobile equipment (buckets and parlors) and fixed milking parlors. **Table 1** gives information on these types of installations in countries where milking machines are developed.

Milking in the Barn or in the Field

As with cows, the first milking machines introduced in dairy goat herds were bucket milking machines, generally used by two animals simultaneously.

When goats are kept in a barn, they are traditionally milked with buckets. One milker can use two or three buckets. Some are completely self-contained with the vacuum pump running with a combustion engine or an electric motor. This kind of milking machine is very popular in emerging countries but also exists in Europe because of its low price.

In a shed, it is also possible to milk goats with a pipeline milking machine. Goats are tied up and the milker walks from one animal to another with the milking unit. One milker can manage three or four units. This type of milking machine is not very common but can be found in Norway, Italy and France.

In a barn, some farmers have built an elevated platform, most often made of wood and about 0.80 m high, on to which goats are moved for milking (**Figure 1**). Simple installations are only equipped with buckets or 'direct-to-can' milking machines. These installations, built in a nonspecific area, are very similar to milking parlors.

In other countries, mobile equipment is popular. Most consist of buckets mounted on a specific carriage with a combustion engine running the vacuum pump. These installations allow farmers to milk their animals directly out on the pasture and relatively far from the farm.

Milking in a Milking Parlor

The most popular milking parlors for goats in developed countries are herringbones types (**Figure 2**) and side-by-side parlors with two platforms (**Figure 3**).

The design of these milking parlors is exactly the same as for cows but takes into account the morphology of goats. In herringbone parlors, animals stand obliquely at a diagonal on the platform and in side-by-side parlors goats stand perpendicularly in a rectangle to the pit. In this last case 0.33 m for each goat is needed instead of 0.70 m for cows in the same position.

Generally, one milker works in small plants with four to six units and up to 12–16. In bigger installations with 20–32 units, two milkers usually work together, often assisted by another person who pushes groups of goats

Table 1 Type and number of milking installations in some countries

Country	*Milk pipelines in sheds*	*Milking parlors*	*Mobile equipment*
France	1800	7300	200
United Kingdom	0	200	0
The Netherlands	0	300	0
Norway	800	50	0
Israel	20	220	20
Italy	800	1500	1000

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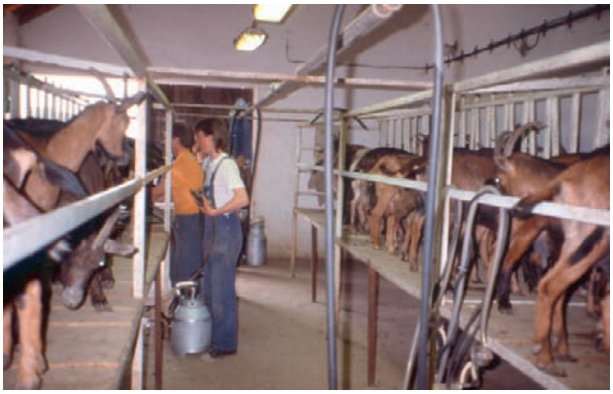

Figure 1 Two elevated platforms and buckets.

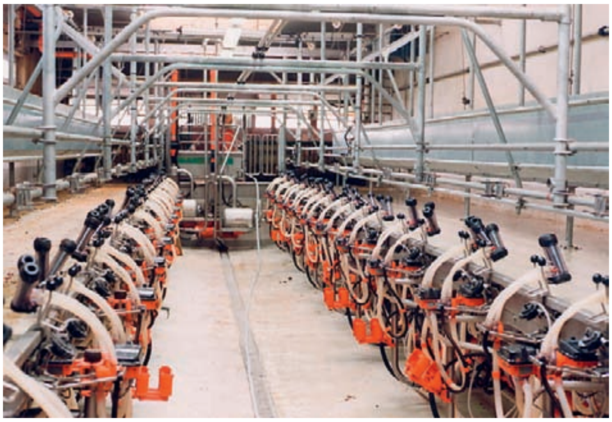

Figure 2 A 2 × 24 herringbone parlor.

on to the platform. In The Netherlands, however, it is common for one milker to manage up to 30 clusters, while most of the large milking parlors are now equipped with automatic cluster removals.

Large herds (in France, Italy, The Netherlands and Spain) are also milked in rotary parlors (**Figure 4**) comprising 20–48 units and sometimes more (up to 60), generally with one or two milkers and a pusher and/or a crowd gate. Automatic cluster removals are more and more popular.

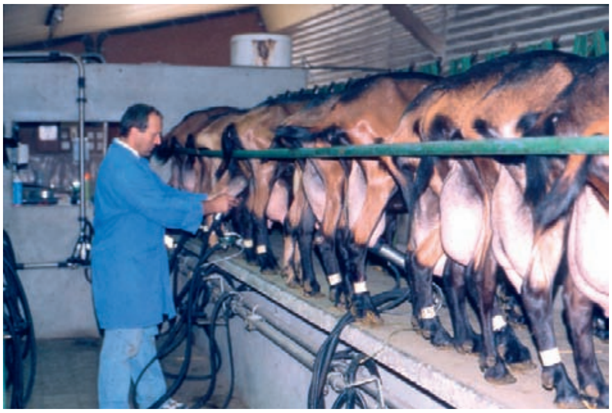

Figure 3 Milking in a side-by-side parlor.

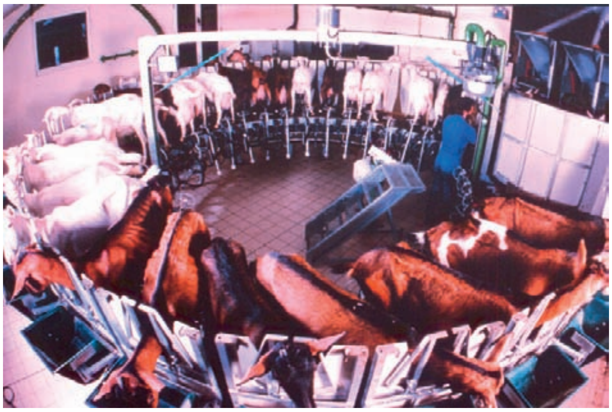

Figure 4 Milking in a rotary milking parlor.

'Tunnels' are not very common but they exist in France and Israel. Animals stand on one or two platforms, free and one behind the other (**Figure 5**). These are very interesting milking parlors that can be used in herds of no more than 200 head with very good throughputs and a lower cost than more sophisticated parlors.

A study carried out in France in medium and large herds showed that one milker could milk around 120 and up to 160 goats per hour in a tunnel parlor with 10–16 units. Large differences in throughputs are observed in different situations depending on the milk yield of goats, the number of groups of animals (in general feeding groups), animal traffic from the barn to the milking parlor, and the milker (**Table 2**).

The same study also revealed that very high throughputs (around 200 goats milked per hour or more) can be reached in herringbones, side-by-side parlors and carousels when they are equipped with at least 15–20 units.

Bigger throughputs (300 goats milked per hour or more) in larger herds can be obtained in milking parlors with 30–32 units (**Table 2**).

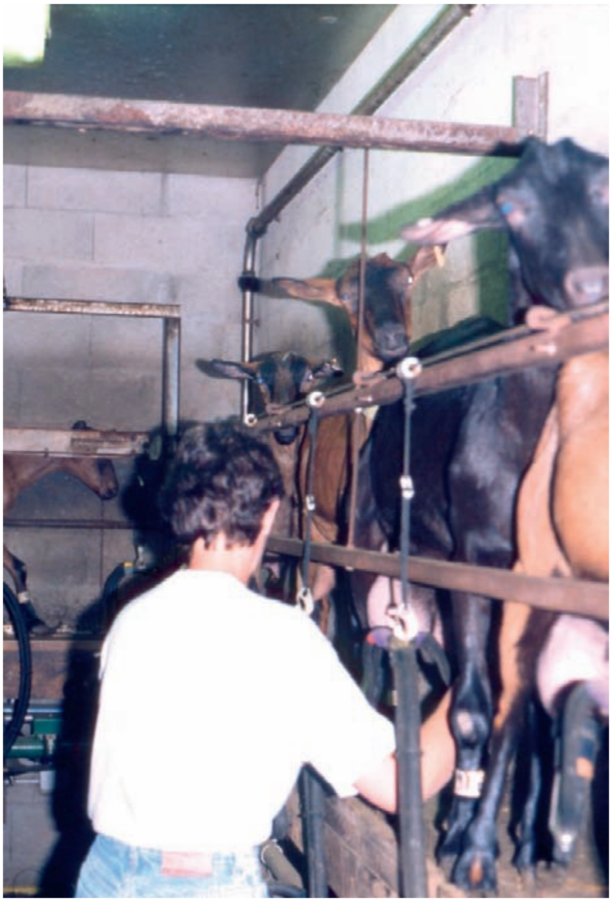

Figure 5 Milking in a tunnel parlor.

Table 2 Average throughputs in large milking parlors

Type of milking installation	*Number of units*	*Number of goats milked >h^{-1}*
Tunnel	10–12	82–150
	14–16	161–164
Herringbone	14–16	109–288
	20	190–249
	30–32	223–312
Side-by-side	14–16	126–211
	18–24	128–217
	30–32	212–331
Carousel	8–2	99–169
	16–20	183–261
	24	208–327

Reproduced with permission from Billon, and Baritaux, (1998).

Most of the above-mentioned milking parlors are equipped with a low-line milk pipe and one unit per two animals. Some parlors are also mounted with one unit per three goats; this is cheaper but general results and labor in the parlor do not seem to be as good, efficient or comfortable as in installations with one unit per two animals.

Herringbone or side-by-side parlors can also be equipped with a high-line milk pipe and one unit for four animals (two on each side of the pit). Such milking parlors are not very common despite the fact that they are not very expensive, but throughputs are relatively poor because milkers have to wait for the end of milking of the two animals standing on one side of the parlor before attaching teat-cups to the two others on the other side. These parlors can be very useful in herds with a small number of goats (50–100, for example) when time is not a problem.

In western Europe in areas where herds are very large and only a few people work on the farm, goat milk producers need large installations and labor organization with a very high level of efficiency.

The above-mentioned French study concluded that one of the best ways of milking 200 goats per hour with minimum labor (only one milker) is the following situation:

- herringbone parlor with 14–16 units
- a collecting yard located close to the milking parlor without a wall between these two rooms in order to gather animals together before milking
- no feeding concentrates in the parlor during milking
- two goats for one unit
- low-line milk pipe.

A similar throughput can also be obtained in a side-by-side parlor equipped with 20–24 units when goats are not fed during milking.

Rotary parlors are generally very expensive and may be reserved for very big herds and high-yielding animals. Small rotaries with only 8–12 units are not very cost-effective. In contrast, carousels with 30–32 units, sometimes more, might be a good solution to milk 300 goats per hour with few milkers. In some situations only one milker in the parlor and one pusher helping the animal traffic can reach this throughput very easily.

Milking Ability of Dairy Goats

Milking machine construction and performances may be directly related to the milking ability of animals in order to milk quickly, completely and gently, to maintain healthy udders and to produce milk with a high quality level.

There are large differences between small ruminant breeds in terms of milking ability. Some goats are milked like sheep, and vice versa. So, it seems very important to carry out studies for particular breeds to improve knowledge of the milking ability of animals in order to give advice to farmers and dealers, particularly in terms of milking technique, cluster handling, stripping and adjustment of milking machines.

These remarks are valid for every milked animal and many studies have been undertaken in the past to improve knowledge of the cow's physiology. These have had a real and important impact on the design of milking machines and on milkers' techniques.

Goats have principally cisternal milk (about 70%) and only 30% comes from the alveoli during milking by the action of oxytocin. Of course, proportions depend on breed: for example, the Canarian breed of dairy goats have nearly their whole quantity of milk in the cistern; this is why in certain cases they are milked only once a day.

A field study was recently carried out in France on the two main breeds kept in that country, Alpine and Saanen breeds, in order to provide more information about how animals give their milk. About 1000 cases of milk ejection kinetics have been recorded in 30 herds and important results have been found.

Dairy goats are characterized by a delay time between the time when the cluster is attached to the teats and time when milk begins flowing through the liner and the claw. The longer this delay, the longer the milking time is and the smaller the average and maximum milk flow rates are.

In the Alpine breed, milk ejection kinetics curves can be divided into three groups:

1. Group A: high milk flow rates (maximum 1.5 l min^{-1} or more), short milking time (around 120 s) and a very short delay time (no more than 5 s)
2. Group B: medium flow rates (maximum between 1.1 and 1.3 l min^{-1}), longer milking time (180–240 s) and longer delay time (5–10 s)
3. Group C: low flow rates (maximum about 0.5 l min^{-1}), very long milking time (300–420 s) and long delay time (15–60 s).

Figure 6 shows the three curves related to the three groups.

In the Saanen breed, such differences were found with a longer milking time and lower flow rates. These results are very useful to calculate the internal diameter of tubes and of milk pipes. They are also helpful in order to determine the maximum number of units that can be used by a milker without overmilking. For example, in a herd where 60–70% of goats belong to groups A and B, this means that the average milking time is about 150 s per animal and the average delay time about 8 s.

Considering that the attachment rate is one unit every 8 s, one milker can only use 20 units maximum. Taking into account some unavoidable incidents during milking (cluster fall-off, mastitis treatment, abnormal behavior of certain animals, bad entry in the parlor), this means that the real number of units per milker is 15–16.

In parlors where premilking hygiene, such as washing and drying udders or predipping, is carried out and where each unit is attached at least every 20 or 25 s, it is advisable to work with two people or more if necessary and to use automatic teat-cup removal.

Milking Machine Requirements

The following sections sum up the most important requirements which are useful for milking machines for dairy goats.

Effective Reserve and Vacuum Pump Capacity

Effective reserve

The International Standards Organization (ISO) Standard 3918 defines the effective reserve as the air flow rate that can be admitted in the installation to induce a vacuum drop of 2 kPa measured when all milking units are working together.

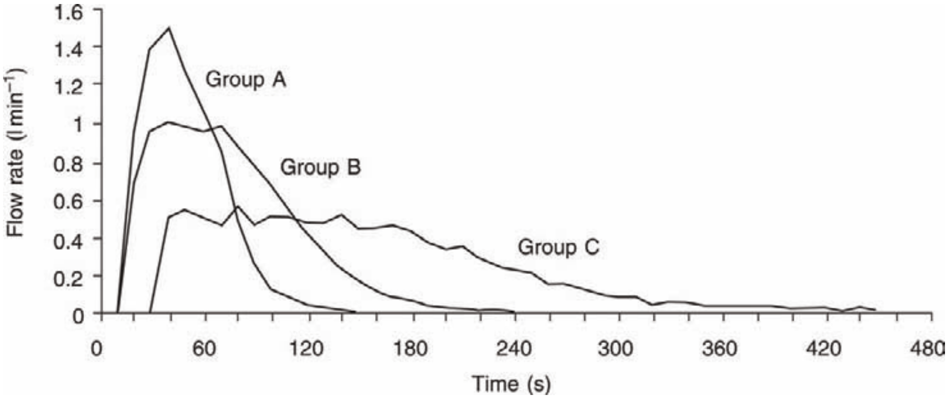

Figure 6 Milk ejection curves in the Alpine breed. Group A, high milk peak flow rate, short milking time and a very short delay time. Group B, medium milk peak flow rate, longer milking time and longer delay time. Group C, low milk peak flow rate, very long milking time and long delay time.

The effective reserve is the difference between the total amount of air pumped out of the milking machine by the vacuum pump and the total air consumption of the different components of the working milking machine. Effective reserve allows the milker to milk in good conditions (i.e. stable vacuum level, low vacuum fluctuations beyond the teat) even if air enters into the installation when attaching or detaching teat cups or in case of liner slips.

A sufficient effective reserve prevents irregular vacuum fluctuations in milk lines and cluster fall-off. Studies showed that the prevalence of mastitis is higher with low effective reserve than with a bigger one in dairy cattle. There is apparently no reason why this phenomenon should not apply to dairy goats.

The above-mentioned survey made by the IDF found that most countries were using a special formula for effective reserve and vacuum pump capacity. Generally, most of them derive from the old ISO Standard 5707 (1986) for cows, generally taking into account a greater amount of air entering into goats' installations during milking and a higher incidence of cluster fall-off.

Therefore, IDF asked its A32 group, which was transformed into the current Standing Committee on Farm Management, to make a proposal for quantitative requirements for milking machines for small ruminants and especially for dairy goats.

Dairy goats are usually milked with three types of clusters: conventional clusters without automatic shut off valve at the claw, conventional clusters with automatic shut off valve at the claw, and nonconventional clusters equipped with automatic teat-cup valves at the liner. An automatic shut off valve is a valve in the milking unit which shuts off the vacuum when a cluster falls (ISO 3918).

An automatic teat-cup valve is a device in the cluster which opens or shuts off vacuum to the liner when being put on, and automatically shuts off when the teat-cup falls down. It also automatically introduces vacuum into the liner when the milker puts the cluster on the teat, or in some cases, the milker only has to pull up the shell in order to admit vacuum under the teat.

Working with these clusters reduces the intake of air to a minimum when handling milking units but most need extra air to work: 20–50 l min^{-1} depending on the model, except if they are mounted with automatic teat-cup removal.

Using clusters with automatic teat-cup valves provides nearly the same milking conditions as those for dairy cows when the milker puts on and/or removes milking units carefully.

The argument is that effective reserve for dairy goats should be relatively similar to the effective reserve for cows, taking into account that claws and liners are different, the volume beyond the teat is smaller, and the frequency of liner slips is certainly higher.

With conventional clusters without an automatic shut off valve in the milking unit, the risk is very high that milkers do not shut off the vacuum at the liner when they attach or when they detach clusters from udders because of poor milking routines due to bad habits and/or the need for high throughputs in large parlors. Then, effective reserves should at least compensate the total air admission of a fully open cluster, which has been evaluated at 600 l min^{-1}. Thus the air admission during units' attachment depends particularly on the number of milkers, unlike with dairy cows.

With conventional clusters with automatic shut off valves, a milker can work more carefully when using these devices. Air admission during attachment, which also depends on the number of milkers, should be similar to the transient air entering into an installation for cows used by a normal operator (200 l min^{-1}).

In addition the effective reserve depends on the type of installation (pipeline or bucket) and on the number of units.

Table 3 and **Figure 7** give examples of effective reserves required for different number of units and different types of clusters.

Vacuum pump capacity

The vacuum pump should be capable of withdrawing all air from the milking plant, whether this is the reserve capacity, air used to run pulsators, air intake, leakage or for any other use.

The capacity of the vacuum pump is estimated as described for dairy cattle and water buffaloes in ISO 5707 and takes into account the influence of altitude, air demand for cleaning, the number of units and of pulsators, the vacuum level at which the machine will work and the minimum effective reserve.

A milking parlor with 12 units with automatic teat-cup valves, six pulsators and a milk line of 48 mm internal diameter, working at 38 kPa, located at an altitude of 1000 m, shall be equipped with a vacuum pump of at least 959 l min^{-1}capacity.

Pulsation Characteristics

Pulsation characteristics do not vary significantly between different countries

Goats are milked with a pulsation rate between 70 and 100 cycles min^{-1}, and with a pulsator ratio about 50–60% (**Table 4**).

Until now, 80 and 90 cycles min^{-1} have seemed to be the most popular rate for numerous breeds worldwide.

Traditionally, goats are milked with simultaneous pulsation (both liners are at the same time in the milking

Table 3 Effective reserves required for bucket milking machines in different situations for one milker (l min^{-1})

	Number of buckets					
Type of cluster	*2*	*3*	*6*	*10*	*12*	*20*
Conventional cluster without automatic shut off valve at the claw[a]	340	360	420	500	520	600
Conventional cluster with automatic shut off valve at the claw[b]	240	260	320	400	420	500
Unconventional cluster with opening and shut off valve at the liner[c]	180	220	340	500	560	800

[a] For each additional milker, add 400 l min^{-1}.

[b] For each additional milker, add 200 l min^{-1}.

[c] Example for clusters which need 20 l min^{-1} extra air.

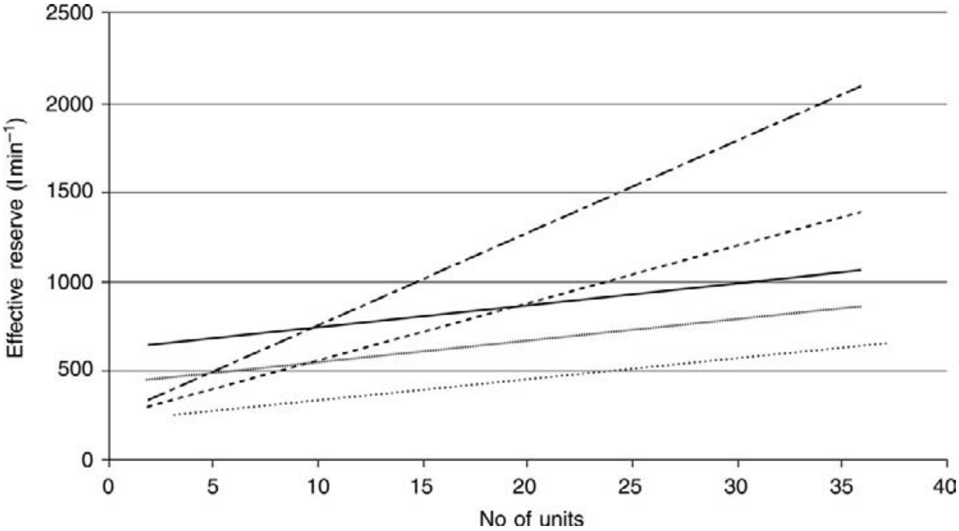

Figure 7 Effective reserve required for pipeline milking machines and one milker. —— Conventional cluster without automatic valve; .... conventional cluster with automatic valve; ----- unconventional cluster and 20 l min^{-1} extra air; ––– conventional cluster and 40 l min^{-1} extra air; .... unconventional cluster and automatic cluster removal.

Table 4 Pulsation characteristics in goat-milking installations in different countries

Countries	*Pulsation rate (cycles min^{-1})*	*Pulsator ratio (%)*
The Netherlands	70–90	50–70
France and Spain	80–90	50–60
Argentina	70–90	50–70
United Kingdom	80–90	60
Ukraine	80–120	66
Israel	90	50–60
Germany	90–120	50–65
Italy	80–90	50
United States	60–90	50–70
Norway	60	≤70
Bulgaria	90	60

Reproduced with permission from Billon *et al*. (1998).

phase and in the rest phase), but alternate pulsation (one liner is in the milking phase while the other is in the rest phase) can be used, particularly with high-yielding and fast-milking animals.

Vacuum Levels

Generally, goats are milked at a lower vacuum level than dairy cows but there are many differences among breeds and/or more especially the type of milking system (**Table 5**). Several breeds (Alpine, Saanen, Canarian) are milked at 36–40 kPa in low-line and bucket installations and at 40–42 kPa in milking machines with high-line milk pipes.

The genetic improvement of the milking ability of certain breeds has led to a general decrease in vacuum levels in milking machines for goats. However, it is very difficult to advise a general vacuum level because of the enormously different situations that can be found around the world.

The adjusted vacuum level of a given milking machine is dependent on many parameters, such as the type of installation (pipeline or bucket), the height of the milk-line, the type of cluster and liner, the milking routine of the milker and of course, the animals themselves.

The tonicity of the sphincter, which closes the streak canal of teats, seems to be stronger for goats than for cows.

Table 5 Working vacuum levels (kPa) in goat-milking installations

	Milking parlors and pipelines		
Country	*High line*	*Low line*	*Buckets*
The Netherlands	43–45	38–40	38–40
France, Spain	40–42	36–40	36–40
United States	44–48	38–42	38–42
Argentina	38–40		33–44
Ukraine		42–50	
Norway	44–48	39–43	38–42
Italy	44	44	44

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For example, studies in the Alpine breed showed that some goats need a 40 kPa vacuum level under the teat just to obtain the first drop of milk. That means that a higher vacuum level is necessary to milk these animals than others that only need 20 or 25 kPa to open their sphincter.

Decreasing the vacuum level leads to longer milking time but improved teat-end conditions, reduced risks of stress during milking and can have a positive influence on mastitis and somatic cell counts.

Installers and advisers have to choose between the better compromise: milking time and animal health and welfare.

Sizing pipes

Air lines

The air line diameters of a milking machine for goats can be figured exactly as for cows, taking into account the amount of air flowing through these pipes, their length and the roughness of the material (galvanized steel or plastic), as described in ISO 5707.

Milklines

Ideally, milk should flow in the lower part of the pipeline with a clear continuous space above for the much larger volume of air to pass over it. This flow condition is known as stratified flow. In practice, flow typically varies between stratified flow and slug flow. Slug flow occurs whenever slugs of milk fill the entire cross section of the milkline.

Milkline vacuum almost always remains stable within ±2 kPa of the receiver vacuum under stratified flow conditions. Therefore, the limit of 2 kPa essentially means that stratified flow should be the normal flow condition in the milkline.

Experimental laboratory studies have been carried out to determine the maximum milk flow rate to ensure that stratified flow is the normal flow condition during dairy cattle milking.

There is apparently no reason why equations applied to cattle should not apply to dairy goats if the ratio between steady air flow and milk flow is chosen according to the real milking conditions of this species.

Calculations are made on the basis of 8 l min^{-1} steady air per unit (air vent at the claw or at liners) and with three milk flow rates: 0.8 l min^{-1} for breeds with low maximum milk flow rates, 1.3 l min^{-1} for breeds with medium maximum milk flow rates and 2.7 l min^{-1} for breeds with high maximum milk flow rates.

The maximum milk flow to ensure that stratified flow is the normal condition during milking should be less than or equal to the real milk flow in the milkline, which can be predicted from typical milk flow rate curves together with the expected average rates of unit attachments.

Referring to milker practices in milking installations for goats, and especially without hygienic treatment before milking, attachment rates of 5 and 10 s seem to be adequate but can last longer if the milker washes and dries the udders before milking (20–30 s).

Figure 8 shows different typical curves of goats used to calculate the minimum internal diameter of a milk line in a milking machine for goats. Calculations were made taking into account the following parameters:

- looped or dead-end milkline
- slope (in %) of the milkline
- type of cluster
- transient air entering into the plant during attachment, detachment or liner slip.

For example, the maximum number of units per slope allowed in a 48.5 mm internal diameter and 1% slope looped milkline for goats with an average milk peak flow rate of 1.3 l min^{-1} and an attachment rate of 5 s is 6, 8 and 13 in installations equipped with conventional clusters without automatic shut off valve, conventional clusters with automatic shut off valve and unconventional clusters with automatic teat-cup valves respectively (**Table 6**). With a similar diameter but in dead-end conditions, the maximum number of units per slope is 3, 6 and 12 respectively.

Increasing the slope increases the number of units. The same looped milkline with a 1.5% slope and in the same conditions will be equipped with a maximum of 8, 13 and 19 units per slope depending on the type of cluster. A similar dead-end milkline will be equipped with a maximum of 6, 8 and 15 units per slope, respectively.

A bigger diameter (60 mm internal diameter) should be necessary in very large installations with 0.5% slope or in medium parlors using clusters without automatic shut off valve when milkers have a poor milking routine.

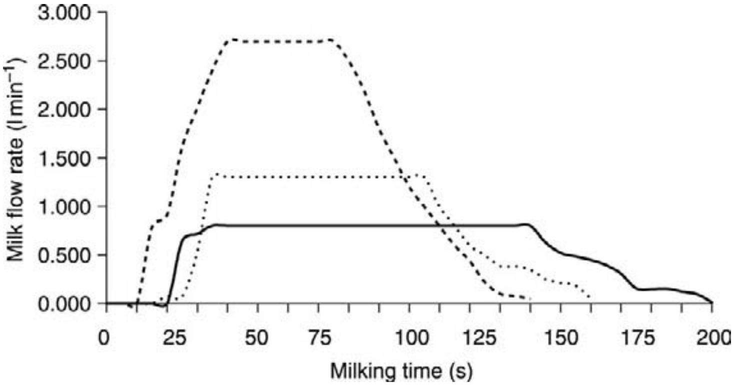

Figure 8 Typical milk flow rate curves for goats (d_{max} milk peak flow rate). —— d_{max}, 0.8 l min^{-1}; ····· d_{max}, 1.3 l min^{-1}; ------ d_{max}, 2.7 l min^{-1}.

Table 6 Maximum number of units per slope in a 48.5-mm internal diameter looped milkline for goats with 1.3 l min^{-1} average milk peak flow rate at 5 s attachment rate (10 s in parentheses)

Slope (%)	*0.5*	*1.0*	*1.5*	*2.0*
Conventional cluster without automatic shut off valve	3 (3)	6 (6)	8 (9)	10 ([a])
Conventional cluster with automatic shut off valve	6 (6)	10 ([a])	13 ([a])	17 ([a])
Unconventional cluster with automatic teat-cup valves	9 (11)	13 ([a])	19 ([a])	[a] ([a])

[a]Unlimited number of units.

A smaller diameter (38 mm internal diameter) should only be used in very small installations with a few number of units per slope (between 3 and 10, depending on the type of cluster) and with a slope of at least 1.5%.

Cluster Assembly

As mentioned above, dairy goats are milked with at least three different types of clusters.

The most common model is a bowl with a volume generally within 100–200 cm^3 without an automatic shut off valve (**Figure 9**). These clusters come directly from dairy cattle milking machine components. Bowls also exist with an automatic shut off valve and these are easier to handle (**Figure 10**). The second model is a simple Y-shaped claw without a bowl but this is less common.

Unconventional clusters are also used, with or without a bowl. Although they are more expensive, these clusters are nowadays used daily by farmers in many countries.

A French study related to the limitation of somatic cell count in goat milk showed that animal health and milk quality can be improved if no air (or a minimum of air) is admitted in the milking machine during milking.

No specific requirements for clusters are available for goats as regards claw or liners. Mouthpiece lip diameters of liners used for goats are generally within the range

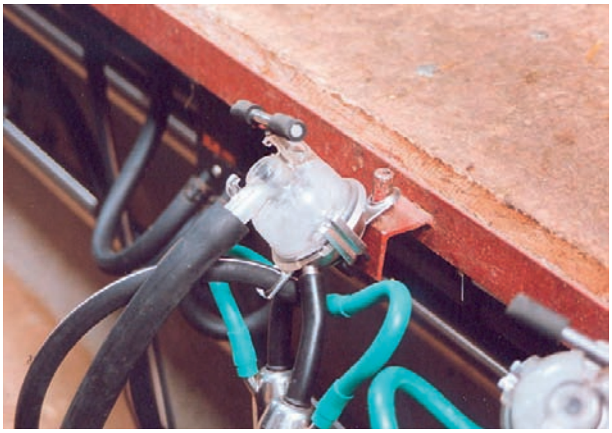

Figure 9 A conventional bowl milking unit.

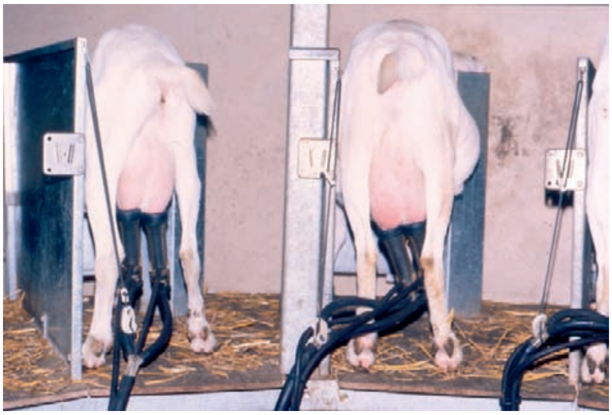

Figure 10 Milking units with automatic teat-cup valves.

20–22 mm for Alpine, Saanen and similar breeds but more research is needed in order to define precisely the exact technical characteristics of the liner and the best adjustments of the milking machine (especially vacuum level and pulsation parameters) for a given breed or a given herd.

Other Equipment

For dairy goat breeds with a good milking ability, machine stripping just before detaching the cluster should be omitted in order to avoid udder health problems and to improve the efficiency of the operator(s). This is automatically done with automatic cluster removal (ACR) systems (**Figure 11**).

Manual cluster removal must be done when no vacuum is admitted into the liner, as for dairy cows. This is why it is recommended to use a claw which is at least equipped with an automatic shut off valve or it is best to use special clusters with automatic teat-cup valves.

ACRs are new on the market but their use is increasing in an effort to reduce labor in large parlors and to avoid excessive overmilking.

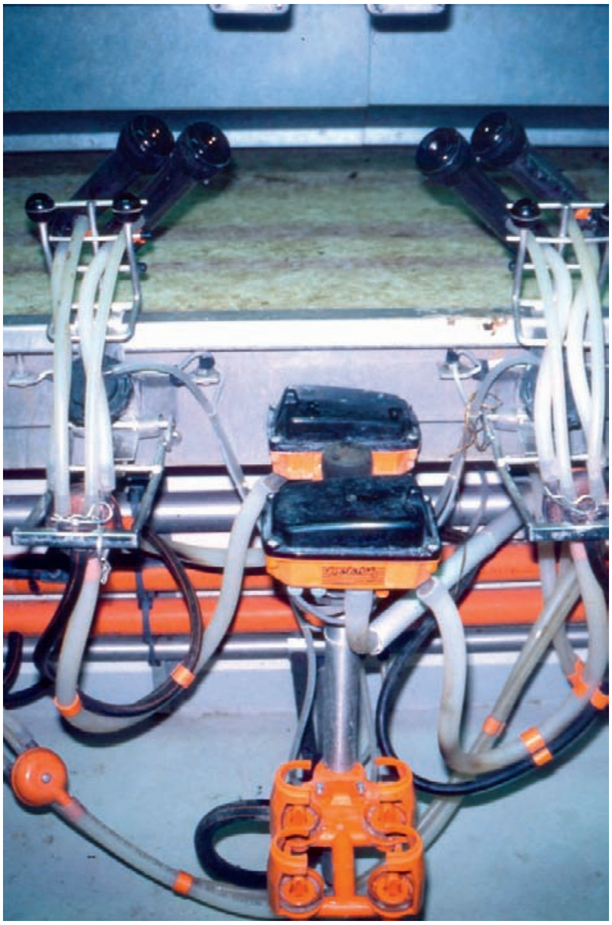

Figure 11 Two units with automatic teat-cup valves and automatic cluster removal (ACR).

The above-mentioned French study on milk ejection kinetics in commercial herds showed that it is not rare that overmilking lasts at least the same time as when milk flows from the udder. Another recent study has shown that using ACRs in a large parlor can reduce the somatic cell count, related to infected and/or stressed udders.

In terms of cleaning and sanitizing milking installations, bulk milk tanks and precooling systems, the same equipment is generally used as for cows (*see* **Milking and Handling of Raw Milk**: Milking Hygiene). In certain cases, when the vacuum level is too low, it is recommended to use two regulators: the first one works during milking at 38 kPa and the second only during the cleaning process at 46–48 kPa.

Milking Routine

Milking hygiene is relatively poor in dairy goat husbandry. In most cases washing and drying udders is omitted and no teat dipping is done after milking. The main reason is the lack of time of operators who want to milk larger and larger herds very quickly (200 goats per hour or more).

Some studies have shown that teat dipping may decrease the new infection rate by about 50%.

Conclusion

Milking machines for goats need more studies in order to make exact adjustments to the different components, taking into account animal behavior and welfare.

Checking the milking machine once a year and changing liners every 2500 h of effective milking should ensure that milkers work in the best way all the time.

See also: **Animals that Produce Dairy Foods**: Goat Breeds. **Husbandry of Dairy Animals**: Goat: Feeding Management; Goat: Multipurpose Management; Goat: Reproductive Management; Sheep: Milking Management. **Milk**: Goat Milk. **Milking and Handling of Raw Milk**: Milking Hygiene. **Milking Machines**: Principles and Design. **Milking Parlors**.

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Goat: Multipurpose Management

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Introduction

Only 12.5% of this planet is land, most of which consists of swamp, hot and cold deserts, fallows, marginal land and mountains. Consequently, there is competition between humans, animals and plants for the land resources available, which is gradually shrinking. The goat, because it is able to utilize less favorable marginal environments and to thrive under difficult conditions, is found almost everywhere. This is attributable to its efficient utilization of food material, disease tolerance, reproductive capacity and its adaptabilty, not least to different farming systems. Apart from the dog, the goat was the first species to be domesticated.

The husbandry of any animal species is dependent on its relationship with humans (governed by socioeconomic, political, religious and cultural factors) and its relationship to the land (governed by geographical and climatic factors). Goat farming, because of its low capital investment and quick economic returns, has been integrated into flexible and vulnerable animal agriculture production systems.

As a source of milk, meat, fiber, skin, hair, horns, manure and other valuable byproducts, goat husbandry calls for multifunctional management systems. These management systems range from the extensive (e.g. free-grazing on common land, ranching) to the intensive (e.g. zero grazing, tethering or stall-feeding and tethering). Many combinations of these systems exist.

Flock size varies with the management system adopted and ranges from singles (tethering) or tens (stall-feeding) to hundreds (group grazing or semi-intensive) or thousands (extensive, zero-input management systems).

Origin and Domestication of Goats

Evidence from areas of modern-day Iraq, Iran, Jordan, Turkey and Palestine shows that goats were domesticated as long ago as 7500–7000 BC. This is supported by the finds of toy goats from the cities of Harappa and Mohenjo-Daro (Pakistan).

Three sources of the domestic goat have been identified:

- the bezoar goat (*Capra aegagrus*) is thought to be the origin of Western Asian goats
- the ibex or wild goat (*Capra ibex*) has been traced as the origin of Asian, East African and European goats
- the markhor (*Capra falconeri*) is thought to be the ancestor of the Cashmere (Pashmina) goats found in Afghanistan, Mongolia, the former USSR, China, Kashmir and across the Karakoram hills.

Archeological and molecular evidence tends to support the above theories, but more recent studies suggest that the goat has Zagoros–Iranian roots. Descriptions from the fifth century bc trace Angora (Mohair) goats to central Anatolia. Ancient Graeco-Roman papers on agronomy and natural history from this period also refer to goats and the references to goats in the Bible, Torah, Koran and other ancient writings point to their existence since ancient times.

The present-day domestic goat was named *Capra hircus* by Linnaeus in 1758, and it was mentioned in a livestock inventory compiled by Buffon in the mid-eighteenth century. Goats were first classified by Sanson in 1886, on the basis of ear shape. However, with the advent of modern cytogenetic and biometric evidence, these old classifications have been replaced by a classification based on genes specific for coat color and for ear or horn variants. Coat-color variants and intersexuality associated with hornlessness are common worldwide.

Distribution and Economic Contribution of Goats

The majority (67%) of the world's goats live in Africa and Asia, but they thrive in all environments, whether temperate, tropical, arid, humid, semihumid, or hot or cold desert. Developing countries with poor economies have more goats (94%) than developed industrialized nations, and most are reared for meat, milk and fiber. Other byproducts, such as hides, bone and hair, are also utilized. The meat and milk contribution from goats for a variety of countries is shown in **Table 1**. The annual growth rate in the production of chevon (goat meat) in Asia and Africa is about 4–6%, while that of milk is 2–3%. It is estimated that about 89% of goats in the world are reared for meat; the nutritional values of goat meat are shown in **Table 2**.

Table 1 Goat meat and milk contributions to diets

Country	*Meat (%)*	*Milk (%)*[a]
Algeria		12.8
Bangladesh	29.6	55.2
China	27.3	3.0
Cyprus	30.8	20.0
Greece	21.7	24.8
Haiti	11.9	56.0
India	42.6	3.0
Indonesia	16.5	28.8
Iran		24.2
Lebanon	14.3	24.4
Mali		42.7
Mexico	2.9	4.1
Nepal	9.6	6.2
Somalia		51.0
Sudan		15.8
Syria	5.4	6.1
Turkey	11.6	9.0

[a] % of all milk produced in the country (FAO, 1993).

Table 2 Nutritional value of chevon (goat meat)

Item	*Content*
Moisture	72.2%
Protein	21.4%
Fat	3.6%
Minerals	1.1%
Calcium	12 mg 100 g^{-1}
Phosphorus	193 mg 100 g^{-1}

Goats' milk is considered wholesome and easy to digest, and its fat and protein contents are similar to those of human milk, making it ideal for human nutrition (**Table 3**). It contains smaller fat globules than cows' milk (3.5 μm compared to 4.5 μm) and has different protein polymorphs and high levels of short-chain and polyunsaturated fatty acids.

Economics of Goat-Keeping

"The goat is a poor man's cow", said Ghandi. Indeed, if investment costs are considered, a goat producing 750 kg of milk annually can be equivalent to a cow producing 3750 kg of milk annually. Goat-keeping requires very little capital investment in equipment and housing and is ideally suited to poor economies. Shelter from the elements vary from a 'kan' box or a brick wall to a bamboo house or a low-cost wire-netting and masonry shed. (**Figure 1** to **4**). Low feed and fodder requirements, early puberty, short gestation and high

Table 3 Composition of goats' milk

Item	*Content*
Total solids (w/w)	12.9%
Lactose	4.4%
Fat (w/w)	4.1%
Protein	3.5%
Casein	3.1%
Minerals	0.8%
Potassium	204 mg l^{-1}
Calcium	134 mg l^{-1}
Phosphorus	111 mg l^{-1}
Sodium	50 mg l^{-1}
Magnesium	14 mg l^{-1}
Zinc	0.3 mg l^{-1}
Vitamins	
Ascorbic acid	1.3 mg l^{-1}
Pantothenic acid	0.31 mg l^{-1}
Niacin	0.28 mg l^{-1}
Riboflavin	0.14 mg l^{-1}
Thiamine	0.048 mg l^{-1}
Vitamin A	185 IU l^{-1}
Fatty acids	
$C_{4:0}$	0.13 g l^{-1}
$C_{6:0}$	0.09 g l^{-1}
$C_{8:0}$	0.10 g l^{-1}
$C_{10:0}$	0.26 g l^{-1}
$C_{12:0}$	0.12 g l^{-1}
$C_{14:0}$	0.32 g l^{-1}
$C_{16:0}$	0.91 g l^{-1}
$C_{18:0}$	0.44 g l^{-1}
$C_{16:1}$	0.08 g l^{-1}
$C_{18:1}$	0.91 g l^{-1}
$C_{18:2}$	0.11 g l^{-1}
Cholesterol	11 mg l^{-1}
PH	6.6

From *US Department of Agriculture Handbook* (1976).

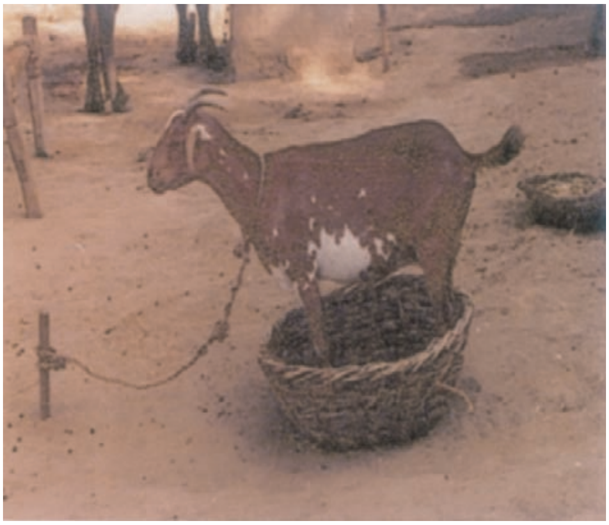

Figure 1 Kan box as goat shelter.

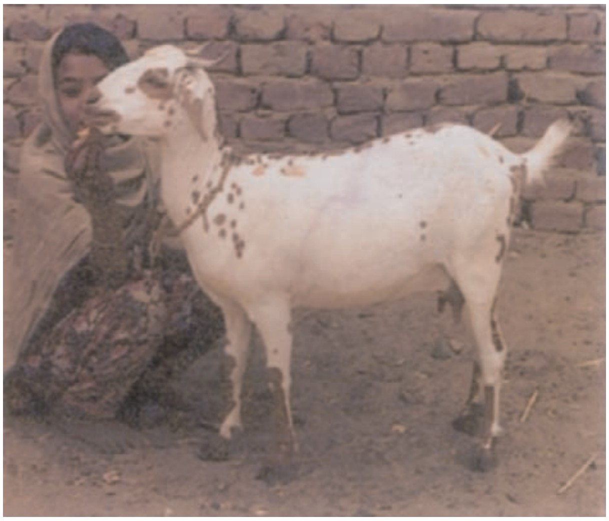

Figure 2 Small brick wall as goat shelter.

Figure 3 Bamboo house as goat shelter.

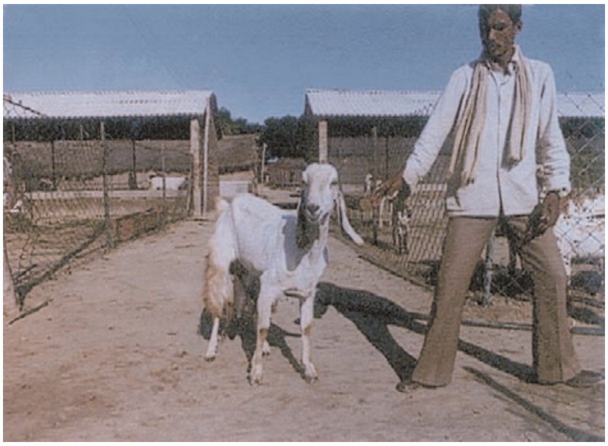

Figure 4 Wire-netting and masonry goat shelter.

prolificacy (i.e. twins, triplets and quadruplets) are added advantages.

The Latur District of Maharashtra in India, after being struck by a devastating earthquake in 1993, focused on goat-farming as part of its rehabilitation programme. It was found that 10–12 female goats yielded sufficient profit (Rs 50–200 per goat) to sustain the unit. Studies in Etawah, also in India, on 64 families in three villages, revealed goat-farming to be a profitable small-scale enterprise. The profitable holding size ranged from 1 to 10 head per unit, and involved 76 adult work-days and 20 child work-days a year. In a study in Bangladesh, 200 households were getting 0.2 kg day^{-1}of milk from rearing goats. With a body weight gain of 25 kg day^{-1}and a kidding rate of 2.0 per goat, this was profitable under a zero-input system.

A study in India of the relative economic benefits of different ruminants revealed goats to be 130% superior to cattle under free-range grazing conditions and 250% superior to sheep under sedentary and migratory systems. Studies in Pakistan showed that goats were 40–60% more profitable than sheep. In Malaysia, the cost of producing 1 liter of goats' milk was half that of 1 liter of cow's milk. A cost–benefit analysis of a dairy-goat agroforestry management system in Guatemala revealed net profits of 13% during the first year, which subsequently increased to 200%, excluding labor and incidental costs.

All goat-farming models tested were profitable and provided employment opportunities; the goats were also a source of protein for the households. Because the farmers involved only held small amounts of land, they were unable to rear larger ruminants. Further experiments have demonstrated that goats produce more meat and milk per unit weight or feed input than sheep, camels or cows. Many studies in Africa, China, India and Mexico have shown goats to be an economically viable option for poor farmers with no resources and for laborers without land. In Mexico, it was found that rearing 50 goats ha^{-1} gave a net return of $US417 ha^{-1}, which was a far better return than other agricultural operations.

Biological Advantages of Goats

Goats mostly occupy marginal lands, with low rainfall and poor soil, that are unfit for cultivation (**Figure 5**). However, they have certain biological advantages over other ruminants. Unlike cattle, which have a large buccal cavity and swallow a large bolus after grazing, goats nibble selectively and swallow a small bolus. They also browse on leaves, which they carefully select rather than biting at the whole plant (**Figure 6**).

As a result, the plant matter eaten by goats contains more nutrients than that eaten by other ruminants. They also obtain more energy per bite and therefore they need a proportionately smaller volume of food. Experiments have shown that, if desirable browse plants are maintained and stocking rates are properly balanced, goats will have little competition from other grazing animals. Any

Figure 5 Goats grazing on fallow lands.

Figure 6 Goats browsing in Rajouri (India).

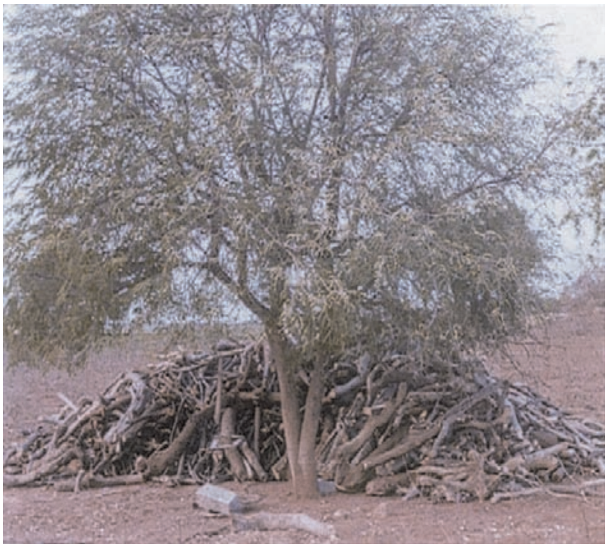

Figure 7 Cut wood: Who is the destroyer, man or goat?

Figure 8 Goats grazing on stubble fields.

deterioration of the environment is caused by unrealistic stocking rates and cutting of wood (**Figure 7**) rather than by the grazing or browsing of goats in areas where crops have been harvested (**Figure 8**). Close-harvesting of ground vegetation is more harmful to the soil (**Figure 9**) than grazing by goats (**Figure 10**).

Soil–Plant–Goat Relationship

This relationship determines the husbandry and management system of a species. Most of the grasslands in the world have been overgrazed by sheep and cattle but only scrub vegetation is utilized by goats. Their feeding habits (see above) make them less destructive to ground vegetation, the loss of which leads to soil erosion. They are lighter in weight than other ruminants and do not cause as much disturbance to the soil. In addition, their urine and droppings enrich the soil and aid plant propagation by dispersing seeds.

It is therefore difficult to see why the goat should be a subject for environmental concerns, given an appropriate stocking rate. A stocking rate of 3 goats ha^{-1}does not affect soil texture under semi-arid and arid conditions, and increased stocking rates may be sustained in temperate pastures and humid, tropical and subtropical conditions.

Recently, goats have been put to beneficial environmental use in the control of herbaceous weeds, shrubs and noxious plants (*Lantana*, *Tithonia*, *Nasella trichotoma*, *Rubus*

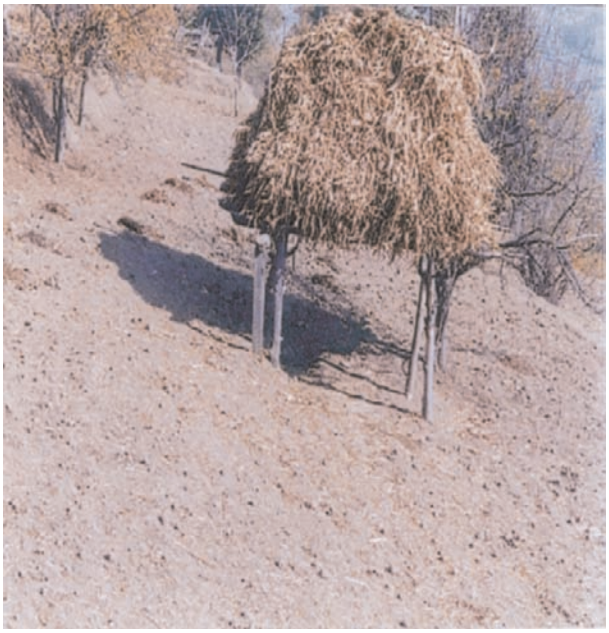

Figure 9 Forest grasses harvested and stocked.

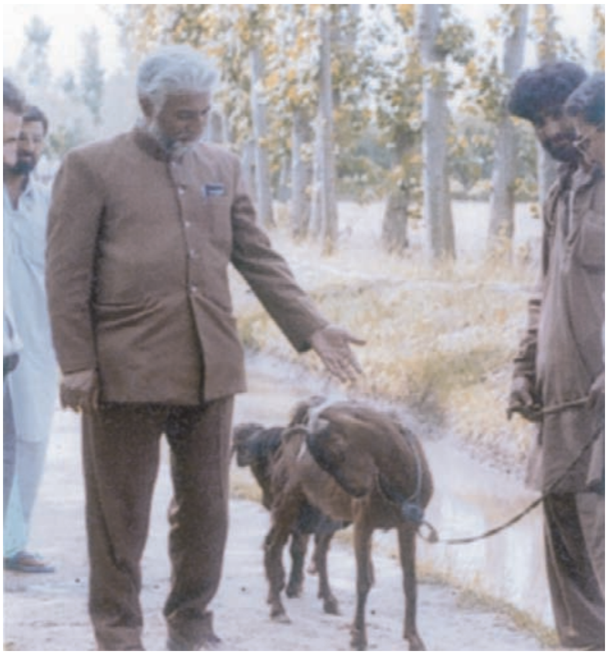

Figure 10 Tethered goat under integrated management.

fruticosus and *Rosa rubiginosus*) in forests, as well as in the creation of firebreaks.

Goat Husbandry: Management Objectives

Goats are husbanded in areas of poor economic conditions with the aims of producing meat, fiber or milk, in varying degrees of importance according to the situation.

Meat and Fiber

Most goats (65%) are found in arid and semiarid areas. The people who rear goats in these situations are either landless or wandering laborers with their unemployed families. These nomadic people require goats which can withstand water deprivation for days and which can digest low-quality forage during their long migrations. Breeds such as the Bedouin of Africa, Khagani of Pakistan, Bakarwal of Kashmir and Cashmere (Pashmina) of Mongolia can take in large amounts of water when it becomes available with no adverse effects on the rumen. The management objective under these situations is mostly meat or fiber, milk and other byproducts being secondary.

Milk

In subhumid parts of the world, where 16% of goats are found, and in some other climatic zones, the farming system is attuned to a tree–crop integrated management system, based on subsistence goat production and management by individuals not groups of people. These individuals range from a few hobby goat-keepers to landless laborers. The management objective of this group is milk for use at home or for limited sale. The goat is also an insurance against hard times or illness.

In this system, laborers work in crop-rearing or plantation establishments during the day and bring home twigs, leaves, byproducts or waste to feed their goats. At times, the goats are tethered in shade or in the farm area where their owners work (**Figure 10**). Such small-scale goat-keeping systems are mostly aimed at milk production.

The 9% of goats reared in humid environments, under a combination of stall-feeding and zero grazing, are also raised for milk.

Meat, Fiber and Milk

In areas of range and pasture land, where goat-rearers have no land tenancy rights, the extensive management system makes optimum use of what is available. In this almost zero-input type of management, meat or fiber for marketing is the objective.

In subsistence, village-based, agropastoral situations, goats are kept for milk and feed on fallow-land vegetation, roadside tree loppings, browse and grazing, supplemented with crop residues. Home consumption rather than marketing is the main objective.

Other Objectives

Apart from the above purposes, goats are also reared as an insurance on future investments. In the event of crop failure, for example, goats can be sold to buy seeds and other goods. They are also kept for religious rituals and other customs, such as the buck-fights held on festive occasions in Mongolia. Their horns and hooves are trimmed to serve as ornaments and aphrodisiacs, and their blood and bones serve as meal for poultry. They are used as pack animals in Nepal, Bhutan, Sikkim and Ladakh. Their silky white hair is used for fishing lines by farmers on the east coast of Mali, and Cashmere and Mohair fiber is highly prized for textiles.

Goat Husbandry: Management Systems

The various management systems have been named differently by different authors. For a better understanding of their purpose and characteristics, the systems have been grouped into four categories, as shown in **Table 4**. The breeds of goat, the countries where they are found, and the management systems and objectives are summarized in **Table 5**.

Subsistence

Subsistence means ‘maintaining existence’. These goats are reared not for commercial purposes but to help and sustain the individual farmer’s domestic needs. Such goat-keepers are mostly smallholders, landless laborers or marginal farmers. This is the predominant system in semi-humid and humid climatic zones, although it is also found in other zones.

In this system, one or a few goats are reared by the farmer. The goat is mostly kept indoors but is also tethered outside for some time during the day. This system is usually associated with an intensive agricultural system, such as rice paddy, wheat or plantation crops, e.g. sugar cane, rubber or fruit trees. Tethering may be combined with grazing, i.e. the goats may be tethered in a grazing area or moved onto roadsides, bunds or river banks for grazing. Usually, the owner finds time during his work on a crop-intensive venture to feed tree leaves or loppings to his goats, which are tied up in the shade of a tree, held in corrals or in the charge of children.

Sometimes, a child may take a group of such goats to graze in stubble fields (**Figure 8**), alongside canals or on common land. Women bringing lunch to workers in the fields may also take charge of the goats for a time and, with their children, also feed the goats on loppings, leaves, cut grass, waste materials or crop residues when at home. In small urban and rural areas, goats are sometimes let loose to browse on roadside trees and hedges.

Hobby goat-keepers also keep their goats housed, after letting them browse in the garden or communal compounds. In India and other developing countries, some urban households rear small goats on kitchen waste and freely available browsing materials.

The Barbari in India is one example of a breed used for this purpose.

Intensive System

This system requires permanent housing and equipment for feeding, watering, milking, etc. A commercial venture, it is mostly aimed at producing milk or milk products, such as cheese (France) and goat-meat products (e.g. kebabs in Turkey). The goats are kept indoors (zero grazing) and stall-fed. This system is common in industrialized countries where waste materials are used to rear goats. It is combined with various agroforestry systems and is applied mainly to dairy goats. In developing countries, it has been propagated through government-owned farms.

The smaller and more compact goats are better suited to this system than the larger, hardy, long-legged or hairy breeds. The Saanen, Toggenburg, Barbari and some East African breeds, for example, are suited to stall-feeding. This intensive production system involves intensive fodder production and supplementation with balanced feeds throughout the year. It also utilizes waste and agricultural byproducts, such as fallen apples and sugarcane waste, all of which are fed to goats after enrichment or fortification. Grazing goats are preferable to high-intensive browsers.

This system is practiced for milk production in southern Germany, France and other parts of Europe, the United States and other temperate countries. In India, in the humid and semihumid conditions of Maharashtra and Kerala, this type of goat-rearing is

Table 4 Goat management systems

System	*Synonyms*	*Purpose*
Subsistence (S)	Tethering, sedentary	Milk
Intensive (I)	Stall-feeding, zero grazing	Meat and milk
Semi-intensive (SI)	Grazing with night penning, browsing with supplementation	Meat, fiber and milk
Extensive (E)	Transhumance, migratory zero-input system, free-grazing system, open-range system, ranching	Meat, fiber and milk

Table 5 Breeds of multipurpose goats and their management systems worldwide

Goat breed	*Country*	*Purpose*[a]	*Management system*[b]
Alpine	France, Switzerland, USA	Mi	I/SI
Altai Mountain	Former USSR	F	SI
Anatolian Black	Turkey	H	SI
Anglo-Nubian	UK	Mi	I
Angora	Turkey	MF	SI
Angora	USA, Turkey, South Africa	F	E
Barbari	India	MMi	SI/S/I
Beetal	India	MMi	SI/S
Benadir	Somalia	MMi	SI
Bhakerwal	India	M	E
Bhuj	Brazil	MMi	SI/I
Black Bengal	India, Bengladesh	MMi	SI/S
Boer	South Africa	MMi	SI
Canary Island	Spain	Mi	SI
Carpathian	Europe	MMi	Si
Cashmere	China, Mongolia, Pakistan	F	F
Chamois	Switzerland	MMi	SI
Changthangi	India	F	SI
Chapper	Pakistan	F	E
Charneguerg	Portugal	M/Mi	SI
Chegde Polled	China	F	Si/E
Chegu	India	F	SI
Chengdu	China	MMi	SI
Corsican	Corsica	Mi	SI
Damascus	Syria, Lebanon	MMi	E
Dera Din Pannah	Pakistan	M	SI/E
Don	Former USSR	F	SI/E
Duan	China	M	E
Dutch White	The Netherlands	Mi	SI
Gaddi	India, Pakistan	M	SI
Ganjam	Pakistan	MMi	SI
Garganica	Italy	MMi	SI
German Improved Fawn	Germany	Mi	I/SI
German Improved White	Germany	Mi	I/SI
Girgentana	Italy	Mi	SI/I
Gohilwadi	India	H	E
Gorki	Former USSR	Mi	SI/I
Greek	Greece	MMi	SI
Huaipi	China	M	SI
Jamnapari	India	MMi	SI
Jining Grey	China	P	SI/E
Kaghani	Pakistan	H/M	E
Kajli	Pakistan	H	E
Kamori	Pakistan	Mi	SI
Kannaiadu	India	M	SI/E
Katjang	Malaysia	M	SI
	Indonesia	M	SI
Khurasani	Pakistan	MMi	SI
Kilis	Turkey	Mi	SI
Kurti	Iraq, Iran	F	E
Kutchi	India	H	E
La Mancha	USA	Mi	I/SI
Lehri	Pakistan	HM	E
Leizho	China	M	SI
Liaoning	China	F	E
Malabari	India	MMi	I/SI
Malaga	Spain	Mi	I/SI
Mamber	Syria Lebanon Israel Jordan	H	E

(*Continued*)

Table 5 (Continued)

Goat breed	*Country*	*Purpose*[a]	*Management system*[b]
Mambrina	Brazil	MMi	SI
Marwari	India	H	E/SI
Matou	China	M	E
Mehsana	India	H	E/SI
Mingrelian	Former USSR	Mi	I/SI
Mongolian	China	F	E/SI
Murcia-Granading	Spain	Mi	I/SI
Nachi	Pakistan	H	E
Nordic	Norway Sweden Finland	Mi	I/SI
Nubian	USA	Mi	I/SI
Oberhasli	USA	Mi	I/SI
Orenburg	Former USSR	F	SI/E
Osmanabadi	India	MMi	SI
Parda Alema	Brazil	MMi	SI
Pashmina	India	F	E
Pygmy	West Africa, USA	MMi	SI
Pyrenean	Spain	MMi	SI
Red	Bosnia	Mi	SI
Red Sokoto	Nigeria	MMi	SI
Russian White	Russia	Mi	I/SI
Saanen	UK	Mi	SI
	Israel	Mi	SI
	Switzerland	Mi	SI/I
	France, USA, New Zealand	Mi	SI/I
Sahelian	Africa	MMi	I/SI
Sardinian	Italy	MMi	I/SI
Serpentina	Portugal	MMi	SI
Serrama	Portugal	MMi	SI
Sind	Pakistan	MMi	SI
Sinhal	Nepal	H	E/SI
Sirohi	India	MMi	SI/E
Small East African	Africa	M	E/SI
Somali	Somalia Kenya	M	E/SI
Soviet Mohair	Former USSR	F	E
Spanish	USA	M	E
Sudanese Nubian	Sudan	MMi	I/SI
Surti	India	MMi	SI
Toggenburg	Switzerland, USA	MMi	I/SI
West African Dwarf	West Africa, USA	M	E/SI
Wuan	China	F	E
Xinjiang	China	F	E
Zalawadi	India	H	E
Zaraibi	Egypt	Mi	SI
Zhongwei	China	P	E
Zuwlin	China	F	E

[a] F, fiber; H, hair; M, meat; Mi, milk; P, pelt.

[b] E, extensive – zero input; I, intensive/stall-feeding; S, subsistence/sedentary; SI, semi-intensive.

gaining in popularity. In Kerala, Malabari goats are fed leaves of jack fruit (*Artocarpus heterophyllus*) and cassava (*Manihot esculenta*). The biggest advantage of this system is the effective utilization of waste or cheap available materials and its conversion into animal proteins. The controlled rearing has no damaging effects on the environment.

The flock size under this system is limited to tens or hundreds, not thousands, as in the case of extensive or semi-intensive management systems. This type of goat-rearing is labor intensive and involves proper hygiene, nutritive care and veterinary attention. These stall-feeding farms breed pedigree goats and supply genetic stocks with proper records to other farms.

Semi-Intensive System

This involves the controlled grazing of goats during the day and keeping them overnight in some form of shelter. Grazing is supplemented with feed or fodder. Almost two-thirds of the goats in the world are reared under some version of this type of management. It is a compromise between the intensive and extensive systems and combines free grazing for a few hours with supplementations during the night. All-purpose goats can be managed under this system.

Some of the traditional ways of practicing this system are as follows:

1. All village goats, about 100–150, are flocked together before dawn and taken to a forest/pasture area for grazing by a traditional goat-herd who knows the area well. He is often accompanied by a herding dog to protect the flock from predators. He brings the flock back at dusk and the goats are housed, fed and milked during the night. Kids are retained and fed at home. The goat-herd is paid either monthly or on a barter system. The villagers provide him with food each day and, on harvesting of the crops, he receives a fixed share per goat to compensate him for days not working.
2. During the summer in temperate areas, dry flocks of a few hundred or thousand are taken to highland pasture by a more experienced goat-herd on a monthly fixed rate per animal. He takes his family with him and is supplied with salt and precautionary medicaments by the owner. After the harvest, the flock is brought back and stall-fed during the winter (or even during a harsh summer).
3. In some areas, even in tropical climates, a few individuals will take their own flock of 20–25 goats out to browse or graze on common land or fallow forest land during the crop-growing season. The goats are brought back in the evening, stalled and given supplementary rations.

Extensive System

This system is practiced in dry, barren lands with low rainfall, and in hot and cold deserts. Such land has a low carrying capacity and is unfit for agriculture, or it consists of steep mountains and difficult terrain.

In temperate and subtropical conditions, this system consists of a summer migration to rich upland pastures and a winter migration to warmer areas where there is no snowfall. The goat-keepers move with their flocks in search of pasture or food resources for their goats. They do not own any land but pay a tax to the government which gives them rights to browse and graze their goats in forest and other available land.

During their upward and downward migrations, many landowners allow the goat-keepers and their flocks to stop-over on their land so that it may be fertilized by the goats. They may also offer them some grass if it is available. Usually, recently harvested intensive-cropping areas are offered for grazing and night rests.

This system is prevalent in regions where pasture, common land or forest land is available. This is a zero-input system and the goat-farmers do not feed or shelter their flocks; feed or fodder is only given to sick or pregnant goats. Generally, a few families in a tribe live and move together. Most of the goats are hardy and they are reared mostly for meat and fiber. Hides and milk are usually considered to be a byproduct. In the rich pastures of temperate Russia, Mongolia, China and Kashmir, milking is routine but the main objective is fiber.

The animals suffer during migrations and casualties due to severe weather conditions are common. The goat-keepers have horses or donkeys to transport tents and camping equipment and dogs to guard the goats in pastures. At times, especially on uphill stretches, goats reared for fiber may be used as pack animals. The system involves little labor. Migration stress may be reduced by using trucks to transport animals up or down the mountains. In dry and tropical climates, these migrations cross areas at times when crops have been harvested and fields are open for grazing, or when leaves are falling in autumn, so that browse is available for the goats.

Factors Affecting Goat Husbandry

Size of Land Holdings

Worldwide, there is an unequal distribution of land, 65% being held by only 2.5% of the population. Landholders fall into three categories: commercial farmers with large holdings, those with small or marginal land holdings, and laborers with no land at all. With the exception of the few who own dairy goats as a hobby, most of the world's goat-rearers belong to the last two categories.

On average, a goat-farmer holds 1.0–2.0 ha of land (this is the size of holdings of 71% of farmers in Asia, 64% in South America and 85% in Africa). In the Philippines, goat-rearing is subsidiary to crop husbandry in almost 80% of households. Similarly, in Fiji, it is subsidiary to sugarcane cropping in 70% of households. Of the goat-farmers in India and in Guatemala, 68% and 88% respectively are smallholders. In Sri Lanka, goat-keepers with families ranging from five to eight in number hold 0.5–2.0 ha of land, while those operating under the intensive system of management own only 0.3 ha. In Costa Rica, more than 40% of small-scale goat-keepers tether their goats, while 26% allow them to graze freely on bush and 27% have crop-combination/open-pasture grazing goats.

labor Requirements

Goat-farming does not involve much labor, requiring three times less labor than crop-farming or cattle-rearing (slightly less for sheep). On average, and varying according to flock size, 40–50 days of total family labor is spent on goat-farming. The women's share in the family labor varies from 20 to 34 days, i.e. marginally more than 50%.

Flock Size

Flock size varies from one to thousands depending on the management objectives (see **Table 4**). Generally, large flocks are maintained for meat and fiber under an extensive management system, medium-sized flocks are kept in semi-intensive and intensive commercial systems, and smaller flocks are kept under a subsistence (tethering) system. However, experimental combinations of these systems are found in various organized and private farms with goat numbers ranging from hundreds to thousands.

Under the subsistence stall-fed system (nonmigratory, 'stationary' farmers), flock sizes are small, e.g. 2–10 in Indonesia, 6–7 in India, 5 in Nigeria and 1–9 in Malaysia. However, under the extensive management systems used for goats reared for fiber, meat or as pack animals, flock sizes are large and are mostly free-range grazers. This zero-input system bears profits but these may be reduced by adverse weather conditions, e.g. floods, drought, forest fires and high winds. In the northern hills of the Philippines, the introduction of night shelters and vaccinations to this system has given good results.

Socioeconomic Conditions of Goat-Farmers

The socioeconomic conditions of farmers rearing goats in Kashmir have been studied in a collaboration between the universities of Hohenheim in Germany and Shalimar in India. Various parameters, e.g. farming system, family status, family income, housing pattern, flock structure, goat housing, landholding size, and grazing land available under multipurpose management systems, were studied.

Family Status

Most of the goat-farmers lived in joint families varying from nine to 12 members. In Malaysia, the Philippines and other developing countries, goat-farmers live in joint families or in group clusters of tens, not in a nuclear family, as is prevalent in developed countries.

Housing

Housing patterns varied between the migratory goat-farmers (using extensive or semi-extensive systems), who are in the majority, and the nonmigratory, sedentary goat-farmers (using subsistence/semi-intensive/stall-feeding systems). Neither owned their own pastureland, although sedentary farmers, especially those rearing goats under a semi-intensive management system, owned some grazing rights in the locality. Goat-farmers managing goats under an extensive, transhumance migratory system also had grazing rights on common land.

Unlike migratory goat-farmers, sedentary farmers tend to till the land and live in their own houses. In India, about 90% of the sedentary goat-rearers shared their house with goats. Most (60–90%) owned some land, which they used to grow cereals and the goats were grazed on forested land.

In Kashmir, some of the sedentary farmers own their own houses. The house is often shared with the goats but, in some places, a separate kacha (mud house) or a permanent shed is available for housing goats during the winter. Goats reared under subsistence or intensive management systems were not the sole livelihood of these families and contributed only 4–15% of the family income. On the other hand, farmers rearing goats under extensive, migratory systems are fully dependent on the goats for their income.

Goat–Farmer/Management Interaction

The four management systems described are not clear cut and variations occur from country to country. Some institutes in Guatemala and India (Central Institute for Research on Goats, Makhdoom, Mathura, India) have experimented on dairy goat/agroforestry management interaction systems, using goat manure in place of fertilizers and tree twigs or routine loppings for feeding the goats. Many of the experiments on zero-grazing management, in which cut-and-carry forage was used, returned profits in spite of the investment on shelters, corrals and dairy equipment. These experimental models were profitable even when the flock size was small (2) and shelters 2–3 m^2 in area and 50 cm off the ground were provided. In these models, flock size (mostly females) varied from 1 to 5 (small holding), 6 to 10 (medium holding) or more than 10 (large holding). All models returned profits. A similar combination of goats and coconut plantations in the Philippines, operated under a semi-intensive management system, also produced profits and was of benefit to the plantations because the goats controlled weeds and undesirable grass growth beneath the trees.

Similarly profitable semi-intensive to intensive goat-rearing proved possible in the Jaffna peninsula of Sri Lanka, where impoverished Tamil laborers rear goats. There, flock size varies between 2 and 10, and the goats are fed tree-loppings from *Gliricidia*, jak or ipil. In Guatemala, forage trees and bushes planted on irrigated land provided 90% of the browse replacement to stall-fed goats.

Many combinations of crop-integrated, semi-intensive to intensive goat management systems exist around the world, e.g. in rice-paddy–goat integrated farming, paddy straw enriched with molasses and urea is fed to the goats. Similarly, many other agricultural wastes (e.g. from the cultivation of mangoes, apples, mulberries, rubber, coconuts) and crop residues can be used as fodder. In Papua New Guinea, flocks of 20–30 stall-fed goats (native goats crossbred with Saanens) are reared on vegetable waste, supplemented with vegetable waste. Similarly, in the Philippines, crosses of the native goat with the Anglo-Nubian, Saanen or Toggenburg are commonly reared on crop residues in backyards. In Korea, intensive stall-feeding on enriched nutrients is profitable and improved varieties of goats are emerging.

Summary

Goat farming, because of its low capital investment and quick economic returns, has been integrated into flexible and vulnerable animal agriculture production systems. As a source of milk, meat, fiber, skin, hair, horns, manure and other valuable byproducts, goat husbandry calls for multi-functional management systems. These management systems range from extensive to the intensive with many combinations. It has been shown that goats are a uniquely successful earner of cash for small farmers or landless laborers. Goats are excellent at utilizing unfavorable marginal environments, and in better terrain can be integrated with crop production as an extra source of profit. Many specialized breeds of goats have been developed to exploit these different opportunities.

See also: **Animals that Produce Dairy Foods**: Goat Breeds. **Dairy Farm Management Systems**: Goats. **Husbandry of Dairy Animals**: Goat: Feeding Management; Goat: Milking Management; Predator Control in Goats and Sheep; Goat: Reproductive Management. **Mammals**. **Milk**: Goat Milk.

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Goat: Replacement Management

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Prenatal Care

Doelings to be raised for replacement should be identified before birth. Since it takes a large investment in feed and labor to raise replacements, only those doelings with genetic superiority should be raised and the remaining kids culled. Doelings should be from does that are good milk producers and have good body conformation. The sires should also be genetically superior for milk production and body conformation.

Prenatal care of the doe begins during the last 3 months of lactation. Does should be fed so that they are in a body condition score of 3.0–3.5 when they are dried off and only need to maintain that body condition during the dry period. Steps should be taken to prevent pregnancy toxemia (maintaining the doe in proper body condition and giving them exercise). Does should be vaccinated for tetanus (*Clostridium tetani*) and overeating (*Clostridium perfringens* type C and D) disease 2–4 weeks before kidding. This will increase the level of antibodies to these pathogens in the colostrum and provide protection against these diseases during the first few weeks of life of the kid. In some areas, it may be appropriate to give an injection of vitamin E and selenium. Hay loses vitamins A and E, so injections for does could be warranted. This will add vitamins to the colostrum, which is rich in antibodies (called immunoglobulins) that will be easily absorbed through the intestinal wall after birth.

Caprine arthritis encephalitis (CAE) virus causes encephalitis, arthritis, progressive pneumonia, and hard udders that do not produce milk. This disease causes serious problems for the dairy goat industry and needs to be eradicated. The major route of transmission is through the milk. The disease can also be spread laterally from infected animals in the herd and through bodily secretions. There are two blood tests (enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID)) available to test for the disease, but it has been documented that animals can carry the virus for years without testing positive (due to not producing antibodies against the virus). Therefore, a negative test result cannot be relied upon to indicate that an animal is not infected. However, animals that test positive are infected. One caveat, a kid that consumed pasteurized milk that contained dead CAE virus may test positive for several months. Eradicating the disease requires a commitment to strict management protocols, including biosecurity.

There are three parts of a CAE eradication program: prevention of the disease in kids, prevention of the disease in the CAE-free herd by lateral transfer, and biosecurity to prevent reintroduction of the disease. Kids need to be taken away from their dam immediately after birth before their dam licks them. The kid should be removed to a separate area that is isolated from contact with adult animals. The kid should be fed heat treated colostrum (heated in a water bath at 56 ° C for 1 h) for the first few feedings and then should be fed pasteurized goat milk, milk replacer, or pasteurized cow milk until weaning. Milk can be pasteurized by heating to 63 ° C for 30 min or 72 ° C for 15 s. Milk must be stirred occasionally as it is heated so that all of it gets up to pasteurization temperature and kills the CAE virus. It is of paramount importance to put safeguards in place so that kids are not accidentally fed unpasteurized milk since several drops may be sufficient to transmit CAE.

The kids that are raised on a CAE-free program should be kept separate from other does/kids to prevent lateral transfer such as by sneezing. Basically, there should be a 2 m wide alley between herds to prevent lateral transfer. When does are milked, the CAE-free herd should be milked first. One needs to be careful to avoid potential transfer of the disease from an infected herd to the CAE-free herd. All replacements raised on a CAE-free program should be tested at 6 months of age to verify that they are truly CAE-free. CAE can be transferred between animals by using the needles or syringes on more than one animal, tattoo equipment, or clippers, so they need to be sanitized between animals. After one works to eradicate the disease, one has to be careful to not introduce the disease back into the herd by bringing new animals into the herd or by taking animals to the show or sale and returning them home.

Neonatal Care

Colostrum is very important for neonate immunity and survival, especially for prevention of scours and pneumonia, as well as for subsequent kid growth. Colostrum is the secretion milked from the udder at the first milking after the doe kids. This milk will have a much higher concentration of antibodies than the second milking. For best results, the kids should receive colostrum in the first 6 h of life when immunoglobulin absorption is highest. Intestinal absorption decreases after 24 h of age. Kids should receive 60 ml of colostrum (heat-treated if on a CAE prevention program) per kg of bodyweight per feeding for three feedings in the first 24 h. Cow colostrum (heat treated) can be used as a substitute. Most commercial colostrum substitutes have not proven to be effective. Successful feeding of colostrum will increase serum total protein to greater than 5.5 g dl^{-1} or serum immunoglobulin concentration to greater than 1200 mg dl^{-1}. The sodium sulfate serum turbidity test can be used to ascertain the adequacy of colostrum feeding. Good prenatal care will help to improve the concentration of immunoglobulins in colostrum. It is useful to have frozen colostrum for emergencies. Colostrum can be frozen in an ice tray and cubes transferred to plastic ziplock bags for storage. Colostrum cubes of appropriate weight can be thawed, heat-treated, and fed to kids. Colostrum should not be thawed in the microwave.

Kids that have a birthweight less than 2.5 kg are at risk for hypothermia and are likely to have a higher mortality than normal-weight kids. Kids that have a birthweight higher than 5 kg are also at risk, because of dystocia. Kids that are suffering from hypothermia lack a sucking reflex and may need to be tube-fed to get colostrum into them to provide energy to aid in warming their bodies. A #14 French urethral catheter can be used for a feeding tube. It is carefully inserted in the esophagus and proper placement is checked by feeling in the esophagus and instillation of a few milliliters of water. Kids can be given 60 ml of colostrum per feeding at 2 h intervals until the kid can suck. If a kid is severely hypothermic (rectal temperature <36.5 °C), he needs to be warmed either in warm water or by blowing warm air such as from a hair dryer or portable electric heater. Hot air should not be used and care should be taken so as to not overheat the animal.

Feeding the Neonate

Commercial dairy goat farms rarely feed their kids with the does' milk because of the cash value of the milk. CAE-free goat milk is superior to milk replacers for raising kids since goat milk contains immunoglobulins at low levels. This may be helpful in protecting the intestinal lining and the respiratory tract from infections. It is possible to raise kids on their dams by allowing kids to nurse during the day and separating them from the does overnight and milking once a day in the morning. Kids with access to their dams only during the day will grow well and have the advantage of learning feeding and social behavior from the dams. Disadvantages are that disease prevention (CAE and Johne's disease) is precluded.

Milk replacers are used to spare milk from the doe for sale and yet provide similar nutrition for the kid. Some milk replacers contain lactose-digesting bacteria or probiotic organisms, which may mitigate digestive upset. Lactose-digesting bacteria can be added to the milk in the form of a heaping tablespoon of yogurt or a dry commercial product. Milk replacers contain 20–28% milk-based protein and 16–24% fat. Since protein is limiting for a young kid, a higher level of protein would be expected to increase growth rate. Fat is used as an energy source for kids to grow and to keep warm. Other important nutrients are fat-soluble vitamins A, D, E, and K, which help maintain the epithelial lining of the skin, gut, and the respiratory systems, which are the physical barriers to infection. Vitamin C, copper levels, and the addition of probiotics should also be listed on the feed tag. Milk replacers should be mixed according to the manufacturer's recommendations.

Farmers have successfully used cow, sheep, and kid milk replacers to raise kids. Artificial rearing is necessary for CAE control. Whey has been fed at 20–45% of the milk with variable results. Some producers mix milk replacer with one-third to one-half goat or cow milk. The kid should be gradually transitioned to different milk sources after the first day of feeding only goat colostrum.

During the first 4 weeks of life, kids have a very high nutrient requirement, especially for protein (see **Figure 1**). Given free-choice access to milk, kids could consume about 700–800 ml each day in their first week of life, 1200 ml each day in the second week, and 1500 ml each day in the third week. Milk intake will be lower when feeding milk only 2 or 3 times per day. However, feeding large quantities of milk at any one time can cause digestive upsets such as bloat or diarrhea. For this reason, it is preferable to feed three or four smaller meals the first 4 weeks of life or to limit feed milk. An alternative is to provide free-choice cold (5–10 °C) or acidified milk, which causes kids to consume numerous small meals throughout the day. Milk or milk replacer can be acidified with dilute formic acid (1 part of 85% formic acid diluted with 9 parts of water) at the rate of 30 ml dilute formic acid to 1 l of milk or milk replacer. This works best if milk is cool at the time of acid addition and the mixture is mixed vigorously. The pH should be maintained between 4 and 4.5 pH paper (test with) to prevent pathogen growth and preserve the milk. Nipple bucket feeders with cooled or acidified milk need only one nipple per three kids in a pen since the kids consume only small meals (**Figure 2**).

There are several protocols for limit feeding of milk replacer, which also reduces digestive upsets in addition to greatly reducing milk replacer costs. With one protocol, 1 kg of milk per day divided into two feedings,

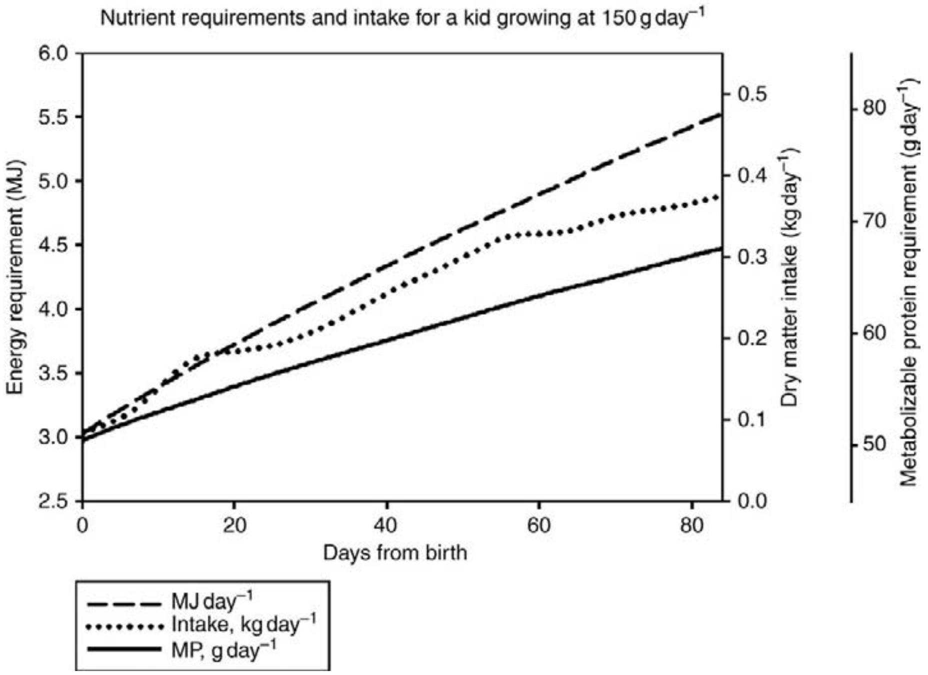

Figure 1 Nutrient requirements and intake of young kids.

beginning at 3 weeks of age, and starter is offered. This resulted in a gain of 150 g day^{-1}, similar to *ad libitum* milk alone to 10 weeks of age. Kids weighed 13.5 kg when weaned at 10 weeks of age and required only 68 l of milk replacer and 7 kg of starter feed. Another protocol fed 400 ml of milk replacer divided into three feedings on days 1–3, 500 ml divided into two feedings on days 4–14, 650 ml divided into two feedings on days 15–21, and fed 780 ml in two feedings on days 22–28 and 780 ml fed in one feeding on days 29–42 and weaned on day 42. A high-quality pelleted starter was offered beginning at 3 days of age. Kids were able to gain the necessary 150 g day^{-1} through 7 months of age and consumed 27.5 l of milk replacer during this time, but starter and hay intake were not measured.

When feeding groups of kids, offer a minimum of one nipple per kid, so that all have an equal chance to feed (**Figure 3**). Automatic milk/milk replacer feeders may be employed to save labor and administer meals free choice or programmed for interval feeding. In large farms, this saves labor. One nipple will severe up to 10 kids (**Figure 4**).

A pelleted starter feed should be introduced at 1 week of age and should contain 18% crude protein (CP), 10.6 MJ of metabolizable energy (ME) per kg, and a coccidiostat such as Rumensin or Deccox (in a recent

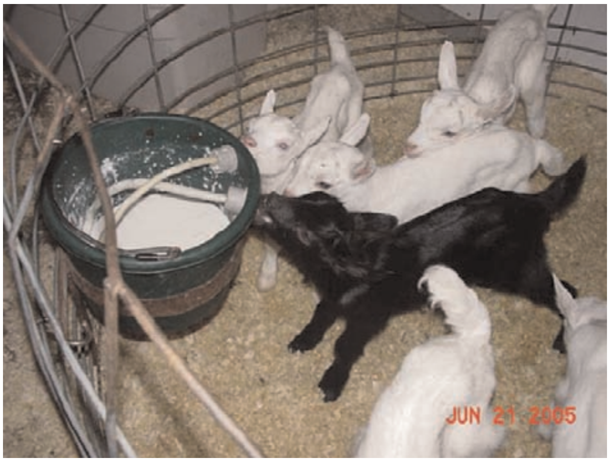

Figure 2 Acidified milk fed free choice with two nipples per pen of six kids.

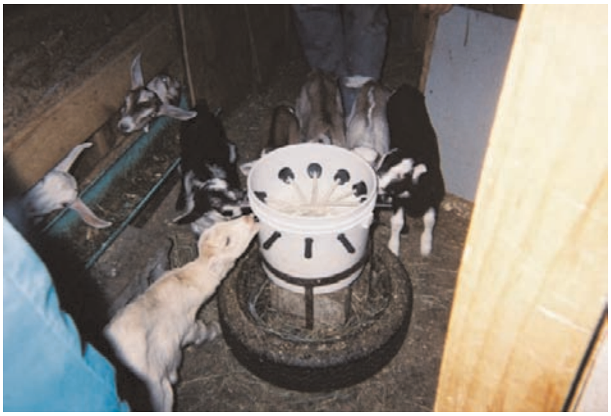

Figure 3 Bucket milk feeding with nipples.

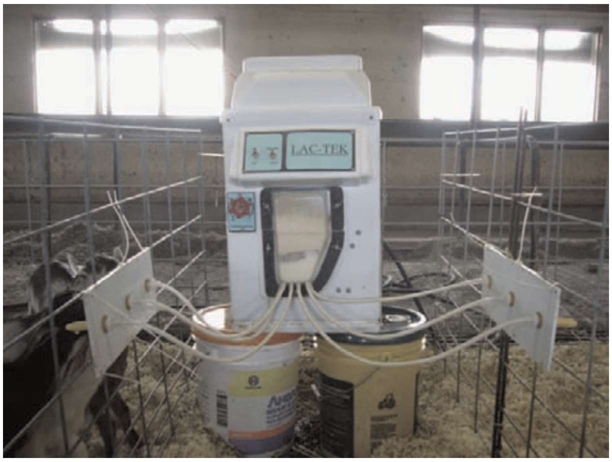

Figure 4 Automatic milk replacer feeder.

work with dairy calves, it was shown that the mash form (crumbled pellets) resulted in greater intake than pelleted starter, which in turn gave greater intake than did ground or meal form). Many calf starters fit these requirements. Hay should be introduced at 2–4 weeks of age and should be of very high quality, such as an immature alfalfa or vegetative stage grass–legume mixed hay. Uneaten hay needs to be replaced twice weekly. Weaning at later ages such as 12 weeks results in a larger kid and ultimately a larger doe, which may have potential to produce more milk, but at a greater feed cost. Many producers wean as early as possible to reduce feed costs since milk (or milk replacer) is expensive. Minimally, a kid must be at least 6 weeks of age to be weaned, should have a starter intake of at least 250 g day^{-1}, and weigh at least 10 kg. It is best to have a several-day transition period during weaning in which milk is reduced to one feeding so that the kid is adapted to consuming starter in place of milk. **Table 1** contains a common feeding regimen for raising newborn kids through weaning to attain breeding weight (60% of adult weight) at 8 months of age.

Raising Bucklings

Buck kids selected for breeding stock may be raised with doelings until about 3–4 months of age with separation at weaning time to prevent unwanted breeding. Buck should be raised together with at least one other buck or wether for company. Bucks will grow fast, weigh at least 25% more than does, and can be used as sires starting at 8 months of age. Mature breeding bucks may be fed solely on high-quality forage, but will need some concentrate beginning at least 2 months before breeding season to improve body condition. Attention should also be given to proper mineral and vitamin nutrition to promote fertility and health. Feeding high levels of grain may result in urinary calculi, which are stones that form and block urination. By feeding a minimum of grain (less than one-fourth of the diet as grain) and by providing free access to clean water, this may be avoided. Other steps that may be taken to reduce the potential for urinary calculi with high-concentrate diets include increasing calcium-to-phosphorus ratio to greater than 2:1, using only sufficient phosphorus to meet nutrient requirements, using urine acidifiers such as ammonium chloride (0.5–2.0%), and the addition of salt up to 3% of the diet to encourage water consumption. Bucklings in the 10–40 kg weight range need a minimum of 8.0 MJ ME kg^{-1} for maintenance and up to 11 MJ ME kg^{-1} for gaining 250 g day^{-1}. The publication by National Research Council provides details on nutrient requirements.

Feeding Doelings

The target weaning weight for large-breed doe kids is 15 kg. The target breeding weight for large breeds like Alpine or Saanen is 35 kg by 7–8 months of age. Although does can breed and conceive at 3–4 months of age and at lower weights, this will stunt their growth and reduce milk production. To prevent this, it is best to separate the buck kids from the doe kids at weaning. To meet the target weaning weights and breeding weights, a kid will need to gain 150 g day^{-1} although many are capable of gaining 175 g day^{-1} before weaning. These weight gain objectives are achievable when a commitment is made to management, feeding, health care, and housing. Slower weight gains may be acceptable and farmers may breed kids at 10–12 months of age, but one must consider seasonal anestrus that occurs in the winter and early spring. **Table 2** has the monthly nutrient requirements and target bodyweights for each month of age. Basically, 0.36 kg concentrate per day is required to maintain growth rate when high-quality hay is provided. Bodyweights should be monitored monthly and nutrition adjusted accordingly.

Kid Health

The greatest threats to the health of the newborn kid are pneumonia, scours, and coccidiosis. Organisms that cause pneumonia are commonly present in the environment. Two practices will help prevent pneumonia: ventilation to reduce humidity and the concentration of pneumonia causing organisms; and the consumption of sufficient colostrum. In addition, the bedding must be kept clean, dry, and free of ammonia odor at animal level. Early detection of pneumonia is critical in its treatment. Animals must be carefully observed for abnormal behavior, such as a kid lying differently from the rest,

Table 1 Artificial feeding recommendations for large-goat breeds from birth to breeding age (8 months)

Age	*Weight*	*Feed (daily intake)*	*Management notes*
Newborn	(ideal)	Colostrum heat-treated	Dip the umbilical cord in a 7% tincture of iodine
Single	4.5 kg	Consumption: 150–300 g in first 24 h	Heat colostrum at 56 °C for 1 h
Twin	4 kg		Give colostrum within 6 h after birth
Triplet	3.5 kg		Give vitamin E/selenium shots if necessary
Quadruplet	3 kg		
One month old	6.8–9 kg	Pasteurized milk, milk replacer, CAE-free raw milk, or Johne's disease-free cow milk Limit the consumption at 3 weeks of age to 2 l day^{-1} Hay,[a] starter, and water available at all times	Daily weight gain, 0–2 months: 180–200 g Have homogeneous groups by weight Sufficient nipples should be available to feed each kid easily
Two months old	12–15 kg	Limit milk to 2–3 l day^{-1} Limit and start to decrease the quantity and frequency of milk Hay[a] intake: 200–300 g day^{-1}; starter intake: 150–200 g day^{-1}	Coccidiosis treatment if necessary Wean kids when starter consumption is at 250–350 g day^{-1} Wean kids over a period of 3–5 days by reducing milk feeding to one time a day
From weaning to breeding			
From weaning to 4 months old	15–24 kg	High-quality hay[a] (<26% ADF) intake: 800 g Concentrate intake: 400 g Concentrate intake: 600 g with lower quality hay (>36% ADF) Access to water May graze or browse	Daily weight gain: 150 g At 4 months old, create weight groups Too much grain will decrease rumen capacity to ingest forages May change from 18% crude protein concentrate to 16% crude protein concentrate Before going to pasture, feeding transition and time in the pasture should be taken into consideration. Even on a pasture system, hay should be offered
At 6–7 months old	26–29 kg	Good-quality hay[a] intake: 1.1–1.4 kg per animal, on a dry matter basis	Daily weight gain: 85–100 g
Breeding period (7–8 months old)	32–34 kg	Hay: 1.2 kg on a dry matter basis Concentrate: 270–450 g	Vitamin A, D, and E injection 15 days prior to breeding
From breeding to kidding		*First 3 months* Hay: 1.2 kg,[a] on a dry matter basis Concentrate: 270–450 g *Last 2 months* High-quality hay[a] Concentrate: 500 g	3 weeks prior kidding: yearlings should consume same forages and concentrate (less quantity) as a lactating goat Avoid any sudden feed changes at kidding

[a] Hay containing 8.76 MJ of metabolizable energy per kg and 12% crude protein (high-quality grass hay or mixed legume hay) to meet nutrient requirements.
ADF, acid detergent fiber.
Adapted from Corcy J-C (1991) *La Chevre*. La Maison Rustique, Paris and *Pole d'Experimentation et de Progres Caprin publications. e.g. L'élevage des chevrettes (des rendez-vous ratés)*. http://www.pep.chambagri.fr/caprins/html/contenu/pdf/mail%20du%202dec08/F.%20chevrette%20g%e9n%e9rale_A3-RV.pdf (accessed April 2010).

separated from the group, not coming up to the kid bar, and not as active as others. Pupil shape may change to round in a sick kid. Heavy breathing, nasal discharge, and coughing are present after the animal has been sick a while. Check the kid's body temperature; a temperature over 40 °C indicates fever. Treatment should follow veterinary protocol. Sometimes, moving kids outside as soon as practical will improve kid health. Overcrowding increases the risk for pneumonia. Animals should be kept healthy since other diseases such as soremouth or coccidiosis will provide opportunity for pneumonia. Stress such as mixing animals should be minimized and good nutrition is also very important.

Neonatal diarrhea is caused by several infectious organisms such as *Escherichia coli*, rotavirus, coronavirus, cryptosporidia, and salmonella. The disease is promoted by a buildup of these organisms in the environment, usually as the kidding season progresses and sanitation is

Table 2 Nutrient requirements for growing replacement kids postweaning

Age (months)	*Weight (kg)*	*Gain ($g\,day^{-1}$)*	*Energy (MJ ME)*	*Protein ($g\,day^{-1}$)*	*Calcium ($g\,day^{-1}$)*	*Phosphorus ($g\,day^{-1}$)*	*Concentrate[a] ($kg\,day^{-1}$)*	*Hay[b]* ($kg\,day^{-1}$)
3	17.3	150	4.55	109	2.65	1.85	0.36	0.48
4	21.8	150	5.41	118	2.91	2.03	0.36	0.68
5	26.3	150	6.23	123	3.17	2.22	0.36	0.77
6	30.8	150	7.01	132	3.43	2.40	0.36	0.86
7	35.3	150	7.72	136	3.66	2.56	0.36	1.0
8	38.3	100	8.26	123	3.54	2.48	0.27	0.95
9	41.3	100	8.74	114	3.52	2.46	0.27	1.05
10	43.3	100	9.06	114	3.63	2.54	0.27	1.05
11	45.3	100	9.37	118	3.76	2.63	0.27	1.09

[a] Quantity of a concentrate diet containing 11.2 MJ ME kg^{-1} and 16% crude protein to meet requirements.

[b] Quantity of hay containing 8.76 MJ ME kg^{-1} and 12% crude protein (high-quality grass hay or mixed legume hay) to meet nutrient requirements.

ME, metabolizable energy.

Data from http://www.luresext.edu/goats/research/nutreqgots.html.

neglected. Kids are more prone to diarrhea when fed large quantities of milk at one time. Insufficient colostrum intake may also be a predisposing factor as is overcrowding. Animals with diarrhea should be strictly isolated to prevent the spread of the organism to other animals. They should be taken care of after healthy animals, and one should be careful not to carry the infection on their clothes or hands to healthy animals. Milk-feeding containers may also spread the disease and need to be thoroughly cleaned and sanitized. Sanitation should be reevaluated and corrected at the first sign of diarrhea. Most of the common causes of diarrhea are self-limiting and therefore antibiotics are seldom used for treatment. Treatment should follow veterinary protocol. Generally, treatment consists of keeping the kid hydrated and warm. Usually, electrolytes are given to the kid in place of milk for several feedings and milk gradually introduced along with the electrolytes. It is best to give milk in small feedings every 2–4 h. Electrolytes may need to be administered subcutaneously to correct dehydration.

Coccidiosis is a protozoal organism that is part of the normal gut microflora and is present in low numbers. Coccidiosis should be suspected in kids over 2 weeks of age that have pasty diarrhea (has white streaks in it) with a history of stress. As the disease progresses, diarrhea becomes watery, causing rapid dehydration of the kid. Goats seldom have bloody feces as observed in other animal species with coccidiosis. Other symptoms of coccidiosis include off feed, listlessness, weakness, and the infected animal may show abdominal pain by crying or getting up again as soon as it lies down. Coccidia parasitize and destroy cells lining the intestinal tract of the goat and the resultant scarring of the small intestine may stunt the kid. The disease is caused by the kid somehow consuming fecal material, such as in the water trough or feedtrough, in combination with a weak, depressed or immature immune system. The immune system can be depressed by the stress of weaning and therefore coccidiosis can be a major problem at weaning. Damp, cool conditions promote the disease because the infective coccidial oocysts survive longer in the environment.

Coccidiosis can be best prevented by keeping the kid from consuming fecal material, usually through contaminated feed, feedtroughs, or water containers. Sanitation and promoting a dry environment aid in the prevention of coccidiosis. Good sanitation can be facilitated by use of slatted floors where feces and urine fall through (**Figure 5**). Feeding acidified milk or yogurt appears to be beneficial. Deccox M can be mixed in the milk and fed for 4 days every 4 weeks as a preventative, or Albon can be mixed in the milk 2 days a week. Good nutrition can help the immune system to prevent coccidiosis. Since older animals have greater immunity to the disease and may shed high levels of oocysts in the feces, young animals should not be housed with older animals. Coccidiostats such as decoquinate (Deccox) and Rumensin are used in feeds to prevent coccidiosis. Animals to be weaned should be fed a feed containing a coccidiostat for 4 weeks before weaning until 3 weeks

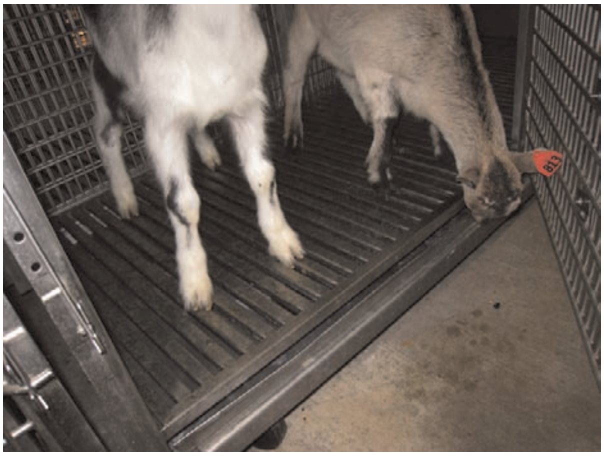

Figure 5 Kids on raised slatted floors.

after weaning. These coccidiostats are best fed when there is a high risk of coccidiosis such as in young animals less than 6 months of age or in animals undergoing a severe stress such as weaning or if there are cool damp conditions. Coccidiosis is generally treated with Albon or Corid.

Colostrum can provide passive immunity to enterotoxemia up to 5 weeks of age if does were vaccinated shortly before parturition. Kids on full feed of milk are at risk for enterotoxemia, especially if the milk is being fed only a few times a day. Sudden changes in feed or feeding routine can also precipitate enterotoxemia. Lush pasture or high-concentrate diets can cause enterotoxemia if the kid is not gradually adapted to them. Feeding high levels of concentrate with insufficient fiber intake from inadequate hay consumption can cause enterotoxemia. The major symptom of enterotoxemia is sudden death. The affected kid has an elevated temperature and severe abdominal pain. As the disease progresses, the animal will lie down on its side with the head down and may have convulsions. It may throw its head over its back. The kid will have diarrhea, and the urine will test positive for glucose if diabetic test strips are used. The disease is best prevented by vaccinating the kid (usually 4, 8, and 12 weeks of age) and by good feed management (gradual changes in feed, sufficient good-quality forage in the diet).

Internal parasites (gastrointestinal nematodes) generally infect kids over a month old. Kids usually acquire infective larvae from grazing, but may acquire some from consumption of bedding. The major strongyles include *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis* with *Strongyloides papillosus* occasionally being a problem, being transmitted through the bedding. Tapeworms (*Moniezia* species) are an ever present problem in young goats raised in semiconfinement (where they have access to grass since a grass mite is necessary for transmission). When kids are allowed to graze during the warm season, *Haemonchus* will be the dominant problem in warmer climates and can be particularly deadly. Beginning at 6 weeks of age, fecal samples should be monitored for roundworm eggs every 3 weeks to determine when there is a problem with internal parasites. Symptoms of parasitism include diarrhea, pale mucous membranes, thin, poor-doing animal, and edema. In temperate and tropical climates, one can monitor FAMACHA eye scores during the warm season, which assesses the degree of anemia, which could be assumed due to infection with *Haemonchus*. One should work with a local veterinarian or parasitologist, who should have a good idea of the species and effective treatments. Deworming all animals by some time schedule promotes dewormer resistance and is unacceptable. In the more humid climates of the United States, there is considerable resistance in parasites to dewormers. Kids raised in confinement where there is no grass growing or those raised on elevated floors are not affected by internal parasites.

Kids need to be protected from external parasites including horseflies, stable flies, horn flies, biting and sucking lice, and mites. Cleanliness and avoiding contact with older animals can help to prevent these problems. Insecticides may be necessary to achieve control of these pests. High populations of these pests can reduce growth and facilitate the spread of disease; therefore, control of these parasites is important.

Housing Environment Considerations

From birth to weaning, the kids should be housed separately from the adults (when removed at day 1) to minimize exposure to an environment with high amounts of potential pathogens. Then, after a successful transition to a diet without milk, the weaned kids may be placed in housing more similar to adults. Therefore, nursing kids should have their own building or at least have an independent ventilation system. For confinement rearing, the floor space and ambient air recommendations can be found in **Tables 3** and **4**.

For kids, up to 2 months of age, it is best to place them in groups of no more than 12–25 kids within 1–2 kg range of weight and no more than 2 weeks range in age. After weaning, kids can be combined in groups of 25–30 allowing for sufficient feeder space, dry bedding, and air quality. One waterer per 25 kids is recommended.

After weaning, kids may be raised outdoors provided that they have shelter from the wind and rain. If raised on browse such as tree saplings, woody shrubs, and broad-leaved plants, internal parasites should not be a problem,

Table 3 Floor space requirements according to age

Surface	*Unit*	*<1 month old*	*1–2 months old*	*2–7 months old*	*7 months old to kidding day*	*Adult*
Floor space	m^2	0.2–3	0.5	1	1.5	1.5–2
Linear feeder space	cm	20	25	35	40	40

Adapted from Le Logement des Troupeaux Caprins du Centre Ouest (2006) France: L'Institute de L'Elevage. http://www.inst-elevage.asso.fr/html1/IMG/pdf_CR_120755014.pdf (accessed October).

Table 4 Ambient air recommendations for two age groups

	Unit	*<1 month*	*>1–7 months*
Temperature range desired	°C	10–18	6–16
Air volume	m^3 per kid	3–4	5–6
Air speed	$m\,s^{-1}$	0.2	0.5
Air renewal			
Winter	$m^3\,h^{-1}$ kid	5	25
Summer	$m^3\,h^{-1}$ kid	25	75
Humidity	%	65–80	
Levels of ammonia	No odor and maximum 5 ppm		

Adapted from Le Logement des Troupeaux Caprins du Centre Ouest (2006) France: L'Institute de L'Elevage. http://www.inst-elevage.asso.fr/html1/IMG/pdf_CR_120755014.pdf (accessed October).

especially if the hay, concentrate, water, and mineral feeding areas are also kept clean of feces. This is done by offering access to these supplements through a feed space where only the kids' heads may enter for eating. Kid, should be kept separate from adults on pasture to reduce infection by internal parasites. Rotation grazing, especially with a long rest period, reduces parasite infection.

Other Management Considerations

Dairy kids need to be disbudded so that they do not develop horns, since they will injure one another with horns and the horns will not fit into feed mangers. Disbudding at an earlier age is easier on the kid and it is easier for the operator also as compared to horn removal later. The most popular method of disbudding kids is to use a hot-iron dehorner. This is best done when kids are less than 2 weeks old. The hair around the bud may be clipped. Lidocaine may be used as an analgesic under veterinary supervision if deemed necessary. The kid is best confined to a kid-holding box during this procedure. A hot iron is applied to burn a copper colored ring (approximately 6 s) around the horn bud. It is possible to apply the dehorner too long and cause brain damage. Proper dehorning eliminates scurs (which are parts of the horn that are not killed by burning and that grow in unusual shapes). If scurs occur, they can be reburned if they are small (check kids a month later). Kids are often tattooed at this time if they are to be registered.

Every kid should be identified by attaching an ear tag or neck chain or collar (**Figures 6** and 7) on their day of birth. Because goats and kids like chewing and playing, they can easily lose their tags. To prevent this, an appropriate tag size is recommended, along with a strong material (i.e., metal) and/or a duplicate tag. For LaMancha breed (earless), if a necklace is unsuitable, the tail may be tattooed on the underside.

Records should include tag number, name of sire and dam, birth date, and any issues observed. The records of the dam should be known to aid the decision of retaining

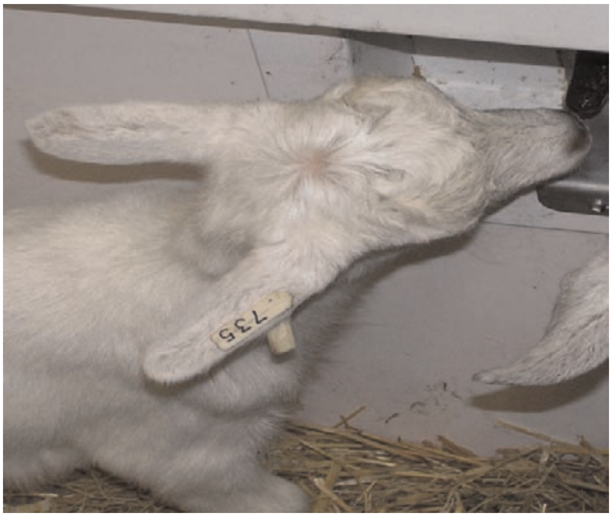

Figure 6 Ear tag.

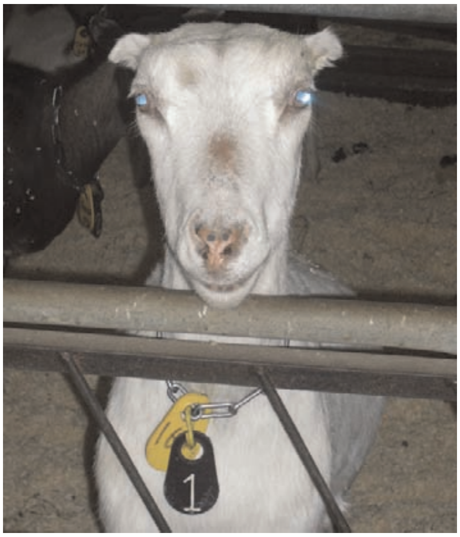

Figure 7 Necklace.

the kid as a replacement. Writing down health records, including disease occurrence, vaccinations and treatments, or symptoms, may help your veterinarian diagnose and treat an ill goat quickly.

Raising replacement kids requires good planning and execution as well as attention to detail. Local veterinary assistance should be involved in the planning stage, especially for health issues. Not only is good nutrition of paramount importance, but also animals should be weighed regularly to verify that kids will be breeding size by breeding season. Raising replacement kids requires much more planning and management than any other class of animals as well as a greater expenditure of time and managerial intensity.

See also: **Animals that Produce Dairy Foods**: Goat Breeds. **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Dairy Farm Management Systems**: Goats. **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease; Parasites, External: Mange, Dermatitis and Dermatosis; Parasites, External: Tick Infestations; Parasites, Internal: Gastrointestinal Nematodes. **Genetics**: Selection: Economic Indices for Genetic Evaluation. **Husbandry of Dairy Animals**: Goat: Feeding Management; Goat: Health Management. **Stress in Dairy Animals**: Cold Stress: Management Considerations. **Vitamins**: Vitamin A; Vitamin E.

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Goat: Reproductive Management

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Introduction

The most important factor affecting the profitability of a goat enterprise is reproduction rate. Poor fertility results in losses due to fewer kids born, fewer kids marketed, lower milk sales, higher semen costs, higher veterinary and medicine costs, and slower genetic progress. Reproduction in goats is influenced primarily by nutritional status, genetic potential, and health status of the animals and also by environmental conditions and photoperiod. There exist reproductive management practices that influence these factors; thus, there is ample margin to increase the reproductive rate of goats under both extensive and intensive conditions.

Fundamental Concepts of Goat Reproduction

In temperate latitudes, dairy goats (does and bucks) initiate sexual activity as the day length decreases, with some variation observed depending on the breed and geographical location. In tropical environments, local goats are able to breed throughout the year. The length of estrous cycle in goats is 20–21 days, and the duration of estrus is typically 24–36 h with ovulation occurring near the end of estrus. Most goat breeds shed more than one egg when ovulating, and the goat's average gestation length is 150 days.

Breeding Management

Managing Replacement Does

In intensive systems, a profitable selection and feeding program must be designed to allow does to reach puberty by 7 months of age and kid at 12–14 months of age.

The average age at puberty can be reduced by increasing the overall plane of nutrition, by exposing prepuberal does to sexually active bucks, and by using crossbred does. On the other hand, age at puberty, subsequent fertility, and mammary gland development can be adversely affected by overfeeding. A doe should reach 65–70% of her mature weight before breeding. Doelings should be grouped by size and body condition for breeding, and these groups should be maintained in place until they kid.

Before the breeding period, it is important to get rid of replacement does with poor body and udder conformation, faulty feet and legs, bad disposition, masculine behavior, polled characteristic (homozygous for this trait), or other anatomical malformations. Additionally, replacement doelings should come from multiparous dams that kid early in the kidding season and that have a high lifetime productivity in the environment in which they will be expected to produce.

Managing Adult Does

To improve reproductive efficiency, infertile, unsound, low-producing, and aged goats should be culled. After pregnancy examination, all does open because of the pathological condition of the reproductive tract should be culled. The decision of culling open does with no pathological problems is based on economic considerations and factors such as poor nutrition, inadequate breeding season, or buck failure are important. Under intensive conditions, it is most economical to cull all open and old goats. However, when replacement costs are high or forage is scarce for goats on pasture, open middle-aged does that may have been nutritionally mismanaged might be retained.

Since undersize goats as well as does incapable of maintaining their body weight during pregnancy are more inclined to abort, animals with the lowest body mass at breeding should be culled. Likewise, does that abort due to nutritional factors under favorable conditions should be culled because this problem seems to be heritable and repeatable. To enhance overall herd performance, all replacement does that fail to become pregnant, as well as aged goats (>6 lactations), should be routinely culled, and more than 70% of the herd should be in the 3- to 6-year-old group.

Nutrition

Body energy reserves of does strongly influence breeding efficiency, young survivability, and milk yield; therefore, body condition scoring (5-grade index: 1 = thin, 5 = obese) should be used by goat producers to evaluate the nutritional status of goats before breeding. Decreased conception and ovulation rate are associated with body condition scores (BCSs) below 2.5 and above 4. For optimum reproductive performance, does should be gaining condition during the breeding period, and should kid in moderate to good body condition (scores 3–3.5). A key time to evaluate condition scores of goats is 1 month before breeding. In this evaluation, thin goats should be sorted out for extra feeding and parasite evaluation. BCS could also be used to determine whether flushing will be of benefit to breeding does. Flushing (increasing the level of energy offered to breeding does 1 month before breeding) increases body weight, ovulation rate, and litter size in does that have low energy reserves (BCS = 2 or lower).

Estrus Synchronization

Estrus synchronization permits the efficient use of artificial insemination and the ET procedure. Additional advantages are the simplification of kid rearing, the control of sexually transmitted diseases, the freshening of goats in the fall, assuring a supply of milk when other does are dry, and the possibility of breeding the does to the highest genetic merit artificial insemination bucks available.

The simplest means of estrus synchronization is the sudden introduction of an intact buck, testosterone-treated does, or androgenized castrated bucks among does with no previous contact with males (**Table 1**). The buck stimulus initiates the ovulatory process few hours after the start of teasing. The proportion of goats 'responding' to the buck exposure will depend principally on the nutritional status of does, 'depth' of anestrus, stage postpartum, and the intensity of the male stimulation.

The most efficient and widespread protocols for estrus synchronization for both breeding and nonbreeding seasons consist of administration of progestagens, via intravaginal polyurethane sponges (30–45 mg fluorogestone acetate (FGA) or 60 mg medroxyprogesterone acetate (MAP)), intravaginal dispensers (CIDR-G®; 330 mg progesterone), or removable subcutaneous implants (1/5–1/2 of the silastic implant used for cattle; 1.2–6 mg norgestomet), during the 9- to 21-day treatment period. Pregnant mare serum gonadotropin (PMSG; 375–750 IU) or human chorionic gonadotropin (hCG)/**PMSG** (250 IU Combination product PG 600®) is given either 2 days before the end of the progestagen treatment (nonbreeding season) or at the time of progestagen withdrawal (breeding season). If the duration of the progestagen treatment is shorter (9- to 11-day treatment) than the luteal phase, it is necessary to add an injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 48 h before the end of the progestagen treatment. The goats are generally in heat within 72 h after the end of the progestagen treatment.

The administration of two intramuscular injections of prostaglandin ($PGF_{2\alpha}$) or its analogs (1.25–15 mg dinoprost tromethamine; 62–250 μg cloprostenol) 11–14 days apart synchronizes estrus only during the breeding period.

Breeding outside the normal breeding season can also be accomplished by artificial lighting, either by itself or in conjunction with melatonin. Such treatments involve application of extra artificial light, with a fixed dawn and a nocturnal lighting, for 2 months, before administration of a melatonin implant for 40–50 days. For timed artificial insemination (TAI), an alternative is the use of 'male effect' and a single 25 mg dose of progesterone given at the time of buck exposure, plus $PGF_{2\alpha}$ 9 days later and TAI 48 h after $PGF_{2\alpha}$. Another alternative is the insertion of an intravaginal sponge for 5–6 days with $PGF_{2\alpha}$ at insertion, equine chorionic gonadotropin (eCG) at withdrawal, and TAI 48 h after sponge withdrawal.

Table 1 Common approaches for controlling heat cycles and inducing ovulation in anovular or cycling goats[a]

Protocol	*Estrus response (%)*	*Onset of standing estrus (h)*	*Conception or kidding rate (%)*
Two injections $PGF_{2\alpha}$ 10–14 days apart	84–94	52–55	55–75
FGA 16 days/eCG 200–250 IU	95		58
FGA 11–21 days/eCG 400 IU/$PGF_{2\alpha}$	96	33	32–67
MAP 13–17 days/eCG 300–500 IU	90–100	35	41–81
MAP 9–19 days/eCG 200–500 IU/$PGF_{2\alpha}$	97	23–81	50–71
CIDR 11 days/eCG 200–600 IU	70	40–50	53–75
60- to 70-day photoperiod (19L:5D)	63–79		63–80
60-day photoperiod (20L:4D)/60 days melatonin	91–93		86–93
Buck stimulus	79–92	110–115	82–85
Buck stimulus/norgestomet 9 days	92	54	78
Norgestomet 9 days/eCG 100 UI	100		80
Sterile copulation/FGA 12 days	70	40–50	53–75
♂ effect/P at ♂ exposure/$PGF_{2\alpha}$ 9 days later/TAI	88	37	66
$PGF_{2\alpha}$ at day 1 + MAP 5 days/250 IU eCG at day 5/TAI	92	54	64

[a]From the literature.

CIDR, controlled internal drug-release device (releases progesterone); eCG, equine chorionic gonadotropin; FGA, fluorogestone acetate; MAP, medroxyprogesterone acetate; P, progesterone; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$ analogs; TAI, timed artificial insemination.

Artificial Insemination

The artificial insemination process is mainly carried out by producers with frozen semen from genetically evaluated sires. Does can also be inseminated with fresh and extended, nonfrozen semen stored chilled for up to 48 h. The most common method used in goats is cervical insemination, which involves deposition of a single dose of 100–200 million frozen–thawed sperm on the uterine side of the cervix (**Figure 1**). Conception rate using this method ranges from 50 to 70%. Females in natural estrus are inseminated 12–18 h after the onset of estrus. For young goats, a pipette that has a needle attached to the end, with the length of the needle approximately the length of most caprine cervices, is preferred. The needle is slightly flexible to allow easier penetration. For pluriparous goats, the insemination gun is simply a smaller version of the gun used to inseminate cattle. A pipette for transcervically conducted bilateral deep cornual insemination, which may be considered as an alternative to laparoscopic insemination, is available.

Embryo Transfer

The ET process comprises (1) estrus synchronization of the donor and recipient, (2) superovulation of the donor, (3) fertilization of the donor, (4) recovery of embryos, and (5) transfer of embryos to recipients. Donor and recipients are synchronized using any of the techniques previously described.

The use of follicle stimulating hormone (FSH) (of goats or sheep origin; goats develop antibodies against porcine FSH and PMSG) is now widely accepted in place of PMSG. A common protocol includes a total of 20 mg FSH-o administered twice daily for 4 days (starting on day 7 after the initiation of a 9-day progestagen treatment) in descending doses of 4, 3, 2, and 1 mg. Acceptable ovulation rates in does following FSH treatment range from 12 to 16 eggs.

The uterus of the donor is flushed 6–8 days following insemination. Traditionally, this is done in goats under anesthesia using a midventral or flank laparotomy, or laparoscopy. Collection via cervix is not advisable because of its complexity and low collection rates.

Following collection, the flushing medium is examined to identify fertilized ova, determine the recovery of embryos (based on the number of corpus luteum), and evaluate embryo quality. Only high-grade embryos should be used for frozen storage; embryos of less quality may be used for fresh transfer. Transference should be carried out via laparoscopy, which gives equivalent or higher fertilization rates than laparotomy and higher fertility than transference through the cervix.

Embryos should be transferred into the uterine horn that is on the same side as the ovary containing the corpus luteum. Multiple transfers into recipients without a corresponding number of corpus luteum are not recommended. Pregnancy rates following transfer of goat embryos range from 45 to 80%, relative to the quality of embryos, nutritional status of the does, and transfer expertise, whereas the number of kids born per donor goat varies from 3 to 4, depending on whether embryos are deep-frozen or not.

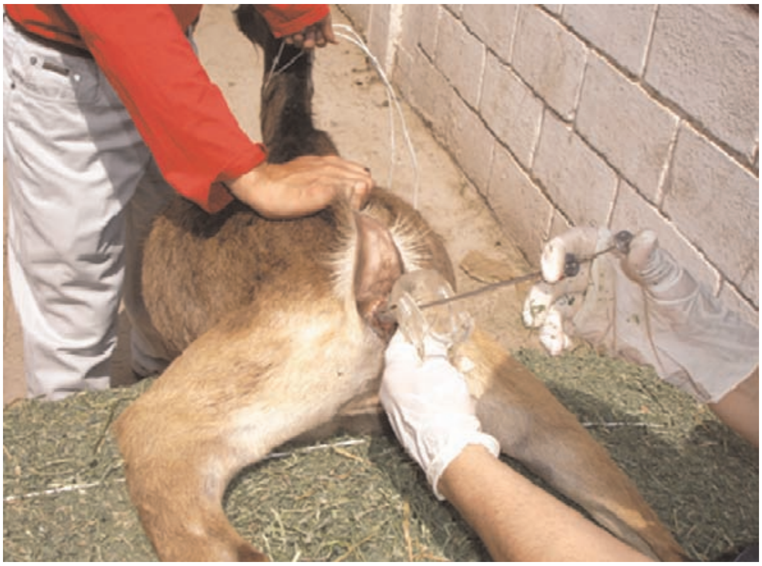

Figure 1 Artificial insemination of does, using alfalfa hay bales to restrain the animal. A plastic speculum previously lubricated has been inserted to visually locate the cervix. The insemination gun is threaded into the opening of the cervix using a circular motion and slight pressure to work the insemination gun through the rings of the cervix.

Management of Natural Serving Bucks

Despite the overwhelming evidence of the genetic advantage of artificial insemination over natural service bucks, many dairy goat producers consider the use of natural service to be beneficial in their reproductive management approach, particularly under extensive conditions. Under these circumstances, bucks should pass a breeding soundness examination and a genetic merit evaluation before being used, and the test should be repeated at the beginning of every breeding season.

The primary objective of a breeding soundness examination is to determine whether a replacement or working buck has the ability, under the circumstances in which it will be used, to successfully mount, intromit the penis into the vagina, and ejaculate semen of sufficient quality to achieve an acceptable pregnancy rate when bred to a certain number of fertile does in a short breeding period. The breeding soundness examination, therefore, must evaluate physical soundness and structural defects that could affect the ability to mount and serve, as well as libido and semen quality.

The general physical examination

Prior to the breeding season, bucks should receive treatment for external and internal parasites, as well as hoof trimming. Foot care is very important since structural faults will hinder the buck from actively seeking out does and fecundate them. Bucks who find it painful to walk or mount will be reluctant to ejaculate even if they show intense libido. Common causes of leg problems include overgrown hooves, foot rot, arthritis, improper foot trimmings, injury, or caprine arthritis encephalitis. The buck's vision should be checked for cataracts, pink eye, conjunctivitis, or seeds or spines lodged in the eyes.

Teeth should be complete and in good condition, because poor teeth can adversely affect the buck's ability to maintain high lipid reserves under extensive conditions. It is necessary to discard polled bucks, because sterile intersexuality condition is linked to the polled trait. Bucks with potentially heritable conditions (e.g., upper or lower brachygnathia, testicular hypoplasia, cryptorchidism) also should be discarded.

To be a satisfactory potential breeder, particularly under extensive conditions, a buck should have high energy reserves (BCS score between 3.5 and 4.0; scale 1–5). An active buck with a high libido stops eating during the breeding season if he remains permanently with the does. An adult buck can be left thin, but a yearling buck can actually be stunted permanently. Therefore, proper prebreeding nutrition is essential to ensure the buck has adequate reserves for a successful breeding season and can tolerate the weight loss (0.5 kg daily under range conditions) without detrimental effects.

Bucks on a good plane of nutrition will have higher testicular mass, exhibit more mounts and ejaculations, and can induce more does to ovulate than those on deficient diets. It is important to note that testicular size, and thus spermatogenesis, responds to improved nutrition even during the nonbreeding season.

The genital examination

Both testes should be oval and cool to the touch (heat may indicate a possible infection), with a smooth, regular outline and a slightly turgid, resilient texture (**Figure 2**). Scrotal circumference correlates highly with testicular and epididymal sperm content, although the direct relationship of bucks' scrotal size to fertility is unknown. The circumference of the scrotum is measured with a flexible tape, at the site of the testicles' largest diameter

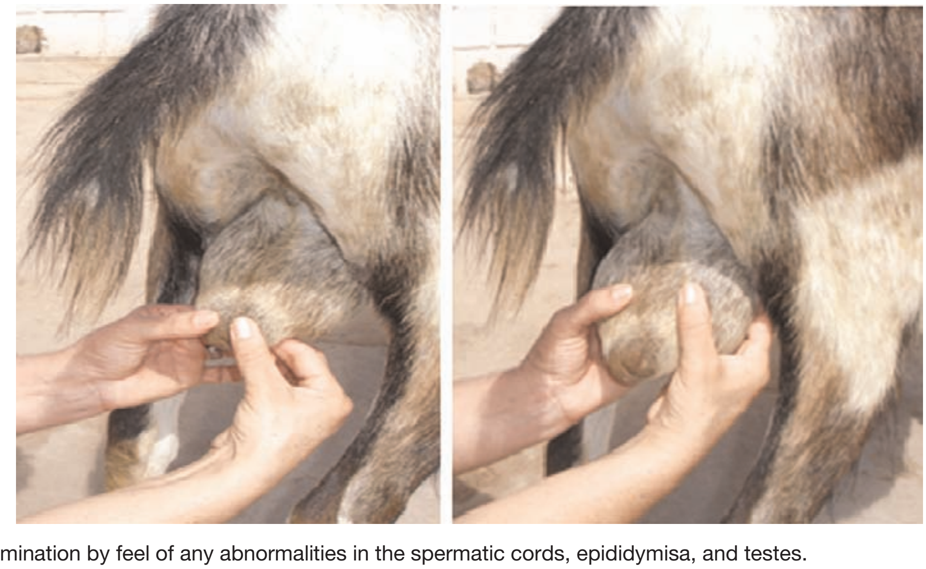

Figure 2 Determination by feel of any abnormalities in the spermatic cords, epididymisa, and testes.

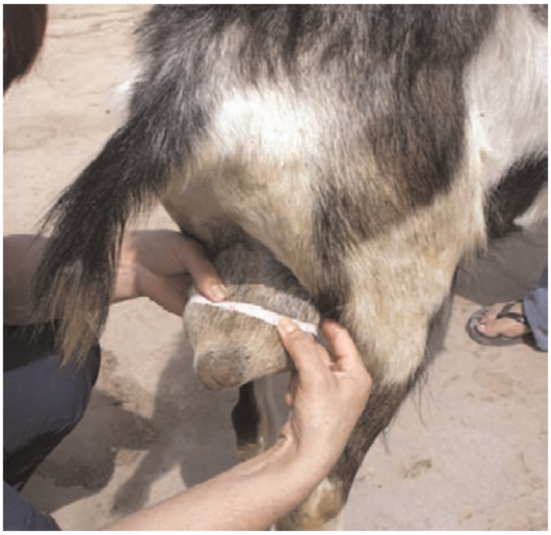

Figure 3 Scrotal circumference measurement by placing a measuring tape around the scrotum at the widest point.

(**Figure 3**). Although criteria for minimally acceptable scrotal size have not been established for bucks, **Table 2** shows typical values for fertile bucks of different breeds used in intensive and extensive dairy production systems. Palpation of the head of the epididymis is particularly important in homozygous polled bucks, because sperm granulomas, as a result of blind efferent tubules, are strongly correlated with the homozygous polled condition.

The penis and prepuce can be examined while semen is being collected. Penis and prepuce should not be adhered together. The skin of the prepuce should be thin and pliable, without inflammatory or proliferative lesions. The penis should be free of hematomas, sores or cuts, or genetic problems such as hipospadias (incompletely formed penis and sheath). Also, the pizzle (the thin process at the end of the penis) should be examined to make sure that no urinary stones are lodged there.

Fertility examination

Fertility examination consists of evaluation of three components: the libido, the ability to mount does, and the quantity and quality of semen. The goat industry has no standardized procedure for evaluating the libido or mounting capacity of bucks. However, sexual behavior can be evaluated during the semen collection process. Typically, a buck with good libido shows immediate and intense desire for does, manifested by restlessness, pawing, intense and tenacious chasing of does if not fully in estrus, urination upon his face, beard, and front legs, and exhibition of the 'flehmen' reaction after placing his nose in the urine stream of does.

Semen collection can be accomplished by using an artificial vagina, a man condom (previously washed to eliminate the lubricant material) inserted into the doe's vagina, or the electroejaculator. The disadvantage of electroejaculation is that it does not allow evaluation of libido or the ability of the buck to mount a doe. Besides, some bucks ejaculate in their penile sheath, as a result of which the penis cannot be examined. Additionally, this collection technique yields larger ejaculate volume and lower sperm motility compared to semen obtained with artificial vagina.

Semen quality is determined basically by evaluation of traits depicted in **Table 2**. Caution should be taken when

Table 2 Selected characteristics of scrotum and semen of mature bucks during the breeding season from well-established breeds utilized in intensive and extensive milk production systems[a]

Breed	*Scrotal circumference (cm)*	*Volume (ml)*	*Sperm concentration ($\times 10^6$ ml^{-1})*	*Sperm motility (%)*	*Abnormal sperm (%)*	*Percentage of live sperm*
Saanen	26.5–27.4	0.7–1.0	2780–3240	90	6.2–12.0	82–85
La Mancha		1.6–2.1	1659–1912	76–79		
Alpine	25.4–30.1	0.6–1.4	2115–3300	67–87	5.0–7.8	90
Toggenburg	26.3	1.0–1.8	1989–2425	75–81	5.6	
Nubian	26.1–28.5	1.1–1.5	1770–2650	82	5.6–9.0	82
Granadina	26.0–27.0	0.6–1.5	1799–2890	71	5.0–6.0	90
Barbari		0.46–1.0	1804–3630	79–85	3.4–4.5	68–84
Jamunapari		0.46–1.1	1500–3650	61–88	1.6–5.0	71–90
Beetal		1.7	2671–2820	80	5.7	81
Mexican Criollo	26.8–27.7	1.0–1.3	2580	73	5.6–6.5	88
Black Bedouin	27.4	1.5	2660	67	22	
Damascus	32.1	1.1	4519	67	22	
Zaraibi	25.9	0.8	3056–5072	65–79	13.5	82
Majorera	26.0	1.8–2.01	3480			

[a] From the literature.

bucks are examined during the nonbreeding season, because testicular mass, libido, and semen quality decrease during this period. Elimination of a buck suspicious of subfertility should be based on at least two examinations of ejaculates. No firm guidelines have been developed to classify bucks according to semen, libido, and testis examination. Thus, bucks would be classified as sound, questionable, or unsatisfactory according to the experience of the examiner.

Mating Management

In general, under extensive grazing systems, a ratio of 1 mature buck in good body condition for every 50 does is appropriate, even if most does come into heat in a 1-week period. This mating load does not compromise kidding rate because bucks are able to increase service rates as number of goats in estrus increases. With hand-mating systems, mature bucks can be individually joined with 60 does in a 30-day breeding period, with no risk of a reduction in the number of pregnant does.

Under grazing conditions, the optimal length of the breeding period is 4 weeks. During this period, if pregnancy fails to be established at the first breeding, most does have a second opportunity of being fecundated. This is so because although introduction of the buck results in an earlier and relatively synchronized ovulation (2.2–2.8 days after the introduction of bucks), a great proportion of does induced to ovulate, particularly the noncyclic animals, present a short ovulation cycle (on average 5.3 days) and do not exhibit estrus. Pretreatment of anovular does with progesterone eliminates premature luteal regression and 'silent' heats in goats 'responding' to the buck stimulus. In those goats with short cycles, the ovaries are restored to normal function at the second ovulation. Kidding rate is not further improved with breeding periods longer than 4 weeks.

The breeding season and the number of mating periods during the year would be dictated fundamentally by the prevailing agro-climatic conditions of the goat operation, the market of the goat's products, and the natural breeding period of the goats utilized.

Gestation Management

Pregnancy Detection

An early assessment of pregnancy and fetal numbers is essential for optimal reproductive results. This information enables the identification of does requiring repeat breeding or insemination and allows the separation of pregnant and open does for differential management.

The most promising and versatile technique currently available for pregnancy detection in goats is the use of real-time ultrasound scanning. The availability of lower cost, portable scanners, which determine pregnancy and fetal number with minimal animal restraint and provide high throughput, has increased the use of this technology in goat farms (**Figure 4**). Transabdominal scanning (linear array and sector scanners) allows reliable pregnancy diagnosis at 35 days of gestation, whereas transrectal examination (5 and 7 MHz linear transducers) will reduce this period further to 25 days, although the correct determination of twinning is possible after day 40.

Another reliable and commercially practical method to detect pregnancy as early as 21 days after insemination under field conditions is the measurement, with commercial kits, of milk or blood serum progesterone levels. The accuracy of the test is high both as an indicator of pregnancy and as an indicator of nonpregnancy (**Table 3**); however, it does not distinguish between pregnancy and pseudopregnancy. Both ultrasonography and progesterone test require skill and experience, and kits and equipment for these procedures are expensive, making their general use prohibitive for goat operations under extensive conditions in developing countries. Therefore, under these circumstances, the easiest and cheapest method for pregnancy detection (or, more accurately, for the detection of estrus) is to expose does to a mature buck impeded to copulate from day 18 to 24 after mating (hand-mating systems) or 30 days after the end of the mating period. In both cases, the accuracy of

Table 3 Comparison of some techniques for pregnancy diagnosis in goats[a]

Technique	*Sensitivity range (days)*	*Fetal numbers*	*Accuracy (%)*	*Practical application*
Exposure to harnessed buck	18–30	No	86	High
Abdominal palpation	61–70	No	70	Moderate
Progesterone assay kit	20–24	No	82–88	Moderate
Real-time ultrasound, transabdominal	25–30	Yes	91–100	High
Real-time ultrasound, transrectal[b]	19–23	Yes	100	High
Abdominal circumference	68–90	No	85	Moderate

[a] From the literature.

[b] 90% accuracy for fetal sex determination.

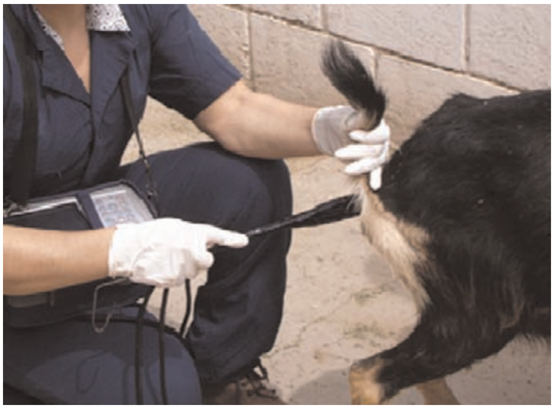

Figure 4 Pregnancy diagnosis by means of real-time ultrasound scanning performed transrectally.

nonpregnancy diagnosis is above 86% (**Table 3**). A serious disadvantage of this method is the failure to detect estrus when goats pass into anestrus immediately after breeding.

Another simple farm-based technique is the detection, through palpation, of fluid buildup and presence of a detectable fetus, but this is applicable only during the late stages of pregnancy.

Pregnancy Losses

Under range conditions, the incidence of abortions of nuttritional etiology can be above 50%. Abortion can be prevented by adequate nutrition during pregnancy (BCS $\geq$3), because this reproductive problem is a temporary reaction to a negative energy balance. To prevent abortions, goats should be free of brucellosis, chlamydiosis, toxoplasmosis, and Q fever. Goats should receive mineral supplement, because deficiencies of selenium, cooper, magnesium, manganese, iodine, and phosphorus provoke abortion. Deworming should be avoided late in gestation because some dewormers (rafoxanide, levamisole, thiabendazole, and febendazole) provoke abortion.

Young goats should not be fecundated before they reach 60% of adult body weight, because growing goats abort if an inadequate diet is maintained. Old goats (>6 kiddings) should be discarded, as they have higher odds of aborting than younger does.

Elimination of aborting goats is necessary because these animals tend to abort in subsequent pregnancies. In addition, it is required to select goats adapted to large fluctuations in nutrient supply and to extreme ambient temperatures, capable to ingest enough essential nutrients to maintain gestation. Under range conditions, lactation concurrent with gestation should be avoided, as this condition is associated with a major fetal loss. Finally, the presence of rams in a flock of goats should be avoided, because matings between rams and does end in abortions.

Reproductive Health Program

The breeding flock (does and bucks) should be dewormed prior to flushing and/or the introduction of the bucks. Does should also be dewormed at kidding, because the doe's hormonal changes will induce gastrointestinal parasites to increase at parturition. The breeding flock should be vaccinated against enterotoxemia (overeating disease; *Clostridium perfringens*, types C and D) and tetanus (*Clostridium tetani*). Breeding does should be vaccinated 4–6 weeks before kidding, so that some immunity will be passed to their offspring. Following birth, kids should be vaccinated against enterotoxemia and tetanus at 8 weeks of age, followed by a booster at 12 weeks of age.

It is necessary to immunize does against diseases that interfere with reproduction and those that are prevalent in the area where the flock is located. For many developing countries, these vaccines include brucellosis, chlamydiosis, campilobacteriosis, and toxoplasmosis. Under extensive conditions, goats should be treated against lice in autumn and before parturition.

See also: **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis. **Replacement Management in Cattle**: Breeding Standards and Pregnancy Management. **Reproduction, Events and management**: Control of Estrous cycles: Synchronization of Estrus; Estrous Cycles: Seasonal Breeders; Mating Management: Artificial Insemination, Utilization; Pregnancy: Parturition.

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Predator Control in Goats and Sheep

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Introduction

Sheep and goats are thought to have been domesticated 12 000 to 10 000 years ago. It seems likely that predation (a mode of life in which food is primarily obtained by killing and consuming other animals) has been a problem since the earliest of times. In some degree this problem is worldwide and has not diminished in seriousness even to the present. For a species of predator to be a problem, it must either become too abundant or unacceptable for man to share that species of prey (an animal that is or may be seized by another to be devoured) with the predator. Although most species of domestic livestock are at times subject to predation, this problem is most serious or has the greatest impact with sheep and goat producers, who frequently shoulder the burden of controlling or reducing wild predator populations. This may be explained in that larger livestock species, due to their size, may have greater resistance to predation and smaller domestic species are more often produced in confinement.

Predation and methods of control are similar for sheep and goats, but if there is a difference, goats may well be more susceptible to losses. This may be partially explained by two situations. Kid (young) goats tend to hide or bed down for long periods of time while the mothers graze some distance away. During this time they are subject to predation by a wide range of predatory species including small mammals as well as birds of prey. This is made more serious if the young become hungry and attract predators because they are both vocal and colorful. In range areas of the United States, it is more difficult to protect kid goats from predation than other groups. In addition, most types of adult sheep have a fleece cover which makes them unattractive to some predator species. They become more susceptible to predation after they have been shorn; lambs, with their scant wool, are also susceptible.

The nature and extent of the problem will vary widely depending on the predator species, their prevalence and the production or management practices employed. In many instances, the livestock management practices utilized are dictated by the threat of predation. It is doubtful that there is any open range in the world in which sheep and goats can be produced without some loss or threat of loss to predation. However, there are large differences in the degree to which predation presents a problem. This problem is currently most serious in parts of Africa and in North America.

There is strong circumstantial evidence that predation or the threat of predation has a major effect on numbers of sheep and goats or the trend in the numbers of these species both within and between countries. Some countries with large numbers of sheep, such as Australia and New Zealand, were from the outset largely free of natural predators. This continues to be the situation with New Zealand, whereas some serious predators have been introduced to Australia. These include the dingo or wild dog, foxes and feral swine. However, because the sheep industry is of great importance in that country, control efforts have received both government and public support. In many other countries, with the United States and Scandinavia as examples, there are ever-increasing restrictions on the methods used and the level of control. As a result, predation is becoming an increasingly important problem for sheep and goat producers in many areas due to the flocks being maintained under extensive conditions with fewer people involved in their care and to the restrictions on control methods or tools available.

Predator Species

The number of predator species, which may at times become a problem, is almost endless and will obviously vary with location. They may range in size from the sea gull to the African lion or the grizzly bear. Some species which may cause problems are listed in **Table 1** somewhat in order of their size, but not necessarily in the order of their contribution to losses. The list is admittedly incomplete, but with a list so extensive one might wonder how anyone produces livestock, especially sheep and goats. Indeed there are large areas of the world where sheep or goats are not produced or are not produced in meaningful numbers precisely due to predation. The damaging effects of predation are much more serious than merely the market value of animals killed. In addition, there is the cost in money and time of efforts to

Table 1 Some of the common predators of sheep and goats

Common name	*Latin name*	*Locations*
Grizzly bear	*Ursus horribilis*	North America
Black bear	*Ursus americanus*	North America
European brown bear	*Ursus arctos*	Europe
African lion	*Panthera leo*	Africa
Leopard	*Panthera pardus*	Africa and Asia
Jaguar	*Panthera onca*	Asia and Central America
Cheetah	*Acinonyx jubatus*	Africa
Mountain lion (puma)	*Felis concolor*	North and South America
Wolf	*Canis lupus*	Worldwide
Dog	*Canis familiaris*	Worldwide
Hyena	*Hyaena* spp.	Africa
Coyote	*Canis latrans*	North America
Cape hunting dog	*Lycaon pictus*	Africa
Jackal	*Canis* spp.	Africa
Bobcat	*Felis rufus*	North America
Lynx	*Felis caracal*	Africa and North America
Wolverine	*Gulo gulo*	Europe and North America
Red fox	*Vulpes vulpes*	Worldwide
Gray fox	*Urocyon cineroargenteus*	Worldwide
Feral or European boar	*Sus scrofa*	North America, Europe and Australia
Golden eagle	*Aquila chrysaetos*	Europe and North America
Others:		
Birds: Eagles, hawks, falcons, vultures		Worldwide
Reptiles: Alligators, crocodiles, pythons		Central and South Asia

protect the animals, and the reduced performance due to altered management practices. Still more important is the decision not to attempt to produce these species in areas where they are well suited due to the certainty that predation will occur. Also, losses to predation can cause emotional turmoil for those entrusted with the responsibility of caring for livestock.

The most serious predator is the one a producer is dealing with in any given situation, and this will vary with time and location. In general the most serious losses over wide areas are caused by the canine and feline groups. The coyote and the domestic dog are the most pervasive problems in the United States, but at specific times and places this distinction may go to the bobcat, mountain lion, wolf, feral swine, etc. The most serious predators in southern Africa are reported to be the lynx and jackal, but this must be after ruling out as unsuitable for sheep and goats areas where larger predators are found. Fox, puma and domestic dogs are major problems in parts of South America, and the jaguar causes losses in Central America. The heavily populated regions of western Europe and the British Isles may have relatively low levels of predation, with foxes and domestic dogs (where they are not controlled) the main predators. Northern Europe and Scandinavia report problems with brown bear, wolverine, wolf, lynx and golden eagle. These species are protected in these areas and their recovery is thought likely to occur at the expense of the sheep and goat industries. In the Near and Middle East, one hears references to wolves as a problem although their density could not be great because of the absence of protected areas in which to hide. Also, in the absence of fencing, the flocks in these areas are almost always under human supervision.

Bears tend to be limited in their distribution, and do not live primarily by predation. Because bears (especially grizzly and brown bears) may represent a threat to humans as well as their livestock, sheep and goats are seldom produced in meaningful numbers in areas inhabited by these species. A somewhat similar situation exists with the large predators of Africa such as the lion, leopard, cheetah, hyena and the African hunting dog.

Predation usually occurs unobserved (at night). In the majority of cases, it is possible for experienced observers to determine with some degree of certainty the species of predator involved, because many of these have very distinct killing and feeding patterns. Bears often kill lactating ewes and feed on the udder containing milk. Domestic dogs are inefficient killers and frequently slash and tear animals as they come in contact with them, which usually results in a large number of injured animals, some of which may be partially eaten without being killed. Animals on which dogs have fed have a poor prognosis for survival. By contrast, coyotes and wolves soon become proficient killers by catching the animals by the lower part of the throat to suffocate their prey. They also leave puncture marks in the skin matching their canine teeth. They ordinarily kill only a few animals at a given time for food, but return regularly enough so that the losses cannot be tolerated. By contrast, most cats bite the animals from the top (head or neck) or may slash with their paws and, of this group, the mountain lion or puma may kill large numbers (the author has seen as many as 70 animals killed in one entrance to the pasture) and may not feed on any of them. Birds of prey (especially eagles) usually leave talon marks in the skin and are very efficient in cleaning the meat off the bones of the carcass.

Management Practices to Reduce Predation

There is no single universal effective method of protecting sheep and goats from predators, and in many situations, none of the approaches taken is totally satisfactory. A number of management practices can be employed that may partially eliminate predator losses, but in many cases, these are not consistent with economic measures that permit the industries to survive.

Confinement

Some species (swine, dairy goats, poultry) are now produced on a commercial scale largely in confinement, and so are some large-scale dairy goat and cattle herds; there is also the feedlot finishing phase for cattle and sheep. However, the ruminant animal is well adapted to utilization of large quantities of roughage and grazing of extensive areas that are not well suited to cultivated agriculture. Thus, even if confinement is effective in preventing predation, it is not a satisfactory method of resolving this problem in many areas where sheep and goat production is important.

Night Confinement

Because most of the losses to predation occur at night, night confinement is often used to prevent or reduce losses. In some areas, this also correlates well with the use of herders because they are able to rest at night. The type of structures required to prevent predation will depend largely on the species of predator(s) involved. Generally, the animal must be prevented from jumping, climbing, digging under, etc. Coyote predation is relatively effectively prevented by night confinement because, in part, they tend to have an aversion to enclosed quarters. Also the escape behavior of prey species is an invitation for predators to give chase, but this is reduced in confinement. Types of fencing required will be discussed later. It might be inferred that night confinement constitutes a solution to this problem, but this is not universally the case. There are a number of drawbacks to night confinement. One is that the animals are subjected to more disease problems if confined for a long period of time. Secondly, the cool hours of the late evening, early morning and sometimes the night are favoured times for grazing in hot environments. Finally, in areas where commercial-scale production (with large numbers) occurs, it may in fact take days and considerable travel to gather the animals, and thus confinement at night is not an option.

Herding

Throughout much of the world, sheep and goats (as well as other types of livestock) are herded, and without this practice, these industries would not persist. Herding, with confinement at night, is often done by children and the increasing efforts to provide public education for the children conflicts with their role as herders. In many places the flocks are small and the option of using paid employees is not a viable alternative. Even with herding, it is unlikely that the flocks can be run in areas subject to predation by the larger predator species. In many areas herders would not be able to afford or would not be permitted to own a gun for protection of themselves or their flocks. One of the major exceptions to some of these conditions is the use of herders in the northwestern region of the United States (Colorado, Idaho, Montana, Utah, etc.) in which a single herder is able (with the assistance of dogs) to control and protect flocks of 2000 + head (1000 ewes plus their lambs) in the mountains during the summer or in the desert in winter. In this case the herders are with the flocks essentially 24 hours a day by spending their nights in tents or tepees which are moved as the flock moves. Although this practice is largely effective in reducing predation, the practice is declining for other reasons including the difficulty of obtaining herders and the restrictions being placed on the use of public lands for livestock grazing.

Use of Guard Animals

The use of guard animals (especially dogs) is an age-old practice in parts of the world, but has only recently been used with commercial-scale flocks or herds in the Western world such as North and South America, although there are examples of guard dogs having been used by native Americans for centuries. In addition to dogs, other animals that have been tried or proposed as protectors of sheep and goats are donkeys (sometimes horses), cattle, llamas and ostriches. Some advantage can be realized from bonding sheep (or goats) with cattle to protect from coyote predation, but several complicating problems prevent widespread use of this practice. Although results from other species are sometimes encouraging, only guard dogs have been shown conclusively to be useful for this purpose. In some areas donkeys are being used, but they do not appear to serve this role as well as dogs. On the other hand, donkeys and llamas are much easier to manage than dogs. Although native Americans often used any type of dog available to them, on a world basis there are a number of breeds of dogs that have been especially developed for this purpose. Most of these evolved in Europe or the Near East and include breeds such as the Great Pyrenean, Komondor, Maremma, the Anatolian Shepherd and others. For many, the Turkish dogs (these are known within Turkey by a number of names such as Kangal, Ak-Bash or Kara-Bash) have worked out best because they may be better adapted to conditions of hot weather and large areas and are often more aggressive in protecting the flocks. Even though dogs serve an excellent role in reducing predation, this practice cannot be viewed as a total solution to the problem. Some of the difficulties encountered are that not all dogs work out as expected or they may be lost for a variety of reasons: the dogs themselves may become predators or may be killed by larger predators (grizzly bears are known to have killed guard dogs). The dogs appear to work best where used to aid herders or when used on private fenced properties where the land area, the animals being protected, the owner or manager, and the production and management system is a constant. Generally speaking, they have not been used widely with large free-ranging flocks or herds, which may spread out under extensive conditions with little supervision or contact with humans. The useful life of the dogs may be short because they are lost or must be replaced for a number of reasons. Most producers use neutered dogs, and thus replacements must be purchased or obtained from breeding kennels. Also there is some training or management skills (such as raising them from an early age with the species they are to protect) which are needed to use dogs effectively. Experience is of great value in using guard dogs.

Early Weaning of Kids or Lambs

The young are generally more subject to predation than older animals. As a result, and when losses are occurring, producers will often wean the lambs or kids earlier than normal and place them in a protected environment such as a feedlot. Although this may not be the preferred practice, the efficiency of weight gains by the young animal may make this practice desirable or feasible from an economic standpoint.

Fencing

Numerous efforts have been expended in the United States to control predation by the use of fencing, with the primary efforts directed at coyotes. Domestic dogs and perhaps wolves would be similarly affected. Fencing may be proposed to protect small areas for night confinement or to encompass larger grazing areas. It must be assumed that fencing for the purpose of night confinement can be made to work, but construction details would depend on the materials available and the type of predators involved. Fencing is more often directed at members of the canine group and less at the feline group because of the latter's ability to climb. In general the animal must be prevented from going through openings, jumping, climbing, or digging under. Usually, this can best be accomplished by the use of net wire fencing which may be largely unavailable or unused in many parts of the world. This fence must be sufficiently tight to prevent passage, high enough to prevent jumping, and have an apron or buried wire to discourage digging. Electric additions are often used (in the form of a trip wire at the bottom to discourage digging or a wire along the top to prevent climbing). The use of this technology is greatly enhanced by the availability of solar fence chargers.

Amount and type of terrain, cost and stocking rate are some major factors that should be considered when making a decision to fence large grazing areas. A high stocking rate or carrying capacity (level of forage production) has a great influence on the ability to use fencing. When the stocking rates are held constant, fencing cost, when expressed as a function of animal units protected, is much more economical when large areas are fenced. For this reason, a group or an area approach may be indicated but is seldom realized. In general, very little use has been made of fencing or refencing large areas of arid lands with low stocking rates, and unfortunately, these are the areas where much of the sheep and goat populations are found. Initial costs and maintenance are major impediments to fencing large areas.

The classical case of the use of fencing to prevent predation is the dingo fence, which crosses much of the continent of Australia. This was built in a much earlier era, perhaps with government assistance, and might be difficult to accomplish at the present. Ordinarily, net wire fences should approach 2 m in height with a portion turned under to discourage digging. These types of fences are usually directed at dogs, coyotes or wolves and may not work for other types of predators. In most economies such a fence is prohibitively costly for extensive areas, but is, at times, used in areas with high carrying capacity. High-tensile electric fencing offers an alternative, but it is also expensive to construct and difficult to maintain. Although new fencing sufficient to exclude predators may be too expensive for widespread use, any good type of net wire fence is likely to contribute to some reduction in losses or to assist in other approaches of predator control or removal.

Methods of Predator Removal

In areas where free-ranging sheep or goats are (or might be) managed under extensive conditions, it is simply not possible to produce these species without some effort at predator control or removal. In the past, this was often accomplished with reasonable success, but is today becoming more difficult due to public involvement or resistance to the practices employed and to the reduced labor supply available at the farm level.

Toxins

There is a large or almost infinite number of toxic agents which could be utilized to remove or control predators. At one time, strychnine in the form of drop baits with beef tallow as a carrier was extensively used in predator control in the United States Strychnine in this form is not selective for either the species or the individual predators. Although other species are at times taken, populations of nontarget species are usually not seriously affected. Unfortunately, this also applied to the target species in that they rebounded by invasion from surrounding areas or by reproduction in subsequent generations.

Another toxin that has been employed extensively is sodium cyanide (potassium cyanide works equally well) in the form of the 'Coyote Getter' or the M-44 device. In this case, the device is placed in the ground with an attractant placed on an exposed pad or mouth piece. When the target animal pulls on the mouth piece a cyanide pellet is fired into its mouth, which results in death within seconds. This tool can be made somewhat selective by the type of attractant used and placement of the device. This tool is currently in use under controlled conditions over much of the range areas in the United States.

Another toxin that has been utilized in several places in the world is sodium monofluoroacetate (Compound 1080). This material is extremely toxic to canids and for this reason can be made somewhat selective for this group. In the past, carcasses of dead animals have been laced with Compound 1080, then placed in areas of concentrations or drift areas for predators (coyotes). This procedure is forbidden in the United States at present. An alternative method for Compound 1080 is in drop baits as described for strychnine. This method is not permitted in the United States at present, but is currently carried out in other areas. Another use of Compound 1080 is in the form of a toxic collar (sometimes known as LPC for Livestock Protecting Collar) in which the compound is placed in plastic packets in a collar around the neck of the prey species, and thus the predator (which attacks the animal by the neck) punctures one or more of these packages and is killed. This method is being applied in the United States at the present time, but under carefully controlled conditions. This procedure is somewhat selective for the actual animals involved in predation, but is difficult to use on a large scale which is often necessary to deal with the problems on a continuing basis. The primary limitations are the large amount of Compound 1080 used in the collar and expense. A large number of animals must be collared in each flock to improve the probability that the collared animal will be struck, or alternatively to remove or corral most of the flock leaving only a few in the pasture. In this case, all of the remaining animals are collared.

Shooting

Where permitted or where cost-effective, most owners or herders will carry guns. This is often a good way to remove domestic dogs that damage livestock. However, opportunistic shooting of wild predators is limited in effectiveness, but should remain an option. Calling (mimicking prey species to attract predators) can at times be practiced to remove predators where dense populations exist, but this is seldom an effective way to remove individual offending animals as they often become very wary of human presence. Shooting from the air can be highly effective and is used extensively in the United States at present. Shooting from helicopters for this purpose is more effective than fixed wing aircraft, but is more expensive.

Hunting Dogs

Hunting dogs or trail hounds and possibly catch dogs were widely used in the United States in earlier times to rid large areas of primary predators such as wolves, mountain lions, coyotes, etc. They may actually kill predators or bay or tree them until handlers arrive to deal with the predator. Even if this approach is not successful in removing the predator, it may cause them to avoid the grazing areas due to harassment by the dogs. This approach is not widely practiced today due to the difficulty of dealing with net fences. Also, few individuals maintain the necessary number of trained dogs. Hounds remain one of the better methods of dealing with large cats such as the mountain lion. Dogs continue to be an important method of predation control utilized in South Africa at present.

Traps and Snares

Steel leg-hold traps have long been a basic component of dealing with predators, but there is rapidly growing opposition to this practice. They can be made somewhat selective for the species or even for the individual offending predator by selection of lures and placement of the traps. It is painful to the trapped animal, and this is the basis of much of the objection to its use. As a result, steel leg-hold traps are being outlawed in some areas. Due to the human time involved, it is an expensive approach to predator removal and requires some experience or training to be successful. Experienced trappers usually have their own secrets in respect to placement and types of lures used. An alternative type of trap known as the 'have a heart' cage-type trap has been used quite successfully with cats such as bobcats, but seldom works with an animal such as the fox or coyote. A chicken (white rooster) to attract bobcats has proven to be quite effective. This type of trap would not be suitable for use with large predators such as wolves or mountain lions.

Where reasonably good fences exist and predators must gain entrance to a pasture by going through a hole or digging under, the use of snares is one of the most economical methods of removing many species of predators. The limitation to this practice is first the requirement that a good fence be in place, and as usual with many practices some individual predators become wary and learn to avoid snares.

Denning

Heavy predation losses often coincide with the presence of adult predators raising young in the pasture or nearby. If this is the case, one should locate these dens (for those predators which den in the ground) and have the young dug out or gassed in the dens. This may permit removing two generations (the adults and the young) at one time. The adults often may be trapped or shot near the dens, and if not, the removal of the young may cause them to vacate and avoid the area. Producers should make an effort to ensure that successful reproduction of problem species does not occur on their property. If this is successfully done on an area or regional basis, the problem with the individual species involved would be largely resolved.

Other Approaches to Dealing with Predation

A large amount of research has been conducted looking to other approaches in dealing with predation. These include the use of aversive agents (lithium chloride for example), repellants (sounds, visual devices, body sprays containing taste or odors thought to repel predators) or chemosterilants (for target species). To date none of these has proved sufficiently effective in reducing predation to suggest widespread use. In addition to the question of effectiveness, there are problems in obtaining approval to use such materials on food-producing animals or their use in the environment.

Policy Issues

In many places in the world there is public resistance to predator control in general or to some of the methods employed. This probably represents the greatest threat to range livestock production, especially for sheep and goats. To the writer, it seems unconscionable that an informed public would attempt to force the large carnivora predators (bear, lions, wolves, etc.) into the environment of people involved in production agriculture in that they constitute both a direct threat to humans and indirectly through their means of support. These large carnivora should be restricted to areas which can be set aside for this purpose, and with an exploding world human population to feed, there must be serious constraints on the amount of land that can be devoted to this purpose. Even within these areas, the population of large predators should be managed, including prevention from extensive movement outside the protected area.

Smaller predators (such as the coyote and the smaller felines), which do not constitute a direct threat to man, will probably continue to enjoy a wider range, but citizens must be given the freedom and tools to protect their interests.

Predators do not respect property boundaries, and some species cover large areas. Thus it is difficult for an individual producer operating under extensive conditions to deal with this problem alone. For this reason, an organized regional or area approach may be necessary. These may be managed or conducted by a governmental agency or by producer organizations.

Livestock producers are not the only ones to gain from control of predators, as they also prey on household pets as well as wild game species (e.g. deer) which may be valued for sport hunting or ecotourism. Some predator species also cause damage to agricultural crops.

The concept of removing the individual offending predator is not usually a workable practice, apart from cases where individual large predators have escaped from protected preserves. In the case of endemic populations, of animals such as wolves or coyotes, one animal may do most of the killing, but if this animal is removed, others will take over this role.

The approach to dealing with predation is greatly influenced by conditions. Throughout much of the world, herding is a most important practice and the herders must be given the tools (e.g. guns and the knowledge to use them) necessary to protect themselves and their herds or flocks.

Where herding and night confinement are not possible or practical and animals are to be allowed free range under extensive conditions, the offending predators must be removed. This is done most efficiently through the use of toxins, shooting from the air, trail hounds, and finally the most expensive way – with traps.

For smaller farm flocks, and where organized control efforts are not available, the most utilized tools are fencing, guard dogs, night confinement, traps and snares, and opportunistic shooting. Management practices such as early weaning are also widely utilized under these conditions.

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Sheep: Feeding Management

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Introduction

Sheep have been milked since time immemorial around the Mediterranean Basin, where they were traditionally fed on natural pastures and cereal stubble, and the lambing season coincided with the emergence of pasture. In these systems, sheep had to be able to buffer phases of extreme underfeeding by depleting body reserves accumulated during phases of nutritional abundance. In the second half of the twentieth century, grain prices decreased relative to sheep milk prices. In addition, owing to a general increase in the standard of living, the demand for sheep cheese increased. This economic conjuncture, together with the limitations of natural pastures as a food source, was the trigger for the intensification of dairy sheep farming. This intensification was featured in all Mediterranean countries by improved sheep nutrition and health status, controlled reproduction and selection, and the promotion of mechanical milking that overall resulted in a marked rise of stock numbers per farm. However, the strategies for upgrading sheep nutrition varied between countries. In the more rainy parts of the Mediterranean, improvement in sheep nutrition was achieved by the establishment of artificial pastures, either rainfed or irrigated, featured by higher herbage production than the natural pastures. In the semi-arid Eastern Mediterranean area, where little water was available for irrigation, and where land tenure legislation was a problematic issue, sheep breeding was progressively disconnected from grazing, sheep were confined, and adapted feeding procedures were implemented.

Why Sheep are Suited to the Intensification of Feeding Management

Dairy sheep are much smaller than cattle, but the size of rumen relative to body weight is similar in these two species. However, the energy requirement is proportional to metabolic weight, i.e. when related to body weight, higher in sheep than in cattle. In other words, nutrient intake, relative to the size of the gastrointestinal tract, must be higher in sheep than in cattle. Because retention time is not higher in the rumen of sheep than in cows, the digestion of roughage generally tends to be lower in sheep compared with cattle. Therefore, sheep are not able to consume as much roughage as cattle per kilogram of metabolic weight. Like other small ruminants, they have to compensate for their small body size by increasing the concentration of dietary energy, which is consequent with their behaving as 'concentrate selectors'. The diet selected by sheep is always more digestible than the average of dietary components available. Sheep can adapt well to starch-rich diets: they are less sensitive to acidic condition in the rumen; their small intestine, where bypass residual starch is digested, is longer; and they digest whole grain more thoroughly than cattle. Owing to these features, they easily adapt to diets rich in concentrates.

Intensive Dairy Sheep Production Systems

Intensive production systems for dairy sheep are concentrated in the European Mediterranean countries (Italy, with particular reference to Sardinia, France, partially Spain and, to a lesser extent, Greece and Portugal), as well as in the Near East (Israel and Cyprus). These systems are based on different genotypes and are featured by intensification levels depending on soil and climate conditions, feeding source, management and production targets. The main features of the most relevant systems are outlined in **Table 1**.

Intensive dairy sheep systems can be grouped into two sectors depending on the main feeding source:

1. Systems based on grazing.
2. Zero grazing systems.

Systems Based on Grazing

Sarda and Lacaune dairy sheep farming systems can be regarded as typical pastoral systems. Although in the 1980s and 1990s Lacaune dairy production has become more and more dependent upon conserved forage and concentrates, lactating ewes still graze for at least 6–7 months during the spring–summer period. The grazing season lasts up to 10–12 months in Sardinia on irrigated lowland farms

Table 1 Features of the main dairy sheep intensive systems

Country	*Region*	*Breed*	*Stock number*	*Number of lactations per year*	*Milk yield per lactation (kg per ewe)*
Italy	Sardinia	Sarda	3 500 000[a]	1	210
	Sicily	Comisana	750 000[a]	1	190
France	Rayon de Roquefort	Lacaune	825 000	1	270
Spain	Castilla–Leon	Crossbreeds[b]	*c*. 800 000	1–1.1	180–200
Israel	—	Assaf and Awassi	46 200	1.1[c], 1.3[d]	530[c], 320[d]

[a] About one-third of sheep managed under intensive conditions.
[b] Mainly based on crosses between Assaf rams and Churra, Castellana or Manchega ewes.
[c] Assaf.
[d] Selected Awassi.

(under very intensive conditions). Production systems based on grazing, even in the case of irrigated pastures, are usually featured by seasonal production. Mature ewe lambings are usually concentrated just before the beginning of the grazing season (late winter in France and late autumn in Sardinia). Herbage production and hence its availability, its quality and the distribution of nutrients on offer throughout the grazing season can be regarded as the main limiting factors for milk production.

In temperate regions, such as the Rayon de Roquefort, pastures mainly consist of perennial forages and herbage quality rarely limits intake and performance through the grazing season. In contrast, limitations due to herbage quality generally occur in the Sarda sheep system, where annual grasses are usually the main feed sources. However in semiarid Mediterranean climates that are under irrigation, these limitations can be offset by adequate forage systems – based on white clover, lucerne (alfalfa) and ryegrass – that allow both high stocking rates (20 ewes ha^{-1}) and remarkable levels of milk yield (250 kg per ewe per lactation).

Systems Based on Zero Grazing

Spanish, Cypriot and Israeli production systems, located in areas featured by harsh climates (dry continental in Spain and arid in Cyprus and Israel), are based on conserved forage, byproducts and concentrate feeding. The quality of these feedstuffs can be regarded as potentially limiting factors under these conditions. In these systems, dairy ewes are confined and separated into groups fed different diets. Group number, size and grouping criteria can play an important role under these circumstances due to the limited extent to which individual ewe selective capability can be expressed.

Pasture and Supplements for Dairy Sheep

When ewes are grazing under intensive conditions, grazing is usually based on artificial pastures that can either exclusively be devoted to grazing or used as multipurpose crops, cutting them at maturity stage for silage- or hay-making. These pastures are rarely offered as a unique food source under intensive conditions management. In fact, even when herbage mass availability is well above the threshold below which the ewes' intake is limited, the variability associated with outdoor conditions (weather, walking distances, abrupt changes in forage quality during grazing bouts, etc.) needs to be offset by the offer of supplements with the aim of increasing milk yield persistency over time.

Temperate Pastures

These mainly consist of perennial ryegrass (*Lolium perenne*) and, to a lesser extent, white clover (*Trifolium repens*), perennial species that feed most of the grazing livestock in the temperate regions of northern Europe. Other species, such as Italian ryegrass (*Lolium multiflorum*) and lucerne (alfalfa) (*Medicago sativa*) provide an important contribution to the forage chain, not only as grazed crops but also as conserved stock (silage and hay). An ancillary contribution comes from other perennial species, such as cocksfoot (orchardgrass) (*Dactylis glomerata*), tall fescue (*Festuca arundinacea*) brome grass (*Bromus inermis*), timothy (*Phleum pratense*) and red clover (*Trifolium pratense*). Overall, these forages are featured by both high mass production and good quality for most of the vegetative cycle, however, their feeding value will be strictly dependent on their management.

In continuously stocked systems, herbage mass, measured by the sward height, is putatively an efficient criterion for grazing management. In mutton sheep, using undisturbed sward height measured by sward stick, intake and production per hectare were optimized when both perennial ryegrass and white clover were maintained at a sward height of 60 mm. These guidelines are acceptable also for dairy sheep grazing the same species under continuous stocking.

Under rotational grazing management, residual stubble height has been shown to be strictly related to intake and performance. Under these dynamic conditions, a stubble height of 40–60 mm warrants adequate intake and performance, in dry and lactating mutton sheep, respectively. According to some authors, a higher stubble sward height (around 80 mm) should be aimed for during autumn, when herbage growth is reduced. These thresholds can also be generalized to dairy ewes grazing perennial swards.

Mediterranean Pastures

Under Mediterranean conditions, dairy sheep flocks mainly live on annual pastures, such as Italian ryegrass, the self-seeding annual ryegrass (*Lolium rigidum*) and winter cereals like barley and oats, which are usually combined as 'space' or 'time' mixtures with high-quality legumes. Among them, berseem clover (*Trifolium alexandrinum*), self-reseeding forages such as burr medic (*Medicago polymorpha*) subterraneum clover (*Trifolium subterraneum*) or short-lived perennial legumes such as sulla (*Hedysarum coronarium*). Under irrigation, these 'winter forages' are usually integrated with spring–summer growing forages, e.g. white clover, lucerne (used mainly for producing hay store) and C_4 cycle forages such as *Sorghum* subsp., millet and maize (for silage-making). When continuously stocked on Italian ryegrass, Sarda dairy sheep had a higher intake and performance when sward was maintained at 60 mm sward height, measured by a weighted disc, as compared with 30 and 90 mm. At 30 mm sward height, individual intake and performance were both depressed, whereas at 90 mm height during spring, quality was markedly reduced (more stems and fewer leaves) and hence performance was impaired. Moreover, the sward was markedly understocked. Sward heights kept within the range 80–90 mm in winter time were the optimal for individual intake and performance, but with a trend to lower stocking rate and performance per hectare (**Figure 1**).

There is a lack of information on rotational grazing for most Mediterranean forage types; however, for annual ryegrass it would be sensible to take into account the stubble height, avoiding depletion of herbage below 60 mm in winter and 40 mm in spring.

The quality of grass becomes limiting during the spring when Mediterranean annual pastures turn from the vegetative to the reproductive stage. In this case, the inclusion of broadleaved species, namely the legumes, can prevent marked decreases in nutrient intake and dairy yield. Furthermore, a timed allowance on sulla pasture restricted to 6 h daily can increase the intake by 30% and the milk yield by 50% during the annual ryegrass heading stage.

Under conditions of irrigation, the available mass during the spring and summer period is usually above sheep requirements. During this period, it is important to balance grass and legumes in the diet. An adequate ratio of protein to energy should be offered in order to reduce both the hazard associated with low-protein diets for milk production (see above) and that of protein waste, which can result in the high urea levels in blood and milk often associated with low fertility in ewes submitted to artificial insemination. Urea levels higher than 45 mg dl^{-1} were indeed found in flocks with low fertility in Sardinia.

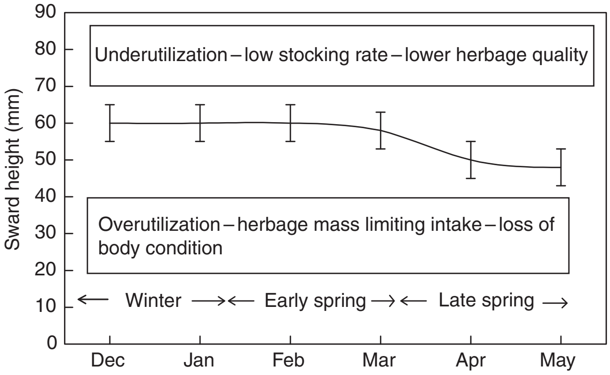

Figure 1 Suggested profile of sward height measured by weighted-disc of Italian ryegrass grazed continuously by dairy ewes during lactation. (Adapted with permission from Molle *et al*. (2001) In: Pulina G (ed.) *Alimentazione degli Ovini da Latte*, pp. 275–304. Bologna: Avenue Media.)

Supplementation of Grazing Dairy Ewes

Supplementing grazing dairy ewes is a practical tool for reducing the variability in milk yield and reproductive performance. Under intensive conditions, moderate levels of high-quality legume hays (300–500 g day^{-1}) are usually offered at night when sheep are housed, particularly during the winter months. Concentrate supplementation is aimed at increasing the individual intake and performance and also at 'saving' the available herbage for additional stock, taking into account the substitution effect. Starchy concentrates, such as cereal grains, are particularly useful for attaining the latter goal due to their high substitution effect. This is modified by herbage availability: substitution rate tends to increase along with herbage availability. Mixed concentrate usually gives lower substitution rates, the effect seems in relationship with the starch quality being higher with low-degradability starch (**Table 2**).

Overall, it is evident from the literature that the responses in terms of milk yield to concentrate supplementation based on starchy concentrate is limited, as expected by the substitution effect and possible reduction of diet digestibility. Recent results show that concentrates high in digestible structural carbohydrate and pectins are more effective for milk production persistency during the last third of lactation than high starch concentrates, either in grazing or in housed conditions. This is probably attributable to a shift of the metabolizable energy consumed by

Table 2 Effect of concentrate-based supplementation on substitution rate and milk yield of dairy ewes grazing Mediterranean pastures

Breed	*Lactation stage (months)*	*Pasture type (grazing system)*	*Herbage mass (kg DM ha^{-1} (Sward height))*	*Supplement type*	*Supplement level (g day^{-1})*	*Substitution rate[a](%)*	*Effect on milk yield[b](%)*
Sarda[h]	2–3	Rotational	nd	Nil	—	—	100
				Hay	*ad lib*.	nd	100
				Barley grain + hay	400 + *ad lib*.	nd	110
Comisana[i]	2–5	Natural pasture (rotational – 5 h day^{-1})	1400–500	Cereal-based concentrate[c] + hay	300 + 500	—	100
				Cereal-based concentrate[c] + hay	600 + 500	43.0	127
Comisana[j]	2–3	Natural pasture (rotational – 5 h day^{-1})	1400 (average)	Maize-based concentrate[d] + hay	350 + 500	—	100
				Maize-based concentrate[d] + hay	700 + 500	58.3	106
				Barley and wheat-based concentrate[e] + hay	350 + 500	—	104
				Barley and wheat-based concentrate[e] + hay	700 + 500	47.4	110
Sarda[k]	2–3	Italian ryegrass – winter	1111 (20 mm)[f]	Nil	—	—	100
				Maize grain	500	56.0[g]	119
		(continuous – all day)	1730 (40 mm)[f]	Nil	—	—	117
				Maize grain	500	88.0[g]	135
			2650 (60 mm)[f]	Nil	—	—	124
				Maize grain	500	96.0[g]	124
Sarda[k]	3–4	Italian ryegrass – spring	1400 (30 mm)[f]	Nil	—	—	100
				Maize grain	500	50.0[g]	105
		(continuous – all day)	2850 (60 mm)[f]	Nil	—	—	140
				Maize grain	500	136.0[g]	144
			3190 (90 mm)[f]	Nil	—	—	142
				Maize grain	500	116.0[g]	150
Sarda[l]	5–6	Italian ryegrass – standing hay	—	Nil	—	—	100
			1200–600	Maize grain	250	74.0[f]	106
		(rotational)		Soya bean meal	270	40.0[f]	114

[a] Marginal substitution rates.

[b] Milk yield as proportion of a reference treatment (within experiment).

[c] Concentrate including 63% of cereals (2/3 barley: 1/3 maize).

[d] Maize-based concentrate (low degradability starch).

[e] Barley and wheat-based concentrate (high degradability starch).

[f] Target sward heights measured by weighted disk.

[g] Absolute substitution rates (i.e. referred to the unsupplemented control).

[h] Sanna and Casu (1971) *Rivista di Zootecnia* 44: 19–26.

[i] D'Urso *et al*. (1993) *Animal Feed Science and Technology* 42: 259–272.

[j] Avondo *et al*. (1995) *Livestock Production Science* 44: 237–244.

[k] Molle *et al*. (1997) *Options Méditerranéennes, Serie* A 34: 65–70.

[l] Molle *et al*. (1995) *Small Ruminant Research* 17: 245–254.

nd, no data.

the ewes receiving the high-starch concentrate toward the fat depot more than toward the mammary gland. In contrast, crude protein-based supplements, particularly if of moderate to low degradability (lupin, soybean meal), support milk yield in late lactation and can improve ovulation rate and prolificacy in dairy sheep grazing on mature grassland.

Feeding Confined Dairy Sheep

Feedstuffs for Confined Dairy Sheep

In Mediterranean areas, grazing sheep have been traditionally supplemented with hay and grain. Using agricultural byproducts for dairy sheep farming is almost as ancient as sheep domestication, and the use of cereal bran as fodder is mentioned in the Bible. The first zero-grazing rations consisted of hay, wheat bran and whole grain.

The development of agriculture and associated industries during the twentieth century provided dairy sheep farmers with new agricultural and industrial byproducts. These products can be classified according to their moisture content and their functional role in rations (**Table 3**). Their classification as concentrate or roughage is risky, because most of them combine both concentrate and roughage features; for example, whole cottonseed is featured by high energy ($13.4\,\mathrm{MJ\,kg^{-1}}$ dry matter) but high ($480\,\mathrm{g\,kg^{-1}}$) neutral detergent fiber (NDF) content, with low content of long fiber. The most widespread industrial byproducts used in sheep nutrition are citrus pulp, brewers' grains, tomato pulp, maize husks, grape pomace and olive cake, which can be fed fresh or ensiled. With the development of irrigated fruit and vegetable production in some Mediterranean areas, new sources of roughage have emerged, e.g. tomato and cantaloupe straw. Onion and garlic peels, residues of the flower industry, or fruit (citrus, apple, peaches, avocado and dates) and vegetables (potatoes, eggplant, cantaloupe, watermelon), which are not marketed because of price-keeping policies, are regularly fed to dairy sheep.

Use and Misuse of Byproducts in Dairy Sheep Feeding

Until the late 1960s, byproducts were either fed *ad libitum* in the trough or provided in controlled, predetermined amounts. The former method was found to be associated with numerous metabolic disorders. When Awassi sheep were provided with citrus pulp *ad libitum*, it resulted in hypocalcemia and overfattening, a ketosis-like syndrome was observed, and subsequent lactation was impaired. The free-feeding of cereal bran, rich in phosphorus and featuring a reversed calcium:phosphorus (Ca:P) ratio, resulted in urolithiasis of Assaf rams and lambs. Correction of the Ca:P ratio and addition of ammonium chloride are necessary under such conditions. Numerous field cases of copper toxicity have been reported in sheep fed brewers' grains, where the brewing vats were made of copper, or in sheep fed copper-rich cottonseed meal. The copper hazard is critical in breeds that are sensitive to copper loading, such as the German Ostfriesland

Table 3 Characteristics of some byproducts used in dairy sheep feeding

		On DM basis		
	DM (g kg^{-1})	*CP (g kg^{-1})*	*NDF (g kg^{-1})*	*ME (MJ kg^{-1})*
Wet byproducts				
Citrus pulp	140	72	223	12.5
Brewers' grains	200	210	525	10.5
Grape pomace	320	72	708	5.0
Tomato pulp	200	180	673	8.4
Olive cake silage	490[a]	50[a]	722[a]	5.36[a]
Dry byproducts				
Cottonseed (whole)	890	220	474	14.6
Wheat bran	890	170	456	10.9
Cottonseed hulls	910	41	810	7.5
Sunflower seed hulls	910	49	860	2.5
Roughages				
Wheat straw	900	22	790	5.8
Cotton-gin trash	900	66	800	6.9
Maize straw (stover)	900	2.9	693	8.8
Groundnut (peanut) straw	890	110	523	7.7
Tomato/cantaloupe straw	910	110	590	5.0

[a] Hadjipanayiotou (1999) *Livestock Production Science* 59: 61–66.

Compiled from INRA, 1989 and NRC (1985) tables of feed composition.

Milchschaf and breeds resulting from crossbreeding, such as the Assaf. Forages grown in some Mediterranean soils are deficient in molybdenum and sulfur, which accounts for the high availability of copper, even in feeds of medium copper content. Calculation of absorbed copper can be carried out, taking into account dietary copper, sulfur and molybdenum, and accumulation in the liver can be calculated as a function of liver size and time of accumulation. Such calculations can lead to the addition of molybdenum and sulfur when necessary, as constituents of added mineral and vitamin mixtures. Selenium deficiency, resulting in the occurrence of white muscle disease in Assaf and Sarda lambs, has been reported in flocks fed whole cottonseeds or cotton-gin trash. Selenium and vitamin E must, therefore, be added, where needed. Loose feces and even chronic diarrhea has been noted when Assaf dairy sheep are fed with tomato straw as the sole roughage, probably caused by excess calcium (more than 3%) and low phosphorus (less than 0.1%), resulting in an extremely impaired Ca:P ratio. Bad conservation procedures are thought to be responsible for the occurrence of botulism and listeriosis in Awassi and Assaf sheep fed fresh brewers' grains and orange pulp.

Practical Implementation of Total Mixed Ration for Dairy Sheep

The advances in mixer-feeder manufacturing in the 1970s, involving digital scales and upgraded weighing software, resulted in widespread adoption of total mixed rations (TMR) in dairy cattle feeding. The new technology was based on the observation that there was less diurnal variation in the ruminal environment in TMR-fed cattle than in cattle fed roughage and concentrates separately. This technique also resulted in a steadier feed intake featured by more small meals. Using TMR allowed the inclusion of well-controlled, diluted amounts of byproducts in ruminant diets, thus improving the safety of feeding byproducts. It also allowed a significant cut in personnel expenses. These advantages were the trigger for the intensifying dairy sheep industry to employ the new technology. However, it soon became apparent that some problems specific to sheep needed to be solved before the use of TMR could become widespread.

The first problem that needed to be tackled was the high rate of residues left by TMR-fed sheep. When sheep are fed TMR prepared according to cattle manufacturing criteria, long-fiber roughage leftovers are left in the trough, i.e. sheep select short-fiber components and reject long-fiber components (**Table 4**), which is compatible with their normal feeding behavior as 'concentrate selectors'. Roughage and, in particular, silage have to be chopped to a length of approx. 20 mm, using the mixer-feeder, before other components are added, in order to decrease selecting behavior. Thorough mixing of TMR fed to sheep must also be implemented in order to decrease selective behavior. Unlike cattle and goats, feeding sheep with TMR *ad libitum* is often associated with decreased motivation to enter the milking parlor. Therefore, some of the concentrate is generally transferred from the TMR to distribution in the milking parlor, which results in decreased energy concentration in the TMR itself.

Matching TMR Composition with Sheep Requirements

The requirement of dairy animals for nutrients is a function of milk component yield, which itself depends on genetic merit and days in milk. Also, dry matter (DM) intake varies with days in milk. Changes in DM intake relative to days in milk depend on breed and genetic excellence within a breed. In most cases, optimizing TMR formulation with individual requirement is not practical, even where milk recording is practiced. Two strategies are possible: (1) using a TMR suitable for high-producing animals, i.e. of high energy and crude protein (CP) content, and distributing limited amounts to

Table 4 Chemical composition (in 1991) of a total mixed ration (TMR) and of residues left by early lactating dairy sheep at different times after meal administration; residual composition indicates positive selection of protein and fat-rich components (i.e. leaves, seeds) and avoidance of fiber-rich components (i.e. stems)

		Composition of residues at different times after TMR administration			
Composition (DM basis)	*TMR*[a]	*2 h*	*8 h*	*12 h*	*24 h (refuse)*
Crude protein (%)	20.7	19.6	15.7	12.3	9.7
Ether extract	4.6	3.8	2.8	2.5	1.8
Crude fiber (%)	21.5	22.6	27.9	30.9	34.6

[a] Ingredients (on fresh matter basis): 39.9% maize silage, 18.3% mixed hay, 11.7% dehydrated alfalfa, 6.7% delinted cottonseed, 23.4% concentrate compound including barley, soybean meal, sugarcane molasses and mineral–vitamin premix.

low-producing animals; or (2) using a TMR of low energy and medium protein content, and supplying extra concentrate to high producers. Both strategies have drawbacks. Strategy (1) enables a proportion of the low producers to overfatten, while part of their group is underfed. Strategy (2) is deleterious to high producers, because decreased energy density is obtained by using bulky components. If roughage is chopped up before it is included in TMR, little selectivity can be applied by high yielders and their intake is decreased, resulting in a decreased milk yield. A compromise is generally achieved whereby TMR of medium (10.5 MJ kg^{-1} DM) energy concentration are fed to dairy ewes. Assuming that the energy requirement of a ewe weighing 70 kg and producing 2.5 kg of milk (containing 65 g kg^{-1} of fat) is 31.3 MJ of ME, and that DM intake is 2.9 kg, 23 MJ is supplied as TMR in 2.2 kg of DM, and the rest is supplied in the milking parlor as concentrate (**Table 5**). Only traces of concentrate are provided to low yielders in the milking parlor.

Practical Formulation of TMR for Dairy Sheep

As stated above, TMR has enabled the safer use of byproducts in dairy sheep nutrition. However, emphasis is still placed on using only safe ingredients; glucosinate-rich rapeseed meal, gossypol-rich cottonseed and nitrate-rich forages must be identified and discarded. Even if there is shortage of scientific information on the inclusion of byproducts in dairy sheep diets, practical experience exists, particularly in Israel. On the Eastern Mediterranean shore, rations are calculated according to Linear Programming (LP), so as to minimize costs, whereas in the French INRA system (INRAtion), which is partially adopted in Italy and Spain also, more emphasis is placed on prediction of intake, taking into account animal factors and interactions between feedstuffs.

Chemical constraints for linear programming must take into account the fact that not all the ration is fed as TMR, and concentrate contribution must be forced into formulation as fixed values. In Israel, feedstuffs matrixes and dietary constraints for formulation include mature equivalent (ME), crude protein, Ca, P, Cu, Mo, S, Ca:P ratio, Cu absorbed (as a function of Cu, Mo and S concentrations). Using tabulated data from *in sacco* trials, fermentable organic matter is used as a predictor of microbial protein contribution, and bypass protein is calculated. Because the number of ingredients may be as high as 20, no attempt is made in Israel to predict feed interactions on sheep intake; the substitution rate between each individual feed and all the others is assumed to be 1. In France, Italy and Spain, the partition of ruminally degradable and nondegradable protein is carried out using the French PDI system. A special component of the French method is a constraint of bulkiness of

Table 5 Practical formulation (a) and typical composition (b) of TMR (in 2001) for dairy sheep in Israel (in addition, 400 g of a commercial concentrate (17% CP) was in the milking parlor and an additional 200 g of a commercial concentrate was distributed for 1 month postpartum)

(a)

Constraints	*Minimum*	*Maximum*	*Actual*
DM (kg)	2.9	2.8	2.9
FM (kg)	Ratio constraint	Ratio constraint	3.8
CP (kg)	0.420	0.500	0.478
ME (MJ)	31	No constraint	31
Roughage (kg)	0.800	No constraint	1.050
Calcium (g)	23.0	No constraint	28.5
Phosphorus (g)	10.0	No constraint	11.4

(b)

Ingredients	*% Dry matter*
Ground maize grain	16.2
Whole barley grain	3.2
Whole oats grain	7.3
Wheat bran	6.5
Chopped wheat silage (35% DM)	32.4
Vetch hay	27.6
Limestone	0.9
Mineral–vitamin premix	5.9

Table 6 Formulation criteria (a) and composition (b) of typical TMR (in 2001) for milked sheep in Italy (in addition, 200–400 g of a commercial concentrate (16% CP) was offered in the milking parlor)[a]

(a)

Criterion	*Minimum*	*Maximum*	*Actual*
DM (kg)	1.9	2.1	2.0
CP (% DM)	15	20[b]	15.9
PDI (% DM)	10.0	13[b]	10.0
NE (feed units kg^{-1} DM)	0.80	0.90	0.85
ME (MJ kg^{-1} DM)	9.5	10.6	10.0
Roughage (kg)	0.600	—	0.760
Calcium (% DM)	0.8	—	0.8
Phosphorus (% DM)	0.3	—	0.3

(b)

Ingredients	*% Dry matter*
Maize grain	17.0
Soybean meal (48% CP)	6.0
Dried sugar-beet pulp	9.0
Chopped maize silage (33% DM)	33.0
Lucerne hay	34.0
Mineral–vitamin premix	1.0

[a] Allowing the sheep receiving this TMR to graze a buffer crop (e.g. Italian ryegrass) in vegetative stage for 3–5 h reduces TMR intake by about one-third and improves milk performance.
[b] Maximum level depending on the current cost of CP and PDI. Reproduced from *Proceedings of the 12th Congress of the National Scientific Association of Animal Production (ASPA)* pp. 255–256.

roughage constituents termed ‘fill unit’. Another feature is calculation of substitution between concentrate and forage. The INRAtion software also takes into account differences in feed intake derived from breed and body condition, in the main breeds of dairy sheep. Special constraints on ingredients, resulting from trial and error, are used to fine-tune LP calculations. An example of TMR for dairy sheep, which was calculated taking the above criteria into account, is shown in **Table 6**. This TMR was formulated for lactating sheep (40–50 kg body weight) with a potential milk yield averaging 1.1 kg day^{-1} per ewe during the whole milking period (about 180 days). The allowance of a complementary pasture for a few hours daily proved a practical tool in reducing the feeding cost and improving feed efficiency of this ‘conventional’ TMR based on good-quality forages and concentrates.

In Israel and Cyprus, where complementary grazing is uncommon and byproducts are frequently used for sheep feeding, a typical TMR formulation for a 65–70 kg dairy sheep (2.2 kg DM, not including concentrate fed at the parlor) would consist, on a DM basis, of no less than 0.8 kg of total roughage (including at least 0.3 kg of palatable hay), no more than 0.3 kg of citrus pulp (or another fruit pomace) and no more than 1.0 kg of cereal silage. At least two grain sources, differing in ruminal degradability, are always included. The vitamin and mineral complementation always contains salt, a calcium contributor, such as calcium carbonate, and a mixture of vitamins A, D and E.

Implications of Intensive Feeding Management on Dairy Sheep Product Quality

Sheep milk is used only for cheesemaking. Therefore, knowledge regarding nutritional effects on milk features should be extended to cheese. Unfortunately, knowledge on this issue is still scarce. In grazing conditions, stocking rate (or herbage on offer per individual) can affect protein content in the milk. If stocking rate is very high, intake becomes limiting, energy balance is markedly negative and under these conditions milk protein decreases as well as casein. This can result in a lower cheese yield. Utilization of starchy concentrates in winter can overcome this problem.

Milk urea is a good practical gauge for monitoring protein nutrition in ewes. If it is too low, milk production can be limited, but on the contrary it is clearly wasted. This nitrogen cannot be recovered in cheese and therefore can become a concern, from a system and environmental viewpoint. Legumes as a complement to grasses can help to keep protein intake through the season as steady as possible, but this is particularly important when grass quality decreases (late spring–summer). In these conditions, urea levels would be lower in tannin-rich legumes such as sulla as compared to burr medic.

Fat content in milk is usually strictly inversely related to milk yield. Its concentration is affected by the offer of roughage only when the concentration of NDF in the diet is particularly low, as typically occurs during the winter period when herbage is immature and scarce and the supplementation level is high. Milk fatty acid composition, which is of great interest to consumers in terms of health, can also be affected by feeding. Unsaturated fatty acids, medium chain length fatty acids and conjugated linoleic acids play different roles in preventing some important human diseases. The use of fat-based supplements, such as full-fat seeds or beans, calcium soaps or byproducts of oil mills, can exert similar effects but, in some circumstances, reduce milk protein and casein. The same forage species has an effect on fatty acid composition. For instance, in sheep, as well in cows, the intake of fresh grass is linearly related to milk conjugated linoleic acids, but this is not the case for some fresh legumes.

Offering legumes, such as sulla, as well as other species, such as daisy plants (e.g. *Chrysanthemum coronarium*), can affect the concentration of volatile compounds (e.g. terpenes) and hence the sensory properties of sheep cheese as compared with grass-based diets. This area of study is still in its infancy and further research is required to elucidate the mechanism through which nutrition can impact the concentration of these nutriceutical and organoleptic components in sheep dairy products.

Conclusions

The intensification of dairy sheep production in the Mediterranean has been a dichotomous process, where food security has been attained by improving pastures or by discontinuing grazing completely. In countries where water resources were not severely limited, traditional pasture systems evolved into well-monitored systems relying on high-yielding grasses and legumes, which were totally or partially devoted to grazing. In more arid countries, where no water or land was available to establish cultivated and irrigated pastures, the feeding of sheep with concentrates and agroindustrial byproducts has developed. Both systems are economically sustainable. The zero grazing system allows the maximization of milk production, but dairy products, even if of good quality, are not well individualized. In contrast, intensified grazing systems, even if less productive, allow for marketing of traditional high-quality cheeses, e.g. the protected designation of origin (PDO) products.

See also: **Dairy Farm Management Systems:** Dry Lot Dairy Cow Breeds; Sheep. **Diseases of Dairy Animals:** Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Milk Fever. **Feed Ingredients:** Feed Concentrates: Co-Product Feeds; Feed Concentrates: Oilseed and Oilseed Meals; Feed Supplements: Macrominerals; Feed Supplements: Microminerals. **Forages and Pastures:** Perennial Forage and Pasture Crops – Species and Varieties; Annual Forage and Pasture Crops – Species and Varieties. **Milk:** Sheep milk.

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Sheep: Health Management

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Introduction

Infectious disease is thought to account for approximately half of the estimated 17–25% total worldwide production losses encountered by sheep owners each year. The situation has undoubtedly worsened recently due to reduced economic margins, intensification and the increasingly more frequent and longer distances moved by livestock. Such circumstances make it easier for infectious organisms to be transmitted from flock to flock and make it crucial that disease control strategies are both efficient and cost-effective. Owners should always be encouraged to adopt proactive attitudes toward flock health management rather than making reactive and often belated responses once problems occur. The purpose of this article is to highlight common diseases suffered by dairy sheep and examine how they can be prevented by developing and implementing a structured flock health management plan.

Common Diseases of Dairy Sheep

Mastitis

Mastitis can be a major problem for dairy flocks and is usually caused by bacteria, e.g. *Pasteurella* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli*, infecting the udder via the teat canal, or occasionally by systemic bacterial or viral infection spreading to the udder via the blood, e.g. *Mycoplasma* spp. and Maedi Visna virus. Treatment of cases caused by bacterial infection depends upon early detection followed by milking out the affected udder and immediate administration of appropriate antibiotics and anti-inflammatory drugs.

Infectious Abortion and Infertility

Infectious abortion and infertility can have significant effects on lambing output and therefore milk yield in a dairy flock. Infectious abortion can be caused by a variety of bacterial, protozoal and viral infections, including *Chlamydophila abortus ovis* (EAE), *Campylobacter* spp. (vibriosis), *Salmonella* spp., *Brucella melitensis*, *Toxoplasma gondii*, *Coxiella burnetii* and border disease virus. Apart from *Toxoplasma* infection, which is spread via feed contaminated with cat feces containing parasite oocysts, most infections enter the flock through purchase of previously infected animals and are spread when infectious organisms are liberated as ewes abort. Control focuses on buying in clean stock, observing strict hygiene when handling aborted ewes, fetuses and placentae and, if suitable vaccines are available, using them to protect susceptible stock.

Infertility in dairy rams may be a problem due to epididymitis arising from infection with bacteria such as *Br. ovis*, *Histophilus ovis* and *Actinobacillus seminis*.

Lameness

Sheep lameness is a major welfare concern throughout the world. The pain suffered by the animal results in a reluctance to move and feed, which has secondary effects, including lowered milk yields and reduced conception rates. Lameness problems usually arise in the foot as a result of bacterial infections. Common conditions include interdigital dermatitis (scald), footrot, contagious ovine digital dermatitis (CODD, supervirulent footrot). Most conditions are infectious so control and treatment require strict segregation of affected animals, foot trimming to remove diseased tissue, foot bathing and topical and systemic antibiotics.

Nutritional and Metabolic Disorders

Dairy ewes are often subjected to a number of nutritional and metabolic stresses throughout their lives. These occur at periods of peak production, such as mid to late pregnancy and early to mid lactation. If dietary requirements are insufficient or poorly balanced at these times then conditions such as pregnancy toxemia (twin-lamb disease), hypocalcemia (lambing sickness) and hypomagnesemia (lactation tetany) may occur. All animals should always be fed the best quality of diet available, which has been analyzed to ensure that it contains the required levels of energy, proteins, minerals, vitamins and trace elements. If certain mineral or trace element deficiencies occur, e.g. copper, selenium, cobalt or vitamin E, then these may have to be given as a supplement in the form of a mineral mix, oral drench or systemic injection. Dietary changes should always be introduced gradually and responses to feeding regimes monitored by regularly weighing or assessing body condition scores while handling.

Pasteurellosis and other Respiratory Infections

Pasteurellosis caused by *Mannheimia (Pasteurella) haemolytica* is the commonest cause of acute pneumonia in sheep, while infection with *Pas. trehalosi* can also cause peracute systemic disease in young and growing lambs. Pasteurellosis outbreaks are usually sporadic but outbreaks may be precipitated by stress associated with handling, transport or housing. Long-acting oxytetracycline antibiotics have been shown to be effective therapeutically and prophylactically under field conditions against both forms of the disease and effective vaccines against the respiratory form of the disease are also available. Other bacterial infections, including *Mycoplasma* spp. (atypical pneumonia), and retroviral infections such as sheep pulmonary adenomatosis (SPA, Jaagsiekte) and Maedi Visna also cause respiratory problems associated with pneumonia and, in the case of SPA, through the production of lung tumours.

Mycobacterial Infections

Dairy sheep may be affected by two chronic diseases caused by mycobacteria. The most common is paratuberculosis (Johne's disease), a chronic enteritis of all ruminants that is found worldwide. Tuberculosis also occurs more rarely but raises important public health concerns, particularly with regard to infection in milk. Johne's disease remains a significant problem because current diagnostic tests and vaccines are unable to deliver effective control. The disease is economically important as a result not only of clinical disease but also because of reduced productivity during the prolonged preclinical stages when weight loss and reduced milk production occur. Infections are spread via the feces and the disease occurs in a wide variety of ruminant wildlife species, as well as some monogastric species, which further complicates the development of effective control strategies.

Clostridial Diseases

Clostridial diseases are probably responsible for causing the greatest mortality in sheep due to infectious disease. Infections with different clostridial bacteria are responsible for causing lamb dysentery and pulpy kidney (*Clostridium perfringens* types B and D), tetanus (*Cl. tetani*) and neonatal infections caused by *Cl. septicum* and *Cl. chauvoei*. Ewes are also susceptible to struck (*Cl. perfringens* type C), braxy (*Cl. septicum*), blackleg (*Cl. chauveoi*), black disease (*Cl. novyi* type B) and abomasitis and enteritis associated with *Cl. sordelli* infection. Clostridia have a ubiquitous distribution in the environment, particularly in soil, and they also occur in small numbers in clinically unaffected animals. Disease is usually precipitated as a result of stress factors ranging from changes in management to traumatic damage to organs or parasitic disease. Once infection is established the course of the disease is so rapid that animals are usually moribund or dead before treatment can be given. Most clostridial diseases are amenable to prevention by vaccination.

Parasitic Conditions

Dairy sheep are susceptible to a number of diseases associated with internal and external parasites. Internal parasite infections caused by gastrointestinal nematodes lead to the condition known as parasitic gastroenteritis (PGE). Common parasite species involved include *Telodorsagia* spp., *Haemonchus contortus*, *Trichostrongylus* spp. and *Nematodirus* spp. Clinical signs of PGE include ill-thrift, weight loss, diarrhea and, with *Haem. contortus*, anemia and peripheral edema. Liver flukes cause liver damage leading to weight loss, anemia, jaundice and peripheral edema and death from liver failure.

Control and treatment of internal parasites rely upon reducing animal exposure to infective larvae by adopting rotational grazing policies and through prophylactic and therapeutic treatment with appropriate anthelmintic and flukicidal drugs. Nematode resistance to anthelmintics is becoming an increasing problem, so products containing different classes of drugs should be selected carefully and rotated according to veterinary advice. External parasite infestations involving flies, mites, lice, ticks, keds and fleas all cause severe skin lesions leading to wool loss and intense pruritus. Sheep scab, caused by the mite *Psoroptes ovis*, and fly strike caused by fly maggot larvae feeding on the host are serious problems in certain parts of the world where they are a major welfare concern. Dipping using an organophosphorous or synthetic pyrethroid-containing product in the spring and late summer or early autumn will eradicate sheep scab mites and help to protect against fly strike, horn flies, ticks and lice. Injectable endectocide anthelmintic preparations containing avermectin or avermectin derivatives also control sheep scab but have no activity against flies, lice or ticks.

Nonparasitic Skin Diseases

Bacterial infections of the skin and fleece caused by *Staphylococcus* spp. (periorbital eczema), *Corynebacterium* spp. (Bolo disease), and *Actinobacillus* spp. (leathery lips, cruels), *Pseudomonas aeruginosa* (fleece rot) and *Dermatophilus congolensis* (mycotic dermatitis) occur sporadically, requiring antibiotic treatment. Ringworm caused by the fungus *Trichophyton verrucosum* also occurs sporadically but appears to have become an increasing problem in recent years. The bacterium *Corynebacterium pseudotuberculosis* causes a more widespread problem known as caseous lymphadenitis (CLA). CLA infections localize to the superficial lymph nodes, usually around the head and neck area, eventually bursting out through the skin to produce suppurating abscesses. The condition is refractory to antibiotic treatments and can spread rapidly through the flock.

Orf (contagious ecthyma) is a viral skin disease caused by a parapoxvirus that affects the mouth, udder and coronet of the hoof. Infections result in the development of scabs, which may become extensive and develop into proliferative wart-like lesions. Although the condition is usually self-limiting, outbreaks in dairy flocks can cause particular problems due to the development of teat lesions that interfere with milking and predispose toward the development of mastitis. Control of orf is extremely difficult due to the ability of the virus to survive for long periods in the environment and the poor immune response generated in the host postinfection. Orf vaccines are available, but should only be used in severe outbreaks because they can contribute to contamination of the environment with live virus.

Transmissible Spongiform Encephalopathies

Transmissible spongiform encephalopathies (TSEs) are a group of diseases that share many common features. They are infectious; incubation periods are always long and asymptomatic, but culminate in an irreversible, progressive and ultimately fatal degeneration of the brain. In sheep, scrapie has long been recognized as a member of this class of diseases and it occurs in adults of both sexes and mostly in sheep 2–5 years of age, although there is no upper age limit. Clinical signs are often variable but include initial behavioral changes followed by incoordination, wasting, hypersensitivity and pruritus, which leads to wool loss and skin lesions due to rubbing. Treatment is impossible and affected cases are usually euthanized on welfare grounds. Infections are spread from sheep to sheep, particularly at lambing time when ewes pass the disease on to their lambs via infected placentae and birth fluids. Control relies upon careful sourcing of animals from flocks free from the disease. Recent work on the genetics associated with the disease has shown that infection is linked to alleles on the *PrP* gene coding for three codons of the PrP protein. As a result, genotyping tests are now available which allow stock to be selected on the basis of their resistance or susceptibility to the disease. Because of concerns that animal TSEs may be a source of infection to humans, veterinary authorities must be notified about suspected scrapie cases in most countries.

Major Plagues

In certain parts of the world, diseases responsible for causing occasional severe widespread epidemics among livestock can devastate farming. The most important of these ‘plague’ diseases affecting sheep include foot-and-mouth disease, sheep pox, bluetongue, Rift valley fever and peste de petits ruminants (PPR). An international surveillance network coordinated by the Office International des Epizooties (OIE) monitors disease outbreaks. Control is brought about by a combination of statutory measures imposed by national veterinary authorities, usually involving compulsory slaughter and/or vaccination, as well as international restrictions on the movements of animals and animal products from affected countries.

Flock Health Planning

In many industrialized countries, where sheep are often reared intensively, the development of a formal written flock health plan outlining routine disease prevention treatments such as vaccinations and antiparasitic treatments is becoming a statutory requirement for all sheep owners. Although the primary intention is to improve welfare by ensuring that appropriate husbandry and health management procedures are carried out at the correct time, additional benefits associated with improved product quality, reduced input costs, drug residues and drug resistance resulting from inappropriate use of veterinary products are also achieved. Providing the necessary economic infrastructure, veterinary expertise and flock owner compliance can be guaranteed, some of these benefits may also be achieved under extensive management systems found in less developed countries by some or all of the measures outlined below.

A key factor in the development of a health plan is to arrange regular veterinary visits to discuss disease-related issues. After each visit, a comprehensive report containing action points should be generated identifying problem areas to be targeted by the flock owner when making future management decisions.

Of equal importance is the development of effective recording systems to allow the rapid identification of problems with flock performance, which may be caused by disease. In dairy flocks, these systems should, as a minimum, include the ability to record animal origin, reproductive data, milking performance, veterinary treatments, culling rates and reasons for culling. Flock owners and their veterinarians should look critically at existing records and satisfy themselves that all relevant herd or flock performance and health information can be easily retrieved. Owners should always be aware of the best management targets and be prepared to respond quickly if underperformance due to disease is suspected.

If necessary, veterinary assistance should be sought as soon as possible and, as part of health plan discussions, owners should try to develop appropriate contingency plans should a disease occur. In a disease outbreak it is much easier to limit losses if risk factors have been identified in advance and treatment can be started immediately.

Animal Purchase

Health management starts before animals arrive. Movement of livestock into the flock and mixing them with existing animals represents the single greatest risk for spreading disease. Such movements may occur as a result of purchasing replacement breeding stock, borrowing or sharing rams, purchase of growing lambs and accommodating animals on rented pastures. To avoid such situations, owners should always try to use management practices that reduce movements to a minimum and keep their flocks as 'closed' as possible, e.g. breed their own replacements, use home-bred rams and keep growing animals completely separate from breeding stock. To avoid risks of inbreeding in closed flocks, replacement rams need to be introduced from time to time but particular care needs to be taken to establish the health status of these animals. If facilities and economics permit, it may be worth considering artificial insemination using accredited semen from a breeding company to minimize the risks.

In situations where bringing in and mixing animals is unavoidable, appropriate procedures, summarized in **Table 1**, should be followed. Throughout the world there are national and local commercial health schemes, which accredit member flocks on the basis of clinical and laboratory testing as being free from certain diseases. These schemes currently cover diseases such as brucellosis, anthelmintic-resistant parasites, fly strike, footrot, Johne's disease, enzootic abortion, Maedi Visna and genetic tests for scrapie susceptibility. Wherever possible, owners should always select animals from suppliers certified under such schemes in preference to animals from flocks whose health status is unknown. Even if accreditation requirements cannot be met on the purchaser's farm or the diseases tested for are not considered a problem, the fact that the stock are already accredited gives an indication of their general health status and provides the impetus to raise standards to allow accreditation to be maintained.

Table 1 Procedures to be followed when purchasing livestock

Assess whether it is absolutely necessary to buy animals in the first place
Try to buy home-bred stock direct from as few farms as possible
Select animals accredited under a health scheme wherever possible
Record the exact origin of every animal
Ask about the disease and health history of the stock to be purchased
Request and examine all documents relating to the health of the animals
Discuss the possibility of prepurchase health screening and examinations with your veterinary surgeon
Check requirements for retaining health scheme accreditation before moving and see if these can be met on your own premises
Always transport animals direct to the farm using your own vehicle or one belonging to a reputable haulage contractor
If buying at auction, always buy through the ring to ensure traceability
If purchasing from abroad, be fully aware of all import legislation requirements
Always be prepared to withdraw if problems or obstructions are encountered at any stage of the buying process

Quarantine and Admission Treatments

On arrival, it is vital that purchased animals undergo a suitable quarantine and treatment regime before being mixed. These requirements should apply irrespective of whether dealing with growing lambs, rams or ewe replacements. These requirements should also apply to animals that are health scheme-accredited or subjected to other prepurchase testing or examination procedures. Buyers should be aware that there are many conditions, such as scab, footrot, anthelmintic-resistant nematodes and CLA, that cannot be easily tested for and may not be evident at the time of purchase, but will cause major problems once animals are mixed.

Quarantine allows time for such problems to emerge and be controlled before mixing occurs. Owners should be prepared to quarantine animals for at least 3 weeks, or longer if management requirements allow, and this needs to be taken into account when planning to buy or borrow animals, including, for example, rams. For replacement breeding stock, the possibility of introducing infectious abortion may make it appropriate to mate these animals separately and to keep them isolated until after lambing. **Table 2** outlines the major factors to consider when setting up and operating a quarantine procedure. If local economics and veterinary resources allow, it is also good practice to carry out a number of prophylactic treatments in addition to quarantine (summarized in **Table 3**) to protect purchased sheep against common diseases as soon as they arrive at the farm. These treatments should be fully completed and recorded before the animals are released from quarantine. Once released, purchased animals should then ideally fall in line with an established flock health management plan tailored to the needs of existing stock.

Table 2 Quarantine requirements for incoming stock

All purchased or borrowed animals must undergo quarantine before mixing with existing stock

Quarantine areas should be capable of housing incoming stock for at least 3 weeks or more

Quarantine means complete isolation, so the area chosen must be completely secure from existing stock

Use separate buildings or walled or double-fenced paddocks with at least 3 m separation from other stock

If adjacent areas must be used, separate them by 2-m wide unbroken hedges, ditches or solid walls that are at least 3 m high

Check regularly that animals do not have any nose-to-nose contact at any point, including gates

Staff should never handle quarantined stock before handling existing stock

Quarantined stock should be examined daily for signs of disease

It may be appropriate to devise strategies to keep replacement breeding stock separate from the rest of the breeding flock until after lambing to prevent the spread of infectious abortion

Table 3 Routine admission treatments for purchased animals

Treat with an anthelmintic known to be effective against anthelmintic-resistant nematodes

Dip sheep in a plunge bath using an ectoparasiticide which is effective against sheep scab, lice and flies

Examine all feet, trim overgrown hooves and put all animals through a copper sulfate, zinc sulfate or 5% formalin footbath

Isolate animals with footrot and treat with parenteral antibiotics as appropriate

Monitor closely for lameness throughout the quarantine period and reexamine all feet before releasing animals from quarantine

Give a full primary course of vaccines to all purchased animals so they are fully covered for the same diseases as existing animals. Seek veterinary advice on vaccinating against clostridial diseases, pasteurellosis, infectious abortion and footrot

Basic Health-Care and Husbandry Programme for Dairy Flocks

Lactating Dairy Ewes

Health management procedures involving the prophylactic administration of medicines to dairy ewes are often severely restricted by requirements for observing appropriate drug withdrawal periods to prevent the risk of drug residues in milk for human consumption. Unlike the situation with dairy cattle, many veterinary products are currently not licensed for use in sheep or, where they are licensed, specific milk withdrawal periods have not been established for dairy ewes. In general these restrictions apply to antimicrobial, anthelmintic, ectoparasitic or analgesic drugs, but also include certain vaccines. Under such circumstances, all medicinal products for lactating dairy ewes should be used with extreme caution and then only under direct veterinary supervision. Where restrictions prevent the administration of prophylactic treatments that have direct welfare consequences, e.g. ectoparasitic treatments during the summer, exceptionally high standards of stock management will be required to ensure that affected animals are identified and given immediate treatment. Once any animal receives a product that requires milk to be withheld, the treatment should be recorded, along with the required withdrawal period, and the ewe clearly marked and separated from the remainder of the milking flock.

Lambing of dairy ewes

Prepare all lambing equipment and collect the necessary veterinary supplies at least a fortnight before the first lambs are due. Ensure the lambing shed or lambing area has sufficient numbers of lambing pens set up to allow them to be properly cleaned and disinfected between successive ewes. There should be at least one pen per eight ewes (or one pen per four ewes if the flock has been synchronized). Maintain the highest standards of hygiene in and around the lambing pens to reduce the risk of neonatal infections in the lambs. Monitor and record all ewes that have problems around lambing time. Ewes that develop vaginal or uterine prolapses or have difficulties lambing should be culled before the next mating.

Those left in charge of lambing ewes should receive proper training from their local agricultural training organization or veterinary surgeon on how to recognize and assist ewes having difficulty lambing and how to care for neonatal lambs. Try to observe as many lambings as possible and only interfere if absolutely necessary. Strict hygiene precautions must be taken when assisting ewes to lamb to prevent uterine infections (metritis) and transmission of zoonotic infections such as enzootic abortion. If initial attempts to lamb the ewe fail, seek veterinary assistance without delay. After every lambing, hygienically check ewes' udders for adequate milk and the presence of mastitis. All lambs should receive at least 50 ml colostrum within the first few hours of life. If there is any doubt about the ewe's ability to provide colostrum or the lamb's ability to suck, supplement natural feeding with a bottle or stomach tube using fresh or frozen ewe or cow colostrum.

Lambs suffering from hypothermia will need to have access to suitable warming facilities and, in cases where starvation has also occurred, may require 20% glucose supplementation given by intraperitoneal injection. Dress the lamb's navel with tincture of iodine as soon as possible after birth to prevent navel infections and joint ill. Where vaccination against orf (contagious ecthyma) is advised, lambs may be vaccinated within the first week of life (**Table 4**). Lambs that are to be retained for rearing either for fattening or as dairy ewe replacements should always be fully vaccinated against clostridial diseases and, if

Table 4 Examples of vaccines and vaccination regimes for use in dairy flocks

Lambs born to vaccinated dairy ewes requiring protection against clostridial diseases only	*Lambs born to vaccinated dairy ewes requiring protection against clostridial diseases and pasteurellosis*	*Lambs born to unvaccinated dairy ewes requiring protection against clostridial diseases only*	*Lambs born to unvaccinated dairy ewes requiring protection against clostridial diseases and pasteurellosis*	*Replacement dairy breeding stock and adult dairy ewes and rams*	*Unvaccinated adults (e.g. purchased animals in quarantine)*
Colostrum protection	Colostrum protection	No colostrum protection	No colostrum protection	Booster-vaccinate ewe replacements 12 months after second lamb vaccination	Give two vaccinations 4–6 weeks apart using a clostridial vaccine or combined clostridial and *Pasteurella* vaccine
Give first clostridial vaccine at 8–12 weeks	Give first clostridial and *Pasteurella* vaccine from 3 weeks of age[a]	Give first clostridial vaccine in first week of life	Give first clostridial and *Pasteurella* vaccine in first week of life, followed by second *Pasteurella* vaccine at 3 weeks of age	Annual booster vaccination for all breeding ewes given 4–6 weeks before lambing to produce maximal antibody levels in colostrum for passive protection of lambs	**Other vaccines that may be administered to sheep as required** Enzootic abortion and *Toxoplasma* abortion vaccination of ewes and ewe replacements premating
Give second clostridial vaccine 4–6 weeks after first vaccination	Give second clostridial and *Pasteurella* vaccine 4–6 weeks after first vaccination	Give second clostridial vaccine 6 weeks after first vaccination	Give second combined clostridial and *Pasteurella* vaccine 6 weeks after first vaccination	Annual booster vaccination of all rams and ram replacements	*Escherichia coli* and erysipelas vaccination of breeding ewes prelambing to provide passive protection against watery mouth and joint ill in lambs
If pulpy kidney is a major problem, lambs may need an additional clostridial vaccine booster in the autumn	If pulpy kidney is a major problem, lambs may need an additional clostridial vaccine booster in the autumn	If pulpy kidney is a major problem, lambs may need an additional clostridial vaccine booster in the autumn	If pulpy kidney is a major problem, lambs may need an additional clostridial vaccine booster in the autumn	If there is a high incidence of pasteurellosis, a supplementary *Pasteurella* vaccination may also be required 2–3 weeks before expected seasonal outbreaks	Footrot vaccination of ewes and rams Orf vaccination of ewes prelambing and lambs in first week of life Louping ill vaccination of susceptible ewes and lambs

[a] Lambs born to ewes vaccinated against clostridial diseases and *Pasteurella* but at high risk of developing pasteurellosis or lambs born to ewes previously vaccinated against clostridial diseases only but requiring immediate protection against *Pasteurella* may also require an additional vaccination against *Pasteurella* in the first week of life.

necessary, pasteurellosis. Timing of primary and booster vaccinations depends on the presence or absence of passive immunity obtained via ewe colostrum. (**Table 4**)

Lactation

Check dairy ewes' udders and milk for the presence of mastitis daily before each milking throughout lactation, as mastitis incidence may increase both at the start and at the end of lactation. If clinical mastitis is detected, intramammary and parenteral antibiotic treatment should be started as soon as possible to save the ewe and to have any chance of preserving future milk production. Pretreatment milk samples should be taken into sterile containers to allow bacteriological culture and antibiotic sensitivity testing. If appropriate laboratory services are available, adopt monthly bulk milk testing to monitor total bacterial counts (TBCs) and somatic cell counts (SCCs) as general indicators of milk hygiene and subclinical mastitis. Use individual animal sampling procedures such as examining foremilk in a strip cup and the California mastitis or ZAL-pine tests, which give a qualitative indication of SCCs in the milk, to detect the presence of subclinical and early clinical mastitis.

If mastitis incidence or bulk milk TBC and SCC results increase suddenly, review milking hygiene procedures such as pre- and postmilking teat preparation as well as overall hygiene of milking equipment. Examine ewes' teats for lesions and abrasions, often associated with orf infection, which can harbor large quantities of mastitis-causing bacteria. Examine teat ends carefully for signs of damage, allowing bacterial entry, which may be caused by poor milking technique or poorly serviced automatic milking equipment. If all other causes can be ruled out, start 'dry-ewe therapy' involving the instillation of long-acting intramammary antibiotics at the end of lactation to reduce the risk of infection being carried over into the next lactation. When using long-acting intramammary preparations, make sure they are administered far enough in advance of lambing to avoid the risk of antibiotic residues still being present in the milk once lactation starts. Monitor ewe feed intake throughout lactation and ensure they are fed on a well-balanced diet to maximize milk yield and maintain body condition.

Premating

Every dairy ewe should be dried off and physically examined at least 2 months before mating to ensure that she is fit to be retained for another year's production. The examination should involve a careful assessment of the ewe's body condition and general health, checking udders for evidence of chronic mastitis and teeth for 'broken' mouth. Examine all feet for signs of ovine interdigital dermatitis or footrot and treat the whole flock as required. Individual records should also be consulted for milk production, health and reproductive information. If deemed fit, ewes should be 'flushed' on good grazing or with supplementary feeding for at least 4 weeks before mating to ensure they attain the required body condition to ensure high conception rates. Vaccinate ewes against enzootic abortion and toxoplasmosis at least 4 weeks before mating if these diseases have occurred in previous years and control by vaccination has been advised (**Table 4**).

Pregnancy

Maintain dairy ewe body condition throughout pregnancy and avoid stress-induced early embryonic death by minimizing all unnecessary treatments and handling in the first 2 months. If possible, ewes should be ultrasound-scanned at about 70–90 days of gestation to determine pregnancy rates, expected lambing dates and the numbers of lambs carried by each ewe. Scanning information allows ewes to be grouped according to nutritional requirements for the remainder of the pregnancy and also gives an early indication of any reproductive problems that may be caused by infertility in the ewes or the rams or the presence of infectious abortion agents that may cause early fetal death and resorption. If ewes are to be housed in late pregnancy, ensure all footrot problems are brought under control and consider worming before bringing inside. However, avoid carrying out excessively stressful handling procedures and treatments that may cause ewes to abort or develop metabolic disorders such as pregnancy toxemia and hypocalcemia.

Calculate required levels of supplementary concentrate rations needed and start feeding in increasing amounts over the last 8 weeks of pregnancy. Ensure feeding levels are adequate and avoid inducing starvation, even for short periods, as this can also lead to pregnancy toxemia. Vaccinate ewes against clostridial diseases and, if necessary, against pasteurellosis, *E. coli* and erysipelas 4–6 weeks before lambing to ensure continued protection for the ewes as well as maximal protection for the lambs via antibodies in colostrum (**Table 4**). If orf infection is a significant problem among the ewes on the farm, it may be necessary to vaccinate ewes no later than 8 weeks before lambing to reduce lamb exposure to the virus (**Table 4**).

Nonlactating Dairy Stock

Dairy Ewe Replacements

All nonlactating stock, including ewe replacements, rams and lambs, need routine protection against ectoparasites throughout the spring and summer. Shearing of all stock, including lactating ewes, should be performed before summer conditions become too warm, using equipment that has been properly disinfected to prevent the spread of infectious skin conditions, including CLA, sheep scab, lice, orf and ringworm. Ewe replacements will require additional clostridial and *Pasteurella* booster vaccinations at 12 months of age to maintain protection until their first prelambing booster and will also need to be inspected and flushed premating along with the lactating ewes. Vaccination against infectious abortion should also be carried out at this stage if required (**Table 4**).

Dairy Rams

Rams should receive a thorough physical examination at least 2 months before mating. Any animals deemed to be unsatisfactory that were not disposed of at the end of the last breeding season should be culled. Examinations should focus on overall body condition, teeth, feet and reproductive organs. If any abnormalities are found when examining the testicles or the penis or if there is concern about the animal's previous reproductive performance, then a fertility examination, involving semen collection and assessment, should be arranged with the veterinary surgeon.

Lameness can severely inhibit a ram's ability to serve ewes and particular attention should be paid to treating and controlling foot problems. Rams should be foot-pared, foot-bathed and, if necessary, vaccinated against footrot before mating starts (**Table 4**). Rams will also benefit from flushing premating and sufficient numbers should be available to allow a ram-to-ewe ratio of no more than 1:30 for experienced rams and 1:15 or 1:20 for ram replacements. If ewe estrus synchronization methods are used, then this ratio will have to be reduced still further to 1:10.

Purchased or borrowed rams should undergo full quarantine procedures as for other stock and should always be blood-tested and possibly semen-tested before use to check for diseases such as border disease, which can be spread to ewes by the venereal route. Outside specific health management for mating, rams should also be included in all vaccination, anthelmintic and ectoparasite control programs, as for other nonlactating stock.

See also: **Analytical Methods**: Microbiological. **Dairy Farm Management Systems**: Sheep. **Diseases of Dairy Animals**: Infectious Diseases: Bluetongue; Infectious Diseases: Foot-and-Mouth Disease; Infectious Diseases: Johne's Disease; Non-Infectious Diseases: Grass Tetany; Non-Infectious Diseases: Milk Fever; Non-Infectious Diseases: Pregnancy Toxemia; Parasites, External: Mange, Dermatitis and Dermatosis; Parasites, External: Tick Infestations; Parasites, Internal: Gastrointestinal Nematodes; Parasites, Internal: Liver Flukes. **Husbandry of Dairy Animals**: Sheep: Feeding Management; Sheep: Milking Management; Sheep: Multipurpose Management; Sheep: Reproductive Management. **Mammary Resistance Mechanisms**: Anatomical; Endogenous. **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens. **Milk**: Sheep Milk. **Milk Quality and Udder Health**: Test Methods and Standards. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Office of International Epizooties**: Mission, Organization and Animal Health Code.

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Sheep: Milking Management

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Sheep Dairying

Sheep have been used for milk production probably longer than any other mammal. They are easy to handle, graze, have wool and meat as by-products and produce a milk exceptionally high in the elements required for healthy living. The high total solids in the milk make it ideal for cheesemaking, thick yogurt and ice cream (*see* **Milk**: Sheep Milk). A wide variety of products can be made: yogurt, cheese and ice cream are made from the milk and the whey is used for ricotta and sweetmeats. In the United Kingdom, the milk is bottled for drinking.

Today, sheep can be milked economically in large numbers, and by careful genetic selection some breeds can produce ewes able to give yields of up to 1000 l in a lactation. Rams from such ewes provide a high genetic potential yield in their daughters.

Sheep Suitable for Milking

All sheep will give milk after lambing, but only some breeds can be used in commercial sheep dairying. Most ewes are left to rear their lambs for 35–60 days after birth before being milked (*see* **Dairy Farm Management Systems**: Sheep). With high-yielding ewes, especially the Friesland, lambs can be taken away soon after birth (0 to 4 days) and artificially reared while the ewes are used for milking.

The main dairy breeds are:

- Friesland (East Friesian)
- Awassi
- Assaf (cross between the Awassi and Friesland)
- Lacaune
- Sarda
- Manchega
- Chios.

There are many other pure and synthetic breeds, mainly developed in the Mediterranean basin, which suit the climate and existing conditions. Yields and lactation lengths of most dairy sheep quoted in the literature vary enormously (*see* **Animals that Produce Dairy Foods**: Sheep Breeds).

The selection of ewes for milking or culling of ewes should follow very specific guidelines. Ewes should be culled that:

- kick
- are slow milkers
- have recurrent mastitis
- are so nervous that they go dry within a few days of initiation of milking
- have unsuitable temperaments and badly shaped udders as these take extra time and tend to make the operator tired and bad tempered.

Udder Shape

The morphology or genetically determined udder shape is of great importance in commercial sheep milking (see **Figure 1**). Dr G. Caja has demonstrated that sheep whose teats are situated near the base of the udder not only evacuate the milk faster but also give more milk. He maintains that selection for yield alone will lead to pendulous udders, making them difficult to milk by machine. Culling such ewes can be a great economic loss initially as they give the most milk, so where the udders are so misshapen that the cups will not attach snugly (i.e. the teats are up the side of the udder) use of a Sagi hook will greatly facilitate milk evacuation (see **Figure 2**). The goat-shaped udder with the teats facing forward is another udder shape which is particularly difficult to machine-milk. Ewes with this type of udder should not be bred from. Conical teats tend to be less prone to teat creep than cylindrical teats (finger shaped) and are therefore less prone to some forms of mastitis.

Unit Size

In the United Kingdom and countries where most of the ewes milked are either Friesland, part Friesland, or improved breeds like the Lacaune, Sarda, Manchega, etc., there are two distinct ways of utilizing their milk:

1. For on-farm manufacture of the milk, the unit size varies from 80 to 1000 ewes according to the product, skills and time of the product maker, and the market.

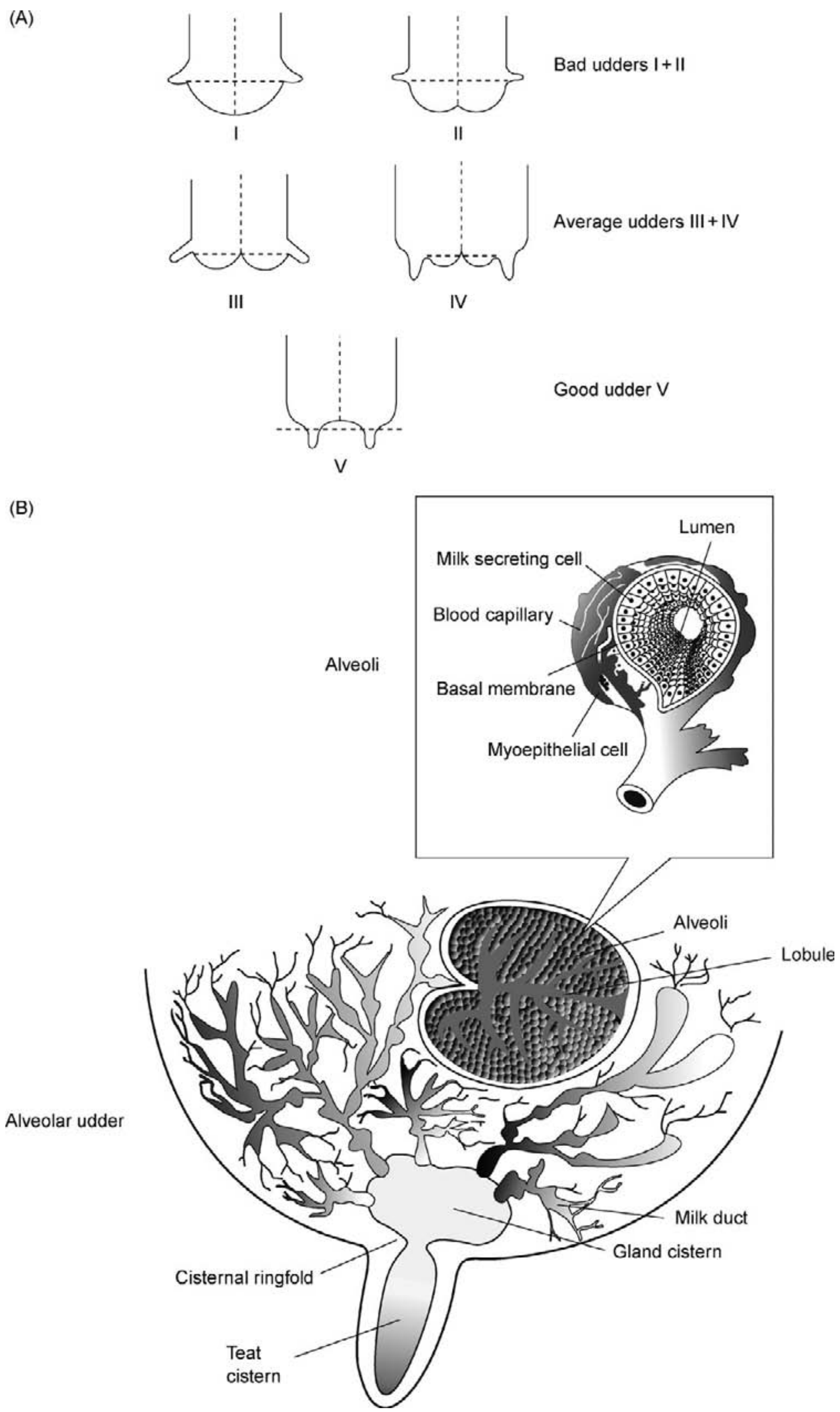
(A)
Bad udders I + II
I
II
Average udders III + IV
III
IV
Good udder V
V
(B)
Lumen
Milk secreting cell
Blood capillary
Alveoli
Basal membrane
Myoepithelial cell
Alveoli
Lobule
Alveolar udder
Milk duct
Gland cistern
Cisternal ringfold
Teat
cistern

Figure 1 (A) Types of sheep udder; (B) sheep udder anatomy.

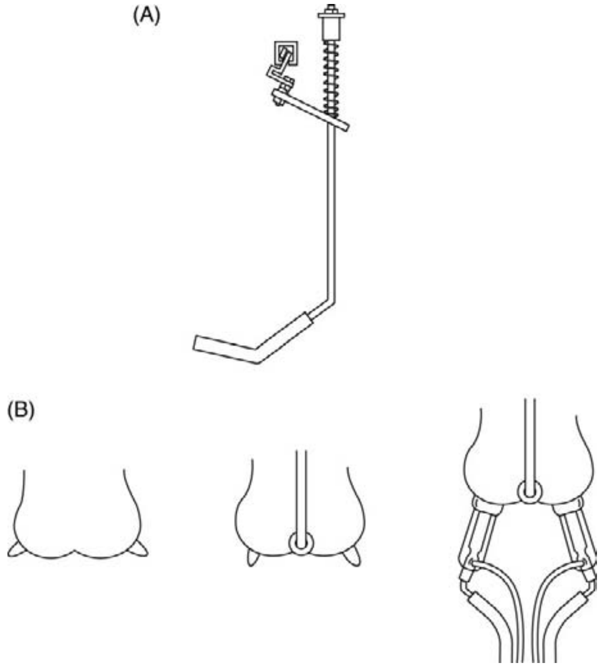

Figure 2 (A) Sagi hook; (B) a Sagi hook in use.

2. For the production of milk alone, a viable unit size starts at about 400 and goes up to 1500 ewes.

It is now common practice for ewes to be milked all year round, so the flocks may be split up into three or four units to maintain the continuous production of lambs and subsequent milk, with the optimum number being milked at any one time.

In other countries, especially where yields are lower, much greater numbers of ewes may need to be milked; however as most of these countries have a Mediterranean climate, milking year round is considerably easier provided labor is cheap and plenty of feed is available.

Yields

Yields vary from breed to breed and from one farm to another (see **Table 1**). Feed as well as genetic selection plays a big part. Most producers tend to use the short cut to greater milk production by crossing their local breed with the Friesland. Although good results can be produced in the short term, a selected and improved pure breed or a scientifically developed synthetic breed may be more reliable in the long term. It would appear that 200–250 l per lactation is the lowest viable yield in modern commercial sheep dairying. However, where there is a good available market for the lambs and the ewes are only milked for the 'third profit' (i.e. after the profit from meat and wool), lower milk yields can be compensated for by the high value of the lambs.

Table 1 Milk production of various breeds in 1990

Breed	*Lactation length (days)*	*Milk yield (liters)*
Friesland	153–260	148–600
Awassi	144–260	104–350
Assaf	100–117	192–287
Lacaune	143–155	143–165
Manchega	150	135
Chios	172–197	151–205

Reproduced with permission from Sukul (1991) *Dairy Sheep Symposium*. University of California.

The 1000 l per lactation ewe is at the top end of the Friesland, Assaf and Awassi breeds, most breeds achieving less than half that amount. Sadly, most ewes are seldom milked to their genetic potential due to poor management, poor or unsuitable feed or climatic conditions. Therefore, giving yield figures for a breed can be very misleading. More information on this can be obtained from the breed society or Flock Book.

The Lactation Curve

Most ewes reach their peak of production at around 35 days and from then on to the end of the lactation there is a steady decline in milk yield. This decline can be rapid or slow according to the breed, nutrition and frequency of milking.

Lactation Length

The lactation length of ewes varies considerably, and some milk recording programs have set lactation length as a comparison tool only. However, anything from 150 to 240 days is to be found as a typical lactation length, while some good ewes will milk for well over a year before having to be bred again, somewhat like goats.

Milking

There are advantages and disadvantages for both hand- and machine-milking. Circumstances vary from country to country but the three main deciding factors are:

- cost of labor
- availability of clean water and electricity
- rapid cooling facilities.

Machine-milking is not necessarily cleaner than hand-milking. The machine-milking equipment must always be kept well cleansed and sterilized. Correct vacuum levels and perishable parts of the equipment should be periodically checked.

Machine-Milking in Parlors

The Parlor

Machine-milking for sheep has been available since the early 1930s, but it has evolved over the years as labor and time have become more costly. The machine-milking equipment and parlor must suit the number of ewes that can be milked within 2 h by any one operator. This will prevent tired operators from leaving cups on for too long or neglecting to detect mastitis.

The principle is the same for all milking parlors. The batch of ewes come in, line up and the cups are put on, and when the milk has all been evacuated the cups are taken off and put on to other ewes in the row. The ewes then all leave to be replaced by the next batch. There are many varieties and improved versions of parlors being researched all the time. The height of the platform is very important as the operators should not have to bend their backs while putting the cups on, but at the same time must be able to see the cups on the teats (see **Figure 3**). Rotary parlors (see **Figure 4**), abreast, double abreast and one behind the other passages have all been developed into very sophisticated semi-automated milking equipment. Almost all parlors are much like miniature cow milking parlors (*see* **Milking Parlors**), but milking machines have two teat-cups per cluster instead of four.

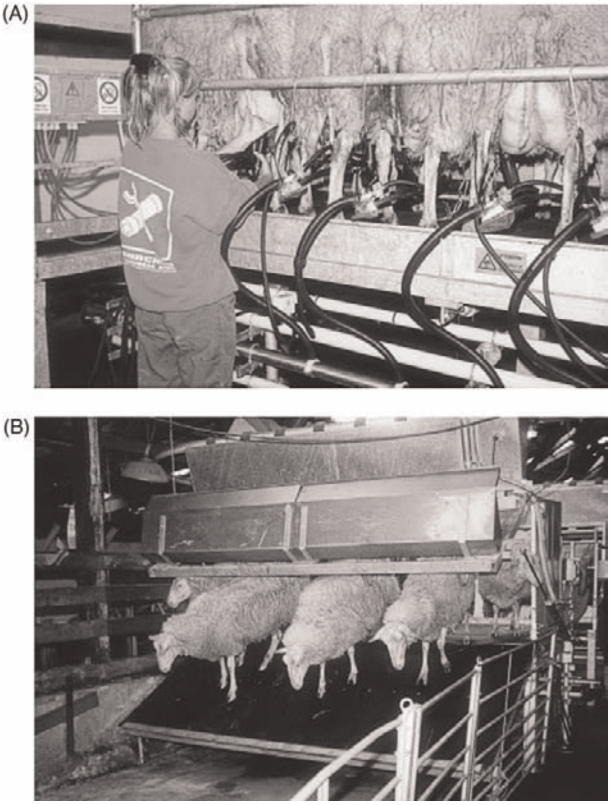

Figure 3 (A) View of one of the new generation of small parlors showing an excellent height for the operator to work at with typical good Friesland udders. (B) The ewes exit down a steep ramp in order to hasten the time taken to fill and empty the platform.

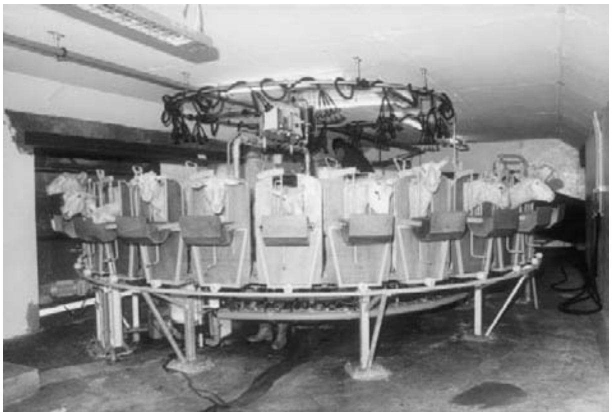

Figure 4 One of the smaller rotary parlors where the operator stands inside the circle.

With the original ‘system casse’ parlor invented by Alfa Laval (**Figures 5** and **6**), the ewes come in and line up on a platform, find a place along a feed trough and are then wheeled back in front of the operator to be milked. With the rotary parlor, the ewes enter, are milked while on board the carousel and then step off at the conclusion of the circle. There is a wide variety of other ideas, all seemingly designed to speed up the milking time. There are both high-line and low-line parlors. The main advantage of high-line is that with a double abreast parlor, the cups can swing over to the opposite side after the first line of ewes have been milked, allowing the second side to be milked while the first side empties and refills with sheep. This is a cheaper option as only one set of cups is needed to milk both sides. Usually a set of cups is used for every one or every other ewe according to the system.

Yokes or restraints are optional. The more amenable breeds of sheep may not require any form of restraint and can be packed into a line along a feed trough and held in place by a cross bar, which is adjustable for the difference in numbers in any one row and the difference in width between shorn and full-wool sheep.

For illustrations of a 6-point standing and a 12-point standing platform see **Figure 7**.

Automatic Cup Removers

Perhaps one of the more useful inventions of modern times has been the automatic cup remover (ACR). This allows one operator to put the cups on, which are then automatically removed, either by the vacuum being cut off at a prescribed point in the rotary or at a set time, and pulled back away from the ewe to a clean and safe

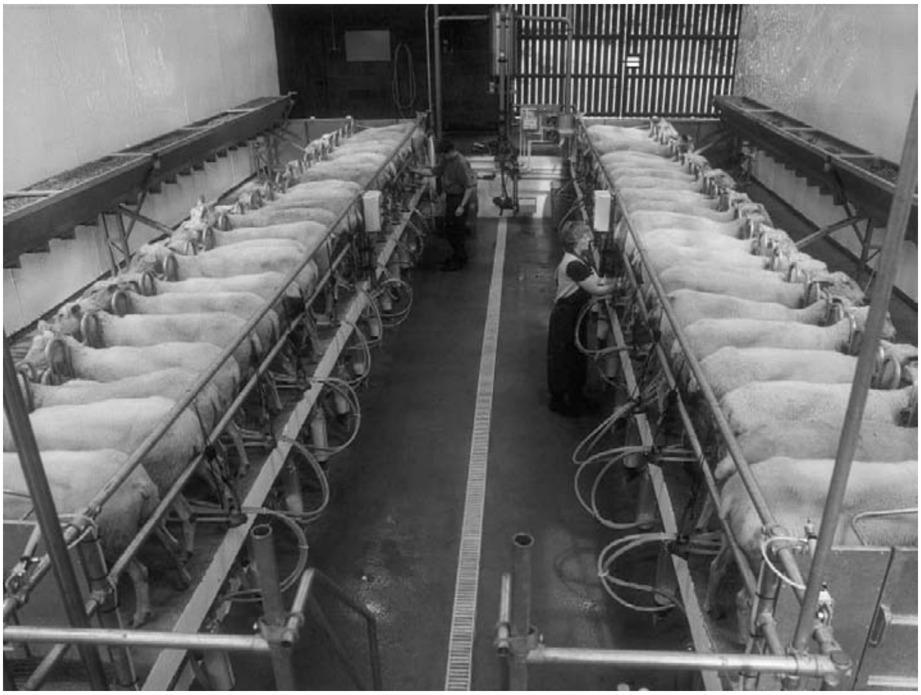

Figure 5 An Alfa Laval double abreast system casse milking parlor, showing automatic feed troughs, individual milk recording jars and low-line milking.

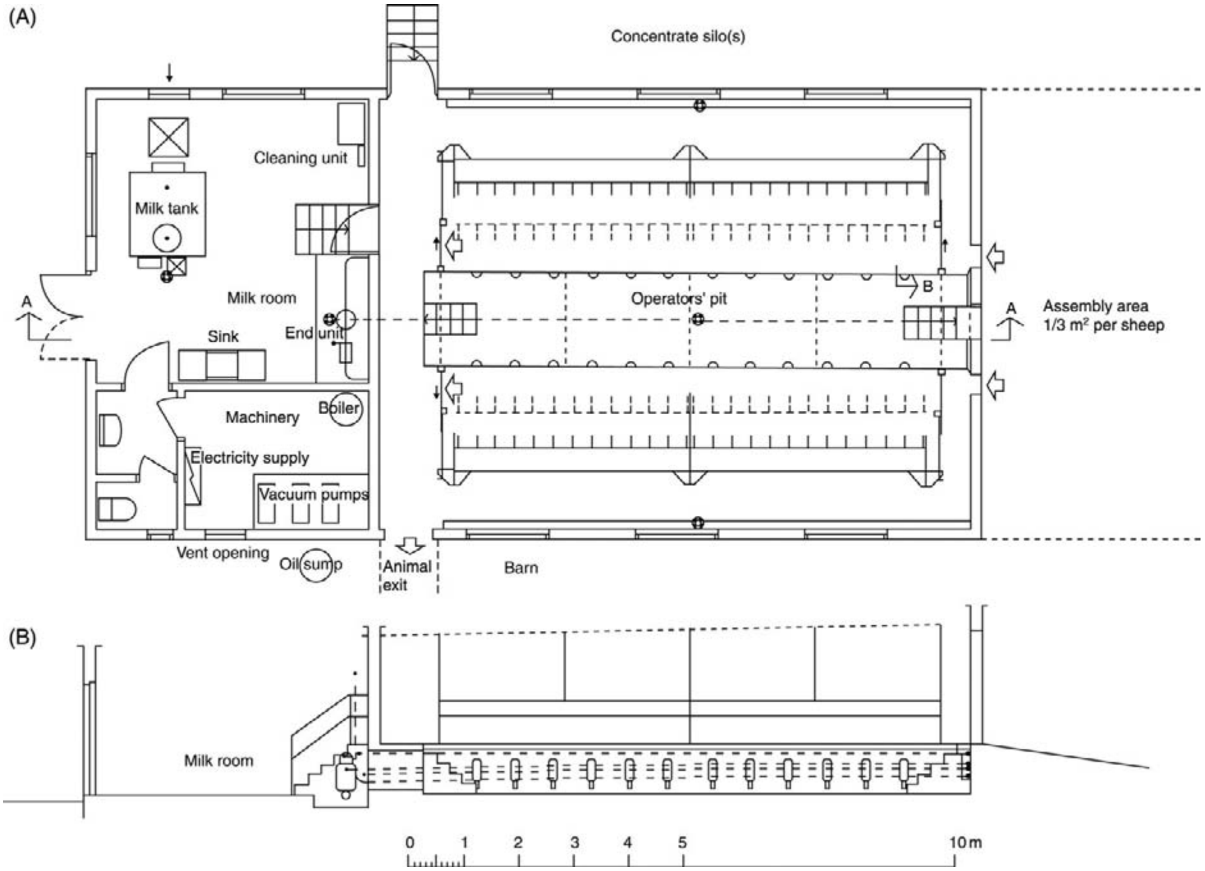
(A)
Concentrate silo(s)
Cleaning unit
Milk tank
Milk room
A
Sink
End unit
Operators' pit
B
A
Assembly area
1/3 m² per sheep
Machinery
Boiler
Electricity supply
Vacuum pumps
Vent opening
Oil sump
Animal exit
Barn
(B)
Milk room
0
1
2
3
4
5
10 m

Figure 6 Parallel stall 2×24 places milking parlor, with recorders. (A) plan view; (B) section A–A.

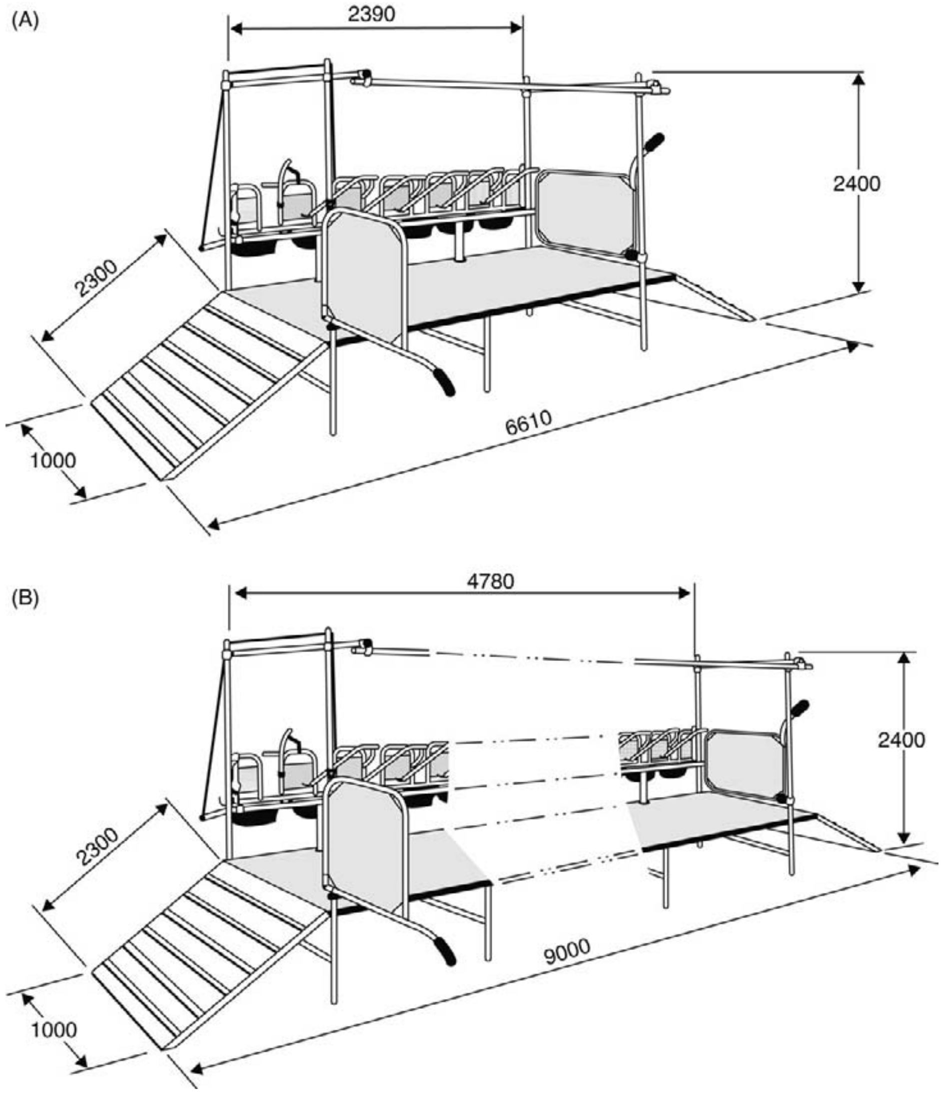

Figure 7 A 6-point standing (A) and a 12-point standing (B) milking platform.

position. These ACRs are usually only fitted in large parlors and there are a number of different versions. Problems can arise when ewes of differing milking capacity are in the same flock, when the cups are left on too long, or pulled off too soon. However, the grouping of ewes in equal yield batches is perfectly feasible.

Feeding

Feeding in the parlor is optional and is a matter of choice and management. Many producers believe that the sheep will not come in to be milked without the incentive of food, but this was also thought to be the case with cows. Milking is much faster without feeding and the ewes will happily go out to be fed in a yard after milking. The ewes need to drink water immediately after eating and should not be disturbed while doing so.

Milk Recording

Milk recording is an important tool in modern milking, but can be very labor intensive. Highly computerized methods are in use, but for the smaller dairy manager, the use of milk jars in the line are usually the preferred method; they are either in place permanently or just on recording days. Milk meters for sheep are also available and some types have been shown to be very accurate.

The Milking Equipment

The main items of equipment for machine-milking, common to all types of parlor, are the vacuum pump, the pulsator, and the shells and liners (cups or cluster) (*see* **Milking Machines**: Principles and Design).

Vacuum levels

Vacuum levels tend to vary from country to country, especially if there is no automatic cut-off of the vacuum should the cups be kicked off. A lower vacuum level than that for cows or goats is most suitable for sheep. The 'claw' is used to control the vacuum to the cups and acts as an attachment for the cluster to be suspended from. The prescribed mean vacuum at the claw is between 32 and 40 kPa.

The vacuum levels on the gauge measure the:

- high-line range from 37 to 42 kPa
- low-line range from 34 to 38 kPa
- ratio 50:50.

It used to be thought that higher vacuum levels would 'glue' on the cups and they would not be kicked off with subsequent loss of vacuum, but this has also been shown to damage the thin udder tissue and cause bruising, teat-end erosion, etc., leading to mastitis. Now, greater vacuum capacity as well as automatic cut-off of the vacuum if the teat-cups come off has solved the problem.

Pulsation

A lamb suckles at 180 pulses per minute (ppm), but until recently, most pulsators for sheep were set at 110–120 ppm. The new generation of electronic pulsators are set at 180 ppm and are found to be faster, less stressful for the ewes, more efficient at evacuating the milk from the udder and show a lower somatic cell count (SCC).

Air bleed

The air bleed should be set to allow 7 l min^{-1} to be milked out.

Cups

The cups come in two lengths. The standard length is suitable for most sheep and a longer version is available for exceptional dairy ewes, such as the Assaf and the Friesland, which have soft udders and longer teats. With a shorter teat-cup, the teats can suffer from teat-end erosion and there may not be enough space at the end of the teat to evacuate the milk. Today, only a few companies manufacture cups, most of which are very light in weight and easy to clean.

Sagi hook

This metal hook suspended on a spring was developed by Professor Ram Sagi in order to lift the center of large udders, so that the teats point downwards making it easier for the cups to fit and to empty the cistern of milk (**Figure 2**).

Hand-Milking

Hand-milking can be very efficient, clean and fast, but usually requires operators whose hands and backs are accustomed to the work. The ewes must be restrained in lines, or close penned. The restraints can be in a milking parlor situation with the ewes yoked at the right height for the operator to milk without bending his back, or at ground level as seen in places like Sardinia where the operator can either stand or sit, or bend over the ewes and milk backwards toward a bucket. In some places, sheep are still milked by hand with the operator sitting on a small stool. Almost all hand-milking is done through the back legs and not from the side as with a cow. For the sake of hygiene, it is important that surplus wool and hair is removed from the back end of the sheep and the receptacle used to collect the milk has, if possible, a hooded cover like those used in the past for the hand-milking of cows. Although extraneous material in the milk can be filtered out afterwards, in this modern culture of extreme hygiene, health inspectors can object to such forms of milking.

General Points

Milking Hygiene

To maintain high standards of hygiene (*see* **Milking and Handling of Raw Milk**: Milking Hygiene), the following should be considered:

1. The operator should wear clean and waterproof clothing.
2. The area of the udder must be kept free of hair and wool either by crutching or shearing.
3. Fear may cause ewes to urinate.
4. Milklines and teat-cups must be renewed at regular intervals (once a year) and access by flies or dust into the milkline prevented when not in use.

The following should be watched out for:

- delayed afterbirth; this is unpleasant for the operator
- excessive diarrhea due to worms; this is a serious hygiene hazard
- dirty wool around teat area; this should be clipped away before milking
- orf on teats (usually the result of suckling) which is highly contagious to the operator
- abscesses, often benign tumors, at the front of udders which can burst unseen
- early onset of mastitis; often detected by the operator handling the udder prior to milking
- poor udder shapes, which can allow vacuum leak at the teats and cause bruising

- large awkward udders needing a Sagi hook (see **Figure 2**)
- uneven halves of the udder, either due to only suckling one lamb or to subclinical mastitis in one half.

Washing of Udders

The washing of udders is not practised in most countries as it is slow and in very dry conditions not necessary. However, if ewes are kept out at pasture, and especially if out at grass in early lactation, the udders may well require washing and drying. Washing should be carried out with either hot water and chlorine or an impregnated damp cloth. Wiping or washing of the udders results in a certain amount of udder stimulation, which provides a stimulus to the udder to encourage milk letdown. Handling of the udder during washing should also indicate to a skilled operator the onset of mastitis, etc.

Taking the Foremilk

In some countries, there are regulations to make this obligatory, but in fact it is seldom done if the udders are wiped or checked prior to milking, as the few milliliters held in the teat are evacuated automatically. Where this procedure is mandatory, it involves pulling the foremilk from each teat into a receptacle for inspection; the foremilk should not be released onto the platform.

Milking Technique

Putting on the cups should be a quick operation without undue escape of vacuum. It is important to realize that the vacuum will act like a vacuum cleaner and the cups can suck in extraneous matter from the back end of the ewe if application is not swift and accurate. Where ewes have awkwardly shaped udders, a Sagi hook is invaluable as it lifts and divides the udder, bringing the teats to the base of the udder so that the cups can fit correctly with no drag. In early lactation and for ewes with pendulous udders, without the benefit of a Sagi hook, many ewes would have to be culled.

The maximum amount of time for the cups to be attached is 1.5 min. If the ewe is a slow milker or has an exceptional amount of milk, the cups should be removed to give her teats a rest from the suction for a few seconds before reapplying them. The vacuum should always be turned off when taking the cups off. The teats, in many breeds of sheep, have delicate skin and the rough pulling off of cups can cause pain and damage leading to mastitis and kicking.

Hand or Machine Stripping

It is common practice to try and evacuate the udder completely. Some ewes have what appears to be two peaks during milking at the start of the lactation (the second peak is called the second letdown) or hold back milk for their lost lambs. Hand stripping is not to be encouraged due to the hygiene aspect, but putting some light pressure on the base of the cluster before taking the cups off may be enough to make sure all the milk has been evacuated. The cups can be reapplied before exiting if the udder has filled up again.

Handling of Raw Milk (Cooling and Storage)

Once milked out, the milk must be cooled rapidly and held in a bulk tank at below 4 °C with fairly constant agitation (every 20–30 min) to prevent a fat layer from forming (*see* **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality). When held at about 1 °C the milk can be bulked up for almost a week. The fat layer can provide an environment for the collection of spoilage organisms and it should be kept broken up while in storage. If no bulk tank is in use, the milk must be cooled rapidly by other methods, e.g. placing it in churns in ice cold water, misting the air around the churn with a fine spray of water, etc. In-churn coolers and other methods are also available. Precooling is frequently needed, especially when adding fresh milk to that which is at least 12 h old, since the length of time taken to cool the milk is vital to its keeping quality.

If milk is to be stored frozen it should be held for 12–24 h at below 4 °C to allow the fat globules to heal after milking; it should then be frozen in 5–10 l blocks. It is best to freeze the milk at −30 °C using a blast freezer with a fan.

Cleaning the Equipment

Sheep milk with its high fat content is vulnerable to attack by many lurking bacteria, which is usually found in the pipework of the milking parlor or bulk tank. Dust and air yeasts from feed should be avoided in the milking area. The following should be carried out:

1. Rinse the whole plant through with tepid, not cold, water and let the water run to waste.
2. Wash through all equipment with a suitable caustic cleaner or acid boiling water.
3. Rinse through with clean chlorinated water and allow to dry out.
4. Remove milkstone about once a month.

The source of water is as important as the washing itself and it should be tested for contaminants. The final rinse must always contain chlorine.

Frequency of Milking

The number of times per day ewes are milked depends largely on the type of ewe, cost of staff and dairy requirements.

1. Where lambs are taken off the ewe after the first 4 days, twice or thrice a day milking will stimulate more milk for a longer lactation length.
2. Where lambs are left on dairy ewes for 35 days or more, twice a day milking is essential for the first month or so and then once a day can be practised if they are higher-yielding ewes.

Normally dairy ewes are milked twice a day until they start to go dry and then milked once a day until drying-off is achieved. Sudden drying-off is practised, but has many disadvantages including the danger of mastitis. All lambs can be artificially reared if milk production is the main aim of the enterprise.

However, some dairies milk three times per day in early lactation to cover the peak period of milk production. This prevents the udders from becoming pendulous and provides more milk per ewe per day. Some farmers switch to once a day after 2–3 months with high-yielding ewes to save money on staff and electricity. In the Carpathians, for example, the ewes are milked three times a day as labor is cheap and the ewes would not give enough milk without constant demand (stimulus). There is evidence that if labor is cheap, milking three times a day will produce a higher lactation yield provided this also involves extra feed.

Expected Yield (Daily and Lactation)

Using the known curve of the milk lactation graph, calculations on the daily yield can give an indication of the total lactation yield. Computerized milk recording can estimate from the daily yield together with the number of days in milk the total lactation yield, but can only do so by referring to the lactation curve built into the recording package. Milk recording taken to 150 days of lactation with three recordings may only be used as a comparative tool. For the total lactation yield, recording must continue for 200+ days with at least six recordings for the improved breeds.

There is often a considerable difference between the genetic potential yield and the actual yield due to breeding, feeding and management, and also to the management before, at and after lambing. This can depend on:

- the date lambs are taken off
- length of lactation
- time of year
- number of hours of light
- general health of flock.

Yield can also depend on:

- time of year the ewes lamb down
- whether milking is toward or away from maximum light hours
- whether night light stimulation is used
- ambient temperature
- availability of water
- excellent understanding of nutrition.

Although tables exist on the expected yield of most breeds of sheep around the world, the area, type of feed and management are usually not taken into account. When taken to other parts of the world, many breeds produce milk differently. Even different operators can affect the milk yield.

Mastitis Detection

Mastitis detectors can be put in the milkline, but often are of limited value for ewes, as the mastitic clot is very short-lived and slips through unnoticed in the early stages (*see* **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk). Much more valuable is hand feel, which is the surest method of detection, but takes a while to develop. The following should be noted:

- any change of temperature, whether hot or very cold
- reddening of the udder
- lumps or soreness to the touch
- the look of the ewe, since mastitis is painful.

Pulling of the foremilk may also give some indication:

- there may be clots in the milk
- it can have changed consistency to a watery substance
- it can have a thick creamy consistency
- it may be pink. (Pink milk does not always indicate mastitis; it may be due to a broken blood vessel in the udder as a result of overstretching in early lactation or an injury. Although blood in the milk may not be related to mastitis, it should not go into the bulk tank.)

When in doubt a Californian mastitis test kit (CMT) can be used and the milk discarded (*see* **Mastitis Therapy and Control**: Medical Therapy Options). The mastitis should be typed and the ewe treated as quickly as possible under veterinary instruction. Treatment by any form of antibiotic must be watched carefully as the withdrawal times are longer for sheep milk than for cows (*see* **Mastitis Therapy and Control**: Medical Therapy Options). The presence of antibiotics in the milk can be a very expensive problem to the milk processor as well as the producer.

Milk Hygiene Testing

Milk should be tested regularly, both from the bulk tank and individually (*see* **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality). Somatic cell counts are one of the best indicators of subclinical mastitis which may not show any symptoms during milking but can reduce the cheese yield. A sure indicator is when the udder is uneven to milk half way through the lactation and may or may not lead to full-blown mastitis at any stress point. The ewe should always be checked at drying-off, in case she develops mastitis at her next lambing.

Mastitis can be expected in ewes that have been suckled by lambs for any length of time, as the lambs carry *Pasteurella haemolytica* on their tonsils.

Any period of stress can produce mastitis in sheep, such as:

- weaning or separating ewes from lambs
- shearing, if the weather is cold afterwards
- dipping when the ewe is in full flood of milk
- very adverse weather conditions.

Extra feed at these times has been shown to help the ewe over the crisis. Sheep are quick to pick up on the mood of the operator; rough handling or bad temper can stress ewes and also cause lower milk yields and mastitis.

Lambing and Milking Year-Round

In today's world, where cheese factories and consumers expect milk to be provided fresh all year round, lambing at several specific times in the year is required with overlapping lactations. To achieve this, ewes need to be kept on a level plane of nutrition, which usually involves being housed while lactating, going out for an hour or two a day for exercise and the opportunity to graze on fresh grass. The following techniques are also used:

1. Light can be used to manipulate estrus and increase milk production in winter months.
2. Ewes can be synchronized by the use of vaginal sponges impregnated with progestagens to aid lambing out of the normal season.
3. Ewes can be remated while still milking if well fed, and should always be shorn, regardless of the time of year, 6 weeks before lambing.

Future Prospects

Sheep dairying is an ecological form of farming. Nowadays, sheep are easy to milk, do not cost much to buy, multiply fast and their milk with its high total solids gives an exceptional cheese yield. They can be induced to produce milk year-round and can live in most environments in the world. Local breeds can be improved by crossing with the specialized dairy sheep breeds, e.g. Friesland, Assaf, Lacaune.

Consumers perceive sheep milk products as organic and natural compared with those from cows' milk. The milk is bland tasting and although higher in fat, this fat is lower in saturated fatty acids than cows' milk and the percentage of small fat globules is much higher, making the milk more easily digestible. A wide variety of cheeses and other products can be made from sheeps' milk, which are top of the range in market value, and with good management can produce as good as or better gross margins than most other farming enterprises.

See also: **Animals that Produce Dairy Foods**: Sheep Breeds. **Dairy Farm Management Systems**: Sheep. **Husbandry of Dairy Animals**: Sheep: Reproductive Management. **Mammary Gland**: Anatomy. **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk; Management Control Options; Medical Therapy Options. **Milk**: Sheep Milk. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Milking Machines**: Principles and Design. **Milking Parlors**.

Further Reading

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Sheep: Multipurpose Management

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Introduction

The multiple-purpose product oriented sheep breeds are largely characteristic of those that have accompanied humans along the track of history throughout the last 100 centuries. They have provided people with food (milk and meat), covered them with their wool and skin and fertilized their fields with their manure. The presence of sheep in ancient civilizations first became evident around 30 centuries when they began to feature in animal paintings, recordings, writings and artistic presentations.

Mesopotamia, Egypt, India, China and farther west Minoan Crete and Mycenaean Greece are all areas that have shown several elements of civilization through sheep farming. We are not referring as yet, however, to the modern conception of multiple-purpose sheep breeds because the productive orientation of the flocks is differentiated in relation to the cultural evolution of the people. In Homer's *Iliad* and *Odyssey* (eighth century) however, we find already the modern definition of multiple-purpose sheep; the Cyclops Polyphemus milked his goats and sheep in wooden tubs and proceeded to cheesemaking in baskets, a practice still existing today in Mediterranean countries.

The Homeric heroes consumed roasted sheep and goat meat while offering their fumes of smoke to the gods. They used the hides in everyday life (wine flasks, garments, etc.) and, of course, the wool. Sheep's manure fertilized the fields, and it was also useful as a fuel.

The multiple-purpose breeds, with the passing of centuries, have been associated with particular phenotypic traits, which refer mainly to their wool and tail as well as to the body constitution. They have covered vast territories of the Earth where land and climatic conditions have been characterized as being rather harsh and their husbandry systems had been traced back to very ancient social, cultural and managerial systems. These are still being maintained in many regions today, although mostly marginalized and in full decline.

Multiple-Purpose Sheep Breeds in Today's World

The multiple productive targets of sheep are today not quite as evident as they once were some decades ago when their uniqueness among all the domesticated species was satisfying many indisputable human needs. This shift in the initially clear definition of the sheep as an animal with many productive targets begins with the decline in the economic importance of wool, which resulted in certain regions (e.g. the Balkans) in the complete devaluation of this product. At the same time, this development led to the conversion of the single-purpose breeds of the past (e.g. the Merino populations raised for wool only) to dual-purpose ones, as evidenced in Oceania, South Africa and South America.

We believe, thus, that the definition of the notion multiple-purpose sheep should include, in addition to production parameters, factors such as the prevailing husbandry systems and those resulting from strong cultural traditions which connect the places where this animal is found to the people that farm with it.

We shall, therefore, define as multiple-purpose sheep those animals whose production refers to at least two products of major importance, one of which is milk, a product that was obtained directly after domestication and which strongly characterizes sheep husbandry in the Old World. This is produced by ewes that have been managed by extensive and semi-extensive methods and that in turn have influenced directly or indirectly the quality of the environment. In addition, these sheep have been associated with very old management practices (nomadism, transhumance) found throughout the Old World, and with other more unique methods of management (e.g. the 'tselingato' system in the Balkans, a joint cooperative-like management of individual flocks).

In delineating the areas where multiple purpose sheep are traditionally raised, we distinguish two major regions: (1) the northern coast of the Mediterranean basin through to Morocco, Algeria, Tunisia and Libya in the south, and (2) the Near East. The regions covering the arch from the Caspian Sea to Mongolia and to India may also be added to these areas. Dominating these regions are characteristic sheep populations such as: the Zackel (Balkans and southern Italy), the carpet-wool sheep (mainly of the Iberian Peninsula and Italy) and the fat-tailed, thin-tailed, hairy and fat-rumped sheep of the Near East and the Asiatic Arch (**Figure 1**). In other areas of the world, such as Southern Africa, the Americas, Oceania and Northern Europe, there are no true multiple-purpose sheep management conditions.

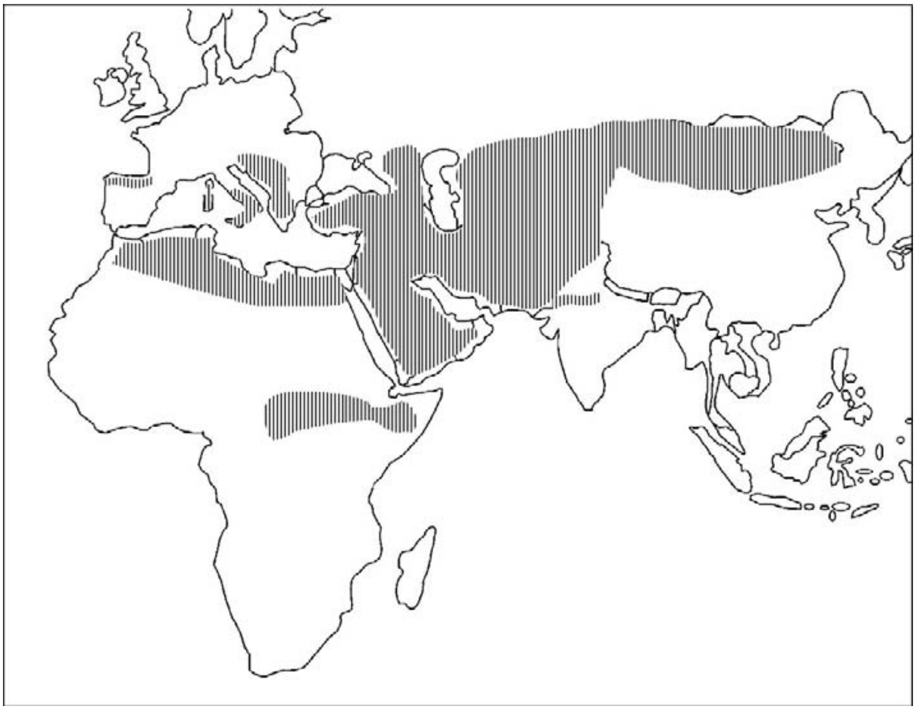

Figure 1 The distribution of multiple-purpose sheep.

Included in these large groups are breeds, many of which according to I.L. Mason are characterized as triple-purpose or, according to the definition proposed here, can be termed as multiple-purpose breeds (**Table 1**). It should be mentioned, however, that the references made to countries and breeds are merely examples and do not constitute detailed descriptions (*see* **Animals that Produce Dairy Foods**: Sheep Breeds).

Environment and Climatic Conditions

Land and climatic conditions in the regions where multiple-purpose breeds are farmed, although greatly varying, present common characteristics inherent to adverse and unfavorable environments. These determined in the past and continue to shape today, for the most part, the development and implementation of the prevailing husbandry systems.

In the Mediterranean basin, the unfavorable environment can be attributed to many elements of differing importance to each country: marginal mountainous and semi-mountainous rangelands, stony or salty soil, lack of rainfall during the summer season and high temperatures are some of those characterizing the northern Mediterranean coastal belt where the multiple-purpose sheep are primarily the carpet-wool dairy breeds.

Arid or semi-arid conditions characterize the environment of the Near East and the Asiatic Arch where infertile mountainous areas alternate with steppes, creating extreme conditions within which human populations and animals have survived and still continue to do so. This was accomplished through the development of specific human traits and the creation of fat-tailed and fat-rumped breeds with special anatomical and physiological properties which have permitted optimal adjustment to the ecosystem and the implementation of special husbandry systems based on nomadic pastoralism and migration in a continuous effort to cover the nutritional requirements of the animals.

Reproductive and Productive Performances

The immense populations within this extended region of our planet include sheep breeds that demonstrate considerable variability as much in their reproductive as in their productive characteristics. This variability is observed between the breeds but also between individuals within breeds. Changing husbandry conditions are considered to be one of the sources of variability.

Fertility rates vary between 55% and 90% and the prolificacy (litter size, number of lambs ÷ number of ewes × 100) from 100% to 150%. Lamb mortality tends to increase under extreme conditions of nomadic husbandry. The primary characteristic of all the breeds, types and varieties is their excellent ability to adjust to extreme

Table 1 Some multiple-purpose sheep breeds, their characteristics and their products

Region	*Country*	*Breed*	*Characteristics*	*Products*[a]	*Husbandry systems*
Mediterranean basin					
1. Northern coast	Portugal	Badana	Carpet-woolled	me–mi–w	Sedentary or small-scale transhumance
	Spain	Churra Lacha	Carpet-woolled	mi–me–w	Sedentary or small-scale transhumance
	France	Manech	Carpet-woolled	mi–me–w	Sedentary or small-scale transhumance
	Italy	Lecce Calabrian	Zackel	mi–me–w	Sedentary or small-scale transhumance
		Sarda Comisana	Carpet-woolled	mi–me–w	
	Yugoslavia	Pramenka	Zackel	mi–me–w	Sedentary or small-scale transhumance
	Albania	Common Albanian	Zackel	mi–me–w	Sedentary or small-scale transhumance
	Greece	Karagouniko Mountain breeds	Zackel	mi–me–w	Sedentary or small-scale transhumance
2. Southern coast	Morocco	Berber Beni Guil	Thin-tailed Carpet-woolled	me–w–mi	Transhumance or nomadism
	Algeria	Arab	Thin-tailed Carpet-woolled	me–mi–w	Nomadism
	Tunisia	Tunisian Barbary	Fat-tailed Carpet-woolled	me–mi–w	Nomadism
	Libya	Libyan Barbary	Fat-tailed Carpet-woolled	me–w–mi	Nomadism
Near East	Egypt	Ausimi Rahmani	Fat-tailed Carpet-woolled	me–w–mi	Sedentary or seminomadic
	Sudan	Sudan desert	Thin-tailed Hairy sheep	mi–me	Transhumance – nomadism
	Ethiopia	Ethiopian	Fat-tailed Hairy sheep with mane	me–mi	Nomadism
	Eritrea	Arrit	Fat base of tail Hairy sheep Carpet-woolled	mi–me	Nomadism
	Turkey	White Karaman Red Karaman Daglic Awassi	Fat-tailed Carpet-woolled	mi–me–w	Sedentary or transhumance
	Syria	Awassi	Fat-tailed Carpet-woolled	me–mi–w	Transhumance or nomadism
	Lebanon	Awassi	Fat-tailed Carpet-woolled	me–mi–w	Transhumance
	Cyprus	Cyprus Fat-tailed	Fat-tailed Carpet-woolled	mi–me–w	Sedentary or transhumance
	Saudi Arabia	Najdi	Fat-tailed	mi–me–w	
	Jordan	Awassi	Fat-tailed Carpet-woolled	me–mi–w	Nomadism
	Iraq	Awassi Hamdani Iraq Kurdi Arabi	Fat-tailed Carpet-woolled	mi–me–w w–me–mi me–w–mi w–me–mi	Nomadism or transhumance
	Iran	Baluchi	Fat-tailed Carpet-woolled	w–mi–me	Sedentary–transhumance
		Grey Shirazi	Fat-tailed Carpet-woolled	me–w–f–mi	Transhumance

(Continued)

Table 1 (Continued)

Region	*Country*	*Breed*	*Characteristics*	*Products*[a]	*Husbandry systems*
		Morhani	Fat-tailed Carpet-woolled	me–w–mi	Transhumance
		Zel	Thin-tailed Carpet-woolled	mi–w–me	Sedentary
		Bakhtiari-Luri	Fat-tailed Carpet-woolled	me–mi–w	Transhumance
		Khorasan-Kurdi	Fat-tailed	me–mi–w	Transhumance
		Makui	Fat-tailed Carpet-woolled	w–me–mi	Sedentary
		Mehraban	Fat-tailed Carpet-woolled	me–mi–w	Sedentary
	Pakistan	Baluchi	Fat-tailed Carpet-woolled	w–mi–me	Transhumance or nomadism
		Damani	Thin-tailed Carpet-woolled	mi–me–w	
		Dumbi	Thin-tailed Carpet-woolled	me–mi–w	
		Harnai	Fat-tailed Carpet-woolled	w–me–mi mi–w–me	
		Kachhi	Thin-tailed Carpet-woolled	me–w–mi	
		Rakhshani	Thin-tailed Carpet-woolled		
	Afghanistan	Karakul	Fat-tailed Carpet-woolled	f–mi–me–w	Transhumance or nomadism
		Ghiljai	Fat-tailed Carpet-woolled	w–me–mi	
		Afgan Arabi	Fat-rumped Carpet-woolled	w–me–mi	
		Turki	Fat-rumped Hairy sheep	me–mi	
		Kandahari	Fat-tailed Carpet-woolled	w–me–mi	
		Hazaragie	Fat-tailed Carpet-woolled	w–me–mi	
		Baluchi	Fat-tailed Carpet-woolled	w–me–mi	
Asiatic Arch	Asiatic	Armenian	Medium	w–me–mi	Sedendary or transhumance
	Regions of	Semicoarse wool	Carpet-woolled		
	the former	Caucasian			
	USSR (Armenia,		Fat-tailed	me–mi–w	
	Georgia, Uzbekistan,	Eric	Carpet-woolled		
	Turkmenistan,		Fat-tailed	mi–w–me	
	Kazakstan)	Karakul	Carpet-woolled		
			Fat-tailed	f–me–w–mi	
		Mazekh	Carpet-woolled		
			Fat-tailed	w–mi–me	
		Edilbaev	Carpet-woolled		
			Fat-rumped	me–mi–w	
			Carpet-woolled		
	Mongolia	Mongolian	Fat-tailed Carpet-woolled	w–me–mi	Nomadism
	China	China (Mongolian)	Fat-tailed Carpet-woolled	w–me–mi	Transhumance
	India	Jalauni		w–me–mi	Sedentary or
		Muzaffarnagri	Thin-tailed	w–me–mi	transhumance
		Patanvadi	Carpet-woolled	w–mi–me	
		Sonadi		w–mi–me	

[a] mi, milk; me, meat; w, wool; f, fur.

environmental conditions. The predominantly arid climate of the Near East is characterized by extended and dry summers with an average rainfall that does not generally exceed 100 mm. In certain particular cases, the very high summer temperatures, with a maximum mean that rises well above the body temperature of the mammals, are accompanied by high humidity, which consequently places the animal under physical stress during the lengthiest period of the year.

Under these conditions, productivity drops numerically to lower but still satisfactory levels, if one takes into consideration the almost permanent deficiency observed in covering the nutritional needs of the animals. The commercialized milk yield varies largely within the limits of 20–160 kg. Meat production, with the exception of the northern Mediterranean coast, refers to lambs slaughtered at heavy weights or to mature animals (mutton); this depends qualitatively on the mean adult live weight of the breed, which varies from 15 to 20 kg for the local Yemen sheep up to 60–65 kg for the Awassi breed. A characteristic trait of the Near East sheep meat is the presence of the fat tail in the carcass, for which the demand in local markets is very strong.

The wool is mainly carpet wool derived from the fat-tailed breeds; it constitutes the basis for the local carpet industries. The hides have not yet been exploited to a satisfactory degree. **Table 2** outlines the reproduction and production traits of some indigenous sheep of the Near East.

Husbandry Systems and the Environment

The classic form of nomadism and of transhumance at any scale constitutes the dominant husbandry system of the multiple-purpose sheep breed in the African and Asiatic traditional zones. There, of course, where conditions permit, this husbandry system coexists with plant production.

In the European zone, nomadism has been virtually extinct since the late nineteenth century. Transhumance still exists but is in a state of continuous decline. Indicatively, the transhuming sheep populations in Greece are shown to have dropped from 30% in 1955 to 6% in 2000. The reasons may be explained more by the change in the social fabric of these regions and less by the change in productive procedures. A result of these evolutions has been the eradication of the tselingato, a sort of cooperative of sheep and goat farmers which flourished in the Balkan countries during the eighteenth and nineteenth centuries and became an example of an optimal human organization of flock management founded on respect for and protection of the environment.

The repercussions of this change in livestock farming systems have been serious and have contributed to the overthrow of the fragile Mediterranean ecosystems due to an unbalanced use of pastureland (overstocked or undergrazed pastures) resulting in an increase in fires, erosion and degradation of extended areas.

On the African side of the Mediterranean, the agriculture is based traditionally on mixed systems of animal and plant production. Exclusive animal farming dominates where the rainfall is too low to support any form of cultivation. Nomadic camel-raising dominates in areas receiving under 200 mm of rainfall per year (desert conditions). Nomadic or transhumant livestock farming (sheep, goats, cattle, camels, equines) is the main feature in regions with rainfall totalling 200–600 mm yearly.

In these regions, humans and animals, relying on traditional knowledge, follow the climatic and ecological changes in order to secure on a daily basis sufficient amounts of pasture and water. A characteristic example is the movement of the Arab people the Baggara from western Sudan to the south during the dry period to appropriate pastureland and afterwards to the north during the rainy season to avoid the mud and biting insects and to take advantage of the brief vegetation of the semidry pastureland.

It should be emphasized that the conception that pastoral systems are unproductive is a totally wrong one. In reality, the opposite holds true, for it has been proved that transhumance has resulted in the production of a greater amount of protein per hectare than has modern ranching in similar regions of the United States and Australia (**Table 3**).

A direct demonstration that the prevailing opinion that the seasonal movement of the flocks constitutes a sort of primitive technique has been shown to be wrong, is shown by many other examples such as the case of Mongolia

Table 2 Reproduction and production traits of some indigenous sheep of the Near East

Breed	*Country*	*Fertility (%)*	*Prolificacy (%)*	*Milk yield (kg)*	*Fleece weight (kg)*
Awassi	Syria	80–90	100–105	110–160	1.4–3.0
Arabi	Iraq	50–60	100–105	20–30	1.5–2.0
Baluchi	Iran	92	115–120	60	2.2–2.7
Daglic	Turkey	80	100–110	35–45	1.8–2.0
Turki	Afghanistan	60–70	108–110	55–65	1.5–2.0

Table 3 Protein production according to livestock system practiced

	USA	*Australia*	*Sahel*	*Sahel*	*Sahel*
Livestock system	Modern ranching	Modern ranching	Nomadism	Transhumance	Sedentary
Protein (kg ha^{-1} yr^{-1})	0.3–0.5	0.4	0.4	0.6–3.2	0.3

Adapted from Brenan and de Wit (1983).

where, until recently, the long migrations have been developed as a strategy for the intensification of production.

The reduction in flock movements during recent years has been accompanied by pasture degradation resulting from the appropriation of land for the cultivation of fodder crops through intensification and mechanical means. Proportional reduction in pasture quality has been observed recently in China following privatization; this was also accompanied by a reduction in flock movement.

In any case, it is extremely difficult to generalize the solutions that could reserve the negative consequences in the medium term.

Multiple-Purpose Sheep in Developing Countries

The role of multiple-purpose sheep – fat-tailed, thin-tailed, fat-rumped, carpet-wool or hairy sheep – is absolutely vital to the economies of all the developing countries located in the huge region of their exploitation. Although this region is inhabited by culturally different people groups with differing social structures, a common point of reference between them is an adverse environment which reaches, in certain cases, barely sustainable limits. They also share in common large unaltered sheep populations traditionally managed and an increased demand for all the animal products (meat, milk, wool, skins). This increasing demand coincides with the human population explosion in all the countries where those sheep types are kept and thus comprises the condition under which every measure for the improvement and development of the farming systems will be undertaken, taking into account the following factors: rapidly increasing human population, increasing small ruminant population, need for improvement of genetic material and of farming techniques, dangers of devaluation from actions detrimental to the environment. It is a notable fact that the human population in the northern African countries will increase during the period 2000–2025 at an average rate of 40%, whereas the corresponding rate for Sudan, Ethiopia, Eritrea and Somalia will approach 80%. Respectively for the two regions, the sheep population increased from 1980 to 2000 by 24% and 146%. The same immense population increase is expected by 2025 for countries such as Iraq, Iran, Afghanistan and Pakistan, at an average of 78%.

Pressure from increasing human population leads to an expansion of cultivated land in grazing areas and this practice is encouraged by the fact that the ownership of land is traditionally secured through cultivation and not via periodic grazing. As a result of these actions, an irreversible degradation of the land has been observed after a period of one to two decades. A further considerable problem is the tendency toward a rising demand for an increased animal population, which constitutes a capital investment in the face of a lack of other opportunities and a method of replacing losses and increasing production. Therefore, every measure employed toward the amelioration of the management of livestock farming should include other parameters in addition to those related to the animal husbandry systems.

Initially, it had become clear that all breeds and types of multiple-purpose sheep are best adapted to a harsh environment and produce the maximum possible amounts under the given conditions. The sought-after upgrading will, therefore, result from the improvement in farm management, after selection.

Before any system is to be introduced, the question of two basic problems must first be addressed, namely feeding and disease control.

Although sheep in African and Asian countries are not all managed according to the nomadic and/or transhuming systems, given that good pasturelands exist in certain countries, inefficient nutrition remains a constant problem. In certain cases, in order to gain better nutrition, sheep have been removed from pasturelands and given harvested animal feed, while there have been efforts undertaken to obtain and make available different agricultural byproducts suitable for animal feeding.

The confrontation and control of diseases constitutes an especially difficult task requiring organization and substantial economic input. The degree of difficulty increases during the constant movement of the flocks, a fact borne out not only in the vast territories of Africa and Asia, but in the much more limited areas of southern Europe.

Genetic improvement of multiple-purpose breeds has as its goal, on the one hand, the upgrading of the breeds and types exploited for all three/four productive traits (meat, milk, wool/fur, hide) and, on the other, the maintenance of their specific phenotypic characteristics (carpet-wool, fat-tail, etc.). The use of other techniques such as estrous synchronization and artificial insemination consists of classical methods with well-proven results. More advanced techniques, however, such as embryo transfer, can be implemented for the time being only in state institutes and well-organized commercial farm enterprises. One would hope that genetic engineering will solve in the future serious problems such as the diagnosis of diseases and the preparation of vaccines.

Finally, it should be emphasized that the evolution from traditional extensive systems (nomadism, transhumance) toward more intensive ones cannot be introduced by force from above nor in a top-stratified manner, thus violating the principles and rules of livestock farming which have survived through the centuries with satisfactory results and whose efficacy cannot be disputed.

See also: **Animals that Produce Dairy Foods**: Sheep Breeds. **Dairy Farm Management Systems**: Sheep. **Husbandry of Dairy Animals**: Sheep: Health Management; Sheep: Milking Management; Sheep: Reproductive Management.

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Sheep: Replacement Management

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Introduction

Depending upon the rate at which adult dairy ewes in the flock are replaced, the replacement ewes generally comprise 15–30% of total ewe flock numbers. These replacement ewes must be fed and managed for more than 1 year in intensive production systems and for more than 2 years in less intensive systems before any financial returns from milk or meat lambs are obtained. Therefore, they need to be raised as economically as possible, but in a manner that optimizes their adult production.

There have been very few scientific studies that have evaluated different methods of managing replacement dairy ewes or have determined which components of replacement dairy ewe management have a significant impact on her milk and lamb production as an adult. A few resources that discuss this topic in more detail are listed in the 'Further Reading' section at the end of this article.

Production and management of replacement dairy ewes can be divided into six stages: (1) selection, (2) prepartum, (3) nursing, (4) prepubertal, (5) first breeding, and (6) first lactation.

Selection

Replacement dairy ewes with the potential for high levels of performance can be produced from parents, primarily sires, of high genetic value for economically important traits. Accurate estimates of genetic merit can be obtained only if animals are individually identified, pedigrees of animals maintained, and objective measures of performance recorded. This information may not be available for commercial flocks. However, such flocks can be genetically improved to a considerable degree by using rams of above-average genetic value from flocks involved in performance recording. Genetic improvement is greater in the entire population if a nucleus of recorded flocks cooperates in order to obtain across-flock estimates of genetic values – called estimated breeding values (EBVs). The flocks in the nucleus have rapid increases in breeding values (BVs) and performance. The commercial flocks in the population also improve in BVs and performance through the use of sires from the nucleus, but their performance legs that of the nucleus.

The selection program of the Lacaune dairy sheep in south central France is an example of a very effective genetic improvement program. The program resulted in a 6 l per year genetic gain in lactation milk yield per ewe when selection was primarily based on milk yield, and has decreased only slightly in recent years to 5 l milk per year when selection based on milk composition (fat and protein percentage) has been added to selection based on milk yield.

Milk yield is moderately heritable (30%), so some genetic progress within a flock can still be made by selecting replacement ewes from dams with high milk yields, even if an organized genetic improvement program is not available for the entire population.

Prepartum

A relatively new area of animal research that holds promise for increasing the productivity of replacement ewes is fetal programming. Research with sheep has shown that some treatments, primarily nutritional, applied to the pregnant female affect the performance and health of the offspring. Undernutrition of pregnant ewes has been found to negatively affect growth and reproduction of the offspring. In addition, the fetal mammary duct area decreases because of undernutrition of the dam during pregnancy, and this could result in poorer milk yield of the progeny as adults.

However, does overfeeding a pregnant ewe have any effect on the milk yield of her daughters? There has been one large trial reported with meat sheep in which pregnant ewes on pasture were fed at maintenance (moderate nutrition level) or *ad libitum* (high nutrition level) and the milk yields of their progeny were measured during first and second lactation. During first lactation, progeny from maintenance dams tended to have higher milk yields than those from *ad libitum* dams, but there was no difference in second lactation milk yields of progeny from the dams of the two nutrition groups. Since these progeny were raised on their dams, the effects of dam nutrition observed in the first lactation of the progeny may have been due to early postnatal effects rather than *in utero* effects (i.e., *ad libitum* dams produced more milk for their nursing progeny than did maintenance dams, and some studies have shown that a higher level of nutrition during the prepubertal period results in lower milk yield as an adult).

There is a need for more studies on dairy ewes, where the resulting progeny are raised on milk replacer to eliminate differential postnatal effects, to determine if under- or overfeeding of the pregnant ewe can have a significant *in utero* effect on the lifetime milk production of the female progeny.

Nursing

It is very important that the newborn lamb receive adequate quantities of colostrum during its first hours of life. Colostrum is the first secretion from the udder, and it is produced for the first 2–4 days of lactation. It is richer in nutrients than milk and is an excellent source of food for the newborn lamb. In addition, colostrum contains specific immunoglobulins that are absorbed directly through the intestinal wall of the newborn lamb. This absorption of immunoglobulins is not possible after approximately 2 days of age, and the rate of absorption is highest during the first few hours after birth. These immunoglobulins provide the lamb with passive immunity to the infectious agents that the dam had been exposed to during her lifetime or to any diseases for which the dam had been vaccinated. This passive immunity protects the lamb against disease organisms in the farm environment until the lamb develops its own active immune system.

If the dam dies during lambing or shortly after or if the dam has inadequate amounts of colostrum for her lamb or lambs, colostrum should be collected from recently lambed ewes with an abundance of colostrum and fed to the lambs that have a deficient supply or no colostrum. These lambs should receive three or four feedings of colostrum per day during their first 1–2 days of life before they are transitioned to milk or milk replacer. Lambs should be fed 4–5% of their body weight in colostrum at each feeding or approximately 150–200 ml per feeding for lambs weighing 3.75–5.00 kg. If the lamb is able to suckle, colostrum can be provided by a bottle with a nipple. Otherwise, the colostrum can be administered through a tube that is passed through the mouth, down the esophagus, and into the stomach. The exposed end of the tube is attached to a 200–400 ml syringe (without the plunger). The colostrum is poured into the syringe and fed to the lamp via gravity flow.

If fresh ewe colostrum is not available, frozen–thawed ewe (best) or cow (good) colostrum that was collected earlier can be administered. Thawing of the colostrum should be done at room temperature or under warm tap water to avoid destruction of the immunoglobulins from high heat. If no ewe or cow colostrum is available, artificial colostrum available commercially can be purchased and used; however, these products are less satisfactory than natural colostrum.

Potential replacement ewes are either raised on their dams or raised artificially on milk replacer. In both cases, the goal of the dairy sheep producer is to wean them off milk or milk replacer and on to dry diets as soon as possible without adversely affecting their growth or health in order to harvest more milk from ewes that raise their lambs or to eliminate the expense of purchasing milk replacer powder for lambs raised artificially.

In more extensive systems that do not provide nursing lambs with supplemental dry feed, lambs should be up to 8 weeks of age before they are weaned from their dams. However, most sheep dairies provide dry feeds to lambs nursing their dams or on milk replacer, so they are able to transition to a completely dry diet at 3–4 weeks of age. Since these young lambs are not fully functioning ruminants, the dry diets need to be low in fiber and high in energy and protein; also, the diets should be processed into a pellet or meal and fed *ad libitum*. An example of a diet for young lambs on milk or milk replacer is given in **Table 1**.

Raising Lambs on Milk Replacer

Since the percentage of fat, protein, and solids is higher in sheep milk than in either cow or goat milk, it is very important to use a milk replacer specifically formulated for lambs. The milk replacer comes in the form of a dried powder to be reconstituted with water before feeding.

Table 1 Example of a simple diet for nursing lambs or lambs on milk replacer

Ingredient	*Percentage of the diet*[a]
Corn grain	61.0
Soybean meal	31.0
Liquid molasses	5.0
Feed-grade limestone	2.0
Ammonium chloride	0.5
Salt-mineral mix formulated for sheep	0.5

[a] Diet estimated to have 22% crude protein and 83% total digestible nutrients on a dry matter basis. Diet should be pelleted or fed in a coarse meal form.

The primary ingredients of milk replacer powder, from the highest to the smallest proportion, generally are dried skim milk, dried whey protein concentrate, dried whey, animal fat, vegetable fat, casein (major protein in milk), various minerals and vitamins, and a preservative. The powder on a dry matter basis should contain a minimum of 23% crude protein, a minimum of 30% crude fat, and a maximum of 0.15% crude fiber. The powder is often reconstituted by adding 1 part of powder to 2–4 parts of water by volume.

For the first 1 or 2 days after a lamb is separated from its dam, it is fed warm milk replacer from a bottle with a nipple 3 or 4 times a day in order to train it to suckle milk replacer through a nipple. Once it is able to suckle efficiently from a nipple, the lamb is trained to suckle from a nipple attached to a larger supply of milk replacer that is continuously available. The milk replacer is initially provided warm but later provided chilled to prevent overconsumption. Each lamb requires 8.00–8.25 kg of milk replacer powder from 1.5 days of age to 4 weeks of age.

The milk replacer can be provided in nipple buckets for smaller groups of lambs or through machines that automatically mix the replacer with water and dispense it on demand through tubes attached to nipples for larger groups of lambs. The most important aspect for successfully rearing lambs artificially is thorough cleaning of the milk replacer dispensing equipment on a daily basis with detergent and hot water.

Prepubertal

The level of feeding and growth rate of replacement ewe lambs from the time of weaning from milk or milk replacer at 4–8 weeks of age until puberty at 6–9 months of age may have an effect on their subsequent milk yield as adults. While relatively little research has been conducted for sheep, there has been significant research for cattle. The majority of well-designed studies have shown that high feeding levels for dairy heifers during the prepubertal period is detrimental to subsequent milk production. Increased feeding levels for beef heifers during their prepubertal period also results in decreased milk production or decreased weaning weights of their calves.

Studies evaluating the effects of the prepubertal feeding level in ewe lambs on udder development, subsequent milk production, or lamb weaning weights are fewer in number and are less conclusive than the studies on cattle. However, a series of studies with relatively small numbers of meat-type lambs where milk production was estimated 1–3 times during the lactation period using the weigh–suckle–weigh technique (weighing lambs before and after suckling to estimate milk production) or by injecting the hormone oxytocin to remove milk have generally found that ewe lambs that were fed for a moderate rather than maximum growth rate during the prepubertal period produced more milk during their first lactation. In contrast, in a large study with dairy ewes where lactation performance was monitored for up to four lactations, ewes that were fed a 13% crude protein diet *ad libitum* had milk yields similar to those of ewes fed the same ration but at 73% of *ad libitum* intake from approximately 50 to 150 days of age.

While the data on sheep are limited and inconclusive regarding the effect of level of nutrition and growth rate during the prepubertal period on subsequent milk production, studies to date show no detrimental effects on milk yield of feeding for moderate growth rates (65–75% of daily gains expected with *ad libitum* feeding) compared to feeding for maximum growth rates and some studies have shown a beneficial effect of moderate growth rates. Therefore, it seems reasonable to recommend that replacement ewe lambs should be fed for moderate, but not maximum, body weight gains from weaning to 5–6 months of age prior to puberty.

Results from a research trial conducted in North America with pen-fed prepubertal ewe lambs of East Friesian and/or Lacaune breeding indicate that restricting ewe lambs to dry matter intake of 4.2% of body weight at younger ages and lighter body weights and to dry matter intake of 2.6% of body weight at older ages and heavier body weights results in average daily gains 75% of the gains observed with *ad libitum*-fed ewe lambs

Table 2 Dry matter intake (percentage of body weight) of pen-fed dairy ewe lambs from 50 to 150 days of age from a trial conducted in North America

	Feeding group[a]	
Body weight kg	Ad libitum *grain mix, average daily gain = 327 g*	*73%* ad libitum *grain mix, average daily gain = 245 g*
17–34	5.10%	4.24%
34–44	3.67%	3.05%
44–50	3.15%	2.55%

[a]Grain mix was 13% crude protein and contained whole shelled corn and a high-protein pellet. Ewes in both groups received 0.18 kg hd^{-1} day^{-1} of dry matter from alfalfa hay.

(**Table 2**). Prepubertal ewe lambs raised on (1) high-quality grass–legume pastures or (2) pastures of lower quality and supplemented with approximately 0.50 kg hd^{-1} day^{-1} of grain would be expected to achieve the recommended body weight gains of 65–75% of maximum gains.

First Breeding

In dairy sheep production systems where nutrition of ewe lambs is poor or where less precocious breeds are utilized, dairy ewes may be approximately 2 years of age before they give birth for the first time and enter their first lactation. However, ewe lambs of many of the dairy breeds have the ability to exhibit estrus by 6–8 months of age if they have been well fed. Therefore, it is a common practice in many dairy sheep operations to mate ewe lambs so that they enter their first lactation at little over 1 year of age.

Ewe lambs should reach at least 60% of their mature weight before they are mated. Generally, they are mated when 8–9 months of age, a month or two after the start of the mating season for the mature ewes. There are several reasons for mating ewe lambs later than the mature ewes:

1. An older age at mating will increase the percentage of ewe lambs that have reached puberty and increase the percentage that will lamb to this first exposure.
2. During the mating season, it is the estrous ewe that seeks out the ram. If young naive ewe lambs are in the same mating group as mature ewes, the mature ewes may monopolize the rams, resulting in a lower than expected conception rate in the ewe lambs.
3. An ewe lamb requires 15–25% more energy per day than a mature ewe during the prebreeding period and during pregnancy in order to meet her requirements for continued growth and pregnancy. Keeping the ewe lambs in a group separate from the mature ewes during mating and pregnancy allows for differential feeding between the groups.
4. More labor may be required to lamb out ewe lambs than mature ewes. Ewe lambs may experience a higher incidence of dystocia than mature ewes, their lambs may need extra colostrum supplementation since ewe lambs produce less colostrum than mature ewes, and ewe lambs may need more human intervention to accept and suckle their lambs than mature ewes. If the majority of the mature ewes have lambed prior to the start of the lambing season for the ewe lambs, labor is available to concentrate on the lambing of the ewe lambs.
5. If the mature ewes have been milking for 1 or 2 months before the ewe lambs enter the milking parlor, the milking routine for the new season has been well established, and it is easier for the inexperienced ewe lambs to be assimilated into the milking routine than if the ewe lambs and mature ewes all start the new milking season at the same time.

First Lactation

The nutrient requirements of lactating dairy ewes less than 2 years of age are not well documented. The *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids* published by the National Research Council in the United States in 2007 presents nutrient requirements for mature dairy ewes during lactation but not for yearling dairy ewes. Yearling dairy ewes have a greater requirement for both energy and protein as a percentage of body weight than mature ewes during lactation because the yearling ewes are growing and lactating while the mature ewes are only lactating.

Since the yearling ewes are 20–30% lighter in body weight than the mature ewes, feeding the yearling ewes the same amount of feed as the mature ewes allows them to continue to grow during their first lactation. In practice, this occurs on most dairy sheep farms. Generally, ewes of all ages are managed as one group during lactation and receive the same amount of supplemental feed in the milking parlor; therefore, the yearling ewes receive more feed as a percentage of their body weight than do mature ewes.

A common feeding regime for lactating dairy ewes when they have *ad libitum* access to high-quality grass–legume hay, silage, or pasture is to provide 0.50 kg of whole grain per ewe per milking (2 milkings per day) in the parlor during early and midlactation, decreasing to 0.25 kg of whole grain per ewe per milking in late lactation. If the stored or grazed forage is of lower quality, a 16% crude protein grain mix is substituted for the whole grain. Since the yearling ewes enter the milking string 1 or 2 months later than the mature ewes, they will still be in midlactation when the mature ewes start into late lactation. It is desirable for the yearling ewes to continue to receive 0.50 kg of concentrate at each milking once the feed for mature ewes has dropped to 0.25 kg in late lactation. The yearling ewes should continue to receive the 0.50 kg of concentrate at each milking through their entire lactation. This requires more labor during milking to top up the amount of feed given to each yearling ewe once the mature ewes are in late lactation, but it results in larger, healthier ewes as 2-year-olds.

See also: **Husbandry of Dairy Animals**: Goat: Replacement Management. **Replacement Management in Cattle:** Breeding Standards and Pregnancy Management; Growth Diets; Growth Standards and

Nutrient Requirements; Health Management; Pre-Ruminant Diets and Weaning Practices.

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Sheep: Reproductive Management

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Introduction

The reproductive efficiency of sheep can be expressed as the number of lambs born annually per ewe put to the ram(s). Age at first mating or insemination, conception rate, seasonality in sexual activity, prolificacy, embryo and fetus viability, length of the postpartum anestrous period, and flock age structure are the main factors governing reproductive efficiency. With an average gestation length of about 5 months and an average prolificacy of 2.5 lambs per lambing in prolific breeds, annual lamb production can go up to 5 lambs per ewe. In practice, however, the reproductive efficiency of sheep flocks is far lower, mainly in the range of 1–2 lambs per ewe. For ewes belonging to low-prolific breeds that are raised under extensive migratory systems, reproductive efficiency may drop to less than 1 lamb per ewe.

Patterns of reproductive management and, consequently, reproductive efficiency vary considerably among sheep flocks. This is mainly due to the diversity of breeds in use (more than 1000 sheep breeds are known) and the diversity in management systems. Flock-feeding strategy, latitude, climatic conditions, economic constraints, and cultural traditions define the latter. In mid- and high-latitude regions where about 60% of the world sheep population is managed (altogether about 1035 million head), ewes generally lamb once a year during a restricted lambing season (**Figure 1**). In low-latitude regions, lambings may be distributed throughout the year.

For a particular breed, pasture availability is the rate-limiting factor for reproductive efficiency in extensive production systems. In semi- and fully intensive systems where supplemented feeds such as grains, hay, silage, and agricultural and industrial by-products are routinely used, reproductive management is intensified with the aim of utilizing the full physiological potential of the ewe. This goal can be achieved under accelerated lambing management, where, by using adequate feeding, controlled environmental conditions, and estrous synchronization, ewes can lamb on average every 8 months.

Summary of Reproductive Events

Puberty

Generally, ewe lambs reach puberty, the time of first ovulation and first estrus, at about 50–70% of their adult body weight. Nutritional level during the growth period, breed affiliation, and seasonality affect age at first mating. Age at first mating may vary from as early as 4–5 months of age in ewe lambs belonging to some prolific breeds to as late as 1.5 years of age in ewe lambs from seasonal breeds raised under suboptimal nutrition. Ram lambs may reach puberty and be introduced into the flock at the age of about 6 months. However, they are usually first put to ewes at about 1–1.5 years of age and it may take some time before they reach their full sexual potential.

Estrus and Ovulation

Cycling ewes exhibit estrus within the natural breeding season at intervals of about 16–17 days and each estrous period may last 18–72 h, depending on the ewe's age, breed, stage of the breeding season, and management type. During estrus, the ewe shows specific behavior patterns and allows the ram to mount her. Ovulation normally occurs late in estrus, about 25–30 h after its onset. In nonprolific breeds multiple ovulation is not frequent, whereas in prolific breeds several ovulating follicles can be detected in both the ovaries and most ovulate within few hours. Besides genotype, ovulation rate may be influenced by age of the ewe, stage of the breeding season, nutritional level before mating, and other environmental conditions, such as type of hormonal treatment.

Pregnancy

Following successful fertilization, the ewe becomes pregnant. Maternal recognition of pregnancy starts at about day 12 of gestation following secretion by the embryo of substances that affect the uterus and suppress the secretion, and hence the luteolytic effect of, prostaglandin F2a (PGF2a). Subsequently, the function of corpus luteum (CL) is maintained and it continues to release progesterone necessary to maintain pregnancy. In the absence of embryonic antiluteolytic signal, the CL regresses and circulating progesterone levels decrease. This is followed by a rise in gonadotropin production by the pituitary gland, initiating a new wave of follicle growth, estradiol secretion, estrus, and finally a new ovulation event.

Implantation and placenta formation take place by day 30 postfertilization. Embryo and fetal losses occur mainly up to day 30 of gestation and can be as high as 30%. Beyond that stage, embryo losses are relatively low,

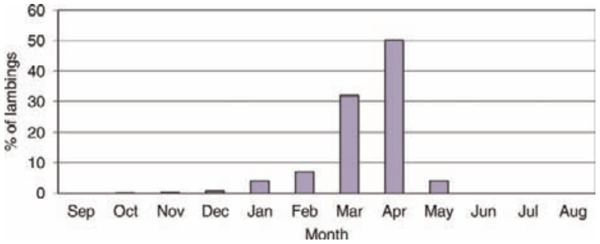

Figure 1 Distribution of lambings of the national flock (about 18 million ewes) in the United Kingdom during 1996 (GE Pollott, personal communication).

estimated to be about 2.5%. Average gestation length in domestic sheep breeds is about 147 days with a few days' variation between breeds. Other main factors that affect gestation length are age of the ewe and litter size. Ewes in their first pregnancy and ewes carrying multiple fetuses have shorter gestations by a few days.

Postpartum Anestrus

Following lambing, the ewe enters a postpartum anestrous period, and it normally takes 6–8 weeks before the uterus recovers, ovaries resume their normal activity, and ewe can be bred again. Several factors influence the duration of postpartum anestrous periods, including stage of the breeding season, nutritional status, suckling, and lactation.

Effect of Milk Yield on Reproduction

In dairy ewes, lactation yield had no significant effect on the lambing-to-estrus interval. However, higher milk yield was found to be associated with a longer lambing-to-conception interval (**Figure 2**) The delay in conception in high-yielding animals is explained by the adverse effect of the negative energy balance at the beginning of lactation on reproductive performance.

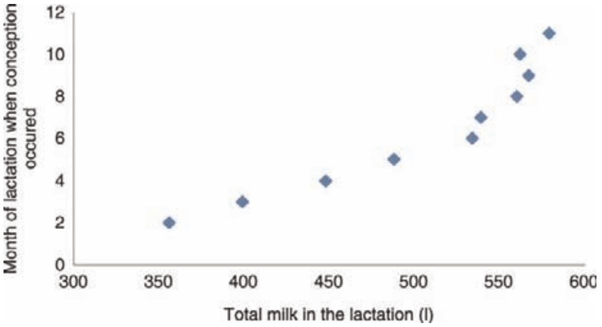

Figure 2 The relationship between milk yield (l) through the lactation and month of the lactation when conception occurred in improved Awassi ewes. Reproduced with permission from Gootwine E and Pollott GE (2000) *Animal Science* 71: 607–615.

Culling

Reproductive efficiency in sheep peaks by the second or third parity, subsequently decreasing with age. Ewes are usually culled on average at the age of 4–6 years. However, there are records of ewes up to 17 years of age that still reproduced.

Nutrition and Reproduction

Monitoring nutrition is an important part of proper reproductive management. Research has indicated that reproductive parameters such as entry into breeding activity, ovulation rate, conception rate, embryo survival, and fetal development are affected by nutritional level and diet composition.

Static, Dynamic, and Immediate Nutritional Effects

Nutritional effects on reproductive performances are classified into three categories: static, dynamic, and immediate. The nutritional effect via body weight or body condition is termed the 'long-term' or 'static' effect. Body condition score reflects the degree of fatness of the animal and is set by feeling the fat cover over and around the bones in the loin region of the back of the sheep or in the tail root. Condition scores are scored on a scale ranging from 1 to 5. While in animals with a condition score of 1 or 2 it is easy to feel individual bones, light or firm pressure is needed to feel bones in animals with a body score of 3 or 4, respectively. In very fat animals that gain a condition score of 5, individual bones or vertebrae cannot be detected, even with firm pressure. The results of several studies indicate that within breeds, the length of the seasonal anestrous period is shorter and ovulation rate is higher for heavier ewes and ewes with better body condition.

Nutritional effects are considered 'short-term' or 'dynamic' effects when ewes change their body condition following 2–4 weeks of treatment. It has long been recognized that improving nutrition for a few weeks prior to mating can increase lambing rate by about 10–20%, by elevating ovulation rate. This practice, known as 'fushing', is applied by introducing the flock to better pastures or by supplementing its feed with good-quality hay or grains. The effect of fushing may not be evident in well-fed ewes. It is therefore recommended that the level of feeding is reduced 2–3 weeks before fushing to maximize the effect of fushing.

An 'immediate' nutritional effect is achieved by altering the ewe's energy and protein supplementation for a few days. Feeds such as cereal grains rich in energy, or lupin grains, rich in both energy and protein, or similar economically available high-energy–high-protein feeds such as beans or oilseed meals are valuable for this purpose. In one study, a 25–30% increase in ovulation rate was achieved after 6 days of supplementing the diet of Merino sheep with 750 g per day of lupin grains.

Pregnancy Toxemia

No major nutritional effect has been observed on postmating embryo survival under regular nutrition. However, high postmating nutrition was found to adversely affect embryo survival, and severe undernutrition during the second and third months of gestation led to fetal losses. Inadequate nutrition throughout the ewe's pregnancy may result in pregnancy toxemia, a metabolic disorder of advanced pregnancy that affects ewes unable to meet simultaneously glucose requirements for body maintenance and glucose demands of the uterus and the growing conceptuses. Multiparous ewes and ewes carrying multiple fetuses are more susceptible to pregnancy toxemia. If not diagnosed and treated in the early stages of the disease, pregnancy toxemia often causes the death of the pregnant ewe.

Phytoestrogens

A special case of nutritional effect on reproduction is the presence of phytoestrogens in the legumes pasture, such as clover or lucerne (alfalfa). Long-term consumption of clover may cause temporary or even permanent infertility.

Ram's Nutrition

Nutritional status has also been found to affect ram libido and semen quality. The recommended practice is to offer the rams a high level of feeding during the premating period to enhance the spermatogenic process, which takes some 46–49 days. Proper feeding may result in an increase in testis size and weight, which are correlated with sperm production ability.

Seasonality in Reproductive Activity

Breed Differences

Differences in the duration of reproductive activity on a yearly basis have been observed among sheep breeds. D'man sheep from Morocco, for example, maintain their ovulatory cyclicity throughout the year. Merino ewes, on the other hand, may experience an anestrous period any time during the year. However, as the time of anestrus varies among animals, at any given time of year some ewes in a Merino flock will be in estrus. Ewes belonging to seasonal breeds such as the Suffolk and Texel display regular estrus behavior only during the breeding season. The rest of the time they do not show estrus behavior, although silent ovulations may occur during the anestrous period.

In the northern hemisphere, the natural breeding season in sheep occurs in late summer, autumn, and winter. Consequently, lambing occurs at the end of winter or in the beginning of spring, when pasture availability and climatic conditions are favorable for lambing, milk production, and lamb growth. Length of the breeding season varies among breeds: in the Soay sheep of the St. Kilda Isles of western Scotland, ewes may experience only a single estrus during the breeding season. In other seasonal breeds such as the Ile-de France or the Awassi, the natural breeding season can last more than 6 months.

Seasonality in Rams

Seasonal breeding activity is also observed in rams. Unlike ewes, rams are sexually active even outside the breeding season. However, during that time, their sperm production decreases, the size and weight of the testicles decrease, and they are less sexually active.

The Photoperiod Effect

When ewes from European seasonal breeds were transported to the southern hemisphere, they adjusted their breeding activity to the new environment. Those and many other experiments in which ewes were subjected to different artificial light regimes have proved that breeding activity in seasonal breeds is controlled by annual photoperiodic changes. Although sheep are known as short-day breeders because their period of sexual activity occurs in the autumn and winter when day length is relatively short, it is not the absolute day length but rather the alternations between long day and short day that stimulate sexual activity. Under a constant short-day light regime, cycling ewes will become refractory and spontaneously stop ovulating. On the other hand, ewes kept continuously under long days will start reproductive activity spontaneously. Further research has shown that, generally, ewes from both seasonal and non-seasonal breeds do not ovulate continuously throughout the year and most ewes experience periods of cessation in sexual activity. Breed differences in seasonality thus lie in the ability of the animals in the flock to respond simultaneously to the photoperiod signal that synchronizes their breeding activity.

Melatonin Secretion

The physiological mechanisms mediating the effect of light on the control of reproduction in sheep are only partially understood. The photoperiod effect has been shown to be mediated by changes in the secretion pattern of melatonin, a hormone that is secreted from the pineal gland during the dark hours. Advancing the breeding season of seasonal ewes to spring or early summer can be achieved by manipulating the photoperiod effect. Exposing ewes to flashes of light during the dark hours can artificially induce a long-day effect. The use of commercially available melatonin implants can mimic the short-day situation.

Artificial changes in day length and the use of exogenous melatonin treatment can also serve to manipulate the breeding activity of rams. This has been practiced in artificial insemination (AI) centers in France to advance puberty in young rams and to maintain a steady level of sexual activity of rams throughout the year.

Control of Reproduction by Estrous Synchronization

The Ram Effect

Introducing rams to a group of ewes that have been kept apart from the rams for a period of time results in the induction of heat about one estrous cycle interval later. This ‘ram effect’ has been used to induce synchronized breeding activity in sheep flocks during the non-seasonal periods. The ram effect is also used to advance the breeding season. As no physical or visual contact between rams and ewes is needed, this effect has been suggested to be mediated by pheromones.

Hormonal Synchronization

The common procedure for artificial estrous synchronization in sheep is the administration of progestagen followed by equine chorionic gonadotropin (eCG) (previously designated pregnant mare’s serum gonadotropin (PMSG)). This procedure is used to synchronize ewes for mating or insemination during both nonbreeding and breeding seasons, and to synchronize donor and recipient ewes in embryo transfer programs. During the breeding season, ewes that fail to conceive following progestagen and eCG treatment will show estrus again after 16–19 days. This usually does not occur during the anestrous period.

Insertion, for 11–14 days, of intravaginal sponges impregnated with progesterone analogues (medroxyprogesterone acetate (MAP) or fluorogestone acetate (FGA)), or insertion of a silastic drug-release device containing progesterone, results in prolongation of the luteal phase and inhibition of ovulation in the ewe as long as the treatment lasts. Following cessation of the progestagen treatment, ewes will come into heat and ovulate. The time interval for the onset of estrus varies with the age of the ewe, time of year, and the type of progestagen treatment and its duration. Intramuscular injection of follicle-stimulating hormone (FSH) in the form of eCG at the end of the progestagen treatment supports follicular growth and ovulation rate, and causes better synchronization of estrus and ovulation.

The recommended dose for treating ewes with eCG is 350–700 international units (IU). Within this range, increasing the dose of the injected eCG is followed by moderate increase in ovulation rate and consequently litter size. In some cases, repeated use of eCG was found to result in the appearance of anti-eCG antibodies in the circulation. Those ewes display lower fertilization rate and lower fertility and prolificacy.

Superovulation

Higher doses of eCG (1000–1200 IU) may be used for superovulation induction in donor ewes in embryo transfer programs. Using eCG for superovulation has the advantage of being relatively inexpensive. However, embryo yield following eCG treatment is relatively low as a large proportion of the follicles that develop fail to ovulate and the excess estrogen produced by those follicles causes lower fertilization rates. The common protocol for superovulation of sheep combines a priming dose of eCG and several injections of commercially available ovine, equine, or porcine pituitary extract containing FSH. Yields following this treatment can increase to 7–9 embryos per donor.

An alternative and different method for estrous synchronization by hormone manipulation is the induction of luteolysis of the CL by injection of PGF2a or one of its analogues. This may result in the onset of estrus in cycling ewes 48 h later. The use of this treatment is limited to the breeding season and as the exact stage in the cycle is not known for each ewe, it is common to apply two injections, 10 days apart.

Breeding Management

Ram-to-Ewe Ratio

Breeding management differs among flocks. For uncontrolled mating management, rams are introduced to the ewes and allowed to remain with them throughout the breeding season. The ram-to-ewe ratio is about 1:50 or less and the age structure of the rams and ewes is considered when the ratio is being decided. Under this system, ewes often mate with more than one ram. Controlled mating is practiced when the identity of the sire is important for genetic breeding purposes. In that case, a single-sire mating system is applied where only one ram is introduced to a group of ewes, or hand mating is performed where mating of each ewe is predetermined and recorded. Hand-mating is often required in breeding fat tail sheep when rams are not trained to lift the fat tail before mounting the ewe. AI is considered to be the most controlled but also the most labor-intensive breeding management system.

Artificial Insemination

AI in sheep using fresh or chilled ram semen is applied mainly to support large-scale breeding programs. Other advantages of AI over natural service are the need for fewer rams and the possibility of extensively using elite rams. Usually, semen is collected from rams using artificial vagina. In each collection, up to 1.0 or 1.5 ml of semen can be collected with a concentration of $2–6 \times 10^9$ spermatozoa per ml. Considerable differences may exist between rams in their sperm quality and quantity. Season, ram age, environmental conditions such as nutritional level and climate, and frequency of ejaculations determine semen characteristics.

Most AI in sheep is via vaginal insemination as the cervix of the ewe is hard to penetrate. Usually, a dose of 0.2–0.3 ml of undiluted or diluted ram semen containing about 500 million spermatozoa is deposited. The optimal time for cervical inseminations is toward the end of estrus, before ovulation takes place. Conception rates following AI are generally somewhat lower than those obtained by natural matings. Applying two consecutive inseminations 12 h apart improves conception rate following AI.

AI with fresh semen was the common reproductive practice in commercial sheep flocks in the former Soviet Union. In each flock, heat detection by teaser rams was practiced every morning during the breeding season and ewes in heat were inseminated twice a day. AI with fresh semen became of practical use in commercial flocks in western countries only after estrous synchronization procedures became available, which enabled fixing the insemination time. Currently, the leading country in applying AI in sheep is France, with about 410 000 AI services performed in 1999, serving the Lacaune breeding scheme only. A procedure for transcervical intrauterine insemination has been described for sheep. However, the fertility rate following those inseminations has been relatively low.

Artificial Insemination Using Frozen Semen

Unlike the situation with dairy cattle where AI using frozen–thawed bull semen has become the major reproductive procedure in commercial herds, AI with frozen ram semen is of limited use in sheep due to the unsatisfactory pregnancy rates obtained by vaginal insemination. It has been found that frozen–thawed ram semen has limitations in passing the cervix. While new diluents that will overcome this problem are being investigated, satisfactory conception rates with frozen–thawed ram semen can be obtained through laparoscopic intrauterine inseminations.

Intrauterine insemination is performed while the ewe is tied upside down on a cradle. Before the operation, the wool of the lower belly area is removed and the skin is sterilized with an antiseptic solution. Following the application of local anesthetic, both an endoscope connected to a light source via fiber-optic cable and an inseminating pipette loaded with semen are inserted into the peritoneal cavity of the ewe. Looking through the endoscope, the operator guides the tip of the inseminating pipette to the uterus and the semen is injected into the uterine lumen. Usually, about 25 million sperm are introduced into each uterine horn. Only few minutes are needed for a skilled and experienced operator to perform an intrauterine insemination. After a rest of 2–3 h, the inseminated ewe can rejoin the flock.

Pregnancy Testing in Sheep

Accurate pregnancy diagnosis performed soon after mating provides a valuable reproductive management tool. Following positive results, pregnant ewes can be grouped and offered appropriate rations based on the number of fetuses they are carrying. Nonpregnant ewes, on the other hand, can be culled early on or maintained on low-cost feeding till they are bred again. A practical way of identifying nonpregnant ewes during the breeding season is simply by observing whether the ewe goes back into estrus after previous mating. Sterile rams or rams fitted with a ewe-marking harness can be used to identify the cycling ewes.

Ultrasonography

Pregnancy testing methods are evaluated simultaneously for their ability to detect pregnancy and number of fetuses as early as possible, their accuracy in detecting both pregnant and nonpregnant ewes, their operational costs, and their simplicity and feasibility for application on-farm. With respect to these parameters, real-time ultrasonic scanning is currently the method of choice. Ultrasonography accurately detects pregnancy in sheep at a reasonable cost as early as 1 month postmating, and an experienced operator can distinguish between one, two, or three or more fetuses (**Figure 3**).

Other Methods

Pregnancy can be confirmed or rejected even sooner, as early as 17 or 18 days after mating, by monitoring plasma progesterone levels using radioimmunoassay. Plasma progesterone levels are relatively lower in cyclic ewes at estrus and relatively higher in pregnant ewes. However, as this method is expensive and results are not obtained immediately, it is used mainly for research

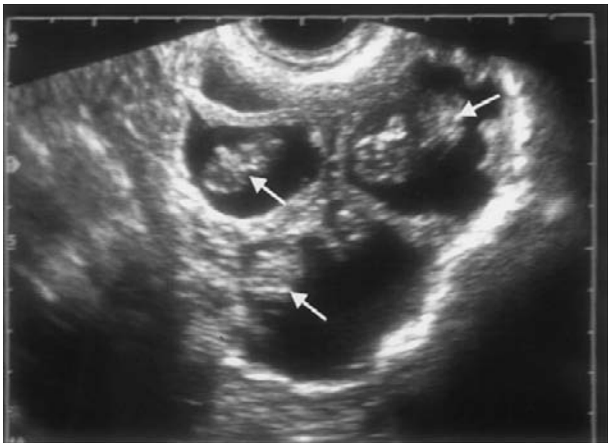

Figure 3 Ultrasonographic scan at 40 days of pregnancy of an Afec-Assaf ewe carrying three fetuses (fetuses are indicated by arrows).

purposes. In addition, the method suffers from false positives, as it does not detect embryo mortality beyond the testing day. Other pregnancy detection methods include hormone assays for placental lactogen, which is secreted only during pregnancy, rectal–abdominal palpation, and monitoring of fetal heart activity. However, these methods can be performed only during advanced stages of pregnancy and are less accurate than the ultrasound scan.

Genetics of Reproduction: Use of Prolific Breeds

Reproductive efficiency of sheep flocks depends largely on the genotype in use. High prolificacy in sheep (about 2.5 lambs born per lambing) is inherited either as a multilocus trait, as the case in Finnsheep, Romanov, and East Friesian breeds, or as a trait controlled by a single autosomal or X-linked major gene, as has been identified in Booroola Merino and Inverdale genes, respectively. Both genes were mapped and identified as members of the TGF-β pathway. Outside of their native environment, prolific breeds are used mainly for crossbreeding or are included in gene introgression breeding schemes. Combining the use of prolific genotypes with a frequent mating regime, hormonal synchronization, and improved lamb survival is the basis for intensive lamb production systems developed in several countries.

Conclusion

Records of introducing new methods of reproductive management go back to biblical times with the interesting story of Jacob, who synchronized sheep and goats by exposing them to peeled rods posted by water troughs (Genesis 30, 37–39). Understanding the environmental, physiological, and genetical bases behind the reproductive activity of sheep has led to establishment of methods for controlling, improving, and intensifying reproductive efficiency. While some methods like AI or pregnancy diagnosis have become part of the day-to-day flock reproductive management routine in many cases, other methods such as embryo transfer or gamete and embryo cryopreservation have so far only limited use in sheep.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Pregnancy Toxemia. **Gamete and Embryo Technology**: Artificial Insemination; Multiple Ovulation and Embryo Transfer. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Estrous Cycles: Characteristics; Estrous Cycles: Postpartum Cyclicity; Estrous Cycles: Puberty; Estrous Cycles: Seasonal Breeders; Mating Management: Artificial Insemination, Utilization; Pregnancy: Characteristics; Pregnancy: Physiology.

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I

ICE CREAM AND DESSERTS

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Ice Cream and Frozen Desserts: Product Types

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Introduction

Frozen dairy desserts are characterized by containing milk solids (which may or may not include milk fat) and being consumed in the frozen state. Typically, freezing is performed while the mix is under shear ('dynamic' freezing, as opposed to quiescently frozen, although there are several exceptions) and the products are also concomitantly whipped such that they contain air and thus can be categorized as a frozen foam (although again there are some notable exceptions, such as frozen pelleted ice cream mix in the form of a bead or sphere). Within the frozen dairy desserts category, ice cream is the most widely consumed product and can be found with many variations: regular ice cream (usually defined by minimum levels of fat and either food solids or milk solids or both, according to the legal definition of the country involved, therefore also including higher-fat premium-type products), low-fat or non-fat versions, no-sugar-added or sugar-free versions, all available in multiple flavors and shapes (including hand-held or impulse products). The category can be further divided according to hard-frozen products, those that contain a second freezing step after the dynamic freezing step, and soft-frozen products, those that are consumed directly after dynamic freezing with no hardening step. Also, frozen dairy desserts include frozen custard, frozen yogurt, and sherbet, all of which could be hard or soft frozen, and frozen milk shakes and smoothies. Frozen desserts in general (again, characterized by being consumed in the frozen state) would also include products that contain no milk-derived ingredients, for example, sorbets, water ices, or plant protein-based products such as soy/tofu frozen desserts, although these are not discussed further. All these products generally contain seven categories of ingredients: fat, milk solids-not-fat (the principal source of protein), sweeteners, stabilizers, emulsifiers, water, and flavors. Manufacturing is performed by the preparation of a suitable liquid mix by blending, pasteurizing, homogenizing, cooling, and aging at 4 °C; subsequently, freezing that mix to −5 °C through a scraped-surface freezer while under shear (which incorporates air and produces small, discrete ice crystals); optionally incorporating any flavoring materials that will remain discrete in the product (fruits, nuts, candy, or bakery pieces); packaging or shaping (as in the case of 'novelty' or 'impulse' products); and then finally blast freezing these products to a temperature of −25 to −30 °C. This article further defines and describes ice cream and the other frozen dairy desserts. Ingredients and processes are

Table 1 *Per caput* consumption of ice cream and related products in selected countries (2002, unless otherwise noted)

Country	*Consumption*
New Zealand	22–23 (2006)
United States	18.3 (2007)
Australia	18 (2007)
Finland	14 (2006)
Sweden	12
Italy	9.2
Ireland	9.0
Denmark	8.7
Canada	8.7 (2007)
United Kingdom	6 (2007)
Chile	5.6
Malaysia	2.0
China	1.9 (2006)

detailed in **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Manufacture.

Global *per caput* consumption of ice cream and related products (it is difficult to know exactly what is included in these data from different countries) is presented in **Table 1**. New Zealand ranks first, followed by the United States and Australia. Although not quite the largest consumer of ice cream, the United States is the largest producer. The US ice cream market in 2007 was $23 billion. The global value of the ice cream industry is US$59 billion, growing on average by 4% per year with the biggest growth in Latin America, eastern Europe, Africa, and the Middle East (all growing at 7–9% per year). More than 90% of American households purchase ice cream. At-home consumption represents $9 billion in sales revenues while away-from-home consumption represents $14 billion. In the United States, frozen dairy dessert production in 2006 could be subdivided as follows: 3700 million liters of regular ice cream, 1500 million liters of low-fat and non-fat ice cream, 260 million liters of frozen yogurt, and 200 million liters of sherbet for a total of 5700 million liters. When classified according to freezing/serving method, 4300 million liters were hard-frozen products and 1400 million liters were soft-serve. Of the hard-frozen products, 76% were regular products and 11.7% were low-fat or non-fat, while of the soft-frozen products, 18% were regular and 69% were low-fat.

Global market share according to point of manufacture/consumption, take-home, impulse, or artisanal products, is shown in **Table 2**. The take-home category is defined as grocery store purchases and home consumption, the impulse category is categorized by hand-held, single-serving eat-on-the-spot products (sometimes referred to as novelty products), while the artisanal category is defined as being manufactured at the site of purchase and consumption or sold directly by the manufacturer to the consumer (e.g., ice cream parlors, street vendors). There are very large differences globally, impacted in large part by the presence of home freezers.

Table 2 Global market share characterized by point of manufacture/consumption (2006)

	Take-home	*Impulse*	*Artisanal*
Global, by volume	41	50	8
Global, by monetary value	26	52	21
Regional, by monetary value			
North America	43	34	21
Western Europe	21	45	34
Australasia	39	49	19
Asia Pacific	10	86	4
Eastern Europe	28	67	5
Latin America	24	63	13

The ice cream industry is very progressive with many new products being introduced annually. Some of the current formulation trends in the industry include a growing interest in 'reduced' or 'no' claims for fat, calories, or sugar; the use of nutritionally functional additives (e.g., vitamins or minerals) or flavors showing added nutritional functionality (e.g., high in antioxidants); a renewed focus on frozen yogurt, particularly with probiotic cultures, although this represents a very small segment of the overall total market of frozen desserts; and formulations approved as kosher or halal compliant. Products are also marketed with a growing social awareness, including fair trade to primary producers, organic products, environmental sustainability, and social responsibility by aligning products with particular social justice causes.

Product Types

Ice Cream

Ice cream composition is highly regulated in almost all legal jurisdictions. The US compositional standards, for example, can be found in the Code of Federal Regulations, Title 21 Food and Drugs, Part 135 Frozen Desserts and subsections for definitions, ice cream and frozen custard, goat's milk ice cream, mellorine (ice cream with non-dairy fat substituted for milk fat), sherbet, and water ices. According to the US standard for frozen desserts, regular ice cream must contain at least 10% milk fat and 20% total milk solids. Ice cream containing 10% milk fat and 10% milk solids-not-fat provides approximately 3.5–4% milk-derived protein. Manufacturers are permitted to substitute up to 25% of the milk solids-not-fat content with the solids of cheese whey.

The regulations for ice cream products within each legal jurisdiction will vary somewhat from those of the

United States. For example, in Canada, regular ice cream must contain at least 10% milk fat and 36% total solids, but there is no minimum for total milk solids. Although countries in the European Union (EU) are attempting to agree on common compositional standards, the current minimum fat content of ice cream ranges from 5% in the United Kingdom to 10% in Germany. Some countries (e.g., United Kingdom and many countries of Asia) permit vegetable fat to replace milk fat, provided that disclosure is by obvious labeling. Most EU countries have a minimum total solids requirement of 29–30% for ice cream. Like the US standard, European standards generally specify the amounts and types of non-dairy ingredients that are permitted, including sweeteners, stabilizers, emulsifiers, colors, and flavorings.

Legal standards also usually exist for the amount of air that can be incorporated into ice cream. ‘Overrun’ is the increase in volume that the manufacturer obtains in the frozen product compared to the volume of the mix. It can be calculated by volume or by weight. For example, if 5000 l of mix is frozen into 9000 l of finished ice cream, the overrun is calculated as (volume of ice cream − volume of mix used to make that ice cream)/volume of mix × 100%, for example, $(9000 - 5000)/5000 \times 100\% = 80\%$. Overrun can also be calculated for individual containers by determining the portion of the mix displaced by air, as follows: % package overrun = (mix weight $(\mathrm{g\,l^{-1}})$ − product weight $(\mathrm{g\,l^{-1}})$)/product weight $(\mathrm{g\,l^{-1}}) \times 100\%$. For example, if a 1 l container of unfrozen mix weighs 1100 g and 1 l of the frozen product weighs only 550 g, the increase in volume is twofold and the overrun is 100%. In the US regulations, ice cream must weigh at least $540\,\mathrm{g\,l^{-1}}$ and contain at least $192\,\mathrm{g}$ food solids $\mathrm{l^{-1}}$. This limits the maximum amount of air that can be whipped into the mix during freezing to approximately100%, or the same volume as the volume of the mix that goes into the package. Canada requires a minimum of 180 g food solids $\mathrm{l^{-1}}$ but no minimum weight per volume. European standards generally do not specify minimum weight per unit of volume.

Standards for ice cream provide opportunities for manufacturers to go beyond the minimum requirements to make a range of product qualities, defined loosely as ‘economy’, ‘regular’, ‘premium’, or ‘superpremium’ ice creams (**Table 3**). Economy ice creams typically are at the minimum requirements for composition and maximum limits for overrun, are made with the most economical ingredients, and sell for the lowest price. Increasing amounts of higher-quality ingredients as well as less overrun characterize standard, premium, and superpremium products, all of which fall within the legal definitions of ‘ice cream’. Whereas economy ice cream may be made using the more concentrated and shelf-stable forms of milk, such as butter, skim milk powder, and dry whey, superpremium products are more likely to be prepared from fresh concentrated milk and cream. Furthermore, economy ice creams tend to contain high amounts of corn sweeteners and stabilizers, and to be flavored artificially, whereas premium and superpremium ice creams may contain mostly sucrose as sweetener, little or no stabilizer, and natural flavors.

Fruits, nuts, chocolate, baked items, confections, and ripple sauces are the favorite flavorings for frozen desserts. Any liquid flavors or colors that are to become homogeneously distributed throughout the ice cream are generally added to the mix prior to freezing whereas those that are discrete in the products are added after dynamic freezing through a rate-controlled ingredient feeder or ripple pump. Consumption by flavor in the United States is shown in **Table 4**. Plain vanilla-flavored

Table 3 Approximate composition (%) of commercial frozen desserts by formulation category

Group	*Milk fat*	*Milk solids-not-fat*	*Sweeteners*[a]	*Stabilizers*[b]	*Total solids*
Non-fat ice cream	<0.5	12–14	18–22	1.0	28–32
Low-fat ice cream	2–5	12–14	18–21	0.8	28–32
Light ice cream	5–7	11–12	18–20	0.5	30–35
Reduced-fat ice cream	7–9	10–12	18–19	0.4	32–36
Economy ice cream	10	10–11	15–17	0.4	35–36
Standard ice cream	10–12	9–10	14–17	0.2–0.4	36–38
Premium ice cream	12–14	8–10	13–16	0.2–0.4	38–40
Superpremium ice cream	14–18	5–8	14–17	0–0.2	40–42
Frozen yogurt: regular	3–6	9–13	15–17	0.5	30–36
Frozen yogurt: non-fat	<0.5	9–14	15–17	0.6	28–32
Sherbet	1–2	1–3	22–28	0.4–0.5	28–34

[a] Includes sucrose, glucose, corn syrup solids, maltodextrins, polydextrose, and other bulking agents, some of which contribute little sweetness.

[b] Includes ingredients such as locust bean gum, guar gum, carrageenan, cellulose gum, and cellulose gel, as stabilizers, and also mono- and diglycerides and polysorbate 80, as emulsifiers.

Table 4 US ice cream consumption by flavor (2006) (%)

Vanilla	30.2
Chocolate	10.0
Butter pecan	4.0
Strawberry	3.7
Chocolate chip mint	3.2
Neapolitan	3.0
Cookies and cream	2.6
Chocolate chip	2.5
Rocky road	1.9
Cookie dough	1.5
French vanilla	1.4
Fudge	1.2
Cherry vanilla	0.9
Peanut butter	0.9
Chocolate marshmallow	0.7
Coffee	0.7

ice cream accounts for 30% of ice cream production; not only is it consumed as is but it is a popular topping for desserts and is used in milk shakes, banana splits, sundaes, and others, within the retailing sector.

Frozen Custard

Formulations for frozen custard, also known as ‘French ice cream’, are generally the same as for other ice creams of the same flavor, except that egg yolk solids are added. In the United States, the minimum amount of egg yolk solids is 1.4%. The French product ‘*glace aux oeufs*’ must contain at least 7% egg yolk solids. The German product called ‘Kremeis’ contains at least 240 g of whole or whipped egg per liter of milk used in the formula.

Reduced-Fat and Nonfat Products

Reduced-fat (‘light’), low-fat, and non-fat products have increasingly gained in popularity with the global obesity epidemic and the desire for consumers to reduce caloric intake while satisfying their demand for sweet and creamy desserts, and also with increasing nutritional knowledge regarding the increased risk of cardiovascular disease with higher intake of saturated fats. The following are the US Food and Drug Administration requirements for labeling of foods with lowered fat content: reduced-fat – at least 25% less fat than in the reference product; light – 50% reduction in total fat compared with the reference product, or one-third reduction in calories if less than 50% of the calories is from fat; low-fat – not more than 3 g of total fat per serving; non-fat or fat-free – less than 0.5 g of fat per serving. A serving is commonly 120 ml. In Canada, lower-fat versions are defined as light ice cream (5–7.5% milk fat), ice milk (3–5% milk fat), or frozen dairy dessert (undefined fat content). Most EU countries permit sales of ice milk containing at least 2.5–5% fat.

Approximate compositions of reduced-fat products are given in **Table 3**. As fat is removed from ice cream formulations, other ingredients must be added to keep the water content within reasonable limits for two reasons: (1) regulations stipulate a minimum concentration of food solids or dry matter, and (2) too much water means too much ice in the frozen product, resulting in a very cold and icy product with poor keeping quality. It is possible to formulate reduced-fat or ‘light’ ice creams, down to about 4–5% fat, with traditional ingredients. The low-temperature extrusion process, which takes ice cream from −5 to −12 °C under defined shear conditions in a screw extruder, enables reduced-fat products with similar textures to their full-fat counterparts, due to reduced ice crystal and air bubble size distributions. However, with low-fat products, less than 4% fat, there exists a greater product development challenge and the need to utilize fat replacers. Traditionally, fat replacers have been classified in relation to the materials that comprise them: carbohydrate-, protein-, or fat-based. Water-soluble carbohydrate polymers typically used in low-fat formulations include cellulose products, starches, dextrins, maltodextrins, and polydextrose. Carbohydrate-type fat replacers contribute bulk and increase viscosity while helping to limit the growth of ice crystals. The common sources of protein-type fat replacers are cheese whey and egg white. These proteins are processed into colloidal particles that vary in diameter from 0.1 to 3.0 mm, a size range that permits them to be sensed on the tongue as creamy. Monoacylglycerols and diacylglycerols are useful at low concentrations (<1.0%) as fat-based fat mimetics. Decreasing the fat content of ice cream decreases the creamy sensation and increases the intensities of flavors of skim milk powder and of corn syrup. Vanilla flavor is perceived more quickly in non-fat ice cream than in ice cream containing 10% milk fat.

No-Sugar-Added/Sugar-Free/Low Glycemic Index Products

The global rise in the prevalence of type II diabetes mirrors the global epidemic of obesity. Diabetic people have an impaired capacity to reduce blood glucose levels after consumption of high-sugar-containing products. For these people, the large amount of sucrose and glucose normally used in ice cream needs to be replaced with an acceptable sweetener, to reduce the glycemic index of the product. The sugar alcohols, or polyols, have been the sweeteners of choice, since they are absorbed much more slowly than glucose. When substituting sweeteners in ice cream formulations, the factors to be considered include sweetness, freezing point depression, and contribution to total solids. Sorbitol (a monosaccharide) has been used for many years but the intake of sorbitol must be restricted because of its laxative nature. Other polyol sweeteners include xylitol or mannitol (both monosaccharides) or maltitol or lactitol (both disaccharides). These sweeteners allow matching of the freezing curves to conventional formulations due to their freezing point depression characteristics. If necessary, sweetness levels can be boosted with a nonnutritive high-potency sweetener such as aspartame or sucralose, but these, by themselves, do not contribute to total solids or freezing point depression. Likewise, total solids or viscosity (bulk) can be enhanced with a product like polydextrose, but this by itself does not contribute either sweetness or freezing point depression. Thus, careful blending of alternative sweeteners is required to provide all of the necessary functional properties when producing no-sugar-added products. Sugar-free products have the added complication of needing to eliminate lactose from the milk solids-not-fat component of the mix, which can be done either by lactose hydrolysis or with the use of milk protein-derived ingredients.

Sherbets

Sherbets are frozen dairy desserts characterized by being low in milk ingredients, high in sugar, and slightly acidified. Milk solids, including milk fat, are usually limited to 5%, while total sweetener content can approach 25% or greater in a product that contains 30–35% total solids (**Table 3**). A general formulation might contain 1–2% milk fat and 3–4% milk solids-not-fat. Sherbets typically have an acidity, calculated as citric acid, of not less than 0.35%. Most sherbets are flavored with fruit, fruit juice or juice concentrates, and artificial flavorings. Citrus flavors (lime, lemon, orange) are quite common. Examples of non-fruit sherbets are those flavored with spices, chocolate, or coffee. Particulates, such as pieces of fruit, may also be added to sherbets.

Frozen Yogurt

Frozen yogurt characteristically has the composition of light or low-fat ice cream (**Table 3**). Additionally, it contains cultures of the two yogurt bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus,* and these have been fermented to produce developed acidity. Typically, these bacteria are added to a portion of milk that has been heated to 85 °C and held for 15 min. The mixture is then incubated at about 42 °C to permit acid and flavor to be produced. This cultured material is then added to the other ingredients of the mix that have been pasteurized. Commonly, 10–20% of culture is added to the yogurt mix to produce a titratable acidity, expressed as lactic acid, of about 0.30%. Some processors have added the probiotic (health-promoting) bacteria, *Bifidobacterium* spp. and/or *Lactobacillus acidophilus*, to frozen yogurt. Both have the potential to become resident in the colon when ingested in high numbers where they provide numerous health benefits. Additionally, prebiotics such as inulin may be added to the product as nutrients for the probiotic bacteria.

Soft-Frozen Products

Sales of dairy desserts soft-frozen at the site of consumption had their beginning in the 1930s and grew quite rapidly after World War II. Any of the above categories of products – ice cream, frozen custard, low-fat ice cream, or frozen yogurt – can be served soft-frozen although there are typically slight variations in soft-serve formulations compared to their hard-frozen counterparts. As shown by the data above, in the United States, in 2006, soft-frozen dairy products accounted for 25% of the total frozen dairy dessert market and about 70% of these were low-fat ice creams. Soft-frozen products are typically prepared at the site of consumption from mixes that have been processed in dairy processing facilities and are packaged and distributed in bulk. Low-fat mixes for soft-serve typically contain 3–5% fat, 11–14% milk solids-not-fat, 13–15% sweetener, and 0.3–0.5% stabilizer/emulsifier. Mixes used for soft-frozen ice cream are relatively low in total solids (30–35%) compared with those used for hard-frozen ice creams (36–40%). However, the amount of overrun in the soft-frozen desserts is commonly 50% versus 90–100% for hard-frozen ice creams. Soft-serve freezers are of the batch type. The refrigeration system maintains a set temperature so that frozen product can be dispensed over an extended period of time. This means that mixes must be formulated to limit churning of fat caused by agitation within the freezer cylinder during times of slow product turnover.

Milk Shakes and Smoothies

Milk shakes, like soft-frozen products, are prepared for consumption at the retail outlet directly from milk shake mixes that have been processed at a dairy processing facility and packaged and shipped in bulk containers. These products are common in fast-food outlets. A typical mix contains 3% milk fat, 13% milk solids-not-fat, 8% sucrose, 3% corn syrup solids, and 0.4% stabilizer, giving a total solids content of 28.4%. Smoothies are a category similar to milk shakes but containing a wider range of other flavoring ingredients. Many formulations exist for smoothies, but the most typical is a blend of whole fruit and/or fruit juice or vegetable juice, cream or yogurt, and crushed ice. The mixture is blended in a high-speed blender and served promptly.

Impulse/Hand-held/'Novelty'/Fancy-Molded Products

This category of products can be made from any of the above categories of formulations – ice cream, frozen custard, low-fat ice cream, or frozen yogurt – but is characterized by the purchase in a single-serving size for immediate consumption while being hand-held. It is also characterized by being 'novel' in shape or design or flavor or presentation (e.g., chocolate enrobed or with a bakery product or in the form of a well-known chocolate bar product), to attract impulse purchases and excitement on the part of the consumer. Products can be directed specifically at various market segments, such as children's or adult novelty products. Examples are quiescently frozen ice cream bars, combinations of ice cream with flavored coatings and confections, cone and cup items, and fancy-molded products. As previously discussed and shown in **Table 2**, in some parts of the world, this category represents a large fraction of the total ice cream market. It also represents more added value than the take-home product market. Novelties can be formed in molds, for example with stick insertion, or a very stiff form of the product can be extruded onto a very cold surface in a variety of shapes or subsequently pressed into a variety of shapes, also with possible stick insertion, and passed through a freezing tunnel. Coatings can also be applied after hard freezing is completed. Among the novelty items intended for multiple servings are fancifully decorated cakes and pies made with frozen desserts. Frequently, these are made in specialty ice cream shops, but they can also be produced on a larger scale in ice cream factories.

Conclusion

Manufacturers of ice cream and related frozen dairy desserts combine in varying proportions a variety of dairy and nondairy ingredients, including sweeteners and flavorings, to make a liquid mix, freeze this mix while stirring to incorporate air and to produce small ice crystals and smooth texture, add flavorings, and serve the resulting products soft-frozen or shape them into novelty items or package them for take-home products, and in either of the previous two cases harden them at very cold temperatures. The wide variety of product types includes ice cream, as defined by the legal jurisdiction, reduced- or low- or non-fat varieties, reduced- or low- or no-sugar varieties, frozen yogurt, sherbet, and others. Milk shakes and smoothies are also considered as frozen dairy desserts. Ice cream can be further subdivided into economy, standard, premium, and superpremium categories. With so many variables in formulations, ingredients, and processes available to the producer, and with opportunities to vary the shape, size, and presentation of the product, consumers are able to enjoy a surprisingly large variety of frozen desserts at a wide range of costs. The industry that started with the addition of flavorings to snow now satisfies the desires of consumers wherever milk is produced and refrigeration is available.

See also: **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Manufacture.

Further Reading

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Ice Cream and Frozen Desserts: Manufacture

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Introduction

Frozen dairy dessert products are a unique frozen food because they are consumed in the frozen state, usually as a scooped product or as a single-serving item (sometimes on a stick and often with other confectionery items). These products rely on a concomitant freezing and whipping process to establish the desired structure and texture. The manufacturing process for most of these products is similar and involves the preparation of a liquid mix; whipping and freezing this mix dynamically under high shear to a soft, semi-frozen slurry; incorporation of flavoring ingredients to this partially frozen mix; shaping and packaging the product; and further freezing (hardening) of the product under static, quiescent conditions (**Figure 1**). The liquid mix is prepared by blending the desired ingredients, followed by pasteurization (batch or continuous), homogenization, and aging. Swept-surface freezers are used for the first freezing step, while forced convection freezers, such as air blast tunnels or rooms, or plate-type conduction freezers are used for the second freezing step.

Ice Cream Ingredients

Ice cream mix and other frozen dairy dessert formulations identify the following as their content: of milk fat, milk solids not fat (MSNF), sweeteners, stabilizers, emulsifiers, and water that are desired (*see* **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Product Types for details of formulations). Dairy and other ingredients used to supply these components are chosen on the basis of availability, cost, and expected quality. The use of fat derived from milk ingredients (e.g., cream and butter) is common in North America and many other parts of the world, while fat derived from non-dairy sources (e.g., coconut oil and palm kernel oil) is more common in parts of Europe and Asia. The triglycerides in milk fat have a wide melting range, 40 to −40 °C. Consequently, at refrigeration temperatures there is always a combination of liquid and crystalline fat within the globules. The resulting solid:liquid ratio at freezer barrel temperatures is important for ice cream structure formation, as crystalline fat is required for partial coalescence. Thus, non-dairy fat sources must also be chosen to provide a suitable solid fat content.

The MSNFs comprise the lactose, casein micelles, whey proteins, minerals (ash), vitamins, and other minor components of the milk or milk products from which they were derived (e.g., condensed skim milk, skim milk powder, and whey-protein-derived ingredients). Proteins contribute much to the development of structure in ice cream, including emulsification, whipping, and water-holding capacity. Emulsification properties of proteins in the mix arise from their adsorption to fat globules at the time of homogenization. Whipping properties of proteins in ice cream contribute to the formation of the initial air bubbles in the mix. The water-holding capacity of proteins enhances the viscosity in the mix, which imparts a beneficial body to the ice cream, increases the meltdown time of ice cream, and contributes to reduced iciness.

In addition to providing sweetness, sweeteners improve the texture and palatability of the ice cream and enhance flavors. Their ability to lower the freezing point of a solution imparts a measure of control over the temperature–hardness relationship (**Figure 2**). In determining the proper blend of sweeteners for an ice cream mix, the total solids required from the sweeteners, the sweetness factor of each sugar, and the combined freezing-point depression of all sugars in solution (including lactose from the MSNF component) must be calculated to achieve the proper solids content, the appropriate sweetness level, and a satisfactory degree of hardness. The most common sweetening agent used is sucrose. However, it has become common practice in the industry to substitute sweeteners derived from starch hydrolysate syrup (glucose syrup) for all or a portion of the sucrose. The use of starch hydrolysis products in ice cream is generally perceived to provide greater smoothness by contributing to a firmer and more chewy body, to provide better meltdown characteristics, to bring out and accentuate fruit flavors, to reduce heat shock potential that improves the shelf life of the finished product, and to provide an economical source of solids.

Ice cream stabilizers are a group of ingredients (usually polysaccharides such as guar, locust bean gum, carboxymethyl cellulose, xanthan, etc.) commonly used in ice cream formulations. The primary purposes for using stabilizers in ice cream are to increase mix viscosity, produce smoothness in body and texture of the frozen product, retard or reduce ice and lactose crystal growth during storage (or mask the effects of crystal growth), especially

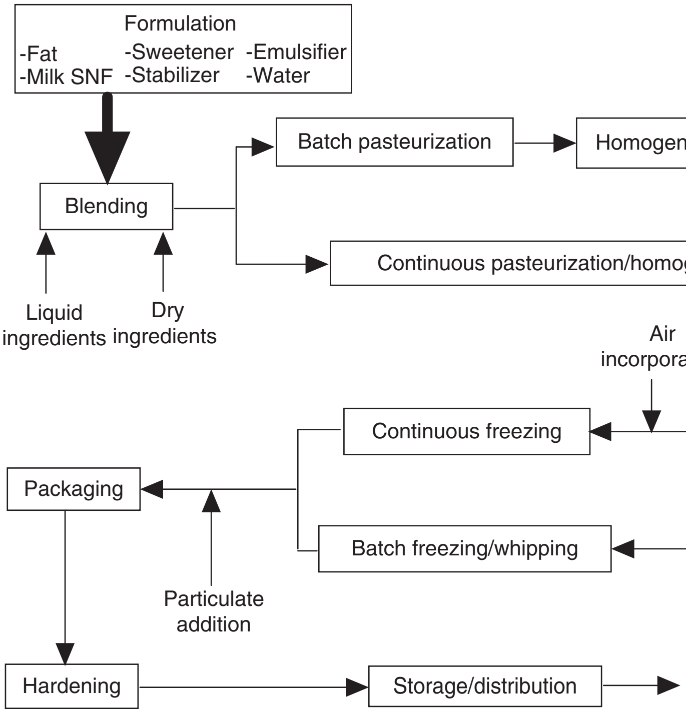
Formulation
-Fat
-Milk SNF
-Sweetener
-Stabilizer
-Emulsifier
-Water
Blending
Liquid ingredients
Dry ingredients
Batch pasteurization
Homogenization
Cooling
Continuous pasteurization/homogenization/cooling
Air incorporation
Continuous freezing
Aging
Batch freezing/whipping
Flavor/color addition
Packaging
Particulate addition
Hardening
Storage/distribution

Figure 1 A schematic illustration of the processing steps in ice cream manufacture.

during periods of temperature fluctuation, known as heat shock, and to hold flavoring ingredients in uniform suspension. The mechanism of action of stabilizers in enhancing frozen stability is related primarily to their effect on the ice and unfrozen serum phases. Stabilized ice cream has been observed by microscopy techniques to have smaller ice crystals than unstabilized ice cream after storage at fluctuating temperatures.

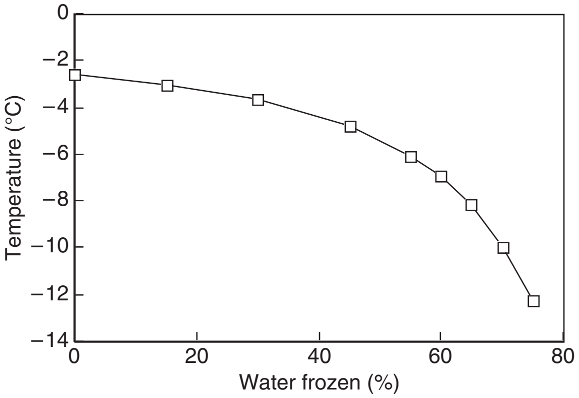

Figure 2 An equilibrium freezing curve for a typical ice cream mix illustrating the relationship between temperature and unfrozen water as determined by freezing-point depression characteristics. This relationship illustrates the maximum amount of ice that may form at any temperature, but due to kinetic considerations, the amount of ice may be considerably less as a result of freezing-rate considerations.

Emulsifiers are sometimes integrated with the stabilizers in proprietary blends but their function and action are very different from the stabilizers. They are used to improve air incorporation in the mix; produce a drier ice cream at extrusion to facilitate shaping, novelty product manufacture, or packaging; provide smoother body and texture in the finished product; and produce a product with good stand-up properties and melt resistance. Emulsifiers used in ice cream manufacture today are of two main types: the mono- and di-glycerides, and the sorbitan esters, such as polysorbate 80, although eggs also provide similar emulsifying properties. Their mode of action is related to their activity at the air–serum and fat–serum interfaces. At the fat–serum interface, they displace proteins from the surface of the fat globules, rendering the fat globules more susceptible to partial coalescence and structure formation during the freezing and whipping process.

Mix Manufacture

Ice cream processing operations can be divided into two distinct stages: mix manufacture and freezing operations. Ice cream mix manufacture consists of the following unit operations: combination and blending of ingredients, batch or continuous pasteurization, homogenization, and mix aging (**Figure 1**). Ingredients are usually pre-blended prior to pasteurization, regardless of the type of pasteurization system used. Blending of ingredients is relatively simple if all ingredients are in the liquid form, as automated metering pumps or tanks on load cells can be used. When dry ingredients are used, powders are added through either a pumping system under high velocity or through a liquifier, a large shear pump with rotating knife blades that chops all ingredients as they are mixed with the liquid.

Pasteurization is designed for the destruction of any pathogenic bacteria that may be present, especially from raw milk sources. In addition, it serves a useful role in reducing the total bacterial load, and in solubilizing some of the components (proteins and stabilizers). Both batch and continuous (high temperature short time, HTST) systems are in common use. In a batch pasteurization system, blending of the proper ingredient amounts is done in large jacketed vats equipped with some means of heating, usually saturated steam or hot water. The product is then heated in the vat to at least 69 °C and held for 30 min to satisfy legal requirements for pasteurization, necessary for the destruction of pathogenic bacteria. Various time–temperature combinations can be used, depending on the legal jurisdiction. Continuous pasteurization is usually performed in an HTST heat exchanger following the blending of ingredients in a large, insulated feed tank. Some preheating, to 30–40 °C, may be necessary for solubilization of the components. Regulations concerning time–temperature combinations for continuous pasteurization usually specify a minimum temperature of 80 °C for at least 25 s.

Following pasteurization, the mix is homogenized using high pressures. Homogenization is responsible for the formation of the fat emulsion by forcing the hot mix through a small orifice under pressures of 15.5–18.9 MPa, depending on the mix composition. The resulting 8–10-fold increase in the surface area of the fat globules is responsible in part for the formation of the fat globule membrane, comprised of adsorbing materials attempting to lower the interfacial free energy of the fat globules. With single-stage homogenizers, fat globules tend to cluster as bare fat surfaces come together or adsorbed molecules are shared, especially in ice cream mix, and this is thought to be less optimal than a monomodal distribution of fat for fat structuring in the ice cream freezing step. Therefore, a second homogenizing valve is frequently placed immediately after the first with applied back pressures of 3.4 MPa, allowing more time for surface adsorption to occur. The net effects of homogenization are in the production of smaller, more uniform fat droplet size, resulting in a greater stability of fat droplets during aging, a better whipping ability, and a smoother, more uniform final product with a greater apparent richness. Homogenization also decreases the potential for undesirable levels of fat churning in the freezer and makes it possible to use products that could not otherwise be used, such as butter and frozen cream.

An aging time of 4 h or greater at 2–4 °C is recommended following mix processing prior to freezing. This allows for hydration of milk proteins and stabilizers (some viscosity increase occurs during the aging period), crystallization of the fat globules, and a membrane rearrangement to produce a smoother texture and better quality product. Non-aged mix exhibits a low viscosity, is very wet at extrusion from the dynamic freezer, and exhibits variable whipping abilities. The appropriate ratio of solid:liquid fat must be attained at this stage, a function of temperature and the triglyceride composition of the fat used, as a partially crystalline emulsion is needed for partial coalescence in the whipping and freezing step. Emulsifiers generally displace milk proteins from the fat surface during the aging period. The whipping qualities of the mix are usually improved with aging. Aging is performed in insulated or refrigerated storage tanks, silos, etc., or in single-walled tanks in chilled rooms, where valves and pipework can also be kept cold. Mix temperature should be maintained as low as possible without freezing.

Freezing Processes

Ice cream freezing also consists of two distinct stages: (1) passing the mix through a swept-surface heat exchanger under high shear conditions to promote extensive ice crystal nucleation and air incorporation, and (2) freezing the packaged ice cream under conditions that promote rapid freezing and small ice crystal sizes (**Figure 1**). The freezing and whipping process is one of the most important unit operations for the development of quality, palatability, and yield of finished product, due to the incorporation of air creating the foam, the formation of the ice phase, and the partial destabilization of the fat emulsion. The objective of ice cream manufacturers is to produce ice crystals that are below the threshold of sensory detection at the time of consumption. This threshold has been suggested to be between 40 and 50 μm. Consequently, the freezing steps of the manufacturing process and the temperature profile throughout the distribution system are critical factors in meeting this objective. Flavoring and coloring can be added as desired to the mix prior to passing through the barrel freezer, and particulate flavoring ingredients, such as nuts, fruits, candy pieces, or ripple sauces, can be added to the semi-frozen product at the exit from the barrel freezer prior to packaging and hardening.

Dynamic Freezing

Continuous freezers dominate the commercial ice cream industry. In this type of process, mix is drawn from the flavoring tank into a scraped-surface heat exchanger, which is jacketed with a liquid, boiling refrigerant (usually ammonia, a chlorinated, fluorinated hydrocarbon (CFC) such as R-12, R-22, or R-502, or one of the newly developed CFC substitutes) (**Figure 3**). Incorporation of air into ice cream, termed the overrun, occurs at this stage (see **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Product Types for details of overrun calculations and variations). Some overrun is necessary to produce desirable body and texture. Two main types of air incorporation systems are used in continuous freezers. In older systems, the pump configuration resulted in a vacuum either at the pump itself or on the mix line entering the pump. Air was then incorporated through a spring-loaded, controllable needle valve. Newer types of freezers use compressed air, which is injected into the mix. This type of air handling system allows for air filtration (0.65 μm micropore filter) prior to entering into the mix.

Following aeration, the water in the mix is partially frozen as the mix and air combination passes through the barrel of the heat exchanger. Ice forms on the inside wall of the heat exchanger from the water in the mix, resulting in a freeze-concentration of the dissolved solutes. Rotating knife blades are responsible for continually scraping this frozen mix off the surface of the heat exchanger wall and mixing it into the bulk flow of freeze-concentrated liquid mix, where these tiny ice crystals grow (**Figure 4**). The dasher keeps the product agitated inside the barrel and provides a more homogeneous mixture of ice and freeze-concentrated liquid (**Figure 3**). Residence time for mix through the annulus of the freezer varies from 0.4 to 2 min, although some products may remain for much longer times; especially with open cage dashers, freezing rates can vary from 5 to 27 °C min^{-1}, and a draw temperature of −6 °C can easily be achieved. The continuous freezer is usually operated under pressure, to minimize the volume of air in the ice cream and thus maximize heat transfer. The air bubbles expand rapidly when the ice cream is drawn through the outlet valve to atmospheric pressure.

The introduction of low-temperature extrusion equipment has been a recent development in ice cream freezing technology. This is a continuous-flow heat exchanger in the form of a screw extruder that reduces the temperature of ice cream from the draw temperature of the barrel freezer, typically −5 °C, to a range of −10 to −14 °C. The ice cream passes at low shear through a twin-screw or single-screw design with a residence time of 1–2 min. Ice cream that results from such processing shows much smaller ice cream and air bubble sizes and the fat is agglomerated into a structure that provides a creamy texture. When the product emerges from the low-temperature extruder, it is still flowable, so some particulates can be added to it and it can be cut and packaged, but it sets up very quickly thereafter to a very firm consistency. Hence, the claim is made that low-temperature extrusion can replace the need for conventional hardening of ice cream.

Batch freezing differs considerably from the continuous systems described previously. The barrel of a batch swept-surface heat exchanger is jacketed with refrigerant and contains a set of dashers and scraper blades inside the barrel. It is filled to about one half volume with the liquid mix. Barrel volumes usually range from 2 to 12 l. The freezing unit and agitators are then activated and the product remains in the barrel for sufficient time to achieve the desired degree of overrun and stiffness. Whipping increases with time and cannot exceed the amount that will fill the barrel with the product (i.e., 100% overrun when starting half full). Batch freezers

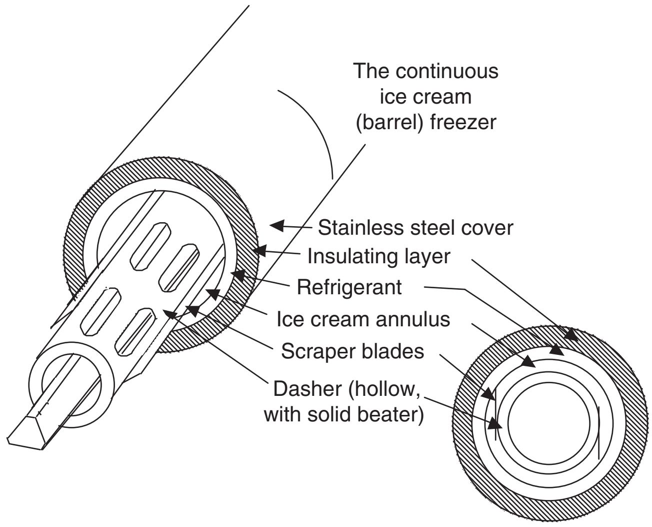

Figure 3 A schematic illustration of the continuous ice cream freezer.

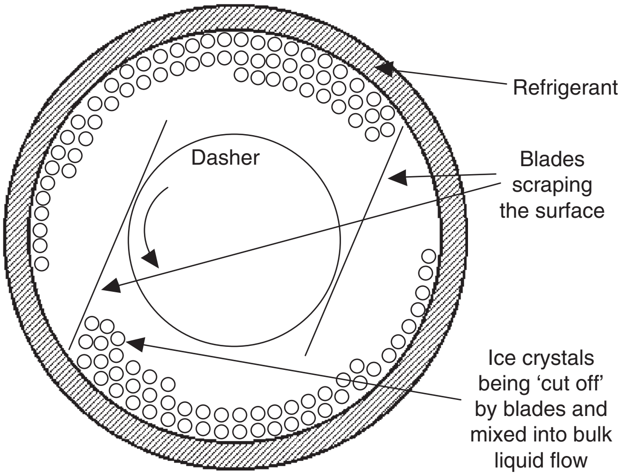
Refrigerant
Dasher
Blades scraping the surface
Ice crystals being 'cut off' by blades and mixed into bulk liquid flow

Figure 4 A cross-sectional view of an ice cream barrel freezer, showing the action of the scraper blades in clearing ice crystals off the surface during rotation.

are used in smaller operations where it is desirable to run individual flavored mixes on a small scale or to retain an element of the 'homemade'-style manufacturing process.

A type of batch freezer is also used for the production of soft-serve-type desserts, but is operated in a semicontinuous mode. In this case, a hopper containing the mix feeds the barrel as the frozen product is removed. Soft-serve freezers may also have pressurized air feed systems to ensure more constant overrun. Soft-serve formulations vary slightly from mixes destined for hardening; typically, the MSNF and emulsifier concentrations are slightly higher whereas the fat and stabilizer concentration may be slightly lower. However, the big distinction between soft-serve products and hard-frozen products is simply that the former are served at an intermediate stage in the production of the latter, and do not go through the hardening process.

The formation of the ice phase during the dynamic freezing stage is responsible in part for the structure and texture of the final product. The crystallization of water to ice involves two major steps: nucleation and crystal growth. Nucleation occurs at the wall of the heat exchanger during start-up. After start-up, the continual scraping action of the blades acts as a seeding mechanism, by providing a source of tiny crystals into the bulk where they grow. Rapid heat removal, which results from low temperatures (large ΔT) in the freezing medium, and rapid agitation, which are both present in the barrel freezer, create numerous, tiny ice crystals. Further temperature reduction during hardening accounts for continued growth of the preformed crystals. As water freezes out of solution in a relatively pure form, the formation of ice acts to concentrate the dissolved sugars, lactose, milk proteins, salts, and hydrocolloids in an ever-decreasing amount of water. Water and its dissolved components are referred to as the serum or matrix of the mix. Because the freezing point of the serum is a function of the concentration of dissolved solids, the formation of more ice concentrates the serum and results in an ever-decreasing freezing temperature for the remaining serum. Thus, at temperatures several degrees below the initial freezing temperature, there is always an unfrozen phase present (**Figure 2**). Ice cream hardness is a function of temperature due to its effect on this conversion from unfrozen water to ice and further concentration of the serum phase surrounding the ice crystals, which helps to give ice cream its ability to be scooped and chewed at freezer temperatures.

Hardening

Ice cream following dynamic freezing, ingredient addition, and packaging is transferred immediately to a hardening chamber (−30 °C or colder, either forced convection or plate-type conduction freezers) where the majority of the remaining water freezes. Rapid initial freezing is essential to set up as many crystal nuclei as possible so that during the maturation or growth stage, their size stays small. In the same context, rapid hardening is also necessary to keep ice crystal sizes small. When hardening is slow, there is too great an opportunity for water still remaining in the ice cream to migrate to crystal centers already formed, resulting in large ice crystals. Many factors need to be considered during the hardening process. The main factors affecting heat transfer are the temperature difference between the product and the freezing medium, the area of product being exposed to the freezing medium, and the heat transfer coefficient for the particular operation. Following rapid hardening, ice cream should be stored at a low, constant temperature, usually −25 °C. Subsequent distribution must maintain these temperatures to protect the structure and textural

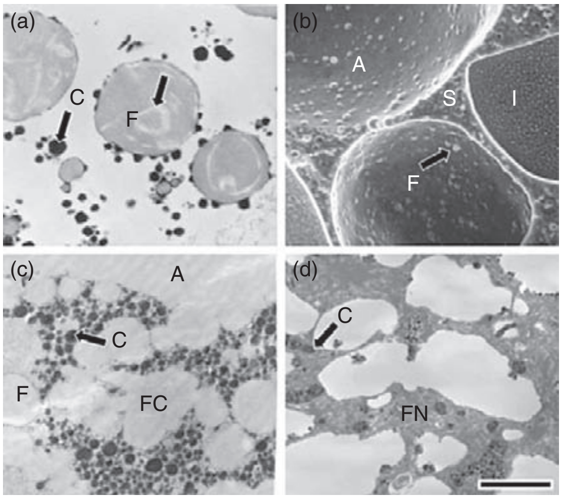

Figure 5 The structure of ice cream mix, ice cream, and melted ice cream. (a) Ice cream mix as viewed by thin-section transmission electron microscopy. arrow = crystalline fat within the globule; c = casein micelle; f = fat globule; bar (shown in (d)) = 0.5 μm. (b) Close-up of an air bubble in ice cream as viewed by low-temperature scanning electron microscopy. a = air bubble; f = fat globule adsorbed to the bubble surface; i = ice crystal; s = freeze-concentrated serum phase; bar (shown in (d)) = 10 μm. (c) Ice cream as viewed by thin-section transmission electron microscopy with freeze substitution and low-temperature embedding. a = air bubble; c = casein micelle; f = fat globule; fc = fat cluster; bar (shown in (d)) = 1 μm. (d) Melted ice cream as viewed by thin-section transmission electron microscopy. c = casein micelle; fn = fat network; bar = 5 μm.

quality of frozen dairy dessert products. Shelf life is also dependent upon temperature of storage and distribution.

Structure Formation during Manufacture

The texture of ice cream is perhaps one of its most important quality attributes. It is the sensory manifestation of structure; thus, establishment of optimal ice cream structure is critical to maximal textural quality. The structure of ice cream begins with the mix as a simple emulsion, with a discrete phase of partially crystalline fat globules surrounded by an interfacial layer comprised of proteins and surfactants (**Figure 5**). The continuous, serum phase consists of the unadsorbed casein micelles in suspension in a solution of sugars, unadsorbed whey proteins, salts, and high-molecular-weight polysaccharides. Ice cream is a complex food colloid in that the mix emulsion is subsequently foamed, creating a dispersed phase of air bubbles, and is frozen, forming another dispersed phase of ice crystals. Air bubbles and ice crystals are usually in the range of 20–50 μm. The serum phase is freeze-concentrated. In addition, the partially crystalline fat phase at refrigerated temperatures undergoes partial coalescence during the concomitant whipping and freezing process, resulting in a network of agglomerated fat, which partially surrounds the air bubbles and gives rise to a solid-like structure (**Figure 5**).

See also: **Homogenization of Milk**: High-Pressure Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification); Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Product Types. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Plant and Equipment**: Pasteurizers, Design and Operation.

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Dairy Desserts

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Introduction and Commercial Scene

In the context of this article, dairy desserts (excluding cultured products and ice creams) comprise a diverse range of consumer ingredients containing significant amounts of dairy ingredients (milk solids). Some of the varied product types and forms are listed in **Table 1**.

In each case, the dessert product is made by transforming milk into a semi-solid, spoonable texture, by using concentrated milk solids and/or texturing ingredients, commonly nondairy ingredients.

Several of the common dairy dessert forms have their origins many generations past, and there are localized variations on a few common themes. A good example is the classic vanilla custard, in essence a starch-thickened milk. Such a product is called 'vanilla custard dessert' in the United Kingdom, 'vanilla vla' in the Netherlands, 'crème dessert' in France, and 'natillas' in Spain.

As a product category, dairy desserts have become increasingly popular in the past two decades. The volume of ready-to-eat (RTE) dairy desserts consumed globally has grown rapidly, with a proliferation of types and variants. This growth has been prompted by several consumer drivers, including convenience, nutrition, and sensory appeal. There is an interesting dichotomy in this food category indulgence and health foods. In fact, dairy desserts represent some of the best examples of true healthy indulgence in a food product.

Simultaneous with these consumer trends, technological developments have expanded product development opportunities for dairy desserts, including

- improved functional ingredients – texture modifiers (thickening, gelation)
- new ultra-high-temperature–short-time (UHTST) processing and aseptic filling systems
- innovative packaging

The global market for dairy desserts is hard to ascertain, because of the diverse range of products involved (as indicated in **Table 1**) and the many local variants around the world. The chilled RTE dairy dessert segment alone is probably worth about US$10 billion (in 2008), and forecasters have predicted that it will have a compound annual growth of about 4%. Europe dominates this segment with nearly 70% of global sales.

In all regions of the world, chocolate is the number one flavor in chilled dairy desserts, with other popular flavors being strawberry, vanilla, and caramel. Different fruit flavors, such as mango, are starting to feature more prominently. In the 12 months to December 2008, over 1300 new products were launched around the world that could be classified as chilled desserts, with 72% of these launches occurring in the European region. Over the 10 years to 2007, there has been a steady increase in the number of chilled dessert launches globally (**Figure 1**), highlighting the increasing popularity of products within this category. As noted above, one of the growth drivers has been health, as seen by the increase in compositional label claims (**Figure 2**).

At a basic level, most dairy desserts involve five key ingredients:

1. water,
2. milk solids-not-fat,
3. fat (dairy and/or vegetable),
4. texture modifiers (thickening/gelling), and
5. flavor and color.

The type/combination of thickening/gelling agents will largely determine the form and functionality of the final product (in combination with the processing regime). Major texture-modifying materials are discussed later, but the two most common ones are starches and carrageenans.

Dairy Dessert Types and Formulations

Ready-to-Eat Creamy and Gelled Desserts

The range of RTE dairy desserts is now immense, and new innovations are being introduced in the market each year. A standard creamy chocolate dairy food is shown in **Figure 3.** Recently, products with complex textures have been particularly popular – multiple layers, inclusions, and so on. Such products require even more attention to the texturizing/stabilizing ingredients than products with a homogeneous texture.

A standard creamy or gelled milk dessert may consist of the following composition: 82% milk (full fat), 10% sucrose, 2% skim milk powder, 3% starch, 0.2% carrageenan, flavor, and color. A milk gel based on gellan gum could contain 86% milk, 13% sucrose, 0.3% disodium hydrogen phosphate (buffering salt), 0.12% gellan gum, 0.1% salt, flavor, and color.

In all cases, the level of starch can be reduced by increasing the level of other hydrocolloids, for example, 0.2% guar gum or locust bean gum, 0.1% xanthan gum, and 2.5% whey protein. Each combination of texturizers

Table 1 Some of the common dairy dessert types and forms

Types	*Selling forms*
Creamy and gelled desserts	Chilled, frozen, canned, shelf stable
Custards/puddings	Chilled, frozen, canned, shelf stable
Sachet desserts	Dry mix
Aerated desserts (mousses)	Chilled, frozen, dry mix
Cheesecakes	Chilled, frozen, dry mix
Others, for example, Dulce de leche, clotted cream, creamed rice	Chilled, frozen, canned

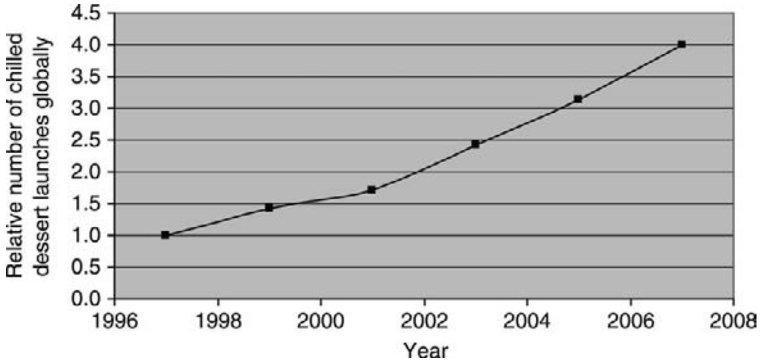

Figure 1 Relative global number of new product launches for chilled desserts (1997–2007) indexed to 1997 at 1.0.

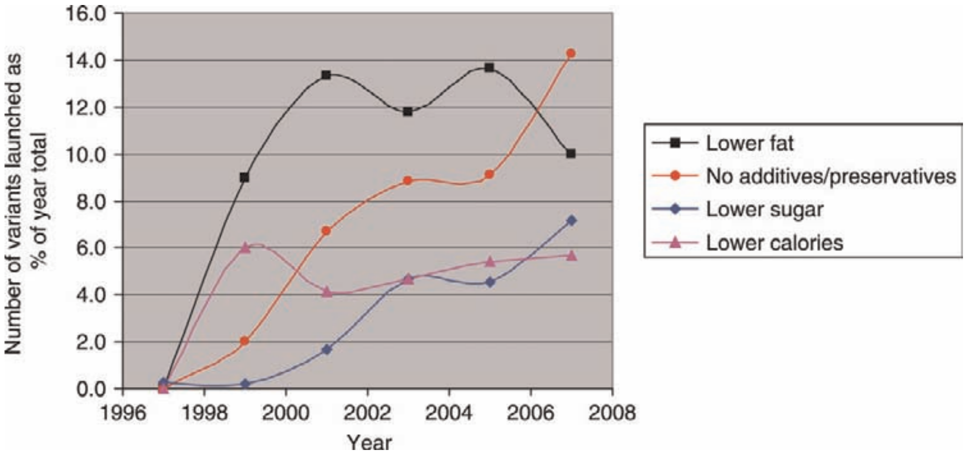

Figure 2 Approximate proportions of global new product launches (% of annual totals) for chilled desserts (1997–2007) where the above composition restrictions were claimed.

will produce a slightly different texture under a given processing regime.

Custards/Puddings

Custards and puddings are thick/creamy products, generally based on milk solids and corn starch. They have traditionally been sold in cans, but now many RTE types are available. Baked custards are produced by heating a custard mixture for an extended period (e.g., 35 min at 90 °C).

A basic vanilla custard (pudding) formulation could contain 1.5% native waxy maize starch, 8% sucrose, 8% skim milk powder, 1.3% milk fat, 2.5% gelling protein (e.g., egg solids, whey protein), and vanillin (with water to 100%).

Sachet Desserts

Sachet dairy desserts that are simply whisked into milk are very cheap to buy and widely available. They generally have a powdered sucrose base, with κ-carrageenan

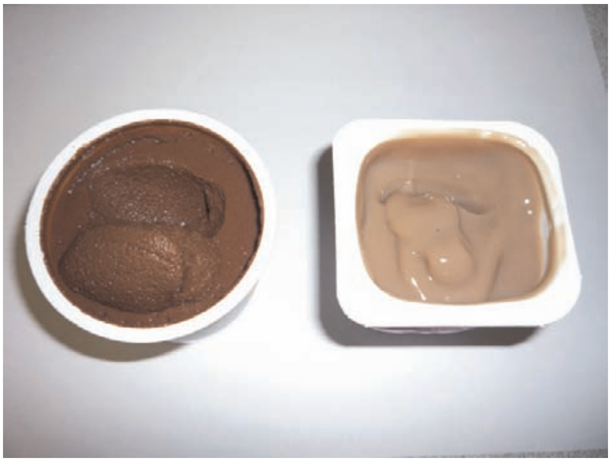

Figure 3 Examples of commercial creamy chocolate dairy food (right) and chocolate dairy mousse (left) (a spoonful of each has been scooped out and placed on the surface).

as the gelling agent, and are manufactured by dry blending the ingredients.

Similar versions containing milk powder that the consumer adds to water (rather than milk) can be manufactured in like fashion.

Aerated Desserts (Mousses)

Dairy-based mousses are aerated products with a stabilized foam structure. They have appealing sensory properties, with a light, refreshing texture, and economic advantages for the manufacturer (less solids per serving). Aerated desserts require both emulsifiers and stabilizers – the emulsifiers aid in air cell structure formation and the stabilizers thicken the liquid phase, preventing air cell wall breakdown/movement. Common emulsifiers are mono-diglycerides and lactic acid esters of mono-diglycerides. Stabilizers include those already mentioned for nonaerated RTE desserts. The most common mousse flavor is chocolate – an example is given in **Figure 3**.

A typical mousse formulation will have 6–8% fat (from cream), 10–12% milk solids-not-fat (from skim milk or skim milk powder), 10–12% sucrose, and 2.5–3.0% emulsifier/stabilizer blend.

Processing of aerated desserts is very similar to other RTE products, except for the inclusion of an aeration step after heat treatment. This is best carried out using a continuous aerator to give an overrun of 60–100%.

Cheesecakes

Traditional cheesecakes consist of a compressed biscuit crumb base under a cream cheese-based filling. Such a filling could consist of 66% cream cheese, 14% whole eggs, 3% milk, 16% sucrose, and 1% flavor (e.g., vanilla, lemon). However, many filling formulations are now based on other dairy ingredients, for example, calcium caseinate.

Baking of the cheesecake may be carried out under either high (wet baking) or low (dry baking) relative humidity. Dry baking produces an appealing golden surface, but the filling can easily crack. For cream cheese-based fillings, cracking is reduced by having the correct protein/fat ratio and moisture content, and through the use of gum blends (e.g., 0.03% locust bean gum and 0.15% guar gum).

Many cheesecakes are now sold frozen, and need to be properly thawed to ensure that the filling has optimal mouthfeel.

Other Dairy Desserts

There are many regional and localized types of dairy desserts made around the world – just three will be mentioned here to show the diversity involved.

Dulce de leche (known by several other country-specific names) is a milk-based syrup/sauce that is particularly popular in Central and South American countries. In its simplest form, sweetened milk is heated gently for several hours to concentrate the solids and cause Maillard browning (commonly misnamed caramelization in this context). The resulting thick material is brown in color and has a rich milky caramel flavor. It is generally not eaten on its own (although it is in some countries), but is used as a filling/topping/spread.

A good example of a less common, regionally localized dairy dessert is clotted cream. This is a traditional product of western England, and is made simply by heating cream (e.g., 54% fat), either over boiling water (scalding, e.g., heating for 45–70 min to 77–85 °C internal) or in an oven (baking, e.g., 60 min at 90 °C). After cooling, the cream sets to a firm consistency. Baking causes more Maillard browning and a more flavorful product than does scalding. Fresh clotted cream has a shelf-life of less than 1 week, but the product may be frozen.

Creamed rice is a popular dessert item in many parts of the world. It is made by cooking short- to medium-grain rice in sweetened milk to give softened, but intact, rice grains in a creamy base. Vanilla is the most popular flavor variant. Domestically, the mixture can be boiled or baked, but commercially the product is usually sold in metal cans processed through a rotary retort.

Dairy Dessert Ingredients

Milk Solids-Not-Fat and Fat

Good quality fresh milk (skim or fat-standardized) of known protein concentration can be used, or milk powder can be recombined/reconstituted. While skim milk powder will enable different fat sources to be used (e.g., vegetable fats), the reconstitution of whole milk powder eliminates the need for an initial homogenization step (and whole milk powder quality, storage stability, etc. are now very good). Skim milk powder is used to adjust the milk solids-not-fat to fat ratio.

The choice of fat and the fat content will affect mouthfeel (creaminess). Around 1.5% fat will give a rich, creamy product, but this level can be reduced through the use of fat replacers (e.g., microparticulated protein, microcrystalline cellulose–guar gum aggregates).

Products based on whey protein concentrates and isolates can also be made. Whey proteins are very good gelling agents, under appropriate conditions, and can be used in place of other non-dairy gelling ingredients.

Flavors and Colors

Sweetness can be provided through the addition of mono/disaccharides and/or artificial sweeteners, in the case of diet products. Monosaccharides can cause Maillard browning during thermal processing, which may or may not be desirable. Increasing the sugar content can also result in an increase in product firmness and stickiness.

Flavors and colors are key product differentiators. As mentioned previously, traditional flavors include chocolate, vanilla, caramel, and strawberry. New products are incorporating more exotic flavors, such as superfruits, along with texture/color variations and inclusions.

The type of cocoa powder used can have important effects on product quality. Up to a certain point, increasing the cocoa powder content will increase product texture, but at higher levels, viscosity/firmness will decrease. This is particularly the case as alkalization level increases.

With food-related health and wellness issues, such as obesity, there is mounting pressure to reduce sugar intake. At the same time, health concerns around artificial sweeteners have highlighted the need for natural alternatives. Available natural intense sweeteners are very limited in number (e.g., thaumatin, citrus dihydrocalcones), but steviol glycosides (primarily rebaudioside A) seem to be the best long-term prospect. Rebaudioside A is around 200–300 times sweeter than sucrose in a food product, with high-purity extracts known as reb A, or rebiana. It is anticipated that steviol glycoside-sweetened dairy desserts will be common beyond 2010.

Fortification

All dairy formats provide excellent bases for fortification with additional vitamins, minerals, and bioactives. While the traditionally indulgent dessert categories have not been obvious targets for health-promoting additives, this situation is changing, as consumers seek health benefits throughout the food product range. Options range from simple calcium fortification to the inclusion of sensitive bioactives (e.g., complex lipids and probiotics).

Thickening and Gelling Agents

As already stated, the texture-modifying ingredients are central to a successful dairy dessert product. The term hydrocolloid or hydrophilic colloid is used to describe a range of materials that thicken and/or gel aqueous systems. Such substances are water-dispersible/soluble, high-molecular-weight polymers. The thickening and gelling ability of each hydrocolloid is dependent on many factors. Thickening is largely dependent on concentration, temperature, and pH, but gelation commonly involves an interaction with specific ions. Key aspects of the hydrocolloids commonly used in dairy desserts are given in **Table 2**.

The most common texturizing ingredient combination for dairy desserts is starch (1–4%) and a carrageenan (0.05–0.4%). Common levels are around 3% starch and 0.2% carrageenan. The starch provides the bulk of the texture (body), while the carrageenan modifies this texture in sensorially appealing ways. In choosing the starch, it is vital to match its gelatinization temperature and shear tolerance with the processing regime. In the past, this has necessitated the use of modified starches. However, native starches are now available with higher processing tolerance, and have significant advantages with clean ingredient labeling and lower cost. If modified starches are used, acetylated waxy maize adipates or hydroxylated waxy maize or tapioca starch phosphates have been recommended. A mixture of 80% modified starch and 20% standard native starch can be used without significant changes in product characteristics (so giving a cost saving).

Whatever the starch used, it must be fully gelatinized to obtain maximum thickening. However, starch granules are much more susceptible to degradation after gelatinization. Greatest mechanical stresses will occur during the homogenization step. Hence, this is generally done before UHTST treatment. For scrapped surface UHTST processing, a single-stage homogenization of 7.5 MPa has been recommended to avoid sandiness.

The type of carrageenan used will depend on the desired texture. Creamy desserts need a smooth, heavy-bodied, short texture, with surface gloss. ι-Carrageenan is generally best in this case. Gelled desserts require

Table 2 Hydrocolloids used in dairy desserts

Hydrocolloid	*Functionality*	*Interactions*
Agar	*Gelation*. Forms thermoreversible gels through H-bonded helices. Gels form at low concentrations, and are not dependent on ionic species, pH, or sugar levels. Large thermal hysteresis effect (e.g., set at 40 °C, melt at 80 °C)	Synergy with LBG (*Gelidium* agar)
Alginates	*Thickening and gelation*. Thickened systems are shear-thinning (pseudoplastic), and viscosity increases with low concentrations of calcium. Heat-stable gels set at ambient temperatures through a reaction between calcium ions and guluronic acid units ('egg-box' model). Gelation can also occur at low pH through H-bonding of homopolymer regions. The derivative, propylene glycol alginate, is functional at pH < 3.5, where alginic acid precipitates	Synergy with high-methoxyl pectin – the pectin will gel at lower solids and a wider pH range
Carrageenans	*Thickening and gelation*. Gelation occurs through cation-induced helix formation: κ-Carrageenan; forms strong rigid gels with K^+ and Ca^{2+} that are susceptible to syneresis: ι-Carrageenan, forms elastic gels with Ca^{2+} that do not undergo syneresis and are freeze–thaw stable: ι-Carrageenan, does not gel, but gives high-viscosity fluids	Synergy between κ-carrageenan and milk proteins (primarily κ-casein), or konjac flour. There is also a strong synergy between ι-carrageenan and starch, but starch can reduce the gel strength of κ-carrageenan
Cellulose derivatives (methyl cellulose, hydroxypropyl cellulose, methyl hydroxypropyl cellulose, CMC)	*Thickening and stabilization*. Thickened fluids are pseudoplastic (and often thixotropic), the viscosity decreasing with increasing temperature, until gelation and flocculation occur. At low temperatures, the fluids show good pH stability	CMC stabilizes milk proteins at acid pH by forming soluble complexes that are stable to heat treatment and storage. A small amount of carrageenan can be used to prevent syneresis in CMC-thickened systems (at 10–20% of the CMC level)
Exudate gums (gum arabic, gum tragacanth, gum karaya)	*Thickening and stabilization*. Gum arabic generates low viscosity, while tragacanth and karaya generate higher viscosities at the same concentration	
Gellan gum	*Thickening and gelation*. Fluids are pseudoplastic, with a large drop in viscosity at 30–35 °C. Gels are thermoreversible and thermostable, formed by cation-induced helix formation	Synergies occur between gellan gum and the following: xanthan gum/LBG, konjac flour, alginate, starch, and gelatin
Gelatin	*Thickening and gelation*. Viscosity decreases with increasing temperature. Gels are formed by random coil–helix reversion, and melt at approximately 37 °C	
Konjac flour	*Thickening and gelation*. Thickened fluids are acid-stable, while gels are thermoreversible (or thermostable if treated with alkali)	Synergy with carrageenans and xanthan gum
Pectins	*Thickening*, *gelation*, *and stabilization*. High-methoxyl pectin will gel only at high sugar concentrations and low pH (e.g., 65% sucrose, pH 3–3.2), where polymer chains are partially dehydrated and have reduced negative charge. As the DE decreases from 75 to 60%, gelation time increases. Low-methoxyl pectin is calcium reactive, with reactivity increasing as the DE decreases. Gelation is thought to be similar to alginate ('egg-box' model). Amidated low-methoxyl pectins are more tolerant of calcium levels, and the gels show greater thermal/shear reversibility	With proteins below their isoelectric points (used to stabilize acid milk beverages)

(*Continued*)

Table 2 (Continued)

Hydrocolloid	*Functionality*	*Interactions*
Seed gums (LBG, guar gum, tara gum)	*Thickening*. Fluids are pseudoplastic, with full shear reversibility. Viscosity increases rapidly with concentration, due to polymer entanglement. Hence, viscosity increases with polymer molecular weight – by a power of 3–5	LBG and xanthan or κ-carrageenan (1:1 ratio) form strong, elastic gels. Interaction decreases with increasing galactose content. Phase separation occurs with proteins at high gum concentrations, particularly with guar gum
Starch (native and modified)	*Thickening and gelation*. Within native starch granules, some of the amylose is crystalline, and the granules themselves are insoluble. Upon heating in the presence of excess water, the granules swell, as the polymers hydrate, and the crystallinity is lost. This process is known as gelatinization. The linear amylose chains have a tendency to recrystallize after gelatinization. This process is termed retrogradation, and is invariably unwanted. As the amylose recrystallizes, there is an increase in opacity and firmness, and water may be expelled from the polymer network (syneresis). Key technological aspects of the starches from different species are the amylose to amylopectin ratio and the gelatinization temperature range. Due to labeling requirements and consumer demands for 'natural' ingredients, there is a tendency now toward the use of unmodified starches. Native starches with increased processing tolerance are now available	Synergy with ι-carrageenan and xanthan gum
Whey proteins	*Thickening*, *gelation*, *and emulsification*. Thickening/gelation occurs when the proteins denature and aggregate. Denaturation is generally induced by heating at >70 °C. Gel characteristics are pH-dependent – at pH >7, gels are firm, elastic, and transparent; at pH 3–5, gels are soft and opaque	
Xanthan gum	*Thickening.* Very high viscosity is produced at low gum concentrations. Fluids are strongly pseudoplastic, but not thixotropic, with significant viscoelasticity (i.e., they exhibit a yield stress). Viscosity is stable over a wide pH, salt, and temperature range	Synergies with seed gums (especially LBG), konjac flour, and starches

CMC, carboxymethyl cellulose; LBG, locust bean gum; DE, degree of esterification.

increased firmness and a homogeneous consistency – κ-carrageenan is then the preferred type.

Milk–carrageenan interaction

The interaction between milk proteins and carrageenan, or more specifically between κ-casein and κ-carrageenan, is important with regard to the texturization of dairy desserts. This interaction is responsible for texture formation in a large proportion of dairy dessert products, particularly the dry mixes. Hence, it warrants special attention.

The molecular basis of this polymer interaction has been extensively studied and is complex. κ-Carrageenan will interact with α_{S1}- and β-caseins in the presence of calcium, but interaction with κ-casein does not require cations. A negatively charged region on κ-casein (between residues 20 and 115) can electrostatically interact with the negative charges on the carrageenan polymer. The effect of this interaction is very dependent on the carrageenan concentration. At high κ-carrageenan levels (>0.1% w/w), milk proteins have little effect on the carrageenan gel network, while at low κ-carrageenan levels (< 0.018%), the presence of milk proteins inhibits carrageenan gelation. Between these limits, the interaction is synergistic, with 5–10 times less carrageenan required to obtain a certain gel strength in milk, versus water.

Manufacturing Methods

Powdered Products

These are produced by dry blending of the various ingredients, principally milk powder, starch, texturizer (generally carrageenan), sugar, flavor, and color. The key to successful dry blending is a uniform particle size to ensure homogeneous mixing and to reduce the chance of deblending during product handling.

Ready-to-Eat Products

The manufacture of RTE dairy desserts involves three basic steps:

1. mixing of the ingredients into a homogeneous fluid,
2. heat treatment of the fluid, and
3. filling of product containers.

Mousse-type products will in addition require an aeration step before container filling.

Effective dispersion of the dry ingredients (particularly the texturizers) is best achieved by dry blending the powders and then making a slurry with a small quantity of milk. The slurry is then diluted with the remaining milk. Complete solubilization of many of the hydrocolloids requires a preheating stage (e.g., at 70 °C) during the mixing.

The type of heat treatment determines the shelf-life of the product, while the combination of heat treatment and container filling dictates the texture of the final product (for a given formulation). Three basic types of heat treatment are used, as detailed below.

Pasteurization

Temperature/time combinations range from 63 °C for 30 min to 72 °C for 15 s, and the product may be hot or cold filled. Shelf-life varies from about 3 to 21 days at 7 °C, being shorter if cold filling is used. Advantages of pasteurization can include lower processing costs and minimal changes to the sensory and nutritional properties of the product.

Retort sterilization

Canned custards are the most important dairy desserts made using this thermal process. Rotary retorts are necessary to ensure even heat penetration. The heat treatment of low-acid canned foods requires very accurate control to ensure product safety (and quality). Generally, F_0 values are used to quantify the process – these values represent the equivalent effect on *Clostridium botulinum* of that number of minutes at 121 °C. Minimum F_0 requirements are commonly legislated. Long treatment times above 100 °C (e.g., 13 min at 115 °C) result in significant chemical, physical, and sensory changes to the product, for example, caramelization and loss of vitamins.

Ultra-high-temperature–short-time processing

The problems with product quality deterioration using traditional retort sterilization can be overcome by processing at a higher temperature for a very short time, for example, 140 °C for 3 s. Such a process requires sophisticated equipment that can increase and then decrease the temperature of the product very rapidly. UHTST equipment has become widespread over the past 20 years, and is now used extensively in the dairy industry and for processing a myriad of liquid and semi-liquid foods. It has the major advantage of being continuous, and can be coupled to a wide range of packing lines.

Prior to UHTST treatment, some form of preheating may be applied. To avoid loss of texture (starch granule breakdown) during the UHTST process, care is required to avoid starch gelatinization during preheating. However, there is a possibility of storage stability being compromised if the preheating is insufficient (due to residual enzyme activity and other mechanisms).

Various UHTST heat exchange systems are used:

1. direct – steam injection and steam infusion and
2. indirect – plate, tubular, and scraped surface (in the order of increasing fluid viscosity limit).

After UHTST treatment, the product is generally aseptically packed, with filling either hot (around 70 °C) or cold (<10 °C). An aseptic canning process has also been developed (the Dole system). Cold filling is more economical from an energy point of view.

Product filling temperature

The filling temperature is critical in determining the texture and stability of the final product. With hot filling, product texture fully develops in the consumer pack, and this can give firm, brittle gels. Generally, slower cooling leads to greater firmness.

With cold filling, shearing during cooling and filling leads to loss of texture, but gives a smooth and creamy product. If a more gelled texture is required, a strongly thixotropic (shear-reversible) carrageenan should be used, which will regel after filling. Pure ι-carrageenan has the strongest thixotropic character.

See also: **Fermented Milks**: Yogurt: Types and Manufacture. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Manufacture; Ice Cream and Frozen Desserts: Product Types.

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IMITATION DAIRY PRODUCTS

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Definition and Product Types

Imitation dairy products range from complete dairy substitutes, with no dairy ingredients, such as margarine and soy milk, to products that have a high percentage of dairy ingredients, but also contain some nondairy constituent, for example, vegetable fat. Food regulations in some countries differentiate between substitutes, which are allowed, and imitations, which are regulated, to protect the dairy industry from what is seen as unfair competition. However, today, imitation dairy products are freely available in the many parts of the world. They appeal to consumers because they are generally cheaper, and may be perceived to have health or dietary benefits. They also employ a much wider range of functional ingredients, and for this reason may have a longer shelf life, more convenient packaging, and be easier to use. These advantages are diminishing as advances in dairy technology allow greater sophistication in the design of real dairy products. Nutritionally, imitation dairy products may be disadvantaged by the lower digestibility of hydrogenated vegetable fats, compared with milk fat, the lower biological value of vegetable proteins, compared to milk proteins, and a lower bioavailability of calcium.

The most important imitation dairy products are imitation milks, condensed milks, and milk powders. These range from filled products, which are based on skim milk, with the butterfat wholly or partially replaced by vegetable fat, to totally synthetic milks using nonmilk proteins, fats, and carbohydrates. Other products that will be covered in this article are coffee creams, coffee whiteners, imitation whipped creams, whipped toppings, and fermented products. Spreads, cheeses, and ice creams are discussed elsewhere in the encyclopedia (**Butter and Other Milk Fat Products**: Fat Replacers; Milk Fat-Based Spreads. **Cheese**: Cheese Analogues. **Ice Cream and Desserts**: Dairy Desserts).

Ingredients

Initially, the production of imitation dairy products was driven by the price advantage gained by using vegetable oils and fats instead of milk fat. These days, the quality of the product is equally important and the fats used in its manufacture are often tailored to meet the functionality of the product. Liquid products, with a short shelf life, can be based on a wide range of oils and fats, as long as they are bland in flavor. Powdered products require fats that are stable to oxidation, do not melt during storage, but at the same time, are not waxy in the mouth. Ideally, they will have the same melting profile as milk fat. Hardened coconut, palm, or soy oils meet these criteria and are widely used.

Milk proteins are often used either in the form of skim milk powder or as sodium caseinate. The most widely used alternatives are soy protein concentrates or isolates.

Most imitation milk products employ additional emulsifiers and stabilizers to improve quality, whether it be to prevent creaming or coalescence, or facilitate whipping, or improve whiteness.

Imitation Milks

Early imitation milks were essentially recombined milks in which the milk fat was replaced by refined, bleached, and deodorized vegetable fat. These products are known as filled milks, and typically comprise pasteurized skim milk, 3–4% vegetable fat, and additives such as mono- and diglyceride emulsifiers, phosphate buffers, carrageenan, and vitamins A, D, and E. As already mentioned, hydrogenated coconut, palm, or soybean oils, with melting points around 35 °C, and a pleasant mouthfeel, neither greasy nor waxy, are well suited for this purpose. The milk is heated to between 70 and 90 °C, mixed with the melted fat, homogenized in a two-stage homogenizer at 15 and 4 MPa, and then cooled and packed. The milk may be either pasteurized, to extend the shelf life by a few weeks, or sterilized for a much longer shelf life. In the latter case, the milk must be very efficiently homogenized (e.g., at 20 MPa) to avoid creaming, and the possible formation of a cream plug, during storage, and then either ultra-high temperature (UHT) treated (e.g., 15 s at 142 °C) and aseptically packed/bottled, or in-bottle sterilized for, say, 12 min at 121 °C.

Imitation milks are common in many Asian countries where cow's milk is not readily available, and recombined skim milk powder is used instead of fresh milk. The skim milk powder (9% total solids) is rehydrated in good quality water for 20 min at 40–50 °C and then heated, mixed with fat, homogenized, cooled, and packed as before.

Skim milk powder may be replaced by other proteins that are cheaper, and sometimes more efficient, emulsifiers, to make the oil/water emulsion. Sodium caseinate, whey protein concentrates, and soy protein isolates are commonly used. Formulations simulate the proximate composition of cow's milk, containing 3–3.5% fat, 2–3.5% protein, and 5–6% carbohydrate. Maltodextrins or glucose syrup solids may be used as substitutes for lactose. These recombined or imitation milks tend to be nutritionally deficient in calcium, but can become unstable when calcium is added to the same level as in cow's milk. Most of the calcium and much of the protein precipitate from the milk. This defect has been overcome by the addition of citrate and mono/diglycerides to the formulation. The citrate solubilizes the calcium, and the emulsifiers stabilize the emulsion droplets. A typical formulation contains 4% hydrogenated coconut oil, 2% sodium caseinate, 0.5% mono/diglyceride (hydrophilic–lipophilic balance between 3 and 5), 0.3% calcium chloride, 0.35% disodium phosphate, and 0.55% potassium citrate.

The nutritional status of imitation milks has been criticized. They tend to be low in lysine and methionine, as well as calcium and magnesium, and the sodium content is generally quite high. However, if soy protein is used, the iron content may be significantly better than in cow's milk. There is also concern about the content of highly saturated hydrogenated fats in these products, which may be atherogenic, and many imitation milks now advertise a minimum content of polyunsaturated fats. In some countries (e.g., the Philippines), this is mandatory.

Soy, Nut, and Grain Milks

Milks based on extracts of soybeans, nuts such as almonds and cashews, or grains such as oats, barley, rice, corn, or wheat have become increasingly available in recent years, and are popular with vegetarians and people allergic to milk or its ingredients. Soy milk is by far the most important of these substitute milks, and is made by triturating soybeans with water, filtering off the aqueous extract, and heat processing the aqueous extract to inactivate enzymes, particularly trypsin inhibitors, and to pasteurize the product. Much the same process is used for grain and nut milks. A more sophisticated process for oat milks has been developed in Scandinavia, which uses amylases to hydrolyze the starch component of the grain. The proximate composition of soy milk is very similar to bovine milk, but it is low in calcium unless it is fortified. Other plant-based milks also tend to be deficient in protein.

Imitation Milk Powders

Imitation milk powders are a natural development from imitation milk, and require some of the same technology. They are reconstituted to milk, or used as cooking ingredients. The powders are generally made by spray drying a concentrated (about 50% solids) emulsion containing from 10 to 15% fat and 3.5 to 15% protein, the balance being carbohydrate. The fat must be of the highest quality, with a low free fatty acid content (<0.4% as oleic acid), and generally has antioxidants such as butylhydroxytoluene (BHT) added. Hydrogenated fats with a short plastic range, which melt at about 40 °C, are preferred, as they are pleasant to taste, but stable to coalescence during shipping and storage. The protein may be milk protein, from skim milk powder, sodium caseinate, or whey protein or a soy protein isolate. Skim milk powder is reconstituted at a concentration of about 40% in water at 40–50 °C. Other proteins are generally used at much lower concentrations, ∼5%, with the balance of the solids made up with lactose, glucose syrup solids, high-fructose corn syrup solids or maltodextrin, and minerals. Other additives to the mix can include emulsifiers, such as mono/diglycerides or lecithin, to improve dispersion of the spray-dried product, phosphate salts to aid solubility, vitamins, particularly A, D, and E, and sometimes cream flavors and colors.

The reconstituted skim milk, or protein/carbohydrate dispersion, is heated to 65–70 °C. The fat, plus any lipid emulsifiers, heated to the same temperature, is stirred in. This process can be batchwise or continuous. The coarse emulsion is then homogenized using a two-stage homogenizer (at ∼20 and 2 MPa) and fed to the spray dryer. To maximize efficiency, the solids concentration of the emulsion is made as high as possible without it becoming too viscous to atomize effectively. A cocurrent flow spray dryer with a conical chamber base is most suitable, and it is generally advantageous to have a two-stage fluidized bed dryer to finish the drying and agglomerate the particles.

Infant Formulae

Infant formulae are a special case of imitation milks and milk powders, and generally have a stricter nutritional specification, particularly with regard to the fat composition. It has been recommended that hydrogenated fats not be used, and that the fats should contain a high proportion of polyunsaturated fatty acids, including linoleic and linolenic acids in the proportion of 6.7:1. The proteins used for emulsification may also have a nutritional focus and include hydrolyzed casein, soy, and whey proteins.

Imitation Evaporated and Sweetened Condensed Milk

Much of the world production of evaporated and condensed milk is made by a recombining process, in which skim milk powder, milk fat, and sugar are combined in the correct proportions. This is particularly the case in southeast Asian countries where fresh cow's milk is often in limited supply. In imitation or filled evaporated milk, vegetable fats are used. A typical formulation is 8% fat and 20% nonfat milk solids. The mix is heated to 60 °C and homogenized in two stages at 14 and 3.4 MPa. After homogenization, mono/disodium phosphate is added to prevent the coagulation of milk and the mix is then sterilized. Sweetened condensed milk is made by a similar process, with the addition of about 45% sucrose to the mix. The high sugar content obviates the need for sterilization. The nonfat milk solids and sucrose are dissolved in water at 40–50 °C, heated to 70 °C, and homogenized at 4 MPa. The thick emulsion is then seeded with solid lactose to initiate the crystallization of the supersaturated lactose in a finely divided form, cooled to 18 °C, and packed in sterile containers under rigorously hygienic conditions.

As with the milks and powders, the skim milk powder can be replaced with other proteins. A completely artificial condensed milk could comprise 8% vegetable fat, 12% soy protein, 5% lactose, 45% sucrose, and 30% water. The soy protein may be partially hydrolyzed to improve its solubility in this system.

Imitation Coffee Creams

Condensed milk is often used as a coffee cream, but purpose-made coffee creams are not sweetened and contain more fat (10–20%). They are frequently sterilized and retailed in portion packs. Like other imitation products, sodium caseinate or soy protein isolate (2%) is used to emulsify the fat (which is, primarily, hydrogenated vegetable oil), and corn syrup solids (10%) are added to give body to the product. These artificial creams have less tendency to produce a scum of denatured protein on the surface of the hot coffee (generally called feathering), which is a problem with real cream, because it contains much more milk protein, the cause of its instability. The temperature of hot coffee is about 85 °C and the pH is generally in the range of 4.7–5.3. Under these conditions, casein may partially coagulate, particularly in the presence of calcium and magnesium ions in hard water. Phosphates (0.3%), to buffer the product and counteract the acidity of the coffee, and polysorbate emulsifiers (up to 0.4%), to stabilize the emulsion, may be added to improve this aspect of the functionality.

The protein, carbohydrate, and salts are dissolved in water at 40–50 °C and then heated to 70 °C, and later the melted fat and emulsifiers are mixed in. The coarse emulsion is then homogenized in a two-stage homogenizer at 11 and 3 MPa, packed, and sterilized at, for example, 115 °C for 15 min. Alternatively, the product may be UHT treated for 10 s at 140 °C and aseptically packed. In this case, it may be necessary to homogenize the product aseptically after the UHT process to redisperse the fat.

Coffee Whiteners

As generally understood, coffee whiteners are spray-dried coffee creamers. Their manufacture follows the pattern of other imitation milk powder products, but special features of their design are fats that have a low iodine value and resist oxidation, have a short plastic range, so that they melt completely in the beverage, and are neither greasy nor waxy, yet have sufficiently high melting points to be stable during shipping and storage. Once added to coffee, the creamer needs to have good light reflectance and emulsion stability under hot acid conditions. Thus, the emulsions are homogenized, before spray drying, at high pressures (~20 MPa) in order to reduce the fat globule size to less than 1 μm in diameter. This ensures maximum light reflectance when the powder is reconstituted in coffee. Sodium caseinate is an excellent emulsifier for these products, but generally requires the addition of phosphate and citrate to buffer the system to counteract 'feathering'. Lipid emulsifiers, such as monoglycerides, lactyl or diacetyl tartaric esters of monoglycerides, or polysorbates, are also thought to improve the functionality.

Following these principles, a typical coffee whitener contains 35% hydrogenated palm oil (m.p. 36 °C), 4% sodium caseinate, 2% emulsifiers, 1.5% dipotassium phosphate, 0.5% potassium citrate, and 57% glucose syrup solids.

Imitation Whipped Creams

Whipped creams are oil/water emulsions that are stable to storage, but easily destabilized by whipping to incorporate air and form a stable foam. For ease of whipping, the fats should have a very high solids content at lower than room temperature, but for good mouthfeel they should melt completely at body temperature. In these respects, hardened vegetable fats can be superior to milk fat, and make a more stable foam. Whipped creams can be pasteurized or UHT sterilized. Sometimes, they are frozen prewhipped. Powdered whipped toppings, for reconstitution with water or skim milk, occupy a large market segment. Because imitation whipping products generally form a more stable whipped foam than real cream, the powders are widely used in the cake and confectionery industry.

The emulsions are generally made from skim milk powder or sodium caseinate, or both, and fats that have a high solids content at the whipping temperature (generally 5 °C), but still melt around body temperature. Like other imitation dairy products, lauric fats, such as hydrogenated coconut or palm kernel oil, are often used. The choice of an appropriate destabilizing emulsifier is crucial to the functionality of imitation whipped creams. The best have been found to be α-tending emulsifiers, which crystallize in the α-form at the oil/water interface, below their melting point. This promotes fat agglomeration during whipping. Monoglyceride acetates and lactates, and propylene glycol palmitate or stearate are all α-tending emulsifiers. Most whipped creams also contain a stabilizer such as carrageenan or sodium alginate to increase the viscosity of the aqueous phase, which retards any tendency to creaming or syneresis. Hydroxypropyl methylcellulose is sometimes added to promote overrun.

A typical liquid whipped cream might contain 29% hardened coconut fat, 6% skim milk powder, 1% lactyl monoglyceride, 10% sucrose, and 0.2% sodium alginate. Recombination should be carried out above 70 °C to avoid interaction between the calcium of the skim milk powder and the alginate. Homogenization at 10–15 MPa (at 75 °C) should be downstream of the pasteurization (15 s at 85 °C) or UHT (4 s at 144 °C) treatments, to reverse any agglomeration of the fat. The liquid should then be cooled to 10 °C as quickly as possible, to minimize the fat crystal size and the viscosity. Liquid products are then aseptically packed. For a frozen product, the cream contains more sucrose, or a mixture of sucrose and glucose syrup, and is kept chilled for 24 h before whipping and freezing.

For a powdered whipped topping, the cream contains more carbohydrate, usually glucose syrup, and is recombined in the same way, before spray drying. The dry powder consists of about 50–55% fat, 8% sodium caseinate, 8% α-tending emulsifiers, and 29–34% glucose syrup solids.

Sour Cream and Yogurt

Imitation sour cream has much the same fat composition as coffee cream, but is often emulsified with skim milk powder. It is acidified to about pH 4.5 with citric or lactic acid, and can be thickened with gelatin, guar gum, or carrageenan. Yogurts can be made from any of the imitation milks, including the vegetable milk substitutes, by the addition of the appropriate bacterial culture and proper fermentation. They can also be made by direct acidification. When the protein content is low, the texture of the yogurt can be improved by the addition of thickeners such as pregelatinized starch or gelatin.

See also: **Butter and Other Milk Fat Products**: Fat Replacers; Milk Fat-Based Spreads. **Cheese:** Cheese Analogues. **Concentrated Dairy Products**: Evaporated Milk; Sweetened Condensed Milk. **Dehydrated Dairy Products**: Infant Formulae; Milk Powder: Types and Manufacture. **Fermented Milks**: Yogurt: Types and Manufacture. **Ice Cream and Desserts**: Dairy Desserts; Ice Cream and Frozen Desserts: Product Types. **Liquid Milk Products**: Recombined and Reconstituted Products.

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LACTATION: Galactopoiesis, Effects of Hormones and Growth Factors; Galactopoiesis, Effect of Treatment with Bovine Somatotropin

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John W. Fuquay, Professor Emeritus of Dairy Science at Mississippi State University, served on the faculty there from 1969 to 1999. His areas of emphasis in teaching and research were environmental physiology and reproductive physiology. He received his BS and MS degrees from North Carolina State University and his PhD degree from Pennsylvania State University, all in the area of dairy science. After completing the PhD degree in 1969, he accepted a teaching and research position at Mississippi State University, where he progressed through the ranks from assistant professor to professor before retiring in 1999. Professor Fuquay served as Coordinator for the Graduate Program in Animal Physiology from 1986 to 1999. He was a Visiting Professor in the Animal Sciences Department, University of California-Davis in 1979 and in 1985–86.

Professor Fuquay was active in his professional society, The American Dairy Science Association. He was a member of the editorial board of *Journal of Dairy Science* for seven years, an editor for four years, and served as the first Editor-in-Chief for six years (1997–2002). For his professional contributions and service to the Association, Professor Fuquay was recognized as a Fellow in the American Dairy Science Association in 2001 and received the Association's Award of Honor in 2002. Other recognitions include the World Association of Animal Production Jean Boyazoglu Award in 2003, the Distinguished Dairy Science Alumnus Award from Pennsylvania State University in 2003, and several teaching and research awards from his university.

Professor Fuquay has participated in a variety of international activities. He has presented short courses and lectures as well as provided consultations in a number of countries, primarily in Asia and Latin America. In addition to his research publications, he is the coauthor of a textbook, *Applied Animal Reproduction* (Prentice Hall), that has been widely used by universities in the United States and internationally. The first edition was published in 1980 and the last (sixth) edition in 2004. In 2010, he published a memoir, *Musings of a Depression-Era Southern Farm Boy* (Vantage Press), which reflects on how the experience of growing up on a farm in the southern United States during the great depression instills one with an understanding of the importance of strong family bonds and a sound work ethic in meeting the challenges of the adult world.

Patrick F. Fox was Professor and Head of the Department of Food Chemistry at University College, Cork (UCC), Ireland, from 1969 to 1997; he retired in December 1997 and is now Emeritus Professor of Food Chemistry at UCC. Prof. Fox received his BSc degree in Dairy Science from UCC in 1959 and PhD degree in Food Chemistry from Cornell University in 1964. After postdoctoral periods in Biochemistry at Michigan State University and in Food Biochemistry at the University of California, Davis, he returned to Ireland in 1967 to take up a research position at the Dairy Products Research Centre at Moorepark before moving to UCC in 1969.

Prof. Fox's research has focused on the biochemistry of cheese, the heat stability of milk, physicochemical properties of milk proteins, and food enzymology. He has authored or coauthored about 520 research and review papers, and authored or edited 25 text books on Dairy Chemistry. He was one of the founding editors of the *International Dairy Journal.*

In recognition of his work, Prof. Fox has received the Research & Innovation Award of the (Irish) National Board for Science and Technology (1983), the Miles-Marschall Award of the American Dairy Science Association (1987), Medal of Honour, University of Helsinki (1991), the DSc degree of the National University of Ireland (1993), the Senior Medal for Agricultural & Food Chemistry of the Royal Society for Chemistry (2000), the ISI Highly Cited Award in Agricultural Science (2002), the International Dairy Federation Award (2002), Gold Medal of the UK Society of Dairy Technology (2007), and an autobiography published in Annual Review of Food Science & Technology (2011).

Prof. Fox has been invited to lecture in various countries around the world. He has served in various capacities with the International Dairy Federation, including President of Commission F (Science, Nutrition and Education) from 1980 to 1983.

Paul McSweeney is Professor of Food Chemistry in the School of Food and Nutritional Sciences, University College, Cork, Ireland (UCC). He graduated with a BSc degree in Food Science and Technology in 1990 and a PhD degree in Food Chemistry from UCC in 1993 and also has an MA in Ancient Classics. He worked for a year in the University of Wisconsin (1991–92) as part of his PhD and as a postdoctoral research scientist in UCC (1993–94). He was appointed to the academic staff of UCC in 1995. The overall theme of his research is dairy biochemistry with particular reference to factors affecting cheese flavor and proteolysis during cheese maturation including the role of non-starter lactic acid bacteria and smear microorganisms, the ripening of hybrid and non-Cheddar varieties, the specificity of proteinases on the caseins, proteolysis and lipolysis in cheese during ripening, and characterization of enzymes important to cheese ripening (proteinases, peptidases, amino acid catabolic enzymes). He is the coauthor or coeditor of eight books, including the third edition of *Cheese: Chemistry, Physics and Microbiology* (Amsterdam, 2004) and the *Advanced Dairy Chemistry Series* (New York, 2003, 2006, 2009), and has published numerous research papers and reviews. Prof. McSweeney is an experienced lecturer and researcher and has successfully managed research projects funded through the Food Industry Research Measure and its predecessors administered by the Irish Department of Agriculture and Food, the EU Framework Programmes, the US–Ireland Co-operative Programme in Agriculture/Food Science and Technology, and Bioresearch Ireland and Industry. He was awarded the Marschall Danisco International Dairy Science Award of the American Dairy Science Association in 2004 and in 2009 a higher doctorate (DSc) on published work by the National University of Ireland.

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GUIDE TO USE OF THE ENCYCLOPEDIA

STRUCTURE OF THE ENCYCLOPEDIA

The material in the Encyclopedia is arranged as a series of entries in alphabetical order. Some entries comprise a single article, whilst entries on more diverse subjects consist of several articles that deal with various aspects of the topic. In the latter case the articles are arranged in a logical sequence within an entry.

To help you realize the full potential of the material in the Encyclopedia we have provided three features to help you find the topic of your choice.

1. CONTENTS LISTS

Your first point of reference will probably be the contents list. The complete contents list appearing in each volume will provide you with both the volume number and the page number of the entry. On the opening page of an entry a contents list is provided so that the full details of the articles within the entry are immediately available.

Alternatively you may choose to browse through a volume using the alphabetical order of the entries as your guide. To assist you in identifying your location within the Encyclopedia a running headline indicates the current entry and the current article within that entry.

2. CROSS REFERENCES

All of the articles in the Encyclopedia have been extensively cross referenced. The cross references, which appear at the end of an article, have been provided at three levels:

i. To indicate if a topic is discussed in greater detail elsewhere.
ii. To draw the reader's attention to parallel discussions in other articles.
iii. To indicate material that broadens the discussion.

Example

The following list of cross references appear at the end of the entry entitled **Bacteria, Beneficial** | Lactic Acid Bacteria: An Overview

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; *Streptococcus thermophilus*; Taxonomy and Biodiversity. **Pathogens in Milk**: Enterobacteriaceae.

3. INDEX

The index will provide you with the volume number and page number of where the material is to be located, and the index entries differentiate between material that is a whole article, is part of an article, or is data presented in a table or figure. Detailed notes are provided on the opening page of the index.

4. COLOR PLATES

The color figures for each volume have been grouped together in a plate section. The location of this section is cited in the contents list. Color versions of black and white figures are cited in figure captions within individual articles.

5. CONTRIBUTORS

A full list of contributors appears at the beginning of each volume.

6. GLOSSARY

A glossary of terms used within the work is provided in Volume Four before the Index.

PREFACE

We are pleased to present the second edition of the *Encyclopedia of Dairy Sciences*. The first edition was published in 2003 by the Major Reference Works Division of Academic Press, now part of Elsevier Sciences, and it comprised 427 articles. The objective was to satisfy the need for an authoritative source of information for people involved in the integrated system of production, manufacture, and distribution of dairy foods. It was realized from the beginning that a program of revision would be needed to keep the Encyclopedia up to date. This goal has been met in the second edition through 503 articles, of which 121 are new articles and 382 are revised articles. We express appreciation to the Editorial Advisory Board for its role in evaluating articles for needed revision, reviewing new and revised articles, and for help in identifying new topics to be included along with appropriate authors. Likewise, we are grateful for the contributions of the many authors who have either revised their articles or prepared new articles.

The main topics related to milk production and dairy technology are addressed in addition to providing information on nutrition, public health, and dairy industry economics including aspects of trade in milk and dairy products. All species that produce milk for human consumption have been included in this work. Some of these species are of regional significance only, but they have been included because of the essential role that their milk plays in the nutrition of people inhabiting various regions of the world. A significant addition to the second edition is four introductory articles addressing the history of Dairy Science and Technology. A synopsis has been prepared for each article in the second edition and will appear with the online listing of the articles in this publication.

The primary aim of the Encyclopedia is to provide a complete resource for researchers, students, and practitioners involved in all aspects of the dairy sciences as well as those involved with economic and nutritional policy and members of the media. We have tried to do this with a writing style that is easily comprehended by persons who are not highly trained in the technical aspects of the Dairy Sciences. Users should be able to access information on topics that are peripheral to their areas of expertise.

We express appreciation to the staff of the Major Reference Works Division, responsible for this Encyclopedia, for their timely responsiveness to the needs of the editors and their essential administrative role in keeping this major reference work on-track toward a satisfactory completion within the desired time schedule. We remember Nancy Maragioglio, Senior Life Sciences Editor, who initiated the work and was ever responsive to queries by the editors, as well as Sera Relton, Esmond Collins, Milo Perkins, and Claire Byrne, Development Editors, and Charlotte (Charlie) Kent, Publishing Administrator, who kept things moving through their communication with editors, authors, and reviewers and who exhibited almost flawless administrative skills. Sera Relton was particularly helpful as she assisted us in moving through the final submission and review stages. Laura Jackson is recognized for her contributions as Production Manager of the Encyclopedia.

Special recognition is due to Ms Anne Cahalane, Senior Executive Assistant, School of Food & Nutritional Sciences, University College, Cork, whose stylized representation of a cow, a milk can, and a wedge of cheese adorns the cover of the first and second editions of the Encyclopedia of Dairy Sciences.

John W. Fuquay
Patrick F. Fox and
Paul L. H. McSweeney

FOREWORD

The cow is the foster mother of the human race. From the days of the ancient Hindoo to this time have the thoughts of men turned to this kindly and beneficent creature as one of the chief sustaining forces of human life.

William Dempster Hoard (1836-1918)
Former governor, state of Wisconsin, USA (1889-1891)
Founder of **Hoard's Dairyman** (1885)

We must never forget that milk and milk products are and will always be important sources of basic food nutrients for humans both young and old. The more scientific facts we can discover, understand, and apply related to producing, processing, and marketing milk and milk products, the better we will serve the nutritional needs of humanity throughout the world.

More than 2000 years ago Aristotle noted, *Everyone honors the wise and excellent.* We are indebted to those *wise* enough to conceptualize and envision the favorable global impact that is certain to follow by bringing together this exhaustive, rich collection of 503 pertinent articles written and reviewed by more than 700 world-renowned disciplinary experts representing 50 countries – persons each of whom bears the mark of *excellence.* Happily these timely topics are now recorded in four informative, important, engaging volumes. We thank, commend, and salute the prodigious efforts of the *wise* and *excellent* authors who generated, compiled, and put the spotlight on the useful information and data, and who now share them through their well-written articles.

One noteworthy value and enduring virtue of these articles is bringing into clear perspective the context of both the state-of-the-art and the future of dairy sciences. When the history and contributions of scholarly publications related to the all-important global dairy industry are recorded, the second edition of the *Encyclopedia of Dairy Sciences* will be cited often and with great respect and appreciation.

Fundamental to continued progress and success in the dairy industry have been the signal service, cooperation, and collective contributions of dedicated scientists, teachers, agricultural advisors/extension workers, and representatives of governments and industries. Additional exciting breakthroughs in applying new findings and developments in research and technology to the production and processing of milk are sure to follow as we move surefootedly through the twenty-first century. This continued growth and success will be aided immensely by the vast and extraordinarily useful knowledge base made available by the idea-rich, insightful authors, editorial advisory board members, editors, and publisher of the second edition of the *Encyclopedia of Dairy Sciences.*

Indeed, by perusing the comprehensive and authoritative articles of this greatly needed and monumental encyclopedia, readers will be made even more aware of the tremendous progress that has occurred in the basic and applied sciences underpinning the global dairy industry.

Ours is an internationally competitive and incredibly technological world. And unless talented, creative scientists continue to work together in researching and applying the most effective and economical ways and means of providing

an abundant, safe supply of milk and milk products for an ever-increasing world population, we will never reach our noble goal of adequately feeding all the earth's people.

May we utilize the comprehensive scientific knowledge base made available through this second edition of the *Encyclopedia of Dairy Sciences* as we pledge to realize advances in the health and well-being of the undernourished millions – including many who need and deserve to be rescued from the ugly grip of hunger – by increasing the availability of nature's most nearly perfect food – milk!

Pure milk from healthy animals is a luxury of the rich, whereas it ought to be the common food of the poor.

Mohandas Gandhi (1869-1948)
Indian nationalist leader

John R. Campbell

John R. Campbell, Ph.D., D.Sc. (Hon.)
President Emeritus and Professor of Animal Science
Oklahoma State University

Dean Emeritus and Professor Emeritus of Animal Sciences
College of Agriculture, University of Illinois

Professor Emeritus of Animal Sciences
University of Missouri

Past President, ADSA (1980-81)

April 2010

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LABELING OF DAIRY PRODUCTS

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Introduction

Food labeling is any written, printed, or graphic matter (a tag, a brand, a mark, a pictorial, and other descriptive material), which is present on the packaging or on the food itself, and which accompanies the food or is displayed near the food, including that for sales and promotion purposes.

Labeling is the principal means of communication between the manufacturer, the food handler, and the purchaser and is therefore an integral, important part of the marketing efforts and it also helps in assuring proper product handling.

In the early days of manufacturing prepackaged foods (sixteenth to seventeenth century), the basic types of information labeled were the product name (what it is, possibly what it is made of), the trade name (who manufactures it), and instructions for use (how to prepare or to use the food).

With increasing diversification of food products and increasing trade, and to measure up against the sophistication of consumer attitudes and education, labeling patterns have become complex and more informative. The purpose, however, has not changed – to present and promote the product and to inform the purchaser in a way that is as faithful, fair, and accurate as possible.

With increasing international trade, the demand for international harmonization of labeling has increased. Due to widespread trade, the dairy sector was the first to initiate the establishment of international labeling rules, primarily to ensure fair-trade practices.

In a historical perspective, labeling regulations protecting consumers appeared rather late and has really been developed in general since the late 1960s.

Most labeling rules of today aim at ensuring objective and informative information to the consumer, assuming that the individual consumer is rational and capable of making informed choices.

More recently, individuals tending to associate themselves with identity groupings made the use of labeling claims explode. Therefore, recent regulatory initiatives within labeling have focused mainly on regulating this area.

This article focuses primarily on the Codex requirements for the labeling of milk products, as these are implemented in national and regional legislation in most countries. Individual countries and regions have implemented or are in the process of establishing additional labeling requirements. Reference to such requirements is made only to a limited extent.

Depending on the needs and requirements, labeling of milk products can be grouped as follows:

1. Mandatory labeling requirements: name of the food, list of ingredients, net content, origin of the product, date marking and storage instructions, and instructions for use, where necessary.
2. Additional mandatory commodity-specific labeling requirements.
3. Optional (and conditional) labeling requirements: nutrient declaration and claims concerning specific attributes.

Note that the purpose of this article is to address and explain the implications of food-labeling requirements and not to provide labeling guidelines.

General Labeling Principles

Three basic and important principles govern all labeling requirements:

- Any label or labeling shall not describe or present a food in a manner that is false, misleading, or deceptive, or is likely to create an erroneous impression regarding its character in any respect.
- Any label or labeling shall not describe or present a food in a way that directly or indirectly refers to or

is suggestive of any other product with which such food might be confused, or in a manner that leads the purchaser or consumer to believe that the food is connected with such other product.

- Statements on food labels shall be correct, verifiable, and must not promise anything that cannot hold.

These principles need commodity-specific interpretation when implemented, with due respect to local perceptions, traditions, and patterns.

Milk and milk products have a high nutritional value and a very good organoleptic image. Therefore, milk products have achieved a significant market position and share of household consumption in most homes. This position makes direct and indirect reference to milk products in the marketing of nondairy foods very tempting. In recent years, this fact is mirrored in the number and nature of misleading practices (**Table 1**).

The Codex General Standard for the Use of Dairy Terms has been established to avoid such misleading practices. However, it also allows for limited exemption from the general labeling principles: names for traditional non-milk products, which due to long-established practices include dairy terms, are recognized and allowed by this standard provided that the exact nature of the product is clear to the purchaser or when the name is clearly used to describe the characteristic quality of the product.

Traditional or historical foods using dairy terms that are generally allowed by this standard include names such as peanut butter, coconut butter, cocoa butter, and crème de cassis.

Additionally, other traditional names of non-milk products have been used locally for a number of years. Examples are the product name ‘filled milk powder’ primarily used in some South East Asian countries and a number of traditional names registered in accordance with the European Council Regulation 1237/2007. It should be noted that it is only the names of these traditional products that can be used and only in countries where the tradition has been established. No other type of representation referring to dairying is allowed.

Table 1 Examples of possibly misleading use of dairy names and terms

Examples relating to designations
• The name ‘butter’ and ‘vegetable butter’ for fat spreads containing vegetable oil
• The designations ‘vegetable oil cheese’ and ‘margarine cheese’
• The margarine names ‘rama’, ‘edelram’, ‘butella’, and ‘beurrine’
• The name ‘coffee creamers’ for coffee whiteners without milk fat
• The name ‘soy milk’ and ‘soy yogurt’ for products based on soy
Examples relating to functionality terms
• The claim ‘made exactly like cheese’
• The claim ‘real alternative to cream’
Examples relating to general presentation
• Pictures of churns, milk cans, etc. on the package of non-milk products

Mandatory Labeling Requirements

Name of the Food

Application in general

The name of any food shall indicate its true nature and shall normally be specific and not generic. Misleading and confusing names, whether they are laid down in legislation or not, are not permitted.

The name applicable to a food shall be chosen using the following hierarchy:

1. The first priority is a name established by Codex Alimentarius. If such a name exists it shall apply. In the case of regional Codex Standards, the name shall apply only in the region to which the standard applies.
2. If no name is established by Codex Alimentarius (globally applicable or applicable in a defined geographical region), priority shall be given to a name established by national legislation (of the country of retail sale). If such a name is defined, it shall apply. Where regional regulation of names has been established, such regulation normally sets a precedent in relation to national names.
3. If no name is established by Codex Alimentarius (globally applicable or applicable in a defined geographical region) or by the legislation of the country of retail sale, the name of the food shall be chosen from among the following options:
   - a common or usual name, including the name specified in the country in which the food originates but is not sold retail, or
   - a descriptive designation.

A fancy name (e.g., brand name) cannot replace the name of the food. However, some fancy names have with time established themselves as ‘common names’. Examples include ‘lasagne’, ‘T-bone steak’, ‘hotdog’, and a vast number of cheese names.

Use of Descriptive Designations

Descriptive designations are designations that in appropriate terms describe the true nature of the food in a way that is transparent and non-misleading to the consumer.

Consequently, descriptive designations cannot be unclear designations such as comparable designations. A descriptive designation shall refer to the raw materials and ingredients used and in the state in which they have been used.

Table 2 Examples of descriptive designations

Misleading descriptive designations	*Appropriate descriptive designations*
'Vegetable butter', 'butter made from vegetable oil', and 'butter blend' for fat spreads containing vegetable oil	When processed after blending oil and cream: 'blended fat made from cream and vegetable oil'. When recombined from butter(oil) and vegetable oil: 'blended fat made from butter and margarine'
'Vegetable oil cheese' and 'margarine cheese'	'Sliceable/spreadable coagulated food prepared from skimmed milk and vegetable oil'
'Vegetable cheese'	'Sliceable coagulated food prepared from soy paste and vegetable oil' (or 'tofu')
'Soy milk' and 'soy yogurt' for foods based on soy with intended use as drinking milk	'Reconstituted soy paste' and 'reconstituted fermented soy paste'

Examples, using imitations of milk products, that illustrate the application of descriptive terms are provided in **Table 2**.

Table 3 Milk products not covered by Codex commodity standards

Preserved milk products	*Fermented milk powders, e.g., yogurt powder and buttermilk powder*
	Whey protein concentrates and other dried individual milk constituents
	Permeate powders
Cheese products	Cheese powder
Fresh milk products	Liquid milk for direct consumption
	Milk-based drinks
	Milk-based desserts

Application to milk products

The name 'milk'

The term 'milk' is defined as the normal, untreated (i.e., raw) mammary secretion of any milking animal obtained from one or more milkings without either addition to it or extraction from it.

The Codex Standard for the Use of Dairy Terms permits the use of the term milk for treated raw milk; however, if the milk is modified or adjusted in composition, the term may only be used as a name if a clear description of the modification made is given in close proximity to it, for instance, 'skim milk', 'lactose-reduced milk', and 'vitaminized milk'.

Naming of milk products

Codex has established names for the milk products of significance in international trade. These products shall be named as specified in the relevant standard, and should comply with the content of it. However, the use of defined variety names of cheeses (e.g., Cheddar, Emmental, and Brie) and of fermented milks (e.g., yogurt and kefir) is optional – the variety name can replace the product designation if the product complies with the corresponding standard(s).

Those milk products to which a name has not been established by Codex shall be named as specified in the national legislation in the country of sale, or by appropriate descriptive designations in the absence of a prescribed name. Milk products not covered by Codex commodity standards are listed in **Table 3**.

A number of other variety names, in particular within the group of fermented milks and cheeses, are regulated according to national commodity standards and/or definitions by national legislation.

Naming of compositionally modified milk products

Alteration of the composition of the milk used for manufacturing is the normal tool to control end product composition. Such alterations may be relatively small (adjustment) or substantial (modification) ('adjustment' means minor changes in composition within natural variation while 'compositional modification' means compositional modification beyond natural variation).

Almost all milk products are subject to adjustment of the main constituents prior to, or during, manufacturing (fat, protein, dry matter, lactose, and/or fat-free dry matter). For instance, the fat and/or protein content of cheese milk is adjusted to meet the compositional requirements of full (reference)-fat versions of the cheese.

The General Standard for the Use of Dairy Terms permits adjustments provided that the compositional standards in the applicable Codex product standard allow for a product resulting from the adjustment.

The use of defined milk product names for compositionally modified milk products (i.e., products that differ in composition from the reference product) is permitted only when complying with the following three conditions:

1. The modification to which the milk product has been subjected is clearly described in association with the name, for example, 'reduced-fat', 'light', 'neutralized', 'demineralized', 'decholesterolized', and 'calcium-enriched'.
2. The essential product characteristics have been maintained, as there is limitation to the extent to which modifications can take place without altering the essential characteristics (basic identity) of the

reference product. Some examples where modifications result in altering the basic identity are 'fat-free butter' (the basic identity of butter is milk fat), 'protein-free cheese' (the basic identity of cheese is a protein structure), and 'protein- and fat-free drinking milk' (the product is milk permeate).

3. The limits of modification are detailed in the specific product standards concerned. If not addressed here, it should be assumed that reference to the milk product name should not occur, even with the use of supplementary descriptors.

Supplementary names

Both Codex standards and most national labeling regulations state the conditional obligation to use appropriate descriptors in addition to the name. These stipulate that, in conjunction with or in close proximity to the name, appropriate additional words or phrases shall appear as necessary to avoid misleading or confusing the consumer with regard to the true nature and physical condition of the food.

In which case the consumer may be considered misled is obviously a question for constant debate; perceptions differ from country to country and with time, depending on tradition.

When deciding which descriptors are required in the name, at least the following three factors need to be taken into account:

1. The type of product;
2. Whether the identity (nature) or condition of the product is different from the usual reference product familiar to the consumer; and
3. Whether the modification made (compared to the reference product) affects the intended use, shelf life, nutritional properties, taste, or similar attributes that change the nature of the reference product.

Examples of descriptors relevant to milk products that may be identified according to the above factors are provided in **Table 4**.

Reference to specific technology

Most foods are prepared by the application of a number of technological processes. It is obvious that not all processes have to be declared in the labeling. However, some processes need to be declared as they may be important for correct information with respect to the true nature of the product.

When deciding which processes should be declared, the following factors need to be taken into account:

- The novelness of the technology;
- Whether the process may affect consumer choice as it affects the intended use, shelf life, nutritional properties, taste, or similar attributes.

Table 4 Examples of descriptors/qualifiers suitable for milk products

Addressing raw materials	• Origin of the milk, e.g., 'made from goats' milk' • Raw materials used, e.g., 'recombined drinking milk'
Addressing changed nutritional properties	• Vitamins added, e.g., 'vitaminized milk powder'
Addressing extended shelflife	• 'Long-life milk'
Addressing a changed physical condition	• Lecithin added, e.g., 'instant milk powder' • Divided into parts, e.g., 'cut cheese' and 'sliced cheese' • 'Whipped'

In the case of milk products, labeling of some processes may be mandatory, such as irradiated, dried, fermented, heat-treated after fermentation, and fractionated.

Labeling of other technologies may not be necessary as it is not anticipated to affect consumer choice, such as coagulation of cheese, fat, and protein adjustment of milk products, microfiltration, ultrafiltration, heat treatments, evaporation, and drying.

Declaration of manufacturing processes should be required only when it serves a justified purpose, such as providing information about the altered character of an end product.

Reconstitution/recombination

For most milk products, the use of any milk-based ingredient as raw material is normally not restricted, and reconstitution as well as recombination is therefore permitted without any restriction.

Consequently, in most cases, it would not be necessary to use the descriptors 'recombination' or 'reconstitution' because

- the identity (nature) of the final food is not different from the corresponding reference product (not reconstituted/recombined);
- the raw materials are listed in the ingredients list; and
- most milk products are subjected to a number of different treatments and processes that in most cases include partial recombination and reconstitution.

If the omission of a statement regarding reconstitution or recombination is considered to be misleading to the consumer, the local authorities may require specific labeling.

Naming of composite milk products

A composite milk product is a product composed of a milk product and another food. The other food is added to provide additional characteristics to the dairy product, such as flavoring foods, spices, flavors, etc., in amounts that retain milk constituents as the essential part.

However, if the other food in part or in whole takes the place of any milk constituent, the product is no longer a composite milk product (or a milk product).

Composite milk products shall be named by combining the name of the milk product constituting the essential part with a description of the added characterizing (or flavoring) ingredients. The description of the other ingredients may be simplified by, for instance, using a group name. Examples are 'fruit yogurt', 'chocolate milk', and 'spiced cheese'.

Ingredient Listing

If the food consists of a single ingredient (e.g., an apple), a list of ingredients is not required. If the food consists of more than a single ingredient, all ingredients shall appear in the labeling in descending order of ongoing weights at the time of manufacture.

Compound ingredients may be declared by 'compound names' provided that (1) the compound name has been established (by Codex or national legislation), (2) the compound constitutes less than 5% of the food, and (3) the compound is not an additive. However, foods that are known to cause hypersensitivity (and components that impact malabsorbants) shall always be declared. Among these are cereals containing gluten, eggs and egg products, tree nuts and nut products, and milk and milk products (including lactose).

Specific 'class names' may be allocated to certain compound ingredients. Such class names may be specified in the ingredient listing instead of the specific names of the ingredients. Generally accepted class names include 'cheese', 'starch' (other than chemically modified starches), 'spices' and 'herbs', 'emulsifying salts', and 'caseinates'.

Additives (their name or number) shall be listed together with their functional class. Additives that are added to serve a technical function during the manufacturing, but which do not serve a function in the end product, need not be declared (see 'processing aids' under 'Additives and Processing Aids').

If the labeling otherwise places special emphasis on the presence of one or more valuable and/or characterizing ingredients, the ongoing percentage of the ingredient shall be declared quantitative ingredient declaration (QUID). This does not apply, where reference is made in the name of the food (e.g., 'cream cheese' and 'fruit yogurt'). QUID is also triggered if the omission of quantitative declaration will be misleading, where an ingredient is essential to characterize the food and its presence is expected by the consumer.

It is not required to label withdrawn constituents. For instance, in the case of ultrafiltration, the withdrawal of milk retentate is, in technical terms, exactly the same as the addition of milk permeate. Similarly, the withdrawal of milk permeate is exactly the same as the addition of milk retentate. Consequently, the ingredients listing of additions of milk permeate and milk retentate may be regarded as unnecessary.

Origin of the Food

Three types of information are normally required for the purpose of informing the consumer and/or for traceability purposes:

- Name and address of the manufacturer, packer, distributor, importer, exporter, or vendor
- Lot identification
- Country of origin, if the omission would mislead or deceive the consumer

Some countries require additional labeling, such as the EC Identification Mark, mainly to meet the needs of public authorities.

Country of origin labeling

The 'country of origin' equals 'country of manufacture' and should not be confused with the country in which the product name first originated (as perceived in countries enforcing legislation concerning protected geographical designations). If the food undergoes substantial transformation (i.e., other than simple treatments such as repackaging, cutting, and slicing) in a second country, the second country would be the country of origin for labeling purposes.

Most consumers believe that, if not informed otherwise, the products they buy are domestically produced. Decisions with respect to whether the omission of information on the country of origin could be misleading are based upon several factors, such as

- any direct or indirect geographical references on the label to geographical places other than those related to the location where the product has not been manufactured (words, pictures);
- use of names of a food that is generally perceived as originating in another country; and
- use of claims indicating a geographical relation of the food, ingredients, or attributes such as 'French style' and 'Mexican taste'.

Where it is generally known to the consumer that a geographical reference does not indicate a specific origin, it may not be misleading to omit country of origin labeling, for instance, in the cases of'Danish pastry' and 'hamburger'.

Date Marking and Storage Instructions

In general, the date of minimum durability shall be declared. There are some exceptions of well-preserved foods, including ripened whole extra hard, hard, and firm cheeses. In order to ensure that adequate information with respect to the age of these cheeses is provided, the date of manufacture should be declared instead.

Where the durability of the product is determined by the consumer storage conditions, these conditions shall be declared as well.

Product shelf life is influenced by a number of factors, such as the microbiological stability, control measures and cooling methods applied, type of packaging (e.g., hermetically sealed and modified atmosphere), the level of postprocess contamination, and type of contaminant.

It is the responsibility of the manufacturer to determine the shelf life of the product and the storage conditions in order to ensure that the safety and suitability of the product can be retained throughout the maximum period specified, taking into consideration the potential for reasonably anticipated temperature abuse during manufacture, storage, distribution, sale, and handling by the consumer.

Reasonably anticipated temperature abuses constitute the normal period of transporting of purchased products to appropriate consumer storage facilities and normal patterns of handling during consumption. For instance, the number and length of periods in which the product is removed from the refrigerator and subjected to ambient temperatures until the whole package has been consumed can be used for durability calculations.

In some countries (e.g., EC), durability information on food may be presented in two forms, as follows:

- A date of minimum durability ('best before') applied to indicate when the food, when properly stored, starts losing its specific (quality) characteristics. Beyond this date, the food may still be perfectly safe and satisfactory. Durability information can be presented in this form on the label of all milk products.
- A use-by date ('use by', 'expire by') applied for highly perishable foods only and to indicate when the food is likely to constitute an immediate microbiological danger to human health. After this date, such highly perishable food is likely to contain hazards at levels that may cause an adverse health effect and sale is not permitted.

Additional Specific Labeling Requirements for Milk Products

Principally, the general labeling rules should be sufficient for the labeling of dairy products provided that these are properly implemented and interpreted. However, some additional labeling requirements specific to some milk products have been found necessary. Such deviations are acceptable when they are fully justified according to scientific or technological evidence, or when severe difficulties occur in practically adhering to the general labeling provisions.

Additional requirements, other than interpretation and/or implementation of general labeling rules, normally include

- Mandatory declaration of (1) the milk fat content of creams and (2) the milk fat in dry matter content of cheese and cheese products, as the milk fat content is essential to the identity of the product.
- Mandatory declaration of the milk fat content of other dairy products, if the omission would be misleading to the consumer. Omission would, for instance, not be misleading if other descriptive material in the labeling addresses the milk fat content.
- Mandatory declaration of the milk protein content of milk powders, cream powders, evaporated milk, and sweetened condensed milk if the omission would be misleading to the consumer. Omission may be misleading if there is a significant variation of protein contents.

Optional (and Conditional) Labeling Requirements

Nutrient Declaration

Declaration of nutrients is generally voluntary, but national legislation in certain countries mandates such declaration for certain foods.

Where applied, the declaration will, as a minimum, include energy value, contents of protein, available carbohydrates, and fat.

However, the use of nutrition claims normally triggers mandatory nutrient declaration, the required content of which may differ from country to country. Examples are provided in **Table 5**.

In some countries, and perhaps recommended by Codex in the future, the use of nutrient claims relating to sugars, fibers, saturated fat, and sodium will also trigger the mandatory nutrient declaration of these substances.

Vitamins and minerals for which recommended intakes have been established may be nutrient declared if present in significant amounts (i.e., above 5% of the recommended daily intake).

Table 5 Examples of nutrient declaration consequences of making claims

Triggering claim	*Nutrient declaration required*
Amount or type of sugar emphasized	Total amount of sugars, possibly also the amount of starch and/or other carbohydrate constituents
Amount or type of fatty acids or cholesterol	Amounts of saturated fatty acids or cholesterol and of polyunsaturated fatty acids

Claims

A claim is any representation that states, suggests, or implies that a food has particular characteristics relating to its origin, nutritional properties, nature, production, processing, composition, or any other quality.

Claims are normally not permitted if they are misleading, if they cannot be substantiated, or if they could give rise to doubt about the safety of similar food or could arouse or exploit fear in the consumer.

Nutrition claims

A nutrition claim is a statement (other than list of ingredients, information included in nutrition labeling, and mandated declaration of certain nutrients and/or ingredients) that suggests, states, or implies that a food has particular nutritional properties. Any nutrition claim triggers mandatory nutrition declaration.

The conditions for using some nutrition claims, as recommended by Codex Alimentarius, are provided in **Table 6**.

Comparative claims

A comparative claim is a claim that compares the nutrient levels and/or energy value of two or more food, such as 'reduced' ('light'), 'less than', 'increased', and 'more than'. Such claims may only be used for different versions of the same food or similar foods, and these have to be clearly identified.

Health claims

A health claim is understood as a statement that suggests, states, or implies that a relationship exists between a food or one of its constituents and health. A health claim must consist of (1) information on the physiological role of the nutrient or on an accepted diet–health relationship and (2) information on the composition of the product relevant to the physiological role of the nutrient or the accepted diet–health relationship.

To avoid the misuse of fear of disease in connection with the sale of foods, health claims have to be based on relevant, scientific substantiation and the level of proof is to be sufficient to substantiate the type of claimed effect and the relationship to health as recognized by generally accepted scientific review of the data. A health claim may not be accepted if not in compliance with the national health policy. Further, it may be considered misleading if the claim is not based upon reasonable quantity of consumption in the context of a normal diet. Health claims

Table 6 Codex recommended conditions for using nutrition claims (CAC/GL 23-1997, rev. 2008)

Component	*Claim*	*Conditions (solid foods)*	*Conditions (liquid foods)*
Energy	Low	Max. 40 kcal (170 kJ) per 100 g	Max. 20 kcal (80 kJ) per 100 ml
	Free	Not relevant	Max. 4 kcal per 100 ml
Fat	Low	Max. 3 g per 100 g	Max. 1.5 g per 100 ml
	Free	Max. 0.5 g per 100 g	Max. 0.5 g per 100 ml
Saturated fat	Low	Max. 1.5 g per 100 g and 10% of energy	Max. 0.75 g per 100 ml and 10% of energy
	Free	Max. 0.1 g per 100 g	Max. 0.1 g per 100 ml
Cholesterol	Low	Max. 0.02 g per 100 g and compliance with the conditions for low in saturated fat	Max. 0.01 g per 100 ml and compliance with the conditions for low in saturated fat
	Free	Max. 0.005 g per 100 g and compliance with the conditions for low in saturated fat	Max. 0.005 g per 100 ml and compliance with the conditions for low in saturated fat
Sugars	Free	Max. 0.5 g per 100 g	Max. 0.5 g per 100 ml
Sodium	Low	Max. 0.12 g per 100 g	
	Very low	Max. 0.04 g per 100 g	
	Free	Max. 0.005 g per 100 g	
Protein	Source	Max. 10% of NRV per 100 g or Max. 5% of NRV per 100 kcal (12% of NRV per 1 MJ) or Max. 10% of NRV per serving	Max. 5% of NRV per 100 ml or Max. 5% of NRV per 100 kcal (12% of NRV per 1 MJ) or Max. 10% of NRV per serving
	High	Max. 2 times the values for 'source'	
Vitamins and minerals	Source	Max. 15% of NRV per 100 g or Max. 5% of NRV per 100 kcal (12% of NRV per 1 MJ) or Max. 15% of NRV per serving	Max. 7.5% of NRV per 100 ml or Max. 5% of NRV per 100 kcal (12% of NRV per 1 MJ) or Max. 15% of NRV per serving
	High	Max. 2 times the values for 'source'	
Dierary fibres	Source	3g per 100g or 1.5g per 100 kcal or 10% of daily (national) reference value per serving	3g per 100g or 1.5g per 100 kcal or 10% of daily (national) reference value per serving
	High	6g per 100g or 3g per 100 kcal or 10% of daily (national) reference value per serving	6g per 100g or 3g per 100 kcal or 10% of daily (national) reference value per serving

that encourage or condone excessive consumption of any food or disparage good dietary practice are not acceptable. Further, it is generally recognized that health claims should not be generally permitted for foods for infants and young children.

Health claims can be divided into three types:

- 'Nutrient function claims' that describe the physiological role of a nutrient in growth, development, and normal functions of the body, such as 'calcium aids in the development of strong bones and teeth. Milk is rich in calcium'.
- 'Other function claims' that concern beneficial specific effects (in the context of the normal diet) of the consumption of the food or constituent on normal body functions or biological activities of the body, such as 'calcium may help to improve bone density. Milk is rich in calcium'.
- 'Reduction of disease risk claims' that relate intake of the food or constituent (in the context of the normal diet) to a reduced risk of developing a disease, such as 'sufficient calcium intake may reduce the risk of osteoporosis in later life. Milk is high in calcium'.

Other claims

A number of other claims may be made in the label. Some of these are

- Religiously derived claims (e.g., kosher and halal). Codex has established general guidelines for the use of the term 'halal'.
- Claims relating to specific production methods (e.g., organic and bio). Codex has established Guidelines for the Production, Processing, Labelling and Marketing of Organically Produced Foods.
- Negative claims that highlight the absence or non-addition of particular substances (e.g., 'no preservatives added'). Such claims are misleading if the same or similar foods do not contain these additives (e.g., not permitted) or if the consumer would normally not expect to find them in the food.
- Terms like 'natural', 'pure', 'fresh', 'home made', and 'artisan'. The use of such terms may be regulated (e.g., 'fresh cheese').

Trends in Labeling Regulation

Regulation of the food sector through labeling is a never-ending story, driven by numerous factors such as new scientific and processing developments, epidemiological research, consumer awareness, environmental protection, etc.

Further, the trend to deregulate commodity-specific legislation, as for instance has been done in many countries on milk products, leaves labeling as the principal means of regulation.

Finally, the World Trade Organization (WTO) Agreement on Technical Barriers to Trade states that technical regulations shall not be more trade-restrictive than necessary to fulfil a legitimate objective; in many cases, the least trade-restrictive measure is a labeling requirement.

Labeling issues that are currently (2010) debated at an international level with a view to establishing new labeling requirements include establishing nutritional reference levels (NRVs) for nutrients other than vitamins and minerals, labeling of foods and ingredients obtained through certain techniques of modern biotechnology, and mandatory nutrition labeling.

See also: **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius; Standards of Identity of Milk and Milk Products.

Further Reading

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Codex Secretariat *Working Documents for Codex Meetings.* Rome: FAO. http://www.codexalimentarius.net/web/index_en.jsp.

Joint FAO/WHO Food Standards Programme *Codex Alimentarius.* Rome: Food and Agriculture Organization of the United Nations; World Health Organization.

LABOR MANAGEMENT ON DAIRY FARMS

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Introduction

Dairy farm managers accomplish their profitability, growth and excellence goals through people. Success with people on dairy farms depends primarily on effectiveness in hiring, orientation, training, communication and motivation of employees. This article will provide an overview of each of these major components of labor management.

Hiring

Success in labor management starts with hiring. An eight-step process for filling a position can guide a successful hiring plan.

Step 1: Determine Labor Needs

What the dairy farm needs rather than what an applicant likes to do should guide the hiring process. An understanding of the goals for the farm business and its current and long-term constraints to progress will help in identifying desirable characteristics for employees.

Step 2: Develop a Current Job Description Based on the Needs Identified in Step 1

Job descriptions help both the employer and employees by answering three questions: What does the jobholder do? How is it done? Under what conditions is it done? The job description has at least four parts:

1. Job title.
2. A brief one or two sentence summary of the job.
3. A listing of the major tasks involved in the job summarized under three to seven general headings.
4. A listing of the knowledge, skills and abilities necessary to do the job.

Job descriptions are typically one page long. The brevity requires a terse, direct writing style. Simple words with single meanings are best. Action verbs in the present tense should be used in defining the job duties, e.g. “feeds young dairy stock”. The job description does not include detailed protocols on how to do the job.

Step 3: Build a Pool of Applicants

Word of mouth and “help wanted” ads are likely to generate the most applicants. Word of mouth involves current employees, customers, neighbors, agribusiness contacts, veterinary surgeons and others who come in contact with potential employees. Word of mouth is fast and low cost. However, it limits the scope of the job search because qualified applicants may not hear about the position. Word of mouth is helped most by the farm’s reputation as an outstanding place to work. Following is a list of guidelines to help build a positive reputation as an employer:

1. Like, enjoy and appreciate employees.
2. Use written job descriptions.
3. Provide training.
4. Trust employees.
5. Catch people doing things right and say thank you.
6. Develop pride in your employees and business.
7. Celebrate farm successes with employees.
8. Communicate clearly and often.
9. Compensate fairly.
10. Provide exceptional monetary benefits.
11. Provide extraordinary nonmonetary informal benefits.
12. Promote from within.
13. Make the business friendly to employees’ families.
14. Be proud of employees who leave to advance their careers in the dairy industry.

“Help wanted” ads in newspapers and on the Internet have the potential of expanding the applicant pool beyond the local community. Following a seven-step process should result in an effective want ad:

1. Lead with a job characteristic that attracts positive attention.
2. Give a job title.
3. Say something positive about the farm.

4. Describe the job.
5. Explain necessary qualifications.
6. Explain that wages and benefits are competitive.
7. Say how to apply for the job.

Step 4: Review Applications and Select those to be Interviewed

The dairy manager uses the application form to exclude some applicants. Ideally, the employer has a remaining pool of at least three people. Disappointing losses from the applicant pool can come from the best person choosing a competing offer and some applicants withdrawing. Interviewing may dramatically change the evaluation of an applicant.

Step 5: Interview

Preparing a list of questions before the interview is critical to interview success. Avoid questions that can be answered yes or no. Instead of yes/no type questions, use open-ended questions that encourage applicants to explain experiences, characteristics and ideas in their own words. The open-ended questions should be geared toward the following general areas: previous job accomplishments and achievements, nonjob accomplishments and achievements, motivation and ambition, hobbies and use of leisure time, and "what if" questions. "What if" questions are based on practical real-world problem situations. The intent is to discover how the applicant would handle the real-world problem.

An interview team can help the dairy manager plan and conduct interviews. The following nine steps provide a logical plan for the interview team to follow.

1. Relax the applicant and build rapport (2–3 min).
2. Give the applicant a copy of the job description and describe the job in considerable detail (3–5 min).
3. Determine the accuracy of the information on the application form (4–7 min).
4. Ask a series of open-ended questions previously prepared (10–15 min).
5. Encourage the applicant to ask questions (2–5 min).
6. Summarize the farm's mission, objectives and business philosophy (2–4 min).
7. Summarize the opportunities the position provides (2–4 min).
8. Encourage the applicant to ask questions (2–10 min).
9. Close with information about plans for making a decision (2–4 min).

Interviewing is difficult. Knowing how to do it well makes it enjoyable. Some dos and don'ts can serve as reminders of how to improve one's interviewing skills.

Do:

- Make sure the applicant does most of the talking
- Listen!
- Concentrate on the interview and what is being said
- Show enthusiasm throughout the interview
- 'Read' nonverbal messages
- Show appreciation for the person being interested in the position
- Show pride in the farm, agriculture and the dairy industry
- Stay in control of the interview.

Don't:

- Project the answer desired from the applicant, e.g. "You do like cows don't you"
- Let note-taking during the interview detract from the flow on the interview
- Read questions to the applicant
- Let facial expressions and other nonverbal responses show dissatisfaction with the applicant's answers
- Add a series of follow-up questions to explore interesting side issues
- Allow an aggressive applicant to ignore questions and talk about things not on the interview agenda
- Go into the interview with the intention of simply confirming that a pre-interview favorite is in fact the best candidate for the position.

Step 6: Check References

References can provide additional information about those applicants still under serious consideration after being interviewed. Some employers skip this step because of previous employers' reluctance to share any useful information out of fear of defamation charges. Persisting with reference checks can still be productive. Personal visits or telephone conversations will be more productive than asking for written comments.

Step 7: Make a Selection

Strive to be as objective as possible given the job description, the knowledge, skills and abilities necessary to do the job and the information available concerning each applicant. If no satisfactory applicant is found, start the process again rather than deciding to take a chance on a doubtful applicant.

Step 8: Hire a Person

Make an oral offer in person or by telephone to the first choice. Follow-up with a written offer that summarizes the key conditions of employment. In making the offer, emphasize that the applicant is the first choice among several qualified people. Show enthusiasm over the hope that this person will soon be joining the farm team.

The First 30 Days: Orienting New Employees

A dairy farm employer has only one opportunity to make a good first impression on new employees. The first day that a new person is on the job provides many 'teachable moments'. Nearly all employees want to get off to a good start.

Orientation is the introduction of a new employee to the industry, the farm, the requirements of the job, the social situation in which he or she will be working and the farm‘s culture. The farm's culture includes its values (shared beliefs), history, traditions, and norms of behavior expressed as dos and don'ts. Orientation is socialization. A new employee who is socialized understands the key points about a farm and its people and why things are done in particular ways.

Introductions and informal interaction should encourage acceptance by other employees. A tour of the facilities should include short stops to hear people talk about their jobs and history of employment at the farm. Opportunity for new employees to ask questions can help people gain acceptance.

Orientation should cover farm characteristics, personnel policies, compensation and benefits, introductions to the management team and coworkers, and an overview of job duties. The specific content of the orientation depends on the size and complexity of the farm. An employee handbook can cover some of the orientation content. Employees can be asked to read the handbook and to discuss follow-up questions with their immediate supervisor.

Training Dairy Farm Workers

No matter how carefully a dairy farm manager hires and orients employees, they will not come to their new jobs with all the necessary knowledge, skills and abilities. Training is essential if employees are to reach their potential. Training should help them feel that they are creating better opportunities for themselves and at the same time helping the dairy farm business accomplish its goals.

Job instruction can be divided into getting ready to train and training. Two important questions guide preparation for training. What is the objective of the training? Define specifically what the learners are to know or be able to do at the conclusion of the training. What are the principal steps in the task and in what sequence should they be done?

The actual instruction can be aided by a five-step teaching method:

1. **Prepare** the learner. Learners are prepared when they are at ease, understand why they need to learn the task, are interested in learning and have the confidence that they can learn and the trainer can teach. The most important part of learner preparation is creating a need to know or desire to learn on the part of the trainee.
2. **Tell** the learner about each step or part of the task.
3. **Show** the learner how to do each step or part of the task. In demonstrating the task, explain each step emphasizing the key points and more difficult steps. Remember the little and seemingly simple parts of the task. Get the learner involved by asking questions about what is being shown.
4. Have the learner **do** each step of the task while the trainer observes. Ask the learner to explain each step as it is performed. If steps or parts of the task are omitted, re-explain the steps and have the learner repeat them. Then have the learner do the task without the trainer observing.
5. **Review** each step or part of the task with the learner, offering encouragement, constructive criticism and additional pointers on how to do the job. Be frank in the appraisal. Encourage the learner toward self-appraisal.

Communication

Communication plays a major role in employer–employee relationships on dairy farms. Although effective communication cannot guarantee success of a dairy, its absence usually assures problems. Communication influences the effectiveness of the hiring and training of employees, motivation of employees, providing daily instructions, performance evaluations and the handling of discipline problems. These are the obvious roles of communication. Communication also affects the willingness of employees to provide useful suggestions.

Communication Model

The communication process starts with a sender who has a message for a receiver. Two or more people are always involved in communication. The sender has the responsibility for the message.

The sender's message travels to the receiver through one or more channels chosen by the sender. The channels may be verbal or nonverbal. They may involve only one of the senses, hearing for example, or they may involve all five of the senses: hearing, sight, touch, smell and taste.

Nonverbal communication, popularly referred to as body language, relies primarily on seeing rather than hearing.

The sending of a message by an appropriate channel to a receiver appears to have completed the communication process or at least the sender's responsibility. Not so: after sending the message, the sender becomes a receiver and the receiver becomes a sender through the process of feedback. Feedback is the receiver's response to the attempt by the sender to send the message. Feedback is the key to determination by the sender of whether or not the message has been received in the intended form. In providing feedback, the receiver of the original message chooses the channel. The channel for feedback may be quite different from the original channel chosen by the sender. A puzzled look may be the feedback to what the sender considered a perfectly clear oral instruction.

Effect on the receiver completes the communication process. Effective communication is the original sender having the desired effect on the receiver.

Barriers to Communication

Muddled messages

Muddled messages are a barrier to communication because the sender leaves the receiver unclear about the intent of the message. Muddled messages have many causes. The sender may be confused in his or her thinking. The message may be little more than a vague idea. The problem may be semantics as for example in this muddled newspaper ad: "Dog for sale. Will eat anything. Especially likes children."

Feedback from the receiver is the best way for a sender to be sure that the message is clear rather than muddled. Clarifying muddled messages is the responsibility of the sender.

Stereotyping

Stereotyping causes people to typify a person, a group, an event or a thing on oversimplified conceptions, beliefs or opinions. Thus, basketball players can be stereotyped as tall, Holsteins better than Jerseys, and people raised on dairy farms as interested in animals. Stereotyping can substitute for thinking, analysis and open-mindedness to a new situation.

Stereotyping is a barrier to communication when it causes people to act as if they already know the message that is coming from the sender or worse, as if no message is necessary because "Everybody already knows." Both senders and listeners should continuously look for and address thinking, conclusions and actions based on stereotypes.

Wrong channel

"Good morning." An oral channel for this message is highly appropriate. On the other hand, a detailed request to a contractor for construction of a heifer barn should be in writing, i.e. non-oral. A long conversation between a manager and a contractor about the dairy barn construction, with neither taking notes, surely will result in confusion and misunderstanding. These simple examples illustrate how the wrong channel can be a barrier to communication.

In choice of a channel, the sender needs to be sensitive to such things as the complexity of the message (good morning versus a construction contract); the consequences of a misunderstanding (medication for a sick animal versus a guess about tomorrow's weather); knowledge, skills and abilities of the receiver (a new employee versus a partner in the business); and immediacy of action to be taken from the message (instructions for this morning's work versus a plan of work for the next 12 months).

Language

Words are not reality. Words as the sender understands them are combined with the perceptions of those words by the receiver. Language represents only part of the whole. Listeners fill in the rest with perceptions. Trying to understand a foreign language easily demonstrates words not being reality.

Each new employee needs to be taught the language of the farm. Until the farm's language is learned, it can be as much a barrier to communication as a foreign language.

Lack of feedback

Feedback is the mirror of communication. Feedback mirrors what the sender has sent. Feedback is the receiver sending back to the sender the message as perceived. Without feedback, communication is one-way.

Feedback should be helpful rather than hurtful. Prompt feedback is more effective that feedback saved up until the 'right' moment. Feedback should deal in specifics rather than generalities.

Poor listening skills

Listening is difficult. A typical speaker says about 125 words per minute. The typical listener can receive 400–600 words per minute. Thus, about 75% of listening time is free time. The free time often sidetracks the listener. The solution is to be an active rather than passive listener.

Tune out thoughts about other people and other problems. Search for meaning in what the person is saying. A mental outline or summary of key thoughts can be very helpful. Avoid interrupting the speaker. Withhold evaluation and judgement until the other person has finished with the message. A listener's premature frown, shaking of the head, or bored look can easily convince the other person that there is no reason to elaborate or try again to communicate his or her excellent idea. Providing feedback is the most important active listening skill. Ask questions. Nod in agreement. Look the person straight in the eye. Lean forward. Be an animated listener. Focus on what the other person is saying. Repeat key points.

Interruptions

A dairy farm is a lively place. Conversations, meetings, instructions and even casual talk about last night's storm are likely to be interrupted. The interruptions may be due to something more pressing, rudeness, lack of privacy for discussion, a drop-in visitor, an emergency, or even the curiosity of someone else wanting to know what two other people are saying.

Regardless of the cause, interruptions are a barrier to communication. In the extreme, there is a reluctance of employees and family members even to attempt discussion with a manager because of the near certainty that the conversation will be interrupted.

Physical distractions

Physical distractions are common on farms. If the phone rings, the tendency is to answer it even if the caller is interrupting a very important or even delicate conversation. A supervisor may give instructions from the driver's seat of a pick-up truck. Talking through an open window and down to an employee makes the truck door a barrier. A person sitting behind a desk, especially if sitting in a large chair, talking across the desk is talking from behind a physical barrier. Two people talking facing each other without a desk or truck-door between them have a much more open and personal sense of communication. Noise is a physical distraction simply because it is hard to concentrate on a conversation if hearing is difficult.

Motivating Employees

Three basic guidelines can help a dairy farm manager develop and maintain motivation among employees:

1. Satisfy employee needs.
2. Provide jobs employees enjoy having.
3. Make employees part of a farm team.

Satisfy Employee Needs

This guideline is based on a simple model of motivation:

1. Employees have needs that they desire to satisfy, which in turn leads to
2. Actions that will fulfill their needs, which in turn lead to
3. Intrinsic rewards from the work and extrinsic rewards from the employer and others, which in turn
4. Reinforces their actions and causes the employees to repeat the actions.

This simple model suggests a basis for deciding which rewards to provide each employee. By providing rewards tied to needs, the employer reinforces the employee's actions thus causing the employee to repeat the action to get more reward. To illustrate, an employee has a need to be thanked and appreciated. An employer, recognizing this need, gives an employee certain tasks. When the employee performs the tasks well, the employer regularly shows appreciation by saying thank you and giving merit increases in pay. The employee's needs are satisfied. To continue having the need for thanks and appreciation satisfied, the employee continues to do the tasks well.

Maslow's Hierarchy of Needs takes the needs approach a step further by dividing needs into five categories: (1) physiological, (2) security, (3) social, (4) esteem and (5) self-fulfillment. According to Maslow, the most basic needs are physiological, e.g. food, shelter and clothing. These most basic needs must be satisfied before the next higher-level needs, the security needs, become important. Security deals with the needs for a safe and secure physical and emotional environment. Social needs are satisfied through belonging. Employees have a need for friends, peers they respect who in turn are accepting, and supervisors with whom they have a positive relationship. Esteem needs relate to the desire to feel good about one's self and to have a positive self-image from job, family and personal experiences. Self-fulfillment is the highest level but most vague of the five needs. Self-fulfillment comes from an employee's job and personal accomplishments, special achievements, increased competence and growth as a person.

Herzberg developed a Two-Factor Theory of Motivation that also deals with needs but in a way different from Maslow. According to Herzberg, two factors affect employee motivation: dissatisfiers and motivators. Examples of dissatisfiers are poor working conditions, exhausting physical work combined with excessively long work days and weeks, unfair pay, disagreeable supervisors, unreasonable rules and policies, and conflict with coworkers. According to Herzberg, these problems must be resolved before motivators can work. Motivators are factors that influence job satisfaction and lead to motivation. Examples include achievement, recognition, satisfying work, responsibility and personal growth through training and new experiences. These factors turn an employee from being neutral about the job into a motivated employee.

Provide Jobs Employees Enjoy Having

Employers can design jobs with employee motivation in mind. Uninteresting or boring jobs will certainly cause motivation problems. Employers can capitalize on the advantages people see in farm work. To illustrate, people who love animals are motivated by the opportunity to work with animals. Some people like machinery much more than animals. Some enjoy repairing machinery more than operating it. Some people like office work; others want to be outdoors.

Managers should first take into consideration the tasks that must be accomplished for the farm to succeed. They can also take into consideration what individuals want in their jobs. Sometimes relatively minor changes in job design can dramatically improve a job in the employee's view, e.g. changing a calf-feeder's job to include explanation of calf care to farm visitors.

Certain job characteristics help motivate employees. First, jobs should be designed whenever possible to encourage employees to use a variety of skills. Second, jobs should be designed whenever possible so that an employee performs a total job, e.g. all aspects of calf raising as contrasted with just feeding. Third, jobs should be designed so that the employee understands the significance of his/her job to the farm. Fourth, jobs should be designed so that each employee has responsibility, challenge, freedom and the opportunity to be creative. Finally, supervisors should incorporate feedback into each job.

Make Employees Part of a Farm Team

Saying "We are a team" is easy. Actually functioning as a team is difficult. Making employees feel important to a farm team takes time. It often starts with how the employer views employees: are employees working managers or are employees managed workers? Employees as working managers suggests that each person in the business has ideas on how to improve the business.

Teams are built through four stages: forming, storming, norming and performing. In the forming stage, farm team members break the ice with each other, become oriented to farm goals and begin to exchange ideas. Storming is the stage of conflict, open disagreement and the surfacing of conflicting ideas. Managers face the challenge of getting disagreements out in the open for discussion and resolution. Norming follows from resolving conflicts. Team harmony and unity arise. By this stage, the leader is clearly identified and team members' roles are clear. By the performing stage, the team is functioning well. The team solves the farm's problems for the good of the farm business.

Concluding Comment

Managing labor on dairy farms is a complex task. This article has provided an overview of five components of labor management. Dairy managers who make labor management one of their important strengths master these five components plus several others. Successful labor management is a combination of positive attitudes toward people in the business, carefully honed interpersonal skills and human resource procedures designed to fit the business. Can each dairy manager learn to be a better human resource manager? The simple and emphatic answer is yes.

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LACTATION

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Lactogenesis

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Introduction

Other than bottle-fed humans and dairy calves, the success of reproduction depends on more than the birth of healthy offspring. For mammals, initiation of milk synthesis and secretion is essential for successful rearing of the neonate, and, ultimately, reproduction. Hormonal and growth factor stimulation of mammary development during gestation allows the generation of abundant glandular alveoli. The subsequent differentiation of the epithelial cells of these alveoli leads to onset of milk synthesis and secretion in conjunction with parturition. This is indeed a biological marvel. Milk of all mammals contains variable amounts of proteins, carbohydrates and fats in an aqueous medium. Although there are marked species differences with regard to details of milk composition, having the birth of the neonate and functionality of the mammary gland coincide is obviously critical. The purpose of this article is to provide an overview of final stages of gestational mammary development and especially the dramatic, acute changes in secretory cell structure and function as the gland prepares for onset of copious milk secretion called lactogenesis.

Overview of Mammary Structure

A mammary whole mount prepared from the mammary gland of a virgin mouse (**Figure 1**) illustrates the mammary ducts that elongate throughout the mammary fat pad in the period around puberty. These ducts provide a framework for the subsequent appearance of side branches and alveolar structures largely during gestation. The situation is grossly similar in ruminants except that the mammary stromal tissue contains many fewer adipocytes than in rodents, and proliferation of ducts into the mammary stroma follows a much more dense, compact developmental pattern such that the stroma is not filled during the peripubertal period. Regardless, during the second half of gestation, alveolar formation predominates mammogenesis as new alveoli are formed and existing alveoli increase in size. Connected via a terminal duct to progressively larger ducts and ultimately the teat or nipple, the epithelial cells of the alveoli synthesize and secrete milk.

Once lactation is established, milk secreted by these epithelial cells is stored in the luminal spaces of the hollow alveoli and ducts between milkings or suckling episodes (**Figure 2**). Milk is also stored in the gland and teat cisterns of those animals with mammary glands arranged into an udder (ruminants). Thus, the single layer of epithelial cells of the internal surface of the alveoli is responsible for all milk synthesis and secretion, and it is the onset of these functions that is the focus of lactogenesis.

Lactogenesis is usually described as a two-stage process. Stage 1 consists of limited structural and functional differentiation of the secretory epithelium during the last third of pregnancy. Stage 2 involves completion

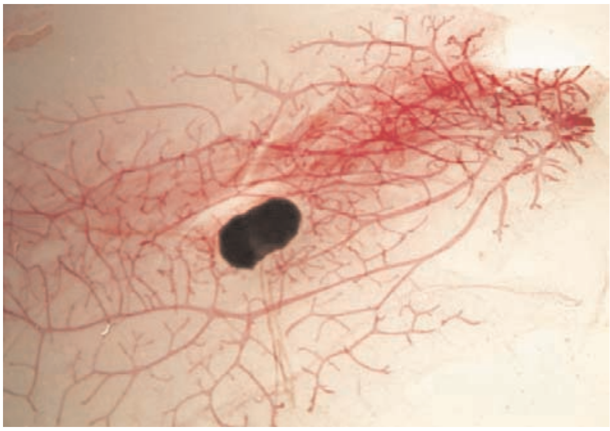

Figure 1 Fixed, defatted and stained whole mount of fourth inguinal mammary gland of a virgin mouse. Note that the gland has been defatted to allow observation of the epithelial elements of the gland. Note network of ducts, which radiate throughout the entire fat pad. Magnification is approximately 3×.

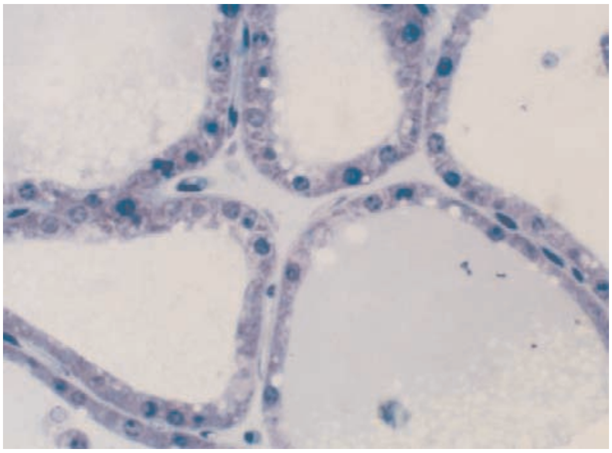

Figure 2 Light microscope view of a section of mammary parenchymal tissue from a lactating cow. Several alveoli cut in cross section are illustrated. Lighter stained areas are alveolar lumina. Secretory cells form a single layer around the periphery of each alveolus. Because of accumulation of secretions within the luminal spaces, alveoli are closely packed together with little apparent stromal or vascular tissue. Magnification is approximately 200×.

of cellular differentiation during the immediate periparturient period coinciding with onset of copious milk synthesis and secretion.

Secretory Cell Differentiation

Successful lactation requires at least three distinct events: (1) the prepartum proliferation of alveolar epithelial cells (2) biochemical and structural differentiation of these cells and (3) synthesis and secretion of milk constituents. During lactation, the secretory cells synthesize and secrete copious amounts of carbohydrate, protein and lipid. Production of this complex mixture of nutrients depends on coordination between biochemical pathways to supply metabolic intermediates and secretory pathways for secretion. For example, the disaccharide lactose is the predominate milk sugar. The enzyme complex necessary for lactose synthesis (membrane-bound galactosyltransferase and the whey protein α-lactalbumin) combine in the Golgi apparatus to form lactose synthetase, which links glucose and galactose, producing lactose. Activation of the α-lactalbumin gene and synthesis of α-lactalbumin is most closely associated with stage 2 of lactogenesis. Indeed, synthesis of lactose is essential to maintain milk volume and composition. This is because lactose trapped in membrane-bound secretory vesicles osmotically draws water into the vesicles. These lactose- and protein-containing vesicles fuse both singly and in chains with the apical plasma membrane and release their contents into the alveolar lumen via exocytosis. Because the plasma membranes of the duct and secretory cells are also impermeable to lactose, the osmolarity of the secretory vesicles and secreted milk is similar. It is generally accepted that some minimal level of production of lactose (or similar molecule) is essential to maintain the relative fluidity of milk for efficient milk removal either by the sucking young or the milking machine. For example, marine mammals in polar environments produce secretions with very little lactose but very large amounts of fat and protein. These dense, energy-rich secretions are particularly important for rapid weight gain in neonates to provide insulation from the cold and to minimize the metabolic use of water. Transgenic mice in which prevention of α-lactalbumin synthesis blocks lactation dramatically illustrate the significance of lactose for terrestrial mammals. Sucking pups are unable to obtain nutrients despite the presence of milk proteins and fat in the alveolar lumina of mammary tissue of lactating mothers.

Certainly, biochemical differentiation of the secretory cells is required for onset of milk secretion. However, the cells must also acquire the structural machinery needed to synthesize, package and secrete milk constituents. When alveolar cells first appear during mid-gestation, they exhibit few of the organelles needed for copious milk biosynthesis or secretion. These cells are characterized by a sparse cytoplasm with few polyribosomes, few clusters of free ribosomes, limited rough endoplasmic reticulum, rudimentary Golgi usually in close apposition to the nucleus, some isolated mitochondria and widely dispersed vesicles. Individual cells often contain large lipid droplets (especially during later stages of gestation) that, along with irregularly shaped nuclei, account for much of the cellular area. Soon after the alveoli appear, luminal spaces accumulate, thereby increasing volumes of fluid containing serum-derived proteins. These secretions result in formation of immunoglobulin rich colostrum, which, depending on the species, may be essential for survival of the offspring.

As parturition approaches, the alveolar cells undergo a dramatic structural transformation. Cell nuclei become more rounded and positioned in the basal area of the cells. Lateral and basal regions of the cells become filled with abundant arrays of rough endoplasmic reticulum and small lipid droplets. The apical cell area becomes populated with swollen arrays of Golgi membranes, developing secretory vesicles and small lipid droplets. Even in the light microscope, these changes are evident (**Figure 3**). A lacy appearance highlights the apical region of the cell because of the abundance of secretory vesicles in contrast with the darkly stained basolateral cytoplasm (ergastoplasm). The fully differentiated cell becomes decidedly polarized. The basolateral area is active in the uptake of precursors and synthesis of proteins and lipids, and the apical cytoplasm, with abundant Golgi, is devoted to posttranslational modification of proteins and packaging of proteins and lactose for secretion from the cell. The capacity of the cells to sequester immunoglobulins essentially disappears as the cells complete their differentiation. Indeed, inhibition of prolactin secretion during the period of lactogenesis promotes accumulation of immunoglobulins and correspondingly increased expression of IgG_1 receptors. Such experiments reinforce realization that complex coordination is necessary to shift mammary function from colostrogenesis to lactogenesis as parturition approaches.

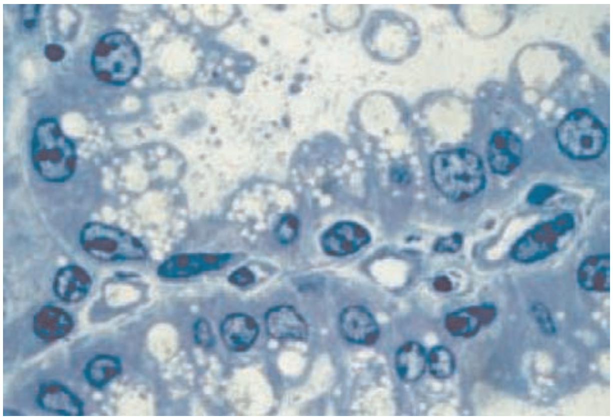

Figure 3 Light microscope view of a mammary parenchymal tissue from a lactating cow. Portions of two alveoli are illustrated. Note the lacy appearance of the apical regions of individual epithelial cells, presence of secretory vesicles and fat droplets. Cell nuclei are generally rounded and basally displaced so that the cells acquire a polarized appearance. Magnification is approximately 2000×.

Control of Lactogenesis

Now confirmed in numerous species, classic mammary explant culture studies demonstrated that the major positive regulators of structural differentiation of the secretory cells are glucocorticoids and prolactin. Mammary tissue explants from pregnant or steroid hormone-primed donors exhibit both biochemical and structural differentiation when incubated in culture medium containing a combination of insulin, glucocorticoids and prolactin. While it is widely believed that insulin is necessary for mammary tissue maintenance in culture, species variation in insulin sensitivity of the mammary gland in intact animals casts some doubt on this belief. Recent data support the idea that insulin-mediated effects on mammary cells in culture may actually represent effects more appropriately ascribed to the insulin-like growth factors (IGF-I and IGF-II). This is because mammary epithelial cells have specific IGF-I receptors, and insulin (especially at higher concentrations typical of culture experiments) is likely to bind to the IGF-I receptor. In general terms, glucocorticoids are most closely associated with development of rough endoplasmic reticulum, and prolactin is more associated with maturation of the Golgi apparatus and appearance of secretory vesicles. Prolactin added to explant cultures incubated with insulin and glucocorticoids dramatically increases the *de novo* synthesis and secretion of α-lactalbumin and caseins.

Application of molecular techniques to mammary gland biology has served to solidify the idea that prolactin and glucocorticoids are primary stimulators of mammary cell differentiation. For example, both prolactin and glucocorticoid response elements are found within the promoter regions of the genes for several mammary-specific milk proteins. Similarly, induction of both mRNA and specific milk proteins in response to addition of prolactin or glucocorticoids in isolated mammary epithelial cells confirms the importance of these hormones in lactogenesis and provides details for mechanisms of action.

On the other hand, despite the continuous presence of both prolactin and glucocorticoids in the circulation during gestation, stage 2 of lactogenesis is held in check until the period just prior to parturition. Assay of serum concentrations of the milk protein α-lactalbumin provides an excellent noninvasive illustration of each of the two phases of lactogenesis. For dairy heifers in their first pregnancy, α-lactalbumin concentrations are undetectable before day 200 prepartum but reach levels of about $20\,\mathrm{ng\,ml^{-1}}$ by 120 days before calving. Concentrations then increase exponentially during the last 30 days prepartum (40 to $\sim 300\,\mathrm{ng\,ml^{-1}}$ within a day of calving). Similar changes are also evident among cows induced into lactation with exogenous hormones.

Essentially, the combined effects of positive stimulators (prolactin, glucocorticoids, growth hormone and estradiol) and the reduced negative influence of progesterone interact to determine the timing of the onset of copious milk secretion. Many studies have reported changes in blood concentrations of these hormones in correspondence with parturition. In dairy ruminants, for example there are consistent large increases in concentrations of serum prolactin in the period around parturition and acute secretion of glucocorticoids in closer association with the actual birth process. Concentrations of estradiol progressively increase during late gestation to a maximum within a few days of parturition. In contrast, in association with luteolysis of the corpus luteum, concentrations of progesterone abruptly decline 3 to 4 days prior to parturition. Changes in other hormones (prostaglandin $F_{2\alpha}$ increases at parturition) as well as changes in hormone and growth factor concentrations in mammary secretions may also serve to regulate stage 2 lactogenesis. There are also marked changes in concentrations of IGF binding proteins during the periparturient period. However, it remains to be seen if IGF-I or IGF-II is acutely involved in lactogenesis. It is also possible that such changes correspond with a decrease in mammary cell proliferation with the onset of lactation, given that IGF-I is a potent stimulator of mammary cell proliferation. In rodents at least, there is also evidence that epidermal growth factor may act to modify the ability of prolactin to stimulate synthesis and secretion of milk proteins. Neither should it be forgotten that many of the growth factors and other biologically active substances in milk, especially during the early postpartum period, might have evolved to play a role in gastrointestinal tract physiology of the neonate quite independent of possible roles in maternal mammary physiology.

Although progesterone can inhibit lactogenesis, simple removal of progesterone does not necessarily induce onset of lactation. For many species, lactogenesis is well under way before the decline in progesterone before parturition. Furthermore, prepartum milking causes premature differentiation of the mammary epithelium and subsequent evolution of mammary secretions of essentially normal composition often several weeks before parturition. Thus, differentiation of the alveolar epithelium and onset of milk synthesis and secretion is possible despite presence of high progesterone concentrations in the blood.

Some of the best evidence for the importance of increased periparturient secretion of prolactin in stage 2 lactogenesis has come from experiments in which the administration of a dopamine agonist has been used to inhibit prolactin secretion and correspondingly impair lactation. In ruminants in which postpartum milking continues, administration of the dopamine agonist α-bromoergocryptine (CB154) reduced basal prolactin concentration about 80% and prevented the usual periparturient rise as well as milking-induced prolactin rise during the first week postpartum. Milk production was reduced 45% during the first 10 days postpartum. Lost milk production was associated with reduced synthesis of α-lactalbumin, lactose and fatty acids, as well as impaired structural differentiation of the mammary secretory cells. Cows treated with exogenous prolactin in addition to the agonist (to replace the periparturient surge in prolactin) showed no loss of milk production or effects on milk component biosynthesis or alveolar cell differentiation. Clearly, prolactin is important in mammary cell differentiation and lactogenesis. That the periparturient period is critical can also be gleaned from experiments in which intramammary infusion of the microtubule inhibitor colchicine during the week prior to parturition also markedly inhibited subsequent structural and biochemical differentiation of the secretory epithelium. This suggests that *in vivo* mammary cell differentiation normally occurs in a relatively short period and that disruption of the process during this time may have long-term consequences on milk production.

Aside from circumstantial evidence related to changes in circulating hormone concentrations, there is a marked increase in numbers of mammary cell receptors for prolactin, IGF-I and cortisol during late gestation. It is also relevant that progesterone receptor concentration is correspondingly reduced with the onset of lactation. Thus, simultaneous, coordinated changes in circulating hormones and receptors appear to regulate the timing of lactogenesis.

Data from a variety of tissue culture experiments also strongly support a role of these hormones in lactogenesis. For example, additions of estradiol or cortisol markedly enhance prolactin-induced secretion of α-lactalbumin by mammary explants taken from pregnant cows. Mammary explants from estrogen-primed or pregnant mice also require insulin, cortisol and prolactin for the accumulation of casein as well as α-lactalbumin. However, some caution is advised with wholesale extrapolation of results from culture to the intact animal as well as uncritical extrapolation between species. For example, induction of the various milk proteins in culture does not necessarily reflect the timing of events *in vivo*. Neither do existing culture methods allow consistent synthesis and secretion of milk lipids. Finally, hormone concentrations employed may not accurately reflect the situation at the level of the mammary cell in the animal. Culture systems by their very nature represent relatively uncomplicated regulation compared with the intact animal.

Despite our seemingly adequate understanding of the process of lactogenesis, study of the mammary gland as a target for a seemingly myriad of hormones and growth regulators remains as fertile subject matter for biochemists, molecular biologists, cancer specialists, biotechnologists and dairy scientists.

See also: **Mammals**. **Mammary Gland**: Anatomy; Growth, Development and Involution.

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Induced Lactation

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Introduction

The interest in inducing nonpregnant dairy animals into lactation has been there for centuries. This interest was enhanced as the modern dairy enterprise became more commercial with the discovery, development, and adoption of technologies like refrigeration, pasteurization, designed mating schemes, and advances in mechanical milking. The advanced dairy farm became more specialized with more emphasis on milk sales, and the nonlactating animal was promptly perceived to be a profit-consuming resource. Dairy farms today face the challenge of minimizing costs of production and maximizing volume of milk produced in order to remain profitable. Profitability may be improved by reducing the number of nonlactating cows and heifers on the farm, increasing the percentage of days-in-milk relative to herd life, and by saving cows of high genetic merit (and high milk production) but reduced fertility. Each of these goals could be accomplished with an approved safe and reliable method to induce cows into lactation. It should be noted that none of the hormonal approaches to induce cows into lactation has been approved for commercial use by the US Food and Drug Administration or the cogent regulatory agencies in Canada and the European Community.

Fundamental Methodology

During the last century, an impressive variety of methods have been used in an attempt to induce cows, goats, and sheep into lactation. These methods have included administration of estrogen (or synthetic estrogen), alone or in combination with progesterone, and administration of hormones orally, via subcutaneous or intramuscular injection, via percutaneous inunction, or by implantation of hormone-containing pellets. Different dosages of the hormone were tested, and the duration of administration varied from as brief as 7 days to over 100 days, the latter to mimic the duration of a significant proportion of normal pregnancy. The milk yield responses to these treatments were generally quite variable, with some animals not producing milk and some others remaining at a low level of milk production.

A number of significant advances in approaches to hormonally induce lactation emerged during the last 30 years. One advance was the demonstration that open dairy cows could be induced into lactation with a simple 7-day treatment with estradiol-17β (50–100 μg kg^{-1} body weight) and progesterone (250 μg kg^{-1} body weight) injected subcutaneously. This simplified the treatment (subsequently referred to as 7-day estrogen–progesterone treatment) but used higher daily doses of estrogen and progesterone than used previously. It was designed to mimic the high levels of these steroid hormones observed in plasma of cows during the last month of pregnancy, when most mammary development occurs. This treatment was very significant in that it was simple, relatively brief, and led to higher milk production than previously reported, but milk production remained variable, with cows producing only 50–80% of their previous production from a postpartum lactation. Another novel contribution during this period was the demonstration that an intravaginal sponge with high doses of estrogen and progesterone could be used to administer these hormones over a 10-day period, with the sponge being physically pulled from the cows after 10 days. This approach was desirable in eliminating injections and presumed animal discomfort, but without adjunct treatments (dexamethasone or reserpine) did not attain the milk yield performance of the 7-day estrogen–progesterone treatment. Many other treatment regimes have been tested with varying levels of success, and there remains ample opportunity for further improvements in treatment protocols.

The patterns of estradiol-17β, progesterone, and prolactin in plasma that result from the 7-day high-dose treatment have been described in several papers. Cows and heifers typically had progesterone levels of <2 ng ml^{-1} at the start of the treatment (although this is dependent upon the stage of the estrous cycle). Progesterone concentrations in plasma increased to 2.5–5 ng ml^{-1} by days 5–8 of the treatment, and then decreased rapidly as no additional hormone treatment was provided. By day 15, progesterone decreased to 1–1.5 ng ml^{-1} and must decrease further to <1 ng ml^{-1} in order for lactogenesis and the initiation of milk secretion to occur between days 18 and 21. Estradiol-17β followed a similar pattern: starting at <30 pg ml^{-1} on day 0, increasing to >400 pg ml^{-1} by days 5–7, and declining to <100 pg ml^{-1} by days 18–21 relative to the start of treatment. The anterior pituitary gland in cows responds to the estrogen–progesterone treatment with an increase in the secretion of endogenous prolactin by days 10–15, and a further increase in prolactin concentration to 40–50 $\mathrm{ng\,ml^{-1}}$ at the initiation of lactation around 18–21 days from the start of the treatment. It is this response in plasma prolactin, along with the significant decrease in progesterone, that is thought to promote mammary differentiation and appears to be one of the key factors in successfully inducing animals to produce milk.

At least two published studies demonstrated that milk yield from cows induced into lactation was quite dependent upon season of the year, as cows induced into lactation in late autumn or winter had significantly reduced milk production when compared to cows induced in the spring or summer months. Correlation analysis suggested that this was partly due to the significantly reduced prolactin secretion that occurs naturally with short day lengths. One can overcome this seasonal effect with environmentally controlled facilities.

The observed relationship between prolactin and subsequent milk production encouraged investigators to add additional treatments including prolactin secretogogues, like thyrotropin-releasing hormone and reserpine. In some cases, but not all, treatments that included drugs to increase prolactin secretion led to significant improvements in milk yield. Other investigators have added glucocorticoid treatments to the induction protocol, which were administered around the start of milking (typically days 18–21 with the 7-day protocol). The results of glucocorticoid treatments have been somewhat variable, with very significant improvements in subsequent milk production in some studies, but not as clear results in others. It may be that glucocorticoid or prolactin secretogogue supplementation was not beneficial to milk production in some studies because of animals that did not have adequate mammary development to respond to these treatments.

Lactation-induced cows have lower somatotropin concentrations in blood than observed in postpartum cows. As bovine somatotropin (bST) has been approved for use in the United States, it is possible to use bST to regulate metabolism in lactation-induced cows to support higher levels of milk production than previously observed. A 15–17% increase in milk yield resulted when cows were treated with somatotropin after being induced into lactation; there were no significant changes in milk composition.

Physiological Responses

The purpose of the estrogen–progesterone treatment to induce lactation is twofold, with the initial mammary response being the stimulation of mammary development and the second response the initiation of lactation. Estrogen and progesterone are the hormones of pregnancy that stimulate mammary ductal growth followed by lobuloalveolar development. Successful induction of lactation also requires biochemical differentiation of mammary cells to initiate milk secretion. Failure of either mammary development or biochemical differentiation will result in poor milk production.

Changes in the gross appearance of the mammary glands of cows hormonally induced into lactation are quite subtle in the first week after initiation of the treatment. During the second week of the 7-day protocol, there is a small but observable increase in the size of mammary gland. During the third week from the start of treatment, the teats increase in the size of and mammary glands become more turgid as they fill with milk. Many animals that will subsequently produce high volumes of milk will develop edema around the udder in the few days prior to milking, just as many cows do at the time of parturition.

Studies have examined the development of mammary glands in hormonally induced animals by histological examination or as assessed by magnetic resonance imaging of mammary tissue. Histological analyses show numerous small ducts and alveoli during the first 2 weeks from the start of treatment. Data suggest that ducts grow into the mammary stromal areas, and that many small alveoli form, but that the degree of histological development was generally less than one would observe in the cow udder within a few days of parturition. Imaging analysis shows that most of the growth of the mammary parenchyma occurs after the period of estrogen–progesterone treatment, and continues into early lactation. Thus, at the initiation of milking in most induced lactation protocols, there is likely concomitant mammary cell proliferation and differentiation in the udder, as has been observed in rat and mouse mammary glands at the time of parturition. A two-dimensional image analysis of udders from cows that were induced into lactation demonstrates a modest 12% increase in udder area by 14 days of treatment when compared to pretreatment area, and a further 30% increase in premilking udder volume on day 28 (**Figure 1**). Some of the increased volume on day 28 is due to accumulation of milk over the previous 12 h, but the remainder reflects increased mammary parenchymal tissue volume. Measurements of DNA, RNA, and enzymatic activities generally reflect the developmental state of the tissue, with activities increasing around the initiation of milking and tissue from animals producing more milk having greater activity than exhibited by tissue from animals producing lower amounts of milk.

A significant and repeatable finding among studies that have used the 7-day estrogen–progesterone treatment to induce lactation is the consistent observation of behavioral estrus activity beginning the second week after the onset of estrogen treatment. This is expected given the normal proestrus effects of estrogen on the brain. It is

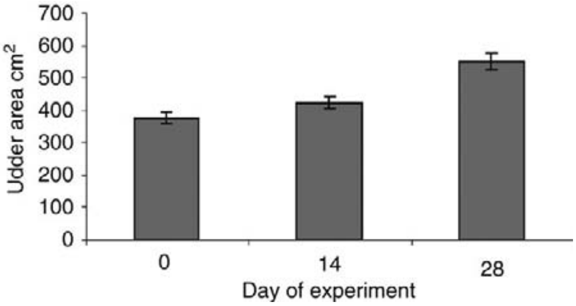

Figure 1 Rear udder dimensions of cows as determined by analysis of digital images captured on days 1, 14, and 28 of hormonally induced lactation. Cows ($n = 28$) were treated with estrogen and progesterone on days 1–7, and milking commenced on day 18 of the experiment.

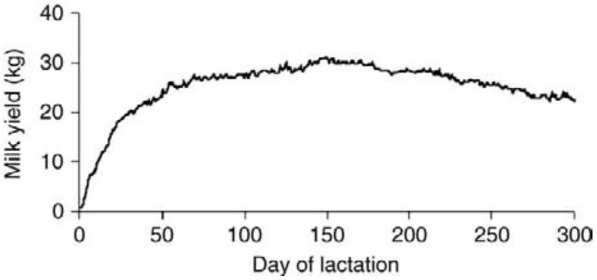

Figure 2 Milk yields over 305 days for cows induced into lactation. Cows ($n = 28$) were treated with estrogen and progesterone for 7 days, milking commenced on day 18 of the experiment, and all cows were treated with bovine somatotropin during lactation.

potentially problematic, however, because the extended duration of intense estrus-like activity, including mounting behavior, makes these animals prone to injury. The cause of such behavior is known.

The levels of estrogen in the circulation during the induction are designed to mimic the concentrations observed in late pregnancy, and will cause the pelvic ligaments and tendons to remodel and loosen as it normally occurs in preparation for parturition. However, as the induced cow has no corpus luteum (or adequate progesterone) to attenuate the estrus behavior, the intense mounting activity in the face of the pelvic changes allows unconfined-grouped cows to injure themselves. Many publications have indicated injuries to some animals allowed to run in groups. This problem can be avoided by simple management practices. Cows should be confined in a tie-stall facility for about 4 weeks after the initiation of the treatment to prevent them from injuring themselves. A goal of new induction protocols may be to refine the delivery of hormones in a manner to achieve the mammary growth effects without eliciting the estrus-behavioral side effects.

Few data are available concerning voluntary appetite of animals hormonally induced into lactation, but casual observation suggests that the appetite of animals is reduced when estrogen concentration peaks in the plasma, and it is evident that appetite increases significantly as milking commences. More data in this regard would be useful for verification, but it appears that the biological controls of appetite are working in the induced cows during lactation the same way as they do in postpartum cows.

Milk Production and Lactation Curve

The composition of colostrum and milk that results from methods for inducing lactation is generally similar to that of normal colostrum and milk ('Milk Composition and Value of Technology'), but the shape of the lactation curve for the cow is somewhat different. Milk production commences very slowly (**Figure 2**), and requires more time to reach the peak milk yield than observed for cows in postpartum lactation. Some studies report 60–100 days before peak milk yield is attained. More recent work using bST indicated that cows did not attain peak milk yield until about 125 days in milk (**Figure 2**). After peak milk production, the induced cows were very persistent, as milk yield remained over 25 kg day^{-1} through almost 300 days, and averaged 27.1 kg day^{-1} (**Figure 2**).

An exciting new opportunity reported recently was that the 7-day estrogen–progesterone treatment when supplemented with placental lactogen, dexamethasone, and prolactin could be used in heifers at 15 months of age to induce lactation. Those heifers produced over 10 kg milk day^{-1} by 9 weeks in lactation, suggesting that opportunities exist to induce pubertal heifers into lactation and have them return income to the farm at a much earlier age. Long-term health implications are not yet known.

Milk Composition and Value of Technology

The composition of the colostrum and milk from cows induced into lactation has been investigated in numerous studies. There is concurrence that the composition is normal given the level of milk production and the fact that the initiation of lactation is more gradual in hormonally induced cows. Protein concentration in the initial colostrum of induced cows can average as high as 20%, with the expected high somatic cell count, and low lactose and fat (**Figure 3**). By day 7 of an induced lactation, most of the transition from colostrum to mature milk has taken place as milk protein has declined to about 5% and lactose and fat have increased to near the concentrations expected for mature Holstein milk (**Figure 3**). There are small but continued decreases in protein and somatic cell

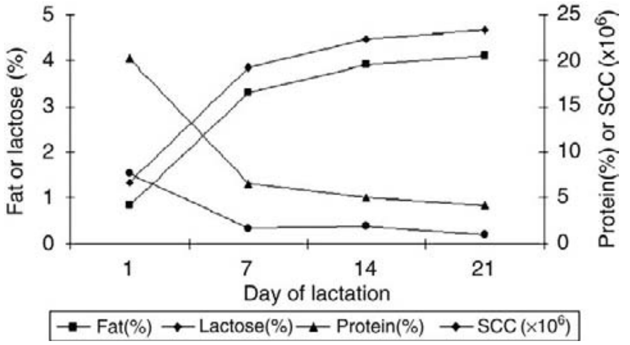

Figure 3 Composition of major milk components on days 1, 7, 14, and 21 of lactation from cows hormonally induced into lactation. Cows ($n = 28$) were treated with estrogen and progesterone on days 1–7, and milking commenced on day 18 of the experiment. SCC, somatic cell count.

count, and increases in lactose and fat, through 21 days in milk, reflecting the previously mentioned gradual increase in milk production. Thus, the only difference in major components between induced cow milk and postpartum cow milk is the more gradual transition from colostrum to mature milk.

The levels of immunoglobulin G (IgG) in the colostrum of induced cow milk are interesting given the desire to ensure adequate passive transfer of immunity to neonatal calves. The very viscous nature of day 1 colostrum from cows induced into lactation is reflected by IgG concentrations that averaged $200\,\mathrm{mg\,dl^{-1}}$, and which declined precipitously to less than $20\,\mathrm{mg\,ml^{-1}}$ by day 7 of lactation, and even further by days 14 and 21 (**Figure 4**). These data reinforce the conclusions above relative to the transition from normal colostrum to mature milk in induced cows, and indicate that cows induced into lactation are an excellent model to study the mechanism of IgG transport into mammary secretion.

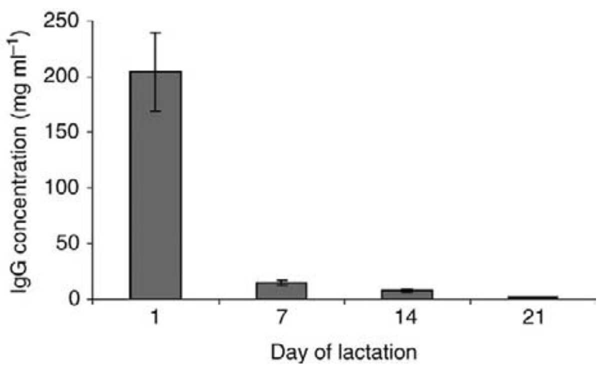

Figure 4 Concentrations of immunoglobulin G as determined by enzyme-linked immunosorbent assay in mammary secretions on days 1, 7, 14, and 21 for cows hormonally induced into lactation. Cows ($n = 28$) were treated with estrogen and progesterone on days 1–7, and milking commenced on day 18 of the experiment.

A number of studies have examined the concentrations of estrogen and progesterone in the colostrum and milk of cows induced into lactation, primarily because of concern for individuals consuming the milk. In general, the concentrations of these hormones in milk are not significantly higher than concentrations observed in the milk of postpartum cows. Some studies report that concentrations are actually lower in milk from induced cows. This is not surprising given the relatively short duration of elevated plasma hormone concentrations seen with modern approaches to induce lactation, such as the 7-day treatment. In contrast, a pregnant cow experiences a significantly longer duration of high concentrations of estrogen and progesterone in plasma.

An analysis of the value of a cow induced into lactation relative to replacing her with a replacement heifer indicates that the hormonally induced cow would have a US$500 net present value advantage over the heifer. Thus, assuming that a commercially approved product was available, there is significant economic incentive for a farmer to retain a nonpregnant but otherwise healthy cow. Similarly, there should be significant economic incentive for private industry to develop this technology and obtain the approval of the appropriate regulatory agencies.

Health Concerns

The literature has numerous statements suggesting that treatments to hormonally induce lactation (1) can lead to abnormal structures on the ovaries, (2) should not be used to try to salvage problem breeders, and (3) will not result in very good subsequent reproductive performance. The reality is that most of those studies were performed with very limited numbers of animals; many of them were performed with cows that were problem breeders prior to the initiation of the treatment; and few studies have allowed cows to complete full lactations and pregnancies to report actual calving data. One study with 28 cows (mostly problem breeders) showed that 21 of 28 calved subsequent to the induced lactation and that was achieved with 1.6 services/conception. Thus, it appears that good reproductive success is possible, and that there need not be any great concern about the subsequent reproductive health of these cows.

The concerns previously stated about excessive and prolonged estrus behavior soon after the estrogen–progesterone treatment are real, as there are reports in the literature of cow injuries. However, simple confinement of animals for a few weeks can eliminate this problem, and there may be modified treatments in the future that do not lead to these behaviors.

Concerns for long-term safety of cows and heifers have not been adequately addressed in the literature simply because few investigators have made the investment in long-term studies to adequately evaluate health and survival data. Scientists should be prepared to defend the technique based upon factual information. Data in the literature suggest that heifers and cows that experience a 9-month gestation are exposed to much more estrogen and progesterone than animals treated with estrogen and progesterone for 7–10 days as is done in many experiments to induce lactation.

An alternate view is worth consideration. Given that the majority of health problems experienced by dairy cattle, including parturient paresis, ketosis, fatty liver, displaced abomasum, new intramammary infection, uterine infection, and nerve damage, occur within 14 days of parturition, it is likely that hormonal induction of lactation (on a herd basis) could actually improve the general health of dairy cows, decrease herd culling rate, and make the entire enterprise more profitable. However, given the complexity of regulation of mammary cell proliferation and lactogenesis, it appears unlikely that scientists will ever develop techniques to hormonally induce lactation that will be superior to the stimulus of pregnancy and parturition. Furthermore, successful reproduction is required to make continued genetic progress. Consequently, induced lactations, assuming techniques are one day approved by regulatory agencies, should be viewed as a tool to use in selected situations to make the dairy enterprise more profitable.

Applications of Induced Lactation

Induced lactation could increase income from commercial dairy farms by reducing the number of heifers raised on the farm. All dairy farms, at some time, come across good cows of high genetic merit that simply express low fertility during a given lactation. Healthy, multiparous open dairy cows could be induced back into lactation after a dry period, thereby reducing the number of replacement heifers needed. Profitability may be enhanced further by use of somatotropin during induced lactation. In addition, farmers could also induce pubertal-age heifers into lactation if the practice proves to be safe and a commercial product is available. This practice would significantly reduce heifer-rearing costs and make them profitable much sooner than current practice allows.

Hormonally induced lactation offers a useful scientific model for mammary gland biology, including the study of mammary development and tumorigenesis, biochemical differentiation of the mammary gland, and the regulation of IgG transport into mammary secretion. Many additional questions about regulation of gene expression and the balance between cellular proliferation and apoptosis might be answered as well.

Hormonal induction of lactation also has great value in other areas. There are numerous projects worldwide that endeavor to utilize transgenic dairy animals to produce commercially valuable proteins in milk, including clotting factors, human hemoglobin, growth factors, enzymes, and human lactoferrin. The great challenge in using dairy cattle for this purpose is their long generational interval (9-month prenatal period and about 24 months before a heifer has her first offspring and produces milk). Hormonally induced lactations could reduce this time. The use of techniques to hormonally induce young transgenic animals into lactation would allow much earlier determination of whether the beast was secreting the protein in milk, the level of expression, and whether it was expressed in a mammary-specific manner. All of these are important questions when one considers the cost of maintaining a herd or flock.

See also: **Lactation:** Galactopoiesis, Effects of Hormones and Growth Factors; Lactogenesis. **Mammary Gland:** Growth, Development and Involution.

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Galactopoiesis, Effects of Hormones and Growth Factors

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Introduction

The term 'galactopoiesis' was originally coined to describe the enhancement of an established lactation. In this sense, only exogenous somatotropin (ST) and thyroid hormone are clearly demonstrated galactopoietic agents in dairy animals, suggesting these hormones are endogenously rate limiting. However, in a more inclusive sense, galactopoiesis has been used to describe the maintenance of lactation. A number of hormones and factors are involved in the maintenance of milk production in dairy animals, and these will be described in this article.

Galactopoietic hormones, growth factors, and regular milk removal are essential for the regulation and maintenance of lactation. The pituitary gland and its hormones are essential integrators of the endocrine regulation of milk secretion. Milk production of goats declines precipitously after hypophysectomy, but it can be fully restored to pre-hypophysectomy levels by the combined administration of prolactin (PRL), ST, glucocorticoids, and triiodothyronine (T_3; **Figure 1**). Although there are clear species differences, endocrine organ ablation/replacement studies, have shown that PRL, ST, glucocorticoids, and thyroid hormones are typically required for the full maintenance of lactation. Still, additional hormones and growth factors (both humoral and local, identified and unidentified) are probably important for the normal physiological maintenance of lactation. In addition to regulatory factors that actively support synthesis of milk components, frequent emptying of the mammary gland is critical. This process is supported by the milking-induced release of oxytocin. Physiological support for both processes – milk synthesis and milk removal – is necessary for maintenance of lactation. Other factors that can affect the maintenance of lactation are those that impinge upon the maintenance of the secretory cell population by decreasing cell loss or by increasing cell proliferation. These factors do not impact the secretory capacity of existing cells but impact the secretory capacity of the mammary gland, and the shape and length of the lactation curve.

Somatotropin

The ability of a crude extract of anterior pituitary to increase milk production in goats and cows was first demonstrated in the 1930s. Identification of ST or growth hormone as the active component occurred in the 1940s. Since then, there has been much progress in understanding the galactopoietic activity of ST in dairy animals, although questions regarding its mode of action remain. This progress was greatly accelerated by the advances in molecular biology that allowed production of commercial quantities of bovine ST.

The clearest galactopoietic activity of ST appears to be in coordinating changes in tissue metabolism that promote a flux of nutrients and energy to the mammary gland. Adipose and liver tissues play prominent roles in this process. These actions appear to be largely mediated by ST receptors in these tissues. In the adipose tissue, ST inhibits lipogenesis when animals are in positive energy balance and promotes lipolysis when animals are in negative energy balance. In the liver, ST promotes gluconeogenesis, which is particularly important in ruminants, in which nutrient absorption provides a negligible percentage of the glucose required for milk synthesis. In other tissues, the galactopoietic activity of ST appears to be mediated by other members of the ST axis, including the insulin-like growth factors (IGFs), most prominently IGF-1, and the insulin-like growth factor binding proteins. In muscle and other body tissues, ST decreases glucose utilization and oxidation of amino acids. The net result of these metabolic changes is to conserve nutrients and energy for synthesis of milk lactose, protein, and lipid. A direct action of ST has not been observed in lactating mammary tissue, and functional ligand binding assays have failed to detect receptor protein. The IGFs and their binding proteins appear the most likely mediators of effects on the lactating mammary gland, whereby ST increases milk synthesis.

For a more complete discussion of the galactopoietic activity of ST and IGFs, see the article **Lactation**: Galactopoiesis, Effect of Treatment with Bovine Somatotropin by Capuco and Akers.

Thyroid Hormones

Involvement of the thyroid in the maintenance of lactation has long been appreciated. In 1918, Grimmer reported that milk yield is reduced in thyroidectomized goats. Subsequently, in 1934, Graham showed that

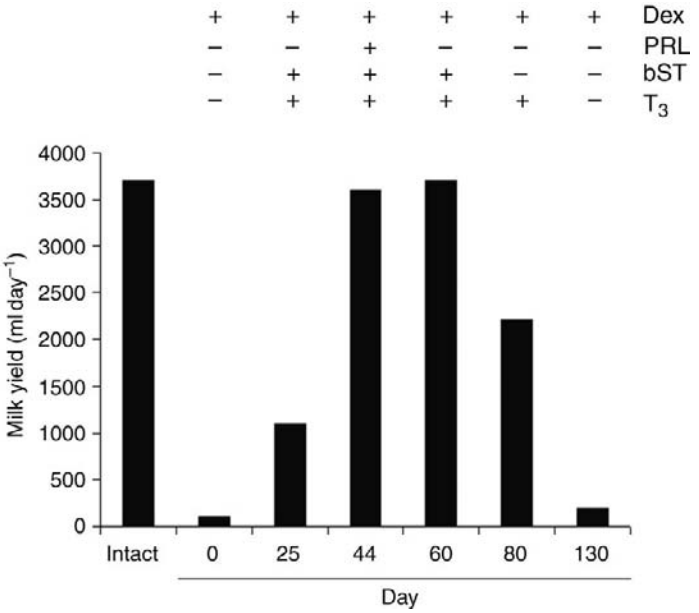

Figure 1 Daily milk yields of a goat after hypophysectomy and during replacement hormone therapy. The goat was producing approximately 3700 ml of milk per day prior to hypophysectomy (intact). After treatment with dexamethasone (Dex) for 2 months, milk production was as shown at time 0 in the figure. Milk production is depicted after addition or removal of the hormones, as illustrated. PRL, prolactin; ST, somatotropin; T_3, triiodothyronine. Adapted from Cowie AT (1969) General hormone factors involved in lactogenesis. In: Reynolds M and Folley SJ (eds.) *Lactogenesis: The Initiation of Milk Secretion at Parturition*, pp. 157–169. Philadelphia: University of Pennsylvania.

thyroidectomy of dairy cows reduced milk yield, and conversely, treatment with the thyroid hormone, thyroxine (T_4), increased milk yield by approximately 20%. Because T_4 is efficacious when fed, these results aroused considerable interest in the practical utilization of the hormone to increase milk production in cattle. This was made economically feasible by the manufacture of T_4 and thyroactive iodinated proteins at reduced cost. However, results of numerous experiments indicated that while feeding T_4 (or iodinated protein) increased milk production by 10–40%, the galactopoietic effect was of variable duration, and milk production returned to normal or below normal levels despite continued treatment. The galactopoietic effect of T_4 supplementation appears due to a general increase in body metabolism. Thus, it is not effective when cows are in early lactation (negative energy balance) and mobilizing body reserves to meet the energy demands of lactation. A general increase in body metabolism at this time would be contrary to meeting the nutrient demands of lactation. It was concluded that T_4 treatment should not be initiated before mid-lactation and that energy density of the diet should be increased during treatment because feed intake does not increase in proportion to increased energy utilization. Furthermore, upon withdrawal of treatment, a hypothyroid condition ensues that exacerbates the decline in milk yield. Despite an initial interest in thyroid hormone supplementation to increase milk yield, the temporary nature of the milk yield response and frequent overshoot below normal production led to the conclusion that its adaptation would be of minimal value.

In addition to a general effect on metabolic rate, thyroid hormones potentiate the activity of other lactogenic and galactopoietic hormones. T_3 enhances the ability of PRL to stimulate lactose synthetase in mouse mammary tissue cultures by approximately fivefold and enhances PRL stimulation of casein synthesis in rabbit mammary tissue culture. Similarly, for estradiol to stimulate lactogenesis in mouse or bovine mammary tissue culture, T_3 must be present in the culture medium.

In contrast to the general increase in metabolism evident with thyroid hormone supplementation, organ-specific changes in thyroid hormone metabolism occur during lactogenesis that may facilitate adaptation to a lactational state by promoting differential rates of energy utilization. Although T_4 is the predominant thyroid hormone in the circulation, it may be viewed as a prohormone because it has little, if any, biological activity. The most metabolically active thyroid hormone, 3,3′,5- T_3, is produced by enzymatic 5′deiodination of T_4 within the thyroid and peripheral tissues. The extra-thyroidal activity of T_4-5′-deiodinase (5′D) is an important regulator of localized T_3 availability in animal tissues during various physiological states. With onset of lactation in rodents and ruminants, there is an increase in 5′D in the mammary gland and a decrease in the liver. In cows, sensitivity of the mammary gland is also supplemented by an increase in thyroid hormone receptor-β1 and a decrease in thyroid hormone degradation. These changes should maintain a euthyroid state in the mammary gland while promoting a hypothyroid condition for the body as a whole. The transfer of iodine, iodinated nonhormonal compounds, and thyroid hormones through the mammary gland into the milk further promotes a systemic hypothyroid condition. Maintenance of a euthyroid state in the lactating mammary gland in the midst of a functional hypothyroid condition is consistent with increasing the metabolic priority of the mammary gland and providing T_3 to potentiate the effect of other galactopoietic hormones.

The opposite organ-specific changes in thyroid hormone metabolism are proportional to lactational intensity in rodents and appear to be involved in eliciting a response to galactopoietic hormones. In mice, thyroid hormones are necessary to obtain milk production increases in response to ST and PRL, and it has been demonstrated that mammary 5′D is uniquely responsive to galactopoietic hormones. Both ST and PRL increase 5′D activity in murine mammary gland, but do not alter activity in the liver or the kidney. Similarly, changes in 5′D activity are hypothesized to mediate or augment the galactopoietic effect of bST in dairy cows. However, results are not as consistent as those in rodents, and further investigation is necessary to clarify the interaction between thyroid hormone metabolism and bST-increased milk production in cattle.

The relationship between ST and thyroid hormones is not limited to ST-induced alterations in 5′D during lactation and galactopoiesis. There is a close relationship between thyroid hormones, thyroid hormone metabolism, ST, and IGF-I synthesis. Mechanistically, T_3 can alter hepatic ST receptor binding and thus enhance ST stimulation of IGF-I synthesis, or T_3 can increase IGF-I synthesis in the absence of ST. It is worth noting that in those situations when ST does not stimulate IGF synthesis (e.g., during food restriction, fetal development, sex-linked dwarfism, and hypothyroidism), there is evidence for T_3 deficiency. In addition, T_3 serves as a regulator of ST synthesis by the pituitary gland. Conversely, ST can alter synthesis of 5′D and the peripheral production of T_3.

Prolactin

The pituitary hormone (PRL) was named in deference to its pro-lactational effects. However, species differ with regard to their sensitivity to PRL and its impact on lactation. In rodents and primates, PRL is mammogenic (particularly during pregnancy), is lactogenic, and is the primary galactopoietic hormone. However, in ruminants, PRL plays a less dominant role in mammary gland physiology. In these dairy animals, it is a primary lactogenic hormone, but its galactopoietic activity is subtle and its mammogenic activity questionable. In ruminants, ST is thought to be the primary galactopoietic hormone and PRL a minor player. The opposite appears to be the case in other species, for example, mice, rats, rabbits, and humans.

It is clear that the mammary epithelium responds directly to PRL. Receptors for the hormone have been identified in mammary epithelial cells of numerous species, and these receptors have been characterized as members of the cytokine receptor superfamily. The receptors do not have intrinsic kinase activity, but associate with the Janus kinase (JAK) family of tyrosine kinases, which, when activated by receptor binding and dimerization, phosphorylates cellular proteins. Of primary importance appears to be phosphorylation of the signal transducer and activator of transcription-5a (Stat5a). Stat5a is a key regulator of casein transcription in keeping with a role for PRL in lactogenesis and galactopoiesis.

In contrast to laboratory rodents, a galactopoietic role for PRL in dairy animals is not easily demonstrable. In lactating mice, rats, and rabbits, administration of ergot alkaloids (inhibitors of pituitary PRL secretion) to decrease circulating concentration of PRL, markedly reduces milk production (50–100%). Conversely, administration of PRL to mice and rats increases milk yield. In lactating ruminants, treatment with ergot alkaloids has a lesser effect than in rodents. There is no effect in lactating dairy cows, and approximately a 10% reduction of milk yield in lactating goats and sheep. (In contrast, preventing the preparturient increase in plasma PRL concentrations markedly reduces milk production during early lactation – demonstrating the importance of PRL for the final stage of lactogenesis. Ergot alkaloids present in endophyte-infected fescue can markedly reduce milk production. Work is needed to determine sensitive periods and mechanisms of action.) Additionally, when PRL treatment of lactating hypophysectomized goats is terminated without eliminating treatment with ST, glucocorticoid, and T_3, there is no apparent decline in milk production (**Figure 1**). Conversely, when dairy cows were treated with PRL for a 14-day period before peak lactation and again after peak lactation, milk production was not affected, nor was milk composition affected except for a 10% increase in α-lactalbumin concentration in the milk of cows treated during early lactation. However, PRL has occasionally increased milk yield in lactating dairy goats. Thus, data suggest that availability of PRL is not rate limiting to lactation of dairy cows, but small increases in milk production may be realized by PRL treatment of dairy goats.

The conclusion that increasing plasma PRL does not increase milk production of dairy cows has been difficult to accept. There is a small correlation of milk production and plasma PRL in dairy cows, but a strong correlation between the milking-induced release of PRL and milk production. As lactation progresses, there is a decline in both milk production and the release of PRL at milking. It is unknown if this relationship is physiologically relevant. Additionally, increased photoperiod significantly increases milk production while increasing plasma PRL. Recently, it has been shown that increased photoperiod also increases plasma IGF-I (without increasing ST), and this has been hypothesized to mediate the milk production effect of photoperiod. Still, the ability of IGF-I to induce an increase in milk production is not certain, and subtle effects of PRL on milk production, particularly long-term effects, are possible.

Regardless of the ability of PRL to increase milk production in a relatively short time frame, recent data suggest that it may help to maintain the population of mammary secretory cells and thus promote lactational persistency (**Figure 2**). This was first proposed by Flint and Gardner, who discovered that treatment of lactating rats with ergot alkaloids decreased milk production by approximately 50%, compromised epithelial tight-junction integrity, and reduced DNA content of the mammary gland by 20–25%. When PRL concentrations were reduced significantly, increased epithelial cell apoptosis occurred, with an accompanying disruption of the blood–milk barrier and decline in number of secretory cells. Maintenance of the mammary epithelial cell population appears to involve an interaction between the PRL and ST axes. However, this requires further investigation in ruminants.

Insulin-Like Growth Factors

IGFs appear to be essential participants in the galactopoietic response to exogenous bST. When the production of IGF-1 is uncoupled from ST regulation, such as the one that occurs during negative energy balance, a milk production response to bST is abrogated. Infusion of IGF-1 into the close arterial supply to the mammary gland of goats rapidly increased milk synthesis. These data strongly support a galactopoietic role for IGF-1. The galactopoietic activity of IGF-II is uncertain.

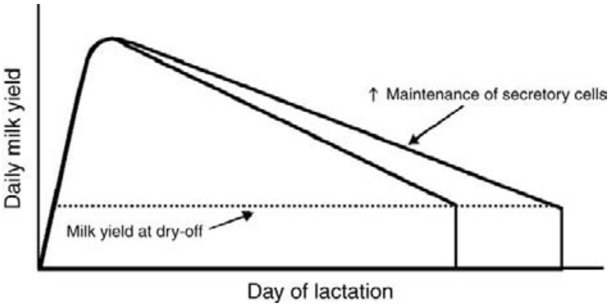

Figure 2 Effect of maintenance of the secretory cell population on the lactation curve. Increased maintenance of the secretory cell population increases the persistency of lactation as evident by a decrease in the slope of the declining phase of milk production. Length of lactation is increased if milking is terminated at a similar daily production.

Additionally, IGF-I is a mammary mitogen and survival factor. The ability of IGF-I to induce cell proliferation has been demonstrated in numerous *in vitro* and *in vivo* mammary model systems. Recently, administration of bST was shown to increase the percentage of mammary epithelial cells expressing Ki-67, a nuclear antigen marker for cell proliferation, approximately threefold. It is proposed that this apparent proliferation response to bST is mediated by IGF-I. Such increased cell renewal would limit the decline in number of mammary epithelial cells that occurs with advancing lactation and accounts for the steady decline in milk production after peak lactation. Regulation of mammary apoptosis in rats seemingly involves an interaction between PRL and IGF-I. Reduction of plasma PRL by ergot alkaloids decreases milk secretion, accompanied by an increase in the incidence of apoptosis in the mammary gland. PRL appears to depress the synthesis of IGF binding protein-5 (IGFBP-5), thus limiting its ability to bind IGF-I and suppress its cell survival activity. Thus, PRL is thought to promote cell survival so that the outcome of PRL insufficiency is increased mammary apoptosis. Whether IGFBP-5 or an analogous IGFBP is regulated by PRL in the mammary glands of ruminants remains to be demonstrated. However, in ruminants, bST increases lactational persistency and maintains mammary cell number as lactation advances – by increasing cell renewal or increasing cell survival.

Insulin

Insulin is clearly a hormone that plays an important role in the regulation of nutrient utilization during lactation. In ruminant dairy animals, insulin has no effect on the mammary uptake of glucose, acetate, β-hydroxybutyrate, and amino acids, but exogenous insulin inhibits milk production by virtue of its metabolic effects on other tissues. For example, in adipose tissue insulin promotes the uptake of glucose and acetate, stimulates lipogenesis while inhibiting lipolysis, and in the liver it inhibits gluconeogenesis. However, the homeorhetic attributes of bST are largely realized by virtue of its ability to inhibit selected processes that are stimulated by insulin (*see* **Lactation**: Galactopoiesis, Effect of Treatment with Bovine Somatotropin). It is unlikely that insulin plays a role in regulating the number of mammary epithelial cells. Mammogenic properties that were historically attributed to insulin on the basis of its effects on mammary cells *in vitro* can be dismissed as artifactual. Due to the use of supraphysiological concentrations of insulin in these systems, the mitogenic activity observed is attributed to the ability of insulin at high concentration to cross-react with IGF receptors and elicit IGF-related responses.

Glucocorticoids

It has been known that in rats and mice, adrenalectomy severely reduces milk yield, and conversely that administration of glucocorticoids to intact animals increases milk yield by retarding the decline that occurs with advancing lactation. Because the decline in milk yield during a murine lactation is due to decreased activity of mammary secretory cells, rather than a decrease in cell number, it follows that the galactopoietic effect of glucocorticoids in rodents occurs because they are rate limiting to milk synthesis. It was determined that glucocorticoids bind to specific glucocorticoid receptors in mammary tissue and regulate the secretion of α-lactalbumin and β-casein. Some of these actions are synergistic with other regulatory hormones such as PRL. For ruminants, there is little evidence that the glucocorticoids are limiting to milk production. However, adrenalectomy reduces milk yield, which is restored by glucocorticoid treatment. Bovine mammary tissue contains glucocorticoid receptors that are present in greater concentration in lactating than in prepartum tissues, and receptor number correlates with glucose uptake. Certainly, glucocorticoids are important for maintenance of milk production in dairy species.

Hormones of Pregnancy

Unlike most species, dairy cows are typically pregnant through the majority of lactation, and goats may be in late lactation when pregnant. Consequently, hormones of pregnancy can impact lactation. One of these hormones, a member of the ST–PRL family, is produced by the placenta (binucleate cells of the trophoblast), and is known as placental lactogen. The relative lactogenic and somatotropic activities of placental lactogen vary with species. In ruminants, placental lactogen has greater homology with PRL than ST, although it binds to both lactogenic and somatogenic receptors. Concentrations of placental lactogen in the maternal circulation of dairy cows are very low, whereas concentrations in the maternal blood of sheep and goats are quite high (100- to 1000-fold greater than for cows). Concentrations peak during the last trimester of pregnancy. Exogenous placental lactogen stimulates milk production by mechanisms that differ from those that mediate the galactopoietic effects of ST. Compared with bST treatment, placental lactogen treatment of dairy cows or ewes increases milk production more slowly and does so without increasing lipolysis. Unlike bST, feed of placental lactogen-treated cows increased rapidly. Its biological function appears related to maternal partitioning of nutrients. Under usual circumstances, the physiological impact of placental lactogen on the mammary gland is likely on mammary growth and lactogenesis in animals that are not lactating when pregnant, and on growth and lactogenesis during the periparturient dry period in animals that are lactating during pregnancy.

In contrast to potential galactopoietic effects of placental lactogen, concomitant pregnancy decreases the persistency of lactation, with an accelerated decline in milk production occurring during the last months of pregnancy. The reasons for this decline are unclear, but certainly the physiological processes that promote fetal growth and vigor take precedence over milk production. Other hormones of pregnancy that likely impact mammary gland function are estrogen and progesterone. High concentrations of estrogens decrease milk yield, while progesterone does not. Although progesterone inhibits lactogenesis partly due to its ability to competitively inhibit binding of glucocorticoid to the glucocorticoid receptor, the inability of exogenous progesterone to impact lactation in many species is due to the lack of progesterone receptors during lactation. In dairy cows, this inability is due to a reduction in number of progesterone receptors rather than an absolute absence of the receptors in the lactating gland. The mechanism by which estrogens decrease milk production is unclear.

Milk Removal

Removal of milk from the mammary gland is a necessary component for maintaining lactation. This is evident from the decreased persistency of lactation with incomplete milking and conversely from the enhanced lactation persistency in response to the facilitation of milk removal by daily injections of oxytocin at milking.

In the absence of milk removal, intramammary pressure increases, blood flow to mammary tissue decreases, and a substance(s) that inhibits milk secretion and promotes apoptosis apparently accumulates within the alveolar milk. In the short term, such as prolonged milking interval, lack of milk removal causes partial inhibition of milk synthesis and secretion; in the long term, it causes the termination of lactation and initiation of mammary involution. Considerable research effort has been expended in attempts to identify products in milk that feedback to inhibit milk secretion. Initial research tentatively identified a substance with appropriate characteristics that was referred to as feedback inhibitor of lactation (FIL), but the substance has not been purified and identified, and its gene is not yet identified. Currently, a naturally produced proteolytic fragment of casein has been identified, and the synthesized peptide sequence has been shown to inhibit milk synthesis. These latter results are preliminary but intriguing. Additionally, milk accumulation produces leakiness of the tight junction complexes between epithelial cells. Artificially increasing tight junction leakiness decreases milk secretion by mechanisms as yet undetermined, but may well involve signal transduction through the cytoskeleton.

The benefits of frequent milking are multifaceted. Increasing milking frequency from twice daily to thrice daily increases milk production by approximately 20%. This increase accrues largely because of the removal of the inhibitory effects of milk accumulation. In the short term, increased milking frequency appears to cause increased cellular activity, whereas in the long term, glands milked more frequently have a greater number of cells. Because these results can be demonstrated by milking glands within the same udder at different frequency, the effects are presumably due to local effects rather than systemic. Limited studies indicate that the rate of decline of milk production after peak lactation (persistency) is the same in 3× and 2× milked cows. Finally, increased milk production can be realized by temporarily utilizing increased milking frequency during early lactation. This carryover effect suggests that the increased milking frequency induces an increase in the number of differentiated mammary epithelial cells and thus an effect that persists through the lactation. Conversely, it was demonstrated over 30 years ago that a temporary reduction in milking frequency during early lactation causes a decrease in milk yield that persists for that lactation. Although the effect of milking frequency appears to be largely mediated by local effects, one should not rule out an interaction with the galactopoietic hormones that are released at each milking in addition to oxytocin. These hormones include PRL, and glucocorticoids, as well as ST in goats and rats, but not in cows or humans.

Other Mitogens and Survival Factors

As mentioned previously, increased expression of mitogenic factors and cell survival factors in the mammary gland can increase milk production by virtue of effects on the size and maintenance of the secretory cell population (**Figure 2**). Beyond the classical hormones and growth factors already discussed, there are many factors that can impact cell turnover. A number of reviews concerning mammary growth factors have been published, and it is beyond the scope of this article to consider the variety of factors that have been investigated. A greater understanding of known factors, and the potential discovery of new factors, will increase our knowledge of processes that can regulate persistency of lactation and provide the tools to regulate.

See also: **Lactation**: Galactopoiesis, Effect of Treatment with Bovine Somatotropin.

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Galactopoiesis, Effect of Treatment[1] with Bovine Somatotropin

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Introduction

In the 1930s, injection of crude extracts from bovine anterior pituitary was shown to increase the milk yield of dairy goats and cows. Approximately 10 years later, the component of these extracts that was responsible for increased milk production was identified as growth hormone or somatotropin. Initial advances toward understanding the myriad of physiological effects of somatotropin were achieved using pituitary-derived somatotropin, culminating in the idea that somatotropin orchestrates coordinated metabolic responses of tissues throughout the body to regulate nutrient partitioning and enhance milk production. Two groups led by Dale Bauman and Ian Hart proposed this concept of 'homeorhetic' control by somatotropin. With the production of recombinantly derived bovine somatotropin (bST), it became feasible to utilize the hormone for increasing lactational performance of dairy cows. Subsequent investigations and commercial use expanded our knowledge of the physiological effects of bST and demonstrated its efficacy and safety as a stimulant of milk production. The efficacy of bST as a stimulant of milk production has also been demonstrated for goats, sheep, and buffaloes. Indeed, somatotropin appears to be the primary galactopoietic hormone (i.e., hormone that increases milk production) in mammals, except for rodents, in which prolactin appears to be the primary galactopoietic hormone. bST signaling pathways include direct signaling by bST and indirect signaling by insulin-like growth factors (IGFs). Homeorhetic control is exerted, in large part, by altering the response to homeostatic signals. While bST exerts homeorhetic regulation, homeostatic regulatory processes that ensure animal well-being, and other homeorhetic mechanisms such as those that support body growth and fetal development during pregnancy, are still operative. Thus, bST exerts an overarching control, but not an overriding control, on processes that support milk production.

Effects of bST on Milk Production

Administration of bST to lactating dairy cows increases the yield and efficiency of milk production. In response to injection of bST, milk secretion increases within a day and is maximized within a week. The increased milk yield is maintained as long as treatment is continued but quickly returns to control levels when bST is discontinued. The milk yield response is dose dependent and the response curve is hyperbolic in shape. At approximately 40 mg of bST per day, nearly maximal response is obtained. Milk yield achieved with near-maximal doses of bST is impressive, with increases reported as high as 30–40%. Typically, bST increases milk production by 4–6 kg day^{-1}, approximately a 10–15% increase in yield. The magnitude of response to a particular dose of bST depends upon biological variation, stage of lactation, and management parameters.

During midlactation, the pattern of bST administration does not affect the magnitude of the galactopoietic response. Similar increases in milk yield are obtained with the same daily dose of bST whether administered as once-daily injections, 4-h pulses, or constant infusion. The bST formulation currently approved for use in the United States is a prolonged-release *n*-methionyl-bST (Posilac, Monsanto Co.) that was approved by the US Food and Drug Administration in November 1993. It is administered at a dose of 500 mg per lactating cow every 2 weeks. Treatment should be initiated after peak lactation at >60 days postcalving, when cows are at or near positive energy balance. During early lactation, response to bST is minimal.

In addition to increasing milk yield, bST increases the efficiency of lactation. Cows treated with bST increase feed intake over the first few weeks to match increased nutrient demands for milk synthesis and thus cows remain in neutral or positive energy balance during the majority of lactation. However, because milk secretion increases more rapidly than voluntary intake, bST-treated cows initially experience a temporary period of negative energy balance. Respiration calorimetry studies demonstrated that the energy requirements for body maintenance and the partial efficiency of milk synthesis from absorbed nutrients were not changed in bST-treated cows. Milk production efficiency is therefore increased by bST treatment because the increased milk production is achieved without nutritional overhead. Assuming an 11% increase in milk production, 9 bST-treated cows can yield

[1] Mention of a trade name or proprietary product does not constitute a guarantee or warranty by the United States Department of Agriculture and does not imply approval to the exclusion of others not mentioned.

the same amount of milk as 10 control cows, and the energy savings would be the maintenance requirements for 1 cow. However, it is important to note that bST is not unique in this regard. There are other methods of increasing milk production, such as increasing milking frequency, that increase the efficiency of lactation because production is increased without increasing energy requirements for maintenance. In contrast, thyroid hormone supplementation increases milk production, but it also increases body metabolism and maintenance requirements and there is no gain in efficiency. A recent analysis demonstrated that bST provides a means to increase dairy production efficiency while reducing environmental impact. Conventional herds supplemented with bST had lower acidification, eutrophication, and global warming potential than conventional herds without bST or herds adhering to organic production guidelines.

Administration of bST typically does not alter the gross composition of milk from cows in positive energy balance. Syntheses of milk proteins, fat, and lactose are all increased proportion in to milk volume and normal milk composition is maintained. Additionally, there is little change in the composition of milk protein or milk lipid when cows are treated with bST. Casein proteins are expressed in the same proportions in milks produced from control and bST-treated cows; whey proteins that have been evaluated appear similarly unaffected by treatment, and the ratio of whey protein to casein protein is unaltered. Lipid classes and fatty acid composition of milk fat are not altered or altered very slightly by bST treatment when cows are in positive or neutral energy balance. For example, there may be a small increase in the relative amount of long-chain fatty acids in the milk of bST-treated cows. For reasons discussed subsequently, the fat content of milk increases, and fatty acid composition may change, if cows are in negative energy balance when bST is administered. The mineral content of milk appears largely unaffected by bST treatment and, although vitamin content has been less thoroughly examined, vitamin concentration also seems to be unaltered.

When bST is provided as a sustained-release formulation, small cyclical effects on milk yield and composition have been noted. With the biweekly injection protocol, milk production peaks 7–9 days after injection and then declines until the next injection, seemingly as a function of bST concentrations in the blood. The concentration of milk lactose follows the same cyclical pattern as milk yield, although the reasons for this effect are unclear. Milk fat and protein cycle in a manner that is out of phase with milk yield, that is, the concentration of milk protein and fat is at a nadir when milk yield peaks. With the biweekly injection protocol, a steady state of the metabolic alterations coordinated by bST is seemingly never fully achieved. Thus, synthetic processes for the synthesis of milk components may not be fully coordinated, resulting in minor and temporary alterations in milk composition. Other changes, such as changes in nutrient balance or changes in mammary blood flow, may also occur in response to biweekly injections of bST. These may produce small changes in the availability of nutrients to the mammary glands and may partially explain the small fluctuations in milk volume and composition. However, it should be noted that these cyclical fluctuations in composition are not apparent in the bulk tank milk because cows within a herd typically calve asynchronously and are injected with bST asynchronously. Thus, this effect is of no importance to milk processors or consumers. Indeed, these variations in milk composition during bST treatment are minor compared with normal variation in milk composition that occurs between herds and within a herd. Milk composition is more strongly influenced by season, stage of lactation, genetics, nutritional management, and energy balance.

Mode of Action of bST

The galactopoietic action of exogenous bST may be the result of a combination of direct and indirect effects: (1) direct stimulation of mammary tissue; (2) indirect stimulation of mammary tissue; (3) direct effects on other tissues to supply nutrients to support increased milk production; and (4) indirect effects on other tissues to supply nutrients to support milk production. There is a preponderance of evidence that suggests that bST enhances milk production largely by partitioning nutrients to support milk production, by both direct and indirect actions; however, bST does not alter the digestibility of nutrients. The effects on the mammary gland appear to be indirect, and whether the direct effects of bST are operative remains to be determined.

It is questionable whether bST has direct effects on the lactating mammary gland. Although bST is galactopoietic *in vivo*, the addition of bST to mammary culture systems has failed to increase the synthesis of milk components, and receptor-binding assays have failed to detect somatotropin receptor in mammary tissue. These early results argued against a direct effect of bST on the mammary gland, and it was presumed that if endocrine stimulation of the mammary gland occurred, it was via bST-induced increases in circulating IGFs. Because bovine mammary epithelial cells have receptors for IGF-I and IGF-II, they appear to be target cells for IGF signaling. Indeed, infusion of IGF-I or IGF-II into the local arterial supply to one of the mammary glands of a goat caused an increase in milk production and blood flow to the infused gland within 2–4 h. Although this increase in milk yield is consistent with a direct galactopoietic effect of IGFs on mammary tissue, it may also have been an indirect outcome of increased blood flow and nutrient supply to the mammary gland. It is interesting to note that *in vitro* treatment with bST increased milk fat synthesis by mammary explants when cocultured with adipose and liver explants, but not in the absence of liver and adipose tissue. This is consistent with a nutrient- and hepatic IGF-mediated effect on milk component synthesis.

More recently, somatotropin receptor mRNA has been detected in lactating bovine mammary tissue by Northern blotting and by *in situ* hybridization. The mRNA for somatotropin receptor was localized in both epithelial and stromal (nonsecretory) elements of mammary tissue, but IGF-I mRNA was restricted to stroma. Assuming that the bST receptor transcripts are translated into protein (as they are in rabbit and rat mammary epithelium, where somatotropin receptor protein has been detected by immunohistochemistry), the results open the possibility that bST may have effects on the mammary gland that are mediated by local production of IGFs.

Several levels of complexity are involved in modulating IGF-regulated functions: the local concentration of IGF, expression of IGF receptors and their downstream signaling pathways, the types and quantities of IGF-binding proteins (IGFBPs), and abundance of, the acid-labile subunit (ALS) protein. The majority of IGF-I circulates in a ternary complex with one of the IGFBPs and ALS. Consequently, ALS has the capacity to regulate the availability of systemic IGF-I to tissues. To date, six high-affinity IGFBPs (IGFBP-1–6) and nine low-affinity IGFBPs, known as IGFBP-related proteins (IGFBP-rp1–9), have been identified. A number of IGFBPs are synthesized by mammary epithelial cells. Depending upon the specific IGFBP, the binding proteins may reduce IGF activity by competing with IGF receptors for ligand, increase IGF activity by serving as delivery vehicles to the target cell, or serve as a reservoir for IGFs, causing their slow release and reducing their turnover. Furthermore, the IGFBPs may have activities that are independent of their interaction with IGFs and they are subject to enzymatic modifications that may alter their various activities. The picture that emerges is one of a highly complex IGF system with multiple levels of regulation, making the specific actions of IGFs during lactation difficult to resolve.

Somatotropin directly or indirectly coordinates metabolic adaptations that promote increased milk production in the lactating dairy cow. These adaptations involve chronic alterations in carbohydrate, lipid, and protein metabolism in a number of tissues and serve to preferentially direct nutrients toward the mammary gland. This coordinated regulation to support the priorities of a physiological state has been termed homeorhetic regulation. The importance of integrated nutrient partitioning is illustrated by udder perfusion studies. Milk secretion by isolated goat udders is dependent on the presence of glucose in the perfusate. Removal of acetate or amino acids allows continued milk synthesis; however, the secretion of fat and protein is reduced. Normal milk composition is dependent upon balanced nutrient supply to the mammary gland.

Lipid metabolism is strongly influenced by bST administration. Although bST has no acute effects on lipogenesis or lipolysis, it has chronic effects on these processes in adipose tissue. When cows are in negative energy balance during bST administration, lipolysis is increased. This is manifested as increases in the level of nonesterified fatty acids (NEFAs) in blood, increased milk fat percentage, and an increase in the percentage of long-chain fatty acids in the milk fat. (In the mammary gland, the long-chain fatty acids incorporated into milk triacylglycerols originate from mobilized fat stores and from dietary sources, whereas short- and medium-chain fatty acids are synthesized within mammary tissue.) When cows are in positive energy balance during bST administration, lipogenesis is inhibited. These effects are achieved by altered responsiveness to key homeostatic signals and changes in the quantity of key enzymes (**Figure 1**).

Modulation of insulin responsiveness provides an important means to regulate lipid metabolism, and bST antagonizes some of the actions of insulin. Insulin is a key homeostatic regulator of nutrient metabolism. It promotes the facilitated transport of glucose into most cells of the body (the central nervous system and mammary glands are not insulin dependent) and inhibits many of the liver enzymes that catalyze gluconeogenesis. It also promotes the synthesis of glycogen and inhibits glycogenolysis. It stimulates the deposition of fat by enhancing the activity of key enzymes of fatty acid synthesis and by inhibiting lipolysis of triacylglycerol. It stimulates protein deposition by enhancing facilitated uptake of amino acids and increasing the activity of some ribosomal enzymes involved in protein synthesis. Only a few of insulin's actions are antagonized by bST treatment. Most importantly, bST inhibits the lipogenic activity of insulin, and the effect appears to be exerted on processes that are downstream in the signaling cascade from the insulin receptor, consistent with the targeted inhibition of a limited number of insulin actions. Lipoprotein lipase (LpL) is an enzyme that is partly regulated by insulin. bST treatment of lactating dairy cows causes a decrease in LpL in adipose tissue but no change in mammary tissue. LpL is an enzyme that hydrolyzes triacylglycerols of very-low-density lipoproteins and chylomicrons in the serum, permitting the uptake of NEFAs by surrounding cells. Reduced LpL in adipose tissue and normal LpL in mammary tissue, along with the inhibition of lipogenesis in adipose tissue, ensure the preferential delivery of NEFAs to the mammary gland for synthesis of milk fat. bST also

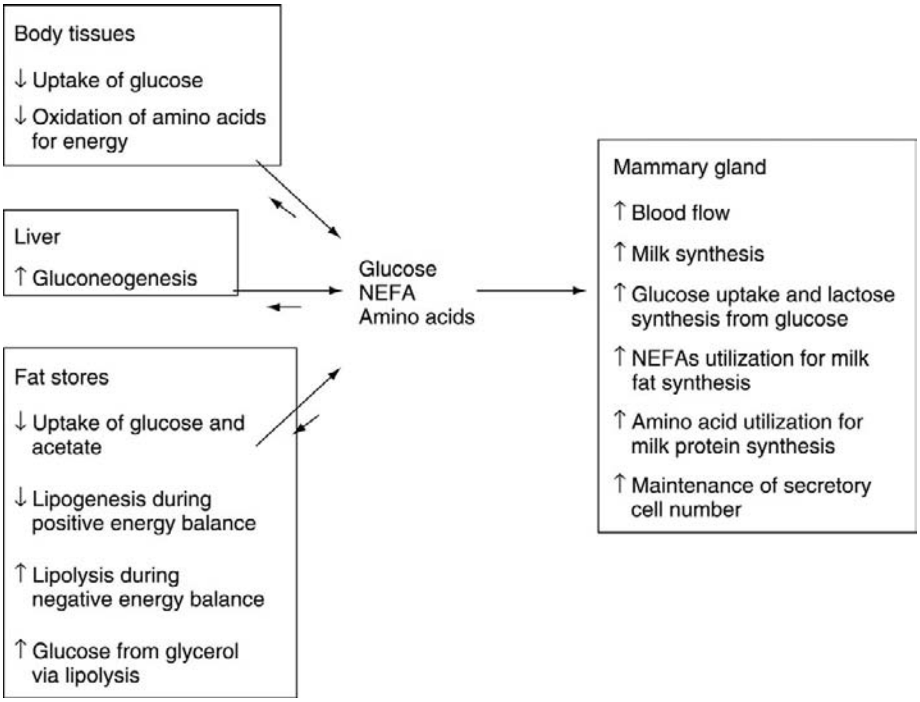
Body tissues
↓ Uptake of glucose
↓ Oxidation of amino acids for energy
Liver
↑ Gluconeogenesis
Fat stores
↓ Uptake of glucose and acetate
↓ Lipogenesis during positive energy balance
↑ Lipolysis during negative energy balance
↑ Glucose from glycerol via lipolysis
Glucose
NEFA
Amino acids
Mammary gland
↑ Blood flow
↑ Milk synthesis
↑ Glucose uptake and lactose synthesis from glucose
↑ NEFAs utilization for milk fat synthesis
↑ Amino acid utilization for milk protein synthesis
↑ Maintenance of secretory cell number

Figure 1 Effect of bovine somatotropin administration on nutrient partitioning to support increased milk production. Metabolism is altered in an organ-specific fashion to establish nutrient flux toward the mammary gland. NEFAs, nonesterified fatty acids.

decreases expression of key enzymes involved in fatty acid synthesis, such as fatty acid synthase and acetyl-CoA carboxylase.

When nutrients are in limited supply, bST enhances lipolysis again by altering the response to homeostatic regulators. Dairy cows treated with bST mobilize considerably more NEFAs following epinephrine challenge than do control cows. However, there is little change in adrenergic receptor numbers, and no change in the stimulatory G proteins and other components of the cyclic-AMP lipolytic signaling pathway. Rather, it has been discovered that bST enhances lipolysis by antagonizing antilipolytic regulators. Treatment with bST decreases the activity of the inhibitory G proteins. Thus, bST promotes lipolysis by chronic inhibition of antilipolytic regulation. The ability to enhance lipolysis often comes into play when bST treatment is initiated. Because cows are typically near neutral energy balance when treatment is initiated and feed intake does not increase immediately, bST induces a period of negative energy balance that requires the mobilization of energy stores. When cows enter positive energy balance, for the majority of lactation, inhibition of lipogenesis is the hallmark of bST action on lipid metabolism.

Carbohydrate metabolism is altered by bST treatment to meet the increased glucose requirement for greater milk secretion. More glucose is made available for milk synthesis by increasing hepatic glucose production and decreasing oxidation by body tissues. In ruminants, the products of rumen fermentation are volatile fatty acids, and only a small percentage (15%) of blood glucose is derived from the diet. Body glucose supply is met by hepatic gluconeogenesis, which can amount to production of 3 kg day^{-1} in a lactating cow. Administration of bST enhances hepatic gluconeogenesis at least in part by antagonizing the ability of insulin to inhibit gluconeogenesis. Glucose serves as the substrate for lactose synthesis in the mammary gland, and in high-producing lactating dairy cows, nearly 85% of total glucose turnover is used for milk synthesis. Treatment with bST increases net utilization of glucose in mammary tissue and decreases glucose utilization by nonmammary tissues. These metabolic adaptations are sufficient to provide the necessary glucose for milk synthesis; no glucose deficit is encountered and ketosis is not induced. Somatotropin treatment also decreases expression of glucose transporters in skeletal muscle and in fat stores, but has no effect on transporters in the mammary gland, thus increasing the proportional flux of glucose into the mammary gland. Other data suggest that the effects on glucose uptake are primarily secondary to nutrient gradients created by metabolic effects on the tissues.

Protein metabolism of bST-treated lactating dairy cows is altered to support the added amino acid requirements for increased milk protein synthesis. This appears to be largely the result of more efficient utilization of amino acids. Whole-body oxidation of amino acids is reduced in bST-treated dairy cows and there is a resulting decrease in the concentration of urea and a decrease in urinary nitrogen loss. Increased milk protein synthesis appears to be supported primarily by increased availability of precursors to the mammary gland due to decreased oxidation of amino acids by other tissues.

bST exerts an overarching control, but not an overriding control, on metabolic processes that support milk production. While bST exerts homeorhetic regulation, homeostatic regulatory processes that ensure animal well-being, and other homeorhetic mechanisms such as those that support body growth or fetal development during pregnancy, are still operative. Increases in milk yield are greater in multiparous than in primiparous cows because the milk response to bST is reduced by an amount that is dictated by the nutrient requirements of continued body growth. Similarly, when bST-treated cows are simultaneously lactating and pregnant, milk production declines normally during the later months of pregnancy and thus minimizes conflict with nutrient demands for fetal growth. Use of bST does not ensure increased milk production to the detriment of a young lactating animal's continued body growth or of a lactating animal's ability to support pregnancy. Normal physiological processes that serve to ensure the well-being of a lactating animal and survival of her fetus are still operative during prolonged use of bST during lactation. Indeed, use of bST over multiple lactations has proven to be safe and effective.

Although much has been learned about the nature of the metabolic alterations induced by bST and the tissue-specific effects of bST, the means by which the hormone signal elicits the biological response is poorly understood. The effects of bST on adipose tissue can be demonstrated *in vitro*, suggesting that these effects are direct and are mediated by the somatotropin receptor. The effects on the liver are presumed to be direct effects, because these too can be mimicked *in vitro*. However, within these tissues, locally acting paracrine or autocrine effects cannot be ruled out. In contrast, the effects of bST on muscle and mammary tissue appear to be primarily mediated by the IGF system. Although the metabolic effects of bST on nonmammary tissues effectively spare nutrients to support milk synthesis, there also appear to be effects at the level of the mammary gland. Infusion of IGFs into the local arterial supply of the mammary gland stimulates milk production and argues for IGF-mediated effects on mammary gland synthetic ability. Associations between energy balance, bST, the IGF system, and milk production suggest the importance of IGF-mediated effects on the mammary gland. Moderate undernutrition causes a muted IGF response to administration of bST and a reduced galactopoietic effect. Thus, during early lactation, when cows are in negative energy balance, IGF response to bST administration is reduced and bST is a less effective stimulator of milk production. During severe undernutrition, there is dissociation between bST and the IGF system, and both IGF response and the milk production response to bST are abolished.

Other than bST, thyroxine is the only other hormone known to increase milk production in dairy cows, and there are numerous interactions between the somatotropic and thyroid hormone axes. Indeed, tissue-specific changes in thyroid hormone metabolism alter the local action of systemic thyroid hormones, which appear to be important for supporting milk production and for modulating the galactopoietic response to prolactin and somatotropin in mice and rats. The situation in cows is less clear and has received scant attention.

In addition to metabolic effects, bST appears to alter population kinetics within the mammary gland. Production data indicate that bST increases the persistency of lactation, and this may be achieved by decreasing the loss of secretory cells during lactation and by increasing cell proliferation. Data for goats indicate that bST administration results in maintenance of cell number as lactation progresses, due to decreased cell loss. Recent data suggest that cell proliferation is increased in the mammary tissue of bST-treated dairy cows and heifers during midlactation. These data are consistent with the *in vitro* mitogenic activity of IGF-I and IGF-II in bovine primary cell culture, mammary tissue slices, and an established line of bovine mammary epithelial cells (MAC-T cells), and suggest that the IGFs mediate this effect. However, decreased cell death and increased cell proliferation assist in the partial maintenance of the population of secretory cells, as previous studies have demonstrated that bST does not actually increase mammary cell number. These effects supplement the metabolic alterations induced by bST and lessen the decline in milk production with advancing lactation.

Galactopoiesis in Other Species

Although dairy cows have been the subject of most investigations, bST has been shown to be an effective galactopoietic hormone in other dairy animals, including sheep, goats, and the Italian water buffaloes. As with dairy cows, substantial increases in milk production are obtained (14–30%) and the composition of milk remains unaffected. The processing attributes of milk are unimpaired. In fact, coagulation time is improved in milk from ewes during late lactation. Because sheep, goats, and buffaloes are seasonal breeders, the ability to increase persistency of lactation is particularly attractive to maintain milk production throughout the year.

Effects on Udder Health

The effect of bST on mastitis has been studied extensively in the 1990s using more than 11 000 cows in 19 investigations. The conclusion of these experiments is that bST treatment does not significantly alter the incidence of mastitis and has negligible effects on milk somatic cell count. In a review of Dairy Herd Improvement records from dairy herds in the northeastern United States (8 years, >80 000 cows and >2 million test days), it was reported that there was no change in the productive life of cows from herds that used bST. Although bST caused a small but significant increase in the milk somatic cell count, the increase is of little biological significance. It is consistent with the small increase in somatic cell count that accompanies increased milk yield, and the increase is considerably lower than the effects of season, parity, breed, and age in control cows. bST helps to maintain milk production in mastitic quarters, and there is evidence that it enhances neutrophil function and quickens the recovery from (coliform) mastitis.

Effects on Reproduction

Dairy cows undergo a period of reduced fertility during early lactation when they are in a state of negative energy balance. Administration of bST is approved for use after 63 days of lactation to minimize its use during this period of negative energy balance. Nonetheless, both heifers and multiparous cows treated with bST often experience a reduction in pregnancy rate and an increase in days open, which is partially due to increased frequency of undetected estrus. Despite these apparent effects, the reproductive performance of bST-treated cows typically does not differ from that of cows with equivalent milk production. However, administration of bST increases the incidence of twinning and follicular development. The influence of the bST/IGF axis on ovarian function is an area of active research that should provide information to improve fertility.

See also: **Lactation:** Galactopoiesis, Effects of Hormones and Growth Factors.

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Galactopoiesis, Seasonal Effects

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Introduction

Enhancing established lactation is referred to as galactopoiesis (from Greek, *gala*, milk, *poiesis*, production). In dairy production, the capacity for mammary tissue to synthesize and secrete milk components is maximal at peak lactation and declines linearly thereafter. The slope of the decline in milk yield following peak lactation is referred to as the persistency of lactation. Lactation persistency is a result of maintaining mammary epithelial cell (MEC) number and cellular secretory activity. Cellular dynamics from early postpartum stages to peak lactation can be characterized by an increase in secretory capacity of MEC rather than gland growth via MEC proliferation. Enhancing established lactation has major economic benefits to dairy producers. Hence, the factors that regulate MEC turnover and secretion rate together with mechanisms mediating mammary nutrient uptake and milk synthesis or secretion have been the major foci of research and are discussed relative to galactopoiesis. Factors affecting yield are reviewed first, followed by issues affecting milk component synthesis.

Galactopoietic Factors

Three management methods consistently increase milk yields in lactating dairy cows during established lactation: the administration of exogenous bovine somatotropin (bST), increased milking frequency, and increased photoperiod. Two of these methods, increased photoperiod and bST, also alter persistency, while the third, increased milking frequency, alters peak milk yield but has little effect on persistency.

Bovine Somatotropin

The galactopoietic response of ruminants to recombinant bST has stimulated much research into its role in nutrient partitioning. Milk yield increases in a dose responsive manner to exogenous bST and the enhancement is influenced by parity, stage of lactation and degree of negative energy balance. Bovine somatotropin stimulates hepatic tissue insulin-like growth factor I (IGF-I) synthesis and secretion, which may act on the mammary gland through an endocrine mode of action in concert with circulating and local IGF-I binding proteins. However, a direct role of IGF-I on galactopoiesis in mammary tissue has not been concretely established. In general, bST coordinates metabolism to alter the delivery of nutrients to the mammary gland. This action of bST is homeorhetic in nature because metabolism is coordinated to favor the flow of nutrients for a specific and dominant physiological function, namely increased milk yield. It carries out this action without creating a metabolic imbalance and thus does not cause physiological problems.

The response to bST is remarkably consistent and increases lactation yields by approximately 900 kg over a 300-day lactation. Typically, treatment begins following peak lactation and is continued until dry-off. The milk yield response is partly influenced by the stage of lactation, and this is primarily because of differences in body condition. For example, more fat is partitioned to the mammary gland in cows receiving bST during early lactation at the expense of lipid synthesis in adipose tissue. During the first 8 weeks of treatment, exogenous bST promotes an increase in milk energy yield; while dry matter intake and digestibility are not affected, the rate of peripheral glucose oxidation is reduced. Treatment with bST increases the circulating concentration and the oxidation rate of nonesterified fatty acids, which suggests that fatty acids are mobilized from adipose tissue to compensate for the decreased energy balance due to the higher milk energy output. There is a concomitant accumulation of fat in milk, predominantly long-chain fatty acids at the expense of *de novo* fatty acid synthesis. These changes apparently occur in the absence of fluctuations in the concentration of circulating insulin or glucose, although, in some studies, administration of bST was accompanied by a hyperglycemic response. Treatment with bST increased mammary uptake of glucose at the expense of that of nonmammary tissue. Glycerol appears to contribute to the synthesis of glucose via gluconeogenesis, whereas the contribution from glycogen, amino acids, and proprionate seem to be negligible. After 8 weeks of treatment, dry matter intake increases, thus compensating for increased milk output in the absence of effects on milk fat content. The exact causes of the increase in milk output are not known, but proposed mechanisms include increased rate of synthesis per cell, reduced rate of cell loss, and an increase in the number of cells actively secreting at any one time. Cumulative evidence to date suggests that increased rate of synthesis per cell and an increase in the number of cells actively secreting are the primary mechanisms because milk yield returns to baseline values when bST treatment ceases, or in other words, there is no carryover effect on milk yield.

Milking Frequency

Increasing the number of times milk is removed from the mammary gland increases milk yield in dairy animals. For example, increasing the milking frequency from 2 to 3 times daily increases milk yield from 15 to 20%. Further increases in milk yield (>20%) have been reported when milking frequency was increased from 3 times (38.5 kg day^{-1}) to 6 times (46.8 kg day^{-1}). However, on commercial dairies, this further increase is more difficult to achieve as time away from feed may adversely affect the response because of large distances to and from the housing barn to the milking parlor. Research indicates that the transition from 3 times to 6 times daily milking was accompanied by an increase in the circulating levels of oxytocin, growth hormone, IGF-I, prolactin, dry matter intake, and nonesterified fatty acids and a decrease in circulating insulin, whereas glucose levels were unaffected. There is also a stage-of-lactation effect on the response to increased milking frequency. Initiating 3 times daily milking at the beginning of lactation results in much greater responses than initiating 3 times daily milking after peak lactation. Furthermore, some reports suggest a positive carryover effect on milk yield if cows are milked 3 times daily beginning immediately after calving but returned to twice a day milking after peak lactation. Increased milking frequency increases mammary growth, and this effect is most pronounced in early lactation when the gland is still growing. Mammary gland DNA continues to increase with a 26% mammary growth for rats during early lactation. Furthermore, it is interesting to note that without a suckling stimulus, the growth in early lactation does not occur for this species.

As milk yield per cow has steadily increased, the percentage increase in milk yield to increased milking frequency has declined. The current US Department of Agriculture adjustments for 3 times daily milking over 2 times milking for lactation records are a 12% increase for the first lactation and 14% for second and later lactations. Increased milking frequency is additive to the increase in milk yield associated with the use of bST, and the increase in milk production associated with increased photoperiod. When utilizing bST and increased milking frequency concomitantly, milk yield is increased in an additive manner. Studies demonstrating the additive effects of milking frequency and bST also show that they work through different mechanisms. A study that utilized midlactation heifers in which udder halves were milked either 2 or 4 times daily and subsequently treated with bST and saline demonstrated clear additive effects. Milk yield was increased by 12.8% by 4 times daily milking over 2 times daily milking, 14% by bST, and 28.5% when 4 times daily milking was combined with bST. These scientists also utilized mammary biopsies to determine which galactopoietic agent (agents) enhances (enhance) mammary growth. Mammary epithelial size tended to increase in glands milked 4 times daily but no effects were detected for bST or the combined treatment (bST and 4 times daily milking).

Photoperiod

Photoperiod is a galactopoietic management tool utilized by dairy producers to increase milk yield. Natural photoperiod is greatly influenced by latitude, and near the equator, there is little variation in photoperiod. However, at latitudes greater than 30° north and south the range in photoperiod is much more pronounced. Increasing photoperiod to 16 h of light and 8 h of darkness from 12 h of light and 12 h of darkness increases milk yield by 8–10%. This response is associated with increased feed intake and increased plasma prolactin concentration and sometimes an increase in plasma IGF-I levels. There appears to be no stage-of-lactation difference in the response to photoperiod, and there is no evidence that increased photoperiod alters mammary growth during lactation or pregnancy. Milk component concentration is generally not affected by long-day photoperiod, although some studies have reported both increased and decreased milk fat percentages. When cattle are exposed to continuous lighting, there is a change in milk yield, and this is hypothesized to be a result of desynchronization of the cows' biological rhythm. The increase in milk yield following increased photoperiod has been shown to be additive to increased milk yields because of exogenous bST and increased milking frequency. This suggests that the three management methods increase milk yields through differing mechanisms. Studies evaluating photoperiod effects during the dry period on milk yield in the subsequent lactation do not show any positive effect of long-day photoperiod. On the contrary, short-day photoperiod treatment of pregnant dry cows resulted in higher milk yields in the subsequent lactation. However, there was no further increase in milk yield by extending photoperiod in the same cows during lactation. In other words, the dairy producer can use either short photoperiods during late gestation or long photoperiods during lactation to achieve the increase in milk yield. Interestingly, during short-day photoperiod of late pregnant goats, there was no increase of IGF-I or prolactin during treatment associated with greater milk yields during lactation.

Factors Affecting Milk Component Yields

Milk protein

After parturition, developmental and hormonal signals induce a number of ontological events that lead to enhanced expression of milk protein genes. Specifically, the lactogenic hormone, prolactin, and glucocorticoids contribute to the activation of expression of caseins and whey proteins. The transfer of amino acids into the mammary gland is primarily from plasma. Although red blood cells contribute modest amounts of amino acids, they account for approximately 14% of the total mammary uptake of methionine. Approximately 15–30% of the mammary uptake of aspartic acid and proline is concentrated in red blood cells as they pass through the mammary gland. The partition of amino acids into milk protein synthesis is influenced by several factors, including the availability of amino acids in the arterial blood, mammary blood flow rate, and the efficiency with which amino acids are extracted by the mammary gland and used for protein synthesis or intracellular metabolism. The efficiency of converting extracted amino acids into milk protein can be expressed by the following equation:

$$K = \left(\frac{\mathrm{AV}}{\mathrm{AC}}\right) \times \mathrm{BF} \times E$$

where K is the fractional use for milk protein synthesis, AV the arteriovenous concentration difference divided by the arterial concentration AC, BF the flood flow rate ($\mathrm{l\,h^{-1}}$), and E the efficiency of utilization of the extracted amino acid.

Total protein synthesis exceeds by approximately twofold the rate of protein secretion, thus suggesting, a high rate of protein turnover by mammary tissue. Protein degradation affects both newly synthesized and mature caseins and contributes to maintaining an intermediary pool of available amino acids at the sites of protein synthesis.

Amino acids, including leucine, valine, isoleucine, arginine, threonine, and histidine, are typically extracted in excess of their milk output, suggesting that the mammary gland may metabolize these amino acids to balance milk protein requirements. In contrast, when maize silage–based diets are fed, amounts of nonessential amino acids and lysine and methionine available for uptake rarely exceed milk output. For example, supplementation of increasing amounts of soybean meal as protein supplement further decreased the absorbable methionine. Duodenal infusion of lysine at peak of lactation (∼5 weeks postpartum) and fed a 70:30 maize silage:concentrate ratio increased the blood extraction rates of amino acids and secretion of milk protein. In contrast, lysine infusion had no effects on milk yield beyond week 8, suggesting that lactational response to supplemental amino acids is influenced by stage of lactation.

Amino acids limiting milk yield and composition vary depending on the type of forage and protein supplements in the basal diet. The pool of amino acids available to the mammary gland is influenced by the profile of the absorbed amino acids and metabolism by nonmammary tissues. For example, approximately 20–30% of absorbed total essential amino acids are metabolized by the portal-drained viscera and liver, and this results in deviations from the absorbed amino acid composition. The amino acids lysine and methionine are the most likely to be limiting in metabolizable protein for milk protein synthesis in diets based on maize silage, whereas histidine has been identified as first-limiting for milk yield of cows fed a grass silage and cereal diet. However, methionine deficiencies seem to influence milk fat more effectively than protein synthesis, whereas lysine may be more important in mammary gland amino acid metabolism. Supplementing maize silage and grass silage diets with ruminally protected methionine effectively increased milk fat content. A possible explanation is that, in mammary tissue, methionine may serve as a methyl donor in the transmethylation reaction of lipid biosynthesis. Therefore, amino acids may influence milk composition beyond their expected role of precursors for protein synthesis.

Modulation of protein synthesis

Milk protein content is relatively constant, although substrate availability may influence, at least in part, protein synthesis by the mammary gland. For example, fat supplementation has been reported to depress protein synthesis because of reduced mammary blood flow. A potential explanation for this phenomenon is that increased levels of circulating fatty acids have a sparing effect on oxidation of acetate, thereby increasing the efficiency of lactose synthesis from glucose. However, the increased availability of glucose and higher milk yields are not paralleled by an increase in amino acid uptake, resulting in a reduction of protein in milk. In contrast, feeding of diets containing a high percentage (70–80%) of concentrate increases the supply of amino acids to mammary tissue or the extraction from blood of amino acids, which sustain increased synthesis of milk proteins.

In general, milk protein production is highly correlated to energy in the diet. Enhanced microbial protein, which is influenced by energy availability, accounts for the largest component of intestinal protein flow. Approximately 11% of adenosine triphosphate (ATP) generated within the mammary gland is utilized for milk protein synthesis.

Studies of the endocrine regulation of milk protein synthesis have reported that treatment with bST increased the yield but not the concentration of milk components. On the other hand, hyperinsulinemia, under conditions of euglycemia markedly increases protein percentage (from 3.13 to 3.44%) in milk from cows fed an alfalfa hay-maize-based diet and receiving an abomasal casein infusion. These data suggest that mammary protein synthesis is endocrine regulated. Consequently, it appears there is approximately 25% additional protein synthetic capacity in bovine mammary tissue that is not presently being utilized.

Milk caseins are subjected to partial hydrolysis during mammary gland involution, whereas the whey proteins α-lactalbumin and β-lactoglobulin are relatively resistant to plasmin digestion. This process probably involved hydrolysis by plasmin and other proteases. The accumulation of milk components at dry-off is one of the factors that contributes to increasing susceptibility to mastitis. Digestion of milk protein leads to the production of biologically active peptides that may be involved in mammary gland involution. Lactoferrin, an important antimicrobial protein found in mammary secretions during involution, may also be a substrate for degradation by plasmin. Digestion products of lactoferrin, lactofericins, have a broad-spectrum antimicrobial activity.

Phagocytosis by leukocytes that invade the mammary gland during involution contribute to clearance of milk components.

Lactose

Lactose is the major osmotic determinant of milk and therefore factors that increase lactose synthesis also increase milk yield. Treating lactating dairy cows with bST increases availability of glucose and rate of synthesis of lactose, leading to an increase in milk yield. However, the increase in milk yield is proportional to the increase in lactose synthesis, resulting in no change in milk lactose content. Likewise, increase milking frequency and increased photoperiod, which also increase milk yield, do not alter milk lactose concentration. Recent information on the feedback regulation of milk synthesis indicates that serotonin, a neurotransmitter is also synthesized in mammary tissue and the expression of its synthesis appears to be under partial control by prolactin. Serotonin downregulates expression of the gene coding for α-lactalbumin, the rate-limiting enzyme for lactose synthesis. Thus, increasing amounts of serotonin in milk due to delayed milking acts to downregulate lactose synthesis, which leads to reduced milk synthetic rate.

Milk fat

Milk from Holstein dairy cows fed standard diets based on maize or grass silage and alfalfa hay contains approximately 35–40 g l^{-1} of fat. Fat content is typically highest immediately following parturition and lowest during peak milk yield. Acetate and β-hydroxybutyrate (rumen-derived volatile fatty acids) are important carbon sources for *de novo* fatty acid synthesis by mammary tissue. Approximately 50% of butyric acid is derived from circulating β-hydroxybutyrate, whereas the remaining fraction is originated by condensation of acetyl units.

Ruminants typically consume a diet rich in polyunsaturated fatty acids, yet milk and other dairy products tend to have higher saturated fatty acid content. This is primarily because unsaturated fatty acids tend to be toxic to rumen microflora and therefore biohydrogenated by rumen microbes. Intermediates of biohydrogenation include fatty acids containing a *trans* double bond, and these (including the saturated end product) are incorporated into ruminant lipids. Dietary fatty acids are packaged and transported via chylomicrons and very low-density lipoproteins (repackaged hepatic triglycerides), and their triglyceride core is hydrolyzed by lipoprotein lipase in mammary capillary beds. Long-chain fatty acids (50% of $C_{16:0}$ and longer) represent approximately 50–60% of the total milk fatty acid pool by weight and 40–50% of the total milk fatty acid pool on a molar basis. Short- and medium-chain fatty acids ($C_{4:0}$ to $C_{14:0}$ and 50% of $C_{16:0}$) are synthesized *de novo* and represent the difference on a weight and molar basis, respectively. Fatty acids containing 18 carbons and less constitute a large majority, but over 400 different fatty acids are found in milk fat. Blood and *de novo*-derived fatty acids (both saturated and unsaturated) can be desaturated by sterol CoA desaturase and along with unique rumen-derived fatty acids (odd, even, and branched chained) contribute to the assortment and variation found in typical milk fat. Fatty acids are esterified onto a glycerol backbone, and the positioning of specific fatty acids within the triglyceride contributes to the physical (fluidity characteristics) and organoleptic qualities (taste, smell, etc.) of milk and milk products.

Modulation of milk fat synthesis

Compared with the relative consistency of milk protein and lactose concentrations, milk fat levels can vary markedly and are heavily influenced by nutrition and environment. Rumen microbial carbohydrate fermentation contributes the precursors (primarily acetate) for milk fat synthesis. Therefore, modifications of the rumen environment can manipulate the mammary supply of milk fat building blocks. Certain dietary situations cause milk fat levels to decline, and this low milk fat syndrome is commonly referred to as milk fat depression (MFD). MFD can be described briefly as milk that contains a reduced fat concentration and thus decreased yield of total milk fat. Milk has traditionally been, in large part, priced on the basis of milk fat content, and, consequently, MFD has been an economic concern for dairy producers for over a century. Many feeding regimes such as high-concentrate, low-fiber diets, or diets that contain high amounts of plant oils, cause MFD.

When cows experience MFD the milk fat content of *trans*-$C_{18:1}$ increases and these unique fatty acids are 100% rumen derived. Detailed analysis of the *trans* fatty acid pattern reveals that diet-induced MFD is specifically associated with an increase in *trans*-10 $C_{18:1}$. The association between increased *trans*-10 $C_{18:1}$ and MFD has now been observed in a number of situations. However, whether or not specific *trans* $C_{18:1}$ monoenes actually cause MFD is still not clear. The putative origin of *trans*-10 $C_{18:1}$ is the hydrogenation of *trans*-10, *cis*-12 conjugated linoleic acid (CLA), and this rumen-derived unique fatty acid markedly reduces milk fat synthesis. However, *cis*-9, *trans*-11 CLA, the primary CLA isomer found in milk fat does not induce MFD. In fact, a variety of CLA isomers have been tested and most of them do not cause MFD. Only the aforementioned CLA isomer and *trans*-9, *cis*-11 CLA and *cis*-10, *trans*-12 CLA negatively affect milk fat synthesis. The CLA isomers that do reduce milk fat levels do it by primarily inhibiting *de novo* milk fatty acid synthesis, although during extensive MFD, preformed fatty acids contribution to milk fat is also reduced.

CLA and human health

Theoretically, a number of CLA isomers that differ in the positions of the double bond pairs (7–9, 8–10, 9–11, 10–12, etc.) are possible. Additional differences can exist in the geometric configuration of the double bond so that *cis–trans*, *trans–cis*, *cis–cis,* or *trans–trans* configurations are possible. Fatty acids with conjugated double bonds were first demonstrated in ruminant food products over 65 years ago and later shown to consist of primarily *cis*-9, *trans*-11 CLA. CLA has been shown to have a 'functional food' role because a variety of CLA isomers possess anticarcinogenic properties in a variety of models (mammary, skin, stomach, intestinal, lung and prostate cancers). Subsequent work has identified additional beneficial health effects and consequently milk CLA has received considerable attention from not only the medical community and human nutritionists but also animal scientists. In general, potential beneficial health effects have been identified using biomedical studies with animal models and utilizing chemically synthesized supplements containing a variety of CLA isomers.

CLA synthesis

Traditionally, *cis*-9, *trans*-11 CLA was thought to originate only from ruminal production. However, the dynamics of rumen biohydrogenation are such that CLA are only transient intermediates, and the reduced products of CLA (*trans*-$C_{18:1}$ isomers) accumulate. Therefore, it was hypothesized that this particular CLA isomer was produced by an additional route. In support of this hypothesis, it has been demonstrated recently that at least 64% of *cis*-9, *trans*-11 CLA originates by endogenous synthesis involving sterol CoA desaturase in the mammary gland. Presumably, desaturation of *trans*-7 $C_{18:1}$ (and other *trans* isomers produced from rumen biohydrogenation) within the mammary gland is also the source of *trans*-7, *cis*-9 CLA, the second most abundant CLA isomer found in ruminant fat. Vaccenic acid (*trans*-11 $C_{18:1}$) is typically the most abundant trans 18:1 monoene in ruminant milk fat, and this isomer is also desaturated by sterol CoA desaturase in human tissues. Consequently, human lipid *cis*-9, *trans*-11 CLA is derived from consuming both *cis*-9, *trans*-11 CLA and vaccenic acid (both of which are unique to ruminant-derived food products).

Seasonal Effects

Pronounced seasonal patterns of milk yield and composition are evident in cattle. These seasonal patterns are largely induced by climatologic variables, breed effects, and management factors, such as feed quality and reproductive management. Month of parturition has a pronounced impact on subsequent milk yield and composition. Highest yields occur following January and February parturition, whereas lowest yields occur following August and September calvings (see **Figure 1**).

This results in correction factors that are used to adjust milk yields to remove effects of season on breeding considerations. The seasonal pattern in milk yield is related to the direct and indirect effects of environment on milk production. Direct effects are related to the effects of elevated temperature on milk yield; indirect effects are a result of photoperiod effects and the negative impact of heat stress, during late pregnancy on maternal and fetal metabolism, and circulating plasma endocrine patterns that are altered by the stress. As is apparent from **Figure 1**, there is also a seasonal pattern in milk protein that parallels the seasonal pattern in milk yield. Interestingly, the milk protein yield pattern appears to be more directly affected by temperature as the nadir occurs during the hottest part of the summer, while the milk yield curve displays some of the carryover effects related to indirect effects on pregnancy and metabolic state of the cow.

The majority of studies published on climatic effects on milk composition and yield have evaluated effects of temperature. Dairy cattle are sensitive to heat stress because of the high metabolic heat production feed intake associated with rumen fermentation and milk yield. Likewise, for the same reasons, dairy cattle are relatively resistant to cold stress. Heat stress in cattle is characterized by increased rectal temperature, elevated respiration rates, and decreased feed intake that subsequently, decreases milk yield. The environmental temperature range from −5 to 23.9 °C has little impact on milk yield and composition and is referred to as the thermoneutral zone for the lactating dairy cow. However, temperatures above 23.9 °C are known to decrease solids-not-fat (SNF), protein, lactose, and fat percentage of milk. Because of its

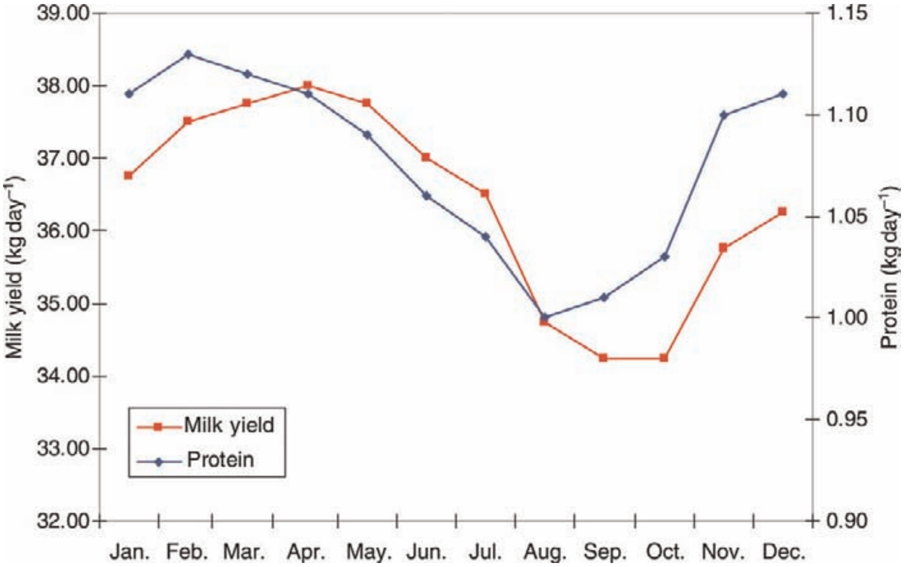

Figure 1 Effect of month of year on milk and protein yield of lactating dairy cows. Adapted from Barash H, Silanikove N, Shamay A, and Ezra E (2001) Interrelationships among ambient temperature, day length, and milk yield in dairy cows under a Mediterranean climate. *Journal of Dairy Science* 84: 2314.

involvement in osmotic regulation of milk, the impact of temperature on lactose and mineral content of milk is much less than that of temperature on protein and fat yields. Generally, in temperate regions, the fat content may average 0.4% lower and the protein content 0.2% lower in summer than in winter months. An alternative approach to evaluating cooling needs in cattle is to use the Temperature Humidity Index (THI). This combined measure of both ambient temperature and relative humidity has been shown to be more effective in evaluating environmental effects on lactating cattle than temperature alone. The original upper limit for cow comfort was established as a THI of 72, using cows producing at or less than 15 kg day^{-1}. Recent data indicate that increasing milk yields of dairy cows to or above 35 kg day^{-1} lowers the upper THI limit to 68.

A final component of milk that displays strong seasonal trends is milk somatic cell content. Milk somatic cell content rises during warm summer months related to increases in pathogen populations in the environment during summer months, decreased disease resistance in cattle associated with prolonged effects of thermal stress, and behavior of the animals that results in animals seeking wet environments with high pathogen populations.

As stated earlier, heat stress is a major contributor to the seasonal pattern of milk yield due to the negative effects of elevated temperature on feed intake and subsequently on milk yield. There is a demonstrated genotype–environment interaction both across and within breeds, indicating that genetic selection for increased heat tolerance is feasible. Furthermore, some traits, such as increased sweat gland number, hair coat characteristics, and increased skin surface area, could be altered without adversely affecting milk yields. However, the time required for achieving a higher level of heat resistance is lengthy. Therefore, the greatest opportunity for increasing milk yield during periods of environmental heat stress has been in the management of the environment surrounding the cow. Dairies in warm climates use a variety of cooling systems, including protection from solar radiation (shades), fans, combinations of fans and misters, and evaporative cooling to produce an environment around the cow, which is preferably in the thermoneutral zone (−5 to 23.9 °C) or a THI below 68.

See also: **Lactation**: Galactopoiesis, Effect of Treatment with Bovine Somatotropin; Galactopoiesis, Effects of Hormones and Growth Factors; Lactogenesis. **Stress in Dairy Animals**: Heat Stress: Effects on Milk Production and Composition.

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LACTIC ACID BACTERIA

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Taxonomy and Biodiversity

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Introduction

Lactic acid bacteria (LAB) are commonly detected in various habitats such as foodstuffs, gut and mucous membranes of humans and animals, and in many environmental niches. In the fermentations of yogurt, cheese, salami, sourdough bread, and wines, LAB are the key organisms providing both desired sensory changes and increased shelf life and product safety. Many *Lactobacillus* and *Lactococcus* spp. play a major role in the fermentations of traditional foods and in an expanding range of novel foods and products designed to have specific nutritional or other health-enhancing benefits. In addition to the 'generally regarded as safe' (GRAS) bacteria, some pathogenic species are included within the LAB. Some species of streptococci and enterococci cause both human and animal infections. Another range of harmful LAB are the psychrotrophic LAB, which are able to grow under refrigerated storage. These bacteria are specific spoilage organisms in modified-atmosphere-packaged meat and fish products.

Determination of a Lactic Acid Bacterium

LAB are a metabolically and physiologically related group of Gram-positive, catalase-negative bacteria, which consist of both cocci and bacilliforms. Typical LAB are nonsporing and nonrespiring, aero- and acid tolerant, and fastidious. They are strictly fermentative, the principal end product of carbohydrate metabolism being lactic acid. There are some exceptions to this typicality. Members of the genus *Sporolactobacillus* form spores and some LAB possess cytochromes and may respire if heme is available.

LAB have a DNA base composition of less than 50 mol% G + C, and so are phylogenetically included in the so-called *Clostridium* branch of Gram-positive organisms. Nowadays, LAB comprise around 20 genera, of which *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* are considered as the principal LAB. The genera *Lactococcus* and *Lactobacillus* are commonly associated with milk and milk products, but none of the genera, with the exception of the wine-bound *Oenococcus*, are associated with only one habitat. Species of *Enterococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* are also commonly detected in milk and milk products.

The phylogenetic tree of LAB is presented in **Figure 1**. Bifidobacteria, which also produce lactate and acetate as the end products of carbohydrate metabolism, have a unique pathway of hexose fermentation, a fructose-6-phosphate shunt that differs from Embden–Meyerhof–Parnas (EMP), and 6-phosphogluconate metabolic routes of LAB. They also have a G + C content of over 50 mol%, and are not related to LAB but to actinobacteria.

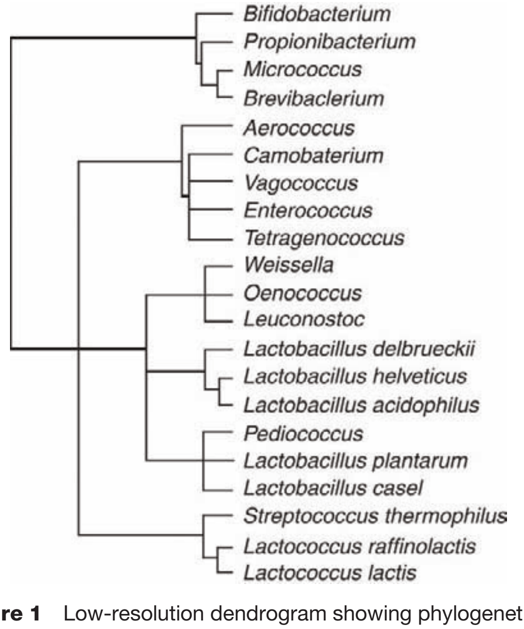

Figure 1 Low-resolution dendrogram showing phylogenetic relationships between bacteria of the high- and low-G+C subdivisions of the Gram-positive division, based primarily on 16S rRNA sequence data. Branchings indicate phylogenies; phylogenetic distances are not shown to scale.

Polyphasic Taxonomy of Lactic Acid Bacteria

Even though *Bacterium lactis* (currently *Lactococcus lactis*) was the first bacterial species described, and LAB were among the first bacterial groups defined, the taxonomy of LAB is still evolving rapidly. This is partly due to the detection of completely new species and partly also to the, sometimes even reversal, rearrangement of the former species within and between genera. This vagueness of species arises from the fact that the former use of classical phenotypical tests rarely led to accurate species definition within LAB.

Species is a basic element of bacterial taxonomy. In 2001, Roselló-Mora and Amann described it as "a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property". This description conforms well to the nowadays widely accepted 'polyphasic approach' of species delineation.

Polyphasic taxonomy utilizes both phenetic (phenotypic and genotypic) and phylogenetic information. In practice, phenetic data are processed by numerical taxonomy based on the concept of overall similarity (resemblance). Analysis of the phylogenetic data (sequences of DNA, RNA, or proteins) is, instead, based on the concept of homology (having a common origin) and parsimony, which means that evolution is assumed to have reached the current situation by the shortest possible route. The aim in phylogenetic analysis is to determine the evolutionary branching pattern. The use of polyphasic approach should, at best, result in delineation that is stable, provides a diagnostic scheme for species differentiation, and reflects phylogenetic relationships.

However, despite the indisputable usefulness of the polyphasic approach, there is still no universal or official concept of polyphasic species delineation. At present, the only definition acknowledged by the International Committee on Systematics of Prokaryotes's (ICSP) Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology is that a species is a group of strains sharing 70% or greater DNA–DNA reassociation values and 5 °C or less ΔT_{m} (difference in the DNA–DNA hybrid melting points). Thus, these methods are the official base for species delineation within all bacteria.

According to the report by the Ad Hoc Committee, all species descriptions should also include an almost complete 16S rRNA encoding gene sequence. As a conserved housekeeping gene, it can be used as a phylogenetic marker in determining the relationship between even distantly related bacteria. In addition, the 16S rRNA encoding gene sequence similarity can be used in species delineation. It has been observed that organisms with

DNA–DNA similarities above 70% usually share more than 97% sequence similarity in their genes encoding 16S rRNA. However, for several reasons, the 16S rRNA encoding gene sequence should not be used as the only method in species delineation. Among LAB, different species with identical or nearly identical 16S sequences exist and thus similarity values can reliably be used only as an excluding criterion.

Also the sequencing of the other housekeeping genes (genes encoding metabolic functions) or other genes has been regarded as a promising method for phylogenetic analyses by the Ad Hoc Committee. The recommendation was that the data should be obtained, as an extension of the multilocus sequence typing (MLST) approach, from the determination of at least five genes located in diverse chromosomal loci and found widely distributed among taxa. However, even though there are MLST schemes for at least streptococcal and enterococcal species identification, the combined analysis of several genes sequenced is rarely used in LAB taxonomy. The sequencing of only one or few genes is far more widely used. For *Enterococcus*, an application of MLST based on RNA polymerase α-subunit (*rpoA*) and phenylalanyl-tRNA synthase (*pheS*) genes has been reported. Genes *recA*, *cpn*60, *tuf*, and *slp* have been used with *Lactobacillus* and *gyrA*, *gyrB*, *sodA*, and *parC* with *Streptococcus*.

Single nucleotide polymorphism (SNP) is a variation of a single nucleotide in specific locations of DNA. SNP can be regarded as a simplified and more cost-effective version of MLST, where small variable fragments of genes are sequenced instead of the whole gene. Unfortunately, this method is also scarcely used within LAB; there are only few reports of its use with streptococci.

Understanding Phylogeny Has Not Necessarily Resulted in Changes at the Genus Level

Omitting the subspecies, close to 180 species names have been proposed to be included in the genus *Lactobacillus*. Some of these names are not used anymore since taxonomic revisions have placed the organisms into other genera, typically *Carnobacterium* or *Weissella*, or one species has been detected to bear several different names. *Lactobacillus* is a very heterogeneous genus. For example, DNA base compositions are in the broad range of about 32–55 mol% G + C. Based on phenotypic and chemotaxonomic characteristics, lactobacilli have been divided into two, three, or four groups. This grouping reflects more the fermentation characteristics of strains but does not correlate with the groups indicated by phylogenetic analysis (**Figure 2**). The inclusion of such diverse bacteria in a single genus is historical, and subdivisions of the genus have been under taxonomic revision for some years but yet it has not been suggested to split this genus based on the polyphasic taxonomy concept. A common mistake of beginners is to consider that all lactobacilli are closely related even though some are more closely related to *Pediococcus*.

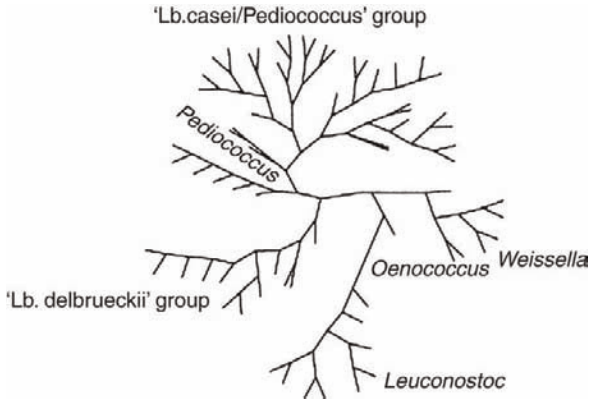

Figure 2 Phylogenetic branching within *Lactobacillus* and its near relatives, showing the three major groups. Branchings indicate phylogenies; phylogenetic distances are not shown to scale. Data used in preparing this figure were derived from the Ribosomal Database Project accessed at Michigan State University (via ftp.cme.msu.edu).

Changes have also affected the genera *Leuconostoc* (split currently between *Leuconostoc* and *Fructobacillus*), *Enterococcus*, and *Weissella*. The researcher must pay attention while selecting strains for taxonomy or biodiversity studies since many novel species have been included in these genera.

Keeping Updated with the Taxonomy Revisions

Keeping up with changes in prokaryotic nomenclature has always been problematic. It has been estimated that since January 2000, bacterial names have undergone a change at a rate approaching 750 validly published names every year. It is good to bear in mind that only the names given to prokaryotes are regulated. No official classification of prokaryotes exists. General considerations, principles, rules, and recommendations that govern the way the names of prokaryotes are to be used are delineated in the *International Code of Nomenclature of Bacteria* (*Bacteriological Code*).

Fortunately, nowadays, many useful sources about bacterial classification are available through the Internet. 'List of Prokaryotic names with Standing in Nomenclature' provides accurate information about the current status of a name, synonyms, and other useful information. The NCBI Taxonomy Browser is helpful in determining full lineages of bacteria and finding the sequences associated with them.

'The All-Species Living Tree' project aims to provide a single 16S rRNA tree harboring all sequenced type strains of the hitherto classified species of archaea and bacteria. This tree will be regularly updated by adding the species with validly published names that appear monthly in the validation and notification lists of the *International Journal of Systematic and Evolutionary Microbiology*. The 'Eztaxon' resembles The All-Species Living Tree project. Within Eztaxon, it is not possible to retrieve the actual database but one can do sequence searches. Retrieving or limiting the work especially within the sequences of type strains is very useful when research associated with identification or characterization of biodiversity is carried out.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: *Enterococcus* in Milk and Dairy Products; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; *Streptococcus thermophilus*.

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http://www.arb-silva.de – SILVA rRNA database project.

Proteolytic Systems

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Introduction

In bacteria, proteolytic systems are well known to play key roles in various functions such as nutrition, virulence, protein turnover, regulatory events, and protein maturation and export. In LAB, generally recognized as safe, and widely used in fermented food manufacturing and especially in the dairy industry, the most documented aspect of the proteolytic system concerns its relationship with nitrogen nutrition. The majority of the knowledge has been acquired in *Lactococcus lactis* considered as the model of the LAB family, which includes the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. In these species, in addition to the proteolytic enzymes characterized for their role in nutrition, a significant number of putative proteolytic enzymes of unknown functions have been identified. Although LAB are not considered to be highly proteolytic as compared to bacilli, for example, a search for clusters of orthologous groups and conserved proteolytic motifs reveals the existence of a total of 40 putative or known peptidases in the *L. lactis* strain IL1403, the genome sequence of which is available (**Table 1**).

Nitrogen Nutrition

The importance of proteolytic systems in the nitrogen nutrition of lactic acid bacteria (LAB) can be explained by citing two main reasons. The first is linked to the incapacity of LAB to synthesize several amino acids for which they are said to be auxotrophic. Generally speaking, *Streptococcus thermophilus* is the less demanding species (a maximum of six amino acids including leucine, valine, and cysteine), while lactobacilli are auxotrophic for a large number of amino acids. The model, *Lactococcus lactis*, is auxotrophic for 7–12 amino acids in a strain-dependent manner; the subspecies *lactis* is less demanding than the subspecies *cremoris*. The amino acids most frequently needed in *L. lactis* are the branched-chain amino acids arginine and methionine. In parallel, several studies have demonstrated that some *L. lactis* strains possess functional biosynthesis pathways for amino acids such as histidine, tryptophan, branched-chain amino acids, or aspartic acid. The genes coding the enzymes involved in these biosynthesis pathways are generally grouped together in large operons. The data concerning the amino acid demands of LAB came from growth experiments in chemically defined media that have been developed for several species and in which different amino acids can be added or omitted. These growth experiments allowed the distinction of amino acids that are essential for growth from those that stimulate growth and from those that are not necessary for growth. The genome sequences, which have been accumulating for several years, constitute an interesting source of information). Their analysis allows prediction of the functionality of biosynthetic pathways and needs for amino acids. As an example, the genome sequence of *Lactobacillus sakei* reveals that only the biosynthetic pathways of asparagine and glutamine would be functional. Auxotrophies are more often linked to punctual mutations than to absence of genes, which suggests that the loss of capacity to synthesize amino acids would be linked to an evolutional adaptation process to media such as milk or meat. The observation that lactococci from plant origin show a limited number of auxotrophies confirms this hypothesis.

The second reason explaining the importance of the proteolytic system in LAB is linked to the composition of their usual growth medium, which is milk. This medium is indeed poor in free amino acids and short peptides that could sustain growth. Consequently, LAB must hydrolyze the caseins, the main milk proteins, to grow. Because optimal growth of LAB in milk is needed for successful fermentation, the part of the proteolytic system involved in nitrogen nutrition has been extensively studied. In all LAB, three steps are necessary: first, casein hydrolysis by a cell envelope-anchored protease; second, transport of peptides into the bacterial cell, and third, intracellular hydrolysis of peptides into free amino acids by a set of peptidases (**Figure 1**).

The Cell Envelope Protease and Extracellular Proteolysis

Extracellular proteolysis is made possible by the presence, in most LAB growing in milk, of a protease commonly called the cell envelope protease/proteinase (CEP or Prt), which is linked to the cell envelope. It is capable of hydrolyzing caseins into oligopeptides, which could be further transported into the bacteria. Prt was first studied

Table 1 List of the putative or known peptidases in the strain *Lactococcus lactis* IL1403. This strain does not possess any cell envelope protease. Predictions of proteolytic enzyme localization were performed with SurfG+ software (Barinov *et al*., 2009)

Gene	*Name*	*Localization*	*Function*	*Regulation*	*References*
clpP	Proteolytic subunit of the Clp protease	Cytoplasm	Stress		Cortes-Perez *et al*. (2006)
comC	Type 4 prepilin-like protein-specific leader peptidase	Membrane	Competence		Wydau *et al*. (2006)
dacA	D-Alanyl-D-alanine carboxypeptidase	Secreted	Cell wall		
dacB	D-Alanyl-D-alanine carboxypeptidase	Secreted	Cell wall		Courtin *et al*. (2006)
ftsH	ATP-dependent metalloprotease	Potentially surface exposed	Stress		
gcp	O-Sialoglycoprotein endopeptidase	Cytoplasm			
htrA	Housekeeping protease	Secreted	Stress		Poquet *et al*. (2000)
lspA	Lipoprotein signal peptidase	Secreted	Maturation		Venema *et al*. (2003)
pepA	Glutamyl aminopeptidase	Cytoplasm	Nutrition		Ianson *et al*. (1995)
pepC	Aminopeptidase C	Cytoplasm	Nutrition	CodY, O_2	Neviani *et al*. (1989)
pepDA	Dipeptidase	Cytoplasm	Nutrition	CodY	
pepDB	Dipeptidase	Cytoplasm			
pepF	Oligoendopeptidase F	Cytoplasm			Monnet *et al*. (1994)
pepM	Methionine aminopeptidase	Cytoplasm	Maturation		
pepN	Aminopeptidase N	Cytoplasm	Nutrition	CodY	Tan *et al*. (1992)
pepO	Oligoendopeptidase O	Cytoplasm	Nutrition	O_2	Mierau *et al*. (1993)
pepP	Aminopeptidase P	Cytoplasm		CcpA	Mars *et al*. (1995)
pepQ	Proline dipeptidase	Cytoplasm	Nutrition	CcpA	Kaminogawa *et al*. (1984)
pepT	Di/tripeptidase	Cytoplasm	Nutrition		Mierau *et al*. (1994)
pepV	Dipeptidase	Cytoplasm			Hellendoorn *et al*. (1997)
pepX	X-Prolyl dipeptidyl aminopeptidase	Cytoplasm	Nutrition		Nardi *et al*. (1991)
pi136	Prohead protease	Cytoplasm			
pi323	Clp putative protease	Cytoplasm			
sipL	Signal peptidase I	Potentially surface exposed	Maturation		
yaiF	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane		CodY	
yaiH	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane		CodY	
yajF	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane			
ybdI	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane			
ybdJ	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane			
ybcH	Putative X-Pro dipeptidyl peptidase	Secreted			
yciA	Metal-dependent amidase/aminoacylase/carboxypeptidase	Cytoplasm			
yjiB	Metal-dependent amidase/aminoacylase/carboxypeptidase	Cytoplasm			
yjgB	γ-Glutamyl-diamino acid-endopeptidase	Secreted	Cell wall		Redko *et al*. (2007)

yueE	Predicted Zn-dependent peptidase	Cytoplasm	
yueF	Protease	Cytoplasm	
yugD	Protease	Cytoplasm	
yuhB	Protease	Cytoplasm	
yvdC	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane	
yvdE	Glutamine amidotransferase with a putative peptidase domain	Cytoplasm	
yvjB (eep)	Zinc metalloprotease (SP2 family)	Potentially surface exposed	
yxdF	Hypothetical protein with a prenyl peptidase domain CAAX	Membrane	CodY

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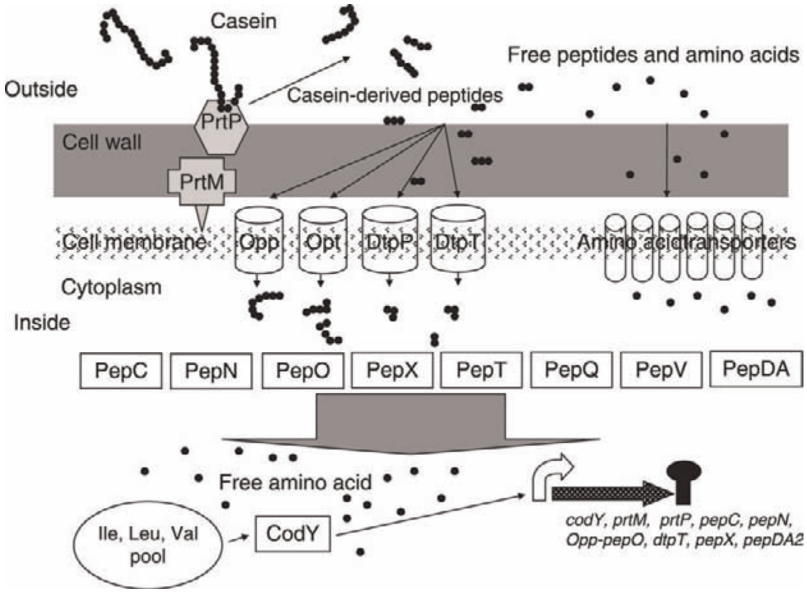
Casein
Outside
Casein-derived peptides
Free peptides and amino acids
PrtP
Cell wall
PrtM
Cell membrane
Opp
Opt
DtpP
DtpT
Amino acid transporters
Cytoplasm
Inside
PepC
PepN
PepO
PepX
PepT
PepQ
PepV
PepDA
Free amino acid
Ile, Leu, Val pool
CodY
codY, prtM, prtP, pepC, pepN,
Opp-pepO, dtpT, pepX, pepDA2

Figure 1 Schematic view of the part of the proteolytic system of *Lactococcus lactis* involved in the nitrogen nutrition process. Only the peptidases known for their role in nitrogen nutrition are represented. (For abbreviations, see text.)

in detail in lactococci (PrtP), and more recently in lactobacilli (PrtB, PrtR, PrtH) and *St. thermophilus* (PrtS).

These large proteases (1900 amino acids, 200 kDa) from the subtilisin family have a serine catalytic mechanism and are anchored to the cell wall via an LPXTG motif. They are composed of several domains exhibiting different functions (localization, anchoring, catalysis, etc.). Eight well-conserved domains are found in the protease from lactococci and *Lactobacillus casei* (**Figure 2**). The CEPs from other species slightly differ from this model: PrtS from *St. thermophilus* does not possess any B domain, probably involved in the stabilization of the catalytic domain; PrtB and PrtH have different anchoring domains as compared to PrtP. The CEP is synthesized as a pre-pro-protein. The CEP acquires its tri-dimensional structure after cleavage of its signal sequence and secretion. This step needs, for PrtP from lactococci, the participation of a lipoprotein, PrtM, which acts as a chaperone. This chaperone is not always present and is not needed, for example, for the maturation of PrtB in *Lactobacillus delbrueckii* subsp. *bulgaricus*. The final maturation of the CEP is obtained after autoproteolysis and release of the prodomain (**Figure 2**).

The cleavage specificity of the CEPs has been studied for several strains of lactococci and lactobacilli using the main caseins (α_{s1}, β, κ), casein fragments (the most commonly used is the fragment 1–23 from α_{s1} casein), or chromogenic substrates. Specificity studies have revealed that even a very limited number of differences in the CEP sequences can lead to significant differences in the specificity of casein hydrolysis. A classification of hydrolysis specificities has been proposed: the proteases with PI specificity hydrolyze mainly β-casein, the chromogenic substrate MS-Arg (methoxy-Suc-Arg-Pro-Tyr-*p*-nitroanilide), and

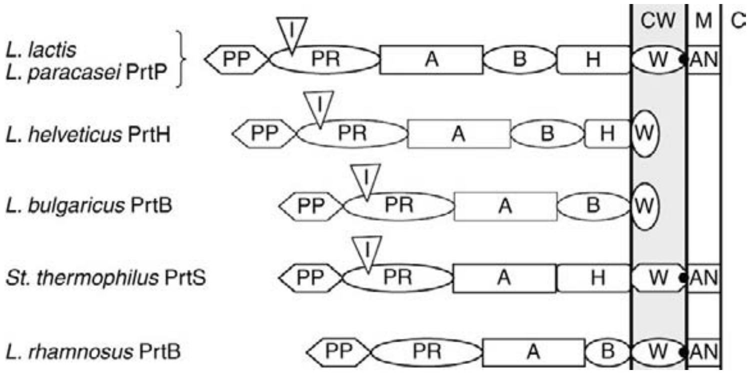
CW
M
C
L. lactis
L. paracasei PrtP
L. helveticus PrtH
L. bulgaricus PrtB
St. thermophilus PrtS
L. rhamnosus PrtB
I
PP
PR
A
B
H
W
AN

Figure 2 Schematic representation of cell envelope proteases of different LAB. CW, cell wall; M, membrane, C, cytoplasm; PP, pre-pro-domain; PR, catalytic domain; I, insert domain; A, A domain; B, B domain; H, helix domain; W, cell wall spacer domain; black dot, sorting signal; AN, anchor domain. Adapted from Siezen R (1999) Multi-domain, cell-envelope proteinases of lactic acid bacteria. *Antonie van Leeuwenhoek* 76: 139–155.

the N-terminal part of the peptide 1–23 from α_{s1}-casein; the proteases with PIII specificity hydrolyze the three main caseins, the S-Glu (Suc-Ala-Glu-Pro-Phe-*p*-nitroanilide) substrate, and the C-terminal part of the peptide 1–23; between these two extreme specificities, a high diversity of specificities has been described.

Usually, LAB possess only one copy of the *prt* gene. In lactococci, the PrtP encoding gene is often carried on a plasmid and sometimes associated with the genes necessary to the use of lactose, the milk sugar. This plasmid known as protease-lactose plasmid therefore carries the genes essential for optimal growth in milk. The strains that are, naturally or after inactivation of the *prt* gene, protease-negative can develop only slightly in milk and only reach a biomass corresponding to 10% of that obtained with a protease-positive strain. The lactococcal protease-negative strains are mainly of vegetable origin. In *St. thermophilus*, PrtS is present only in a limited number of strains and is homologous to the C5a peptidase from pathogenic streptococci. A recent work showed that, even without a CEP, *St. thermophilus* can grow optimally in milk in the presence of a *L. delbrueckii* subsp. *bulgaricus* strain endowed with a CEP and which provides *St. thermophilus* with the oligopeptides necessary for its growth. The genome sequences of lactobacilli reveal that a CEP encoding gene is not always present. This observation was made for the *Lb. sakei* 23K and *Lactobacillus plantarum* WCFS1 genomes, for example. When present, it is chromosomally encoded in lactobacilli.

Amino Acid and Peptide Transport

The oligopeptides released by the CEP are, with the peptides and free amino acids present in milk, the main source of amino acids for LAB. These nitrogen compounds are transported into the bacteria via several transport systems, which belong to three groups: (1) the permeases working with the proton motive force called PTR (peptide transporters); (2) the ATP-binding cassette (ABC)-transporters that get their energy via ATP hydrolysis; and (3) the antiports, which use concentration gradients to import one molecule against the export of another. Amino acid transporters are generally specialized for a single amino acid or a family of amino acids. This specialization probably makes the adjustment of amino acid balance easier or facilitates the response to a stress such as the osmotic stress, which induces glycine betaine transport.

The capacity of LAB to grow in a medium in which an essential amino acid is given in a peptide indicates that LAB have the capacity to transport peptides. At the specificity level, we distinguish di- and tripeptide transporters from oligopeptide transporters, which sometimes also transport tripeptides. The transporter DtpT, initially characterized in *L. lactis* but also identified in *St. thermophilus* and lactobacilli, uses the proton motive force. It preferentially transports hydrophobic di- and tripeptides with a high affinity for dipeptides. The other PTR, namely, Dpp, importing preferentially the hydrophobic di- and tripeptides, or the oligopeptide transporters (Opp, Opt, Ami) are ABC transporters (**Figure 1**). All the oligopeptide transporters have the same organization: (1) two proteins at the inner face of the membrane in charge of ATP hydrolysis; (2) two membrane proteins that form the channel allowing peptide internalization; and (3) several binding proteins (usually very homologous) anchored to the membrane. The reason why there are several copies of the binding proteins remains poorly understood. The example of one *St. thermophilus* strain shows that the genes coding for the binding proteins (three in this case) are located at different places on the chromosome: one is in the operon with the genes coding the other proteins of the transporter, and the two others are isolated at other chromosomal locations. Whole oligopeptide transport systems are frequently duplicated. For example, the strains *L. lactis* SK11 and Wg2 possess two functional transport systems: Opp and Opt. Oligopeptide transport plays a key role in the nitrogen nutrition and is essential for optimal growth in milk. An Opp-negative mutant exhibits the same growth phenotype as a Prt-negative mutant and only reaches a low cellular density.

Intracellular Proteolysis

Various peptidases (aminopeptidases, di- and tripeptidases, endopeptidases) have been identified and characterized inside LAB (up to 13 in *L. lactis*). They exhibit different substrate preferences (oligo-, di-, or tripeptides) and cleavage specificities (general or specific for some amino acids). It is noticeable that several peptidases specifically cleave peptide bonds involving a proline, which demonstrates that LAB are well adapted to caseins, which are proteins with a high proline content. It has to be remembered that proline is formally a secondary amine and acts as a structure disruptor in proteins. Therefore, peptide bonds involving proline are rather resistant to proteolysis.

The involvement of peptidases in the nitrogen nutrition process has been evaluated by growth rate measurements of negative mutants during growth in milk. Because the peptidase cleavage specificities often overlap, it was necessary to construct multiple mutants to observe a clear effect on growth. In *L. lactis*, nitrogen nutrition is ensured by several peptidases exhibiting either general (the aminopeptidases PepC and PepN, the oligopeptidase PepO, the tripeptidase PepT) or proline (the prolidase PepQ, the dipeptidase PepV, and the X-prolyl dipeptidyl aminopeptidase PepX) specificities. The role of other peptidases such as the proline-specific aminopeptidase PepP or the glutamic acid – specific aminopeptidase PepA is not yet clear. Some peptidases are specific for some LAB species and have no counterparts in lactococci: the aminopeptidase PepS in thermophilic LAB, and the peptidases PepL, PepG, and PepI in lactobacilli.

Regulation of the Proteolysis Linked to the Nitrogen Nutrition

In *L. lactis*, recent studies have demonstrated that the expression of the main genes coding enzymes and proteins necessary to nitrogen nutrition, namely, *prtP*, *prtM*, *opp*, *pepO*, *pepD*, *pepN*, *pepC*, and *pepX*, is negatively controlled by a rich nitrogen source. More precisely, the transcriptional regulator CodY senses the intracellular branched-chain amino acid pool and, when this is high, represses (between 5- and 150-fold) the expression of the genes coding the main components cited above. CodY was found to modulate the expression of up to 100 genes in *L. lactis* including its own gene. From these results, a consensus CodY box has been identified. It is present in the promoter region of *pepC*, *pepN*, and *opp*. A direct interaction between CodY and the promoter region of *oppD* was demonstrated. Three out of seven genes encoding CAAX proteases in *L. lactis* were also identified as being strongly regulated by CodY. These proteases, also named prenyl endopeptidases, are involved in the post-translational modification of the CAAX (C, Cysteine; A, aliphatic; X, any amino acid) sequence at the C-terminus of some proteins. They release the tripeptide AAX, while a prenyl (i.e., an alkenyl) group is linked to the cysteine. Although the role of CAAX proteases in bacteria is still unknown, it is believed that the modification they are involved in could be necessary for protein-anchoring to cell membrane. These proteases are widespread both in eukaryotes and prokaryotes and seem to have accumulated in *L. lactis* by horizontal transfer.

The expression of *pepP* and *pepQ* is under the control of the catabolic repressor CcpA (catabolic control protein A). This repressor was shown to regulate the expression of genes coding the proteins involved in carbon assimilation, but the possible link between these proline-specific peptidases and carbon metabolism is not yet understood (**Figure 1**). Finally, the peptidases PepO and PepC were found to be more abundant under conditions permitting aeration and respiration, in a CodY-independent manner.

Consequences and Applications of Casein Hydrolysis

The hydrolysis of caseins and especially that of β-casein by LAB CEPs generates hydrophobic peptides that, when they accumulate, are responsible for bitterness, considered as a defect in cheese. The phenomenon is more pronounced with CEPs with PI specificity and is dependent on the capacity of bacteria to hydrolyze further the bitter peptides into short peptides and free amino acids via its peptidases. The use of negative mutants for the peptidases PepN, PepO, and PepC demonstrated that these peptidases are involved in the degradation of bitter peptides. Overexpression of peptidase activity in lactococci or lactobacilli leads to a decrease in bitterness and acceleration of ripening. However, it is clear that, although proteolysis has an impact on bitterness and the release of free amino acids, it is not the limiting factor for flavor development, which depends on the first step of modification of amino acids into aroma compounds. The second possible application of casein hydrolysis by LAB is the production of peptides endowed with health-promoting activities. In this respect, caseins are of particular interest since they contain many bioactive peptide sequences that can be released by proteolysis. The release of such peptides by lactococci and lactobacilli has been demonstrated in fermented foods. However, it is not clear if and how these peptides remain intact or active after ingestion. Peptides endowed with antihypertensive activity are the most documented. The two most famous are the tripeptides IPP and VPP probably resistant to proteolysis due to their high proline content. They are present in several commercial fermented milks and their efficiency has been demonstrated in clinical studies.

Degradation of Abnormal Proteins

Throughout their use in food industry, LAB have to face various stresses during which abnormal proteins are produced. Three proteolytic enzymes have been described as involved in the hydrolysis of proteins damaged by stresses: Clp, HtrA, and FtsH. Although not homologous to the housekeeping protease Lon from *Escherichia coli*, HtrA seems to play the same role in degrading abnormal proteins. The proteolytic subunit of Clp, namely, ClpP, and the associated regulation subunits ClpC, ClpE, and ClpB are regulated by the stress response regulator CtsR. In addition, the inactivation of the gene *trmA* induces the degradation of abnormal proteins via a Clp-independent pathway, indicating that TrmA is a negative regulator of proteolysis in *L. lactis*.

Maturation and Secretion of Proteins

Following a general bacterial maturation and secretion scheme, we can postulate that, in LAB, like in other bacteria, nascent polypeptides are first maturated by cleavage of the N-terminal methionine. A putative peptidase dedicated to this task, an aminopeptidase PepM, has been identified on the basis of its homology with aminopeptidases M from other bacteria whose activity has been already demonstrated.

Concerning proteins that are secreted, they are guided by the presence of a signal peptide, which can be cleaved by the membrane-located signal peptidase I for all secreted proteins except lipoproteins, or signal peptidase

II, dedicated to the lipoproteins. In *L. lactis*, 146 among 2310 predicted proteins possess a putative signal peptide with an average size of 33 amino acids. Secretion process releases signal peptides, which need to be hydrolyzed and recycled. The way these peptides are hydrolyzed is not completely clear in any bacteria and *a fortiori* in LAB. However, a recent study indicates that the oligoendopeptidase PepF could be involved in signal peptide degradation in *L. lactis*.

Regulation via Pheromones

A specific proteolysis, the regulatory intra-membrane proteolysis (RIP), allows the release of regulatory peptides or pheromones in the membrane. Pheromones are released either from signal peptides of lipoproteins or from larger peptides. A family of membrane metalloproteases, S2P, has been identified in many bacteria and is involved in RIP proteolysis. Eep (for enhanced expression of pheromone) is one of these enzymes in *Enterococcus faecalis*. By hydrolyzing signal peptides from lipoproteins, Eep releases seven short amino acid peptides, which act as pheromones and induce the conjugation process. Eep from *L. lactis* is also necessary for the correct maturation of lipoproteins and could also play a role in the release of pheromones. This domain has been poorly investigated in LAB, although there is a growing interest in regulation via peptides.

Synthesis or Hydrolysis of Peptidoglycan

Due to the presence of peptide bridges in the peptidoglycan structure, peptidases are logically involved in both the synthesis and the hydrolysis of peptidoglycans. The peptidases DacA and DacB, which are actually transpeptidases, are known to be involved in the last step of peptidoglycan synthesis in bacteria. In *L. lactis*, DacB has been characterized and its involvement in peptidoglycan maturation demonstrated. Another peptidase, YjgB is one of the five peptidoglycan hydrolases in *L. lactis* and is capable of hydrolyzing peptidoglycan peptides with the γ-D-Gln-L-Lys sequence.

Conclusions and Perspectives

The role and the potential of the proteolytic system of LAB have been extensively investigated in the field of nitrogen nutrition. The different steps allowing LAB to obtain the necessary amino acids are known. However, most putative peptidases detected in the genome sequences, as well as a part of the characterized peptidases, are still of unknown function. It is predictable that they are essential in various posttranslational modifications or regulation processes, not yet identified. A part of these poorly known peptidases belong to the CAAX family and are predicted to be membrane-located. They seem to have accumulated in *L. lactis* and constitute, with the other membrane-located proteases, a challenging topic of research for the future.

See also: **Cheese**: Biochemistry of Cheese Ripening. **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development; Taxonomy and Biodiversity.

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Physiology and Stress Resistance

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Introduction

Abiotic stress in lactic acid bacteria (LAB) is an ever-present consideration in milk processing. Dairy processing provides a series of stresses designed to reduce, control, and intervene in microbial metabolism. Unfermented products, such as fluid milk, use temperature (pasteurization) stress followed by cold stress to reduce and control pathogenic and spoilage microbes. Fermented liquid products have the additional stresses of acid and refrigeration. In the case of surface-ripened cheeses, yeast and mold also bring about new stress conditions for LAB. Cheese undergoes yet more stress treatments with the combined stresses of heat, oxidation, osmotic, acid, carbohydrate starvation, and refrigeration during production and aging. LAB must respond to this suite of stresses to survive, grow, and produce metabolic end products that impart desirable flavor compounds important to consumers.

Milk-associated LAB have a relatively limited metabolic capability in comparison to other microbes; yet they have been selected to resist and persist under the stresses of fermented products. In cheese, much attention is paid to nonstarter LAB (NSLAB), which are largely lactobacilli, because they resist stress, grow, and persist in cheese during aging to high cell numbers. Lactococci intentionally added at a very high cell density to initiate the formentation respond to stress to maintain populations and produce many flavor compounds. In fact, lactococcal strains used in dairy fermentations were distinguished into subspecies based on phenotypic response to stress – resistance to heat and salt, and arginine utilization. Understanding of individual stress responses beyond phenotypic traits is rapidly expanding largely due to genome sequencing. Defining the systematic and simultaneous stress load provided by dairy processing is critical to understanding survival and stress-induced metabolic capabilities for production of acceptable fermented products. It is also critical for starter culture strain selection for use in dairy processing.

Stresses in milk fermentations can be divided into biotic and abiotic. Biotic stresses include metabolites from the microbial community (e.g., acids, bacteriocins), bacteriophage (phage), and enzymes that degrade the cell wall (e.g., proteases, muramidase aka lysozyme) from other bacteria in the community. In some cases, biotic stress also leads to DNA damage or genome evolution due to phage and prophage integration. Evolution of dairy starter cultures is directly linked to stress-induced traits – phage resistance is one of the most commonly selected traits in lactococci – as it is directly linked to acid production, which also induces DNA damage and potentiates genetic evolution via a complex set of DNA repair mechanisms that are often divergent and prone to mistakes.

As a whole, processing-associated stresses occur in series and are maintained in combination. For example, during the aging of hard cheese, LAB are exposed to acid (pH 5.2), salt (about 4.5–5% in the moisture), sugar starvation, and refrigeration (storage at about 14 °C) for an extended period of time (months to years). In an effort to define the effect of each stress, studies are often conducted as single events with follow-up studies to define additional stress resistance. This approach is often limiting in providing a comprehensive understanding of the cellular events needed to cope with multiple and long-term stresses. A global or systemwide change must occur in a very short time period for the LAB cell to survive, persist, and produce flavor changes during aging. Stress response induces changes throughout the cell, ranging from DNA damage to disruption of metabolism, membrane composition, and cell wall construction. Consequently, a system-level approach will more accurately assess the vast number of changes induced and propagated throughout the cell. The phenotypic changes often considered are increased survival and modified metabolism, which in lactococci are associated with various flavor and texture defects if the cell populations become too high or are unbalanced with a single dominating organism.

Techniques used to study stress response have also evolved. Initially, growth was used to monitor resistance to specific stresses, such as phage and temperature resistance. This approach led to isolation of many strains for use in the laboratory and in the factory. Proteomic and metabolic tools subsequently provided a more systemic view of the proteins involved in stress. More recently with the public availability of more than 60 LAB genome sequences, the use of genomics and gene expression has brought a new capability and technological basis to the study of LAB stress and its role in fermented products.

The genetic composition of stress response and the induced regulatory events is dramatically better elucidated with the use of genomics. With the comparative genomics capability using multiple genomes of *Lactococcus* and *Lactobacillus*, additional information can be derived for genome stability and stress-induced genome rearrangement. Another advance enabled by the use of genome sequence is metabolic reconstruction. This approach permits simultaneous examination of the entire metabolic potential of a cell individually or in a complex community. Overlaying gene, protein, and metabolite profiles provides a multifaceted perspective of how a cell deals with stress and how specific flavor compounds are produced. Abiotic stresses associated with fermented dairy products will be the focus of this article.

Tools to Study Systemwide Stress Response

Many studies use a single approach to study the mechanisms of stress. However, use of multiple ' -omics' tools provides many benefits and is greater than the sum of the individual tools (**Figure 1**). Monitoring individual gene or a handful of genes is easily done using PCR or quantitative PCR, but it gives a limited perspective of the entire cellular response. Using microbial genome sequences enables one to examine the entire expression profile of individual microbes using whole genome expression arrays. If appropriately designed, expression of small

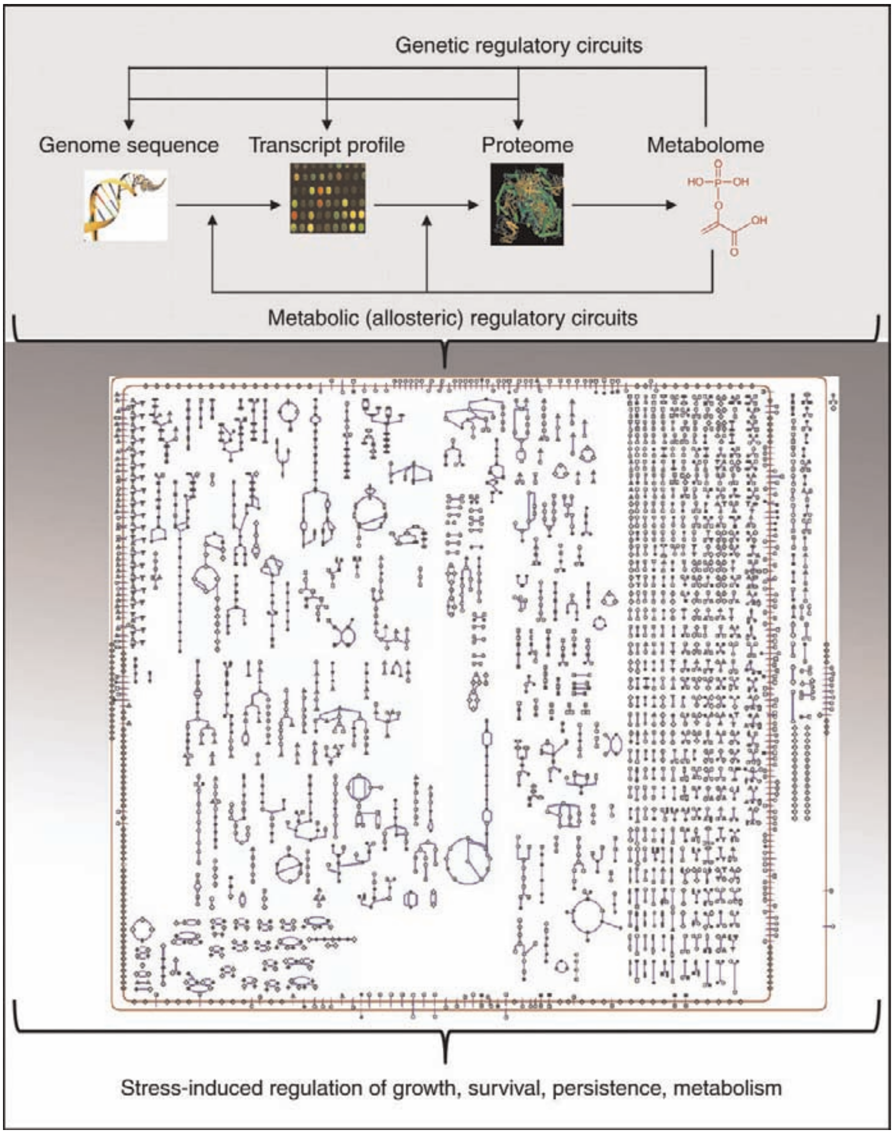
Genetic regulatory circuits
Genome sequence
Transcript profile
Proteome
Metabolome
HO–P–OH
OH
O
Metabolic (allosteric) regulatory circuits
Stress-induced regulation of growth, survival, persistence, metabolism

Figure 1 Schematic representation of systems biology of stress response – genome to phenotype via metabolism and gene regulation. The wire diagram represents the metabolic reconstruction map of *Lactococcus lactis* ssp. *cremoris* SK11 using the genome sequence from Pro Cyc.

RNAs can also be examined using gene chips with intergenic spaces included so as to define the genetic regulation induced by stress. Based on genome sequences, the molecular-level study of bacterial stress became possible at a scale not previously used – the entire genomic system. Use of gene expression arrays gained ground with each bacterial genome sequenced. DNA and RNA extraction limitations were overcome, which led to new breaths of information on the role of bacterial genes in cheese manufacture.

Proteomic analysis provides a view of the most dominant proteins expressed by the cell at any point in time. However, proteins produced at lower concentrations are not normally observable unless additional isolation measures are taken to find specific classes or types of proteins in the mixture. This is also true for community proteomics. Without a genome with which to compare the specific protein sequence, it is difficult to distinguish individual proteins originating from an individual in a community, such as fermenting cheese, making it difficult to distinguish which community member contributes which specific attributes. Two-dimensional gel electrophoresis and mass spectrometry can be used with pure cultures, but is less useful within the cheese matrix due to the relatively low concentration of bacterial proteins as compared to the casein matrix. This limits the direct study of stress response in the cheese matrix using proteomics, but provides an opportunity to measure the proteolytic changes of caseins by various microbes.

Metabolite study is well advanced in dairy products. Many studies have created metabolic profiles of chemicals found in dairy products with the goal of flavor identification. Currently, metabolomic profiling is marching forward to provide 50–2000 molecular profiles simultaneously. Use of these lists with the genetic potential of the organism brings out new insights into the metabolic actuation due to growth and stress directly in the product. The combination of metabolic reconstruction maps derived from the genomes, gene expression profiles and metabolite analysis is a powerful tool for understanding stress response in the entire organism. This approach is possible with genomic resources and the widely available high-throughput analytical biochemistry and bioinformatics-based tools.

Genomic Evolution and Stress

Genomic drift and evolution is occurring rapidly in LAB. Lactobacilli dominate the LAB genome sequences, being represented by four organisms, thus surpassing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, and a mixed phenotype, largely due to the emerging realization that lactobacilli are commonly associated with humans. Lactococci are also found on human and animal skin, as well as on plants, demonstrating their survival capacity. Their adaptation to milk fermentation is linked directly to lactose utilization and casein proteolytic capability in combination with their ability to survive stress.

Lactococcus has chromosomal and plasmid genes to mediate stress. The presence of dairy-associated genes for lactose utilization, citrate metabolism, and proteolysis is unmistakable evidence of the importance of extrachromosomal DNA in stress response and metabolism. These functions have a direct role as the cornerstone in dairy fermentations. The involvement of plasmids in lactococcal genomic evolution complicates the understanding of the stress response, because strains share plasmid DNA, making strain variation a substantial confounding factor in the delineation of stress response. It also complicates the strain differences and genomic evolution of LAB. A classic example of stress-induced changes is that of arginine (Arg) metabolism via the Arg deiminase pathway. Phage derivation results in the isolation of strains with varying Arg utilization, which often reverts to the wild-type phenotype with multiple passages. This is one of the critical differentiating traits between *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. This metabolic difference is associated with gene regulation and single nucleotide polymorphism (SNP) accumulation in specific genes or in the regulatory regions in the intergenic spaces between coding sequences during biotic stress. It also changes the ability of LAB to survive in cheese since it changes pH and ATP production.

The large number of mobile elements (e.g., prophage, phage, insertion sequence (IS) elements, transposase, integrase, resolvase) in LAB genomes leads to questions about the role of stress in genome drift and DNA exchange. For example, *Lc. lactis* subsp. *cremoris* SK11 contains more than 350 mobile elements between the chromosome and the plasmid pool. The five plasmids in this genome contain IS elements and conjugative elements that allow chromosomal integration and mobilization surrounding stress-associated and metabolic genes. Industrial use of plasmids to confer phage resistance is common and leads to new stress responses. For example, four different lactococcal transporter genes encoded on different plasmids were found in the genome sequence of *Lactobacillus helveticus* DPC 4571. To examine this among the sequenced LAB, a phylogenomic grouping based on stress functions was done to find stress response functions that differentiate closely related strains (**Figure 2**). For example, the lactococcal genomes lacking plasmids were grouped together, but closely to the strain *Lc. lactis* subsp. *cremoris* SK11, which contains five plasmids. Interestingly, the proteolytic operon (*opp*) is located on the genome of *Lc. lactis* subsp. *lactis* IL1403, but mutations in the upstream regulatory region prohibit expression in normal and stress conditions. This perspective of the relationship between stress response and the genome plasticity may be important to predict common and differential responses to stress based on common gene sets but

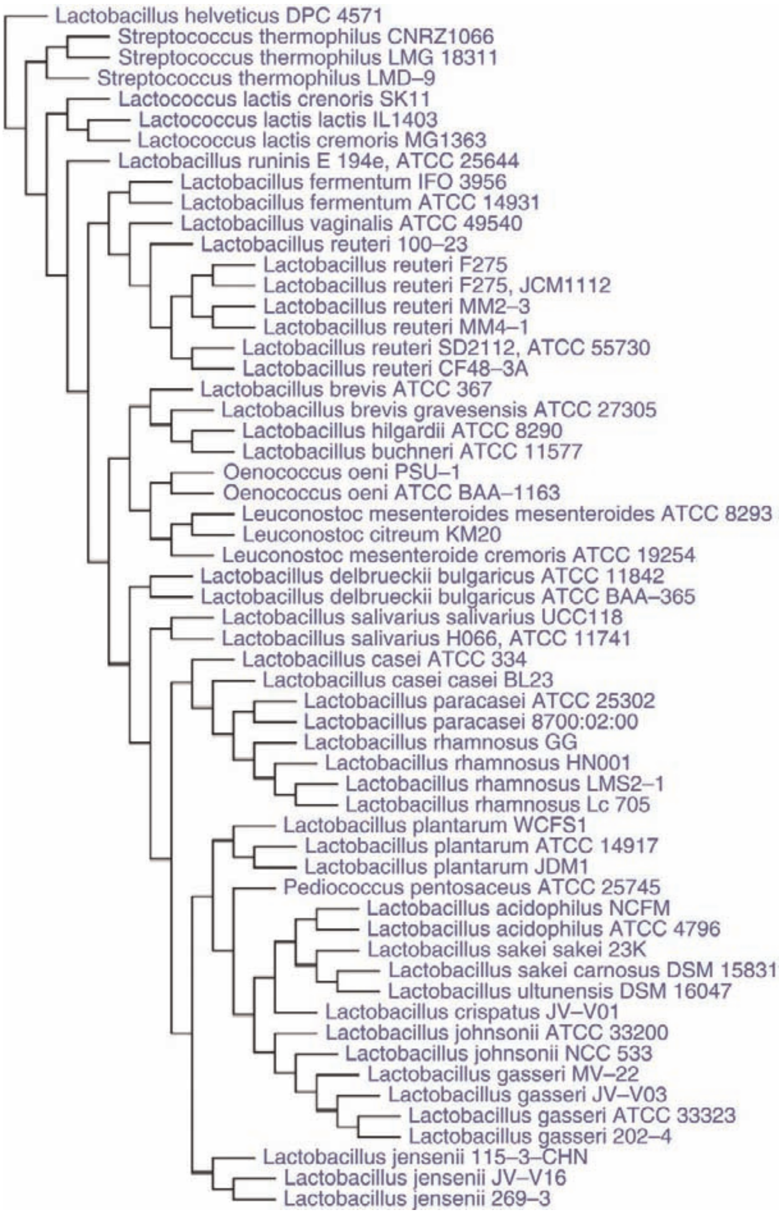
Lactobacillus helveticus DPC 4571
Streptococcus thermophilus CNRZ1066
Streptococcus thermophilus LMG 18311
Streptococcus thermophilus LMD–9
Lactococcus lactis crenoris SK11
Lactococcus lactis lactis IL1403
Lactococcus lactis cremoris MG1363
Lactobacillus runinis E 194e, ATCC 25644
Lactobacillus fermentum IFO 3956
Lactobacillus fermentum ATCC 14931
Lactobacillus vaginalis ATCC 49540
Lactobacillus reuteri 100–23
Lactobacillus reuteri F275
Lactobacillus reuteri F275, JCM1112
Lactobacillus reuteri MM2–3
Lactobacillus reuteri MM4–1
Lactobacillus reuteri SD2112, ATCC 55730
Lactobacillus reuteri CF48–3A
Lactobacillus brevis ATCC 367
Lactobacillus brevis gravesensis ATCC 27305
Lactobacillus hilgardii ATCC 8290
Lactobacillus buchneri ATCC 11577
Oenococcus oeni PSU–1
Oenococcus oeni ATCC BAA–1163
Leuconostoc mesenteroides mesenteroides ATCC 8293
Leuconostoc citreum KM20
Leuconostoc mesenteroide cremoris ATCC 19254
Lactobacillus delbrueckii bulgaricus ATCC 11842
Lactobacillus delbrueckii bulgaricus ATCC BAA–365
Lactobacillus salivarius salivarius UCC118
Lactobacillus salivarius H066, ATCC 11741
Lactobacillus casei ATCC 334
Lactobacillus casei casei BL23
Lactobacillus paracasei ATCC 25302
Lactobacillus paracasei 8700:02:00
Lactobacillus rhamnosus GG
Lactobacillus rhamnosus HN001
Lactobacillus rhamnosus LMS2–1
Lactobacillus rhamnosus Lc 705
Lactobacillus plantarum WCFS1
Lactobacillus plantarum ATCC 14917
Lactobacillus plantarum JDM1
Pediococcus pentosaceus ATCC 25745
Lactobacillus acidophilus NCFM
Lactobacillus acidophilus ATCC 4796
Lactobacillus sakei sakei 23K
Lactobacillus sakei carnosus DSM 15831
Lactobacillus ultunensis DSM 16047
Lactobacillus crispatus JV–V01
Lactobacillus johnsonii ATCC 33200
Lactobacillus johnsonii NCC 533
Lactobacillus gasseri MV–22
Lactobacillus gasseri JV–V03
Lactobacillus gasseri ATCC 33323
Lactobacillus gasseri 202–4
Lactobacillus jensenii 115–3–CHN
Lactobacillus jensenii JV–V16
Lactobacillus jensenii 269–3

Figure 2 Phylogenomic relatedness of LAB using complete genome sequence based on stress-related gene functions. The tree was created using IMG.

different regulatory elements. This is yet to be confirmed in comparative experiments in the laboratory, but seems to support antidotal strain selection strategies.

Genomic evolution due to stress and IS elements is likely. However, the genome structure is very highly conserved within lactococci and *Streptococcus thermophilus* (**Figure 3**). Lactococci have genome content modification and organizational inversion. In all cases, inversions are flanked by mobile elements. Additional genome sequences will provide increasing amounts of data to prove the genomic content and organization. In the absence of sequences, comparative genomic hybridization is a tool available for comparing stress gene distribution between different strains.

Bioinformatic comparison of the finished genomes for genes associated with stress reveals that each organism has similar gene contents for five classes of stress proteins. Interestingly, lactococci have more cold stress genes than lactobacilli, while *Lactobacillus plantarum* and *Lactobacillus casei* have more heat stress proteins than lactococci (**Table 1**). This analysis suggests that genome mutability is mediated by modification of the cytoskeleton, and cold shock and heat shock responses. With advanced comparative genomics and experimental evidence, the

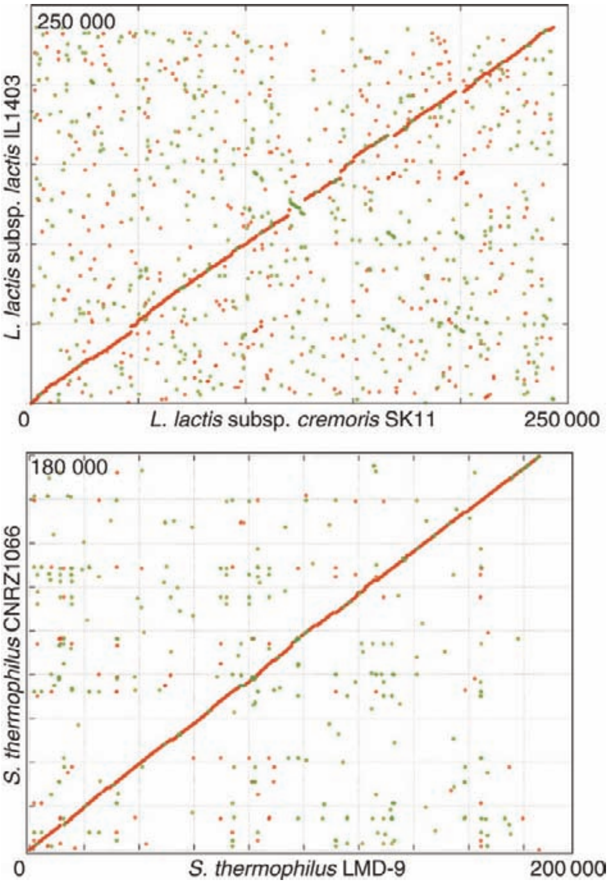

Figure 3 Mummer plots of the genomes demonstrating the genomic relatedness between strains of lactococci and streptococci. Spots forming the diagonal line indicate a consistent structural alignment between the two strains. Spots off the diagonal line and those that create a perpendicular line indicate missing homologues and genome inversions, respectively.

role of stress is likely to be directly linked to genomic modification due to stress.

Stress Conditions in Cheese

Fermented dairy products provide abiotic stress conditions that induce gene expression changes in LAB. The cell response changes between short-term and prolonged exposure to stress. For example, cooking temperatures used for hard cheese last for relatively short periods of time as compared to acid, cold, and salt stresses during ripening, which last for months rather than minutes. Nutrient stress is often produced with a slow utilization of the substrate allowing adequate time for gene regulation to bring about new capabilities. Oxidative and osmotic stress can be brought on quickly, but usually last for long periods. Gene regulation induced by stress directly influences the fermentation process by changing bacterial survival, metabolism, and flavor compound generation.

Over 145 regulatory genes are predicted in bioinformatic analysis of LAB genome sequences. Of those, over half are yet to be characterized. One of the most common stress response regulators is the universal stress protein UspA. The copy number of this protein ranges from 11 in *Lb. plantarum* JDM1 to 2 in *Lb. acidophilus* NCFM. LAB genomes were thought to be devoid of sigma factors, which control gene expression in many near phylogenetic neighbors. However, upon genome sequencing of many LAB, at least six DNA-directed regulatory genes (*rpoABCDE*, *ytgE*, *com*, and *sigX*) were found in many genomes. LAB still lack a full repertoire of *rpo* regulatory elements as compared to other microbes, which still leaves one wondering about the exact methods by which LAB control gene expression and modulate response to their environment using these regulatory elements. However, there are many regulators that fulfill additional roles not directly linked to *rpo* regulatory elements. For example, *codY*, *codZ*, and *ccpA* are pleiotropic transcriptional repressors found in lactococci that regulate the metabolic switch between sugar and protein utilization and mediate the stringent response. Induction of *codY* is complex and occurs under various stress conditions, including nutrient and pH stress. This protein plays a central role in lactococci in inducing the stringent response via amino acid starvation and is allosterically regulated by branched-chain amino acids interacting with the protein to control peptidase production and induction of new amino acid transporters, while switching between two important proteolytic systems in lactococci (*opp* to *dtp*). Often cold, nutrient, and pH stresses also induce the stringent response. The following sections provide a brief overview of the genes associated with specific stresses important in dairy products. In many cases, relatively few regulatory genes lead to extensive physiological changes in LAB. Each section will focus on the needs for survival under a specific stress and how it is regulated. It is not intended to be an exhaustive list of all changes associated with each stress.

Temperature Stress

Temperature treatments are very common in dairy processing. Thermal stress is one of the first stresses to which LAB are subjected to during milk processing. Since NSLAB are common in cheese, a few studies have defined the bacterial response needed to survive. Cell count reduction occurs, but there tends to be an increase in stationary phase cells. This is also true for many other food-associated spoilage and pathogenic bacteria. Since the heat treatment is short, most studies focus on the heat shock response.

Table 1 Stress gene comparison between LAB genomes. Numbers indicate the number of genes contained in each organism within the stress category. Only genomes with a finished sequence were included in the analysis. The analysis was done using IMG available at the Joint Genome Institute web site

LAB genome	*DNA translocase FtsK (ITERM:00138)*	*Sigma 54 modulation protein (ITERM:02225)*	*Cold shock DNA-binding protein family (ITERM:02239)*	*MEROPS family M50B metallopeptidase (ITERM:03736)*	*Heat shock protein Hsp20 (ITERM:05062)*
Lactobacillus acidophilus NCFM	1	1	1	1	1
Lactobacillus brevis ATCC367	0	1	2	1	1
Lactobacillus casei ATCC334	0	1	3	1	2
Lactobacillus casei subsp. *casei* BL23	0	0	3	1	2
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC11842	1	1	2	1	1
Lactobacillus delbrueckii subsp. *bulgaricus* ATCCBAA-365	1	1	2	1	1
Lactobacillus fermentum IFO3956	0	0	1	1	1
Lactobacillus gasseri ATCC33323	1	1	1	1	1
Lactobacillus helveticus DPC4571	1	1	2	1	1
Lactobacillus johnsonii ATCC33200	1	1	1	1	1
Lactobacillus johnsonii NCC533	1	1	1	1	1
Lactobacillus plantarum JDM1	0	1	3	1	3
Lactobacillus plantarum WCFS1	0	1	3	1	3
Lactobacillus reuteri F2750	1	2	1	1	
Lactobacillus reuteri F275, JCM1112	0	0	2	1	0
Lactobacillus rhamnosus GG	0	0	0	1	0
Lactobacillus rhamnosus Lc705	0	0	0	1	0
Lactobacillus sakei subsp. *sakei* 23K	0	1	4	1	1
Lactobacillus salivarius subsp. *salivarius* UCC118	0	1	1	1	0
Lactococcus lactis subsp. *cremoris* MG1363	1	1	6	1	0

(*Continued*)

Table 1 (*Continued*)

LAB genome	*DNA translocase FtsK (ITERM:00138)*	*Sigma 54 modulation protein (ITERM:02225)*	*Cold shock DNA-binding protein family (ITERM:02239)*	*MEROPS family M50B metallopeptidase (ITERM:03736)*	*Heat shock protein Hsp20 (ITERM:05062)*
Lactococcus lactis subsp. *cremoris* SK11	1	1	5	1	0
Lactococcus lactis subsp. *lactis* IL1403	1	1	2	1	0
Leuconostoc citreum KM20	0	1	2	1	0
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC8293	0	1	2	1	1
Oenococcus oeni PSU-1	0	1	1	1	1
Pediococcus pentosaceus ATCC25745	0	1	2	1	1
Streptococcus thermophilus CNRZ1066	0	1	2	1	0
Streptococcus thermophilus LMD-9	0	1	2	1	1
Streptococcus thermophilus LMG18311	0	1	2	1	0

Heat shock has been studied extensively in many bacteria, including LAB, especially lactococci and lactobacilli. The phase of growth and the presence of other abiotic stress conditions change the cells' response to heat. Heat response is classified based on the regulatory motif. Class I genes are controlled by HrcA, class II by Rpo proteins, and class III by CtsR, and class IV genes are those of undefined regulation. LAB have all of these classes, with some better defined than others.

In many bacteria, thermal stress results in the establishment of the stringent response, which often leads to the production of proteins that also protect the cell against other abiotic stresses as well. This is observed by an increase in resistance to other stresses that cause cell death. Initial regulation of specific response genes often leads to additional gene products that mitigate additional damage. One example of this is the subsequent induction of *relA*, which initiates recombination if DNA damage is detected with single-strand replacement or rearrangement. Lactococci are particularly susceptible to *relA* changes in DNA structure due to the many stresses they encounter during processing, but also because some strains have mutations in another repair system that replaces base mismatches (*mutLS*). Many other stresses also cause entry into resistance pathways. Preadaptation with a short sublethal temperature stress or another stress often leads to an increased resistance to temperature stress in LAB. This observation is more easily understood once one realizes that sets of common genes are inducted by stress in LAB.

The stress-related genes are highly conserved in LAB. Of the over 150 (COG K) response regulators, more than 20 are dedicated to stress response regulation that commonly leads to the induction of *dnaJ*, *grpE*, *dnaK* (aka *hsp70*), and *groELS* – the core set of chaperone proteins in many bacteria, but especially in LAB. The complements of chaperones and proteases are commonly used to tag and degrade misfolded or denatured protein by refolding or degradation, respectively. In addition, proteases (*clp*) are also commonly induced to degrade denatured proteins. A relatively newly discovered gene in *Lc. lactis* subsp. *cremoris* MG1363 – *hslO* (*hsp33*; aka *yudG* in *Lc. lactis* subsp. *cremoris* SK11 and *Lc. lactis* subsp. *lactis* IL1403) – is a disulfide bond chaperone heat shock protein. Interestingly, lactococci and *Lactobacillus salivarius* subsp. *salivarius* UCC118 lack the small heat shock protein Hsp20, while other LAB contain between one and three copies of this gene (**Table 1**).

Short exposure to heat stress (e.g., 15–30 min) induces expression of a large number of genes. Many of the stress proteins and response regulators have multiple functions. For example, *dnaK* (*hsp70*) also has a role in DNA repair as part of the SOS response to repair DNA damage and SNPs via DNA binding and base replacement, allowing ribosome and transcription factor binding to progress. The gene induction is transient, with relief from the stress-induced expression occurring within 15–45 min.

Cold stress is mediated by the *csp* genes along with *dnaK*. The csp cluster is complex and varies between strains of lactococci. The strain *Lc. lactis* subsp. *cremoris* MG1363 has six genes – *cspA*, *B*, *C*, *D*, D_2, and *E* – while *Lc. lactis* subsp. *cremoris* SK11 and *Lc. lactis* subsp. *lactis* Il1403 have only *cspD* and *E*. It is likely that *cspE* is the most important gene in cold shock regulation. Lactobacilli contain between one and four cold shock genes. Generally, *csp* regulates the cytoplasmic membrane composition, DNA maintenance, RNA stability, and the stringent response via the stress-modified ribosome components, and (p)ppGpp levels. In lactococci, the stringent response is also regulated via *codY* in cooperation with *relA*, which produces (p)ppGpp. Increasing amounts of (p)ppGpp lead to increasing levels of cold stress resistance and an increase in the magnitude of the response. The genes needed for cold stress response are widely conserved in bacteria.

pH Stress

Acid stress and cellular damage are particularly important for dairy products and LAB. The influence of pH stress is widely characterized in bacteria. Since LAB produce large amounts of lactic acid to reduce the pH to around 4.0 for lactobacilli and around 4.7 for lactococci, response to acid stress is predictable; damage is mitigated by a series of metabolic changes to maintain a constant intracellular pH. Commercial production of lactococci usually involves addition of a base to neutralize the acid produced. This avoids acid-induced damage and maintains higher viability before adding the product to a dairy fermentation.

Preexposure to a sublethal level of acid induces bacterial survival in lactococci and lactobacilli, as well as in many other microbes. In lactococci, preexposure to acid improves the survival to almost 100% for a subsequent exposure to a higher level of acid for an extended time. The transcriptional regulator *ahrC*, which is a regulator of Arg metabolism via the Arg deiminase pathway, is induced by acid shock and stress. This leads to Arg catabolism to produce ammonia and carbon dioxide along with ATP. The intracellular and the extracellular pH are regulated to be differential, as they do not need to be the same. Many transporters export H^+, but the F_0F_1 ATPase is unique to Gram-positive bacteria, including LAB. In addition, amino acid transporters are induced. Acid stress also induces the stringent response via ribose metabolism (*deoB*), hypoxanthine and guanine metabolism (*hpt*, *guaA*), and *relA*. Along with Arg metabolism via carbamyl phosphate, this cluster of genes brings in the role of nucleoside metabolism as well as being one of a few reactions using substrate-level phosphorylation to produce energy. This is linked to phosphate transport (*pstS*, *B*) as well. Decarboxylation reactions (e.g., *gadCB*) are also induced by acid stress. With the induction of the stringent response, *codY* is also involved, which leads to the induction of new amino acid transport proteins and repression of the *opp* operon coupled to the induction of *dfp*. These mechanisms are also involved in the transition to stationary phase, which leads to increased resistance to acid stress.

Sugar Starvation

Sugar starvation is common in fermented dairy products that require aging. Lactose in hard cheese is depleted within 30 days of ripening. Consequently, bacteria in the curd, to survive and produce flavor compounds, must find an alternative carbon source. In cheese, the protein matrix supplies the needed substrate. The shift from using sugar to using protein and amino acids as the carbon source is mediated by a set of regulatory elements. One regulatory gene, *cstA*, is present in lactococci, but not in lactobacilli. In addition, the *ccpA* regulator that binds to the *cis*-regulatory element (CRE) is also important in carbon starvation response. Conservatively, there are approximately 1000 binding regions in LAB for this regulator if 1–2 mismatches are allowed. The CRE box regulates sugar catabolism broadly – ribose, mannitol, galactose, maltose, gluconate – and the genes needed to degrade complex carbohydrates (α- and β-glucosidases). This regulatory pair also regulates citrate metabolism. Amino acid catabolism, and nucleic acid (stringent response) and peptidases production are also regulated during carbon starvation. This indicates that *ccpA*/CRE is key to survival of LAB during carbon starvation. Recently, this regulator was also implicated in the transition to stationary phase and further to a nonculturable state in lactococci: cells lose their ability to form colonies on agar, but remain metabolically active, by repression of *ftsZ*, which initiates cell division by forming the Z-ring. During this transition, Arg and branched-chain amino acid catabolism increase, while sugar transport via the phosphotransferase system (PTS) is repressed due to the depletion of phosphoenolpyruvate (PEP) and repression of HPr. This physiological state leads to the induction of new metabolic capabilities to produce branched-chain fatty acids from branched-chain amino acids, which is produced only during carbon starvation in lactococci. Again, *codY* plays a role in combination with *ccpA* in inducing new metabolic capabilities in LAB via the stringent response; this is a good example of the importance of the stringent response and redundant systems in improving persistence during stress.

Oxidative Stress

Oxidation in LAB is common during dairy processing. Pumping of milk infuses oxygen into the milk, which is ultimately reduced due to LAB metabolism. However, addition of heme to the medium, which will be present in milk, leads lactococci to respire. A newly found regulator *ythA* is yet to be characterized for its possible role in oxidative stress response. The induction of this state is linked to *ccpA* and *relA*, which leads to increased survival in high oxygen-containing media. Menaquinones (MK-8 to MK-10) are produced by lactococci during growth. Transition to MK-3 and dimethylMK-3 occurs during the transition to stationary phase growth.

Addition of hydrogen peroxide is common in some raw milk cheeses. This leads to oxidative stress due to reactive oxygen species and nitrous oxide, which links it to nitrogen metabolism using superoxide dismutase (*sodA*). The OxyR regulon and *relA* also play a role in oxidative stress response in LAB. The protease *ftsH* is also important in oxidative stress response, but its exact role is not yet defined in LAB. In *Lb. plantarum*, FtsH has a dual role of protease and chaperone, and is involved in protein turnover during oxidative and heat stress. The role of thioredoxin (*trxB*$_{1,2}$) in oxidative stress, to mitigate the stress from reactive oxygen, is yet to be fully determined in LAB.

Osmotic Stress

During cheese production addition of salt is common. In hard cheeses, NaCl is usually added up to a concentration of approximately 4.5–5.0% of the moisture phase, meaning that LAB must be resistant to such concentrations of Na^+. LAB have limited ability to produce compatible solutes, which leads the cells to induce a betaine–glycine transporter (*busAA*, *AB*). Osmotic stress induces nucleotide salvage, and the biosynthesis of glutamate, lysine, and peptidoglycan, but represses genes associated with fatty acid production for inclusion in the cell membrane. The heat shock gene, *dnaK*, is induced, as is the stringent response. The role of the mechanoreceptor *mscL* is still unclear, but it is also thought to be important to osmotic stress response. The regulator *osmC* is also induced by osmotic stress. Characterization of the stress and metabolic changes due to sequential and combined stress is needed to fully understand the impact of stress in LAB, yet this is a very difficult task without the '-omics' tools (**Figure 4**). This important stress is yet to be fully characterized for the survival of and metabolic changes in LAB.

Conclusions

LAB contain multiple genetic systems to handle the stress conditions in dairy processing. As more genome sequences are characterized, additional systems and

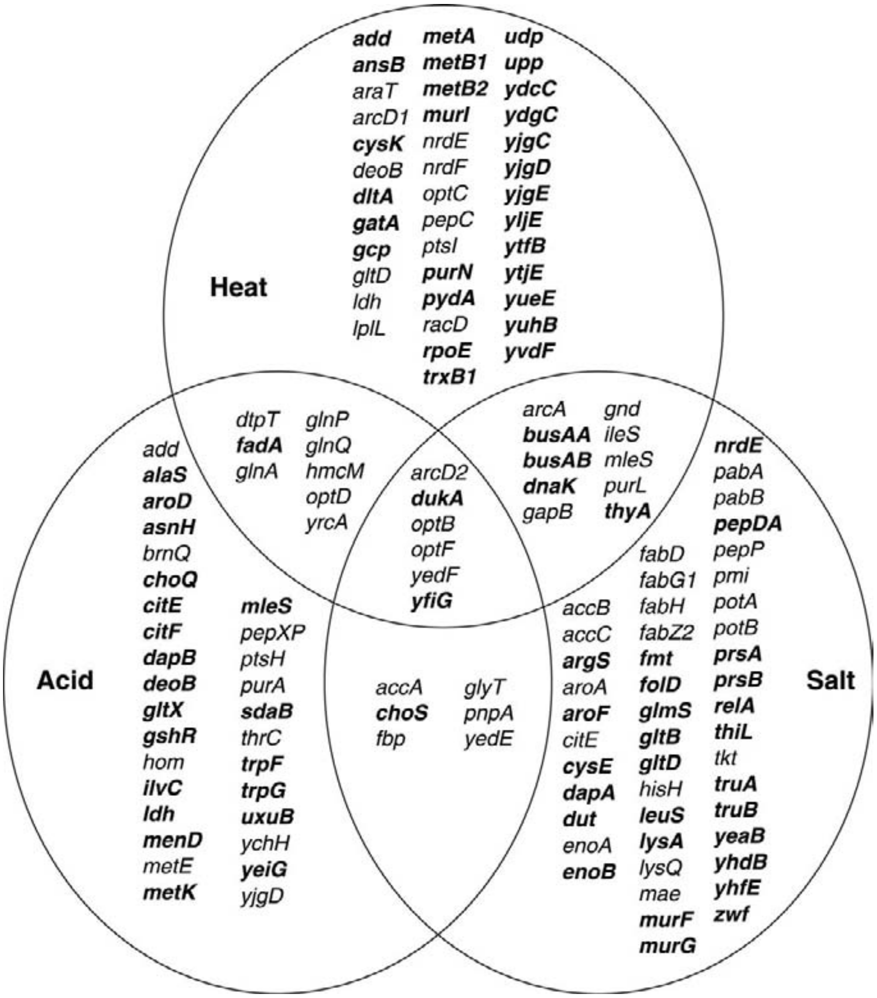
Heat
add **ansB** araT arcD1 **cysK** deoB **dltA** **gatA** **gcp** gltD ldh lplL
metA **metB1** **metB2** **murI** nrdE nrdF optC pepC ptsI **purN** **pydA** racD **rpoE** **trxB1**
udp **upp** **ydcC** **ydgC** **yjgC** **yjgD** **yjgE** **yljE** **ytfB** **ytjE** **yueE** **yuhB** **yvdF**
dtpT **fadA** glnA
glnP glnQ hmcM optD yrcA
arcD2 **dukA** optB optF yedF **yfiG**
arcA **busAA** **busAB** **dnaK** gapB
gnd ileS mleS purL **thyA**
Acid
add **alaS** **aroD** **asnH** brnQ **choQ** **citE** **citF** **dapB** **deoB** **gltX** **gshR** hom **ilvC** **ldh** **menD** metE **metK**
mleS pepXP ptsH purA **sdaB** thrC **trpF** **trpG** **uxuB** ychH **yeiG** yjgD
accA **choS** fbp
glyT pnpA yedE
Salt
accB accC **argS** aroA **aroF** citE **cysE** **dapA** **dut** enoA **enoB**
fabD fabG1 fabH fabZ2 **fmt** **folD** **glmS** **gltB** **gltD** hisH **leuS** **lysA** lysQ mae **murF** **murG**
nrdE pabA pabB **pepDA** pepP pmi potA potB **prsA** **prsB** **relA** **thiL** tkt **truA** **truB** **yeaB** **yhdB** **yhfE** **zwf**

Figure 4 Genes regulated by three stress treatments in lactococci identified using gene expression profiling. Bold lettering indicates induction and normal font indicates repression.

their regulation are being uncovered. Overlapping stress responses provide protection against multiple stress conditions. Common regulatory elements lead to multiple cellular, metabolic, and genotypic changes. The stringent response along with the SOS response seems to be common among LAB. The use of *codY* seems to be unique to lactococci. The large number of mobile elements and the induction of genes associated with stress suggest that abiotic stress treatments used in dairy processing promote genome mutability. This is supported by the genetic variation between strains, which include specific changes in genetic mutation and genome rearrangement in isolates that lead to phenotypic variation. Such variations bring substantial complexity and challenges to understanding the entire stress response of LAB.

See also: **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview. **Cheese**: Biochemistry of Cheese Ripening; Starter Cultures: General Aspects; Starter Cultures: Specific Properties. **Fermented Milks**: Starter Cultures. **Flavors and Off-Flavors in Dairy Foods**. **Lactic Acid Bacteria**: Genomics, Genetic Engineering.

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Genomics, Genetic Engineering

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Introduction

The lactic acid bacteria (LAB) encompass a group of phylogenetically related genera that are obligatory fermentative and produce lactic acid as the major end product. Sometimes, the genus *Bifidobacterium* has been included in the LAB, as they also are obligatory fermentative and produce lactic acid as the major end product. While they do share many phenotypic characteristics, it is important to note that they are phylogenetically distinct and are taxonomically part of the *Actinobacteria* (**Figure 1**). Given their many phenotypic similarities to the LAB and their common role as probiotic cultures of particular interest to the dairy fermentation industry, they will be included in this discussion of the genomics and genetic engineering of the LAB.

Lactococcus and *Lactobacillus*

The two best known and most studied genera of the LAB are *Lactococcus* and *Lactobacillus*. Members of both these genera are vital to the food industry, with *Lactococcus lactis* the undisputed workhorse of the cheese production industry and different species of *Lactobacillus* essential to numerous fermented foods, such as yogurts, some cheeses, fermented milks (kefir, koumiss, etc.), fermented vegetables (sauerkraut, pickles, olives, etc.), fermented cereals (soy sauce, sourdough, etc.), and fermented meats (salami, summer sausage, etc.). Other genera of LAB that are important for the food fermentation industry include *Pediococcus*, *Leuconostoc*, *Carnobacterium*, and *Oenococcus*. *Streptococcus thermophilus* is the only member of the streptococci with an important role in food production.

Probiotics

In addition to applications in the food fermentation industry, the LAB also have an important role in probiotics, which is the ingestion of live microorganisms for the improvement of intestinal and overall health. While the ecosystem of the gastrointestinal (GI) tract is extremely complex, certain species of *Lactobacillus* are believed to be important for maintaining a healthy gut, especially in the small intestine and the vaginal cavity. Some species of *Bifidobacterium* are believed to be helpful in the large intestine, in part by modulating the dominance of other less desirable microbial groups, and are therefore very popular probiotic cultures. Given the commercial significance of the LAB and bifidobacteria, it is not surprising that they have attracted major research interest, and figure prominently in the areas of genomics and genetic engineering.

Genetic Analysis of the LAB

Genetic studies of the LAB were initiated in earnest following the discovery of plasmids in the 1950s, with the term being proposed by the Lederberg laboratory in 1952. It was subsequently found that *Lc. lactis* harbored numerous plasmids, and many phenotypes important for this bacterium to ferment lactose and grow in milk were eventually linked to plasmids. This explained the relative instability of these traits in this bacterium, which was a major problem considering their important role in cheese production. Over the past 40 years, numerous LAB plasmids have been characterized and many have been converted into cloning and expression vectors for the genetic analysis and engineering of this group of bacteria. **Table 1** lists representative cloning vectors that were constructed from plasmids originating in the different LAB. Vectors derived from one of the LAB genera frequently replicate in other LAB, given the genetic similarity of the group. However, LAB vectors do not replicate in bifidobacteria, with the reverse also being true, reflecting the separate genetic lineage of these bacteria.

Gene Transfer in the LAB

The genetic manipulation of any organism requires effective gene transfer into the organism of interest. While some bacteria, such as many members of the genera *Streptococcus* and *Bacillus*, readily take up DNA using competence proteins, the LAB were not as fortunate and transformation was very difficult. This was improved with the development of protoplast transformation in 1982 in Larry McKay's laboratory at the University of Minnesota. Prior to this, transduction was found to be more useful in the study of the LAB. All these methodologies were eventually superseded by the advent of electroporation, whereby electrically induced pores enable the uptake of DNA into the cell. Today, electroporation procedures for the transfer

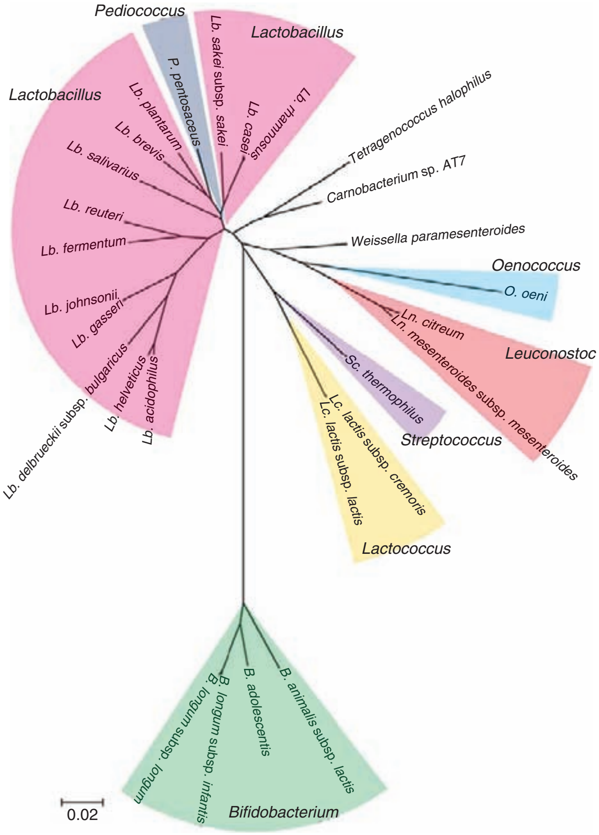
Pediococcus
Lactobacillus
Lactobacillus
P. pentosaceus
Lb. sakei subsp. sakei
Lb. casei
Lb. rhamnosus
Lb. plantarum
Lb. brevis
Lb. salivarius
Lb. reuteri
Lb. fermentum
Lb. johnsonii
Lb. gasseri
Lb. delbrueckii subsp. bulgaricus
Lb. helveticus
Lb. acidophilus
Tetragenococcus halophilus
Carnobacterium sp. AT7
Weissella paramesenteroides
Oenococcus
O. oeni
Ln. citreum
Ln. mesenteroides subsp. mesenteroides
Leuconostoc
Sc. thermophilus
Streptococcus
Lc. lactis subsp. cremoris
Lc. lactis subsp. lactis
Lactococcus
B. longum subsp. longum
B. longum subsp. infantis
B. adolescentis
B. animalis subsp. lactis
Bifidobacterium
0.02

Figure 1 Phylogenetic tree based on the 16S rRNA gene sequence, illustrating the genetic clustering of the LAB and the genetic divergence of bifidobacteria.

of DNA into all the major LAB and bifidobacteria have been developed.

Conjugation was first described for the LAB in 1979 with the transfer of plasmids from different strains of *Lc. lactis*. This enabled specific traits present in one strain to be transferred to another strain using a completely natural process. Given that many commercially important traits in *Lc. lactis* are encoded on plasmids, this provided a means of genetically engineering selected strains of this species with traits of interest. Examples of traits encoded on plasmids in *Lc. lactis* include lactose uptake and metabolism, protease and peptidase production, peptide uptake, bacteriophage resistance (including restriction modification systems, abortive infection systems, absorption resistance systems, and injection blocking systems), heavy metal resistance, bacteriocin production, and citrate metabolism. While the conjugal transfer of plasmids from one bacterium to another is a form of genetic engineering, it does not fall under the legal umbrella of 'genetically modified foods' (often called GM foods) as the process is natural and does not involve modern biotechnological approaches. Given the sensitivity of GM issues in the food industry, particularly in Europe, the natural genetic engineering approaches have proven very useful.

Genetic Engineering Accomplishments of the LAB

While classical genetic engineering of the LAB has proven to be very useful commercially as discussed above, modern biotechnological techniques have also enabled important accomplishments. Paramount to these accomplishments was the development of specialized expression vectors for heterologous gene expression. **Table 2** lists expression vectors developed for the LAB, including vectors with secretory signals and ones designed to express proteins on the surface of the cell. The early genetic engineering accomplishments were mostly concentrated

Table 1 Representative cloning vectors constructed from different species of LAB and bifidobacteria

Vector	*Original host*	*Characteristics*	*Size (kb)*
pTRK159	*Lactobacillus acidophilus*	pPM4 replicon; Cm^{r},[a] Em^{r},[b] Tc^{r} [c]	10.3
pAZ20	*Lb. casei*	pNCDO151 replicon; Cm^{r}, Ap^{r} [d]	8.3
pJK355	*Lb. curvatus*	RCR[e]; pLC2 replicon; Cm^{r}	3.2
pDOJ4	*Lb. delbrueckii*	Theta replication; *lacZ*; Cm^{r}	13.3
pcaT	*Lb. plantarum*	pCAT replicon; Cm^{r}	8.5
pRV566	*Lb. sakei*	Theta replication; pRV500 replicon; Em^{r}, Ap^{r}	7.3
pGK12	*Lactococcus lactis*	RCR; pWV01 replicon; Cm^{r}, Em^{r}	4.4
pCI431	*Leuconostoc lactis*	RCR; pCI411 replicon; Cm^{r}	5.8
pFBYC051	*Ln. mesenteroides*	Theta replication; pTXL1 replicon; Ap^{r}, Em^{r}	5.6
pND913	*Streptococcus thermophilus*	RCR; pND103 replicon; Em^{r}, Ap^{r}	6.4
pUCB825	*Tetragenococcus halophila*	Theta replication; pUCL287 replicon; Em^{r}	6.9
pDOJHR	*Bifidobacterium longum*	Theta replication; p15A replicon; Cm^{r}	8.6

[a] Chloramphenicol resistance.
[b] Erythromycin resistance.
[c] Tetracycline resistance.
[d] Ampicillin resistance.
[e] Rolling circle replication.

Table 2 Expression vectors developed for heterologous gene expression in the LAB

Vector	*Target host*	*Characteristics*
Expression within cells		
pLNG1363, pUK500, pNZ9530, pNZ8008	*nisRK* containing hosts: *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*	*nisA* promoter; nisin induction
pMSP3535, pNZ9520	*Streptococcus*, *Lactococcus*	*nisA* promoter; *nisRK* genes in vector; nisin induction
pRBE4	*Lactobacillus*	*xylA* promoter; xylose induction
pNZ2119, pNZ2118	*Lactococcus*	*lac* promoter; lactose induction
pLEB604	*Lactobacillus*	*pepR* constitutive promoter
pTRK391	*Lactococcus*	Ø31 promoter; phage infection induction
pSOD4	*Lactococcus*	*sodA* promoter, aeration induction
pNZ544, pNZ554	*Lactococcus*	*prtP* and *prtM* promoters; low peptide induction
pLET1	*Lactococcus* harboring pIL227 (*lac* promoter controlling T7 RNA polymerase)	T7 promoter; lactose induction of T7 RNA polymerase
pTREX series	*Lactococcus*	pLET1 plus a broad-range replicon (pAMβ1)
pGM4	*Lactobacillus*	*sppIP* promoter; sakacin P incomplete operon (*sppKRTE*) in vector; pheromone peptide (IP-673) induction
pKRV3	*Lactobacillus*	*sapIP* promoter; sakacin A incomplete operon with mutated *sapIP* (*sapIP**-*sapKR*) in vector; inducing peptide (IP) induction
Expression and secretion		
pNZ8110	*Lactococcus* harboring *nisRK*	*nisA* promoter; nisin induction; signal leader segment of *usp45*
pL2MIL2	*Lactococcus* harboring pILPol (*lac* promoter controlling T7 RNA polymerase)	T7 promoter; signal leader segment of *usp45*
pNZ123	*Lactococcus*	*dnaJ* promoter; signal leader segment of *usp45*; high temperature inducible
pLET2	*Lactococcus* with pIL227 (*lac* promoter controlling T7 RNA polymerase)	T7 promoter; lactose induction of T7 RNA polymerase; signal leader segment of *usp45*
Expression and cell surface anchored		
pLET4	*Lactococcus* with pIL227 (*lacA* promoter controlling T7 RNA polymerase)	T7 promoter; lactose induction of T7 RNA polymerase; cell wall-associated motif of *prtP*; signal leader segment of *usp45*
pSVac	*Lactococcus*	T7 promoter; cell wall-binding motif of *acmA*

on *Lc. lactis* because of its economic significance and its amenability to genetic manipulation, compared to many of the other LAB. This was also facilitated by the construction of laboratory strains of *Lc. lactis* that were cured of their plasmids to allow genetic analysis of their plasmid-encoded traits.

Food-Grade Selection Markers

To introduce any vector into a cell, a selection marker is required for selection of cells that contain the vector. The most common selection markers are antibiotic resistance genes, as it readily facilitates the selection of the plasmid in a targeted host by incorporating the relevant antibiotic in the selection medium. While this is a user-friendly approach for selection of vectors in a host, it does not lend itself to food or environmental applications as the dissemination of antibiotic resistance genes can accelerate the evolution of antibiotic resistance in bacterial communities. Given the complexity of the microbial community in the GI tract, which includes many pathogenic types, it is clearly not a good idea. For this purpose, numerous food-grade selection markers have been evaluated for the successful selection of a plasmid in a target host. This has led to the development of food-grade cloning vectors. **Table 3** lists the different food-grade cloning vectors that have been developed for the LAB.

Metabolic Engineering

Metabolic engineering in the LAB also requires the ability to specifically inactivate genes of interest. Site-specific approaches for removing and/or replacing specific DNA

Table 3 Food-grade cloning vectors developed for use in the LAB

Food-grade vector	*Target host*	*Characteristics*
Using heterologous genes		
pLP3537-xyl	*Lactobacillus*, *Escherichia coli*	D-Xylose metabolism
pGIP series	*Lactobacillus*, *Enterococcus*	Starch metabolism
pLPEW1, pLPEW2	*Lactobacillus*, *E. coli*	Inulin metabolism
pTRK434	*Lactobacillus*	Lactacin F resistance
pRAF800	*Lactococcus*, *Pediococcus*	Melibiose metabolism
pLEB590	*Lactococcus*, *Lactobacillus*	Nisin immunity (*nisI*)
pVS40, pFM011, pFK012	*Lactococcus*, *Lactobacillus*	Nisin resistance (*nsr*)
pAH90	*Lactococcus*	Cadmium resistance (*cadA*)
pDBORO	*Lactococcus*	5-Fluoroorotate (pyrimidine analogue) sensitivity
pSMB74	*Pediococcus*	Pediocin resistance
pSt04, pHRM1	*Streptococcus*	Heat and acid resistance
pOC13	*Lactobacillus*, *Lactococcus*, *Leuconostoc*	Cellulose metabolism
Complementation-based markers		
pJAG5	*Lactococcus lactis* threonine mutants	Threonine complementation
pFG1	*Lc. lactis* purine mutants	Purine complementation
pFG200	*Lc. lactis* spp. *cremoris* with amber mutation in pyrimidine synthesis (*pyrF*)	Amber suppressor gene (*supD*)
pNZ7120	*Lactobacillus* and *Lactococcus* with alanine racemase (*alr*) gene mutation	D-Alanine complementation
pNZ2104, pNZ2105, pNZ2106, pNZ2107	*Lc. lactis* with chromosomal deletion of the *lacF* gene	Complementation of the *lacF* gene
pLEB600	*Lactobacillus casei* with chromosomal deletion of the *lacG* gene	Complementation of the *lacG* gene
pPR602	*Streptococcus thermophilus* thymidylate synthase (*thyA*) mutants	Complementation of the *thyA* gene
pFG1	*Lc. lactis* with nonsense codon in a purine biosynthetic gene	Ochre suppressor gene (*supB*)
pSt04	*Sc. thermophilus* lacking plasmid encoding *shsp* gene	Complementation of the small heat shock protein (*shsp*) gene
pVEC1	*Lc. lactis* harboring pCOM1 (*regB*-deficient vector with erythromycin resistance)	Complementation of *regB*

fragments have been developed for many of the LAB. These involve inserting DNA into a target region by using identical DNA to facilitate homologous recombination. This can enable the insertion of a new DNA fragment in a single crossover event and can also result in replacing the original DNA with a second crossover event. In practice, the frequency of these events is low, thus facilitating the development of strategies to improve it. The simplest approach is to use a nonreplicating plasmid, such that expression of the selective marker in the cell would require homologous recombination to occur as that is the only way the plasmid can exist in the cell. However, this requires good DNA transfer efficiency as the frequency of insertion is low. It has worked for certain targets in *Lc. lactis*, but is not very practical for all members of the LAB.

An improved approach is the use of conditional functioning plasmid replicons that enable the stable introduction of the plasmid into the cell, but when the plasmid-encoding cell is exposed to conditions that do not support its replication, the plasmid will be forced to undergo homologous replication. An example is heat-sensitive plasmid replicons, such as the pGhost family of vectors that are stable in *Lc. lactis* at normal growth temperatures, but are unstable at temperatures above 37 °C. These plasmids can be used for site-specific DNA integration by incorporating a specific homologous DNA target in the vector. They have also been used for random, nonhomologous integration by incorporating an insertion element (ISS1). In the latter case, the plasmid is forced to randomly integrate via the insertion sequence (IS) element when the growth temperature of the plasmid-containing host is increased above 37 °C.

Another approach that has been used is to supply the *repA* gene of a plasmid vector *in trans* on another vector that is heat sensitive and readily lost, thus facilitating the integration of the selection vector. An example is the pORI vectors, where the *repA* gene is on a helper heat-sensitive plasmid, pVE6007, thus enabling the stable introduction of the integrative pORI-based vector into the cell. When the cell is moved to the nonpermissive temperature, the helper plasmid is lost and the selection of the pORI vector forces its integration. The advantage of this dual-vector system over the single-plasmid pGhost system is that a more stable integration event occurs, as when the helper plasmid is lost there is no possibility of the pORI-based vector to excise from the chromosome and replicate in the cell, whereas in the pGhost case, the plasmid can excise at lower temperatures as its replication can function again.

A novel site-directed mutagenesis approach for different bacteria that has been developed and commercialized evolved from a group II intron that was found in a conjugative plasmid from *Lc. lactis*. Introns are regions within genes that are transcribed, but are excised from the mRNA prior to translation. They are characteristic of eukaryotic cells, but very rare in bacteria. By changing specific nucleotides in the intron and replacing them with nucleotides from another DNA site, they can move or 'home' to the new site. This 'rehoming' of this group II intron was perfected by comparing its insertion sites in *Escherichia coli* and can be developed to 'rehome' to specific sites in the LAB. While some successful integrants have been achieved in *Lc. lactis* using the 'rehoming' data from *E. coli*, currently there are no sufficient data on the preferred homing sequences in the LAB for effective use of this system.

Overproduction of flavor and aroma compounds

Engineering of the metabolic pathways can alter the amounts of the different fermentation end products, such that a desired product can be greatly increased. As the wild-type *Lc. lactis* cell channels nearly all the carbon from its fermentation substrates into lactic acid, simply inactivating the gene encoding lactate dehydrogenase prevents the conversion of pyruvate into lactic acid. This forces the cell to convert pyruvate into other end products, such as diacetyl, acetaldehyde, acetoin, and ethanol. Diacetyl is a very useful food ingredient as it is the major flavoring compound in buttermilk and adds a butter-like flavor to numerous foods. A diacetyl overproducing culture was obtained by inactivating the genes for lactate dehydrogenase and α-acetolactate decarboxylase. Another strategy to alter the amount of lactic acid produced in favor of other fermentation products is to express NADH oxidase in the cell. This reduces the need to regenerate all its NADH coenzyme by conversion of pyruvate to lactic acid. This was found to increase diacetyl and acetoin.

Heterologous Gene Expression

The development of an array of expression vectors for the LAB, as shown in **Tables 2** and **3**, has facilitated the production of novel metabolites by this versatile group of bacteria. These have both food and nonfood uses. For example, a surface-anchored α-amylase from *Streptococcus bovis* was expressed in *Lactobacillus casei*, creating a strain with significant soluble starch degradation abilities. Sorbitol, which is a low-calorie sweetener and a prebiotic, was produced by a strain of *Lb. plantarum* that was engineered to express sorbitol-6-phosphate dehydrogenase. An exciting application is the surface expression of antigens against known pathogens, such that their passage through the GI tract will trigger an immune response. This was tested with tetanus in a mouse model with some success. This is an area of tremendous interest as it would greatly facilitate the vaccination of large populations using an oral and cost-effective approach. Another exciting application is to utilize engineered strains of bifidobacteria to treat tumors. These are obligate anaerobes and if engineered to express cytotoxic compounds could grow in tumors and kill tumor cells. This was tested in mice by expressing cytosine deaminase in *Bifidobacterium infantis* and injecting this strain with 5-fluorocytosine into melanomas resulting in some tumor shrinkage.

Genomics of LAB

The era of genomics has revolutionized the genetic understanding of all living organisms. The era officially began in 1995 with the publication of the first complete genome sequence of a living organism, the Gram-negative bacterium *Haemophilus influenzae*, by Craig Venter and colleagues. Genome sequence projects at that time, including the human genome project, were utilizing a directed sequencing approach, whereby mapped regions of a genome were individually sequenced in an ordered fashion. Venter's group showed that a shotgun sequencing approach, whereby shotgun libraries of entire randomly sheared genomes are constructed and their sequences are assembled using sequence scaffolding, could accomplish the task much more quickly and changed the direction of genome sequence projects.

The first LAB to be completely sequenced was *Lc. lactis* subsp. *lactis* IL1403 in 2001. This is a plasmid-cured strain and is used for genetic understanding of different aspects of this important subspecies of *Lc. lactis*. Currently (August 2009), there are three complete genome sequences available for *Lc. lactis*, including the plasmid-cured *Lc. lactis* subsp. *cremoris* MG1363 and the commercially relevant wild-type strain *Lc. lactis* subsp. *cremoris* SK11. Following the publication of the genome sequence of IL1403, the complete genome sequences of isolates representing all the major genera of the LAB and bifidobacteria were deciphered and published. This was greatly facilitated by the Lactic Acid Bacteria Genome Consortium (LABGC), which is a group of 11 prominent LAB researchers from 7 US universities that selected 9

LAB important for the food fermentation industry, together with a *Bifidobacterium* and a *Brevibacterium* isolate, and completed their genome sequences. **Table 4** depicts the completed and ongoing genome projects for all the LAB and bifidobacteria relevant to foods. The nine LAB genome sequences from the LABGC were published in 2006 and their comparative analysis revealed novel insights into the evolution of the LAB group of organisms. This analysis revealed that extensive genome reduction occurred over their evolution

Table 4 Completed and ongoing genome projects of the LAB and bifidobacteria as of August 2009

Family	*Genus*	*Species*	*Complete*	*Incomplete*	*Total*
Streptococcaceae	*Streptococcus*	*thermophilus*	3	1	4
	Lactococcus	*garvieae*	0	2	2
		lactis subsp. *cremoris*	2	0	2
		lactis subsp. *lactis*	1	1	2
Lactobacillaceae	*Lactobacillus*	*acidophilus*	1	1	2
		amylolyticus	0	1	1
		antri	0	1	1
		brevis	1	2	3
		buchneri	0	2	2
		casei	2	2	4
		coleohominis	0	2	2
		crispatus	0	8	8
		delbrueckii subsp. *bulgaricus*	2	0	2
		delbrueckii subsp. *lactis*	0	1	1
		fermentum	1	1	2
		gasseri	1	4	5
		helveticus	2	2	4
		hilgardii	0	1	1
		iners	0	1	1
		jensenii	1	6	7
		johnsonii	1	2	3
		paracasei	0	3	3
		plantarum	2	1	3
		reuteri	2	5	7
		rhamnosus	0	7	7
		ruminis	0	1	1
		sakei subsp. *carnosus*	0	1	1
		sakei subsp. *sakei*	1	0	1
		salivarius subsp. *salivarius*	1	1	2
		ultunensis	0	1	1
		vaginalis	0	1	1
	Pediococcus	*acidilactici*	0	2	2
		pentosaceus	1	0	1
Leuconostocaceae	*Leuconostoc*	*citreum*	1	0	1
		gasicomitatum	0	1	1
		mesenteroides subsp. *mesenteroides*	1	1	2
	Oenococcus	*oeni*	1	2	3
	Weissella	*paramesenteroides*	0	1	1
Enterococcaceae	*Tetragenococcus*	*halophilus*	0	1	1
Carnobacteriaceae	*Carnobacterium*	*divergens*	0	1	1
Bifidobacteriaceae	*Bifidobacterium*	*adolescentis*	1	1	2
		angulatum	0	2	2
		animalis subsp. *lactis*	3	3	6
		bifidum	0	4	4
		breve	0	3	3
		catenulatum	0	2	2
		dentium	0	6	6
		gallicum	0	1	1
		longum subsp. *longum*	2	1	3
		longum subsp. *infantis*	1	5	6
		pseudocatenulatum	0	1	1
		scardovii	0	1	1
Total			35	101	136

from a common ancestor from the class Bacilli. Genome reduction over evolutionary periods appears to occur as organisms adapt to new habitats that demand less phenotypic capabilities to be successful. An example is biosynthetic capabilities, where the LAB have lost extensive genetic regions involved in biosynthesis, likely reflecting their nutrient-rich habitat where the need to synthesize all amino acids and vitamins is not essential. To better adapt to this nutrient-rich habitat, the LAB have obtained a wide range of transporters to improve their abilities at utilizing the available nutrients in their habitat. The genome analysis reflects that this increase in transporter abilities likely evolved through horizontal gene transfer, whereby DNA is acquired from diverse organisms. As a group, they are very well adapted to nutrient-rich habitats and as a result can dominate in those habitats. **Table 5** depicts the general characteristics of the completed genome sequences of the LAB and bifidobacteria.

Lactobacillus Genomics

There are 18 available complete genome sequences for *Lactobacillus*, including representatives from 12 species important for the food industry. Analysis of the genomes can reveal the complete metabolic pathways of the different species. **Figure 2** depicts the metabolic pathways as deciphered from the genome sequences of the homofermentative *Lb. acidophilus* and the heterofermentative *Lb. brevis*.

Lactobacillus plantarum contains the largest genome and this is proposed to reflect its adaptation to a very heterogeneous habitat compared to the other lactobacilli. This is substantiated by the highest number of transcriptional

Table 5 General characteristics of the completed genomes from the different species of the LAB and bifidobacteria

Genus	*Species*	*Strain*	*Plasmids*	*Size (kb)*	*GC (%)*	*ORFs*[b]	*Pseudogenes*
Lactobacillus	*acidophilus*	NCFM	0	1993	34.7	1936	0
	brevis	ATCC 367	2	2291 (49)[a]	46.2	2314	49
	casei	ATCC 334	1	2895 (29)	46.6	2909	82
	delbrueckii subsp. *bulgaricus*	ATCC BAA-365	0	1857	49.7	2040	192
	fermentum	IFO 3956	0	2098	51.5	1912	0
	gasseri	ATCC 33323	0	1894	35.3	1898	48
	helveticus	DPC 4571	0	2080	37.1	1838	155
	johnsonii	NCC 533	0	1992	34.6	1918	0
	plantarum	WCFS1	3	3308 (40)	44.5	3135	42
	reuteri	JCM 1112	0	2039	38.9	1820	0
	sakei subsp. *sakei*	23K	0	1884	41.3	1963	30
	salivarius subsp. *salivarius*	UCC118	3	1827 (306)	32.9	1864	49
Lactococcus	*lactis* subsp. *lactis*	IL1403	0	2365	35.3	2425	1
	lactis subsp. *cremoris*	SK11	5	2438 (158)	35.9	2610	144
Leuconostoc	*citreum*	KM20	4	1796 (100)	39.0	1785	1
	mesenteroides subsp. *mesenteroides*	ATCC 8293	1	2038 (37)	37.7	2073	19
Oenococcus	*oeni*	PSU-1	0	1780	37.9	1864	122
Pediococcus	*pentosaceus*	ATCC 25745	0	1832	37.4	1847	20
Streptococcus	*thermophilus*	LMD-9	2	1856 (7)	39.1	2003	206
Bifidobacterium	*longum* subsp. *longum*	DJO10A	2	2375 (14)	60.2	1990	0
	longum subsp. *infantis*	ATCC 15697	0	2833	59.9	2416	78
	adolescentis	ATCC 15703	0	2090	59.2	1631	0
	animalis subsp. *lactis*	AD011	0	1934	60.5	1528	17

[a] Total size of plasmids.

[b] ORFs, Open Reading Frames.

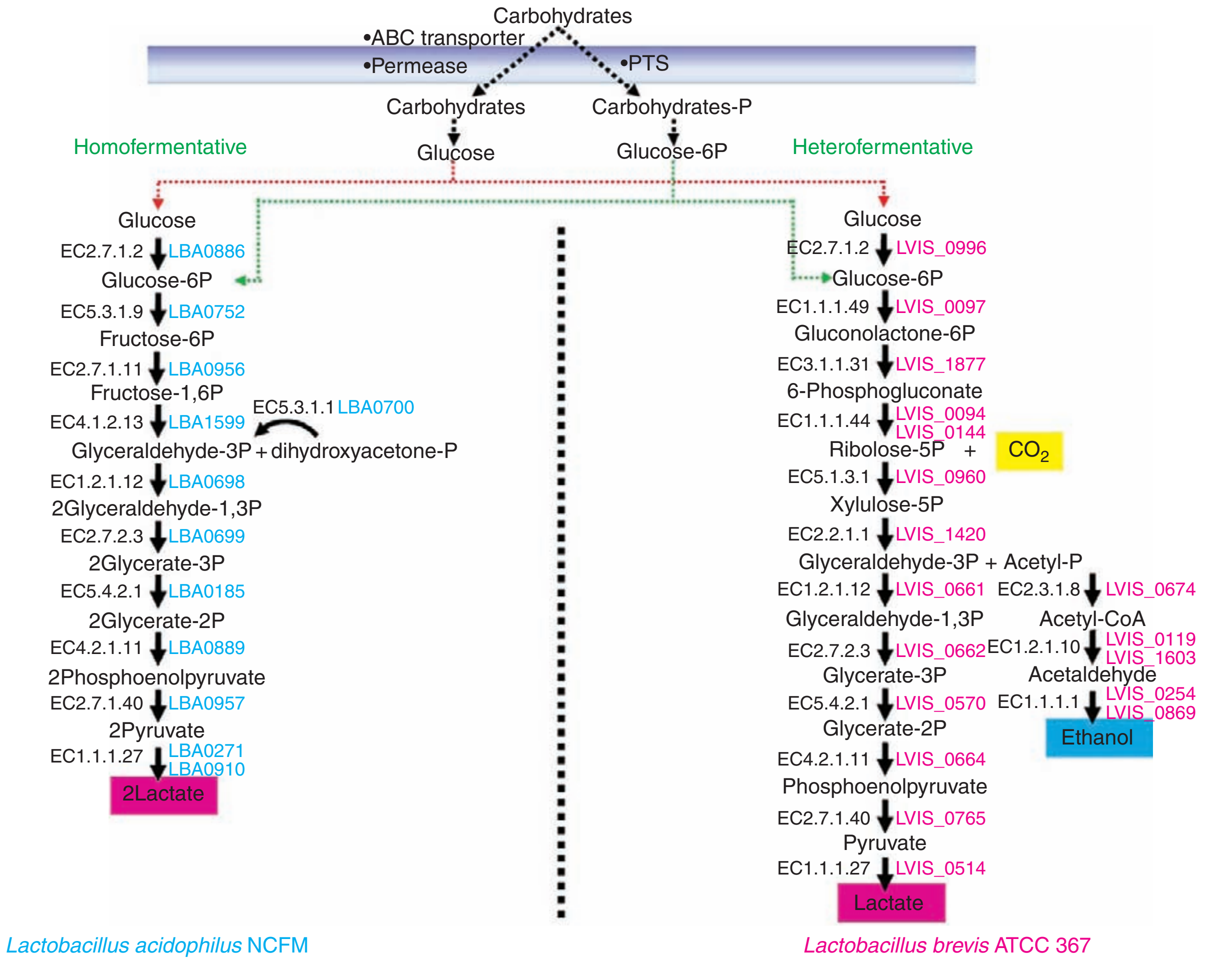
Carbohydrates
•ABC transporter
•Permease
•PTS
Carbohydrates
Carbohydrates-P
Homofermentative
Glucose
Glucose-6P
Heterofermentative
Glucose
EC2.7.1.2 LBA0886
Glucose-6P
EC5.3.1.9 LBA0752
Fructose-6P
EC2.7.1.11 LBA0956
Fructose-1,6P
EC5.3.1.1 LBA0700
EC4.1.2.13 LBA1599
Glyceraldehyde-3P + dihydroxyacetone-P
EC1.2.1.12 LBA0698
2Glyceraldehyde-1,3P
EC2.7.2.3 LBA0699
2Glycerate-3P
EC5.4.2.1 LBA0185
2Glycerate-2P
EC4.2.1.11 LBA0889
2Phosphoenolpyruvate
EC2.7.1.40 LBA0957
2Pyruvate
EC1.1.1.27 LBA0271 LBA0910
2Lactate
Glucose
EC2.7.1.2 LVIS_0996
Glucose-6P
EC1.1.1.49 LVIS_0097
Gluconolactone-6P
EC3.1.1.31 LVIS_1877
6-Phosphogluconate
EC1.1.1.44 LVIS_0094 LVIS_0144
Ribolose-5P + CO_2
EC5.1.3.1 LVIS_0960
Xylulose-5P
EC2.2.1.1 LVIS_1420
Glyceraldehyde-3P + Acetyl-P
EC1.2.1.12 LVIS_0661
EC2.3.1.8 LVIS_0674
Glyceraldehyde-1,3P
Acetyl-CoA
EC2.7.2.3 LVIS_0662
EC1.2.1.10 LVIS_0119 LVIS_1603
Glycerate-3P
Acetaldehyde
EC5.4.2.1 LVIS_0570
EC1.1.1.1 LVIS_0254 LVIS_0869
Glycerate-2P
Ethanol
EC4.2.1.11 LVIS_0664
Phosphoenolpyruvate
EC2.7.1.40 LVIS_0765
Pyruvate
EC1.1.1.27 LVIS_0514
Lactate
Lactobacillus acidophilus NCFM
Lactobacillus brevis ATCC 367

Figure 2 Schematic representation of the metabolic pathways of the homofermentative *Lactobacillus acidophilus* and the heterofermentative *Lb. brevis* as represented in their genome sequence analysis. The EC number for each enzyme is listed as well as their cognate gene number from the genome sequences, blue for *Lb. acidophilus* and red for *Lb. brevis*.

regulators and carbohydrate utilization and transport systems, including 25 dedicated sugar phosphotransferase systems (PTSs). The genome sequence of the psychrotrophic *Lb. sakei* revealed its adaptation to a meat and fish environment, as it contains nucleoside metabolic pathways that enable it to utilize meat and fish nucleosides for energy once the limited amount of glucose is utilized. *Lactobacillus salivarius* genome was unique in that it contained a megaplasmid (242 kb) in addition to its chromosome. This plasmid encodes its bacteriocin production and immunity as well as some genes for the pentose phosphate pathway and bile salt hydrolase. Given that the plasmid also contains conjugative features, it may have been obtained by horizontal gene transfer at a distant evolutionary time and retained because of the competitive advantages it likely imparts this intestinal isolate. It is also possible that the plasmid evolved from the chromosome of the host to increase gene dosage of certain genes, as its GC content is similar to that of the chromosome.

The complete genome sequence of *Lb. reuteri* revealed a large genomic island that is involved in the production of cobalamin (vitamin B_{12}) and reuterin; reuterin is a very broad-spectrum antimicrobial compound produced from glycerol metabolism. Both these features are believed to be important for the probiotic properties of this intestinal bacterium. It is interesting that these features are encoded on a genomic island as this reflects a recent evolutionary event. Some genomes of pathogenic bacteria contain genomic islands encoding virulence genes. *Lactobacillus reuteri* is the first example of a bacterium containing a genomic island predicted to be involved in probiotic characteristics. **Figure 3** depicts a comparison of some features of all the LAB and bifidobacteria genomes completed to date.

Lactococcus Genomics

Prophage are observed in most bacterial genomes, but *Lc. lactis* contains the highest number among the LAB.

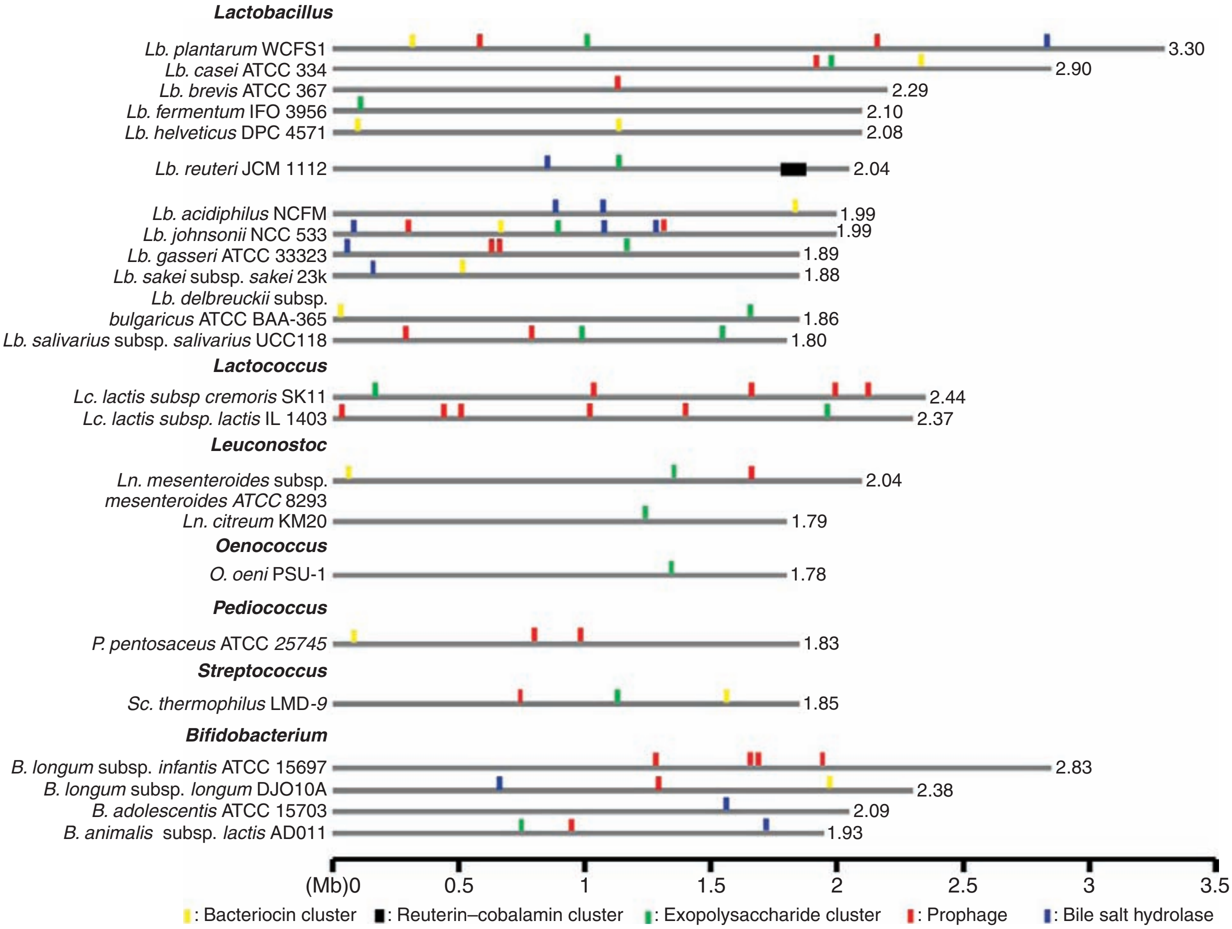

Figure 3 Comparative genome alignment of the completed genomes representing each species of the LAB and bifidobacteria. Gene clusters representing bacteriocin production, reuterin and cobalamin production, exopolysaccharide production, prophage, and bile salt hydrolases are indicated by colored boxes.

This may reflect their long history in milk fermentation environments where phage are known to be prevalent. Their genomes are also unique as many traits needed for survival in their habitat are encoded on plasmids. The genome of *Lc. lactis* subsp. *cremoris* MG1363 contains an integration hot spot, which contains 20% of the genomes IS elements and a mobile sex factor. This suggests a genome that is still in an active evolutionary state, as it can readily accommodate new DNA. The future analysis of the genome sequence of the wild-type *Lc. lactis* subsp. *cremoris* SK11 will shed more light on the background of this unique species.

Streptococcus Genomics

Streptococcus thermophilus is an important dairy fermentation culture and its genome sequence revealed that it does not contain any of the virulence regions common to many species of *Streptococcus*. This likely reflects adaptation by genome reduction to a dairy environment. These changes from its pathogenic relatives reinforce the safety of these cultures and are substantiated by the small size of its genome relative to the other species of this genus. It is also intriguing that it has a similar size of genome to *Lb. delbrueckii* subsp. *bulgaricus*, which itself has the smallest total genome size of the lactobacilli, suggesting their likely coevolution in a milk environment, consistent with their long history in yogurt fermentation.

Leuconostoc, *Pediococcus*, and *Oenococcus* Genomics

Complete genome sequences for two species of *Leuconostoc* and one species each of *Pediococcus* and *Oenococcus* have been obtained, but limited bioinformatic analysis of their genomes is currently available. **Table 5** and **Figure 2** depict the general characteristics of their genomes, which have lots of similarities to the other LAB genomes.

Bifidobacterium Genomics

The first genome sequence of the bifidobacteria was from *B. longum* NCC2705, a Nestlé culture-collection isolate, and was published in 2002. This revealed an extensive capacity to utilize complex carbohydrates, such as oligosaccharides, when compared to other genomes. This reflects their adaptation to the large intestine where utilization of this class of nutrients is believed to give them a competitive advantage, given the lack of simple carbohydrates, which are absorbed in the small intestine. A subsequent comparative genome analysis of this culture-collection strain, with that of an intestinal strain of *B. longum* (i.e., a strain that has been minimally cultured outside the intestine), revealed an even greater oligosaccharide utilization capacity in the intestinal strain. This analysis also revealed that several other features believed to be important for survival and competition in the large intestine were also present in the intestinal strain, such as sugar alcohol utilization, arsenic resistance, and bacteriocin production. Bioinformatic tools predicted that the DNA regions encoding these traits were likely lost during recent evolutionary events in the culture-collection strain. This was proven experimentally when the intestinal strain was cultured in a fermentation environment for 80 generations and was found to have lost two significant DNA regions, including the region encoding bacteriocin production, in a deletion event very similar to the culture-collection strain. This highlighted the need to minimally culture bifidobacteria outside the intestine to minimize loss of DNA regions that are predicted to be important for their probiotic functionality. While current commercial bifidobacteria may be compromised in this regard, their use may still have benefits from other characteristics. For example, exopolysaccharide-producing bifidobacteria can still have laxative effects on the intestine during their transient passage in sufficient numbers through the GI tract. In the United States, this is the only health related claim of bifidobacteria containing yogurts or fermented milks currently being promoted. This illustrates that a probiotic effect can still be achieved using a culture that has lost significant abilities to compete in the GI tract. However, attaining the same probiotic effect with cultures that still have the ability to modulate the GI microflora is a more desirable goal.

The genome sequence of *B. longum* subsp. *infantis* was significantly larger than the genome sequence of its relative *B. longum* subsp. *longum*. This likely reflects its ability to dominate in the intestines of breast-fed infants, where the sole dietary components come from breast milk. Relative to other mammals, human breast milk contains an unusually large number of different oligosaccharides and *B. longum* subsp. *infantis* is very well adapted to utilizing these. The extra genome content of this subspecies reflects its superior ability compared to *B. longum* subsp. *longum* at utilizing human milk oligosaccharides. As the infant's diet changes to a more adult diet, *B. longum* subsp. *infantis* is not found as often as *B. longum* subsp. *longum*, likely reflecting the lack of human milk oligosaccharides in the adult diet.

The completed genome sequence for a strain of *B. animalis* subsp. *lactis* was also obtained, given its commercial importance for dairy bifidobacteria probiotics. This subspecies evolved from *B. animalis* by adapting to a dairy fermentation environment. This gives it better stress tolerance than other bifidobacteria, making it attractive for food applications. Its genome is the smallest genome of the sequenced bifidobacteria, reflecting the genome reduction trend that occurs in pure culture habitats. It is also noteworthy that it also contains the lowest number of carbohydrate metabolic genes, also reflecting adaptation to a carbohydrate homogeneous habitat. Another notable feature is the reduction in ribosomal RNA operons (two compared to four in *B. longum* and five in *B. adolescentis*). This also reflects adaptation to a homogeneous stable habitat, as microbes have more ribosomal RNA operons in heterogeneous habitats to enable a faster adaptation to new nutrient sources.

Conclusion

The genetic analysis of the LAB and bifidobacteria has greatly improved our understanding of how these organisms function in fermentations and in the GI tract. There is a bright future for the genetic engineering of these organisms to produce value-added food ingredients and other nonfood products, as well as their potential for effective oral vaccines against a wide range of infectious diseases. The genome sequences for many of these bacteria have only recently become available and this has provided a new understanding on the evolutionary background of this group of bacteria. The genome sequences also reaffirmed the nonpathogenic potential of these organisms, which are so essential for food production. This is also reassuring for the probiotic field where large numbers of specific strains are deliberately ingested, and a genome analysis is now an essential step when considering the safety of any bacterium for human use.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Morphology and Physiology; Lactic Acid Bacteria: An Overview; Probiotics, Applications in Dairy Products. **Cheese**: Starter Cultures: General Aspects. **Fermented Milks**: Starter Cultures. **Genetics**: Cattle Genomics. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*;

Lactobacillus spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; Taxonomy and Biodiversity.

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Lactobacillus spp.: General Characteristics

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Introduction

Lactobacillus is the largest genus within the group of lactic acid bacteria. To date it contains 166 species (see LPSN website), which are mainly used in the manufacture of fermented dairy, sourdough, meat, and vegetable foods, or as probiotics. The general utility of the *Lactobacillus* species is related to their GRAS (generally recognized as safe) status and will be dependent on the availability of cost-effective methods for production and delivery of viable cultures. In dairy products such as fermented milks and cheeses, the lactobacilli have multiple effects depending on the species, strain, manufacturing conditions, and product. The two most obvious beneficial roles of lactobacilli are as starter cultures (to produce acid rapidly) and as probiotic cultures. However, they may also synthesize bacteriocins and exopolysaccharides and contribute to the flavor of different dairy products, and, in some circumstances, may also cause flavor and texture defects.

Lactobacilli are not found in milk drawn aseptically from the udder. Nevertheless, during normal milking procedures, contamination by lactobacilli may occur from soil, manure, grass, silage, and other feeds adhering to the udder, as well as from milking equipment and milk storage.

Taxonomy of *Lactobacillus*

The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order II *Lactobacillales*, and family *Lactobacillaceae.* Lactobacilli are Gram-positive, catalase-negative, non-spore-forming, rod-shaped bacteria that produce lactic acid as the major end product of fermentation. They have complex nutritional requirements, needing to be supplied with carbohydrates, fatty acids or fatty acid esters, salts, nucleic acid derivatives, and vitamins. The genus *Lactobacillus* comprises a group of bacteria charecterized by a wide range of G + C ratios and little DNA–DNA homology between many of the species. The genus is most closely related to the genera *Leuconostoc* and *Pediococcus* (**Figure 1**) with a more distant relationship to streptococci, *Carnobacterium*, and the aerobic *Bacillus* species. Traditionally, the genus is split into three distinct groups based on carbohydrate fermentation pathways: (1) obligate homofermentative lactobacilli, which ferment hexoses almost entirely to lactic acid, while pentoses or gluconate is not fermented; (2) facultative heterofermentative lactobacilli, which ferment hexoses either almost entirely to lactic acid or, under glucose-limiting conditions, to lactic acid, acetic acid, ethanol, and formic acid, and pentoses to lactic acid and acetic acid; and (3) obligate heterofermentative lactobacilli, which ferment hexoses to lactic acid, CO_2, acetic acid, and/or ethanol, and pentoses to lactic acid and acetic acid. Lactobacilli commonly found in cheeses are listed in **Table 1**.

Examination of lactobacilli by genetic methods such as DNA–DNA homology and 16S rRNA sequencing has led to the reclassification of a number of lactobacilli. The phylogenetic relatedness of lactobacilli was investigated by using the 16S rRNA sequences of all species validly described up to now. This permitted allotment of the lactobacilli to the following groups: *Lb. buchneri* (bu), *Lb. casei* (ca), *Lb. delbrueckii* (de), *Lb. plantarum* (pl), *Lb. reuteri* (re), *Lb. sakei* (sa), and *Lb. salivarius* (sl) (see **Table 1** and **Figure 1**). As the relationships between the groups could not always be resolved unambiguously, the branching is indicated by multifurcations starting from one ancestor. On the other hand, *Lb. brevis* and *Lb. perolens*, as well as the related species *Lb. bifermentans* and *Lb. coryneformis*, are uniquely positioned among the lactobacilli. In general, the G + C content of the species within most of the subgroups is rather widespread. This fact may be explained by changes in codon usages stemming from the degeneracy of the genetic code. The *Lb. buchneri* group contains only obligately heterofermentative lactobacilli, except for *Lb. homohiochii*, which has been described to be facultatively heterofermentative. The *Lb. casei* group consists of both obligately homofermentative and facultatively heterofermentative bacteria. The latter are allotted to the highly related species *Lb. casei*, *Lb. paracasei*, *Lb. zeae*, and *Lb. rhamnosus*; their taxonomic status is currently a controversial subject. The *Lb. delbrueckii* group contains mainly obligate homofermentative bacteria, and the G + C content of most of the species is <40 mol%. *Lactobacillus delbrueckii* contains three subspecies, which cannot be differentiated by rRNA sequence analysis. The *Lb. plantarum* group consists of 12 *Lactobacillus* species, and all three types of carbohydrate fermentation are

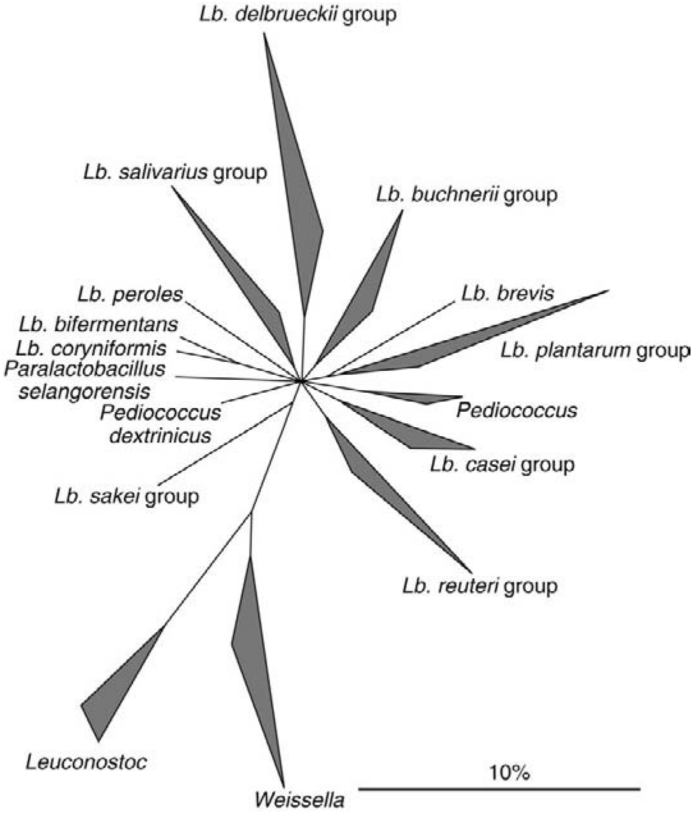

Figure 1 Phylogenetic tree depicting groups of the family *Lactobacillaceae*. The consensus tree is based on maximum parsimony analyses of all available, at least 90% complete 16S rRNA sequences of the depicted genera and/or species. Alignment positions that share identical residues in at least 50% of all sequences of the depicted genera were considered. Multifurcations indicate that a common branching order could not be significantly determined or was not supported when performing different alternative treeing approaches. Adapted from Hammes WP and Hertel CH (2006) The genera *Lactobacillus* and *Carnobacterium*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, and Stackebrandt E (eds.) *Prokaryotes*, Vol. 4, 3rd edn., pp. 320–403. New York: Springer.

represented. Remarkably, within this group, many species show very high 16S rRNA sequence similarity, namely, *Lb. plantarum*, *Lb. pentosus*, and *Lb. paraplantarum* (99.7–99.9%); *Lb. kimchii* and *Lb. paralimentarius* (99.9%); and *Lactobacillus mindensis* and *Lactobacillus farciminis* (99.9%). Besides, the G + C content of the above species is rather similar. The *Lb. reuteri* group contains exclusively obligate heterofermentative bacteria, and members of this group show marked differences with respect to their DNA composition (36–54 mol%). The species *Lb. durianis* and *Lb. vaccinostercus* exhibit a very high 16S rRNA sequence similarity (99.7%), but were shown to differ significantly in their G + C content (36 and 43 mol%, respectively); as compared to the other highly related species of the genus *Lactobacillus*, this difference in the G + C content is unique. The *Lb. sakei* group is the smallest and comprises only facultatively heterofermentative lactobacilli. Both *Lb. curvatus* and *Lb. sakei* contain two subspecies, which can be distinguished only by molecular typing methods, the interlaboratory reliability of which is not given; hence, the subspecies status is questionable. The *Lb. salivarius* group contains obligate homofermentative and facultative heterofermentative bacteria. Again, *Lb. animalis* and *Lb. murinus* (99.7%), as well as *Lb. cypricasei* and *Lb. acidipiscis* (99.7%), show 16S rRNA sequence similarities usually found among different species of the genus *Lactobacillus*. *Lactobacillus salivarius* contains two subspecies, which cannot be differentiated by rRNA sequence analysis. A number of traditional lactobacilli have been transferred to other genera (e.g., *Lb. viridescens* to *Weissella viridescens*, *Lb. minutus* to *Atopobium minutum*, and *Lb. carnis* to *Carnobacterium piscicola*).

Isolation and Enumeration of *Lactobacillus*

Media for the growth of lactobacilli must provide for their complex nutritional requirements and their preference for microaerophilic conditions. The most commonly used medium for the nonselective enumeration of lactobacilli is MRS agar; APT agar is a similar medium, which may also be used to grow lactobacilli. For anaerobic

Table 1 List of the cheese-related species of the genus *Lactobacillus* and genome sequencing progress to date (December 2009)

Species	*Type of glucose fermentation*	*Main habitat/function*	*PG*	*Strain with identified genome*	*Genome size (Mb)*	*GenBank accession number*
Lb. acidophilus	A	Associated with human and/or animals; probiotic; dominant NSLAB in some cheeses (e.g., Camembert)	de	NCFM	1.99	NC_006814
Lb. brevis	C	Probiotic; NSLAB in some cheeses (e.g., Canestrato Pugliese, Cheddar, Ricotta Forte)	u	ATCC 367	2.34	NC_008497
Lb. buchneri	C	NSLAB in some cheeses (e.g., Canestrato Pugliese, Ricotta Forte)	bu	ATCC 11577	~ 2.85	In progress
Lb. casei	B	Associated with human and/or animals; probiotic; NSLAB in some cheeses (e.g., Canestrato Pugliese, Camembert, Cheddar, Grana Padano, Gruyère, Idiazabal, Parmigiano-Reggiano, Ricotta Forte, Roncal, Serra da Estrela)	ca	ATCC 334	2.89	NC_008526
Lb. coryniformis	B	NSLAB in some cheeses (e.g., goat cheese, Iraqi cheese)	u	NA	NA	NA
Lb. curvatus	B	NSLAB in some cheeses (e.g., Canestrato Pugliese, Cheddar, Fiore Sardo, Fossa, Idiazabal, Montasio, Mozzarella, Pecorino Romano, Pecorino Sardo, Roncal)	sa	NA	NA	NA
Lb. cypricasei	B	NSLAB in some cheeses (e.g., Idiazabal, Pecorino Toscano, Roncal)	sl	NA	NA	NA
Lb. delbrueckii subsp. *bulgaricus*	A	Probiotic; primary thermophilic natural and commercial starter for Italian (e.g., Grana, Pecorino, Mozzarella) and Swiss (e.g., Emmental, Sbrinz, Gruyère) cheese varieties; starter culture for fermented milks	de	ATCC 11842 ATCC BAA-365	1.86 1.85	NC_008054 NC_008529
Lb. delbrueckii subsp. *lactis*	A	Probiotic; primary thermophilic natural and commercial starter for Italian (e.g., Grana, Pecorino, Mozzarella) and Swiss (e.g., Emmental, Sbrinz, Gruyère) cheese varieties; starter culture for fermented milks	de	NA	NA	NA
Lb. diolivorans	C	Associated with fermented foods	bu	NA	NA	NA
Lb. fermentum	C	Probiotic; NSLAB in some cheeses (e.g., Comtè, Ragusano)	re	IFO 3956	2.09	NC_010610
Lb. gasseri	A	Associated with human and/or animals; probiotic	de	ATCC 33323	1.89	NC_008530
Lb. helveticus	A	Probiotic; primary thermophilic natural and commercial starter for Italian (e.g., Grana, Pecorino, Mozzarella) and Swiss (e.g., Emmental, Sbrinz, Gruyère) cheese varieties	de	DPC 4571	2.08	NC_010080
Lb. hilgardii	C	NSLAB in some cheeses (e.g., Mozzarella)	bu	ATCC 8290	~ 2.6	In progress
Lb. johnsonii	A	Associated with human and/or animals; probiotic	de	NCC 533	1.99	NC_005362
Lb. kefiranofacies	A	Associated with fermented foods (fermented milk)	de	NA	NA	NA
Lb. kefiri	C	Associated with fermented foods (fermented milk)	bu	NA	NA	NA
Lb. parabuchneri	C	NSLAB in some cheeses (e.g., Caciocavallo Silano)	bu	NA	NA	NA

Lb. paracasei subsp. *paracasei*	B	Associated with human and/or animals; probiotic; NSLAB in some cheeses (e.g., Arzua, Batzos, Caciocavallo Silano, Caciocavallo Pugliese, Camembert, Canestrato Pugliese, Cheddar, Comté, Emmental, Fiore Sardo, Fontina, Fossa, Grana Padano, Kefalotyri, Parmigiano-Reggiano, Pecorino Toscano, Ricotta Forte, Serra da Estrela)	ca	8700:2	~ 2.97	In progress
Lb. paracasei subsp. *tolerans*	B	Associated with human and/or animals; probiotic; NSLAB in some cheeses (e.g., Canestrato Pugliese, Camembert, Cheddar, Grana Padano, Gruyère, Idiazabal, Parmigiano-Reggiano, Ricotta Forte, Roncal, Serra da Estrela)	ca	NA	NA	NA
Lb. paraplantarum	B	NSLAB in some cheeses (e.g., Batzos)	pl	NA	NA	NA
Lb. pentosus	B	NSLAB in some cheeses (e.g., Batzos, Canestrato Pugliese, Fiore Sardo, Fossa)	pl	NA		
Lb. plantarum	B	Associated with human and/or animals; probiotic; NSLAB in some cheeses (e.g., Arzua, Caciocavallo Pugliese, Camembert, Canestrato Pugliese, Cheddar, Fontina, Fossa, Kefalotyri, Mahòn, Manchego, Mozzarella, Pecorino Toscano, Roncal, Serra da Estrela)	pl	WCFS1	3.31	NC_004567
Lb. reuteri	C	Associated with human and/or animals; probiotic; NSLAB in some cheeses (e.g., Cheddar, Grana Padano, Gruyére, Idiazabal, Parmigiano-Reggiano, Roncal, Toma)	re	DSM 20016 JMC1112	1.99 2.04	NC_009513; NC_010609
Lb. rhamnosus	B	Associated with human and/or animals; probiotic	ca	HN001	~ 2.4	In progress
Lb. sakei	B	NSLAB in some cheeses (e.g., Iranian traditional Lighvan cheese)	sa	23K	1.88	NC_007576

Lb., *Lactobacillus*; A, obligately homofermentative; B, facultatively heterofermentative; C, obligately heterofermentative; PG, phylogenetic group; bu, *Lb. buchneri* group; ca, *Lb. casei* group; de, *Lb. delbrueckii* group; Pl, *Lb. plantarum* group; re, *Lb. reuteri* group; sa, *Lb. sakei* group; NSLAB, nonstarter lactic acid bacteria; NA, not available.

lactobacilli, 0.05% cysteine should be added to the medium to help reduce the oxygen tension. The agar plates should be incubated anaerobically, usually for 48 h at 30 or 37 °C. Selective enumeration of lactobacilli relies on their aciduric nature for differentiation from other bacteria. The most commonly used medium for selective enumeration of lactobacilli in dairy products is Rogosa agar (SL or LBS agar). The pH of this medium should be adjusted to 5.3–5.0 to prevent the growth of lactococci, enterococci, and streptococci. MRS agar with pH adjusted to 5.0 may also be used for selective enumeration of lactobacilli. Plates of both media should be incubated anaerobically either for 3 days at 37 °C or for 5 days at 30 °C. However, neither of these media is totally selective, because *Leuconostoc* (cocci in pairs and short chains), *Pediococcus* (cocci in tetrads and clusters), and some yeast are also able to grow on these media at pH 5.0. Microscopic examination of the isolates may be necessary to determine whether the colonies are lactobacilli.

Methods for Identification of *Lactobacillus*

Traditionally, lactobacilli are classified based on phenotypic properties such as morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, fermentation of various carbohydrates, type of methyl esters from fatty acids, and pattern of proteins in the cell wall or in the whole cell (SDS–PAGE). Modern approaches of phenotypic identification include the study of a very large fermentative profile (95 different carbon sources) by the Biolog system, as well as another approach for a rapid identification based on spectroscopic techniques (pyrolysis mass spectrometry (PyMS), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Fourier transform infrared spectroscopy (FT-IR), and Raman spectroscopy). Unfortunately, phenotypic methods have some limitations, such as their poor reproducibility owing to the plasticity of bacterial growth and environmental conditions that may affect gene expression (e.g., growth under laboratory conditions). Genotypic methods are increasingly applied for the identification of lactobacilli, and the polyphasic approach, a combination of phenotypic and genotypic methods, is considered to provide the most reliable identification.

Genotyping techniques may be applied as a tool for either the identification of the species or differentiating strains of lactobacilli at the clonal level. Owing to the availability of a comprehensive data set of 16S and 23S rRNA sequences, application of rRNA technology has become of great importance for bacterial identification. Complete or at least partial 16S rRNA sequences are available for all *Lactobacillus* species. Comparative analysis of the 16S rRNA sequences may therefore be used for reliable identification of the strain. On the other hand, the 16S rRNA sequence may be too well-conserved to differentiate between closely related species. Within the genus *Lactobacillus*, several species have very high 16S rRNA sequence similarity, for example, *Lb. plantarum*, *Lb. pentosus*, and *Lb. paraplantarum* (99.7–99.9%), *Lb. kimchii* and *Lb. paralimentarius* (99.9%), *Lb. mindensis* and *Lb. farciminis* (99.9%), *Lb. animalis* and *Lb. murinus* (99.7%), and *Lb. durianis* and *Lb. vaccinostercus* (99.7%). Taking these remarks into account, the comparative analysis of partial 16S rRNA sequences (approximately the first 900 bases) is a fast tool to gain insight into the taxonomic position of an unknown *Lactobacillus* isolate. Comparative analysis of 16S and 23S rRNA sequences reveals evolutionarily less conserved regions that have a diagnostic value for species, genus, or groups of phylogenetically related organisms. Specific rRNA-targeted probes were already in use in fluorescent *in situ* hybridization (FISH), where the specific rRNA sequences are detected within morphologically intact cells. Among cultivation-dependent techniques, DNA fingerprinting techniques, such as pulsed-field gel electrophoresis (PFGE) of rare-cutting restriction enzyme fragments, ribotyping, randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP), are extensively used for intraspecific identification and for genotyping lactobacilli isolated from several fermented foods as well as from the human gastrointestinal tract. Molecular biological methods are developed for the culture-independent analysis of the diversity of complex microbial communities in dairy products. A flow chart of the current molecular approaches used singularly or in combination to analyze cheese microbial communities is shown in **Figure 2**. rRNA or total DNA is extracted from cheese samples, and fragments of this rRNA or DNA are amplified using universal primers. The separation of PCR amplicons, which are similar in size but different in sequence, is performed by denaturing gradient gel electrophoresis (DGGE) or temporal temperature gradient electrophoresis (TTGE). To investigate the metabolically active species during cheese manufacture, real-time (RT) PCR followed by DGGE of the 16S rRNA gene is used. Terminal-restriction fragment length polymorphism (T-RFLP) and single-strand conformation polymorphism (SSCP) analyses are based on specific target sites for restriction enzymes and on the secondary structure of single-stranded DNA, respectively. These techniques have completely revolutionized the detection of DNA/RNA in microbial ecological studies, because they are not influenced by the physiological state of the cells and remain constant during cell growth. The selection of the most appropriate typing method depends on cost, throughput capacity, and reproducibility of fingerprints.

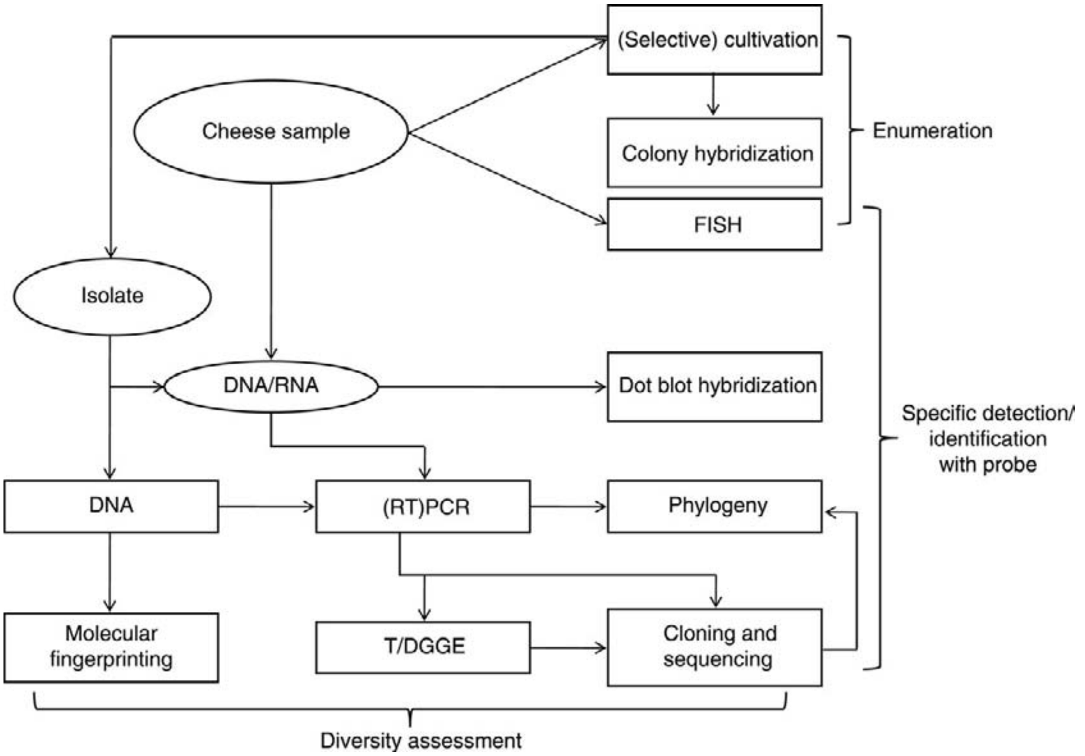

Figure 2 Flow chart of current molecular approaches used alone or in combination to analyze cheese microbial communities. FISH, fluorescence *in situ* hybridization; (RT)PCR, (real-time) polymerase chain reaction; T/DGGE, temperature/denaturing gradient gel electrophoresis. Adapted from Randazzo CL, Caggia C, and Neviani E (2009) Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. *Journal of Microbiological Methods* 78: 1–9.

Lactobacillus as Starter Cultures in the Manufacture of Fermented Milks and Cheeses

Starter cultures are so called because they 'start' the production of lactic acid from lactose, which occurs in the early phase of manufacture of fermented milks and cheeses. A useful rule is to decrease the value of pH to <5.3 in milk in approximately 6 h at 30–37 °C, depending on the dairy product. The low pH suppresses or kills undesirable organisms, and, in fermented milks, it is the main factor in the formation of the gel. Starters may also impart other benefits. Lactobacilli are also often added as adjuncts rather than starters, where either the conditions or the inoculum level ensures that other added cultures are responsible for the starter function of acid production. Increasingly, lactobacilli adjuncts are added to cheese milk to contribute to the ripening phase only. The species and strain of lactobacilli; the inoculum level; the presence and densities of other bacteria; the type, composition, and heat treatment of the milk base; presence of additives; and fermentation conditions all contribute to the effects of lactobacilli in dairy products.

Fermented Milk

The main starter culture used in fermented milks is a mixture of *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The ratio used varies according to the dairy product. A 3% inoculum of a bulk starter at an optimum temperature of 40–45 °C may lower the pH sufficiently (pH 4.0–4.5) within 3 h. The two different starter organisms provide each other with beneficial growth compounds (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group). In some drinking yogurts, a mixture of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. paracasei* subsp. *paracasei* slowly brings down the pH over 150 h to minimize gel formation. Other lactobacilli used in fermented milks include probiotic *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. reuteri*, and *Lb. paracasei* (**Table 1**). In many dairy products, these species are added for their health features, as, in general, they do not rapidly acidify milk. Different lactobacilli may impart other benefits to fermented milks in addition to their acid production and health features. For instance, the synthesis of exopolysaccharides improves the rheology, bacteriocins are considered additional preservatives, and the synthesis of acetaldehyde and diacetyl characterizes the flavor of fermented milks.

Cheese

Lactobacillus. delbrueckii subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. helveticus* are the main lactobacilli starter cultures used in cheesemaking (**Table 1**). These are usually used in combination with *Sc. thermophilus* for the acid development in cheese varieties with high cooking temperatures, such as Mozzarella, Parmigiano, and Swiss-type cheeses. These thermophilic lactobacilli survive the high cooking temperatures and continue to make acid in the cooling curd. The species and strain of the thermophilic lactobacilli, the inoculum levels, and the ratio of the starter used with *Sc. thermophilus* are important for controlling the pH profile during curd manufacture. *Lactobacillus helveticus* strains and some strains of *Lb. delbrueckii* subsp. *lactis* have an additional role in some cheese varieties where removal of the galactose produced by *Sc. thermophilus* in the starter is important. Galactose may cause undesirable browning or secondary fermentations in the final cheese.

Inhibitors of Starter Activity

There are industrial examples of bacteriophage (phage) for lactobacilli causing poor coagulation during milk fermentation. Literature searches led to the identification of 231 *Lactobacillus* phages, 186 of which are observable by electron microscopy, with 109 belonging to the *Siphoviridae* family, 76 to the *Myoviridae* family, and 1 to the *Podoviridae* family. Phages of thermophilic lactobacilli (e.g., *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, *Lb. acidophilus*) are responsible for the difficulties in acidification encountered in the preparation of fermented milks and cheeses. Besides, virulent phages specific for some probiotics (e.g., *Lb. casei* and *Lb. paracasei*) were isolated from functional dairy products. The focus of interest was the characterization of different phage resistance mechanisms in lactobacilli. The natural defense mechanisms in lactic acid bacteria against phage infections are often plasmid coded and may be classified into four broad categories: adsorption interference, injection blocking, restriction/modification (R/M) systems, and abortive infection. R/M systems are further classified into Type I, Type II, and Type III on the basis of their composition and cofactor requirements, nature of their target sequence, and the position of the site of DNA cleavage with respect to the target sequence. Some thermal, chemical, and photocatalytic treatments were also proposed for the inactivation of phages (e.g., *Lb. plantarum* bacteriophage).

Natural milk inhibitors (e.g., the peroxidase system), the presence of high somatic cell counts, residues of antibiotics and cleaning agents, microbiologically generated or added hydrogen peroxide, and heat-induced inhibitors are other important factors inhibiting starter activity. Compatibility of lactobacilli with their costarters is important, as a number of strains from different starter species may synthesize bacteriocins that inhibit other starters.

Lactobacillus as Nonstarter Lactic Acid Bacteria

Lactobacilli are one of the few contaminant bacteria that are able to grow in cheese after manufacture. Lactobacilli of nonstarter origin originate either from the milk or from the manufacturing environment. Pasteurization usually eliminates lactobacilli from cheese milk, but recontamination from equipment, personnel, and the environment during cheese manufacture is more difficult to prevent. Hence it is normal for lactobacilli to be present in cheese manufactured from both raw and pasteurized milk, and, given time and a suitable ripening temperature, the initially low levels of lactobacilli in the cheese may increase to high levels (**Figure 3**). However, not all lactobacilli are able to thrive in the low-pH and moderately high-salt environment of cheese, or to grow at normal ripening temperatures, or to utilize the various substrates available in cheese. A relatively limited number of species show all these features, which include *Lb. casei*, *Lb. paracasei*, *Lb. plantarum*, and *Lb. rhamnosus*, which may grow in most cheeses, and *Lb. coryneformis*, *Lb. curvatus*, *Lb. brevis*, *Lb. buchneri*, *Lb. fermentum*, and *Lb. bifermentans*, which grow when the ripening temperature and salt-in-moisture level are suitable. Other species are found in cheese soon after manufacture, but do not tend to dominate the nonstarter microbiota. Most lactobacilli will make some contribution to flavor during the acid development stage and more during cheese ripening.

Significance of NSLAB in Cheeses

In young cheese (ripened <4 months) with modest levels of salt (salt-in-moisture level of 5.2–5.5%), low pH

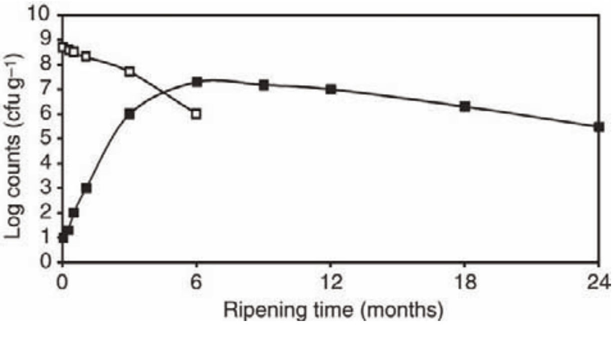

Figure 3 Growth of starter and nonstarter lactic acid bacteria (NSLAB) in Cheddar cheese manufactured from pasteurized milk. □, Starter; ■, NSLAB.

(5.2−5.5), and low ripening temperatures (<10 °C), NSLAB generally have little or no effect unless the lactobacilli are present at high levels soon after manufacture. However, some manufacturing practices, such as returning trimmings and broken blocks from the previous day's manufacture back into the vat, may create a recycling loop. This seeds the cheese leading to relatively high levels of lactobacilli early in the ripening process. Manufacturers that recycle curd tend to produce cheese with a certain strain dominating, and in the matured cheese this may contribute to a distinct flavor typical of the factory (e.g., green-onion flavored or port wine flavored cheese). In mature cheese (ripened >6 months), NSLAB may increasingly influence flavor development. This influence is strain specific, and, more often in matured cheese, the effects may be either desirable and contributing to the flavor development or neutral and so not detracting from or enhancing the flavor development. A number of strains of NSLAB from the *Lb. casei* group have been identified as producing flavor attributes. These strains are increasingly added to vat milk as flavor adjuncts to provide only flavor attributes from the NSLAB, as they outcompete the adventitious NSLAB (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus casei* Group). However, many flavor defects in matured cheese occur due to the adventitious microbiota, normally the NSLAB. Heterofermentative lactobacilli in particular produce undesirable effects in cheese, such as texture problems (crystals and slits), health risks (biogenic amines), and flavor defects (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: Other Species).

Flavor Development

Lactobacilli as starters and NSLAB may play different roles in the metabolism of lactose, lactate, and citrate, and in proteolysis and lipolysis, which are considered the primary events for characterizing cheese flavor. The flavor-generating reactions described in the following paragraphs may be carried out by lactobacilli during cheese ripening. Nevertheless, in many dairy fermentations, whether they contribute significantly to the flavor or not will depend on a large number of other factors (e.g., product type and composition, strain type and density, storage temperature and time).

Metabolism of Lactose

Apart from when milk is treated with β-galactosidase or supplemented with other carbohydrates, lactose is the only carbohydrate available to lactobacilli for rapid growth and acid production. The way lactose is initially utilized by lactobacilli is species- and sometimes strain-dependent and is determined by a combination of the transport system and the lactose hydrolyzing enzyme. The details of how lactose is fermented by lactobacilli have not been established for many lactobacilli species, but there are essentially three possibilities:

1. Many of the lactobacilli transport lactose into the cell via a permease and then hydrolyze the lactose to glucose and galactose by the enzyme β-galactosidase. Glucose and galactose are then metabolized either via the Embden–Meyerhof pathway (or glycolytic pathway) forming mainly lactic acid as the fermentation end product or via the 6-phosphogluconate pathway forming lactic acid, CO_2, and acetate and/or ethanol.
2. Some lactobacilli (e.g., strains of *Lb. delbrueckii* subsp. *bulgaricus*) have a transport system similar to the well-studied system in *Sc. thermophilus* (*see* **Lactic Acid Bacteria**: *Streptococcus thermophilus*). Lactose is transported into the cell in association with the expulsion of galactose via an antiport system. Lactose is hydrolyzed again by a β-galactosidase, but only glucose is further metabolized; galactose is expelled in the lactose transport system.
3. Lactose is transported into the cell as lactose-6-phosphate in some strains of *Lb. paracasei.* This lactose derivative is phosphorylated using phosphoenolpyruvate (PEP) during membrane translocation by the PEP-dependent phosphotransferase system. Lactose-6-phosphate is hydrolyzed by β-phosphogalactosidase into glucose and galactose-6-phosphate. Galactose-6-phosphate is metabolized via the tagatose-6-phosphate pathway and joins glucose, midway along the Embden–Meyerhof pathway, to produce lactic acid. The presence or absence of galactose in the milk fermented with the strain, along with the activity of β-galactosidase and β-phosphogalactosidase in the strain extract, indicates the possible lactose pathway(s) involved.

Metabolism of Lactate

Lactate is an important substrate for a range of reactions that contribute either positively or negatively to cheese ripening. Cheddar cheese contains a considerable concentration of D-lactate, which could be formed by racemization of the L-lactate produced by starter cultures. Racemization of L-lactate is likely to occur more rapidly in cheese made from raw milk due to high numbers of NSLAB. Racemization presumably involves oxidation of L-lactate by L-lactate dehydrogenase (L-LDH) to pyruvate, which, in turn, is reduced to D-lactate by D-LDH. The solubility of Ca-DL-lactate is less than that of pure Ca-L-lactate, so racemization of lactate favors the development of crystals in cheese. Lactate may be metabolized by lactic acid bacteria, depending on the strain, to acetate, ethanol, formate, and CO_2. The oxidation of lactate to acetate in cheese depends on the NSLAB population and on the availability of O_2, which is determined by the size of the block and oxygen permeability of the packaging material.

Metabolism of Citrate

Although citrate is present at low levels in milk ($\sim$8 mmol l^{-1}), its concentration in the aqueous phase of the curd is approximately 3 times higher than that in the whey, presumably reflecting the concentration of colloidal citrate. Cheddar cheese contains 0.2–0.5% citrate. Citrate is not metabolized by most primary starter Cit^- strains of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, but is metabolized by the Cit^+ strains of lactococci, *Leuconostoc* sp. or by thermophilic lactobacilli, with the production of diacetyl, acetate, acetoin, 2,3-butylene glycol, and CO_2. The CO_2 produced is responsible for the characteristic eyes of Dutch-type cheeses and the undesirable openness in Cheddar cheese. Mainly due to the formation of diacetyl, citrate metabolism is very significant in flavor development in Cottage, Quarg, and Dutch-type cheeses. Citrate may be metabolized by some strains of facultatively heterofermentative lactobacilli to acetoin, acetate, and probably diacetyl. It was shown that citrate in Cheddar cheese decreased slowly to almost zero in 6 months, presumably as a result of the metabolism of the growing NSLAB.

Proteolysis

The concentrations of amino acids in milk are below the nutritional requirements for the growth of auxotrophic lactobacilli, and, therefore, their complex proteolytic system degrades mainly caseins into small peptides and amino acids, which fulfill their nutritional needs and inadvertently contribute to the cheese flavor. The main components of the proteolytic system are cell envelope-associated proteinases (although intracellular proteinases have been reported), amino acid and peptide transport systems, and a range of intracellular peptidases (**Figure 4**). During growth in milk, the initial step in casein degradation is carried out by proteinases, and the short (4–40 amino acid residues) peptides produced are taken up by the cell via peptide transport systems. Further degradation to amino acids is catalyzed by a number of intracellular peptidases. Many different peptidases have been characterized from lactobacilli; these include endopeptidases, which degrade oligopeptides to shorter peptides, and exopeptidases, which release one or two amino acids at a time from short peptides.

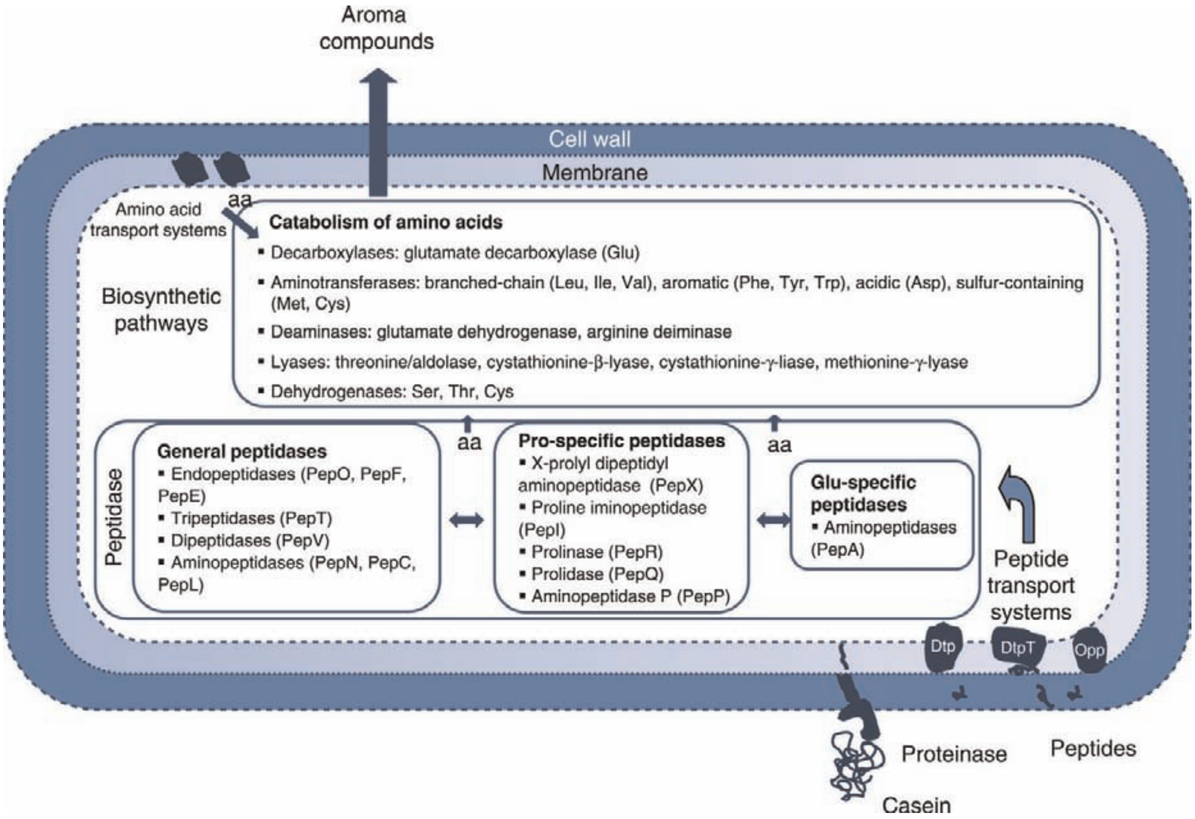
Aroma compounds
Cell wall
Membrane
aa
Amino acid transport systems
Catabolism of amino acids
▪ Decarboxylases: glutamate decarboxylase (Glu)
▪ Aminotransferases: branched-chain (Leu, Ile, Val), aromatic (Phe, Tyr, Trp), acidic (Asp), sulfur-containing (Met, Cys)
▪ Deaminases: glutamate dehydrogenase, arginine deiminase
▪ Lyases: threonine/aldolase, cystathionine-β-lyase, cystathionine-γ-liase, methionine-γ-lyase
▪ Dehydrogenases: Ser, Thr, Cys
Biosynthetic pathways
aa
aa
Peptidase
General peptidases
▪ Endopeptidases (PepO, PepF, PepE)
▪ Tripeptidases (PepT)
▪ Dipeptidases (PepV)
▪ Aminopeptidases (PepN, PepC, PepL)
Pro-specific peptidases
▪ X-prolyl dipeptidyl aminopeptidase (PepX)
▪ Proline iminopeptidase (PepI)
▪ Prolinase (PepR)
▪ Prolidase (PepQ)
▪ Aminopeptidase P (PepP)
Glu-specific peptidases
▪ Aminopeptidases (PepA)
Peptide transport systems
Dtp
DtpT
Opp
Proteinase
Peptides
Casein

Figure 4 Schematic representation of proteolytic enzymes of *Lactobacillus* that contribute to cheese ripening.

On the basis of substrate specificity, lactic acid bacteria possess three types of endopeptidases: PepO, which is capable of hydrolyzing several casein fragments but not di-, tri-, and tetrapeptides; PepF, which specifically cleaves Phe–Ser bond; and PepE, the general properties of which indicate a substrate specificity different from those of the other two metalloendopeptidases. Tripeptidases (PepT) and general dipeptidase (PepV) of lactic acid bacteria have a broad specificity and are capable of hydrolyzing specifically tri- and dipeptides, respectively. Carboxypeptidases are exopeptidases, which catalyze the hydrolysis of peptides from the C-terminal end. No carboxypeptidase activity has been detected in lactococci, but some activity toward N-terminal-blocked peptides has been reported in strains of lactobacilli. The most thoroughly studied exopeptidase from lactic acid bacteria is the general aminopeptidase (PepN). It has a broad specificity, being capable of hydrolyzing a wide range of peptides differing in both size and amino acid composition. Substrates with a hydrophobic or basic amino acid residue at the N-terminal end are hydrolyzed preferentially. PepC in lactic acid bacteria is a metal-independent general aminopeptidase. It shows broad specificity including little activity on peptides with positively charged amino acid residues. PepA is a narrow-specificity peptidase that releases only an N-terminal Glu or Asp from di-, tri-, and oligopeptides with up to 10 amino acid residues. Glutamate is a well-recognized flavor enhancer and, therefore, the role of PepA in the development of flavor in cheese may be of marked importance. The presence of more than one leucyl-aminopeptidase (PepL) has been reported in lactic acid bacteria. PepL preferentially hydrolyzes dipeptides and some tripeptides with an N-terminal leucyl residue.

Caseins are rich in the amino acid proline. Because of its cyclic structure, specialized peptidases are required to hydrolyze peptide bonds involving proline, thus making peptides accessible to the action of other peptidases. Several proline-specific peptidases with distinct substrate specificities have been found in lactic acid bacteria. X-prolyl dipeptidyl aminopeptidase (PepX) is a peptide hydrolase capable of releasing X-Pro and sometimes X-Ala dipeptides from the N-terminal end of oligopeptides. This enzyme influences proteolysis and sensorial characteristics of Gruyere cheese. Proline iminopeptidase (PepI) catalyzes the release of an N-terminal proline residue from di-, tri-, and oligopeptides. Prolinase (PepR) and prolidase (PepQ) are specific dipeptidases that hydrolyze dipeptides with the sequence Pro-X and X-Pro, respectively. Aminopeptidase P (PepP) is a specific aminopeptidase that catalyzes the removal of the N-terminal amino acid from oligopeptides having the sequence X-Pro-Pro-(X)*n* or X-Pro-(X)*n*. The combination of proteinase and peptidase activities may contribute to flavor changes through the peptides and amino acids produced both in cheeses and in fermented milks.

Catabolism of Free Amino Acids

Catabolism of free amino acids plays a major role in flavor development during cheese ripening. Lactobacilli may utilize a large variety of amino acids that are likely to contribute to flavor changes in dairy products. The first step in amino acid catabolism could be a decarboxylation, deamination, or elimination carried out by an enzyme from one of the following five groups: decarboxylases, transaminases, deaminases, lyases, and dehydratases (see **Figure 4**). The decarboxylation reaction (e.g., by glutamate decarboxylases, GAD) regulates the intracellular pH. GAD enzymes are found in some lactobacilli such as *Lb. plantarum*, *Lb. paracasei*, and *Lb. brevis*. During cheesemaking experiments, a correlation was found between the use of raw milk and the decarboxylation activities on Glu to yield γ-aminobutyric acid (GABA), as well as on Tyr and His to yield tyramine and histamine, respectively. Aminotransferase transfers the amino group from an amino acid to an α-keto acid (usually α-ketoglutaric acid) and this results in the synthesis of new α-keto acids and amino acids (usually Glu). α-Keto acids produced by the transamination of aromatic and branched-chain amino acids and Met are further degraded to other compounds (e.g., aldehydes, alcohols) by enzyme-catalyzed or chemical reactions. Aminotransferases from lactobacilli are specific to different groups of amino acids such as branched-chain (Leu, Ile, Val), aromatic (Phe, Tyr, Trp), sulfur-containing (Cys, Met), or acidic (Asp) amino acids. Glutamate dehydrogenase (GDH) is the key enzyme for the catabolism of amino acids by producing α-ketoglutaric acid from Glu. Lactobacilli use Arg to produce ATP, ornithine, carbamoyl phosphate, and NH_3 by the arginine deiminase pathway (ADI). Amino acid lyases cleave the side chains of amino acids. These pathways are particularly important for the catabolism of aromatic amino acids and Met. Other pathways by which amino acids may be catabolized include the activation of deaminases and dehydratases. There are also specific pathways for the metabolism of Glu and Arg, and Ser, Thr, and Cys, respectively. Transamination was found in *Lb. helveticus*; it seems to be the first and the main step for the conversion of free amino acids by *Lb. helveticus*, with Phe and Tyr being converted most efficiently. Aminotransferase activity was also shown in NSLAB. *Lactobacillus paracasei* subsp. *paracasei* strains generate aldehydes, alcohols, and acids from branched-chain amino acids, Phe, and Met when grown in media containing casamino acids or lactalbumin hydrolysate. Volatile sulfur compounds are found in most cheeses and are important components for flavor. As Met is present in the caseins at a higher concentration than Cys, sulfur compounds in cheese presumably originate mainly from Met. The major aroma compounds produced from Met are methional, methanethiol and its oxidation products, dimethylsulfide, and dimethyltrisulfide. Methionine-γ-lyase catalyzes the conversion of methionine to α-ketobutyrate, methanethiol, and ammonia. Cystathionine-β-lyase and cystathionine-γ-lyase catalyze the conversion of cystathionine to homocysteine, pyruvate, and ammonia, and to cysteine, ammonia, and α-ketobutyrate, respectively. Homocysteine is in turn converted to Met by the activity of homocysteine methyltransferase. Several primary starters such as *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus* are capable of degrading Met to methanethiol, dimethylsulfide, and dimethyltrisulfide. The ability of NSLAB strains of *Lb. casei* and *Lb. plantarum* to produce flavor compounds was investigated. Some strains were found to be capable of transaminating methionine to α-keto-γ-methylthiobutyrate, but no methionine lyase or amino acid decarboxylase activities were detected. Moreover, *Lb. fermentum* and *Lb. reuteri* produce aroma compounds from sulfur amino acids, but *Lb. brevis*, *Lb. paracasei*, and *Lb. curvatus* do not. *Lactobacillus fermentum* DT41 was isolated from a natural starter for Parmigiano-Reggiano cheese; it contains a cystathionine-γ-lyase that retains activity under cheese-ripening conditions. The same enzyme was purified from *Lb. reuteri* DSM 20016. This microorganism, together with other lactobacilli, was used as an adjunct in the manufacture of Canestrato Pugliese-type cheese, and cheeses containing an adjunct composed of *Lb. fermentum* DT41 and *Lb. reuteri* DSM 20016 had the highest levels of methanethiol, dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide. Dehydratases are active in anaerobic environment, such as the interior of cheese during ripening, hydrolyzing amino acids containing OH or SH groups (Ser, Thr, Cys).

Aromatic amino acid catabolism by lactobacilli may produce undesirable flavors including *para*-cresol, indole, and skatole, and harmful biogenic amines such as tryptamine and tyramine.

Lipolysis

Lipids play an important role in cheese flavor by acting as a source of free fatty acids, which in turn may be catabolized to other flavor compounds (e.g., methyl ketones), and as the solvent for sapid compounds produced from lipids or other precursors. Lactic acid bacteria possess intracellular esterolytic/lipolytic enzymes capable of hydrolyzing a range of derivatives of free fatty acids. Lipases and esterases of lactic acid bacteria seem to be the principal lipolytic agents in Cheddar and Dutch-type cheeses made from pasteurized milk. Evidence for this comes from studies on aseptic starter-free cheeses acidified with gluconic acid-δ-lactone, where very low levels of free fatty acids are released during ripening, and from the relationship between autolysis of primary starter cells and free fatty acid levels during ripening. Intracellular esterolytic activities were found in *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus*. The presence of lipase and esterase activities was shown in NSLAB also. In the majority of strains, activities increased as the carbon chain length of the fatty acid decreased. Intracellular lipases and esterases were purified and characterized from *Lb. plantarum*, *Lb. casei*, and *Lb. fermentum*. The response of esterase activity to the effects of salt, temperature, and pH is strain dependent. *Lactobacillus* spp. are weakly lipolytic in comparison to species such as *Pseudomonas* and *Flavobacterium*, and because they are present in large numbers over an extended ripening period, lactic acid bacteria are responsible for the liberation of significant levels of free fatty acids in many cheese varieties that do not have a strongly lipolytic enzyme and/or secondary microbiota.

Claims of Probiotic Effects

Probiotics are described as “live microorganisms which when administered in adequate numbers confer a health benefit on the host”. The delivery of probiotics in dairy products was traditionally via fermented milks such as yogurt, but more recently milk powders and cheeses are also being considered as possible hosts. Although strictly strain dependent, the probiotic lactobacilli include *Lb. acidophilus*, *Lb. brevis*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis, Lb. fermentum*, *Lb. gasseri*, *Lb. helveticus*, *Lb. johnsonii*, *Lb. paracasei* subsp. *paracasei*, *Lb. paracasei* subsp. *tolerans, Lb. plantarum*, *Lb. reuteri*, and *Lb. rhamnosus* (see **Table 1**). The evidence and mechanisms of the claimed beneficial health effects from the use of probiotic lactobacilli cultures have been intensively investigated. The standard of evidence varies from strain to strain as well as between the many health claims. The long history of the safe use of probiotic cultures delivered in dairy foods remains the best proof of their safety, particularly when comparing the many side effects from the use of antibiotics. Probiotics, particularly *Lactobacillus* species, are used to improve gut health, where often the mechanism is believed to be the improvement of the microecology of the gut; the synthesis of antibacterial substances may be important as well. Lactose tolerance is improved with acidophilus milk, and the same probiotic, *Lb. acidophilus*, decreases serum cholesterol. The probiotic lactobacilli, in addition to influencing the gut microbiota and preventing colonization by pathogens, may also influence the occurrence of infections in tissues besides the gut by enhancement of immune function. Other beneficial health effects such as antitumor and antimutagenic effects are under investigation. The most extensively studied probiotic strains include *Lb. acidophilus* LA1, *Lb. paracasei* biovar shirota, and *Lb. rhamnosus* GG. Often the cultures in dairy foods include the well-known probiotic strains of *Bifidobacterium* and the yogurt starter *Sc. thermophilus*. A probiotic culture delivered in a dairy food should be viable and abundant (at least 10^7 cfu g^{-1}).

Production of Bacteriocins by Lactobacilli

Lactobacilli produce a number of substances, including lactic acid, hydrogen peroxide, diacetyl, and bacteriocins, that are inhibitory to other bacteria. Bacteriocins may be loosely defined as proteins produced by bacteria that are bactericidal (or bacteriostatic) to closely related bacteria. However, many Gram-positive bacteria synthesize bacteriocins that are also active against unrelated bacteria. Bacteriocins of lactic acid bacteria are heterogeneous peptides and were previously classified into five groups; based on the recent classification, they are divided in to two classes. Class I consists of lanthionine-containing bacteriocins, or lantibiotics, which include both single-peptide (nisin, mersacidin, lacticin 481) and two-peptide (lacticin 3147, cytolysin) lantibiotics. Class II is comprised of a very large group of non-lanthionine-containing bacteriocins and is further divided into four subclasses: class IIa includes pediocin-like peptides, such as pediocin PA-1(AcH) and leucocin A; class IIb consists of two-peptide bacteriocins, such as lactacin F; class IIc consists of cyclic bacteriocins, such as enterocin AS48 and reuterin 6; and class IId consists of nonpediocin single linear peptides, such as lactococcin A and divergin A. Bacteriocins produced by lactobacilli are nontoxic and meet the requirements for food preservatives. For a selection of bacteriocins synthesized by lactobacilli and the bacteria they are active against, *see* **Bacteriocins**. Bacteriocins have two main applications in cheese manufacture: (1) to improve the safety of the cheese through inhibition of pathogenic microorganisms and (2) to improve the quality of cheese products, achieved through inhibition of undesirable and spoilage microorganisms or through starter culture lysis. With regard to the inhibition of pathogens, the addition of the pediocin PA1(AcH)-producing *Lb. plantarum* strain led to an almost complete inhibition of *Listeria monocytogenes* when the pathogen was added at 10^2 cfu ml^{-1} of salt brine solution to red smear cheeses. *Listeria monocytogenes* may be a persistent problem in smear cheese varieties such as Tilsit, Limburg, Dambo, and Munster, and soft cheeses such as Cottage cheese. This pathogen survives over a wide pH range, at refrigeration temperatures, and is tolerant of salt concentrations as high as 20%. Some *Listeria*-active bacteriocins produced by lactobacilli and nisin produced by lactococci were found to be very effective in controlling *L. monocytogenes* as well as other pathogens such as *Clostridium sporogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. The bacteriocin culture may be either added to the vat at the time of cheesemaking or sprayed onto the surface of the cheese after manufacture. The use of bacteriocin-producing lactobacilli was also tested in brine-salted cheese to prevent 'late blowing' caused by *Clostridium*. The *Lactobacillus* culture must not inhibit the starter culture during cheesemaking, but must reach sufficient numbers in the first few days after manufacture to produce inhibitory levels of bacteriocin. If this does not occur within the first few days, then *Clostridium* can increase in numbers to a level where the bacteriocin becomes ineffective. Bacteriocin-producing strains must be chosen carefully, as the presence of high levels of lactobacilli early in the ripening process will have an effect on cheese flavor.

Bacteriocins may also be used to control undesirable microbial populations. Spoilage microorganisms (e.g., Gram-negative bacteria) and also some NSLAB are associated with a number of defects including the development of off-flavors, the formation of calcium lactate crystals, and slit formation in cheese. The control of undesirable microbial populations is undoubtedly desirable and leads to the development of a more consistent and predictable end product in cheesemaking. For instance, a lacticin 3147-producing *Lc. lactis* reduces NSLAB population in full-fat cheese by at least 100-fold as compared to the control cheese manufactured with a bacteriocin-negative strain. As stated above, some lactobacilli NSLAB contribute positively to the flavor and quality of cheese. Consequently, a starter culture system that uses lacticin 3147-producing starters in combination with 3147-tolerant NSLAB (e.g., *Lb. paracasei* subsp. *paracasei* DPC5336) was developed.

There is an important link between the rate of starter cell lysis (autolysis) and the development of cheese flavor. Consequently, there has been a tremendous effort to increase the rate of autolysis, including through the elevation of ripening temperature, the use of lytic bacteriophage, and the addition of exogenous enzymes. Bacteriocins may also be used in the acceleration of cheese ripening through a bacterolytic action that results in lysis of the target strain. For instance, the use of plantaricin-producing *Lb. plantarum* strains in combination with *Lb. helveticus* resulted in an acceleration in the release of intracellular aminopeptidases. Overall, the use of bacteriocins in dairy products is increasingly being investigated, as bacteriocins produced by cultures in dairy products are not considered to be an additive.

See also: **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products. **Bacteriocins**. **Cheese**: Cheese Flavor. **Fermented Milks**: Yogurt: Role of Starter Culture. **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii*

Group; *Lactobacillus* spp.: Other Species; *Streptococcus thermophilus*; Taxonomy and Biodiversity.

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Lactobacillus spp.: *Lactobacillus acidophilus*

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Introduction and History

The genus *Lactobacillus* was first proposed more than 100 years ago in 1901 by M. W. Beijerinck. The species *acidophilus* (meaning "acid loving") was so named perhaps because, historically, lactobacilli are isolated from the intestinal tract and vagina of humans and animals, where the environment can be quite acidic. *Lactobacillus acidophilus* is one of the most commonly recognized species of the genus *Lactobacillus*. This is mainly due to the commercialization of this species in various health-promoting fermented foods and dietary supplements.

Classification and Taxonomy

Since its first description as *Bacillus acidophilus* (from infant feces; Moro, 1900), lactobacillus-type bacteria have been isolated from the mouth, vagina, and gastrointestinal (GI) tract of many animals including humans and until 1970 were classified as *Lb. acidophilus* based on classical microbiological techniques. *Lactobacillus acidophilus* was adequately described for the first time in 1970 by Hansen and Mocquot, who designated a neotype strain (ATCC 4356) for it according to the recommendations of the International Subcommittee on Taxonomy of Lactobacilli and Closely Related Organisms. In 1980, based on DNA hybridization studies, six homologous groups were distinguished among *Lb. acidophilus* and designated as A-1, A-2, A-3, A-4, B-1, and B-2. The group A strains have 20–30% intergroup homology but very low homology with groups B-1 and B-2. DNA/DNA homology values of about 20–50% are found between individual strains of different groups. This later led to definition of the species *Lb. acidophilus* (neotype strain ATCC 4356), *Lb. amylovorus*, *Lb. crispatus*, *Lb. gallinarum*, *Lb. gasseri*, and *Lb. johnsonii*.

First described in 1991, the 16S rRNA sequences of lactobacilli help to build a picture of the phylogenetic relatedness of the various species. Phylogenetically, *Lb. acidophilus* is most closely related to *Lb. helveticus*. There is little correlation between physiologically or morphologically based subdivision of lactobacilli and their phylogenetic relatedness. It has also been proposed that the genus *Lactobacillus* and the related genera be allocated to three groups, and that *Lb. acidophilus*, along with *Lb. delbrueckii* and the other obligately homofermentative lactobacilli, be classified together in Group 1.

Microarray analyses of the *Lb. acidophilus* complex for inter- and intraspecies diversity have recently been conducted and complemented with multilocus sequence analysis, DNA typing, and comparative genomics. The use of modern whole-genome-based techniques demonstrated a remarkably consistent pattern of similarity within the *Lb. acidophilus* complex despite highly variable genomic G+C contents.

The current taxonomy of *Lactobacillus acidophilus* is as follows:

Phylum: Fermicutes
Class: Bacilli
Order: Lactobacillales
Family: Lactobacillaceae
Genus: *Lactobacillus*
Species: *Lactobacillus acidophilus*

Morphology and Growth Conditions

Microscopic Morphology

Morphologically, the *Lb. acidophilus* group is Gram-positive, non-spore-forming rods with rounded ends, generally 0.6–0.9 × 1.5–6 μm, that occur singly, in pairs, and in short chains.

Culture Morphology

Colonies on agar media are usually small (2–5 mm) with well defined margins, convex, smooth, glistening, and opaque without pigments. Growth in liquid media generally occurs throughout the liquid. Pellicles are never formed.

Growth Conditions

With rare exceptions, good growth occurs at 45 °C, in slightly acidic media with an initial pH of 6.4–4.5. Although most strains are fairly aerotolerant, optimal growth is achieved under microaerophilic or anaerobic conditions. The members of the *Lb. acidophilus* group are fastidious organisms adapted to growth on complex organic substrates. The species requires not only carbohydrates as carbon source but also nucleotides, amino acids, and vitamins. Calcium pentothenate, folic acid, niacin, and riboflavin are essential; pyridoxal, thiamine, thymidine, and vitamin B_{12} are not required.

General Characteristics

The *Lb. acidophilus* group contains mainly obligately homofermentative lactobacilli, but a few are facultative heterofermenters. Initially, the group was classified as lactic acid thermobacteria because of their ability to grow at 45 °C and their homofermentative metabolism. The mol% G+C of the DNA is 32–37 (Bd, Tm). Electron micrographs of thin sections reveal a typical Gram-positive cell wall profile. The cross-linkage type observed in cell wall peptidoglycan is Lys-D-Asp.

Biochemical Properties

Carbohydrate utilization pattern of *Lb. acidophilus* and other physiological and biochemical characteristics are summarized in **Tables 1** and **2**.

Isolation and Enrichment

The isolation of *Lb. acidophilus* from any source must take into account the acidophilic nature, complex nutritional requirement, and the preference for microaerophilic conditions of the organism. If the predominant microorganisms in the source material are lactobacilli, a nonselective medium such as MRS agar can be used for isolation. However, if *Lb. acidophilus* is part of a mixed population of different genera of microorganisms, a selective medium must be employed for isolation. The most commonly used selective medium for this purpose is the acetate medium of Rogosa. This medium is not completely selective and will support growth of other lactic acid bacteria such as pediococci, bifidobacteria, and enterococci. Yeasts and molds can be eliminated by addition of cycloheximide at a concentration of $100\,\mu g\,ml^{-1}$. Supplementation with specific growth factors such as sodium acetate, bile oxgall, or tomato juice will assist growth of *Lb. acidophilus*. When isolating *Lb. acidophilus* from sources such as feces, the mouth, or the vagina, addition of 0.05% (w/v) cysteine to the medium is recommended. Lactobacilli prefer anaerobic conditions, and growth is stimulated in broth or agar under a standard anaerobic environment of 90% nitrogen (or hydrogen) and 10% carbon dioxide.

Identification and Characterization

With increasing interest in the health-promoting properties of lactobacilli, it is critically important to have a reliable means of identifying the strain of interest. Unequivocal characterization of bacterial strains to be used for human consumption is extremely important for proprietary reasons (i.e., protection of intellectual property) and for safety reasons (e.g., GRAS status). This is especially important for establishing the contribution of the added strains to the indigenous human intestinal microbiota and for controlling any unique beneficial properties.

In the past decade, major advances have been made in molecular taxonomy of lactobacilli, and an increasing number of techniques are becoming available for reliable characterization of new lactobacilli strains including species of *Lb. acidophilus*. These techniques include

Table 1 Carbohydrate fermentation pattern for *Lactobacillus acidophilus*

Amy	*Ara*	*Cell*	*Esc*	*Fru*	*Gal*	*Glu*	*Lac*	*Mal*	*Man*	*MelT*	*MelB*	*Raf*	*Rha*	*Rib*	*Sal*	*Sor*	*Suc*	*Tre*	*Xyl*
+	–	+	+	+	+	+	+	+	+	–	d	d	–	–	+	–	+	d	–

The carbohydrates are amygdlin (Amy), arabinose (Ara), cellulose (Cell), esculin (Esc), fructose (Fru), galactose (Gal), glucose (Glu), lactose (Lac), maltose (Mal), mannose (Man), meltriose (MelT), melbiose (MelB), raffinose (Raf), rhamnose (Rha), ribose (Rib), salicin (Sal), sorbitol (Sor), sucrose (Suc), trehalose (Tre), and xylose (Xyl).
+, 90% or more strains positive; –, 90% or more strains negative; d, 11–89% strains positive.

Table 2 Physiological and biochemical characteristics of *Lactobacillus acidophilus*

		Electrophoretic mobility				
Peptidoglycan type	*Teichoic acid*	*D-LDH*	*L-LDH*	*Lactic acid isomer*	*Growth at 15 °C*	*NH_3 from arginine*
Lys-D-Asp	Glycerol	1.5[a]	1.3	DL[b]	No	No

[a] Some strains give more than one band. Distance recorded is from type strain.
[b] 25–75% of the total lactic acid is of L- configuration.

sequencing of the 16S or 23S rRNA regions, and the 16S–23S intergenic spacer region, which is used to define identity at the species level. The use of synthetic 16S and 23S rRNA-targeted genus- and species-specific hybridization probes enable specific detection, enumeration, and identification *in situ* or after differential plating. Pulsed-field gel electrophoresis, although a labor-intensive technique, is often very effective in discriminating between strains on the basis of overall genomic organization. Description of any new member of the lactobacilli should take a polyphasic approach, that is, should be based on the integration of phenotypic, genotypic, and phylogenetic information.

Enumeration and Maintenance

The method for enumerating *Lb. acidophilus* in yogurts and other dairy products as recommended by the International Dairy Federation is MRS agar at pH 5.4. Under these conditions, the growth of starter cultures such as *Streptococcus thermophilus* is inhibited, but other lactobacilli present in the product will grow in this medium. It is advisable to check the recovery of the particular strain of *Lb. acidophilus* used in the yogurt on MRS agar at pH 5.4 relative to standard MRS agar, as some strains give reduced recoveries at the lower pH.

When probiotic cultures of lactobacilli are added to a particular dairy product, it may be necessary to check the identity of the strains on the isolation medium by carbohydrate fermentation patterns and a more specific DNA-based method.

For short-term storage, *Lb. acidophilus* cultures can be propagated on MRS agar stabs, and, once growth is visible, stored at 4 °C. Freeze-drying is the method of choice for longer-term storage. In this, cultures are grown to late log phase and the cells are then resuspended in sterile skim milk, freeze-dried, and stored in sealed ampoules under nitrogen. In this form, the cultures can be preserved for 10–20 years.

Bacteriocins

Bacteriocins are proteinaceous substances with bactericidal activity, and many strains of *Lb. acidophilus* have been reported to produce bacteriocins. The antimicrobial activity of these strains, in some instances, can be due to the production of lactic acid and/or hydrogen peroxide. Because some strains of *Lb. acidophilus* are strong producers of hydrogen peroxide, care must be taken not to confuse this characteristic with bacteriocin production. Lactocidin, first isolated from *Lb. acidophilus*, was reported to have a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, but these activities were later shown to be due to a combination of hydrogen peroxide, organic acid, and an antibiotic-type substance. The bacteriocins produced by the *Lb. acidophilus* group include lactocin B, lactacin F, acidocin A, and acidocin B.

Lactocin B is produced by the *Lb. acidophilus* N2. Its molecular mass is estimated to be 6200 Da, but it can form aggregates with molecular masses of ~100 000 Da. The spectrum of activity of this bacteriocin includes *Lb. bulgaricus*, *Lb. helveticus*, *Lb. lactis*, and *Lb. leichmannii.*

Lactacin F is produced by *Lb. acidophilus* 11088 (NCK88), and has a molecular mass of 2500 Da. The spectrum of activity of this bacteriocin includes *Enterococcus faecalis* and *Lb. fermentum* in addition to the four strains found for lactacin B.

The activity spectrum of acidocin B is quite different from those of lactocin B and lactocin F. Acidocin B is active against pathogens such as *Listeria monocytogenes*, *Clostridium sporogenes*, and *Brochthrix thermosphacta*, but shows no activity against lactobacilli.

Bacteriocins may be important physiologically in providing a competitive advantage to a particular strain in an ecological niche. This property may be of specific relevance to probiotic activity when these strains are ingested by humans, thereby enabling them to compete with pathogens in the gastrointestinal tract.

Significance of *Lactobacillus acidophilus* in Fermented Dairy Products and Human Health

Eli Metchnikoff, a Nobel Prize-winning Russian scientist, is credited with initiating the concept of the benefits of ingestion of lactic acid bacteria in human health. This concept has given rise to a new field of research termed as 'probiotics'. The most accepted definition of probiotics is "live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host". Many strains of the genera *Lactobacillus* and *Bifidobacterium* have been shown to produce prophylactic as well as therapeutic effects in humans. As a result, these strains are increasingly incorporated into various food products, specifically fermented and nonfermented dairy products. The discussion in this section concentrates on the use of *Lb. acidophilus* strains in dairy products.

Lactobacillus acidophilus in Fermented Dairy Products

Lactobacillus acidophilus strains have been used in the manufacture of acidophilus milk, yogurt, yogurt drinks, miru-miru, kefir, and koumiss. It is very important to maintain a minimum level of viability for a given strain of *Lb. acidophilus* in these products because the products are often sold with a claim, for example, "product will deliver, a given number of live cells of a particular strain, per serving". The final pH appears to play an important role in determining the stability and survival of *Lb. acidophilus* in these products. Higher pH values allow a longer survival of *Lb. acidophilus*. The storage temperature is another factor that has an impact on the survival of *Lb. acidophilus*. Survival is generally higher at lower temperatures (e.g., refrigerator temperatures of 5–9 °C) than at room temperature (25 °C).

During the production of yogurt and yogurt drinks, the chosen strain of *Lb. acidophilus* is used in combination with the yogurt starter strains of *Str. thermophilus*. The associative growth of these two species in milk is important. The growth rate of *Str. thermophilus* is often much higher than that of *Lb. acidophilus*. However, the growth of *Lb. acidophilus* in milk benefits from the metabolites such as formic acid, and possibly carbon dioxide and pyruvate, produced by *Str. thermophilus*. It is important to keep these factors in mind when fermenting milk with a combination of two different strains, because the strain composition of the final fermented product will depend on these factors. This means that the strain pairing and ratio of the two species must be optimized in the different milk fermentations to produce a product with a defined number of *Lb. acidophilus*. It has been suggested that instability of the *Lb. acidophilus* cells added to yogurt can be caused by the production of hydrogen peroxide by the lactobacilli in yogurt starter cultures. Inclusion of reducing agents such as thioglycolate, cysteine, and dithiothreitol confers greater stability on *Lb. acidophilus*, perhaps via direct interaction with the –SH group of key enzymes involved in the pathway of hydrogen peroxide production.

Sometimes, when milk is fermented with strains of *Lb. acidophilus* alone, the product lacks the characteristic buttery flavor of yogurt (due to a lack of production of acetaldehyde). As a result, the fermented acidophilus milk is tart and plain. To improve the flavor of such products, fruit juices are often mixed in varying ratios. However, certain fruit juices, such as strawberry, have been reported to have a negative effect on the viability of *Lb. acidophilus*.

Lactobacillus acidophilus can also be delivered through dry dairy products such as milk powders. Such products usually require a much longer shelf life, often at higher temperatures. An important factor to keep in mind for such products is the water activity of the milk powders; strains usually survive better at lower water activity.

Lactobacillus acidophilus in Human Health

Lactobacilli are generally accepted as human probiotics, and many strains of *Lb. acidophilus* with health benefits have been described. The health benefits associated with these strains include reduction of gastrointestinal symptoms in lactose-intolerant individuals, relief from symptoms of constipation, treatment of infantile diarrhea, prevention of travelers' diarrhea, and activity against *Helicobacter pylori*.

Lactobacillus acidophilus strain NCFM is a well-studied probiotic strain that has been commercialized. This is the only strain of *Lb. acidophilus* whose genome has been fully sequenced and annotated. A number of health benefits for this strain have been described. Some of the more interesting findings with this strain include an analgesic effect in the intestine and the induction of opioid and cannabinoid receptors. Evidence for the benefits of some other published strains includes the immune-enhancing effects of *Lb. acidophilus* HN017 in a mouse model and the stimulation of phagocytosis by *Lb. johnsonii* (La-1) in humans.

Traditionally, the quality of published studies in the area of probiotic effects of various strains of lactic acid bacteria has been quite variable. In recent years, well-designed clinical studies that are double-blind and placebo-controlled have started to appear in peer-reviewed scientific journals, validating some of the health benefits of different probiotic strains.

See also: **Lactic Acid Bacteria:** *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species.

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Lactobacillus spp.: *Lactobacillus casei* Group

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Introduction

Lactobacillus casei was proposed as *Streptobacterium casei* by Orla-Jensen in 1916. The isolate that was first described was found in cheese, but *Lb. casei* has since been shown to be a widely distributed group of bacteria. It can be found in various habitats including decaying plant material, silage, the human intestinal tract, the mouth and vagina, sewage, and various dairy products. Because of its aciduric nature, it is found in many fermented products from both plant and animal origin.

The two common dairy species are *Lb. paracasei* subsp. *paracasei* (*Lb. paracasei*) and *Lb. rhamnosus*. They are most often identified as the main adventitious bacteria (commonly called the nonstarter lactic acid bacteria) present and growing in ripening cheese. As adventitious bacteria, they can contribute to flavor attributes or defects or have no impact on cheese ripening depending on the strain, the cell densities, and the cheese conditions. Since the last decade, selected strains of *Lb. casei* group have been increasingly used as adjuncts for cheese ripening or as probiotic cultures in different foods, especially cheeses and fermented milks.

Characteristics of the *Lb. casei* Group

Members of the *Lb. casei* group are nonmotile, nonsporeforming, catalase-negative, Gram-positive rods, and generally $0.7 \pm 1.1\ \mu m \times 2.0 \pm 4.0\ \mu m$, occurring singly, in pairs, or in chains. The G + C ratio of the DNA is between 45 and 47 mol%. The peptidoglycan of the cell wall is of the Lys-D-Asp type. No teichoic acid is found bound to the cell wall. The enzyme urease is not produced and ammonia is not produced from arginine. Riboflavin, folic acid, calcium, pantothenate, and niacin are essential for growth, and pyridoxal or pyridoxamine may be required for growth by some strains.

All species in the group are facultatively heterofermentative lactobacilli that produce lactic acid as the major end product of fermentation and do not produce gas from glucose. Under carbohydrate-limiting conditions, mainly acetic acid and ethanol, followed by butyric acid, diacetyl, and formic acid, are produced in addition to lactic acid. Pentoses are fermented to lactic acid and acetic acid. Usually only the L(+) isomer of lactic acid is produced, although some strains of *Lb. paracasei* can form an equal mixture of the L(+) and D(−) isomers.

Division of the *Lb. casei* Group

Members of the *Lb. casei* group exhibit considerable phenotypic and genotypic heterogeneity, which has led to various attempts to classify the group. In the past, all members were classified as *Lb. casei* with five subspecies (*alactosus*, *casei*, *pseudoplantarum*, *rhamnosus,* and *tolerans*) in the Approved List of Bacterial Names. This classification was based on phenotypic properties, particularly carbohydrate fermentations that are often unreliable for differentiation of members of the group. Thanks to genetic techniques, such as DNA–DNA homologies and 16S rRNA sequencing, species of the *Lb. casei* group were reclassified into three species: (1) *Lb. casei* (formerly *Lb. casei* subsp. *casei*); (2) *Lb. paracasei* with two subspecies *paracasei* (including the former *Lb. casei* subsp. *alactosus* and subsp. *pseudoplantarum*) and *tolerans* (including the former *Lb. casei* subsp. *tolerans*); (3) *Lb. rhamnosus* (formerly *Lb. casei* subsp. *rhamnosus*).

This classification, however, initiated a stream of controversial results, largely due to the failure of differentiation between most of *Lb. paracasei* and *Lb. casei* strains even by molecular techniques. For example, all strains of *Lb. paracasei* studied in depth showed a very high genetic similarity to a reference strain (ATCC 334) of *Lb. casei*. Furthermore, a high DNA–DNA homology (82%) between the type strain *Lb. casei* ATCC 393^T and the type strain *Lb. zeae* ATCC 15820^T (first named *Lactobacterium zeae* Kuznetsov 1959) was observed. All this led some researchers to request the Judicial Commission of the International Committee on Systematics of Bacteria for an opinion on the following proposals: (1) to include ATCC 393^T in *Lb. zeae*; (2) to accept ATCC 334 as the new type strain for *Lb. casei*; (3) to reject the name *Lb. paracasei*.

Although the Judicial Commission recognized the data presented by those researchers, together with previous publications, as scientifically sound, the proposals were rejected substantially because the Judicial Commission can rule on matters of nomenclature, not of classification. Consequently, the Judicial Commission affirmed that typification of *Lb. casei* had to be based on ATCC 393^T, that ATCC 334 had to be allotted to a different taxon

(*Lb. paracasei*), and that the name *Lb. paracasei* had not to be rejected. On the contrary the Judicial Commission rejected the revival of the name *Lb. zeae*.

In conclusion, it seems appropriate to clarify that the currently accepted nomenclature and taxonomic division of the *Lb. casei* group is (1) *Lb. casei* (type strain: ATCC 393^T alias NCDO 161^T); (2) *Lb. paracasei* subsp. *paracasei* (type strain: ATCC 25302^T) and *Lb. paracasei* subsp. *tolerans* (type strain: ATCC 25599^T); (3) *Lb. rhamnosus* (type strain: ATCC 7469^T alias NCDO 243^T). Therefore, the name '*Lb. zeae*' should not be used. All papers accepted for publication before the opinion of the Judicial Commission had been issued should be interpreted carefully, because some authors had already abandoned the name '*Lb. paracasei*', whereas others had not. In the following sections, where possible, the generic '*Lb. casei* group' will be used. Alternatively, the nomenclature adopted by the authors of the studies cited will be used.

Distinguishing Features of the *Lb. casei* Group

Lactobacillus casei

Few strains of *Lb. casei* have been studied in depth. Those strains that have been studied can be distinguished from other members of the group by their inability to ferment lactose and sucrose (**Table 1**). Growth at 10 °C, but not at 45 °C, is in common with *Lb. paracasei*, and lack of growth at 45 °C distinguishes these species from *Lb. rhamnosus*. It should be noted that in much of the older literature strains of *Lb. casei* are probably *Lb. paracasei*.

Lactobacillus paracasei subsp. *paracasei*

After a study published in 1989, the majority of strains formerly classified as *Lb. casei* were reclassified as *Lb. paracasei* subsp. *paracasei*. Carbohydrates fermented by *Lb. paracasei* are listed in **Table 1**. Growth at 10 °C but not at 45 °C is in common with *Lb. casei* and *Lb. paracasei* subsp. *tolerans*, and lack of growth at 45 °C and the inability to ferment rhamnose distinguish these species from *Lb. rhamnosus*. A few strains produce racemic lactic acid due to the activity of both the L(+) and D(−) lactate dehydrogenases.

Lactobacillus paracasei subsp. *tolerans*

The main distinguishing feature of this subspecies is its ability to survive heating at 72 °C for 40 s. As with *Lb. casei* and *Lb. paracasei* subsp. *paracasei*, this subspecies grows at 10 °C but not at 45 °C. It ferments fewer carbohydrates than the others in the group (**Table 1**) and gives a very different fermentation profile in most identification kits.

Lactobacillus rhamnosus

Lactobacillus rhamnosus can be distinguished from the other members of the *Lb. casei* group by its ability to produce acid from rhamnose and its ability to grow at 45 °C. All strains grow at 15 °C and most strains grow at 10 °C.

Isolation and Identification

Isolation of the *Lb. casei* group generally relies on pH of the medium and/or acetate concentration to inhibit the growth of other bacteria. Both Rogosa agar at pH 5.35 ± 0.05 and MRS agar at pH 5.4 will allow the growth of *Lb. casei* and inhibit many other bacteria found in similar environments such as lactococci and *Streptococcus thermophilus*. MRS agar with 100 $\mu g\,ml^{-1}$ vancomycin will prevent the growth of lactococci, *Sc. thermophilus,* and some lactobacilli including the *Lb. delbrueckii* group and *Lb. helveticus*.

As neither Rogosa agar nor MRS agar will prevent the growth of all other lactobacilli, pediococci, or *Leuconostoc*, confirmation of isolates is required by either carbohydrate fermentation or polymerase chain reaction (PCR) methods. The members of the *Lb. casei* group and other common homofermentative and facultative heterofermentative dairy lactobacilli can be differentiated from one another using the criteria in **Table 2**. However, it is unwise to rely on carbohydrate fermentations alone for the identification of lactobacilli. Genetic techniques such as the use of species-specific probes and/or randomly amplified polymorphic DNA (RAPD) techniques provide more reliable and reproducible identification.

The *Lb. casei* Group in Cheese

Sources of the *Lb. casei* Group in Cheese

Strains of the *Lb. casei* group, notably either *Lb. paracasei* or *Lb. rhamnosus*, are found in cheese either as a contaminant (often becoming the dominant species of the nonstarter lactic acid bacteria (NSLAB) population) or as added adjunct cultures.

Nonstarter Lactic Acid Bacteria

Lactobacilli are common contaminants of raw milk but generally make up a small proportion of its flora. They are generally present at a few per milliliter to a few thousand per milliliter in bulk milk. In raw milk cheeses, lactobacilli will be present in the curd after manufacture. In cheeses made from pasteurized milk the presence of lactobacilli in the vat milk will depend on the heat sensitivity of the strains present and any postpasteurization contamination.

Table 1 Carbohydrate fermentation by species of the *Lb. casei* group

		Lb. paracasei	Lb. paracasei	
Acid produced from	Lb. casei	*subsp.* paracasei	*subsp.* tolerans	Lb. rhamnosus
N-acetyl-glucosamine	+	+	+	+
Adonitol	–	d	–	–
Amygdalin	+	+	–	+
D-Arabinose	–	d	–	d
L-Arabinose	–	–	–	–
D-Arabitol	–	–	–	–
L-Arabitol	–	–	–	–
Arbutin	+	+	–	+
Cellobiose	+	+	–	+
Dulcitol	–	d	–	d
Erythritol	–	–	–	–
Esculin	+	+	–	+
D-Fructose+	+	+	+	
D-Fucose	–	d	–	–
L-Fucose	D	–	–	d
Galactose	+	+	+	+
b-Gentibiose	–	+	–	+
Gluconate	+	+	+	+
D-Glucose	+	+	+	+
Glycerol	–	d	–	D
Glycogen	–	–	–	–
Inositol	–	d	–	+
Inulin	–	d	–	–
2-Keto-gluconate	–	–	–	–
5-Keto-gluconate	–	–	–	–
Lactose	–	D	+	+
D-Lyxose	–	d	–	D
Maltose	D	+	–	+
Mannitol	+	+	–	+
D-Mannose	+	+	–	+
Melezitose	+	+	–	+
Melibiose	–	–	–	–
α-Methyl glucoside	–	D	–	+
α-Methyl mannoside	–	–	–	–
β-Methyl xyloside	–	–	–	–
Raffnose	–	–	–	–
Rhamnose	–	–	–	+
Ribose	–	+	–	+
Salicin	+	+	–	+
Sorbitol	–	D	–	+
L-Sorbose	–	d	–	D
Starch	–	d	–	–
Sucrose	–	+	–	+
D-Tagatose	+	+	+	+
Trehalose	+	+	–	+
D-Turanose	–	+	–	+
Xylitol	–	–	–	–
D-Xylose	–	–	–	–
L-Xylose	–	–	–	–

d, 10±49% of strains; D, 50±90% of strains produce acid.

Lactobacillus paracasei subsp. *tolerans* will survive pasteurization. However, it has not been commonly isolated from cheese, probably because it is able to use much fewer carbohydrates than other taxa of the *Lb. casei* group (see **Table 1**). The heat resistance of *Lb. paracasei* and *Lb. rhamnosus* is variable. Some strains such as *Lb. paracasei* S203 have a *D*-value (time for a log reduction in numbers) at 72 °C of <0.2 s and some strains such as *Lb. paracasei* S372 have a *D*-value at 72 °C of >5 s (**Figure 1**). These more heat-resistant strains would survive pasteurization and be present in the cheese milk at low levels.

Contamination of the curd occurs during the manufacturing process even in modern mechanized cheese manufacturing plants. Major causes include contaminated

Table 2 Differentiation of common dairy lactobacilli

	Growth at 15 °C	*Growth at 45 °C*	*Lactic acid isomers*	*Galactose*	*Lactose*	*Maltose*	*Mannitol*	*Raffnose*	*Rhamnose*	*Sucrose*
Lb. acidophilus	−	+	DL	+	+	+	−	d	−	+
Lb. delbrueckii subsp. *bulgaricus*	−	+	D	−	+	−	−	−	−	−
Lb. delbrueckii subsp. *lactis*	−	+	D	d	+	+	−	−	−	+
Lb. helleticus	−	+	DL	+	+	d	−	−	−	−
Lb. casei	+	−	L	+	−	+	+	−	−	
Lb. paracasei subsp. *paracasei*	+	−	L[a]	+	+	+	+	−	−	+
Lb. paracasei subsp. *tolerans*	+	−	L	+	+	−	−	−	−	−
Lb. rhamnosus	+	+	L	+	+	+	+	−	+	+
Lb. plantarum	+	−	DL	+	+	+	+	+	−	+

d, 10±49% of strains produce acid; a, some strains produce L(+) and D(−) lactic acid.

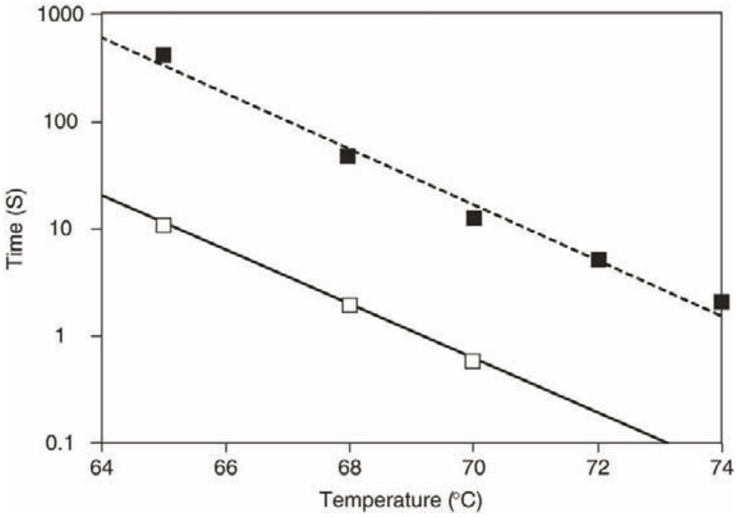

Figure 1 Time for one log kill (*D*-value) of *Lb. paracasei* S203 (■) and S372 (□) in skim milk.

starter cultures, handling of the curd by cheesemakers, and recycling of curd (particularly trimmings and damaged blocks) and/or whey back into the milk or fresh curd. Other sources of contamination include dirty equipment (including vats, whey draining screens, draining belts, mills, blockformers, hoops, presses, conveyors, and various utensils), aerial contamination, wash water, and brine. Sometimes the same strains of *Lb. casei* group dominate NSLAB microbiota of cheeses produced in the same factory but in different seasons, suggesting that the cheese factory environment might be an important source of such microorganisms.

Adjuncts

Selected strains from the *Lb. casei* group are increasingly being added to cheese milk at low numbers (usually from 10^4 to 10^6 cfu ml^{-1}) as adjuncts. These adjunct strains do not contribute significantly to acid production but will normally grow in the ripening curd to high numbers (from 10^7 to 10^8 cfu g^{-1}) and maintain high numbers for sustained periods. Provided the adjunct strains are matched to the cheese type and the curd is manufactured under good hygienic conditions with low initial NSLAB densities, the adjunct cultures will likely dominate the adventitious NSLAB population during cheese ripening. In such ripening curd, the adjuncts can provide controlled and/or accelerated ripening or produce specific flavors. For example, the adjunct single culture of *Lb. paracasei* subsp. *paracasei* in the manufacturing of a typical Egyptian cheese (Ras) produced higher level of free amino acids than any other adjunct culture singly used in the same cheese. That cheese also received a high score for sensory evaluation.

Some adjunct strains are added to produce bacteriocins in the cheese, which will prevent the growth of undesirable organisms such as clostridia, mold, other lactobacilli, or pathogens. Experimental Italian cheeses (Fiore Sardo PDO) produced in different farms by mixing selected autochthonous strains of *Lactococcus lactis* subsp. *lactis*, *Lb. casei* subsp. *casei*, and *Lb. plantarum* were characterized by a balanced ratio of free amino acids and free fatty acids, by a reduced number of spoilage microorganisms, and by an overall standardized good quality. Adjuncts can also be added as inactivated (attenuated – usually by heat shock, freeze shock, spray-drying, or freeze-drying) cultures that usually do not grow but are a source of enzymes in the cheese.

Growth of the *Lb. casei* Group in Cheese

Most strains of the *Lb. casei* group are capable of growing at the redox potential, pH, and salt concentration of cheese. They increase in numbers from low levels (often <10 cfu g^{-1}) to high (10^7–10^8 cfu g^{-1}) given sufficient time and a suitable ripening temperature. The rate of decline in numbers after the peak has been reached depends on the strains and the ripening temperature.

Ripening temperature is the variable that most influences the growth rate of the *Lb. casei* group in cheese. The minimum growth temperature varies from strain to strain. Many strains of *Lb. paracasei* will grow at 4 °C in cheese but *Lb. rhamnosus* will usually be outgrown below 10 °C. At higher ripening temperatures, the time required to reach peak numbers decreases, but the subsequent die-off rate also increases.

The effect of salt levels in cheese on the growth rate of the *Lb. casei* group is strain dependent. The growth rates of many strains of the *Lb. casei* group are not affected by

4–6% salt-in-moisture, whereas these salt levels significantly slow the growth of other NSLAB species. Some strains of *Lb. casei* can grow at salt-in-moisture concentrations greater than 10%. In hypertonic environments, such as the ripening curd, *Lb. casei* might use a balance of protease and peptidases activities to obtain an intracellular pool of amino acids and/or di- and tripeptides to act as osmoprotectants alternative to the widespread glycine–betaine and carnitine.

During cheese ripening lactobacilli can grow on various substrates. Usually lactose is mostly used by the starter during manufacture or in the first few days after manufacture. Nevertheless, residual lactose might represent an energy source for many strains of the *Lb. casei* group, because only about 2 mg g^{-1} would be required for growth to 10^7–10^8 cfu g^{-1}. Alternative substrates to lactose include lactate, oligosaccharides, citrate, protein breakdown products (peptides, amino acids), lipids and their breakdown products (phospholipids, lipoproteins, diglycerides, monoglycerides, fatty acids), sugars in the milk fat globule membrane and various components of lysed starter cells. Although capable of using substrates alternative to most common carbohydrates, strains of the *Lb. casei* group show a metabolic versatility inferior to that of *Lb. plantarum*, another important species normally grouped in the NSLAB microbiota.

General Ripening Effects of the *Lb. casei* Group in Cheese

Often, the major contaminating lactobacilli in cheese are made up of a number of strains from the *Lb. casei* group and will collectively contribute to the quality of ripening. It is therefore difficult to assign particular ripening properties to a single strain. Some strains produce undesirable effects such as bitterness, other off-flavors, and an open or crumbly texture. Other strains contribute either positively or very little to flavor. The overall contribution to flavor by the contaminating lactobacilli is difficult to control because of the random nature of the dominant strains.

The obvious benefit of proper use of adjuncts from the *Lb. casei* group is having only defined selected strains of NSLAB contributing to ripening. The ripening by *Lb. casei* group adjuncts is additional to the significant contribution from starters, coagulant, and endogenous enzymes. In the early weeks of ripening, the densities and contributions to ripening of the *Lb. casei* group are usually insignificant compared with those of the starters. With increasing time and temperature, the densities of the NSLAB will be high relative to that of the starters and as the starter enzymes become less active, the ripening effects from NSLAB will become progressively more significant.

Proteolytic Activities of the *Lb. casei* Group in Cheese

The proteolytic enzymes of a number of strains from the *Lb. casei* group have been purified and characterized. Although not as proteolytic as *Lb. helveticus*, most strains of *Lb. paracasei* and *Lb. rhamnosus* have at least one cell envelope-associated, serine-type proteinase. Specificity on casein and oligopeptides is strain dependent. The proteinases from both *Lb. paracasei* and *Lb. rhamnosus* are less inhibited by cheese conditions such as salt and pH than lactococcal proteinases.

A large number of peptidases (e.g., specific and general aminopeptidases, dipeptidases, tripeptidases, and endopeptidases) from this group have been identified and studied. The peptidase specificity types and activities are strain dependent and, where studied carefully, the enzymes appear to be mainly located intracellularly. Peptidases may be active also after cell lysis eventually occurring in cheese.

It is not clear what the critical features of the proteolytic system of the *Lb. casei* group are for improved flavor development. Some strains have an imbalance in their proteolytic system that produces a bitter defect in the cheese. One of the most consistent observations from different groups studying the use of adjuncts from the *Lb. casei* group for cheese ripening is the effect on proteolysis. Improved cheese flavor is usually accompanied by altered proteolysis leading to higher concentrations of amino acids and small peptides and changes in the peptide profile.

Other Ripening Effects of the *Lb. casei* Group in Cheese

The activity, numbers, and specificity of esterolytic enzymes vary between strains of the *Lb. casei* group. Although the activity is generally low (similar to that of lactococcal strains), the products have high flavor impact; thus their activities probably contribute to cheese flavor. Flavor contributions can be by the short-chain (C_4–C_{12}) fatty acids, derived from the hydrolysis of the mono-, di-, and triglycerides, as well as by producing fruity esters when ethanol or other alcohols are present at sufficient concentrations.

The *Lb. casei* group has considerable diversity in its ability to utilize other substrates (e.g., citrate, lactate, and amino acids) in cheese to give products that have the potential to influence the quality of the ripening cheese. Some strains of *Lb. paracasei* and all strains of *Lb. rhamnosus* metabolize citrate if sugars are also present, and the carbon dioxide formed in cheese may contribute to an open texture. Moreover, during long-term ripening of a Dutch-type cheese, *Lb. paracasei* subsp. *paracasei* INF448 showed the capacity to degrade citrate into lactate, diacetyl, and acetoin. Lactate racemization is possible by some strains of *Lb. paracasei*, but not by any strains of *Lb. rhamnosus*, and when it occurs in nonwashed cheeses such as Cheddar, lactate crystal problems can occur.

During cheese ripening, the *Lb. casei* group can metabolize a wide range of amino acids (e.g., serine, alanine, glutamate, the sulfur-containing, branched-chain, and aromatic amino acids) with the products often giving either positive or negative effects on flavor. Methionine is the major sulfur-containing amino acid in milk proteins and can act as the precursor of cheesy, cabbagy, and garlic flavors of cheeses. Few strains of *Lb. casei* group showed a methionine amino transferase activity that could lead to methanethiol formation. Furthermore, *Lb. paracasei* subsp. *paracasei* CHCC 4256 showing amino transferase activity toward branched-chain amino acids (leucine, isoleucine, and valine), aspartic acid, and phenylalanine was used as adjunct in the production of a reduced-fat, semi-hard, bovine cheese. The adjunct increased the content of flavor compounds, such as 3-methylbutanoic acid and its corresponding alcohol and aldehyde from leucine, and diacetyl from aspartic acid. Remarkably, degradation of amino acids by means of transamination is strain dependent. α-Ketoglutarate produced from glutamate dehydrogenase (GDH) is considered to play a key role in amino acid catabolism, since it serves as the amino group acceptor during transamination. Thus, the production of α-ketoglutarate is considered the rate-limiting step in cheese ripening. GDH was found in some strains of the *Lb. casei* group. Moreover, a number of strains have been shown to possess enzymes that act on aromatic amino acids (tyrosine, phenylalanine, and tryptophan), producing compounds which, upon subsequent chemical degradation to indole and skatole, can impart off-flavors to cheese. Some strains have decarboxylases that have the potential to produce the potentially toxic biogenic amines from amino acids such as histidine and tryptophan.

A balance between compounds derived from catabolism of amino acid is important for different cheese flavors. Since catabolism of amino acids during cheese ripening is a very complex phenomenon involving starter lactic acid bacteria, eventual secondary starters (e.g., molds), NSLAB, and other adventitious microorganisms (e.g., bacteria of the smear), the details and the contributions in different cheeses by the *Lb. casei* group are currently being investigated.

The *Lb. casei* Group in Probiotic Foods

The *Lb. casei* group contains several probiotic bacteria. For example, *Lb. rhamnosus* 35 constitutes the active substance of different pharmaceutical products that have been successfully used in the treatment and prevention of diarrhea for more than four decades. *Lactobacillus rhamnosus* GG is commonly used in a number of probiotic dietary supplements or foods. One of the most studied probiotic microorganisms of the *Lb. casei* group is *Lb. casei* strain Shirota. This strain exhibits antitumor, immunostimulatory, and antimicrobial activities. Furthermore, *Lb. casei* strain Shirota orally administered to animals infected with *Listeria monocytogenes* or *Escherichia coli* O157:H7 or *Helicobacter pylori* confers enhanced resistance against those pathogens. Different mechanisms have been shown to explain the improvement of the immune system due to probiotics. One of those mechanisms could be the ability to differentially modulate expression of cytokines and costimulatory molecules, such as interleukins (IL) and interferon-γ (IFN-γ). Some probiotic bacteria belonging to the *Lb. casei* group are able to induce or potentiate production of IL-6 and/or IL-12.

Sometimes probiotic microorganisms may positively influence the health status of the host even when not viable, through the production of biogenic compounds. For example, some strains of the *Lb. casei* group are able to form free conjugated linoleic acid (CLA) from linoleic acid in cultured dairy products. Since CLA may prevent carcinogenesis and atherosclerosis, may modulate immune response, and may reduce body fat, consumption of such dairy products may have potential health or nutritional benefits.

As a result of the growing popularity of probiotics, numerous so-called probiotic foods have been marketed, in most cases without proper trials, which has raised concern about their real safety and efficacy. The latter two properties are strain specific and thus cannot be extrapolated to a whole genus or species. Foods such as fermented milks, Cheddar cheese, Gouda cheese, Cottage cheese, Crescenza cheese, frozen yogurts, and ice cream have been studied as carriers of probiotic microorganisms. *Lactobacillus rhamnosus* and *Lb. paracasei* can be added to fermented milks for their health features as, in general, they do not rapidly acidify milk. The commercial probiotic fermented milk Yakult® may contain high cell densities of the aforesaid *Lb. casei* strain Shirota. Probiotic strains of *Lb. casei* are used in addition to *Lb. acidophilus* and bifidobacteria in the so-called 'ABC' yogurt. However, fermented milks may not be optimal for the maintenance of recommended cell densities of some probiotic strains. Therefore, cell immobilization, use of oxygen-impermeable containers, and stress adaptation have been proposed for improving the viability of probiotics. Fruit pieces used in immobilization of *Lb. casei* cells may be an effective support for survival of such probiotics during shelf-life of fermented milks and, thanks to indigestible fiber contained in fruit, they may eventually protect probiotic microorganisms during gastrointestinal transit.

Cheeses have a number of advantages over fermented milks as a delivery system for viable probiotic to gastrointestinal tract, as they tend to have higher pH, more solid consistency, and relatively higher fat content. These offer protection to probiotic bacteria during storage and passage through the gastrointestinal tract. Cheeses also have higher buffering capacity than fermented milks. Probiotic strains of the *Lb. casei* group may be successfully used as adjunct of a number of cheeses, although it appears that the capability to survive cheese manufacture and ripening vary from strain to strain. Most studies concern potentially probiotic Cheddar cheese. For example, *Lb. casei* 279 or *Lb. paracasei* LAFTI® L26 singly used as a probiotic adjunct in Cheddar cheeses survived the cheesemaking process at high levels. After 6 months of ripening at 4 °C, cheeses maintained the level of probiotic organisms at values higher than 10^8 cfu g^{-1}. Furthermore, differences in proteolytic pattern and organic acid profile between probiotic Cheddar and a Cheddar produced without adjunct (control) were observed. In detail, probiotic cheeses were characterized by much higher hydrolysis of casein and concentrations of free amino acids and by higher acetic acid concentration than the control. Interestingly, some strains of the *Lb. casei* group isolated from cheeses may be resistant to some conditions of the gastrointestinal environment such as low pH and different amounts of bile salts and acids. Furthermore, most of those strains may show anti-carcinogenesis activity, being capable to prevent DNA damage by genotoxins.

Besides fermented milks and cheeses, milk powders added with freeze-dried *Lb. paracasei* and *Lb. rhamnosus* are available to produce an instant probiotic drink. Probiotic strains of the *Lb. casei* group may be also used along with the inulin for manufacturing fermented milks or cheeses with prebiotics thus having a higher added value than the probiotic food alone. Indeed inulin increases activity of probiotics, helps in digestion of high-protein diets, decreases fat absorption, prevents constipation, and boosts the body's natural defenses.

See also: **Bacteria, Beneficial:** Probiotics, Applications in Dairy Products. **Lactic Acid Bacteria:** *Lactobacillus* spp.: General Characteristics.

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Lactobacillus spp.: *Lactobacillus helveticus*

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Introduction

Lactobacillus helveticus, paired in some cases with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, is used as a starter primarily in cheese manufactured at elevated cooking temperatures, such as hard and extrahard Italian and Swiss varieties. In most of the cases, it is the major component of natural whey starters. As a starter it also contributes to flavor development. More recently, *Lb. helveticus* is increasingly being used as a flavor adjunct in cheeses (e.g., Cheddar) to which thermophilic lactobacilli are traditionally not added. In the past, there was some confusion in the naming of the thermophilic lactobacilli used in the dairy industry, often resulting in mistaken identification, especially between *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus*. These two species are clearly separated by genetic criteria, but for practical purposes as dairy starters some strains from the two species are similar. Overall, *Lb. helveticus* is a galactose-positive thermophilic *Lactobacillus* that produces D(−) and L(+) lactic acid and does not contain metachromatic granules. In contrast, *Lb. delbrueckii* subsp. *bulgaricus* is galactose-negative, produces only the D(−) isomer of lactic acid, and contains metachromatic granules.

General Characteristics

Lb. helveticus is a Gram-positive, catalase-negative, non-spore-forming, and rod-shaped (diameter from 0.5 to 1.1 μm) thermophilic lactic acid bacterium. Previously known as *Thermobacterium helveticus*, *Lb. helveticus* is mainly isolated from dairy sources such as sour milks and, especially, cheeses. *Lactobacillus helveticus* does not grow at 15 °C, but will grow well at 45 °C with most strains showing growth also at 50–52 °C. It requires niacin, riboflavin, calcium pantothenate, and either pyridoxamine or pyridoxal as essential growth factors. No strains utilize arginine to produce ammonia.

Genetics

Lb. helveticus belongs to the largest of the eight proposed phylogenetic *Lactobacillus* subgroups, the *Lb. delbrueckii* group. The complete genome sequence of the cheese culture *Lb. helveticus* DPC 4571 was recently determined. Strain DPC 4571 seemed to have an excellent aptitude for cheesemakimg. The main technological features concerned rapid autolysis and intense peptidase activities. Sequencing of *Lb. helveticus* DPC 4571 showed a 2.08 Mb genome saturated with insertion sequence (IS) elements and confirmed the close genetic relationship between this dairy culture and lactobacilli that inhabit the gastrointestinal tract, such as *Lactobacillus acidophilus*. The 16S sequences of these two bacteria differ by 1.6% only, and 75% of the predicted DPC 4571 ORFs have orthologues in the genome of *Lb. acidophilus* NCFM. Five hundred predicted genes from the genome of *Lb. helveticus* DPC 4571 were not conserved in the genome of *Lb. acidophilus* NCFM. Both these strains are located in a separate branch from the sequenced genomes of dairy *Lb. delbrueckii* and probiotic species such as *Lactobacillus gasseri* and *Lactobacillus johnsonii*. One of the main distinguishing features of the genome of strain DPC 4571 with respect to other related lactobacilli is the 213 IS elements, which are ∼10 times more than in other lactobacilli.

Proteomics

A proteomic study was carried out mimicking the heat stress to which cells of *Lb. helveticus* PR4 are subjected during natural whey propagation for making Grana cheeses. After an initial exposure of 35 min to 55 °C followed by decreases in temperature to 40 (3 h), 30 (5 h 30 min), and 20 °C (13 h 30 min), a transient induction of the levels of expression of several proteins was found with respect to cells grown for the same time durations at a constant temperature of 42 °C. Expression of most of these proteins increased following cooling from 55 to 40 °C (3 h). Differences were attributed mainly to heat stress response. Heat shock proteins (HSPs) such as DnaK and GroEL, low-molecular-weight HSPs, ATP-dependent protease, ATP synthase, and 50 ribosomal proteins such as L7-L12 and L13 were found. Glycolytic enzymes were also induced in *Lb. helveticus* PR4. The resulting population of *Lb. helveticus* at the end of propagation in whey under a temperature gradient could be considered as a mixture of cells that had been subjected to transient heat adaptation and may show differences in technological properties. Proteinase and peptidase activities were found to be higher for cells harvested when the temperature reached 40 °C. The same was observed for the acidification rate in skim milk. It seemed that exposure to a transient decrease in temperature from 55 to 40 °C for ~3 h was needed to express the highest proteolytic and acidification activities. Another proteomic study revealed that among the most abundant proteins expressed by *Lb. helveticus* ITG LH1 grown in milk medium, there were seven peptidases: two general aminopeptidases (PepN, PepC), three dipeptidases (PepDA, PepV, PepQ), and two endopeptidases (PepO, PepO3). The induction of several stress proteins and glycolytic enzymes was also confirmed in this study.

Enumeration and Isolation

The medium most commonly used for the enumeration of *Lb. helveticus* is MRS agar, incubated anaerobically at 37 °C. However, this medium is nonselective and will recover other starter bacteria such as *Lactococcus lactis* and *Str. thermophilus*. Adjusting the pH of MRS agar to 5.4 will prevent the growth of these starter bacteria, but will not inhibit the growth of other lactobacilli that are present in the dairy product. When probiotic cultures of lactobacilli are added to dairy products, or in ripening cheese when the nonstarter lactobacilli are increased to levels similar to or higher than those of the starter lactobacilli, it will be necessary to check the identity of the colonies. *Lb. helveticus* can be identified from these other lactobacilli on the isolation medium by carbohydrate fermentation or using genetic tools.

Genetic Identification and Typing

All routine genetic analyses (e.g., 16S rDNA) are frequently used for the identification of *Lb. heleveticus*. Nevertheless, the abundance of IS sequences in terms of both number (213 intact) and diversity (21 types) is a unique feature of *Lb. helveticus*. PCR-based IS fingerprinting was recently shown as a useful and rapid method for identification. Multiplex-PCR using all 10 primers targeting 5 of the most numerous IS elements (ISL*1201*, ISLhe*65*, ISLhe*2*, ISLhe*15*, and ISL*2*) gave fingerprints in the range 0.5–3 kb that were strain-specific and reproducible.

Several molecular methods are used to discriminate between *Lb. helveticus* strains and to investigate whether the findings concerning phenotypic discrimination are consistent with the genotypes. These methods include analyses of the total genomic DNA by restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), or randomly amplified polymorphic DNA (RAPD), or analyses of ribosomal RNA genes (ribotyping, PCR-ribotyping, rDNA sequence). Multilocus restriction typing (MLRT), which considers the restriction analysis of PCR products generated from several loci of selected housekeeping genes, was shown to be a discriminatory and rapid method to characterize *Lb. helveticus* isolates from different dairy products.

In situ molecular methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient gel electrophoresis (TTGE) have been used successfully to analyze the biodiversity of *Lb. helveticus* in cheese samples.

Fermentation Properties

Lb. helveticus is an obligately homofermentative species, fermenting hexoses mainly to lactic acid. It does not ferment pentoses or gluconate. All strains possess a mixture of L(+) and D(−) lactate dehydrogenases, resulting in L(+) (~25–80%) and D(−) lactic acid as the major end product from fermentation of carbohydrates. The gene for D(−) lactate dehydrogenase was inactivated in a strain of *Lb. helveticus* to get a biotype that synthesizes only the L(+) isomer of lactic acid. All strains ferment lactose, galactose, and glucose, and some strains ferment fructose, maltose, mannose, and trehalose. The synthesis of β-galactosidase is induced in the presence of lactose, and there is an absence of phospho-β-galactosidase. This indicates that lactose is transported into the cell via a permease and that lactose is then hydrolyzed to glucose and galactose. Glucose and galactose are then both metabolized via the Embden–Meyerhof–Parnas (glycolysis) pathway to produce mainly lactic acid.

Bacteriocins and Synthesis of Exopolysaccharides

Some strains of *Lb. helveticus* synthesize bacteriocins. These bacteriocins are active against a limited number of lactobacilli, including other strains of *Lb. helveticus*, and *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. acidophilus*. Their limited activity range means that these bacteriocins are not used commercially. However, it is important when pairing these lactobacilli as starters that bacteriocin production by *Lb. helveticus* be checked for compatibility problems. Two bacteriocins from *Lb. helveticus*, lactocin 27 and helveticin J, have been characterized in some detail.

Some strains of *Lb. helveticus* synthesize exopolysaccharides (EPSs). A number of EPSs have been structurally analyzed and are branched polysaccharides made up of galactose and glucose in different ratios.

Although yogurt manufacture remains the most important commercial application of these polymers, some attempts were made to introduce EPS-producing strains as adjunct cultures to improve low-fat and partly skim milk cheeses. Thermophilic EPS-producing starters and/or adjunct cultures were also used in the manufacture of reduced-fat and low-fat Mozzarella to increase moisture retention. The enzymes involved in sugar nucleotide biosynthesis of *Lb. helveticus* ATCC 15807 were studied. Batch fermentations using lactose as energy source resulted in a higher synthesis of EPSs at pH 4.5 instead of pH 6.2, and the enzyme α-phosphoglucomutase (α-PGM) was related with EPS production. When glucose was used instead of lactose, the synthesis of EPSs was found to be reduced due to a decrease in α-PGM and galactose-1-phosphate-uridyltransferase (GalT) activities. These two enzymes are needed to synthesize UDP-glucose and UDP-galactose to supply the corresponding monomers for synthesis of EPSs. The monomeric composition of EPSs was independent of the carbon source.

Bacteriophage

The bacteriophage of *Lb. helveticus* may be a problem in cheese factories where strains are used as starters with large volumes of milk. The bacteriophage from different strains are genetically related closely to each other but are unrelated to those from the other thermophilic lactobacilli (*Lb. delbrueckii* group) used in the dairy industry. To avoid poor acid development from the use of *Lb. helveticus* as starters, it is important to practice the well-established good starter production and manufacturing procedures that may avoid problems from bacteriophage.

Proteolytic Enzymes

Lb. helveticus strains have more general proteolytic activity toward milk proteins and peptides than the other dairy lactobacilli. The high activities and the balance of proteinases and peptidases vary between strains. The proteolytic activity of *Lb. helveticus* strains is an important property in determining their usefulness in cheese ripening. Two cell wall-associated proteinases (CEPs) and possibly an intracellular proteinase were identified in a number of strains. Indeed, the presence of two or more different genes encoding CEPs was reported. The *prtH* gene was identified in *Lb. helveticus* CNRZ32, as well as the presence of a second CEP encoding gene, named *prtH2*, was shown. Two other putative genes that encode CEPs, *prtH3* and *prtH5*, were characterized in the same strain showing that they were markedly induced during growth in milk as compared to growth in MRS medium. The diversity of the genes encoding CEPs was studied in a collection of 29 strains of *Lb. helveticus*. The *prtH2* gene was ubiquitous in all the 29 strains, whereas only 18 strains exhibited the *prtH* gene. CEPs from different strains have different specificities. For instance, some hydrolyze the α_{s1}- and β-caseins without preference; some others hydrolyze only β-casein; and still others are more active on α_{s1}-casein.

A number of general and specific aminopeptidases and an endopeptidase were identified in different strains of *Lb. helveticus*. All are likely to be located intracellularly and include a number of general di- and tripeptidases, imidodipeptidase (prolidase), proline iminodipeptidase (prolinase), and proline iminopeptidase. The relative proportions of the different peptidase activities are strain-dependent and in some strains, particularly strain CNRZ 32, the biochemical and genetic properties of some of the peptidases were studied in detail.

Other Flavor Activities

Lb. helveticus strains have other biological activities that could contribute to flavor development in cheese, but such activities have not been studied in the same detail as their proteolytic activities. The esterase activity (hydrolyzing substrates in solution) is higher than the lipase activity (hydrolyzing substrates in emulsion). The lipase activity of *Lb. helveticus* has a different specificity than that of the *Lb. delbrueckii* group, as it is more active on triglycerides than on monoglycerides.

Incubation of *Lb. helveticus* with amino acids produces a number of aroma and flavor compounds but their contribution to cheese ripening has not been studied in detail. A number of strains have a tryptophan decarboxylase, but not all these strains may use tryptophan under conditions similar to those in cheese. A cell extract of a strain may produce benzaldehyde and phenolpyruvic acid from phenylalanine. In cheeses whose manufacture is associated with the use of *Lb. helveticus* as starter, appreciable levels of furanones such as 2,5-dimethyl-4-hydroxy-3[2*H*]-furanone (DMHF) and 4-hydroxy-5-methyl-3[2*H*]-furanone (MHF) were identified. MHF and DMHF are considered important flavor compounds, especially for Parmigiano Reggiano cheese. Besides, furanones are naturally occurring compounds associated with a variety of diverse biological phenomena. In particular, they may act as signaling molecules in the mechanisms of cell-to-cell communication and/or in the mechanisms of stress response. The possible role of furanones in the induction of autolysis was also studied.

Significance in Fermented Milks

Lb. helveticus is occasionally used as a starter, usually paired with *Str. thermophilus*, in some yogurt and yogurt-like products, but not nearly in the volume and diversity of products as the traditional thermophilic starter *Lb. delbrueckii* subsp. *bulgaricus*. Overall, *Lb. helveticus* strains produce a lower final pH in milk than *Lb. delbrueckii* subsp. *bulgaricus*. *Lb. helveticus* produces less D(−) lactic acid than produced by *Lb. delbrueckii* subsp. *bulgaricus* during milk fermentations. In some yogurt products for infants where the presence of the D(−) isomer may be a problem, *Lb. helveticus* is the preferred *Lactobacillus* starter. Associative growth between *Str. thermophilus* and *Lb. helveticus* occurs but is not as well studied as that between *Lb. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus*.

Significance in Cheesemaking

In the Swiss and Italian cheese varieties manufactured with elevated cooking temperatures of 45–60 °C, *Lb. helveticus* may be used as a thermophilic *Lactobacillus* starter. In some cases, *Lb. helveticus* is paired with *Str. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Different ratios of cocci to rods in the cheese milk and curd are required to match the cheese type and manufacturing requirements. The ratio of cocci to rods defines the rate of acid development, influences the final pH, and, in a number of cheeses, is important for determining the level of residual galactose. In hard cheeses made with thermophilic starters and subsequently dried and grated for their final use, or for Mozzarella, the presence of galactose at concentrations >2–5 mmol kg^{-1} cheese may cause undesirable browning before or during the heat process. Of the thermophilic starters used in these types of curd manufacture, *Str. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, and many of the *Lb. delbrueckii* subsp. *lactis* strains all produce galactose during curd acidification but do not use galactose during curd ripening. The use of *Lb. helveticus* as thermophilic starter can ensure that the levels of galactose produced by *Str. thermophilus* are negligible in the young curd provided the ratio of cocci to rods added as starter is correct.

As Starters in Swiss-Type Cheese

In Swiss-type cheese manufactured with *Str. thermophilus* and galactose-negative lactobacilli, the galactose is removed during warm room ripening when the propionibacteria and adventitious flora are active. However, the flavor is different from that of the same cheese made with galactose-positive *Lb. helveticus*. It is not known if the flavor differences are due only to consequences of galactose utilization or whether there are other interactions between the two lactobacilli starters that contribute to the flavor differences. The greater proteolytic activity and/or the ability to produce L(+) lactic acid as well as D(−) lactic acid of *Lb. helveticus* as compared with *Lb. delbrueckii* subsp. *bulgaricus* may influence flavor directly or via the influence on propionibacteria. The L(+) isomer is preferred by propionibacteria, and there are other unknown factors produced by the thermophilic lactobacilli in curd that influence the growth of propionibacteria.

As Starters in Hard and Extrahard Italian Cheese Varieties

It is well established that natural whey starters for hard Italian cheeses are dominated by thermophilic lactic acid bacteria (~10^9 cfu ml^{-1}). Depending on the variety, *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Str. thermophilus* may dominate. The natural whey cultures used as starters for Parmigiano Reggiano and Grana Padano cheeses are prepared from the whey from the previous cheesemaking, which is held under a gradient of temperature from ~50 to ~35 °C for ~24 h. The microbial composition of the natural starter is very complex and subject to various environmental factors. Nevertheless, *Lb. helveticus* is usually the dominant species. Marked differences concerning the proteolytic and acidifying activities were found among *Lb. helveticus* strains isolated from several natural whey cultures. The biodiversity of *Lb. helveticus* strains isolated from different natural whey starters for Parmigiano Reggiano, Grana Padano, and Provolone was studied. Strains isolated from Parmigiano Reggiano whey cultures were distinguishable from those isolated from Grana Padano and natural Provolone whey. The presence of specific biotypes of *Lb. helveticus* is considered to be one of the main features that contribute to typical cheese varieties.

Contribution to Ripening as Starters

Lb. helveticus, when used as a starter, will contribute not only to acid production but also to flavor and texture development during cheese ripening. The use of *Lb. helveticus* versus *Lb. delbrueckii* subsp. *bulgaricus* and the use of different ratios of proteinase-positive to proteinase-negative *Lb. helveticus* as starters for Mozzarella influence the melt and stretch properties. Different strains of *Lb. helveticus* used as starters in Swiss-type and Parmigiano Reggiano cheeses may cause flavor differences, which are in large part due to the release of the intracellular enzymes such as peptidases. Autolysis of *Lb. helveticus* is an important factor that influences the flavor quality through its contribution to proteolysis.

Catabolism of amino acids, including sulfur-containing amino acids, by lactic acid bacteria is a major contributor to the development of flavor compounds in cheese during ripening. One of the two microbial pathways leading to amino acid conversion into flavor compounds is initiated by transamination. *Lb. helveticus* was incubated with a mixture of amino acids (phenylalanine, tyrosine, methionine, leucine, valine, and isoleucine) in ratios similar to those found in Emmental cheese. It was shown that transamination is the first and the main step for the conversion of free amino acids by *Lb. helveticus*, with Phe and Tyr being converted most efficiently. The role of cystathionine β-lyase (CBL) in the production of volatile sulfur compounds from sulfur-containing amino acids/derivatives (e.g., methionine, cysteine, and cystathionine) was studied in *Lb. helveticus* CNRZ 32. When methionine was used as the substrate, overexpression of CBL resulted in the synthesis of larger quantities of volatile sulfur compounds as compared to wild-type *Lb. helveticus* CNRZ 32 or CBL-null mutant. When cystathionine was used as the substrate, methanethiol production was detected from the CBL-overexpression variant and complementation of the CBL-null mutant (CNRZ 32 Δ*cbl*), implying that CBL may be involved in the conversion of cystathionine to methanethiol. When cysteine was used as the substrate, no differences in the synthesis of volatile sulfur compounds were found between the wild type and genetic variants, indicating that CBL does not contribute to the conversion of cysteine.

Significance as Adjunct Starter

The traditional and major dairy use of *Lb. helveticus* as a cheese starter in high-cook cheeses has expanded in the last 10–15 years to this species being used increasingly in lower-cook cheeses as an adjunct for flavor. *Lb. helveticus* is added to cheese milk either as a live active culture or more often as an attenuated culture. For both applications, its main contribution to flavor is through its proteolytic system accelerating and/or controlling (debittering) proteolysis. Several studies elucidated the debittering activity of the peptidases of *Lb. helveticus* WSU19 when used as an adjunct starter in the manufacture of Cougar Gold cheese. Degradation of α_{s1}-CN f1–23 plays a role in the production of free amino acids and flavor development. Although some activity of peptidases was also shown, it is the proteinases from the starter that mainly degrade α_{s1}-CN f1–23. Some peptides derived from α_{s1}-CN f1–23 were shown to be bitter (e.g., α_{s1}-CN f1–7, f1–9, f1–13, f11–14, f14–17, f17–21, and f21–23). Recently, the substrate specificity of aminopeptidase N (PepN), endopeptidase E (PepE), endopeptidase O (PepO), endopeptidase O2 (PepO2), and endopeptidase O3 (PepO3) from *Lb. helveticus* WSU19 toward peptides generated from α_{s1}-CN f1–23 was investigated. PepN exhibited activity on α_{s1}-CN f1–23 only in the presence of PepO-like endopeptidases. PepO, PepO2, and PepO3 cleaved α_{s1}-CN f1–23 mainly at the position Glu_{14}-Val_{15}, forming the bitter peptide α_{s1}-CN f1–14. PepE cleaved α_{s1}-CN f1–23 primarily at Lys_3-His_4, suggesting a debittering actvity. Combinations of PepE/PepO and PepE/PepO2 also had the potential of decreasing the accumulation of α_{s1}-CN f1–14. When *Lb. helveticus* is added as a live adjunct culture it contributes very little to acid production. This is because of the combination of its lower inoculum level, the lower manufacturing temperatures, and the greater acid development activity of the mesophilic starters. Provided that *Lb. helveticus* reaches maximum densities of $>10^7\,\text{cfu}\,\text{g}^{-1}$ cheese, the culture can potentially contribute to flavor development via its proteolytic activity. In a number of cheeses, including Gouda, Cheddar, and low-fat cheeses, the use of attenuated cultures of *Lb. helveticus* has successfully accelerated flavor development. Methods for attenuation of *Lb. helveticus* usually involve either heat-shocking or freeze-shocking of the cells. Different strains require different methods for optimum attenuation. For some strains, it has been shown that a rapid freeze/thaw is the preferred method. Optimum attenuation aims at prevention of acid development while retaining maximum activity of the desired ripening reactions. For *Lb. helveticus*, heat attenuation of some strains prevents autolysis, which is a desirable property for its proteolytic contribution. The inactivation of acid development in attenuated *Lb. helveticus* cultures is desired as it allows the addition of large biomass without influencing the desired acid development rate.

Health-Promoting Effects

Lb. helveticus is considered to be one of the most efficient lactic acid bacteria in generating various bioactive peptides from caseins. Some of these peptides were produced in fermented milks and cheeses, and they showed mainly *in vitro* and *in vivo* antihypertensive and antimicrobial activities. Other studies carried out in mouse models demonstrated that bioactive compounds released in milk fermented by *Lb. helveticus* R389 had immunoenhancing and antitumor properties.

See also: **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species.

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Lactobacillus spp.: *Lactobacillus plantarum*

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Taxonomy, Identification, and Genetics of the Species

Lactobacillus plantarum is a Gram-positive, nonmotile, non-spore-forming, microaerophilic, and mesophilic bacterium with growth occurring at 10–15 °C but not at 45 °C. Cells are straight rods with rounded ends, 0.9–1.2 × 3.0–8.0 μm, occurring singly, in pairs, or in short chains. It has been found that under special conditions, a few strains of *Lb. plantarum* possess true catalase and manganese-containing pseudocatalase activities. Some strains also exhibit nitrate- and hematin-dependent nitrite reductases.

The cell wall contains either ribitol or glycerol teichoic acid although some strains have an unusual teichoic acid. Peptidoglycan of the cell wall is of the *meso*-diaminopimelic acid (DAP) type.

On the basis of 16S rRNA-based phylogeny, recently supported by whole-genome DNA, DNA–RNA hybridization, and GC content studies, *Lb. plantarum* belongs to the *Lactobacillus casei–Pediococcus* group; DNA–DNA hybridization studies have demonstrated that *Lb. plantarum* forms two genomically related groups, in their turn separated by other streptobacteria and lactobacilli containing DAP in the cell wall. Even if simple methods like random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) have been used for both strain typing and differentiation from similar *Lactobacillus* spp., a polyphasic strategy based on physiological tests and more than one molecular technique is necessary for the correct species designation.

Lactobacillus plantarum, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum* form a closely related group known as the '*Lactobacillus plantarum* group', the species of which cannot be distinguished from each other by 16S rRNA gene sequence analysis because of 99.7–99.9% sequence similarity; the species of the group also show a very similar fermentation pattern. Discrimination among these species can be obtained using species-specific PCR primers based on *rec*A gene and by employing repetitive element sequence-based polymerase chain reaction (Rep-PCR) moreover, strain differentiation between *Lb. plantarum* and *Lb. pentosus* could be achieved using RFLP–PFGE, 16S ARDRA, AFLP using restriction enzymes *Hin*dIII and *Mse*I, as well as ribotyping and RAPD–PCR. Multilocus sequence typing (MLST) based on *pgm*, *ddl*, *gyr* B, *pur*K1, *gdh*, and *mut*S housekeeping genes can be useful to determine the genetic relatedness and to discriminate among *Lb. plantarum* strains. In recent years, culture-independent methods have been developed and applied to the direct identification of bacteria from many matrices: DGGE and TGGE are the most useful techniques for this purpose and were successfully applied to identify *Lb. plantarum* in cheese and dairy products.

By means of *rec*A gene sequencing and Southern hybridization with *pyr* probe on *Bgl*I digestion of chromosomal DNA, *Lb. plantarum* has been subdivided into subspecies *Lb. plantarum* subsp. *plantarum* and *Lb. plantarum* subsp. *argentoratensis*.

The complete genome of *Lb. plantarum* WCFS1, a single colony isolated from *Lb. plantarum* NCIMB8826 (National Collection of Industrial and Marine Bacteria, Aberdeen, UK), has been sequenced and deposited in EMBL database with accession number AL935263. *Lactobacillus plantarum* WCFS1 contains a single circular chromosome of 3 308 274 bp (**Figure 1**), which can be considered one of the largest genome sizes known for lactic acid bacteria; two small cryptic plasmids of 1917 and 2365 bp and one large plasmid of 36 069 bp encoding genes involved in conjugal plasmid transfer as well as other functions have also been found. The G + C content of the chromosome is 44.5% while the plasmids show a lower, from 34.3 to 40.8%, G + C content.

The *Lb. plantarum* genome contains five rRNA operons distributed around the chromosome, displaying a very limited number of sequence polymorphisms; 3502 predicted protein-encoding genes are present in the chromosome and putative biological functions could be assigned to 2120 of the predicted proteins, whereas the three above-mentioned plasmids contain, in the order of length, 3, 4, and 43 genes, respectively. Microarray analysis revealed the presence of two large regions of flexibility inside the chromosome, which run from 2.70 to 2.85 Mb and from 3.10 to 3.29 Mb; 293 genes, organized in clusters of 3–6 genes, have been predicted in these regions, and most of these are involved in sugar transport

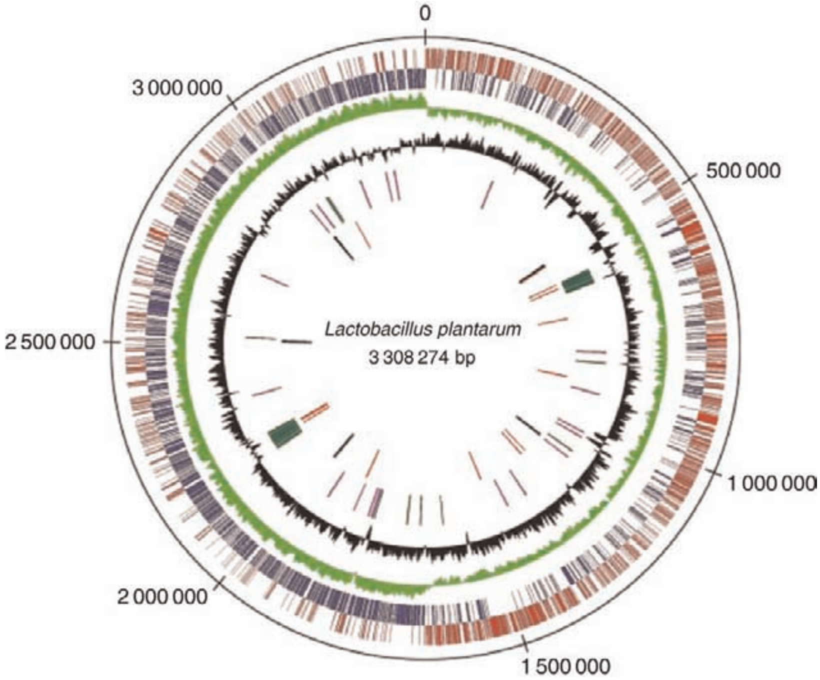

Figure 1 Genome-atlas view of the *Lactobacillus plantarum* WCFS1 chromosome, with the predicted origin of replication at the top. The circles show (from outer to inner) (1) positive strand ORFs (red), (2) negative strand ORFs (blue), (3) GC skew (green), (4) G + C content (black), (5) prophage-related functions (green) and IS-like elements (purple), and (6) rDNA operons (black) and tRNA encoding genes (red). The GC% and GC skew (C − G)/(C + G) were calculated in a window of 4000 nt, in steps of 75 nt. The G + C percentage was plotted as the number of G + C nucleotides in the plus strand divided by the window size, that is, (G + C)/4000; the lowest and highest values are 30.8 and 51.8%, respectively. The upper and lower values of the GC skew are 0.22 and 0.27, respectively. Reproduced from Kleerebezem M, Boekhorst J, van Kranenburg R, *et al*. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences of the United States of America* 100(4): 1990–1995. www.pnas.org_cgi_doi_10.1073_pnas.0337704100. Copyright (2009) National Academy of Sciences, U.S.A.

(phosphotransferase system (PTS)) and catabolism. Moreover, many genes in this region show an unusual base composition compared with the rest of the genome, which could be an indicator of horizontal gene transfer. On the basis of these findings, it has been hypothesized that this part of the *Lb. plantarum* genome represents a 'lifestyle adaptation island' of the species to different environmental niches.

The type strain of the species is *Lb. plantarum* ATCC 14917.

Metabolism and Enzymes

Lactobacillus plantarum is included in the group of facultatively heterofermentative lactobacilli; hexoses are converted almost entirely to lactic acid via the Embden–Meyerhof–Parnas pathway, while pentoses are converted to lactic and acetic acids via the 6-phosphogluconate/phosphoketolase pathway by the induction of phosphoketolase. Both L- and D-lactic acid are produced by NAD-dependent lactate dehydrogenases (LDHs); amygdalin, arabinose, cellobiose, fructose, galactose, glucose, gluconate, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, salicin, sorbitol, sucrose, and trehalose are used as carbon and energy sources. Sugars are usually transported into the cell by permeases and are phosphorylated in the cytoplasm; the presence of 25 complete phosphoenolpyruvate–phosphotransferase (PEP–PET) sugar transport systems, as deduced by the complete sequence of the chromosome of *Lb. plantarum* WCFS1, reflects the adaptative behavior of the species.

Besides lactic acid, many secondary end products can be produced from pyruvate (**Figure 2**), the nature of which depends on the strain and culture parameters. For example, in the presence of oxygen, glucose is converted to acetate from pyruvate via acetylphosphate; the same end product is obtained in anaerobiosis, but in the presence of a neutral or alkaline pH or an external electron acceptor (e.g., citrate). The formation of acetate via acetylphosphate increases the synthesis of ATP through acetate kinase activity. Ethanol too can be produced by some *Lb. plantarum* strains under different culture conditions, such as the aerobic and anaerobic catabolism of glucose or from external electron acceptors (e.g., citrate or acetate) during the anaerobic catabolism of mannitol.

In addition, *Lb. plantarum* can produce a variety of secondary end products, such as diacetyl, acetoin, and

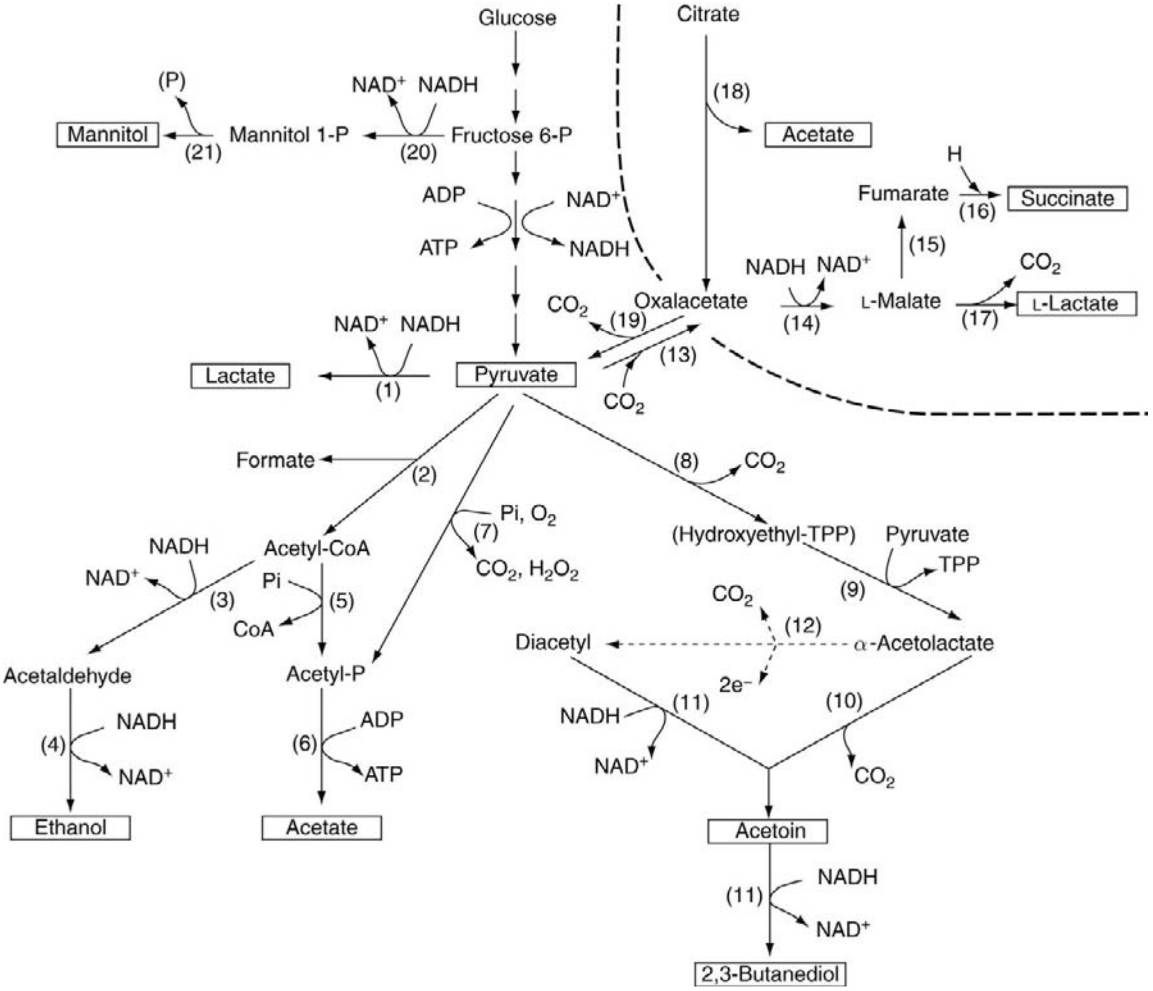
Glucose
Citrate
(P)
NAD+ NADH
Mannitol
(21)
Mannitol 1-P
(20)
Fructose 6-P
(18)
Acetate
H
ADP
ATP
NAD+
NADH
Fumarate
(16)
Succinate
(15)
NADH NAD+
CO2
CO2
Oxalacetate
(14)
L-Malate
(17)
L-Lactate
(19)
(13)
NAD+ NADH
Lactate
(1)
Pyruvate
CO2
Formate
(2)
(8)
CO2
Pi, O2
(7)
CO2, H2O2
NADH
Acetyl-CoA
(Hydroxyethyl-TPP)
Pyruvate
TPP
NAD+
Pi
(3)
(5)
CoA
CO2
(9)
(12)
Diacetyl
α-Acetolactate
Acetaldehyde
Acetyl-P
2e−
NADH
(11)
(10)
(4)
NADH
NAD+
(6)
ADP
ATP
NAD+
CO2
Ethanol
Acetate
Acetoin
(11)
NADH
NAD+
2,3-Butanediol

Figure 2 Glucose and citrate degradation pathways proposed for the *Lactobacillus plantarum* wild-type strain (NCIMB8826) and LDH-defective strain (TF103). (1) LDH; (2) pyruvate formate-lyase; (3) acetaldehde dehydrogenase; (4) alcohol dehydrogenase; (5) phosphotransacetylase; (6) acetate kinase; (7) pyruvate oxidase; (8) pyruvate decarboxylase; (9) α-acetolactate synthase; (10) α-acetolactate decarboxylase; (11) 2,3-butanediol dehydrogenase; (12) nonenzymatic decarboxylation; (13) pyruvate carboxylase; (14) malate dehydrogenase; (15) fumarase; (16) fumarate reductase; (17) malolactic enzyme; (18) citrate lyase; (19) oxalacetate decarboxylase; (20) mannitol-1-phosphate dehydrogenase; (21) mannitol-1-phosphatase–enzyme IIMtl. CoA, coenzyme A; $2e^-$, electrons transferred to an unknown acceptor; Tpp, thiamine PPi. Reproduced with permission from Ferain T, Schanck AN, and Delcour J (1996) ^{13}C nuclear magnetic resonance analysis of glucose and citrate end products in a *ldhL-ldhD* double-knockout strain of *Lactobacillus plantarum*. *Journal of Bacteriology* 178: 7311–7315.

2,3-butanediol, which represent key flavor compounds in many dairy products, from glucose and citrate metabolism via α-acetolactate. Other end products from citrate metabolism can be lactic, acetic, and succinic acids as well as CO_2. Citrate metabolism of *Lb. plantarum* is greatly affected by external pH (optimum pH is 4.5) and by the physiological state of the cells (e.g., growing or not growing). It has been reported that in growing cells, metabolism of citrate is higher in cofermentation with galactose than in the presence of glucose or lactose, whereas in nongrowing cells, metabolism is not significantly enhanced by the presence of fermentable sugars. Moreover, citrate is metabolized more rapidly than sugar by nongrowing cells; on the contrary, growing cells show a more rapid sugar consumption. Malic and tartaric acids too are metabolized directly or in cofermentation with carbohydrates, to produce CO_2, lactic and acetic acids, and other by-products. Through the malolactic fermentation, *Lb. plantarum* converts malic acid into lactic acid and CO_2, which represents an important metabolism in the production of some type of wine.

On the basis of the sequenced genome of *Lb. plantarum* WCFS1, metabolic pathways have been reconstructed. LacplantCyc includes 129 pathways and 704 predicted reactions involving 670 chemical species and 710 enzymes. It represents the most extensively curated pathway genome for Gram-positive bacteria.

Reconstruction of the metabolic pathways of *Lb. plantarum* WCFS1 confirmed the prototrophy of the species toward folic acid, thiamine, and pyridoxal-5-phosphate. The same study showed that most pathways for amino acid biosynthesis are complete. Only arginine, glutamate, tryptophan, and branched-chain amino acids (isoleucine, leucine, and valine) are essential for growth of the species. Auxotrophy for glutamate is caused by an incomplete TCA cycle and hence no supply of α-ketoglutarate. Synthesis of the tryptophan is regulated by a feedback mechanism in the presence of other amino acids such as phenylalanine and tyrosine, and for that reason, tryptophan is required for growth. The auxotrophy for arginine has not yet been clarified.

Presence of glucose increases the conversion of methionine, and the aminotransferase activity shows variable specificity for the amino group acceptors glyoxylate, ketoglutarate, oxalacetate, and pyruvate. In *Lb. plantarum*, serine is deaminated to pyruvate and ammonia probably by serine dehydratase. Pyruvate is then catalyzed by pyruvate–formate lyase to acetate, formate, and CO_2; acetoin is also produced in small quantities from serine.

Lactobacillus plantarum possesses an arginine–deiminase (ADI) pathway. Through this pathway, citrulline, ornithine, and ammonia are formed from arginine, driving an energy supplement for the cell and contributing to pH homeostasis because of the NH_3, which combines with protons. Moreover, ornithine is a precursor for the formation of volatile compounds such as 2-acetyl-1-pyrroline, which represents a key flavor compound in bread crust. Benzaldehyde is formed from phenylpyruvic acid, derived by the aminotransferase activity on phenylalanine, in the presence of high levels of Mn^{2+}, and contributes to the generation of flavor compound during cheese ripening. Mn^{2+} also presents a pseudocatalase activity, which replaces SOD in a defense mechanism of the cell against oxygen toxicity.

When compared with other mesophilic lactobacilli, *Lb. plantarum* shows a lower proteolytic activity, higher on β-casein as on α_{s1}-casein, than reported for some strains. Nevertheless, a high proteolytic activity has been reported for the strain *Lb. plantarum* DBPZ1015 during sourdough fermentation, with the formation of consistent amount of free amino acids. Proteinase and peptidase activities have been detected in the cell lysates of *Lb. plantarum* isolated from cheese, and studies conducted on cell-free extracts of *Lb. plantarum* demonstrated higher specific activity toward hydrophobic as compared with hydrophilic dipeptides. Peptides are hydrolyzed by intracellular peptidases of *Lb. plantarum*, several of which were characterized at the biochemical and genetic level.

Compared with other cheese-related mesophilic and thermophilic *Lactobacillus* spp., *Lb. plantarum* shows high levels of esterolytic and lipolytic activity related to strain-specific intracellular and/or extracellular lipases and intracellular esterases. An intracellular tributyrin esterase of 85 kDa and an intracellular lipase of 65 kDa have been purified and characterized from *Lb. plantarum* DPC 2739 isolated from Cheddar cheese. Lactobacilli mainly or only hydrolyze esters containing C_4–C_6 fatty acids. The esterase activity of some *Lb. plantarum* strains is highest on β-naphthyl butyrate, but β-naphthyl esters of C_{16}, C_{18}, and $C_{18:1}$ fatty acids are also hydrolyzed. In general, lipase activity of the strains decreases progressively as the length of the fatty acid chain increases from tributyrin to tripalmitin, even if a strain isolated from dry fermented sausages was found to produce an extracellular lipase active on triolein. On the other hand, recent studies conducted on strains isolated from salted meat reported that of 17 strains of *Lb. plantarum* tested, none was found to possess lipolytic activity.

Proteins (12) and enzymes (30), mainly hydrolases and transglycosylases, have been analyzed and are predicted to be involved in adherence to host components such as mucin and collagen; this finding suggests that they could play a role in substrate degradation and can maintain the growth of *Lb. plantarum* in different environmental niches.

Antimicrobial Substances and Other Compounds with Potential Impact on Food Quality

Besides organic acids, lactic acid bacteria can produce many inhibitory compounds with activity against other microorganisms. Bacteriocins, phenyllactic acid, peptides, and fatty acids are the most active ones and could be of interest in relation to food biopreservation.

Bacteriocins are ribosomally synthesized proteins or peptides, often cationic, amphiphilic, and membrane permeabilizing, that inhibit growth of other bacteria. They are usually divided into three classes on the basis of common, mainly structural, characteristics. Class I is of small, heat-stable peptides containing thioether amino acids, such as lanthionine, and is hence named lantibiotics. Class II is of small, hydrophobic, heat-stable, nonmodified bacteriocins consisting of either a single peptide with antilisterial activity (class IIa) or two polypeptide chains (class IIb), and also includes other peptide bacteriocins (class IIc). Class III is of large, hydrophilic, heat-labile proteins. Bacteriocins produced from *Lb. plantarum,* generally indicated as plantaricins, have been included in both classes I and II. Some examples are plantaricin C and W (class I); plantaricin C19 and 423 and pediocin AcH (class IIa); plantaricin EF, JK, S, and NC8 (class IIb); and plantaricin 1.25β (class IIc). Production of plantaricins is dependent on pH and temperature, and maximum yield is generally obtained at neutral pH and on incubating the producer strains at

30 °C. Strains of *Lb. plantarum* that produce bacteriocins have been isolated from various vegetal and animal matrices such as cereals, sourdough, wine, meat, and dairy products. Examples of bacteriocins produced by *Lb. plantarum* isolated from dairy products are plantaricin C, plantaricin TF711, and pediocin AcH.

In recent years, PCR-based methods have been developed for the identification of bacteriocin-encoding genes; several genes, including *pln*A, *pln*EF, *pln*J, *pln*K, *pln*JKLR, *pln*MNOP, *pln*ABCD, *pln*EFI, and *pln*GHSTUV, have been associated with plantaricin production. Genes *pln*EF and *pln*J have been reported to encode two-peptide bacteriocins; the *pln* locus of bacteriocin produced from the strains *Lb. plantarum* C11, NC8, J23, and J51 showed different patterns of operons involved in transport, secretion of induction factor, and response regulation of the bacteriocin biosynthesis. It is a general opinion that production of several bacteriocins from *Lb. plantarum* is quorum-sensing regulated in the presence of a threshold cell density in the environment, which determines the secretion of the induction factor.

Other nonbacteriocin antimicrobial proteins or peptides are produced by lactic acid bacteria; *Lb. plantarum* MiLAB14 produces two cyclic peptides (cyclo(L-Phe-trans-4-OH-L-Pro) and cyclo(L-Phe-L-Pro)) and one protein, all showing antifungal activity at a concentration on the order of milligram per milliliter; the same strain produces antifungal hydroxylated fatty acids active at 10 μg ml^{-1}.

The major antifungal compounds of some *Lactobacillus* spp., including *Lb. plantarum,* are represented by phenyllactic acid (PLA) and 4-hydroxyphenyllactic acid (OH-PLA), which are derived from the catabolism of phenylalanine; these compounds showed inhibitory activity against *Fusarium* spp., *Penicillium* spp., and mycelial growth of *Aspergillus niger.*

Lactic acid bacteria also produce high amounts and a great variety of homo- and hetero-exopolysaccharides (EPSs). These compounds play an important role in the manufacture of fermented dairy products as safe additives to improve texture, viscosity, and stability.

The biosynthesis of bacterial EPSs is complex and unstable, and a large number of genes are involved. Molecular approaches have been used to characterize the genes encoding EPSs. The presence of a glycosyltransferase gene has been reported for *Lb. plantarum.*

EPSs are generally produced from *Lb. plantarum* during the exponential phase of growth and reach the maximum amount at the beginning of the stationary phase. Some strains of *Lb. plantarum* can produce more than one type of EPS, differing in terms of molecular mass and sugar composition. A study carried out on the strain *Lb. plantarum* EP56 revealed the production of two polymers of 8.5×10^5 and 4×10^4 Da, differing in the content of *N*-acetyl galactosamine and rhamnose, respectively.

Carbon source and temperature greatly influence EPS synthesis by *Lb. plantarum*; compared with glucose, galactose, fructose, and sucrose, lactose is the most efficient carbon source for EPS biosynthesis by strain EP56. To optimize the fermentation parameters for EPS production by *Lb. plantarum*, statistical and computational methods such as artificial neuronal networks have been applied.

Functional Activities and Health Benefits

A large number of lactic acid bacteria have been classified as probiotics. According to the definition adopted by the World Health Organization, probiotics are "live microorganisms that when administered in adequate amounts confer a health benefit to the host". Probiotics are either incorporated into functional foods or marketed as lyophilized cells; criteria for selection of a probiotic include its ability to reach the final destination in a high live number, adhere to mucus and epithelial cells, and survive at low pH values and high bile salt concentrations. Susceptibility to antibiotics, β-galactosidase activity, and ability to utilize fructooligosaccharides (FOSs) are other important characteristics to evaluate. With regard to this point, it has been recently reported that different types of FOSs, recognized as prebiotics, can be fermented by *Lb. plantarum* because of the presence of specific transporters and hydrolases; genetic studies related this ability to the presence of operons possessing different architecture, which suggests that these genes were acquired after evolutionary divergence of the species.

Even though it has been demonstrated that colonization of gastrointestinal (GI) tract can be dependent on the individual, the complete sequence of the genome of *Lb. plantarum* WCFS1 can be useful to elucidate many molecular mechanisms related to the intestinal properties of this species. The large number of genes encoding surface proteins (217 predicted proteins) could be involved in the environmental adaptation of some strains, because several of those genes show homology to mucus-binding, aggregation-promoting, and intracellular adhesion proteins. *Lactobacillus plantarum* strain 299v (DSM 9843) is marketed as a probiotic since 1999; this strain showed an ability to adhere to human cells in a mannose-inhibited manner (that is indicative of binding to a mannosylated cell-bound receptor). Studies performed using DNA microarray analysis on the strain *Lb. plantarum* WCFS1 revealed the presence of two genes involved in mannose adhesion. This finding could be of interest considering that mannose residues are commonly found on the surface of eukaryotic cells. A daily consumption for 3 weeks of *Lb. plantarum* 299v gives rise to a significant increase in the fecal concentration of short-chain fatty acids (from 83 to 113 μmol g^{-1} wet feces), as well as of acetic and propionic acids.

Moreover, a significant decrease in Gram-negative anaerobic bacterial count has been observed in jejunum and rectum of healthy volunteers after daily intake of *Lb. plantarum* 299v and *Lb. plantarum* E98. Studies on the effectiveness of *Lb. plantarum* against intestinal infections and pathogenic bacteria also indicated a protective effect against *Escherichia coli* and *Clostridium difficile*, and more than one possible mechanism of action has been supposed to explain this effect. *Lactobacillus plantarum* 299v also showed an ability to reduce LDL-cholesterol and fibrinogen levels in the blood. Administration of another strain, *Lb. plantarum* PH04, reduced serum cholesterol by 7% and triglycerides by 10%, decreasing considerably coronary heart diseases in rats. Moreover, a possible mechanism of stimulation of the immune response in mice has been reported after oral administration of *Lb. plantarum* CRL 936.

Resistance to antibiotic action has been studied for many *Lb. plantarum* strains isolated from dairy products. Several strains of *Lb. plantarum* isolated from raw milk showed a broad spectrum of resistance to many antibiotics such as tetracycline, erythromycin, ampicillin, penicillin G, ofloxacin, and vancomycin. A certain susceptibility has been shown by some strains to norfloxacin, gentamicin, amikacin, cefuroxime, and streptomycin. *Lactobacillus plantarum* 423, tested as probiotic in infant milk formulations, showed resistance to norfloxacin and ciprofloxacin. Moreover, a high susceptibility of *Lb. plantarum* to inhibitors of protein synthesis has been demonstrated.

Many authors suggested that the use of *Lb. plantarum* as a probiotic could be preferred above other lactic acid bacteria because of convenience in production, high-level genetic accessibility, and high performance in the GI tract.

Other Characteristics of *Lactobacillus plantarum* Possibly Related to Health Benefits

In the recent years, some work has been done to evaluate the possibility of reducing the toxicity of some proteins such as gluten by selected lactic acid bacteria: indeed, the presence of residual proline and glycine polypeptides is considered to be responsible for celiac disease. A probiotic named VSL#3, obtained with mixed cultures of *Lb. plantarum*, other *Lactobacillus* spp., as well as *Streptococcus thermophilus* and bifidobacteria, was successfully applied for the hydrolysis of gliadin during dough leavening, and the resulting bread was tolerated by a patient suffering from celiac disease. With relation to the same food, as phytic acid and *myo*-inositol hexaphosphate (IP_6) represent antinutritional factors in baked products because of their property to chelate divalent dietary minerals, a mixed starter culture including *Lb. plantarum*, *Lactobacillus brevis*, and *Lactobacillus curvatus* was successfully used to reduce IP_6 content by about 80–90% in sourdough after 12 h of fermentation.

Recently, conjugated linoleic acids (CLAs), especially the isomers *cis*-9, *trans*-11-octadecadienoic acid and *trans*-10, *cis*-12-octadecadienoic acid, have attracted much attention because of their beneficial effects, such as reduction of carcinogenesis, arteriosclerosis, and body fat. In that context, it has been demonstrated that *Lb. plantarum* is able to produce considerable amounts of *cis*-9, *trans*-11-octadecadienoic acid and *trans*-9, *trans*-11-octadecadienoic acid, the two main bioactive CLA isomers, by the bioconversion of linoleic and ricinoleic acids.

Another challenge is represented by the use of *Lb. plantarum* as a live vaccine to deliver heterologous antigens to the mucosal immune system. For this purpose, *Lb. plantarum* NCIMB 8826 has been studied as a model; some studies have also exploited the ability of *Lb. plantarum* to secrete bioactive molecules that evoke an immune response. Advantages of the LAB over traditional vaccines are represented by the noninvasiveness and the possibility to induce both a systemic and mucosal immune response.

Milk and Dairy Products

Lactobacilli are microorganisms traditionally regarded as useful for dairy technology, and interest in their applications is reinforced by documented probiotic and functional properties. *Lactobacillus plantarum* has been frequently isolated from dairy products, and it is a member of a group of mesophilic lactobacilli referred to as ‘nonstarter lactic acid bacteria’ (NSLAB), which represent an adventitious secondary microflora that can survive pasteurization and enter milk or curd as a contaminant and become dominant, reaching levels of 10^6–10^8 cfu g^{-1}, during cheese ripening. It has been reported that the count of *Lb. plantarum* is higher during the first stage of cheese ripening (from 1 week to 3–4 months), reaching often more than 10^7 cfu g^{-1}; during prolonged aging (6–7 months), the count of the species generally decreases to 10^4 cfu g^{-1}, and often other *Lactobacillus* spp. become the dominant microbial population. The growth rate and final population density are affected by pH, salt, and moisture levels of the curd and by the temperature of ripening. The presence of *Lb. plantarum* has been documented in many cheese varieties made throughout the world, using either pasteurized or raw cows’, ewes’, or goats’ milk, as well as in traditional fermented milks and milk-based beverages. Some examples of European cheeses include Italian varieties of Pecorino cheese and Gorgonzola, the Spanish Manchego and Roncal, the Portuguese Picant, and Irish Cheddar cheese. Among cheeses produced worldwide and fermented milks, one can mention Tibetan Qula cheese, Kenian fermented milk, Himalayan ethnic fermented milk, Moroccan camel milk, and New Zealand Cheddar cheese.

In Feta and Teleme cheeses (Greece), *Lb. plantarum* represents 47.8 and 65.8% of the isolated lactobacilli, respectively; it constitutes 56.9% of the main species isolated from Tenerife goats' milk cheese, and it is predominant in cave and pit (Fossa) ripened cheeses. A study on Irish Cheddar cheese indicated that *Lb. plantarum* represented 28% of the NSLAB, while a similar study on mature cheese revealed *Lb. plantarum* as 2.1% of the population. Another study on the population dynamics of Cheddar cheese referred to a mixture of *Lb. plantarum*, *Lb. paracasei*, and *Lb. rhamnosus* till 6 weeks of maturation; thereafter, no strains of *Lb. plantarum* were found.

Many laboratory-scale studies have been carried out in model cheese systems to elucidate the dynamics and impact of an 'NSLAB starter' formulation. Model studies applied to Cheddar cheese revealed that NSLAB produce desirable flavor and reduce harshness and bitterness associated with some starter cultures; moreover, an extension of shelf life of the cheeses was observed as well as a major agreement at sensory analysis with respect to cheeses made with traditional starter cultures.

Because the starter microorganisms rapidly deplete all the lactose, the ability to use residual substrates such as lactate, citrate, pyruvate, proteins, and lipids is necessary for NSLAB. Various studies reported the ability of *Lb. plantarum* to metabolize citrate in the presence or absence of other fermentable sugar; citrate represents the precursor of important flavor components of cheeses such as diacetyl, acetate, acetoin, and 2,3-butanediol. Recent studies evaluating the interactions between starter bacteria and adjunct *Lb. plantarum* showed that citrate degradation in cheese varied according to the type of starter used; *Lb. plantarum* degraded citrate to acetoin and diacetyl, especially in the presence of a Cit^- mesophilic homofermentative starter. Moreover, the same adjunct strain degraded citrate to succinic acid in the presence of a thermophilic starter.

In general, NSLAB show intense secondary proteolysis, as evidenced by high oligoendopeptidase and aminopeptidase activities, leading to high levels of free amino acids contributing directly or as precursors to cheese flavor. *Lactobacillus plantarum* can catabolize aromatic and branched-chain amino acids via transamination, which represents a key step in the amino acid conversion to aroma compounds in cheeses. The mechanism is initiated by a transamination reaction and is catalyzed by aminotransferases, because the degradation occurs only in the presence of an α-ketoacid, which is used as an amino group acceptor; flavor compounds such as hydroxyacids, aldehydes, alcohols, and carboxylic acids are further produced from α-ketoacids.

Some studies have been focused on the purification and characterization of lipases and esterases from *Lb. plantarum*, indicating the potential significance of some strains to lipolysis during cheese ripening and highlighting the need for selecting appropriate starters to produce enzyme-modified cheese as well as accelerated ripened cheese because of wide variations in activity between strains. It has been reported that strains of *Lb. plantarum* isolated from Argentinean goats' milk and cheeses also showed high specific activity on α-naphthol butyrate, caproate, and acetate, and it was found that some strains presented more than one esterase; for example, *Lb. plantarum* O236 showed four enzymes that hydrolyze carboxyl ester linkages with different specificities. Moreover, the intracellular and extracellular fractions of the above strain were also able to hydrolyze tributyrin. On the other hand, another strain (*Lb. plantarum* O155) did not hydrolyze triglycerides.

Mesophilic lactobacilli also possess some glycoside hydrolase activity, and it has been shown in model systems that they can utilize sugars from glycoproteins of the milk fat globule membrane.

Even if the role of NSLAB in cheese ripening continues to be under investigation, the above-mentioned finding corroborates the hypothesis of a useful impact of selected *Lb. plantarum* strains on the overall quality of many dairy products.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks. **Fermented Milks**: Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Kefir; Koumiss; Middle Eastern Fermented Milks; Nordic Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture. **Nutrients, Digestion and Absorption**: Fermentation in the Rumen.

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Lactobacillus spp.: *Lactobacillus delbrueckii* Group

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Introduction

Based on the first phylogenetic analysis of lactobacilli performed in the early 1990s, the genus *Lactobacillus* was divided into three groups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei pediococcus* group, and the *Leuconostoc* group. The phylogenetic *Lb. delbrueckii* group referred to *Lb. delbrueckii* and other species such as *Lb. gasseri* and *Lb. acidophilus*. The description of a large number of species in recent years and the following phylogenetic reexamination of the genus have contributed to the splitting of the three lactobacilli groups into smaller groups more feasible.

In this section, only the *Lb. delbrueckii* species is described. The *Lb. delbrueckii* species consists of what were previously considered to be four separate species with very similar phenotypes (*Lb. delbrueckii*, *Lb. leichmanni*, *Lb. lactis*, and *Lb. bulgaricus*). The genotypes of these four previous species show 80% DNA homology and, because of this similarity, they are now retained as a single species with three subspecies. The subspecies, which are not separated by rRNA sequence analysis, are *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* (includes the previous species *Lb. lactis* and *Lb. leichmanni*), *Lb. delbrueckii* subsp. *delbrueckii*, and the novel subspecies *Lb. delbrueckii* subsp. *indicus*.

Two of the four subspecies of *Lb. delbrueckii* are important thermophilic lactobacilli starters in dairy fermentations. *Lactobacillus delbrueckii* subsp. *bulgaricus* is used extensively as a starter for yogurt manufacture. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* are two of the three thermophilic lactobacilli used in cheese manufactured with elevated cooking temperatures. They are usually paired with *Streptococcus thermophilus*, show associative growth with this starter, and contribute other attributes, including flavor and texture modifications, to the fermented dairy products in addition to rapid acid development.

General Characteristics

The strains from the *Lb. delbrueckii* group form single or short chains of rods with rounded ends, have only slight growth at <10 °C, have an optimum growth in milk between 40 and 45 °C, and are still able to grow at 50–55 °C. Their DNA ranges from 49 to 51 mol% G+C and the cell surface is characterized by a lysine-D-aspartyl-type peptidoglycan and a glycerol-based teichoic acid.

Enumeration, Isolation, and Identification

The medium most commonly used for the enumeration of *Lb. delbrueckii* is MRS (de Man, Rogosa, and Sharpe) agar incubated anaerobically at 37 °C. However, this medium is nonselective and will recover other starter bacteria such as *Sc. thermophilus*.

The method for enumeration of *Lb. delbrueckii* in yogurt as recommended by the International Dairy Federation is on MRS agar at pH 5.4. This will prevent the growth of *Sc. thermophilus*, but will not inhibit the growth of other lactobacilli that can be present in the yogurt. It is advisable to check the recovery of the particular strain of *Lb. delbrueckii* used in the yogurt on MRS agar at pH 5.4, compared with standard MRS, as some strains give reduced recoveries at the lower pH.

When probiotic cultures of lactobacilli are added to yogurt, it may be necessary to check the identity of strains on the isolation medium by carbohydrate fermentation or genotypic techniques that exhibit various levels of discriminatory power, from species level to differentiation of individual strains.

Identification of *Lb. delbrueckii* strains by genotypic methods includes PCR with specific primers, determination of gene sequences (16S rRNA, 23S rRNA, and 16S 23S rRNA intergenic spacer region), restriction enzyme analysis (REA) of DNA or PCR amplicons, randomly primed (RAPD)-PCR, or culture-independent methods such as DNA hybridization using labeled probes or DGGE (denaturing gradient gel electrophoresis) analysis of PCR amplicons. Characteristics that distinguish the four subspecies of *Lb. delbrueckii* are summarized in **Table 1**.

Lactobacillus delbrueckii subsp. *bulgaricus*

This subspecies is isolated from cheese and fermented milk products and ferments the smallest range of carbohydrates (glucose, lactose, and fructose) of the three

Table 1 Characteristics that distinguish the four subspecies of *Lactobacillus delbrueckii*

	Fermentation pattern									
Subspecies	N-*Acetyl glucosamine*	*Maltose*	*Lactose*	*Sucrose*	*Trehalose*	*ARDRA* Eco*RI*	*Subspecies-specific PCR*	lacZ *gene*	*Regulation of the* lac *operon*	galT *gene*
Lb. bulgaricus	–	–	+	–[a]	–	2	B	+	C	–
Lb. lactis	+	+	+	+	+	1	A	+	I	+
Lb. delbrueckii	–	–	–	+	–	1	NA	–	ND	–
Lb. indicus	–[a]	–	+	+	–	1	NA	–	C	+

[a] Except one strain.

+, positive; −, negative. ARDRA *Eco*RI sites in the 16S rRNA gene sequence: 1, one site at 60 bp; 2, two sites at 60 and 690 bp. Subspecies-specific PCR assay: NA, no amplification; A, one 1600 bp amplicon; B, one 1065 bp amplicon. *lacZ* is the β-galactosidase gene: +, presence of gene; −, absence of gene.

Adapted from Dellaglio F, Felis GE, Castioni A, Torriani S, and Germond JE (2005) *Lactobacillus delbrueckii* subsp. *indicus* subsp. nov., isolated from Indian dairy products. *International Journal of Systematic and Evolutionary Microbiology* 55: 401–404.

subspecies from the *Lb. delbrueckii* group. The D(−) lactate dehydrogenase from this subspecies migrates faster in gel electrophoresis than the enzyme from the other two subspecies. No strains ferment arginine.

The genetics and the proteolytic enzymes of *Lb. delbrueckii* subsp. *bulgaricus* have been studied in more detail than the other two subspecies. Despite the difficulty of transforming this subspecies, some of the specific genes of lactose metabolism have been cloned into *Escherichia coli* and sequenced.

Lactobacillus delbrueckii subsp. *lactis*

These strains are isolated from a range of dairy products and grain mash. Of the three subspecies, *Lb. delbrueckii* subsp. *lactis* ferments the widest range of carbohydrates (esculin, fructose, glucose, lactose, maltose, mannose, salicin, sucrose, and trehalose), and some strains ferment cellobiose and galactose. Arginine is fermented by a number of strains.

Lactobacillus delbrueckii subsp. *delbrueckii*

The strains from this subspecies are isolated from fermented plant material. *Lactobacillus delbrueckii* subsp. *delbrueckii* does not ferment lactose and is not important in dairy products either as a starter or as an adventitious bacterium. All strains ferment glucose, fructose, mannose, and sucrose and a number of strains ferment cellobiose, maltose, and trehalose.

Lactobacillus delbrueckii subsp. *indicus*

This subspecies, isolated from Indian dairy products, could not be assigned to another definite subspecies because molecular identification and phenotypic traits did not agree with those of recognized subspecies of *Lb. delbrueckii.* Glucose, fructose, mannose, and lactose are fermented by all strains, while fermentation of sucrose and *N*-acetylglucosamine is more variable. Glycerol, arabinose, ribose, xylose, galactose, rhamnose, mannitol, sorbitol, maltose, and inulin are not fermented. Arginine is not deaminated. All the strains grow in MRS at pH 3.4 and 5 but not at 7.8.

Lactose Fermentation

The *Lb. delbrueckii* group are obligately homofermentative lactobacilli with the major fermentation product from a variety of carbohydrates being the D(−) isomer of lactic acid. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* ferment lactose and possess β-galactosidase to hydrolyze lactose. The chromosomally encoded β-galactosidase nucleotide sequence from *Lb. delbrueckii* subsp. *bulgaricus* has been determined. *Lactobacillus delbrueckii* subsp. *bulgaricus* is galactose negative. When growing in milk, it transports the lactose into the cell in association with the expulsion of galactose via an antiport system, similar to that in *Sc. thermophilus.* Inside the cell, lactose is hydrolyzed by β-galactosidase with only glucose being fermented to D(−) lactate via the Embden–Meyerhof pathway. Many of the *Lb. delbrueckii* subsp. *lactis* strains are also galactose negative and, by implication, may use lactose in a similar way.

Bacteriophage

Lactobacillus delbrueckii subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* have lytic and temperate bacteriophages that can be a problem during milk fermentations in both cheese and yogurt products. The genetics, life cycle, and morphology of bacteriophage of a number of *Lb. delbrueckii* subsp. *bulgaricus* and *lactis* strains have been documented. It is important to use good starter production and manufacturing practices to eliminate or control bacteriophage attack and associated poor acid production. Phage monitoring is particularly important in the dairy industry. The detection of lytic bacteriophages and the presence of the prophage *int* gene in *Lb. delbrueckii* strains can be carried out by PCR protocols. Phylogenetic analysis of partial *int* gene sequences revealed a high similarity within strain- and phage-derived sequences, which suggests a common evolution in the starter culture ecosystem. To minimize phage disseminations in dairy plants, various strategies are applied, such as strain rotation programs, direct vat inoculation of starters, optimized sanitation, and use and improvement of phage-resistant starter cultures.

Significance of *Lactobacillus delbrueckii* subsp. *bulgaricus* in Yogurt and Yogurt-Like Products

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the economically most important lactic acid bacteria, with a worldwide application in the production of fermented milks including yogurt. Fermented milks have long been recognized as a nutritious, natural, and safe component of a healthy diet and are the basis of the concept of probiotics. A well-documented health benefit of the consumption of yogurt containing live *Lb. delbrueckii* subsp. *bulgaricus* and *Sc. thermophilus* is an attenuation of lactose intolerance. In addition, immune modulation and diarrhea-alleviating effects have been reported, and both *Lb. delbrueckii* subsp. *bulgaricus* and *Sc. thermophilus* have been implicated in these effects. For more details on the use of *Lb. delbrueckii* subsp. *bulgaricus* in yogurt and yogurt-like products, *see* **Fermented Milks**: Yogurt: Role of Starter Culture.

Associative Growth

During yogurt fermentations, protocooperation between *Lb. delbrueckii* subsp. *bulgaricus* and *Sc. thermophilus* results in accelerated acidification, but the mechanisms involved are not completely understood. The associative growth between these two species in milk is important, such that the rate of acid production is greater with the correct mixture of the two species. The strain pairing and ratio of the two species must be optimized in the different milk fermentations.

Lactobacillus delbrueckii subsp. *bulgaricus* is more proteolytic than *Sc. thermophilus* and in milk provides a range of amino acids that stimulate the growth of *Sc. thermophilus* strains. The growth of *Lb. delbrueckii* subsp. *bulgaricus* in milk benefits from the stimulation of formic acid and possibly carbon dioxide and pyruvate produced by *Sc. thermophilus*.

Other Factors Influencing Growth in Milk

Some strains of *Lb. delbrueckii* subsp. *bulgaricus* are more sensitive than *Sc. thermophilus* to antibiotics like streptomycin. The presence of antibiotics in milk can break down the associative growth between the two species, resulting in lower rates of acid development.

Strains of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* can produce bacteriocins with different activities. Lacticin A and B are high-molecular-weight-type bacteriocins produced by strains of *Lb. delbrueckii* subsp. *lactis* that inhibit the growth of only *Lb. delbrueckii* subsp. *bulgaricus* strains. In contrast, the thermostable bacteriocin bulgarican, produced by a strain of *Lb. delbrueckii* subsp. *bulgaricus*, has a broad spectrum, inhibiting different species of both Gram-positive and Gram-negative bacteria. For practical purposes, pairing of bacteriocin and nonbacteriocin producers must be checked to ensure that rapid acid production is not compromised.

Flavor Production

Acetaldehyde, an important flavor compound in milk fermentations such as yogurt, is derived mainly from the metabolism of threonine by the enzyme threonine aldolase, produced by *Lb. delbrueckii* subsp. *bulgaricus*. The activity of this enzyme varies according to the strain, pH of milk, salt, and divalent cations.

Other flavor compounds that contribute to yogurt flavor include other carbonyl products (diacetyl, acetoin, and acetone), acids (lactic, succinic, butyric, formic, and acetic), other volatiles (ethyl esters, ethanol, and butanone), and a mixture of amino acids. *Lactobacillus delbrueckii* subsp. *bulgaricus* will produce some of these compounds depending on the strain and milk fermentation conditions.

The extensive proteinase and peptidase activities of this species will contribute to the amino acid content of fermented milks when used as a major starter and may play a role in the gel properties of casein. Although *Lb. delbrueckii* subsp. *bulgaricus* can use a number of amino acids in addition to threonine, this is unlikely to occur to a significant extent in the final low pH of most yogurts.

Exopolysaccharide Production

Strains from the *Lb. delbrueckii* group, and in particular *Lb. delbrueckii* subsp. *bulgaricus*, can produce exopolysaccharides (EPS) under a variety of conditions. A number of commercial yogurts have *Lb. delbrueckii* subsp. *bulgaricus* starters that produce significant concentrations of EPS. These affect rheological properties, particularly increasing the viscosity, that improve the texture and associated mouthfeel of yogurt and yogurt-like products.

The specific physical properties of the EPS have not been well studied. The concentration and the composition of EPS are influenced by many factors including temperature, pH, vitamins, growth phase, carbohydrate source, and strains. The composition and the structure of EPS produced by a number of strains have been determined. Most commonly, EPS are made up of galactose and lesser concentrations of glucose and rhamnose, but can include mannose, fructose, and *N*-acetyl-D-galactosamine.

Significance of the *Lactobacillus delbrueckii* Group in Cheese

Acid Production

Strains of *Lb. delbrueckii* subsp. *lactis* and *Lb. delbrueckii* subsp. *bulgaricus* are used as starters when elevated cooking temperatures of 45–60 °C are used in curd preparation for Swiss-type cheeses and the very hard Italian-type cheeses. The strains, used at 0.2–2%, are generally paired with *Sc. thermophilus* and will grow or survive the elevated temperatures and continue to produce acid in the cooling curd. The paired starter inoculum ratio of rods to cocci usually ranges from 10:90 to 90:10. The ratio of the two species and the inoculum level into the vat milk must take into account the strain differences, their interactions, and the presence of inhibitors so that the desired acid production rates can be achieved with the specific manufacturing parameters.

The presence of H_2O_2 in milk from the activity of microorganisms and its activation of the lactoperoxidase/thiocyanate system present naturally in cheese milk can inhibit the growth of *Lb. delbrueckii* subsp.

bulgaricus. Strains of this species can, to varying degrees, produce H_2O_2 via NADH oxidase and remove H_2O_2 via NADH peroxidase. Therefore, in milk fermentations, the use of different strains of *Lb. delbrueckii* subsp. *bulgaricus* must take into account the milk used with respect to the lactoperoxidase system and the H_2O_2 concentration.

In many of the paired thermophilic starter systems used in high-cooked cheeses, *Lb. helveticus* (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus helveticus*) may be used rather than either of the two subspecies from the *Lb. delbrueckii* group. *Lactobacillus delbrueckii* subsp. *bulgaricus* and most strains of *Lb. delbrueckii* subsp. *lactis* paired with *Sc. thermophilus* will produce galactose from lactose and will not ferment it further. In Swiss-type cheeses, the added propionibacteria will use the galactose, and the lactobacilli starter can thus help the growth of the propionibacteria strain in the ripening cheese in addition to the associative growth with the *Sc. thermophilus* starter. In other cheeses made with the same starters, the galactose will remain in the ripened cheese. The residual galactose can be used in undesirable fermentations by adventitious bacteria or cause undesirable browning, particularly when the cheese is further heat-processed into a dried grated product or with Mozzarella on melting.

The use of proteinase-deficient and proteinase-positive strains of *Lb. delbrueckii* subsp. *bulgaricus* with or without *Sc. thermophilus* as starters in the manufacture of Mozzarella cheese can influence not only the rate of acid production but also some of the functional properties such as stretch and melt. The use of proteinase-deficient strains of *Lb. delbrueckii* subsp. *bulgaricus* reduces the browning effect during cooking.

After the cooking temperature, the lactobacilli starters, more so than the *Sc. thermophilus* starters, usually continue to grow at pH <5.6 and ferment the remaining lactose to lactic acid and galactose, particularly for cheese that is brined. For dry-salted cheeses, the salt concentration can prevent some lactose from being fermented by the stressed lactobacilli starters.

Flavor Development

Although the *Lb. delbrueckii* group is selected mainly for acid production in the high-cooked curds, important contributions to flavor development during ripening are also made. However, its contribution to ripening is not well understood. In addition, many factors, including other starters, adjuncts, enzymes, and adventitious bacteria, also contribute to ripening. Unlike the contribution to flavor during yogurt manufacture, in cheese the contribution by the *Lb. delbrueckii* group will be more important after curd manufacture because of the longer ripening times and the higher pH of cheese. Their proteinases and peptidases will be active during the long-term period of ripening and the autolysis of these starters will influence these activities. The autolysis of lactobacilli starters in cheese is not well understood compared with the autolysis and ripening effects of the mesophilic starters. Some of the proteolytic enzymes from the *Lb. delbrueckii* group have been purified and characterized and the genes cloned (e.g., tripeptidase, leucyl aminopeptidase (PepL), and imidodipeptidase (prolidase)); however, their detailed contribution in cheese ripening needs further study. The *Lb. delbrueckii* subsp. *bulgaricus* genomes encode a large set of enzymes involved in amino acid catabolism. The esterolytic activity of the *Lb. delbrueckii* group and the ability to use amino acids, such as threonine and the aromatic and branched-chain amino acids, have been demonstrated in the laboratory. These activities will occur in cheese, but the extent and the overall contribution to ripening are not established.

Functional Properties

Recent findings provide potential basis to develop healthy or functional foods fermented by *Lb. delbrueckii*. Selected *Lb. delbrueckii* subsp. *bulgaricus* SS1 produced fermented milk that contained bioactive peptides (angiotensin-converting enzyme (ACE) inhibitory). Some other strains belonging to *Lb. delbrueckii* subsp. *bulgaricus* show an ability to synthesize high levels of γ-aminobutyric acid (GABA) during cheese ripening and milk fermentation, a nonprotein amino acid with different physiological functions such as neurotransmission, induction of hypotension, and diuretic and tranquilizer effects. Furthermore, it has been demonstrated that probiotic strains of *Lb. delbrueckii* stimulate the production of inflammatory cytokines and induce macrophage activation and Th1 differentiation in peripheral blood mononuclear cells (PBMCs) in humans; their use as adjuvants in vaccination protocols has been proposed.

See also: **Cheese**: Biochemistry of Cheese Ripening; Swiss-Type Cheeses. **Fermented Milks**: Yogurt: Role of Starter Culture. **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus helveticus*.

Further Reading

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Lactobacillus spp.: Other Species

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Introduction

Lactobacillus is the largest genus within the group of lactic acid bacteria. To date (July 2010), it contains 168 species, some of which are used in the manufacture of fermented dairy, sourdough, meat, and vegetable foods, or used as probiotics. The genus *Lactobacillus* belongs to the phylum Firmicutes, class Bacilli, order II Lactobacillales, and family Lactobacillaceae. Lactobacilli are Gram-positive, catalase-negative, non-spore-forming, rod-shaped bacteria that produce lactic acid as the major end product of fermentation. The phylogenetic relatedness of lactobacilli was investigated by sequencing the 16S rRNA of all species successfully described up to now. This permitted the allotment of the lactobacilli to the following groups: *Lactobacillus buchneri* group (bu), *Lactobacillus casei* group (ca), *Lactobacillus delbrueckii* group (de), *Lactobacillus plantarum* group (pl), *Lactobacillus reuteri* group (re), *Lactobacillus sakei* group (sa), and *Lactobacillus salivarius* group (sl). On the other hand, *Lactobacillus brevis* and *Lactobacillus perolens* as well as the related species *Lactobacillus bifermentans* and *Lactobacillus coryneformis* are uniquely positioned among the lactobacilli.

Various lactobacilli are used in cheesemaking. As lactose is the only fermentable carbohydrate, low water activity and oxygen tension limit the growth of several species. However, some microorganisms are still able to grow under these environmental conditions and to influence maturation. Lactobacilli used in cheesemaking are either mesophilic or thermophilic. Several species of lactobacilli used in dairy fermentations as starters, flavor adjuncts, probiotics, or adventitious nonstarter lactic acid bacteria (NSLAB) are covered in other articles. These include the *Lb. casei* group (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus casei* Group), the *Lb. delbrueckii* group (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group), *Lb. helveticus* (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus helveticus*), *Lb. acidophilus* (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus acidophilus*), and *Lb. plantarum* (*see* **Lactic Acid Bacteria:** *Lactobacillus* spp.: *Lactobacillus plantarum*). Besides, other species of lactobacilli are commonly used in fermentations. These include facultatively and obligately heterofermentative species such as *Lb. brevis*, *Lactobacillus fermentum*, *Lb. buchneri*, *Lactobacillus kefiri*, *Lactobacillus rhamnosus*, *Lactobacillus curvatus*, *Lb. reuteri*, *Lb. coryneformis*, *Lb. hilgardii*, and *Lb. sakei.* Most of them are not well characterized as the other dairy lactobacilli. The above species are not important as starters for acidification but are mainly used in some dairy products for flavor, probiotic, antimicrobial, or textural attributes. Nonstarter lactobacilli originate from either the milk or the cheese manufacturing environment. Compared to the modern manufacture of fermented milks, cheesemaking is less aseptic and thus it may resemble the original production conditions. The effect of nonstarter lactobacilli on cheese quality may be positive, negative, or neutral depending on the strains that predominate and on their role during ripening. Several of the above species were studied because of the negative impact on flavor and texture of cheese when they are present as adventitious NSLAB. This article mainly focuses on the following species: *Lb. brevis*, *Lb. buchneri*, *Lb. fermentum*, *Lb. reuteri*, *Lb. kefiri*, *Lb. rhamnosus*, and *Lb. curvatus.*

Proteolysis

Lactobacilli are fastidious microorganisms that require for growth an exogenous nitrogen source such as amino acids or better peptides. These nitrogen sources are provided through proteolysis of casein, the most abundant protein in milk. Overall, NSLAB grow poorly in milk, but they are able to grow in fresh cheeses. Lactobacilli contribute to proteolysis in cheese mainly via their peptidase activity, increasing the level of small peptides and free amino acids (FAA). Overall, the hydrolysis of casein by lactic acid bacteria is initiated by the cell envelope proteinase (CEP) that degrades proteins into oligopeptides that are subsequently taken up by the cells via specific peptide transport systems for further degradation into shorter peptides and FAA by the concerted action of various intracellular peptidases. Although many strains of lactic acid bacteria contain a CEP (e.g., primary starters), some strains of the NSLAB population do not. Therefore, they rely on starter lactic acid bacteria for the liberation of peptides and FAA. Five different types of CEP were cloned and characterized in lactic acid bacteria, including the PrtR of *Lb. rhamnosus.* The latter seemed to be markedly different from the other CEP of starter lactic acid bacteria. The hydrophilic W domain is more homologous to certain cell-surface antigens expressed by oral and vaginal streptococci, and its anchor domain contains an atypical sorting signal. Several NSLAB possess key enzymes for generating cheese flavor: peptidases and enzymes responsible for the catabolism of FAA. The criteria that NSLAB should meet to become a suitable adjunct culture include the capability (1) to reach and maintain high levels of cell density during ripening; (2) to not cause defects in the product; and (3) to impact positively the overall cheese quality. The effect of a given adjunct culture in different cheese models may differ, as the growth and biochemical expression rely on the technology and the starter used. In addition, some biochemical activities may be considered negative in one cheese but desirable in others (e.g., CO_2 production, L-lactate isomerization, aldehyde production).

Proteolysis is important not only for cheesemaking but also for the liberation of bioactive peptides (BP), which are thought to promote health beyond the basic nutrition. Caseins are of particular interest, as they are known to harbor latent BP that are released by proteolysis. Strains of *Lb. rhamnosus* GG (LGG) have been shown to liberate BP.

Lactic Acid Fermentation

Those we define as other species of lactobacilli include facultative and obligate heterofermentative species. The facultative heterofermentative lactobacilli ferment hexoses by the Embden–Meyerhof–Parnas (EMP) pathway almost entirely to a mixture of L(+)- and D(−)-lactate. Under glucose-limiting conditions, they ferment hexoses to lactic acid, acetic acid, ethanol, and formic acid, and are also able to degrade pentoses. The obligate heterofermentative lactobacilli ferment hexoses to lactic acid, CO_2, acetic acid, and/or ethanol via the 6-phosphogluconate pathway, which involves the key enzyme pentose phosphoketolase, which is absent in obligative but present in facultative heterofermentative species. The obligate heterofermentative strains lack the key enzyme of the homofermentative pathway, fructose-1,6-bisphosphate aldolase. The metabolism of carbohydrates may lead to various end products that are influenced by the presence of oxidants. These may cause the formation of end products more oxidized than lactic acid (e.g., CO_2 plus acetate, acetoin, or diacetyl). In the presence of oxygen, the formation of ethanol is reduced in favor of acetate. Overall, testing for obligately heterofermentative lactobacilli involves the detection of gas (e.g., CO_2). Except for certain fermented milk products (e.g., kefir), heterofermentative lactic acid bacteria are rarely used or appreciated in cheesemaking, even though they are not uncommon in milk and dairy products. If allowed to grow to significant numbers, they may cause defects such as slits in hard cheeses or bloated packaging in other dairy products.

Lactose is fermented by *Lb. fermentum*, *Lb. kefiri*, and *Lb. reuteri*, and by most strains of *Lb. brevis* and *Lb. buchneri*. However, only limited information is available on the lactose fermentation of the above species. *Lactobacillus fermentum* ferments galactose and has constitutive β-galactosidase but not phospho-β-galactosidase activity. This implies that lactose is taken by a permease transport system and hydrolyzed so that both glucose and galactose are fermented. Of the few *Lb. brevis* strains studied, cell extracts contain no detectable β-galactosidase activity, but phospho-β-galactosidase activity was found, which is inducible by lactose and galactose. It is assumed, but not proved, that lactose in these strains is transported by the phosphoenolpyruvate-dependent phosphotransferase system into the cell with the galactose-6-phosphate derived from hydrolysis of the phosphorylated derivative metabolized by the tagatose-6-phosphate pathway.

Arginine Metabolism

Dairy heterofermentative lactobacilli species may derive supplementary energy from the catabolism of arginine. In addition to the ATP synthesized through glycolysis, a further ATP-generating step may involve the breakdown of arginine via the arginine deiminase pathway, whereby carbamate kinase synthesizes ATP from carbamoyl phosphate with the synthesis of ammonia and CO_2. This could be important during ripening of cheese, where fermentable carbohydrates available for growth are either limited or absent. The catabolism of arginine was studied in depth on *Lb. buchneri*; the three enzymes of the arginine deiminase pathway were purified and their properties studied. The three enzymes are (1) arginine deiminase, which hydrolyzes arginine to citrulline and ammonia; (2) ornithine transcarbamylase, which catalyzes the phosphorylation of citrulline to produce ornithine and carbamoyl phosphate; and (3) carbamate kinase. A protein responsible for the antiport between arginine and ornithine is the fourth component of the system. The enzymes are inducible with arginine, are not repressed by glucose, and are produced at high levels in cells growing on galactose.

Description of the Species

Lactobacillus brevis

The strains of *Lb. brevis*, which was previousaly known as *Betabacterium breve*, are isolated from dairy products, silage, and animals. *Lactobacillus brevis* may be used as a probiotic culture and as an adjunct in some cheese varieties (e.g., Canestrato Pugliese, Cheddar, and Ricotta Forte). Strains of this species are obligately heterofermentative. Type strain is ATCC 14869, and the genome was sequenced for strain ATCC 367, which has a size of 2.34 Mb. Based on the comparative analysis of 16S rRNA sequences, *Lb. brevis* strains are uniquely positioned among the lactobacilli without inclusion in the seven specific groups. *Lactobacillus brevis* occurs as single or short chains of rods with rounded ends (0.7–1.0 × 2–4 μm), does not grow at 45 °C but grows at 15 °C, and has 44–47% G+C content (mol%) and the lysine-D-aspartyl-type peptidoglycan. Strains ferment esculin, galactose, maltose, lactose, raffinose, sucrose, and xylose. Acetate formation increased with glycerol and glucose as substrates. Glycerol may be reduced to propane-1,3-diol via 3-hydroxypropanal as the intermediate. *Lactobacillus brevis* consumes numerous carbon sources simultaneously and appears to lack the normal hierarchical control for carbohydrate utilization. Analyses on several isolates of *Lb. brevis* indicated that co-utilization of xylose and glucose is a common trait for this species. Proteomic analysis of *Lb. brevis* grown on glucose, xylose, or a glucose/xylose mixture showed the constitutive expression of the enzymes of the heterofermentative pathway. Fermentative mass balances between mixed sugar inputs and the end products indicated that both glucose and xylose are simultaneously metabolized through the heterofermentative pathway. Genes of the *xyl* operon are expressed during growth on xylose or glucose/xylose, but not in the presence of glucose alone. Nevertheless, the level of expression of XylA and XylB proteins in cells grown on a glucose/xylose mixture decreased 2.7-fold with respect to cells grown on xylose alone. *Lactobacillus brevis* lacks phosphate acetyltransferase and alcohol dehydrogenase, and fructose is used as an oxidant, which is reduced to mannitol. In the presence of oxygen, it produces superoxide (O_2^-), H_2O_2, or H_2O. *Lactobacillus brevis* possesses a true catalase activity to remove hydrogen peroxide.

Lactobacillus buchneri

Lactobacillus buchneri, and was named in honor of E. Buchner, a German bacteriologist, is an obligately heterofermentative species and was previously known as *Bacillus buchneri*. Based on the analysis of the 16S rRNA sequences, the *Lb. buchneri* group was created within the family Lactobacillaceae. This group contains only obligately heterofermentative lactobacilli, except for *Lactobacillus homohiochii*, which is facultatively heterofermentative. *Lactobacillus buchneri* was isolated from some cheese varieties (e.g., Canestrato Pugliese, Ricotta Forte). Type strain is ATCC 4005, and the genome was sequenced for strain ATCC 11577 having a size of 2.85 Mb. Large similarities were found between *Lb. buchneri* and *Lb. brevis*, including the source of isolation. *Lactobacillus buchneri* occurs as single or short chains of rods with rounded ends (0.7–1.0 × 2–4 μm), does not grow at 45 °C but grows at 15 °C, and has 44–46% G+C content (mol%) and the lysine-D-aspartyl-type peptidoglycan. Strains variously ferment esculin, galactose, lactose, raffinose, sucrose, and xylose. The slower migration of the two lactate dehydrogenases from *Lb. buchneri* and the ability of strains of this species to ferment melezitose distinguish this species from *Lb. brevis*.

Lactobacillus fermentum

Lactobacillus fermentum, so named because it causes fermentation, is an obligately heterofermentative species. It may be used as a probiotic culture, and it is found as a member of the NSLAB population in some cheese varieties (e.g., Comté, Ragusano). *Lactobacillus fermentum* is one of the most abundant species found in natural whey cultures for Caciocavallo Silano, a hard 'pasta filata' cheese, and Parmigiano Reggiano cheese. *Lactobacillus fermentum* is a member of the *Lb. reuteri* group. It does not grow at 15 °C but grows at 45 °C. Type strain is ATCC 14931, and the genome was sequenced for strain IFO3956 having a size of 2.09 Mb. The single or pairs of *Lb. fermentum* rods are highly variable in length. *Lactobacillus fermentum* uses several carbohydrates (arabinose, cellobiose, galactose, maltose, mannose, melibiose, raffinose, ribose, sucrose, trehalose, and xylose), but fermentation is strain-dependent. *Lactobacillus fermentum* has 52–54% G+C content (mol%) and the D-aspartyl-L-ornithine-type peptidoglycan. True catalase activity to remove hydrogen peroxide is exhibited.

Lactobacillus reuteri

Lactobacillus reuteri was previously known as *Lb. fermentum* Type II, which reflects that it is physiologically similar to *Lb. fermentum*. Based on the analysis of its 16S rRNA, the *Lb. reuteri* group was constructed within the Lactobacillaceae. This group contains exclusively obligate heterofermentative bacteria, and members of the group show marked differences with respect to their DNA composition. Type strain is ATCC 23272. The genome was sequenced for two strains of *Lb. reuteri*, DSM20016 and JMC1112, with sizes of 1.99 and 2.04 Mb, respectively. *Lactobacillus reuteri* does not grow at 15 °C but grows at 45 °C. Strains are isolated from dairy and meat products and from animal sources. *Lactobacillus reuteri* is a probiotic culture and a member of the NSLAB population in several cheese varieties (e.g., Cheddar, Grana Padano, Gruyère, Idiazabal, Parmigiano Reggiano, Roncal, and Toma). The single pairs, or clusters of *Lb. reuteri* rods are often irregular in shape. *Lactobacillus reuteri* ferments arabinose, galactose, maltose, melibiose, raffinose, ribose, and sucrose. It has 40–42% G+C content (mol%) of the DNA and the D-aspartyl-L-lysine-type peptidoglycan. There is some evidence that drug resistance is linked to plasmids in *Lb. reuteri*, with one strain having a 5–7 MDa plasmid that codes for erythromycin resistance. One study showed that there is intergeneric conjugation of a plasmid from *Lb. reuteri* to *Enterococcus faecalis* in the gut of germ-free mice. Plasmids with limited host range replicons were identified in *Lb. reuteri*.

Lactobacillus kefiri

Lactobacillus kefiri was isolated from more limited sources than were other main dairy heterofermentative lactobacilli; it was mainly found in beer, kefir drink, and kefir grains. It grows at 15 °C but not at 45 °C. The *Lb. kefiri* rods with rounded ends form chains of either short rods (3 μm) or long filaments (15 μm). *Lactobacillus kefiri* has 40% DNA homology with *Lb. buchneri* and belongs to the *Lb. buchneri* group. *Lactobacillus kefiri* has 41–42% G+C content (mol%) and has the lysine-D-aspartyl-type peptidoglycan. Type strain is ATCC 35411. *Lactobacillus kefiri* is mainly associated with fermented milk products in the Northern Caucasus along with *Lactobacillus parakefiri* and *Lactobacillus kefirigranum*. Recently, it was found as NSLAB in Camembert cheese.

Lactobacillus rhamnosus

Lactobacillus rhamnosus was previously known as *Lb. casei* subsp. *rhamnosus*, etymologically pertaining to rhamnose. *Lactobacillus rhamnosus* is a facultative heterofermentative bacterium, and is included with other highly related species in the *Lb. casei* group. Cells have the form of rods, singly or in short chains. Type strain is ATCC 7469. The genome was sequenced for LGG and *Lb. rhamnosus* 53103. The sizes were 3.010 and 3.005 Mb, respectively. The genome of *Lb. rhamnosus* ATCC 53103 is 5 kb shorter than that of LGG. Furthermore, an alignment analysis of genome sequence of both strains shows that the 8.9-kb region (genome coordinates 618415 to 627294) of *Lb. rhamnosus* ATCC 53103 is inverted. *Lactobacillus rhamnosus* has 45–47% G+C content (mol%) and the lysine-D-aspartyl-type peptidoglycan. *Lactobacillus rhamnosus* grows at both 15 and 45 °C, and produces the L-lactic acid isomer. It ferments carbohydrates such as arabinose, cellobiose, esculin, ribose, sorbitol, and sucrose. LGG is one of the most extensively studied and widely used probiotic culture. Recently, it was demonstrated that the proteome of LGG grown in hydrolyzed whey-based medium markedly differed from that of the same strain grown in a rich MRS laboratory medium. This clearly demonstrated the fundamental effect of the culture conditions on the proteomic features and properties of the strain GG. Indeed, the adhesion properties of GG, which are important probiotic features, were dependent on the culture medium.

Lactobacillus curvatus

Lactobacillus curvatus is a facultatively heterofermentative bacterium included in the *Lb. sakei* group. The name reflects its shape: single rods with a slight moon-shaped curve. *Lactobacillus curvatus* is a member of the NSLAB population in many cheese varieties (e.g., Canestrato Pugliese, Cheddar, Fiore Sardo, Fossa, Idiazabal, Montasio, Mozzarella, Pecorino Romano, Pecorino Sardo, and Roncal). *Lactobacillus curvatus* contains two subspecies: *curvatus* and *melibiosus*. These subspecies may be distinguished only by molecular typing methods. *Lactobacillus curvatus* subsp. *curvatus* and subsp. *melibiosus* have 42–44% G+C content (mol%) and the lysine-D-aspartyl-type peptidoglycan. The type strain is ATCC 25601. *Lactobacillus curvatus* grows at 15 °C but not at 45 °C. Some strains may grow at 2–4 °C. It produces both the D- and L-lactic acid isomers. Fermentation is mainly restricted to cellobiose, esculin, gluconate, melibiose, ribose, and sucrose.

Desirable Properties in Dairy Products

Antimicrobial Effects

During fermentation of dairy products, lactic acid bacteria cultures mainly metabolize lactose to lactic acid. Acid production lowers the pH and creates an environment unfavorable to pathogens and spoilage organisms. Moreover, the low pH of fermented foods increases the antimicrobial effects of organic acids. The specific antimicrobial mechanisms of lactic acid bacteria that were exploited in the biopreservation of foods include syntheses of organic acids, hydrogen peroxide, carbon dioxide, diacetyl, and broad-spectrum antimicrobials such as reuterin and bacteriocins. Propionic acid is a well-known compound inhibitory to fungi and bacteria. It is contained in a commercial product where the use of *Propionibacterium freudenreichii* along with *Lb. rhamnosus* increases the inhibitory activity against fungi and some Gram-positive bacteria.

Bacteriocin-producing species were identified among almost all the genera that comprise the lactic acid bacteria. A number of *Lb. fermentum* strains produce bacteriocins, although they have a limited host range, usually against strains of the species *Lb. fermentum* and *Lb. acidophilus*. One strain of *Lb. fermentum* produces a bacteriocin similar to lactocin 27 synthesized by *Lactobacillus helveticus*. *Lactobacillus reuteri* excretes an antimicrobial compound, termed reuterin, when fermenting carbohydrates and using glycerol as the hydrogen acceptor. Reuterin is a low-molecular-mass, soluble compound made up of a mixture of mono- and dimeric forms of 3-hydroxypropanaldehyde synthesized from glycerol. Reuterin inhibits a wide range of Gram-positive and Gram-negative bacteria, yeasts, fungi, and protozoa. It was hypothesized that the inhibitory activity is addressed to DNA synthesis and inhibition of ribonucleotide reductase. Because of its nonproteinaceous nature and extremely broad-spectrum antimicrobial activity, reuterin is not classified as a bacteriocin. It is active in a wide range of pH (2–8) and resistant to proteolytic and lipolytic enzymes. Reuterin was proposed for the biopreservation of milk and Cottage cheese due to its inhibitory action on pathogenic bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Echerichia coli*. A milk fermented with *Lb. reuteri* and containing reuterin is available on the market.

Probiotic Effects

Dairy products with probiotic bacteria are mostly fermented milks, such as yogurt. However, other milk products such as cheese, ice cream, and milk powder may also be used as carriers of probiotic cultures. The majority of probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium*. Fermented milks as well as cheeses may contain LGG and *Lb. reuteri* as probiotic cultures. Salting, the long period of ripening, the scalding temperature were shown to be not insurmountable obstacles for the manufacture of probiotic ripened cheeses with these species. Probiotics are added to cheese milk as adjuncts together with or immediately after starters.

Protective effects of *Lb. rhamnosus* strains have been demonstrated against *E. coli* infection and uropathogen growth on silicone rubber after incubation in urine. The health-promoting effects discovered associated with the consumption of LGG include a reduction in the risk of acute diarrhea in children and atopic diseases in infants, as well as relief from milk allergy/atopic dermatitis in infants and a reduction in the risk of respiratory infections and the occurrence of dental caries.

Lactobacillus reuteri is one of the dominant heterofermentative lactobacilli of the human intestinal tract. It was used safely for many years as a probiotic dietary supplement in adults, and recently, it was demonstrated safe after long-term dietary supplementation for newborn infants. Probiotic *Lb. reuteri* has a positive effect toward intestinal disorders such as constipation and diarrhea, improves colicky symptoms in breast-fed infants; increases the intestinal protection from infection, and has the capacity to modulate immune responses.

Although not specifically used in probiotic preparations, some strains of *Lb. brevis* possess potential probiotic activity. It was shown that oral administration of live *Lb. brevis* ssp. *coagulans* strains significantly stimulated the host immunity system by increasing the synthesis of interferon-α and the anti-inflammatory activity of the digestive system. Although strains of *Lb. brevis* are not suitable for fermenting yogurt, supplementation of yogurt with these strains had no negative effects on taste or preservation.

Flavor Formation

The quality of fermented dairy products is largely determined by sensory perception, which is a complex process, influenced by many factors, such as the content of flavor components, texture, and appearance. Flavor compounds in cheese arise from the activity of enzymes from rennet, milk, the secondary starter, and nonstarter lactic acid bacteria, together with nonenzymatic conversions.

Lactobacillus kefiri, along with yeast and other lactic acid bacteria, including *Lb. brevis*, is used in fermented milk products such as kefir, where it contributes to the synthesis of lactic acid, alcohol, CO_2, and sensory compounds. *Lactobacillus kefiri* also contributes to the synthesis of exopolysaccharides, the important constituent of the kefir grains where bacteria and yeast cells are embedded.

Overall, *Lb. fermentum* and *Lb. reuteri* synthesize flavor compounds from sulfur-containing amino acids. *Lactobacillus fermentum* DT41, isolated from the natural whey starter for Parmigiano Reggiano cheese, possesses cystathionine-γ-lyase, the first enzyme responsible for the catabolism of sulfur-containing amino acids. The enzyme retains activity under cheese ripening conditions and contributes to the biosynthesis of sulfur-containing compounds. The same enzyme was purified from *Lb. reuteri* DSM20016. This bacterium was used as an adjunct starter in the manufacture of Canestrato Pugliese-type cheese. Cheeses containing the adjunct of *Lb. fermentum* DT41 or *Lb. reuteri* DSM20016 had the highest levels of methanethiol, dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide.

Free fatty acids make an important contribution to the development of the characteristic flavor during ripening of several cheese varieties. Hard Italian cheese varieties (e.g., Pecorino Romano and Provolone) and blue-veined cheeses are characterized by extensive lipolysis. The pregastric esterase contained in rennet paste is the main agent of lipolysis for several cheese varieties. As considerable cell densities are reached by NSLAB during cheese ripening, a moderate contribution to lipolysis by adventitious lactic acid bacteria cannot be excluded. *Lactobacillus fermentum* DT41 has a cell-surface-associated esterase that may contribute to flavor development in ripened Parmigiano Reggiano cheese. The enzyme showed the highest activity on *S*-naphthyl butyrate, and the *S*-naphthyl esters of C2–C10 fatty acids were hydrolyzed. Among the triglycerides, only tributyrin was degraded. *Lactobacillus fermentum* may also produce ethanol in cheese. The synthesis of ethanol is probably the rate-limiting reaction in the production of ethyl esters of short-chain fatty acids that produce desirable fruity flavors in some hard Italian-style cheeses. *Lactobacillus fermentum* has more esterolytic activity than most other dairy lactobacilli, which could also contribute to the fruity flavors in cheese. However, *Lb. fermentum* is not often added to cheese milk for these benefits, because of the downside of its gas production and the associated split defects in many cheese types.

Recently, it was shown that *Lb. rhamnosus* is one of the dominant species of adventitious lactobacilli during late ripening of Parmigiano Reggiano cheese. Contribution of this species to the sensory properties of the cheese is under investigation.

Undesirable Properties in Dairy Products

Defects from Gas Production

Lactobacillus brevis, *Lb. fermentum*, and *Lb. buchneri* represent the main adventitious heterofermentative lactobacilli that have been identified in different cheeses. This group does not represent the most common adventitious NSLAB found in most cheeses manufactured from pasteurized milk. Often in cheese made from pasteurized milk under good hygienic conditions, heterofermentative lactobacilli are not detected during ripening. Occasionally, strains are detected in low numbers in young (<1-month-old) cheese and then are not detected as the cheese ages, probably because the other lactobacilli, especially *Lb. paracasei* and *Lb. plantarum*, outgrow the heterofermentative lactobacilli that remain at low numbers. The cell density of heterofermentative lactobacilli may increase among adventitious NSLAB in cheeses made from raw milk. When the conditions are suitable (e.g., high temperatures and high initial numbers compared to other adventitious lactobacilli), heterofermentative lactobacilli grow to and remain at high cell densities ($>10^7$ cfu g^{-1}) throughout cheese ripening. They often cause a number of defects. In most cases, however, strains enter the cheese after pasteurization. For instance, as these gas-forming lactobacilli can enter the cheese during brining, their numbers in brine must be minimized by good hygiene, particularly by maintaining the temperature low, the brine levels at >16% NaCl, and the pH at <4.5.

Slits, the structural defects, in Cheddar cheese were often attributed to the growth of heterofermentative lactobacilli. In most cases, the slits are caused by the formation of CO_2, presumably by the heterofermentative lactobacilli fermenting citrate, lactose, galactose, amino acids, or a combination of these substrates during cheese ripening. *Lactobacillus curvatus* appeared to be very common in Cheddar cheese exhibiting the slit defect. *Lactobacillus brevis* may produce excessive CO_2 in cheese giving rise to unwanted gas pockets and blowing of packaged cheeses. Orange-pigmented strains (*Lb. brevis* subsp. *rudensis*) may multiply in hard and white-brined cheeses. When exceeding the usual low numbers, *Lb. fermentum* and *Lb. brevis* may also cause blowing in Italian Grana cheeses.

Biogenic Amines

Biogenic amines are found in cheeses, and lactobacilli have been identified as their main agents of formation. Biogenic amines such as histamine, tryptamine, and tyramine are formed from the decarboxylation of the amino acids histidine, tryptophan, and tyrosine, respectively. Amine intoxication is associated with headache, nausea, and urticaria. Several outbreaks of apparent amine intoxication were associated with the consumption of cheese containing toxic levels of biogenic amines. The availability of amino acids and the high density of specific amine-producing bacteria during cheese ripening are the key factors in determining the concentration of toxic levels of amines. The most common amine-producing bacteria found at sufficiently high densities in dairy foods that could produce biogenic amines are two species of adventitious heterofermentative lactobacilli, *Lb. brevis* and *Lb. buchneri*. *Lactobacillus buchneri* was isolated from Swiss cheese containing a high level of histamine. From Gouda cheese were isolated strains of *Lb. buchneri* and *Lb. brevis*, which produced histamine and tyramine, respectively.

Flavor and Odor Defects

For the manufacture of most cheese varieties, it is important to avoid contamination by heterofermentative lactobacilli or to minimize their growth. Aromatic amino acid catabolism by heterofermentative lactobacilli may produce undesirable flavor compounds including *p*-cresol, indole, and skatole. In Gouda and Cheddar cheeses, defective sulfur, phenolic, fecal, and mealy flavors and odors, as well as gas formation, were related to the catabolism of amino acids by *Lb. brevis* and *Lb. buchneri*. It is believed that excessive degradation of the aromatic and sulfur amino acids is responsible for these defects. The heterofermentative lactobacilli as a group appear to be more active and to have a wider range of fermentable amino acids to be used than the other adventitious lactobacilli commonly found in cheese.

Lactate Crystal Formation

In cheeses, such as Cheddar, where the curd is not washed, the total lactate (usually the L(+) isomer) is at a concentration close to the saturation point. This means that crystals of calcium lactate pentahydrate can be formed under the influence of several parameters (e.g., low temperatures, high total lactate concentration). Crystals are visually undesirable and may block filters during cheese manufacture. Crystal formation in cheeses commonly occurs when the L(+) isomer of lactate becomes an equal mixture (a racemic mixture) of the L(+) and D(–) isomers. The racemic mixture of lactate is more insoluble than the individual isomers. In cheese, the undesirable racemization of lactate is carried out by a number of adventitious

NSLAB species. *Lactobacillus fermentum* and *Lb. brevis* can racemize lactate under laboratory conditions and in cheese. Once strains of these species reach high densities in Cheddar, racemization will occur and lactate crystal problems are very likely. The formation of biofilm during the manufacture of cheeses was related to the formation of defects. Cleaning and sanitizing are efficient tools to eliminate microbial biofilm.

See also: **Lactic Acid Bacteria**: *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*.

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Lactococcus lactis

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Introduction

Lactococcus lactis is a member of the mesophilic group of bacteria forming one of the main constituents in both industrial and artisanal starter cultures. The principal role of lactococci in dairy fermentations involves the metabolism of lactose to lactic acid and the conversion of milk proteins into flavor compounds. In terms of cell morphology, lactococci have spherical or ovoid-shaped cells and occur singly or in chains (**Figure 1**). They are Gram-positive, catalase-negative, facultatively anaerobic, nonmotile, and non-spore-forming. They can grow at 10 °C but not at 45 °C, and ferment glucose by the hexose diphosphate pathway producing L(+)-lactic acid. Three lactococcal genomes have been completely sequenced to date and range in size from 2.37 to 2.53 Mbp, with a G+C content of ~35 mol%.

Classification

Lc. lactis cultures found in dairy fermentations are classified as subspecies *cremoris*, *lactis*, and *lactis* biovar *diacetylactis*. Differentiation of *cremoris* and *lactis* is generally based on a few phenotypic traits: *Lc. lactis* subsp. *lactis* produces ammonia from arginine and is tolerant to 40 °C and 4% NaCl, whereas *Lc. lactis* subsp. *cremoris* is unable to produce ammonia from arginine and has a low tolerance for elevated temperatures and salt concentrations. Glutamate decarboxylase (GAD) activity, which has been observed in *Lc. lactis* subsp. *lactis* but not in subsp. *cremoris*, can also serve as a mechanism for differentiation. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* strains are characterized by their ability to utilize citrate. Genotyping is also used for classification, although, interestingly, it has been found that some strains that are phenotypically *Lc. lactis* subsp. *lactis* appear genotypically as *Lc. lactis* subsp. *cremoris* and vice versa. An example is the well known strain *Lc. lactis* subsp. *cremoris* MG1363, which has a subspecies *cremoris*-like genotype and a subspecies *lactis* phenotype. Three genetic typing methods (partial small subunit (SSU) rRNA gene sequence analysis, $(GTG)_5$-PCR genomic fingerprinting analysis, and multilocus sequence analysis (MLSA) scheme) recently applied to a collection of lactococcal strains from various dairy and plant fermentations and a wide range of geographic locations revealed two major distinct genomic lineages within the species, which are dissimilar to groupings based on phenotypic analysis. The first is the *Lc. lactis* subsp. *cremoris* type-strain-like genotype lineage, which includes both *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* isolates. The second major lineage includes *Lc. lactis* subsp. *lactis* type-strain-like genotype, which is composed of *Lc. lactis* subsp. *lactis* isolates only. A novel third genomic lineage represents two *Lc. lactis* subsp. *lactis* isolates of nondairy origin as their SSU rRNA gene sequences and five-locus MLSA sequences grouped separately from the other *Lc. lactis* isolates. It has been suggested that the current phenotypic classification be amended with a 'type-strain-like-genotype' classification, providing a direct subspecific phylogenetic reference. Thus, the strain *Lc. lactis* subsp. *cremoris* MG1363 is defined as *Lc. lactis* subsp. *cremoris* type-strain-like-genotype and *Lc. lactis* subsp. *lactis* phenotype, while the other well-known dairy strain *Lc. lactis* subsp. *lactis* IL1403 is defined as *Lc. lactis* subsp. *lactis* type-strain-like-genotype and *Lc. lactis* subsp. *lactis* phenotype.

Habitat

As well as being associated with the dairy environment, *Lc. lactis* subsp. *lactis* is commonly associated with plant material where it generally occurs as an early colonizer, being replaced by species that are more resistant to low pH. Nondairy isolates of lactococci have been found to differ from dairy isolates by their inability to degrade caseins, production of high levels of antimicrobial compounds, low acidification activity on lactose, and a low level of amino acid auxotrophies. Few reports have documented the isolation of *cremoris* and *diacetylactis* strains from nondairy environments.

Genomics

Genomes and Plasmids

The genomic organization of *Lc. lactis* is considered to be highly variable. The complete genome sequences of three lactococcal strains have been published to date: *Lc. lactis* subsp. *lactis* IL1403 (plasmid-cured derivative) (gi = 15671982), *Lc. lactis* subsp. *cremoris* MG1363

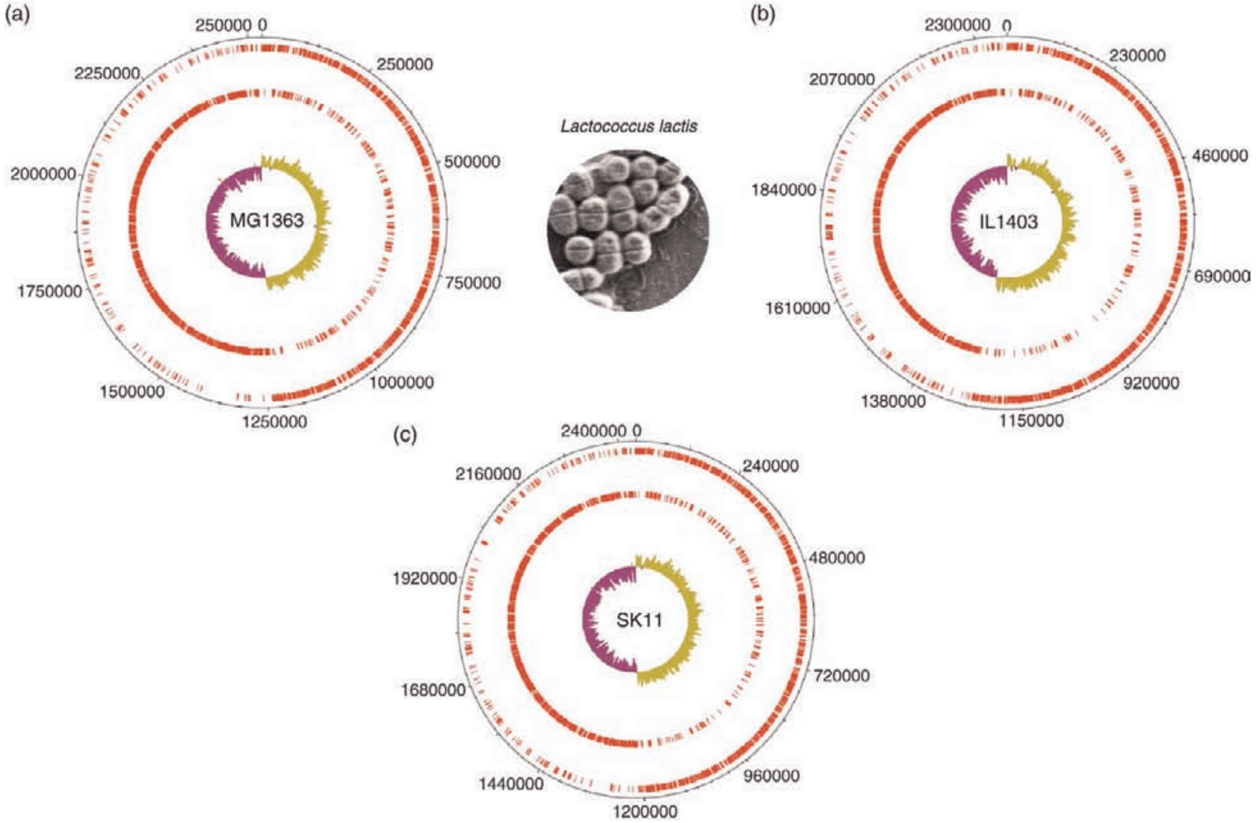

Figure 1 Circular genomes of *Lactococcus lactis* MG1363 (a), IL1403 (b), and SK11 (c), generated with DNA Plotter Software package (http://www.sanger.ac.uk/Software/Artemis/circular/). Outer red lines represent forward-reading open reading frames, inner red lines represent reverse-reading open reading frames. Green/purple region represents GC content; green indicates GC content which is above average, and purple indicates GC content which is below average. Inset: scanning electron micrograph of *Lactococcus lactis*.

(plasmid-cured derivative) (gi = 25622882) and the 'true' *cremoris* strain, *Lc. lactis* subsp. *cremoris* SK11 (gi = 116510843). The genomes of the two *cremoris* strains are each larger than the genome of *Lc. lactis* IL1403 (~2.37 Mbp), with *Lc. lactis* MG1363 containing the largest genome size of ~2.53 Mbp followed by *Lc. lactis* SK11 with a genome size of 2.44 Mbp (**Figure 1**). Approximately 85% DNA sequence identity has been observed between the coding domains (CDs) of *Lc. lactis* IL1403 and *Lc. lactis* MG1363, whereas 97.7% identity has been observed between the two subsp. *cremoris* strains. Approximately 2597 genes were identified in the MG1363 genome of which 2434 were recorded as protein-coding, representing a coding density of ~82%. A total of 2610 genes were identified in the SK11 genome, with 2384 protein-coding genes (81% coding density), whereas *Lc. lactis* IL1403, which contains the smallest genome of the three, contains 2425 genes of which 2321 are protein-coding, representing a coding density of 85%. Both MG1363 and SK11 contain 82 and 81 structural RNAs, respectively, whereas IL1403 contains 79. A comparison of the location of the open reading frames of IL1403 with MG1363 illustrated the presence of a large genome inversion represented by 48% of the MG1363 genome, whereas a 73-kb inversion was observed in the SK11 genome when compared with IL1403. In total, 92 insertion sequence (IS) elements have been identified on the genome of MG1363 compared to 52 on the genome of IL1403. A total of 134 kb of prophage DNA was found on the genome of MG1363 compared to 175 kb on the genome of IL1403. A complete set of competence genes was observed on the IL1403 genome, indicating that the strain may have the ability to undergo natural DNA transformation. The sex factor, a unique mobile genetic element, was also observed on the genome of MG1363, which has been shown to conjugate into IL1403. Reconstruction of the metabolic network of *Lc. lactis* IL1403 based on the annotated genome sequence established a total of 621 reactions and 509 metabolites, representing the overall metabolism of *Lc. lactis*.

Plasmids isolated from lactococci range in size from 3 to 130 kb, have a G+C content of 30–40 mol% and vary in function and distribution with most strains carrying between 4 and 7 plasmids per cell. The difference in G+C content between lactococcal plasmids and genomes suggests that many of these plasmids may be recent additions to the genome enabling their host to grow efficiently in milk. Lactococcal plasmids replicate by either theta replication or rolling-circle replication (RCR). Naturally occurring *Lc. lactis* strains do not seem to harbor more than one RCR plasmid presumably due to the fact that RCR plasmids are less stable than theta-replicating plasmids.

It has been suggested that nondairy lactococci have adapted to the dairy environment through the acquisition of plasmids encoding dairy-related traits such as lactose utilization, citrate metabolism, phage resistance, bacteriocin production, exopolysaccharide (EPS) production, and proteolytic enzymes. Indeed in many cases, the individuality observed among different lactococcal strains can be attributed to the plasmidosome (plasmid complement), especially in terms of most industrially important traits observed among different lactococcal strains. Conjugal plasmids are commonly found in starter lactococci and can be transferred to recipient strains at frequencies as high as 10^{-2} per recipient cell. Since conjugation is regarded as a food-grade process, it has been widely exploited by scientists since the 1980s to generate superior lactococcal starter cultures.

Identification of *Lactococcus*

The analysis of small ribosomal DNA (rDNA) gene sequences, such as the 16S rDNA gene, provides a reliable highly discriminatory method for microbial identification. In this case, amplified, sequenced 16S rDNA genes can be compared (using the bioinformatic basic local alignment search tool (BLAST)) with the nonredundant nucleotide databases for species/strain similarity/identity. The technique of amplified ribosomal DNA restriction analysis (ARDRA) has also been applied to the 16S rDNA gene of *Lc. lactis* wherein restriction endonuclease digestion of the amplified gene generates distinct electrophoretic patterns that correspond to individual strains enabling subspecies identification. Randomly amplified polymorphic DNA (RAPD) fingerprinting can also provide a reliable and fast technique for genotypic identification based on cluster analysis as well as for assessing subspecific diversity. Rep-PCR takes advantage of repetitive DNA elements randomly distributed over the genome, and Rep-PCR fingerprinting with the $(GTG)_5$ primer was recently revealed as a reliable and fast means of identifying *Lc. lactis* subsp. *lactis*. The technique of pulsed field gel electrophoresis (PFGE), which involves restriction digestion of unsheared genomic DNA followed by separation on agarose gels using a constantly reorienting electric field, has also been utilized for species and strain identification based on cluster analysis. The genomic fingerprints generated through PFGE also provide a powerful tool for direct comparison of strains for determining genomic relatedness.

Industrially Significant Properties

Lactose Utilization

Lactococci used as dairy starter cultures generally metabolize lactose by an unusual pathway involving the use of the plasmid-encoded enzyme phospho-β-galactosidase. In this case, lactose is taken up by the enzymes of the phosphoenolpyruvate pathway (PEP-PTS), a system that catalyzes the synthesis of lactose-phosphate. Lactose-phosphate then accumulates intracellularly and is hydrolyzed to glucose and galactose-6-phosphate by the enzyme phospho-β-galactosidase as part of the tagatose pathway. The genes encoding the PEP-PTS and the tagatose-6-phosphate pathway are located on plasmids, where they are organized as an operon. The transcriptional regulator (LacR) of the lac operon is positioned upstream and in a divergent orientation to the operon such that the two promoters are in a back-to-back configuration. The lac operon is induced up to 10-fold by growth on lactose; *lacR* is induced during growth on glucose. A second mechanism is more common in prokaryotes, and transports lactose into the cell using integral membrane proteins called permeases. These enzymes translocate lactose into the cytoplasm without chemical modification where lactose transport is coupled to proton symport. The lactose is then metabolized to glucose and galactose by β-galactosidase as part of the Leloir pathway. The contribution of each transport mechanism to overall sugar transport remains unknown, but it appears that intensive industrial use of lactococci has selected for strains that utilize the plasmid-encoded enzyme phospho-β-galactosidase. In lactococci the internalized glucose is converted to pyruvate via glycolysis, with the resultant production of ATP occurring through substrate-level phosphorylation. The environment is gradually acidified through the conversion of pyruvate to lactate by lactate dehydrogenase (LDH), a conversion essential for regeneration of the NAD^+ consumed during glycolysis. A number of chemostat studies on lactococci have reported the shift from homolactic (lactate production) to a mixed acid fermentation resulting in the production of compounds such as ethanol, acetate, and formate in glucose-limited environments during the metabolism of galactose or maltose (heterolactic fermentation). However, despite the wealth of information that exists, the mechanism(s) governing the metabolic switch is(are) not fully apparent.

Casein Breakdown and Flavor Production from Amino Acids

Lc. lactis requires an exogenous source of amino acids or peptides, which it acquires through the proteolysis of casein, the most abundant protein in milk. The proteolytic system is composed generally of a plasmid-encoded cell envelope proteinase involved in the initial cleavage of casein, specific transport systems involved in the uptake of small peptides and amino acids, and finally peptidases that hydrolyze the transported peptides to smaller peptides and amino acids. These peptides and amino acids along with their derivatives (various alcohols, aldehydes, acids, esters, and sulfur compounds) play a major role in the flavor and texture of fermented products (*see* **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development).

Bacteriophage (Phage) Resistance

Within lactococci there are four main cellular defenses that interfere with different stages of the phage lytic cycle, namely, adsorption inhibition (Ads), injection blocking, restriction modification (R/M), and abortive infection (Abi). The Ads phenotype prevents the adsorption of the phage particle to the cell surface, as a result of phage receptor masking or competition, and has been attributed to various substances including galactosyl-containing lipoteichoic acid, galactose/rhamnose or galactose/glucuronic acid polymer, and cell wall proteins. Bacteria that exhibit this phenotype are referred to as bacteriophage-insensitive mutants (BIMs) and can be generated and isolated under laboratory conditions by exposing the phage-sensitive bacterial culture to a high titer of phage that can result in the proliferation of mutants resistant to the infecting phage. Injection blocking describes the prevention of phage DNA injection into the cell and comes into play only after the phage has successfully attached to the cell surface. R/M systems digest foreign (phage) DNA that has entered the cytoplasm. These enzymes are classified into three groups based on molecular structure, sequence recognition, cleavage position, and the co-factors required, and are commonly associated with lactococcal plasmids. Type I R/M systems consist of three subunits, each with the designation Hsd standing for host specificity determinant. The HsdR subunit represents the restriction endonuclease that digests the foreign DNA. HsdM represents the methylase that methylates the host DNA, thus protecting it from digestion with the endonuclease. The HsdS subunit determines the specificity of the endonuclease or the methylase, directing either enzyme to the target DNA, recognizing a bipartite or disrupted target site. Many lactococcal plasmids encode an *hsdS* locus without having the cognate *hsdR* and *hsdM* subunits. These *HsdS* subunits by themselves function *in trans*, capable of interaction with *HsdR* and *HsdM* subunits encoded on other DNA elements. A typical type II R/M system is composed of two distinct gene products, one of which encodes a sequence-specific endonuclease and the other a cognate methyltransferase. Type III R/M systems generally consist of two distinct structural genes (*mod* and *res*) which encode subunits of a multifunctional restriction complex, possessing both restriction and modification activities. Abi comes into play after the injection of phage DNA and includes a broad range of defenses that can interfere with phage genome replication, transcription, translation, and packaging, or assembly of phage particles. This interruption of phage development leads to the release of few or no phage and to the death of the infected cell; thus further propagation of the phage is prevented and the bacterial population survives. Most Abis identified in *Lactococcus* to date are plasmid-encoded and range from AbiA, AbiB, and so on to AbiV. The phenotype is most often conferred by a single gene, although there are a few exceptions where the involvement of two genes has been proposed (AbiE, G, L, and T). Protein homology has rarely been observed between lactococcal Abis, and no homology with known proteins has been found, rendering it difficult to elucidate their mode(s) of action.

In many cases, the plasmids encoding these phage resistance mechanisms can be mobilized to other strains via conjugation to generate phage-resistant derivatives. Access to phage-resistant derivatives or BIMs of starter cultures is extremely important in the event of a phage buildup during fermentation, which can result in the formation of an inferior product. These phage-resistant alternatives can be used as part of a starter culture rotation strategy whereby the sensitive strain is replaced with a strain that differs only in its susceptibility to phage infection. Screening for phage resistance mechanisms in *Lactococcus* is most readily performed by exposing the cells to phage cocktails and assessing the level of phage resistance phenotypically. Hybridization and PCR assays can also be exploited to scan the plasmid complement of strains for Abi genes and R/M systems. As the availability of an adequate bank of phage can be a limiting factor for this method, it is also feasible to transform a plasmid-free *Lc. lactis* derivative (for which an adequate bank of phage exists) with plasmid DNA from other strains to identify the plasmids harboring phage resistance mechanisms.

Bacteriocin Production

Bacteriocins are polypeptides produced by bacteria and can have a bacteriocidal or bacteriostatic effect on other bacteria. In general, bacteriocins lead to cell death by inhibiting cell wall biosynthesis and/or by disrupting the membrane through pore formation. The bacteriocins of LAB have been classified into as many as five groups but have recently been reclassified into two distinct categories: the lanthionine-containing bacteriocins (class I) and the non-lanthionine containing bacteriocins (class II). The lanthionine-containing bacteriocins, also known as lantibiotics, contain posttranslationally modified amino acids such as lanthionine and β-methyllanthionine, and the dehydrated residues dehydroalanine and dehydrobutyrine. The single-peptide lactococcal bacteriocin nisin and the two-peptide lactococcal bacteriocin lacticin 3147 are apt examples of class I bacteriocins. The class II bacteriocins are also small (<10 kDa), but, unlike the class I bacteriocins, they are not subject to extensive posttranslational modification. Bacteriocins are usually synthesized as an inactive prepeptide that includes an N-terminal leader sequence generally cleaved off during export from the cell. The relevant genes encoding the prepeptides are generally closely associated with genes that encode the products involved in bacteriocin regulation, export, immunity, and, in the case of the lantibiotics, modification. In addition, bacteriocins are often encoded on mobilizable elements such as transposons (e.g., Tn5481), which encode production of and immunity to nisin, or conjugative plasmids such as pMRC01, which encode the genetic machinery for production of and immunity to the bacteriocin lacticin 3147, and thus can deliberately be transferred to nonproducing strains. In general, most bacteriocins have a narrow spectrum of inhibition (against closely related species and other lactococcal strains). However, some bacteriocins such as lacticin 3147 have a broader range of activity and are thus useful as natural antimicrobial agents. Such bacteriocins can be introduced into the food matrix by three approaches: via the starter culture or an adjunct culture producing the bacteriocin; as a purified or semipurified preparation such as nisin; or as an ingredient based on a fermentate of a bacteriocin-producing strain. Production of antimicrobial compounds can be detected by various methods including the agar well diffusion assay and the colony overlay method, or by examining the effect of lactococcal fermentate on the growth of indicator strains. The detection and identification of bacteriocins in producing strains has also been greatly aided by PCR amplification of putative bacteriocin genes using specific primers designed to detect known bacteriocins.

Exopolysaccharide Production

Two forms of EPSs are produced by LAB: capsular and unattached. Capsular EPSs (CPSs) form capsules around the cell wall and are not secreted into the medium. EPSs (unattached) are secreted outside the cell wall of the producer and are responsible for the ropy phenotype observed in fermented milks. Microbial polysaccharides are divided into two groups based on their sugar composition: homopolysaccharides and heteropolysaccharides. Homopolysaccharides contain only one sugar type, either D-glucopyranose or D-fructofuranose. Heteropolysaccharides are composed of a repeating unit that contains two or more different monosaccharides. The genetic machinery responsible for EPS production in lactococci is generally encoded on large operons containing more than 10 genes. Indeed, in the case of the 42-kb mobilizable plasmid pNZ4000, the EPS gene cluster is composed of 14 coordinately transcribed genes that express the glucosyltransferases (GTF) involved in synthesis and assembly of the EPS repeating unit. As the producing strains are food-grade, the EPSs are also considered food-grade. In addition, it has been suggested that these EPSs are active as prebiotics, cholesterol-lowering neutraceuticals, and immunomodulants.

EPSs can also be important factors in phage resistance; for example, by surrounding the cell surface they provide a physical barrier against adsorption of phage, which has been observed for the sugars galactose and rhamnose as discussed previously. However, EPS production does not necessarily protect producing cultures from phage infection.

Numerous screening techniques have been used in the search for EPS-producing LAB. Most methods involve growth of the cultures in media conducive to EPS production, in particular, media containing high concentrations of carbohydrates such as glucose, maltose, fructose, and sucrose. Ropiness of the producing cultures can be identified in colonies on agar media or by monitoring for the resistance to flow through graduated pipettes. Ultrafiltration and gel permeation chromatography have also recently been used to purify and characterize EPS from culture media. EPS screening using PCR can also be performed using primers designed to amplify different genes involved in EPS production. However, many nonproducing strains can give rise to PCR products, indicating that the phenotypic approach is more reliable.

See also: **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development.

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Leuconostoc spp.

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Introduction

Leuconostocs (predominantly *Leuconostoc mesenteroides* subsp. *cremoris*) are the most commonly used heterofermentative dairy lactic acid bacteria. They are present in dairy environments, are closely related to the heterofermentative lactobacilli, and are used as flavor-producing starter or adjunct cultures in a number of fermented milk products and cheese types. Leuconostocs are generally used in mixed dairy starter cultures where lactococcal strains are the main acid-producing starters.

The fermentation of citrate by some *Leuconostoc* is important, providing both diacetyl as an important flavor compound in dairy products (cheese, butter) and CO_2 for the eye formation that is desirable in some cheese types. Other important properties of some *Leuconostoc* strains include the removal of acetaldehyde and the production of dextrans and bacteriocins.

Taxonomy and General Characteristics

Taxonomy

Leuconostoc spp. are widespread in the environment, and have been isolated from plant matter, human clinical sources, and foods such as chill-stored and fermented meats, fermented vegetables (e.g., sauerkraut, kimchi), and fermented dairy products (e.g., cheese, kefir, yogurt). A limited number of *Leuconostoc* spp. associated with human infections have been reported; however, the associated strains are regarded as opportunistic pathogens of susceptible immunocompromised individuals and the genus is 'generally recognized as safe' (GRAS).

The taxonomy of the genus *Leuconostoc* is continually evolving and there continues to be substantial morphological, biochemical, and phylogenetic heterogeneity within the genus as it is currently constituted. While phenotypic characteristics have provided the classical basis for species differentiation, phylogenetic analysis based on 16S rRNA gene sequence is now being used to refine species differentiation. The genus *Leuconostoc* is currently assigned to 10 species (**Table 1**). Two species previously classified as *Ln. oenos* and *Ln. paramesenteroides* have been reclassified as *Oenococcus oeni* and *Weissella paramesenteroides*, respectively. Four species previously classified as *Ln. durionis*, *Ln. ficulneum*, *Ln. pseudoficulneum*, and *Ln. fructosum* have been assigned to a novel genus *Fructobacillus*. *Leuconostoc argentinum* has been reclassified as *Ln. lactis*.

The exact species of *Leuconostoc* occurring in dairy starter cultures are not always defined, but generally there are only two: *Ln. lactis* and *Ln. mesenteroides* (subsp. *mesenteroides*, subsp. *cremoris*, and subsp. *dextranicum*). *Leuconostoc mesenteroides* subsp. *cremoris* is the subspecies most frequently isolated from mesophilic mixed-strain dairy starter cultures and from fermented dairy products. In addition, *Ln. citreum* has been reported as an isolate from cheese.

General Characteristics

Leuconostocs are mesophilic (optimum growth ≈25 °C), Gram-positive, catalase-negative, nonmotile, aerotolerant, obligately heterofermentative cocci, often ellipsoidal. They usually occur in pairs and chains. Leuconostocs are nutritionally fastidious, requiring a source of amino acids and vitamins as well as a fermentable carbohydrate for energy. Usually they grow well in de Man, Rogosa, and Sharpe (MRS) broth but poorly in milk, often requiring supplements of B vitamins, minerals, and amino acids for growth. Specific amino acids that they require are aspartate, glutamate, valine, leucine, isoleucine and, depending on the strain, histidine, methionine, tryptophan, arginine, and cysteine. Mg^{2+} and Mn^{2+} stimulate their growth. Leuconostocs are unable to metabolize arginine. Leuconostocs usually grow associatively with lactococci in milk, although strains of *Ln. lactis* can grow independently and produce acid in milk. Thus, a cooperative relationship appears to exist between the two groups of lactic acid bacteria, although the nature of the association has not been elucidated. There may also be physical associations between lactococci and exopolysaccharide-producing leuconostocs (e.g., *Ln. mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum*) mediated by exopolysaccharides.

Other characteristics of dairy leuconostocs are given in **Table 2**. In addition, *Ln. lactis* has higher heat resistance than other leuconostocs and may survive treatment at

Table 1 Species and subspecies of the genus *Leuconostoc*

Species	*Primary source*
Leuconostoc mesenteroides	
subsp. *mesenteroides*	Plants/raw-milk cheeses/ meats
subsp. *dextranicum*	Plants/raw-milk cheeses
subsp. *cremoris*	Dairy products
Leuconostoc lactis	Dairy products
Leuconostoc carnosum	Chill-stored meats
Leuconostoc gasicomitatum	Chill-stored meats
Leuconostoc gelidum	Chill-stored meats
Leuconostoc fallax	Sauerkraut
Leuconostoc inhae	Kimchi
Leuconostoc kimchi	Kimchi
Leuconostoc citreum	Human clinical sources
Leuconostoc pseudomesenteroides	Human clinical sources

60 °C for 30 min. Selective agar media containing vancomycin or tetracycline are commonly used to isolate and enumerate leuconostocs from fermented dairy products and mixed-strain starter cultures. However, some lactobacilli are also resistant to vancomycin. In the presence of lactobacilli and/or yeasts, a more complex medium containing vancomycin, tetracycline, sodium azide, and sorbic acid can be used to isolate and enumerate leuconostocs.

Dairy Cultures

Leuconostocs (predominantly *Ln. mesenteroides* subsp. *cremoris*) are not generally used for acid production in dairy fermentations, but are used primarily as flavor producers in combination with pure or mixed strains of *Lactococcus lactis* subsp. *lactis* and/or *Lc. lactis* subsp. *cremoris* (mesophilic acidifying starters) in the manufacture of a number of fermented dairy products. These products include several cheese types (Cottage and Cream cheeses, Feta, Gouda, Edam, Havarti, quark, and blue-veined cheeses), ripened (lactic) butter, cultured buttermilk, cultured sour cream, and Scandinavian fermented milks (*see* **Butter and Other Milk Fat Products**: The Product and its Manufacture. **Cheese**: Cheddar-Type Cheeses; Dutch-Type Cheeses. **Fermented Milks**: Buttermilk; Nordic Fermented Milks).

When present, leuconostocs may constitute up to 10% of the total culture in mesophilic mixed-strain dairy starter cultures. Mesophilic mixed-strain cultures are traditionally divided into four types:

1. D cultures, containing only citrate-utilizing strains of *Lc. lactis* as the flavor producer;
2. L cultures (previously known as B cultures), containing only citrate-utilizing leuconostocs as the flavor producers;

Table 2 Characteristics of the dairy leuconostocs

Characteristics	Ln. lactis	Ln. mesenteroides *subsp.* cremoris	Ln. mesenteroides *subsp.* mesenteroides	Ln. mesenteroides *subsp.* dextranicum
Cell shape	Cocci	Cocci	Cocci	Cocci
NH_3 from arginine	−	−	−	−
Citrate metabolism	+	+	+	+
Sugar fermentation	Hetero	Hetero	Hetero	Hetero
Lactate isomer formed	D(−)	D(−)	D(−)	D(−)
Utilization of				
Glucose	+	+	+	+
Galactose	+	+	+	+
Lactose	+	+	+	+
Mannose	±	−	nd	nd
Raffinose	±	−	nd	nd
Mannitol	−	−	−	−
Arabinose	−	−	+	−
Growth at				
10 °C	+	+	+	+
45 °C	−	−	−	−
Lactate production in milk	<0.5%	0.2%	nd	nd
Litmus reduction in milk	−	−	−	−
Catalase	−	−	−	−
Growth in 6.5% NaCl	−	−	+	−
Dextran from sucrose	−	−	+	+
Bacteriocin production	nd	nd	+	nd
Growth at pH 4.8	−	−	−	−

+, positive reactions; −, negative reactions; ±, variable reactions; nd, not defined; hetero, heterolactic fermentation.

3. DL cultures (formerly referred to as BD cultures), containing both D and L cultures; and
4. O cultures (formerly referred to as N cultures), containing no flavor producers.

In addition, *Ln. mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum* are part of the kefir grains (starter cultures) used in kefir manufacture.

Besides the deliberate addition of leuconostocs as starter or adjunct cultures, leuconostocs such as *Ln. lactis* can enter dairy products as part of the adventitious microflora. Some species can survive pasteurization during dairy processing.

The primary function of *Ln. mesenteroides* subsp. *cremoris* is to impart buttery flavor to dairy products via the formation of diacetyl from the metabolism of citrate. Diacetyl is formed only at acid pH, and coculture with acidifying starter cultures (e.g., *Lc. lactis*) is required to potentiate citrate utilization. Another product of citrate metabolism is CO_2, which is important both for eye formation in Gouda and Edam cheeses and to expand mechanical openings in Blue-veined cheeses. Another purported function is to remove the excess acetaldehyde (causing the 'green flavor' defect) produced by lactococcal starters. *Leuconostoc mesenteroides* subsp. *cremoris* and subsp. *dextranicum* can be applied as adjuncts in yogurt manufacture to modify the acetaldehyde flavor and to produce diacetyl flavor. Acetic acid, ethanol, and CO_2 produced from sugars by leuconostocs can also contribute to the overall flavor and character of the product.

Citrate Fermentation

Milk contains small amounts (≈0.2%, $8\,\text{mmol}\,l^{-1}$) of citrate. The ability to metabolize citrate is one of the most important attributes of leuconostocs used as dairy starter or adjunct cultures (although not all leuconostocs utilize citrate). The metabolism of citrate by dairy lactic acid bacteria has been extensively studied. As with citrate-utilizing lactococci, leuconostocs cannot use citrate as the sole energy source; a fermentable sugar is also required. Leuconostocs metabolize citrate using the same biochemical pathway as citrate-positive strains of *Lc. lactis*. The pathway of citrate metabolism consists of the following steps: (1) uptake of citrate facilitated by citrate permease; (2) splitting of citrate into acetate and oxaloacetate by citrate lyase; (3) formation of pyruvate by decarboxylation of oxaloacetate; and (4) reduction of pyruvate to lactate and/or conversion of excess pyruvate into acetate, diacetyl, acetoin, and 2,3-butanediol. Diacetyl is a key volatile flavor compound in many fermented dairy products. The CO_2 produced during citrate fermentation is responsible for eye formation in certain cheese types and for the effervescence of cultured milks. The cometabolism of citrate with a sugar leads to growth stimulation due to the production of additional energy (adenosine triphosphate (ATP)) via the acetate kinase pathway (*see* **Lactic Acid Bacteria**: Citrate fermentation by Lactic Acid Bacteria).

Primary Fermentations

Leuconostocs produce energy only by fermentation, as they lack functional cytochromes and some enzymes of the Krebs cycle. Leuconostocs use the phosphoketolase pathway (also known as the heterofermentative pathway) for the metabolism of hexoses (e.g., glucose and galactose), producing D(−)-lactate, ethanol, and CO_2 as the end products:

$$\text{glucose (galactose)} \rightarrow \text{D}(-)\text{-lactate} + \text{ethanol} + CO_2 + \text{ATP}$$

In the presence of external electron acceptors such as acetaldehyde and pyruvate, acetic acid is formed instead of ethanol and additional ATP is produced. β-Galactosidase is the principal enzyme involved in the hydrolysis of lactose by leuconostocs. Lactose transport into the cell is facilitated by a permease. Inside the cell, lactose is hydrolyzed by β-galactosidase into glucose and galactose. Glucose is then phosphorylated and metabolized via the phosphoketolase pathway. Galactose is first phosphorylated to galactose-1-phosphate, which is then transformed into glucose-1-phosphate and metabolized in the same way as glucose.

Leuconostocs metabolize pentoses via the pentose phosphate pathway:

$$\text{pentose} \rightarrow \text{lactate} + \text{acetate} + 2\text{ATP}$$

There is no formation of ethanol and CO_2.

In addition, strains of *Ln. mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum*, and *Ln. citreum* produce an extracellular dextransucrase, induced by the presence of sucrose in the growth media. This enzyme catalyzes the formation of dextrans (linear D-glucose polymers with α-(1–6) linkages) from sucrose, with the concurrent release of fructose monomer. The presence of disaccharide acceptor carbohydrates such as maltose and lactose can modulate the polymerization, resulting in the formation of dextrans of lower molecular weight, and also the production of oligosaccharides. These reactions offer both textural (hydrocolloid) and functional (prebiotic) applications in fermented foods, including dairy products.

Leuconostocs are generally regarded as nonproteolytic and nonlipolytic. However, leuconostocs do exhibit intracellular and cell wall-associated proteolytic, peptidolytic, and esterolytic activities. The intracellular proteinase and peptidase activities of leuconostocs are considerably lower than those of lactococci. The proteolytic activity of a *Leuconostoc* strain isolated from Feta cheese has been found to be more active on β-casein than α_s-casein, and was salt sensitive. Thus the proteolytic impact of leuconostocs in cheese is likely to be small. The intracellular esterase/lipase activities of leuconostocs are similar to those of lactococci with a preference for esters of short-chain fatty acids. Formation of ethyl and butyl esters has also been reported.

Secondary Fermentations

Leuconostocs require amino acids for growth. Amino acids are produced as a result of proteolysis by and autolysis of starter bacteria. There is a lack of information on the catabolism and biosynthesis of amino acids by leuconostocs. There is evidence that citrate metabolism is involved in amino acid biosynthesis in *Ln. mesenteroides* subsp. *mesenteroides*. Oxaloacetate produced from citrate can be converted to aspartate by transamination. This transamination reaction may also be involved in the biosynthesis of other amino acids from α-ketoacids (glutamate from α-ketoglutarate and leucine from α-ketoisocaproate).

Acetaldehyde produced by dairy starter cultures such as lactococci and lactobacilli is a major flavor compound in young cheeses, cultured cream products, and fermented milks. Excess acetaldehyde causes a flavor defect referred to as 'green apple-like' in these products. Leuconostocs can use acetaldehyde as an electron acceptor, reducing it to ethanol. Alternatively, leuconostocs can oxidize acetaldehyde to acetic acid.

Leuconostocs can use oxygen as an electron acceptor during sugar fermentation, resulting in more acetic acid and ATP (enhanced cell yield) but less ethanol formation.

Bacteriocin Production

Bacteriocins are proteins or polypeptides with bactericidal activity. Dairy leuconostocs are known to produce antimicrobial compounds against pathogenic and spoilage microorganisms. However, the inhibitory activity is generally attributed to the action of organic acids such as acetic acid, CO_2, and H_2O_2 that they produce. Little information is available on the purification and characterization of bacteriocins produced by dairy leuconostocs, although the production of bacteriocins by dairy leuconostocs has been implied.

Strains of *Ln. mesenteroides* subsp. *dextranicum* produce antagonistic activity attributable to bacteriocins, but the compounds involved have not been purified and characterized. In general, bacteriocins produced by leuconostocs may not necessarily be active against lactic acid bacteria, but are active against *Listeria monocytogenes*, a major food pathogen. *Leuconostoc mesenteroides* subsp. *mesenteroides* Y105 isolated from goats' milk produces an exclusively antilisterial bacteriocin, mesentericin Y105, which is relatively heat labile (60 °C, 120 min, pH 4.5). *Leuconostoc mesenteroides* subsp. *mesenteroides* UL5 isolated from Cheddar cheese produces a heat stable (100 °C, 30 min) bacteriocin (mesentericin 5), which is also antilisterial. The commercial application of bacteriocins or bacteriocin-producing leuconostocs to dairy products is not documented; however, they may well be useful in developing a multihurdle approach to dairy product stability. By contrast, there is a wealth of information on the bacteriocins produced by leuconostocs from meat and other environments; for example, a broad-spectrum bacteriocin (kimchicin GJ7) has been described from *Ln. citreum* (*see* **Bacteriocins**).

Bacteriophages

A number of bacteriophages (*see* **Bacteriophage**: Biological Aspects; Technological Importance in the Diary Industry) that attack some dairy leuconostocs have been isolated. However, most leuconostocs are insensitive to bacteriophages. There are no reports of bacteriophage attack in dairy fermentations associated with leuconostocs. This may be because their slow growth and low numbers make them less vulnerable to bacteriophage attack. Alternatively, it may be that attacks by bacteriophages have been overlooked, since the failure of the *Leuconostoc* culture will impact on some aspects of flavor but not on acid production.

Genome

There have been rapid advances in the genomic characterization of leuconostocs with whole genome sequencing. Gene sequences have been completed for *Ln. mesenteroides* subsp. *mesenteroides* ATCC8293 (US Department of Energy Joint Genome Institute, Walnut Creek, CA) and for *Ln. citreum* KM20. A large proportion of the genome encodes for carbohydrate transport and utilization, with a complete set of genes for heterolactic fermentation by the phosphoketolase pathway. The gene sequences also contain phage remnants and mobile genetic elements. *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC8293 has a chromosome of 2 Mb with 1924 protein-coding genes predicted from the sequence. *Leuconostoc citreum* KM20 has a chromosome of 1.8 Mb, with 1702 protein-coding genes. *Leuconostoc citreum* is a dextran-producing species and is considered to have potential as a probiotic strain because of a putative plasmid-encoded cell wall-anchored protein containing five mucus-binding domains known to be important in gastrointestinal colonization.

The strain is also known to suppress the growth of some pathogenic microorganisms and of HT29 adenocarcinoma cells.

Leuconostocs have a limited range (1–6) of plasmids (molecular masses ranging from 1 to 76 MDa), most of which are cryptic. However, genes involved in lactose transport and hydrolysis, and bacteriocin production and resistance, are plasmid encoded in some species of this genus. The plasmid encoding for lactose metabolism in *Ln. lactis* contains two partially overlapping genes encoding β-galactosidase. The permease involved in citrate uptake is also plasmid encoded in the dairy leuconostocs. The bacteriocin mesentericin Y105 is encoded on a plasmid in *Ln. mesenteroides* subsp. *mesenteroides* Y105. Insertion sequence elements are present at low copy number in some dairy leuconostocs.

See also: **Bacteriocins**. **Bacteriophage:** Biological Aspects; Technological Importance in the Diary Industry. **Butter and Other Milk Fat Products**: The Product and Its Manufacture. **Cheese**: Cheddar-Type Cheeses; Dutch-Type Cheeses. **Fermented Milks**: Buttermilk; Nordic Fermented Milks. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria.

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Streptococcus thermophilus

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Introduction

Streptococcus thermophilus belongs to the thermophilic group of lactic acid bacteria. It is traditionally used in association with one or several *Lactobacillus* species as a starter culture in the production of yogurt and the manufacture of Swiss and Italian cheeses such as Emmentaler and Mozzarella. *Streptococcus thermophilus* is ubiquitous in raw milk, and since the 1980s, increased throughput in dairy plants has seen the appearance of *S. thermophilus* in a new and unwelcome role as a successful colonizer of plate heat exchangers.

Characteristics

Streptococcus thermophilus is a Gram-positive spherical to ovoid nonmotile coccus, 0.7–0.9 μm in diameter, occurring in pairs and chains, some of which can be very long. The bacterium has an optimum growth temperature of 40–45 °C, a minimum of 20–25 °C, and a maximum near 47–50 °C. *Streptococcus thermophilus* does not hydrolyze arginine. It ferments a limited number of sugars including lactose, fructose, sucrose, and glucose. *Streptococcus thermophilus* does not ferment galactose during lactose metabolism. *Streptococcus thermophilus* is also characterized by being relatively sensitive to antibiotics and sanitizers and having low proteolytic activity. It is unique among the streptococci in having no group-specific antigen.

Classification

Streptococcus thermophilus shares many phenotypic and genetic properties with the other lactic acid bacteria, but does not fit conveniently into any systematic grouping. It is, for example, fairly closely related by DNA:DNA hybridization to *Streptococcus salivarius*, a common oral bacterium. This finding led to the reclassification of the organism as a new subspecies, *S. salivarius* ssp. *thermophilus*. Recently, it has been returned to the species level again following a more detailed DNA hybridization study. *Streptococcus thermophilus* is now a distinct streptococcal species that can be readily distinguished from the mesophilic lactic acid bacteria by its ability to grow at 45 °C and confirmation of cocci in chains by microscopic analysis.

Closely Related Species

Streptococcus waius was a recently described thermophilic *Streptococcus* originally isolated from stainless steel exposed to pasteurized milk. Based on genetic and phenotypic studies, it has been shown that *S. waius* belongs to the previously described species *Streptococcus macedonicus*.

Habitat

Streptococcus thermophilus is highly adapted to the dairy environment and, in the wild, can only be isolated from milk. It is found at low levels in raw milks obtained from a variety of animals. When colonies selected by growing at >40 °C are isolated from raw milks, a proportion will often prove to be *S. thermophilus*. At present, incubation temperature is the only selective means for enriching *S. thermophilus* from a mixed population. The agar bromocresol purple (BCP) is also very useful in selectively isolating *S. thermophilus* from a mixed population. With the development of species-specific DNA probes, positive identification of suspect clones can now be made readily. Raw milk has been established as a carrier of new *S. thermophilus* phage that appear in cheese plants that use thermophilic starters. No ‘original’, on-farm source of these thermophilic bacteria has yet been identified.

Streptococcus thermophilus and *S. macedonicus* are frequently found growing as biofilms in the regeneration sections of plate heat exchanger pasteurizers in dairy manufacturing plants.

Growth

There is great diversity in the growth of *S. thermophilus* strains. Some are rapid acidifiers of milk and other strains have complex needs for effective growth in milk. *Streptococcus thermophilus* strains require free amino acids for growth. These include glutamic acid, histidine, methionine, cystine, valine, leucine, isoleucine, tryptophan, arginine, and tyrosine. These bacteria grow well on media containing hydrolyzed protein or yeast extracts.

The low level of available nitrogen in raw cows' milk is usually insufficient to support good growth and supplementation is required, by either

- heating milk for yogurt manufacture (sufficient to precipitate whey proteins), or
- pairing the coccus with a suitable (proteolytic) *Lactobacillus* culture.

Streptococcus thermophilus grows rapidly in sterilized reconstituted nonfat dried milk resulting in a firm coagulum with no gas.

Metabolism

Streptococcus thermophilus is a homofermentative bacterium, fermenting lactose via the Embden–Meyerhof pathway (EMP) to L(+) lactate. When grown in milk, lactose is transported into the cell in association with the expulsion of galactose via an antiport system. Lactose is hydrolyzed by β-galactosidase, but only glucose is metabolized further via the EMP to L(+) lactate. Overall, 1 mol of lactose is fermented to 2 mol of lactate plus 1 mol of galactose. This is in contrast to the mesophilic lactic acid bacteria where lactose is transported as lactose-6-phosphate and completely metabolized to 4 moles of lactate.

Some strains of *S. thermophilus* will use a small percentage of the galactose excreted and others will not. With strains that have been investigated in detail, galactose-using strains can be obtained either by prolonged (>72 h) incubation on galactose, or more readily from growth in a chemostat on lactose limitation. This selects variants or mutants able to grow on the excreted galactose. Strains with the Gal$^+$ phenotype are unstable, and unless cultured in a galactose-based medium will quickly lose this phenotype. Although the normal *S. thermophilus* strains (Gal$^-$) have the genes of the Leloir pathway, these strains are normally expressed at such low levels that little or no galactose is used during lactose fermentation.

All lactic starter bacteria have complex peptidase systems enabling them to hydrolyze peptides to amino acids. Several such enzymes have been described for *S. thermophilus*. These include a number of aminopeptidases, a dipeptidase, a tripeptidase, and an endopeptidase. The proteolytic system of *S. thermophilus* is more limited than that of most other dairy starters, resulting in the usual practice of pairing with the more proteolytic thermophilic *Lactobacillus* to obtain maximum acid production.

Metabolites

Strains of *S. thermophilus* that produce bacteriocins are rare, and those that have been isolated have not been well characterized. Studies have shown that only closely related strains are inhibited although a bacteriocin produced by strain 81, a peptide of 32 amino acids, was active against *Bacillus*, *Listeria*, *Salmonella*, *Escherichia coli*, and *Yersinia* species.

Probiotic

It is interesting to note that although it has been claimed that yogurt *per se* and various *Lactobacillus* strains have probiotic activity, no probiotic claims have yet been made for *S. thermophilus*.

Streptococcus thermophilus strains have been used as probiotics for farm animals. Although not formally recognized as probiotic, these strains have the ability to modulate immune responses.

The species is not bile tolerant and was thought to be unable to grow or survive in the intestine. Recent studies in humans and animals have shown that they can survive as a transient population without colonization. The delivery system (such as yogurt) is also important. Conversely, there is no evidence that *S. thermophilus* has any significant ability to act as an opportunistic pathogen.

Some people lack sufficient β-galactosidase in the small intestine to fully digest the 4–5% lactose normally present in milk (lactose maldigestion). The symptoms can appear following milk consumption. Yogurt fermentation uses only a small proportion of the milk lactose and, as a result, yogurt normally has 3–4% lactose. The presence of (initially) viable starter cultures in yogurt can, however, be beneficial to lactose utilization. In the intestine, bile increases the cellular permeability of both *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Lactose is taken up by these bacteria and hydrolyzed by the intracellular β-galactosidases.

Genetics

The *S. thermophilus* ST1 and A054 genomes comprise an average 1.75 and 1.82 Mbp, respectively, based on summing the total restriction fragment sizes obtained with appropriate enzymes. The *S. thermophilus* genome is thus significantly smaller than the 2.35 Mbp of *Lactococcus lactis*.

In a comparative genome investigation of 47 *S. thermophilus* strains, it was shown that *S. thermophilus* evolves mainly via recombination with other *S. thermophilus* strains. A core genome of 1271 genes was detected in all 47 strains. A concept has been proposed consisting of the core genes plus a subset of genes that can be drawn from a gene pool that encode for essential functions.

Plasmids

Streptococcus thermophilus tends to have fewer plasmids than other lactic acid bacteria; the plasmid-free state is more common and observed plasmids are small. In contrast to mesophilic lactococci where plasmids determine metabolic functions critical for use in dairy fermentations, *S. thermophilus* plasmids are usually cryptic.

Recent studies on the molecular properties of *S. thermophilus* plasmids have added a number of new functions. These include a protein for rolling-circle replication, and low-molecular-weight heat stress proteins (Hsps). These proteins may aid in survival at elevated temperatures or low pH. Protection against bacteriophage attack via a type IC restriction-modification system from *S. thermophilus* plasmid pER35 may be beneficial. This system is very similar to those encoded on plasmids in *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. This could indicate the potential for horizontal transfer of these genes between different species of lactic acid bacteria.

Significance in Dairy Products

The symbiotic relationship between *S. thermophilus* and lactobacilli has long been used in the manufacture of fermented milks and various Swiss and Italian cheeses. Compatible strains have been consistently observed to grow faster and produce more acid and flavor than the individual components. The streptococci are stimulated by the casein breakdown products produced by *Lactobacillus* proteinases and, in turn, contribute formate and carbon dioxide to stimulate the *Lactobacillus*. With yogurt, the dependence of each species on the other for growth leads to a stable ratio being maintained between them at the appropriate incubation temperature.

Cheese Starter

The *S. thermophilus* component of thermophilic cheese starters is responsible for much of the early acid production. The inability of *S. thermophilus* to metabolize galactose leads to its excretion from the cell and accumulation in the cheese. The thermophilic lactobacilli (*Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis*) can play an important role in using this galactose, provided manufacturing conditions are optimum, particularly having a significant population of the starter as rods. If the *Lactobacillus* strains in the cheese as starter are unable to use the galactose, atypical cheese can result. The residual galactose in cheese allows opportunity for nonstarter *Lactobacillus* to flourish. This can result in flavor and texture defects in the cheese. 'Pink' discoloration of cheese through Maillard reactions between galactose and protein residues has also been reported.

Streptococcus thermophilus has also been used together with lactococci in the 'short method' of Cheddar manufacture. Starter growth is accentuated by using a higher cooking temperature. Significant levels of galactose in the curd are removed by the adventitious nonstarter flora or by a deliberately added *Lactobacillus* adjunct culture. Galactose is a major contributor to the browning that occurs when cheese is heated in applications such as the manufacture of pizzas or processed cheese.

Streptococcus thermophilus also tends to give atypical flavors in 'long hold' ripened cheese. This is thought to be due to the lack of proteolytic enzymes.

Yogurt

Streptococcus thermophilus is traditionally paired with *Lb. delbrueckii* subsp. *bulgaricus* in the manufacture of yogurt. The typical flavor of yogurt is due to the lactic acid in combination with various carbonyls and other compounds produced by thermal degradation of lipids, lactose, and proteins during the heat treatment of the milk. A major metabolite of *S. thermophilus* and of yogurt flavor is acetaldehyde. The acetaldehyde:acetone ratio in the yogurt is important in determining flavor balance.

Exocellular Polysaccharide Production

In common with other lactic acid bacteria, some strains of *S. thermophilus* produce exocellular polysaccharides (EPS). The EPS produced by *S. thermophilus* is critically important for the final texture in most yogurt processes. The use of EPS-producing cultures is particularly important in countries where stabilizers are not permitted. EPS properties are often used as a substitute for fat in many modern yogurts.

The production of EPS in milk is very dependent on incubation temperature, and EPS-producing *S. thermophilus* cultures are often unstable. Characterizing polymers produced by *S. thermophilus* has revealed a range of compositions. The polymer of one strain comprised galactose, glucose, and *N*-acetylgalactosamine in the ratio of 2:1:1. The molecular mass of the polymer was 1×10^3 kDa. In practice, the quantity of polysaccharide produced does not necessarily correlate with the corresponding viscosity.

This may reflect conformation changes and/or interactions with precipitated caseins at low pH values. The instability may be partially due to the presence of glycohydrolases that can hydrolyze the polymer. In contrast to the lactococci and lactobacilli where ropiness is plasmid mediated, *S. thermophilus* ropy strains are plasmid free.

Bacteriophage

All *S. thermophilus* bacteriophage (phage), whether virulent or temperate, contain a genome of double-stranded DNA ranging in size from 31 to 45 kb. They have the same basic morphology of isometric heads (65 nm in diameter) and long noncontractile tails (230–260 nm long), usually with a small base plate. The morphological group is Bradley's group B (siphoviridae). This contrasts with the morphological variety within the phage for the mesophilic lactococci. Studies on phage isolated from dairy plants in the Europe and the United States reveal only two major groups with considerable cross-reactivity within each group. At the genetic level, all *S. thermophilus* phage belong to a single DNA homology group.

Recent studies have shown that *S. thermophilus* can integrate short stretches of phage-derived sequences to become phage resistant.

There does appear to be geographic diversity between *S. thermophilus* phage. Studies in Switzerland have found that milk entering cheese plants can have *S. thermophilus* phage titers ranging from 10 to 130 pfu ml^{-1}. Studies have shown that 'new' industrial phage are not derived from the existing phage population but are identical to the incoming raw milk phage. There has been little work on the variability of phage in their natural nonindustrial environment.

Commercial fermentations with *S. thermophilus* are susceptible to strain-specific phage infection. Contamination by virulent phage can lyse sufficient starter to slow or even halt acid production with consequent loss of product. Even if the phage infection is insufficient to delay acid production, a decrease in the *S. thermophilus* component of a yogurt starter can give a lower flavor score and have a major impact on the texture of the resulting yogurt. *Streptococcus thermophilus* phage tend to be less troublesome in modern yogurt plants compared with cheese plants because of the following reasons:

- High heat treatment of yogurt milk kills most raw milk phage.
- There is no whey removal. In cheesemaking, removal of whey and its separation promotes phage dispersal throughout the environment.
- Yogurt plants generally have fewer refills of the fermentation tanks in a processing day.

The ubiquitous nature of phage in the dairy plants has led to research efforts focusing on controlling dairy phage rather than trying to eradicate them.

Fast detection methods for *S. thermophilus* bacteriophage are an important tool to avoid phage attack in dairy factories. With increasing demand for quantitative, more sensitive, and quicker procedures, the emphasis has been on development of real-time quantitative polymerase chain reaction (qPCR).

Temperate Phage

Initial efforts to induce infective temperate phage from *S. thermophilus* strains by mitomycin C treatment or UV irradiation were unsuccessful, suggesting that induction conditions, choice of indicators, or choice of host to induce was inappropriate. More recently, a number of temperate phage have been isolated and fully sequenced. One of these is the mitomycin-induced phage φO1205 (43 075 bp). This phage appears to be packaged by the so-called headful mechanism. The genomic organization and structure resemble several temperate lactococcal phage that display a life-cycle-specific organization. Genes involved in the lysogenic life cycle are clustered and arranged in an orientation opposite to the open reading frames that are involved in the lytic cycle. A 45 kbp defective (tailless) *S. thermophilus* phage with homology to lytic phage P55 has also been induced by mitomycin C.

Thermization and Pasteurization

Thermization, a heating process less severe than pasteurization, is used to improve the keeping quality of milk before cheesemaking. Heating is at 63–65°C for 15–20s. Such treatment has negligible effect on *S. thermophilus* present in the incoming raw milk. As a result, the organism is able to establish itself as a biofilm in the appropriate temperature portion of the regenerative section. At the higher temperature used for pasteurization (72°C×15s), the kill is detectable but slight and varies with different wild-type strains of *S. thermophilus* in raw milk (**Figure 1**). With both treatments, the longer the run before cleaning and sanitizing the heat exchanger, the greater the buildup of *S. thermophilus* and the higher the contamination of the treated milk. In general, wild-type *S. thermophilus* strains are more heat resistant than strains selected as starters (**Figure 2**).

Streptococcus thermophilus Biofilms

Biofilms are aggregations of microbial cells and their associated extracellular polymeric substances that adhere to, and grow on, a surface. Bacteria within biofilms are

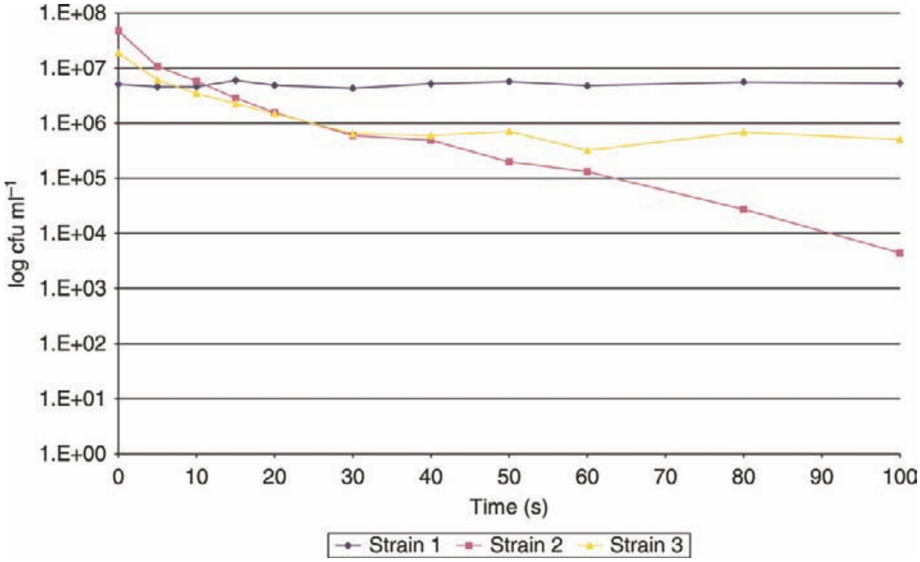

Figure 1 Survival of wild-type strains of *Streptococcus thermophilus* in whey at pH 6.5, after heating for varying times at 72 °C. From Hill and Smythe, unpublished data.

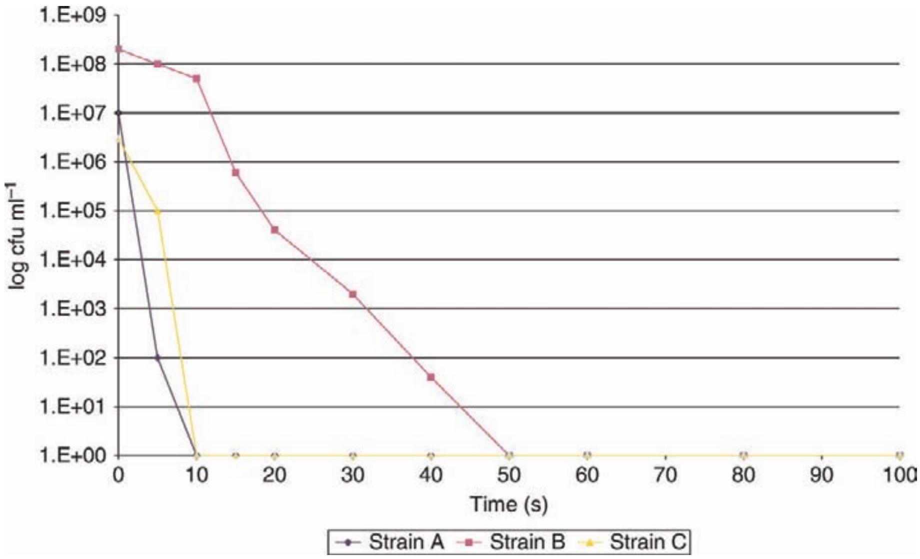

Figure 2 Survival of starter-type strains of *Streptococcus thermophilus* in whey at pH 6.0, after heating for varying times at 72 °C. From Caddick and Heap, unpublished data.

more difficult to eliminate than free-living cells. Cells from bacteria colonizing the surface of milk processing equipment as biofilms can spread throughout the equipment and thus be a significant source of contamination of dairy products. The stainless-steel surface in the cooling section of pasteurizers (30–50 °C temperature range) can harbor levels of 10^7 cells cm^{-2}. Under these conditions, levels of *S. thermophilus* released from a pasteurizer can be $>10^6$ cfu ml^{-1} after 7–8 h operation.

The most frequently reported biofilm problem in dairy manufacturing plants is the development of *S. thermophilus* in pasteurizers and thermalizers. Contamination of milk intended for Cheddar or Gouda cheesemaking, for example, with high levels of *S. thermophilus* may result in problems in cheese quality (**Figure 3**).

Isolates obtained from dairy manufacturing – whether from milk, milk contact surfaces, or products from manufacturing plants experiencing contamination problems – are highly hydrophobic. In contrast, isolates from other sources produce mixed results. This suggests that hydrophobicity is important in the development of *S. thermophilus* biofilms. It is believed that hydrophobicity predisposes the cells to colonizing stainless steel, with the more hydrophobic cells forming a stronger bond with the surface or being more likely to resist aqueous chemical cleaners than the more hydrophilic cells.

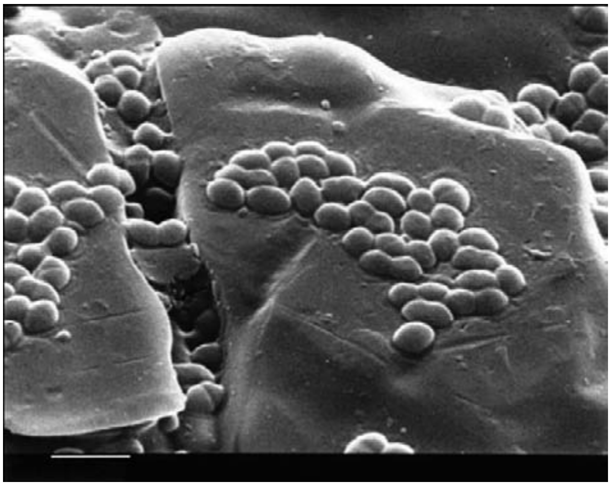

Figure 3 Scanning electron micrograph of an actively growing *S. thermophilus* biofilm on grade 316 2B stainless steel. Scale bar = 2 μm. Reproduced with permission from Dr Steve Flint.

Cell-surface proteins are important in the processes leading to adhesion and biofilm formation. Once established, *S. thermophilus* biofilms are resistant to routine caustic and acid cleaning, as well as to the normal strengths of sanitizers commonly used in cleaning dairy manufacturing plants. The greatest and the most consistent reduction in biofilm cells (>100-fold) is obtained using proteolytic cleaners. Plant sanitation can then be effectively carried out once the biofilm has been removed.

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Pediococcus spp.

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Introduction

Pediococcus spp. are lactic acid bacteria that occur in plant environments along with *Lactobacillus* and *Leuconostoc.* Consequently, they are often found in foods from plant fermentation processes such as beer, cider, silage, sauerkraut, and other fermented vegetables. They are also found in other fermented foods such as cheese as well as in cured meats, raw sausages, and fresh and marinated fish.

Pediococcus spp. are morphologically similar to *Tetragenococcus* spp. as they divide in two planes at 90°, forming tetrads. As a consequence, pediococci never form chains. However, the two genera are only distantly related and the genus *Pediococcus* most closely relates to *Lactobacillus casei* and *Lb. paracasei*, both phenotypically and genotypically.

Pediococcus spp. are not well studied compared with many of the dairy lactic acid bacteria, nor are they as widely used in dairy fermentations. They are found in some cheeses as adventitious bacteria and are occasionally added to fermented milk or cheese products. They are more commonly used as starters in fermented vegetable and meat products.

Taxonomy

Pediococci are Gram-positive spherical cocci forming pairs and tetrads but not chains. Their morphology is often best viewed in a wet mount under phase contrast. They are catalase-negative (although some strains display a pseudocatalase activity), non-spore-forming, nonmotile facultative anaerobes. Pediococci can be distinguished from aerococci by their ability to grow anaerobically, ability to grow at pH 5.0, and by their resistance to vancomycin.

The taxonomy of the genus has been changing rapidly in recent years with the application of phylogenetic analyses (16S rRNA, 16S–23S internally transcribed spacing region, and HSP60 sequence analysis). Currently, 12 species are recognized: *Pc. acidilactici*, *Pc. argentinicus*, *Pc. cellicola*, *Pc. claussenii*, *Pc. damnosus*, *Pc. ethanolidurans*, *Pc. inopinatus*, *Pc. lolii*, *Pc. parvulus*, *Pc. pentosaceus*, *Pc. siamensis*, and *Pc. stilesii* (*Pc. stilesii* is notable for its ability to grow at pH 9.0). The species are phylogenetically heterogeneous; however, the type species, *Pc. damnosus*, is closely related to *Pc. acidilactici*, *Pc. pentosaceus*, and *Pc. parvulus*. Species can be differentiated phenotypically according to their growth temperature, the pH at which growth occurs, carbohydrate fermentations, and the isomers of lactic acid formed (**Table 1**). The species formerly called *Pc. halophilus* has been reclassified as *Tetragenococcus halophilus*, and strains of *Pc. cerevisiae* have been reclassified as *Pc. acidilactici.* Additional species reclassified from pediococci are *Pc. dextrinicus* (reclassified as *Lactobacillus*) and *Pc. urinaeequi* (reclassified as *Aerococcus*).

Strains from only two species, *Pc. acidilactici* and *Pc. pentosaceus*, are associated with dairy products. These two species are closely related and, although they may not be differentiated by phenotypic properties, they can be differentiated by rRNA sequence analysis. The major fermentation product of these species is a mixture of L(+)-lactate and D(−)-lactate when glucose and lactose are the fermentative substrates. Lactose is transported into the cell via a permease and then hydrolyzed to glucose and galactose by the enzyme β-galactosidase. *Pediococcus pentosaceus* produces equimolar concentrations of lactate and acetate from pentoses.

Genome sequencing has been completed for *Pc. pentosaceus* ATCC 25745 (US Department of Energy Joint Genome Institute).

Isolation and Enumeration

Media for the growth of pediococci from dairy products are the same as those for lactobacilli. The media must provide for their complex nutritional requirements and their preference for microaerophilic conditions. The most commonly used medium for the nonselective enumeration of pediococci is MRS (de Man, Rogosa, Sharpe) agar. APT (all-purpose Tween) agar is a similar medium that may also be used to grow pediococci. The agar plates should be incubated anaerobically, usually for 48 h at 30 or 37 °C.

It is difficult to select exclusively for pediococci, as their physiological properties are very similar to those of

Table 1 Phenotypic differentiation of *Pediococcus*

	Growth at 35 °C	*Growth at 45 °C*	*Growth at pH 4.5*	*Growth at pH 9.0*	*Lactic acid isomer(s)*	*Lactose*	*Maltose*	*Ribose*
Pc. acidilactici	+	+	+	−	DL	d	−	+
Pc. argentinicus	+	−	+	−	L (+)	−	+	ND
Pc. cellicola	+	+	+	−	DL	+	+	+
Pc. claussenii	+	−	+	−	L(+)	−	d	+
Pc. damnosus	−	−	+	−	DL	−	d	−
Pc. ethanolidurans	+	+	+	−	DL	d	ND	d
Pc. inopinatus	+	−	+	−	DL	+	+	−
Pc. lolii	+	ND	+	−	DL	−	−	ND
Pc. parvulus	+	−	+	−	DL	−	+	−
Pc. pentosaceus	+	−	+	−	DL	+	+	+
Pc. siamensis	ND	+	ND	−	DL	+	−	−
Pc. stilesii	+	+	+	+	DL	−	+	+

For growth at different temperatures: −, no growth; +, growth. For carbohydrate fermentation acid is produced by: +, greater than 90% of strains; −, less than 10% of strains; d, 10–89% of strains. ND, no data.

lactobacilli and *Leuconostoc* spp. Selective enumeration relies on their aciduric nature for differentiation from other bacteria. The most commonly used medium for the selective enumeration of pediococci in dairy products is Rogosa agar (either Rogosa SL or Rogosa LBS). The pH of this medium should be adjusted to 5.3–5.4 to prevent the growth of lactococci, enterococci, and streptococci. MRS agar with the pH adjusted to 5.4 may also be used for the selective enumeration of pediococci. Plates of both media should be incubated anaerobically for either 3 days at 37 °C or 5 days at 30 °C. However, neither of these media is totally selective, as *Leuconostoc* (cocci in pairs and short chains), *Lactobacillus* (rod shaped), and some yeasts are also able to grow on these media at pH 5.4. Microscopic examination and phylogenetic analysis of the isolates will be necessary to determine if the colonies are pediococci.

Bacteriocin Production

Pediococcus spp. have been shown to produce a number of bacteriocins (pediocins) active against closely related bacteria and some Gram-positive pathogens. Pediocins are heat-stable bactericidal substances, and are produced during the growth phase of cultures. Pediocin A from *Pc. pentosaceus* and pediocin AcH and pediocin PA-1 from *Pc. acidilactici* have been studied in some detail. These bacteriocins have a wider spectrum of activity than most bacteriocins and are active against strains of *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Enterococcus*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Lactococcus*, *Propionibacterium*, and *Listeria*. Other pediocins produced include pediocin 5 and pediocin RZS C8.

The mode of action of the bacteriocin appears to be its attachment to the cell wall, which, in sensitive strains, causes a conformational change in the cell wall that leads to a loss of barrier function. This allows the bacteriocin to penetrate the cell wall and disrupt the cytoplasmic membrane.

While many applications of pediocins have been considered for the meat industry, a number of uses of pediocins have also been proposed for dairy products. Pediocins have been shown to initially kill a significant proportion of *Listeria monocytogenes* in cream, milk, ice cream, and cheese sauce. However, those that survive do recover and grow. Pediocins have been shown to inhibit the growth of *Li. monocytogenes* on the surface of smear-ripened cheese when the cheese is sprayed with a culture of pediocin-producing bacteria (*see* **Bacteriocins**).

Exopolysaccharides and Probiotic Properties

Exopolysaccharide (EPS) production by lactic acid bacteria has been investigated because of the growing interest in the use of cultures to confer textural and rheological attributes to foods *in situ* through natural fermentations rather than by incorporation of additives. Strains of *Pc. parvulus* and *Pc. pentosaceus* are known to produce EPS. A 2-substituted (1,3)-β-D-glucan from *Pc. parvulus* 2.6 has been described in detail. The glucan has a high molecular mass (>10^6 Da), is thermostable up to 220–230 °C, and has been demonstrated to be a useful biothickener capable of enhancing the viscosity of an oat-based ferment.

Some strains of *Pediococcus* are proposed to have probiotic effects due to their ability to survive and adhere to the gastrointestinal tract and also due to reported immune modulation capability. (1,3)-β-D-Glucan from *Pc. parvulus* 2.6 is implicated in the probiotic mechanism. The EPS is involved in the formation of biofilms, strongly supporting its involvement in adhesion to human epithelial cells. *Pediococcus parvulus* 2.6 is also proposed to have a probiotic impact by promoting antitumor and antimicrobial activity through EPS-mediated activation of macrophages, dendritic cells, and other leukocytes.

Pediococcus spp. in Fermented Milks

Pediococcus acidilactici strains have been used occasionally in dairy products such as bioyogurts, where the culture is used for acidification and health reasons. Their use as 'health' cultures has primarily been around the antimicrobial activity of some strains. However, *Pediococcus* spp. have been reisolated from feces following feeding, and *in vitro* testing has demonstrated the ability of some strains to withstand the conditions found in the gastrointestinal tract, stimulating a growing interest in their probiotic potential. This aspect is in its infancy compared to the extensively studied probiotic strains from the genera of *Lactobacillus* and *Bifidobacterium.*

Pediococcus spp. in Cheese

Pediococcus spp. as Adventitious Bacteria in Cheese

Nonstarter lactic acid bacteria (NSLAB) are present in most ripened cheeses and originate from either the milk or the factory environment. The most common types of NSLAB identified in cheese include the homofermentative and heterofermentative lactobacilli (*see* **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species).

Pediococci have been found in only a small number of cheeses studied and are usually not the predominant NSLAB. Most bacteria from cheese that are isolated from selective agar such as Rogosa LBS agar are identified as pediococci if they have the characteristic tetrad formation and spherical shape. If identified further, they are generally strains of *Pc. acidilactici* and *Pc. pentosaceus.* In Cheddar, they can be detected in young cheese (<1 month old), along with lactobacilli, and then are usually not detected during a further 12–15 months of ripening, as the lactobacilli become the predominant NSLAB. They can be detected in some of these cheeses on further ripening, and may become the predominant NSLAB in mature Cheddar (>15 months) as the lactobacilli decrease in number.

Ripening Effects of *Pediococcus* spp. in Cheese

Cheese isolates of *Pediococcus* spp. have not been studied in any detail compared with the more common lactobacilli isolates present in cheese either as adventitious NSLAB or as added adjuncts. *Pediococcus* spp. found in cheese are homofermentative. Their proteolytic and lipolytic enzyme activities and ability to ferment amino acids are equal to, or lower than, those of the lactobacilli isolates, and so far, no studies have identified their importance in cheese ripening. *Pediococcus acidilactici* ATTC 8042 is reported to have at least three different extracellular proteolytic enzymes.

However, pediococci have been associated with defects in some industrial cheeses. In cheese made with lactococcal starter strains, only the L(+)-lactate isomer is produced from lactose. In Cheddar and other nonwashed curd cheeses, this lactate concentration can be sufficiently high to form undesirable calcium lactate crystals. The crystals are undesirable either from a visual point of view or because they can block filters when the cheese is used in processed cheese plants. In cheeses in which the adventitious pediococci reach high densities, the situation is potentially worse. Pediococci have two stereospecific lactate dehydrogenases (LDHs), L(+)-LDH and D(−)-LDH, which will produce a mixture of L(+) and D(−) isomers of lactic acid during lactose fermentation in broth culture. The ratio of the isomers is strain dependent. In cheese, although lactose is absent by the time the pediococci reach high densities, the action of their two LDHs will cause the undesirable racemization of lactate. This racemizing activity of pediococci equilibrates the L(+)-lactate to an equal mixture of the two isomers, the Ca salt which is more insoluble than the separate isomers. This results in a higher risk of the formation of calcium lactate crystals.

Pediococcus spp., along with some other NSLAB, have oxidative activity in Cheddar cheese, with strains of *Pc. pentosaceus* able to oxidize lactose, peptides, and L(+)- and D(−)-lactate. For *Pc. pentosaceus*, it is believed that lactate oxidation is the most important oxidative activity in cheese, with acetate and CO_2 as the products. At high concentrations, the acetate and CO_2 can cause flavor and slit defects, respectively. The extent of lactate oxidation is normally minimal because of limited O_2 availability, as most cheese-wrapping films have low permeability to O_2.

Pediococcus spp. as Adjuncts in Cheese

Pediococci have been reported to be used occasionally as adjuncts in Cheddar cheese. Some strains have improved the flavor and other strains have had no noticeable effect on the flavor. Their ripening mechanism as adjuncts has not been studied. However, their minimal oxidative activity in cheese wrapped in the normal way may be important in maintaining a low redox potential, which is considered to have a positive effect on cheese flavor. Pediococci have also been used in washed curd cheese to improve flavor, but with variable results.

Although for many consumers the lactate-racemizing activity of pediococci and the associated increased possibility of the formation of calcium lactate crystals in nonwashed curd cheeses are defects, for some they can be attributes. Cheddar cheese has been made on occasion with pediococci so that the lactate crystals give a crunchy texture. This is not done routinely on a commercial scale.

See also: **Bacteriocins**. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Leuconostoc* spp.

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Enterococcus in Milk and Dairy Products

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Characteristics of Enterococci

Until 1984, enterococci were included in the Lancefield group D of the *Streptococcus* genus, but DNA–DNA hybridization and 16S rRNA techniques showed that they are significantly different from other groups of streptococci. Thus, a new genus, '*Enterococcus*', was proposed. The genus *Enterococcus* belongs to the lactic acid bacteria (LAB), in spite of, as its name evokes, a fecal origin. Enterococci are Gram-positive, non-spore-forming, catalase-negative (some strains exhibit pseudocatalase activity), oxidase-negative, almost all nonmotile, facultative anaerobic cocci. Under the microscope, they appear as single cells or forming pairs or chains. Their metabolism is fermentative, and the predominant end product of glucose fermentation is L(+)-lactic acid. Within the ~30 species recognized at present, *Enterococcus faecalis* and *Enterococcus faecium* are the most relevant to dairy products. Both are ubiquitous and particularly resistant to hostile environmental conditions. They usually grow at 10 and 45 °C, in 6.5% NaCl and at pH 9.6, and survive heating at 60 °C for 30 min. They are among the most thermoresistant of non-spore-forming bacteria. As a consequence, they are commonly found in many raw or heat-treated foods, such as raw or pasteurized milk, cheeses, and other dairy products.

Enterococci and Flavor

Enterococci occur and grow in many foods, sometimes contributing to their flavors, mainly in fermented foods, and particularly in cheeses produced in the Mediterranean area, made from either raw or pasteurized milk. The effect of enterococci on flavor development in dairy products is basically due to citrate metabolism, proteolysis, and lipolysis.

Citrate metabolism shows great strain-to-strain variation, although the majority of *Ec. faecalis* strains use it as an energy source. This variability is explained by several factors. Citrate-metabolizing ability is linked to plasmids, the presence of other carbohydrates that may or may not inhibit citrate catabolism, and the ability of some strains to utilize glucose first and to use citrate only when this monosaccharide is consumed. The same has been observed with fructose, although not with sucrose or galactose, in model systems.

The catabolism of citrate may produce substances such as acetaldehyde, diacetyl, acetoin, 2,3-butanediol, formate, and acetate, relevant to the flavor of cheese and other products. It must be considered that lactic acid is commonly coproduced by enterococci in dairy products due to lactose metabolism and, obviously, lactic acid influences food flavor, although enterococci exhibit lower milk-acidifying ability than other LAB.

The ability to degrade proteins is strain and species-dependent. *Enterococcus faecalis* is usually more proteolytic than other species, but, generally speaking, the activity may be comparable to that of other LAB, with one exception. Formerly, *Ec. faecalis* subsp. *liquefaciens* was taxonomically recognized; its name derived from the ability of this organism to liquefy gelatine or milk gels due to the intense proteolytic activity of a ~30 kDa extracellular proteinase. The proteolytic activity of *Enterococcus* spp. has been observed on the most important milk proteins (caseins, α-lactalbumin, β-lactoglobulin, and bovine serum albumin). Certainly, casein hydrolysis is of maximum relevance to cheese flavor. *Enterococcus faecalis* proteinase hydrolyzes gliadin (proteins involved in celiac disease) in both agar medium and liquid-fermenting doughs. In the latter case, gliadin decreases to less than half of its original concentration.

Concerning the lipolytic activity of enterococci, limited and often contradictory data exist. Enterococci may have lipases and/or esterases, but the presence of such enzymes is strain dependent. Generally, *Ec. faecalis* exhibits higher lipolytic activity than *Ec. faecium* and *Ec. durans*, whereas data are contradictory about which species of the three is the most esterolytic, indicating that this trait is probably strain dependent. Investigation of lipolytic and esterolytic activity is usually carried out with synthetic substrates and many strains hydrolyze them; the majority of enterococci hydrolyze all substrates from tributyrin (C4) to tristearin (C18) to a decreasing extent. Concerning esterases, it is very likely that any strain is active on synthetic substrates from C2 (4-nitrophenyl acetate) to C18. However, activity on milk fat is more controversial, and it is doubtful whether enterococci play any role in cheese flavor through their lipolytic and/or esterolytic activity, although it may not be ruled out because some strains show pronounced lipolytic activity.

Enterococci may participate in the aroma and flavor development of foods in other ways. These organisms may synthesize esters, although there is great variability among strains. In general, enterococci may produce esters via esterification (free fatty acids and alcohol) and alcoholysis (reacting triglycerides or partial glycerides and ethanol). Ester-producing strains may be recommended as adjunct cultures for small ruminant dairy products, as they could contribute to the development of fruity flavor notes and possibly aid in the generation of specific or even new flavors.

Enterocin Production

Enterococci produce a wider variety of bacteriocins than produced by other LAB; bacteriocins produced by enterococci are called enterocins. A large number of these antimicrobial substances have been isolated and characterized from *Ec. faecalis* and *Ec. faecium*, and other species from dairy products, fermented sausages, fish, vegetables, silage, water, animals, and humans.

On the basis of structural, physicochemical, and molecular properties, bacteriocins are classified into three classes and several subclasses. According to this classification, the enterocins have been assigned to class I (lantibiotics: i.e., small, cationic, hydrophobic, and heat-resistant peptides, which typically contain unusual amino acids), class IIa (pediocin-like, with strong anti-*Listeria* activity), class IIc (bacteriocins not classified as a or b are included here; being small, cationic, hydrophobic, heat-stable peptides that are not posttranslationally modified, except for cleavage of a leader peptide from the prebacteriocin), and class III (large, hydrophilic, heat-labile proteins). Recently, a new classification scheme has been proposed for enterocins, based mostly on structural differences and amino acid sequences. Accordingly, enterocins would be grouped into class I, lantibiotic enterocins; class II.1, pediocin-like enterocins; class II.2, enterocins synthesized without a leader peptide; class II.3, other linear, non-pediocin-type enterocins; class III, cyclic antibacterial peptidic enterocins; and class IV, large proteins.

The primary target of enterocins, as that of almost all bacteriocins, is the cytoplasmic membrane. They form pores in the cell membrane, depleting the transmembrane potential and the pH gradient, provoking the escape of intracellular contents and cell inactivation. Enterocins are usually active against foodborne pathogens such as *Listeria* spp. and *Clostridium* spp., and also work against Gram-negative pathogens such as *Escherichia coli* and *Vibrio cholerae*, and one enterocin, CRL35, is active even against the herpes viruses.

The genes *entA, entB, entP, entQ, entAS-48, entL50A/B, bac31,* and *cylL* encode enterocins A, B, P, Q, AS-48, L50A/B, bacteriocin 31, and cytolysin L, respectively. These genes may be present alone or in combination in the same strain. One of the most interesting enterocins, from a practical point of view, is AS-48, a cationic and cyclic peptide of 7149 Da, owing to its stability and broad activity spectrum. It inhibits Gram-positive and Gram-negative bacteria, is produced *ex situ* or *in situ* in foods, and has shown inhibitory activity against *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* in skim milk and unripened cheeses.

Enterocins also work actively against *Clostridium tyrobutyricum*. Potentially, this may be used to preserve packaged cheeses where the growth of *Cl. tyrobutyricum* may cause blowing of the packages.

In general, the enterocins and/or the producer strains offer an interesting alternative to be used for biopreservation in ripened foods, such as cheeses and dry fermented sausages, especially those in which *Li. monocytogenes* is the target organism. Although inhibition of this bacterium in a dairy manufacturing plant should be accomplished through conventional hygiene procedures, bacteriocin-producing enterococci could be used as a secondary anti-*Listeria* agent. Furthermore, a number of strains have been shown to produce active enterocins against Gram-positive and Gram-negative bacteria. This fact suggests that these enterococci or their bacteriocins have a promising potential for enhancing food safety.

Probiotic properties

Strains used as probiotics usually belong to the genus *Lactobacillus* or *Bifidobacterium.* However, other species, among them *Ec. faecalis* and *Ec. faecium*, are also used as probiotics not only for humans, but also as veterinary feed supplements. Nine *Ec. faecalis* strains are authorized as additives in feed stuffs by the European Union.

The *Ec. faecalis* strain SF68 was isolated originally in Sweden and patented in Switzerland, where it is produced by F. Hoffmann–La Roche Ltd. Its effectiveness can be attributed to the fact that it is a commensal of the intestine with a short lag phase and a short generation time (20 min). SF68 is resistant to low pH and bile salts, and individuals show high tolerance to it with no side effects. It is clinically effective in the prevention of antibiotic-associated diarrhea and in the treatment of diarrhea in children. This strain has an inhibitory effect *in vitro* on the growth of *E. coli*, *Salmonella*, *Shigella*, and *Enterobacter* species. It has been considered to be an alternative to antibiotics for the treatment of diarrhea, with clinical studies showing statistically significant decreases in the duration of diarrhea in both adults and children. For instance, an acute diarrheic process was shortened in patients treated with SF68 recovering in 1–3 days versus 6 days taken by untreated patients, in a study undertaken in Belgium in the 1990s. SF68 has also been studied as feed probiotic, and it has been observed, when used in dry dog food, to enhance immunity. A hypocholesterolemic effect has been attributed to the SF68 strain, although a long-term study failed to show any cholesterol reduction.

Other enterococci strains have also been tested as probiotics. *Enterococcus faecium* CRL183 in combination with *Lactobacillus jugurti* reduced cholesterol in more than 40% in an *in vitro* study. The consumption of *Ec. faecium* PR88 relieves the symptoms of irritable bowel syndrome in humans. Another property detected in some enterococci is the formation of conjugated linoleic acid, which may be used in dairy products and other foods.

Use of enterococci as probiotics is still controversial. Although the probiotic benefits are well established, the emergence and the increased association with human illness and multiple antibiotic resistances have raised concerns regarding its use as a probiotic. In this regard, Canada has prohibited the use of enterococci as probiotics in 2004. There is a need to ensure the safety of enterococci before using them in food and/or probiotic preparations.

Enterococci and pathogenesis

Enterococci are part of the food microbiota, have been consumed for centuries, and were not considered as hazardous organisms; nevertheless, they have now emerged as a major cause of nosocomial infections worldwide. The concern for the manufacturer of dairy products is whether pathogenic enterococci can be transmitted by foods and cause disease. *Enterococcus faecalis* and *Ec. faecium* have been suspected, but never confirmed, as causative agents of foodborne illnesses. However, enterococci are currently the third most common bacterial pathogen associated with nosocomial infections, after staphylococci and *E. coli*. These organisms have been involved in cases of endocarditis, bacteremia, and infections of the urinary tract and central nervous system, as well as in intra-abdominal and pelvic infections, and also affect burns and surgical wounds. A large outbreak of vancomycin-resistant *Ec. faecium* in a German university hospital during late 2004 and early 2005, with an estimated cost of ~€1 million, is well documented. In the future, this potential may cause these bacteria to be considered undesirable in food. However, the incidence of enterococci resulting in human disease does not correlate with the incidence of these organisms in foods. It is important to note that the pathogenicity of these organisms has not been proven by Koch's postulates; therefore, their involvement in human infection is, at best, circumstantial. There is enough proof about the pathogenicity of enterococci in the scientific literature to be considered seriously. The most clinically relevant strains are *Ec. faecium* clonal cluster 17 (CC17) and *Ec. faecalis* CC2, CC9, and CC40. *Enterococcus faecalis* seems to have a higher pathogenic potential than *Ec. faecium*, as more than 60% of the enterococci associated with human infections have been *Ec. faecalis*. This may be due to *Ec. faecalis* usually being more antibiotic resistant and possessing more genes encoding for virulent traits than *Ec. faecium*. The characteristics of enterococci relevant to its pathogenicity are detailed below.

Antibiotic Resistance

Enterococci are multidrug-resistant organisms possessing resistance, either intrinsic or acquired, to a broad spectrum of antibiotics. Within the intrinsic group, resistance to streptogramins, isoxazolyl penicillins, cephalosporins, monobactams, aminoglycosides, lincosamides, and polymyxins to different extents and in several *Ec. faecalis* strains has been observed. On the other hand, the resistance to ampicillin (mainly *Ec. faecium*), tetracyclines, macrolides, aminoglycosides, chloramphenicol, cotrimoxazole, quinolones, glycopeptides (vancomycins), and streptogramins (not in *Ec. faecalis*) may be acquired.

Resistance to ampicillin, gentamicin, and vancomycin is very important because these three antibiotics are indicated in the treatment of infections caused by multidrug-resistant strains. The increase of nosocomial processes caused by antibiotic-resistant enterococci may be due to the inherent ability of these organisms to acquire resistance genes from other bacteria. In this regard, transfer of resistance genes from enterococci of animal origin to 'human' enterococci has been shown in healthy persons. However, resistance of animal origin enterococci to antimicrobials has decreased after the prohibition of using antibiotics as growth promoters, and nowadays, it is uncommon to find vamcomycin-resistant enterococci in animals.

Antibiotic-resistant enterococci are ubiquitous in foods. They may be found in dairy products as well as in many other foods, and even some strains with probiotic potential present a certain degree of antibiotic resistance. However, and generally speaking, food strains are not resistant to clinically relevant antibiotics. Another concern is the possible role that enterococci may play as a natural food reservoir of antibiotic resistance, especially against vancomycin. It is emphasized that a critical criterion for the use of a strain in foods is to check for the absence of transferable antibiotic resistance. Indeed, concern grows when considering that antibiotic resistance genes may be transferred between microorganisms in foods. In this respect, it has been shown that various transposons may be responsible for the spread of tetracycline resistance in enterococci. This finding supports the hypothesis that *Enterococcus* spp. are sources of antibiotic resistance genes for potentially pathogenic bacteria occurring in the food chain. However, it must be considered that the likelihood of finding antibiotic-sensitive strains in foods is very high. It is quite frequent to detect, among enterococci isolated from foods, only organisms sensitive to clinically relevant antibiotics, although they may present quite a variable resistance to a number of other drugs. According to food safety guidelines, the use of enterococci as starters, as probiotics, or in any other role in food production needs a case-by-case evaluation of each potential technological strain.

Virulence Factors

A virulence factor is a molecule that enhances the ability of a microorganism to cause disease beyond that intrinsic to the species background. However, the presence of virulence factors does not mean that the holder strain is pathogenic. Enterococci may colonize tissues; *Ec. faecalis* and *Ec. faecium* are efficient colonizers of host tissues as they bind readily to specific extracellular matrix proteins, possibly through specific adhesin–ligand interactions as well as hydrophobic ones. A large proportion of enterococcal infections may have their origin in the intestinal tract, as *Ec. faecalis* may go through an intact epithelium and cause systemic infection. Pathological changes associated with enterococci include acute inflammation, with many pathogenic strains producing cytolysin, which induces tissue damage. Some enterococci also produce hyaluronidase, which damages tissues as well.

The properties of *Ec. faecalis* considered as virulence factors are the production of adhesins (aggregate substance, antigen A, the extracellular surface protein, and an adhesin to collagen), proteases (gelatinase and serine proteinase), secreted factors (cytolisin/hemolysin and glucose starvation-induced protein), exopolysaccharides, and five transcriptional regulators. Adhesion and/or colonization are achieved by the aggregation substance, antigen A, surface proteins, and adhesin to collagen. Other substances (proteases, cytolysin, and hyaluronidase) cause tissue damage, whereas aggregation substance, glucose starvation-induced protein, and the capsular exopolysaccharides increase the strain resistance to the host defenses. Virulence factors of *Ec. faecium* have been studied less extensively. This species produces at least two adhesins: Acm (collagen adhesin) and Esp (surface protein), which are involved in biofilm formation and transiently aggravate experimental urinary tract infections. Both *Ec. faecalis* and *Ec. faecium* form biofilms, a key factor for pathogenesis and antibiotic resistance.

Since the 1950s, it has been known that some enterococci strains are β-hemolytic. More recently, researchers have noticed that the hemolysin secreted by *Ec. faecalis* also has a bactericidal activity; that is, it is a bifunctional substance, hemolysin and bacteriocin at the same time, and is called cytolysin. It belongs to type A lantibiotics, and is the only one, among these compounds, able to lyse eukaryotic cells in addition to its bactericidal effect. Cytolysin is encoded by two genes: *cyl*$\mathrm{L_L}$ and *cyl*$\mathrm{L_S}$. Only strains that are able to express and secrete both are hemolytic. These genes are located in a very transmissible pheromone-responsive conjugative plasmid. Pheromones are peptides, chromosomally encoded, that promote conjugative transfer of plasmids between strains. Cytolysin is considered to be a virulence factor in animal models, although the role of this factor in enterococci pathogenesis remains unclear.

Aggregation substance (Agg) is a surface protein found exclusively in *Ec. faecalis*, with a high prevalence among strains isolated from food. The activity of this substance is the mediation in enterococci donor–recipient contact to assist plasmid transfer. It is supposed that this trait contributes to pathogenesis in various ways. Cells expressing Agg form large aggregates, although the relevance of this fact to pathogenesis has gone unstudied.

Gelatinase is an extracellular metalloendopeptidase able to hydrolyze gelatine, collagen, and hemoglobin. Capacity to produce this protease has been considered a virulence factor because proteolytic strains induce caries formation in rats, whereas nonproteolytic strains do not. Furthermore, gelatinase activity has been shown to be a virulence factor for peritonitis in mice. The presence of the gelatinase gene in *Ec. faecalis* isolated from food is common, although gelatinase expression is less frequent.

Expression of virulence genes is influenced by extrinsic factors. For instance, sublethal environmental stress enhances their expression, and serum induces the production of some adhesins. Effects of these and other factors should be considered when selecting enterococci for use in foods, not only their set of virulence genes and the source from which they were isolated. Actually, it seems very unlikely to find no virulence factor genes in *Ec. faecalis* or *Ec. faecium* of any origin. The environmental signals responsible for development of pathogenicity in these species remain unclear. No virulence factors have been described in other enterococcal species.

Commonly, clinical strains possess a higher number of virulence factors than possessed by food strains, although occasionally both have similar traits. It must be considered that development of pathogenesis is affected by environmental signals in certain, and, as yet, not fully understood, ways. This may be one of the reasons for explaining why a food strain may be as hazardous as a clinical strain, in terms of virulence factors.

Biogenic Amine Production

Biogenic amines are commonly found in ripened foods and are associated with allergic response, blood pressure rise, and migraine. There is evidence that the accumulation of biogenic amines, especially putrescine, cadaverine, tryptamine, phenylethylamine, and tyramine, are influenced by the presence of *Enterobacteriaceae* and enterococci in ripened foods such as raw fermented sausages and cheeses.

Enterococcus faecium shows a broader decarboxylating capacity than *Ec. faecalis*. In one study, *Ec. faecium* decarboxylated tyrosine, ornithine, lysine, and histidine, whereas *Ec. faecalis* released only tyramine.

Enterococci in Dairy Products

Unlike most LAB, the genus *Enterococcus* is not considered to be 'generally recognized as safe' (GRAS) and, as it may be deduced from this text, safety assessment of enterococci remains controversial. Although some enterococci are considered useful in cheese technology and used as probiotics, others cause nosocomial processes.

Enterococci are ubiquitous. They are natural inhabitants of many ecosystems, such as milk and cheeses, soil, vegetables, insects, and many raw and processed foods, as well as forming part of the microbiota of the human and animal gastrointestinal tract. The common presence of these bacteria in foods is not related with direct fecal contamination. If milking, milk storing, and transport are conducted under clean conditions, bovine feces is not an important source of enterococci for contamination of raw milk. Study of the microbiota of raw milk has concluded that some aerobic sporeformers and micrococci are isolated only from milking machines, whereas enterococci, coliforms, yeasts, and *Pseudomonas* spp. may also be detected in milk samples. The European Union, considering these facts, does not limit the enterococci number in some foods, adducing that these organisms have little value as hygiene indicators in industrial food processing, in contrast with coliforms and *E. coli*, both of which are used as indicators. There are many studies demonstrating the negligible importance of fecal enterococci for contamination of foods. Another possible source of enterococci contamination of potential relevance is the housefly.

The resistance of enterococci to pasteurization and the very wide set of conditions that allow for their growth imply that enterococci are going to be found in many food products manufactured with raw materials, and/or in those processed under nonsterilizing conditions. In addition, they can contaminate finished products.

Enterococci are a common inhabitant of raw milk and, although the majority is inactivated by pasteurization, some do survive through to the final dairy products. The presence of enterococci is very frequent in cheeses of the Mediterranean area, produced from goat, sheep, cow, or water buffalo milk, either from pasteurized or from raw milk. Likewise, enterococci are also detected in cheeses from other parts of the world, although with a lesser prevalence. Enterococci are often identified as constituents of the indigenous microbiota of raw milk artisanal cheeses and are believed to contribute to the unique organoleptic qualities of these products. Levels of enterococci range from 10^4 to 10^6 cfu g^{-1} in different cheese curds and from 10^5 to 10^7 cfu g^{-1} in fully ripened cheeses. Numbers vary with cheese type, ripening time, and season of production. In some cheeses, enterococci develop to become the predominant microorganisms in the fully ripened product. In such cheeses, when manufactured with raw milk, lactococci usually are the predominant microbiota in the early stages of ripening. It is likely that lactobacilli predominate later, owing to their higher tolerance to acidity. Eventually, enterococci, favored for their general resistance (acidity, relatively low water activity, and high salt concentration) and their adaptability to unfavorable environments, may become the most numerous organisms in a very ripened cheese. In this regard, enterococci have been found to be predominant in fully ripened Manchego, Kefalotyri, Picante de Beira Baxa, Cebreiro, Comté, Domiati, and Kashar cheeses. The growth of these organisms in some varieties is deemed to be desirable and may play a major role in aroma development. In this respect, a positive influence of enterococci strains on cheese production and ripening has been shown in many varieties (Manchego, Armada, Cebreiro, Picante, Majorero, Feta, Teleme, Mozzarella, Monte Veronese, Fontina, Caprino, Serra, Venaco, and Comté). Some strains of enterococci are more proteolytic than other LAB, which may be a crucial factor for cheese ripening and flavor development. Another beneficial property of enterococci is esterase activity, which may produce flavor compounds. In addition, enterococci produce typical flavor substances such as acetaldehyde, acetoin, and diacetyl from citrate metabolism.

Product Spoilage

Enterococci are occasionally related with food spoilage. High levels of these organisms can lead to the deterioration of sensory properties in some cheeses. For instance, levels of enterococci of $\sim 10^5$ cfu g^{-1} were associated with an excessively closed texture, unusual mold tastes, undesirable colors, and appearance of the rind in traditional Spanish blue cheese.

Enterococci as starters or adjuncts

Researchers have understood the possible relevance of the biochemical and technological properties of enterococci in the dairy industry, and the number of reports that claim the use of these organisms as starter cultures or adjuncts has increased in recent years. The investigations have focused mainly on cheeses and, to a lesser extent, on raw fermented sausages. Of course, the selection of strains is a hard task, due to the virulence factors and antibiotic resistance shown by many strains. It is assumed that the bacteriocin-producing *Enterococcus* strains lacking hemolytic activity and vancomycin resistance genes may be safely exploited as starter cultures, cocultures, or probiotic bacteria.

The effect of enterococci as starters or adjuncts has been studied in Cheddar, water buffalo Mozarella, Venaco, Hispanico, Feta, and Cebreiro cheeses. *Enterococcus faecalis* has been used in a greater number of studies than were *Ec. faecium* and *Ec. durans*, even though the British Advisory Committee on Novel Foods and Processes had ruled, in 1996, that *Ec. faecium* strain K77D was acceptable as a starter culture for cheese manufacture. Generally speaking, enterococci used as starter or costarter increase the level of proteolysis. Cheeses manufactured with *Ec. faecalis* show a deeper proteolysis, which may improve the flavor, although, in some cases, an excess of proteolysis may cause defects, as observed in the study of starter for the Cebreiro cheese, in which a moderately proteolytic strain was eventually recommended. These starters may also contribute to the increase of the volatile and long-chain free fatty acids, and acetoin and diacetyl. *Ec. faecalis* and *Ec. durans* have been used as adjunct costarters for the manufacture of Feta cheese, improving the taste, aroma, color, and structure of the fully ripened cheeses. Nevertheless, the generally low acidifying ability of enterococci may be a limitation for their use as a primary starter culture for many cheese varieties, although they may be recommended as a costarter.

On the other hand, use of enterocin-producing strains as a costarter for preserving the hygienic-cheese status has been studied on a pilot scale in different cheeses (Camembert, Taleggio, Manchego, Hispano, Saint Paulin, Feta, and Cheddar). Direct addition of bacteriocins has also been studied, on the same scale, in a model dairy system producing Mozzarella and goat cheese, and other dairy products such as Bryndza, a traditional Slovak dairy product made from sheep milk. Reports on the optimization of enterocin production and growth of enterococci during fermentation are scarce. The applicability of enterocins in cheese production is controversial. Some studies have shown that enterocins are stable throughout the cheese ripening process, and, furthermore, enterocin production is *in situ*, recommending its use as a protective tool against listeria or other target organisms in all phases of cheese ripening; however, even though the stability of enterocins under cheese ripening conditions is a fact *in vitro*, sometimes, the inhibitory activity is not detected in cheeses. It has been suggested, as an explanation, that the heterogeneous cheese microbiota and the proteolytic activity of rennet may inhibit enterocin activity. Another argument is the possible adsorption of the enterocin to the cheese matrix, which would prevent its detection in the laboratory, although enterocin could remain active.

The inhibition of listeria by enterocins is very promising; however, there are limitations to the usefulness of enterocins as antilisterial agents because full suppression of the pathogen is rarely achieved in fermented foods. Finally, it must be considered that there is a lack of information about the functionality of enterocins or producer strains on an industrial scale. Hence, their use in the production of cheese or other dairy products still needs to be studied.

Enumeration, Isolation, and Characterization of Enterococci

Considering the significance of enterococci in foods and medicine, it is obvious that their detection, enumeration, and characterization are important issues. Many culture media and methods are available, although none of them is useful for any requirements.

Enterococci usually need biotin, nicotinate, pantothenate, riboflavin, and pyridoxine for growth, and *Ec. faecium* (but not *Ec. faecalis*) also requires folic acid. If pyruvate is used as the energy source, lipoate is necessary for the growth of many *Ec. faecalis* strains. At least 10 amino acids are essential for growth, and others show an additional stimulatory effect. Single L-amino acids support growth better than casein hydrolysates. As a consequence of these requirements, enterococci hardly grow in synthetic media. Plentiful growth is achieved only on rich complex media, such as brain heart infusion (BHI).

More than 100 selective media have been described for enterococci, although most of them allow the growth of certain streptococci and/or other LAB. There is no ideal selective medium to enumerate and isolate enterococci in foods or other substrates. All present drawbacks in terms of recovery or selectivity. The relative insensitiveness to antibiotics (e.g., tetracyclines, aminoglycosides, sulfonamides, several penicillins, and lincosamides) of these organisms allows formulation of such drugs to improve the selectivity of media. Perhaps the most used media are *Enterococcus* selective agar (SB, after the authors Slanetz and Bartley) and kanamycin aesculin azide (KAA). The latter combines the activity of kanamycin and sodium azide to select the target organisms. To improve selectivity, the incubation temperature may be increased from 35–37 °C to 45 °C, a condition very common when analyzing enterococci in dairy products.

The activity of the most relevant substances included in the selective media may be summarized as follows:

- Sodium azide inhibits enzyme systems (catalase and cytochrome *c* oxidase) in electron transport and avoids the growth of Gram-negative bacteria.

- Thallous acetate is used to inhibit the background microbiota, although its action mechanism remains uncertain.
- Antibiotics such as kanamycin, gentamicin, nalidixic acid, and oxolinic acid may be used in selective media. It is common to combine them with sodium azide.
- Aesculin is hydrolyzed by aesculinase or β-D-glucosidase of enterococci and other bacteria, releasing aesculetin, which reacts with Fe^{3+}, forming a dark brown or black complex.
- Triphenyl tetrazolium chloride may be reduced, depending on the pH, to the insoluble, deep red, formazan. At pH 6.0, *Ec. faealis* appears as red-purple colonies, whereas *Ec. faecium* colonies are weakly rosy, but at pH 7.0 both yield formazan to a similar extent.
- 4-Methylumbelliferyl-β-D-glucoside is hydrolyzed by aesculinase, yielding 4-methylumbelliferone, a blue-green substance fluorescent to UV light.
- 5-Bromo-4-chloro-indolyl-β-D-glucopyranoside, a chromogenic substrate, also allows detection of aesculinase activity. After hydrolysis, bromo-chloro-indigo is formed, and a blue color is developed.

Among others, selective media nowadays proposed and used are bile aesculin azide agar, bromocresol purple azide broth, cephalexin–aztreonam–arabinose agar (in this, *Ec. faecium* can be distinguished from *Ec. faecalis* and *Ec. durans* by its ability to ferment arabinose), citrate azide Tween® carbonate medium (this also distinguishes *Ec. faecalis* from *Ec. faecium* because of the more intense formazan production by *Ec. faecalis* species), Columbia (blood) colistin nalidixic acid agar, fluorogenic gentamicin thallous carbonate agar, thallium acetate medium, oxolinic acid aesculin azide agar, and thallous acetate tetrazolium glucose medium.

Variations of selective media are used to isolate vancomycin-resistant enterococci, which differ with regard to the antibiotic used. For instance, bile-aesculin-azide-agar (BEA or Enterococcosel agar) supplemented with different amounts of vancomycin has been used successfully.

It is obvious that the detection rate of strains in all the above mentioned media is improved by previous enrichment.

Use of enterococci as starters or probiotics creates the need for a method to distinguish between such strains and contaminants, mainly those that may represent a health risk. For this purpose, phenotypic and genotypic methods are necessary. While the conventional phenotypic typing schemes are enough for identification of *Enterococcus* species, others such as polyacrylamide gel electrophoresis, multilocus enzyme electrophoresis, serotyping, opsonophagocytic assay, pyrolysis mass spectrometry, vibrational spectroscopy, chromosomal restriction analysis, plasmid profiles, pulsed-field gel electrophoresis (PFGE), ribotyping, PCR systems, and nucleic acid hybridization may be needed for a deeper characterization. One of the most promising methods to differentiate between clinical and nonclinical strains is the opsonophagocytic assay. The difference in the susceptibility to opsonic killing among the clinical and nonclinical *Enterococcus* isolates is attributed to the presence of a capsule in the former, which protects them against the opsonophagocytic killing. This method may be an important tool for discriminating between safe and hazardous strains, a differentiation not achieved by other modern techniques. For instance, the comparison of PFGE profiles from human and food isolates resulted in clusters of genetically closely related strains. Another method that is able to discriminate between hospital-adapted populations and those found in other environments is the genetic fingerprints determined by LH-PCR.

See also: **Lactic Acid Bacteria**: Taxonomy and Biodiversity. **Mastitis Pathogens**: Environmental Pathogens. **Microorganisms Associated with Milk.**

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Lactic Acid Bacteria in Flavor Development

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Introduction

Lactic acid bacteria (LAB) play a crucial role in the development of flavor (taste, retronasal odor, and orthonasal aroma) of several dairy systems, but because of the time frame over which ripening occurs, aged cheese presents by far the most complex system in terms of both flavor pathways and range of flavor outcomes. Consequently, it is from cheese studies that much of our knowledge of the diversity of flavor pathways of dairy LAB has been derived. However, studies on yogurt have also contributed significantly. The book *Improving the Flavor of Cheese* edited by Weimer (see 'Further Reading') covers flavor formation in cheese extremely well and refers extensively to the role of LAB, while information on LAB-directed flavor formation in yogurt is described within the book *Tamime and Robinson's Yogurt. Science and Technology* (see 'Further Reading'). It is worth noting that enology also provides insights into the diversity of flavor compound formation by LAB, insights that, aspirationally, could be applied to dairy fermentations targeted to the generation of novel all-dairy flavor ingredients, rather than traditional dairy products.

Contextualizing Flavor Generation by LAB

The important LAB genera in dairy fermentations are *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. However, for dairy systems, it must be remembered that the LAB are not solely responsible for generating flavor. Flavors can also be generated in products by using rennet (in cheese) and other added enzymes, indigenous milk enzymes and chemical reactions, added microflora other than LAB, raw milk microflora, or derivative enzymes. The levels of both raw milk flora and their enzymes are dependent on how the milk is handled from milking to holding tanks and on the processing of milk (whether or not this includes pasteurization). Flavor molecules can even be passed on from the feed.

Flavors generated by the LAB can be primed or otherwise influenced not only by these factors but also by processing and ripening parameters such as pH, salt levels, water activity, and temperature. These parameters influence the growth, metabolic state, viability, and rate of lysis of the LAB, and the activity and half-life of enzymes released upon lysis. It is worth noting here that there are some misconceptions and dogma regarding the longevity of both LAB and their enzymes in dairy systems. This is especially true in cheese, where the LAB can undoubtedly influence flavor development long after the ability to culture them from the cheese is lost, and subsequently their enzymes, albeit decompartmentalized from any cofactors on eventual lysis of the bacteria, are likely to be far more stable in the high-solids environment than in *in vitro* aqueous buffer systems. Further influences in cheese can be the presence of other microorganisms, such as surface-ripening microflora, and even the physical dimensions of the cheese and the manner of its packaging. The influences on, and the control points or levers for, flavor generation by LAB in different dairy systems (e.g., cheese vs. yogurt) are therefore very different.

Flavor compounds give rise to both positive attributes and defects, depending on absolute and relative levels and on the product in which they are contained and presented. Ultimately, how flavor is defined depends on how it is perceived by the consumer, and so it is not simply which volatile and nonvolatile compounds are present and their levels and interactions that govern flavor but how the compounds are released (and therefore detected by the mouth and nose) on preparation, presentation, and consumption. In this respect, other factors such as composition and texture have a marked impact on flavor perception, and this is further confounded by the wide variation in how individuals perceive flavor.

It is not the scope of this article to discuss flavor release and perception, or the interactions between LAB and non-LAB flavor compound pathways, or the impact of manufacturing conditions on flavor development, nor is it to delve into the microbial physiology of the flavor pathways. The scope is to provide an overview of the complexity, the range, and the potential of flavor compound production by LAB, highlight the current level of knowledge of the pathways, and focus on how to harness them to a useful end point. A point to note is that there is often no single standard definition of the flavor of a chemical compound (the perceptions of which often change on dilution or are dependent on presentation) and with this article many readers may take issue with descriptors used for the different flavor compounds. In working with flavors, one of the initial hurdles is to agree on a single lexicon between collaborators, customers, and sensory panels.

Diversity of Milk as a Raw Material and of LAB as Flavor Factories

Although milk can be viewed as having four major components (apart from water) (i.e., caseins, whey proteins, lactose, and milk fat), it is *in toto* a very complex biological fluid with multiplicity in protein and fatty acid identities and containing a plethora of 'minor components' (including proteins, peptides, lipids, sugars, oligosaccharides, glycoconjugates, hormones, vitamins, and minerals). Holistically, milk is an extremely variable substrate, the composition of which not only differs from species to species, but is also influenced by a range of factors such as season, feed, stage of lactation, and animal health. Because some dairy flavor compounds have extremely low flavor thresholds (e.g., readily tasted at concentrations less than $10^{-12}\,\mathrm{g\,g^{-1}}$ water), the consequence of such variation in substrate quality on flavor compound production, and therefore the flavor profile of the product, is often enormous. A further complication is that many flavor compounds may well be present in concentrations below their individual taste thresholds, but then act synergistically to deliver a flavor impact detectable by an individual consumer.

Some flavors are labeled as 'characteristic' of dairy products and illustrative tables have been produced that indicate characteristic flavors and 'impact compounds', for example, for different cheeses. However, these can be misleading: flavors as preferred by the consumer reflect a broad, balanced profile with a blend of top, middle, and base notes, rather than single-compound dominance. Furthermore, there is no 'standard' product in any category – what is perceived as a quality product to one market region in the globe can often be rejected by another as being unacceptable. For many reasons, therefore, it is not surprising that the overall goals for the dairy industry – consistency in quality flavor delivery, free from flavor defects, with predictability and targeting to consumer preferences – remain elusive.

Considering cheese as the model, insights into the potential complexity and diversity of flavor development can be gained by considering the range of cheeses available around the globe, whether produced on an artisanal scale or on an (automated) industrial scale. In addition, within a cheese, there can be literally hundreds of flavor compounds. It is the ability to understand this and subsequently manipulate the relative levels and balance of the myriad components that will help realize the full commercial potential of the fermentations.

In products such as yogurt, there is no 'ripening' period and indeed no loss of fermentable substrates (and therefore flavor compound potential) through whey draining. The flavors of the base yogurt matrix (i.e., before the addition of any fruit or other flavorings) essentially depend on a balance of carbonyls delivered by the fermenting LAB. With respect to cheese, however, much discourse on flavor development tends to separate the roles of 'starter' and 'nonstarter' (NS) LAB – the former being those added primarily to generate lactic acid, and the latter being those that either enter the cheesemaking process in an uncontrolled fashion during manufacture (adventitious NSLAB) or are deliberately added to control and direct the ripening process (adjunct NSLAB). However, starter properties are such that their impact on the development of flavor extends well beyond acid production and indeed contribute to the 'priming' of the cheese system in terms of both subsequent rate and direction of flavor development. In this respect, and in terms of taking flavor pathways out of the cheese block and into flavor ingredient fermentations, all LAB, whether starters or adventitious or adjunct nonstarters, can be considered 'finishers'.

Given the complexity of milk as a substrate, flavor production by LAB in dairy systems is, for practical reasons, generally viewed as starting with the breakdown of the predominant carbohydrate, protein, and lipid components of milk. The lactic acid and the various amino and fatty acids that are the predominant products, together with citrate, then become the substrates for further metabolism to many flavor compounds.

Flavor Generation from Carbohydrates by LAB

A good summary of the potential for flavor generation from milk carbohydrates by LAB is contained within the article by Wilkinson and Kilcawley (see 'Further Reading'). Homolactic fermentation of lactose, the main carbohydrate component of milk, gives rise almost exclusively to L-, D-, or DL-isomers of lactic acid (which has a characteristic taste and what consumers often describe as a sour aroma), depending on the LAB, whereas heterolactic fermentation gives both lactic and acetic acids (the latter described as having a sharp, pungent, sour, vinegary odor with a sour, acid taste). When combined with citrate fermentation, this provides the basis for the production of diacetyl (strong butter, caramel), acetoin (sweet, buttery, creamy), acetaldehyde (fruity aroma, pungent, ethereal, green apple, nutty), butane-2,3-diol (fruit, onion), butan-2-one (sharp, butterscotch odor, ether-like, acetone-like, fruity), ethanol (strong alcoholic, medical), formic acid (pungent, vinegar, sour, formyl odors), and CO_2. All these compounds have significant flavor impact in themselves. However, these compounds can also be metabolized further or become involved in chemical reactions to give rise to other intensely flavored compounds. An example of this is the esterification of ethanol with free fatty acids to give a range of ethyl esters, which have variously fruity, sweet, floral, green, and butterscotch odors. Another example is the involvement of pyruvate as an α-keto acid acceptor in amino acid catabolism, which is a major source of flavor compounds in dairy products.

One facet of flavor compound production involving carbohydrate metabolism by LAB that is deleterious in some cheeses (e.g., Swiss), but has positive attributes in others (e.g., Mozzarella) and in other dairy-derived products (e.g., *dulce de leche*) is the Maillard reaction. With galactose-negative LAB, the levels of galactose that accumulate on lactose hydrolysis are such that significant heat-catalyzed browning occurs through the reaction of the carbonyl group of the galactose and the amino group of amino acids. This type of reaction has the potential to yield a wide variety of flavor compounds not catalyzed *per se* by LAB but illustrating that the deliberate choice of particular LAB for certain fermentations can be a lever to (re)direct flavor formation.

Flavor Generation from Amino Acids by LAB

Amino acids are essentially the end points of LAB-catalyzed proteolysis of caseins. Certain peptide intermediates of this proteolysis and the amino acids themselves do have associated flavors, such as sweet, brothy, and bitter, but the intensities of these are generally low. While often described as 'background' flavors in cheese, they can be the dominant flavors in other dairy systems and by their very nature are often the source of undesirable flavor notes and therefore considered as defects, especially bitterness and, to a lesser extent and in certain applications, brothy notes.

The amino acid catabolic pathways of LAB in cheese (and, therefore, utilizing casein protein as substrates) have been well described in the article by Ganesan and Weimer (see 'Further Reading'). Note that whey protein degradation by LAB is far more limited than that of caseins; only a few reports are available and these essentially pertain to lactobacilli and yogurt systems, so the flavor impact would be expected to be minimal. Amino acids are the substrates for a multiplicity of pathways of flavor compound formation that are not fully clarified, yet provide a high proportion of the most intense flavors that are delivered into dairy products by LAB, in conjunction with nonenzymatic reactions. Through these various pathways, amino acids are decarboxylated (to amines and CO_2), transaminated (to new amino acids), deaminated (to α-keto acids and ammonia), and desulfated (to form various sulfur compounds), and further metabolized into a range of short- and branched-chain fatty acids, esters, sulfur compounds, aldehydes, ketones, and lactones that provide a wide range of flavor notes of various intensities. The capability of LAB to metabolize amino acids varies between species and between strains. The known pathways for some of the amino acids have been studied in far more depth than others. Genetic studies suggest the existence of pathways that have not yet been proved active.

The group of amino acids with aliphatic side chains includes glycine and alanine, the branched-chain amino acids valine, leucine, and isoleucine, and proline (sometimes referred to as an imino acid, but it does not contain a C=N double bond). Serine and threonine contain aliphatic hydroxyl side chains. Glycine arises from both protein breakdown and biosynthetically, with 3-phosphoglycerate as a precursor, and its importance in flavor compound generation by LAB lies in single-carbon transfer reactions and its role as a serine precursor (which itself is a precursor of cysteine and methionine). This pathway is induced in lactococci where serine is excreted during carbohydrate exhaustion. Through various precursors, LAB also have the capacity to generate aldehydes and ketones from alanine, which include the important flavor compounds diacetyl and acetaldehyde as well as acetone (solvent, ethereal, apple, pear) and butane-2,3-diol. However, these amino acids are also the precursors for a range of fatty acids such as acetic, propionic (acidic, nutty), butyric (sweaty, butter, cheese, acid, sour-rancid), *iso*-butyric (sour, acidic), 3-methylbutyric (pungent 'Roquefort', rancid, sweaty, putrid), valeric (acidic, sweaty, rancid, sickening, putrid), *iso*-valeric (sweaty, rancid), caproic (sweaty, goat), and lauric (fatty, coconut) acids. In addition, many of these compounds can be further metabolized to give molecules delivering very different flavor sensations: for instance, esters of valeric acid are fruity, whereas 2-methylpropanal (*iso*-butyraldehyde) is described as fresh, sweet, fruity, malty, and chocolate-like and 2-methylbutanal as musty, cocoa, coffee, and nutty.

The aromatic amino acids, phenylalanine, tryptophan, and tyrosine, can be metabolized by LAB to *p*-cresol (phenolic, tarry, smoky, medicinal), indole (animal, fecal, mothball), and skatole (animal, fecal, barny), but also to aromatic acids, aromatic aldehydes, and aromatic alcohols that can provide floral (e.g., rosy) and fruity flavor notes. Benzaldehyde (sharp, bitter almond oil odor, sweet cherry taste) is a well-known example of an aromatic aldehyde.

The basic amino acid arginine can give rise to putrescine (rotten meat), ammonia (sharp, pungent), and CO_2, whereas lysine can be catabolized to fatty acids. The contribution of the third basic amino acid, histidine, to flavor compound generation is yet to be established, although genetic studies have shown its potential to be degraded to glutamate. Glutamate and aspartate have acidic side chains, negatively charged at physiological pH, whereas their amide derivatives, asparagine and glutamine, respectively, are uncharged. Again, depending on the species and strain, these four amino acids can be catabolized by LAB to acetic, propionic, butyric, *iso*-butyric, valeric, *iso*-valeric, and caproic acids.

The two sulfur-containing amino acids, cysteine and methionine, give rise to a wide range of volatile sulfur compounds (VSCs) that have a significant impact on flavor formation and give characteristic notes to some aged cheese products. In cheese, VSC production by LAB is limited to mainly methionine catabolism, as α_{S1}- and β-caseins do not contain cysteine, although there is limited evidence for sulfur fixation. In milk fermentations, the cysteine residues of κ-casein and whey proteins would potentially be available for conversion into VSCs. Because of their importance in cheese flavor, the biochemistry of VSC production has been the subject of much study. In addition, the breakdown product of methionine, methanethiol, undergoes further conversions with a range of fatty acids, aldehydes, and ketones. Although these pathways are not fully elucidated, the genetic potential of LAB is evident.

In terms of flavors derived from sulfur-containing amino acids and their catabolism, cysteine has a sulfury odor, whereas methionine presents an acidic note. Methanethiol presents a decomposing cabbage, garlic odor; methional is described as musty, earthy, cooked potato; and the organic sulfides (dimethyl sulfide, trimethyl sulfide) give sulfury, onion, cabbagy, tomato, green flavor notes. Thioesters (e.g., *S*-methylthiobutyrate) and thiocarbonyls also give sulfury, garlic, putrid cabbage notes, whereas spermine is ammoniacal.

Many of the compounds derived from amino acids have odor descriptors that would imply unpleasant flavors, and indeed, this is the case individually and above certain concentrations. However, it would be more accurate to consider that these compounds reflect the breadth of contributors and the balance of flavors required to provide the overall flavor perception and provide a positive impact both directly and through enhancement of other flavors at low concentrations, but a vulnerability to development of unacceptable 'off-flavors' when an imbalance occurs.

Flavor Generation from Milk Fat by LAB

Generally, LAB are only weakly lipolytic, but in long-term flavor generation in dairy systems such as in an aged cheese, this is not such a limiting technological feature. However, it can be envisaged that this would be a distinct disadvantage where accelerated ripening of cheese is the target or in any other dairy system where rapid generation of lipolytic flavors is desired – in such cases, supplementation with lipases from other sources is indeed required.

The lipolytic activities of LAB do not include true lipase enzymes that act at the oil–water interface; all such activities described so far are due to soluble esterases. The latter not only catalyze the hydrolysis of fatty acid esters, monoacylglycerides, and diacylglycerides (but not intact milk fat – a lipase substrate), but certain esterases also catalyze an alcoholysis reaction to produce esters from (predominantly) mono- and diacylglycerides and ethanol. Therefore, with milk fat as the substrate, these enzymes generate a range of short-, medium-, and long-chain fatty acids that impart rancid, cheesy, pungent, goaty, fatty, soapy, or waxy flavor notes with relatively high flavor perception thresholds (levels of parts per million). However, the flavor perception thresholds of the esters derived from the corresponding free fatty acids (e.g., ethyl butanoate, ethyl hexanoate) tend to be three orders of magnitude lower and impart fruity flavor notes such as apple, banana, pear, pineapple, and strawberry. Such flavors can mask fatty acid flavor defects, but again an application-dependent balance is required, and fruity flavor notes are often perceived as defects in Cheddar cheese, for example. Nevertheless, the studies of Holland and coworkers are an example of how the outcome of a flavor fermentation can be manipulated by supplementation with an otherwise rate-limiting substrate (in this case ethanol), either through direct addition or by selection of 'complementary' LAB.

The Future

There is still much to understand about the biochemistry of LAB with respect to flavor compound formation, and the extent to which fermentation systems can be manipulated to produce flavor profiles of interest. Successful industrialization of product manufacture essentially has consistency of product quality as a prerequisite, and scientific understanding underpins the delivery of this consistency. However, the tolerance limits for variation 'on the supermarket shelf' are low, which means much tighter control and selection of strains used, with a consequent loss of diversity. Fortunately, there are extensive, untapped pools of genetic diversity in LAB that are found within various culture collections and the artisanal dairy industry. Furthermore, the possibilities for coculturing, albeit between LAB and non-LAB organisms, both bacterial and yeasts, or LAB and selected enzymes, are extensive and offer many options to control flavor composition.

In considering manipulation of fermentations, cheese presents some interesting insights, as it is essentially an environment in which the LAB are very much at suboptimal growth conditions and can be considered to be under pH, salt, low temperature, and nutrient starvation stresses. Challenging LAB fermentations in different ways may therefore provide the means to not only gain knowledge of the biochemistry of the flavor pathways but also optimize them for targeted flavor delivery. Use of carbohydrate limitation may be a delivery method that potentiates cultures to provide new flavor compounds found in cheese, but not found in the laboratory. For instance, induction of amino acid metabolism during carbohydrate limitation has been shown to produce branched-chain fatty acids not found in milk fat. Also, metabolism of *Lactobacillus casei* under conditions of lactose starvation, lactose limitation, and nonlimiting lactose (albeit in a semidefined medium, rather than in a dairy stream) results in differences in the identities and relative proportions of both major and minor metabolites produced (although the identities of some components need to be confirmed). Some of the minor components, such as propionic acid (pungent, sour milk, nutty), 3-(methylthio)propionic acid (chocolate, roasted), and γ-hexalactone (herbal, coconut, sweet, coumarin), were ubiquitous to all culture conditions. However, production of other compounds, including heptan-2-one (fruity, spicy, sweet, herbal, coconut, woody), nonan-2-one (fresh, sweet, green, weedy, herbal), nonan-2-ol (waxy, green, creamy, citrus), and *iso*-valeric acid (3-methylbutanoic acid), was not seen in cultures grown with nonlimiting lactose, whereas ethyl lactate (2-hydroxy propionic acid ethyl ester; sharp, tart, fruity, buttery, butterscotch) was identified only under such culture conditions. Furfuryl alcohol (2-furanmethanol; nutty) was identified in cultures containing lactose.

With regard to the potential for increasing the diversity of flavors that can be derived from fermentations of LAB, it is interesting to see that studies on both culture collections and more regional and artisanal products are being carried out, although ultimately more needs to be done to establish the key flavor compounds and identify novel flavor compounds.

Pertinent 'artisanal' examples are the recent studies on Leben, on Pecorino Siciliano cheese, and on a range of dairy and nondairy *Lactococcus lactis* strains. Leben is made by culturing milk with starters and then churning out the butter, leaving a product with characteristics similar to unsweetened yogurt. A broader range of volatiles was detected in the Leben produced with artisanal strains of *Lc. lactis* than in the industrial product. In the study on Leben, it was noted that Tunisian consumers prefer the traditionally manufactured product and consider the industrially produced Leben product to have inferior flavor, but no direct correlation between specific flavor compounds and consumer preference was made. Similarly, in Pecorino Siciliano cheeses made with selected artisanal strains, there was a wider range of volatiles produced from a different profile of strains than in the industrial cheeses.

In the study on *Lc. lactis* strains, aroma compound generation by strains isolated from artisanal raw milk cheeses and industrial starter cultures and nondairy sources was compared. Again, in general, industrial strains presented a lower intensity for aroma descriptors than the other strains, but an aroma potentiation effect was observed with mixtures of strains isolated from different sources.

The last observation touches on the potential that coculturing of LAB can present, whether with other LAB strains or with, for example, non-LAB bacteria or yeasts. In cheese, this has been done empirically for millennia, but the deliberate selection of starter and nonstarter strains for targeted cheese ripening based on biochemical understanding of the flavor pathways is comparatively recent. One specific example of combining lactococcal strains to take advantage of the different properties of strains has demonstrated the formation of high levels of branched-chain amino acid derivatives using a strain characterized by its α-keto acid decarboxylase and one with high branched-chain aminotransferase activity (see 'Further Reading').

In 'crystal-ball gazing' for the future of flavor production in, or using, dairy systems with LAB, the possibilities for genetic manipulation always need to be considered, but several caveats are immediately relevant. Although LAB can be readily considered to be 'flavor factories', and there is potential for those factories to be genetically designed from both homologous and heterologous strategies, two main caveats are consumer acceptance and limitations of milk as a substrate, while the third is application relevance, and the fourth is cost. To illustrate this point, while incorporation of genetic engines for fruit flavor pathways into LAB is possible, present-day consumers may well shy away from any flavors or flavor ingredients thus produced, successful fermentations may require supplementary substrates to be added to the milk (affecting labeling freedom), fruity ingredients based on dairy may have limited application scope, and the cost of the dairy-based ingredients, compared with other flavor sources, may be prohibitive.

From a science perspective, how widely fermentations using LAB with dairy substrates can be diversified in terms of flavor generation is an intriguing question: for instance, the potential to deliver new savory, fishy, and chocolate flavors has been seen. However, from a commercial perspective, cost is the most significant driver, and unless an 'all-dairy' label can attract a premium for a dairy-based flavor ingredient, or unless such ingredients become more cost-effective than flavors from other sources, the future for LAB flavor fermentations lies within dairy systems that already use their technological properties. In this arena, it is consistency, predictability, targeting, and acceleration that are the drivers of technological success.

See also: **Cheese:** Accelerated Cheese Ripening; Biochemistry of Cheese Ripening; Cheese Flavor; Enzyme-Modified Cheese; Low-Fat and Reduced-Fat Cheese; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Overview; Secondary Cultures; Starter Cultures: General Aspects; Starter Cultures: Specific Properties.

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Citrate Fermentation by Lactic Acid Bacteria

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Introduction

Citrate can be used by a range of lactic acid bacteria (LAB) including examples of starter and nonstarter flora. As a result of citrate metabolism by starter bacteria, specific metabolic end products are formed such as acetoin, diacetyl, acetaldehyde, acetate, and carbon dioxide, contributing directly (diacetyl, acetaldehyde, acetate) or indirectly (acetoin) to flavor or to the texture (carbon dioxide) of the dairy product. In this article, all aspects of citrate metabolism by starter bacteria that are relevant for dairy fermentation will be discussed. First, the starter bacteria that can metabolize citrate will be introduced and the growth and environmental conditions favoring this specific metabolism will be discussed. Subsequently, the bacterial properties that are required for citrate metabolism will be described, such as the citrate plasmid encoding for the citrate permease. In addition, the exact metabolic pathways leading to flavor formation will be shown in detail including the strategies that have been employed to increase flavor production. Finally, the possibilities of application of this knowledge to control or improve citrate metabolism and flavor formation will be evaluated.

Citrate in Milk and Dairy Products

Citrate is present in cows' milk at an average concentration of 1700 mg l^{-1}, varying between 900 and 2900 mg l^{-1}. The actual citrate level in cows' milk depends on several factors such as the season of the year, the lactation period of the cow (with maximum levels of up to 5000 mg l^{-1} reached immediately after parturition), the breed of the cow, and the feeding regime of the cow. Milks of various species of mammals contain various amounts of citrate. Some mammals produce milk containing less than 100 mg l^{-1} of citrate, in human milk the citrate content ranges from 200 to 800 mg l^{-1}, in goat milk citrate varies between 400 and 1400 mg l^{-1}, while in the milk of the rabbit the highest citrate content of 3000–4000 mg l^{-1} has been recorded. Citrate in milk originates from the citrate formed in the mammary secretory cell where it is a central intermediate in the citric acid cycle, used for oxidation of fatty acids, acetic acid, and pyruvate. In fermented dairy products where mixed mesophilic starters are used, such as Gouda and Cheddar cheese, butter, and buttermilk, all citrate is metabolized by the starter bacteria. In fermented dairy products such as yogurt and some Italian-type cheeses, where thermophilic LAB are used, such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, all the original citrate in the milk is still present in the yogurt or cheese.

Metabolism of Citrate by LAB

Almost all knowledge of citrate metabolism has been derived from practical experience in dairy fermentations. More than a century ago, the first aroma-producing bacteria were recognized in the ripening of cream. In the decades that followed, these bacteria were identified as either betacocci, later renamed *Leuconostoc* spp., or *Streptococcus* spp., nowadays called *Lactococcus* spp. The latter microorganism was originally designated as a separate species, *Streptococcus diacetylactis*, but was later reclassified as a biological variant within the *Lactococcus lactis* species, leading to the present name of *Lc. lactis* subsp. *lactis* biovar *diacetylactis*. The two groups of aroma bacteria were both discovered to have specific citrate-utilizing abilities. In the late 1950s and early 1960s, the specific enzymes involved in citrate metabolism leading to flavor production were discovered, and in the subsequent decades, research mainly focused on the location and regulation of the genes responsible for citrate utilization and for diacetyl production. Most of this research has been conducted on *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, which will be referred to, in the rest of this article, as citrate-utilizing *Lc. lactis*, for simplicity.

Citrate Transport and Energetics of Citrate Metabolism

The essential role of the permease in citrate metabolism is evident not only from the strict coupling of citrate metabolism to the presence of a plasmid encoding the citrate permease, but also from the narrow pH dependence of citrate metabolism. This pH dependence is completely determined by the pH optimum of the permease, which is between pH 5.0 and 6.0. The practical implication of this pH optimum is that citrate utilization occurs only in slightly acidified fermented milk. This means that during, for instance, cheese manufacture, citrate utilization will start only after considerable acidification has already taken place. In practice, this means that a large part of the citrate metabolism actually occurs in the already-made cheese, and that end products of citrate metabolism, such as carbon dioxide, acetoin, and diacetyl, are, for a large part, included in the final cheese.

The citrate permease translocates a citrate molecule in symport with a proton. Thus, the process is driven by the electrochemical proton gradient, the proton motive force. Several observations on the energetics of citrate metabolism, such as the observed growth on citrate and the relative high rates of citrate transport in readily metabolizing *Lc. lactis* cells, have revealed the occurrence of an additional transport reaction by the citrate permease. The permease can also act as an exchanger between a citrate molecule on the outside and a lactate molecule in the cytoplasm of the lactococcal cell (**Figure 1**). The energetic consequence of this reaction is that not only is the abundant end product of fermentation efficiently excreted, but also in the process the gradient for lactate is utilized as a driving force for the uptake of citrate.

Furthermore, by exchanging a bi- or trivalent anion with two or three negative charges with a single charged anion, a net membrane potential is generated, which can serve as a driving force for other processes like ATP synthesis and uptake of essential nutrients. In *Leuconostoc* spp., stimulation of growth has also been observed as a result of citrate metabolism. Since the citrate permease is almost identical in these organisms as in *Lactococcus*, it can be presumed that energy generation occurs in a similar way in these LAB. However, an additional energy-generating route has been described as well. The heterofermentative *Leuconostoc* spp. produce one lactate, one C_2-component, usually ethanol, and one carbon dioxide from the breakdown of a hexose molecule. In the presence of citrate, production of ethanol is replaced by production of acetate as a result of citrate serving as an electron acceptor. Acetate production results in energy conservation in the form of ATP via the acetate kinase reaction. So, more energy is produced during sugar fermentation, resulting in higher growth yield and more rapid growth of *Leuconostoc* spp.

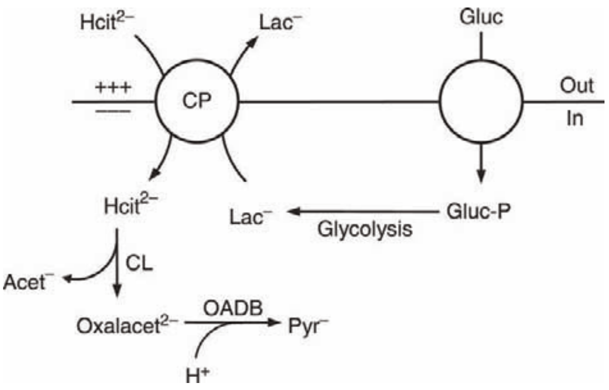

Figure 1 Proposed mechanism of chemiosmotic energy generation by exchange of citrate and lactate through the citrate permease. CP, citrate permease; CL, citrate lyase; OADB, oxaloacetate decarboxylase.

Metabolic Pathways

Once citrate has been taken up by the LAB, it is converted, in two steps, to pyruvate (**Figure 2** (**a**)). The initial step is the cleavage of citrate into oxaloacetate and acetate, catalyzed by the enzyme citrate lyase. This is the reverse of the reaction occurring in the citric acid cycle (Krebs cycle), as present in most aerobic living cells, where, ultimately, acetate is oxidized to carbon dioxide via the intermediate citrate. The cleavage of citrate is catalyzed by citrate lyase, and the presence of this enzyme is limited to the citrate-utilizing LAB. It seems to be chromosomally encoded and while early work indicated that it was constitutively expressed in *Lc. lactis*, more recent data have demonstrated that the genes of the citrate lyase complex are induced under acidic growth conditions. In *Leuconostoc* spp., the enzyme activity is induced by the presence of citrate as described below. One of the products of citrate cleavage, oxaloacetate, is further decarboxylated to pyruvate. This decarboxylation is catalyzed by the enzyme oxaloacetate decarboxylase. This enzyme does not seem to be unique to citrate-utilizing LAB, since in *Lc. lactis* the enzyme could clearly be identified, although at lower activity than in the citrate-utilizing *Lc. lactis*. The enzyme does not appear to be involved in energy generation as the Na^{+}-pumping and membrane-associated enzyme in other bacteria such as *Klebsiella aerogenes*; however, mutations to the oxaloacetate decarboxylase gene perturbed citrate metabolism and reduced the benefits of its utilization during growth under acidic conditions. The pyruvate, thus formed from citrate breakdown, is subsequently metabolized in different ways by the homofermentative LAB (*Lc. lactis* and *Lb. plantarum*; **Figure 2**(**a**)) and heterofermentative *Leuconostoc* spp. (**Figure 2**(**b**)).

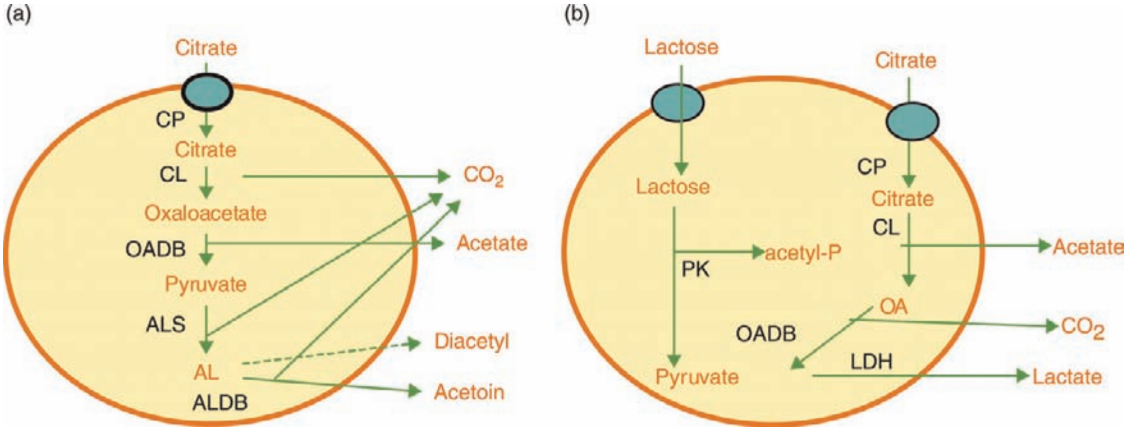

Figure 2 Citrate metabolism in (a) *Lactococcus lactis* and (b) *Leuconostoc* spp. CP, citrate permease; CL, citrate lyase; OADB, oxaloacetate decarboxylase; ALS, acetolactate synthase; ALDB, α-acetolactate decarboxylase; PK, phosphoketolase; OA, oxaloacetate; LDH, lactate dehydrogenase.

The homofermentative LAB possess a specific metabolic pathway for dealing with accumulating pyruvate. When, stoichiometrically, less NADH is generated than pyruvate, as is the case during citrate metabolism, pyruvate is converted directly to the C_5-component, α-acetolactate. This product is formed by a reaction involving two pyruvate molecules, catalyzed by the enzyme α-acetolactate synthase (ALS). This enzyme reaction is dependent on the presence of the cofactor thiamine pyrophosphate (TPP) and involves the formation of the intermediate acetaldehyde-TPP, a decarboxylation, and the reaction of acetaldehyde-TPP with another pyruvate molecule, ultimately leading to α-acetolactate formation. All these reactions are catalyzed by this one enzyme. α-Acetolactate is an unstable metabolic intermediate and is, usually, enzymatically or chemically decarboxylated yielding a final metabolic product acetoin. Thus, in homofermentative LAB, two molecules of citrate are converted into one molecule of acetoin, two molecules of acetate, and four molecules of carbon dioxide: 2 citrate $\rightarrow$ 1 acetoin + 2 acetate + 4 CO_2. For one thing, this illustrates the importance of citrate metabolism in gas formation in fermented dairy products such as Gouda cheese and buttermilk. Heterofermentative LAB, in general, follow a different pathway for conversion of pyruvate. These bacteria tend to reduce the pyruvate to lactate, although no NADH is produced in the breakdown of citrate. The extra NADH is generated by the production of acetate, instead of the usual ethanol, in the heterofermentative sugar conversion. Thus, citrate conversion will always occur simultaneously, in cofermentation with sugar (lactose). In the complete absence of lactose, as in, for instance, a ripening cheese, the pyruvate originating from citrate breakdown will also be converted, slowly, to α-acetolactate and, subsequently to acetoin. In the heterofermentative *Leuconostoc*, this process occurs much more slowly than in the homofermentative *Lactococcus*.

Genetics of Citrate Metabolism

In the early 1950s, it was discovered that citrate utilization was an unstable trait in *Lactococcus* spp. It was shown that the loss of the citrate-utilizing ability was a direct result of the loss of the ability to transport citrate. The first genetic work on LAB in the 1970s demonstrated that the basis for the instability was the presence of a citrate plasmid, coding for the citrate permease. Later genetic work demonstrated also that the enzyme citrate lyase is uniquely present in citrate-utilizing lactococci, but that the genes coding for this enzyme are not located on a plasmid.

More recent genetic work has concentrated on the regulation of citrate fermentation in both *Lactococcus* spp. and *Leuconostoc* spp. In *Lactococcus* spp., there are strong indications that expression of the *citP*, coding for the citrate permease, is transcriptionally regulated by the external pH and subjected to posttranscriptional regulation by specific cleavages of a complex RNA structure including the *citQRP* operon. While early research suggested that expression of citrate lyase and that of other chromosomally encoded enzymes involved in citrate fermentation were not regulated in *Lactococcus* spp., recent data have demonstrated that at least for *Lc. lactis* CRL264, the genes are induced at the level of transcription by acidic conditions.

In *Leuconostoc* spp., the gene coding for citrate transport, *citP*, is almost identical (99% identity in DNA sequence) to the gene from *Lactococcus* spp. and is also encoded on a plasmid in *Leuconostoc mesenteroides*. Also, a regulatory gene, *citR*, showing homology with the *Lactococcus citR*, has been identified in this microorganism although its function in regulation has not been confirmed. In *Leuconostoc paramesenteroides*, all the genes involved in citrate metabolism, including *citP*, are on a plasmid-located operon (**Figure 3**). Expression of this operon is induced by the presence of citrate. Apparently, the citrate operon

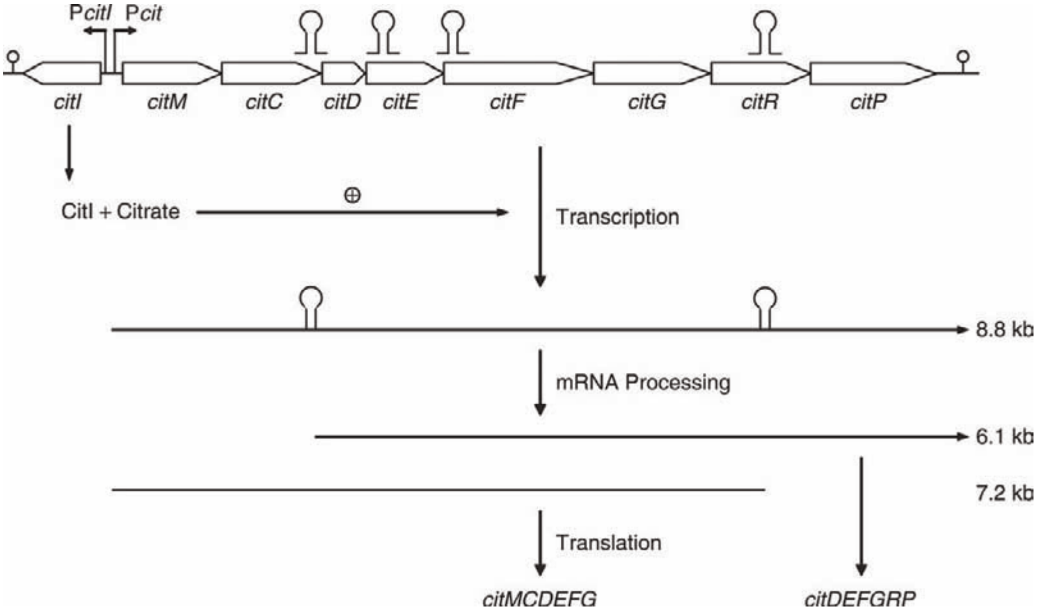

Figure 3 Schematic representation of the *citMCDEFGRP* operon in *Leuconostoc paramesenteroides*. The largest stem–loop structures include the processing sites of the specific *cit* mRNA synthesized. In the mRNA, only the major processing sites are depicted. The symbol '(+)' indicates induction. P*cit*, promoter of the citrate operon; P*citI*, promoter of the *citI* gene.

containing the genes encoding for citrate lyase (*citDEF*), a citrate lyase ligase (*citC*), a malic enzyme (*citM*), and citrate permease (*citP*) and some genes with unknown function including a possible regulator (*citR*) is activated, at the level of transcription, via a regulatory protein CitI, encoded by the *citI* gene. This is a completely different mode of regulation from that found in *Lactococcus*. The functional implication could be that in *Lactococcus* spp. citrate metabolism is turned on only at low pH to serve as a pH-controlling mechanism, while in *Leuconostoc* spp. citrate metabolism is turned on whenever citrate is present to maximize the growth rate of this lactic acid bacterium.

Flavor Formation by Citrate Fermentation

Diacetyl and Acetoin

A side reaction that occurs during the production of acetoin from citrate is the formation of diacetyl, instead of acetoin. The unstable metabolic intermediate α-acetolactate can be decarboxylated spontaneously to acetoin, as already mentioned, but also to the butter aroma compound diacetyl, in the presence of molecular oxygen. This is the actual process resulting in the formation of butter flavor by the citrate-utilizing aroma bacteria in (aerobic) dairy products such as buttermilk and butter. Earlier claims that these aroma bacteria contain an enzyme, diacetyl synthase, that catalyzes the direct synthesis of diacetyl from metabolic intermediates such as acetyl-CoA have never been substantiated. Specific citrate-utilizing *Lc. lactis* strains have been isolated from dairy cultures producing relatively high levels of diacetyl during milk fermentation. Detailed analyses of these diacetyl-producing strains have shown that the strains actually accumulate large amounts of α-acetolactate during citrate metabolism as a result of the absence of the enzyme acetolactate decarboxylase (ALDB). In dairy fermentation, these mutants are responsible for the production of relatively high levels of diacetyl, especially in butter and buttermilk, where aerobic and acidic conditions together form the ideal situation for oxidative decarboxylation of α-acetolactate to diacetyl.

Acetoin is a compound that, in contrast to diacetyl, has no flavor on its own. It is one of the major end products of citrate fermentation and can be found at levels of 500 mg kg^{-1} or more in (fresh) cheese and at 450 mg kg^{-1} (approximately 5 mmol l^{-1}) in fermented buttermilk. Although there is no direct evidence linking the presence of acetoin to flavor formation, it can be presumed that acetoin will react with the amine and thiol groups of the free amino acids (and their degradation products) that are gradually produced during the ripening process of cheese. Indeed, there are numerous observations in the practice of cheese manufacture indicating that citrate-utilizing starter strains clearly indicating contribute to flavor generation in cheese while diacetyl production is not taking place.

Acetate and Acetaldehyde

Quantitatively, acetate is the major end product of citrate metabolism. From every molecule of citrate converted, one molecule of acetate is formed through direct cleavage. During milk fermentation, approximately 10 mmol l^{-1} (600 mg kg^{-1}) acetate is formed in cheese or in fermented buttermilk. Owing to the relatively high pK_a of acetic acid (4.8), a relatively large part of this acetate will be present in the undissociated form (acetic acid) with its typical vinegar odor. This is specifically the case with fermented milks such as buttermilk, with a pH of 4.6, and also in (fresh) cheeses with a relatively low pH such as Cottage cheese.

Acetaldehyde formation is also associated with citrate fermentation. This compound is a very potent aroma compound and is extremely volatile, giving it a prominent presence in flavor perception of fermented dairy products. As with diacetyl, levels as low as a single ppm can have a pronounced effect on flavor perception in cheese and yogurt. In cheese, ‘green’ flavor is associated with the formation of acetaldehyde and is reported to be a direct consequence of citrate fermentation by *Lc. lactis*. The metabolic reactions leading to acetaldehyde formation from citrate remain unclear. A theoretical possibility is the formation of (traces of) acetaldehyde during the formation of α-acetolactate from pyruvate, catalyzed by ALS. As mentioned above, in this reaction, an enzyme-bound metabolic intermediate is formed, acetaldehyde-TPP, and it is possible that some free acetaldehyde is released in the process. Studies with purified ALS from *Lc. lactis* have not given any indication of the existence of this side reaction, but this enzyme, like all other enzymes, could behave differently *in vivo*.

Engineering of Flavor Formation

As mentioned above, diacetyl is formed as a by-product during citrate fermentation by the citrate-utilizing LAB. It is produced by oxidative decarboxylation of the metabolic intermediate α-acetolactate. In specific butter and buttermilk starters, strains of *Lc. lactis* have been identified that accumulate high amounts of α-acetolactate and, thus, are responsible for the formation of diacetyl during milk fermentation. These strains lack the enzyme ALDB. Subsequently, new selection methods and engineering strategies have been developed to select for, or develop, mutations or complete disruptions of the *aldB* gene coding for this enzyme. Many new strains with the ability to form diacetyl from citrate are now available.

Based on the knowledge of the pathways involved in diacetyl production, several other metabolic engineering strategies have been designed to improve diacetyl production by LAB. Since citric acid is only a minor component in milk, much effort has been directed at converting the milk sugar, lactose, into diacetyl. Studies on the overproduction of ALS (coded by the *als* or *ilvBN* genes), inactivation of lactate dehydrogenase (LDH), pyruvate-formate lyase (PFL), or ALDB, or a combination of these strategies have yielded very low levels of diacetyl production but efficient conversion of lactose and glucose into acetoin, especially when LDH is inactivated.

However, attempts to combine both LDH and ALDB inactivation in order to maximize rerouting toward α-acetolactate and diacetyl have so far been unsuccessful. Recent studies have demonstrated that overproduction of the *Streptococcus mutans* NADH oxidase (NOX) in *Lc. lactis* resulted in a phenotype similar to that of the LDH-deficient strain described earlier. In aerated cultures of *Lc. lactis*, more than 80% of the fermented sugar (glucose) was converted into acetoin. As the most recent development in metabolic engineering, the combination of NOX overproduction with ALDB inactivation in *Lc. lactis* has recently been described. Under aerobic conditions, the engineered strain can convert glucose into diacetyl far more efficiently than any recombinant strain described to date. NOX overproduction in the presence of oxygen results in a large reduction in the intracellular pool of NADH + (O_2 + NADH → H_2O + NAD^+). Consequently, the pyruvate pool is rerouted toward NADH-independent pathways (ALS, ALDB, phosphotransacetylase, acetate kinase) and through the NAD^+-dependent pyruvate dehydrogenase complex (PDHC) (**Figure 4**), resulting in a mixture of acetoin and acetate.

The combination of NOX overproduction with ALDB inactivation resulted in high production of the metabolic intermediate α-acetolactate and, ultimately, after chemical oxidation/decarboxylation, in high production of diacetyl. By preventing the reduction of diacetyl to acetoin, catalyzed by the diacetyl reductase enzyme, an even higher efficiency of diacetyl production should be possible.

Application Aspects

Starter Composition

Citrate fermentation, leading to flavor (diacetyl and acetaldehyde) and gas production, is an essential part of manufacture of dairy products such as butter, buttermilk, and Gouda cheese. It is essential that the starters used in these dairy fermentations have the required activity for performing the citrate conversion. In the production of these starters, this is routinely checked by using bacterial enumeration techniques that are specific for citrate-utilizing LAB. These techniques, based on the plating of diluted bacterial samples on solid growth media that are specific for citrate utilizers, can even be used for differentiating between *Leuconostoc* and *Lactococcus* by adjusting the growth temperature (25 °C for *Leuconostoc* and *Lactococcus*, 37 °C for only *Lactococcus*) or the medium composition (addition of Mn^{2+} for detection of *Leuconostoc*). One of these specific plating techniques, whey permeate agar with calcium lactate and casiton (WACCA), is based on the clearing of the calcium citrate precipitation as a result of citrate consumption. Individual cells that can convert

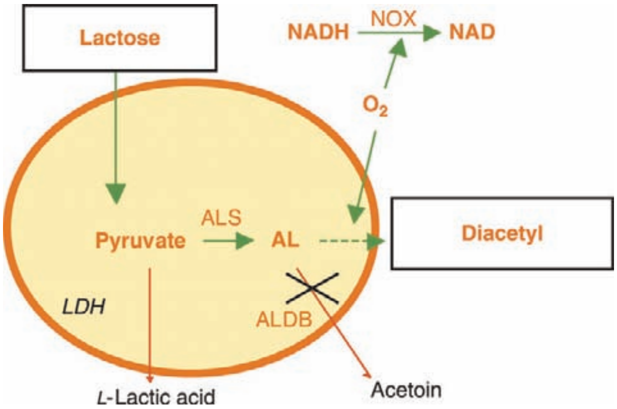

Figure 4 Metabolic engineering of diacetyl production from lactose in *Lactococcus lactis*. The chemical oxidative decarboxylation of α-acetolactate into diacetyl is displayed by a dotted arrow. ALDB, α-acetolactate decarboxylase; AL, α-acetolactate; ALS, α-acetolactate synthase; NOX, NADH oxidase; LDH, lactase dehydrogenase.

citrate develop colonies and utilize all the citrate in the vicinity leading to the formation of clearing zones.

The Dutch dairy industry traditionally uses complex mixed starter cultures for the production of butter, buttermilk, and Gouda cheese. A major part of these starter cultures consists of *Lc. lactis* subsp. *cremoris* strains, which are not able to use citrate. A small part of the culture consists of the so-called aroma bacteria, which are either citrate-utilizing *Lc. lactis* strains (subsp. *lactis* biovar *diacetylactis*) or *Leuconostoc* strains. The starter and cheese industry even uses specific categories L, D, or LD for identifying the nature of the aroma bacteria present in the starter (L for *Leuconostoc* and D for *diacetylactis*). Interestingly, the number of citrate utilizers seems to remain constant in these complex mixtures and varies between 10 and 20% of the total culture (**Table 1**). This stability is the result of the special niche that is occupied by the aroma bacteria. The presence of citrate ensures growth of these bacteria to fixed numbers, while in the absence of citrate they would be overgrown by the non-citrate-utilizing *Lactococcus* strains. The composition of the aromabacteria (L, D, or LD), however, seems to be affected by the continuous competition for citrate. Stable LD starters are rare and they tend to become either L or D starters in time. This can be either a result of the more rapid growth (and citrate utilization) of *Lactococcus* spp. compared to *Leuconostoc* spp. or a result of specific inhibition of citrate-utilizing *Lc. lactis* strains by *Leuconostoc* spp.

Cheese Manufacture

These complicated interactions between aroma bacteria make it difficult, in cheese manufacture, to stimulate citrate fermentation by changing the culture composition. Increased gas or flavor production by increasing the number of aroma bacteria is only possible for carefully selected combinations of strains and starters. Other strategies to increase the amount or the rate of citrate conversion by the aroma bacteria are addition of extra citrate or lowering the initial pH of milk. Increasing the initial citrate concentration in milk, indeed, leads to more gas formation in cheese, but many other crucial properties of the cheese are negatively affected by citrate addition. Additional citrate increases the buffering capacity of milk, leading to decreased acidification rates and lower moisture retention in the cheese. In addition, in its activity as a metal chelator, extra citrate will withdraw Ca^{2+} ions from the (cheese) milk preventing or inhibiting the normal, and essential, process of casein coagulation. Thus, the overall improvement in gas production leading to eye formation

Table 1 Distribution of aromabacteria in some Dutch mixed strain starters

Starter name	*Dairy product*	*Starter type*	Lactococcus lactis *biovar diacetylactis* *(%)*	Leuconostoc *spp.* *(%)*
4/25	Butter	D	9	0
BOS	Cheese	L/D	20	7
A	Butter, buttermilk	L/D	4	9
BK2	Cheese	L/D	2	15
Ur	Cheese	L(/D)	0.000 1	26
Fr18	Cheese	L	0	11
Fr19	Cheese, buttermilk	L	0	10

is completely counteracted by the changes in moisture content and composition of the curd.

Butter Production

For the production of butter and buttermilk, it is essential that diacetyl is formed during the citrate fermentation. This can be achieved by using specific aromatic starters containing ALDB-negative mutants, as described above, and/or by introducing oxygen in the manufacturing process to ensure oxidation of the intermediate α-acetolactate to diacetyl. Even with these measures, it is difficult to control the final diacetyl levels in these fermentations, especially in the traditional production of ripened-cream butter. For this reason, an alternative butter production process has been developed, often referred to as the NIZO butter process. In this split-stream process, the butter flavor, diacetyl, is generated in a separate fermentation process and added, as an aromatic starter, to the ready-made, sweet-cream butter. The aromatic starter contains high numbers of ALDB-negative mutants and intensive aeration is used during the fermentation to ensure maximal diacetyl content. The butter factories using this NIZO butter process have much better control over their butter production and the average quality of butter, indicated by the diacetyl level, has increased significantly since introduction of this alternative butter process.

See also: **Butter and Other Milk Fat Products**: The Product and Its Manufacture; Properties and Analysis. **Cheese**: Cheddar-Type Cheeses; Dutch-Type Cheeses. **Fermented Milks**: Buttermilk. **Lactic Acid Bacteria**: Lactic Acid Bacteria in Flavor Development; *Leuconostoc* spp.

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LACTOSE AND OLIGOSACCHARIDES

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Lactose: Production, Applications
Lactose: Derivatives
Lactose: Galacto-Oligosaccharides
Maillard Reaction
Lactose Intolerance
Indigenous Oligosaccharides in Milk

Lactose: Chemistry, Properties

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Introduction

Lactose, a reducing disaccharide, composed of galactose and glucose linked by a $\beta 1 \rightarrow 4$ glycosidic bond (**Figure 1**), is the principal carbohydrate in the milk of most mammals; its concentration ranges from 0 to ~10% (w/w), and milk is the only known significant source of lactose. The concentration of lactose in milk is inversely related to the concentration of lipids (**Figure 2**) and to the concentration of casein (**Figure 3**). The principal function of lactose and lipids in milk is as sources of energy; since lipids are ~2.2 times more energy-dense than lactose, when a highly caloric milk is required, for example, by animals in a cold environment (marine mammals or polar bears), this is achieved by increasing the fat content of the milk. The inverse relationship between the concentrations of lactose and casein reflects the fact that the synthesis of lactose draws water into the Golgi vesicles, thereby diluting the concentration of casein. Research on lactose commenced with the work of Carl Scheele about 1780; its chemistry and physicochemical properties have been described thoroughly. The objective of this article is to provide a general overview of the chemistry and physicochemical properties of lactose. Some other aspects of lactose are described in **Lactose and Oligosaccharides**: Lactose Intolerance; Lactose: Crystallization; Lactose: Derivatives; Lactose: Production, Applications; Maillard Reactions.

Lactose is synthesized in the epithelial mammary cells from two molecules of glucose absorbed from the blood. One molecule of glucose is converted (epimerized) to galactose via the Leloir pathway, which is widespread in animal tissues and bacterial cells. The galactose is phosphorylated and condensed with a second molecule of glucose through the action of a unique two-component enzyme, lactose synthase. One component is UDP-galactosyl transferase (EC 2.4.1.22), which transfers galactose from UDP-galactose to any of several acceptor molecules in the biosynthesis of glycoproteins and glycolipids. The specificity of the transferase is controlled and modified by one of the principal milk proteins, α-lactalbumin (α-La), which reduces the K_M for glucose 1000-fold, and in its presence, most of the galactose is transferred to glucose, with the synthesis of lactose. There is a positive correlation between the concentrations of lactose and α-La in milk; the milk of the California sea lion and the hooded seal is devoid of lactose and α-La.

The concentrations of lactose in mature bovine, buffalo, ovine, and caprine milk are about 4.8. 4.8, 4.6, and 4.1% (w/w), respectively; the concentration increases slightly during the early stages of lactation, but then it decreases to about 70% of the maximum value at the end of lactation. In contrast, the lactose content of the milk of the equidae (horse, donkey, and zebra), increases during lactation, reaching values in the range of 6.0–7.4% (w/w), with considerable interindividual variation. Human milk contains ~7.5% (w/w) lactose.

Milk contains several sugars in addition to lactose, generally at low concentrations. These include glucose

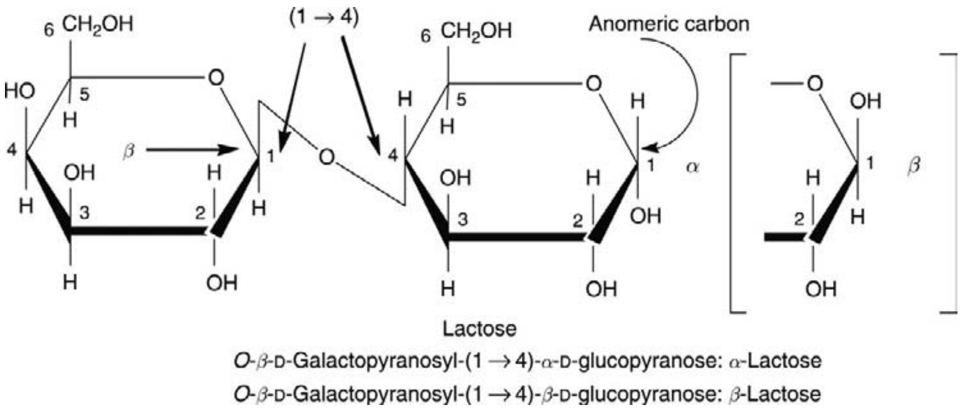
6 CH2OH
HO
5
H
4
OH
H
3
H
β
(1 → 4)
1
H
2
H
OH
O
O
4
6 CH2OH
Anomeric carbon
H
5
H
OH
3
H
H
O
H
1
α
2
OH
OH
—O
OH
1
β
H
2
H
OH
Lactose
O-β-D-Galactopyranosyl-(1 → 4)-α-D-glucopyranose: α-Lactose
O-β-D-Galactopyranosyl-(1 → 4)-β-D-glucopyranose: β-Lactose

Figure 1 Structural formula of α- and β-lactose.

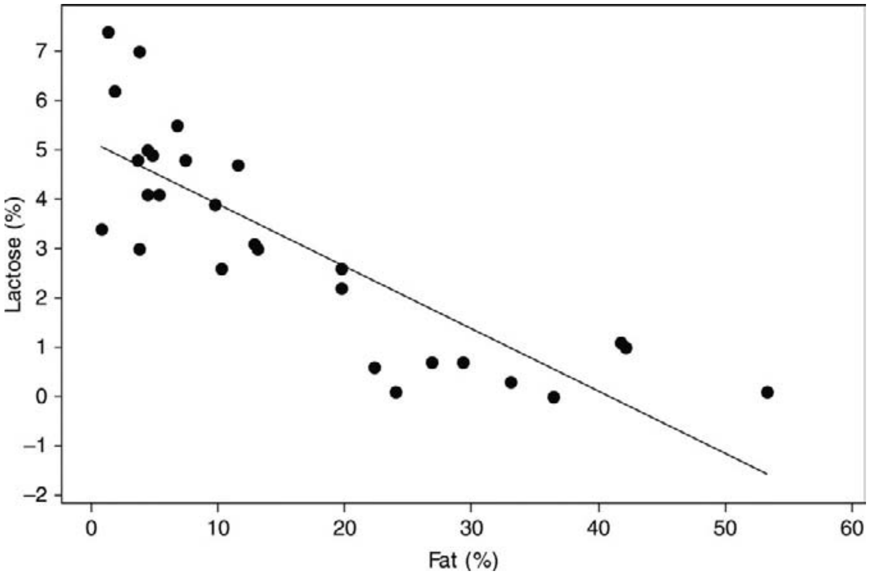

Figure 2 Correlation between fat and lactose in the milk of 23 species. Redrawn from Jenness R and Sloan RE (1970) The composition of milk of various species: A review. *Dairy Sciences Abstract* 32: 599–612.

(~50 mg l^{-1} in bovine milk) and *N*-acetylglucosamine (bifidus factor I, which stimulates the growth of *Bifidobacterium bifidum* and is present at quite a high level in human milk). The milk of most, probably all, species contains oligosaccharides, containing 3–10 monosaccharides, which may be linear or branched. Lactose occupies the reducing end of the oligosaccharides and most contain fucose and/or *N*-acetylneuraminic acid. Human milk contains ~130 oligosaccharides at a total concentration of ~15 g l^{-1}; the milk of marsupials, bears, and elephants also contains high levels of oligosaccharides, which are believed to play several important functions, including brain development and bactericidal activity; they are indigestible, at least by humans, and thus affect the intestinal microflora. The oligosaccharides in milk and their significance are discussed in **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk.

Lactose serves two important functions in milk: it is a ready source of energy for the neonate and is responsible for about 50% of the osmotic pressure of milk, which is isotonic with blood and hence is essentially constant. The synthesis of lactose draws water osmotically into the Golgi vesicles and hence affects the volume of milk and the concentration of casein, which is packaged in the Golgi vesicles, in milk, and is inversely correlated with the concentrations of lactose and casein in milk (**Figure 3**).

For milk with a low level of lactose, the concentration of inorganic salts is high to maintain the osmotic pressure at the desired level. There is a strong inverse relationship between the concentration of lactose and the osmolarity of milk (**Figure 4**), but for the milk of individual species,

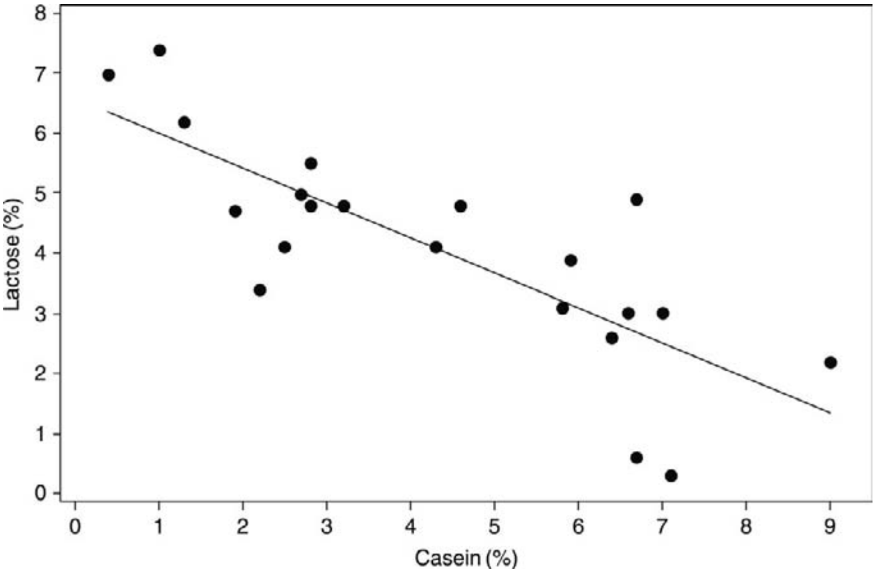

Figure 3 Correlation between lactose and casein in the milk of 23 species. Redrawn from Jenness R and Sloan RE (1970) The composition of milk of various species: A review. *Dairy Sciences Abstract* 32: 599–612.

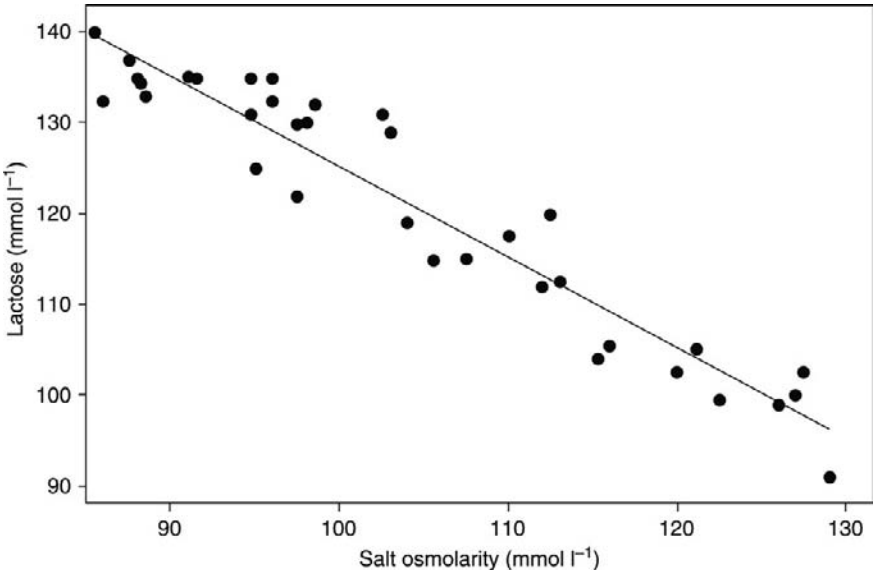

Figure 4 Relationship between concentration of lactose (mmol l^{-1}) and osmolarity (mmol l^{-1}) due to salts. Redrawn from the data of Holt C (1985) The milk salts: Their secretion, concentrations and physical chemistry. In: Fox PF (ed.) *Developments in Dairy Chemistry, Vol. 3: Lactose and Minor Constituents*, pp. 143–181. London: Elsevier Applied Science Publishers.

the inverse relationship between the concentrations of lactose and of ash is weak (**Figure 5**), probably because much of the ash arises from colloidal salts. During mastitis or in late lactation, the integrity of the mammocyte cell membranes is damaged and there is an influx of blood constituents into milk; consequently, the osmotic pressure increases, and to adjust this, the concentration of lactose is reduced. This relationship is expressed as the Koesler number (KN):

$$\mathrm{KN} = \frac{\%\mathrm{chloride}}{\%\mathrm{lactose}} \times 100$$

which is a diagnostic indicator of mastitis (normally, milk has a KN <2 and a value >3 is considered abnormal). Since the development of rapid methods for the enumeration of somatic cells in milk, the KN is rarely used as a diagnostic test for mastitis; however, the electrical conductivity of milk, which depends mainly on the milk salts

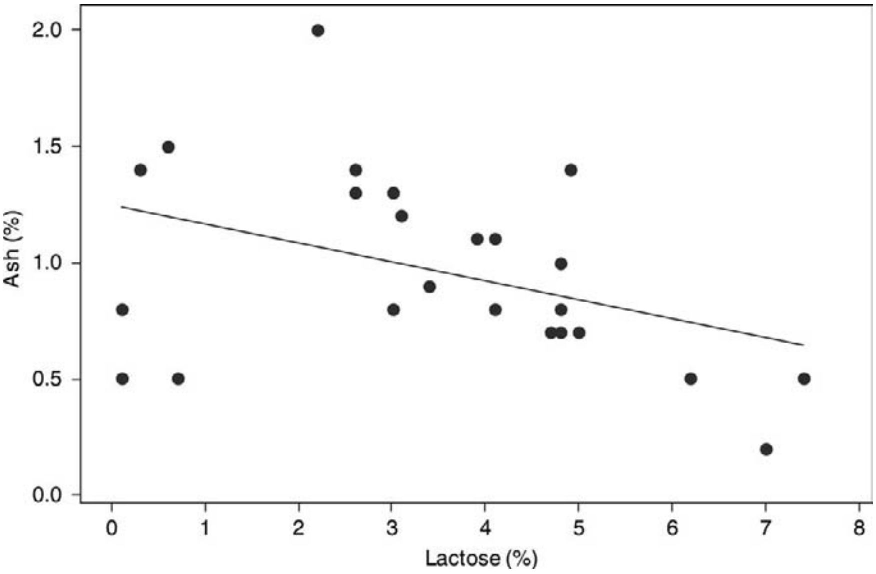

Figure 5 Correlation between ash and lactose in the milk of 23 species. Redrawn from Jenness R and Sloan RE (1970) The composition of milk of various species: A review. *Dairy Sciences Abstract* 32: 599–612.

and can be measured in-line during milking, is commonly used as an index of mastitis.

Why milk contains lactose rather than some other sugar(s) is not clear. The presence of a disaccharide rather than a monosaccharide can be explained on the basis that twice the mass of a disaccharide as a monosaccharide can be accommodated in milk for any particular increment in osmotic pressure. Maltose, which consists of two molecules of glucose, would seem to be the obvious choice of disaccharide. Since energy is expended in converting glucose to galactose, some benefit must accrue from this conversion; a possible benefit is that galactose or derivatives thereof occur in some physiologically important lipids and proteins, and a galactose-containing sugar in milk provides the neonate with a ready supply of this important monosaccharide.

Properties of Lactose

While the properties of lactose are generally similar to those of other sugars, it differs in some technologically important respects. Some important characteristics of lactose are as follows:

- It is a reducing sugar, that is, it has a free, or potentially free, carbonyl group (an aldehyde group in the case of lactose).
- Like other reducing sugars, lactose exists partially as an open-chain form with an aldehyde group, which can form a hemiacetal and, thus, a ring structure. The formation of a hemiacetal creates a new chiral center (asymmetric carbon), which may exist as two enantiomorphs, α or β. By alternatively opening and forming the ring structure, the molecule can interchange between α and β anomers, a process known as mutarotation.
- α- and β-lactose have very different properties, the most important of which are specific rotation, $[\alpha]_D^{20}$ (+89 and +35° for α- and β-lactose, respectively), and solubility in water (70 and 500 g l^{-1} at 20 °C, for α- and β-lactose, respectively).
- Lactose can participate in the Maillard (nonenzymatic browning) reaction, resulting in the production of (off-)flavor compounds and brown polymers. The Maillard reaction contributes positively to the flavor and color of many foods, for example, crust of bread, toast, and deep-fried products, but the effects in dairy products are usually negative and must be avoided. (The Maillard reaction and its consequences are discussed in **Lactose and Oligosaccharides**: Maillard Reactions)
- Redox titration using alkaline $CuSO_4$ (Fehling's solution) or chloramine-T is the principal standard method for the quantitative determination of lactose, although in large laboratories it is now usually determined by infrared spectrophotometry. It may also be determined by polarimetry, spectrophotometry at a visible wavelength after reaction with phenol or anthrone in strongly acid solution, enzymatic reactions, or high-performance liquid chromatography.
- Among sugars, lactose, especially the α enantiomorph, has low solubility in water, but when in solution, it is difficult to crystallize, which may cause problems in lactose-rich dairy products, for example, skimmed milk powder and whey powder, unless precautions are taken

to induce and control crystallization (*see* **Lactose and Oligosaccharides**: Lactose: Crystallization).

- α- and β-lactose are soluble in water at 20 °C to the extent of about 70 and 500 g l^{-1}, respectively; at equilibrium, the ratio of α-lactose:β-lactose is about 37:63, giving a total solubility of about 180 g l^{-1} at 20 °C. The solubility of α-lactose is more temperature dependent than that of β-lactose and α-lactose is the more soluble anomer at >93.5 °C (**Figure 6**). Hence, α-lactose is the form of lactose that crystallizes at <93.5 °C and is the usual commercial form of lactose; β-lactose may be prepared by crystallization at >94 °C.
- α-Lactose crystallizes as a monohydrate, while β-lactose forms anhydrous crystals; thus, the yield of α-lactose is ~5% higher than that of β-lactose.
- When milk or whey is spray dried, any lactose that has not been precrystallized forms an amorphous glass, which is stable if the moisture content of the powder is maintained low. However, if the moisture content increases to >6%, the lactose crystallizes as α-hydrate, the crystals of which form interlocking masses and clumps, which may render the powder unusable if very extensive, that is, inadequately crystallized powder is hygroscopic. The problem can be avoided by adequate crystallization of lactose before drying or by using effective packaging.
- Crystalline lactose has very low hygroscopicity and is used in icing sugar blends.
- Among sugars, lactose has a low level of sweetness; it is only about 16% as sweet as sucrose at 1% in solution and hence has limited value as a sweetening agent, the principal application of sugars in foods. However, it is a useful bulking agent when excessive sweetness is undesirable.
- Lactose is important in the manufacture of fermented dairy products, where it serves as a carbon source for lactic acid bacteria, which produce lactic acid.

Modification of the Concentration of Lactose in Milk through Genetic Engineering

There is interest in reducing the lactose content of milk, by genetic engineering, for at least the following reasons:

- Lactose is the least valuable constituent in milk but it costs energy on the part of the animal to synthesize it; therefore, it would be economically advantageous to reduce the lactose content of milk.
- Since lactose effectively controls the water content of milk and most dairy processes require the removal of water, it would be advantageous to reduce the amount of water in milk by reducing the level of lactose. However, if the level of lactose is reduced too much, the viscosity of the milk will be too high for easy expression. Obviously, this problem could be overcome by reducing the level of lactose rather than eliminating it. Alternatively, it may be possible to modify the mechanism of milk secretion to produce a more useful, or at least a less problematic, sugar than lactose, for example, glucose, maltose, or lactulose (which is a laxative and a prebiotic), or it might be possible to increase the concentration of salts in milk.
- Most adult humans are unable to digest lactose (*see* **Lactose and Oligosaccharides**: Lactose Intolerance); hence, lactose-free or lactose-reduced milk would be nutritionally desirable.

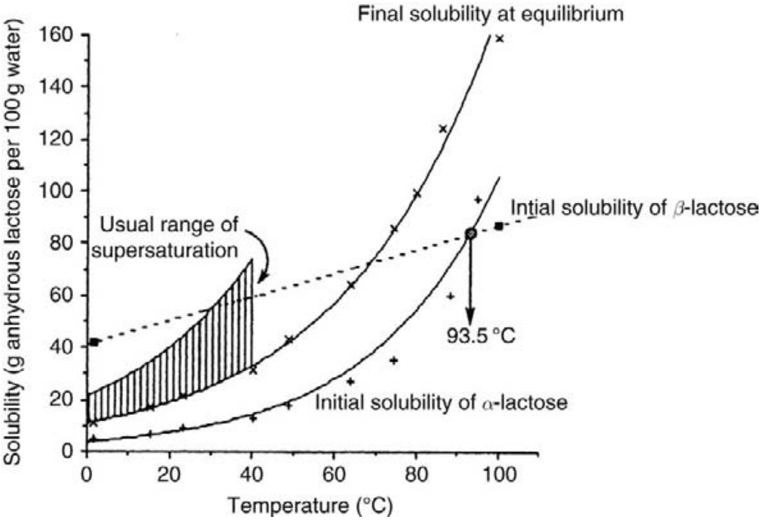

Figure 6 Solubility of α- and β-lactose as a function of temperature. Reproduced from Fox PF and McSweeney PLH (1998) *Dairy Chemistry and Biochemistry*. London: Chapman & Hall.

Since the concentration of lactose is controlled by the concentration of α-La in the secretory cells, the approach to changing the concentration of lactose involves altering the level of this protein.

Nutritional Problems Associated with Lactose

Most adult humans are unable to ingest lactose with impunity, owing to an insufficiency of intestinal β-galactosidase (*see* **Lactose and Oligosaccharides**: Lactose Intolerance). This causes many people to exclude lactose-containing foods from their diet. However, lactose may be removed from milk and dairy products by membrane technology (*see* **Liquid Milk Products**: Liquid Milk Products: Membrane-Processed Liquid Milk) or by prehydrolysis by exogenous β-galactosidase (*see* **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase).

Production and Utilization of Lactose

Previously, whey, from cheese or casein production, was considered a waste material and was fed to farm animals, irrigated on land, or disposed into sewers. Economic and environmental considerations now dictate that whey be used efficiently. The principal products produced from whey are various whey powders, whey protein products, produced mainly by membrane technology, and lactose and its derivatives. Membrane technology is being used increasingly to concentrate and fractionate milk; the resulting permeate has a number of applications, including the production of lactose.

Lactose is prepared commercially by crystallization from concentrated whey or ultrafiltrate. The crystals are usually recovered by centrifugation; this process is essentially similar to that used for sucrose or other sugars. About 400 000 tonnes of crystalline lactose are produced annually (compared to $\sim 10^8$ tonnes of sucrose per annum). The production and utilization of lactose are discussed in **Lactose and Oligosaccharides**: Lactose: Production, Applications.

Owing to the relatively low sweetness and low solubility of lactose, its applications are much more limited than those of sucrose or glucose. Its principal application is in the production of 'humanized' infant formulae based on cows' milk (human milk contains ~7% lactose compared with ~4.8% in bovine milk). The lactose used may be a crystalline product or demineralized whey (for physiological reasons, it is necessary to reduce the concentration of inorganic salts in bovine whey).

Lactose has a number of low-volume, special applications in the food industry, for example, as a free-flowing or agglomerating agent, to accentuate/enhance the flavor of some foods, to improve the functionality of shortenings, and as a diluent for pigments, flavors, or enzymes. It is widely used in the tabletting of drugs in the pharmaceutical industry where low hygroscopicity is a critical property.

Derivatives of Lactose

Several more valuable food-grade derivatives can be produced from lactose, of which the following are the most significant:

- Glucose–galactose syrups, which are ~3 times as sweet as lactose, can be produced by hydrolysis by a strong acid but are usually prepared by using β-galactosidase. The technology for the production of glucose–galactose syrups, by acid or β-galactosidase, is well developed (*see* **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase) but the products are not cost-competitive with other sweeteners (sucrose, glucose, high-fructose syrups, or synthetic sweeteners).
- The several alcohol groups of sugars, including lactose, are very reactive and permit the production of many derivatives. However, the chemical derivatives of lactose are not being produced commercially, probably because similar products can be produced from other, cheaper, sugars; for example, a trichlorinated derivative of sucrose, sucralose, commercialized under the trade name 'Splenda' (E955), is a very successful artificial sweetener (up to 1000 times as sweet as sucrose, 2 times as sweet as saccharin, and 4 times as sweet as aspartame).
- The most commercially successful derivative of lactose is lactulose (galactose–fructose) produced by the epimerization of the glucose moiety of lactose to fructose under mildly alkaline conditions. Lactulose has many applications, including use as a prebiotic and a mild laxative.
- The carbonyl group of lactose can be reduced to lactitol or oxidized to lactobionic acid. Lactitol, which has applications as a low-energy sweetener, suitable for diabetics, can be esterified with various fatty acids and the products can be used as emulsifiers analogous to sorbitans (esters of sorbitol).
- Lactobionic acid is a sweet-tasting acid, which is a very rare property and can be exploited in processed foods. It can be produced by electrochemical oxidation, by enzymatic reactions, using glucose–fructose oxidoreductase, or by living microorganisms.
- Tagatose, the keto analogue of galactose, occurs at a low level in the gum of the evergreen tree *Sterculia setigera*, in severely heated milk, or in stored milk powder. It can be produced by treating β-galactosidase-hydrolyzed

lactose with a weak alkali, for example, $Ca(OH)_2$, which converts galactose to tagatose, which can be purified by demineralization and chromatography. Tagatose is nearly as sweet as sucrose, has a good-quality sweet taste, and enhances the flavor of other sweeteners. It is absorbed poorly from the small intestine, serves as a prebiotic, and has little effect on blood glucose. It is fermented in the lower intestine to short-chain fatty acids, which are absorbed and provide ~35% of the energy obtained from sugars catabolized in the normal way. Tagatose has generally recognized as safe (GRAS) status and is produced commercially by SweetGredients, a company formed by Arla Dairies and Nordzuker (Denmark).

- β-Galactosidase normally acts as a hydrolase, but it also has transferase activity and, under certain conditions, this activity predominates, with the production of oligosaccharides, containing 2–10 monosaccharides. These oligosaccharides have interesting physicochemical properties and may be useful as food ingredients but most attention today is focused on their prebiotic properties (*see* **Lactose and Oligosaccharides**: Lactose: Galacto-Oligosaccharides). The oligosaccharides produced by β-galactosidase should not be confused with the indigenous oligosaccharides in milk, which are described in **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk.
- Lactose can serve as a substrate for the production of various fermentation products (**Figure 7**), of which ethanol, lactic acid, acetic acid, propionic acid, and lactic acid-based plastics are the most important.

The production of ethanol from lactose by fermentation using *Kluyveromyces lactis* or *K. fragilis* has been at a commercial level for at least 30 years. If the ethanol is used in potable products, this process is economically viable but whey-derived ethanol is not classified as potable in some countries. The increased interest in recent years in bioenergy sources may offer new opportunities for lactose-derived ethanol but such applications may not be cost-competitive and will depend strongly on local taxation policy.

The oxidation of ethanol by *Acetobacter* spp. to acetic acid for vinegar or other applications is technically feasible but in most cases is not cost effective.

The *in situ* fermentation of lactose by lactic acid bacteria to lactic acid is widespread in the production of

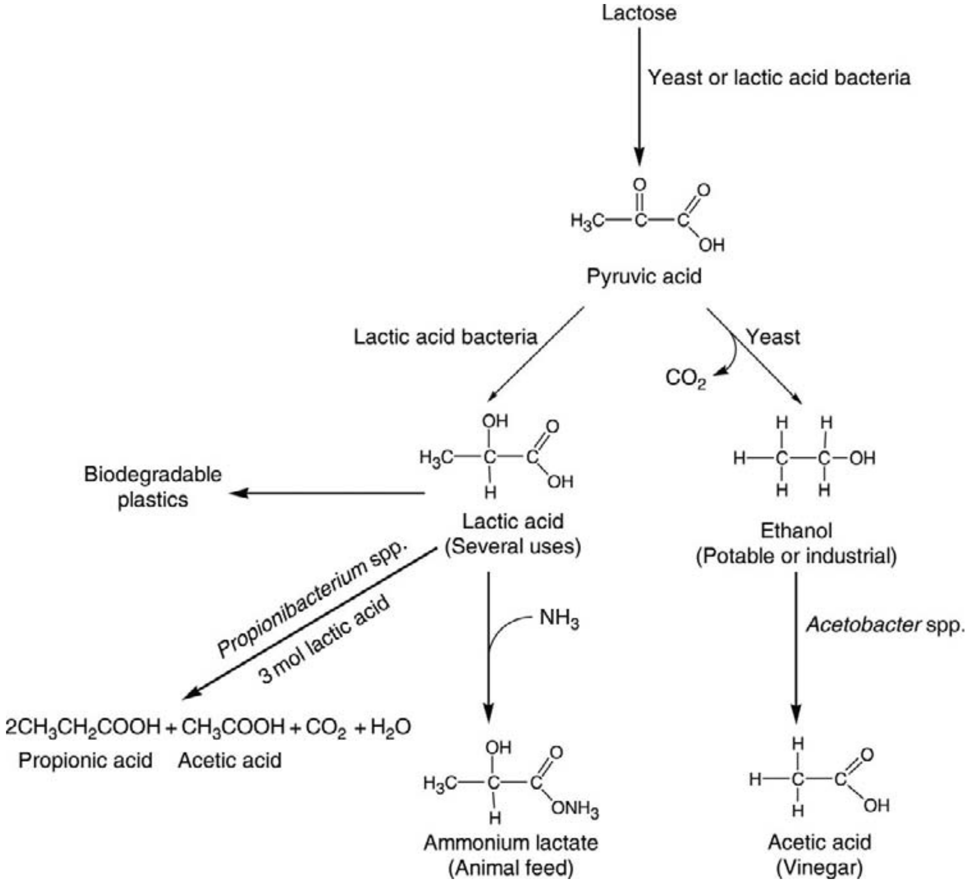
Lactose
Yeast or lactic acid bacteria
Pyruvic acid
Lactic acid bacteria
Yeast
CO2
Biodegradable plastics
Lactic acid (Several uses)
Ethanol (Potable or industrial)
Propionibacterium spp.
3 mol lactic acid
NH3
Acetobacter spp.
2CH3CH2COOH + CH3COOH + CO2 + H2O
Propionic acid
Acetic acid
Ammonium lactate (Animal feed)
Acetic acid (Vinegar)

Figure 7 Fermentation products from lactose.

fermented dairy products. The fermentation of lactose to lactic acid for food or industrial applications (including the biodegradable plastic, polylactic acid) is technically feasible but not cost competitive with production by the fermentation of other sugars or by chemical synthesis.

Lactic acid can be converted by *Propionibacterium* spp. to propionic acid (with acetic acid, CO_2, and H_2O as by-products), which is used as a fungicide in the food industry, but like the other fermentation products described here, this conversion may not be economically feasible.

Significance of Lactose in Dairy Products

Owing to its low solubility, crystallization behavior, and hygroscopicity, lactose causes problems in concentrated, dehydrated, and frozen dairy products. These problems can be avoided by the application of appropriate processing techniques, which have been developed over many years.

Although commercially less important than in concentrated and dehydrated dairy products, these physicochemical properties of lactose also cause problems in the production of frozen milk, which may be economical under certain circumstances, especially if the milk is preconcentrated. However, the casein micelles are destabilized during frozen storage, and aggregate on thawing. Destabilization is caused by a decrease in pH and an increase in $[Ca^{2+}]$, both due to the formation of $Ca_3(PO_4)_2$ from $CaHPO_4$ and $Ca(HPO_4)_2$ following a reduction in the amount of solvent water as a consequence of ice formation, which is further reduced by the crystallization of α-lactose monohydrate. Destabilization can be avoided by prehydrolyzing the lactose or freezing rapidly to $<-30\,°C$. The aggregated casein can be redispersed by heating the thawed milk to $\sim 50\,°C$; the properties of the re-formed micelles have not been studied in detail. The cryoprecipitation of casein may be exploited for the commercial production of casein.

As a reducing sugar, lactose can participate in the Maillard reaction, principally with the ε-amino group of lysine, resulting in the formation of brown-colored pigments or volatile flavored compounds and impaired functionality and nutritional value. The Maillard reaction is the subject of **Lactose and Oligosaccharides**: Maillard Reactions. The reaction is particularly severe in heated products but occurs also in milk powders, especially during storage under adverse conditions of temperature and humidity. It may be a problem in cheese subjected to severe heating, for example, Mozzarella, or in grated cheese during storage.

The use of *Streptococcus thermophilus*, which cannot metabolize galactose, and galactose-negative strains of lactobacilli may produce sufficient galactose to cause browning-related problems in certain types of cheese and especially in whey therefrom during drying. The crystallization characteristics of galactose are quite different from those of lactose, and may cause problems in whey powders.

The monosaccharides glucose and galactose are much more reactive than lactose, and hence dairy products containing hydrolyzed lactose are particularly susceptible to Maillard browning. The hydrolysis of lactose by β-galactosidase markedly increases the heat stability of milk and concentrated milk, especially around the pH of minimum solubility; hydrolysis of $\geq\sim 20\%$ has a significant effect on heat stability. The mechanism of stabilization has not been elucidated fully but is probably due to the carbonyls formed in the Maillard reaction; unfortunately, such lactose-hydrolyzed milk products are very susceptible to intense browning, which may render them nonviable commercially.

Conclusion

Lactose is one of the principal naturally occurring disaccharides. For reasons that are not fully clear, lactose is the principal saccharide in mammalian milks, which are the only significant sources of lactose. As for other sugars, the chemical and physicochemical properties of lactose are well established after more than 200 years of research. However, lactose continues to be the subject of considerable research and new discoveries continue to be made. Lactose has become a valuable commodity, which stimulates further studies.

See also: **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase. **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk; Lactose Intolerance; Lactose: Crystallization; Lactose: Derivatives; Lactose: Galacto-Oligosaccharides; Lactose: Production, Applications; Maillard Reactions. **Liquid Milk Products**: Liquid Milk Products: Membrane-Processed Liquid Milk.

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Lactose: Crystallization

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Introduction

Whey is the liquid dairy product obtained during the manufacture of cheese, casein, or similar products through separation of the curd following coagulation of milk and/or derivative products of milk (**Figure 1**). Whey was for many years regarded as a by-product, the use of which was limited to animal feed and fertilizers. However, the quantity of whey released corresponds to 9 times the weight of the final cheese manufactured, and whey contains not less than 50% of the dry matter of the milk transformed into cheese.

The whey treatment industry has developed considerably in the past 20 years, and the production of whey powders in Europe doubled between 1985 and 2004. This doubling of production is explained mainly by a more than 33% increase in cheesemaking from which the whey results. However, the increase is also explained by the appreciation of the value of whey and its derivatives, owing to their technofunctional (texturing, confectionery, animal feeds, etc.) and nutritional qualities (high protein content, rich in amino acids), as ingredients in the formulation of diet foods, pharmaceuticals, and infant milk products.

Spray-Drying of Whey

As it is constituted mainly of water (>93%), whey requires dehydration when used as an ingredient in the manufacture of other products, and the key objective of drying dairy products in general, and whey in particular, is to stabilize these products at both the microbiological and the physicochemical level by reducing the water activity (a_w). Moreover, concentration of the dry matter allows reduction of the transport and storage costs. Improvements in drying processes and powder stability and the emergence of membrane separation technologies, which have provided qualitative and quantitative improvements in various products, are factors that have contributed to the increased use of whey in dehydrated form.

Main Stages in the Process

Dehydration of whey involves a succession of several unit operations (**Figure 2**): (1) preprocessing (pasteurization, demineralization, filtration); (2) concentration by vacuum evaporation; (3) lactose crystallization; and (4) spray-drying and fluidization. Evaporation removes some of the water (85–95%) from whey by boiling under vacuum and increases the dry matter from 6.5 to 50–65% (w/w). At this stage of concentration, whey is a highly concentrated product, but the concentration is not enough to prevent chemical reactions or changes in state taking place. The water activity (a_w) is close to 0.95–0.99. Therefore, lactose, the concentration of which is much higher than its solubility level, changes state during crystallization. This change in state is not instantaneous and depends on complex kinetics related to the many factors described in this article. The crystallized whey concentrate is then dehydrated more completely by spray-drying combined with fluidization.

Critical Points

Mastering the milk dehydration process does not necessarily mean that the same techniques can be applied to the many derivatives of milk, particularly in the case of whey, which has very varied physicochemical properties. It is therefore not easy to extrapolate the technological properties of one product to another, and difficulties can be encountered in industrial processing at each level. Major problems can be encountered during the lactose crystallization stage according to the nature of the whey (e.g., crystallization rates, crystal size, stirring conditions). For example, the thickening of the lactic acid concentrate during the same stage, which is represented industrially by a considerable increase in viscosity, is not yet resolved. During spray-drying, the problems associated with stickiness are more frequent when treating whey than when treating milk, and caking of whey powders during storage is a frequent problem. Amorphous (noncrystallized) lactose appears to have a significant role in the last two examples due to its hygroscopicity and glass transition temperature. Finally, the size of the lactose crystal can also influence several physical properties (flowability, floodability, density, particle size, color, etc.) and rehydration properties (solubility, dispersibility, wettability, sinkability, instantization, hygroscopicity, etc.) of whey powders.

The lactose crystallization process is a key stage in the processing of whey and lactose powders. Controlling this appears to be fundamental during the drying stage and storage, since the transition of lactose to a crystallized form decreases the proportions of hygroscopic compounds (amorphous lactose). Moreover, management of this stage, particularly through a greater understanding of the respective roles of the product and the process in the kinetics of crystallization or size of the crystals, should allow progress in the future (1) in the management of

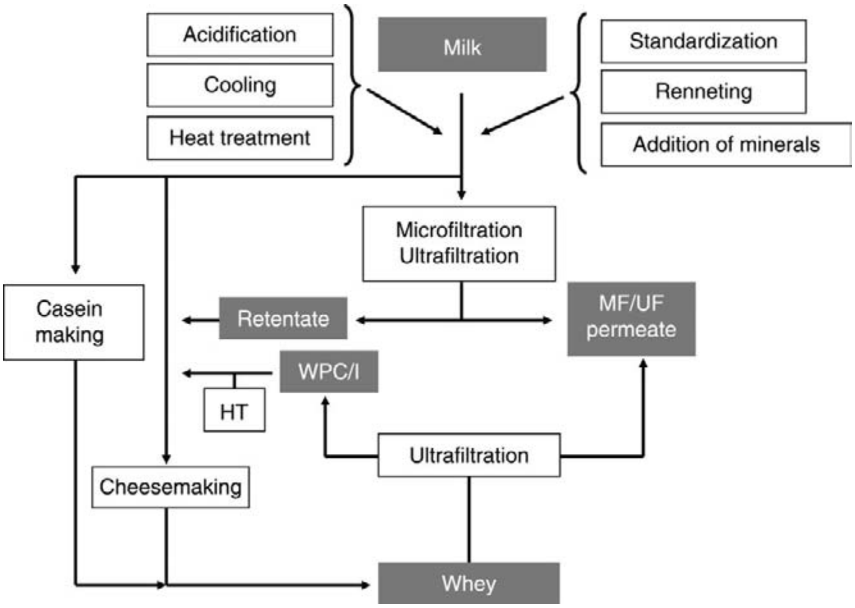
Acidification
Cooling
Heat treatment
Milk
Standardization
Renneting
Addition of minerals
Microfiltration
Ultrafiltration
Casein making
Retentate
MF/UF permeate
WPC/I
HT
Ultrafiltration
Cheesemaking
Whey

Figure 1 Technological processes used to produce various wheys. HT, heat treatment; MF, microfiltration; UF, ultrafiltration; WPC, whey protein concentrate; WPI, whey protein isolate.

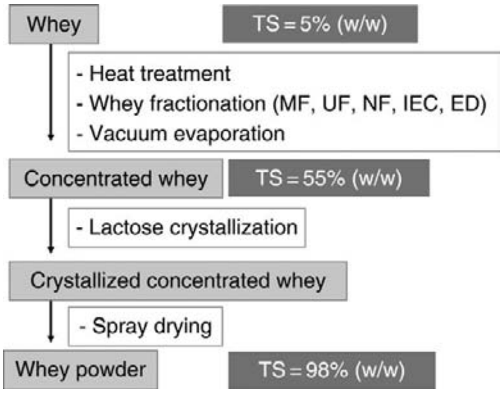
Whey
TS = 5% (w/w)
- Heat treatment
- Whey fractionation (MF, UF, NF, IEC, ED)
- Vacuum evaporation
Concentrated whey
TS = 55% (w/w)
- Lactose crystallization
Crystallized concentrated whey
- Spray drying
Whey powder
TS = 98% (w/w)

Figure 2 Technological process for the production of whey powder.

production by anticipating the duration of the unit operation according to the raw materials and technological parameters involved, and (2) in the production of powders with crystals of given sizes and the development of techno-functional properties specific to certain applications.

Crystallization from Solution: Principles Illustrated by the Example of Lactose Crystallization in α-Monohydrated Form

The concepts of crystallization from an aqueous solution are briefly presented in this section, illustrated by examples taken from studies focusing on lactose in α-monohydrated form.

Solubility

The solubility of a solute in a solvent can be defined as the concentration of the solution in the liquid phase in equilibrium with a solid crystalline phase. From a thermodynamic point of view, when equilibrium is reached there is a balance between the chemical potential of the crystal and that of the solution, and of course the balance depends on the temperature and pressure. In practice, the solubility of a solute in a solvent is defined by the maximum quantity of the solute that can be dissolved at a given temperature and pressure. The solution is then considered saturated, or to be at saturation. The concentration of a solute in a solvent, and hence solubility, can be expressed in different ways. Three main types of unit have been proposed: ratio of mass (or moles) of the solute to mass (moles) of the solvent, ratio of mass of the solute to mass of the solution, and ratio of mass of the solute to volume of the solution.

Many factors can influence solubility, including the chemical nature of the solute–solvent combination and the intensity of their interactions (electrostatic or dispersion energy). It should be noted that, even for nonionic species, electrostatic interactions have a significant role in relation to the possible existence of dipole moments in such species resulting from the asymmetrical distribution of the electrical charges. The dependence of solubility on the nature of the solute–solvent combination means that solubility can vary with the nature of the solvent for a given solute. For example, sugars and carbohydrates are not readily soluble in alcohols, in particular lactose, which is insoluble in pure methanol. Temperature has a significant effect on solubility, and solubility increases to a lesser or greater degree for most species with an increase in temperature. However, the solubility of some substances can be reversed by an increase in temperature. For example, the solubility of calcium phosphate decreases with an increase in temperature. Solubility is also to some extent dependent on pressure under usual conditions.

The presence of other constituents in the solution (e.g., impurities or additives) can have varying effects on the solubility of a component. The presence of certain food components (proteins, starches, sugars) can reduce the solubility of sucrose and other sugars, and other solutes competing for water as solvent are typically a factor that reduces the solubility of a substance. Some authors have suggested that electrolytes can modify the solubility of a substance by affecting the structure of water. In the case of lactose, the α and β anomeric forms, which differ in the position of the hydrogen bond in C1 in relation to the molecular structure (**Figure 3**), are present in the solution. The β form causes a slight reduction in the solubility of the α form, according to its concentration. The solubility of lactose is also appreciably reduced by the presence of sucrose. In 1996, Bhargava and Jelen demonstrated that the solubility of lactose can vary with the presence of certain ionic elements, and these in particular are present in whey. Potassium phosphate appears to increase solubility slightly, whereas calcium lactate, magnesium sulfate, and lithium chloride have the opposite effect. Finally, lactose solubility is not significantly modified in the ultrafiltration permeates of sweet or acid whey, where the positive and the negative effects on solubility appear to compensate each other.

Other solutes can also modify the solubility of a species, not by interaction with the solvent, but by reacting directly with the species. For example, sometimes complexes can be formed between the initial solute and another species that are more soluble than the original solute. In contrast, the addition of another species in solution can result in a reduction in the solubility of a compound by any of the following ways:

- forming a less soluble complex (e.g., reduction in calcium solubility in the presence of phosphate groups);
- contribution of common ions, which affect the balance of dissociation toward increased precipitation, which decreases solubility (e.g., the addition of calcium to a saturated solution of calcium phosphate enhances the precipitation of calcium phosphate); or
- causing a change in pH, which can lead to the precipitation of a substance either directly (e.g., reduction in the solubility of a protein at its isoelectric pH) or indirectly by modifying the concentrations of the species involved in the dissociation–precipitation balance by modification of the acid–base equilibrium. Again, using the above example, an increase in pH results in deprotonation of the phosphate groups, due to their greater affinity for calcium, which forms complexes in increasing quantities. As these complexes are insoluble, calcium and phosphate become less soluble when there is an increase in pH.

Moreover, it is important to recognize that it is even more difficult to determine the solubility of a substance in concentrated solutions. For example, solubility is always influenced by the so-called salt effect, an increase in ionic strength resulting in an increase in the solubility of several substances. This positive effect of ionic strength on solubility compensates for, and often masks, the effects of adding a common ion.

Lactose, which has two anomeric forms in solution (α and β) (**Figure 3**), has a final solubility (i.e., at mutarotation equilibrium) equal to the sum of the concentration of the α form, corresponding to its intrinsic solubility, on the one hand, and the concentration of the β form, confirming the mutarotation equilibrium, on the other hand. It should be noted that the final solubility is not the sum of the intrinsic solubility of each anomeric form; the intrinsic solubility of the β form is in fact much higher ($50\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ water at 15 °C). The solubility of the α form and the final solubility increase with temperature, with the mutarotation equilibrium constant changing little with temperature. For example, the solubility of the α form and final solubility are 9.65 and $24.81\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ water at 30 °C, and 23.36 and $58.40\,\mathrm{g}\,100\,\mathrm{g}^{-1}$ water at 60 °C, respectively. The influence of certain impurities on the final solubility of lactose may in fact be the result of an indirect effect on the value of the mutarotation constant.

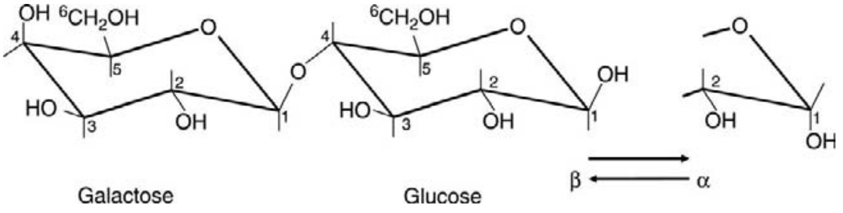

Figure 3 Molecular formula of α- and β-lactose.

Displacement of the mutarotation equilibrium involved (a reduction in the β:α ratio, for example) would result in a decrease in the final solubility of lactose without modifying the solubility of the α form.

Understanding the factors that influence the solubility of a substance is very important in the study of solid–liquid balance. Modification of these factors changes the solubility and is at the origin of changes in phase and the appearance of crystals.

Supersaturation

A supersaturated solution is one in which the concentration of the solute exceeds its solubility. From a thermodynamic point of view, a change in phase occurs when there is a difference between the chemical potential of the solution (supersaturated solution) and the chemical potential of the solution at equilibrium, that is, when the concentration of the solute is equal to its solubility. The potential difference is the driving force for the change of phase. This potential difference depends on the difference between the concentration of the aqueous solution and the solubility calculated at the same temperature and same pressure. This difference is called supersaturation and can be defined, depending on the case, as the difference (absolute supersaturation) or ratio (relative supersaturation) between concentration and solubility. The difference in chemical potential is, strictly speaking, expressed according to the chemical activity, which is difficult to evaluate. However, for organic substances, activity is comparable with concentration in the majority of cases.

Creating supersaturation is thus an essential condition for change of state. Supersaturation is achieved mainly by either increasing the concentration of the aqueous solution (evaporation of solvent) or reducing the solubility of the solute (cooling, change in the composition of solvent, chemical reaction between soluble species leading to the formation of an insoluble compound) (**Figure 4**). In practice, the choice of the method by which supersaturation is obtained depends on the nature of the system. For example, when the solubility of a species does not vary with temperature, evaporation of the solvent is preferable to cooling. When solubility varies substantially with temperature, cooling is preferable. In intermediate cases, cooling and evaporation may be implemented together, as in the manufacture of whey powders.

In the case of α-lactose-H_2O crystallization (monohydrated form of α-lactose) in the processing of whey and derivative powders, supersaturation is performed initially by evaporation of the solvent (i.e., concentration of total solids to 50–65% (w/w) at the end of vacuum evaporation, corresponding to a lactose concentration of 65–158 g 100 g^{-1} water, according to the nature of the whey/permeate) (**Table 1**), then by fast cooling to 25–35 °C using a flash cooler, and finally by slow cooling to 20–25 °C (or not). Under these pressure (atmospheric pressure) and temperature conditions (<93.5 °C), the most thermodynamically stable solid form (i.e., that corresponding to minimum free enthalpy) is the lactose crystal in α-monohydrated form. Thus, although both α and β forms are supersaturated, only the α form crystallizes (in the α-monohydrated form).

One last point to be emphasized for lactose is the influence of mutarotation kinetics on the supersaturation of the α form during crystallization. Two cases to illustrate this are

- *Very slow mutarotation.* The α form is consumed much more quickly by crystal growth during crystallization than by mutarotation of the β form. In this case the slowness of the mutarotation stage does not allow the α form to remain supersaturated for very long.
- *Very fast mutarotation.* In this case, as soon as an α-lactose molecule is consumed by crystallization, the molecule is instantly replaced by a new molecule produced by mutarotation of a β-lactose molecule, thus maintaining a high level of supersaturation.

These two cases illustrate the influence of mutarotation kinetics on supersaturation of the crystallizing aqueous solution at each moment (*t*), and this is a determining factor.

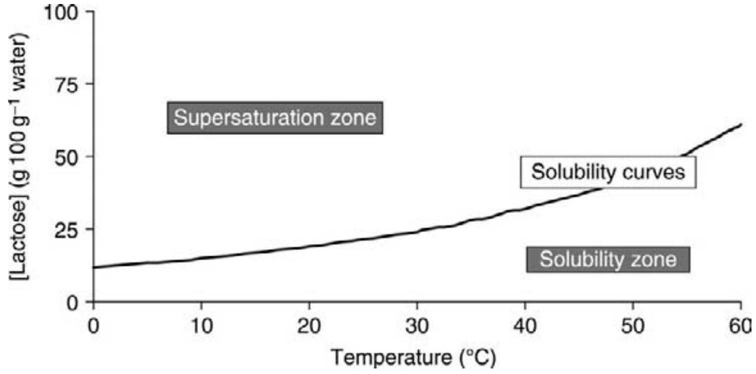

Figure 4 Solubility curve of lactose.

Table 1 Soluble lactose content (SL_w) expressed as g 100 g^{-1} water according to the type of whey (lactic acid, sweet, or permeate) or the soluble lactose content (SL_{TS}) expressed as g 100 g^{-1} total solid (TS) and the TS content of the concentrate expressed as g 100 g^{-1} concentrate

		Soluble lactose content (SL_{TS}) (g 100 g^{-1} TS)		
		Lactic acid whey	*Sweet whey*	*UF permeate*
		65	*75*	*85*
		Soluble lactose content, SL_w, as g 100 g^{-1} water		
Total solids, TS (g 100 g^{-1})	50	65	75	85
	55	79	92	104
	60	98	113	128
	65	121	139	158

SLw is calculated by the equation $SL_w = \dfrac{TS \times SL_{TS}}{(100-TS)}$

Metastability, Induction Time, and Nucleation

Stable, metastable, and unstable zones

Creation of supersaturation is a requirement for a phase shift, but it is not the only condition. Let us see the example of cooling of an undersaturated solution (**Figure 5**). By cooling the solution from A to B, the system slightly exceeds the solubility curve. If the system is left in state B, a day, or even a year, could pass without the onset of crystallization. If another sample, initially in state A, is cooled until it reaches state C, and then maintained in this state, crystals will appear within a few hours. If the cooling is even greater (D), and beyond a certain limit, crystals will appear immediately. Moreover, in the case of state B or C, if crystals are added to the solution they will grow. The states B and C characterize supersaturated solutions considered to be stable, at least for a given time, whereas they are obviously in non-thermodynamic equilibrium (added crystals grow). Such states are called metastable states. The limit of supersaturation, which is the phase at which crystallization begins instantaneously, is called the limit of the metastable zone. However, this limit and consequently the breadth of the metastable zone are not defined by the thermodynamics or the physical properties of the system, as (in contrast) solubility is. At constant temperature and pressure the limit of the metastable zone will depend on several other conditions of the system. It will, for instance, be closer to the solubility curve when the system is stirred. Similarly, the chemical nature of the solute–solvent combination, temperature, the mode and the kinetics by which supersaturation is obtained, the nature and quantity of impurities, thermal and mechanical shocks, and so on will influence the breadth of the metastable zone. Moreover, the breadth of the metastable zone is likely to vary according to the sensitivity of the experimental techniques used for the detection of solids.

Induction time

The duration of the states B and C (**Figure 5**) influences the onset of supersaturation and the beginning of the phase shift. This duration, which decreases with an increase in supersaturation and ceases beyond the limit

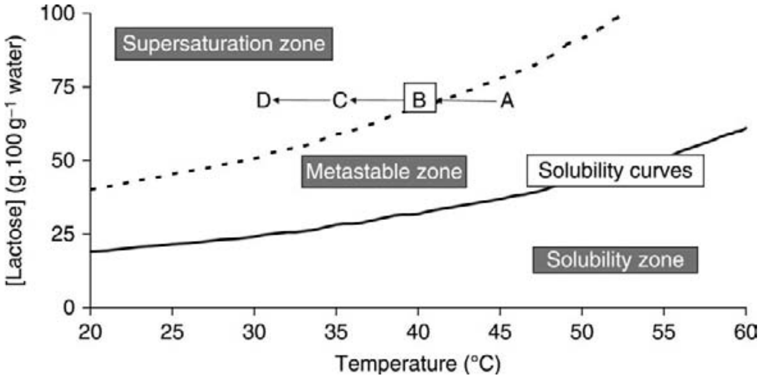

Figure 5 Solubility curves of lactose obtained when cooling an undersaturated solution of lactose in water.

of the metastable zone, is called the induction time; the greater the stability of the system, the longer the induction time. Any factors that reduce the breadth of the metastable zone will similarly reduce the induction time.

Nucleation and metastability

What are the reasons for a supersaturation zone in which a change of state does not start or begins slowly, or a limiting supersaturation zone beyond which transition to a solid state begins suddenly? The answer lies in the kinetics of the mechanism that controls the first stage of the formation of the solid phase, known as nucleation. The mechanisms themselves are complex and still poorly understood. Classical theory distinguishes various types of nucleation:

- Primary nucleation, describing the creation of nuclei of a species in the absence of crystals of this species in a suspension. This is referred to as 'homogeneous' in a pure solution and 'heterogeneous' in the presence of soluble or solid impurities.
- Secondary nucleation, describing a species that develops in the presence of crystals of the same species.

Homogeneous primary nucleation

Being based on the assumption of total purity of a solution, this process is seldom observed in practice. However, it is the basis of the theory of nucleation that proposes a process of random accretion of clusters of aqueous molecules, some being transformed into nuclei that then grow and become crystals and others dissolving, the former passing beyond a critical size compared to the latter. This critical size corresponds to a maximum free enthalpy that must be exceeded to stabilize the nucleus, and the maximum is the result of two antagonistic variations in energy (**Figure 6**). When positive it is disadvantageous, the creation of crystals being accompanied by a solid–liquid interface enthalpic variation (ΔG_s) proportional to the square of the size of the nucleus and the crystal–solution interface tension, and when negative it is advantageous, resulting from the latent heat released by the change in state and being proportional to the volume. The variation in free enthalpy associated with the formation of a nucleus of critical radius (r_{crit}, ΔG_{crit}) is then written as

$$\Delta G_{\text{crit}} = \frac{16\cdot\pi\cdot\sigma^3\cdot V^2}{3\cdot(k\cdot T\cdot\ln\,\nu)^2} \qquad [1]$$

where σ is the crystal–solution interface tension, V the molar volume, ν the supersaturation, k a constant, and T the temperature.

The kinetics or frequency of homogeneous primary nucleation corresponding to the number of nuclei formed per unit volume and unit time obeys Arrhenius's law and is written as

$$B_0 = A\cdot\exp\left(-\frac{\Delta G_{\text{crit}}}{k\cdot T}\right) \qquad [2]$$

or, by combining eqns [1] and [2]

$$B_0 = A\cdot\exp\left(-\frac{16\cdot\pi\cdot\sigma^3\cdot V^2}{3\cdot(k\cdot T)^3\cdot(\ln\,\nu)^2}\right) \qquad [3]$$

This equation shows that the nucleation kinetics are related to the nature of the solute–solvent combination via the value of the crystal–solution interface tension, σ, or molar volume, V, as well as via the expression of the preexponential factor A. The kinetics of B_0 nucleation are also strongly influenced by temperature (T) and particularly by supersaturation (ν). When the latter increases, the nucleation kinetics increase. More precisely, due to the functional nature of supersaturation (ν) in eqn [3], the nucleation kinetics remain weak when the supersaturation value is below a certain critical value but increase strongly above this critical supersaturation. In other words, the time necessary for the appearance of a sufficient number of nuclei to start the change of state process substantially (the induction time defined earlier) is considerably reduced when supersaturation slightly

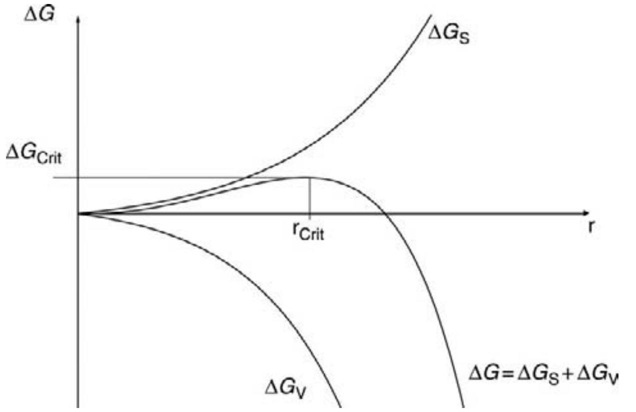

Figure 6 Enthalpic variation (ΔG_s) of nucleus versus radius (r) during crystallization.

exceeds its critical value. It is the superseding (or not) of the critical supersaturation point that is at the origin of the concept of metastability: (1) just below the part of the metastability zone corresponding to critical supersaturation the induction time is still important because the nucleation kinetics are weak, and (2) after the critical supersaturation point the induction time is close to zero and crystallization is spontaneous.

It is important to bear in mind that in practice, and contrary to the prediction of the nucleation kinetics, with strong supersaturation the solution becomes too viscous and nucleation is rapidly limited by molecular movement.

Heterogeneous primary nucleation

Heterogeneous nucleation is the main source of primary nuclei in current applications because extraneous substances (impurities, dust, etc.) are often present, and they reduce the amount of energy required for the formation of stable nuclei. Thus, the kinetics of heterogeneous nucleation are more rapid than those of homogeneous nucleation of weaker supersaturations. The presence of impurities generally leads to a reduction in the breadth of the metastable zone. Although the mechanism of catalysis of primary nucleation by extraneous species is still poorly understood, the most probable hypothesis involves crystal–solution, crystal–impurity, and solution–impurity interface energy. This hypothesis assumes that when the contact angle of a nucleus with an impurity tends to zero (i.e., the nucleus humidifies the impurity more and more), variation in free enthalpy associated with the formation of a nucleus with a critical radius (ΔG_{crit}) tends toward zero. The kinetics of primary nucleation thus affect the induction time. This is often used in experiments as a simple macroscopic method of measuring the kinetics of primary nucleation. Induction time is then taken to be proportional to the inverse of the nucleation kinetics, implying that the factors that influence induction time or the breadth of the metastable zone are the same as those that influence the kinetics of primary nucleation.

- *Intrinsic factors due to the chemical nature of the solute*: solubility (related to supersaturation level), nucleus–solution interface tension, degree of analogy with impurities.
- *Factors related to the composition of the solution*: nature and quantity of impurities.
- *Factors related to the operating conditions*: supersaturation, temperature (directly and via the supersaturation level), solvents used, stirring conditions.

The impact of supersaturation at various temperatures on the induction time of monohydrated α-lactose was first studied in non-stirred systems. The results were in agreement with the above theory: The induction time decreases with supersaturation up to a certain point, beyond which it again increases (at high concentrations and low temperature), illustrating a nucleation limit resulting from a decrease in molecular mobility. At the lowest concentrations (30–40 g lactose 100 g^{-1} water) at any temperature between 0 and 50 °C, the induction time was always longer than 10 h. It was also measured at a higher concentration (80 g lactose 100 g^{-1} water) and was minimal at 30 °C, where it was about 30 min. These results thus suggest a broad zone of metastability for lactose.

Secondary nucleation

Within the metastable zone in many systems (i.e., within a zone where the change in state does not occur spontaneously) there is a supersaturation zone in which the addition of solute crystals causes immediate nucleation of the aqueous solution. This is called secondary nucleation and requires a critical energy to stabilize one nucleus that is lower than that required in primary nucleation. This type of nucleation can thus occur at lower degrees of supersaturation. The mechanism is extremely complex. In stirred crystallizers, most nuclei are generated by a mechanism known as contact nucleation. New nuclei are generated within the solution by contact between crystals, between crystals and stirrers, or between crystals and the walls of the crystallizer. Shock can normally cause cracking of the many protuberances present on the surface of a crystal, giving rise to a multitude of small crystals of about the size of the nucleus (microattrition) or may dislodge aggregates or clusters of molecules of the solute from the outer layer of the crystal surface and transfer these clusters into the solution. If they exceed the critical size they will form new crystals (limited layer process). In practice, the simple friction of a crystal on a glassy surface is sufficient to produce hundreds or even thousands of secondary nuclei in systems such as sucrose or lactose.

In view of the complexity of the mechanisms, the description of the kinetics of secondary nucleation is simplified. An empirical power law is generally proposed to explain the experimental results:

$$\frac{\mathrm{d}N}{\mathrm{d}t} = k_{\mathrm{N}} \cdot M \cdot [C - C_{\mathrm{s}}]^{i} \qquad [4]$$

where N is the number of crystals per unit of solvent mass; M the crystal density of the suspension expressed in mass of crystals per unit mass of the solvent; $(C - C_{\mathrm{s}})$ the absolute supersaturation (C is the concentration of the aqueous solution and C_{s} its solubility); i the order of the reaction; and k_{N} the kinetics constant of secondary nucleation, which is dependent on stirring velocity and temperature, following an activation law of the Arrhenius type

$$k_{\mathrm{N}} = \varphi \cdot \exp\left(-\frac{E_{\mathrm{A}}}{RT}\right) \qquad [5]$$

where E_{A} is the activation energy, R the gas constant, T the temperature, and ϕ a constant. In addition to the impact of supersaturation (also encountered in the description of the kinetics of primary nucleation), the expression of the kinetics of secondary nucleation (eqn [4]) highlights the effects of crystals in the aqueous solution via the M value and the influence of the stirring velocity on which k_N depends. The order of value of the term M is generally 1, and that of supersaturation is generally lower than that for primary nucleation (typically between 0.5 and 2.5). To summarize, the factors influencing secondary nucleation are those that influence primary nucleation (supersaturation, temperature (which also affects supersaturation), and the kinetics constant (eqn [5])) and also the crystal density in the solution, the mechanical power provided to the system, and the hardness of the crystals and their roughness.

Few studies have focused on secondary nucleation of monohydrated α-lactose. Using a microphotographic technique, Shi and colleagues defined the various zones of the supersaturation diagram first presented by Nickerson. They demonstrated that only a very low level of energy is necessary to cause contact nucleation, the critical concentration beyond which contact nucleation occurs being estimated at approximately 30 g 100 g^{-1} water at 30 °C, which is very low. In the same study the authors proposed an estimated reaction of secondary contact nucleation of order 2 (i.e., $i = 2$). In another study, using an MSMPR (mixed suspension mixed product removal) approach in which the prevailing nucleation mechanism is secondary contact nucleation, a value of the order of 1.9 was proposed. The activation energy describing the dependence of the constant nucleation kinetics on temperature T (eqn [3]) is estimated to be 17 kcal mol^{-1} K^{-1}. However, it should be noted that no study has been reported on the influence of stirring velocity or presence of impurities on the secondary nucleation kinetics of monohydrated α-lactose.

To summarize this brief description of nucleation mechanisms and their kinetics, the multiplicity of factors involved and the complexity of the phenomena mean that controlling nucleation is very difficult, particularly in an industrial context. However, nucleation is a key stage in quality control of crystallization and of the products obtained. The number, mean size and size distribution, and the shapes of the crystals, which are controlled by nucleation kinetics, have a significant role, particularly in relation to the quality of food products (texture, conservation).

Growth

When a nucleus reaches its critical size, it grows larger according to the mother aqueous solution from which it draws the molecules. Crystal growth is the result of several stages:

- Diffusion of molecules from the aqueous solution toward the solid interface
- Dissolution of molecules in the aqueous solution
- Contradiffusion of the solvent molecules to the solution
- Adsorption of molecules on the crystal surface
- Migration of molecules on the crystal surface to suitable incorporation sites
- Incorporation of growth units into the crystal network

The first three stages are known as diffusional stages; the last three are related to the adsorption layer. There are two theories regarding the mechanism: growth by two-dimensional nucleation and growth originating from dislocation.

Growth by two-dimensional nucleation

According to this theory, nuclei of critical size are formed on the crystal surface according to a mechanism similar to that of primary nucleation but in two dimensions and not in three dimensions. A nucleus appears and forms a single layer of molecules that covers the entire surface; the construction of a further layer requires the formation of a new nucleus – the mononuclear model. In the polynuclear model, several nuclei appear, but do not spread, and a sufficient number of nuclei are required to cover the entire surface. These two mechanisms are combined in the birth-and-spread model, in which several nuclei grow at constant kinetics but finish simultaneously on the surface, and other layers are formed by the creation of new nuclei on nuclei that are already spreading.

Growth originating from dislocation

The Burton–Cabrera–Frank (BCF) theory proposes a model of more regular growth in which, after adsorption on the surface, the aqueous solution molecules migrate toward incorporation sites located close to dislocations. These dislocations are considered to develop continuously in self-maintaining spirals (**Figure 7**). In this model the dislocation initiates the formation of the new layer, and two-dimensional nucleation is not necessary.

Diffusion theory

The equations expressing the growth kinetics in the above models are often complex and not easily applied to modeling of the operation of a crystallizer. Another theory is therefore used for this purpose: diffusion theory, which describes growth as two stages in series (**Figure 8**):

1. the diffusion stage, during which the aqueous solution molecules diffuse from the center of the solution into the immediate vicinity of the crystal surface, and
2. the integration or surface reaction stage, during which the molecules in solution are integrated into the crystal network.

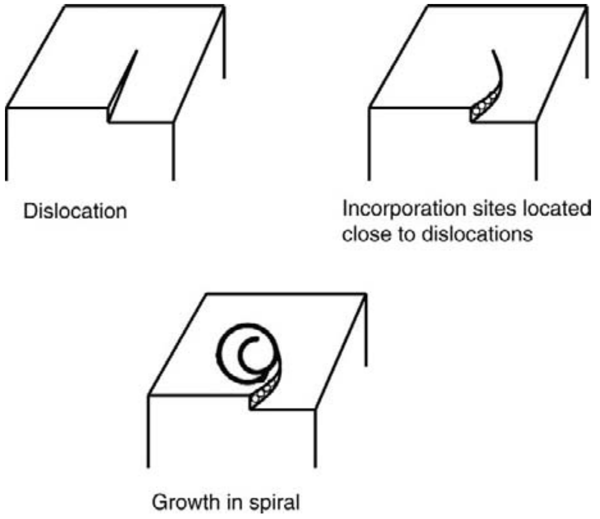

Figure 7 Growth model from dislocation.

The basis of this theory is the existence of a diffusion layer that separates the solution with concentration C and an adsorption layer of maximum concentration C_i. The difference $C - C_i$ constitutes the driving force of the diffusion stage, which can be written as

$$\frac{dM}{dt} = k_d \cdot S \cdot [C - C_i] \qquad [6]$$

where k_d is the diffusion transfer coefficient (which can be obtained by the measurement of dissolution kinetics) and S the surface of the crystal–solution interface. The surface reaction stage is described by

$$\frac{dM}{dt} = k_r \cdot S \cdot [C_i - C_s]^g \qquad [7]$$

where C_s is the solubility of the solute, k_r the kinetics constant of the surface reaction, and g the reaction order. The growth kinetics, $\frac{dM}{dt}$, are expressed as the mass of aqueous solution deposited on the crystal per unit time. However, the interface concentration C_i is not accessible in experiments, and the growth kinetics are therefore expressed according to supersaturation concentration as $C - C_s$:

$$\frac{dM}{dt} = k_c \cdot S \cdot [C - C_s]^n \qquad [8]$$

where k_c and n are the total growth kinetics constant and the total order of growth, respectively. According to the situation, the diffusion stage may be limiting, where, for instance, the surface reaction is very fast, the mass transfer is limited, or the growth is limited by the surface reaction. In the first case, $n = 1$ and $k_c = k_d$; in the second, $n = g$ and $k_c = k_r$; and in the intermediate case, where neither of the two steps is limiting, k_c and n are complex functions of k_d, k_r, and g. Growth kinetics can also be defined as the increase in the solute mass deposited per unit of time and crystal surface $\frac{1}{S} \cdot \frac{dM}{dt}$, or as the increase in a dimension characteristic of a crystal λ (which, e.g., can be the equivalent diameter or the dimension obtained by sifting). In this case the growth kinetics are written as

$$\frac{d\lambda}{dt} = \frac{1}{S} \cdot \frac{f_s}{3 \cdot \rho \cdot f_v} \cdot \frac{dM}{dt} \qquad [9]$$

where f_s is the factor of surface form and f_v the factor of volume form.

To summarize the main points regarding the mechanisms and kinetics of the crystal growth stage of monohydrated α-lactose,

- The diffusion stage is not kinetically limiting, except in particular cases when the growth kinetics are significant at high temperatures and supersaturations (>60 °C).

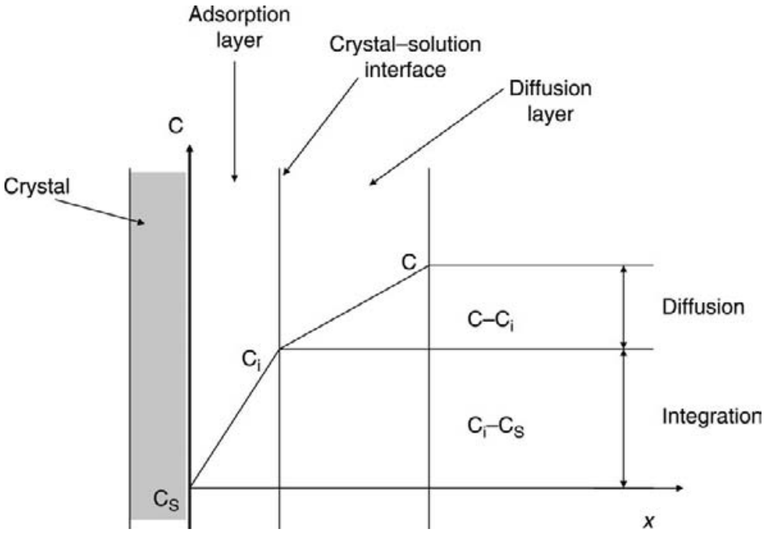

Figure 8 Concentration gradient at the crystal surface in growth.

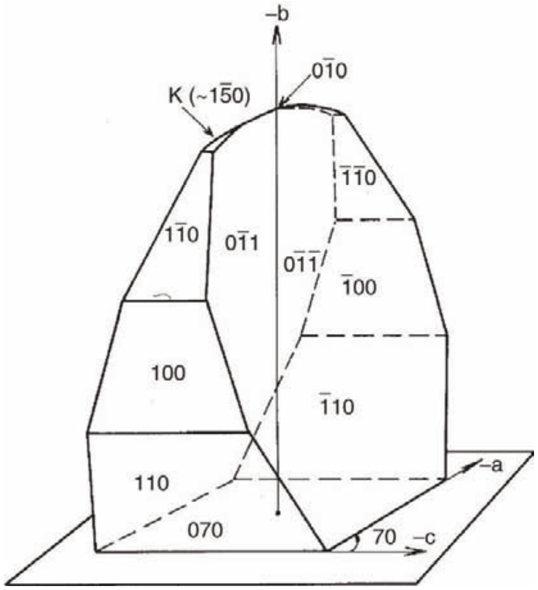

Figure 9 Morphology of α-lactose monohydrate crystal.

- By measuring the growth kinetics of the faces (010) and (100) of nonionic lactose in the $+b$ direction (**Figure 9**), it has been shown that there are two growth mechanisms related to the supersaturation value. The BCF model applies to values less than 1.35, and the two-dimensional birth-and-spread nucleation model to values above 1.5.
- Two separate studies, one using pharmaceutical lactose and the other using nonionic pharmaceutical lactose, have provided correlation of the experimental findings with eqn [3] over a wide range of supersaturations. In the latter study the growth reaction was estimated to be in the order of $n = 2.8$, and the growth kinetics constant k_c was estimated to be 0.7×10^{-3} g monohydrated α-lactose $\text{m}^{-2}\,\text{min}^{-1}$ (g α-lactose $100\,\text{g}^{-1}$ water)$^{-2.8}$ in both the studies.

Technological parameters and crystal growth kinetics

Following the example of nucleation, the growth kinetics generally depend on factors related to the nature of the aqueous solution and factors related to the operating conditions (supersaturation, temperature, presence of impurities).

Supersaturation

Growth kinetics increase with supersaturation, and growth typically varies in the order of 1–2. As in the case of nucleation, it should be noted that too high a level of supersaturation results in a reduction in the speed of growth due to the reduced molecular mobility. The growth of monohydrated α-lactose does not seem to be an exception to this rule, although little has been published on this.

Temperature

Growth kinetics increase with an increase in temperature at constant supersaturation. The kinetics growth constant, k_c, generally depends on temperature, while following Arrhenius's law, as does eqn [5]. The activation energy associated with the growth of monohydrated α-lactose has been estimated at 22.1 kcal $\text{mol}^{-1}\,\text{K}^{-1}$. It is important to remember that the activation energy of the surface reaction is generally quite a bit higher than that of the diffusion stage. This implies that the limiting stage can change according to the temperature and that at a high temperature the diffusion stage is likely to become limiting. This is the case for sucrose above 40 °C with a relative supersaturation of 1.09. The same tendency has been reported in the case of monohydrated α-lactose, but at much higher temperatures and supersaturations.

Stirring

Stirring conditions and the related crystal–solution kinetics can affect resistance to diffusion transfer and thus increase the growth kinetics when the latter is limited by diffusion. Nevertheless, when the diffusion stage is not limiting, as in the case of lactose, total immobility of the system does not permit the supersaturated solution sufficient access to the crystal surface, resulting in reduced growth kinetics. However, this is not due to a reduction of the kinetics constant or order of growth, but appears to result from a reduced area of the crystal–solution interface (see eqn [6]).

Interactions between nucleation kinetics and growth

It is important to note that the kinetics of the nucleation and growth stages influence each other. The growth kinetic depend closely on the crystal surface (eqn [6]), which itself depends closely on the nucleation kinetics – rapid nucleation kinetics leading to a large number of crystals developing a large surface area. This implies that all the factors that influence nucleation kinetics also influence growth kinetics. On the other hand, the presence of the variable M (crystal mass) in eqn [4] describing nucleation kinetics suggests that nucleation kinetics are influenced by the kinetics of the growth stage.

Influence of impurities

The majority of food products contain many other species in addition to the aqueous crystallization solution. Most of the crystallization occurs in matrices rich in impurities of various types. In the case under consideration here (lactose crystallization in whey concentrates), the main crystallization occurs in the presence of whey proteins and caseins, lactic acid, various electrolytes (calcium, sodium, magnesium, potassium, chloride, lactate, phosphate, citrate, etc.), and the β-lactose anomer present at concentrations higher than the concentration of the α form (as described above).

The presence of impurities in a solution can have an indirect influence on the growth kinetics stage by modifying solubility and hence supersaturation. The impurities can also affect the growth kinetics by modifying the surface of the crystal–solution interface via the acceleration or deceleration of the nucleation stage. Finally, certain impurities are likely to modify the crystallization kinetics constant directly. They then have several modes of influence such as

- *Modification of the diffusion transfer constant*: In particular, the largest molecules can impede growth when it is limited (or becomes limited) mainly by the diffusion stage.
- *Inhibition of the surface reaction by adsorption onto the crystal–solution interface*: The surface reaction is then slowed down as the impurities need to be removed before adsorption and incorporation of the crystallizing molecules of solute. Adsorption of the impurities can be selective when it involves certain crystal faces rather than others. It can be reversible or, when it results in incorporation of the impurity within the crystal network, irreversible. Molecules such as proteins, or even biopolymers (oligonucleotides, dextrans), can get incorporated into monohydrated α-lactose crystals.

Macromolecules

Even a very low dose of a macromolecule, such as gelatin (10 mg l^{-1}), is able to inhibit the growth of lactose crystals, even at a high lactose supersaturation. This suggests a mode of inhibition by non-selective adsorption on all crystal faces. This property is also used with alginate, carrageenans, and various vegetable gums to inhibit the growth of lactose crystals in dairy ice cream formulations in order to prevent the development of the sandy character of such products.

Similar additives

The case of structurally similar impurities or additives, referred to as 'tailor-made', is particularly interesting. These molecules have the structural characteristic of being analogous on a molecular scale to the crystallizing solute, and this enhances interactions with the crystal surface. In view of the differences in structural organization between faces on a molecular scale, such additives are believed to adsorb selectively on some surfaces, with a subsequent slowing of growth kinetics in certain crystallographic directions and changes in crystal morphology. Several examples of this type of inhibition are encountered in the growth of monohydrated α-lactose crystals.

The β-lactose anomer is the major form, accounting for approximately 60% of lactose present at equilibrium at 30 °C, and it differs from the α form only in the position of the hydrogen bond of the 4-O-glucose unit (**Figure 3**). The galactosyl unit of both the anomers is identical. Supersaturated lactose solutions thus inherently contain an impurity that is structurally similar to α-lactose (i.e., the crystallizing solute) in large amounts. Several research groups have investigated the inhibitory action of the β anomer. Such studies have concluded that there is a selective inhibition in certain crystallographic directions, leading to a 'tomahawk' morphology (**Figures 9** and **10**). It was suggested that molecular modeling could be used to establish that the tomahawk morphology is explained by the specific adsorption of the β anomer on the faces $(0\bar{1}1)$ and $(0\bar{1}\bar{1})$, and this has subsequently been confirmed. At a molecular level, inhibition by β-lactose is attributed to the insertion of the galactosyl unit common to the crystal network, the dissimilarity of the other half of the molecule preventing continuation of growth at the β-lactose incorporation sites. It is of note that only a few grams of β-lactose are required for 100 g of water in order to achieve a significant result, and that such inhibition is effective even with high levels of supersaturation of the α form. However, above a β:α ratio of 1, the inhibiting effect is not further increased by increasing the concentration of the β anomer, meaning that, in practice, starting from a supersaturated solution (β:α ratio >1.5), inhibition by the β form during α-lactose crystallization is a constant whatever the operating conditions.

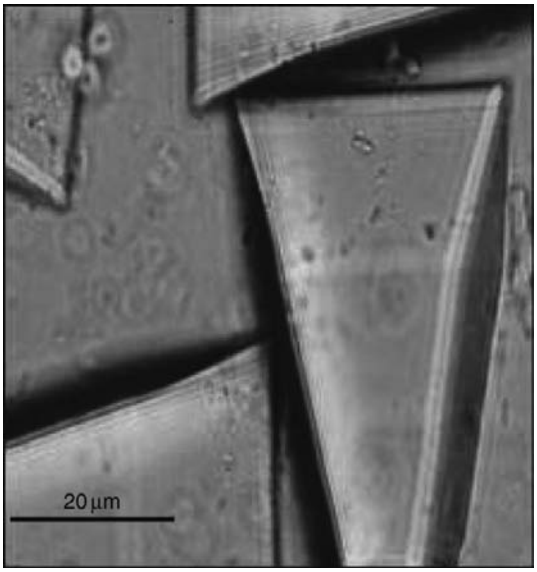

Figure 10 Photomicrograph of α-lactose monohydrate crystal. Scale=10 μm.

The influence of other structurally close additives (α-galactose, β-cellobiose, maltitol, α-glucosamine hydrochlorate) on the growth of monohydrated α-lactose was reported recently. The hypothesis proposed to explain the ability of such additives to be adsorbed on the surface, and their inhibitory capacity, was the number of hydrogen bonds that can be created with the three-dimensional network of molecules of monohydrated α-lactose. This study showed that α-galactose, β-cellobiose, and maltitol can be adsorbed selectively on some faces, leading to a flattened morphology of the monohydrated α-lactose crystals. In contrast, the presence of α-glucosamine hydrochlorate results in a lengthened morphology by selectively inhibiting growth on the faces ($1k0$).

Lactose phosphate impurities have an effect on lactose crystallization. This impurity is in fact a mixture of several lactose phosphate shapes that differ by the position of the phosphate group on the lactose molecule. Ninety percent of the molecules of this mixture have their phosphate group on the galactose moiety, the glucose moiety remaining identical to that of the α-lactose molecules. As their preferential incorporation into monohydrated α-lactose crystals during successive crystallizations yields pharmaceutical lactose crystals, they are more commonly found in pharmaceutical products than in a whey concentrate (a few hundred milligrams per liter). This impurity has a powerful inhibitory capacity even at low concentrations (i.e., $100\,\mathrm{mg\,l^{-1}}$ is necessary for maximum inhibition) on the (010) crystal face in the $+b$ direction, which points to the galactosyl unit in this direction (**Figure 9**). On a molecular scale, the molecules of lactose phosphates are incorporated without difficulty into the α-lactose molecules of the crystal network, due to their common glucose unit, but the galactose phosphate unit on the surface prevents further growth at the level of the impurity incorporation sites.

As lactose phosphate (Lp) is acidic (acid activity), its presence in pharmaceutical lactose powder explains the natural pH (3–4) of the solutions based on these powders. It should be noted that the protonated H_2Lp forms and the double deprotonated Lp^{2-} forms are much less inhibitory to the growth of (010). The first level of acidity having a pK_a close to 1 explains the increase in growth kinetics observed at very acidic pH, minimum kinetics at pH close to 2.5, and the slow increase in the growth of faces (010) and (110) at the time of an increase in pH related to the basal–acidic equilibrium of lactose phosphates. These variations were not observed during the crystallization of nonionic lactose, that is, without the impurity.

Lactose phosphate also complexes with certain cations. The reduction in the inhibitory ability of these complexes explains the increase in the growth kinetics of the face (010) when a supersaturated solution originating from pharmaceutical lactose is used with KCl, NaCl, $CaCl_2$, and $MgCl_2$.

These results therefore indicate that the results of studies on the influence of other impurities (particularly ions) on the growth kinetics of lactose when the studies use pharmaceutical lactose cannot be easily extrapolated to the growth of crystals in a whey concentrate, and must be interpreted in the light of possible interactions with lactose phosphate impurities.

Riboflavin

Riboflavin is present at levels of a few milligrams per liter in whey concentrates. It is adsorbed onto and incorporated in lactose crystals and is responsible for the yellow color of edible lactose. Along with lactose phosphate, riboflavin is a very effective inhibitor. A few milligrams per liter is enough to very effectively slow down the growth of monohydrated α-lactose crystals in the $+a$ direction. However, the precise mechanism of inhibition by riboflavin is still not understood.

Comments

Several comments can be made on the influence of impurities generally:

- The influence of impurities, and particularly their inhibitory capacity, depends on the degree of α-lactose supersaturation. Inhibition is often much weaker with high supersaturation. To explain the reduction in the inhibitory capacity of the β form in relation to supersaturation of the α form, it has been suggested that higher levels of lactose supersaturation might involve a reduction in selectivity during molecule identification. Thus, β-lactose molecules might be adsorbed effectively, but would not prevent the incorporation of an α-lactose molecule. The β-lactose molecules would then be incorporated into the crystal. The consistent presence of the β form in monohydrated α-lactose crystals tends to confirm this hypothesis. More generally, as reported by various authors, the reduction in growth inhibition by impurities when there is a high degree of supersaturation of the crystallizing solute might result from greater competition for adsorption between the solute and the impurity molecules, to the detriment of the latter because they are present in smaller numbers.
- Impurities seldom have a single mode of influence. It is not out of the question that the same impurity may at the same time affect the solubility of the crystallizing aqueous solution, increase its nucleation kinetics, and inhibit its adsorption on a crystal surface. It would thus be extremely difficult to predict its impact. In the case of lactose, the impurities can, in addition, influence the equilibrium or the mutarotation kinetics, on which supersaturation and thus the nucleation and growth kinetics depend.

Conclusions

Lactose crystallization in whey is a key stage in the manufacture of whey powders. Controlling this stage at an industrial level should increase the prospects of improving the process and also the physicochemical qualities of the powders.

Lactose crystallization occurs in highly supersaturated solutions, indicating that the phenomena of nucleation and crystal growth can take place simultaneously. In addition, in the case of lactose a preliminary stage of mutarotation between anomeric forms, each with its own kinetics, is present. The literature shows clearly that the laws of kinetics that control these phenomena are different, but interact closely and are interdependent. Although the lactose crystallization mechanisms have been described widely by many authors, and the parameters of the kinetics (orders and constants) calculated for the mutarotation, nucleation, and lactose crystal growth stages, few investigations have been undertaken into the interactions between the kinetics using integrated approaches.

At an industrial level, lactose crystallization is also performed in a medium of complex chemical composition. In particular, certain macromolecules, such as the whey proteins, the influence of which on the kinetics of lactose crystallization has received very little attention, are also present. The literature indicates that the kinetics can be modified by the presence of other components at each change of state. In order to understand their influence fully, it is necessary to understand their specific effects on lactose solubility and the laws of the kinetics controlling the stages of mutarotation, nucleation, and growth.

Awareness of the general principles of crystallization in solution should make it possible to provide greater control of the unit operation of lactose crystallization prior to the drying process. These principles should also shed light on the physicochemical modifications involved in changes in the state of components other than lactose present at significant concentrations in whey concentrates.

See also: **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk; Lactose Intolerance; Lactose: Chemistry, Properties; Lactose: Derivatives; Lactose: Galacto-Oligosaccharides; Lactose: Production, Applications; Maillard Reactions.

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Lactose: Production, Applications

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Lactose Source

Lactose, the main carbohydrate in mammalian milk, is a by-product that remains in the whey when cheese or casein is produced from milk. The current world production of cheese is approximately 17 million tonne annually. This quantity of cheese equates to an estimated 157 million tonne of whey, from which 6.3 million tonne of lactose could be extracted. 3A Business Consulting have estimated that the current world lactose production is only 0.89 million tonne. Thus, there is a large excess of potentially available lactose. This surplus in supply means that the long-term average price of lactose remains at a value that justifies the investment and processing costs required for its recovery. **Figure 1** shows the price of lactose for the period from 2003 to 2008. The peak in 2007 is because of a temporary shortage of lactose that occurred when dairy companies bought lactose on the open market to standardize their milk powders to the CODEX-allowed protein limit. This made sense, as lactose could be bought for €300 tonne^{-1}, added to milk powder, and sold at €1442 tonne^{-1}. The high price for edible grade lactose seen in 2007 reflects this economics. This demand was quickly met by higher lactose production and hence the price has again dropped to more traditional levels. The price in early 2009 (€233 tonne^{-1}) reflects this and has been amplified by the downturn in dairy commodity prices. Prices as low as €90 tonne^{-1} were recorded in Asia in early 2009 as US dairy companies dumped lactose onto the market to clear space in their warehouses for other, higher value, dairy products.

Applications of Lactose

To understand the production of lactose, it is useful to understand the markets that exist for it and their requirements for quality of product. A breakdown of the uses of lactose in the European Union for 2005 is summarized in **Figure 2**. The main markets for lactose are as a supplement for infant formula milk powders and as an ingredient in confectionary, baking, and other foods.

Henry Nestlé was the first to realize that cow's milk, which typically has a lactose content of around 4.5%, would be more nutritious if the lactose content was raised to 7%, which is the same as in human milk. He produced the first infant formula with the increased lactose content and found that the problems previously associated with feeding human babies with milk made directly from whole milk powder made from cows' milk were generally overcome because of the higher energy (lactose) content.

This desire to make infant formulae more like human milk is opening up a new market for lactose as lactose is the logical starting point for making galacto-oligosaccharides. It has been realized for some years that the number of oligosaccharides in human milk is between 50 and 200, depending on the mother. The oligosaccharides in human milk make up about 5–8 g l^{-1}, while there is only a trace amount in bovine milk. These have been identified as the reason for the difference in stool quality between formula-fed babies and breast-fed babies. Recent research has shown that these oligosaccharides also have anti-infection properties in the lower gut as well as probiotic effects. Thus, there is a drive to start including a mixture of oligosaccharides into infant formulae. The research is still being carried out to determine which oligosaccharides provide benefits to the infant and what is the best source of β-galactosidase enzyme for producing oligosaccharides from lactose.

In baking, lactose is used for its ability to survive yeast fermentation and hence provide a reducing sugar for the Maillard browning reaction at the surface of the bread, which enables the bakers to form desirable brown crusts. In the production of some confectionary products, it is the ability of lactose to make superb caramel that makes lactose a more attractive choice than other sugars. Another reason for choosing lactose in baking and confectionary production is that it is not as sweet as sucrose and offers better mouthfeel. The lactose required for these and other food products needs to be of an edible standard, but it does not need to be ultrapure, leading to the general grade of lactose known as edible lactose. This market currently uses around 780 000 tonne per annum.

The other main use of lactose is in the pharmaceutical industry where it is used as an excipient for making tablets and as a carrier in dry power inhalers. There are three main lactose products in the pharmaceutical area: alpha lactose monohydrate, anhydrous (beta) lactose, and spray-dried lactose. All three are used to make tablets with the choice being decided by the desired powder properties of the manufacturer. Anhydrous lactose is known to make stronger tablets, and spray-dried lactose, which has good flow properties, produces good tablets. For inhaler grade lactose, pharmaceutical grade alpha lactose monohydrate crystals are sterile sifted to produce

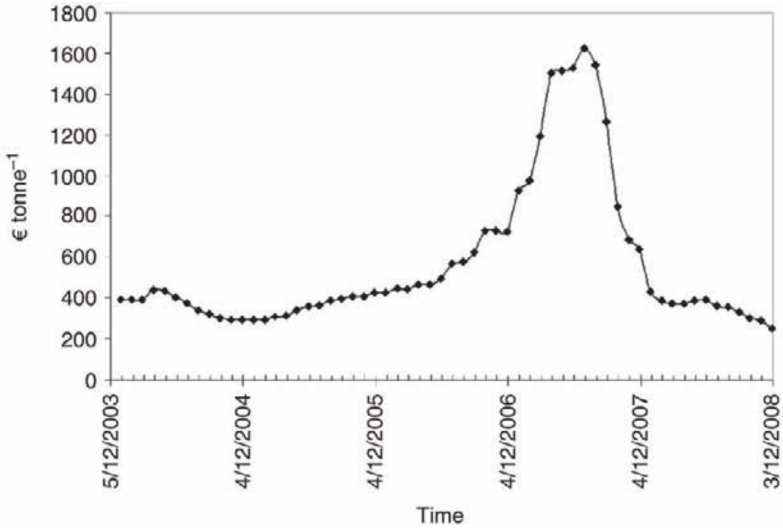

Figure 1 Edible lactose price from 2003 to 2008 in euros per tonne. Data taken from Clal.it web page http://www.clal.it/en/index.php?section=lattosio_usa.

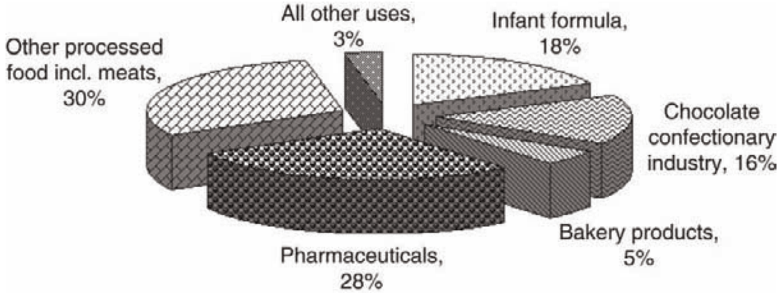

Figure 2 EU market structure for lactose in 2005. Reproduced from Affertsholt-Allen T (2007) Market developments and industry challenges for lactose and lactose derivatives. *IDF Symposium 'Lactose and Its Derivatives'*. Moscow, Russia.

a product with a very narrow particle size around the 90 μm range.

The total pharmaceutical grade lactose market was estimated by 3A Business Consulting to have been 110 000 tonne in 2008.

Edible Grade Lactose Production Process

The first description of the edible lactose production process was provided by Weisberg in 1954. **Figure 3** shows a typical modern edible grade lactose plant. In the late 1970s, ultrafiltration membranes were developed to remove whey proteins to produce whey protein concentrate (WPC) from the whey before it goes to the lactose plant. The stream that reaches the lactose plant is now typically referred to as whey permeate (the stream that has been allowed to permeate (pass) through the ultrafiltration (UF) membranes) and is primarily made up of lactose and milk salts. Whey protein has been found to be a highly valued protein with its main areas of consumption being in sports drinks and in body building supplements. When the whey permeate arrives, it should have its lactose content measured so that the lactose yield through the plant can be monitored. Often, this is not done and general historical lactose levels must be used for the yield calculations.

The basic steps in the production process are as follows.

Whey Permeate Concentration

This is usually accomplished by a combination of reverse osmosis followed by evaporation up to around 110 g lactose per 100 g water (58% total solids (TS) for whey permeates). The higher the TS achieved in the evaporator, the greater the lactose yield that can be achieved from the following crystallization step.

Crystallization

Most lactose plants use a batch cooling crystallizer. The saturated mother liquor that arrives from the evaporator is at around 70 °C and is cooled to about 15 °C.

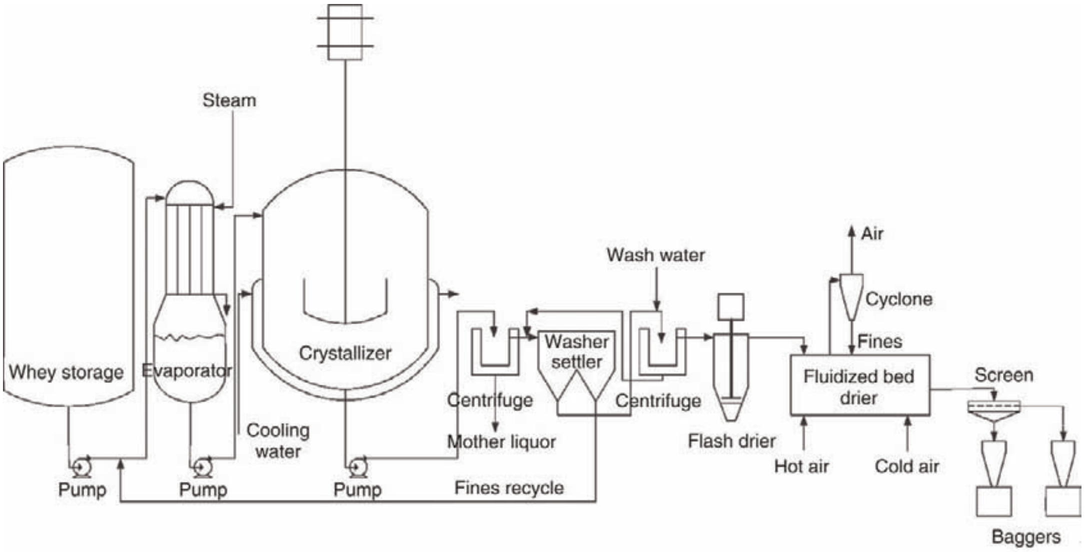

Figure 3 Process flow diagram of a plant producing edible grade alpha lactose monohydrate crystals. Reproduced from Paterson AHJ (2009) Production and uses of lactose. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry*, Vol. 3. Springer Science+Business Media, LLC.

The final temperature usually depends on the ambient conditions of the plant and the cooling streams available. If chilled water is available, then a further lower temperature may be targeted provided sufficient time is available in the crystallizers to allow for the extra growth to occur. There is a direct correlation between the yield of lactose obtained in the plant and the final temperature achieved.

Some plants use evaporating crystallizers, and these allow higher TS and yield to be achieved. In these systems, the majority of the nucleation and growth of the crystals occurs in the evaporator, reducing the risk of showering due to the creation of excessive supersaturation.

The crystallization step is the heart of the lactose production process and it is the area that is considered the hardest to get right. There are many variables that affect the process and these often interact, making it hard to determine exactly what effect each variable has; therefore, many people regard the whole area as an art rather than a science. There have been several university studies clarifying some aspects of the lactose crystallization process. It is now obvious that the critical step is the nucleation step at the start of the crystallization process and this needs to be controlled if the right-sized crystals are to be produced. Current research is focusing on this area.

Crystal Separation and Washing

The crystals produced in the crystallizer are separated from the mother liquor using centrifuges. Some plants carry out a washing step in the centrifuge, but most pass the crystals into a washing section where they are washed with fresh water to remove any mother liquor still adhering to the crystals.

The effectiveness of washing is affected by the crystal size obtained in the crystallizers. When a large amount of fines is produced, then the washing stages are less effective and poor-quality product results. The increased difficulty in separating these fines from the liquid streams also leads to much lower yields.

Drying and Packaging

The crystals are recovered from the washing stream by another centrifuge and are dropped into a flash drier at 4–10% free moisture content (this does not include the 5% moisture associated with the water of hydration bound in the crystal). In the flash drier, they come in contact with hot air (120–150 °C), which rapidly dries the lactose syrup on the surface of the crystals, creating a small layer of amorphous lactose on the surface of the crystals. If this amorphous layer remains intact through the rest of the drying and packaging process, then, unless its moisture content is maintained low enough, it can lead to caking problems. From the flash drier, the crystals travel into a fluid bed drier. A fluid bed drier usually has two sections. The first section is the final drying section, which dries the lactose from about 1% free moisture down to the final targeted moisture content. The second section of the fluid bed drier is used to cool the product before sifting and packaging. Failure to cool the product before it goes into the bag leads to caking in the bag induced by the temperature gradient. To avoid caking, the final moisture content should be low enough to ensure that the water activity (a_w) of the final product is below 0.3 and the temperature of the product is below 30 °C.

Pharmaceutical Grade Lactose

Pharmaceutical grade alpha lactose monohydrate is produced from edible grade lactose crystals by redissolving them in clean water. The resultant solution is mixed with activated carbon and a flocculent and then filtered. The clean lactose solution is then concentrated and crystallized. Both batch and continuous crystallizers have been used for this process. **Figure 4** shows the process.

An alternative process that uses ion exchange, nanofiltration, chromatography, evaporation, and crystallization, without the need for a second crystallization step, with a yield approaching 95% for producing pharmaceutical grade lactose monohydrate directly from whey permeate has been patented. It is understood that at least one European company has a license agreement to build a plant using this technology.

The second grade of pharmaceutical lactose is anhydrous lactose. This is the lactose crystal in the beta form, which is the sweeter of the two lactose isomers. To produce beta lactose crystals, lactose must be crystallized at temperatures above 93.5 °C. A clean lactose solution, produced as in the manufacture of pharmaceutical grade lactose monohydrate, is sprayed onto a hot roller drier. The result of the rapid crystallization at temperatures above 93.5 °C is a cake of very fine crystals containing typically 70–80% beta lactose and 20–30% alpha lactose molecules with no water of crystallization. The cake is then milled and sieved to produce particles of the desired range and the product is packaged.

The third pharmaceutical lactose product is spray-dried lactose. Spray-dried powders of lactose are produced from pharmaceutical grade alpha lactose monohydrate crystals by suspending them in clean water and then spray drying the mixture. This results in spherical crystal agglomerates bound together by amorphous lactose. The amorphous lactose tends to be at the center of the agglomerates, reducing the problems with caking that occur when it is present on the surface. This and the spherical particle shape mean that the product is free flowing and forms very good tablets.

Typical Problems Encountered during the Production of Lactose

Fouling of the Evaporators

This is usually caused by the precipitation of calcium salts, an effect that can be made worse by the coprecipitation of the residue protein still present in the whey permeate. Ways of minimizing this problem have included treatment of the whey with chelating agents that bind calcium and removal of the calcium by ion exchange before the preconcentrated whey is passed to the evaporators.

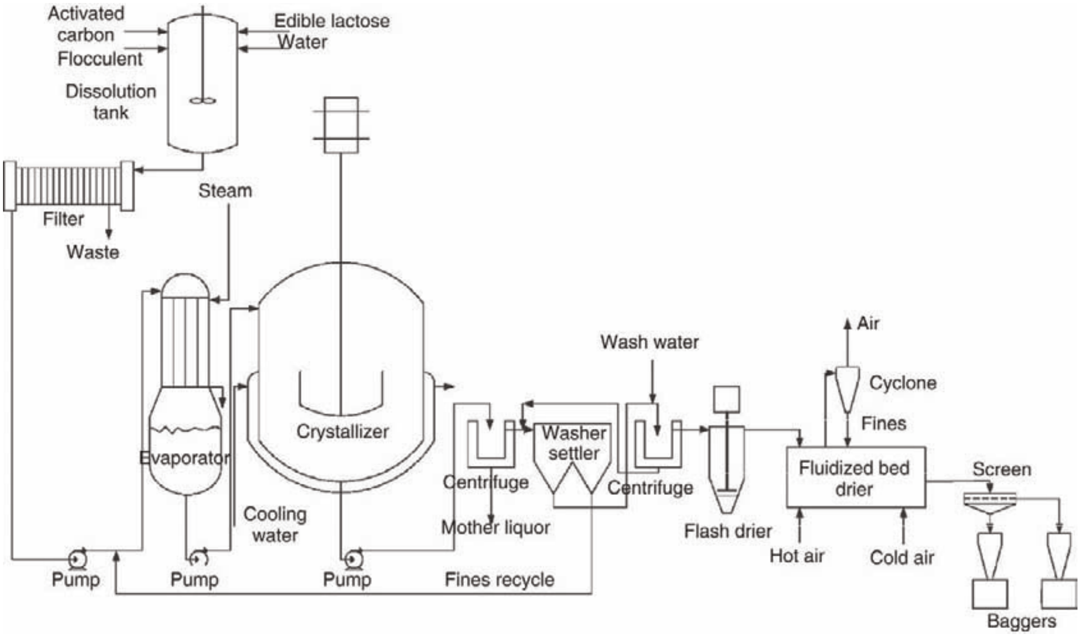

Figure 4 Process flow diagram of a plant producing pharmaceutical grade alpha lactose monohydrate crystals. Reproduced from Paterson AHJ (2009) Production and uses of lactose. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry*, Vol. 3. Springer Science+Business Media, LLC.

Fermentation of the Whey

This can occur due to the growth of lactic acid bacteria in the holding tanks or during the transportation of the whey to the lactose plant. The result is a drop in the pH of the whey and the production of substances that can inhibit crystallization. This inhibition can lead to the production of fines during the crystallization. The fermentation also directly leads to a loss of lactose yield.

Fines Generation in the Crystallizers

There are many factors that can lead to the production of fines in the crystallizers. Most can be traced back to an inadequate nucleation event at the start of the crystallization, which leads to the late generation of massive numbers of nuclei in the solution. These nuclei form from a secondary nucleation event that occurs when the lactose supersaturation builds up because the first nucleation event did not produce enough crystal surface to take the lactose out of solution fast enough for the lactose concentration to follow the cooling curve. Thus, the key to having consistent crystal particle size distributions coming from the crystallizers is to control the initial nucleation event. This is not easily done as there are many variables that influence this event. The two most important are the supersaturation of the lactose solution coming from the evaporator to the point at which the nucleation happens, and the intensity of mixing that occurs at the nucleation point. Usually, the second one is attended to at the design phase. Many plant designers do not understand the importance of this variable on the overall performance of the plant, affecting both the rate of nucleation and the system homogeneity. The concentration, on the other hand, seems to be a variable that often varies considerably during the plant operation, much to the detriment of the overall lactose plant yield.

The formation of too many fines in the crystallizers affects the yield in two ways. The first is the loss of production and the second is that when the fines are recycled back through the plant, water is used to dissolve them and hence lactose is lost as mother liquor. A side effect of fines is that more mother liquor accompanies the crystals into the wash stage, leading to less efficient washing and hence a lower-quality product.

The effect of fermentation by-products on the crystallization process has been speculated upon, but there is very little published work that quantifies these effects. However, plants that run with good cleaning routines have fewer fines generation problems than plants that are experiencing fermentation problems upstream. It is an area open for further research.

Caking

Caking in the lactose industry is an ongoing problem; it tends to be more prevalent for edible-grade lactose, with many customers complaining of receiving 900 kg bricks rather than 900 kg of free-flowing powder. The usual cause of caking is that the lactose has not been dried enough. This, combined with moisture movement within the bag caused by temperature gradients that have arisen during transportation or at the time of packing, causes capillary condensation between the particles leading to lumping and then caking. The solution is to make sure that the lactose product is dried to sufficiently low moisture content and then cooled with dehumidified air before it is packed. A a_w of 0.3 is required if a temperature gradient of 30 °C is to be encountered. a_w is used instead of moisture content, as it more accurately reflects the state of free moisture available within the product. The moisture content is related to the a_w of the product by the adsorption isotherm. The isotherm for alpha lactose monohydrate with no amorphous lactose present is presented in **Figure 5**.

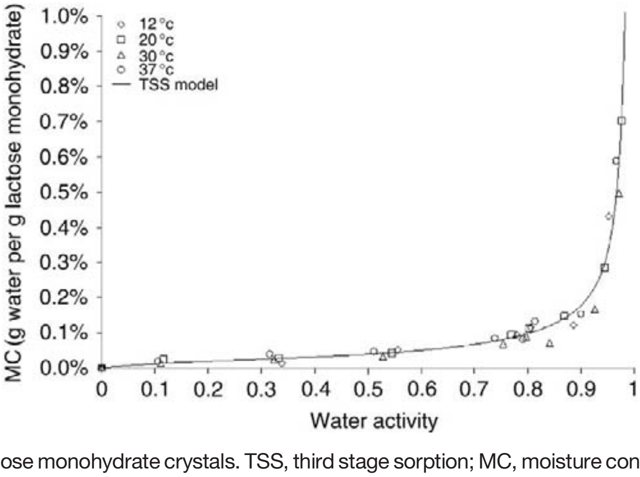

Figure 5 Isotherm for alpha lactose monohydrate crystals. TSS, third stage sorption; MC, moisture content. Reproduced from Bronlund J and Paterson T (2004) Moisture sorption isotherms for crystalline, amorphous and predominately crystalline lactose powders. *International Dairy Journal* 14: 247–254.

This shows that a small change in free moisture content leads to a large change in a_w, especially for $a_w < 0.5$. For this reason, it is recommended that a_w be measured instead of moisture content when determining whether a product is safe to ship.

Future of Lactose

There is an untapped supply of lactose available in raw whey. Currently, much of this is turned into whey permeate powder and sold as a commodity product at around €340 tonne^{-1}. Various derivatives of lactose have been made, including galacto-oligosaccharides, lactulose, lactitol, lactobionic acid, hydrolyzed lactose, and tagatose. These have found niche markets with various uses. The areas where current research indicates that significant amounts of lactose might be needed to meet demand are in the production of galacto-oligosaccharides for addition to infant formulae to make them more like human milk and in the production of tagatose, which has the potential to be a sugar replacement. More details on these are covered in other sections of this encyclopedia (*see* **Lactose and Oligosaccharides**: Lactose: Derivatives; Lactose: Galacto-Oligosaccharides).

If the price of lactose remains low, then it is possible that lactose could become a substrate for the production of bioethanol for use in transportation fuels or a substrate for other fermentation products.

See also: **Lactose and Oligosaccharides**: Lactose: Derivatives; Lactose: Galacto-Oligosaccharides.

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Lactose: Derivatives

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Introduction

Lactose derivatives are obtained from lactose by chemical, enzymatic, or microbial modifications. An overview of the chemical or enzymatic processes used for conversion of lactose is shown in **Figure 1**. Lactobionic acid, lactitol, lactulose, and lactosucrose are commercially produced for a variety of food and pharmaceutical applications (**Table 1**). Predominant food applications of established or experimental lactose derivatives include their use as laxatives and prebiotic carbohydrates. Comparable to dietary fiber, prebiotic carbohydrates are not digested in the small intestine and are converted to short-chain fatty acids, CO_2, and H_2 by the colonic microflora. Prebiotic carbohydrates additionally allow the selective stimulation of growth and/or the activity of beneficial members of the intestinal microbiota, particularly bifidobacteria.

Prebiotic metabolism is understood best for fructo-oligosaccharides and galacto-oligosaccharides. Galacto-oligosaccharides consist of β-linked galactose moieties with galactose or glucose at the reducing end. Their properties and principles of their enzymatic synthesis from lactose are discussed in a separate article (*see* **Lactose and Oligosaccharides**: Lactose: Galacto-Oligosaccharides). In direct analogy to the production of galacto-oligosaccharides by enzymatic transgalactosylation of lactose or galactose, oligosaccharides consisting of β-linked galactose moieties with fructose, mannose, pentose, fucose, or other monosaccharides or disaccharides at the reducing end are produced by β-galactosidases from lactose when the appropriate acceptor carbohydrate is present. Although the enzymatic production, chemical structure, and biological activity of these β-galactosylated oligosaccharides are very similar to those of galacto-oligosaccharides, these compounds are generally not included in the definition of galacto-oligosaccharides and are referred to as hetero-oligosaccharides. This article presents the structure and biotechnological production of commercially produced lactose derivatives as well as the potential for application of some of the experimental lactose derivatives obtained by transfructosylation or transglucosylation of lactose, or by transgalactosylation of other acceptor compounds.

Human milk contains 1–1.3% oligosaccharides of over 200 different structures; the content and the structural complexity of bovine milk oligosaccharides are substantially lower. The majority of oligosaccharides in the milk of mammals have lactose at the reducing end; oligosaccharide structures are extended by glucose, galactose, fucose, *N*-acetylglucosamine, sialic acid, or *N*-glycolylneuraminic acid. They serve a variety of nutritional and protective functions and can be regarded as the natural template for the development of prebiotic carbohydrates as well as other biological activities of the synthetic galacto-oligosaccharides or hetero-oligosaccharides. On the basis of their structure, milk oligosaccharides can be considered as naturally occurring lactose derivatives; however, because their chemical structure, occurrence, and function differ from those of currently established or experimental lactose derivatives, they are not discussed in this article (*see* **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk).

Lactose Derivatives Produced on a Commercial Scale

Lactobionic Acid

Lactobionic acid (β-4′galactosylglucuronic acid) is produced by chemical oxidation of lactose. Biotechnological alternatives for the production of lactobionic acid include enzymatic conversion by glucose–fructose dehydrogenase or cellobiose dehydrogenase with concomitant use of laccase to achieve cofactor regeneration, and fermentative oxidation by *Pseudomonas* species. Low concentrations ($45\,\mathrm{mg\,kg^{-1}}$) of lactobionic acid were identified in yogurt-type dairy products fermented with lactococci and *Acetobacter orientalis*.

Lactobionate is a strong chelator of calcium and other divalent cations. It is commercially applied as an ion sequestrant in detergent solutions, and as a calcium chelator in dietary supplements. Applications in the cosmetic industry include its use as a humectant in skin care products. The use of lactobionate at $100\,\mathrm{mmol\,l^{-1}}$ acid in preservation solutions for the storage of transplant organs represents the most prominent application in the pharmaceutical industry. The iron-chelating properties of lactobionate prevent the formation of reactive oxygen species and reduce oxidative tissue injury during storage. Lactobionate is cell impermeant and thus prevents hypothermically induced cell swelling.

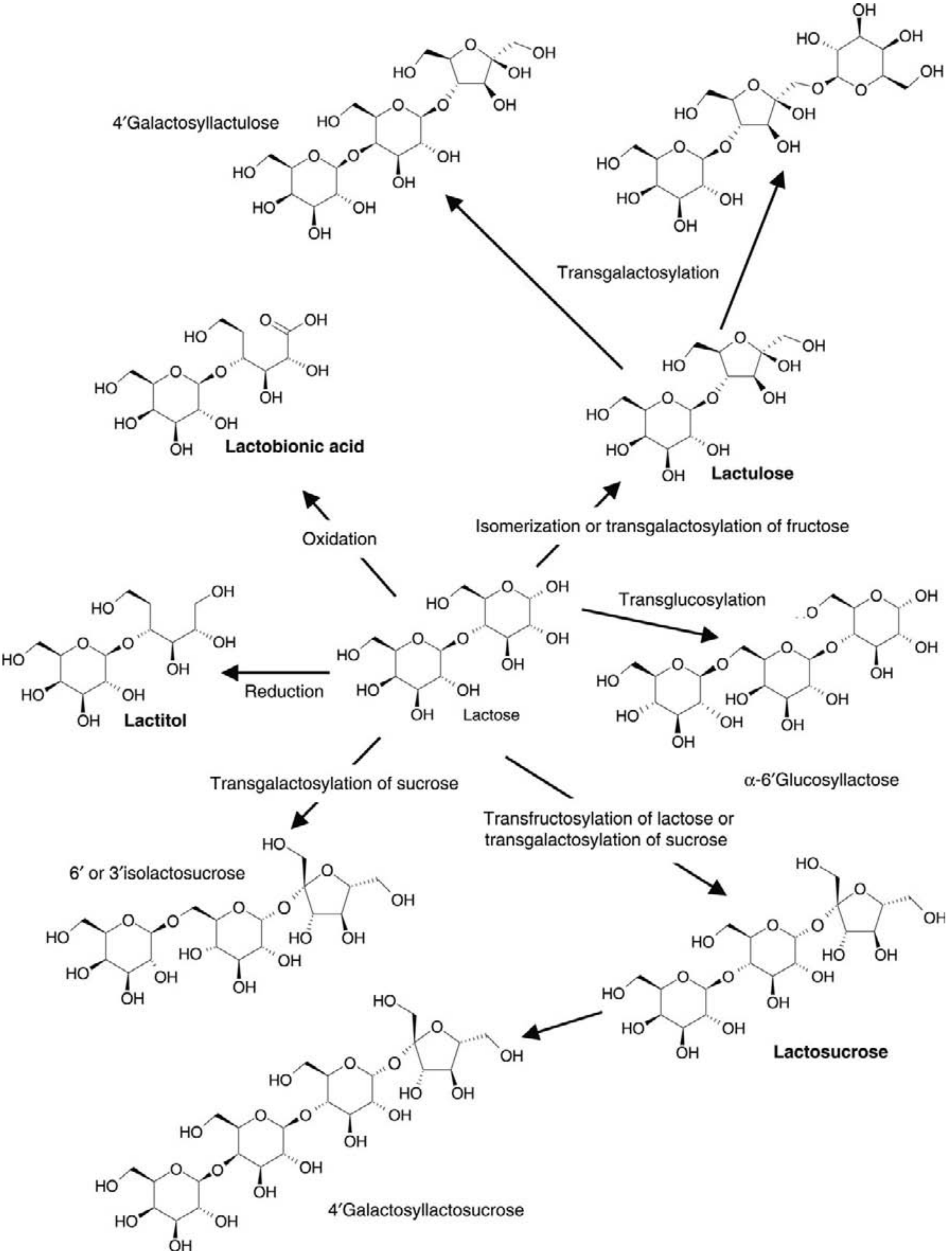
4′Galactosyllactulose
Transgalactosylation
Lactobionic acid
Lactulose
Oxidation
Isomerization or transgalactosylation of fructose
Transglucosylation
Lactitol
Reduction
Lactose
α-6′Glucosyllactose
Transgalactosylation of sucrose
Transfructosylation of lactose or transgalactosylation of sucrose
6′ or 3′isolactosucrose
Lactosucrose
4′Galactosyllactosucrose

Figure 1 An overview of chemical and enzymatic modifications of lactose for production of lactose derivatives. Commercially available lactose derivatives are printed in bold. Modified from Gänzle MG, Haase G, and Jelen P (2008) Lactose – crystallisation, hydrolysis and value-added derivatives. *International Dairy Journal* 18: 685–694.

Table 1 Lactose derivatives and their commercial applications

Compound	*Current use*
Lactobionic acid (β-4′galactosylglucuronic acid)	Metal chelator, pharmaceutical use for dietary supplements, as a humectant in cosmetic applications, and in organ preservation
Lactitol (β-4′galactosylsorbitol)	Alternative sweetener in food applications, medical use as a laxative and for treatment of hepatic encephalopathy
Lactulose (β-4′galactosylfructose)	Medical use as a laxative and for treatment of hepatic encephalopathy
Lactosucrose (β-4′galactosylsucrose)	A functional food ingredient in Japan

Lactitol

Lactitol (β-4′galactosylsorbitol) is produced by catalytic hydrogenation of lactose. The relative sweetness of lactitol is 0.3–0.4 compared to sucrose. Lactitol is not hydrolyzed by human digestive enzymes; dietary energy is derived from hydrolysis to galactose and sorbitol, followed by galactose absorption by the host or fermentation to short-chain fatty acids by the intestinal microbiota. Comparable to other polyols and nondigestible carbohydrates, the caloric value of lactitol is estimated at 2–2.5 kcal g^{-1}. Also in analogy to other nondigestible carbohydrates and polyols, intestinal fermentation of lactitol to short-chain fatty acids has a laxative effect, and the consumption of high levels of lactitol induces flatulence, osmotic diarrhea, and gastrointestinal discomfort. A dose of lactitol of 5–10 g day^{-1} is generally tolerated with a mild laxative effect but without severe adverse symptoms. Lactitol is also used in the treatment of hepatic encephalopathy (see below).

Lactulose

Lactulose (β-4′galactosylfructose) is an isomer of lactose and was the first commercially available lactose derivative. The relative sweetness of lactulose is 0.6 in comparison with sucrose. Lactulose is produced industrially by isomerization of lactose via a 1,2-enediol intermediate. During heating to more than 70 °C in alkaline solution, lactose isomerizes to an equilibrium mixture of 20–30% lactulose and 70–80% lactose. Borate or aluminate shifts the isomerization equilibrium to 70–80% lactulose, and prevents thermal degradation of lactulose. In direct analogy to the enzymatic synthesis of galacto-oligosaccharides, the enzymatic production of lactulose is achieved by transgalactosylation with β-galactosidases using lactose as galactosyl donor and fructose as galactosyl acceptor.

The pH of milk, 6.7, is well below the optimum condition for lactose isomerization to lactulose; nevertheless, between 0.5 and 3.5% of lactose isomerizes to lactulose during UHT processing or during sterilization of milk. The lactulose concentration in fluid milk can therefore be used as an indicator of the intensity of the heat treatment of milk.

To date, lactulose has not been used in food applications but is applied at a dose of about 10 g day^{-1} as a laxative in the treatment of constipation. Moreover, lactulose and lactitol are used in the treatment of chronic hepatic encephalopathy. Hepatic encephalopathy (HE) is a neuropsychiatric disorder that may complicate liver dysfunction. Therapeutic intervention aims to reduce the production of ammonia in the colon. Lactulose and lactitol are nondigestible carbohydrates that are fermented to short-chain fatty acids in the colon. The resulting modifications of the colonic microbiota in favor of saccharolytic bacteria, which produce low amounts of ammonia, and the decrease in luminal pH are thought to reduce ammonia resorption from the colon. Although the benefit of lactulose or lactitol is questionable when compared to the administration of antibiotics, lactitol or lactulose at levels of 30–110 g day^{-1} remains the standard treatment for hepatic encephalopathy.

Lactulose is hydrolyzed by microbial β-galactosidases and is rapidly fermented by *Bifidobacterium* species and other intestinal bacteria. Studies on humans have shown that the consumption of lactulose at 4–10 g day^{-1} increases the proportion of bifidobacteria in fecal microbiota. Lactulose thus meets the criteria for prebiotic carbohydrates, which are defined as nondigestible food ingredients that selectively stimulate growth or activity of beneficial intestinal bacteria. Some preliminary evidence for health benefits associated with prebiotic fermentation, particularly increased calcium adsorption upon lactulose consumption and protective effects in inflammatory bowel disease, has been provided by animal studies.

Lactosucrose

The trisaccharide lactosucrose (β-4′galactosylsucrose) is produced by transfructosylation of lactose, or by transgalactosylation of sucrose. Transgalactosylation of sucrose is carried out with β-galactosidase using lactose as galactosyl donor and sucrose as galactosyl acceptor. In addition to lactosucrose, isolactosucrose (β-3′galactosylsucrose and β-6′galactosylsucrose), galactosyllactosucrose (β-4′digalactosylsucrose), and β-4′galactosyllactose are obtained as reaction products. Transfructosylation of lactose is carried out with fungal or bacterial fructosyltransferases (fructansucrases) using sucrose as fructosyl donor and lactose as fructosyl acceptor. Fructansucrases are classified in the glycoside hydrolase (GH) family GH68. Comparable to β-galactosidases, fructansucrases use a retaining catalytic mechanism and form a covalent intermediate with fructose. The fructose moiety is subsequently linked to an acceptor molecule. Fructosyl transfer to H_2O results in sucrose hydrolysis; transfer to sucrose or other mono- or disaccharides results in oligosaccharide formation; and fructosyl transfer to a growing fructan chain results in fructan synthesis from sucrose (**Figure 2**). Depending on the fructan formed, fructansucrases are termed levansucrase (predominant linkage type in the polymer is β-(2→6)) or inulosucrase (predominant linkage type in the polymer is β-(1 → 2)). Fructansucrases are highly specific for sucrose or raffinose as fructosyl donors, but accept a wide range of carbohydrates, including sucrose, lactose, and galactose, as fructosyl acceptors (**Figure 2**). Fructansucrases are used in the commercial production of fructo-oligosaccharides. High yields of lactosucrose over fructose or fructan are obtained when the lactose and sucrose are present at equimolar concentrations, and when sucrose turnover by levansucrase is coupled to glucose removal by glucose oxidase. Lactosucrose is subject to hydrolysis by levansucrases and accumulates as a kinetic intermediate.

Lactosucrose is commercially produced in Japan and is recognized under the Japanese Foods for Specific Health Use (FOSHU) legislation. Its relative sweetness compared to sucrose is 0.3–0.6. Lactosucrose is not absorbed in the upper intestine and is thus available for hydrolysis and metabolism to short-chain fatty acids by the colonic microflora. Lactosucrose is hydrolyzed by bacterial β-galactosidases in concert with invertase and β-fructofuranidase. Bifidobacteria exhibit extracellular β-galactosidases and β-fructofuranidase, grow rapidly on lactosucrose, and prefer lactosucrose as carbon source over fructo-oligosaccharides. Lactobacilli are capable of lactosucrose metabolism through extracellular fructansucrases, lactose transport, and intracellular β-galactosidase activity. A prebiotic (bifidogenic) effect of lactosucrose was demonstrated in some *in vitro* experiments, animal data, and small-scale human trials but is not as

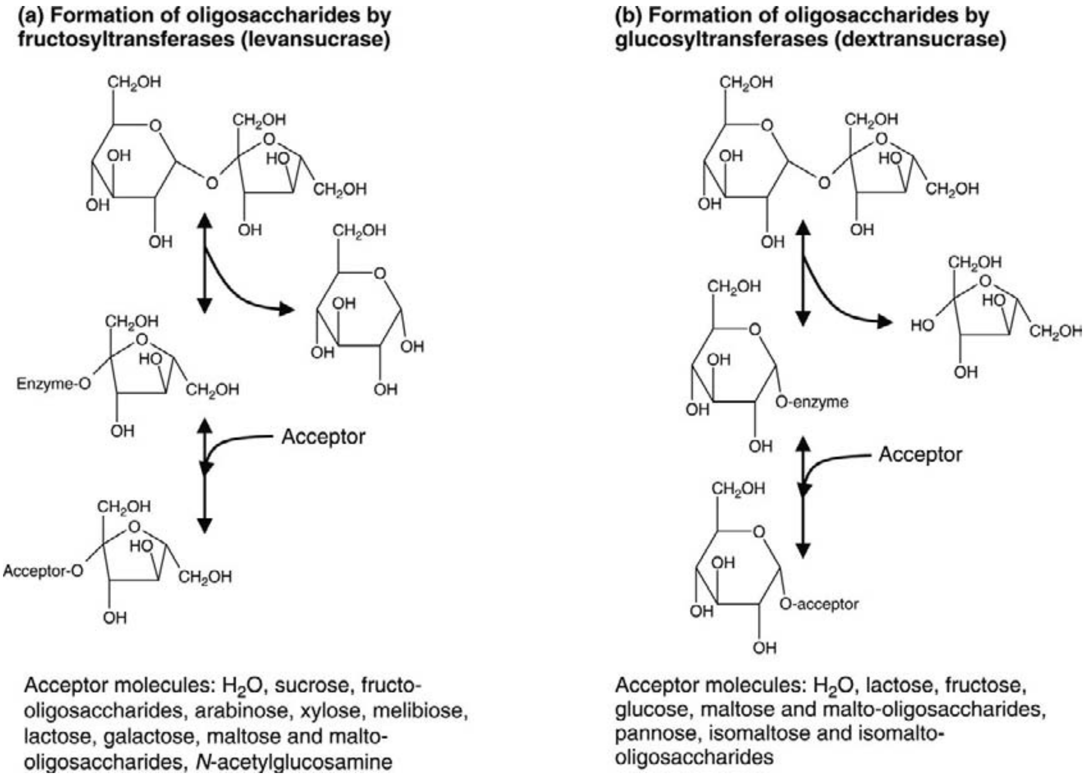
(a) Formation of oligosaccharides by fructosyltransferases (levansucrase)
CH₂OH
O
OH
OH
OH
O
CH₂OH
HO
CH₂OH
OH
Enzyme-O
Acceptor
Acceptor-O
Acceptor molecules: H₂O, sucrose, fructo-oligosaccharides, arabinose, xylose, melibiose, lactose, galactose, maltose and malto-oligosaccharides, N-acetylglucosamine
(b) Formation of oligosaccharides by glucosyltransferases (dextransucrase)
O-enzyme
O-acceptor
Acceptor
Acceptor molecules: H₂O, lactose, fructose, glucose, maltose and malto-oligosaccharides, pannose, isomaltose and isomalto-oligosaccharides

Figure 2 Enzymatic synthesis of lactose derivatives by transfructosylation with sucrose as fructosyl donor and lactose or galactose as fructosyl acceptor (a), or by transglucosylation with sucrose as glucosyl donor and lactose as glucosyl acceptor (b). Modified from Gänzle MG, Zhang C, Sekwati Monang B, Lee V, and Schwab C (2009) Novel metabolites from cereal-associated lactobacilli – novel functionalities for cereal products? *Food Microbiology* 26: 712–719.

well-documented as in the case of fructo-oligosaccharides or galacto-oligosaccharides.

Experimental Lactose Derivatives

Lactose Derivatives Produced by Transglucosylation of Lactose

In analogy to the transfructosylation of lactose, transglucosylation is achieved with bacterial glucosyltransferases (glucansucrases) using sucrose as glucosyl donor and lactose as glucosyl acceptor. Glucansucrases are included in the GH70 family of enzymes. Glucansucrases catalyze the formation of glucan polymers from sucrose, and depending on the main linkage type in the polymers formed, they are termed dextransucrase (predominantly α-(1 → 6) linkages), reuteransucrase (α-(1 → 6) and α-(1 → 4) linkages), mutansucrase (predominantly α-(1 → 3) linkages), and alternansucrase (alternating α-(1 → 3) and α-(1 → 6) linkages). Comparable to β-galactosidases and fructansucrases, glucansucrases use a retaining catalytic mechanism (**Figure 2**) and accept H_2O or other mono- or disaccharides as glucosyl acceptors to form glucose and fructose, or fructose and oligosaccharides. Maltose, isomaltose, and lactose are known glucosyl acceptors to dextransucrases (**Figure 2**) and dextransucrase activity with lactose as glucosyl acceptor results in the formation of α-6′glucosyllactose.

Glucansucrase activity is commonly found in oral streptococci, which employ glucansucrases to produce the carbohydrate matrix of the dental biofilms. Moreover, food-fermenting strains of *Leuconostoc* species, *Weissella* species, and *Lactobacillus* species, particularly *Lactobacillus reuteri*, frequently exhibit glucansucrase activity. Formation of glucosyllactose to levels of up to 1% is observed in dairy fermentations if sucrose and a dextransucrase-positive starter culture are present.

Lactose Derivatives Produced by Transgalactosylation

In addition to the transgalactosylation of sucrose, fructose, and lactose, which are applied in the industrial production of the lactose derivatives lactosucrose, lactulose, and galacto-oligosaccharides, respectively, numerous monosaccharides, disaccharides, or other primary alcohols were shown to act as galactosyl acceptor for β-galactosidases. Oligosaccharide structures on mammalian cells play essential roles in cellular recognition, immunological responses, and host–pathogen interactions, as well as in the internalization of bacterial toxins. The specific enzymatic or chemo-enzymatic synthesis of oligosaccharides or glycoconjugates with high yield and purity is a prerequisite for studies on their structure–function relationships as well as the development of therapeutic applications. Regio-specific or stereo-specific chemical synthesis of oligosaccharide structures is possible but requires numerous synthetic steps, protection and deprotection steps to achieve selectivity, and gives only low final yields. Glycosyltransferases, which catalyze the regio-specific and stereo-specific transfer of glycosides, require activated sugar precursors as substrates, and the enzymes are rather expensive and unstable. In comparison, glycosylhydrolases such as β-galactosidase are relatively cheap, use disaccharides such as lactose as glycosyl donor, and the principles of their catalytic mechanism and industrial application are well understood. β-Galactosidases with lactose as galactosyl donor have thus emerged as a powerful tool for the enzymatic or chemo-enzymatic synthesis of numerous biologically active oligosaccharides, glycoconjugates, or glycoproteins. Moreover, glycosylation of biologically active molecules such as vitamins or toxins can increase their solubility or stability. **Table 2** lists carbohydrates, amino acids and peptides, and primary alcohols that are known galactosyl acceptors for β-galactosidases. The biological activity of many of the products has not been elucidated; some aspects are discussed below.

The degree of polymerization as well as the chemical structure and the linkage type of prebiotic (bifidogenic) carbohydrates determines their metabolism by intestinal microorganisms. Prebiotic properties of β-(1 → 2)-linked fructo-oligosaccharides with a degree of polymerization between 4 and 40 and β-(1 → 4)- or β-(1 → 6)-linked galacto-oligosaccharides with a degree of polymerization between 3 and 8 were well-established in human and animal trials; however, *in vitro* data on the metabolism of oligosaccharides as well as animal data indicate that other galactosylated carbohydrates are metabolized preferentially by *Bifidobacterium* species. Isolactosucrose, β-(1 → 6)- and β-(1 → 1)-linked galactosylated lactulose derivatives, and β-(1 → 3)-linked galacto-oligosaccharides (**Table 2** and **Figure 1**) were synthesized from lactose and the respective acceptor carbohydrates to enable the study of structure–function relationships of prebiotic carbohydrates *in vitro* and *in vivo.* Although it is possible to galactosylate fucose and *N*-acetylglucosamine with β-galactosidases (**Table 2**), the structure of the resulting oligosaccharides differs from the structure of human milk oligosaccharides containing fucose and *N*-acetylglucosamine. Oligosaccharides generated by β-galactosidases are hydrolyzed by microbial β-galactosidases, whereas the hydrolysis and metabolism of human milk oligosaccharides require glycosylhydrolases specific for fucose, sialic acid, and *N*-acetylglucosamine.

Galactosylation of biologically active compounds was performed with vitamins, 2-fluoroethanol, and kojic acid. 2-Fluoroethanol is used to control mammalian pests; however, the toxin is volatile and odorous, and thus

Table 2 Examples of carbohydrates and other primary alcohols used as galactosyl acceptor for enzymatic or chemo-enzymatic synthesis of oligosaccharides or glycoconjugates

Galactosyl acceptor	*Products*
Carbohydrates	
Mannose[a]	β-3′, 4′, and 6′galactosylmannose
Fructose[b]	Lactulose and galactosyllactulose
Fucose[c]	Mono- and digalactosylfucose
Xylose[d]	2′, 3′ and 4′ galactosylxylose
N-Acetylglucosamine and derivatives[e]	β-Gal-(1 → 3)-β-GlcNAc
N-Acetylgalactosamine[f]	β-Gal-(1 → 3)-β-GalNAc
Glucoronic acid[g]	β-Gal-(1 → 3)-glucoronateβ-Gal-(1 → 4)-β-Gal-(1 → 3)-glucoronate
Sucrose[h]	Lactosucrose, 3′ and 6′isolactosucrose
Malto-oligosaccharides[i]	β-4′ galactosylmaltotriose β-4′ galactosylmaltotetraose
Cycloisomaltooctaose[j]	β-6′galactosylcyclomaltooctaose
Rubsoside[k]	13-O-[β-Gal-(1 → 4)- β-Glc]-19-O-β-Glc-steviols
Amino acids and peptides	
N-Protected L-serine methyl esters[l]	3-*O*-β-Gal-L-Ser derivatives
N-Protected dipeptide esters[m]	β-(1 → 3)-digalactosylpeptides
Alcohols	
Alcohols[n]	Alkyl β-galactose
2-Fluoroethanol[o]	2-Fluoroethyl-β-Gal and derivatives
Allyl alcohol[p]	Allyl β-Gal
Kojic acid[q]	Galactosylkojic acid

Compiled with data from:

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detected and avoided by the target pests. Galactosylation of 2-fluoroethanol converts the toxin to an odorless compound; the bioactive 2-fluoroethanol is generated by hydrolysis *in vivo* upon ingestion. Galactosylation of kojic acid, an antimicrobial and metal-chelating metabolite of *Aspergillus oryzae*, maintains its biological activity and improves solubility and stability.

Conclusion

The catalytic oxidation, reduction, or isomerization of lactose generates lactose derivatives with a variety of food and medical applications. The understanding of the catalytic mechanisms of β-galactosidases and the use of transgalactosylation for the production of biologically active oligosaccharides, which has been derived primarily from the study of galacto-oligosaccharide formation from lactose, generate a large variety of additional lactose derivatives. Though most of these oligosaccharides or glycoconjugates currently have no known application, they will undoubtedly contribute to the understanding of structure–function relationships of oligosaccharides and glycoconjugates, and may enable future application development for lactose-derived glycans.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology; Lactic Acid Bacteria: An Overview. **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk; Lactose: Galacto-Oligosaccharides. **Milk**: Human Milk.

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Lactose: Galacto-Oligosaccharides

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Introduction

The term galacto-oligosaccharides (GOS) refers to oligosaccharides consisting of β-linked galactose moieties with galactose or glucose at the reducing end. Known compounds have a degree of polymerization (DP) of 3–8. The terms transoligosaccharides or transgalactooligosaccharides (TOS) are used as synonyms for GOS. GOS occur naturally in the milk of some mammals, and are synthesized through transgalactosylation from lactose by β-galactosidases (β-gal). GOS are commercially applied as prebiotic functional food ingredients. This article presents the structure and natural occurrence of GOS, describes the mechanisms of enzymatic synthesis of GOS from lactose, and outlines the established or emerging biological activities of GOS in food and feed applications. The current use of the term GOS generally excludes the analogous α-GOS, which consist of α-linked galactose moieties and are synthesized enzymatically by α-gal. α-GOS with a terminal sucrose, for example, raffinose (DP 3), stachyose (DP 4), and verbascose (DP 5), are widespread in plants. Because seeds of the plant family Fabaceae have high contents of stachyose and verbascose, these compounds have been referred to as pulse oligosaccharides or soybean oligosaccharides and are not discussed in this article. Oligosaccharides consisting of β-linked galactose moieties with carbohydrates other than glucose or galactose at the reducing end are referred to as heterooligosaccharides and are described in the article **Lactose and Oligosaccharides**: Lactose: Derivatives.

Structure and Occurrence of GOS

Lactose analogs and GOS with known structures are compiled in **Table 1**; selected compounds are depicted in **Figure 1**. GOS occur naturally in the colostrum and milk of mammals but generally are minor constituents when compared to lactose, α-GOS, and sialylated or fucosylated oligosaccharides. Bovine milk or colostrum contains β-3′-galactosyllactose, β-4′-galactosyllactose, and β-6′-galactosyllactose; ovine colostrum contains β-3′-galactosyllactose and β-6′-galactosyllactose; β-3′-galactosyllactose and β-6′-galactosyllactose are present in trace amounts in human milk. Remarkably, α-3′-galactosyllactose is 10-fold more abundant than GOS in bovine milk; elephant milk contains more than $4\,\mathrm{g\,l^{-1}}$ of α-3′-galactosyllactose but no GOS. However, GOS occur naturally as the dominant oligosaccharide species in the milk of some marsupials. Milk of the tammar wallaby contains up to 13% oligosaccharides; β-3′-galactosyllactose and the corresponding GOS with a DP of 4–7 are the major natural oligosaccharides.

Enzymatic Synthesis of GOS

GOS with galactose at the reducing end are released by endogalactosidases from plant galactans. Digestion of plant arabinogalactan with GalA, a bifidobacterial endogalactosidase of the glycosyl hydrolase family (GH) 35, yields β-(1→4) GOS. The enzymatic synthesis of GOS from lactose is catalyzed by β-gal, and GOS carry glucose or galactose at the reducing end. β-Gal are categorized as GH1, GH2, GH35, and GH42; they are retaining glycosyl hydrolases with a glutamate residue as catalytic nucleophile and a transgalactosylic mechanism of catalysis. Enzymes in the families GH1 and GH2 predominantly use lactose as substrate and most enzymes characterized to date have been characterized in Enterobacteriaceae, lactic acid bacteria, and bifidobacteria. GH35 and GH42 enzymes prefer β-(1→3)- or β-(1→4)-linked galactans or GOS over lactose, and most enzymes characterized to date have been described in bifidobacteria and bacteria associated with habitats that do not contain lactose.

β-Gal form a galactosyl-enzyme intermediate during the hydrolysis reaction with galactose covalently bound to the nucleophile at the catalytic site (Glu537 in the LacZ of *Escherichia coli*). Transgalactosylation with H_2O as the acceptor molecule results in lactose hydrolysis. The presence of lactose, glucose, or galactose as alternative galactosyl acceptors results in GOS formation; transgalactosylation of other acceptor carbohydrates generates heterooligosaccharides. The transgalactosylation reaction is favored over lactose hydrolysis at high lactose concentrations. The main products of transgalactosylation are

Table 1 An overview of galactooligosaccharides

Disaccharides	*Trivial name*
β-D-Gal (1→2)-D-Glc[a,b]	
β-D-Gal (1→3)-D-Glc[a,b]	
β-D-Gal (1→4)-D-Glc[a,b]	Lactose
β-D-Gal (1→6)-D-Glc[a,b,c,d,e]	Allolactose
β-D-Gal (1→3)-D-Gal[a,b]	
β-D-Gal (1→4)-D-Gal[f]	4′-Galactobiose
β-D-Gal (1→6)-D-Gal[a,b,c,d,e]	6′-Galactobiose
Trisaccharides	
β-D-Gal (1→4)-β-D-Gal-(1→2)-D-Glc[g]	
β-D-Gal (1→6)-β-D-Gal-(1→2)-D-Glc[g]	
β-D-Gal (1→4)-β-D-Gal-(1→3)-D-Glc[g]	
β-D-Gal (1→6)-β-D-Gal-(1→3)-D-Glc[g]	
β-D-Gal (1→3)-β-D-Gal-(1→4)-D-Glc[b,e]	3′-Galactosyllactose
β-D-Gal (1→4)-β-D-Gal-(1→4)-D-Glc[b,e]4′-Galactosyllactose	β-D-Gal (1→6)-β-D-Gal-(1→4)-D-Glc[b,c,d,e]6′-Galactosyllactose
β-D-Gal (1→2)-β-D-Gal-(1→6)-D-Glc[h]	2′-Galactosylallolactose
β-D-Gal (1→4)-β-D-Gal-(1→6)-D-Glc[a]	4′-Galactosylallolactose
β-D-Gal (1→6)-β-D-Gal-(1→6)-D-Glc[b,c,d,e]	6′-Galactosylallolactose
β-D-Gal (1→4)-β-D-Gal-(1→4)-D-Gal[f]	4′-Galactotriose
β-D-Gal (1→6)-β-D-Gal-(1→6)-D-Gal[b,c,d,e]	6′-Galactotriose
Tetrasaccharides	
β-D-Gal (1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Glc[h]	3′-Digalactosyllactose
β-D-Gal (1→6)-β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Glc[b,d,e]	6′,3′-Digalactosyllactose
β-D-Gal (1→4)-β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc[f]	4′-Digalactosyllactose
β-D-Gal (1→3)-β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc[e,b]	3′,6′-Digalactosyllactose
β-D-Gal (1→6)-β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc[b,c,e]	6′-Digalactosyllactose
β-D-Gal (1→6)-β-D-Gal-(1→6)-β-D-Gal-(1→6)-D-Gal[d,f]	6′-Galactotetraose
Higher oligosaccharides (n = 3, m = 4–7)	
[β-D-Gal (1→3)]$_m$-β-D-Gal-(1→4)-D-Glc[h,i]	
[β-D-Gal (1→4)]$_m$-D-Glc[f]	
[β-D-Gal (1→6)]$_n$-β-D-Gal-(1→4)-D-Glc[b,e]	
[β-D-Gal (1→6)]$_m$-D-Glc[f]	

Galactooligosaccharides with defined structures formed by transgalactosylation of lactose, glucose, or galactose; enzymatic hydrolysis of galactan; or are naturally present in the milk of mammals

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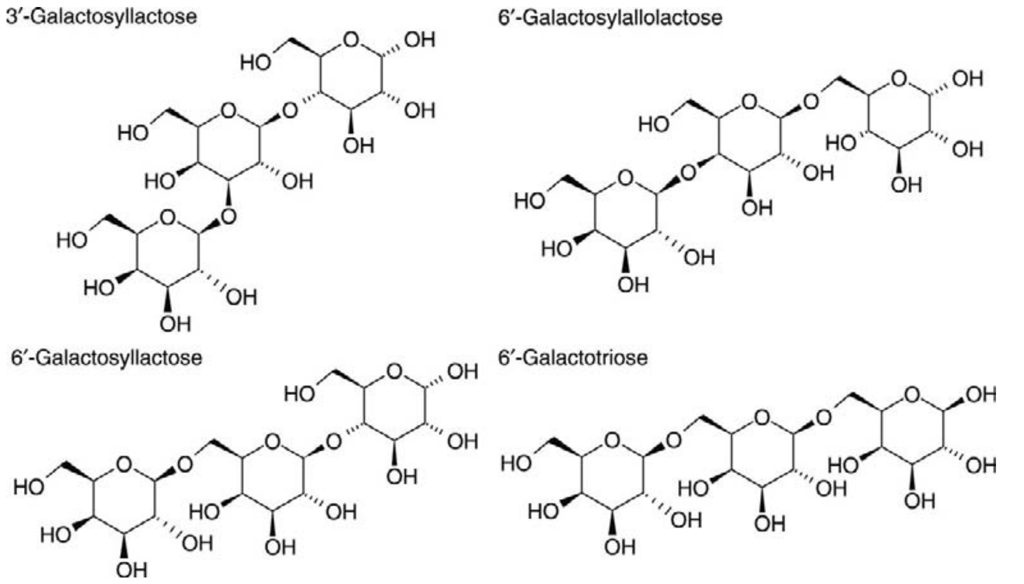
Gal, galactose; Glc, glucose.


3′-Galactosyllactose

6′-Galactosylallolactose

6′-Galactosyllactose

6′-Galactotriose

Figure 1 Structure of selected galactooligosaccharides.

di- and tri-saccharides, but these also act as galactosyl acceptors to form oligosaccharides with a DP of up to 8 (**Table 1** and **Figure 2**).

GOS formation has been described in LacA-type GH42 β-gal of *Bifidobacterium infantis* and *Geobacillus stearothermophilus*, as well as in GH2 β-gal of *Kluyveromyces lactis*, Enterobacteriaceae, lactic acid bacteria, and bifidobacteria (all LacZ type), and in LacLM-type enzymes of lactobacilli. Transgalactosylation by β-gal retains the stereochemistry of the substrate; however, depending on the enzyme source, the resulting di- and oligo-saccharides have one, two, or three different linkages. LacZ of *E. coli* predominantly forms β-(1→6) linkages; β-galI of *B. infantis* forms β-(1→3) linkages; and *Sterigmatomyces elviae* CBS8119 forms β-(1→4)-linked oligosaccharides. GOS with β-(1→3) and β-(1→6) linkages are formed by β-gal from *Lactobacillus reuteri* and *Bifidobacterium bifidum*; β-(1→3) and β-(1→4) linkages are formed by β-gal from *G. stearothermophilus*; and β-gal from *Bacillus circulans* form GOS with β-(1→2), β-(1→3), and β-(1→6) linkages. Because the alternative galactosyl acceptors glucose, galactose, and GOS are produced by β-gal activity from lactose and several linkage types are introduced by some β-gal, the use of lactose as the sole substrate can result in the formation of more than a dozen different disaccharides and GOS (**Table 1**).

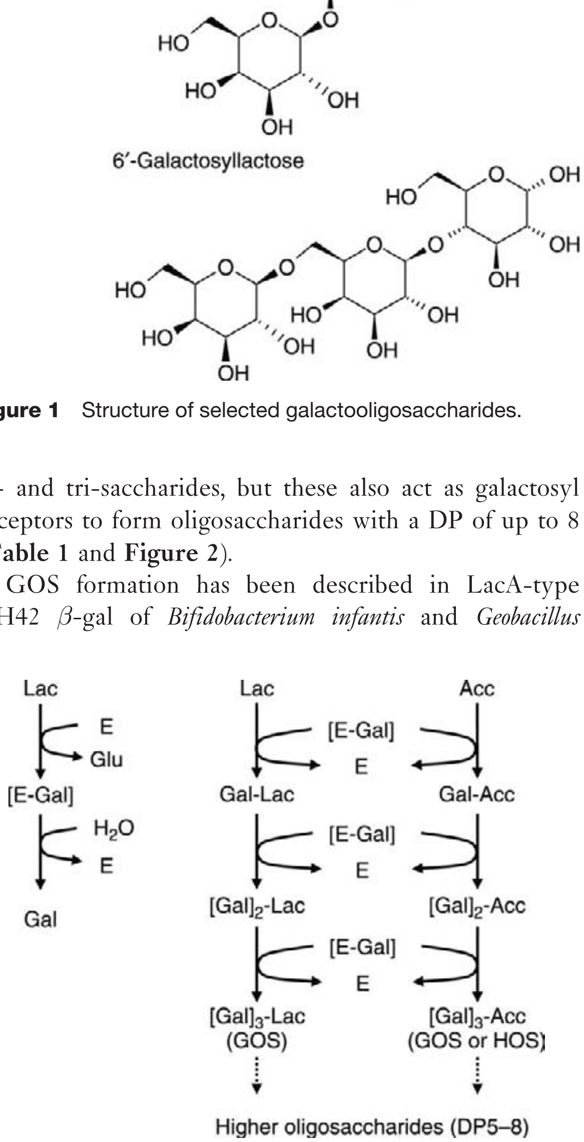


Figure 2 A schematic overview of the hydrolysis and acceptor reaction catalyzed by β-gal. Left panel: Hydrolysis of lactose (Lac) to galactose (Gal) and glucose (Glu) by β-gal (E) proceeds through a double-displacement mechanism. The galactosyl moiety is covalently bound to the nucleophile in the catalytic site of the enzyme before it is transferred to H_2O acting as galactosyl acceptor. Middle panel: Galactosyl transfer from the E-Gal catalytic intermediate to lactose forms trisaccharides, which in turn act as galactosyl acceptors to form GOS with a degree of polymerization (DP) of 4–8. Right panel: Carbohydrates other than lactose (Acc) also act as nucleophile acceptor molecules for galactosyl transfer by β-gal. Resulting oligosaccharides are referred to as GOS if glucose or galactose acts as acceptor molecules, or as heterooligosaccharides (HOS) if other hexoses are used as galactosyl acceptors.

Industrial Production of GOS and Optimization of Enzymatic Synthesis

Quantitative conversion of lactose to GOS is achieved with a theoretical yield of 75% GOS relative to the initial lactose, assuming that all of the lactose is utilized and that 50% of the lactose serves as galactosyl donor with concomitant liberation of glucose, while the remaining lactose is transgalactosylated to trisaccharides. Practical yields are limited to 10–40% GOS relative to the initial lactose because water, glucose, and GOS all compete with lactose as galactosyl acceptors. Moreover, GOS are degraded by β-gal and accumulate only as intermediate products before they are hydrolyzed to glucose and galactose upon depletion of lactose. Maximum GOS levels are generally obtained after conversion of 50–80% of lactose.

Industrial production of GOS is currently achieved with β-gal from *Cryptococcus laurentii* or *B. circulans*, which produce predominantly β-(1→4) linked di- and oligo-saccharides, or enzymes from *Aspergillus oryzae* or *Streptococcus thermophilus* to produce predominantly β-(1→6)-linked disaccharides and GOS. Commercial GOS preparations generally contain glucose, residual lactose, and other disaccharides at concentrations equivalent to, or exceeding, the GOS content. The yield of GOS from lactose, as well as the purity of the resulting GOS preparations, can be improved by strategies aiming to select suitable biocatalysts and to optimize reaction conditions as outlined below.

Solvents, Reaction Conditions, and Reaction Kinetics

GOS formation is dependent on the availability of high concentrations of lactose as galactosyl acceptor, and the highest GOS yields are generally observed at lactose concentrations close to saturation. The optimum pH depends on the individual enzymes, but LacZ-type enzymes generally require 50–100 m mol l^{-1} Na^{+} or K^{+} and 1–2 m mol l^{-1} Mg^{2+} or Mn^{2+} for optimal activity. Studies on the crystal structure of LacZ of *E. coli* have identified sodium and magnesium ion binding sites in the active site of the enzyme.

Water and lactose compete as alternative galactosyl acceptors to yield monosaccharides and GOS, respectively; removal of water from the reaction buffer is therefore in principle an elegant solution to favor transgalactosylation over hydrolysis. Only a limited amount of water can be replaced directly by the substrate lactose because of the low solubility of lactose (see below). Displacement of water by water-miscible organic solvents reduces the overall enzyme activity of β-gal but increases the GOS yield when the water activity is adjusted to values of 0.4–0.6.

Substrate and Product Inhibition

β-Gal of the GH42 family are inhibited at lactose concentrations exceeding 20 g l^{-1} and synthesize only low amounts of GOS. LacZ-type enzymes are inhibited by the hydrolysis products, galactose and glucose, at concentrations of 2–10%. Galactose is considered a competitive inhibitor that competes with lactose for binding at the active site of the enzyme. Fermentative removal of mono- and disaccharides by *Kluyveromyces marxianus* or enzymatic removal of glucose by glucose oxidase was successfully employed to eliminate product inhibition, and to eliminate mono- and disaccharides from GOS preparations.

Temperature

Temperature has a twofold influence on GOS formation by β-gal. In addition to the direct influence of temperature on β-gal activity, the solubility of lactose and hence its availability as galactosyl acceptor are dependent on temperature. At 37 °C, the solubility of lactose is 25% (w/w), which increases to 60 and 120% at 60 and 88 °C, respectively. The use of β-glycosidases from the hyperthermophilic archae *Sulfolobus solfataricus* and *Pyrococcus furiosus* allows for enzymatic GOS synthesis at a temperature of 100 °C and a lactose concentration of 100% (w/w). The yield of GOS, calculated on the basis of the initial lactose concentration, increased from 20 to 40% by increasing the lactose concentration from 20 to 90%. Heat-stable β-gal were also purified from *G. stearothermophilus* and the yeast *St. elviae*, but these enzymes have not yet been evaluated with respect to their catalytic properties in saturated lactose solutions at high temperatures.

Enzyme Selection, Enzyme Immobilization, and Protein Engineering

The ratio of hydrolase to transgalactosylation activity differs strongly between different β-gal. Enzymes in the GH42 family generally have only low transgalactosylation activity, but substantial variation in relative transgalactosylation activity also occurs between enzymes of different origin in the GH2 family.

The immobilization of β-gal to achieve lactose hydrolysis in dairy products is well established on an industrial scale. The major benefits of immobilization are reduced cost of enzymes as immobilized enzymes are available for repeated use, the development of continuous processes, and enhanced stability of enzymes at extremes of temperature and pH. The disadvantages of immobilized enzymes include reduced enzyme activity based on loss of activity upon covalent linkage to the carrier as well as reduced volumetric productivity due to mass transfer limitations. The carriers used for immobilizing β-gal for the production of GOS include absorption on ion exchange resins and celite resins, covalent coupling to cotton, chitosan beads, and controlled pore glass or epoxy-activated acrylic beads, or enzyme cross-linking with glutaraldehyde. Generally, immobilization increases the stability of enzymes at low pH or high temperature conditions but decreases the overall enzyme activity. GOS synthesis can be achieved by immobilized enzymes, with yields comparable to those of free enzyme reactions, but in specific cases immobilization strongly reduces GOS synthesis relative to the hydrolase activity of β-gal.

Protein engineering or directed evolution of enzymes has emerged as a powerful tool to increase the activity, stability, or catalytic properties of carbohydrate-active enzymes. The first step in achieving directed evolution of protein functionality is the generation of genetic diversity by DNA shuffling, mutagenesis, error-prone PCR, or assembly of large clone libraries from environmental DNA. Genes expressed from recombinant or nonrecombinant DNA are then subjected to screening and selection with appropriate high-throughput *in vitro* technologies. Automated screening of clone libraries in a 100 µl volume on microtiter plates is achieved at a rate of about 10^4 events per day; *in vitro* compartmentalization of droplets in an oil phase allows screening at a nanoliter scale at rates exceeding 10^7 events per day. Selected variant genes are reintroduced to the subsequent rounds of mutation and selection. Laboratory evolution increased the thermostability and altered the pH optimum of α-glucan-active enzymes. Directed evolution of glucansucrases, enzymes of the GH70 family that catalyze sucrose hydrolysis or the formation of oligosaccharides and polysaccharides from sucrose, achieved the isolation of mutant enzymes producing polymers with altered properties, for example, linkage type and degree of branching, or a higher preference for the formation of isomaltooligosaccharides with glucose as glucosyl acceptor. N-terminal truncation of Bif3, a β-gal in the GH2 family with exceptionally low transgalactosylation activity, resulted in enzyme variants that utilize 90% of the lactose for transgalactosylation even at low lactose concentrations. Directed evolution of β-gal was carried out with BgaB from *G. stearothermophilus*, a thermostable enzyme in the GH42 family. Wild-type BgaB is inhibited by lactose concentrations exceeding 15 mmol l^{-1} and exhibits only low transgalactosylation activity. Mutant enzymes are not inhibited by lactose and convert nearly 80% of the lactose to GOS, with 3′-galactosyllactose and 4′-galactosyllactose being the major products of lactose conversion.

Synthesis of Food-Grade GOS

Streptococcus thermophilus and *Lactobacillus* species employed as starter cultures in dairy fermentations generally express β-gal with transgalactosylation activity. However, GOS formation to appreciable levels does not occur in dairy fermentations because the lactose concentration in milk is too low to support the accumulation of GOS. Synthesis of food-grade GOS is achieved by the use of reconstituted whey permeate as a source of lactose and crude cellular extracts of lactic acid bacteria as a source of β-gal. GOS synthesis by crude cellular extracts of *Lactobacillus delbrueckii* and *S. thermophilus* is equivalent to that of purified enzymes when the protein concentration in the enzyme preparations is adjusted to the same activity on lactose. Mechanical disruption of the cells of lactic acid bacteria thus provides a food-grade and low-cost source of β-gal suitable for the conversion of lactose to GOS.

Whey permeate is produced in large quantities as a by-product of the manufacture of cheese followed by recovery of whey proteins by ultrafiltration (UF). Lactose is the major component of whey permeates and accounts for 70–80% of the dry matter content; other constituents include mono- and di-valent cations. The mineral composition of UF whey permeate matches the requirements of β-gal for optimal activity, and GOS yields obtained in reconstituted whey permeate are equivalent to the yields obtained in buffered lactose solutions. However, the mineral composition of whey permeates depends on the cheesemaking technology; in particular, the high calcium content of whey from acid-coagulated cheeses may inhibit β-gal activity.

Biological Activities of GOS

Biological Activities of Oligosaccharides: An Overview

The structural diversity of oligosaccharides is matched by the multitude of biological activities mediated by oligosaccharides. The biological activity of naturally occurring oligosaccharides can be inferred from the context of their natural source; biological activities of synthetic oligosaccharides such as the majority of GOS have been established *in vitro* and *in vivo* by analogy to naturally occurring oligosaccharides.

Fructooligosaccharides and α-GOS of the raffinose family mediate protection against desiccation and cold stress. Protective effects of fructooligosaccharides and α-GOS are attributed to their high glass transition temperature, a requirement for glass formation in seeds, and to the specific interactions with biological membranes that maintain membrane function during desiccation and cold stress. Fructooligosaccharides are also effective as cryoprotectants in the preservation of freeze-dried starter cultures, but corresponding activities have not been described for GOS.

In some plants, oligosaccharides serve as the major storage carbohydrates. The presence of oligosaccharides as a significant proportion of the total milk solids in the milk of some mammals, particularly marsupials, implies that they also serve primarily as an energy source for the infant. Suckling tammar wallabys are likely capable of direct utilization of 3′-GOS and other oligosaccharides in wallaby milk through β-gal, neuraminidase, and *N*-acetylglucosaminidase activities in the enterocytes. However, human digestive enzymes degrade only a few oligosaccharides, and supplementation of dietary energy by oligosaccharides occurs only indirectly through absorption of the short-chain fatty acids (SCFAs) produced by colonic microbiota.

Human milk oligosaccharides likely have no nutritional function but mediate the protection of the newborn by at least two different mechanisms. First, the diverse structures of human milk oligosaccharides mimic the variety of carbohydrate structures on cellular surfaces recognized by viruses, bacterial pathogens, and bacterial toxins. Ligand–receptor recognition is an important early event in pathogenicity and the saturation of carbohydrate receptors with soluble oligosaccharides acting as receptor analogs protects against infection or toxin activity. Second, human milk oligosaccharides mediate the enrichment of protective commensal bacteria in the intestinal microbiota of the infant. *Bifidobacterium longum* spp. *infantis* has uniquely adapted to the use of human milk oligosaccharides as carbon sources, an ability absent in other *Bifidobacterium* species. The ability of human milk oligosaccharides to act as soluble receptor analogs as well as their *in vivo* bifidogenic properties served as conceptual templates for application development of GOS.

Sweetness, Hygroscopicity, and Caloric Content of GOS

Commercial GOS preparations have a relative sweetness of 30–40% as compared to sucrose; however, the sweetness is mediated by the high lactose and glucose contents rather than GOS. GOS are not digested by human digestive enzymes; similar to the case of other nondigestible carbohydrates, the caloric content of GOS is about 50% as compared to glucose.

Utilization of GOS by Lactobacilli and Bifidobacteria

Prebiotic applications of GOS aim to confer beneficial effects on the host through selective stimulation of specific bacterial taxa in the gastrointestinal microbiota. Current practice measures prebiotic activity of nondigestible oligosaccharides mainly on the basis of increased numbers of intestinal *Bifidobacterium* spp. or *Lactobacillus* spp. upon consumption.

Lactobacilli generally do not ferment GOS. Although β-gal of lactobacilli recognize GOS as a substrate, they are located in the cytoplasm and the organisms appear to lack efficient transport mechanisms to internalize GOS with a DP of 3 or higher. However, commercial GOS preparations generally contain significant levels of disaccharides, which are readily transported and metabolized by lactobacilli. In contrast, *Bifidobacterium* species have evolved toward competitiveness in intestinal ecosystems, where complex polysaccharides are the only source of fermentable carbohydrates. Up to 8% of the genomic information in *Bifidobacterium* spp. is dedicated to the degradation and metabolism of polysaccharides. Bifidobacteria generally harbor several enzymes with activity on lactose or GOS. In addition to β-GalI, an intracellular enzyme with preferential activity on lactose, bifidobacteria express the extracellular enzyme β-GalII with preferential activity on GOS, and endogalactosidase, which degrades β-(1→4)-linked plant galactans. Different from other bifidobacteria, *B. longum* subsp. *infantis* is specifically adapted to the infant microbiome and harbors an array of glycosidases, extracellular carbohydrate-binding proteins, and permeases that enable the utilization of sialylated and fucosylated human milk oligosaccharides as a carbon source.

Intestinal Fermentation of GOS and Potential Health Benefits for the Host

The human intestinal microbiome comprises about 10^{14} bacterial cells belonging to several hundred bacterial genera and several thousand bacterial species. The important functions of the colonic microbiota include the conversion of nondigestible carbohydrates to SCFAs available as energy sources to the host, the synthesis of vitamins and essential amino acids, interaction with and training of the gut-associated lymphoid tissue; and resistance against colonization by enteric pathogens. An aberrant or immature composition of the colonic microbiota is a prerequisite for intestinal colonization by the toxin-forming organisms *Clostridium difficile* and *Clostridium botulinum*. Moreover, in concert with genetic and environmental factors, dysfunctional intestinal microbiota contributes to the development of chronic inflammatory bowel diseases.

The majority of bacterial species in the adult colonic microbiota belong to the bacterial divisions *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. Bifidobacteria, classified in the division *Actinobacteria*, are stable and abundant members of the adult colonic microbiota and typically comprise 2–20% of the total bacterial population. The relative abundance of lactobacilli, classified in the division *Firmicutes*, in the adult colonic microbiota is typically less than 0.01%. In comparison to the adult microbiota, the infant microbiota exhibits higher interindividual variation and less temporal stability. The dual function of human milk oligosaccharides to specifically stimulate *B. longum* ssp. *infantis* and to prevent pathogen adhesion to intestinal surfaces emphasizes the importance of rapidly establishing a stable commensal microbiota after birth for infant health. In the first month of life, the infant colonic microbiota is dominated by bifidobacteria and the facultative anaerobic genera *Staphylococcus*, *Streptococcus*, and Enterobacteriaceae. Convergence to an individual and stable microbiome in which obligate anaerobic *Firmicutes* and *Bacteroidetes* prevail is observed only after weaning.

Health benefits and adverse effects of GOS are mediated mainly by their colonic fermentation. An overview on the known and some of the putative health benefits of GOS is given in **Table 2**. GOS are not degraded by human digestive enzymes but are metabolized by colonic bacteria to lactate, SCFAs, CO_2, and H_2. Lactate does not accumulate in the intestinal lumen but is converted to butyrate or acetate by the strictly anaerobic members of the clostridial clusters IV and XIV. More than 95% of

Table 2 The known and putative health benefits of GOS

Target functions	*Supportive evidence*
Functions demonstrated in a large number of human studies with consistent results	
Dietary fiber, fermentation to short-chain fatty acids	Resistant to hydrolysis of human digestive enzymes, fermentation to short-chain fatty acids in the colon
Caloric value	Nondigestible, the caloric value is estimated at 50% as compared to glucose
Bowel functions, stool production, and improved stool consistency	Bulking effect, regulation of stool production, and relief of constipation
Colonic microflora: selective stimulation of bifidobacteria	Increased counts of bifidobacteria in the stool. Supplementation of infant formulae with GOS results in fecal abundance of bifidobacteria comparable to that in breast-fed infants
Functions supported by preliminary animal trials, or proposed in analogy to other oligosaccharides	
Increased gastrointestinal absorption of Ca and Mg	Promising human and animal data for fructooligosaccharides, very limited evidence for GOS
Reduction of triglyceridemia and regulation of total and LDL cholesterol levels	Preliminary human and animal data for fructooligosaccharides, limited and inconclusive data for GOS
Inhibition of adhesion of enteropathogenic *E. coli*	Preliminary *in vitro* data for GOS; mechanistic information and animal data for other oligosaccharides

Table compiled with data provided by van Loo J, Cummings J, Delzenne N, *et al.* (1999) Functional food properties of non-digestible oligosaccharides: A consensus report from the ENDO project (DGXII AIRII-CT94-1095). *British Journal of Nutrtion* 81: 121–132; Veereman-Wauters G (2005) Application of prebiotics in infant foods. *British Journal of Nutrition* 93: S57–S60; Roberfroid MB (2005) Introducing inulin-type fructans. *British Journal of Nutrition* 93: S13–S25; Macfarlane GT, Steed H, and Macfarlane S (2007) Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology* 104: 305–344. Gibson GR and Roberfroid MB (eds.) (2008) *Handbook of Probiotics*. Boca Raton, FL: CRC Press; Shoaf K, Mulvey GL, Armstrong GD, and Hutkins RW (2006) Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infection and Immunity 74: 6920–6928.

SCFAs are absorbed by the host and metabolized by colonocytes or in the liver. The low caloric content of GOS relative to glucose is attributed to the energy loss during bacterial fermentation, the formation of bacterial biomass, and the reduced efficiency of uptake and conversion of SCFAs by the host. The formation of SCFAs contributes to the beneficial effects of GOS. Butyrate in particular is a major energy source for colonocytes and controls several physiological functions of epithelial and immune cells.

GOS modulate bowel habits and stool production, and moderate amounts of GOS relieve constipation. The physiological effects are partially mediated by the stimulation of microbial fermentation of GOS to SCFAs and gas. Most human studies on the physiological effects of GOS were carried out with daily dosages of 5–15 g GOS day^{-1}. Consumption of more than 20 g GOS day^{-1} stimulates vigorous intestinal fermentation and gas production, resulting in gastrointestinal discomfort essentially comparable to the effects of lactose overconsumption in lactose-intolerant individuals.

GOS are fermented by a majority of the numerically dominant members of colonic microbiota, including the obligate anaerobic *Firmicutes* and *Bacteroidetes*; nevertheless, many human studies demonstrate that the proportion of *Bifidobacterium* spp. in the intestinal microbiota is significantly increased upon uptake of GOS. Inclusion of GOS in infant formulae increases the bifidobacteria in the feces of infants and toddlers to levels comparable to those for breast-fed infants. Increased levels of bifidobacteria are hypothesized to contribute to the health benefits of GOS, and to increase the resistance to enteric infections. However, bifidogenic effects of GOS are generally measured at the genus level and the specificity of human milk oligosaccharides, which stimulate the subspecies *B. longum* spp. *infantis*, remains unmatched by GOS. Moreover, a majority of the studies do not specify the linkage type and the DP of the GOS employed. As outlined above, GOS metabolism by lactobacilli is limited to disaccharides or compounds with a DP of 3. Emerging evidence indicates that the linkage type and the DP also determine preferential metabolism of GOS by bifidobacteria, which may enable the design of more specific prebiotic preparations in future applications.

GOS and Adhesion of Pathogens

Recognition of carbohydrate receptors on the surface of host cells is an important step in the adherence of intestinal pathogens and the internalization of bacterial toxins. Receptor recognition is pathogen specific, and several organisms bind to different fucosylated or sialylated oligosaccharide structures. P-fimbriated uropathogenic and enterotoxigenic *E. coli* bind to Gal α-(1→3)-Gal motifs, and the shiga-like toxins produced by enterohemorrhagic *E. coli* bind to natural or synthetic α-4′-galactosyllactose ligands. The protective effect of human milk against infectious diseases in infants is partially attributed to the presence of structurally diverse oligosaccharides, which block binding of pathogens and toxins to host carbohydrate receptors and thus prevent infection or toxin activity. Synthetic or natural oligosaccharides that block carbohydrate binding sites on the surface of bacterial pathogens or toxins are emerging as novel therapeutic tools to prevent or to treat infectious diseases in adults. Initial *in vitro* data indicate that GOS reduce adherence of enteropathogenic *E. coli* to intestinal cell lines, providing proof of concept that GOS may exert protective effects against specific pathogens.

Conclusions

GOS are naturally present in the milk of some mammals, and β-(1→4)- or β-(1→6)-linked GOS are produced industrially by enzymatic synthesis from lactose. Synthesis of food-grade GOS is enabled by the use of crude cellular extracts from lactic acid bacteria as a biocatalyst and UF whey permeate as the source of lactose. GOS are currently applied as a prebiotic food ingredient and are particularly relevant as adjuncts in infant formulae to substitute the bifidogenic effects of human milk oligosaccharides. Emerging concepts related to the optimization of β-gal for use as biocatalysts in GOS production and the specific biological activities of GOS with defined linkage type and DP will enable improved production methods and more targeted applications to achieve improved biological activity.

See also: **Lactose and Oligosaccharides**: Lactose: Derivatives; Lactose Intolerance; Indigenous Oligosaccharides in Milk.

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Maillard Reaction

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Chemistry

There are three main mechanisms by which nonenzymic browning occurs in foods: the Maillard reaction, caramelization, and ascorbic acid oxidation. The first is the most important, involving a reactive aldehyde (usually a reducing sugar) and an amine (usually an amino acid, peptide, or protein). The second is undergone by sugars without amines, but, in consequence, normally requires considerably higher temperatures. The third is based on ascorbic acid, which acts as a particularly reactive sugar, but can also be oxidized enzymically and so leads on to polyphenolic compounds, the normal type of substrate for enzymic browning in foods. Enzymic browning is virtually of no consequence for dairy products, but is important for beverages, such as tea, coffee, and cocoa, which are likely to come into contact with milk and dairy products.

The chemistry of the Maillard reaction is very complex and can be thought to comprise caramelization and ascorbic acid oxidation as special cases. Even a simple example of the Maillard reaction, that between glucose and ammonia, leads to more than 15 compounds and glucose and glycine give more than 24, and that is without the use of modern chromatographic methods. Using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) on solvent-soluble material only (0.1% (w/w) of reactants only), about 100 components can be detected as reaction products from xylose and glycine.

In order to understand something so complex, simplification is required. This was achieved remarkably successfully in 1953 through Hodge's scheme (**Figure 1**).

Hodge subdivided the Maillard reaction as follows:

1. Initial stage: products colorless, without absorption in the ultraviolet (about 280 nm).
   A. Sugar–amine condensation
   B. Amadori rearrangement
2. Intermediate stage: products colorless or yellow, with strong absorption in the ultraviolet.
   C. Sugar dehydration
   D. Sugar fragmentation
   E. Amino acid degradation (Strecker degradation)
3. Final stage: products highly colored.
   F. Aldol condensation
   G. Aldehyde–amine condensation and formation of heterocyclic nitrogen compounds

Others have called the three stages early, advanced, and final Maillard reactions, respectively. The way these reactions fit together is outlined in **Figure 1**. The final products of nonenzymic browning are called melanoidins to distinguish them from the melanins produced by enzymic browning. Theoretically, the distinction is clear, but in practice it is very difficult to classify the dark brown products formed in foods, since they tend to be very complex mixtures and chemically relatively intractable.

Oxygen plays an essential part in enzymic browning, but it is not essential for nonenzymic browning. In fact, it may help in the oxidation of reductones, such as ascorbic acid to dehydroascorbic acid, but it may also hinder the progress of the reaction, for example, in oxidizing pyruvaldehyde to pyruvic acid.

Table 1 lists 12 symptoms of nonenzymic browning and shows how these develop in relation to the three stages of it. Note in particular that as far as browning itself is concerned, and also (off-)flavor production, there is an induction period.

The eight reactions (A–G; for H, see under G) of Hodge's scheme, outlined above, are further considered below.

A. Sugar–Amine Condensation

This reaction is reversible. The amine can be a protein, and it has been shown that insulin will react with glucose at a significant rate even at room temperature.

There is no fundamental reason why the glycosylamine should not act as an amine for a further molecule of aldose, thus giving a diglycosylamine.

Lysine locked up as ε-glycosylamine appears to be nutritionally available.

B. Amadori Rearrangement

This reaction, which is thought to be acid catalyzed, can be depicted as in **Figure 2**.

It is important to note that, overall, the Amadori rearrangement is not reversible. The reaction goes spontaneously even at 25 °C. Support for the above mechanism comes from the fact that, if the hydroxy group on C-2 is blocked by, for example, a methyl group, rearrangement becomes impossible. Further support comes from the fact that 11 fructoseamino acids and 2 difructoseamino acids have been found in stored,

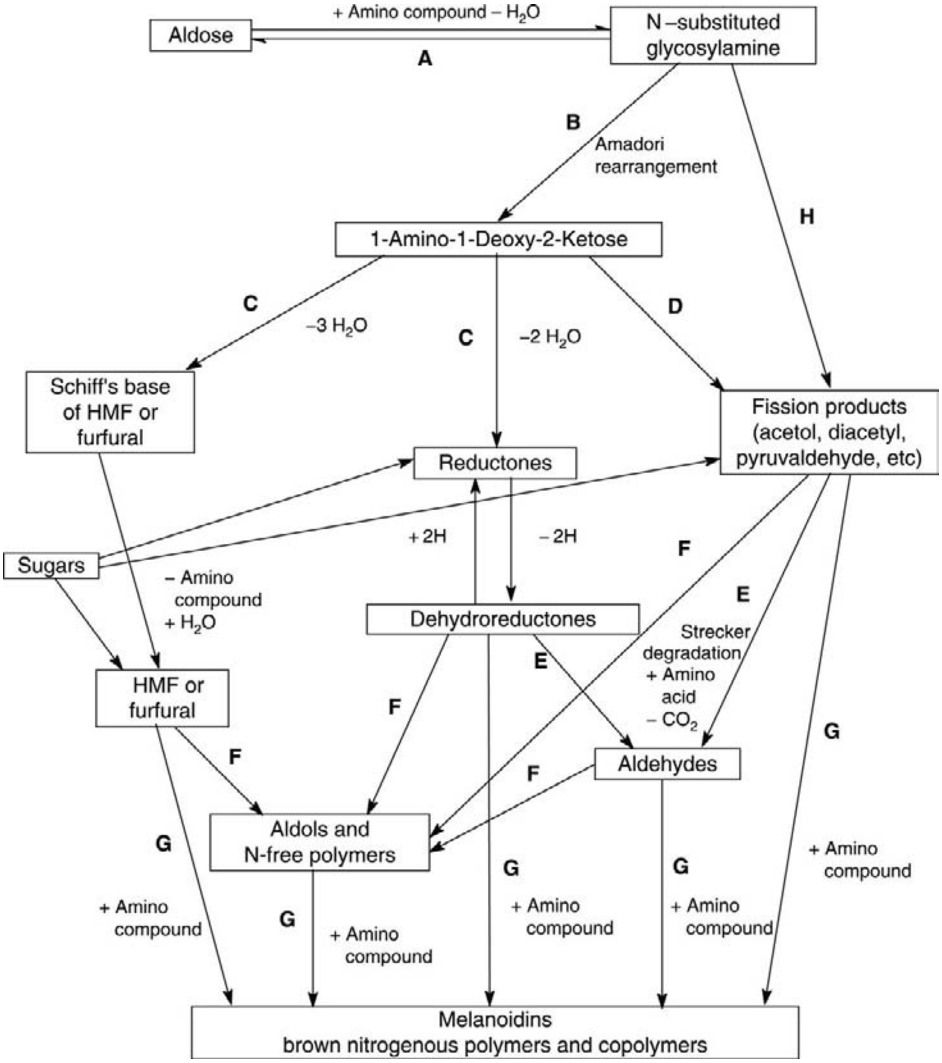

Aldose
+ Amino compound – H_2O
A
N–substituted glycosylamine
B
Amadori rearrangement
H
1-Amino-1-Deoxy-2-Ketose
C
–3 H_2O
C
–2 H_2O
D
Schiff's base of HMF or furfural
Reductones
Fission products (acetol, diacetyl, pyruvaldehyde, etc)
+2H
–2H
Sugars
– Amino compound + H_2O
F
E
Dehydroreductones
Strecker degradation + Amino acid – CO_2
E
HMF or furfural
F
F
G
Aldehydes
F
Aldols and N-free polymers
G
G
G
+ Amino compound
+ Amino compound
G
+ Amino compound
+ Amino compound
+ Amino compound
+ Amino compound
Melanoidins brown nitrogenous polymers and copolymers

Figure 1 Nonenzymic browning. HMF, hydroxymethylfurfural. Based on Hodge JE (1953) Chemistry of browning reactions in model systems. *Journal of Agricultural and Food Chemistry* 1: 928–943.

Table 1 Nonenzymic browning

Symptoms		*Stage*		
		Initial	*Intermediate*	*Final*
1	Browning	–	+	+++
2	(Off-)flavor production	–	+	++
3	Production of water	+	+	+
4	Production of carbon dioxide	?	+	?
5	pH lowering	?	?	?
6	Reducing power (antioxidant activity)	+	+	+
7	Solubility loss	–	–	+
8	Loss of vitamin C	+	–	–
9	Loss of biological value of protein	+	+	+
10	Chelation of metals	–	?	?
11	Toxicity	–	–	?
12	Fluorescence	–	+	–

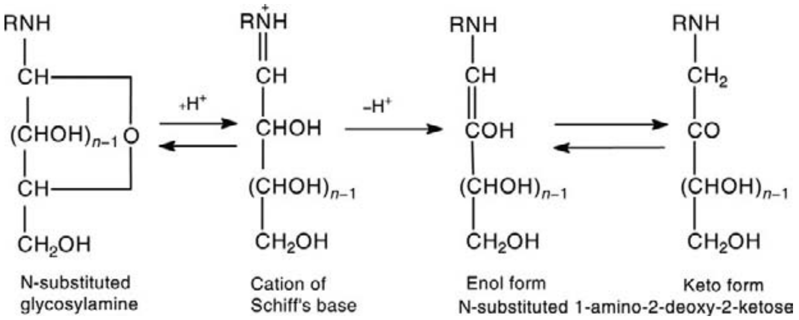


Figure 2 Amadori rearrangement leading to the Amadori compound, the N-substituted 1-amino-2-deoxy-2-ketose.

freeze-dried apricots and peaches and some have also been detected in dehydrated carrots, cabbage, spray-dried tomato powder, tea (glutamic acid, theanine), beet molasses, liquorice, roasted meat, and hog liver extracts.

Compared with the *N*-substituted glycosylamines, the 1-amino-l-deoxy-2-ketoses are more stable to moist acid atmospheres, but are still heat labile and decompose rapidly in mild alkali. They exert greater reducing power, although less than reductones. They brown more easily with amino acids. Acid hydrolysis gives much, compared with little, hydroxymethylfurfural (HMF), but no hexose is recovered, in keeping with the reactions being irreversible.

Ketoses undergo a similar series of reactions, leading to 2-amino-2-deoxyaldoses (Heyns rearrangement). However, browning reactions of fructose differ from those of glucose, for example, loss of amino acid or of free amino groups (casein) is much lower. It is worth noting that lactulose stimulates the growth of bifidobacteria in the gut.

Even when milk is exposed to only very mild heating (60 °C, 20 s), β-lactoglobulin is modified, whereas both α-lactalbumin and β-casein are left unaffected. On HPLC, not only β-lactoglobulin appears with its two genetic variants, A and B, but also each variant has a higher molecular mass component following it. When HPLC is combined with electrospray mass spectrometry (MS), the difference in mass for each pair was shown to be 324 Da, corresponding to the lactulosyl group. The lactulose was found to be attached to Lys47. When the Maillard reaction was made more intense by storing the freeze-dried reaction mixture at 65% relative humidity (RH), 2–11 lactulose molecules became attached in 22 h at 50 °C, with 1–7 molecules in the first 10 h. Lactose became attached to 14 of the 15 lysine residues (not Lys101) and to the α-amino group, 6.5 lactulosyl groups being bound per β-lactoglobulin molecule on average at 50 °C, but only 0.5 in the more familiar aqueous systems. The order of reactivity was Lys47, 91 ($t = 0$ h) > α-amino, Lys15, 70, 100 ($t = 2$ h) > Lys60, 69, 75, 77, 83, 135, 138 ($t = 6$ h) > Lys8, 141 ($t = 10$ h).

β-Lactoglobulin can also clearly be seen in its two genetic variants by capillary electrophoresis (CE). β-Lactoglobulin from skim milk powder exhibits not just such a double peak, but a broad tail, which can be resolved into three or more progressively weaker pairs of peaks. By electrospray MS, the difference in mass between successive pairs has been shown to be 324 Da, corresponding, as above, to the attachment of a lactulose moiety. Native β-lactoglobulin has been shown by capillary isoelectric focusing to fall from 70 to below 20% of total β-lactoglobulin of the whey protein fraction when milk powder was kept for 1 day at 37 °C and 86% RH, making this a fast and convenient method for monitoring storage conditions.

Matrix-assisted laser-desorption ionization coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) is also a powerful technique, especially when used after protein hydrolysis with endoproteinases to perform what is called peptide mapping, which can allow the identification of specific individual amino acid residues that have been modified. In this way, β-lactoglobulin heated with lactose has been shown to be lactulosylated at Lys47 and Lys138 or Lys141. α-Lactalbumin similarly led to attachment of a lactulosyl group to Lys5. Longer reaction oxidized the lactulosyllysine to carboxymethyllysine (CML).

Examining milk and dairy products by such methods has far-reaching implications for elucidating and controlling quality parameters. One of the most important is that lysine locked up in ε-Amadori compounds becomes nutritionally unavailable; this poses considerable analytical problems (see below).

C. Sugar Dehydration

There are two ways in which this occurs; under acid conditions, furfurals are produced, whereas in neutral or alkaline conditions and/or in the presence of amines in nearly anhydrous systems, 6-carbon and other reductones are produced (see **Figure 3**).

1. *Furfural formation*: Various compounds can accelerate furfural formation, for example, glycine accelerates both the conversion of xylose to furfural and that of glucose to HMF. The reason seems to be that the

Amadori product dehydrates more readily than the original aldose or *N*-substituted glycosylamine, giving the Schiff's base of furfural, which is then hydrolyzed, re-liberating part of the amine, but also condensing to melanoidins. It is generally thought that HMF is of low browning potential and does not lie on the main pathway to melanoidins.

2. *Reductone formation*: Reductones can be thought of as products formed from sugars by the loss of only two molecules of water as compared with the loss of three, which leads to furfurals. Reductones are compounds that contain the group -CO-C(OH):C(OH)-, as in ascorbic acid, and a hexose can readily be converted on paper into the vinylogue of a reductone. Compounds such as reductones explain the reducing power that develops during browning, but they take part in browning in the dehydro form and therefore need oxygen to be converted into it. Like furfurals, they brown more readily in the presence of amines.

D. Sugar Fragmentation

The mechanism by which sugar fragmentation occurs is accepted to be principally dealdolization, although oxidative fission is also thought to play a role. It should be recalled that dealdolization is an important part of the EM (Embden–Meyerhof) glycolytic pathway, where fructose-1,6-diphosphate is split into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The sort of reactions that occur are illustrated in **Figure 4**.

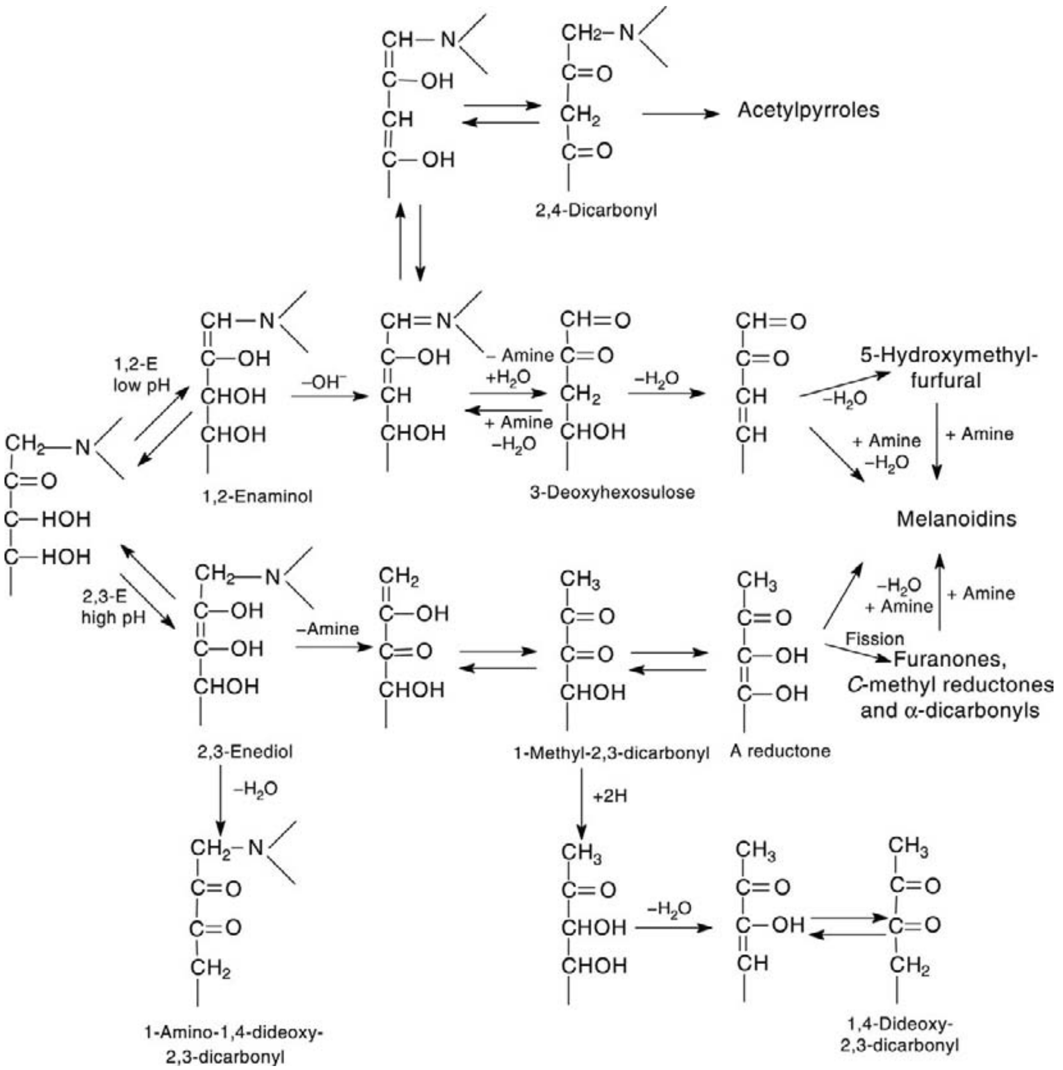

Acetylpyrroles
2,4-Dicarbonyl
1,2-E
low pH
–OH⁻
1,2-Enaminol
– Amine
+H₂O
+ Amine
–H₂O
3-Deoxyhexosulose
–H₂O
5-Hydroxymethyl-
furfural
–H₂O
+ Amine
–H₂O
+ Amine
Melanoidins
2,3-E
high pH
–Amine
2,3-Enediol
1-Methyl-2,3-dicarbonyl
A reductone
–H₂O
+ Amine
+ Amine
Fission
Furanones,
C-methyl reductones
and α-dicarbonyls
–H₂O
1-Amino-1,4-dideoxy-
2,3-dicarbonyl
+2H
–H₂O
1,4-Dideoxy-
2,3-dicarbonyl

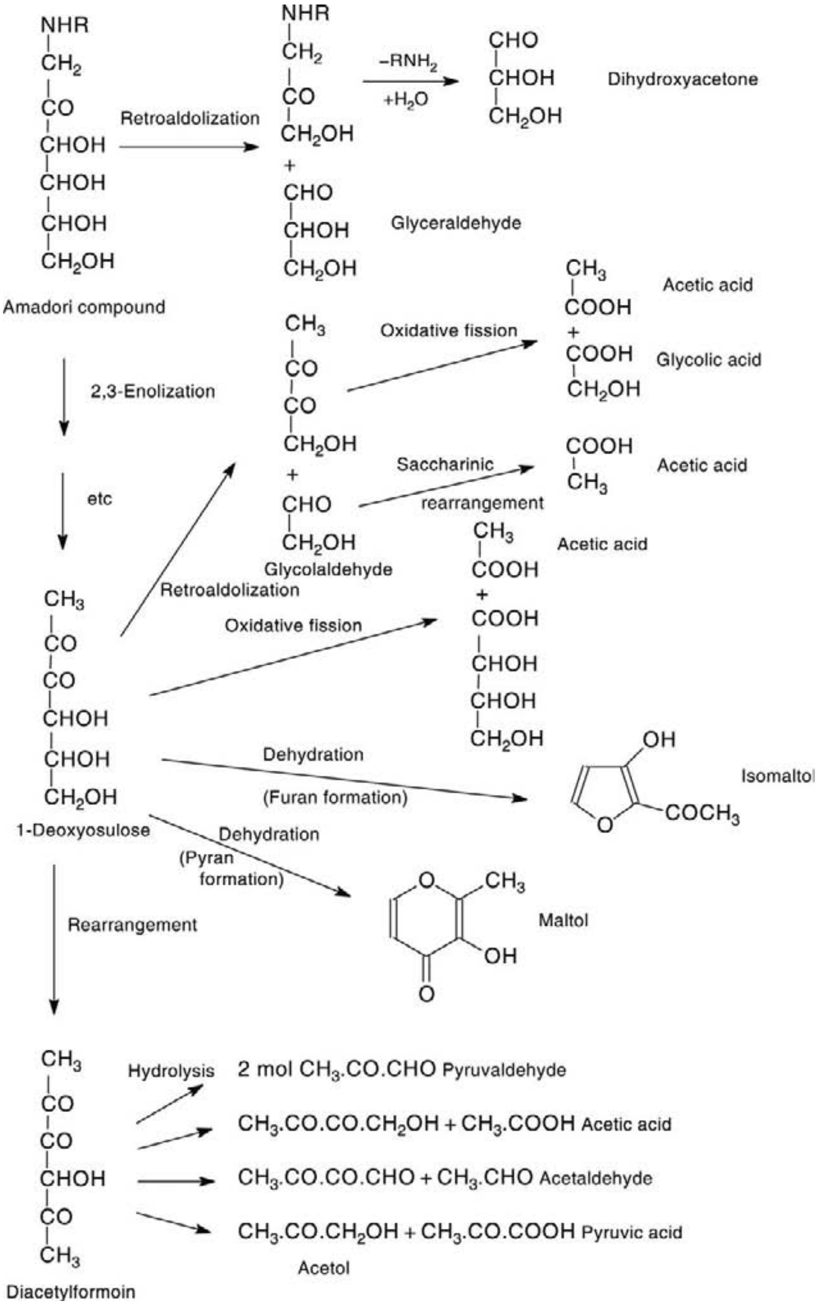
Figure 3 Maillard reactions: the two major pathways from Amadori compounds to melanoidins. Based on Hodge JE (1967) Origin of flavor in foods. Nonenzymatic browning reactions. In: Schultz HW, Day EA, and Libbey LM (eds.) *The Chemistry and Physiology of Flavors*, pp. 465–491. Westport, CT: AVI.

Fragments that retain the α-hydroxymethylcarbonyl grouping will undergo browning alone in aqueous solution, but this will be accelerated greatly by the presence of amines. The relative reactivities of α-hydroxymethylcarbonyl compounds and other sugar fragments are given in decreasing order in **Table 2**.

Recently, model systems of glycine and 1-, 2-, or 5-^{13}C-xylose were used at pH 6 and 8 and 90 and 120 °C to show that acetic acid was derived mainly (77–87%) from C-1–C-2 of the sugar, with 9–15% from C-4–C-5. The results support β-dicarbonyl cleavage of 1-deoxypento-2,4-diulose. Use of γ-aminobutyric acid and 1-^{13}C-arabinose gave a very similar ratio of 82:18 for the labeled:unlabeled acetic acid formed (see below).

E. Strecker Degradation

This is a reaction of α-amino acids, in which they are oxidized to the corresponding aldehyde, along with the formation of carbon dioxide and ammonia. The ammonia formed is transferred to other components of the system, very little being liberated as such. The reaction is initiated



NHR–CH2–CO–CHOH–CHOH–CHOH–CH2OH
Amadori compound
Retroaldolization
NHR–CH2–CO–CH2OH
+
CHO–CHOH–CH2OH
Glyceraldehyde
–RNH2
+H2O
CHO–CHOH–CH2OH
Dihydroxyacetone
2,3-Enolization
etc
CH3–CO–CO–CH2OH
+
CHO–CH2OH
Glycolaldehyde
Oxidative fission
CH3–COOH
Acetic acid
+
COOH–CH2OH
Glycolic acid
Saccharinic rearrangement
COOH–CH3
Acetic acid
Retroaldolization
Oxidative fission
CH3–COOH
Acetic acid
+
COOH–CHOH–CHOH–CH2OH
CH3–CO–CO–CHOH–CHOH–CH2OH
1-Deoxyosulose
Dehydration
(Furan formation)
OH
O
COCH3
Isomaltol
Dehydration
(Pyran formation)
O
CH3
OH
O
Maltol
Rearrangement
CH3–CO–CO–CHOH–CO–CH3
Diacetylformoin
Hydrolysis
2 mol CH3.CO.CHO Pyruvaldehyde
CH3.CO.CO.CH2OH + CH3.COOH Acetic acid
CH3.CO.CO.CHO + CH3.CHO Acetaldehyde
CH3.CO.CH2OH + CH3.CO.COOH Pyruvic acid
Acetol

Figure 4 Some examples of sugar fragmentation.

Table 2 Sugar fragmentation products in decreasing order of reactivity

Sugar fragmentation product	*Formula*	*Reactivity*
Glycolaldehyde	$CH_2OH.CHO$	Highest
Glyceraldehyde	$CH_2OH.CHOH.CHO$	
Pyruvaldehyde	$CH_3.CO.CHO$	
Acetol	$CH_3.CO.CH_2OH$	
Dihydroxyacetone	$CH_2OH.CO.CH_2OH$	
Acetoin	$CH_3.CHOH.CO.CH_3$	
Diacetyl	$CH_3.CO.CO.CH_3$	
Acetaldehyde	$CH_3.CHO$	Slightly lower
Aldol	$CH_3.CHOH.CH_2.CHO$	Still lower
Propionaldehyde	$CH_3.CH_2.CHO$	Very low
Pyruvic acid	$CH_3.CO.COOH$	Even lower
Levulinic acid	$CH_3.CO.CH_2.CH_2.COOH$	
Saccharinic acid	For example, $CH_2OH.(CHOH)_2.CH_2.CHOH.COOH$	Not at all
Lactic acid	$CH_3.CHOH.COOH$	
Acetic acid	$CH_3.COOH$	
Formic acid	$H.COOH$	
Formaldehyde	$H.CHO$	Inhibits

by compounds such as α-dicarbonyl compounds and their vinylogues, or compounds that can give rise to them readily, such as reductones by dehydrogenation or imino analogues by hydrolysis. The reaction may therefore be represented as follows:

$$R.CHNH_2.COOH + R'.CO.CO.R' \rightarrow R.CHO + CO_2 + R'.CHNH_2.CO.R'$$

Studies with radioactive carbon have shown that the carbon dioxide liberated in the Maillard reaction does indeed originate from amino acids.

Glyoxal is a very reactive α-dicarbonyl. Recently, it has been shown in model experiments that cysteine residues play an important role: in the absence of cysteine, glyoxal reacts mainly with arginine residues, leaving lysine residues unaffected, but in the presence of cysteine residues, glyoxal attacks lysine residues, arginine remaining completely protected from reaction.

The Strecker degradation enters browning reactions in two ways: on the one hand, the aldehyde formed can take part in aldol condensation leading to nitrogen-free polymers or, on the other hand, it can react with amino compounds to give melanoidins via aldimines, but this is not thought to be a major color-producing reaction, because glycine can at times give more browning with sugars than alanine, yet glycine produces formaldehyde by the Strecker degradation, which has a negative effect on browning. Furthermore, amino acids other than the α-amino acids can also produce melanoidins, but cannot undergo the Strecker degradation. On the other hand, the dehydroreductones, derived from Amadori products by dehydration and dehydrogenation, or the dicarbonyl fission products, pick up the amino acid nitrogen and go on to form melanoidins.

Dehydroascorbic acid (see later) and quinones formed enzymically from polyphenols can also take the part of the dicarbonyl compound in the reaction. Diacetyl is a well known fermentation product.

Being volatile, the aldehydes formed in the Strecker degradation have often been thought to be important contributors to the aroma of foodstuffs and many patents have been granted for ‘reaction flavors’, which use the Strecker degradation to produce flavoring materials of various types, such as maple, chocolate, coffee, tea, honey, mushroom, and bread.

F. Aldol Condensation

Aldehydes can arise by reactions C–E, and they can then react with each other by the aldol condensation. Amines (and particularly their salts), including peptones and egg albumen, are effective catalysts. Additional carbonyl compounds that can participate in the condensation may be derived by the oxidation of fats.

Browning has been demonstrated for pyruvaldehyde alone, furfurals, and pyruvate plus furfural. Less browning occurs with sugars or with aldol itself.

Two molecules of diacetyl can undergo a similar reaction twice to form 2,5-dimethylbenzoquinone. Benzoquinones can act as dicarbonyl components in the Strecker reaction; they readily form imines in the cold and can be involved in the production of melanoidins.

G. Aldehyde–Amine Condensation heading

Aldehydes, particularly α,β-unsaturated aldehydes, react readily at low temperature with amines to give ‘polymeric’ high-molecular-mass colored products of unknown structure. Heterocyclic rings, such as those of pyridines, pyrazines, pyrroles, and imidazoles, have been shown to be present. Melanoidins usually contain 3–4% nitrogen.

The constitution of melanoidins differs somewhat, depending on how they have been produced, for example,

furfural + glycine	high ether content
glucose + glycine	high alcoholic hydroxyl content
pyruvaldehyde + glycine	high enolic hydroxyl and low ether content

As the condensations proceed, higher molecular mass products are formed, long-period products being nondialyzable. The results in **Table 3** bear on this.

1. In relation to high-molecular-mass material, temperature seems much more important than time. The proportion of high-molecular-mass material increased with temperature.
2. Loss of material, presumably H_2O and CO_2, is much greater at 100 °C (54%) than at 68 °C (30%).
3. The composition of the retentate formed at 22 °C corresponds quite closely to the loss of 3 mol H_2O: C, 49.1%; H, 5.3%; N, 8.2%, that is, all N appears to be retained. N is lost subsequently, some presumably as volatiles.
4. It is not clear to what extent, if any, these high-molecular-mass materials are colored.

Melanoidins are also being studied in other ways.

The carbon nuclear magnetic resonance (^{13}C-NMR) spectrum of high-molecular-mass water-soluble material from glucose and glycine has been found to be very similar to that of the corresponding Amadori compound, there being no evidence of unsaturated or aromatic carbon compounds. The material is very difficult to hydrolyze, suggesting that the glucose units are linked by C–C bonds.

The products of the interaction of α- or β-alanine with arabinose give rise to electron-spin resonance (ESR) spectra with 17 and 23 lines, respectively. These signals were attributed to the presence of the *N,N'*-dialkylpyrazine cation radicals. The radicals are detected before the Amadori compound and hence it was thought that a new pathway (H) for browning had been discovered.

Some of the above reactions have been carried out in D_2O or tritiated (T_2O, radioactive) water to study the extent of H exchange:

$$\text{D}-\text{glucose}-\underset{(3\ \text{mol l}^{-1}\ \text{HCl})}{\text{strong acid}}\rightarrow\text{HMF}\quad\text{no incorporation of D into C}-\text{H bonds}$$

$$\text{D}-\text{xylose}-\underset{(3\ \text{mol l}^{-1}\ \text{HCl})}{\text{strong acid}}\rightarrow\text{furfural}\quad\text{no incorporation of T into C}-\text{H bonds}$$

$$\text{Amadori compounds from glucose}\xrightarrow{1\ \text{mol l}^{-1}\text{HCl}}\text{no incorporation at C-3, but some at times at C-1(aldehydic C}-\text{H)}$$

$$\xrightarrow{2\ \text{mol l}^{-1}\ \text{HOAc}}\text{strong incorporation at both C-3 andr C-1}$$

It therefore seems that Amadori compounds can undergo 1,2-enolization much more easily and under milder acid conditions than the original sugar.

It is probable that the Amadori forms of the 1,2-enediol, of its derivative formed by loss of a hydroxyl ion, and of the 3-deoxyhexosulose (normally unstable to strong acid) are all relatively more stable/have longer lifetimes, thus allowing them to equilibrate and to undergo side reactions.

4-Hydroxy-5-methyl-3(2*H*)-furanone is an important contributor to cooked beef flavor, although it has a caramel-type odor. It can be obtained by heating xylose, ribose, or ribose phosphate with amine salts and is formed by 2,3-enolization and dehydration in a way similar to that shown in **Figure 4** for the formation of isomaltol from a hexose. The furanone can be taken as symptomatic of 2,3-enolization just as furfural and HMF are symptomatic of 1,2-enolization.

Table 4 shows clearly that low pH favors the formation of 2-furaldehyde (1,2-enolization), whereas higher pH favors formation of the furanone (2,3-enolization). It also illustrates how basicity of the amine can affect the

Table 3 Composition of retentate of melanoidins from xylose and glycine (molar ratio 1:1)

Reaction conditions		*Yield (%)*[a]		*Microanalytical data (%)*		
Temperature (°C)	Time	Retentate	Diffusate	C	H	N
22	9 months	4.2		50.3	5.3	8.0
68	6 weeks	39.6	30.0	57.0	6.1	7.3
100	38 h	30.7	15.8	57.8	5.4	6.7

[a] Yield based on total reactants. Membrane cutoff, 12 kDa.

Table 4 Conditions affecting 1,2-enolization and 2,3-enolization

	2 mol l^{-1} H_2SO_4 at 100 °C		*pH 7 at 100 °C*	
Amadori from	*2-Furaldehyde*	*Furanone*	*2-Furaldehyde*	*Furanone*
Glucuronic acid + 1 mol dibenzylamine (derivative of fructuronic acid)	16%	Traces	0	Present
Glucuronic acid + 1 mol benzylamine (derivative of fructuronic acid)	23%	0	0	Present
Xylose + 1 mol benzylamine (derivative of xylulose)	41%	0	0	Present

route; the least basic (dibenzylamine) is least likely to be protonated and therefore more liable to give 2,3-enolization.

Use of 1-^{14}C has shown that the methyl group is derived from C-1 of the hexuronic acid or the pentose. If prepared in D_2O, NMR shows hydrogen exchange from both C-2 and CH_3. This accords with CH_2:C(OH)... intermediates (on the 2,3-enolization route).

Caramelization

Sugars, polysaccharides, polyhydroxycarboxylic acids, reductones, α-dicarbonyl compounds, and quinones will undergo browning in the absence of amino compounds.

Such reactions, even in the absence of catalysts, are important in the food industry, but they require high temperatures, not often encountered. For example, glucose decomposes only above 150 °C. Caramelization is accelerated by carboxylic acids and their salts, phosphates, and metallic ions, but even when so catalyzed, the energy requirements exceed those of sugar–amine reactions.

As with the Maillard reaction, odorous compounds are formed, water and carbon dioxide are liberated, the pH drops during the reaction, color formation is markedly increased by increasing the pH, oxygen has only a slight enhancing effect on color production, and the reaction is inhibited by sulfur dioxide.

The main reactions are 1,2-enolization (Lobry de Bruyn–Alberda van Ekenstein rearrangement, cf. section ‘Amadori Rearrangement’), dehydration to furfurals, and fission.

The volatiles produced by sugar degradation can make an important contribution to flavor. Glucose decomposition at 300 °C has been shown to give more than 130 volatile degradation products, including maltol, which is also present in a variety of food products.

Ascorbic Acid Oxidation

Ascorbic acid alone will brown in aqueous solutions above 98 °C, giving furfural and carbon dioxide. Even in the presence of glycine, the carbon dioxide comes essentially from ascorbic acid (cf. section ‘Strecker Degradation’). The browning with ascorbic acid also increases with pH, and above pH 7, autoxidation and browning occur even at 25 °C. Other reductones will react similarly. Glucose and fructose decrease the rate of browning and so do amino acids initially, although later they increase it.

Ascorbic acid is converted into furfural via a pentose by loss of carbon dioxide. The dehydro form ring-opens into 2,3-diketogulonic acid, which degrades readily, also leading to browning.

Effect on Product Quality

The symptoms listed in **Table 1** are closely linked to qualities important in dairy products. They will therefore be considered in turn.

Color

Although color is the named characteristic in nonenzymic browning, much remains to be discovered about this aspect of the Maillard reaction. There are two fundamental questions: from where does the color come, that is, what chromophore(s) is formed? and where does the color reside in the high- and/or the low-molecular-mass material formed? Contemporary work is trying hard to answer both of them.

As regards chromophore(s), the answer is definitely in the plural and a range of relevant compounds identified mainly among the low-molecular-mass fraction of model Maillard systems are depicted in **Figure 5**. Several of the molecules are worthy of comment.

It should be noted that compounds **1** and **2** are built up of two C_5 moieties, each derived, apparently without chain fragmentation, from xylose, but one is formed by 1,2-enolization and the other by 2,3-enolization of the Amadori compound (see **Figure 3**). Since these reactions require low and moderate pH, respectively, pH of the medium must be a key factor and the nature of the amino component of the Amadori compound will play an important role. The amino acid clearly forms a component of

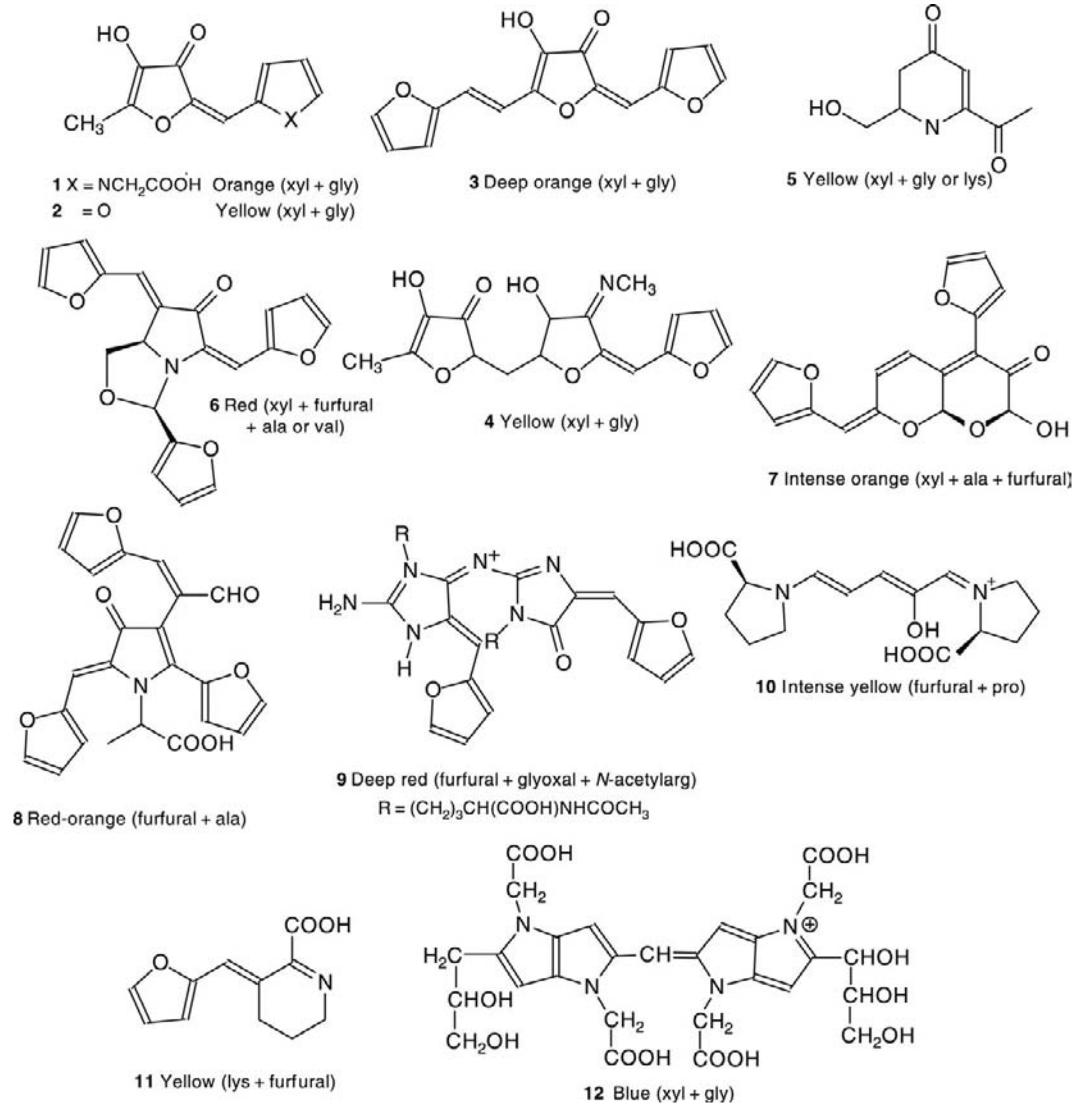

1 X = NCH2COOH Orange (xyl + gly)
2 = O Yellow (xyl + gly)
3 Deep orange (xyl + gly)
5 Yellow (xyl + gly or lys)
6 Red (xyl + furfural + ala or val)
4 Yellow (xyl + gly)
7 Intense orange (xyl + ala + furfural)
8 Red-orange (furfural + ala)
9 Deep red (furfural + glyoxal + N-acetylarg)
R = (CH2)3CH(COOH)NHCOCH3
10 Intense yellow (furfural + pro)
11 Yellow (lys + furfural)
12 Blue (xyl + gly)

Figure 5 The structures of some low-molecular-mass colored compounds formed in model Maillard reactions. ala, alanine; arg, arginine; gly, glycine; lys, lysine; pro, proline; val, valine; xyl, xylose.

compound **1**, but, as regards compound **2**, its role is catalytic.

Compound **3** is built up of three C_5 moieties, but, whereas the central furanone moiety is bifunctional, furfural is monofunctional and thus acts as a capping group. It can be seen to fulfill the same function in other compounds. Compound **4** is also built up of three C_5 moieties, but could readily react further at its active methyl group.

Compound **5** possesses a chain of eight carbon atoms, which must involve fragmentation and reassembly of the xylose skeleton (cf. **Figure 4**). Here, the amino acid supplies only the nitrogen and so both glycine and lysine have given rise to it through interaction with xylose. Similarly, alanine or valine supplies only the nitrogen for compound **6**, for which four C_5 units are required. Compound **7** is built up from a C_2 fragment and three C_5 units, but without the amino acid's nitrogen. On the other hand, compound **8** does involve alanine alongside four C_5 units.

Other amino acids can give rise to different types of chromophore. Thus, arginine can lead to compound **9** and proline (an imino acid) to compound **10**. Note that here furfural provides an open chain of five carbon atoms, as well as in Compound **8**. Lysine supplies all of its atoms, bar one nitrogen, to compound **11**. Compound **12** was considered to be built up from 4 mol Amadori compound by loss of 9 mol H_2O and 1 mol each of H_2 and CO_2.

The above illustrates quite a range of chromophores, but all of these were part of low-molecular-mass compounds. What about high-molecular-mass compounds?

When 5:1 mixtures of glucose and glycine or alanine in phosphate buffer, pH 7, are heated for 4 h at 95 °C and then separated by ultrafiltration through a series of membranes with progressively smaller pore size, the molecular mass of almost 80% of the product is <1000 Da and nearly 20% at 1000–3000 Da, representing 97–99 and 3–1% of the recovered color, respectively, that is, very little high-molecular-mass material had been formed and by far the greatest proportion of the color resides in low-molecular-mass material. When the amino acid was replaced by an equivalent amount of β-casein, the result was very different: the molecular mass of 43% of the product is >100 kDa, 18% at 50–100 kDa, and 24% at <1000 Da, representing 90, 9, and <0.1% of the recovered color, respectively, that is, over 60% of the material is >50 kDa and represents virtually all of the recovered color. The β-casein (molecular mass ~24 kDa) is cross-linked into dimeric and progressively larger entities, presumably through carbohydrate-derived cross-links between appropriate amino acid side chains, as illustrated by some of the structures in **Figure 5**. When sodium caseinate was heated with lactose or glucose in a milk–salt solution at 110–150 °C starting at pH 6.65, most of the browning was also due to pigments bound to protein.

Volatile Compounds

About 3500 volatile compounds have been reported to be formed by the Maillard reaction. Some rationalization is thus required. Accordingly, the volatile products of the Maillard reaction can be arranged in three groups, as listed in **Table 5**.

Not only is the type of amino acid involved of importance, but also whether it is free or part of a peptide or protein. Recently, it has been shown that heating γ-aminobutyric acid, a heptapeptide, or β-casein with 1-^{13}C-arabinose leads to different proportions of isotopomers of acetic acid and norfuraneol with the ratio of methyl-labeled:unlabeled

Table 5 Volatile products of the Maillard reaction

Group 1: 'Simple' sugar dehydration/fragmentation products	
Furans	(e.g., hydroxymethylfurfural)
Pyrones	(e.g., maltol)
Cyclopentenes	(e.g., methylcyclopentenolone)
Carbonyls	(e.g., $CH_3COCOCH_3$)
Acids	(e.g., CH_3COOH)
Group 2: 'Simple' amino acid degradation products	
Aldehydes (cf. section 'Strecker Degradation')	
Glycine	CH_2O
Alanine	CH_3CHO
Valine	$(CH_3)_2.CH.CHO$
Leucine	$(CH_3)_2.CH.CH_2.CHO$
Isoleucine	$CH_3.CH_2.CH(CH_3).CHO$
Phenylalanine	$C_6H_5.CH_2CHO$
Tyrosine	Aspartic acid
Glutamic acid	
Lysine	
Arginine	
Histidine	
Tryptophan	
Serine	$[CH_2OH.CHO]$
Threonine	$[CH_3CHOH.CHO]$
Cystine	$[CH_2SH.CHO]$
Methionine	$CH_3S.CH_2CH_2CHO$, methional
Proline	Cannot
Hydroxyproline	Cannot
Sulfur compounds	
Cystine	H_2S
Methional	$CH_3SH + CH_2{:}CH.CHO$
Group 3: Volatiles produced by further interactions	
Pyrroles	
Pyridines	
Imidazoles	
Pyrazines	
Oxazoles	
Thiazoles	
Compounds ex aldol condensations	

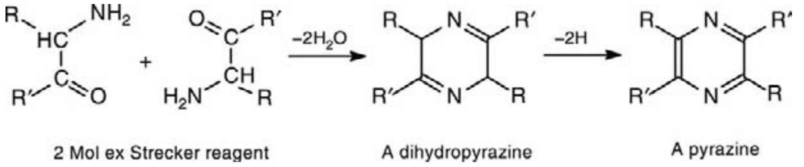


Figure 6 Pyrazine formation from the product of the Strecker degradation.

being 82:18, 26:74, and 5:95 and 80:20, 78:22, and 60:40, respectively.

From a flavor point of view, pyrazines are the most important compounds of Group 3. They are derived from the dicarbonyl compound, which picks up ammonia in the Strecker degradation, as shown in the equation in section 'Strecker Degradation'. The mechanism is shown in **Figure 6**. Pyrazine formation is favored by higher temperature and pH, but intermediate a_w (0.75). Pyrroles can be formed by replacement of the ring oxygen with ammonia or amines in parallel to the nonvolatile, but colored products included in **Figure 5**.

The Maillard reaction can lead to some nonvolatile compounds with bitter attributes, but these have not so far been found to have significance in milk and dairy products.

a_w

The Maillard reaction produces water and so can be a cause of raised a_w, but the effect of a_w on the Maillard reaction is of much greater interest. Since loss of water is part of the Maillard reaction, the mass action effect of water at high a_w will tend to hinder the reaction, as will the dilution of the reagents. On the other hand, as systems become more concentrated, mobility of the reagents becomes reduced. Hence Maillard browning tends to exhibit a maximum at intermediate a_w (e.g., whey powder at ca. 0.44, dried milk at 0.68).

pH and Loss of CO_2

During the Maillard reaction, pH is lowered, both by the production of acids (see **Figure 4**) and by the conversion of amines to cyclic nitrogen compounds, although these changes would be counteracted to some extent by the loss of carbon dioxide.

Antioxidant Activity (Reducing Power)

Most sugars have some reducing power and this increases progressively as they are converted into glycosylamines, Amadori compounds, and reductones. Maillard reaction products themselves also possess antioxidant activity. Their abilities to complex metals and to scavenge free radicals also have relevance here.

Maillard reaction products from all three sugars examined, fructose, glucose, and xylose, were very effective when combined with histidine, which is a known metal chelator, in suppressing hexanal formation through linoleic acid oxidation. Only xylose–arginine produced a comparable result. The products from lysine were somewhat less effective. Equimolar amounts of the reactants are probably to be preferred.

Ultrafiltration of the products led to the greatest activity for the xylose–glycine fraction of about 4500 Da, its effect being greater than that of butylated hydroxyanisole (BHA), but less than that of butylated hydroxytoluene (BHT) on an equal weight basis. There was a strong synergistic effect between it and BHA. With glucose–histidine, the fraction >1000 Da had 6 times the activity of that of the crude reaction mixture.

Allowing β-lactoglobulin to react with xylobiose for 7 days led to a Maillard product containing 108 $\mu g\,mg^{-1}$ xylobiose, 60% of the available ε-amino groups becoming blocked in the process. The product possessed higher radical-scavenging activity (DPPH, α,α-diphenyl-β-picrylhydrazyl) than β-lactoglobulin or its conjugate with lactose.

When pasteurized skim milk was heated at 80, 90, or 120 °C for up to 24 h, the naturally occurring antioxidative activity was depleted initially and some prooxidants were formed. Only severe heat treatment, associated with browning, led to recovery of, or even an increase in, antioxidative activity. These results have implications not only for dairy products, but also for food formulations including milk as an ingredient.

Much still needs to be discovered in this area, which is also of physiological importance, and continues to be an active subject of research.

Solubility Loss

Melanoidin formation is invariably accompanied by an increase in molecular size, which almost always results in a decrease in solubility. However, the stability of milk upon lowering of pH and in the presence of calcium is improved by preheating, which is attributed to the modification of lysine residues and the consequent increase in negative charge on casein.

Loss of Vitamin C

Ascorbic acid is a reductone and is involved in nonenzymic browning, as has been outlined above. Milk is a significant source of vitamin C and any loss would detract from its nutritional value. It follows that storage of milk fortified with vitamin C presents a particular problem. The nature of the packaging plays a leading role. Use of a three-layered opaque bottle led to complete oxidation after 1 month, whereas a six-layered bottle with an oxygen barrier resulted in a slower decrease in vitamin C, 25% being left after 4 months. Furosine content and FAST index (fluorescence of advanced Maillard products and soluble tryptophan; see section 'Fluorescence' of this main section) were markedly higher in in-bottle sterilized milk than in ultra-high temperature (UHT) samples and in fortified compared with nonfortified milk. On storage, furosine content increased, but FAST index decreased.

Loss of Biological Value

Lysine is an essential amino acid and so its loss through the Maillard reaction seriously detracts from the nutritional value of milk, which is a good source of lysine. Such loss becomes even more important in the case of infant formulae.

Determination of nutritionally blocked lysine

As described in section 'Amadori Rearrangement', the Amadori rearrangement of ε-*N*-glucosyllysine irreversibly gives ε-*N*-deoxyfructosyllysine (I). Thus, lysine has become nutritionally no longer available from that point onward.

Amino acid analysis of proteins normally involves hydrolysis in 6 mol l^{-1} HCl. The products of hydrolysis of deoxyfructosyllysine with such strong acid are shown in **Figure 7**. In amino acid analysis by ion exchange, pyridosine elutes ahead of lysine, and furosine elutes well after arginine.

Because of the partial recovery of lysine, interpreting the results of analysis is complicated. It is important to appreciate that lysine locked up in the Amadori compound, although partially recoverable by amino acid analysis, is no longer nutritionally available.

When lactose reacts with protein in dairy products, lactulosyllysine residues are formed. On hydrolysis, these give yields of 40% recovered lysine and 32% furosine. The lysine residues nutritionally blocked can then be calculated as follows:

$$\%\ \text{Lys nutritionally blocked} = \frac{3.1\ \text{furosine}}{\text{total Lys} + 1.87\text{furosine}} \times 100$$

The factors are derived thus: $100/32 = 3.1$ and $60/32 = 1.87$. Total Lys represents the total lysine recovered in the analysis, that is, unreacted lysine plus lysine recovered from lactulosyllysine residues.

Table 6 gives results that have been obtained in this way for lysine damage under good manufacturing practice. For UHT milks, the ratio of pyridosine to furosine was 0.36. UHT milk with furosine >50 mg l^{-1} should be regarded as overprocessed. N^{ε}-Carboxymethyllysine (see below and **Figure 8**) correlates well with furosine ($r = 0.96$), from which it is probably derived by oxidation. HMF, which is more readily determined by gas chromatography (GC), correlates with furosine equally well ($r = 0.96$) for pilot plant UHT milk, but less well ($r = 0.85$) for commercial UHT milk.

Determination of furosine in acid hydrolysates by use of reversed-phase HPLC in conjunction with an external standard of 2-acetylfuran is about 10 times as sensitive and leads to values more than double those given above.

A detailed kinetic study of the formation of HMF, lactulose, and furosine when milk is heated nonisothermally at 90–140 °C has been performed and a variety of models has been explored. Using a pseudo-zero-order model, that is, concentration increases linearly with time, the values for E_a were 90.2, 99.1, and 88.7 kJ mol^{-1},

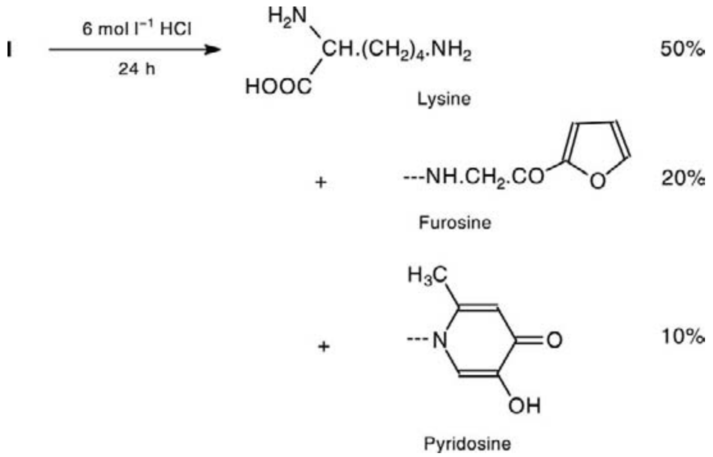


Figure 7 The products of hydrolysis of ϵ-*N*-deoxyfructosyllysine under protein analysis conditions.

Table 6 Lysine damage in good manufacturing practice

Process	*Lysine damage (%)*
Raw or freeze-dried milk	0
Pasteurized (74 °C, 40 s)	0–2
Ultra-high temperature-sterilized (135–150 °C, a few seconds)	0–3
Spray-dried powder	0–3
Sweetened condensed	0–3
In-container sterilized fluid	8–12
Roller-dried (without precondensation)	10–15
Evaporated	15–20
Roller-dried (conventional)	20–30

Reproduced from Mauron J (1981) The Maillard reaction in food: A critical review from the nutritional standpoint. In: Eriksson C (ed.) *Maillard Reactions in Food: Chemical, Physiological and Technological Aspects*, pp. 5–35. Oxford: Pergamon Press.

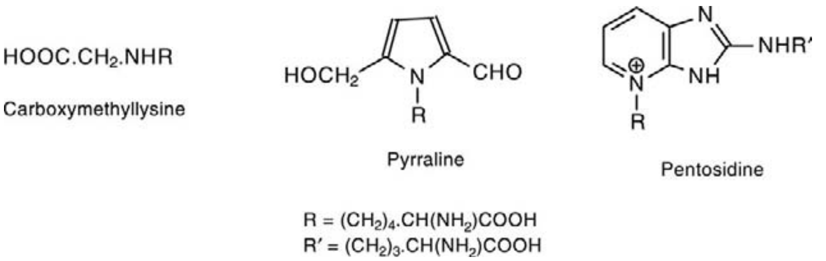


Figure 8 Some amino acids combined with intermediate Maillard products and determined in milk and dairy products.

respectively, with $k_{110\,°\mathrm{C}}$ 1.2 μmol l^{-1} min^{-1}, 51.5 mg l^{-1} min^{-1}, and 16.3 mg 100 g^{-1} protein min^{-1}, respectively.

Milk and dairy products can also be hydrolyzed by a succession of selected enzymes and the resultant hydrolysates examined by amino acid analyzer, when lactuloselysine elutes shortly after phenylalanine. The values for % lysine modified obtained in this way for nine samples of different infant formulae were 3–5.5 times as high as those obtained by the furosine method. This reinforces the view that the results given above are considerable underestimates.

Modification of specific lysine residues in β-lactoglobulin has been relatively quantified for a range of dairy products. Lactulosylation at Lys47 was highest in powdered infant formulae, whereas modification at Lys138/141 was highest in condensed milk. Galactooligosaccharides are well known prebiotic ingredients. They too have the capacity to glycate β-lactoglobulin. Thus, after 23 days at a_w 0.44 and 40 °C, the mean molecular mass had increased 21%, which may have implications for functional dairy products. Prebiotic activity of fructooligosaccharides and inulin was little changed by the Maillard reaction (up to 6 h at 85 °C with 1% glycine).

Modified whey protein concentrate, made from whey by lowering the pH to 3.35 and heating at 80 °C for 3 h, has improved functional properties, particularly as an aid to cold-set thickening. Its β-lactoglobulin is 2.5 times less lactosylated compared with that of commercial whey protein concentrate, which is manufactured at a higher pH. Accordingly, it offers more scope to improve further its functionality, for example, in emulsification, by reaction with other carbohydrates, such as dextran.

Pigs were fed successively with two experimental preparations containing dried skim milk that had been lyophilized or heated to block 50% of the lysine through formation of lactulosyllysine. Blood concentration and flux of individual amino acids, glucose, galactose, and fructosyllysine for 12 h, following ingestion, and excretion of lysine, fructosyllysine, and amino acids for 72 h were determined. Loss in the nutritive value of the protein was mainly due, and proportional, to lysine deterioration, the decrease in the digestibility of some essential amino acids playing a lesser role. Absorption of sugars and amino acids was considered to be relatively little affected in comparison with the extent of the Maillard reaction. Common processed foods, such as milk, infant formulae, bakery products, and pasta, are subjected to much milder Maillard reactions and so the nutritional consequences of the Maillard reaction are likely to be negligible for these foods.

In the absence of cysteine derivatives, glyoxal gives hardly any CML, but preferentially reacts with arginine residues, whereas in their presence arginine residues remain practically unaffected, with noticeable formation of CML. Thus, glyoxal reacts with β-casein, which contains no cysteine residues, to give predominantly arginine derivatives, leaving lysine residues unaffected.

Lactose not only participates in the Maillard reaction, but can also undergo a variety of other reactions, of which isomerization to lactulose (Lobry de Bruyn–Alberda van Ekenstein rearrangement) is the most important. Accordingly, lactulose concentration is a useful index of exposure to heat, even though the smallest amount determinable is quite high (30 mg l^{-1}). Levels found in milk are as follows: pasteurized, 0–82 mg l^{-1}; UHT direct, 41–670 mg l^{-1}; UHT indirect, 120–1430 mg l^{-1}; and sterilized, 412–1840 mg l^{-1}. The different classes of milk are therefore not clearly differentiated by lactulose content alone. There is little increase in lactulose, if any, on normal storage and lactulose does not appear to be formed during milk drying, but lactulose correlates extremely well with furosine for fresh UHT milk ($r = 0.99$). There is thus scope for the ratio of furosine to lactulose to provide additional information on the thermal history of a sample, particularly one of sterilized milk.

Lactosylated α-, β-, and κ-caseins have been shown to migrate shortly behind the unmodified proteins on CE, allowing lactosylated β-casein to be monitored not only in milk and milk powders, but also in processed cheese. The formation of lactosylated β-casein A1 and A2 during the heating of processed cheese was found to correlate with the furosine content. Thus, CE of casein may well be suitable for assessing the extent of the early Maillard reaction in dairy products.

Indeed, CE proved to be a fast, easy, and sensitive method for monitoring the quality of milk powders during storage in comparison with isoelectric focusing (IEF), amino acid analysis, and determination of furosine, HMF, browning index, and available lysine.

The ε-*N*-deoxyfructosyl groups, which on acid hydrolysis give rise to furosine and pyridosine, can undergo a number of reactions (cf. **Figure 4**), leading, for example, to acetic acid. If this were to remain attached to the ε-amino group, CML would result (**Figure 8**). CML can be determined by reversed-phase HPLC with fluorescence detection and pre-column derivatization with *o*-phthalaldehyde, reproducibility being very good (relative standard deviation (RSD) 2.2%). CML was not detected in UHT milk, most flavored milks, and all cheeses ($n = 49$), except for a whey cheese, which had the highest amount (1691 mg kg^{-1}), followed by an evaporated milk (1015 mg kg^{-1}, $n = 9$, median 499 mg kg^{-1}). A single sterilized milk sample out of 13 had 343 mg kg^{-1} protein. CML is questionable as a marker of heat damage in conventional milk products, whereas furosine allows evaluation of the early stages of the Maillard reaction. Determination of CML becomes useful for a range of products: severely heat-treated samples, products where a long shelf life is required and where stability is achieved with salt additions, milk products made with components that have already been compromised (e.g., cocoa products), and other products such as 'follow-on' milks.

Industrial production of infant formulae involves considerable exposure to heat, which leads to many Maillard products. CML is a convenient one to follow. The median concentration of CML in infant formulae was 70 times that in human breast milk. It was higher in hydrolyzed formulae than unhydrolyzed formulae. Plasma CML levels were 46% higher and urinary excretion was 60 times higher in the formula-fed infants than in the breast-fed infants. Thus, CML is absorbed into the bloodstream, but is rapidly excreted in the urine.

Using HPLC with UV detection led to finding furosine at levels of 1.3–1.6 g 100 g^{-1} protein in infant formulae and 0.9–1.2 g 100 g^{-1} protein in follow-on formulae, whereas the mean level in human milk was <0.006 g 100 g^{-1} protein. The lysine blocked in formulae ranged from 19 to 34%. As a consequence, it was suggested that furosine contents should be given on product labels and a maximum value (say 0.7 g 100 g^{-1} protein) should be established.

HMF is an important breakdown product of hexoses via the Amadori compound and 1,2-enolization. One can imagine it reacting with ε-amino groups to give pyrraline (**Figure 8**). Pyrraline can be determined by reversed-phase HPLC with electrochemical detection and it has been shown to increase progressively in the deproteinized whey fraction of non-fat dried milk over 6 h at 80 °C from 2 to 133 ppm. It has also been found in a range of processed foods, including a cheese gravy. During storage of freeze-dried milk at 70 °C, pyrraline (determined after enzymic hydrolysis) increased almost linearly with time and the amount formed increased with moisture content, reaching more than 5000 mg kg^{-1} protein in 50 h at 9% moisture. Values up to 3100 mg kg^{-1} protein were found in some samples of milk or whey powder, attributable to overprocessing and/or storage under adverse conditions.

Once the Maillard reaction has led to the conversion of an ε-sugar residue to an α-diketo compound, such as a 3-deoxy-1,2-osulose, reaction with an arginine residue becomes possible, cross-linking the protein. Pentosidine (**Figure 8**) is a compound formed in such a way. It has been found in small amounts (2–5 mg kg^{-1} protein) in acid hydrolysates of sterilized milk and in evaporated milk.

It appears that aminoreductones are the principal compounds that protect riboflavin (lactoflavin, vitamin B_2) from photodegradation in UHT milk.

Chelation of Metals

Melanoidins have metal-chelating properties. Including 10% of the Maillard reaction product from glucose–glutamate in the diet of rats has been shown to lead to severe diarrhea and nephrocalcinosis. Rats similarly fed diets containing 0.5% of the Maillard reaction product from glucose–glutamate did not show significant changes in the retention of calcium, magnesium, copper, or iron, but zinc retention was reduced by about a third. Zinc is one of the metals in which human diets tend to be marginal.

Toxicity

The content of 4-methylimidazole in ammonia caramel has a legal limit of 200 mg kg^{-1}. Abnormalities associated with its ingestion by rats include lymphocytopenia, but its control is more as an indicator of good manufacturing practice than because of a threat of toxicity. Mutagenicity in Maillard products has been traced to compounds such as imidazoquinolines and imidazoquinoxalines, the formation of which involves creatine and/or creatinine, compounds present primarily in muscle foods. Acrylamide is a probable carcinogen in humans and has been found in foods, where it is principally derived from asparagine at relatively high temperatures. High acrylamide content is not associated with dairy products, but with foods such as potato chips/crisps and crispbread, made from wheat/rye.

Fluorescence

Although fluorescence has been noted in Maillard products, particularly at the intermediate stage, no systematic study has been made of it. Fluorescence has proved useful, especially in examining sections under the microscope. Several molecules derived from cross-links obtained under physiological conditions fluoresce (Ex$_{\text{max}}$ 320–380, Em$_{\text{max}}$ 380–465 nm), providing specific data to supplement the relatively characterless visible absorption spectra. However, quenching of fluorescence constitutes a potential drawback.

Nevertheless, fluorescence has been investigated in various ways to assess product quality. Thus, the relationship between fluorescence (Ex 347 nm, Em 415 nm) and heat treatment of milk was explored using % relative fluorescence in the form of a fluorescence index. Mean results for Spanish milks were as follows: pasteurized, 20 ± 2% ($n = 9$); UHT, 28 ± 4% ($n = 36$); in-bottle sterilized, 44 ± 5% ($n = 6$). For the UHT samples, there was a good linear correlation with HMF content ($r = 0.935$). The behavior of the fluorescence on storage of the milks was not studied and may well confound the results. In model systems, lactose in the absence of casein and whey proteins gave 500–1000 times less fluorescence than when either was present.

Another fluorometric method was developed also to estimate the intensity of heat treatments to which milk had been exposed and was given the acronym FAST. It is based on the accumulation of fluorescent Maillard reaction products (Ex 330, Em 420 nm) and the quantification of protein denaturation by tryptophan fluorescence (Ex 290, Em 340 nm) in the transparent pH 4.6-soluble fraction of the milk. The former is an indicator of high-temperature treatment of milk, as in indirect UHT processing, and the latter is a marker of low-temperature treatment, as in pasteurization. The method was validated with milk heated under various industrial processing conditions (40–140 °C, up to 10 min). Results from FAST assay were compared with those of three HPLC methods for quantification of β-lactoglobulin, furosine, and lactulose, respectively. FAST assay proved as efficient as the three HPLC methods for predicting the effects caused by pasteurization and sterilization and was better at discriminating between the types of heat process applied. It is worth noting that the index is not storage sensitive. From an examination of 80 samples, ranging from raw to indirect UHT-treated milk, it was concluded that the method allows prompt distinction between various types of milk and could form the basis of a rapid quality control system in plants heat-treating milk.

The third method is front-face fluorescence spectroscopy (FFFS). It uses an angle of incidence of 56° in place of the normal 90°, which allows milk samples to be examined directly in spite of their turbidity and thus avoids the additional steps of preparing pH 4.6-soluble fractions. Emission fluorescence spectra of tryptophan and emission and excitation fluorescence spectra of Maillard products were recorded on UHT samples, both normal and overheated. Processing the spectra by principal component analysis and principal component regression led to the simultaneous determination of the furosine and lactulose content. Correlation with reference methods (HPLC for furosine and an enzymic method for lactulose) was good. Optic fibers may make the method suitable for use online.

Practical Applications

Color

The development of color due to the Maillard reactions is usually detrimental for dairy products and therefore normally becomes a question of avoidance or moderation. This is done by using, where feasible, lower temperatures, shorter processing and storage times, and lower pH, and avoiding intermediate a_{w}.

Color parameters and HMF content were monitored during storage of UHT-sterilized milk at 4, 8, or 20 °C for 24 weeks. Levels of total and free HMF increased with duration and temperature. Close correlations were obtained between the course of the Maillard reaction, as indicated by the level of total and free HMF, and ΔE, rather than the b^* values.

Browning is the principal mode of deterioration of sweet whey powder (SWP) and this was investigated using three commercial samples. They were exposed to normal storage conditions (21 °C, 35% RH) and accelerated ones (up to 55 °C). The commercial samples deteriorated at different rates, the spectrophotometrically fastest browning one showing also the fastest lowering of the Hunter Lab L^* value (lightness). However, flavor and aroma were maintained throughout the ambient storage period (19 months), 12 months being the shelf life typically quoted by suppliers. Moisture content does increase on ambient storage and so functional properties, such as flowability and dispersibility, should be examined.

General principles do not necessarily apply in special situations. SWP normally has a pH >6, but longer holding times may lead to lowering of the pH. To check whether this has a significant effect on color, spray-dried SWP (pH 6.3) and SWP acidified to pH 4.9 and 4.2 by exposure to acetic acid vapor were stored at 40, 60, and 80 °C in sealed containers. In addition to color, HMF and lysine contents were determined. In general, browning increased with temperature, storage time, and lower pH. The last goes against what was said previously and seems to be connected with the low a_w of SWP (0.182). HMF was highest at the lowest pH, which also led to the lowest final lysine content.

Volatile Compounds

Individual samples of milk and dairy products have been found to yield up to 400 volatile components, most of which are derived from milk lipids or microbial action. The Maillard reaction becomes important when heat is applied or on longer storage.

HMF is a product of the intermediate stage of the Maillard reaction and is volatile. However, the total amounts formed are variable and do not correlate too well with processing conditions, with values ranging from traces to 24.1 μmol l^{-1} over raw, UHT (direct and indirect), and in-can sterilized milks. Nevertheless, HMF has been used to follow successfully indirectly heated UHT cow/buffalo (1:1) milk through storage at 37 or 22 °C, total HMF falling initially from 18.3 to 15.0 and 12.8 μmol l^{-1} after 9 days, in parallel with the decrease in sulfhydryl groups, and then increasing progressively to 26.8 and 21.9 μmol l^{-1}, respectively, after 33 days, as residual oxygen continues to be depleted. The product became unacceptable sensorily after about 25 days.

At least 67 volatile compounds can be detected in the headspace above whey protein concentrate equilibrated to 75% RH and kept under accelerated storage conditions of 70 °C for 4 days. Most of the 33 compounds identified were clearly lipid breakdown products, but 2-methylbutanal can be attributed to the Strecker degradation, 2-furanmethanol to carbohydrate degradation, methyl- and 2,6-dimethylpyrazine to the Maillard reaction (Group 3), and dimethyl disulfide and dimethyl trisulfide to methionine breakdown.

Block milk or white crumb is an intermediate product in the manufacture of milk chocolate and white chocolate. It is made from concentrated milk and sugar and more than 30 volatiles have been identified in the headspace. Of these, again, most are clearly lipid breakdown products as with the whey protein concentrate above, but they also included 2- and 3-methylbutanal, dihydro-2-methyl-3(2*H*)-furanone, 2/3-furfural, 1-(2-furanyl)etha none, 2/3-furanmethanol, pyrazine, methylpyrazine, pyrrole, dimethyl disulfide, dimethyl trisulfide, formic acid, and 2- and 3-methylbutanoic acid. Quantitative descriptive analysis resulted in a list of nine flavor and five taste attributes, including milk, nutty, caramel, and burnt. Considerable further work is required.

Functionality

Furosine levels are useful indicators of heat treatment and of storage conditions. With milk, there is little change on pasteurization and 8.6 mg furosine 100 g^{-1} protein is thought to be a useful upper limit for pasteurized, peroxidase-positive milk. Seven commercial samples of such milk from Germany gave values in the range of 6.0–7.3 mg 100 g^{-1} protein. Twenty-six samples of Italian pasteurized milk out of 124 examined gave higher results (some even exceeding 100 mg 100 g^{-1} protein), implying the presence of reconstituted milk powder. Turkish pasteurized milks gave acceptable furosine values (3.79–5.88 mg 100 mg^{-1} protein) and UHT milks gave a wide range of higher values (49.7–213.4 mg 100 g^{-1} protein).

UHT treatment brings about a more than 10-fold increase in furosine over raw milk, and in in-bottle sterilized milk the increase is more than 50-fold. Formation of furosine continues on storage, for example, in pasteurized milk at 6–8 °C at 1 mg 100 g^{-1} protein every 16 days.

In order to predict safety and quality parameters for UHT-processed milks (4–58 s at 120–150 °C), lethality (F_0) and microbial inactivation value (B^*), on the one hand, and chemical change (C^*), thiamine loss, lactulose formation, Maillard browning, and HMF formation, on the other hand, were assessed and incorporated into a spreadsheet. For one set of optimized conditions, $B^* = 1.24$ and $C^* = 0.55$. The sensory attributes of this milk were similar to milk with $C^* = 1.5$, but different from the one with $C^* = 6$. The activation energy giving the best agreement between predicted and experimental results for lactulose was 122 kJ mol^{-1}. Browning was noticeable immediately after processing only in the sample most severely heat-treated, but became apparent in others on storage at 30 °C and above.

Lactose crystallization plays an important role in quality deterioration of whole milk powder (4.4% (w/w) moisture, a_w 0.23) under mildly accelerated storage (37–55 °C). The deterioration was strongly temperature dependent, with energies of activation close to

200 kJ mol^{-1}. Glass transition (T_g 48 °C) did not perturb temperature dependence, but lactose crystallization liberated water (a_w increased to 0.46) with a modest time delay (about 2 days at 55 °C), browning (surface Hunter *b* value) and HMF (HPLC) increasing concomitantly, while furosine formation occurred progressively, beginning prior to crystallization. Why the different indicators of the Maillard reaction behave differently needs to be elucidated. There was no evidence of the Maillard reaction products having antioxidant activity.

With skim milk powder, there is again little sensitivity to pasteurization conditions, but a greater than 10-fold increase in furosine content on drying, during which the whey protein nitrogen index (WPNI, a measure of the heat treatment applied to milk during processing to milk powder) changes little. During drying, the powder passes through the region of a_w of maximum Maillard reactivity, resulting in a range of furosine values from 55 mg 100 g^{-1} protein for extra low-heat samples to 350 mg 100 g^{-1} protein for high-heat samples. Similar results have been obtained for skim milk powder by determining lactulosyllysine after enzyme hydrolysis.

Lactulosylation, as detected by CE, is extensive in skim milk powder, spray dried between 185 and 90 °C (inlet and outlet temperatures).

Optimization to minimize lactulosylation led to keeping the outlet temperature low (preferably <80 °C), since this parameter affected the degree of lactulosylation most. The inlet temperature was set to achieve the best compromise between low lactulosylation and high drying rate (170–175 °C). Both temperature and moisture content affected the progress of lactulosylation on storage. Control samples stored (in the dark) at +52 °C turned brown and had developed a caramel odor already in 3.5 weeks. In powders stored at +37 °C, both β-lactoglobulin and β-casein were more extensively modified than in samples stored at lower temperatures from the start of the second week. To prevent lactulosylation of low-lactulose skim milk powder, further drying to <2.5% (w/w) moisture content and storage at −20 °C were required.

Because of the increased furosine content of milk powder, furosine determination can indicate the adulteration of fresh milk with reconstituted milk. The ratio of furosine to lactulose can similarly indicate the adulteration of UHT milk with reconstituted milk.

Furosine has been used to indicate the degree to which lysine has been damaged in milk-based foods with the following results: condensed milk, 36.3; milk nougat, 34.3; soft nougat, 33.0; milk chocolate, 27.1; white chocolate, 18.5; chocolate cream, 14.8; milk tablet 1, 13.4; rice cream, 11.2; milk tablet 2, 11.0; cooked cream, 2.6; yogurt mousse, 2.6; and dietetic meal, 2.5%.

For most infant formulae, the proportion of lysine blocked (13–27%) is not high enough to reduce the lysine intake to below the FAO suggested value of 102 mg kg^{-1} day^{-1}. The amount of lysine blocked is lowered by substituting lactose with glucose syrup of dextrose equivalent (DE) <15. The production of dietetic milks by enzymic hydrolysis of lactose can double the concentration of reducing sugars and is thus liable to increase the amount of lysine blocked, but in none of the milks analyzed was the level of free lysine residues lowered so far as to make lysine the limiting amino acid. It is important that 'lactose-free' milk, in particular, is stored at <4 °C.

When skim milk powder was stored at 20–30 °C for 6 months, a further 15% lysine ceased to be available, as determined by the dye-binding method. During storage, whey protein solubility changes little, whereas furosine levels can more than double in 10 months, in accord with changes in rennet coagulation time and curd tension. Values of up to 1200 mg furosine 100 g^{-1} protein were obtained for atomized skim milk powder on storage at 30 °C and 0.44 a_w for 90 days.

Dulce de leche is a dairy-based confectionery product traditionally prepared by heat concentration of whole milk and sucrose. Seven different formulations commonly used for manufacture were examined for available lysine (*o*-phthaldialdehyde spectroscopic method), using skim milk powder, whole milk powder, or lactose-hydrolyzed milk powder and different combinations of sucrose, glucose, and fructose. Skim milk led to a low-fat product, but with the same available lysine content as the traditional product; however, lactose-hydrolyzed milk gave a very low available lysine value. Replacement of 10% sucrose by glucose increased the rate of lysine glycation by 90%, while fructose gave an increase of only 30%. It was concluded that, in order to minimize loss of lysine, while avoiding sucrose crystallization on storage, dulce de leche should be prepared from sucrose, with fructose added at the final stage of the process.

The effect of the kind of carbohydrate present in sweetened milk products has been investigated in a model system of lactose-free milk with added carbohydrate and batch pasteurized at 80 °C, color formation and furosine being determined. Glucose and fructose were most active, lactose and syrups with high maltose content caused moderate browning, and sucrose and low-DE syrups limited reactivity. In the pH range studied (5.6–7.6), color formation was strongly pH dependent, whereas furosine formation was affected less. Relatively low concentrations of urea considerably reduced furosine formation.

Industrial production of Mozzarella cheese involves only limited exposure to heat and so does not produce furosine values greater than 8 mg 100 g^{-1} protein. Data for European commercial cheeses ($n = 73$) gave 4–38 mg 100 g^{-1} protein, suggesting some use of raw materials other than fresh liquid milk.

Rennet casein is the protein of choice in the manufacture of Mozzarella cheese analogues having melt and flow characteristics suitable for pizza toppings. Their functionality in this regard can be very variable. The furosine levels were found to range from 6.2 to 123.8 mg 100 g^{-1} protein and correlated with hydration characteristics, such as time taken to reach maximum viscosity index. The variability may be due to the temperature and duration of washing the curd and of drying, the heating effects of grinding or milling, and the storage conditions.

The functional properties (solubility, thermal stability, and emulsification and foaming properties) of β-lactoglobulin can be improved by glycation, arabinose, galactose, glucose, lactose, rhamnose, and ribose having been tried (3 days at 60 °C). Glycation shifted the minimum solubility to lower pH and improved the stability to heat at pH 5. Arabinose and ribose, the most reactive sugars, improved emulsification, whereas glucose and galactose led to better foaming properties.

The Maillard reaction products of alanine/xylose (500 ppm) and lysine/lactose (1000 ppm) possessed greater antioxidant activity in relation to butter stored at 6 °C than BHT (200 ppm), whereas other Maillard reaction products, such as those of alanine/xylose (1000 ppm), exhibited a slight prooxidant effect. Development of acidity was retarded most by the Maillard reaction products of proline/xylose and lysine/xylose (both 1000 ppm).

The Maillard reaction is a major deteriorative factor in the storage of whey powders and therefore the effect of the physical structure of the matrix (either porous or mechanically compressed) on it has been examined. Browning decreased in the order sweet whey > reduced-mineral whey > whey protein concentrate > whey protein isolate (WPI). The slope of the second linear segment of the thermogravimetric curve was attributed to the loss of water formed by the Maillard reaction and stood in good relationship with browning. In the glassy state, the compressed systems developed higher rates of browning and of weight loss than the porous systems.

In order to improve its functional properties, WPI was glycated with maltopentaose (50 °C, pH 8.0, 3 days, sugar content increased to almost 20%) and the product was phosphorylated by dry heating with pyrophosphate (85 °C, pH 4.0, 5 days, P content increased to >1%). The stability of WPI to heat-induced insolubility at pH 7.0 increased on conjugation and again on phosphorylation. Although the emulsifying activity of WPI was hardly affected by either process, the stability of emulsions with WPI increased 2.2-fold on its phosphorylation. The properties of heat-induced WPI gels (e.g., hardness, resilience, and water-holding capacity) were markedly improved, phosphorylation leading to transparent gels. Calcium phosphate-solubilizing ability was also enhanced by phosphorylation.

Toxicity

The presence of imidazole mutagens has not been reported for dairy products, although some mutagenicity will develop in dairy products as in many other foods on severe heat treatment. There is no mutagenic activity in high-temperature–short-time (HTST) pasteurized milk, in UHT milk, or in in-bottle sterilized milk. In fact, mutagen binding to casein may reduce the mutagenicity of some food systems.

Allergenicity of β-lactoglobulin may be enhanced by the Maillard reaction, so in manufacturing hypoallergenic milk formulae it is advisable to reduce the concentration of lactose.

Fluorescence

The practical applications have already been covered in section 'Effect on Product Quality'.

Conclusion

From the above, it can be seen that the network of the Maillard reactions is well established, but that a great deal of detail needs to be filled in as regards to most of its aspects, chemical, quality-related, and applied.

See also: **Concentrated Dairy Products**: Dulce de Leche; Evaporated Milk. **Dehydrated Dairy Products**: Infant Formulae. **Flavors and Off-Flavors in Dairy Foods**. **Heat Treatment of Milk**: Heat Stability of Milk; Non-Thermal Technologies: High Pressure Processing; Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems. **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk; Lactose: Chemistry, Properties; Lactose: Derivatives; Lactose: Galacto-Oligosaccharides; Lactose: Production, Applications. **Liquid Milk Products**: Liquid Milk Products: Flavored Milks; Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: UHT Sterilized Milks; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects; Recombined and Reconstituted Products. **Milk Lipids**: Lipid Oxidation. **Milk Protein Products**: Functional Properties of Milk Proteins; Milk Protein Concentrate; Whey Protein Products. **Milk Proteins**: α-Lactalbumin; β-Lactoglobulin. **Molecular Genetics and Dairy Foods**. **Nutrition and Health**: Effects of Processing on Protein Quality of Milk and Milk Products. **Plant and Equipment**: Milk Dryers: Drying Principles.

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Lactose Intolerance

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Background

The major carbohydrate component of milk, the disaccharide lactose, is cleaved into its constituent absorbable monosaccharides, glucose and galactose, by the small intestinal enzyme, lactase. Lactase is thus essential for the nourishment of newborn mammals, whose sole source of nutrition is milk. The enzyme is located on the villi of the small intestine, on the apical 'brush border surface' of the absorptive cells known as enterocytes (**Figure 1**), with the body of the enzyme molecule, including the catalytic site, protruding into the lumen of the gut. The glucose and galactose produced are then taken up into the enterocytes via a specific transporter molecule.

In adult mammals other than humans, lactase production decreases significantly following weaning, when milk is no longer part of the diet. It has been known for some 40 years that in humans, who often consume milk from other species in adult life, the situation is more complicated: lactase production persists into adult life in some people but not in others.

Symptoms of Lactose Intolerance

People in whom lactase persists at a high level throughout adult life are said to be lactase persistent and are lactose digesters, and can consume copious quantities of fresh milk without difficulty. Those with little lactase as adults are described as lactase non-persistent (also referred to in the literature as primary adult hypolactasia) and are lactose 'maldigesters'. Maldigestion can lead to symptoms of lactose intolerance, thus restricting the consumption of fresh milk. The symptoms arise from the transit of undigested lactose from the small intestine into the colon. Two things then occur. The osmotic effect of the lactose can lead to diarrhea, and/or the lactose is fermented by colonic bacteria, leading to the production of fatty acids and gases, the latter potentially giving symptoms of flatulence.

However, most lactase non-persistent individuals can tolerate small amounts of milk (as in tea or coffee), and some can consume a lot without ill effects. Variation in the composition of the gut flora between individuals, as well as a psychosomatic component, may account for some of the inter-personal variations in symptoms.

Determination of Lactase Persistence Status

Since taking intestinal biopsies from healthy people is invasive and not acceptable unless the person is having other investigations, lactase persistence status is often inferred by an indirect method that depends on lactose digestion. This difference in digestion is measured by a test traditionally known as 'lactose tolerance test' and thus the terms 'tolerant' and 'intolerant' are sometimes used, although this can be confused with dietary intolerance and are best avoided.

The lactose tolerance test usually involves giving a lactose load after an overnight fast and then measuring blood glucose or breath hydrogen. A baseline measurement of blood glucose or breath hydrogen is taken before ingestion of the lactose, and then at various time intervals thereafter. An increase in blood glucose indicates lactose digestion (glucose produced from lactose hydrolysis is taken up into the bloodstream), and no increase or a 'flat line' is indicative of a lactose maldigester (probable lactase non-persistent) phenotype. An increase in breath hydrogen also indicates maldigestion and reflects the colonic fermentation of the lactose, described above. These physiological tests, however, have an intrinsic error level, leading to both false negatives and false positives. For example, some people do not have hydrogen-producing bacteria, and some have high blood glucose at the start of the test.

Lactase levels can also be reduced by gastrointestinal disease, leading to secondary lactose intolerance. There are ways of improving the quality of the diagnosis. These include retesting, and giving a dose of a nondigestible carbohydrate, lactulose, to test for the presence of hydrogen-producing bacteria. However, the most accurate diagnosis is made by examination of biopsy material to histologically identify any disease changes, and to confirm the normal expression of brush border sucrase, which acts as an internal control.

The very rare cases of congenital hypolactasia or lifelong deficiency of lactase, present with symptoms of failure to thrive and diarrhea soon after birth, would normally be diagnosed this way, and indeed urgent diagnosis is needed since milk is the sole source of nutrition for newborn babies. In contrast, lactase non-persistence in adulthood rarely presents serious problems; changes in diet due to changes in cultural environment and

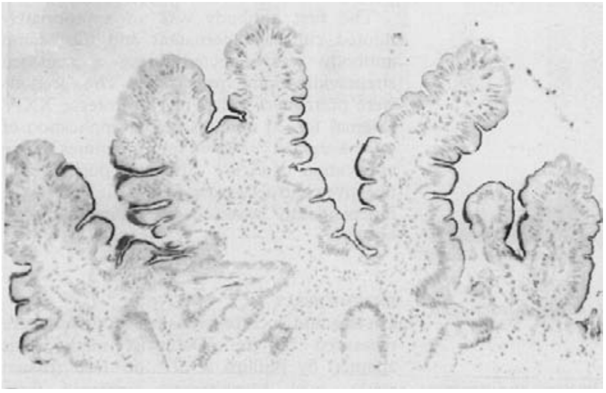

Figure 1 Immunohistological detection of lactase on the villus surface of a human small intestinal sample. Reproduced with permission from Harvey CA, Wang Y, Hughes LA, *et al*. (1995) Studies on the expression of intestinal lactase in different individuals. *Gut* 36: 28–33; Figure 3(a).

ignorance of lactose tolerance can, however, potentially cause debilitating symptoms if the problem is not recognized.

Worldwide Distribution of Lactase Persistence

The global distribution of lactase persistence is now fairly well characterized (**Figure 2**) and reveals that lactase non-persistence is the most common phenotype in humans (~ 65%), with frequent occurrence of lactase persistence tending to be restricted to populations with a long history of pastoralism and milking. Lactase persistence is at the highest frequency in northwestern Europe, with a decreasing cline to the south and east. In Africa and the Middle East, the distribution is patchy, with some pastoralist nomadic tribes having significantly higher frequencies of lactase persistence than neighboring (non-milk-drinking) groups living in the same country (**Figure 3**). The noted correlation of lactase persistence phenotype with the cultural practice of milking suggests that this trait has been subject to adaptive evolution, namely strong positive selection.

Causes of Lactase Persistence

There is good evidence that lactase persistence polymorphism in humans is genetically determined, and is inherited in an autosomal dominant manner. Studies using messenger RNA showed that the polymorphism is attributable to variation in the expression of the lactase gene and that genetic differences in sequences close to the lactase gene are responsible. A likely causative single nucleotide change of C to T (-13910^{*}T) was first identified in Europeans and is located 13.9 kb upstream of the lactase gene transcription initiation site, in an intron of an adjacent gene, known as *MCM6*.

In contrast to the high frequency in Europe, -13910^{*}T is rare in sub-Saharan African populations, even in those populations where the frequency of lactase persistence is reported to be high, and it is also rare in the Bedouins of the Arabian peninsula, who are also frequent lactose digesters. Subsequent studies revealed several new sequence variants in very close proximity to -13910^{*}T, three of which are clearly associated with lactase

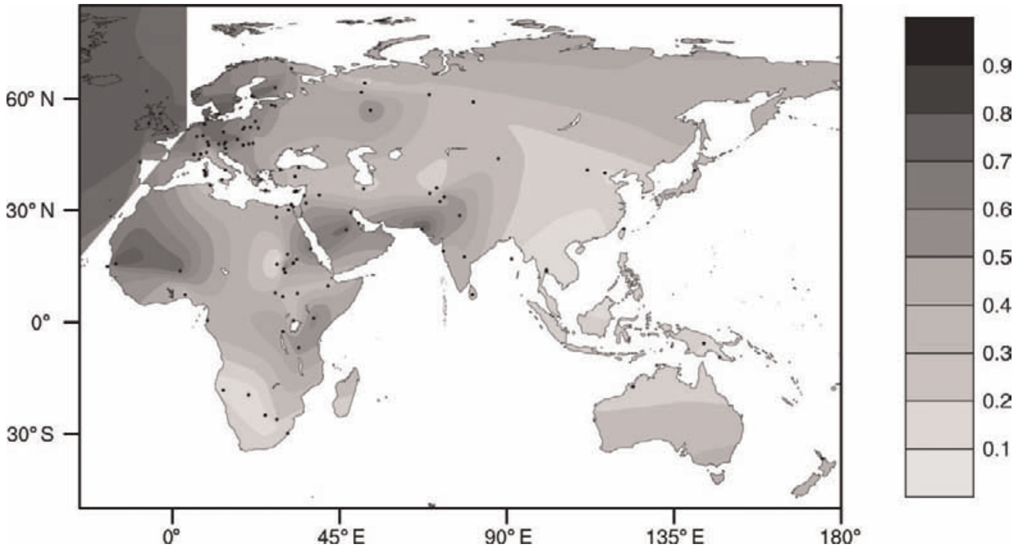

Figure 2 Worldwide frequency of lactase persistence (as assessed by lactose tolerance tests). Reproduced with permission from Ingram CJ, Mulcare CA, Itan Y, Thomas MG, and Swallow DM (2009) Lactose digestion and the evolutionary genetics of lactase persistence. *Human Genetics* 124: 579–591; Figure 1(a).

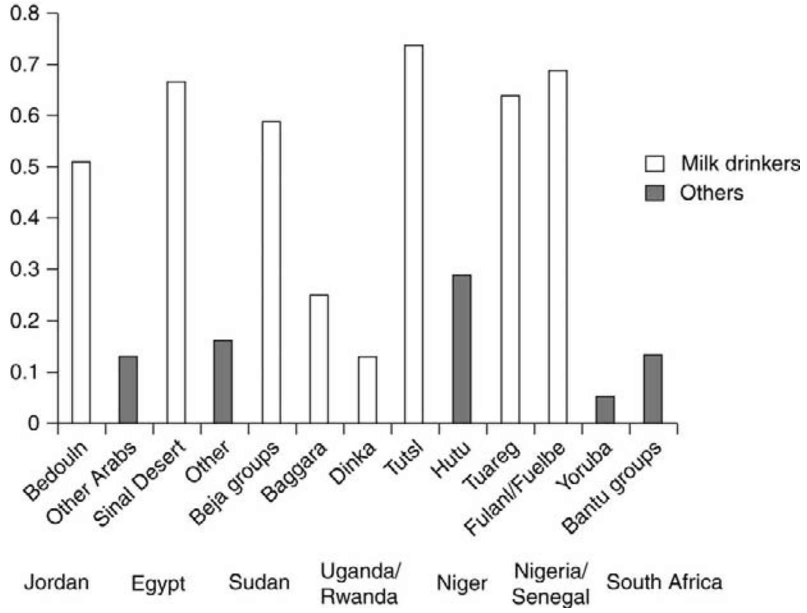

Figure 3 Examples of neighboring groups in which lactase persistence frequencies differ, as well as lifestyle. The pastoralist milk drinkers have a much higher frequency of lactase persistence. Reprinted with permission from Swallow DM (2003) Genetics of lactase persistence and lactose intolerance. *Annual Reviews of Genetics* 37: 197–219; Figure 1(c).

persistence in different parts of East Africa and the Middle East (−13915*G, −14010*C, and −13907*G).

Evidence for the Function of −13910*T, 13915*G, −14010*C, and −13907*G

In vitro studies using promoter–reporter construct assays in cell lines provide some, but not conclusive, evidence that the various clustered alleles affect 'enhancer' or regulatory activity of this sequence region, although currently the way that this alters the pattern of downregulation *in vivo* is far from clear. At least three of the mutations are associated with a different ancestral sequence background (haplotype) showing that a single associated functional mutation cannot be responsible, and that mutations conferring lactase persistence have therefore emerged several times during human evolutionary history.

Another complexity is that, even taking these additional variants into account, and supposing all of them to be functional, association with phenotype is not complete. There are digesters who carry no putative causative allele in this genomic region, indicating that there may be more, as yet unidentified, causal variants.

Role of Other Factors Influencing Lactase Expression

There is a considerable amount of variation in the immediate promoter of *LCT* (the gene encoding lactose) and it is just possible that variations in these single nucleotide polymorphisms affect expression under certain circumstances or at certain developmental stages. Although it has been well established that regulation of *LCT* is predominantly under genetically determined transcriptional control, there is evidence that other factors influence inter-individual differences in the expression of the enzyme. Heterogeneity of the lactase non-persistence phenotype has been reported by a number of research groups, which may reflect epigenetic or nongenetic influences. Other studies show a decline in lactose tolerance with age, which may be due to lowering of lactase levels.

Evolutionary Considerations

Because of the geographic distribution of lactase persistence and the generally coinciding pattern of historically milk-drinking populations, Simoons and McCracken independently suggested, more than 30 years ago, that milk dependence created strong selection for lactase persistence. However, the correlation is not absolute and there are exceptions in both directions. For example, there are some ethnic groups who rely heavily on milk products and for whom cows or camels play a very important role in their lifestyle, but who have a low reported frequency of lactase persistence, for example, the Dinka and Nuer in Sudan and the Somali in Ethiopia. This could, however, be explained by some lactase persistent populations having recently stopped milking or conversely having only recently adopted the habit, therefore allowing insufficient time for lactase persistence to be driven to high frequency. Population migration may also have played an important role. In addition, the cultural practice of milk fermentation (e.g., to yogurt or cheese) reduces lactose content allowing non-persistent individuals to benefit from milk products.

Notwithstanding these complexities, the original proposal of the role of positive selection for lactase persistence has now been supported by molecular evidence. At least two of the lactase persistence-associated alleles show unusually extended haplotype background and restricted sequence diversity, consistent with a rapid increase in frequency in the last 10 000 years.

What Were the Evolutionary Forces?

This 'culture historical hypothesis' suggests that the rise in lactase persistence co-evolved alongside the cultural adaptation of milk drinking, and its associated nutritional benefits. Most analyses conclude that high-frequency lactose digestion capacity never 'evolved' without the prior presence of milking.

Since significant quantities of fresh milk are consumed by many who are lactase non-persistent, apparently without any adverse effects, and it is likely that adaptation of the colonic bacterial flora allows digestion of lactose by these people, it seems that strong selection is likely to operate only at certain times and under more extreme circumstances such as drought and famine. Indeed it has been speculated that in desert climates (i.e., Middle and Near East) where water and food were scarce, nomadic groups could survive by utilizing milk as a food source, and in particular, as a source of clean, uncontaminated fluid. The benefits to persistent individuals may have become more pronounced during outbreaks of diarrheal disease, when non-persistent individuals would be unable to utilize milk as a water source without exacerbating their condition.

Another consideration is the suitability of climate for raising livestock. The frequency of lactose malabsorption is greater in populations where environmental conditions, such as endemic cattle disease, made dairying difficult. Nevertheless, a number of African groups with high lactase persistence frequency managed to circumvent harsh environmental conditions by adopting a semi-nomadic way of life.

In northern Europe, the advantage of improved calcium absorption has been suggested to explain the distribution of the trait. The low light levels experienced at high latitudes are associated with an increased risk of developing rickets and osteomalacia due to a lack of vitamin D production (which is synthesized by the skin in the presence of sunlight). Vitamin D is involved in the gut absorption of calcium, which is itself an essential mineral required for bone health. In addition, calcium may help to prevent rickets by impairing the breakdown of vitamin D in the liver. Hence, the ability to drink fresh milk, which contains calcium and also components that stimulate its uptake (including small amounts of vitamin D), may have provided an advantage to persistent individuals.

Health and Medical Considerations

Consumption of milk and milk products by those who cannot digest lactose is usually consciously or unconsciously adjusted to avoid adverse consequences but is nevertheless a relatively common cause of irritable bowel syndrome in Europe and the United States. Many commercial dairy products and other foods (including yogurts) contain high concentrations of lactose introduced in manufacturing, so that lactose is more widespread in the diet than it was for that same person's ancestors.

Lactose malabsorption can be distinguished from milk protein allergy, both by the range of symptoms and by the fact that dairy products low in lactose should fail to produce symptoms. Lactose tolerance testing can be a useful way of confirming lactose malabsorption and enabling avoidance of the cause, but DNA testing is not yet particularly useful for non-Europeans.

In countries such as Finland, where there is a high frequency of lactase non-persistence in comparison with the rest of northern Europe, commercial low-lactose products are readily available.

Many association studies have attempted to demonstrate the health benefits of milk consumption in lactase-persistent people, for example, by providing protection against osteoporosis, and others have claimed adverse effects of lactase persistence and associated high milk consumption (e.g., cataracts, ovarian cancer, heart disease, and diabetes). The often-contradictory findings are difficult to evaluate because of the high risk of confounding effects such as mixed ancestry, dietary intake, and variation in gut flora.

Conclusion

Lactase persistence is one of the leading examples of evolutionary adaptation in humans, and illustrates an important inter-individual variation in the ability to cope with a component of human diet. While milk products provide important nutrients, high lactose concentrations in fresh milk and in certain commercial products are impediments to their consumption by some people.

See also: **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose. **Milk**: Introduction; Milk in Human Health and Nutrition; Milk of Marine

Mammals. **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins. **Vitamins**: Vitamin D.

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Indigenous Oligosaccharides in Milk

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Introduction

Mammalian milk/colostrum contains from trace to ~13% of carbohydrate, of which lactose (Gal(β1-4)Glc) usually constitutes more than 80%. Milk/colostrum of most mammals also contains a variety of oligosaccharides, many of which have *N*-acetylglucosamine, galactose, fucose, and/or sialic acid residues attached to lactose, which is always located at the reducing end.

The ratio of milk oligosaccharides to free lactose in milk/colostrum varies, depending on the mammalian species. For example, in mature human milk, lactose and milk oligosaccharides constitute 80 and 20%, respectively, of the carbohydrate fraction, whereas mature bovine milk contains only trace amounts of oligosaccharides. Exceptionally, the milk of a few species such as monotremes (platypus and echidna) and marsupials (kangaroo, wallaby, possum, koala, wombat, etc.), and a few species of Carnivora (eutherians) such as the Canoidea (other than dog), contains greater amounts of oligosaccharides than of lactose.

Most human milk oligosaccharides (HMOs) cannot be digested within the small intestine, and reach the colon where they stimulate the growth of bifidobacteria and act as possible anti-adhesion factors against pathogenic microorganisms. The biological functions of milk oligosaccharides have recently been an active field of research. Since mature human milk contains about 1.3% of oligosaccharides, whereas bovine milk contains only trace amounts, infant formulae based on bovine milk should preferentially be supplemented with oligosaccharides that have effects similar to those of HMOs.

In this article, we describe the chemical structures and methods for the structural analysis of milk oligosaccharides, quantitative aspects and methods for their quantification, their gastrointestinal digestion and biosynthesis, and their biological significance as prebiotics, anti-infection factors, receptor analogues, and immunomodulators, with special reference to the oligosaccharides of human milk. We also describe the milk oligosaccharides of domestic farm animals and discuss comparative aspects of milk oligosaccharides among mammalian species, as well as future trends and the industrial utilization of milk oligosaccharides.

The Chemical Structures of Human Milk Oligosaccharides

The structures of at least 93 HMOs have been determined to date (**Table 1**), while mass spectrometry (MS) data have suggested the presence of almost 130 oligosaccharides in human milk or colostrum. Moreover, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analyses suggest that polysaccharides, consisting of more than 50 monosaccharide residues, as indicated by size-exclusion chromatography, are also present in human milk. Therefore, considerably more than 130 different saccharides are probably present in human milk.

It has been shown that human milk and colostrum are rich in oligosaccharides, in both concentration and variety, compared with the milk/colostrum of the cow and of many other species. As described below, these oligosaccharides are considered to play a significant role in the protection of human neonates against infection. It is likely that, because of their immaturity, human neonates are relatively susceptible to infection by pathogenic microorganisms; the presence of significant amounts and a large variety of milk oligosaccharides may therefore be advantageous insofar as they supplement the other, known, anti-infection properties of the milk/colostrum. In this connection, it is notable that the milk of species such as monotremes and marsupials, and a few species of eutherians, including Ursidae, that produce very altricial neonates, also contains relatively high concentrations of oligosaccharides, with lesser amounts of free lactose.

The 93 HMOs, the structures of which have been determined to date, can be grouped into 12 series based on their core units, as in **Figure 1**. The many oligosaccharides are constructed by the addition of a Neu5Acα2-3/2-6 residue to Gal or GlcNAc, and of Fucα1-2/1-3/1-4 to Gal, GlcNAc, or a reducing Glc of the core units.

The main structural features of HMOs are the presence of oligosaccharides containing the type I unit (Gal(β1-3)GlcNAc) as well as those containing the type II unit (Gal(β1-4)GlcNAc), and the fact that type I oligosaccharides predominate over type II. The milk oligosaccharides of other species mostly have the type II

Table 1 Chemical structures of human milk oligosaccharides

No.	Oligosaccharide	Structure
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	3-FL	Gal(β1-4)Glc | Fuc(α1-3)
3	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
4	β4′-GL	Gal(β1-4)Gal(β1-4)Glc
5	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
6	LNTri II	GlcNAc(β1-3)Gal(β1-4)Glc
7	DF-L(LDFT)	Fuc(α1-2)Gal(β1-4)Glc | Fuc(α1-3)
8	LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
9	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
10	LNFP I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
11	LNFP II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
12	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
13	LNFP V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
14	LNDFH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
15	LNDFH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-4) Fuc(α1-3)
16	LNH	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) /
17	LNnH	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
18	F-LNH I	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) /
19	F-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) /
20	F-LNnH	Fuc(α1- { Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
21	F-*para*-LNH I	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
22	F-*para*-LNH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)

(*Continued*)

Table 1 (Continued)

No.	Oligosaccharide	Structure
23	DF-LNH II	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) / Fuc(α1-4)
24	DF-LNH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) /
25	DF-LNnH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) / Fuc(α1-3)
26	DF-*para*-LNH	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4) Fuc(α1-3)
27	DF-*para*-LNnH	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3) Fuc(α1-3)
28	TF-LNH	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) / Fuc(α1-4)
29	TF-*para*-LNH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4) Fuc(α1-3)
30	TF-*para*-LNH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4) Fuc(α1-3) Fuc(α1-3)
31	TF-*para*-LNnH	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3) Fuc(α1-3) Fuc(α1-3)
32	F-LNO	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) /
33	F-LNnO	Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
34	F-*iso*-LNO	Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) /
35	DF-LNO I	Fuc(α1-3) Fuc(α1-3) Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) /

(Continued)

Table 1 (Continued)

No.	Oligosaccharide	Structure
36	DF-LNO II	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) / Fuc(α1-4)
37	DF-LNnO I	Fuc(α1-4) Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
38	DF-LNnO II	Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) / Fuc(α1-3)
39	DF-*iso*-LNO I	Fuc(α1-4) Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) /
40	DF-*iso*-LNO II	Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) /
41	TF-LNO	Fuc(α1-3) Fuc(α1-3) Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) / Fuc(α1-4)
42	TF-LNnO	Fuc(α1-4) Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) / Fuc(α1-3)
43	TF-*iso*-LNO I	Fuc(α1-3) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) /
44	TF-*iso*-LNO II	Fuc(α1-4) Fuc(α1-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) / Fuc(α1-4)
45	Tetra-F-*iso*-LNO	Fuc(α1-3) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) / Fuc(α1-4)
46	Tetra-F-*para*-LNO	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4) Fuc(α1-3) Fuc(α1-3)

(Continued)

Table 1 (Continued)

No.	Oligosaccharide	Structure
47	Penta-F-*iso*-LNO	Fuc(α1-4) Fuc(α1-3) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) ╱ Fuc(α1-4)
48	F-LND	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-3)GlcNAc(β1-3) ╱ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ╱
Acidic oligosaccharides		
49	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
50	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
51	F-SL	Neu5Ac(α2-3)Gal(β1-4)Glc Fuc(α1-3)
52	LST a	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
53	LST b	Neu5Ac(α2-6) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
54	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
55	F-LST a	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4)
56	F-LST b	Neu5Ac(α2-6) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
57	F-LST d	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
58	S-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ╱
59	S-LNnH I	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ╱
60	S-LNnH II	Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ╱
61	FS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) ╱
62	FS-LNH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ╱ Neu5Ac(α2-6)
63	FS-LNH II	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) ╱

(*Continued*)

Table 1 (Continued)

No.	***Oligosaccharide***	***Structure***
47	Penta-F-*iso*-LNO	Fuc(α1-4) Fuc(α1-3) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) Fuc(α1-4)
48	F-LND	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) Gal(β1-3)GlcNAc(β1-3)
Acidic oligosaccharides		
49	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
50	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
51	F-SL	Neu5Ac(α2-3)Gal(β1-4)Glc Fuc(α1-3)
52	LST a	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
53	LST b	Neu5Ac(α2-6) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
54	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
55	F-LST a	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4)
56	F-LST b	Neu5Ac(α2-6) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
57	F-LST d	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
58	S-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3)
59	S-LNnH I	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3)
60	S-LNnH II	Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
61	FS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)
62	FS-LNH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) Neu5Ac(α2-6)
63	FS-LNH II	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ⟩ Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)

(*Continued*)

Table 1 (Continued)

No.	*Oligosaccharide*	*Structure*
76	TFS-*iso*-LNO	Fuc(α1-3) Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) Fuc(α1-4)
77	DS-LNT	Neu5Ac(α2-6) Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
78	FDS-LNT I	Neu5Ac(α2-6) Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4)
79	DS-LNT II	Neu5Ac(α2-6) Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
80	DS-LNH I	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)
81	DS-LNH II	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) Neu5Ac(α2-6)
82	DS-LNnH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
83	FDS-LNH I	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) Neu5Ac(α2-6)
84	FDS-LNH II	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) Neu5Ac(α2-6)
85	FDS-LNH III	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) Fuc(α1-4)
86	FDS-LNnH	Neu5Ac(α2-3/α2-6)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3/α2-6)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
87	TS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) Neu5Ac(α2-6)
88	-	Fuc(α1-4) Neu5Ac(α2-3)Gal(β1-3)GlcNAc
89	-	Fuc(α1-4) Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal

(Continued)

Table 1 (Continued)

No.	Oligosaccharide	Structure
90	FS-*novo*-LNP I	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Neu5Ac(α2-3)Gal(β1-3) ⟋
91	DF-*para*-LNH sulfate I	6S | Fuc (α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
92	DF-*para*-LNH sulfate II	6S | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-4) Fuc(α1-3)
93	TF-*para*-LNH sulfate	6S | Fuc (α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-4) Fuc(α1-3)

F, fucose; L, lactose; S, sialyl; DF, difucosyl; DS, disialyl; TS, trisialyl; FS, fucosyl sialyl; DFS, difucosyl sialyl; TFS, trifucosyl sialyl; FDS, fucosyl disialyl; DGal, digalactosyl; FL fucosyllactose; GL, galactosyllactose; LDFT, lacto-difucotetraose; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; LNFP, lacto-*N*-fucopentaose; LNP, lacto-*N*-pentaose; LNDFH, lacto-*N*-difucohexaose; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; LNO, lacto-*N*-octaose; LNnO, lacto-*N*-neooctaose; LND, lacto-*N*-decaose.

Gal(β1-4)Glc	Lactose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-*N*-tetraose
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-*N*-neotetraose
Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ⟋	Lacto-*N*-hexaose
Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ⟋	Lacto-*N*-neohexaose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	*para*-Lacto-*N*-hexaose
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	*para*-Lacto-*N*-neohexaose
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ⟋	Lacto-*N*-octaose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ⟋	Lacto-*N*-neooctaose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ⟋	*iso*-Lacto-*N*-octaose
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	*para*-Lacto-*N*-octaose
Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-3)GlcNAc(β1-3) ⟋ Gal(β1-4)Glc Gal(β1-3)GlcNAc(β1-3) ⟋	Lacto-*N*-decaose

Figure 1 The 12 core units of human milk oligosaccharides.

but not the type I unit. The many varieties of oligosaccharides in human milk and colostrum are produced by the addition of Neu5Ac and/or Fuc residues to these two units.

The presence of a great variety of oligosaccharides in human milk depends on the activity of many different specific enzymes in the lactating gland. For example, an α1-2-fucosyltransferase is expressed in about 77% of all Caucasian women; these are classified as secretors. Therefore, the oligosaccharides in the milk from these women are characterized by the presence of α1-2-fucosylated components, for example, Fuc(α1-2)Gal(β1-4)Glc (2′-fucosyllactose (2′-FL)) and Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc (lacto-*N*-fucopentaose I (LNFP I)), or more complex oligosaccharides, all possessing Fuc(α1-2)Gal(β1-3)GlcNAc residues. In the mammary glands of Lewis (a+b−) individuals, who produce non-secretor milk, this enzyme activity is absent; as a result, the major fucosylated oligosaccharide is Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc (lacto-*N*-fucopentaose II (LNFP II)). This pattern of HMO is found in about 20% of the population. In about 5% of the population who belong to blood group Lewis (a−b−), LNFP II is absent from the milk because of the absence of α1-4-fucosyltransferase activity.

Methods for the Characterization of Milk Oligosaccharides

Prior to detailed structural characterization, each neutral and acidic milk oligosaccharide usually needs to be purified. The carbohydrate fraction can be separated from the milk by liquid–liquid extraction with four volumes of chloroform/methanol (2:1, v/v) or by precipitation with ethanol, and the oligosaccharides can then be separated from lactose by gel filtration or by stepwise elution from a column of activated charcoal using ethanol. The separation of acidic from neutral oligosaccharides is usually performed by anion exchange column chromatography with DEAE-Sephadex A-50 or other resins, or by anion exchange solid-phase column chromatography on Bond Elut NH_2.

More recently, isolation of the milk oligosaccharides has been accomplished by normal-phase or reverse-phase high-performance liquid chromatography (HPLC) or by high-pH anion exchange chromatography with pulsed amperometric detection (HPEAC-PAD). It is noteworthy, for example, that several novel minor oligosaccharides were isolated by HPLC using triethylamine as an ion pair reagent. On the other hand, the selective separation of specific epitopes has been carried out by affinity chromatography using a monoclonal antibody or lectin. For example, the oligosaccharides containing sialyl Le^a(Neu5Ac(α2-3)Gal(β1-3)[Fuc(α1-4)]GlcNac), a tumor-related carbohydrate epitope, were selectively separated by affinity column chromatography in which MSW113, the monoclonal antibody specific for this unit, was bound to Protein A–Sepharose GL4B as a ligand: the oligosaccharides containing sialyl Le^a were adsorbed by the column and were then eluted by raising the pH of the eluent.

Normal-phase HPLC can be performed using a TSKgel Amide-80 column, with a gradient elution system of acetonitrile/15 mmol l^{-1} phosphate buffer (pH 5.2). UV absorbance at 195 nm is used to monitor the eluate for the separation of acidic milk oligosaccharides. Reverse-phase HPLC is performed with a similar column, the gradient elution system being acetonitrile/ion exchange water; evaporative light scattering is used for the detection of neutral oligosaccharides. In addition, HPLC with a Thermo Hypersil column can be used to separate neutral oligosaccharides, the gradient elution system and detection method being as described above.

As many as 200 different HMOs have been separated and studied by microfluidic HPLC-Chip/MS technology. The oligosaccharide fraction was reduced with sodium borohydride and a hypercarb porous graphitized carbon column was used, gradient elution being performed with 0.1% formic acid/water and 90% acetonitrile/ion exchange water plus 0.1% formic acid.

Structural studies have been performed by analyses with MALDI-TOFMS, fast atom bombardment MS (FAB-MS), or nanoelectrospray ionization MS (nano-ESI-MS). Nuclear magnetic resonance (NMR) techniques with reporter group assignment using one-dimensional ^{1}H-NMR, or signal assignment using one-dimensional ^{13}C-NMR, ^{1}H–^{13}C correlated spectrometry (^{1}H–^{13}C COSY), heteronuclear single quantum coherence (HSQC) experiments, ^{1}H–^{1}H homonuclear Hartmann–Hahn (^{1}H–^{1}H HOHAHA) experiments, or heteronuclear multiple bond correlation (HMBC) experiments, and other experiments, have also been used. MS generally requires less material than NMR spectroscopy, but has the disadvantage that it is usually unable to provide detailed information on the linkages and anomeric configurations. Therefore, to differentiate between isomers with identical masses, structural determination of oligosaccharides using MS sometimes needs to be supplemented by digestion with specific exoglycosidases.

Quantitative Aspects of Human Milk Oligosaccharides

Milk oligosaccharides can be quantified using reverse-phase or normal-phase HPLC subsequent to pre- or post-column labeling techniques. Derivatizations are often performed by condensation of 2-aminopyridine, 2-aminobenzamide, 2-aminobenzoic acid, or 1-phenyl-3-methyl-5-pyrazolone to the reducing end of the sugar aldehyde.

Mature human milk and colostrum contain 12–13 and 22–24 g l^{-1} of oligosaccharides, respectively. Oligosaccharides constitute the third-largest component, after lactose and lipids, of the dry matter of human milk.

The concentration of neutral oligosaccharides in human milk and colostrum is greater than that of acidic oligosaccharides. The neutral fraction contains many fucosyl oligosaccharides, including significant amounts of 2′-FL, LNFP I, lacto-*N*-difucohexaose I (DFLNH I; Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4) Glc), and lacto-*N*-tetraose (LNT; Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc).

Twelve neutral milk oligosaccharides from Mexican women were determined using borohydride reduction of the carbohydrate fraction, followed by separation of the neutral from the acidic oligosaccharides by anion exchange chromatography and HPLC with a Rainin Microsorb C-8 column using acetonitrile/ion exchange water as the elution buffer after *O*-benzoyl derivatization. In addition, 10 neutral milk oligosaccharides from Japanese women were determined using liquid–liquid extraction of the carbohydrate fraction, gel filtration for separation of oligosaccharides and lactose, and reverse-phase HPLC with an ODS column after derivatization with 2-aminopyridine or 1-phenyl-3-methyl-5-pyrazolone.

Representative acidic oligosaccharides of human milk are sialyl lacto-*N*-neotetraose c (LST c; Neu5Ac (α2-6)Gal(β1-4) GlcNAc(β1-3)Gal(β1-4)Glc), disialyl lacto-*N*-tetraose (DSLNT; Neu5Ac(α2-3)Gal(β1-3)[Neu5Ac(α2-6)] Gal(β1-4)Glc), 3′-*N*-acetylneuraminyllactose (3′-SL; Neu5Ac(α2-3)Gal(β1-4)Glc), and 6′-*N*-acetylneuraminyllactose (6′-SL; Neu5Ac(α2-6)Gal(β1-4)Glc).

The concentrations of 12 acidic milk oligosaccharides obtained from American women were determined using capillary electrophoresis with 55% 200 mmol l^{-1} NaH$_2$PO$_4$ (pH 7.05) in water containing 100 mmol l^{-1} sodium dodecyl sulfate (SDS) mixed with 45% methanol as a running buffer. The principal acidic HMO was DSLNT in the milk/colostrum collected on days 3–5 and 9–21 of lactation, followed by 6′-SL and LST c. The concentration of DSLNT was 2–4 times higher than that of 6′-SL. In addition, the concentrations of nine acidic colostrum oligosaccharides obtained from Japanese women using anion exchange solid-phase extraction were determined by reverse-phase HPLC after derivatization with 1-phenyl-3-methyl-5-pyrazolone. The principal acidic oligosaccharide during days 1–3 of lactation was LNT c, followed by DSLNT, 6′-SL, and 3′-SL.

There are some substantial variations in the published data for the concentrations of both neutral and acidic oligosaccharides in human milk (**Tables 2** and **3**). At present, it is unclear whether these variations are due to the different ethnicities of donors, different times of sample collection post-partum, or differences in the methodologies used in these studies.

Changes in the concentrations of the following saccharides during the course of lactation may be important with respect to the biological significance of human milk/colostrum for infants: 2′-FL, 3-fucosyllactose (3-FL; Gal(β1-4) [Fuc(α1-3)]Glc), LNT, lacto-*N*-neotetraose (LNnT; Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc), LNFP I, LNFP II, la9lcto-*N*-fucopentaose III (LNFP III; Gal(β1-4)[Fuc(α1-3)] GlcNAc(β1-3)Gal(β1-4)Glc), DFLNH I, lacto-*N*-difucohexaose II (DFLHN II; Gal(β1-3)[Fuc(α1-4)] GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)Glc]), lacto-*N*-hexaose

Table 2 Concentrations of representative neutral human milk oligosaccharides

	Concentration (g l^{-1})					
Oligosaccharide	*Kunz* et al.	*Chaturvedi* et al.	*Thurl* et al.	*Coppa* et al. *(day 4)*	*Coppa* et al. *(day 60)*	*Asakuma* et al. *(day 2)*
2′-FL	0.45 ± 0.43	2.43 ± 0.26	1.84	3.93 ± 1.11	1.84 ± 0.39	2.01 ± 1.07
3-FL	0.07 ± 0.08	0.86 ± 0.10	0.46	0.34 ± 0.06	0.71 ± 0.07	0.28 ± 0.26
LDFT		0.43 ± 0.04	0.17			0.28 ± 0.30
LNT	1.09 ± 0.47	0.55 ± 0.08	0.86	0.84 ± 0.29	1.56 ± 0.57	1.44 ± 0.70
LNnT	Tr	0.17 ± 0.03	0.11	2.04 ± 0.55	0.95 ± 0.83	0.54 ± 0.14
LNFP I	1.26 ± 1.11	1.14 ± 0.18	0.67	1.36 ± 0.18	0.97 ± 0.61	2.08 ± 1.67
LNFP II			0.20	0.29 ± 0.22	0.29 ± 0.16	
LNFP III			0.28			
LNDFH I		0.50 ± 0.06	0.58	0.79 ± 0.25	1.18 ± 0.22	1.87 ± 1.55
LNDFH II	0.16 ± 0.11	0.09 ± 0.01	0.25			0.020 ± 0.025
LNH			0.13	0.07 ± 0.07	0.09 ± 0.02	

Kunz C, Rudloff S, Baier W, *et al.* (2000) *Annual Review of Nutrition* 20: 699–722.
Chaturvedi P, Warren CD, Altaye M, *et al*. (2001) *Glycobiology* 11: 365–372.
Thurl S, Muller-Werner B, and Sawatzki G (1996) *Analytical Biochemistry* 235: 202–206.
Coppa GV, Pierani P, Zampini L, *et al.* (1999) *Acta Paediatrica. Supplementum* 430: 89–94.
Asakuma S, Urashima T, Akahori M, *et al*. (2008) *European Journal of Clinical Nutrition* 62: 488–494.
The structures of oligosaccharides are given in **Table 1**.

Table 3 Concentrations of representative acidic human milk oligosaccharides

	Concentration (g l^{-1})				
Oligosaccharide	*Kunz* et al.	*Martin-Sosa* et al.	*Bao* et al. *(days 3–50)*	*Bao* et al. *(days 9–21)*	*Asakuma* et al. *(days 1–3)*
3′-Neu5AcL	0.300–0.500	0.100–0.300	0.097±0.038	0.076±0.014	0.297±0.096
6′-Neu5AcL	0.100–0.300	0.200–0.300	0.335±0.033	0.396±0.054	0.370±0.108
LST a	0.030–0.200	1.700–3.800	0.026±0.011		0.141±0.107
LST b			0.131±0.064	0.074±0.026	0.065±0.025
LST c	0.100–0.600	1.400–3.000	0.232±0.058	0.148±0.060	0.686±0.025
SLNFP I					0.078±0.053
SLNFP II				0.025±0.018	
DSLNT	0.200–0.600	0.700–1.500	1.274±0.503	0.795±0.234	0462±0.128

Kunz C, Rudloff S, Baier W, *et al*. (2000) *Annual Review of Nutrition* 20: 699–722.
Martin-Sosa S, Martin MJ, Garcia-Pardo LA, *et al.* (2003) *Journal of Dairy Science* 86: 52–59.
Bao Y, Zhu L, and Newburg DS (2007) *Analytical Biochemistry* 370: 206–214.
Asakuma S, Akahori M, Kimura K, *et al*. (2007) *Bioscience, Biotechnology, and Biochemistry* 71: 1447–1451.
The structures of oligosaccharides are given in **Table 1**.

(LNH; Gal(β1-3)GlcNAc(β1-3)[Gal(β1-4)GlcNAc](β1-6)Gal (β1-4) Glc), lacto-*N*-neohexaose (LNnH; Gal(β1-4) GlcNAc (β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), 6′-SL, 3′-SL, sialyl lacto-*N*-tetraose a (LST a; Neu5Ac(α2-3) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc), sialyl lacto-*N*-tetraose b (LST b; Gal(β1-3)[Neu5Ac(α2-6)] GlcNAc(β1-3)Gal(β1-4)Glc), LST c, and DSLNT.

The concentrations of oligosaccharides in milk or colostrum, as well as changes in their concentrations during the course of lactation, are likely to be significant with respect to their ability to act as anti-infection agents and to stimulate the growth of bifidobacteria in the infant colon, as described below.

It is of interest that, among mammals whose milk oligosaccharides have been investigated, humans are the only species in which oligosaccharides containing lacto-*N*-biose I (LNB; Gal(β1-3)GlcNAc) dominate over those containing *N*-acetyllactosamine (Gal(β1-4)GlcNAc).

Biosynthesis of Milk Oligosaccharides

As almost all milk oligosaccharides contain a lactose unit at their reducing end, it is generally considered that they are synthesized by the action of a variety of glycosyltransferases acting on free lactose as the acceptor. The exact number of glycosyltransferases involved in the biosynthesis of the oligosaccharides is still uncertain. These enzymes are known to be very specific, their specificity being directed toward both the type of linkage and the acceptor molecule. For example, in addition to the above-mentioned β-galactosyltransferase, there are probably at least two other human mammary β-galactosyltransferases, the actions of which are independent of α-lactalbumin; they catalyze the synthesis of Gal(β1-3)GlcNAc-R and Gal(β1-4)GlcNAc-R structures such as those shown in **Table 1**. Although human milk contains traces of three different galactosyllactoses, it is possible that these trisaccharides are formed by the transferase action of β-galactosidase rather than by three specific galactosyltransferases. Judging from the variety of structures among the HMOs (**Table 1**), one can assume that the human mammary gland probably contains at least three β-*N*-acetylglucosaminyltransferases, three α-fucosyltransferases, and two sialyltransferases.

Additional glycosyltransferases are found in lactating mammary glands of nonhuman species. An α-galactosyltransferase synthesizes α-3′-galactosyllactose (Gal(α1-4)Gal(β1-4)Glc, isoglobotriose), a trisaccharide that is present in bovine, ovine, and caprine colostrum and the milk or colostrum of several other species but not in human milk or colostrum. The synthesis of α-4′-galactosyllactose (Gal(α1-4)Gal(β1-4)Glc, globotriose), which has been found in the colostrum of the bottle-nosed dolphin, is presumably catalyzed by a different α-galactosyltransferase. Lactating mammary glands of the tammar wallaby contain a very active β-galactosyltransferase that is involved in the synthesis of a series of β(1-3)-linked galactosyllactoses that are unique to the milk of marsupials. The mammary glands of this species also contain an unusual β-*N*-acetylglucosaminyltransferase that attaches an *N*-acetylglucosaminyl residue to the trisaccharide β-3′-galactosyllactose (Gal(β1-3)Gal(β1-4)Glc,β3′-GL) and to the tetrasaccharide β-3′,3″-digalactosyllactose (Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc).

Gastrointestinal Digestion and Absorption of Milk Oligosaccharides

When infants consume milk, its free lactose is split into galactose and glucose by intestinal lactase (neutral β-galactosidase, lactose–phlorizin hydrolase), an enzyme that is located in the membrane of the microvilli of the brush border of the small intestine. The two monosaccharides are transported into the enterocytes by a specific mechanism, whereupon the glucose enters the circulation and is used as an energy source while most of the galactose is converted to glucose in the liver, to be used as an energy source as well.

Much less is known about the exact fate of HMOs. These are resistant to enzymatic hydrolysis by the brush border intestinal lactase and there is evidence that the majority survive passage through the small intestine and enter the colon where they are fermented by colonic bacteria. Evidently, the brush border of the small intestine does not contain glycosidases, such as sialidase, fucosidase, or *N*-acetylglucosaminidase, that can remove sialic acid, fucose, or *N*-acetylglucosamine residues, respectively, from the lactose units of the milk oligosaccharides. A small fraction of HMOs, however, is absorbed intact, perhaps by receptor-mediated endocytosis, some of which is excreted in the urine. It is unclear what proportion and exactly which of the ingested milk oligosaccharides are absorbed, but there is evidence suggesting that circulating oligosaccharides may have immunological effects on endothelial cells.

It has been of interest to investigate whether the sialic acid of sialyl milk oligosaccharides can be absorbed and utilized as a precursor for the biosynthesis of brain gangliosides and sialoglycoproteins. Rat milk contains significant amounts of sialyllactose, which can be hydrolyzed to sialic acid and lactose by a very active small intestinal neuraminidase that is present in suckling rats. Since this enzyme has a low pH optimum and is absent from the brush border, it is probably of lysosomal, that is, intracellular origin. An intracellular location for this neuraminidase implies that the ingested sialyllactose has to be transferred into the enterocytes before it can be digested within lysosomes or supranuclear vacuoles; the most likely mechanism for this transfer is pinocytosis or endocytosis. It has been found that, when adult rats were fed sialyllactose, there was an increase in brain ganglioside including GM3 content.

It has also been reported that the amount of sialic acid bound to brain gangliosides and sialoglycoproteins is significantly higher in breast-fed than in formula-fed infants and that this may be due to a difference between human milk and infant formula with respect to their content of sialic acid. These observations support the notion that the sialic acid of HMOs and also of sialoglycopeptides can be absorbed and utilized, but they present a paradox. On the one hand, HMOs are considered to be largely indigestible within the infant's small intestine because of the absence of the requisite glycosidases. On the other hand, there is the above-described evidence that the sialic acid of milk oligosaccharides can be absorbed. Is there a mechanism for the digestion/absorption of human milk sialo-oligosaccharides similar to that proposed for the absorption of sialyllactose in infant rats? If so, to what extent can other monosaccharides, such as fucose, that are constituents of the neutral milk oligosaccharides be similarly absorbed and used as biosynthetic precursors?

Brain-Stimulating Activity by Milk Glycoconjugates and Oligosaccharides Containing Sialic Acid

Mature human milk contains 0.7 g l^{-1} sialic acid, the concentration of which is significantly higher than that (0–0.2 g l^{-1}) in milk replacers, which are based on bovine milk. As described above, it has been suggested that the sialic acid of milk oligosaccharides and/or glycoconjugates is absorbed by the infant and utilized for the formation of brain components.

In a study using piglets as a model animal, the feeding of sialic acid-rich glycoconjugates elevated the level of sialic acid in brain glycoproteins and glycolipids as well as the levels of the mRNAs for polysialyltransferase IV (ST8SIA4), a key enzyme for sialyl glycoconjugate biosynthesis, and for UDP-*N*-acetylglucosamine-2-epimerase (GNA), which is a rate-controlling enzyme for *de novo* synthesis of sialic acid. In addition, the learning performance and memory of the piglets were found to have improved after feeding of the above glycoconjugates. Piglets were allocated to one of four groups, of which one was a control group while the other three were fed sow milk, supplemented with increasing amounts of sialic acid as casein glycomacropeptide, for 35 days. Learning performance and memory were assessed using both easy and difficult visual cues, in an eight-arm radial maze. The piglets were required to find the feed milk, which was located in one of the arms. The abilities of the piglets were evaluated by counting the number of times they found or failed to find the feed. The supplemented groups learned in significantly fewer trials than did the control group, with a dose–response relation for the difficult task but not for the easy task. In the hippocampus, significant dose–response relations were observed between the degree of sialic acid supplementation and mRNA levels for ST8SIA4 and GNE, corresponding to proportionate increases in protein-bound sialic acid concentrations in the frontal cortex.

These results suggest that the sialic acid of the caseinoglycomacropeptide supplement was absorbed and then utilized to form brain sialyl glycopeptides and gangliosides, which may have enhanced the learning abilities in this model animal. As above, the mechanism by which the caseinoglycomacropeptide is absorbed remains an open question.

Effects of Human Milk Oligosaccharides on the Growth of *Bifidobacterium*: Lacto-*N*-Biose Hypothesis

Bifidobacteria constitute around 95–99.9% of the total colonic microflora within 1 week after birth in breast-fed infants. This proportion is lower in the flora of bottle-fed infants even when prebiotic oligosaccharides such as lactulose, galacto-oligosaccharides, or fructo-oligosaccharides are present in the milk replacer. The feces of 700 breast-fed infants, 232 formula-fed infants, and 98 infants who had received a combination of breastfeeding and formula feeding were collected at 1 month postpartum, and then the colonic microflora was studied using real-time PCR quantitation analysis after extraction of the microbial DNA. Both the breast-fed and the formula-fed infants had a predominance of bifidobacteria in their colonic microflora, but the formula-fed infants more often contained *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* group, and *Lactobacillus* spp. in their colonic microflora than their breast-fed counterparts. It was suggested that milk components stimulate the growth of colonic bifidobacteria. As mentioned above, HMOs are believed to act as prebiotics that stimulate the growth of bifidobacteria in the lower intestine; this reduces the colonic pH, which would tend to inhibit the growth of pathogenic bacteria such as *E. coli*.

Which of the more than 130 different HMOs stimulate the growth of the infant's colonic bifidobacteria? Kitaoka and coworkers have proposed that milk oligosaccharides that contain LNB are specific growth factors for some species of *Bifidobacterium*. This lacto-*N*-biose hypothesis is based on the published genome information relating to bifidobacteria and the cloning of glycohydrolases, a glycophosphorylase, and an ABC transporter from a strain of *Bifidobacterium longum*. The hypothesis was initially based on the discovery of an enzyme, in the cell extracts of *B. bifidum*, that phosphorolyzes lacto-*N*-biose, the products being galactose-1-phosphate and *N*-acetylglucosamine: this enzyme was subsequently purified, and its gene, *lnp A*, from *B. longum* was cloned. The *lnp A* gene was found to be located in a novel operon for galactose metabolism, which also includes genes for a mucin desulfatase and for Gal-1-P uridyltransferase and UDP-Glc-4-epimerase as well as a gene for an ATP-binding cassette sugar transporter (see **Figure 2**). In addition, it was demonstrated that lacto-*N*-biose phosphorylase can also utilize galacto-*N*-biose (GNB; Gal(*β*1-3)GalNAc), which is a mucin core unit, as a substrate, but not *N*-acetyllactosamine (LacNAc, Gal(*β*1-4)GlcNAc).

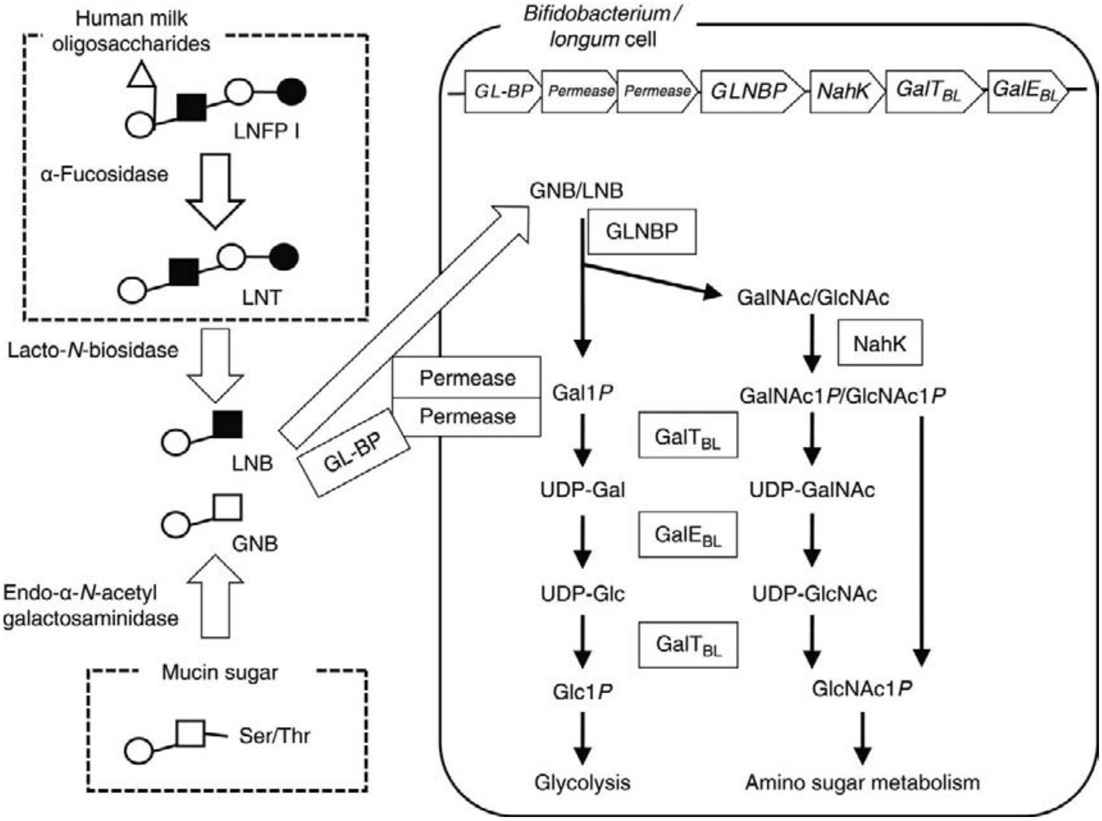
Human milk oligosaccharides
LNFP I
α-Fucosidase
LNT
Lacto-*N*-biosidase
LNB
GNB
Endo-α-*N*-acetyl galactosaminidase
Mucin sugar
Ser/Thr
GL-BP
Permease
Permease
Bifidobacterium / longum cell
GL-BP
Permease
Permease
GLNBP
NahK
GalT$_{BL}$
GalE$_{BL}$
GNB/LNB
GLNBP
GalNAc/GlcNAc
NahK
Gal1*P*
GalNAc1*P*/GlcNAc1*P*
GalT$_{BL}$
UDP-Gal
UDP-GalNAc
GalE$_{BL}$
UDP-Glc
UDP-GlcNAc
GalT$_{BL}$
Glc1*P*
GlcNAc1*P*
Glycolysis
Amino sugar metabolism

Figure 2 Lacto-*N*-biose hypothesis. BL, *Bifidobacterium longum*; GL-BP, galacto-*N*-biose/lacto-*N*-biose I-binding protein; GLNBP, galacto-*N*-biose/lacto-*N*-biose I phosphorylase.

The high content and increase in the concentrations of LNB-containing oligosaccharides such as LNT, LNFP I, and LNDFH I in early-stage milk/colostrum could significantly affect the formation of bifidus flora in the infant colon. The metabolism of LNFP I, which is a dominant HMO, by *B. bifidum* JCM1254 was clarified by the cloning of an α-fucosidase, which catalyzes the release of non-reducing fucose from LNFP I, and a lacto-*N*-biosidase, which catalyzes the hydrolysis of LNT to produce LNB and lactose, and by the purification and crystallization of an ABC-type transporter that delivers LNB through the cell membrane. Since lacto-*N*-biose phosphorylase was purified from an extract of this bifidobacterial strain, it has been suggested that its α-fucosidase produces LNT from LNFP I, while its lacto-*N*-biosidase liberates LNB from LNT, which is followed by the uptake of LNB through the cell membrane; the LNB is then metabolized to galactose-1-phosphate and *N*-acetylglucosamine, as in **Figure 2**. It can confidently be expected that a form of symbiosis between such a *B. bifidum* strain and another bifidobacterial strain that has the ABC-type transporter and lacto-*N*-biose phosphorylase, but does not have the α-fucosidase and lacto-*N*-biosidase, can be demonstrated. A possible metabolic pathway for the *N*-acetylglucosamine produced from LNFP I or LNT (see above) is suggested, based on the analysis of gene products of the galactose operon of *B. longum* ssp. *longum* NCC2705; *N*-acetylglucosamine is converted to *N*-acetylglucosamine-1-phosphate by the novel enzyme *N*-acetylhexosamine 1-kinase and then to UDP-*N*-acetylglucosamine by UDP-glucose hexose 1-phosphate uridyltransferase, finally entering the metabolic pathway of amino sugars. From these findings, the growth-stimulating effect of HMOs on some strains of bifidobacteria, such as *B. bifidum*, can be partially explained.

As already mentioned, the dominant oligosaccharides of human milk/colostrum are 2′-FL, LNFP I, LNDFH I, and LNT, the last three of which are type I oligosaccharides, which contain the LNB unit; they constitute 25–30% of the total amount of milk oligosaccharides. The milk or colostrum of almost all nonhuman mammals, however, contains only type II oligosaccharides, which contain *N*-acetyllactosamine (Gal(β1-4)GlcNAc). The milk oligosaccharides of species that are closely related to humans, namely the great apes (chimpanzee, bonobo, gorilla, and orangutan) and a lesser ape (siamang), have recently been studied. It was found that gorilla and siamang milk, like that of other nonhuman mammals, contains only type II oligosaccharides, whereas chimpanzee, bonobo, and orangutan milk or colostrum contains both type I and type II, but with type II oligosaccharides predominating over type I. These findings show that the predominance of type I oligosaccharides in milk/colostrum is a feature that is specific to humans and suggest that the acquisition of this predominance may have had a selective advantage for humans in relation to the bifidus flora in their infants.

In Vitro Studies on Effects of Human Milk Oligosaccharides on the Growth of Bifidobacteria and Their Metabolism

Growth tests with *B. longum* ssp. *infantis* ATCC 15697, *B. longum* ssp. *longum* ATCC 15707, *B. adolescentis* ATCC 15703, *B. breve* ATCC 27539, or *B. bifidum* ATCC 29521 were performed using a medium containing 1% HMOs but no lactose, which was incubated at 37 °C for 350 h. This resulted in a cell density of *B. longum* ssp. *infantis* strains that was 3 times higher than that of other strains. When oligosaccharide and monosaccharide profiles in the medium containing HMOs were studied using high-performance thin-layer chromatography, a clear change was observed after incubation with *B. longum* ssp. *infantis* when compared with the control, which was the medium with no inoculation. This change was not found in the medium after incubation with *B. breve* and *B. adolescentis*. These results showed that *B. longum* ssp. *infantis* is able to grow with HMOs as the only carbon source.

The degradation of HMOs was studied during the growth of *B. longum* ssp. *infantis* ATCC 15697, *B. breve* ATCC 15700, or *B. longum* ssp. *longum* DJO010A in broth containing oligosaccharides but no lactose, by observation of the molecular mass of ions in Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). In this work, tri- to heptasaccharides were degraded completely during the growth of *B. longum* ssp. *infantis* ATCC 15697 over 25 and 50 h of incubation, while higher oligosaccharides were partially degraded. On the other hand, only 24.4 and 35.2% of LNT were degraded during the growth of *B. breve* and *B. longum*, respectively. It was speculated that *B. longum* ssp. *infantis* ATCC 15697 consumes smaller, in preference to larger, oligosaccharides. In addition, α-fucosidase and sialidase activities were found in the *B. longum* ssp. *infantis* strain but not in *B. longum* ssp. *longum* and *B. breve*. As suggested, the presence or absence of these enzyme activities may affect the utilization of HMOs by bifidobacteria.

The *B. longum* ssp. *infantis* ATCC 15697 genome was fully sequenced to permit investigations into the genetic basis and molecular mechanisms underlying this phenotype. Many of its genomic features encode enzymes that are active on HMOs and include a novel 43-kbp region dedicated to oligosaccharide utilization. Subsequent biochemical and molecular characterization of HMO-related glycosidases and transport proteins has further resolved the mechanism by which this strain imports and catabolizes milk oligosaccharides. The glycosidases were identified to be sialidase, fucosidase, β-galactosidase, β-hexosaminidase, and LNB phosphorylase, but lacto-*N*-biosidase was not found. This approach suggested the existence of a pathway for the metabolism of milk oligosaccharides in which they are transported into the bifidobacterial cell and then degraded from the nonreducing end by the above glycosidases. This pathway differs from that proposed by Kitaoka and coworkers, suggesting that there is more than one possible pathway for the metabolism of milk oligosaccharides among bifidobacteria. The existence of these metabolic pathways may be relevant to the concept of symbiosis between many strains including *B. bifidum*, *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, and *B. breve*.

Using *B. infantis* ATCC 15697 as a reference strain, comparative genomic hybridization (CGH) analyses have been performed on 15 other bifidobacterial strains with various HMO consumption profiles. Preliminary analysis of the CGH data has revealed that these strains could be differentiated into two different groups, one of which consists of strains with moderate or little ability to grow on HMOs whereas the other, consisting of *B. infantis* strains, achieved good growth on HMOs.

Antipathogenic Effect of Human Milk Oligosaccharides

The following antiadhesion phenomena have been observed for human or other milk oligosaccharides or glycoconjugates. A trisaccharide unit, Gal(β1-4)GlcNAc(β1-3)Gal(β1-, which is found in LNnT and other compounds, inhibits the adherence of *Streptococcus pneumoniae* to buccal epithelial cells. The bacteria that are inhibited from binding to colonocytes by fucosylated oligosaccharides include *Campylobacter jejuni*, strains of *E. coli* and their heat-stable toxin, and enteropathogenic *E. coli.*

The observation that 2′-FL inhibits the binding of *C. jejuni* to H(O) antigen (Fuc(α1-2)Gal(β1-4)GlcNAc) in the human intestinal mucosa *ex vivo* is noteworthy, because this trisaccharide is the most abundant oligosaccharide in human milk. Intestinal infection by *C. jejuni* is one of the most common causes of diarrhea worldwide. An inverse correlation has been observed between the concentration of 2′-FL in breast milk and the frequency of Campylobacter diarrhea in breast-fed infants, supporting the view that 2′-FL reduces the pathogenicity of *C. jejuni*. The concentration of 2′-FL in human colostrum was 2.5 g l^{-1} at the start of lactation and decreased during the 2 subsequent days, suggesting that inhibition by 2′-FL of the adhesion of *C. jejuni* to the colonic mucosa is most significant immediately after birth. An inverse correlation has also been observed between the concentration of LNDFH I in breast milk and the frequency of Calicivirus diarrhea in breast-fed infants. Sialylated oligosaccharides, at physiological concentrations, strongly inhibit the binding of influenza A virus and S-fimbriated enteropathogenic *E. coli* to their respective host target cells. It is recognized that sialyl Le^x (Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc) or Le^b (Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc) epitopes have affinity for a lectin, a carbohydrate-binding protein, that is found on the surface of *Helicobacter pylori*, a Gram-negative bacterium whose main host is man. *Helicobacter pylori* resides in the gastric mucosa and adheres to the epithelial cells lining the stomach. Some 50% of the world population is infected by this organism, with a higher incidence in developing countries. *Helicobacter pylori* is associated with the development of peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma.

The binding of *H. pylori* to various carbohydrate structures is mediated by two adhesins, Bab A and Sab A, which are expressed on its surface. As Bab A and Sab A recognize and bind Le^b and sialyl Le^x, respectively, it is likely that oligosaccharides or glycoconjugates containing Le^b or sialyl Le^x inhibit the attachment of *H. pylori* to gastric epithelial cells, thus preventing its colonization within the stomach. Recently, an adhesion assay was used to investigate the capacity of pig milk to inhibit the binding of *H. pylori* to a neoglycoprotein that has Le^b or sialyl-di-Le^x units conjugated to human serum albumin. α1,3/4-Fucosyltransferase transgenic FVB/N mice, known to express Le^b and sialyl Le^x in their gastric epithelium, were colonized by *H. pylori* and subsequently treated with porcine milk or with water. The expression of the Le^b and sialyl Le^x carbohydrate epitopes on pig milk proteins was breed- and individual-specific and correlated with the ability of porcine milk to inhibit the adhesion of *H. pylori* to the gastric mucosa. As HMOs such as DFLNH I or 3-fucosyl-3′-*N*-acetylneuraminyllactose (Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]Glc) contain Le^b or sialyl Le^x, it seems likely that these effects may be achieved also by HMOs.

Another study has reported on the interaction of *H. pylori* with sialylated glycans. The preferred interaction is with α3-linked sialic acid, while glycans having α6-linked Neu5Ac are nonbinding. For example, 50% inhibition by *H. pylori* of hemagglutination of human erythrocytes was observed at a low concentration of some sialylated saccharides. The data show that Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer (S-3-PG), Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc, 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine (Neu5Ac(α2-3)Gal(β1-4)GlcNAc), as well as 3-*N*-acetylneuraminyl-lacto-*N*-neotetraose (Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc) all bound to *H. pylori* CCUG17874 at similar strength. 3′-SL also bound to this organism but its binding ability was somewhat weaker than that of the above saccharides. It has also been reported that LST a, an HMO, was able to bind to another strain, *H. pylori* J99.

The binding of 3′-SL to *H. pylori* CCUG17874 is noteworthy because this saccharide is found in human milk and in bovine colostrum. It was found that at the start of lactation the concentration of 3′-SL in human colostrum was similar to that of 6′-SL. However, the concentration of 3′-SL decreased during the 2 subsequent days of lactation, whereas that of 6′-SL did not. This suggests that, very early in lactation, 3′-SL may be more significant in the prevention of transmission of *H. pylori* from mother to infant than later on.

Recent studies on the ability of various fractions of HMOs to inhibit the adhesion of three intestinal microorganisms (enteropathogenic *E. coli* serotype O119, *Vibrio cholerae*, and *Salmonella fyris*) to differentiated Caco-2 cells have shown that the acidic fraction had an antiadhesive effect on all three pathogenic strains. The neutral high-molecular-weight fraction significantly inhibited the adhesion of *E. coli* O119 and *V. cholerae*, but not that of *S. fyris*; the neutral low-molecular-weight fraction was effective toward *E. coli* O119 and *S. fyris* but not *V. cholerae.* This demonstrated that HMOs inhibit the adhesion to epithelial cells not only of common pathogens such as *E. coli* but also of other aggressive bacteria such as *V. cholerae* and *S. fyris*. Thus, oligosaccharides may be important factors in human milk that defend against acute diarrhea in breast-fed infants.

Breastfeeding is the predominant route for the postnatal transmission of HIV-1 infection in children. However, a majority of breast-fed infants do not become HIV-infected despite continuous exposure to the virus through their mothers' milk. Entry of HIV-1 across the infant's mucosal barrier is partially mediated through binding of the HIV-1 surface glycoprotein gp120 to dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) on human dendritic cells (DCs). Lewis antigen glycans, present in human milk, bind to DC-SIGN and inhibit HIV-1 transfer to CD4+ T lymphocytes. It was hypothesized that HMOs, which carry one or more Lewis antigen glycans, compete with gp120 for DC-SIGN binding. It was shown in two independent assays that physiological concentrations of HMOs significantly reduce gp120 binding to DC-SIGN by more than 80%. These results may provide an additional explanation for the inhibitory effects of human milk on the transmission of HIV-1 from mother to child.

It is recognized that breast-fed babies are less likely than bottle-fed babies to have asthma, lower and upper respiratory infections, and ear infections. A pilot study tested the relationship between the consumption of oligosaccharides, oligosaccharide content of feces, and subsequent disease in breast-fed infants. In this study, the concentration of LNFP II was determined in breast milk and in the feces of breast-fed babies, and the relationship between these concentrations and the frequencies of respiratory and gastrointestinal infection of the babies was evaluated. The results showed that the LNFP II levels in the feces collected at 2 weeks in those babies that had symptoms of respiratory disease within 6 or 24 weeks after birth were significantly lower than those of infants that had had no symptoms. Also, the levels of LNFP II in the milk of mothers whose babies had these symptoms at the same ages were significantly lower than those of other mothers whose babies had had no symptoms. Although the mechanism by which HMOs prevent respiratory disease in infants is unknown, an immunomodulating effect by a prebiotic mechanism was suggested; alternatively, some of the milk oligosaccharides may have been absorbed into the systemic circulation and acted as inhibitory receptor analogues within the respiratory tract.

Immunomodulating Effect of Milk Oligosaccharides

Although direct detection of HMOs in the blood of infants has not yet been reported, it nevertheless seems very likely, on the basis of their observed urinary excretion, that small amounts of intact HMOs are normally absorbed from the gastrointestinal tract and enter the systemic circulation. It follows that they may alter protein–carbohydrate interactions also at a systemic level. Recent studies suggest that HMOs interfere with the adhesion of neutrophils to vascular endothelial cells and to platelets. These effects appear to be based on the structural resemblance of some HMOs to the glycoprotein ligands of selectins. Selectins are transmembrane proteins that are involved in cell–cell interactions in the immune system. P-selectin mediates leukocyte deceleration (rolling) on activated endothelial cells and initiates leukocyte extravasation at sites of inflammation. P-selectin is also involved in the formation of platelet–neutrophil complexes (PNCs), a subpopulation of highly activated neutrophils primed for adhesion, phagocytosis, and enhanced production of reactive oxygen species. The studies suggest that oligosaccharides containing sialyl Le^x or its stereoisomer sialyl Le^a, which resemble the P-selectin ligand, inhibit the binding of selectin ligands to the surface of endothelial cells and platelets; this interferes with the formation of PNCs, the effect of which is anti-inflammatory. The following oligosaccharide fractions were tested *in vitro* to establish whether they reduce leukocyte deceleration on U937 cells, which express the P-selectin ligand: total HMOs, neutral oligosaccharides, total acidic oligosaccharides, neutral oligosaccharides with a polymerization degree of 4, fucosylated oligosaccharides, and DSLNT. The acidic oligosaccharide fraction produced a slight but definite reduction of P-selectin ligand binding, similar to that of standard sialyl Le^x, whereas the total neutral oligosaccharides and neutral fucosylated oligosaccharide fractions did not. These results support the notion of anti-inflammatory effects of acidic HMOs.

However, another study showed that, *in vitro*, the neutral milk oligosaccharide fraction can inhibit the binding of neutrophils to tumor necrosis factor (TNF)-stimulated endothelium and that the whole-milk oligosaccharide fraction enhanced the formation of PNCs.

It has been reported that the incidence of necrotizing enterocolitis, a condition that is considered to be an exaggerated immune response, is about 85% lower in breast-fed than in formula-fed infants. This is consistent with an anti-inflammatory effect of absorbed HMOs.

Another potential HMO target could be DC-SIGN. This is expressed on DCs in the intestine and other tissues and is involved in the capture of different pathogens, including HIV-1, hepatitis C, cytomegalovirus, dengue virus, *Mycobacterium*, and *Candida albicans*. Unidentified components in human milk bind to DC-SIGN and inhibit HIV-1 transfer to CD4+ T lymphocytes. DC-SIGN has high affinity for Le^x, which is a unit contained in a few HMOs, suggesting that the unknown inhibitory milk components could be milk oligosaccharides. The putative complex oligosaccharides with multiple Le^x determinants may inhibit DC-SIGN-mediated interactions similar to the multivalent binding hypothesis for selectins.

The question of whether HMOs influence cytokine production and activation of cord blood T cells has recently been investigated. Cord blood mono-nuclear cells from randomly chosen healthy newborns were co-cultured for 20 days with acidic or neutral oligosaccharides, and intracellular cytokine production and surface marker expression of T cells were studied using flow cytometry. The authors used concentrations of HMOs (neutral oligosaccharides, $10\,\mu\mathrm{g\,ml^{-1}}$; acidic oligosaccharides, $1\,\mu\mathrm{g\,ml^{-1}}$) that were considered by them to mimic physiological conditions, although these concentrations are considerably lower than the calculated values of $100–200\,\mathrm{mg\,l^{-1}}$ for circulating HMOs. The acidic fraction, but not the neutral oligosaccharide fraction, increased the percentage of interferon-γ-producing CD3+CD4+ and CD3+CD8+ cells, and of interleukin-13 (IL-13) production in CD3+CD8+ cells, and significantly elevated CD25+ expression in CD3+CD4+ cells. These results showed that HMOs affect cytokine production and activation of cord blood-derived T cells *in vitro*. Oligosaccharides and, in particular, acidic milk oligosaccharides may therefore influence lymphocyte maturation in breast-fed newborns. It was concluded that HMOs can modulate the immune system of the maturing infant.

Induction of Growth Inhibition of Intestinal Cells by Human Milk Oligosaccharides

It has been reported that HMOs affect the proliferation and differentiation of intestinal cells *in vitro*. The direct effects of milk oligosaccharide fractions or of individual oligosaccharides on the proliferation, differentiation, and apoptosis of transformed human intestinal cells (HT-29 and Caco-2), and of nontransformed small intestinal epithelial crypt cells of fetal origin (human intestinal epithelial cells (HIECs)), were investigated. The growth inhibition induced by neutral and acidic oligosaccharide fractions on HT-29, Caco-2, and HIECs was observed in a dose-dependent manner. The addition of neutral oligosaccharides to the medium at $15\,\mathrm{mg\,ml^{-1}}$ inhibited the growth of HT-29, Caco-2, and HIECs by 47, 63, and 77%, respectively, while acidic oligosaccharides at similar concentrations inhibited the growth of these cells by 32, 54, and 66%, respectively. Growth inhibition was observed for HT-29 by 3′-SL, LST a, or LST c, and for HIECs by LST a, LST b, DSLNT, 3′-SL, 6′-SL, or LST c. In these experiments, alkaline phosphatase (AP) activity, which was used as a biomarker for cell differentiation, was enhanced in HT-29 and HIECs by 123 and 119%, respectively, in the presence of neutral oligosaccharides at $15\,\mathrm{mg\,ml^{-1}}$, compared with the control. It was enhanced by 145 and 171%, respectively, by acidic oligosaccharides at a similar concentration. The addition of LNDFH II at $1\,\mathrm{mg\,ml^{-1}}$ slightly enhanced this activity in HT-29 cells, while sialyllactose slightly enhanced it in both HT-29 and HIECs. In addition, caspase-3 activity, which was used as an early apoptosis marker, was enhanced in HT-29 and HIECs by 300 and 200%, respectively, by neutral oligosaccharides at $15\,\mathrm{mg\,ml^{-1}}$, compared with the control, while it was not enhanced by acidic oligosaccharides. These *in vitro* experiments suggested that HMOs induce growth inhibition of intestinal cells through two different mechanisms: by suppressing cell cycle progression through the induction of differentiation and/or by influencing apoptosis. As the development and maturation of the digestive and absorptive process depends on differentiation, it appears that milk oligosaccharides may influence various stages of gastrointestinal development, but the relevance of these *in vitro* observations to conditions *in vivo* remains to be investigated. In this connection, it may be worth noting that intestinal permeability has been found to decrease significantly more rapidly in breast-fed than in formula-fed babies. The mechanism or cause of this effect is unknown, but if gut closure occurs earlier in breast-fed babies one can speculate that this may be because their small intestinal mucosa has been exposed to milk oligosaccharides since birth, which would be consistent with the above-mentioned *in vitro* studies.

Chemical Structures and Features of Bovine Milk Oligosaccharides; Milk Oligosaccharides of Other Domestic Farm Animals

The structures of 15 acidic and 13 neutral bovine milk oligosaccharides that have been elucidated to date are shown in **Table 4**. Most of these had been isolated from colostrum, which contains more than 1 g l^{-1} of oligosaccharides, the majority of which are acidic, whereas the mature milk contains only trace amounts. The low concentration of oligosaccharides in mature bovine milk makes it difficult to use it in the production of human infant formulae designed to provide prebiotics and receptor analogues (see above). This means that certain chemically produced oligosaccharides that are not normally found in milk, but which have functions similar to those of HMOs, could with advantage be incorporated into infant formulae.

Most of the oligosaccharide fraction of bovine colostrum consists of 3′-SL, 6′-SL, 6′-SLN Neu5Ac(α2-6)Gal(β1-4)GluNAc, and DSL Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc, with 3′-SL constituting 70% of the total oligosaccharide content. Changes in the levels of 3′-SL, 6′-SL, and 6′-SLN in Holstein colostrum pre-partum to 1 week postpartum were investigated. The levels were maximal immediately after parturition, rapidly decreasing by 48 h post-partum. In another study, the concentrations of 3′-SL, 6′-SL, 6′-SLN, and DSL were found to be 681, 243, 239, and 201 mg l^{-1}, respectively, in Holstein colostrum and 867, 136, 220, and 283 mg l^{-1}, respectively, in Jersey colostrum immediately after parturition.

Among the neutral oligosaccharides, the following are characteristic of bovine colostrum, insofar as they have not so far been found in the milk or colostrum of other mammals: GalNAc(β1-3)Gal(β1-4)Glc, GalNAc(α1-3)Gal(β1-4)Glc, and GalNAc(β1-4)Glc. It is noteworthy that both α(1-3)- and β(1-3)-linked galactosyllactose and *N*-acetylgalactosaminyllactose have been found among the bovine oligosaccharides.

As shown in **Table 4**, some bovine milk oligosaccharides have an *N*-acetyllactosamine (Gal(β1-4)GlcNAc) unit at their reducing end, in contrast to HMOs, of which almost all have a lactose residue in that position. The core units of most bovine milk oligosaccharides are lactose or *N*-acetyllactosamine, unlike HMOs, whose core units are LNT, LNnT, LNH, LNnH, and other substances. Both the variety and the concentration of fucosylated oligosaccharides in bovine colostrum and milk are very low; this is in contrast to HMOs, many of which are fucosylated.

Recently, 39 oligosaccharides have been detected in bovine colostrum using microchip liquid chromatography separation and high-performance MS, including Fourier transform ion cyclotron resonance (FTICR) and time-of-flight (TOF) analysis. Although there was no information on the anomeric configuration and linkage position of each residue, the presence of LNnT, LNnH, lacto-*N*-novopentaose I(Gal(β1-3)[Gal(β1-4)GluNAc(β1-6)]Gal(β1-4)Glc), and their *N*-acetylneuraminyl or *N*-glycolylneuraminyl derivatives was suggested by this method.

It can be expected that milk oligosaccharides of other domestic farm animals, such as goats and sheep, will be used as biofunctional materials. The concentration of milk oligosaccharides in goat milk is 0.25–0.30 g l^{-1}, which is higher than that in bovine (0.03–0.06 g l^{-1}) or ovine (0.02–0.04 g l^{-1}) milk. In addition, the variety of oligosaccharides is greater in goat milk than in bovine or ovine milk, as shown by the profiles of HPEAC analysis. Colostrum from Japanese Saanen goats contains more 6′-SL than 3′-SL; it also contains 6′-*N*-glycolylneuraminyllactose, 6′-SLN, isoglobotriose, 3′-GL, Gal(β1-6)Gal(β1-4)Glc (6′-GL), and 2′-FL. Another study has shown that mature milk from Spanish goats contains 6′-SL, 3′-SL, DSL, *N*-glycolylneuraminyllactose, 3′-GL, *N*-acetylglucosaminyllactose, LNH, and additional high-molecular oligosaccharides, as demonstrated by analysis with FAB-MS. Ovine colostrum contains more 3′-*N*-glycolylneuraminyllactose than 3′-SL and 6′-SL and, notably, contains Neu5Gc in preference to Neu5Ac.

Comparative Aspects of Milk Oligosaccharides among Other Mammalian Species

Prior to about 1980, most studies on the oligosaccharides of nonhuman eutherian mammals were concerned with bovine colostrum or milk. Recently, milk or colostrum oligosaccharides of the following species have been studied and characterized, as listed in **Table 4**: platypus (monotreme), echidna (monotreme), tammar wallaby (marsupial), cow, buffalo, horse, goat, sheep, dog, Ezo brown bear, Japanese black bear, polar bear, giant panda, white-nosed coati, mink, crabeater seal, hooded seal, harbor seal, bearded seal, minke whale, beluga, bottlenose dolphin, Bryde's whale, Sei whale, Asian elephant, African elephant, rat, brown capuchin, giant anteater, hyena, chimpanzee, bonobo, gorilla, orangutan, and siamang.

The concentration of oligosaccharides in milk varies between mammalian species and depends on the time post-partum. For example, bovine colostrum collected immediately post-partum contains about 1 g l^{-1}, but the content rapidly decreases at 48 h post-partum and mature bovine milk contains only trace amounts. By contrast, mature human milk contains 12–13 g l^{-1} of oligosaccharides, a more than 10-fold difference

Table 4 Chemical structures of milk oligosaccharides of nonhuman mammals

No.	*Oligosaccharide*	*Structure*
Brown capuchin colostrum		
Neutral oligosaccharides		
1	3-FL	Gal(β1-4)Glc | Fuc(α1-3)
2	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
3	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
4	*novo*-LNP I	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc GlcNAc(β1-3) /
5	LNnH	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
Acidic oligosaccharides		
6	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
Chimpanzee milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	3-FL	Gal(β1-4)Glc | Fuc(α1-3)
3	LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
4	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
5	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
Acidic oligosaccharides		
6	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
7	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
Bonobo milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	3-FL	Gal(β1-4)Glc | Fuc(α1-3)
3	LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
4	A-tetrasaccharide	GalNAc(α1-3)Gal(β1-4)Glc | Fuc(α1-2)
5	LNFP I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
6	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
Acidic oligosaccharides		
7	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc

(*Continued*)

Table 4 (Continued)

No.	***Oligosaccharide***	***Structure***
8	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
9	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
Gorilla milk or colostrum		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
3	B-tetrasaccharide	Gal(α1-3)Gal(β1-4)Glc Fuc(α1-2)
Acidic oligosaccharides		
4	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
5	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
Orangutan colostrum		
Neutral oligosaccharides		
1	3-FL	Gal(β1-4)Glc Fuc(α1-3)
2	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
3	LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
4	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
5	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
6	DF-LNnH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ╱ Fuc(α1-3)
Acidic oligosaccharides		
7	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
8	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
9	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
10	LST b	Neu5Ac(α2-6) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
11	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
12	Fs-LNnH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-3) ╲ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ╱

(*Continued*)

Table 4 (Continued)

No.	*Oligosaccharide*	*Structure*
Siamang milk		
Neutral oligosaccharides		
1	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
2	LNnH	Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ╱
Acidic oligosaccharides		
3	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
4	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
5	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
6	SLNnH II	Gal(β1-4)GlcNAc(β1-3) ╲ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ╱
Bovine colostrum or milk		
Neutral oligosaccharides		
1	*N*-Acetylgalactosaminylglucose	GalNAc(β1-4)Glc
2	LacNAc	Gal(β1-4)GlcNAc
3	Le^x trisaccharide	Gal(β1-4)GlcNAc | Fuc(α1-3)
4	α3′-GalNAcL	GalNAc(α1-3)Gal(β1-4)Glc
5	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
6	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
7	β4′-GL	Gal(β1-4)Gal(β1-4)Glc
8	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
9	*novo*-LNP I	Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-3) ╱
10	β3′-GalNAcL	GalNAc(β1-3)Gal(β1-4)Glc
11	β6′-GlcNAcL	GlcNAc(β1-6)Gal(β1-4)Glc
12	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
13	LNnH	Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ╱
Acidic oligosaccharides		
14	3′-*O*-Lac phosphate	Gal(β1-4)Glc-3′-PO_4
15	3S-Gal	Neu5Ac(α2-3)Gal
16	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
17	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
18	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
19	6′-Neu5GcL	Neu5Gc(α2-6)Gal(β1-4)Glc

(*Continued*)

Table 4 (Continued)

No.	Oligosaccharide	Structure
20	6′-SLacNAc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc
21	6′-Neu5GcLacNAc	Neu5Gc(α2-6)Gal(β1-4)GlcNAc
22	-	Neu5Ac(α2-3)Gal(1-3)Gal(1-4)Glc
23	DSL	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc
24	6′-SLacNAc-1-*O*-phosphate	Neu5Ac(α2-6)Gal(β1-4)GlcNAc-1-PO_4
25	6′-SLacNAc-6-*O*-phosphate	Neu5Ac(α2-6)Gal(β1-4)GlcNAc-6-PO_4
26	3′-SLacNAc	Neu5Ac(α2-3)Gal(β1-4)GlcNAc
27	3′-S- β6′-GL	Gal(β1-6)Gal(β1-4)Glc | Neu5Ac(α2-3)
28	6′-S- β3′-GL	Gal(β1-3)Gal(β1-4)Glc | Neu5Ac(α2-6)
Buffalo colostrum		
Acidic oligosaccharides		
1	DSL	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc
Equine colostrum		
Neutral oligosaccharides		
1	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
2	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
3	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
4	*iso*-LNnT	Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glc
5	*novo*-LNP I	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-3) /
6	LNnH	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
Acidic oligosaccharides		
7	LacNAc-1-*O*-phosphate	Gal(β1-4)GlcNAc-1-PO_4
8	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
Caprine colostrum or milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
3	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
4	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
Acidic oligosaccharides		
5	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
6	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc

(*Continued*)

Table 4 (Continued)

No.	Oligosaccharide	Structure
7	6′-Neu5GcL	Neu5Gc(α2-6)Gal(β1-4)Glc
8	6′-SLacNAc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc
9	3′-S- β6′-GL	Gal(β1-6)Gal(β1-4)Glc | Neu5Ac(α2-3)
10	6′-S- β3′-GL	Gal(β1-3)Gal(β1-4)Glc | Neu5Ac(α2-6)
Ovine colostrum		
Neutral oligosaccharides		
1	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
2	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
3	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
Acidic oligosaccharides		
4	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
5	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
6	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
Ezo brown bear milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
3	DF-LNnT	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
4	Gal-LNFP III	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
5	Tetra-F-LNnH	Fuc(α1-3) | Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) ╱ | Fuc(α1-3)
6	DF D Gal-LNnH	Fuc(α1-3) | Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) ╲ Gal(β1-4)Glc Gal(α1-3)Gal(β1-4)GlcNAc(β1-3) ╱ | Fuc(α1-3)
Japanese black bear milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
3	3-F-isoglobotriose	Gal(α1-3)Gal(β1-4)Glc | Fuc(α1-3)
4	B-tetrasaccharide	Gal(α1-3)Gal(β1-4)Glc | Fuc(α1-2)

(*Continued*)

Table 4 (Continued)

No.	*Oligosaccharide*	*Structure*
5	B-pentasaccharide	Gal(α1-3)Gal(β1-4)Glc Fuc(α1-2) Fuc(α1-3)
6	Gal-LNFP III	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
7	B-heptasaccharide type II chain	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-2) Fuc(α1-3)
8	DF DGal-LNnT	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3) Fuc(α1-3)
9	DF DGal-LNnH	Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(α1-3)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
10	TF DGal-LNnH a	Fuc(α1-2) Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(α1-3)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
11	TF DGal-LNnH b	Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(α1-3)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-2) Fuc(α1-3)
Acidic oligosaccharides		
12	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
13	FS Gal-LNnH	Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
14	DFSGal-LNnH	Fuc(α1-2) Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
15	DS-LNnH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
Polar bear milk		
Neutral oligosaccharides		
1	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
2	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
3	B-tetrasaccharide	Gal(α1-3)Gal(β1-4)Glc Fuc(α1-2)
4	3-F-isoglobotriose	Gal(α1-3)Gal(β1-4)Glc Fuc(α1-3)

(*Continued*)

Table 4 (Continued)

No.	Oligosaccharide	Structure
5	A-tetrasaccharide	GalNAc(α1-3)Gal(β1-4)Glc Fuc(α1-2)
6	A-pentasaccharide	GalNAc(α1-3)Gal(β1-4)Glc Fuc(α1-2) Fuc(α1-3)
7	Galilipentasaccharide	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
8	B-hexasaccharide type II chain	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-2)
9	Gal-LNFP III	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
10	DF Gal-LNnT	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3) Fuc(α1-3)
11	DGal-LNnH	Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)
12	DF DGal-LNnH	Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(α1-3)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
White-nosed coati milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
3	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
4	LNFP IV	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
5	Galilipentasaccharide	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
6	F Gal-LNnH	Fuc(α1-2) { Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal (α1-3) { Gal(β1-4)GlcNAc(β1-3)
Asian elephant milk		
Neutral oligosaccharides		
1	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
Acidic oligosaccharides		
2	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
3	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
4	3′-S-3-FL	Neu5Ac(α2-3)Gal(β1-4)Glc Fuc(α1-3)
5	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
6	SGal-LNnH	Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
7	FsGal-LNnH	Fuc(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)

(*Continued*)

Table 4 (Continued)

No.	*Oligosaccharide*	*Structure*
8	FS-LNnT I	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
9	S-*para*-LNnH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
10	FS-*para*-LNnH	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-3) Fuc(α1-3)
African elephant milk		
Neutral oligosaccharides		
1	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
2	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
3	GalLNFP III	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
4	DF-*para*-LNnH	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-3) Fuc(α1-3)
5	DFGal-*para*-LNnH	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-3) Fuc(α1-3)
Acidic oligosaccharides		
6	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
7	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
8	3′-S-3-FL	Neu5Ac(α2-3)Gal(β1-4)Glc | Fuc(α1-3)
9	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
10	FS-LNnT I	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
11	FS-LNnT II	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
12	S-LNnH II	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
13	FS-LNnH I	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)
14	FS-*para*-LNnH II	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
15	FSGal-LNnH	Fuc(α1-3) | Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)

(Continued)

Table 4 (Continued)

No.	***Oligosaccharide***	***Structure***
Rat milk		
Acidic oligosaccharides		
1	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
2	3′-SL-6′-*O*-sulfate	Neu5Ac(α2-3)Gal(β1-4)Glc-6′-SO$_3$
3	6′-L-*O*-sulfate	Gal(β1-4)Glc-6′-SO$_3$
4	β6′galactosyl-*myo*-inositol	Gal(β1-6)-*myo*-inositol
Dog milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
Acidic oligosaccharides		
2	3′-L-*O*-sulfate	Gal(β1-4)Glc-3′-SO$_3$
Echidna milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	LDFT	Fuc(α1-2)Gal(β1-4)Glc Fuc(α1-3)
Acidic oligosaccharides		
3	4-*O*-Ac-3′-SL	Neu4,5Ac$_2$(α2-3)Gal(β1-4)Glc
Giant anteater milk		
Neutral oligosaccharides		
1	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
Acidic oligosaccharides		
2	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
3	6′-Neu5GcL	Neu5Gc(α2-6)Gal(β1-4)Glc
4	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
5	LST c(Neu5Gc)	Neu5Gc(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Spotted hyena milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
3	B-tetrasaccharide	Gal(α1-3)Gal(β1-4)Glc Fuc(α1-2)
Acidic oligosaccharides		
4	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc

(*Continued*)

Table 4 (Continued)

No.	*Oligosaccharide*	*Structure*
Platypus milk		
Neutral oligosaccharides		
1	LDFT	Fuc(α1-2)Gal(β1-4)Glc Fuc(α1-3)
2	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
3	LNDFH III	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3)
4	DF-LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-3) Fuc(α1-3)
5	LNDFH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc Fuc(α1-4)
6	DF-LNnH I	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
7	TF-LNnH a	Fuc(α1-3) Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
8	TF-LNnH b	Fuc(α1-3) Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-3)
Tammar wallaby milk		
Neutral oligosaccharides		
1	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
2	Di-GalL	Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
3	Tri-GalL	Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
4	Tetra-GalL	Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
5	Penta-GalL	Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
6	*novo*-LNT	GlcNAc(β1-6) Gal(β1-4)Glc Gal(β1-3)
7	*novo*-LNP I	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(β1-3)
8	Gal-*novo*-LNP I	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)Glc Gal(β1-3)Gal(β1-3)
9	-	Ra or Rb Ra or Rb Gal(β1-3) [Gal(β1-3)] nGal(β1-4)Glc

(*Continued*)

Table 4 (Continued)

No.	***Oligosaccharide***	***Structure***
10	-	Ra Ra Gal(β1-3) [Gal(β1-3)] nGal(β1-4)Glc Ra=Neu5Ac(α2-3/ α2-6)Gal(β1-4)GlcNAc(β1-6) Rb=Gal(β1-4)GlcNAc(β1-6)
Giant panda milk		
Neutral oligosaccharides		
1	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
2	3-F-isoglobotriose	Gal(α1-3)Gal(β1-4)Glc Fuc(α1-3)
Acidic oligosaccharides		
3	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
4	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
5	3′-S-3-FL	Neu5Ac(α2-3)Gal(β1-4)Glc Fuc(α1-3)
Beluga milk		
Acidic oligosaccharides		
1	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
Minke whale milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
3	A-tetrasaccharide	GalNAc(α1-3)Gal(β1-4)Glc Fuc(α1-2)
4	*para*-LNnH	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Acidic oligosaccharides		
5	3‴-S-LNnT	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
6	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
7	S-*para*-LNnH	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Bottlenose dolphin milk		
Neutral oligosaccharides		
1	Globotriose	Gal(α1-4)Gal(β1-4)Glc
Acidic oligosaccharides		
2	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
3	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
4	GM_2 tetrasaccharide	GlcNAc(β1-4) \ Gal(β1-4)Glc Neu5Ac(α2-3) /

(Continued)

Table 4 (Continued)

No.	*Oligosaccharide*	*Structure*
Brydes whale milk		
Acidic oligosaccharides		
1	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
2	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
3	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Sei whale milk		
Acidic oligosaccharides		
1	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
2	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
3	LST c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Crabeater seal milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
Hooded seal milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
3	LNFP Ⅳ	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
4	LNnH	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
5	F-LNnH a	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) /
6	F-LNnH b	Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) /
7	DF-LNnH Ⅱ	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) /
8	*para*-LNnH	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
9	F-*para*-LNnH	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
Bearded seal milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	LNFP IV	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
3	DF-LNnH II	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) \\ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) /
Arctic harbor seal milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc

(*Continued*)

Table 4 (Continued)

No.	Oligosaccharide	Structure
3	LNnH	Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ⟋
4	F-LNnH a	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ⟋
5	F-LNnH b	Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) ⟋
6	DF-LNnH II	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3) ⟋
Acidic oligosaccharides		
7	S-LNnH II	Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ⟋
8	FS-LNnH	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ⟋
9	DS-LNnH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ⟋
Mink milk		
Neutral oligosaccharides		
1	2′-FL	Fuc(α1-2)Gal(β1-4)Glc
2	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
3	LNnT	Gal(β1-4)GlcNAc(β1-3) Gal(β1-4)Glc
4	LNFP IV	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
5	Galilipentasaccharide	Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
6	LNnH	Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ⟋
7	F-LNnH	Fuc(α1-2) { Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Gal(β1-4)GlcNAc(β1-3) ⟋
8	FS-LNnH	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ⟋
9	SGal-LNnH	Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) ⟍ Gal(β1-4)Glc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) ⟋

F, fucose; L, lactose; S, sialyl; DF, difucosyl; TF, trifucosyl; DS, disialyl; TS, trisialyl; FS, fucosyl sialyl; DFS, difucosyl sialyl; TFS, trifucosyl sialyl; FDS, fucosyl disialyl; DGal, digalactosyl; FL fucosyllactose; GL, galactosyllactose; LDFT, lacto-difucotetraose; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; LNFP, lacto-*N*-fucopentaose; LNP, lacto-*N*-pentaose; LNDFH, lacto-*N*-difucohexaose; LNH, lacto-*N*-hexaose; LNnH, lacto-*N*-neohexaose; LNO, lacto-*N*-octaose; LND, lacto-*N*-decaose.

between cow and human. As already mentioned, the low concentration of oligosaccharides in mature bovine milk presents a problem with respect to the production of milk replacers for human infants when using components of this milk as a starting material. In future, it may be possible to utilize other substances such as LNB, with biological effects similar to those of HMOs, in the formulation of infant formulae (see below).

Although lactose is the dominant saccharide in the milk of many mammalian species, the milk of monotremes and marsupials contains considerably more oligosaccharides than lactose and the free lactose concentration is low. Among eutherians, the milks of several species of the order Carnivora (Ursidae, Phocidae, Procyonidae, and Mustelidae) contain relatively high ratios of milk oligosaccharides to lactose; in particular, those of Ursidae contain considerably more oligosaccharides than lactose.

There are also differences between species with respect to the content of fucosyl oligosaccharides in their milk. Milk of the platypus and echidna is rich in fucosyl oligosaccharides, which are also found in the milks of three species of bears and three species of seals. Milk oligosaccharides of the tammar wallaby and other marsupials do not, however, contain fucose. It is noteworthy that seal fucosyl oligosaccharides contain H antigen (Fuc(α1-2)Gal) as the only fucosyl unit, while most of the higher oligosaccharides of bears contain Lewis x (Gal(β1-4)[Fuc(α1-3)]GlcNAc). The types of linkages involved in the fucosylation, namely Fuc(α1-2)Gal, Fuc(α1-3)Glc(NAc), or Fuc(α1-4)GlcNAc, are also very different between mammalian species.

Oligosaccharides containing the type II chain dominate over those containing the type I chain in the milk of most species other than human. Many milk oligosaccharides of the platypus, bears, and seals, and some of humans, contain LNnT, LNnH, or *para*-lacto-*N*-neohexaose (Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc), as well as lactose, as core units. In contrast, the major tammar wallaby milk oligosaccharides consist of (β1-3)-linked Gal chains, ranging from trisaccharides to at least octasaccharides; with the exception of the trisaccharide Gal(β1-3)Gal(β1-4)Glc, this is a type of structure that has never been found in the milk or colostrum of other species. Eutherian milk oligosaccharides therefore resemble those of monotremes more than they do those of marsupials.

The species specificity of milk oligosaccharides resides mainly in the structures of their nonreducing end units. For example, many seal milk oligosaccharides contain H antigen at their nonreducing ends, while some of the bear milk oligosaccharides contain A (GalNAc(α1-3)[Fuc(α1-2)Gal]) or B (Gal(α1-3)[Fuc(α1-2)]Gal) antigens. On the other hand, some of the milk oligosaccharides of bear, the white-nosed coati, mink, cow, goat, sheep, and elephant contain the α-Gal epitope (Gal(α1-3)Gal(β1-4)Glc(NAc)). The milk/colostrum of these species contains isoglobotriose, while bottlenose dolphin colostrum contains globotriose but no isoglobotriose.

A few milk oligosaccharides of some species including dog, rat, human, and bearded seal contain sulfate, while those of cow and horse contain phosphate.

Although the milk of almost all species contains oligosaccharides in addition to lactose, that of otariid pinnipeds exceptionally contains neither, because of the absence of a functioning α-lactalbumin from their lactating mammary glands.

Concluding Remarks

Novel milk oligosaccharides are yet to be discovered as a result of improvements in the methods for structural characterization, using milk samples that are smaller than those required at present. The significance of individual HMOs will be further clarified, based on the accumulation of more data on their concentrations in milk at different stages of lactation in a variety of ethnic groups. The metabolism of milk oligosaccharides by bifidobacteria will be further elucidated. Studies on the interaction between milk oligosaccharides and pathogenic bacteria, viruses, and protozoa, and on immunomodulating effects by milk oligosaccharides, are still in their early stages. The industrial isolation of milk oligosaccharides of domestic farm animals, for their utilization as drugs or biofunctional foodstuffs, is still in its infancy but may have a great future.

See also: **Analytical Methods**: Mass Spectrometric Methods; Nuclear Magnetic Resonance: Principles. **Bacteria, Beneficial**: *Bifidobacterium* spp.: Morphology and Physiology. **Lactose and Oligosaccharides**: Lactose: Derivatives; Lactose: Galacto-Oligosaccharides. **Milk**: Human Milk. **Milk Proteins**: Interspecies Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity; α-Lactalbumin. **Prebiotics**: Functions.

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LIQUID MILK PRODUCTS

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Liquid Milk Products: Pasteurized Milk

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Introduction

The objectives of milk pasteurization are to ensure the safety of fluid milk by killing pathogens known to occur in milk and to prolong shelf life by destroying undesirable enzymes as well as reduce the number of viable spoilage microorganisms. The target of pasteurization is to achieve 99.999% (5-log) reduction in viable microorganisms. Pasteurization should not be confused with commercial sterilization, where the objective is to kill all microorganisms (pathogenic and spoilage) in the food. Pasteurization is a mild type of heat treatment that causes only minor changes in milk flavor and nutritional quality.

The common types of pasteurized milk are whole milk, reduced-fat milk, and non-fat/skim milk. Milk beverages fortified with vitamins, minerals, and other nutraceutical compounds are also common. These frequently target population groups with unique dietary requirements. Other speciality products include organic milk, flavored milk, and low-lactose milk.

History

Pasteurization was named after Louis Pasteur, who applied heat treatment to improve the shelf life of wine. Later, the process was applied to milk. Originally, the temperature–time combination for pasteurization was based on the amount of heat treatment required to destroy *Mycobacterium tuberculosis* var. *bovis*, which caused tuberculosis and was considered to be the most heat-resistant pathogen in milk at the time. Currently, the temperature–time setting is based on thermal death time studies for the most heat-resistant pathogen found in milk, *Coxiella burnetii*, which causes Q fever. In 1908, Chicago was the first major city in the United States that required mandatory pasteurization for milk sold in the city. In 1987, the Food and Drug Administration (FDA) banned interstate sale of raw milk and issued mandatory pasteurization of milk and milk products for human consumption for interstate commerce (21 CFR 1240.61).

Regulations and Definitions

The Code of Federal Regulations (CFR) defines milk as: "The lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows. Milk that is in the final package form for beverage use shall have been pasteurized or ultrapasteurized, and shall contain not less than 8.25% milk solids-nonfat and not less than 3.25% milk fat. Milk may have been adjusted by separating part of the milk fat therefrom, or by adding thereto cream, concentrated milk, dry whole milk, skim milk, concentrated skim milk, or nonfat dry milk. Milk may be homogenized."

Production of fluid milk in the United States is regulated by the FDA and milk production guidelines are outlined in the Pasteurized Milk Ordinance (PMO). The PMO

Table 1 Temperature (°C)–time (min or s) relationships for pasteurization as specified by US FDA Grade 'A' Pasteurized Milk Ordinance

Temperature	*Time*	*Pasteurization type*
63 °C (145 °F)[a]	30 min	Vat pasteurization. Low-temperature, long-time pasteurization (LTLT)
72 °C (161 °F)[a]	15 s	High-temperature, short-time pasteurization (HTST)
89 °C (191 °F)	1 s	Higher-heat, shorter-time pasteurization (HHST)
90 °C (194 °F)	0.5 s	HHST
94 °C (201 °F)	0.1 s	HHST
96 °C (204 °F)	0.05 s	HHST
100 °C (212 °F)	0.01 s	HHST
137.8 °C (280 °F)	2.0 s	Ultrapasteurized (UP)

[a] If the fat content of the milk product is 10%, or if it contains added sweeteners, the specified temperature shall be increased by 3 °C (5 °F).

specifies pasteurization conditions, milk quality, antibiotics testing, and current good manufacturing practices.

Specifically, the PMO defines pasteurization as "the process of heating every particle of milk or milk product, in properly designed and operated equipment, to one of the temperatures given in the following table and holding continuously at or above that temperature for at least the corresponding specified time" see **Table 1**.

The International Dairy Federation defines pasteurization as "a process applied to a product with the object of minimizing possible health hazards arising from pathogenic microorganisms associated with milk, by heat treatment which is consistent with minimal chemical, physical, and organoleptic changes in the product".

The alkaline phosphatase enzyme (present in raw milk) is slightly more heat resistant than *C. burnetii* and a test for phosphatase activity is therefore used to demonstrate that the milk has been adequately pasteurized. In the United States, this is sufficient to demonstrate proper pasteurization. However, according to some European regulations, pasteurization is defined by both minimum and maximum heat treatments. Pasteurized milk must exhibit both a phosphatase-negative test and lactoperoxidase (more heat resistant than alkaline phosphatase)-positive result. The upper limit is set to prevent over-heating of milk.

Batch versus Continuous Pasteurization

Milk and milk products may be pasteurized by batch or continuous method. The batch method or low-temperature–long-time (LTLT) method uses a minimum temperature–time combination of 63 °C for 30 min (i.e., every particle of milk shall be held at a minimum of 63 °C for at least 30 min, with the atmosphere above the milk held at not less than 66 °C, that is, 3 °C higher than the required minimum). The milk is stirred relatively vigorously to promote rapid heating and cooling and to minimize temperature differential among milk particles during pasteurization (all milk should be within 0.5 °C during pasteurization to prevent cold spots within the pasteurizer or outlet valve). The advantage of batch pasteurization is the simplicity of the method – heat–hold–cool – which can be done with only a double-jacketed tank, although a typical licensed batch pasteurizer is quite sophisticated. However, this is a slow and inefficient process. To increase efficiency and to reduce energy costs, most fluid milk plants use plate heat exchangers that continuously heat milk to a minimum of 72 °C for at least 15 s. The continuous method is referred to as high-temperature–short-time (HTST) method. Energy efficiency is improved in the HTST pasteurizer by using regeneration of heat. Warm pasteurized milk gives off heat across separating plates to the incoming cold, raw milk within the regeneration section of the pasteurizer. Regeneration efficiencies of 90% or above are typically obtained, making these systems much more energy efficient than the batch pasteurizer.

Fluid Milk Processing

Pasteurized milk processing varies from plant to plant and among countries. Usually, raw milk is stored in silo tanks before processing. In the United States, raw milk can be stored for up to 72 h at refrigeration temperature (legally below 7.2 °C but preferably at or below 4 °C). From the silo(s), raw milk is clarified, preheated, separated, standardized, homogenized, pasteurized, and cooled. Finally, the cooled pasteurized milk is pumped to a storage tank until packaged.

Several features help assure the safety of HTST pasteurization. A flow diversion valve (FDV), controlled by a temperature detector, automatically diverts milk back to the balance tank if the milk is not at the required temperature at the exit of (or at the entrance to) the holding tube. Furthermore, use of a positive displacement pump ensures that the pressure of the pasteurized milk is higher than that of the raw milk, thereby prevents the mixing of raw milk into the pasteurized milk stream within the regeneration section of the pasteurizer. Other pumps can also be used to generate the pressure differential, which should be

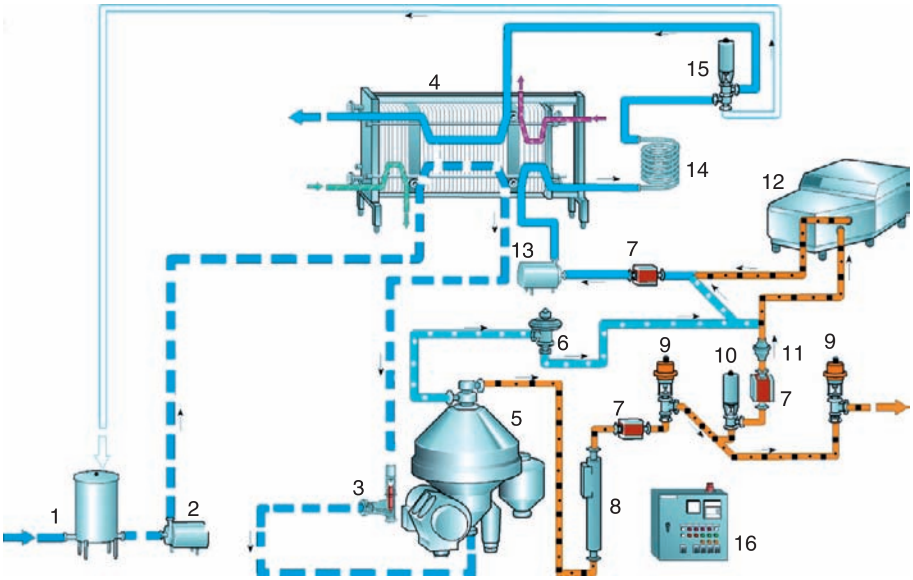

Figure 1 Production line for pasteurized milk. Milk passes balance tank, regeneration section, separator, online standardization with homogenized cream, heating section, holding tube, regeneration section, and cooling section. ▬ ▬ milk, ▬▪▬▪ cream, ▬•▬• skim milk, ▬ standardized milk, ▨ cooling medium, ▨ heating medium, ═ diverted flow. 1, Balance tank; 2, product feed pump; 3, flow controller; 4, plate heat exchanger; 5, separator; 6, constant pressure valve; 7, flow transmitter; 8, density transmitter; 9, regulating valve; 10, shutoff valve; 11, check valve; 12, homogenizer; 13, booster pump; 14, holding tube; 15, flow diversion valve; 16, process control. Source: Tetra Pak, Inc.

monitored continuously. Additional safety features include an indicator thermometer, positively sloped holding tube, vacuum break, and placement of the balance tank below the inlet valve to the system. While the process described above, and illustrated in **Figure 1**, is typical for milk pasteurization, there are many alternatives. The milk may be cold separated before pasteurizing. Not all milk is standardized for fat content online prior to homogenization. Energy can be saved if the cream flow is homogenized immediately following the separator and then added into the skim milk flow. Occasionally, whole milk is not homogenized, as some consumers prefer a cream layer on the top of the milk. This cream layer is particularly obvious when milk is bottled in clear glass containers.

Effects of Pasteurization

Microbial: As mentioned above, the time–temperature parameters in **Table 1** were developed to sufficiently destroy *C. burnetii*, which is considered the most heat-resistant non-spore-forming pathogen likely to be present in milk. Destruction of microorganisms by pasteurization is described by first-order kinetics. Pasteurization kills most of the microorganisms in milk but does not render milk sterile. Spores and thermoduric bacteria can be especially difficult to destroy by pasteurization. Thus, pasteurized milk must be kept under proper refrigeration (preferably below 4 °C) throughout distribution and storage. The upper limit for the standard plate count (SPC) of pasteurized milk is 20 000 cfu ml^{-1} in the United States. However, the actual aerobic bacterial count in freshly pasteurized milk tends to be much lower than this.

Nutritional: Pasteurization causes only minor nutritional losses in milk. The approximate loss of vitamins is summarized in **Table 2**. It should be noted that the extent of vitamin loss during pasteurization depends on several other factors, notably temperature–time combinations during pasteurization and oxygen concentration. Some denaturation of whey proteins (3–5%) can also be expected. Most heat-induced chemical reactions, such as Maillard browning reactions and lactulose formation, occur primarily at temperatures above those typical for pasteurization.

Enzymatic: Several indigenous milk enzymes are destroyed. The denaturation of lipase and some proteases limits the formation of off-flavors in the pasteurized milk and thus contributes to extending shelf life. In contrast, some lipases and proteases of bacterial origin are heat resistant and cannot be inactivated by pasteurization. Plasmin is heat resistant as well, and its activity is actually increased by pasteurization due to the inactivation of an inhibitor, which inhibits the transformation of plasminogen into plasmin.

Packaging

The package protects milk from microbial contamination and the intentional or unintentional addition of foreign

Table 2 Typical loss (%) of some vitamins on pasteurization of milk

Vitamins	*A*	*B_1*	*B_6*	*B_9*	*B_{12}*	*C*	*D*
Loss (%)	NS	10	1–5	3–5	1–10	5–20	NS

NS, not significant.

From Lewis M and Heppell N (2000) Pasteurisation. In: Lewis M and Heppell N (eds.) *Continuous Thermal Processing of Foods: Pasteurisation and UHT Sterilization*, pp. 193–231. Gaithersburg, MD: Aspen Publishers, Inc.; Walstra A, Geurts TS, Noomen A, Jellema A, and van Boekel MAJS (1999) *Dairy Technology: Principles of Milk Properties and Processes*. New York: Marcel Dekker Inc.; Wong N (1984) *Fundamentals of Dairy Chemistry*. New York: Van Nostrand Reinhold Co.

objects. Furthermore, it should block the passage of ultraviolet (UV) and visible light, which could lead to sunlight-oxidized off-flavors in the milk and to the loss of light-sensitive vitamins. Last, but not the least, the packaging provides an important opportunity for communicating with customers.

Glass bottles, used for milk packaging, are becoming rare. They are heavy and pose a safety hazard in the processing environment. Glass also permits full light penetration. Glass and polycarbonate (Lexan)$^{\text{TM}}$ bottles may be cleaned and reused. While this is beneficial from a recycling perspective, great care must be taken to ensure proper cleaning of the returned containers.

Paperboard milk cartons, frequently gable tops, with polyethylene coating are excellent light barriers and are popular due to the efficient, low-cost carton packaging systems. Disadvantages include difficulty of opening and resealing; however, newer cartons have spouts with resealable caps. These containers are easier for consumers to open and reseal.

High-density polyethylene (HDPE) is the plastic of choice for the dairy industry. In many dairy plants, the plastic containers are blow-molded at the facility in a room or building adjacent to the bottling room. Polyethylene terephthalate (PET) is gaining popularity for use in single-serve blow-molded milk containers because it has high clarity and consumer appeal. PET also has excellent oxygen and water vapor resistance. To prevent light penetration, the plastic can be tinted with UV barriers or covered with a printable film.

Shelf Life

One of the objectives of pasteurization is to increase the shelf life of milk by reducing spoilage organisms and inactivating several milk enzymes. Raw milk spoils within a few days even when stored at refrigeration temperature, while pasteurized milk can have a shelf life between 7 and 28 days, or even longer. Shelf life is influenced by the quality of the raw milk, post-pasteurization contamination, especially at packaging, and temperature control during storage and distribution.

Shelf life is usually determined by microbial analysis and/or sensory analysis by consumer or trained panelists. Both methods are time consuming. Microbial tests are usually done after a certain storage time and temperature, and the result is correlated with the actual shelf life. The Moseley Keeping Quality Test involves plating freshly processed milk and milk samples stored at 7 °C for 5–7 days on SPC. A large increase of microbial growth between the first and second platings indicates limited shelf life. The Virginia Tech Shelf Life test uses a preliminary incubation at 21 °C for 18 h followed by plating on SPC plates or Petrifilm and incubating for 24 or 48 h at 21 °C, respectively.

Post-pasteurization contamination is the contamination of milk following the holding tube of the pasteurizer, due to improperly cleaned and sanitized cooling sections, process lines, valves, tanks, fillers, and packaging materials. Post-pasteurization contamination is a major cause of milk spoilage. Contamination by psychrotrophic bacteria, such as *Pseudomonas* and *Bacillus* species, is most undesirable. Good process design, cleaning and sanitation of both equipment and environment, and product temperature control from the point the milk leaves the holding tube until consumption are required to obtain a good shelf life. However, since the process is not aseptic, a certain amount of post-pasteurization contamination is inevitable. The coliform bacteria count can be used as a test for post-pasteurization contamination because coliforms are killed by pasteurization.

Flavor and Flavor Defects of Pasteurized Milk

Milk should have a slightly sweet flavor with little or no foretaste and aftertaste, have a homogeneous appearance, and should be practically odorless. Raw milk of poor quality or processing and distribution problems may occasionally lead to flavor defects in milk. The Committee on Flavor Nomenclature and Reference Standards of the American Dairy Science Association has proposed seven categories of off-flavors in milk:

1. Heated off-flavor, also described as cooked, nutty, burnt, scorched, or caramel. The intensity depends on the time–temperature of heating, product composition, heating and cooling rates, and burn-on of milk residues on the heat exchanger surfaces.

2. Light-induced, cabbage-like, burnt-protein, or burnt-feather is due to photooxidation of methionine to methanal when milk is exposed directly to sunlight or fluorescent light.
3. Lipolyzed or rancid, also called hydrolytic rancidity, is caused by milk fat hydrolysis by milk lipase, which results in free fatty acids and partial glycerides.
4. Oxidized off-flavor or oxidative rancidity, also described as metallic, tallow, cardboardy, or fishy, results from a chemical change in unsaturated fatty acids and/or phospholipids. Metal ions, such as copper and iron, act as catalysts.
5. Transmitted off-flavors, usually called feed, barny, or weed off-flavors, originate from feed and/or the odor of air breathed by the cows, which is transmitted to the milk.
6. Microbial off-flavors, of which there are two types. The first type is acid, high acid, and sour, malty off-flavor milk, and is usually caused by bacteria and occasionally by yeast or molds. It usually occurs in raw milk and is due to unsanitary practices and improper cooling of milk. The second type is usually putrid, fruity, unclean, and sometimes bitter, and is commonly caused by psychrotrophic bacteria.
7. Miscellaneous off-flavors, which can be caused by various factors, such as certain medications, disinfecting agents, or sanitizing agents. Some examples of off-flavors in this category are stale, salty, bitter, chalky, chemical, flat, and foreign.

Types of Pasteurized Milks

Thirty years ago, the milk beverage of choice in North America was whole milk, which contains a minimum of 3.25% fat. Consumer concerns over high-fat diets have led to a shift in milk consumption away from whole milk toward reduced-fat, low-fat, or skim milk (**Figure 2**). The nutritional quality of low-fat milk is similar to that of whole milk, although there is a decrease in fat-soluble vitamins and in caloric value. Skim milk has different sensory characteristics (whiteness, mouthfeel, flavor) than whole milk. Therefore, many skim milks have been developed with extra solids content, water-binding stabilizers, and other ingredients to imitate the texture and mouthfeel of whole milk. Vitamin D-fortified milk was introduced to the United States in 1933 to enhance calcium absorption and reduce rickets and it is common to fortify reduced- and low-fat milks with both vitamins A and D. Basically, these fat-soluble vitamins are added back to make up for vitamins removed in the cream. Vitamins A and D are added to obtain levels of at least 2000 and 400 international units (IU) per quart (~0.95 l) milk, respectively.

Reduced-lactose milks are commonly prepared by adding β-galactosidase (lactase). The product is targeted toward lactose-intolerant consumers (*see* **Lactose and Oligosaccharides**: Lactose Intolerance). Ultrafiltration techniques can also be used to remove lactose in the permeate.

Probiotic bacteria such as *Lactobacillus acidophilus* can be added to milk at refrigeration temperatures. Due to the low storage temperature, the milk is not fermented but serves as a delivery system for the probiotic cultures. There are few US standards for probiotic milk products, and only a few states have regulations on minimum content of viable bacteria in acidophilus milk; for example, in Oregon, acidophilus milk must contain above 2×10^6 viable bacteria per milliliter.

Even though organic milk remains a niche product, growth rates in this market category are significant, especially when compared to the stagnant sales of traditional milk. Organic milk is produced and processed in facilities that follow organic guidelines (*see* **Organic Dairy Production**).

The sale of flavored milks is increasing rapidly. Single-serve flavored milk is the fastest-growing market segment of the US fluid milk market. Over the past 10 years, annual sales increases in the United States have averaged 74 000 tonnes. Chocolate milk is the most popular flavor followed by strawberry milk. While chocolate and strawberry flavors are developed and marketed primarily

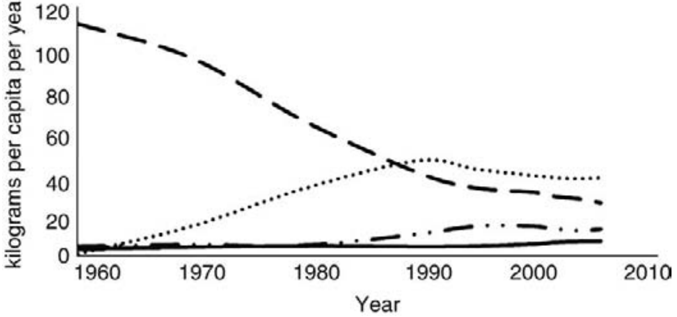

Figure 2 Per caput consumption of whole milk (– – –), low- and reduced-fat milk (..........), skim milk (· · — · ·), and flavored milk and drinks (———), in the United States from 1960 to 2006. Source: USDA Economics, Statistics, and Market Information System.

Table 3 US beverage market (millions of liters)

	1995	*1999*	*2004*	*2007*
Carbonated soft drinks	52 055	57 729	58 165	55 668
Bottled water	10 916	16 105	21 936	33 147
Milk	24 049	24 394	23 774	23 662
Coffee	23 955	22 706	24 035	24 805
Beer	21 919	23 043	24 206	25 089
Fruit beverages[a]	15 159	15 636	15 849	14 760
Tea	9958	9826	8134	9404
Wine	1783	2010	2413	2662

[a] Includes liquid fruit juice and fruit drinks; excludes powdered fruit drinks and vegetable juices.

Source: Reproduced with permission from Beverage Marketing Corporation, NY.

toward children, other flavors such as coffee-flavored products are directed toward the adult market. One reason for the success of flavored milk is the packaging in single-serve containers with eye-catching graphics. These products compete well against soft drinks, juices, and sports drinks, and are well adapted for sale in special milk-vending machines. The increase in ultrapasteurized (UP) processing lines indicates that the development of flavored milk will focus on extended shelf life products and not on pasteurized products.

The beverage market is experiencing rapid changes. Consumption of bottled water has surpassed milk consumption (**Table 3**). Health and wellness trends drive sales of single-serve beverages. Energy drinks and beverages offering functional advantages are selling well. This increase has, in part, been credited to commercialization at multiple locations such as vending machines, convenience stores, and supermarkets. In response, the dairy industry is focusing on the development of new products and innovative packaging design. Full shrink sleeves (polyvinyl chloride) provide the opportunity for eye-catching graphics on single-serve bottles. The dairy industry is transforming from milk bottlers to beverage producers. Milk is being diversified from a plain white product to include formulated products targeted toward meeting emotional and nutritional needs of specific population segments. Milk beverages fortified with multiple vitamins, minerals, and neutraceuticals are appearing. Package sizes have been adapted to promote consumption anytime and anywhere.

Pasteurized Milk versus Extended Shelf Life Milk

Pasteurized milk must be stored under refrigeration and has a relatively short shelf life. In contrast, UP milk (heated to 125–138 °C for 2–4 s; *see* **Liquid Milk Products**: Liquid Milk Products: UHT Sterilized Milks) can be stored for up to 3 months under refrigeration and milk subjected to ultra-high temperature (UHT) treatment (heated to 135–140 °C for a few seconds; *see* **Liquid Milk Products**: Liquid Milk Products: UHT Sterilized Milks) can be stored for 3–6 months at ambient temperatures. In some countries, the convenience of longer shelf life and less refrigeration capacities have led to a significant shift away from pasteurized milk toward UHT and UP milks. Studies with consumer panels have revealed that US consumers can distinguish between pasteurized and UHT milk. Most consumers prefer pasteurized milk because of the flavor (UHT milk tends to have a cooked flavor) and the 'fresh' image associated with pasteurized milk.

Alternative Technologies

Traditional pasteurization in HTST equipment is energy efficient due to regeneration, and conditions are optimized to maximize microbial kill while minimizing chemical changes in the milk. Nevertheless, there are other technologies currently being researched that may eventually replace the pasteurizer. These include microfiltration, high-pressure, and pulsed electric field technologies. Briefly, microfiltration involves removing the large bacterial cells from skim milk in retentate, which is either discarded or pasteurized prior to reincorporation into the skim milk permeate. High-pressure processing involves exposing the milk to high pressure above 400–500 MPa and pulsed electric fields expose the milk to short electric pulses. Both treatments damage bacterial cells. Currently, none of these techniques are as efficient and effective as pasteurization and regulatory changes would be required prior to the adaptation of an alternative technology. Therefore, it appears likely that pasteurized milk will remain the primary fluid milk product on the US market for the foreseeable future.

See also: **Lactose and Oligosaccharides**: Lactose Intolerance. **Liquid Milk Products**: Liquid Milk Products: UHT Sterilized Milks. **Organic Dairy Production**.

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Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk)

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Introduction

The keeping quality or shelf life of fluid milk products is a problem in many of the world's markets, due to inadequate refrigeration, poor raw material, and/or insufficient processing and filling technologies. Milk pasteurized by traditional high-temperature–short-time (HTST) processing and filling technologies is limited to a shelf life of approximately 1–3 weeks at refrigerated temperatures, thus limiting transportation to regional locations. Until recently, the only solution has been to manufacture ultra-high temperature (UHT) milk with a shelf life of 3–6 months at ambient temperature. However, UHT processing alters the sensory properties of the milk, contributing a pronounced cooked or scorched flavor and associated darker color.

In an effort to prevent thermal alteration of the sensory properties, extend the shelf life beyond that of HTST pasteurized milk, and expand the ability to transport milk without spoilage, milk can be processed at temperatures above those used for pasteurization yet below those used for UHT processing. Milk subjected to this treatment is referred to as ultrapasteurized, superpasteurized, extended shelf life (ESL), or extended long-life milk. The terms 'superpasteurized' and 'ESL milk' will be used interchangeably in this article. Superpasteurized milk is designed to have a shelf life of up to 90 days at refrigeration temperatures and have sensory properties superior to those of UHT milk. However, limited data are available in the scientific literature on the safety, sensory qualities, or shelf life of superpasteurized milk.

Product Stability

Shelf Life

Product shelf life is generally defined as the time between processing and the point at which the quality of the product falls below an acceptable level. This definition depends on the perception of 'minimum acceptable quality'. In some applications of pasteurized milk, a microbial count exceeding 10^7 organisms per ml is associated with a minimum acceptable quality. More practically, the onset of sensorially defined off-flavors, such as the development of rancidity, denotes the end of shelf life.

The shelf life of superpasteurized milk is influenced by raw material quality, processing and packaging conditions, and environmental conditions during distribution and storage. Although chemical reactions resulting from metal- and light-induced oxidation may have dramatic influences, outgrowth of spoilage microorganisms is thought to be the greatest limiting factor for the shelf life of superpasteurized milk.

Flavor Defects of ESL Milk

One of the major challenges of producing ESL milk is elimination of spoilage bacteria without compromising flavor, vitamin content, textural character, or appearance of the final product. The reduction of cooked or scorched flavors is perhaps one of the main issues facing processors. Additionally, there are other off-flavors that can be manifest in ESL milk, such as those resulting from lipid oxidation, Maillard reactions, and the adsorption of volatiles from the packaging materials used in milk containers.

Cooked flavor (usually related to the formation of sulfur compounds from the decomposition of sulfur-containing proteins) is a dominant flavor note associated with high-temperature treatment of milk products. The strategies used to reduce the generation of cooked flavors involve the use of high temperatures for shorter periods, thus limiting the off-flavor-producing reaction mechanism. Maillard reactions are very common in heat-treated milk products and can lead to the generation of a variety of off-flavors (described as burnt candy, cabbage, baked potato) and some discoloration. The oxidation of unsaturated fatty acids yields aroma-active aldehydes and ketones capable of inducing cardboard-like flavors. High-temperature processing, exposure to light, irradiation, and the presence of metals (copper and iron) are the factors that can potentiate oxidation.

A number of strategies have been proposed to minimize the defects caused by thermal treatment. The main goal of such methods is to use heat treatment sufficient to cause bacterial death, yet limited to avoid undesired degradation reactions. Non-thermal approaches such as microfiltration technologies may hold promise for achieving ESL milk with sensory and chemical properties more similar to those of HTST milk. The use of pulsed electric fields to obtain ESL milk has also been reported to be a method that yields no apparent changes in the olfactory or visual characteristics of the product when compared to HTST processing.

Texture and Appearance Defects

Another quality problem with ESL milk products is related to textural changes that can occur after long periods of storage. The most common textural changes involve the separation of the lipid phase, sedimentation of denatured proteins, and age gelation. Age gelation is an irreversible phenomenon that occurs during the storage of sterilized milk, transforming the product into a gel. Gelation is considered a critical quality problem associated with sterilized milk products and is affected by a multitude of factors such as the severity of heat treatment, proteolysis during storage, milk composition and quality, seasonal milk production factors, and storage temperature. Gelation is regarded as a two-stage process involving the formation of a β-lactoglobulin–κ-casein complex, which cross-links to form a protein network gel. Gelation can be minimized by selecting high-quality milk, inactivating proteinases, increasing the degree of heat treatment, storing the milk at a temperature below or above the optimum range for gelation (25–30 °C), and/or adding protein-solubilizing agents such as citrates or phosphates.

Factors That Affect the Shelf Life of ESL Products

Storage temperature

Storage temperature of the product after pasteurization is one of the most important factors involved in the extension of the shelf life of pasteurized milk. As a general rule, each 3 °C decrease in the storage temperature doubles the shelf life of pasteurized milk. In 1991, Cromie reported that the spoilage level of pasteurized milk, represented by 10^7 microorganisms per ml and unacceptable flavor scores, was reached at 12 °C after 14 days, at 7 °C after 25 days, and at 3 °C after 50 days of storage. As the temperature falls below the optimum growth temperature of 20–40 °C of most spoilage microorganisms, it causes an increase of the lag phase (period of adaptation to new conditions before logarithmic growth ensues) and a decrease in the growth rate of the microorganisms.

Microbial spoilage of superpasteurized milk is most commonly associated with inadequate control of post-pasteurization storage temperature. It has been reported that in the United States, the overall range of temperature in retail dairy display cases is 2–14 °C. However, given the longer shelf life of superpasteurized milk, it is critical that the storage temperature is held well below 7 °C. Psychrotrophic spore-forming microorganisms, which pose a potential spoilage and health risk in ESL milk, grow well at 8–10 °C, whereas their activity is suppressed at 2–5 °C.

Thermal treatment parameters

Typically, superpasteurized milk is processed at approximately 138 °C/2 s, which is below the temperature used in UHT processes (145 °C/3 s) and above the HTST pasteurization temperature (72–75 °C/15 s). Superpasteurized milk processing temperature is intended to extend the shelf life of HTST milk by significantly reducing the microbial load, yet limiting the deterioration of sensory properties caused by extreme thermal treatment.

Even though little information is available on the safety and absolute shelf life of ESL milk, there have been some reports that microbial counts for ESL milk may be higher than initially expected. The following explanations for these unexpected reports were linked to the elevated temperature used in ESL processing: (1) Most of the microorganisms are killed; therefore, the influence of competitive microflora is suppressed. As a result, heat-resistant microorganisms, which are found at low levels, and/or post-pasteurization microbial contaminants grow relatively unhindered. (2) Some bacterial spores may be activated by the temperature used in ESL processing. (3) The natural antimicrobial systems in milk may be destroyed.

Raw milk quality

In general, raw milk must be free from any impurity or distortion and have low somatic cell and total microbial counts. The quantity, type, and activity of microorganisms present in raw milk prior to processing and packaging are critical for the shelf life and flavor of superpasteurized milk.

Heat-resistant microorganisms

Some psychrotrophic spore-forming microorganisms present in raw milk have considerable spoilage potential in ESL milk. Certain thermally resistant bacterial spores not only survive heat treatments used in ESL processing, but are in fact activated by these thermal treatments. Additionally, the elevated temperature used in ESL processing may enhance the growth environment for psychrotrophic spore formers via elimination of competitive microflora and/or the natural antimicrobial systems in milk such as the lactoperoxidase system. Although inhibition of heat-resistant spore-forming psychrotrophic microorganisms is achieved at a storage temperature below 7 °C, it must be emphasized that there exists great potential for the product to experience temperature abuse over the course of transportation and the long shelf life period.

Some candidate heat-resistant spore-formers include *Bacillus circulans* and *Bacillus cereus*. *Bacillus circulans* is capable of metabolizing lactose to lactic acid, resulting in acid milk. *Bacillus cereus* is a recognized food-borne pathogen. Dairy products have rarely been implicated in outbreaks related to *B. cereus*, potentially due to an elevated rate of rancidity resulting from *B. cereus* enzymes that act on the milk fat globule membrane, thus making the milk unpalatable.

Heat-resistant enzymes

Raw milk containing high counts of any *Pseudomonas* species is not recommended for ESL processing. *Pseudomonas* species, particularly *Pseudomonas fluorescens,* produce lipases resistant to heat treatments used in ESL processing. These enzymes, which can remain active during long storage periods at refrigeration temperatures, degrade the fat globule membrane and associated lipids, resulting in rancid off-flavors.

Post-pasteurization contamination

Postpasteurization contamination with Gram-negative psychrotrophs is considered a main cause of spoilage of superpasteurized milk produced from raw material of good quality. Species routinely associated with milk spoilage include *Pseudomonas, Enterobacter*, *Klebsiella*, and *Flavobacterium.*

In order for superpasteurized milk to reach the intended shelf life, it is essential to practically eliminate postpasteurization contamination. The absence of competitive microflora in ESL milk may lead to rapid outgrowth of psychrotrophic spoilage organisms. Postprocess contaminants originate from numerous sources, including the atmosphere, milk piping, packaging, and other food contact surfaces. Thus, it is critical that superpasteurization processing utilizes aseptic packaging and filling technologies as well as strictly controlled storage conditions.

Sensory and Nutrient Qualities of ESL Milk

Relative to UHT milk, the lower temperature of ESL processing results in improved sensory properties. However, HTST milk may still be regarded as having superior flavor to ESL milk. Reflective of the heat treatment, ESL milk achieves intermediate consumer panel ratings relative to HTST and UHT processed milk (**Figure 1**).

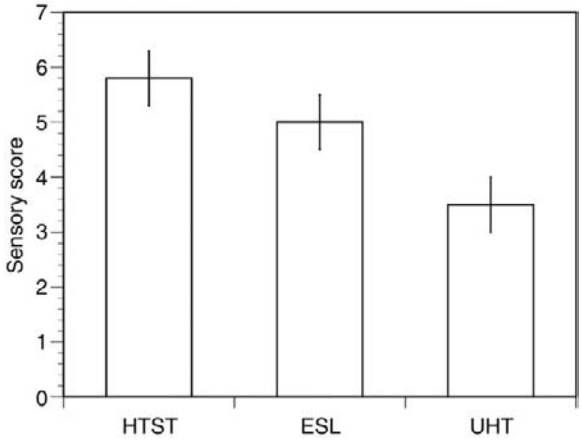

Figure 1 Average consumer acceptance (hedonic rating with 1 = extreme dislike, 9 = extreme like) comparing high-temperature–short-time (HTST; 74 °C for 16 s), extended shelf life (ESL; 134 °C for 4 s, direct steam injection), and ultra-high temperature (UHT; indirect plate exchange heating) processed milk. Adapted with permission from Blake MR, Weimer BC, McMahon DJ, and Savello PA (1995) Sensory and microbial quality of milk processed for extended shelf life by direct steam heat injection. *Journal of Food Protection* 58: 1007–1013.

Due to the extended duration of retail display, suitable packaging is an important factor in limiting light-induced degradation of nutritional and sensory qualities of ESL milk. Vitamins A and B_2 (riboflavin) can be reduced substantially when exposed to the high-intensity fluorescent lighting typical of many retail display cases. Light-induced flavor has been demonstrated to strongly diminish consumer preference. Paper cartons allow less than 1% transmission of oxidation-inducing wavelengths of light, while untinted, high-density polyethylene materials have substantially inferior light barrier characteristics. Incorporation of air and the associated oxidative potential are also detrimental to the flavor and shelf life of ESL milk. Removal of absorbed air improves flavor stability, nutrient retention, and container fill uniformity by limiting foaming at the filler. Retention of light-sensitive vitamins, inhibition of light-induced off-flavor, and minimal external flavor absorption are all achieved by using coated paperboard and multilayer laminates. These laminates are the most suitable materials for packaging ESL milk.

Production of Superpasteurized Milk

In general, successful superpasteurized milk processing is the combination of appropriate thermal treatment and aseptic filling technologies. The thermal treatment may be one of a variety of time and temperature combinations designed to achieve the desired microbial lethality and enzymatic destruction. Typical temperature/time combinations for superpasteurized milks fall in the range of 125–145 °C for 2–4 s. The US Food and Drug Administration defines ultrapasteurization as a process in which a dairy product is thermally processed at or above 138 °C for at least 2 s, either before or after packaging, so as to produce a product that has an ESL under refrigerated conditions. ESL milk affords many advantages including increased flexibility in production and distribution schedules, an increase in allowable shipping distances, and a potential increase in point of sale quality to the consumer.

Thermal Technologies

Typical heat transfer devices for ESL milks may include both direct and indirect heating technologies. Direct methods include infusion, where the product is sprayed into a steam atmosphere, and injection, where steam is injected directly into the product. Indirect methods include plate and tubular heat exchangers, which do not involve direct contact between the product and heating medium.

Direct heat exchange systems

The primary advantage of direct steam heating systems is the rapid attainment of process temperature. Infusion and injection are the two most common methods for direct heat exchange. The infusion method involves spraying the product into an environment of sufficient culinary quality steam (**Figure 2(a)**). An infuser creates a milk aerosol, thus dramatically increasing the surface area and rate of heat transfer. Injection involves spraying steam directly into the stream of liquid milk (**Figure 2(b)**). Steam injectors are operated at pressures to ensure that the steam is fully condensed into the product prior to its entry into the holding tube.

Infusion and injection systems follow a similar approach. Incoming raw milk is first heated to an intermediate temperature between 68 and 85 °C and then transferred to an infuser or steam injector module. The rapid direct heating allows a high sterilization temperature to be achieved readily with hold times in the 2- to 4-s range.

After holding, the product is sent through several cooling stages. The first stage involves 'flashing' the product into a vacuum chamber where the residual water gained during the infusion or injection process is removed, thus ensuring that there is no dilution of the finished product. If the product contains milk fat, the discharge from the vacuum chamber is homogenized using an aseptic homogenizer prior to being cooled to container filling temperature. Direct steam systems, particularly infusion, are more complex to operate than indirect systems, making automated control of sterilization, production, and cleaning essential.

Indirect heat exchange systems

While limited to processing temperatures and pressures below those of more advanced heat transfer technologies, plate heat exchange systems represent a proven, relatively low-cost means of thermal processing. The high ratio of heat transferred to product volume yields a relatively low residence time. Plate heat exchangers are comprised of a series of parallel, intimately spaced, stainless-steel corrugated plates, which are compressed together in a plate frame (**Figure 3(a)**). Milk is distributed through narrow passages, producing a turbulent flow and a high rate of heat exchange with minimal contact time. Ports within the plate assembly direct the product and heating/cooling medium to alternate sides of the corrugated plate.

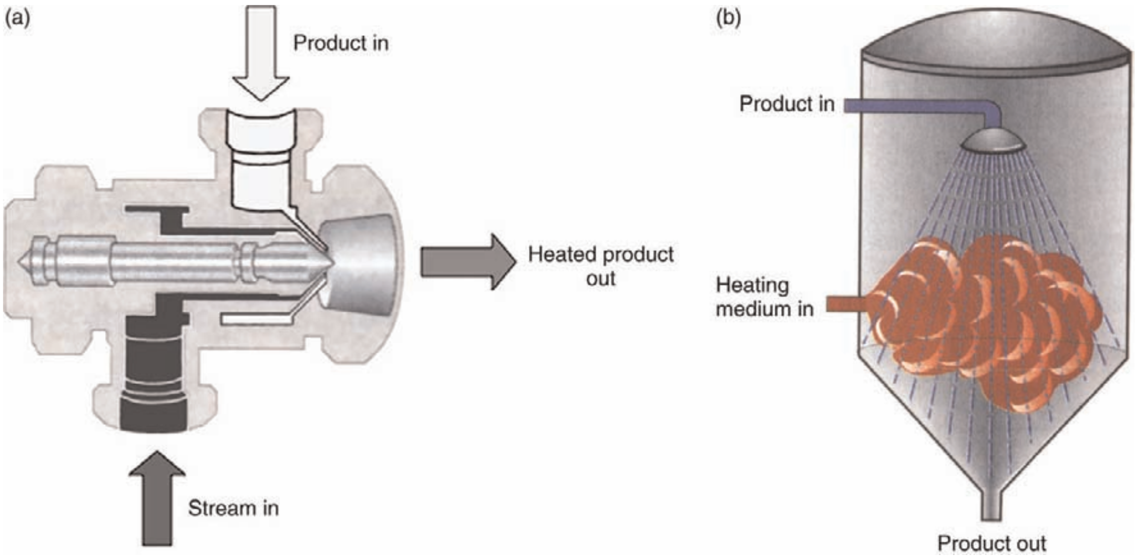

Figure 2 Cursory details of two direct heating systems used for the production of extended shelf life milk: (a) injection port and (b) infuser system. Adapted with permission from Anonymous Long life milk. In: *APV Dairy Processing Handbook*, p. 224. Lund, Sweden: APV Crepaco, Inc.

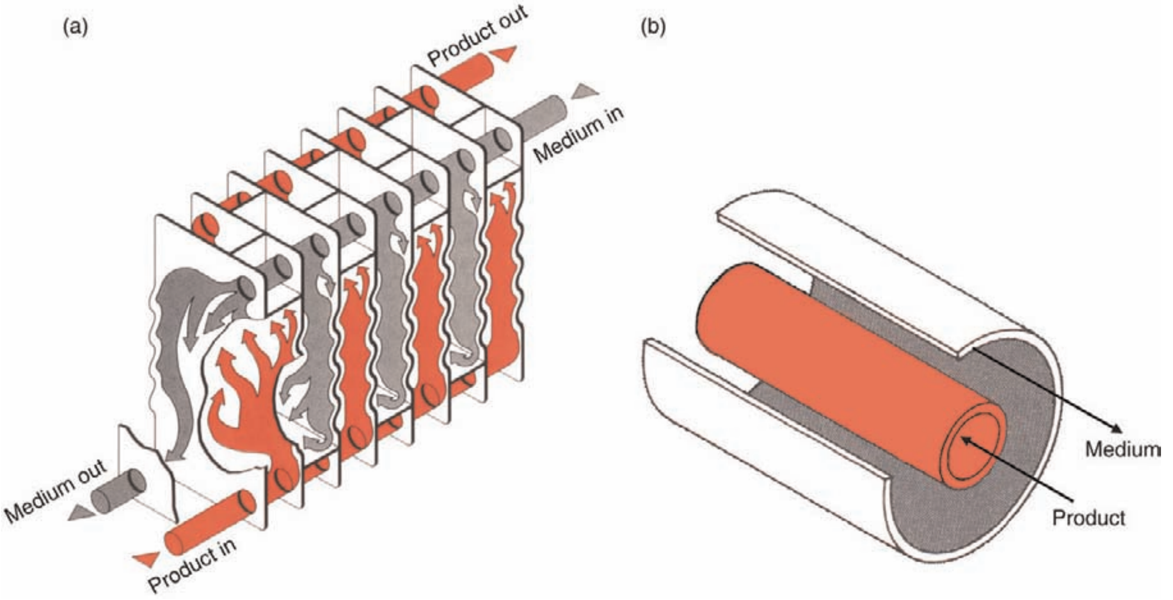

Figure 3 Cursory details of two indirect heat exchangers: (a) high-temperature–short-time plate system and (b) tube-in-tube module. Adapted with permission from Anonymous Introduction to aseptic/ESL systems. In: *APV Aseptic/Extended Shelf Life Processing Handbook*, pp. 18, 23. Lund, Sweden: APV Crepaco, Inc. APH292.

A connector grid may be incorporated to allow several independent sections, such as regeneration, heating, and cooling, to be placed on the same frame assembly. Because of the high surface areas and torturous product paths, plate heat exchange systems are more susceptible to fouling than tubular units.

There are essentially three different types of tubular heat exchangers: coiled tube, tube-in-tube, and multiple tube-in-tube heat exchangers (**Figure 3**(**b**)). In brief, these technologies involve pumping milk through tubes that are surrounded by a heating medium. A tubular process module can be operated at a pressure in excess of 275 bar and at a variety of product velocities.

Equipment Sanitation

ESL processing systems typically utilize a cleaned-in-place (CIP) protocol, which involves circulating rinse water/caustic and acid solutions at defined concentrations, temperatures, and time periods. Some processes are given an intermediate clean at a flow rate and temperature identical to those used during normal processing, thus allowing a plant to retain sterility and switch from one product to another. Following cleaning, the entire system is sterilized with hot water to eliminate microorganisms in the aseptic side of the system. Water is heated to a minimum of 132 °C and pumped through the holding tube, cooling system, and the filler (or surge tank) before being cooled for recirculation.

Aseptic Packaging and Filling

Unlike many traditional HTST processes using rotary filling devices open to the plant atmosphere, ESL milk containers must be filled using aseptic technologies to reduce/eliminate post-process microbial contaminants and to achieve the intended shelf life. Aseptic packaging and filling essentially seals a sterile product into commercially sterile containers.

There are several methods of aseptic packaging and filling. Aseptic cartoning systems sterilize a laminated fiberboard material with hydrogen peroxide; a flow of sterile hot air removes residual hydrogen peroxide prior to filling. Aseptic form–fill–seal fillers then heat-form a plastic container within a sterile cabinet under a curtain of sterile laminar-flow air. The container is filled with the product, covered with a sterile foil laminate lid, and sealed. Pre-formed plastic containers can be sterilized with hydrogen peroxide and filled aseptically in a similar fashion.

Some systems utilize clean-room technology using a combination of pressurized high-efficiency particulate air (HEPA)-filtered air, hydrogen peroxide mist, superheated air, and/or UV lamps to sterilize the product containers and filling environment; superheated air activates hydrogen peroxide for increased microbial lethality.

There are two additional methods for aseptic packaging and filling: bottle-shaped plastic containers and bag-in-box systems. Bottle-shaped plastic containers can be formed and sterilized by blow-molding and are filled and capped in an aseptic laminar-flow cabinet. Bag-in-box systems are used for larger (>4 l) container applications; milk is filled into laminated plastic bags, which have been sealed and then presterilized with irradiation. The filled bags are held within wood, plastic, or cardboard outer cases or within steel drums.

Non-thermal Technologies

Several alternatives to thermal treatment have been proposed for the production of ESL milk without compromising the sensory attributes of the product due to heat treatment. Methods such as bactofugation and microfiltration, the use of pulsed electric fields, plasma, or UV lights, or a combination of these with mild thermal treatments have also been evaluated as potential technologies to extend the shelf life of milk. The major challenges for the application of these novel technologies in the elaboration of ESL are the energy costs, the attainment of production rates similar to those of conventional systems, and the validation of the effectiveness in destroying specific pathogenic and spoilage microorganisms and enzymes in milk by recognized authorities.

Microfiltration

The microfiltration process consists of the removal of bacterial cells and spores from the milk mechanically using membrane processing where milk constituents are separated based on particle size. A main limitation for the application of this technology to ESL processing is that the particle size distribution of bacterial cells and spores is similar to that of milk fat globules, thus limiting the application of this method to non-fat milk. Also, the overlap of the particle size distribution of cells and spores with that of casein micelles requires a compromise in the pore size used. Microfiltration is carried out with a ceramic membrane; membranes with average pore diameters of 0.8–1.4 μm are commonly used commercially. To prevent membrane fouling and preserve a high and constant flux, special circulation systems capable of achieving a spore reduction of about three logarithmic cycles have been proposed. Multi-layered membranes with the same average pore size but a narrower size distribution have enabled a spore reduction of 4–5 log10 steps. The initial content of spores in the milk has a significant influence on the content of spores in the microfiltered milk. Microfiltered milk is marketed in several countries as more 'pure' and 'natural' than standard heat-treated milk and may have a higher price as a branded product.

Pulsed Electric Fields

The use of pulsed electric fields (PEFs), a technology consisting of a treatment of foods with very short electric pulses at high electric field intensities and moderate temperatures, has also been presented as a viable alternative for the production of ESL milk without altering its sensory and nutritional attributes. Milk is reported to be the first electrically pasteurized food product. Systems to process milk electrically involve voltages of 3000–4000 V. In the 1990s, several studies were performed in order to develop different types of equipment for the application of high-intensity PEF for the pasteurization of milk. Some research efforts have demonstrated that PEF technology by itself is able to extend the shelf life of fluid milk stored at refrigeration temperatures for up to 2 weeks without causing changes to the physical or chemical properties of milk or to its sensory attributes. The application of PEF in combination with a mild thermal treatment has been shown to be a more effective preservation strategy, capable of extending the shelf life of fluid milk for up to 4 weeks without compromising its quality. However, the commercial application of PEF technology is yet to be implemented mainly due to lack of regulatory approval, high initial investment, and elevated processing costs.

See also: **Heat Treatment of Milk**: Ultra-High Temperature Treatment (UHT): Aseptic Packaging. **Liquid Milk Products**: Liquid Milk Products: Flavored Milks; Liquid Milk Products: Membrane-Processed Liquid Milk; Liquid Milk Products: Modified Milks; Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: UHT Sterilized Milks; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects; Recombined and Reconstituted Products.

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Liquid Milk Products: UHT Sterilized Milks

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Introduction

Quality attributes, acceptability and shelf-life of sterilized milk products are affected and determined by the combined influence of milk composition, storage history and quality, by the sterilization process configuration and conditions and by the conditions under which the final product is stored. Although the prime target of the sterilization process is the microbiological population in the processed milk, it also significantly affects the physical and chemical stability, flavor, color and nutritional value of the product. The overall quality of sterilized milk products is determined not only by the effect of heat treatment during the manufacturing process but rather continues to be significantly influenced by a broad array of physicochemical phenomena and some enzymatic reactions that occur during storage. The mode and extent to which the influence of process and storage variables is manifested in a given product are affected by composition, properties and quality of the milk, prior to processing.

Factors affecting the Quality of Sterilized Milk Products

High temperatures used in the manufacture of sterilized milk products induce a broad array of physical and chemical changes (**Table 1**). These affect some of the principal quality attributes of milk, such as flavor, color, odor and nutritional value and thus the keeping quality, commercial value and acceptability of the product. A properly designed sterilization process has to be based on introducing time–temperature conditions that are needed to attain the desired extent of commercial sterility, from the microbiological and enzymatic points of view, while minimizing the adverse effects on all other quality attributes. Retort-sterilized milk or concentrated milk products are shelf-stable for a long period of time and exhibit different levels of browning, development of cooked and/or caramelized flavor and loss of nutritional value. These products are not considered desirable as beverages and are more suitable as ingredients in various formulations or as coffee milk. Sterilized milks prepared using the direct UHT method are shelf-stable for up to 12 and 6 months at a storage temperature of 4 and 20 °C, respectively, and that prepared using the indirect process configuration is shelf-stable for more than 12 months at 4 °C and up to 1 year at 20 °C. UHT processes achieve a sporicidal effect that is at least equal to that obtained with retort sterilization while maintaining the extent of adverse effects on the color, flavor and nutritional value significantly lower than that obtained with the retort processes. The keeping quality of UHT milk is determined by the manifestation of phenomena affecting the physical stability of the product during storage. Among these are, potentially, protein sedimentation, fat separation and gelation. Flavor and color changes that can, potentially, occur during processing and storage of UHT products may significantly impact the sensorial characteristics of the product; however, the extent to which these events occur can be controlled through adjustment of process configuration and conditions. Fat separation can be controlled by proper homogenization and heat treatment conditions and the manifestation of sedimentation can be minimized by properly adjusting the sequence of unit operations. It has been established that with UHT processed milk, physical phenomena, especially gelation during storage, are the most detrimental to the keeping quality.

Physicochemical Changes

All major milk constituents (proteins, lipids, minerals and carbohydrates) are significantly affected during the manufacture and storage of sterilized milk and concentrated milk (**Table 1**). Effects of heat treatment continue to influence the properties and thus the quality of the product pending consumption. Physicochemical changes that occur during heat treatment trigger complex cascades of different reactions which manifest themselves during storage and collectively determine the shelf-life and quality of the product. A detailed discussion on the effect of heat treatment is beyond the scope of this article and only some aspects are discussed (*see* **Heat Treatment of Milk**: Heat Stability of Milk; Thermization of Milk; Ultra-High Temperature Treatment (UHT): Heating Systems. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Plant and Equipment**: Pasteurizers, Design and Operation). Heat treatment during the sterilization of milk or concentrated milk leads to

Table 1 Some chemical, enzymatic and physical phenomena that occur during the manufacture and storage of sterilized milk products

Component or property	*Phenomena*
Proteins	Unfolding, denaturation (50–85%), formation of complexes with κ-casein at the surface of the casein micelles; increase in size of casein micelles, some disintegration of the micelles, increase in the proportion of non-sedimentable casein, proteolysis during storage, increase in the proportion of non-protein and non-casein nitrogen, polymerization during storage; formation of lactulosyl lysine and fructosyl lysine during storage
Minerals	Decrease in the proportion of ionic Ca and Mg due to precipitation as phosphates during processing; partial reversal of the latter during storage
Lactose	Maillard reaction, isomerization to lactulose
Rennet coagulation time	Increases with both UHT and retort sterilization, decreases during storage of UHT milk
Sensitivity to alcohol	UHT milk: increases during storage Retort-sterilized milk: unchanged during storage
Sensitivity to calcium	UHT milk: significant increase during storage Retort-sterilized milk: some increase during storage
Lipids	Lipolysis by heat-resistant or reactivated lipases during storage

denaturation of whey proteins and interactions between the latter and caseins. Denaturation of β-lactoglobulin depends on the severity of the heat treatment and ranges between 50% to more than 85%. The sterilization process leads to irreversible formation of disulfide bond-mediated complexes between β-lactoglobulin and κ-casein at the surface of the casein micelles (**Figure 1**) that change the zeta potential and aggregation properties of the casein micelle system. Sterilization of milk or concentrated milk results in various extents of temperature- and pH-dependent dissociation of caseins from the micelle system and disintegration of micelles. UHT processes result in some rearrangements of casein components in the casein micelle through both aggregation and disintegration reactions (**Table 1**). Side chain groups of amino acids and possibly terminal groups of proteins undergo different reactions at high temperatures that reduce protein solubility. The sterilization process can also lead to the liberation of different active compounds such as HS$^-$, NH$_4$, HPO$_4^{2-}$, etc. which may influence the sensorial and physicochemical properties of the product.

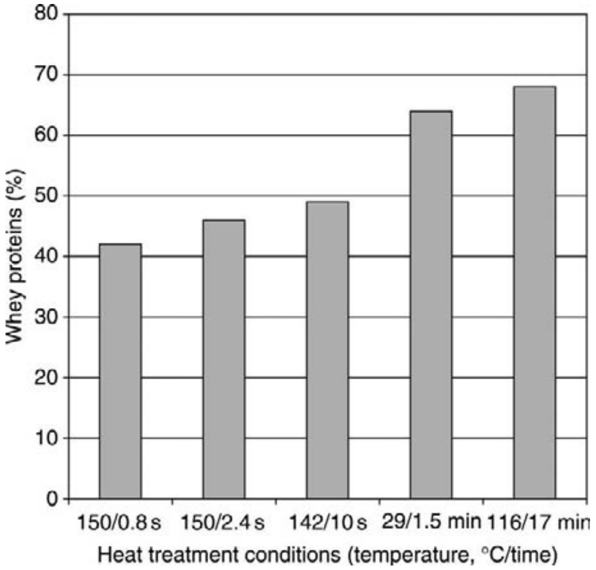

Figure 1 Effect of heat treatment conditions on the proportion of whey proteins that become associated with the casein micelles. (After Renner and Schmidt, 1981.)

Lactose

Lactose undergoes heat-induced reactions during milk sterilization which have a significant effect on the physico-chemical and sensorial characteristics of the product. Lactose interacts with amino residues of milk proteins, mainly with lysine, through the Maillard reaction and also undergoes isomerization that parallels the Maillard reaction or can occur at a slow rate in the absence of amino groups. Isomerization results in the accumulation of 300–1000 mg l^{-1} lactulose and minute quantities of epilactose in sterilized milks. Isomerization reactions that occur during sterilization are reversible and may lead to additional directions. Changes in mineral balance and distribution occur during both sterilization and storage and affect the aggregation and stability properties of the casein system in the product (**Table 1**).

Sediment Formation

Formation of sediment, although typical to UHT milk, does not represent a significant quality problem. Sediment formation has been suggested to represent fouling material generated during the process. When it appears, the amount of sediment is small and proportionally related to the severity of the heat treatment as well as to the proportion of ionic calcium in milk, and is inversely related to pH, milk quality and homogenization pressure. Indirect-heat UHT processing results in less sediment formation than the direct-heat process.

Age Gelation of Sterilized Milk Products

Age gelation, an irreversible phenomenon that occurs during the storage of sterilized milk and which ultimately transforms the product into a gel, is considered the most important single quality problem associated with this type of product, and, when fully manifested, signals the end of the product's shelf-life. Age gelation in retort-sterilized milk is not a common problem. The viscosity of UHT milk or concentrated milk undergoes significant changes during storage. Typically, some thinning is evident during the early stages of storage and is followed by a long period during which no significant changes in consistency are observed. Then, a steep increase in viscosity occurs and indicates the onset of irreversible gelation that occurs within 1–3 weeks. Syneresis is not evident on gelation, but it may occur during post-gelation storage. Gelation of UHT milk is less critical than that in UHT concentrated milk. Although investigated by many, the physico-chemical and mechanistic aspects of age gelation have not been fully understood and elucidated yet.

Age gelation of UHT sterilized milk and concentrated milk is affected by the combined influence of a multitude of variables related to milk composition and quality as well as to process and storage conditions. Although similar factors affect the gelation of concentrated and unconcentrated UHT milk, the extent of the specific influence varies. The severity of the heat treatment, both prior to and during the sterilization process, critically affects age gelation in UHT milk products. The resistance to gelation of a given product, reflected by the duration of 'gelation-free' time during storage, is proportionally related to the severity of the sterilization conditions, due to the influence of heat-induced changes in the state and distribution of the proteins. The formation, mainly through disulfide bonds, of complexes between β-lactoglobulin and κ-casein at the surface of the casein micelles (**Figure 1**) renders the micelles more resistant to gelation during storage. The protection against gelation is proportionally related to the extent to which whey proteins are attached to the surface of the casein micelles. The role of the reported increase in casein micelle size during UHT processes in affecting the propensity to gelation is unclear. Although the changes in calcium distribution during sterilization and storage (**Table 1**) are likely to affect the stability of the protein system in milk, the specific influence on the gelation of sterilized milk is unclear.

Storage Conditions

Both process conditions and additives that may be added to the processed milk affect the age gelation of sterilized milk (**Tables 2** and **3**; **Figure 2**, **Figure 3 and Figure 4**). Sterilized milk produced by the direct-heat UHT process is more prone to gelation than that prepared using the indirect method, probably due to the better control over the severity of heat treatment given in the latter. Homogenization and the sequence of unit operations also affect the gelation of UHT milk products. The rate of gelation of sterilized (HTST or UHT) concentrated milk during storage is proportionally related to the total solids content of the product, in particular to the proportion of non-fat constituents (**Figure 3**). The effect of heat treatment in retarding gelation during storage is influenced by the total solids content of the product. Under given sterilization conditions, the gelation-free storage time of concentrated milk is inversely related to total solids while at a given total solids, the time to gelation is inversely related to the severity of the heat treatment. In the manufacture of concentrated UHT milk, preheating the concentrate at >110 °C for several minutes enhances the stability of the product; cold storage of concentrated milk prior to sterilization accelerates age gelation, probably due to effects of oxidation/reduction reactions that modify the stability of the protein system. Seasonality, breed, stage in lactation cycle and health of the animal affect gelation properties of sterilized milk products, through their influence on milk composition and physico-chemical properties. The gelation-free storage time of sterilized milk is inversely related to the microbiological load in the milk, especially the presence of

Table 2 Effects of some processing variables on the age gelation of sterilized milk products

Variable	*Effect*
Sterilization temperature	Gelation-free shelf-life increases with sterilization temperature
Heating time	Gelation-free shelf-life increases with sterilization time
Preheating	Delays gelation
Process configuration	Indirect heating provides better resistance to age gelation than direct heating
Forewarming	Delays gelation of in-container sterilized concentrated milk
Sequence of unit operations	Homogenization prior to concentration and heat treatment reduces the resistance to gelation of sterilized concentrated milk; concentration prior to sterilization enhances resistance to gelation
Holding the sterile concentrate at 94 °C followed by homogenization	Retards gelation if carried out at optimal viscosity; homogenization at lower or higher viscosities leads to gelation or sedimentation, respectively

Table 3 Effects of some additives on the age gelation of sterilized milk products

Additive	*Effect*
Sodium phosphate, sodium citrate	Enhances gelation of UHT milk and concentrated milk; improves heat stability during retort sterilization of concentrated milk
Polyphosphates	Delay gelation of UHT milk and concentrated milk Efficacy increases with chain length and concentration of the polyphosphate
Orthophosphates	Enhance age gelation
Cyclic condensed phosphates	Delay the gelation of UHT milk and concentrated milk More effective than polyphosphates
Mixture of monophosphates and polyphosphate	Enhances gelation
Polyhydric compounds (lactose, sucrose, sorbitol)	Delay the gelation of sterilized concentrate
Sulfhydryl blocking agents	Delay the gelation of sterilized concentrate
Disulfide reducing agents	Promote the gelation of sterilized concentrate
Hydrogen peroxide	Enhances the gelation of UHT sterilized skim milk concentrate

microorganisms that produce heat-stable proteases, such as those originating from psychrotrophs.

Different additives to the milk have been shown to be effective in influencing the gelation properties of sterilized milk products (**Table 3**, **Figure 2**) through their influence on the protein and mineral constituents of the product. Storage temperature greatly affects the gelation-free time of sterilized milk products (**Figure 4**); however, conflicting information about the relationship between temperature and gelation of sterilized milk exists and the reported Q_{10} varies significantly (>1–4). There is an inverse relationship between storage temperature and the gelation-free storage time of sterilized concentrated milk. For both concentrated and unconcentrated products, the significant differences between reported data, relating storage temperature and gelation, suggest that this relationship is critically dependent on the history of the product. The difficulty in drawing clear and consistent correlations depicting relationships between storage temperature and age gelation may be attributed to the fact that the actual gelation reflects the manifestation of the potential for gelation that has been introduced prior to storage. This potential is, in turn, the overall result of a multitude of physico-chemical phenomena that are known to be dependent on milk and process conditions. It is thus clear that products with inherent different gelation potentials would gel to varying extent at a given storage temperature.

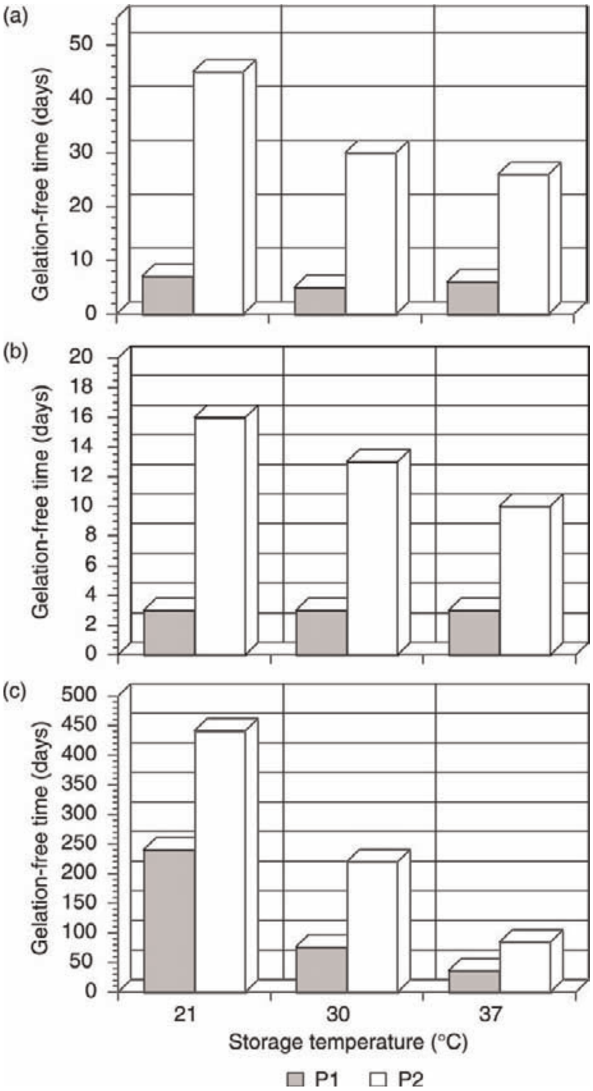

Figure 2 Some effects of additives and process configuration on gelation-free shelf-life of sterilized milk stored at different temperatures. P1, process consisting of forewarming, homogenization, additives, sterilization, and concentration. P2, process consisting of forewarming, concentration, additives, sterilization and homogenization. Additives: (A) none, (B) monophosphates and (C) polyphosphates. In all cases: total solids, 36%; sterilization, 138 °C for 15 s. (After Harwalkar, 1992.)

Role of Proteins and Minerals

Changes in state, distribution and stability of both protein and mineral constituents of sterilized milk during storage (**Table 1**) significantly affect the manifestation of gelation. Changes that affect the stability of the colloidal dispersion of caseins and minerals have been correlated with gelation during storage. Changes with storage time and temperature that influence the charge, size, mineral content and

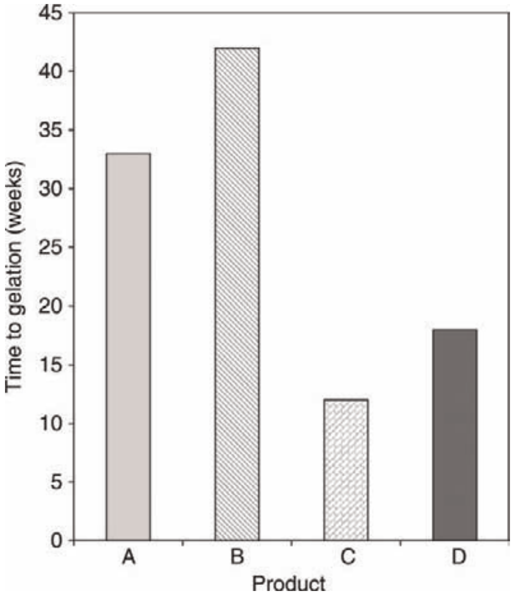

Figure 3 Effect of composition and sterilization time on gelation-free time during storage at ambient temperature of sterilized concentrated milk. Total solids: 26% (A, B) and 36% (C, D). Sterilization time at 132 °C: 35 s (A, C) and 70 s (B, D). (After Harwalkar, 1992.)

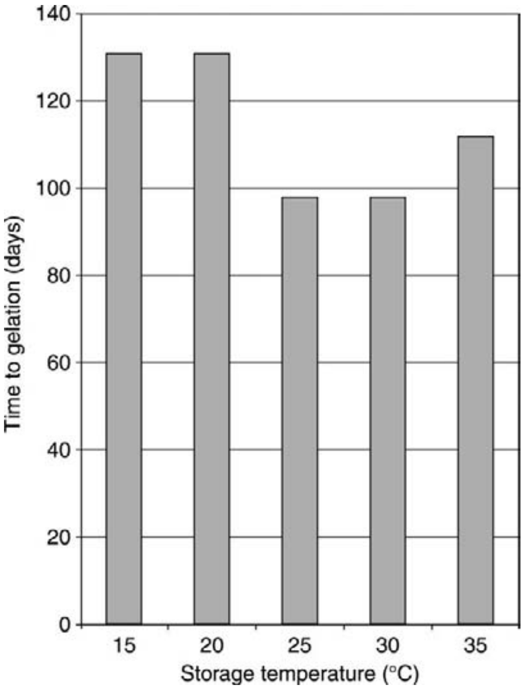

Figure 4 Influence of storage temperature on gelation-free time of UHT milk sterilized at 140 °C for 3 s. (After Harwalkar, 1992.)

composition of the casein micelles affect the association properties of the micelles. The degree of association between casein micelles increases during storage and parallels the changes in product viscosity.

Proteolysis, due to residual activity or reactivation of microbial and milk-derived proteases evident during storage of sterilized milk, is influenced by both milk quality and process variables and has been correlated, in many cases, with age gelation of UHT milk products. Gelation-free time is inversely related to the level of heat-resistant proteases, especially those derived from psychrotrophic bacteria, in the original milk. At a given milk quality, UHT milk produced by the 'direct' method exhibits a higher degree of proteolysis during storage than that prepared by either 'indirect' UHT or in-container sterilization processes. Proteolysis in UHT concentrated milk during storage is slower than in UHT milk, but the former gels sooner than the latter. Although the proteolysis-derived increase in the non-protein nitrogen content of UHT milk products has been correlated with gelation time, a critical level of proteolysis needed for the initiation of gelation has not been established.

Mechanistic Aspects

The mechanistic aspects of gelation during storage have not been elucidated fully and understood yet. It has been established that age gelation represents the ultimate result of sequential events that collectively destabilize the colloidal dispersion of the casein micelles in milk by modifying the surface of the micelles and allowing the micelles to interact with each other to form a gel consisting of a three-dimensional network of aggregated modified casein micelles. Attempts to model the events leading to gelation have been based on either assuming a significant role for proteolysis or physico-chemical phenomena.

In light of the evident proteolysis during the storage of sterilized milk products and the observed profile of changes in sensitivity to calcium as well as the observed changes in viscosity, a mechanism consisting of an enzymatic triggering stage followed by a non-enzymatic aggregation phase has been suggested to govern age gelation. This assumption is supported by the short gelation-free shelf-life of UHT milk prepared from poor-quality milk or from milk that has been subjected to a long cold storage prior to processing. The activity of heat-stable proteases has been suggested, among other things, to trigger age gelation. However, this hypothesis failed to explain age gelation in cases where proteolytic activity was not evident, cases where proteolysis was deliberately inhibited and cases where age gelation was not evident in products that exhibited proteolytic activities. It has been postulated that although proteolysis is involved in age gelation, non-enzymatic mechanisms play a major role in governing the phenomenon. It has been hypothesized that changes in the surface properties of casein micelles, due to non-enzymatic physico-chemical phenomena, especially those affecting interactions between caseins and whey proteins, govern age gelation. The exact nature of these mechanisms is not fully understood yet;

however, this hypothesis is supported by the aforementioned effects of sterilization on structure, composition and properties of the casein micelles and by the evident relationships between the process-dependent extent of these modifications and the manifestation of age gelation. Modifications of the casein micelles by Maillard reactions, disulfide bonding or physical disintegration of the micelles have been also implicated with age gelation. However, consistent information to substantiate these assumptions has not been developed yet.

Color and Flavor of Sterilized Milk

Temperatures used for milk sterilization introduce a whitening effect which has been attributed to the time- and temperature-dependent effect on mineral distribution and especially on denaturation of whey proteins, especially β-lactoglobulin and the formation of complexes between the latter and κ-casein at the surface of the casein micelles. Such interactions modify, through their effect on the size of the casein micelles, the light reflectance properties of milk and hence make sterilized milk whiter. At a pH lower than 6.55, there is a decrease in whiteness due to sedimentation. The color of sterilized milk, especially in the case of in-container-sterilized milk, is affected by the accumulation of dark-colored compounds, melanoidins, as a result of non-enzymatic browning via the Maillard reaction. The extent of browning is dependent on pH and time–temperature conditions during both sterilization and storage. Over the temperature range 25–150 °C, the browning reaction has an activation energy of 107 kJ mol^{-1}. In the temperature ranges 95–120 °C and 100–150 °C, the overall browning has a Q_{10} of 2.41 and a z value of 26.2 °C and Q_{10} of 2.26 and a z value of 28.2 °C, respectively. Browning reactions occurs in the aqueous phase of milk; however, the overall effect on a product's color is influenced by the fat content of the product. At given time–temperature conditions, the extent of perceived browning is inversely related to the fat content. In homogenized, sterilized milk, the ultimate color is also affected by the whitening effect that is dependent on homogenization conditions, through its influence on particle size distribution of milk lipids.

Storage Effects

Flavor and odor profiles of UHT and in-container-sterilized milk products change during storage (**Table 4**) and represent complex and dynamic systems that are affected by the

Table 4 Stages in development of flavor characteristics of sterilized milk and some of the suggested constituent flavor compounds

Stage	*Characteristics*	
Primary, phase 1	Heating flavor along with sulfhydryl odor	
Primary, phase 2	Weaker sulfhydryl odor and cooked flavor	
Primary, phase 3	Acceptable flavor; residual cooked flavor note	
Secondary, phase 1	Acceptable flavor and odor	
Secondary, phase 2	Acceptable, notes of oxidized flavor and odor	
Secondary, phase 3	Development of oxidized and rancid flavor and odor; possible bitterness	
Type of flavor	*Flavor compounds: diacetyl, lactones, alcohol ketones, maltol, vanillin, benzaldehyde, acetophenone*	
	Compound	*Concentration (mg kg^{-1})*
Sterilized flavor	2-Methyl-1-propanethiol	0.008
	2-Heptanone	0.4
	2-Nonanone	0.21
	Benzothiazole	0.005
	γ-Octalactone	0.025
Suggested UHT flavor	2-Undecanone	0.18
	δ-Decalactone	0.65
	Hydrogen sulfide	0.03
	Diacetyl	0.005
	Dimethyl disulfide	0.002
	γ-Dodecalactone	0.025
	δ-Dodecalactone	0.1
	Methanethiol	0.002
	2-Heptanone	0.29
	Methyl isothiocyanate	0.01
	Ethyl isothiocyanate	0.01
	Maltol	10.0

composition and quality of the processed milk, the severity and configuration of the heat treatment and storage conditions. At a temperature above 70 °C, a ‘heated flavor’ is developed and is associated with the formation of free –SH groups due to denaturation of the β-lactoglobulin. The free –SH groups can be oxidized to the volatile hydrogen sulfide which is responsible for the characteristic odor of freshly heated milk. The level of free –SH groups decreases with time, due to oxidation during the early stages of storage of UHT-sterilized milk. This change alters the flavor and odour of the product and results in the formation of a new flavor, called ‘degenerate heated flavor’. At temperatures above 90 °C, the level of free –SH groups decreases and a different flavor, ‘sterilized flavor’, characteristic of sterilized milk, appears, probably due to the Maillard reaction. The intensity of the ‘sterilized flavor’ increases with storage time, even at room temperature. The contribution of free –SH to the flavor characteristics of freshly processed UHT milk may be affected by fat content. The proportions of ascorbic acid in milk, the redox potential and the level of O_2 also affect the accumulation and disappearance of free –SH groups and thus the flavor of UHT milk. The rate of disappearance is proportionally related to the level of O_2 and inversely related to the storage temperature. A wide variety of compounds, originating from the effect of high temperature on milk proteins and lipids, have been identified and some have been suggested to be associated with the sterilized flavor (**Table 4**). The flavor and odor of UHT sterilized milk change with storage time in a way that reflects the combined effects of process history, level of oxygen, exposure to light and of storage conditions. Fresh UHT milk is characterized by a poor flavor, described as a noticeable ‘heated’ flavor, and by a sulfurous odor note. The initial sensorial characteristics of UHT milk disappear within a few days of storage at a proper temperature and a characteristic UHT milk flavor develops with storage time. Acceptable flavor and odor profiles can be accomplished by wisely adjusting process and storage conditions and by controlling milk quality. Efforts to identify and quantify compounds responsible for the typical flavor of UHT milk have been made and a ‘synthetic UHT flavor’ has been composed (**Table 4**). The significant differences between the sterilized flavor and the ‘mature’ typical flavor of UHT milk have suggested that some of the flavor compounds associated with the latter serve as precursors of the former. Intensity of flavor notes typical of UHT and in-container sterilized milk have been shown to correlate well with the accumulation of lactulose. For a given severity of heat treatment, the flavor acceptability of UHT milk prepared by the direct-heat method is comparable to that manufactured by the indirect-heat process. However, flavor differences can be anticipated in cases where process-associated differences in severity of heat treatment exist. Flavor acceptability of sterilized milk is critically dependent on level of free –SH groups, originating from the effect of heat treatment on whey proteins, and are responsible for the strong hydrogen sulfide odor of fresh sterilized milk. The level of these compounds declines rapidly, due to oxidation, with storage time at a rate that is proportionally related to the level of oxygen in the product. The rate and extent of the Maillard reaction that occurs during storage contributes to the decline of flavor quality of UHT milk.

Proteolysis and Lipolysis

Proteolysis and lipolysis during the storage of sterilized milk, due to the activity of residual or reactivated milk and bacterial proteases and lipases, can introduce different off-flavors and odors. Bitterness has been related to the effect of proteolysis while lipolysis-related flavor and odor deterioration is dependent on the relative amounts and type of free short-chain fatty acids that are liberated. With relatively high levels of oxygen and upon exposure to light, especially in the absence of significant amounts of the reduced form of ascorbic acid, the oxidation of milk lipids occurs and leads to the development of strong rancid (‘painty’) flavor and odor notes.

Nutritional Value

Severe heat treatment can, potentially, lead to the destruction of important nutrients such as vitamins. Nutritional loss in sterilized milk products is governed by the severity of the heat treatment, process configuration and storage conditions. Additionally, the loss of nutrients such as vitamins is also affected by exposure to light, the level of oxygen in the product and by interactions between vitamins and other constituents, as well as by interactions between vitamins. In general, although different process configuration-dependent data have been reported, vitamin loss in UHT processed products is relatively small and is significantly smaller than that observed with in-container sterilized product (**Table 5**, **Figure 5**). Oil-soluble vitamins (A, D and E) as well as some water-soluble vitamins (riboflavin, nicotinic acid, biotin) are heat-stable and are not adversely affected by either in-container or UHT processes. Vitamins such as folic acid, vitamin B_{12} and ascorbic acid are lost, to different extents. With some vitamins, such as vitamin C, the detectable loss in sterilized milk products represents not only the effect of heat treatment but also the influence of chemical reactions, such as oxidation. The detectable loss of ascorbic acid depends on the extent to which the natural, reduced form of the vitamin has been oxidized by dissolved oxygen prior to heat treatment. The oxidized form is heat labile and is likely to be destroyed completely

Table 5 Loss of some vitamins during storage of UHT milk under different conditions

Storage conditions	*Vitamin loss*
Airtight container, 3–6 months storage at 15–25 °C in the dark	Thiamin: 0–10% Riboflavin: 0–10% Nicotinic acid: 0–20% Vitamin B_6: 35–50% Vitamin B_{12}: 15–40%
Airtight container in the dark, 8 mg l^{-1} O_2,[a] 15–20 °C	Vitamin C: >90% after 7 days Folic acid: 100% after 30 days
Airtight container in the dark, 1 mg l^{-1} O_2, 15–20 °C	Vitamin C: 13% and 15% after 30 and 60 days, respectively Folic acid: <10% after 60 days
Airtight container in the dark, 8 mg l^{-1} O_2, 23 °C	Vitamin B_{12}: 20% after 9 weeks
Airtight container in the dark, 1 mg l^{-1} O_2, 23 °C	Vitamin B_{12}: 10% after 9 weeks

[a] Initial oxygen level in the product.
Data from Ford and Thompson (1981) and Burton (1988)

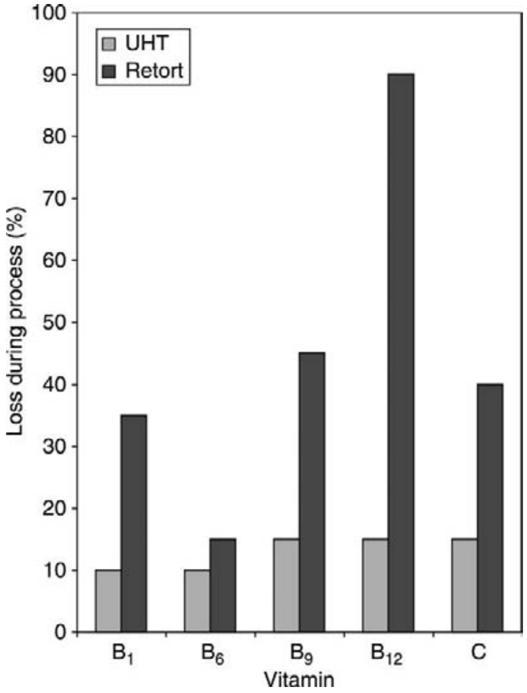

Figure 5 Representative values for vitamin loss during milk sterilization by UHT or retort processes. (After Schaafsma, 1989.)

during UHT processing while loss of only 10–20% of the reduced form can be anticipated. The loss of vitamin B_{12} is linked to the oxidative destruction of vitamin C. The loss of folic acid is limited by protection provided by the reduced form of vitamin C and thus oxidative destruction of the latter will affect the loss of the former.

Oxygen Levels

The nutritional value of sterilized milk can deteriorate during storage to an extent that is highly dependent on the oxygen level in the product, temperature and exposure to light. When stored in the dark, the oil-soluble vitamins in UHT milk are stable but on exposure to light, a rapid temperature- and time-dependent loss can be expected. The loss of water-soluble vitamins during storage of UHT milk varies among the vitamins (**Table 5**) and a significant loss can be expected on exposure to light. The most significant loss of vitamins during storage has been reported for ascorbic and folic acids. The loss of the reduced form of vitamin C (that survived the heat treatment) during storage is highly dependent on the level of oxygen in the product. The latter, in turn, is significantly influenced by the UHT process configuration and gas-barrier properties of the packaging material. The level of oxygen in freshly prepared UHT sterilized milk is about 8–9 mg l^{-1} and about 1 mg l^{-1} for products prepared by the indirect and direct process configuration, respectively. The stability of vitamin C in the stored product is inversely related to the level of oxygen. At an oxygen level of 1 mg l^{-1}, loss of ascorbic and folic acids during storage of UHT milk packaged in an oxygen-impermeable container in the dark occurs to only a very limited extent while at a higher oxygen level, a very significant loss occurs. The oxygen level also affects the loss of vitamin B_{12}. Although exposure to light during storage accelerates nutritional deterioration, oxygen levels have a very detrimental effect. In light of the above, a high nutritional value of UHT sterilized milks can be attained by selecting process configuration that allow reaching a low (1 mg l^{-1}) level of oxygen, selecting packaging materials with effective oxygen barrier properties and preventing exposure to light by using opaque containers. In high-quality UHT milk products, the decrease in available lysine due to the Maillard reaction is small and does not represent a significant loss in nutritional value. Similarly, oxidation- and lipolysis-related changes in milk lipids are small and do not present difficulties.

Conclusions

The properties, acceptability and thus the commercial value of sterilized milk products are affected by the combined influence of a multitude of aspects extending from the time of milking (and probably earlier) to consumption. Technologies for the manufacture of high-quality sterilized milk products exist, but a wise selection and storage of milk prior to processing and a proper adjustment of process and storage conditions are required in order to maintain high product quality. Although some of the detailed information and basic understanding pertaining to physical, chemical and enzymatic events that affect quality attributes has not been fully developed yet, existing practical knowledge suggests that implementation of some basic approaches can minimize the rate and extent of quality deterioration in sterilized milk products. Among these are:

- utilization of high-quality milk
- minimizing cold storage prior to processing
- application of thermization or microfiltration in cases where cold storage prior to processing is needed
- avoidance of milk containing a high level of plasmin (e.g. from cows recovering from mastitis)
- adjusting heat treatment conditions to provide the highest severity possible while preventing adverse effect on sensorial properties
- use of legally permitted additives that are known to delay or limit age gelation
- when possible, storing sterilized milk at a temperature not higher than 20 °C
- selecting a process configuration that gives a low level of O_2 in the product
- use of packaging materials with effective oxygen-barrier properties
- preventing exposure of product to light during storage and delivery.

See also: **Heat Treatment of Milk**: Heat Stability of Milk; Thermization of Milk; Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Plant and Equipment**: Pasteurizers, Design and Operation.

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Liquid Milk Products: Modified Milks

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Introduction

Modified milks refer to milk-based beverages in which fluid milk is altered to deliver a more comprehensive nutritional composition and, in some cases, to enhance digestion. Modified milks include vitamin- or protein-enriched milk, prebiotic-fortified milk, nutritionally balanced milk, lactose-reduced or lactose-free milk, and flavored milk. Modified milks are increasing in popularity, with new products being introduced to the market on a regular basis. For example, milk that has been ultrafiltered to remove 20% of the water contains a higher concentration of natural calcium and natural protein than regular milk. Milk protein composition can also be modified by genetic engineering to increase or reduce the content of certain proteins, for example, the levels of α-lactalbumin and lactoferrin can be increased and β-lactoglobulin content reduced for formulation of infant formula. Milk plays an important role in human diet, especially in the diet of inhabitants of Western countries, providing food energy, fat, protein, calcium, phosphorus, vitamins A and D, and riboflavin. Milk is also a perfect vehicle for the formulation of fortified nutritional beverages. Using fluid milk as a base from which to create modified milk products provides additional ways to offer nutritious milk to consumers.

Vitamin-Enriched Milk

Vitamin-enriched milk is a product to which vitamins have been added during processing, and includes all the market milk products sold in the United States. Vitamins A and D are usually added to all fluid and powdered milk in the United States. The rationale behind vitamin enrichment of milk is based on the following issues:

- insufficient nutrient levels in the natural product;
- nutrients that are destroyed or removed during processing; and
- a need to supply adequate nutrient levels when milk serves as an important nutrient source for an individual or a population segment.

Vitamin enrichment of milk has a long history and continues to be a widespread practice. At present, the nutrient fortification of both liquid and dry milk with vitamins A and D is practiced as fat-soluble vitamins are removed with the fat or cream phase in the manufacture of skim or low-fat milk. Additionally, since non-fat dry milk provides very little indigenous vitamin E, it makes an excellent carrier for vitamin E fortification in developing countries.

Multivitamin- and mineral-fortified milk has been produced, with additions made to adjust the natural levels of these nutrients in milk to levels that are considered to be more in nutritional balance with human requirements. Mineral fortification of milk is limited today, although calcium is sometimes used. Unfortunately, it is difficult to prevent nutrient interactions and/or flavor changes in milk following the addition of some mineral additives.

Since World War II, virtually all homogenized fluid whole milk and evaporated milk in the United States have been fortified with vitamin D. The marked decline in rickets in the United States has been attributed, in part, to the fortification of milk with vitamin D. Another benefit of vitamin D supplementation is a lower rate of osteomalacia in the elderly, which is largely responsible for bone fractures. Recent studies showed that vitamin D might have other health benefits including improvement of the immune response. Cows' milk, as secreted, is deficient in vitamin D and does not meet the needs of humans, in particular children and infants. Cows' milk contains 47–105 IU (1.2–2.6 μg) of vitamin D per liter, and since the recommended daily allowance (RDA; daily reference intake (DRI)) is 400 IU (10 μg), milk is fortified with 400 IU of cholecalciferol per liter. Vitamin D fortification of milk began with the feeding of vitamin D-enriched yeast to the cow. This was followed by a UV irradiation process for milk, and currently a vitamin concentrate is added to milk. This allows for better control of vitamin D level with a concomitant decrease in flavor problems.

The current level of vitamin A fortification of fluid milk is 2000 IU (606 μg) l^{-1} and that of vitamin D fortification 400 IU (10 μg) l^{-1}. In the United States, fluid milk products are allowed a minimum of 100% and a maximum of 150% of required levels to be in compliance with good manufacturing practice. Over-fortification with vitamin A to more than 6000 IU (1818 μg) l^{-1}, and with vitamin D to more than 800 IU (20 μg) l^{-1}, is considered harmful. Many measures are taken to ensure the accuracy of these levels, for example, both vitamins can be included in the concentrate, so that only one addition to milk is required to produce the resultant vitamin A- and D-fortified milk. Vitamin levels in milks from different plants may vary depending on

- the type of vitamin concentrate used,
- the point of addition of the vitamins,
- uniformity of addition in continuous operations, and
- subsequent blending operations.

Vitamin A is stable during heat treatment of milk, but it is very sensitive to light, which leads to its potential loss during the storage of milk packaged in transparent containers. Additionally, vitamin A added to milk is even more susceptible to destruction by light than the naturally occurring form. The introduction of opaque containers has mitigated the development of off-flavors due to light exposure and protects light-sensitive vitamins.

In addition to vitamins A and D, dairy manufacturers sometimes fortify milk with other vitamins. For example, milk is fortified with vitamin C to provide up to 25% of the RDA of vitamin C in each 236-ml serving of fluid milk. Along with the loss of vitamin A, loss of riboflavin and vitamin C can occur in enriched milk upon prolonged exposure to light. Exposure to light can also cause a hay-like flavor in vitamin A-fortified milk under certain conditions. The use of opaque containers can retard the development of off-flavors in fluid milk. The technology involved in the production of vitamin-enriched milk includes the following steps:

- raw milk reception;
- clarification/removal of impurities;
- standardization;
- fortification with vitamins A and D;
- pasteurization;
- homogenization;
- filling/packaging; and
- distribution.

Protein-Enriched Milk

Protein-enriched milk is a beverage product to which additional milk proteins have been added, or milk that has had some of the water removed, leaving milk with a higher concentration of protein and some other naturally occurring nutrients.

One liter of milk supplies approximately half of an adult's estimated daily requirement of the essential amino acids. Approximately 23% of the total protein in the US diet is derived from dairy products. Dairy proteins are considered complete since they contain high levels of all the essential amino acids required by humans. The distribution of amino acids in milk protein closely resembles the pattern required by adult humans. Therefore, milk proteins are an excellent source of amino acids for human nutrition, and milk is also an excellent vehicle for the delivery of these nutrients. Several protein-enriched milks have been introduced onto the US market. These milks are calcium-fortified and iron-rich, and contain a higher protein level than traditional milk. This is accomplished through an ultrafiltration process that removes some of the water in milk, leaving milk with increased levels of natural calcium and protein. Immunoglobulin-enriched milk contains antibodies that are protective to humans. To produce immunoglobulin-enriched milk, cows are first immunized with specific antigens. They produce antibodies (G-type immunoglobulins) that are secreted into the milk and which are specific for certain antigens and are capable of neutralizing a broad spectrum of pathogens, including *Shigella*, *Salmonella*, and *Streptococcus*. This immunoglobulin-enriched milk can be consumed directly or it can be concentrated and dried into a powder for use as a functional ingredient in foods and beverages.

Prebiotic-Fortified Milk

Milk is one of the best natural foods available. It could be better balanced if fortified with prebiotics/functional fibers. Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's intestinal balance. Consumption of prebiotics may also increase the absorption of minerals, such as calcium and magnesium. All prebiotics to date are carbohydrates, ranging in size from small sugar alcohols and disaccharides, to oligosaccharides and large polysaccharides. The defining criteria for prebiotics are as follows: (1) A prebiotic should be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract. (2) It should be a selective substrate for one or more potentially beneficial bacteria in the large intestine. Colonization by an exogenous probiotic could be enhanced and extended by simultaneous administration of a prebiotic that the probiotic can utilize in the intestinal tract. As such, it should stimulate that bacterium to divide, become metabolically active, or both. (3) It should alter the colonic microenvironment toward a healthier composition. (4) It should induce luminal or systemic effects that are advantageous to the host. The most-studied nondigestible oligomers are galactooligomers, such as soy-derived raffinose and stachyose, and the fructooligomers or fructans. Two commonly used prebiotics are chicory inulin and oligofructose (FOS). Inulin is suitable for whole milk, low-fat milk, and skim milk fortification since its addition will not sweeten the milk. Inulin may improve the color of low-fat milk and skim milk. FOS may be used for flavored milk due to its sweetness. Based on the data from the author's laboratory, prebiotic milk preparation includes the following steps:

- raw milk reception;
- clarification/removal of impurities;
- standardization;
- fortification with inulin or FOS;
- pasteurization;
- homogenization;
- filling/packaging; and
- distribution.

Lactose-Reduced Milk

Lactose-reduced and lactose-free milk are those products in which some or all of the lactose has been hydrolyzed by β-galactosidase to its constituent monosaccharides, glucose and galactose. Lactose-reduced and lactose-free milk are appropriate for individuals who suffer from lactose intolerance or lactose malabsorption. Lactose is the most abundant sugar in milk, constituting approximately 5% of cow's milk (*see* **Lactose and Oligosaccharides**: Lactose: Production, Applications). In the small intestine, it is cleaved by the enzyme β-galactosidase (lactase) to its constituent monosaccharides, which are absorbed readily. Lactose facilitates the absorption of calcium and may enhance the absorption of other minerals (*see* **Lactose and Oligosaccharides**: Lactose: Derivatives).

Up to an estimated 70% of the world population, and 28% of Americans, have low levels of lactase in the intestine and therefore have a genetically controlled inability to digest lactose, which enters the large intestine and causes such symptoms as gas, cramps, and diarrhea upon milk consumption (*see* **Lactose and Oligosaccharides**: Lactose Intolerance). The problems caused by insufficient intestinal β-galactosidases can be overcome by prehydrolyzing the lactose using exogenous β-galactosidases (*see* **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase). The steps involved in the production of lactose-free and lactose-reduced milk for small to medium operations include

- raw milk reception,
- clarification,
- standardization,
- pasteurization,
- homogenization,
- enzymatic hydrolysis,
- heating and cooling,
- filling/packaging, and
- distribution.

Nutritionally Balanced Milk

Nutritionally balanced milk is a product to which additional vitamins, minerals, fat in the form of vegetable oils, proteins, and sometimes fiber have been added. Using a base of fluid milk, the various nutrients are added to give a product that is considered nutritionally balanced for human consumption. Nutritionally balanced milk is designed primarily as an oral nutritional supplement to be used in conjunction with meals or as a snack, or as a meal replacement in appropriate amounts. Many are lactose- and gluten-free and are suitable for use in modified diets, including low-cholesterol diets. Nutritionally balanced milks are also suitable for elderly patients at nutritional risk, patients with involuntary weight loss, individuals recovering from illness or surgery, and patients who require a low-residue diet.

The protein in nutritionally balanced milk is generally a blend of three high biological value proteins: casein, whey, and soy. The fat is a blend of high-oleic safflower, canola, and maize oils. Corn syrup, maltodextrin, and sucrose provide the carbohydrates. The technology for the production of sterilized, shelf-stable nutritionally balanced milks includes the following steps:

- raw milk reception;
- clarification;
- standardization;
- addition of proteins, oils, vitamins, minerals, and perhaps fiber;
- homogenization;
- filling/packaging;
- sterilization; and
- storage.

Milk Drinks

Milk drinks are ready-to-consume flavored beverages made from milk. They are created when specific flavoring ingredients are added to fluid milk in order to enhance the variety and popularity of milk as a beverage and to appeal to more consumers. Milk drinks are homogeneously mixed products containing different levels of fat and additives, including sugar, cocoa, fruit concentrates, coffee, and other food ingredients that contribute to flavor. The final product contains not more than 30% additives; the most popular flavors are chocolate, orange, strawberry, and banana.

Traditionally, chocolate milk, chocolate drink, and eggnog have been the most important from the standpoint of volume, although other flavored milk drinks have been marketed from time to time, with few becoming established as permanent products. Chocolate drinks with different fat content (0.5, 1.0, or 2.0%) are prepared, whereas chocolate milk is made with whole milk (3.5% fat). The principal problem faced when preparing fruit-flavored milks is the acidity of fruit juices (if used), which coagulates the caseins. Therefore, whey protein is a better milk ingredient, suitable for mixing and processing with fruit juices, as it is not coagulated by acid.

Milk drinks are considered 'dairy-based products'. They have been developed as a result of increasing consumer demands for variety in taste. Additionally, they can provide children with a wider array of milk-based beverages, which can be beneficial for school milk programs. The increasing demand for these products has resulted in a significant market share for milk-based beverages in the United States and other countries. Milk drinks are marketed as pasteurized, ultraheat-treated (UHT), or sterilized products. In addition to flavors, other ingredients, such as stabilizers, that can contribute to a homogeneous structure and a homogeneous distribution of additives, are added. Basic products can be enriched with milk proteins, and whey and ultrafiltrate (permeate) are added to produce refreshing beverages. The steps for processing a chocolate-flavored milk include the following:

- raw milk reception;
- clarification;
- standardization;
- homogenization;
- addition of 1:1:90 parts cocoa:sucrose:milk;
- heating, with stirring, to 65 °C;
- addition of sodium alginate (0.2%) and sucrose (0.2:8 ratio);
- heating at 65 °C for 30 min;
- rapid cooling;
- filling/bottling; and
- storage.

Simulated milk products are beverages that may be made with or without some of the components of milk. Thus, these 'imitation milks' resemble milk but one or more of the classes of milk components have been replaced by non-milk ingredients. Usually, skim milk or sodium caseinate is combined with vegetable fats. When sodium caseinate is used as the protein source, corn syrup or lactose may be used as carbohydrate. These types of products have been most successful in countries with a limited supply of dairy products and ample vegetable components to facilitate the production of beverages that can mitigate the occurrence of malnutrition.

One important characteristic of simulated milk products is that milk fat is generally not used, as it is expensive. Typically, vegetable fats are used, but there is no completely comparable substitute for milk fat; thus, simulated milk products tend to lack the natural flavors of milk, even though structurally they resemble milk. The only completely satisfactory protein for simulated products is milk protein, and sodium caseinate is the protein of choice due to its ready availability, reasonable price, and its pleasing, mild flavor.

Filled milks are milk-based products to which any fat or oil other than milk fat has been added to create a product that resembles milk or cream. Filled milk products resemble milk more closely in terms of nutritional value than nondairy products, and they are usually cheaper, because vegetable oils are less expensive than milk fat.

See also: **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase. **Lactose and Oligosaccharides**: Lactose: Derivatives; Lactose Intolerance; Lactose: Production, Applications.

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Liquid Milk Products: Flavored Milks

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Markets and Importance of Flavored Milks

Flavored milks cater to the desire of the consumers for variety and a different experience in flavor. Some consumers do not like the flavor of natural milk but appreciate the healthy nutritional value of milk in the form of flavored milk. It can also help encourage children to consume more milk, and sometimes flavored milk is used in school milk programs. Originally, flavored milks were prepared as milk shakes in restaurants, fast food shops, and households. Later they became established products manufactured by dairy companies and sold in family-size beverage cartons (Tetra Pak, SIG Combibloc, Pure Pak) or as single-service portions in beverage cartons or high-density polyethylene (HDPE) bottles on the supermarket shelves.

Separate statistical data are mostly not available for this group of dairy products. It is normally included in groups such as 'fresh dairy products', 'milk drinks and fermented products', or 'milk drinks, yogurt, and other fermented milks'. For these groups the consumption is growing nearly everywhere worldwide (**Table 1**). Exceptions are countries where the consumption level is already high, like Scandinavia. The total production of these product ranges has increased by 9 million tonnes to 20 million tonnes over the last decade, which averages to a growth of 6.2% annually. On the contrary, the group 'liquid milk' has stagnated or decreased slightly in consumption, except in countries like China where the consumption of liquid milk is increasing quite rapidly. The group 'milk drinks, yogurt, and other fermented products' is a product group with higher nutritional benefits than, for example, soft drinks. Often with a reduced fat content, the liquid products within this group are enriched with probiotic bacteria and other added nutritional value and/or fruit puree; have an increasing popularity among modern consumers; and increasingly take their place as a meal replacement.

According to market research, the consumption of flavored milk also shows growth rates above those for normal liquid milk. From 2000 to 2002, the growth rate in Europe was 4% and worldwide even 13%. The worldwide consumption volume in 2002 was 8277 million liters according to that study. Where specific statistical data for flavored milks are available (Switzerland and United Kingdom) quite a high increase can be observed. Some companies are successful in launching new products in this field with the support of marketing campaigns and building on the healthy nutritional value and image of milk, which attracts new groups of consumers. Milk-based coffee beverages in Europe, India, Australia, and other parts of the world are an example of this. In a newer type of flavored milk with fruit puree, the healthy nutritional value of milk and the added probiotic bacteria better known from fermented milks are combined with caffeine and sulfonic acid, taurine, often used in energy drinks.

Definition of Flavored Milks

Flavored milks are ready-to-drink products made from unfermented milk of different fat contents, mixed with ingredients like sugar or other sweetener, cocoa powder, fruit juice, coffee, aroma agents, and/or other ingredients and additives. Besides the most important flavors, chocolate and coffee, the other popular flavors are vanilla, strawberry, malt extract and chocolate, and banana. The added ingredients, often limited to 30% (e.g., in European Union, Switzerland). According to Codex Alimentarius, the milk constituent has to be an essential part in terms of quantity in the final product and the milk constituents not derived from milk have to be not intended to take the place, in part or in whole, of any milk constituent. Fat content is full fat (3.5%), semi-skimmed (1.5–1.8%), or skimmed (<0.5% fat). Fat content of the milk influences the creaminess and texture of the product. It also lightens the color, for example, of chocolate milk. As milk fat masks the cocoa flavor, chocolate milk based on skimmed milk tastes more chocolaty than its full-fat equivalent. Flavored milks are pasteurized, ultra-pasteurized, or ultra-high temperature-treated (UHT). Sometimes, conventional in-container sterilization is applied. Additional ingredients are thickeners such as carrageenan (European additive number E 407), pectin (E 440), sodium alginate (E 401), and carboxymethylcellulose (E 466), and stabilizing agents such as sodium phosphate (E 339) or diphosphate (E 450) and sodium

Table 1 Consumption of flavored and liquid milk in selected markets, per caput per year

	Unit	*2000*	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*
Flavored milk									
EU27[a]	kg	16.1	16.6	18.1	18.7	18.9	19.6	20.0	20.3
Denmark[b]				40.7	43.0	44.6	45.9	50.0	48.2
United Kingdom[c]	l	1.5	1.7	2.0	2.1	1.1	1.2	1.2	
Germany[b]	kg			27.0	28.5	27.9	29.8	29.8	30.8
Switzerland[d]	kg	3.5	3.5	3.7	4.7	6.2	8.0	8.7	8.6
Switzerland[b]				23.7	25.6	27.0	30.7	31.4	n.a.
Italy[b]					6.5	6.3	7.5	8.4	8.3
Liquid milk consumption including milk drinks, yogurt, and other fermented products									
EU25[e]	kg			92.1	92.6	92.8	93.2	93.1	93.5
EU15[e]	kg			97.3	98.0	97.2	96.7	97.1	98.0
EU27[f]	kg	68.7	69.6	69.2	69.3	69.0	68.9	68.6	68.6
Denmark[f]	kg			135.7	136.7	137.2	135.7	139.7	138.0
United Kingdom[e]	kg			111.4	111.6	108.9	103.9	104.8	105.1
Germany[e]	kg			91.0	93.9	91.9	92.7	95.0	94.7
Germany[f]	kg	63.4		64.0		62.0		64.6	
Switzerland[e]							80.7	81.1	79.0
Switzerland[f]	kg	70.6	69.6	68.8	67.2	67.0	65.5	65.7	64.4
Italy[e]				64.0	63.9	63.1	57.9	57.2	56.1

[a] Milk drinks, yogurt, and other fermented milk (International Dairy Federation (IDF), Eurostat)
[b] Milk drinks, yogurt, and other fermented milk (IDF)
[c] Milk drinks and other milks: includes flavored milk, buttermilk, goats milk, ready-to-drink milk shakes, milk smoothies (not fruit-based or yogurt-based) and non-yogurt probiotic drinks such as Yakult (DEFRA, UK)
[d] TSM-GmbH Switzerland
[e] IDF
[f] Without milk drinks, yogurt, and other fermented products
n.a.: data not available

hydroxide (E 524) for products subjected to a more intense heat treatment.

pH Stabilization and Ionic Strength of Flavored Milks

It is a big technological challenge to stabilize milk protein, particularly the casein micelles, outside its normal range of pH and ionic strength. The stabilizers sodium phosphate and biphosphate and also sodium hydroxide are additives used to stabilize and correct the pH during heat treatment and in the product flavored milk. During intense heat treatment (UHT, sterilization) the pH of dairy products without stabilizing additives decreases temporarily up to about 1 pH unit due to a reduction of the true solubility of calcium and hydrogen phosphate leading to the formation of colloidal calcium phosphate and the liberation of H^+. This drop in pH could destabilize the casein and, therefore, the flavored milk drinks could curdle. Prevention of this by the above-mentioned stabilizers is especially important for flavored milks with acidic ingredients such as coffee or fruit juices. In addition, they influence the ionic strength. Sodium phosphate and biphosphate also chelate minerals such as calcium and magnesium and allow adjusting the ionic strength to the requirement of the thickener.

Stabilization of Milk Protein in Acidic Flavored Milks

Flavored milks with a high percentage of fruit juice (≥15%) are stabilized with about 0.3% (w/w) high-methoxy pectin to avoid flocculation of milk proteins and subsequent macroscopic whey separation. Sometimes for sterilized milk drinks, heat-stable cellulose derivatives are recommended: 0.2% carboxymethylcellulose prevents protein coagulation in milk drinks containing orange juice or other fruit juices. Pectin is an important structural element of higher terrestrial plants. Its concentration is the highest in the middle lamella between the cell formations in fruits and vegetables. It belongs to the group of heteropolysaccharides. The main component of pectin is polygalacturonic acid, which is partly esterified with methanol. Pectin with a degree of esterification of more than 50% is called high-methoxy pectin. Pectin is produced from vegetable raw material with a high natural pectin content, such as apple pomace or citrus peel.

At the normal pH of milk, 6.7, κ-casein chains protrude from the surface of the casein micelles in order to maximize their entropy. This same tendency to maximize the entropy of κ-casein chains causes the micelles to have a repulsive mutual interaction (**Figure 1**). A complete loss of the overall negative charge of the casein micelles and especially of the κ-casein on the surface of the casein

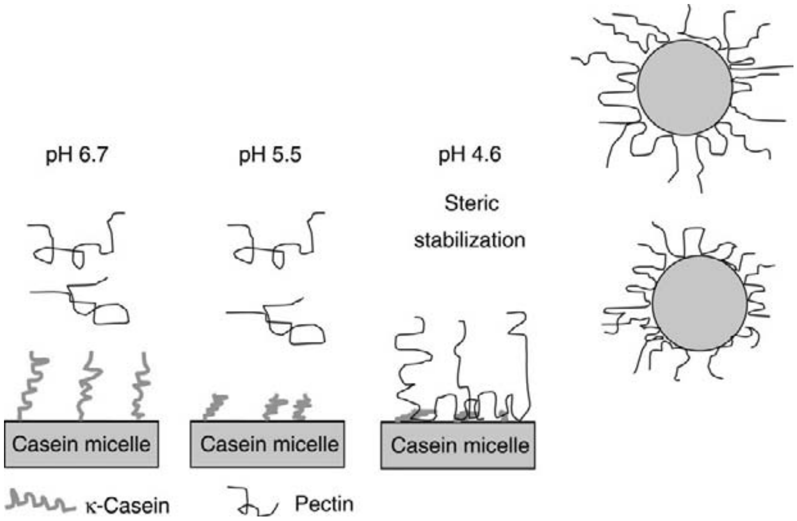

Figure 1 Schematic picture of the loss of hydration of the κ-casein on the surface of the casein micelle with decreasing pH. Added high-methoxy pectin adsorbs onto the casein micelle surface and reintroduces a steric repulsion. Left-hand side: high magnification, right-hand side: low magnification. Adapted from Tromp RH, de Kruif CG, van Eijk M, and Rolin C (2004) On the mechanism of stabilization of acidified milk drinks by pectin. *Food Hydrocolloids* 18(4): 565–572, with permission.

micelles at pH 4.6 and below leads to the loss of surface hydration. Therefore, the casein micelles lose their steric repulsion and this leads to destabilization of the casein and to aggregation and flocculation. This has to be prevented by addition of a stabilizer, say, pectin. Pectin directly interacts with the casein micelles and reintroduces the steric repulsion between the micelles. Pectin adsorbs on to the casein micelles as the result of an electrostatic interaction of the charged blocks of the pectin chain. The uncharged stretches in between form entropy-rich loops that extend into the solution. These loops cause a repulsive interaction between the micelles at a low pH in the same way as κ-casein chains do at pH 6.7, as shown schematically in **Figure 1**. A large part of the added pectin, up to 90%, is in the serum phase and not directly adsorbed on to the surface of the casein micelles. It is linked to the network of pectin-coated casein micelles but does not contribute to the stability of the system. The amount of ineffective pectin can be reduced by a more intense mixing or homogenization step after the addition of pectin.

Stabilization of Neutral Flavored Milks with Carrageenan

Carrageenan for the Formation of a Weak Gel

For the stabilization of flavored milk drinks with a pH of about 6.7, carrageenan is usually used as a thickening and gelling agent. Carrageenans are anionic polysaccharides with a high molecular weight extracted from different species of red seaweeds (Rhodophyta) such as *Chondrus*, *Gigartina*, *Eucheuma*, *Furcellaria*, and *Phyllophora*. They are linear polymers made up of repeating galactose and 3,6-anhydro-galactose units joined by alternating α-1,3- and β-1,4-glucosidic linkages. They are highly sulfated, and the different types of carrageenan vary mainly in the number and positions of the sulfate groups on the repeating galactose disaccharide. Three generic carrageenan families are especially important for the food industry: κ-, ι-, and λ-carrageenan. Two of them, κ- and ι-carrageenan, are able to form gels; λ-carrageenan is unable to gel owing to the large amount of sulfate groups. κ-Carrageenan is most often considered as suitable for the stabilization of dairy products. It should be mentioned that most commercially applied carrageenans are mixtures of all three types, even if they carry the name of only one. At elevated temperatures (>50 °C, depending on salt concentration), κ-carragenan exists in solution as a random coil (disordered state). When the temperature is lowered sufficiently, the polymer undergoes a transition from a coil to a helix (ordered state). The formation of the helix and the subsequent aggregation of neighboring helices facilitate the formation of a three-dimensional network and thus lead to gelation. Ions also play a vital role in the formation of the κ-carrageenan helix and in gelation. Cations, such as potassium and calcium, affect the transition temperature as well as aiding in helical aggregation; certain anions, such as iodide, affect gelation by interacting with the helix and inhibiting helical aggregation, thus preventing gel formation. In the ionic environment of milk, with 10.2 mmol l^{-1} calcium and 33.3 mmol l^{-1} potassium, the κ-carrageenan polymer is in an environment favorable for gelation, provided the κ-carrageenan concentration is high enough. Many commercial carrageenans contain mixes of cations to improve gelation properties.

κ-Carrageenan–κ-Casein Interaction

By means of dynamic light scattering, it was found that the diameter of casein micelles increases in the presence of κ-carrageenan as the temperature is lowered below the coil-to-helix transition temperature. This indicates adsorption of κ-carrageenan to the casein micelles and a molecular interaction. It was shown that an electrostatic attraction occurs between κ-carrageenan and κ-casein, but not with α_s- or β-casein. The association of κ-carrageenan with casein micelles at neutral pH is driven by attraction of the negatively charged carrageenan to the positive patch between amino acid residues 97 and 112 of κ-casein, although at this pH the overall charge of the casein micelle is negative. Increased ionic concentration above the normal value of milk decreases the interactions between casein micelles and κ-carrageenan. The open porous surface of casein micelles allows a large polymer like carrageenan to penetrate the hairy layer and adsorb to the surface of the micelles. The helical state of carrageenan results in an increase in charge density, as the sulfate groups are brought closer together, and this facilitates the electrostatic interaction between the casein micelle surface and κ-carrageenan. It has been established that in the presence of casein micelles, gelation occurs at relatively low κ-carrageenan concentrations and at temperatures below the carrageenan coil-to-helix transition temperature. In systems that are not strongly gelled, κ-carrageenan is able to inhibit visual phase separation between casein micelles and polysaccharides, which otherwise occurs readily due to biopolymer incompatibility. It has been shown, however, that such 'stable' systems remain microscopically phase-separated in an emulsion-like structure, with discrete casein micelle-enriched microdomains constituted in a continuous casein micelle-depleted phase. The κ-carrageenan in helical form attached to the surface of casein micelles at the periphery of the microdomains and subsequent interaction between neighboring κ-carrageean helices on adjacent microdomains stabilize the microdomains against flocculation and coalescence, and eventual separation. The three-dimensional network formed entraps cocoa particles and prevents them from sedimenting. Casein micelles also bind to the surface of the cocoa particles. Cross-linking of the biopolymers casein and κ-carrageenan with multivalent ions like calcium improves the assembly of a composite weak gel (**Figure 2**).

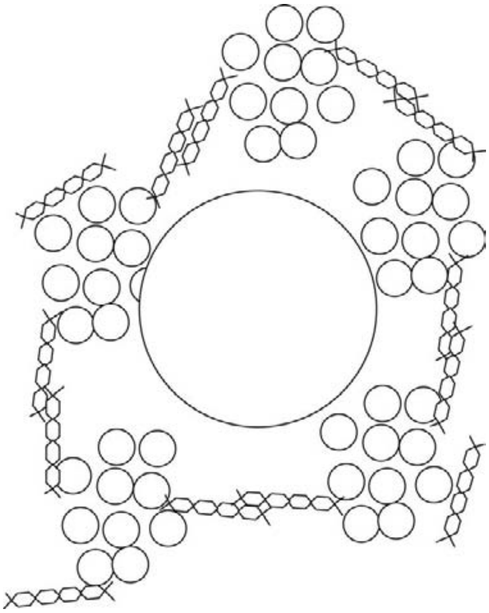

Figure 2 A cocoa particle in the middle is kept in suspension within the three-dimensional network of κ-carragenan and the microdomains of concentrated casein micelles. Interactions between κ-carrageenan helices and casein micelles at the periphery of the microdomains and subsequent interaction between neighboring κ-carrageenan helices on adjacent microdomains stabilize the whole network. Adapted from Spagnuolo PA, Dalgleish DG, Goff HD, and Morris ER (2005) Kappa-carrageenan interactions in systems containing casein micelles and polysaccharide stabilizers. *Food Hydrocolloids* 19: 371–377, with permission.

Dosage of Carrageenan, Temperature, and Shear

The minimum gelling concentration of κ-carrageenan in the above system was found to be 0.03%. At concentrations below that needed to produce a strong gel, κ-carrageenan interactions are fundamental in stabilizing milk protein-based dispersions and suspensions like chocolate milk. κ-Carrageenan concentrations of 0.01–0.05% are recommended for the stabilization of chocolate milk. A weak gel is necessary to prevent sedimentation of cocoa particles; an increase in viscosity delays sedimentation but it still occurs. Only carrageenan is able to form such weak gels in milk systems. These gels are easily broken down by stirring, pumping, shaking, or drinking, and they re-form if left undisturbed. This is called 'thixotropic' behavior. The initial mixing temperature of skim milk and κ-carrageenan dispersions strongly affects the particle size distribution of the aggregates. Mixing at 25 °C gives larger aggregates and a wider particle size distribution. Mixing at 60 °C, which is above the coil-to-helix transition temperature, gives smaller particles and a narrower particle size distribution. Shearing influences the particle size distribution and the viscoelastic properties. By mixing κ-carrageenan and casein micelles at 60 °C, followed by cooling to 25 °C without shearing, a gel structure can be formed, even with a κ-carrageenan concentration of 0.025%. Cooling the same mixture under shear results in a homogeneous liquid, containing aggregates of casein micelles with κ-carrageenan, but without a gel structure. Higher shear rates give smaller particle sizes.

If too much κ-carrageenan is used, it will cause the chocolate milk to form a stronger gel, resulting in an inhomogeneous or even custard-like product. A high dosage of κ-carrageenan can also lead to phase separation. On the other hand, if the concentration of κ-carrageenan is too low, cocoa powder sedimentation still occurs. As the protein-covered fat globules of the homogenized milk drinks participate in the stabilizing network with the κ-carrageenan, chocolate milks with a lower fat content need a higher concentration of κ-carrageenan for stabilization. Flavor release and flavor authenticity of chocolate milk stabilized with carrageenan was found to be clean and favorable.

Influence of Heat Treatment on Protein and Particle Stabilization

Severe heating, and therefore increased whey protein denaturation; lowering of the pH; or the use of κ-carrageenan instead of ι-carrageenan leads to excessive gelation during long storage of UHT-treated milk drinks. The gel strength probably is determined by the balance between carrageenan–carrageenan interactions and carrageenan–protein interactions. If the interactions between carrageenan and the casein micelles are reduced, more carrageenan is available for carrageenan–carrageenan interactions, leading to the formation of a stronger gel. This is the case if κ-carrageenan is used instead of ι-carrageenan, because the former's interactions with proteins are weaker than those of the latter. It is also the case if more severe heating is applied or in milk drinks with a reduced pH because both these conditions increase the attachment of whey proteins to the casein micelle surface, hindering the attachment of carrageenan to the micelles. In UHT-treated milk drinks, gel strength can increase upon storage. Particle size increase and viscosity increase indicated that upon storage weak carrageenan–protein aggregates are formed. The firming of the gel is probably related to the slow structural arrangement of the gel. This and the reduced heat stability with added cocoa powder indicate the necessity to limit the heat treatment.

Other Thickeners Used for the Stabilization of Neutral Flavored Milks

Sometimes, sodium alginate at a concentration of 0.2–0.5% is used as a thickener. At similar viscosities, κ-carrageenan systems showed better flavor-releasing properties than alginates. In comparison with κ-carrageenan, the concentration of alginate needed to get a similar viscosity is about 10-fold higher. Sometimes, guar gum is used in combination with carrageenan to give a rich creamy mouthfeel to flavored milks.

Chocolate Milk and Cocoa Powder

For chocolate milk a main ingredient is cocoa powder. Sometimes, chocolate powder also is added. Cocoa powder used in chocolate milk is usually produced from defatted cocoa, from which cocoa butter has already been separated by pressing. The cocoa powder thus prepared has a fat content of about 11%. The particle size of the cocoa powder used in chocolate milk has to be as small as possible: 10–30 μm is recommended, and less than 0.5% with a particle size above 75 μm. According to Stokes' law, in addition to the increased viscosity of the liquid phase, this is an important factor to reduce sedimentation. The chosen cocoa powder must provide a good chocolate flavor and color. Alkalized types of cocoa powder are generally preferred. The more alkalized the powder, the darker the color and the stronger the flavor obtained. The strength of the bond between the casein micelles and cocoa particles increases at higher levels of alkalization. Cocoa powder has to be free from enzymes such as heat-stable lipases and proteases and have a shell content of <1.5%. The higher the cocoa powder content, the lower the heat stability. As it is a critical point, especially for heat-treated products with a defined shelf life, cocoa particles must possess good bacterial quality with low counts of heat-resistant bacterial spores. Only 30–40% of the cocoa powder is soluble. As described above, the rest has to be kept in suspension by the thickener or gelling agent added as their density is higher than that of milk. Usually, an indication is given on the container to shake the beverage well, before use, to redistribute sedimented cocoa particles. The amount of cocoa and/or chocolate powder added varies between 1 and 15%, depending on the quality and price range of the product. The other ingredients are sucrose (5–8%) or other sugars and often vanillin or vanilla extract.

Processing of Chocolate Milk

For a good swelling of the cocoa particles and therefore an improved dispersability, the cocoa particles are first mixed into milk at a ratio of 1:2 and held for 2–3 h. Sometimes, this mixture is heated up to 80–90 °C for 30 min instead. This also allows air incorporated in the cocoa particles to escape. Carrageenan is premixed with 3–5 parts of sugar before being added to the milk, considering its tendency to form lumps. The concentrated suspension of cocoa in milk, the premixed carragenan, and the other ingredients are mixed into the main part of the milk at 40 °C. Intense mixing keeps the cocoa particles in suspension. For full-fat and semi-skimmed flavored milks, homogenization is applied either before (pasteurization, ultra-pasteurization) or after the heat treatment (UHT). Homogenization at the recommended pressure of 180–200 bar reduces the size of the fat globules but not the size of the cocoa particles. Heat treatment according to the defined conditions is followed by cooling and filling. Above 60 °C, the carrageenan molecules dissolve properly and interact more with the casein micelles. Pasteurized and ultra-pasteurized products are cooled to 4 °C and filled ultra-clean into HDPE plastic bottles or beverage cartons. UHT products are cooled to 20 °C and filled aseptically. After reaching a temperature below 35 °C, the carrageenan helices interact with neighboring helices and form a three-dimensional network leading to weak gelation. This will only happen if the flavored milk is left undisturbed. The processing equipment can be designed without the need to consider viscosity buildup. Nevertheless, once the flavored milk is cooled below the gelling temperature of carrageenan (around 35 °C), it should not be pumped or stirred for long. Sterilized flavored milks are heat-treated after filling. Cooling the milk before filling to a lower temperature increases the strength of the gel. The filling temperature should not be higher than 20 °C. If a higher filling temperature is used, there will not be sufficient thixotropy present to obtain full stability, and cocoa sedimentation may occur. Even for UHT-treated chocolate milk, cooling to 5 °C is advised to prevent sedimentation, knowing that storage will be at room temperature. Sometimes, guar gum is added in addition to carrageenan to prevent sedimentation of cocoa powder during cooling. In-container-sterilized flavored milks must be rotated during cooling to allow the forming network to entrap the cocoa particles and to keep them in suspension.

Important quality criteria for chocolate milk include a homogeneous appearance without phase separation or too much sediment, an appealing brown color, a good authentic chocolate flavor and clean flavor release, a viscosity high enough to give creaminess and a favorable mouthfeel but avoiding a strong gel, low Enterobacteriaceae and aerobic mesophilic colony counts, a good dispersability of a possible sediment, a fat content according to definition, a pH value in the range 6.2–7.0, and stability during a storage test for the defined shelf life.

See also: **Additives in Dairy Foods**: Legislation; Types and Functions of Additives in Dairy Products. **Chocolate**: Milk Chocolate. **Flavors and Off-Flavors in Dairy Foods**. **Hazard Analysis and Critical Control Points**: Processing Plants. **Heat Treatment of Milk**: Heat Stability of Milk. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Liquid Milk Products: UHT Sterilized Milks. **Milk**: Physical and Physico-Chemical Properties of Milk. **Milk Proteins**: Casein, Micellar Structure. **Rheology of Liquid and Semi-Solid Milk Products**.

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Liquid Milk Products: Membrane-Processed Liquid Milk

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Introduction

Laboratory curiosities until the late 1960s, membrane technologies started to become an industrial reality with the pioneering work of Loeb and Sourirajan (1963) who have developed the first anisotropic membranes, made from cellulose acetate, able to deliver reasonable fluxes and permeabilities for sea water desalination by reverse osmosis. Then, remarkable progress was also accomplished in the development of more robust membranes and better designed equipment as in the applications of this ubiquitous family of technologies which include separation of molecules or particles based on size differences: reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF), and microfiltration (MF); separation based on ionic charge: electrodialysis (ED); and separation based on chemical potential difference (pervaporation).

Among the food industries, dairying is undoubtedly that which has known the largest introduction of most of the membrane technologies, MF, UF, NF, and RO (the total installed area was in 2007 more than 500 000 m^2 according to Gezan-Guiziou, 2007), except for pervaporation which has, to our knowledge, no application in milk treatment. There are numerous reasons for this success: deep knowledge of the biochemical characteristics of milk and of the co-products (mostly whey) which helped greatly the optimization of the desired differential separation; the dynamism of several research teams; the operating temperature which did not cause irreversible damage to the biological properties of milk components; the high unacceptable environmental pollution caused by the discharge of cheese whey, etc. The presence of membrane equipment in dairy plants is nowadays as common as the presence of a cream separator in many countries worldwide. Concerning drinking liquid milks, the main applications are bacteria removal by MF, which has known worldwide success and adjustment of the protein content by UF. There has also been some use of RO for the concentration of milk for the production of yogurts or powders and some recent studies have been devoted to the specific separation of somatic cells (SC), described below, and to the separation of milk fat globules according to their size which has not yet been applied industrially.

Bacteria Removal by MF

Decontamination of collected milk is generally achieved through heat treatments: thermization, pasteurization, or sterilization by autoclaving or ultra-high temperature (UHT) treatment, using various combinations of time–temperature parameters to obtain the desired bactericidal effect. While these heat treatments ensure the safety of milk and dairy products, they almost always induce irreversible modifications of milk components, alter calcium salts–protein equilibrium, and also adversely affect the organoleptic quality of fluid milk and dairy products as well as the cheesemaking ability. Moreover, the cells of dead bacteria remain in heated milk with their potentially active enzymes which, with the metabolic activity of remaining thermoduric bacteria, will cause alterations of liquid milk during storage, thus reducing commercial shelf life.

Tangential membrane microfiltration offers an interesting alternative to heat treatments. Initially proposed in 1984, it has led to the technology and equipment called 'Bactocatch®' by the Tetra Laval Company. Numerous studies, done in Sweden and in France and summarized by Saboya and Maubois (2000), have optimized the original parameters described in the patent of Holm *et al.* (1984). Nowadays, skim milk heated to 50 °C is circulated at a velocity of 7.2 m s^{-1} along a ceramic membrane made of porous alumina and having an average pore size of 1.4 μm (Sterilox® or equivalent) supported by a thick porous layer also of alumina. The process is carried out according to the hydraulic concept of a uniform transmembrane pressure (UTP), approximately 0.5 bar, obtained either by recirculation of the permeate or by specially designed MF membrane with a continuous variation in the porosity of its support (Membralox GP®) or continuous variation of the thickness of the membrane layer (Isoflux®). All the somatic cells and most of the residual fat and contaminating microorganisms present in the incoming milk are concentrated 20 times in the MF retentate. In MF industrial equipment, this retentate is then concentrated 10-fold more in a second MF apparatus, thus leading to a volume concentration factor (VCF) of 200. Fluxes obtained industrially for the microfilterated skim milk are in the order of 500 l h^{-1} m^{-2} during 10 h. For VCFs 20 and 200, the observed permeation rates are, respectively, for proteins 99.0 and 99.4% and for total solids 99.5 and 99.9%. Average observed decimal reduction (DR) of bacteria is above 3.5 for milk collected in most developed dairy countries (initial total count (TC) <200 000 cfu ml^{-1});

it can be higher than 6.0 in milk of poor bacteriological quality collected in some developing countries. Spore-forming bacteria, which represent the main surviving species to pasteurization, are highly retained by MF membrane (DR > 4.5) because of their large apparent cellular volume when they are in milk. Synthesis of the different studies done by Pasteur Institute and INRA have shown for *Listeria monocytogenes, Brucella abortus, Salmonella typhimurium,* and *Mycobacterium tuberculosis* respective DR of 3.4, 4.0, 3.5, and 3.7. Considering the usually described contamination of milk at farm level, such results will assure that skim milk microfiltered through 1.4 μm membranes will contain less than 1 cfu l^{-1} of these pathogenic bacteria which means 1.4 μm MF milk can be considered as safe as pasteurized milk.

France is the only country which has officially allowed the commercialization of extended shelf life (ESL) MF raw milk. The MF raw skim milk is mixed with an amount of heated cream (95 °C for 20 s) required for fat standardization; the mixture is homogenized and aseptically filled. The authorized shelf life of the packed milk, legally raw, because it has a positive phosphatase reaction, at 4–6 °C is 3 weeks. The annual volume of this MF milk, to our knowledge, produced by only one dairy company under the trade mark 'Marguerite®' (see **Figure 1**) reached more than 10 million liters in 2008. Other plants as in many countries in the world homogenize the mixture, before a high temperature short time (HTST) (72 °C for 20 s) pasteurization step leading to a claimed shelf life of 5 weeks. The commercial success experienced by MF milk is great in many countries because of its improved flavor (no cooked taste) and because it is long storable. In some plants, MF through 1.4 μm membranes has been used as a pretreatment in the production of UHT milk in order to reduce the intensity of heat treatment (reduced to 140 °C for 4 s or less instead of 150 °C for 5 s) with consequently less cooked taste and improved storage arising from the removal by MF of thermostable enzymes present in dead bacterial cells and in somatic cells.

Use of MF membranes with a smaller pore diameter (0.8 μm instead of 1.4 μm) originally proposed by Lindquist (1998) was studied in Sweden, France (AFSSA, 2002), and Canada. At 50 °C, the flux obtained was approximately 400 l h^{-1} m^{-2} and the observed DR with this MF 0.8 μm membrane was higher than 13 on *Clostridium botulinum*, a value which means sterility of the product. After mixing with UHT (142 °C to 4 s) cream for fat standardization, followed by homogenization at 80 °C, a heat treatment at 95 °C for 6 s is applied with the only purpose of inactivating endogenous milk enzymes; then aseptic packaging is done at 20 °C. The resulting milk called 'Ultima®' milk by the Tetra Laval Company was recognized as commercially sterile and was allowed to be commercialized. Indeed, it is stable at 40 °C for 62 days and for more than 8 months at room temperature. Its organoleptic quality was judged to be similar to that of a HTST pasteurized milk. Its lactulose content was reduced by 71% compared to commercial UHT milk. But until now, to our knowledge, the 'Ultima®' process has not been developed commercially by the Tetra Pak Co, for unknown reasons. Nevertheless, nowadays, in some dairy plants, the 1.4 μm MF membrane is substituted by a 0.8 μm membrane for the production of ESL MF pasteurized milk in order to extend storage ability to more than 5 weeks.

Protein Standardization by UF

Ultrafiltration offers the possibility of adjusting the protein content of consumer milk either by their concentration or by the addition of UF permeate to the

Marguerite
1 Litre
La laiterie
Lait Frais
Microfiltré

Figure 1 Microfilterated raw milk.

milk in order to overcome natural variations in milk composition depending on cows' breed, their feed, the season, and their stage of lactation. Surprisingly, whilst fat standardization is commonly accepted and legally authorized for many years, the proposal to deliver consumer milk with a defined protein content has encountered incomprehensible and illogical (protein content is one of the payment criteria to milk producers!) opposition and until now, to our knowledge, no country in the world has modified its legislation to allow protein standardization of consumer milk although adjustment is allowed for milk and whey powders. Questions raised by protein standardization of consumer milk could be summarized as follows: ethical acceptance of fat standardization, one unique level (for example, $32\,\mathrm{g\,l^{-1}}$ or several ranging from $29\,\mathrm{g\,l^{-1}}$: minimum defined in EU and required from a nutritional point of view to $34\,\mathrm{g\,l^{-1}}$ (content found in many developed countries)), technologies to be used, and economical consequences.

Removal of Somatic Cells by MF

SCs which have a size ranging from 6 to 15 μm, contain numerous thermo-resistant enzymes (protease, lipase, catalase). They are very sensitive to mechanical treatments and consequently are able to release their enzymes into the milk with a potential impact on the quality of the dairy products derived from that milk (pasteurized and UHT milks). They have been shown to protect *Listeria monocytogenes* during heat treatment and it has been suggested that milk leukocytes could also contain BSE prions but no demonstration of this hypothesis has been made either in milk or in colostrum. Specific removal of SC from raw whole milk by MF membranes having an average pore size ranging from 12 to 5 μm was studied by our group. Permeation fluxes between $2000\,\mathrm{l\,h^{-1}\,m^{-2}}$ and $1460\,\mathrm{l\,h^{-1}\,m^{-2}}$ were, respectively, obtained over a running time of 8 h. SC of 93–100% was retained in the MF retentate which represented 4–5% of the volume of treated milk. Permeation rates of the globular fat were, respectively, 89 and 83%. Moreover, in order to be the solution for treating milk if presence of prion is eventually demonstrated, these results open new avenues for research such as the specific effects of varied numbers of SC in normal milk (most of the published studies have been done with mastitic milk the composition of which is highly modified) on the stability of UHT milk in comparison to the residual activity of the endogenous milk plasmin or of the proteases of *Pseudomonas*, the potential creation of micro-heterogeneity in the microstructure of cheese, and on the other hand to be used as tracers for identifying cows or herds which produced the used milk raw material because all their genetic patrimony is contained in the SC.

See also: **Cheese**: Membrane Processing in Cheese Manufacture; Preparation of Cheese Milk; Raw Milk Cheeses. **Heat Treatment of Milk**: Non-Thermal Technologies: Introduction. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: UHT Sterilized Milks. **Milk Proteins**: Nutritional Quality of Milk Proteins. **Nutrition and Health**: Effects of Processing on Protein Quality of Milk and Milk Products. **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products.

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Pasteurization of Liquid Milk Products: Principles, Public Health Aspects

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Introduction

Milk can be defined as the lacteal secretion, practically free of colostrum, obtained by the complete milking of one or more cows, (parallel definitions apply also to milk from sheep, goats, and other lactating animals). However, from a microbiological perspective, milk can be viewed as a highly nutritious growth medium for beneficial organisms (e.g., lactic acid bacteria) as well as numerous spoilage organisms (e.g., Gram-negative psychrotrophs) and bacterial pathogens (e.g., *Salmonella, Listeria*). Although bovine milk, as secreted by a healthy, nonmastitic cow, is sterile, contamination from the udder and teat surface as well as soil, bedding, manure, feed, milking equipment, and/or milk handlers invariably leads to the introduction of psychrotrophic and mesophilic bacteria at reasonably high numbers.

Problems associated with the microbial spoilage of milk are best handled by maintaining the milk at a temperature slightly above 0 °C until the time of consumption. However, since microbial pathogens are not eliminated during cold storage, milk, cream, and other fluid milk products are heat-treated to reduce the levels of microorganisms. Three general categories of heat treatment are currently recognized: thermization, sterilization, and pasteurization. Thermization, a process used to improve keeping quality rather than eliminate pathogens, typically involves heating milk at 57–68 °C for 15 s. Sterilization of milk, achieved by heating the product at 112 to 140 °C for 20–35 min, eliminates all pathogenic microorganisms and most vegetative spoilage organisms with only a few spore-forming bacteria surviving. Pasteurization, the most widely used thermal treatment for milk and fluid dairy products, is specifically designed to minimize possible health hazards arising from pathogenic microorganisms and also to minimize chemical, physical, and organoleptic changes that occur in milk during heating.

Principles of Pasteurization

Pasteurization derives its principles and its name from the famous French scientist Louis Pasteur, who in 1864–65 found that heating wine at 50–60 °C would destroy microorganisms responsible for abnormal fermentations. He made similar studies on beer and later reported that souring of milk was similar to spoilage of wine and beer, as all of which result from the growth of undesirable microorganisms. Commercial thermal processing of milk began in the late 1880s, with one of the primary goals being the inactivation of *Mycobacterium tuberculosis*, the causative agent of tuberculosis in humans, which was endemic in the raw milk supply. Interest in minimizing the public health hazards associated with milk consumption grew steadily during the early 1900s, with pasteurization almost universally adopted by developed countries after the end of World War II. Having been used for more than 120 years, pasteurization has been, currently is, and will likely remain the treatment of choice to render milk, cream, and other dairy products safe for consumption.

By definition, pasteurized milk and fluid dairy products must have received a heat treatment sufficient to minimize possible health hazards arising from pathogenic microorganisms associated with the raw product as well as adverse chemical, physical, and organoleptic changes. All pasteurized dairy products, which by definition are negative for alkaline phosphatase (a heat-labile enzyme in milk) immediately after processing, must be cooled rapidly and packaged without delay under conditions that minimize contamination.

As previously mentioned, the primary purpose of pasteurization is to reduce the numbers of bacterial pathogens in milk to levels that do not constitute a risk to human health. The process has been designed to destroy the most heat-resistant non-spore-forming pathogens found in raw milk, including *Coxiella burnetii*, *M. tuberculosis*, and *Listeria monocytogenes*. Other less heat-resistant pathogens of public health concern such as *Brucella*, *Staphylococcus aureus*, *Salmonella*, *Campylobacter*, and *Escherichia coli* O157:H7 are even more readily inactivated by pasteurization. However, thermoduric non-spore-forming bacteria (e.g., *Lactococcus* and *Streptococcus*), as well as spore-forming bacteria (e.g., *Bacillus* and *Clostridium*), can survive pasteurization, and growth of these organisms adversely affect product quality during refrigerated storage.

Thermal inactivation of any microorganism is based on exposure to a sufficiently high temperature for a sufficient length of time, with the heating menstruum also playing a major role. Hence, different time/temperature treatments have been established for fluid milk and cream based on fat content. Three different types of thermal processes have been developed that meet the current requirements for pasteurization. Before continuous pasteurization systems became popular in the 1940s, pasteurization regulations required that milk be held at not less than 62.8 °C for not less than 30 min. This time/temperature treatment, termed in-vat pasteurization or low-temperature–long-time (LTLT) pasteurization, was the first time/temperature treatment developed to render milk safe from microbial pathogens, including *M. tuberculosis*, the most heat-resistant pathogen of major public health concern in milk. Of the three pasteurization processes, LTLT pasteurization requires the simplest equipment, is the simplest to operate, and is still used today, primarily by small-scale cheese manufacturers. High-temperature–short-time (HTST) pasteurization, a continuous process in which milk is held at not less than 71.1 °C for a minimum of 15 s (or equivalent conditions at a higher temperature for a shorter time period), is now the norm for processing fluid milk and cream. Most vegetative bacterial cells are destroyed by both LTLT and HTST pasteurization. However, some thermoduric lactic acid bacteria and spore-forming organisms will survive, which necessitates prompt cooling and refrigerated storage of the product to minimize the growth of psychrotrophic bacteria involved in spoilage. Another continuous process known as ultra-high temperature (UHT) pasteurization, in which milk is heated to at least 135 °C for a minimum of 1 s, has been adopted by some segments of the dairy industry, particularly in Europe. When combined with aseptic processing/packaging technologies, the high heat treatment involved in UHT pasteurization produces a shelf-stable product that can be kept unrefrigerated until opened. Unlike other products, UHT-pasteurized milk and cream are not subject to microbial spoilage with proteolysis of the milk by certain heat-stable enzymes, generally the determining factor in product shelf life. Although several cold-pasteurization methods, including microfiltration, high-pressure processing, and ultraviolet light, are now attracting some attention, none of these non-thermal microbial inactivation strategies are currently in widespread commercial use.

Public Health Aspects

The public health importance of various dairy products has changed dramatically over the last 100 years, with routine adoption of pasteurization virtually ensuring that milk consumed by the general public is safe. Consumption of raw milk and cream was the leading cause of dairy-related illness prior to World War II, with numerous outbreaks of typhoid and scarlet fever being reported. However, major improvements in sanitation, milk-handling procedures, and animal health along with routine pasteurization of milk and cream have eliminated the threat of milk-borne typhoid and scarlet fever in all but a few developing countries. Today, about one-third of all dairy-related outbreaks involve the consumption of raw milk, which in some cases can still be sold legally or obtained through cow-share programs in the United States. Given the continued availability of raw milk, a total of 39 outbreaks were traced to the consumption of raw milk (or raw-milk cheese) in the United States from 1998 to 2005, which resulted in 831 cases of illness, 66 hospitalizations and 1 fatality. Homemade ice cream and to a lesser extent cheese (particularly soft unripened cheese prepared from raw milk) have been responsible for most other dairy-related outbreaks, with butter, dried milk, and dried whey seldom implicated. Except for two unusually large outbreaks – one in the Chicago area in 1985 and the other in Japan in 1998 – few additional outbreaks have been linked to pasteurized milk in recent years.

Changes in milk production practices have altered the types of etiological agents involved in milk-borne illnesses. However, more than 90% of all dairy-related illnesses continue to be of bacterial origin, with at least 22 milk-borne or potentially milk-borne diseases recognized (**Table 1**). Prior to 1940, typhoid fever and scarlet fever accounted for most cases of milk-borne illness with milk, typically raw milk, contaminated during or after milking by human carriers of *Salmonella typhi* or *Streptococcus pyogenes*, the bacteria responsible for typhoid and scarlet fever, respectively. During and shortly after World War II, brucellosis, staphylococcal poisoning, and salmonellosis emerged as major threats to public health, with non-typhoid *Salmonella* still currently responsible for the largest number of dairy-related illnesses. Since the 1970s, campylobacteriosis has been identified as an ongoing health concern for those individuals who still consume raw milk. From 1973 to 1992, *Campylobacter* accounted for 26 of 46 raw milk-associated outbreaks in the United States, with 8 of 18 raw milk outbreaks from 2000 to 2007 also traced to *Campylobacter*. A similarly high incidence of such campylobacteriosis cases was also reported in England and Scotland. In 1985, as many as 85 people in California died of cheese-borne listeriosis, a rare illness that was epidemiologically linked to the consumption of pasteurized milk in Massachusetts 2 years earlier. More recently, *E. coli* O157:H7 has emerged as a serious threat to the dairy industry with outbreaks of potentially fatal hemolytic uremic syndrome traced to the consumption of raw milk in the United States, Canada, and England. While able to cause potentially serious health problems, the rickettsiae, parasites, and viruses are each responsible for less than 1% of all dairy-related illnesses, with mycotoxins such as aflatoxin

Table 1 Historical, current, and uncommon/emerging public health concerns regarding milk and dairy products

Historical	*Current*	*Uncommon/emerging*
Diphtheria	*Bacillus cereus* poisoning	Aflatoxin
Poliomyelitis	Botulism	*Cronobacter sakazakii*
		Citrobacter freundii
Scarlet fever	Brucellosis	*Corynebacterium ulcerans*
Septic sore throat	Campylobacteriosis	Creutzfeldt–Jakob disease
Tuberculosis	Enteropathogenic *Escherichia coli*	Cryptosporidiosis
Typhoid	Enterohemorrhagic *E. coli*	Haverhill fever
	Listeriosis	Histamine poisoning
	Salmonellosis	Infectious hepatitis
	Staphylococcal poisoning	Johne's and Crohn's disease
	Yersiniosis	Mycotoxins
		Q fever
		Shigellosis
	Streptococcus zooepidemicus	
		Tick-borne encephalitis
		Toxoplasmosis

AFM_1 also posing minimal public health concerns in developed countries. In response to the emergence of 'mad cow disease', the safety of the milk supply has been questioned again. However, currently, there is no evidence for shedding of the prion – the infectious protein responsible for fatal new variant Creutzfeldt–Jakob disease (nvCJD) in humans – in milk.

Pathogens of Concern

The presence of pathogenic bacteria in raw milk continues to pose a major public health concern to the dairy industry. The common pre-World War II pathogens, including *S. typhi*, *S. pyogenes*, and *Corynebacterium diphtheriae*, have been replaced by organisms of more immediate concern such as *Bacillus cereus*, *Campylobacter jejuni*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, *Salmonella*, *S. aureus*, and *Yersinia enterocolitica*, all of which are responsible for gastroenteritis of various durations with nausea, vomiting, and/or diarrhea as predominant symptoms. Other milk-borne pathogens including *Brucella* spp., *Clostridium botulinum*, enterohemorrhagic *E. coli* (*E. coli* O157:H7), and *L. monocytogenes* are of particular importance because of their disease severity and high fatality rate.

Bacillus cereus, a common environmental contaminant of raw milk, is both a public health and a spoilage concern, being responsible for gastroenteritis and 'sweet curdling' of milk. Heat-resistant spores of *B. cereus* will survive pasteurization, with germination and outgrowth of this psychrotrophic organism occurring in milk during refrigerated storage. Several large outbreaks of gastroenteritis involving infant formulae also attest to the ability of this pathogen to survive spray-drying and grow to hazardous levels in the reconstituted product.

Botulism, among the most fatal of milk-borne diseases, results from ingesting minute amounts of a pre-formed neurotoxin produced by *C. botulinum*. Symptoms of botulism are particularly severe, progressing from initial diarrhea and vomiting to blurred vision, difficulty swallowing, muscle paralysis, and eventual death due to respiratory failure. Spores produced by this anaerobic Gram-positive organism are widely distributed in the environment with soil serving as a primary reservoir. While spores of *C. botulinum* frequently contaminate raw milk and survive both LTLT and HTST pasteurization, toxin production in raw and drinkable milk does not occur because of the product's short refrigerated shelf-life and the organism's inability to compete with the native psychrotrophic background flora. Consequently, reports of dairy-related botulism remain very rare, with only 13 such outbreaks (12 traced to various cheeses and 1 to yogurt) recorded since 1899.

Human brucellosis, a classic zoonosis, is primarily acquired through direct or indirect contact with infected animals harboring three of six bacterial species belonging to the genus *Brucella*. Two of these species, *Brucella melitensis* and *Brucella abortus*, cause disease in goats/sheep and cattle, respectively, and are consequently a major concern to the dairy industry. However, LTLT and HTST pasteurization are again sufficient to eliminate this pathogen from raw milk. In humans, symptoms of brucellosis typically occur after an incubation period of 3–21 days and may range from a mild flu-like illness to undulant fever, with the latter characterized by sweating, chills, chest and joint pain, weight loss, and anorexia, which can lead to numerous debilitating complications. Dairy-related cases of brucellosis remain a rare occurrence in most well-developed nations; however, some Mediterranean and Middle Eastern countries are seeing a resurgence in human cases due to infections in domestic livestock. Most of these human cases have been associated with the consumption of various raw-milk cheeses that were prepared from the milk of infected animals.

Campylobacter jejuni, a leading cause of food-borne gastroenteritis worldwide, has a propensity for young children. Flu-like symptoms including mild fever and profuse diarrhea predominate. Young children may exhibit appendicitis-like symptoms that lead to unnecessary appendectomies, with some elderly and immuno-compromised adults developing other serious complications requiring medical intervention. While most often associated with poultry, up to 10% of the raw milk supply can reportedly harbor *Campylobacter* at any given time. Natural shedding of this pathogen in raw milk as a result of mastitis is considered rare, with fecal contamination of the milk during or after milking now regarded as the primary route of contamination. However, being a delicate organism, readily destroyed by pasteurization, milk-borne outbreaks of campylobacteriosis have been invariably associated with raw or inadequately pasteurized milk.

Escherichia coli is a common contaminant of raw milk. While easily destroyed by pasteurization, *E. coli* frequently enters pasteurized milk and other finished products as a post-pasteurization contaminant. Most *E. coli* strains are harmless commensals common to the gastrointestinal tract of humans and animals. However, three categories of *E. coli* – enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) – have been responsible for dairy-related illnesses. ETEC strains produce one or more enterotoxins responsible for 'traveler's diarrhea', which is characterized by a low-grade fever, abdominal cramps, diarrhea, and vomiting. Two outbreaks involving ETEC have been traced to cheese. Unlike ETEC, strains classified as EIEC invade the lining of the colon to produce a severe bloody diarrhea accompanied by fever and abdominal cramps. Reports of dairy-related EIEC infections are confined to a single outbreak involving cheese.

EHEC, which includes *E. coli* O157:H7, has recently emerged as the most serious *E. coli* threat to the dairy industry. Unlike other pathogenic strains of *E. coli*, dairy cattle are now recognized as a major reservoir for EHEC. Frequent shedding of this pathogen in cattle feces has led to cross-contamination of milk on the farm, with raw milk contamination rates of up to 10% being reported. Compared to most other food-borne illnesses, infections involving *E. coli* O157:H7, the most commonly identified strain of EHEC in the United States and many other countries, are particularly serious, with manifestations ranging from mild, non-bloody diarrhea to hemorrhagic colitis (bloody diarrhea and severe appendicitis-like abdominal pain), hemolytic uremic syndrome (kidney failure, seizures-coma, terminal in 3–10% of cases), and thrombotic thrombocytopenic purpura (fatal strokes). Like *Campylobacter*, numerous dairy-related outbreaks involving EHEC have been traced to the consumption of raw milk, particularly among small children visiting dairy farms, with several additional outbreaks traced to cheese.

In 1985, *L. monocytogenes* emerged as a major threat to the dairy industry with the report of up to 300 cases of listeriosis (including 85 fatalities) in southern California that were linked directly to the consumption of contaminated Mexican-style cheese. Additional outbreaks have also involved pasteurized milk, chocolate milk, various surface-ripened cheeses, and butter. Unlike other food-borne pathogens, this Gram-positive, non-spore-forming organism is acid tolerant, salt tolerant, and capable of growing in fluid milk and certain high-pH specialty surface-ripened cheeses, such as Camembert, at refrigeration temperatures. Listeriosis, the disease caused by *L. monocytogenes*, is relatively rare, with approximately 2500 cases reported annually in the United States and with similar incidence rates in other developed countries. Most listeriosis cases are confined to pregnant women, newborn infants, the elderly, and immuno-compromised individuals (i.e., cancer, organ transplant, and HIVAIDS patients), with healthy adults seldom affected. In contrast to most other food-borne infections, typical manifestations of listeriosis include meningitis, abortion, and perinatal septicemia (mortality rate of 20–30%), with one large outbreak of non-fatal gastroenteritis also having been traced to the consumption of highly contaminated chocolate milk. *Listeria monocytogenes* is widespread in the farm environment with dairy cattle and other ruminants also capable of developing listeriosis and shedding the pathogen symptomatically or asymptomatically in milk and feces for extended periods of time. While more heat resistant than most other milk-borne pathogens, standard LTLT and HTST pasteurization practices are sufficient to inactivate this pathogen in fluid milk, with most dairy-related outbreaks traced to products that were either prepared from raw milk or contaminated after manufacture.

Salmonella and *Campylobacter* are the two leading causes of dairy-related illness in the United States and western Europe, with infection rates being particularly high in areas of the world where raw milk is neither pasteurized nor boiled. All salmonellae are of public health importance given their propensity to produce manifestations ranging from mild self-limiting gastroenteritis (nausea, vomiting, diarrhea) to life-threatening septicemia and localized tissue infections. In addition to humans, dairy cattle and other ruminants are also prone to *Salmonella* infections. Symptomatic and asymptomatic shedding of this pathogen in feces frequently leads to contamination of raw milk, with 2–6% of bulk tank samples normally testing positive. Standard HTST and LTLT pasteurization will destroy expected levels of salmonellae (e.g., <100 cfu ml^{-1}) in milk with a wide margin of safety. While numerous outbreaks have been traced to the consumption of raw milk (as was also true for *Campylobacter*), inadequate pasteurization and post-processing contamination have resulted in occasional outbreaks involving fluid milk, ice cream, and various cheeses (i.e., Cheddar, Mozzarella, Mexican-style, goat's milk). The United States experienced two particularly large outbreaks. In 1985, at least 16 000 culture-confirmed salmonellosis cases in the Chicago area were traced to one particular brand of pasteurized 2% fat milk. A later follow-up survey estimated the total number of cases at nearly 200 000, making this the second largest food-borne outbreak of salmonellosis ever recorded. The largest outbreak occurred 9 years later in Minnesota, Wisconsin, and South Dakota, with an estimated 240 000 cases of gastroenteritis traced to ice cream containing *Salmonella enteritidis*, an organism typically associated with poultry and eggs. Follow-up investigations revealed that tankers used to haul raw eggs were also used to haul pasteurized ice cream mix to the ice cream factory where the mix was not re-pasteurized.

Dairy products are well-known vehicles of staphylococcal poisoning, a classic food-borne intoxication that results from ingesting a preformed, heat-stable toxin (termed enterotoxin) produced during the growth of *S. aureus* at temperatures above 15 °C. This food-borne intoxication develops suddenly but is of short duration. Nausea, vomiting, diarrhea, and abdominal cramps typically appear 1–6 h after ingesting the preformed toxin, with these symptoms generally persisting for 1–8 h. Staphylococci are frequent contaminants of raw milk, with *S. aureus* widely recognized as a common cause of clinical and sub-clinical mastitis in dairy cattle, sheep, and goats. While the mammary gland represents an important reservoir for *S. aureus*, this pathogen is also commonly found on human skin, with both of these sources contributing to contamination of raw milk. Staphylococcal poisoning was the major milk-borne illness from the 1930s to the 1960s with outbreaks traced to raw milk, pasteurized milk, cheese, ice cream, butter, and non-fat dry milk. However, except for one isolated outbreak in Japan, in which more than 14 000 cases were traced to non-fat dry milk in 1999, the role of dairy products in staphylococcal poisoning has generally declined in importance.

Yersinia enterocolitica was not widely recognized as a food-borne pathogen until the 1970s, with most cases being linked to pork, particularly chitterlings, because hogs are the primary reservoir of human pathogenic strains. Yersiniosis, the disease cased by infection with *Y. enterocolitica*, most often presents as gastroenteritis in children less than 7 years of age with symptoms of low fever, diarrhea, and severe abdominal pain that can mimic appendicitis. However, septicemic infections accompanied by various secondary complications have also been reported, particularly in the elderly. Since 1972, at least seven dairy-related outbreaks of yersiniosis have been documented in the United States and Canada, with raw milk, powdered milk, chocolate milk, and pasteurized milk confirmed as vehicles of infection. While capable of growing in milk during refrigeration, *Y. enterocolitica* is generally regarded as an unusual cause of milk-borne illness due to the low incidence of human pathogenic strains in raw milk and the organism's high susceptibility to pasteurization.

Uncommon and Emerging Concerns

The aforementioned bacterial pathogens easily account for more than 95% of all dairy-related illnesses of known cause. However, the list of 'new' and 'emerging' milk-borne pathogens continues to evolve and now includes such previously unrecognized concerns as Creutzfeldt–Jakob disease (CJD), cryptosporidiosis, and Johne's/Crohn's disease, and *Cronobacter* (formerly *Enterobacter*) *sakazakii*.

CJD, the principal form of human spongiform encephalopathy, is an extremely rare and fatal neurodegenerative disorder. In 1996, a somewhat different form of CJD, termed nvCJD, emerged in the United Kingdom, and this disease was subsequently shown to be related to bovine spongiform encephalopathy (BSE) – a disease diagnosed in British cattle 10 years earlier. Transmission of nvCJD is assumed to result from consuming animal tissues containing high levels of an infectious protein, termed a 'prion'. There has been no single case of nvCJD linked directly to consumption of BSE-infected beef or related animal by-products in the United Kingdom or elsewhere. While the safety of the milk supply has been questioned, no cases of nvCJD have been associated with milk or dairy products and currently there is no evidence for shedding of the infectious prion in milk. Consequently, the risk of contracting nvCJD from dairy products appears to be minimal.

Cryptosporidiosis, one of the most common self-limiting gastrointestinal infections in healthy individuals, is caused by protozoan parasites from the genus *Cryptosporidium*. Ruminants, including cows, sheep, and goats, are susceptible to such infections and can shed high numbers of *Cryptosporidium* oocysts, a highly resistant form of the organism, in feces, with subsequent contamination of raw milk reported. Evidence for the involvement of *Cryptosporidium* in milk-borne illness is growing, with at least five major outbreaks of gastroenteritis traced to the consumption of raw milk or improperly pasteurized milk. However, HTST pasteurization is sufficient to render any oocysts in milk non-infectious.

Johne's disease is an economically devastating affliction in cattle, characterized by ileocolitis that eventually leads to diarrhea, weight loss, debilitation, and death. High numbers of the causative bacterium *Mycobacterium avium subsp. paratuberculosis* are shed in feces, with up to 35% of clinically infected cows and 12% of asymptomatic carriers yielding positive milk samples. A possible association between Johne's disease in ruminants and Crohn's disease, a nearly identical form of ileocolitis in humans often requiring surgical intervention, has generated considerable interest. Evidence for complete inactivation of *M. paratuberculosis* in milk by HTST pasteurization is conflicting, with many genetic-based studies also reporting the presence of DNA from this pathogen in commercially pasteurized milk. However, the importance of *M. paratuberculosis* as a milk-borne pathogen remains unclear given the lack of a definitive link between Johne's disease in ruminants and Crohn's disease in humans.

Most recently, *C. sakazakii*, a Gram-negative enteric bacterium formerly known as *Enterobacter sakazakii*, has been identified as a rare cause of sepsis, meningitis, and necrotizing enterocolitis in neonates and infants who have consumed rehydrated infant formula. Given a mortality rate of 20–50% and the severe long-term neurological disorders encountered by surviving victims, *C. sakazakii* is now a leading concern among manufacturers of infant formulae. Although typically not present in raw milk and easily inactivated by pasteurization, *C. sakazakii* is widespread in many dairy processing environments, with this opportunistic pathogen entering UHT milk and dried infant formulae as a post-pasteurization contaminant. Hence, appropriate control strategies need to be followed to minimize post-processing contamination.

See also: **Cheese**: Public Health Aspects. **Diseases of Dairy Animals**: Infectious Diseases: Listeriosis. **Mastitis Pathogens**: Environmental Pathogens.

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Recombined and Reconstituted Products

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Background

Recombined and reconstituted milk and milk products are made from preserved dairy ingredients (e.g., skim-milk powder, whole-milk powder, anhydrous milk fat, unsalted frozen butter), which are processed in such a way as to resemble products made from fresh milk supplies. As a result, recombined and reconstituted milk and milk products provide a nutritious and high-quality source of dairy products in areas where a fresh raw-milk supply is not readily available or is in short supply. Because refrigeration and transportation may not be readily available in some regions, utilization of preserved milk ingredients may be the only viable means of producing dairy products. Originally, recombined milk products were developed to supply the need for milk of US Armed Forces during World War II. However, in the 1950s, regions of the world with limited fresh milk, such as Japan, southeast Asia, the Middle East, Africa, South America, and Central America, quickly adopted this technology to provide dairy products for the general public. In recent years, reconstituted milk products are used as concentrated sources of milk components for standardization of milk supplies to improve the consistency and efficiency of manufacturing operations. Other food manufacturers prefer to reconstitute or recombine milk for use as a food ingredient rather than invest in raw-milk procurement and processing capabilities and expertise. Today, products produced from reconstituted and recombined milk include liquid milk products, specialty beverages, creams, concentrated milk products, fermented milks, cheeses, and desserts. Approximately one-third of the total world production of milk powders is utilized for recombined dairy products. Vegetable oils and other alternative dairy (e.g., whey protein concentrate) and non-dairy ingredients are combined to produce some beverages and other food products.

As with any dairy product, it is critical that ingredients with the appropriate microbiological, sensory, and functional attributes be used in recombined and reconstituted milk products. It is also essential that manufacturing conditions be established and controlled to ensure consistency in the attributes of the finished product.

Recombined Milk and Related Liquid Products

Products

Sterilized liquid milk products with the level of fat and solids-not-fat appropriate to meet the regulations for the area were the first products to be produced from recombined milk. A typical composition is 3% fat, 10% solids-not-fat, and 13% total solids. In some cases, additional skim-milk powder is added to enhance the nutritional value of the recombined milk product. However, ultra-high temperature (UHT)-processed and aseptically filled recombined milk products have less scorched flavor than sterilized milk products and have gained popularity. More recently, with the continued improvement in the quality of the preserved milk supplies and consumer demand for more 'fresh'-tasting milk products worldwide, recombined pasteurized milk products are becoming available.

In other parts of the world, to take advantage of lower-cost ingredients and/or available raw materials, some filled, imitation or substitute dairy products are produced which contain vegetable oils and other non-dairy ingredients. Also, non-standardized (no standard of identity) drinks or specialty foods (e.g., infant formulae, nutritional beverages, and blends with fruit juices, coffee, or other extracts) are produced. More acidic conditions and/or the addition of minerals and other nutrients in these non-standardized products can create stability and quality challenges for the food manufacturer.

Processes

The general production protocol for recombined milk products involves the following unit operations: blending, dispersing (aeration should be minimized), deaerating, hydrating, dissolving, emulsifying, thermal processing, homogenizing, and packaging. The mixture is filtered to remove any undissolved material prior to thermal processing. Reconstitution equipment can be as simple as a tank with a propeller-type agitator and recirculation pump, to a venturi jet mixer with a recirculation system, to more sophisticated systems containing high-speed blenders (e.g., Tri-blender). Water temperature is critical and can influence the required hydration time prior to subsequent processing. Generally, water at a temperature of 40–50 °C is used most commonly. Originally, reconstituted and recombined milk products destined for retail packaging were sterilized in the bottle or can (120 °C for 12 min). However, UHT processing (135–150 °C for 3 s) followed by aseptic filling produces a product that has equivalent shelf life and a less scorched flavor.

Ingredients

One key goal in the manufacture of recombined liquid milk products is to produce a beverage with a sensory profile as similar as possible to a corresponding product manufactured from a high-quality fresh raw-milk supply. As a result, it is desirable to use low-heat or medium-heat milk powders (whey protein nitrogen index (WPNI) >3.5) to ensure the best sensory profile for the recombined product. However, low-heat or medium-heat milk powders generally are not as heat stable as high-heat milk powders. Hence, in some cases a trade-off must be made between optimizing heat stability and the sensory properties of the product. In some cases, undesirable heat-induced changes in product stability can be minimized by the addition of phosphates. In addition, the fat source, whether it be from whole-milk powder, anhydrous milk fat, or vegetable fats, must not have an excessive level of oxidized flavors. Since the reconstituted products generally contain 85–90% water, it is important to have a high-quality water source. Excessively hard water can lead to difficulties in powder solubility and stability.

For the manufacture of shelf-stable products (sterilized or UHT products) it is important to obtain ingredients that do not contain sufficient heat-stable enzymes and/or microorganisms (e.g., spores) that can survive the heat treatments and degrade the product quality during storage (e.g., causing age-thickening). Age-thickening or age gelation is an unacceptable increase in viscosity which is associated with aggregation, gelation, and/or precipitation of milk proteins and occurs due to proteolysis and/or exposure to elevated processing and storage temperatures.

Recombined Concentrated/Evaporated Milk

Products

Recombined concentrated/evaporated whole-milk products range in composition from 25.9 to 31% total solids, 7.9 to 9.0% fat, and 18 to 22% solids-not-fat. Recombined concentrated/evaporated skim milk or low-fat milk is also available. Such products are traditionally and most commonly in-can-sterilized products but some UHT products are available.

Processes

In contrast to conventional evaporated milk processing, there is usually no significant removal of water under vacuum in the manufacture of recombined evaporated milk. In this case, the mixture is formulated from dry ingredients and an appropriate amount of water is used to achieve the final solids content of the final product. After all the ingredients have been blended and hydrated to the proper solids content (as described above for recombined milk), the mixture is homogenized and subsequently filled into cans or bottles (for sterilization in a batch or continuous retort) or UHT-processed and filled aseptically. Homogenization conditions can affect heat stability and final product viscosity.

Ingredients

Because of the higher concentration of solids in these products, they can be more unstable than unconcentrated products during heat processing. It is important to select a milk powder for use which will not result in a product with excessive age-thickening. High-heat (WPNI <1.5) heat-stable milk powder is commonly used. Highly heat-stable milk powder is prepared by exposing the milk (prior to evaporation and drying) to an additional heat treatment (approximately 90–95 °C for 5–15 min or 115–120 °C for 2–4 min). In addition, selected phosphates are added to adjust the pH to help ensure good protein stability (prevent sedimentation). Carrageenan may also be added to modify product viscosity and minimize creaming (fat separation). The products are usually fortified with vitamins A and D prior to homogenization.

Recombined Sweetened Condensed Milk

Products

Recombined sweetened condensed milk products contain approximately 7–9% fat, 20–22% solids-not-fat, and 43.5–45% sucrose. Additionally, creamers containing sucrose, milk powder replacers, whey-based ingredients, and vegetable fats and oils are produced as lower-cost alternative recombined products.

Processes

After all the dry ingredients have been dispersed in the water portion of the formulation, the mixture is preheated, filtered, homogenized, pasteurized, and then vacuum-cooled and seeded with lactose (usually less than 5–10 μm). In the more traditional approach to the manufacture of this product, 10% more water is usually added during the mixing step and then subsequently removed during the vacuum cooling and seeding process. A relatively new approach involves the formulation of the mixture to the final solids level coupled with continuous lactose seeding in a scraped surface heat exchanger. Seeding allows for sufficient nuclei to help regulate the size of lactose crystals and the ultimate product viscosity.

Ingredients

It has been demonstrated that the milk powder used in the formulation is one of the most important factors in the production of recombined sweetened condensed milk. As with recombined evaporated milk, optimizing the viscosity throughout the shelf life of recombined sweetened condensed milk is of primary interest. If the product is of low viscosity, it can lead to rapid fat separation and sedimentation of components such as crystals of lactose and calcium phosphate. If the product is too viscous, it can become difficult to pour from the container. While WPNI values can provide some starting point guidelines for milk powder selection, pilot-scale trials may be needed to ensure good performance of a given milk powder in recombined sweetened condensed milk. Maillard reactions (nonenzymatic browning reactions) are also accelerated in these concentrated products which have a high content of reducing sugars.

Recombined Cheese

Products

A wide variety of cheeses are made from 100% recombined milk or fresh milk supplemented with reconstituted skim-milk powder. However, because of changes which occur during concentration and drying of milk, the cheesemaking properties of such milks may be altered – most notably coagulation and whey expulsion/drainage. Generally, reconstituted milk coagulates more slowly on renneting, and forms a softer gel which is more difficult to drain than cheese made from fresh pasteurized or raw milk. Nevertheless, cheesemaking conditions can be modified or other ingredients can be added to allow the successful production of most cheese varieties (e.g., cottage cheese, Cheddar, Mozzarella, Camembert, Tilsit, Feta, and Edam) from high-quality reconstituted/recombined milk.

Processes

The steps in cheesemaking basically follow the procedure used to manufacture the particular cheese variety from fresh pasteurized milk. However, sufficient time should be allowed to hydrate the milk powder fully, reequilibrate the mineral balance, and release any dissolved air from the reconstituted or recombined milk. Additional calcium chloride can be added 12–18 h before renneting to ensure sufficient ionic calcium for effective coagulation. Other slight modifications in cheese manufacture can be made for variances in the amount and timing of moisture expulsion/retention and acidification rate due to the use of recombined or reconstituted milk.

The use of reconstituted milk with a higher solids content and/or fresh milk supplemented with a concentrated source of milk solids, such as milk powder, is an effective means for standardizing the protein-to-fat ratio for optimum cheese manufacture. In some cases, standardization to a level of higher total milk solids is advantageous for cheese yield and productivity (weight of cheese produced per turnover of the cheese vat). However, if other modifications are not made in cheese manufacture, excessive acid production in cheese curd occurs due to retention of a higher lactose level in the curd. This can lead to acid cheese of poor quality. In some cases, the use of membrane-concentrated dairy ingredients or milk which has been processed (diafiltration) to reduce the lactose content can minimize this problem. In addition, higher retention of whey protein in cheese can modify the texture, ripening rate, and functional properties of cheese.

Ingredients

Low-heat milk powder (WPNI >6.0) is preferred for cheese manufacture to minimize the tendency for poor curd firmness and excessive moisture retention associated with high-heat-treated milk and milk powders. In addition, attention should be paid to the casein content of the milk powder (not just total solids) since it is critical for coagulation and fat retention in cheese. If cheese is being made from 100% recombined milk, great care should be exercised in homogenization of the reconstituted milk, since homogenization adversely affects moisture expulsion and curd firmness during cheese manufacture. Excessive free fat in the milk can also cause defects in cheese (e.g., reduced fat recovery, poor curd fusion, poor coagulation, and lower cheese yield).

Recombined Yogurt and Related Fermented Milk Products

Products

Recombined fermented milk products vary in fat content, bacterial fermentation, and level of total solids in the finished product. However, in all cases, a lactic fermentation promotes the acidification of the milk, other metabolic products from fermentation, an increase in viscosity, and the presence of viable lactic acid bacteria. Stirred yogurts are fermented in large tanks (bulk set), cooled, and subsequently packaged. Set yogurts are fermented in the final package (cup set) and cooled.

Processes

The basic steps in the manufacture of recombined yogurt and other fermented milk products involve the reconstitution of the milk powder and other ingredients, preheating, homogenization, pasteurization, fermentation, cooling, and packaging. The pasteurization temperature is typically higher than the minimum pasteurization temperature to promote the denaturation of whey proteins to increase viscosity and improve water-holding capacity (minimize wheying-off of the set yogurt).

Ingredients

As with any fermented dairy product, the ingredients must be free of inhibitory substances that could slow or prevent optimum fermentation rates. In addition, milk powders used for recombined fermented milk products must have good water-binding properties and promote viscosity (usually medium- to high-heat milk powders). However, if processing steps include a high pasteurization temperature, low-heat milk powders can also be used effectively for such products. Since the structure of many fermented milk products requires a fine network of caseins, the casein content of the milk powder should be monitored closely. The requirements for the cultures and other ingredients for recombined fermented milk products are not different from those for yogurt made from fresh milk supplies.

Other Recombined Dairy Products

Many other dairy products can be made using recombination technology. Higher-fat-containing products (including butter and cream) are produced effectively by recombining anhydrous milk fat with skim-milk powder, buttermilk, or other milk protein sources. Such ingredients can also be used for the formulation and manufacture of recombined dairy desserts, including ice cream. In higher-fat-containing products, the dairy ingredients must provide good emulsifying properties and/or other emulsifiers must be added to ensure good emulsion stability.

See also: **Cheese**: Overview. **Concentrated Dairy Products**: Evaporated Milk. **Cream**: Products. **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture. **Fermented Milks**: Types and Standards of Identity. **Heat Treatment of Milk**: Heat Stability of Milk; Ultra-High Temperature Treatment (UHT): Heating Systems. **Homogenization of Milk**: High-Pressure Homogenizers; Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers; Other Types of Homogenizer (High-Speed Mixing, Ultrasonics, Microfluidizers, Membrane Emulsification). **Ice Cream and Desserts**: Dairy Desserts. **Lactose and Oligosaccharides**: Maillard Reaction. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milk Protein Products**: Membrane-Based Fractionation. **Plant and Equipment**: Milk Dryers: Drying Principles.

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MAMMALS

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Introduction

Mammals are defined by the ability to produce milk for the nurture of their offspring. Living members of the class Mammalia belong to one of three groups: the egg-laying monotremes (the duck-billed platypus and the echidna) in the subclass Prototheria and in the subclass Theriiformes (therians), the marsupials and the eutherian (placental) mammals. The basic structure of the mammary glands is remarkably similar in all these groups, although monotreme glands do not have nipples. Instead of being suckled, the hatched young lick milk from a specialized skin area.

The early mammal-like reptiles appear in the fossil record (**Table 1**) during the Permian and Triassic Periods. Mammals are thought to have evolved at the close of the Triassic from small therapsid reptiles, called cynodonts. These are found from the late Permian and already share some bony features with mammals, such as specialization of teeth and a hard palate (see 'Mammalian Characteristics', below). Progression to producing milk is suggested to involve the development of cutaneous glands in egg incubation patches producing antimicrobial secretions to first protect the eggs and later supply nutrients to the hatchlings. Part of the argument for this progression comes from the presence in milk of a number of microbial inhibitors (immunoglobulins, lactoferrin, peroxidase and xanthine oxidase). In addition, the milk protein α-lactalbumin, a component of the enzyme lactose synthetase, is thought to have evolved by gene duplication from the bacteriocidal protein lysozyme.

About 5000 mammalian genera are currently known, some 80% of them extinct. Many new fossils have been discovered only in the last decade. In addition, there is a growing database initially from immunological comparisons and more recently from protein sequences and from the nuclear and mitochondrial genome. Some molecular techniques may even become accessible in suitable fossil material. The analysis of molecular data requires sophisticated mathematical and statistical approaches and the use of powerful computer programs. Morphological and molecular evidence are not always easy to reconcile. The traditional view of mammalian relationships (and therefore mammalian taxonomy) is being challenged and the timing of the major events in mammalian evolution is also being reconsidered.

Discussed here are the anatomical and physiological characteristics of mammals, the part played by varied patterns of lactation in adapting mammals to different environments, the taxonomic and evolutionary position of mammals used for milk production and the processes of domestication.

Mammalian Characteristics

In addition to the ability to produce milk from specialized skin glands, the mammary glands, mammals share a number of other anatomical features. These can be considered under two headings, hard parts accessible in both living mammals and fossils, and soft-tissue anatomy very rarely directly accessible and in general uncertain in fossils.

Of the many skeletal characteristics shared by mammals, the one that is generally considered critical and of most practical use in defining them is the structure of the lower jaw. In reptiles, the lower jaw consists of six paired bones. The dentary carries the teeth and the articular forms the joint with the quadrate of the upper jaw. A single bone, the columella auris (equivalent to the stapes of the mammalian ear), is responsible for conducting sound. In mammals, the lower jaw consists of a single bone, the dentary. The other elements of the reptilian jaw have either been lost or, together with the quadrate

Table 1 The geological time-scale, in millions of years ago (mya)

Era	*Period*	*Epoch*
Paleozoic 540-245 mya	Cambrian (540-505 mya) to Permian (286-245 mya)	—
Mesozoic 245-66 mya 'Age of Reptiles'	Triassic (245-210 mya) Jurassic (210-144 mya) Cretaceous (144-66 mya)	—
Cenozoic 66 mya – present 'Age of Mammals'	Tertiary (66-1.8 mya)	Paleocene (66-57 mya) Eocene (57-36 mya) Oligocene (36-23 mya) Miocene (23-5 mya) Pliocene (5-2 mya)
	Quaternary (1.8 mya – present)	Pleistocene (2 mya-10 000 ya) Holocene (10 000 ya – present)

of the upper jaw (now incus) and the articular (malleus), have been incorporated into the middle ear. These changes have resulted in mammalian hearing of acute sensitivity.

All modern mammals have hair. Recent work in China has identified fossilized hair in beds of the Late Paleocene Epoch dated to 55 million years ago, associated with four different mammalian groups, including multituberculates, an ancient group of small herbivores. The evolutionary position of multituberculates is contested. They may be related to either monotremes or therians, or they may be an even earlier, more basal mammalian branch. Either way, this discovery marks hair as a very ancient feature of mammals that may even have been shared by late mammal-like reptiles (cynodonts show evidence of whiskers). Although much reduced in some groups, such as whales, hair provides most mammals with the insulation that helps them to be efficient endotherms, that is to generate and to control effectively their internal body temperature.

Other mammalian features include a muscular diaphragm separating the pleural and abdominal cavities, large lungs of complex structure and a complete division of the heart into four chambers. Mammals have a secondary palate, separating air and food passages so that breathing and mastication can occur simultaneously. This feature is also necessary for effective intake of milk from the mammary glands by young mammals, and has enabled teeth to become specialized.

These efficient physiological adaptations, together with a large and complex brain, have enabled mammals to achieve a wide diversification in size, shape, diet and behaviors. The smallest mammal, the Etruscan shrew, weighs only 2 g as an adult. The largest, the blue whale, can weigh 200 000 kg. Thanks to their sophisticated control over all aspects of their internal milieu, mammals can survive across a wide range of environments, latitudes and altitudes and be active at night as well as by day. They inhabit all continents except Antarctica and all oceans.

Lactation as an Adaptive Character

The possession of mammary glands is diagnostic of mammals but to meet the needs of different species, there are wide variations in patterns of lactation and in the composition of milk. The length of lactation varies from a few days to several years. A major determinant is the body weight of the mother. In general, larger mammals have longer lactations, but there are notable exceptions.

Shorter lactations than body weight would predict occur in mammals that produce milk while fasting. This strategy has been adopted in two different groups of marine mammals, the true or earless seals (Pinnipedia, family Phocidae) and the baleen whales (Cetacea, suborder Mysticeti). An extreme example is the hooded seal (body weight 179 kg) which suckles its young for only 4 days, giving birth on unstable ice floes and taking no food while lactating. By contrast, the other major group of pinnipeds, the sea lions and fur seals (family Otariidae), and the toothed whales and dolphins (Cetacea, suborder Odontoceti) have more extended lactations during which the mothers feed. Remarkably, in cold climates, black, brown and polar bears give birth in dens in mid-winter and start their lactations while dormant or hibernating, neither eating nor drinking during this period. The mother and cubs emerge only 2 or 3 months later, when lactation continues and the mother can start to feed and replace lost body reserves.

Lactations 50% longer than body weight would predict are characteristic of marsupials, bats and primates. In marsupials, this represents a major maternal investment in lactation after a very short pregnancy, shorter than or similar in length to one estrous cycle. The joey is at first continuously attached to a teat in the pouch, then releases the teat at intervals and finally leaves the pouch but returns regularly for milk until finally weaned as much as a year after birth. An interesting aspect of this strategy is that two adjacent mammary glands can be simultaneously producing milks of quite different composition.

The pouch young receives a limited volume of dilute milk high in carbohydrate and low in fat and protein. The offspring leaving the pouch receives more concentrated milk high in fat and protein and lower in carbohydrate. In primates, long lactations provide for an extended period of contact between mother and young, increasing the opportunities for the young to learn social and survival strategies.

Within lactations of average length, mammals vary widely in contact time between mother and young. Rats spend up to 18 h a day with pups attached to the nipples in the 2 weeks after birth. Each day up to 100 milk-ejection episodes of 15 s can occur at intervals of 3–10 min. By contrast, rabbits and hares visit their young once a day, taking only 3–4 min to feed the litter. This is thought to reduce the danger of attracting predators and gives the doe time to forage.

As indicated above for marsupials, the composition of milk varies widely both within and between species. Data for some representative placental mammals at mid-lactation are given in **Table 2**. The adaptive significance of these differences is not always clear, but may relate to a number of factors including the requirements of the offspring, the dictates of maternal metabolism and the constraints on the secretory process itself. One important factor is the state of the neonate at birth, related to reproductive strategy. Some mammals like rodents, termed *r*-selecting species, produce large numbers of very immature (altricial) young after a short gestation period. Others are termed *K*-selecting (many ungulates and primates) and produce a few well-developed (precocial) young. The milk of *K*-species is lower in protein than that of *r*-species, a difference which can be related to slower growth rates. Another important factor is the capacity of the mother to support the metabolic demands of lactation. Over a wide range of species, the daily milk yield (or the output of energy in milk) at peak lactation is correlated with the metabolic body weight of the mother (i.e. body weight raised to the power of 0.75). Nevertheless, there are substantial species differences, which can be related to litter size, mammals with large litters producing proportionally more milk than those with single young. The major carbohydrate in most milks is the disaccharide lactose, which is also the major osmotically active component, drawing water into the secretion and so controlling milk volume. Concentrations of fat and protein largely depend on their secretion rates relative to lactose (and so water). Thus, over many species fat and protein are negatively correlated with lactose, while fat and protein are positively correlated. In early marsupial milk, lactose is replaced by complex carbohydrates that may increase energy content without an effect on the tiny volumes of milk the pouch young can ingest. Lactose is also low in

Table 2 Average concentration (%) of major constituents in milks of some species of placental mammals at mid-lactation. Where no value is shown, data are not available. Methods of analysis may be nonspecific and values based on only limited sampling from wild species

Species		*Fat*	*Protein*	*Sugar*	*Ash*
Lagomorpha					
	Rabbit	15.2	10.3	1.8	1.8
Rodentia					
	Brown rat	8.8	8.1	3.8	1.2
	House mouse	13.1	9.0	3.0	1.5
Primates					
	Baboons	4.6	1.5	7.7	0.3
	Human	4.1	0.8	6.8	0.2
Carnivora					
	Dog	9.5	7.5	3.8	1.1
	Brown bear	18.5	8.5	2.3	1.5
	Hooded seal	61.0	5.1	1.0	—
	Northern fur seal	49.4	10.2	0.1	0.5
Proboscidea					
	African elephant	5.0	4.0	5.3	0.7
Perissodactyla					
	Horse	1.3	1.9	6.9	0.4
Cetacea					
	Blue whale	39.4	11.3	1.3	1.4
	Common dolphin	30.0	10.3	—	0.8
Artiodactyla					
	Cow	3.7	3.2	4.6	0.7
	Sheep	7.3	4.1	5.0	0.8
	Reindeer	10.9	9.5	3.4	1.3

Data largely from Oftedal in Peaker *et al*. (1984).

the milk of species which fast in lactation (bears, true seals and baleen whales), probably representing a water conservation measure.

Quantitatively the most important proteins are the caseins, phosphoproteins specific to milk. They contain a high proportion of essential amino acids, the amino acids which the body cannot make and must be supplied by the diet. They also perform an important function in transporting minerals, calcium, phosphate and magnesium, needed for skeletal development. Indeed, across species calcium and phosphorus are positively correlated with protein concentration. At the amino acid level, caseins evolve very rapidly, presumably because evolutionary constraints are limited on proteins with a nutritional, rather than an enzymic role.

Milk fat consists mainly of triacylglycerols and is among the most variable components, differing between species, but also with stage of lactation, diet and breed. The high fat content of the milk of mammals fasting in lactation (bears, seals and baleen whales) probably reflects the need to switch from glucose to fat mobilization as primary energy source, in order to conserve maternal body protein and water. The very high fat content in the milks of many aquatic mammals is also likely to satisfy the needs of the young for a high-energy-value food to maintain body temperature and to deposit subcutaneous fat for the purposes of insulation.

Evolutionary Divergence of Placental Mammals

The classical view, derived from paleontology, is that a radiation of placental mammals occurred rapidly about 65 million years ago, at the Cretaceous–Tertiary boundary (**Figure 1A**). Relatively few placental mammals are known from Cretaceous Period deposits (144 to 66 million years ago) and most orders of living placental mammals appear as fossils in the early Epochs of the Tertiary Period, the Paleocene and Eocene (66 to 36 million years ago). This rapid diversification, from a few lineages of small carnivores and herbivores to many mammalian orders varying widely in size and habitat, coincides with the extinction of the dinosaurs. While available ecological niches were occupied by dinosaurs, mammals may have been unable to diversify. The extinction of the dinosaurs provided ecological opportunities for placental mammals to radiate adaptively.

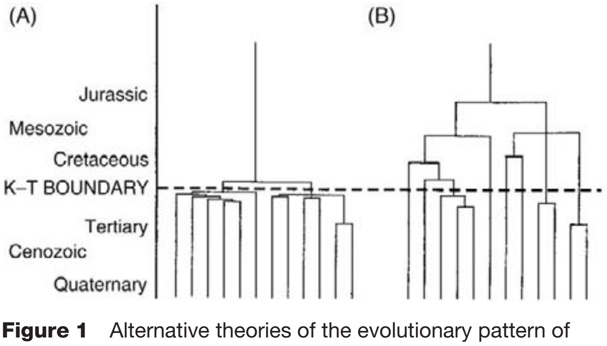


Figure 1 Alternative theories of the evolutionary pattern of placental mammals. (A) The Cretaceous/Tertiary (K–T)-boundary radiation theory indicated by the fossil record; (B) the early branching theory indicated by comparison of molecular sequences. (Reproduced with permission from Easteal, 1999.)

However, calculations of evolutionary rates made by comparing nucleotide and amino acid sequences of DNA and proteins in different species suggest longer timescales and a different pattern. Instead of a rapid radiation over a relatively short period of geological time, molecular evidence suggests a branching pattern of evolution, in which divergences are separated in time and began well before the end of the Cretaceous Period (**Figure 1B**). In this view, the phylogenetic separation of placental mammals into orders occurred before their morphological adaptive radiation. This theory is consistent with biogeographic evidence and suggests that fragmentation of emergent land areas during the Cretaceous may have provided suitable conditions for the diversification of mammalian orders.

The role that biogeography can play in mammalian evolution is well illustrated by the history of the marsupials. These are thought to have originated in North America and spread very widely before the separation of continents. Forms isolated in Australia after its separation in the early Tertiary underwent a major radiation to fill the available ecological niches. South America developed a mixed marsupial/placental fauna, while marsupials became extinct in other parts of the world.

The Classification of Mammals

Taxonomy, the science of classification, is not usually regarded as one of the most exciting areas of biology. However, classification is more complex and much more controversial than might at first be apparent.

Principles of Classification

The system of classification in current use in biology has its origins in the work of the Swedish naturalist Carl Linnaeus (1707–78). He introduced the use of Latin binomial naming, the first (capitalized) name being that of the genus, the second (lower case) of the species. Genera can be combined into higher groupings. For example, domestic sheep (*Ovis aries*), goats (*Capra hircus*) and cattle (*Bos indicus*, zebu or humped cattle and *Bos taurus*, taurine cattle without humps) all belong to the family Bovidae (having hollow, continuously growing horns), suborder Ruminantia (ruminating or chewing the cud), order

Artiodactyla (uniting all even-toed hoofed mammals), grandorder Ungulata (hoofed mammals).

Since the publication of Darwin's *On the Origin of Species* in 1859, the aim of most classifications has been not simply to put living organisms into a convenient 'filing system', based on phenotypic similarities, but to achieve a family tree, reflecting the relationships of common ancestry. Classification based on comparing the anatomy of living and fossil mammals has many pitfalls. Apart from the incomplete and fragmentary nature of the fossil record, there are the problems of parallel and convergent evolution. In order to meet the same challenges of adaptation, different groups may develop similar solutions to the same problem quite independently. As an example, an extinct group of South American mammals, the litopterns, adapted to a life of grazing and the need to escape from predation in open grassland by elongation of their legs and loss of all toes except for the middle one which developed as a hoof. In the nineteenth century, they were proposed as the ancestors of all horses, but are in fact quite unrelated. Moreover, the inheritance of morphological characteristics is generally complex. As we have already seen, the sometimes different views provided by rapidly accumulating molecular information has also to be accommodated, although the interpretation of molecular data is potentially as problematical as of morphological findings.

The strictest form of classification is cladistic taxonomy, the criterion for grouping organisms together being their degree of relatedness, or relative recency of common ancestry. The aim is to identify natural clades, or monophyletic groups. A clade contains a set of organisms descended from a particular common ancestor, which is not an ancestor of any nonmember of the group. Mammals are defined as including the most recent common ancestor of the three living groups (monotremes, marsupials and placentals) and all that ancestor's descendants. Both the class Mammalia and eutherian mammals are thought to be monophyletic. Such a classification will, of course, be subject to continual modification in the light of the latest discoveries. An example of a comprehensive phylogenetic tree is illustrated in **Figure 2**. We are clearly still some way from the ultimate aim of identifying the single true phylogenetic tree, but accumulating information on DNA and protein sequences may make this a realizable goal in the longer term.

Mammals Used by Humans for Milk

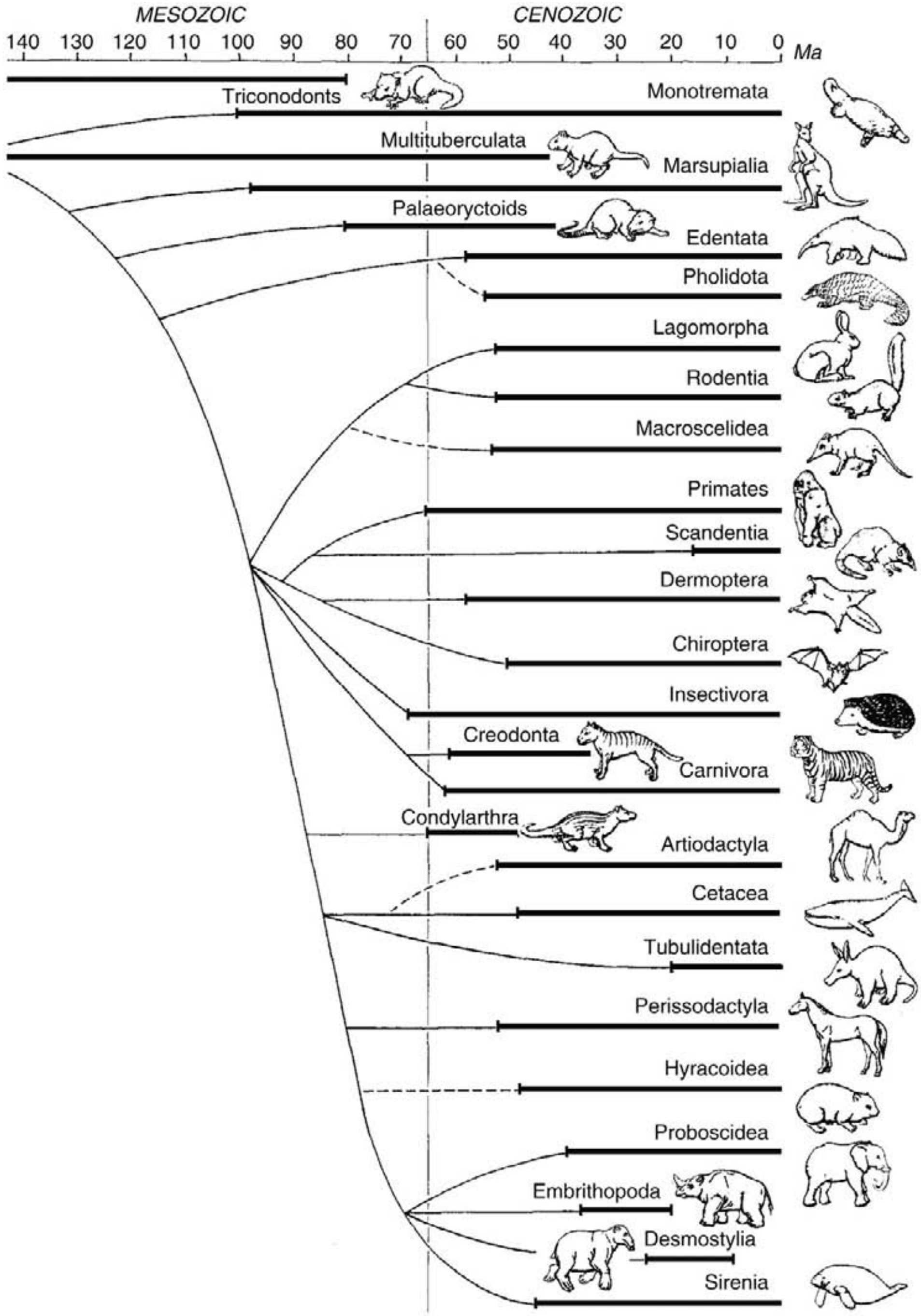
Among about 3000 species of living mammals, a very restricted number of ungulates are used to produce milk for human use. These fall into two taxonomic groups, the Artiodactyla and the Perissodactyla. For many years the standard reference in mammalian classification has been that of George Gaylord Simpson, published in 1945. He divided placental mammals into four cohorts, both Artiodactyla and Perissodactyla being included in the cohort Ferungulata which also included carnivores. This is, however, only one among several attempts to construct a higher classification of mammals based on morphology and these in many respects disagree with each other (see also **Figure 2**).

Order Artiodactyla, even-toed ungulates

The order Artiodactyla is very large with over 180 different species, most of them (123) in the family Bovidae. The majority of dairy animals are artiodactyls. Included are cattle (*see* **Animals that Produce Dairy Foods**: Bos Indicus Breeds and Bos Indicus × Bos Taurus Crosses), water buffalo (*see* **Animals that Produce Dairy Foods**: Water Buffalo), yaks (*see* **Animals that Produce Dairy Foods**: Yak), goats (*see* **Animals that Produce Dairy Foods**: Goat Breeds) and sheep (*see* **Animals that Produce Dairy Foods**: Sheep Breeds) (family Bovidae), camels (*see* **Animals that Produce Dairy Foods**: Camel) (family Camelidae) and reindeer (*see* **Animals that Produce Dairy Foods**: Reindeer) (family Cervidae).

One of the more dramatic revisions to classification in recent years has suggested a close relationship between artiodactyls and cetaceans (whales, dolphins and porpoises). This association is supported by both morphology and molecular studies and leads to the recognition of a monophyletic clade of cetartiodactyls.

However, which artiodactyl group is closest in relationship to cetaceans has been disputed. Living artiodactyls have commonly been divided into three suborders: Suiformes (pigs, peccaries and hippopotamuses), Ruminantia (bovids, deer, mouse-deer and giraffes) and Tylopoda (camels). This classification is now disputed in recent work using short and long interposed elements or retroposons. These are pieces of chromosomal DNA that were at some point transcribed into RNA, then back into DNA and then reincorporated into a new position in the genome. Because these are irreversible events, retroposons make useful tools in phylogeny. A schematic representation of retropositional events at one of the loci studied, the INO locus, is shown in **Figure 3**. The conclusion (**Figure 4**) is that hippopotamuses (formerly classed with pigs and peccaries) are closest to whales and form with whales a monophyletic group. Whales and hippopotamuses share aquatic adaptations including lack of hair, lack of sebaceous glands and underwater vocalizations. These were thought to be convergent adaptations to life in water, but now appear to be shared derived characters or synapomorphies. Camels may form the basal lineage in this group, but more information is needed to confirm this. Among ruminants, hornless chevrotains appear to be the basal group.


MESOZOIC
CENOZOIC
140
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Monotremata
Multituberculata
Marsupialia
Palaeoryctoids
Edentata
Pholidota
Lagomorpha
Rodentia
Macroscelidea
Primates
Scandentia
Dermoptera
Chiroptera
Insectivora
Creodonta
Carnivora
Condylarthra
Artiodactyla
Cetacea
Tubulidentata
Perissodactyla
Hyracoidea
Proboscidea
Embrithopoda
Desmostylia
Sirenia

Figure 2 A phylogenetic tree illustrating relationships among the major mammalian clades. Solid horizontal bars indicate the age range of the clade based on first appearance in the fossil record. Dashed lines indicate more ambiguous relationships. (Reproduced with permission from Novacek, 1992.)

Order Perissodactyla, odd-toed ungulates

The order Perissodactyla is quite small with only 17 extant species. This is the grouping to which horses belong (*see* **Animals that Produce Dairy Foods**: Horse), together with donkeys, rhinoceroses and tapirs. In addition to the domestic horse and donkey, there are six extant species in the genus *Equus*, two species of ass, three species of zebra and Przewalski's horse, which survived extinction in captivity and is now being reintroduced into the wild.

Molecular studies have confirmed that the Perissodactyla are monophyletic. They have also clarified the relationships of the small Middle Eastern and African mammals, the hyraxes, and confirmed their position in the Paenungulata,

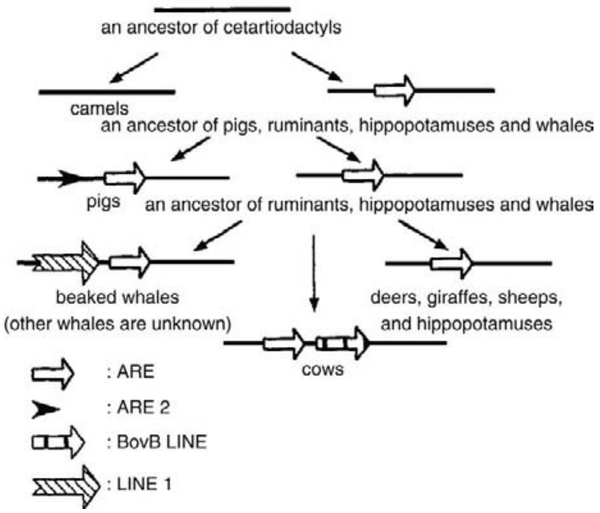
an ancestor of cetartiodactyls
camels
an ancestor of pigs, ruminants, hippopotamuses and whales
pigs
an ancestor of ruminants, hippopotamuses and whales
beaked whales
(other whales are unknown)
deers, giraffes, sheeps, and hippopotamuses
cows
: ARE
: ARE 2
: BovB LINE
: LINE 1

Figure 3 Schematic representation of retropositional events at the INO locus. ARE, artiodactyl repetitive element; LINE, long interspersed element. (Reproduced with permission from Nikaido *et al*., 1999.)

a group including the elephants and sirenia (sea cows and manatees) as its other members. Some morphological work had suggested hyraxes as more closely related to perissodactyls, but this finds no support in protein sequence studies.

From mitochondrial DNA sequences, the evolutionary divergence between the families Equidae and Rhinocerotidae is dated at 50 million years ago.

Domestication

The process of domestication is thought to have been a gradual one, progressing from hunting to herd-following, to management of herds, controlled breeding and finally selection for desired characteristics. The dog had been domesticated by Neolithic hunters about 12 000 years ago and domestication of reindeer may also have been an early event. The origin of the common farm animals is dated to the appearance of livestock bones in Neolithic sites in western Asia about 10 000 to 8000 years ago. Goats and sheep were the first to be domesticated in this area, followed later by cattle and pigs. Migration from the founder groups took place slowly northwest into Europe, west and south into Africa and east to the Indian subcontinent.

For successful domestication, species need to have suitable behaviors. These can be summarized as flocking, breeding in captivity, a wide home range and a short flight distance. Young and smaller animals were probably also selected initially as easier to handle. The ancestor of the domestic goat is the beozar goat, *Capra aegagrus*, of western Asia. Domestic sheep origins are less certain, but their ancestor was probably similar to the mouflon, *Ovis musimon*, both having a diploid chromosome number (2*n*) of 54, the chromosome number of other wild sheep ranging from 52 to 58. All modern domesticated cattle breeds, with the exception of Bali cattle (*Bos javanicus*) and mithan, are believed to be derived from the wild ox or aurochs, *Bos primigenius*. The aurochs only became extinct in Poland in 1627. Although named as different species, the two major types of cattle, the humped zebu (*Bos indicus*) and taurine cattle without humps (*Bos taurus*) are completely cross-fertile and as such may be better considered as subspecies. The general view is that *Bos taurus* was domesticated from the Asian race of aurochs, *Bos primigenius namadicus*. Early remains of small size, so thought to be domestic, have been found in Anatolia dating to 7800 years ago. Zebu breeds were then believed to have developed from taurines as

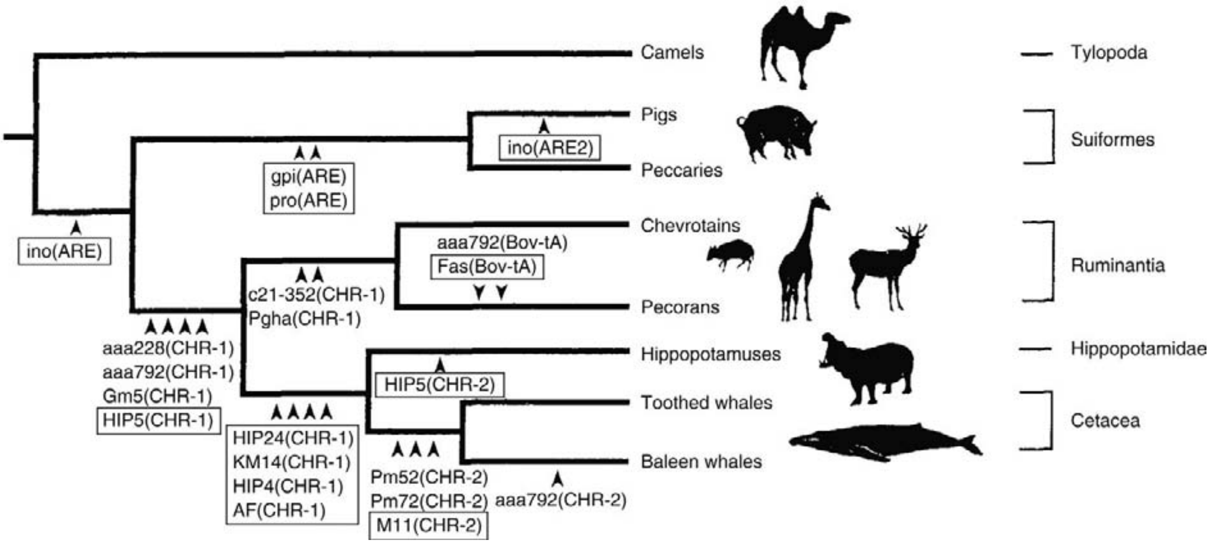
Camels
Tylopoda
Pigs
Peccaries
Suiformes
ino(ARE2)
gpi(ARE)
pro(ARE)
ino(ARE)
Chevrotains
Pecorans
Ruminantia
aaa792(Bov-tA)
Fas(Bov-tA)
c21-352(CHR-1)
Pgha(CHR-1)
aaa228(CHR-1)
aaa792(CHR-1)
Gm5(CHR-1)
HIP5(CHR-1)
Hippopotamuses
Hippopotamidae
HIP5(CHR-2)
Toothed whales
Baleen whales
Cetacea
HIP24(CHR-1)
KM14(CHR-1)
HIP4(CHR-1)
AF(CHR-1)
Pm52(CHR-2)
Pm72(CHR-2)
M11(CHR-2)
aaa792(CHR-2)

Figure 4 Phylogenetic relationships among the major certartiodactyl subgroups, based on evidence from short and long interspersed elements. The insertion sites characterized are mapped on the phylogeny. (Reproduced with permission from Nikaido *et al*., 1999.)

arid-adapted animals after the first domestication event. However, recent work based on mitochondrial DNA suggests two independent domestications of cattle, perhaps from different subspecies of auroch. The situation in African cattle is, however, complex, since they cluster with European breeds with respect to their (maternally inherited) mitochondrial genome, although three of the four African breeds examined were of zebu character. It is suggested that the zebu characteristics were introduced by crossing with *Bos indicus* males.

Water buffalo (*Bubalus bubalis*) can be classified on the basis of morphology, behavior and chromosome number into two types, the river buffalo ($2n = 50$) of the Indian subcontinent, also found in the Balkans and Italy, and the swamp buffalo ($2n = 48$) of southeast Asia, resembling in morphology the wild Asian buffalo, *Bos arnee*. Domestication was some 5000 years ago in India and in China is variously estimated as 4000 or 7000 years ago.

The many and varied breeds of domestic horse belong to a single species, *Equus caballus*. It has a chromosome number ($2n$) of 64, different from any extant wild equid. It is thought to have originated from *Equus ferus*, which was widely distributed in Europe and Asia at the end of the Pleistocene Epoch. Two subspecies are known, a western form, the tarpan, *Equus ferus ferus*, now extinct, and the Mongolian Przewalski's horse, *Equus ferus przewalski*, which has a chromosome number of 66 and forms fertile hybrids with the domestic horse. From a domestication in Turkestan about 4500 years ago, horses spread into Macedonia and reached Egypt about 1500 BC. In China, domestication is known from 1300 BC. It is possible that European and Chinese horses derived respectively from the tarpan and Przewalski's horse, but exchange of horses and so genetic mixing occurred at least 2000 years ago.

See also: **Animals that Produce Dairy Foods**: *Bos indicus* Breeds and *Bos indicus* × *Bos taurus* Crosses; Camel; Donkey; Goat Breeds; Horse; Major Bos Taurus Breeds; Minor and Dual-Purpose Bos Taurus Breeds; Reindeer; Sheep Breeds; Water Buffalo; Yak.

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MAMMARY GLAND

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Anatomy
Growth, Development and Involution
Gene Networks Controlling Development and Involution

Anatomy

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R M Akers, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

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Introduction

Dramatic development of the mammary gland during gestation and subsequent differentiation of alveolar cells to allow onset of milk synthesis and secretion in precise correspondence with parturition is indeed a biological marvel. The initial mammary secretion produced after parturition is called colostrum. Colostrum and the mature milk subsequently produced provide the neonate with a spectrum of nutrients and antibodies necessary for good health and early development. Nutritionally, milk of all mammals contains variable amounts of proteins, carbohydrates, and fats suspended in an aqueous medium. Thus, milk provides each of the major classes of nutrients to the neonate. Although there are species differences in milk composition, having the birth of the offspring and functionality of the mammary gland coincide is clearly critical.

The mammary gland evolved in all mammalian species to nourish the newborn young. However, in dairy animals such as the cow, through genetic selection and advances in milking technology, the mammary gland or udder now yields far more milk than a calf can consume and far greater quantities than the original organ was designed to accommodate. The selection for greater milk production and the removal of the product by machine milking impose unnatural stresses on the bovine udder. Thus, a basic understanding of mammary gland anatomy, supporting structures, milk storage, and the processes involved in milk secretion, letdown, and removal from the udder should aid in the development of procedures to efficiently harvest large volumes of milk from the mammary gland. For the placental mammals, the number of mammary glands varies markedly between classes and species. However, among those studied to date, each mammary gland has a teat or nipple. It is nonetheless worth remembering that only a few of the known mammals have been studied. Because the dairy cow is the most important milk-producing animal from an economic standpoint, the following information is primarily based on the bovine mammary gland. However, some consideration is given to differences in udder development among dairy ruminants.

Gross Anatomy

Regardless of the specific arrangement or number of mammary glands for a given mammal, milk synthesis and secretion require development of a functionally mature mammary gland. In reproductively competent animals, the mature mammary gland consists of a teat or nipple, associated ducts, which provide for passage of milk to the outside, and alveoli composed of epithelial secretory cells and supporting tissues. The epithelial cells are arranged to form the internal lining of the spherical alveoli, and these cells synthesize and secrete milk. Secretions are stored within the internal space of the hollow alveoli and larger ducts between suckling episodes.

Given the variety of mammals and the environmental niches occupied, it is no surprise that there is much variation in the number of mammary glands, location, and composition of secretions. Unlike common dairy species (cows, goats, or sheep), aquatic mammals, especially those in cold environments, produce milk very high in lipid content with relatively less lactose. High lipid content is essential for the suckling young to rapidly produce

Table 1 Variation in location, number, and nipple openings of mammary glands of some common species

Order	*Common name*	*Position of glands*			*Total glands*	*Opening per teat*
		Thoracic	*Abdominal*	*Inguinal*		
Artiodactyla	Cattle			4	4	1
Artiodactyla	Goat			2	2	1
Artiodactyla	Pig	4	6	2	12	2
Artiodactyla	Sheep			2	2	1
Carnivora	Domestic dog	2	6	2	10	8–14
Carnivora	House cat	2	6		8	3–7
Cetacea	Whale			2	2	1
Lagomorpha	Rabbit	4	4	2	10	8–10
Marsupialia	Opossum		13		13	8
Marsupialia	Red kangaroo		4		4	15
Perissodactyla	Horse			2	2	2
Primate	Man	2			2	15–25
Proboscidea	Elephant	2			2	10–11
Rodentia	House mouse	4	2	4	10	1
Rodentia	Norway rat	4	4	4	12	1

a layer of insulating fat to protect them from the cold and to provide a source of metabolically derived water. This illustrates the relevance of lactation to provide a strategy for survival of offspring and for securing reproductive success. **Table 1** illustrates some of the variations found in the number and location of mammary glands in some common species.

Although the basics of mammary development are generally similar among species, the unique anatomy of the udder deserves special attention. In the cow and other ruminants, the mammary glands are clustered together into groups of two (goats or sheep) or four (cattle) mammary glands to create the udder. This arrangement provides a practical advantage. Because the mammary glands and teats are close together, the portion of the milking machine attached to the animal (teat cups and teat cluster) can be relatively compact. For those not familiar with milking and management of modern dairy cows, the udder of a lactating Holstein cow for example can be rather massive. It is not unusual for a single cow to yield 25 kg or more of milk at a single milking. Combined with the mass of the udder tissues, this means that the connective tissue elements and supporting structures of the mammary glands have to support as much as 70 kg of tissue and stored milk just before milking. Given the ventral inguinal orientation of the udder, this is no trivial matter. Support is provided by strong, flat suspensory ligaments, which are attached to the pelvic bone and to the strong tendons of the abdominal muscles in the pelvic area.

In the cow and other ruminants, the udder is divided into two distinct halves, separated by the medial or median suspensory ligaments, which provide most of the strength to hold the udder attached to the ventral body wall. Fibers of the lateral suspensory ligaments are continuous with the median ligaments but spread over either side of the udder so that the gland appears to be held in a sling of connective tissue. The median ligaments are somewhat elastic but the lateral ligaments are not. As the milk accumulates in the udder, the normally vertical orientation of the teats is lost as teats progressively protrude laterally. As animals age, excessive degradation of the fibers of the median suspensory ligament can reduce its support capacity so that the udder becomes pendulous irrespective of time relative to milking. This can lead to problems with milking, that is, difficulty maintaining attachment of teat cups as well as problems with teat injury and increased mastitis risk. The mammary glands of the udder are directly connected to the abdominal cavity only via passage through the inguinal canals. These are paired narrow oblique passages through the abdominal wall on either side of the midline, just above the udder. These canals allow passage of blood and lymph vessels and nerves into the udder.

Interestingly, the two halves of the udder can easily be dissected by cutting along the median suspensory ligament, but there are no evident gross anatomical barriers between the front and rear glands (quarters) on either side of the udder; only a thin connective tissue septum is present. Additionally, there are no direct connections between front and rear quarters. This is easily demonstrated following the injection of dye into the teat opening of one of the mammary glands. The dye stains only the tissue of the gland that is injected. This demonstrates that the mammary glands of the udder are independent. This is sometimes an advantage in some experimental situations since one mammary gland, or more often, one udder half, can be given an experimental treatment with the opposite side serving as a control. This of course is relevant only if treatments can be shown to have only local effects.

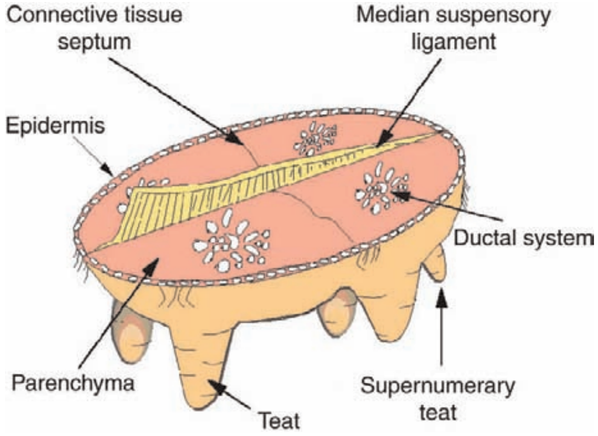

Figure 1 Diagrammatic cross section of the four quarters of the udder illustrating the gross anatomy.

The surface epidermis of the udder is composed of a stratified squamous epithelium and is covered with fine hair; however, the teats are hairless. Although the fore teats are usually longer than the rear teats, the capacity of the rear quarters is greater than that of the fore quarters; the ratio is approximately 60:40 (**Figure 1**).

The dairy goat and dairy sheep industries in the United States are not extensive, but in many parts of the world, these ruminants provide a much greater portion of the milk and dairy products to the local economy than dairy cows. In the case of dairy sheep, international protocols for evaluation of the udder were developed in the early 1980s. Using standardized protocols, the udder structure and development in many dairy breeds have been systemically studied, especially relative to machine milking. Milk production and milk composition are of course critical elements, but udder shape, teat length and size, and ease of machine milking in sheep are also important. Comparisons of external udder morphology and typology are used to standardize groups of ewes for machine milking, choice of animals to create a milking flock, or for culling of breeding animals. A number of researchers have suggested that an ideal udder of a lactating dairy sheep should have the following characteristics: (1) large volume with a globular shape and clearly defined teats; (2) soft and elastic tissues, with an evident, palpable gland cistern; (3) moderate udder height, no lower than the hock; (4) an apparent demarcation or groove between udder halves; and (5) teats of medium size (length and width), oriented in a nearly vertical position. When morphological traits are related to milk production, udder width and height are usually positively correlated with milk yield, confirming the importance of udder volume to milk yield. Interestingly, mammary cistern cavity size in some dairy sheep is nearly as large as that in cows, but of course, total gland size is much smaller than that in cows. This does, however, suggest that a proportionally greater amount of the milk obtained at milking for dairy ewes comes from cisternal storage rather than the alveolar storage. Differences in udder anatomy reflect greater daily milk yields in Lacaune ($1.9\,\mathrm{l\,day^{-1}}$) sheep compared with Manchega sheep ($0.9\,\mathrm{l\,day^{-1}}$). For example, cisternal milk volume and tissue area are more than doubled in Lacaune ewes but alveolar milk yields are essentially identical. This suggests that differences in udder anatomy are important determinants of lactation performance, and in this case, the cistern capacity is especially important. Moreover, it may be that the relative need for oxytocin release at milking might differ among dairy sheep breeds or perhaps among various dairy ruminants depending on the proportion of milk obtained from alveolar compared with cisternal storage.

Patterns of milk flow during an individual milking can be characterized as occurring in 1, 2, or 3, or more peaks. However, there seems to be little if any relationship between the number of peaks and total milk yield. There are also apparent differences in the patterns of oxytocin release among animals within breed as well as average differences in oxytocin release among breeds in response to machine milking. Regardless, it is difficult to define an optimal pattern of oxytocin release since some animals in both high-yielding and low-yielding breeds show minimal secretion of oxytocin but apparently normal milk yields. On the other hand, it is generally accepted that the volume of milk obtained during the primary phase of machine milking (prior to stripping) is greater in animals with more oxytocin release and if there is a bimodal release of oxytocin. These observations simply indicate that relationships among udder anatomy (alveolar vs. cisternal space), effectiveness of udder stimulation or milking to cause oxytocin release, and lactation performance are complex. Considering differences in the degree of selection for milk yield among breeds and differences among dairy ruminants (cows, goats, sheep, and camels), this finding is hardly surprising.

In the dairy cow, each udder half is nearly independent and has its own vascular system, nerve supply, and suspensory apparatus. Teats vary in shape from cylindrical to conical, and teat length is extremely variable. Both characteristics are independent of the shape or size of the udder. The teat skin is thin and devoid of sebaceous glands; however, supernumerary teats are commonly found, mainly on rear quarters. It has been estimated that 40% of cows have one or more supernumerary teats, which tend to be nonfunctional and should be removed because they can become infected with mastitis-causing bacteria.

Supporting Structures

The mammary gland is attached to the cow's body wall under the pelvis by several strong, flat ligaments. Median and lateral suspensory ligaments provide the main support by forming a sling for the udder. The median suspensory ligaments are attached to the pelvic bone and to the tendons of the external oblique abdominal muscles in the region of the pelvis. These ligaments run parallel to each other and pass ventrally between the two udder halves, forming the intermammary groove at the ventral surface, which separates the right and left quarters. These two layers of ligaments are joined by loose areolar connective tissue. The ligaments then separate to cover the anterior, posterior, and ventral areas of the glandular tissue on each udder half but terminate at the base of the teats. The two median suspensory ligaments fuse with the two lateral suspensory ligaments at the anterior, posterior, and ventral borders of each udder half (**Figure 2**).

The lateral suspensory ligaments of each udder half originate at the subpubic and prepubic tendons of the body wall and travel vertically, covering the outer sides of the mammary gland. Both the median and lateral suspensory ligaments have lateral branches (lamellar plates) that are inserted into the glandular tissue and become continuous with the connective tissue stroma supporting the lobules and lobes of parenchyma. The median ligaments are composed of both yellow elastic and fibrous connective tissues. Because of the elastic fibers, this ligament will stretch to absorb the shock as the cow moves about. In addition, the elasticity of these ligaments allows for the increase in udder size between milkings. As a cow matures and the udder increases in weight, the median suspensory ligaments often stretch, weaken, and lose tone, allowing teats to point outward. The lateral ligaments are mainly composed of white fibrous connective tissue (collagen) and do not stretch as much; hence, they provide support in the absence of much elasticity. If both the median and lateral suspensory ligaments weaken, the udder becomes pendulous and is vulnerable to injury and mastitis.

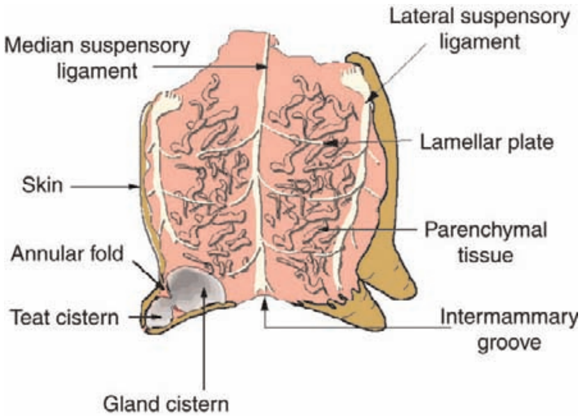

Figure 2 Diagram of longitudinal section of the cow's udder illustrating gross structure and suspensory apparatus.

Microscopic Anatomy

Synthetic and Secretory Tissues

Each of the four quarters functions as a separate gland within the udder and has its own milk secretory (parenchymal) tissues. The parenchyma is composed of alveoli, ducts, and connective tissue; the connective tissue supports and protects the delicate synthetic tissues. The millions of alveoli are the milk-producing units of the udder (*see* **Mammary Gland**: Growth, Development and Involution). These are microscopic globe-like structures that are 50–250 μm in diameter, depending upon the volume of accumulated milk. A single layer of cuboidal to columnar epithelial cells lines the peripheral borders. Milk component precursors are absorbed from blood capillaries adjacent to the alveoli by mammary epithelial cells and are converted into milk protein, lactose, and butterfat. These components are released with other milk components into the lumen or interior of the alveolus for storage between milkings (**Figure 3**).

As milk accumulates in the alveolar lumenal spaces between milkings, the pressure on the epithelial lining causes the secretory cells to become flattened. This signals the cells to stop synthesizing milk and releasing it into the lumen. In addition, capillaries surrounding the alveoli collapse because of the expanding lumenal space, and the supply of milk precursors is reduced. Just prior to milking, approximately 60% of the milk synthesized by the udder is held in the alveoli and small ducts, and 40% is stored in the cisterns and large ducts. After milking, the alveolar lumena are no longer filled with milk, and secretory cells assume a columnar shape as the alveolar lining collapses; capillaries also assume their normal shape. A network of smooth muscle cells called myoepithelial cells immediately surrounds each alveolus. Myoepithelial cells also surround the small ducts, running in a lengthwise direction, and upon contraction they shorten the ducts, thereby increasing the diameter of the ductal lumena, which permits maximum milk flow.

The alveolar epithelial cells, limited by a cell membrane, contain the organelles necessary to convert precursors absorbed from the blood into milk constituents. The interior of the cell is composed of cytoplasm in which organelles such as the nucleus, rough endoplasmic reticulum, mitochondria, and Golgi apparatus are dispersed. The portions of the cytoplasm adjacent to the basement membrane and near the nucleus are occupied by parallel cisternae of rough endoplasmic reticulum (ergastoplasm). The Golgi apparatus is located in a supranuclear position, between the nucleus and apical cell membrane, and is composed of parallel cisternae of smooth-surfaced endoplasmic reticulum with terminal swellings that pinch off as casein-containing secretory vesicles. Butterfat droplets and secretory vesicles

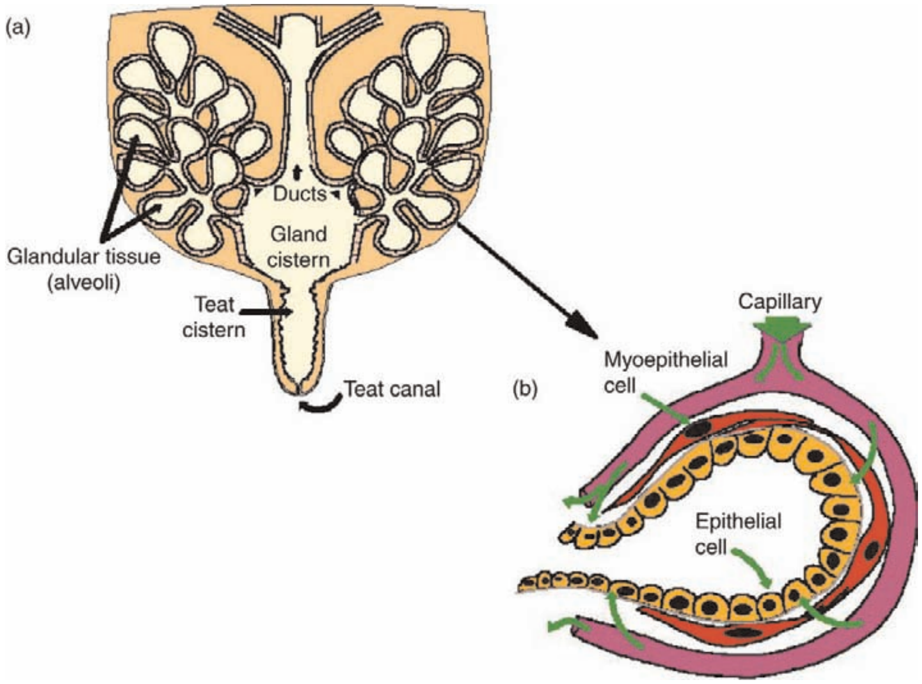

Figure 3 (a) Diagram of a mammary quarter illustrating the glandular tissue (alveoli are drawn out of scale), ducts, gland and teat cisterns, and teat canal. (b) Diagrammatic cross section of an alveolus illustrating mammary epithelial cells, myoepithelial cells, and capillary network.

populate the apical cytoplasm, and microtubules are oriented perpendicular to the plasma membrane to guide the flow of secretory products toward the alveolar lumen. Mitochondria and free ribosomes are found throughout the cytoplasm (**Figure 4**).

Milk protein, most of which is casein, is composed of amino acids that are taken up by cells from the blood. Casein is synthesized in the rough endoplasmic reticulum and transported to the Golgi apparatus, where it is concentrated and packaged in secretory vesicles for export from the cell to the alveolar lumen. Lactose is synthesized in the Golgi apparatus and is secreted from the cells in the same vesicles that transport casein. Calcium, magnesium, and other ions are also secreted via secretory vesicles originating from the Golgi apparatus. Butterfat is synthesized in areas of the cytoplasm occupied by rough endoplasmic reticulum. The size of fat droplets increases from the basal to apical cytoplasm, and many small droplets probably coalesce to form larger droplets. During secretion, the droplets push through the apical cell membrane and are pinched off and released into the lumen, with each droplet limited by a unit membrane that originated from the apical cell membrane. For a more complete discussion of component synthesis and secretion as well as an electron micrograph of a lactating cell, see **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents.

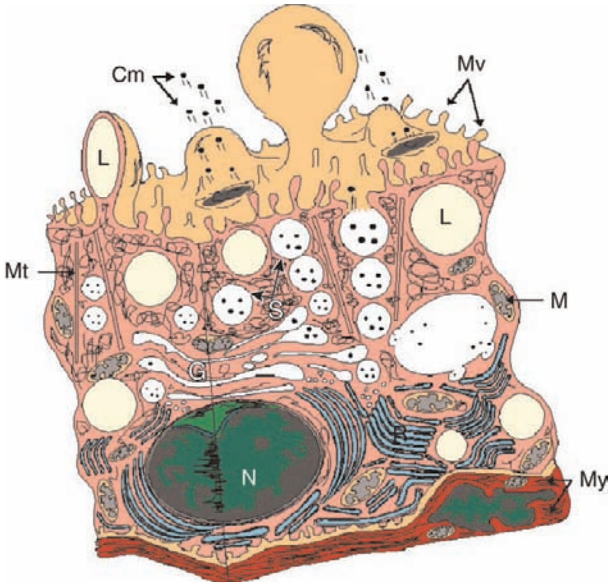

Figure 4 Diagram of an alveolar epithelial cell typical of the lactating bovine mammary gland illustrating an extensive rough endoplasmic reticulum (R), secretory vesicles (S) and numerous casein-containing Golgi secretory vesicles (G) typical of the active milk-producing cell. Other structures include mitochondria (M), microtubules (Mt), nucleus (N), microvilli (Mv), and myoepithelial cells (My). The casein micelles (Cm) and lipid droplets (L) are synthesized within the cell cytoplasm and released into the alveolar lumen for storage between milkings.

The alveoli are drained by small ducts that possess some synthetic activity. Some alveoli have a common opening into a duct, or they may open directly into other alveoli. A cluster of alveoli separated from other clusters of alveoli by fibrous connective tissue is referred to as a lobule, and the ducts draining alveoli converge into a common larger intralobular duct. A cluster of lobules forms a lobe that is drained by a common interlobular duct, and the lobes make up the glandular tissue of a quarter. Each lobe is surrounded by fibrous connective tissue to separate it from other lobes. Within each lobe, the intralobular ducts merge to form a single intralobar duct, which becomes the interlobar duct as it emerges from the lobe. This combination of alveoli and the tubular ducts supported in a connective tissue framework (stroma) classifies each quarter as a tubulo-alveolar gland.

The ducts draining lobes of milk-producing tissues are composed of a double-layered epithelium and are surrounded by myoepithelial cells. These ducts converge into larger ducts that eventually drain into the collecting spaces (cisterns) near the ventral surface of the quarter. From 5 to 20 large ducts empty into the gland cistern of the udder. Gland cisterns are extremely variable in size and shape within an udder, and hold from 100 to 2000 ml of milk. The shape of the gland cistern ranges from a spherical hollow cavity to one composed of folds or divisions, exhibiting a honeycomb appearance. A double-layered epithelium forms the lining of the gland cistern, and lobes of secretory tissue are found immediately adjacent to the lining.

Teat

The gland cistern empties ventrally into the teat cistern, and, at their union, there may be a slight constriction known as the annular fold. The teat cistern is also lined by a double-layered epithelium; however, the superficial (lumenal) epithelial cells are more columnar than cuboidal, and the basal cells are smaller and cuboidal. This cistern holds 10–50 ml of milk, and the surface structure varies greatly. It may be smooth or it may exhibit longitudinal and horizontal folds, giving a pocketed or honeycombed appearance. Lobules of secretory tissue are sometimes present adjacent to the teat cistern lining, which drain directly into the teat cistern (**Figure 5**).

The teat cistern terminates distally at the teat canal, the opening through which milk is removed. The teat canal terminates distally at the teat meatus or orifice. Just above the union of the teat cistern and teat duct, the 6–10 longitudinal folds of the cistern lining converge to form Fürstenberg's rosette. The tissue folds appear to provide no mechanical function in preventing milk leakage as previously theorized. The increased epithelial surface area and connective tissue stroma provided by the folds, however, appear to recruit protective leukocyte populations, especially lymphocytes and plasma cells, which may function in the local defense against mastitis-causing organisms.

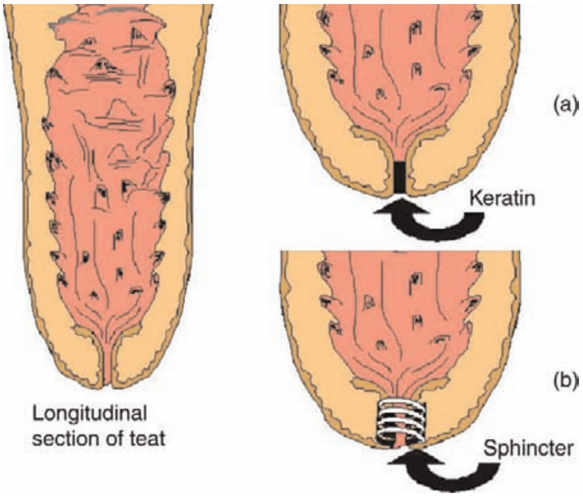

Figure 5 Diagrams of longitudinal sections of the teat highlighting the teat canal keratin (a) and sphincter muscle (b).

Teat Canal

The teat canal is 5–13 mm in length and averages about 8.5 mm. The diameter ranges from 0.4 mm at the distal end to 1.63 mm at the proximal end and averages 0.46 mm at its midportion. With advanced lactation age, the teat canal lengthens and increases in diameter. At the union of the teat cistern and teat canal at Fürstenberg's rosette, the double-layered epithelium abruptly changes ventrally to a stratified squamous epithelium, which is continuous with that of the outer teat skin. Continued desquamation of the cells surrounding the teat canal lumen results in the formation of keratin, which occludes the canal lumen between milkings, serving as a barrier to bacterial penetration.

If keratin is lost or removed, the effective barrier is compromised, and the teat canal may be unable to resist bacterial invasion. For example, if the milk flow-induced shear stress is excessive because of prolonged machine milking time, excessive vacuum, or improper pulsation, some of the keratin may be lost. In addition, the keratin barrier may be compromised by the method of infusing antibiotics into a mammary quarter to treat mastitis. Full insertion of the antibiotic treatment syringe cannula may push portions of keratin colonized by bacteria into the teat cistern and induce an intramammary infection in addition to the one for which therapy was directed. In addition, keratin could be forced against the interior teat duct wall by the syringe cannula, creating a larger than normal opening, thereby enhancing bacterial penetration. The conventional syringe cannula averages 3.1 mm in diameter, and teat duct diameters range from 0.46 to 1.63 mm for distal through proximal portions of the duct. Full insertion of a commercial cannula can result in temporary dilation of the duct lumen beyond the normal diameter (up to 8 times). Likewise, tissue trauma caused by full insertion of the cannula may cause gaps or spaces in keratin, providing areas in which bacteria can adhere and colonize. A comparison of histological cross sections of teat ducts that were inserted with a syringe cannula by partial or full insertion revealed that teat ducts inserted partially had a thicker keratin layer compared with teat ducts infused by full insertion. The latter exhibited partial loss of keratin, which decreased resistance to intramammary infection. Commercial syringes are now available to accommodate partial insertion by providing a twist-off tip, which when removed allows the protrusion of approximately 3.0 mm of the syringe cannula, and at the same time, forms a seal with the teat orifice to provide support during infusion and to ensure upward movement of the antibiotic.

The teat canal is surrounded by bundles of smooth muscle fibers. Fibers are arranged longitudinally immediately adjacent to the epithelial lining and in a circular fashion around the canal deeper in the connective tissue. The circular smooth muscles in their contracted state function to maintain tight closure of the canal between milkings to prevent leakage and to keep keratin occluding the canal lumen compressed as an aid in preventing bacteria from progressing upward into the teat cistern. Teats with weak, relaxed, or incompetent circular smooth muscle bundles (sphincters) are termed ‘patent’ or ‘leaky’. Cows having such teats milk out in 2–3 min, but the incidence of mastitis is higher in quarters with patent teat canals. Cows having teats with tight sphincters are called ‘hard milkers’ because milk is expressed as a fine spray and milk flow is very slow, thereby extending milking time.

Vascular System

Arterial Supply

The vascular system reaches the udder via the right and left inguinal canals in the abdominal wall. Arterial blood from the heart is supplied initially through the posterior dorsal aorta, which becomes the abdominal posterior dorsal aorta after entering the abdominal cavity. This vessel runs parallel to the vertebral column until it reaches the sixth lumbar vertebrae, and then it diverges into the right and left iliac arteries, which in turn diverge into the internal and external iliacs. The external pudendal or mammary artery arises from the external iliac and passes through the inguinal canal to the dorsal surface of the udder. Upon emerging from the inguinal canal, the mammary artery and the associated mammary vein follow a tortuous route forming an S-shaped curve. This allows for the lengthening of the blood vessels as the median suspensory ligaments stretch to accommodate the full and distended udder that gravitates downward.

The mammary arteries enter the right and left halves of the udder just anterior to the rear teats and divide into the anterior and posterior mammary arteries, branching into arterioles that supply the fore and rear quarters, respectively. The subcutaneous abdominal artery usually arises from the mammary artery before it divides into the anterior and posterior branches. This artery supplies blood to the anterior dorsal portion of each side of the udder. The anterior and posterior mammary arteries spread vertically through the parenchyma of the fore and rear quarters of each side, respectively, and divide, ultimately terminating in capillaries that form a network surrounding the alveoli (**Figure 6**).

The mammary arteries also give rise to the papillary arteries of the teats. The vascular tissues of the teat composed of the papillary arteries and venous plexis are collectively termed the corpus cavernosum. The right and left udder halves generally have their own arterial supply; however, some small arterial connections pass from one half to the other. Blood also reaches the udder, to a lesser degree, via the cranial epigastric and perineal arteries that supply, in part, the anterior and posterior portions of the udder, respectively. The arterial blood flow pathway leads from the heart to the udder.

The primary purpose of the arterial system is to provide a continuous supply of nutrients to the milk-synthesizing cells so as to produce milk. The arterial vessels have heavy muscular walls, which aid in driving blood away from the heart to peripheral tissues. In a 500 kg cow, about 71 000 l of blood flow through the

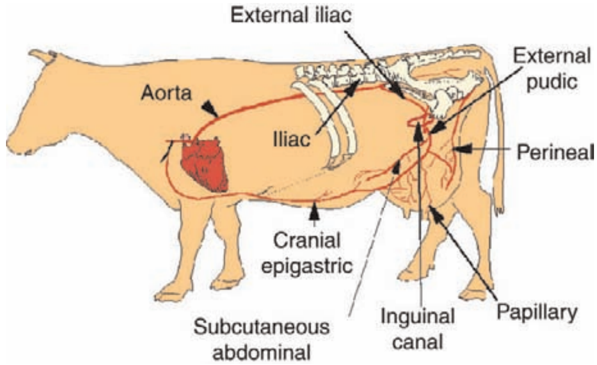

Figure 6 Diagram of the arteries supplying the udder with blood.

udder each day. Approximately 400 volumes of blood pass through the mammary gland to produce 1 volume of milk.

Venous Drainage

After passing through the capillaries surrounding the alveoli, and the interchange between blood and tissue fluids takes place, blood reaches the small veins or venules. These venules run in a dorsal direction and unite to form the larger mammary veins at the base of the udder, forming the venous circle. Veins have thin connective tissue walls and exhibit little change in diameter because venous pressure does not vary greatly. Papillary veins of the teat also course upward to meet the mammary veins and converge upon the venous circle at the base of the udder. The external pudendal vein follows the course of the external pudendal artery, passes through the inguinal canal, and becomes the external iliac vein, which then drains into the posterior vena cava (**Figure 7**).

Anterior extensions of the mammary veins on both sides of the udder are the very prominent and turgid subcutaneous abdominal veins, also known as milk veins in the mature lactating cow. These travel along the ventral surface in a rather tortuous route under the skin but exterior to the abdominal wall. The two veins from each side form an anastomosis in front of the udder and enter through the rectus abdominis muscle near the breast bone to become the internal abdominal veins. They penetrate the diaphragm to become the internal thoracics, which drain into the anterior vena cava. The two main routes by which blood exits the mammary gland are the external pudendal and the subcutaneous abdominal veins. Approximately two-thirds of the blood exits the udder via the external pudendal veins and one-third exits via the subcutaneous abdominal veins. Some blood may leave the rear quarters via the perineal veins. The pathway of venous blood flow is from the udder to the heart.

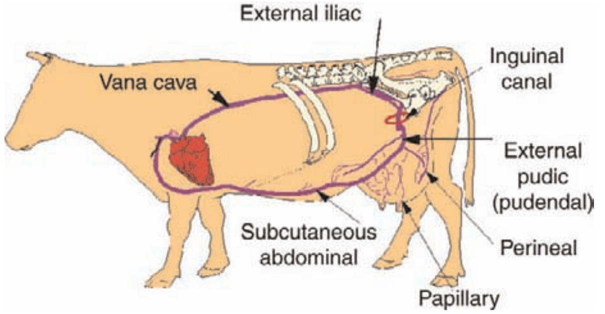

Figure 7 Diagram of the veins draining blood from the udder.

Lymphatic System

Interstitial fluids originating from capillaries that nourish mammary parenchymal cells recirculate via the lymphatic system, which carries waste products away from the udder. The composition of lymph is similar to blood plasma but has half the protein and no red blood cells. Lymph vessels are very thin walled and begin as small capillaries dispersed among the connective tissues of the teat and milk secretory parenchyma. These small vessels converge upon larger lymphatics toward the dorsal portions of the udder, terminating at the supramammary lymph nodes on the right and left halves of the mammary gland. These nodes are located dorsal to the rear quarters, and each side of the udder may have from one to three nodes. The nodes serve as filters that remove or destroy foreign substances and also provide a source of lymphocytes to fight infection. Lymph is filtered through the nodes by entering at the peripheral border, passing through a network of sinuses, and exiting at the hilus via large vessels that pass through the inguinal canal. Vessels may then branch, and the fluid is passed through the inguinal, iliac, and prefemoral lymph nodes before joining the lumbar lymph trunk. The fluid continues to the thoracic duct and empties into the anterior vena cava (**Figure 8**).

Movement of lymph in vessels of the udder is always in a dorsal direction, toward the supramammary lymph nodes. When the udder becomes edematous during the periparturient period, the udder surface can be massaged in the direction of the supramammary lymph nodes as an aid in alleviating the subcutaneous buildup of lymphatic fluids. Lymphatic vessels are equipped with one-way valves to maintain the direction of flow; however, movement is slow because there is no pump to circulate the fluid. The forces behind lymph flow include muscle movement, swaying of the udder as the cow moves about, and breathing; for example, with each inspiration, lymph is drawn forward and emptied into vena cava.

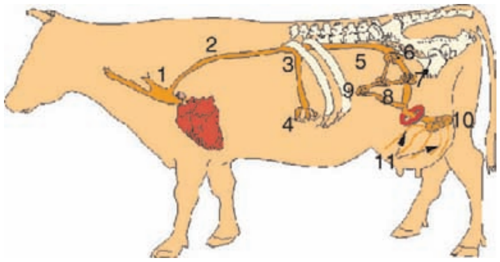

Figure 8 Diagram of the mammary lymphatic system. 1, vena cava; 2, thoracic duct; 3, cisterna chyli; 4, lacteals; 5, lumbar trunk; 6, internal iliac node; 7, inguinal node; 8, external iliac node; 9, prefemoral node; 10, supramammary lymph node; 11, mammary lymphatics.

Nervous System

The major nerves of the udder are the sensory nerves that carry impulses from the four quarters to the brain. Other nerves are sympathetic and are composed of motor fibers to the smooth muscles of arterial walls and those of the teat sphincter. These fibers control the rates of blood flow through the udder by regulating the diameter of arteries and are involved in inhibiting the milk ejection reflex.

The main spinal nerves are the first, second, third, and fourth lumbar nerves and the external spermatic nerves, which become inguinal nerves as they pass through the inguinal canal; these nerves are distributed to the glands and skin via anterior and posterior fibers. The first lumbar nerve supplies the anterior portion of the udder but does not innervate the parenchyma. The second lumbar nerve joins the third lumbar nerve, which fuses with the second and fourth lumbar nerves, composing the inguinal nerve. The perineal nerve, derived from the second, third, and fourth sacral nerves, feeds the caudal portion of the udder. Afferent fibers of the inguinal nerve send signals from the udder to the spinal cord and brain, while the efferent fibers send signals from the brain and spinal cord to the udder via the ventral root ganglia.

Each quarter is supplied with nerves terminating in the dermis of the udder skin and teats, which lead to the spinal column and brain. Innervation of the udder is greatest in the dermis of the teats where pressure-sensitive receptors have been identified. These terminal endings are sensitive to physical stimuli such as pressure, touch, and stretching, and they tend to be more numerous at the proximal end of the teat and close to the surface. The precise nature of the nerve endings has not been established, but the highly specialized sensory nerve endings present in the teats of some species have not been documented in the cow. Impulses travel via afferent fibers through the mammary nerves to the inguinal nerve, which courses through the inguinal canal to the second, third, and fourth lumbar nerves, and the dorsal roots of these nerves carry the afferent signal along the spinal cord to the brain (**Figure 9**).

Nerves also arise from the spinal column and terminate in the muscles of the teat and arteries. The circular smooth muscle bundles surrounding the teat canal undergo continuous rhythmic contractions between milkings via impulses from the sympathetic nervous system. When these nerves are severed or blocked, the cow tends to leak milk. During milking, impulses from the brain and spinal cord cause the muscle bundles to relax, allowing the teat canal to dilate for the flow of milk.

The nervous system has no direct involvement in the synthesis and secretion of milk or in milk removal (ejection) from the udder. These processes are controlled directly by hormones circulating in blood. However, the nervous system is essential to the milking process itself because it triggers the mechanisms of hormone release from the brain to the mammary tissue.

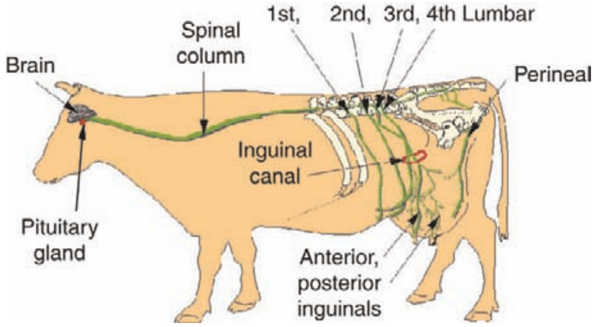

Figure 9 Diagram of the nerves supplying the udder.

See also: **Mammary Gland**: Growth, Development and involution. **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents. **Milk Quality and Udder Health**: Test Methods and Standards.

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Growth, Development and Involution

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Introduction

Mammary glands are accessory reproductive organs that develop to nourish the young. Mammary gland development and lactation may occur multiple times in a mammal's life. In fact, the mammary gland is one of a few body organs that undergo repeated cycles of structural development, functional differentiation, and regression. Careful management of this cycle is the basis for successful lactation in dairy animals. Growth and development of the mammary gland (mammogenesis) occur through a series of phases that are intimately associated with the specific physiology of the animal's growth and reproduction. Each mammary growth phase is regulated systemically by hormones and locally by growth factors produced in the gland. The specific hormones responsible for mammary growth vary with the developmental phase. To understand mammary gland growth and regulation, one needs to have a clear picture of the tissue components that give rise to the growing gland.

Mammary Tissue Components

Mammary parenchyma of the lactating gland is composed of epithelial structures, such as alveoli and ducts, and the associated stromal connective tissue surrounding those structures. Stroma of a lactating gland is primarily composed of cellular and noncellular components of the connective tissue surrounding the epithelial structures. Cellular components of the stroma include fibroblasts, endothelial cells associated with blood vessels, and leukocytes localized in the tissue, while noncellular components include collagen and other connective tissue proteins. In contrast to the lactating gland, considerable white adipose tissue exists in the gland from the early phases of fetal development and it extends through much of pregnancy. This fat pad is often included as part of the stroma of the developing gland, but is considered extraparenchymal tissue.

Alveoli are the basic structures that produce milk during lactation (**Figure 1**). The extensive system of mammary ducts provides a pathway for removal of milk from alveoli. Ducts and alveoli are the defining structures of the mammary gland. Understanding their development has been the primary historical focus of research. Groups of alveoli are organized into clusters, with each cluster constituting a lobule (**Figure 2**). Each alveolus in a lobule drains into an intralobular duct, and intralobular ducts in turn are connected by interlobular ducts. Development of this lobular structure is a fundamental process of mammary growth. Prior to puberty, ductules arising from rudimentary epithelial structures near the base of the teat will elongate by growing into the fat pad. After puberty, these ductules continue to elongate in the cycling heifer, but also begin branching to form structures called terminal ductule lobular units (TDLUs). These structures are characteristic of postpubertal mammary development in the human breast. Similar structures can be observed in the ruminant udder (**Figure 3**). A TDLU makes up a developmental and functional unit of parenchymal tissue. Epithelial components of a TDLU are held in place by loose intralobular connective tissue and are surrounded by a denser interlobular connective tissue sheath. While the lobular organization of mammary tissue is apparent in the cycling heifer, it is during pregnancy that a TDLU will develop further to form a cluster of alveoli in what is recognizable histologically as a lobuloalveolar unit (**Figure 2**).

Development of the epithelial components of TDLU and subsequent differentiation into functioning alveoli are essential elements of mammary development. The importance of nonepithelial tissue components has been highlighted by recent research. For example, growth of mammary epithelium requires the presence of the fat pad in order to form ductal structures (**Figure 4**(**a**)). The ultimate number of epithelial cells in the gland is regulated to a great extent by the fat pad.

The ruminant's mammary fat pad has an abundance of interlobular fibroblastic connective tissue. Of particular note is the network of connective tissue fibrils, which are distributed through the adipose tissue as thick interconnected sheets (**Figure 4**(**b**)). These connective tissue sheets contain extensive networks of collagen, fibroblasts, and blood vessels. Growing ducts and their associated lobular epithelial structures are thought to grow by advancing preferentially through these fibrous connective tissue sheets. Ducts and TDLUs are often surrounded by layers of connective tissue (**Figures 4**(**b**) and **4**(**c**)). This contrasts with mammary development in the mouse, which has often been used as a model to study mammary growth. The mouse mammary fat pad is composed of thin fibrous connective tissue septa, which are irregularly

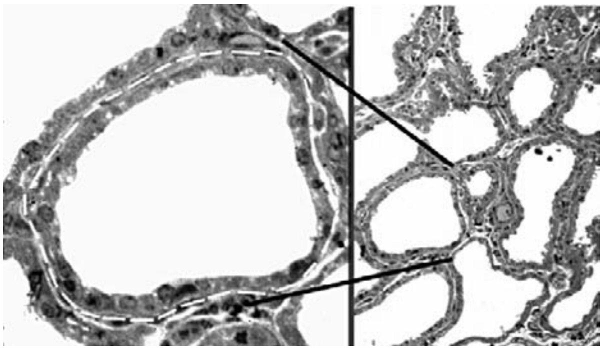

Figure 1 Histological structure of mammary alveoli from a lactating cow. A single alveolus (outlined with a white dotted line in the left panel) is composed of a lumen surrounded by a single layer of epithelial cells. The dotted line is approximately defining the interalveolar connective tissue between adjacent alveoli. Myoepithelial cells (contractile cells) would be between the basal side of the epithelial cells and the basement membrane, but are not typically visible in this type of histological section. The right panel is a group of alveoli within a lobule.

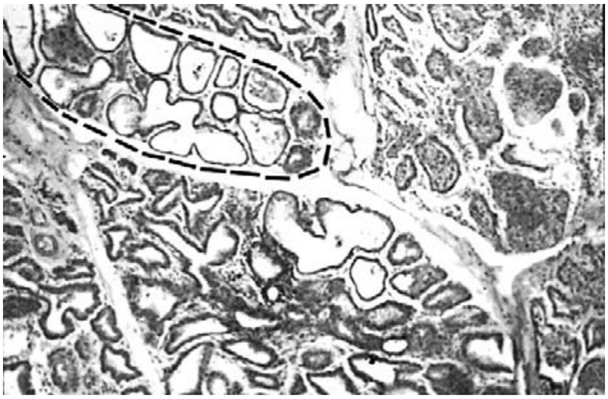

Figure 2 Histological structure of mammary lobular structure from a lactating cow. One lobule is outlined with a dotted line, which is drawn over the interlobular connective tissue. There are portions of as many as five additional lobules present in this section, each with numerous alveoli.

interspersed among the adipocytes. Duct elongation in the mouse results from actively growing epithelial structures in close proximity to adipose cells and surrounded by relatively little fibrous connective tissue.

During the early postnatal period of mammary gland development, ruminant mammary parenchymal tissue grows into the fat pad as a dense mass, replacing the adipose tissue as it progresses. Lipids from the adipocytes are mobilized as the parenchymal structures grow into the fat pad. As the parenchymal mass gets larger, continued ductal elongation seems to occur by epithelial cell proliferation at the periphery of the growing terminal lobule structures. The interactions between epithelial cells and stromal tissue are central to mammary gland development and its regulation by hormones.

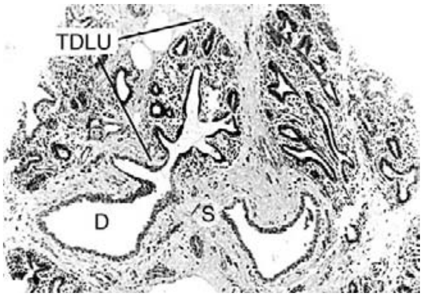

Figure 3 Histological section of a structure similar to a terminal ductule lobular unit (TDLU). This tissue was collected by needle biopsy from a Holstein cow approximately 24 h after milking. This cow was about 15 months into her first lactation, not pregnant, and averaging approximately 13 kg of milk per day. An interlobular duct (D) has side branchings, one of which shows a TDLU in cross section with a cluster of small ductules branching from the central TDLU ductule. Note the distinct staining pattern of the 'loose' intralobular connective tissue of the TDLU compared with the more fibrous connective tissue in the interlobular stroma (S). The presence of these immature structures in a lactating cow suggests that not all TDLUs may develop into milk-secreting lobuloalveolar units during late pregnancy or early lactation. Alternatively, additional TDLUs may develop during lactation, but not differentiate into milk-secreting lobuloalveolar units.

Hormones and Growth Factors

Mammogenic hormones establish the conditions for specific growth patterns in mammary tissue. For example, concurrently elevated blood concentrations of estrogen and progesterone observed in late gestation result in exponential parenchymal growth and in the formation of alveoli, whereas the cyclic changes of hormones associated with estrous cycles result primarily in duct elongation and some lobular tissue formation, but not in the formation of functional alveoli. Mammary development is usually driven by a complex of hormones acting in concert. The effects of many mammogenic hormones are thought to be mediated through stroma-derived growth factors, which act in a paracrine manner by eliciting mitogenic responses in the adjacent epithelial structures (**Figure 4**(**c**)). Much of our current understanding of how mammogenic hormones and growth factors function has been derived from research in rodents. However, similarities have been noted in ruminants, and understanding of hormone action in rodents can help in understanding similar processes in ruminants.

Estrogen is an important mammogenic factor, particularly in the postpubertal female. Estrogen receptors appear in the gland around the time of puberty, coinciding with the period when the gland becomes exposed to cyclic elevations in the blood concentrations of estrogen. Estrogen acts on its receptors in the stromal tissue to stimulate production of growth factors, which in turn stimulate ductal development. Interestingly, the

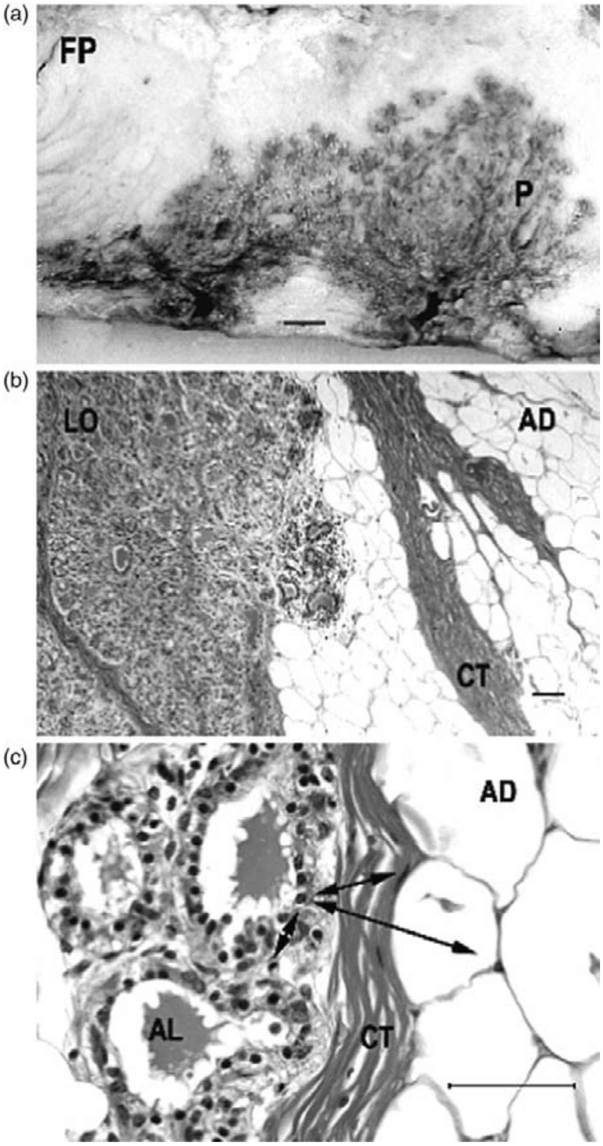

Figure 4 Gross and histological structure of the fat pad and parenchymal tissue in the developing mammary gland. (a) Cross section through fore and rear quarters of a postpubertal beef heifer. Note the branching and elongation of parenchyma (P; ducts, lobules, and associated connective tissue) into the mammary fat pad (FP), and the gland cisterns at the base of the parenchymal tissue. Scale = 1 cm. (b) Low-magnification histological section of developing mammary gland (gilt during early pregnancy) illustrating fibrous connective tissue (CT) sheaths that form a meshwork interspersed among the adipoctyes (AD). Note the development of lobules (LO) associated with the connective tissue sheaths. Scale = 50 μm. (c) High-magnification histological section of developing mammary gland (gilt during early pregnancy) illustrating the structural relationship among alveoli (AL), fibrous connective tissue (CT), and adipocytes (AD). The double-headed arrow represents cross talk of paracrine factors between epithelial cells, cells in connective tissue, and adipocytes. Scale = 50 μm.

prepubertal bovine mammary gland seems to be responsive to estrogen, as well. This effect also is probably mediated through estrogen's effects on the mammary fat pad. The impact of mammogenic hormones on the heifer calf is an emerging area of research.

Progesterone is another ovarian steroid hormone that plays a key role in mammary development. While progesterone receptors have been difficult to identify in mammary fat pad, administration of progesterone can result in proliferation of stromal cells under some physiological conditions. The stimulatory effect of progesterone on DNA synthesis in ductal epithelium is probably mediated indirectly through its effects on stromal cells. The major mammogenic effect of progesterone is the stimulation of ductal side-branching or the formation of alveolar buds, which are the hallmarks of postpubertal mammary development. Estrogen stimulation of progesterone receptor expression in cells is required for this progesterone effect. Progesterone, therefore, has a major role in alveolar morphogenesis and a lesser role in ductal morphogenesis. During estrous cycles, duct elongation and expansion of the parenchymal tissue into the fat pad occur in limited bursts associated with the period of elevated estrogen. During the luteal phase of elevated progesterone in ruminants, relatively little further expansion occurs, but formation and maintenance of lobular structures may be stimulated by progesterone, with little ductal regression occurring between cycles.

Synergy between estrogen and progesterone is observed during pregnancy when both hormones are present in blood at high concentrations. Elevated blood concentrations of estrogen and of progesterone together establish the conditions required for the exponential cell growth that occurs during pregnancy. Lobuloalveolar development represents the greatest increase in mammary gland tissue mass during pregnancy. In the cow, progesterone is elevated throughout gestation, while estrogen concentrations increase gradually and are particularly elevated during the later phase of gestation, coinciding with the period of greatest increase in mammary tissue mass.

Growth hormone (somatotropin) administration to cattle is known to stimulate milk production during lactation. This effect is in part indirect in that growth hormone stimulates secretion of insulin-like growth factor-I (IGF-I) from the liver, which in turn mediates many of the galactopoietic effects of growth hormone during lactation. Growth hormone also acts as a mammogenic hormone and can stimulate mammary growth at all stages of development. Growth hormone may act on ruminant mammary tissue by stimulating stromal production of IGF-I, which is mitogenic for mammary epithelial cells. The highest level of IGF-I expression in mammary tissue occurs in the fat pad and is greatest during the prepubertal allometric growth phase and during late pregnancy. Mammary expression of IGF-I is regulated by growth hormone, estrogen, and positive feedback stimulation from proliferating epithelial cells.

Prolactin is often associated with initiation of lactation and galactopoiesis, but it also has mammogenic effects. Prolactin receptors are present in the fat pad of some species, as well as in the epithelium. Prolactin may act on both epithelial and stromal components of the growing mammary tissue. Inhibition of prolactin secretion inhibits mammary gland development in pregnant goats, pigs, and other species. Blood concentrations of prolactin are normally low during pregnancy. Mammary development during pregnancy may not be limited by the normal blood concentrations of prolactin.

Placental lactogens are secreted from the placenta and they may have prolactin- or growth hormone-like activities, depending upon the species. In pregnant goats, placental lactogen in the maternal blood is closely correlated with the number of fetuses present. This graded concentration of placental lactogen, in combination with other mammogenic hormones, may regulate the extent of mammary development during late pregnancy. In the dairy cow, there is a relationship between placental mass and subsequent milk production. However, the concentration of placental lactogen in the maternal blood of the dairy cow is low, and the effect of placental mass may result from other placental hormones, including estrogen.

Other hormones are also required for mammary growth, including glucocorticoids, thyroid hormones, and insulin. Severely diabetic mice given estrogen and progesterone will develop extensive lobuloalveolar structures. Nevertheless, insulin synergizes with estrogen and progesterone to increase mammary development. Normal blood concentrations of insulin are probably not limiting for normal mammary development.

In addition to the IGFs, other growth factors have positive or negative effects on mammary gland development. Local mammary production of transforming growth factor-β (TGF-β) inhibits mammary growth, during the prepubertal period and between estrous cycles. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) stimulate mammary cell proliferation. Both EGF and TGF-α bind to the EGF receptor. The mammogenic action of estrogen and progesterone occurs in part by decreasing local production of the inhibitory TGF-β, while increasing local production of TGF-α and the levels of EGF receptor in the epithelium. Stromal EGF receptors are also necessary for normal ductal growth. Several additional growth factors produced by stromal cells are known epithelial cell mitogens and may be involved in mediating the effects of mammogenic hormones.

Mammary epithelial structures are often seen growing into a lipid-rich environment of the fat pad. Fatty acids, particularly unsaturated fatty acids, stimulate mammary epithelial cell growth and can substantially enhance the *in vitro* effects of other growth factors such as IGF-I and EGF. Mammary stromal cells are also involved in dissolving the connective tissue collagen, and hence the epithelial structures can continue to grow. Several proteases involved in tissue remodeling and growth of parenchymal tissue are derived from stromal tissue. Extracellular matrix components, which are important for mammary tissue growth and function, are produced by both epithelial cells and stromal cells.

Phases of Mammary Development

Fetal Development

Mammary development begins when the animal is a fetus. The first discernible group of embryonic ectodermal cells that is destined to form the mammary gland is called the mammary band, which runs on either side of the trunk of the fetus from the upper limb to the lower limb. The mammary band appears in the bovine embryo at about 32 days after conception. Mammary bands undergo several further stages of development, ultimately giving rise to the mammary buds. In the bovine species, there are two discrete mammary buds on each side of the embryo's ventral midline in the inguinal region. These give rise to the fore and rear quarters. Mammary buds form early in the second month after conception. The mammary bud stage marks the beginning of differential development patterns of male and female glands. The mammary bud stage is followed by teat development. At the same time, mammary bud cells are invaginating into the surrounding mesenchyme, resulting in formation of a solid core of ectodermal cells, called the primary sprout. The primary sprout ultimately gives rise to the teat and gland cisterns. The mammary fat pad also begins to develop at this time. Limited branching of the primary sprout occurs as the ectodermal cells continue to divide and grow into the surrounding mesenchyme, resulting in cords of epithelial cells called secondary and tertiary sprouts. During secondary sprout formation, the rapidly expanding core of epithelial cells of the primary sprout leads to the formation of a lumen by a process called canalization.

Most major prenatal developmental changes of the gland occur by midgestation. By the time the calf is born, teats are well developed; secondary sprouts are canalized, but still have the solid core of cells at the end; growth of the sprouts is limited to the area around the gland cistern; median suspensory ligament formed; and mammary connective tissue, fat pad, blood vessels, and lymph vessels have formed. There is no development of mammary secretory or glandular structures during the fetal stage of development. In the calf, the mammary fat pad makes up the majority of the udder's mass.

Postnatal Development

In the neonatal ruminant, the parenchymal tissue of each gland, or udder quarter, includes a single primary duct, which branches to form several secondary and tertiary ducts. At the ends of the secondary and tertiary ducts are clusters of ductules, which will give rise to the TDLU-like structures after puberty.

Growth of mammary parenchyma of the heifer calf occurs at the same rate as general body growth (isometric growth) for the first 2–3 months after birth. Mammary parenchyma then begins growing faster than the body (allometric growth) until about 9 months of age or a short time beyond puberty, after which mammary growth returns to an isometric rate. The major increase in total udder size during the prepubertal period results from continued growth of the fat pad.

Individual differences in udder shape and size are observed at birth; however, palpation of the gland from birth to 6 months of age is a poor predictor of the potential for future milk production of the mature animal. Correlations between mammary DNA and subsequent milk production are low in young heifers. Nevertheless, the prepubertal period marks an important phase for mammary gland development and subsequent milk production. Nutrition and growth rate of prepubertal heifers can significantly affect mammary development during this phase and may affect milk yield once the heifer begins lactation. Underfeeding during the prepubertal period decreases subsequent milk yield. The effect of feeding for high growth rates in prepubertal heifers, but not postpubertal heifers, may have a negative impact on mammary gland development and subsequent lactation. However, not all studies have shown these negative effects of feeding for high growth rates. Prepubertal nutrition may also affect the response of the developing mammary gland to mammogenic hormones. The mechanism by which prepubertal feeding may affect subsequent milk yield remains an active area of investigation.

Growth hormone concentrations in the blood of prepubertal heifers on restricted intake (slower growth rates) are increased compared with heifers having *ad libitum* access to feed. Mammary parenchymal tissue mass is positively correlated with growth hormone levels and negatively correlated with mammary adipose tissue. This is consistent with the high level of IGF-I expression in mammary tissue during the prepubertal allometric growth phase, as noted above. Growth hormone administration during the prepubertal period increases mammary parenchyma and decreases extraparenchymal tissue compared with controls, but does not seem to increase milk yield during the first lactation in heifers. The limited increase in mammary development in the prepubertal period may be overshadowed by the exponential growth occurring during late pregnancy.

After puberty, the gland is exposed to cyclic changes in ovarian steroid hormones. In postpubertal ruminants, mammary development occurs in bursts of duct elongation and development (**Figure 4**(**a**)), and development of the TDLU. The postestrus decline in parenchymal tissue is irregular. There is a linear relationship between increased udder weight and increasing age of the heifer up to about 30 months. This is partly due to increased body weight and partly due to accumulation of udder fat as heifers put on body conditioning with advancing age.

Pregnancy

Once the animal becomes pregnant, development of the mammary gland accelerates at an exponential rate. This means that mammary growth is greatest during the later stages of pregnancy, coinciding with the most rapid period of fetal growth. Extensive lobuloalveolar development occurs only during pregnancy. This period is important in determining the number of secretory cells in the lactating gland and the subsequent production of milk. Correlations between total DNA in a lactating gland and milk yield range between 0.50 and 0.85, depending on the species and other factors.

Lactation

Mammary cell numbers continue to increase after parturition. The impact of postpartum mammary growth on milk production can be substantial in litter-bearing species such as the rat and pig, where total mammary DNA can increase by over 100% during lactation depending upon litter size. In contrast, postpartum cell proliferation in dairy ruminants is thought to occur only in the early postpartum period. Mammary DNA in lactating goats increases by about 25% during the first 3 weeks of lactation. Because milk production does not peak until about 8 weeks of lactation in goats, the postpartum increase in mammary cell numbers is thought to contribute only a portion of the ascending phase of the lactation curve, with increased milk synthesis per cell being the major contributor. In cows, mammary DNA increases by 65% between 10 days prepartum and 10 days postpartum, although how much of this increase occurred prepartum versus postpartum is unclear. Cell numbers in the mammary gland of the cow have not been determined throughout the lactating period.

Mammary Gland Involution

The dairy cow requires a nonlactating period prior to an impending parturition in order to optimize milk production in the subsequent lactation. This period is called the dry period and it includes the period between cessation of milk removal at dry-off and the initiation of milking at the subsequent calving. If the dry period is less than 40 days, then milk yield in the next lactation is typically decreased. Physiology of the mammary gland during the dry period differs markedly from that during lactation. The dry period can be divided into three phases: active involution, steady-state involution (mid-dry period), and redevelopment and colostrogenesis. Active involution begins with cessation of periodic milk removal, either by drying off the cow or by weaning the young. It is the phase when the mammary gland makes the transition from a lactating to a nonlactating state and it marks the final phase of a lactation cycle.

Mammary gland involution is initiated when milk is no longer removed from the gland. Mammary involution in a species like the mouse is characterized by a rapid loss of tissue function, cell death, and degeneration of alveolar structure resulting from loss of epithelial cells. The number of cells in the gland at any given time is a function of the balance between cell division and apoptosis (programmed cell death or cell suicide). Loss of mammary cells by apoptosis also occurs in the mammary tissue of goats and cows, indicating that mammary epithelial cells are indeed lost during involution in these species. In contrast to the involuting mouse mammary gland, loss of epithelial cells from bovine mammary parenchyma is gradual during involution, requiring 3 or 4 weeks to be completed. General structural integrity of lobular units in the tissue seems to be retained throughout involution. Redevelopment of the mammary gland in cows during the dry period, as defined by an increase in cell division, begins 5–6 weeks prior to calving. Interestingly, some evidence indicates that apoptosis is actually increased in very early lactation, perhaps as a means of eliminating dysfunctional cells in the tissue.

Systemic and locally produced IGF-I play important roles in the survival of mammary epithelial cells. Activity of IGF-I in tissues is modulated by local production of IGF binding proteins (IGFBPs), a family of proteins that may inhibit or enhance IGF activity depending on a number of factors. Research in rodents and sows indicates that specific IGFBPs produced by mammary tissue during early involution are involved in inhibiting IGF-mediated cell survival. This may be part of the mechanism for increased apoptotic cell death during involution. Some IGFBPs may have a similar function in cattle and sheep.

Histological and ultrastructural changes associated with involution begin within 48 h after cessation of milk removal in bovine mammary tissue. The most apparent change is the formation of large stasis vacuoles in the epithelial cells (**Figure 5**(**b**)), formed largely as a result of intracellular accumulation of milk fat droplets and secretory vesicles. These vacuoles persist for at least 2 weeks of involution, but typically are absent by the fourth week. Alveolar lumenal area declines during this period, while interalveolar stromal area increases (**Figure 5**(**c**)).

A substantial reduction in fluid volume in the gland occurs between days 3 and 7 after drying-off. Fluid volume continues to decrease through about 4 weeks of involution. Concentrations of milk proteins, fat, and lactose decline during the initial 1–2 weeks of the dry period. Concentrations of several protective factors, such as lactoferrin and leukocytes, increase during early involution. The protective factors are important for the gland's resistance to intramammary infection.

The length of the steady-state period depends on the total length of the dry period. If active involution takes about 3–4 weeks to complete in the dairy cow and the redevelopment stage takes about 3–4 weeks, then the gland spends little time in the steady-state involution phase during the recommended 45- to 60-day dry period.

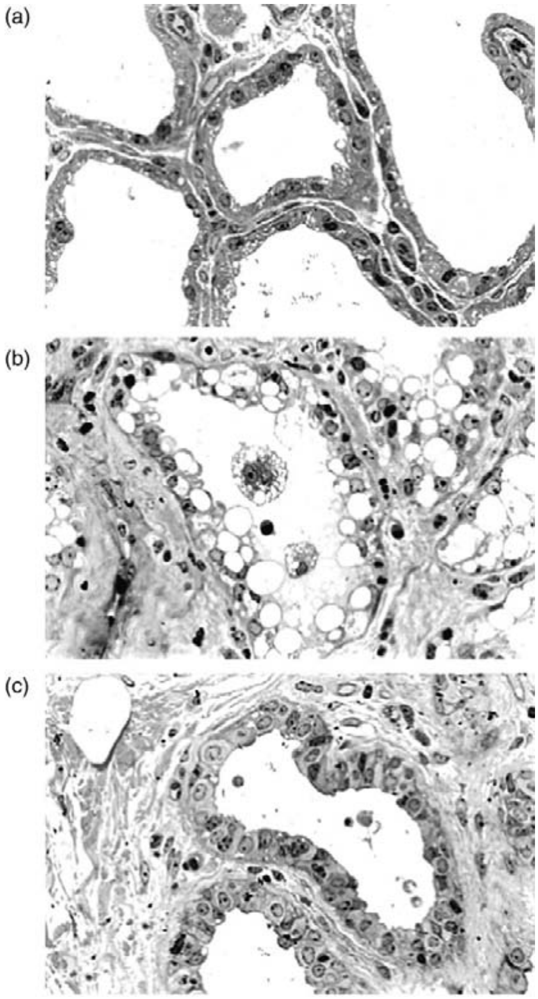

Figure 5 Histological structure of bovine mammary gland tissue during involution. (a) Alveoli of a lactating mammary gland with large lumens. Minimal stromal tissue is present in the lactating tissue. (b) Mammary tissue 4 days after drying-off. Alveolar epithelial cells contain large vacuoles. Interalveolar connective tissue is increased compared with lactating tissue. Note leukocytes in the alveolar lumen; leukocyte concentration is increased in mammary secretions during involution. (c) Mammary tissue 21 days after drying-off. Vacuoles are no longer present. Extensive interalveolar connective tissue is present. Integrity of epithelial structures remains intact with a single layer of cells.

Synthesis of DNA in mammary tissue begins as much as 35 days prepartum in the pregnant dry cow. This marks the phase of the dry period when the gland begins the transition from the nonlactating state to the lactating state. Selective transport of IgG_1 is a major activity of epithelial cells during the last 2–3 weeks prior to parturition and is one of the hallmarks of colostrum formation. Concentrations of major milk components begin to increase from about 2 weeks prepartum, and then increase markedly from 3 to 5 days prepartum leading up to the subsequent initiation of lactation.

Functional Genomics of Mammary Gland Development

Characterization of gene expression by microarray analysis of tissue RNA from mammary glands during developmental stages, lactation, and involution is beginning to provide important insights into the complex interplay of genes involved in mammary gland development. Early studies using microarray technology support the concept that responses of mammary fat pad to estrogen may occur through the secretion of paracrine factors that stimulate stem cell growth. Further studies of mammary development in preweaning dairy heifer calves confirm that even at that very young age there is a differential expression of cytokines and growth factors in parenchymal and fat pad tissues of the mammary gland. These factors are involved in tissue development and cell cycle functions. This preweaning mammary growth may also be affected by the nutritional status of the calf, although the significance of this effect on future milk production remains to be fully demonstrated. Additional studies of gene expression in involuting mammary gland tissue of cows using microarray technology confirm that milk stasis causes diminished milk synthesis, and also initiates a decline in the metabolic function of the gland as it transforms from a secretory to a nonsecretory tissue. Genes related to lipid metabolism are downregulated after cessation of milking. Expression of genes associated with apoptosis, cell growth and proliferation, and inflammation is increased during involution of the bovine mammary gland, suggesting a coordinated expression of genes in these functional pathways. Functional genomic studies of bovine mammary gland tissue at defined stages of development and under controlled physiological conditions will offer further important insights into the cellular and molecular mechanisms that control growth and development of this rapidly changing tissue.

Conclusion

Mammary gland development and lactation may occur multiple times in a mammal's life. Development of mammary gland epithelial structures relies on the presence of a mammary fat pad and other stromal components of the tissue. Many mammogenic hormones function through their effects on stromal tissue, leading to the secretion of growth factors that control epithelial development. Development of lobular epithelial structures in the gland begins after puberty in response to cyclic changes in ovarian steroid hormones. Exponential growth of lobular structures and development of alveoli within the lobules occur during pregnancy in response to high blood concentrations of estrogen and progesterone.

Further mammary gland growth after parturition is limited in dairy ruminants, although production of milk continues to increase up to peak lactation. When milk removal is stopped at the end of lactation, the mammary gland undergoes a process of involution, which returns the tissue to a nonlactating state that is prepared for the next cycle of mammary growth, lactation, and regression. A greater understanding of the growth regulatory factors at each phase of development, as well as an understanding of the relationship among the developmental phases, is essential for enhancing mammary gland growth. Current studies using genome-enabled technologies (transcriptomics, proteomics, gene silencing) will contribute to the understanding of mammary gland development.

See also: **Mammary Gland**: Anatomy; Gene Networks Controlling Development and Involution.

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Gene Networks Controlling Development and Involution

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Introduction

Structural and functional developments of the mammary gland occur in multiple cycles during the life of the animal. The mammary gland is one of the few tissues in the body that undergo repeated cycles of structural development, functional differentiation, and regression. Regulation of these dramatic cyclic changes in the tissue occurs through an orchestrated set of cellular metabolism, cell physiology, cell growth, and cell death. Most, if not all, of the molecular events required for mammary development during the life cycle are under the control of genes that are encoded by DNA molecules. Expression of genes occurs via the synthesis of RNA, which is a complementary copy of the DNA encoding each gene in the animal's genome. External stimuli (e.g., nutrients, hormones, growth factors) and internal control mechanisms regulate the expression of genes. RNA molecules are translated into proteins, which perform biological functions in the cells. Historically, investigators of mammary biology have been limited by the research tools available to them in their quest to understand the mammary gland. Several newly developed technologies for gene expression analysis are providing the opportunity to study the mammary gland through a more precise and expansive lens that encompasses the expression of extremely large numbers of genes. Such a global view of what is happening in the mammary gland is beginning to change the way in which we think about this important tissue.

Microarray Technology

DNA microarray technology allows quantitative and simultaneous monitoring of the expression of thousands of genes in tissues or cells. This technology has made its impact upon many basic scientific disciplines including cancer biology, developmental biology, toxicology, investigation of growth factor and hormonal signaling, and the applied areas of disease diagnostics and drug development. Application of microarray technology to the exploration of genomic plasticity in tissues of economically important livestock animals is still in its infancy.

Monitoring gene expression by microarray is achieved through the ability of single-stranded DNA molecules to hybridize with a complementary molecule of single-stranded DNA. Microarray 'chips' can contain thousands of DNA sequences encoding highly specific regions of target RNA. To measure the abundance of each RNA molecule in a sample, RNA extracted from a biological sample of interest is reverse-transcribed into complementary DNA (cDNA) molecules that ideally represent a quantitative copy of RNA at the time of sample collection. During the synthesis and processing of cDNA, fluorescent dyes are incorporated into the molecules. The cDNA molecules produced can then hybridize with cDNA or aligonucleotide molecules in the microarray chips. Subsequently, the abundance of each specific cDNA in the sample of interest can be measured through evaluation of fluorescent signal intensities.

In essence, a cDNA microarray experiment involves competitive hybridization between a sample that is labeled with a red fluorescent dye (cyanine 5, Cy5) and a sample that is labeled with a green fluorescent dye (cyanine 3, Cy3). The signal intensity (i.e., measure of gene expression) values that are captured digitally are analyzed by means of commercial software to obtain numerical data. When a 'reference sample' design is used for microarray studies, all comparisons are made against the reference RNA sample (i.e., a mixture of RNA from different animals and/or tissues) and the final data for each sample of interest are the average of the signal intensity of the sample divided by the signal intensity of the reference sample across both dye combinations (i.e., Cy3 and Cy5). This final ratio is used to compare RNA abundance among samples. The resulting data set is then subjected to statistical analysis to generate a list of differentially expressed genes, that is, genes that are affected by changes in physiological state (e.g., pregnancy to lactation) or by hormonal or nutritional treatments.

Construction of microarray chips is generally dependent on information gained from genome sequencing or expressed sequence tag (EST) projects that provide large sets of annotated clones and RNA sequences. Currently available types of microarray platforms include cDNA microarrays (usually pure products from polymerase chain reaction (PCR) amplification of cDNA and EST clones that are 100–2000 nucleotides long), oligonucleotide microarrays (60–70 nucleotides per DNA synthesized from single-stranded probes on the basis of sequence information in databases), and Affymetrix GeneChips (35–200 nucleotides per DNA), which have multiple probes per gene. Probes are printed onto glass slides by robotics (cDNA and oligonucleotide platforms) or synthesized *in situ* by photolithographic synthesis (oligonucleotide or Affymetrix GeneChips).

Data Mining

Extrapolation of biological meaning from the vast amounts of data obtained by microarray analysis constitutes a fascinating challenge for scientists. Fortunately, there are commercially and freely available bioinformatic tools to help with visualization and mining of microarray data. Bioinformatics involves the use of computer resources that encompass mathematics, statistics, and biochemistry in order to solve biological problems at the molecular level and on a scale of magnitude that is too large for direct human achievement. Data mining can encompass manual compilation of functionally annotated genes from publicly available online resources (e.g., National Center for Biotechnology Information). Scientists can browse detailed information on groups of genes that is available in the published scientific literature. Knowledge that can be obtained for groups of genes includes specific molecular functions, well-described biochemical pathways, and functional or physical relationships with other genes.

Functional Genomics of the Mammary Gland

Pregnancy through Lactation

While functional genomics resources for laboratory animals are relatively mature, there is increasing attention toward their development and use in livestock animals. Early studies of bovine mammary gene expression during the periparturient period and different developmental stages have been reported. Often, those studies suffered from limited biological replication, which reduces the inference power of the results obtained. A general approach to circumvent this problem is the sampling of multiple animals to ensure that effects that do achieve statistical significance are real and will be reproducible in different settings. Despite their limitations, those early studies provided the basis for designing appropriate experiments to study mammary genomic plasticity with microarrays as has been done in laboratory animals.

The elucidation of integrated networks of signaling events controlling development of the bovine mammary gland is under way. Hormones and growth factors activate a myriad of receptors including steroid receptors as well as cell surface receptors, in addition to other receptor families. Engagement of these receptors then sets in motion complex intracellular signaling networks, ultimately leading to activation of transcription factors to regulate gene expression. A few examples of the use of functional genomics to characterize these networks are presented here. Characterization of bovine mammary transcript profiles during the transition from late pregnancy through lactation (**Figure 1**) is helping to define stage-specific gene sets, as already accomplished in the mouse. Compared to other biological functions (e.g., molecular transport, metabolism), microarray analysis of bovine mammary tissue has revealed that immune-related genes appear to decrease in expression as lactation progresses (i.e., from parturition through end of subsequent lactation). This analysis has also allowed the discovery of more than 100 transcription factors whose expression changes significantly throughout lactation. A transcription factor is a protein that binds to specific sequences of DNA, thereby controlling the transfer (or transcription) of genetic information from DNA to RNA. Transcription factors perform this function, alone or in complex with other proteins, by promoting (as an activator) or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that activates the transcription of genetic information from DNA to RNA) to specific genes. It is likely that the transcription factors identified in the bovine mammary transcript profiles regulate the transcriptomics adaptations observed. These results not only provide much-needed molecular information but also form the basis for more detailed functional studies in the future. From the same data, more than 500 genes had a consistent upregulation and more than 500 genes, a consistent downregulation in expression during lactation compared with late pregnancy. The upregulated genes are primarily involved in protein synthesis, vesicle-mediated transport, and apoptosis (cell death). The major portion of the downregulated genes are associated with cell/tissue development, cell differentiation, and cell cycle.

Currently, several approaches are being used to mine large-scale mammary microarray data. Clustering of genes into different groups allows the identification of those elements that share some common biological function. Gene network analysis also is an alternative approach to establish relationships among genes within large microarray data sets. Such gene networks can be used to model or describe all possible regulatory interactions occurring under any condition. It is often practical, however, to study in greater detail smaller portions of the network that can be considered autonomous. In the case of the mammary gland, such a subnetwork unit (i.e., a module) could encompass the reconstruction of metabolic networks associated with milk synthesis. Through the use of RNA expression, milk composition and yield, and published data from the nonruminant literature, milk fat synthesis and

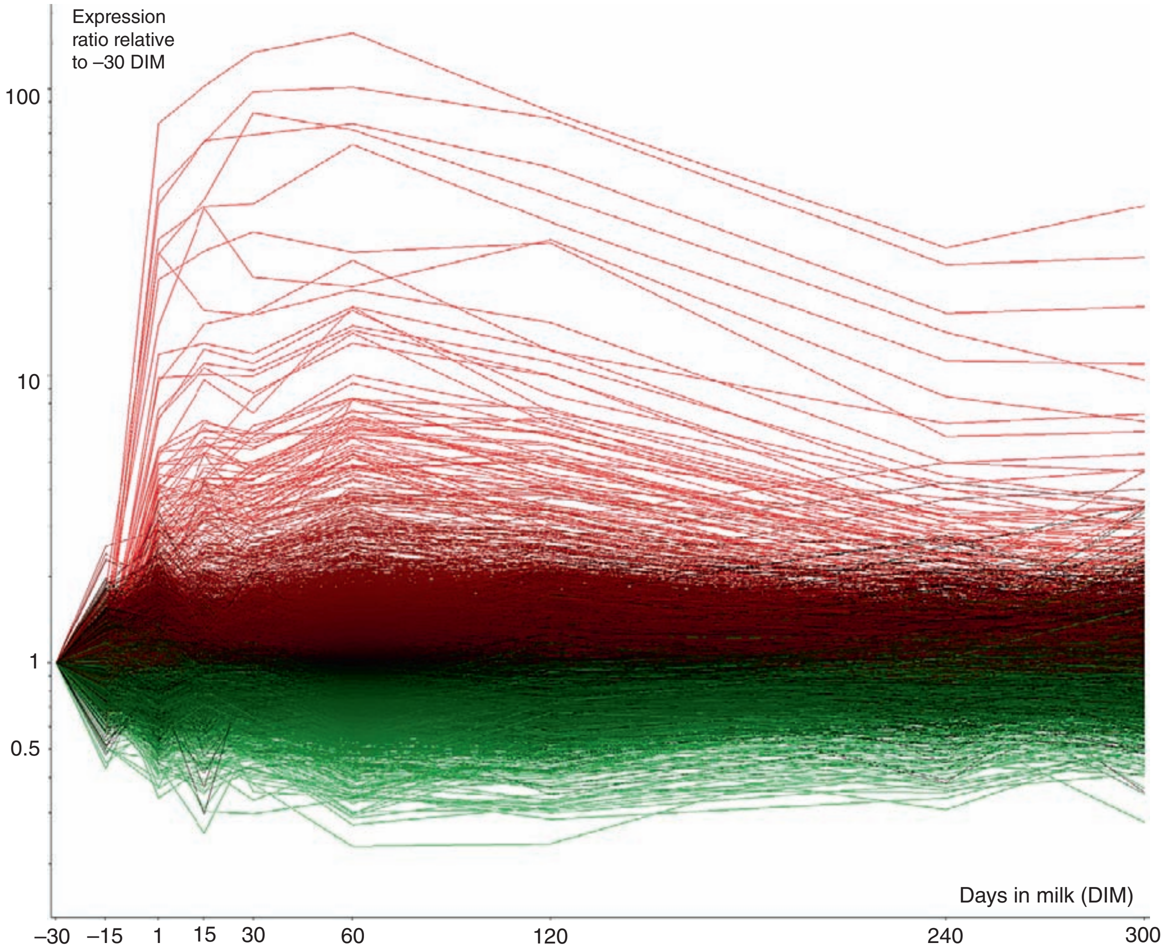

Figure 1 Transcriptomics adaptation of bovine mammary gland tissue from late pregnancy to the end of subsequent lactation (approximately −30 days from parturition through 300 days postpartum). Mammary gland tissue from eight Holstein dairy cows was biopsied during the study at various days in milk (DIM). Expression ratio (*Y*-axis) indicates for each gene the ratio between the level of expression at each time point relative to the expression at −30 DIM. Expression ratios >1 are upregulated expression of the gene compared with the expression level at −30 days from parturition; normalized ratios <1 are downregulated expression of the gene relative to the expression level at −30 days from parturition. Data shown include temporal gene expression pattern of over 6000 differentially expressed genes.

secretion networks in bovine mammary tissue have been reconstructed through the lactation cycle. Genes with potential roles in mammary *de novo* fatty acid synthesis, triglyceride synthesis, phospholipid synthesis, fatty acid import and trafficking, and lipid droplet secretion were used to reconstruct the mammary lipid synthesis network in Holstein cows (**Figure 2**). RNA expression was evaluated in mammary gland tissue biopsied from 2 weeks prepartum through 8 months of lactation via quantitative PCR. Results from this study reinforced the hypothesis that a network of genes controlled by several transcription factors participates in coordinating milk fat synthesis and secretion, as opposed to a single transcription factor being central for milk fat synthesis regulation. This gene network also identifies other candidate genes for future studies of mammary lipid synthesis regulation.

Involution

The molecular events associated with involution of the mammary gland are also being studied with microarray technology. Involution is the process by which the mammary gland returns to its nonlactating state. This process could occur in a gradual way (after peak lactation) or in an abrupt way (after cessation of milking), and it is regulated by environmental factors, such as milking or suckling. At the cellular level, the process of involution involves invasion of leukocytes, increased epithelial cell death (through apoptosis or autophagy), and/or proliferation of connective tissue. In general terms, morphological changes during involution in cows are more a reflection of a change in secretory state than a reflection of tissue regression perhaps due to the fact that the dairy cow is usually pregnant during 'normal' involution, and pregnancy may in itself inhibit epithelial cell apoptosis.

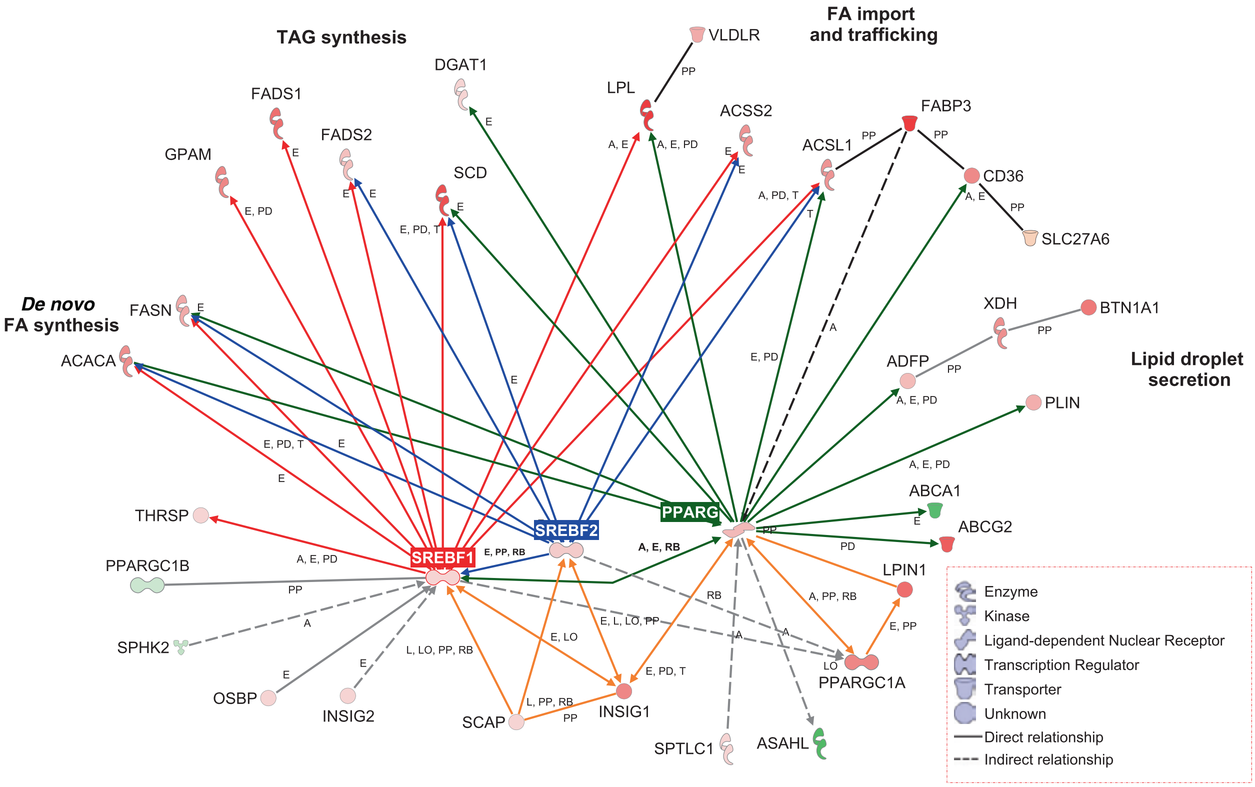
TAG synthesis
FA import and trafficking
De novo FA synthesis
Lipid droplet secretion
VLDLR
LPL
DGAT1
FADS1
FADS2
GPAM
SCD
ACSS2
ACSL1
FABP3
CD36
SLC27A6
FASN
ACACA
XDH
BTN1A1
ADFP
PLIN
ABCA1
ABCG2
PPARG
SREBF2
SREBF1
THRSP
PPARGC1B
SPHK2
OSBP
INSIG2
SCAP
INSIG1
SPTLC1
ASAHL
LPIN1
PPARGC1A
PP
E
A, E
A, E, PD
E, PD
E, PD, T
A, PD, T
T
A
E, PP, RB
A, E, RB
A, E, PD
L, LO, PP, RB
E, LO
E, L, LO, PP
L, PP, RB
RB
A, PP, RB
E, PP
LO
Enzyme
Kinase
Ligand-dependent Nuclear Receptor
Transcription Regulator
Transporter
Unknown
Direct relationship
Indirect relationship

Figure 2 Gene networks regulating milk fat synthesis in the mammary gland. Networks were developed using results from studies in bovine mammary tissue performed by quantitative polymerase chain reaction. Red nodes denote positive fold changes and green nodes negative fold changes in expression at peak lactation relative to end of pregnancy. Red, blue, and green edges denote genes whose transcription is under the control of SREBF1, SREBF2, and PPARG, respectively. Highlighted in orange is the network encompassing PPARG, PPARGC1A, LPIN1, INSIG1, and SCAP, which controls the expression/function of SREBF proteins. Letters along the edges denote effects on activity (A), expression (E), localization (LO), proteolysis (L), RNA binding (RB), protein–DNA binding (PD), and protein–protein binding (PP). Genes are grouped based on their primary function during milk fat synthesis.

Using microarray analysis, it has been observed that forced cessation of milking in nonpregnant dairy cows after peak lactation results in gradual increases in the expression of genes associated with tissue remodeling, oxidative stress, inflammation, and immune response. Forced involution also is characterized by increases in the expression of mammary tissue host defense proteins along with decreased expression of milk protein and milk fat genes. These initial results confirm that the involution process in the bovine mammary gland is associated with substantial extracellular matrix degradation as a necessary response to allow for tissue remodeling. Furthermore, the reduction in the expression of genes required for milk fat and protein synthesis reflects the transition of the involuting mammary gland from a secretory to a nonsecretory organ.

Prepubertal Development

Another fundamental area of interest in the field of mammary gland biology relates to the role of the fat pad and the parenchymal tissue of the prepubertal mammary gland in mammary development. Microarray studies have explored the interaction between mammary fat pad and parenchyma at the transcriptome level. Differences in gene expression profiles in both tissues after estrogen treatment support the necessity of the extracellular matrix for parenchymal growth and differentiation, and indicate that the mammary fat pad might affect parenchymal cell proliferation through the secretion of paracrine stimulators. This cross talk between mammary fat pad tissue and parenchymal tissue has been further characterized through their tissue-specific transcriptomic signatures in preweaning dairy heifer calves. The gene expression profile of the mammary gland fat pad is characteristic of adipose tissue, and the major functions of the differentially expressed genes include lipid metabolism and transport. In contrast, the profile of the parenchyma is characteristic of an epithelial tissue with marked enrichment of genes involved in cell apoptosis, proliferation and growth, movement of cells, and cell adhesion. The differential expression of cytokines and growth factors secreted by these two tissues is thought to be involved in the process of development of the mammary gland at this

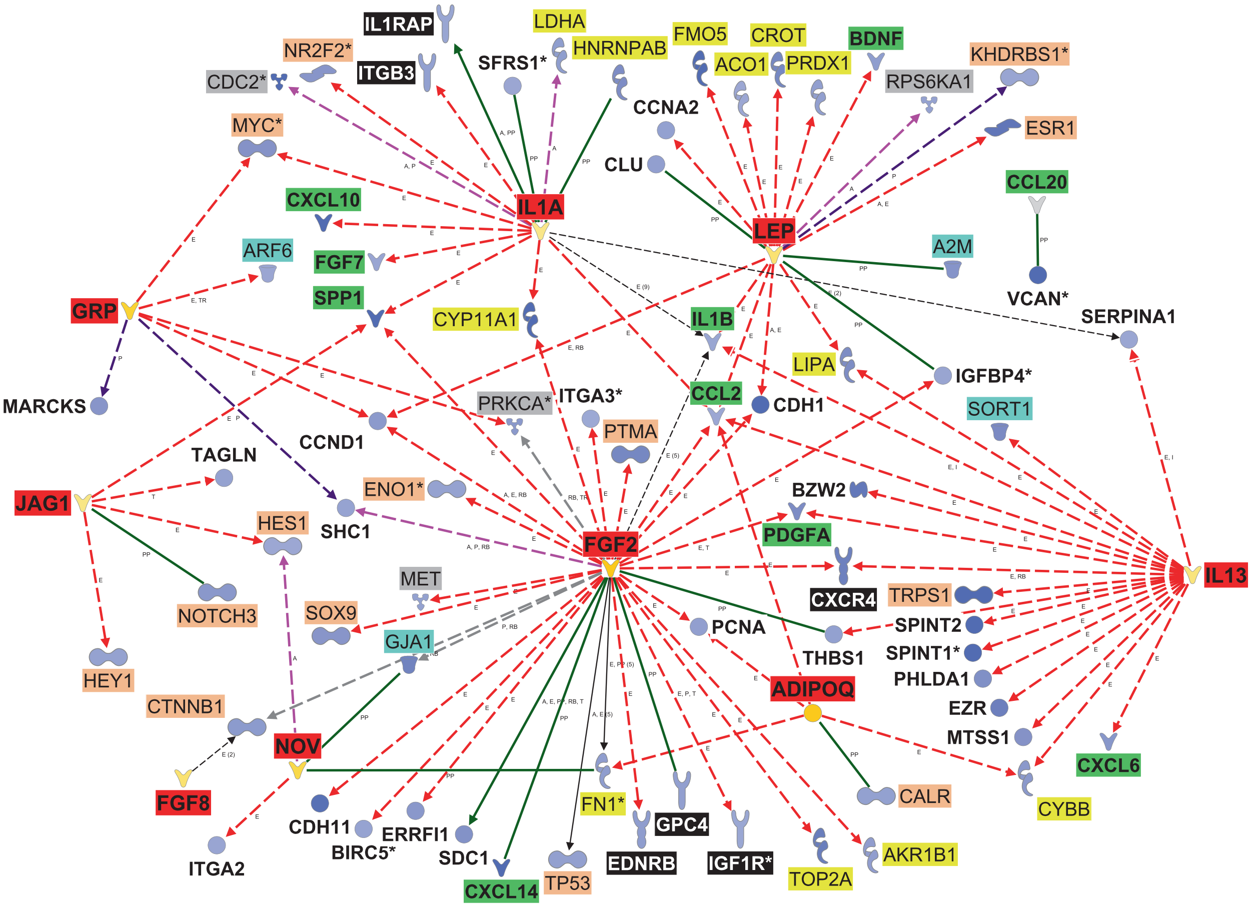
IL1RAP
LDHA
NR2F2*
ITGB3
SFRS1*
HNRNPAB
FMO5
CROT
BDNF
ACO1
PRDX1
KHDRBS1*
CDC2*
CCNA2
RPS6KA1
MYC*
CLU
ESR1
CXCL10
IL1A
CCL20
LEP
ARF6
FGF7
A2M
GRP
SPP1
VCAN*
CYP11A1
IL1B
SERPINA1
LIPA
IGFBP4*
MARCKS
PRKCA*
ITGA3*
CCL2
CDH1
SORT1
CCND1
PTMA
TAGLN
JAG1
ENO1*
BZW2
SHC1
HES1
PDGFA
FGF2
IL13
MET
CXCR4
TRPS1
NOTCH3
SOX9
SPINT2
PCNA
GJA1
THBS1
SPINT1*
HEY1
PHLDA1
ADIPOQ
CTNNB1
EZR
MTSS1
NOV
CXCL6
FGF8
CALR
CYBB
CDH11
ERRFI1
FN1*
GPC4
BIRC5*
SDC1
ITGA2
EDNRB
IGF1R*
AKR1B1
TP53
CXCL14
TOP2A

Figure 3 Putative interaction of cytokines and growth factors highly expressed in fat pad vs. parenchyma with differentially expressed genes (DEGs) highly expressed in parenchyma vs. fat pad. Shapes of molecules have a shade of blue when highly expressed in parenchyma vs. fat pad and orange when the expression is higher in fat pad vs. parenchyma. The intensity of the color relates to the fold difference in one tissue vs. the other. In the figure, several molecular types have background highlighted: enzymes in yellow, cytokines and growth factors potentially secreted by tissues in red, cytokines and growth factors highly expressed in tissues and potentially affected by cytokines and growth factors released by the other tissue in light green, membrane and G-protein-coupled receptors in black (white font), phosphatase and kinases in gray, and transcription factors and nuclear-dependent transcription regulators in dark pink. All the other molecules have white background. Red arrows denote effect on gene expression, purple arrows denote activation, dark violet arrows denote phosphorylation, and green arrows denote protein–protein interaction. Solid lines denote direct relations and dashed lines indirect relations. Letters along the edges denote are expression (E), activation (A), modification (m), protein–protein interaction (PP), protein–RNA interaction (PR), phosphorylation (P), and translocation (TR).

early stage of development (**Figure 3**). Some of the cytokines and growth factors revealed through these microarray studies have already been identified in the literature, but almost none have been researched in the prepubertal heifer model, and certainly not in the preweaning heifer calf. This model has led to a better understanding of the process of mammary development from the perspective of epithelial-fat pad interactions.

Additional characterization of gene expression profiles of mammary fat pad and parenchymal tissue in preweaning calves has indicated that the level of nutrition can alter those profiles even at that very young age. Prepubertal plane of nutrition is an important stimulus that affects bovine mammary RNA expression and the rate of tissue development, and it also has potential long-lasting effects on productivity and profitability. In one study, Holstein heifer calves were fed one of four milk replacers during the first 2 months of age. Interestingly, these dietary treatments stimulated differential expression of only a limited number of genes. However, those transcriptomics adaptations are in line with the moderate biological responses observed in mammary tissue weights and blood plasma metabolites, and hormones. The results indicated that a high-protein milk replacer fed at a level resembling the current industry standards had the highest impact on mammary parenchyma and fat pad transcriptome, whereas supplemental fat or higher intake rate for the milk replacer showed no additional effects on gene expression. Whether the observed changes in gene expression profiles are related to subsequent mammary gland growth and function when the animal lactates remains to be demonstrated.

Conclusion

The use of high-throughput technologies such as microarrays has opened up the possibility of a biological holistic view of a complex system such as the bovine mammary gland. This technology has enabled the discovery of previously unrecognized molecular functions and pathways that are important during development and lactation. Particularly important is the discovery of interacting networks of genes, which is suggestive of functional interactions as well as common-regulated functions. Better understanding of the significance and regulation of those interactions is the next challenge for livestock mammary biologists. Besides microarray technology, other techniques will need to be implemented in the near future to more fully understand these complex interactions. The discovery and functional characterization of transcription factors involved in mammary gland adaptations to a new physiological state could result in long-term practical applications. These molecules can be controlled by additional factors (i.e., effectors) such as nutrients, hormones, and/or growth factors. Therefore, controlling (increasing, decreasing) the availability of these effectors in the mammary gland might allow for greater regulation of the system.

See also: **Mammary Gland**: Anatomy; Growth, Development and Involution.

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MAMMARY GLAND, MILK BIOSYNTHESIS AND SECRETION

Contents
Milk Fat
Milk Protein
Lactose
Secretion of Milk Constituents

Milk Fat

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Introduction

Fat is the most variable component in the milk of ruminants. The concentration of fat in milk varies among individual cows and is influenced by animal and environmental factors such as breed, diet, stage of lactation, season of year, ambient temperature, and body condition. Over 95% of the fat content of milk is triacylglycerol, with phospholipids, cholesterol, diacylglycerols, monoacylglycerols, and free fatty acids constituting the remainder (**Table 1**).

Biosynthesis of Milk Fat

Ruminants are estimated to have over 400 different fatty acids comprising milk fat, but the majority of the fatty acids have chain lengths between 4 and 18 carbons. Ruminant milk fat is unique among mammals in that it contains a high proportion of short-chain fatty acids (4, 6, 8, and 10 carbons). These fatty acids are not present in typical feedstuffs and are not found in the milk fat of nonruminant species (except in the rabbit) or the body fat of any species. In ruminants, milk fatty acids arise from two sources – *de novo* synthesis in the mammary gland and the mammary uptake of preformed long-chain fatty acids (**Figure 1**). The fatty acid-synthesizing system (*de novo* synthesis) in the mammary gland of the cow produces even-numbered fatty acids that are 4–16 carbons in chain length. *De novo* fatty acid synthesis accounts for approximately 45 and 60% of the total milk fatty acids on a weight and molar basis, respectively. The other fatty acids, which include approximately half of the 16 carbon and all those 18 carbons or greater in length, are taken up preformed from the blood.

De Novo Synthesis

Ruminants primarily use acetate (C_2) and β-hydroxybutyrate (C_4) as the carbon source for milk fat synthesis, and this is in contrast to monogastric animals, which use glucose. Acetate results from carbohydrate fermentation in the rumen. The rumen bacteria also produce butyric acid during fermentation, which is predominantly converted into β-hydroxybutyrate by the rumen wall and liver. Acetate and β-hydroxybutyrate are extracted from the blood by the mammary gland. Once in the mammary cell, acetate and β-hydroxybutyrate are activated to a coenzyme A (CoA) derivative so that they can undergo further metabolism. Acetyl-CoA carboxylase 1 (ACC1) catalyzes the formation of malonyl-CoA from acetyl-CoA, the first committed step in fatty acid synthesis. The activation of β-hydroxybutyrate

Table 1 Lipids in bovine milk

Class of lipid	*% of total lipid (g/100 g)*
Triacylglycerol	95.80
1,2-Diacylglycerol	2.25
Phospholipids[a]	1.11
Cholesterol	0.46
Free fatty acids	0.28
Monoacylglycerol	0.08

[a] Includes sphingomyelin.

Adapted from Jensen RG and Newburg DS (1995) Bovine milk lipids. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 543–575. San Diego, CA: Academic Press.

does not lead to the formation of acetyl-CoA or malonyl-CoA, but instead to β-hydroxybutryl-CoA, which serves only as a 'primer' in the initiation of the synthetic process. Acetate and β-hydroxybutyrate contribute equally to the first four carbons of fatty acids; however, acetate is the source of all other carbons in *de novo*-synthesized fatty acids. Thus, it is estimated that β-hydroxybutyrate contributes only about 8% of the total carbon in milk fatty acids. For acetate to initiate the process, malonyl-CoA is condensed with acetyl-CoA by the enzyme fatty acid synthase (FASN) to produce the first 4-carbon acyl unit. Additional malonyl-CoAs are then condensed with the growing acyl chain to produce longer-chain fatty acids. The mammary gland FASN creates a range of fatty acids with chain lengths of 4–16 carbons. Mechanisms regulating chain length termination are not clearly understood, but an acylthioesterase present in mammary tissue cleaves fatty acids of different lengths from the FASN complex.

Preformed Fatty Acids

Mammary uptake of circulating long-chain fatty acids is the other source of fatty acids for milk fat synthesis. Circulating fatty acids originate from lipids absorbed from the digestive tract and mobilized from body fat reserves. Dietary triacylglycerols are not soluble in water but are packaged in lipoproteins within the blood. The specific lipoproteins that transport dietary triacylglycerols to the mammary gland are the very low-density lipoproteins (VLDLs). Lipoprotein lipase, an enzyme residing within the capillary wall in the mammary gland, cleaves the VLDL triacylglycerols into glycerol and nonesterified fatty acids (NEFAs) that are then taken up by the mammary epithelial cell. Plasma NEFAs also originate from mobilization of body adipose triglycerides (adipocyte hormone-sensitive lipase) and are also taken up by the mammary gland. Movement of NEFAs across the cell membrane and intracellular transport are not well described, but fatty acid transport proteins (FATPs) and fatty acid-binding proteins (FABP) are thought to play key roles. Once in the mammary cell, the preformed fatty acids become activated to CoA esters and glycerol is converted into glycerol phosphate. Although plasma triacylglycerols and NEFAs represent less than 3% of total plasma lipid, their

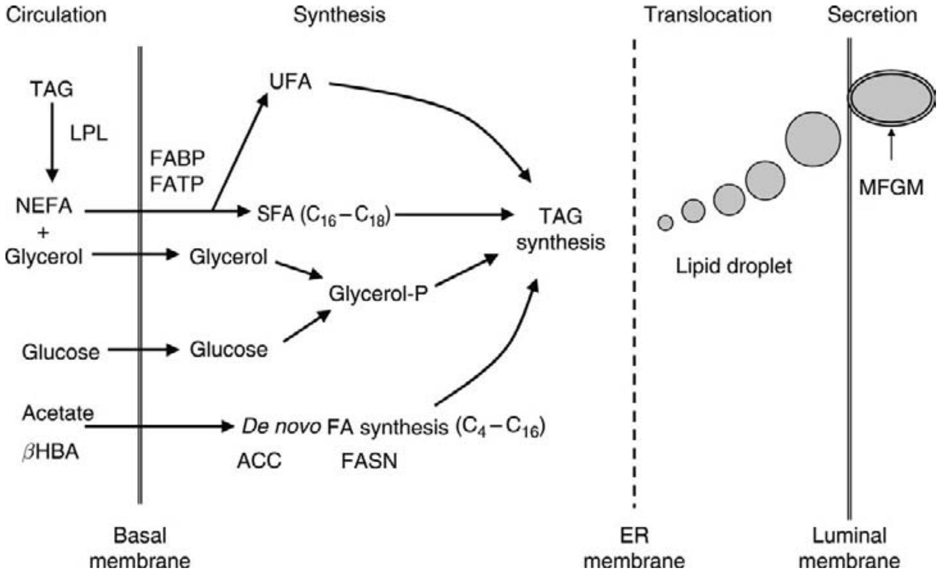

Figure 1 The synthesis of milk fat in the mammary gland of dairy cows includes substrate uptake, *de novo* fatty acid synthesis, desaturation, triacylglyceride synthesis, and milk fat secretion. The Δ^9-desaturase enzyme is capable of inserting a double bond into both saturated and unsaturated fatty acids. A key role of glucose is to provide reducing equivalents (NADPH) for *de novo* fatty acid synthesis (not shown). ACC, acetyl CoA carboxylase; βHBA, β-hydroxybutyrate; ER, endoplasmic reticulum; FABP, fatty acid–binding protein; FASN, fatty acid synthase; FATP, fatty acid transport protein; glycerol-P, glycerol phosphate; LPL, lipoprotein lipase; MFGM, milk fat globule membrane; NEFA, nonesterified fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol; UFA, unsaturated fatty acid.

contribution to total milk fat is approximately 55% by weight and 40% on a molar basis. Mammary uptake of plasma NEFA is proportional to its plasma concentration and the plasma concentration varies by energy state. Thus, the contribution of plasma NEFA to milk fat varies according to physiological state and energy balance. For example, early lactation cows in negative energy balance mobilize fatty acid reserves at high rates, resulting in increased plasma NEFA concentrations and an increased contribution of preformed fatty acids to milk fat.

Triacylglycerol Synthesis

Milk fat is composed mostly of triacylglycerols, and thus esterification of the fatty acids is also an important step in the synthesis of milk fat. The short- and long-chain fatty acids are attached to the glycerol molecule in an orderly and systematic fashion. There are three sites of attachment to the glycerol molecule. Some fatty acids are positioned at random onto glycerol, whereas others occupy a specific position. For example, lauric acid ($C_{12:0}$) is randomly assigned, whereas unsaturated fatty acids are selectively esterified at the second carbon (*sn*-2) and butyric acid (C_4) is positioned primarily on the third carbon (*sn*-3) of the glycerol structure. Once the triacylglycerols are formed, they coalesce into lipid droplets, which move through the epithelial cell toward the luminal side. The droplets are engulfed by a portion of the cell membrane and pinched off into the lumen. Thus, a membrane referred to as the ‘milk fat globule membrane’ surrounds the fat droplets present in milk. The proteins of the milk fat globule membrane have been well studied and include an enrichment of approximately six proteins, some of which have been demonstrated to be essential to secretion of the milk fat in genomic studies with mouse models.

Milk Fatty Acids

As stated earlier, milk fat contains a multitude of fatty acids (**Table 2**). Saturated, monounsaturated, and polyunsaturated fatty acids are all present in bovine milk fat. The variety of fatty acids allows the mammary gland to produce triacylglycerols with a range of fluidity so that the mammary cell can secrete the milk fat. The fluidity of the triacylglycerol is increased by use of short- and medium-chain fatty acids that arise from *de novo* synthesis as well as long-chain unsaturated fatty acids. The mammary gland also has an additional means to regulate the fluidity of the milk fat via the enzyme Δ^9-desaturase (stearoyl-CoA desaturase). This enzyme is very active in cow mammary cells and inserts a double bond into a variety of saturated and monounsaturated fatty acids. The increased unsaturation of the resulting fatty acids decreases the melting point of the fatty acids present in milk. This is critical for the maintenance of the fluidity of both milk fat and cellular membranes. The main action of the Δ^9-desaturase enzyme is to convert $C_{18:0}$ into $C_{18:1n\text{-}9}$. Oleic acid constitutes over 20% of total milk fatty acids, and estimates are that 60% of milk fat oleic acid is derived from stearic acid via Δ^9-desaturase. However, Δ^9-desaturase is also important in the production of *cis*-9, *trans*-11 $C_{18:2}$ (conjugated linoleic acid; CLA), and this is discussed later.

Table 2 Fatty acids in bovine milk fat as determined by gas–liquid chromatography with capillary columns

Saturated fatty acids		*Monounsaturated fatty acids*		*Polyunsaturated fatty acids*	
Fatty acid	*g/100 g total fatty acids*	*Fatty acid*	*g/100 g total fatty acids*	*Fatty acid*	*g/100 g total fatty acids*
4:0	4.5	14:1*n*-5	0.9	18:2*t*	0.4
6:0	2.3	15:1	0.3	18:2*n*-6	2.9
8:0	1.3	16:1*n*-7	1.8	18:3*n*-3	0.3
10:0	2.7	17:1	0.4	20:4*n*-6	0.2
11:0	0.3	18:1*t*	1.7		
12:0	3.0	18:1*n*-9	21.4		
13:0	0.2	20:1*n*-9	0.6		
14:0*i*	0.1				
14:0	10.6				
15:0*i*	0.7				
15:0	1.0				
16:0	28.2				
17:0*i*	0.7				
17:0	0.6				
18:0	12.6				
20:0	0.2				

Adapted from Jensen RG and Newburg DS (1995) Bovine milk lipids. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 543–575. San Diego, CA: Academic Press.

Environmental Effects Including Diet

The high genetic correlation between fat yield and the yield of milk and other milk components makes it difficult to use genetic selection to alter milk fat independent of other milk components. However, milk fat is affected markedly by physiological and environmental factors. Physiological factors generally involve changes in energy balance (i.e., stage of lactation) and offer little potential as a practical means of manipulating milk fat. However, nutrition is the predominant environmental factor affecting milk fat and represents a practical tool to alter its yield and composition.

Effect of Diet on Milk Fat Percentage and Composition

Nutrition is the predominant factor affecting milk fat and provides a practical tool to alter the yield and composition of milk fat. However, in contrast to nonruminants, the composition of dietary fat has only a minor effect on the milk fatty acid composition in ruminants. In ruminants, dietary fat undergoes two important processes in the rumen. First, the esterified fatty acids are hydrolyzed by the rumen bacteria to yield free fatty acids. Second, the free unsaturated fatty acids are biohydrogenated because they are toxic to many rumen bacteria and would adversely affect rates of fermentation. The major fatty acids in typical ruminant feedstuffs are linolenic acid ($C_{18:3}$) predominately from the forage components and linoleic acid ($C_{18:2}$) from concentrates and seed oils. Rumen hydrolysis and biohydrogenation are extensive so rumen outflow of lipids is mainly saturated free fatty acids, with the largest portion being stearic acid. The major rumen pathways for the biohydrogenation of linolenic and linoleic acids to stearic acid ($C_{18:0}$) are shown in **Figure 2**. Although most linoleic and linolenic acids are completely hydrogenated to stearic acid, rumen outflow also contains small quantities of biohydrogenation intermediates, and these are also absorbed and incorporated into body fat and milk fat. Recent studies with labeled substrates have shown that the pathways of rumen biohydrogenation are much more complex than the simple depiction in **Figure 2**, so trace quantities of many *trans*-18:1 and conjugated linoleic acid (CLA) isomers are found in rumen outflow and ruminant fat.

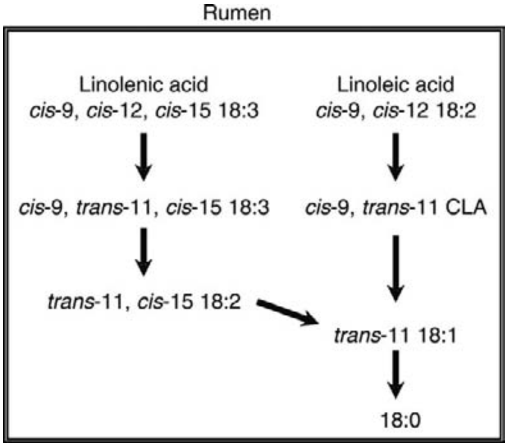

Figure 2 Pathways of microbial biohydrogenation of linoleic and linolenic acids in the rumen. Note CLA and *trans* fatty acids as intermediates. CLA, conjugated linoleic acid. Adapted from Bauman DE and Lock AL (2006) Conjugated linoleic acid: Biosynthesis and nutritional significant. In: Fox PF and Sweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 2: Lipids*, pp. 93–136. New York: Springer.

Various methods of protecting lipid supplements have been developed in an attempt to bypass rumen fermentation. Examples of these technologies include the formation of Ca salts or amides of unsaturated fatty acids and the use of encapsulation methods. These formulation methods reduce the amount of unsaturated fatty acids available in the rumen and decrease the adverse effects of unsaturated fatty acids on rumen fermentation, especially fiber digestion. To the extent these formulations also protect from rumen biohydrogenation, they offer a means to supply unsaturated fatty acids that could be used for milk fat synthesis. Indeed, rumen-protected fatty acid supplements have been used to modestly enhance the milk content of oleic acid, linoleic acid, CLA, and omega-3 fatty acids. However, rumen protection methods differ in their efficacy, and, to date, these approaches have had only limited commercial use for modification of milk fatty acid profile.

The most dramatic example of nutritional effects on milk fat is the low-fat milk syndrome, typically referred to as milk fat depression (MFD). First observed over 150 years ago, diet-induced MFD remains a challenge in modern dairy production, and the decrease in milk fat concentration and yield can be substantial. In the first half of the twentieth century when the feeding of dairy cows began to follow ‘scientific principles’, reductions in milk fat yield were observed for a range of common diets, including those supplemented with fish or plant oils, diets high in concentrates and low in fiber, and diets low in ‘effective fiber’ (e.g., grinding or pelleting of the roughage). The investigation of diet-induced MFD has a rich history that has included many theories to explain reduced milk fat synthesis. Most of these theories postulated that limitations in substrate supply for milk fat synthesis caused MFD, generally based on changes in absorbed metabolites as a consequence of alterations in ruminal fermentation. Over several decades, researchers have tested theories based on substrate limitations and found little to no evidence in their support. However, from these investigations, several general characteristics were recognized. First, milk fat was specifically reduced by up to 50% with no change in the yield of milk or other milk components. Second, the yield of all individual fatty acids was reduced during MFD, but the decline was greatest for short- and medium-chain fatty acids that are synthesized in the mammary gland. Third, when studies were examined more broadly, it became apparent that two conditions are needed for MFD: (1) the diet must alter the rumen environment, thereby cause changes in ruminal microbial processes, and (2) the diet must contain at least a modest level of unsaturated fatty acids. MFD did not occur if either of these conditions was absent. Thus, the etiology of diet-induced MFD involves products of rumen bacteria that are produced as a consequence of the diet-induced shifts in rumen microbial processes and the presence of unsaturated fatty acids.

The cause of MFD perplexed scientists and producers for over a century, but key insight was provided by recognition that increases in milk *trans*-18:1 fatty acids (TFAs) concentration was associated with MFD. TFAs are formed as intermediates in rumen biohydrogenation and *trans*-11 18:1 (vaccenic acid) is the predominant isomer produced, as illustrated by the pathway for the biohydrogenation of linoleic acid (**Figure 2**). However, in some studies, the increases were poorly correlated to milk fat yield. Thus, the basis by which certain diets cause a reduction in milk fat yield had to be more complex than a simple relationship to the rumen production of TFA. As analytical techniques improved, it was discovered that it was a shift in the pattern of TFA isomers rather than total TFA that was correlated with MFD. The net effect was that under certain dietary situations a portion of the linoleic acid undergoes biohydrogenation via different pathways that produce unusual TFA isomers (**Figure 3**). On the basis of these results, the 'biohydrogenation theory' was proposed as a unifying concept to explain diet-induced MFD; this theory hypothesized that under certain dietary conditions the pathways of rumen biohydrogenation are altered to produce unique fatty acid intermediates that are potent inhibitors of milk fat synthesis. Subsequent studies have validated this theory and established that diet-induced MFD coincides with a marked shift in the milk fat concentration of many biohydrogenation intermediates. The first of these to be identified as regulating milk fat synthesis was *trans*-10, *cis*-12 CLA. Treatment with purified *trans*-10, *cis*-12 CLA induces MFD with the same phenotype as diet-induced MFD. Thus, *trans*-10, *cis*-12 CLA provided a clear demonstration of the interrelationship between digestive processes in the rumen and metabolism in the mammary gland, where a specific fatty acid produced naturally by rumen bacteria affects mammary gene expression, thereby regulating rates of milk fat synthesis.

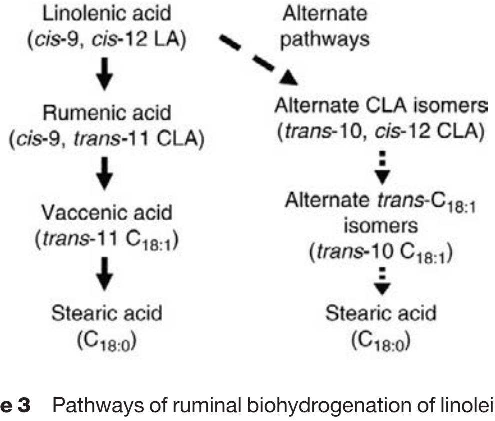

Figure 3 Pathways of ruminal biohydrogenation of linoleic acid and CLA under normal and altered ruminal fermentation. CLA, conjugated linoleic acid. Adapted from Harvatine KJ, Boisclair YR, and Bauman DE (2009) Recent advances in the regulation of milk fat synthesis. *Animal* 3: 40–54.

Trans-10, *cis*-12 CLA is a potent inhibitor of milk fat synthesis (**Figure 4**); effects are dose dependent and as little as 2.5 g day^{-1} leaving the rumen is sufficient to cause a 25% reduction in milk fat production. The mechanism by which *trans*-10, *cis*-12 CLA causes a reduction in milk fat synthesis has been investigated, and it involves regulation of gene expression in the mammary epithelial cells that results in a coordinated reduction in key enzymes involved in pathways of milk fat synthesis. Consistent with the biohydrogenation theory, several lines of evidence suggested that there must be additional fatty acid intermediates that reduce the synthesis of milk fat. This is an active area of research, and, to date, two additional CLA isomers that regulate milk fat synthesis have been identified, *trans*-9, *cis*-11 and *cis*-10, *trans*-12. The predominant CLA isomer in milk fat is *cis*-9, *trans*-11 CLA (trivial name: rumenic acid (RA)), typically constituting about 75–90% of total CLA isomers in milk fat. It is interesting that RA has no effect on milk fat synthesis, but *trans*-9, *cis*-11 CLA is a potent inhibitor. Although the double bonds are in the same position in both of these isomers (carbons 9 and 11), their orientation has been reversed (*trans*/*cis* vs. *cis*/*trans*). This emphasizes the critical importance of bond position and orientation in determining the biological activity of a fatty acid.

Rumenic acid is only a minor component of milk fatty acids, but there is widespread interest in RA because of its potential benefits to human health and the prevention of chronic diseases. Biomedical studies with animal models have demonstrated that RA has both anticarcinogenic and antiatherogenic activity, and over 90% of the natural CLA in human diets comes from ruminant-source foods. Although RA is an intermediate in the rumen biohydrogenation of linoleic acid (**Figure 2**), most RA in milk and meat fat is derived

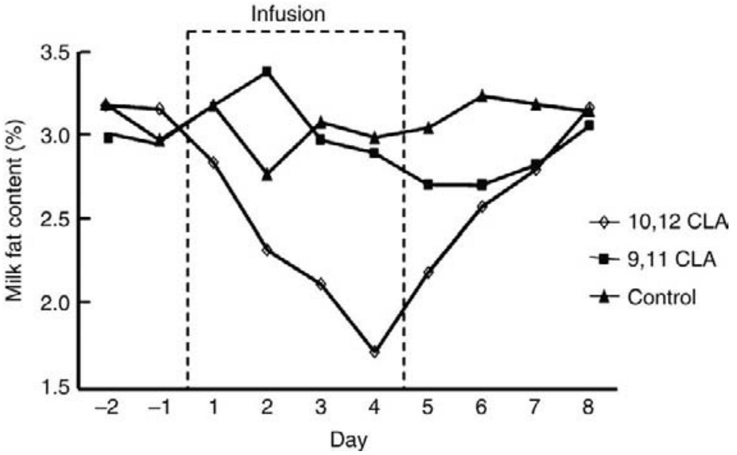

Figure 4 Effect of abomasal infusion of CLA supplements of milk fat synthesis in dairy cows. Abomasal infusion is a convenient experimental approach to bypass possible alterations by rumen microbial fermentation and treatments were control or CLA isomers (10 g day^{-1}) *cis*-9, *trans*-11 and *trans*-10, *cis*-12. CLA, conjugated linoleic acid. From Baumgard LH, Corl BA, Dwyer DA, Saebo A, and Bauman DE (2000) Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. *The American Journal of Physiology* 278: R179–R184, used with permission.

from endogenous synthesis involving the enzyme Δ^9-desaturase, with the substrate being vaccenic acid, rumen biohydrogenation intermediate produced from both linoleic and linolenic acids. Of special importance, research has established that the level of RA in milk fat can be markedly enhanced by controlling the nutrition of the cow. Diets formulated with oil seeds or plant and fish oils high in polyunsaturated fatty acids are especially effective. Thus, this is an active area of research by animal scientists and biomedical scientists.

Insights Gained from Milk Fat Depression

Research in the regulation of milk fat synthesis has focused on investigations of MFD rather than on situations or models where milk fat synthesis is enhanced. Nevertheless, MFD represents a biologically significant and physiologically relevant example in which a metabolite(s) produced in digestive processes regulates metabolism, and the basis for this regulation can be explained at the molecular level. Knowledge of the basis for MFD allows the development of feeding strategies and provides the opportunity to troubleshoot commercial problems in low milk fat production. Milk fat depression continues to be a real-world condition that reduces the efficiency and productivity of dairy cows, but understanding its fundamental basis allows for effective management and intervention strategies.

Under certain marketing systems and management schemes, it may be advantageous to reduce milk fat yield, and in some feeding and management systems, the reduction in milk fat yield has allowed for a repartitioning of nutrients to support increased milk and milk protein yield. Producers may also find it advantageous to induce MFD during periods of limited feedstuff availability such as inadequate rainfall in pasture-based systems or for a short period while breeding. Inducing MFD during breeding periods may also be a useful management practice to improve short-term energy balance and subsequently reproductive efficiency, although caution is important in application of classical MFD diets.

Climate Considerations

Milk fat percentage is typically higher in the winter than in the summer for the northern hemisphere. One could attribute the changes in milk fat percentage to differences in nutrient intake or specific effects of climate (i.e., environmental temperature). Certainly changes in carbohydrate and polyunsaturated fatty acid intake may be the driving force behind some changes associated with seasonal effects as pasture available in the summer is replaced with preserved forages and grains in the winter. However, milk fat percentage still varies similarly in pasture-fed herds compared with commercial herds without access to pasture. Concentrations of unsaturated fatty acids in milk including vaccenic acid, oleic acid, CLA, and linolenic acid are greatest in the summer. The coordinate increase in vaccenic acid and CLA suggests an enhancement of Δ^9-desaturase activity. The changes in saturated fatty acids are inverse to those of unsaturated fatty acids, suggesting a reduction in *de novo* synthesis during the summer months. The factors causing these changes in milk fat percentage and fatty acids beyond nutrition are unknown.

The specific effects of temperature (i.e., heat or cold stress) on milk fat, however, are not clear. Some have speculated that heat stress would cause a condition similar to ruminal acidosis that should lead to a reduction in milk fat percentage. Direct assessment of the effect of heat stress through the use of environmental chambers failed to detect an effect on milk fat percentage even though obvious effects of heat stress (i.e., increased body temperature and respiration rate and decreased feed intake) were apparent. Thus, it is likely that the changes during the summer are not attributable to warmer environmental temperatures. The impact of cold stress may again not be a direct effect of climate on milk fat synthesis but more an indirect effect on nutrient utilization. Milk production is reduced in cows exposed to temperatures below −5 °C. As milk yield declines, milk fat percentage increases. The changes are probably related more to the utilization of energy for maintenance of body temperature, leading to reduced energy available for milk synthesis. However, milk fat synthesis is maintained or declines less than milk yield, and thus, milk fat percentage increases. Few data are available regarding the effects of cold stress on fatty acid composition of milk.

See also: **Feed Ingredients**: Feed Supplements: Fats and Protected Fats. **Milk Lipids**: Conjugated Linoleic Acid; Fatty Acids; General Characteristics; Nutritional Significance. **Stress in Dairy Animals**: Heat Stress: Effects on Milk Production and Composition.

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Milk Protein

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Introduction

Milk provides the neonate with a readily available complete diet, supplying all essential nutrients. Colostrum, that is, the first milk after parturition, has a considerably higher protein content than later milk. The primary reason for this is the high content of blood-derived immunoglobulins in colostrum compared to milk. Unlike other species such as humans, ruminants lack the ability to transfer immunoglobulins to the fetus *in utero* and are therefore born without immunity. Hence, colostrum, being rich in immunoglobulin proteins, is essential to the early survival of the young calf. In normal milk, the protein content varies considerably between species, from a low of $1\,\mathrm{g\,l^{-1}}$ in humans to a high of $145\,\mathrm{g\,l^{-1}}$ in the black bear, and cow's milk contains approximately $32–35\,\mathrm{g\,l^{-1}}$ (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose, **Table 1**).

Through improved nutrition, breeding, and management, milk production of the modern dairy cow is well in excess of that required to feed its offspring. Milk is an important product to man, in terms of both nutritional and economic values. At present, protein, and in particular casein, is the most valued milk component, being the principal ingredient of cheese. Until recently, whey proteins were considered to be of limited value and were often disposed of as waste or used as an animal feed. However, various specific whey proteins are now being valued for their bioactive properties.

Milk Protein Composition

Milk protein is made up of a large number of different small and large proteins. Most milk proteins are mammary-derived, synthesized within the secretory epithelium of the mammary gland and secreted into the milk pool within the alveolar lumen. The mammary-derived milk proteins can be further divided into two broad categories: casein and whey proteins. The classical method to distinguish between the two is by acid precipitation of defatted milk. Caseins precipitate when milk is acidified to a pH of approximately 4.6, but the whey proteins remain in solution. Ruminant milk contains four different caseins: α-casein, β-casein, κ-casein, and γ-casein (**Table 1**). The first three caseins are expressed by different genes, and different cows may carry different genetic variants of each of the caseins. γ-Caseins are the breakdown products cleaved from β-casein by the major milk proteolytic enzyme plasmin (EC 3.4.21.7). In milk, casein molecules appear as micelles, which are spherical structures consisting of many thousands of individual casein molecules linked together and encapsulating significant amounts of calcium and phosphate and to a lesser extent citrate. The major whey proteins synthesized by the ruminant mammary gland are α-lactalbumin and β-lactoglobulin (**Table 1**). Interestingly, α-lactalbumin plays a crucial role in milk lactose synthesis as part of the enzyme lactose synthetase (EC 2.4.1.22; *see* **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose). In addition to milk proteins, the mammary gland also synthesizes a number of constitutive proteins, such as structural proteins, enzymes, hormones, and growth factors. These proteins play an important role in mammary function, and they may be secreted into milk, but are mostly turned over within the mammary gland.

Finally, in addition to proteins that are synthesized within the mammary gland, the whey fraction of milk contains a large number of smaller proteins that are taken up from the blood and transported without further processing across the secretory cell into the milk, via either a transcellular route or a paracellular (i.e., between adjacent mammary epithelial cells) route. Examples of such blood-derived and mostly immune-related proteins are immunoglobulins, lactoferrin, lactoperoxidase, and serum albumin. Some are taken up into the mammary cell by active transport mechanisms, whereas others enter by passive diffusion or by a process of internalization, and for some it is not yet known exactly how they enter the secretory cells of the mammary gland.

Biosynthesis and Secretion

The milk protein substrates (amino acids) are supplied to the mammary gland by the blood. Arteriovenous difference studies have demonstrated the remarkable capacity of the mammary gland for extracting amino acids from the blood. The actual movement of amino acids through the basolateral membrane of the secretory cell is facilitated by several sodium-dependent or sodium-independent amino-acid transport systems, with different transporters being specific to the transport of different groups of amino acids.

Table 1 The content and composition of protein in creamery milk in south-west Scotland

		Skim milk (g l^{-1})		*Total protein (%)*		*Total casein (%)*		*Total milk serum protein (%)*	
	Mammary or blood derived	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Total protein	-	32.71	1.80	-	-	-	-	-	-
Total casein	-	26.92	1.54	82.2	0.6	-	-	-	-
Total milk serum protein	-	5.79	0.32	17.8	0.6	-	-	-	-
α_{s1}-Casein	Milk	10.25	0.57	31.3	0.5	38.1	0.5	-	-
β-Casein	Milk	9.60	0.50	29.3	0.6	35.7	0.8	-	-
κ-Casein	Milk	3.45	0.32	10.5	0.5	12.8	0.6	-	-
α_{s2}-Casein	Milk	2.74	0.21	8.4	0.5	10.2	0.8	-	-
γ-Casein	Milk	0.88	0.15	2.7	0.4	3.2	0.4	-	-
β-Lactoglobulin	Milk	3.14	0.19	9.6	0.4	-	-	54.2	1.3
α-Lactalbumin	Milk	1.23	0.09	3.8	0.3	-	-	54.2	1.3
IPL	Blood	0.97	0.10	3.0	0.2	-	-	21.2	1.4
Bovine serum albumin	Blood	0.45	0.04	1.4	0.1	-	-	7.8	0.6

Values are the mean of 29 samples from five creameries.

IPL, immunoglobulins, proteose-peptone component 3, and lactoferrin; SD, standard deviation.

Adapted from Davies DT and Law AJR (1980) The content and composition of protein in creamery milks in south-west Scotland. *Journal of Dairy Research* 47: 83–90.

Once inside the secretory epithelium, the basic process of using the amino acids for protein synthesis in the mammary gland does not differ from that occurring in other tissues within the body (**Figure 1**). Milk proteins, as any other proteins, are encoded by different genes that help make up the genome. The biosynthesis of proteins is initiated by factors (e.g., hormones) that induce gene expression. The actual initiation of gene expression is a complex process involving interactions between hormone-induced nuclear transcription factors and the promoter area or specific enhancer regions of the DNA upstream of the promoter. Gene expression occurs in all tissues and is not unique to the mammary gland. A detailed description of these processes is therefore beyond the scope of this article.

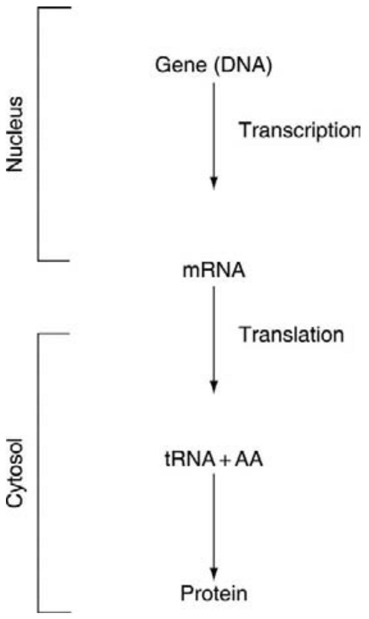

Figure 1 General pathway for protein synthesis. Transcription occurs in the nucleus of the cell, whereas translation occurs on the ribosomes on the rough endoplasmic reticulum. AA, amino acids; mRNA, messenger RNA; tRNA, transfer RNA.

Expression of the gene encoding a protein starts with making a mirror image of the gene's DNA template with the help of the enzyme RNA polymerase (EC 2.7.7.6). The resulting image or messenger RNA (mRNA) is complementary to the DNA template, but differs slightly in that RNA contains the base uracil instead of thymidine. The resulting mRNA now forms the blueprint for the protein and determines the sequence of the amino acids that make up the particular protein. Thus far, the process has occurred in the nucleus of the cell; however, the mRNA then moves from the nucleus into the cytosol and to the ribosomes on the rough endoplasmic reticulum (RER). On the ribosomes, the mRNA is translated into protein. Each successive nucleotide triplet in the mRNA codes for a specific amino acid and as the mRNA moves through the ribosome the appropriate amino acids are linked together with the aid of transfer RNA (tRNA).

Interestingly, evidence is emerging that in addition to the classical regulation of gene expression, as described before, milk protein expression may also be under epigenetic regulation. It was recently shown that DNA methylation at specific sites on the αs1-casein promoter was able to downregulate the expression of αs1-casein during certain physiological conditions.

Milk proteins are secretory proteins and have to be exported from the cell into the milk pool in the alveolar lumen. To facilitate the movement of a secretory protein into the RER, an amino-terminal signal sequence of approximately 20 hydrophobic amino acids is added to the protein to guide it through a membrane channel in the wall of the RER. Even before translation of the protein is completed, the signal peptide is cleaved from the protein by a proteolytic enzyme (signal peptidase; EC 3.4.21.89) and is inserted into the membrane channel. After completion of the translation process and following translocation into the inside of the RER, the (milk) protein has now been formed; however, this does not necessarily mean that it is also a functional protein. In order for the protein to become functional, it must be folded into its appropriate three-dimensional structure. Moreover, other molecules such as carbohydrates (in the case of glycoproteins), phosphate groups, and ions may be attached to the protein. In the case of caseins, many casein molecules (as many as 25 000) group together to form large micelles. These processes occur en route to and in the Golgi apparatus, from where the proteins, together with lactose, are encapsulated in secretory vesicles that bud off the Golgi and are moved with the aid of the cytoskeleton toward the apical membrane. There, the secretory vesicles fuse with the apical membrane and release their contents including proteins, lactose, ions, and water into the milk pool of the alveolar lumen.

Factors Affecting Milk Protein Synthesis

Diet

Like any other tissue where protein synthesis occurs, the mammary gland has a requirement for nonessential and essential amino acids. The only difference with other tissues is that the mammary gland is a secretory organ that can produce and export into the milk vast amounts of milk protein, and as such has a very substantial demand for substrates. The required substrates are supplied via the mammary arterial blood supply and the diet determines to a large extent the amount and type of substrate available for mammary uptake. However, it is pertinent to point out that there is a distinct difference in dietary protein digestion between ruminants and nonruminants. In the latter, the breakdown of proteins starts in the stomach with the production of hydrochloric acid and the enzyme pepsin (EC 3.4.23.1), and is completed in the small intestine where a series of other proteolytic enzymes such as trypsin (EC 3.4.21.4) and amino- and carboxypeptidases further break down the dietary protein. The ultimate breakdown products, amino acids and small peptide fractions, are absorbed across the intestinal wall and used for protein synthesis and/or gluconeogenesis. The composition of the diet determines directly which proteins are presented for digestion. Although the same processes occur in ruminants, all food has to pass first through the rumen before it moves on to the stomach and intestines. Approximately two-thirds of all dietary protein is broken down in the rumen by bacteria and protozoa and converted into microbial protein. It is this microbial protein that is presented to the lower digestive tract, together with the roughly one-third of dietary protein that escapes degradation in the rumen, the so called bypass protein. Although it may not appear to be all that efficient to have this extra compartment (i.e., the rumen) where dietary protein is converted first to microbial protein before it enters the stomach, one must remember that this unique system allows ruminants to digest large amounts of plant material, sometimes of low quality, and convert it into high-quality microbial protein. This also means that in ruminants the protein composition of the diet has relatively less effect on milk protein composition and that the essential amino-acid composition of the diet is less critical, because of the ability of the rumen microbes to synthesize essential amino acids.

This does not mean that diet cannot influence milk protein production. To the contrary, simply increasing the crude protein content of the diet may increase milk protein yield, but different experiments do not always give consistent results, due to differences in the level and composition of the basal diets. Experiments to determine the effect of extra dietary protein on milk protein production often involve the infusion of casein protein postruminally. Using casein, one is assured that the extra amino acids supplied by the diet are the right ones for milk protein synthesis, and infusing them postruminally prevents the conversion of the dietary casein protein into microbial protein. A positive effect on milk protein synthesis of such additional dietary protein depends very much on the composition of the basal diet, and is observed only when the basal diet is energy deficient or is low in protein. Similarly, the best response is seen in very high-yielding cows, which are more likely to be in a negative energy balance. Increasing the degradable carbohydrate fraction of the diet may also have a positive impact on milk protein synthesis, either through supplying more energy to the rumen microbes and thus enhancing microbial protein production and substrate supply to the mammary gland, or by increasing the supply of energy substrates to the mammary gland, allowing for increased amino-acid utilization.

In summary, the success, in terms of increasing milk protein output, of any dietary manipulation depends on delivering the right substrates to the mammary gland. For example, methionine is generally considered a limiting amino acid in ruminants, and including protected methionine to bypass rumen degradation increases milk protein content, in particular in cows on low-protein corn-based rations. Lysine is often considered to be another limiting amino acid in ruminants, but the effects of additional lysine on mammary protein synthesis are less straightforward since the net mammary uptake often exceeds the output into milk, but this may be due to substantial intramammary oxidation of lysine.

Endocrine Control

Endocrine factors play an important role in the induction and regulation of gene expression in all tissues, and the mammary gland is no exception. The results from *in vitro* experiments with either mammary cell or explant cultures, and also those from *in vivo* experiments with predominantly rodents, indicate that prolactin, insulin, and cortisol (hydrocortisone) are the essential hormones required for milk protein synthesis. In contrast, progesterone has been shown to decrease casein and α-lactalbumin accumulation in mammary explants of mice. This, however, may be related to progesterone's role as a general inhibitor of lactation, rather than a direct effect on protein secretion *per se.* Similarly, continuous infusion of glucagon into early lactation dairy cows showed a transient negative effect on milk yield and milk protein content. Again this is unlikely to be a direct effect of glucagon on milk protein gene expression, but rather relates to the effect of glucagon on amino-acid availability to the mammary gland. A concurrent transient decrease in feed intake and an increase in gluconeogenesis from amino acids by the liver are likely responsible for the decrease in substrate supply.

Studies at Cornell University, examining the role of insulin in mammary protein synthesis in cows, demonstrated quite clearly that even in high-yielding dairy cows protein synthesis has not yet reached its maximum capacity. These studies, which used the hyperinsulinemic-euglycemic clamp technique, showed that insulin was able to increase mammary protein output in addition to an increase resulting from abdominally infused casein protein (**Figure 2**). Thus, even after optimizing dietary conditions, further gains may be achieved through endocrine manipulation.

How exactly endocrine factors can enhance milk protein synthesis is not clear. Increasing substrate supply by increasing mammary blood flow, both at the arterial and microvascular level is, however, a distinct possibility, as is increasing mammary uptake of substrates via effects on membrane transporter systems. However, another important area may be the reduction of intramammary protein turnover. The turnover of constitutive proteins (e.g., enzymes) and casein in the mammary gland may be as high as 40–70%. This means that a significant proportion of synthesized milk proteins are not secreted, which clearly contributes to the rather low overall efficiency of 20–30% with which dietary nitrogen is converted into milk protein.

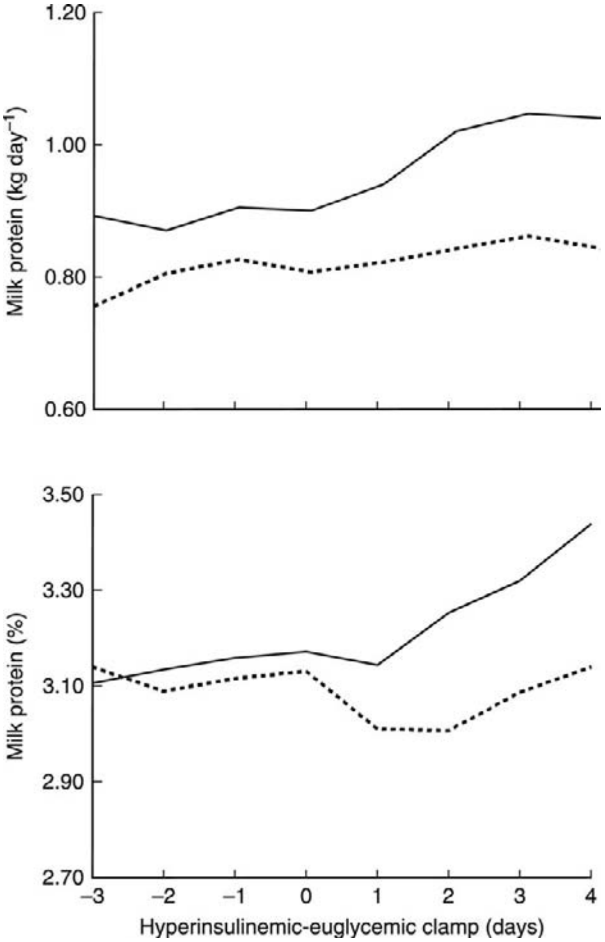

Figure 2 Protein yield and concentration in milk from cows ($n = 5$) during the baseline interval (−3 to 0 days) and 4-day (0–4 days) exposure to an insulin infusion (1 μg per kg body weight per hour) using a hyperinsulinemic-euglycemic clamp. In addition, throughout the experiment, cows received abomasal infusions of water (dashed lines) or casein (solid lines). Casein infusion improves mammary protein synthesis, but infusion of insulin improves protein synthesis even further. (Insulin clamp technique: insulin acts to maintain constant blood sugar levels, and infusion of insulin would result in a dangerous drop in the blood glucose level; therefore, during the clamp technique, glucose is infused concurrently to maintain the blood glucose level, while insulin levels are elevated.) From Griinari JM, McGuire MA, Dwyer DA, Bauman DE, Barbano DE, and House WA (1997) The role of insulin in the regulation of milk protein synthesis in dairy cows. *Journal of Dairy Science* 80: 2361–2371.

Temperature

Ambient temperatures on either side of the thermoneutral zone affect milk protein synthesis. Numerous studies report that heat stress has an adverse effect on mammary protein synthesis, whereas providing shade during hot weather conditions may prevent the drop in protein production. It is not clear if this is the result of a direct effect on mammary protein synthesis, or instead due to an indirect effect of altered mammary substrate supply.

During hot weather, food intake is reduced and the metabolic rate is lowered to prevent the body from overheating. Endocrine changes also occur, most notably systemic reductions in thyroxine and cortisol, and an increase in prolactin. Given the importance of prolactin for mammary protein synthesis, these contradictory effects would argue against a direct effect of heat stress on milk protein synthesis. In contrast to studies examining the effects of heat stress on milk production, there are considerably fewer studies examining the effects of cold stress. However, the limited available data suggest an increase in milk protein when animals are exposed to below 0 °C temperatures. Systemic prolactin concentrations are reduced during cold exposure, arguing again against a direct effect on milk protein synthesis. Feed intake increases during cold stress and the levels of the stress hormone epinephrine also increase, causing an increased rate of lipolysis. All these increase the energy supply to support an increased metabolic rate and probably increase mammary substrate availability.

Milking Frequency

Tight junctions are the cellular structures that prevent the intercellular movement of blood and/or milk components between adjacent secretory cells of the mammary epithelium. As discussed in more detail in **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose, more or less frequent milking than twice a day will result, respectively, in tighter or 'leaky' tight junctions. Leaky tight junctions will affect the whey protein composition of the milk, as small mammary-derived milk proteins, such as α-lactalbumin, will 'leak' from the milk via this paracellular route into the blood, whereas small blood proteins, such as proteolytic enzymes (e.g., plasmin), serum albumin, lactoferrin, and immunoglobulins, may leak into the milk pool. More frequent milking tends to tighten the tight junctions and prevents these changes from occurring.

Stage of Lactation

The protein content of milk changes considerably during lactation. The first milk after calving (i.e., colostrum) is very high in protein (approximately 230 g l^{-1}). This is mainly due to the large amount of immunoglobulins in colostrum. Within days of parturition, colostrum changes into regular milk, which is of much lower protein content. The milk protein content is lowest around 8 weeks into lactation, which coincides with the maximum negative energy balance of the cow, that is, with the maximum gap between energy intake from the feed and output via the milk is the largest. Hence, the low rate of mammary protein synthesis at this time is most likely related to insufficient substrate supplies to the mammary gland. As lactation progresses and the animal switches to a positive energy balance, milk protein content gradually increases. Toward the end of lactation, the whey content of milk starts to increase. This is the result of a gradual loosening of the tight junctions between the epithelial cells and is indicative of the onset of gradual mammary gland involution.

Mastitis

Mastitis is an inflammation of the mammary gland and is, in most cases, the result of a pathogenic infection. As such, there is a rapid influx of immune cells into the gland and subsequently into the milk. Most notably, the level of neutrophils increases rapidly with the onset of mastitis. The increase in cells in milk manifests itself in an elevated level of somatic cells. Indeed, the most commonly used on-farm indicator of mastitis is an elevated milk somatic cell count. In addition to immune cells, mastitic milk contains a vast array of peptides and proteins representing both the adaptive immune system (e.g., immunoglobulins, complement) and the innate immune system (e.g., cytokines, lactoferrin, lactoperoxidase, RNases, acute-phase proteins). Not only do these host defense peptides and proteins markedly change the whey protein composition of milk, the casein proteins may also be affected by mastitis. The casein content of milk will be reduced as mastitis causes an increase in plasmin activity in milk, which, as pointed out at the beginning of this article, degrades β-casein, resulting in an increase in γ-caseins.

Breed Differences

The previous factors have all been environmental, that is nongenetic factors, but it is well known that on average, milk composition, and in particular milk protein content, varies among the major dairy breeds. The protein content of milk from Jersey cows is significantly higher than the protein content of milk from Holstein-Friesian cows. However, the latter produce more milk, such that total milk protein production is higher in Holstein-Friesian cattle.

As mentioned earlier, different genetic variants exist for each of the major whey and casein proteins. In most dairying countries, there is considerable interest in exploiting such within- and among-breed polymorphisms, as increased contents of certain variants may have beneficial influences on the processing properties or may result in increased cheese yields. For example, milk from β-lactoglobulin AA cows contains more whey protein, whereas that from BB cows has more casein, and also better processing properties, such as higher curd tensions. Indeed, BB milk has been shown to give higher yields of certain cheeses (e.g., Parmesan and Gouda cheese).

Table 2 Examples of substances with biological activity present in milk[a]

Bioactive function	*Bioactive component*	*Chemical nature*	*Remarks*
Antibacterial	Lactoferrin, lactoperoxidase, lysozyme, defensin	Peptide	Can be added to infant formula, tooth paste, and cosmetics. May also be used to increase the shelf life of products
Gastrointestinal function (intestinal motility, emptying, absorption)	Casomorphin, lactorphin	Peptide	Casein-derived fragments with opioid agonistic activity
	Casoxin	Peptide	Casein-derived fragments with opioid antagonistic activity
	Serophin	Peptide	Opioid activity; derived from serum albumin
	Lactoferroxin	Peptide	Opioid antagonistic activity; lactoferrin-derived
	β-Lactotensin	Peptide	Affects the smooth muscle of the gut; β-lactoglobulin-derived
	Albutensin	Peptide	Affects the smooth muscle of the gut; serum albumin-derived
	Caseinomacropeptide	Peptide	Increases gut motility and CCK and gastrin release; κ-casein-derived
Cell growth and repair	Growth factors (e.g., IGF-I, EGF, TGF-α), growth inhibitory factors (MDGI, TGF-β)	Peptide	Play a role in the regulation of cell growth and repair in many different tissues (e.g., intestines); also used as supplements in cell culture media
	β-Casein-derived fragments	Peptide	Cell growth promoting
	Glutamylcysteine	Peptide	Stimulates glutathione, an antioxidant involved in cell protection and repair
	Lactoferrin	Peptide	Involved in cell protection and repair (antioxidant)
Hypertension lowering	ACE inhibitors	Peptide	Inhibits ACE, preventing the conversion of angiotensin-I into the active vasoconstrictor angiotensin-II
	Calcium	Mineral	
Mineral utilization	α_{s1}-, α_{s2}-, and β-casein-derived phosphopeptides	Peptide	Sequester minerals in soluble complexes for easy intestinal absorption (e.g., calcium, iron, manganese, selenium)
Bone synthesis	Calcium	Mineral	Promotes bone growth
	Hormone (PTHrP)	Peptide	Calcium uptake
Immunoregulation	Lactoferrin		
	Immunoglobulins	Peptide	Provide passive immunity. Bovine milk is a rich source of IgG and to a lesser extent IgA
	α- and β-casein-derived fragments	Peptide	Immune-enhancing properties
	Cytokines	Peptide	Stimulate lymphocyte trafficking and development of immune system
	Minerals (zinc, iron, copper, selenium) and vitamins (A, β-carotene, B$_6$, C, E)		Cofactors in many immune processes, and as such immunostimulatory
Anticarcinogenic	CLA	Lipid	Anticarcinogenic properties (in particular against mammary cancer)
	Sphingolipids (sphingomyelin, ceramides, gangliosides)	Glycolipids	Phospholipids of cell membranes. Inhibit cell growth and may suppress tumor growth
Increasing lean body mass	CLA	Lipid	Reduces body fat and enhances lean body mass
Prebiotics/probiotics	Galacto-oligosaccharides	Carbohydrate	Enhance the growth of beneficial bifidobacteria in the gut. Lactose derived; the disaccharide lactose is extended with β-galactosidase to form oligosaccharides
Atherosclerosis	CLA	Lipid	There is some evidence from animal studies that CLAs may lower cholesterol

[a] Information collected from various refereed and nonrefereed publications.

ACE, angiotensin converting enzyme; CCK,cholecystokinin; CLA, conjugated linoleic acid; EGF, epidermal growth factor; IGF, insulin-like growth factor; MDGI, mammary-derived growth inhibitor (homologous to fatty acid binding protein); PTHrP, parathyroid-related peptide; TGF, transforming growth factor.

Bioactive Milk Proteins

Bioactives are substances that consist of protein, lipid, and/or carbohydrate molecules and are capable of eliciting a biological response, such as reducing hypertension, preventing cancer, enhancing lean body mass, stimulating the immune system, and killing bacteria. Increasingly, milk is considered to be a rich source of such bioactives (**Table 2**). The first published results of proteomic analysis of milk, using powerful proteomic tools, revealed more than 90 different minor milk peptides and proteins, and many are thought to play a role in mammary host defense. Many of them are proteins or peptides that exist in very low concentrations in the whey fraction of milk. Some of them are blood-derived, whereas others may be synthesized within the mammary gland either directly or indirectly via breakdown of casein molecules (e.g., casomorphins).

Traditionally, caseins have been considered the most valuable proteins in milk given their importance for cheese manufacturing. Whey proteins, on the other hand, have long been considered to be of little value and were often disposed of as waste or returned to the farmer to be used as calf or pig feed; however, this has changed now and there is a market for whey products such as whey protein concentrates and lactoferrin. Bovine milk-derived lactoferrin is now a common ingredient in human and pet foods, cosmetics, or oral and skin care products. Moreover, bovine colostrum is now widely available as an immune-stimulating health supplement for human consumption. The importance of milk whey peptides and proteins is likely to increase further with the development of more advanced technology to detect and extract bioactives from milk. Although the yields of bioactive proteins are very low compared to the major milk proteins, they are included in products at the high end of the market (e.g., cosmetics, infant formula, biomedical products, and sport enhancing and health food products) yielding high financial returns. Such value-added products will certainly have positive impact on future milk prices. Whether casein will ever become a by-product of whey, as has been suggested, remains to be seen, but there is no doubt that milk-derived bioactive products will play an important role in the future success of the dairy industry.

Health Disorders

Humans may suffer from milk protein allergies after consuming dairy products. Allergies against milk proteins can occur in patients of all ages, but are most common in infants. Double blind studies have shown that up to 2% of infants may suffer from such allergies. Bovine milk proteins and their fragments are foreign to the human body, in particular β-lactoglobulin, which does not occur in human milk, and are recognized by the immune system as any other antigen, and as such can elicit an immune response. The prerequisite is, however, that the milk proteins or fractions thereof cross the intestinal wall into the bloodstream without being digested into amino acids. This occurs only in patients who suffer from a 'leaky gut' or compromised intestinal wall as a result of gastroenteritis, Crohn's disease, or gastrointestinal bleeding. In infants, it is probably related to insufficient or a delay in the natural process of closure of the intestinal wall after birth. Processing steps, such as hydrolysis, may help to lessen the allergenicity of milk proteins. Some claim that infants that are allergic to cow's milk are better off drinking goat milk-based products; however, the proteins in goat's milk are just as foreign to the body as those in cow's milk, and the same holds true for soy protein-based infant formula. That goat milk appears to be overall less allergenic than cow's milk may be related to the fact that the former contains a lower level of αS1-casein. Moreover, goat β-lactoglobulin appears to be broken down more effectively by humans than its bovine counterpart. Ironically, it has become increasingly evident from experiments that milk and colostrum components (bioactives) have beneficial effects on intestinal development and on the prevention of compromised gut syndrome (**Table 2**). There is even some evidence emerging from international epidemiological studies suggesting that raw, unpasteurized, bovine milk may have antiallergenic properties.

See also: **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose.

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Lactose

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Introduction

Milk provides an essential and complete diet for the newborn, which initially is unable to collect, chew, or digest solid food. Milk consists of a large number of both major and minor components. Lactose is one of the major components of milk, ranging in concentration from as little as $1\,\mathrm{g\,l^{-1}}$ in fur seals to more than $70\,\mathrm{g\,l^{-1}}$ in humans and donkeys (**Table 1**). In ruminants, the concentration of lactose in milk appears to be between 40 and $50\,\mathrm{g\,l^{-1}}$. In fact, of all milk components, lactose content is the least variable, and, as will be discussed in greater detail later, it is the major osmole in milk that determines to a large extent milk volume or the amount of water in the milk. A lower content of lactose in milk tends to be associated with less water in the milk or a higher content of milk solids. The advantages of this to dairy manufacturers in terms of lower storage and transportation costs are obvious. This is the reason why, despite it being one of the major milk components, not many countries consider the value of lactose in their milk pricing system. Nevertheless, from a biological point of view, lactose is an essential milk component.

Biosynthesis and Secretion

Lactose is a disaccharide consisting of two six-carbon carbohydrates: a glucose and a galactose molecule. Since the latter is also derived from glucose, glucose is the sole precursor of lactose in both ruminant and nonruminant species. The mammary gland itself does not produce glucose, because it lacks the crucial glucose-6-phosphatase (EC 3.1.3.9) enzyme, and all glucose must be supplied via the mammary arterial blood supply. During full lactation in dairy species, as much as 85% of the circulating blood glucose supply may be extracted by the mammary gland.

There is a distinct difference in glucose metabolism between ruminants and nonruminants. In the latter, food carbohydrates are enzymatically broken down in the digestive tract into simple sugars, and glucose is one of the major breakdown products readily absorbed from the small intestine into the bloodstream. In ruminants, on the other hand, the vast majority of food carbohydrates are broken down in the rumen by bacteria and protozoa to satisfy their own energy demand. They in turn produce volatile fatty acids such as butyrate, acetate, and propionate, which are absorbed through the rumen wall into the blood. In the liver, propionate is converted to glucose via gluconeogenesis. This means that ruminants are in a state of continuous gluconeogenesis and that blood glucose levels in ruminants are less than those in nonruminants. However, in both, glucose is essential for the maintenance of critical body functions, which means that the large drain of glucose by the lactating mammary gland potentially has greater repercussions on the ruminant. Potentially, ruminants have adopted a number of 'glucose-sparing' strategies. Instead of glucose, they can also use ruminally produced acetate as an energy source. Furthermore, in contrast to nonruminants, in which glucose is the major precursor for fatty acid synthesis in the mammary gland, in ruminants the required acetyl CoA carboxylase comes instead from acetate, thus leaving more of the precious glucose available for lactose synthesis.

The fact that all glucose, as the sole precursor of lactose, has to come from the blood raises the following question: How glucose gets into mammary secretory cells? High glucose concentrations in the blood and very low intracellular glucose levels imply a large concentration gradient. Formerly, it was generally believed that glucose entered the cell from the blood via a passive process of diffusion. However, it is now clear that transmembrane glucose transport is facilitated by at least two active transport systems. A family of facilitated glucose transporters (GLUTs) is expressed in the bovine mammary gland, with GLUT1 being the key transporter; several other members of the GLUT family are also expressed in the bovine mammary gland. Moreover, expression of the sodium/glucose cotransporters (SGLT1 and SGLT2) has been demonstrated in bovine mammary tissue, and the SGLT1 protein has been detected in the plasma membrane fraction. Significant upregulation of both GLUT and SGLT at the onset of lactation, compared with the nonlactating gland during gestation, clearly demonstrates a significant role of these transport systems in the uptake of glucose by the mammary epithelium.

Once in the cytosol, glucose has to move into the Golgi apparatus where the actual synthesis of lactose occurs. *In vitro* studies in the early 1980s with purified Golgi membrane vesicles from rat mammary cells suggest that glucose can freely enter the Golgi from the cytosol, but

Table 1 Gross composition of milks of various species

		Percentage by weight				
Species		*Water*	*Fat*	*Protein*	*Lactose*	*Ash*
Aardvark	*Orycteropus afer*	68.5	12.1	14.3	4.6	1.4
Black bear	*Ursus americanus*	55.5	24.5	14.5	0.4	1.8
Camel	*Camelus dromedarius*	86.5	4	3.6	5	0.8
Cow	*Bos taurus*	87.3	3.9	3.2	4.6	0.7
Dog	*Canis familiaris*	76.4	10.7	7.4	3.3	1.2
Dolphin	*Tursiops truncatus*	58.3	33	6.8	1.1	0.7
Donkey	*Equus asinus*	88.3	1.4	2	7.4	0.5
Fringed bat	*Myotis thysanodes*	59.5	17.9	12.1	3.4	1.6
Fur seal	*Callorhinus ursinus*	34.6	53.3	8.9	0.1	0.5
Goat	*Capra hircus*	86.7	4.5	3.2	4.3	0.8
Gray squirrel	*Sciurus carolinensis*	60.4	24.7	7.4	3.7	1
Guinea pig	*Cavia porcellus*	83.6	3.9	8.1	3	0.8
Hedgehog	*Erinaceus europaeus*	79.4	10.1	7.2	2	2.3
Horse	*Equus caballus*	88.8	1.9	2.5	6.2	0.5
Human	*Homo sapiens*	87.1	4.5	1	7.1	0.2
Indian elephant	*Elephas maximus*	78.1	11.6	4.9	4.7	0.7
Manatee	*Trichechus manatus*	87	6.9	6.3	0.3	1
Opossum	*Didelphis virginiana*	76.8	11.3	8.4	1.6	1.7
Pig	*Sus scrofa*	81.2	6.8	4.8	5.5	1
Rabbit	*Oryctolagus cuniculus*	67.2	15.3	13.8	2.1	1.8
Rat	*Rattus norvegicus*	72.4	9.3	8.2	3.7	1.4
Red kangaroo	*Macropus rufus*	80	3.4	4.6	6.7	1.4
Reindeer	*Rangifer tarandus*	66.7	18	10.1	2.8	1.5
Sheep	*Ovis aries*	82	7.2	4.6	4.8	0.9
Sloth	*Bradypus variegatus*	83.1	2.7	6.5	2.8	0.9
Tree shrew	*Tupaia belangeri*	59.6	25.6	10.4	1.5	
Water buffalo	*Bubalus bubalis*	82.8	7.4	3.8	4.8	0.8
Yak	*Bos grunniens*	82.7	6.5	5.8	4.6	0.9
Zebu	*Bos indicus*	86.5	4.7	3.2	4.7	0.7

Reproduced from Jenness R (1986) Lactational performance of various mammalian species. *Journal of Dairy Science* 69: 869–885.

work on mammary GLUT1 expression in the mouse mammary gland demonstrates targeting of this GLUT to the Golgi membrane, which obviously contradicts earlier observations of unrestricted entry of glucose into the Golgi. The mode of entry of glucose into the Golgi has not yet been resolved, but the presence of GLUT1 strongly suggests an active transport mechanism for glucose.

The synthesis of lactose from glucose requires a number of enzyme-mediated steps (**Table 2**). The initial steps occur in the cytosol, whereas the final step takes place inside the Golgi. The fact that UDP-galactose is formed in the cytosol means that it too needs to be transported into the Golgi and, as for glucose, there appears to be an active transport mechanism for UDP-galactose in the Golgi membrane. The final step of combining glucose and galactose takes place inside the Golgi, and the reaction is catalyzed by the enzyme lactose synthetase (EC 2.4.1.22). This critical enzyme consists of two proteins, the enzyme galactosyltransferase (EC 2.4.1.22) and α-lactalbumin, one of the major whey proteins in milk that is produced in the rough endoplasmic reticulum. Despite galactosyltransferase being bound to the inside of the Golgi membrane, the enzyme is not specific to

Table 2 Enzyme-mediated steps in lactose synthesis

1	Glucose + ATP	$\xrightarrow{(1)}$	Glucose-6-P + ATP
2	Glucose-6-P	$\xrightarrow{(2)}$	Glucose-1-P
3	Glucose-1-P + UTP	$\xrightarrow{(3)}$	UDP-glucose + PP_i
4	UDP-glucose	$\xrightarrow{(4)}$	UDP-galactose
5	UDP-galactose + glucose	$\xrightarrow{(5)}$	Lactose + UDP

(1) hexokinase (EC 2.7.1.1), (2) phosphoglucomutase (EC 5.4.2.2; formerly EC 2.7.5.1), (3) UDP-glucose pyrophosphorylase (EC 2.7.7.9), (4) UDP-galactose-4-epimerase (EC 5.1.3.2), (5) lactose synthetase (EC 2.4.1.22); UDP, uridine diphosphate; UTP, uridine triphosphate.

mammary cells. Only after forming a complex with α-lactalbumin, which increases the affinity of the enzyme for glucose, is it able to facilitate the formation of lactose, a process that is unique to the mammary gland. Although both galactosyltransferase and α-lactalbumin are essential components of lactose synthetase, the enzyme complex can be activated only in the presence of bivalent cations, probably manganese, zinc, and/or calcium.

Once synthesized, lactose cannot 'escape' from the Golgi organeller other than in vesicles budding from the inner Golgi membrane. These vesicles, called secretory vesicles, also contain milk proteins and move with the help of the microtubular component of the cytoskeleton through the cytosol toward the apical membrane of the cell. Here they fuse with the apical membrane and release their contents, including lactose, into the milk pool inside the alveolar lumen. However, because lactose makes the inside of the vesicles hypertonic with respect to the cytosol, water is drawn from the cytosol into the secretory vesicles until they are in equilibrium with the cytosol. Hence, an increase in lactose synthesis and thus higher concentrations inside the secretory vesicles result in more water being drawn in. This explains why the lactose content of milk varies very little within species, but it also means higher milk production. In other words, lactose determines to a large extent milk volume or the amount of water in the milk. Although ions and other small solutes will also contribute to the hypertonic environment within the secretory vesicles, lactose is by far the major osmotic constituent.

Reducing Milk Lactose Content

Lactose obviously plays a major role in determining milk volume, which would be of great benefit to the farmer if it were not for the fact that it does so solely by increasing water content and thus increasing milk transportation and handling costs. For this reason, many have considered lowering the lactose content of milk in an attempt to produce more concentrated milk, or even producing milk without lactose. The latter would make milk and dairy products, in general, more acceptable to people who are lactose intolerant. However, the biology of milk synthesis and secretion is such that this is impossible, atleast until the advent of modern biotechnology. This issue can be addressed through transgenics and gene knockout technology, albeit in laboratory animals.

Gene knockout experiments in mice not only demonstrate irrefutably the absolute requirement for α-lactalbumin for lactose synthesis, but also show that reducing or eliminating lactose synthesis will lead to a significantly lower, but more concentrated milk, yield. Moreover, milk production can be restored by introducing the gene for human α-lactalbumin (**Table 3**). These experiments not only show that α-lactalbumin is not species specific, but also demonstrate that it is not possible to produce lactose-free milk. Despite the fact that mice had both lactose alleles knocked out, they produced viable offspring, but the pups could not survive if left with the mother, presumably because the 'milk' was too concentrated to be removed from the mammary glands.

It should probably be pointed out that currently dairy processors would have to struggle to deal with milks of different levels of viscosity, because factories are set up to handle large amounts of milk with a consistent solids content of approximately 12%. A more realistic scenario, considered in some countries where milk has to be transported over long distances, is to set up regional substations where milk could be condensed to a desired solids level before it is transported. This would, however, require drastic changes in milk reception areas of dairy factories.

Table 3 The effect of α-lactalbumin gene knockout and gene replacement on milk yield and composition in mice

	Genotype			
Parameter	*α-lac^{m}/α-lac^{m}*	*α-lac^{m}/α-lac^{-}*	*α-lac^{-}/α-lac^{-}*	*α-lac^{h}/α-lac^{h}*
Fat (%) (vol/vol)	28.23 ± 1.65	29.6 ± 1.3	45.25 ± 2.15***	21.2 ± 0.23*
Protein (mg ml^{-1})	87.52 ± 5.82	95.81 ± 9.5	164.63 ± 13.92***	77.7 ± 1.05
Lactose (mmol l^{-1})	62.44 ± 9.27	42.7 ± 4.2	0.7 ± 0.34**	56.85 ± 3.8
Milk yield (g day^{-1})	7.51 ± 0.44	6.7 ± 0.38	1.37 ± 0.48***	9.94 ± 0.65*

Reproduced with permission from Stacy A, Schnieke A, Kerr M, *et al*. (1995) Lactation is disrupted by α-lactalbumin deficiency and can be restored by human α-lactalbumin gene replacement in mice. *Proceedings of the National Academy of Science of the United States of America* 92: 2835–2839.

Values given are the mean ± SE; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired *t* test. α-lac^{m}/α-lac^{m} = wild-type mice, with both α-lactalbumin alleles intact; α-lac^{m}/α-lac^{-} = mice deficient for one α-lactalbumin allele; α-lac^{-}/α-lac^{-} = mice deficient for both α-lactalbumin alleles; α-lac^{h}/α-lac^{h} = mice carrying two human α-lactalbumin alleles.

Factors Affecting Milk Lactose Synthesis

Diet and Environmental Effects

A more practical and less dramatic approach to manipulating milk lactose content would be through environmental and/or dietary measures. However, one must keep in mind that due to its role as the major determinant of milk volume, the lactose concentration in milk is very stable. Lactose yield, on the other hand, may fluctuate considerably, but this is a direct result of corresponding changes in milk yield. Nutritional factors, such as feeding frequency and level and order of concentrate and forage feeding, can significantly increase or reduce lactose yield by affecting arterial glucose supply to the mammary gland (i.e., changes in blood glucose concentration and/or mammary blood flow), changes in mammary uptake of glucose, and changes in the metabolic rate within the mammary gland. None of these factors, however, will have much of an impact on milk lactose content.

Environmental stress factors can similarly affect lactose yield. Temperatures on either side of the thermoneutral zone and high relative humidity have been shown to lower milk lactose yield. These observations are consistent with the results from experiments on the effects of providing shade during hot weather, which had shown that shade can prevent the adverse effects of high ambient temperatures on lactose production. In addition to these physical stress factors, it is well known that psychological stressors, such as noise, transport, novel environments, and fear, can adversely affect milk yield and thus lactose yield. None of these factors are likely to have any major direct effect on lactose synthesis in the mammary gland, but they act indirectly through reduced substrate supply due to directing away of glucose from the mammary gland to supply energy for other more acute body functions to maintain body temperature (e.g., shivering, cooling). Under psychological stress, critical tissues such as brain, heart, and skeletal muscle have an increased energy demand and levels of epinephrine, increase. These ensure that glycogenolysis increases in the liver and skeletal muscles, and glucose supply to the mammary gland is reduced, to favor of adequate supply to other critical tissues.

One interesting difference between nutritional factors and environmental factors in terms of their effect on lactose is that with the former, milk lactose content remains constant, whereas stress, both physical and psychological, results in lower concentrations of lactose in the milk. This is an interesting phenomenon given that lactose is the major osmotic factor in milk. The only way by which lactose content can decrease is by ‘leaking’ from the milk pool between adjacent alveolar cells into the bloodstream. Normally, this so-called paracellular pathway is blocked by tight junctions, the ‘gasket-like’ structures surrounding each secretory cell in the mammary gland, but there is now good evidence that stress can temporarily impair these tight junctions, resulting in elevated levels of milk lactose in the blood. Once in the blood, lactose is not metabolized, but it is cleared rapidly by the kidneys. The elimination half-life of lactose is approximately 40 min in cows.

Milking Frequency

Milk lactose content may also fluctuate as a result of milking frequency. Compared to twice-daily milking, the concentration of lactose in milk may be higher with thrice-daily milking. In contrast, there is ample evidence that once-daily milking results in lower levels of lactose in the milk. Given that milk cells cannot secrete lactose through the basolateral membrane, the only way the concentration of lactose in milk secreted into the alveolar lumen can decrease is by ‘leaking’ between secretory cells via open tight junctions into, the interstitial fluid and blood, as pointed out in the previous section. In fact, the measurement of lactose in plasma provides a good *in vivo* indicator of tight junction patency.

Once-daily milking decreases milk yield by 15–20%, and one of the processes occurring during the first 24 h, after switching from twice-daily milking to once-daily milking, is the opening of tight junctions after approximately 18 h of milk accumulation. This is demonstrated by a large increase in the level of plasma lactose (**Figure 1**). It has been shown that tight junctions are closely connected to the cytoskeleton of the cell, and that components of the tight junction are directly involved in gene expression through interaction with transcription factors. Thus, disruption of tight junctions, via disruption of their interaction with the cytoskeleton, may restrict the movement of secretory vesicles from the Golgi to the apical membrane, which would explain the decrease in milk yield with once-daily milking. Alternatively, with thrice-daily milking, tight junctions appear to be tighter than with twice-daily milking, and milk yield is increased correspondingly.

Milking frequency may also have a direct effect on lactose synthesis. A putative feedback inhibitory protein isolated from milk decreases lactose synthesis *in vitro* and decreases milk yield *in vivo* when administered via the teat to goats. It is proposed that with once-daily milking, this protein may act to decrease lactose synthesis and thus milk yield, whereas with more frequent milking the feedback inhibitory activity of the protein is reduced or prevented, allowing for increased milk yield. However, the exact mode of action of this protein and its sequence is yet to be established.

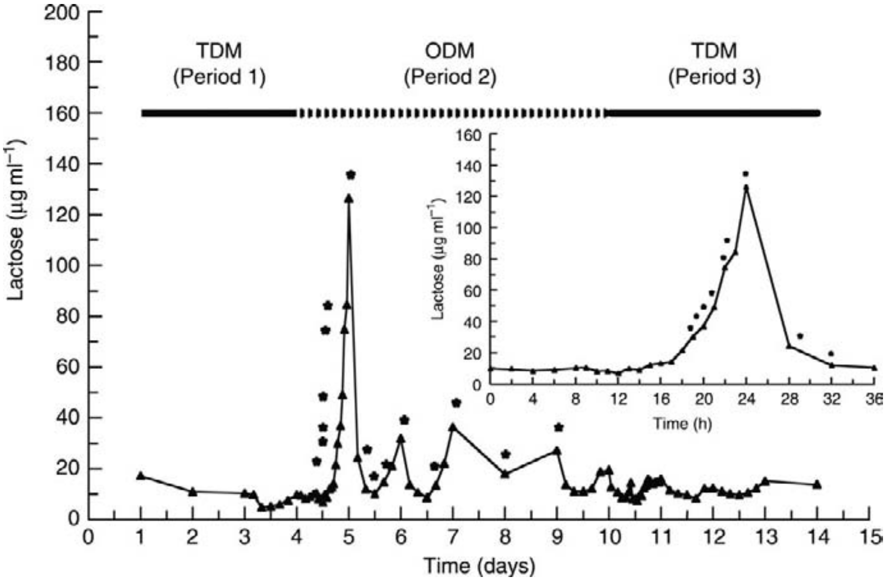

Figure 1 Effect of twice-daily (TDM) and once-daily (ODM) milking of Jersey cows on the concentration of lactose in blood plasma. Plasma lactose levels start to increase after approximately 18 h of milk accumulation, indicating that tight junctions between adjacent mammary secretory cells open. *Significantly increased compared to baseline levels ($P < 0.05$). Reproduced with permission from Stelwagen K, Farr VC, McFadden HA, Prosser CG, and Davis SR (1997) Time course of milk accumulation-induced opening of mammary tight junctions and blood clearance of milk components. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* 273: R379–R386.

In conclusion, there are a number of different mechanisms operating in the mammary gland that regulate milk yield in response to changes in milking frequency. Ultimately, the effects of these mechanisms appear to be mediated by lactose synthesis.

Uses of Lactose

Although lactose plays a crucial role in determining milk volume, not many countries include lactose in their milk payout scheme. Lactose, however, does hold value as a milk component in its own right. Globally, milk lactose is being used as a fermentation precursor to produce ethanol. This use of lactose may become increasingly important as demand for ethanol as an alternative to fossil fuel continues to increase. Traditionally, lactose has also been valued by the pharmaceutical industry as a filler compound in many common drugs. Finally, lactose is also the precursor of lactulose, an inert sugar molecule that is used as a prebiotic to stimulate gastrointestinal function and also as a treatment for constipation.

Health Considerations

Ketosis or acetonemia is a metabolic disorder that is caused by a sudden excessive influx of fatty acids into the liver as a result of lipolysis. There are several factors that may induce ketosis, but they are all related to a situation in which energy output exceeds energy intake. In high-producing cows, especially during early lactation, the sharp increase in mammary glucose uptake from the blood to facilitate lactose biosynthesis may cause the animal to become hypoglycemic and develop ketosis. Intravenously administered glucose and/or propylene glycol solutions may be used to treat hypoglycemia and ketosis. Glucocorticoids may also be an effective treatment. In ruminants, glucocorticoids reduce mammary utilization of glucose, thereby maintain higher systemic levels, whereas in nonruminants, glucocorticoids appear to enhance glucose production.

In certain human populations, in particular those of Asian and African origin, lactose intolerance is a common problem. Although this problem is not directly related to lactose biosynthesis in the mammary gland as such, it is an important consideration when milk-derived products are used for human consumption. People affected by lactose intolerance lack the enzyme lactase (EC 3.2.1.108). Lactase, produced by the small intestine, breaks down lactose into glucose and galactose, which can then be readily absorbed through the intestinal wall. In the case of lactase deficiency, lactose will not be broken down and absorbed, but instead it provides an excellent nutrient for bacteria residing in the gastrointestinal tract, resulting in increased carbon dioxide production, which leads to excessive flatulence. Excess lactose may also upset the osmotic balance in the intestine and interfere with water absorption from the intestine, causing, among other things, diarrhea. These properties, however, may also be used to our advantage, for example, by using lactulose, for which lactose is the precursor, as a stool softener to alleviate constipation in humans. Although the effects of lactose intolerance are not life-threatening, they can cause significant discomfort. However, it should be pointed out, that lactose intolerance is not a disorder. It could even be considered a normal developmental phenomenon. In many people, the production of lactase starts to decline after 2 years of age, when the diet changes from a milk-based to a nonmilk-based diet. In fact, humans are the only mammals that rely on milk-based foods as a major part of their adult diet. Therefore, perhaps lactose-tolerant people are the odd ones out, in a biological sense.

Conclusions

Glucose is the sole precursor for the biosynthesis of lactose in the mammary gland, and it is converted into lactose via a number of enzymatic steps. The final step, linking glucose and galactose, is mediated by lactose synthetase, which requires galactosyltransferase to form a complex with α-lactalbumin in order to increase the affinity for glucose. Because lactose is the predominant osmoregulatory component in the secretory vesicles produced within the secretory cells, it draws in water from the surrounding cytosol and, as such, determines milk volume when the vesicles fuse with the apical membrane and release their contents into the alveolar lumen. Management factors and environmental factors can affect lactose synthesis and, as a result, milk volume. However, most of these effects are mediated indirectly by affecting substrate (i.e., glucose) availability for mammary uptake.

See also: **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Crystallization; Lactose: Derivatives; Lactose: Galacto-Oligosaccharides; Lactose Intolerance; Lactose: Production, Applications; Indigenous Oligosaccharides in Milk; Maillard Reaction.

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Secretion of Milk Constituents

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Introduction

The major constituents of milk are either directly synthesized and secreted from the mammary epithelial cells into the alveolar lumen or transported across the epithelial barrier from other sources. At least five pathways are recognized (**Figure 1**): (1) lipid droplets bud from the cell apex and are secreted into milk with a membrane derived from intracellular sources and the cell surface (Pathway 1); (2) mammary epithelial cell-specific proteins such as the caseins, α-lactalbumin, and β-lactoglobulin, the disaccharide lactose, and at least some of the minerals and water are secreted by exocytosis (Pathway 2); (3) some proteins such as serum albumin and immunoglobulins, peptide hormones, and other constituents that originate from cells other than milk-secreting cells are conveyed across the mammary epithelium by transcytosis (Pathway 3); (4) a fraction of the minerals, small molecules, and water is transported across the basal/lateral and apical membranes by membrane-bound transporters (Pathway 4); and (5) constituents equilibrate between cells by a paracellular route during times when the epithelial tight junctions are permeable (Pathway 5). The last pathway may be important in some species during established lactation, and in production animals like cows during the earliest (colostral) and perhaps very late stages of lactation. In cows with healthy mammary glands the paracellular pathway appears to play a minor role during an established lactation because the junctional complexes are tight and effectively seal off the epithelium to extracellular small molecules.

This article will focus on the first four major pathways for milk secretion in ruminants and laboratory rodents. As far as is known, secretory mechanisms involved in milk production are the same or generally similar across species, judged primarily by morphological observations and analysis of milk composition. However, milk secretion has been studied in detail in a very few species; hence, the general conclusions discussed in this article should be considered with this caveat in mind.

Milk Lipid Secretion (Pathway 1)

Most of the lipid in cow's milk is present in globules that range in diameter from less than 0.5 to more than 8 μm. Each globule consists of a triacylglycerol-rich core surrounded by a membrane known as the milk lipid or milk fat globule membrane (MFGM) (*see* **Milk Lipids**: Milk Fat Globule Membrane). Within the mammary epithelial cells, milk lipid globules originate from triacylglycerols synthesized in the rough endoplasmic reticulum (ER) and are then assembled into microlipid droplets, each of which comprises a triacylglycerol-rich core surrounded by a surface coat composed of proteins and polar lipids. The details of how such droplets are initially formed in any species are unclear and the suggested mechanisms are contradictory and controversial.

The current paradigm for the genesis of lipid droplets in many cells, including the mammary epithelium, is that nascent triacylglycerols associate between the two leaflets within the hydrophobic core of the ER membrane. In the mammary gland, accretion of neutral lipid between the two phospholipid monolayers is presumed to lead to the formation of small droplets of triacylglycerols ($\leq$0.5 μm in diameter) that are released into the cytoplasm coated with the cytoplasmic-facing leaflet of the ER membrane. Thus, the droplets are assumed to acquire a monolayer of phospholipid and some proteins directly from the ER. Evidence for this mechanism comes from electron micrographs showing specialized areas of the membrane with osmiophilic material (presumptive nascent lipid droplets) accumulated between the bilayer halves. In addition, ER-associated phospholipids and resident ER proteins have been detected in lipid droplet fractions from isolated lactating mouse mammary tissue by lipid and proteomic analysis. That ER proteins are associated with secreted milk lipid droplets has been recently confirmed by proteomic analysis of the MFGM (*see* **Milk Lipids**: Milk Fat Globule Membrane). However, specialized budding sites within the ER membrane have not been routinely identified, and especially not at the frequency expected in the many cell types in which lipid is extensively synthesized and turned over.

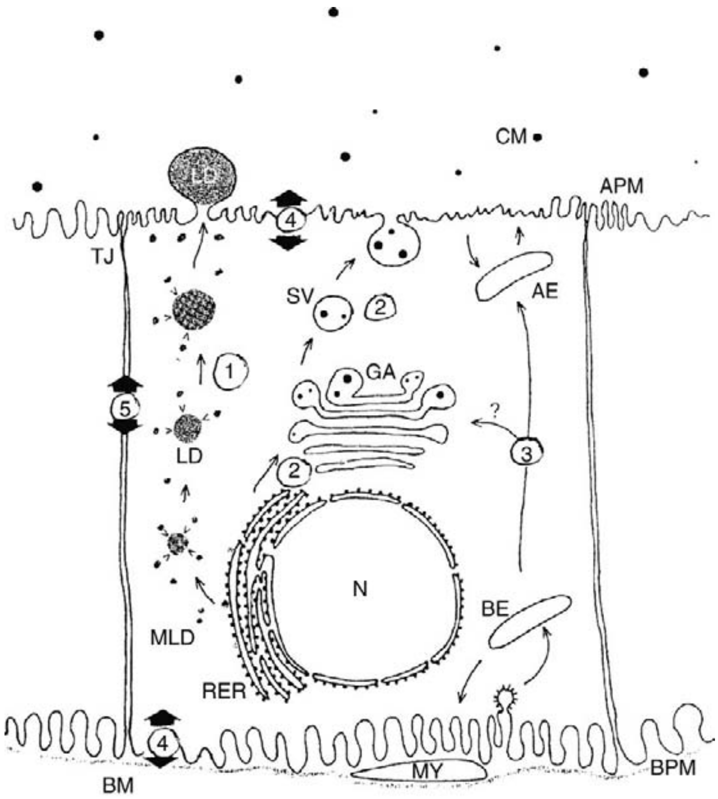

Figure 1 Diagram showing the major pathways for the secretion of milk constituents, discussed in the text. Pathway 1: Lipid secretion. Lipid droplets originate from the rough endoplasmic reticulum (RER) as small droplets called microlipid droplets (MLDs). Droplets can grow in volume by fusion with each other to form larger droplets (LDs), especially at the apical surface, and are secreted by envelopment in apical plasma membrane (APM), giving rise to milk lipid droplets. Pathway 2: Protein secretion. Proteins, lactose, water, and some ions are synthesized and processed through the classical secretory pathway, involving the RER, Golgi apparatus (GA), and secretory vesicles (SV). Pathway 3: Transcytosis. Molecules may transit the cell in either direction in vesicles formed at either the APM or basal/lateral plasma membranes (BPM). Basal (BE) and apical (AE) endosomes serve as sorting stations. Pathway 4: Ion transport. Ions and water are transported into and across the cell by specific transporters and channels. Pathway 5: Paracellular transport. Constituents may pass between the cells by a paracellular route if the tight junctions (TJ) are permeable. Pathway not shown: Secretion of membrane-bounded 'exosomes', which are presumed to be formed either by direct blebbing from the apical surface or by exocytosis of vesicles from multivesicular bodies. BM, basement membrane; CM, casein micelle; MY, myoepithelial cell. Reproduced with modifications by permission from Mather IH and Keenan TW (1998) Origin and secretion of milk lipids. *Journal of Mammary Gland Biology and Neoplasia* 3: 259–273.

An alternative possibility is that lipid droplets initially form in the cytoplasm close to the ER membrane surface. In one recent freeze-fracture study from Robenek's laboratory, presumptive nascent lipid droplets in macrophages were identified in proximity to the ER membrane, which formed egg-cup-shaped folds around the droplets. The lipid droplet-associated protein, adipophilin (ADPH), was localized by immunolabeling of the fractured surfaces on both the lipid droplet surface and the cytoplasmic leaflet of the ER adjacent to the droplets. These specialized regions of the ER were postulated to be the sites of lipid droplet assembly, and ADPH was suggested to play a role in facilitating the transport of triacylglycerols and fatty acids between the ER and the forming droplets. ADPH may also promote lipid accretion by sterically blocking lipase-mediated turnover. Whether the many other proteins and enzymes required for the assembly of nascent lipid droplets are also concentrated in such specialized areas of the ER is unknown, but this could be evaluated by similar immunolabeling techniques using lactating mammary tissue.

The size range of the lipid globules in milk argues that lipid droplets can grow substantially in volume after their formation. Globules with diameters >1 μm account for 90% or more of the total volume of lipid in cow's milk even though 80% or more of the droplets are <1 μm in diameter. Given this distribution, one can infer that many droplets grow little in volume, if at all, after their initial formation, but that the larger droplets must grow appreciably before secretion. Whether droplet growth occurs randomly or is a controlled process is unknown. Potential mechanisms include droplet expansion by the self-synthesis of triacylglycerols through the activity of droplet-associated lipogenic enzymes, or by the incorporation of triacylglycerols shuttled from sites of synthesis in the ER to the droplets by lipid carrier proteins. However, the only mechanism for which there is morphological and biochemical evidence is the direct fusion of microlipid droplets with each other. Fusion appears to be restricted to small droplets; these can fuse with each other and with larger droplets, but large droplets over ~2 μm in diameter do not appear capable of fusing with each other. From morphometric studies, it is apparent that lipid droplets can grow appreciably in volume just before and even during the secretion process as the droplet is being coated with the apical plasma membrane. That fusion may be a regulated process is suggested by observations that calcium promotes fusion and that small-molecular-weight GTP-binding proteins are present on droplet surfaces.

Lipid droplets traverse the cell from their sites of origin, primarily in basal and medial/lateral regions, to the cell apex, from whence they are secreted. Judging from electron micrographs of fixed tissue, droplets may associate with other organelles besides the ER during transit, including mitochondria, the Golgi complex, and secretory vesicles. Whether these associations are fortuitous, or are functionally important to droplet assembly, is unclear. In this context, proteomic analysis of the MFGM from several species has identified protein constituents from several intracellular sources in the secreted membrane, indicating that such associations may well modify the composition of the MFGM (*see* **Milk Lipids**: Milk Fat Globule Membrane; also see below).

Transit of the lipid droplets is almost certainly guided by cytoskeletal elements, but exactly how they function is not clear. The potential involvement of microtubules is suggested by live-cell imaging studies of the trafficking of lipid droplets in other cells that show the association and movement of lipid droplets along microtubule tracks. Microtubules are abundant in milk-secreting cells, and proteomic analysis has identified microtubule motors and microtubule-associated proteins in fractions of cytoplasmic lipid droplets from lactating mouse mammary gland. Drugs that disrupt microtubules or interfere with microtubule assembly suppressed milk lipid secretion when infused into the mammary gland via the teat canal. In glands treated with such drugs lipid synthesis was not inhibited and the cytosolic lipid droplets were larger than those in contralateral untreated control glands. Unfortunately, the secretion of milk serum was also inhibited, which precluded any definitive conclusions. The potential role of microtubules in the trafficking of lipid droplets in mammary epithelial cells deserves further study especially using live-cell imaging approaches to follow the movement in real time of droplet-associated proteins tagged with green fluorescent protein analogues. Actin-containing microfilaments and keratin intermediate filaments are also abundant in milk-secreting cells and keratin filaments show a strong propensity to bind lipids. In adipocytes, intermediate filaments form a cage around the lipid droplets. However, the potential role of such elements in lipid droplet assembly and trafficking in mammary cells has not been investigated.

Upon arrival at the cell apex, lipid droplets are discharged into the alveolar lumen by budding from the apical surface, such that the droplets are entirely coated with membrane (**Figures 1** and **2**(**a**)). During this process, a 10–20 nm thick electron-dense layer becomes visible in electron micrographs between the lipid droplet surface and the plasma membrane bilayer. This coat is presumed to consist of the cytoplasmic tails of integral plasma membrane proteins and peripheral proteins derived from multiple sources, including the lipid droplet surface, the cytoplasmic face of the apical membrane, and the cytoplasm. In static electron micrographs, droplets of all size classes, from very small to large, appear to be secreted by the same mechanism. In some cases, cytoplasm and even whole organelles may become trapped between the plasma membrane and the droplet, resulting in the formation of a structure known as a cytoplasmic crescent (*see* **Milk Lipids**: Milk Fat Globule Membrane). Crescent formation appears to be much less common in cows than it is in some other species.

In an alternative mechanism, Wooding has proposed that lipid droplets are surrounded by secretory vesicles in the cytoplasm. Progressive fusion of neighboring vesicles on the surface of the droplets is postulated to generate vacuoles, in which the lipid droplets are enveloped in secretory vesicle membrane. The contents of such vacuoles, which would include lipid droplets and skim milk components, are then presumed to be released by exocytosis from the apical surface. A combination of both mechanisms is also possible, with contributions to the MFGM coming from both the apical surface and the secretory vesicle membrane as the droplets engage with both membranes at the cell surface.

The bulk of available morphological and biochemical evidence favors the direct plasma membrane mechanism because some of the major integral proteins of the MFGM, such as butyrophilin and the mucin, MUC-1, are concentrated in the apical plasma membrane but not in secretory vesicle membranes. However, the possibility that both mechanisms may operate, perhaps with one or the other being more prevalent at different stages of lactation, cannot be excluded.

Little is known about the actual processes involved in recognition between lipid droplets and the plasma membrane, and what forces are required to expel the droplets from the cell. Several molecular mechanisms have been proposed based on the phenotypic analysis of knockout mouse strains, immunohistochemistry of selected MFGM proteins, and protein–protein interaction assays. Most models are based on the assumption that interaction between the lipid droplet and membrane occurs between

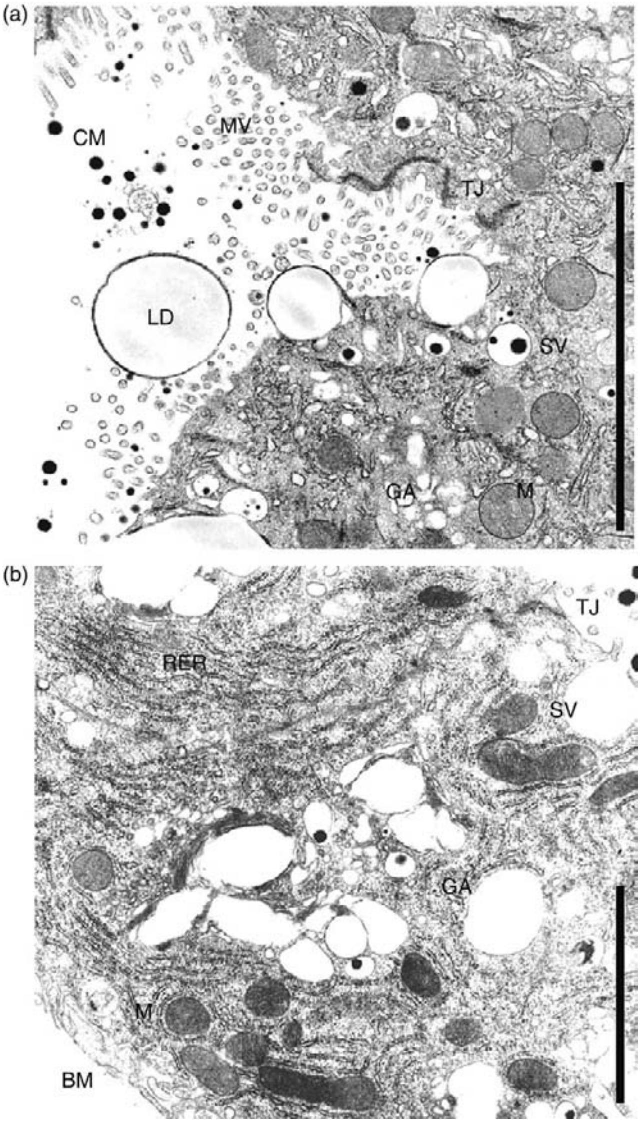

Figure 2 Electron micrographs of milk-secreting cells. (a) Lipid droplet secretion from the apical surface. One lipid droplet has been secreted and two are budding from the apical plasma membrane. (b) Entire cell showing centrally located Golgi apparatus with the associated swollen secretory vesicles and casein micelles. A secretory vesicle at the apical surface has fused with the apical plasma membrane. BM, basement membrane; CM, casein micelle; GA, Golgi apparatus; LD, lipid droplet; M, mitochondrion; MV, microvilli; RER, rough endoplasmic reticulum; SV, secretory vesicle; TJ, tight junction. Scale = 5 μm. Part (**a**) is reproduced with modifications by permission of the National Academy of Sciences, USA, from Ogg SL, Weldon AK, Dobbie L, and Mather IH (2004) Expression of butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. *Proceedings of the National Academy of Sciences of the United States of America* 101: 10084–10089.

proteins and protein domains exposed on the inner face of the apical plasma membrane, those on the droplet surface, and proteins from the cytoplasm. These protein complexes are presumed to give rise to the electron-dense layer sandwiched between the lipid droplet surface and the outer membrane bilayer visible in electron micrographs of osmium-fixed tissue. Proteins that contribute to this coat complex include butyrophilin 1A1 (BTN), xanthine dehydrogenase/oxidase (XDH/XO), and ADPH, which are all major constituents of the MFGM in many species. Knockout of the BTN (*Btn*) and XDH/XO (*Xdh*) genes severely disrupts the secretion of lipid droplets in *Btn*$^{-/-}$ and *Xdh*$^{+/-}$ mice, and large lipid droplets accumulate in the epithelial cell cytoplasm. In *in vitro* assays, XDH/XO binds to the B30.2 domain of BTN in the cytoplasmic tail, and in one model it is postulated to aggregate BTN in the plane of the lipid bilayer to form a multimeric complex with other proteins including ADPH on the lipid droplet surface. Thus BTN is seen to function as a transmembrane scaffold linking the membrane bilayer to the droplet surface. In distinct contrast, Robenek has proposed that formation of the MFGM is mediated through homophilic interactions between BTN on the lipid droplet and BTN in the outer membrane. However, this latter model requires the direct association of BTN, which is an integral transmembrane protein, with the lipid droplet in an unconventional topology. In a third model, ADPH on the lipid droplet surface is proposed to directly bind to the lipid bilayer via a putative hydrophobic cleft in the C-terminus. There is some evidence that lipid globule secretion may be controlled by protein kinases or phosphatases, by calcium, and by GTP-binding proteins, although nothing is known about how such regulatory molecules may function. Resolution of all the above issues would be aided by analysis of lipid secretion in cultured mammary cells *in vitro*. Unfortunately, no established mammary cell lines that secrete membrane-coated lipid droplets in a regulated manner are available.

Following secretion, the MFGM undergoes structural rearrangements, and some membrane vesiculates and is lost to the skim milk phase (*see* **Milk Lipids**: Milk Fat Globule Membrane).

Exocytosis (Pathway 2)

Unlike the mechanism of lipid secretion, which is unique to mammary cells, milk protein transport and secretion follow the universal secretory pathway. Milk proteins synthesized in the rough ER are processed through the Golgi complex, packaged into secretory vesicles, and secreted from the apical surface by exocytosis (**Figure 1**).

Major secretory proteins of cow's milk comprise the caseins (α_{s1}-, α_{s2}-, β-, and κ-), α-lactalbumin, and β-lactoglobulin (*see* **Milk Proteins**: Casein Nomenclature, Structure, and Association). All of these proteins are synthesized with N-terminal signal peptides, which target the respective mRNAs to the ER for translocation of the nascent peptides across the ER membrane. Within the ER lumen, the proteins are co- and posttranslationally modified and the signal peptides are proteolytically removed. The secretory proteins are then transported to the *cis* face of the Golgi apparatus in vesicles, which, by analogy with other better-characterized secretory systems, are most likely coated with COPII proteins. These coat proteins serve to recruit protein cargo to specialized exit sites in the ER and form a cage on the cytoplasmic side of the membrane, thus inducing curvature. Completed vesicles are released, which then transit to the *cis* face of the Golgi apparatus via an intermediate sorting station comprising tubular–vesicular elements, which is commonly referred to as the intermediate compartment. Whether such vesicles and tubules fuse together to form new Golgi cisternae or shuttle to preexisting Golgi membranes is currently the subject of intense debate. This point is yet to be studied in mammary epithelial cells.

Transport of proteins through the Golgi stack to the *trans* side is accompanied by further processing, although the exact sites for such reactions in mammary cells are unknown. At some point, the caseins are phosphorylated, and the *O*-linked glycan chains associated with κ-casein terminally sialylated. The caseins begin to assemble into micelles as they transit the Golgi apparatus and as they are packaged into secretory vesicles (*see* **Milk Proteins**: Caseins, Micellar Structure). Calcium, which is needed for casein micelle formation, is actively transported across Golgi membranes by Ca^{2+}-stimulated ATPases. However, in a recent paradigm shift, the bulk of the calcium in milk appears to be transported directly across the apical surface by a plasma membrane type Ca^{2+} ATPase (see section 'Secretion of Minerals and Water (Pathway 4)'). This implies that casein micelle formation may well continue in the alveolar lumen following secretion.

Following processing, the caseins and other secretory proteins are packaged into secretory vesicles. Whether such vesicles selectively contain just caseins or whey proteins, or both, has not been firmly established, although in one study rat caseins and α-lactalbumin were identified in the same secretory vesicles. Compared with secretory granules in other cell types, mature vesicles in mammary cells are swollen and distended, and as such they constitute a morphological hallmark of milk-secreting cells during lactation (**Figures 2**(**a**) and **2**(**b**)). Extensive swelling of the vesicles is presumed to be due to the presence of lactose, which is synthesized in the Golgi apparatus and draws water and ions into the vesicles by osmosis.

After formation, the secretory vesicles transit to the apical pole, possibly guided by microtubules. However, as is the case with the transport of lipid droplets, the experimental evidence for this is equivocal. Secretion of vesicle contents is accomplished by exocytosis, during which the vesicles fuse with the apical plasma membrane and the contents empty into the alveolar lumen. By analogy with secretion in more widely characterized secretory systems, the targeting and fusion of the vesicle membrane will be mediated by SNAREs, which form four-helix protein bundles (SNAREpins) that drive fusion of the apposing lipid bilayers. The identity of the SNAREs responsible in bovine mammary cells is uncertain. The mammalian plasma membrane/vesicle SNAREs, SNAP 23, syntaxin 3, and VAMP 2, have all been identified in bovine MFGM by proteomics, thus plausibly suggesting that they may function in secretory vesicle–plasma membrane fusion before being incorporated into the MFGM by default.

Exocytosis may occur by either simple or compound mechanisms. In simple exocytosis, single vesicles individually fuse with the plasma membrane. In compound exocytosis, vesicles in the cytoplasm interconnect in chains that fuse with each other. Thus, the contents of a number of vesicles can be released from the cell in a single vesicle–plasma membrane fusion event. Although both mechanisms have been observed in mammary cells by electron microscopy, simple exocytosis appears to be the most prevalent mechanism (**Figures 1** and **2**(**b**)).

Secretion from most cells can be classified into one of two main types: constitutive or regulated. Constitutive secretion is typically a continuous 'housekeeping' process, which is common to most cells. In regulated secretion, secretory protein is stored in membrane-bounded vesicles as highly condensed aggregates, which are secreted only when an external signal stimulates rapid exocytosis of the vesicle contents in a secretory burst. Milk protein secretion defies such a straightforward classification. On the one hand, it appears to be continuous, apparently needing no regulatory signal and thus could be considered constitutive. On the other hand, mature casein micelles are morphologically like regulated protein aggregates, even though they are surrounded by milk serum in swollen atypical vesicles. By the use of isolated primary acinar preparations, Burgoyne and colleagues have shown that casein secretion may occur at basal levels by an apparently constitutive mechanism. However, addition of calcium to these preparations stimulated secretion in an apparently regulated manner. This suggests that milk protein secretion may occur by both constitutive and acute calcium-regulated mechanisms, but, as yet, no external regulator of cytosolic calcium levels has been identified.

For many years, it was assumed that the secretory vesicle membrane added to the plasma membrane during exocytotic fusion serves to replenish that portion of the plasma membrane expended in fat globule envelopment. Although this may occur, it is obvious from the size and surface area of secretory vesicles and the volume of secreted skim milk that more vesicle membrane is added to the surface than is needed for the formation of the MFGM. Thus, a large fraction of the added membrane is most likely retrieved by endocytosis and is possibly recycled for further use, as occurs in many other cell types. Nothing is currently known about such possibilities in mammary epithelial cells. Membrane may also vesiculate at the surface and bleb off into the lumen, and contribute to the skim milk membrane fraction. In this context, vesicles derived from mammary epithelial cells and containing marker proteins such as BTN, XDH/XO, and immune molecules have been isolated and characterized from human breast milk. Such 'exosomes' are assumed to play immunomodulatory roles in protecting the gland from infections.

Transcytosis (Pathway 3)

Proteins found in milk that originate from cells other than milk-secreting cells most probably cross the mammary epithelium by transcytosis. Such proteins may include the immunoglobulins, transferrin, serum albumin, and prolactin. Similarly one can infer that at least some of the other peptide hormones, in addition to prolactin, found in milk cross the mammary epithelium by similar means. The only other possible route is between the cells by paracellular transport (Pathway 5), which, as indicated in the section, Introduction, is unlikely to occur in an established lactation because the epithelial tight junctions are sealed, at least in cows and mice.

Transcytosis begins by the uptake of extracellular material by endocytosis. Uptake into membrane-bounded vesicles can occur by either a clathrin-dependent or a clathrin-independent mechanism. Either mechanism may be receptor-mediated, or involve nonspecific uptake of extracellular fluid, the latter of which is known as fluid-phase endocytosis or pinocytosis. If the transport is in a basal to apical direction, the material is first delivered into a basally located sorting endosome (**Figure 1**) and is then re-sorted for delivery in a second vesicle through the cytoplasm to an apical endosomal sorting station. A final sorting step ensures delivery to the apical surface and secretion of the vesicle contents to the other side of the cell. During transit through the cell some constituents may be delivered to intracellular organelles for processing. For example, in rabbit and mouse mammary cells, transferrin and prolactin are taken up into coated vesicles, transported to basal endosomes, and then delivered either to multivesicular bodies or to the Golgi complex, wherein they enter the apically directed secretory pathway.

In the mammary gland, transcytosis is critically important for the transport of immunoglobulins, especially during the formation of colostrum, but also at lower levels throughout lactation. In cows, IgG_1 is selectively taken up from the serum via the interstitial fluid into vesicles by a specific receptor, which is most likely to be the neonatal Fc receptor (FcRn), a receptor that is widely expressed in epithelia that transport IgGs. Although the exact intracellular route remains unclear, it is assumed that the internalized IgG_1 is transported without modification to the apical side of the epithelium for secretion into colostrum or milk. IgG_2 is presumed to be taken up nonselectively into either the same IgG_1-containing vesicle, or a different class of vesicle by fluid-phase endocytosis and similarly transported. Thus IgG_1 is selectively concentrated four- to fivefold over levels in the serum, compared to IgG_2, whose concentration is more than threefold higher in the serum. In contrast, IgA is synthesized in plasma cells underlying the mammary epithelium, secreted as a dimer in association with

J chain, and taken up by the secretory cells into clathrin-coated vesicles by binding to the polyimmunoglobulin receptor. At some point during transcytosis, the exoplasmic portion of the polyimmunoglobulin receptor is cleaved and subsequently secreted as 'secretory component' bound to the IgA dimer, thus giving rise to secretory IgA (sIgA) in colostrum and milk.

Cells can transport material by transcytosis in either direction (apical to basal, or basal to apical). Depending upon cell type and physiological need, the amount of material transported in either direction may be substantially different. In mammary cells, it is assumed that most material (e.g., IgG, sIgA, transferrin, serum albumin) is transported in a basal to apical direction. However, there is fragmentary experimental evidence for the uptake of material from the milk and transport to the basal side of the cell. The potential physiological importance and magnitude of this pathway is unclear. Unfortunately, we know little else about the transcytosis pathway as it operates specifically in the mammary gland.

Secretion of Minerals and Water (Pathway 4)

Water is the most abundant constituent of cow's milk and, as indicated above, is most probably drawn into secretory vesicles in the Golgi complex by osmosis following the synthesis of lactose. In many cells, water transport is facilitated by the aquaporins, a family of integral membrane proteins that form pores in membrane bilayers. Aquaporin 3 has been histochemically localized to the basal and lateral plasma membranes of secretory and ductal epithelial cells in the rat mammary glands, but apparently no such proteins were detected in Golgi membranes, secretory vesicles, or the apical surface. Therefore, aquaporin 3 most probably functions in the uptake of water from the interstitial fluid across basal membranes. The secretion of water across apical membranes is presumed to be driven by ion gradients (see below).

There is an extensive history of investigation of transport mechanisms for univalent and divalent ions into milk, which is beyond the scope of this review. Briefly, the K^+/Na^+ ratio in secretory cells is maintained by a classical Na^+/K^+ ATPase on basal/lateral membranes that maintains an excess of K^+ over Na^+ in the secretory cell cytoplasm. This same K^+/Na^+ ratio is maintained in milk, albeit at lower total concentrations, by transport of the two ions across the apical membrane. A Na^+–K^+–Cl^- cotransporter on basal/lateral and possibly apical membranes serves as an inward-facing Cl^- pump and ensures that the concentration of Cl^- in the cytoplasm is higher than in milk. Thus milk is electrically positive relative to the cell cytoplasm, and it is this electrical gradient across the apical plasma membrane that is presumed to be the driving force for the secretion of water into milk. Osmotic balance between the cell cytoplasm and milk is modulated by the level of lactose, such that for any decrease in the amount of milk lactose there is a compensatory rise in the amount of K^+ and Na^+ in milk, on a day-to-day basis.

Ca^{2+} is the most abundant divalent cation in many milks, with total concentrations of 31 and 7.5 mmol l^{-1} in bovine and human milk, respectively. It was long thought that the major pathway for the transport of Ca^{2+} into milk was via a Golgi-located Ca^{2+} ATPase, which provided more than enough Ca^{2+} for the formation of casein micelles in Golgi-derived vesicles. However, recent work by VanHouten and Wysolmerski has shown that the principal transporter of Ca^{2+} in the mouse mammary gland is a spliced variant of the plasma membrane Ca^{2+} ATPase isoform 2 (PMCA2bw), which is targeted to the apical plasma membrane. This transporter is estimated to account for about 70% of the Ca^{2+} secreted into mouse milk, whereas the two Golgi-located transporters, called secretory pathway Ca^{2+} ATPases 1 and 2 (SPCA1 and 2), account for the remaining 30%.

Conclusions and Perspective

Today there is a good understanding of the general pathways by which lipids, milk-specific proteins, lactose, and some of the ions and water of milk are secreted, and we have a general idea of how non-milk-specific proteins can transverse the mammary epithelium to enter milk. What is missing is specific information about the mechanisms and molecular events involved in any of these processes in mammary epithelial cells. The revised paradigm for Ca^{2+} secretion discussed in the last section neatly highlights the potential surprises that may be in store.

Perhaps because the mechanism of lipid secretion is unique to the mammary gland, this has been studied in some detail, and we may be on the verge of understanding the molecular and regulatory processes involved. On the other hand, possible mechanisms for the exocytotic secretion of skim milk proteins and the molecules involved have been formulated by analogy with better-characterized model secretory systems (e.g., yeast and the neuronal synapse). In addition, much of what we know about mammary gland secretion is based on the examination of static electron micrographs and from some dated biochemical studies. There is no reason, *a priori*, to suppose that secretory mechanisms will be any different in mammary epithelial cells than those in other cells. However, several activities are unique to mammary cells, notably the synthesis of lactose, the assembly of casein micelles, and the secretion of membrane-coated lipid droplets followed by the continual loss of apical membrane that this process entails. Furthermore, intracellular and apical membrane may be lost in the form of 'exosomes' and contribute to the skim milk membrane fraction. The synthesis of lactose stimulates water transport into Golgi vesicles, which alters the morphology of the entire apparatus (**Figure 2**(**b**)). How this might affect transport of material through the Golgi stack is unknown. Mammary cells are ripe for study using contemporary live-cell imaging approaches and by the application of other modern cellular and molecular biological approaches. It remains a pity that we have so little specific information about the secretion of a fluid that is so critical for the survival of mammalian life.

See also: **Lactation**: Galactopoiesis, Effects of Hormones and Growth Factors; Galactopoiesis, Effect of Treatment with Bovine Somatotropin; Lactogenesis. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat; Milk Protein; Lactose. **Milk Lipids**: Milk Fat Globule Membrane. **Milk Proteins**: Casein, Micellar Structure; Casein Nomenclature, Structure, and Association; Immunoglobulins; α-Lactalbumin; β-Lactoglobulin.

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MAMMARY RESISTANCE MECHANISMS

Contents
Anatomical
Endogenous

Anatomical

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Introduction

Tissues associated with the teat duct or teat canal form the first barrier against mastitis-causing bacteria and include the exterior skin, sphincter muscle, stratified squamous epithelium of the teat canal lining, and keratin (**Figure 1**). These structures provide both a physical barrier and a chemical deterrent to bacterial colonization. Physical properties of the teat, such as length, width, and shape, also influence the incidence of mastitis as do teat end shape, milk flow rate, and presence of teat end lesions. Similarly, udder conformation and position are associated with mammary health. Fortunately, some of these traits are heritable, and to a certain degree, selection for resistance to mastitis is possible.

Teat Skin

The healthy exterior teat skin composed of stratified squamous epithelium serves as a defense mechanism by providing a hostile environment for microbial survival, including an impenetrable keratinized layer as well as bacteriostatic fatty acids. However, abnormalities such as cuts, abrasions, lesions, and chapping provide an environment for bacterial growth, especially the staphylococci, for example, *Staphylococcus aureus* and *Staphylococcus* spp. Proper milking machine function and use of appropriate teat dips containing teat skin conditioning agents, such as emollients and humectants, are important in maintaining a healthy, smooth, and intact teat skin.

Teat Canal Keratin

The teat canal is approximately 8.5 mm (5–13 mm) in length and between 0.4 and 1.63 mm in diameter, averaging 0.46 mm at its midportion. Keratin is a gummy substance produced by the stratified squamous epithelium that lines the teat canal. This epithelial layer is actually continuous with the external skin and results from an invagination of the epidermis during fetal development. This lining terminates abruptly at Furstenberg's rosette at which the stratified squamous epithelium becomes a double-layered epithelium. The stratum corneum of the teat canal epithelium is synonymous with keratin. Below this layer lie the stratum granulosum, stratum spinosum, and stratum germinativum. The epithelium is arranged in a series of longitudinal folds that interlock to form a seal as the sphincter muscle contracts after milking.

In cross-section, the teat canal lumen has a mesh-like appearance where it is occluded with keratin, and is surrounded by layers of epithelium. The keratinized cells are derived from the stratum granulosum, and this dedifferentiation is associated with loss of nuclei and cellular organelles. One function of keratin is to provide bacteriostatic and bactericidal lipids and proteins to repress growth of microorganisms in the teat canal. The other function of keratin is to help block the teat orifice, serving as a physical barrier to bacterial penetration between milkings.

During lactation, keratin exists in a dynamic state of generation and degradation as it sloughs from the stratified squamous epithelial lining. Portions of keratin are removed during each milking, probably as a result of the shearing forces of milk through the teat canal, but the substance is regenerated during the intermilking period within 24–60 h. Thus, immediately after milking, some degree of teat canal patency exists because of keratin removal, and this is the period during which the teat canal is most susceptible to penetration by mastitis-causing miroorganisms. However, keratin sloughing and removal are important because these processes help to remove colonized bacteria as does the flow of milk through the teat canal during machine milking. In fact, the mere flushing action of milk as it jets through the

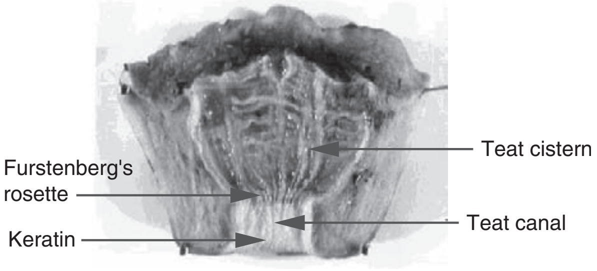

Figure 1 Longitudinal section through the distal teat end with lateral teat walls stretched and pinned to expose the teat canal and teat cistern. Furstenberg's rosette is found at the junction of the canal and cistern where numerous longitudinal tissue folds of the cistern converge. Keratin is observed as a white gummy substance lining the teat canal lumen.

teat canal is a defense mechanism because it removes colonized bacteria. Cows milked 3 or 4 times a day are generally less susceptible to mastitis than are cows milked twice a day. In addition, when the frequency of milking increases, the pressure within the udder is reduced, especially just before the next milking. This reduction in pressure is important because an excess of pressure within the teat cistern shortens the teat canal and increases the susceptibility to intramammary infection by mastitis-causing bacteria.

The loss of the keratin lining may be accentuated during conventional pulsation milking through the cyclic opening and closing of the teat canal, which tends to loosen the surface keratinized cells and promotes desquamation. In fact, it has been shown that more keratin is retained in the teat canal after pulsationless milking as compared with conventional pulsation milking. In one study, 50% more keratin was removed by pulsation milking as compared with milking without pulsation. However, by using pulsationless milking, the patency of the teat canal increases, making the quarter markedly more susceptible to intramammary infection. A decrease in the removal of keratin, as well as the bacteria entrapped in this substance, caused by milking without pulsation may also contribute to increased susceptibility to mastitis (**Figure 2**).

Physical properties of the teat end are influenced by the milking machine, which affect susceptibility to infection. Teat congestion or edema, as measured by teat end thickness, usually decreases after conventional milking, but where there exists a milking-induced increase in teat end thickness that exceeds 5%, colonization with mastitis-causing bacteria increases significantly. Likewise, teat end thickness increases after pulsationless milking, thereby increasing susceptibility to infection. In addition, the reduced blood flow to the teat end that accompanies edema may alter the redox potential of the teat end tissues and compromise defense mechanisms such as the activity of xanthine oxidase.

Within 2–3 weeks after drying off, a keratin plug completely occludes the teat duct and inhibits bacterial penetration (**Figure 3**). Research has shown that the udder is highly resistant to mastitis at this time. Keratin also contains certain proteins and fatty acids harmful to microorganisms; however, bacteria have been shown to survive in teat duct keratin for months. Thus, the value of these antimicrobial substances is questionable, and keratin serving as a barrier probably plays a greater part in host resistance to mastitis than the antimicrobial proteins and fatty acids.

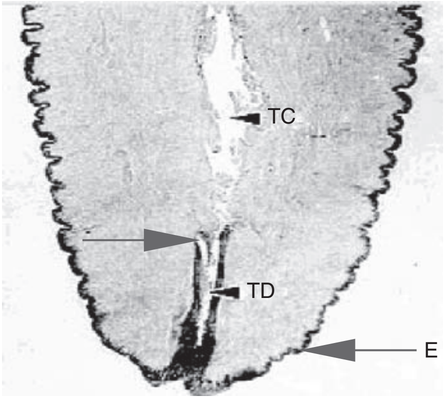

Figure 2 Longitudinal histological section of the teat illustrating the teat duct (TD) and teat cistern (TC). The junction of these two structures at Furstenberg's rosette is observed at the arrow. Note that the exterior teat skin (epidermis-E) is continuous with the teat duct.

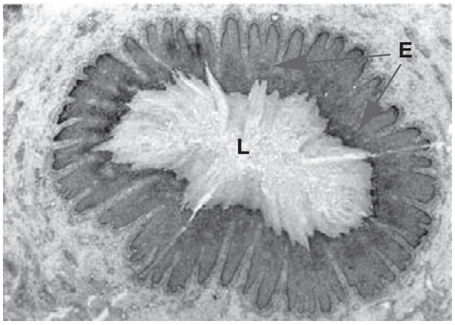

Figure 3 Cross section through the midportion of the teat canal illustrating the lumen (L) occluded with mesh-like keratin and the stratified squamous epithelium (E).

Sphincter Muscle

The teat canal is surrounded by bundles of smooth muscle fibers. The fibers are arranged longitudinally immediately adjacent to the epithelial lining, and in a circular manner around the canal deeper in the connective tissue. The circular smooth muscles in their contracted state function to maintain tight closure of the teat canal between milkings to prevent leakage, and to keep keratin occluding the canal lumen compressed as an aid in preventing bacteria from progressing upward into the teat cistern. The elastic fibers in the dermis associated with the teat end also aid in closing the teat canal.

During milking, the barrel of the teat elongates and the teat canal becomes dilated and shortened. However, after milking, contraction of the teat sphincter leads to a shortening of the teat barrel and lengthening of the teat canal. Shortened teats are less prone to injury, and the lengthened and closed teat canal is less prone to bacterial penetration. For a diagram of the teat sphincter (*see* **Mammary Gland**: Anatomy).

Teats with weak, relaxed, or incompetent sphincters are termed 'patent' or 'leaky'. Cows having such teats milk out fast in 2–3 min, but the incidence of mastitis is greater in quarters with patent teat canals. This is likely inherited, and such cows will be more susceptible to mastitis. Cows having teats with tight sphincters are called 'hard or slow milkers' because milk is expressed as a fine spray and rate of milk flow is reduced, thus they take longer to milk. The teat duct may remain dilated for 0.5–2 h after milking, and feeding the cows during this period keeps them on their feet, keeps the teats clean, and provides time for the sphincter muscle to tighten and close around the duct, thereby preventing bacterial entry.

Because the teat canal remains dilated for a period of time after machine removal, the dipping of teats into a germicide is recommended to reduce bacterial populations at the teat end and to prevent subsequent colonization and infection. If the milking machine malfunctions due to excessive vacuum or faulty pulsation, then teat end congestion or edema may develop as mentioned above. Such edema decreases tissue elasticity as well as muscle contractions at the teat end, which may enhance bacterial penetration.

Factors Affecting Anatomical Resistance Mechanisms

To maintain healthy teat end tissues, it is necessary to maintain recommended milking machine function and operation, namely, proper teat end vacuum level, optimum pulsation ratio, appropriate milking time, and proper teat cup removal. Awareness of these protective tissues of the teat end also becomes important when administering therapy by infusing antibiotics into the mammary gland. Bacteria living in or colonizing teat duct keratin require mechanical assistance to penetrate into the teat and the gland cisternal areas and cause an intramammary infection.

Full insertion of the antibiotic treatment syringe cannula may push portions of keratin colonized by bacteria into the teat cistern and induce intramammary infection. In addition, keratin could be forced against the interior teat duct wall by the syringe cannula, creating a larger than normal opening, thereby enhancing bacterial penetration. The conventional syringe cannula averages 3.1 mm in diameter, and teat duct diameters range from 0.40 to 1.63 mm for distal through proximal portions of the duct. Full insertion of a commercial cannula can result in temporary dilation of the duct lumen beyond the normal diameter (up to 8 times the normal diameter of the teat duct if the lower diameter range is considered). Tissue trauma caused by full insertion of the cannula may cause gaps or spaces in keratin, providing areas in which bacteria can adhere and colonize. A comparison of the histological cross sections of teat ducts that were inserted with a syringe cannula by partial or by full insertion revealed that the teat ducts inserted partially had a thicker keratin layer as compared with the teat ducts infused by full insertion. The latter exhibited partial loss of keratin, and it has been shown that removal of keratin decreases resistance to intramammary infection. Similarly, glands susceptible to infection have been shown to have canal keratin that was thinner, less dense, and detached from the epithelium in many areas.

Sanitation of teat ends prior to antibiotic infusion destroys many bacteria, but organisms lodged in microscopic cracks and crevices near the teat orifice and in the teat duct may be protected and may survive. In these sites, they could be carried upward as full insertion of the cannula is applied. If bacteria gaining access to the teat cistern by these various aforementioned routes are resistant or inaccessible to the infused drug, a new intramammary infection may result.

Studies designed to compare methods of cannula insertion for administration of intramammary drug therapy at dry off showed that the depth of cannula insertion had an effect on number of new intramammary infections at freshening. A 58.8% reduction in the number of new intramammary infections with *Streptococcus uberis*, *S. aureus*, *Streptococcus agalactiae*, and the coliforms at calving was found in quarters treated by partial insertion (2–3 mm) of the cannula as compared with those treated with full insertion.

Commercial syringes are now available that facilitate partial insertion by providing a twist-off tip, which when removed, allows the protrusion of ~3.0 mm of the syringe cannula, and at the same time forms a seal with the teat orifice to provide support during infusion and to ensure upward movement of the antibiotic (**Figure 4**). Use of such cannulas has reduced the incidence of new intramammary infections caused by *S. aureus* and *Str. uberis* by 40–50%.

Teat orifice lesions also affect resistance to mastitis, and include abnormalities such as eversions, hyperkeratosis, and hemorrhagic blisters. Such lesions are a result of leaving the milking machine on the cow for long periods of time (overmilking), excessive vacuum levels,

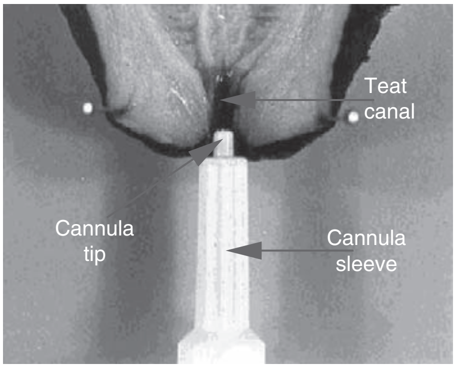

Figure 4 Syringe with removable tip, allowing partial insertion into teat canal.

inadequate pulsation, or a combination of these factors. Lesions are readily colonized by pathogenic bacteria such as *S. aureus*, and an increase in teat end lesions is associated with an outbreak of mastitis. The tissues lining the teat cistern can likewise be irritated by hard-liner mouthpieces under conditions of excessive vacuum, which leads to teat cup crawl or its upward movement on the teat barrel. This leads to a pinching-off of the teat cistern at the point where the teat attaches to the ventral surface of the udder, leading to constriction and tissue irritation as the opposite walls of the teat cistern lining rub against one another resulting in lesions. As stated above, such lesions are readily colonized by pathogenic bacteria and lead to intramammary infection.

Use of functionally adequate milking machines is also important to udder health. Research has confirmed that the milking machine can be a vector for transferring mastitis organisms from teat to teat and from cow to cow, and can serve as a means of transferring those organisms through the teat canal and into the udder. Every effort should be made to ensure that milking machines meet functional standards as well as operator use such as meeting internationally accepted design and installation standards, providing a relatively stable milking vacuum level of 275–300 mm of mercury or 37–41 kPa at the claw during peak milk flow, avoiding slipping or squawking teat cup liners during milking, and shutting off the vacuum to the claw before removing the teat cups. Milking machine vacuum fluctuations and inadequate vacuum reserve may lead to teat cup liner slippage. When this occurs, the teat cup liner drops or slips down on the teat barrel as milk volume and pressure in the teat cistern decreases, often resulting in a squawking sound as air enters the space between the liner and outer teat wall. This air travels down the liner, through the short milk tube, and across the cluster. Here, the air agitates milk in the cluster and forms tiny droplets of milk that are carried by the air to the opposite short milk tube and teat cup liner of the claw, ultimately impacting the opposite teat end. These impacting droplets are carried with such a high velocity that they penetrate the teat canal and enter the teat cistern. If such droplets are contaminated with bacteria from other quarters of the cow being milked or with bacteria that contaminate the milk tubes and cluster from previously milked mastitic cows, they can cause a new infection in the quarter into which they are propelled. Cluster removal under vacuum also may lead to droplet impacts.

Hereditary Factors

Heritability of udder and teat shape is moderate to high, and selection for cows with desirable traits may reduce the incidence of mastitis. For example, studies have demonstrated that deep or pendulous udders are more susceptible to intramammary infections and have higher somatic cell counts (SCCs) than shallow, tight, nonpendulous udders. The correlation between udder height and incidence of clinical mastitis is -0.13, while that between udder height and SCCs is -0.11. The heritability of udder depth is ~ 0.25.

Teats, according to their shape, are generally classified as funnel-shaped, cylindrical, or bottle-shaped. Research suggests that cows with funnel-shaped teats have lower incidence of intramammary infection than those with cylindrically shaped teats. The former may offer greater resistance to teat cup crawl, pinching-off of the teat cistern during milking, and subsequent tissue damage to the delicate tissues of teat end. In fact, teat end erosion is more common in cylindrically shaped than in funnel-shaped teats. Udders with funnel-shaped teats also have been shown to produce more milk, milk out more completely, and exhibit lower SCC.

Teat length and diameter are also associated with mastitis, and these traits are heritable. Teats with smaller diameters milk out more completely and are probably less prone to mastitis, whereas teats of larger diameter have larger orifices and are more susceptible to infection. One study indicated that the heritability of teat diameter may be as high as 0.67. The heritability of teat length ranges from 0.25 to 0.60; shorter teats are less prone to teat-treading injuries, milk out faster and more completely, and are associated with greater milk yield.

Teat end shape also affects resistance and appears to be highly heritable as well. Teat end shape is generally classified as pointed, round, flat (disk or plate-shaped), or inverted. Pointed teats tend to be longer and are predisposed to damage of the orifice. Inverted or disk-shaped teat ends are associated with larger-diameter teat ducts, which are less resistant to bacterial invasion. One study showed that as teat end shape varied from pointed to inverted, milk flow rate increased, which suggests larger-diameter teat canals. It has been suggested that after milking, milk may have a greater tendency to adhere to the bottom of the cone of inverted teat ends and serve as a source of nutrients for bacterial growth and subsequent penetration. In general, cows with round teat ends have lower incidence of infection and lower SCC than those with pointed, inverted, or flat teat ends.

As stated above, wider-diameter teat canals with increased milk flow rates are more susceptible to infection, whereas narrow canals are more resistant. The speed of milking is positively correlated with yield, that is, greater yields mean greater flow rates; thus, increasing the selection for yield will increase milk flow rates. However, the keratin that occludes the teat canal between milkings serves as the primary defense by forming a physical barrier, and research has shown that bacteriostatic lipids and proteins associated with this substance are also heritable.

See also: **Mammary Gland**: Anatomy. **Mastitis Therapy and Control**: Management Control Options; Role of Milking Machines in Control of Mastitis. **Milking Machines**: Principles and Design.

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Endogenous

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General Features

The mammary gland is protected by a variety of defense mechanisms, which can be separated into two distinct categories: innate and acquired immunity. Innate immunity, also referred to as nonspecific responsiveness, includes a set of resistance mechanisms that are not necessarily specific to a particular antigen. The generalized responses of innate immunity can be localized within affected tissues or activated and quickly mobilized to the site of infection by numerous stimuli; however, they are not augmented by repeated exposure to the same insult. Innate responses of the mammary gland are mediated by the physical barrier of the teat end, macrophages, neutrophils, natural killer (NK) cells, and by certain soluble and physiological factors. Conversely, the acquired or adaptive immune system recognizes specific antigenic determinants and can activate selective elimination of a pathogen. Recognition of specific pathogenic factors is mediated by antibody molecules, pathogen recognition transmembrane proteins, macrophages, and several lymphoid populations. Because of the 'memory' of certain lymphocytes, acquired immune responses can be augmented by repeated exposure to a particular pathogen. While it is convenient to discuss the highly complex nature of mammary gland defense in terms of nonspecific and specific responses, it should be emphasized that innate and acquired immunity do not operate independent of each other. In the mammary gland, it is necessary for both innate and acquired protective factors to be highly interactive and coordinated to provide optimal protection from mastitis.

Innate Immunity

Innate immunity is the predominant defense when the mammary gland is first exposed to mastitis-causing pathogens and before the adaptive immune system is activated. The development of acute or chronic inflammatory responses, for example, is often associated with several aspects of innate defense mechanisms in response to bacterial infection. Most pathogens are readily eliminated within a short period of time, and inflammation is resolved if nonspecific defense mechanisms function adequately during the early stages of disease pathogenesis. Rapid elimination of bacteria often will not result in noticeable changes in milk quality or quantity. Innate defenses of the mammary gland consist of anatomical barriers provided by the teat, tissue and milk leukocytes, cellular receptors located on different mammary cell populations, and a variety of noncellular physiological factors (**Table 1**).

Teat End Defense and Bacteriostatic Fatty Acids

Since pathogens must gain entrance to the mammary gland in order to cause mastitis, the teat end is considered the first line of localized defense against invading bacteria. The teat end contains sphincter muscles that surround the teat end opening and maintain a tight closure between milkings to hinder bacterial penetration. The teat duct is also lined with keratin that is derived from the epithelial lining. Accumulation of keratin can provide a physical obstruction to bacteria, particularly during the nonlactating period when the duct can become completely occluded. The lipid components of keratin also contain antibacterial factors in the form of bacteriostatic and bactericidal fatty acids. In general, Gram-positive bacteria are more susceptible than Gram-negative bacteria to the bacteriostatic and bactericidal effects of keratin. While the proposed mechanisms for keratin's antibacterial activity are still a subject of debate, there is evidence to suggest that the long-chain fatty acids disrupt bacterial lipid membranes, resulting in bacterial cell perforation. Differences in the composition of Gram-positive and Gram-negative bacterial cell walls may explain the differential antibacterial effects of keratin with respect to bacterial species. Through several mechanisms, the teat end can prevent the penetration of mastitis-causing pathogens and inhibit most bacterial growth. However, increased patency of the smooth muscles in the teat end or removal of the keratin lining is directly related to increased incidence of mastitis.

Table 1 Summary of innate mammary defenses

Type	*Factor*	*Mechanism*
Anatomical	Teat end	Sphincter muscles provide a mechanical barrier to hinder bacterial entry Keratin impedes bacterial growth and entry into the gland Furstenberg's rosette is densely populated with leukocytes, but the significance to host defense is not defined
	Neutrophils and macrophages	Phagocytosis and intracellular killing of bacteria; secretion of antibacterial factors; formation of NETs
	Natural killer cells	Nonimmune lymphocytes that secrete antibacterial proteins upon activation
	Epithelial cells	Expression of TLRs that sense the presence of bacteria in the mammary gland
	Cytokines	Proinflammatory factors that enhance the magnitude and activity of mammary leukocytes
	Complement	Bacteriolytic and/or facilitates phagocytosis

NETs, neutrophil extracellular traps; TLR, toll-like receptor.

Innate Cellular Defenses

If bacteria are successful at overcoming the local defenses of the teat end, the antibacterial activities of the mammary gland microenvironment can serve to inhibit the establishment of disease. Both resident and newly recruited mammary gland leukocytes play an essential role during the early stages of pathogenesis. Lymphocytes and macrophages are the predominant leukocyte types found within uninfected mammary tissues, whereas neutrophils are rarely present. In the milk of healthy mammary glands, total somatic cell counts (SCCs) are often $<10^5$ cells ml^{-1} and the distribution of leukocytes in the milk varies as a function of lactation stage. However, total SCC can increase to $>10^6$ cells ml^{-1} of milk within just a few hours of bacterial penetration and the major leukocyte types are neutrophils. The promptness and magnitude of neutrophil migration into mammary gland tissues and milk is considered to be a major determining factor for the establishment of new intramammary infections.

An important innate defense mechanism facilitated by mammary gland leukocytes is the ingestion and killing of bacteria, a process referred to as phagocytosis. In the mammary gland, phagocytosis is carried out primarily by neutrophils and macrophages. During phagocytosis, the leukocyte's plasma membrane extends to form pseudopodia that entrap bacteria and results in the formation of vacuoles called phagosomes. The newly formed phagosome moves toward other vesicles (lysosomes) within the cytoplasm where they fuse to form a phagolysosome. Bacteria are destroyed within these phagolysosomes by both oxygen-dependent and oxygen-independent factors and these will be described in more detail later. The degraded bacteria are then exocytosed from the neutrophil or macrophage. Mammary gland neutrophils and macrophages can ingest fat, casein, and other milk components, which render them less effective at phagocytizing bacteria. The phagocytic and bactericidal activities of these cells are especially diminished during the periparturient period and are thought to be an underlying cause of increased susceptibility to mastitis during this stage of lactation. However, the phagocytic and bactericidal capabilities of neutrophils and macrophages can be increased substantially in the presence of opsonic antibody for specific pathogens.

Neutrophil extracellular trap (NET) formation is an additional antimicrobial defense mechanism in the innate immune system. Pathogen stimulation of neutrophils triggers the release of nuclear material (DNA, histones) as well as granular proteins and extracellular fibers that function to trap and kill microbes. NET formation may be of particular importance to the mammary gland due to the ability of NETs to function in the presence of milk in contrast to other neutrophil functions that can be suppressed in that environment.

Another potentially important innate defense of the mammary gland is facilitated by a subpopulation of lymphocytes called NK cells. NK cells are characterized as large granular lymphocytes that have cytotoxic activity independent of the major histocompatibility complex (MHC). A unique aspect of NK cells is their ability to utilize Fc receptors to participate in antibody-dependent, cell-mediated cytotoxicity. For example, NK cells can bind tumor cells or virus-infected cells and then destroy the target cell by membrane disruption through the release of perforin. Cytokine-stimulated NK cells, however, are also capable of killing bacteria by releasing bactericidal proteins that belong to the saposin-like protein family. Specifically, bovine mammary tissue NK cells exhibit bactericidal activity against *Staphylococcus aureus* and, therefore, these lymphoid populations could be an important aspect of innate defense in preventing mastitis. Changes in this cell population during the periparturient period have not been studied extensively, but the potent bactericidal activity of these cells makes them worthy of future study.

Leukocytes are not the only cell type within the mammary gland that can contribute to innate immunity. The ability to sense the presence of bacteria within the mammary gland is an essential component of early host defense and any cell capable of facilitating this recognition can effectively stimulate the innate immune response. Toll-like receptors (TLRs) are a family of transmembrane proteins that recognize and bind to specific sequences of pathogens, termed pathogen-associated molecular patterns (PAMPs). Receptor expression is predominantly on antigen-presenting cells associated with acquired immune responses, but TLRs are also expressed on endothelial cells, epithelial cells, fibroblasts, and adipocytes. TLR-2 and TLR-4 are of particular importance to mammary defense as these receptors recognize components of Gram-positive (peptidoglycans) and Gram-negative (lipopolysaccharide) bacteria, respectively. Following pathogen recognition and binding, TLRs transmit intracellular signals to initiate innate immune responses. Activation of TLRs may also serve to link the innate and acquired immune responses by providing some pathogen specificity to the responses.

Innate Physiological Factors

Neutrophils are the predominant leukocyte type during the initial inflammatory response and, therefore, both the oxygen-dependent and oxygen-independent killing mechanisms of this cell type play a critical role in the establishment of disease (**Table 2**). The oxygen-dependent system is operative during the ingestion process in which there is a major burst of oxidative metabolism. The increased oxygen consumption results in the production of reactive oxygen and nitrogen intermediates, which are produced through a metabolic process known as respiratory burst. These microbicidal oxidizing agents are located within phagolysosomes and can oxidize bacterial membrane lipids and cause pathogen destruction. The primary enzymes involved in catalyzing the oxidation process are myeloperoxidase and superoxide dismutase.

In addition, bacteria may become exposed to and destroyed by several oxygen-independent reactants, including lysozyme, a variety of cationic proteins, and lactoferrin (**Table 2**). These antimicrobial elements of neutrophils are also stored within large cytoplasmic granules unique to bovine neutrophils. For example, a number of cationic peptides have been isolated from these large granules of bovine neutrophils and have been studied and described experimentally. Bovine neutrophil cationic proteins are a heterogeneous group including cathelicidin family members and the β-defensins, which display antibacterial activity against pathogens associated with mammary gland infection. As characterization of these proteins progresses, recombinant proteins or synthetic homologues may become available for study as potential therapeutics for the treatment of mastitis.

Lactoferrin is among the better-characterized antimicrobial proteins and is the most common iron-binding protein found in the exocrine secretions of mammals. Lactoferrin levels in milk fluctuate during the lactation cycle, with the highest concentrations observed in the fully involuted gland. The ability of lactoferrin, in the presence of bicarbonate, to bind soluble iron in milk is the basis of its most important biological activities. First, it can act as a transport protein by moving the bound iron to a different area within the host. In addition, the iron-binding capability of lactoferrin greatly reduces soluble ferric iron available to multiplying bacteria. Withholding this essential element prevents the production of dismutase, an enzyme produced by bacteria to counteract superoxide radicals generated by the host. Collectively, the iron-binding capacity of lactoferrin results in a bacteriostatic effect, resulting in greatly reduced bacterial multiplication rates. Lactoferrin can also have direct bactericidal effects on certain mastitis-causing pathogens and is known to play a role in normal lymphocyte and macrophage functions. However, the bacteriostatic and antibacterial properties of lactoferrin are depressed in the presence of citrate, a natural buffer produced by the mammary epithelium and which chelates iron into a form that bacteria can utilize. Citrate levels in milk tend to be very low during involution but increase substantially at calving. There exists a direct correlation between changes

Table 2 Neutrophil bactericidal factors

Type	*Factors*	*Mechanism*
Oxygen dependent	Superoxide anion ($O_2^{\,-}$) Hydrogen peroxide (H_2O_2) Hydroxyl radicals (OH^-) Myeloperoxidase (MPO)	Unstable molecules that bind to and damage bacterial cell walls and membranes
Oxygen independent	Lysozyme	Cleaves carbon bonds and disrupts bacterial cell walls
	Bactenecins (BAC 5, BAC 7), indolicidin	Cathelicidin family members that pass through bacterial membranes; can inhibit intracellular synthetic processes
	β-Defensins (BNBD 1–13)	Mechanisms yet to be defined
	Lactoferrin	Sequesters iron to prevent bacterial uptake; disrupts bacterial cell wall; regulates mammary leukocyte activity

in citrate and lactoferrin ratios in lacteal secretions and susceptibility to new intramammary infections.

There are several other soluble factors associated with innate defenses of the mammary gland. Complement is a collection of proteins present in serum and milk that can impact both innate and acquired immunity. Many of the biological activities of complement are mediated through complement receptors located on a variety of cells. Complement activation can occur through three different pathways resulting in lysis of bacterial target cells. Gram-negative bacteria such as *Escherichia coli* are especially sensitive to complement-mediated lysis. Complement also functions in concert with a specific antibody as an opsonin, which will promote bacterial phagocytosis and intracellular killing by neutrophils and macrophages. Concentrations of complement are highest in colostrum, inflamed mammary glands, and during involution. In contrast, concentrations of complement are lowest during lactation. Therefore, because of its intermittent presence in milk, complement is thought to play only a minor bactericidal role in the mammary gland.

Cytokines are a heterogeneous group of low-molecular-weight glycoproteins secreted by both immune and nonimmune cells under a variety of circumstances. The immunoregulatory capacity of the cytokine network is complex and these molecules are known to regulate essentially every aspect of inflammation and immunity. Cytokines bind to receptors on target cell membranes and can exert an autocrine, paracrine, or endocrine action. Individual cytokines can interact with other cytokines synergistically, additively, or antagonistically on multiple cellular targets. Cytokines regulate the intensity and duration of the host response to infection by regulating (enhancing or inhibiting) the activation, proliferation, and differentiation of cells involved in the immune response. Therefore, cytokines can influence both innate and acquired immunity in the mammary gland.

Cytokines can indirectly influence the severity and duration of mastitis by regulating the promptness of the leukocyte migratory response and the efficiency of phagocytes at the site of infection. Macrophages become activated in response to bacterial toxins and other factors associated with bacterial colonization. Upon activation, macrophages are capable of producing a variety of cytokines. Certain macrophage-derived cytokines, including tumor necrosis factor (TNF), interleukin 1 (IL1), IL6, and IL8, regulate the magnitude and duration of leukocyte infiltration into mammary tissues at several stages of the inflammatory response (**Table 3**). If neutrophils are able to migrate rapidly from the bloodstream to the mammary gland and effectively eliminate the bacteria, then the recruitment of leukocytes will cease and SCC will return to healthful levels. However, if bacteria persist, then the inflammatory response will continue into a chronic state. Prolonged diapedesis of leukocytes causes considerable damage to mammary parenchymal tissues and this will result in reduced milk production. The overexpression of TNF and IL1 during acute inflammation is also directly correlated with morbidity and mortality associated with coliform mastitis. One of the most potent activators of neutrophil and macrophage functions is interferon-gamma (IFN-γ), which is produced to a large extent by activated T lymphocytes.

Acquired Immunity

Acquired or specific immunity is triggered if a pathogen is able to evade or is not completely eliminated by the innate defense system. Specific immune responses are elicited to particular antigenic challenges associated with bacterial pathogens or any other foreign bodies. An amazing feature of the immune system is the ability of a host to recognize and respond to billions of unique antigens that they may encounter. If a host should encounter the same antigen more than one time, a heightened state of immune reactivity would occur as a consequence of immunological memory. In comparison with the first exposure to a particular antigen, a memory response will be much faster, considerably stronger, last longer, and often be more effective in clearing the pathogen. It is on this feature of the specific immune response that vaccination protocols are based. It is also important that an inappropriate specific immune response does not occur against the host's own antigens. For this reason, the immune system is able to distinguish self from nonself and selectively react to only foreign antigens. Genetically diverse, membrane-bound proteins called

Table 3 Cytokines that influence mammary gland inflammation

Major event	*Biological function*	*Cytokines*
Vascular changes	Vasodilation, increased blood flow, adhesion molecule expression	TNF, IL1, IL6, IL8
Chemotaxis	Leukocyte attraction to the vascular endothelium, margination and extravasation to the site of infection	TNF, IL1, IL6, IL8
Leukocyte activation	Phagocytosis and intracellular killing, cytokine production	IFN-γ, TNF
Metabolic changes	Fever, induce synthesis of acute-phase proteins, cachexia	TNF, IL1, IL6

IFN-γ, interferon-gamma; IL, interleukin; TNF, tumor necrosis factor.

MHC molecules assist in this recognition. A specific immune response will occur only if antigens are combined with an MHC molecule on the surface of certain cells, a process referred to as antigen presentation.

Acquired Cellular Defenses

Generation of effective specific immunity involves two types of cells: lymphocytes and antigen-presenting cells. Lymphocytes recognize bacterial antigens through membrane receptors specific to the invading pathogen. These are the cells that mediate the defining attributes of adaptive immunity including specificity, diversity, memory, and self/nonself recognition. The T and B lymphocytes are two distinct subsets of lymphocytes and they differ in function and protein products. The T lymphocytes can be further subdivided into $\alpha\beta$ T lymphocytes, which include CD4+ (T helper) and CD8+ (T cytotoxic and T suppressor) lymphocytes, and $\gamma\delta$ T lymphocytes (**Table 4**). Depending on the stage of lactation and tissue location, the percentages of these cells can vary significantly.

The T helper lymphocytes produce cytokines in response to recognition of antigen–MHC complexes on antigen-presenting cells (B lymphocytes and macrophages). When activated, T helper cells produce a variety of immunoregulatory cytokines. Through the ability to secrete certain cytokines, T helper cells play an important role in activating both T and B lymphocytes, macrophages, neutrophils, and various other cells that participate in the immune response. Differences in the particular pattern of cytokines produced by activated T helper cells result in different types of immune responses. For example, the cytokines IFN-γ and IL2 are thought to enhance some nonspecific cellular activities such as phagocytosis and intracellular killing.

The CD8+ lymphocytes can exert either a cytotoxic or a suppressor function when activated. Cytotoxic T lymphocytes recognize and eliminate altered self-cells via antigen presentation in conjunction with MHC class I molecules. There is some speculation that cytotoxic T lymphocytes may act as scavengers that remove old or damaged secretory cells in the mammary gland. Similar to T helper lymphocytes, suppressor T lymphocytes are thought to control or modulate the immune response by the repertoire of cytokines that they produce. Suppressor T lymphocytes can become activated in infected mammary glands and during the periparturient period. This process possibly contributes to impaired local defenses under these circumstances.

The biological functions of $\gamma\delta$ T lymphocytes have been the subject of much speculation. Their functions are primarily associated with the protection of epithelial surfaces. There are indications that $\gamma\delta$ T lymphocytes can mediate cytotoxicity with variable involvement of MHC. These cells may also play a role in infectious diseases and therefore provide an important line of defense against bacterial diseases. Mammary secretions and tissues express a higher percentage of $\gamma\delta$ T lymphocytes compared to the peripheral blood. The fact that the percentages of these cells decrease significantly in the mammary gland during times of increased susceptibility to disease suggests that these lymphocytes may constitute an essential line of defense against mastitis-causing pathogens.

The primary role of B lymphocytes is to produce antibodies against invading pathogens. B lymphocytes utilize membrane-bound antibody molecules to recognize specific pathogens. Following recognition, B lymphocytes can internalize, process, and present antigen in the context of MHC class II molecules to T helper lymphocytes. The T helper lymphocytes become activated and begin to secrete certain cytokines including IL2, which in turn induce proliferation and differentiation of B lymphocytes into antibody-producing plasma cells or memory B lymphocytes. In contrast to T lymphocytes, the percentages of B lymphocytes remain fairly constant between stages of lactation.

Table 4 Mammary gland lymphoid populations

Type	*Subpopulations*	*Function*
T lymphocytes	$\alpha\beta$ T lymphocytes	
	CD4+ (T helper)	Production of immunoregulatory cytokines following antigen recognition with MHC class II molecules; memory cells following antigen recognition
	CD8+ (T cytotoxic)	Lysis of altered or damaged host cells when complexed with MHC class I molecules; production of cytokines that can downregulate certain leukocyte functions
	$\gamma\delta$ T lymphocytes	Biological role in the mammary gland is speculative
B lymphocytes	Mature B lymphocyte	Displays membrane-bound antibody molecules to facilitate antigen presentation; memory cells following antigen interactions
	Plasma cell	Terminally differentiated B lymphocytes that synthesize and secrete antibody against a specific antigen
Natural killer cells		Large granular lymphoid cells that can synthesize and secrete antibacterial proteins following activation

Macrophages are the predominant cell type found in the milk and tissues of healthy, involuted, and lactating mammary glands. While these cells have a role in early nonspecific defense as phagocytes and regulators of inflammation, they also play a key role in antigen processing and presentation. Antigens from ingested bacteria are processed within macrophages and appear on the membrane in association with MHC class II molecules. When a naive T helper lymphocyte encounters antigen complexed with MHC molecules, it can proliferate and differentiate into memory cells and cytokine-producing effector cells.

Specific Soluble Defenses

Immunoglobulins (Ig) function as the soluble effector of specific or humoral immune responses. These proteins are produced by antigen-activated B lymphocytes that subsequently proliferate and differentiate into antibody-secreting plasma cells. Antibodies in lacteal secretions are synthesized locally or are selectively transported or transudated from serum. Four classes of Igs are known to influence mammary gland defense against bacteria causing mastitis: IgG_1, IgG_2, IgA, and IgM. Each of these classes differs in physiochemical and biological properties (**Table 5**). The concentration of each Ig class in mammary secretion varies depending on the stage of lactation and infection status of the mammary gland. In healthy glands, the concentration of Ig is low during lactation but slowly increases during the nonlactating periods and reaches peak concentrations during colostrogenesis. High concentrations of Ig also occur in the mammary gland during inflammation. The concentration of Ig in the gland is dependent upon the degree of permeability of secretory tissue and the number of Ig-producing cells that are present in the mammary gland.

Table 5 The role of immunoglobulins in mammary gland defense

Isotype	*Biological function*
IgG_1	Selectively transported into the mammary secretions; opsonizes bacteria to enhance phagocytosis
IgG_2	Transported into secretions during neutrophil diapedesis; opsonizes bacteria to enhance phagocytosis
IgA	Associated with the fat portion of milk; does not bind complement or opsonize particles; can cause agglutination and toxin neutralization, and prevent bacterial adhesion
IgM	Efficient at complement fixation, opsonization, agglutination, and toxin neutralization; only opsonic for neutrophils in the presence of complement

Conclusion

Dairy cattle are especially susceptible to mastitis during certain phases of the lactation cycle. The frequency of new intramammary infections is greatest during the early dry period, lower in the fully involuted mammary gland, and dramatically increases during the periparturient period. Changes in the incidence of mastitis with respect to lactation stage are directly related to changes in the composition, magnitude, and efficiency of the mammary gland defense system. The development of innovative strategies that can enhance an otherwise impaired immune response during periods of increased susceptibility to disease could have a major impact on the incidence of mastitis. The challenge that confronts researchers now is to gain a better understanding of the complex interactions between the pathogenesis of bacteria, host responses needed to eliminate the pathogens from the mammary gland, and methods to enhance the immune potential of these factors before disease is established.

See also: **Mammary Resistance Mechanisms**: Anatomical. **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk; Management Control Options; Medical Therapy Options; Role of Milking Machines in Control of Mastitis. **Nutrition and Health**: Nutraceuticals from Milk. **Milk Proteins**: Lactoferrin; Immunoglobulins.

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Introduction

Production practices on dairy facilities continue to change due to a number of influencing factors. Over the last few decades, facilities have increased size and production through development and implementation of new technologies. Technologies to improve milking and reduce labor, mix and deliver feed, and manage information through animal identification–data capture systems have reduced labor necessary to harvest milk. Greater knowledge of the feed value of nontraditional feedstuffs and mechanization of feed delivery systems have aided in the ability to increase herd size without the need to grow more feedstuffs locally. The number of milking and dry cows in an average herd in the United States has increased from 25 in 1975 to 82 in 1999 to 143 in 2008. The average herd size in the year 2000 for California, Wisconsin, and New York were 533, 61, and 85 cows, respectively. These herd sizes increased to 824, 88, and 110 in 2007. Ironically, one of the factors that has encouraged concentration of cattle onto fewer but larger farms is environmental regulation. In order to comply with environmental regulations and the resulting increased management requirements, many farms have found it necessary to become larger to generate additional income to cover the cost of improved facilities and personnel to manage them.

Information on environmental management systems will continue to inundate dairy operators. Changes in federal law have created a market for consultants to help plan and manage nutrient management systems on dairies. A myriad of water and air quality regulations exist, and more are proposed for animal operations. Future changes in manure collection, handling, storage, treatment, and utilization systems will result as more stringent regulations are promulgated.

Design of Waste Handling Systems

Dairy design is critical to minimizing the impact of an animal feeding operation on the environment. Local water and air quality concerns should be considered during the planning stages of dairy development and subsequent expansion processes. Environmental impact from animal facilities can be minimized by regular collection and proper storage, treatment, and utilization of manure. Site selection and location of the animal feeding operation on the site should be made with consideration of soil type, depth to water table, distance to surface water, and ability of the land to utilize nutrients. Consideration should also be made concerning the location of neighbors and roads in order to avoid conflicts over odor emissions. Animal housing largely dictates the method of manure collection. Manure from animals housed in open lots, loafing barns, or stanchion barns is usually collected in a solid form. Manure from animals housed in freestalls can be collected as a semisolid, slurry, or liquid.

In warmer climates, flushing with water (usually recycled from an anaerobic lagoon) is the most popular form of manure collection. It requires little labor and a minimum of mechanical equipment (usually one pump). Flushing works best with anaerobic lagoons since the dilute top layer of lagoon water can be recycled to flush the housing lanes. One disadvantage of flushing systems is that with an anaerobic lagoon, much of the nitrogen in the manure is volatilized and is thus unavailable for crop production while the phosphorus remains in the system. Over time, this can lead to a buildup of phosphorus in the fields where manure is applied. Other complicating factors include the popularity of sand as bedding for the cows and the buildup of undigested solids in the bottom of lagoons. Sand is an excellent bedding material for cow comfort, but it is a problem if allowed to get into a lagoon as it is very difficult to remove. It is heavy and very abrasive to pumping and handling equipment. This sand, along with other undigested solids, must be removed periodically in order for the lagoon to function properly, and solids removal is usually a difficult and expensive procedure.

In colder climates, scraper systems are more popular since they work better in low temperatures and they allow manure to be handled as slurry since there is no need to add extra water to flush. With no need for extra water, the nutrients are more concentrated and have a nitrogen/phosphorus ratio that is more balanced with crop needs. Two disadvantages of these systems are that there are more mechanical systems (automated scrapers or tractors with scraper blades) that must be maintained and odors may be significantly worse from slurry manure storages.

Regardless of the housing and manure management system used, control of rain runoff from animal housing and manure storage areas as well as land used for nutrient application is essential. Additionally, potential groundwater contamination from infiltration of nutrients from animal housing areas, manure, and wet feed storage areas must be considered. **Figure 1** illustrates some of the ways by which collection, storage, treatment, and utilization of manure can potentially affect the environment. It is important to carefully weigh environmental considerations (soil, water, and air) before manure management decisions are made. As an example, when solid manure is composted as a means to reduce pathogens, viable weed seeds, and volume, ammonia is emitted. Conversely, when anaerobic treatment occurs, methane is produced. If anaerobic digestion is incomplete, volatile acids (some are odiferous) will be emitted. Planners as well as regulators must become more aware of how solving one problem (i.e., water quality) can adversely affect another area (i.e., air quality).

Method of manure management should be considered during the design process. Odors are generally reduced when manure is either dried or submerged in water. Facilities that are designed and managed to minimize the depth of dry manure in open lots should minimize primary particulates (dust) due to animal activity and adverse air conditions. It is critical that not only the designer of the system understand how it is to be operated, but that the person who will be managing the facility understand how management decisions can affect the performance of the system, both economically and environmentally.

Changing Trends in System Designs

The need to reduce emissions or increase nutrient density has resulted in rethinking manure collection and treatment technologies. When manure is seen as something that must be disposed of, the tendency is to design systems that remove as much organic matter and nitrogen as possible (i.e., anaerobic waste lagoons). The result is a dilute waste stream that is difficult to transport long distances, and therefore must be applied through irrigation. Since nitrogen has been reduced in these systems, the manure has a poor nitrogen/phosphorous ratio, which may result in a buildup of phosphorous in soils over time.

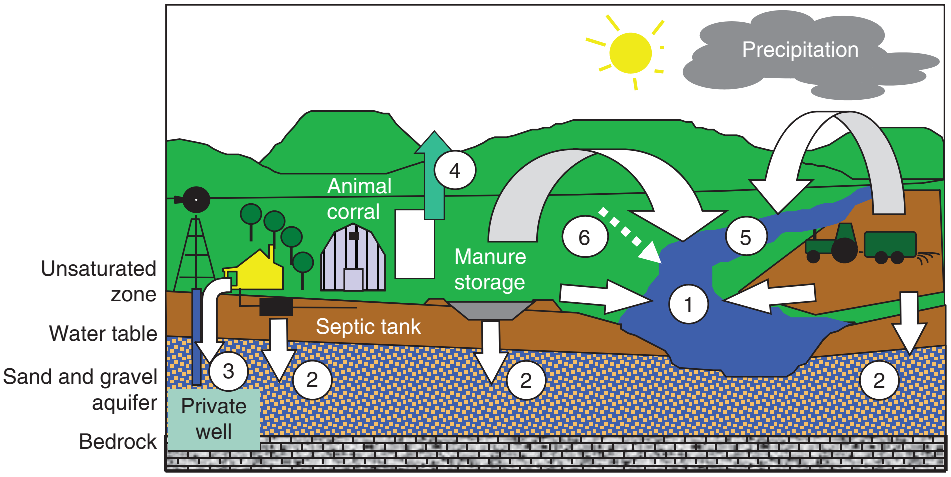

Figure 1 Potential routes of environmental impact from manure. 1, Surface runoff; 2, leaching to groundwater; 3, well casings as conduit to groundwater; 4, gaseous and particulate emissions; 5, ammonia deposition; 6, macrospore flow. Adapted from Livestock and Poultry Environmental Stewardship Curriculum. 2001. Lesson 1 authored by Rick Koelsch, University of Nebraska, courtesy of MidWest Plan Service, Iowa State University, Ames, IA 50011-3080.

When manure is seen as a resource that should be used efficiently, the tendency is to preserve the nutrients (i.e., a slurry system). The result is better nutrient utilization, but also the potential for increased odor production. Anaerobic digestion is one solution to the odor control issue, and increases in fuel prices have made this technology feasible for more farms, yet caution must be exercised before making the large investment necessary to install this technology. Many site-specific factors affect whether digestion will be successful economically for a given operation.

Numerous companies have developed proprietary additives for manure systems that claim to virtually eliminate the negative aspects of manure such as odor and sludge buildup. Because of the numerous uncontrollable variables that affect manure systems on farms, it is very difficult if not impossible to scientifically verify these claims. Some of the products seem to work at some locations but not at others or seem to work for a while, but not consistently. These products must be used with caution, recognizing the limitations of scientific understanding for most of them. Multimedia implications must also be considered. (Does it help air quality at the expense of soil or water quality?)

Much effort and funding from federal sources, as well as funds obtained through litigation, have been put toward finding alternatives to the current norm of anaerobic lagoon systems, especially in the swine industry. For example, the agreement between the NC Attorney General and Smithfield Farms and Premium Standard Farms provided $15 million in industry funds to try and find alternative environmentally superior technologies to replace the lagoon/spray field systems that dominate the North Carolina swine industry. As another example, the Farm Pilot Project Coordination (FPPC), a program supported by federal funds, has evaluated a number of technologies on farms that were designed to reduce the impacts of concentrated animal feeding operations (CAFOs) on the environment. While some of the technologies developed under these efforts do work and show promise that they may be economical on some farms and in certain situations, no magic bullet that will successfully replace current systems across a broad number of farms has yet been found. Each farm must look at what will work under the unique circumstances under which they must operate. As an example, anaerobic digestion works well for some farms where they have sufficient income streams from the sale of energy, savings on bedding and fertilizer, tipping fees for the addition of other waste streams, and carbon exchange credits. Anaerobic digestion has failed at other locations when the circumstances were not right and/or the personnel managing the system were not totally dedicated to making it work.

One trend that is currently gaining popularity in the United States is that of using more grazing in dairy operations. Years ago, most dairies were grass-based systems where cows primarily grazed pastures and received supplemental nutrition from grain at the time of milking. Increase in herd size has led to more totally confined systems because of the logistics of getting a large number of cows from pasture to barn and the associated problems with mud in travel lanes and holding areas. Currently, a number of dairies are going back to more of a grazing-centered system. Using intensive grazing management (placing a large number of cows in a small area for a short time interval) reduces inputs by letting the cows harvest their feed rather than using mechanical systems for harvesting, storing, and delivering feed to the cows. While milk production rates are generally lower, the goal of these systems is to reduce costs more than income in order to increase profitability. There are still challenges that must be overcome in introducing a new system, but the challenge appears to be one that is worth pursuing.

Concerning waste management, one of the main advantages of grazing operations is that the animals distribute much of the manure themselves, and thus a smaller portion of the manure produced must be collected, stored, and distributed. Through intensive grazing management, the manure is distributed more evenly over the crop area, and the crops are better utilized than when cows are left on a larger area for a longer period of time. In warm climates where grazing is possible year-round, the collected manure can be applied year-round onto grazing paddocks rather than being stored for several months and then applied in a short time to meet the needs of crops grown for hay, silage, or grain. This type of management offers tremendous cost savings since the manure can be applied at a lower rate requiring lower horsepower and smaller pipes and application equipment. Also, the small amount of stored manure can be easily agitated to suspend solids in the material being pumped. Smaller storage structures cost less initially and cost less to maintain. Agitation of large storage structures is one of the difficult and expensive factors experienced with conventional manure management systems.

Water Quality

Dairy Manure Management and Water Quality

Increase in the size of dairies has led to large numbers of dairy cows and the waste associated with dairy production concentrated in small regional areas. The industry has developed many different methods of handling the waste from these large operations. Regardless of the waste management system, by far the most common utilization of the waste is land application to crop land or pastures as fertilizer.

Dairy manure is a resource that when applied properly can improve water quality by increasing soil tilth and infiltration and lessen soil erosion and runoff volumes from crop lands and pastures by increasing crop growth and ground cover. However, if manure is applied improperly or overapplied, nutrients can leach into groundwater or run off into surface water. Dairy manure contains nitrates, which can easily leach into groundwater where if consumed in high enough concentrations can cause major health problems or even death in humans and livestock. Nutrient enrichment of surface waters (also known as eutrophication) causes excessive growth of plants/algae, which leads to oxygen depletion, fish kills, shifts in aquatic species, negative recreational impacts, and taste and odor issues. Because of the water quality issues associated with livestock production, regulations have been passed to help assure manures are properly applied and water quality is preserved.

Regulatory History

The Clean Water Act (CWA), as amended in 1972, focused on point source (PS) pollutants resulting in contamination of surface waters (PL 92-500, 1972). Section 303(d) of the act mandated that states identify beneficial uses of water resources and that water bodies that do not meet the requirements for these beneficial uses be identified. Additionally, the act identified CAFOs as PS. Dairies with 700 milking and dry cows or between 200 and 699 milking and dry cows that discharged into surface waters were defined as a PS, and thus were required to obtain a National Pollutant Discharge Elimination System (NPDES) permit and comply with effluent limitations guidelines (ELGs), which define the legal discharge parameters (volume and chemical composition) as defined in industry-specific ELGs. For the dairy and livestock industries, the NPDES permit strictly prohibited discharges to surface waters from permitted facilities. It was only legal to discharge the water in excess of the 25-year, 24-h or chronic storm event.

Dairy operations below 200 milking and dry cows that do not discharge to surface waters are not required to get an NPDES permit. However, these operations are still regulated by the CWA and cannot discharge into waters of the United States. The US Environmental Protection Agency (EPA) has the authority to require any dairy operation to obtain an individual NPDES permit if they determine an operation is polluting waters of the United States. It is also important to note that there may be state-specific permitting and compliance regulations for dairies. The individual states' department of agriculture and/or the state environmental regulatory agency are great resources to determine state-specific requirements.

In March 1999, the US EPA along with the United States Department of Agriculture Natural Resources Conservation Service released the Joint Unified Animal Feeding Operation Strategy. This document identified comprehensive nutrient management plans (CNMPs) as a management tool to assist in proper application and tracking nutrients for an operation. The strategy has served as a roadmap for producers to predict where US EPA may travel with respect to the NPDES permit system.

In February 2003, EPA issued revised permitting requirements for CAFOs. The revised regulation expanded the number of operations required to seek NPDES permit coverage as well as requirements for land application of animal waste. EPA was forced into litigation over the new regulations and was directed by the court to make changes. Due to the litigation, implementation deadlines were extended from July 2007 to February 2009. The revised EPA CAFO regulation became effective from December 2008. State-level EPA-delegated agencies were given a year to bring their regulations into compliance with the new federal regulations. Regulations developed at the state level must be, at a minimum, as stringent as the EPA regulations.

Current Regulations

The current EPA CAFO regulations, which went into effect in December 2008, maintain the CAFO size threshold of 700 dairy cows (milking or dry) for all dairy operations. All CAFOs that discharge or propose to discharge must seek coverage under an NPDES permit. These operations must obtain a nutrient management plan (NMP) and submit that plan with their application for permit coverage. The permitting authority must review the NMP and provide the public an opportunity for review and comment. The NMP is considered part of the NPDES permit and it must be implemented, along with documentation that the plan is being followed.

EPA clarifies that "a CAFO proposes to discharge if it is designed, constructed, operated, or maintained such that a discharge will occur". If a dairy operation does not discharge or propose to discharge, it is not required to seek coverage under an NPDES permit. However, if a discharge occurs from a no discharge operation, it is in violation both for discharge and for failure to seek permit coverage. To determine whether an operation proposes to discharge, a factual objective assessment must be conducted. One option in the new regulations is for a farm to seek a 'no discharge certification.' In order to certify as a no discharge operation, the owner/operator must develop and implement an NMP and keep records of NMP implementation. Additional certification requirements include documentation of open manure storage design capacity as determined by NRCS Animal Waste Management Software (or equivalent). Design inputs must include all volume additions including rain fall based on the previous 30 years of climate data. To determine if manure storage is adequate, it must be evaluated using the most recent version of the soil plant air water hydrology tool including the past 100 years of climate data.

Nutrient Management Plans

For both the permitted and certified no discharge operations, NMPs must be implemented, and NMPs should be implemented for all dairies. The goal of nutrient management planning is to properly utilize the nutrients in the animal manure for agronomic crops while at the same time protecting water quality. One important aspect of nutrient management planning is making sure enough land is available to efficiently utilize the nutrients in the manure. As more nontraditional feedstuffs are used to feed dairy cows, more nutrients are being brought onto the farm. This situation can lead to nutrient imbalance unless plans carefully consider this and include strategies to export excess nutrients.

Nutrient management consists of several steps:

- testing the soil to determine the ability of the soil to supply nutrients to the crops
- determining the recommended amounts of nutrients needed to produce the desired yields
- accounting for nutrient inputs from other sources, such as legumes
- analyzing manures, composts, and irrigation water to determine their nutrient content
- applying manures or composts at recommended rates and based on the critical nutrient (usually either nitrogen or phosphorus)
- applying the additional inorganic nutrients as needed
- keeping records so that evaluations and adjustments can be made
- being aware of one's surrounding landscape so that sensitive areas can be protected

Each state has specific criteria for NMP development. NMPs are farm and field specific and at a minimum should include nutrient production, crop rotation, application rates, and best management practices. In most states, application rates are determined based on nitrogen needs as modified by a phosphorus index, which determines the risk of phosphorus losses from a field. Once application rates are determined for each field, a whole-farm nutrient budget can be developed to determine the amount of manure if any that must be transported off farm.

Implications of CAFO Regulations

For most CAFO size dairy producers, the current federal regulations require nutrient management planning, records of NMP implementation, and having the NMP made available to the public for review and comment. Although the regulations do not specify how public notification must be handled, environmental groups, animal welfare groups, and concerned neighbors can potentially influence the permitting process. Nutrient management planning and records of plan implementation are good farm management tools and a great way to demonstrate environmental compliance and stewardship.

Development and implementation of water quality regulation for CAFOs has always been a dynamic process. As public perception and policies change, regulations will change and adapt to remain relevant. Therefore, it is important for the dairy industry and dairy producers to stay informed on current and developing issues, perceptions, and policies/regulations. It is also important for the industry to be environmentally proactive and demonstrate to the general public that environmental stewardship is important to all aspects of dairy production.

It is very important to keep in mind that in addition to federal regulation changes, state and local regulations and ordinances will also change. State and local regulations must be as strict as the federal regulations but can be more stringent, and these regulations supersede federal regulations. State regulations for dairy operations can usually be obtained from the state department of agriculture or environmental regulation agency and local regulations and ordnances can be obtained from local county courts.

Air Quality

Regulatory History

The first federal legislation concerning air pollution was the Air Pollution Control Act of 1955, which primarily provided funding for research on air quality. The Clean Air Act of 1963 extended this authority to monitor and control air pollution. The Air Quality Act of 1967 extended the federal government's authority to control air pollution, especially where pollution crossed state lines. The Clean Air Act of 1970 represented a significant increase in the federal government's authority to control air quality. It established National Ambient Air Quality Standards (NAAQS) for various pollutants and other regulatory devices that could require states to enforce standards on industries within their borders. Major amendments were added to the act in 1977 and 1990 to further strengthen federal authority. Air pollutants identified in the Clean Air Act included a number of chemicals and particulate matter (PM). Initial efforts were aimed at PM_{10} (particles with a diameter smaller than 10 μm). Later, more concern was directed toward $PM_{2.5}$ (sometimes called PM_{fine}, particles with a diameter less than 2.5 μm), and in 1997, NAAQS were introduced to control $PM_{2.5}$, and PM_{10} NAAQS were modified.

Regulation in Agriculture

As in water quality regulations, initial air quality efforts were aimed at industrial and municipal sources, but as progress was made in those areas, agriculture began to be scrutinized more closely. The pollutants most commonly discussed in animal agriculture are ammonia, sulfur dioxide, and PM. Odor has driven much public concern over agricultural operations, but federal regulations have not been developed to regulate it at this point. Regulation of air quality has lagged behind water quality regulation partially because of the more easily identifiable environmental impacts of impaired water quality, but primarily because of the complexity of defining air quality. A number of problems exist when it comes to defining air quality issues.

1. Water always flows downhill, so it is relatively easy to determine with some degree of certainty where water quality problems originate. Air, on the other hand, can move in multiple directions depending on shifting wind directions, so air quality problems can originate from multiple locations. Also, because of the variability of wind direction and speed, it has been difficult to accurately determine how much of a particular quantity of air pollutant is emitted from a typical animal production facility.
2. One of the main air quality complaints against animal agriculture is odor, and odor is a very subjective quantity. It has been said that the sense of smell is the most complicated of the human senses and the most difficult to emulate. The most readily accepted scientific method of measuring odor is through the use of olfactometry, the use of a panel of humans who employ scientific methods to sniff samples of air to determine the strength and qualities of the odors in the air. This method is very expensive because of the training and number of people involved and has the limitation that dust must be removed from the air sample before testing in order to prevent contamination of the equipment. Dust is an important factor in how odors are perceived. In addition, while olfactometry is the best available method for measuring odors, it is limited by the fact that it cannot completely eliminate the subjectivity involved.
3. Odors from animal production facilities result from a combination of many compounds. Some of the most common gases that have been targeted for regulation are ammonia and hydrogen sulfide, but many more compounds are involved, some of which can be sensed at levels several orders of magnitude lower than ammonia or hydrogen sulfide. Most of these compounds are organic in nature (volatile organic compounds (VOCs)) and result from the partial decomposition of manure, feed, skin cells, feathers, and other waste products from animal production. Methane is often cited as a gas of concern, but it is actually odorless. Methane is of concern because it is a powerful greenhouse gas and not because it smells bad. Nitrogen can be volatilized in the form of ammonia (NH_3), which is a precursor to acid rain and can be of environmental concern. It can also be volatilized into nitrogen gas (N_2). The Earth's atmosphere is made up of 78% nitrogen gas, so adding a small quantity of nitrogen is not thought to be of particular concern.

Because of these and other issues, regulation of air quality has been slow to develop. State and local authorities have passed regulations attempting to deal with odor concerns, but they have primarily relied on setbacks from property lines to keep odor sources away from neighbors.

Current Focus

A publication was released in 2003 by the National Academy of Science entitled 'Air Emissions from Animal Feeding Operations'. The document pointed out the need for more accurate information on emissions from agricultural operations and provided the incentive for a National Air Emissions Study, which was authorized in 2006 and funded primarily by the US animal agriculture industries and overseen by EPA. The study monitored actual farms for a period of 2 years in order to better understand how emissions vary seasonally, diurnally, and with animal age and management changes. Some of these studies have been completed, but data analysis is ongoing, and the final reports have not yet been released. When they are, they will provide a much more realistic picture of emissions from farms.

One attempt at regulating air quality was to apply the regulations developed under the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA) and the Emergency Planning and Community Right-to-Know Act of 1986 (EPCRA) to animal production facilities. Provisions in these acts required the reporting of any spills or releases to the atmosphere of significant amounts (usually over 45 kg day^{-1} (100 lb day^{-1})) of certain identified hazardous compounds including ammonia and hydrogen sulfide. The purpose was to inform local emergency personnel of any release of hazardous material so that they could warn neighbors or take other appropriate action. Although the release of these compounds from the natural breakdown of manure at agricultural operations was not considered when these regulations were written, agriculture was not specifically exempted from the rules; thus, an extended court battle ensued over whether agriculture should be required to follow these reporting requirements.

As a result of court rulings, a clarification was finally developed and released effective from 20 January 2009. The rules at that point clearly said that agricultural operations are exempt from reporting emissions under CERCLA (reporting to federal authorities) and that smaller agricultural facilities (those not large enough to require an NPDES permit) are exempt from reporting under EPCRA (local reporting). Large CAFOs do have to report if they determine through a good faith estimate that they are emitting over 45 kg of either ammonia or hydrogen sulfide on any given day during the year. They must report this once each year to local and state emergency management personnel. Several methods have been suggested for determining the good faith estimate, but the method for determination will probably be better specified once the results of the National Air Emissions Monitoring Study (NAEMS) are completed.

Implications for the Future

Much effort and funding has been focused on determining emission rates for typical animal agriculture facilities. The need to shift this emphasis toward finding and documenting affordable methods of reducing these emissions is evident. Measuring emissions is expensive and time consuming, and thus measuring and documenting improvements in emissions as a result of introduced technologies is difficult. Therefore, the need to search for ways to monitor emissions more economically is evident. Emphasis needs to be placed on emission reduction solutions that improve air quality within the production facilities as well as the air that is emitted from the facilities. By improving air in the facility, the animals may benefit from the improved conditions and this will hopefully result in better performance and more efficient production. In that way, improving air quality can be an advantage for the producer as well as the general public.

See also: **Forages and Pastures**: Grazing Management. **Manure/Effluent Management**: Nutrient Recycling. **Utilities and Effluent Treatment**: Reducing the Negative Impact of the Dairy Industry on the Environment.

Further Reading

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Nutrient Recycling

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Introduction

Overapplication of nutrients to land leads to losses of fertilizer nutrients and is a threat to the environmental standards that we want, especially with respect to water quality. Nutrient accumulation on intensive food animal production farms stems from farm imports of elemental nutrients in purchased feeds being greater than nutrient exports in food animal products. Manure management and application specifically has been targeted by regulatory agencies in recent years to try to assure that losses, especially of nitrogen (N) or phosphorus (P), are low and to avoid environmental consequences off-site (*see* **Manure/Effluent Management**: Systems Design and Government Regulations). Monitoring of potassium (K) also is encouraged.

This article focuses on the development of manure nutrient management budgets and needed nutrient management strategies. Too often in the past it was decided that manure nutrient recovery costs were greater than value and, thus, manure nutrients were not fully utilized. Accumulation of unused nutrients in storage or on fields increased risk of nutrient leakage to the environment. Now, environmental concerns mandate manure utilization regardless of costs. If balancing production and use is not possible on-farm, it will be necessary to quantify the nutrient excess that must be exported off-farm. The tables herein are designed as worksheets to be entered into a computer spreadsheet to invite easy recalculation in order to assess effects of possible changes on overall nutrient budgets and plans.

Objectives

The objectives of this article are the following:

1. To show a practical method to estimate manure nutrient excretions and show how dietary changes in nutrient contents affect excretions.
2. To estimate manure nutrient recoveries, predict crop production (acreage and yields) needed to recycle manure nutrients, decide if manure applications to fields will be based on N or P, and identify cash value of manure nutrients recovered that can help pay costs to manage manure.
3. To explore alternatives available to the dairyman if nutrients are in excess of potential to utilize them on-farm.

How Much N, P and K Are Excreted?

Manure is what is excreted in the form of urine and feces after the animal has digested and utilized all that it could from the ration provided to it. Digestibility is the percentage of the dry matter (DM) or particular nutrient in the diet that the animal could absorb from the digestive tract and have available for use for maintaining life and producing offspring, milk and meat. Knowing digestibility and, hence, indigestibility of the ration DM and organic matter permits us to estimate the amounts of DM and organic matter excreted, components that determine manure volume.

If animals are consuming N, P and K at maintenance levels, they will excrete, on average over time, the same amount of N, P and K they consume except for small amounts of nutrients in shed hair and sloughed tissues; and those are usually collected with the manure. When animals are accumulating N, P and K in body weight gain, offspring, milk and meat, the amounts excreted in fresh manure (feces plus urine) differs from what is fed by the amounts in products produced. Thus, manure N, P and K for dairy cows are accurately predicted by subtracting outputs of N, P or K in products produced from dietary inputs. A very important reason for using a dietary-based approach to predict nutrient excretions is that accurate feed and production data usually are available on the farm and the method shows how much reductions of dietary inputs, when feasible, will reduce manure nutrient excretions.

Needed data are average dry matter intake (DMI), milk yield, milk nutrient compositions, body weight gains and body weight gain compositions. Some key conversion factors are as follows: dietary protein is 16% N, milk protein 15.5% N, milk P averages 0.10% with little variation, and milk K 0.15%. Similarly, body weight gain for cows averages about 1.2% N, 0.70% P and 0.20% K.

Equations that predict composition of gain can be used, if desired. The gain calculations are used herein primarily to show that nutrient outputs in gain are small relative to outputs in milk on dairy farms. If manure DM estimates are desired to predict manure volume, they can be derived from DMI and diet DM digestibility estimates. If DM digestibilities for example in **Table 1** were 65%, the amount of DM in feces is 35% of DMI (1 minus digestibility); an estimate of DM excreted in urine is 5% of DMI for a total of 40% of DMI excreted in feces plus urine. If organic matter content of manure is desired, data show original DM excretion of dairy cow manure to be about 83% organic matter. However, both DM and organic matter in manure decrease with time after excretion because organic matter is digested, either anaerobically or aerobically, in storage.

Calculations of N, P and K excretion estimates are shown in **Table 1**. In Example 1, lactating cows were grazing 60% of the time and in confinement 40% of the time. Dry cows and manure from dry cows were assumed to be managed at a separate location, probably pasture, where a separate nutrient budget would be needed. The manure nutrient budget plan for Example 1 is for the 40% of the manure collected in the milking parlor, adjacent feeding barn and holding pens. Discussion of the 60% of the manure deposited on pasture is included in a later

Table 1 Calculating manure N, P and K excretions of milking and dry cows

			Example 2		
Line number	*Example 1*	*Units*	*While milking*	*Total*	*While dry*
Herd information (yearly averages on a per day basis)					
1. Average number milking cows day^{-1}	100	cows	100		100
2. Average DIM or days dry	365	days	310		55
3. Average DMI day^{-1}	20.0	kg day^{-1}	22.0		12.0
4. Average diet CP% (DM basis)	17.0	% CP	19.0		14.0
5. Average diet N% = CP% × 0.16 =	2.72	% N	3.04		2.24
6. Average diet P% (DM basis)	0.41	% P	0.50		0.35
7. Average diet K% (DM basis)	1.20	% K	1.20		1.20
8. Milk yield day^{-1} (average across all DIM)	25	kg day^{-1}	30		0
9. Milk protein percentage	3.2	%	3.2		
10. Milk nitrogen (N)% = protein% × 0.155 =	0.496	% N	0.496		
11. Milk phosphorus (P)%	0.10	% P	0.10		
12. Milk potassium (K)%	0.15	% K	0.15		
13. Average net body weight gain day^{-1}	0	g day^{-1}	100		
14. Average N% of weight gain	1.20	% N	1.20		
15. Average P% of weight gain	0.70	% P	0.70		
16. Average K% of weight gain	0.20	% K	0.20		
Daily balances per cow Nitrogen (N)					
17. Input: kg DMI × 1000 g kg^{-1} × diet N%/100 =	544	g day^{-1}	669		269
18. Export: Milk yield day^{-1} × milk N%/100 =	124	g N day^{-1}	149		0
19. Export: g day^{-1} gain × gain N%/100 =	0	g N day^{-1}	1		17
20. Difference (manure estimate) = input − export =	420	g N day^{-1}	519		252
21. Yearly manure N per cow = difference × DIM or days DRY =	153	kg N yr^{-1}	161		14
Phophorus (P)					
22. Input: kg DMI day^{-1} × 1000 g kg^{-1} × diet P%/100 =	82	g P day^{-1}	110		42
23. Export: kg milk day^{-1} × 1000 g kg^{-1} × milk P%100 =	25	g P day^{-1}	30		0
24. Export: g day^{-1} gain × gain P%/100 =	0	g P day^{-1}	1		10
25. Difference (manure estimate) = input − export =	57	g P day^{-1}	79		32
26. Yearly manure P per cow = difference × DIM or days DRY =	21	kg P yr^{-1}	25		2
Potassium (K)					
27. Input: kg DMI day^{-1} × 1000 g kg^{-1} × diet K%/100 =	240	g K day^{-1}	264		144
28. Export: kg milk day^{-1} × 1000 g kg^{-1} × milk K%/100 =	38	g K day^{-1}	45		0
29. Export: g day^{-1} gain × gain K%/100 =	0	g K day^{-1}	0		3
30. Difference (manure estimate) = input − export =	203	g K day^{-1}	219		141
31. Yearly manure K = cows × difference × 365 =	74	kg K yr^{-1}	68		8
32. % manure collected (% of time in area)	40	%	100		100
33. Yearly kg N per herd (cows × kg N yr^{-1} × % collected)	6132	kg N yr^{-1}		17 469	
34. Yearly kg P per herd (cows × kg P yr^{-1} × % collected)	832	kg P yr^{-1}		2 635	
35. Yearly kg K per herd (cows × kg K yr^{-1} × % collected)	2957	kg K yr^{-1}		7 559	

DIM, days in milk; DM, dry matter; CP, crude protein.

section on pasture budgets. Example 2 is for cows in total confinement (milking and dry) where 100% of manure is collected within the manure management system.

The percentage of time that cows spend in areas where manure is collected versus pastures is important. Available data suggest that manure flow through the digestive tract is relatively steady throughout the day. Therefore, it is estimated that cows distribute their manure equal to the percentage of time they spend in different areas, e.g. freestall barns, holding pens, milking parlor, feed barns, pastures.

How Much of the Excreted Nutrients Are Recovered for Fertilizer?

It is necessary to determine the quantities of manure N, P and K recovered in order to fertilize crops correctly and to determine the cash value realized from manure nutrients applied. Weighing enough loads of manure solids hauled to the fields to estimate amount and analysing enough samples to predict N, P and K composition are necessary. Nutrient recoveries are obtained by multiplying concentrations by load weights and number. If an irrigation system is used to distribute wastewater from a lagoon or holding pond, wastewater analyses are needed to go with the volume of wastewater distributed. Volume meters on irrigation pumps are important; if not available, volume pumped must be estimated from the hours of pumping and expected liters per minute based on pump specifications.

With relatively hard data in hand on nutrient excretion and nutrient recovery for fertilizer use, recovery percentages can be calculated, and by difference, nutrient losses that occurred in the manure management system can be estimated. Example 2 in **Table 2** provides amounts of manure products collected and composition analyses that estimated recoveries of 42.4% of excreted N, 93.9% of P and 85.3% of K.

If amounts and composition have not been measured, it is essential to estimate nutrient recovery percentages in order to develop preliminary nutrient management plans. For dairy cows, about half of the original N excretion is urea (from urine) or other easily degraded N compounds in feces that yield ammonia. Volatilization losses of 50% or more of N are expected unless manure is moved to fields very quickly after excretions are collected. Losses up to 80% of excreted N are not uncommon. Recoveries of P and K should be high, 90% or more for P and close to that for K because P and K are not lost to the air. Apparent losses of P should be questioned if they were large, e.g. greater than 20%. If anaerobic lagoons are utilized, the sludge at the bottom of the lagoon may be a place where P is sequestered.

Table 2 Manure N, P and K recovered for fertilizer use

Line number	*Example 1*	*Units*	*Example 2*
Manure solids utilized yr^{-1} (manure spread)			
36. Number loads yr^{-1}	[Data not collected for Example 1]		200
37. Average weight per load		kg	5 000
38. Average DM% of loads		% DM	25.0
39. Average N% of manure DM		% N	2.00
40. Average P% of manure DM		% P	0.75
41. Average K% of manure DM		% K	1.7
42. Manure DM = loads × kg per load × % DM/100 ÷ 1000 kg Mg^{-1}		Mg yr^{-1}	250
43. Yearly N recovered = DM Mg × 1000 kg Mg^{-1} × N%/100 =		kg N yr^{-1}	5 000
44. Yearly P recovered = DM Mg × 1000 kg Mg^{-1} × P%/100 =		kg P yr^{-1}	1 875
45. Yearly K recovered = DM Mg × 1000 kg Mg^{-1} × K%/100 =		kg K yr^{-1}	4 250
Wastewater utilized yr^{-1}			
46. Cubic meters for irrigation		m^3	20 000
47. Average N mg kg^{-1}		mg kg^{-1}	120
48. Average P mg kg^{-1}		mg kg^{-1}	30
49. Average K mg kg^{-1}		mg kg^{-1}	110
50. Yearly N recovered = m^3 × 1000 l m^{-3} × N mg kg^{-1}/1 000 000		kgN yr^{-1}	2 400
51. Yearly P recovered = m^3 × 1000 l m^{-3} × N mg kg^{-1}/1 000 000		kg P yr^{-1}	600
52. Yearly K recovered = m^3 × 1000 l m^{-3} × N mg kg^{-1}/1 000 000		kgK yr^{-1}	2 200
Total N, P and K recovered yr^{-1}			
53. N recovered = solids spread + irrigation = (line 43 + line 50)		kgN yr^{-1}	7 400
54. % recovered of collected N excreted = (line 53 ÷ line 33)		%	42.4
55. P recovered = solids + irrigation = (line 44 + line 51)		kg P yr^{-1}	2 475
56. % recovered of collected P excreted = (line 55 ÷ line 34)		%	93.9
57. K recovered = solids + irrigation = (line 45 + line 52)		kgK yr^{-1}	6 450
58. % recovered of collected K excreted = (line 57 ÷ line 35)		%	85.3

DM, dry matter.

That P will need to be budgeted and managed sometime later when sludge is emptied from the lagoon. Solids that settle in large holding ponds also are a reservoir for P. Some suggested estimates to use if nutrients recovered for crop fertilization have not been measured are:

- With quick application and incorporation, for example, irrigation of flushed manure within 5 days after excretion to crops grown under sprayfield, N recovery..65%
- Application of wastewaters from anaerobic lagoon with a 21-day or longer holding time, N recovery 20–30%
- An average recovery for N 40%
- For P, estimate recovery of 90% or more unless an anaerobic lagoon is used and discount for what probably remains in the sludge in bottom of the lagoon. That could be as much as 50% in lagoons with 21 day or more average retention time. Usual recovery for P 90%
- For K, estimate recovery of 80–90%

For Example 1 in **Tables 2 and 3**, the grazing scenario assumed that 40% of total manure excreted was collected during the 40% of time cows spent in holding, milking and feeding areas and this manure was stored in an

Table 3 Budgeting use of recovered manure N, P and K

Category	*Example 1*	*Units*	*Example 2*
Nutrient recoveries (%)			
59. N recovered % (from Table 2, line 53, or estimated)	25.0	%	42.4
60. P recovered % (from Table 2, line 55, or estimated)	50.0	%	93.9
61. K recovered % (from Table 2, line 57, or estimated)	80.0	%	85.3
Nutrient recoveries (kg)			
62. N recovered (line 53 or line 59×line 33)	1533	kg yr^{-1}	7 400
63. P recovered (line 55 or line 60×line 34)	416	kg yr^{-1}	2 475
64. K recovered (line 57 or line 61×line 35)	2365	kg yr^{-1}	6 450
Crop application rates (from Table 4)			
65. Crop system 1 (maize silage) N	310	kgN/ha	310
66. Crop system 1 (maize silage) actual P	50	kgP/ha	50
67. Crop system 1 (maize silage) actual K	198	kgK/ha	198
68. Crop system 2 (bermudagrass hay) N	400	kgN/ha	400
69. Crop system 2 (bermudagrass hay) actual P	45	kgP/ha	45
70. Crop system 2 (bermudagrass hay) actual K	228	kgK/ha	228
71. Crop system 3 (alfalfa hay) N	620	kgN/ha	620
72. Crop system 3 (alfalfa hay) actual P	75	kgP/ha	75
73. Crop system 3 (alfalfa hay) actual K	605	kgK/ha	605
74. Crop system 4 (maize, bermudagrass, rye) N	695	kgN/ha	695
75. Crop system 4 (maize, bermudagrass, rye) actual P	85	kgP/ha	85
76. Crop system 4 (maize, bermudagrass, rye) actual K	338	kgK/ha	338
Calculated acreage needed for crop system examples			
77. Crop system 1 (maize silage) N	4.9	ha	23.9
78. Crop system 1 (maize silage) P	8.3	ha	49.5
79. Crop system 1 (maize silage) K	11.9	ha	32.6
80. Crop system 2 (bermudagrass hay) N	3.8	ha	18.5
81. Crop system 2 (bermudagrass hay) P	9.2	ha	55.0
82. Crop system 2 (bermudagrass hay) K	10.4	ha	28.3
83. Crop system 3 (alfalfa hay) N	2.5	ha	11.9
84. Crop system 3 (alfalfa hay) P	5.5	ha	33.0
85. Crop system 3 (alfalfa hay) K	3.9	ha	10.7
86. Crop system 4 (maize, bermudagrass, rye) N	2.2	ha	10.6
87. Crop system 4 (maize, bermudagrass, rye) P	4.9	ha	29.1
88. Crop system 4 (maize, bermudagrass, rye) K	7.0	ha	19.1
Commercial fertilizer values			
89. Value kg^{-1} N	$0.79	\$ kg^{-1} N	$0.79
90. Value lb^{-1} P_2O_5	$0.70	\$ kg^{-1} P_2O_5	$0.70
91. Value lb^{-1} P (2.29×P_2O_5)	$1.61	\$ kg^{-1} P	$1.61
92. Value lb^{-1} K_2O	$0.33	\$ kg^{-1} K_2O	$0.33
93. Value lb^{-1} K (1.20×K_2O)	$0.40	\$ kg^{-1} K	$0.40
Manure fertilizer values recovered yr^{-1}			
94. With P budget (all recovered N, P, K used)	$2822	\$ yr^{-1}	$12 405
95. With N budget (all recovered N used, 40% of P, 90% of K)	$2325	\$ yr^{-1}	$9 755

anaerobic lagoon 21 days or more before the effluent was distributed to cropland. It was estimated that 25% of the N, 50% of the P and 80% of the K from the 40% of manure collected were recovered for fertilizer use. The recovered P% was discounted to 50% because it was estimated that 50% of P was retained in sedimented sludge that will have to be managed separately in the future when the lagoon is cleaned out.

Budgeting Use of Recovered Manure Nutrients

After estimating total manure nutrient excretion and accounting for losses in the manure management system, the next step is to determine how much crop production will be necessary to utilize recovered manure N, P and K (**Table 3**). If sufficient acreage and crop production potential exists, it is likely that the optimum use of the fertilizer value in the manures will be to develop budgets based on P and, thus, manure N will be supplemented with additional N from commercial fertilizer. Optimum crops probably will be high-energy crops that are conducive to high per-animal milk production. If acreage is short and soil storage of excess P is permitted, multiple cropping with forages and maximum application of N may be required. Irrigation is almost essential for consistent maximum production and nutrient utilization per hectare in most regions. If excess nutrients exist after cropping system needs are met, they will have to be exported to an off-farm site.

For evaluation of crop production needs, four cropping systems were selected from **Table 4** (maize for silage, bermudagrass hay, lucerne (alfalfa) hay and a multiple cropping system). If expected yields and N and P compositions differ appreciably from those listed in **Table 4**, one should change to yield and compositions documented for the farm or use other documented yield and composition data appropriate for the region.

A key question is, what fertilizer application rates are needed to achieve the N and P removals expected in those crops? Obviously, application rates must be greater than nutrients removed in quantities harvested unless soils have high fertility stores of N and P. In **Table 4** examples, N removals were multiplied by 1.3 which allows 20% more than crop removals for denitrification and volatilization and 10% for losses to ground and surface waters. For P and K, crop removals were multiplied by 1.1 which allows for 10% more than crop removal for losses to surface waters.

An alternative to calculating nutrient application rates is to use accepted agronomic recommendations for commercial fertilizer applications. If those are greater than determined in **Table 4**, use those. There should not be risk in applying manure nutrients up to those levels (certainly no more risk than with commercial fertilizers). Most nonammonia manure N is stable organic N from the feces which requires extra time to decompose and become available and may not be available for the first crop grown after application. The degradation rates are somewhat climate and region dependent and, therefore, the extra amount should be determined based on appropriate factors determined by agronomists experienced in manure utilization. While the N in dry feces scraped from an unpaved dry lot may be only 40% available during the first month or two after application (depending upon climate and soil type, as noted), N in lagoon or holding pond effluent is often 80% or more inorganic (depending upon the amount of sludge pumped) and essentially as available as commercial sources. However, this N may also be somewhat less effective than expected for one or two seasons as the addition of water and organic matter may stimulate denitrification and other soil microbial changes that require time to equilibrate.

Many dairy farmers apply manure at established per-hectare rates year after year and the amount of carryover averages out so that it is unimportant for budget calculations. Also, this likely will be the case if a manure budgeting plan is for land that has had routine manure applications in the past. However, for the first year of manure application, especially when manure is solid or semisolid, it is important to discount the availability of the organic N and assume, for example, that about 30% of that will not be available during the first year. Thus, some extra N from commercial fertilizer during the first year may be needed.

It should be kept in mind that the N budgeting application rates assume good production conditions or the availability of supplemental irrigation. A weather-related crop failure will (assuming drought rather than flood) result in nutrient carryover to the next crop. This will require downward adjustment in the application rate for the next crop in order to assure that soil nitrate does not leach to groundwater. If all available acreage is required to utilize manure and drought is a common occurrence, an irrigation system is almost essential for near optimum manure nutrient management.

Calculating the Value of Recovered N, P and K

Animal nutritionists and most dairymen use and think in terms of actual N, P and K. Agronomists and the fertilizer industry use actual N, but generally refer to P in terms of P2O5 and K in terms of K2O. The P2O5 actually contains 43.6% P (0.436) and K2O is 83% K (0.83). Thus, 10–10–10 fertilizer units are equivalent to 10–4.36–8.3 units of actual N, P and K.

Table 4 Estimating N, P and K application rates to use or compare with other agronomic rate recommendations

				Composition (% of DM)				*Crop removals (kg ha^{-1})*			*A calculated application rate*[a] *(kg ha^{-1})*				
Crop	*Wet (Mg ha^{-1})*	*DM (%)*	*DM (Mg ha^{-1})*	*CP%*	*N%*	*P%*	*K%*	*N*	*P*	*K*	*N*	*P*	*K*	*P_2O_5*[b]	*K_2O_5*[b]
Maize silage	50.0	30	15.0	10.0	1.60	0.30	1.2	240	45	180	310	50	198	110	240
Rye or wheat haylage	15.7	40	6.3	20.0	3.20	0.30	1.0	202	19	63	260	21	69	50	85
Lucerne (alfalfa) hay	25.0	88	22.0	22.0	3.52	0.31	2.5	774	68	550	620	75	605	170	730
Bermudagrass hay	16.0	86	13.8	14.0	2.24	0.30	1.5	309	41	207	400	45	228	105	275
Bermudagrass hay after no-till maize for silage	5.0	86	4.3	14.0	2.24	0.30	1.5	96	13	64	125	14	71	30	85
Orchardgrass hay	8.0	86	6.9	12.0	1.92	0.30	1.5	265	41	207	340	45	228	100	270
Forage sorghum silage (after maize)	45.0	28	12.6	9.0	1.44	0.30	1.2	181	38	151	235	42	166	95	200
Bermudagrass pasture	45.0	20	9.0	16.0	2.56	0.30	2.0	230	27	180	300	30	198	70	240
Rye pasture	30.0	15	4.5	22.0	3.52	0.30	1.5	158	14	68	205	15	74	20	90
Multiple crop totals															
Maize silage – bermudagrass hay – rye silage	63.6		25.6					536	77	308	695	85	338	195	405
Maize silage – forage sorghum – rye silage	110.0		33.7					617	102	390	800	112	429	260	520
Maize silage – bermudagrass hay	59.0		19.3					337	55	244	440	60	268	140	325

				Composition (% of DM)				*Crop removals (kg ha^{-1})*			*A calculated application rate (kg ha^{-1})*				
Calculations for farm or other crops	*Wet (Mg ha^{-1})*	*DM (%)*	*DM (Mg ha^{-1})*	*CP%*	*N%*	*P%*	*K%*	*N*	*P*	*K*	*N*	*P*	*K*	*P_2O_5*	*K_2O*

[a] Calculated application rates (rounded to nearest 5 kg for N, P_2O_5 and K_2O) are developed from crop removals plus expected losses. For N, removals were multiplied by 1.3 which allows 20% more than crop removals for denitrification and volatilization and 10% for losses to ground and surface waters. For P and K, crop removals were multiplied by 1.1 which allows for 10% more than crop removal for losses to surface waters.

[b] The N application rate for alfalfa is discounted to 80% of removal because legumes will not completely stop N fixation when N is applied from manure or other fertilizer. The best recommendation for applying manure nutrients to alfalfa is to apply manure based on fertilizer P needs.

DM, dry matter; CP, crude protein.

Note that the application rates used in **Table 3** were based on actual P and K from **Table 4**. Conversions often are needed for pricing as well. For example (as used in **Table 3**, Lines 89 to 93), quotes obtained from a fertilizer dealer on a N-only fertilizer, converted to \$0.79 kg^{-1} actual N, a phosphate, converted to \$0.70 kg^{-1} P2O5 and a K source converted to \$0.33 kg^{-1} K2O. Equivalent values per kilogram of actual N, P and K are \$0.79, \$1.61 and \$0.40 (P2O5 values divided by 0.436 and K2O values divided by 0.83).

Analysis of the Preliminary Budget

The worksheet in **Table 3** shows the variation in hectares needed to utilize manure N, P or K for two example dairies with four different cropping system scenarios. Maize silage and alfalfa were included as single crop examples because yields and feed quality are high. Alfalfa was included as an example in case acreage is short and it is necessary to use alfalfa acreage as a place to recycle manure nutrients. Because it is a legume, alfalfa does not need N fertilization because Rhizobium bacteria in root nodules fix atmospheric N; however, the plant will utilize fertilizer N in preference to fixation if it is available, up to a point. The N application rates used assumed that 80% of expected N removal could be applied for recycling N, if necessary. However, the best use of manure on alfalfa will be to limit manure applications to P needs which also will supply K needs (compare Lines 83 and 85, **Table 3**). Most single crop systems will not use nutrients enough of the year to meet most dairymen's manure management needs. Therefore, multiple crop systems are advised, when possible. Sod-based no-till systems are preferred to assure that active roots are present in the soil year-round to intercept N, especially.

Although only examples, several points can be made. Two to three times as many hectares will be needed when manure applications are based on P versus N (Lines 77 to 88, **Table 3**). When P is not sequestered (Example 2), acreages based on K fall in between acreage based on N or P. When N recoveries are low and manure is applied on N basis, K will be overapplied (Example 1) which may lead to high K content in forage when applied to forages that luxury consume K. Dairy managers wish to avoid high-K forages for dry cows.

In Example 1, P budget acreages were closer to N budget acreages than in Example 2. There are two major reasons for this: dietary P was 0.41% in Example 1 versus 0.50% in Example 2 and 50% of the collected manure P in Example 1 was estimated to remain in sludge in the lagoon. Additionally, DMI was lower in the grazing example.

Will Manure Applications Be Limited to the P Budget?

In some regions, legislation mandates that the nutrient management plan be based on P because of demonstrated environmental impacts of P moving into surface waters. In other regions, this may depend on the P status in soils as described by a P index. If not already required, it is likely that at some time in the future, applications will be restricted to P removals or even less if runoff risk is high. Dairymen should prepare a plan of what they will do if they must apply manure based on P budgets.

Can the dairyman make good use of the feed produced if all the acres needed to utilize the P are used to produce forage? In Example 1, it would take only 0.083 ha per cow (8.3 ha per 100 cows, Line 78, **Table 3**) of maize silage producing 15 Mg of DM ha^{-1} to utilize manure P. This translates to cows needing to eat an average of 3.4 kg DM day^{-1} year around from maize silage to utilize all of the forage, an amount that could be utilized and still graze 60% of the time. However, Example 2 cows would require 0.495 ha per cow of maize silage to utilize manure P. This computes to cows needing to eat an average 20.3 kg DM day^{-1} from maize silage to utilize all of the forage produced. This is more than can be utilized by the cow herd. Although acreages vary somewhat with the other example forage programs, DM production and, hence, consumption needs are very similar to utilize produced forages.

The acreage required for the N budget would allow most dairymen to utilize the manure and forage produced, e.g. with maize silage, consumption of 9.8 kg day^{-1} DM is needed for Example 2. Consumption needs with other forage scenarios based on N were about 7 kg DM per cow daily.

How Could these Worksheets Be Used for a Pasture Budget?

If in Example 2, we were interested in some analysis of the pasture budget, we could recalculate the worksheets with a change in the percentage time in the manure collection area (Line 32, **Table 1**) to 60%, the amount of time the cows were on the pastures. Additionally, let us assume our pasture system is described by the bermudagrass and rye pasture data in **Table 4**. This would be managed as bermudagrass overseeded with rye in the fall, thus, double-cropping. The total calculated application rates per hectare for these two crops (from **Table 1**) is 1247 kg N, 111 kg P and 672 kg K. For this example, it was assumed that 40% of N, 90% of P and 80% of K dropped on those pastures was recovered as fertilizer. Putting those application rates and recovery percentages into **Table 3** in place of one of the systems illustrated gives

7.3 ha (N basis), 25.0 ha (P basis) or 13.0 ha (K basis) of pasture needed.

Obviously 7.3 ha based on N is not enough acres to feed 100 cows. How many hectares are needed? If we assumed that there was pasture to graze 90% of the year and that cows ate 7 kg DM day^{-1} from pasture, that computes to 2300 kg DM per cow yearly with supplemental feed needed in place of pasture 10% of the year. **Table 4** assumptions for those pastures were for 13.5 Mg (13 500 kg) DM grazed per hectare. Dividing 13 500 kg ha^{-1} by 2300 kg per cow gives 5.89 cows per hectare as an average stocking rate or 100 cows divided by 5.89 cows per hectare equals 17.0 ha. If the number of hectares to have in pasture is based on estimated DM consumption, relative N, P and K availability can be estimated from the ratios of calculated hectares needed to utilize N, P or K to hectares needed for DM. For example for N, 7.3/17.0 = 43%, which is an estimate that manure would supply only 43% of the total N fertilizer needed for 17.0 hectares. The ratio for P of 25.0/17.0 = 1.47 indicates that manure would be supplying about 1.5 times as much P as recommended. Thus, soil P storage amounts would be expected to increase. The K ratio, 13.0/17.0 is about 0.76 which indicates some K application is likely to be needed.

This pasture example helps support some general conclusions about pasture budgets. First, if pastures are truly grazed, as in Example 1, and not simply used as a sodded dry lot, additional N from commercial fertilizer generally will be needed beyond that supplied by manure N. Second, additional P from commercial fertilizer will not be needed unless soil P storage previous to current grazing year was below soil fertility recommendations based on soil tests. Third, soil tests are recommended to help decide if K fertilization is needed.

Alternatives to Consider if P Budgets Are Required

Step 1: Reduce Dietary P as Much as Possible

The US National Research Council recently increased estimates of P absorption from feeds and supplements compared with 0.50 absorption used in the previous NRC publication. The revised estimates of P absorption are in line with other feeding standards used worldwide (*see* **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins). If dietary P for the milking cows in Example 2 was reduced to 0.38% of DM, which would still meet minimum P requirements, the expected daily excretion of P would be reduced from 79 to 53 g for 310 days of lactation and total yearly excretion from 27 to 18 kg P. The area of maize silage needed for manure P could be reduced from 49.5 ha to 34.1 ha per 100 cows and an average daily DMI of 14.0 kg day^{-1} by all cows in the herd would utilize all of it. This still may be more maize silage than many dairymen prefer to feed but some of that amount probably can be fed to other animals on the farm making production of 34.1 ha of maize silage per 100 cows a feasible amount.

If sufficient land is available to apply manure based on a P budget, more fertilizer value will be realized. For Example 1 (**Table 3**, Lines 94 and 95), 21% more value was estimated if manure was applied to crop-producing acreage utilizing all of the P than if applied based on a N budget. For Example 2, the advantage was 28% ($12 405 versus $9755 per 100 cows). These values (Example 2) were with 42.4% of the N being recovered. How many dollars could have been saved by utilizing better N conservation management practices in manure handling? We do not have an exact answer but if we use a 65% N recovery in Example 2 along with P reduction for lactating cows to 0.38% of DM, the manure fertilizer value recovered increases to $14 298 ($143 per cow) if we utilize all of the P and K. Additionally, acreages needed with N and P budgets were nearly equal. These examples suggest that in the future most dairies will need to reduce P excretion as much as possible and save more N if possible, to make dairy manure nutrient management systems balance well with crop production, economics and the environment.

Step 2: Decide How to Export Nutrients Off-Farm

Flushed manure will not export well. Dry manure is best and economics will lead to hauling it to the closest neighbor who can use it profitably when applied at environmentally acceptable agronomic rates. Hauling wet, solid manure is next best but dairymen may have difficulty finding takers for odorous manure that will bring complaints from their neighbors. That encourages consideration of methods that concentrate manure P for easier export, e.g. composting and use of chemicals and flocculants that precipitate P. These methods will be expensive but it may become necessary to use one of them for dairymen to stay in business in some locations and situations. More than likely, dairy managers will use as much manure as possible on-farm and will choose to process only enough manure with these methods to export the required amount of P.

How Much P Will Need to Be Exported?

Let us presume that a dairy manager has been able to increase forage production sufficiently to utilize manure N accountably and now is forced to shift to P budgeting without the option of increasing crop production. Looking at Example 2 (**Table 3**), it was calculated that 23.9 ha of maize silage needed to be produced to utilize all manure N. If that is all the available acreage and manure must be applied on P basis, 23.9 ha fertilized at 50 kg P ha^{-1} would utilize 1195 kg P. With dietary P at 0.50% of DM in Example 2, it was estimated that 2475 kg of manure P needed to be utilized (**Table 3**, line 63) meaning that 1280 kg P per 100 cows would need to be exported (2475−1195). If dietary P were reduced to 0.38% of DM, recovered P would be estimated at 1706 kg and only 511 kg P would need to be exported (1706−1195).

Many dairy managers feed more dietary P than is required by dairy cows. Being able to reduce export requirements will be a significant incentive to cut dietary P content back to required amounts especially compared with cutting back on cow numbers, an alternative to meeting imposed environmental standards for P. Reducing cow numbers almost never is the best option economically.

Summary

Nutrient excretions by lactating dairy cows, especially N and P, are easily estimated accurately when DMI, nutrient composition of the diet and milk yields are known. Dairy managers must quantify manure nutrients recovered from the waste management system to accurately develop a whole-farm nutrient budget. That means weighing loads of manure, measuring gallons of wastewaters used in irrigation and having samples analyzed for nutrient composition. Losses of more than 50% of the excreted N are common. If an average recovery must be used for preliminary calculations, 40% is suggested for N (60% lost), 90% for P and 80% for K. Pasture budgets will almost always show need for supplemental N from commercial fertilizer N with no supplemental P needed from commercial fertilizer.

Most dairy manure management systems recover enough P to fertilize two to three times as many hectares of crop production based on P as can be fertilized appropriately with manure N. Practical goals to realize the most value from dairy manure usually include: (1) reducing dietary P to minimum dietary requirements to reduce diet cost and manure P excretion, (2) applying manure based on P budgets and (3) applying manure to growing crops as soon as possible after excretion to minimize N volatilization losses.

See also: **Feeds, Ration Formulation**: Systems Describing Nutritional Requirements of Dairy Cows. **Manure/Effluent Management**: Systems Design and Government Regulations. **Nutrients, Digestion and Absorption**: Absorpotion of Minerals and Vitamins.

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MASTITIS PATHOGENS

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Contagious Pathogens

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Introduction

There are over 140 different microorganisms that cause mastitis, and they live on the cow and in her environment. Thus, mastitis is the result of the interaction between the cow, her environment, and microorganisms. These microorganisms are microscopic forms of life and include bacteria, mycoplasmas, yeasts, algae, and, on rare occasions, viruses. However, bacteria are, by far, the major cause of intramammary infections in dairy cows.

The microorganisms that most frequently cause mastitis can be grouped into four categories of pathogens as follows: (1) contagious; (2) environmental; (3) opportunistic; and (4) other. A pathogen is a microorganism that causes an adverse reaction in the animal it is infecting. Some pathogens elicit a very strong inflammatory reaction in the udder, which results in a very high somatic cell count (SCC). Other microorganisms are referred to as minor pathogens because they cause only a slight elevation in SCC.

Most mammary gland infections of consequence are caused by only a few types of bacteria, which include (1) streptococci, (2) staphylococci, and (3) coliforms. Contagious pathogens are spread from infected to uninfected quarters and cows. The major source of contagious microorganisms is the milk from infected quarters. These microorganisms, usually bacteria, are spread from cow to cow during the milking process via milking machine teat cup liners and clusters, milkers' hands, and udder wash cloths.

Environmental pathogens, as the term suggests, arise from the environment in which the cow lives. Such microorganisms gain access to the teat canal and enter the interior of the udder between milkings when the teats are exposed to mud, manure, and dirty bedding materials. Most contagious and environmental pathogens elicit an elevated SCC, and are referred to as major pathogens.

Opportunistic microorganisms are the most prevalent microorganisms isolated from infected quarters, but they cause only mild inflammation in udder tissues, and are referred to as minor pathogens. They thrive on the surface of the udder, teats, and teat canal keratin in large numbers and are consequently a constant source of intramammary infection. Other less common microorganisms also cause mastitis and include fungi, yeast, and algae.

Contagious Microorganisms

The most important contagious mastitis-causing microorganisms are the pathogenic bacteria *Streptococcus agalactiae* and *Staphylococcus aureus* as well as the less pathogenic *Corynebacterium bovis*. *Mycoplasma bovis* are intermediate in size between bacteria and viruses, lack a cell wall, and are important contagious pathogens, but fortunately far less prevalent as mastitis pathogens as compared with *S. aureus*, *Str. agalactiae*, and *C. bovis*.

Infected mammary glands of lactating cows are the chief reservoirs of contagious microorganisms, and transmission from infected to uninfected quarters occurs during milking. Most of these pathogens are well adapted to surviving and growing within the udder. They typically establish subclinical infections of long duration, often called chronic infections, and the microorganisms are shed in milk from infected quarters in large numbers.

Streptococcus agalactiae

Streptococcus agalactiae are spherical Gram-positive bacteria that grow in chains. The Latin term *Streptococcus* *agalactiae* can be literally translated as *strepto*, meaning chains; *coccus*, meaning spheres; and *agalactiae*, meaning without milk. The only important reservoir of *Str. agalactiae* on a dairy farm is milk from infected mammary quarters. These bacteria can, however, be found on surfaces that have had recent contact with contaminated milk, including milking equipment, milkers' hands, and bedding materials. Young dairy heifers have been shown to become infected from nursing each other in group pens or when fed colostrum or milk infected with *Str. agalactiae*.

These bacteria are shed in very high numbers in milk from infected quarters. It has been documented that one infected quarter of one cow in a 100-cow herd can elevate the bacterial count of bulk tank milk to more than 100 000 ml^{-1}. The spread of *Str. agalactiae* to uninfected quarters occurs mainly during milking. In the absence of good udder hygiene and effective control measures, *Str. agalactiae* can spread rapidly throughout a herd. Incomplete milking of infected quarters may increase the severity of *Str. agalactiae* mastitis, because large numbers of bacteria remain in infected quarters for potential transmittance to other cows.

Quarters infected with *Str. agalactiae* typically have a high SCC that can markedly elevate the bulk tank cell count. Individual quarter SCC may range between 1 and 10 million ml^{-1}. Clinical signs include slightly off-colored milk and presence of clots and flakes. This microorganism is sensitive to penicillin and cephalosporin and can be eradicated from individual dairy herds. Subclinical infections can become chronic if not treated successfully with antibiotics, resulting in nonfunctional or blind quarters.

Staphylococcus aureus

Staphylococcus aureus microorganisms are Gram-positive bacteria that grow in grape-like clusters. The term *Staphylococcus aureus* is translated as *staphylo*, meaning clusters; *coccus*, meaning spheres; and *aureus*, meaning golden (as it appears to the naked eye on culture medium) – hence, a golden cluster of spheres. These bacteria may be found on healthy teat skin, and readily colonize or grow in teat canal keratin. Teat skin chapping, resulting in lesions or sores, promotes colonization. *Staphylococcus aureus* growing in these sites are in an ideal location for infecting the udder, and are transmitted to uninfected quarters by milking machine teat cup liners, udder wash cloths, and milkers' hands. These bacteria may survive in other sites on the cow's body as well.

Bacteria from infected quarters can also be introduced to uninfected quarters through droplet impacts created by liner slips. Once *S. aureus* establishes an infection in the milk-producing tissues of the udder, a chronic inflammation results along with an elevated SCC. Firm, fibrotic areas of scar tissue may be found upon palpation. Most often, infections caused by *S. aureus* are subclinical in nature with periodic flare-ups of clinical symptoms. Such clinically infected quarters usually exhibit moderate swelling and obvious clots and flakes when forestripping takes place. Chronic infections are extremely difficult to cure with antibiotic therapy because of the development of scar tissue at multiple sites. These areas impede the distribution of antibiotics within the affected quarter after infusion and thus protect the staphylococci. As a result, antibiotics do not come into contact with the bacteria, the infection remains established, and the affected cow must be culled from the herd to prevent the spread of the disease to other animals.

With acute clinical cases, quarters are usually hot and swollen, and the cow exhibits an elevated temperature of 103–106 °F (39.4–41.1 °C). A few infections become gangrenous. The affected quarters become cold to the touch due to a loss in blood supply, a condition referred to as 'blue bag'.

In some herds, *S. aureus* causes intramammary infections in breeding age heifers, which become chronic and persist through calving. Therefore, cases of *S. aureus* mastitis in heifers that have recently calved can serve as a source of new infections entering the milking herd.

By implementing the excellent mastitis control programs that are now available, *S. aureus* can be reduced to very low levels, and even eradicated in some herds. Unfortunately, *S. aureus* and *Str. agalactiae* are still widespread in some dairy herds throughout the world and continue to cause serious economic losses. This is especially true in herds not practicing effective teat dipping and nonlactating cow treatment programs.

Mycoplasma bovis

Mycoplasma species do not stain with Gram stain, are intermediate in size between bacteria and viruses, and do not have a cell wall. These microorganisms are also known as pleuropneumonia-like organisms. Mastitis caused by *Mycoplasma* should be suspected when milk samples from cows with clinical symptoms, often in multiple quarters, are negative after repeated culture using standard microbiological methods. In addition, mycoplasmal mastitis is characterized by (1) a sudden onset, (2) formation of a purulent secretion in affected quarters, (3) rapid transmission throughout the herd, (4) a marked reduction in milk yield, and (5) resistance to antibiotic therapy.

In spite of the severe local reaction in the udder, the affected cow does not generally develop systemic symptoms. Treatment is ineffective, and serious herd problems will result if the level of infection is not controlled, leading to the slaughter of affected cows. Mycoplasmas can also be cultured from manure, blood, respiratory tract, eyes, and uterus of infected cows. Improper treatment procedures, specifically multiple use of syringes or improper teat end sanitization, may be the cause of new infections. This disease can be introduced to a herd via purchased replacement animals as well.

Although numerous species of *Mycoplasma* exist, the most common is *Mycoplasma bovis* followed by *Mycoplasma californicum*. Culture methods require special media and incubation times to identify milk infected with any *Mycoplasma* species.

Corynebacterium bovis

Corynebacterium bovis are Gram-positive, club-shaped or coryneform bacteria. Mammary gland infections with these bacteria are usually mild with a slight elevation in SCC, ranging from 200 000 to 400 000 ml^{-1}, but which can at times exceed 1 million ml^{-1}. Mastitis outbreaks caused by *C. bovis* have been reported most commonly in herds that do not practice postmilking teat dipping and nonlactating cow therapy. Primary reservoirs of these microorganisms are infected udders and teat ducts.

It has been reported that quarters infected with *C. bovis* are less susceptible to infections by *S. aureus* but more susceptible to infections by *Str. agalactiae* and the environmental streptococci.

Control of Contagious Microorganisms

The principal sources of the contagious pathogens, such as *Str. agalactiae*, *S. aureus*, *M. bovis*, and *C. bovis*, are infected mammary glands. The spread of these pathogens within a herd occurs almost exclusively during milking. However, *S. aureus* readily colonizes teat skin and the teat canal, and has also been isolated from the teat skin, teat canal, and mammary secretions of heifers that have never calved. These organisms, with the exception of *Mycoplasma*, are usually controlled quite easily by (1) proper udder hygiene, (2) correct use of functionally adequate milking machines, (3) teat dipping after milking, and (4) treatment of all quarters of all cows at the end of lactation.

Streptococcus agalactiae

The sole reservoir of *Str. agalactiae* is the mammary gland; thus, the spread of this microorganism occurs during milking. Transmission from cow to cow can be reduced by (1) excellent milking hygiene practices, (2) correct use of functionally adequate milking machines, and (3) use of an effective postmilking teat dip. Infections already present in the herd can be eliminated during lactation by using an antibiotic therapy because *Str. agalactiae* organisms are killed readily by most intramammary antibiotic preparations, especially penicillin. These microorganisms inhabit the duct system of an infected quarter; thus, they are easily accessible to antibiotics. The small percentage of chronic infections that resist therapy should be treated at drying off or the infected cow culled from the herd. *Streptococcus agalactiae* can often be eradicated from a herd within 2–3 years if a control program involving good milking management, teat dipping, total dry cow therapy, and not purchasing infected animals is applied.

If a herd exhibits a high prevalence of *Str. agalactiae* mastitis, and the bulk tank SCC and bacterial count are also elevated, then culturing is necessary. All lactating cows should be sampled and cultured, and all infected quarters treated with penicillin or other approved infusion products. In most cases, 90% of the infected quarters will usually respond to therapy. Teat dipping and dry cow therapy should be continued, and follow-up herd cultures must be performed if eradication of this microorganism from the herd is to be achieved. Prevention of *Str. agalactiae* mastitis is based on maintaining a closed herd. All new additions to the herd, including fresh heifers, should be cultured before they are added to the milking string. The following program was developed for eradicating this organism from a dairy herd.

1. A herd infected with *Str. agalactiae* is identified by culturing a sample of bulk tank milk on a regular basis.
2. Prior to collecting milk samples from each lactating animal in the herd, a qualified person should visit the farm at milking time to evaluate the following:
   - Are teats clean and dry when teat cups are attached?
   - Is a predip that has been proven effective being used properly?
   - Do teat cups slip down on teats during milking leading to squawking? If so, the milking machine dealer should be contacted to correct the problem.
   - What intramammary treatment procedures are being followed? Recommend changes if necessary.
   - What postmilking teat dip is being used and has it been proven effective?
   - Is teat spraying being used? If so, recommend that teat dipping be initiated.
   - Are all cows treated at drying off? If so, is it with an acceptable product?
   - Discuss the significance of other mastitis pathogens found in the herd milk samples.
3. Emphasize changes needed prior to initiating a *Str. agalactiae* eradication program.
4. Evaluate SCC records and emphasize the importance of reducing the SCC to the minimum practical level in order to reduce economic losses resulting from mastitis.
5. Discuss the process of collecting sterile milk samples to detect *Str. agalactiae*.
6. Set a date to collect composite milk samples from all lactating animals for culture.
7. Prepare a list of all *Str. agalactiae*-infected cows.

8. Recommend that infected cows be segregated from the rest of the herd and milked last.
9. Emphasize the importance of using proper intramammary treatment procedures.
10. Recommend that all infected cows be treated twice at 12-h intervals in each quarter with a commercial tube containing 100 000 units of penicillin, which should eliminate more than 90% of the infections.
11. Withhold milk from treated cows according to the manufacturer's recommendations.
12. Discuss how to handle cows that are infected with other pathogens.
13. Collect a bulk milk tank sample the day before treated animals are placed back in the herd and culture to determine if all *Str. agalactiae*-infected cows have been identified and treated. If the bulk tank milk is positive for *Str. agalactiae*, it will be necessary to collect composite milk samples from all lactating cows to identify the infected animals. Follow the above recommendations in treating such animals.
14. Reculture all *Str. agalactiae*-treated animals 14–21 days after the initial treatment. If any cows remain infected with *Str. agalactiae*, they should be re-treated with a commercial tube containing penicillin or sodium cloxacillin. The maximum number of treatments recommended by the manufacturer should be administered at 12- or 24-h intervals. Discard milk as per the manufacturer's recommendations.
15. Collect samples from the bulk milk tank once a week for 3 months, and monthly thereafter, to monitor for reinfection of the herd. If *Str. agalactiae* reappears in the bulk milk, repeat the procedures outlined above to identify and treat infected animals. Animals infected with *Str. agalactiae* that were not cured by the two treatment regimens should be culled.
16. Collect samples from all cows and heifers at calving for culture to determine if fresh animals are infected with *Str. agalactiae*. Any animals that are infected should be treated immediately using the procedures outlined above.
17. Continue to monitor milking management changes made earlier to make certain that recommendations are still being followed.

It is strongly recommended that dairy farmers work closely with their herd veterinarian on a *Str. agalactiae* eradication program. Other professionals such as milk plant fieldsmen, extension specialists, county agents, laboratory technicians, milking machine dealers, and others may also be of assistance.

Staphylococcus aureus

As with *Str. agalactiae*, the spread of these contagious bacteria occurs during milking. Mastitis caused by *S. aureus* is almost impossible to control by antibiotic therapy alone. Thus, control depends upon preventing the spread of this contagious microorganism from infected to uninfected quarters using proper milking hygiene and dry cow therapy, as well as by culling chronically infected cows that do not respond to treatment.

Spread can be minimized by using recommended milking hygiene practices, the most important of which are ensuring clean, dry teats before milking with properly functioning milking machines, and dipping teats after milking with a safe and effective product. Teats and udders should be prepared for milking, and milking machines should be maintained and used as described in the article on milking hygiene (*see* **Milking and Handling of Raw Milk**: Milking Hygiene). Use of an effective teat dip product after milking will greatly reduce the number of *S. aureus* (originating from the milk of previously milked cows) remaining on the skin and in the teat canal after the unit is removed, thereby reducing the potential for these bacteria to enter the udder after milking when the teat canal is still dilated.

Dry cow treatment of all cows that are drying off is beneficial in curing the existing *S. aureus* infections and preventing new ones from occurring during the early dry period. The effectiveness of dry cow therapy ranges from 20 to 70%, depending on whether the infections are chronic and accompanied by extensive fibrosis and scar tissue formation. Many dry period infections persist, however, resulting in clinical flare-ups at freshening. Such animals eventually should be culled from the herd, as they represent a significant reservoir of *S. aureus* for infecting other cows.

Antibiotic therapy during lactation using conventional treatment regimens is of little benefit. Only 10–30% of infected quarters will be cured. Infusion of drugs may temporarily reduce SCC and improve the appearance of milk in clinically infected quarters, but it often does not eliminate bacteria from the gland. This is because these microorganisms become walled off by scar tissue that protects them from the action of antibiotics. *Staphylococcus aureus* may also develop resistance to certain antibiotics and can produce an enzyme that inactivates penicillin. Thus, treatment of subclinical infections using conventional therapy during lactation is generally not recommended. However, some success in treating chronic *S. aureus* mastitis has been realized by combining intramammary infusions with intramuscular injections of antibiotics, as well as through extended therapy.

The spread of *S. aureus* within a dairy herd can be reduced by milking (1) first-lactation animals before older cows, (2) uninfected cows next, and (3) known infected cows last. The use of vaccines against this organism in adult lactating cows has been somewhat effective in lowering the SCC and increasing the spontaneous cure rate, but immunization does not prevent new *S. aureus* infections. Thus, vaccination of cows should be viewed as an adjunct to the control programs given immediately above, rather than as a replacement for those programs.

As outlined above, *S. aureus* can cause intramammary infections in breeding age and pregnant heifers, which become chronic and persist through calving, serving as sources of new infections entering the milking herd. Use of nonlactating cow antibiotics infused during pregnancy and no sooner than 45 days prepartum can result in *S. aureus* cure rates approaching 100%. In addition, SCCs are reduced at calving, and milk production during the first lactation is increased by 10% in comparison to infected untreated herdmates. Vaccination is another control measure that holds promise. By immunizing animals with a commercial bacterin starting at 6 months of age followed by a 14th-day booster injection and immunizing thereafter every 6 months through calving, the incidence of *S. aureus* mastitis at calving can be reduced by 45–60%. In one study, SCCs at calving were reduced by 50%; culling rate during the first lactation was reduced by 33%; and milk production, milk fat, milk protein, and days in milk were increased during the first lactation in vaccinated heifers as compared with controls. Herds subjected to some form of fly control have also been shown to have a lower prevalence of *S. aureus* mastitis in heifers.

Mycoplasma bovis

Mycoplasma almost always enters into an expanding herd via purchased heifers. Some specialists believe that heifer calves can become infected with this organism simply by drinking milk from infected cows, including colostrum. Infected calves may develop joint, ear, or lung infections, and the disease may spread from calf to calf via aerosolized droplets. When infected animals calve, they may spread the disease via nasal discharge. Metritis may also result, and vaginal discharge may inoculate teats resulting in infected udders that then spread the disease to calves via colostrum. *Mycoplasma bovis* is the most frequently isolated species.

Animals infected with *Mycoplasma* species usually have SCCs well in excess of 1 million ml^{-1}. In herds that do not have a history of infection with this organism and which suddenly become positive, one should suspect purchased animals or newly calved heifers as the source. It is not uncommon for herds that have experienced an outbreak of mycoplasmal mastitis to be free of the organism for 2 years and again have a problem when calves that were born during the previous outbreak calve and enter the herd. Even if a dairy herd is not currently experiencing mycoplasmal mastitis, these microorganisms are likely present within the herd, and the possibility of an outbreak always exists.

The organisms are highly contagious and may spread rapidly throughout a herd with devastating consequences. The disease should be suspected when (1) more than one quarter (oftentimes all four) has clinical mastitis, (2) affected quarters do not respond to treatment, (3) milk yield decreases significantly, and (4) abnormal secretions are observed ranging from watery milk with a few clots to colostrum-like material. Chronically infected cows may show tan-colored secretions with sandy or flaky sediment, which resembles cooked cereal in a whey-like fluid. Udder secretions may become purulent, and symptoms may last for several weeks. Herds that routinely cull such animals and practice excellent milking management with effective teat dipping can avoid, or at least minimize, spread of the infection.

Even though *Mycoplasma* species infecting mammary glands are not sensitive to intramammary therapeutics, commercially prepared mastitis tubes should be used when infusing drugs into the udder to treat other infections. This is because home- or vet-prepared bottle mixes may get contaminated with *Mycoplasma.* In addition, excellent hygiene should be followed when preparing teats for treatment as *Mycoplasma* at the teat orifice may be pushed up into the gland.

Control of this contagious pathogen is through the prevention of new infections based on the use of rigid milking hygiene because treatment is ineffective with the drugs currently available. Specific management procedures include backflushing or heat treatment of the milking cluster between cows to reduce the spread of mycoplasma, and segregation of cows with clinical mastitis, but such animals should not be commingled with fresh cows. Great care with milking equipment maintenance can also minimize the incidence of new infections with this microorganism. In herds experiencing *M. bovis* outbreaks, all milking cows must be cultured and affected animals segregated or culled from the herd.

Culturing of bulk milk on a monthly basis and culturing clinical cases of mastitis should be initiated to monitor the herd infection status. All fresh heifers and cows should be tested before they enter the milking herd, and they should be segregated if found positive. Cows that are intermittent shedders of *M. bovis* should be culled at the end of lactation. Some animals will clear the infection spontaneously and undergo a normal lactation.

Corynebacterium bovis

Corynebacterium bovis rarely causes clinical mastitis and causes only a modest increase in the SCC, although on occasion, the SCC may approach or exceed 1 million ml^{-1}. New infections caused by *C. bovis* are easily prevented by postmilking teat dipping. In addition, this microorganism is highly susceptible (more than 90%) to nonlactating cow therapy, especially penicillin. However, dry cow therapy has no effect on new infections that develop around the time of calving. Herds having a high prevalence of *C. bovis* usually have not maintained adequate teat dipping and nonlactating cow therapy programs. Backflushing and bucket sanitization of the milking cluster between cows are helpful procedures in preventing new infections.

Segregation

Segregation, or milking of first-calf heifers first, uninfected cows next, and infected cows last, may also be of some benefit. For example, the level of mastitis is low in first-calf heifers as compared with older cows. Thus, the number of mastitis-causing microorganisms left in milking units would likely be minimized after milking first-calf heifers. If older cows with a higher level of infection were milked first, more mastitis microorganisms could be left in the milking unit, which could potentially infect the younger, mastitis-free cows.

Because infected animals can be identified only by culture of all cows in the herd, and the segregation of infected cows is inconvenient in many housing situations, this recommendation is often impractical. With the use of effective hygiene procedures, segregation of cows becomes less important. Nevertheless, when control of infections caused by certain contagious pathogens such as *Mycoplasma* species or *S. aureus* is necessary, culture and subsequent segregation of infected cows can be an important part of the control program. In one study, segregation of *S. aureus*-infected cows reduced the prevalence of this organism from 29.5 to 16.3% and the SCC from 600 000 to 345 000 ml^{-1} in 2 years.

Purchased replacements, especially cows that have been milked in other herds, should be regarded as potential sources of contagious pathogens and segregated from the herd until their health status has been determined. If replacements must be purchased, heifers that have not yet calved should be chosen, because they are generally less likely than older milking cows to be sources of infection; one exception is pregnant heifers chronically infected with *S. aureus*. The purchased animals should be cultured before they enter the milking herd, and only cows and heifers free of contagious mastitis should be allowed to join the herd.

Backflushing

Teat cup liners of the milking machine clusters used to milk infected quarters can become heavily contaminated with contagious bacteria. These pathogens may be transferred to udders of cows milked subsequently. Backflushing or the automated rinsing of milking clusters with water after milking machine removal, followed by rinsing with a disinfectant and a blast of air, can reduce the spread of contagious mastitis pathogens. However, the benefit of backflushing the milking units will likely be small if an effective postmilking teat dip is used. Likewise, the benefits of backflushing may not be sufficient to justify the additional costs and labor involved.

Dipping clusters into a bucket of disinfectant after milking can be an effective procedure for reducing the spread of infections, if performed correctly. For example, immediately after detachment, the cluster should first be rinsed by immersion in a bucket of warm water (by dipping two teat cups at a time to avoid the airlock and ensure liquid contact with teat cup liners) to prerinse off milk residues from the teat cup liner surfaces. This is followed by immersion (two teat cups at a time) into a germicidal solution of iodine or chlorine, and a 30-s drip dry time. When the buckets of water and sanitizer become cloudy with milk residues or organic matter, they should be replaced.

Conclusions

Regardless of the contagious pathogen causing mastitis in a herd, it is imperative that the new infection rate be controlled. Otherwise, new infections will be occurring as rapidly as old infections are being eliminated by treatment or culling, and the incidence of clinical mastitis and the bulk tank SCC level will not drop. The practice of sampling fresh cows is very useful in monitoring mastitis in a herd and will help to reduce and maintain infection levels and SCC at an acceptable point. Screening of fresh cows with a California mastitis test (CMT) paddle and checking for abnormal milk are useful methods for determining which cows should be sampled and cultured. Contaminated hands are a potential source of spreading pathogens from cow to cow, and the use of disposable gloves helps reduce such spread. The practice is especially valuable for employees who work with animals diverted to the sick pen. Lastly, if lactating cow replacements are needed, it is important to culture the animals prior to purchase. If this is not done, such animals should be isolated, and samples collected and cultured before adding the animals to the lactating herd.

See also: **Feeds, Ration Formulation**: Dry Period Rations in Cattle. **Mastitis Pathogens**: Environmental Pathogens. **Mastitis Therapy and Control**: Management Control Options; Medical Therapy Options; Role of Milking Machines in Control of Mastitis. **Milking and Handling of Raw Milk**: Milking Hygiene. **Pathogens in Milk**: *Staphylococcus aureus* - Molecular.

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Environmental Pathogens

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Introduction

Mastitis, an inflammation of the mammary gland caused by bacterial infection, trauma, or injury to the udder, remains the most common and most expensive disease affecting dairy cattle throughout the world. Mastitis is caused by several different bacteria that can invade the udder, multiply there, and produce harmful substances that result in inflammation. The NMC (formerly the National Mastitis Council) estimates that mastitis costs dairy producers in the United States over $2 billions annually. Consequently, mastitis continues to be one of the most (if not the most) significant limiting factors to profitable dairy production.

Mastitis organisms are categorized as contagious or environmental pathogens. Contagious pathogens live and multiply on and in the cow's mammary gland and they spread from cow to cow primarily during milking. Contagious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* species, and *Corynebacterium bovis*, and are described in considerable detail in the article **Mastitis Pathogens**: Contagious Pathogens of this volume. Environmental mastitis pathogens reside in the environment where cows live and consist of a heterogeneous group of bacterial genera and species.

Current mastitis control programs devised primarily in the 1960s are based on hygiene including pre- and post-milking teat disinfection, antibiotic therapy during lactation and at drying off, and culling of chronically infected cows. Acceptance and application of these measures have led to considerable progress in controlling contagious mastitis pathogens. However, these mastitis control procedures are less effective against environmental mastitis pathogens. Studies have shown that as the prevalence of contagious mastitis pathogens reduced, the proportion of intramammary infections (IMIs) by environmental pathogens increased markedly. Therefore, it is not surprising that environmental mastitis has become a major problem in many well-managed dairy farms that have successfully controlled contagious pathogens. In these herds, environmental mastitis pathogens account for a significant number of both subclinical and clinical IMIs in lactating and nonlactating cows. The purpose of this article is to summarize what is known on the etiology, diagnosis, epidemiology, and pathogenesis of environmental mastitis pathogens.

Environmental Mastitis Pathogens

The most frequently isolated environmental pathogens are several species of streptococci other than *S. agalactiae*, collectively referred to as environmental streptococci, and Gram-negative bacteria (**Table 1**) The primary source of environmental mastitis pathogens is the environment of the cow. Infections generally occur between milkings and during the milking process. Some characteristics of herds with an environmental mastitis problem include (1) a low prevalence of IMI during lactation, (2) a low bulk tank somatic cell count, (3) infections of short duration, (4) many IMIs resulting in clinical mastitis, and (5) a high prevalence of infection during the dry period.

Pathogenesis

Some Gram-negative mastitis pathogens such as *Escherichia coli*, *Klebsiella*, and *Enterobacter* ferment lactose efficiently. The high lactose content of bovine milk, coupled with the warm, anaerobic environment within the mammary gland, promotes rapid growth of these coliform bacteria. Bacterial numbers can reach peak levels within 5–16 h. This rapid growth, coupled with the subsequent release of toxins and the response of the host, can initiate an unlimited inflammatory reaction and result in acute clinical mastitis. Cardinal signs of inflammation characterize acute mastitis; the infected quarter will be red, swollen, hot, and painful. Milk may also be watery and contain clots. Milk production will be reduced markedly as a result of tissue damage from the inflammatory response including the influx of blood components into the mammary gland and release of reactive oxygen species from neutrophils and macrophages. In fewer than 5% of the cases, the infection can develop into endotoxemia, a systemic reaction characterized by elevated body temperature, depression, dehydration, appetite loss, diarrhea, and possibly death.

The classical paradigm has been that *E. coli* IMIs are of short duration, resulting in either rapid bacterial clearance or death of the cow. However, as early as 1979, research described some *E. coli* IMIs in which the course of infection was persistent and recurrent, rather than acute and transient. Field observations since then have confirmed this behavior. Genotyping of *E. coli* strains involved in persistent IMI has shown that these are indeed recurrent or persistent *E. coli* infections caused by the same strain. Given the pathogenesis of *E. coli* infections in several other sites (e.g., enteric and urinary tract) in mammalian hosts, adaptation of coliform bacteria to the mammary gland of the cow is not surprising. Recently published data suggest that adhesion and internalization into mammary epithelial cells and subsequent intracellular survival may be prerequisites for persistency of host-adapted *E. coli* strains.

The risk of developing mastitis caused by Gram-negative organisms is about 4 times greater during the dry period. Susceptibility of the udder to new IMI is highest 2 weeks after drying off and again during the period that starts 2 weeks before and, ends 2 weeks after calving. With each subsequent dry period, this risk increases. The greater incidence of mastitis during the initial 2 weeks of the dry period is associated with milk cessation and the subsequent physiological changes that occur when the udder is undergoing marked transition from lactation to involution. Manure, bedding, and other contamination are not removed at each milking, and this increases the bacterial load at the teat end. Without milk removal, the gland enlarges and the teat end dilates, allowing environmental mastitis pathogens easier access into the mammary gland. For high-producing dairy cows that have not yet reduced milk production, this aspect may further increase the likelihood of environmental mastitis occurring. Compounding the increased bacterial load and ease of penetrating the teat end, is the fact that bacteria are not flushed out every 8–12 h by regular milking.

Dairy cows are also susceptible to environmental mastitis during the first 90 days of lactation. However, susceptibility and severity are greatest during the nonlactating and periparturient periods. At this time, overall immune defenses are compromised due to factors such as calving stress, hormonal changes, glucocorticoid release, increased lactation demands, and insufficient energy intake. In particular, a threefold reduction in neutrophil migration and in addition lower chemotaxis, phagocytosis, and reactive oxygen species generation are especially problematic as neutrophil influx and killing are critical to resolving an infection. Not only immune responses are reduced, allowing greater bacterial growth, but also resident macrophages within the mammary gland are primed to produce greater tumor necrosis factor concentrations during the periparturient period. At first, this may seem beneficial, but may contribute to the severity of coliform infections observed at this time.

A high proportion of Gram-negative and environmental streptococcal infections that originate during the dry period or during lactation result in clinical mastitis during early lactation. This points out the importance of the dry period in the control of environmental mastitis. The prevalence or percentage of quarter infections with environmental pathogens at any point of time during lactation is generally quite low (<7–8% of quarters). Environmental pathogen infections, particularly infections caused by Gram-negative bacteria, are typically of short duration, which is most likely associated with the low prevalence of infection in dairy herds. On the other hand, a high proportion of environmental pathogen IMI during lactation results in clinical mastitis. Thus, conventional methods of monitoring herd mastitis such as bulk tank and individual cow somatic cell count, and herd survey and bulk tank bacteriologic culturing do not accurately reflect the degree of environmental mastitis in dairy herds. A more precise method of monitoring environmental mastitis in herds is to record new clinical cases of mastitis and to culture milk from clinically infected mammary glands prior to treatment.

Environmental *Streptococcus* Species

The genus *Streptococcus* has been recently separated into three genera: *Streptococcus*, *Enterococcus*, and *Lactococcus*. In addition, several new species and better-defined descriptions of previously described species have been reported. Environmental *Streptococcus* species involved in bovine mastitis (**Table 1**) are *Streptococcus uberis*, *Streptococcus dysgalactiae* ssp. *dysgalactiae*, *Streptococcus acidominimus*, *Streptococcus alactolyticus*, *Streptococcus canis*, *Streptococcus equi*, *Streptococcus equinus* (formerly referred to as *Streptococcus bovis*), and *Streptococcus parauberis* (formerly referred to as

Table 1 Environmental pathogens that cause mastitis in dairy cows

Streptococcus *species*	*Gram-negative bacteria*	Enterococcus *species*
S. acidominimus	*Escherichia coli*	*E. faecium*
S. alactolyticus	*Klebsiella oxytoca*	*E. faecalis*
S. canis	*Klebsiella pneumoniae*	*E. durans*
S. dysgalactiae	*Enterobacter* spp.	*E. saccharolyticus*
S. equi	*Serratia* spp.	
S. equinus	*Pseudomonas* spp.	
S. parauberis	*Proteus* spp.	
S. uberis	*Pasteurella* spp.	

S. uberis genotype II). *Enterococcus* species involved in bovine mastitis include *Enterococcus durans*, *Enterococcus faecalis* (formerly *Streptococcus faecalis*), *Enterococcus faecium* (formerly *Streptococcus faecium*), and *Enterococcus saccharolyticus* (formerly *Streptococcus saccharolyticus*). Among the environmental streptococci, *S. uberis* and *S. dysgalactiae* ssp. *dysgalactiae* appear to be the most prevalent, infecting mammary glands as favorable conditions arise. The following information on *Streptococcus* and *Enterococcus* species is intended to provide the reader with a broad overview of these types of mastitis pathogens.

The colonies of streptococci and enterococci on blood agar are small (1–3 mm in diameter), smooth, translucent, and convex. Bacteria may be surrounded by a zone of greenish, discolored erythrocytes (referred to as alpha hemolysis), surrounded by a clear zone of lysed erythrocytes (referred to as beta hemolysis), or nonhemolytic (also referred to as gamma hemolysis). Addition of aesculin to blood agar can be used to differentiate *S. agalactiae* from the many other streptococci that hydrolyze aesculin. Other mastitis-causing bacteria may have a similar colony morphology on blood agar. These colonies should be Gram stained and tested for catalase production. Streptococci and enterococci are catalase negative. Only catalase-negative, Gram-positive cocci should be selected for subsequent testing to differentiate streptococcal and enterococcal species. Several tests have been developed for differentiation of *Streptococcus* and *Enterococcus* species. Tests that are useful include methods to identify serological groups, commercial microbial identification systems, the CAMP test, hydrolysis of aesculin and sodium hippurate, acid production in broth containing various carbohydrates, and growth in broth containing 6.5% NaCl.

Streptococcus uberis is a Gram-positive coccus that occurs in chains. The bacteria appear as small (1–3 mm in diameter) colonies on blood agar; the colonies are moist, convex with dense centers, and translucent. Most are alpha-hemolytic (green) on blood agar, but can be nonhemolytic (gamma). *Streptococcus uberis* is catalase-negative, CAMP variable (primarily CAMP-negative), aesculin positive, sodium hippurate positive, primarily inulin positive, and is not Lancefield groupable (some strains are reported to belong to Lancefield group E, G, P, or U).

Streptococcus dysgalactiae belongs to Lancefield serological group C. Taxonomic studies have classified organisms designated previously as *Streptococcus equisimilis* as well as group L and human group G as *S. dysgalactiae*. Based on electrophoresis of cell wall proteins and physiological tests, *S. dysgalactiae* ssp. *dysgalactiae* was proposed for strains of animal origin that belong to Lancefield serogroups C and L, and *S. dysgalactiae* ssp. *equisimilis* was proposed for human isolates that belong to Lancefield serogroups C and G. Group L streptococci, *S. equisimilis*, and human group G streptococci are isolated infrequently from bovine mammary glands. Bovine strains of *S. dysgalactiae* are a homogeneous group of nonhemolytic or alpha-hemolytic cocci that should be distinguished from beta-hemolytic strains of group L, human group G, and *S. equisimilis*.

Streptococcus dysgalactiae ssp. *dysgalactiae* is a Gram-positive coccus that occurs in chains. The bacteria appear as small (1–2 mm in diameter) colonies on blood agar; the colonies are moist, convex, and translucent. Most are nonhemolytic (gamma) on blood agar, but they can be alpha-hemolytic (green). *Streptococcus dysgalactiae* ssp. *dysgalactiae* is catalase negative, CAMP negative, primarily aesculin negative, and belongs to Lancefield serological group C. *Streptococcus dysgalactiae* ssp. *dysgalactiae* can be identified by serotyping and trehalose utilization.

Other *Streptococcus* species appear as small (1–3 mm in diameter) colonies on blood agar; the colonies are moist, convex with dense centers, and translucent. These colonies can be nonhemolytic (gamma) or alpha-hemolytic (green). *Streptococcus canis* produces a wide zone of beta-hemolysis. Other distinguishing characteristics include Gram positive cocci occurring in chains, catalase negative, CAMP variable (primarily CAMP-negative), primarily aesculin positive, Lancefield serological group C, D, or G (many are not groupable), and primarily sodium hippurate-positive. *Streptococcus canis* produces a false-positive club-shaped type of CAMP reaction.

Enterococcus species appear as small (1–3 mm in diameter) colonies on blood agar; the colonies are moist, convex, and semitranslucent. The colonies can be nonhemolytic (gamma) or alpha-hemolytic (green). Other distinguishing characteristics include Gram positive cocci occurring in chains, catalase negative, CAMP-negative, aesculin positive, primarily Lancefield serological group D (some may not be groupable), sodium hippurate-positive, growth in 6.5% NaCl broth, and bile-aesculin-positive.

Characteristics of *Streptococcus uberis*

Streptococcus uberis was first described in 1932. Using DNA hybridization, two *S. uberis* genotypes designated types I and II were observed. Nucleotide sequences of 16S ribosomal RNA of *S. uberis* genotypes I and II showed that the two genotypes were phylogenetically distinct, and it was proposed that *S. uberis* genotype II be designated *S. parauberis*. However, differentiation of *S. uberis* from *S. parauberis* was possible only by DNA hybridization or 16S rRNA sequencing, since cultural, morphological, biochemical, and serological characteristics of the two closely related species are indistinguishable. Techniques were developed for differentiating *S. uberis* from *S. parauberis* based on DNA fingerprinting. This technique was expanded to include 10 additional *Streptococcus* species capable of causing mastitis in dairy cows. Thus, techniques are now available for conducting molecular epidemiological investigations, and they could identify new avenues for better control of mastitis due to *Streptococcus* species by revealing important reservoirs, prevalence, and mode(s) of transmission of the various *Streptococcus* species.

Streptococcus uberis is an amazingly versatile mastitis pathogen that can affect lactating cows, dry cows, heifers, and multiparous cows, cause clinical or subclinical mastitis, and can even cause persistent colonization without an elevation in the somatic cell count. It is a major cause of mastitis in housed cattle with year-round calving and in pasture-based management systems with seasonal calving. *Streptococcus uberis* has been described as an environmental pathogen and as a potentially contagious pathogen. It has the ability to survive in several diverse environments such as bedding, pastures, feces, various body sites of cows, and in the mammary gland. Thus, *S. uberis* isolated from the mammary gland of a cow with mastitis is likely able to (1) colonize and grow in the rumen and/or other areas of the gastrointestinal system of cows, (2) survive in the cows' environment after excretion in feces, and (3) grow to sufficient numbers in the environment to gain access to the mammary gland. Once inside the mammary gland, *S. uberis* must grow in milk and nonlactating mammary secretions, and avoid a number of host defense mechanisms.

Streptococcus uberis is ubiquitous in the cows' environment. Consequently, mammary glands are exposed continuously to *S. uberis* during lactation and the nonlactating period. The prevalence of *S. uberis* IMI does not appear to be influenced by season of year or breed, but was the predominant cause of mastitis in unmilked cows, and *S. uberis* mastitis increased significantly in older cows. In the absence of antibiotic dry cow therapy, the number of new *S. uberis* IMI during the nonlactating period increased markedly, especially during the early dry period and near parturition. Antibiotic therapy at drying off reduced the rate of new *S. uberis* IMI during the early dry period, but had no effect on preventing *S. uberis* IMI at the end of the nonlactating period. Thus, logically, the nonlactating period is a time for developing strategies for controlling *S. uberis* mastitis.

Virulence factors associated with the pathogenesis of *S. uberis* mastitis are not well understood, which constitutes a major obstacle for development of strategies to control this important mastitis pathogen. Recent research suggests that adherence to and subsequent internalization of mastitis pathogens into mammary epithelial cells is an important early event in the establishment of new IMI in dairy cows. A molecule referred to as *S. uberis* adhesion molecule (SUAM) that is involved in adherence to, internalization into, and persistence of *S. uberis* in bovine mammary epithelial cells was identified and partially characterized. Antibodies against SUAM significantly reduced adherence to and internalization of *S. uberis* into bovine mammary epithelial cells. It has been proposed that SUAM is an important virulence factor of *S. uberis* and may be a promising antigen that could be used to control *S. uberis* mastitis, particularly during the nonlactating and periparturient periods.

Characteristics of *Streptococcus dysgalactiae* ssp. *dysgalactiae*

Information regarding the epidemiology of *S. dysgalactiae* ssp. *dysgalactiae* in dairy herds is scarce. *Streptococcus dysgalactiae* ssp. *dysgalactiae* is a rather unique mastitis pathogen because it behaves as a contagious as well as an environmental pathogen and has characteristics of both groups. *Streptococcus dysgalactiae* ssp. *dysgalactiae* has been isolated from infected mammary glands and teat injuries and is transmitted primarily during milking. However, detection of several potential extramammary reservoirs such as cattle tonsils, mouth, and vagina, and occurrence of *S. dysgalactiae* ssp. *dysgalactiae* IMI during the nonlactating period in herds with no previous history of *S. dysgalactiae* ssp. *dysgalactiae* IMI suggest that the organism also behaves as an environmental pathogen. In addition, *S. dysgalactiae* ssp. *dysgalactiae* is involved in the multietiological clinical entity referred to as summer mastitis, which affects dry cows and heifers during summer months, primarily in northern Europe and Japan. This organism, among other causative agents, has been isolated from the common cattle fly *Hydrotaea irritans*, which appears to play a significant role in the establishment and maintenance of bacterial contamination of teats of healthy cattle. Considering the etiology of summer mastitis, *S. dysgalactiae* ssp. *dysgalactiae* is considered to be the first bacterial species to colonize the bovine teat, subsequently providing a favorable environment for colonization by *Arcanobacterium pyogenes* and anaerobic bacteria such as *Peptostreptococcus indolicus* and *Fusobacterium necrophorum.*

Gram-Negative Mastitis Pathogens

Many IMIs caused by Gram-negative bacteria develop into clinical mastitis. It is estimated that 30–40% of all clinical cases of mastitis are due to Gram-negative mastitis pathogens. Recent studies have shown an increase in the importance of *E. coli* IMIs among all mastitis pathogens, and these studies indicated that *E. coli* has become a major problem in many well-managed dairy farms resulting in high milk loss and frequent culling, and/or death of infected cows.

Gram-negative bacteria naturally inhabit the soil and the intestinal tract of animals; they can accumulate and multiply in manure, as well as in contaminated water and bedding. Other environmental pathogens are common in soil, plant material, and bedding, and some can be isolated from various body sites of animals. On a practical basis, environmental mastitis pathogens cannot be eliminated from the cow's environment, but it is possible to keep pathogen numbers low by maintaining a clean and dry environment. This is a fundamentally important concept since the greater the bacterial load present in the cow's environment, the greater the risk of infection and mastitis. Gram-negative mastitis pathogens are isolated from approximately 1% of all infected quarters and the majority of these infections develop into clinical cases. The most common Gram-negative pathogens isolated are *Escherichia*, *Klebsiella*, *Pseudomonas*, *Serratia*, and *Enterobacter*. Of these, *E. coli*, *Klebsiella* species, and *Enterobacter* form a subgroup of Gram-negative organisms termed coliforms. Milk losses and mortality due to *Klebsiella* mastitis tend to be higher than those due to *E. coli* mastitis. IMIs caused by Gram-negative mastitis pathogens generally are of short duration; approximately 50% last less than 10 days and 70% last less than 30 days. However, chronic coliform infections of long duration caused by *E. coli* and *Klebsiella* species can occur. Recent studies suggest that strains of *E. coli* isolated from cows with chronic mastitis have adapted well to the mammary gland and have developed virulence mechanisms to evade local defense mechanisms.

Characteristics of *Escherichia coli*

Escherichia coli found within the soil, manure, and water in the surroundings of the animal represents the most common species of coliform isolated from clinical cases of mastitis. As with most environmental Gram-negative organisms, infection rates are greatest during the periparturient period and decrease throughout lactation. In general, these infections last less than 10 days and respond poorly to antibiotic therapy. These Gram-negative rods form small (3–5 mm) colonies that are gray, moist, and typically have a fecal odor. Fewer than 15% of colonies demonstrate hemolysis on blood agar plates. On MacConkey agar plates, *E. coli* forms pink to red, dry, flat colonies (2–4 mm) surrounded by a pink zone of precipitated bile salts. *Escherichia coli* also ferment lactose, produce acid slants and butts with gas production on triple sugar iron reactions, are motile, do not grow on Simmons citrate agar, and have negative cytochrome oxidase reactions.

Characteristics of *Klebsiella* Species

Klebsiella pneumoniae and *Klebsiella oxytoca* are important etiologic agents of clinical mastitis and can cause significant losses in milk production and mortality of affected cows. Antimicrobial therapy and vaccination are of limited value. A high prevalence of mastitis caused by *Klebsiella* organisms can occur when organic bedding materials, in particular sawdust, are used as this provides the primary reservoir for this species. This is a particular problem during summer months when higher ambient temperatures and humidity provide an ideal growth environment for these organisms. As with *E. coli*, these infections are most prominent during the periparturient period and respond poorly to antibiotic therapy. Unlike infections caused by *E. coli*, which are relatively short in duration, *Klebsiella* infections can persist for several weeks or months. These Gram-negative rods form small (3–5 mm) colonies that are gray, moist, often mucoid, and nonhemolytic on blood agar plates. On MacConkey agar plates, *Klebsiella* species form pink-yellow mucoid colonies. *Klebsiella* species also have negative cytochrome oxidase reactions, ferment lactose, produce acid slants and butts with gas production on triple sugar iron reaction, grow on Simmons citrate agar, and are nonmotile.

Characteristics of Other Gram-Negative Mastitis Pathogens

Other less commonly isolated Gram-negative mastitis pathogens include *Enterobacter*, *Serratia*, *Pseudomonas*, *Pasteurella*, and *Proteus* species. Overall, the etiology and prevention of IMI from these bacteria are similar to Gram-negative mastitis pathogens described above. However, some important characteristics that separate these strains are outlined below. *Serratia* species often cause chronic infections that last several lactations. More importantly, some strains of *Serratia* are resistant to germicides containing chlorhexidine gluconate. *Pseudomonas*, another bacterial species that causes chronic infections, often leads to culling of that animal from the herd. Often, the primary reservoir is contaminated water or milk hoses, as *Pseudomonas* species are resistant to certain sanitizers.

Prevention, Control, and Therapy

Control of environmental mastitis pathogens is best achieved by maintaining a clean, dry environment for lactating and nonlactating cows, and heifers. An achievable goal is to reduce clinical mastitis to less than 3% of milking cows per month. Any procedure that reduces the number of bacteria to which the teat end is exposed will likely be beneficial. Premilking and postmilking teat disinfection are recommended. Use of massive amounts of water in the milking parlor should be avoided. Wet milking of cows is likely to increase the incidence of environmental mastitis. Postmilking teat disinfection with effective products that have been evaluated following NMC protocols is highly recommended. In general, effective germicidal teat disinfectants provide some protection against environmental streptococci, but are uniformly less effective against Gram-negative mastitis pathogens. Barrier teat disinfectants are relatively new in the marketplace, and use of effective barrier teat disinfectants in herds with an environmental mastitis problem may be advantageous. Mastitis incidence increases with liner slips during milking, which lead to a series of events that generate droplets of contaminated milk from the claw to impact against the teat end. In the open phase of the liner, these bacteria-laden droplets are propelled through the teat canal, allowing bacteria to enter the mammary gland and initiate a new infection. By properly maintaining the milking system, overall udder health will be promoted and mastitis incidence reduced.

The environment is critical in the control of environmental mastitis. Cows housed in total confinement are at greater risk of environmental mastitis than pastured cows. Overcrowding of cows; poor ventilation; poorly maintained free stalls, alleyways, feeding areas, and exercise lots; access to farm ponds or muddy lots; and general lack of farm cleanliness and sanitation can all contribute to environmental mastitis problems. Types of bedding and bedding management are also extremely important. Bedding materials such as sawdust, shavings, peanut hulls, and straw typically contain low numbers of environmental bacteria when placed in stalls. However, these bedding materials quickly become contaminated with urine and feces, and can be a significant source of teat end exposure to environmental pathogens. Damp and humid conditions in association with high ambient temperatures are likely to exacerbate the problem. Thus, proper stall and bedding management should be an important management priority. Herd environments (total confinement housing and pastures) should be as clean and dry as possible. It is important for dairy producers to recognize that the environment of dry cows and pregnant heifers is just as important as that of lactating cows.

Enhancing host immune responses will also reduce overall disease incidence. Central to the development of an effective immune response is adequate nutrition. Lack of specific nutrients and insufficient energy can significantly impair the ability of the immune system to mount an effective immune response. A deficiency of two nutrients in particular, vitamin E and selenium, has been associated with an increased risk of mastitis. Vitamin E and selenium supplementation has been demonstrated to reduce the incidence of mastitis by reducing the time required for neutrophil migration from the blood to the mammary gland, as well as by increasing neutrophil function. Supplementation is necessary in regions such as the northeastern United States that have low selenium levels in the soil and hence crops. Supplementation of dry cows and heifers approaching calving that are exposed to increased bacterial loads and are undergoing increasing levels of stress is particularly important.

Another means of increasing disease resistance is immunization. In general, development of effective mastitis vaccines has been problematic due to the wide variation in organisms that cause mastitis. However, with respect to Gram-negative bacteria, a vaccine that reduces the severity of clinical coliform mastitis cases three- to fourfold in field studies when compared to unvaccinated controls has been developed. This vaccine was developed from the J5 mutant *E. coli* strain that lacked variable external antigens, but still maintained the core lipopolysaccharide that is highly conserved among Gram-negative bacteria. Experimental challenge of vaccinated cows reduced the clinical severity of infection, as well as the number of bacteria isolated from milk, compared with unvaccinated controls. As such, vaccination may constitute an important means of decreasing the severity of mastitis due to Gram-negative mastitis pathogens in a herd, but cannot be expected to provide any benefits under poor sanitary conditions.

Antibiotic dry cow therapy is also recommended. Dry cow therapy helps to control during the early dry period new infections caused by environmental streptococci. However, dry cow therapy with most antibiotics approved for use in nonlactating cows has little effectiveness in controlling Gram-negative mastitis pathogens and is not effective in preventing new infections that occur near calving. If a coliform infection does occur, antibiotic therapy effectively treats 10–20% of the cases. This is not unexpected as therapy is initiated following observation of clinical signs, which occur after peak bacterial numbers have been reached and the infection is in the process of curing itself. Ceftiofur hydrochloride was recently introduced in the United States for intramammary use and is indicated for the treatment of clinical mastitis caused by *E. coli* in lactating dairy cows. Therapeutic regimens should also include fluid, an anti-inflammatory agent, and calcium to ameliorate clinical symptoms, as well as frequent milking to remove bacteria and toxins.

Conclusion

Environmental mastitis pathogens have become increasingly important during the last decade, particularly in well-managed dairy herds applying measures to control contagious mastitis pathogens. Among the environmental mastitis pathogens, *S. uberis*, *S. dysgalactiae* ssp. *dysgalactiae*, *E. coli*, and *Klebsiella* species are isolated most frequently. The epidemiology of environmental mastitis differs markedly from mastitis caused by contagious mastitis pathogens. Methods of mastitis prevention and control that are effective against contagious pathogens are less effective against environmental pathogens. As current methods of mastitis control receive greater acceptance, more commercial dairy herds will likely experience increased problems with environmental mastitis. Control of environmental mastitis is best approached by preventing new infection through pre- and postmilking teat disinfection, antibiotic therapy at drying off, vaccination, adequate nutrition, and maintenance of a clean, dry environment for lactating cows, dry cows, and pregnant heifers. Further studies are needed to identify procedures that are more effective against environmental pathogens if additional mastitis control is to be achieved.

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See also: **Mastitis Pathogens**: Contagious Pathogens. **Mastitis Therapy and Control**: Automated Online Detection of Abnormal milk; Management Control Options; Role of Milking Machines in Control of Mastitis. **Milking and Handling of Raw Milk**: Milking Hygiene. **Pathogens in Milk**: *Escherichia Coli*. **Milk Proteins**: Lactoferrin; Immunoglobulins.

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Relevant Websites

http://www.nmconline.org – National Mastitis Council Website.

MASTITIS THERAPY AND CONTROL

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Automated Online Detection of Abnormal Milk
Management Control Options
Medical Therapy Options
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Automated Online Detection of Abnormal Milk

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Introduction

There are many aspects to milk quality. Mastitis is associated with two of the milk quality aspects that are used in most dairy-producing countries: somatic cell count (SCC) and, if mastitis is clinical, visibly abnormal milk (in the remaining part of this article referred to as abnormal milk). In most dairy systems, it is assumed that the farmer, informed by the official organizations in his country, has the responsibility to deliver milk of sufficient quality. In order to deliver milk with a low SCC, the focus should be on adequate detection and prevention of mastitis. Efficient detection of clinical mastitis is therefore important. A well-established method to detect clinical mastitis is to strip before milking and check the foremilk for abnormalities. Discarding of abnormal milk is part of the EU Milk Hygiene Directive (EC/92/46). Milk from diseased cows or milk that is visually abnormal should not be delivered. Discarding of abnormal milk is also mandatory in the United States, according to the Grade ‘A’ Pasteurized Milk Ordinance. Checking of foremilk is thus important to detect clinical mastitis, depending on the regulations of a country, to meet regulations (*see* **Milk Quality and Udder Health**: Test Methods and Standards; Effect on Processing Characteristics. **Milking and Handling of Raw Milk**: Milking Hygiene).

From the mid-1980s, work has been carried out in order to automate the detection of mastitis by means of sensors. When detection of clinical mastitis is carried out automatically, the task of the milker becomes easier and the capacity of milking parlors can be increased. Although sensors for detection of mastitis became commercially available in the beginning of the 1990s, they were never applied on a large scale. Because of the fact that with automatic milking no milker is present at the time of milking, the need for sensors to detect clinical mastitis and abnormal milk was high when automatic milking systems were commercially introduced. Moreover, because the number of milking clusters in an automatic milking system is much lower than in a comparable milking parlor, the cost of application of sensors is also lower in an automatic milking system. Therefore, interest in the application of sensors to detect mastitis and abnormal milk is gaining importance (*see* **Milking Machines**: Robotic Milking).

This article describes the current status of sensors to detect abnormal milk and mastitis (subclinical as well as clinical).

Demands for Automatic Detection of Mastitis and Abnormal Milk

Test Requirements

Sensors for detection of mastitis and/or abnormal milk can be seen as diagnostic tests, which can be characterized by epidemiological parameters. The two most important parameters are sensitivity and specificity. The sensitivity refers to the probability that a milking with abnormal milk (or a cow with clinical mastitis) will be classified as such (positive test result). The specificity refers to the probability that a milking without abnormalities (or a cow without clinical mastitis) will be classified as normal (negative test result). Sensitivity and specificity are interdependent. If the threshold of a test is increased, the number of positive outcomes and thus the sensitivity will decrease. On the other hand, the specificity will increase. Therefore, thresholds have to be set in such a way that the performance of a sensor in terms of sensitivity and specificity is optimized. However, for practical evaluation, the predictive values (positive and negative) of a test are more important. The predictive value depends on sensitivity, specificity, and prevalence of a disease. When discussing methods to detect clinical mastitis and abnormal milk, the test characteristics described above should be taken into account. Moreover, the goal for which the test is used is important in the evaluation of the test characteristics. Therefore, the usefulness of tests may differ for the detection of clinical mastitis to treat the cow and the detection of abnormal milk to automatically separate this milk. The prevalence of clinical mastitis, subclinical mastitis, and abnormal milk is very low. The prevalence of clinical mastitis and abnormal milk is approximately 0.04% (4 cases per 10 000 milkings). The prevalence of subclinical mastitis is much higher: 100–5000 cases per 10 000 milkings. This low prevalence, especially for clinical mastitis, will have effects on the interpretation of sensor data. In the following three sections, the demands for tests to detect clinical mastitis, subclinical mastitis, and abnormal milk are described.

Clinical Mastitis

The primary goal of online detection of clinical mastitis is to be able to cure the diseased cow. After an alert signal, the herdsman will check the cow first to confirm the mastitis before deciding on treatment. The advantage of using a sensor system to detect clinical mastitis is the management by exception principle. Only those cows requiring attention will get it. Because mastitis case will be confirmed first, a reasonable amount of false-positive test results will not be a problem. The only costs that are incurred are the labor costs of the herdsman performing checks. Alerts can be given directly in the milking parlor, decreasing the time that is needed to milk one cow. When the person applying treatments is not in the milking parlor (as is the case with automatic milking), the alerts of the sensor system may be placed on an alert list. The herdsman checks this list regularly and checks the cows on it. It is important that as many cows with clinical mastitis as possible (preferably all) be identified, requiring a high sensitivity. At least cows with severe clinical mastitis (grave systemic and local symptoms) must be detected (*see* **Mastitis Therapy and Control**: Management Control Options).

Subclinical Mastitis

Treatment of subclinical mastitis during lactation is not a common practice. However, in controlled experiments, the efficacy of treatment improves when cows with an intramammary infection are treated before the occurrence of signs of clinical mastitis (preclinical mastitis). When treating cows based on alerts of a sensor system before clinical signs appear, a very low proportion of false-positive test results can be allowed. Treating a cow when there is really no need for treatment is a waste of antibiotics, milk, and labor. On the other hand, there are no very high demands for sensitivity. A high sensitivity is useful, but will not be necessary. In order to define demands for a test for subclinical mastitis, it is important to know what the actions are to be undertaken when a case of subclinical mastitis is found (*see* **Mastitis Therapy and Control**: Management Control Options).

Abnormal Milk

Almost all abnormal milk is a result of clinical mastitis. Abnormal milk might also be caused, for instance, by the presence of blood in milk. While the goal of detection of clinical mastitis is to be able to cure a diseased cow, the goal of detection of abnormal milk is to discard this milk, as is required by regulations in most milk-producing countries. If the task of discarding abnormal milk has to be carried out automatically as is the case with an automatic milking system, it is not sufficient to use alert lists. A signal of abnormal milk must be followed directly by automatic separation of the milk. The management information system must detect as much milkings with abnormal milk as possible (preferably all), requiring a high sensitivity. Since each milking that is unnecessarily separated costs money, the number of false positives should be low.

Sensors to Detect Mastitis and Abnormal Milk

Because of the pathophysiological changes in the udder, intramammary infections lead to major alterations in the composition of milk. Clinical mastitis is as per definition an intramammary infection where visible changes in the appearance of the milk, the udder, or both occur as an effect of the inflammation process. Among others, a rapid influx of polymorphonuclear leukocytes leads to an increase of the SCC. The SCC is the basis of many milk quality programs worldwide. Besides the influx of polymorphonuclear leukocytes, there are other changes in milk composition. Sensors have been developed that can detect some of these changes online. This article presents the current knowledge on sensors that have been developed for online detection of mastitis, abnormal milk, or both.

Electrical Conductivity

General

Electrical conductivity (EC) is a measure of the resistance of a particular material to an electric current. In milk, ions present are the main components conducting electricity. Active and passive transport systems in the secretory cells of the mammary gland keep the sodium to potassium ratio in the milk at approximately 1:3, whereas it is 30:1 in the extracellular fluid or blood. The chloride concentration in milk is much lower than in blood. The mammary ducts are impermeable to ions (**Figure 1** (**a**)). Mastitis leads to a change in blood capillary permeability, destruction of tight junctions, and destruction of the active ion-pumping systems. As a result, the ion concentrations in milk change. Since milk is iso-osmotic with blood, the secretory cells of the mammary gland will stabilize the osmotic pressure leading to a change in EC (**Figure 1** (**b**)) (*see* **Mammary Gland**: Anatomy).

Because the principle of measuring EC is relatively simple, sensors for measuring EC are commercially available for a number of years. Basically, two types of systems are available: (1) systems that measure the conductivity of the whole milk, located for instance in the electronic milk meter, and (2) systems measuring the conductivity per udder quarter, located in the claw of the milking cluster (traditional milking systems) or in the long milk tube (automatic milking systems). Since mastitis is an event that occurs on udder quarter, EC measurements at the quarter level give the possibility to compare udder quarters, thus increasing the test characteristics.

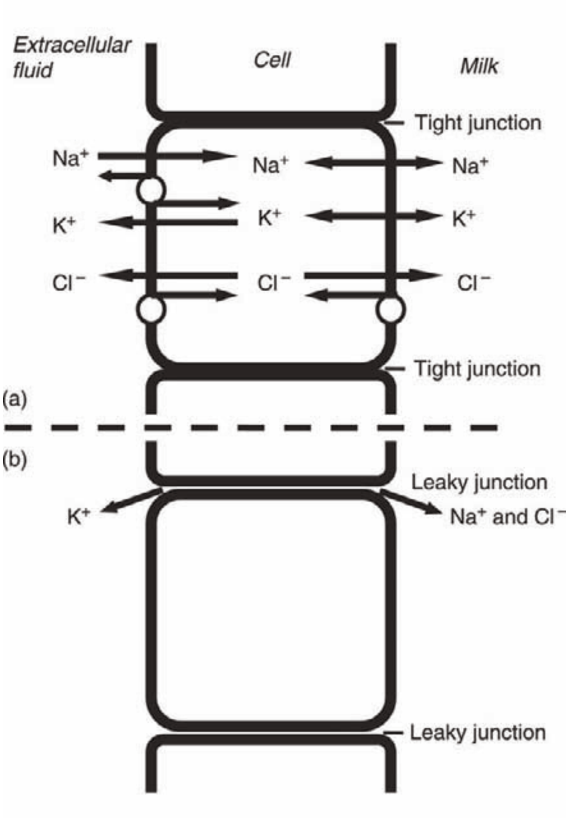

Figure 1 Schematic overview of the pathways of ion transport in the mammary secretory epithelium. (a) Situation in a normal functioning mammary gland and (b) some of the changes caused, among others, by mastitis. Based on Linzell JL and Peaker M (1971) Mechanism of milk secretion. *Physiological Reviews* 51: 564–597.

Many studies have been carried out to evaluate the use of EC to detect mastitis. **Table 1** gives results from two meta-analyses. It can be noticed that there is a large variation in results between the studies. Moreover, the test characteristics seem to differ between the reference methods used. The best results in sensitivity as well as specificity were found when bacteriology was used as a reference.

Clinical mastitis

Although some laboratory studies found a sensitivity of 100% when using EC to detect clinical mastitis, average test results given in **Table 1** are not very good. Especially the sensitivity is too low. The average sensitivity for clinical mastitis signs was 68%, varying from 34 to 95%. However, the studies summarized in **Table 1** were rather diverse, and the algorithms used could be improved (see further in this article for more on this subject).

Subclinical mastitis

The performance of EC to detect subclinical mastitis (defined as increased SCC, positive bacteriology, or both) varies between studies. Although subclinical mastitis can be detected, the specificity is not more than 95%. With the relatively low prevalence of subclinical mastitis, in practice this will lead to an unacceptable high number of false-positive test results. To make treatment (with antibiotics or in another way) based upon EC efficient, the number of false-positive test results should be lowered. For the same reason, early treatment of clinical mastitis (before clinical signs occur) based upon EC is not efficient. Small-scale application of new algorithms (fuzzy logic) showed a large reduction of false-positive test results. When this type of algorithms give the same performance online, the number of false-positive test results might be reduced to such an extent that treatment of early mastitis or subclinical mastitis based upon EC could become an efficient option.

Abnormal milk

There is not much work done on the use of EC to detect abnormal milk. For instance, detection of abnormal milk, using commercially available EC sensors and software on six dairy farms, has been reported with an average sensitivity of 39%, varying from 13 to 50%. When decreasing

Table 1 Results from two meta-analyses on the test characteristics of electrical conductivity in mastitis detection

Reference	*No. of studies*	*Median*	*Mean*	*Min.*	*Max.*	*Source*
Somatic cell count	23					Nielen (1994)
Sensitivity		57		22	100	
Specificity		94		0	99	
Bacteriology	41					Nielen (1994)
Sensitivity		75		16	100	
Specificity		95		67	100	
Somatic cell count and bacteriology	12					Nielen (1994)
Sensitivity		60		6	100	
Specificity		91		1	100	
Clinical mastitis signs	17					Hamann and Zecconi (1998)
Sensitivity			68	34	95	
Specificity			81	71	100	

Source: Nielen M (1994) Detection of Bovine Mastitis Based on Milking Parlour Data, 165pp. PhD Thesis, Utrecht University, Utrecht, The Netherlands; Hamann J and Zecconi A (1998) Evaluation of the electrical conductivity of milk as a mastitis indicator. *Bulletin of the International Dairy Federation No. 334*. Brussels, Belgium: IDF.

the detection threshold, the sensitivity would improve greatly, but the number of false-positive test results would increase to unacceptable levels. Although this study was rather limited (138 cows were followed for 48 h), the results are not unexpected. After all, there is a large correlation between abnormal milk and clinical mastitis, and the results for detection of abnormal milk will probably not deviate very much from the results presented above. With those results, EC is not sufficient to withhold abnormal milk automatically. Too many milkings with abnormal milk will still be delivered and too much milk will be discarded unnecessarily leading to economic losses. Improvement of sensors and algorithms might make automatic separation of abnormal milk possible in the future.

L-Lactate dehydrogenase

L-Lactate dehydrogenase (LDH) is an enzyme found in the cytoplasm of all cells and tissues in the body and it plays an important role in the glycolytic pathway. LDH is a responsive indicator of mastitis. A biosensor, based on LDH and using dry-stick technology, is commercially available. A model, using LDH, milk yield, EC, and cow-specific information, has been developed. This model appears to be robust and gave a sensitivity and specificity of 82 and 99%, respectively.

Color

A direct measure of the physical characteristics of abnormal milk (mostly due to clinical mastitis) will most likely offer better detection results than measurement of an indirect indicator of mastitis or abnormal milk. One of the visible aspects of milk is its color. Recently, a sensor for online color measurement is on the market.

The principle of the sensor is based on the reflection of light generated by a light-emitting diode (LED). The whiter the milk, the more the light reflected. Three different wavelengths of light are measured by the sensor: red, green, and blue.

In a first study under laboratory circumstances, using homogenized quarter milk samples from eight cows with clinical mastitis, the potential to detect mastitis from color measurements was estimated. The milk samples of the suspected quarters of all eight cows with clinical mastitis showed lower color values than homogenized milk.

In a detailed study on the predictive potential of EC and color measurements, it became clear that most information to distinguish udder quarters with abnormal milk and clinical mastitis from other udder quarters could be found in EC measurements. The potential of color measurements did add but not very much. This means that color sensors should always be used in combination with other sensors.

Somatic Cell Count

The best-known and most widely applied parameter related to mastitis and used for its detection is the SCC. Rapid reliable measurement of SCC is carried out routinely in laboratories, and can be used to monitor udder health. Therefore, SCC is used as an important tool for the control of (subclinical) mastitis. There are a few results of online measurement of SCC by means of near-infrared (NIR) technology. The results of these limited experiments are promising for future application of NIR.

Sensors that measure SCC online are commercially available on automatic milking systems. One of these sensors utilizes the gel formation process of the Californian mastitis test (**Figure 2**). The potential value of this sensor has been studied at the cow level in

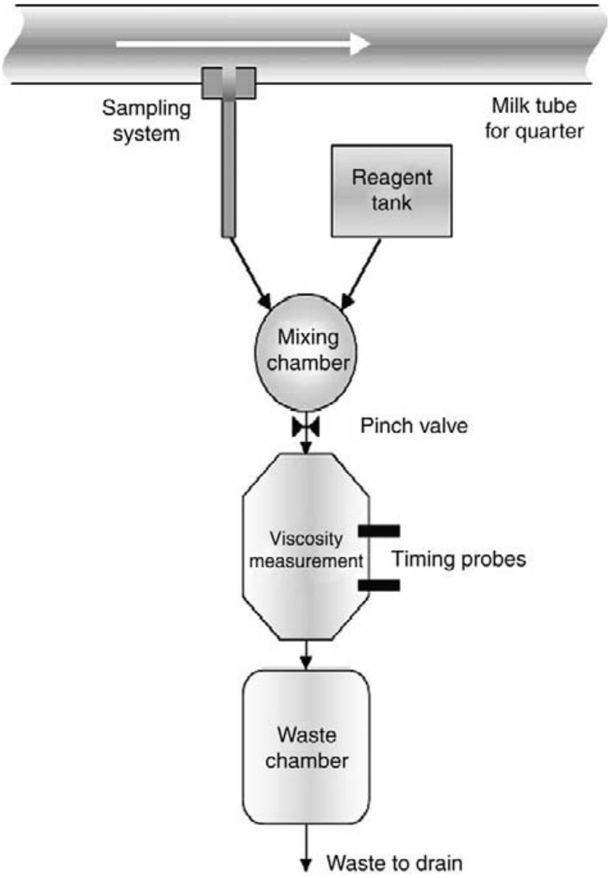

Figure 2 Representation of online somatic cell count sensor based on gel formation as utilized by the Californian mastitis test.

combination with quarter-based EC. With thresholds set in such a way that the sensitivity of the test was 80%, online SCC measurement gave similar results as EC (using standard algorithms). The test based on online SCC measurement gave a probability of 13% that a positive sensor outcome was a case of clinical mastitis. When combining EC and SCC measurements, the probability that a positive sensor outcome was a case of clinical mastitis increased to 33%. These results suggested that estimating SCC from a composite cow milking contributes to an automatic sensing system for the detection of clinical mastitis by reducing the number of false-positive attentions while keeping the sensitivity of detection at a reasonable level. Recent, not yet published, data show that measuring SCC at the quarter level gives better detection performance than measuring SCC at the cow level (*see* California Mastitis Test; Somatic Cell Count).

Other Methods for the Detection of Abnormal Milk and Mastitis

Mastitis has effects on the body temperature of the cow, and thus on the milk, activity of the cow, and milk yield. Equipment to measure these parameters online in milk is commercially available. However, these parameters are not distinctive enough for detection of mastitis or abnormal milk. More recent work describes the use of thermal cameras to measure the temperature of the udder. Temperature changes of 1–1.5 °C can be detected this way. However, most of the clinical mastitis cases do not affect the general condition of the cow.

Besides EC, there are many other changes in milk composition, caused by the disease-combating response of the cow, the reduced synthetic ability of the mammary gland, or tissue damage and blood capillary permeability. Changes in anion and cation concentration in milk are the basis for EC measurement. But also changes in the levels of enzymes such as catalase and *N*-acetyl-β-D-glucosaminidase (NAG-ase) are of interest.

Recently, a large number of new possibilities to detect mastitis online are described. Among these is a chemical array-based sensor that was able to detect chloride, potassium, and sodium ions released during mastitis. Another development is the measurement of volatile metabolites produced by mastitis pathogens in milk. Biosensors have also been developed to detect NAG-ase, haptoglobin, and hemoglobin in milk. Besides these recently described developments, outside the dairy industry biosensors are developed for a wide range of compounds and applications. The commercial success of some of these applications indicates that this is a viable and robust technology that might be very useful for the dairy industry, and in the future new developments are being expected.

Many sensors for detection of mastitis and abnormal milk measure an indirect indicator of mastitis. Any change in milk composition, caused by mastitis, either clinical or subclinical, can be used as the basis for a sensor. However, especially for the discarding of abnormal milk, a direct indicator might be useful. Abnormal milk, in the sense that it is meant in European and American legislation, is visibly abnormal. Visible abnormality of the milk occurs also in most clinical mastitis cases. Color is one part of visible abnormalities. A method to detect deviations in milk color has been described above. The other part is the appearance of milk emulsion: clots and flocks in the milk are abnormal and this is a sign of clinical mastitis. Patents have been filed for direct clot sensors but so far they have not been implemented in a commercial milking system (*see* Analysis of Milk and Dairy Products; **Analytical Methods**: Biosensors; Somatic Cells in Milk; Spectrophotometric Techniques).

Algorithms

Sensors, how advanced they might be, deliver data. Many online sensors deliver a large amount of data. There are

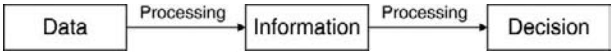

Figure 3 The path from data to decision.

many measurements per milking, sometimes per udder quarter and during many milkings. These data in themselves are not informative. These data should be processed to generate information (**Figure 3**). An example of information is the probability that this cow has mastitis during this milking (alerts). Many algorithms have been proposed, developed, and described to perform this task. A very straightforward example of an algorithm is the use of a threshold: if the measurement is above that threshold, an alert is generated. Because of the low prevalence of the event that has to be detected (clinical mastitis, abnormal milk, or subclinical mastitis), there are high demands on the processing of sensor data. A good algorithm is essential to optimize the online sensor data in an interpretable value. Algorithms can make a huge difference in the performance of a sensor system. Described algorithms include the use of thresholds, moving averages, dynamic linear models (Kalman filters), fuzzy logic, and data mining. The information should finally be processed into action. Many times this latter step is carried out by the herdsman, although there are possibilities to support this with automation (automated decision support).

Because mastitis is associated with many changes in the cow and milk, a combination of more than one sensor has been proven to be useful. For instance, the combination of EC with LDH or SCC measurements has been shown to give better performance than the use of EC, LDH, or SCC alone. A final suggestion is the combination of sensor outcomes with cow-specific information such as mastitis history, age of the cow, and lactation stage. The usefulness of this latter combination is not yet proven.

To evaluate the performance of a mastitis detection algorithm, the alerts created by the algorithm used and sensor output must be compared to a gold standard describing the animal status to be detected (clinical mastitis, subclinical mastitis, or abnormal milk). When comparing the alert with the observation of the gold standard, the time between the alert and the observation of the gold standard is important. In published studies on performance of sensors to detect mastitis, time windows between 0 and 28 days are found. It is obvious that the performance of the sensors improves with a larger time window. However, it should be noted that the usefulness of alerts decreases with increasing time windows.

Besides the development of algorithms to evaluate the outcome of a sensor, for a practical implementation of sensors, it is also important that calibration schemes are made and systems are developed that detect malfunctioning sensors. Maintenance is often underestimated.

Conclusion

Automated online detection of mastitis and abnormal milk is an important subject in the dairy industry, especially because of the introduction of automatic milking systems. Because of the demands for performance, automatic detection systems should be evaluated separately for clinical mastitis, subclinical mastitis, and abnormal milk. EC is the most applied sensor to detect mastitis. The use of EC might be sufficient to produce alert lists for clinical mastitis. The herdsman then has to interpret the results and check cows manually. The use of EC is insufficient to detect and separate abnormal milk automatically. New sensors, measuring the color of milk, SCC, or LDH, are commercially available. These sensors can be especially useful when combining them with EC. The increasing complexity of sensors requires complex algorithms to optimize the utilization of the data measured by the sensors.

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Biosensors; Mass Spectrometric Methods. **Mammary Gland**: Anatomy. **Mastitis Therapy and Control**: Management Control Options. **Milking and Handling of Raw Milk**: Milking Hygiene. **Milking Machines**: Robotic Milking. **Milk Quality and Udder Health**: Effect on Processing Characteristics; Test Methods and Standards; **Policy Schemes and Trade in Dairy Products:** Agricultural Policy Schemes: European Union's Common Agricultural Policy; Agricultural Policy Schemes: Other Systems; Agricultural Policy Schemes: United States' Agricultural System.

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Management Control Options

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Introduction

Even though therapy with antimicrobials is beneficial in curing existing infections during lactation and at drying off, the goal of any mastitis control program is to prevent the occurrence of new infections. The dairyman has several management control options that have proven useful in reducing the rate of new cases of mastitis; these include genetic selection for disease resistance, proper nutrition, alleviation of stress, and promoting proper hygiene.

Genetic Selection

Breeding for mastitis resistance is difficult because this characteristic is regulated by genes at numerous loci and is greatly influenced by environmental factors. Likewise, although the resistance is based on the status of the immune system, which is controlled by the major histocompatibility complex (MHC), other genes outside the MHC may also affect disease resistance.

Breeding programs in dairy cows have resulted in great genetic improvement for milk yield but have led to increased susceptibility to mastitis owing to a negative correlation between yield and disease resistance. Assuming a genetic correlation between yield and mastitis of 0.30, mastitis incidence increases ∼0.4% per year as a result of the current trends for milk yield. Although this yearly rate is low, it becomes quite high over the long term; thus, mastitis resistance should be considered in breeding programs.

Somatic cell count (SCC) data are used as an indirect measure of this disease because intramammary infection is associated with an increase in SCC. The genetic correlation between SCC and mastitis is 60–80%; thus, selection for cows with low SCC reduces the incidence of this disease. Somatic cell score (SCS) is a logarithmic transformation of SCC and has been in use as a trait for selection. Disease traits have relatively low heritability; however, reasonable estimates of transmitting ability can be obtained for artificial insemination (AI) bull sires. Based on estimates of genetic variation, the incidence of mastitis in daughters of the worst 5% of bulls would range 10–15% higher than that in daughters of the best 5% of bulls. Thus, consideration should be given to removing bulls from breeding programs when their daughters are predisposed to elevated SCS.

Anatomical mechanisms also play a role in defense against mastitis. For example, pendulous udders are susceptible to inflammation, and selecting against this trait, that is, selecting AI bulls that sire heifers with shallow, well-attached udders, reduces the incidence of the disease. Similarly, cows with short teat canal length and wide canal diameter have higher incidences of mastitis than those with a longer teat canal length and narrower canal diameter. Estimates of heritability for continuous markers such as udder and teat traits are high (**Table 1**). The genetic interrelationship between these traits and mastitis is given in **Table 2**, which indicates that the coefficients of genetic correlation are negative between teat tip to floor distance for all mastitis parameters, suggesting that the shorter the distance (i.e., pendulous udder), the greater the chances for mastitis.

The bovine lymphocyte antigen (BoLA) genes may be suitable markers to include as selection criteria, and associations between specific BoLA alleles and mastitis have been reported. One study reported an association between a bull's breeding value on the progeny test for disease resistance and BoLA Class II alleles, for example, the DQ^{1A} haplotype, which is associated with increased susceptibility to clinical mastitis. Also, cows with BoLA-w16 are susceptible to mastitis, whereas cows with BoLA-w2 are resistant to this disease. In one study, it was concluded that the presence of the BoLA Class I allele CA42 increased the susceptibility of cows to *Staphylococcus aureus* mastitis; however, heritability estimates of susceptibility to *Staph. aureus* infection were low.

Biochemical markers that may be used to predict susceptibility to mastitis are available. The M blood group system may be closely linked to the BoLA system, and its presence is associated with an increased incidence of mastitis. Cows carrying the M-factor (M/M and M/-) exhibit higher frequencies of mastitis than exhibited by cows lacking this factor. Likewise, M-positive cows have higher SCC than that observed in M-negative cows.

Nutrition

Diet plays a role in resistance to udder infection because certain micronutrients affect mammary defense mechanisms such as leukocyte function, and deficiencies in these micronutrients are related to an increase in incidence, duration, and severity of mastitis. Leukocytes are a

Table 1 Estimates of heritability for continuous markers

Teat tip to floor distance	0.40
Teat length	0.50
Distance between front teats	0.40
Milk flow rate	0.23

Adapted from Jensen NE, *et al*. (1985) *Kieler Milchwirtschaftliche Forschungsberichte* 37: 506–509.

Table 3 Levels of blood/selenium, glutathione peroxidase, and serum vitamin E of cows from low- and high-SCC herds

Item	*Low SCC*	*High SCC*
Blood Se (μg ml^{-1})	0.133	0.074[a]
Blood GSH-Px (mU mg^{-1} of Hb)	35.6	20.2[a]
Serum vitamin E (μg (100 ml)$^{-1}$)	484.6	421.3

Adapted from Erskine RJ *et al*. (1987) *Journal of the American Veterinary Medical Association* 190: 1417–1421.

[a] Significantly different ($P < 0.01$).

major defense mechanism of the bovine mammary gland, and nutritional effects on leukocyte function can have a profound effect on mammary immunity. Antioxidants have been implicated as critical to the promotion of efficient leukocytes. Following oxidation-dependent bacterial killing, oxygen radicals enter the cytosol, resulting in oxidative damage to the host cell. In response, an array of antioxidant defenses derived from micronutrients protects the host cells. Cytosolic enzymes such as selenium (Se)-dependent glutathione peroxidase (GSH-Px) and copper (Cu)- and zinc (Zn)-dependent superoxide dismutases protect leukocytes from oxidation by degrading the potential substrates for oxygen radical production. Vitamins E and C, and β-carotene also reduce oxygen radicals, augmenting the cytosolic enzyme systems.

The Cu plasma protein, ceruloplasmin, is an acute-phase protein, and cows with clinical mastitis exhibit elevated concentrations of plasma ceruloplasmin as compared with nonmastitic cows. Ceruloplasmin reduces *in vitro* adhesion of activated neutrophils to endothelial cells and scavenges extracellular superoxide as well. Neutrophils from Cu-deficient animals exhibit reduced ability to kill *Staph. aureus.*

Vitamin E and Se supplementation of dairy cattle diets enhances the ability of blood neutrophils to kill ingested *Staph. aureus* and *Escherichia coli.* Whole blood concentrations of vitamin E and Se, and GSH-Px activity tend to be higher in low-SCC herds than in high-herds (**Table 3**) and are associated with reduced incidence of clinical mastitis. In the udder, peak bacterial concentrations in milk and duration of infection were found to be higher in Se-deficient than in Se-supplemented heifers. In addition, milk loss resulting from coliform infection was significantly higher in Se-deficient than in Se-supplemented heifers. Dietary supplementation with vitamin E (50–100 ppm) and Se (0.3 ppm) 60 days prepartum and throughout lactation in addition to an injection of 50 mg Se 3 weeks prepartum reduced staphylococcal and coliform infections at calving by 42.2%; duration of infection by organisms other than *Corynebacterium bovis* was reduced by 40–50%. Clinical mastitis was reduced in early lactation (57.2%) as well as throughout lactation (32.1%), and mean SCC was lower.

Vitamin A is a colorless, fat-soluble, long-chain unsaturated alcohol (retinol) involved in maintaining a functional epithelium (which provides a physical barrier to the entrance of pathogens) and in enhancement of cellular and humoral immunity. β-Carotene (provitamin A) also has been shown to enhance immune function and disease resistance. Results of several studies suggest that the highest incidence of mastitis coincides with a period of poor vitamin A and β-carotene status in dairy cows, and that vitamin A and/or β-carotene play a role in regulating host defense system during periods of increased disease susceptibility. In one study, β-carotene concentrations in healthy cows (SCC $<100 \times 10^3$ cells ml^{-1}) averaged 6.1 μg ml^{-1}, whereas the values in mastitic cows (SCC $>500 \times 10^3$ cells ml^{-1}) averaged 3.66 μg ml^{-1}. Another experiment showed a lower incidence of intramammary infection in cows supplemented with a low level of vitamin A and β-carotene (27% incidence), when compared with cows supplemented with a higher level of vitamin A (50% incidence) alone. Exogenous β-carotene stimulates *in vitro* phagocytosis in blood neutrophils and stimulates killing of *Staph. aureus*. However, phagocytosis or killing of *Staph. aureus* does not differ between phagocytes collected from cows fed adequate vitamin A (53 000 IU day^{-1}), supplemental

Table 2 Genetic correlations between mastitis diagnosis and continuous markers

	Teat tip to floor distance	*Teat length*	*Distance between front teats*	*Milk flow rate*
Clinical mastitis	−0.49	0.52	0.33	0.25
Infectious mastitis	−0.89	0.85	−0.15	0.09
Aseptic mastitis	−0.24	0.71	0.42	0.38
Logarithm of cell number	−0.72	0.45	0.39	0.38

Adapted from Jensen NE, *et al*. (1985) *Kieler Milchwirtschaftliche Forschungsberichte* 37: 506–509.

vitamin A (213 000 IU day^{-1}), or a combination of vitamin A (53 000 IU day^{-1}) and β-carotene (400 mg day^{-1}).

Zinc deficiency leads to increased susceptibility to infections in dairy cattle, and use of organic Zn complexes in the diet has been found to decrease SCC. Zinc is required for the maintenance of a healthy skin and is instrumental in the synthesis of the keratin plug that provides a protective barrier at the teat end against mastitis-causing organisms. Zinc methionine is typically included in the diet to provide 180 mg of Zn and 360 mg methionine, so as to yield 20 ppm of Zn in the diet. The amount of Zn methionine is doubled temporarily to provide 360 mg Zn and 720 mg methionine in herds experiencing elevated SCC. In one study, SCC decreased from 560×10^3 to 282×10^3 ml^{-1}, and in another study, SCC decreased from 242×10^3 to 115×10^3 ml^{-1} owing to a twofold increase in the amount of daily dietary Zn.

Research indicates that supplemented chromium (Cr) may also have immunostimulating properties and some production-enhancing abilities. Dairy cows fed diets supplemented with chelated Cr (0.5 ppm day^{-1}) from 6 weeks prior to calving until 16 weeks postcalving showed significantly higher antibody responses to a number of antigens and a higher lymphocyte proliferation upon mitogen stimulation compared with nonsupplemented controls. In addition, Cr supplementation was associated with increased milk yield, particularly among primiparous cows.

Studies with the iron (Fe) chelator deferoxamine indicate that Fe binding may have a possible benefit in reducing tissue damage resulting from ischemic events in the mammary gland, but the effect of Fe chelator therapy on the severity of acute mastitis remains speculative.

Stress

The dairy industry imposes severe stress on cows: animals calve once every year, are pregnant and lactating for 7 months, and are machine milked 2 or 3 times daily for 305 days. As dairymen demand more from their animals by increasing milk yield and improving efficiency, greater stress is placed upon the productive capacity of the bovine. Hot and humid environmental conditions along with solar radiation, animal crowding, insect pests, and poor ventilation add to this stress and are associated with a reduction in milk production and increased mastitis.

As the environmental temperature and relative humidity rise, heat loss from the cow via radiation, conduction, and convection decreases. Thus, body temperature rises and other mechanisms of heat loss, for example, decreased feed intake, increased sweating and respiration, and behavioral changes become observable in attempts to maintain thermoneutrality. For instance, a cow may seek shade to control radiation, move into the wind to control convection, or lie down in a cold wet spot to control conduction. These mechanisms are necessary to maintain homeostasis but negatively impact feed intake, milk yield, and mastitis resistance.

Thermal stress significantly affects milk yield and indirectly leads to an elevation in mastitis and SCC during the summer months. Mean SCC for the hot summer months of June to August increases dramatically owing to the temperature–humidity index, and there is a carryover effect into the cooler months of September, October, and November.

This seasonal increase is likely owing to differences in exposure of teat ends to bacteria during hot and humid conditions, which favor bacterial growth and teat end contamination. However, herd SCC increases do not necessarily indicate a response of healthy quarters to heat stress, and although various forms of thermal stress cause depression in yields, SCC is not affected in uninfected quarters. Serum corticoid concentrations are elevated, along with circulating leukocytes, in response to heat stress and are known to suppress immune function. These hormones interfere with neutrophil adhesiveness, chemotaxis, receptor activity, lysosomal enzyme release, bactericidal activity, and phagocytosis, which may explain, in part, why incidence of infection is higher during periods of thermal stress.

Flies are vectors of bacterial diseases, and biting flies greatly contribute to the level of stress in a herd of cows and may cause milk production to drop as well as spreading mastitis. Flies are instrumental in the establishment of *Staph. aureus* and coagulase-negative staphylococcal teat canal colonizations in dairy heifers, which lead to intramammary infections with these organisms. Therefore, fly control is especially important during hot, humid weather, when conditions are optimal for insect multiplication.

Methods to Manage Stress

Shade

Trees provide a good source of shade, and their leaves absorb heat from cows' body. However, high cow density can kill trees in a matter of months, and muddy, wet areas around trees where manure accumulates are a good source of environmental mastitis pathogens. In corral systems, locating shade structures over the feed mangers is problematic because cows occupy the area for feeding as well as for shade; thus, the area becomes overloaded with manure, cows become dirty, and mastitis results. Hence, it is recommended that additional shaded areas be provided away from feed mangers.

Cooling

Cows drink 50% more water when the temperature is 26.7 °C, than when it is 4.4 °C. In addition, this elevated temperature requires them to cool themselves through respired moisture and body sweat. Chilling drinking water to 10 °C alleviates heat stress, resulting in increased feed intake, milk yield, and rumen mobility, with decreased respiration and body temperature.

Commercial coolers combine air turbulence and high-pressure water injectors to effectively lower temperature under shades. One study showed that coolers increased milk production by about 10% and lowered the death rate, as cooled cows recovered more rapidly from mastitis when heat stress was tempered. The use of corral manger misters on heat-stressed cows may increase milk production and reduce death loss among fresh cows having had recent cases of mastitis. In humid environments, use of low-speed fans with misting is highly recommended to carry away the humid air.

A shower-and-fanning station adjacent to the milking parlor has been quite successful. Water is sprayed with high-capacity overhead sprinklers for about 30 s every 5 min or with low-capacity sprinklers operating continuously; fans remove the warm humid air from animal surfaces to dry them. Cows can also be wet down again in the exit lane, spraying only the top and sides so that the postmilking germicidal teat dip is not washed off. In this way, cows are temporarily relieved from the sun, and, instead of returning immediately to the shade, they follow their normal cool-weather practice of eating and drinking after each milking. This practice keeps animals on their feet and allows time for teat duct closure before contact with soil and manure, which may lead to mastitis.

Another management strategy for cooling cows and reducing mastitis involves the use of cooling ponds. During periods of maximal heat stress, well water is continuously pumped into ponds and the overflow is carried into contained settling areas. Reduction in mastitis occurs probably owing to enhanced resistance to infection resulting from reduced heat stress, as well as from improved udder preparation afforded by cleaner cows at milking time. Cows from pond access are cleaner upon entering milking parlors and require less premilking preparation. In addition, cows with pond access are less likely to seek relief from the heat by lying down in the mud.

Hygiene

The underlying principle on which the control of mastitis rests involves prevention of the disease. This is best accomplished by minimizing the number of mastitis pathogens to which teats are exposed during the premilking, milking, postmilking, and intermilking periods. Transmission often occurs during the milking process via milker's hands, udder cloths or sponges, and teat cup liners. Microorganisms most likely to be transmitted at this time are contagious pathogens such as *Staph. aureus*, *Streptococcus agalactiae*, and *Mycoplasma* species. Transmission of the environmental streptococci (e.g., *Streptococcus uberis*) and coliforms (e.g., *E. coli)* occurs during the intervals between milkings.

Disinfection of Milker's Hands

Hands are an important means of transmitting mastitis pathogens to the teats of cows. Contamination of hands can occur when stripping foremilk to detect clinical mastitis, handling and attaching teat cups, or touching any contaminated object in the milking center. Rinsing hands in a disinfectant eliminates many mastitis pathogens, but not all. The wearing of disposable, surgical-type, plastic gloves, combined with the practice of rinsing hands frequently in disinfectant, further reduces the transfer of microorganisms during udder preparation.

Disinfection of Teats and Predipping

The primary objective of premilking udder preparation and teat sanitation is to achieve an acceptable level of decontamination, which reduces the spread of microorganisms and the incidence of mastitis. It is important to use a minimum amount of water, if any, and to concentrate attention primarily on cleaning and sanitizing only the portion of the teats actually touched by teat cup liners. Forestripping, or the removal of 2–3 ml of milk prior to machine attachment, reduces new infections by flushing mastitis organisms from teat canals. Teats should be dried subsequently with single-service paper or cloth towels prior to teat cup attachment. If water is present on the flanks or udder of cows, it drains down and pools at the top of teat cup liners, collecting additional microorganisms and leading to mastitis.

Predipping is a practice in which teats are immersed in a germicide (such as iodophor, chlorhexidine, and sodium chlorite) prior to milking to reduce the mastitis-causing bacterial load on teat skin. Recommended predipping procedure is as follows: clean the teats, forestrip, predip, wait for 20–30 s as recommended by the product manufacturer, dry teats with a single-service paper towel or a clean cloth towel, and attach the teat cups. Research on predipping has shown that the incidence of new infections caused by environmental streptococci and coliforms is reduced by about 50%. Care must be exercised to prevent germicide residues from contaminating the milk supply, but if the teats are dried carefully after predipping, the amount of additional residue present in the milk is negligible.

Adjusting Milking Units and Teat Cup Removal

During milking, units should be observed closely while attached to the udders to ensure that they are adjusted correctly and to aid in preventing liner slips. Liner slips are the result of improper liner design, absence of hose support arms, overmilking of individual quarters, milking wet teats, milking short or small teats, cluster weight, vacuum level, and vacuum fluctuations. If liner slips occur at the same time the liner opens, milk droplets impact against the teat end, which may contain mastitis-causing organisms and penetrate the teat canal. Also, teat cups seated excessively high on teats cause irritation to the lining of the teats and may contribute to the development of mastitis.

At the end of milking, the goal should be to remove the teat cups just as the last quarter milks out, but the vacuum should always be shut off before detaching teat cups; an increased risk of infection exists when teat cups are removed while under vacuum.

Postdipping

If the incidence of mastitis is to be reduced, it is important that the vast majority of organisms present on teats after milking be destroyed. The dipping of teats after milking (postdipping) in a suitable germicidal product (such as chlorhexidine, iodophor, and sodium chlorite) is the single most important practice to prevent new intramammary infections, especially those caused by contagious pathogens such as *Staph. aureus* and *Strept. agalactiae.* The correct application of a good teat postdip will reduce the rate of new infection by at least 50%.

Teat spraying is as effective as teat dipping if it is done properly, but, to be as effective, the entire barrel of the teat contacting the teat cup liner must be covered with germicide. This is rarely accomplished because milkers usually apply teat spray only on one side of the teats rather than to the entire surface. Moreover, teat spraying requires more time and more disinfectant than required by dipping; thus, most authorities recommend dipping rather than spraying.

The two basic types of teat dips are germicidal and barrier products. Germicidal products enjoy the major share of the teat dip market, and products are highly effective in reducing populations of mastitis organisms remaining on teats at the end of milking. The persistency of germicidal activity is limited when used as a postdip, however, because it is partially neutralized by milk and by organic matter in the environment. The theory behind barrier teat dips is that they form a physical obstruction between the teats and the environment, thus reducing infections during the intermilking period, especially with microorganisms of environmental origin.

Disinfection of Teat Cup Liners

Teat cup liners are a potential means for spreading microorganisms both within and among cows. One method for reducing the number of microorganisms is to immerse teat cups into a disinfectant two at a time to permit the disinfectant solution to have contact with the full length of the liners. However, the most effective method for disinfecting liners between cows is backflushing, which incorporates a water rinse to flush milk residues from the inside surface of the liners, claw, and milk hose; a rinse with a disinfectant solution; a short time delay to destroy microorganisms; another rinse with water to remove disinfectant residues; and a blast of air to remove residual water.

Importance of Excellent Environmental Hygiene

Regardless of whether cows are placed in pastures, open corrals, free stalls, open housing, or other types of housing, with or without bedding, the provision of a clean and comfortable environment will increase milk production, assist with maintaining a healthy immune system, and reduce mastitis. When cows lie down in their environment, teats are exposed to mastitis pathogens, especially *Strept. uberis* and *E. coli*, which may be present in concentrations ranging from 1000 to 1 billion per gram of bedding. Udders should be clipped or singed as necessary to remove long hair and to reduce the amount of bacteria in adhered dirt, manure, and bedding.

Conclusion

The objective of a mastitis control program is the prevention of new infections. In efforts to (1) breed dairy cows for disease resistance, (2) supply appropriate nutrition including micronutrients, (3) reduce the level of environmental stress imposed, and (4) provide proper milking time hygiene, the dairyman is taking the necessary steps to reduce the susceptibility of his herd to mastitis-causing bacteria, promote herd health, and increase milk quality.

See also: **Mammary Gland**: Anatomy; **Milking Machines**: Principles and Design.

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Medical Therapy Options

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Introduction

The control of bovine mastitis is best accomplished by a preventative program including pre- and postmilking teat antisepsis, proper maintenance of milking equipment, control of environmental exposure to mastitis pathogens, management of environmental stress on animals, and vaccination against certain pathogens. Despite the proven effectiveness of these procedures, new intramammary infections (IMIs) will occur and must be managed properly to prevent a herd mastitis problem from developing.

Once an infection is established, the task is to eliminate the infection from the individual cow, if possible, and to protect other cows in the herd from infection. Options for eliminating infections from the herd include (1) culling of infected animals, (2) treating with antibiotics during lactation, (3) treating with antibiotics at the beginning of the dry period, and (4) supportive care and management of the infection to enhance spontaneous cure. Of these, antibiotic therapy is the principal method used to treat IMIs.

The primary aim of antibiotic therapy is to cure the existing infection and return the cow to profitability as soon as possible. Additional goals include reducing the sources of new infection in the herd, preventing existing chronic cases from worsening, preventing cow mortality in severe cases, minimizing damage to secretory tissue, and preventing new infections from developing during the dry period.

Treatment during Lactation

When mastitis occurs during lactation, the dairy farmer can hope for a spontaneous cure, cull the infected animal, treat the infection during lactation with antibiotic therapy, or terminate lactation early and treat with a nonlactating cow antibiotic preparation.

Factors to consider in deciding whether to treat during lactation include the economic worth of the cow, whether the organism is one that can reasonably be expected to respond to treatment, and whether the risk of antibiotic residues in milk can be minimized to an acceptable level.

Therapy of *Streptococcus agalactiae*

Treatment in lactation is always indicated when cows are infected with *S. agalactiae*. This organism is highly contagious and is spread from cow to cow during milking. Fortunately, this organism responds well to antibiotic therapy during lactation, with cure rates usually in the range 90–95%. This organism is generally considered to be susceptible to all of the antibiotics commercially available to treat mastitis.

Identification of all infected quarters in the herd is of paramount importance when treating a *S. agalactiae* problem. If infected cows are left untreated, they will serve as sources of bacteria for infecting other cows. Because of this, *S. agalactiae* is often treated by 'blitz' therapy whereby all infected cows are treated in all quarters simultaneously in an attempt to eliminate all infections from the herd. This procedure can be effective; however, care must be taken to ensure that all infected quarters are treated and follow-up culturing is performed to identify treatment failures. Those cows failing to respond to therapy should be culled from the herd. In addition to antibiotic therapy during lactation, in herds infected with *S. agalactiae*, strict milking time hygiene, effective teat dipping program, and dry cow antibiotic therapy must be followed on all quarters of all cows at the end of each lactation.

Therapy of *Staphylococcus aureus*

Staphylococcus aureus is another contagious mastitis pathogen that is spread during milking. Unlike *S. agalactiae*, however, *S. aureus* does not respond well to treatment during lactation. Conventional therapy with commercially available mastitis preparations generally results in cure rates between 20 and 30% following label instructions. Because of this poor success, it is generally not recommended to treat *S. aureus* mastitis during lactation. This organism is best treated during the dry period with an approved dry cow antibiotic. There are times, however, when it becomes necessary for the dairyman to consider treatment of *S. aureus* infections during lactation. When high somatic cell counts (SCCs) threaten the milk market and other options such as culling and early dry-off have been exhausted, it may be necessary to attempt treatment of *S. aureus* infections during lactation.

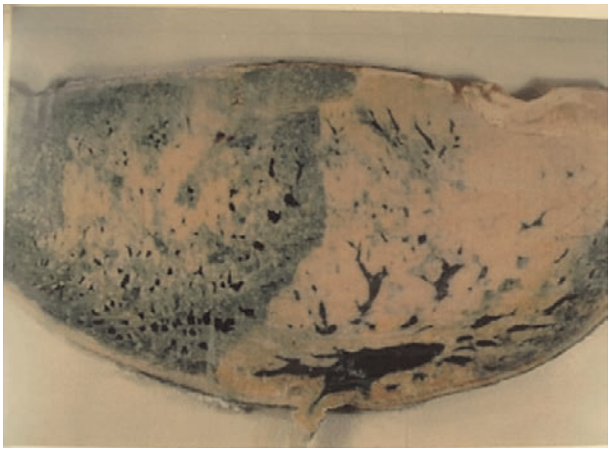

Figure 1 Cross-sectional view of a mammary gland chronically infected with *Staphylococcus aureus*. The gland has been infused with green dye and the unstained areas indicate portions of the gland inaccessible to antibiotic due to blockage of milk ducts.

The poor response of *S. aureus* to lactational therapy is believed to be caused by the combination of several factors. This organism tends to produce a chronic type of mammary inflammation, with foci of infection deep within the mammary tissue, resulting in scarring, clot formation, and swelling. This in turn results in the occlusion of milk ducts draining the infected area, which impedes the distribution of infused antibiotic to the site of infection. Contact between the antibiotic and the infecting bacteria is minimized, and thus treatment failures occur. **Figure 1** shows a cross-sectional view of a mammary gland chronically infected with *S. aureus*. The gland has been infused with green dye and the unstained areas indicate portions of the gland inaccessible to antibiotic due to blockage of milk ducts. To enhance the effectiveness of antibiotic therapy against *S. aureus*, several new strategies that increase the chances of cure have been devised (discussed below).

Combination Therapy

A combination of intramuscular injections together with intramammary infusions has resulted in much higher tissue antibiotic concentrations and a higher cure rate. In one study, 49 cows with 78 quarters subclinically infected with *S. aureus* were tested. One group of cows received intramammary infusion at each milking for six milkings with a lactating cow product containing 62.5 mg amoxicillin. Another group of cows received the same intramammary infusion regimen plus intramuscular injections of 6 million units of procaine penicillin G after each milking for 3 days.

The combination of intramammary and intramuscular treatment cured 51% of quarters compared with 25% of quarters treated by intramammary infusion alone. Thus, combination therapy was twice as effective as conventional infusion alone, and it allowed more antibiotic to penetrate deep areas of infection, and increased the cure rate. The SCCs taken prior to initiation of therapy from milk of cured quarters averaged 2 500 000 ml^{-1} and they were less than SCCs from quarters that failed therapy (4 000 000 ml^{-1}). By 8 days after treatment, SCC for cured quarters was 340 000 ml^{-1} compared with 1 900 000 ml^{-1} for failed quarters, and at 21 days, SCC was 224 000 ml^{-1} for cured compared with 1 975 000 ml^{-1} for failed quarters.

Extended Therapy

Another alternative for enhancing cure rates when treating chronic mastitis involves the use of pirlimycin hydrochloride and is called extended therapy. Pirlimycin has excellent activity against *S. aureus* and it penetrates scar tissue quite well. Approximately 50% of the drug is absorbed from the udder into the bloodstream and 50% of that amount is then re-excreted back into the udder. This helps the drug in reaching infected tissues.

A treatment protocol using three series of on-label treatments, separated by 36-h milk discard periods, was developed. Milk was salable for human consumption 36 h following the third series of treatments. In one investigation in a research herd using the three-treatment regimen, the drug was administered to quarters that had failed repeated therapy attempts. The results showed that 41% of quarters were cured. In a second investigation using a commercial dairy herd in which extensive treatment of subclinical *S. aureus* infections had not been practiced during lactation, 86% of quarters were cured using the three-treatment regimen. The SCCs were monitored in the infected quarters in both investigations, and a decrease from 3 400 000 to 280 000 ml^{-1} was observed by 10 days after treatment in quarters that cured. Similar improvement in cure rates using this treatment regimen was noted with a penicillin–novobiocin product, but no improvement was seen with this regimen using an erythromycin-containing product.

Combining Antibiotic Therapy with Vaccination

More recently, vaccination has been employed to amplify the cow's immune system against *S. aureus* antigens and augment the effectiveness of antibiotic therapy. In one study, vaccination in combination with intramammary therapy was evaluated over a 1-year period in a 48-cow herd with a *S. aureus* prevalence of 58.3%. After selected *S. aureus*-infected cows were dried off or culled, 20 animals were vaccinated at 2 and 14 days prior to antibiotic therapy. Vaccinations were subcutaneous in the area of the supramammary lymph node with a bacterin containing an encapsulated strain of *S. aureus* plus an autogenous strain isolated from the herd. Each cow was then treated using extended therapy with pirlimycin as described above and vaccinated again 7 days later. The results demonstrated that all but three quarters responded to this vaccine/treatment program, and by 5 months after trial initiation, *S. aureus* was eliminated from the herd. The herd SCC was reduced to $492 \times 10^3\,\text{ml}^{-1}$ 1 year later. All herds would not be expected to exhibit this degree of success, but this strategy may be extremely useful for certain *S. aureus*-infected herds.

No matter what treatment strategy is used, the probability of curing existing infections is reduced under the following conditions: older cows, high SCC, rear quarters infected, cows in early- to mid-lactation, and presence of multiple quarter infections within an udder. Because of the low probability of cure, it is important for dairy producers and veterinarians to take great care in selecting *S. aureus*-infected cows for treatment in lactation.

Therapy of Acute Clinical Mastitis

Acute clinical mastitis is characterized in the cow by systemic symptoms resulting from the toxic effects of the bacteria causing the infection. The primary cause of this severe form of mastitis is the coliform group of organisms including *Escherichia coli* and *Klebsiella pneumoniae*. These Gram-negative bacteria produce endotoxin, which results in systemic symptoms typified by dehydration, depression, inability to stand, diarrhea, and shock. Therapy must be directed toward alleviating these symptoms and countering the effects of the endotoxin. In most cases, the bacteria reach very high numbers in the mammary gland quickly and then die off rapidly. Often the bacteria that are responsible for the infection are no longer viable when symptoms begin to become apparent, resulting in cultures that are negative for bacteria despite obvious clinical symptoms. As the bacteria die, the endotoxin component of their cell wall is released and this causes the toxic effects. Because the bacterial cause of the symptoms is often no longer present, antibiotic therapy is often of little or no benefit. Therapy must be supportive and directed to counter the effects of the endotoxin in the bloodstream, and includes frequent stripping of the quarter and administering electrolytes, anti-inflammatory agents, glucose, bicarbonate, and calcium. Often large amounts of fluids are needed, and severely affected cows may require 40–60 l of fluids to be administered intravenously on the first day of the onset of symptoms.

Care must be taken when using anti-inflammatory products to manage toxemia, inflammation, and shock associated with acute clinical mastitis. Products such as prednisone, prednisolone, methylprednisolone, and isoflupredone can be effective but should be administered for no longer than 2 or 3 days. These products can suppress the immune system and can induce premature calving, retained placenta, and uterine infection when used in the third trimester of pregnancy.

Oxytocin is another product that may be considered as an adjunct to therapy of acute clinical mastitis. This hormone is normally released from the brain into the bloodstream in response to milking stimuli. Oxytocin causes contractions of the alveoli in the mammary gland, resulting in expulsion of milk. The therapeutic administration of exogenous oxytocin is believed to cause additional contraction of alveoli in infected and inflamed mammary tissue, resulting in increased expulsion of clots, bacteria, inflammatory products, and toxins.

The therapeutic recommendations for oxytocin include frequent stripping of infected quarters (6–8 times per day) following oxytocin administration (40–50 units intramammarily). Clinical studies with oxytocin have yielded variable results. Often a clinical response is noted initially only to be followed in some cases by relapse. In one study, relapses were observed more often in clinical cases caused by environmental streptococci than by coliforms.

Therapy of Subacute Clinical Mastitis

The majority of clinical mastitis cases do not progress to the acute toxic form discussed above and are characterized as subacute. In most clinical cases, the milk is visibly abnormal and exhibits clots, flakes, or a watery consistency. The quarter may be swollen, but no systemic signs are apparent.

Antibiotic therapy with an approved infusion product is generally recommended for cases of subacute clinical mastitis. Therapy should be continued for a minimum of 3 days and should continue for at least 1 day beyond obvious clinical improvement. Discontinuation of therapy too soon can result in relapses that may evolve into chronic cases that will not respond to further treatment. Successful antibiotic therapy of clinical mastitis is dependent on several factors including the severity of the infection, how long the infection has persisted, the organism causing the infection, and the overall health of the cow.

Culture of the milk from the infected quarter to identify the causative organism is important in cases of clinical mastitis. Knowing which organisms are present can help determine which treatment procedures have the best chance of success. If routine culture methods fail to identify the cause of the infection, alternative culture techniques may be necessary. Often mastitis pathogens are present in low numbers in clinical cases and routine culture methods are not sensitive enough to detect the organisms. Alternatively, some mastitis pathogens require specialized media and culture conditions. The coliform organisms that are responsible for many clinical mastitis cases are often present in detectable numbers for the first

12–18 h of an infection. Milk samples collected after this time often contain no or very low numbers of these pathogens, resulting in culture-negative clinical samples. Increasing the volume of milk cultured can enhance the recovery of pathogens present in low numbers. The presence of numerous culture-negative milk samples (50%) may indicate the presence of *Mycoplasma* species. These organisms cause a highly contagious type of mastitis that is unresponsive to antibiotic therapy. *Mycoplasma* species will not grow on routine culture media and require specialized media, enhanced CO_2 atmosphere, and typically 5–10 days to grow.

Treatment at Dry-Off

Dry cow therapy is a major component of a comprehensive mastitis control program. Infusion of an approved dry cow antibiotic into every quarter of every cow at the end of each lactation is the most effective use of antibiotic therapy against mastitis. The advantages of dry cow therapy are as follows: cure rates are higher than for treatment in lactation, higher concentrations of antibiotics can be used, the incidence of new infections is reduced, number of clinical mastitis cases at calving are reduced, salable milk is not discarded, unrecognized infections are treated, laboratory culturing is not required, and damaged tissue has time to redevelop prior to the next lactation if therapy is successful. Treatment at dry-off not only treats existing infections, but also provides protection from new infections during the early dry period. Some dairy farmers use selective dry cow therapy in which they treat only quarters of cows with known cases of mastitis. This often proves to be false economy because it is difficult to know the presence of all infections in a herd and some undiagnosed infections will go untreated. Also, the protection from new infections that may occur in the early dry period is lost when no dry cow therapy is administered. Research has shown that treatment of all quarters of all cows is the most effective method for using dry cow antibiotics. Some concern has been expressed that the constant exposure of microorganisms to the antibiotics commonly used to treat mastitis will encourage the emergence of strains of bacteria that are resistant to these antibiotics. However, despite widespread use of these products for many years, no evidence of resistance development associated with these practices has emerged.

Recently, a new procedure for dry cow treatment has been developed, called teat sealing. An inert paste-type product made of 65% bismuth subnitrate in heavy liquid paraffin is infused into the gland at dry-off. The product forms an inert seal in the lower portion of the teat cistern and in the teat canal, and this prevents new IMIs by serving as a physical barrier. Data indicate that the product is effective at preventing new IMIs when used in uninfected mammary glands.

Current recommendations are to use this product immediately after administration of a dry cow antibiotic product, and is not intended as a replacement for dry cow antibiotic therapy. Also, care must be taken at calving to ensure complete removal of the sealant before returning the cow to the milking herd.

Heifer Mastitis

The realization in recent years that dairy heifers are at risk of contracting mastitis prior to their first lactation has led to research aimed at treating and eliminating these infections during the prepartum period. The results of this research indicate that IMIs present in dairy heifers respond well to administration of nonlactating cow antibiotic preparations infused intramammarily >45 days prior to calving. The staphylococci are the predominant organisms isolated from heifer secretions and *S. aureus* can account for a substantial number of these infections. Left untreated, these infections may persist into the first lactation where they become much more difficult to cure. In addition, the presence of IMI prior to and during the first lactation can damage developing mammary tissue, reducing potential milk production in subsequent lactations.

Cure rates for *S. aureus* IMI in heifers treated prepartum with approved dry cow products exceed 90%. Prepartum therapy of heifers is more effective, in part, due to the smaller volume of tissue in the developing heifer mammary gland compared to the adult cow, which results in much higher concentrations of antibiotic and greater contact with the infecting bacteria. **Figure 2** shows a cross-sectional comparison of a heifer gland relative to that of an adult cow. Recent research aimed at determining the

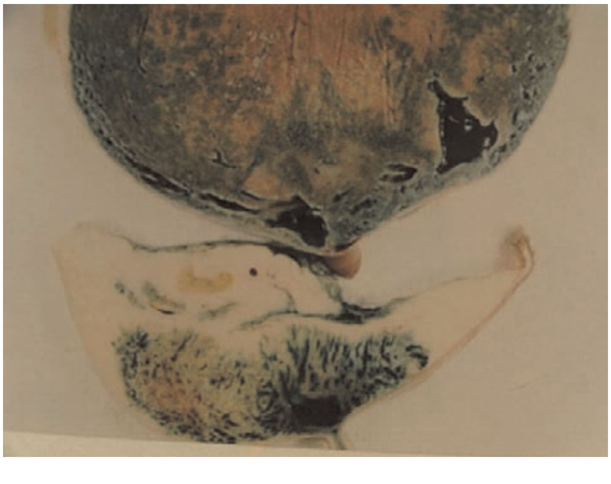

Figure 2 Cross-sectional comparison of a heifer gland to an adult cow.

optimum time prepartum to treat heifers found no difference in treatment efficacy when treatment was administered during the first, second, or third trimesters. However, treating during the third trimester resulted in fewer new infections occurring after treatment and prior to calving, suggesting that treatment in the third trimester reached the most infections. Dry cow treatment of heifers is an effective way to control *S. aureus* mastitis in herds with high numbers of heifers calving with mastitis. If a problem is suspected, secretion samples should be collected from a representative number of heifers and cultured to determine if the problem exists and if therapy is warranted.

Treatment Procedures

Care must be taken to follow correct procedures when infusing antibiotics into the mammary gland via the teat end. A cotton ball soaked in 70% alcohol or a prepackaged alcohol pledget is used to thoroughly cleanse the teat end prior to infusion. The teat end often contains large numbers of bacteria that may have originated from the environment; these organisms can be pushed into the mammary gland via the treatment syringe cannula during treatment if the teat end is not properly sanitized. Some of these organisms such as *Nocardia* spp., *Bacillus* spp., *Prototheca* spp., and fungi can cause severe mastitis that does not respond to antibiotic therapy.

It is recommended that gloves be worn when treating cows if highly contagious microorganisms, such as *S. agalactiae* or *Mycoplasma bovis*, are present in the herd. In addition, hands should be dipped in a sanitizing solution between treating animals.

The method of drug infusion can actually cause mastitis by inadvertently introducing microorganisms through the teat canal. Full insertion of the conventional mastitis tube syringe cannula can result in temporary dilation of the teat sphincter muscle. In addition, the keratin plug that normally occludes the teat canal is either pushed aside or partially removed. Both of these situations create a larger than normal teat canal opening, allowing entry of microorganisms. The syringe cannula may also push microorganisms that are colonized in keratin into the teat cistern. If microorganisms gaining access to the teat cistern by these routes are resistant to the infused antibiotic, then a new infection may result, which may be more severe than the one for which treatment was intended.

Studies designed to compare conventional full insertion with partial insertion of only the first 2–3 mm of the cannula tip at drying off have demonstrated that mastitis at calving was reduced markedly by using the partial insertion technique. Several types of syringe cannulas have since been developed to aid in inserting only the tip of the cannula into the teat canal and to form a seal against the teat opening to provide support during infusion. The use of the partial insertion technique may reduce new infections by major mastitis-causing microorganisms at calving by 50% or more.

Treated animals must be clearly identified in some obvious manner with neck chains or leg bands. In addition, they should be kept separate from untreated animals to aid in preventing their milk from being commingled with milk from untreated animals.

See also: **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens.

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Role of Milking Machines in Control of Mastitis

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Introduction

Machine milking plays a role in the incidence of mastitis. The milking machine is an important vector of bacteria, both between cows and within the cow, from teat to teat, and can play an active role in the penetration of bacteria into the teat canal, teat sinus, or gland sinus, and may cause trauma to the teat rendering it more susceptible to colonization and infection (**Table 1**). With the current knowledge, it is not possible to quantify the effects of the different infection mechanisms on new intramammary infection (*see* **Milking Machines**: Principles and Design).

Transfer of Bacteria

The milking process offers multiple opportunities for bacteria to be transferred between cows and quarters. The number of pathogenic bacteria on the teat skin and particularly the teat orifice will affect the rate of new infections. Bacteria may be transferred from one cow to another and between quarters via the hands of the milker, liners, clusters, and towels (**Figure 1**). Poor barn hygiene in between milkings and improper teat preparation increase contamination and transmission of bacteria (*see* **Milking and Handling of Raw Milk**: Milking Hygiene).

During milking, vacuum fluctuations in the claw cause milk to move between teat cups. If the cow being milked has one or more infected quarters, this process transfers pathogenic bacteria to the surfaces of other teats. After a cow has been milked, the liner surfaces carry bacteria originating from the teat surface and milk of that cow. These bacteria are transferred to the next cow when the machine is applied. Mastitis-infected cows should be milked last or in separate milker units used only for infected cows.

Proper premilking teat preparation and the use of predipping can lower the number of bacteria at the teat end. Teat cup flushing between milkings can reduce the amount of bacteria on liners and claws; however, the effect on new infection rate is not clear (*see* **Mastitis Therapy and Control**: Management Control Options and **Milking and Handling of Raw Milk**: Milking Hygiene).

Increase Bacterial Penetration of Teat Duct

The action of the milking machine can cause bacteria to be propelled directly from the exterior of the teat into the teat sinus. Vacuum fluctuations in the claw can cause impacts: air and droplets of milk, including pathogens, are propelled toward the teat tip. The impact mechanism is the only known means by which vacuum fluctuations are capable of increasing infection rate under both experimental and field conditions. Vacuum fluctuations can be characterized in terms of rate of change and magnitude and are the result of milk flowing in the system, pulsation, and admission of air. Large fluctuations in vacuum have earlier been shown to increase intramammary infections, but the rate of change and magnitude were not well defined in these older studies.

During cluster attachment or removal, air is admitted through the teat cup and this air is sucked toward the claw. Liners should be applied to teats with minimal vacuum leakage by keeping the short milk tube bent over the claw ferrule until the liner slides onto the teat. Cluster takeoff should be done after vacuum shutoff. Liner slippage is an important cause of vacuum fluctuations, which occurs primarily at the end of milking. The long milk hose should be adjusted during the milking process to avoid liner slippage and/or uneven milkout. Milking with high vacuum and the use of liners with a good grip on the teat result in less liner slippage. Some udder and teat conformations increase the probability of liner slip: rear quarters lower than front quarters, wider teats, larger teat diameters, and longer teats.

Vacuum stability in milkline systems can be achieved by using adequate claw air bleeds, pipeline diameters, claw volume, vacuum pump capacity as well as a well-functioning vacuum regulator. Shields in the liner or valved claws intercepts impact and reduce new infection rates. However, they have never been a success in practice.

Besides factors associated with the milking machine, factors related to the cow also play a role; for example, quarters with a high peak flow rate ($>1.6\,\mathrm{kg\,min^{-1}}$) are more susceptible to infection probably because of larger teat canal diameter.

Besides vacuum fluctuations, reverse pressure gradients (RPGs) between liner space and teat cistern may cause milk contaminated with pathogens to move from the exterior into the teat, but the effect of this mechanism is not known.

Table 1 Machine milking mechanisms potentially affecting new infection

Mode of infection	*Main milking-related mechanisms*	*General evidence*
1. Transfer of bacteria	Transfer of bacteria from (a) Environment to teat (b) Cow to cow (c) Teat to teat (same cow)	Teat disinfection reduces bacterial numbers on the teat skin and orifice and decreases new infection rate (NIR) Experimental bacterial challenges increase NIR
2. Increase bacterial penetration of teat duct	By causing impacts of bacteria-laden milk droplets and dispersing pathogens from (a) Teat canal to the teat sinus (b) Teat sinus to the gland sinus and/or ducts	Endotoxin, *Escherichia coli*, and dyes have been jetted through the teat canal Shields or valves reduced NIR High-velocity air/liquid flows toward the teat end increased NIR Few infections occur if bacteria placed within the teat sinus are carefully removed by stripping but bacteria placed within the gland sinus frequently cause NIR In some experiments, presquirting of teats before milking reduced milking
3. Frequency and/or degree of udder evacuation	By changing (a) Susceptibility of the gland to invading pathogens (b) Concentrations of pathogens on the teat end (c) Duration of exposure to pathogens	NIRs are higher in dried off cows at the start of the dry period Incomplete milking or omittance of milking tends to increase NIR
4. Resistance of teat to bacterial invasion	By affecting (a) Teat canal integrity (b) Teat congestion and/or edema (c) Increasing skin and/or orifice lesions	NIR is increased by reaming keratin from the teat canal and by visible teat canal injuries NIR is increased when pulsation is ineffective Clinical mastitis is increased with increasing teat end callosity or lesions

Adapted from Bramley AJ, Dodd FH, Mein GA, and Bramley JA (1992*) Machine Milking and Lactation*. Berkshire, UK: Insight Books; IDF (1987) Machine milking and mastitis. *International Dairy Federation Bulletin No. 215*.

Frequency and/or Degree of Udder Evacuation

Good premilking procedures and adequate lag-time before attachment of the teat cups will allow cows with a milk yield of 10 kg milk per milking to milk in about 5 min (±0.5 min). To make optimum use of milk letdown and to ensure that milking of empty teats is avoided, the lag-time between first teat manipulation during premilking treatment and applying the teat cups must be 30–90 s. Attachment of teat cups, before milk letdown occurs, results in teat cup crawling up during the first minutes of milking and less complete and efficient harvest of milk.

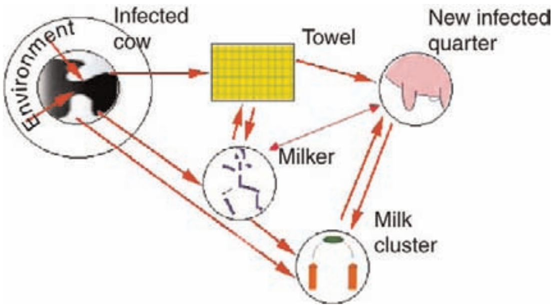

Figure 1 Role of machine milking in the transmission of bacteria.

Cases of poor type or condition of the liner, mismatch between claw inlet and short milk tube, a too light cluster, not evenly hanging cluster, high milking vacuum levels, or malfunctioning pulsators may lead to inadequate milk removal and this incompleteness of milking can cause problems. Incomplete milking or omitted milking tends to increase new infection rates or the progression of subclinical infections to clinical stage. Many bacteria are removed by the flushing action of milk as it moves through the teat duct during milking.

Increasing cluster weight and the use of a mechanical device to align the unit squarely on the udder reduce strip yields. Early removal of the milking unit (at a flow rate of 400 vs. 200 g min^{-1}) does not affect milk yield or composition, and the incidence and prevalence of subclinical mastitis, but machine-on time is shortened and teat condition improved.

Although overmilking can damage the epithelial lining of the teat sinus as a result of teat tissue being rubbed together during liner collapse, little teat damage occurs from moderate overmilking when milking machines are installed correctly and operated properly. Overmilking in combination with inadequate pulsation, high vacuum, or excessive vacuum fluctuation increases new infection rate.

Resistance of Teat to Bacterial Invasion

Teat Condition

The teat canal is a strong and important primary barrier against invasion of mastitis pathogens into the udder. Teat tissue changes by machine milking can be seen as teat swelling (teat end, base, or top), teat flattening, color changes, openness of the teat orifice, vascular damage (hemorrhages), teat lesions, teat end callosity, and loss of teat canal keratin (*see* **Mammary Resistance Mechanisms**: Anatomical).

Changes of the teat canal integrity and teat tissue pliability may favor penetration of bacteria into the udder. Trauma to the mucous membranes lining the teat sinus may provide an environment favoring bacterial colonization or multiplication. Local pain may lead to neurohormonal responses that suppress immune function and increase the likelihood of disease.

Teat swelling

Vacuum opens the teat canal and milk flows out but also blood and lymph are drawn into the teat tissue. The collapsing liner exerts a mechanical force on the teat end (compressive load), causing the teat canal closure and transport of blood and lymph back to the udder.

During milking, the thickness of the teat ends decreases during the high flow rate phase, increases during the low flow rate and overmilking phases, and again decreases as soon as milking is finished. The differences in the increase of teat end thickness are influenced by overmilking, use of wide-bore liners, high vacuum, pulsation failure, insufficient rest phase of pulsation, short A- and C-phases of pulsation, or liner mouthpiece chamber. Vacuum level seems to have a greater effect than the duration of milking.

For teats milked without pulsation or with liner-less teat cups, the increase in teat end thickness is significantly greater than for conventional liners and pulsation settings. Decreases in teat end thickness have been observed for positive pulsation, calf suckling, and hand milking. Milking with narrow-bore liners that have a soft mouthpiece lip reduces congestion and edema. It is generally agreed that the internal diameter of the liner should be at least 1 mm smaller than the average teat diameter in a herd before milking.

Teat orifice openness

Factors associated with greater openness of the teat orifice include high milking vacuum, overmilking, design of the liner mouthpiece, or unusually heavy cluster weight, or excessively high mounting tension of the liner.

Teat lesions

Injured teat skins are a common site for bacteria and viruses to multiply. Bacteria, in particular *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae*, are often associated with teat lesions. The risk of infection is amplified when lesions are at, or near, the teat orifice.

Overmilking, pulsation ratios above 70%, inappropriate housing conditions, and insufficient hoof care are associated with an increased incidence of teat lesions. The associations of pulsation ratio and overmilking suggest that factors that predispose to teat end edema and/or irritation during milking might cause teat lesions.

Vascular damage

The proportion of teats with petechial hemorrhages gives an indication of the extent of any vascular damage. Vascular damage usually reflects some type of pulsation failure, wide pulsation ratios (>80%) often associated with high vacuum and/or prolonged overmilking.

Teat end callosity

The long-term effects of repeated use of the milking machine are changes in teat end tissue, resulting in a callous ring around the teat orifice. Changes in teat end tissue result from mechanical forces exerted by vacuum and the collapsing liner during machine milking. Teat end callosity increases during lactation and parity. High-yielding cows are more often affected, probably because machine-on time is longer. The differences between farms can be explained in part by the differences between the cows and in part by the differences in the characteristics of the milking machine and management like use of high vacuum, wide pulsation ratios, use of teat dip, and so on.

Most of the systems that have been developed to classify teat end condition either do not differentiate between smooth and rough callosity rings or do include scabs or lesions. The adapted classification system of The Netherlands shows marked differences in the thickness of the callosity ring, which is transformed into five classes: none (N), slight (A), moderate (B), thick (C), and extreme (D). Additionally, the ring is classified as smooth (1) or rough (2) (**Figure 2**). A more practical classification is to define teat end callosity into four categories: none (N), smooth (S), rough (R), and very rough (VR).

Teat end callosity involves hyperkeratosis (stratum corneum) (**Figure 3**). Thicker callosity rings show parakeratosis and nuclei are observed in the cells. Teats with a higher score of teat end callosity show perivascular reaction: infiltration of lymphocytes, granulocytes, or erytrocytes.

With thick callosity, the outside portion of the teat canal cannot close tightly and microorganisms may penetrate the teat easily. On rough callosity, it is easier for bacteria to anchor and reproduce, contributing to the incidence of mastitis. Mild hyperplasia is a basic physiological response to the forces imposed by milking.

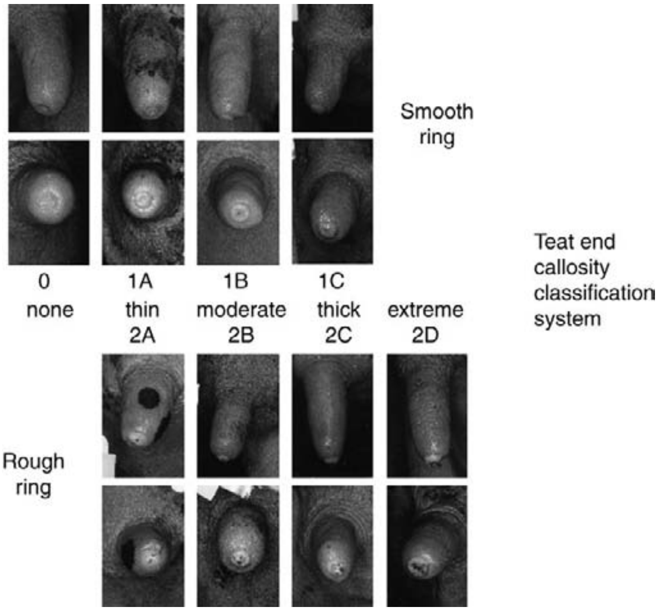

Figure 2 Teat end callosity classification system. Adapted from Neijenhuis F, Barkema HW, Hogeveen H, and Noordhuizen JPTM (2000) Classification and longitudinal examination of callused tead ends in dairy cows. *Journal of Dairy Science* 83:2795–2804.

Teat canal keratin

Milking removes keratin out of the teat canal, and this may adversely affect teat canal defense as keratin has a protective role. Loose keratin traps invading bacteria and these are removed by the flushing action of milking. Up to 40% of the keratin is lost from the teat canal wall during milking. The loss is greater for milking with pulsation than for milking without pulsation. But milking without pulsation increases new infection rates, apparently removing an inadequate amount of keratin. Higher losses of keratin can also increase new infection rates, as occurring in very high flow rates. Under the right milking conditions, there will be a healthy balance between the degree of desquamation per milking and the rate of regeneration.

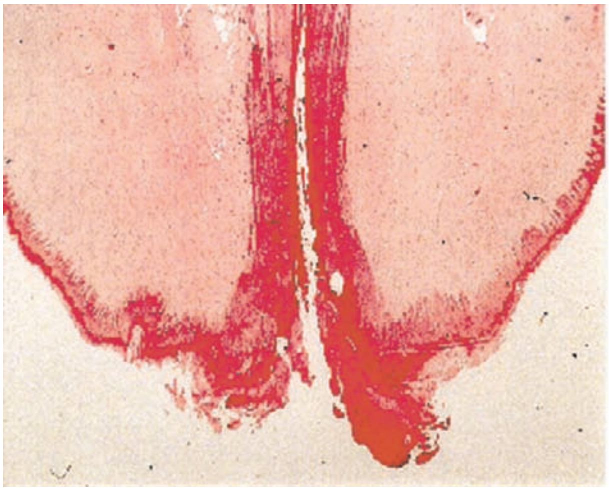

Figure 3 Microscopic view of a teat end with a thick rough callosity ring (2C). Courtesy of Dr. A de Man, Dr. YH Schukken, and Dr. JP Koeman, Utrecht University.

Practical guide to teat condition

An international group of teat condition experts made a list of teat conditions and their target criteria (**Table 2**). It is recommended to score at least all teats of 80 randomly selected cows or 20% of the herd, whichever is larger. If the target criteria are not met, further investigations to find the cause of deterioration of teat condition should be made.

Machine Milking Factors Affecting Teat Condition

The mechanical forces during machine milking that result in changes in teat end tissue are milking vacuum, pulsation vacuum, and liner type in combination with teat shape and milking time. **Table 3** summarizes the common

Table 2 Teat condition measures and criteria to be met

Teat condition measure	*Criteria*
1. Color	>20% visibly reddened or blue
2. Swelling at the teat base	>20% swelling or palpable rings
3. Swelling at the teat end	>20% firm, hard, or swollen
4. Openness	>20% classified as open
5. Vascular damage	>10% petechiations
6. Teat end roughness	>20% rough and very rough
7. Open lesions	>5% open lesions or cracked skin

Reproduced from National Mastitis Council (2001) *Proceedings of 2nd International Symposium on Mastitis and Milk Quality*. Vancouver, BC, Canada, 10–15 September.

Table 3 Some of the common primary causes or exacerbating influences on short-, medium-, and long-term machine-induced teat condition

	No. of teats affected	*Teat color*	*Swelling at the base*	*Firmness/ hardness of the teat end*		*Open orifice*	*Hemorrhages*	*Hyperkeratosis*
Observation		Red/blue	Ringing	Hard	Wedge	Diameter	Petechiations	Callosity ring and roughness
Duration		Short	Short	Short	Short	Short	Medium	Long
Machine factors								
High milking vacuum	All	✓		✓		✓	✓	✓
Faulty pulsation	All	✓		✓	✓		✓	
Short d-phase	2–4	✓		✓				
Long d-phase	2–4				✓			✓
Liners								
Wide bore	All	✓		✓		✓	✓	
Tapered	All							
Aged	All	✓	✓					
High compression	All				✓			✓
High tension	All	✓			✓	✓		✓
Mouthpiece								
Large chamber	Mostly front	✓	✓					
Large height	Mostly rear	✓	✓					
Small lip diameter	All	✓	✓					
Stiff lip	All		✓	✓				
Mismatch of liner and teats	All	✓						
Milking management								
Long dribble times	Mostly rear	✓	✓	✓				✓
Overmilking	Variable with milk out	✓	✓	✓		✓	✓	✓
Teat cup crawling	All		✓					

Ohnstad I, Mein GA, Baines JR, *et al*. (2007) Addressing teat condition problems. *National Mastitis Council Annual Meeting Proceedings.*

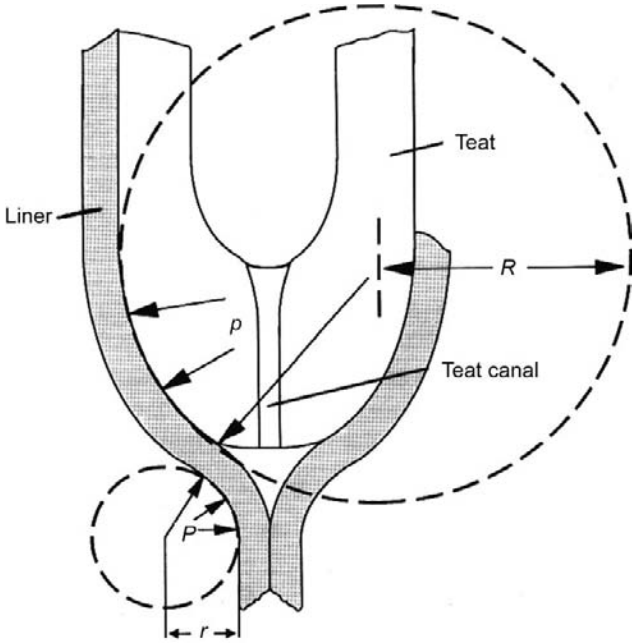

Figure 4 Sketch of a section of the liner bent around the teat end along the plane of collapse. Reproduced from Williams D and Mein GA (1980) Effects of pulsation and pulsation failure on the bovine teat canal. In: *Proceedings of the International Workshop on Milking Machines and Mastitis*, pp. 73–81. Fermoy, Eire.

primary causes of the short-, medium-, and long-term teat condition (*see* **Milking Machines**: Principles and Design).

Liner and teat

The pressure of the liner on the teat during the d-phase of pulsation is believed to relieve congestion of the teat tissue but excessive pressure seems to contribute to the development of teat end callosity or hyperkeratosis.

The compressive load of the liner on the teat can be estimated from the shape of the curved liner and the pressure difference across the liner just underneath the teat (**Figure 4**):

$$pR = Pr, \quad \text{i.e.,} \quad p = \frac{Pr}{R} \text{ kPa}$$

$$\text{if } P = 50\,\text{kPa}, \quad \text{then } p = \frac{50r}{R} \text{ kPa}$$

where R is the radius of curvature of the liner around the teat (mm), p the pressure (kPa above atmosphere) between the liner and the teat, r the radius of curvature of the liner just underneath the teat (mm), and P the pressure difference across the liner just under the teat (kPa).

On average, r:R ratio can be assumed to be 1:5; thus, the pressure on the teat would be around 10 kPa. Very soft, thin-walled liners will show a ratio of 1:15, indicating a compressive load of 3 or 4 kPa. Thick-walled liners under high tension show a ratio of 1:2, indicating a compressive load of 25 kPa. The compressive load applied by the liner on the teat should be 8–12 kPa. This approximates the bovine arterial pressure of about 12 kPa.

Another way to estimate the effect of the liner and vacuum on the teat is by measuring the pulsation vacuum at which the milk flow starts or stops. This is called overpressure or liner compression. Another important factor that determines the response of the teat to milking is milking time.

Vacuum

Increasing the milking vacuum at the teat end results in shortened milking times. On the other hand, milking at high vacuum can increase strip yields, teat end callosity, and teat thickness. Local trauma of the mucous membranes lining the teat sinus by excessive vacuum may provide an environment that favors bacterial colonization or multiplication. Milking at low vacuum increases machine-on time and the frequency of liner slip and decreases milk flow rate, but has a positive influence on teat condition and udder health compared to milking at high vacuum. Vacuum underneath the teat should not exceed 40–42 kPa (<13 inches) during the peak milk flow. This results in nominal vacuum setting of 40–46 kPa for low lines and 47–50 kPa for high lines.

Pulsation

If the duration of liner collapse is inadequate or if the liner is too short so that collapse does not occur at the teat end, there will be an increase in infection possibly through loss of mature stratum corneum and the exposure of an immature (desmosome-bound) surface. The closing phase of the liner (d-phase) should be more than 150 ms per cycle.

See also: **Mammary Resistance Mechanisms**: Anatomical. **Mastitis Therapy and Control**: Management Control Options. **Milking and Handling of Raw Milk**: Milking Hygiene. **Milking Machines**: Principles and Design; Robotic Milking.

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MICROORGANISMS ASSOCIATED WITH MILK

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Introduction

Milk is a good growth medium for many microorganisms because of its near-neutral pH, complex biochemical composition, and high water content. Although in the absence of mastitis it is secreted free of microorganisms, it is subject to microbial contamination from a number of sources (**Figure 1**). Some microorganisms move up the teat canal causing aseptically drawn milk to be contaminated. These contaminants, called udder commensals, are present in small numbers and are mainly lactic acid bacteria. Their numbers are limited by the animal's immune system and antimicrobial agents secreted into the milk. Therefore, contaminants external to the udder from locations such as udder skin, hide, milking equipment, and utensils form the vast majority of microorganisms in raw milk. Although technology has allowed the production of milk with very low microbial levels, product quality still depends on controlling entry and growth of microorganisms in milk from farm to the consumer.

The number and types of microorganisms present in milk are influenced by season, farm hygiene, feed, and efficiency of cooling. The number of bacteria in milk ranges from a few hundred to thousands per milliliter of freshly drawn milk from healthy cows. Four physiological groups of spoilage bacteria are commonly found in raw milk: producers of lactic acid, propionic acid, butyric acid, and degradative enzymes (primarily proteases and lipases). In addition, raw milk may contain pathogens whose multiplication depends mainly on the temperature and competing microflora. The main criteria for high-quality raw milk are the presence of a low number of spoilage microorganisms and the absence of animal pathogens. The sources of microorganisms found in milk, their characteristics, and their growth are discussed below.

Microorganisms Found in Milk

The microorganisms found in milk can be divided into three groups: animal pathogens and toxin producers, spoilage agents (saprophytes), and microorganisms used to produce fermented products. Some overlap among these groups occurs; for example, *Bacillus cereus* is a toxin producer and is also involved in spoilage, and lactic acid bacteria can cause spoilage and be used in fermentations. **Figure 2** shows the different morphological groups commonly found in raw milk.

Pathogenic Microorganisms

Pathogenic bacteria are those capable of causing infection or intoxication. Milk is a good growth and protective medium for most pathogens. The growth of pathogens in milk is inhibited by cooling and the growth of nonpathogenic competing microorganisms. Many pathogens, such as *Mycobacterium tuberculosis* and *Brucella* species, grow very slowly in milk, and others, such as viruses, do not multiply at all. Therefore, the threat associated with their presence in milk will depend on the initial load of milk contamination. Although the potential hazard of pathogens has been minimized by modern milk production and technology practices, disease outbreaks caused by *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, and *Escherichia coli* O157:H7 have occurred in recent years.

Staphylococcus spp.

Staphylococcus spp. are nonmotile, non-spore-forming, Gram-positive, catalase-positive cocci. They grow well in media containing 10% NaCl and produce proteases, lipases, and esterases. *Staphylococcus aureus* is the most important species of this genus associated with milk. It produces heat-stable enterotoxins that are responsible for food poisoning. Its natural habitat includes the skin and mucous membranes of mammals. Most contamination occurs during milking and originates with the cow. About 40% of bulk tank milk from a nationally representative sample of 542 dairies tested positive for *Staph. aureus*. It has been reported that 16 and 18% of the cows from organic and conventional farms, respectively, harbored oxacillin-resistant staphylococci, which indicated the presence of the methicillin-resistant gene (*mecA*). Although the presence of *Staph. aureus* in milk is difficult to prevent, appropriate cleaning and sanitation of the cow and milking equipment controls initial numbers and

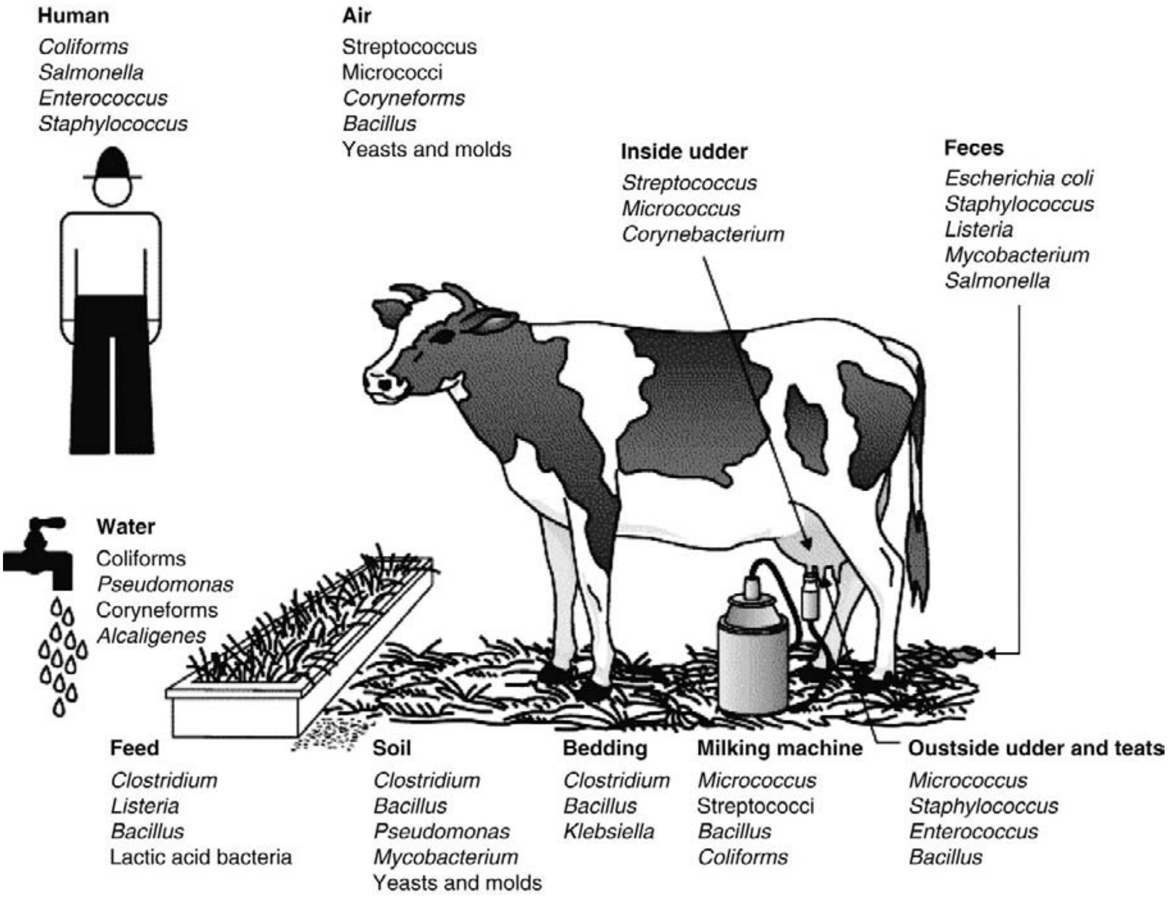
Human
Coliforms
Salmonella
Enterococcus
Staphylococcus
Air
Streptococcus
Micrococci
Coryneforms
Bacillus
Yeasts and molds
Inside udder
Streptococcus
Micrococcus
Corynebacterium
Feces
Escherichia coli
Staphylococcus
Listeria
Mycobacterium
Salmonella
Water
Coliforms
Pseudomonas
Coryneforms
Alcaligenes
Feed
Clostridium
Listeria
Bacillus
Lactic acid bacteria
Soil
Clostridium
Bacillus
Pseudomonas
Mycobacterium
Yeasts and molds
Bedding
Clostridium
Bacillus
Klebsiella
Milking machine
Micrococcus
Streptococci
Bacillus
Coliforms
Oustside udder and teats
Micrococcus
Staphylococcus
Enterococcus
Bacillus

Figure 1 Sources of milk contamination at the dairy farm.

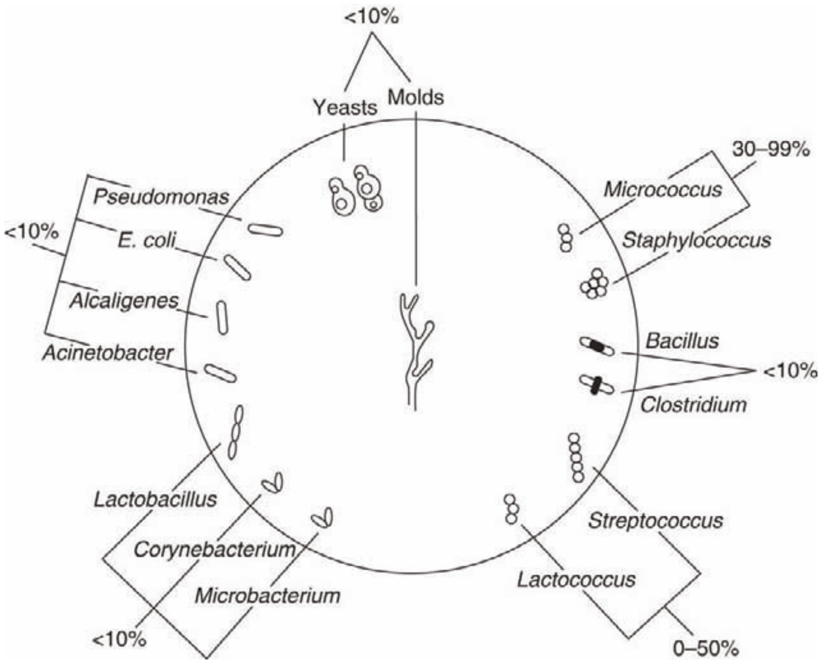
<10%
Yeasts
Molds
30–99%
Micrococcus
Staphylococcus
Pseudomonas
<10%
E. coli
Alcaligenes
Acinetobacter
Bacillus
<10%
Clostridium
Lactobacillus
Corynebacterium
Microbacterium
<10%
Streptococcus
Lactococcus
0–50%

Figure 2 Different morphological groups of microorganisms commonly found in raw milk.

cooling the milk prevents growth, toxin production, and spreading of bacteria to humans via the milk chain. *Staphylococcus hyicus* has been isolated from the skin of cows and from the milk of cows with mastitis. Some strains are enterotoxigenic.

Streptococcus spp.

Streptococcus pyogenes is pathogenic to humans and some animals. It resists phagocytosis and produces an erythrogenic toxin that causes scarlet fever. *Streptococcus agalactiae* belongs to group B streptococci (GBS) and is an important agent of bovine mastitis in some countries and a cause of diseases in humans. The gastrointestinal tract is the main human reservoir. *Streptococcus dysgalactiae* belongs to Lancefield group C streptococci. It is found mainly in mastitic bovine udders. *Streptococcus uberis* is found in the lips and skin of cows, udder tissue, and milk. It is the main cause of mastitis in some countries, especially during the winter. The only phenotypic criterion that differentiates between *Sc. uberis* and *Sc. parauberis* is the ability of the former to produce β-D-glucuronidase. *Streptococcus equi* subsp. *equi* and *Sc. equi* subsp. *zooepidemicus* are β-hemolytic group C *Streptococcus* species that infect animals and humans, respectively.

Campylobacter jejuni

Campylobacter jejuni is a common cause of human gastroenteritis. It is a Gram-negative, curved, slender, motile rod and is found in food, feces, and water. Although it does not grow in milk, the consumption of fresh raw milk has caused numerous outbreaks of gastroenteritis (*see* **Pathogens in Milk**: *Campylobacter* spp.). Results from NAHMS Dairy 2002 indicated that the percentage of cows shedding *Campylobacter* spp. was 51.4%, while the percentage of dairy herds with at least one cow shedding *Campylobacter* was 97.9%.

Yersinia spp.

The genus *Yersinia* belongs to the family Enterobacteriaceae. Members of this genus are Gram-negative, motile rods, facultatively anaerobic at 28–29 °C but strictly aerobic at 37 °C, which can grow at 2–4 °C. *Yersinia enterocolitica* is the only species of importance in milk. The most common source of contamination is the rinsing water used at the farm. *Yersinia* species grow well in milk and cause an enteric infection that mimics appendicitis (*see* **Pathogens in Milk**: *Yersinia enterocolitica*).

Salmonella spp.

The genus *Salmonella* (family Enterobacteriaceae) consists of small, Gram-negative, non-spore-forming rods. Most strains are motile. The primary habitat is the intestinal tract. Cows suffering from salmonellosis can excrete large numbers of salmonellae in their feces and occasionally directly into milk. Milk can be contaminated with *Salmonella* from feces, livestock feed, humans, water, and dust. Direct contact from other herds can result in the introduction of *Salmonella* spp. into dairy farms. Many outbreaks of salmonellosis have been attributed to milk and milk products. Unlike staphylococcal food poisoning, the ingestion of viable cells of *Salmonella* is necessary for salmonellosis. Raw milk or dairy products made from raw milk have been the cause of outbreaks of salmonellosis in the Western world. Nontyphoid *Salmonella enterica* infects an estimated 2–4 million people every year in the United States. Growth of *Salmonella* in milk is not necessary as ingestion of only a few cells can cause the disease (*see* **Pathogens in Milk**: *Salmonella* spp.).

Escherichia coli

Escherichia coli is a member of the family Enterobacteriaceae. It is a facultatively anaerobic, Gram-negative rod, and ferments lactose. Four categories of pathogenic *E. coli* are recognized: enteropathogenic, enterotoxigenic, enteroinvasive, and enterohemorrhagic. Enterotoxigenic *E. coli* produces enterotoxins that cause diarrhea. Humans are the main reservoir. To cause diarrhea, sufficient numbers of toxigenic *E. coli* must be ingested and they must adhere to the small intestine. In recent years, enterohemorrhagic *E. coli*, mainly *E. coli* O157:H7, have caused milk-borne illness. These pathogens cause hemorrhagic colitis (bloody diarrhea), an illness that can lead to renal failure in children. The dairy cow gut is a reservoir for *E. coli* O157:H7. Hemorrhagic strains of *E. coli* do not ferment sorbitol and the incubation temperature used to detect fecal coliforms (44–45 °C) will not allow *E. coli* O157:H7 to grow (*see* **Pathogens in Milk**: *Escherichia coli*). Non-O157 shiga toxin-producing *E. coli* (STEC) was also associated with raw milk and raw milk cheese.

Listeria spp.

Within the genus *Listeria*, only *Li. monocytogenes* and *Li. ivanovii* are considered virulent, with *Li. monocytogenes* being the only species of public health concern. *Listeria innocua* is the most frequently encountered nonpathogenic *Listeria* species. *Listeria monocytogenes* is a Gram-positive, short rod with rounded ends that occurs singly, in parallel, or in V shapes. It grows slowly in milk at refrigeration temperature. *Listeria monocytogenes* causes mastitis and abortion in animals. In humans, it causes miscarriages and meningitis in its severe form and flu-like symptoms in its mild form. Raw milk can be contaminated with *Li. monocytogenes* from feces, cows suffering from mastitis, poor-quality silage, and milking equipment (*see* **Pathogens in Milk**: *Listeria monocytogenes*).

Mycobacterium spp.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is one of the most heat-resistant pathogenic microorganisms likely to be present in milk. *Mycobacterium tuberculosis* may gain access to milk from infected animals through milk secretion and fecal contamination or from milkers and other environmental sources such as cow alleyways, manure storage, calving area, sick cow pen, water runoff, and postweaned calves areas. The organism does not multiply in milk but survives in unpasteurized milk and milk products.

MAP causes the infection commonly known as Johne's disease. Detectable numbers of this organism have been reported in the milk of both clinically affected and asymptomatic cattle. Pasteurization inactivates MAP when present at levels associated with milk from asymptomatic cattle. It is unknown whether this microorganism is pathogenic for man.

Mycobacterium bovis causes infection in animals and humans. Compared with *M. tuberculosis*, growth of *M. bovis* is less luxuriant and it adapts to aerobic growth after repeated subculturing. Contaminated milk, feces, soil, and water are the main sources of infection (*see* **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease; Infectious Diseases: Tuberculosis).

Brucella spp.

Brucella species are Gram-negative, aerobic, short rods that do not ferment carbohydrates. They survive well in milk and dairy products and are pathogenic to humans and animals. The most important causes of brucellosis in milk are *Br. abortus* and *Br. melitensis* (*see* **Pathogens in Milk**: *Brucella* spp.).

Coxiella burnetii

Coxiella burnetii is a rickettsia (family Rickettsiaceae), which causes Q fever in humans. Rickettsiae are obligate intracellular parasites. *Coxiella burnetii* is a small pleomorphic, sometimes filamentous, nonmotile rod that produces a capsule-like structure. It is highly resistant to chemical and physical disinfectants and is the most heat-resistant pathogen found in milk (*see* **Pathogens in Milk**: *Coxiella burnetii*).

Aeromonas spp.

Aeromonas species are facultatively anaerobic, Gram-negative, motile cocci or rods that grow in media containing up to 5% salt. *Aeromonas hydrophila* is an opportunistic pathogen found in the feces of healthy animals.

Bacillus spp.

Bacillus species are Gram-positive, aerobic, spore-forming rods belonging to the family Bacillaceae. Their spores make them resistant to heat and other destructive agents. Strains can be psychrotrophic, mesophilic, or thermophilic. *Bacillus cereus* is a common soil bacterium that is often found in raw milk. It is capable of producing enterotoxins (especially in starchy foods), which cause food poisoning. *Bacillus anthracis* causes anthrax, which is a deadly acute infectious disease. Purposeful contamination of foods by *B. anthracis* was considered a potential biological weapon. The presence of *Bacillus* in raw milk shows marked seasonal variation. Soil, feces, bedding, air, silage (possibly via feces), and the milking equipment are the main sources of *Bacillus* in milk (*see* **Pathogens in Milk**: *Bacillus cereus*).

Clostridium spp.

Clostridia are Gram-positive, spore-forming rods, which are usually found in sediments, the intestinal tract of humans and animals, soil, dust, feces, and silage. They are anaerobic and often poisoned by atmospheric oxygen unless in the spore form.

Clostridium perfringens causes food poisoning and gas gangrene in humans and a variety of diseases in animals. Milk and milk products provide a ready medium for transmission of *Cl. perfringens*. Vegetative cells sporulate in the gut and release exterotoxin, which is responsible for symptoms such as profuse diarrhea, sudden acute inflammation of the abdomen, and severe abdominal pain. Large numbers of cells must be consumed for illness to occur.

Botulism is an intoxication attacking the nervous system caused by ingestion of toxins produced by *Cl. botulinum*. Very low levels (<1 spore ml^{-1}) of these organisms are common in milk. They do not germinate or grow in raw or pasteurized milk, but cheese and cheese products can support growth if they are not sufficiently acidic (*see* **Pathogens in Milk**: *Clostridium* spp.).

Xanthomonas maltophilia

Xanthomonas maltophilia, previously known as *Pseudomonas maltophilia*, is an aerobic rod of the family Pseudomonadaceae. It is one of several pseudomonad-like organisms in hospital laboratories associated with serious infections. It has been isolated from milk and water.

Klebsiella pneumoniae

Klebsiella pneumoniae belongs to the family Enterobacteriaceae and is an agent for pneumonia, urinary tract infection, and gastroenteritis and mastitis. It is present in animal bedding materials and water. Fecal shedding of *Kl. pneumoniae* is considered one of the sources of this microorganism in milk. Inorganic bedding such as sand reduces its occurrence.

Serratia spp.

Serratia species are Gram-negative, facultative, anaerobic rods of the family Enterobacteriaceae. They are distributed in soil, air, water, and plants. Some strains produce hemolysins and infected animals produce contaminated milk. Their presence in pasteurized dairy products indicates poor sanitation practices since they are heat sensitive. *Serratia marcescens* is the most important species and causes mastitis.

Proteus spp.

Proteus species are Gram-negative, non-spore-forming rods belonging to the family Enterobacteriaceae. They are commonly found in the feces of humans and animals, soil, and plants. They frequently cause a variety of infections in humans, especially urinary tract infections. *Proteus mirabilis* is the most common species followed by *Pr. vulgaris* and *Pr. penneri*.

Enterobacter sakazakii

Enterobacter sakazakii belongs to the family Enterobacteriaceae. Although rarely found, it can cause life-threatening neonatal meningitis. It does not survive pasteurization but is found in ultra-high temperature (UHT) milk and milk powder that have been subjected to postprocessing contamination. Its natural habitat is not known.

Hafnia alvei

Hafnia alvei, which belongs to the family Enterobacteriaceae, resembles *Salmonella*. Some strains cause mild gastroenteritis. It is found in sewage, soil, water, and feces. *Hafnia* species can grow on the surface of Camembert cheese (*see* **Pathogens in Milk**: Enterobacteriaceae).

Actinomyces spp.

Actinomyces species are irregular, non-spore-forming, Gram-positive rods that occur mainly in the oral cavity and on the mucous membranes of warm-blooded vertebrates. They cause pyogenic infections. *Actinomyces bovis* and *Ac. pyogenes* are the important species in milk.

Leptospira spp.

Species of the genus *Leptospira* (family Leptospiraceae) are Gram-negative, flexible, helicoidal rods. *Leptospira interrogans* causes leptospirosis in animals and humans. The kidney is the natural habitat and contaminated urine is the main source of contamination.

Molds and Yeasts

Many different genera of molds such as *Aspergillus*, *Penicillium*, and *Fusarium* produce mycotoxins in milk and dairy products. Mycotoxins are secondary metabolites that are acutely toxic, carcinogenic, emetic, estrogenic, hallucinogenic, mutagenic, or teratogenic. *Aspergillus flavus* and some other *Aspergillus* species produce aflatoxins when growing on cheeses. Some strains of *Geotrichum* might cause infection in humans. *Candida albicans* is pathogenic to humans and animals (*see* **Yeasts and Molds**: Spoilage Molds in Dairy Products; **Yeasts and Molds**: Yeasts in Milk and Dairy Products).

Viruses

Viruses are genetic elements that replicate inside cells and also have an extracellular nonreplicating state. Replication of the virus often destroys the host cell causing illness. The extracellular form of the virus is a submicroscopic particle and can be transmitted between hosts. Viruses do not multiply in milk but some may survive for long periods. Viruses can be infective at very low doses and most that are found in milk produce gastroenteritis and originate with the cow. Viruses in milk are inactivated by pasteurization.

Rotaviruses can produce severe gastrointestinal illnesses. Milk may be a vector for this virus but detection in raw milk is prevented by the presence of antiviral antibodies (immunoglobulins; IgG). The antibody/virus complex is dissociated in the highly acidic conditions of the stomach.

Hepatitis A virus enters milk through fecal contamination or polluted water. This virus causes symptoms of fever, vomiting, nausea, and abdominal discomfort. The virus eventually affects the liver, causing enlargement and jaundice.

Poliovirus causes the acute infectious disease poliomyelitis, which is characterized by fever, vomiting, and headache. The poliovirus might infect the central nervous system and cause paralysis. It can be transmitted to milk through fecal contamination.

Milk can serve as a vector for the spread of the foot-and-mouth disease virus. This virus, which is nonenveloped, belongs to the Picornaviridae family, which also includes rhinoviruses and enteroviruses.

Protozoa

Toxoplasma gondii is a parasite of humans and many warm-blooded animals, especially dairy goats. Infection occurs as a result of ingestion of foods or water contaminated with oocysts. Contaminated milk is considered a potential source of human toxoplasmosis.

Cryptosporidiosis is an infection caused by *Cryptosporidium muris* and *Cr. parvum.* Diarrhea is the primary symptom of infection. Cows can shed *Cr. muris* oocysts in their feces, which may lead to contamination of milk. Drinking water is the most common vehicle for transmission of this disease.

Entamoeba histolytica is a protozoan that causes amoebiasis, the third most common cause of death by parasites in the world. Transmission occurs via ingestion of cysts in contaminated foods or water or through poor personal hygiene.

Giardia intestinalis is a flagellated protozoan that inhabits the intestinal tracts of humans and animals. Cysts survive well in the environment and are transmitted via contaminated hands, drinking water, and food contaminated with feces.

Spoilage Microorganisms

Spoilage of milk and dairy products is manifest as off-flavors and odors and changes in texture and appearance. The most important spoilage microorganisms of milk and dairy products are the Gram-negative, rod-shaped bacteria (e.g., *Pseudomonas* and coliforms), Gram-positive, spore-forming bacteria (*Bacillus* and *Clostridium*), lactic acid bacteria, members of coryneform group, yeasts, and molds.

Psychrotrophic Bacteria

Most psychrotrophic bacteria present in raw milk belong to the family Pseudomonadaceae, which includes Gram-negative, non-spore-forming, nonfermentative, aerobic rods. *Pseudomonas fluorescens* is the species most commonly isolated from milk, with *Ps. fragi* and *Ps. putida* being of less importance. Soil, water, animals, and plant material are the natural habitat of psychrotrophic bacteria found in milk. Contaminated milk equipment and air provide an additional source of contamination, especially after processing. As little as 1 cfu ml^{-1} of raw milk may be enough to spoil milk during cold storage within 5 days. Some *Pseudomonas* spp. grow at cold and warm temperatures, and others found in milk cannot propagate at temperatures greater than 25 °C. Defects associated with the growth of these organisms in milk include development of bitter and fruity off-flavors. Although psychrotrophic, Gram-negative bacteria are not heat resistant, they produce heat-stable extracellular proteolytic and lipolytic enzymes that can spoil heat-treated products. However, not all Gram-negative isolates from raw milk display proteolytic activities at 7 or 20 °C. Other spoilage Gram-negative rods include *Acinetobacter*, *Psychrobacter*, *Flavobacterium*, *Shewanella putrefaciens*, and *Alcaligenes* spp. Cold-tolerant endospore-forming bacteria such as *Bacillus* and *Paenibacillus* spp. in raw milk are considered a major cause of spoilage of pasteurized milk.

Family Moraxellaceae

Moraxellaceae are Gram-negative, psychrotrophic, non-pigmented, nonmotile, aerobic coccobacilli. There are three genera: *Acinetobacter* are nonmotile, oxidase-negative organisms, *Moraxella* are similar but oxidase-positive, and the third genus is *Psychrobacter*. Moraxellaceae seldom spoil milk because they lack sufficient biochemical activities such as proteolysis and lipolysis that cause off-odors. In addition, they are overgrown by *Pseudomonas* during cold storage.

Shewanella putrefaciens

Shewanella putrefaciens, previously known as *Pseudomonas putrefaciens* or *Alteromonas putrefaciens*, is a Gram-negative rod. It is found in water and soil and contaminates milk and milk products causing spoilage such as surface taint in butter.

Flavobacterium spp.

Flavobacterium species are Gram-negative organisms found in water, soil, and milk. They hydrolyze casein and cause spoilage in milk and dairy products during cold storage. Important species are *Fb. maloloris*, *Fb. aquatile*, and *Fb. lutensis*.

Alcaligenes spp

Alcaligenes species are Gram-negative, short, motile rods. They are aerobic and do not use lactose. They are found in water, soil, and milk. *Alcaligenes faecalis* produces the exopolysaccharide curdlan and is a potential contaminant of dairy products. *Alcaligenes* can grow under refrigeration conditions, producing off-flavors and reducing the shelf life of milk.

Coliforms

Coliforms are Gram-negative, oxidase-negative, non-spore-forming rods that grow aerobically or facultatively anaerobically in the presence of bile salts. They also ferment lactose to produce acid and gas within 48 h at 37 °C. Coliforms include the genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus*, *Serratia*, and *Citrobacter*. They do not survive pasteurization. Gas production may result in early blowing of hard cheese and a poor curd structure in Cottage cheese. Approximately 10^7 cfu g^{-1} of coliforms is needed for a gassy defect to be noticed. Other types of spoilage include acid production, slime production in Cottage cheese, bitter flavors, and grassy, unclean, medicinal, or fecal odors. Cheese varieties in which acid production is delayed are more susceptible to coliform growth. *Klebsiella* (*Kl. pneumoniae* and *Kl. oxytoca*) and *Enterobacter aerogenes* are responsible for gas formation in cheese, which can lead to swelling of the package. *Serratia marcescens* produces proteolytic enzymes that might cause gelation of UHT milk. In addition, they produce methanethiol in Cheddar cheese. Soiled udders and teats are common sources of coliforms. Coliforms can also grow and contaminate milk from poorly sanitized milk contact surfaces and equipment.

Spore-Forming, Gram-Positive Rods

Two genera in this group are associated with milk: *Bacillus* and *Clostridium*. They are unique spoilage microorganisms because of their ability to form heat- and chemical-resistant spores and their ability to grow over a wide temperature range (>0–75 °C). Their main habitat is soil, but they can also be found in water, air, fodder, and feed. New subtyping methods for DNA sequencing of the *rpoB* gene have reclassified some *Bacillus* into new genera such as *Paenibacillus* and *Alicyclobacillus*.

Bacillus cereus causes defects in high-temperature-treated milk and cream including sweet curdling (curd formation with no acid production). It also produces lecithinase, an enzyme that degrades fat globule membranes resulting in fat aggregation in cream (bitty cream). *Bacillus subtilis* survives pasteurization, and milk concentration and perhaps UHT treatment and produces a variety of enzymes that are capable of hydrolyzing casein and polysaccharides. *Bacillus licheniformis* is a common cause of spoilage in UHT and pasteurized milk. *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) is extremely heat resistant; it is more resistant than *Cl. botulinum* spores. It can survive canning and can cause flat sour spoilage and sweet curdling defects with little or no gas formation. This microorganism is also used to detect antibiotics in milk through growth inhibition assays. *Bacillus coagulans* has also been implicated in the spoilage of UHT and concentrated milk; it also produces chymosin-like enzymes, which cause sweet curdling. *Bacillus circulans* is a psychrotrophic bacterium, which causes an acid defect in aseptically packaged heat-treated milk. *Bacillus macerans* causes a flat sour defect in canned condensed milk.

Clostridium species are important spoilage microorganisms in cheese and canned milk products. Late blowing of some cheese varieties that have a relatively high pH, a high moisture content, and a low interior salt content, such as Emmental, Gouda, and Edam, is caused by these microorganisms. *Clostridium tyrobutyricum* and, occasionally, *Cl. sporogenes* and *Cl. butyricum* are involved. These clostridia produce carbon dioxide, hydrogen gas, and butyric and acetic acids.

Lactic Acid Bacteria and Related Organisms

Many species of lactic acid bacteria cause souring, off-flavors, and texture defects (ropiness, curdling) in milk and dairy products. The general characteristics of lactic acid bacteria will be discussed later.

Lactobacillus spp.

Heterofermentative lactobacilli such as *Lb. brevis* and *Lb. casei* subsp. *pseudoplantarum* cause an open texture in Cheddar and Mozzarella cheeses due to gas production. Lactobacilli also convert L(+)-lactate to D(−)-lactate, which reacts with calcium to form calcium lactate, which appears as white insoluble crystals in ripened Cheddar and other cheeses. Salt-tolerant lactobacilli produce phenolic and putrid sulfide-like flavors during ripening. *Lactobacillus casei* subsp. *casei* causes a soft body in Mozzarella cheese due to proteolysis. *Lactobacillus delbrueckii* subsp. *bulgaricus* can produce a pink discoloration in Italian cheese. *Lactobacillus casei* species sometimes produce a phenolic flavor in Cheddar cheese.

Lactococcus spp.

Lactococcus lactis is commonly used as a starter culture for cheese and fermented milks. These strains are carefully selected so that they do not produce off-flavors or other defects. Wild strains of lactococci often contaminate raw milk, and if the milk is not kept sufficiently cold they will produce sourness and other off-flavors and odors. One variant, *Lc. lactis* subsp. *lactis* var. *maltigenes*, produces a malty flavor in fluid milk due to the production of 3-methylbutanal. Wild strains of lactococci may also contaminate cheeses and grow during manufacture and aging. Some of these strains produce fruity flavors due to the production of esters such as ethyl hexanoate and ethyl butyrate.

Propionibacterium spp.

Pigmented strains of *Propionibacterium* cause pink spots in Swiss cheese. Split formation is influenced by *Prop. freudenreichii* subsp. *shermanii* strains used in making Swiss cheese.

Enterococcus spp.

Enterococcus faecalis, *Ec. faecium*, and *Ec. durans* are part of the normal microflora of many cheeses and are sometimes used as starter cultures. Some strains produce undesirable flavors and high levels of amines during cheese ripening.

Some *enterococci* are also considered probiotic. Two species, *Ec. faecalis* and *Ec. faecium*, were formerly classified as fecal streptococci (*see* **Lactic Acid Bacteria**: *Enterococcus* in Milk and Dairy Products).

Micrococcus spp.

Some strains of *Micrococcus* species are able to survive pasteurization, but spoilage of heat-treated products is also caused by postprocessing contamination. *Micrococcus* species can produce swelling in UHT milk packs and have the ability to grow on some cheese varieties. Their growth may be either beneficial or detrimental to cheese flavor, depending on the cheese variety and strains involved.

Yeasts

The presence of large numbers of yeasts on a cheese surface may result in a slimy rind, discolored appearance, and undesirable flavors. Non-lactose-fermenting *Saccharomyces* produce gas in fruit yogurt. Their source is usually a contaminated processing plant environment or unpasteurized fruits. *Saccharomyces cerevisiae* can spoil sweetened and acid dairy products.

Kluyveromyces marxianus var. *lactis*, *K. marxianus* var. *marxianus*, *Debaryomyces hansenii*, and *Yarrowia lipolytica* spoil acidified refrigerated dairy products such as yogurt. *Yarrowia lipolytica* does not utilize lactose but metabolizes fat, protein, and organic acids, allowing it to spoil high-fat products such as butter and cream. It liberates amino and fatty acids and usually causes off-odors and softening of cheese curd. In addition, it produces melanin pigment from tyrosine and causes discoloration of cheese surfaces.

Candida species are potential dairy spoilage organisms because of their ability to digest casein and fat, grow at storage temperatures, and ferment lactose and sucrose. They form surface slime on cream cheese. In yogurt, they cause yeasty and bitter flavors and a gassy texture. The important spoilage species in cheese is *Can. famata* and in yogurt *Can. famata*, *Can. versatilis*, and *Can. lusitaniae.*

Molds

Mucor, *Rhizopus*, *Penicillium*, and *Aspergillus* grow at the yogurt–air interface of undisturbed packages. One mold spore can spoil a carton of yogurt by producing a visible colony. Molds also produce off-flavors in contaminated dairy products. *Scopulariopsis brevicaulis* causes odor defects in mold-ripened cheese. *Sporendonema sebi* grows on sweet condensed milk forming discrete colonies (mold buttons). *Geotrichum* grows on most dairy products, especially cheese and butter. Cheese varieties with low salt content are often spoiled by *Mucor* species.

Beneficial Microorganisms in the Dairy Industry

Selected lactic acid bacteria and other microorganisms are used by the dairy industry to produce fermented products such as yogurt and cheese. Commercially available starter cultures have been selected for a variety of desirable properties such as flavor production, lack of associated off-flavors, fast acid production, bacteriophage resistance, salt tolerance, exopolysaccharide production, bacteriocin production, and heat sensitivity. These microbial preparations may consist of different species of lactic acid bacteria, propionibacteria, surface-ripening bacteria, yeasts, and molds.

Lactococcus spp.

Lactococci are the major acid-producing microorganisms used for dairy fermentations, *Lc. lactis* being the main species of importance. *Lactococcus lactis* is homofermentative and weakly proteolytic. There are two subspecies: *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. Citrate-positive strains of *Lc. lactis* (*Lc. lactis* subsp. *lactis* biovar *diacetylactis*) utilize citrate to produce diacetyl, carbon dioxide, and other compounds in cheeses and cultured milks. Some strains are highly ropy.

Streptococcus spp.

Streptococcus thermophilus is used to produce acid in dairy products subjected to high temperatures during fermentation (yogurt and Mozzarella cheese). It is weakly proteolytic and some strains produce extracellular polysaccharides.

Leuconostoc spp.

Leuconostoc species are heterofermentative, Gram-positive cocci. They produce flavor compounds such as diacetyl and acetoin, and some strains produce exopolysaccharides. *Leuconostoc mesenteroides* subsp. *cremoris* is used in the production of cottage and cream cheese and cultured milks. *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Ln. mesenteroides* subsp. *dextranicum* often produce slime (glucan, formerly known as dextran) from sucrose. *Leuconostoc paramesenteroides* does not produce glucan and is used in making brined cheese. *Leuconostoc lactis* is also found in milk and dairy products. *Leuconostoc* species reduce an undesirable 'green' flavor in cultured dairy products by converting acetaldehyde to diacetyl, but grow only slowly in milk.

Lactobacillus spp.

Lactobacillus species are a genetically and physiologically diverse group of rod-shaped lactic acid bacteria. They are the most acid tolerant of the lactic acid bacteria and produce numerous proteolytic enzymes. There are three groups based on the type of fermentation end products: homofermentative (*Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. acidophilus*, *Lb. helveticus*, and *Lb. helveticus* subsp. *jugurti*), facultatively heterofermentative (*Lb. casei*, *Lb. curvatus*, and *Lb. plantarum*), and obligately heterofermentative (*Lb. kefir*, *Lb. fermentum*, and *Lb. brevis*). Some lactobacilli, such as *Lb. acidophilus*, are claimed to confer health benefits. Homofermentative lactobacilli are usually thermophilic and are used to produce fermented milk and some cheese varieties that require the use of high temperature during manufacturing. *Lactobacillus casei* and *Lb. plantarum* cannot grow at high temperatures (at 45 °C). They are involved in the ripening of Cheddar and other cheese varieties.

Propionibacterium spp.

Propionibacteria produce propionic and acetic acids and carbon dioxide from sugars and lactic acid. They are responsible for eye formation in Swiss cheese. *Propionibacterium freudenreichii*, *Prop. jensenii*, *Prop. thoenii*, and *Prop. acidipropionici* are the species commonly found in cheese.

Coryneform Bacteria

Coryneform bacteria are a group of irregular, non-spore-forming, Gram-positive rods, often found growing on the surface of surface-ripened cheese. From a taxonomic viewpoint, dairy species differ from the reference pathogenic species. Cheese smear coryneform bacteria are mostly psychrotrophic and do not grow at 37 °C, whereas pathogenic coryneform bacteria are facultative anaerobes and grow at 37 °C. The cheese smear group includes *Brevibacterium*, *Arthrobacter*, *Microbacterium*, *Aureobacterium*, *Brachybacterium*, *Rhodococcus*, and *Corynebacterium*. These microorganisms are responsible for the viscous, red-orange surface (smear) on surface-ripened cheese varieties. They survive high salt concentrations and produce various proteolytic enzymes. Their presence in large numbers causes a slimy rind, discolored appearance, and undesirable flavors in cheese. Currently, the genus *Brevibacterium* is restricted to four species: *Brev. linens*, *Brev. iodinum*, *Brev. casei*, and *Brev. epidermidis*. *Arthrobacter* species include *Ab. globiformis*, *Ab. nicotianae*, *Ab. protophormiae*, *Ab. agilis*, *Ab. sulphurous*, and *Ab. citreus*. The genus *Microbacterium* contains *Microb. lacticum*, *Microb. laevaniformans*, *Microb. imperiale*, and *Microb. arborescens*. The genus *Aureobacterium* contains *Au. liquefaciens* and *Au. testaceum*. *Brachybacterium alimentarium* and *Brach. tyrofermentans* are two new species of coryneform bacteria isolated from the surface of Gruyère and Beaufort cheeses and they tolerate up to 20% salt. *Corynebacterium* species found in dairy products include *C. ammoniagenes* and *C. variabilis*. *Rhodococcus fascians* was also isolated from cheese. The habitats of coryneform bacteria are cheese and human skin (*see* **Bacteria, Beneficial**: *Brevibacterium Linens*, *Brevibacterium Aurantiacum* and Other Smear Microorganisms).

Bifidobacterium spp.

Bifidobacterium species are inhabitants of the intestinal tract, which are added to dairy products for probiotic applications. The species most often used are *Bif. longum*, *Bif. bifidum*, and *Bif. animalis*. *Bifidobacterium* species grow poorly in milk (*see* **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology).

Pediococcus spp.

Pediococcus species are salt-tolerant members of the family Streptococcaceae, which usually grow as tetrads of cells. Most species can grow at 4–6.5% NaCl. They produce acetic acid and small amounts of lactic acid when growing aerobically and mostly lactic acid when growing anaerobically. The most important species used in the dairy industry are *Pc. acidilactici* and *Pc. pentosaceus* (*see* **Lactic Acid Bacteria**: *Pediococcus* spp.).

Micrococcus spp.

Micrococcus species are the predominant microorganisms found in raw milk drawn aseptically from the udder. They are the constituents of the natural microflora of teat skin and have also been isolated from soil, water, and dust. Based on phylogenetic and chemotaxonomic analysis, many micrococci (except *Mc. luteus* and *Mc. lylae*) have been renamed. The new names are *Kocuria varians*, *Kc. roseus*, *Kc. kristinae*, *Dermacoccus nishinomiyaensis*, *Kytococcus sedentarius*, *Ab. agilis*, and *Nesterenkonia halobia*. *Micrococcus* species have proteolytic, lipolytic, and esterase activities, which make them important in the ripening of various cheeses, especially those that are surface-ripened.

Molds

Penicillium species are widely distributed in the environment. This genus includes four subgenera. The surface of soft white cheese can be colonized by *P. camemberti* Thom to produce many different cheese varieties such as Brie and Camembert. This mold produces a white layer of the mycelium on the cheese surface, while spores (candida) are embedded in the curd. The mold metabolizes some of lactic acid thereby raising the pH, which allows growth of *Brev. linens* and other ripening microflora. In blue-veined cheese varieties, *P. roqueforti* (blue mold) is used in cheese ripening. *Penicillium roqueforti* has a relatively low oxygen requirement for growth (<4.2%) and tolerates 6–10% salt. Both *P. camemberti* and *P. roqueforti* produce lipolytic and proteolytic enzymes. They produce additional compounds that give cheese its distinctive flavor and aroma. *Penicillium casei* is similar to *P. roqueforti* and is found in Swiss cheese. *Penicillium nalgiovense* (Lava), another white mold, tolerates up to 8% salt and is used in some cheese varieties.

Aspergillus niger and *A. awamori* produce lipase and contribute to the flavor of some cheese varieties. *Geotrichum candidum* is often found in raw milk, on milking and dairy processing equipment, and on the surface of ripened cheese. It can be used in starter cultures for Brie and Camembert cheese when combined with *Penicillium* species. *Rhizomucor miehei* and *Rm. pusillus* produce chymosin-like enzymes, which are used in cheese manufacture.

Yeasts

Saccharomyces species are ascosporogenous yeasts with a capability for vigorous anaerobic or semianaerobic fermentation of sugar to produce ethanol and carbon dioxide. None of them utilizes lactose. *Saccharomyces cerevisiae* is the most important species and can be found on the surface of mold-ripened cheeses. *Saccharomyces cerevisiae* metabolizes hexoses, lactic acid, and other organic acids. The optimum pH for the growth of *Saccharomyces* species is 4.5–6.5. Oxygen is important to maintain viability but they survive under microaerophilic conditions.

Kluyveromyces marxianus and *K. lactis* can ferment lactose and produce various hydrolytic enzymes. *Kluyveromyces* species are often isolated from Nordic fermented milks. They produce lactase, which can be used to reduce the lactose content of milk and assist in the digestion of milk by lactose maldigesters.

Debaryomyces species are isolated from soil, water, and plants. *Debaryomyces hansenii* is involved in the ripening of mold-ripened soft cheeses. It tolerates high salt concentrations, utilizes lactic acid, produces protease and lipase, and grows well at low temperature. *Candida kefyr* is present in the microflora of the fermented milk kefir.

Microbial Population Dynamics in Milk

Microbial populations in milk are continually changing and interacting with the milk components and with each other. The Pasteurized Milk Ordinance in the United States requires grade A milk to have a total bacterial count of less than 100 000 cfu ml^{-1}. The initial microbial population of raw milk is affected by factors such as equipment and animal cleanliness, season, feed, and cow health. The initial microflora of good quality milk mainly consists of organisms found within the udder and the normal teat skin flora. These microorganisms include non-thermoduric micrococci and streptococci, which do not grow well at low temperatures. Pathogens will predominate in milk from mastitic cows. Seasonal factors include feed source and housing, both of which influence the level of bacterial spores in milk. Psychrotrophic bacteria (those that grow at low temperatures) are at low or even undetectable levels in the best quality fresh raw milk. They originate from the water and milking equipment. After collection, milk is cooled, which initiates a change in microbial population from Gram-positive rods and cocci, which do not grow, to Gram-negative rods, which can grow slowly at low temperatures. These Gram-negative rods are primarily *Pseudomonas* spp. and are capable of causing various off-flavors when populations exceed 10^6 cells ml^{-1}.

When raw milk is not kept cold, lactic acid bacteria will grow. The resulting increase in acidity will inhibit the growth of many other microorganisms including spore-forming *Bacillus* and *Clostridium* species and Gram-negative *Pseudomonas* species and coliforms. Growth of lactic acid bacteria is rapid at 20 °C and slows as the temperature decreases. Many strains of lactic acid bacteria produce bacteriocins, which inhibit other lactic acid bacteria as well as some pathogens such as *Listeria* and *E. coli*.

Both the lactic acid bacteria and the psychrotrophic bacteria that grow in raw milk are heat sensitive, so when milk is pasteurized, microorganisms that have previously grown in the product are greatly reduced in number. The surviving microflora mainly consists of spore-forming bacteria, micrococci, and lactobacilli. Pasteurized milk that is not recontaminated with microorganisms will often keep at refrigeration temperature for 3 weeks or longer, because these surviving microorganisms grow slowly or not at all in the cold milk. Spoilage may occur more rapidly if significant numbers of psychrotrophic *B. cereus* are present in the raw milk. Most pasteurized milk is recontaminated with low numbers of psychrotrophic bacteria, resulting in a shorter shelf life.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology; *Brevibacterium linens*, *Brevibacterium Aurantiacum* and Other Smear Microorganisms. **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease; Infectious Diseases: Tuberculosis. **Lactic Acid Bacteria**: *Enterococcus* in Milk and Dairy Products; *Pediococcus* spp.. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Pathogens in Milk**: *Bacillus cereus*; *Brucella* spp.; *Campylobacter* spp.; *Clostridium* spp.; *Coxiella burnetii*; Enterobacteriaceae; *Escherichia coli*; *Listeria monocytogenes*; *Salmonella* spp.; *Yersinia enterocolitica*. **Yeasts and Molds**: Spoilage Molds in Dairy Products; Yeasts in Milk and Dairy Products.

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MILK

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Introduction

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Introduction

Milk is a fluid secreted by the female of all mammals, of which there are about 4500 species, for the nutrition of its offspring. In addition to meeting the complete nutritional requirements of the neonate, milk serves several physiological functions: immunoglobulins and other antibacterial agents present in milk have a protective effect; enzymes and enzyme inhibitors, and binding or carrier proteins of the milk aid digestion; and growth factors/ hormones of milk have growth-promoting activity. Because the nutritional and physiological requirements of each species are more or less unique, there are marked interspecies differences in the composition of milk; however, the milks of only about 200 species have been analyzed and, of these, the data for only about 50 species are considered to be reliable (due to sufficient number of samples, representative sampling, adequate coverage of the lactation period and health and stress status of the animal). Not surprisingly, the milks of the principal dairying species – cow, goat, sheep, and water buffalo – and humans are among those that are well characterized. The gross composition of milk from some species, selected to demonstrate the diversity, is shown in **Table 1**. Its gross composition belies the great complexity of milk. For example, it has been reported that bovine milk fat contains up to 400 fatty acids (FAs; more than any other fat), resulting in several thousand triglycerides (TGs) and complex lipids. In addition to the eight main proteins (α_{S1}-, α_{S2}-, β-, and κ-caseins, β-lactoglobulin, α-lactalbumin, blood serum albumin, and immunoglobulins), there are at least 80 minor proteins, including about 60 enzymes. Milk contains at least 30 inorganic elements, which are important from technological, stability, and nutritional viewpoints. It contains all known vitamins, some at quite high concentrations, presumably reflecting the needs of the particular species. There are separate articles in this encyclopedia on all important constituents of milk and on the milk of all major and minor commercial dairy species, as well as some extreme species

Table 1 Composition (%) of the milk of selected species

Species	*Total solids*	*Fat*	*Protein*	*Lactose*	*Ash*
Human	12.2	3.8	1.0	7.0	0.2
Chimpanzee	10.4	1.1	1.0	8.0	-
Cow	12.7	3.7	3.4	4.8	0.7
Buffalo	16.8	7.4	3.8	4.8	0.8
Goat	12.3	4.8	2.9	4.1	0.8
Sheep	19.3	7.4	4.5	4.8	1.0
Camel	15.0	5.4	3.8	5.2	0.7
Yak	19.3	7.9	5.3	5.2	1.0
Horse	11.2	1.9	2.5	6.2	0.5
Donkey	11.7	1.4	2.0	7.4	0.5
Reindeer	33.1	16.9	11.5	2.8	-
Pig	18.8	6.8	4.8	5.5	1.0
Rabbit	32.8	18.3	11.9	2.1	1.8
Rat	21.0	10.3	8.4	2.6	1.3
African elephant	17.7	6.7	4.7	3.9	0.7
Rhinoceros	7.8	0.2	1.6	6.1	0.3
Polar bear	47.6	33.1	10.9	0.3	1.4
Gray seal	67.7	53.1	11.2	0.7	-
Blue whale	55.0	40.9	11.9	1.3	1.4

(*see* **Animals That Produce Dairy Foods**: Camel; Goat Breeds; Major *Bos taurus* Breeds; Minor and Dual-Purpose *Bos taurus* Breeds; Sheep Breeds; Water Buffalo; **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Production, Applications; **Milk**: Milk of Marine Mammals; Milk of Monotremes and Marsupials; **Milk Lipids**: General Characteristics; **Milk Proteins**: Heterogeneity, Fractionation, and Isolation; **Vitamins**: General Introduction).

Since milk supplies all the nutritional requirements of the neonatal mammal during the period of its most rapid growth, it is not surprising that milk is the most nearly complete natural food; only the whole carcass of an animal, including bones, meets the nutritional requirements of humans better than milk. The early antecedents of *Homo sapiens* were probably vegetarians but became omnivorous at an early stage in evolutionary history – anthropologists suggest that the practice of eating meat was one of the factors that facilitated human evolution because meat provided a concentrated source of nutrients that was largely independent of the season. Until relatively recently, human beings were hunter-gatherers who killed small, and occasionally larger, animals, probably ate carrion, and collected roots, fruits, and nuts when available. It is very unlikely that humans consumed the milk of other species until animals had been domesticated, that is, about 8000 BC. Sheep and goats were domesticated early during the agricultural revolution, 8000–10 000 years ago. Cattle were domesticated later but have become the principal dairying species in the most intense dairying areas, although sheep and goats are very important in arid regions, especially around the Mediterranean. Buffaloes are especially important in India, Pakistan, and Egypt and less important elsewhere. Equine milk is used extensively in central Asia and is receiving attention in Europe for special dietary purposes since its composition is closer to that of human milk than is bovine milk. Donkey milk, the composition of which is also relatively close to that of human milk, is attracting attention for special dietary purposes. Donkey milk appears to have been used for cheese production in classical Greece and was considered preferable to cows' milk for the artificial feeding of infants in the eighteenth and nineteenth centuries, and probably earlier. Other dairying species include the camel, which is quite important in the desert areas of North and East Africa and the Middle East, yak in Mongolia and Tibet, and the reindeer in Lapland. The husbandry of these minor species will be discussed in separate articles in this encyclopedia (*see* **Animals That Produce Dairy Foods**: Camel; Donkey; Horse; Reindeer; Water Buffalo; Yak).

Classification of Mammals

Mammals evolved from premammalian egg-laying reptiles about 200 million years ago. It has been suggested that premammalian reptiles secreted a fluid from sebaceous glands to maintain their shell-less eggs moist and to prevent microbial infection. The hatchlings would have licked off and ingested some of this fluid, which provided nutritional benefits, eventually becoming the principal function of such secretions, leading ultimately to milk production (*see* **Mammals**). The essential distinguishing characteristic of mammals is the ability of the female of the species to produce milk in specialized organs (mammary glands) for the nutrition of its newborn.

It is estimated that about 80% of all mammalian species are extinct (*see* **Mammals**).

The class Mammalia is divided into two subclasses: Prototheria and Theria.

Prototheria

This subclass contains only one order, Monotremata, the species of which are egg-laying mammals, that is, the duck-billed platypus and four species of echidna, all of which are indigenous only to the Australian mainland, Tasmania, and New Guinea. Monotremes were the earliest mammals and, presumably, many species have become extinct. They possess perhaps as many as 200 mammary glands grouped in two areas of the abdomen; the glands do not terminate in a teat and the secreted milk is licked by the young from the surface of the gland. Not surprisingly, the milk of monotremes is very different from that of most other mammals and is described, along with that of marsupials, in the article **Milk**: Milk of Monotremes and Marsupials.

Theria

The young of the subclass Theria are born live (viviparous) and fall into two groups: Metatheria and Eutheria.

Metatheria (Marsupials)

Marsupials (about 200 species) do not have a placenta and the young are born alive after a short gestation and are very premature at birth, to a greater or lesser degree, depending on the species. After birth, the young are transferred to a pouch where they reach maturity. Marsupials include kangaroo, wallaby, and koala.

In marsupials, the mammary glands, which vary in number, are located within the pouch and terminate in a teat. The mother may nurse two offspring, differing widely in age, simultaneously from different mammary glands that secrete milk of very different composition, designed to meet the different specific requirements of each offspring (*see* **Milk**: Milk of Monotremes and Marsupials).

Eutherians (True Placental Mammals)

About 95% of all mammals belong to this group. The developing embryo *in utero* receives nourishment via the placental blood supply (hence, they are referred to as placental mammals) and is born at a high, but variable, species-related state of maturity. Although all eutherians secrete milk, the young of some species are born sufficiently mature to survive and develop without milk.

The number and location of mammary glands vary with the species, from 2 (human, goat, and sheep) to 14–16 for the pig or up to 22 in primitive insectivores. Each gland is anatomically and physiologically separate and is emptied via a teat (*see* **Mammary Gland**: Anatomy; Gene Networks Controlling Development and Involution; Growth, Development, and Involution).

The wide interspecies variations in the composition of milk (**Table 1**) and in the chemistry of its constituents render milk species-specific, that is, designed to meet the requirements of the young of that species. However, in spite of the large interspecies differences, the milk of one species is tolerated moderately well by the young of other species after the first few critical days postpartum. There is also a good relationship between milk yield and maternal body weight; species bred for commercial milk production, for example, dairy cow and goat, fall above the line. The modern dairy cow is the result of selective breeding for high milk yield and produces several times more milk than is required, or even can be consumed, by its offspring.

Structure and Development of Mammary Tissue

The mammary gland of all species has the same basic structure and in all species it is located external to the body cavity, which facilitates research on milk biosynthesis. The structure, development, and control of the mammary gland are quite well understood (*see* **Mammary Gland**: Anatomy; Growth, Development, and Involution).

The principal constituents of milk are synthesized in the specialized cells (mammocytes) in the mammary gland from components obtained from the blood and transported across the basal (outer) membrane of the secretory cell. Within the mammocytes, these components are modified, rearranged, and/or polymerized as they pass inward through the cell; the finished milk constituents are excreted into the lumen across the luminal (apical) membrane. Some constituents are transferred unchanged from the blood to milk, for example, immunoglobulins, blood serum albumin, and vitamins, sometimes against a concentration gradient, indicating active transport. Various techniques have been developed to study the biosynthesis of milk constituents, for example, arteriovenous concentration differences, isotope studies, perfusion of excised gland, tissue slices, cell homogenates, and tissue culture, each of which has advantages and limitations. The synthesis of principal constituents of milk is well established and is discussed in several articles in this encyclopedia (*see* **Mammary Gland, Milk**

Biosynthesis and Secretion: Lactose; Milk Fat; Milk Protein; Secretion of Milk Constituents).

The constituents of milk can be grouped into four general classes according to their source:

- organ- (mammary gland) and species-specific constituents (most proteins and lipids)
- organ- but not species-specific constituents (lactose)
- species- but not organ-specific constituents (some proteins)
- neither organ- nor species-specific constituents (water, salts, vitamins)

The lactating mammary gland is by far the most metabolically active organ of the body of many species. For many small mammals, the energy input required for the milk secreted in a single day may exceed that required to develop a whole litter *in utero*. A cow yielding 50 kg of milk per day secretes approximately 2.4 kg of lactose, 1.8 kg of fat, and 1.7 kg of protein per day. This compares with the daily weight gain for a beef animal of 1–1.5 kg day^{-1}, 60–70% of which is water. In large measure, a high-yielding mammal is subservient to the needs of its mammary gland, to which it must supply not only the precursors for the synthesis of milk constituents but also an adequate level of high energy-yielding substrates (adenosine triphosphate, uridine triphosphate, etc.) required to drive the necessary synthetic reactions. In addition, minor constituents (vitamins and minerals) must be supplied.

Constituents and Variability of Milk

With the exception of water and vitamins, the principal constituents of milk are unique to milk and were designed for nutritional purposes. However, milk is an important raw material for the production of a wide range of foods, the production and properties of which depend on certain characteristics of the macroconstituents of milk, which have been studied in depth. Most natural foods contain the same types of constituents, water, lipids, proteins, and carbohydrates, but at very different concentrations and proportions, and the physicochemical properties of the constituents vary widely. The physicochemical and technological properties of foods are determined by the properties of their main constituents, especially the carbohydrates and proteins, which are unique to each group of foods.

Lactose is the principal (0–10%) carbohydrate in the milk of most species and it is unique to milk; why lactose, rather than some other sugar, is the principal sugar in milk is a matter of conjecture, as discussed in the article **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties. However, milk contains several other sugars in addition to lactose, either as components of glycoproteins or glycolipids or as oligosaccharides, which are major components of the milk of some species, for example, humans, and are considered to be very important from nutritional and physiological viewpoints (*see* **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk).

The milk of all species that have been examined contains lipids (~1 to >50%), of which the principal class is TGs (~98% for most species). The FAs in milk lipids are not synthesized in the mammary gland but are obtained, via the blood, from dietary lipids or are synthesized in the liver. The FA profile is unique at the species level and especially at the genus level; the lipids of ruminants are the only natural lipids that contain butanoic acid (C4:0). The mammary gland is the only organ of the animal body that excretes large quantities of lipids from the secretory cells. Since the hydrophobic lipids are exported into an aqueous hydrophilic system, milk serum, with which they are incompatible, they must be formed into a stable emulsion for colloidal stability. This is done as the TGs are exported from the mammocytes by surrounding them with the apical cell membrane, which consists of special proteins and polar lipids. The composition and structure of the membrane are discussed in the article **Husbandry of Dairy Animals**: Predator Control in Goats and Sheep. The lipids of milk supply the neonate with essential FAs, fat-soluble vitamins, and energy; they are important for the flavor and textural properties of dairy products.

The total concentration of protein in milk ranges from ~1 to ~25%; there are eight principal proteins and perhaps 80 others at low or trace levels. The principal proteins fall into two groups: caseins and whey (serum) proteins (WPs). The caseins, a family of phosphoproteins that are insoluble at their isoelectric points (at pH ~4.5), are unique to milk, in which their function is to supply amino acids, calcium, and phosphates to the neonate and to prevent pathological calcification of the mammary gland. Most of the WPs have some biological function (in addition to supplying amino acids). In addition to differences in the total concentration of proteins, there are marked interspecies differences in the ratio of caseins to WPs and in the concentration of individual proteins. The technological properties of milk are mainly due to the proteins, which are described in the articles **Milk Proteins**: Analytical Methods; Casein Nomenclature, Structure, and Association; Caseins, Micellar Structure; Heterogeneity, Fractionation, and Isolation; Immunoglobulins; Inter-Species Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity; α-Lactalbumin; β-Lactoglobulin; Lactoferrin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; Nutritional Quality of Milk Proteins; Proteomics; **Milk Protein Products**:; Bioactive Peptides; Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory

Aspects; Functional Properties of Milk Proteins; Membrane-Based Fractionation; Milk Protein Concentrate; Whey Protein Products.

The minor constituents of milk, especially the vitamins and inorganic elements, are disproportionately important from a nutritional viewpoint and many of them from the technological viewpoint also. They are discussed in the articles **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance; **Vitamins**: Biotin (Vitamin B_7); Folates; General Introduction; Niacin; Pantothenic Acid; Riboflavin; Thiamine; Vitamin A; Vitamin B_{12}; Vitamin B_6; Vitamin C; Vitamin D; Vitamin E; Vitamin K.

The principal constituent in the milk of most species is water, the significance of which is considered in the articles **Water in Dairy Products**: Analysis and Measurement of Water Activity; Water in Dairy Products: Significance.

Milk is a very variable biological fluid. In addition to interspecies differences (**Table 1**), the milk of any particular species varies with the individual animal, breed (in the case of commercial dairying species), health (mastitis and other diseases), nutritional status, stage of lactation, age, interval between milkings, and other factors. In a bulked factory milk supply, variability due to many of these factors is evened out but some variability persists and may be quite large when milk production is seasonal. In addition to variations in the concentrations of the principal and minor constituents due to the above factors, the actual structure of some of the constituents also varies, for example, the FA profile is influenced strongly by diet. Variations in the concentration and properties of the constituents of milk cause variations in the processing properties of milk and hence in the quality of dairy products. Some of the variability in the nature of milk can be minimized, adjusted, or counteracted by animal husbandry practices or processing technology but some differences may persist. The variability of milk and the consequent problems will become apparent from several articles throughout the encyclopedia.

From a physicochemical viewpoint, milk is a very complex fluid, the constituents of which occur in three phases. About 40% of the dry matter of bovine milk is in true solution, that is, lactose (sugar), organic and inorganic salts, vitamins, and other small molecules in water. In this aqueous solution are dispersed proteins, some (WPs) at a molecular level, others (the caseins) as large colloidal aggregates (micelles), ranging in diameter from 50 to 300 nm, and lipids, which exist in an emulsified state, as globules ranging in diameter from 0.1 to 20 μm and stabilized by a lipoprotein membrane, the milk fat globule membrane (MFGM). Milk has a slightly sweet taste due to lactose, which has a low level of sweetness in comparison with other common sugars, for example, fructose, glucose, galactose, sucrose, or maltose. The concentration of lactose in milk ranges from 0 to ~10% but the four commercially important milks – cow, goat, sheep, and buffalo milks – contain 4.5–5.0%; human milk contains 7% lactose. The pleasant mouthfeel of milk is due to its colloidal constituents, casein and fat. The white color (appearance) of milk is due to the scattering of light by the colloidal particles, especially the casein micelles. The rheological properties and surface activity of milk and dairy products also are determined primarily by the colloidal constituents. Other physical properties of milk, for example, freezing point, boiling point, osmotic pressure, electrical conductivity, pH, and redox potential (E_h), are determined mainly by the low-molecular-mass compounds present in true solution.

Milk is a dynamic system owing to

- the instability of many of its structures, for example, the MFGM and casein micelles
- changes in the solubility of many constituents, especially inorganic salts, but also proteins, with temperature and/or pH
- the presence of various enzymes, which can modify constituents through lipolysis, proteolysis, or oxidation/reduction
- the growth of microorganisms, which can cause major changes, either directly through their growth, for example, changes in pH or E_h, or through enzymes they excrete
- the interchange of gases with the atmosphere, especially CO_2

Milk was intended to be consumed directly from the mammary gland and to be expressed from the gland at frequent intervals. However, in dairying operations, milk is stored for various periods, ranging from a few hours to several days, during which it is cooled (and perhaps heated) and agitated to various degrees. These treatments will cause at least some physical changes and permit some enzymatic and microbiological changes, which may alter the processing properties of milk. It may be possible to counteract some of these changes.

History of Dairy Science and Technology

Milk is probably the best characterized of the principal food groups in terms of chemical and physicochemical properties. Research on milk dates from the eighteenth century and dairy science/technology is a well-established discipline for technological study and is serviced by several professional societies and associations (*see* **Dairy Science Societies, and Associations**). There is a very ancient and now voluminous literature on various aspects of dairy science. Several Roman writers, for example, Cato, Varo, Pliny the Elder, and Columella, wrote on various aspects of agriculture, including dairying and dairy products. Books on various aspects of dairying were published at the end of the nineteenth century, perhaps the oldest of which is *Lehrbuch der Milchwirtschaft* by W Fleischmann, published in 1870. The early interest and progress in dairy science can be attributed to many factors, and some are listed below:

1. While milk can be consumed without any treatment or processing, its principal constituents separate during storage, without human intervention, creating the opportunity for the production of new products.
2. The principal constituents of milk (lipids, proteins, and lactose) can be fractionated and purified rather easily in comparison with other major food groups, for example, meat or cereals, and were amenable to the application of new analytical techniques as these were developed.
3. The perishability of milk necessitated and encouraged the development of methods to conserve it, which, in turn, encouraged the study of the properties of milk.
4. The properties of some milk constituents are very interesting – in fact, unique – which has encouraged their study. The properties of some constituents permit the manufacture of new products; for example, coagulability of caseins by enzymes or acids permits the production of cheeses and fermented milks; the very high heat stability of milk permits the manufacture of a wide range of pasteurized and sterilized products without major physical changes.
5. The fact that milk is a liquid that can be readily converted to a solid renders milk a very flexible raw material.

As will become apparent from several articles throughout this encyclopedia (*see* **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Production, Applications; **Milk Lipids**: General Characteristics; **Milk Proteins**: Heterogeneity, Fractionation, and Isolation), the constituents of milk are well characterized at the chemical, physical, and genetic levels. Certainly, knowledge of the chemical and physical properties will be extended and refined in the future but the greatest opportunities for advancement are in the area of molecular genetics, through which the yield of milk will be increased, its composition modified (e.g., lactose deleted, replaced, or modified), properties of milk constituents modified (properties of milk proteins modified via point mutations), or new constituents introduced. Genetic engineering opens up the possibility of converting the cow from a food-producing animal to a producer of pharmaceuticals (*see* **Molecular Genetics and Dairy Foods**).

Dairy cows have been scientifically bred for at least 150 years and casually for much longer. The objectives of such breeding programs have been to increase milk yield and fat content and recently protein content also. Inadvertently, some technological properties may have been improved (and perhaps disimproved) but there has been no concerted effort in this regard. Advances in classical genetics are discussed in the articles **Genetics**: Selection: Concepts; International Flow of Genes; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. Developments in molecular biology make it possible to design milk for specific nutritional or technological applications, including milk as a source of nutraceuticals; these developments are described in the article **Molecular Genetics and Dairy Foods**.

Production and Utilization of Milk

Probably, some milk and dairy products are consumed in all regions of the world but they are major dietary items in Europe, North and South America, Australia, New Zealand, India, and some Middle Eastern countries. Total recorded milk production is about 600 million tonnes. In addition to domestic consumption, there is a substantial international trade in dairy products, aspects of which are discussed in the articles **Policy Schemes and Trade in Dairy Products**: Trade in Milk and Dairy Products, International Standards: Harmonized systems; Trade in Milk and Dairy Products, International Standards: World Trade Organization. Data on the consumption of milk and dairy products in member countries are published at regular intervals by the Food and Agriculture Organization (FAO) and the International Dairy Federation (IDF). Milk and dairy products are quite important in several other countries that are not members of the IDF. Approximately 85, 11, 2, and 2% of global milk production is produced from cows, water buffaloes, sheep, and goats, respectively; camel, yak, reindeer, horse, and donkey are important in certain regions, but are of very little significance globally.

Milk and dairy products are major dietary items in Europe and North America, where they contribute 20–30% of dietary protein, 15% of dietary lipids, and 80% of calcium. An adequate intake of calcium, especially in childhood, is critical for bone development and the prevention of osteoporosis in later life, especially in women; hence, milk and calcium-rich dairy products, for example, rennet-coagulated cheeses, are very important (**Milk Salts**: Macroelements, Nutritional Significance). Milk plays a key role in infant nutrition. While human milk is probably best for human infants, most survive and grow very well on bovine milk. Although bovine milk was probably fed to human infants in medieval and even ancient times, the first formulated infant food was produced by Justus von Liebig in 1856. The development of sweetened condensed milk and sterilized evaporated milk in the mid-nineteenth century was a major factor in reducing infant mortality during the early years of the twentieth century. The production of modified (humanized) formulae is now a widespread, important, and profitable sector of the dairy industry (*see* **Dehydrated Dairy Products**: Infant Formulae).

Because milk is perishable and its production was traditionally seasonal, milk that was surplus to immediate requirements was converted to more stable products, traditional examples being butter or ghee, fermented milk, and cheese; small amounts of dried milk were produced by sun-drying. These traditional products are still very important, their production is expanding, and many new variants thereof have been introduced. In addition, several new products have been developed during the past 150 years, for example, sweetened condensed milk, sterilized concentrated milk, a range of milk powders, ultraheat-treated (UHT) sterilized milk, ice creams, infant foods, and milk protein products.

One of the important developments in dairy technology in recent years has been the fractionation of milk into its principal constituents, for example, lactose, milk fat fractions, and milk protein products (caseins, caseinates, WP concentrates, WP isolates, mainly for use as functional proteins but more recently as nutraceuticals, that is, proteins for specific physiological and/or nutritional functions, e.g., lactotransferrin, immunoglobulins). All these products will be considered in individual articles in this encyclopedia (*see* **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Production, Applications; **Milk Protein Products**: Whey Protein Products; **Milk Proteins**: Casein Nomenclature, Structure and Association; Immunoglobulins; Lactoferrin; **Nutrition and Health**: Nutraceuticals from Milk).

As a raw material, milk has many attractive features:

1. Milk was designed for animal nutrition and hence contains the necessary nutrients in easily digestible forms (although the balance is designed for the young of a particular species) and free of toxins. No other single food, except the whole carcass of an animal, including the bones, contains the complete range of nutrients at adequate concentrations.
2. The principal constituents of milk – lipids, proteins, and carbohydrates – can be fractionated readily and purified by relatively simple methods for use as food ingredients.
3. Milk itself is readily converted into products with highly desirable organoleptic and physical characteristics and its constituents have many very desirable and some unique physicochemical (functional) properties.
4. The modern dairy cow is a very efficient converter of plant material; average national yields of about 8000 kg per annum are common, with individual cows producing up to 20 000 kg per annum. In terms of kilograms protein that can be produced per hectare, milk production, especially by modern cows, is much more efficient than meat production but less efficient than some plants (e.g., cereals and soybeans). However, the functional and nutritional properties of milk proteins are superior to those of soy protein and since cattle, and especially sheep and goats, can thrive under farming conditions not suitable for growing cereals or soybeans, dairy animals need not be competitors with humans for use of land, although high-yielding dairy cows are fed products that could be used for human foods. In any case, dairy products improve the quality of life, which is a desirable objective *per se*.
5. One of the limitations of milk as a raw material is its perishability – it is an excellent source of nutrients for microorganisms as well as for humans. However, this perishability is readily overcome by a well-organized, efficient dairy industry at both the farm and factory levels. Milk is probably the most adaptable and flexible of all food materials, as will be apparent from **Table 2**, which shows the principal families of milk-based foods. Some of these families, for example, cheese, contain several hundred different products. However, a few families of products dominate the dairy sector, for example, 40% of total milk production is used as beverage (liquid) milk, 35% is converted into cheese, and 10% is converted into whole-milk powder.

Many of the processes to which milk is subjected cause major changes in the composition, physical state, stability, and nutritional and sensory attributes of the product; some of these changes will be discussed in separate articles (*see* **Analytical Methods**: Principles and Significance in Assessing Rheological and Textural Properties; Rheology of Liquid and Semi-Solid Milk Products).

Conclusion

Milk is a unique biological fluid, intended for a specific purpose, that is, the nutrition of neonatal mammals. However, due to its high nutritional value and its suitability as a raw material for the production of some unique products, it is a major item in the human diet in many regions of the world and is a major item of international trade.

See also: **Analytical Methods**: Principles and Significance in Assessing Rheological and Textural Properties. **Animals That Produce Dairy Foods**:

Table 2 Diversity of dairy products

Process	*Primary product*	*Further products*
Centrifugal separation	Cream	Butter, anhydrous milk fat, ghee, creams of various fat content (10–50%) (pasteurized, ultraheat-treated sterilized, in-container stabilized) Cream cheeses
	Skim milk	Powders, casein, cheese, milk protein concentrates
Concentration by thermal evaporation or reverse osmosis		In-container or ultraheat-sterilized concentrated milks; sweetened condensed milk
Concentration and drying		Whole-milk powders; infant formulae; dietary products
Enzymatic coagulation	Cheese	Numerous varieties; further products, for example, processed cheese, cheese-based ingredients
	Rennet casein	Cheese analogues
	Whey	Whey powders, demineralized whey powders, whey protein concentrates, whey protein isolates, individual whey proteins, whey protein hydrolysates, nutraceuticals Lactose and lactose derivatives
Acid coagulation	Cheese	Fresh cheeses and cheese-based products
	Acid caseins/ caseinates	Functional applications, for example, coffee creamers, meat extenders; nutritional applications
	Whey	As for enzymatic coagulation
Fermentation		Various fermented milk products, for example, yogurt, buttermilk, acidophilus milk, bioyogurt
Freezing		Ice cream (numerous types and formulations)
Miscellaneous		Chocolate products

Modified from Fox PF and McSweeney PLH (1998) *Dairy Chemistry and Biochemistry*. London: Chapman & Hall.

Camel; Donkey; Goat Breeds; Horse; Major *Bos taurus* Breeds; Minor and Dual-Purpose *Bos taurus* Breeds; Reindeer; Sheep Breeds; Water Buffalo; Yak. **Cheese**: Accelerated Cheese Ripening; Acid- and Acid/Heat Coagulated Cheese; Avoidance of Gas Blowing; Biochemistry of Cheese Ripening; Blue Mold Cheese; Camembert, Brie, and Related Varieties; Cheddar-Type Cheeses; Cheese Analogues; Cheese as a Food Ingredient; Cheese Flavor; Cheese Rheology; Cheese with Added Herbs, Spices and Condiments; Cheeses Matured in Brine; Gel Firmness and Its Measurement; Curd Syneresis; Current Legislation for Cheeses; Dutch-Type Cheeses; Enzyme-Modified Cheese; Hard Italian Cheeses; Low-Fat and Reduced-Fat Cheese; Mechanization of Cheesemaking; Membrane Processing in Cheese Manufacture; Microbiology of Cheese; Non-Starter Lactic Acid Bacteria; Pasta-Filata Cheeses: Low-Moisture Part-Skim Mozzarella (Pizza Cheese); Pasta-Filata Cheeses: Traditional Pasta-Filata Cheese; Pasteurized Processed Cheese Products; Public Health Aspects; Raw Milk Cheeses; Rennet-Induced Coagulation of Milk; Rennets and Coagulants; Salting of Cheese; Secondary Cultures; Smear-Ripened Cheeses; Starter Cultures: Specific Properties; Swiss-Type Cheeses; Use of Microbial DNA Fingerprinting. **Concentrated Dairy Products**: *Dulce de Leche*; Evaporated Milk; Khoa; Sweetened Condensed Milk. **Cream**: Manufacture; Products. **Dairy Science Societies, and Associations**. **Dehydrated Dairy Products**: Dairy Ingredients in Non-Dairy Foods; Infant Formulae; Milk Powder: Physical and Functional Properties of Milk Powders; Milk Powder: Types and Manufacture. **Dehydrated Dairy Products**: Infant Formulae. **Genetics**: International Flow of Genes; Selection: Concepts; Selection: Economic Indices for Genetic Evaluation; Selection: Evaluation and Methods. **Husbandry of Dairy Animals**: Predator Control in Goats and Sheep. **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk; Lactose: Chemistry, Properties; Lactose: Production, Applications. **Mammals**. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose; Milk Fat; Milk Protein; Secretion of Milk Constituents. **Mammary Gland**: Anatomy; Gene Networks Controlling Development and Involution; Growth, Development and Involution. **Milk Lipids**: General Characteristics. **Milk Protein Products**: Bioactive Peptides; Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Functional Properties of Milk Proteins; Membrane-Based Fractionation; Milk Protein Concentrate; Whey Protein Products. **Milk Proteins**: Analytical Methods; Casein Nomenclature, Structure, and Association; Caseins, Micellar Structure; Heterogeneity, Fractionation, and Isolation; Immunoglobulins; Inter-Species Comparison of Milk Proteins: Quantitative

Variability and Molecular Diversity; α-Lactalbumin; β-Lactalbumin; Lactoferrin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; Nutritional Quality of Milk Proteins; Proteomics. **Milk**: Milk of Marine Mammals; Milk of Monotremes and Marsupials. **Molecular Genetics and Dairy Foods**. **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance. **Nutrition and Health**: Nutraceuticals from Milk. **Policy Schemes and Trade in Dairy Products**: Trade in Milk and Dairy Products, International Standards: Harmonized Systems; Trade in Milk and Dairy Products, International Standards: World Trade Organisation. **Rheology of Liquid and Semi-Solid Milk Products**. **Vitamins**: Biotin (Vitamin B_7); Folates; General Introduction; Niacin; Pantothenic Acid; Riboflavin; Thiamine; Vitamin A; Vitamin B_6; Vitamin B_{12}; Vitamin C; Vitamin D; Vitamin E; Vitamin K. **Water in Dairy Products**: Analysis and Measurement of Water Activity; Water in Dairy Products: Significance.

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Physical and Physico-Chemical Properties of Milk

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Introduction

Milk is a complex colloidal dispersion of fat globules, casein micelles, and whey proteins in an aqueous solution of lactose, minerals, and a number of minor compounds. Its physical and physico-chemical properties depend on intrinsic compositional and structural factors, extrinsic factors such as temperature, and postmilking treatment.

There is no clear dividing line between physical properties and physico-chemical properties. However, physical properties may be thought of as measures of the bulk behavior of milk and of how milk interacts with energy, while physico-chemical properties are measures of how bulk behavior and energy interactions depend on milk's constituent colloidal particles, molecules, atoms, and ions.

Knowledge of physical properties is of importance particularly in the technological and engineering design and control of milk processes and processing equipment. Knowledge of physico-chemical properties provides a basis for the design of modern methods of milk analysis, determination of milk microstructures, and elucidation of the complex chemical reactions that occur in milk.

In this article, recognizably physical properties, such as rheological properties, density, and thermal properties, are discussed first. Then, properties that fall within the realms of both physics and physical chemistry are described: surface tension, acoustic properties, electrical and dielectric properties, and optical properties. Finally, recognizably physico-chemical properties – the colligative properties, and acid–base and oxidation–reduction equilibria – are discussed.

Rheological Properties

The rheological behavior of milk is in accord with that of emulsions and suspensions in general. Milk may exhibit Newtonian or non-Newtonian behavior depending on composition, prior treatment, and measurement conditions (especially shear rate and temperature).

At moderate to high shear rates, Newtonian behavior is exhibited by skim milk, by whole milk at temperatures >40 °C (milk fat completely molten, no cold agglutination), and by whole milk at temperatures <40 °C when cold agglutination is absent.

Non-Newtonian behavior manifests itself in raw whole milk under conditions that favor cold agglutination (temperatures <40 °C) and at low shear rates. Shear thinning is the predominant rheological behavior. Skim milk shows shear-thinning behavior at low shear rates and at temperatures <30 °C.

As milk is concentrated by heat evaporation or by membrane processes, viscosity increases, shear thinning becomes more pronounced, and deviation from Newtonian behavior persists to higher shear rates. Thixotropy (time-dependent shear thinning) appears at a sufficiently high concentration, or after a sufficiently long storage time (at concentrations and temperatures above certain minima), during which structure development occurs. Such structure development, and the consequent steady viscosity increase, is known as age thickening.

Viscosity of milk is greatly influenced by the concentration and state of the fat and casein, and thus on factors that affect these. Such factors include temperature, and technological treatments such as homogenization, heat treatment, renneting, and acidification.

A full discussion of the rheological properties of milk may be found in the article **Rheology of Liquid and Semi-Solid Milk Products**.

Density and Specific Gravity (Relative Density)

The density ($\mathrm{kg\,m^{-3}}$) of milk is identified for a given measurement temperature as, for example, ρ^{20}, where the superscript is the temperature in °C. Specific gravity (SG), defined as $\rho_{\text{milk}}/\rho_{\text{water}}$, is identified in a similar way, for example, SG_{20}^{20} or SG_{4}^{20}, where the superscript is the temperature at which the milk density was measured, and the subscript the temperature at which the density of water was determined. Density and specific gravity (more properly called 'relative density') are related by the equation

$$\rho^{\theta_1} = \mathrm{SG}_{\theta_2}^{\theta_1} \times \rho_{\text{water}}^{\theta_1} \qquad (1)$$

where θ is the temperature (°C) and, usually, $\theta_1 = \theta_2$.

ρ^{20} of whole milk is about $1030\,\mathrm{kg\,m^{-3}}$ and usually ranges from 1027 to $1033\,\mathrm{kg\,m^{-3}}$ depending mainly on fat content. $\mathrm{SG}_{15.5}^{15.5}$ ranges from 1.030 to 1.035 for mixed-herd milk, and a commonly quoted average value is 1.032. Inter-breed variations in density and specific gravity are small.

The density and specific gravity of milk depend on composition, temperature, and, in the melting point range of the milk triglycerides (–40 to +40 °C), thermal history; thermal history determines the ratio of liquid fat (lower density) to solid fat (higher density). Other factors, such as the stage of lactation and nutritional status of the cow, are important only insofar as they affect composition.

Density data are needed for converting mass to volume and vice versa, and for calculating the values of physical properties such as kinematic viscosity and thermal diffusivity. The density of milk may be predicted, if the milk's proximate composition is known, by using the equation

$$\frac{1}{\rho} = \sum\left(\frac{x_i}{\rho_i}\right) \qquad (2)$$

where x_i and ρ_i are the mass fraction and density, respectively, of the ith component; $\Sigma(x_i) = 1$ for the non-gaseous components. One of the non-gaseous components will be ice if the temperature is below the (initial) freezing point of the milk.

The value of ρ^{20} of milk can be calculated using the values of component densities given in **Table 1**. The value of 918 kg m^{-3} for milk fat given in the table assumes that the fat is a supercooled liquid, but allows for the higher density of the milk fat globule membrane.

If milk contains dispersed gas, such as air, the actual density will be lower than that predicted by eqn [2]:

$$\rho^{\theta}_{\text{actual}} = (1 - \varepsilon_a)\,\rho^{\theta}_{\text{predicted}} \qquad (3)$$

where ε_a is the volume fraction occupied by dispersed gas, which is assumed to have zero mass. θ is the temperature of measurement or prediction. Dispersed gas considerably lowers density, which then becomes pressure dependent.

There have been many attempts to develop empirical equations relating milk density to fat content and temperature, the two most important determining variables. One such equation is as follows:

$$\begin{aligned}\rho = {} & (-2.307\times10^{-3}\theta^2) - (0.2655\theta) + 1040.51 \\ & -[F(-4.78\times10^{-5}\theta^2 + 9.69\times10^{-3}\theta + 0.967)] \\ & (\text{kg m}^{-3})\end{aligned} \qquad (4)$$

Table 1 Density of milk components at 20 °C

Milk component	*ρ^{20} (kg m^{-3})*
Water	998.2
Fat	918
Protein	1400
Lactose	1780
Residual components	1850

From Walstra P and Jenness R (1984) *Dairy Chemistry and Physics*. New York: Wiley.

where θ is the temperature (°C) and F is the % fat (w/w). Equation [4] is valid for milk and cream with fat contents in the range 0–15% (w/w), at temperature in the range 65–140 °C. The temperature dependence of milk's specific gravity is very slight, which is the principal advantage of expressing density in this way.

Processing operations such as homogenization, pasteurization, and sterilization have negligible effects on milk density.

Thermal Properties

Specific Heat Capacity and Enthalpy

The specific heat capacity of milk determines the quantity of thermal energy that has to be added to or removed from milk to effect a given temperature change according to the following equation:

$$q = \Delta h = \int_{\theta_1}^{\theta_2} c(\theta)\,\mathrm{d}\theta\,(\text{J kg}^{-1}) \qquad (5)$$

where q is the quantity of heat energy per kg (J kg^{-1}), Δh is the change in milk enthalpy (J kg^{-1}), θ is the temperature (°C), $(\theta_2 - \theta_1)$ is the temperature change (θ_1 to θ_2 or θ_2 to θ_1), and $c(\theta)$ is the specific heat capacity (J kg^{-1} K^{-1}) expressed as some function of temperature. $c(\theta)$ is the specific heat capacity at constant pressure, rather than at constant volume. As milk is a liquid, the two can be considered equal, except perhaps under the conditions used in high-pressure processing. The equality of q and Δh is valid under isobaric conditions and in the absence of phase transitions, chemical reactions, changes in composition, and forms of work other than displacement.

Equation [5] is valid for temperatures >40 °C, the upper end of the milk fat melting point range. At temperatures between the (initial) freezing point of milk and 40 °C, sensible heat demands are confounded with the latent heat demands caused by phase changes in the milk fat, and eqn [5] must be written as

$$\Delta h = \int_{\theta_1}^{\theta_2} c_{\text{app}}(\theta)\,\mathrm{d}\theta \qquad (6)$$

where $c_{\text{app}}(\theta)$ is the apparent specific heat capacity (J kg^{-1}) expressed as some function of temperature. $c_{\text{app}}(\theta)$ depends on fat content, triacylglycerol composition, and thermal history. There is a maximum (~4000 J kg^{-1} K^{-1}) in the temperature range 15–20 °C and a minimum (~3880 J kg^{-1} K^{-1}) at about 40 °C. At temperatures away from the maximum, specific heat capacity is inversely related to fat content. At temperatures above 40 °C, where the milk fat is completely molten and there are no phase changes, specific heat capacity increases gradually with temperature, reaching ~3940 J kg^{-1} K^{-1} at 100 °C and ~4000 J kg^{-1} K^{-1} at 140 °C.

The specific heat capacity of skim milk, which includes no significant latent heat effects due to phase changes in milk fat, increases essentially linearly but slowly with temperature over a wide range. Representative values are 3899 and 3988 J kg^{-1} K^{-1} at 0 and 50 °C, respectively.

For skim milk, and for whole milk with 4% fat, specific heat capacity may be predicted as a function of temperature (at temperatures above the milk fat melting point range) by the following equations:

$$\text{Skim milk at } 52-143\ ^{\circ}\text{ C: } c = 2.814\theta + 3942 \ \ (\text{J kg}^{-1}\text{K}^{-1}) \tag{7}$$

$$\text{Whole milk at } 53-153\ ^{\circ}\text{C: } c = 2.976\theta + 3692 \ \ (\text{J kg}^{-1}\text{K}^{-1}) \tag{8}$$

The ability to predict the enthalpy change between two chosen temperatures is, from a technological or engineering point of view, more useful than the ability to predict specific heat capacity at specific temperatures. The following general expression is valid for any temperature range, any fat content, and any total solids content:

$$\Delta h_{\theta_1 \leftrightarrow \theta_2} = \Delta h_{\text{fat}} x_{\text{fat}} + \Delta h_{\text{nonfat}} x_{\text{nonfat}} \tag{9}$$

where Δh_{fat} is the enthalpy change (J kg^{-1}) in milk fat between θ_1 and θ_2, Δh_{nonfat} is the enthalpy change in the non-fat portion of the product between θ_1 and θ_2, x_{fat} is the fraction of milk fat in the product, and $x_{\text{non-fat}}$ is the mass fraction of the nonfat portion.

Δh_{fat} must be found from a suitable set of published empirical data. $\Delta h_{\text{non-fat}}$ can be predicted from

$$\begin{aligned}\Delta h_{\text{nonfat}} &= \Delta h_{\text{milk plasma}} = \int_{\theta_1}^{\theta_2} \sum (c_i x_i)\, \mathrm{d}\theta \\ &= \sum (c_i x_i)(\theta_1 - \theta_2)\end{aligned} \tag{10}$$

where c_i is the specific heat of the ith component, x_i is the mass fraction of the ith component, and $\Sigma(c_i x_i)$ is the specific heat of milk plasma. Suitable values of c_i for water, lactose, protein, and ash are 4200, 1400, 1600, and 800 J kg^{-1} K^{-1}, respectively. The slight temperature dependence of the specific heat capacity of these non-fat components may usually be ignored for practical purposes, as indicated by eqn [10].

When milk is heat-concentrated, the enthalpy demand is almost wholly the latent heat of evaporation of water (h_{fg}) that must be supplied. This demand can be expressed as kilograms of water evaporated per kilogram of unconcentrated milk × h_{fg} (J kg^{-1}), where h_{fg} is read from steam tables at the pressure at which evaporation occurs. (There is an additional, though relatively small, sensible heat demand, as milk boiling point increases during concentration, but this demand is moderated by a concomitant decrease in specific heat capacity.)

In calculating enthalpy changes over a temperature range that straddles or lies below the initial freezing point of milk, it is necessary to account for latent heat effects due to phase change in the water substance (water or ice) as well as in the milk fat. There are ways of doing this, but milk is rarely frozen.

Thermal Conductivity and Thermal Diffusivity

The thermal conductivity of skim milk, whole milk, and cream increases slowly with increasing temperature. It decreases with increasing %TS or increasing fat content, the effects being generally greater at higher temperatures. The following semi-empirical relationship, although developed specifically for creams of fat content ≥20% (w/w), is accurate to within 10% for milk and cream with a fat content in the range 0.1–42% (w/w), and for the temperature range 5–75 °C:

$$\begin{aligned}\lambda = {} & (0.5279 + 2.13 \times 10^{-3}\theta - 7.32 \times 10^{-6}\theta^2) \\ & [1 - (0.843 + 1.9 \times 10^{-3}\theta)\,\phi_{\text{fat}}](\text{W m}^{-1}\text{ K}^{-1})\end{aligned} \tag{11}$$

where λ is the thermal conductivity (W m^{-1} K^{-1}), ϕ_{fat} is the fat volume fraction, and θ is the temperature (°C). Representative values of thermal conductivity at 20 °C calculated using eqn [11] are compared with the thermal conductivity of water in **Table 2**.

The thermal conductivity of whole milk heat concentrates may be predicted using the following equation:

$$\lambda = (0.59 + 0.0012\theta)\ (1 - 0.0078\text{TS})\ \ (\text{W m}^{-1}\text{ K}^{-1}) \tag{12}$$

The equation is valid for the temperature range $40 < \theta < 90$ °C and the %TS range $37 < \text{TS} < 72\%$; it is accurate to ±0.88%.

The thermal diffusivity of milk may be calculated using the equation:

$$\alpha = \frac{\lambda}{\rho c} \quad (\text{m}^2\text{ s}^{-1}) \tag{13}$$

Table 2 Representative values of the thermal conductivity of fluid milk products compared with the thermal conductivity of water, at 20 °C

Fluid	*Thermal conductivity (W m^{-1} K^{-1})*
Water	0.603
Skim milk (0.1% fat)	0.568
Whole milk (3.9% fat)	0.548
Cream (42% fat)	0.357

From Fernandez-Martin F and Montes F (1972) *Milchwissenschaft* 27: 772–776.

where α is the thermal diffusivity ($\mathrm{m^2\,s^{-1}}$), ρ is the density ($\mathrm{kg\,m^{-3}}$), and c is the specific heat capacity ($\mathrm{J\,kg^{-1}\,K^{-1}}$). λ, ρ, and c can be predicted as described above, and α, then, can be calculated. For example, at 20 °C, $\lambda \approx 0.548\,\mathrm{W\,m^{-1}\,K^{-1}}$, $\rho \approx 1030\,\mathrm{kg\,m^{-3}}$, and $c \approx 3980\,\mathrm{J\,kg^{-1}\,K^{-1}}$, giving a value for α of $\sim 1.34 \times 10^{-7}\,\mathrm{m^2\,s^{-1}}$.

Surface Tension

The surface tension of milk lies in the range 40–60 $\mathrm{mN\,m^{-1}}$ (average: $\sim 52\,\mathrm{mN\,m^{-1}}$) at 20 °C. Comparable values for some milk fractions are 72.8 (water), 51–52 (rennet whey), 52–52.5 (skim milk), 42–45 (25% fat cream), and 39–40 (sweet-cream buttermilk).

Surface tension decreases with increasing temperature. For whole milk, the relationship is

$$\gamma = 1.8 \times 10^{-4}\theta^2 - 0.163\theta + 55.6 \qquad (14)$$

where γ is the surface tension ($\mathrm{mN\,m^{-1}}$) and θ is the temperature (°C).

Surface tension is influenced by fat content, homogenization, and temperature history. It decreases with increasing fat content up to ~4%, but no further decrease occurs at higher fat contents. Homogenization of pasteurized milk increases surface tension. Homogenization of raw milk, however, can lead to a decrease in surface tension owing to the release of free fatty acids by lipase activity. The surface tension of milk held at 5 °C and brought to 20 °C is lower than that of milk cooled to 20 °C when measured immediately. Heat treatment of milk at sterilization temperatures can cause a small increase in surface tension, probably the result of denaturation of whey proteins, a change that reduces their surface activity.

Acoustic Properties

Acoustics is the science of sound (mechanical vibrations at frequencies in the range 1.6×10^{-2} to 20 kHz, which are detectable by the human ear), infrasound (frequencies of $<1.6 \times 10^{-2}$ kHz), and ultrasound (frequencies of >20 kHz). Only low-power ultrasound (which involves power levels $<1\,\mathrm{W\,cm^{-2}}$) is of significance in dairy science and technology. It is used principally as an analytical tool for indirectly measuring food characteristics and as an in-line method for measuring flow rate.

The ultrasonic properties of a material most frequently measured are the ultrasonic velocity ($\mathrm{m\,s^{-1}}$), the attenuation coefficient ($\mathrm{Np\,m^{-1}}$), and the acoustic impedance ($\mathrm{kg\,m^{-2}\,s^{-1}}$). These depend on other physical properties of the material.

The ultrasonic velocity (the distance traveled by an ultrasonic wave in unit time) is related to the elastic modulus, E, and density, ρ:

$$\left(\frac{k}{\omega}\right) = \frac{\rho}{E} \qquad (15)$$

Here, ω is the angular frequency ($\mathrm{rad\,s^{-1}}$) and k is the complex wave number $= (\omega/c) + \mathrm{i}\alpha$, where c is the ultrasonic velocity ($\mathrm{m\,s^{-1}}$), α is the attenuation coefficient ($\mathrm{Np\,m^{-1}}$), and $\mathrm{i} = \sqrt{-1}$.

For low-attenuating materials (i.e., $\alpha << \omega/c$), eqn [15] can be written as

$$\frac{1}{c^2} = \frac{\rho}{E} \qquad (16)$$

where ρ is the density ($\mathrm{kg\,m^{-3}}$) and E is the modulus of elasticity (Pa).

In the case of milk, E is the bulk modulus, as liquids are normally subjected to a compressive (as opposed to a shearing) ultrasonic wave.

The attenuation coefficient, α, is a measure of the decrease in the amplitude of an ultrasound wave as it travels through the material. It is given by the equation

$$A = A_0\,\mathrm{e}^{-\alpha x} \qquad (17)$$

where A_0 is the initial amplitude of the wave and A is the amplitude after the wave has traveled a distance x through the material. Attenuation results from absorption and scattering.

The acoustic impedance is a measure of the fraction of an ultrasonic wave reflected from a material's surface. The (complex) specific acoustic impedance, Z, is defined as

$$Z = \frac{\omega\rho}{k} \qquad (18)$$

where $Z = R_Z + \mathrm{i}X_Z$, with R_Z being the resistive (real) part of the complex impedance and X_Z the reactive (imaginary) part of the complex impedance.

For low-attenuating materials (i.e., $\alpha << \omega/c$), eqn [18] can be written as

$$Z = R_Z = \rho c \qquad (19)$$

Values of the three ultrasonic properties of skim milk (at 1 mHz and 28 °C) are as follows:
velocity, $c = 1522\,\mathrm{m\,s^{-1}}$,
attenuation coefficient, $\alpha = 23\,\mathrm{Np\,m^{-1}}$, and
impedance, $Z = 1.5 \times 10^{-6}\,\mathrm{kg\,m^{-2}\,s^{-1}}$.

Ultrasonic properties, which are frequency and temperature dependent, are most commonly measured using the pulse-echo technique. Enhanced information about the characteristics of the sample can be gained by carrying out measurements over a range of frequencies and also a range of temperatures.

The ultrasonic properties of milk are fundamental properties, but they are not of interest by themselves. Their measurement is useful only insofar as it is possible to establish relationships between them and other physico-chemical characteristics such as composition, structure, and state – characteristics that determine how ultrasound interacts with the sample. Such relationships can be developed empirically by means of calibration experiments, or theoretically.

Ultrasound has been used to measure the composition of milk in terms of fat and solids-not-fat, the particle size distributions of milk fat globules and casein micelles, and the rate of coagulation of milk by chymosin. Ultrasonic imaging has been used to measure teat milk flow rate, and to non-invasively monitor the microbial spoilage of packaged UHT milk. The measurement of creaming profiles, the principle of which is the measurement of the ultrasound velocity and/or attenuation coefficient as a function of sample height and time, has obvious potential for milk and other liquid dairy products.

Apart from being non-invasive and non-destructive, ultrasound can be used to analyze opaque as well as concentrated systems, and is inexpensive, rapid, and accurate.

Electrical and Dielectric Properties

Electrical Conductivity

Electrical conductivity (EC), σ, is a measure of a material's ability to carry an electrical current. It ranges in value from 10^{-18} to $10^{7}\,\mathrm{S\,m^{-1}}$ (Siemen per meter), depending on the material. The EC of normal whole milk is about $0.460\,\mathrm{S\,m^{-1}}$.

EC is most easily measured by applying a known DC voltage across a pair of parallel electrodes immersed in the sample, measuring the current produced, and calculating the resistance of the specimen (the volume bounded by the electrodes):

$$\sigma = \frac{1}{R}\frac{l}{A} = G\frac{l}{A}\,(\mathrm{S\,m^{-1}}) \qquad (20)$$

where R is the resistance (Ω), G the conductance (S), l the distance between the electrodes (m), and A the electrode area ($\mathrm{m^2}$).

Equation [20] shows that EC and electrical conductance (the reciprocal of resistance) are related via the dimensions of the specimen.

EC is generally measured in practice by impedance (or, admittance) spectroscopy, in which an AC rather than a DC voltage is applied to the sample. Impedance and admittance (the reciprocal of impedance) are complex properties, the real parts of which are, respectively, resistance and conductance. Measurements are best carried out at frequencies >10 kHz; at and above this frequency the measured value is a property of the bulk milk and not of the milk–electrode interface.

The EC of milk is determined mainly by the charged species present, particularly the salts. There is very little contribution from lactose; casein, also, makes a much smaller contribution than do the milk salts. The main effect of milk proteins in general is to hinder the migration of ions and thus depress EC. However, the release of calcium ions from the casein micelles as a result of a decrease in pH, caused by either deliberate acidification or bacterial growth, results in an increase in EC. A drop in milk pH to about 5 causes all of the colloidal calcium phosphate to dissolve, and the equilibria of milk buffer systems to change, resulting in saturation of the EC to a constant maximum value. This phenomenon is the basis of the automatic monitoring of the growth of lactic acid bacteria by conductimetric methods.

Mastitis in a quarter of a cow's udder results in decreases in the concentrations of lactose and $\mathrm{K^+}$ in the milk secreted, and corresponding increases in the concentrations of $\mathrm{Na^+}$ and $\mathrm{Cl^-}$ that preserve the milk's isoosmolality with the cow's blood. The net effect is an increase in the milk's EC. This phenomenon has led to much research over the last 60 years aimed at finding a reliable way of using in-line EC measurement at milking to detect both subclinical and clinical mastitis in the quarters of individual cows. Although the EC of milk from individual quarters can be monitored easily and accurately, achieving this aim has been hampered by the fact that variation in EC depends not only on the level of infection, but also on numerous other factors such as breed, parity, estrus, lactation stage, presence of other diseases, milking interval, time of day, and milk composition. In 1998, an extensive analysis of published data carried out by the International Dairy Federation concluded that EC measurement could not identify mastitic quarters or mastitic cows, nor detect subclinical mastitis, with sufficient accuracy to be useful. Currently, most automatic milking systems and some manual systems incorporate sensors for measuring the EC of quarter milk and software for processing the data generated. The development of sophisticated statistical modeling of the data, which involves comparisons between the quarters of the individual cow over successive milkings, has led to an improvement in the sensitivity of detection of subclinical and clinical mastitis. In the case of automatic milking systems, the farmer is dependent on EC measurement for detecting mastitic cows.

The EC of fresh milk and cream decreases with increasing fat content because the fat globules (themselves nonconducting) occupy volume that would otherwise be filled with the conducting aqueous phase of the product, thus impeding the mobility of the conducting ions and increasing the distance that migrating ions have to travel. For a fat content in the range

0.15–51% (w/w), the EC of milks and creams is related to the volume fraction of fat, ϕ_{fat}, as follows:

$$\sigma = \sigma_{\text{skim}}(1-\phi_{\text{fat}})^{\delta}\,(1+q\,\phi_{\text{fat}}^{2})^{\delta} \tag{21}$$

where $\delta = 1.56 \pm 0.04$, and q varies between 3.0 and 3.5, depending on the batch of milk.

Measurement of the admittance of milk has the potential to allow estimation of milk fat content if milk water content is known, and vice versa. The latter utility of the measurement could provide a means of detecting adulteration of milk with water.

The EC of stored whole milk reaches a higher saturation value than that of stored skim milk, because for whole milk the EC-depressing effect of the presence of fat is more than compensated for by the production of free fatty acids and the release of phosphate ions from the milk fat globule membrane.

The EC of milk increases markedly with temperature, as illustrated by the following empirical relationship for buffalo milk, valid for the temperature range 5–70 °C:

$$\sigma = 1.71\times10^{-1} + 6.32\times10^{-3}\theta + 9.01\times10^{-6}\theta^{2} \quad (\text{S m}^{-1}) \tag{22}$$

The EC and the viscosity of milk have been shown to be related. This relationship is thought to be due to the fact that the ion content of milk affects both the EC and the conformation of milk proteins, the latter influencing viscosity.

Dielectric Properties

The dielectric properties of a material, the permittivity (ε') and the dielectric loss factor (ε''), are the real and imaginary parts, respectively, of the complex permittivity (ε):

$$\varepsilon(\omega) = \varepsilon'(\omega) - j\varepsilon''(\omega) \tag{23}$$

where ω is the frequency (Hz) and j $=\sqrt{-1}$.
$\varepsilon'$is a measure of the ability of the material to store electromagnetic energy. ε'' is a measure of the material's ability to dissipate electromagnetic energy as heat. The latter phenomenon is exploited in microwave and radio frequency heating.

Permittivity and loss factor are frequency and temperature dependent. Representative values of ε' and ε'' for whole milk (3.25% fat) at 20 °C and 2.45 GHz (the frequency most commonly used for microwave heating) are 67.9 and 17.6, respectively, relative to the values for free space. Corresponding values for water are 80.2 and 13.4.

The permittivity at 20 °C of skim milk, low-fat milk, and whole milk decreases with increasing frequency in the range 1–20 GHz. The loss factor, on the other hand, exhibits a net increase with frequency over this range, but goes through a minimum near the lower end and a maximum near the higher end. At a given frequency, the permittivity of whole milk decreases slowly with increasing temperature, whereas the loss factor is almost temperature-independent.

The dielectric property spectra of whole milks of varying total solids content can be modeled using the following theoretical equations:

$$\varepsilon'(\omega) = \varepsilon'_{\infty} + \frac{\varepsilon'_{S} - \varepsilon'_{\infty}}{1+\omega^{2}\tau^{2}} \tag{24}$$

$$\varepsilon''(\omega) = \frac{(\varepsilon'_{S} - \varepsilon'_{\infty})\,(\omega t)}{1+\omega^{2}\tau^{2}} + \frac{\sigma}{\omega\varepsilon_{0}} \tag{25}$$

where ε'_{∞} is the real permittivity at very high frequencies, ε'_{S} is the real static (low frequency) permittivity, $\tau = 1/2\pi f_{R}$, where f_{R} is the relaxation frequency, σ the electrical conductivity, and ε_{0} the permittivity of free space ($= 8.854\times10^{-12}$ F m^{-1}).
Modeling shows that the complex permittivity of milk is essentially that of water, with perturbations caused by the ionic and nonionic milk components. Analysis of the spectral data relating to permittivity (ε'_{S}, ε'_{∞}, and τ) and those relating to loss factor (($\varepsilon'_{S} - \varepsilon'_{\infty}$), τ , and σ) shows that the perturbations are sensitive to the presence of fat, carbohydrate, protein, and ionic species. For example, permittivity is depressed by the nonionic components fat, lactose, and casein. Measurement of dielectric property spectra could potentially be useful in monitoring the gross composition of large quantities of milk in real time.

Optical Properties

The term 'optical properties' comprises properties with respect to electromagnetic radiation not only in the visible region of the spectrum (380–760 nm), but also in the infrared (IR, 760 nm to 1 mm) and ultraviolet (UV, 5–380 nm) regions that lie on either side of it. Visible, IR, or UV light incident on milk is absorbed, absorbed and reemitted (producing fluorescence), scattered, or transmitted, depending on wavelength.

Milk, as a complex colloidal dispersion of fat globules, casein micelles, and whey proteins in an aqueous solution containing a number of solutes, not only absorbs light at several wavelengths but also scatters light, as well as transmitting it. In the visible region, riboflavin in milk absorbs strongly near 470 nm (giving the yellow-green color of whey) and emits fluorescent radiation with a maximum at 530 nm. The β-carotene in milk fat absorbs near 460 nm, which is responsible for the yellow color of the fat.

In the UV region, aromatic amino acid residues of proteins (tyrosine and tryptophan) absorb strongly near 280 nm, and a part of the UV radiation is emitted as fluorescence at 340 nm. Measurement of the intensity of this fluorescence has been used to quantify the protein content of milk. The double bonds of the milk fat absorb very strongly near 220 nm.

In the IR region, absorption is primarily by the amide (II) groups (CONH) of proteins at 6465 nm, the hydroxyl groups (OH) of lactose at 9610 nm, and the ester carbonyl groups (C=O) of lipids at 5723 nm. This is the basis for the instrumental compositional analysis of milk using infrared analyzers. Milk samples are first homogenized to reduce the size of fat globules to <1 μm, to prevent their presence interfering with the measurement.

Both fat globules and casein micelles scatter light. It is possible to measure the intensities of both the scattered light and the light transmitted by the sample. Both measurements form the basis of the determination of the fat content of milk and of the size distributions of fat globules and casein micelles. Instruments for fat determination are based on measurement of turbidity, that is, the attenuation of the incident beam, usually expressed as optical density or absorbance.

The surface light scattering (diffuse reflection) and absorption properties of milk strongly affect the visual appearance of milk. The creamy color of whole milk is due to the β-carotene in the fat. Casein micelles scatter blue light (shorter wavelength) more effectively than they scatter red (longer wavelength), giving skim milk its bluish color. Homogenization of whole milk makes milk whiter by increasing the diffuse reflection (e.g., by some 19% at 550 nm). Heating has the same effect initially, but severe heating causes nonenzymic browning.

The penetration of light into milk can be important with respect to light-induced reactions. At 580 nm, light incident on whole milk loses 90 and 99% of its intensity at penetration depths of 8 and 24 mm, respectively.

The refractive index of milk varies with temperature and wavelength. The value for bovine milk at 589.3 nm (the D line of the sodium spectrum) at 20 °C, n_{D}^{20}, lies in the range 1.344 0–1.348 5. This may be compared with the value of 1.333 0 for water. The difference (Δn) between the values of n_{D}^{20} for milk and water reflects the presence of dissolved substances (lactose, minerals, etc.) and colloidal substances (casein micelles and whey proteins):

$$\Delta n = \rho \Sigma (mr) \tag{26}$$

where ρ is the density of the aqueous part of milk (kg m^{-3}), and m and r are, respectively, the mass fractions and specific refractive increments (m^3 kg^{-1}) of the individual substances. Values of r for the main determinants of Δn are 2.07×10^{-4} (casein micelles; m^3 kg^{-1} of dry casein), 1.87×10^{-4} (whey proteins), 1.40×10^{-4} (lactose), and $\sim 1.70 \times 10^{-4}$ (other dissolved substances). Fat globules and air bubbles do not contribute to the refractive index of milk.

Colligative Properties: Freezing Point, Boiling Point, and Osmotic Pressure

The part of milk comprising water and low molecular weight solutes obeys Raoult's law approximately. This law in one form states that the relative lowering of the solvent vapor pressure resulting from the presence of a solute is equal to the mole fraction of the solute in the solution. Consequences of the lowering of vapor pressure (the equilibrium relative humidity of milk at ambient temperature is ~99.3%) are a depression of freezing point ($\Delta\theta_{\mathrm{f}}$) and an elevation of boiling point ($\Delta\theta_{\mathrm{b}}$). In addition, the presence of the solute gives rise to an osmotic pressure.

For very dilute solutions

$$\Delta\theta_{\mathrm{f}} = K_{\mathrm{f}} M \tag{27}$$

and

$$\Delta\theta_{\mathrm{b}} = K_{\mathrm{b}} M \tag{28}$$

where K_{f} and K_{b} are the cryoscopic constant and ebullioscopic constant, respectively, and M is the molality of the solution.

The freezing point of milk is less than 0 °C, and the boiling point higher than 100 °C (at atmospheric pressure). Equations [27] and [28] can be used to roughly calculate the freezing point depression and boiling point elevation of milk as functions of the concentrations of water-soluble milk components.

Lactose, chloride salts, and other components (calcium, potassium, magnesium, lactate, phosphate, citrate, etc.) contribute about 55, 25, and 20%, respectively, to the freezing point depression. The freezing point of the vast majority of individual milks lies in the range −0.512 to −0.550 °C, and few fall outside the range −0.520 to −0.512 °C. The constancy of the freezing point depression is a reflection of the constant osmolality of milk. Although the concentrations of individual water-soluble components can vary, the total molality of fully dissociated species remains fairly constant. As the milk secretion process dictates that the osmotic pressure of milk be kept equal to that of blood (~687 kPa), a constant osmolality is maintained in milk by the passage of blood constituents into the mammary gland; any change in the concentration of lactose in milk is compensated for by changes in the concentrations of sodium and chloride. Milk with a freezing point of −0.53 °C has an osmolality of 0.285 osmol kg^{-1} water.

The determination of freezing point is extensively used to assess whether or not milk has been adulterated by the addition of water, and to quantify the amount added. Milk with a freezing point ≤ -0.525 °C is usually assumed to be unadulterated.

Acid–Base Equilibria

pH of Milk

The pH of bovine milk at 25 °C usually lies in the range 6.5–6.7, with 6.6 being the most common value. The pH decreases with increasing temperature. This implies temperature-induced changes in milk's complex buffering system rather than an increase in milk's acidity. At a given temperature, differences in pH and buffering capacity between individual lots of fresh milk reflect compositional variation. The pH of colostrum can be as low as 6.0 and that of mastitic and end-of-lactation milks as high as 7.5. High pH can be due to increases in $[\mathrm{Na}^{+}]$ and $[\mathrm{Cl}^{-}]$ (and possibly in the concentrations of other ions), a reduction in the lactose content, and a reduction in the concentration of soluble inorganic P – changes that alter buffering capacity in this pH range.

Buffering Constitutents of Milk

Milk, because of its buffering system, resists change in pH when it is titrated against a strong acid or a strong base. The extent of resistance, that is, the buffering action, at a given pH is called the 'buffering index'. The buffering index, $\mathrm{d}B/\mathrm{dpH}$, is the derivative (slope) of the titration curve (the plot of moles of strong acid or strong base added to milk vs. pH). It is calculated as follows:

$$\frac{\mathrm{d}B}{\mathrm{dpH}} = \frac{(\text{volume of acid or base solution added}) \times (\text{normality of solution})}{(\text{average volume of sample}) \times (\text{pH change produced})} \tag{29}$$

The resulting value of $\mathrm{d}B/\mathrm{dpH}$ is assigned to the pH at the mid-point of the pH change that has occurred.

Buffering action is due to the presence in milk of acid buffers (weak acid–conjugate base pairs) and base buffers (weak base–conjugate acid pairs). Milk contains many acidic and alkaline groups that result in buffering action over a wide pH range. The main groups are listed in **Table 3**. The principal buffer components in milk are soluble phosphate, colloidal calcium phosphate (CCP), citrate, bicarbonate, and casein.

The relationship between pH and $\mathrm{p}K_{\mathrm{a}}$ (the negative logarithm of the acidity constant K_{a}) for the titration of a weak acid against a strong base is given by the Henderson–Hasselbalch equation:

$$\mathrm{pH} \approx \mathrm{p}K_{\mathrm{a}} + \log\frac{[\mathrm{A}^{-}]}{[\mathrm{HA}]} \tag{30}$$

Maximum buffering occurs at $\mathrm{pH} = \mathrm{p}K_{\mathrm{a}}$. In the case of a weak base–conjugate acid buffer, maximum buffering action likewise exists when $\mathrm{pH} = \mathrm{p}K_{\mathrm{a}}\ (= 14 - \mathrm{p}K_{\mathrm{b}})$. K_{b} is the basicity constant and $\mathrm{p}K_{\mathrm{b}}$ is its negative logarithm.

In some cases several values are given for $\mathrm{p}K_{\mathrm{a}}$ in **Table 3**; values for many milk constituents are uncertain because of the complexity of the overall system.

Table 3 Principal buffering groups in milk

Group	*Approximate concentration (mmol l^{-1})*	*Expected pK_a*	*pK_a (in milk)*
Salts			
Inorganic phosphate	21.0	2.1, 7.2, 12.3	3, 5.8, 6.6
Citrate	9.0–9.2	3.1, 4.7, 5.4	3, 4.1, 4.8
Organic phosphate esters	2.5–3.5	1.4, 6.6	1.7, 5.9
Carbonate	2.0	6.4, 10.1	6.4, 10.1
Lactic acid	<0.4	3.9	3.9
Formic acid	0.2–1.8	3.6	3.6
Acetic acid	0.05–0.8	4.7	4.8
Various amines	1.5	~7.6	7.6
Protein-bound residues	*Titratable group*	*Expected pK_a*	*pK_a (in milk)*
Aspartic acid	β-COOH	4.6	4.1
Glutamic acid	α-COOH	4.6	4.6
Histidine	Imidazole	6.1	6.5
Tyrosine	Phenol	9.7	-
Lysine	ϵ-NH_3^+	10.4	10.5
Phosphoserine	Phosphate	1.5, 6.5	2, 6
N-Acetylneuraminic acid	COOH	2.6	5.0
Terminal carboxyl	α-COOH	3.6	3.7
Terminal amino	α-NH_3^+	7.9	7.9

From McCarthy OJ and Singh H (2009) Physico-chemical properties of milk. In: McSweeney PLH and Fox PF (eds.) *Advanced Dairy Chemistry, Vol. 3: Lactose, Water Salts and Minor Constituents*, 3rd edn., pp. 691–758. New York: Springer.

The shape of a titration curve, and consequently the relationship between buffering index and pH, depends on the experimental methodology used; titration and back-titration curves of milk do not coincide (**Figures 1** and **2**). When milk is acidified (**Figure 1**), maximum buffering (a maximum in d*B*/dpH) occurs at about pH 5.1, but when acidified milk is back-titrated with a base, a low extent of buffering exists at pH 5.1 and a maximum at pH ~6.3. The acid part of the titration curve is important because milk is acidified in the manufacture of products such as yogurt.

When milk is titrated to pH 11.0 (**Figure 2**), buffering action is relatively slight at pH 8–9 but increases at higher pH values. There is little difference between the curves for titration with alkali and back-titration with acid. On back-titration, a buffering peak is not observed at pH 6.3 (cf. **Figure 1**). Although there is a peak at about pH 5.1, it is weaker and less distinct than the peak obtained when normal milk is first acidified (**Figure 1**).

The maximum at pH 5.1 on acidification is due to dissolution of CCP and the consequent formation of HPO_4^{2-} and $H_2PO_4^-$. On back-titration, buffering action is low at this pH because the CCP is already solubilized. The maximum at pH 6.3 is due to the formation of calcium phosphate, resulting in the release of H^+ (from HPO_4^{2-} and $H_2PO_4^-$), which can combine with OH^-.

The buffering action of milk at high pH values is probably due to lysine residues and carbonate. When milk is titrated to high pH, all of the calcium phosphate precipitates. The precipitate dissolves again on subsequent acidification, resulting in buffering action over a wide pH range.

Colostrum has a much higher buffering capacity than that of normal milk over the pH range 6.6–3.0. The buffering peak at pH ~5.1 for mastitic milk is slightly higher than that for normal milk.

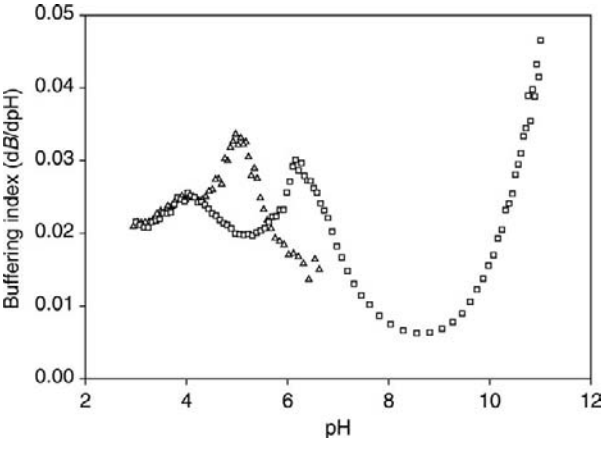

Figure 1 Buffering curves of milk titrated from the initial pH (6.6) to pH 3.0 with 0.1 M HCl (Δ) and then back-titrated to pH 11.0 with 0.5 M NaOH (□). Data supplied by J. A. Lucey, Department of Food Science, University of Wisconsin–Madison.

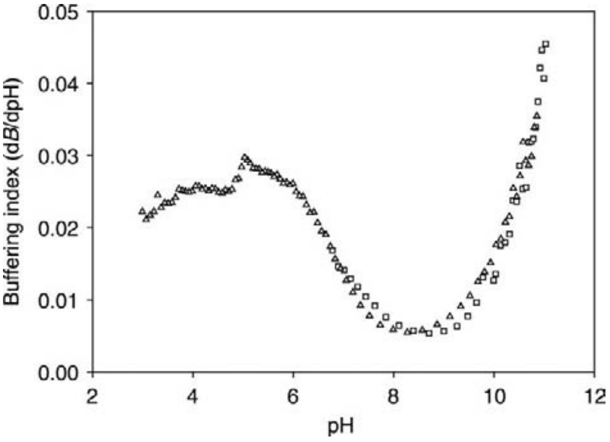

Figure 2 Buffering curves of milk titrated from the initial pH (6.6) to pH 11.0 with 0.5 M NaOH (□) and then back-titrated to pH 3.0 with 0.5 M HCl (Δ). Data supplied by J. A. Lucey, Department of Food Science, University of Wisconsin–Madison.

Titratable Acidity of Milk

The alkaline range of the titration curve is important because of the widespread use of titratable acidity to characterize milk. The titratable acidity is the buffering capacity of milk between its own pH (6.6) and pH 8.3 (the phenolphthalein end point). The measurement of titratable acidity (usually expressed, somewhat arbitrarily, as percentage lactic acid) is useful for determining the freshness of milk and for controlling the manufacture of fermented dairy products. The titratable acidity of fresh milk seldom falls outside the range 0.14–0.16%.

Effects of Milk Treatment on Acid–base Equilibria

Heating

Heat treating milk (e.g., by holding at 100 °C for 10 min) results in an increase in buffering action at pH ~5.0 during acid titration. This is probably caused by an increase in the concentration of CCP owing to the formation of heat-precipitated calcium phosphate. More severe heat treatment causes a shift of the peak from pH 5.0 to pH 4.4, and the buffering action in the pH range 5.0–4.0 is much stronger than that of unheated or less severely heated milk. The shift in the pH of the peak may be due to a change in the structure and composition of CCP during the severe heat treatment.

Freezing

The pH of milk decreases to as low as 5.8 during slow freezing, probably owing to the precipitation of calcium phosphate and the consequent release of H^+ in the unfrozen phase as this phase becomes increasingly concentrated.

Little change occurs on rapid freezing, possibly owing to insufficient time available for precipitation to occur.

Dilution and concentration

Concentration of milk lowers milk pH owing to precipitation of calcium phosphate; dilution has the opposite effect.

During the ultrafiltration concentration of milk, the buffering capacity of the retentate increases steadily owing to the increasing concentrations of casein, whey proteins, and milk salts.

Oxidation–Reduction Equilibria

Oxidation and reduction are, respectively, the loss and gain of electrons. The potential of a system to transfer electrons, that is, the oxidation–reduction or redox potential (E_h), is measured relative to the hydrogen electrode, and expressed in volts. For a simple binary oxidation–reduction system, E_h is related to the concentrations of the oxidized and reduced forms of the substance involved by the Nernst equation:

$$E_h = E_0 + \frac{RT}{nF}\ln\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) \tag{31}$$

where E_0 is the standard redox potential (V) (i.e., the potential when both the oxidized and reduced forms are at unit activity); [Ox] and [Red] are the activities of the oxidized and reduced forms, respectively; n is the number of electrons transferred per molecule; R is the gas constant ($8.314\,\mathrm{J\,K^{-1}\,mol^{-1}}$); T is the absolute temperature (K); and F is the Faraday constant ($96.5\,\mathrm{kJ\,V^{-1}\,mol^{-1}}$). Systems with a positive value of E_h are oxidizing systems, whereas those with a negative value are reducing ones.

At 25 °C and for a one-electron transfer, eqn [23] becomes

$$E_h = E_0 + 0.059\log\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) \tag{32}$$

In many biological systems, H^+ are involved in the overall oxidation reaction. In this case, again, for a one-electron transfer,

$$E_h = E_0 + 0.059\log\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) - 0.059\mathrm{pH} \tag{33}$$

An oxidation system exhibits poising capacity, analogous to buffering capacity, at values of [Ox]/[Red] close to unity, that is, at E_h values close to the E_0 value.

The redox potentials of individual milk samples in equilibrium with air fall in the range +0.25 to +0.35 V at 25 °C at the normal milk pH. The redox potential is inversely related to pH, ranging from about +0.20 at pH 10 to about +0.395 at pH 3.5 for raw milk. Little information is available on the considerable poising capacity of milk, because of measurement difficulties. These difficulties are the result of sluggishness of some oxidation–reduction systems in milk in coming to equilibrium (redox potentials pertain to equilibrium conditions), incomplete reversibility of some systems, and back- diffusion of oxygen during measurement.

O_2, ascorbate, and riboflavin are the principal systems determining the E_h of milk. The thiol–disulfide system contributes to the E_h of heated milk. All the ascorbate in freshly drawn milk is in the reduced form, but reversible oxidation to dehydroascorbate occurs at a rate dependent on the concentrations of Cu^{2+}, Fe^{3+}, and O_2; the E_h values of all three of these are higher than that of ascorbate. Preservation of ascorbate depends on deaeration and prevention of contamination by copper.

Oxidation–reduction reactions in milk are influenced by heat treatment, bacterial activity, and exposure to light, as well as by O_2 and the presence of metal ions. Heating lowers the E_h of milk, the more so if high temperature–short time conditions are used and prior deaeration carried out; the result is better retention of reducing substances such as ascorbic acid.

Bacterial activity reduces the E_h by consumption of available oxygen in the milk. The decrease in potential can lead to the reduction and loss of color of certain added dyes, and is the basis of the methylene blue test for the bacterial status of fresh milk.

The riboflavin in milk does not contribute significantly to E_h or to poising. However, the absorption of visible light by riboflavin (at 470 nm) results in excitation. Excited-state riboflavin can oxidize a number of compounds in milk, thereby itself becoming reduced. This is an example of photooxidation, and it results in the production of off-flavors in milk exposed to sufficiently intense visible light, for example, direct sunlight.

Conclusions

Some of the physical and physico-chemical properties of milk, such as density, thermal properties, freezing point, acid–base equilibria, and oxidation–reduction equilibria, have been well studied and are well understood. Others, such as acoustic properties and dielectric properties, have been studied to a relatively limited extent. This is a reflection of the relative importance, historically, of the different properties with respect to production, analysis, handling, and processing of milk.

Milk, a complex biological fluid, is by no means fully understood. There is no doubt that future advances in our understanding of its nature and attributes will depend to a significant extent on the use of measurement techniques whose development will be built upon and will extend our current knowledge of physical and physico-chemical properties.

See also: **Analytical Methods**: Differential Scanning Calorimetry; Infrared Spectroscopy in Dairy Analysis; Light Scattering Techniques; Physical Methods; Rheological Methods: Instrumentation; Principles and Significance in Assessing Rheological and Textural Properties; Spectroscopy, Overview; Ultrasonic Techniques. **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk. **Rheology of Liquid and Semi-Solid Milk Products**.

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Bovine Milk

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Introduction

While milk for human consumption is obtained from several species, breeds of the genus *Bos*, especially *Bos taurus* (*see* **Animals that Produce Dairy Foods**: Major *Bos taurus* Breeds; Minor and Dual-Purpose *Bos taurus* Breeds), predominate in milk production. Bovine milk represents about 85% of all milk offered for sale, compared to 11% represented by buffalo milk and 2% each by ovine and caprine milk. For several hundred years, some breeds of *B. taurus* have been selected and bred specifically for milk production, and consequently are now extremely efficient for this purpose. Owing to its commercial importance, bovine milk has been the subject of research for more than 100 years and is now the most thoroughly characterized food system. The word milk, when unqualified, is assumed to refer to bovine milk.

The milks of all species have similar constituents and those of the commercial dairying species are quite similar, but there are significant interspecies, even interbreed, differences, which affect the processability of milk. The general characteristics of milk are described in the article **Milk**: Introduction. The specific unique properties of bovine milk are described in an introductory way in this article, while the milks of other commercially important or interesting species are described in the articles **Milk**: Buffalo Milk; Camel Milk; Equid Milk; Goat Milk; Human Milk; Milk of Marine Mammals; Milks of Non-Dairy Mammals; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.); Sheep Milk. This set of articles is intended to provide overviews, and especially interspecies comparisons, of milk. The properties of milk constituents, especially those of bovine milk, and the effects thereof on milk processing are considered in several articles throughout this encyclopedia.

Milk contains several hundred compounds, mostly at trace levels. These constituents can be classified into four categories:

- organ- and species-specific constituents (lipids and the principal proteins)
- organ- but not species-specific constituents (lactose)
- species- but not organ-specific constituents (some whey proteins (WPs))
- neither organ- nor species-specific constituents, although the profile and concentrations are species specific (water, salts, vitamins)

Like the milk of probably all species, the composition, and hence the properties, of bovine milk varies with several factors, especially breed, stage of lactation, and health, nutritional status, and individuality of the animal – the differences observed may be very large and consequently milk should be selected carefully for processing. The influence of animal husbandry practices on the production and composition of milk is described in several articles throughout this encyclopedia. Although the modern dairy cow is very highly bred and selected, intrabreed differences persist, even within single herds under essentially identical management.

The principal and some of the minor constituents of milk are the subject of at least one dedicated article. This article presents a brief general overview of the principal constituents, lactose, lipids, proteins, and salts, and properties of bovine milk.

Lactose

Lactose, one of the principal constituents of the milk of most species, is a reducing disaccharide unique to milk. Bovine milk typically contains ~4.8% lactose, a value that decreases with advancing lactation and mastitic infection. The concentration of lactose in the milk of the principal dairying species, cow, buffalo, goat, and sheep, is approximately the same, at 4.5–5.0%, and there is little effect of breed or individuality or nutrition of the animal on the lactose content of its milk, owing to the relationship between lactose concentration and the osmotic pressure of blood, which is fixed (*see* **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties).

Lactose is a slightly sweet-reducing disaccharide, which occurs in the milk of most species. Because of its low solubility, crystallization behavior, and hygroscopicity, it causes problems in concentrated, dehydrated, and frozen products; these problems are largely confined to bovine milk since very little of such products are produced from the milk of other species. The chemistry, technologically important properties, production, and applications of lactose are described in the articles **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Derivatives; Lactose: Galacto-Oligosaccharides; Lactose Intolerance; Lactose: Production, Applications; Maillard Reactions.

Oligosaccharides

Oligosaccharides (OSs) containing 3–10 monosaccharides, including fucose and *N*-acetylneuraminic acid, are believed to have been the principal carbohydrates in milk during the early stages of mammalian evolution; they are considered to have had a protective rather than a nutritional role (*see* **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk). In comparison to the milk of many other species (e.g., humans, elephants, bears, marsupials, and monotremes), bovine milk contains low levels of OSs, which have no known technological significance but are believed to be nutritionally and physiologically significant. The OSs partition into the whey during cheese production and there is commercial interest in isolating them for the supplementation of infant formulae. They can be concentrated and purified by membrane fractionation, and the charged OSs, recovered by ion-exchange chromatography.

Lipids

Until recently, the lipids of bovine milk were considered to be its most valuable constituents, being used for the production of butter and ghee and being very important to the quality of cheese, liquid milk, and ice cream. The use of milk fat in spreads has now declined and has been replaced by hydrogenated plant oils. The significance of milk fat in various dairy products is discussed in several articles throughout this encyclopedia (*see* **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Fat Replacers; Modified Butters; Milk Fat-Based Spreads; Properties and Analysis; The Product and Its Manufacture; **Cream**: Manufacture; Products).

Bovine milk contains ~3–6% lipids, the value depending on breed, individuality, stage of lactation, interval between milking, point during milking at which the sample is taken, nutritional and health status of the animals, and other minor factors. The milk of Jersey and Guernsey cows has the highest fat content and that of Friesian and Holstein the lowest; within any breed, there is considerable variation between individual animals in the fat content of milk. Since the fat content of milk is genetically controlled, breeding programs strive to increase it. The fat content of bovine milk decreases during the first 4–6 weeks postpartum, but then increases considerably, especially toward the end of lactation. The point during milking at which a sample is taken has a very large effect on its fat content, which increases from ~1% at the start of milking to about 10% at the end; this effect is attributed to the retardation of fat globules in the small ducts of the mammary gland – a similar effect is not observed for the soluble constituents, proteins and lactose. Assuming that the cow is thoroughly milked, this factor is not commercially significant. If the interval between milkings is different, the fat content of milk is inversely related to the interval between milkings; this effect may be commercially important, especially if an incorrect sampling procedure is used. If the animal suffers from malnutrition, the fat content of its milk will be reduced, especially if the diet is deficient in roughage, because about 50% of the fatty acids (FAs) in milk are synthesized by rumen microbes acting on cellulose; a diet rich in lipids, especially unsaturated lipids, depresses the fat content of milk (*see* **Feed Ingredients**: Feed Concentrates: Cereal Grains; Feed Concentrates: Cereal Grains; Feed Concentrates: Oilseed and Oilseed Meals; **Feeds, Prediction of Energy and Proteins**: Feed Energy; Feed Proteins; **Feeds, Ration Formulation**: General Nutritive Requirements of Sheep and Goats; Lactation Rations for Dairy Cattle on Dry Lot Systems; Models in Nutritional Management; Models in Nutritional Research; Systems Describing Nutritional Requirements of Dairy Cows; **Feed Supplements**: Feed Supplements: Anionic Salts; Feed Supplements: Fats and Protected Fats; Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Organic-Chelated Minerals; Feed Supplements: Ruminally Protected Amino Acids; Vitamins).

The lipids in milk are principally (98%) triglycerides, with smaller amounts of phospholipids, other polar lipids, diglycerides, sterols (principally cholesterol), carotenoids, fat-soluble vitamins, and traces of FAs and hydrocarbons.

The FAs in milk lipids are not synthesized in the mammary gland but originate from the diet or from blood lipids synthesized *de novo* in the liver; in the case of ruminants, β-hydroxybutanoic acid, produced by rumen bacteria, supplies a substantial part of the lipid carbon, being converted to butanoic acid and incorporated directly into triglycerides or converted to butanyl CoA used instead of acetyl CoA in the biosynthesis of FAs (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat). Hence, ruminant milk fats contain substantial (~20 mol%) amounts of C4:0, C6:0, C8:0, and C:10:0 – they are the only lipids that contain C4:0. The proportions of these short-chain FAs, which vary with the species of ruminants, have major effects on the physical properties and flavor-generating aspects of these lipids. Ruminant milk fats contain low levels of polyunsaturated fatty acids (PUFAs) because dietary PUFAs are biohydrogenated in the rumen. Ruminant milk fats contain a relatively high level of saturated FAs, which are considered to be nutritionally undesirable. However, opinions on the nutritional significance of FAs are changing (*see* **Milk Lipids**: Bioactive Lipids; **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Fatty Acids of Milk and Cardiovascular Disease). However, contain substantial amounts of Δ 9, 11 *cis, trans* octadecadienoic acid (conjugated linoleic acid (CLA)), produced by incomplete biohydrogenation of linoleic acid in the rumen. The level of CLA, which has anticarcinogenic properties, in ruminant milk fats is affected by the levels of PUFAs in the diet and is higher in bovine than in other ruminant milk fats (*see* **Milk Lipids**: Conjugated Linoleic Acid).

Cattle transfer dietary carotenoids to milk, at a level that is strongly dependent on the breed of animal, the plants in the forage, and season of the year; other ruminants do not do this. Consequently, bovine milk and products produced therefrom are markedly yellow in comparison with buffalo, sheep, or goat milk. The yellow color of products produced from bovine milk is unattractive to many consumers accustomed to products produced from buffalo, sheep, or goat milk. The carotenoids may be bleached by benzoyl peroxide or masked using TiO_2 or chlorophyll.

Lipids are insoluble in the aqueous environment of milk; they occur as an emulsion stabilized by a special membrane, the milk fat globule membrane (MFGM), which consists of polar lipids and special proteins, acquired during the expression of fat globules from the mammocytes (*see* **Milk Lipids**: Milk Fat Globule Membrane). One of the special proteins is xanthine oxidoreductase (XOR), which is present on the inner surface of the MFGM and somehow initiates blebbing of the fat globules through the apical membrane of the mammocyte. The mechanism of this action of XOR is unknown but it is not enzymatic. Bovine milk possesses much higher XOR activity than the milk of any other species studied, apparently due to a very low level of molybdenum in the milk of the other species studied – how this affects the release of fat globules is not known. The fat globules in bovine milk range in diameter from 0.1 to 20 µm, with an average of 3–4 µm. The average diameter decreases throughout lactation and is higher in high-fat (Jersey and Guernsey) milk than in Friesian or Holstein milk. The MFGM partitions into buttermilk on churning for butter manufacture, which is, therefore, rich in phospholipids and other polar lipids which have good emulsifying and nutritional properties (*see* **Milk Lipids**: Buttermilk and Milk Fat Globule Membrane Fractions).

The fat globules in milk are less dense than the skim milk phase and, consequently, would be expected to rise to the surface and form a cream layer in accordance with Stokes' Law. However, the fat globules in bovine milk cream much faster and more copiously than Stokes' Law predicts. The difference is due to the presence of certain globulins (referred as cryoglobulins) that are insoluble at low temperatures and precipitate onto the fat globules, causing them to form clumps, which cream very rapidly due to their large diameter. The milk of the other dairying species lacks cryoglobulins and consequently creams very slowly. The nutritional and physiological significance, if any, of cryoglobulins in bovine milk is not known.

The MFGM contains mucoproteins (mucins), MUC 1 and MUC 15, which in human and equine milk form long (5–20 µm) tendrils; in the case of bovine milk, these tendrils are very unstable and are shed quickly. Pathogenic bacteria attach to the mucins of the tendrils, which appear to have an antibacterial function; they may also improve the digestibility of lipids. The stability of the tendrils of the MFGM of other species has not been reported.

Proteins

Bovine milk contains ~3.5% protein, but this level varies substantially with breed, individuality, stage of lactation, and health and nutritional status of the animal. The technological properties of milk, indeed the very existence of most dairy products, are determined mainly by the unique properties of some of its proteins, which have been very well characterized.

The milk proteins of all species that have been studied fall into two groups: caseins (phosphoproteins that are insoluble at pH ~4.6 and 20 °C, and which are unique to milk) and whey (serum) proteins, the principal of which are milk-specific, with several minor ones derived from the blood (*see* **Milk Proteins**: Heterogeneity, Fractionation, and Isolation). The ratio of caseins to WPs is characteristic of the species. In bovine, ovine, caprine, and buffalo milk, and probably in the milk of other ruminants, the caseins represent ~80% of total proteins, while in human, equine, asinine, porcine, and elephant milk, they are 50% or less. However, the milk of rodents, cats, dogs, and rabbits also has a high proportion of casein and there seems to be no obvious pattern among species, although species that produce a high level of protein in their milk have a high proportion of casein, which presumably reflects the high growth rate of the neonate; animals that grow rapidly tend to have a high casein content in their milk. In addition to supplying amino acids to the neonate, the caseins supply calcium and phosphorus (see below), which are essential for the rapidly growing neonate.

Caseins

Bovine casein comprises four proteins, α_{s1}-, α_{s2}-, β-, and κ-caseins, in the approximate proportions of 38, 10, 35, and 12, respectively, each of which exhibits microheterogeneity owing to genetic polymorphism, variations in the degree of phosphorylation or glycosylation and perhaps deamidation, and in the case of α_{s2}- and κ-caseins, disulfide-linked polymerization. The milk of buffalo, sheep, and goat and many other ruminants also contains these four caseins but human milk contains little or no α_s-casein and equine milk contains little κ-casein. The caseins are relatively small (20–25 kDa) molecules with many rather unique characteristics. All are phosphorylated but the level of phosphorylation varies from 1 to 13; some equine and human caseins are not phosphorylated. All caseins, regardless of species, have a high level of proline (16% for bovine β-casein), which interrupts secondary structures, resulting in the caseins being unstructured, open, rheomorphic proteins; this renders the caseins very surface active and readily digestible, which conforms with the natural function of the caseins as sources of amino acids. The caseins are amiphatic and have high surface activity (*see* **Milk Proteins**: Casein Nomenclature, Structure, and Association).

α_{s1}-, α_{s2}-, and β-caseins bind Ca^{2+} and other polyvalent cations strongly and are rendered insoluble at $[Ca^{2+}]$ >6 mmol l^{-1} at 20 °C. However, the solubility of κ-casein is not affected by Ca^{2+} and it can stabilize up to 10 times of its mass of Ca-sensitive caseins by forming micelles (*see* **Milk Proteins**: Caseins, Micellar Structure). The stability, and destabilization under certain circumstances, of the micelles is critical to dairy technology; in fact, the dairy industry depends on certain properties of the casein micelles. Therefore, the structure and properties of the casein micelles have been studied intensely over the past 100 years and there is general agreement thereon (*see* **Milk Proteins**: Caseins, Micellar Structure). The caseins in the milk of all species appear to form micelles, which are generally similar to those of bovine milk but there are significant differences, as will be apparent from the species-specific articles (*see* **Milk**: Buffalo Milk; Camel Milk; Colostrum; Equid Milk; Goat Milk; Human Milk; Milk of Marine Mammals; Milks of Nondairy Land Mammals; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.); Sheep Milk; Seasonal Effects on Processing Properties of Cows' Milk).

Whey Proteins

The WPs are typical globular proteins, with high levels of secondary, tertiary, and, in some cases, quaternary structures. Bovine milk contains four principal WPs, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), blood serum albumin (BSA), and immunoglobulins (Igs), which represent ~60, 20, 10, and 10% of total WP, respectively, and several minor proteins, mostly at trace levels, many of which have a biological function, including ~60 indigenous enzymes. The WPs of bovine milk are generally similar to those of other species, although there are many minor interspecies differences in amino acid sequences and properties. Human and rodent milk contains no β-Lg and the milk of some marine mammals lacks α-La; the properties and significance of α-La and β-Lg are described in the articles **Milk Proteins**: α-Lactalbumin; β-Lactoglobulin, respectively.

BSA is a leakage protein from blood and has no biological or technological significance in milk. The principal Ig in bovine, ovine, caprine, and buffalo milk is IgG1, which is transported selectively from the blood and is present at ~10% in colostrum and bestows passive immunity on the young of these species who do not receive Ig *in utero*; Ig has no special significance in mature milk. The minor proteins include lactoferrin, vitamin-binding proteins, peptide hormones, and enzymes, and are probably common to most species although the concentration of many shows large interspecies differences; only a few minor proteins in non-bovine milks have been studied. For unknown reasons, the indigenous enzymes are probably the most variable constituents in milk. Bovine milk contains high levels of XOR, lactoperoxidase (LPO), and ribonuclease but a low level of lysozyme. Several of the indigenous enzymes have significant functions: for example, deterioration of quality (e.g., lipase, plasmin, acid phosphatase, XOR), preservation of quality (lysozyme, superoxide dismutase, LPO), as indicators of thermal history (e.g., alkaline phosphatase, catalase, γ-glutamine-transpeptidase, LPO), or as indicators of mastitis (e.g., *N*-acetylglucosaminidase, catalase).

Milk Salts

When bovine milk is heated at a very high temperature, >500 °C, a residue, ash, representing 0.7–0.8% (w/w) of the milk remains. Some of the ash derives from the proteins, a little from the phospholipids, and a trace from sugar phosphates, but the majority is derived from inorganic salts, especially chlorides, phosphates, sulfates, and carbonates of Na, K, Ca, and Mg (macroelements). In addition, milk contains ~30 elements at trace concentrations (microelements). The inorganic elements are present in the milk as salts, some of which are in true solution, some associated with proteins, especially caseins (i.e., colloidal), and a small proportion in the MFGM as a component of some proteins. The inorganic elements are important for nutrition, spoilage (lipid oxidation), structure, and properties of many of the proteins and the activity of several enzymes. Although the inorganic salts are quantitatively minor constituents, they are very important from a nutritional viewpoint and for the technological properties of milk, as discussed in the articles **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace

Elements, Nutritional Significance. Qualitatively, bovine milk contains the same range of inorganic elements as the milk of other species but there are very substantial quantitative differences, which affect the processability of milk.

Technological Properties of Bovine Milk

It will be evident from the preceding discussion that although there are interspecies differences in the composition and chemical and physical properties of milk, these differences are quite small, especially between the milk of species of the same family, for example, Bovinae. However, these small differences are reflected in the processing properties of milk. Perhaps the most important technological differences are FA profile of the lipids, rate of creaming, rennet coagulability, syneresis of rennet-induced gel, and heat stability of milk. These properties are discussed more extensively in separate articles in this encyclopedia.

As described earlier, bovine milk creams much faster than the milk of other species due to the aggregation of the fat globules. This property is not important today (in fact creaming is often prevented by homogenizing the milk) but in the past it was important for the preparation of cream for buttermaking, prior to the invention of the centrifugal cream separator by Gustav de Laval in 1878. Milk was designed to coagulate in the neonatal stomach, which is achieved through limited proteolysis of the micelle-stabilizing protein, κ-casein, by a specific gastric proteinase, chymosin (*see* **Cheese**: Rennet-Induced Coagulation of Milk); coagulation delays discharge from the stomach into the intestine and thereby improves digestibility and levels out the supply of nutrients. The firmness of the coagulum is strongly dependent on the protein content of the milk – human, camel, and equine milks form protein flocks rather than a gel and are probably more readily digested than bovine, caprine, and ovine milks.

The rennet-induced coagulation of milk is exploited in the production of cheese, 75% of which is produced from rennet-coagulated casein. Bovine milk has good rennet coagulability, as do the milks of goat, sheep, and buffalo, due partly to their relatively high casein content (*see* **Milk**: Buffalo Milk; Goat Milk; Sheep Milk).

Milk also gels on acidification to pH ~4.6, that is, to the isoelectric point of the caseins. This property is exploited in the production of acid-coagulated (fresh) cheeses, fermented milks, and acid casein (an important functional protein) (*see* **Cheese**: Swiss-Type Cheeses; **Fermented Milks**: Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Kefir; Koumiss; Middle Eastern Fermented Milks; Nordic Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture; **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects). As for the rennet-induced coagulation of milk, the firmness of an acid-induced milk gel is strongly related to the casein content of the milk, and the four principal dairying species, especially sheep, are very suitable for this application.

Bovine milk is a very heat-stable system (*see* **Heat Treatment of Milk**: Heat Stability of Milk), which permits the production of a range of heat-treated products without major apparent changes in the appearance and properties of milk. The heat stability of milk is highly dependent on its composition; bovine milk is more heat stable than the milk of other dairying species due, at least in part, to the concentration of calcium in the milk.

Conclusion

Bovine milk is a very complex and very well-characterized biological system. Most of the topics introduced in this article will be explained in greater detail in other articles throughout the encyclopedia.

See also: **Animals that Produce Dairy Foods**: Major *Bos taurus* Breeds; Minor and Dual-Purpose *Bos taurus* Breeds. **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Fat Replacers; Milk Fat-Based Spreads; Modified Butters; Properties and Analysis; The Product and Its Manufacture. **Cheese**: Rennet-Induced Coagulation of Milk; Swiss-Type Cheeses. **Cream**: Manufacture; Products. **Feed Ingredients**: Feed Concentrates: Co-Product Feeds; Feed Concentrates: Cereal Grains; Feed Concentrates: Oilseed and Oilseed Meals. **Feeds, Prediction of Energy and Proteins**: Feed Energy; Feed Proteins; Feed Supplements: Anionic Salts; Feed Supplements: Fats and Protected Fats; Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Organic-Chelated Minerals; Feed Supplements: Ruminally Protected Amino Acids; Feed Supplements: Vitamins. **Feeds, Ration Formulation:** Lactation Rations for Dairy Cattle on Dry Lot Systems; Models in Nutritional Management; Models in Nutritional Research; Systems Describing Nutritional Requirements of Dairy Cows. **Fermented Milks**: Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Kefir; Koumiss; Middle Eastern Fermented Milks; Nordic Fermented Milks; Starter Cultures; Types and Standards of Identity; Yogurt: Role of Starter Culture; Yogurt: Types and Manufacture. **Heat Treatment of Milk**: Heat Stability of Milk. **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk; Lactose: Chemistry, Properties; Lactose: Derivatives; Lactose:

Galacto-Oligosaccharides; Lactose Intolerance; Lactose: Production, Applications; Maillard Reactions. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat. **Milk**: Buffalo Milk; Camel Milk; Colostrum; Equid Milk; Goat Milk; Human Milk; Introduction; Milk of Marine Mammals; Milks of Non-Dairy Mammals; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.); Physical and Physico-Chemical Properties of Milk; Sheep Milk; Seasonal Effects on Processing Properties of Cows' Milk. **Milk Lipids**: Buttermilk and Milk Fat Globule Membrane Fractions; Conjugated Linoleic Acid; Milk Fat Globule Membrane. **Milk Proteins**: Casein Nomenclature, Structure, and Association; Caseins, Micellar Structure; Heterogeneity, Fractionation, and Isolation; α-Lactalbumin; β-Lactoglobulin. **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects. **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance. **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Fatty Acids of Milk and Cardiovascular Disease.

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Goat Milk

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Introduction

Goats' (*Capra hircus*) milk ranks fourth after cows', buffaloes', and sheep's milks in terms of world milk production. Although goats' milk production (14.80 metric ton (MT), for 2007) accounts for only 2.2% of the total world milk production, goat-keeping has a significant economic importance in countries where climatic conditions are not favorable for cattle-keeping. The world leaders in goats' milk production are listed in **Table 1**.

The production of goats' milk is likely to be much greater than the official statistics indicate because a large amount of milk that is used for home consumption goes unreported. Especially in developing countries, the production of this type of milk has become a useful strategy to deal with malnutrition, especially among the infant population. Europe, which produced 2.58 Mt of goats' milk in 2007, contributes about 17.5% of the world's goats' milk supply. Countries around the Mediterranean region have the most developed dairy goat industries, with France, Greece, Spain, and Italy among the main goats' milk-producing countries. In recent years, there has been an increased interest in goats' milk production and conversion to value-added products, encouraged within the European Union countries by the introduction of quotas on cows' milk in 1984. Moreover, there are four aspects to the increase in goats' milk production compared to other farm animals: (1) domestic consumption; (2) the connoisseur interest in goats' milk products, especially cheeses and yogurt, in many developed countries; (3) the affliction of people with cows' milk allergies and other gastrointestinal problems; and (4) the potential health benefits associated with the consumption of goats' milk, related to, for example, its higher oligosaccharide and nucleotide content.

Composition and Physical Properties of Goats' Milk

The composition of goats' milk varies widely and is influenced by breed, nutritional and environmental factors, stage of lactation, parity, and season. To add to this complexity, there is a wide variability in composition among individual animals of the same breed attributed to an extensive and complex genetic polymorphism of the goats' milk caseins. **Table 2** shows the average gross composition of goats' milk as well as the ranges of the main constituents. The composition of goats' milk is similar to that of cows' milk in terms of total solids (TS), fat, crude protein, lactose, and ash contents, but there are important differences in the individual components, as detailed below.

The density of goats' milk is comparable to that of cows' milk but is lower than that of sheep's milk, whereas both goats' and sheep's milks have higher specific gravity, viscosity, and titratable acidity but lower refractive index and freezing point than cows' milk (**Table 3**).

Carbohydrates

Lactose is the major carbohydrate in goats' milk, but it is approximately 0.2–0.5% less than that in cows' milk. Other carbohydrates in goats' milk include oligosaccharides, glycopeptides, glycoproteins, and nucleotide sugars. Goats' milk has a remarkably high oligosaccharide content, and the diversity found in goats' milk oligosaccharides is important. Cows' milk contains low levels of monosaccharides and oligosaccharides. Milk oligosaccharides have considerable antigenic properties and are valuable in promoting the growth of the intestinal flora of the newborn. Oligosaccharides promote the growth of bifidobacteria in the neonate and play a role as intestinal mucosal cell protectors against pathogens. Finally, they may play a role in neonatal brain development. There is 4 times as much sialic acid in goats' milk (~230 mg sialic acid per kg fresh milk) as in cows' milk (60 mg sialic acid per kg fresh milk). Moreover, nucleotide sugars in milk are of particular interest since they are the glycosyl donors for glycosyltransferase in milk and mammary gland, and are the precursors of glycoproteins, glycolipids, and oligosaccharides in the biosynthesis of milk. Goats' milk has a high nucleotide content, about 154 μmol $100\,\mathrm{ml}^{-1}$ milk, followed by sheep's milk (93 μmol $100\,\mathrm{ml}^{-1}$) and cows' milk (68 μmol $100\,\mathrm{ml}^{-1}$), while human mik contains only 5 μmol $100\,\mathrm{ml}^{-1}$.

Table 1 Top 10 goats' milk-producing countries in the world during 2007

Goats' milk production		
Position	*Country*	*Mt*
1	India	3.82
2	Bangladesh	2.01
3	Sudan	1.45
4	Pakistan	0.70
5	France	0.59
6	Greece	0.50
7	Spain	0.49
8	Somalia	0.39
9	Islamic Republic of Iran	0.37
10	China	0.27
	World goats' milk production	14.80

Data from FAO electronic statistical service.

Table 2 Gross composition of goats' milk

	Average	*Range*
	(%, w/w)	*(%, w/w)*
Total solids	12.90	9.95–21.5
Fat	4.10	2.46–7.76
Crude protein[a]	3.50	2.49–5.06
Casein	2.90	2.33–4.63
Lactose	4.50	3.62–6.30
Ash	0.80	0.69–0.89

[a] Protein values represent crude protein, that is, total nitrogen multiplied by 6.38, and thus are approximately 0.25% higher than true protein (total nitrogen minus nonprotein nitrogen).
Ranges are based on the results from different authors.

Lipid Fraction

Fats are one of the most important components of goats' milk in terms of cost, nutrition, and the physical and sensory characteristics that they impart to goat dairy products. The fatty acid (FA) composition of goats' milk shows substantial differences from that of cows' milk. Goats' milk fat is rich in short-chain fatty acids (SCFAs) (e.g., caproic ($C_{6:0}$), caprylic ($C_{8:0}$), and capric ($C_{10:0}$) acids) and medium-chain fatty acids (MCFAs) (e.g., lauric acid ($C_{12:0}$)). SCFAs represent up to 15–18% of the FAs in goats' milk, but only 5–9% in cows' milk, because of differences in the polymerization of the acetate produced by the rumen bacteria in goat, and are associated with the characteristic odor and flavor of goats' milk cheeses (**Table 4**).

Another noteworthy aspect concerning the FA profile of goats' milk is its content of branched-chain free FAs with less than 11 atoms of carbon, which are virtually non-existent in cows' milk and which give the characteristic goaty flavors to goat dairy products. The most novel aspect reported to date concerning the composition of ruminant milk fats concerns their content of conjugated linoleic acids (CLAs). These are a series of positional and geometric isomers of linoleic acid, which contain a conjugated double bond system. Data from animal models have been used to suggest that the main CLA isomer, *cis*-9 *trans*-11 $C_{18:2}$, is responsible for the anticarcinogenic properties of CLA, as well as antiatherogenic effects.

The triacylglycerol (TAG) structure of milk fat is responsible for its rheological properties and its melting and crystallization behavior. Although the composition and particularly the dominant position of the triacylglycerides are not unlike those of cows' milk, a larger concentration of free FAs is found in goats' milk. The free FAs are the result of hydrolysis and their presence could lead to the development of desirable or undesirable flavors, depending on their type and concentration. In goats' milk, the free FAs are the main source of the characteristic flavor. A number of lipases have been identified and are considered responsible for the hydrolysis. **Table 5** shows the average TAG composition of goats' milk fat, which presents a wide range of molecular weights and chain lengths with acyl carbon atom numbers between C_{26} and C_{54}. The distribution of acyl carbon numbers shows a maximum at C_{40}–C_{42}. The percentage

Table 3 Some physical properties of goats' milk

Properties	*Goat*	*Cow*
Specific gravity (density)	1.029–1.039	1.023 1–1.039 8
Viscosity (cP)	2.12	2.0
Surface tension (dyn cm^{-1})	52.0	42.3–52.1
Conductivity (Ω^{-1} $cm^{-1)}$	0.004 3–0.013 9	0.004 0–0.005 5
Refractive index	1.450 ± 0.39	1.451 ± 0.35
Freezing point (−°C)	0.540–0.573	0.530–0.570
Acidity (lactic acid %)	0.14–0.23	0.15–0.18
pH	6.50–6.80	6.65–6.71

Data from Juárez M and Ramos M (1986) Physico-chemical characteristics of goats' milk as distinct from those of cow milk. Bulletin of the *International Dairy Federation* 202: 54–67.

Table 4 The main fatty acids (percentage of total fatty acid methyl esters) in goats' milk

Fatty acid	*Mean*	*Range*
Butyric ($C_{4:0}$)	2.18	1.97–2.44
Caproic ($C_{6:0}$)	2.39	2.03–2.70
Caprylic ($C_{8:0}$)	2.73	2.28–3.04
Capric ($C_{10:0}$)	9.97	8.85–11.00
Decenoic ($C_{10:1}$)	0.24	0.19–0.38
Lauric ($C_{12:0}$)	4.99	3.87–6.18
Dodecenoic ($C_{12:1}$)	0.19	0.10–0.40
Tridecanoic ($C_{13:0}$)	0.15	0.06–0.28
Myristic ($C_{14:0}$)	9.81	7.71–11.20
iso Pentadecanoic ($C_{15:0}$)	0.13	0.12–0.15
anteiso Pentadecanoic ($C_{15:0}$)	0.21	0.17–0.24
Myristoleic ($C_{14:1}$)	0.18	0.17–0.20
Pentadecanoic ($C_{15:0}$)	0.71	0.46–0.85
iso Palmitic ($C_{16:0}$)	0.24	0.17–0.40
Palmitic ($C_{16:0}$)	28.00	23.20–34.80
iso Heptadecanoic ($C_{17:0}$)	0.35	0.24–0.52
anteiso Heptadecanoic ($C_{17:0}$)	0.42	0.30–0.50
Palmitololeic ($C_{16:1}$)	1.59	1.00–2.70
Heptadecanoic ($C_{17:0}$)	0.72	0.52–0.90
Heptadecenoic ($C_{17:1}$)	0.39	0.24–0.48
Stearic ($C_{18:0}$)	8.88	5.77–13.20
Oleic[a] ($C_{18:1}$)	19.3	15.40–27.70
Linoleic[a] ($C_{18:2}$)	3.19	2.49–4.34
Eicosanoic ($C_{20:0}$)	0.15	0.08–0.35
Linolenic[a] ($C_{18:3}$)	0.42	0.19–0.87
Linoleic conjugated[a] ($C_{18:2}$)	0.70	0.32–1.17

[a] All isomers.
Ranges are based on the results from different authors.

Table 5 Triacylglycerol composition of goats' milk fat

	Goats' milk fat
Triacylglyceride CN[a]	*(wt.%)*
C_{26}	0.49
C_{28}	1.23
C_{30}	2.47
C_{32}	4.06
C_{34}	6.20
C_{36}	9.40
C_{38}	12.10
C_{40}	12.60
C_{42}	12.50
C_{44}	11.60
C_{46}	8.10
C_{48}	5.84
C_{50}	5.85
C_{52}	4.92
C_{54}	2.01

[a] Carbon number.
Data from Fontecha J, Fraga MJ, and Juárez M (1998) Triglyceride analysis by GC in assessment of authenticity of goats' milk fat. *Journal of the American Oil Chemists' Society* 75: 1893–1896.

of C_{38}–C_{44} TAG is much higher in goats' milk fat than in cows' milk fat (49 vs. 36%, respectively), which is related to the need for a TAG composition with appropriate melting points for fat secretion. Goats' milk fat is rich in medium-chain triacylglycerides (MCTs), made up preferentially of SCFAs. Therefore, the MCTs of goats' milk are of special interest, indeed, from a therapeutic point of view, because of their particular metabolism and hence their application for certain cases of metabolic illness.

Analysis of the positional distribution of FAs in goats' milk triglycerides shows that a majority of the SCFAs are esterified at the *sn*-3 position of the glycerol, whereas the long chain FAs are esterified at *sn*-2 position. This specific placement of FAs implies that triglycerides are synthesized from a pool of long-chain 1,2-diglycerides.

Lipids in goats' milk are present in the form of globules, which naturally do not aggregate upon cooling because they lack agglutinin, responsible for the aggregation of fat globules in cows' milk. Besides, the goats' milk fat globules are characteristically abundant and smaller than those of cows' milk. The average diameter of the individual fat globules in goats' milk is 2.76 μm and is smaller than the mean diameter of 3.51 μm for cows' milk. This is advantageous for digestibility and a more efficient lipid metabolism compared with cows' milk fat.

The milk fat globule membrane (MFGM) consists of plasma membrane of the secretory cell and contains protein constituents (some of which are enzymes), phospholipids, cerebrosides, and gangliosides. So far, studies suggest that the protein and phospholipid compositions of the MFGM are similar in goats and cows. About 60% of the total phospholipids in goats' milk are on the MFGM. All the classes found in cows' milk are present – phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin.

Goats' milk fat contains significant concentrations of aliphatic δ-lactones or their hydroxy acid precursors. These compounds are associated with the development of flavors in heated and stored dairy products.

Proteins

The principal proteins in goats' milk are the same as in the milk of other species, namely the caseins (κ-, β-, α_{s1}-, α_{s2}-, and γ-CN) and the whey proteins, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), serum albumin, and immunoglobulins.

The amino acid composition and sequence of the goat caseins and whey proteins have been determined from analyses of the cDNA sequence and are shown in **Table 6**.

A homology of 80–90% exists between goats' and cows' milk proteins. The relative proportion of the four

Table 6 Amino acid composition of goats' milk caseins and whey proteins

Amino acid	*α_{s1}-CN B*	*α_{s2}-CN*	*β_1-CN A*	*β_2-CN A*	*κ-CN A*	*β-Lg*	*α-La*
Aspartic acid	7	5	4	4	7	8	14
Alanine	12	9	4	4	16	16	6
Arginine	7	7	3	3	5	3	1
Asparagine	11	13	4	4	9	6	8
Cysteine	0	2	0	0	3	5	8
Glutamic acid	20	25	19	19	11	15	6
Glutamine	14	16	21	21	15	9	7
Glycine	9	2	5	5	1	5	5
Histidine	4	5	5	5	4	2	3
Isoleucine	9	12	9	9	10	10	8
Leucine	17	10	22	22	8	21	13
Lysine	13	24	12	12	8	16	13
Methionine	5	4	6	6	1	4	0
Phenylalanine	7	7	9	9	4	4	4
Proline	19	13	33	33	19	8	2
Serine	9	5	10	9	11	6	6
Serine phosphate	9	9	5	6	2		
Threonine	5	14	11	11	15	8	6
Tryptophan	2	3	1	1	1	2	4
Tyrosine	11	12	3	3	9	4	4
Valine	9	11	21	21	12	10	5
Total amino acids	199	208	207	207	171	162	123

major caseins in caprine milk varies widely between individual animals. This variation is due largely to an unusual and complex genetic variability that exists on the casein loci (**Table 7**). In the polymorphism of α_{s1}-CN, more than 18 alleles have been identified, which are distributed among ten different classes of protein variants (α_{s1}-CN A, B1, B2, B3, C, D, E, F, G and O) and associated with four levels of α_{s1}-CN expression. Variant α_{s1}-CN B1 is believed to be the original type of the species because it has the closest homology with the bovine and ovine counterparts. The allele frequencies at the α_{s1}-CN locus vary with breed. In French and Italian Alpine and Saanen goats, alleles E and F predominate. In the Italian goat breed, Sarda, the strong A and B alleles predominate, whereas the majority of Verzaschese goats have the weak F variant. In French and Spanish breeds, allele E is the most frequently encountered, whereas in Swiss breeds there is a high frequency of the defective mutants E, F, and O in the order of decreasing frequency. This has enormous implications because the genes of the Alpine, Saanen, and Toggenburg breeds have been spread worldwide.

Table 7 Protein composition of goats' milk

	Concentration
Protein	*(%)*
Total casein	2.33–4.63
β-Casein[a]	0[b]– 64.0
κ-Casein[a]	15.0–29.0
α_{s1}-Casein[a]	0[b]–28.0
α_{s2}-casein[a]	10.0–25.0
Whey proteins	0.37–0.70
β-Lactoglobulin[c]	39.2–72.1
α-Lactalbumin[c]	17.8–33.3
Serum albumin/lactoferrin[c]	5.1–21.5
Immunoglobulins[c]	4.6–21.4

[a] Percentage of total casein.
[b] Absence of β- or α_{s1}-caseins in milk from animals carrying the respective null alleles.
[c] Percentage of total whey.
Individual caseins and whey proteins are expressed as percentage of the total casein and total whey, respectively.

There is also polymorphism at the α_{s2}-CN locus, for which seven alleles have been identified, and which are also associated with three different levels of casein synthesis.

Caprine β-CN is encountered at two different phosphorylation levels, β_1-CN and β_2-CN, with six and five phosphoseryl residues, respectively. β-CN is the main casein fraction in goats' milk, and for a long time was considered monomorphic. However, a null allele was detected in the Garganica, Creole de Guadeloupe, and Granadina breeds.

So far, seven variants of goat κ-CN have been identified. Alleles A and B are the most frequent variants, occurring in the majority of breeds, with the highest prevalence of the B variant, except for the Canarian breed, where the A allele is more frequent. Sequence data suggest that the F variant is the original type of goat κ-CN, other alleles being derived from it, following two different tracks by successive mutations. Variants F and G are found only in the Italian breeds Teramana, Girgentana, and Sarda, at a relatively high frequency.

Compared with cows' milk, goats' milk has a higher β-CN and a lower α_{s1}-CN content. However, there are a number of important differences in the individual caseins between the two milks, which affect their processing properties and are responsible for the characteristic textural and sensory attributes of goats' milk products.

Two genetic variants of goat β-Lg have been identified. Goat β-Lgs have a lower net charge than the bovine counterparts. Differences also occur in the charged amino acids of α-La, and two variants have been reported having Lys or Ala as N-terminal residues, although the B variant is rare. Recently, an additional silent allele at the goat α-La locus has been detected.

The percentage of β-Lg in goat acid whey is lower than in sheep or cow acid whey, and the goat whey also has a low percentage of α-La. The α-Las in sheep's and goats' milks are closely homologous to α-La in cows' milk.

Minor Proteins and Non-protein Nitrogen Compounds

Goats' milk, like cows' milk, contains 20–200 mg l^{-1} of lactoferrin and transferrin. Prolactin has also been isolated (~44 μg l^{-1}) as well as immunoglobulins (IgGs, IgA, IgM). A minor whey protein (glycoprotein) that binds folate has also been identified.

The non-protein nitrogen (NPN) contents of goat and human milks are higher than that of cows' milk. NPN is composed of several nitrogenous compounds, including nucleosides and nucleotides; goats' and sheep's milks principally contain UMP, AMP, and CMP but also some UDP.

Enzymes

The lysozyme content of goats' milk averages 25 μg l^{-1}, which is in the low-range characteristic of the milk of artiodactyls. Ribonuclease, lipase, and xanthine oxidase activities in goats' milk are lower than those in cows' milk. The levels of lactic hydrogenase and malic dehydrogenase in goats' milk are comparable to those of cows' milk. Raw goats' milk contains substantially lower levels of alkaline phosphatase than cows' milk. Inactivation of this enzyme serves as the basis for the evaluation of the effectiveness of pasteurization in cows' milk. The low level of alkaline phosphatase in goats' milk precludes its use as an effective index of pasteurization of goats' milk.

Minerals and Trace Elements

The mineral composition of goats' milk is shown in **Table 8**. There is great variability in the reported values as a result of genetic effects, diet, stage of lactation, and analytical procedures. Overall, goats' milk has a higher level of calcium, phosphorus, potassium, magnesium, and chlorine, and a lower level of sodium than cows' milk. However, the repartition of calcium, phosphorus, and magnesium between the soluble and colloidal phases of milk is similar for cows' and goats' milks. Goats' milk generally contains more magnesium than cows' milk and lower or comparable levels of iodine and copper. More than 50% of the magnesium is found in the soluble phase. Trace elements are part of essential substances such as hemoglobin and vitamin B_{12}. Many enzymes also require specific metallic activators. Colostrum has much higher levels of trace elements than mid-lactation milk. The concentration of some elements, such as zinc, molybdenum, and strontium, varies greatly with their concentration in the diet. Zinc and manganese are found largely in the micellar phase, whereas copper and iron are most abundant in the soluble phase of milk.

Table 8 Concentration of minerals and vitamins in goats' and cows' milk (amounts in 100 g)

	Goat	*Cow*
Minerals		
Ca (mg)	134	122
Mg (mg)	16	12
Na (mg)	41	58
K (mg)	181	152
P (mg)	121	119
Cl (mg)	150	100
S (mg)	28	32
Fe (mg)	0.07	0.08
Zn (mg)	0.56	0.53
Cu (mg)	0.05	0.06
Mn (mg)	0.032	0.002
I (mg)	0.022	0.020
Se (μg)	1.33	0.96
Vitamins		
Vitamin A (IU)	185	126
Vitamin D (IU)	2.30	2.0
Vitamin C (mg)	4.16	0.94
Thiamin (B_1) (μg)	68	45
Riboflavin (B_2) (mg)	0.21	0.16
Niacin (mg)	0.27	0.08
Pantothenic acid (mg)	0. 31	0.32
Biotin (μg)	1.50	2.0
Vitamin B_{12} (μg)	0.065	0.36
Folic acid (μg)	1.29	5.0
Vitamin E (μg)	120	

From Park YW, Juárez M, Ramos M, and Haenlein GW (2007) Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research* 68: 88–113.

Vitamins

Goats' milk contains a higher level of vitamin A than cows' milk because goats convert all carotenes into vitamin A, which gives the milk a whitish color. In contrast, goats' milk is poor in folic acid and vitamin E.

Variability in Milk Composition

Factors Affecting Milk Yield

Milk yield is closely linked to the breed, lactation number and lactation period, season of kidding, and litter size. Genetic selection has resulted in improved breeds, such as Alpine and Saanen, with yields several orders of magnitude higher than local unimproved breeds. Maximum yield is usually attained during the third to fourth lactations, and early kidding favors milk production.

Litter size is also positively correlated to milk yield. Nutritional and environmental factors, such as a diet supplemented with concentrate, husbandry conditions, and flock management can influence milk yield. Milk yield is inversely related to the fat content, TS, and crude protein content in milk.

Lactation

In most of the relevant studies, the lactation effects are confounded with nutritional and seasonal effects. During the first month after parturition, milk has a higher content of fat, TS, protein, and nonfat solids than milk at later stages of lactation. As a general consensus, a curvilinear response in total milk solids, fat, total casein, and total whey protein content during the lactation period has been reported (**Figures 1** and **2**). There is a gradual decrease in total milk solids, fat, and protein content, reaching a minimum around week 20–25 of lactation, followed by an increase toward the end of lactation. The proportion of the individual caseins does not seem to change during lactation but among the whey proteins, β-Lg seems to vary more than α-La.

The total fat content is highest on the first days after parturition, and then declines until day 110 of lactation. The fat composition changes greatly during lactation. After parturition, when body fat deposits start to be mobilized, a high content of $C_{18:0}$ and $C_{18:1}$ FAs and a relatively low content of $C_{16:0}$ FAs are found in milk. A high proportion of $C_{14:1}$ and $C_{16:1}$ FAs in the colostrum is probably of the same origin. As lactation advances, there is a shift in metabolism to utilize substrates derived entirely from the diet rather than from body stores. The proportion of milk fat derived from the *de novo* synthesis then increases and citrate decreases.

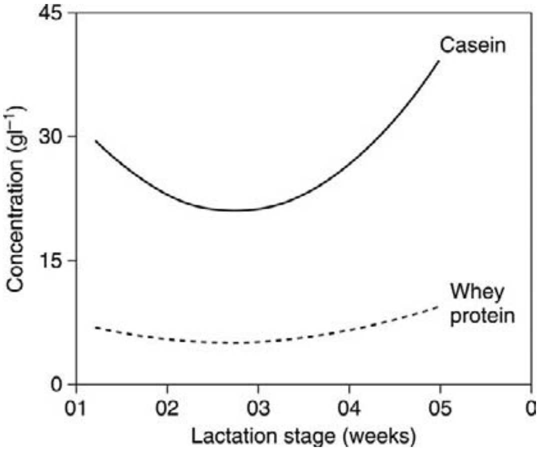

Figure 1 Changes in the concentration of the major goats' milk proteins during lactation.

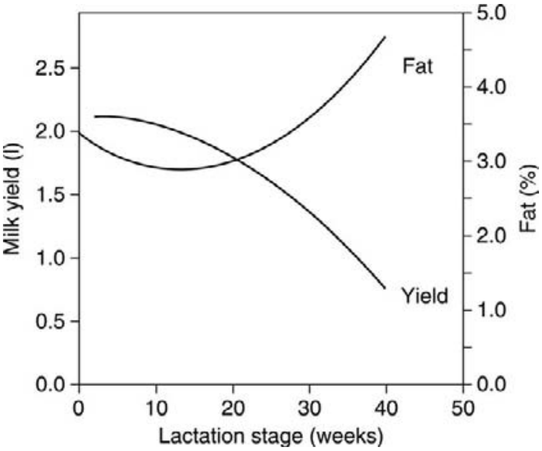

Figure 2 Changes in the milk yield and fat content of goats' milk during lactation.

The SCFAs $C_{4:0}$, $C_{6:0}$, and $C_{8:0}$ decrease during lactation as well as the long-chain FAs $C_{18:1}$ and $C_{18:3}$ while the levels of $C_{10:0}$ and $C_{14:0}$ FAs increase.

Diet

Dietary manipulation constitutes a natural and economical way to markedly and rapidly change the milk FA composition. There is a great challenge for farmers and for scientists working on ruminants to be able to modulate the milk FA composition, increasing those FAs of greatest interest for human nutrition such as *cis* monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs), including CLA. The largest changes can be obtained either by changing the forages in the diets of ruminants, particularly pasture, or by adding plant or marine lipid supplements to the diet. These dietary FAs are metabolized extensively and biohydrogenated in the rumen, resulting in the production of a wide range of isomers of PUFAs and MUFAs, especially *trans* FAs and CLAs. Some FA intermediates biohydrogenated in the rumen, for example, *trans* vacenic acid ($t11\text{-}C_{18:1}$, TVA), are transformed by body tissues, especially by the mammary gland where the δ-9 desaturase acts by adding a *cis*-9-double bond to different FAs, which partly reduces the saturation level and the melting point of milk fat.

The goat responds very differently to dietary factors compared to the cow, both in milk fat secretion and in some aspects of milk FA composition, which are of interest for consumers of goat dairy products. Unlike the situation in dairy cows, there is no decrease in milk fat content when diets supplemented with PUFAs are fed to dairy goats. It has been suggested that the inhibition of acetyl-CoA carboxylase and *de novo* lipogenesis elicited by long-chain FAs (especially with a high level of unsaturation) seems less strong in goat mammary gland metabolism. Therefore, potential inhibitors of lipogenesis present in the rations would be less active in goats than in dairy cows. With regard to milk protein, it has been observed that the addition of fat to the diet of goats results in no change in milk protein content and may, in fact, lead to an increase.

Although the CLA content in dairy products is affected by many factors, animal feeding strategies and specifically diets with oil seed or oil supplements rich in PUFAs have been effective in enriching the milk of dairy goats with CLA. Effects of feeding fresh forages and free oils and the seasonal changes of Mediterranean natural pastures on FAs, especially on CLA composition and its precursors, in goats' milk have been reported. Generally, free oils are more effective than whole or treated oilseeds.

A slight increase in the level of *n*-3 FAs in goats' milk fat occurred when goats were fed fish oil, but the levels of these supplements in the diet should be low and protected against ruminal biohydrogenation in order to avoid a decrease in milk yield and fat and protein contents. To date, data on CLA enhancement in goats' milk by adding fish oil supplements are, however, limited.

Breed

Goats' milk composition varies within breeds (**Table 9**). Undoubtedly, some of the variation in the reported values arises also from differences in sampling and analytical procedures. The casein number (casein N/total N) ranges from 0.69 to 0.82, with an average of 0.76, which is similar

Table 9 Variation in the gross composition of goats' milk from different breeds

		Total solids	*Fat*	*Protein*	*Casein*	*Lactose*	*Ash*
Country	*Breed*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
Britain	Anglo-Nubian		4.94	3.60		4.51	
	British Saanen	11.6	3.48	2.61	2.30	4.30	0.80
	British Alpine		3.77	2.74			
	Toggenburg		3.69	2.72			
Cyprus	Damascus	13.20	4.33	3.75	2.97		0.83
France	Alpine		3.38	3.08	2.33		
Finland	Finnish	12.60	3.90	3.52		4.48	0.84
Greece	Native (*Capra prisca*)	14.80	5.63	3.77	3.05	4.76	0.73
	Saanen	11.10	3.00	3.10			0.84
India	Barbari	14.93	5.69	4.05	2.64	4.31	0.88
	Beetal	13.62	4.74	3.74	2.57	4.32	0.82
	Black Bengal	18.07	7.93	4.97	3.48	4.28	0.89
	Jamunaparati	14.69	5.59	3.85	2.66	4.40	0.85
	Parbatsar	14.57	5.00	3.32		4.31	
Italy	Sardinian		5.10	3.90			0.71
Libya	Maltese cross	12.94	4.00	3.76		4.40	0.88
Nigeria	African Dwarf	18.00	6.9	3.90		6.30	0.82
	Red Sokoto	15.28	4.86	4.38		4.72	
South Arabia	Aardi	11.15	2.83	3.28		4.12	0.82
	Masri	11.13	3.06	3.40		4.12	0.77
Spain	Murciano-Granadina			4.09	3.21		
	Verata	13.40	4.54	3.45			
USA	Alpine	12.93	4.79	3.02	2.43		
	La Mancha	13.67	4.95	3.34	2.70		
	Nubian	16.02	7.02	3.59	2.77		
	Oberhasli	11.70	3.29	3.35	2.28		
	Saanen	12.98	4.64	3.03	2.48		
	Toggenburg	11.83	4.27	2.76	2.44		

to that of cows' milk. The greatest variation is observed in the TS level and fat content.

Unusually high levels of fat, TS, and protein content have been reported for the West African dwarf goat, Pygmy goat of Oregon, and Black Bengal and Barbari in India. Indigenous goats in Greece (*Capra prisca*) and Sardinian have been reported to produce milks richer in TS, fat, and protein content. Generally, Alpine and Saanen milks have a lower fat, protein, and casein content. This is not unexpected given the higher milk yields of these breeds. Statistical analysis of the composition of the milk fat of individual goats suggests the existence of genetic differences in FA composition.

Nubian and Anglo-Nubian goats produce milk with higher fat and protein levels than animals of the Alpine breed kept at the same experimental station.

In Libyan goats (Maltese cross), it has been reported that the concentrations of C_6–C_{18} FAs are similar to those found in the Masri breed of South Arabia (which they resemble closely).

Genotype

It is well established that the casein composition in goats' milk is influenced by genetic polymorphism on the casein loci. Polymorphism of α_{s1}-CN is the most interesting and extensively studied because it controls the level of α_{s1}-CN excretion in milk. Four levels of α_{s1}-CN expression ranging from 0 (null allele α_{s1}-CN^0) to 3.5 g l^{-1} per copy of each A, B, or C (strong) allele have been reported. Alleles F and G (weak) are associated with low contents of α_{s1}-CN (0.45 g l^{-1} per allele) and alleles E and I (medium) are associated with an intermediate content of α_{s1}-CN (1.1–1.7 g l^{-1} per allele).

The α_{s1}-CN genotype influences the total protein, total casein, and fat contents and is linked to the technological behavior of goats' milk. Milk from animals with the strong alleles has higher levels of fat, TS, and protein. However, α_{s1}-CN genatype does not interfere with the level of expression of the other caseins or whey proteins. Differences have been found in the micellar size distribution and their calcium content in milks from animals of different α_{s1}-CN genotypes. Thus, F-type milk has, on average, larger micelles than milks with α_{s1}-CN A, B, or E.

Technologically, milk from animals with the strong alleles is considered superior to milk from animals with weak alleles, with higher cheese yields and firmer curds. Organoleptically, cheeses made from F-type milks are characterized by a 'strong' goaty flavor.

There is also polymorphism at the α_{s2}-CN locus, which is associated with three different casein synthesis levels. A, B, C, E, and F alleles produce 'normal' α_{s2}-CN contents (2.5 g l^{-1}), the D allele causes reduced α_{s2}-CN contents, and the 0 allele has no detectable amounts of this casein in goats' milk.

Moreover, the total casein content in the milk of animals with the null allele for goat β-CN has been reported to be extremely low and its clotting ability poor.

Characteristic Properties of Goats' Milk

An important characteristic of goats' milk is the unique 'goaty' flavor. This is attributed to differences in the fat fraction of goats' milk. Other investigators attributed the characteristic 'goaty' flavor to the relatively higher proportion of SCFAs and MCFAs.

From the technological point of view, it is clear that the differences in chemical composition and particularly the impact of casein genotype on the structure of the goat casein micelles are sufficiently pronounced to explain the differences in the textural characteristics of cheeses and fermented milk products. Goats' milk has better digestibility and buffering capacity than cows' or human milk, as well as certain therapeutic values in medicine and human nutrition. Recently, goat β-Lg and α_{s2}-CN have been reported as sources of bioactive peptides with angiotensin converting enzyme (ACE) inhibitory properties.

Use of Goats' Milk in the Production of Dairy Products

In the Mediterranean and many eastern European countries, goats' milk has gained economic importance mainly via its utilization in cheese making. In the European Community, the originality of goats' and sheep's milk products is protected by the legislation on the Protected Designation of Origin (PDO) and the Protected Geographic Indications (PGI). International rules are opportune in order to protect the typical products, their specific characteristics, and their biodiversity, encouraging the permanent residence of the population in the rural areas, especially in the less favorite areas. Greece and France have by far the largest goat cheese production and, along with Italy and Spain, are also noted for their sheep milk cheeses. Excellent cheeses are made from sheep's or goats' milk or from mixtures of the two. Differences in the FA composition of the fat give a unique flavor to the cheeses. Ripened goats' milk cheeses are characterized by a piquant and peppery-sharp flavor, while sheep milk cheese is smooth, mellow, and aromatic. Goat cheeses are white because goats' milk lacks carotene. Best cheeses are obtained from early lactation milks. **Table 10** gives some compositional

Table 10 Composition of some traditional goats' milk cheeses

			Fat in dry matter	*Moisture*	
Country	*Cheese variety*	*Consistency*	*(%)*	*(%)*	*Raw material*
France	Brocciu	Semisoft	25		Sheep's and/or goats' milk whey
	Chabichou du Poitou	Soft	45	50 max.	Goats' milk
	Crotin de Chavignol	Semihard	45	53 max.	Goats' milk
	Le-Mothe St-Heraye	Soft	45	55 max.	Goats' milk
	Saint-Maure de Touraine	Soft	45	55 max.	Goats' milk
	Selles sur Cher	Soft	45	41 max.	Goats' milk
	Picodon de la Drôme	Soft	45		Goats' milk
	Pouligny Saint Pierre	Soft	45	40 max.	Goats' milk
	Rocamadour		45		Goats' milk
	Valencay	Soft	45	55 max.	Goats' milk
Greece	Anthotyros	Soft or dry	65, 65	70, 40	Sheep and/or goat whey
	Feta	Soft	45	53	Sheep's and 5–10% goats' milk
	Formaella	Hard	50	33	Sheep's and/or goats' milk
	Galotyri	Soft	47	71	Goats' and/or sheep's milk
	Graviera of Crete	Hard	38	40 min.	Goats' and sheep's milk
	Kaseri	Semihard	45	42 avg.	Goats' and sheep's milk
	Kopanisti	Soft	48	60% avg.	Cows'/sheep's/goats' milk or mixtures
	Ladotyri	Hard	47	34	Sheep's and/or goats' milk
	Manouri	Soft	52	76	Sheep and/or goat whey
	Metsovone	Hard	44	42	Sheep's and/or goats' milk
India	Paneer type	Soft	53		Goats' milk
Israel	Goats' cheese	Soft	45	66	Goats' milk
Italy	Apulian Cacioricotta	Soft	29		Goats' milk
	Cacioricotta	Soft	31		Sheep's and/or goats' milk
	Riccotta	Semisoft	25		Sheep's and/or goats' milk whey
Norway	Gammelost		5		Goats' milk
	Gjetost	Semisoft	38	13.4	Goats' milk whey
Spain	Cabrales	Soft	31		Blend cows'/sheep's/goats' milk
	Iberico	Hard/oily			Blend cows'/sheep's/goats' milk
	Ibores	Hard	59		Goats' milk
	Majorero	Hard	50		Goats' milk
	Trochon	Semisoft			Sheep's and/or goats' milk
	Valdeteja	Hard	73		Goats' milk
Mexico	Anejo Enchilado	Hard			Goats' milk

information on the most popular goats' milk cheeses in the world.

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See also: **Animals that Produce Dairy Foods**: Goat Breeds. **Cheese**: Overview; **Dairy Farm Management Systems**: Goats. **Husbandry of Dairy Animals**: Goat: Feeding Management; Predator Control in Goats and Sheep. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose; Milk fat. **Milk Lipids**: General Characteristics. **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance.

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Sheep Milk

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Introduction

On a global scale, the annual production of sheep's milk, approximately eight million tonnes, is of marginal importance when compared to cow's milk in quantitative terms (2% of the total); nevertheless, it is of major importance in the Middle Eastern and Mediterranean countries, such as France, Italy, Spain, and Greece. The numbers of sheep do not fully reflect the amount of milk produced, as sheep are often used for other purposes such as meat and wool. Although sheep milk is richer in nutrients than cow's milk, it is rarely used for consumption as liquid milk. In general, sheep's milk is essentially used for cheese making, but in some countries a part of it is used for making yogurt or whey cheeses.

Chemical Composition and Physical Properties

Milk is a mixture in equilibrium of proteins, fat, lactose, and minerals in various states of dispersion in water, such as emulsions, colloidal suspensions, and true solutions. Sheep's milk is more viscous than cow's milk and is more resistant to the proliferation of bacteria in the first hours after milking, thanks to its greater immunological activity. It has a smell that is characteristic of the animal that produces it, and is an opaque white in color.

Table 1 shows the typical composition of sheep's milk, which generally contains higher amounts of total solids and major nutrients than goat and cow milks. Sheep's milk is an excellent source of high-quality protein, calcium, phosphorus, and lipids. There is a good balance between the protein, fat, and carbohydrate components, each being present in approximately similar amounts. The supply of nutrients is high in relation to the calorie content of the food. Fat and protein are the principal components of the dry matter, constituting 69% in sheep's milk as compared to 56% in cow's milk. The levels of fat and protein are higher than in cow's milk, and therefore the yield of cheese is also higher (approximately 15% for sheep's milk as compared with 10% for cow's milk).

As has been reported for cow's milk, the composition of sheep milk varies with diet, breed, animals within breed, parity, season, feeding, management and environmental conditions, locality, and stage of lactation. The principal factor is breed; breeds selected for their high milk yield produce milk with a lower level of total solids. Fat shows greater variation within a breed than do proteins. Another factor to consider is the stage of lactation. Sheep colostrum in the early post-partum period also contains approximately 13.0% fat, 11.8% protein, 3.3% lactose, 0.9% minerals, and 28.9% total solids, and thus has a much higher basic nutrient concentration than cow colostrum. The fat and protein contents of the milk itself decline during the first few weeks, then gradually increase after the first month until lactation ends and the yield decreases. At the end of seven months of lactation, the fat content may be as high as 8.1–10% and the protein content as high as 6.8–8.9%. The lactose content falls slightly throughout lactation, and more sharply at the end (4.1 to 3.4%).

Sheep milk has higher specific gravity, viscosity, and titratable acidity, but lower refractive index and freezing point than typical cow milk (**Table 2**). Sheep's milk is slightly more acidic than cow's milk, possibly due to its higher protein content.

Proteins in Sheep Milk

Sheep's milk contains 0.7–1% nitrogen. This is distributed in fractions, the importance of which varies in terms of dairy technology and nutrition. Proteins account for approximately 95% of the total nitrogen, and 5% is non-protein nitrogen. The principal proteins in sheep are the same as those in cow milk. Milk proteins occur in two distinct phases. One is a micellar phase composed of caseins, the micelles averaging about 190 nm in diameter. The submicelles are interlinked by calcium phosphate and small amounts of magnesium, sodium, and potassium citrate, which diffuse light and give skimmed milk its opaque white appearance. The other type of protein is the soluble whey proteins. The caseins precipitate at pH 4.6 at room temperature, whereas under the same conditions the whey proteins (β-lactoglobulin, α-lactalbumin, and serum albumin) remain soluble.

Caseins in Sheep Milk

Caseins (CNs) are the majority proteins in sheep milk (76–83% of total proteins) and are also present in most types of its cheeses. The denomination 'casein' embraces

Table 1 Composition of ewe's milk

Component	*Average content (%)*	*Range (%)*	*Average of dry matter (%)*
Water	81.60	79.27–83.80	
Lactose	4.61	4.10–4.95	25.0
Fat	7.09	5.10–8.70	38.5
Crude Protein (Total nitrogen × 6.38)	5.72	4.75–6.60	31.1
Casein	4.44		
Whey proteins	0.98		
Non-protein nitrogen	0.047		
Ash	0.91	0.70–1.10	4.9
Total solids	18.40	16.20–20.73	
Nonfat solids	11.31		

Average values calculated on the results given by different authors.

Table 2 Physical properties of sheep and cow milks

Properties	*Sheep milk*	*Cow milk*
Specific gravity	1.034 7–1.038 4	1.023 1–1.039 8
Viscosity, C_p	2.86–3.93	2.0
Surface tension (dynes/cm)	44.94–48.70	42.3–52.1
Conductivity(ohm^{-1} cm^{-1})	0.003 8	0.004 0–0.005 5
Refractive index	1.349 2–1.349 7	1.451 ± 0.35
Freezing point (−°C)	0.570	0.530–0.570
Acidity (lactic acid%)	0.22–0.25	0.15–0.18
pH	6.51–6.85	6.65–6.71

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four types of polypeptide chains: α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN. The heterogeneity of caseins is determined either by the presence of genetic variants or by other factors such as a discrete level of phosphorylation, variation in the extent of glycosylation of the κ-CN fraction, and the coexistence of protein forms having different chain lengths.

It has been demonstrated that four ovine casein genes, α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN, are polymorphic and are linked on both ovine and bovine genomes. In recent years, five variants of α_{s1}-CN have been described in bovine caseins, designated A, B, C, D, and E, and the corresponding nomenclature is proposed for goat milk caseins. α_{s1}-CN D was described initially and was called the Welsh variant because of its discovery in the Welsh mountains by King in 1966. This is the least phosphorylated variant. The lower number of phosphate groups explains the longer migration time of this variant by capillary electrophoresis at acid pH and the slow migration in alkaline polyacrylamide gels. The variant α_{s1}-CN C differs from the variant α_{s1}-CN A by the substitution of Ser for Pro at position 13, which explains the loss of the phosphate group on site 12 of the protein chain. Two variants of α_{s2}-CN, A and B, have been described, and they differ in that Asn_{49} and Lys_{200} are replaced by Asp_{49} and Asn_{200}. In addition, a variant with high electrophoretic mobility and low molecular weight has been found in the Manchega breed.

Ovine β-CN consists of 209 amino acids. For β-CN, there is a nongenetic polymorphism due to varying degree of phosphorylation, with six and five phosphate groups found in β_1-CN and β_2-CN, respectively. In addition, three genetic variants, designated A, B and C, have been described. The only sequence difference found between A and C is the substitution of the Glu at position 2 in variant A for Gln in variant C – no sequence data for the variant B are available yet. Both the stability of the casein micelle, and the availability and distribution of Ca are affected by the extent of phosphorylation of the caseins.

Ovine κ-casein contains 171 amino acid residues. No genetic variants have been found for ovine κ-CN, but it shows non-genetic polymorphism due to varying degrees of glycosylation at three different Thr residues (positions 135, 137, and 138) and two phosphorylation sites (Ser P^{151} and Ser P^{168}). The casein fraction also contains γ-caseins,

Table 3 Positions and amino acid differences in genetic variations of ovine milk proteins

		Position and amino acid in the protein						
Protein	*Variant*	*12*	*13*	*64*	*66*	*68*		
α_{s1}-CN	A	Ser P	Ser	Ser P	Ser P	Ser P		
	B							
	C	Ser	Pro					
	D			Ser	Ser	Asn		
	E[a]							
		49						200
α_{s2}-CN	A	Asn						Lys
	B	Asp						Asn
	Fast[a]							
		2						
β-CN	A	Glu						
	B[a]							
	C	Gln						
		20					148	
β-LG	A	Tyr					Arg	
	B	His						
	C						Gln	

[a] No sequence data are available.

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the product of the breakdown of β-caseins by plasmin. **Table 3** shows the positions and the differences in genetic variations of sheep's milk proteins.

Whey Proteins

Sheep milk whey proteins account for 17–22% of the total proteins. The major whey proteins are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Immunoglobulins, serum albumin, and proteose-peptones are present at lower concentrations. The latter are products of the breakdown of β-casein by plasmin. Another soluble protein found in small amounts and possessing antibacterial properties is lactoferrin. Serum albumin and immunoglobulins are not specific to milk and are considered to be the same as those found in blood. In the case of rennet whey, caseinomacropeptide is also present, produced by chymosin action on bond 105–106 of κ-CN.

β-Lactoglobulin, the major protein in whey, consists of a polypeptide chain of 162 amino acids. Three genetic variants have been described in sheep's milk: β-Lg A, β-Lg B, and β-Lg C. Ovine β-Lg B and A variants differ only by a single amino acid exchange, His for Tyr at position 20. β-Lg C is a subtype of ovine β-Lg A with a single exchange, Arg for Gln at position 14. The three variants can be resolved by isoelectric focusing and by capillary electrophoresis. Of the three variants described for ovine β-Lg, alleles A and B were present in almost all breeds analyzed, whereas allele C is rather rare and confined to specific breeds. α-Lactalbumin is closely homologous to bovine α-lactalbumin. It is a metalloprotein containing one atom of Ca per molecule and is important from a biological standpoint in that it is involved in lactose synthesis. Two genetic variants, A and B, have been described, although the B variant is very uncommon.

Non-protein Nitrogen

Non-protein nitrogen accounts for 5–6.8% of the total nitrogen in sheep milk. Non-protein nitrogen compounds are urea (45%), free amino acids (16%), creatine (2.4%), creatinin (1.7%), ammonium nitrogen (1%), uric acid (2.1%), and other indeterminate compounds. Sheep's milk contains more urea and uric acid than found in cow's milk. Among the free amino acids taurine and carnitine are important because of their essential physiological functions in the newborn. Sheep's milk contains significantly more taurine (14 μmol/100 ml) than that contained in cow's milk (1 μmol/100 ml) but less than that contained in human milk (30 μmol/100 ml).

Lipid Fraction

Lipids are one of the most important components of milk in terms of cost, nutrition, and the physical and sensory characteristics they impart to dairy products. They are present in the form of globules and are characteristically abundant in sizes of less than 3.5 μm. No appreciable differences have been found in the mechanism of fat globule secretion in sheep and cows; the structure and composition of the membrane are similar in both species and represent approximately 1% of the total milk fat volume. The phospholipid profile in both species is similar to that of the plasma membrane, which tends to confirm their common origin.

Along with triacylglycerols, other simple lipids (diacylglycerols, monoacylglycerols), complex lipids (phospholipids), and lipo-soluble compounds (sterols, cholesterol esters, hydrocarbons) are contained in the lipid fraction of sheep's milk. Triacylglycerols constitute the largest group (nearly 98%), including a large number of esterified fatty acids.

Fatty Acids

Most fatty acids, from butanoic to eicosanoic acid, contain an even number of carbon atoms. The five most important fatty acids in quantitative terms ($C_{16:0}$, $C_{18:1}$, $C_{10:0}$, $C_{14:0}$, and $C_{18:0}$) account for >75% of the total fatty acids in sheep's milk. Sheep's milk contains more caproic ($C_{6:0}$; 2.9%), caprylic ($C_{8:0}$; 2.6%), and capric ($C_{10:0}$; 7.8%) acids than does cow milk. These fatty acids are associated with the characteristic flavor of cheeses and can also be used to detect mixtures of milk from different species (**Table 4**).

The most important factor among the intrinsic and extrinsic variables that modulate milk fatty acid composition is the feed, in particular, adding lipid supplement to the diet.

To increase the polyunsaturated fatty acid content in milk fat, a high forage-to-concentrate ratio or the inclusion of a polyunsaturated oil-seed rich in polyunsaturated fatty acids or a rich, protected fat are recommended for

Table 4 Mean values and the minimum (Min) and maximum (Max) contents of the main fatty acids in sheep's and cow's milk fat (% in total fatty acid methyl esters)

	Sheep		*Cow*	
Fatty acid	*Mean*	*Min/Max*	*Mean*	*Min/Max*
Butyric ($C_{4:0}$)	3.5	3.1–3.9	3.9	3.1–4.4
Caproic ($C_{6:0}$)	2.9	2.7–3.4	2.5	1.8–2.7
Caprylic ($C_{8:0}$)	2.6	2.1–3.3	1.5	1.0–1.7
Capric ($C_{10:0}$)	7.8	5.5–9.7	3.2	2.2–3.8
Decenoic ($C_{10:1}$)	0.3	0.2–0.3	0.2	
Lauric ($C_{12:0}$)	4.4	3.5–4.9	3.6	2.6–4.2
Tridecanoic ($C_{13:0}$)	0.2	0.1–0.2	0.2	
Myristic ($C_{14:0}$)	10.4	9.9–10.7	11.1	9.1–11.9
iso Pentadecanoic ($C_{15:0}$)	0.3	0.3–0.4	0.4	
anteiso Pentadecanoic ($C_{15:0}$)	0.5	0.3–0.6	0.4	
Myristoleic ($C_{14:1}$)	0.3	0.2–0.5	0.8	0.5–1.1
Pentadecanoic ($C_{15:0}$)	1.0	0.9–1.1	1.2	0.9–1.4
iso Palmitic ($C_{16:0}$)	0.2	0.2–0.3	0.4	
Palmitic (C_{16})	25.9	22.5–28.2	27.9	23.6–31.4
iso Heptadecanoic ($C_{17:0}$)	0.5	0.4–0.6	0.5	
anteiso Heptadecanoic ($C_{17:0}$)	0.3	0.3–0.4	0.5	
Palmitololeic ($C_{16:1}$)	1.0	0.7–1.3	1.5	1.4–2.0
Heptadecanoic ($C_{17:0}$)	0.6	0.6–0.7	0.6	
Heptadecenoic ($_{17:1}$)	0.2	0.2–0.3	0.4	
Stearic ($C_{18:0}$)	9.6	8.5–11.0	12.2	10.4–14.6
Oleic[a] ($C_{18:1}$)	18.2	15.3–19.8	17.2	14.9–22.0
trans Octadecenoic ($C_{18:1}$)	2.9	2.5–3.2	3.9	
Linoleic[a] ($C_{18:2}$)	2.3	1.9–2.5	1.4	1.2–1.7
Eicosanoic ($C_{20:0}$)	0.5	0.4–0.5	0.4	
Linolenic[a] ($C_{18:3}$)	0.8	0.5–1.0	1.0	0.9–1.2
Linoleic conjugated[a] ($C_{18:2}$)	0.7	0.6–1.0	1.1	0.8–1.5

[a] All isomers.

Reproduced with permission from Goudjil H, Fontecha J, Luna P, De la Fuente MA, Alonso L, and Juárez M (2004) Quantitative characterization of unsaturated and trans fatty acids in ewe's milk fat. *Le Lait* 84: 473–482 and McGibbon AKH and Taylor MW (2006) Composition and structure of bovine milk lipids. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol 2: Lipids*, 3rd edn., pp. 1–42. New York: Springer.

sheep feed. Changes in the fatty acid profile of ovine milk fat should not substantially differ from the pattern previously described for cow milk.

Other minor components of milk fat are branched-chain and odd-numbered chain saturated fatty acids. The main point of quantifying these components is that volatile branched-chain fatty acids give characteristic flavors to many dairy foods. The amount of these compounds contained in a cheese is dependent largely on the composition of the milk fat substrate. The concentration of branched-chain fatty acids reported in sheep's milk fat (2% of the total fatty acids) was made up of six different acids: *iso*-C14, *iso*- and *anteiso*-C15, *iso*- and *anteiso*-C17, and *iso*-C16. These branched-chain fatty acids are also predominant in bovine milk.

Monounsaturated *trans* fatty acids in milk fat range from 2.5 to 5% of the total fatty acids, depending on the species and the season. In general, sheep's milk contains the highest quantities, followed by cow's and, finally, goat's milk fat. The pattern of *trans* C18:1 isomer distribution is quantitatively identical in the three species, and the individual *trans* fatty acid isomers could have differing physiological effects. The importance of *trans*-11 C18:1, vaccenic acid, the predominant *trans* fatty acid in milk, lies in its role as a precursor of the main isomer of conjugated linoleic acid (CLA), rumenic acid (*cis*−9 *trans*−11 C18:2), physiologically the most relevant bioactive compound present in milk fat. This synthesis occurs not only in the bovine mammary gland but also in human tissues.

Milk CLA concentration in different ruminant species seems to decrease in the order sheep > cow > goat milk fat, accounting for 1.08, 1.01, and 0.65% of the total fatty acids, respectively, and varies with the season, mainly due to variations in feeding factors. The greatest seasonal differences in CLA content were measured in sheep's milk, 1.28% in summer and 0.54% at the end of winter. The relative composition of CLA isomers in sheep's milk is similar to that reported in cow's milk. Data from *in vitro* studies and animal models suggest that the rumenic acid isomer is responsible for the anticarcinogenic and anti-atherogenic properties of CLA, as well as for a multiplicity of potentially beneficial effects on human health.

Triacylglycerols

The triacylglycerol structure of milk fat is responsible for the rheological properties of the fat and its behavior during melting and crystallization. Their composition is also of interest given that it can be used to verify the authenticity of milk fat.

Triacylglycerols are almost invariably accompanied by small amounts of di- and mono-acylglycerols, mainly at positions 1 and 2, which are therefore probably intermediates in the biosynthesis of triacylglycerols. The distribution of fatty acids in the triacylglycerol molecule, as determined by stereospecific analysis, differs slightly from that in cow's milk, but the distribution of acids in the molecule is not random. On the other hand, in both sheep's and cow's milk, butyric acid ($C_{4:0}$) and other short-chain fatty acids ($C_{6:0}$–$C_{8:0}$) are esterified mainly at position 3 of the glycerol molecule and only one short-chain acid ($C_{4:0}$ or $C_{6:0}$) occurs per triacylglycerol molecule; the distribution of the other fatty acids (C_{10} or greater) exhibits no such marked specificity for positions 1 or 2.

Table 5 shows the average triacylglycerol composition of sheep's milk as compared with cow's milk. The triacylglycerols in sheep's milk show a wide range of molecular weights when distributed according to the number of carbon atoms (taking into account the carbon atoms of the three acyl radicals), with two peaks at C38–C40 and C50–C52, and a minimum at C44–C46. The chromatographic triacylglycerol profile of sheep's milk shows similarities to that reported for cow's milk. However, as a result of the differences in fatty acid composition between the two species, sheep milk fat has a higher percentage of short-chain triacylglycerols (C26–C36) than cow's milk (24 vs. 21%). Percentage of medium-chain triacylglycerols (C38–C44) is also higher in sheep's milk than in cow's milk (42 vs. 36%). However, percentages of long-chain triacylglycerols (C46–C54) and unsaturated triacylglycerols are lower in sheep's than in cow's milk (34 vs. 43% and 51 vs. 55%, respectively). These differences relate to the

Table 5 Triacylglycerol composition of ewe and cow's milk fat (wt%)

Triacylglycerol	*Ewe*[a]	*Cow*[b]
C_{26}	0.72[c]	0.22
C_{28}	1.6	0.6
C_{30}	2.5	1.1
C_{32}	3.6	2.6
C_{34}	6.0	6.0
C_{36}	9.6	10.8
C_{38}	12.8	12.5
C_{40}	12.0	9.9
C_{42}	9.0	6.9
C_{44}	8.1	6.5
C_{46}	6.8	7.3
C_{48}	6.7	9.1
C_{50}	7.6	11.3
C_{52}	8.4	10.0
C_{54}	4.5	5.0

[a] Reproduced with permission from Goudjil, Fontecha, Fraga, and Juárez (2003) *Journal American Oil Chemist Society* 80: 219–222.
[b] Reproduced with permission from Precht (1992) *Zeitschrift für Lebensmittel Untersuchung und-Forschung* 194: 1–8.
[c] Cholesterol included.

need for a triacylglycerol composition with an appropriate melting point to allow the fat to be secreted.

It has been established that the principal triacylglycerols in sheep's milk fat are composed largely of three fatty acids C_{14}, $C_{16,}$ and $C_{18:1}$ combined with the short-chain fatty acids $C_{4:0}$ and $C_{6:0}$. The largest triacylglycerols are composed primarily of three fatty acids $C_{4:0}$, $C_{16:0}$, and $C_{18:1}$, the same as those in cow's milk.

Unsaponifiable Lipids

The unsaponifiable fraction of milk fat is composed largely of sterols, with a smaller proportion of hydrocarbons, basically squalene, and trace amounts of a number of normal- and branched-chain hydrocarbons in the range C_{17}–C_{48} with odd and even numbers of carbon atoms, lipo-soluble vitamins, and aliphatic alcohols. Ovine milk contains virtually no tocopherol or β-carotene.

Sterols are a minor fraction of the total milk fat, the main component being cholesterol (270–350 mg per 100 g of fat, equivalent to approx. 20 mg per 100 ml of sheep's milk), but small amounts of other sterols, implicated in cholesterol biosynthesis, have also been found in ovine milk: lanosterol (5–15 mg per 100 g of fat) and, in still smaller proportions, dihydrolanosterol, desmosterol, and lathosterol. The variation is wide but comparable to that found in cow's milk fat. The minor sterols represent 3–5% of the total sterol fraction.

Phospholipids

The major phospholipids in both sheep's and cow's milk are phosphatidyl-ethanolamine, phosphatidyl-choline, phosphatidyl-serine, phosphatidyl-inositol, and sphingomyeline. These account for roughly 0.8% of the total lipid fraction, and the proportions of corresponding phospholipid classes in sheep milk are remarkably similar to those in the milks of other ruminants.

Carbohydrates

Lactose, the major carbohydrate in ovine milk, is composed of glucose and galactose bonded by a β 1–4 glycosidic linkage. The lactose content in sheep milk is similar to that in bovine milk. Sheep's milk contains 4.5–5.0 g lactose per kilogram of milk, and lactose accounts for 22–27% of dry matter versus 33–40% in cow's milk. The lower lactose content does not present a problem in cheesemaking, as enough lactose is available to ensure lactic fermentation.

Lactose is a valuable nutrient because it favors intestinal absorption of calcium, magnesium, and phosphorus, and the utilization of vitamin C. Carbohydrates other than lactose, such as glycopeptides, glycoproteins, and oligosaccharides are also found in ovine milk. The amount of oligosaccharides in ovine milk is in the range 20–30 mg l^{-1}, and their content, as in other mammalian milks, is considerably higher in colostrums. Three neutral milk oligosaccharides, isomers of galactosyllactose, Gal (α1-3) Gal (β1-4) Glc, Gal (β1-3) Gal (β1-4) Glc, and Gal (β1-6) Gal (β1-4) Glc, and three acidic milk oligosaccharides from the ovine colostrums have been isolated and identified by ^{1}H-NMR. These acidic milk oligosaccharides contain sialic acid. Oligosaccharides promote the growth of bifidobacteria in the newborn and play a role as intestinal mucosal cell protectors against pathogens.

Mineral Elements and Vitamins

Sheep's milk contains mineral salts (around 0.9% of ash). The most abundant elements are Ca, P, K, Na, and Mg. Ca and P are the most important of the major constituents, both in nutritional terms and for their role in the structure of casein micelles and hence in the behavior of the caseins during milk processing. The most abundant of the trace elements are Zn, Fe, Cu, and Mn. **Table 6** shows the average concentrations of major elements and

Table 6 Vitamin and mineral contents of sheep's and cow's milk (amount in 100 g)

Vitamins	*Sheep*	*Cow*
Vitamin A (IU)	146	126
Vitamin D (IU)	0.18 μg	2.0
Thiamin (B1) (μg)	68	45
Riboflavin (B2) (mg)	0.38	0.16
Niacin (mg)	0.42	0.08
Pantothenic acid (mg)	0.41	0.32
Biotin (μg)	0.92	2.0
Vitamin B_{12} (μg)	0.71	0.36
Vitamin C (mg)	4.16	0.94
Folic acid (μg)	5.0	5.0
Vitamin E (μg)	120	
Minerals		
Ca (mg)	193	122
Mg (mg)	18	12
Na (mg)	44	58
K (mg)	136	152
P (mg)	121	119
Cl (mg)	160	100
S (mg)	29	32
Fe (mg)	0.08	0.08
Zn (mg)	0.57	0.53
Cu (mg)	0.04	0.06
Mn (mg)	0.007	0.002
I (mg)	0.020	0.020
Se (μg)	1.00	0.96
Al (mg)	0.05–0.18	

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trace elements in sheep's milk. With the exception of Na, the concentrations of these elements are higher than those found in cow's milk. Sheep's milk contains an average of 2 g Kg^{-1} of citrate, which is slightly higher than that in cow's milk. The mineral content of sheep's milk is much higher than that of human milk. The concentrations of minerals are not constant but are influenced by a number of factors such as stage of lactation, nutritional status of the animal, and environmental and genetic factors due to feeding differences and seasonal variations.

The percentages of Ca and P in the soluble phase are, respectively, 20–25% and 35–40% of the total. These proportions are lower than those in cow's milk, but the absolute concentrations in the soluble phase are comparable for the two species. Therefore, the concentrations of both elements in the colloidal phase of sheep milk are much higher than those in cow's milk, given the higher levels of casein found in sheep's milk. Na and K are the major elements in the soluble phase. Of the trace elements mentioned, Zn, Mn, Fe, and Cu, the first two are associated mainly (around 90%) with the colloidal fraction of the milk, whereas Fe and Cu are associated to a lesser extent (70 and 64%, respectively). In addition, of all the elements considered here, Fe is probably the one that is bound in the highest proportion to the lipid fraction. About the rest of the trace elements, it should be noted that availability of Se in sheep milk appears to be significantly lower than that in bovine milk.

Most of the known vitamins are contained in ovine milk, and for some of them this foodstuff is a rich source. From the literature it is concluded that sheep's milk is richer than cow's milk in most of the vitamins. However, documented research data on vitamins in sheep's milk are too sparse to offer a definitive picture.

The average values and the ranges of variation are shown in **Table 6**.

Enzymes

Enzymes are constituents of the mammary gland that pass into the milk during the secretion process. The enzymes found in milk are highly specific and are chiefly oxidoreductases, transferases, and hydrolases. Particularly important for technological purposes are proteases and lipases. Lipase, lysozyme, ribonuclease, and xanthinoe-oxidase are less active in sheep's milk, whereas alkaline phosphatase, despite possessing the same molecular weight and the same properties, is more active than in cow's milk. The most abundant enzyme is lactoperoxidase, which has proved impossible to distinguish from the bovine form but appears to be more thermolabile.

The native proteinases include aminopeptidases, thermolabile neutral acid proteinases, and heat-resistant serine proteinases. Serine proteinase, also known as alkaline or plasmin proteinase, is heat-stable and hydrolyzes Lys–Xy and Arg–X bonds. It becomes more active after thermal treatment.

Renneting Properties

The renneting properties of sheep's milk are affected by a number of factors such as pH, physicochemical composition, micellar system, salts equilibria, calcium concentration, temperature and time of heating. Sheep's milk coagulates well and is indeed very suitable for making high-quality cheeses. Coagulation is faster than in cow's milk, and less rennet is needed to coagulate in the same time as for cow's milk. Curd formation is also faster than in cow's milk, but syneresis takes longer. The reason for this difference may be that sheep's milk contains more casein, ionic calcium, and colloidal calcium than those contained in cow's milk. Renneting time and rate of firming are practically unaffected by the addition of calcium.

The coagulation properties of sheep's milk are less affected than those of cow's milk by storage at 4 °C or by heat treatment. In fact, although severe treatments (90 °C, 10 min) delay coagulation of sheep's milk, gelation does take place. In cow's milk, on the other hand, heating for 1 min at 90 °C prevents gelation. The reason for this difference in the behavior of cow's and sheep's milks could be that in the latter, when heated to 85 or 90 °C, the micelles grow considerably in size, attaining 1.75 times the size of the micelles in fresh milk.

Bioactive Components

Enzymatic hydrolysis of milk proteins can release fragments able to exert specific biological activities, such as antihypertensive, antimicrobial, opioid, antioxidant, immunomodulatory, or mineral-binding. Such protein fragments, known as bioactive peptides, are formed from the precursor inactive proteins during gastrointestinal digestion and/or during food processing. Owing to their physiological and physicochemical versatility, milk peptides are regarded as highly prominent components for health-promoting foods or pharmaceutical applications. Research in the field of bioactive peptides has focused mainly on milk proteins of bovine origin. However, during recent years, research has been extended to milk proteins from other mammals, including ovine and caprine species.

A proline-rich peptide, called colostrinin, which was originally found as a fraction accompanying ovine immunoglobulins, was found to promote T cell maturation. This peptide promotes procognitive functions in experimental animal models, indicating prevention of pathological processes in the central nervous system. In humans, the therapeutic benefit of colostrinin in Alzheimer's disease patients, by delaying the progress of the disease, has been demonstrated.

Cheeses

Practically all sheep's milk is used to make cheeses, most of which are craft products. Some have an appellation of origin, so that production is confined to specific regions , and only a minority are made on an industrial basis. There are six categories of sheep's milk cheese based on the technology used: fresh, white brined (pickled), blue-veined, semi-hard, hard, and whey cheeses. **Table 7** shows some of the principal sheep's milk cheeses produced in different countries, indicating the type of consistency and the global characteristics of composition.

The world's principal sheep milk cheese in terms of output and general availability is Roquefort. This is a blue-veined cheese made from raw milk inoculated with *Penicillium roqueforti* spores. It undergoes high levels of proteolysis, 50% water-soluble nitrogen (WSN) in total nitrogen, and lipolysis, 8–10% of total fatty acids, after 5 months of ripening. Spain produces a blue-veined variety with similar characteristics: Cabrales cheese. This has a very good reputation, although the raw material is a mixture of cow's, sheep's, and goat's milk.

Feta cheese is a white-brined cheese originally from Greece, and is one of the most popular internationally. Traditionally, it is made from raw milk and is ripened in barrels with brine (6–8% NaCl) for around 1 month at 8–10 °C; it is then kept chilled for at least 2 months prior to consumption. It is now made mainly from pasteurized milk. There is a variety similar to Feta called Teleme cheese, originally from Romania, which is generally made from a mixture of milks of all three species. The white-brined category also includes Halloumi, a cheese produced chiefly in Cyprus from raw sheep's milk.

Pecorino is an Italian semi-hard or hard cheese. There are several varieties with appellations of origin, including Roma, Sicilia, and Fiore Sardo. The Roma variety accounts for 50% of all production. This cheese is made with raw or pasteurized milk, in most cases coagulated with lamb or kid rennet paste. Proteolysis is moderate (≈20% WSN), but there is intense lipolysis due to the use of pregastric esterases in the traditional curd pastes used. Kefalotiri is a variety similar to Pecorino produced in Greece, although curd pastes are not normally used for coagulation.

Kachkaval has been produced in various European countries, particularly in the Balkans, at least since the eleventh century. This is a stretched-curd cheese which undergoes moderate proteolysis even after 3 months of ripening (≈13% WSN).

The most characteristic Spanish semi-hard/hard sheep's milk cheeses are Manchego, Zamorano, Roncal, and Idiazabal, traditionally made from raw milk. These are generally ripened for 3–6 months, although Manchego is matured in olive oil for a longer period. Proteolysis is medium/high, 20–45% WSN, whereas lipolysis is low. Idiazabal cheese is ripened for 1–2 months and then smoked.

Some sheep's milk cheeses are made in Portugal and Spain using vegetable rennet extracted from the flower of the chard *Cynara cardunculus*. Examples include Serra da Estrela in Portugal, and La Serena, Los Pedroches, and Torta del Casar in Spain. These are marketed after relatively short ripening (30–60 days), but proteolysis levels are high (40–50% WSN) due to the strong proteolytic activity in the curds.

Spain produces two highly popular fresh cheeses, Burgos and Villalón. These are now made from pasteurized milk using animal rennet, but without a starter culture. They must be kept in chilled storage and consumed within less than 6 days.

Finally, in various countries, the whey from sheep's milk cheesemaking is used on its own or mixed with milk to manufacture products of high nutritional value. The whey contains mainly water-soluble proteins (≈1%), fat

Table 7 Average content of fat, protein, calcium, and phosphorus in cheeses from ewe's milk in different countries

Country	*Name*	*Type of cheese*	*Fat content (% total solids)*	*Protein (%)*	*Ca (%)*	*P (%)*
Bulgaria	Kaschkaval	Stretched curd	45	20		
Cyprus	Halloumi	Brined	48	24		
France	Roquefort	Blue-veined	50	21	0.62	0.42
Greece	Feta	Brined	40	18	0.65	0.40
Italy	Pecorino	Hard	42	29	0.40	0.32
Portugal	Serra da Estrela	Semihard	56	196	0.65	0.53
Romania	Teleme	Brined	50	18	0.53	0.40
Spain	Manchego	Semi-hard	50–55	23	0.68	0.54

Average values calculated on the results given by different authors.

(<1%), lactose, minerals, non-nitrogen substances, and vitamins. These whey cheeses are the result of heat-induced coagulation of the whey protein (which occludes fat). The best-known whey cheeses are Ricotta from Italy, Manouri and Myzithra from Greece, and Requesón from Spain.

Besides the cheeses mentioned here, many countries produce cheeses, generally semihard varieties, in which the raw material is a mixture of sheep's, cow's, and goat's milk.

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See also: **Animals that Produce Dairy Foods**: Sheep Breeds. **Cheese**: Cheeses Matured in Brine; Hard Italian Cheeses; Smear-Ripened Cheeses. **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Production, Applications. **Milk Lipids**: General Characteristics. **Milk Proteins**: Heterogeneity, Fractionation, and Isolation; Nutritional Quality of Milk Proteins. **Milk Salts**: Interaction with Caseins.

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Buffalo Milk

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Introduction

Buffalo milk constitutes over 12% of the global milk production, which is second only to cow's milk; however, in hot and humid regions of the world, buffaloes contribute more significantly than cows to the total milk production because of their ability to thrive under more severe conditions, being more hardy and disease-resistant, and being better converters of coarse roughages. In the Indian subcontinent, the greater part of the milk produced is by buffaloes. Buffalo milk represents >53% of the total milk production in India, increasing at a rate of ~3.6% per annum, and >68% in Pakistan; this growth rate will continue for the immediate future. India and Pakistan together account for ~90% of the total worldwide production of buffalo milk. Buffalo milk is also produced in other Asian countries, but outside Asia only around 2 million tonnes is produced in Egypt and small volumes in Europe (notably in Italy, Bulgaria, Romania, and Germany). Buffalo milk provides more energy per unit volume than provided by cow's milk due to its higher fat and protein content, and it is more economical for producers, processors, and consumers. Planners, researchers, and development agencies of the world are now paying more attention to the efficient utilization of this previously neglected resource.

Gross Composition

Buffalo milk is known for its richness and creaminess. Its composition is influenced by a number of factors that may be genetic, physiological, nutritional, seasonal, or related to the stage of lactation and animal husbandry practices. The general chemical composition of buffalo milk in comparison to the milk of zebu and western cattle is given in **Table 1**. Buffalo milk contains 82–83% water, 6–12% fat, 4–5% proteins, 4–5.5% lactose, and 0.80% ash; it contains higher total solids, fat, proteins, and ash than found in cow's milk.

Milk Proteins

Buffalo milk contains about 4.0% casein and 0.9% whey proteins (**Table 2**). Of the 4.0% total casein, 1.4–1.8% is α_{S1}-casein, 0.2–0.28% α_{S2}-casein, 1.25–1.6% β-casein, 0.15–0.16% γ-casein, and 0.4–0.5% κ-casein. Of the 0.9% whey proteins, about 0.15% is α-lactalbumin, 0.40% β-lactoglobulin, 0.32% proteose-peptone, 0.03% serum albumin, and 0.03% lactoferrin. In comparison to cow's milk, both caseins and whey proteins are present at higher concentrations in buffalo milk. The average proportions of α_{S1}-, α_{S2}-, β-, and κ-caseins in buffalo milk are 40, 6.3, 35, and 12%, respectively. The concentration of nitrogen, calcium, and phosphorus in buffalo casein is about 12.0, 3.5, and 4.5%, respectively. The sialic acid content is 2.0 mg g^{-1}, hexose 2.54 mg g^{-1}, and hexosamine about 1.81 mg g^{-1} of casein. Almost all the casein in buffalo milk is in micellar form. The diameter of the micelles is 80–250 nm, the majority of the micelles being 110–160 nm as compared to 70–110 nm in bovine milk. The voluminosity of the micelles is 2.68–3.72 ml g^{-1} in the temperature range of 25–37 °C. Solvation of the micelles, calculated from voluminosity, is 2.60–2.90 g water g^{-1} casein. This is much lower than the solvation of the casein micelles in cow milk. The opacity of buffalo milk micelles is higher than that of cow's milk casein micelles.

α_{S1}-Casein

Buffalo α_{S1}-casein is a single polypeptide and has no disulfide bridges. Its amino acid composition is similar to that of bovine α_{S1}-casein. The C-terminal and N-terminal sequences are alike in the two species, except that the N-terminal sequence of buffalo α_{S1}-casein has lysine at the fourth position, whereas it is histidine in bovine milk casein. The reverse is true at position 8. The C-terminal sequence of α_{S1}-casein is Leu-Trp-Thr. Like its bovine counterpart, water buffalo α_{S1}-casein has 199 amino acid residues with a theoretical molecular mass of 22 798 Da. The nitrogen and phosphorus content of the protein is 15 and 1.1%, respectively. No genetic polymorphism has been observed in buffalo α_{S1}-casein. On the basis of the number of phosphoserine residues, it can be separated into α_{S1}-Frac.I (six phosphate groups molecule^{-1}) with a molecular mass of 23 276.2 ± 6.1 Da; α_{S1}-Frac.II (seven phosphate groups molecule^{-1}) with a molecular mass of 23 360.6 ± 4.6 Da; and α_{S0}-CN (eight phosphate groups molecule^{-1}) with a molecular mass of 23 437.9 ± 4.1 Da.

α_{S2}-Casein

Two subfractions of buffalo α_{S2}-casein have been identified, containing 10 and 11 phosphoserine residues, respectively. The two fractions are similar in amino acid composition except for lysine and glutamic acid. The

Table 1 Concentration (% w/w) of the major constituents in buffalo's and cow's milk

		Cow's milk	
Nutrient	*Buffalo's milk*	*Indian (Zebu)*	*Western*
Water	83.2	85.3	87.2
Total solids	16.3	13.8	12.8
Solids-not-fat	10.0	9.1	9.1
Fat	6.7	4.6	3.7
Protein	4.7	3.3	3.5
Lactose	4.6	4.4	4.9
Total ash	0.8	0. 7	0.7

Table 2 Concentration of various protein fractions in buffalo's and cow's milk casein fractions

	Concentration in milk ($g\ 100\,ml^{-1}$)	
Polypeptide	*Buffalo's milk*	*Cow's milk*
α_{s1}-Casein	1.44–1.8	1.04–1.3
α_{s2}-Casein	0.22–0.28	0.26–0.34
β-Casein	1.26–1.58	0.94
γ-Casein	0.16	0.15
κ-Casein	0.43–0.54	0.3–0.37
β-Lactoglobulin	0.39	0.31–0.38
α-Lactalbumin	0.14	0.071–0.1
Proteose-peptone	0.33	0.24
Serum albumin	0.029	
Lactoferrin	0.032	0.005

calcium sensitivity of the protein at 30 °C is maximum at 2–4 mmol l^{-1} calcium. There is no genetic polymorphism in α_{S2}-casein.

β-Casein, γ-casein, and proteose-peptone

The primary structures of β-casein, γ-casein, and proteose-peptone (PP) are known. The γ-casein and PP-5, PP-8F, and PP-8S are fragments of β-casein; therefore, it is recommended that these should be called β-casein fragments. Chymosin action on buffalo milk produces three peptides from β-casein, namely, β-casein I, β-casein II, and β-casein III. Buffalo β-casein is more sensitive to calcium than bovine β-casein. Its amino acid composition is similar to that of bovine β-casein; it is devoid of carbohydrates, and no genetic polymorphism has been observed. Its pI is 5.3, the absorbance of a 1% solution in a 1-cm cell at 280 mm is 4.9, and its partial specific volume is 0.764 ml g^{-1}. The β-casein content of serum casein and smaller micelles is lower than that of large micelles. Buffalo β-casein is more sensitive to heat in the presence of calcium than bovine β-casein. The hydrolysis of buffalo β-casein by trypsin is slower than that of bovine β-casein. Buffalo β-casein gives three subfractions on anion exchange chromatography and two bands on gel electrophoresis. Recently, genetic polymorphism of buffalo β-casein has been reported; the molecular mass of the A and B variants is 24 048.8 ± 2.2 and 23 981.2 ± 1.3 Da and they have six and five phosphate groups per molecule, respectively. Buffalo β-casein A and B variants differ by three amino acid substitutions.

The proteose-peptone content of buffalo milk is about 330 mg 100 ml^{-1} compared to about 240 mg 100 ml^{-1} in bovine milk. Heating the milk and hydrolysis with trypsin lead to an increase in the concentration of proteose-peptone in buffalo milk. PP-3, PP-5, and PP-8 – like polypeptides with molecular weights of 25 120, 23 170, and 20 980 Da, respectively – have been isolated from buffalo milk. These fractions are low in aromatic amino acids and have arginine as the N-terminal amino acid. Component 3 is rich in carbohydrates but poor in phosphorus.

γ-Casein represents 4.7% of the total casein in buffalo milk; it gives three bands on SDS-PAGE corresponding to molecular weights of 23 300, 16 400, and 11 200 Da, respectively.

κ-Casein

Buffalo κ-casein is heterogeneous; eight subfractions have been isolated from colostrum. All the fractions are similar in phosphorus content but differ in carbohydrate content; no genetic polymorphism has been observed. Its amino acid composition is similar to that of bovine κ-casein, but its sialic acid content is lower. The release of sialic acid from buffalo κ-casein on rennet action is less than that from bovine milk. The stabilizing capacity of κ-casein for α-casein is low. However, its aggregation and disaggregation in the presence of $CaCl_2$ during heating are similar to bovine κ-casein. This fraction contains almost all the carbohydrates of casein and is rich in nitrogen but poor in phosphorus. Its pI value is 5.8–6.4, its extinction value at pH 8.6 (1% solution in a 1-cm cell at 280 nm) is 10.75, and its partial specific volume is 0.689 ml g^{-1}. Its concentration is higher in serum casein and in smaller micelles than in large micelles. In buffalo colostrum, this fraction is highly glycosylated and is devoid of carbohydrate-free κ-casein.

Whey proteins

Although the concentration of whey proteins in buffalo milk is higher than that in bovine milk, the proportions of different proteins are similar. The individual whey proteins are discussed below.

β-Lactoglobulin

The amino acid composition of buffalo β-lactoglobulin is identical to that of bovine β-lactoglobulin. However, buffalo β-lactoglobulin does not exhibit genetic polymorphism and is identical to bovine β-lactoglobulin B in electrophoretic mobility, sedimentation, and titration behavior. Sedimentation data under different conditions of pH and protein concentration show little difference from those obtained with bovine β-lactoglobulin. Based on the sedimentation coefficient, the molecular weight of buffalo β-lactoglobulin is 38 500 Da, that is, it exists as a dimer. The intrinsic viscosity of a solution of the protein at pH 5.2 and 37 °C is 0.034–0.035 dl g^{-1}, and is independent of pH. The molecule exists as a dimer at pH 5.2, but as a monomer at pH below 3.5 or above 6.5. At a low temperature and at a pH of 4.5, the molecule is present as a tetramer. At pH 12, the molecule is completely denatured and aggregates to form dimers, trimers, tetramers, pentamers, and hexamers. The surface tension of a 0.2% solution of the protein is 40–45 dyn cm^{-1}; at high concentrations, the surface tension is independent of concentration.

α-*Lactalbumin*

There is no genetic polymorphism in buffalo α-lactalbumin, which corresponds to the A variant of bovine α-lactalbumin. Chromatofocusing resolves buffalo α-lactalbumin into one major and three minor components. The Stoke's radius, as determined by gel filtration, is 1.86 nm and the degree of hydration, determined viscometrically, is very low (0.52 g g^{-1}). The partial specific volume is 0.73 ml g^{-1} and the molecular weight, determined by SDS-PAGE, is 14 400–14 500 Da, but gel filtration indicates that it is 2000–3000 units higher. The major component and one of the minor components with a slower electrophoretic mobility are devoid of carbohydrates, while the two other minor components are similar in hexose and hexosamine content; but only one of these fractions contains sialic acid. All the components are active in modifying the activity of galactosyl transferase in the synthesis of lactose. The physicochemical properties of buffalo and bovine α-lactalbumin are similar: they have the same crystalline form, and a similar nitrogen content, specific extinction coefficient at 280 nm, and tyrosine and tryptophan content. Their sedimentation coefficient is within the range of $1.87–1.99 \times 10^{-13}$ s.

Immunoglobulins

Buffalo milk contains about 10 mg of immunoglobulins per 100 ml of milk. In colostrum, their concentration is very high, being 43 and 32% of the total proteins in the first and second milking, respectively. The immunobiological role of buffalo immunoglobulins has generated considerable interest. Due to the high levels of these proteins in buffalo colostrum, efforts are being made to isolate and utilize them in infant foods as bioprotective additives. Four classes of these proteins, namely, IgG, IgA$_1$, IgA$_2$, and IgM, have been identified in buffalo colostrum, and IgG predominates.

Lactoferrin

Buffalo milk contains about 32 mg 100 ml^{-1} lactoferrin, compared with 15 mg 100 ml^{-1} for bovine milk; buffalo colostrum contains about 750 mg 100 ml^{-1} lactoferrin. The molecular weight of the protein is 73 500–74 000 Da. Total binding sites and other properties of the protein are similar to those of bovine lactoferrin.

Enzymes

Over 20 enzymes have been isolated from bovine milk and characterized. However, information on enzymes in buffalo milk is very limited. The important enzymes that have been characterized in some detail are given in **Table 3**. The major difference is in the activity of lactoperoxidase, which is nearly 1.76-fold higher in buffalo milk than in bovine milk.

Lipids

The proportions of mono-, di-, and tri-glycerides in buffalo milk fat are 0.7, 0.4, and 95%, respectively, which is similar to those in bovine milk fat. Significantly lower levels of free fatty acids have been found in buffalo milk and ghee (anhydrous milk fat) as compared to cow's milk and ghee.

The unsaponifiable matter is less in buffalo milk (390–400 mg 100 ml^{-1}) than in bovine milk (416–450 mg 100 ml^{-1}). Its content is higher in spring than in summer or winter.

Table 3 Concentration or activity of some enzymes in buffalo and bovine milk

	Activity/concentration in milk	
Enzyme	*Buffalo milk*	*Bovine milk*
Lipase (units ml^{-1})	0.16–1.13	0.1–0.6
Alkaline phosphatase (units ml^{-1})	0.12–0.18	0.08–0.12
Xanthine oxidase (units ml^{-1})	0.075	0.092
Lysozyme (μg ml^{-1})	0.152	0.18
Lactoperoxidase (units ml^{-1})	5.2–9.8	4.36–7.16
Ribonuclease (μg ml^{-1})	9.78	8.23
Protease (units ml^{-1})	0.78	0.68

Cholesterol

Buffalo milk fat has a lower concentration of total ($275\,mg\,100\,g^{-1}$) and free cholesterol ($212\,mg\,100\,g^{-1}$) than bovine milk fat (330 and $280\,mg\,100\,g^{-1}$, respectively). On the other hand, there are about $64\,mg\,100\,g^{-1}$ esterified cholesterol in buffalo milk fat compared to $48\,mg\,100\,g^{-1}$ in bovine milk fat. The cholesterol content is higher in foremilk than in strippings. On heating ghee (milk fat), thermal oxidation of cholesterol occurs, resulting in a lowered cholesterol level. Colostrum and mastitis milk contain more cholesterol than normal milk.

Phospholipids

The concentrations of phospholipids in bovine and buffalo milk are the same (about $39\,mg\,100\,ml^{-1}$ milk). However, due to the much higher fat content in buffalo milk, the phospholipid content of buffalo milk fat is much lower than that of bovine milk fat. Therefore, buffalo butter and ghee are poor in phospholipids. Winter ghee and the solid fraction obtained at 29 °C are rich in phospholipids.

Minor components

Buffalo milk fat contains about $8\,\mu g\,g^{-1}$ squalene compared to $5.9\,\mu g\,g^{-1}$ in cow's milk fat. Buffalo milk fat is also rich in ubiquinone, about $6.5\,\mu g\,g^{-1}$ compared to $5\,\mu g\,g^{-1}$ in cow's milk fat. However, buffalo milk fat is a poor source of lutein and lanosterol, which are present at 3.0 and $83\,\mu g\,g^{-1}$, respectively, compared to 4 and $93\,\mu g\,g^{-1}$ in bovine milk fat. The concentration of ethers is also lower in buffalo milk fat, about $0.8\,\mu g\,g^{-1}$, compared to $0.9\,\mu g\,g^{-1}$ in bovine milk fat. The concentration of total carbonyls is $9.8\,\mu g\,g^{-1}$ fat in buffalo milk. Alkanols (ethanol, methanol, and butanol) are present in lower concentrations in buffalo milk fat, being $1.85\,\mu g\,g^{-1}$ compared to $2.29\,\mu g\,g^{-1}$ in bovine milk fat. Ketoglycerides are also present in buffalo milk fat.

Fatty acid composition

The fatty acid composition of buffalo and bovine milk fat is given in **Table 4**. The proportions of $C_{4:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$ fatty acids are higher but those of $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, and $C_{14:1}$ fatty acids are lower in buffalo milk fat than in bovine milk fat. In summer, the proportions of monounsaturated fatty acids, namely, $C_{14:1}$, $C_{16:1}$, and $C_{18:1}$, are higher, but the proportions of saturated fatty acids, namely, $C_{4:0}$, $C_{12:0}$, $C_{14:0}$, and $C_{16:0}$, are lower than the corresponding proportions in winter. Colostrum and late-lactation milk are rich in unsaturated fatty acids but poor in saturated fatty acids. Buffalo milk fat is richer in tetraenoic and pentaenoic acids but poorer in dienoic and trienoic fatty acids than bovine milk fat (**Table 4**).

Table 4 Fatty acid composition (% w/w) of buffalo and bovine milk fat

Fatty acid	*Buffalo milk*	*Bovine milk*
$C_{4:0}$	4.36	3.20
$C_{6:0}$	1.51	2.11
$C_{8:0}$	0.78	1.16
$C_{10:0}$	1.28	2.57
$C_{10:1}$	-	0.31
$C_{12:0}$	1.78	2.78
$C_{14:0}$	10.81	11.93
$C_{14:1}$	1.27	2.12
$C_{15:0}$	1.29	1.23
$C_{16:0}$ Br	0.18	0.30
$C_{16:0}$	33.08	29.95
$C_{16:1}$	1.99	2.16
$C_{17:0}$	0.58	0.34
$C_{18:0}$ Br	0.24	0.35
$C_{18:0}$	11.97	10.07
$C_{18:1}$	27.15	27.42
$C_{18:2}$	1.51	1.49
$C_{18:3}$	0.47	0.59

Br, branched chain.

Table 5 Polyunsaturated fatty acid composition of buffalo and bovine milk fat

	Concentration (% w/w of total fat)	
Fatty acid	*Buffalo*	*Bovine*
Dienoic		
Conjugated	0. 725	0.821
Nonconjugated	0.942	1.10
Total	0.022	1.921
Trienoic		
Conjugated	0.022	0.027
Nonconjugated	0.502	0.538
Total	0.524	0.565
Tetraenoic		
Conjugated	0.006	0.003
Nonconjugated	0.196	0.131
Total	0.202	0.134
Pentaenoic		
Conjugated	0.002	0.0
Nonconjugated	0.105	0.062
Total	0.107	0.062

Colostrum and late-lactation milk are rich in polyunsaturated fatty acids (PUFA) (**Table 5**).

Glyceride structure

Separation of triglycerides based on crystallization behavior into high-melting triglycerides (HMT), medium-melting triglycerides (MMT), and low-melting triglycerides (LMT) reveals that buffalo milk fat contains a higher proportion (9–12%) of HMT than cow's milk fat (5–6%). Separation of triglycerides on the basis of molecular weight into higher-molecular-weight triglycerides (HMWT), medium-molecular-weight triglycerides (MMWT), and lower-molecular-weight triglycerides (LMWT) reveals that their proportions in buffalo milk fat are 42.5, 17.0 and, 40.5%, respectively, compared to 52.9, 18.9, and 28.2% in bovine milk fat. About 90% of the fatty acids in HMWT are $C_{14:0}$, $C_{16:0}$, $C_{18:1}$, and $C_{18:2}$. The principal fatty acids in MMWT are $C_{6:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:1}$, and those in LMWT are $C_{4:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{18:1}$.

Physicochemical constants

Buffalo milk fat has a higher melting point, specific gravity, saponification value and Reichert–Meissl value and larger grain size, but lower BR reading, refractive index, acid value, iodine value, and Polenske value compared to cow's milk fat.

Fat globules and fat globule membrane

The fat globules in buffalo milk are bigger, with an average volume of 4.15–4.6 μm compared to 3.36–4.15 μm for bovine milk. Buffalo milk fat globules are rendered chargeless at a much higher pH (4.5–4.6) as compared to bovine milk fat globules (pH 4.3). Buffalo fat globule membrane has an average lipid content of 38.5% compared to 35.7% in bovine milk. The total neutral lipids are higher in buffalo milk (74.4%) than in bovine milk (65.7%) fat globule membrane. On the other hand, the phospholipid content is almost the same in both species. Except for triglycerides and diglycerides, which are higher in buffalo milk (52.7 and 7.4%, respectively) than in bovine milk (35.5 and 5.5%), the concentration of other fractions is similar in both the milks.

Minerals

Major minerals

Buffalo milk is rich in calcium and magnesium (divalent cations) but poor in monovalent cations (sodium and potassium) and monovalent anions (chloride) as compared to bovine milk. However, the concentration of divalent anions (phosphate and citrate) is similar in both the milks. The average concentration and the partitioning of different salts between the dissolved and the colloidal phases in buffalo milk and bovine milk are given in **Table 6**. The concentrations of calcium and magnesium in the dissolved phase of buffalo and cow's milk are almost similar (about 39–40 mg of calcium and 8 mg of magnesium per 100 ml of milk). However, due to the higher concentrations of these minerals in buffalo milk, only about 20–22% of calcium and 45–46% of magnesium are in the dissolved state in buffalo milk, compared to about 33% of calcium and 75% of magnesium in bovine milk. On the other hand, in buffalo milk nearly twice as much divalent cations (about 172 mg 100 ml^{-1}) are present in the colloidal state, compared to 78 mg 100 ml^{-1} in bovine milk. The proportions of phosphate and citrate are also higher in the colloidal phase of buffalo milk than in bovine milk.

Trace elements

The trace elements in buffalo milk have not been studied as extensively as the major minerals. However, their concentrations reported in the literature are iron (1.3–1.94 mg kg^{-1}), copper (0.29–0.35 mg kg^{-1}), zinc (4.96–5.9 mg kg^{-1}), boron (0.52–1.36 mg kg^{-1}), and sulfur (157–314 mg kg^{-1}).

Table 6 Concentration of salt constituents in milk and its dissolved phase

Parameter		*Buffalo*	*Cow*
Calcium	Total (mg 100 ml^{-1})	182.6	114.2
	Dissolved (mg 100 ml^{-1})	39.75 (22)	39.2 (34)
Magnesium	Total (mg 100 ml^{-1})	18.04	11.0
	Dissolved (mg 100 ml^{-1})	8.30 (46)	7.8 (10)
Sodium	Total (mg 100 ml^{-1})	43.85	50
	Dissolved (mg 100 ml^{-1})	41.59 (95)	47 (94)
Potassium	Total (mg 100 ml^{-1})	106.6	148
	Dissolved (mg 100 ml^{-1})	100.96 (95)	143 (98)
Phosphorus	Total (mg 100 ml^{-1})	82.39	84.8
	Dissolved (mg 100 ml^{-1})	26.20 (31)	38.4 (45)
Citric acid	Total (mg 100 ml^{-1})	159.2	166.0
	Dissolved (mg 100 ml^{-1})	114.69 (84)	152.0 (96)
Chloride	Total (mg 100 ml^{-1})	57.69	106.3
	Dissolved (mg 100 ml^{-1})	57.03 (99)	106.2 (100)

Values in parentheses are the percentage of total in the dissolved phase.

Vitamins

The concentrations of vitamins in buffalo and bovine milk are given in **Table 7**. Except for vitamin A and tocopherol, the concentrations of vitamins in buffalo milk are similar to their concentrations in bovine milk. However, due to the absence of carotenoids and the high fat content of buffalo milk, its total potential level of vitamin A is lower than that of bovine milk. Similarly, there is a higher concentration of tocopherol in buffalo milk, but due to its higher fat content buffalo milk fat is poorer in tocopherol (about 26 μg g^{-1}) as compared to cow's milk fat (about 35 μg g^{-1}).

Pigments in Buffalo Milk

Buffalo milk contains a blue-green pigment, biliverdin IX alpha, which is not found in cow's milk. The average concentrations of biliverdin in skim milk of Murrah and Surti buffalo are about 52 and 65 μg 100 ml^{-1}, respectively, and are influenced by the stage of lactation. Biliverdin in buffalo milk is associated with the caseins. On souring of milk, the pigment is dissociated and undergoes a rapid chemical reduction to a fat-soluble yellow pigment, bilirubin IX alpha. This pigment binds milk lipids and imparts the characteristic greenish-yellow appearance to ghee and butter prepared by the traditional method.

Table 7 Concentration of vitamins in buffalo and bovine milk

	Concentration in milk	
Vitamin	*Buffalo*	*Bovine*
Vitamin A (IU ml^{-1})	340	230
Thiamine (μg ml^{-1})	0.2–0.5	0.2
Riboflavin (μg ml^{-1})	1.59	2.33
Pyridoxine (μg ml^{-1})	3.25	2.6–3.0
Ascorbic acid (mg 100 g^{-1})	2.2	1.94
Tocopherol (μg g^{-1})	334.2	312.2

Miscellaneous Constituents

Non-protein nitrogen

About 5% of the total nitrogen in buffalo milk is non-protein nitrogen, the same as in bovine milk. Non-protein nitrogen compounds in buffalo milk include amides, urea, amino acids, creatine, creatinine, uric acid, and ammonia. The concentrations of these compounds in buffalo milk are similar to their concentrations in bovine milk except for urea, which is present at a much lower concentration (17–22 mg 100 ml^{-1}) than in bovine milk (37–40 mg 100 ml^{-1}). Buffalo milk has an average taurine content of 23 mg l^{-1}.

Physicochemical Properties of Buffalo Milk

Due to quantitative and qualitative differences in various constituents, buffalo milk differs from bovine milk in physicochemical properties. The physicochemical properties of buffalo and bovine milk are compared in **Table 8**.

Heat Stability

The heat stability of buffalo milk is similar to that of bovine milk, and no correlation has been observed between its heat stability and its natural pH or between heat stability and any of its constituents. However, alteration in its natural pH due to souring or addition of acid or alkali leads to a considerable change in heat stability. All buffaloes produce type A milk with its natural pH coinciding with the pH of maximum stability. Therefore, any alteration in its pH results in the destabilization of buffalo milk. Addition of urea, glyceraldehyde, glyoxal, and deoxyribose stabilizes buffalo milk in the region of maximum stability while the addition of acid causes its destabilization. Colostrum, dry secretion, mastitic milk,

Table 8 Physical properties of bovine and buffalo milk

Property	*Buffalo milk*	*Cow's milk*
Specific gravity (at 20 °C)	1.0323	1.0317
Viscosity (cP at 27 °C)	2.245	1.450
Curd tension (g)	32.85	28.54
Surface tension (dyn cm^{-1} at 20 °C)	45.50	42.50
Freezing point (°C)	−0.545 4	−0.530
pH (at 20 °C)	6.7	6.6
Acidity (as % lactic acid)	0.16	0.15
Redox potential (V)	+0.310	+0.258
Electrical conductivity (mΩ)	3.22–6.67	4.00–5.50
Thermal conductivity (kcal h^{-1}m^{-1} °C^{-1} at 37 °C)	0.569	0.46
Heat capacity (cal g^{-1} °C^{-1} at 30 °C)	0.852	0.93–0.95
Heat stability (min at 130 °C)	31	32
Rennet stability (min)	25.6	28.2

and milk from tuberculosis-infected animals are very unstable. No correlation between the heat stability of buffalo milk and its casein number, casein solvation, and voluminosity has been observed.

Comparatively much less work has been done on the heat stability of concentrated buffalo milk. Concentrated buffalo milk is much less stable than cow's milk at its natural pH due to a greater shift in its pH of maximum stability toward the acidic side. In the case of concentrated bovine milk, the pH of maximum stability is usually on the alkaline side of the natural pH. Therefore, addition of substances that cause an increase in its pH, such as disodium phosphate or trisodium citrate, leads to stabilization. On the other hand, due to the location of the pH of maximum stability on the acidic side of the natural pH, the above compounds, instead of stabilization, cause very strong destabilization of concentrated buffalo milk. Similarly, removal of calcium by cation exchangers destabilizes buffalo milk concentrates. For these reasons, problems were encountered in the manufacture of evaporated milk from buffalo milk. However, with the addition of an optimum amount of either NaH_2PO_4 or $CaCl_2$, evaporated milk can be prepared from buffalo milk. The heat stability of buffalo milk concentrate at the pH of maximum stability is as good as that of cow's milk, except that cow's milk is on the acidic side of the natural pH while cow's milk concentrate is on the alkaline side. The heat coagulation time (HCT) versus pH curves of buffalo milk and its concentrate are presented in **Figures 1** and **2**.

Rennet and Ethanol Stability

Under similar conditions, the rennet coagulation time of buffalo milk is shorter than that of cow's milk and is inversely proportional to its calcium content.

Ethanol stability, expressed as the concentration of ethanol in aqueous solution that coagulates an equal volume of milk, is 60–72%, which is lower than 70–80% for the milk of Zebu cattle. Addition of ethylenediaminetetraacetic acid (EDTA), disodium phosphate, and trisodium phosphate; forewarming at 90 °C for 30 min; and dilution with water increase the ethanol stability of buffalo milk. On the other hand, addition of calcium chloride reduces ethanol stability.

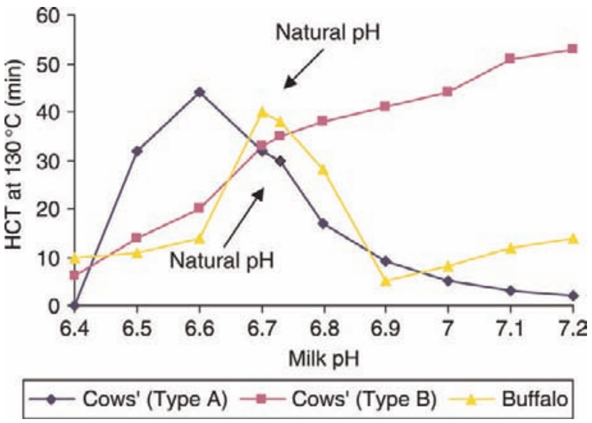

Figure 1 Effect of pH on the heat stability of buffalo's and cow's milk.

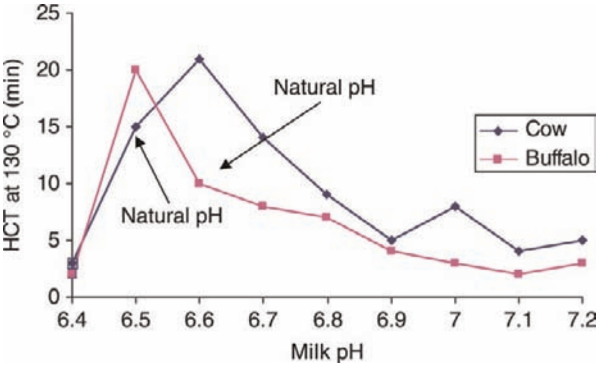

Figure 2 Effect of pH on the heat stability of concentrated milk (buffalo's and cow's).

Density and Specific Gravity

The density of buffalo milk at 20 °C is about 1.034 1 kg l^{-1}, which decreases to 1.027 2 and 1.026 3 kg l^{-1} at 27 and 29 °C, respectively. The specific gravity of the milk is about 1.032 3 at 20 °C and 1.031 9 at 30 °C.

pH, Acidity, and Buffering Capacity

The pH of fresh buffalo milk is in the range of 6.63–6.8. It is highest during January and lowest during May–June. The titratable acidity of buffalo milk is 0.16%, compared to 0.15% for cow's milk. The development of acidity is faster in buffalo milk than in cow's milk. The buffering capacity of buffalo milk is highest in the acidic pH range, with the peak at pH 4.8–5.2; on either side of this range it shows a steep fall. The buffering capacity at 60 °C is maximum at about pH 4.6, which shifts to around pH 5.7 at 20 °C. The addition of disodium phosphate or trisodium citrate causes an increase in buffering capacity but the addition of calcium chloride reduces it.

Freezing Point

The freezing point of buffalo milk is in the range of −0.545 4 to −0.544 3 °C. This range is higher for milk from the Egyptian buffaloes, being −0.552 to −0.558 °C. These values for the freezing point are much lower than the freezing point of cow's milk, which is −0.522 °C.

Viscosity and Surface Tension

The viscosity of buffalo milk at 20 °C is about 2.245 cP, which is much higher than that of cow's milk (1.450 cP at 20 °C). This is due to the higher protein and fat content of buffalo milk. The surface tension of buffalo milk at 20 °C is in the range of 45.5–48.7 dyn cm^{-1}, which is somewhat higher than the surface tension of cow's milk (42.3–42.5 dyn cm^{-1}).

Curd Tension

The curd tension of buffalo milk is 32–85 g, which is much higher than that of cow's milk, which is in the range of 28–54 g. The higher curd tension of buffalo milk may be due to its higher total solids, particularly the casein and calcium.

Oxidation–Reduction Potential and Electrical Conductivity

The oxidation–reduction potential (Eh) of buffalo milk is in the range of +0.129 to +0.469 V, with an average of +0.310 V at 30 °C. This value is higher than the Eh of cow's milk, which is +0.258 V. There is no appreciable difference in the electrical conductivity of milk from the two species; for buffalo milk the value is 3.22 m℧ and for cow's milk it is 4.0–5.5 m℧.

Thermal Expansion and Thermal Conductance

The volume of buffalo milk increases by 1.26% when heated from 4 to 40 °C and by 6% when heated up to 95 °C. The increase in volume is less for skim milk than for whole milk. Furthermore, the increase is greater for buffalo milk than for cow's milk. For whole milk the coefficient of thermal expansion is 4.106×10^{-4} °C^{-1} in the temperature range of 20–25 °C. The thermal conductance of buffalo milk is in the range of 0.548 7–0.593 7 kcal h^{-1} m^{-1} °C^{-1} with an average of 0.568 9 kcal h^{-1} m^{-1} °C^{-1} at 37 °C. This value is much higher than the thermal conductance of 0.46 kcal h^{-1} m^{-1} °C^{-1} for cow's milk.

Heat Capacity

The heat capacity of buffalo milk is 0.852 0 cal g^{-1} °C^{-1} at 30 °C and is lower than that of cow's milk, which is in the range of 0.933–0.954 cal g^{-1} °C^{-1} at the same temperature.

Color and Flavor

Fresh buffalo milk is whiter than cow's milk and has a slight blue-green hue. This may be attributed to the larger size of casein micelles and fat globules, the presence of biliverdin, and the absence of carotenoids in buffalo milk. Buffalo milk has a smoother, more pleasant, slightly sweeter, and richer flavor than cow's milk apparently due to higher total solids and fat content.

Inherent Advantages and Problems Encountered during the Processing of Buffalo Milk

Inherent Advantages of Buffalo Milk

Due to higher levels of total solids, fat, proteins, and colloidal calcium, and the larger fat globules in buffalo milk, fermented products like dahi (curd), lassi, shrikhand, shrikhand wadi (indigenous Indian products), leben, cermir (Iraq), lule kaymagl (Turkey), zabadi (Egypt), buttermilk (Bulgaria), and yogurt made from buffalo milk have a better body and texture than similar products made from cow's milk. For yogurt, the need to concentrate milk or to add milk powder is avoided due to the higher total solids content. The yield of cream, butter, and ghee from buffalo milk is higher due to higher fat, and the separation of cream and its churning into butter are easier due to the larger size of the fat globules and the higher proportion of solid fat. The fat losses in skim milk and buttermilk are lower. The texture of ghee is better due to the bigger grain size, which, in turn, may be due to the higher proportions (9–12%) of HMT compared to only 5% in cow's milk fat. The quality of khoa (a heat desiccated indigenous Indian product) from buffalo milk is superior to that of khoa from cow's milk, which has a moist surface, a sticky and sandy texture, and a salty taste. Similarly, the quality of buffalo milk paneer (an acid-coagulated unripened cheese) is superior, as the cow's milk product is too soft, weak, and fragile, and after cooking, its pieces lose their identity. The yield of these products is also higher from buffalo milk than from cow's milk.

The manufacture of edible casein and caseinate is easier from buffalo milk due to its higher casein content. The yield of these products is also higher than the yield from cow's milk, as the losses in the whey are less due to larger size, less hydration, and lower voluminosity of the casein micelles in buffalo milk.

Better absorption of fat due to superior emulsifying capacity; a higher calcium-to-phosphorus ratio; higher concentrations of whey proteins, calcium, magnesium, lactoferrin, esterified cholesterol, and taurine; and lower concentrations of sodium, potassium, chloride, urea, and total and free cholesterol in buffalo milk as compared to cow's milk are beneficial in human nutrition. These attributes make buffalo milk superior to cow's milk as an ingredient for health foods and, with slight modification, for infant foods.

Certain varieties of cheese, like Mozzarella and Domiati, are superior in quality when made from buffalo milk. Other prominent varieties of cheese made from buffalo milk are Feta, Bjalo salamureno sirene (Bulgaria), Beli-sir-u-kriskama (Yugoslavia), Teleme (Romania), Lori, Imeretinskii, Limanskii, Osetinskii (Russia), and Brizna (Israel).

Problems during the Processing of Buffalo Milk

Due to the quantitative and qualitative differences of various constituents and physicochemical properties of the milk from the two species, the processing technology developed in Western countries for cow's milk is not suitable for processing buffalo milk. Therefore, buffalo milk poses some problems during processing to some products; a brief description of these problems is given below.

The lower heat capacity, higher thermal conductivity, and higher thermal expansion of buffalo milk clearly indicate that a lower quantum of heat energy is required to achieve certain desired heat effects in buffalo milk. Therefore, time–temperature combinations for its processing may have to be standardized and suitably modified.

Buffalo milk is considered not suitable for the manufacture of certain ripened cheese varieties, namely, Cheddar, Gouda, and Emmental. These ripened varieties of cheeses are characterized by their soft, mellow and velvety body and texture and a rich pleasing flavor. The cheese made from buffalo milk has a flat flavor and a hard, rubbery and dry body and texture. These problems in buffalo milk cheese may be due to slower acidity development, a lower retention of moisture, and a lower rate of glycolysis, proteolysis, and lipolysis, as compared to cow's milk cheeses. However, with modifications in the process, Cheddar, Gouda, and Emmental cheeses of acceptable quality can be made from buffalo milk.

The quality of buffalo milk chhana (an acid-coagulated Indian product used as an ingredient in sweetmeats) is not comparable to that of cow's milk chhana as its texture is hard and cohesiveness is lower, which makes it unsuitable for making rasogolla. Also, butter made from buffalo milk is harder than cow's milk butter. As a result it creates problems during working and has poor spreadability on bread. The oxidative stability of buffalo ghee is poor because the amounts of tocopherol and phospholipids present are low.

See also: **Animals that Produce Dairy Foods**: Water Buffalo. **Husbandry of Dairy Animals**: Buffalo: Asia; Buffalo: Mediterranean Region.

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Camel Milk

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Introduction

According to FAO (Food and Agriculture Organization of the United Nations) statistics, there are about 19 million camels in the world, of which 15 million are found in Africa and 4 million in Asia. Of this estimated world population, 17 million are believed to be one-humped dromedary camels (*Camelus dromedarius*) and 2 million two-humped (*Camelus bactrianus*). Approximately 15 million dromedaries, representing two-thirds of the world camel population, are in the arid areas of Africa, particularly in northeast Africa, that is, Somalia, Sudan, Ethiopia, and Kenya. This article is concerned entirely with the one-humped camels. The term 'camel' should therefore be taken to refer to this species unless specifically stated otherwise.

Camels are kept by pastoralists in subsistence production systems. They are very reliable milk producers during dry seasons and drought years when milk from cattle, sheep, and goats is scarce. At such times, camel can contribute up to 50% of the nutrient intake of the pastoralists. Camel meat is also an important by-product mainly as a source of income. Sale of live camels, usually males and unproductive females for slaughter, is very common in East Africa and there are now increasing numbers of camel butcheries in many urban centers. There is also a growing export trade of slaughter camels to the Arabian Peninsula. The camel is also a means of transportation and serves domestic needs such as drawing water from wells, rivers, and dams.

From a global perspective, the economic significance of camel production is minimal in comparison with that of other domestic animals. Nevertheless, in Africa, especially in East Africa and Sahel countries, the camel population makes a significant contribution to national economies. However, it is difficult to evaluate this economic contribution as most of the camel products are traded in the informal sector. Owing to the increasing human population and declining per caput production of food, there is an urgent need to develop marginal resources, such as arid land, and optimize their utilization through appropriate livestock production systems of which camel production is the most suitable without doubt.

Milk Production

It is difficult to estimate the daily milk yield of a camel under pastoralist conditions, owing to the inconsistency of milking frequency. Milk yield also varies with species, breed, stage of lactation, feeding, and management conditions.

The length of lactation can vary from 9 to 18 months. This depends mainly on the husbandry practices, which are determined largely by the need for milk, more being required in the dry months than in the wet months when other sources of food are available. In general, both udder halves are milked at the same time by two herdsmen, but before milking, the calf is allowed to suckle until the milk starts to flow and the camel can be milked. Without this stimulation, the dam cannot be milked. If the calf dies, the dam dries up if milking is not stimulated. Often it is sufficient for the dam to see the skin of her calf for milk secretion to be stimulated.

Estimates of milk yields from various countries are given in **Table 1**. The data should be considered as guidelines for milk yields under pastoral conditions. It must also be noted that throughout lactation calves are still suckling and therefore the actual volume of milk secreted is higher than the figures presented in the table.

Camels are slow reproducers. A female camel is sexually mature at the age of 4–5 years. Pregnancy is just over 12 months and the calving interval in pastoral production systems is normally 24 months or more. Female camels can remain fertile up to the age of 25 years and it is often reported that they produce 8–10 calves during a lifetime. In pastoral production systems, however, only a small fraction of the breeding female can reach this production performance.

Besides this natural productivity limitation, the main factor affecting milk production is calf mortality. Newborn mortality in camels is very common and losses as high as 50% have been reported in different parts of eastern and northern Africa.

In the surveyed areas, deaths of newborn and calves up to 1 month were sixfold compared to grown-up camels with 3% of losses. The main reason given by the herders was feeding of colostrum, which leads, according to their belief, to diarrhea. For this reason, the colostrum is milked onto the ground, leaving only a relatively small quantity for suckling by the calf. This indicates that low colostrum

Table 1 Milk yield of camels reported from various sources

Country	*Average daily yield (kg)*	*Lactation length (months)*	*Calculated yield (kg yr^{-1})*
Algeria	3.0	9–16	1460
Ethiopia	5.0	12–18	1825
India	6.8	18	2482
Kenya	4.5	11–16	1643
Pakistan	10.0	16–18	2920
Somalia	5.0	9–18	1825
Tunisia	4.0	9–16	1460

intake, and consequently low immunization, of the newborn calf is presumably one of the main causes of early calf mortality.

Camel milk is generally opaque white. It has a sweet and sharp taste, but sometimes it can be salty. The taste generally depends on the type of fodder and availability of drinking water. The pH of camel's milk ranges from 6.2 to 6.5 and the density from 1.026 to 1.035. Both density and pH are lower than those of cow's milk.

Compared to cow's milk, camel's milk sours very slowly and can be kept longer without refrigeration.

Milk Composition and Main Components

The composition of camel milk quoted from various sources and the corresponding values from other animal species are presented in **Table 2**. There are greater variations in the constituents of camel milk than in cow milk. Camels are known to produce diluted milk in hot weather when water is scarce. The main difference between cow's and camel's milk lies in the different physicochemical characteristics of the individual components.

Proteins

The average casein content and the whey protein content of camel milk vary between 1.9 and 2.3%, and 0.7 and 1.0%, respectively. Comparison with bovine milk proteins reveals pronounced differences in the quantitative distribution of casein and whey proteins. β-Casein is found in higher concentration (65% of total casein) than in bovine milk (39% of total casein), whereas κ-casein, which is about 13% of total caseins, in bovine milk amounts to only 3.5% of the casein fraction of camel milk (**Figure 1**).

Camel milk casein differs from cow milk casein in terms of micellar size distribution. Electron microscopic studies showed a relatively broad size distribution of casein micelles in camel milk with a greater number of large micelles than cow milk.

Most casein particles in cow milk have a diameter of 40–160 nm. In camel milk, casein particles range in diameter from 20 to more than 300 nm (**Figure 2**).

Nearly 90% of the whey proteins consist of α-lactalbumin, serum albumin, immunoglobulin, and lactophorin, the rest being minor proteins such as peptidoglycan recognition proteins (PGRPs), lactoferrin, and whey acidic protein.

β-Lactoglobulin, the main whey protein of bovine milk, is not found in camel milk.

Milk Fat

The fat content of camel milk varies between 2.9 and 5.4% and the average size of the fat globules is about the same as fat globules of cow milk. According to our present knowledge, the main differences in fat between cow milk and camel milk are as follows:

1. Natural creaming of camel milk differs markedly from that of cow milk. On standing, camel milk creams less rapidly and less completely than cow milk and no skimmable cream can be obtained even after standing for several days.

Table 2 Gross composition (%) of milk from various animal species

Species	*Moisture*	*Fat*	*Lactose*	*Protein*	*Ash*
Camel	86–88	2.9–5.4	3.3–5.8	3.0–3.9	0.6–1.0
Cow	86–88	3.7–4.4	4.8–4.9	3.2–3.8	0.7–0.8
Goat	87–88	4.0–4.5	3.6–4.2	2.9–3.7	0.8–0.9
Sheep	79–82	6.9–8.6	4.3–4.7	5.6–6.7	0.9–1.0
Human	80–88	3.3–4.7	6.8–6.9	1.1–1.3	0.2–0.3

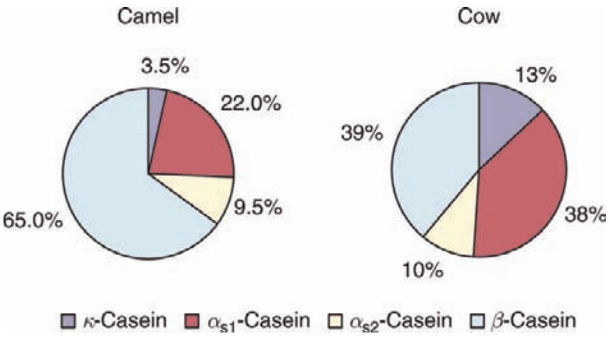

Figure 1 Proportion of individual caseins in camel and cow milk.

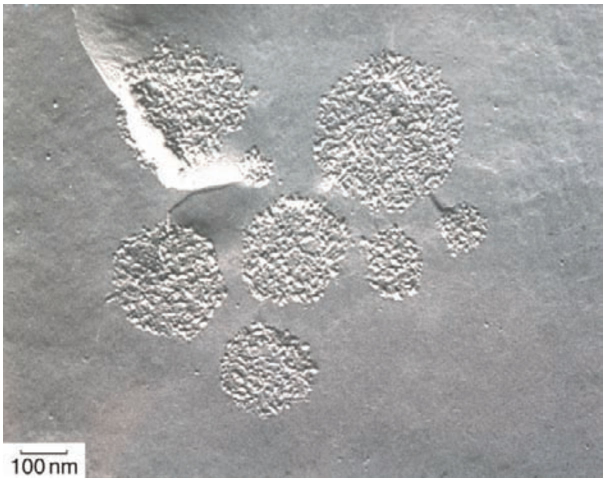

Figure 2 Electron micrograph (×75 000) of freeze-fractured casein micelles in camel milk. Scale = 100 nm.

2. Compared to cow milk fat, camel milk fat contains less short-chain fatty acids. Long-chain unsaturated fatty acids occur to about the same extent in both.
3. Butter can be obtained from camel milk only at a high churning temperature of 20–25 °C. These values are considerably higher than the value for cow milk, which normally varies between 8 and 12 °C.
4. The mean melting point of camel milk butter is around 41.5 °C and is on average 8 °C higher than the corresponding value for cow milk butter.

Lactose

Lactose is the major carbohydrate in the milk of all mammals and it is generally accepted that there are no non-mammalian sources of lactose.

The lactose content of camel milk ranges from 4.8 to 5.8% and is slightly higher than the lactose content of cow milk. It seems that the lactose content of camel milk is relatively constant throughout lactation.

Studies on the effect of drought on the composition of camel milk found that the lactose content was low at birth being around 2.8% but within 24 h it was increased to 3.8%. There was a further increase up to 5% as long as drinking water was available. Dehydration of the animals led to a decline in milk lactose content to as low as 2.6%. According to these studies, this change in lactose concentration would account for the milk being described sometimes as sweet and other times as bitter.

Mineral Salts and Vitamins

Only fragmentary information is available on minerals and vitamins in camel milk.

Milk mineral salts are mainly chlorides, phosphates, and citrates of potassium sodium, calcium, and magnesium. Although salts comprise less than 1% of the milk, they influence the physical state and stability of milk proteins, particularly the phosphocaseinate complex. The mineral content of camel milk expressed as ash ranges from 0.6 to 0.8%. Although the salt composition of milk is influenced by factors such as health status of the udder and stage of lactation, the major salt constituents of camel milk seem to be similar to those of cow milk.

Camel milk contains less vitamin A and E, thiamine, riboflavin, folic acid, and pantothenic acid than cow milk, while the content of pyridoxine and B_{12} is about the same. The content of niacin and vitamin C is higher than that of cow milk. The availability of a relatively fair amount of vitamin C (range reported in the literature is 25–60 mg l^{-1}) in camel milk is significant from the nutritional standpoint in arid areas.

Technologically Relevant Properties of Milk

Enzymatic Coagulation

Most attempts to make cheese from camel milk have revealed major difficulties in getting the milk to coagulate. With the same amount of calf rennet, the coagulation time of camel milk is two- to threefold longer than cow milk. The action of rennet on camel milk leads to coagulation in the form of flocks, with no firm coagulum. Typical formagraph recordings of duplicate cow and camel milk samples are shown in **Figure 3**.

The coagulation time (*R*) is determined by measuring the distance from the origin to the point where the baseline begins to increase in width and curd formation is expressed as the time from the start of gel development until a width of 20 mm is reached. Following this definition, no curd firmness could be measured in camel milk as this width of formagraph was not reached, due to the failure of curd formation.

One reason for this weak coagulum is presumably the low amount of κ-casein in camel milk, which can lead to weak curd formation. Another possible reason is the fact that the mean diameter of camel casein micelles is larger than

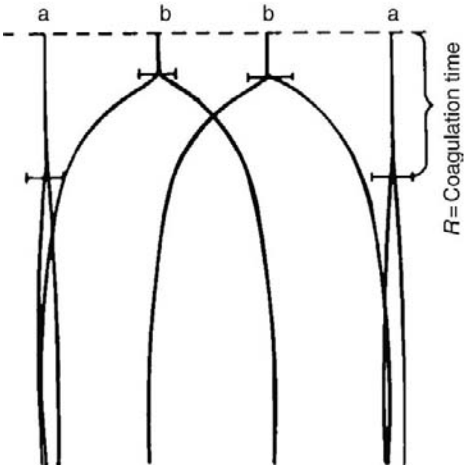

Figure 3 Formagram of duplicate camel (a) and cow (b) milk samples (*R* cow = 330 s, *R* camel = 930 s).

that of casein micelles in bovine milk. This can contribute to the formation of weak coagulum, as normally smaller diameter micelles give firmer curd than larger micelles.

It is well known that the clotting enzyme from one species is more effective and specific for milk from the same species. For example, chymosin from lamb was found to be more effective on sheep milk than on cow milk. This suggests an adaptation between the proteolytic specificities of the gastric proteases and the structure of the caseins. Accordingly, it can be expected that camel chymosin would be more effective on camel milk than calf chymosin.

In a recent study, the production of camel chymosin was reported. In the first step, the genetic information (in the form of the so-called mRNA) needed for the production of chymosin was obtained from the stomach of a young camel. This information was then inserted into a so-called expression vector, a piece of genetic sequence needed for introduction (transformation) of the chymosin information into a fungus. The fungus was now able to produce and secrete camel chymosin. The high-yielding fungal strains were selected for production of the enzyme. After centrifugation of the fungus and purification of the supernatant, the enzyme was ready for use. The process is patented. Field and laboratory studies for making cheese from camel milk were successfully carried out in different countries and the camel chymosin in now available on the market (**Figure 4**).

Effect of Heat on Milk

There are very few studies on the effect of heat treatment on camel milk proteins. Available data indicate that the whey proteins in camel milk are more heat resistant than those in cow milk. In this study, the milk was pasteurized to 63, 80, and 90 °C for 30 min, and the effect of heat on the whey proteins was determined by polyacrylamide gel electrophoresis. Under the selected experimental conditions, the rate of heat denaturation of camel milk whey proteins was approximately twofold lower than cow milk whey proteins. This indicates that camel milk can be pasteurized. In fact, there are commercial small- and middle-scale camel milk processing plants for the production of pasteurized milk in Mauritania, Kenya, and Gulf states.

Figure 4 Fresh cheese from camel milk manufactured using camel chymosin.

Reported investigations aiming to study the ability of camel milk to withstand ultra-high temperature (UHT) processing showed phase separation and the formation of deposit after storage. This heat instability of camel milk at high processing temperatures can be due to the low content of κ-casein and the total absence of β-lactoglobulin in camel milk. Both proteins play an important role in the heat stability of bovine milk.

Milk Commercialization and Hygiene

In eastern African countries (Kenya, Somalia, Sudan, and Ethiopia) where most of the world's camel populations are kept, camel milk plays a central role in food security.

In the context of advancing urbanization, camel milk, in the form of raw or fermented milk, is increasingly commercialized and consumed in urban areas. The camel milk suppliers are nomadic pastoralists. Traditionally, nomadic settlements are transient and their movements depend on where they can find adequate pasture and water for their herds. However, this picture of 'moving' nomads has changed in recent decades. With growing urbanization, the demand for milk among the city population has been increasing. On the other hand, the demand for a number of goods such as grain, oil, sugar, and clothes has increased among the pastoralists and milk sales have become the most important source of cash income for many camel-owning pastoralists. Studies on the camel milk market in some eastern African countries showed how camel milk outweighed other milk types in rural pastoral areas during the dry season.

In these studies, two types of camel-oriented dairy systems are described. One consists of wide-ranging nomadic herders who from time to time during their seasonal migratory movement pass through the 'milk catchment areas' surrounding settlements, where they sell their surplus milk. The other is more intensive camel dairying and is based on semi-stationary camel herds established near urban centres around towns in regions with adequate pasture and water. In a herd, there are approximately 10–100 lactating camels and the milk is marketed through urban milk traders who collect the milk and sell it in the urban centers. These semi-stationary herds regularly exchange camels with dry herds that utilize more remote grazing areas to ensure that limited pasture near markets is used by lactating camels.

Camel milk production areas are often located far from markets. Distances to provincial markets range from 20 to 90 km and may be up to 400 km for distant urban markets. During periods of milk surplus (rainy season), transport on dirt roads is unreliable, resulting in breakdowns and delays in milk delivery. Storage in unhygienic containers, pooling of milk from different suppliers, prolonged transport times, and high environmental temperatures all increase contamination and spoilage of milk.

This results in considerable losses in marketed camel milk caused by frequent spoilage due to the absence of appropriate conservation and storage methods. In addition, the presence of zoonotic infectious diseases in camels results in a potentially high health risk to the public.

The monitoring of hygienic quality of camel milk from pastoral production areas has serious logistical problems because of distances to the laboratory.

Field studies showed that raw milk directly milked into clean containers has a total bacterial count (TBC) of about 10^4–10^5 cfu ml^{-1}, but it deteriorates rapidly as it enters the informal marketing chain, turning sour in less than 24 h at 25 °C or in less than 12 h under hot conditions (35 °C).

Optimizing milk hygiene under pastoral conditions is difficult; however, the introduction of simple interventions showed measurable results in field studies. One of the most promising methods is the activation of the lactoperoxidase system (LPS) in milk.

The LPS is composed of lactoperoxidase, thiocyanate, and hydrogen peroxide.

Lactoperoxidase is always present in milk at an adequate concentration. The concentration of thiocyanate is more variable as it is dependent on feed and stage of lactation. The third component of the system, hydrogen peroxide, is not normally detected in milk. When active, the LPS produces hypothiocyanite ions, a strong antimicrobial agent. The system can be activated by adding a defined quantity of thiocyanate and hydrogen peroxide to milk.

In field tests performed in rural Kenya using an FAO- and WHO-approved LPS kit, TBC of untreated fresh milk was 1×10^5 cfu ml^{-1}. After LPS treatment and transport for 6 h at 30 °C, the TBC of untreated milk increased to 3×10^7 cfu ml^{-1}, while LPS-treated milk remained unchanged.

Based on these results, it was concluded that lactoperoxidase treatment can be effective in extending the shelf life of raw camel milk following the same procedure as for cow milk.

Furthermore, the introduction of the following simple milk handling techniques proved to be effective:

- replacement of cheap plastic with quality stainless steel containers for storage and transport;
- filtration of milk through disposable clean gauze/paper filters;
- rotation of sealed sanitized containers between production areas and dairy processors;
- short heating/flash boiling of raw milk at primary collection sites;
- reduction of milk temperature at collection sites using solar- or gas-powered refrigerators, by evaporative cooling from charcoal-walled cooling chambers, or simply by wrapping milk containers in a moist cloth; providing shade/cooling boxes during vehicle transport;
- training and extension to increase awareness among producers on clean milking and handling practice; accelerated transport from production to market;
- exclusion of milk from processing if the animal is infected with brucellosis, mastitis, or tuberculosis. Also, the milk must not be used if it contains colostrum or veterinary drug residues and if the animal has been vaccinated within the previous 24 h;
- before milking, washing the udder of the animal with lukewarm water and disinfectant and wiping clean-dry with a cloth used for this and no other purpose. The milker's hands must also be washed and disinfected.

Concluding Remarks

Camel husbandry is becoming increasingly important in the arid zones. The camel has long been the mainstay of pastoral societies due to its drought tolerance, reliable milk supply, and ability to feed on a wide variety of plants.

The interest in research on camel milk is gradually increasing; however, much of the research work so far is being carried out by individuals from different institutions with little impact on the development efforts.

Attention must be given to practically oriented research by developing market-oriented milk products of high quality and prolonged shelf life.

This will get the support of the private investors interested in the development of camel dairies in countries with large camel populations and satisfy the demand for camel milk among the growing urban population.

See also: **Enzymes Indigenous to Milk**: Lactoperoxidase. **Fermented Milks**: Types and Standards of Identity. **Milking and Handling of Raw Milk**: Milking Hygiene.

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Equid Milk

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Introduction

More than 200 species of ungulate (hoofed) animals exist today and are grouped into two orders Artiodactyla (even-toed ungulates) and Perissodactyla (odd-toed ungulates). There are about 190 species of Artiodactyla and 15 species of Perissodactyla; the latter includes three families, Equidae, Rhinocerotidae, and Tapiridae (**Figure 1**). Four of the equidae species, the African ass, onager, mountain zebra, and Grévy's zebra (**Figure 2**), are considered endangered, while the kiang and plains zebra are threatened in the wild. *Equus przewalskii* is often listed as a subspecies of the domestic horse or as the last surviving wild horse *Equus ferus przewalskii*. The domestic donkey (*Equus asinus*) is often classified as a subspecies of its presumed wild ancestor, the African wild ass (*Equus africanus*). The fact that the horse is spread throughout the world, has been domesticated, is relatively easily handled, and produces large amounts of milk, which can be obtained relatively easily, makes equine milk a relatively easy subject for research. Although the donkey is now less widely distributed than the horse, its milk, also, can be obtained readily. However, the zebra has not been domesticated and although it is widespread in the wild in Africa and in captivity elsewhere, apparently it is very difficult to obtain milk from zebras, even from captive animals. As far as is known, the zebra has never been used for milk production, nor have the onager and kiang.

The horse is a nonruminant herbivore and initially digests food enzymatically in the foregut and then ferments it in the very large sacculated hindgut. The equine digestive system is designed to process small amounts of food consumed frequently. Equids rarely fast for more than 2–4 h and naturally forage for 16–18 h per day. Donkeys, like horses, generally survive on a diet high in fiber and low in soluble carbohydrates and proteins. While donkeys are not as efficient as ruminants at digesting cell wall components, they are far more efficient than horses and can increase forage intake to compensate for a low-quality diet. Donkeys have a much lower requirement for water per unit weight than any other mammal, except the camel, and can rehydrate quickly by consuming a large volume of water, without complications. The zebra, on the other hand, needs a constant supply of water for survival.

Composition of Equid Milks

Young mammals are born at very different stages of maturity and their mothers' milk differs greatly in composition, although milk from species in the same taxonomic order tends to be reasonably similar. Horses produce milk that is low in protein and fat but high in lactose and has a low energy value (**Table 1**). Among species studied, only the rhinoceros produces milk with a lower level of total solids, protein, and gross energy than equid species. All equid species studied to date produce milk containing 10–11% total solids, 1–2% fat, 1.6–1.8% true protein, and 6–7% lactose, with little difference between the species throughout lactation (**Table 1**). The high metabolic needs of the foal are met through frequent feeding. The average weight gain for a foal weighing 40–50 kg at birth is about 1 kg per day and its birth weight doubles in 40–60 days, depending on the breed. Equine colostrum contains >15 g protein (primarily immunoglobulin (Ig)) per 100 g immediately postpartum, but the concentration decreases to <4 g per 100 g after 24 h and to <2 g per 100 g after 4 weeks. The casein to whey protein ratio in equine colostrum is 0.2:1 immediately postpartum and this changes to ~1.1:1 within 1 week. Equid milks are relatively rich in lactose and the concentration more than doubles during the first 24 h postpartum and subsequently increases steadily throughout lactation, a trend that is different from that observed for bovine milk, in which the lactose content decreases with advancing lactation. The lipid content of equine milk decreases with advancing lactation, whereas that of bovine milk shows a distinct minimum after ~3 months of lactation and increases thereafter.

Lactose

Although the lactose content of equid milks is high, the physicochemical properties of lactose that cause problems in the processing of bovine milk are of no consequence for equid milks, which are consumed either fresh or in fermented form. Lactose intolerance is less of a problem with fermented equine milk, koumiss, as some lactose is converted into lactic acid, ethanol, and carbon dioxide during microbial fermentation.

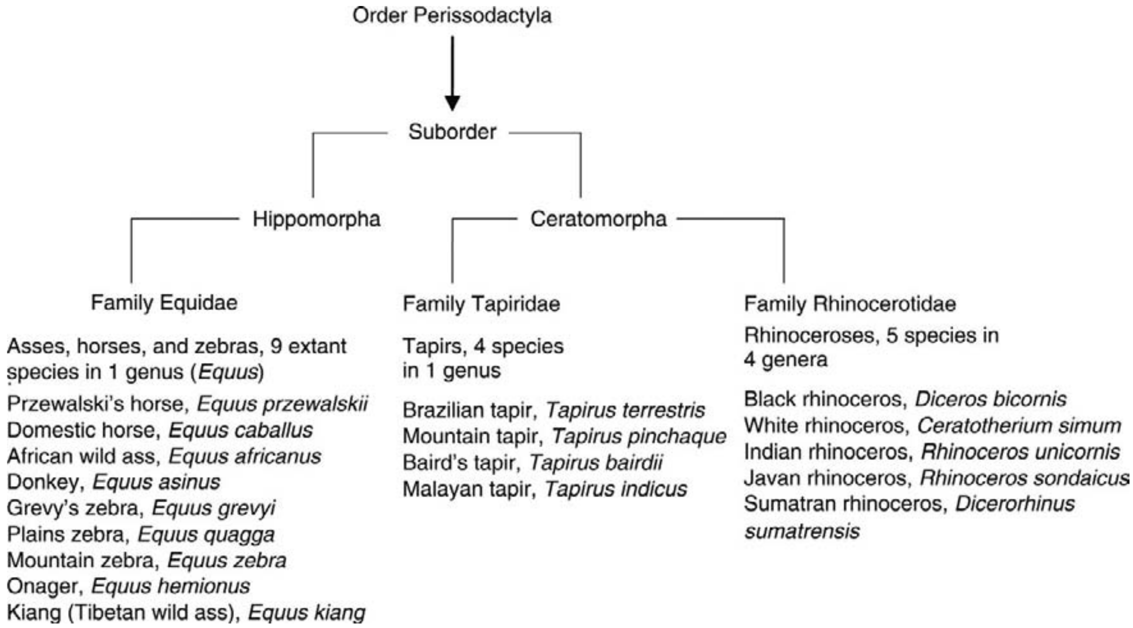
Order Perissodactyla
Suborder
Hippomorpha
Ceratomorpha
Family Equidae
Asses, horses, and zebras, 9 extant species in 1 genus (*Equus*)
Przewalski's horse, *Equus przewalskii*
Domestic horse, *Equus caballus*
African wild ass, *Equus africanus*
Donkey, *Equus asinus*
Grevy's zebra, *Equus grevyi*
Plains zebra, *Equus quagga*
Mountain zebra, *Equus zebra*
Onager, *Equus hemionus*
Kiang (Tibetan wild ass), *Equus kiang*
Family Tapiridae
Tapirs, 4 species in 1 genus
Brazilian tapir, *Tapirus terrestris*
Mountain tapir, *Tapirus pinchaque*
Baird's tapir, *Tapirus bairdii*
Malayan tapir, *Tapirus indicus*
Family Rhinocerotidae
Rhinoceroses, 5 species in 4 genera
Black rhinoceros, *Diceros bicornis*
White rhinoceros, *Ceratotherium simum*
Indian rhinoceros, *Rhinoceros unicornis*
Javan rhinoceros, *Rhinoceros sondaicus*
Sumatran rhinoceros, *Dicerorhinus sumatrensis*

Figure 1 Phylogenetically related families of the order Perissodactyla.

Oligosaccharides

The oligosaccharides identified in equine colostrum are summarized in **Table 2**. The oligosaccharides in mature equine milk have not been reported but it can be assumed that levels are considerably lower than in colostrum. The neutral oligosaccharides lacto-*N*-neotetraose and lacto-*N*-neohexaose identified in equine colostrum are also abundant in human milk, while iso-lacto-*N*-neotetraose and lacto-*N*-novopentanose 1 are not, but have been identified in bovine colostrum; lacto-*N*-novopentanose 1 has been identified also in the milk of the Tammar wallaby and brown capuchin monkey.

Proteins of Equid Milk

Like the milk of all species that have been studied, equine milk contains two principal classes of proteins: caseins and whey proteins. While caseins are the predominant group of proteins in bovine milk (~80% of total milk protein), equine milk contains much less casein and more whey proteins. The casein to whey protein ratio in equine milk is ~0.2:1 immediately postpartum but changes to ~1.1:1 during the first week of lactation. The concentrations of caseins and whey proteins in equine and asinine milk are shown in **Table 3**, with comparative data for human and bovine milk.

Whey Proteins

The principal whey proteins in equine milk are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), Igs, serum albumin, lactoferrin (LF), and lysozyme, all of which are also present in bovine milk and all, except β-Lg, in human milk; the relative amounts differ considerably between equine, bovine, and human milk. Equine and asinine milk have a high content of lysozyme compared to bovine milk (**Table 3**).

β-Lactoglobulin

β-Lg is the major whey protein in the milk of most mammals but is absent from the milk of humans, lagomorphs, rodents, and camels. Two distinct forms of equine β-Lg have been isolated, designated β-Lg I and II, which contain 162 and 163 amino acids, respectively. Asinine milk also has two forms of β-Lg, β-Lg I and II, although a recent study on Sicilian donkey milk found that >23% of the animals lacked β-Lg II. Two genetic variants of asinine β-Lg I, A and B, and three genetic variants of asinine β-Lg II, A, B, and C, have been identified. Equine β-Lg I has a molecular mass of 18 500 Da and an isoelectric pH of 4.85, whereas equine β-Lg II has a molecular mass of 18 262 Da, despite having an extra amino acid, and an isoelectric pH of 4.7. Unlike bovine β-Lg, equine β-Lg contains no free sulfhydryl group. In ruminant milk, β-Lg exists as a dimer at natural pH, whereas in equine milk, β-Lg occurs in the monomeric form.

α-Lactalbumin

α-La occurs in the milk of most mammals and is homologous with type C lysozymes. It is a calcium metalloprotein, in which the Ca^{2+} plays a crucial role in

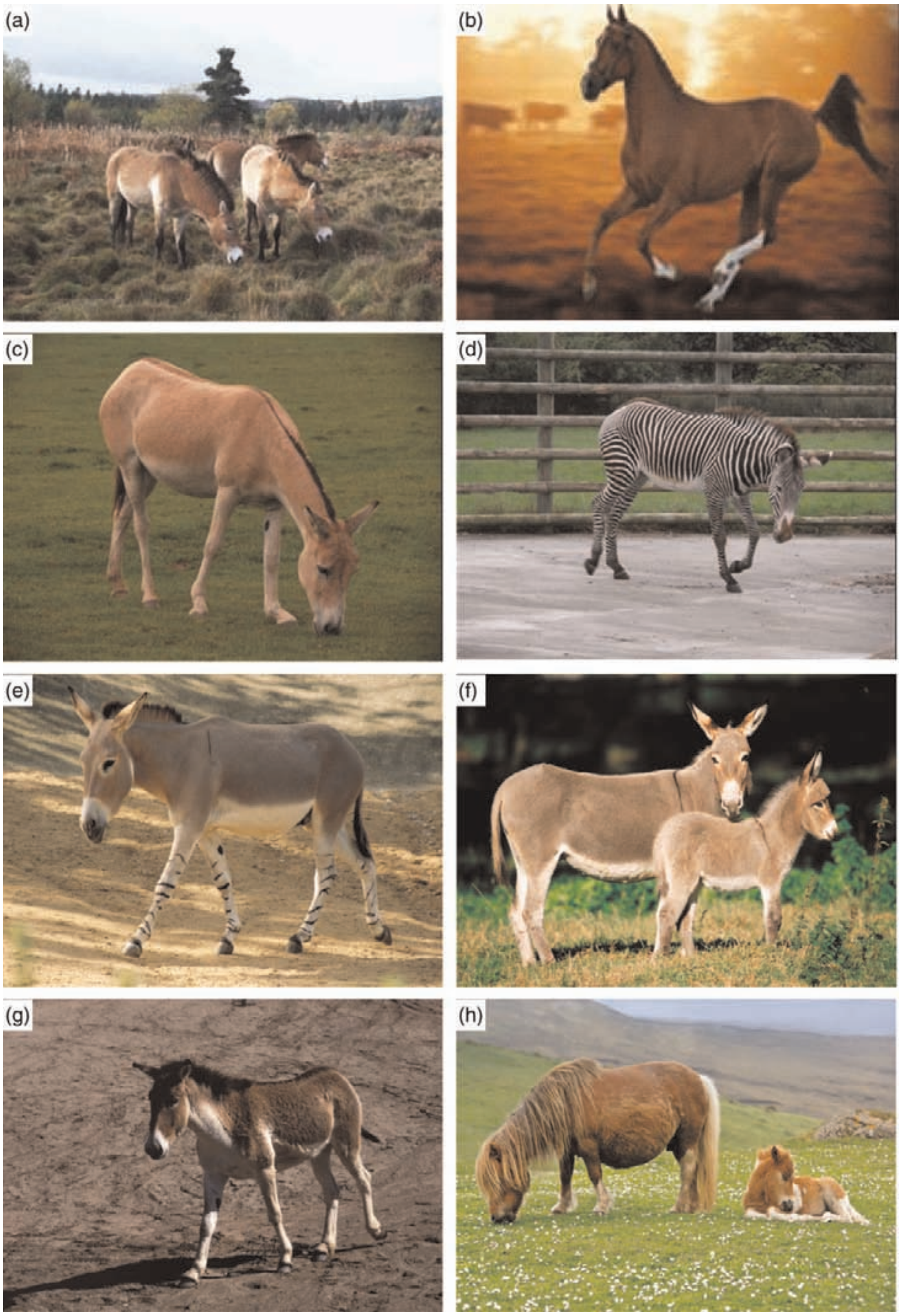

Figure 2 Equid species: (a)Przewalski's horse (*Equus przewalskii*); (b) horse (*Equus caballus*); (c) onager (*Equus hemionus*); (d) Grévsy's zebra (*Equus grevyi*); (e) Kiang or Tibetan wild ass (*Equus kiang*); (f) donkey (*Equus asinus*); (g) African wild ass (*Equus africanus*); (h) Shetland pony (*Equus caballus*). Photographs (a), (c), and (d) printed with kind permission from the North of England Zoological Society (Chester Zoo); photograph (b) supplied by Orchids Paardenmelkerij, Zeeland, the Netherlands; photograph (e), © Pat Morris/ardea.com; photograph (f), © Duncan Usher/ardea.com; photograph (g), © Brent Huffman/Ultimate Ungulates Images; photograph (h), © Steffan & Alexandra Sailer/ardea.com.

folding and structure. α-La regulates the synthesis of lactose in the lactating mammary gland. α-La of the cow, buffalo, sheep, goat, horse, donkey, camel, and human contains 123 amino acids. The primary structures of equine, bovine, and human α-La differ by only a small number of single amino acid replacements, as a result of which the proteins have similar properties. Equine α-La occurs as three genetic variants, A, B, and C. The A variant differs from the B variant at positions 7, 33, 54, 78, and 95, at which equine α-La A contains Glu, Ser,

Table 1 Gross composition (g per 100 g) of the milks of some equid species (bovine and human milk are included for comparison)

	Total solids	*Fat*	*Protein*	*Casein/whey ratio*	*Lactose*	*Ash*	
Gross energy (kJ)	Donkey[a]	9.5	1.4	1.6	~1.0	6.5	
0.4	271						
Mountain zebra	10.0	1.0	1.6		6.9	0.3	200
Plains zebra	11.3	2.2	1.6		7.0	0.4	240
Przewalski's horse	10.5	1.5	1.6	1.1	6.7	0.3	210
Pony	10.4	1.5	1.8	1.1	6.7	0.5	220
Cow	12.7	3.7	3.4	4.7	4.8	0.7	270
Human	12.4	3.8	0.9	0.3	7.0	0.2	275

[a] Values averaged from five recent publications.

Table 2 The principal oligosaccharides of equine colostrum

Oligosaccharide	*Concentration (mg l^{-1})*
Acidic	
Neu5Ac(α2-3)Gal(β1-4)Glc	***n/a***
Gal(β1-4)GlcNAcα1-diphosphate (*N*-acetyllactosamine-α1-phosphate)	n/a
Neutral	
Gal(β1-3)Gal(β1-4)Glc (β3′-galactosyllactose)	7.8
Gal(β1-6)Gal(β1-4)Glc (β6′-galactosyllactose)	4.8
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc (lacto-*N*-neotetraose)	n/a
Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glc (iso-lacto-*N*-neotetraose)	0.5
Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]Gal(β1-4)Glc (lacto-*N*-novopentanose 1)	1.1
Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc (lacto-*N*-neohexaose)	1.1
Gal(β1-4)GlcNAc-1-phosphate (*N*-acetyllactosamine-1-0-phosphate)	n/a
Neu5Ac(α2-3)Gal(β1-4)Glc (3′-*N*-acetylneuraminyllactose)	n/a

Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetylglucosamine; Neu5Ac, *N*-acetylneuraminic acid; n/a, not available.

Table 3 Distribution of caseins and whey proteins in equine and asinine milk compared to human and bovine milk

	Equine	*Asinine*	*Human*	*Bovine*
Total casein (g per100 g)	1.4	0.78	0.25	2.6
α_{s1}-Casein	0.25	Identified, no value	0.08	1.07
α_{s2}-Casein	0.02	Unknown	Unknown	0.28
β-Casein	1.1	Identified, no value	>85% of total casein	0.86
κ-Casein	0.025	Unknown	<15% of total casein	0.31
γ-Casein	High?	Unknown		0.08
Total whey protein (g per100 g)	0.83	0.58	0.64	0.63
β-Lactoglobulin	0.3	0.33		0.32
α-Lactalbumin	0.33	0.19	0.26	0.12
Serum albumin	0.037	0.038	0.05	0.04
Proteose peptone				0.08
IgG	0.039		0.004	0.072
IgA	0.048		0.1	0.013
IgM	0.003		0.01	0.004
Lactoferrin	0.08	0.037	0.17	0.01
Lysozyme	0.11	0.1	0.05	Trace
Nonprotein nitrogen (mg l^{-1})	376	455	454	266
Casein micelle diameter (nm)	255		64	182

The data are compiled from 14 references from 1984 to 2008.

Gln, Asp, and Ile residues, respectively, and equine α-La B contains Gln, Asp, Glu, Asn, and Asp residues, respectively. The B and C variants of equine α-La differ only at position 95, where the C variant contains Ile. Equine α-La A, B, and C have an isoelectric point at pH 4.95, 4.95, and 5.11, respectively. Two forms of asinine α-La have been identified with isoelectric points differing by 0.23 pH units. Bovine, equine, asinine, and human α-La contain four intramolecular disulfide bonds and lack a sulfhydryl group.

Lactoferrin

The level of LF in equine milk is similar to that in human milk and considerably higher than that in asinine and bovine milk (**Table 3**). Equine LF contains 689 amino acid residues, similar to bovine LF, whereas human LF contains 692 residues. Equine and human LF contain 17 and 16 intramolecular disulfide bonds, respectively. Compared to most other milk proteins, LF has a high isoelectric point, that is, at pH 8.32, 8.67, and 8.47 for equine, bovine, and human LF, respectively. Consequently, LF is positively charged at the natural pH of milk and may associate with negatively charged proteins via electrostatic interactions. The iron-binding capacity of equine, bovine, and human LF is equivalent, although the pH dependence of the iron-binding capacity of bovine LF differs from that of the equine and human proteins.

Immunoglobulins

Igs form part of the humoral-mediated aspect of the immune system. In some species, for example, man, maternal IgG is responsible for neonatal passive systemic immunity and is transferred to the fetus from maternal circulation *in utero*; the intestines of a full-term baby are relatively impermeable to maternal proteins. In ruminants, pig, and the horse, IgGs are not transferred *in utero* but are contained in colostrum and transferred through the intestine, which remains permeable to proteins for 2–3 days after birth; the intestine then ‘closes’ and further significant passage of proteins is prevented. Three classes of Igs are commonly found in milk, IgG, IgA, and IgM, the relative proportions of which differ between species (**Table 3**).

Denaturation of Whey Proteins

Whey proteins are thermolabile. Equine LF and serum albumin are the most heat sensitive of the equine whey proteins that have been studied (no reports are available on the thermal sensitivity of equine Igs) and have similar thermal sensitivities to their bovine counterparts; in both species, a temperature of up to 130 °C for 1 min is required for complete denaturation of the whey proteins. Equine β-Lg and α-La are much more thermostable than their bovine counterparts and do not undergo significant denaturation at temperatures <100 °C. The high thermal stability of equine β-Lg may be related to its lack of a sulfhydryl group. Equine lysozyme is very heat stable and retains 68% of its antimicrobial activity after 15 min at 82 °C and undergoes significant denaturation only at >90 °C.

Caseins

The caseins are the principal protein class in the milk of ruminants and that of most other mammals. The caseins of most species are four gene products: α_{s1}-, α_{s2}-, β-, and κ-caseins. Of these, α_{s1}-, α_{s2}-, and β-caseins are calcium-sensitive proteins. All the caseins exhibit microheterogeneity caused by genetic polymorphism and posttranslational modifications, for example, phosphorylation, glycosylation, and proteolytic cleavage. Equine β-casein and α-La undergo spontaneous deamidation, which has been reported also for canine milk lysozyme and human LF. Caseins represent ~55% of the total protein in mature equine milk, the principal proteins being α- and β-caseins (~50:50), both of which are very heterogeneous due to variations in the degree of phosphorylation; however, equine milk contains little or no κ-casein. As in the milk of other species, the caseins in equine milk occur as large colloidal aggregates, called micelles, the properties of which have been studied only superficially.

α_{s1}-Casein

Equine α_{s1}-casein contains 205 amino acids and has a molecular mass of 24 614.4 Da prior to posttranslational modification: that is, it is slightly larger than its bovine counterpart. Equine α_{s1}-casein contains six potential phosphorylation sites, five of which are in very close proximity and can form a phosphorylation center, which is important in the structure of casein micelles. Bovine α_{s1}-casein contains eight or nine phosphorylation sites in two groups. Like bovine α_{s1}-casein, equine α_{s1}-casein has three hydrophobic regions, including the N-terminal region, and therefore, it has properties similar to those of bovine α_{s1}-casein.

α_{s2}-Casein

Only the 12–15 amino acids of the N-terminal region of equine α_{s2}-casein have been identified and the sequences reported thus far differ between different studies. Isoelectric focusing of equine α_{s2}-casein shows two major bands, with isoelectric points in the pH range 4.3–5.1.

β-Casein

The amino acid sequence of equine β-casein, deduced from its cDNA, comprises 226 amino acids with a molecular mass of 25 496.3 Da. Bovine and human β-casein contain 209 and 211 amino acid residues, respectively. Two smaller genetic variants of equine β-casein, which probably result from exon skipping during transcription, have been reported. In equine β-casein, the first 28 C-terminal amino acids contain 7 potential phosphorylation sites (Ser$_9$, Ser$_{15}$, Ser$_{18}$, Ser$_{23}$, Ser$_{24}$, Ser$_{25}$, Ser$_{28}$), 3–7 of which are phosphorylated, giving a family of proteins with pI values varying from 4.74 to 5.30. Extensive deamidation of equine β-casein occurs at Asn$_{135}$; deamidation of bovine or human β-casein has not been observed. γ-Casein and proteose peptone 5 have been identified in equine milk. Bovine plasmin readily hydrolyzes the Lys$_{55}$-Ile$_{56}$ bond of equine β-casein, but the corresponding Lys$_{48}$-Ile$_{49}$ bond in bovine β-casein has not been reported to be hydrolyzed by plasmin. Equine β-casein is readily hydrolyzed by chymosin at Leu$_{198}$-Tyr$_{199}$.

κ-Casein

The presence of κ-casein in equine milk has been an issue of debate for several years; several authors have reported its absence but recent studies indicate its presence, albeit at a very low level. The primary structure of equine κ-casein, deduced from its cDNA sequence, contains 165 amino acid residues, that is, 4 less than bovine κ-casein but 3 more than human κ-casein. The molecular mass of equine κ-casein prior to posttranslational modification is 18 844.7 Da. Equine and human κ-casein have a considerably higher pI, 8.03 and 8.68, respectively, than bovine κ-casein at 5.93, indicating that the former have a net positive charge at physiological pH, whereas bovine κ-casein has a net negative charge. Equine κ-casein appears to be less hydrophilic than its bovine counterpart, which has a hydrophilic C-terminus, which is important for the stability of bovine casein micelles. No direct information is available on the glycosylation of equine κ-casein, but lectin-binding studies suggest that equine κ-casein is glycosylated at Thr$_{123}$, Thr$_{127}$, Thr$_{131}$, Thr$_{149}$, and Thr$_{153}$.

Casein Micelles

Like the milk of (probably) all species, the caseins in equine milk occur as large aggregates, called micelles. Equine casein micelles are larger than bovine or human casein micelles (**Table 3**). Electron microscopic studies have shown that bovine and equine micelles have a similar 'spongy' appearance, while human micelles seem to have a much 'looser', more open structure. There are no specific reports on the substructure of the equine casein micelle although equine milk does contain ~10.1 mmol l^{-1} micellar calcium and ~2.6 mmol l^{-1} micellar inorganic phosphorus, suggesting a micellar calcium:casein ratio of >20:1, which, on a molar basis, far exceeds the calcium-binding capacity of equine casein molecules. Hence, it may be assumed that equine micelles, like bovine casein micelles, contain nanoclusters of calcium phosphate. Since equine milk contains little or no κ-casein, dephosphorylated β-casein may play a role in micellar stability.

Casein Micelle Stability

Equine casein micelles at natural pH are not susceptible to rennet-induced gelation although it has been reported that isolated equine κ-casein is hydrolyzed slowly by calf chymosin at the Phe$_{97}$-Ile$_{98}$ bond. On renneting, equine milk forms fine flocs after several hours, but it does not form a gel. Preliminary research suggests that asinine milk is coagulable by chymosin, forming a very weak gel (G' of 10–15 Pa, after 60 min at 30 °C, compared to 180–200 Pa for bovine milk coagulum under similar conditions). No cheese is produced from equine or asinine milk. Acidifying equine milk to pH 4.2 (the point of minimum solubility of equine caseins) causes very limited flocculation and no gelation and as a consequence, no yogurt-type products are produced from equine milk.

At 140 °C, the heat coagulation time (HCT) of equine milk increases with pH, giving a sigmoidal pH–HCT profile with a midpoint around pH 6.7; the HCT is <2 min at pH 6.3–6.9, but is >60 min at pH 7–7.3. The low heat stability of equine milk at pH <6.8 is probably related to its very high level of ionic calcium, ~2.6–3.0 mmol l^{-1}, compared to bovine milk, ~1.8 mmol l^{-1}, and its very low level of κ-casein.

The ethanol stability of bovine milk, defined as the minimum concentration of added aqueous ethanol that causes it to coagulate, at its natural pH (~6.7), is 70–75% (added 1:1 to milk), whereas the ethanol stability of equine milk (pH ~7.2) is 40–45%. The high concentration of ionic calcium and low level of κ-casein in equine milk probably contribute to its low ethanol stability.

Nonprotein Nitrogen

Nonprotein nitrogen (NPN) constitutes 10–15% of the total nitrogen in mature equine milk, which is intermediate between values for human (~25%) and ruminant (~3–5%) milk (**Table 3**). The NPN is composed primarily of urea, creatine, creatinine, small peptides, amino acids, and ammonia.

Indigenous Enzymes

Milk contains many indigenous enzymes, which originate from the milk fat globule membrane (MFGM), mammary cell cytoplasm, or blood. The indigenous enzymes in bovine and human milk have been studied extensively, but the enzymes in the milk of other species have been studied only sporadically. Equine milk probably contains all the enzymes that have been identified in bovine milk, but relatively few studies have been reported. Lactoperoxidase (LPO), catalase, amylase, proteinase (plasmin), lipase, lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) have been reported in equine milk. Bovine milk is a rich source of xanthine oxidoreductase (XOR), but the milks of other species for which data are available have much lower XOR activity, because in nonbovine species, most (up to 98%) of the enzyme molecules lack Mo and are inactive. XOR activity has not been reported in equine milk, but considering the role of XOR in the excretion of fat globules from the secretory cells, it is highly likely that the MFGM in equine milk contains XOR. Equine milk contains quite a high level of Mo, which presumably is present exclusively in XOR.

Lysozyme (EC 3.1.2.17) occurs at high levels in equine, asinine, and human milk (**Table 3**). Human and equine milk contain 3000 and 6000 times more lysozyme, respectively, than bovine milk; lysozyme represents 4 and 3%, respectively, of the total protein in human and equine milk. Equine milk lysozyme contains 129 amino acid residues and, like the lysozyme of other species, is highly homologous with α-La. Unlike α-La, most lysozymes cannot bind Ca^{2+}, but equine lysozyme is an exception in this regard and binds Ca^{2+} tightly, making it particularly stable. Equine milk has very good antibacterial activity, presumably due to its high level of lysozyme.

Lipids

The milk of all equidae contains a low level of lipids, and lactose, rather than lipids, is the principal source of energy. Human and bovine milk, which are relatively rich in fat, provide about 53 and 51%, respectively, of energy as fat, compared to ~25% for equine milk. The fat content of equine milk decreases during lactation to about 50% of its initial value. Triglycerides (TGs) represent only 80–85% of the lipids in equine milk compared with ~98% in most other milks; ~9.4% are free fatty acids (FFAs) and phospholipids account for ~5% (**Table 4**).

The levels of monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids in the milk fat of some species are shown in **Table 5**. The milk fat of nonruminants contains substantially higher levels of PUFAs than ruminant milk, and equine milk has a considerably higher level of PUFAs than asinine milk. The higher content of conjugated linoleic acid (CLA) in ruminant milk fat compared to that of nonruminants is inversely correlated with the level of PUFAs and weakly correlated with the content of MUFAs.

Table 4 Lipid classes in human, bovine, and equine milk

	Equine	*Bovine*	*Human*
Total lipids (g per 100 g)	1.21	3.61	3.64
Constituents (percentage of total lipids)			
Triglycerides	81.1	97	98
Phospholipids	5.0	1.5	1.3
Unsaponifiable matter (sterols)	4.5	1.5	0.7
Free fatty acids	9.4	Trace	Trace

Table 5 Monounsaturated and polyunsaturated fatty acids (percentage of total fatty acids) in the milk fat of some ruminants and nonruminants

	MUFAs	*PUFAs*	*CLA*
Nonruminants			
Equine	31.20	22.70	0.09
Asinine	15.30	16.00	
Porcine	51.80	12.40	0.23
Human	39.40	15.27	0.39
Ruminants			
Caprine	26.90	2.58	0.65
Bovine	24.65	5.82	1.01
Ovine	23.00	3.85	1.08

CLA, conjugated linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

The fatty acid profile of equid milk differs from that of bovine and human milk fat in a number of respects (**Table 6**). It contains virtually no butyric acid (C_4), which is present at high levels in bovine and other ruminant milk fats. All equid milks contain high proportions of middle-chain fatty acids (20–35% of all fatty acids contain <16C) but are poor in both short- and long-chain saturated fatty acids. Equid milk fats contain a very high level of α-linolenic acid (ALA), which reflects the high level of PUFAs in the diet and the lack of biohydrogenation, as occurs with ruminants.

Equine and asinine milk have similar fatty acid profiles, although equine milk has a significantly higher content of MUFAs (**Tables 5** and **6**). The ratio of $n-6{:}n-3$ (1.16:1) in asinine milk compared to 3.14:1 in equine milk makes it an interesting product for human nutrition. Small amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) occur in asinine milk, whereas equine milk has only trace amounts. EPA and DHA are important in infant nutrition but their absence from equine milk is not considered to be a problem as an infant's liver can desaturate linoleic acid (LA) and ALA to form EPA and DHA. Furthermore, the ratio of LA to ALA in equine milk is regarded as optimal for absorption by the human neonate, who can quickly convert them into EPA and DHA.

Table 6 Average fatty acid composition in the milks of some species (percentage of total fatty acids)

Fatty acid	*Common name*	*Asinine*	*Equine*	*Zebra*	*Bovine*	*Human*
Saturates						
$C_{4:0}$	Butyric	0.60	0.09		3.90	0.19
$C_{6:0}$	Caproic	1.22	0.24		2.50	0.15
$C_{8:0}$	Caprylic	12.80	3.15	8.20	1.50	0.46
$C_{10:0}$	Capric	18.65	6.48	15.30	3.20	1.03
$C_{12:0}$	Lauric	10.67	6.65	9.20	3.60	4.40
$C_{13i:0}$		0.22				
$C_{13:0}$		3.92	0.17		0.19	0.06
$C_{14i:0}$		0.12				0.04
$C_{14:0}$	Myristic	5.77	7.04	6.50	11.10	6.27
$C_{15a:0}$						0.21
$C_{15\ i:0}$		0.07	0.16			
$C_{15:0}$	Pentadecanoic	0.32	0.39	0.50	1.20	0.43
$C_{16i:0}$		0.12				0.17
$C_{16:0}$	Palmitic	11.47	20.43	13.30	27.90	22.00
$C_{17i:0}$		0.20	0.31			0.23
$C_{17:0}$	Margaric	0.22	0.38		0.60	0.58
$C_{18i:0}$						0.11
$C_{18:0}$	Stearic	1.12	1.18	2.00	12.20	8.06
$C_{20:0}$		0.12			0.35	0.44
$C_{21:0}$					0.04	0.13
$C_{22:0}$		0.05			0.20	0.12
$C_{24:0}$					0.14	0.25
Total		67.66	46.67	55.00	68.62	45.33
Monounsaturates						
$C_{10:1}$		2.20	1.46		0.15	
$C_{12:1}$		0.25	0.20		0.06	
$C_{14:1c\ n-5}$	Myristoleic	0.22	0.52		0.80	0.41
$C_{14:1t\ n-5}$						0.07
$C_{15:1}$	Pentadecanoic		0.22		0.30	0.11
$C_{16:1c\ n-7}$	Palmitoleic	2.37	5.68	4.20	1.50	3.29
$C_{16:1c\ n-9}$			0.56			
$C_{16:1t\ n-7}$						0.36
$C_{17:1}$	Heptadecanoic	0.27	0.62		0.36	0.37
$C_{18:1c\ n-9}$	Oleic	9.65	20.26	20.40	17.20	31.30
$C_{18:1c\ n-11}$			1.31			
$C_{18:1t\ n-9}$						2.67
$C_{18:1t\ n-11}$	Vaccenic				3.90	
$C_{20:1\ n-9}$			0.40		0.32	0.67
$C_{20:1\ n-11}$		0.35				
$C_{22:1\ n-9}$					0.06	0.08
$C_{24:1\ n-9}$						0.12
Total		15.31	31.23	24.65	24.65	39.45
Polyunsaturates						
n−6 series						
$C_{18:2cc}$	Linoleic	8.15	16.04	10.10	1.40	10.85
$C_{18:2\ \mathrm{conj.}}$	Conjugated linoleic		0.07		1.10	
$C_{18:2tt}$						0.46
$C_{18:2ct}$						0.69
$C_{18:3}$	γ-Linolenic	0.15	0.61		1.00	0.25
$C_{20:2}$		0.35	0.37		0.07	0.27
$C_{20:3}$	Dihomo-γ-linolenic		0.10		0.10	0.32
$C_{20:4}$	Arachidonic		0.11		0.14	0.46
$C_{22:2}$					0.04	0.11
$C_{22:4}$					0.03	0.09
$C_{22:5}$					0.04	0.09
n−3 series						
$C_{18:3}$	α-Linolenic	6.32	5.31	8.00	1.80	1.03
$C_{18:4}$		0.22				
$C_{20:3}$		0.12				
$C_{20:4}$		0.07				0.09

(*Continued*)

Table 6 Continued

Fatty acid	*Common name*	*Asinine*	*Equine*	*Zebra*	*Bovine*	*Human*
$C_{20:5}$	Eicosapentaenoic	0.27	0.02		0.09	0.12
$C_{22:5}$		0.07	0.10			0.19
$C_{22:6}$	Docosahexaenoic	0.30	0.04		0.01	0.25
Total PUFAs		16.02	22.77	18.00	5.82	15.27
Ratio $n-6$:$n-3$		1.17	3.14	1.26	2.06	8.09
Ratio $C_{18:2}$:$C_{18:3}$		1.28	2.72	1.26	1.55	9.37

c, cis; *t*, trans; *i*, iso; *a*, anteiso; PUFA, polyunsaturated fatty acid.

Structure of Triglycerides

The distribution of fatty acids in animal TGs is nonrandom, apparently so that the TGs will be liquid at body temperature. Interspecies comparison of the positional distribution of fatty acids has been made, using fatty acid and stereospecific analysis, for 11 species, the echidna, koala, Tammar wallaby, guinea pig, dog, cat, Weddell seal, horse, pig, cow, and human. Generally, the positional distribution of fatty acids is similar, except for the echidna, with short-chain fatty acids preferentially esterified at *sn*-3, saturated fatty acids at *sn*-1, and unsaturated fatty acids generally found at *sn*-2. In equine milk fat, $C_{10:0}$ occurs at the *sn*-3 position, whereas in bovine milk fat, more $C_{10:0}$ is found at the *sn*-2 than at the *sn*-3 position. In human and equine milk, $C_{16:0}$ is located preferentially at the *sn*-2 position, which is regarded as favorable for assimilation by infants and children, whereas in bovine milk, $C_{16:0}$ is equally distributed between the *sn*-1 and *sn*-2 positions. In the milk fat of the Weddell seal and horse, $C_{18:1}$ is esterified preferentially at *sn*-1, but for all other species studied it occurs mainly at *sn*-3.

Fat Globules

The fat in milk is emulsified as globules, 2–3, 3–5, and 4 μm in diameter, in equine, bovine, and human milk, respectively. The fat globules are surrounded and stabilized by a very complex emulsifying layer, consisting of phospholipids and proteins, called the MFGM. Many of the indigenous enzymes in milk are concentrated in the MFGM. The glycoproteins in the MFGM of human, rhesus monkey, chimpanzee, dog, sheep, goat, cow, gray seal, camel, horse, and alpaca have been studied; large intra- and interspecies differences have been found. Very highly glycosylated proteins occur in the MFGM of primates, horse, donkey, camel, and dog. Long (0.5–1 mm) filamentous structures, comprised of mucins (highly glycosylated proteins), extend from the surface of the fat globules in equine and human milk. These filaments dissociate from the surface into the milk serum on cooling and are lost on heating. For unknown reasons, the filaments on bovine milk fat globules are lost much more easily than those in equine and human milk. The filaments facilitate the adherence of fat globules to the intestinal epithelium and probably improve the digestion of fat. The mucins prevent bacterial adhesion and may protect mammary tissue against tumors (mammary tumors are very rare in the cow).

The temperature-dependent melting characteristics of bovine milk fat have been studied thoroughly, but since equid milk is not used for the production of butter, the spreadability, rheology, and melting characteristics of these fats have not been studied in detail. Considering the rather unusual fatty acid profile of equid milk fat, it should have interesting melting and rheological properties. One study has shown that equine milk fat has a lower melting point than bovine milk fat (20–22 vs. 26–34 °C), a higher iodine number (92–101 vs. 25–40), and a lower saponification value (209 vs. 232).

No studies on the chemical spoilage of equid milk fat have been reported. Considering the high content of PUFAs in these fats, they are probably quite susceptible to oxidation. Since equine milk contains lipase, hydrolytic rancidity would be expected under certain conditions.

Vitamins

Vitamin levels in the milks of some species are shown in **Table 7**. Equine milk contains a significantly higher level of vitamin C than other milks shown, except for ovine milk. The levels of vitamins A, D_3, K, and C are affected by stage of lactation and are significantly higher in equine colostrum compared to equine milk, whereas vitamin E concentration remains unchanged throughout lactation. Fat-soluble vitamin concentrations are generally similar in equine and bovine milk (**Table 7**).

Minerals

The ash content of equine milk is higher than that of human milk but only about half that of bovine milk (**Table 1**). As in the milk of other species studied to

Table 7 Vitamin levels (mg l^{-1}) in the milk of some species

Vitamin	*Buffalo*	*Goat*	*Sheep*	*Donkey*	*Cow*	*Horse*	*Human*
Water-soluble							
Thiamine, B_1	0.5	0.49	0.48	0.41	0.37	0.3	0.15
Riboflavin, B_2	1.0	1.5	2.3	0.64	1.8	0.3	0.38
Niacin, B_3	0.8	3.2	4.5	0.74	0.9	1.4	1.7
Pantothenic acid, B_5	3.7	3.1	3.5		3.5	3	2.7
Pyridoxine, B_6	0.25	0.27	0.27		0.64	0.3	0.14
Biotin, B_7	0.11	0.039	0.09		0.035		0.006
Folic acid, B_9					0.18		0.16
Cobalamin, B_{12}	3.0	0.7	5.1	1.1	0.004	0.003	0.5
Ascorbic acid, C		9.0	220		21	150	43
Fat-soluble							
Vitamin A and β-carotene		0.5	0.5		0.32–0.50	0.12	2.0
Cholecalciferol, D_3					0.003	0.003	0.001
α-Tocopherol, E					0.98–1.28	1.128	6.6
Phylloquinone, K					0.011	0.020	0.002

Table 8 Concentrations (mmol l^{-1}) of minerals in the milk of some species

Species	*Calcium*	*Magnesium*	*Sodium*	*Potassium*	*Phosphorus (inorganic)*	*Citrate*	*Chloride*
Horse	16.5	1.6	5.7	11.9	6.7	3.1	6.6
Donkey	16.9	1.5	9.5	13.0	15.7		9.5
Cow	29.4	5.1	24.2	34.7	20.9	9.2	30.2
Human	7.8	1.5	5.0	16.5	2.5	2.8	6.2
Goat	23.1	5.0	20.5	46.6	15.6	5.4	34.2
Sheep	56.8	9.0	20.5	31.7	39.7	4.9	17.0
Pig	104.1	9.6	14.4	31.4	51.2	8.9	28.7
Rat	80.4	8.8	38.3	43.6	93.3	0.1	36.1
Rabbit	214.4	19.5	83.7	89.5	54.2	17.4	80.0

The data are compiled from various sources.

date, the principal salts in equine milk are phosphates, chlorides, carbonates, and citrates of potassium, sodium, calcium, and magnesium. However, there are considerable quantitative interspecies differences (**Table 8**). Compared to human milk, calcium and phosphorus levels in equine and especially asinine milk are high. For human infant nutrition, a Ca:P ratio of ~2:1 is considered optimal; in bovine milk, the ratio is ~1:1, but in equine milk it is about 2:1, and very close to that of human milk. The concentrations of all macroelements, except potassium, are higher in equine milk than in human milk, but all are considerably lower than the concentrations in bovine, caprine, ovine, or porcine milk.

The concentration of macroelements in equid milks is strongly influenced by the stage of lactation; the concentrations of calcium, magnesium, phosphorus, sodium, and potassium decrease progressively as lactation advances. A comparison of the concentrations of trace elements in equine, bovine, and human milk is shown in **Table 9**. Compared to bovine milk, equine milk contains noticeably higher levels of aluminum, copper, iron, and titanium but lower levels of boron, barium, lithium, molybdenum, manganese, silicon, and zinc.

Table 9 Concentrations (mg l^{-1}) of trace elements in equine, bovine, and human milk

	Species		
Element	*Horse*	*Cow*	*Human*
Aluminum	0.123	0.098	0.125
Boron	0.097	0.333	0.273
Barium	0.076	0.188	0.149
Copper	0.155	0.052	0.314
Iron	0.224	0.194	0.26
Lithium	0.015	0.024	0.007
Molybdenum	0.016	0.022	0.017
Manganese	0.014	0.021	0.007
Silicon	0.161	0.434	0.472
Strontium	0.442	0.417	0.06
Titanium	0.145	0.111	0.025
Zinc	1.835	3.96	2.15

Physical Properties of Equid Milk

The physical properties of the milk of some species are compared in **Table 10**. The lower freezing point of equine milk compared to bovine milk is related to its

Table 10 Physical properties of the milk of some species

Property	*Equine milk*	*Equine colostrum*	*Bovine milk*	*Human milk*	*Asinine milk*
Freezing point (°C)	−0.554 to −0.548		−0.512 to −0.550		
pH (25 °C)	7.1–7.3		6.5–6.7	6.8	7.0–7.2
Density (kg m^{-3}) (20 °C)	1032	1080	1027–1033	1031	1029
Refractive index, $n_D{}^{20}$	1.3394	1.340–1.354	1.344–1.349		
Viscosity (mPa s)	1.5031		1.6314		
Zeta potential (mV)	−10.3		−20.0		
Color, L^*, a^*, b^*	86.52,−2.34, −0.15		79.12,−7.46, 2.31		80.88,−2.27, −3.53

higher lactose content. The density of equine colostrum shows a significant linear correlation with its IgG content, and is higher than that of equine milk. The higher refractive index of equine colostrum compared to that of equine milk is probably related to the higher total solids content of colostrum, since refractive index increases with the increasing mass fraction of each solute. Differences in the pH of equid and bovine milk are presumably related to differences in protein and salt composition. Equid milks are less viscous than bovine milk due to a lower total solids content. Bovine casein micelles have a net negative charge of approximately −20 mV, which contributes to their stability and is dependent on pH and temperature. Equine casein micelles have a lower average zeta potential, approximately −10.3 mV, probably due to their larger micellar size, compared to bovine micelles, and a very low, if any, surface coverage of κ-casein.

Equid milk should be less white than bovine milk due to its low protein content and large casein micelles but this is not the case and equid milk appears considerably whiter; this is due to the presence of β-carotene in bovine milk fat, which confers a yellow color to the milk.

Equid Milks in Human Nutrition

Currently, 84% of recorded milk production is bovine, 12.3% is buffalo, 2% is caprine, 1.3% is ovine, and ~0.2% is from other species, that is, horses, donkeys, camels, yaks, and reindeer. Equine milk for human consumption is an ancient practice and can be traced back to ~2000 BC in early Chinese records, which describe the preparation of koumiss, fermented equine milk. Equine milk is regarded as having nutritional and therapeutic properties that are beneficial to the elderly, convalescents, and infants. Similar to human milk, it is low in protein, has a low casein to whey protein ratio, high concentrations of lactose and PUFAs, and low levels of cholesterol. In patients with compromised digestive systems, equine milk is often recommended due to its ease of digestion; in the stomach, high-casein milks, such as bovine milk, form firm clots that are digested relatively slowly (3–5 h), whereas the coagulum formed from human or equine casein is soft and flocculant, with an evacuation time of ~2 h. Equine milk and products therefrom (e.g., koumiss) have, traditionally, been used widely in Russia (for the treatment of tuberculosis) and eastern and central Asia. In Mongolia, koumiss (Airag) is the national drink and distilled or double-distilled versions are also produced. Per caput consumption of koumiss in Mongolia is about 50 l per annum.

Koumiss belongs to the yeast–lactic fermentation group of beverages, where alcoholic fermentation using yeasts is used in combination with lactic acid fermentation and is the principal product from equine milk. Koumiss is thought to be more effective than raw equine milk in disease treatment due to the additional peptides and bactericidal substances produced by microbial metabolism.

A decade ago, equine milk was produced only in isolated small holdings in parts of eastern Europe and Mongolia, but now there are large-scale operations in France, Belgium, Germany, Austria, and the Netherlands. Estimates suggest that more than 30 million people worldwide drink equine milk regularly, with that figure increasing significantly in recent years. Currently, there is considerable interest in the use of equine milk in human nutrition in western Europe where supply now falls short of demand.

Asinine milk is consumed mainly in countries where donkeys are bred traditionally (Asia, Africa, and eastern Europe), but consumption in western European countries is increasing due, like equine milk, to the similarity of its composition to human milk. Both milks are considered good substitutes for bovine milk for children with severe IgE-mediated cows' milk allergy (CMA). The high lactose content of these equid milks improves palatability and the absorption of calcium, which is essential for bone mineralization in children.

In health food shops and some pharmacies in western Europe, equine milk is sold frozen or in the form of capsules of lyophilized milk. Powdered equine milk, which may be fortified with vitamins, is also widely available in pharmacies in Germany, France, and Italy, where it is consumed directly or after rehydration. Producers of equine milk in Belgium and the Netherlands sell equine milk fresh or as a koumiss-type product. Supporting literature for many of the products claim relief from metabolic and intestinal problems, help in gut cleansing and 'repair' of the intestinal flora, prevent stomach ulcers, high blood pressure, high cholesterol, and liver problems, and serve as a complementary aid in the treatment of cancer patients. For healthy individuals, the products are recommended as a preventative for the ailments listed. The recommended dose is ∼250 ml day^{-1}. No cheese products are produced from equine milk because, as discussed earlier, it is not coagulable by the methods traditionally used for bovine milk. The production of cosmetics incorporating equine milk is a recent innovation, and soaps, creams, shampoos, and moisturizers are now available commercially.

See also: **Animals that Produce Dairy Foods:** Donkey; Horse. **Enzymes Indigenous to Milk:** Lactoperoxidases; Lipases and Esterases; Other Enzymes; Plasmin System in Milk; Xanthine Oxidoreductase. **Feed Ingredients:** Feed Supplements: Vitamins. **Fermented Milks:** Koumiss. **Lactose and Oligosaccharides:** Indigenous Oligosaccharides in Milk. **Mammary Gland, Milk Biosynthesis and Secretion:** Lactose. **Milk:** Introduction. **Milk Lipids:** Fatty Acids; General Characteristics. **Milk Proteins:** Casein Nomenclature, Structure, and Association; Caseins, Micellar Structure; Immunoglobulins, α-Lactalbumin; Lactoferrin; β-Lactoglobulin. **Milk Salts:** Distribution and Analysis; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance.

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Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.)

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Introduction

Information on the milk of domesticated species is not readily available, since the milk of some of these mammals is produced for human consumption only in certain regions of the world and only limited studies have been conducted. However, their milk is important for human consumption, or nutritional and health research.

Reports on the composition of milk of wild and domesticated minor species vary in accuracy due to the lack of sample numbers, difficulties in defining stage of lactation, bias introduced during sampling, and different analytical procedures. The objective of this article is to discuss the milk of sow, yak, reindeer, musk ox, llama, alpaca, and other minor dairy species.

General Composition of Milk of Minor Domesticated or Wild Mammals

The chemical composition of the milk of different species is designed by natural selection to provide the nutritional needs of the neonate of the specific species. There are considerable differences in the basic composition of milk among different domesticated and wild mammals (**Table 1**). Frequently, little is known about the stage of lactation and milk sampling time from the gland, which can result in misleading interspecies differences. Even under standard conditions of milk sampling, there are substantial short-term (diurnal and day-to-day) variations in composition, which are due to environmental conditions, feeding, management, season, locality, disease, and yield per day, as is also the case for the major domestic milk producers – the cow, buffalo, camel, goat, and sheep.

Colostrum contains much higher levels of total solids, protein, and ash than mature milk obtained 2 or 3 weeks after parturition in all species. The high protein level in colostrum is due to the globulins, which contain the antibodies. Since the antibody titer of blood of the newborn is low, mammals such as cows, sheep, goats, horses, and pigs acquire passive immunity from colostrum and its immunoglobulins.

The amount of dietary intake of roughage is important for the level of milk fat in ruminants. A decrease in roughage intake depresses the milk fat content and causes changes in rumen fermentation and parakeratosis. The daily ruminant diet should contain at least 17% crude fiber in order to prevent the decrease in the molar percentage of acetic acid and the increase in propionic acid in the rumen, which results in a low fat content in the milk.

Production and Composition of Milk from Minor Species

Sow Milk

Little has been documented on the production and utilization of sow milk, while a large amount of data have been reported on the composition of sow milk. Since humans and pigs have similar digestive and physiological systems, research data on the production and composition of sow milk are useful and applicable for the nutritional and medical well-being of humans.

The yield of sow milk is affected by the stage of lactation, suckling frequency, sow body weight, metabolic state, litter size, ambient temperature, and amount of mammary tissue. The daily yield of sow milk may range from 400 to 2000 ml. Peak milk yield is at about 3 weeks post-partum and then declines. Increased suckling frequency plays a role in increased mammary gland mass and milk production.

The composition and quantity of sow milk depend on the body condition of the sow at farrowing and early lactation. The efficiency of utilizing dietary nutrients depends on litter size, suggesting that sows nursing large litters utilize dietary nutrients more efficiently than sows nursing small litters. Since ambient temperature influences the proportion of blood flow into skin capillaries for dissipation of body heat, it also affects the composition and yield of milk during nursing.

The fat and protein levels of sow milk vary in a curvilinear manner during lactation. Sow milk fat is highest at the onset of lactation and lowest at around day 20 (**Figure 1**). The level of proteins in sow milk follows a similar pattern, but does not vary as much as fat content. The fat and protein levels in the milk of ruminants, such as cows, sheep, and goats, follow a somewhat different trend, usually increasing with advancing lactation (**Figure 2**).

The essential and semi-essential amino acids of sow milk constitute 45.5% of the total amino acid content, and the ratio of Lys to Met plus Cys is 1:0.5. The amino acid composition of sow and bovine caseins (CNs) is shown in **Table 2**. Bovine CN contains lower levels of Glu, Pro, Val, Ala, Phe, Lys, Tyr, and His than sow CN, while human milk proteins are reported to have less Glu, Met,

Table 1 Average composition (g per 100 ml) of milk of minor mammalian species

Mammals	*Days of lactation*	*No. of samples*	*Total solids*	*Protein*	*Lactose*	*Fat*	*Ash*
Ass	60–120	9	9.1	1.6	6.2	1.00.4	
Bison	?	2	13.7	4.2	4.7	1.7	1.0
Caribou	?	3	23.6	7.6	3.7	11.0	1.3
Dromedary	?	15	13.6	3.6	5.0	4.5	0.7
Elk	14–77	28	19.0	5.7	4.2	6.7	1.3
Llama	2–120	54	13.1	3.4	6.5	2.7	0.5
Moose	>2	15	23.6	11.0	3.3	8.5	1.5
Musk ox		1	19.0	5.3	3.8	8.2	1.7
Pig[a]	21	18	19.2	6.0	4.5	6.65	0.79

[a] Farreira AS, de Assuncao Costa PM, Sant Anna R, and Comes JC (1988) Composition of sows milk. *Revista de Sociediade Brasileira de Zootaxa* 17: 212–220.

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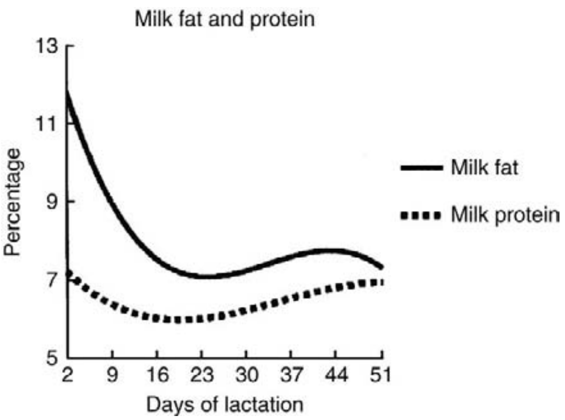

Figure 1 Profiles of fat and protein levels in sow milk through 51 days of lactation (cubic order regression). Adapted from Garst AS, Ball SF, Williams BL, *et al*. (1999) Influence of pig substitution on milk yield, litter weights, and milk composition of machine milked sows. *Journal of Animal Science* 77: 1624–1630.

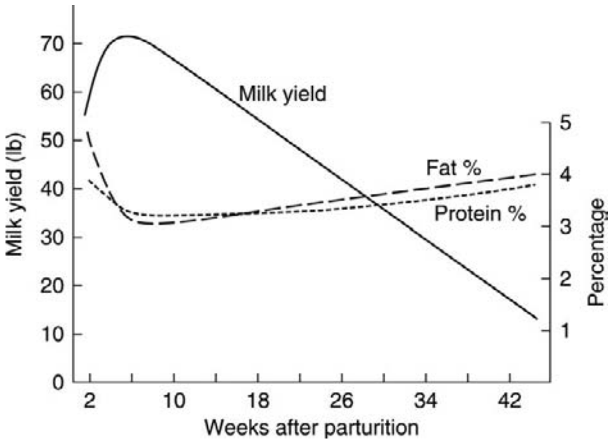

Figure 2 Lactation curves of milk yield, and milk fat and protein percentages of Holstein cows. Adapted from Schmidt GH (1971) Mammary gland anatomy. *Biology of Lactation*, pp. 6–35. San Francisco, CA: W.H. Freeman and Company.

Met, Tyr, Lys, and His and more Cys and Trp than sow milk proteins.

In light of fatty acid composition, the differences between sow and bovine milks are primarily in short-chain fatty acid levels and the degree of saturation. Sow milk fat contains C4–C12 fatty acids at barely detectable levels and these fatty acids represent only 0.2% of the total, whereas in bovine milk these fatty acids represent 8% of the total. Bovine milk fat contains more myristic, palmitic, and stearic acids, and far less palmitoleic, oleic, linoleic, and linolenic acids than sow milk.

There is little information available on the effect of genetics on mineral concentrations in sow milk. The level of cholesterol is less than 0.5% of total lipids in cow and sow milk. Somatic cell counts in sow milk are affected by litter replacement and oxytocin administration and may increase from about 8×10^6 cells ml^{-1} milk on day 2 to more than 12×10^6 cells ml^{-1} on day 51 post-partum.

Yak Milk

Origin and distribution of yak

Yak is called 'the ship of cold regions' and serves many needs related to food, fiber, and transportation of the people in the cold mountainous regions of northern China, Mongolia, Tajikistan, Uzbekistan, Russia, Nepal, and Bhutan. Yaks are members of the subfamily of Bovinae and are classified as *Bos grunniens* or *Poephagus grunniens*. They originated in the Qinghai-Tibetan Plateau and were first domesticated about 4500 years ago. Yak have a unique ability to live under extreme environmental conditions and are able to survive unsheltered through the winter months, exposed to harsh snowstorms and temperatures below −40 °C at high altitudes, 3000–5000 m above sea level. China has an estimated 13 million yaks, with the largest numbers in the Sichuan Province, followed by Qinghai Province, the Tibet Autonomous Region, Gansu, Xinjiang, Inner Mongolia, and Yunnan Province. Outside of China, the largest yak

Table 2 Amino acid composition of sow and bovine casein (g per 100 g protein)

Amino acid	*Sow casein*[a]	*Bovine casein*[b]
Asp	7.2	7.4
Thr	4.8	4.3
Ser	5.3	6.6
Glu	29.5	23.2
Pro	12.4	11.8
Gly	1.8	2.1
Ala	3.8	3.4
Val	8.1	7.5
Met	1.3	3.2
Ile	5.8	6.6
Leu	10.2	10.0
Tyr	6.2	5.8
Phe	5.7	5.4
Lys	8.7	8.1
His	3.9	3.2
Arg	3.9	4.1
Trp	1.5	1.3

[a] Nake T and Kataokak (1973) Comparative studies on the milk constituents of various mammals in Japan. IV. Comparison in composition of milk proteins from various mammals. *Japanese Journal of Dairy Science* 22: A20–A28.

[b] Davendra C (1980) Milk production in goats compared to buffalo and cattle in humid tropics. *Journal of Dairy science* 63: 1755–1767.

Gallagher DP, Cotter PF, and Mulvhill DM (1997) Porcine milk proteins: A review. *International Dairy Journal* 7: 99–118.

population is in Mongolia with approximately 600 000 head. Wild populations of yak are estimated to be about 150 000.

Yak breeds and milk production

On average, yaks produce less milk than dairy cows, approximately 1.5 kg day^{-1} after suckling the calf, or 300 kg per lactation, with a lactation period of 5–6 months. Milk production by the Chauri and Jom yak breeds exceeds the yield of pure-bred yak. Jom can produce 1.5–3 l milk per day. When yaks are artificially inseminated to Brown Swiss (BS) cattle, the female hybrids (BS Joms) produce about 3–4 l day^{-1}. Chauri are considered more versatile than pure-bred yak throughout Nepal and are more manageable draught and pack animals, tolerating lower altitudes. Jhopkyo yak can carry between 60 and 80 kg loads.

Yak milk composition

Yak milk is high in total solids and has a sweet fragrance and golden rich color. Because of its higher fat and protein content than bovine milk, yak milk quality is often referred as ‘thick’ or ‘rich’. Research data on the composition of yak milk produced in China, Mongolia, and Nepal are shown in **Table 3**. The fat content of yak milk is about twice that of average cow milk, ranging from 5.45 to 8.60%, depending on season, time of milking, stage of lactation, and availability of supplementary feeds.

Yak milk contains lower levels of short- and medium-chain fatty acids than caprine or bovine milks. The major fatty acids in yak milk are C16:0, C18:1, C18:0, and C14:0; oleic acid is the most abundant among the unsaturated fatty acids. Semihard cheeses made from yak milk reared in the highlands of the Nepalese Himalayas contain lower myristic and palmitic acids, higher total long-chain

Table 3 Composition (range) of normal milk and colostrum of yak at different regions

	Normal milk		*Colostrum*	
	China	*Mongolia*	*Nepal*	*China*
Total solids (%)	15.70–18.36	16.00	17.40	33.01
Fat (%)	5.45–8.60	5.60	6.50	14.00
Protein (%)	4.20–6.40	4.23	5.40	16.14
Lactose (%)	3.30–5.80	5.29	4.65	1.86
Ash (%)	0.40–0.90	0.91	0.85	1.01
Energy (kcal l^{-1})	871–957			
α-Lactalbumin (% of protein)	3.8			
β-Lactoglobulin (% of protein)	15.3			
Serum albumin (% of protein)	2.2			
Calcium (mmol l^{-1})	36.8			
Phosphorus (mmol l^{-1})	24.8			
Potassium (mmol l^{-1})	27.6			
Sodium (mmol l^{-1})	20.8			
Magnesium (mmol l^{-1})	2.5			

Pu Jiabi (2004) Yak milk production in China. *Southwest Agricultural University Report*, p. 4. Chengdu, China.

Silk TM, Guo MR, Haenlein GFW, and Park YW (2006) Yak milk. In: Park YW and Haenlein GFW (eds.) *Handbook of Milk of Non-Bovine Mammals*. pp. 345–354. Ames, Lowa; Oxford, England: Blackwell Publishers.

saturated fatty acids, and much higher polyunsaturated fatty acids than Cheddar cheese made from cow milk.

The levels of protein and total solids in yak milk from China and Nepal range from 4 to 7% and 16 to 18%, respectively. As in bovine milk, there is an inverse relationship between milk yield and fat and protein levels. Lactose and ash contents of yak milk range from 3 to 6% and 0.4 to 0.9%, respectively (**Table 3**).

Yak milk has six major proteins comparable with bovine: α-lactalbumin (α-La), β-lactoglobulin (β-Lg), β-CN, α_s-CN, bovine serum albumin (BSA), and lactoferrin. The major proteins in yak milk are CNs, which account for over 60% of total proteins. The relative percentage of α-La in yak milk is significantly lower ($P < 0.05$) than that in bovine milk, while differences in other proteins between yak and bovine milk including CNs, immunoglobulins, lactoferrin, BSA, and β-Lg are not significant. Yak milk has a good level of essential amino acids. Mean lysine and methionine contents are 80.0 and 30.6 (mg^{-1} g protein), respectively, and glutamic acid is the most abundant (184 mg^{-1} g protein) amino acid in Mongolian yak milk.

Yak milk products

Yak milk has been a major traditional staple food for yak herders and their families in the highland plains of Tibet. Yak milk is consumed as full-fat milk by children and the elderly, and is used to manufacture butter, ghee, cheese, and yogurt. In some areas, it is processed into powder, which can provide up to 60% of the total income for yak herders. 'Milk tea', a mixture of boiled tea and yak milk, is popular and is consumed throughout the year. It is made by boiling tea leaves (cut from a tea brick) in water and then adding yak milk and boiling for a few more minutes.

A Swiss Gruyere-type cheese has been made from yak milk since 1952, supported by the Food and Agriculture Organization (FAO) of the United Nations, transferring Swiss cheese technology from the Alps to Nepal.

Butter is a major product from yak milk, and it represents one of the staple foods of yak-raising people in China. Milk is first heated to about 35 °C and then filtered to separate cream. The cream is naturally fermented for a 1 or 2 days and then transferred to a wooden churn, where it is stirred/churned by rotating the stick until the fat solidifies. 'Butter tea' is especially popular with yak herders in China and Tibet.

Yak milk cake is made from whole yak milk or sometimes skimmed yak milk. It is hard in texture and looks like a cake. It is usually consumed with butter and sugar, which are used to enhance flavor, by yak herdsmen and their guests. Milk curd is also widely consumed as a dessert by yak herdsmen on the Tibetan Plateau. Yak milk whey is used as pig feed or for traditional leather processing.

Reindeer

Reindeer husbandry and milking evolved at least 2000 years ago on the Taiga region of eastern Siberia around Lake Baikal and spread to nearby ethnic groups. Reindeer milking regimen developed along the borders of Russia, Mongolia, and China. This was probably triggered by cultural exchange and expansion of pastoral nomads living on the northern fringe of the Asian steppe. The Yakuts, famous horse and cattle breeders, adapted reindeer raising as they pushed north and introduced an advanced milking culture into the region.

Reindeer (*Rangifer tarandus tarandus*) and caribou (*Rangifer tarandus granti*) are closely related in species and ancient associations in northern lands. For indigenous Northern Americans, caribou have provided meat, skins for clothing and shelter of exceptional warmth and lightness, and implements made from bones and antlers. The fur trade led to early exploitation of North American caribou.

Reindeer milking also evolved in Scandinavia independently by the Nordic Saami people of Lapland. Saami reindeer herding families in the late 1800s practiced small-scale reindeer pastoralism, and the milk was used to manufacture cheese and butter for their own consumption and for sale. As new technology developed, intensive production in larger herds became possible by using snow mobiles.

Reindeer milking starts usually a month after calving during May, depending on latitude and environmental conditions, and continues up to the rut in late September and early October. Among advanced southeastern Siberian reindeer herders, the calves and females are normally tethered during alternate periods to ensure that both remain close to the campsite, but are kept separated during part of the day. The females are milked up to 3 times daily and the separation time of the calves from their mother varies with the stage of lactation. The most efficient but labor-intensive form of Saami milking regime is called 'kjevle' of the calf, in which small herds are driven into a restricted or fenced compound, where the calves are caught and a small wooden stick is placed in their mouth to prevent them from sucking, but this does not hamper grazing seriously after milking.

Yield of Reindeer milk

The pattern of the lactation curve of reindeer is similar to that of other ungulates (**Figure 3**), where the peak of lactation occurs during weeks 2–4 with a daily output of around 1 l, depending on the weight of the animal, with a total milk yield of about 100 kg, but also less depending on the nutritional state of the female. Isotope tracer techniques have been used to study the potential milk production of reindeer and caribou, which are accurate, but elaborate and expensive.

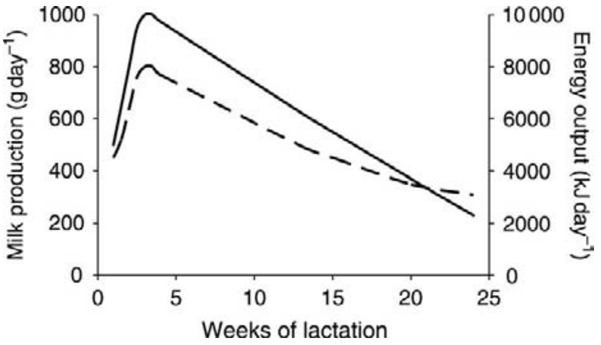

Figure 3 Lactation curves of reindeer for potential daily mean milk production (solid line) and energy output (dotted line) of an average female (75 kg) through lactation. Adapted from Holand Ø, Aikio P, Gjøstein H, Nieminen M, Hore K, and White RG (2002) Modern reindeer dairy farming – the influence of machine milking on udder health, milk yield and composition. *Small Ruminant Research* 44: 65–73.

The lactation curve of reindeer has been determined on the basis of milk yield during a few selected stages of lactation. The estimated total milk yield of reindeer is lower than that of red deer (150 kg) and Iberian red deer (224 kg), but slightly higher than that of black-tailed deer (93 kg).

Composition of Reindeer milk

It is difficult to give a typical composition of reindeer milk, due to limited sample size, individual variation, difference in sampling techniques, feeding regime, and variations in the lactation stage among different studies. In general, wild and semi-domestic ruminants give richer milk, particularly in late lactation, than the domesticated species. Reindeer milk at peak and mid-lactation has a relatively high level of fat (11–15%) and protein (7–10%), but a moderately low level of lactose (about 3.5%) (**Table 4**).

The protein content of reindeer milk increases from around 9% in early lactation to about 11% in late lactation, while the relative levels of different amino acids are rather constant throughout lactation. The amino acid profile resembles those in sheep and goats, except for low cysteine and high tyrosine contents. CN is the predominant protein fraction in reindeer milk, with a content of 7–9%. β-Lg is the main whey protein: it contains 162 amino acids. Reindeer milk contains high levels of non-protein nitrogen (NPN), urea, ammonium, and carnitine (at 84–118 mg per 100 ml). Urea content is about 48 mg per 100 ml and free carnitine 71 mg kg^{-1}.

Fat is the major energy component in reindeer milk, representing 67% of the energy content at peak lactation and 75% at late lactation. Fatty acids of reindeer milk are dominated by palmitic acid (16:0), accounting for one-third of the total fat, and stearic (18:0), oleic (18:1), and myristic acids (14:0), contributing around 13%. The levels of short-chain fatty acids, especially butyric (4:0) and capric (6:0) acids, are higher in reindeer than in red deer, roe deer, and fallow deer milk. Reindeer milk contains about 3–3.5% lactose at peak lactation, which is lower than in other wild ungulates, and also has small amounts of oligosaccharides.

The mineral content of reindeer milk is moderate to high (1–1.5%), compared to most other ungulates. Reindeer milk is high in fat- and water-soluble vitamins. Vitamin C content is around 2 mg per 100 ml, which is similar to that in red deer and fallow deer milk. Reindeer milk is several times higher in vitamin D_3 (0.5–2.0 mg kg^{-1}) as compared to cow milk.

Utilization of reindeer milk

Reindeer milk is consumed as fluid and processed products. Children drink fresh milk and adults consume it in tea and coffee. The milk is also dried and processed into cheese, butter, and sour cream, as well as used in medications (**Table 5**). The milk is curdled and often mixed with tasty herbs (*Oxyria* spp. and *Angelica* spp.). Reindeer milk is also stored frozen and often mixed with berries (*Vaccinium* spp., *Empetrum nigrum*).

Normally, reindeer milk from the first part of lactation is consumed fresh, the second part is mainly used for cheese production, and the last part of lactation is used more appropriately for churning butter. Reindeer milk and its products are highly priced, and are also used for medical purposes such as cures for digestive problems, owing to its anti-diarrheic properties and for healing wounds. Fat oozed by heat from reindeer cheese is used to cure nursing pains, frostbites, and other injuries. Colostrum is used for children's ailments.

Table 4 Gross composition (%) of reindeer milk at peak lactation, 3–5 weeks post-partum

Weeks of lactation	*No.*	*Total solids*	*Fat*	*Protein*	*Lactose*	*Ash*
4–5	8	27.1	11.1	11.1	3.0	1.5
4	3	23.7	10.2	7.5	3.7	1.2
5	5	38.1	19.6	13.0	3.7	2.7
3–5	7	31.6	15.5	10.7	3.7	1.3
5	2	32.8	17.1	10.9	2.8	1.5

Holand Ø, Gjøstein H, and Nieminen M (2006) Reindeer milk. In: Park YW and Haenlein GFW (eds.) *Handbook of Milk of Non-Bovine Mammals*, pp. 355–370. Ames, IA; Oxford, UK: Blackwell Publishing.

Table 5 Traditional products and uses of reindeer milk

Fresh milk	Consumed by children (often diluted with water), used in tea and coffee and in medical treatments
Stored milk	
Frozen	Stored for consumption during winter, ice cream mixed with berries
Fermented	
Short	Sour cream and cultured milk inoculated with a bacterial starter, for consumption
Long	Stored in wooden containers often mixed with herbs, curdled, consumed during winter and spring migration, both the liquid and solid phases
Dried	Dried in stomach compartment, reticulum, for winter consumption
Manufactured milk product	
Cheese	Curdled by heating or by adding dried abomasum, dried and stored for consumption and sale
Butter	Churned from both fresh and fermented milk, for consumption and sale
Other products	Buttermilk and whey, consumed fresh and reduced and eaten as soup

Holand Ø, Gjøstein H, and Nieminen M (2006) Reindeer milk. In: Park YW and Haenlein GFW (eds.) *Handbook of Milk of Non-Bovine Mammals*, pp. 355–370. Ames, IA; Oxford, UK: Blackwell Publishing.

Musk Ox

Musk ox ranges across northern Alaska, Canada, and Greenland. Musk ox was extinct in Alaska by the mid-1800s, possibly due to hunting by native people, explorers, and whalers. In the early 1900s, the musk oxen population in the world was estimated to be as few as 5000 animals, which was considered to be in danger of extinction. Beginning in 1917, the Canadian Government enforced protection of the species from hunting and made efforts to save musk oxen. The species was translocated from Greenland to Alaska and held in captivity in Fairbanks for feeding, growth, and breeding studies until 1935–36, and then released into the wild on Nunivak Island.

Although the lactational strategies differ in length, musk oxen and caribou live in similar arctic environments, and often eat similar forages. Healthy musk oxen continue to nurse their young throughout the rutting period, until December to February, and they may lactate throughout the winter in the field.

Musk oxen have three recognized subspecies: *Ovibos moschatus moschatus*, *Ovibos moschatus niphoecus*, and *Ovibos moschatus wardi*. Musk oxen in Alaska are descendants of animals transplanted from Greenland (*O. moschatus wardi*). A comparative study of allozyme electrophoresis has shown that little genetic variation exists within and between populations of Alaskan and Greenlandic Musk Oxen.

The gross composition of musk ox milk is shown in **Tables 1** and **6**. Although there are some variations between reports, musk ox milk contains a much higher level of total solids than cow milk. This is due to the higher fat and protein, which is a common characteristics of the milk of Arctic and sub-Arctic species. The gross composition of musk ox milk such as total solids, solid-not-fat, and lactose contents increases from day 1 to 3 months of lactation. Musk oxen have the highest milk production at 3 weeks postpartum, and production remains high for about 1 month and then tapers off gradually.

Table 6 Gross composition of musk ox milk

	Study 1[a]	*Study 2*[b]
Total solids (%)	27.1	21.5
Water (%)	72.9	78.5
Fat (%)	10.9	11.0
Solid-not-fat (%)	16.2	10.6
Ash (%)	1.20	1.8
Protein (%) ($N \times 6.38$)	11.9	5.3
Lactose (%)	2.1	3.6
Specific gravity	1.023	-
pH	5.4	-
Vitatmin B_{12} ($\mu g\, l^{-1}$)	-	3.4

[a] Baker BK, Cook HW, and Teal JJ (1970) musk ox (*Ovibos Moschatus*) milk. I. Gross composition, fatty acid, and mineral constitution. *Canadian Journal of Zoology* 48:1345–1347.

[b] Tener JS (1956) Gross composition of musk ox milk. *Canadian Journal of Zoology* 34:569–571.

The fatty acid composition of musk ox milk reveals that approximately 38% of the total fatty acids is oleic acid, and only a trace amount of butyric acid is detected. The levels of small- and medium-chain fatty acids (C4–C14) of musk ox milk range from 9.8 to 17.4%, which are significantly lower than those of bovine or caprine milk. Must ox milk also contains lower levels of long-chain fatty acids (above C18) compared to the milk of other Arctic species.

Llama Milk

Milk Yield

Llama has a milk secretory system consisting of four mammary glands, similar in structure to the cow, with four teats each having two streak canals, which enter into separate teat and gland cisterns. Milk is collected by variable numbers and sizes of milk ducts from the gland and emptied into the gland cistern. The amount of milk produced daily by individual llamas varies significantly and ranges from 16 to 413 ml per animal, with a median of 62 ml per animal.

Milk composition

Llama milk contains an average of 13.1% total solids, 6.5% lactose, 3.4% protein, and 2.7% fat. The energy content of llama milk varies between 50.0 and 95.8 kcal per 100 g with an average of 70.0 kcal per 100 g, which is lower than that of bovine (85.2 kcal per 100 g), caprine (103.6 kcal per 100 g), and ovine milk (155.6 kcal per 100 g). Llama milk has a density of 1.033 g ml^{-1}, a milk fat density of 0.935 g ml^{-1}, and a pH of 6.52 at 20 °C.

The mineral content of llama milk is different from human and bovine milk, in which potassium is the most abundant mineral, while calcium is the main mineral in llama milk, followed by phosphorus and potassium. The calcium content of llama milk is higher (1310–2210 mg kg^{-1}) than that of human, cow, and goat milk (280, 1120, and 1400 mg kg^{-1}, respectively), but similar to that of camel milk. The concentration of sodium in llama milk (193–413 mg kg^{-1}) is lower than in cow milk (530 mg kg^{-1}), but higher than in human milk (180 mg kg^{-1}).

The most abundant trace element in llama milk is zinc, similar to that in milk of other species. The mean zinc content of llama milk (about 4.2 mg kg^{-1}) is higher than that of human milk (1.2 mg kg^{-1}), but similar to cow milk (3.9 mg kg^{-1}) and camel milk (4.0–5.0 mg kg^{-1}). Barium concentration in llama milk (0.278 mg kg^{-1}) is higher than that of cow milk (188 mg kg^{-1}). The mean copper concentration of llama milk (0.109 mg kg^{-1}) appears to be lower than that of mare (0.155 mg kg^{-1}), human (0.250–0.314 mg kg^{-1}), or guinea pig (0.500 mg kg^{-1}) milk. The relatively low copper concentration in llama milk agrees with low blood serum copper concentration of llamas compared to other domestic animals. Mean iron concentration of llama milk (0.65 mg kg^{-1}) is comparable to that of cow milk (0.50 mg kg^{-1}), but higher than that of human milk (0.3 mg kg^{-1}) and lower than that of camel milk (1.3–2.5 mg kg^{-1}). Llama milk proteins contain significant proportions of phosphorus, being 0.36, 0.45, 0.30, and 0.15% for α-CN, β-CN, γ-CN, and the proteose peptone fraction, respectively.

Alpaca

There are four species of South American camelids: vicuna, guanaco, llamas, and alpacas. The vicuna and guanaco are wild species. The vicuna is native to the altiplano regions of Chile, Bolivia, and Peru. The guanaco is native to the Patagonia regions of southern Chile and Argentina. Llamas and alpacas are the two domesticated species in this camelid family.

Alpacas are highly important for the village economy in Chile, Bolivia, Peru, Ecuador, and Argentina. Alpacas are an essential source of income and provide food and wool for the native people, who live on the high altiplano regions. Alpaca-producing regions in these countries have a conception rate of 50% or lower and a 20% mortality rate of the young called ‘cria’. Nutritional inadequacies, infectious diseases, and changes in the environment may cause these reproductive problems.

Studies on the composition of alpaca colostrum at 48 h postpartum for Andean high plateau and Patagonia regions (**Table 7**) showed 21 and 19% total solids, 9.8 and 9.2% protein, 4.8 and 2.7% fat, 4.4 and 5.3% lactose, 1.6 and 1.8% ash, respectively. The colostrum of Andean altiplano alpaca has higher dry matter, protein, and fat, but lower lactose than Patagonia region alpaca. A similar trend was observed for the major constituents of mature of milk alpaca from 1 to 5 months of lactation (**Table 7**). The higher fat content of alpaca milk at the Andean high plateau compared to the lower altitude Patagonia region may suggest that the animals at the higher altitude may

Table 7 Comparison of gross compositions of colostrum and mature milk of alpaca from two regions of Chile

	Dry matter	*Protein*	*Fat*[a]	*Lactose*[a]	*Ash*
Colostrum					
AHP[b]	20.66 ± 1.3	9.84 ± 0.6	4.80 ± 1.2	4.41 ± 0.1	1.63 ± 0.0
Patagonia[c]	19.06 ± 0.5	9.24 ± 0.5	2.71 ± 0.6	5.33 ± 0.1	1.78 ± 0.1
Mature milk					
AHP[b]	16.8 ± 0.7	6.9 ± 0.3	3.8 ± 0.6	4.4 ± 0.5	1.7 ± 0.3
Patagonia[c]	15.8 ± 0.6	6.5 ± 0.3	2.6 ± 0.5	5.2 ± 0.5	1.4 ± 0.1

[a] Differences in fat and lactose contents of both colostrum and mature milk between the two regions are significant ($P < 0.05$).

[b] AHP: Andean high plateau region (4400 m above sea level); 24 alpacas were tested.

[c] Patagonia: Patagonia region (12 m above sea level); 18 alpacas were tested.

Data were reorganized from Parraguez VH, Thenot M, Latorre E, Fernando G, and Raggi LA (2003) Milk composition in alpaca (*Lamo pacos*): Comparative study in two regions of chile. Archivos de Zootechia 52:431–439.

require more energy for body maintenance by natural selection compared to those at the lower altitude. The differences in fat and lactose composition between the two regions could also be explained by the pasture composition and availability of the regions as well as the grazing behavior of the alpacas.

The main immunoglobulin of colostrum found in alpaca crias is IgG. Mean IgG concentrations are similar in llama (2370 mg per 100 ml) and alpaca crias (2340 mg per 100 ml), and are not different between males and females. Llama and alpaca crias are born agammaglobulinemic, with IgG concentrations increasing after suckling.

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Milks of Non-Dairy Mammals

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Introduction

The use of milk and dairy products is such a daily routine that mankind does not realize that it is using the milk from another species. Knowledge therefore reaches no further than the assumption that milk from all species is the same. Furthermore, mankind has become so used to dairy products such as butter, cheese, and yogurt, which have been discovered merely by chance, owing to abusive handling and storage of milk, that no thought is given about the consequences that would have been faced if milk had a different composition. Only when malnutrition among humans has been observed during feeding of infants with cow's milk, and when differences in dairy product properties have been observed when milks from different domestic species are used, has an interest sprouted in the composition of the milks of non-dairy species. Although the milks of all mammals contain the same components – water, salts, vitamins, fat, carbohydrates, and proteins – it was realized that this does not imply that all milks are the same.

The objective of this article is to provide a brief comparison of milks, and to describe certain characteristics that are unique to milks from certain animals, which are not to be expected by extrapolation of the knowledge of well-studied species. Very little data are available on milks of non-dairy animals, because studies in this area have been limited. Further limitations are the inaccuracy of data for many species owing to the small numbers of samples, small sample volumes, difficulties in defining the stage of lactation, biases during sampling, and flaws in analytical procedures. It is therefore attempted here to present the data from multiple samples of which the stage of lactation was specified; the analytical procedures used are currently regarded as accurate.

Gross Composition of Milk

By comparing the milk composition of different species (**Table 1**) it may be concluded that, in general, the milks of some primates and Perissodactyla (horses and rhinoceroses) are dilute, low-fat milks, whereas bears, Pinnipedia, and Cetacea produce high-fat and energy-dense milks. The milks of marsupials are rich in carbohydrates, especially oligosaccharides, whereas in the milks of Pinnipedia these carbohydrates are present in trace amounts. Deviations from these general observations are noticed, and may be ascribed to adaptation to special needs, such as to the environment or special dietary requirements. Among the Bovidae, the total dry matter of the milks of ibex, dall sheep, muskox, dorcas gazelle, and springbok is above 20%, as compared to the figures of 12–20% for the other members, possibly owing to the adaptation to arid environments. Among the Felidae, the total dry matter may vary; for serval milks it is 29.5%, whereas for cheetah milk it is 16.6%. Adaptation to the environment cannot be considered as the reason, as these two cats coexist in the same regions. Each milk, therefore, provides nutrients and bioreactive components in the correct amount and form, which is the result of adaptation to environmental circumstances and physiological constraints.

Proteins

Of all the milk nutrients, proteins are the least studied in species other than humans and a few domestic mammals. The proteins of all milks are subdivided into whey proteins and caseins. 'Whey protein' is a collective description for a wide variety of proteins and includes enzymes and immunoglobulins, as well as the major proteins β-lactoglobulin and α-lactalbumin. The caseins are α_{s1}-, α_{s2}-, β-, and κ-caseins, of which different subtypes may occur.

The proteins occur in different amounts. In cow's milk caseins are the major proteins at 2.6%, with whey proteins at 0.7%. In human milk, the respective amounts are 0.2 and 0.6%. Subfractionation of milk proteins of only a few species has been reported; so phylogenetic relationships cannot be established. In the milks of all ruminants, a ratio of whey proteins to caseins similar to that in cow's milk is observed. In non-ruminant species, the whey proteins are not always the major fraction. A major problem in subfractionation lies in the methods applied, which are based on selective precipitation of the caseins with acid and subsequent separation of the whey proteins. It is then assumed that milk proteins from other species will react in the same manner as the bovine proteins for which the methods were developed, which is the case quite often, but not always. Actually a separation method should be developed for the milk proteins of each species; however, this is not possible when only small quantities of milk are available from wild animals.

Table 1 Gross composition of mammalian milks

Species		*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Lactose and sugars (%)*
Monotremata					
Tachyglossidae					
Short-beaked echidna	*Tachyglossus aculeatus*	48.9	31.0	12.4	2.3
Qrnithorhynchidae					
Platypus	*Ornithorynchus anatinus*	39.1	22.2	8.2	3.7
Marsupialia					
Dasyuridae					
Eastern native quoll	*Dasyurus viverrinus*	29.6	10.9	7.3	5.6
Phalangeridae					
Brushtail possum	*Trichosurus vulpecula*	24.0	4.4	7.0	11.0
Potoroidae					
Long-nosed potoroo	*Potorous tridactylus*	27.0	3.0	10.0	14.0
Macropodidae					
Tammar wallaby	*Macropus eugeni*	25.0	4.0	6.0	12.5
Red kangaroo	*Macropus rufus*	24.1	10.3	7.0	6.2
Soricomorpha					
Soricidae					
Greater white-toothed shrew	*Crocidura russula*	51.0	30.0	9.4	3.0
Chiroptera					
Vespertilionidae					
Little brown bat	*Myotis lucifugus*	27.1	15.8	8.5	4.0
Cave bat	*Myotis velifer*	25.4	19.9	10.7	4.4
Primata					
Lemuridae					
Brown lemur	*Eulemur fulvus*	9.6	0.9	1.3	8.5
Mongoose lemur	*Eulemur mongoz*	9.8	0.7	1.3	7.9
Galagidae					
Thick-tailed bushbaby	*Otolemur crassicuadatu*	18.6	8.0	4.8	6.4
Lorisidae					
Slow loris	*Nycticebus coucang*	16.3	7.0	3.9	6.6
Callitrichidae					
Common marmoset	*Callithrix jacchus*	12.7	2.3	2.2	8.0
Cebidae					
Bolivian squirrel monkey	*Saimiri boliviensis boliviensis*	16.6	4.6	3.6	7.0
Atelidae					
Mantled howler	*Alouatta palliate*	11.7	1.6	2.2	6.7
Cercopithecidae	*Macaca fascicularis*				
Crab-eating macaque	*Macaca fascicularis*	12.2	5.2	1.6	-
Rhesus macaque	*Macaca mulatta*	15.2	4.4	1.8	7.5
Vervet monkey	*Chlorocebus pygerythrus*	9.9	3.1	1.3	8.6
Carnivora					
Canidae					
Dog (domestic)	*Canis familiaris*	22.7	9.5	7.5	3.8
Ursidae					
Black bear	*Ursus americanus*	37.6	25.1	7.0	3.0
Mustelidae					
American mink	*Neovison vison*	21.7	7.3	5.6	4.5
Felidae					
Cheetah	*Acinonyx jubatus*	16.6	6.5	10.1	4.0
Serval	*Felis serval*	29.5	15.2	15.8	0.7
Pinnipedia					
Otariidae					
Cape fur seal	*Arctocephalus pusillus pusillus*	42.0	23.2	10.8	-
	Arctocephalus australis				
Northern fur seal	*Callorhinus ursinus*	63.3	50.7	10.3	0.1

(*Continued*)

Table 1 (Continued)

Species		*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Lactose and sugars (%)*
Australian sea lion	*Neophoca cinerea*	37.6	25.4	10.5	-
California sea lion	*Zalophus californianus*	41.0	31.7	8.6	0.3
Phocidae					
Hooded seal	*Cystophora cristata*	69.8	61.1	4.9	1.0
Harp seal	*Phoca groenlandica*	65.7	53.5	7.7	0.8
Cetacea					
Delphinidae					
Bottlenose dolphin	*Tursiops truncates*	27	12.8	8.9	1.0
Balaenopteridae					
Fin whale	*Balaenoptera physalus*	46.5	33.2	10.5	2.3
Proboscidea					
Elephantidae					
Asian elephant	*Elephas maximus*	17.7	7.3	4.5	5.2
African elephant	*Loxodonta Africana*	20.7	10.6	5.59	18.4
Perissodactyla					
Equidae					
Plains zebra	*Equus quagga burchelli*	11.3	2.2	1.6	7.0
Przewalski horse	*Equus ferus przewalskii*	10.5	1.5	1.6	6.7
Tapiridae					
Baird's tapir	*Tapirus bairdii*	13.3	1.9	4.6	5.3
Rhinocerotidae					
Black rhinoceros	*Diceros bicornis*	8.8	0.2	1.4	6.6
Artiodactyla					
Tayassuidae					
Collared peccary	*Tayassu tajacu*	16.2	4.2	5.1	6.2
Camelidae					
Bactrian camel	*Camelus bactrianus*	15.5	5.4	4.4	4.8
Dromedary camel	*Camelus dromedaries*	12.2	3.8	3.2	4.5
Cervidae					
North American elk	*Cervus Canadensis*	19.0	6.7	5.7	4.2
Mule deer	*Odocoileus hemionus*	18.5	5.5	7.0	4.5
Giraffidae					
Giraffe	*Giraffa camelopardalis*	14.5	4.8	4.0	-
Bovidae					
Springbok	*Antidorcas marsupialis*	25.5	14.5	7.4	4.3
Ibex	*Capra ibex*	23.3	12.4	5.7	-
Black wildebeest	*Connochaetes gnou*	12.0	5.5	4.3	4.1
Blue wildebeest	*Connochaetes taurinus taurinus*	13.4	7.5	4.1	5.3
Dorcas gazelle	*Gazella dorcas*	24.1	8.8	8.8	-
Sable antelope	*Hippotragus niger*	13.4	7.8	5.3	8.7
Dall sheep	*Ovis dalli*	22.9	9.5	7.2	5.3
Muskox	*Ovibos moschatus*	28.5	14.3	8.7	3.6
Rodentia					
Castoridae					
European beaver	*Castor fiber*	34.1	19.0	11.2	1.7
Muridae					
Brown or Norway rat	*Rattus norvegicus*	22.1	8.8	8.1[N*]	3.8
Caviidae					
Guinea pig	*Cavia porcellus*	17.5	5.7	6.3	4.8
Chinchillidae					
Chinchilla	*Chinchilla lanigera*	-	11.2	7.3	1.7
Echimyidae					
Punare	*Thricomys apereoides*	-	22.3	11.0	4.4
Sciuridae					
Chipmunk	*Tamias amoenus*	50.0	28.3	9.0	3.3

(*Continued*)

Table 1 (Continued)

Species		Dry matter (%)	Fat (%)	Crude protein (%)	Lactose and sugars (%)
Lagomorpha					
Leporidae					
European brown hare	*Lepus europaeus*	32.5	15.6	10.0	1.5
Rabbit	*Oryctolagus cuniculus*	31.2	15.2	10.3	1.8

Oftedal OT and Iverson SJ (1995) Comparative analysis of non-human milks: Phylogenetic variation in the gross composition of milks. In: Jensen RG (ed.). *Handbook of Milk Composition*, pp. 749–788. New York: Academic Press; Osthoff G, Hugo A, and De Wit M (2007) The composition of serval (*Felis serval*) milk. *Comparative Biochemistry and Physiology. Part B* 147: 237–241; Osthoff G, Hugo A, and De Wit M (2008) Composition of a free-ranging white rhinoceros (Ceratotherium simum) during late lactation. *Mammalian Biology* 73: 245–248; Osthoff G, Dickens L, Urashima T, Bonnet SL, Uemura Y, Van der Westhuizen JH (2008) Structural characterization of oligosaccharides in the milk of an African elephant (*Loxodonta africana africana*).*Comparative Biochemistry and Physiology. Part B* 150: 74–84; Osthoff G, Hugo A, and De Wit M (2009) Comparison of the milk composition of free-ranging blesbok, black wildebeest and blue wildebeest of the subfamily Alcelaphinae (family: Bovidae). *Comparative Biochemistry and Physiology* 154: 48–54; Park YW and Haenlein FW (2006) *Handbook of Milk of Non-Bovine Mammals*. Oxford: Blackwell.

Milk proteins may be separated further by polyacrylamide gel electrophoresis (PAGE). The technique may be adapted to effect separation by size, charge, or both. When the proteins are stained, quantification by densitometry is possible. However, this method is also not foolproof, as unrelated proteins with similar molecular weight or charge may not be separated, and furthermore not all proteins are stained at equal intensity. In **Figure 1**, the electrophoretic separation of milk proteins by urea-PAGE of a number of species is shown. Among the Bovidae the electrophoretic migration of the caseins is very similar, whereas that of the whey proteins, α-lactalbumin for example, varies to a great extent. Variation in electrophoretic mobility is the result of differences in amino acid composition or post-translational modification. The migration of β-caseins is very similar when compared to cow's milk protein, whereas the migration of the α-type caseins is slightly different. The κ-casein of cow's milk is clearly separated from the β-caseins; an almost similar separation is observed for kudu milk and springbok milk. This is indicative of only minor differences in amino acid composition or post-translational modification of these proteins. For the other five antelopes, the κ- and β-caseins are not clearly separated. Among all these example species, the migration of the α-lactalbumin band may also be slower or faster than its bovine equivalent.

Apart from these major protein bands, many other bands that are distinct for each species are visible. These may be enzymes and globulins which occur at different concentrations in each species. Among closely related species similarities may occur, but also there are differences. Good examples in **Figure 1** are the three representatives from the subfamily Alcelaphinae. Although the genetic distance between the two wildebeest species is 0.02 ± 0.014 (determined with 29 protein encoding loci), and between these and the blesbok is 0.121 ± 0.066, the electrophoretograms seem to contradict this, especially regarding the migration of α-lactalbumin. The same is observed for the κ-casein of kudu and eland of the subfamily Bovinae.

The electrophoretograms of non-ruminant species differ substantially from those of the ruminants. The β-caseins have a slower mobility, while some proteins,

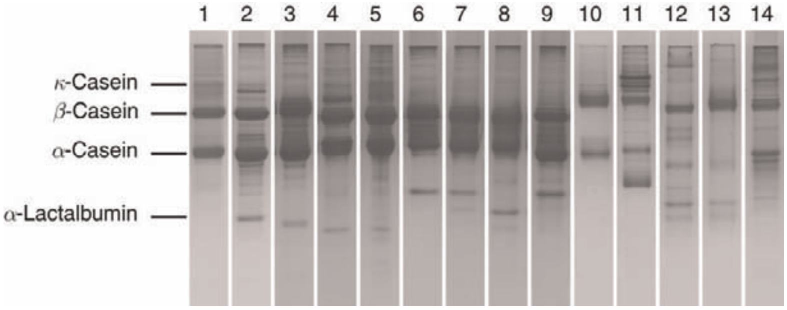


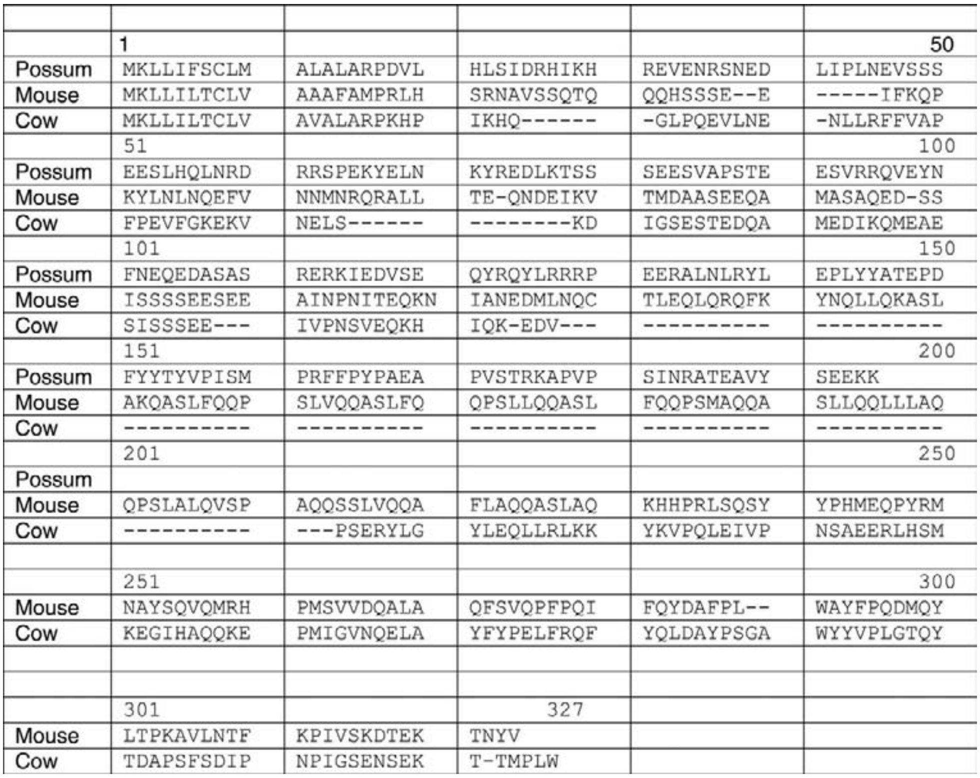
Figure 1 Urea–polyacrylamide gel electrophoretograms of sodium caseinate (lane 1), and milk of cow (2), springbok (3), kudu (4), eland (5), blesbok (*Damaliscus dorcas phillipsi*) (6), black wildebeest (7), blue wildebeest (8), sable antelope (9), white rhinoceros (10), African elephant (11), vervet monkey (12), rhesus macaque (13), and human (14).

such as the α-type casein, may be absent. The African elephant milk has a short migration distance for β-casein and contains high amounts of other (whey) proteins, while the α-caseins of the white rhinoceros have a similar migration distance as their bovine equivalent.

Comparison of the electrophoretograms of the primate milks shows the β-casein at a shorter migration distance than the bovine equivalent. However, among the primate milks shown, the electrophoretic migration of the β-casein does not vary as much as that of the whey proteins – a similar observation as for the Bovidae described above. It has been established that the α-type casein is absent in human milk. The protein bands at the migration position equivalent to that of the bovine α-type casein have been identified as lactoglobulin and lactoferrin. High amounts of immunoglobulins are present in human milk. Owing to their high molecular weights they migrate short distances. When the milks of vervet monkey and macaque are compared to human milk, the absence of α-type casein becomes obvious. The migration of the lactalbumin bands is similar, which seems logical considering the close relationship between these species within the Cercopithecidae family. In spite of this close relationship, however, differences in the electrophoretic properties of the proteins in general are also observed.

A more specific comparison of the caseins regarding molecular mass and post-translational modification may be explained by the amino acid sequence, phosphorylation, and glycosylation of the caseins from some marsupials and ruminants. The caseins of the marsupials differ substantially from those of eutherian species (**Figure 2**). Marsupial milk contains only one type of α-casein, of which the amino acid sequence shows low levels of similarity with that of eutherian species, and, furthermore, it is glycosylated. The β-caseins have no similarity to their eutherian equivalents.

Among the eutherian species, high levels of amino acid sequence identity for the α_{s1}-casein are observed within closely related species. Among ruminants, the identity is >85%, and among rodents >80%. Lower levels of identity are observed between unrelated groups, for example, 45% between bovine and pig, and 29% between bovine and rat α_{s1}-caseins. This explains the difference in electrophoretic migration of these proteins from those of Bovidae and other species (**Figure 1**). Multiple forms of these proteins may occur, which have been described for bovine and goat species. The β-caseins show a similar degree of divergence across species as the α_{s1}-caseins, and multiple forms have also been reported. The multiple forms of these two caseins are indicative of hereditary lineage, which in cattle may be traced to *Bos indicus* or



	1				50
Possum	MKLLIFSCLM	ALALARPDVL	HLSIDRHIKH	REVENRSNED	LIPLNEVSSS
Mouse	MKLLILTCLV	AAAFAMPRLH	SRNAVSSQTQ	QQHSSSE--E	-----IFKQP
Cow	MKLLILTCLV	AVALARPKHP	IKHQ------	-GLPQEVLNE	-NLLRFFVAP
	51				100
Possum	EESLHQLNRD	RRSPEKYELN	KYREDLKTSS	SEESVAPSTE	ESVRRQVEYN
Mouse	KYLNLNQEFV	NNMNRQRALL	TE-QNDEIKV	TMDAASEEQA	MASAQED-SS
Cow	FPEVFGKEKV	NELS------	--------KD	IGSESTEDQA	MEDIKQMEAE
	101				150
Possum	FNEQEDASAS	RERKIEDVSE	QYRQYLRRRP	EERALNLRYL	EPLYYATEPD
Mouse	ISSSSEESEE	AINPNITEQKN	IANEDMLNQC	TLEQLQRQFK	YNQLLQKASL
Cow	SISSSEE---	IVPNSVEQKH	IQK-EDV---	----------	----------
	151				200
Possum	FYYTYVPISM	PRFFPYPAEA	PVSTRKAPVP	SINRATEAVY	SEEKK
Mouse	AKQASLFQQP	SLVQQASLFQ	QPSLLQQASL	FQQPSMAQQA	SLLQQLLLAQ
Cow	----------	----------	----------	----------	----------
	201				250
Possum					
Mouse	QPSLALQVSP	AQQSSLVQQA	FLAQQASLAQ	KHHPRLSQSY	YPHMEQPYRM
Cow	----------	---PSERYLG	YLEQLLRLKK	YKVPQLEIVP	NSAEERLHSM
	251				300
Mouse	NAYSQVQMRH	PMSVVDQALA	QFSVQPFPQI	FQYDAFPL--	WAYFPQDMQY
Cow	KEGIHAQQKE	PMIGVNQELA	YFYPELFRQF	YQLDAYPSGA	WYYVPLGTQY
	301		327		
Mouse	LTPKAVLNTF	KPIVSKDTEK	TNYV		
Cow	TDAPSFSDIP	NPIGSENSEK	T-TMPLW		

Figure 2 Alignment of the amino acid sequences of the α_{s1}-caseins of possum (*Trichosurus vulpecula*), mouse (*Mus musculus*), and cow (*Bos taurus*) to show the difference between marsupial and eutherian proteins, and partial similarity between eutherian proteins. From Ginger MR and Grigor MR (1999) Comparative aspects of milk caseins. *Comparative Biochemistry and Physiology. Part B* 124: 133–145.

Bos taurus lineages. In general, the α_{s2}-like caseins are more disparate among different species. The level of sequence identity between bovine and pig proteins is 64%, and between rabbit and mouse it is <40%.

The α- and β-type caseins share several common properties, which include partial amino acid sequence and calcium sensitivity, which suggests that they may have originated from a common protein ancestor. κ-Casein is not related to the above proteins. It is the only eutherian casein that contains carbohydrate moieties, does not display calcium sensitivity, and is susceptible to cleavage by the aspartate protease, chymosin (rennin). Human milk κ-casein is more highly glycosylated than bovine and ovine proteins and it differs from these with the presence of fucose and *N*-acetylglucosamine.

In terms of the cleavage of κ-casein by chymosin, mammals may be separated in two groups. In group I, κ-casein is cleaved at a specific Phe–Met bond, and this group is found mainly in the ruminants Cervidae, Giraffidae, and Bovidae. In group II, κ-casein is cleaved at Phe–Ile or Phe–Leu, and this group is mainly found in non-ruminants such as Primata, Cetacea, Perissodactyla, Suidae, Tayassuidae, and Camelidae. It seems the division on the basis of ruminant or non-ruminant is not definite. Both the κ-casein groups are represented in the Rodentia, with the guinea pig (Caviidae) producing group I, and the mouse and rat (Muridae) producing group II κ-caseins, while the rabbit (Lagomorpha) also produces group I κ-caseins. The resulting difference in cleavage affects the clotting mechanism of the two milks. Milks of the Equidae family contain very low levels of κ-casein, the effect being that equine milks do not coagulate on renneting or form very weak curd.

Of the whey proteins, α-lactalbumin has been characterized in great detail. It occurs in the milk of most mammals and is involved in the synthesis of lactose. The amounts of this protein in the mammary gland differ among species, which in turn governs the amounts of lactose in the milk. Small amounts are found in monotremes and marsupials, while high amounts are observed in most eutherians. While there is a homology between the α-lactalbumins of monotremes, marsupials, and eutherians, the protein of the former two also shows a high degree of homology with eutherian lysozyme with regard to amino acid sequence, secondary and tertiary structure, as well as arrangement of the genes. It was therefore proposed that α-lactalbumin has evolved from lysozyme. In spite of the homology of lysozymes between species, substantial differences occur, which may explain the large variation in electrophoretic mobility of this protein observed in **Figure 1**.

The difference of proteins between species is of course not restricted only to the milk proteins, but also extends to other functional proteins such as enzymes. Consequently, there will be differences in the reaction products produced by such enzymes, which, in milk, will be reflected in the composition of fats and saccharides, as is described next.

Lipids

Fatty Acid Composition

The triacylglycerides in milk are synthesized in the mammary cells. The fatty acids are obtained from three sources: (1) *de novo* synthesis of fatty acids from metabolites such as NADPH and acetate, (2) direct uptake from fatty acids circulated in blood, and (3) further modification of fatty acids in the mammary gland by desaturation and elongation. In monogastric animals, the fatty acid composition of the milk reflects to a high degree the circulating fatty acids, most of which normally stem from the diet of the mother. This is not the case in species with foregut fermentation, such as ruminants, where the ingested fatty acids are changed by microbial fermentation.

In *de novo* synthesis the length of the fatty acids produced depends on several factors such as the ability to utilize specific molecules as sources and the specific properties of enzymes. While glucose is the source for fatty acid synthesis in non-ruminants, it is not used effectively in ruminants. Instead, lactate, acetate, and 3-hydroxybutyrate, which are metabolites of the fermentation of carbohydrates by the rumen bacteria, are utilized. The medium-chain fatty acids (8:0–12:0) are synthesized *de novo* by the mammary tissue. Fatty acids are synthesized by fatty acid synthetase in an elongation process. In the liver and adipose tissue, the fatty acids attain a chain length of 16 or more. In the mammary gland, the synthesis process is terminated by a thioesterase after the addition of up to 16 carbons. In some mammals this termination is effected after the addition of 8 to 14 carbons. Short-chain fatty acids (4:0 and 6:0) are characteristic of ruminant milk.

Fatty acids from the diet are obtained from triacylglycerols, which are carried to the mammary cells by lipoproteins, where the fatty acids are released. Generally, these fatty acids are more than 14 carbons long, mainly 16 or 18 carbons. In ruminants, approximately half of the fatty acids in milk are derived in this way, including about one-third of the 16-carbon acids and most of the 18-carbon acids. Plant foods contain high amounts of unsaturated fatty acids. In ruminants, these are altered by the rumen bacteria through hydrogenation to produce more saturated fatty acids, resulting in more saturated ones being incorporated in milk fat than found in nonruminant milks.

In the liver and adipose tissue, fatty acids are normally not elongated beyond the length of 16 carbons. However, in some species 16- and 18-carbon acids are used as substrates to be elongated up to 24 carbons in the liver and mammary gland. A further modification process of fatty acids is desaturation. In ruminants, 18:0 and 16:0 may get changed to 18:1 and 16:1. Unsaturated fatty acids such as linoleic acid ($18{:}2n-6$) and α-linoleic acid ($18{:}3n-3$) cannot be synthesized *de novo*, and have to be obtained through the diet such as plant material. In ruminants α-linoleic acid may also be produced by the rumen bacteria. Through elongation and further desaturation of these two fatty acids, unsaturated fatty acids up to 22 carbons long are formed. The substrates for elongation and desaturation may be obtained from plant material.

A phylogenetic relationship has been noted for the preference of which lipid synthesis mechanisms are used. Milk of Artiodactyla, specifically the ruminant families Cervidae, Giraffidae, and Bovidae, is characterized by the presence of up to 15% short-chain fatty acids (4:0 and 6:0) (**Table 2**). In the Camelidae these fatty acids constitute approximately 1%, and in the Suidae they are absent. Medium-chain fatty acids (8:0–12:0) are present in different amounts, and occur along family lines. The milk of goat and sheep (subfamily Caprinae) may contain up to 19% of these, whereas the milk of blesbok and the black and blue wildebeest (subfamily Alcinaphinae) contain 20–30%. The milks of the latter three animals also contain up to 20% $C_{14:0}$, which in almost all other species occurs at amounts below 13%. The single sample of okapi milk analyzed may suggest that higher amounts of medium-chain fatty acids are also found in the milk of the Giraffidae.

The milks of non-ruminant mammals may have varying amounts of medium-chain fatty acids. Among the Rodentia, the species seem to vary in their secretion of these fatty acids, and phylogenetic trends are not clear. While they may be absent in the milk of gerbil, cloud rat, guinea pig, and acouchi, they may reach 16–37% in the Norway rat and wood rat, and within the genus *Peromyscus* (deer mouse) they may be present or absent.

Some non-ruminant herbivores have extraordinarily high contents of medium-chain fatty acids (8:0–12:0). Of the Lagomorpha, rabbit milk was found to contain more than 62% of these fatty acids, and hare milk 25%. Of the Perissodactyla, the horse, tapir, and rhinoceros synthesize large proportions of medium-chain fatty acids, the content of which may change over lactation time; in the Indian rhinoceros, 10:0 was found to change from 18 to 36%.

Elephants (Proboscidae) produce most of their milk fatty acids by *de novo* synthesis of medium chain, which may reach amounts of up to 90%. The levels of these change drastically over lactation time. The amounts of 8:0 and 10:0 increase from, respectively, 3 and 30% to 12 and 60%, while 12:0 and 14:0 decrease from approximately 25 to 10%. These are the highest level of change in fatty acid composition over lactation observed for any mammal and are not related to changes in the diet. It is not known what the reason of this change in medium-chain fatty acid composition over lactation is, that is, what the benefit is to the mother animal or to nutrition.

Synthesis of medium-chain fatty acids is also a property of the primates, and specific phylogenetic relationships are found. The milks of lemurs and lorises seem to contain no 8:0, but relatively high amounts of 12:0 (4–19%) and 14:0 (6–20%), while the Callitrichidae may contain up to 2% 8:0 and higher levels of 10:0 (8–22%) and 12:0 (8–17%). Milk of the Cebidae seems to contain high amounts of 8:0 (3%) and 10:0 (12–23%) and less of 12:0 and 14:0. The Cercopithecidae and Hylobatidae seem to secrete the highest levels of 8:0 and 10:0 and low levels of 12:0 and 14:0. In the Hominidae, the bonobo, chimpanzee, Bornean orangutan, gorilla, and human low amounts of 8:0 and 10:0 are observed, which shows that there is less emphasis on *de novo* synthesis of fatty acids. In all primates, the diet plays a significant role in the fatty acid composition of the milk, and great variation in long-chain fatty acids are observed accordingly.

The milks of all the other mammalian orders not discussed above do not appear to contain short- or medium-chain fatty acids, and the fatty acid composition is highly dependent on the diet. Marine mammals such as the Pinnipedia and Cetacea are characterized by high amounts of long-chain polyunsaturated fatty acids which are obtained from fish, and the insectivorous Monotremata, Insectivora, and Chiroptera contain high levels of $18{:}3n-3$. The milk fatty acid composition of the Carnivora follows the same trend of being affected by the diet, which is best illustrated by the level of $18{:}3n-3$, which is normally low, but may reach 15% in black bear when consuming vegetable material.

Melting Properties of Fat

The above discussion and **Table 2** show that the milk fat composition of animals varies extensively with regard to chain length and degree of unsaturation. This has a great effect on the physical properties of the fat. Animal fats, including milk fat, contain more saturated fatty acids than plant fats do. The effect of fatty acid composition on the physical properties is best demonstrated with plant fats being liquid oils at room temperature while the animal fats, butter and lard, are solid. However, in the living mammal, the milk fat is not in a solid state because of the high body temperature (~ 37 °C) being maintained. The melting properties of the different milk fats give insight into the degree of fluidity at different temperatures as affected by the fatty acids. In **Figure 3** the melting thermograms of a few representative species as determined by differential scanning calorimetry (DSC) are shown, and compared to butterfat.

Table 2 Fatty acid composition of the milks of a selection of mammalian species

Species		*Weight % of fatty acids*																			
		4:0	*6:0*	*8:0*	*10:0*	*12:0*	*14:0*	*14:1*	*16:0*	*16:1*	*16:x*	*15:0 and 17:0*	*15:1 and 17:1*	*18:0*	*18:1*	*18:2n−6*	*18:3n−3*	*18:4*	*18:x*	*>20:0*	*Others*
Monotremata																					
Short beaked echidna	*Tachyglossus aculeatus*					1.0	1.4	0.4	15.9	6.2		0.7	0.0	3.9	61.2	5.1	0.8			0.5	3.0
Ornithorynchidae																					
Platypus	*Ornithorynchus anatinus*						1.6	0.7	19.8	13.9		2.5	1.5	3.9	22.7	5.4	7.6		0.6	14.4	5.3
Marsupialia																					
Dasyuridae																					
Eastern quol	*Dasyuridae viverrinus*						1.7		24.6	4.0				8.7	36.1	15.4	2.2			1.6	
Phalageridae																					
Brushtail possum	*Trichosurus vulpecula*						1.0		18.9			0.8		2.8	18.2	23.8	24.7				8.2
Potoroidae																					
Long-nised potoroo	*Potorous tridactylus*						2.1	0.1	28.0	6.7		2.6	0.6	5.6	36.5	13.9	1.4				
Macropodidae																					
Tamar wallaby	*Macropus eugenii*					tr	4.4	0.5	54.5	8.4		2.1	0.9	2.8	16.6	6.0	0.8				3.0
Red kangaroo	*Macropus rufus 1–4 days pp*	tr	tr	tr	tr	0.6	3.3	1.2	51.4	7.6		1.5	0.0	2.2	15.6	11.1	0.7			2.9	1.9
	100 days pp	tr	tr	tr	tr	tr	1.7	0.4	25.3	5.4		1.4	0.5	10.2	45.3	5.4	1.6			1.1	1.7
Phascolarctidae																					
Koala	*Phascolarctos cinereus*					0.1	3.3	0.2	24.4	4.3		1.8	0.8	5.2	16.8	10.7	32.5				0.0
Chiroptera																					
Big brown bat	*Eptesicus fuscus*					3.9	1.5		21.5	8.3				7.4	37.9	15.7	2.1				1.9
Little brown bat	*Myotis lucifugus*					4.1	3.3		21.0	11.6				5.4	24.7	12.0	11.0				7.0
Primata																					
Lemuridae																					
Brown lemur	*Eulemur fulvus*			0.1	5.5	19	20.4	2.3	17.4	4.3		tr	0.5	1.5	17.8	8.7	0.5			1.7	0.3
Mongoose lemur	*Eulemur mongoz*			0.0	0.7	2.2	5.3	0.3	26.3	6.5		0.3	0.4	2.3	35.8	14.8	0.8			4.3	0
Lorisidae																					
Slow loris	*Nycticebus coucang*			0.0	0.4	5.8	10.6	0.1	30.7	2.5		0.8	0.6	4.9	27.2	13.3	0.6			2.5	0

(*Continued*)

Table 2 (Continued)

Species		*Weight % of fatty acids*																			
		4:0	*6:0*	*8:0*	*10:0*	*12:0*	*14:0*	*14:1*	*16:0*	*16:1*	*16:x*	*15:0 and 17:0*	*15:1 and 17:1*	*18:0*	*18:1*	*18:2n−6*	*18:3n−3*	*18:4*	*18:x*	*>20:0*	*Others*
Callitrichidae																					
Common marmoset	*Callithrix jacchus*				8.0	8.5	7.7	18.1	5.5					3.4	29.6	10.9	0.9			8	0.7
Cebidae																					
Mantled howler	*Alouatta palliata*	0.0	0.0	2.2	11.9	5.7	2.9	0.0	23.4	2.2	0.0	1.2	0.6	4.7	14.7	12.2	17.8			0.5	0.2
Cercopithecidae																					
Vervet monkey	*Chlorocebus pygerythrus*	0.0	0.4	5.8	9.4	2.7	1.7	0.1	22.3	2.1	0.0	0.5	0.3	6.2	19.2	26.5	1			1.6	0.8
Crab eating macaque	*Macaca fascicularis*		1.1	8.1	7.9	1.8	2.1		22.1	5.4				4.9	28.9	15.8	0.8				1.1
Hylobatidae																					
White handed Gibbon	*Hylobates lar*	0.0	0.0	0.8	10.3	6.9	3.3	0.1	19.3	2.5	0.0	0.3	0.4	5.4	36.6	10.7	1.1			2	0.3
Hominoidea																					
Mountain gorilla	*Gorilla beringei*			0.0	0.4	0.1	3.8	0.5	27.1	2.2	0.0	0.0	0.0	5.7	27.1	10.6	16.3			3.7	0
Carnivora																					
Canidae																					
Domestic dog	*Canis familiaris*	0.0	0.0	0.0	0.0	0.2	3.3	0.4	27.9	6.4		0.7	0.6	4.3	40.7	11.9	0.5	0.3		2.7	0.5
Ursidae																					
Black bear	*Ursus americanus*	0.0	0.0	0.0	0.0	0.2	3.2	0.5	23.5	3.3	tr	0.8	0.5	5.8	39.9	15.7	5.1	0.2		0.8	0.5
Mustelidae																					
American mink	*Neovison vison*				tr	0.2	2.1	0.1	27	3.4		0.1	0.4	10.2	39.3	9.2	0.9			6.7	0.5
Felidae																					
Cheetah	*Acynonix jubatus*	0.0	0.0	0.0	0.0	0.1	2.7	0.2	21.0	5.8		0.7	0.4	4.5	32.4	15.3	10.2			3.1	0.0
Serval	*Felis serval*	0.0	0.0	0.0	0.0	0.0	0.89	0.0	24.4	0.2		0.8	0.2	8.1	34.0	27.1	1.3	0.0		3.2	0.0
Pinnipedia																					
Otariidae																					
Northern fur seal	*Callorhinus ursinus*					0.3	6.6	0.3	20.0	10.3		0.6	1.3	1.9	34.5	1.9	0.2			21.2	0.9
Australian sea lion	*Neophoca cinerea*	0.0	0.0	0.0	0.0	0.2	7.0	0.2	18.2	6.9	1.0	1.8	0.7	2.5	19.0	1.7	1.0	1.1	0.2	36.9	1.3
California sea lion	*Zalophus californianus*	0.0	0.0	0.0	0.0	0.1	4.5	0.1	18.1	6.5	0.8	1.0	0.2	3.0	23.2	1.6	0.9	1.0	0.4	38.6	0.0
Phocidae																					
Hooded seal	*Cystophora cristata*	0.0	0.0	0.0	0.0	0.1	4.4	0.2	11.7	13.4	1.0	0.3	0.2	2.1	27.0	1.4	0.4	1.2	0.2	36.3	0.2

Cetacea																					
Delphinidae																					
Bottle-nosed dolphin	*Tursiops truncates t.*	0.0	0.0	0.0	0.0	0.3	3.2	0.2	21.1	13.3	1.1	0.8	0.7	3.3	23.1	1.2	0.2	0.2	0.2	29.2	2.1
Balaenopteridae																					
Fin whale	*Balaenoptera physalus*	0.0	0.0	0.0	0.0	0.1	5.5	0.2	22.9	6.5	0.9	0.6	0.2	3.9	24.7	1.1	0.6	1.1	0.1	29.4	1.8
Proboscidae																					
Elephantidae																					
Indian elephant	*Elephas maximus*	0.0	0.4	5.8	43.4	21.5	3.5		9.1	1.9				0.5	9.8	2.3	0.5			0.5	1.4
African elephant	*Loxodonta Africana*			9.7	64.5	17.4	1.2		2.6	0.5				tr	3.4	0.1	0.1				
Perissodactyla																					
Equidae																					
Horse	*Equus caballus*			6.8	14.1	12.9	9.2		17.9	4.8				1.1	10.1	3.8	9.6				
Rhinocerotidae																					
White rhinoceros	*Ceratotherium simum*			3.0	25.5	16.5	9.6		15.7	1.2		0.8		8.9	8.6	3.7	2.5	0.0		1.2	0.0
Artiodactyla																					
Tayassuidae																					
Collared peccary	*Tayassu tajacu*					tr	2.8	tr	29.4	6.0				6.2	36.8	17.2	1.6				
Camelidae																					
Dromedary camel	*Camelus dromedarius*	tr	0.1	0.1	0.1	0.7	9.8	1.4	25.7	10.5		2.8		11.9	27.1	3.8	3.7			1.1	1.4
Cervidae																					
Mule deer	*Odocoileus hemionus*	7.8	2.1	0.3	0.8	0.7	12.7		35.6	1.1				15	14.7	1.4	2.1				5.5
Giraffidae																					
Okapi	*Okapia johnstoni*	0.6	1.4	1.9	7.0	1.1	14		30.5	4.4				11.8	20.9	3.5	0.0				2.8
Bovidae																					
Springbok	*Antidorcas marsupialis*	0.8	0.8	0.5	1.0	1.4	14.7	0.6	23.1	1.8		3.1	0.2	18.7	27.6	3.4	1.3			0.9	
Blackbuck antelope	*Antilope cervicapra*	4.3	3.4	1.7	4.6	3.3	15.9		37.2	2.3				6.8	19.4	1.6					
Black wildebeest	*Connochaetes gnou*	0.2	2.0	5.0	15.3	5.2	13.2	0.7	20	0.5		1.9	0.0	12	20	2.7	1.0				
Blue wildebeest	*Connochaetes taurinus taurinus*	0.2	2.0	5.6	20.7	9	20.6	0.4	21.5	0.6		2.3	0.0	5.5	8.6	1.6	0.6				
Gazelle	*Gazella granti*	3.4	3.1	2.7	5.0	3.1	15.5		33.2	2.7				5.8	15.8	6.4	0.0				3.3
Sable antelope	*Hippotragus niger*	0.5	1.3	2.1	8.6	2.9	11.4	0.4	24.7	1.6		3.0	0.2	18.8	21.1	1.9	1.0			1.0	0.3

(*Continued*)

Table 2 (Continued)

		Weight % of fatty acids																			
Species		*4:0*	*6:0*	*8:0*	*10:0*	*12:0*	*14:0*	*14:1*	*16:0*	*16:1*	*16:x*	*15:0 and 17:0*	*15:1 and 17:1*	*18:0*	*18:1*	*18:2n−6*	*18:3n−3*	*18:4*	*18:x*	*>20:0*	*Others*
Bighorn sheep	*Ovis canadensis*	2.6	1.2	0.5	2.5	1.9	11.2		24.7	2.2				9.6	36.8	4.7	2.0				0.0
Rodentia																					
Muridae																					
Gerbil	*Meriones unguiculatus*	0.0	0.0	0.0	0.0	0.0	0.7		13.4	0.7				5.9	24.5	46.8	0				8.1
Slender tailed cloud rat	*Ploeomys cumingi*	0.0	0.0	0.0	0.0	0.0	4.2	29.4	4.4					3.8	46.6	7.9	0				3.7
Brown Norway rat	*Rattus norvegicus*			15.8	12.8	8.9	11.7		19.6	1.7				3.2	12.3	9.4	0.8		0.2	2.6	0.2
Caviidae																					
Guinea pig	*Cavia porcellus*	0.0	0.0	0.0	Tr	0.1	1.6		32.5	1.5				3.4	32.3	24.1	4.4				
Dasyproctidae																					
Acouchi	*Myoprocta pratti*	0.0	0.0	0.0	0.0	0.0	4.9		54.9	8.6				2.4	21.9	6.8	0.6				
Lagomorpha																					
Leporidae																					
European hare	*Lepus europaeus*		tr	6.8	13.1	4.8	5.2		27.4	5.5		0.8	0.4	3.6	17.5	12.8	2.0			tr	0.2
Rabbit	*Oryctolagus cuniculus*		tr	27.1	28.9	5.6	1.8		9.6	1.3		0.4	tr	2.1	9.8	11.1	2.3			tr	

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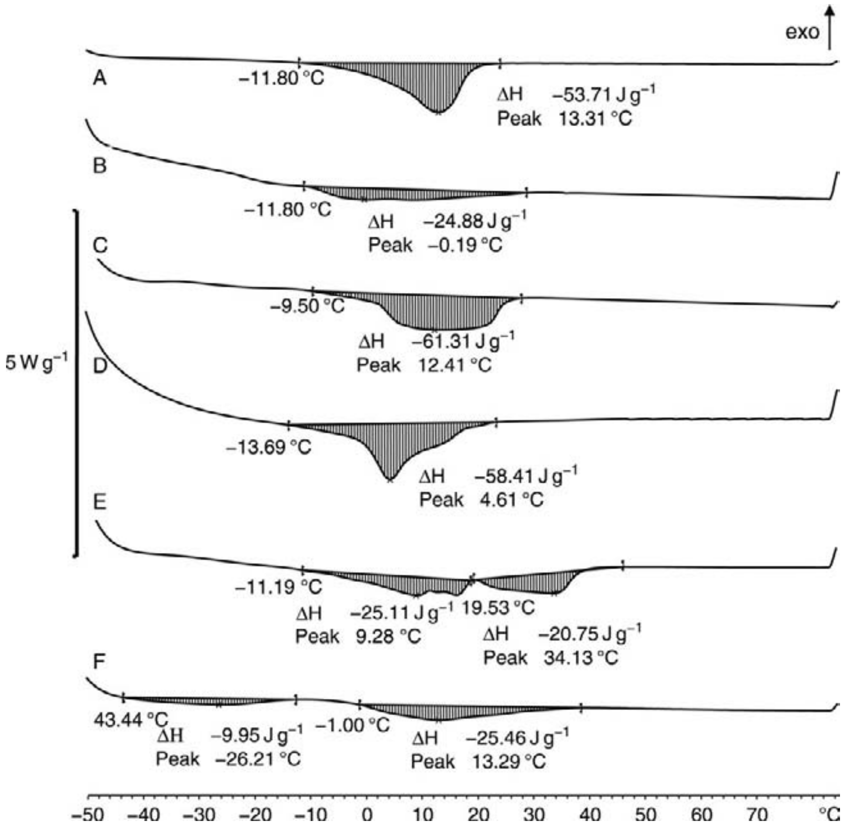

Figure 3 Differential scanning calorimetry thermograms of milk fat from African elephant (A), cheetah (B), blue wildebeest (C), vervet monkey (D), domestic cow (E), and bottlenose dolphin (F).

A distinct melting curve is observed for each species. The first aspect to be noted is the initial and final melting points. A high initial, as well as final, melting point is indicative of high amounts of saturated long-chain fatty acids, and a low initial, as well as final, melting point is the result of high amounts of short-chain or polyunsaturated fatty acids. The final melting point of all milk fats is below 40 °C, but for a few species, such as the cow, a small percentage of the fat may be solid above that temperature. This shows that milk fat in all species is in a liquid state at body temperature.

The second aspect is the different melting peaks observed for the same milk fat, which is the result of triglycerides containing different types of fatty acids. Elephant milk fat shows only one melting peak between −11 and 24 °C, owing to the lipids containing up to 90% medium-chain fatty acids. The lipids in cow's milk occur in two groups. One group contains high amounts of polyunsaturated fatty acids and the other more of saturated ones. The effect is that these two groups of molecules melt between −11 and 20 °C, and between 20 and 46 °C, respectively. Similarly, two groups are observed for the milk fat of the bottlenose dolphin, which melt between −48 and −4 °C, and between −1 and 38 °C, the low melting peak being ascribed to the high levels of polyunsaturated fatty acids. As the milk fats of most ruminants have a very similar fatty acid composition (**Table 2**), similar melting properties are observed. The milk fat of ruminants such as the blue wildebeest, however, contains higher amounts of medium-chain fatty acids, which results in a lower maximum melting point. Furthermore, if the fatty acids are more evenly distributed among the lipid molecules, a narrower melting range, resembling that of the elephant milk fat, is observed.

The technological implication of the fatty acid composition and melting properties is that cow's milk fat results in a butter that is hard at 4 °C due to approximately 75% of the fat being in a solid state, but soft and spreadable at 25 °C, due to approximately 60% of the lipids being in a liquid state and being entrapped by the 40% solid molecules. In comparison, the other milk fats whose melting properties are discussed above do not result in butter with the same properties? The milk fats of elephant, blue wildebeest, and vervet monkey have the same solid character as cow's milk fat at 4 °C, but are liquid oils at 25 °C. The milk fat of cheetah is soft at 4 °C and liquid at 25 °C, and that of bottlenose dolphin is very soft at 4 °C and almost completely liquid at 25 °C. Concurrently, will these fats also present (structural characteristics and mouthfeel) to food products such as ice cream and baked goods as cow's butterfat?

Carbohydrates

Carbohydrates that are found in milk are lactose and oligosaccharides, and the monomer constituents, namely, galactose, glucose, fucose, and to a lesser extent sialylic acid and *N*-acetylglucosamine. The major carbohydrate in the milk of most eutherian species is lactose.

Of over 200 species the milks of which have been analyzed, the specific contents of lactose and the other saccharides have been determined for only a few, the reason being the specificity and sensitivity of the methods of analysis that have been used. Older methods, such as the phenol–sulfuric acid method, were nonspecific and not very sensitive. The total carbohydrate content was actually recorded, while the lactose content was reported based on the assumption that lactose is the only carbohydrate in all milks. The latest techniques rely on chromatographic separation, such as gas chromatography and high-performance liquid chromatography, and quantification relative to standards, which enables specific quantification. Nevertheless, it was recognized with the aid of paper chromatography that milk of a broad range of species contains carbohydrates of larger molecular weight than lactose. In the milk of monotremes and marsupials, and also a few eutherian species, the major carbohydrates were shown to be oligosaccharides.

These observations initiated the research on the mechanism of oligosaccharide synthesis in the mammary gland. Lactose is synthesized by lactose synthase, which consists of a complex of β-4-galactosyltransferase I and α-lactalbumin. The latter controls the rate of lactose synthesis. Oligosaccharide synthesis is catalyzed by a variety of glucosyl transferases which transfer monosaccharide residues (galactose, glucose, fucose, sialylic acid, or *N*-acetylglucosamine) either to free lactose or to another saccharide residue attached to lactose. In species with high levels of milk oligosaccharides, α-lactalbumin is present at a very low level.

Based on the homology of α-lactalbumin to lysozyme, it was proposed that the ability to secrete lactose is linked to the evolutionary development of α-lactalbumin. Prior to the presence of α-lactalbumin in the mammary gland, the rate of lactose synthesis was not controlled and almost all of the lactose was utilized for the synthesis of oligosaccharides by glycosyl transferases. This is observed in monotremes and marsupials. The biological role of the oligosaccharides in milk also seems to have changed. Oligosaccharides are not only a source of energy, but act mainly as anti-infection factors against pathogenic organisms. In eutherian species, this role has been lost to a large extent where oligosaccharides have been replaced by lactose, which acts as a source of energy.

The milks of most eutherian species contain lactose as the dominant saccharide. The concentration of lactose changes over the lactation period. In general an increase is observed in early lactation, after which it may stay stable or decrease slightly over a prolonged time. In the milk of the African elephant, a steady decrease is observed. The highest lactose concentrations (5–9%) are found in the milk of primates, Perissodactyla, and some ruminants; intermediate levels (3–5%) in Chiroptera and Rodentia; and very low levels in Pinnipedia, Cetacea, bears, monotremes, and marsupials. Great variation in lactose content is observed among, as well as within, the Carnivora families. This has best been demonstrated for the felines, where the milk lactose of the lion was determined as 3.6%, of the cheetah 4.0%, and of the serval 6.9%.

In monotremes and marsupials, the lactose content is low, but the oligosaccharide content is high (2–12%) owing to the preferred synthesis of oligosaccharides over lactose, because of the absence of α-lactalbumin. Milk of eutherian species also contains oligosaccharides, the varying amounts of which have a phylogenetic relationship. Elephant milk contains up to 2.6% oligosaccharides, which decrease over the lactation period to around 1%. This decrease is surpassed by the decrease of lactose; so oligosaccharides become the major saccharide fraction during later lactation. Human and other hominid ape milks contain substantial amounts of oligosaccharides. Human colostrum and mature milk contain 2.2–2.4% and 1.2–1.3% oligosaccharides, respectively, while in gorilla milk, 0.7% was detected. The milks of other anthropoids which have been analyzed for oligosaccharides – Hylobatidae, Cercopithecidae, and Cebidae – contain less than 0.1% of these sugars. Ruminant milks, in general, contain 0.1% oligosaccharides or less, although 0.4% has been recorded for the sable antelope.

Bears are exceptional among eutherian species, as their milk contains little lactose and a relatively high concentration (2–3%) of oligosaccharides. Among the Pinnipedia, it was found that milk of the hooded seal, a phocid pinniped, contains small amounts of lactose together with oligosaccharides, while those of the Australian and northern fur seals and Californian sea lion, otariid pinnipeds, contain no saccharides. In the latter group, saccharides are not being synthesized; this is due to the absence of α-lactalbumin, which is not expressed. In these species the nutritional role of lactose has been lost, and lactose has been replaced by fat as the energy source.

Phylogenetic relationships are observed not only in the amounts of oligosaccharides in milk, but also in their structure. The oligosaccharides may consist of 3–10 monomeric units, and their structures may be linear or branched. Phylogenetic comparison shows that oligosaccharides in the milk of marsupials are represented by three structural groups, one of which consists of sialyl oligosaccharides, which have two *N*-acetyllactosamine

(Gal(β1-4)GlcNAc) branched units, while a second one consists of β(1-3)Gal sequences. Some of the oligosaccharides are found exclusively in marsupial milks.

In the milks of eutherian species, the oligosaccharides are divided into 12 groups. Phylogenetic differences in the occurrence of oligosaccharides have been noted. Further, the exclusive occurrence of certain oligosaccharides in some species has been noted, although this exclusiveness seems to become less as more species are being studied. A great number of oligosaccharides and chain types seem to be present in most milks, but a specific difference between human and other eutherian species is notable. In human milk oligosaccharides containing the type I chain (Gal(β1-3)GlcNAc) are more prominent than those containing the type II chain (Gal(β1-4)GlcNAc), whereas the milks of some other species, including some great apes, may even contain only the type II chain oligosaccharides.

The knowledge gained thus far shows that milk saccharides play a role not only in the provision of energy, but also in protection. It seems the need for the latter role differs between species, inferred from the amounts of saccharides present in the milk, as well as the oligosaccharide chain types. In humans, the oligosaccharides act as prebiotics to promote the establishment of beneficial intestinal bacteria, lactobacilli and bifidobacteria. The oligosaccharide structures are similar to glycoconjugates on the surface of gastrointestinal cells to which pathogenic organisms may attach. When the free milk oligosaccharides bind to the pathogens, attachment is prevented. It is suggested that lacto-*N*-tetraose, as well as fucosylated oligosaccharides, specifically promotes the establishment of bifidobacteria in the human intestine. Since oligosaccharides in general, and fucosylated oligosaccharides in particular, occur at low levels, and lacto-*N*-tetraose is absent in the ruminant milks consumed by humans, they cannot provide the same protection to human infants. One of the aims of improving dairy products is therefore enrichment with prebiotic oligosaccharides, and finding sources of oligosaccharides other than milk that can fulfill the role of the natural product.

Conclusion

The composition of the milk of each species is complex and unlikely to be duplicated. This uniqueness in composition stems from evolutionary development and adaptation to provide nutrients and protection for the offspring as demanded by the circumstances experienced by each species. Regarding proteins, lipids, and carbohydrates, the most unique properties may perhaps be observed in the following examples. Milk of the Equidae contains a very low level of κ-casein. Elephants (Proboscidae) produce most of their milk fatty acids by *de novo* synthesis of medium-chain length, which may reach levels of up to 90%, the contents of which change drastically over the lactation period, independent of the diet. Milks of monotremes and marsupials, and of eutherians, the bears and seals, contain very small amounts of lactose due to the absence of α-lactalbumin, but high levels of oligosaccharides for protective purposes.

If one or more of these properties were characteristic of the milk of the known domestic species, the impact on technological aspects would have been severe. Without κ-casein, such as in the Equidae, milk does not coagulate on renneting or forms a very weak curd. Without lactose, as in monotremes, marsupials, bears, and seals, fermentation is not possible. Milk fat with high levels of medium-chain fatty acids, as found in elephant milk, would not only affect the taste of dairy products, but would govern melting properties, which in turn affect mouthfeel. With this in mind it seems to be a real coincidence that, from all the wild animals, the most docile milk-producing species that were employed in agriculture produce milk with the best properties for multipurpose processing! Furthermore, cow's milk butterfat was used as model for physical properties to be achieved by chemically modified vegetable fats!

With regard to the foster milk used for human nutrition, the milk of the domesticated species lacks sufficient amounts of oligosaccharides for protection against pathogenic microorganisms in the intestine. Species such as bears, elephants, and the great apes can shed more light on this theme.

Differences in nutrient composition of milk among species mainly show a shift of one source of energy to another or one source of protective mechanism to another. However, it is not yet understood why a great change in fatty acid composition over lactation period, in favor of shorter *de novo* synthesized fatty acids, is observed only in the Perissodactyla and Proboscidae and not in other monogastric mammals, and what the nutritional benefit of such change is to the lactating mother or the suckling young.

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See also: **Milk**: Milk of Marine Mammals.

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Milk of Monotremes and Marsupials

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Introduction

Lactation has evolved from an ancient reproductive strategy that appears to have been there long before the evolution of extant mammals. The ability to lactate is a feature found only among mammals and involves a facet of maternal care where mothers secrete nutrient-rich milk, which is delivered to the young by the mammary gland. Evolutionary studies indicate that lactation was established prior to the divergence of extant mammalian lineages. It also seems that lactation arose long before gestation. Secretions of ancestral mammary glands may have had antimicrobial properties that protected either the eggs or the hatchlings and contained organic components that supplemented offspring nutrition. Over the course of evolution, lactation became a highly efficient, effective, and adaptable means of providing maternal care for neonates. The evolution of a placenta in eutherian mammals resulted in more extensive intrauterine development of an embryo, and the ability to lactate after giving birth became a critical part of the reproductive strategy of mammalian species. Following the development of highly nutritious milks, evolution produced diversity in milk composition and function, quantity of milk output, length of lactation period, length of intervals between nursing, and contributions of lactation to offspring nutrition.

Monotreme Reproduction and Lactation Strategy

Monotremes diverged from therians ~231–217 million years ago in the Middle or Late Triassic. Consequently, monotreme milk may be regarded as an ancestral form of milk and may be used to trace the progenitors of the better-characterized marsupial and eutherian milk protein genes to provide insight into the evolution of function of these proteins. Living monotremes are classified into three genera confined to the Australian region: *Ornithorhynchus* (represented only by the duck-billed platypus) and the echidna genera, *Tachyglossys* and *Zoglossus* (two species found in Australia). *Ornithorhynchus* is confined to the Australian subcontinent. Monotreme young are born from small eggs covered by a leathery shell, and the tiny hatchlings are highly altricial. Monotreme young are completely dependent on milk as their source of nutrition, and the period of suckling is prolonged relative to gestation and incubation. A large amount of development of the young occurs during the suckling period.

Some characteristics of monotreme lactation are suggested to be ancestral to extant mammals, with marsupials and eutherians later evolving divergent lactation strategies. Extant monotremes, *Ornithorhynchus anatinus* (duck-billed platypus), and *Tachyglossys aculeatus* and *Zoglossus bruijni* (echidna genera), are the only mammals that begin early developmental stages in an external egg (echidna 14–15 mm; platypus 16.5 mm × 15 mm) covered by a soft leathery shell, which is incubated outside the mother's body.

Eggs are carried by mothers in an external skin flap or pouch where pouch secretions are predicted to keep eggs moist and protected against bacterial infection. The small and altricial hatchlings emerging from the eggs are completely dependent on milk as the sole source of nutrition during the suckling period and undergo a significant amount of development during this stage (**Figure 1**). Thus, monotremes invest only minimally in development in gestation in comparison with development during lactation, exchanging the placenta as the path of nutrition and developmental signals for milk during lactation. Relative to gestation, the lactation periods in echidnas and platypus are extensive and are characterized by intermittent suckling lasting up to 200 days and at least 120 days, respectively. In contrast to the tammar pouch young, which remains attached to the teat for the first 100 days post-partum, monotreme young suck from the areolae, as teats are absent in all monotreme species. In monotremes, glands appear as paired structures consisting of discrete elongated lobules that fan out as oval clusters beneath the skin, where they are surrounded by connective tissue. Ducts connecting to the glands open on specialized skin patches, areolae, with a well-defined structure. Despite monotremes lacking a nipple, monotreme young exhibit true sucking behavior.

Marsupial Reproduction and Lactation Strategy

Marsupials diverged ~193–186 million years ago in the Early Jurassic Period. The reproductive strategy of marsupial differs from that of monotremes; they give birth to an altricial young that emerges from the uterus. Similar to

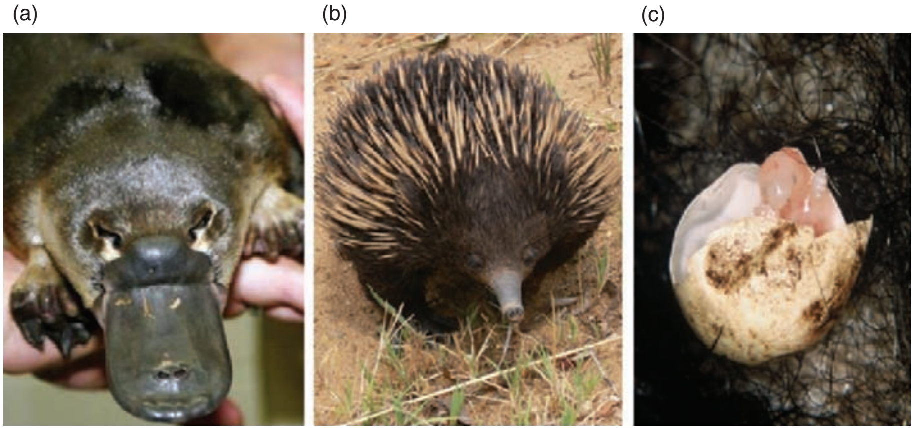

Figure 1 Reproductive strategy of the monotremes. The platypus (a) and echidna (b) lay eggs. Young hatch in a highly altricial state (c) and rely solely on milk as a source of nutrition and for developmental cues.

monotremes, marsupials rely solely on milk as the source of nourishment for the young during a long lactation period (up to 300 days, depending on the species). Much of the work on marsupial lactation has been performed using the tammar wallaby (*Macropus eugenii*) as a model. Many of the features of the tammar lactation cycle are present in other marsupials such as brushtail possums (*Trichosurus vulpecula*), eastern quolls (*Dasyurus viverrinus*), northern bandicoots (*Isoodon macrourus*), potoroos (*Potorous tridactylus*), red-necked wallabies (*Macropus rufogriseus banksianus*), Tasmanian bettongs (*Bettongia gaimardi*), and North American opossums (*Monodelphis domestica*). Like the offsprings of all other marsupials, the tammar wallaby pouch young is born altricial and relies solely on the mother's milk for growth and development during the initial 200 days of its life (**Figure 2**). During this long lactation period, the milk composition changes progressively to meet the developing nutritional requirements of the young.

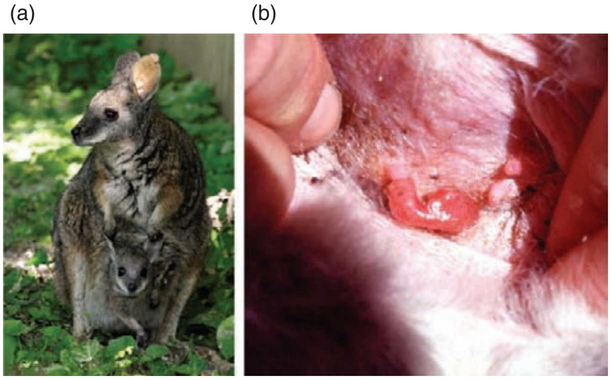

Figure 2 Reproductive strategy of the tammar wallaby. The tammar wallaby (a) gives birth to a highly altricial young (b) which climbs into the pouch, attaches to the teat, and remains there for a further 200 days during the lactation period, where it receives nutrition and developmental signal supplied in the milk.

Lactation in the tammar wallaby is divided into three main phases and was first characterized by suckling stimulus (**Figure 3**). Phase 1 comprises a 26- to 27-day gestation period, followed by birth of the altricial pouch young. The subsequent 100-day period (phase 2A) is characterized by lactogenesis and the secretion of dilute milk. During this phase the young is permanently attached to the teat. During phase 2B the young relinquishes the teat, sucking intermittently and less frequently for an additional 90–100 days. Phase 3 commences at about day 200 when the young begins periodically exiting the pouch, sucking less frequently but more vigorously. At this stage the young begins the consumption of herbage as well as milk.

Composition of Monotreme and Marsupial Milk

The amounts of total solids in both monotreme and marsupial milks (12%) at the time of hatching and birth, respectively, are much lower than that in mature milk (30–55%). Comparative studies have revealed marked differences between species and between mammalian subclasses in the quantity and combination of milk constituents. However, such studies also indicate that different milks exhibit a number of common features, which include lipid globules that consist largely of triacylglycerols, casein phosphoproteins, some whey proteins, and carbohydrates. The most marked difference between these groups is in the progressive changes in milk composition during the lactation period. Eutherians have a longer gestation relative to their lactation period and produce milk of a relatively constant composition after the expression of the initial colostrum, whereas marsupials such as the tammar wallaby have a short gestation and give birth to a highly immature young. This is

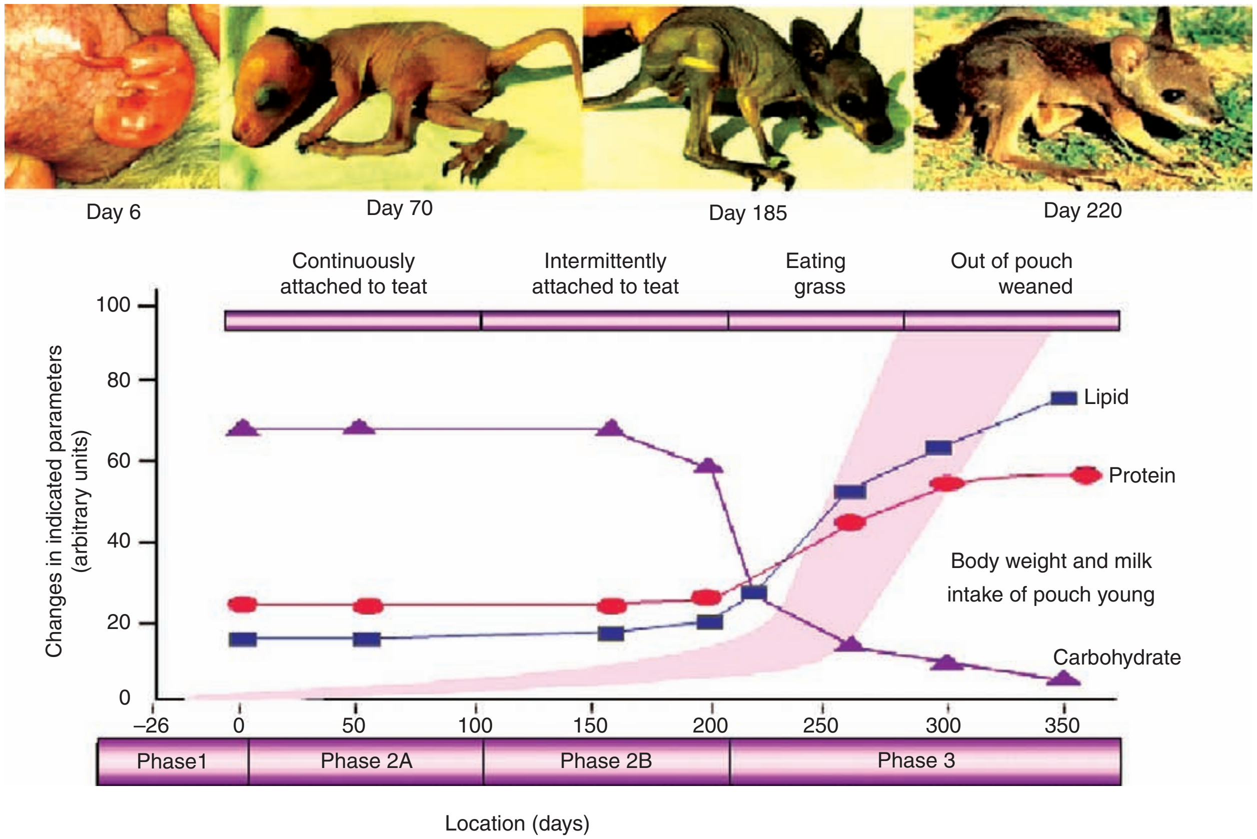

Figure 3 Lactation strategy of the tammar wallaby. The top panel shows the developmental stages of the pouch young throughout lactation. The bottom right panel represents the profile of milk composition – carbohydrate, protein, and lipid – across the long lactation period.

followed by a long lactation during which milk production changes progressively in composition to suit the needs of the developing young. Tammar wallaby milk produced in early lactation (phases 1 and 2) shows an enrichment of complex carbohydrates, which is the nutrient most easily utilized by the undeveloped young, and low concentrations of proteins and lipids. As the young becomes endothermic later in lactation (phase 3), the mother's milk composition changes markedly to become a highly concentrated source of energy. There is a sharp decrease in carbohydrate levels, which also changes to monosaccharides, and an increase in protein and lipid, which together become the major components of the milk (**Figure 3**). This milk is similar in composition to that of eutherian mammals, and thus the transition from marsupial lactation phase 2B to 3 has been described as equivalent to eutherian lactation. For example, milk production increases with a corresponding increase of the young's milk intake and weight gain, which is similar to weight gain in the eutherian neonate. Thus, the milk of phase 3 is suited to assisting maximal growth of the young, whereas the characteristics of phase 2 milk, unique to marsupials, are ideal for maximal physiological development of the young, and limited growth.

Like marsupial milk, echidna milk also changes in composition over the lactation period, but platypus milk remains unchanged. Very little is known about the changes in composition of monotreme milk, which has not been studied as extensively as milk from marsupials such as the tammar wallaby.

Milk Carbohydrate

The main carbohydrate in mammalian milk is generally the disaccharide lactose (Gal(β1-4)Glc), which is synthesized in the lactating mammary gland from UDP-Gal (donor) and Glc (acceptor) by a transgalactosylation catalyzed by the lactose synthase complex comprised of β-1,4galactosyltransferese (β-1,4GalT) and α-lactalbumin (modifer). The modifying action of α-lactalbumin lowers the K_M of β-1,4GalT for glucose 1000-fold and changes β-1,4GalT's acceptor from GlcNAc to Glc.

When lactation commences in phase 2A of the tammar wallaby lactation cycle, carbohydrates are the primary energy source for the tammar young. During the first 5 days of lactation in the tammar the major carbohydrate is lactose, but by about day 6 this disaccharide is replaced by complex oligosaccharides, and this remains so for the remainder of phase 2. It is likely that at birth the young's gut does not express the appropriate enzymes to break down complex oligosaccharides; hence disaccharides are present in the milk immediately post-partum. Presumably, the complex oligosaccharides are utilized as energy source more efficiently than is lactose, without significantly increasing the osmolarity of milk. Oligosaccharides have also been shown to have numerous bioactivities, particularly in the development of the neonate. The presence of complex oligosaccharides in milk at this stage of lactation may therefore also be beneficial to the development of the young. Formation of the complex oligosaccharides occurs owing to the catalytic action of galactosyltransferases, including β-1,3 galactosyltransferase (β-1,3GalT), which uses lactose as an acceptor for galactose and appears to be active in the mammary gland only during phase 2.

At the transition to phase 3, β-1,3GalT activity declines, resulting in the loss of complex oligosaccharides. At this stage of lactation only monosaccharides are secreted into the milk; the lactose synthesized with UDP-galactose by β-1,4GalT and α-lactalbumin complex is hydrolyzed to monosaccharides following the induction of β-galactosidase. In addition, the total carbohydrate concentration decreases, most likely owing to a high UDP-galactose hydrolase activity in phase 3 mammary tissue. UDP-galactose hydrolase cleaves UDP-galactose, the substrate for lactose synthesis. Thus, even though β-1,4GalT activity is vital for the formation of lactose (a simple sugar and building block for oligosaccharides), the composition of the complex carbohydrates is controlled largely by the transient activity of galactosyltransferases such as β-1,3GalT in phase 2 of lactation.

Studies have shown that oligosaccharides very similar to or identical with those found in tammar wallaby milk are present in the milk of all other marsupials so far studied, including the milks of red-necked wallaby and several nonmacropod marsupials such as the eastern quoll, brushtail possum, and the American opossum. The available evidence thus strongly suggests that the presence of high concentrations of a variety of oligosaccharides, and low concentrations of free lactose, in their milk is characteristic of marsupials.

The carbohydrate content of monotreme milk is distinguished from that of both marsupial and placental mammal milks by its high fucose content. It is therefore likely that suckling monotremes, unlike the sucklings of other mammals, utilize fucose as an energy source, or that fucose has an unknown specific biological significance for these animals. The principal neutral carbohydrates of echidna and platypus milks are a trisaccharide, fucosyllactose, and a tetrasaccharide, difucosyllactose, respectively. Studies have also shown that platypus milk contains low concentrations of other oligosaccharides and echidna milk contains sialyllactose, characterized by the unique presence of an *O*-acetyl group at C-4 of its Neu5Ac residue. Monotreme milk also differs from other milks in lactose content. Free lactose is found only in small amounts in both monotreme species. This is most likely due to very low concentration of α-lactalbumin in the milk compared with the concentrations of α-lactalbumin in the milks of other species. The main oligosaccharide (50%) in monotreme milk is 4-*O*-acetyl-α-*N*-acetylneuraminyl-(2-3)-lactose.

There is no evidence for quantitative or qualitative changes in milk carbohydrates in echidna and platypus during the course of the lactation season, except for a small decline in total hexose toward the end of lactation in platypus.

Lipids

Major quantitative and qualitative changes in milk lipids occur during lactation in marsupials. The crude lipid content of the milk is relatively low at the beginning of lactation and increases gradually, but toward late lactation it increases rapidly. The triglyceride fraction of the lipid at early lactation contains a large amount of palmitic acid and relatively little oleic acid, whereas mature milk contains little palmitic and more oleic acid. Very little is known about the lipid content of monotreme milk.

Milk Protein

The protein composition of tammar milk changes considerably during the lactation cycle (**Figure 4**). Protein levels are low during phase 2 of lactation, but increase dramatically in an inverse relationship with carbohydrate during phase 3. Though many of the milk proteins are common to eutherians and marsupials, a number of whey proteins appear to be novel to marsupials. Marsupial milk protein secretion has been studied primarily in the tammar and the possum, which practice similar reproductive patterns. In the tammar, some milk protein genes are induced at parturition and expressed consistently during the entire lactation, whereas some other milk protein genes are asynchronously expressed throughout lactation (**Figure 5**). At parturition there is a coordinated induction of the genes encoding β-casein, α-casein, α-lactalbumin, and β-lactoglobulin (BLG). Early-lactation protein (ELP) gene is induced at parturition and is downregulated during the transition to phase 2B when whey acidic protein (WAP) gene expression is induced. During the transition from phase 2B to 3, there is an increase in total milk protein and there is also a considerable change in protein composition. During this phase the *WAP* gene is downregulated, and late-lactation protein-A (LLP-A),

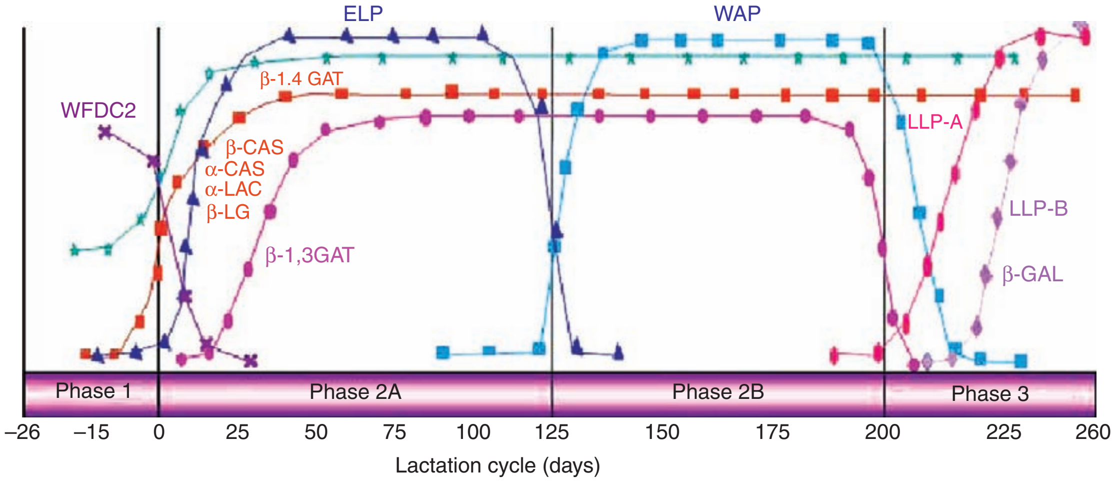

Figure 4 Milk protein gene expression during the lactation cycle. Profile of tammar milk protein composition across the lactation cycle showing changes in secretion of the major milk proteins α-casein (α-CAS), β-casein (β-CAS), α-lactalbumin (α-LAC), and β-lactoglobulin (β-LG), early-lactation protein (ELP), whey acid protein (WAP), late-lactation protein-A (LLP-A), and late-lactation protein-B (LLP-B) corresponding to the major phases of milk secretion.

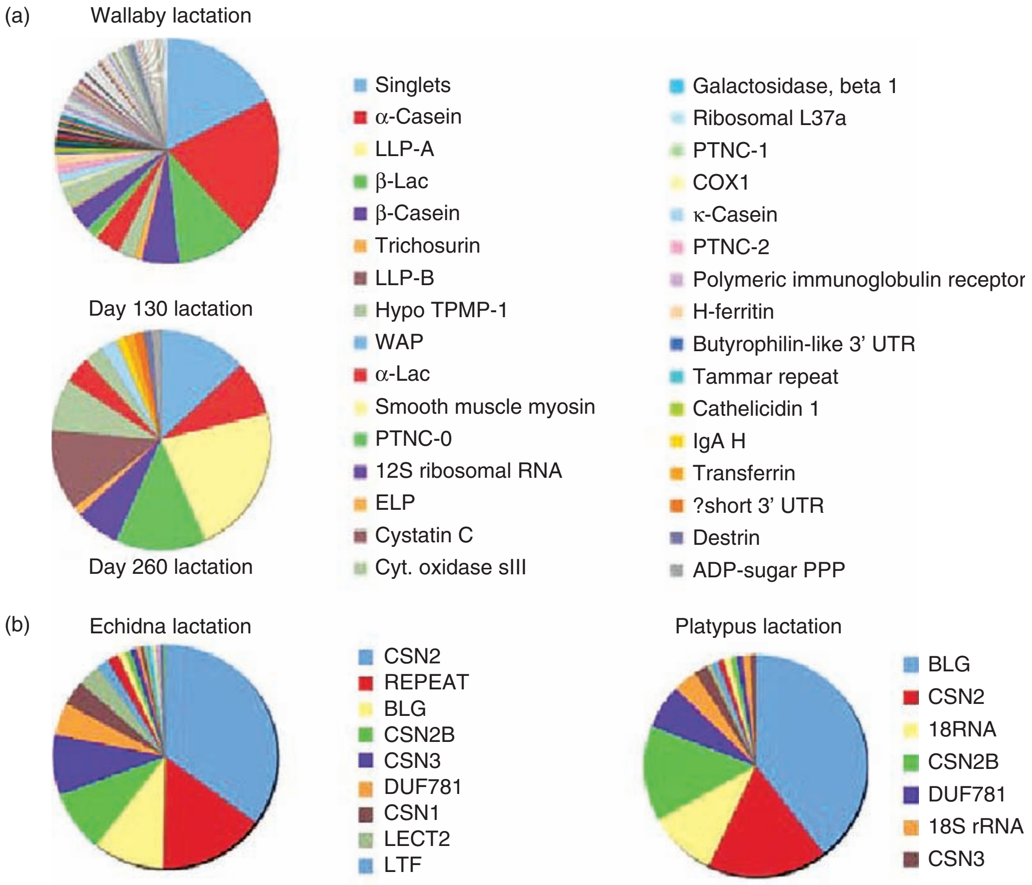

Figure 5 Transcriptome analysis of representative stages within the wallaby, echidna, and platypus lactation cycles. (a) The tammar wallaby mammary gland produces a wide variety of transcripts during lactation. At day 150 lactation (phase 2B) and day 260 lactation (phase 3), the mammary gland shows an abundance of major transcripts relating to the various stages of milk secretion. (b) The echidna and platypus milk cell transcriptomes show that the major milk protein transcripts, BLG, caseins, and lactoferrin, are abundant in the milk.

β-galactosidase, and lactoferrin are upregulated, and late-lactation protein-B (LLP-B) is induced. A similar profile of milk protein gene expression is reported in the possum, with the exception that no β-1,3GalT activity has been identified, and two of the phase 3 whey proteins, LLP and lactoferrin, are expressed transiently for 4–5 days post-partum.

The complete gene expression profile in the tammar wallaby mammary gland during the entire lactation period has been investigated by use of a comprehensive set of cDNA libraries derived from lactating tissues throughout the lactation cycle. A total of 14 837 express sequence tags (ESTs) were produced by cDNA sequencing and analysis. Sequence assembly was used to construct a comprehensive catalog of mammary transcripts representing about 25% of the tammar genome. This analysis enabled the construction of a comprehensive list of 213 secreted milk proteins produced by the tammar mammary gland and reiterated the known changes in milk protein gene expression between the different stages of lactation (**Figure 5(a)**). This analysis also revealed a number of potential new milk proteins including PTMP-1, a predicted 132- amino acid protein, which appears to be highly specific to the tammar, as no similarities could be found in the opossum or other available genome sequences, and PTMP-2-GlyCAM, a protein with no clear similarity to any known proteins except for a predicted glycosylation-dependent cell adhesion motif.

The transcriptome of monotreme milk cells, harvested from the milks of the echidna and platypus, has also been studied recently, revealing a partial set of milk protein genes (**Figure 5(b)**). Analysis of the casein genes from these monotremes revealed the presence of a second β-casein gene, which suggests that a recent duplication of β-casein has occurred in the monotreme lineage. In the monotreme it appears that the most abundant milk protein is BLG, whereas in the wallaby, generally the caseins appear more abundant (depending on the phase). Interestingly, BLG is also the major whey protein in ruminant milk, but its function remains unknown.

Immune-Related Proteins

The immunological significance of milk is a well-studied area in eutherians. Immunoglobulins are usually secreted in high amounts in colostrum of eutherians. These immunoglobulins may be absorbed by the eutherian neonate's gastrointestinal system and may be transferred into their circulation in some species, thus providing passive immunity. This absorption is limited by the time taken for the gastrointestinal system's epithelium to close, which ranges from 24 h in some ungulates to up to 19 days in rodents. Immunoglobulin levels in marsupial milk are much lower than those seen in eutherian milk. However, in marsupials absorption of immunoglobulins, other proteins, and possibly cells in the milk continues throughout the entire lactation period. Consequently, marsupials must rely upon more efficient uptake and different immunoglobulin isotypes to help meet the immunological challenges of the young. Evaluation of the expression of immunoglobulin genes in the tammar mammary gland throughout lactation suggests that there are two periods of increased expression of immunoglobulin genes corresponding to two periods of immune transfer to the young. This has also been observed in the brushtail possum lactation cycle. These periods of immune transfer coincide with the periods of greatest immune challenge for the pouch young, which include the periods following parturition and leaving the pouch. Immunoglobulins and other immune components such as the neonatal Fc alpha receptor (FcRn) and the iron regulatory proteins ferritin and transferrin in the brushtail possum are transferred at this time. Consequently, differential regulation of expression of immune components in the mammary gland may help the marsupial neonate to survive periods of immune stress though still being relatively immunocompromised.

Proteins known to be associated with immunological protection of the pouch young have also been identified across all phases of tammar lactation. A total of 14 proteins, detected by proteomics studies, were identified and were proposed to play a role in host defense, whilst a number of other proteins had proposed secondary roles in immune protection. An important immunological protein, cathelicidin, is expressed in tammar milk from day 0 to 137. Cathelicidins are small peptides (<50 amino acids) that share a conserved precursor peptide sequence, although the mature peptide can vary enormously in sequence, length, and secondary structure. Cathelicidins are important in mucosal defenses in both adults and infants owing to their antimicrobial and immunoregulatory roles and play a role in host defense as effector molecules and as modulators of innate immunity. Antimicrobial peptides, such as cathelicidin, are an evolutionarily old defense system. However, they have more complex actions than just simply their antimicrobial effects, which include immunoregulation and interaction with the adaptive immune system. A total of seven novel tammar cathelicidin peptides have been identified from the tammar mammary gland, reflecting the ability for diverse immune and antimicrobial protection provided by milk.

Few immune-related proteins have been identified in monotreme milk. However, transcript analysis of echidna milk cells by Sharp and Nicholas has suggested that echidna milk contains LECT2, a protein that has previously been shown to be associated with the acute-phase response of the innate immune system, and polymeric immunoglobulin receptor (PIGR), a protein that plays a major role in the transport mechanism of IgA and IgM.

The presence of these proteins suggests that like marsupial and eutherian milk, monotreme milk also plays a role in immune protection of the young.

Egg Proteins

A plausible hypothesis has suggested that mammary secretions originated to provide moisture and nutrients to hatchlings of monotremes, allowing a progressive decline in egg size, which was accompanied by the young hatchlings becoming more altricial. This hypothesis can be extended to include the possibility that ancient mammary secretions may have resembled egg white, enabling hatchlings to emerge from the egg at an earlier stage without compromising on consumption of nutrients or growth factors. Analysis of ESTs within echidna and platypus milk cells shows that ovostatin-2 (OVOS2), a minor protein present in avian egg white, is secreted by these cells into milk and gives support to this hypothesis.

Biological Activity of Milk

Milk not only does provide nutrition to the suckling young, but also has a more comprehensive role in programming and regulating growth and development of the suckling young, and an autocrine impact on the mammary gland so that it functions appropriately during the lactation cycle. Many of the tammar wallaby genes encode secreted proteins that are differentially expressed at specific phases of lactation and have a variety of bioactivities. Many studies have shown that the progressive changes in the milk profile across the long lactation are necessary to meet the nutritional needs of the growing pouch young, making the tammar wallaby a unique model to study the role of these proteins. Indeed, cross-fostering experiments in which younger pouch young at day 60 were fostered onto mothers secreting milk at day 120 of lactation, suggesting that the tammar milk may contain factors that dramatically accelerated growth of the fostered pouch young.

Identification of potential bioactives has been possible in the tammar wallaby by interrogation of the tammar EST database and microarray data to identify cDNAs coding for proteins that contained a signal peptide. This approach identified 75 novel genes that were differentially expressed during different phases of lactation and therefore had the potential to have different bioactive properties. To determine the functional significance of these genes, individual proteins encoded by each cDNA were synthesized *in vitro* and subsequently shown to have activity for immune modulation, inflammatory responses, growth, and differentiation effects. Some of these synthesized proteins showed a capacity to alter the development of mammospheres – a culture model that employs primary mammary cells to generate three-dimensional (3D) structures that functionally and morphologically resemble the secretory unit of the mammary gland – by increasing the size of the mammospheres and the rate of mammosphere formation. This raised the possibility that these milk proteins may contribute to growth and function of the mammary gland during lactation.

Tammar milk has also been observed to affect the gut maturation of the young. The normal stomach is lined with a self-renewing epithelial monolayer that is organized into pit/gland units, and each pit/gland contains a heterogeneous population of cells that originate from the stem cells the cytodifferentiation of which is influenced by factors from the external milieu. In the tammar wallaby, it has been reported that dramatic changes in stomach tissue morphology take place during the suckling period. Changes in fore-stomach morphology during the development of tammar wallaby have been correlated with significant changes in milk composition, raising the possibility that these processes may be regulated by specific milk components. In a recent study, tammar wallaby pouch young at day 120 were cross-fostered to host mothers at day 170 of lactation. In the fore-stomach of the fostered pouch young, the parietal cell population was significantly reduced and the expression of gastric glandular phenotype markers (*ATP4A*, *GKN2*, *GHRL*, and *NDRG2*) downregulated, suggesting downregulation of the gastric phenotype. However, the expression of cardia glandular phenotype genes (*MUC4*, *KRT20*, *CSTB*, *ITLN2*, and *LPLUNC1*) was not affected. There was also an increased apoptosis, but no change in cell proliferation. These data suggest that fore-stomach maturation is driven by milk composition and proceeds via two temporally distinct processes: downregulation of gastric glandular phenotype and initiation of cardia glandular phenotype. In the fostered pouch young, these two processes appear uncoupled, as gastric glandular phenotype is downregulated, but cardia glandular phenotype is not initiated. It remains to be determined what factors regulate these two apparently distinct processes.

Functions of milk proteins such as BLG and WAP have remained obscure; however, the divergent nature of *WAP* gene structure and expression profile across various monotreme, marsupial, and eutherian species suggests that the protein is still evolving. In some species such as marsupials, monotremes, and specific eutherians, WAP is a major whey protein, whereas it is completely absent in others species such as ruminants. In eutherians that express *WAP*, the protein is produced consistently throughout lactation; in contrast, *WAP* is expressed only transiently during the lactation period of the tammar. Its presence in the milk of other marsupials such as the brushtail possum, red kangaroo, and stripe-faced dunnart, which also demonstrate significant changes in milk composition during lactation, suggests that in these species it may be regulated in a similar way as in tammar. Although the amino acid sequences of WAP from the two monotremes, platypus and echidna, have also been reported, the pattern of secretion in these species is yet to be established. The transient expression pattern of *WAP* in the tammar suggests that it may either play a role in development of the mammary gland or influence development in the young.

The WAP proteins identified from all species show moderate sequence similarity at the amino acid level and contain a signal peptide and a varying number of four-disulfide core (4-DSC) domains, which are characteristic of the Kunitz family of protease inhibitors. The Kunitz family has over 150 members that contain from one to three 4-DSC domains. Although a large functional diversity exists between these proteins, typically the 4-DSC domain is associated with protease inhibitor activity. The families of proteins containing these domains are grouped according to tissue specificity and functions, such as serine protease inhibitors, pancreatic trypsin inhibitors, inter-alpha-trypsin inhibitors, elastase inhibitors, chymotrypsin inhibitors, and hepatocyte growth factor activator inhibitors. However, WAP has not been shown to possess any of these properties.

Interestingly, the number of 4-DSC domains differs between eutherian, marsupial, and monotreme WAPs. Eutherian WAP encodes two 4-DSC domains, designated Domain I and Domain II (DI-DII). WAP DI has a characteristic SSSE sequence, which is conserved in most species and is absent in DII. Marsupial WAP encodes an additional 4-DSC domain, designated Domain III (DIII), at the N-terminus of the WAP protein, so that the domain structure has the configuration DIII-DI-DII. Monotreme WAPs also encode the DIII; however, there is limited similarity within the other domains. Echidna WAP has the structure DIII-DII, whereas platypus WAP has the structure DIII-DII-DII (**Figure 6**).

In mice, WAP has been implicated in alveolar development and has been shown to impair the proliferation of mouse epithelial cells and mammary tumor cell lines *in vitro* by inhibiting cell cycle progression. The inhibitory action of WAP on the proliferation of mouse epithelial cells was also demonstrated to be enhanced by the presence of extracellular matrix. This is consistent with studies showing that over-expression of WAP in transgenic mice inhibits development of the mammary gland and secretion of milk. In contrast, in studies at Deakin University laboratory, Geelong,VIC, Australia, using full-length tammar WAP and DIII added to culture media stimulated proliferation of tammar mammary epithelial cells. In addition, WAP increases expression of the cell cycle genes cyclin D1 and CDK-4, stimulates DNA synthesis in primary cultures of mammary epithelial cells, and promotes *in vitro* growth of 3D alveoli-like structures (or mammospheres), which mimic *in vivo* mammary gland development and function. These studies also showed that this stimulation is entirely due to the presence of the third tammar WAP domain (DIII), which is absent in eutherian *WAP* genes. Earlier studies have shown that DNA synthesis in the tammar mammary gland is higher in phase 2 than in phase 3, which is consistent with a potential role of tammar WAP in the development of the mammary gland. As tammar WAP is a major milk protein secreted during the middle third of lactation when the pouch young's diet comprises only milk, it could be speculated that this protein plays a specific role in the development of both the mammary gland and the suckling young at this time. Interestingly, studies in *WAP* knockout mice have demonstrated that the expression of the *WAP* gene is not required for alveolar specification and functional differentiation, and is dispensable as a protease inhibitor to maintain the stability of secretory proteins in milk. This study, however, found that pups failed to thrive in the second half of lactation, suggesting that WAP may be essential for the adequate nourishment of the growing young. From the above-mentioned studies it is clear that marsupial WAP and eutherian WAP have quite different roles in milk; the role of WAP in monotreme milk is yet to be determined. However, WAP is not found in all eutherian milk; it is absent in the milks of cows, sheep, and humans. The role of WAP in milk is therefore species specific, and it appears that this role is dispensable in some species. As a consequence, it has been postulated that the WAP protein is slowly being lost during the process of evolution in eutherians.

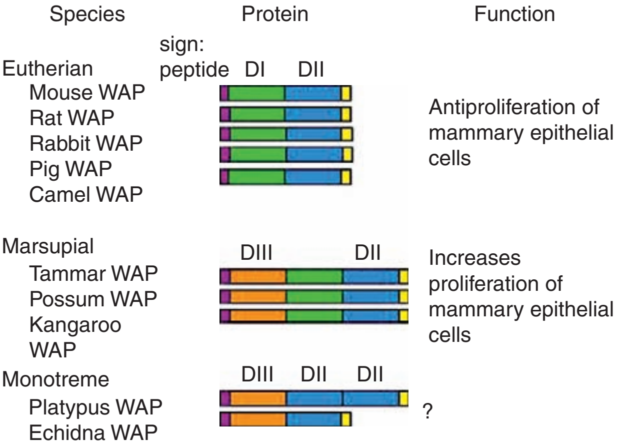

Figure 6 The diverse structure and function of eutherian, marsupial, and monotreme WAP proteins. Eutherian WAP contains two domains, whereas the marsupial and monotreme WAPs contain three domains. The difference in protein domain structure has been shown to account for the difference in WAP function between lineages.

Autocrine Factors in Milk

Milk-borne factors involved in the control of mammary gland function have been reported in a number of species, including the tammar wallaby. There is increasing evidence to suggest that milk plays an important role in regulating mammary epithelial function and survival, and this is particularly evident during involution. For example, apoptosis has been induced preferentially in the sealed teats of lactating mice, whereas the litter suckled successfully on the remaining teats, indicating that cell death is stimulated by an intramammary mechanism sensitive to milk accumulation. A protein known as feedback inhibitor of lactation (FIL) has been identified in the tammar wallaby as well as in a number of domestic species including cows, goats, and rabbits, and is thought to control milk production by responding to the frequency and completeness of milk removal, acting specifically through interaction with the apical surface of the mammary epithelial cell to reduce milk secretion.

Recent studies using small pieces of mammary tissue maintained in culture, known as the explant culture model, have confirmed a role for milk in the process of involution showing that milk directly acts on the mammary tissue during involution. Mammary explants from pregnant tammar wallabies were cultured with lactogenic hormones (insulin, cortisol, and prolactin) to induce milk protein gene expression; subsequent removal of all hormones for 10 days downregulated the expression of the milk protein genes and mimicked involution. Surprisingly, the explants retained the same level of lactogenic response during a subsequent challenge with lactogenic hormones. The maintenance of epithelial cell viability and hormone responsiveness in explants cultured in the absence of hormones is consistent with the idea that accumulation of local factors in the milk is the primary stimulus for apoptosis of mammary epithelial cells in the tammar wallaby mammary gland. This model shows how hormones and mammary gland involution are uncoupled, and provides evidence for the extraordinary capacity for survival and maintenance of hormone responsiveness exhibited by mammary epithelial cells cultured in a chemically defined medium in the absence of exogenous hormones and growth factors.

The authors have recently identified an apoptotic role for another key milk protein, α-lactalbumin, which is proposed to regulate mammary function by causing apoptosis of the gland following milk stasis. We have already discussed the α-lactalbumin protein in relation to lactose synthesis; thus it appears that α-lactalbumin possesses dual functionality in the lactation process. Analysis of function shows that α-lactalbumin has apoptotic effects on a variety of mammary epithelial cells, including tammar wallaby mammary cells grown in cell culture, and acts to initiate mammary gland regression at the end of lactation. The tammar wallaby has an unusual α-lactalbumin expression profile. As previously discussed, the wallaby milk during peak lactation is devoid of lactose, but α-lactalbumin is secreted in wallaby milk at levels similar to those in other mammal milks throughout lactation. Examination of expression levels of the wallaby α-lactalbumin gene also revealed levels similar to those in other mammals such as pigs and mice during peak lactation. The wallaby, therefore, is a species that has uncoupled α-lactalbumin secretion and lactose production. The continued secretion of α-lactalbumin during wallaby lactation is predicted to be required solely for rapid initiation of mammary gland involution at weaning.

Conclusion

Milk production has evolved over the course of evolution. As such, milk has developed as the most efficient, effective, and adaptable medium for nutrient provision that has risen among vertebrates. Milk has played an important role in the worldwide adaptive radiation of mammals. It has conferred a primary advantage on mammals: the ability to reproduce and nourish their young in any environment that supports the health and well-being of the adult. Milks from species such as the marsupial and the monotreme, whose young rely solely on milk at a time when regulated development is paramount, provide useful insight into the function of milk.

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Milk of Marine Mammals

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The Origin and Evolution of Marine Mammal Lactation

Lactation is a diagnostic mammalian trait, involving the synthesis of a nutrient-rich secretion by specialized mammary glands. Since its initial evolution during the Mesozoic, perhaps more than 200 million years ago (mya), lactation has undergone profound evolutionary divergence among mammalian taxa, resulting in a diversity of lactation patterns characterized by major differences in milk composition, milk yield, suckling patterns, and duration of lactation. In comparison to terrestrial mammals, nearly all species of marine mammals, including seals (**Figure 1**) and whales, produce exceptionally rich milks high in fat and energy. For example, in the order Carnivora, the true seals (family Phocidae) typically produce milks that contain 48–60% fat, while dolphins and whales (order Cetacea) produce milks that typically contain 15–50% fat.

The species included as marine mammals do not form a single phylogenetic group but represent several distinct lineages that made the transition from terrestrial to marine life at different points in time. Given that seals, whales, and manatees are not closely related to each other, one would not expect them, *a priori*, to produce milks similar in composition. Any similarities that do occur represent convergent evolution.

The marine mammals include three major lineages. The dolphins and whales (order Cetacea) derive from the even-toed ungulates (order Artiodactyla). Genetic analysis indicates that the cetaceans are nested within the Artiodactyla, which is therefore sometimes termed the Cetartiodactyla, including cetaceans. The stem group of ancestral cetaceans, the 'Archaeocetes', appears in the fossil record from early to middle Eocene, 52–42 mya. Thus, the milks of cetaceans and other artiodactyls have diverged for at least 52 million years. Subsequently, two major groups of cetaceans, the odontocetes or toothed whales and dolphins, and the mysticetes or baleen whales, diverged about 35 mya. Among living mammals, the odontocetes are much more species-rich (71 species) than the baleen whales (13 species) and typically have longer lactations (**Table 1**). Among mammals, lactation duration typically increases in length with body mass, but the largest mammals of all, the large baleen whales, depart from this trend and have lactation durations of only 5–12 months, as compared to 1–4 years or longer in much smaller odontocetes. All cetaceans give birth in the water to single offspring, which nurse from nipples buried within mammary slits on the posterior abdomen. At least fragmentary data are available on the milk composition of 17 odontocete species and 8 mysticete species (**Table 1**).

A second lineage of marine mammals, the order Sirenia (manatees and dugongs), first appears as early and middle Eocene fossils from 50 mya, or shortly after the appearance of the 'Archaeocetes'. The herbivorous Sirenia are not closely related to either cetaceans or marine taxa of the Carnivora, but are rather nested within a group of African mammals known as the Afrotheria. Among living taxa, the sirenians are most closely related to the Proboscidea (elephants). The placement of the single pair of mammae in the axillary region (armpits) is distinctive. Although there are only four living species of Sirenia (three manatees and the dugong), another much larger species, the *c.* 4–10 tonne Steller's sea cow (*Hydrodamalis gigas*), grazed on algae in the North Pacific until discovered by Europeans in 1741 and then hunted to extinction by 1768. Apparently, milk was never collected from this 'sea cow'. Milk composition has been reported only for the West Indian manatee.

A third lineage of marine mammals evolved within the order Carnivora. The seals, sea lions, and walruses (collectively termed pinnipeds) are represented by three families, Phocidae (true seals), Otariidae (fur seals and sea lions), and Odobenidae (walrus). The pinniped lineage diverged from arctoid carnivores in the late Oligocene (27–25 mya); pinnipeds are most closely related to raccoons (Procyonidae), bears (Ursidae), and weasels (Mustelidae). The phocids appear in the late Oligocene, the walruses (formerly a more diverse group) in the middle Miocene (16–14 mya), and the otariids in the late Miocene (11 mya). The phocids, otariids, and odobenids are currently represented by 18, 16, and 1 species, respectively; the Caribbean monk seal is extinct (**Table 1**). Lactation duration varies greatly among species, from 4 days in the hooded seal to 17 months in Australian sea lions and 24 or more months in the walrus; in general, otariids have longer lactation periods than phocids (**Table 1**). Pinnipeds have 2–6 mammae, primarily abdominal in location and typically inverted. All pinnipeds give birth on land or ice, and as all except the walrus nurse their pups out of the water, they are more accessible for lactation research than other marine mammals. Lactation has been more thoroughly studied in pinnipeds than in any other wild mammal group. At least fragmentary milk composition data

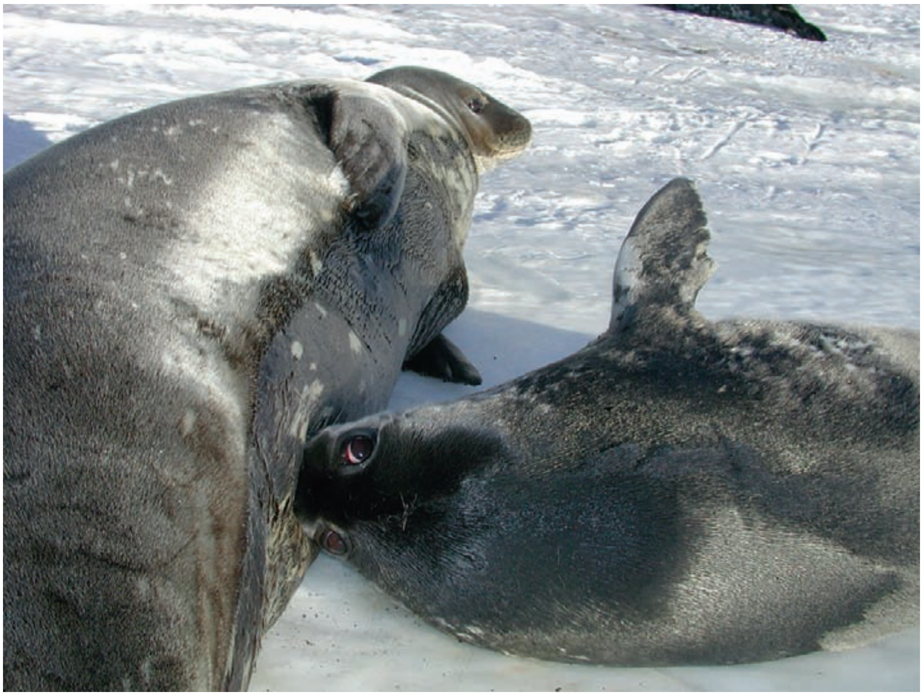

Figure 1 The Weddell seal, the world's southernmost milk-producing animal. Mother and suckling pup at Hutton Cliffs, Ross Island, McMurdo Sound, Ross Sea, Antarctica (78° S, 166° E). Photograph taken by Olav Oftedal under marine mammal permit 763-1485-00 issued by U.S. National Marine Fisheries Service.

Table 1 Lactation in marine mammals

Family	Common name	Scientific binomial	Milk samples	Age at first major solids	Duration of lactation
Order Carnivora (carnivores, 286 spp.)					
Mustelidae (weasels, otters, 59 spp., 2 marine)					
	Sea otter[a]	*Enhydra lutris*	Yes	1 month	6 months
	Marine otter	*Lontra felina*	No	??	??
Ursidae (bears, 8 spp., 1 marine)					
	Polar bear	*Ursus maritimus*	Yes	3–6 months	18–34 months
Odobenidae (walruses, 1 sp., marine)					
	Walrus	*Odobenus rosmarus*	Yes	5–6 months	12–24 months
Otariidae (fur seals, sea lions, 16 spp., all marine)					
	South American fur seal	*Arctocephalus australis*	Yes	??	12 months
	New Zealand fur seal	*Arctocephalus forsteri*	Yes	??	12 months
	Galapagos fur seal	*Arctocephalus galapagoensis*	Yes	??	11 months
	Antarctic fur seal	*Arctocephalus gazella*	Yes	At weaning	4 months
	Juan Fernandez fur seal	*Arctocephalus philippii*	Yes	??	7 months
	Australian/Cape fur seal	*Arctocephalus pusillus*	Yes	4–7 months	9 months
	Subantarctic fur seal	*Arctocephalus tropicalis*	Yes	??	10 months
	Northern fur seal	*Callorhinus ursinus*	Yes	At weaning	4 months
	Steller sea lion	*Eumetopias jubatus*	Yes	??	4–12 months
	Australian sea lion	*Neophoca cinerea*	Yes	??	17 months
	California sea lion	*Zalophus californianus*	Yes	4–5 months	11 months
	Galapagos sea lion	*Zalophus wollebaeki*	Yes	??	24 months
Phocidae (true seals. 18 spp., all marine or freshwater)					
	Hooded seal	*Cystophora cristata*	Yes	Postweaning	4 days
	Bearded seal	*Erignathus barbatus*	Yes	13–14 days	24 days
	Gray seal	*Halichoerus grypus*	Yes	Postweaning	17 days

(*Continued*)

Table 1 (Continued)

Family	*Common name*	*Scientific binomial*	*Milk samples*	*Age at first major solids*	*Duration of lactation*
	Weddell seal	*Leptonychotes weddellii*	Yes	45 days?	40–50 days
	N. elephant seal	*Mirounga angustirostris*	Yes	Postweaning	26 days
	S. elephant seal	*Mirounga leonina*	Yes	Postweaning	23 days
	Mediterranean monk seal[b]	*Monachus monachus*	No	??	119 days
	Harp seal	*Pagophilus groenlandicus*	Yes	Postweaning	12 days
	Harbor seal	*Phoca vitulina*	Yes	Postweaning	27 days
	Ringed seal	*Pusa hispida*	Yes	??	39 days
Order Cetacea (dolphins and whales, 84 spp., all marine or freshwater)					
Suborder Odontoceti (toothed whales, 71 spp.)					
Delphinidae (dolphins, 34 spp.)					
	Common dolphin	*Delphinus delphis*	Yes	??	16 months
	Long-finned pilot whale	*Globicephala melas*	Yes	6–9 months	24 months?
	Humpback dolphin	*Sousa chinensis*	Yes	??	??
	Spotted dolphin	*Stenella attenuata*	Yes	3–7 months	20 months
	Atlantic spotted dolphin	*Stenella frontalis*	Yes	??	??
	Spinner dolphin	*Stenella longirostris*	Yes	??	11–34 months
	Bottlenose dolphin	*Tursiops truncatus*	Yes	4–11 months	19 months
Iniidae (river dolphins, 3 spp.)					
	Amazon River dolphin	*Inia geoffrensis*	Yes	??	??
	Franciscana dolphin[c]	*Pontoporia blainvillei*	Yes	??	9 months
Monodontidae (beluga, narwhal, 2 spp.)					
	Beluga whale	*Delphinapterus leucas*	Yes	12 months	20–24 months
Phocoenidae (porpoises, 6 spp.)					
	Harbor porpoise	*Phocoena phocoena*	Yes	2–3 months	8–12 months
	Dall's porpoise	*Phocoenoides dalli*	Yes	??	??
Physeteridae (sperm whales, 3 spp.)					
	Pygmy sperm whale	*Kogia breviceps*	Yes	??	??
	Dwarf sperm whale	*Kogia simus*	Yes	??	??
	Great sperm whale	*Physeter macrocephalus*	Yes	12 months	25 months
Ziphiidae (beaked whales, 21 spp.)					
	Bottlenose whale	*Hyperoodon* sp.	Yes	??	??
	Stejneger's beaked whale	*Mesoplodon stejnegeri*	Yes	??	12 months
Suborder Mysticeti (baleen whales, 13 spp.)					
Balaenidae (right whales, 4 spp.)					
	Bowhead whale	*Balaena mysticetus*	Yes	??	9–12 months
Balaenopteridae (rorquals, 7 spp.)					
	Minke whale	*Balaenoptera acutorostrata*	Yes	5–6 months	5–6 months
	Sei whale	*Balaenoptera borealis*	Yes	6–7 months	6–7 months
	Bryde's whale	*Balaenoptera edeni*	Yes	??	??
	Blue whale	*Balaenoptera musculus*	Yes	6–7 months	6–7 months
	Fin whale	*Balaenoptera physalus*	Yes	6–7 months	6–7 months
	Humpback whale	*Megaptera novaeangliae*	Yes	5–6 months	10–11 months
Eschrichtiidae (gray whale, 1 sp.)					
	Gray whale	*Eschrichtius robustus*	Yes	6–7 months	7–8 months
Order Sirenia (manatees, dugongs, 5 spp., all marine or freshwater)					
Trichechidae (manatees, 3 spp.)					
	West Indian manatee	*Trichechus manatus*	Yes	3–5 months	12–24 months

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[b] Aguilar A, Cappozzo LH, Gazo M, Pastor T, Forcada J, and Grau E (2007) Lactation and mother–pup behaviour in the Mediterranean monk seal *Monachus monachus*: An unusual pattern for a phocid. *Journal of the Marine Biological Association of the United Kingdom* 87: 93–99.

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are available for 12 of 16 otariids, 9 of the 18 phocids, and on the sole odobenid (the walrus) that is, on 63% of the extant pinniped species. By comparison, among the approximately 250 species of non-pinniped carnivores, in only 12 species (5%) have at least 3 samples of mid-lactation milk been assayed (**Table 2**).

The order Carnivora also includes three 'newer' marine mammals, the marine and sea otters and the polar bear (**Table 1**). They arose in the Pleistocene (*c.* 1–3 mya), but remain closely related to other lutrine otters and other bears of the genus *Ursus*, respectively. Milk data are available for sea otters and polar bears.

Factors Affecting the Composition of Marine Mammal Milks

The collection of milk samples from wild mammals requires that animals be captured and restrained (**Figure 2**), or chemically immobilized. Marine mammals often react adversely to chemical immobilization, but whether this affects the composition of collected milk is not known. During the heyday of whaling, milk samples were taken during the processing of carcasses, and samples have been obtained from stranded animals. For such samples, there is always concern that post-mortem or pathological changes may result in abnormal results, especially for labile constituents such as carbohydrates or constituents that may diffuse into or out of damaged mammary glands, such as sodium and potassium. More recently, captive animals, such as bottlenose dolphins, have been trained to present for milk collection. Even in this situation, seawater contamination can occur, leading to elevated levels of water, sodium, and magnesium. Another potential error could arise from incomplete mammary evacuation, which in some terrestrial species can lead to underestimation of milk fat. In seals, milk composition does not change during the course of mammary evacuation, but this has not been examined in otters, bears, cetaceans, or sirenians. Thus, the magnitude of sampling bias in the data in **Tables 2–4** is unknown.

Marine mammal milks change in composition according to lactation stage. In most species of pinnipeds and cetaceans, fat and energy contents rise, and water content falls, from early to mid-lactation (**Figure 3**). In some species, fat and energy contents then remain high to the end of lactation, while in others a modest decline in fat and energy content may occur toward the end of lactation. In polar bears, milk fat content is constant at about 32–36% from 3 to 4 months until weaning, or may decline when bears return onto land from the sea ice.

Other constituents may also change with lactation stage. The protein content of pinniped and cetacean milks may rise somewhat over lactation, but the degree and direction of change vary among species and may even vary with maternal condition. In general, milk composition should be compared across species at similar stages of lactation to avoid confounding effects.

An additional complication occurs in sea lions and fur seals (family Otariidae). After a short (*c.* 1 week) post-parturient period ashore, mothers undertake foraging trips to sea. The pups remain on land or in shallow pools, fasting, until the mothers return. Foraging trip length varies by species, breeding population, and maternal condition, with trips lasting less than a day in some sea lions or as much as 23 days in the sub-antarctic fur seal. During the foraging trip, the rate of milk protein synthesis and overall milk production declines and the composition of secreted milk changes. On return to the breeding colony, the first milk provided to the pup is considerably higher in fat and protein than milk provided on subsequent days ashore. Some authors consider this a case of two separate milks (milk on arrival vs. milk obtained by subsequent suckling), but it is more likely a continuous process whereby secretory processes are up- and down-regulated according to the stage of the foraging/nursing cycle. Nonetheless, this complicates determination of the 'average' milk composition.

The Proximate Composition of Marine Mammal Milks

The summary data in **Tables 2–5** were selected from available data as those that best represent milk composition at the period of maximal milk production or peak lactation. Although milk production has been measured at different lactation stages only in a few pinniped species, peak production can be inferred from the pattern of offspring growth and behavior. Offspring growth can be sustained only if the increased energy needs associated with increasing size are matched by an increase in milk yield, an increase in milk energy/nutrient density, or the ingestion of solid foods. Peak lactation is estimated as the period preceding the onset of substantial food intake by the young (**Table 1**), as determined by observation or examination of stomach contents.

The adaptation to marine life has apparently entailed changes in milk composition, but to discriminate such changes from simple phylogenetic variation due to long periods of separate evolution, milk composition needs to be compared to the closest living relatives of marine mammals. Thus in **Tables 2–4** compositional data are provided for terrestrial carnivores, a subset of the artiodactyls, and elephants as the closest living relatives of marine carnivores, cetaceans, and sirenians, respectively.

Both the polar bear and sea otter, species that have recently diverged from terrestrial counterparts, produce milks that are higher in fat and energy than other

Table 2 Milk composition of terrestrial and marine Carnivora

Family and species	*N*	*Lactation stage (days)*	*Water (%)*	*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Sugar (%)*	*Ash (%)*	*Gross energy MJ kg^{-1}*
Canidae (wolves, foxes, 35 spp.)									
Arctic fox	∼100	Mid?	71.4	28.6	13.5	11.1	3.0	1.0	8
Dog (domestic)	25	7–37	77.3	22.7	9.5	7.5	3.8	1.1	6
Raccoon dog	22	7–59	81.4	18.6	3.4	7.8		1.1	
Red fox	3	28–35	81.9	18.1	5.8	6.7	4.6	0.9	5
Ursidae (bears, 8 spp.)									
Brown bear	9	60–98	68.1	31.9	17.1	9.2	2.2	1.5	9
Black bear	6	60–90	62.4	37.6	25.1	7.0	3.0	1.8	12
Polar bear[a]	**7**	**210–550**	**52.0**	**48.0**	**33.0**	**11.1**	**0.3**	**1.4**	**16**
Mephitidae (skunks, 13 spp.)									
Striped skunk	15	20–48	69.4	30.6	13.8	9.9	3.0		8
Mustelidae (weasels, otters, 59 spp.)									
Ferret	18	11–25			9.7	6.9	3.8		6
American mink	20	10–27	78.3	21.7	7.3	5.6	4.5	1.0	5
Sea otter[b]	**3**	**Mid-late**	**59.8**	**40.2**	**23.7**	**11.7**	**0.9**	**0.8**	**12**
Felidae (cats, 40 spp.)									
Cat (domestic)	15	6–38			10.8	10.6	3.7	1.0	7
Serval	3	56–77	70.7	29.3	15.3	15.8	0.7		10
African lion	6	45–90	73.2	26.8	8.7	11.8	3.2		7
Odobenidae (walruses)									
Walrus[c]	**3**	**Early (<30)**	**61.0**	**39.0**	**24.1**	**9.2**	**0.0**	**0.6**	**12**
Otariidae (fur seals, sea lions)									
South American fur seal	**4**	**∼150**	**45.6**	**54.4**	**44.4**	**9.7**			**20**
Antarctic fur seal[d]	**27**	**∼20–100**	**42.3**	**57.7**	**42.4**	**10.5**	**0.1**		**19**
Juan Fernandez fur seal[e]	**23–44**	**1–110**	**44.9**	**55.1**	**41.4**	**11.9**	**1.2**	**0.7**	**19**
Australian fur seal[f]	**>20**	**∼80–150**	**43.6**	**56.4**	**42.8**	**10.2**		**0.8**	**19**
Subantarctic fur seal[g]	**74–103**	**20–180**	**38.2**	**61.8**	**47.1**	**12.8**			**21**
Northern fur seal	**5**	**30–120**	**36.7**	**63.3**	**50.7**	**10.3**	**0.1**		**22**
Australian sea lion	**20–38**	**14–125**	**62.4**	**37.6**	**25.4**	**10.5**		**0.9**	**12**
Southern sea lion[h]	**4**	**18–20**	**63.5**		**25.8**	**8.6**		**1.1**	**12**
California sea lion	**12**	**90–120**	**45.8**	**54.2**	**43.7**	**8.9**	**0.6**		**19**
Phocidae (true seals)									
Hooded seal[i]	**15**	**2–4**	**30.2**	**69.8**	**61.1**	**4.9**	**1.0**	**0.5**	**25**
Bearded seal[j]	**3**	**∼10–20**	**41.0**	**59.0**	**48.0**	**10.0**			**21**
Gray seal	**13**	**8–15**	**28.9**	**71.1**	**59.8**	**8.9**			**25**
Weddell seal	**7**	**10–45**	**33.8**	**66.2**	**53.6**	**8.9**	**0.0**	**-**	**23**

(*Continued*)

Table 2 (Continued)

Family and species	*N*	*Lactation stage (days)*	*Water (%)*	*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Sugar (%)*	*Ash (%)*	*Gross energy MJ kg^{-1}*
N. elephant seal	20–24	20–28	34.2	65.8	51.9	10.2	<0.025		23
S. elephant seal[k]	26	10–20	37.0	63.0	47.9	9.6	2.6	0.7	21
Harp seal[l]	8	10–13	34.3	65.7	53.5	7.7	0.8		23
Harbor seal[m]	42	7–21	37.8	62.2	49.8	9.1			22
Ringed seal[n]	3	~15–35	48.6	51.4	38.1	9.9		1.0	17

[a] Derocher AE, Andriashek D, and Arnould JPY (1993) Aspects of milk composition and lactation in polar bears. *Canadian Journal of Zoology* 71: 561–567.

[b] Jenness R, Williams DT, and Mullin RJ (1981) Composition of milk of the sea otter (*Enhydra lutris*). *Comparative Biochemistry and Physiology* 70A: 375–379.

[c] Fay FH (1982) *Ecology and Biology of the Pacific Walrus*, Odobenus rosmarus divergens. North American Fauna No. 74, pp. 1–279. Washington, DC: U.S. Fish and Wildlife Service.

[d] Arnould JPY and Boyd IL (1995) Inter- and intra-annual variation in milk composition in Antarctic fur seals. *Physiological Zoology* 68: 1164–1180.

[e] Ochoa-Acuña H, Francis JM, and Oftedal OT (1999) Influence of long intersuckling interval on composition of milk in the Juan Fernandez fur seal, *Arctocephalus philippii*. *Journal of Mammalogy* 80: 758–767.

[f] Arnould JPY and Hindell MA (1999) The composition of Australian fur seal (*Arctocephalus pusillus doriferus*) milk throughout lactation. *Physiological and Biochemical Zoology* 72: 605–612.

[g] Georges J-Y, Groscolas R, Guinet C, and Robin J-P (2001) Milking strategy in subantarctic fur seals *Arctocephalus tropicalis* breeding on Amsterdam Island: Evidence from changes in milk composition. *Physiological and Biochemical Zoology* 74: 548–559.

[h] Werner W, Figueroa-Carranza A-L, and Ortiz CL (1996) Composition and energy content of milk from Southern sea lions (*Otaria flavescens*). *Marine Mammal Science* 12: 313–317.

[i] Oftedal OT, Boness DJ, and Bowen WD (1988) The composition of hooded seal (*Cystophora cristata*) milk: An adaptation to postnatal fattening. *Canadian Journal of Zoology* 66: 318–322. Lydersen C, Kovacs KM, and Hamill MO (1997) Energetics during nursing and early postweaning fasting in hooded seal (*Cystophora cristata*) pups from the Gulf of St. Lawrence, Canada. *Journal of Comparative Physiology B* 167: 81–88.

[j] Lydersen C, Kovacs KM, Hammill MO, and Gjertz I (1996) Energy intake and utilisation by nursing bearded seal (*Erignathus barbatus*) pups from Svalbard, Norway. *Journal of Comparative Physiology B* 166: 405–411.

[k] Carlini AR, Marquez MEI, Soave G, Vergani DF, and Ronayne de Ferrer PA (1994) Southern elephant seal *Mirounga leonina*: Composition of milk during lactation. *Polar Biology* 14: 37–42.

[l] Oftedal OT, Bowen WD, and Boness DJ (1996) Lactation performance and nutrient deposition in pups of the harp seal, *Phoca groenlandica*, on ice floes off southeast Labrador. *Physiological Zoology* 69: 635–657.

[m] Lang SLC, Iverson SJ, and Bowen WD (2005) Individual variation in milk composition over lactation in harbour seals (*Phoca vitulina*) and the potential consequences of intermittent attendance. *Canadian Journal of Zoology* 83: 1525–1531.

[n] Lydersen C, Hammill MO, and Ryg MS (1992) Water flux and mass gain during lactation in free-living ringed seal (*Phoca hispida*) pups. *Journal of Zoology, London* 228: 361–369.

Unless otherwise specified, data from Oftedal OT and Iverson SJ (1995) Phylogenetic variation in the gross composition of milks. In: Jensen R (ed.) *Handbook of Milk Composition*, pp. 749–789. New York: Academic Press.

Species in bold are considered marine mammals. See **Table 1** for scientific binomials. *N* refers to number of milk samples assayed.

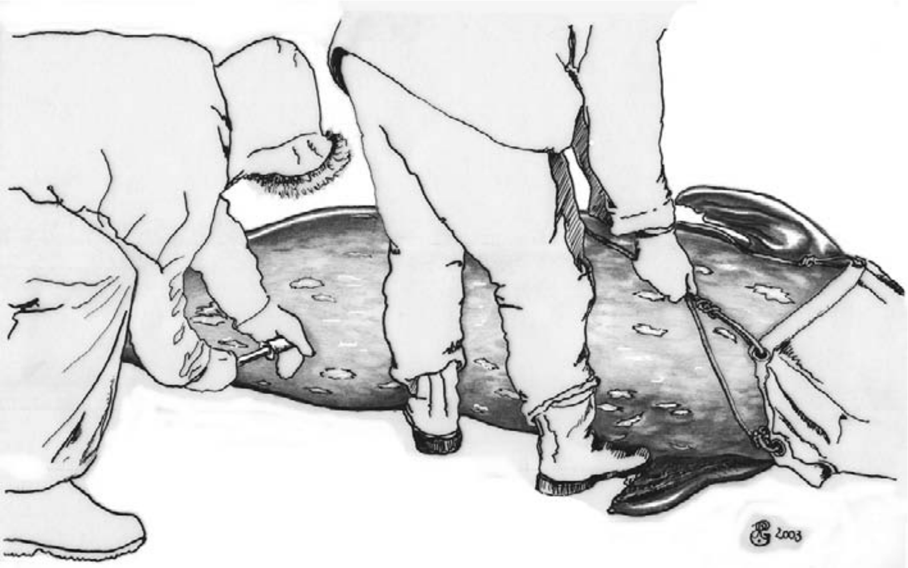

Figure 2 Milk collection from the Weddell seal in Antarctica. Lactating seal (*c.* 450 kg) is manually restrained in a head bag, injected intramuscularly with oxytocin, and milk is collected by pumping a cutoff 60 ml syringe placed over the inverted nipple. Drawing by Regina Eisert.

terrestrial carnivores, except bears (**Table 2**). The high fat and low sugar contents of bear milk are believed to be important to preservation of lean body mass during 'hibernation', when bears are simultaneously lactating and fasting. Bear milk was thus 'preadapted' to a marine lifestyle (see below); such a process is termed exaptation in evolutionary biology. Unfortunately, there are no reliable data on milks of other otters with which to compare sea otters, although mink, which are quite aquatic, do not have high milk fat levels.

Seven of nine otariids (fur seals and sea lions) and eight of nine phocids (true seals) that have been studied produce extremely high-fat (40–60%) and high-energy (19+ MJ kg^{-1}) milks (**Table 2**). No other terrestrial mammals produce milks that come close to these levels, although some shrews, tree shrews, bears, and bats produce milks containing about 30% fat and 12–15 MJ kg^{-1} energy. Even walrus, Australian sea lion, and southern sea lion milks, although not as energy dense as other otariids or phocids, are still high in fat (24–26%) and energy (12 MJ kg^{-1}) compared to terrestrial carnivores. Pinniped milks are less variable in protein content (about 9–12%) and these levels are matched by such terrestrial carnivores as Arctic fox, striped skunk, and various cats (**Table 2**). The reported sugar levels are usually low (0.1–1.2%), although at least one species appears to have more than 2% (**Table 2**).

The milks of the cetaceans differ greatly from those of the terrestrial Artiodactyla to which they are related (**Table 3**). Artiodactyl milks typically contain 4–10% fat and 3–8 MJ kg^{-1} energy, as compared to 10–30% fat and 7–14 MJ kg^{-1} energy in dolphins and toothed whales (Odontoceti), and 30–44% fat and 15–19 MJ kg^{-1} energy in baleen whales (Mysticeti) (**Table 3**). Like the pinnipeds, cetaceans produce milks containing about 8–12% protein (as compared to 3–9% in artiodactyls). Cetacean milks are also low in total sugar (1–2.5%).

Manatee milk is higher in fat, protein, and energy, but lower in sugar, than the milks of elephants, to which it is related (**Table 4**); manatee milk resembles the milks of dolphins and toothed whales in gross composition (**Table 3**).

Thus, all marine mammals produce milks that are higher in fat and energy than the terrestrial taxa to which they are most closely related. It is likely that this is a necessity due to the thermoregulatory challenge of a marine environment. A newborn mammal is not only small but typically has less subcutaneous body fat that can provide insulation against heat loss upon immersion in water. High-fat, high-energy milks allow rapid postnatal fat deposition, generating insulation, and also supply energy that can be used to sustain high metabolic rates in a thermally demanding environment. Polar bears use snow dens to provide a more benign climate for neonates. Many pinnipeds live in cold temperate or polar environments and pups delay water entry until a substantial blubber layer is developed. Some polar cetaceans, such as the large baleen whales, migrate to warm temperate or subtropical waters for parturition and early nursing. The distribution of manatees is restricted by cold water temperatures.

The highest milk fats (48–61%) are found in phocid seals, which have relatively short lactations, and deposit fat rapidly after birth. Phocid seals that pup on unstable floating ice (such as hooded and harp seals) have particularly

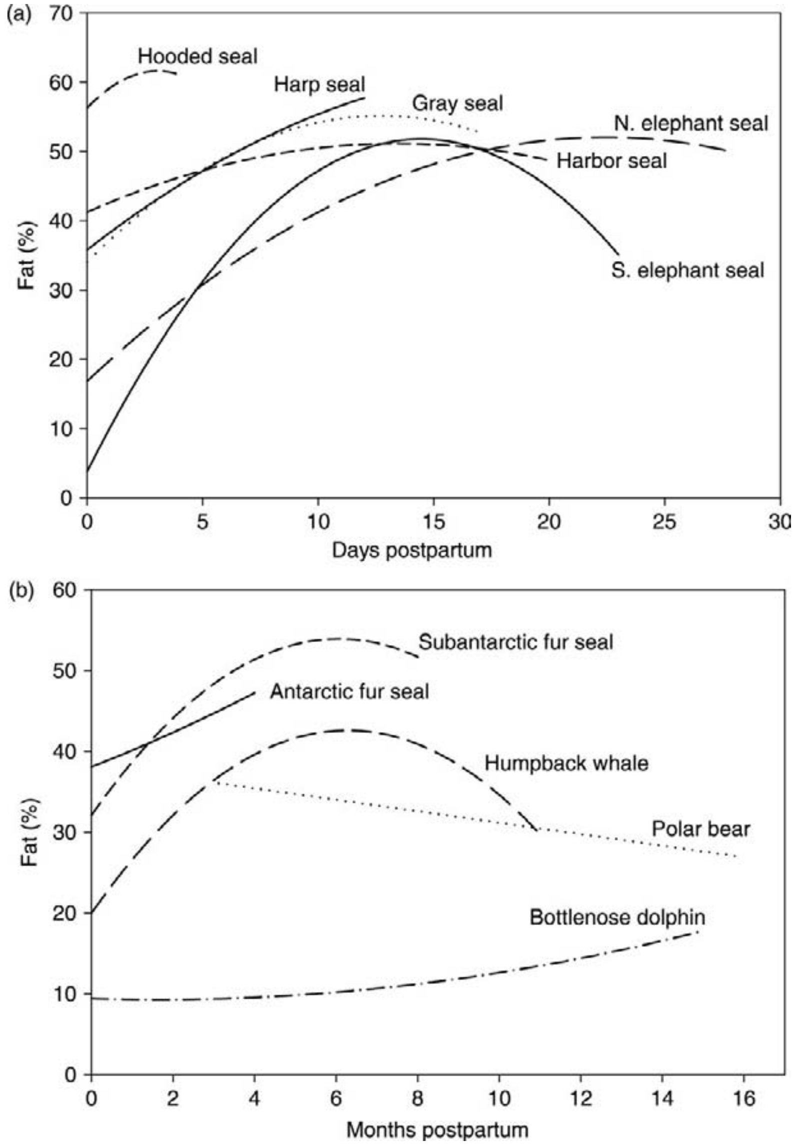

Figure 3 Changes in milk fat composition over the course of lactation in marine mammals. (a) Phocid seals with short lactation periods (<1 month). (b) Other marine mammals with long lactation periods (4–16 months). Data are from references listed for these species in **Tables 2** and **3**, plus Kretzmann MB, Costa DP, and Le Boeuf BJ (1993) Maternal energy investment in elephant seal pups: Evidence for sexual equality? *The American Naturalist* 141: 466–480; Lydersen C and Kovacs KM (1996) Energetics of lactation in harp seals (*Phoca groenlandica*) from the Gulf of St. Lawrence, Canada. *Journal of Comparative Physiology B* 166: 295–304; Mellish JE, Iverson SJ, and Bowen WD (1999) Variation in milk production and lactation performance in grey seals and consequences for pup growth and weaning characteristics. *Physiological and Biochemical Zoology* 72: 677–690.

short and intensive lactations. Hooded seal pups gain 7 kg mass per day, most of which is fat, and are weaned in 4 days, the shortest lactation of any mammal. Hooded seals also produce milk which has the highest fat content (61%) of any mammalian milk. As the mothers of many phocid species fast during lactation, the secretion of high-fat, low-sugar milk may also be important in reducing the glucose demand of the mammary gland, thereby conserving gluconeogenic substrates (especially body proteins).

Most fur seals and sea lions also have high-fat, high-energy milks (**Table 1**). This may be related, in part, to the fact that mothers undertake extended foraging trips to sea during the long lactation period. The pups of otariid seals do not deposit fat as rapidly as phocid pups, partly because when their mothers depart to sea they must use stored energy to cover metabolic costs until she returns. In some species (such as subantarctic and Juan Fernandez fur seals), these maternal foraging trips may be 2–3 weeks in duration, as the mother travels hundreds of kilometers to find suitable prey densities. The high-fat, high-energy milks of otariid seals allow rapid energy transfer during the short period the mother is on land with her pup. Thus,

Table 3 Milk composition of the Artiodactyla and Cetacea

Species	*Scientific binomial*	*N*	*Lactation stage (days)*	*Water (%)*	*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Sugar (%)*	*Ash (%)*	*Gross energy MJ kg^{-1}*
Order Artiodactyla (even-toed ungulates, 240 spp.)										
Bovidae (antelope, goats, sheep, etc., 143 spp.)										
Gayal	*Bos frontalis*	>4	11–50	80.0	20.0	7.0	6.3	5.2		5
Cattle (domestic)	*Bos taurus*[a]	>2000	Mature	87.6	12.4	3.7	3.2	4.6	0.7	3
Goat (domestic)	*Capra hircus*[a]	120	14–56	88.0	12.0	3.8	2.9	4.7	0.8	3
Sable antelope	*Hippotragus niger*	6–8	~30–107	82.1	17.9	5.0	6.2	5.3	0.9	4
Muskox	*Ovibus moschatus*	6	~100	71.5	28.5	14.3	8.7	3.6	1.2	8
Rocky mountain goat	*Oreamnos americanus*	28	14–35	82.0	18.0	7.0	6.5	4.5	0.7	5
+7 spp.										
Camelidae (camels, 4 spp.)										
Bactrian camel	*Camelus bactrianus*	30	23–91	84.8	15.2	4.3	4.3		0.9	
Cervidae (deer, 51 spp.)										
N. American elk	*Cervus elaphus*	28	14–77	81.0	19.0	6.7	5.7	4.2	1.3	5
Mule deer	*Odocoileus hemionus*	24	14–35	81.5	18.5	5.5	7.0	4.5	1.4	5
Reindeer	*Rangifer tarandus*	6	21–30	73.7	26.3	10.9	9.5	3.4	1.3	7
+3 spp.										
Giraffidae (giraffe, okapi, 2 spp.)										
Giraffe	*Giraffa camelopardalis*	3	Mid	85.5	14.5	4.8	4.0		0.8	
Suidae (pigs, 19 spp.)										
Pig (domestic)	*Sus scrofa*[a]	>300	14–35	79.9	20.1	8.3	5.6	5.0	0.9	5
Tayassuidae (peccaries, 3 spp.)										
Collared peccary	*Tayassu tajacu*	4	21–48	83.8	16.2	4.2	5.1	6.2		4
Order Cetacea (84 spp.)										
Suborder Odontoceti (toothed whales)										
Delphinidae (dolphins, *n* = 34 spp.)										
Common dolphin	***Delphinus delphis***	**1**	**Mid**	**58.6**	**41.4**	**30.0**	**10.3**		**0.8**	**14**
Humpback dolphin	***Sousa chinensis***	**1**	**Mid**	**76.7**	**23.3**	**10.2**	**11.3**		**0.8**	**7**
Spotted dolphin	***Stenella attenuata***	**3**	**Mid-late**			**22.5**	**8.4**	**1.2**		**11**
Bottlenose dolphin	***Tursiops truncatus***[b]	**17**	**210–360**	**73.0**	**27.0**	**12.8**	**8.9**	**1.0**		**7**
Iniidae (river dolphins, *n* = 3 spp.)										
Franciscana dolphin	***Pontoporia blainvillei***[c]	**3**	**Mid-late**			**15.6**	**10.3**	**2.5**		**9**
Physeteridae (sperm whales, *n* = 3 spp.)										

(*Continued*)

Table 3 (Continued)

Species	*Scientific binomial*	*N*	*Lactation stage (days)*	*Water (%)*	*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Sugar (%)*	*Ash (%)*	*Gross energy MJ kg^{-1}*
Pygmy sperm whale	***Kogia breviceps***	**1**	**Mid**	**74.3**	**25.7**	**15.3**	**8.2**	**2.2**	**0.8**	**8**
Dwarf Sperm whale	***Kogia simus***	**1**	**Early-mid**	**62.2**	**37.8**	**18.5**	**10.6**		**1.1**	**10**
Great sperm whale	***Physeter macrocephalus***	**7**	**90–180**	**63.8**	**36.2**	**25.7**	**8.5**		**0.6**	**12**
Suborder Mysticeti (baleen whales, *n* = 13 spp.)										
Balaenopteridae (rorquals, *n* = 7 spp.)										
Minke whale	***Balaenoptera acutorostrata***	**16**	**~120–150**	**51.9**	**48.1**	**30.2**	**13.6**		**1.7**	**15**
Blue whale	***Balaenoptera musculus***	**4–7**	**~150–210**	**45.5**	**54.5**	**39.4**	**11.3**	**1.3**	**1.4**	**18**
Fin whale	***Balaenoptera physalus***	**10–12**	**180–210**	**53.2**	**46.8**	**33.4**	**10.6**	**2.1**	**1.2**	**16**
Humpback whale	***Megaptera novaeangliae***	**~3–5**	**~120–210**	**42.9**	**57.1**	**43.8**	**9.1**	**0.7**	**2.1**	**19**

[a] Oftedal OT (1984) Milk composition, milk yield and energy output at peak lactation. A comparative review. *Symposia of the Zoological Society of London* 51: 33–85.

[b] West KL, Oftedal OT, Carpenter C, Krames BJ, Campbell M, and Sweeney JC (2007) Effect of lactation stage and concurrent pregnancy on milk composition in the bottlenose dolphin (*Tursiops truncatus*). *Journal of Zoology* 273: 148–160.

[c] Caon G, Secchi ER, Capp E, and Kucharski LC (2008) Milk composition of franciscana dolphin (*Pontoporia blainvillei*) from Rio Grande do Sul, southern Brazil. *Journal of the Marine Biological Association of the United Kingdom* 88: 1099–1101.

Unless otherwise specified, data from Oftedal OT and Iverson SJ (1995) Phylogenetic variation in the gross composition of milks. In: Jensen R (ed.) *Handbook of Milk Composition*, pp. 749–789. New York: Academic Press; Oftedal OT (1997) Lactation in whales and dolphins: Evidence of divergence between baleen- and toothed-species. *Journal of Mammary Gland Biology and Neoplasia* 2: 205–230.

Marine mammals are indicated in bold.

Table 4 Milk composition of the Sirenia and Proboscidea

Species	*Scientific binomial*	*N*	*Lactation stage (days)*	*Water (%)*	*Dry matter (%)*	*Fat (%)*	*Crude protein (%)*	*Sugar (%)*	*Ash (%)*	*Gross energy MJ kg^{-1}*
Order Proboscidea										
Elephantidae										
Asian elephant	*Elephas maximus*	3	60–120	82.3	17.7	7.3	4.5	5.2	0.6	5
African elephant	*Loxodonta africana*	6	60–80	82.7	17.3	5.0	4.0	5.3	0.7	4
Order Sirenia										
Trichechidae										
West Indian manatee[a]	***Trichechus manatus***	**5**	**210–720**	**76.9**	**23.1**	**14.8**	**8.1**	**0.4**	**1.0**	**8**

[a] Bachman KC and Irvine AB (1979) Composition of milk from the Florida manatee, *Trichechus manatus latirostris*. *Comparative Biochemistry and Physiology A* 62: 873–878. Pervaiz S and Brew K (1986) Composition of the milks of the bottlenose dolphin (*Tursiops truncatus*) and the Florida manatee (*Trichechus manatus latirostris*). *Comparative Biochemistry and Physiology A* 84: 357–360.

Unless otherwise specified, data from Oftedal OT and Iverson SJ (1995) Phylogenetic variation in the gross composition of milks. In: Jensen R (ed.) *Handbook of Milk Composition*, pp. 749–789. New York: Academic Press. Marine mammals are indicated in bold.

otariid pups experience a feast–famine–feast pattern throughout lactation; those species with the longest foraging trips tend to produce the highest fat milks. The high fat and energy density of otariid milks may also be important in maximizing milk energy storage in the mammary glands during the prolonged foraging trips. It appears that rates of milk protein synthesis and milk production decline during otariid foraging trips, but recover once suckling is reinitiated upon return to the pup. The amazing fact that otariid mammary glands do not involute during these prolonged periods between suckling may be related to milk composition (see below). In contrast, phocid mammaries begin to involute if not suckled for a day or more.

Both cetaceans and manatees give birth in the water, and the calf accompanies its mother while she forages. High-fat milk is presumably important both for deposition of insulating blubber and for supporting the energetic (and thermoregulatory) costs of living in water. Among the cetaceans, the toothed species (odontocetes) have long lactations and grow slowly, and therefore fat deposition is also slow. As there is no added burden of a concurrent fast by mother or calf, and no need for rapid fat accumulation by calves prior to weaning, it is perhaps to be expected that the fat and energy levels in odontocete milks are not as high as in most pinniped milks. The baleen whales, including the gargantuan blue whale (the largest living vertebrate), have higher milk fat levels than odontocetes, but unlike odontocetes most species eat little if anything during the first 6 months of lactation. During this period, milk production derives mostly from body reserves, and high-fat, low-sugar milks may limit protein catabolism via minimizing mammary glucose demand, as in phocids and polar bears that fast while lactating.

Other Milk Constituents

Most recent research on marine mammals has focused on understanding reproductive energetics and relatively little attention has been paid to milk constituents other than the primary energy sources.

Lipids

Lipids are by far the most abundant organic component of marine mammal milks, and in some cases the lipids exceed water content (**Tables 2** and **3**). As in other mammals, the milk lipids are packaged into membrane-bound milk fat globules, and at least in the Weddell seal these milk fat globules are relatively large. There has been considerable interest in the fatty acid composition of the lipids (**Table 5**), in part because this may provide indirect information on the prey species eaten by lactating females. Most marine mammal milks are high in long-chain polyunsaturated lipids, including such omega-3 fatty acids as 18:3n3, 20:4n3, 20:5n3, 22:5n3, and 22:6n3, as well as such omega-6 fatty acids as 18:2n6 and 20:4n6 (**Table 5**). The differences among species may reflect differences in diet more than species differences in lipid processing, since the fatty acid composition of marine mammal milks typically reflects both the fatty acid composition in the diet and the fatty acid composition of stored lipids (which reflect previously consumed lipid). The extent to which milk or blubber fatty acids are altered by lipogenesis, preferential mobilization, chain elongation, or desaturation is debated, but this is undoubtedly important in some species, particularly those that ingest low-fat diets (such as manatees). Manatee milk appears to lack the long-chain (C20 and longer) polyunsaturated fatty acids characteristic of pinniped and cetacean milks, but does have short-chain saturates such as 8:0, 10:0, 12:0, and 14:0 (not shown in **Table 5**), as might be expected of a herbivore that generates volatile fatty acids via intestinal fermentation.

Proteins

The crude protein in marine mammal milks represents about 8–12% of the milk by mass, which may seem high by comparison to most terrestrial mammals but is actually low relative to total milk energy. The proportion of milk energy provided by protein is lower in odontocetes and mysticetes than in artiodactyls, and lower in otariids and phocids than in terrestrial carnivores (**Figure 4**). The particularly low percentage of energy provided by protein in phocid milks relates to the pattern of post-natal pup growth, which entails high rates of fat deposition rather than high rates of lean mass gain. About 3–9% of the total nitrogen in marine mammal milks is non-protein nitrogen, and thus crude protein somewhat overestimates true protein.

Marine mammal milks contain both caseins and a variety of whey proteins, as do the milks of terrestrial mammals. The relative proportions of caseins:whey proteins range from 30:70 (in the southern elephant seal) to 70:30 (in fin and blue whales); sea otters (35:65) and polar bears (61:39) are intermediate. It is not known if these differences are associated with differences in the formation and retention of curds in the stomach of the young, although gastric retention of milk solids may be important to allow gastric lipolysis of high-fat milks. Gene transcript sequencing has identified αS1, αS2, β, and κ caseins in fur seal mammaries, although the proportions in milk are not known. Genes for the whey proteins β-lactoglobulin 1 and lysozyme C have also been identified in fur seal mammary tissue. Electrophoretic bands with the mobility of β-lactoglobulin have been found in milks of the northern fur seal, Stejneger's beaked whale, and bowhead whale, and two variants of β-lactoglobulin

Table 5 Fatty acid composition of milk lipids of marine mammals, expressed as mass% of total fatty acids

Family and species	*N*	*Lactation stage (days)*	*14:0*	*16:0*	*16:1*	*18:0*	*18:1*	*18:2n6*	*18:3n3*	*18:4n3*	*20:1*	*20:4n3*	*20:4n6*	*20:5n3*	*22:1*	*22:5n3*	*22:6n3*
Order Carnivora (carnivores)																	
Otariidae (fur seals, sea lions)																	
New Zealand fur seal[a]	160	?	3.8	20.2	5.2	2.9	28.2	1.5	nr	0.4	7.6	1.2	1.2	3.6	0.6	2.4	13.4
Antarctic fur seal	8	15–25	10.3	20.6	12.5	1.6	25.2	1.4	0.3	0.4	2.4	0.6	0.5	11.6	0.5	2.2	6.3
Juan Fernandez fur seal[b]	15	1–110	2.9	18.1	5.4	2.8	29.2	1.1	0.5	0.3	2.3	0.7	1.2	3.7	0.2	3.1	21.3
Australian fur seal	1	90.0	7.0	18.2	6.9	2.5	19.0	1.7	1.0	1.1	6.1	2.3	1.2	5.6	2.3	2.5	16.2
California sea lion	2	30–60	4.5	18.1	6.5	3.0	23.2	1.6	0.9	1.0	2.7	1.2	1.0	8.5	1.3	3.9	19.2
Phocidae (true seals)																	
Hooded seal	10	0–4	4.4	11.7	13.4	2.1	27.0	1.4	0.4	1.2	14.8	0.7	0.3	6.8	4.9	1.9	6.5
Gray seal[c]	18	∼7	4.6	13.1	13.6	1.9	30.2	1.4	0.5	1.1	10.4	nr	0.8	6.7	nr	4.5	10.0
Weddell seal	5	10–18	8.7	13.3	11.8	2.0	38.8	1.7	0.4	1.0	7.5	0.4	0.2	4.7	1.6	0.9	4.1
N. elephant seal	3	25–26	2.7	11.3	4.7	3.1	37.9	1.5	0.3	tr	21.0	0.2	0.4	1.1	6.9	0.9	4.2
S. elephant seal[d]	53	1–23	2.7	10.4	5.0	2.8	30.5	1.3	0.4	1.7	8.2	1.7	0.9	8.4	1.5	5.3	15.9
Harp seal	5	4–9	4.6	8.8	17.4	1.6	23.0	1.1	0.3	0.8	17.2	0.3	0.4	6.1	5.9	3.2	6.5
Order Cetacea (dolphins and whales)																	
Suborder Odontoceti (toothed whales)																	
Delphinidae (dolphins)																	
Bottlenose dolphin	1	Late	3.2	21.1	13.3	3.3	23.1	1.2	0.2	0.2	9.0	0.4	1.4	6.0	2.8	2.0	6.4
Suborder Mysticeti (baleen whales)																	
Balaenopteridae (rorqual whales)																	
Fin whale	1	Late	5.5	22.9	6.5	3.9	24.7	1.1	0.6	1.1	3.1	0.7	0.5	13.9	1.9	3.0	5.7
Order Sirenia																	
Trichechidae (manatees)																	
West Indian manatee[e]	5	>365	6.3	20.2	11.6	0.5	47.0	1.8	2.2								

[a] Baylis AMM and Nichols PD (2009) Milk fatty acids predict the foraging locations of the New Zealand fur seal: Continental shelf versus oceanic waters. *Marine Ecology Progress Series* 380: 271–286.

[b] Ochoa-Acuña H, Francis JM, and Oftedal OT (1999) Influence of long intersuckling interval on composition of milk in the Juan Fernandez fur seal, *Arctocephalus philippii*. *Journal of Mammalogy* 80: 758–767.

[c] Grahl-Nielsen O, Hammill MO, Lydersen C, and Wahlstrøm S (2000) Transfer of fatty acids from female seal blubber via milk to pup blubber. *Journal of Comparative Physiology B* 170: 277–283.

[d] Brown DJ, Boyd IL, Cripps GC, and Butler PJ (1999) Fatty acid signature analysis from the milk of Antarctic fur seals and Southern elephant seals from South Georgia: Implications for diet determination. *Marine Ecology Progress Series* 187: 251–263.

[e] Manatee milk also contains shorter-chain fatty acids (see text).

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nr, not reported, i.e. <0.5%.

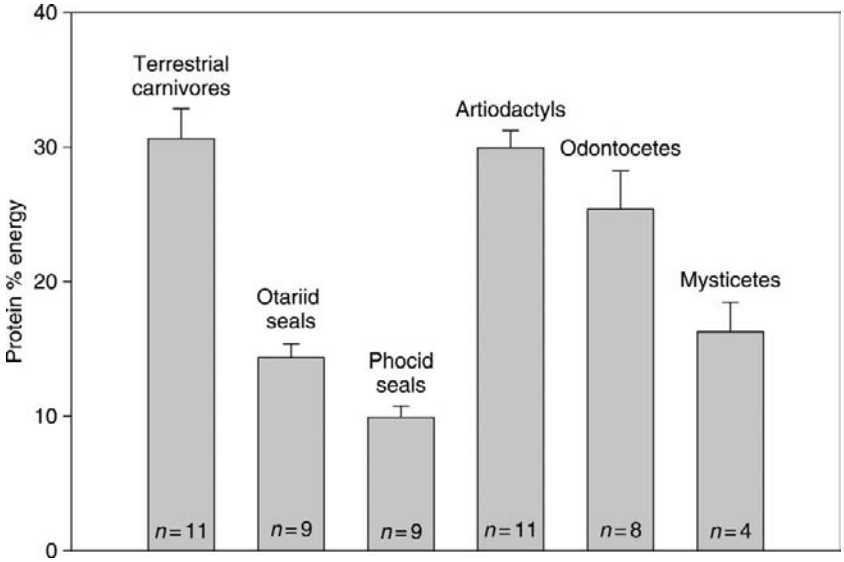

Figure 4 Percentage of milk energy provided by protein in different mammalian groups. Bars represent means plus standard errors. Number of species is indicated at the bottom of each bar. Values are calculated from data in **Tables 2** and **3**.

have been isolated and partially sequenced from bottlenose dolphin milk. Electrophoretic evidence for the presence of serum albumin has been observed in gray and southern elephant seals. However, other whey proteins observed via electrophoresis of marine mammal milks have not been characterized. Activity of the enzyme bile salt-stimulated lipase has been observed in hooded seal milk. The milk fat globule membrane of the Weddell seal contains the proteins butyrophilin and xanthine oxidoreductase.

Of particular interest is the apparent lack of lactose synthase activity in otariid mammary glands. This appears to be due both to mutations causing aberrant splicing during the transcription of the α-lactalbumin gene and to great reduction in the transcription and translation of α-lactalbumin, which is an essential component of the lactose synthase complex. Without α-lactalbumin, the β1,4-galactosyltransferase does not utilize glucose as a receptor for galactose, and thus lactose is not formed. As noted below, otariid milks lack both free lactose and oligosaccharides that incorporate lactose. The failure to produce α-lactalbumin appears to be important in preventing mammary involution during the long foraging trips that lactating otariids may undertake. The milk of Stejneger's beaked whale, which lacks lactose, also lacks α-lactalbumin in amounts detectable by electrophoresis.

Carbohydrates

Marine mammal milks are typically much lower in total sugar content than the milks of related terrestrial taxa (**Tables 1–3**). Most marine mammals, including polar bears, phocid seals, and at least some dolphins and whales, produce milks with low levels of both lactose and other more complex sugars (**Table 6**). These complex sugars are synthesized by the addition of other monosaccharide units to lactose (Gal(β1-4)Glc), such as the trisaccharides isoglobotriose (Gal (α1-3)Gal (β1-4)Glc), 2′fucosyllactose (Fuc(α1-2)Gal(β1-4)Glc), and 3′sialyllactose (Neu5Ac(α2-3)Gal(β1-4)Glc). Of these, isoglobotriose is the predominant sugar in polar bear milk (and in the milk of some other bears), and 2′fucosyllactose is a codominant sugar (with lactose) in at least some phocid seals (hooded seal, harbor seal) and some baleen whales (minke whale).

Most marine mammals with the ability to synthesize lactose also synthesize an array of longer chain oligosaccharides (up to octa- and nonasaccharides) that include lactose (i.e., Gal(β1-4)Glc) at the reducing end as well as varying combinations of galactose, *N*-acetyl galactosamine, *N*-acetyl glucosamine, fucose, and sialic acid (or *N*-acetyl neuraminic acid (Neu5Ac)) (**Table 6**). Thus, 7–10 oligosaccharides have been identified in the milks of polar bears, hooded seals, harbor seals, and minke whales, but not the same oligosaccharides in each species. The biological significance of this array of oligosaccharides is not clear, although by analogy to human milk oligosaccharides, antimicrobial functions in either the mammary gland or the neonatal digestive tract have been hypothesized.

Some marine mammals, including otariids, the walrus, and Stejneger's beaked whale, appear to produce milks devoid of lactose, the primary sugar in the milks of most mammals. In otariids and the walrus, gene mutations and/or changes in transcription rates effectively

Table 6 Oligosaccharides in marine mammal milks

	Number of sugar units per oligosaccharide							*Marine mammal species*								
	Total of sugar units	*Glucose*	*Galactose*	N-*Acetyl glucosamine*	N-*Acetyl galactosamine*	*Fucose*	N-*Acetyl neuraminic acid*	*Polar bear*[a]	*Australian fur seal*[b]	*Northern fur seal*[c]	*Hooded seal*[b]	*Bearded seal*[d]	*Harbor seal*[e]	*Bottlenose dolphin*[f]	*Beluga whale*[g]	*Minke whale*[g]
Inositol	1								xx	xx	x	x	x			
Lactose	2	1	1					x			xx	xx	xx	xx	trace	xx
Isoglobotriose	3	1	2					xx								
Globotriose	3	1	2											x		
2-Fucosyllactose	3	1	1			1		x			xx	xx	xx			xx
3′-Sialyllactose	3	1	1				1	x						x	x	
6′-Sialyllactose	3	1	1				1							x		
Lacto-*N*-neotetraose	4	1	2	1							x		x			x
B-Tetrasaccharide	4	1	2			1		x								
3′-Fucosylisoglobotriose	4	1	2			1		x								
A-Tetrasaccharide	4	1	1		1	1		x								x
Monosialyltetrasaccharide	4	1	1		1		1							x		
A-Pentasaccharide	5	1	1		1	2		x								
B-Pentasaccharide	5	1	2			2		x								
Sialyl lacto-*N*-neotetraosea	5	1	2	1			1						x			x
Lacto-*N*-fucopentaose IV	5	1	2	1		1						x				
Sialyl lacto-*N*-neotetraose c[b]	5	1	2	1			1									x
Lacto-*N*-neohexaose	6	1	3	2							x		x			
Para lacto-*N*-neohexaose	6	1	3	2							x					x
Monofucosyl para lacto-*N*-neohexaose	7	1	3	2		1					x					
Monosialyl lacto-*N*-neohexaose	7	1	3	2			1						x			

(*Continued*)

Table 6 (Continued)

	Number of sugar units per oligosaccharide							*Marine mammal species*								
	Total of sugar units	*Glucose*	*Galactose*	N-*Acetyl glucosamine*	N-*Acetyl galactosamine*	*Fucose*	N-*Acetyl neuraminic acid*	*Polar bear*[a]	*Australian fur seal*[b]	*Northern fur seal*[c]	*Hooded seal*[b]	*Bearded seal*[d]	*Harbor seal*[e]	*Bottlenose dolphin*[f]	*Beluga whale*[g]	*Minke whale*[g]
Sialyl para lacto-*N*-neohexaose	7	1	3	2			1									x
Monofucosyl lacto-*N*-neohexaose a	7	1	3	2		1					x		x			
Monofucosyl lacto-*N*-neohexaose b	7	1	3	2		1					x		x			
Difucosyl lacto-*N*-neohexaose	8	1	3	2		2					x	x	x			
Monosialyl monofucosyl lacto-*N*-neohexaose	8	1	3	2		1	1						x			
Disialyl lacto-*N*-neohexaose	8	1	3	2			2						x			
Monosialyl difucosyl lacto-*N*-neohexaose	9	1	3	2		2	1					x	x			
Difucosyldecasaccharide	10	1	4	3		2						x				
Unnamed	11	1	4	3		2	1					x				

[a] Urashima T, Nagata H, Nakamura T, *et al*. (2003) Differences in oligosaccharide pattern of a sample of polar bear colostrum and mid-lactation milk. *Comparative Biochemistry and Physiology B* 136: 887–896.

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Predominant sugar is identified by xx; others by x.

produce milks lacking functional α-lactalbumin, and α-lactalbumin is reported missing from Stejneger's beaked whale. The West Indian manatee also produces milk in which lactose was not detected by paper chromatography or enzymatic methods, although trace levels of lactose were found by high-performance liquid chromatography (HPLC) and very low levels of α-lactalbumin could be detected. It is not known if the low level of sugar in manatee milk (**Table 3**) represents free sugars or covalently bound sugars that were hydrolyzed from glycolipids or glycoproteins during analysis.

Sea lions and fur seals that lack the ability to synthesize lactose produce milks devoid of lactose-containing oligosaccharides. It appears that the primary free sugar in these milks is the monosaccharide inositol (**Table 6**). Whether inositol or other small organic osmolytes replace the osmotic role of lactose in the secretion of the aqueous phase of otariid milks is not known.

Mineral Elements

The milks of marine mammals presumably supply the wide variety of macrominerals, trace elements, and vitamins that growing young require for tissue growth and metabolic function, but these constituents are not well studied. Some of the early work on whale milks collected during whaling operations used what are now outdated methodologies, and as the results may not be reliable they are not included herein. As noted above, contamination of milks with water, sodium, and magnesium from seawater is of concern, and contamination of milk with dirt or fecal material may be hard to avoid when working with land-breeding seals in large colonies.

Among terrestrial mammals, the ratio of calcium to phosphorus (Ca:P) in milk is typically above 1:1, reflecting a greater post-natal requirement for calcium than for phosphorus, primarily due to calcium deposition in growing bone. Ca:P ratios above 1 are also found in the milks of the polar bear, sea otter, some dolphins and whales, and the West Indian manatee (**Table 7**). However, both the

Table 7 Major mineral elements in marine mammal milks

		N	*Ca* *g kg^{-1}*	*P* *g kg^{-1}*	*Ca:P* *ratio*	*K* *g kg^{-1}*	*Na* *g kg^{-1}*	*K:Na* *ratio*
Carnivora								
Ursidae	Polar bear[a]	7	0.29	0.23	1.26			
Mustelidae	Sea otter[b]	5	1.07	0.84	1.27			
Otariidae	Galapagos fur seal	17	0.63			1.67		
	Northern fur seal	1	0.57	1.19	0.48	0.84	0.52	1.61
	Southern sea lion[c]	4	0.40	1.00	0.40			
	California sea lion	7	0.80	1.03	0.78	1.21	0.89	1.36
Phocidae	Weddell seal					0.57	0.53	1.08
	N. elephant seal	7–12	0.51	0.94	0.54	0.56	0.86	0.65
	S. elephant seal					1.36	0.99	1.37
	Harp seal	4	0.72	1.20	0.60	1.08	0.62	1.75
Cetacea								
Delphinidae	Common dolphin	2	1.5	2.3	0.65			
	Humpback dolphin	1	1.7	1.4	1.21			
	Spotted dolphin	8		1.2				
	Atlantic spotted dolphin	1	1.5			1.7	1	1.70
	Bottlenose dolphin	3–4	1.3	1.1	1.18	0.8	1.6	0.50
Physeteridae	Pygmy sperm whale	1	1.5	1.7	0.88			
Ziphiidae	Stejneger's beaked whale	1	2.2	0.7	3.14	1.1	1.3	0.85
Balaenopteridae	Blue whale	2	3.3	2.1	1.57	1.5	1	1.50
	Fin whale	1	3.2	4.1	0.78			
Sirenia								
Trichechidae	West Indian manatee[d]	1	0.26	0.22	1.18	0.51	3.1	0.16

[a] Jenness R, Erickson AW, and Craighead JJ (1972) Some comparative aspects of milk from four species of bears. *Journal of Mammalogy* 53: 34–47.

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Data are presented on a whole milk basis.

otariids and the phocids produce milks with an inverse Ca:P ratio, that is, more phosphorus than calcium. The functional significance of this is not known. In captivity, seal pups allowed to suckle beyond normal weaning dates may develop pathological bone demineralization.

In terrestrial mammals, milk is higher in potassium than sodium, reflecting the existence of sodium and potassium gradients maintained across the basal membranes of mammary secretory epithelial cells. The same is probably true of marine mammals, although data are limited (**Table 7**). The high sodium values reported for some cetacean and manatee milks are suspect due to the possibility of seawater contamination. Based on data on a handful of species, marine mammal milks are reported to contain about 1000–1700 mg kg^{-1} chloride, 120–200 mg kg^{-1} magnesium, and trace levels of iron (5–36 mg kg^{-1}), copper (2–3 mg kg^{-1}), zinc (1–8 mg kg^{-1}), and selenium (0.4–0.5 mg kg^{-1}).

Vitamin data on marine mammal milks are sparse. The milks of blue and fin whales reportedly contain, per kg fresh milk, the following vitamins: 3100–7800 IU vitamin A, 1.1–1.6 mg total thiamin, 0.2–1.6 mg riboflavin, 7–26 mg niacin, 0.9–1.1 mg vitamin B_6, 3–18 mg pantothenic acid, 12–65 μg biotin, and 8–130 μg vitamin B_{12}. Fairly fresh (<6 h post-mortem) fin whale milk reportedly contains 70 mg l^{-1} ascorbic acid (vitamin C). In harp and hooded seals, vitamin E content of colostrum is high but then falls to stable levels of about 20–50 mg kg^{-1} at mid-lactation; by contrast, vitamin A levels are constant throughout lactation. Similar patterns have been seen in gray seals. Further research is needed on freshly collected and well-preserved marine mammal milks to determine if the above values are representative or include errors due to vitamin losses during collection, storage, and analysis.

See also: **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk. **Mammary Gland**: Growth, Development and Involution. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose; Milk Fat; Milk Protein; Secretion of Milk Constituents. **Milk:** Milk of Monotremes and Marsupials; Milks of Non-Dairy Mammals; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.). **Milk Lipids**: Fatty Acids; General Characteristics; Nutritional Significance. **Milk Proteins**: Casein Nomenclature, Structure, and Association; Interspecies Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity; Nutritional Quality of Milk Proteins; α-Lactalbumin; **Milk Salts**: Macroelements, Nutritional Significance; Trace elements, Nutritional Significance.

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Human Milk

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Introduction

The primary function of mammalian milk is to nourish the neonate in the most efficient manner possible from the mother's perspective, while providing the neonate with additional benefits from enhanced immune protection, and a source of nonnutrient growth promoters, hormones, and other bioactive components that serve as biochemical messengers within the body. In addition, several components in human milk help to maintain the integrity of mammary tissue, optimizing milk output and composition.

Human milk, a biologically complex fluid containing many hundreds of different constituents, is ideally suited to meet the term infant's nutritional needs during the first 4–6 months of life, provided that the mother is also healthy and well nourished. For this reason, infant formula manufacturers often refer to the gross composition of human milk as the 'gold standard'. However, it is important to remember that, unlike infant formula, the composition of human milk is not uniform. Significant changes in the composition of breast milk occur not only between individual women, but also within a single feed, between feeds, throughout the day, throughout the lactation period, with changing maternal diet, and as a result of other external factors such as exercise or metabolic illness. Implicit in these findings is the understanding that a single description of human milk is somewhat erroneous. Nevertheless, it is important to establish data on the composition of human milk at various key stages of lactation.

Gross Nutrient Composition

There are three stages in human lactation that are clearly identified by changes in specific components in the milk, such as the whey proteins and lactose. These compositional changes appear uniquely to match the changing physiological needs of the infant, and can be defined based on the following time periods: 1–5 days post-partum, colostrum; 5–21 days postpartum, transitional milk; and >21 days post-partum, mature milk. Nutrient information relating to human milk at the predominant stages of lactation is provided in **Table 1**.

Human colostrum differs from mature milk in both the types and amounts of individual components. Colostrum has a much higher protein content and a lower lactose content than mature milk. The specific gravity of colostrum is 1.040 compared with 1.060 for mature milk, and the mean energy content of colostrum is also lower at 67 kcal 100 ml^{-1} compared with mature milk, which supplies 75 kcal 100 ml^{-1} of energy. Although the fat and ash contents of colostrum and mature milk do not differ significantly, variations in specific micronutrients are apparent, with the concentrations of sodium, chloride, and magnesium being higher, and that of potassium and calcium lower, in colostrum.

Component Composition

Human milk contains hundreds of different components; **Table 2** lists some of these grouped according to their physicochemical properties. The water-soluble constituents found in the aqueous phase account for 87% of milk volume, and include some salts, vitamins and trace elements, lactose, oligosaccharides, whey proteins, and non-protein nitrogen (NPN) components. Complementing the aqueous phase is a colloidal dispersion (0.3% of volume) containing casein proteins held in micellar form, which contributes to the white color of milk. Calcium phosphate and some other proteins are also linked to the casein micelles. A further 6% of milk volume is made up of an emulsion containing fat globules, fat globule membranes, triglycerides, and other lipid components, including the fat-soluble vitamins. The remainder consists of cellular components, non-peptide hormones, and other minor fractions.

Proteins

Many of the functional properties of human milk are delivered via the milk proteins. Provision of essential amino acids for growth is the most obvious role of milk proteins. Determining the gross amino acid composition of human milk will accurately describe amino acid

Table 1 Gross nutrient content (g 100 ml^{-1}) of human milk at different stages in lactation

Nutrient	*Colostrum (1–5 days)*	*Transitional milk (5–21 days)*	*Mature milk (21 days+)*
Protein	1.5–1.7	0.9	0.8–0.9
Non-protein nitrogen	0.5		0.45
Lactose	4.1–5.8	5.4	6.8
Glucose	0.2–1.0		0.2–0.3
Oligosaccharides	2.4		1.3
Total lipid	2–3	3–4	3–5
Ash	0.25		0.2

Table 2 Components of human milk grouped according to physicochemical properties

Proteins	*Non-protein nitrogen*
β-Casein	Urea
α-Lactalbumin	Glucosamine
Lactoferrin	Creatine/creatinine
Lysozyme	Uric acid
Serum albumin	Nucleotides
Secretory immunoglobulin A	Nucleic acids
	Polyamines
Immunoglobulin A	
Immunoglobulin G	
Immunoglobulin M	*Lipids*
Peptide hormones	Fatty acids
Enzymes	Phospholipids
Growth factors	Triacylglycerols
Binding proteins	Fat-soluble vitamins (A, D, E, K)
	Sterols
Carbohydrates	
Lactose	*Other vitamins*
Oligosaccharides	Biotin
Glycoproteins	Choline
	Folic acid
Minerals	Inositol
Calcium	Nicotinic acid
Phosphate	Pantothenic acid
Potassium	Riboflavin
Sodium	Thiamine
Sulfate	Vitamin B_1
Magnesium	Vitamin B_2
Chloride	Vitamin B_6
	Vitamin B_{12}
Trace minerals	Vitamin C
Chromium	
Cobalt	*Cells*
Copper	Epithelial cells
Iodine	Leukocytes
Iron	Lymphocytes
Manganese	Macrophages
Molybdenum	Neutrophils
Nickel	
Selenium	*Others*
Zinc	Bicarbonate
	Citrate
	Taurine
	Glutamine
	Carnitine
	Nonpeptide hormones

content, and in the past this figure has been used to define the amount of protein available to the infant for growth and development. However, milk proteins also provide the infant with immune protection, increased digestive capacity, and enhanced micronutrient availability, and, as many bioactive components of human milk are of protein origin, implicit in their functionality is a resistance to digestion. Investigation of the nutritive value of human milk protein reveals that although most (95%) milk protein is available, a proportion does not contribute significantly to amino acid nutrition. To determine accurately the amounts of amino acids, and therefore protein, used for nutrition by the breast-fed infant, a correction needs to be made for bioavailability. A comparison of gross and available amino acid composition of mature human milk is made in **Table 3**.

The total protein content of human milk consists of two major groups, caseins and whey proteins, as well as

Table 3 The gross and available amino acid[a] composition of mature human milk (mg 100 g^{-1} protein[b])

Amino acid	*Gross*	*Available*
Aspartic acid	9.2	9.1
Threonine	4.7	4.6
Serine	5.0	4.2
Glutamic acid	18.4	16.8
Proline	8.5	8.4
Glycine	2.3	2.1
Alanine	3.8	3.4
Valine	6.4	5.1
Isoleucine	5.9	5.1
Leucine	9.7	9.3
Tyrosine	4.2	3.8
Phenylalanine	3.9	3.8
Histidine	2.3	2.3
Lysine	6.8	6.2
Arginine	4.0	3.1
Cysteine	2.2	2.6[c]
Methionine	1.6	1.4

[a] Includes free amino acids and peptides.

[b] Calculated from total g N × 6.38.

[c] Value for available is higher than gross due to correction for significant losses during the acid hydrolysis stage of amino acid determination.

Table 4 The types and amounts of protein (g 100 ml^{-1}) and non-protein nitrogen (mg 100 ml^{-1}) in human colostrum and mature milk

	Colostrum	*Mature milk*
Casein proteins		
β-Casein	0.26	0.3–0.5
κ-Casein	0.12	0.1–0.3
Whey proteins		
α-Lactalbumin	0.36	0.2–0.3
Serum albumin	0.04	0.03
Lactoferrin	0.35	0.1–0.3
Lysozyme		0.05
Secretory immunoglobulin A/ immunoglobulin A	0.2	0.05–0.1
Immunoglobulin G	0.034	0.001
Immunoglobulin M	0.012	0.002
Non-protein nitrogen (N)	48	50
Urea N	12.1	15–25
Creatine N		3.7
Creatinine N		3.5
Uric acid N	0.5	0.5
Glucosamine	14.2	4.7
α-Amino N	4.5	13
Ammonia N	0.2–0.8	0.2

some minor components (**Table 1**) and proteins present in the milk fat globule membrane. The ratio of whey proteins to casein changes during the initial stages of lactation, from 90:10 immediately post-partum, to 60:40 in mature milk and 50:50 in late lactation. These ratios directly reflect a decrease in the content of immune proteins (**Table 4**) in line with development of the infant's own immune system.

Casein

Human milk contains two (β and κ) caseins, and is devoid of α-casein, the predominant casein found in bovine milk. The casein pattern differs depending on the stage of lactation, with κ-casein detectable in human milk only from day 3–4 post-partum onward. A unique feature of human milk is the high degree of glycosylation of κ-casein (40–60% glycosylated) and other milk proteins (e.g., immunoglobulins (Igs) and lactoferrin). Glycosylated proteins exhibit anti-infective properties and enhance the absorption of some micronutrients. As part of the normal digestive process, the caseins in human milk release smaller peptides into the gut lumen. These casein fragments are thought to enhance the absorption of calcium by keeping it in solution in the gut lumen. Other casein-derived peptides have been linked to the regulation of intestinal motility and to the growth promotion of beneficial bacteria in the infant's gut.

Whey proteins

α-Lactalbumin, lactoferrin, serum albumin, and secretory IgA (sIgA) are the main whey proteins found in human milk. β-Lactoglobulin, prominent in bovine milk, does not occur in human milk.

The predominant sources of amino acids for nutrition among the whey proteins are α-lactalbumin and serum albumin. The other whey proteins, while contributing to nutrition to some extent, have a primary function in other roles. Lactoferrin, an iron-binding protein, has bacteriostatic properties that may impact on both the infant and the mammary gland. Lysozyme is capable of cleaving proteoglycans in the cell wall of certain bacteria, providing an antibacterial effect. The Igs in human milk are distinct from those found in blood serum, indicating selective synthesis in the mammary gland. sIgA, formed by the linking of two serum IgA molecules by disulfide bonds, is present at very high concentrations in colostrum, but the sIgA content declines rapidly to a much lower level within 14 days of lactation. sIgA protects the infant by blocking the attachment of pathogenic bacteria to the gut epithelium. Other Igs in human milk provide immune protection through activation of the complement system, or by promoting phagocytosis.

Other proteins

In addition to casein and whey proteins, human milk contains other proteins, such as serum proteins, most of which are in the aqueous phase. The milk fat globule membrane proteins surround the lipid droplets, but are a minor fraction quantitatively (1–3% of total protein). However, these minor proteins can contribute in many ways to the health and well-being of the infant, for example, by facilitating transport of certain vitamins and minerals. Milk-borne peptide hormones and enzymes assist in the digestion of other milk components (e.g., bile salt-stimulated lipase (BSSL) and amylase). A detailed list of the hormones, both peptide and non-peptide, and enzymes found in human milk is given in **Table 5**.

Non-protein Nitrogen

Compared with the milk of other mammalian species, human milk is notably different with regard to the NPN fraction. In human milk, the NPN fraction represents a very high percentage (20–25%) of total nitrogen (N). Urea N accounts for almost 50% of the NPN in human milk, with more than 200 compounds, including free amino acids, carnitine, taurine, amino sugars, nucleic acids, nucleotides, and polyamines, making up the remainder. In general, the total NPN of human milk remains constant throughout the lactation period, although there may be some variation in individual NPN components (**Table 4**).

Table 5 Hormones and enzymes found in mature human milk

Hormones			
Peptide		*Non-peptide*	
Growth factors	Epidermal growth factor	Thyroid	Thyroxine
	Insulin		Triiodothyronine
	Insulin-like growth factor 1		
	Nerve growth factor	Adrenal	Cortisol
	Transforming growth factor-α		
	Transforming growth factor-β		
		Sexual	Progesterone
			Pregnane-3(α)20(β)-diol
			Estrogen
Gastrointestinal regulators	Gastrin		
	Gastric inhibitory polypeptide		
	Gastric-releasing peptide		
	Neurotensin		
	Peptide histidine-methionine		
	Peptide tyrosine–tyrosine		
	Somatostatin		
	Vasoactive intestinal peptide		
Hypothalamus/hypophyseal hormones	Gonadotropin-releasing hormone		
	Growth-releasing factor		
	Growth hormone		
	Prolactin		
	Thyrotropin-releasing hormone		
	Thyroid-stimulating hormone		
Thyroid–parathyroid group	Calcitonin-like hormone		
	Parathyroid hormone		
	Parathyroid hormone-related peptide		
Enzymes			
Phosphoglucomutase		Lactose synthase	
Fatty acid synthase		Thioesterase	
Galactosyltransferase		Amylase	
Bile salt-stimulated lipase		Proteases	
Lysozyme		Peroxidase	
γ-Glutamyltransferase		Platelet-activating factor acetylhydrolase	
Protease inhibitors		Sulfhydryl oxidase	
Lipoprotein lipase		Glutathione peroxidase	
Alkaline phosphatase		Xanthine oxidase	

Given that the total protein content of human milk is low compared to other species, yet supports adequate growth of the breast-fed infant, the suggestion has been made that the urea found in the NPN fraction of human milk is used by the infant for protein synthesis. This would have major implications for infant formula manufacturers, necessitating a total reevaluation of infants' protein requirements. Isotopic tracer experiments with infants have shown that as much as 40% of labeled urea N was retained within the body. However, this may not directly reflect the incorporation of urea N into body protein, as NH exchanges occurring during transamination may in part explain the appearance of urea ^{15}N in body proteins. This remains an important area for future investigation of infant protein metabolism.

Taurine and glutamine, two predominant free amino acids in human milk, are considered conditionally essential, as the infant appears to have a requirement for these amino acids above what the body can synthesize. Taurine plays an integral part in fat digestion by conjugating with bile acids to form bile salts. Although no direct link has been made between a deficiency in taurine and abnormal development of retinal and brain tissue in infants, the addition of taurine to infant formulae is now routine. Glutamine is involved in the cellular metabolism of enterocytes and in the immune response associated with inflammation and sepsis.

During periods of rapid growth or tissue repair, a dietary source of nucleotides may be beneficial, as *de novo* synthesis of nucleotides can be a limiting factor.

Table 6 Nucleotide content of human milk, and the amounts of specific nucleotides approved for inclusion in infant formulae

Nucleotide	*In human milk (mg 100 ml^{-1})*	*Maximum to be added to infant formula (mg 100 kcal^{-1})*
Adenosine diphosphate (ADP)	69	
Adenosine monophosphate (AMP)	175	1.5
Cytidine diphosphate (CDP)	474	
Cytidine monophosphate (CMP)	461	2.5
Guanosine diphosphate (GDP)	96	
Guanosine monophosphate (GMP)	138	0.5
Inosine monophosphate (IMP)	228	1.0
Uridine diphosphate (UDP)	174	
Uridine monophosphate (UMP)	179	1.75

Adapted from Lawrence and Lawrence (1994); infant formula inclusion rates from Rudloff S and Kunz C (1997) Protein and nonprotein nitrogen components in human milk, bovine milk, and infant formula: Quantitative and qualitative aspects in infant nutrition. *Journal of Pediatric Gastroenterology and Nutrition* 24: 328–344.

The infant is in a state of rapid growth and cellular development, and human milk is rich in nucleotides (**Table 6**). To date, however, no link between nucleotides in human milk and enhanced cellular function in the infant has been made. Despite this lack of direct evidence, some regulatory authorities have approved the inclusion of nucleotides in infant formulae (**Table 6**).

Carbohydrate

Besides water, lactose constitutes the largest component of human milk (**Table 1**); lactose concentration decreases as lactation progresses, in line with the decline in milk volume.

Oligosaccharides constitute the next greatest fraction of carbohydrate in human milk, representing more than 27% of the carbohydrate in colostrum. This gradually decreases to 15–16% of carbohydrate as oligosaccharides in mature human milk. Most oligosaccharides found in human milk contain lactose at the reducing end, and may contain fucose or sialic acid at the non-reducing end. Compared to other mammalian milks, human milk is unique due to its content of complex oligosaccharides. Milk oligosaccharides provide a low osmolar source of calories for the infant, and have been classed as 'soluble' fiber because they appear to remain largely undigested in the infant's gut. Human milk oligosaccharides contain significant quantities of sialic acid, which is essential for brain development, and they are also thought to stimulate the growth of bifidus bacteria and to inhibit the adhesion of pathogenic bacteria to epithelial surfaces. Approximately 130 oligosaccharides have been identified in human milk, some of which are listed in **Table 7**. Glucose and galactose are present in much smaller quantities in human milk, as are other carbohydrates such as monosaccharides, and peptide-bound and protein-bound carbohydrates.

Table 7 Quantities of some carbohydrates found in human milk

carbohydrate oligosaccharid	*Concentration mg 100 ml^{-1}*
Lactose	6800
Oligosaccharides	1200–1300
Lacto-*N*-tetraose	5–15
Lacto-*N*-fucopentaose I	12–17
Lacto-*N*-fucopentaose II	3–10
Lacto-*N*-fucopentaose III	0.1–2
Lacto-*N*-difucohexaose I	1–2
NeuAc(α2–6)lactose	3–5
NeuAc(α2–3)lactose	1–3
NeuAc-lacto-*N*-tetraose a	0.3–2
NeuAc-lacto-*N*-tetraose b	1–6
NeuAc$_2$-lacto-tetraose	2–6

Adapted from Kunz C, Rodriguez-Palmero M, Koletzko B, and Jensen R (1999) Nutritional and biochemical properties of human milk. 1. General aspects, proteins, and carbohydrates. *Clinics in Perinatology* 26: 307.

Lipids

The lipids in human milk occur in globular form, emulsified in the aqueous phase, and provide the infant with a major proportion (45–55%) of the energy needed to support growth. They also contain bioactive components important to the infant's retinal and neural tissue development.

The triacylglycerols (TGs) account for 98% of total fat, and 90% of this is fatty acids (FAs). Phospholipids (0.8%), cholesterol (0.5%), and other minor lipid components make up the remainder of the fat, which includes fat-soluble vitamins.

In the first few weeks of lactation, the total lipid content increases from around 2 to 3.5–5% (**Table 8**). As the fat content increases, so too does the average size of the milk fat globules, which in turn results in a reduction in the ratio of phospholipids and cholesterol in TGs.

Table 8 Lipid content of human colostrum and milk

Lipid component	*Colostrum*	*Mature milk*
Total lipid (percentage of milk volume)	2	3–5
Phospholipid (percentage of total lipid)	1.1	0.8
Triacylglycerol (percentage of total lipid)	97–98	97–98
Cholesterol (percentage of total lipid)	1.3	0.5

Table 9 An example of the fatty acid composition of human milk (wt%)

Fatty acid	*Colostrum*	*Mature milk*
Saturated		
10:0	0.5	1.1
12:0	2.3	4.8
14:0	5.3	6.7
15:0	0.3	0.3
16:0	26.2	21.8
17:0	0.4	0.3
18:0	7.5	7.5
20:0	0.3	0.2
22:0	0.2	0.1
24:0	0.3	0.1
Monounsaturated		
14:1$n-9$	0.1	0.3
16:1$n-9$	2.1	2.7
18:1$n-9$	34.7	33.0
20:1$n-9$	1.3	0.6
22:1$n-9$	0.3	0.1
24:1	0.5	0.1
Polyunsaturated		
18:3$n-3$	0.8	1.0
20:3$n-3$	0.2	0.1
20:5$n-3$	0.04	0.04
22:5$n-3$	0.2	0.1
22:6$n-3$	0.6	0.2
18:2$n-6$	8.8	10.7
18:3$n-6$	0.1	0.2
20:2$n-6$	0.6	0.3
20:3$n-6$	0.6	0.4
20:4$n-6$	0.8	0.5
22:4$n-6$	0.2	0.1
22:5$n-6$	0.4	0.2

The TG composition in human milk is determined by the types and quantities of FAs esterified to the glycerol molecule. Human milk contains eight major FAs in amounts greater than 1% (**Table 9**), and is an excellent source of the essential FAs $C_{18:2n-6}$ and $C_{18:3n-3}$ and their long-chain derivatives $C_{20:4n-6}$ (arachidonic acid) and $C_{22:6n-3}$ (docosahexaenoic acid). The latter FAs, although not classed as essential for adults, are the only FAs utilized by the brain and are important structural components of the membrane systems of all tissues.

Approximately 60% of the $C_{16:0}$ FAs in human milk are situated at the *sn*-2 position on the TG, which is a unique feature of the FAs in human milk. Lipolytic hydrolysis cleaves FAs from the *sn*-1 and *sn*-3 positions, and a $C_{16:0}$ remains as a monoacylglycerol. This facilitates the absorption of $C_{16:0}$ across the infant's gut epithelium, improving overall lipid utilization to around 90–95%.

Lipid digestion and absorption are enhanced further by four enzyme systems. These are, in the order of utilization, the gastric phase with lingual lipase and gastric lipase, and the intestinal phase with pancreatic lipase and BSSL. The gastric phase is important because the milk fat globule would otherwise resist the action of pancreatic lipase and BSSL. Unlike the other lipases, which are endogenous, BSSL is secreted in human milk specifically to assist the infant with milk fat digestion.

Of all the components in human milk, the lipid fraction is the most variable. Changes in fat content occur not only during the first few weeks of lactation (**Table 8**), but also in milk secretions throughout the day, and even within a single feed, with the hindmilk being significantly higher in fat content than the foremilk. Variations also exist in the fat content of milk from individual women, although by far the greatest variation occurs in the types and amounts of FAs.

Micronutrients

Vitamins

Most vitamins found in human milk increase in concentration as lactation shifts from colostrum to a mature secretion (**Table 10**), with the exception of carotenoids (α-carotene, β-carotene, lutein, cryptoxanthin, and lycopene), retinol, and vitamin E, which decrease. Reduction in the concentration of some vitamins may not alter the total amount available to the infant, however, as any reduction may be offset by an increase in the quantity of breast milk consumed.

The milk from healthy, well-nourished women usually contains sufficient quantities of most of the vitamins required by the infant. There are a few exceptions, however, when the amount in milk may not be adequate. Deficiencies in vitamin D, with consequent development of rickets, have been reported in breast-fed infants, directly linked to the mother's diet and the infant's lack of exposure to sunlight. To meet physiological requirements, vitamin K is routinely given to newborn babies because transplacental transfer is limited, and the level in milk is low.

Minerals

In general, the macromineral content of human milk remains stable during the lactation period after an initial decrease in the concentration of chloride, potassium,

Table 10 Micronutrient content of human colostrum and milk

Micronutrient	*Units* l^{-1}	*Colostrum*	*Mature milk*
Water-soluble vitamins			
Ascorbic acid	mg	60	50–100
Biotin	μg	0.6	6
Folic acid	μg	5	80–133
Niacin	mg	0.6	1.6–6.0
Pantothenic acid	mg	1.8	2.0–2.5
Riboflavin	μg	300	400–600
Thiamine	μg	19.5	200
Vitamin B_6	mg		0.1–0.3
Vitamin B_{12}	μg	0.5	1.0
Fat-soluble vitamins			
Carotenoids	mg	2	0.2–0.6
Retinol	mg	1.8	0.3–0.7
Vitamin D	μg		0.4
Vitamin E	mg	10–15	3–8
Vitamin K	μg	2–5	3–15
Major minerals			
Calcium	mg	300	278
Chloride	mg	813	426
Citrate	mg		500[a]
Magnesium	mg	36	35
Phosphorus	mg	140	140
Potassium	mg	652	530
Sodium	mg	502	180
Sulfur	mg	277	142
Trace minerals			
Chromium	ng		200–400
Cobalt	ng		100–200
Copper	mg	0.4–0.8	0.2–0.4
Fluoride	μg		4–15
Iodine	μg		146
Iron	mg	0.75	0.72
Manganese	μg	5–12	3–6
Molybdenum	mg		1–2
Nickel	mg		0.5–2
Selenium	μg	40	10–20
Zinc	mg	8–12	1–3

[a] Citrate is not classed as a mineral, but is soluble in water and binds some minerals, so is included in this table.

sodium, and sulfur (**Table 10**). Most of the sulfur in human milk is found in the sulfur-containing amino acids methionine and cysteine, with only a small fraction (10%) existing in salt form. The sulfur amino acids predominate in whey proteins, so a reduction in whey protein content during the first few days of lactation is matched by a similar reduction in sulfur content.

Human milk has a higher calcium-to-phosphorus ratio (2:1) than the milk from other mammalian species (e.g., in bovine milk, Ca:P ratio is 1.1:1), although the absolute amounts of calcium and phosphorus are lower. A low phosphorus content suits the infant's limited renal capacity. In addition, the contribution of phosphate to the buffering capacity of human milk is such that a low pH results, promoting the growth of beneficial gut bacteria.

Human milk contains what would initially appear to be an inadequate level of iron, yet breast-fed infants rarely deplete their iron stores until after 4–6 months of age. Maximum utilization of the iron in human milk is achieved with the assistance of lactoferrin, most likely via specific protein receptors in the gut that link with lactoferrin and therefore facilitate iron absorption.

Bioactive Factors

Human milk contains an optimal balance of the nutrients required by the infant and, in addition, it contains an array of bioactive factors. In the first instance, these factors assist in the production and secretion of milk components and provide immune protection of mammary tissue. Once ingested by the infant, however, bioactive factors actively promote growth and development, and afford the infant immune protection until its own defense system has matured. Even the major components in milk may have specific bioactive functions either before being digested or as a result of releasing bioactive components during the digestive process.

The main sites of action for these bioactive factors are mucosal surfaces, although some may enter the circulation and have a systemic effect elsewhere in the body. The various physiological roles of milk components and their derivatives, either within the mammary gland or in the infant, are listed in **Table 11**.

Intact cellular components are found in human milk (**Table 12**), and although their exact role within either the mammary gland or the recipient infant is not clearly defined, there does appear to be a degree of biological activity associated with them. The total count of cells is high in colostrum, with the numbers decreasing as lactation becomes established. More than 90% of the cells in human milk are macrophages and neutrophils, while the remainder are lymphocytes (80% of which are T cells) and epithelial cells.

The primary function of milk leukocytes is thought to be as a means of defense within the mammary gland, and not necessarily to transfer maternal immunocompetence to the infant. Macrophages,however, have been suggested as a potential vehicle for the storage and transport of milk-borne Igs, and may be involved in the formation of lactoperoxidase and other factors that enhance the growth of intestinal epithelium and digestive capacity. The lymphocytes present in human milk are part of the immunological system that synthesizes IgA, and considerable research data are available to support the suggestion that lymphocytes in colostrum and milk provide the infant with immunological benefits.

Table 11 Physiological roles of components in human milk, either within the mammary gland or following ingestion by the infant

Physiological function	*Components in human milk*
Within the mammary gland	
Component synthesis/secretion	α-Lactalbumin, lipoprotein lipase, xanthine oxidase
Immune effect/anti-infection/ anti-inflammatory	Lactoferrin, lysozyme, γ-glutamyltransferase, cytokines
Within the infant	
Nutrition	β- and κ-casein, whey proteins, lactose, fats, minerals, and vitamins
Enhancing nutrient availability[a]	Bile salt-stimulated lipase, milk amylase, lactoferrin (Fe), glutamine (Zn), α-lactalbumin (Ca), casein phosphopeptides (Ca), folate-binding protein
Gut colonization	Bifidus factor peptide, κ-casein oligosaccharides
Gastrointestinal development	Epidermal growth factor, insulin-like growth factor, growth factor-binding proteins, hormones, enzymes, nucleotides, oligosaccharides, amino sugars
Brain development	Docosahexaenoic acid, other long-chain polyunsaturated fatty acids, taurine, carnitine
Immunity	Immunoglobulins, leukocyte-stimulating factors, T lymphocytes, macrophages, immunopeptides
Anti-infective	Lactoferrin, lysozyme, free fatty acids, cell adhesion molecules, cytokines, mucins, glycoproteins, glycopeptides, oligosaccharides
Anti-inflammatory	Vitamins, prostaglandins, growth factors, cytokines (interleukin-10), transforming growth factor-β
Immunomodulating	Interleukins, tumor necrosis factor-α

[a] Nutrient that has increased availability due to a particular component in human milk is given in parentheses.

Table 12 Cellular components (number per ml) in human colostrum and milk

Cell type	*Colostrum*	*Mature milk*
Total cells	2840	51
Macrophages	1490	52
Neutrophils	1375	8
Lymphocytes	250	1

Adapted from Jensen R (ed.) (1995) *Handbook of Milk Composition*. San Diego, CA: Academic Press.

Factors that Influence Milk Volume and Composition

Factors Affecting Milk Volume

In women, the principal determinant of milk volume is infant demand. Comprehensive review of milk volume from exclusively breast-feeding women throughout the world has revealed that milk volume, 6 months into lactation, is incredibly constant, with an average of 800 ml (range 500–1200 ml) of milk transferred from mother to infant each day. The potential for milk production far exceeds this amount, however, given that mothers of twins or triplets can adequately nourish their children with human milk.

The energy density of human milk, in part, influences milk production, as infants consuming lower calorific milk will suckle more to obtain the energy required, in turn stimulating greater milk production. In one study, women with a low body fat content produced as much as 15% more milk as a result of increased infant demand. As soon as the weaning process begins, and human milk is substituted by either supplementary formula or complementary solids, milk production declines, indicating yet again the dominant role the infant plays in controlling milk volume.

Major Factors Affecting Milk Composition

Stage of lactation

Major compositional changes that occur from parturition through to day 21 of lactation have been discussed. The composition of human milk continues to change throughout lactation, however, with a gradual reduction in component content, although some components, for example, lysozyme, may increase.

During weaning, changes in composition can occur depending on the time taken to wean. When weaning is rapid (several days/weeks), lactose and potassium contents decrease, while sodium, chloride, fat, and total protein (specifically the whey proteins) increase. Gradual weaning (over several months) results in an increase in sodium (220% from baseline), iron (172%), and protein (142%), no change in calcium, and a decrease in zinc (58% from plateau). Both rapid and gradual weaning produce a decrease in lactose content.

Prematurity

Relative to normal human milk, the milk of mothers delivering prematurely contains higher concentrations of protein (1.8–2.4 g 100 ml^{-1}), short-, medium-, and long-chain FAs, calcium, phosphorus, sodium, chloride, magnesium, and iron, which is related to the low volume produced. Cellular components are also significantly greater in premature colostrum (total cells 6800 per ml). Levels of the other components in premature milk do not differ markedly from those found in normal human milk.

Changes during a feed and during the day

Changes that occur in composition during a feed relate more to the physicochemical nature of fractions in the milk, rather than a selective change in individual components. In general, most components remain relatively constant throughout a feed. The lipid content increases rapidly toward the end of a feed, however, reflecting a propensity of the mammary gland tissue to retain the lipid globules.

Subjective evidence from breast-feeding women suggests that milk composition changes during the day, especially toward evening, when the infant appears to be less satisfied by milk consumed. An unsettled infant is more likely to be due to insufficient milk volume, however, as lipid content actually increases toward the end of the day (by two- to fivefold). No significant changes occur during the day in other components.

Nationality/age/parity

The composition of milk from women from different geographic, ethnic, and socioeconomic backgrounds is remarkably similar, particularly in reference to the macronutrients. Any differences observed are more likely to be the result of dietary variation, rather than genetic modification of composition.

Milk from teenagers has a significantly lower concentration of lactose and some macrominerals compared to adults, but diet may play an important part in this difference as the diet of teenagers is often suboptimal.

Parity may have a significant effect on milk composition, with primiparous women having higher concentrations of protein and fat, and multiparous women experiencing a reduction in the content of major components in milk with each successive lactation.

Maternal diet

Two of the most significant factors influencing milk composition are the nutritional status of the mother and her ongoing diet. An inadequate nutritional status will adversely affect milk volume and the content of milk minerals such as iron and selenium. Severe malnutrition and diminished nutritional status will cause a decrease in the protein and fat content of milk.

The components most affected by maternal diet are FAs, vitamins, and some macro- and micro-minerals. The FA content of human milk can undergo pronounced changes that directly reflect the FA content of the diet. For example, the $C_{18:2n-6}$ content of milk from vegetarians is high compared to the milk from women consuming an omnivorous diet. Also, a low-fat diet will result in a marked increase in medium-chain FAs as the *de novo* synthesis of FAs in the mammary gland increases.

Exercise

Recent evidence suggests that the lactic acid content of human milk can change following exercise, with a decline in infant acceptance of milk secreted immediately after exercise. Exercise regimens at 100, 75, or 50% of $VO_{2\ \max}$ resulted in increases in the lactic acid content of milk sampled immediately after exercise, although only 100% intensity exercise produced milk with a lactic acid content significantly different from baseline. Thus, breast-feeding women can complete a moderate exercise program without the fear that the infant will reject their milk.

Illness and metabolic disorders

Mastitis

Infection of the mammary tissue, with cellulitis and, more rarely, abscess formation, can alter milk composition to such an extent that the infant's nutrition is compromised. During a bout of mastitis, milk volume and lactose content decrease, while the levels of sodium and chloride increase, giving the milk a much saltier taste. Immune factors such as sIgA, lysozyme, and lactoferrin also increase in milk from mastitic tissue, as part of the mammary gland immune response.

Insulin-dependent diabetes mellitus

The delayed lactogenesis associated with mothers with insulin-dependent diabetes mellitus (IDDM) results in a lower volume of milk being consumed by the infant in the first few days following birth. Initially (<5 days' lactation), the milk from an IDDM mother has a composition different from that of normal milk, with less lactose and fat, and more total nitrogen. Once lactation has been established, however, most of these differences diminish. The FA content of milk from IDDM women continues to differ throughout the lactation, with more C_{10}, C_{12}, and C_{14}, and less C_{20} and C_{22}, FAs.

Drug use

The degree to which a drug taken by the mother will affect the breast-fed infant depends entirely on the class of drug. Detailed information on the transfer of pharmaceutical drugs in human milk is available. Recreational drugs, such as alcohol, caffeine, nicotine, and marijuana, also appear in human milk and can affect both milk volume and composition. Human milk from a mother who smokes not only contains many chemical by-products, including nitrates, nitrites, lead, and cadmium, but is also likely to have lower levels of certain vitamins. Exposure of an infant to drugs (both medical and recreational) in milk can be minimized by advising the mother to take any medication immediately after a breast-feed, and/or just before the infant is due to have a lengthy sleep period.

Human Milk Banking

Human milk is usually collected and stored in milk banks to assist in the nourishment of premature infants. Implicit in this action of 'banking' milk is the need to maintain functional integrity in the milk. Numerous steps in the process can affect the composition of human milk, including the suitability of the donor, and her dietary regimen; the method of milk collection and the type of container chosen to collect the milk; the length of time and temperature at which the milk is stored; and the methods used to thaw, mix, and reheat the milk prior to a feed. The Human Milk Banking Association of North America has compiled a set of guidelines for the establishment and operation of a donor milk bank.

See also: **Dehydrated Dairy Products**: Infant Formulae. **Mammals**. **Milk**: Colostrum.

Further Reading

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Colostrum

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Introduction

Colostrum is the initial mammary secretion after parturition. In the cow, it is secreted for approximately the first 5–7 days after delivery. The biological function of this early milk is to provide the newborn with immune protection against pathogens at its most vulnerable phase of life and to boost its physiological performance, growth, and development. Thus, the composition of colostrum is different from that of mature milk. Colostrum contains plenty of immunologically and physiologically active components. These include immunoglobulins (Igs), leukocytes, lactoferrin (LF), lysozyme (LZM), cytokines and other (casein-derived) immunomodulatory factors, growth factors, hormones, and oligosaccharides. Therefore, the commercial exploitation of colostrum for pharmaceutical and dietary use has attracted a great deal of attention of late.

Composition and Changes during Lactation

Cow's colostrum contains more dry matter, mineral salts, and protein and less lactose than mature milk. Also, the fat content is often, but not always, higher than that of milk (**Table 1**). Moreover, the composition of colostrum varies more between individuals than that of mature milk. The concentration of the elements Ca, Na, Mg, P, and Cl is higher and that of K is lower in colostrum than in milk. The main change from colostrum to normal milk occurs in the first few milkings after parturition and continues at reduced rates for approximately 5 weeks. Thereafter, the fat and protein contents rise gradually, and toward the end of lactation they increase more sharply (see **Table 1**).

The proportion of whey proteins is substantially higher in colostrum than in milk. The foremost protein fraction in colostrum consists of Igs (IgG, IgM, IgA). In the first milking, the total amount of Ig varies between 20 and 200 $\mathrm{g\,l^{-1}}$ and the IgG concentration post-partum ranges from 15 to 180 $\mathrm{g\,l^{-1}}$, the mean being approximately 60 $\mathrm{g\,l^{-1}}$. Thereafter, the IgG concentration falls sharply (**Figure 1**). Cows at their first lactation produce significantly less (total yield about half or less) IgG than cows in later lactations due to both a lower IgG concentration and a smaller volume of colostrum. The levels of both α-lactalbumin and β-lactoglobulin are highest in the first milkings of bovine colostrum and decrease sharply thereafter. The protein composition of human colostrum and milk differs considerably from that of bovine colostrum. Human colostrum does not contain β-lactoglobulin and the major Ig class is IgA. The major casein component of human colostrum is β-casein instead of α_{s1}-casein, which is the predominant casein fraction in bovine colostrum.

The fatty acid composition of human and bovine colostrum is markedly influenced by maternal dietary composition. Changes in nutrition have an effect on the colostral fatty acid profile. Both human and bovine colostra typically contain more polyunsaturated fatty acids than mature milk. In colostrum the proportions of short chain fatty acids (C4-C10) are typically lower than in mature milk (**Table 2**). The fat fraction of bovine colostrum contains significantly higher concentrations of phospholipids, cholesterol, and fat globule membrane material than mature milk.

The concentrations of vitamins vary in bovine and human colostra and milk according to stage of lactation and maternal dietary intake. However, much of the variation in reported values in the literature is due to analytical difficulties rather than variable patterns of secretion. In bovine colostrum, the concentrations of fat-soluble vitamins A, D, and E and carotenoids, as well as those of water-soluble vitamins thiamine, riboflavin, vitamins B_6 and B_{12}, folate, and vitamin C, are higher than in mature milk. The contents of pantothenic acid and biotin are lower in colostrum and that of niacin about the same as in mature milk (see **Table 1**). In human colostrum, the levels of vitamins A and E and carotenoids are higher than those in milk. The content of vitamin K in the colostrum is equal to that in milk. (Most of the water-soluble vitamins (thiamine, riboflavin, vitamin B_6, folate, vitamin C, pantothenic acid, biotin, and niacin) exist at lower concentrations in colostrum than in mature milk, whereas the vitamin B_{12} content is higher in colostrum.

Immune Protection by Colostrum

Passively acquired immunity from colostrum is important for the health of a neonate. Newborn calves and piglets that do not receive colostrum show high mortality and poor weight gain during the first weeks of life. Calves deprived of colostrum or given colostrum of poor quality

Table 1 Composition of colostrum and milk of Holstein cows

	Colostrum			*Milk*
Milking postpartum	*1th*	*2th*	*3th*	*6th*
Parameter				
Specific gravity	1.056	1.040	1.035	1.032
Total solids (%)	23.9	17.9	14.1	12.9
Fat (%)	6.7	5.4	3.9	4.0
Total protein (%)	14.0	8.4	5.1	3.1
Casein (%)	4.8	4.3	3.8	2.5
Albumin (%)	6.0	4.2	2.4	0.5
Total Igs (%)	6.0	4.2	2.4	0.09
IgG (g l^{-1})	32	25	15	0.6
Lactose (%)	2.7	3.9	4.4	5.0
IGF-I (μg l^{-1})	341	242	144	15
Insulin (μg l^{-1})	65.9	34.8	15.8	1.1
Ash (%)	1.11	0.95	0.87	0.74
Calcium (%)	0.26	0.15	0.15	0.13
Magnesium (%)	0.04	0.01	0.01	0.01
Zinc (mg l^{-1})	12.2		6.2	3
Manganese (mg l^{-1})	0.2		0.1	0.04
Iron (mg 100 g^{-1})	0.2			0.05
Cobalt (μg 100 g^{-1})	0.5			0.10
Vitamin A (mg l^{-1})	2.95	1.90	1.13	0.34
Vitamin E (μg g^{-1} fat)	84	76	56	15
Riboflavin (mg l^{-1})	4.83	2.71	1.85	1.47
Vitamin B_{12} (μg l^{-1})	49		25	6
Folic acid (μg l^{-1})	8		2	2
Choline (mg ml^{-1})	0.7	0.34	0.23	0.13

Ig, immunoglobulin; IGF-I, insulin-like growth factor 1.

Reproduced with permission from Godden S (2008) Colostrum management for dairy calves: A review. *The Veterinary Clinics of North America: Food Animal Practice* 24: 19–39.

suffer severe long scour episodes. Colostrum from the first two milkings is most important since the concentrations of Igs and LF decrease steeply after the first milkings (see **Figure 1**). The gut of a newborn calf is non-selective and open to the transport of a variety of macromolecules from 12 to 36 h matching with the time of the highest IgG content of colostrum. A newborn calf needs about 2–4 l of colostrum (100–200 g Ig) to protect it against scouring.

Most farm animals do not have *in utero* transport of Igs to the fetus and their young are born virtually without protective antibodies. In humans, IgG transport via the placenta is very efficient and therefore the human infant is born with a serum concentration of IgG equaling that of the mother. Mammals can be categorized into species with extensive placental transfer (e.g., human and rabbit), those with no placental transfer but extensive lacteal transport (e.g., pig, horse, cow, sheep, and goat), and those that use both pathways (e.g., dog, mouse, and rat).

The transport of Ig from serum to colostrum is selective, favoring homologous IgG in most species. Specific receptors are involved in the process (see **Figure 2**). This enables the selective concentration of IgG isotypes in colostrum. For information on the absorption of antibodies, *see* **Milk Proteins**: Immunoglobulins.

Colostrum also contains an active complement system that participates in immune defense of the udder. It is not known whether the colostral complement system provides any immunological protection to the newborn. There is some evidence that feeding colostrum or colostral whey concentrate to a calf increases the lytic activities of both antibody-dependent classical and antibody-independent alternative pathways of complement in the serum of the calf.

Colostral Ig and complement proteins are quite resistant to gastric acids but sensitive to trypsin. On the other hand, bovine colostrum contains a trypsin inhibitor that neutralizes trypsin activity and allows absorption of Ig.

Living leukocytes are an integral part of normal bovine and human colostrum and milk. Their concentration is highest in the colostrum and declines gradually in transition to normal milk. The leukocytes defend the breast or udder against pathogens. It has been established that maternal leukocytes cross the neonatal gut and circulate in the newborn calf. This may result in the

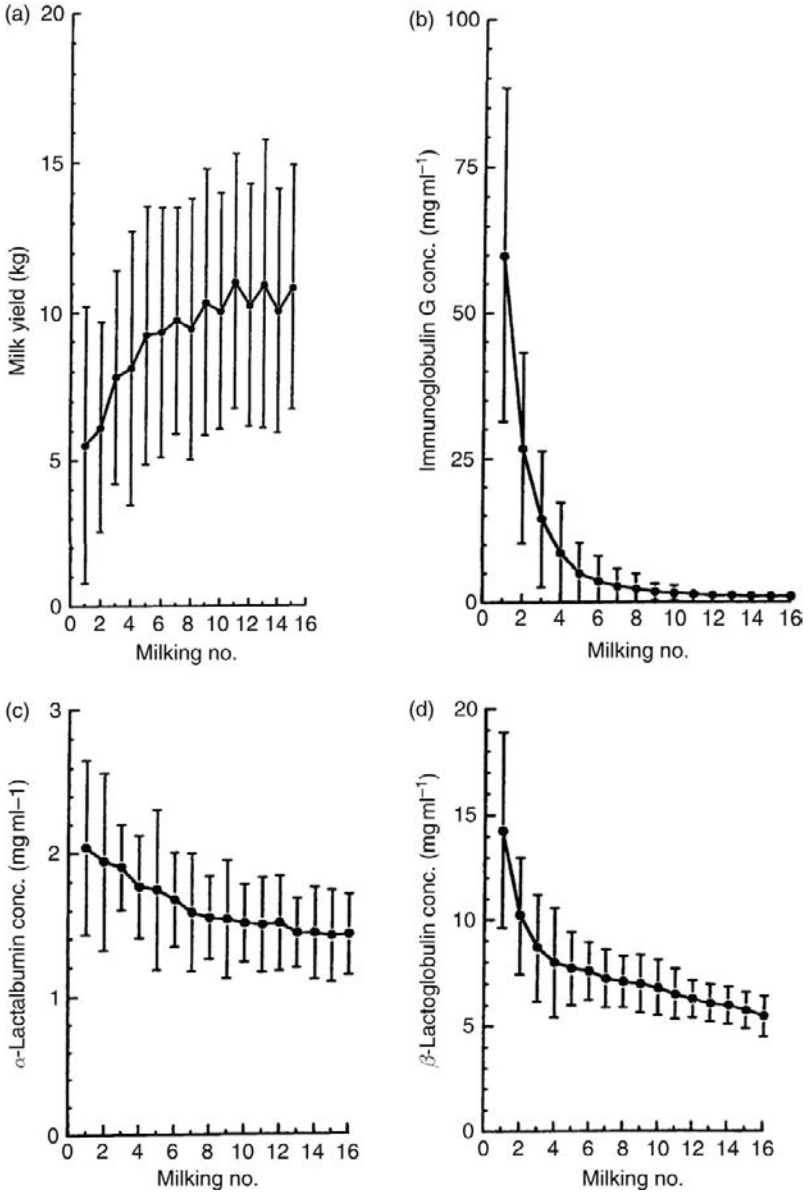

Figure 1 (a) Milk yield and concentrations of (b) immunoglobulin G, (c) α-lactalbumin, and (d) β-lactoglobulin of the first milkings after calving in 60 Holstein-Friesian cows (mean values with SD indicated by vertical bars). Adapted with permission from Levieux D and Ollier A (1999) Bovine immunoglobulin G, β-lactoglobulin, α-lactalbumin and serum albumin in colostrum and milk during the early post partum period. *Journal of Dairy Research* 66: 421–430.

transfer of antigen-specific cellular immunity to the calf. Recent studies have shown that these maternal leukocytes have an important role in the development and maturation of neonatal cellular immunity. However, so far, only little is known about the mechanisms of the immunological interactions of maternal leukocytes with the neonate.

Colostral Ig and lymphocytes represent the mother's specific immune response to antigens encountered in the past. This immunological memory is mediated to the next generation via colostrum. In addition, colostrum also contains components that carry nonspecific antimicrobial and immune-enhancing properties. Such factors are LF, LZM, lactoperoxidase (LPO), and leukocytes (**Table 3**).

Human colostrum is particularly rich in LF, with the concentration ranging from 5 to 10 $g\,l^{-1}$. The concentration decreases in transition to mature milk to about 0.1 $g\,l^{-1}$. In cow's colostrum, the LF concentration varies from 1 to 2 $g\,l^{-1}$, decreasing soon after parturition to a level of about 0.1 $g\,l^{-1}$ in normal milk (see **Table 3**). LF is synthesized in the mammary gland, but also in other exocrine glands. LF and especially its cleavage products have a broad bacteriostatic and bactericidal spectrum; in particular, LF has a synergistic antibacterial effect with

Table 2 Fatty acid composition in colostrum and milk of Swedish Friesian cows

	Total fatty acid content (wt.%)			
	Time post-partum			
Fatty acid	*0–10 h*	*11–30 h*	*31–50 h*	*Mature milk*
C_4	3.7	3.7	5.1	4.7
C_6	2.0	1.9	2.3	2.8
C_8	1.0	0.9	1.1	1.5
C_{10}	2.1	2.0	2.2	3.1
C_{12}	3.3	2.9	2.7	3.8
C_{14}	13.0	11.7	10.3	11.3
C_{15}	0.8	0.7	0.8	0.9
C_{16}	37.8	37.4	33.2	30.3
C_{17}	0.4	0.5	0.6	0.4
C_{18}	5.5	7.7	9.2	11.5
C_{20}	0.2	0.1	0.1	0.2
C_{22}	0.1	n.d.	n.d.	n.d.
Total saturated fatty acids	69.9	69.5	67.6	70.4
$C_{16:1}$ *trans*	<0.1	<0.1	<0.1	0.1
$C_{18:1}$ *trans*	1.2	1.5	1.9	2.2
$C_{18:2}$ *trans*	0.1	0.1	0.1	0.2
Total *trans* fatty acids	1.3	1.6	2.0	2.5
$C_{10:1}$	0.1	<0.1	0.1	0.3
$C_{14:1}$	1.2	0.8	0.6	0.6
$C_{16:1}$ *cis*	3.1	3.1	2.7	1.5
$C_{17:1}$	0.2	0.4	0.3	0.1
$C_{18:1}$ *cis*	20.7	21.6	23.5	21.6
$C_{22:1}$	0.2	0.3	0.3	n.d.
Total *cis* monounsaturated fatty acids	25.5	26.1	27.4	24.4
$C_{18:2}$ *cis*	2.2	2.0	1.8	1.5
$C_{18:3}$ *cis*	0.8	0.7	0.7	0.6
Total *cis* polyunsaturated fatty acids	3.0	2.7	2.5	2.1
Total *cis* unsaturated fatty acids	28.5	28.9	30.0	26.5
Others	0.3	0.0	0.4	0.6

n.d., not determined.
Data from M. Paulsson and H. Lindmark-Månsson (unpublished results).

LZM. LF also regulates immune defense by inhibiting the progressive inflammatory cascade. In the mouse, orally administered bovine milk LF increases the numbers of CD4+ and CD8+ lymphocytes and natural killer (NK) cells and regulates cytokine secretion in gastrointestinal lymphoid tissue. The systemic effects of orally administered LF include increased cell numbers in spleen and lymph nodes, enhanced cell activity of phagocytes and NK cells, and modulation of lymphocyte functions (*see* **Milk Proteins**: Lactoferrin).

Soluble CD14 receptors (sCD14) belong to the family of pattern-recognizing receptors and bind lipopolysaccharides (LPS). It is reported to exist in human colostrum ($20 \pm 9\,\mathrm{mg\,l^{-1}}$ (mean ± SD)) and mature milk (about $14 \pm 6\,\mathrm{mg\,l^{-1}}$ (mean ± SD)) at a remarkably higher concentration than in serum (about $3\,\mathrm{mg\,l^{-1}}$). Quantification of sCD14 in bovine milk has been difficult due to the lack of a specific assay. Nevertheless, it is estimated to be present at slightly lower concentrations than in human milk and the levels are elevated during mastitis or intramammary challenge with LPS. It contributes to the 'education' and maturation of the neonatal immune system for recognizing and responding to microbial components.

LPO is an effective antimicrobial oxidoreductase enzyme that catalyzes the oxidation of thiocyanate and halide ions (mainly chloride, iodide, or fluoride) to corresponding hypohalides to generate highly reactive oxidizing agents having a broad spectrum of antimicrobial effects. Cow's colostrum contains about $30\,\mathrm{mg\,l^{-1}}$ LPO, the same level as in mature milk. In human colostrum, practically all of the peroxidase activity is derived from myeloperoxidase of leukocyte origin, and only a trace amount of it is from LPO. LPO plays an important role in protecting the lactating mammary gland and the intestinal tract of the newborn against pathogenic microbes. Feeding of LPO-supplemented milk to calves reduces the incidence of scouring and improves growth in newborn calves. LPO can also be exploited for preservation of raw milk. In human infants, salivary peroxidase may compensate the paucity of LPO in colostrum.

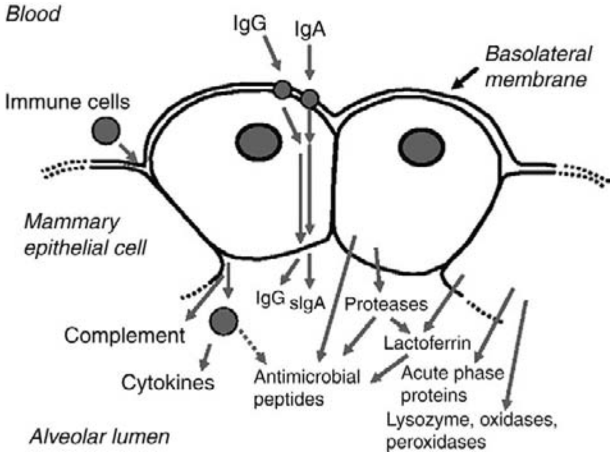

Figure 2 Schematic diagram showing some of the principal known proteinaceous components of the host defense system in milk and colostrum. IgA and IgG, immunoglobulins A and G; sIgA, secretory IgA. Reproduced with permission from Wheeler TT, Hodgkinson AJ, Prosser CG, and Davis SR (2007) Immune components of colostrum and milk – a historical perspective. *Journal of Mammary Gland Biology and Neoplasia* 12(4): 237–247.

LZM is a potent antibacterial component in human colostrum and milk and influences the intestinal bacterial flora of the neonate. Human colostrum contains LZM at about $0.36\,\mathrm{g\,l^{-1}}$; the concentration of LZM decreases to $0.30\,\mathrm{g\,l^{-1}}$ in transition and mature milk. In bovine colostrum, the LZM level ranges from 0.3 to $0.8\,\mathrm{mg\,l^{-1}}$, declining to $0.1\,\mathrm{mg\,l^{-1}}$ in mature milk.

Growth Factors

Colostrum and milk contain many factors that influence cell growth and differentiation. These non-nutrient components contribute to the specific stimulation of jejunal and skeletal muscle development in colostrum-fed neonatal pigs and calves as well as humans. Results from animal studies suggest that feeding of trace amounts of colostral growth factors augments intestinal absorptive capacity as well as protein and fat metabolism and exerts beneficial effects on the endocrine system of neonatal calves. Also, it is suggested that colostral growth factors stimulate brain and heart protein synthesis in colostrum-fed neonatal animals. Furthermore, some non-peptide constituents of colostrum (e.g., glutamine, polyamines, and nucleotides) play an important role in maintaining the gastrointestinal mucosal mass and in modulating the immune system via multiple mechanisms, for example, by modulating the gastrointestinal microflora and

Table 3 Concentration and biological functions of main proteins in bovine and human colostrum and milk

	Concentration				
Protein	*Bovine colostrum*	*Bovine milk*	*Human colostrum*	*Human milk*	*Function*
Caseins (α_s, β, κ) ($\mathrm{g\,l^{-1}}$)	26	28	Trace amounts	2.7	Transport of minerals and trace elements (Ca, PO_4, Fe, Zn, Cu), precursors of bioactive peptides
α-Lactalbumin ($\mathrm{g\,l^{-1}}$)	1–3	1.4	4.9	3.4	Lactose synthesis in mammary gland, calcium transport, immunomodulation, anticarcinogenic properties, precursor of bioactive peptides, enhancement of serotonin release
β-Lactoglobulin ($\mathrm{g\,l^{-1}}$)	6–14	3	None	None	Binding of fatty acids, retinol transport, precursor of bioactive peptides
Immunoglobulins (IgG, IgM, IgA) ($\mathrm{g\,l^{-1}}$)	20–200	0.7	6.3–48	0.26–1.8	Transfer of passive immunity to offspring, immune protection of mammary gland
Lactoferrin ($\mathrm{mg\,l^{-1}}$)	1–2	0.1	5–10	0.1	Antimicrobial, immunomodulatory, and anticarcinogenic effects, iron binding and transport
Lactoperoxidase ($\mathrm{mg\,l^{-1}}$)	20–40	30	Trace amounts	Trace amounts	Antimicrobial function, synergistic effect with immunoglobulins and lactoferrin
Lysozyme ($\mathrm{mg\,l^{-1}}$)	0.3–0.8	0.1	360	300	Antibacterial effects, synergistic effect with immunoglobulins and lactoferrin

Data compiled from Jensen RG (ed.) (1995) *Handbook of Milk Composition*. San Diego, CA: Academic Press, Inc.; Korhonen H, Pihlanto-Leppälä A, Rantamäki P, and Tupasela T (1998) The functional and biological properties of whey proteins: Prospects for the development of functional foods: A review. *Agricultural and Food Science in Finland* 7: 283–296; Pakkanen R and Aalto J (1997) Growth factors and antimicrobial factors of bovine colostrums: A review. *International Dairy Journal* 7: 285–297; Velona T, Abbiati L, Beretta B, *et al*. (1999) Protein profiles in breast milk from mothers delivering term and preterm babies. *Pediatric Research* 45(5 Pt. 1): 658–663.

influencing the actions of growth factors. In general, the growth factor concentration in colostrum is highest during the first hours after calving and declines substantially thereafter.

In bovine colostrum, the epidermal growth factor (EGF) and betacellulin belong to the EGF family. Members of the EGF family stimulate the proliferation of epithelial and epidermal cells, act as differentiation factors for some cell types, and modulate the synthesis of a number of hormones. EGF inhibits the secretion of gastric acid. In bovine colostrum, the concentration of EGF varies from 4 to 320 $\mu g\,l^{-1}$ and it ranges from 2 to 155 $\mu g\,l^{-1}$ in mature milk. In human colostrum, the total EGF concentration is about 35–330 $\mu g\,l^{-1}$ and ranges from 3 to 60 $\mu g\,l^{-1}$ in milk. Betacellulin is present in bovine colostrum (2.30 $\mu g\,l^{-1}$) and in cheese whey (2.59 $\mu g\,l^{-1}$), suggesting that it plays a role in accelerating the growth and maturation of the calf and/or in mammary gland function. Human EGF is degraded in both gastric and duodenal lumen. Bovine milk is reported to contain peptidase inhibitors that protect human EGF from proteolytic degradation in human gastrointestinal fluids.

Insulin-like growth factors 1 and 2 (IGF-I and IGF-II) are single-chain polypeptides structurally resembling insulin. They are widely distributed and promote cell proliferation and differentiation and have *in vitro* acute anabolic effects on protein and carbohydrate metabolism. In serum and milk, the IGFs are found almost entirely bound to high-affinity binding proteins (IGFBPs). In bovine colostrum, IGFBP-3 is the major binding protein, but IGFBP-2 is also abundant. The IGF peptide is released from binding proteins by acid treatment. Bovine colostrum contains significantly higher concentrations of IGF-I than does human colostrum (32–800 $\mu g\,l^{-1}$ as compared with 17–52 $\mu g\,l^{-1}$), with lower concentrations in mature bovine milk (4–27 $\mu g\,l^{-1}$). In neonatal piglets, parenteral IGF-I administration enhances the thickness of epithelial, submucosal, and smooth muscle layers, promotes gastrointestinal tract growth, and improves the absorption of sodium and glucose. IGF-I in bovine colostrum is an anabolic agent and is at least partly responsible for mediating the growth-promoting activity of the growth hormone (GH), which has been detected in bovine colostrum at a low concentration of 0.17 $\mu g\,l^{-1}$ but is absent from mature milk. Although many proteins are absorbed effectively within the first 24–48 h after birth, only negligible or no absorption of insulin or IGF-I is detected in the blood circulation of calves within the first 24 h after birth. IGF-II is present in bovine colostrum and milk at concentrations of 150–600 and 2–100 $\mu g\,l^{-1}$, respectively. IGF-II has anabolic activity and has been shown to reduce the catabolic state in starved animals.

LF may also participate in regulation of the gut and in bone growth in developing neonates as it stimulates *in vitro* the growth of fibroblasts, intestinal epithelial cells, leukocytes in the gastrointestinal tract-associated lymphoid tissue, and osteoblasts. Receptors for LF are expressed by several cell types, including T and B lymphocytes, platelets, and intestinal epithelial cells.

Transforming growth factor-α (TGF-α) is present in human colostrum and milk at concentrations of 2.2–7.2 $\mu g\,l^{-1}$. Most studies suggest that the major physiological role of TGF-α is to act as a mucosal integrity peptide, maintaining normal epithelial function in the nondamaged mucosa.

The TGF-β family comprises multi-functional growth and differentiation factors acting on most cell types, stimulating the proliferation of connective tissue cells and acting as growth inhibitors of some other cell types such as lymphocytes and epithelial cells. TGF-β1 is detected in bovine colostrum and milk (12–43 and 0.8–4 $\mu g\,l^{-1}$, respectively) but TGF-β2 is the predominant isoform present at high concentrations in bovine colostrum and milk (150–1150 and 13–71 $\mu g\,l^{-1}$, respectively). The physiological function of TGF-β in bovine colostrum and milk is obscure, but the high concentrations suggest that TGF-β in colostrum may be a mediator of mucosal immunity and may contribute to epithelial differentiation of gastrointestinal tract of suckling neonates.

Sialyl oligosaccharides in colostrum and milk prevent adhesion of pathogenic microbes. Sialic acid is probably used in the biosynthesis of neonate brain gangliosides and sialyl glycoproteins. In the cow, the total sialic acid content is 2–4 times higher in colostrum (0.3–0.8 $g\,kg^{-1}$) than in transition and mature milk (0.1–0.2 $g\,kg^{-1}$).

Other growth factors, cytokines, and hormones are also found in human and bovine colostrum, but their physiological significance remains obscure. These are, for example, the vascular endothelial growth factor, fibroblast growth factors, the platelet-derived growth factor, GH and its releasing factor, insulin, granulocyte, macrophage, and granulocyte–macrophage colony-stimulating factors, interleukins 1β, 6, 10, and 18, and tumor necrosis factor-α.

Recent studies suggest that colostral fractions, or individual peptides present in colostrum, might be useful for the regulation/modulation of a wide variety of gastrointestinal functions. It is likely that in the future, additional growth factors will be identified in colostrum and milk. For additional information on these factors and their extraction and bioavailability from colostrum and milk, see the 'Further Reading' section.

Industrial Utilization of Colostrum

Colostrum is normally produced in amounts that exceed the need of the newborn. At present, dairy or food industries do not exploit bovine colostrum commercially on a large scale due to technical and hygienic problems related to collection and processing. In many countries, colostrum must be excluded from bulk milk collection during the first 5 days post-partum. The low clotting temperature of colostrum interferes with pasteurization and the high protein content leads to problems in industrial processes. Furthermore, the high content of antimicrobial components in colostrum may slow down or inhibit fermentation processes. This also affects the antibiotic residue tests based on microbial growth, causing false-positive results.

Bovine colostrum is utilized on a minor industrial scale and in households in many countries, for example, in Scandinavia, in the form of cheese baked in an oven. Also, the casein fraction of renneted colostrum is baked and used as fresh cheese.

Cheese whey-based and colostral whey-based antibody preparations are commercially available in many countries as feed supplements or colostrum substitutes for farm animals. They are prepared mainly for neonate calves and pigs and have shown variable efficacy in the prevention of diarrheal diseases caused by pathogens such as rotaviruses and enterotoxigenic *Escherichia coli* strains. There is increasing interest in the development of so-called immune milk preparations made from colostrum of hyperimmunized cows. Such preparations may target both humans and domestic animals for prevention and treatment of specific gastrointestinal diseases. A few preparations, for example, those against rotavirus and *E. coli*, are already on the market.

The pharmaceutical and biotechnological industries have recently shown interest in bovine colostrum as a source of growth factors and other specific bioactive components. Colostrum-based growth media have been developed for culturing cells. Also, a multitude of health products and foods made from various colostrum fractions, for example, drinks and chewing gums for sportsmen, have been launched on the market. Scientific studies have shown some evidence that orally administered bovine colostral supplements can increase lean body mass and have a small improving effect on exercise performance and/or recovery but the mechanisms still remain elusive. A few studies suggest that orally administered bovine colostral whey preparations could reduce the incidence of upper respiratory tract infections and have beneficial effects on cholesterol level and type 2 diabetes in humans. However, more research is needed to verify these effects. Currently, there is increasing pharmaceutical research under way to examine the potential related to the use of colostrum-derived preparations for a wide range of gastroenterological conditions.

See also: **Milk Proteins**: Immunoglobulins; Lactoferrin.

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Seasonal Effects on Processing Properties of Cows' Milk

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Introduction

Milk production may be described in terms of two distinct cow herd management systems; one system is defined by a comparatively even milk production pattern throughout the year, whereas the other system is defined by milk production peaking at a specific time of year (e.g., April–May) and decreasing steadily until end of lactation (e.g., December). The consistent milk production system observed in most European countries is characterized by a relatively consistent calving pattern throughout the year. For example, in Norway, the month with greatest incidence of calving is September with fewer than 14% of the total cow population calving during that month. Alternatively, the seasonal milk production system is characterized by a compact calving pattern where a large proportion of cows calve between January and April, for example, with as many as 60% of total cows calving in the February–March period in Ireland.

Some dairy industries such as that of Ireland and New Zealand are based around the use of pasture as a natural low-cost feed source, which has led to the wide adoption of seasonal calving to maximize pasture utilization. Milk producers continually adjust the date of calving, so that most cows calve in early spring, and are dried off for 8–10 weeks during winter. Though this system maximizes cost efficiency from a grass-based production perspective, the seasonal milk production system creates two constraints for the milk processing industry; that is, an irregular supply of milk for product manufacture during the winter months (when milk production ceases) and an irregular quality of milk during the autumn/winter period when cows have reached an advanced stage of lactation (>250 days). When the national herd is predominately spring-calving and a management strategy of compact calving is applied, the composition of the milk varies as the milk of an individual cow would throughout her lactation. This phenomenon may be less noticeable in countries where a more consistent calving pattern is practiced throughout the year. Although seasonal variation in quality is most evident and extreme in seasonal calving milk production systems, seasonal variation in composition and quality is also notable in milk from consistent milk production systems. Seasonal variation in the processing quality of milk is manifested through stage of lactation and nutritional and physiological effects.

Seasonality of milk may be defined simply as the changes in composition, quality, and suitability for processing into dairy-based products throughout the calendar year. For compact spring-calved herds on pasture-based feeding systems, advance in season coincides with advance in stage of lactation (e.g., typically start of lactation in February to end of lactation in December in the Northern Hemisphere). This is less so in the case of milk produced by herds with a consistent calving pattern over the year, where milk is produced all year round.

The main factors affecting seasonal changes in milk composition and quality are stage of lactation and nutritional status/environment. Lactation changes in milk composition may be defined as those that take place during the period of milk production, between parturition and drying-off, mainly as a result of physiological changes occurring in the mammary gland in healthy cows fed on standard good-quality diets. On the other hand, seasonal changes in milk may be defined as those arising due to lactation and the superimposed effects of other factors such as variations in diet, illness (e.g., subclinical mastitis), environmental factors, and climate.

The aforementioned effects of stage of lactation occur in milk from both calving systems (i.e., compact spring-calving and consistent calving) but are not as evident in the overall milk pool of the latter because of the masking by milk contributed from cows at different stages of lactation. Indeed, the late-lactation effects of individual cow milk may be more extreme in consistent-calved herds than spring-calved herds owing to the herd management/practices applied (e.g., somatic cell count as influenced by hygiene issues associated with the production of milk indoors, and days of lactation of individual cows at drying-off). However, other factors influencing milk composition/quality include, *inter alia*, breed, health status, age of the cow, and the counts and types of somatic cells and bacteria in the milk.

The processing properties of cows' milk may be described as the characteristics that enable it to meet the criteria required for efficient manufacture and production of a quality product. This article describes the effect of season on milk processing properties and its suitability for product manufacture, focusing on three main areas: cheese-making properties, heat stability, and alcohol stability of milk, with particular emphasis on the former.

It is noteworthy that cheese manufacture accounts for a significant portion of total milk produced globally (i.e., ~25%), even though it varies widely with region.

Cheese

Principles of Cheese Manufacture

Cheese is a concentrated protein gel, which occludes fat and moisture. Its manufacture essentially involves gelation of cheesemilk, dehydration of the gel to form a curd, and treatment of the curd (e.g., dry stirring, cheddaring, texturization, salting, molding, pressing). The molded curds may be consumed fresh (shortly after manufacture, e.g., within 1 week) or matured for periods of ~2 weeks to 2 years to form a ripened cheese. The gelation of milk may be induced by:

- selective hydrolysis of the κ-casein by the addition of acid proteinases, referred to generically as rennets (e.g., chymosin, pepsin);
- acidification (e.g., using starter cultures or food-grade acids and/or acidogens), at a temperature of 20–40°C, to a pH value ~4.6; and/or
- a combination of acid and heat (e.g., heating milk at ~pH 5.6 to ~90°C).

Essentially, cheesemaking is a process involving protein (of which casein is the largest component) aggregation and gel formation, and dehydration of the resultant gel. Thus, the degree of casein aggregation is a critical parameter controlling the properties and quality of the final cheese. The most important milk-quality characteristics for cheese manufacture are those that promote:

- aggregation of the casein to form a gel that is sufficiently firm to cut within an acceptable time frame (typically, 30–50 min for rennet-curd cheese; 4–14 h for acid-curd cheeses);
- continued aggregation together with whey expulsion during the remaining cheesemaking operations after gelation;
- a gel structure and rheology that at all stages of manufacture provides (a) a robustness that maximizes the retention of fat and casein in the curd and curd yield and (b) the desired texture in the final cheese, at the point of use (secondary processing or consumption).

These attributes are prerequisite to the formation of a fresh curd with desired composition, structure, texture, and yield. Characteristics of the milk that are generally positively correlated with enhanced rennet coagulation include:

- high values for casein number, intact casein content, contents of total casein, individual (α_{s1}-, β-, and κ-) caseins, calcium-to-casein ratio, and ratios of κ-casein to total casein and to individual caseins (α_{s2}- and β-caseins);
- low values for serum casein, micelle size, and degree of κ-casein glycosylation.

Quality Requirements of Milk for Cheese Manufacture

In an overall context, the quality of milk for cheese may be defined as its characteristics that fulfill the requirements of its users, direct (the cheese manufacturer) and indirect (the cheese user, consumer). The quality requirements of the manufacturer/processor are discussed below.

1. Compositional/nutritional, which indicate the conformity to minimum levels of particular components (e.g., fat, protein, casein, calcium) that make it suitable for cheese manufacture (e.g., enable the milk to form a gel suitable for cutting within a certain time after addition of rennet); to give desired manufacturing efficiency (e.g., percentage recovery of fat and casein; product yield), composition (e.g., levels of protein, calcium, moisture), and sensory characteristics.
2. Intact casein level (an index of the wholeness of the milk casein, the absence of milk protein hydrolysis) reflecting the ability of the milk to form a strong intact gel, capable of occluding protein, fat and moisture during manufacture, maximizing the recovery of milk fat and water to cheese, and imparting the desired texture and functional properties to the final cheese (*see* **Cheese**: Cheese as a Food Ingredient; Cheese Rheology). Native bovine milk contains proteinases from a number of sources, the indigenous milk trypsin-like proteinase (plasmin), lysosomal proteinases of somatic cells, and bacterial proteinases of bacteria (especially psychrotrophic bacteria such as *Pseudomonas* or *Bacillus*). These proteinases hydrolyze caseins, are complex in their regulation, and vary in activity according to factors such as stage of lactation and mastitis status. Hydrolysis of the milk casein, apart from its effects associated with reduction in intact casein level, reduces protein recovery to cheese during the manufacturing process, an effect that reduces the overall manufacturing efficiency.
3. Fat stability, an index of the intactness of the fat globule (with little or no damage to the milk fat globule membrane, or MFGM) and absence of lipolysis of fat triacylglycerols (to products such as di-/mono-acylglycerols and free fatty acids, FFA). Bovine milk contains lipases which hydrolyze the fat into FFA, which cause unpleasant rancid or bitter off-flavors in many dairy products. Milk from cows in late lactation has a higher FFA level than milk from cows in early lactation (0.68 and 0.28 mM 100 g^{-1} fat, respectively). This may be related to the type of feed (pasture or dry

feed indoors) and/or quality of feed, particularly in regions where cows are compact spring-calved and late lactation corresponds with a time of year when good-quality feed is in short supply. Alternatively, the increased milk lipolysis levels may be due to mechanical damage of the fat globule membrane (which encloses the fat) by excessive mixing of air into the relatively small volume of milk, especially during late lactation.

Effect of Milk Composition and Quality on Cheese

Variation in the fat content of the raw milk is generally of little practical significance, as milk for cheese manufacture is easily standardized to a protein-to-fat ratio (PFR) within a defined range (e.g., ~0.85–0.90 for Cheddar cheese), as part of a standard operating procedure, by the appropriate removal of fat *via* mechanical separation (skimming) (e.g., for low-moisture part-skim Mozzarella) or the addition of cream (e.g., for cream cheese). However, where milk is not standardized, seasonal variation in the PFR of milk (**Figure 1**) can have significant effects on cheese composition. Increasing the PFR in the range 0.70–1.15 has been found to significantly increase the level of moisture but reduce the level of moisture-in-non-fat substances (MNFS), fat, fat-in-dry matter (FDM), and salt-in-moisture (S/M).

Similarly, the protein content, or more specifically the casein content, of raw milk has little effect when the protein content of the cheesemilk is standardized to a defined level, for example, by low concentration factor (1–1.5 times) ultrafiltration of the raw milk or by addition of a milk protein supplement. However, protein standardization is not a universal practice, and consequently variation in milk protein level can have significant effects on cheese composition, yield, and quality.

Apart from variations in the levels of gross constituents, seasonal variation can also occur in the functionality or 'quality' of the protein in terms of its ability to form a gel with satisfactory curd firming and syneretic (wheying-off) properties and to produce cheese curd of satisfactory moisture content and texture. Late-lactation milk generally gives poor rennet coagulability (low curd firmness), impaired curd syneresis, high-moisture Cheddar cheese, and lower recovery of milk fat in cheese. These defects coincide with low levels (approximately ≤4.3%, w/w) of milk lactose, low casein number (<72, casein as a percentage of true protein), and increased levels of serum casein (>40 g 100 g^{-1} total protein), non-sedimentable at 30 000 × *g*. In this context, it is noteworthy that low lactose levels in milk generally coincide with high somatic cell count (SCC) and level of plasmin activity and may be indicative of udder infection and increased excretion of blood constituents into the milk. The extent of these cheesemaking defects in late-lactation spring-calved herds is accentuated when both the plane of nutrition of the cow and the milk yield at drying-off are low (e.g., high stocking density on pasture in October and November without dietary supplementation in the Northern Hemisphere, and <6 l of milk per cow per day).

Seasonal Changes in Milk Composition

There are notable changes in the concentrations of fat, protein, casein, and lactose and individual minerals associated with seasonality (**Figures 2** and **3**). These changes in turn affect the rennet gelation characteristics (**Figure 4**) of milk and its cheesemaking properties. The seasonal changes are affected by a number of factors, especially stage of lactation and cow nutrition/environment.

Effect of Stage of Lactation on Milk Composition

The composition of milk changes markedly with stage of lactation. After parturition, yield increases during the first 6 weeks and thereafter declines steadily to the end of lactation. The colostrum produced at parturition has a very high concentration of total solids (i.e., ~25% compared with 13% in mid-lactation). The increase in milk

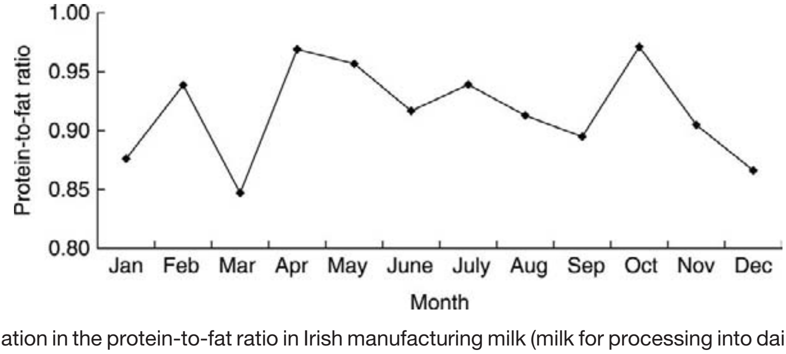

Figure 1 Seasonal variation in the protein-to-fat ratio in Irish manufacturing milk (milk for processing into dairy products rather than for sale as pasteurised drinking milk).

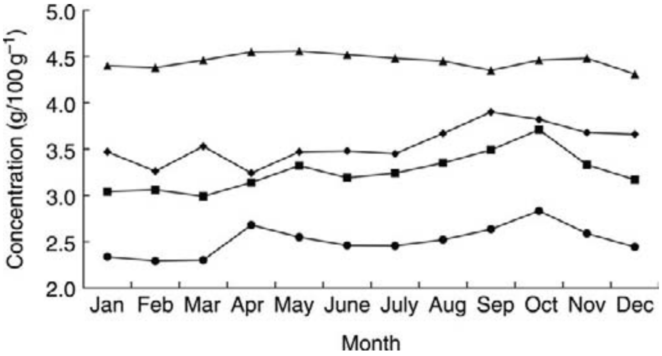

Figure 2 Seasonal variation in the concentration of fat (◆), protein (■), lactose (▲), and casein (•) in Irish manufacturing milk.

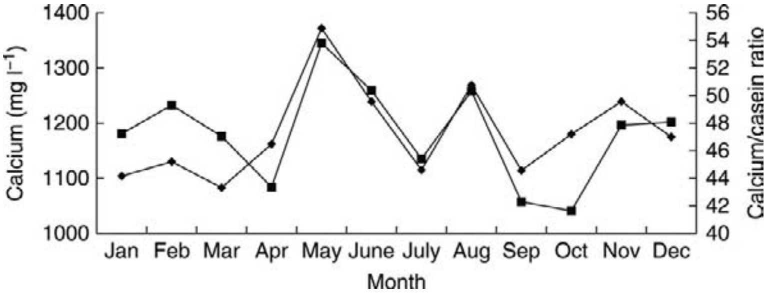

Figure 3 Seasonal variation in the concentration of calcium (◆) and calcium-to-casein ratio (■) in Irish manufacturing milk.

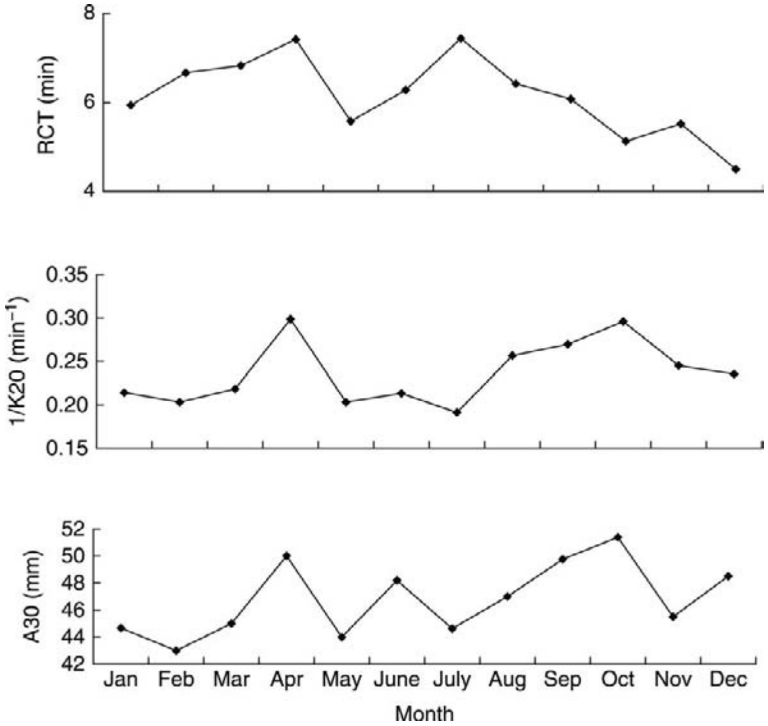

Figure 4 Seasonal variation in rennet coagulation time (RCT), curd-firming rate (1/K20), and curd firmness at 30 min (A30) of Irish manufacturing milk at natural pH (~6.6–6.7) and at 31 °C.

yield to its peak level in early lactation (~6 weeks) is paralleled by reductions in the levels of total solids, protein, casein, and fat and an increase in the concentration of lactose. In contrast, the subsequent steady decrease in milk yield to late lactation (i.e., 285 days) coincides with gradual increases in total solids, fat, protein, and casein and a reduction in lactose level. Lactation effects on gross composition are more pronounced at the beginning (first 6–8 weeks) and end of lactation (increasingly so from 225 days onward). During the latter period, the proportional increases in total protein and fat are similar, but that for casein is less than that of total protein (as reflected by the decrease in casein number). Hence, the casein-to-fat ratio decreases rather dramatically, emphasizing the need for accurate milk standardization for cheese manufacture to maintain optimum composition and cheese yield. Whereas the cheese-yielding potential of acceptable-quality milk increases progressively in the late-lactation period (days 225–285) due to the increase in casein level, the decrease in casein number indicates that for a given protein level, the cheese-yielding capacity is lower compared with that at mid-lactation.

Moreover, the proportions of different caseins and whey proteins change over the course of lactation. α_{s1}-Casein as a percentage of total casein drops rapidly during the first few days of lactation, then increases, and thereafter remains more or less constant throughout the remainder of lactation. In contrast, the proportion of β-casein is low at the initial stages of lactation, then increases to ~200 days and thereafter decreases as the levels of γ-caseins increase. Although no information is available on the effects of the differences in the proportions of individual caseins on cheese yield, it is expected that the dry matter cheese yield per 100 kg of casein- and fat-adjusted milk would decrease slightly toward the end of lactation with the increase in the proportion of γ-caseins and the concomitant reductions in intact casein level and protein recovery from milk to cheese. The proteose peptones, produced on hydrolysis of β-casein to γ-caseins, are soluble in the serum phase and are likely to be only partly, if at all, recovered with the curd during cheese manufacture. The total proteose peptone fraction increases during lactation from ~20 to 50 mg N 100 g^{-1} milk. Moreover, changes in the proportions of different caseins are likely to affect protein interactions (ratio of hydrophobic to electrostatic), gel strength, and the physical properties of the resultant cheeses.

Effect of Cow Nutrition on Milk Composition

One alternative to influence the manufacturing potential of milk is through the nutrition of the cow, but the response may vary depending on the stage of lactation.

Early lactation

In pasture-fed systems, calving date is targeted to commence with the start of the grass herbage-growing season. However, supplementing spring-calved cows on grass silage and concentrates with grazed grass in late February to late April (by allowing cows on pasture for 2–4 h per day) was found to significantly improve the gelation properties of milk, an effect concomitant with numerical increases in protein concentration (e.g., 3.06–3.17%, w/w).

Mid-lactation

Studies have shown that increasing herbage allowance from 16 to 24 kg grass dry matter (DM) in mid-lactation significantly increased both the yields and concentrations of total protein (e.g., from 3.2 to 3.4%, w/w), casein (2.43–2.61%, w/w), and lactose (4.60–4.65%, w/w) but did not significantly affect the concentrations of calcium and phosphorus, rennet gelation properties, and the alcohol stability of the milk. Similarly, the cheesemaking characteristics were not influenced, as reflected by the absence of significant effects of herbage allowance on the gross composition, rheologic characteristics, or cooking properties of the resultant Mozzarella cheese. However, the moisture-adjusted cheese yield increased by ≥0.5 kg 100 kg^{-1} milk, an effect that coincided with the higher level of milk casein (~0.1%, w/w) at the higher herbage rates. The above trends were corroborated by further studies, showing that an increase in stocking density above a standard system (defined as post-grazing grass height of 60 mm) resulted in significant reductions in the concentrations of total protein (e.g., 3.22 vs. 3.40%, w/w), casein (2.48 vs. 2.67%, w/w), and whey proteins, and a deterioration in rennet coagulability. Imposing concentrate supplementation on the standard system increased the levels of total protein (e.g., 3.40 vs. 3.49%, w/w), casein (e.g., 2.67 vs. 2.76%, w/w), and whey protein but generally did not affect processing characteristics, including rennet coagulability and alcohol stability (stable to 76 vs. 79% ethanol). The supplemented diet also resulted in an increase in moisture-adjusted cheese yield of 1.1 kg 100 kg^{-1} milk, an effect that coincided with the higher level of casein (~0.25%, w/w).

Late lactation

Maintenance of a high plane of nutrition has also been found to maintain satisfactory milk processing (composition, N fractions, free fatty acid levels, alcohol stability, rennet coagulability) and Mozzarella cheesemaking characteristics of milk from spring-calved herds into late lactation (278 days in lactation), as reflected by acceptable cheese yield, composition, texture, and cooking properties. Some characteristics of this milk were lactose ≥4.3% (w/w); protein = 3.6% (w/w); casein = 2.8% (w/w); rennet gelation parameters (Formagraph Type 1170; Foss Electric, Denmark): curd firmness A60 = 42.1 mm at 60 min.

Seasonal Changes in Milk Quality

In addition to composition, seasonality also affects the state (quality) of the protein and fat in milk and their suitability for processing into products, including cheese.

Protein Hydrolysis (Proteolysis)

Excessive proteolytic activity is undesirable as it hydrolyzes caseins to water-soluble peptides that are lost to varying degrees in whey and not recovered fully during the manufacture of products such as casein or cheese. Moreover, hydrolysis may alter the functionality (e.g., molecular mass, charge) and interactivity of the remaining (recovered) protein and thus the techno-functionality of the resultant products, such as the ability of the resultant cheese to shred or grate, or the ability of casein to hydrate, form gels, or impart structure/texture to products in which it is used as an ingredient (e.g., gluten substitute in bakery products, imitation cheese, and processed cheese products).

There are three main pathways by which proteolysis occurs: native milk proteinase (plasmin), proteinases of somatic cells, and proteinases of psychrotrophic bacteria.

The native proteinase system of milk comprises plasmin as the active enzyme, its zymogen (plasminogen), and enzyme activators/inhibitors. Whereas plasminogen, plasminogen activator, and plasmin are all very heat stable, the plasmin inhibitor is heat labile. Plasmin and plasminogen in milk fully survive pasteurization at pH 6.8. Plasmin is associated with the casein micelles and readily hydrolyzes α_{s1}-, α_{s2}-, and β-caseins, resulting in an increase in γ-caseins. κ-Casein can also undergo some degree of hydrolysis by plasmin, but reports differ on the extent of hydrolysis, which may be due to environmental conditions or the concentrations of enzyme and substrates used.

High levels of plasmin activity and corresponding proteolysis (e.g., >40 to 50% of total α_{s1}- and β-caseins) as affected by addition of plasmin to milk have generally been found to give longer rennet gelation times and markedly lower gel firmness. The impaired rennet gelation characteristics coincide with a more porous, open-structured gel and less connectivity between the particles and clusters making up the gel matrix.

The lysosomes of somatic cells in milk are a significant source of proteinases (e.g., cathepsins D and B). The level of cathepsin D in milk is correlated significantly with SCC, which is an indicator of the intensity of the cellular immune defense in cows.

Although the refrigerated storage of raw milk is used to prolong shelf life and reduce spoilage by mesophilic bacteria, it favors the growth of psychrotrophic microorganisms, which produce heat-resistant extracellular enzymes such as proteinases and lipases. These proteinases hydrolyze the caseins in milk, to a degree dependent on temperature (2–7 °C) and duration of cold storage. The contribution of this source may be particularly important during autumn and winter when milk volumes are low, resulting in extended storage time on-farm. The caseins are particularly susceptible to hydrolysis at low temperatures because of the solubilization of colloidal calcium phosphate, lower degree of hydrophobic-induced casein interactions, loosening of the micelle structure, and the solubilization and dissociation of all caseins, especially β-casein, into the serum phase.

Effect of stage of lactation on proteolysis

The levels of plasmin and plasminogen of milk have been found to increase significantly with advancing lactation. However, the levels of both are affected by husbandry practices such as nutritional status and milking frequency, udder health, and by the onset of involution. The process of involution involves para-cellular leakage from the blood, assisted by disruption of tight junctions between mammary epithelial cells. It is suggested that there may be a positive correlation between loosening of the mammary tight junctions and plasmin and plasminogen-derived activity in milk. This phenomenon is normally associated with significant reductions in both milk yield and lactose content of milk.

Milk SCC generally increases with stage of lactation, a trend that may be associated with a reduction in the milk yield and/or increased infection where late-lactation milk coincides with a deterioration in environmental conditions of the animal (e.g., wet lying conditions). Infection with pathogens such as *S. aureus*, *S. chromogenes*, *E. coli*, and *S. dysgalactiae* is conducive to the development of mastitis and udder inflammation in the cow. Cellular damage at the site of this infection initiates chemical signals that attract white blood cells to the area of infection. Some of these cells are transferred to milk and, therefore, the SCC of milk increases during mastitis. An increase in SCC of milk is associated with a higher rennet clotting time and decreases in curd firmness, cheese yield, and the percentage fat recovered from milk to cheese. The magnitude of these effects varies with SCC and cheese type. An SCC standard of 400×10^3 cells ml^{-1} for bulk milk is adopted in European milk quality schemes with many milk purchasers now applying bonus payments for milk with $\leq 200 \times 10^3$ cells ml^{-1}, and this has reduced the effects of mastitis and high SCC on product quality.

Modern farm and milk collection practices have resulted in milk being cooled rapidly to <8 °C after milking and a relatively low frequency of milk collection from the farm, for example, every 2 or 3 days. Moreover, cold milk is hauled over long distances and is often cold-stored at the cheese plant for 1–3 days depending on time of year and the manufacturing schedules; hence, milk can be cold-stored for 2–5 days prior to processing, particularly during winter. Psychrotrophic bacteria grow during refrigerated storage of milk and produce proteolytic enzymes, which can have a major effect on the quality of products. Hydrolysis of casein by proteinases from psychrotrophic bacteria is undesirable because of the deterioration in cheesemaking quality of the milk, especially when counts of the psychrotophic bacteria are high (e.g., $>1\,000\ \times 10^3$ cfu ml^{-1}). Adverse effects include increased rennet coagulation time, reduced curd firmness, higher losses of protein in cheese whey, lower cheese yield, higher cheese moisture, and development of off-flavors such as bitterness and rancidity. The extent of these defects is exacerbated by an increase in the temperature of cold storage in the range 2–10 °C, as a consequence of higher levels of bacterial growth and activities of lipases and proteinases, especially during the first 48 h.

Fat Hydrolysis

Inadvertent lipolysis in milk leads to elevated levels of FFAs, which are carried over into the cheese and result in off-flavors (rancid, soapy, bitter) and flavor inconsistency. This is undesirable in all cheeses, even in those where the cheesemaking procedure is designed to promote controlled hydrolysis of the milk fat (triacylglycerols) by the addition of exogenous lipases/esterases and/or lipolytic cultures (e.g., *Penicillium roqueforti* in Blue-type cheese). The addition of the latter enzymes to the cheesemilk for cheeses such as Pecorino and Blue-type promotes the development of the desired flavors (e.g., piccante, peppery, ketone) and sensory properties during maturation (*see* **Cheese**: Blue Mold Cheese; Hard Italian Cheeses).

Most lipolysis in milk is caused by the native lipoprotein lipase (LPL). LPL acts on the milk fat triacylglycerols, with the resultant production of FFAs, which are carried over to the cheese. Lipolysis in milk can be broadly described as induced or spontaneous depending on the means of activation of the LPL. Induced lipolysis is defined as lipolysis promoted by both mechanical damage to and temperature changes of the milk. Mechanical damage to milk involves disruption of the membrane surrounding the fat globule, thus allowing direct contact between fat and LPL. Such damage may be accelerated by subjecting the milk to mechanical processes and/or temperature cycling (cooling/reheating). Physical actions that promote mechanically induced lipolysis include excessive shear/whipping and cavitation, which may be caused by a number of factors such as over-agitation and pumping, air incorporation, and poor design of milk-handling systems; the resultant damage may be accentuated by freezing and thawing of milk. Milk may be particularly vulnerable to these actions when volumes are low, depending on time of year.

Temperature-activated lipolysis is induced by temperature cycling, which can occur at several stages on the farm, during milk collection, and at assembly at the factory. Milk as it leaves the cow is at ~37 °C. The activity of LPL on milk fat is at a maximum at 30 °C and markedly decreases at temperatures >37 °C and <12 °C. However, change in temperature can also promote the development of lipolysis, for example, cooling to 5 °C followed by rewarming to 25–37 °C and recooling.

Alternatively, spontaneous lipolysis is defined as that which develops in the milk of some cows during cold storage without being subjected to any physical or mechanical treatment. Lipolysis in this milk is initiated just by prompt cooling of the milk after removal from the cow. Milk from individual cows differs in its tendency to develop spontaneous rancidity depending on the biochemical factors in milk and several predisposing factors in the animal. The main biochemical factors include the amount of lipase activity, the integrity of the MFGM, and the balance of lipolysis activating and inhibiting factors. The major predisposing factors associated with spontaneous lipolysis in the cow are late stage of lactation, poor-quality feed, and mastitis.

Effect of stage of lactation on lipolysis

A number of studies have reported that milk from cows in late lactation has a higher FFA level than that from cows in early lactation. This may be due to changes in the lipolytic activity of the milk and/or greater damage to the MFGM, especially when milk volumes are relatively small, making the milk more susceptible to agitation, pumping, and freezing (freezing onto the cooling surface of direct expansion milk storage tanks). Moreover, diet also has a significant impact on lipolysis in late lactation, as cows are generally under nutritional stress at this time, resulting in milk with relatively high FFA levels.

Psychrotrophic bacteria produce heat-stable lipases that survive pasteurization and ultra-high temperature (UHT) treatments. Even though the bacterial lipase is not inactivated by pasteurization (unlike indigenous LPL), the psychrotrophic bacteria that produced them are destroyed. This has implications in that the bacterial lipase may be carried through to the manufactured cheese where it contributes to off-flavors (rancidity, soapiness, bitterness) during advanced maturation. However, lipolysis associated with psychrotrophic lipases in cold-stored milk is generally only a problem where bacterial counts are very high ($>1 \times 10^6$ to 1×10^7 cfu ml^{-1}) and not an issue with modern milk production practices, except perhaps where holding times prior to processing are long (eg., > 5days). EU Regulation 853/2004 of 2004 (Annex III, Section IX) specifies a plate count requirement (at 30 °C) of $\leq 100 \times 10^3$ colony forming units (cfu) ml^{-1} for cows' milk, corresponding with the rolling geometric average over a 2-month period, with at least two samples per month.

Heat Stability of Milk

A further important parameter of milk quality is that of heat stability, which represents the susceptibility of the milk proteins to aggregate on heating, especially at high temperatures (e.g., >80 °C), as a consequence of protein-protein interactions and hydrophobic bonding. In contrast with cheese manufacture, where casein aggregation, as promoted by enzymatic treatment and/or acidification, is desirable, heat-induced aggregation of milk during processing is highly undesirable in most situations. Raw milk is generally heated during the processing of most products. However, the type of heat treatment varies depending on the product (e.g., concentration and dehydration processes). If the raw milk is not heat stable, problems can arise during processing, such as coagulation on equipment (e.g., heat exchanger). Many factors affect heat stability such as concentrations of urea and ionic calcium, salt concentration, pH, β-lactoglobulin to κ-casein ratio, ratio of different caseins, and casein hydrolysis. Many of these milk components change in absolute and/or relative contents with season and may be responsible for the seasonal variation in heat stability of milk. Various studies have shown that the heat stability of milk is highest during the summer; minimum heat stability was observed during autumn and winter.

Alcohol Stability of Milk

Milk ethanol stability can be defined as the minimum added aqueous ethanol concentration that gives rise to milk coagulation. Knowledge of ethanol stability is critical for the formation and stability of dairy products such as cream liqueurs or alcoholic beverages, in which casein hydration is central to providing its fat-emulsification role. Various studies have shown seasonal variation in the ethanol stability of milk; lower stability has been observed both in autumn or spring periods and during winter time (e.g., Ireland). The stability of milk proteins to alcohol is influenced by various parameters including ionic strength, concentrations of milk salts, and pH. A high salt balance ratio (expressed as [Ca and Mg] to [inorganic phosphate and citrate]) may be a contributor to reduced ethanol stability of late-lactation milk.

Conclusion

Different products require different processing regimes for manufacture (e.g., aggregation of protein into a gel, enclosing fat and moisture, in the case of cheese, or stability to heat in the case of UHT milk manufacture). In all cases, it is desirable that the components (e.g., casein, fat, minerals) in milk are as close as possible to the native state when leaving the udder of a healthy cow, on a good plane of nutrition, in a period excluding the extremes of early and late lactation. Such milk lends itself to the desired degree of alteration (e.g., controlled limited destabilization of the protein phase) on the application of unit operations (e.g., heat, acidification, and enzymatic treatment) during manufacture to give products with the desired quality. Satisfactory milk processing characteristics are conditional on a number of factors including the breed, health status, age, plane of nutrition, and stage of lactation of the cow; the somatic cell count and bacterial count of the milk; associated enzymatic activities; season of year; and milk production practices.

The optimum 'designer' milk for product manufacture is most naturally and cheaply obtained through 'best farm and cow management practices'. Changes in characteristics particularly influenced by season (e.g., composition, protein hydrolysis, and fat lipolysis) may be minimized by maintaining a consistently adequate diet to the cow throughout lactation and ceasing to milk cows at low yields, while also maintaining clinical and subclinical mastitis at a minimum, ensuring minimum storage time of milk between production and processing, treating milk gently when pumping or agitating, and having a consistent supply of milk throughout the year.

See also: **Cheese**: Blue Mold Cheese; Cheese as a Food Ingredient; Cheese Rheology; Hard Italian Cheeses.

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Milk in Human Health and Nutrition

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Introduction

Milk is by far the best single food available to man. Regular consumption of it is an easy way for one to help ensure the adequacy of her/his nutrition. The nutritive value of milk has been very extensively investigated for over a 100 years in laboratories throughout the world. As a consequence, one can have a great deal of confidence in the reliability of the published information about it. This massive research effort is a reflection of the highly important role milk, both human and bovine milk, plays in man's health and well-being. While a variety of animal species are used by man for their milk, the dairy cow is by far the most important. This arises from her ability to produce large volumes of milk and to support the process using crude plant materials as feed. The cow has been appropriately and colorfully described as 'the foster mother of the human race'. In this article, the major contributions of cow's milk to human nutrition are described.

In order to fully appreciate the many contributions of milk to the nutritional well-being of humans, one must first understand a few fundamental aspects of milk, in particular, how it is made and what its purposes are. Milk is made by female mammals for the nourishment of their young. Mammals are a large group of animals with warm blood and include, among others, man and the species he has domesticated, such as horses, dogs, cats, cattle, sheep, goats, and pigs. Newborn mammals are in urgent need of food. Environmentally available plant material is not suitable for them at that early stage of life. The food supplied to them via their mothers' mammary glands, milk, is a complete food. It is specifically designed to support life in new individuals, and is also an invaluable immune support for the newborn. No other food or supplement is required. As nursing proceeds day after day, it can be seen that the limbs are growing and that the baby animal is getting larger, but most importantly, all the organs, tissues, and bones that the animal was born with are being kept healthy and functional. There is a tendency for people to think of milk as exclusively a growth promoter. One often hears, 'It's for kids.' In addition to being a growth promoter, milk is an excellent maintenance food, not just for kids but for humans at any age. It maintains the body in a healthy, functional condition.

Milk and Evolution

Milk as made by a mammal mother is a complete food for her young. An important question is how did it get that way? The most plausible answer is by evolution. Any mammal mother that produces milk of inadequate nutritional quality will produce young that become sickly and either die young or will be incapable of producing healthy offspring. In this situation, the mother's genes for producing milk are not perpetuated. However, the mother that by virtue of her abundant, nutritious milk rears normal healthy offspring will have her genetic facility for making such milk passed on to the succeeding generations. As a result of such selectivity, one can see that nature is constantly perfecting the nutritive value of milk for each species.

It is possible at this point to raise an objection: while cows may have perfected milk for their calves, it does not necessarily mean that such milk is good for humans. Nonetheless, it appears to be. The public's general experience with cows' milk for hundreds of years is supportive of its value as a human food. There has been no convincing evidence, either short or long term, of a cause-and-effect relationship between milk consumption and toxicity or disease. Of course, some humans may experience allergy to cows' milk, but in the case of infants and children so afflicted, most usually outgrow the situation quickly.

A Historical Perspective

If a country has had extensive favorable long-term experience with a particular food, that is reliable evidence of the food's wholesomeness. People will not continue to consume something that shows obvious evidence of making them sick. On the other hand, there will be a strong continuing demand for foods that make them strong and healthy. The experience with milk and milk products in the United States is instructive in that regard. Having had favorable experience with milk, our European forbearers promptly brought dairy cattle to this country in the early 1600s. From that point on, paralleling development of the human culture itself, milk grew to be a decided success. One might say that man and the dairy industry tended to make each other prosper. This brings up the matter of milk's quality and stability.

Since milk of animals has been used as food by man for many thousands of years and in various areas of the world, one of the earliest observations about it must have concerned its tendency to deteriorate. On even brief storage, it spoiled quickly. In time this led to cooling of milk, which discouraged the growth of spoilage bacteria, and next to pasteurization, which simply kills most of the bacteria and, most importantly, those causing diseases. The application of these two processes together with other quality control and sanitation measures has enabled the dairy industry to make available one of the cleanest, safest, most uniform food products known.

Gross Composition of Milk

Details about the many components in milk are provided elsewhere in this encyclopedia. However, it is helpful to have a general idea of the composition of milk. This is provided in **Table 1**, which also affords a comparison of bovine and human milk. The significantly higher protein and ash, especially calcium, content of cow's milk arises from the much greater need of the calf for protein and calcium to support its rapid increase in bone, tissue, and total mass as compared to the human infant. For this reason, babies who are breastfed generally tend to gain weight less rapidly than those nourished on cow's milk or formulae based on it. However, there is nothing unhealthy about this slower rate of gain.

With specific reference to cow's milk, it is a good source of sodium, potassium, and most other mineral elements. It is an outstanding source of calcium, needed especially to make and maintain strong bones. Supplementation of milk with other sources of iron, copper, manganese, and magnesium is appropriate. The fat and carbohydrate of milk serve mainly as a source of calories (energy) and, in general, are not unlike those components in other foods. The aqueous (skim milk) phase contains significant amounts of all B-vitamins and vitamin C. While in fresh raw milk the content of vitamin C is appreciable (28 mg qt^{-1}), the concentration may be lowered appreciably by exposing milk to heat (pasteurization), light, or air. Thus, milk should not be relied on as source of vitamin C. It is also deficient in iron. These two nutrients are well supplied by fruits and red meat, respectively. Because milk is liquid and not chewable, one might be led to think that it is not much of a food. Milk is better than any other food as a source of nutrients that a human needs. Milk does form solid particles called curd in the human stomach. Milk protein is of very good nutritional quality and contains substantial amounts of essential amino acids.

The sugar of milk is known as lactose. While it is only about one-fifth as sweet as ordinary table sugar (sucrose), the two are equivalent in generating energy in the human body. On hydrolysis in the gut, lactose yields one molecule each of glucose and galactose. Both are absorbed into the circulation and eventually the galactose is converted to another molecule of glucose.

Completeness of Milk as a Food

A classical proposition of human nutrition is the so-called 'balanced' diet. It assumes that steady consumption of no single food or small group of foods will be adequate to supply the many essential nutrients humans require. Thus, it is recommended that different kinds and amounts of foods should be eaten so that all the needs are met. The balanced diet idea was conceived following discoveries of the many vitamins, amino acids, and minerals humans must have to maintain health and vigor. The advice to eat a balanced diet has been with us for over 50 years. Another form of this instruction is that we should regularly, if not at every meal, consume foods from each of the main categories: meats, dairy, fruits, and vegetables. A few single foods consumed exclusively such as meat, milk, or eggs come close to fulfilling all nutrient requirements of humans. In brief, milk by itself is designed to sustain life in the young growing animal. Meat is what animals are composed of. So it is not too surprising that we can digest meat and use the resulting products to replenish ourselves. Eggs contain everything to support the development of a young bird. So again one has a relatively complete food in the egg.

As foods, milk and meat share considerable features in common. Both are animal products containing very high-quality protein, both include a full spectrum of vitamins and minerals required by humans, and both contain a similar (relatively saturated) type of fat.

Table 1 Proximate composition of milk

	Fat (%)	*Protein (%)*	*Carbohydrate[a] (%)*	*Ash (%)*	*Water (%)*
Cow	3.5	3.3	5.0	0.7	87.5
Human	4.4	1.0	6.9	0.2	87.5

[a] Refers to lactose content.

Flavor

It is known that flavor plays a critical role in consumer acceptance of a food. Of course, no nutritional value is delivered if the food is rejected because of its flavor. Normal milk is bland tasting and not unpleasant. Nonetheless, there are a few who object to its faint characteristic flavor, the way it feels in the mouth, or to a perceived aftertaste. This is unfortunate but no different from the varied human response to all other foods. In the case of milk, which has so much good nutritional value to deliver and potentialities for weight control, one can only hope that objectors will keep giving it another try. In the case of some foods, one needs to develop a taste for them.

Another notable aspect of milk flavor is its susceptibility to change. In part, this is because normally it has so little flavor that it makes any kind of a newly developed flavor quite noticeable. In addition, exposure of milk to heat, light, or air may bring about a change in flavor that is objectionable. Aberrations in the cow's metabolism or what she is fed can also produce milk with abnormal flavor. Experimentation has shown that inhaling the odor of onions by a cow can impart their flavor to her milk in less than a minute. It is a minor miracle that the dairy industry has learned so well how to successfully manage the challenging situation that milk presents with regard to flavor. Much of the needed information for this has been derived from state and federally sponsored research programs.

Vitamin D

Milk represents a special situation with respect to vitamin D. With the exception of oily fish, that is, sardines, salmon, tuna, and mackerel, most foods contain little or no vitamin D. There are some other foods such as cereals, margarine, some cheeses, and orange juice that are fortified with this vitamin. However, since the 1930s, it has been required by law in most areas of the country that milk be fortified with vitamin D at a level of 400 IU per quart. This was brought about in order to prevent the bone deforming disease known as rickets (osteomalacia), the most obvious ultimate symptom of which is bowed legs. With high levels of naturally occurring calcium in milk, mandated additions of vitamin D made it the ideal food for supporting bone maintenance and development.

One might wonder if milk is so relatively low in vitamin D, how does the calf manage to develop its substantial bony structure? The answer seems to be that it has a diversity of sources that together provide an adequate supply. These include vitamin D the mother transfers to her fetus before birth and in her milk after birth, that which the newborn synthesizes through its exposure to light, and that consumed in its feed, often vitamin D-fortified. Actually the question is worthy of research. It would be of interest to know if there are other substances in milk that, similar to vitamin D, help to catalyze bone formation.

In more recent years, scientists have come to recognize other beneficial effects of vitamin D on human health, including reduced risks of heart disease, diabetes, and various forms of cancer and strengthening of the immune system. Another reported function of the vitamin is its ability to suppress the development of new fat cells (adipocytes) in the body. This is of particular interest in light of obesity as a growing public health problem. Considering its multiple beneficial effects, some researchers feel that the recommended daily intake of vitamin D should be raised from the current 400 IU to 800 or 1000 IU. They are concerned that worldwide there is insufficiency of vitamin D in many populations. It should be noted that consumption of vitamin D above 2000 units per day can be toxic.

Myths and Facts about Milk

Because milk is such a key food and of great importance in many countries and cultures, it is constantly being evaluated and sometimes criticized, without unequivocal clinical or epidemiological evidence to support the criticism.

Saturated Fat/Cholesterol

The most damaging misinformation about milk in recent years is that regarding saturated fat in the diet. In the early 1950s, Ancel Keys, a researcher at the University of Minnesota, observed that men with heart disease had cholesterol deposits in the walls of their arteries. By inference this quickly came to mean that cholesterol circulating in the blood was depositing in coronary arteries, thickening their walls, thereby choking off the circulation and causing heart attacks. A more refined later version was that deposition of cholesterol weakened the walls of the arteries making them susceptible to injury, which could give rise to clots with the potential to restrict blood flow if not plug the vessel shut. Further studies showed that saturated fats in the diet, that is, primarily those of milk, milk products, and meat, raise circulating cholesterol. So based on these associations, and without it ever having been demonstrated as a cause-and-effect relationship, consumption of milk fat was implicated as a factor in heart disease. This led to the widely disseminated advice, "Avoid milk fat (milk, cream, ice cream, butter, etc.) because it will raise your 'bad' cholesterol, which is associated with heart disease."

This recommendation has been purveyed by major medical groups, agencies of the federal government, and news media for years. One was always reminded in any news article involving diet/heart disease to reduce or eliminate consumption of saturated fats, that is, milk or meat. The great body of evidence, not just a few studies, indicating that saturated fats are not problematic was either overlooked or ignored. At this point, there are at least three major medical/nutritional reviews of the literature that make it very clear that saturated fat in the diet is not a problem in relation to heart disease.

One of the truly misleading tangents of this half a century of advice about diet and heart disease has been the implication that everyone is susceptible to if not suffering from an elevated cholesterol pathology. This is said even though there are studies showing that in older people higher cholesterol is associated with better health. It seems evident that the only people with a truly problematic high blood level of cholesterol are those afflicted with the genetic disorder known as familial hypercholesterolemia, and they make up far less than 1% of the population.

Cancer

Various antimilk groups often resort to the warning, 'Milk causes cancer!' No cause-and-effect relationship has ever been shown between milk consumption and any form of cancer. On the contrary, milk contains a large array of cancer antagonists, including conjugated linoleic acid, butyric acid, vitamins A and D, carotene, sphingomyelin, ether lipids, and whey proteins. In addition, there are impressive studies of thousands of women showing that regular consumption of milk is associated with reduced incidence of breast cancer. The results from one of these studies, showing that the incidence of breast cancer was lowered in proportion to the amount of milk consumed, are presented in **Figure 1**.

Digestion of Lactose

Human milk, like cow's milk, contains lactose (**Table 1**). So all humans start off being able to digest and utilize lactose. However, this capability does not persist in all individuals, nor to the same degree. If milk is discontinued in the diet, such as at weaning, normally the ability to digest lactose is lost, and in time the individual will exhibit what is known as lactose intolerance. However, in the United States and Europe among other areas, milk and milk products continue to be major items in the human diet throughout childhood and the rest of life. Thus, lactose is well utilized by most people in those areas.

When lactose is not digested, it moves along with the food residue to an area of the lower intestine where it is fermented by bacteria with the production of acid and gas (these, when substantial, can cause discomfort). Here again, those opposed to milk consumption imply that many people have a very grave problem in this regard. This clearly is not the case. For those relatively few people who have a real problem digesting lactose, there are a number of simple solutions:

Purchase milk in which the lactose has been hydrolyzed; such milk is available in many supermarkets.
Purchase a lactase enzyme preparation that can be added to milk to digest the lactose.
Avoid quickly downing large glasses of milk and, instead, ingest smaller portions of it over several hours.
Consume some yogurt with (or in) your milk. It contains lactase enzyme naturally.

Because people of certain nationalities and ethnic groups have lactose intolerance, it is assumed that genetics plays an important role in their limitation. However, a total and continuing lack of lactose in their diets may be more important in the development of their intolerance than their genetics. Interestingly, milk was recently introduced into schools for Chinese children without apparent problem. This suggests that lactase enzymatic activity is inducible.

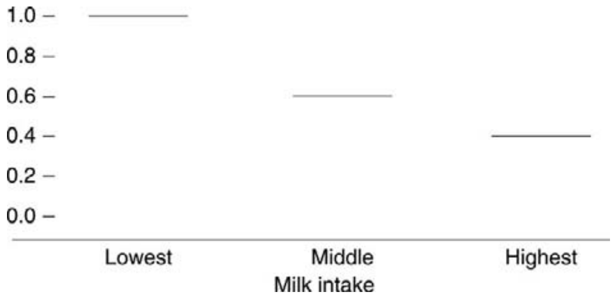

Figure 1 Relative risk of breast cancer as a function of milk consumption. Breast cancer risk is reduced ($p < 0.003$) in relation to milk intake (low = 486, middle = 679, and high = 1060 g of dairy products per day). Data adapted by DE Bauman from Knekt P, Järvinen R, Seppänen R, Pukkala E, and Aromaa A (1996) Intake of dairy products and the risk of breast cancer. *British Journal of Cancer* 73: 687–691.

Of course, what one accomplishes by drinking smaller amounts of milk incrementally is avoidance of burdening the digestive system. This is always a good idea with any food, or as they say, ‘not too much of anything’. On the other hand, in the interest of getting the excellent nutritive value milk offers, it is well worth one’s adopting one or more of the mentioned measures if that is essential for milk to be accommodated comfortably in one’s diet. Due to misunderstandings about the need and the ability to digest lactose, many people have self-diagnosed incorrectly that they have a serious unsolvable problem. The phenomenon and genetics of lactose intolerance have been extensively covered in a number of reviews.

Asthma

There are unsupported claims that milk causes asthma, a complicated disorder of the lungs and respiratory tract. At least one study has shown that milk consumption is not a factor in asthma.

Types of Milk Commercially Available

Due to the growing concern about human health and well-being, milk is now being scrutinized in ways and to degrees that were never considered necessary in the past. Consumers are demanding that variations in the way milk is produced be defined and labeled on the carton. So now we have organic milk, and, in some countries such as the United States, we have milk produced with or without the aid of additional bovine growth hormone (BGH; also called bovine somatotropin (BST)) and milk produced by cows either treated or not treated with antibiotics. Other variables, such as whether the animals were grazing pastures or not, may also come to be considered. While the consumers’ interest in these matters is understandable, they are beyond the scope of this article because they are not currently relevant to the well-established nutritional properties of milk.

Homogenized Whole Milk

In the United States, for the past 70 years or so, there has been one principal marketed form of milk, that is, homogenized whole milk. Its fat content as defined by law is somewhere in the 3.0–3.5% range, which approximates the average fat content of milk as secreted by the cow. It was and still is produced under very strict government regulations and industrial standards. All things considered, it is an extremely uniform product. Normally, in the United States, no matter where one obtains it, restaurant, supermarket, schools, or home delivery, milk will exhibit little or no difference in its highly acceptable odor, flavor, or appearance. There are many factors that have contributed to this success story, but one of the most important has been the informed and demanding legal requirements at local, state, and federal levels for milk production, processing, and distribution.

Raw Milk

In view of humanity’s long and continuing association with raw milk, that is, unaltered milk as withdrawn from the udder of the cow, it deserves special considerations. Until the adoption and mandatory application of pasteurization to milk, the human experience for thousands of years had been with raw milk exclusively. Considering the ability of raw milk to support the growth of bacteria, including pathogens, it is not surprising it came to be recognized as a potential menace to health. The development of pasteurization technology to deal with that problem is perhaps the greatest milestone of progress ever achieved by the dairy industry. The minimal safe heat treatment for the destruction of milk-borne pathogens was established and equipment designed to carry out the necessary heating and cooling process in application to huge volumes of milk. A remarkable feature of pasteurization is that the flavor and appearance of the milk remain essentially unchanged. There are only very minor effects on the nutritive value. Mainly, about half of the vitamin C content is destroyed and slight losses, <10%, of vitamins B_1 and B_{12} occur. It is speculated that pasteurization destroys enzymes, vegetative forms of native bacteria, and other milk components of possible value to human health. In the opinion of health authorities, these minor concerns in no way justify doing away with the process, which they view as having tremendous public health benefits. Industrial production of pasteurized milk commenced in earnest between 1900 and 1920 when most of the laws mandating it were passed by municipalities and states. Generally speaking, we can be very thankful for pasteurization. It is still not uncommon to read notices in the news that lack of pasteurization of milk or milk products led to a communicable disease outbreak.

Despite the huge legal and health momentum supporting pasteurized milk, there are many people in the United States who recognize that a market exists for raw milk. The commercial supply of the product, known as certified raw milk, was initiated in the 1930s. It was permitted only in areas specifying extremely demanding standards of animal health and cleanliness in the circumstances surrounding production and processing of the milk. Under such conditions, it is possible to produce milk with very low bacterial counts.

Raw milk is legally available in many parts of the United States today. The current environmentalist trend in our culture has developed partisans for it. In their opinion, it has not been debased by man and is more natural and therefore better. Controlled human feeding studies comparing raw and pasteurized milks might be worth pursuing. That should be a good way to reveal whether there is a difference in their nutritive value and if so how significant it is.

See also: **Milk Quality and Udder Health**: Effect on Processing Characteristics; Test Methods and Standards. **Nutrition and Health**: Diabetes Mellitus and Consumption of Milk and Dairy Products; Effects of Processing on Protein Quality of Milk and Milk Products; Galactosemia; Milk Allergy; Nutraceuticals from Milk; Nutrigenomics and Nutrigenetics; Nutritional and Oral Health-Promoting Properties of Dairy Products: Caries Prevention and Oral Health; Nutritional and Health-Promoting Properties of Dairy Products: Fatty Acids of Milk and Cardiovascular Disease.

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Milk of Primates

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Introduction

Primate is a member of the mammalian order Primates (prime, first rank), which contains a total of ~431 species of lemurs (100 spp.), lorisids (9 spp.), galagos (19 spp.), tarsiers (9 spp.), monkeys (139 spp. of New World monkeys, Platyrrhini; 135 spp. of Old World monkeys, Cercopithecoidea), and apes (20 spp. in the superfamily Hominoidea). The number of species reported varies depending on the classification criteria used. The superfamily Hominoidea comprises two families: Hylobatidea (13 species in 4 genera of gibbons or lesser apes) and Hominidae (4 genera of Great Apes: *Pongo* (2 species of orangutan), *Gorilla* (2 species), *Pan* (2 species and 3 subspecies of *Pan troglodytes*), and *Homo* (1 extant species, *Homo sapiens*)). A phylogenetic tree of the primates is shown in **Figure 1**, which reflects current understanding of the evolutionary relationships among primates.

It is generally assumed that human milk has evolved as the most appropriate food for nutrition of the human infant. The composition and constituents of human milk are quite different from those of the principal dairy species (cattle, buffalo, sheep, and goat; *see* **Milk**: Bovine Milk; Buffalo Milk; Goat Milk; Introduction; Sheep Milk). The milks most like human milk in macronutrient composition are those of the horse and donkey (*see* **Milk**: Equid Milk), which is rather surprising since *Homo sapiens* is phylogenetically quite distant from *Equus caballus* and *Equus asinus*. In this article, human milk is compared with that of our closest relatives, the Great Apes, and, to a lesser extent, with the milk of primates in general. Unfortunately, the milk of most primates has not been studied at all, and the milk of those species that have been studied has been the subject of relatively few studies; according to Oftedal and Iverson, the milk of only 16 primate species had been studied till 1995 in sufficient detail as to be reliable; since then, the number of species studied has doubled and considerable information is now available on some species. However, in comparison with the milk of the commercial dairying species (cow, buffalo, sheep, and goat), the horse, pig, and human, the milk of nonhuman primates has been studied only superficially. Many reports on primate milk have focused on gross composition, mainly with the objective of improving the nutrition of captive species. However, there is a lack of information on the chemistry of the constituents of primate milks, especially of the proteins.

The composition of milk is significantly affected by the animal's diet. The human diet is quite different from that of the three other Great Apes: the human diet includes a high proportion of animal-derived products (meat, fish, milk, and eggs), whereas diets of the three other genera of Great Apes is mainly or exclusively vegetarian. The human diet was probably vegetarian when the genus *Homo* first evolved, but meat eating has been common for a long time and is considered to have been a major factor in human evolution. Also, most items in the human diet are cooked, which alters digestibility and energy yield, and may affect milk synthesis.

Gross Composition

Mammals differ with respect to the gross composition of their milk, the frequency of neonatal feeding, and the duration of lactation. Differences in the nutrient requirements of young mammals arise from differences in postnatal growth rate, the stage of maturity at birth, body composition at birth, and environmental peculiarities of their natural habitat. Factors that directly affect the lactating female, for example, litter size, lactation load, maternal diet, and nursing schedule, may affect the gross composition of her milk.

The gross composition of the milk of the four Great Apes and several other primate species is shown in **Table 1**. Although only 31 of the 431 primate species are included in this table, a number of general features are apparent: generally, the milk of primates is dilute with a high concentration of lactose and low concentrations of fat and protein. This is true even for callitrichid species, the smallest primates (lion tamarin and pygmy marmoset at ~700 and 100 g body weight, respectively), which would be expected to produce milk with higher levels of fat and protein, but, in fact, produce milk approximately similar to the milk of larger primates, although the milk shows large individual variations, for example, 1–10% fat. There is some evidence that maternal condition affects milk composition in these primates: small marmoset and tamarin mothers with twins or triplets produce milk lower in energy and infants are weaned early (within ~2 months). Nursing style also affects the overall composition of milk: prosimians that carry their young throughout the day produce more dilute milk than those that leave their young unattended for periods. Lorises and bush babies produce relatively rich milk. It has been

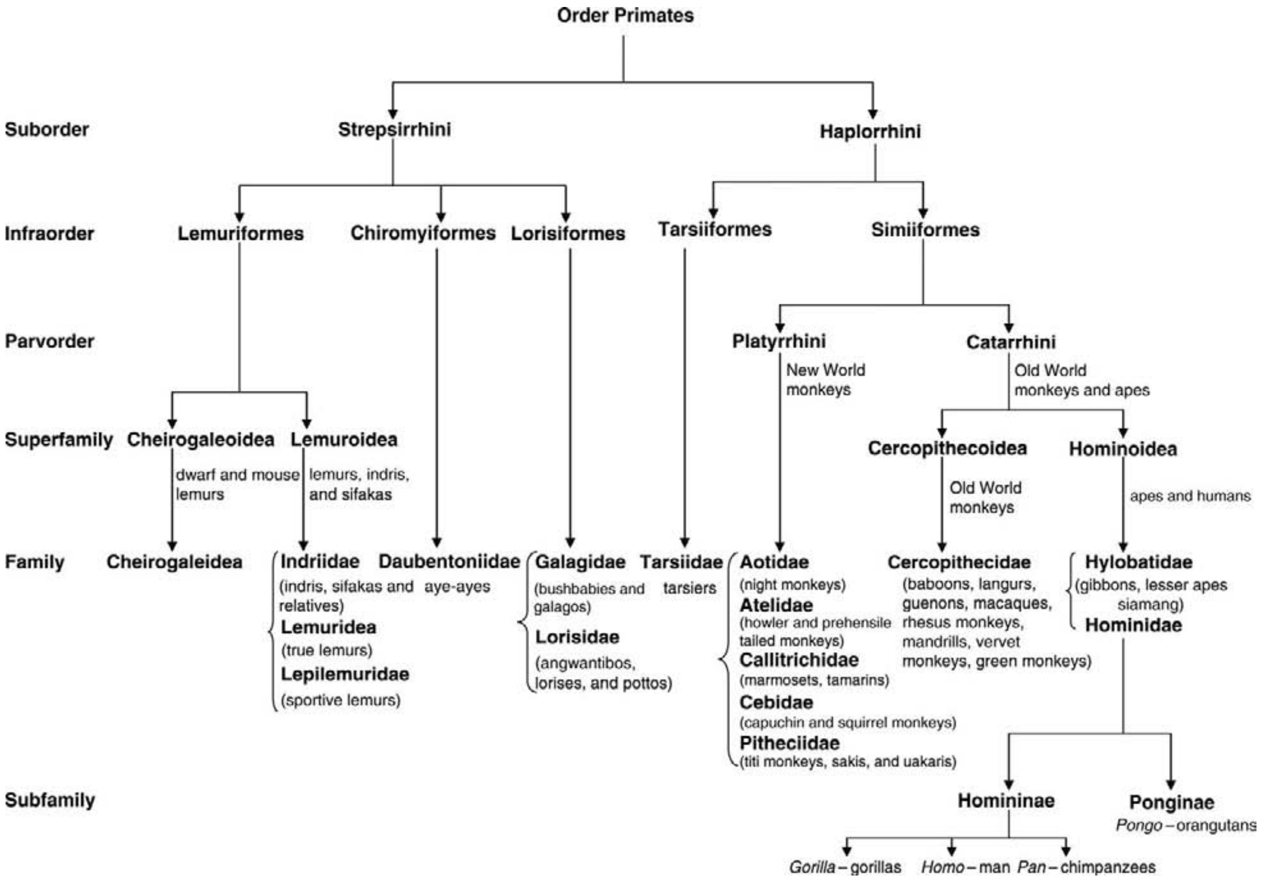
Order Primates
Suborder
Strepsirrhini
Haplorrhini
Infraorder
Lemuriformes
Chiromyiformes
Lorisiformes
Tarsiiformes
Simiiformes
Parvorder
Platyrrhini
New World monkeys
Catarrhini
Old World monkeys and apes
Superfamily
Cheirogaleoidea
dwarf and mouse lemurs
Lemuroidea
lemurs, indris, and sifakas
Cercopithecoidea
Old World monkeys
Hominoidea
apes and humans
Family
Cheirogaleidea
Indriidae
(indris, sifakas and relatives)
Lemuridea
(true lemurs)
Lepilemuridae
(sportive lemurs)
Daubentoniidae
aye-ayes
Galagidae
(bushbabies and galagos)
Lorisidae
(angwantibos, lorises, and pottos)
Tarsiidae
tarsiers
Aotidae
(night monkeys)
Atelidae
(howler and prehensile tailed monkeys)
Callitrichidae
(marmosets, tamarins)
Cebidae
(capuchin and squirrel monkeys)
Pitheciidae
(titi monkeys, sakis, and uakaris)
Cercopithecidae
(baboons, langurs, guenons, macaques, rhesus monkeys, mandrills, vervet monkeys, green monkeys)
Hylobatidae
(gibbons, lesser apes siamang)
Hominidae
Subfamily
Homininae
Ponginae
Pongo – orangutans
Gorilla – gorillas
Homo – man
Pan – chimpanzees

Figure 1 Taxonomic classification of the order Primates.

suggested that as primates in the wild live in hot humid climates, they require a large intake of water for evaporative cooling, which is supplied by milk during the early period postpartum – hence the provision of dilute milk. The high environmental temperature avoids the need for a high-energy diet, unlike marine mammals and land animals that live in a cold environment.

In human milk, the protein content has a pronounced lactation pattern and is high in early lactation (15 g kg^{-1}) and lower in mature milk (9 g kg^{-1}), whereas in primate milks, in general, the decrease in protein concentration is relatively slight (Rhesus monkey: 24.9 g kg^{-1} in colostrum compared to 23.5 g kg^{-1} in milk). The fat and lactose contents of Rhesus monkey milk do not vary significantly over lactation, but for human milk, fat content increases ($\sim$23 g kg^{-1} in colostrum to $\sim$35 g kg^{-1} in milk), as does lactose ($\sim$41 g kg^{-1} in colostrum to $\sim$68 g kg^{-1} in milk).

Across the primate species, the proportion of gross energy from protein varies considerably (**Table 2**) but correlates negatively with the rate of growth; for example, the percentage of energy from milk protein is lowest in human milk and the time to reach maturity is the longest among primates. In some lemurs and lorises, the proportion of energy from protein is high and reflects the comparatively rapid development of their infants.

Infants of low-ranking Rhesus macaque mothers nurse frequently for short bouts, and their milk is more dilute than that of higher-ranking species. Furthermore, Rhesus mothers who are nursing sons produce milk with higher gross energy than those rearing daughters, although the milk yield of the former is lower and, overall, the available energy is equal whether the offspring is female or male.

It has been suggested that the unique pattern of human brain growth is due to the unique composition of human milk. Using an encephalization quotient (EQ) (brain size divided by that expected for an animal of the same body size), humans are twice as encephalized as chimpanzees. At birth, the human neonate has an EQ similar to that of other primates (the brain is $\sim$12% of body weight at birth), but the critical period of rapid brain growth is longer in humans than in other primates. Rapid postnatal brain growth slows down at about 2 months in Rhesus infants, at 6 months in chimpanzees, but continues for up to 12 months in human infants.

The pH of mature baboon milk is 7.17 and its specific gravity is 1.027, which are comparable to the values for human milk, 6.9–7.0 and 1.031, respectively.

Table 1 Gross composition (g kg^{-1}) of the milk of various primate species

Species	*Total solids*	*Protein*	*Fat*	*Lactose*	*Ash*	*Gross energy (kJ kg^{-1})*
Hominidae						
Man (*Homo sapiens*)	124	9	38	70	2	2753
Chimpanzee (*Pan troglodytes*)	119	12	36	69	2	2711
Lowland gorilla (*Gorilla gorilla gorilla*)[a]	94	22	14	62	3	1933
Orangutan (*Pongo pygmaeus*)	115	14	35	60	2	2573
White-handed gibbon (*Hylobates lar*)[a]	113	13	24	83		2510
Cercopithecidea						
Baboon (*Papio* sp.)	144	16	50	73	3	3372
Talapoin monkey (*Cercopithecus talapoin*)	123	21	29	72	3	2648
Crab-eating macaque (*Macaca fascicularis*)	122	16	52		4	
Japanese macaque (*Macaca fuscata*)	140	16	42	62		2887
Rhesus macaque (*Macaca mulata*)	125	13	33	90	4	2966
Green monkey (*Chlorocebus sabaeus*)	164	31	40	102	6	3732
Vervet monkey (*Chlorocebus pygerythrus*)	99	16	31	85		2858
Callitrichidae						
Golden lion tamarin (*Leontopithecus rosalia*)	161	26	52	72	8	3598
Common marmoset (*Callithrix jacchus*) – captive	140	27	35	74	3	3008
Common marmoset (*Callithrix jacchus*) – wild	127	22	23	80		2573
Cotton-top tamarin (*Saguinus oedipus*)	131	38	31	58	4	2774
Pygmy marmoset (*Cebuella pygmaea*)	137	29	37	78		3598
Atelidae						
Red howler (*Alouatta seniculus*)	113	19	11	66		1837
Mantled howler (*Aloautta palliata*)	117	22	16	67		2092
Cebidae						
Squirrel monkey (*Saimiri sciureus*)	122	30	10	70	2	2050
Squirrel monkey (*Saimiri boliviensis boliviensis*)	166	36	46	70		3507
Tufted capuchin (*Cebus apella*)	165	24	52	69		3515
Lemuridae						
Brown lemur (*Eulemur fulvus*)	96	13	9	85	2	1979
Black lemur (*Eulemur macaco*)	101	15	11	84	3	2071
Ring-tailed lemur (*Lemur catta*)	109	20	18	81	4	2368
Lesser bamboo lemur (*Hapalemur griseus*)	120	19	27	80		2673
Red-bellied lemur (*Eulemur rubriventer*)	103	11	8	89	2	1975
Mongoose lemur (*Eulemur mongoz*)	98	13	7	79	2	1803
Black and white ruffed lemur (*Varecia variegate*)	140	42	32	77	4	3197
Galagidae						
Garnett's bushbaby (*Otolemur garnetti*)	185	52	73	66	6	4724
Thick-tailed bushbaby (*Otolemur crassicaudatus*)	186	48	80	64	6	4887
Mohul bushbaby (*Galago moholi*)	243	75	126	42		6703
Lorisidae						
Slow loris (*Nycticebus coucang*)	163	39	70	66	7	4393

[a] Single sample.
Data from various sources.

Saccharides

Lactose, a reducing disaccharide composed of galactose and glucose linked by a β1-4-glycosidic bond, is unique to milk. It is the principal sugar in the milk of most species, exceptions being a few species of seal, which lack lactose. The concentration of lactose, which serves as a ready source of energy, in primate milk ranges from ~5% for galago to >10% for the African green monkey (vervet; the highest concentration reported); the value for all the Great Apes is ~7%. The milk of gorilla also contains relatively high concentrations of glucose, galactose, and fucose.

The milk of all species examined contains oligosaccharides (OSs), the concentration of which is particularly high in the milk of humans, bears, elephants, and marsupials (*see* **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk). The OSs serve mainly nonnutritional roles, for example, antibacterial or bifidogenic. Human, gorilla, and macaque monkey milk contain approximately 15, 7, and 0.1 g kg^{-1} OSs, respectively, and, in the case of human milk, OSs are the third largest solid constituent. The OS-to-lactose ratio in chimpanzee milk, bonobo milk, gorilla colostrum, gorilla milk, orangutan colostrum, and siamang (a lesser ape) milk is 1:4, 1:5,

Table 2 Percentage of calories from protein in the milk of some primate genera

Family	*Genus*	*Gross energy ($kJ\ kg^{-1}$)*	*Protein %*	*% of gross energy from protein*
Hominidae	*Homo*	2552	0.9	5.9
Cercopithecidae	*Papio*	3305	1.6	9.2
	Macaca	3347	1.8	11.6
	Cercopithecus	2803	2.1	16.2
Cebidae	*Alouatta*	2050	2.0	21.3
Callitrichidae	*Callithrix*	3221	2.7	19.0
	Cebuella	3347	2.9	19.3
	Leontopithecus	3765	2.6	17.3
Lemuridae	*Eulemur*	2008	1.3	12.8
	Varecia	3472	4.2	27.9
Lorisidae	*Nycticebus*	4644	3.9	19.2
	Otolemur	5146	5.0	22.5

Adapted from Power ML, Oftedal OT, and Tardif SD (2002) Does the milk of callitrichid monkeys differ from that of larger antropoids? *American Journal of Primatology* 56: 117–127.

1:20, 1:7, 1:0.8, and 1:3, respectively. The ratio in human milk, at peak lactation, is 1:2.7.

Human milk contains over 100 different OSs, the core units of which are shown in **Table 3**. The types of OSs in the milk of nonhuman apes are different from those in human milk – OSs containing Gal(β1-4)GlcNAc (Type II) units are found exclusively in the milk of the gorilla, siamang, and brown capuchin monkey and predominantly in the milk of the chimpanzee, bonobo, and orangutan, whereas Gal(β1-3)GlcNAc (Type I) units are found only in human milk. It has been reported that Type I, and not Type II, OSs promote the growth of a beneficial bifidus flora in the infant intestinal tract. The milk of Great Apes and human milk contain fucosylated OSs, whereas siamang milk does not. **Table 3** lists the principal OSs that have been identified in the milks of some primates.

Lipids

The fat content of primate milk is reported to vary from $\sim$10 to $\sim$40 $g\ kg^{-1}$ (**Table 1**); exceptions are species of Lorisidae, the milk of which is reported to contain $\sim$70 $g\ kg^{-1}$ fat. The milk of all the Great Apes contains 30–40 $g\ kg^{-1}$ fat. The relatively low concentration of fat in the milk of primates probably reflects the need for low dietary calories.

Species-specific physiological mechanisms and maternal diet determine the concentration of fatty acids (FAs) in milk. The FA profile of the milk fat of a number of primate species has been reported; it is probably the most extensively studied feature of primate milks and the results are summarized in **Table 4**. Notable features are the relatively high concentrations of the middle-chain acids, $C_{8:0}$ and $C_{10:0}$; the very high concentrations of polyunsaturated fatty acids (PUFAs), especially $C_{18:2}$; and the substantial concentrations of C_{20} and C_{22}. In Cebidae and Cercopithecidae monkeys, the levels of $C_{8:0}$ and $C_{10:0}$ are much higher than those in the gorilla: these FAs are synthesized in the mammary gland and are not diet related (hence values for captive and wild species are similar). The milk fat of lemur (three species) contains exceptionally high concentrations of $C_{12:0}$ and $C_{14:0}$, in some cases up to 50% of the total FAs. There are large, in some cases very large, interspecies and interstudy differences in FA concentrations, which may reflect differences in diet and whether the animals were wild or captive, as well as representative sampling and the use of unreliable analytical methods. The high levels of PUFAs probably reflect the plant-based diet of most of the primate species and have implications for the nutrition of captive primates.

FA synthesis in the mammary gland varies across mammals, and the distribution of FAs over three possible stereospecific positions may be important in neonatal nutrition. $C_{8:0}$, $C_{18:0}$, and $C_{18:0}$ are esterified mainly at the *sn*-1 and *sn*-3 positions of glycerol in the triglycerides in the milk fat of four species of monkey (macaque (2), Rhesus, and mangabey), whereas $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$ are concentrated at the *sn*-2 position. In a somewhat similar study by Myher and collaborators, the unhydrolyzed lipids of eight prosimian primates were studied by gas chromatography (GC). Triglycerides represented 88–95% of the lipids; free fatty acids (FFAs), 0.5–10%; and diglycerides (DGs), 5–10%; monoglycerides were absent (the high levels of FFAs and DGs may suggest lipolysis in some samples, perhaps as a consequence of damage to the milk fat globule membrane (MFGM)). The acyl carbon number of the TGs was mainly in the range C40–C49, and of the FFAs was $C_{8:0}$–$C_{18:0}$. Considerable information on the structure of the lipids in the milk fat is available.

A noteworthy feature in **Table 4** is the relative proportions of $n-6$ to $n-3$ fatty acids. In the wild primate

Table 3 The principal oligosaccharides in the milk of some primates

Species	*Sample*	*Oligosaccharide*
Human[a] (*Homo sapiens*)	Milk	Gal(β1-4)Glc (lactose)
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc(lacto-*N*-tetraose)
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(lacto-*N*-neotetraose)
		Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)Glc]Gal(β1-3)GlcNAc(β1-3) (lacto-*N*-hexaose)
		Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)Glc]Gal(β1-4)GlcNAc(β1-3) (lacto-*N*-neohexaose)
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(*p*-lacto-*N*-hexaose)
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(*p*-lacto-*N*-neohexaose)
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)Glc]Gal(β1-3)GlcNAc(β1-3)(lacto-*N*-octaose)
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)Glc]Gal(β1-4)GlcNAc(β1-3)(lacto-*N*-neooctaose)
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)Glc]Gal(β1-3)GlcNAc(β1-3)(Iso-lacto-*N*-octaose)
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(*p*-lacto-*N*-octaose)
		Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc[Gal(β1-3)GlcNAc(β1-3)(lacto-*N*-decaose)
Brown capuchin (*Cebus apella*)	Colostrum	Gal(β1-3)Gal(β1-4)Glc(β3′-galactosyllactose)
		Gal(β1-6)Gal(β1-4)Glc(β6′-galactosyllactose)
		Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]Gal(β1-4)Glc(lacto-*N*-novopentanose 1)
		Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc (lacto-*N*-neohexaose)
		Neu5Ac(α2-3)Gal(β1-4)Glc(3′-*N*-acetylneuraminyllactose)
		Gal(β1-4)[Fuc(α1-3)]Glc(3′-fucosyllactose)
Chimpanzee (*Pan troglodytes*)	Milk	Gal(β1-4)Glc(lactose)
		Fuc(α1-2)Gal(β1-4)Glc(2′-fucosyllactose)
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)[Fuc(α1-3)]Glc(3′-fucosyllactose)
		Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc
		Neu5Ac(α2-3)Gal(β1-4)Glc
		Neu5Gc(α2-3)Gal(β1-4)Glc
Bonobo (*Pan paniscus*)	Milk	Gal(β1-4)Glc(lactose)
		Fuc(α1-2)Gal(β1-4)Glc(2′-fucosyllactose)
		Gal(β1-4)[Fuc(α1-3)]Glc(3′-fucosyllactose)
		GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-4)Glc
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc
		Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
		Neu5Ac(α2-3)Gal(β1-4)Glc
		Neu5Ac(α2-6)Gal(β1-4)Glc
		Neu5Gc(α2-3)Gal(β1-4)Glc
		Neu5Gc(α2-3)Gal(β1-4)Glc1-4 lactone

(Continued)

Table 3 (Continued)

Species	*Sample*	*Oligosaccharide*
Gorilla (*Gorilla gorilla*)	Milk	Gal(β1-4)Glc (lactose)
		Fuc(α1-2)Gal(β1-4)Glc(2′-fucosyllactose)
		Gal(α1-3)[Fuc(α 1-2)] Gal(β1-4)Glc
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
	Colostrum	Neu5Ac(α2-3)Gal(β1-4)Glc
		Neu5Gc(α2-3)Gal(β1-4)Glc
Orangutan (*Pongo pygmaeus*)	Milk	Gal(β1-4)Glc (lactose)
		Gal(β1-4)[Fuc(α1-3)]Glc
		Gal(β1-6)Gal(β1-4)Glc
		Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)Gal(β1-4)Glc
		Neu5Ac(α2-3)Gal(β1-4)Glc
		Neu5Ac(α2-6)Gal(β1-4)Glc
		Neu5Gc(α2-3)Gal(β1-4)Glc
		Gal(β1-3)[Neu5Ac(α2-6)]GlcNAc(β1-3)Gal(β1-4)Glc
		Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
		Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal (β1-4)[Fuc(α1-3)]GlcNAc(β1-6)Gal(β1-4)Glc
Siamang (*Symphalangus syndactylus*)	Milk	Gal(β1-4)Glc
		Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
		Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc
		Neu5Ac(α2-3)Gal(β1-4)Glc
		Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
		Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)[Gal (β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc

[a] Core oligosaccharides – variations include the addition of a NeuAc α2-3/2-6 to Gal or GlcNAc, and of Fuc α1-2/1-3/1-4 to Gal, GlcNAc, or a reducing Glc of the core unit.

Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetylglucosamine; Neu5Ac, *N*-acetylneuraminic acid.

Human milk data from Urashima T, Saito T, Nakamura T, and Messer M (2001) Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal* 18: 357–371.

All other data from Urashima T, Odaka G, Asakuma S, *et al*. (2009b) Chemical characterization of oligosaccharides in chimpanzee, bonobo, gorilla, orangutan, and siamang milk or colostrum. *Glycobiology* 15: 499–508 and Urashima T, Kitaoka M, Asakuma S, and Messer M (2009a) Milk oligosaccharides. In: McSweeney PLH and Fox PF (eds.) *Advanced Dairy Chemistry, Vol. 3: Lactose, Water, Salts and Minor Constituents*, 3rd edn., pp. 295–349. New York: Springer.

Table 4 The principal fatty acids of primate milks

Species	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{16:1}$	$C_{18:1}$[a]	$C_{20:1}$[b]	$C_{18:2}$	$C_{20:3}$	$C_{20:4}$	$C_{18:3}$	$C_{20:5}$	$C_{22:5}$	$C_{22:6}$	*Ratio n−6:n−3*
Cotton-top marmoset (*Saguinus oedipus*)	2.40	14.70	15.70	12.10	21.50	3.40	2.20	19.60		8.00							
Common marmoset (*Callithrix jacchus*) (wild species)	16.61	16.71	0.02	10.37	19.86	3.68	2.41	22.71	0.28	3.29	0.23	0.30	2.63	0.08	0.21	0.14	1.25
Common marmoset (*Callithrix jacchus*)		8.01	8.48	7.71	18.09	3.40	5.54	29.63	1.18	10.85	0.41	0.11	0.86	1.26	0.78	2.46	2.13
Golden-lion tamarin (*Leontopithecus rosalia*) (wild species)	13.79	16.18	0.02	13.55	20.58	3.52	1.72	20.81	0.41	1.10			1.94	0.11	0.24	0.24	0.43
Golden-lion tamarin[c] (*Leontopithecus rosalia*) (species)	18.60	15.56	0.02	9.93	15.53	3.38	0.96	20.43	0.57	11.50	0.86	0.36	0.61	0.03	0.16	0.12	13.93
Ring-tailed lemur (*Eulemur catta*)	0.70	8.10	5.90	14.90	28.70	3.20	3.70	26.20		6.30			1.60				3.94
Brown lemur (*Eulemur fulvus*)	0.20	7.60	22.50	21.20	16.10	1.30	4.00	14.90	0.30	7.30	0.30	0.20	0.40	0.10	0.10	0.10	11.43
Black lemur (*Eulemur macaco*)	0.20	3.40	15.60	19.50	28.00	1.50	5.30	14.70	0.30	7.20	0.30	0.50	0.40	0.10	0.20	0.20	9.22
Mongoose lemur (*Eulemur mongoz*)	0.00	1.10	2.90	6.20	27.40	2.20	6.80	33.90	0.30	14.10	0.30	0.40	0.80	0.40	0.40	0.80	6.33
Red-bellied lemur (*Eulemur rubriventer*)	1.00	8.50	15.40	16.60	20.60	2.00	4.50	16.00	0.30	9.90	0.50	0.50	0.60	0.20	0.30	0.40	7.60
Gentle bamboo lemur (*Hapalemur griseus*)	1.60	8.20	9.60	11.40	22.00	1.40	6.50	23.00	0.30	8.70	0.30	0.40	1.00	0.10	2.40	1.00	2.13
Ruffed lemur (*Varecia variegata*)	0.00	1.70	5.60	9.00	24.90	2.30	6.40	41.70	0.40	5.40	0.10	0.10	0.30	0.20	0.20	0.10	7.25
Greater galago (*Otolemur garnettii*)	0.60	3.80	5.80	7.30	22.60	2.60	5.90	35.30	0.80	9.20	0.50	0.90	0.80	0.10	0.40	0.50	6.11
Thick-tailed galago[c] (*Galago crassicaudatus*)	0.80	6.20	7.90	7.40	19.80	4.10	3.20	30.50		18.80			1.20				15.67
Slow loris[c] (*Nycticebus coucang*)	1.90	6.60	7.50	11.10	31.10	2.50	4.20	30.40		1.10							
Grivet[c] (*Cercopithecus aethiops*)	6.40	9.60	2.70	1.60	25.10	7.20	4.40	34.60		7.60							
Howler monkey (*Alouatta paliatta*) (wild species)	12.33	4.88	0.01	2.71	24.97	5.04	2.77	17.57	0.28	12.59	0.06	0.68	15.06	0.06	0.16	0.03	0.87
Green monkey (*Chlorocebus sabaeus*)	5.00	5.40	1.90	1.40	21.20	5.90	1.30	32.80		22.10							
Common squirrel monkey (*Saimiri sciureus*)	4.40	6.60	6.00	7.20	29.20	3.90	4.20	30.00		7.80							
Brown capuchin (*Cebus apella*)	2.34	4.99	0.01	3.82	17.78	5.53	1.38	26.56	0.54	30.63	0.37	0.92	2.91	0.06	0.28	0.31	8.99

(Continued)

Table 4 (Continued)

Species	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{16:1}$	$C_{18:1}{}^{a}$	$C_{20:1}{}^{b}$	$C_{18:2}$	$C_{20:3}$	$C_{20:4}$	$C_{18:3}$	$C_{20:5}$	$C_{22:5}$	$C_{22:6}$	*Ratio n−6:n−3*
Bolivian squirrel monkey (*Saimiri boliviensis boliviensis*) (captive species)	5.38	3.80	0.01	2.60	17.54	4.31	2.29	29.47	0.04	28.78	0.30	0.89	2.08	0.11	0.22	0.45	10.54
Toque macaque (*Macaca sinica*) (wild species)	8.87	6.79	0.01	4.12	26.79	5.21	5.29	30.47	0.31	8.03	0.16	0.32	2.55	0.11	0.19	0.12	2.88
Rhesus macaque (*Macaca mulatta*) (captive species)	4.70	9.66	3.04	1.23	20.25	5.08	1.57	20.03		28.99		0.37	2.93	0.04	0.07	0.08	9.43
Vervet monkey (*Chlorocebus pygerythrus*) (captive species)	5.77	9.43	2.66	1.74	22.29	6.21	2.05	19.14		26.46		0.32	0.98	0.05	0.05	0.10	22.88
Baboon (*Papio* spp.)	5.10	7.90	2.30	1.30	16.50	4.20	1.20	22.70		37.60							
Siamang[c] (*Symphalangus syndactylus*)	16.22	12.13		3.99	14.39	4.73	1.19	18.83	0.46	22.76	0.21	0.44	2.02	0.10	0.38	0.25	8.53
Mountain gorilla (*Gorilla beringei beringei*) (wild species)	0.03	0.36	0.13	3.77	27.10	5.67	2.23	27.14	1.04	10.56	0.13	2.08	16.31	0.22	0.69	0.09	0.74
Gorilla[c] (*Gorilla gorilla gorilla*) (captive species)	0.05	0.24	0.80	5.42	33.28	8.41	2.37	28.82	0.53	13.60		0.56	1.80	0.03	0.11	0.13	6.89
White-handed gibbon[c] (*Hylobates lar*) (captive species)	0.83	10.33	6.90	3.28	19.26	5.42	2.54	36.68	0.27	10.71		0.46	1.12	0.01	0.02	0.27	7.90
Bornean orangutan (*Pongo pygmaeus*)	2.36	2.95		1.60	26.38	5.79	1.25	26.86	0.54	27.45	0.13	0.70	7.87	0.02	0.22	0.07	3.46
Common chimpanzee (*Pan paniscus*)	0.18	0.95	0.02	5.34	25.99	6.00	1.44	25.10	0.54	24.74	0.40	0.59	2.32	0.08	0.24	0.21	9.05
Human (*Homo sapiens*)	0.46	1.03	4.40	6.27	22.00	8.06	3.29	31.30	0.67	10.85	0.32	0.46	1.03	0.12	0.19	0.25	7.44

[a] Sum $C_{18:1}$ $n-9$ + $C_{18:1}$ $n-7$.

[b] Sum $C_{20:1}$ $n-9$ + $C_{20:1}$ $n-11$.

[c] Data from single species.

Data from various sources.

species studied, this ratio is very low, whereas in captive species and human milk, it is high. Today, the Western human diet is generally deficient in $n-3$ acids, with an $n-6$-to-$n-3$ ratio in the range 7.5:1–17:1. Such diets have been shown to promote many cardiovascular and autoimmune diseases. Compared to captive species, the diet of wild primates contains ~5 times more PUFAs, of which ~4% are $n-3$ acids, resulting in a favorably low $n-6$ to $n-3$ ratio.

The fat in milk exists as a water-in-oil emulsion, stabilized by a special emulsifier referred to as the MFGM. In bovine milk, the diameter of the fat globules is in the range 0.1–20 μm (mean 3–4 μm) (*see* **Milk Lipids**: Fat Globules in Milk); we have found no information on the size of fat globules in primate milk. The MFGM is a very complex structure comprised principally of phospholipids and specific proteins; the inner layer of phospholipids and proteins (including xanthine oxidoreductase and butyrophilin, which associate, and are involved in the expression of the fat globules from the mammocytes) is acquired within the mammocyte, and this is surrounded by a trilaminar structure acquired as the globule is expressed from the mammocyte through the apical membrane into the lumen of the alveolus (*see* **Milk Lipids**: Milk Fat Globule Membrane). This structure contains mucoproteins, mucins (MUC 1 and MUC 15), which increase the hydrophilicity of the membrane. In human and equine milk, the mucins form filaments, 0.5–1 μm long, which are lost on mild heating. It has been suggested that the filaments retard the movement of fat globules through the intestine and facilitate the action of lipase, thereby improving digestibility. SDS-PAGE shows that the MFGM of chimpanzee and Rhesus monkey contains high-molecular-weight glycoproteins like human and equine milk fat, but the electron micrographs of these globules have not been reported, and it is not known if the fat globules in these milks have filaments.

Proteins

The protein content of primate milk is generally low (~10 to ~40 g kg^{-1}, with a few exceptions), especially that of the milks of the Great Apes. This low level of protein reflects the slow growth rate of the Great Apes, all of which are born in an immature state, have a long nursing period, grow and mature slowly, and have a relatively long life. Like in all other species, the milk proteins of primates fall into two groups, the caseins and whey proteins (WPs), the ratio of which varies considerably between primate species, ranging from about 30:70 for human milk (the lowest proportion of all mammalian species reported) to 70:30 for some small primates (similar to that for the Bovidae). According to Bounous and collaborators, WPs enhance the humoral immune response in humans and promote longevity. These authors claim that WP-based formulae are nutritionally better for human infants than casein-based formulae (*see* **Dehydrated Dairy Products**: Infant Formulae); this hypothesis warrants further investigation. It should be noted that all WPs have a biological function, whereas the function of the caseins is nutritional.

Table 5 is a summary of the distribution of casein and WPs in the milks of some primate species and includes bovine milk for comparative purposes. Both caseins and WPs are very heterogeneous, but the milk proteins of only a few primates have been analyzed by modern electrophoretic techniques. Urea-polyacrylamide gel electrophoretograms (PAGE) of milks of various species are shown in **Figure 2**; work in this area and the application of 2D electrophoresis to the study of primate milk proteins are warranted.

Bovine milk and the milk of many other ruminants contain four caseins (α_{s1}, α_{s2}, β, and κ) and two principal WPs (α-lactalbumin (α-La) and β-lactoglobulin (β-Lg)) with lesser amounts of blood serum albumin and immunoglobulins (Igs), and perhaps 100 other proteins at very low or trace levels, including about 70 enzymes (*see* **Milk Proteins**: Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins). The milk protein system of other commercially important species is also very heterogeneous and has been studied thoroughly (*see* **Milk**: Buffalo Milk; Camel Milk; Equid Milk; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.); Sheep Milk). However, there are very few studies on the milk protein systems of primates other than humans.

Electrophoresis is the best technique for studying the heterogeneity of protein systems and identifying the constituents. Probably, the first interspecies (40 species, including one sample from *Homo sapiens* and one sample from *Macaca mulatta*) study of milk proteins is that of Sloan and colleagues in 1961, who used zone electrophoresis on paper at pH 8.6; the identification of the protein zones was uncertain, but the Rhesus and human electrophoretograms were generally similar and both appeared to lack β-Lg and α-casein. Subsequently, β-Lg has been identified in the milk of Rhesus monkeys. The casein-to-WP ratio in baboon milk changes from 0.3:1 after parturition to ~1.0:1 after 5 days; the caseins and WPs were shown by electrophoresis on cellulose acetate to be heterogeneous. Cynomolgus monkey (*Macaca fascicularis*) milk contains β- and κ-caseins but apparently no α-casein; the β-casein consists of nine components, which are resolved by SDS-PAGE. The microheterogeneity is probably due to different levels of phosphorylation, as it disappears on treatment with potato acid phosphate. Monkey κ-casein is highly glycosylated (~50% CH_2O); the constituent monosaccharides are galactose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid.

Table 5 Distribution of caseins and whey proteins in some primate species with bovine milk included for comparative purposes

	Cynomolgus monkey[a] (Macaca fascicularis)	*Rhesus monkey*[b] (Macaca mulatta)	*Vervet monkey*[b] (Chlorocebus pygerythrus)	*Baboon*[c] (Papio cynocephalus anubis)	*Gorilla*[b] (Gorilla gorilla gorilla)[d]	*White-handed gibbon*[b] (Hylobates lar)[d]	*Human*[e] (Homo sapiens)	*Bovine*[f] (Bos taurus)
Total casein ($g\ kg^{-1}$)		5.9	7.2	4.7	13.3	14.8	2.5	26
α_{s1}-Casein	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Reported[g]	10.7
α_{s2}-Casein	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	2.8
β-Casein	Reported (nine variants)	Reported (major component)	Reported	Reported	Reported	Reported	(>85% of total casein) (six variants)	8.6
κ-Casein	Reported (highly glycosylated)	Reported	Reported (substantial)	Not reported	Reported (low)	Not reported	(<15% of total casein) (highly glycosylated)	3.1
γ-Casein	Not reported	Reported	Substantial	Not reported	Reported (low)	Not reported	Not reported	0.8
Total whey protein ($g\ kg^{-1}$)		7.1	8.5	5.1	9.1	8.5	6.4	6.3
β-Lactoglobulin	Reported	2.5	Reported (<rhesus)	Reported (3 variants)	Not Reported	Not Reported	Not Reported	3.2
α-Lactalbumin	Reported	Reported (major protein)	Reported (<rhesus)	Reported (two variants)	Not reported	Not reported	3.2	1.2
Serum albumin	Not reported	Reported (substantial)	Reported (<rhesus)	Not reported	Reported (substantial)	Not reported	0.41	1.2
Proteose peptone	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	0.8
IgG	0.04	Reported (very low)	Not reported	Not reported	Not reported	Not reported	0.05	0.72
IgA	2.4–2.6	2.4	Not reported	Reported (significant)	Not reported	Not reported	1.0	0.13

IgM	0.05	Reported (very low)	Not reported	Not reported	Not reported	Not reported	0.2	0.04
Lactoferrin	Not reported	>2.0	Not reported	Reported	Not reported	Not reported	1.9	0.1
Lysozyme	Not reported	Reported	Not reported	~5% of TN	Not reported	Not reported	0.5% of TN	trace
Casein-to-whey protein ratio	48:52	45:55		48:52			30:70	80:20
NPN (mg kg^{-1})	Not reported	700	600	~20% TN	300	100	460	266
Casein micelle diameter (nm)	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	64	182

[a] From Azuma N and Yamauchi K (1991) Identification of α-lactalbumin and β-lactoglobulin in cynomolgus (*Macaca fascicularis*) milk. *Comparative Biochemistry and Physiology* 99B: 917–921; Cole MF and Bowen WM (1976) Immunoglobulins A, G and M in serum and some secretions of monkeys (*Macaca fascicularis* sym irus). *Infection Immunology* 13: 1354–1359; Yamauchi K, Azuma N, and Shimizu M (1983) Comparison of casein from cynomolgus monkey (*Macaca fascicularis*) with human casein. *Comparative Biochemistry and Physiology* 75B: 287–292.

[b] From Kunz C and Lönnerdal B (1993) Protein composition of Rhesus monkey milk: Comparison to human milk. *Comparative Biochemistry and Physiology* 104A: 793–797; Kunz C and Lönnerdal B (1994) Isolation and characterization of a 21 kDa protein in Rhesus monkey (*Macaca mulatta*) milk. *Comparative Biochemistry and Physiology* 108B: 463–469; Osthoff G, Hugo A, de Wit M, Nguyen TPM, and Seier J (2009) Milk composition of captive vervet monkey (*Chlorocebus pygerythrus*) and Rhesus macaque (*Macaca mulatta*) with observations on gorilla (*Gorilla gorilla gorilla*) and white handed gibbon (*Hylobates lar*). *Comparative Biochemistry and Physiology* 152B: 332–338; Eitzman DV (1970) Immunoglobulin levels in the *Macaca mulatta*. *Folia Primatology* 12: 313–316; Davidson LA and Lönnerdal B (1986) Isolation and characterization of Rhesus monkey milk lactoferrin. *Pediatric Research* 20: 197–201; Davidson LA and Lönnerdal B (1988) Specific binding of lactoferrin to the intestinal brush border membrane: Ontogeny and effect of glycan chain. *American Journal of Primatology* 254: G580–G585; Takenaka A and Takenaka O (1982) Postnatal changes in the blood of Japanese monkeys (*Macaca fuscata*): Immunoglobulins IgG, IgM and IgA. *Primates* 23: 298–302.

[c] From Buss DH (1978) Studies on baboon milk proteins. *Comparative Biochemistry and Physiology* 59B: 5–8; Buss DH (1971) Isolation and partial characterization of lysozyme from baboon milk. *Biochimica et Biophysica Acta* 236: 587–592; Quarfoth GT and Jenness R (1975) Isolation, composition and functional properties of α-lactalbumin. *Biochimica et Biophysica Acta* 379: 476–487; Hall AJ, Massel A, Bell K, Halliday JA, Shaw DC, and Vandeberg JL (2001) Characterization of baboon (*Papio hamadryas*) milk proteins. *Biochemical Genetics* 39: 59–71; Aschaffenburg R, Fenna RE, Phillips DC, *et al*. (1979) Crystallography of α-lactalbumin. III. Crystals of baboon milk α-lactalbumin. *Journal of Molecular Biology* 127: 135–137.

[d] Single species. TN, total nitrogen; Ig, Immunoglobulin.

[e] From Lönnerdal B and Atkinson SA (1995) Nitrogenous components of milk. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 351–368. San Diego, CA: Academic Press, Inc.; Hambræus L (1984) Human milk composition. Nutrition abstracts and reviews. *Clinical Nutrition – Series A* 54: 219–236.

[f] From Walstra P, Wouters JTM, and Geurts TJ (2006) *Dairy Science and Technology*, 2nd edn. Boca Raton, FL: CRC Press.

[g] From Rasmussen LK, Due HA, and Petersen TE (1995) Human α_{s1}-casein: Purification and characterization. *Comparative Biochemistry and Physiology* 111B: 75–81.

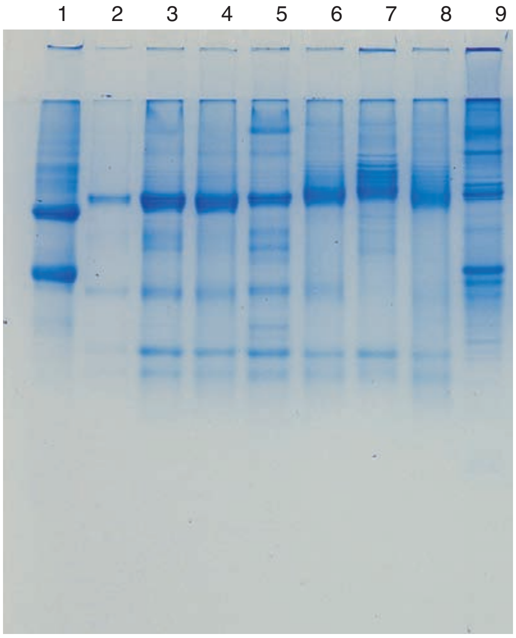

Figure 2 Urea-polyacrylamide gel electrophoretograms of four individual vervet monkey milks, three macaque monkey milks, and a human milk sample. Lane identification: 1, NaCaseinate (bovine); 2–5, Vervet monkeys; 6–8, Macaque monkeys; 9, Human.

Urea-PAGE shows that the milk proteins of Rhesus, and especially vervet monkeys, are very heterogeneous (**Figure 2**, *see* **Milk**: Milks of Non-Dairy Mammals). They appear to contain α-La, β-Lg, and β-casein, but lack α-casein; several other proteins present have not been identified.

Caseins

As far as we are aware, no casein has been isolated from nonhuman primate milk and characterized with respect to amino acid composition and sequence, mass, degree of phosphorylation and/or glycosylation, genetic polymorphism, sensitivity to Ca^{2+}, and so on. Human milk contains β- and κ-casein; although it is generally regarded as being devoid of α_s-casein, there are some reports of its presence. κ-Casein represents only $\sim$12% of bovine casein, but it is critical for many of the physicochemical and technologically important properties of the casein system, and indeed of milk. Apart from a high level of glycosylation reported for the κ-casein of cynomolgus monkey and humans milks, we are not aware of any reports on the properties of other nonhuman primate κ-casein.

Whey proteins

The WPs of primate milk are better characterized than the caseins. SDS-PAGE showed that baboon milk contains α-La, lysozyme, and β-Lg (which is well characterized, see below) and a group of uncharacterized proteins (probably caseins).

α-Lactalbumin

Considering the high concentration of lactose in primate milks, the presence of α-La (which is involved in the biosynthesis of lactose, *see* **Milk Proteins**: α-Lactalbumin) in primate milk is expected. α-La has been isolated from the milk of chimpanzee, baboon, cynomolgus, and Rhesus monkey; the amino acid composition is virtually identical and the sequence of the 20 N-terminal residues is identical to that of human α-La, and they have a good activity in lactose synthesis; baboon α-La has been crystallized.

β-Lactoglobulin

β-Lg, the principal WP in the milk of most species, but which is absent from the milk of humans, guinea pig, rodents, and rabbit, is a very well-characterized protein (*see* **Milk Proteins**: β-Lactoglobulin). β-Lg has been identified in the milk of baboon, cynomolgus, Rhesus, and vervet monkey. The sequence of the 26 N-terminal residues of cynomolgus monkey β-Lg is 54% homologous with bovine β-Lg. Baboon β-Lg occurs as three genetic variants, A, B, and C, with pIs of 5.25, 5.45, and 5.75, respectively. It contains 168 amino acid residues, compared with 162 for the bovine protein; the difference is due to the insertion of eight amino acids between residues 61 and 62 (bovine numbering) and the deletion of residues 109 and 112. It has an apparent mass (by SDS-PAGE) of 20 750 Da (about the same MW as Rhesus β-Lg), compared with 18 281 Da for bovine β-Lg. Baboon β-Lg is more similar to human glycodelin protein (a glycosylated homologue of β-Lg found in the uterus of humans and baboon and thought to have a protective role as the embryo is embedded) than it is to bovine β-LgA.

β-Lg of some species has a free –SH group, whereas those of others do not; some exist as a dimer at neutral pH, others do not (these characteristics are not related) (*see* **Milk Proteins**: β-Lactoglobulin). These properties have not been reported for primate β-Lg.

The homology (%) of baboon β-Lg with the corresponding protein from other species is sheep, 51.3; reindeer, 51.8; pig, 48.7; horse, 55.6; cat, 56.8; and dolphin, 54.9 (*see* **Milk Proteins**: β-Lactoglobulin).

We are not aware of reports of the presence of β-Lg in the milk of the Great Apes. Considering the absence of this protein from human milk, it is expected that it is also absent from the milk of nonhuman apes. Extending the range of primates for which the status of β-Lg is known is desirable.

Immunoglobulins

Mammal neonates acquire immunity either *in utero* or postpartum through the action of maternal Igs; however, the mode of transfer of Igs from mother to offspring is species specific. Ungulates transfer Igs, mainly IgG1, postpartum via colostrum (first colostrum of bovines contains ~10% Ig), whereas humans and other advanced primates and lagomorphs transfer Igs *in utero*; some species, for example, rodents, use both mechanisms. Whether Igs are passed transplacentally or via milk depends on the structure of the placenta, and the transfer process becomes increasingly more elaborate from prosimians to Old World monkeys and the Great Apes. In hominoids, the placenta is hemochorial, and maternal blood comes in direct contact with the placenta trophoblast; Igs (mainly IgG, which is relatively small) reach the fetal bloodstream by direct transfer and provide sufficient passive immunity against pathogens until the neonatal immune system has matured. Colostrum in these primates contains predominately IgA (the primary function of which is protection of the newborn's intestine from bacterial pathogens). The production of IgA, IgM, IgE, cytokines, growth factors, and other immune-related substances is delayed in human infants and presumably in other nonhuman primates. Placentation is more primitive (epitheliochorial) in Strepsirrhini primates and maternal Igs are transferred only via colostrum postpartum.

In Rhesus macaques, serum IgG levels in the neonate have been measured and are comparable to those of the adult. The concentration of IgG in the blood serum of neonatal Japanese macaque is ~70% of that of the adult; the concentrations of IgA and IgM are very low at birth but increase thereafter, presumably as the neonate encounters antigens. Macaque colostrum/early milk contains a substantial amount (mean $2.4\,\mathrm{g\,l^{-1}}$) of IgA (probably sIgA), though the amount is lower than that of human early milk, but it contains very little IgG or IgM.

Lactoferrin

Lactoferrin (Lf) is a nonheme, iron-binding protein with bactericidal and other biological functions (*see* **Milk Proteins**: Lactoferrin). Rhesus monkey milk contains a considerable concentration of Lf, which has been isolated and characterized. We are not aware of reports on Lf in the milk of other nonhuman primates, but it seems reasonable to conclude that it is a general constituent (Lf has been found in the milk of all species investigated, and human milk is a very rich source – about 20% of total N).

Casein micelles

Considering that the milk of all primates for which data are available is white, it seems reasonable to conclude that the caseins of all species exist as micelles capable of scattering light. The casein micelles in human milk are much smaller (~60 nm) and more porous than bovine casein micelles (mean diameter ~150 nm). We are not aware of any work on the casein micelles of other primates; work in this area is warranted.

An important property of milk is its rennet-induced coagulation in the neonatal stomach following the specific and limited proteolysis of κ-casein, a property that delays the discharge of milk from the stomach into the small intestine, thereby improving digestibility. The proteins of human milk form flocs, rather than a gel, on renneting and are more easily digested than bovine milk, which forms a firm gel, by the human baby. Milk proteins of primate species generally have a casein content of $\leq$60% of total protein and form soft clots in the stomach, which should be an important consideration in the selection of a substitute for maternal milk when hand-rearing is necessary. We are not aware of any work on the rennet-induced coagulation of the milk of nonhuman primates.

Neither are we aware of any work on other technologically important properties of the proteins in primate milk, for example, acid-induced coagulation, ethanol-induced coagulation, surface activity, water binding, or rheological properties.

Total amino acids

Human and chimpanzee (*Pan troglodytes*) milks have significantly lower concentrations of total amino acids (protein-bound plus free) than the milks of other primate and nonprimate species, though concentrations are similar between the gorilla (*Gorilla gorilla gorilla*) and lower primates (**Table 6**). The low protein, and hence low total amino acid concentration, is not unique to human milk but occurs across primates generally, but in nonprimates it is observed only in the horse (*Equus caballus*). There is some commonality in the general amino acid patterns of primate and nonprimate species (**Table 6**) despite differences in total amino acid concentrations. In all species studied, total amino acid concentration is much greater in colostrum than that in mature milk, and a decrease of ~75% in total amino acids occurs between colostrum and milk for humans and horses, but the decrease in baboons and Rhesus monkeys is ~34 and 21%, respectively. Glutamate, leucine, and proline comprise 40, 10, and 10%, respectively, of the milks of primate and nonprimate species, and no phylogenetic trend is found across species for these amino acids, except perhaps, leucine, which is high in primate milk. There is some phylogenetic trend between primate and nonprimate species in the concentration of cystine and methionine, as primates have lower methionine and higher cystine levels than nonprimates. Across primate species, humans and Great Apes have lower methionine and higher cystine than lower primates. Total essential amino acids concentration is similar across primate and nonprimate species and represents ~40% of total amino acids in both.

Nonprotein nitrogen and free amino acids

About 3–5% of the total nitrogen in bovine milk is nonprotein nitrogen (NPN; soluble in 12% TCA), comprised

Table 6 Total (g l^{-1})[a], essential amino acids (mg g^{-1} total) and some important amino acids (mg g^{-1} total) in the milk of some primate and nonprimate species

Primate	*Total*	*Total essential amino acids*	*Glutamate*[b]	*Leucine*	*Proline*	*Methionine*	*Cystine*	*Glycine*	*Serine*	*Arginine*
Human (*Homo sapiens*)	8.5	400	190	104	95	16.1	20.2	22.0	61.0	36.0
Chimpanzee (*Pan troglodytes*)	9.2	392	221	104	104	17.0	16.2	20.0	41.0	35.0
Gorilla (*Gorilla gorilla gorilla*)	11.5	408	203	102	99	19.8	15.5	22.0	47.0	35.0
Baboon (*Papio cynocephalus anubis*)	11.5	408	194	105	107	21.2	10.1	14.0	53.0	56.0
Rhesus monkey (*Macaca mulatta*)	11.6	421	191	111	112	24.8	11.7	14.0	48.0	47.0
Nonprimate										
Cow (*Bos taurus*)	33.6	427	208	99	100	26.3	8.9	18.0	56.0	34.0
Goat (*Capra hircus*)	25.7	433	209	96	106	25.5	8.6	18.0	49.0	29.0
Sheep (*Ovis aries*)	54.1	422	203	90	102	28.7	7.5	18.0	52.0	34.0
Llama (*Lama glama*)	29.6	443	220	99	102	31.1	7.3	14.0	41.0	36.0
Pig (*Sus scrofa*)	35.0	379	208	89	117	21.7	15.6	32.0	51.0	44.0
Horse (*Equus caballus*)	15.8	377	217	93	91	22.0	11.3	16.0	52.0	60.0
Elephant (*Elephas maximus*)	37.1	411	195	98	102	21.8	10.6	13.0	68.0	48.0
Cat (*Felix catus*)	75.7	400	208	118	94	32.0	12.1	10.0	44.0	64.0
Rat (*Rattus norvegicus*)	86.9	371	221	92	75	25.0	25.7	15.0	85.0	33.0

[a] Amino acids in proteins plus free amino acids.

[b] Glutamate plus glutamine.

Modified from Davis TA, Nguyen HV, Garcia-Bravo R, *et al*. (1994) Amino acid composition of human milk is not unique. *The Journal of Nutrition* 454: 1126–1132.

mainly of urea and amino acids, whereas the corresponding value for the milk of human, Rhesus monkey, and baboon is 25, 6–13, and 20%, respectively. Of the Lemuridae species studied, *Eulemur fulvus* has 10.4% and *Eulemur macaco* has 13.9% NPN as percentage of total nitrogen (TN), whereas for one species from the Galagidae family, *Otolemur garnetti*, the NPN is only 2.8% of TN. The free amino acids may be important from a nutritional point of view and, in the case of bovine milk, they also sustain the initial growth of lactic acid bacteria in fermented milk products. The high concentration of free amino acids in human milk is assumed to have a beneficial role in postnatal development, and, across mammals, it is assumed that each species has a characteristic free amino acid pattern. Of the 13 species studied by Sarwar and colleagues, in 1998, the concentration of free amino acids was the highest in the milk of pinnipeds (up to 20 mmol l^{-1}) and lowest in bovine milk. The milk of human, chimpanzee, baboon, and Rhesus monkey contains ~0.6 to ~4 mmol l^{-1} free amino acids (**Table 7**). Glutamic acid is the most abundant free amino acid in the milk of primates and nonprimates. Taurine, which is considered to be important for brain development in the neonate, is the free amino acid present at the highest or second highest concentration in the milk of many species, including human, chimpanzee, baboon, Java monkey, and Rhesus monkey (**Table 7**).

Urea, which represents ~50% of the NPN of bovine milk (~6 mmol l^{-1}), is also present at substantial concentrations in the milk of other species, including primates.

Milk Salts

Bovine milk contains 4 principal (macro) inorganic cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}), 3 macro inorganic anions (PO_4^{3-}, Cl^{-}, SO_4^{2-}), 1 macro organic anion (citrate), and about 20 elements at low or trace (micro) levels (*see* **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance). These elements are present as salts in solution or associated with proteins in the colloidal phase. Although quantitatively minor constituents, these inorganic elements play very important physicochemical, technological, and nutritional roles. The concentration of the inorganic elements can be determined by various techniques, and the total concentration can be estimated as the ash remaining after incineration at 550 °C × 5 h. The amount of ash obtained from the milk of most primates is very low, 2–3 g kg^{-1}, which is as would be expected considering the dilute nature of these milks. The concentration of minerals and trace elements in the milk of some primates is shown in **Table 8**. In human milk, as lactation progresses, the concentration of

Table 7 The free amino acids (μmol l^{-1}) of some primate milks

Amino acid	*Rhesus monkey (*Macaca mulata*)*[a]	*Baboon (Papio sp.)*[a]	*Chimpanzee (*Pan troglodytes*)*[a]	*Java monkey (*Presbytis comata*)*[a]	*Human (*Homo sapiens*)*[b]	*Bovine*[a] *(*Bos taurus*)*
Alanine	106	108	194	32	228	30
Arginine	29	60	22	7	35	10
Aspartic acid	58	81	185	25	183	15
Cystine	47	61	44		56	21
Glutamic acid	314	439	2645	291	1184	117
Glutamine	70	97	27	22	285	12
Glycine	106	194	177	50	125	88
Histidine	27	37	61	4	8	9
Isoleucine	19	23	17		33	3
Leucine	33	46	38		56	3
Lysine	35	64	15	2	39	15
Methionine	6	13	9		9	~0
Ornithine	34	33	14	6		3
Phenylalanine	21	33	30		24	
Proline					64	23
Serine	125	148	146	43	274	13
Taurine	561	380	264	135	301	16
Threonine	45	49	45	10	98	0.3
Tyrosine	19	29	19		3	5.0
Valine	37	68	42	4	73	
Total	1692	1963	3994	623	3078	578

[a] Rassin DK, Sturman JA, and Gaull GE (1978) Taurine and other free amino acids in milk of man and other mammals. *Early Human Development* 2(1): 1–13.

[b] Agostini C, Carratù B, Boniglia C, Riva E, and Sanzini E (2000) Free amino acid content in standard infant formulas: Comparison with human milk. *Journal of the American College of Nutrition* 19: 434–438.

Table 8 Concentrations (mmol l^{-1}) of minerals and trace elements in the milk of some primate species

Species	*Calcium*	*Magnesium*	*Sodium*	*Potassium*	*Phosphorus (inorganic)*	*Citrate*	*Chloride*	*Iron*	*Copper*	*Zinc*	*Manganese*
Man (*Homo sapiens*)	6.5	1.4	6.0	12.1	4.6	2.6	12.9	0.01	0.004	0.026	0.006
Common marmoset (*Callithrix jacchus*)	22.15	2.0	10.4	14.70	7.13		16.6				
Rhesus monkey (*Macaca mulatta*)	9.8	1.26	3.57	7.1				0.02	0.007	0.028	0.02
Ringtailed lemur (*Eulemur catta*)	16.75		19.7	5.6	20.0		5.5				
Black lemur (*Eulemur macaco*)	7.5		10.0	2.5	7.1		4.8				
Brown lemur (*Eulemur fulvus*)	22.0		33.0	9.46	24.0		16.0				

Data from various resources.

individual minerals changes. Na, K, and Cl decrease, whereas Ca increases initially but decreases again by ~6 months lactation. Magnesium concentration remains fairly constant throughout lactation with a small decrease at 6 months, and P increases slightly over the first 3 months of lactation and subsequently decreases. Fe, Cu, and Zn, which are relatively high in colostrum, decrease significantly as lactation progresses. The pattern described above has been observed also for the minerals of Rhesus monkey milk over the course of lactation. Overall, the levels of iron and copper in Rhesus monkey milk are significantly higher than those in human milk.

Indigenous Enzymes

Milk contains numerous indigenous enzymes, which originate from the blood (leukocytes or serum), mammocyte cytoplasm, and the apical membrane of mammocytes. These enzymes may cause spoilage, have a bactericidal effect, or assist in digestion. About 70 indigenous enzymes have been identified in bovine milk, and the most important of these have been isolated and characterized. The milk of all species probably contains the same range of enzymes as bovine milk, but only a few enzymes have been studied in most species, an exception being human milk in which 30 enzymes have been identified. There are large interspecies differences in the levels of many enzymes in milk. Unfortunately, there are very few studies on the indigenous enzymes in the milk of nonhuman primates. An interesting exception is bile salts-stimulated lipase (BSSL). The principal lipase in the milk of all species studied is lipoprotein lipase (LPL) from blood serum. However, in 1928, E. Freudenberg showed that human milk contains a lipase activated by bile salts, and hence was called BSSL. In 1966, Freudenberg showed that gorilla milk also contains BSSL and that the milk of several other species lacked this enzyme. BSSL is identical to pancreatic carboxyl ester hydrolase (CEL), and was shown to be present in the milk of human, gorilla, squirrel monkey, and African green monkey, but absent from the milk of all other species studied, including other apes and monkeys. Initially, it was thought that the milk of most species lack CEL, but it is now known that it occurs widely in the pancreas and mammary gland, including in the human, gorilla, chimpanzee, orangutan, and macaque. Gene duplication has resulted in the evolution of a pseudogene in most species. The structure and organization of the human and gorilla CEL gene have been studied, and the primary structure of the enzyme determined; they show 97% homology. CEL contributes to the digestion of lipids in human infants, who have limited pancreatic function; presumably, it serves a similar function in other species. Baboon milk contains both lipase and esterase activities, but the enzymes were not separated.

In 1923, Alexander Fleming identified a bactericidal agent in nasal fluid, tears, sputum, saliva, and other body fluids, which lysed bacteria (*Micrococcus lysodeikticus* was used for the assay and hence the name lysozyme, which was shown to be an enzyme). He did not report the presence of lysozyme in milk, but shortly thereafter Bordet and Bordet reported the presence of lysozyme in the milk of several species and that human milk is a particularly rich source; the enzyme has been isolated from the milk of several species. Human and equine milks are exceptionally rich sources, containing 400 and 800 mg l^{-1}, respectively (3000 and 6000 times the level found in bovine milk, or 4 or 3% of the total protein in human or equine milk). Lysozyme represents ~5% of the total N of baboon milk; the enzyme does not survive passage through the gastrointestinal tract (GIT; active lysozyme is not found in the feces of the infant baboon). The enzyme has been purified from baboon milk and characterized. Human milk lysozyme contains 130 amino acid residues, and 129 residues are found in egg white lysozyme, but the two enzymes differ at 14 positions.

Hanke and colleagues compared the lysozyme in the milk of 19 primate species using antibodies raised against human or baboon lysozyme; the milk of all 19 species contained lysozyme (activity not quantified) that was immunologically related to human lysozyme approximately, according to the phylogenetic relationship among primates; human and chimpanzee lysozyme were immunologically indistinguishable.

Vitamins

There appear to have been very few studies on the vitamins in primate milk, which is surprising because many of the studies on these milks have concerned the artificial feeding of primates. The concentrations of most B-vitamins in the milk of captive baboons (*Papio cynocephalus*) throughout lactation are intermediate between those in human and bovine milk (**Table 9**), except for niacin and pyridoxine, which are higher in baboon milk than in human and bovine milk, and folate, which is lower. This was the only study on vitamins in the milk of nonhuman primates, and there seems to have been no reports since.

Conclusions and Prospects

The gross composition of about 30 primate species is known; they contain the same types of constituents as all other mammalian species examined but at different

Table 9 Concentration of water-soluble vitamins (μg l^{-1}) in baboon (*Papio cynocephalus*), human (*Homo sapiens*), and bovine (*Bos taurus*) milk

Vitamin	*Baboon*[a]	*Human*[b]	*Bovine*[c]
Thiamine (B_1)	180	166	410
Riboflavin (B_2)	740	475	1730
Niacin (B_3)	3200	2300	922
Pantothenic acid (B_5)	2630	2500	3349
Pyridoxine (B_6)	700	310	556
Biotin (B_7)	6.5	7.9	29
Folate (B_9)	30	79	56
Cobalamin (B_{12})	2	0.97	4

[a] From Buss DH, Ford JE, and Scott KJ (1977) The B-vitamin content of baboon (*Papio cynocephalus*) milk. *The British Journal of Nutrition* 38: 507–511.
[b] From Picciano MF (1995) Vitamins in milk (A). Water-soluble vitamins in human milk. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 675–688. San Diego, CA: Academic Press, Inc.
[c] Data averaged from Jensen RG (1995) Vitamins in milk (B). Water-soluble vitamins in bovine milk. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 688–692. San Diego, CA: Academic Press, Inc.

concentrations. With a few exceptions, the milk of primates is dilute, with low levels of fat, protein, and inorganic salts, and a high level of lactose. In-depth knowledge of nonhuman primate milk composition is of fundamental importance for selecting formulae for hand-rearing animals in captivity. Furthermore, the formula should reflect the natural proportions of proteins (both casein and WPs), fats, and sugars in maternal milk, and should be appropriate for an individual's digestive capability (i.e., frequency of feeding, amount fed, and gastric clot formation should be similar to the corresponding traits of normal maternal feeding). Detailed information on the constituents, especially the proteins, is lacking for primate milks. The OSs of a few primate species have been studied; though the constituent monosaccharides are common with those of other species, the glycosidic bonds are somewhat different. The FAs in the lipids seem to be common with all other species, but the lipids of many primates contain high levels of $C_{8:0}$ and $C_{10:0}$, and very high levels of PUFAs; there appear to be no unique FAs. Like the milk of other species, primate milks contain caseins and WPs; the principal WPs are those present in the milk of other orders, namely, α-La, β-Lg, Igs, and Lf, and no novel WPs have been reported. Unfortunately, the milk proteins of only a few primate species have been examined by high-resolution electrophoretic techniques; available data indicate that, like human milk, the milk of nonhuman primates contain β- and κ-caseins, but the situation of the α-casein is unclear. Primate milk contains several minor proteins that have not been identified. A thorough proteomic study of primate milks is warranted.

There is essentially no information on the colloidal aspects of primate milks, that is, of the fat globules and casein micelles, and there are no data on the colloidal stability of primate milks. A number of primates, for example, chimpanzee, gorilla, orangutan, baboon, and Rhesus and other macaque monkeys, are readily available in captivity (in primate centers and zoos) or in the wild, and it should be relatively easy to obtain milk samples. An extensive interspecies electrophoretic study of the proteins in primate milk should provide interesting information on the evolution of primates, which have been evolving and undergoing speciation for about 65 million years; the most recent species, *Homo sapiens*, evolved only ~200 000 years ago.

See also: **Dehydrated Dairy Products**: Infant Formulae. **Lactose and Oligosaccharides**: Indigenous Oligosaccharides in Milk. **Milk**: Bovine Milk; Buffalo Milk; Camel Milk; Equid Milk; Goat Milk; Introduction; Milks of Non-Dairy Mammals; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.); Sheep Milk. **Milk Lipids**: Fat Globules in Milk; Milk Fat Globule Membrane. **Milk Proteins**: Lactoferrin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; α-Lactalbumin; β-Lactoglobulin. **Milk Salts**: Distribution and Analysis; Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance.

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MILKING AND HANDLING OF RAW MILK

Contents

Milking Hygiene
Influence on Free Fatty Acids
Effect of Storage and Transport on Milk Quality

Milking Hygiene

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Microbiological Contamination

The following main sources of microbial contamination of milk can be distinguished: (1) interior of the udder, (2) exterior of the teats and the udder, and (3) milking and storage equipment.

The interior of the udder is of minor importance as long as cows are healthy: primary infection of milk is very low. Housing conditions and the exterior of the teats are permanent although highly variable sources of contamination. Also organic residues on the inner surface of the milking equipment including the bulk milk tank contribute to microbiological contamination of fresh milk.

The two main sources of bacteria in raw bulk milk are organisms transported from the environment into the milking machine and mastitis organisms from within the udder. Bacteria deposited in milk handling equipment will multiply and can become a major source of contamination if this equipment is not cleaned and sanitized properly.

Microbiological contamination of these main sources can be minimized by: (1) post- and premilking teat disinfection, (2) premilking cleaning and disinfection of udders, and (3) cleaning and disinfection of equipment.

Post- and Premilking Teat Disinfection

Teat dipping, or better teat disinfection, reduces bacterial numbers on the teat skin and orifice (*see* **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens). Postmilking teat disinfection is an effective procedure for reducing the rate of subclinical and clinical mastitis during lactation. While postmilking teat disinfection with an effective germicide reduces intramammary infections caused by contagious mastitis pathogens, it is less effective in controlling mastitis due to environmental pathogens.

Premilking teat disinfection was developed as a potential method to control environmental pathogens by reducing bacterial populations on teat skin before milking. Teat disinfection can be performed by dipping the teats in a dipping solution or by spraying the disinfectant on the teats by a hand spray or by automatic spraying devices.

The disinfectants used for pre- and postmilking disinfection can be based on:

1. Chlorhexidine gluconate solutions.
2. Iodine solutions varying in concentrations from 0.1% to 1% active iodine, in many cases mixed with glycerine or other emollients.
3. Linear dodecyl benzene sulfonic acid.
4. Sodium chlorite and lactic acid.
5. Sodium hypochlorite.

The first two mentioned disinfectants are used in most commercially available products.

Although teat disinfection reduces the number of bacteria, there may be problems with possible residues in the milk.

Premilking Cleaning and Disinfection of Udders

Between milkings, cows' teats may become soiled with feces, mud and bedding materials (e.g. straw, sawdust, wood shavings or sand). If not removed beforehand, this dirt, together with the large number of microorganisms associated with it, may be washed into the milk during milking. A first step in hygienic milking is cow hygiene in stables and outdoors. This influences the effectiveness of cleaning the udder. Differences in teat contamination can be found between housed cows and cows at pasture (see **Table 1**). Contamination of teats is lower when cows are at pasture. When cows are housed, bedding material and feedstuffs can be sources of contamination. Feces and urine are also important sources of microbiological contamination of teats and udders.

Therefore, before milking, udder preparation should take place to remove visible dirt and reduce bacterial contamination of the milk. This udder preparation can be done in different ways:

1. Cleaning with a dry towel (textile or paper).
2. Cleaning with a wet towel (towel put in water with disinfectant between cows).
3. Washing with water, left wet.
4. Washing with water, dried with towel (textile or paper).
5. Washing with water including disinfectant, left wet.
6. Washing with water including disinfectant, dried with towel (textile or paper).

The effect of different methods of udder preparation is shown in **Table 2**.

With the last most intensive way of udder preparation, a maximum reduction of teat contamination of 90% can be reached for total bacterial and spore count. If teats are left wet, the effect may also be negative because more bacteria are found on the teats. In practice the time for drying is very short and not always done effectively. Therefore in many cases a dry cleaning is advised.

During udder preparation not only is cleaning of the teats important, but also the milk letdown that is stimulated by massage of the teats. In the case of automatic milking the premilking udder cleaning and disinfection is done automatically in different ways, depending on the brand of milking system. There are systems with rotating brushes, with possible addition of disinfectant during or after preparation. Also washing of teats in teat cup liners combined with drying with air is possible in some systems. There are systems with separate udder preparation and systems without no separate udder preparation. Although automatic detection of dirt is in theory possible, all current automatic milking systems do not adapt to the dirtiness of the teats.

Cleaning and Disinfection of Equipment

Cleaning of milk handling equipment is accomplished by a combination of chemical, thermal and physical processes. Recommended cleaning and sanitizing practices are a balance between the cleaning temperatures, cleaning chemical concentration, contact time and mechanical action (see **Figure 1**). Any one of these factors can be intensified to make up for lack in another, up to a point.

Although system designs vary considerably, typical features of milking parlor cleaning-in-place (CIP) systems are shown in **Figure 2**. Cleaning solutions are transported from the wash vat through the sanitary parts of the system and back to the wash vat during the process.

A cardinal rule for an efficient and effective CIP system design is to keep pipe lengths and number of fittings to a minimum. This will reduce the installation and

Table 1 Contamination of teats of housed cows and of cows at pasture

Conditions	*Total plate count (log cfu per swab ± SD)*	*Aerobic spore counts (log cfu per swab)*
Housed cows	5.12 ± 0.50	4.42 ± 0.51
Pastured cows	3.69 ± 0.34	3.29 ± 0.42

Adapted from Slaghuis *et al*. (1991) *Milchwissenschaft* 46(9): 574–578.

Table 2 Effect of different udder preparation methods on total bacterial counts and number of spores of butyric acid bacteria of teat swabs

Udder preparation method	*n*	*Total plate count (median mean; cfu per swab)*	*Spores of butyric acid bacteria (median mean; cfu per swab)*
Washing with hand shower, drying with paper towel	24	1.3×10^4	2.5×10^1
Washing with water from bucket, drying with paper towel	20	1.1×10^5	1.4×10^2
Cleaning with dry paper towel (1 towel per cow)	20	2.7×10^5	2.5×10^2
Cleaning with wet cotton towel (10 cows per towel)	28	9.8×10^5	6.0×10^2

Adapted from De Vries and Stadhouders (1997).

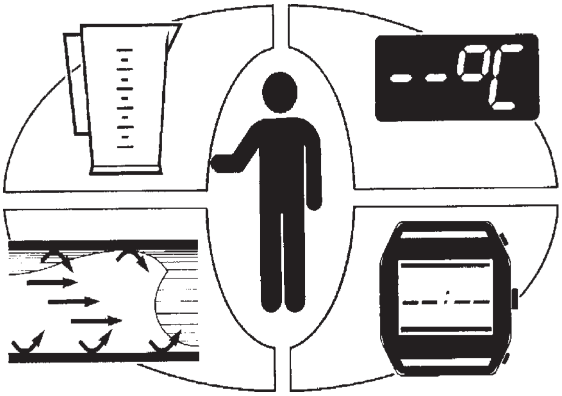

Figure 1 Factors influencing the result of the cleaning process of milking equipment.

operational cost of the CIP system as well as improving both milking and cleaning performance.

Temperature, Time and Concentration of Chemicals

Water Rinses

A water rinse should be performed immediately after milking is completed, to remove most of the residual milk remaining in the system or between chemical washes to remove residual cleaning chemicals. The temperature of these water rinses is usually between 38 °C and 55 °C. The upper limit has been specified in the belief that proteins may be 'baked' on to surfaces at higher temperatures. The lower limit is set above the melting point of milk fat to ensure that fats will be removed and not redeposited. A second benefit of the initial rinse is to warm the equipment so as to reduce the temperature drop during the subsequent cycles. This benefit is marginal, however, when the subsequent cycles are not started within 5 min after the rinse. These rinses should not be recirculated. Water rinses are also recommended after every chemical cycle to remove residues from the milking machine.

Alkaline Detergent

Chlorinated alkaline detergents are used to remove organic soils such as milk fat and proteins. Most detergents have a working temperature range between 43 and 77 °C, but there are also low-temperature formulations available. The cleaning effectiveness of detergents improves as temperature is increased and as water hardness decreases. The detergent concentration must be adjusted to account for these factors.

Acid Rinse

An acid rinse cycle may be performed to remove mineral deposits from water and milk. This may be a cold or warm rinses. The required frequency of acid rinses depends on the quality of the water used for cleaning.

Acidified Boiling Water

The acidified boiling water method is used in some parts of the world. An acid detergent solution is used at a temperature of nearly 100 °C. The wash solution makes

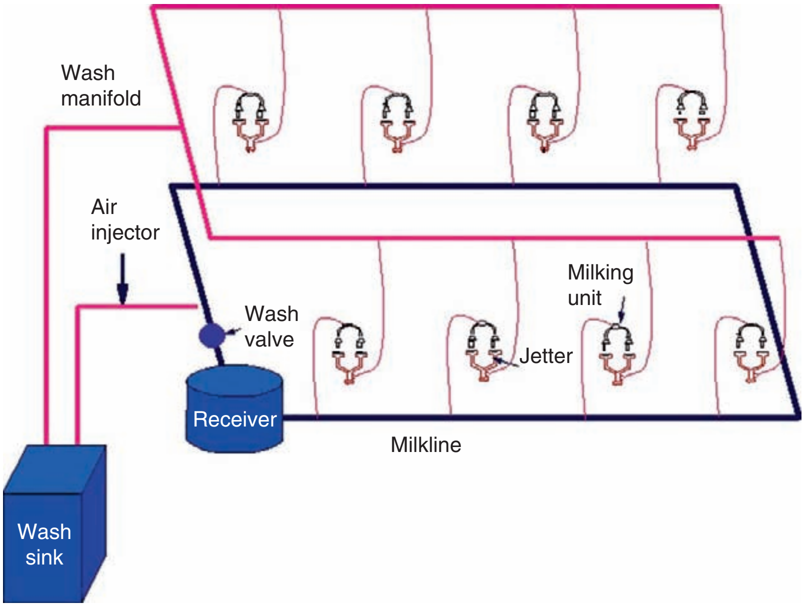

Figure 2 Basic cleaning-in-place (CIP) system for milking parlors.

a single pass through the system and is not recirculated. The objective is to maintain all surfaces at a temperature above 77 °C for at least 2 min. This method replaces the chemical action of alkaline detergents with intensified thermal action. It requires special equipment to achieve elevated water temperatures and milking system components that can withstand these high temperatures.

Chlorine Sanitizers

Chlorine-based sanitizers are circulated immediately before milking to kill bacteria that have survived the cleaning process. This premilking sanitizing step is performed on milking machines that stand idle for several hours between the previous wash cycle and the subsequent milking. A premilking sanitize is not practiced in many areas where a sanitizer is combined with either the detergent or acid rinse cycles and where water rinsing after each chemical treatment cycle is enforced.

The chemical processes described above are used in various combinations around the world. The most common routine in the United States is a combination of: prerinse, alkaline detergent, acid rinse (frequency depending on water hardness), and premilking sanitize. In The Netherlands and many other European countries, the standard cleaning of milking equipment consists of three phases: prerinse, combined alkaline detergent and chlorine sanitizer, followed by a postrinse. A common practice in New Zealand is an acidified boiling-water wash (alternated periodically with alkaline detergent) followed by a cold water rinse. A conventional Nordic procedure is composed of prerinse, alkaline detergent, cold water rinse, acid rinse, and then a pure water rinse immediately before milking.

The choice of which cleaning regime is used depends strongly on the habits and regulations of each country, the relative cost of energy for heating water, chemical cost and availability and effectiveness of the process.

Mechanical Action

Assuring an adequate mechanical cleaning action typically requires very little added cost but relies on the skill of the equipment installer. As milking machines become more complex the task of assuring adequate mechanical cleaning action in all parts of the milking machine becomes increasingly complex.

Milklines must be sloped between 1% and 2% toward the receiver jar to prevent slugging in milklines during milking. All pipelines, hoses and components must also be installed so that they will drain by gravity between cleaning cycles. Drainage is an important aspect of cleaning, because any standing water in the system increases the risk of bacterial growth between milkings and mixing of different cleaning chemicals during cleaning. Different mechanical processes are discussed.

Manual Cleaning

Simple milking machines such as bucket milkers are typically disassembled and cleaned by hand. Even in the most complex machines using circulation cleaning, however, there are still components that must be disassembled and cleaned by hand. The source of the mechanical cleaning action is typically a brush used to remove milk solids deposits. The use of extended contact times (soaking parts in a sink) is also widely practiced for small components.

Flooded Flow

The milk transfer line (from the milk pump to the wash sink) is cleaned in a flooded condition with the milk pump providing the fluid velocity. Milking machines with milkline diameter less than 48 mm have used flooded flow to circulate cleaning solutions through milklines. Flooded flow is also encountered in components such as hoses in milking units in which internal diameters are small. The desired flow velocity for flooded components is about 3 m s^{-1}.

Milk pump capacity is often the limiting factor in CIP systems. The distribution of water flow between units should be as uniform as possible to make the most efficient use of water and air when cleaning milking equipment and to avoid exceeding the capacity of the milk pump. Adding flow restrictors is an effective way of optimizing water flow rates for cleaning and balancing the flow between units. Systems with milk meters or weigh jars require 4.5 to 6 l min^{-1} for effective cleaning.

Steady Air Admission

The amount of water required to fully flood a milking system with very long and/or large diameter milklines is impractical. The power available to achieve adequate flow velocity is also limited with the equipment available as part of the milking machine. Air admission has been used to produce two-phase (air/water) flow and overcome these limitations. Steady air admission is practiced on some small-diameter milklines (<48 mm) to reduce the required water volume and increase flow velocities. Some milking units and milk meters are also designed to introduce steady air admission during cleaning. This steady air admission will improve mechanical cleaning action slightly but makes balancing water flow through units more difficult and increases the vacuum pump capacity required for cleaning.

Cycled Air Admission and Slug Flow

Cycled air admission is commonly practiced on milking machines with milkline diameter of 48 mm or greater. The objective in air-injected flow is to form a 'slug' of cleaning solution and move this slug around the entire pipeline. The slugs may vary from a few centimeters up to several meters in length. The area between the slugs contains a slower-moving liquid film in the bottom of the pipe. The slug velocities developed with air injected two-phase flow can be three to five times higher, and the wall shear stress developed 10 to 20 times higher than those in flooded CIP circuits. The contact time between the slug and pipe wall is significantly reduced, however.

Slug velocities of 7–10 m s^{-1} maximize the wall shear stress developed while minimizing the variation of shear stress along the pipe. The rate of air admission to the milkline should be controlled to achieve these slug velocities. Air admission rates above this maximum will result in reduced slug density and reduced mechanical cleaning action in the milkline.

Spray Cleaning

Bulk tanks and other large vessels are cleaned by covering their inner surfaces with a sprayed sheet of water. The mechanical action and surface temperatures are significantly lower than for circulation cleaning. The chemical concentrations and wash water temperatures are therefore of critical importance for successful cleaning.

Very small herds (fewer than 30 cows) tend have a high degree of manual cleaning and disinfecting. This usually involves hand cleaning of some or all of the milk harvesting and storage equipment. Small to medium herds (30 to 500 cows) commonly use automatic washing equipment. This equipment will automatically mix the chemicals with the appropriate water volume and temperature and circulate these solutions through the milking machine. On large farms (1000 cows or more), an attendant may be present to mix chemical solutions and operate valves for circulation.

Environmental Issues

There are several environmental issues that are beginning to influence cleaning practices in some parts of the world. The discharge of cleaning and disinfecting chemicals into the environment can be an issue, with elements such as phosphorous and chlorine. The uses of enzyme cleaners and ozone sanitizers has been investigated but in general these have not reached the market yet.

The volume of water used for cleaning and discharged into the environment can be an issue in areas with water shortages. The energy used for heating and pumping water also has environmental implications. Various methods to reduce water volume, chemical volume and energy requirements for cleaning through efficient system design and control and through reuse of cleaning solutions have been reported.

Cleaning Assessment Methods

Visual Inspection

Cleaning failures usually result in a visible build-up or residual film on some part of the milk harvesting or storage equipment. Parts that can be controlled are for example: milking claws, milk production meters and the milkline, especially at an end of a line.

Some of these residual films have a characteristic appearance, which can help determine the cause of the cleaning failure. There are two broad categories of residual films: organic films such as fat and protein, and inorganic films such as hard water minerals, iron and silica. Discoloration may also occur due to corrosion and/or pitting of surfaces. Protein films can appear as a brownish slime ('applesauce') when wet. Mineral films usually have a rough porous texture and are invisible when wet. Organic films are generally alkaline soluble whereas inorganic films are generally acid soluble. Protein films are soluble in chlorine. Films can be diagnosed by scrubbing a small area with concentrated acid and/or detergent solutions.

Bulk Milk Cultures

Some form of testing for bacterial contamination is done periodically on all farms to assure compliance with national, state and local requirements.

The most common method used to assess the bacterial quality of raw milk is the standard plate count (SPC) or total bacteria count (TBC). These are broad-spectrum tests that do not identify the types of organisms present. These tests provide an overall measure of milk quality but they have little diagnostic value in determining the source of bacterial contamination.

ATP Measurements

Cleanliness of the equipment can also be tested by ATP measurement. ATP produced by bacteria or organic residues can be determined by using special swabs with reagents. Measuring light emission gives an indication about the hygiene in the equipment.

See also: **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens. **Mastitis Therapy and Control**: Management Control Options. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Milking Machines**: Principles and Design; Robotic Milking.

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Influence on Free Fatty Acids

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Introduction

Around 60–70% of the free fatty acid (FFA) concentration in the final pasteurized milk product is produced during milking and milk transfer to the bulk tank. Therefore, increased FFA concentration is one of the most frequent farm-related defects in drinking milk. FFAs are formed by the hydrolysis of triglycerides by the enzyme lipoprotein lipase (LPL). Large concentrations of FFAs result in the appearance of rancid off-flavors in dairy products. The rancid flavor developing from lipolysis in milk is often described as gouty or soapy. The sensory threshold for lipolytic rancidity in milk is not precisely established, but it is estimated to be in the range of 1–1.5 meq per 100 g fat, because the development of rancid flavors is greatly affected by the composition of the FFA. It is mainly the fatty acids with a chain length of 4–12 that contribute to the rancid flavor; for example, the concentration for off-flavor threshold of C_{14} is more than 10 times that for C_4.

Since the milk quality payment globally focuses on criteria such as freezing point and bacteria and somatic cell count, milk producers do not pay much attention to the problems of accumulation of FFAs in the milk. However, in the Netherlands, the farm milk is tested twice a year for the level of FFAs, and if the concentration is above 1.0 meq per 100 g fat, a reduction in payment is executed.

Lipolysis

LPL is the enzyme mainly responsible for lipolysis in raw milk. It originates from the mammary gland, where it is involved in the uptake of blood lipids for milk synthesis. The enzyme is active in lipid–water interfaces. Its optimum temperature is 33 °C and pH optimum is about 8.5. It is a relatively heat-labile enzyme and is mostly inactivated by a high-temperature short-time heat treatment. In milk, LPL is mainly associated with the casein micelles. LPL is brought into contact with the triglycerides when the milk fat globule (MFG) membrane is disrupted and casein coats the formed lipid–water interface. The enzyme is activated by apolipoprotein CII from the blood, which assists LPL to bind onto the fat globule. In spite of the high amount of LPL in milk, lipolysis is limited since milk fat is protected by the membrane and raw milk is normally stored at temperatures far below the optimum temperature of LPL. Furthermore, the products formed by the hydrolysis of triglycerides, the FFAs, inhibit the enzyme, presumably by binding to the enzyme.

The activity of LPL in whole milk is not correlated to the FFA content in raw milk. However, studies have shown that the activity of LPL in the cream fraction is related to the level of lipolysis. Consequently, the formation of FFAs is assumed to be dependent on the susceptibility of MFG to the action of lipases. LPL preferentially hydrolyzes fatty acids in positions sn-1 and sn-3. The fatty acids placed at high frequencies in positions sn-1 and sn-3 are C_4, C_6, C_{18}, and $C_{18:1}$. The development of a rancid flavor in milk is greatly affected by the composition of FFAs. It is mainly the fatty acids with a chain length of 4–12 that contribute to the rancid flavor.

Lipases synthesized by bacteria such as *Pseudomonas* and *Bacillus* species can also be developed in milk. Flavor defects can be detected in milk only when the bacterial counts reach approximately 10^6–10^7 CFU ml^{-1}. Therefore, if milk is properly stored and has an acceptable hygienic quality, microbial lipases are not an important factor for lipolysis until after several days of storage.

The classic differentiation of lipolysis in milk is between spontaneous and induced lipolysis. The factors affecting spontaneous lipolysis include milking frequencies, udder health, and stage of lactation. Induced lipolysis is caused by homogenization, pumping, and temperature fluctuations.

Factors Affecting FFAs in Milking Systems

Air Intake

In milking systems, the milk is mixed with air, especially when air is used as a transport medium for the milk. The stability of the MFG is lowered by mixing with air or any other gas during pumping or agitation of the milk. The contact between an MFG and an air bubble results in rupture of the MFG, since the membrane material and part of the core fat will spread over the air/milk plasma interface and will be released into the milk plasma when air bubbles collapse or coalesce. The total air admission in the cluster should be between 4 and 12 l min^{-1}; however, in automatic milking systems (AMSs), it is often higher. Increasing air intake has great impact on the formation of FFAs, and even if milk and air are mixed only for a short pumping distance, it causes damage.

Pumping

Mechanical treatments of the milk such as pumping and stirring subject MFG to physical stress. Higher flow velocities during pumping in pipes result in a greater friction in the liquid itself and between the liquid and the pipe wall. These relative differences in flow velocity perpendicular to the flow direction are called shear rates. The shear rate depends on the diameter of the pipe and the flow velocity. The presence of air, the temperature of the milk, and the fat content affect the stability of the MFG during mechanical treatments of milk.

The milk temperature is also a very important factor when milk is exposed to mechanical treatments. Several studies have reported that the maximum accumulation of FFAs upon agitation is at a temperature of ∼15 °C and again after ∼30 °C, with low formation of FFAs between 20 and 30 °C. At low temperatures, the milk fat is more resistant to mechanical stress, since large parts of the triglycerides in the MFGs are crystallized, and this stabilizes the globules against coalescence. One minor factor is that the temperature affects the activity of LPL.

The average diameter of MFGs can be affected through the feeding of cows. Diets with high levels of saturated fat supplements result in high milk fat production and in MFGs with large average diameters. These milk types with large MFGs are more susceptible toward coalescence and lipolysis during pumping compared with milk from cows fed a low-fat diet or unsaturated fat supplements. The reason for the lesser stability of large milk globules during pumping is presumably that the surface potential is lower for large globules than it is for smaller globules. Therefore, less energy is needed to coalesce large globules, and at the same time the collision energy is greater for large MFGs.

Figure 1 shows that a cooling system consisting of a plate cooling exchanger placed in the milking parlor close to the milking unit significantly decreased the formation of FFAs in the milk. Moreover, the milk flow in the milking systems was controlled by a variable frequency pump in order to control the cooling more precisely and economically. The figure indicates that the right cooling strategy in a milking system can improve the milk quality regarding lipolysis.

Milking Frequencies

One advantage of AMSs is that the cows are more frequently milked, since they voluntarily attend the robot. Increased milking frequency enhances the milk yield; for example, with thrice-daily milking, the milk output is increased by ∼18% when compared to twice-daily milking. The average milking frequency in AMSs is between 2.4 and 2.6 per day. This implies that a large share of cows attend the milking robot 3 or 4 times per day. **Table 2** shows that one drawback of increasing milking frequency to 3 or 4 times a day is a higher concentration of FFAs in milk. In the studies presented in **Table 1**, the effect differs among studies, which can be ascribed to different analytical methods; however, the trend of an increased concentration of FFAs upon increased milking frequency is clear.

A study in our laboratory demonstrated that the difference in the concentration of FFAs is not present immediately after milking, but only after 24 h of storage,

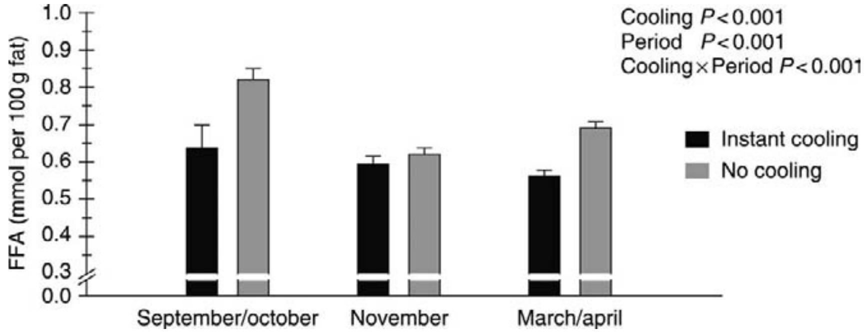

Figure 1 The effect of cooling of milk in the milking parlor compared with no cooling. The milk was cooled down to 4 °C by a plate heat exchanger before being further pumped to the bulk tank. The milk flow in the milking systems was controlled by a variable frequency pump in order to control the cooling more precisely and economically. The samples were collected just before the inlet to the bulk tank by a small separation pump, which was regulated by the main milk pump frequency, so the sample represents milk from the whole milking session of 120 Danish Holsteins cows. The distance between the milking parlor and the bulk tank was only 22 m. Samples were collected during three periods. The concentration of FFAs was analyzed 24 h after milking by using the BDI method. Reproduced with permission from Wiking L and Nielsen JH (2007) Effect of automatic milking systems on milk quality. *Journal of Animal and Feed Sciences* 16(supplement): 108–116.

Table 1 Effect of milking 3 or 4 times a day compared with 2 times a day on FFA concentration in milk

	2×	*3×*	*4×*	*Year of study*
FFA (meq per 100 g fat)	1.14		1.49	2006[a]
	0.72	0.99		1997[b]
	0.42	0.71		2004[c]
	0.33	0.44		1986[c]

[a]Used the autoanalyzer method.
[b]Used the copper soap method.
[c]Used the BDI method for FFA content analysis.

which indicates that the increased concentration of FFAs in milk is caused by a weakness of the MFG membrane. We also observed that the average diameter of MFG was significantly larger in milk obtained by milking 4 times than in milk obtained by milking two times. This could be one of the explanations for the weakness of the MFG membrane.

Cool Storage in the Milk Tank

Cooling

The initial FFA concentration in fresh drawn milk is around 0.15–0.5 mmol per 100 g fat. During cool storage of the milk in the tank, the concentration of FFAs increases. The increase in the concentration of FFAs is largest during the first 24 h after milking. The cooling itself renders plasma proteins to adsorb to the MFG membrane. Hereby, the LPL is brought into contact with the lipids, since it is bound to the caseins. Furthermore, crystallization of the triglycerides will occur, which will result in some structural changes of the MFG membrane. The crystallization range for milk fat is broad due to the large variety of triglycerides. Crystallization begins from the edges moving inward to the core. The membrane mainly consists of phospholipids, which are unsaturated and therefore crystallizing only at very low temperatures. However, volume changes caused by crystallization of the core fat will affect the structure of the membrane. In addition, some proteins and Cu are released from the membrane to the serum phase during cooling. On the whole, the composition and the structure of the MFG membrane are altered when raw milk is subjected to cooling. The relation between the structural changes of the MFG membrane during cooling and the access of LPL is not clearly understood (*see* **Milk Lipids**: Lipolysis and Hydrolytic Rancidity).

Temperature fluctuations in the bulk milk tank are a risk if the milk is not precooled before it enters the tank. Mixing warm fresh milk with cold milk from the previous milking results in a fast temperature increase before cooling starts again. Temperature fluctuations in milk can cause major increases in the concentration of FFAs. In some modern milking systems, the milk is precooled in a plate cooling exchanger or buffer tank, so temperature fluctuations are avoided in the bulk milk tank. This will also reduce energy costs of cooling in the bulk tank.

Stirring

A wrong dimension of the stirring unit in the bulk tank results in inexpedient mechanical treatments of milk, which again leads to churning of the MFGs and thereby susceptibility of fat to lipolysis. Incorporation of air into milk using wrongly dimensioned stirring unit also contributes to the rupture of MFGs. The problem is presumably largest when the milk volume in the tank is low and the stirring unit therefore touches only the milk surface.

FFAs in Milk from Different Milking Systems

High pipeline milking replaced bucket milking in stanchion barns many years ago, but lifting of the milk from the udder to the milkline requires inlet of air in the claw. Milking parlors and carousels (*see* **Milking Parlors**) are normally equipped with milklines mounted below cow level and milk flows directly from the cow into the milkline without air bubbling into the milk. **Table 2**, which is from a large Danish study, shows that milk from automatic milking and high pipeline systems contains greater concentration of FFAs than milk from systems with low lines. Similar observations are made regarding greater concentrations in milk from automatic milking in other West European countries since the introduction of the AMS, and this problem is still not solved. In the following section, the possible factors causing higher level of lipolysis in automatic milking are described.

Automatic Milking Systems

A recent survey of AMSs in Denmark showed that the most frequent faults in AMS herds with large concentrations of FFAs in bulk milk were stirring (79%), pumping (67%), and cooling of milk (58%). The milk flow into the bulk tank in AMSs is different from that in conventional systems due to the continuous flow of milk all day instead of just from two milking sessions. This also means that in some periods, such as after collection and cleaning, the milk volume in the tank is low. In some tanks, the stirring unit and cooling capacity are wrongly dimensioned in relation to a slow inlet of milk, causing churning of MFGs and mixing with air. On the other hand, too fast jacket cooling causes freezing of the milk to the shell of the bulk tank, which damages the MFG membrane. Furthermore, some AMSs have a buffer tank with a capacity to store up to 300 l milk, where the milk is precooled

Table 2 Concentration of FFAs in milk from different milking systems

Milking systems	*Number of herds*	*FFA (meq per 100 g fat)*
Automatic milking	219	0.889
Pipeline in traditional cow houses	1339	0.888
Milking parlor	1618	0.615
Rotary parlor	99	0.585

Reproduced from Danish Dairy Board, 2007.

before it is pumped into the bulk tank. However, during periods with low milking intensity, there is a risk that the milk may be stored in the buffer tank for a long time.

As mentioned earlier, the milking frequency in AMSs is often higher than that in other systems, and this fact contributes to the large concentration of FFAs in the milk. AMSs have greater air intakes than conventional milking systems since the milking units of AMSs are individual quarter milkers with rather long 'short milk tubes', individual shutoff valves, foremilk separators, and different kinds of flow meters, and the milk normally has to be lifted several times before it enters the receiver. Such technical constructions may require different amounts of air for transportation of the milk compared to conventional milking units.

High Pipeline Milking Systems

In high pipeline systems, high air intake from the claw or the pumps is also a main reason for a relatively high accumulation of FFAs in milk. Furthermore, the milk is often lifted in the pipes above cow level, which increases the pressure on the MFGs and thereby increases the degree of damages. A continuous flow of milk in the milkline to the bulk tank instead of a controlled milk flow is also a factor that increases FFA concentration in the milk. The most frequent faults in conventional herds in a Danish study were leakage (71% of the herds) and intake of too much air in the cluster (61%), whereas pumping and stirring faults occurred on 29% of the farms.

Many of the high pipeline milking systems used today are rather old and have leakages into the milk stream. This may also be an explanation for the fact that the average FFA concentration in milk from high pipeline systems is high in surveys comparing milking systems. As a consequence of better animal welfare conditions, the number of stanchion barns with high pipeline systems is decreasing at the expense of free barn systems with milking parlors.

Conclusion

A decrease in the number of farms with high pipeline milking systems will improve milk quality pertaining to FFAs, but the increasing number of AMSs may increase the risk for greater concentrations of FFAs in silo milk. Thus, the margin to the threshold of rancid flavor of milk becomes smaller.

The factors that contribute to elevated FFA concentration are virtually elucidated. Therefore, technical solutions such as correct cooling and gentle mechanical treatment could be implemented in modern milking systems.

Generally, in the future, more attention should be given to the effect on FFAs during development and implementation of new milking technologies.

See also: **Milk Lipids**: Lipolysis and Hydrolytic Rancidity. **Milking Machines**: Robotic Milking. **Milking Parlors**.

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Effect of Storage and Transport on Milk Quality

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Introduction

Never has there been a time when the quality and safety of our milk supply have been more critical. Like most perishable foods, the quality of finished dairy products begins with that of the raw milk. The quality of the raw milk received by the processing plant depends on a number of key factors, such as

1. health of the animal;
2. feed consumed;
3. milking conditions and procedures;
4. cleanliness of milking equipment, lines, and bulk tank, and freedom from residual sanitizer;
5. temperature control from bulk tank through to the silo storage tank at the processing plant;
6. cleanliness of tanker, lines, and silo; and
7. good handling practices at all points in the process.

In the United States, the usual way for raw milk to be handled is for the milk to be pumped from the milking parlor to the bulk tank. The product is then cooled rapidly to 7 °C or lower within 2 h of milking. The legal maximum temperature at which milk can be maintained, either raw or pasteurized, is 7 °C. Milk is picked up by a bulk hauler on a daily or every-other-day basis. It is transported mostly in tank trucks with a storage capacity of typically 6200 gallons (can be larger or smaller). The milk can then be taken (1) directly to a dairy processing plant, depending on the type of dairy food being produced at that facility; or (2) to a transfer or pump-over station. The trend in the United States appears to be more toward milk being transported directly to the processing plant. This trend is based on several factors such as a shift toward larger but fewer dairy farms and dairy processing plants, higher transportation costs, and fewer but larger cooperatives. This basic process is outlined in **Figure 1**.

Assuming that milk is taken directly to the dairy processing plant, several quality and safety tests should be run on the milk before unloading it from the tank truck. Other tests are then run on the milk after unloading it, but before it is processed. Raw milk is pumped into refrigerated silo storage tanks, where it is maintained at temperatures that are normally lower than 4 °C. The sizes of these silo tanks typically range from 10 000 to 80 000 gallons or more (tanks of 200 000-gallon capacity are not unknown) for some plants that manufacture dairy products such as cheese. It is important that the haulers start their pickup route each day with a tank truck that has been thoroughly cleaned. The trucks are cleaned after the milk is removed. The trucks are usually cleaned and sanitized at the dairy plant (but not in the receiving area) to which the milk was delivered or at an intermediate wash station. At either place, the truck must be tagged to indicate that this cleaning has indeed occurred. This process is critical to ensure the supply of high-quality raw milk.

Raw Milk Testing

While most dairy processing plants have their own lists of tests to be routinely performed on the raw milk received by them, there are tests that should be done to ensure a safe and high-quality milk supply. A suggested listing of these tests follows and this has been arbitrarily divided into tests to be performed 'prior to unloading' the milk and 'after unloading' the milk. The latter tests are also classified as 'troubleshooting' tests (**Tables 1** and **2**).

With regard to the tests to be run before unloading, the direct microscopic count (DMC) appears to be an old-fashioned laboratory technique; however, it is probably the only economical test currently available to processing plants that can estimate the microbiological quality of milk in the time allowed prior to tanker unloading. To facilitate milk unloading, all testing needs to be completed within 30–45 min. Other technologies are being developed and optimized, but the DMC has an advantage of low cost per sample. Technician error can be minimized by training and attention to detail. The DMC shows living and dead cells, which normally results in higher counts than the standard plate count (SPC) when run on the same sample.

There are several screening tests designed to detect the presence of inhibitory substances, especially antibiotics. No milk should be accepted by a dairy processing plant unless a 'negative' is received. If a 'positive' is obtained, state or federal regulations must be followed, to ensure that contaminated milk does not get into products offered for sale to the consuming public.

While temperature measurement is very simple, decisions as to whether or not to accept the milk on the basis of a temperature reading can sometimes be difficult. This is especially true in times of a reduced supply of milk. One needs to remember that as the temperature of milk increases, even slightly, the generation time of the bacteria present is decreased. The psychrotrophic

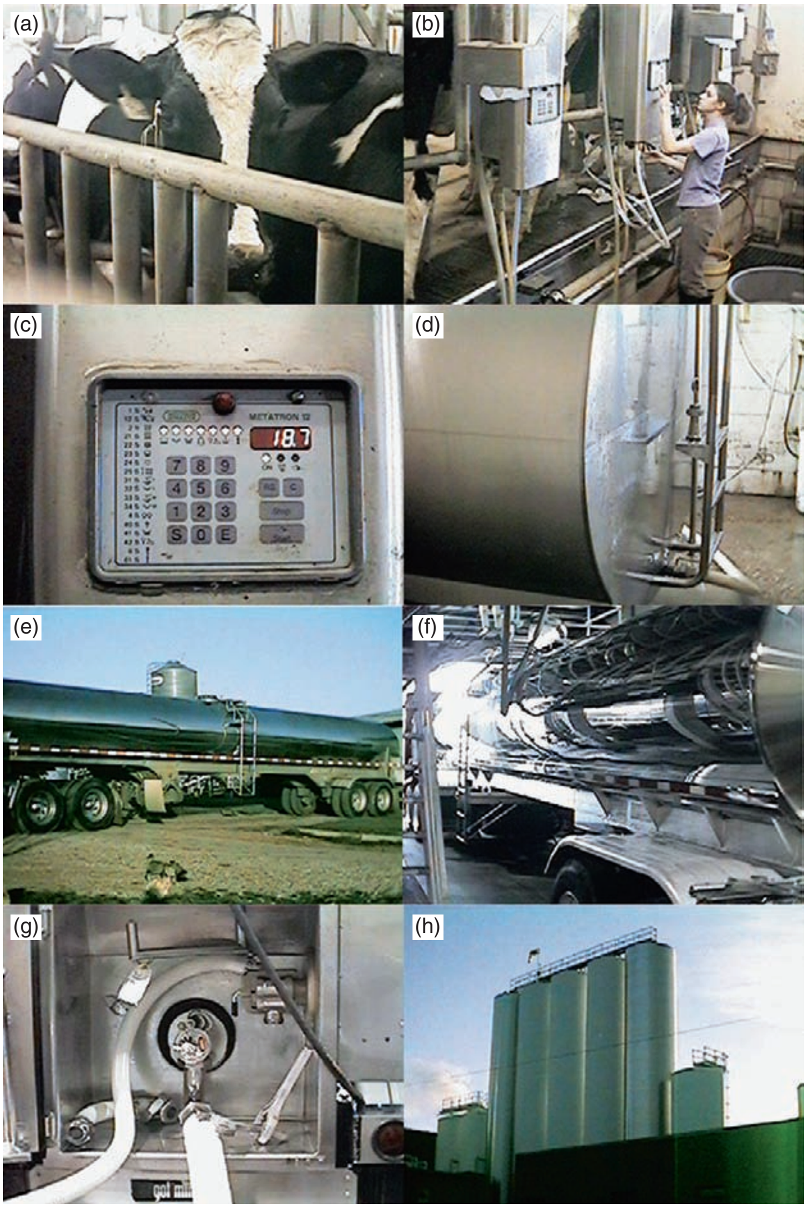

Figure 1 Typical flow of milk from dairy farm to processing plant appearing left to right in rows: (a) cows; (b) milking parlor; (c) milk meter in parlor; (d) bulk tank in milk room of parlor; (e) tank at farm; (f) tank truck at processing plant; (g) off-loading milk at processing plant; and (h) milk storage silos.

Table 1 Tests to be run on all raw milk prior to unloading from the tanker

	Test	*Standard (goal)*
1	Direct microscopic count (DMC)	<100 000 clumps ml^{-1}
2	Presence of inhibitory substance (antibiotic)	None detected with screening test
3	Temperature	Legal = .7 °C; desired = .4 °C
4	Freezing point (cryoscope)	Legal = lower than −0.530 °C
5	Sensory evaluation	No off-odors or off-flavors
6	Sediment test	('2' (1.50-mg equivalent) or '1')
7	Preliminary incubation count (PIC)	<100 000 cfu ml^{-1}

Table 2 Tests to be run on raw milk after unloading at the processing plant (and 'troubleshooting' tests)

	Test	*Standard (goal)*
1	Standard plate count (SPC) (only on 'high' DMC loads)	<100 000 cfu ml^{-1}
2	Direct microscopic somatic cell count (DMSCC)	Legal = <750 000 ml^{-1}
3	Acid degree value (ADV)	<0.90
4	Laboratory pasteurization count (LPC) (troubleshooting only)	<500 cfu ml^{-1}
5	Fat and total solids	NA
6	Coliform	<200 cfu ml^{-1}
7	Presence of aflatoxins	<0.5 ppb
8	HR-3 (test for Gram-positive bacteria)	Insignificant number of Gram-positive bacteria
9	Titratable acidity	>0.17%

bacteria – bacteria that are capable of fairly rapid growth at refrigeration temperatures – increase dramatically as the temperature is increased even from 4 to 7 °C.

Checking the freezing point of the raw milk indicates whether or not water has been added (either deliberately or inadvertently). Typical cryoscope values for tanker milk fall between −0.530 and −0.550 °C. Higher-solids milk yields values closer to the −0.550 °C end of the range. The closer the value gets to 0 °C (the freezing point of water), the more it proves that water has been added to the milk. These values can be calculated on a percentage basis.

Flavors (Taste or Odors) of Raw Milk

Desirable sensory properties are essential for high-quality raw milk. The milk should be checked for off-odors while still in the farm bulk tank. At the processing facility, the receiving room personnel should be the only ones to open the dome lid and carefully check for odors. The tanker should have been cleaned prior to picking up the milk. When the samples are being taken (aseptically) for acceptance testing, a sample can actually be laboratory pasteurized (heated up to 73–75 °C for 15–20 s), then cooled rapidly to 21–32 °C, and tested for the presence of off-flavors. This is especially critical for milk that has a suspicious odor. A 'feed' flavor is normal but should not be too strong or high in intensity. Other flavors that may be encountered occasionally are listed in **Table 3**.

While there are other off-flavors that may rarely be found in raw milk, those described above are probably the ones most commonly seen.

The sediment test can be run at the receiving plant by pouring 470 ml of raw milk through a white disk and checking for the amount of residue, that is, the sediment retained on the disk. This is a measure of the care taken during milking and pumping of the milk from the farm. While the milk is either run through a 'sock' filter as it is being pumped to the silo tank or run through an in-line separator before pasteurization, the presence of sediment in milk is unacceptable and should be avoided.

The SPC is simply a measure of the aerobic bacteria capable of growth on plate count agar at 32 °C. It is not a 'total' count but is a good indication of the 'total' microflora present in the milk sample. The SPC is accepted as an 'official' estimate of the microbial quality of a sample of raw milk. The problem in using it as a screening test for raw tank milk is the fact that the plates must be incubated for 48 h before the results are known; hence, we have the most logical reason for using the DMC. When an unacceptable DMC is obtained, the SPC should be run on that tanker for documentation purposes. In the United States, the legal maximum for individual producer milk is 100 000 cfu ml^{-1}, and for commingled milk it is 300 000 cfu ml^{-1}.

The direct microscopic somatic cell count (DMSCC) is the standard method for estimating the number of somatic cells in raw milk. This count provides a fairly

Table 3 Off-flavors occasionally encountered in raw milk

Flavor	*Cause*
Malty (do not accept)	Bacterial – normally traced to refrigeration problems at farm
Acidic (do not accept)	Bacterial – normally traced to refrigeration problems at farm
Onion (do not accept)	Feed-related
Flat (if definite, do not accept)	Mostly related to water getting into milk
Light-activated (do not accept)	Milk exposed to sunlight (ultraviolet light)
Rancid (do not accept)	Normally excessive pumping with air incorporated into milk; can be bacterial
Sanitizer (do not accept)	Excessive sanitizer in lines, bulk tank (or dipper)
Unclean (do not accept)	Primarily psychrotrophic bacteria

accurate picture of the health of the cows, especially with regard to bovine mastitis.

The acid degree value (ADV) is actually a measure of the free fatty acids present in the raw milk. If rancidity is a problem, incoming loads of raw milk should be screened with the ADV test since butyric acid is the fatty acid commonly associated with rancidity. The range of ADVs and how they should be interpreted are given below:

$$<0.7 = \text{normal milk}$$

$$0.7-1.1 = \text{slightly hydrolyzed}$$

$$1.2-1.4 = \text{definitely hydrolyzed}$$

$$>1.4 = \text{extremely hydrolyzed}$$

The laboratory pasteurization count (LPC) is a good test to run when shelf life problems are occurring and there is a need to know more about the quality of the incoming raw milk. The LPC indicates whether or not there are significant numbers of pasteurization-resistant bacteria present in the raw milk. When the LPC exceeds 500 cfu ml^{-1} or especially 1000 cfu ml^{-1}, there is a definite thermoduric bacterial problem in the raw milk.

The HR-3 test is a color-reaction test designed to estimate the number of Gram-positive bacteria in raw milk. Many Gram-positive bacteria can survive pasteurization. With the increased emphasis on the extended shelf life (ESL) of fluid milk, it becomes critical to know if certain raw milk samples have high numbers of Gram-positive bacteria that can cause shelf life problems when the milk is in the later stages of storage or distribution.

The fat, protein, and total solids content of the raw milk must be accurately measured. This importance is not just from a payment (to the farmer) standpoint, but – in the case of cheese plants – a major factor in calculating the cheese yield. Rapid and accurate measurements are readily available with the laboratory instruments being used. Regular maintenance and calibration make this critical area controllable.

While coliforms are not a legal standard in raw milk, in most states in the United States, they do give valuable information as to the sanitary methods by which the milk was taken from the cow, stored at the farm, and transported to the processing plant. Coliforms are 'indicator' organisms, in that their presence in the milk indicates that conditions are suitable for the presence of enteric pathogens. The legal standard for coliforms in pasteurized milk in the United States is <10 cfu ml^{-1}. A suggested standard in raw milk might be <200 cfu ml^{-1}.

The molds *Aspergillus flavus* and *Aspergillus parasiticus* produce a group of highly toxic secondary metabolites that are referred to as aflatoxins. These molds may grow on wet feed or grains and produce the aflatoxins, which could be consumed by the cow and thereby gain entrance into the milk. Raw milk should be routinely screened for the presence of aflatoxins owing to the inherent danger to humans consuming the milk.

Microbiology of Raw Milk

The microorganisms found in raw milk may come from several sources: the milk as it is excreted from the teat, the environment (water, soil, etc.), milking equipment, milk lines, farm bulk tank, the tank (transfer) truck, lines and pumps at the processing plant, and the raw storage silo tanks at the processing plant. Cows with mastitis can shed high numbers of bacteria and somatic cells. An elevated somatic cell count (SCC) can adversely impact milk quality.

According to the International Commission on Microbiological Specifications for Food (ICMSF; 2000), "The normal flora of the udder includes streptococci, staphylococci, and micrococci (normally >50%) followed by *Corynebacterium* spp., *Escherichia coli*, and others."

The main types of aerobic mesophiles include micrococci, streptococci, and asporogenous Gram-positive rods, such as *Microbacterium*, *Corynebacterium*, and others; spore-formers, *Bacillus* spp.; Gram-negative rods, such as *Pseudomonas*, *Flavobacterium*, and others; and miscellaneous, such as yeasts and molds. In reflecting on the various microorganisms in raw milk, the ones with which we are most concerned are those bacteria that can survive the pasteurization process, that is, the thermodurics, and those bacteria that can grow, although slowly, at refrigeration temperatures, that is, the psychrotrophs.

Psychrotrophs that have been isolated from raw milk include the following genera: *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Enterobacter*, *Alcaligenes*, *Achromobacter*, *Klebsiella*, *Aeromonas*, and *Bacillus*. Genera of thermoduric bacteria found in raw milk are *Microbacterium*, *Micrococcus*, *Alcaligenes*, *Bacillus* (spore-former), and *Clostridium* (spore-former). Several genera are both thermoduric and psychrotrophic. These bacteria are *Bacillus*, *Clostridium*, *Microbacterium*, *Micrococcus*, and *Corynebacterium*. In general, the psychrotrophs are Gram-negative bacteria, with the thermodurics tending to stain Gram-positive. The psychrotrophs can be very detrimental to milk quality, especially as related to heat-stable enzymes produced by many of these psychrotrophic bacteria. Many of the proteases produced by common psychrotrophs, such as *Pseudomonas*, are extremely heat-stable and can degrade the milk proteins especially upon prolonged storage of raw milk. Consequently, even though the bacteria may be killed upon pasteurization of the milk, these enzymes can easily survive and cause decreases in shelf life, quality, and yield (of cheese). Researchers have shown that protease from *Pseudomonas fluorescens* P26 retained 71% of its original activity after being heated at 71.4 °C for 60 min. When the enzyme was added to milk, both cottage cheese and Cheddar cheese were made. Significant proteolysis occurred in both products with a definite reduction in quality (especially flavor). It has been demonstrated that psychrotrophic development during milk storage adversely affects cheese yield and quality. Proteinase-producing Gram-negative psychrotrophic bacteria are often isolated from milk. The fact that psychrotrophs are present in raw milk, coupled with the facts that heat-stable enzymes may be produced and liberated in the milk by these psychrotrophs and these enzymes degrade the quality of milk and milk products, demands that raw milk be stored for no longer than necessary. The 72-h maximum time for a US dairy processor to hold milk prior to pasteurizing is justified and should not be violated. If anything, this time should be minimized. The two factors of temperature and time are constantly at work hurting milk quality. Unless the milk has been held at temperatures below 5 °C and comes into the processing plant at 5 °C or lower, its quality is in jeopardy. This is true since the generation times of typical psychrotrophs are only 9–18 h (obviously based on growth temperature). At any rate, the higher the level of psychrotrophs in the raw milk upon arrival at the processing plant, the higher the potential number of bacteria at the pasteurizer. Since high-temperature, short-time (HTST) pasteurization is a percentage kill, the higher the numbers in raw milk, the greater the numbers surviving.

The predominant contaminants of processed dairy products have their origins in the raw milk supply. While recognizing that there are many pathogens that could be linked to raw milk, some specific foodborne illnesses attributed to dairy products are caused by *Salmonella*, *Listeria monocytogenes*, and *Yersinia enterocolitica*. An important issue with *Listeria* and *Yersinia* is that they are both psychrotrophic pathogens. Major outbreaks have been caused by these bacteria, resulting in many illnesses and even deaths. Even though the milk is pasteurized before consumption, the potential for cross-contamination in the dairy processing plant stresses the importance of the raw milk being of high quality, to minimize these occurrences and the high numbers of pathogens. Some pathogens associated with raw milk are as follows:

Gram-Negative Pathogens:

Escherichia coli, Salmonella, Y. enterocolitica, Campylobacter jejuni, Aeromonas hydrophila (another psychrotrophic pathogen), and so on are Gram-negative pathogens.

Gram-Positive Pathogens:

Bacillus cereus, *Bacillus anthracis*, and *Clostridium perfringens* – spore-formers

Staphylococcus aureus, Streptococcus agalactiae, and Streptococcus pyogenes – cocci

Listeria monocytogenes, Mycobacterium tuberculosis, and so on – ‘other’

Rickettsia

Coxiella burnetii

Viruses

Enteroviruses, foot-and-mouth disease (FMD) virus, and so on

Fungi

Molds, such as *A. flavus*

Protozoa

Entamoeba histolytica, Giardia lamblia, and *Toxoplasma gondii*

While many of us have drunk raw milk, one cannot rule out the possibility that human pathogens could be present. The dairy industry compares very favorably with other segments of the total food industry regarding safety to the consumer; nevertheless, the industry must continue to be diligent in eliminating any known threat.

As a means of pinpointing the exact source of contamination of high-count milk from a specific form, an in-line sampling technique to measure the bacterial contamination of milk during milking has been studied. The in-line milk (ILM) sampler could be used in conjunction with appropriate culture media to compare bacterial contamination at the clawpiece (this would represent contamination from mastitic milk or milk at the teat surface), at the end of the milking pipeline, and in the final bulk tank. Improperly cleaned bulk tanks represent the major source of contamination, especially with regard to psychrotrophic bacteria. Studies indicate that the types and numbers of organisms introduced into the milk from milk handling equipment (including the bulk tank) depend largely on the efficiency of cleaning and sanitizing. When psychrotrophs are introduced into the milk, there is evidence that – generally within 2–3 days of the transfer of the milk from transport tankers – the microflora of the milk is dominated by psychrotrophs, whereas the thermoduric microflora does not increase and changes little in composition.

Researchers have found that storage at 4.0̣ 1 °C for 48̣ 2 h caused significant increases in the numbers of lipolytic and proteolytic bacteria ($p < 0.01$). The observed dominance of psychrotrophs after 2 days’ storage indicated their significance in bacterial counts in raw milk, when bulk storage at refrigeration temperature was practiced.

Realizing that psychrotrophs are in almost all raw milk, one must also accept the fact that spore-forming bacteria are present. It was reported that *Bacillus* species account for 95% of the total spore-forming bacteria in milk (*Clostridium*, 5%). This indicated that in the United States, 43% of the *Bacillus* bacteria are *B. licheniformis* and 37% are *B. cereus* (in other countries, *B. cereus* may be predominant). As there is more and more demand for the ultra-heat treatment (UHT) of milk and for ESL products, the presence of spore-forming bacteria takes on added importance. In the future, incoming raw milk should also be tested for an estimation of the numbers of spore-forming bacteria and the numbers of psychrotrophic bacteria. This must be in addition to the DMC – a rough estimation of the total number of bacteria present.

Typical Flow Pattern at the Processing Plant

After milk is pumped from the truck to silo tanks at a processing plant, a recommended practice is to pump the milk through a cloth filter (sock filter) as a way of reducing sediments in the milk. The normal process for 'processing' the milk after refrigerated storage (silo tanks) is as follows:

1. Separation and clarification – The separation process produces three fractions: skim milk, cream, and sediment (separator or clarifier >slime=). Clarification removes suspended particulate matter and adhering microorganisms by centrifugation. In the United States, most fluid milk plants run milk through in-line separators, which do an efficient job of removing sludge. Most of these plants do not have clarifiers.
2. Pasteurization and homogenization – In the United States, most of the milk is pasteurized by the HTST method, with treatments ranging from 73 to 83 °C and with holding times typically ranging from 20 to 30 s.
3. Specific product treatment – This totally depends on the type of product being made, for example, fluid milk products versus cheese.
4. Packaging.
5. Finished product storage.
6. Distribution.

Future Considerations

There is a greater and greater tendency to process milk using membrane processing. This is already being done to some extent in the United States at the farm level. Either reverse osmosis or ultrafiltration is being used to concentrate the milk. There are probably two basic reasons for this:

1. to take advantage of the functionality of the individual milk components in further processing; and
2. to reduce the transportation costs by removing water. With the extremely long-distance shipping of raw milk in some milk distribution markets, any step to reduce these costs must be considered as an advantage.

The use of membrane processing designed specifically for concentrating or fractionating the milk – either at the farm or at the central plant – thus appears to be a logical step for the dairy industry. With that in mind, this places an even bigger need for high-quality raw milk.

One final consideration is spurred by recent terrorist attacks. These events have made the entire food industry reevaluate the way things are done. Bioterrorism has become a word with real meaning, even to the dairy industry. Specifically for raw milk, we need to consider each step to reduce or eliminate any possibility of contamination of our raw milk supply. Examples that must be considered include things like bulk tanks being locked when the dairy farmer is not present and the domes on the tank trucks being locked or sealed when the hauler or driver is not present. The entire process must be carefully scrutinized by all those involved, to ensure a safe milk supply.

The dairy industry is very special, as the entire world depends heavily on dairy products. It is a vital food for infants and children, as well as for adults. We must continually do everything we can to enhance and maintain high quality.

See also: **Mastitis Pathogens**: Contagious Pathogens; Environmental Pathogens. **Milking and Handling of Raw Milk**: Milking Hygiene.

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MILK LIPIDS

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General Characteristics

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Introduction

Milk fat is composed largely of triacylglycerols (about 98% of the total fat), with minor contributions from phospholipids, cholesterol, free fatty acids, and mono- and diacylglycerols. There are also trace amounts of β-carotene, fat-soluble vitamins (A, D, E, and K), and flavoring compounds. Milk fat melts over a wide temperature range, from −35 to +38 °C, with a substantial proportion of the fat melting between 10 and 20 °C. These melting characteristics, which are due to the complex mixture of triacylglycerols in milk fat, can markedly affect the functional properties of dairy products. The desirable flavor of milk fat is one of its main attributes. The flavor profile is complex, with a large number of volatile compounds contributing to the overall aroma and taste. Two deteriorative reactions, namely oxidation and lipolysis, can occur in milk fat. These reactions produce off-flavors, which can destroy the pleasant, delicate flavor of milk fat.

A lipid can be broadly defined as any molecule of intermediate molecular weight that contains a substantial portion of aliphatic or aromatic hydrocarbon; included in this group are hydrocarbons, phospholipids, sterols, and triacylglycerols.

There is a tendency to use the terms ‘lipid’ and ‘fat’ more or less interchangeably, although a fat is considered to consist largely of triacylglycerols, with minor contributions from other lipid classes, whereas a mixture of lipids can have significant contributions from a number of different lipid classes.

The lipid (fat) fraction of milk is composed mainly of triacylglycerols, with minor contributions from diacylglycerols, monoacylglycerols, free (unesterified) fatty acids, phospholipids, and sterols. Trace amounts of fat-soluble vitamins, β-carotene, and fat-soluble flavoring compounds are also present (**Table 1**). These lipids exist in milk in the form of minute globules (average diameter 3–4 µm) surrounded by a membrane, which maintains the integrity of

Table 1 Main classes of lipids in milk

Lipid class	*Amount (wt%)*
Triacylglycerols	98.3
Diacylglycerols	0.3
Monoacylglycerols	0.03
Free fatty acids	0.1
Phospholipids	0.8
Sterols	0.3
Carotenoids	Trace
Fat-soluble vitamins	Trace
Flavor compounds	Trace

From Walstra P and Jenness R (1984) *Dairy Chemistry and Physics*. New York: John Wiley.

the globule. Although the vast bulk of the lipids are in the core of the globule, some are found in the fat globule membrane, for example, the majority of the phospholipids and small amounts of triacylglycerols and sterols.

Classes of Lipids in Milk Fat

Triacylglycerols

Triacylglycerols are relatively simple molecules consisting of a glycerol backbone to which are esterified three fatty acid molecules (**Figure 1**). They are nonpolar molecules and hence are insoluble in water. Although not highly reactive, triacylglycerols can undergo hydrolytic and oxidative reactions at ambient temperature. They are by far the most important class of compounds in milk fat, from a quantitative viewpoint, accounting for about 98% of the total fat (**Table 1**). As a result, the triacylglycerols have a major influence on the properties of milk fat, such as density and melting profile.

There are many different types of triacylglycerols in milk fat, which vary considerably in molecular weight and degree of unsaturation. This complex mixture is a direct result of the large number and wide variety of constituent fatty acids, which vary in chain length from 4 to 18 carbons.

The distribution of fatty acids at the three positions of the triacylglycerol molecule (positions *sn*-1, *sn*-2, and *sn*-3) (**Figure 1**) is not random. This is because mechanisms involved in the biosynthesis of triacylglycerols in the mammary gland exert some selectivity over the placement of fatty acids. Indeed, one of the distinctive features of milk fat is the almost exclusive esterification of the short-chain fatty acids, butyric and caproic, at position *sn* 3. As a result of this highly specific distribution, the major triacylglycerols consist of two broad structural types: those containing various combinations of three long-chain fatty acids and those containing two long-chain fatty acids and a short-chain fatty acid.

Phospholipids

Phospholipids are a small but important lipid class in milk. They are present in the fat globule membrane (about 60% of the total) and in other membranous material of the skim milk phase (about 40% of the total). Their composition is shown in **Table 2**. The glycerophospholipids constitute about 75% of the total, while the sphingolipids account for most of the remainder.

Glycerophospholipids consist of a glycerol backbone to which are attached two fatty acids at positions *sn*-1 and *sn*-2 and a phosphate group at position *sn*-3. Different side chains are attached to the phosphate group. The major glycerophospholipids are phosphatidylcholine (**Figure 2**) and phosphatidylethanolamine. Two other glycerophospholipids, namely phosphatidylserine and phosphatidylinositol, are present in small amounts.

Table 2 Phospholipid composition of milk

Phospholipid	*Amount (wt%)*
Phosphatidylcholine	34.5
Phosphatidylethanolamine	31.8
Phosphatidylserine	3.1
Phosphatidylinositol	4.7
Lysophospholipids	0.8
Sphingomyelin	25.2
Cerebrosides	Trace
Gangliosides	Trace

From Jensen RG and Clark RW (1988) Lipid composition and properties. In: Wong NP, Jenness R, Keeney M, and Marth EH (eds.) *Fundamentals of Dairy Chemistry*, 3rd edn., pp. 171–213. New York: Van Nostrand.

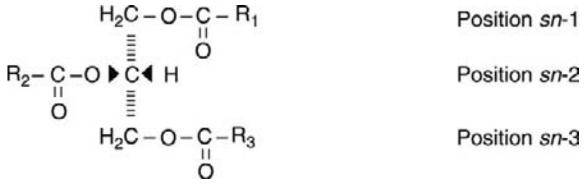

Figure 1 Triacylglycerol: Fischer projection diagram using the stereospecific numbering (*sn*-) convention.

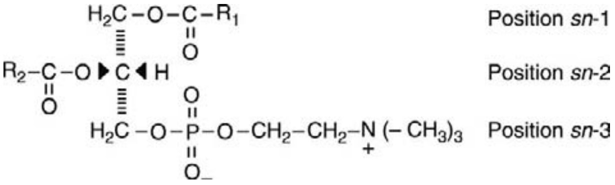

Figure 2 Phosphatidylcholine: Fischer projection diagram using the stereospecific numbering (*sn*-) convention.

The two charged groups in the glycerophospholipids give the molecules a polar part. The remaining part is nonpolar and hence they are amphiphilic and can accumulate at an oil–water interface. This explains their presence in membrane material.

The glycerophospholipids (phosphatidylethanolamine and phosphatidylcholine) have greater proportions of unsaturated fatty acids (47% $C_{18:1}$ and 13% $C_{18:2}$ in the case of phosphatidylethanolamine) than triacylglycerols (25% $C_{18:1}$ and 2% $C_{18:2}$) and hence they are more susceptible to oxidation. This has led to the postulation that fat oxidation in milk may begin with the glycerophospholipids in the fat globule membrane.

Sphingolipids contain a ceramide, which consists of a sphingosine base and a fatty acid. When a phosphorylcholine group is esterified at position 1 of the ceramide, sphingomyelin, which is the major sphingolipid in milk, is formed. Sphingomyelin also exhibits amphiphilic properties.

Trace amounts of glycosphingolipids (cerebrosides and gangliosides) are also present in milk. Cerebrosides consist of a ceramide linked to a sugar group, while the more complex gangliosides contain a ceramide, a number of sugar groups, and sialic acid. These molecules are important biological compounds.

Diacylglycerols, Monoacylglycerols, and Free Fatty Acids

Directly after milking, milk contains only small amounts of di- and monoacylglycerols and free fatty acids (**Table 1**). On storage, however, triacylglycerols in milk fat may be subjected to enzymatic hydrolysis by either milk lipase or bacterial lipases, which can significantly increase the quantities of these compounds. For example, free fatty acids can increase from 0.1% to greater than 1.0%. Such an increase normally leads to a rancid flavor in milk, which is caused by the lipases releasing short-chain fatty acids.

The small proportion of diacylglycerols present in fresh milk are largely *sn*-1,2 diacylglycerols. Since the *sn*-3 position is the last to be acylated during the biosynthesis of triacylglycerols, these diacylglycerols are probably intermediates in this process. They are unlikely to be the products of lipolysis because the lipases preferentially attack the *sn*-1 and *sn*-3 positions of the triacylglycerol molecule, thereby forming a mixture of *sn*-1,2 and *sn*-2,3 diacylglycerols.

The profile of free fatty acids in freshly drawn milk differs somewhat from the profile of the fatty acids esterified to the triacylglycerols (e.g., there appears to be very little free butyric acid), indicating that they also are unlikely to be the products of lipolysis.

Although diacylglycerols have a free hydroxyl group, their physical properties are broadly similar to those of triacylglycerols in that they are only slightly polar and show little surface activity. In contrast, monoacylglycerols can be considered relatively polar molecules. Those containing long-chain fatty acids are amphiphilic, and thus surface active, while those with a short-chain fatty acid are partially soluble in water. Free fatty acids in the undissociated state have properties broadly similar to monoacylglycerols; long-chain free fatty acids are surface active, while short-chain free fatty acids are partially soluble in water. However, at the pH of fresh milk (6.7), free fatty acids are predominantly in the dissociated form, which markedly increases their solubility in water.

Sterols

Although sterols are a minor component of milk fat (0.3%), they are the major part of the unsaponifiable material. Cholesterol is the principal sterol component, accounting for about 95% of the total sterols. Small amounts of other sterols, such as lanosterol and β-sitosterol, are also present. It should be noted that a small proportion of cholesterol exists in the esterified form and that these cholesterol esters are saponifiable.

The basic structure of sterols is a four-membered ring system, termed the cyclopentanophenanthrene ring. Various side chains are attached to this ring system to give different sterols (**Figure 3**). These compounds are very nonpolar but, interestingly, readily associate with phospholipids.

Cholesterol is an important biological compound that appears to play a role in controlling lipid and protein transitions and conformations in membranes. It is also a precursor for a number of hormones. Cholesterol has been implicated in a number of diseases that afflict humans, such as atherosclerosis, cholesterol gallstones, and certain inherited disorders.

An interesting aspect of cholesterol is that, in direct contrast to cows' milk fat and other animal fats, there are

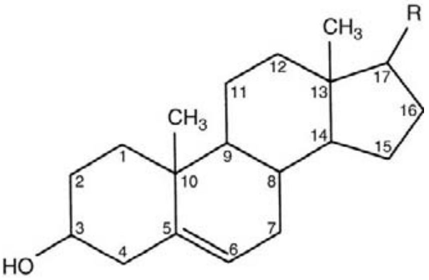

Figure 3 Basic structure of sterols: For cholesterol, $R = -\underset{\substack{| \\ CH_3}}{CH} - CH_2 - CH_2 - CH_2 - CH(-CH_3)_2$.

only trace amounts of cholesterol in vegetable oils. This has important commercial implications, since food products containing only vegetable oils can be labeled as cholesterol-free.

Carotenoids

The principal color component in milk fat is β-carotene, accounting for about 95% of the total carotenoids present. It is a highly unsaturated hydrocarbon, containing a series of conjugated double bonds which absorb at about 450 nm. The concentration of β-carotene in milk fat varies considerably, with levels ranging from 2.5 to 8.5 $\mu g\,g^{-1}$ fat.

The β-carotene content is dependent upon diet and breed of cow. Jersey cows produce milk fat with a higher color than Friesian cows, while cows fed a diet of hay and concentrate tend to produce milk fat with a lower color than pasture-fed cows. This latter finding is not surprising as carotenoid pigments are abundant in green plant material.

Fat-Soluble Vitamins

A significant nutritional aspect of fats and oils is that the vitamins A, D, E, and K are dissolved in the liquid fat or oil phase. Milk fat is considered to be a major source of vitamin A but is a poor source of vitamins D, E, and K.

Vitamin A is considered to exist in two forms: preformed vitamin A (retinol and retinyl esters) and provitamin A (β-carotene). The vitamin A activity of these different compounds varies considerably, ranging from 100% for all-*trans* retinol, 75% for 13-*cis*-retinol, and just 17% for β-carotene. Levels of all-*trans*-retinol in milk fat range from 8 to 14 $\mu g\,g^{-1}$ fat with typical values of about 10 $\mu g\,g^{-1}$ fat. The value for 13-*cis*-retinol is much lower, about 1 $\mu g\,g^{-1}$ fat. The total vitamin A activity can be obtained by summation of the concentrations and activities of all-*trans*-retinol, 13-*cis*-retinol, and β-carotene. This gives an average value for milk fat of approximately 11.5 retinol equivalent per gram of fat or 40 IU g^{-1} fat.

Vitamin D activity is derived from a number of compounds, namely 25-hydroxycholecalciferol (25-hydroxy vitamin D_3), cholecalciferol (vitamin D_3), and ergocalciferol (vitamin D_2), with 25-hydroxycholecalciferol accounting for about 65% of the total activity. The level of vitamin D in milk fat is very low, about 0.01 0.02 $\mu g\,g^{-1}$ fat, which equates to 0.4–0.8 IU g^{-1} fat. There is evidence to suggest that vitamin D levels in cows are affected by the amount of sunlight to which cows are exposed, with greater levels present in summer than during winter months.

A series of compounds termed tocopherols and tocotrienols contribute to vitamin E activity. In milk fat, α-tocopherol accounts for virtually all the vitamin E and is present at a concentration of about 35 $\mu g\,g^{-1}$ fat (the range is 25–50 $\mu g\,g^{-1}$ fat). This is equivalent to approximately 0.05 IU g^{-1} fat. The amount of tocopherol can vary during the dairying season: lush spring pastures result in greater levels than mature summer pastures. It is generally accepted that tocopherols act as antioxidants by reacting with free radicals, thereby protecting unsaturated fatty acids and β-carotene from oxidation.

Vitamin K, which is required for the synthesis of blood-clotting compounds, is present in milk at low concentrations. The amount of phylloquinone (vitamin K_1) is about 0.1–0.2 $\mu g\,g^{-1}$ fat.

Flavor Compounds

The desirable flavor of milk fat is one of its main attributes. The flavor is very complex, with a large number of compounds contributing to the overall aroma and taste. Approximately 120 volatile compounds have been identified in milk fat and, although many are present at concentrations below their individual threshold values, some may interact in a synergistic manner and contribute to the overall flavor profile. Variations in the concentration of individual volatile compounds can also affect the perceived flavor; for example, some aldehydes are thought to contribute buttery flavors at very low concentrations but are the source of oxidized flavors at higher levels.

The main classes of volatile compounds that are considered to contribute to the overall flavor of butter are fatty acids, aldehydes, lactones, and methyl ketones.

Short- and medium-chain fatty acids (C_4–C_{12}), which are present in fresh milk fat at concentrations at or below their flavor threshold values, are thought to act synergistically to provide key flavor components in milk fat. Most of these fatty acids tend to impart unpleasant flavors at higher concentrations and hence lipolysis of triacylglycerols, which releases fatty acids, will inevitably lead to undesirable, rancid flavors.

Aliphatic aldehydes in milk fat originate from the oxidation of unsaturated fatty acids in triacylglycerols. Thus, the concentrations of these aldehydes in high-quality, fresh milk fat are extremely low. However, these aldehydes have very low flavor threshold values, in the range of 0.05–1.0 $\mu g\,g^{-1}$ fat for saturated aliphatic aldehydes and as low as 0.001 $\mu g\,g^{-1}$ fat for unsaturated aldehydes. Thus, slight oxidation of milk fat can increase the concentrations of aldehydes to levels where they can contribute sensorily. Generally, aldehydes are the source of 'oxidized' flavors, but there is evidence that certain aldehydes, at very low concentrations (i.e., at or below their flavor threshold levels), may contribute buttery and deep-fried flavors.

In milk fat, small amounts of 4- and 5-hydroxy acids are esterified in triacylglycerols. These acids act as precursors for a series of four-carbon ring (γ) and five-carbon ring (δ) lactones, which are important flavor components. A proportion of these hydroxy acids rearrange spontaneously at ambient temperature to give a concentration of lactones in the range of 10–30 μg g^{-1} fat in fresh milk fat. Research suggests that three lactones, namely δC_8 lactone, δC_{10} lactone, and γC_{12} lactone, which have flavor threshold values between 1 and 3 μg g^{-1} fat, are present at sufficient concentrations to impart sweet, fruity flavors to fresh milk fat. When milk fat is heated, all hydroxy acids are converted to lactones to give a total concentration of 80–150 μg g^{-1}. Virtually all the lactones exceed their flavor threshold values and contribute to the rich flavor associated with baked goods containing butter.

Milk fat also contains trace amounts of β-keto acids esterified to triacylglycerols. At high temperatures, they are hydrolyzed and decarboxylated to form methyl ketones, which, at low concentrations, are thought to contribute to cooked butter flavors.

In addition to the four classes of compounds discussed above, there are several other volatiles that can contribute to the aroma and taste of milk fat. They include diacetyl (a diketone), phenol and cresol (aromatic alcohols), and skatole.

Physical Properties of Milk Fat

Crystallization and Melting Characteristics

The crystallization and melting characteristics of the triacylglycerols of milk fat have a marked effect on the functional properties of dairy products, particularly the consistency of butter. These characteristics are influenced to a large degree by the fatty acid composition of the triacylglycerols and to a lesser extent by the physical processes of polymorphism and mixed crystal formation.

Triacylglycerols can crystallize in different polymorphic forms, termed α, β', and β. The main difference between the forms is in the packing of the hydrocarbon chains of the triacylglycerols in crystal lattices, which leads to differences in melting points and can also influence the size of crystals.

Triacylglycerols can also form mixed (compound) crystals in which two or more different molecular species of triacylglycerols pack together in the same crystal lattice. The formation of mixed crystals affects the melting characteristics of milk fat: the greater the degree of mixed crystal formation, the more crystalline (solid) fat formed at a given temperature.

These two physical processes are dependent on the thermal history of the fat. The rate at which liquid milk fat is cooled and the temperature to which it is cooled will affect both polymorphism and mixed crystal formation. For example, rapid cooling causes a greater amount of mixed crystal formation than slow cooling.

The fatty acid composition of triacylglycerols influences melting characteristics. Triacylglycerols containing three long-chain saturated fatty acids have closely packed crystal structures and relatively high melting points. The replacement of a long-chain saturated fatty acid in a triacylglycerol molecule with either a *cis*-unsaturated fatty acid or a medium- or short-chain fatty acid gives triacylglycerols that have less closely packed crystal structures and lower melting points. In other words, the melting point of triacylglycerols decreases as the degree of unsaturation of triacylglycerols increases and as their molecular weight decreases.

Milk fat contains a large number of different types of triacylglycerols, which vary considerably in molecular weight and degree of unsaturation. It therefore follows that milk fat will melt over a wide temperature range. This is evident from the melting profile of milk fat, which shows a range from −35 to +38 °C (**Figure 4**). There is a main melting peak at about 17 °C, a smaller broad peak centered at about 7 °C, and a plateau from 22 to 36 °C. It can be seen that a substantial proportion of milk fat melts between 10 and 20 °C. (The area under the differential melting curve is approximately equal to the amount of melted fat.)

As noted earlier, a number of factors, such as the rate of cooling of milk fat and its fatty acid composition, can affect the melting profile. In New Zealand, spring milk fat has greater amounts of triacylglycerols containing unsaturated fatty acids and short-chain fatty acids than summer milk fat. As a consequence, spring milk fat has greater amounts of lower melting triacylglycerols, as can be seen in the melting profiles of the two milk fats (**Figure 4**).

Specific and Latent Heats

The specific heat of completely liquid milk fat (above +40 °C) is about 2.1 kJ kg^{-1} K^{-1}, while completely solid

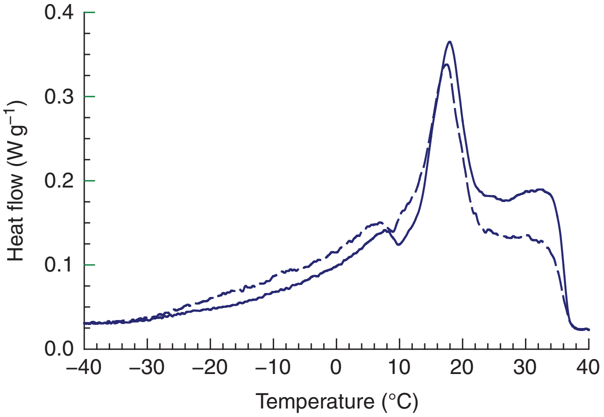

Figure 4 Melting profiles of spring (- - -) and summer (—) milk fat, determined by differential scanning calorimetry. From AKH MacGibbon (personal communication): Seasonal survey of New Zealand milk fat.

milk fat (below $-40\,^\circ\mathrm{C}$) has a value of approximately $1.7\,\mathrm{kJ\,kg^{-1}\,K^{-1}}$. These values vary slightly with temperature, increasing slightly as the temperature is increased.

The latent heat of fusion (solid-to-liquid transition) for milk fat has an average value of about $80\,\mathrm{kJ\,kg^{-1}}$. The latent heat value for milk fat varies according to temperature because higher melting triacylglycerols have greater latent heats of fusion than lower melting triacylglycerols. The values are also dependent on the polymorphic form of the crystals.

Measurements of specific heat made at temperatures within the melting range of milk fat, which will include both sensible and latent heats, are termed apparent specific heats. These values show marked variations through the temperature range because of the different amounts of melting that occur at different temperatures (**Figure 4**). For example, at 0 and 15 °C, milk fat has apparent specific heat values of 3.34 and $5.43\,\mathrm{kJ\,kg^{-1}\,K^{-1}}$, respectively, indicating that a greater amount of melting occurs at 15 °C than at 0 °C.

Other Physical Properties

The refractive index of a fat or oil is influenced by its fatty acid composition; the refractive index increases as the degree of unsaturation increases and as the length of the hydrocarbon chains increases. As a consequence, the refractive index of milk fat varies during the dairying season; in northern European countries, values for refractive index tend to be higher in summer, when there are more unsaturated fatty acids in milk fat, than in winter. Typically, values lie within the range of 1.453 0–1.455 0.

The density of liquid milk fat is $890\,\mathrm{kg\,m^{-3}}$ at 60 °C, with a coefficient of expansion of $0.78\,\mathrm{kg\,m^{-3}\,K^{-1}}$ for the temperature range 40–60 °C. This value is fairly typical for fats and oils; for example, lard has a density of $895\,\mathrm{kg\,m^{-3}}$ at 40 °C, while the value for olive oil is $915\,\mathrm{kg\,m^{-3}}$ at 15 °C.

In a similar manner to other fats and oils, the viscosity of milk fat decreases slightly as the temperature increases, with a viscosity of 31, 22, and 17 cP at 40, 50, and 60 °C, respectively.

Milk fat is a poor conductor of both heat and electricity. It has a thermal conductivity of about $1.7 \times 10^{-4}\,\mathrm{kJ\,m^{-1}\,s^{-1}\,K^{-1}}$ at room temperature and an electrical conductivity of about $10\,\Omega\,\mathrm{cm^{-1}}$.

Deteriorative Reactions of Milk Fat

Triacylglycerols are considered not to be highly reactive. Nevertheless, these compounds can undergo two deteriorative reactions, namely oxidation and lipolysis, which can result in unpleasant flavors in milk fat.

Lipolysis is the hydrolysis of the ester linkage between a fatty acid and the glycerol backbone of a triacylglycerol. The reaction is catalyzed by lipolytic enzymes (lipases), either the indigenous milk lipase or lipases produced by psychrotrophic bacteria. While the indigenous milk lipase is deactivated by pasteurization, bacterial lipases are often heat stable and retain at least some activity after pasteurization. The short- and medium-chain fatty acids released by lipolysis will contribute to undesirable off-flavors once their concentrations increase to levels slightly above their flavor threshold values.

Oxidation is the reaction between oxygen and the unsaturated fatty acids in either triacylglycerols or phospholipids. The reaction, which involves the formation of free radicals, initially produces hydroperoxides. These compounds subsequently break down to form highly flavored carbonyl compounds, such as saturated and unsaturated aldehydes. Even at very low concentrations (i.e., $<1.0\,\mu\mathrm{g\,g^{-1}}$ fat), these aldehydes impart metallic and tallowy flavors to milk fat. The reaction is catalyzed by light and by transition metals such as copper. Antioxidants, such as α-tocopherol, can inhibit the reaction by reacting with the free radicals formed during the reaction. The removal of virtually all air from products containing milk fat will also slow down the reaction.

See also: **Analytical Methods**: Sensory Evaluation. **Flavours and Off-Flavours in Dairy Foods**. **Milk Lipids**: Analytical Methods; Fatty Acids; Lipid Oxidation; Lipolysis and Hydrolytic Rancidity; Nutritional Significance; Phospholipids; Rheological Properties and Their Modification; Triacylglycerols.

Further Reading

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Fatty Acids

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Introduction

Bovine milk fat contains a large number of fatty acids. However, many of these are found in only extremely small quantities and only 15 or 16 fatty acids are present at concentrations equal to or greater than 1%. The main saturated fatty acids, which account for 62–70% of the total fatty acids, are straight-chain molecules, which vary in length from 4 to 18 carbon atoms. The principal unsaturated fatty acid is oleic acid (about 20% of the total). The acid composition of milk fat is affected by factors such as the diet of the cow and the stage of lactation. As a consequence, the proportions of the fatty acids vary throughout the dairying season, in some instances quite markedly.

The fatty acid composition of bovine milk fat is to a large extent the fatty acid composition of its constituent triacylglycerols because the triacylglycerols make up approximately 98% of milk fat. Small quantities of fatty acids are also esterified to mono- and di-cylglycerols and to phospholipids and there is about 0.1% unesterified or free fatty acids in milk fat. The fatty acids of triacylglycerols account for a large proportion of the molecular weight of the triacylglycerol molecule (85–95%). Furthermore, unsaturated fatty acids contain double bonds, which are reactive sites. Thus, it is not surprising that the fatty acids have a marked effect on the chemical and physical properties of triacylglycerols, and consequently on the properties of milk fat. Fatty acids influence the melting characteristics of milk fat and the rate at which fat oxidation occurs.

Bovine milk fat is regarded as one of the most complex naturally occurring fats and oils because of the large number and wide variety of fatty acids it contains. Using a combination of chromatographic and spectroscopic techniques, research workers have identified approximately 400 fatty acids in milk fat. The common and systematic names of the 16 'major' fatty acids, which constitute about 95% of the fatty acids, are presented in **Table 1**, together with their proportions in milk fat. It should be noted that these proportions are typical values for cows grazed on pasture; the fatty acid composition is affected by factors such as diet and stage of lactation and consequently the proportions of some fatty acids can show marked variations.

There is an empirical classification of these major fatty acids on the basis of the length of the hydrocarbon chain, namely short-chain (C_4–C_6), medium-chain (C_8–C_{14}), and long-chain (C_{16}–C_{18}) fatty acids. The magnitude of the difference in chain length between butyric acid (4:0) and stearic acid (18:0) can be seen in **Figure 1**.

Origins of the Fatty Acids

The fatty acids of bovine milk fat arise from two sources: from *de novo* synthesis in the mammary gland and from the plasma lipids. The types of fatty acids from these two sources are very different (**Figure 2**). The fatty acids synthesized *de novo* are of short- to medium-chain length, from 4:0 to 14:0, and also some 16:0. In contrast, the C_{18} fatty acids and some 16:0 arise from the plasma lipids.

The *de novo* synthesis of fatty acids in the mammary gland utilizes mainly acetate, which is provided by the fermentation of dietary carbohydrates in the rumen. The mechanism essentially involves the carboxylation of acetyl-CoA to malonyl-CoA, which is then used in a stepwise, chain elongation process. This leads to a series of short- and medium-chain length fatty acids that differ by two CH_2 groups, for example, 4:0, 6:0, and 8:0.

The blood lipids come mainly from the diet although they can also include fatty acids released from adipose tissue. This suggests that dietary lipids will have a large effect on the fatty acid composition of milk fat. Indeed, the inclusion of 16:0, 18:0, and 18:1 in the cow's diet increases the proportions of these fatty acids in milk fat. However, changes in the amounts of polyunsaturated fatty acids in the diet of the cow normally affect the levels of only 18:0 and 18:1 in milk fat. This is because polyunsaturated fatty acids are extensively hydrogenated in the rumen by microorganisms. An additional reaction, namely the desaturation of 18:0 to 18:1 by a desaturase in the mammary gland, can also influence the levels of 18:0 and 18:1. The net result is that changes to the diet of the cow normally affect the proportions of 16:0, 18:0, and 18:1 in milk fat but have very little effect on 18:2 or 18:3.

Although the sources of fatty acids in the milk of other species are essentially the same as those for the cow (i.e., *de novo* synthesis and blood lipids), there are some important differences between the fatty acid

Table 1 Major fatty acids of bovine milk fat

			Typical composition[a]	
Fatty acid	*Common name*	*Systematic name*	*wt%*	*Mol%*
4:0	Butyric	Butanoic	3.7	9.6
6:0	Caproic	Hexanoic	2.4	4.7
8:0	Caprylic	Octanoic	1.5	2.3
10:0	Capric	Decanoic	3.2	4.2
12:0	Laurie	Dodecanoic	3.6	4.1
14:0	Myristic	Tetradecanoic	11.1	11.0
14:1	Myristoleic	*cis*-9-Tetradecenoic	0.9	0.9
15:0	—	Pentadecanoic	1.2	1.1
16:0	Palmitic	Hexadecanoic	28.3	25.3
16:1	Palmitoleic	*cis*-9-Hexadecenoic	1.6	1.5
18:0	Stearic	Octadecanoic	11.8	9.5
18:1	Oleic	*cis*-9-Octadecenoic	18.8	15.4
18:1	Vaccenic	*trans*-11-Octadecenoic	4.2	3.4
18:2	Linoleic	*cis*, *cis*-9,12-Octadecadienoic	1.4	1.1
18:2c	Linoleic (conjugated)	*cis*, *trans*-9,11-Octadecadienoic	1.1	0.9
18:3	Linolenic (alpha)	*cis*, *cis*, *cis*-9,12,15-Octadecatrienoic	0.9	0.8
	Minor acids		4.4	3.7

[a] From A. K. H. MacGibbon (personal communication), Fonterra Research Centre seasonal survey.

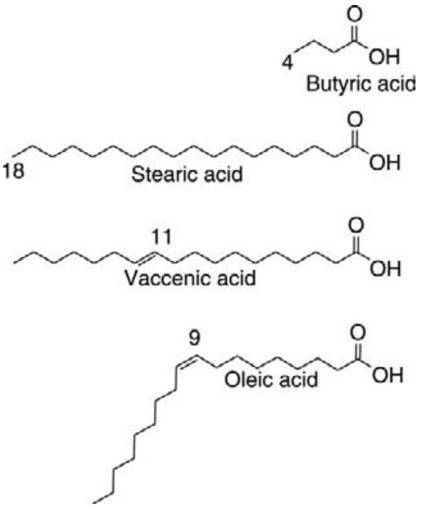

Figure 1 Skeletal structures of some fatty acids

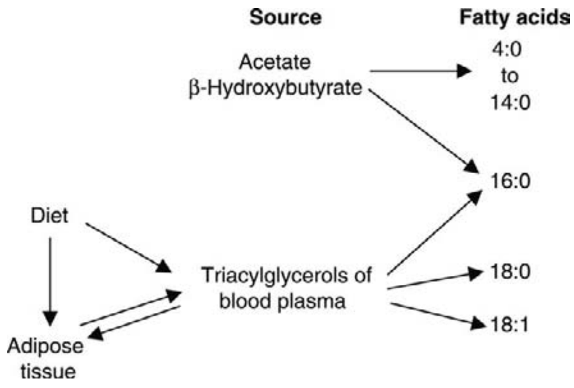

Figure 2 Sources of the fatty acids of milk fat

composition of the milk fat of ruminants and nonruminants. *De novo* synthesis in the mammary gland produces relatively high concentrations of short-chain fatty acids (i.e., 4:0 and 6:0) in ruminants but only small amounts in non-ruminants. Unsaturated dietary lipids are not subjected to biohydrogenation reactions in non-ruminants. Hence, long-chain fatty acids in the milk of non-ruminants are a direct reflection of dietary lipids and consequently the milk fat of non-ruminants often contains appreciable amounts of polyunsaturated fatty acids.

Different Types of Fatty Acids

Saturated Fatty Acids

The major saturated fatty acids are molecules with unbranched hydrocarbon chains containing an even number of carbon atoms; the length of the molecule varies from 4 to 18 carbons. These saturated fatty acids account for approximately 65–70% (w/w) of the total fatty acids. The most important saturated fatty acid from a quantitative viewpoint is 16:0, which accounts for about 25–30% of the total, while two other fatty acids, 14:0 and 18:0, constitute 10–12% each (**Table 1**).

The amounts of the short-chain fatty acids, 4:0 and 6:0, are reasonably high when their proportions are expressed as molar percentages (approximately 9.5 and 4.5%, respectively). Interestingly, these short-chain fatty acids are esterified almost exclusively at position *sn-3* on the glycerol molecule, which means that approximately 42% (3 × 14%) of bovine milk fat triacylglycerols contain a short-chain fatty acid in combination with two other fatty acids.

Two reasons have been postulated to explain the relatively high proportions of short- and medium-chain fatty acids in bovine milk fat. First, these fatty acids are absorbed rapidly as free fatty acids from the intestine into the portal bloodstream and are quickly metabolized in the liver. Hence, they can make a rapid and direct contribution to the energy metabolism of the newborn calf. Second, short-chain fatty acids and, to a lesser extent, medium-chain fatty acids lower the melting point of triacylglycerols and thus their presence helps to keep milk fat liquid at physiological temperatures.

Unsaturated Fatty Acids

The principal monounsaturated fatty acid is oleic acid (*cis*-9-octadecenoic acid), which constitutes 17–25% (w/w) of the total fatty acids. There are also relatively small but significant contributions from other monounsaturated fatty acids, namely 14:1 (about 1.0%), 16:1 (about 1.5%), and vaccenic acid (*trans*-11-octadecenoic acid) (**Table 1**). Vaccenic acid is formed as a result of biohydrogenation in the rumen and its proportion in milk fat is markedly affected by the cow's diet; a mixture of hay and concentrate gives a low level (about 1.5%), whereas pasture results in a high value (about 5.0%).

Because of the biohydrogenation reactions in the rumen, there are only low concentrations of the polyunsaturated fatty acids, linoleic and linolenic, in milk fat. The level of linoleic acid is of interest because it is an essential fatty acid. Despite the low level of linolenic acid (about 1.0–1.5%), the newborn calf appears to be able to build up reserves of this acid. However, there have been concerns that an infant formula made solely from whole milk may not contain sufficient 18:2 to meet the requirements of the human infant. Thus, commercial infant formulae are supplemented with vegetable oils to raise the level of 18:2.

An interesting polyunsaturated fatty acid that is present in relatively small amounts (about 1%) is conjugated linoleic acid. While this acid can exist as a range of isomers of linoleic acid, which possess conjugated double bonds, the principal isomer in milk fat is *cis*-9, *trans*-11-octadecadienoic acid. This fatty acid has been the subject of extensive recent research as it appears to impart a wide range of health benefits. Trials involving animals have shown that conjugated linoleic acid may provide some protection against the development of mammary tumors and may enhance immune functions. In addition, the acid may be able to regulate energy metabolism so that body fat is reduced.

An important difference between *cis* and *trans* unsaturated fatty acids is that the *cis* configuration of the double bond puts a significant 'kink' in the hydrocarbon chain, whereas the *trans* configuration causes only a slight distortion (**Figure 1**). This has a major impact on the packing density of triacylglycerols in crystal lattices. Triacylglycerols containing *cis* unsaturated fatty acids have a lower packing density than triacylglycerols containing either *trans* unsaturated or saturated fatty acids and as a result have lower melting points. Thus, the relative levels of *cis* and *trans* unsaturated fatty acids can exert a considerable influence on the melting characteristics of milk fat.

Minor Fatty Acids

As stated previously, there are a very large number of minor fatty acids, many of which are present at extremely low concentrations (less than 0.02%). As a consequence, most of these fatty acids are of little practical importance.

Among the minor saturated fatty acids are odd-numbered and branched-chain fatty acids. Examples of odd-numbered fatty acids are 13:0 (0.1%) and 17:0 (0.5%). The monomethyl branched-chain fatty acids are quite significant, accounting for about 2.5% of the total fatty acids. Examples are the C_{15} branched-chain acids 13-methyl tetradecanoic acid (the *iso* configuration) and 12-methyl tetradecanoic acid (the *anteiso* configuration), which together make up about 1% of milk fat.

There are only trace amounts of minor unsaturated fatty acids. Among these are approximately 150 positional isomers of monoenoic and dienoic fatty acids; there are, for example, 11 *trans* isomers of 18:1. Trace amounts of hydroxy and keto acids are also found in milk fat.

Variations in Fatty Acid Composition

The fatty acid composition of bovine milk fat is affected by factors such as diet and stage of lactation and as a result the proportions of some fatty acids can vary considerably. For example, values for 16:0 can range from 21 to 36%, while 18:1 can vary from 19 to 31%.

Normal Feeding Regimes

Variations in the normal diet fed to cows throughout the dairying season can lead to relatively small but distinct variations in the proportions of fatty acids in milk fat.

In northern Europe, where the general pattern of dairy husbandry is to house cows in winter, it has been shown that the change from winter feed of hay and concentrate to a diet of fresh grass in spring and summer causes an increase in the proportions of 18:0 and 18:1 and a decrease in the proportions of 14:0 and 16:0.

In contrast, in Australia and New Zealand, where cows are generally grazed on pasture throughout the year, milk fat from cows grazed on mature summer grass contains lower proportions of 18:0 and 18:1 and a greater proportion of 16:0 than milk fat from cows fed lush spring grass.

These observations that 16:0, 18:0, and 18:1 vary as a result of dietary changes are not unexpected when the source of these fatty acids is taken into account.

Lactation

The proportions of fatty acids also show relatively small but distinct variations as lactation progresses. The proportion of 4:0 declines gradually throughout lactation, the fatty acids 6:0 to 14:0 increase from relatively low levels at the beginning of lactation to a maximum 8–12 weeks after calving and decline slowly thereafter, 16:0 shows a slight increase as lactation progresses, and 18:0 and 18:1 decline for the first 4–6 weeks and then increase slightly toward the end of lactation.

Seasonal Effects

The seasonal variation in the fatty acid composition of milk fat is affected by a number of factors such as composition of the diet, plane of nutrition, and stage of lactation. Despite this complexity, studies in different countries have shown a seasonal pattern of fatty acid variation that consistently reoccurs.

In northern Europe, the seasonal variation in fatty acid composition shows the following general trends: 4:0 to 12:0 show no consistent seasonal pattern; 14:0 and 16:0 are lower in summer than in winter; and 18:0 and 18:1 are higher in summer than in winter. The fatty acid composition of samples of winter and summer milk fat from Belgium, which are presented in **Table 2**, shows these trends. The variations in 14:0, 16:0, 18:0, and 18:1 appear to be caused by the change in feeding conditions described above, while the lack of a consistent seasonal pattern in the fatty acids 4:0 to 12:0 may be attributed to the practice of not confining calving to spring.

In New Zealand, where cows calve in spring and are fed on pasture, the seasonal variation shows the following pattern: 4:0 shows a gradual decline throughout the season; 6:0 to 14:0 show a slight increase from low values in early spring to a maximum during early summer and a decline thereafter throughout the rest of the season; 16:0 increases significantly from relatively low values in spring to high values during summer and then decreases in autumn; and both 18:0 and 18:1 show a seasonal trend that is the reverse of that exhibited by 16:0 (**Table 2**). The changes in the proportions of fatty acids of chain length C_4–C_{14} follow the pattern resulting from lactational effects, whereas the seasonal trends in 16:0, 18:0, and 18:1 appear to be the result of dietary effects.

Table 2 Seasonal variation in the fatty acid composition of milk fats from different countries

	Fatty acid composition (wt%)			
	Belgian[a]		*New Zealand*[b]	
Fatty acid	*Winter*	*Summer*	*Spring*	*Summer*
4:0	3.9	3.7	4.2	3.9
6:0	2.1	2.1	2.5	2.5
8:0	1.2	1.1	1.6	1.6
10:0	2.8	2.2	3.3	3.5
12:0	3.7	2.7	3.6	4.0
14:0	11.5	9.8	10.6	12.4
14:1	1.5	1.3	0.6	1.0
15:0	1.2	1.1	1.1	1.3
16:0	28.1	24.7	26.4	31.2
16:1	2.9	2.3	1.8	1.7
18:0	10.0	12.5	14.3	12.2
18:1	27.0	31.3	26.5	21.3
18:2	2.3	2.3	2.5	2.4
18:3	1.7	2.7	1.1	1.0

[a]From Hughebaert A and Hendrickx H (1971) Studies on Belgian butterfat. 3. The fatty acid composition. *Milchurissenschaft* 26: 613–617.

[b]From A. K. H. MacGibbon (personal communication), Fonterra Research Centre seasonal survey.

The differences, described above, in the seasonal variations in the fatty acid composition of milk fats in different countries appear to be a consequence of different dairy husbandry practices.

Fat Supplementation of Diets

For many years, fats and oils have been included in the concentrate mixtures for dairy cows largely because of their high calorific value and their potential to alter the fatty acid composition of milk fat. One of the limitations of this type of supplementation is that a relatively high level of dietary fats/oils impairs digestion and metabolism in the rumen, which in certain instances can lead to a reduction in the overall yield of fat in milk.

Fat supplementation of the cow's diet generally produces two broad trends in the fatty acid composition of milk fat. First, there is a decrease in the amounts of fatty acids that are produced by *de novo* synthesis in the mammary gland (i.e., C_4–C_{16}), which occurs as a consequence of the suppression of rumen metabolism. In one such trial, using soybeans as the fat supplement, there was an overall decrease in these fatty acids from 389 to 313 g day^{-1} (**Table 3**). Second, there is an increase in the amounts of 18:0 and 18:1, but no appreciable increase in the levels of polyunsaturated fatty acids (**Table 3**). The increased levels of 18:0 and 18:1 could be simply from an

Table 3 Effect of dietary supplements of soybean oil or protected soybean oil on the yields of fatty acids in milk fat

	Yield (g day^{-1})		
Fatty acid	*Basal diet*	*Basal diet + soybean oil*	*Basal diet + protected soybean oil*
4:0	23	31	30
6:0	12	12	13
8:0	6	6	7
10:0	22	16	20
12:0	28	18	21
14:0	83	67	70
14:1	12	11	7
16:0	194	145	153
16:1	9	7	5
18:0	37	76	77
18:1 *cis*	77	120	126
18:1 *trans*	12	34	14
18:2	18	27	76
Total	533	570	619

Calculated from Banks W, Clapperton JL, and Girdler AK (1990) Effect of dietary unsaturated fatty acids in various forms on the *de novo* synthesis of fatty acids in the bovine mammary gland. *Journal of Dairy Research* 57: 179–185.

increased dietary intake of these acids or indirectly through biohydrogenation in the rumen. Furthermore, increased 18:1 could result from desaturation of 18:0 to 18:1. The absence of any appreciable increase in polyunsaturated fatty acids is a result of ruminal biohydrogenation reactions.

The rumen metabolism and the biohydrogenation reactions can be prevented by treating fat supplements with a mixture of protein and formaldehyde, which largely prevents the release of fat into the rumen. The inclusion of these 'protected fat' supplements in the diet invariably leads to an increase in the yield of milk fat and, if polyunsaturated oils are used as the supplement, a marked increase in polyunsatured fatty acids (**Table 3**).

Milk fat from cows fed a 'protected fat' supplement, which contained a 'high level' of 18:2, has been made into butter. This butter was found to be considerably softer than ordinary butter and was spreadable at domestic refrigerator temperatures. This would indicate that the feeding of 'protected fat' supplements has considerable potential as a technique for dramatically altering the fatty acid composition of milk fat and the rheological properties of butter. However, current trends have seen the blending of milk fat with vegetable oils to achieve changes in the properties.

See also: **Feed Ingredients**: Feed Supplements: Fats and Protected Fats. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat. **Milk Lipids**: Analytical Methods; Conjugated Linoleic Acid; Nutritional Significance; Triaclglycerols.

Further Reading

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Conjugated Linoleic Acid

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Introduction

Nutritional quality is an important consideration when making food choices; milk is an excellent source of many essential nutrients and supplies the body with energy, high-quality protein, and a variety of the required vitamins and minerals. In addition, consumers are increasingly aware of the link between diet, health, and disease prevention and a recent National Academy of Sciences report identified research on bioactive food components as a key focus area to enhance human health through nutrition. Traditionally, specific components in fruits and vegetables have been featured for their health-promoting properties; however, recent investigations have established that bioactive components are also present in animal-derived foods, including milk and dairy products. One such bioactive component that has created widespread interest is conjugated linoleic acid (CLA), a group of naturally occurring 18-carbon fatty acids.

CLAs are a mixture of positional and geometric isomers of linoleic acid, and some CLA isomers have been shown to offer benefits in relation to health maintenance and disease prevention. In nature, polyunsaturated fatty acids (PUFAs) typically contain *cis* double bonds that are separated by an interceding carbon (methylene group). Linoleic acid (*cis*-9, *cis*-12 18:2) found in many plant oils is an example (**Figure 1**). In contrast, the double bonds in CLA are adjacent with no interceding methylene group. Furthermore, the geometric orientation of the conjugated double bonds may be *cis–trans, cis–cis, trans–cis*, or *trans–trans* (e.g., the CLA isomer *cis*-9, *trans*-11 18:2) (**Figure 1**). The first recognition of milk fatty acids with a conjugated double bond system dates back to the 1930s when English scientists reported an increase in the spectrophotometric absorption at 230 nm for butter made from summer milk as compared to butter from winter milk. Then, in 1977, PW Parodi identified the *cis*-9, *trans*-11 CLA isomer in milk fat. Subsequent research established that milk fat contains trace amounts of many CLA isomers, but *cis*-9, *trans*-11 CLA is the predominant isomer in milk fat, representing about 75–90% of the total CLA. This is also true for the CLA in ruminant meat fat, and thus, the *cis*-9, *trans*-11 CLA isomer has appropriately been given the common name 'rumenic acid' (RA). Of special interest to nutritionists, ruminant-derived foods are the major dietary source of CLA in human diets, with dairy products and ruminant meats representing over 90% of human intake (**Figure 2**). In the following sections we will discuss the origin of CLA in ruminants and results from biomedical studies demonstrating the potential role of RA as a natural functional food component with anticarcinogenic and antiatherogenic effects.

Origin of RA and Other CLA Isomers

The presence of CLA in dairy products is related to rumen fermentation of dietary PUFAs. The rumen is essentially a large fermentation vat and in dairy cows it has a 50 l volume and contains about 10^{11} bacteria and 10^{6} protozoa per milliliter. Rumen biohydrogenation occurs because dietary PUFAs are toxic to some bacteria. Thus, only a few bacterial species carry out biohydrogenation and most of them have the ability to carry out only some of the steps involved in biohydrogenation.

When dietary lipids enter the rumen, the first major transformation they undergo is hydrolysis of their ester linkages, resulting in free fatty acids. Hydrolysis is catalyzed by enzymes produced by rumen microflora and it results in the liberation of over 85% of esterified fatty acids. Hydrolysis is a prerequisite for the second major rumen transformation, biohydrogenation of unsaturated fatty acids. The predominant dietary PUFAs that are biohydrogenated in the rumen are linoleic (*cis*-9, *cis*-12 18:2) and linolenic (*cis*-9, *cis*-12, *cis*-15 18:3) acids. The first step in the biohydrogenation of linoleic acid is an isomerization of the *cis*-12 double bond to form RA, and this is followed by hydrogenation of the *cis*-9 double bond, producing vaccenic acid (VA; *trans*-11 18:1). The structures of these intermediates are shown in **Figure 1** and the pathway of their formation is presented in **Figure 3**. In contrast to linoleic acid, RA is not an intermediate in the biohydrogenation of linolenic acid; however, VA is an intermediate formed from both dietary fatty acids. The final step in biohydrogenation is a second hydrogenation to produce stearic acid, a saturated fatty acid (**Figure 3**).

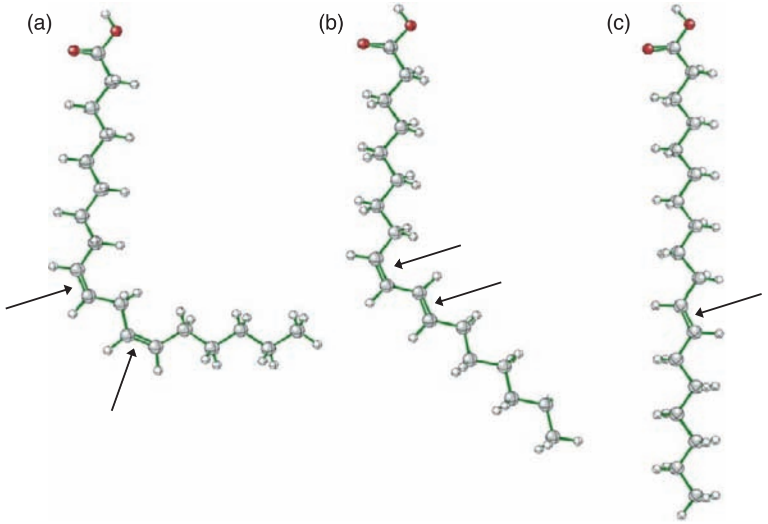

Figure 1 Comparison of the structures of (a) linoleic acid (*cis*-9, *cis*-12 18:2), (b) *cis*-9, *trans*-11 conjugated linoleic acid, and (c) vaccenic acid (*trans*-11 18:1). Double bonds are denoted by arrows. Structures are adapted from Bauman DE and Lock AL (2006) Conjugated linoleic acid: Biosynthesis and nutritional significance. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 2: Lipids*, 3rd edn., pp. 93–136. New York: Springer.

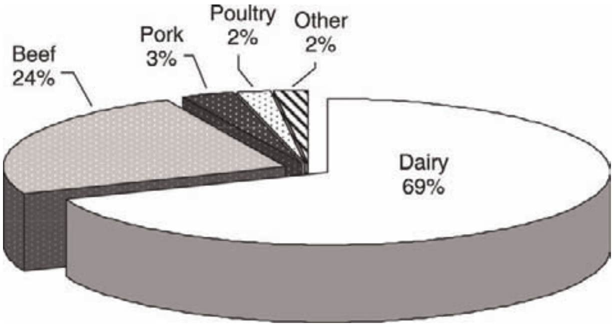

Figure 2 Distribution of conjugated linoleic acid (CLA) sources in the US diet. Based on data from Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, and McGuire MA (2001) Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. *Journal of Nutrition* 131: 1548–1554.

Biohydrogenation in the rumen is relatively complete and this represents the basis for ruminant fat having a relatively high content of saturated fatty acids. Nevertheless, small amounts of intermediates can pass from the rumen before they are completely biohydrogenated, and it was assumed initially that this was the primary source of the RA in milk fat. However, *in vitro* rumen fermentation studies revealed that RA was only a transitory product during biohydrogenation, and VA was the major intermediate that accumulated in the rumen. Based on this and additional lines of evidence, it was hypothesized that RA in ruminant fat could originate from endogenous synthesis whereby VA that escaped complete biohydrogenation would be absorbed in the small intestine and converted to RA in the cow's tissues by the enzyme Δ^9-desaturase (**Figure 3**). This was indeed the case, and investigations using several different approaches across a range of diets and management systems have clearly established that endogenous synthesis is the predominant source of RA in ruminant fat.

Endogenous synthesis of RA via Δ^9-desaturase in the mammary gland (milk fat during lactation) and adipose tissue (body fat during growth) is the reason why RA is the predominant CLA isomer in ruminant-derived foods. Synthesis of RA relies heavily on the activity of this enzyme as well as the tissue uptake of its precursor, VA. Studies have consistently shown an enzymatic equilibration between VA and RA, usually in about a 3:1 ratio, providing evidence of the importance of VA in RA synthesis. The second most prevalent CLA isomer in ruminant fat is most often *trans*-7, *cis*-9 CLA (~5–10% of total CLA) and it also arises from endogenous synthesis involving Δ^9-desaturase with the precursor being *trans*-7 18:1 produced in the rumen. There are, however, no analogous desaturases for endogenous synthesis of other CLA isomers. Hence, other individual CLA isomers are produced in the rumen via minor pathways of biohydrogenation, but they are present in milk and meat fat only in trace amounts, with each of these CLA isomers typically representing <1% of total CLA isomers.

Altering the CLA Content of Dairy Products

Diet is the most significant factor affecting the CLA content of milk fat. CLA concentration can be enhanced several-fold by using combinations of feed ingredients that affect the dietary supply of PUFAs and/or affect

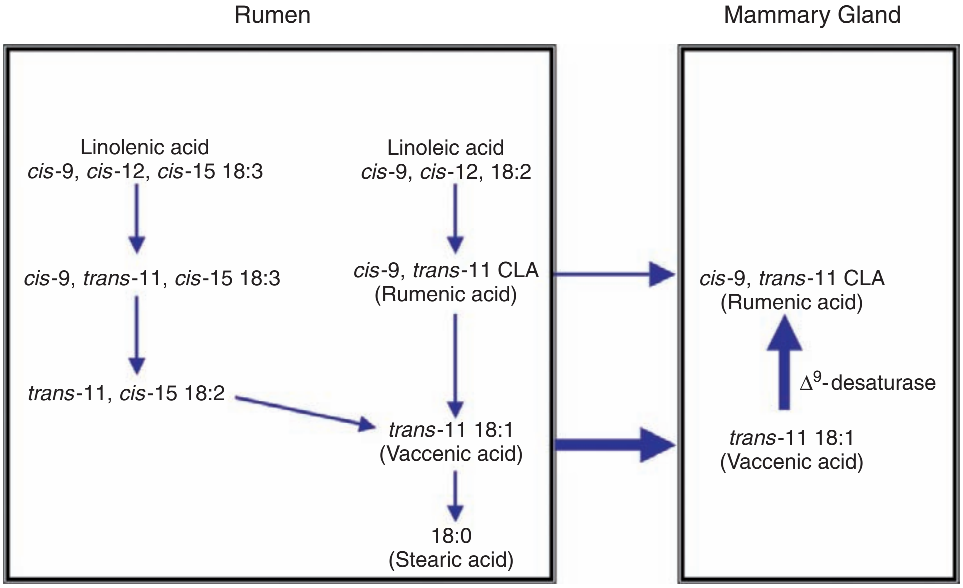

Figure 3 Pathways for ruminal biohydrogenation of linoleic and linolenic acid and the endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in the dairy cow. Adapted from Bauman DE, Lock AL, Corl BA, Ip C, Salter, AM, and Parodi PW (2006) Milk fatty acids and human health: Potential role of conjugated linoleic acid and *trans* fatty acids. In: Sejrsen K, Hvelplund T, and Nielsen MO (eds.) *Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress*, pp. 523–555. Wageningen, The Netherlands: Wageningen Academic Publishers.

the rumen environment to alter the rate and completeness of biohydrogenation. For example, consumption of lush pasture increases the content of RA in milk fat about two-fold, consistent with this forage source providing linolenic acid for rumen biohydrogenation and the formation of VA. Likewise, feeding total mixed diets that provide seed oils that are high in linoleic acid content (e.g., corn, soybeans, distillers grains) can increase the content of RA in milk fat three-fold or more. Feeding fish oil supplements is also effective in increasing the RA concentration in milk fat, but these have a different mechanism; in this case, the long-chain omega-3 fatty acids in fish oil act to inhibit the final step in rumen biohydrogenation of linoleic and linolenic acids and, as a consequence, the rumen outflow of VA is increased, which can then be used for endogenous synthesis of RA (**Figure 3**).

The RA content of milk fat is also influenced by physiological factors, although to a lesser extent than dietary effects. Individual animal variation is the physiological factor that has the greatest effect, and typically there is about three-fold range among cows. Furthermore, the hierarchy in RA content of milk fat among cows is maintained even when cows are switched between diets that are designed to result in very different average concentrations of RA in milk fat. This genetic variation relates to animal differences in rumen fermentation and/or tissue activity of Δ^9-desaturase, and it provides an opportunity for genetic selection of cows that naturally produce high levels of RA in milk fat. Some studies have also reported that the RA content of milk fat differs according to dairy breed, stage of lactation, and parity, but these effects are minor compared to the effects of individual animal variation and diet.

Functional Food Implications

'Functional foods' is a collective term for any food or food component that provides additional health benefits beyond the traditional nutrient content of the food product. To be considered a functional food, the bioactive component(s) must be consumed as a natural food component rather than taken as a dietary supplement, and the emphasis is on health maintenance and the prevention of chronic diseases as opposed to the pharmaceutical treatment of disease. RA is a bioactive fatty acid, and the functional food role of milk and meat products from ruminant animals relates to these foods being the major source of CLA in human diets and the fact that RA is the predominant CLA isomer in ruminant fat.

The biological effects of CLA were first recognized in the 1980s when M Pariza and colleagues (University of Wisconsin, USA) discovered that partially purified beef extracts could inhibit tumor growth in mice; the anticarcinogenic factors were identified as conjugated 18:2 fatty acids and they coined the term CLA. Subsequent investigations expanded the use of biomedical models, and additional health-related effects were identified for CLA, including anti-obesity, anti-diabetogenic, and anti-atherogenic effects. Although the initial research used mixed isomers of CLA, more recent studies with pure RA and *trans*-10, *cis*-12 CLA, a minor CLA isomer in milk fat, have shown potent bioactivity in biological models, indicating clear differences in biological effects. From a functional food perspective, RA was shown to have anti-carcinogenic and anti-atherogenic effects, while *trans*-10, *cis*-12 CLA has antiobesity and anti-diabetogenic effects as well as anti-carcinogenic effects; however, *trans*-10, *cis*-12 CLA has little potential as a functional food component because only trace quantities of this CLA isomer are naturally present in foods.

There are biomedical models for most types of cancer and many of them have been used to investigate the role of CLA. They have included cell lines, tumor transplant models, and models of *in situ* organ carcinogenesis; in virtually all cases, CLA has been shown to be a potent inhibitor of chemically induced carcinogenesis. In summarizing the overall effects, a report by the National Academy of Sciences on *Carcinogens and Anticarcinogens in Foods* concluded, "conjugated linoleic acid (CLA) is the only fatty acid shown to unequivocally inhibit carcinogenesis in experimental animals".

The anti-carcinogenic effect of RA is particularly impressive in studies of mammary cancer, and a landmark study demonstrated that RA was able to mitigate chemically induced mammary carcinogenesis when provided as either a synthetic supplement or a natural component of the diet, in this case an RA-enriched butter (**Table 1**). A striking observation was that rats fed the RA-enriched butter also had higher levels of RA in their mammary tissue and this was a direct result of the conversion of VA to RA in the tissues via Δ^9-desaturase. As mentioned previously, VA and RA are present roughly in a 3:1 ratio in dairy products, and in humans approximately 20% of the dietary intake of VA is converted to RA. Thus, VA is also an anti-carcinogenic component of dairy products due to its conversion to RA.

Investigations of the anti-atherogenic effects of CLA have also used animal models. When fed an appropriate diet, rabbits, hamsters, and mice develop atherosclerotic lesions in the aorta that exhibit similarities to the early lesions seen in the arteries of humans. Several studies have shown that RA is effective in reducing the development of atherogenic lesions in these animal models, although there are some inconsistencies, especially in studies that supplement with mixed isomers of CLA and those that use lipoprotein-related biomarkers as end points. Furthermore, a recent human clinical investigation reported that an RA supplement produced changes in plasma lipids that are indicative of a reduced risk in cardiovascular disease (CVD).

Studies of the functional food implications have examined the effects of an RA-enriched butter on biomarkers of CVD in the cholesterol-fed Golden Syrian hamster. This animal model is often used in biomedical studies on CVD because its lipoprotein metabolism and low rates endogenous cholesterol synthesis are similar to those humans. The results indicated that hamsters consuming RA-enriched butter had a more favorable plasma lipoprotein profile than hamsters consuming a typical western fat diet, including a reduction in plasma total cholesterol and low-density lipoprotein (LDL)-cholesterol, as well as reductions in the total cholesterol:high-density lipoprotein (HDL)-cholesterol and LDL:HDL ratios (**Figure 4**). The total cholesterol:HDL-cholesterol ratio is considered a particularly effective biomarker for the risk of CVD. While data are limited, several studies provide support that some of the cardioprotective effects of RA-enriched

Table 1 Effect of natural and synthetic CLA on tumor development in a rat model of mammary carcinogenesis

Treatment group	*Tumor incidence*	*Tumor number*
Control	28/30 (93%)^a	92^a
Synthetic *cis*-9, *trans*-11 CLA	16/30 (53%)^b	46^b
VA/RA-enriched butter	15/30 (50%)^b	43^b

$p < 0.05$ for treatment effects, with significant differences indicated by differences in superscript letters (a, b).

CLA, conjugated linoleic acid; RA, rumenic acid (*cis*-9, *trans*-11 CLA); VA, vaccenic acid.

Adapted from Ip C, Banni S, Angioni E, *et al*. (1999) Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *Journal of Nutrition* 129: 2135–2142.

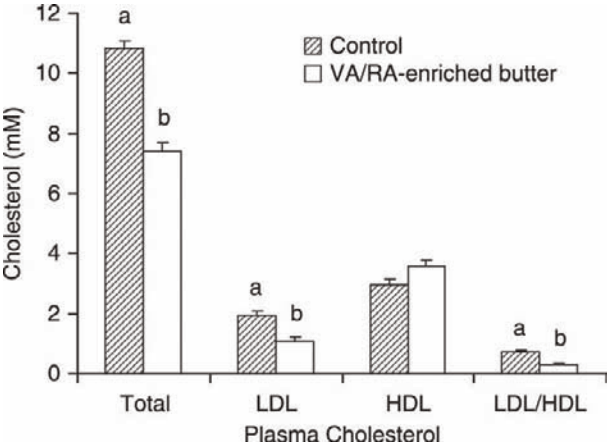

Figure 4 Effect of VA/RA-enriched butter on plasma cholesterol levels in the cholesterol-fed hamster. Based on data from Lock AL, Horne CAM, Bauman DE, and Salter AM (2005) Butter naturally enriched in conjugated linoleic acid, and vaccenic acid alters tissue fatty acids and improves the plasma lipoprotein profile in cholesterol-fed hamster. *Journal of Nutrition* 135: 1934–1939. Standard error is indicated by error bars over each column. $p < 0.01$ for treatment effects with significant differences indicated by differences in letters (a, b) over the columns. LDL, low-density lipoprotein; HDL, high-density lipoprotein; RA, rumenic acid; VA, vaccenic acid.

dairy products reported in animal models will extend to humans.

Consumer acceptability and processing considerations are also important aspects in evaluating the functional food potential of 'CLA-enriched' products. Studies have found no differences in consumer acceptability and shelf life between regular and CLA-enriched dairy products. In addition, the processing of milk has little or no effect on CLA and the end product content is essentially the same as the RA content of the original milk fat. However, it is important to remember that since RA is a fatty acid, the amount in foods will vary according to the product's total fat content. Therefore, dairy products like cheese, ice cream, butter, and whole milk will provide most CLA to the human diet. Current dietary recommendations are to maintain an appropriate fat intake and a healthy balance of saturated and unsaturated fats. By consuming dairy products with an enhanced content of RA and VA, higher levels of these beneficial fatty acids can be obtained while contributing to this balance and not increasing the intake of saturated fat.

Finally, extending the results from biomedical models to implications for RA as a functional food component in humans is challenging and problematic. First, many chronic diseases such as cancer and CVD have long latency periods and lack consensus biomarkers; measuring the potential of a functional food component thus proves difficult and costly in extended studies on humans. Second, the RA and VA content of dairy products is highly variable even when expressed on a fat basis, and there are difficulties in the analysis of these fatty acids in foods; thus, it is problematic to achieve an accurate estimate of intake of RA over the long latency period of these diseases. Overall, the results from biomedical studies with animal models continue to provide exciting evidence of the beneficial health effects of RA, but the functional food role of RA in health maintenance and the prevention of chronic diseases in humans remains to be clearly established.

See also: **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat. **Milk**: Milk in Human Health and Nutrition. **Milk Lipids**: Fatty Acids; Nutritional Significance. **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake.

Further Reading

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Triacylglycerols

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Introduction

Bovine milk fat consists of a complex mixture of triacylglycerols, which vary considerably in molecular weight and degree of unsaturation. Despite the complexity, the major triacylglycerols can be broadly categorized into two structural types, those containing combinations of long-chain fatty acids and those containing two long-chain fatty acids and a short-chain fatty acid esterified at position *sn*-3. The latter type is found in significant quantities only in ruminant milk fats. The melting properties of milk fat are determined largely by the fatty acid composition of its constituent triacylglycerols.

The most important class of compounds in bovine milk fat, from a quantitative viewpoint, are by far triacylglycerols, accounting for 98% of the total fat. Triacylglycerols are relatively simple, nonpolar molecules, consisting of a glycerol backbone to which are esterified three fatty acid molecules (**Figure 1**). Although triacylglycerols are not highly reactive, they do undergo two important chemical reactions, namely hydrolysis and oxidation, which cause deterioration in the quality of food (*see* **Milk Lipids**: Lipolysis and Hydrolytic Rancidity; Lipid Oxidation).

The complex nature of triacylglycerols of bovine milk fat is a direct result of the large number and wide variety of fatty acids that make up the triacylglycerols. Although the distribution of the fatty acids on the triacylglycerol molecules is not entirely random, the number of distinct triacylglycerols arising from the combination of a large number of fatty acids is in the order of several hundred. For example, if only the 14 acids that are present at concentrations above 1% are considered and if the position on the triacylglycerol molecule is ignored, then there are a possible 560 compositionally different triacylglycerols (this figure is derived from the equation $N=(n(n+1)\times(n+2)/6)$, where N is the number of triacylglycerols and n is the number of fatty acids). Because of this complexity, the determination of individual triacylglycerols is a very difficult task.

The general composition of milk triacylglycerols, determined by capillary gas chromatography, is presented in **Table 1**. The triacylglycerols show a wide range of molecular weight (from carbon number 28 to 56), which arises from the large difference in the chain length of the constituent fatty acids (from C4 to C18). Interestingly, the range of values for the different carbon numbers is considerable, indicating that there is significant variation in triacylglycerol composition throughout the dairying season.

It should be noted that there are a number of individual triacylglycerols having the same carbon number; for example, C50 could possibly include the triacylglycerols 16:0, 16:0, 18:0; 14:0, 18:0, 18:0; 16:0, 16:0, 18:1; and 14:0, 18:0, 18:1.

Structure of Triacylglycerols

An initial examination of the glycerol molecule may suggest that positions *sn*-1 and *sn*-3 are equivalent (**Figure 2**). However, further examination of configurations A and B shows that when the three-dimensional arrangement of the bonds involving the central carbon is taken into account, the two configurations cannot be superimposed. Thus the carbon atom at position sn-1, in relation to the other groups attached to the central carbon, is different from the carbon atom at position sn-3.

Triacylglycerols are synthesized in the mammary gland by enzymatic reactions that normally involve multi-point attachment, and consequently the spatial arrangement of neighboring groups is critical. This means that it is possible for enzymes to distinguish between positions *sn*-1 and *sn*-3 and the enzymic mechanisms involved in the biosynthesis of triacylglycerols exert some selectivity over the placement of fatty acids at the different positions.

Stereospecific analytical procedures have been developed that have permitted the determination of the positional distribution of fatty acids on the triacylglycerols. Results obtained by several workers have shown that there is a general pattern of stereospecific distribution of fatty acids in the triacylglycerols of bovine milk fat (**Table 2**). For cows fed a normal diet, the fatty acids 4:0 and 6:0 are esterified almost entirely at position *sn*-3. In contrast, 12:0 and 14:0 are esterified preferentially at position *sn*-2 while 16:0 is incorporated preferentially at positions *sn*-1 and *sn*-2. 18:0 is esterified preferentially at position *sn*-1 and 18:1 shows a preference for positions *sn*-1 and *sn*-3. This overall pattern of fatty acid distribution does not change significantly either throughout the dairying season or between countries. This indicates that

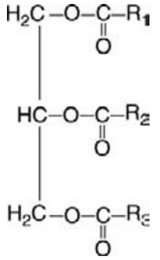
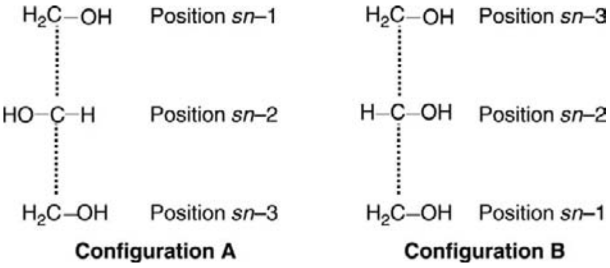


Figure 1 Triacylglycerol molecule.

Table 1 Composition of triacylglycerols of milk fat

	Triacylglycerol composition (mol%)	
Carbon number^a	*Typical*	*Range*^{b}
C28	0.8	0.5–1.1
C30	1.5	1.0–1.8
C32	3.1	2.3–3.6
C34	6.8	5.3–7.5
C36	12.5	10.6–13.3
C38	14.5	13.2–15.9
C40	11.2	10.2–12.6
C42	7.4	6.6–7.9
C44	6.5	5.4–7.0
C46	6.7	5.2–7.2
C48	7.7	6.2–9.0
C50	9.3	8.4–11.4
C52	7.9	6.4–10.9
C54	3.8	2.7–5.9
C56	0.4	0.3–0.5

[a] Carbon number is the sum of the acyl carbons on the triacylglycerol molecule.
[b] Range of values over a dairying season.
A.K.H. MacGibbon (personal communication), Fonterra Research Centre seasonal survey.



H_2C–OH Position *sn*–1
HO–C–H Position *sn*–2
H_2C–OH Position *sn*–3
Configuration A
H_2C–OH Position *sn*–3
H–C–OH Position *sn*–2
H_2C–OH Position *sn*–1
Configuration B

Figure 2 Fischer projection diagrams of glycerol using the stereospecific numbering (*sn*-) convention. Configuration B is obtained from configuration A by rotating the molecule through 180 °.

in normal milk fats, in which fatty acids vary to some extent as a result of dietary and lactational effects, the overall pattern of positional specificity of fatty acids is relatively constant.

When a cow is placed on a restricted diet, there is a decrease in the fatty acids that are synthesized de novo in the mammary gland (4:0 to 16:0) and an increase in the fatty acids 18:0 and 18:1 as a result of the mobilization of body reserves. The net effect is a marked increase in the proportion of 18:1 at position *sn*-3 from 17.1 to 47.6%, which is accompanied by reductions in the proportions of 4:0 to 16:0 at this position (**Table 2**). Despite these marked changes caused by the restricted diet, the overall pattern of stereospecific distribution of fatty acids is altered only slightly.

Composition of Triacylglycerols

Although milk fat contains a very complex mixture of triacylglycerols, it is possible to fractionate these triacylglycerols into different classes according to their molecular weight and degree of unsaturation using a combination of chromatographic methods. This approach has enabled a detailed analysis of the different classes of triacylglycerols in milk fat.

Milk fats from different countries have been separated in this manner and the proportions of the different fractions are given in **Table 3**. The proportion of saturated triacylglycerols ranges from 33 to 39% and is high relative to other fats and oils. For example, many vegetable oils contain only very small amounts of saturated triacylglycerols, and even palm oil, which contains about 40% 16:0, has only 8–10% saturated triacylglycerols. A further feature of bovine milk fat is the high proportion of low-molecular-weight triacylglycerols (31–44%). These triacylglycerols largely consist of two long-chain fatty acids in combination with a short-chain fatty acid and are found in significant quantities only in ruminant milk fats.

The proportions of corresponding classes (i.e., similar molecular weight and the same degree of unsaturation) in the three milk fats are broadly similar, with saturated and monoene triacylglycerols being the dominant classes. One interesting exception is the lower amount of high-molecular-weight saturated triacylglycerols reported in Canadian milk fat.

Stereospecific analyses of the different triacylglycerol classes have shown that the general pattern of positional distribution of fatty acids of chain length C4–C16 in these classes is similar to that in normal milk fat. However, the pattern of distribution of 18:0 and 18:1 varies according to the molecular weight of the triacylglycerols; these fatty acids tend to be esterified preferentially at positions *sn*-1 and *sn*-3 in triacylglycerols of high molecular weight and concentrated at position *sn*-1 in triacylglycerols of medium and low molecular weight where 4:0 and 6:0 occupy much of the *sn*-3 position. A further interesting point is that the *sn*-3 position in unsaturated triacylglycerols of

Table 2 Positional distribution of fatty acids in the triacylglycerols of milk fat

	Fatty acid composition (mol%)					
	Normal diet			*Restricted diet*		
Fatty acid	sn-1	sn-2	sn-3	sn-1	sn-2	sn-3
4:0		0.4	30.6	0.4		26.8
6:0		0.7	13.8	0.7	0.4	9.2
8:0	0.3	3.5	4.2	0.7	2.6	1.8
10:0	1.4	8.1	7.5	1.1	5.6	2.4
12:0	3.5	9.5	4.5	2.1	6.7	
14:0	13.1	25.6	6.9	7.4	17.4	
16:0	43.8	38.9	9.3	32.6	40.7	2.1
18:0	17.6	4.6	6.0	20.9	5.6	10.1
18:1	19.7	8.4	17.1	34.0	21.1	47.6

Calculated from the data of Parodi PW (1979) Stereospecific distribution of fatty acids in the triglyceride classes of milk fat. *Journal of Dairy Research* 49: 75–81.

Table 3 Proportions of triacylglycerol classes in bovine milk fat from different countries[a]

Nature of triacylglycerols		*Proportions of triacylglycerols (percentage in milk fat)*		
Molecular weight	*Level of unsaturation*[a]	*New Zealand milk fat*	*Australian milk fat*	*Canadian milk fat*
High	Saturated	11.4	12.7	6.4
	Monoene	15.6	19.8	14.4
	Diene	8.7	10.1	10.8
	Triene	4.2	3.7	5.0
	Polyene			2.4
	Total	39.9	46.3	39.0
Medium	Saturated	7.7	9.3	6.6
	Monoene	6.3	8.6	6.6
	Diene	1.7	2.5	2.5
	Triene	1.2	2.3	1.5
	Polyene			
	Total	16.9	22.7	17.2
Low	Saturated	20.1	14.5	19.7
	Monoene	16.1	12.0	16.7
	Diene	4.3	2.8	5.1
	Triene	2.7	1.7	2.3
	Polyene			
	Total	43.2	31.0	43.8
Milk fat	Saturated	39.2	36.5	32.7
	Monoene	38.0	40.4	37.7
	Diene	14.7	15.4	18.4
	Triene	8.1	7.7	8.8
	Polyene			2.4
	Total	100.0	100.0	100.0

[a]Saturated, monoene, diene, and triene triacylglycerols contain zero, one, two, and three carbon–carbon double bonds, respectively. Data from Hawke JC and Taylor MW (1995) Influence of nutritional factors on the yield, composition and physical properties of milk fat. In: Fox PF (ed.) *Advanced Dairy Chemistry, Vol. 2: Lipids*, 2nd edn., pp. 37–88. London: Chapman & Hall.

low molecular weight consists largely of short-chain fatty acids and 18:1. The *sn*-3 position is the last to be acylated during triacylglycerol biosynthesis and it has been postulated that the placement of these fatty acids at this position may well be to keep the fat liquid at physiological temperatures.

In recent times, the use of combinations of chromatographic techniques has been refined further to permit the separation and identification of many of the triacylglycerols in milk fat. Complex mixtures of triacylglycerols are subdivided into much simpler mixtures prior to the separation and identification of individual triacylglycerols.

In one study, milk fat was separated by normal-phase high-performance liquid chromatography and electrospray ionization mass spectrometry (HPLC-ESI-MS) of ammonium ion adducts to enable the identification of over 400 triacylglycerols species in 184 quantified peaks. This exhaustive study included the use of enzymatic *sn*-2 analysis, thin-layer chromatography, and solid-phase extraction (and gas chromatography analysis) to isolate a wide range of fractions for analysis by HPLC-ESI-MS. This allowed their proportion in milk fat, and in many cases the stereoisomeric positions, to be determined. The vast majority of these triacylglycerols were present at very low levels, that is, less than 0.2 mol%. However, a few were present at surprisingly high amounts: for example, in the order of increasing acyl carbon number from quantified peaks 16:0/14:0/4:0 + 18:0/12:0/4:0 (3.4%),16:0/16:0/4:0 + 18:0/12:0/6:0(5.2%), 18:1/16:0/4:0 (4.1%), 18:0/16:0/4:0 (2.9%), 18:1/14:0/16:0 + 16:0/16:1/16:0 (3.1%), 16:0/16:0/16:0 + 18:0/14:1/16:0 (2.4%), 18:1/16:0/18:1 + 18:2/18:0/16:0 (3.7%). These species account for approximately 25% of the milk fat sample analyzed.

These sophisticated separation techniques, in combination with stereospecific analyses, have enabled the major triacylglycerols and many of the minor triacylglycerols in milk fat to be determined. The major triacylglycerols are presented in a somewhat simplistic manner in **Figure 3**. The major high-molecular-weight triacylglycerols comprise combinations of four long-chain fatty acids (14:0, 16:0, 18:0, and 18:1), whereas the major triacylglycerols of medium and low molecular weight contain two of these four long-chain fatty acids and a short-chain fatty acid (either 4:0 or 6:0) esterified at position *sn*-3.

Although these painstaking studies have identified a large number of triacylglycerols in milk fat, the time-consuming nature of these investigations means that it is currently impractical to measure the level of individual triacylglycerols in milk fat on a routine basis.

Crystallization and Melting Behavior of Triacylglycerols

The crystallization and melting characteristics of triacylglycerols of milk fat have a marked effect on the functional properties of dairy products, particularly the consistency of butter. These characteristics are influenced to a large degree by the fatty acid composition of the triacylglycerols and to a lesser extent by the physical processes of polymorphism and mixed crystal formation (see **Milk Lipids:** Rheological Properties and Their Modification).

Triacylglycerols form crystals when they solidify. Generally, triacylglycerols crystallize in layered structures, with glycerol groups and methylene groups in one plane and the axes of the hydrocarbon chains straight and parallel to each other within a layer. The attractive forces acting between molecules are lateral van der Waals' interactions between hydrocarbon chains, van der Waals' interactions of methyl end groups, and polar interactions of ester groups. The melting points of triacylglycerols are determined largely by the packing density of the hydrocarbon chains in the crystal lattice; the greater the packing density, the closer the hydrocarbon chains and the greater the attractive van der Waals' forces, and hence the higher the melting point.

The fatty acid composition of triacylglycerols has a marked effect on their molecular arrangement in the crystal lattice, which in turn affects their packing density. Triacylglycerols consisting of three long, 'straight-chain', saturated fatty acids have a closely packed lattice structure and a high melting point. The replacement of a saturated fatty acid with a 'bent-chain' *cis*-unsaturated fatty acid has a large effect on the molecular arrangement of triacylglycerols in the lattice, which in turn reduces the packing density of hydrocarbon chains and hence lowers the melting point of *cis*-unsaturated triacylglycerols compared to saturated triacylglycerols. In a similar manner, the inclusion of short- and medium-chain fatty acids lowers the melting points of

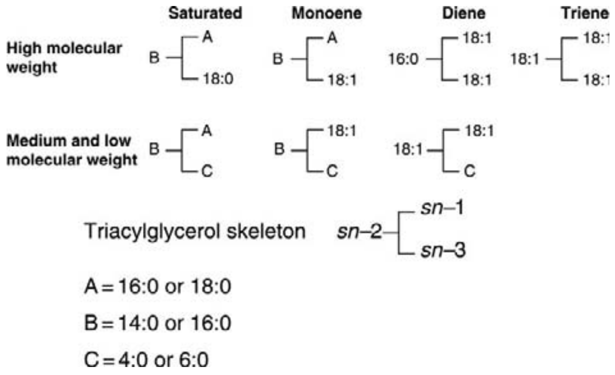


Figure 3 Major triacylglycerols of milk fat.

low-molecular-weight triacylglycerols compared to high-molecular-weight triacylglycerols.

As noted previously, milk fat triacylglycerols vary considerably in both molecular weight and degree of unsaturation and as a result they melt over a wide range of temperature. Indeed, milk fat has a very broad melting range, from −40 to +40 °C. A further consequence of the above trend in melting points is that the higher the proportion of low-molecular-weight and unsaturated triacylglycerols in milk fat, the greater the proportion of low-melting triglycerides and hence the softer the milk fat. New Zealand spring and summer milk fat shows this trend. Spring milk fat contains greater proportions of triacylglycerols containing 4:0 and 18:1 than summer milk fat, which leads to a higher proportion of low-melting triacylglycerols in the former. As a consequence, spring butter is softer than summer butter. Similarly, in Europe, summer butter contains greater proportions of triacylglycerols containing 18:1 than winter butter and consequently is softer.

See also: **Milk Lipids**: Fatty Acids; Lipid Oxidation; Lipolysis and Hydrolytic Rancidity; Rheological Properties and Their Modification.

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Phospholipids

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Introduction

Although polar lipids are only a very small proportion of the lipids in milk, they play an important role due to their mixed hydrophilic and hydrophobic nature. Their dual hydrophilic and hydrophobic structure contributes largely to stabilization of the suspension of milk fat in the aqueous environment of liquid milk, thus allowing the relatively high concentrations of milk fat and protein to coexist in the same solution. This is accomplished by the integral part that polar lipids play in the milk fat globule membrane (MFGM) and, to a lesser extent, the general emulsification properties in the aqueous solution. The major structural features involved are the large nonpolar (hydrophobic) fatty acid chains and the polar (hydrophilic) charged head group residues of the phospholipids.

General Features

Lipids are usually classified on the basis of polarity as neutral lipids or polar lipids. However, an alternative classification often quoted is 'simple lipids' and 'complex lipids' (simple lipids yield only two types of primary products on hydrolysis, while complex lipids yield three or more primary products). Under this second definition, polar lipids are complex lipids while neutral lipids are simple lipids.

Polar lipids can contain a variety of polar groups that contribute to the charged nature of the molecule. Phospholipids contain a charged phosphate group (**Figure 1**), whereas glycolipids contain polar carbohydrate groups, which increase the solubility in water (**Figure 2**). While phospholipids are the major polar lipid components of milk, glycolipids also need to be considered as they have important biological functions, especially in nerve and brain function. In humans, they are involved in a number of disease states such as Tay–Sachs disease, which is characterized by an enzyme deficiency causing the accumulation of a specific ganglioside. Common abbreviations for polar lipids are given in **Table 1**.

Structure

The structures of polar lipids found in milk are shown in **Figures 1** and **2**. Phosphatidic acid has fatty acids at positions *sn*-1 and *sn*-2 and a phosphate group at the *sn*-3 position. The glycerol phospholipids (e.g., phosphatidylcholine and phosphatidylethanolamine) are based on phosphatidic acid with a moiety attached to the phosphate at the *sn*-3 position. Plasmalogens (not shown in the figures) have a similar structure to phosphatidylcholine and phosphatidylethanolamine but with an ether linkage rather than an ester linkage at the *sn*-1 position. Lysophospholipids have only one fatty acid in the glycerophospholipid. These are the products of phospholipases, enzymes that can degrade phospholipids. The sphingophospholipid, sphingomyelin, consists of a ceramide (a fatty acid linked to a sphingosyl base through an amide linkage) and a phosphorylcholine group. Sphingomyelin is usually included as a phospholipid.

The ionic properties of phospholipids are important in their function as emulsifiers. **Table 2** shows the typical ionization constants of glycerophospholipids, illustrating the charge state of the various phospholipids. At the normal pH of milk (pH 6.7), the properties can range from a zwitterion, with no net charge (phosphatidylcholine, phosphatidylethanolamine), to an acid with a net negative charge (phosphatidylserine, phosphatidylinositol).

Glycoceramides (glycosphingolipids) have one or more hexose sugar units attached at the 1-position of the ceramide (**Figure 2**). Gangliosides are complex ceramide polyhexosides, which contain one or more sialic acid groups (*N*-acetylneuraminic acid (NANA)). The specific names of the gangliosides identify their structure (the letter G followed by M, D, T, or Q designates the mono-, di-, tri-, or quatrasialic acid group and the number indicates the carbohydrate sequence that is attached to the ceramide (5−*n*, where *n* is the number of neutral sugar residues)). For example, GM3 is a ganglioside with one NANA unit and two neutral sugar residues (**Figure 2**).

A number of gangliosides have been isolated from bovine milk (GM3, GM2, GM1, GD3, GD2, GD1). The major gangliosides are GD3 (50%) and GM3 (20%) (**Figure 2**).

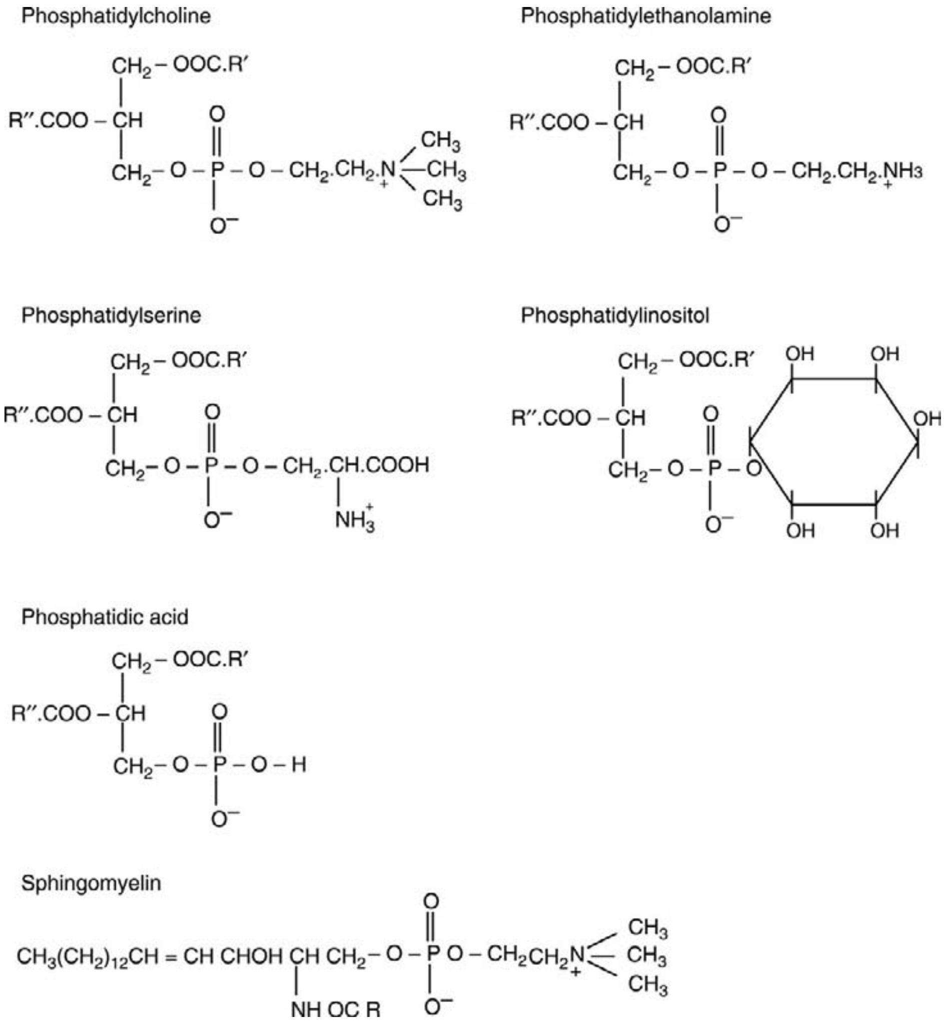

Figure 1 Phospholipid structures. R′ and R″ are fatty acids at *sn*-1 and *sn*-2 positions, respectively.

Source

The MFGM that surrounds the milk fat droplets is derived from the apical plasma membrane of secretory cells in the lactating mammary gland. The MFGM is composed of phospholipids and glycolipids as well as protein, glycoprotein, enzymes, triacylglycerols, and minor components. Estimates of the proportion of phospholipids in the MFGM vary from 15 to 30%.

About 60–65% of the phospholipids in milk are associated with the MFGM. The remaining 35–40% are found in the aqueous phase associated with protein/membrane fragment material in solution rather than still attached to the MFGM.

Polar lipids constitute about 0.5–1% of total milk lipids. This percentage of phospholipids does vary a little with the stage of lactation. Phospholipid levels in milk tend to decline during lactation, although they can increase near the end. While there is a small change in the percentage of phospholipids, the ratio of major phospholipids remains relatively constant, suggesting a constant ratio in the MFGM.

Table 3 shows the percentage of polar lipids in bovine milk. Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are the major components and they contribute equally to the total polar lipid content. Phosphatidylserine and phosphatidylinositol are present at lower levels and there are only trace (but important) amounts of ceramides and gangliosides.

As the milk is processed, the phospholipids are partitioned differently from the neutral lipids (**Table 4**). When the whole milk is separated, the phospholipids tightly associated with the MFGM go into the cream with the neutral lipids, while those associated with the protein/membrane fragments in the aqueous phase are retained in the skim milk. Hence, the phospholipids-to-total fat ratio is greater in whole milk than in cream. Furthermore, during buttermaking, a greater proportion of the phospholipids than the

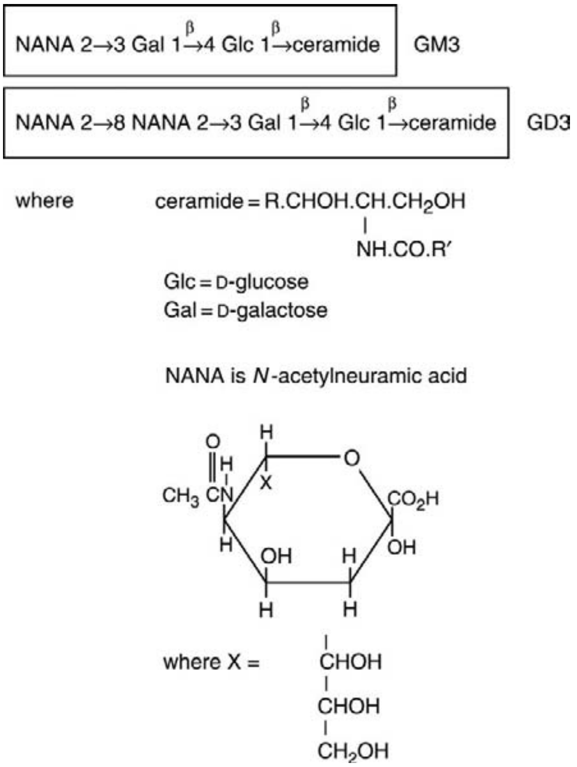
NANA 2→3 Gal 1→4 Glc 1→ceramide
β
β
GM3
NANA 2→8 NANA 2→3 Gal 1→4 Glc 1→ceramide
GD3
where
ceramide = R.CHOH.CH.CH2OH
NH.CO.R′
Glc = D-glucose
Gal = D-galactose
NANA is N-acetylneuramic acid
O
H
CH3
C
N
X
O
CO2H
OH
OH
H
where X =
CHOH
CHOH
CH2OH

Figure 2 Structure of gangliosides (GM3 and GD3).

Table 1 Common abbreviations for polar lipids

Abbreviation	*Full name*
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PI	Phosphatidylinositol
PA	Phosphatidic acid
Sph (or SM)	Sphingomyelin
CMH	Ceramide monohexoside
CDH	Ceramide dihexoside
NANA	*N*-acetylneuraminic acid (sialic acid)
MFGM	Milk fat globule membrane

neutral lipids from the cream are retained in the buttermilk, leading to a high ratio of phospholipids to total fat.

Chemical Properties

The positional distribution of fatty acids in the major phospholipids of bovine milk (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) is shown in **Table 5**. Unlike triacylglycerols, phospholipids do not have short-chain fatty acids ($C_{14:0}$ being the shortest chain fatty acid that is significant). In glycerophospholipids, the unsaturated fatty acids, especially the polyunsaturated fatty acids, tend to be preferentially esterified at the *sn*-2 position, while the saturated fatty acids are at the *sn*-1 position. There is a range of degree of unsaturation among the specific phospholipids. Phosphatidylethanolamine has a high content of unsaturated fatty acids, especially linoleic acid and even linolenic acid, far higher than that found in the milk fat triacylglycerols. The unsaturated fatty acids are found predominantly at the *sn*-2 position, while the $C_{18:1}$ is fairly evenly distributed and the $C_{18:0}$ and $C_{16:0}$ are at the *sn*-1 position. On the other hand, phosphatidylcholine is more saturated than phosphatidylethanolamine, and the distribution of saturated and unsaturated fatty acids is less distinct between the *sn*-1 and *sn*-2 positions, although the $C_{18:0}$ and the polyunsaturated fatty acids still show the preference described above.

Sphingomyelin has a different structure (**Figure 1**). The major fatty acid moiety is either $C_{16:0}$ or a longer-chain fatty acid ($C_{22:0}$ to $C_{24:0}$), producing an almost completely saturated fatty acid composition. However, the major sphingoid base is sphingosine ($C_{18:1}$), which introduces unsaturation into the molecule.

Analysis

Milk phospholipids can be extracted with slightly polar solvents such as methanol, ethanol, or chloroform.

Table 2 Typical ionization constants (pK_a) of the polar groups of phospholipids, ionic strength 0.1 NaCl

Ionizable group	*Phosphatidyl choline*	*Phosphatidylethanolamine*	*Phosphatidylserine*	*Phosphatidylinositol*	*Phosphatidic acid*
Phosphate	1.0	1.7	2.6	2.5	3.0
Phosphate (2)					8.0
Carboxylic			5.5		
Ammonium		11.2	11.5		
pH range of neutral charge	3–12	4–9	4		
Type	Zwitterion	Zwitterion	Weakly acidic	Acidic	Acidic

Values determined in 0.1 mol l^{-1} NaCl. Values will be altered for higher ionic strength or in emulsions and membranes.

Table 3 Polar lipid composition of bovine milk

Polar lipid	*Percentage of total polar lipids*[a]
Phosphatidylcholine	34.5
Phosphatidylethanolamine	31.8
Phosphatidylserine	3.1
Phosphatidylinositol	4.7
Sphingomyelin	25.2
Plasmalogens	3.0
Ceramides	Trace
Gangliosides	Trace

[a] Polar lipids account for 0.5–1% of total lipids in milk.
From Patten and Jensen (1976).

Acetone is usually a poor solvent for phospholipids and can be used for precipitation and concentration.

Thin-layer chromatography is a simple and useful technique for the qualitative determination of phospholipid groups. With the use of appropriate stains and standards, the position of most of the specific phospholipids can be determined. More quantitative analysis can be carried out by high-performance liquid chromatography (HPLC) and mass spectroscopy. The fatty acid composition of glycerophospholipids can be determined by fatty acid methyl ester (FAME) analysis, as for triacylglycerols. The fatty acids are hydrolyzed from glycerol, esterified, and then analyzed by gas chromatography. However, the fatty acid of the sphingophospholipid sphingomyelin is more difficult to release due to the attachment by an amide bond and requires extensive acid hydrolysis prior to methylation of the released fatty acid.

Features

Dairy phospholipids are of major importance because they are able to stabilize emulsions and foams, and contribute to the formation of micelles and membranes. Phospholipids also have the potential to be prooxidants, because they contain mono- and polyunsaturated fatty acids and have the ability to attract metal ions. Mono- and polyunsaturated fatty acids and metal ions are known to accelerate lipid oxidation, especially when heat is applied; hence, the phospholipids can be lost

Table 4 Approximate lipid content of different milk products

Product	*Total fat wt%*	*Phospholipids wt%*	*Phospholipids/total fat %*
Whole milk	4	0.035	0.9
Skim milk	0.06	0.015	25
Cream 40%	40	0.21	0.5
Buttermilk	0.6	0.13	22

Adapted from Mulder H and Walstra P (1974) *The Milk Fat Globule*. Farnham Royal, UK: Commonwealth Agriculture Bureaux.

Table 5 Fatty acid composition of the major phospholipids of bovine milk

	Phosphatidylcholine		*Phosphatidylethanolamine*		
Fatty acid	sn-*1*	sn-*2*	sn-*1*	sn-*2*	*Sphingomyelin*[a,b]
14:0	5.6	10.8	1.9	1.3	2.6
16:0	41.9	30.6	19.7	4.7	22.9
16:1	0.6	1.2	1.2	2.2	0.8
18:0	17.5	2.4	19.0	1.3	4.7
18:1	20.3	27.8	45.8	47.8	5.2
18:2	2.7	9.2	2.9	21.4	0.9
18:3	0.8	1.8	1.1	4.5	
20:3		1.6	0.2	2.2	
20:4	0.2	1.2	0.2	3.0	
22:0					15.2
23:0					28.0
23:1					1.0
24:0					15.4
25:0					0.6

[a] From Morrison WE, Jack EL, and Smith LM (1965) Fatty acids of bovine milk glycolipids and phospholipids and their specific distribution in the diacylglycerophospholipids. *Journal of the American Oil Chemical Society* 42: 1142–1147.
[b] Fatty acid linked to amide nitrogen of the base.

during processing. However, in dairy products, the situation is complex and it appears that the phospholipids act as both prooxidants and antioxidants, depending on pH and the water content and phospholipid species.

In addition to their importance in cell membranes and cell signaling, specific polar lipids are reputed to have a number of positive health effects related to immune system, heart health, brain health, and cancer. These are related to either the polar lipids themselves or their breakdown products.

Sphingomyelin, gangliosides, phospholipids, and plasmalogen are implicated in different aspects of prevention of cancer. Further research will elucidate whether dairy polar lipids have unique properties related to their fatty acid composition or are simply another source of generic polar lipids.

See also: **Milk Lipids**: General Characteristics; Milk Fat Globule Membrane; Nutritional Significance.

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Fat Globules in Milk

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Introduction

By definition, lipids (fats and oils) are insoluble in water and, since they are less dense than water, a mixture of water and oil will form two immiscible layers, with the lipid layer on top. If the mixture is subjected to shearing forces, the mass of oil is dispersed into spherical droplets (globules), which, when shearing ceases, coalesce and reform a surface layer. The mutual insolubility of oil and water results in interfacial tension between the phases, which is responsible for the spherical shape of the globules (a sphere has the smallest surface area-to-volume ratio of all geometrical shapes). If the interfacial tension is reduced by adding a surface-active agent (detergent, surfactant, emulsifier), the globules coalesce slowly or not at all, although they still rise to the surface (cream) due to the difference in density between the fat and aqueous phases. This system is an oil-in-water (o/w) emulsion, that is, a continuous water phase in which is dispersed a discontinuous oil phase; although these two phases are immiscible, they are made compatible by a small volume of a third phase, an emulsifier. Milk and cream are examples of an o/w emulsion. In butter, the continuous phase is oil in which are dispersed droplets of water, some intact fat globules, fat crystals, air bubbles, and some protein; thus, butter is a water-in-oil (w/o) emulsion.

In 1674, Anton van Leeuwenhoek reported that the fat in milk exists as microscopic globules, that is, as an o/w emulsion. Since then, the properties of milk fat globules have been studied extensively and have been found to have a marked influence on many properties of milk, for example, color, mouthfeel, and viscosity. The globules in bovine milk range in diameter from 0.1 to 20 μm, with a mean of 3–4 μm (the range and mean vary with the species, breed and health of the animal, stage of lactation, etc.). The size and size distribution of fat globules in milk may be determined by light microscopic examination, light scattering, for example, using a Malvern Mastersizer, or electronic counting devices, for example, the Coulter counter. In bovine milk, 75% of all globules have a diameter <1 μm but these represent only a small proportion of the total volume or mass of the fat. The mean fat globule size in milk from Channel Island breeds (Jersey and Guernsey) is larger than the mean fat globule size in milk from other breeds (the fat content of the milk from Channel Island breeds is also higher), and for all breeds, the size decreases by about 25% throughout lactation.

Milk contains 15×10^9 globules ml^{-1}, with a total interfacial area of 1.2–2.5 m^2 g^{-1} fat. This very large interfacial area means that the properties of the interfacial emulsifying layer play a very important role in determining the behavior of the fat globules. Emulsification of the fat in milk is achieved not by shearing but rather by a process in which the triglycerides synthesized within the mammary cells acquire a layer of emulsifier (protein) and become surrounded by the apical membrane of the secretory cell as they are excreted from the cell into the lumen of the alveolus (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat). Thus, the emulsifying layer in the natural milk emulsion is much more complex than the emulsifying layer formed by an artificial emulsifier (*see* **Milk Lipids**: Milk Fat Globule Membrane).

As far as is known, the fat in the milk of all species occurs as globules and the properties of these globules are generally similar for all species, although there are some significant interspecies differences. This article will focus on some properties of the fat globules in bovine milk; some properties of the milk lipids in other dairying species are described briefly in a number of other articles (*see* **Milk**: Buffalo Milk, Equid Milk, Goat Milk, and Sheep Milk).

Stability of the Milk Fat Emulsion

Owing to interfacial tension, lipid emulsions are inherently unstable systems and are susceptible to flocculation, coalescence, deemulsification, and phase inversion. Although interfacial tension is reduced by the use of an emulsifier, the interfacial film may be imperfect. When two globules collide, they may adhere (flocculate), for example, by sharing emulsifier, or they may coalesce due to the Laplace principle, which states that the pressure is greater inside small globules than inside large globules and hence there is a tendency for large fat globules (or gas bubbles) to grow at the expense of smaller ones. Taken to the extreme, this will lead to the formation of a continuous mass of fat. The rate of destabilization is influenced by the fat content, shear rate (motion), liquid-to-solid fat ratio, inclusion of air, and globule size.

Another destabilizing factor arises from the difference in density between the lipid and aqueous phases (0.9 and

1.036 g cm^{-3}, respectively, for milk), which causes the fat globules to float or cream according to the Stokes' equation:

$$V = \frac{2r^2(\rho_1 - \rho_2)g}{9\eta} \quad (1)$$

where V is the rate of creaming, r the radius of fat globules, ρ_1 and ρ_2 are the densities of the continuous and dispersed phases, respectively, g is the acceleration due to gravity, and η the viscosity of the system. If creaming is not accompanied by other changes, it is readily reversible by gentle agitation.

The stability, or instability, of the milk fat emulsion is very significant with respect to many physical and chemical characteristics of milk and dairy products. The stability of the emulsion depends strongly on the integrity of the milk fat globule membrane (MFGM), which is quite fragile and is more or less extensively changed during dairy processing operations (*see* **Milk Lipids:** Milk Fat Globule Membrane).

In the following, some of the principal aspects and problems related to or arising from the stability of the milk fat emulsion are discussed. Some of these relate to the inherent instability of emulsions in general, while others are specifically related to the milk system.

The Creaming Process in Milk

A cream layer may be evident in milk within 20 min of milking. If formed as a result of the rise of individual globules of 4 μm diameter according to Stokes' equation, it would take approximately 50 h for a cream layer to form. The very rapid rate of creaming in milk is caused by the clustering of globules to form approximate spheres, up to 1 mm in diameter. As milk is drawn from the cow, the fat exists as individual globules and the initial rate of rise is proportional to the radius of the individual globules. Cluster formation is promoted by the disparity in the size of the fat globules in milk. Initially, the larger globules rise several times faster than the smaller ones and consequently overtake, collide with, and flocculate with the slower-moving small globules, forming clusters, which rise at an increased rate, collide with and aggregate with more globules, and continue to rise at a rate commensurate with the increased radius. The rate of creaming of clusters only approximates to Stokes' equation, since they are irregular in geometry and contain occluded serum, and therefore the difference between the aqueous and lipid phases is variable.

In 1889, Babcock postulated that creaming of cows' milk results from an agglutination type of reaction, similar to the agglutination of red blood cells; this hypothesis has been confirmed. Creaming is enhanced by adding blood serum or colostrum to milk; the responsible agents are immunoglobulins (Igs, which are present at high levels in colostrum), especially IgM. Because these Igs aggregate and precipitate at temperatures <37 °C and redisperse on warming, they are often referred to as cryoglobulins. When milk is cooled, the cryoglobulins precipitate on to the surface of the fat globules, causing them to agglutinate, probably through a reduction in surface (electrokinetic) potential. The cryoprecipitated globulins may also form a network in which the fat globules are entrapped. The clusters can be dispersed by gentle stirring and are completely dispersed on warming to 37 °C or higher. Creaming is strongly dependent on temperature and does not occur above 37 °C. The fat globules in the milk of buffaloes, sheep, and goats do not exhibit flocculation and these milks cream very slowly. The milk of some cows also exhibits little or no flocculation; apparently, this is a genetic trait, probably related to a deficiency of cryoglobulins.

The rate of creaming and the depth of the cream layer vary considerably. The concentration of cryoglobulins might be expected to influence the rate of creaming and although colostrum (rich in Ig) creams well and late-lactation milk (deficient in Ig) creams poorly, there is no correlation in mid-lactation milks between Ig concentration and the rate of creaming. An uncharacterized lipoprotein appears to act synergistically with cryoglobulin in promoting clustering. The rate of creaming is increased by increasing the ionic strength and is retarded by acidification. High-fat milk, which also tends to have a higher proportion of larger fat globules, creams quickly, probably because the probability of collisions between globules is greater and because large globules tend to form larger aggregates. The depth of the cream layer in high-fat milks is also greater than might be expected, owing to the greater dead space in the interstices of aggregates formed from large globules.

The rate of creaming and the depth of the cream layer are markedly influenced by processing operations. Creaming is faster and more complete at low temperatures, due to the temperature-dependent precipitation of the cryoglobulins. Gentle agitation during the initial stages of creaming promotes cluster formation and creaming, possibly because of an increased probability of collisions, provided that the globules adhere when they collide. It would be expected that stirring cold milk would lead to the deposition of all the cryoglobulin on to the fat globule surfaces and that rapid creaming, without a time lag, would occur when stirring ceased. However, milk treated in this way does not cream at all or creams only slightly after a prolonged lag period. If cold, creamed milk is agitated gently, the clusters are dispersed and do not reform unless the milk is rewarmed to 40 °C and then recooled, that is, the whole cycle is repeated. Vigorous agitation is detrimental to creaming due to denaturation of the cryoglobulins and/or alteration to the fat globule surface. If milk is separated at ≥40 °C, the cryoglobulins are present predominantly in the serum, while they are in the cream produced at a lower temperature. Agglutination and creaming are impaired or prevented by heating (e.g., 70 °C for 30 min or 77 °C for 20 s) owing to denaturation of the cryoglobulins; addition of Igs to heated milk restores creaming (except after very severe heat treatment, e.g., 2 min at 95 °C or equivalent). Homogenization prevents creaming due to the reduction of fat globule size, possibly due to the denaturation of Ig and due to the increased density of the fat globules owing to the newly attached casein membrane. Two types of Ig appear to be involved in agglutination, of which one is denatured by heating and the other by homogenization. A blend of unhomogenized cream and homogenized skim milk does not cream well.

Influence of Processing Operations on the Fat Globule Membrane

The MFGM is relatively fragile and susceptible to damage during any of several processing operations; consequently, emulsion stability is reduced by dislodging interfacial material by agitation, homogenization, heat treatment, concentration, drying, or freezing. Rearrangement and/or loss of the membrane increases the susceptibility of the fat to hydrolytic rancidity, light-activated flavors, and oiling-off of the fat, but reduces susceptibility to metal-catalyzed oxidation. The influence of the principal dairy processing operations on the MFGM and concomitant defects are discussed below.

Milk Supply: Hydrolytic Rancidity

The production of milk on the farm and transportation to the processing plant can potentially cause major damage to the MFGM. The membrane may be damaged at several stages of the milking operation: foaming due to air sucked in at teat cups, agitation due to vertical sections (risers) in milk pipelines, constrictions and/or expansion in pipelines, pumps (especially if operating below capacity), surface coolers, agitators in the bulk tank, and freezing of milk on the walls of bulk tanks. While some oiling-off and perhaps other physical damage to the milk fat emulsion may accrue from such damage, by far the most serious consequence is the development of hydrolytic rancidity caused by lipoprotein lipase (*see* **Enzymes Indigenous to Milk**: Lipases and Esterases and **Milk Lipids**: Lipolysis and Hydrolytic Rancidity).

Mechanical Separation of Milk

Until 1878, gravity creaming was the only means by which the fat in milk could be recovered for the manufacture of butter or for other purposes. Gravity creaming is relatively efficient, especially in the cold (a fat content of 0.1% in the skim milk phase may be obtained). Milk for the manufacture of Grana Padano and Parmigiano Reggiano cheeses is still partially skimmed by gravity creaming. Incidentally, up to 90% of the bacteria in milk are occluded in the cream layer and are removed from the cheese milk. However, gravity creaming is slow and inconvenient for industrial-scale operations. The centrifugal milk separator, which was developed by Gustav de Laval in Sweden in 1878, permitted the rapid and continuous separation of the fat from milk and revolutionized dairy technology – it was the principal factor responsible for the industrialization of milk processing.

In centrifugal separation, *g* in Stokes' equation is replaced by centrifugal force, $\omega^2 R$, where ω is the speed of angular rotation in rad s^{-1} (2π rad = 360°) and *R* is the distance (cm) of the particle from the axis of rotation. Hence,

$$V = \frac{2r^2(\rho_1 - \rho_2)\omega^2 R}{9\eta} \qquad (2)$$

Thus, the rate of separation is influenced by the radius of the fat globules, the radius and speed of the separator, the difference in the density of the continuous and dispersed phases, and the viscosity of the milk; temperature influences r, $(\rho_1 - \rho_2)$, and η (*see* **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Centrifuges and Separators: Types and Design).

Fat globules <2 μm in diameter are not completely removed by cream separators and since the average size of fat globules decreases with advancing lactation, the efficiency of separation decreases concomitantly. The fat content of cream is regulated by manipulating the ratio of cream to skim milk streams from the separator, which regulates back-pressure. With any particular separator operating under more or less fixed conditions, temperature is the most important variable affecting the efficiency of separation; η decreases with increasing temperature, while r, ρ_1, and ρ_2 change with temperature due to the temperature-dependent cubical expansion. The efficiency of separation increases with temperature, especially in the range 20–40 °C. In the past, separation was usually performed at 40 °C, but modern separators are very efficient, even at low temperatures.

Since the cryoglobulins are entirely in the serum phase at temperatures above 37 °C, cream prepared at such temperatures has poor natural creaming properties and the skim milk foams copiously due to the cryoglobulins. Following separation at low temperatures, most of the cryoglobulins are in the cream phase. Assuming that the cryoglobulins are not denatured and are associated mainly with the fat globules, the viscosity of cream produced by low-temperature separation is much higher than that produced at higher temperatures.

Homogenization

Homogenization is widely practiced in the manufacture of liquid milk and milk products (*see* **Homogenization of Milk**: Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Value Homogenizers). The process involves forcing milk through a small orifice at high pressure (13–20 MPa), usually at about 40 °C (at this temperature, the fat is liquid; homogenization is less effective at a lower temperature when the fat is partially solid). The principal effect of homogenization is to reduce the average diameter of the fat globules to <1 μm (the vast majority of the globules in homogenized milk are <2 μm). Size reduction is achieved through the combined action of shearing, impingement, distension, and cavitation. Following a single passage of milk through a homogenizer, the small fat globules occur in clumps, causing an increase in viscosity; a second-stage homogenization at a lower pressure (e.g., 3.5 MPa) disperses the clumps and reduces the viscosity. Clumping arises from incomplete coverage of the greatly increased emulsion interfacial area during the short passage time through the homogenizer valve, resulting in the sharing of casein micelles by neighboring globules.

By reducing the average diameter of the fat globules, homogenization causes a concomitant increase (four- to sixfold) in the fat–plasma interface. There is insufficient natural membrane to coat the newly formed surface completely or insufficient time for complete coverage to occur, and consequently the globules in homogenized milk are coated by a membrane that consists mostly of casein, with some whey proteins. The membrane of homogenized milk contains 2.3 g protein per 100 g fat (10 mg protein per m^2), which is considerably higher than the level of protein in the natural membrane (0.5–0.8 g per 100 g fat), and is estimated to be 15 nm thick. The casein content in the serum phase of homogenized milk is reduced by about 6–8%.

Homogenization causes several major changes in the properties of milk:

1. Homogenized milk does not cream naturally and the fat is recovered only poorly by mechanical separation. This is due in part to the smaller average size of the fat globules, but failure of the globules in homogenized milk to aggregate is mainly due to the agitation-induced denaturation of some Igs.
2. Homogenized milk is very susceptible to hydrolytic rancidity because the artificial membrane does not isolate the fat from the lipase, which is associated mainly with the casein micelle; consequently, homogenized milk must be pasteurized prior to or immediately after homogenization.
3. Homogenized milk is more susceptible to sunlight-oxidized flavor, which is due to the production of methional from methionine, but is less susceptible to metal-catalyzed lipid oxidation, presumably because the phospholipids, which are very susceptible to oxidation (they are rich in polyunsaturated fatty acids) and are located largely in the natural membrane (which contains prooxidants, e.g., xanthine oxidoreductase and metals), are distributed more uniformly after homogenization and, therefore, are less likely to propagate lipid oxidation.
4. Homogenized milk is whiter due to finer dispersion of the fat (and thus greater light scattering) and its flavor is more bland.
5. The heat stability of whole milk is reduced by homogenization, as is the strength (curd tension) of rennet-induced gels. Viscosity is increased for unknown reasons, probably independent of changes in globule size.
6. Homogenized milk has improved foaming characteristics, perhaps due to the release of foam-promoting proteins from the natural membrane or due to the reduction in fat globule size – small globules are less likely to damage foam lamellae. Homogenization reduces surface tension, possibly due to the inclusion of very surface-active proteins in the artificial membrane and due to the changes in the fat globule surface.
7. Homogenized milk drains cleanly from the sides of a glass bottle or drinking glass, probably due to the disruption of large protein and lipoprotein complexes.

The efficiency of homogenization may be assessed by microscopic examination or, more effectively, by a particle sizer, for example, Malvern Mastersizer.

Recently, high-pressure homogenizers have been developed, capable of reaching operating pressures of up to 300 MPa. High-pressure homogenizers are more effective than conventional valve homogenizers. The average diameter of the fat globules is about the same in both cases but high-pressure homogenizers give a narrower size distribution.

Heating

Normal high-temperature, short-time (HTST) pasteurization causes very little change in the fat globule membrane or in the characteristics of milk fat dependent on the membrane. However, excessively high pasteurization temperatures denature the cryoglobulins, and aggregation of the fat globules and creaming are impaired or prevented. Severe treatments, for example, 80 °C for 15 min, remove lipid and protein material from the membrane; the fat globules are partially denuded and may coalesce, forming large clumps of nonglobular (free) fat and resulting in defects such as 'cream plug' in milk or cream.

Processes such as thermal concentration also damage the MFGM with the formation of free fat, especially since many of these treatments also involve vigorous agitation in high-velocity heating systems. Dehydration, especially roller-drying, causes extensive damage to the MFGM, resulting in the formation of free fat. Since milk for concentrated and dehydrated milk products is normally homogenized, damage to the natural membrane is of little significance. Freezing milk, and especially cream, causes extensive damage to the MFGM due to physicochemical changes to the lipoprotein complexes and physical damage by the ice crystals. On thawing, much of the fat is released as nonglobular (free) fat.

See also: **Enzymes Indigenous to Milk**: Lipases and Esterases. **Homogenization of Milk**: Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Value Homogenizers. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat. **Milk**: Buffalo Milk; Equid Milk; Goat Milk; Sheep Milk. **Milk Lipids**: Lipolysis and Hydrolytic Rancidity; Milk Fat Globule Membrane. **Milk Proteins**: Immunoglobulins. **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Centrifuges and Separators: Types and Design.

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Milk Fat Globule Membrane

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Introduction

Membrane material surrounding the lipid globules of milk is called the milk lipid or milk fat globule membrane (MFGM). This membrane originates from material associated with the fat droplets within milk-secreting cells, and from material acquired during the process of secretion of fat globules from the cell (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents). Herein we summarize current information on the composition and structure of the MFGM. The MFGM of bovine milk is discussed most extensively because research has focused primarily on the membrane from this species.

Origin of MFGM

Materials that constitute the MFGM originate from different sites within the milk-secreting cell. Fat globules are assembled in, or close to, the endoplasmic reticulum (ER), wherein the synthesis of the triacylglycerol core lipid is completed by the transfer of fatty acyl chains to diacylglycerols by ER-bound terminal transferases. During or following synthesis, the nascent microlipid droplets are coated with surface material composed primarily of proteins and polar lipids derived from the ER and cytoplasm. Adipophilin (ADPH), a constituent of the MFGM, is a component of this initial coat, and the phospholipids are presumed to form a monolayer in direct contact with the underlying core of neutral lipids. These nascent droplets are then transported from their sites of origin to the apical cytoplasm, during which time they may fuse together to form larger droplets and interact with membranes associated with the Golgi complex, mitochondria, secretory vesicles, and other organelles. Lipid is finally secreted from the cell apex as each droplet is progressively enveloped with the plasma membrane, until the membrane pinches off behind the droplet, expelling it into the alveolar lumen. During this process, a 10–20 nm-thick layer of protein largely derived from the cytoplasmic face of the apical plasma membrane and the surface of the droplet forms between the outer membrane bilayer and the core lipid. Thus, milk lipid droplets are coated with a multilayered envelope comprising a monolayer of protein and lipid acquired from the cytoplasm, ER, and possibly other intracellular membranes, an interstitial layer or protein coat, and an outer bilayer with an associated glycocalyx (**Figure 1**). The origin and secretion of milk fat globules are discussed in detail elsewhere (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents).

Isolation of MFGM

The amount of MFGM in milk can vary considerably, depending upon both the fat content of the milk and the size distribution of the milk fat globules. Bovine milk fat globules range in diameter from under 1 μm to well over 10 μm. While very small globules contain only a small amount of the total milk fat, they contribute a large proportion to the total surface area of the membrane.

Several factors influence the composition and properties of the MFGM material isolated from milk. Following secretion, areas of the initial membrane condense to form islands or lenses on the droplet surface and some of the MFGM vesiculates and is lost to the skim milk phase, either within the mammary gland or following removal of milk from the animal. Estimates of the extent of this loss vary considerably. What factors cause it and whether it is progressive are unknown. Possible causes include aging of the milk post secretion and methods of handling the raw milk and separated cream. In the milk of many species, some globules have cytosolic material, including membranes, entrained between the globule core and the surrounding outer membrane. These inclusions are called ‘crescents’ because of their two-dimensional appearance in electron micrographs (**Figure 2**). Application of most methods for isolation of the MFGM will lead to the inclusion of membrane material from these crescents and therefore affect the composition of the final membrane preparations. However, in cows’ milk, only a small number of globules have crescents, compared to some other species.

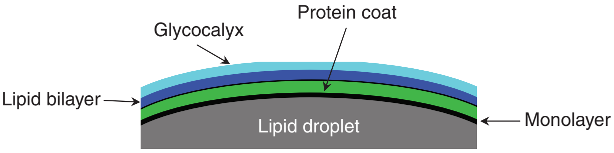

Figure 1 Structure of the milk fat globule membrane. Schematic diagram of the four layers of membrane discussed in the text: monolayer (black); protein coat (green); lipid bilayer (dark blue); and glycocalyx (light blue).

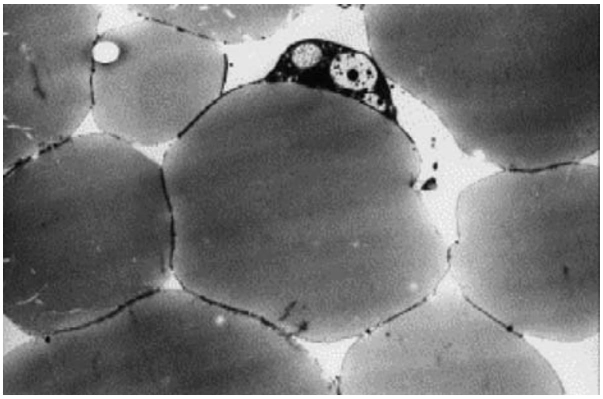

Figure 2 Electron micrograph of fat globules from cows' milk. One of the globules has a cytoplasmic crescent that contains three secretory vesicles as well as densely stained cytosolic material. These globules were fixed simultaneously with glutaraldehyde and osmium tetroxide.

Many different methods have been used to isolate MFGM. The most common approach is to float fat globules centrifugally to the surface, recover the cream layer, and then disperse the cream in water or buffer and refloat to remove entrained skim milk constituents. The membrane can be released from aqueous dispersions of globules by physical methods such as agitation (churning), freezing and thawing, and exposure to ultrasound. Chemical methods include the addition of mild detergents or the dispersion of globules in polar aprotic solvents. Following release of membrane material from the globules, the membrane is usually collected by centrifugal sedimentation.

The isolation method used influences the composition of the resultant MFGM. Washing cream extensively may remove extrinsic proteins from the globule surfaces. Conversely, poorly washed globules, especially if just distilled water is used, will yield membranes contaminated heavily with casein micelles. One gentle method that combines washing and globule recovery in one step is to increase the density of milk with sucrose, place the density-modified milk under buffer, and then centrifuge to hasten flotation of the globules through the buffer layer. However, it is possible that cells, cell fragments, and membranous material in skim milk may be entrained with the globules during centrifugal flotation, in which case particulate material from cells and cell fragments will be present in the isolated MFGM. During the globule washing and membrane collection steps, large proportions of some peripheral proteins originally associated with the globules are lost into the aqueous medium (hereafter referred to as MFGM supernatant).

Gross Composition

At least 95% of the total lipids in milk from cows are present in the globule fraction collected by centrifugal separation, and 95% or more of the lipids in these globules are triacylglycerols. What percentage of the total mass of fat globules is accounted for by MFGM material has not been established with certainty. From available data, membrane-associated materials can comprise from about 2 to 6% of the globule mass. Available information on the gross composition of MFGM is presented in **Table 1**. Proteins and lipids together account for over 90% of the membrane dry weight. Relative proportions of proteins and lipids measured have varied widely in different studies. Neutral lipids, especially triacylglycerols, are the most variable component (**Table 1**), presumably because of differences in the methods used to physically strip the membrane from the core lipid. The phospholipid content of MFGM is less variable, averaging about 0.25 mg mg^{-1} protein. The glycosphingolipid content of MFGM is about 13 µg mg^{-1} protein. RNA has been identified in cow MFGM preparations, about half of which can be removed by extraction with high-salt buffers. Whether

Table 1 Gross composition of milk fat globule membranes

Constituent group	*Amount*
Proteins	25–60% by weight
Total lipids	0.5–1.1 mg mg^{-1} protein
Neutral lipids	0.25–0.88 mg mg^{-1} protein
Phospholipids	0.13–0.34 mg mg^{-1} protein
Glycosphingolipids	13 µg mg^{-1} protein
Hexoses	108 µg mg^{-1} protein
Hexosamines	66 µg mg^{-1} protein
Sialic acids	20 µg mg^{-1} protein
RNA	20 µg mg^{-1} protein
Glycosaminoglycans	0.1 µg mg^{-1} protein

Data compiled from Keenan TW, Mather IH, and Dylewski DP (1988) Physical equilibria: Lipid phase. In: Wong NP, Jenness R, Keeney M, and Marth EH (eds.) *Fundamentals of Dairy Chemistry*, 3rd edn., pp. 511–582. New York: Van Nostrand Reinhold; and Keenan TW and Mather IH (2006) Intracellular origin of milk fat globules and nature of the milk fat globule membrane. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 2: Lipids*, 3rd edn., pp. 137–165. New York: Springer Science and Business Media Inc.

this RNA is a constituent of the primary MFGM or is present in contaminating cytoplasmic crescents is unknown. DNA has not been reported in cow MFGM. Hexoses, hexosamines, and sialic acids are found in the MFGM at about 0.2 mg mg^{-1} protein. Probably, most MFGM-associated carbohydrates are linked covalently to proteins and lipids. The glycosaminoglycans, hyaluronic acid, chondroitin sulfate, and heparin sulfate have been found in MFGM preparations.

Lipid Composition

Triacylglycerols are the most abundant lipids in the MFGM (**Table 2**), but methods used for preparing the membrane have a major influence on the triacylglycerol content. MFGM-associated triacylglycerols contain higher proportions of long-chain saturated fatty acids than do the triacylglycerols of core fat. Triacylglycerols with high melting points may associate selectively with membrane material during globule formation or during processing. When globules are destabilized at elevated temperatures, the MFGM is not enriched in high melting point triacylglycerols. Di- and monoacylglycerols are also found in the MFGM, but whether these are products of lipolysis or are true membrane constituents is not known. Sterols and sterol esters account for 0.3 to over 2% of the total membrane-associated lipids. Cholesterol is the major sterol and is most likely incorporated directly into the MFGM as a constituent of the plasma membrane-derived outer bilayer. Esterified sterols account for 10% or less of the total sterols in the membrane.

Table 2 Lipid composition of milk fat globule membranes

Constituent class	*Percentage of total lipids*
Triacylglycerols	62
Diacylglycerols	9
Monacylglycerols	0–0.5
Sterols	0.2–2.0
Sterol esters	0.1–0.3
Hydrocarbons	1.2
Unesterifiedfatty acids	0.6–6.0
Phospholipids[a]	26–31
Sphingomyelin	22
Phosphatidylcholine	36
Phosphatidylethanolamine	27
Phosphatidylinositol	11
Phosphatidylserine	4
Lysophosphatidylcholine	2

[a]Phospholipid classes given as percentage of total phospholipids.
Data compiled from Patton S and Keenan TW (1975) Milk-fat globule membrane. *Biochimica et Biophysica Acta* 415: 273–309; Keenan TW, Mather IH, and Dylewski DP (1988) Physical equilibria: Lipid phase. In: Wong NP, Jenness R, Keeney M, and Marth EH (eds.) *Fundamentals of Dairy Chemistry*, 3rd edn., pp. 511–582. New York: Van Nostrand Reinhold; and Keenan TW and Mather IH (2006) Intracellular origin of milk fat globules and nature of the milk fat globule membrane. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 2: Lipids*, 3rd edn., pp. 137–165. New York: Springer Science and Business Media Inc.

Milk phospholipids are distributed in an approximately 60:40 ratio between the MFGM and skim milk membrane fractions. The five major phospholipids of MFGM are sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (**Table 2**). Lyso derivatives of phosphatidylcholine and phosphatidylethanolamine have also been found in the MFGM, but are minor constituents in samples handled in ways that minimize lipolytic degradation. Alkyl and alkenyl ethers have been identified as constituents of the choline and ethanolamine phosphoglyceride fractions from cows' whole milk but what proportions of the various phosphoglyceride classes of MFGM are alkyl or alkenyl ethers has not been determined (*see* **Milk Lipids**: Phospholipids).

Glycosphingolipids, although minor constituents of the MFGM (**Table 1**), have been investigated extensively. Interest in milk glycolipids was stimulated by the recognition that glycosphingolipids and some of their degradation products function in a number of biological phenomena, such as cell–cell interaction, differentiation, and transmembrane signaling, and as receptors. MFGM contains two major neutral glycosphingolipids, glucosyl and lactosyl ceramides, in nearly equimolar proportions. Neutral glycosphingolipids with more complex oligosaccharide structures have not been reported. Nine different gangliosides, including mono-, di-, and tri-sialogangliosides, have been identified. The membrane-associated glycosphingolipids and sphingomyelin contain primarily long-chain fatty acids, especially palmitate (16:0), stearate (18:0), behenate (22:0), tricosanoate (23:0), and tetracosaenoate (24:1).

Protein Composition

Proteins account for 25–60% of the mass of membrane material recovered from milk fat globules (**Table 1**). Variation in the amount of protein in the MFGM almost certainly is due to differences in sample history and MFGM preparative techniques. The membrane-associated proteins have been studied extensively over the past 40 years, especially with respect to the primary sequences derived from the corresponding cDNAs, glycosylation profiles, and in some cases, the three-dimensional structures obtained by X-ray crystallography.

Upon one-dimensional separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), MFGM is resolved into 7–8 major bands of protein (**Figure 3**). In addition, many minor

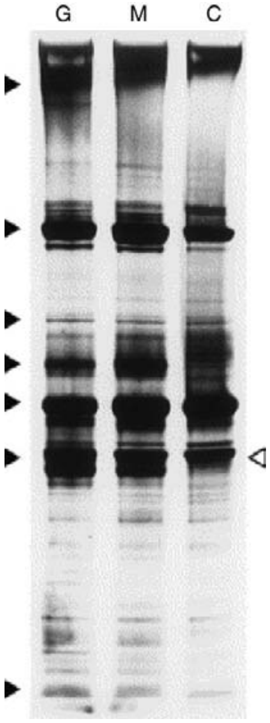

Figure 3 SDS-polyacrylamide gel electrophoretic separation of polypeptides of washed milk fat globules (G), milk fat globule membranes (M), and the Triton X-100- and 1.5 mol l^{-1} NaCl-insoluble milk fat globule membrane coat (C). Arrowheads from top to bottom along the left lane denote the positions of MUC-1, XDH/XO, MUC-15, CD36, BTN, PAS 6/7 combined with ADPH, and FABP. The open arrowhead along the right lane denotes ADPH after the quantitative removal of PAS 6/7 with detergent/high-salt mixtures. Separation was in a commercially prepared 4–12% NuPage electrophoretic gel (Novex, San Diego, CA) run with MOPS buffer. The gel was stained with silver.

polypeptides can be identified, especially when SDS-PAGE gels are overloaded with sample. More recent proteomic techniques have revealed well over 100 MFGM-associated proteins (see **Table 3** for a partial list). Historically, major MFGM proteins were designated according to their relative mobilities during SDS-PAGE and whether they stained with the periodic acid/Schiff (PAS) reagent used for the detection of glycosylated proteins. This led to confusion, because different numbering systems were used by different investigators. The current nomenclature for MFGM proteins recommended by the Milk Protein Nomenclature Committee of the American Dairy Science Association has been used and limited database information is included for the bovine proteins. Extensive information for many species, including cow, is now readily available by accessing the respective genome databases.

The major proteins of MFGM, in the order of increasing mobility upon SDS-PAGE separation, are the mucin MUC-1, the redox enzyme xanthine dehydrogenase/oxidase (XDH/XO), the mucin MUC-15 (previously called PAS III), cluster of differentiation 36 (CD36), butyrophilin 1A1 (BTN), an overlapping group of bands comprising ADPH and glycosylated variants of PAS 6/7, and in a separate band of much higher mobility, fatty acid-binding protein (FABP). Of these major proteins, six (XDH/XO, CD36, BTN, ADPH, PAS 6/7, and FABP) are stained readily with Coomassie blue. The mucins MUC-1 and MUC-15 are not stained with Coomassie blue but can be detected either with the PAS reagent or by silver staining.

Table 3 Enzymes and other selected constituents reported in bovine milk fat globule membranes

Enzyme/enzymic activity	*Enzyme commission number*
Retinal short-chain dehydrogenase/reductase	1.1.-.-
Alcohol dehydrogenase (NADP$^+$)	1.1.1.2
Glycerol-3-phosphate dehydrogenase (NAD$^+$), cytoplasmic	1.1.1.8
Malate dehydrogenase, cytoplasmic	1.1.1.37
Isocitrate dehydrogenase, cytoplasmic	1.1.1.42
Sterol-4-α-carboxylate 3-dehydrogenase, decarboxylating	1.1.1.170
Xanthine dehydrogenase/oxidase	1.2.3.1
Lipoamide dehydrogenase (diaphorase)	1.8.1.4
Cytochrome b_5 reductase	1.6.2.2
NADPH/cytochrome *c* reductase	1.6.99.1
NADH/cytochrome *c* reductase	1.6.99.3
Catalase	1.11.1.6
Lactoperoxidase	1.11.1.7
Fatty acid synthase	2.3.1.85
γ-Glutamyl transpeptidase	2.3.2.2
Galactosyl transferase(s)	2.4.1.-
Pyridoxal kinase	2.7.1.35
Patatin-like phospholipase domain-containing protein 2	3.1.1.3
Cholinesterase	3.1.1.7 (or 8?)
6-Phosphogluconlactonase	3.1.1.31

(*Continued*)

Table 3 (Continued)

Enzyme/enzymic activity	*Enzyme commission number*
Ribonuclease I	3.1.27.5
Alkaline phosphatase	3.1.3.1
Acid phosphatase	3.1.3.2
Phosphatidic acid phosphatase	3.1.3.4
5′-Nucleotidase	3.1.3.5
Glucose-6-phosphatase	3.1.3.9
Phosphodiesterase I	3.1.4.1
UDP-glycosyl hydrolases	3.2.1.-
β-Glucosidase	3.2.1.21
β-Galactosidase	3.2.1.23
N-Acetyl-β-D-glucosaminidase (hexosaminidase)	3.2.1.52
Plasminogen/plasmin	3.4.21.7
Transitional endoplasmic reticulum ATPase	3.6.-.-
Inorganic pyrophosphatase	3.6.1.1
Adenosine triphosphatase (activated by K^+/Mg^{2+})	3.6.1.3
Thiamine pyrophosphatase (nucleoside diphosphatase)	3.6.1.6
Nucleotide pyrophosphatase	3.6.1.9
Probable cation transporting ATPase 13A3	3.6.3.-
Plasma membrane Ca^{2+} transporting ATPase 2 (PMCA2)	3.6.3.8
G protein subunits: α-11, α-13, α-14, G(i) α-2, Gs α-	3.6.5.1
GTP-binding proteins: ARF1; rab 1A, 1B, 2A, 3A, 3B, 3C, 5B, 5C, 7, 10, 11A, 11B, 13, 18, 22A, 27B, 35; ral B; rap 1A, 1B; rheb 1, 2; rho A, C, F; rit1; Sar 1b; Sara	3.6.5.2
Aldolase	4.1.2.13
Carbonic anhydrase	4.2.1.1
Petidyl-prolyl *cis-trans* isomerase (cyclophilin 1)	5.2.1.8
Protein disulfide isomerase A3	5.3.4.1
Tryptophanyl-tRNA synthetase, cytoplasmic	6.1.1.2
Long-chain fatty acid CoA ligase 1	6.2.1.3
Acetyl-CoA carboxylase	6.4.1.2
Actin, cytoplasmic 1	-
Angiogenin	-
Annexins: III, V	-
Apolipoproteins: A1, E	-
Basic fibroblast-like growth factor	-
Calnexin	-
CD14	-
Galectin 7	-
Gelsolin	-
Glial fibrillary acidic protein	-
Cytochrome b_5	-
Cytochrome P-420	-
Heat shock protein 90-β	-
Presenilin1	-
Secretory carrier-associated membrane protein 2 (SCAMP2)	-
Serum amyloid A	-
SNAREs: endobrevin, SNAP23, syntaxin 3, VAMP2, Ykt6	-
STAT5	-
Transporters: excitatory amino acid transporter 3, Na^+/Cl^--creatine 1, Na^+-glucose (SGLT1), Na^+-phosphate	-
Toll-like receptors 2, 4	-
Uromodulin	-

Adapted from Mather IH (2000) A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *Journal of Dairy Science* 83: 203–247; Reinhardt TA and Lippolis JD (2006) Bovine milk fat globule proteome. *Journal of Dairy Research* 73: 406–416, Fong BY, Norris CS, and MacGibbon AKH (2007) Protein and lipid composition of bovine milk-fat globule membrane. *International Dairy Journal* 17: 275–288, and other sources.

The supernatant fraction recovered after disruption of the globules and centrifugal removal of the MFGM contains 20% or more of the protein originally associated with the fat globules. Major constituents in this supernatant fraction include MUC-1, XDH/XO, PAS 6/7, and FABP (*see* **Milk Lipids**: Buttermilk and Milk Fat Globule Membrane Fractions).

MUC-1

MUC-1 is a heavily glycosylated mucin found in the MFGMs of many ruminant, rodent, and primate species. MUC-1 mucins have single type 1 membrane anchors, short cytoplasmic tails, and extensively glycosylated exoplasmic domains with a variable number of tandem repeats (VNTRs) in the peptide sequences. Ruminant (cow, sheep, goat) and human MUC-1 mucins display allelic polymorphism. Each allele encodes a 20 amino acid tandem repeat sequence of specific length. Alleles are expressed in a codominant manner such that each gives rise to equal quantities of protein. At least nine alleles have been identified in bovine MUC-1 with molecular weights for the predicted polypeptide backbones ranging from 50 400 to 78 700 Da.

MUC-1 is found in both the cream and skim fractions of cows' milk, at an estimated total level of up to 40 mg l^{-1}. When cream is treated to release MFGM, some MUC-1 is found in the MFGM supernatant. In milk stored in a bulk tank, an appreciable amount of MUC-1 is recovered in skim milk. Soluble forms of human MUC-1, lacking the membrane anchor, have been described; they arise either from alternatively spliced mRNA variants or from proteolytic processing. In some cultured cells, proteolytic cleavage in the exoplasmic domain close to the membrane anchor gives rise to a heterodimer in which the exoplasmic domain is bound noncovalently to the membrane-anchored C-terminal fragment. This could explain why some of the membrane-associated MUC-1 is shed into the milk serum upon cooling and agitation, if such proteolytic cleavage occurs in the MFGM.

The biological functions of MUC-1 are not known with certainty. MUC-1 is expressed on the apical plasma membrane and is incorporated into the MFGM upon budding and release of fat droplets from the cells. The exoplasmic domain of MUC-1 appears to be present in filament-like structures extending 0.5–1.0 μm above the MFGM bilayer, well beyond the estimated 10 nm confines of the glycocalyx. Thus, MUC-1 on the cell surface may protect against physical damage and invasive microorganisms. MUC-1 may also have an immunoprotective role in the suckling neonate by binding and sequestering microorganisms in the gut lumen. Notably, *Muc1* null mice are more susceptible to gastritis and infections by *Helicobacter pylori* than wild-type animals.

The consensus structure of MUC-1 (ruminants and primates) has an N-terminal cleavable signal sequence, a repetitive domain, an SEA (sea urchin sperm protein, enterokinase, and agrin) module, a single transmembrane anchor, and a short cytoplasmic tail (**Figure 4**). In humans, the VNTR is repeated 21–125 times in at least 30 alleles. The deduced amino acid sequence of cow MUC-1 included a tandem repeat region with 11 partially conserved repeats of 20 amino acids each (EMBL: AJ400824). The mature secreted protein was deduced to have 558 amino acid residues and an average mass of 55 688 Da.

Cow MUC-1 contains about 60% carbohydrate by weight, and fucose, galactose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid(s) have been identified. From the abundance of each carbohydrate, it is most likely that the predominant glycans comprise the sialyl T antigen (Neu5Ac(α2-3)-Gal(β1-3)-GalNAcα1) linked through O-glycosidic bonds to serine and threonine residues within the repetitive domain and flanking regions. Five asparagine residues in the exoplasmic domain are within the sequon NXS/T, which specifies N-linked glycosylation. That one or more of these sites are most likely glycosylated is indicated by the presence of *N*-acetylglucosamine, mannose, and fucose in purified preparations and sensitivity to digestion with the enzyme protein: *N*-glycosidase.

XDH/XO

XDH/XO is an abundant protein of milk fat globules, comprising about 20% of the globule-associated protein and is concentrated in the protein coat (**Figure 1**), between the outer membrane bilayer and lipid droplet surface. Upon destabilization of washed cream, about half of the globule-associated XDH/XO is released into the MFGM supernatant. Of that remaining associated with MFGM, a little over half can be removed from the membrane by washing with buffers containing either nonionic detergents or high concentrations of salt.

XDH/XO is a complex iron–sulfur- and molybdenum-containing redox protein and the only major component with enzymic activity in bovine MFGM (EMBL: X83508). The enzymatically active form of the enzyme is a homodimer of 293 600 Da, which may be identified by SDS-PAGE as a monomer of approximately 150 000 Da. The three-dimensional structure of the bovine milk enzyme has been determined by X-ray crystallography and the mechanism of enzyme action characterized in detail. Mammalian XDH/XO exists as both a dehydrogenase and an oxidase. The dehydrogenase form utilizes NAD^+ preferentially as an acceptor of reducing equivalents, but can also use molecular oxygen in the absence of NAD^+. The oxidase form transfers reducing equivalents to molecular oxygen, forming hydrogen peroxide and superoxide radical. Under hypoxic conditions, the enzyme can also reduce inorganic nitrite leading to the formation of nitric oxide. XDH/XO is expressed predominantly as a dehydrogenase in tissues and can be converted to the oxidase form by oxidation of cysteine residues or by limited

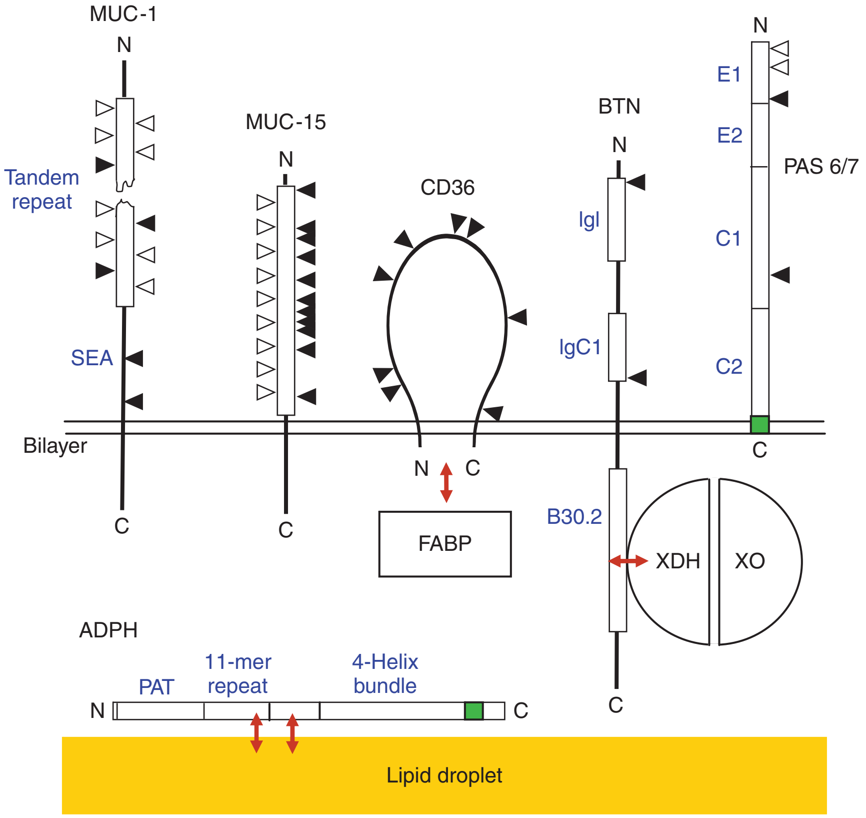

Figure 4 Topology of the major bovine milk fat globule membrane proteins. Shown are the orientations of MUC-1, MUC-15, CD36, BTN, and PAS 6/7 in the lipid bilayer and the locations of XDH/XO, FABP, and ADPH between the bilayer and the lipid droplet surface (yellow band). Confirmed interactions between proteins are indicated by double-headed red arrows, specific protein domains by blue lettering, the amphipathic helix in PAS 6/7 and hydrophobic cleft in ADPH by green boxes, N-linked glycans by black arrowheads, and O-linked glycans by open arrowheads.

proteolysis. XDH/XO of freshly collected milk has been estimated to be 50–70% in the oxidase form, and it is rapidly and completely converted to the oxidase form on storage.

XDH/XO may have multiple functions depending on the physiological context. In many organisms, XDH/XO plays a key role in the terminal steps of purine metabolism, catalyzing the oxidation of hypoxanthine and xanthine to xanthine and uric acid, respectively. In mammals, XDH/XO may also play a key role in milk lipid secretion because ablation of the *Xdh* gene in mice disrupts the regulated secretion of lipid droplets in heterozygous animals (lactation in *Xdh* null mice cannot be analyzed because the mice do not survive to sexual maturity). XDH/XO may function in concert with BTN by forming a protein complex, which is required for the formation of the MFGM (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents). The enzyme may also serve as a signaling molecule through the generation of reactive oxygen and nitrogen species that function as second messengers and activate downstream targets. In yet a further role, the production of reactive oxygen and nitrogen species may protect the mammary gland and the neonatal gut from bacterial infections. Both superoxide radical and nitric oxide are bacteriocidal and together form peroxynitrite, a highly effective antimicrobial agent.

XDH/XOs are not known to be glycosylated or phosphorylated. However, the bovine enzyme may be acylated with long-chain fatty acids (*see* **Enzymes Indigenous to Milk**: Xanthine Oxidoreductase).

MUC-15

MUC-15 (previously called PAS III) is a mucin with a membrane anchor that is found both associated with the membrane and in the MFGM supernatant. Upon separation by SDS-PAGE, MUC-15 migrates as a diffuse PAS-positive band with an apparent molecular weight of 95 000 to over 100 000 Da. The protein comprises 330 amino acids, including a cleavable N-terminal leader sequence of 23 residues (EMBL: AJ417816), and is inserted into membranes as a type 1 glycoprotein with an extensive exoplasmic domain (**Figure 4**). A splice variant that is secreted because it lacks the membrane anchor has also been described. The exoplasmic domain is heavily glycosylated with both O- and N-linked glycans, and carbohydrates constitute 65% of the total molecular weight. The majority of the O-linked glycans are based on core 1-type structures with smaller amounts of more complex oligosaccharides possibly containing poly-*N*-acetyllactosamine. N-linked glycans are principally of the hybrid type. A human homologue with 67% similarity and similar topological features has been characterized from a cloned cDNA and equivalent mucins recently identified in sheep, goat, pig, and buffalo milk. MUC-15 is widely expressed in many tissues on the surface of epithelial cells, as well as hematopoietic and immune cells. In the mammary gland, MUC-15 is concentrated on the apical surfaces of epithelial cells in pre-lactating and lactating glands, making it a useful marker for ductal and secretory epithelia.

CD36

CD36 is an integral protein of the MFGM with an apparent molecular weight of 77 000 Da on SDS gels. Most of it remains associated with the MFGM upon destabilization of cream. CD36 has been implicated in diverse functions such as a receptor for collagen and thrombospondin, as a scavenger receptor for apoptotic cells, and a transporter of long-chain fatty acids. However, what specific functional roles are served by CD36 on the apical plasma membrane of mammary epithelial cells and the MFGM remain unclear.

CD36 appears to be synthesized with an uncleaved hydrophobic N-terminal signal sequence that is hydrophobic and serves as a membrane anchor. Near the C-terminus is another hydrophobic sequence that presumably serves as a stop transfer signal (EMBL: X91503). As a consequence, short sequences in both the N- and C-terminal regions of CD36 are on the cytosolic face of the plasma membrane, and there is a large exoplasmic heavily glycosylated domain (**Figure 4**).

In human CD36, two cysteine residues near the N-terminus and two other cysteine residues near the C-terminus are acylated with palmitate. It is not known if cow MFGM CD36 is palmitoylated, but these particular cysteine residues are conserved in all characterized mammalian CD36 proteins. Excluding sialic acids, carbohydrate accounts for about 24% of the weight of CD36 from cow MFGM. Most of the glycans of CD36 are N-linked and comprise high-mannose-, hybrid-, and complex-type chains. Interestingly, both human and mouse CD36 contain rare O-linked α-2,8-linked polysialic acid glycans, which appear to be specific to CD36 expressed in the mammary gland (e.g., CD36 from platelets is not polysialylated). Such highly charged anionic residues may play a protective role in milk and in the gut of the suckling neonate by binding to and sequestering pathogenic microorganisms.

BTN

BTN migrates during SDS-PAGE with an apparent molecular weight of about 66 000 Da. It is the most abundant integral membrane protein in bovine MFGM and together with XDH/XO comprises over 50% by weight of the globule-associated protein. Upon destabilization of fat globules, most of the BTN remains associated with the membrane. BTN resists extraction from MFGM with solutions of high ionic strength, chaotropic agents, and with a variety of detergents, but is solubilized by the addition of thiol-reducing agents. BTN thus behaves as an integral membrane protein and its association with the membrane is probably stabilized by intramolecular disulfide bonds and by disulfide bonding with other MFGM proteins.

Bovine BTN is synthesized as a polypeptide of 526 amino acids that includes an N-terminal 26-residue signal peptide, which is cleaved *in vivo*, leaving a protein with a calculated molecular weight of 56 460 Da (EMBL: M35551). BTN is a member of a subfamily of BTN-like proteins in the immunoglobulin (Ig) superfamily, a large group of adhesive proteins, receptors, and constituents of the immune system. Canonically, the BTNs comprise type 1 membrane glycoproteins with two or more Ig folds in the exoplasmic domains, single transmembrane anchors, and cytoplasmic regions, which are dominated by the B30.2 or PRY/SPRY domain. The latter domain is predicted to comprise two antiparallel β-strands folded into a β-sandwich and is assumed to function as an interactive binding domain in many proteins. BTN has two exoplasmic Ig folds, one of the intermediate type (IgI) and one of the constant C1 type (IgC1), and a B30.2/PRY/SPRY domain, which binds tightly to XDH/XO in a pH- and salt-sensitive manner (**Figure 4**).

BTN may function in the secretion of milk lipid because its expression on the apical plasma membrane is highly upregulated during lactation and the secretion of milk lipid droplets is compromised in BTN knockout mice. In several current models for milk fat secretion, interactions between BTN and XDH/XO are postulated to form a linker protein complex, which is necessary for the budding and release of lipid globules from the apical surface and formation of the MFGM (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents). BTN may also function in the immune system as a coinhibitor of T-cell activation via interactions between the exoplasmic Ig folds and a presumptive receptor on the T-cell surface.

Bovine BTN is glycosylated at two sites in the exoplasmic domain (**Figure 4**). The glycans linked to BTN have been structurally characterized and comprise bi-, tri-, and tetra-antennary sugar chains. No O-linked glycans have been identified. There is evidence that BTN is acylated with medium- and long-chain fatty acids. BTN contains several potential phosphorylation sites, and it is phosphorylated upon incubation of isolated MFGM with ATP. However, whether BTN is phosphorylated *in vivo* remains unclear.

ADPH

ADPH is a major constituent of the insoluble material remaining after MFGM is extracted with salts, chaotropic agents, and nonionic detergents. ADPH was overlooked for a long time as a constituent of MFGM because its position on gels after SDS-PAGE separations overlaps with that of the PAS 6/7 glycoprotein, and because it is not readily solubilized using conventional methods of sample preparation for electrophoresis.

Originally, ADPH was identified as one of the earliest proteins expressed in cells undergoing adipogenic differentiation and it was named adipose differentiation-related protein (ADRP). Subsequently, ADPH was found in association with storage lipid droplets in a wide variety of cultured cell types, and through sequence comparisons with other proteins it was recognized as a member of a family of lipid droplet-associated proteins, called the PAT family (after three constituent members: perilipin/adipophilin/TIP-47). Structurally, PAT family proteins are characterized by an N-terminal PAT domain, which is the most conserved region across family members, an overlapping 11-mer repeat region, which is predicted to fold into a series of amphipathic helices, followed by a predicted four-helix bundle of amphipathic helices (**Figure 4**; see EMBL: AJ011680 for the bovine sequence). A highly conserved region of 14 amino acids toward the C- terminus forms a hydrophobic cleft, which was identified in the crystal structure of TIP-47, and is assumed to form a similar structure in other family members, including ADPH.

ADPH associates with the surface of lipid droplets during the early stage of their formation in regions of the cytoplasm close to the ER and may play a role in facilitating the transport of triacylglycerols and fatty acids between the ER and forming droplets. It plays a major role in regulating lipid droplet turnover, by sterically blocking the action of lipases on the cell surface. As discussed elsewhere (*see* **Mammary Gland, Milk Biosynthesis and Secretion**: Secretion of Milk Constituents), ADPH may also function in the secretion of lipid droplets at the apical surface, either by forming a protein complex with XDH/XO and BTN, or by direct insertion of the hydrophobic cleft in the C-terminus of ADPH into the phospholipid bilayer of the apical plasma membrane.

Little is known about post-translational modifications of ADPH. It does not have an N-terminal signal peptide, and is either expressed as an unstable protein in the cytoplasm in the absence of lipid droplets, or stably associated with the surface of lipid droplets in their presence. ADPH does not appear to be glycosylated. There is evidence that ADPH may be acylated with long-chain fatty acids.

PAS 6/7

PAS 6/7 appears as a doublet in SDS-polyacrylamide gel profiles of MFGM in the approximate molecular weight range of 48 000–54 000 Da (**Figure 3**). It consists of a single polypeptide chain that is differentially glycosylated, leading to its resolution as a doublet during SDS-PAGE. PAS 6/7 is an extrinsic protein of MFGM and can be displaced from intact globules or membrane preparations by extraction with buffers containing high concentrations of salt or with chaotropic agents. Because of its peripheral nature, PAS 6/7 is a major protein constituent of the MFGM supernatant fraction. The N-terminal region of PAS 6/7 contains two epidermal growth factor (EGF)-like domains, followed by repeat domains that are similar to the C1 and C2 domains of blood clotting factors V and VIII (EMBL: X91895) (**Figure 4**). PAS 6/7 is most probably bound to anionic phospholipids in the MFGM via a C-terminal amphipathic α-helix.

One major modification of PAS 6/7 is the addition of both N- and O-linked glycans. Removal of N-linked glycans converts both PAS 6/7 isoforms to a protein of about 50 000 Da. The higher molecular weight isoform, PAS 6, was found to be N-glycosylated on two residues and O-glycosylated on one residue, and the PAS 7 form was found to be N-glycosylated on one residue and O-glycosylated on one residue. During development, the glycosylation patterns of PAS 6/7 change appreciably, especially in the early stages of lactation. Other post-translational modifications of PAS 6/7 have not been reported.

The name lactadherin has been assigned to the human form of PAS 6/7 to reflect the protein's presence in milk and its adhesive properties. The glycans associated with lactadherin may bind rotaviruses, potentially protecting the neonatal gut from infection. In addition, PAS 6/7 contains the integrin-binding motif 'RGD' in the second EGF-like domain. This property, and its widespread distribution in cells and tissues, suggests a role for PAS 6/7 in adhesion between cells. In this capacity, PAS 6/7 and its human (lactadherin) and mouse (MFG-E8) homologues play a major role in the process of programmed cell death (apoptosis) by linking phagocytic cells (through the integrin-binding domain) to phosphatidylserine displayed on the surface of apoptotic cells (through the C-terminal lipid-binding amphipathic α-helix). Expression of PAS 6/7 is upregulated in late lactation and thus is presumed to play a major role in the apoptosis of mammary epithelial cells during involution.

FABP

FABP has been identified as an approximately 13 000 Da constituent of MFGM and is recovered in both the membrane and MFGM supernatant fraction. A fraction enriched in FABP protein from lactating bovine mammary gland was originally shown to inhibit the growth of tumor cell lines and the protein was called mammary-derived growth inhibitor (MDGI). However, subsequent work showed that the tissue form of MDGI was a mixture of heart and adipose FABP. By proteomic analysis it has been recently confirmed that MFGM contains the heart form, and adipose FABP is presumed to be associated with tissue adipocytes.

FABP in MFGM occurs in at least two major and one minor isoelectric forms, suggesting the possibility of post-translational modifications. The protein is phosphorylated upon incubation of MFGM with ATP, and there is evidence that insulin stimulates phosphorylation of FABP in rat mammary cells. There is no evidence that FABP in MFGM is glycosylated. Other post-translational modifications of FABP have not been reported.

What roles, if any, FABP may play in milk fat globule formation or secretion is unclear. Within mammary epithelial cells, FABP may serve to transport fatty acids in the cytoplasm and it is bound to the integral fatty acid transporter CD36 in isolated MFGM preparations (**Figure 4**).

Enzymes and Other Constituent Proteins

Of the above major proteins, only XDH/XO is known to be an enzyme. Historically, the more-minor proteins of the MFGM were identified by enzyme assay and in some cases linked to specific electrophoretic bands of protein on SDS gels. During the last decade, more comprehensive proteomic approaches and two-dimensional electrophoretic separations have revealed many minor components (**Table 3**).

Close to 80% of the enzymic activities found in bovine MFGM are members of the hydrolase class, including a large number of ras-related GTP-binding proteins. Oxidoreductases are next in abundance, followed by transferases. The only lyases reported are the cytoplasmic enzymes aldolase and carbonic anhydrase. Cytoplasmic proteins constituted 24% of all the proteins identified in a recent proteomic analysis. Many of these soluble proteins may be incorporated into the secreted globules in cytoplasmic crescents.

Several enzymes with high specific activities in MFGM, such as cation-stimulated ATPase, phosphodiesterase I, and 5′-nucleotidase, are enriched in plasma membranes. XDH/XO, which is a soluble cytoplasmic protein, clearly is an abundant enzyme of the MFGM and most probably associates with the membrane by binding to the B30.2 domain of BTN (**Figure 4**). Other enzymic activities detected in the MFGM are associated with intracellular membranes such as the Golgi apparatus (glycosyltransferases), ER (glucose-6-phosphatase), and lysosomes (acid phosphatase, glycosidases). Ras-related GTPases are numerous in bovine MFGM. Why this is the case is unclear because they are typically localized to specific membrane compartments and the cytoskeleton and play diverse roles in the vesicular trafficking of membranes throughout cells. Many of these minor proteins (and the above organelle-associated enzymes) may bind to lipid droplets during their transit to the apical surface and during formation of the MFGM. Proteins that function in the trafficking of membranes and proteins in cells constituted the largest class of identified components (23%) in a recent proteomic analysis of bovine MFGM. Besides the GTPases and cytoskeletal elements, such trafficking proteins include those involved in the machinery of vesicle membrane fusion such as SNAREs and annexins. More detailed functional analysis of these proteins, especially the rabs, rhos, and SNAREs, may reveal the molecular details of lipid droplet trafficking and secretion. Industrial studies of MFGM-associated enzymes have focused on their use as markers of membrane damage and examination of their involvement in degradative events that alter processing characteristics, flavor, or the texture of milk products. Thermal inactivation of alkaline phosphatase is widely used to monitor adequate pasteurization.

Molecular Organization

The current view of the MFGM is that it initially comprises a true bilayer membrane that has a dense, proteinaceous coat of 10–20 nm thickness oriented on the inner membrane face, plus an innermost layer, which existed on the surface of droplets before secretion (**Figure 1**). Following secretion, the membrane undergoes some structural changes such that the membrane condenses into areas akin to islands and continents on the globule surface. Molecular mechanisms underlying these changes are unknown.

Early biochemical and morphological studies showed that there is a distinct asymmetric orientation of the glycans, enzyme active sites, and proteins of the MFGM, and this has been confirmed by molecular biological approaches (**Figure 4**). With respect to proteins, it is now firmly established that PAS 6/7 is an externally disposed, extrinsic MFGM constituent and that XDH/XO is an internal constituent of the protein coat. MUC-1, MUC-15B, and BTN are type 1 transmembrane proteins with externally oriented N-termini. CD36 also is a transmembrane protein but with short internally disposed N and C-termini and a large exoplasmic heavily glycosylated loop. Interactions have been confirmed or inferred between the B30.2 domain of BTN and XDH/XO, FABP and CD36, and the lipid droplet surface and ADPH. As discussed above, several models describing how XDH/XO, BTN, and ADPH may interact with each other during the milk lipid globule budding process have been proposed. Phospholipids are asymmetrically arranged with respect to the plane of the lipid bilayer in many cellular membranes, with phosphatidylcholine and sphingomyelin in the outer leaflet and phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol in the inner leaflet. However, as yet this disposition has not been confirmed for the phospholipids of bovine MFGM.

Perspectives

There has been a substantial increase in the knowledge of the MFGM and of its protein constituents in recent years. However, we still have little specific information on the cellular mechanisms involved in the formation of the MFGM, and on what roles the constituents of this membrane play in globule secretion, in nurturing of the neonates, and in affecting processing parameters.

See also: **Enzymes Indigenous to Milk**: Xanthine Oxidoreductase. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Fat; Secretion of Milk Constituents. **Milk Lipids**: Buttermilk and Milk Fat Globule Membrane Fractions; Fat Globules in Milk; Lipolysis and Hydrolytic Rancidity; Phospholipids. **Milk Proteins**: Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins.

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Buttermilk and Milk Fat Globule Membrane Fractions

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Introduction

During butter manufacture, milk fat globules are destabilized, and an aqueous by-product, buttermilk, containing milk-derived components as well as the milk fat globule membrane (MFGM) is produced. Buttermilk has received increased interest, as food science and nutrition research turns to develop products with not only increased nutritional value but also health-promoting properties. This by-product of butter-making can be a source of highly valuable functional ingredients, from both the technological and nutritional prospectives. Unfortunately, buttermilk is often subjected to extensive processing not necessarily aimed at preserving the quality of its components. The lack of consistency between buttermilk sources is perhaps the reason why the research strategies aimed at determining the benefits associated with its use as an ingredient or the benefits of fractionation of its components have not always been successful.

The MFGM is complex in composition as well as in its supramolecular structure. To date, the nature and functional properties of many of its components are at least partially understood. Buttermilk can be employed to isolate MFGM material and to prepare valuable ingredients; however, processes have to be designed to produce products with consistent composition and functionality. MFGM components are, in fact, very sensitive to environmental, processing, and storage changes, and it is not yet clear which processing conditions need to be controlled to best preserve their functional properties. In addition, more research has to be conducted to determine the benefits associated with the use and consumption of ingredients prepared from buttermilk.

Buttermilk and MFGM Composition

As a result of cream churning during butter manufacture, the aqueous phase is released and recovered as a by-product, commonly named buttermilk. While the original material (cream) is composed mainly of milk-derived proteins (caseins, whey proteins, etc.) and fat globules polydisperse in size, buttermilk contains skim milk proteins and is also rich in proteins and phospholipids derived from the original MFGM. As a consequence, buttermilk can contain up to 7 times more phospholipids than whole milk.

In raw milk, fat globule sizes vary from about 0.2 to 20 μm in diameter. The size distribution depends on a large number of factors including the breed of cow and the stage of lactation. While the majority of the fat globules are less than 1 μm in diameter, most of the triglycerides are transported by a relatively small number of large oil droplets in the milk. On the other hand, small fat globules contain relatively less triacylglycerides and relatively more phospholipids, glycoproteins, and enzymes as they contain more membrane material per unit fat than large globules.

The milk fat globules are enveloped by the MFGM, which protects them from coalescence and enzymatic degradation by lipases. This membrane originates from the mammary cell, and is composed, in native fat globules, of (1) an inner polar lipid monolayer surrounding the lipid core of the fat droplet, (2) a proteinaceous coat (appearing electrodense in microscopic images), (3) a bilayer membrane of polar lipids and proteins, and (4) an externally oriented glycocalyx. The MFGM is not homogeneous, and the various components are differentially distributed throughout the membrane. While several membrane proteins are located on the outer or inner face, the carbohydrate groups of the glycoproteins and gangliosides are exposed mainly on the globule surface. The lipids are also asymmetrically arranged: the choline-containing phospholipids, phosphatidylcholine (35%) and sphingomyelin (25%), and the glycolipids are located mainly on the outer layer, whereas phosphatidylethanolamine (30%), phosphatidylserine (3%), and phosphatidylinositol (5%) are concentrated mostly on the inner surface. In addition to the different distributions of the components, the amount and composition of MFGM can vary considerably depending on the fat content, fat globule size, and processing applied during and after milk extraction (e.g., agitation, heating, cooling, homogenization, evaporation, spray-drying).

In addition to sweet or commercial buttermilk produced during the manufacture of sweet cream (uncultured) butter, other sources of MFGM include cultured or sour buttermilk and whey buttermilk. These two refer to the products obtained from churning cultured cream and whey cream, respectively, and they both represent potential ingredients for the food industry. While the composition of regular buttermilk and cultured buttermilk is very similar to that of skim milk, the composition of whey buttermilk is closer to that of whey, but with a higher fat content, as a result of the presence of MFGM material.

It has been reported that the processing functionalities of whey buttermilk and regular or cultured buttermilk are different because of their different compositions. For example, whey buttermilk has higher emulsifying properties but lower foaming capacity than regular or cultured buttermilk, as well as different pH stability.

Compared to raw whole milk, the distribution of polar lipids in different dairy products clearly shows that buttermilk and butter serum are among the best sources for the isolation of MFGM material. The polar/neutral lipid ratio, on a dry matter basis, in skim milk, cream, butter, acid whey buttermilk, buttermilk, and butter serum (the aqueous portion obtained from melted butter) is 1.2, 1.7, 0.7, 8.0, 8.8, and 50.1 times that in raw milk, respectively.

In addition to proteins derived from skim milk (caseins, whey proteins), proteins derived from MFGM are also present in buttermilk. A recent study on the MFGM proteome identified up to 120 proteins, of which 71% are membrane-associated proteins, 24% are cytoplasmic, and 5% are secreted. Moreover, from the identified proteins, it was found that almost 50% of the proteins are associated with membrane protein trafficking and cell signaling, with the remainder having different functions including immune response (4%), transport and fat metabolism (20%), and protein synthesis/folding (7%). The functions of some proteins (21%) are not yet known. An important finding of this report was the identification of a large number of proteins, constituting part of the apical membrane of the mammary secretory cells, that are associated with cell signaling and infection detection (CD14, Toll-like receptors 2 and 4).

Moreover, as a result of progress in cloning and proteomics, most of the major proteins present in the MFGM have been sequenced. Proteins account for 25–60% of the mass of the membrane material and this percentage varies according to the method of isolation and sample history. Major proteins include MUC1 and MUC15 (mucins), the redox enzyme xanthine dehydrogenase/oxidase (XDH/XO), glycoproteins PAS III and PAS 6/7, cluster of differentiation 36 (CD36) and butyrophilin (two integral proteins), fatty acid-binding protein, and adipophilin, a lipid-binding protein. Other minor proteinaceous components, such as the proteose peptone fraction (PP3), have also been associated with the MFGM, although it is not clear whether they form part of the membrane or are a result of processing or the isolation procedure. All these proteins are recovered in buttermilk fractions.

Changes Occurring to MFGM during Processing and Storage

The supramolecular structure of the MFGM changes from the moment the fat globule leaves the mammary secretory cell. Depending on the type and extent of the processing treatment, various physicochemical changes may occur. Post-harvest changes (not physiological, i.e., due to diet, breed, or stage of lactation) can be chemical, enzymatic, or physical (i.e., due to mechanical stress). Handling during and after harvesting of milk, agitation, changes in temperature, and processing treatment such as heating, homogenization, and concentration all affect the supramolecular structure as well as the composition of the MFGM. Most work so far has failed to quantify these changes as the different purification procedures are themselves a source of the variation. However, there seems to be general agreement that homogenization under standard conditions results in very little loss of MFGM. Studies have in fact demonstrated that homogenization does not change the distribution of individual phospholipids in globules, although it alters the morphology of the membrane. In fact, because of changes in the size of the fat globules, the MFGM cannot completely cover the newly formed globules; hence, a significant amount of skim milk-derived proteins needs to adsorb to stabilize the interface.

Several methods have been used in attempts to quantify the changes occurring to the milk fat globule and its membrane during processing. These methods include the determination of free fat or free fatty acids by titration, or the changes in ζ-potential of the fat globules. Dairy processing greatly affects the properties and composition of the MFGM. Some of these changes are summarized in **Table 1**. Heating and physical agitation or homogenization, at temperatures as low as 65 °C, can cause changes in the MFGM by increasing the binding of serum proteins. Furthermore, heating at 80 °C results in a total loss of the PAS 6 protein and partial loss of PAS 7, and may result in a loss of phospholipids. Studies on heated washed creams at higher temperatures show the formation of high-molecular-weight complexes between XO and butyrophilin. The temperature at which XO and butyrophilin aggregate is lower than the denaturation temperatures reported for β-lactoglobulin and α-lactalbumin.

Table 1 Fat globule size distribution parameters in raw and homogenized cow milk

Parameters	*Raw milk*	*Homogenized milk*
Volume-surface average diameter (μm)	2.5–4.6	0.13–1.7
Volume average diameter (μm)	2.9–5.9	0.3–2.8
Surface area per 1 g of fat (m^2)	1.4–2.9	4–50
Surface area per 1 ml of milk (m^2)	0.06–0.12	0.13–1.75
Mean free distance between fat globules (μm)	6–25	1–8

Data compiled from Michalski *et al*. (2002), Mulder and Walstra (1974), Rüegg and Blanc (1981), and Walstra (1969).

In addition, a major role is played by the MFGM during heat treatment, causing the release of volatile sulfur compounds including hydrogen sulfide, carbon disulfide, dimethyl sulfide, and methanethiol. Although exposition of sulfhydryl groups of whey proteins by denaturation is considered to be the principal mechanism involved in milk flavor development, it has been suggested that the serine and methionine residues present in the protein fraction of MFGM also have a major effect.

Moreover, mechanical agitation is an important factor affecting the changes in the composition of the MFGM; in a study comparing ultra-high temperature heating of whole milk by direct steam injection or by indirect heating, with or without homogenization, it was demonstrated that agitation has a greater effect than temperature on the changes in the size of the fat globules.

Therefore, damage to the milk fat globule and its membrane can greatly affect the future quality of the derived products, as there will be higher susceptibility to oxidation, coalescence, changes in biological functionality, and interactions with other proteins. Hence, the processing steps to which the raw material and/or the by-products are subjected are a fundamental key to the quality of the isolated products. All these steps may affect the processing and biological functionality of the MFGM material.

Isolation of MFGM

The separation and isolation of MFGM from cream, buttermilk, and whole milk has been studied widely, and the laboratory methodologies typically involve four major steps: (1) separation of fat globules from whole milk by centrifugation; (2) washing of the fat globules to remove undesired components including caseins and whey proteins; (3) disruption of the fat globules and release of the membrane material; and (4) recovery of the membrane fractions. As previously mentioned, the processing history of the material used for the isolation will profoundly affect the final results.

Disruption of the fat globules can be carried out using a number of processes, including freezing and thawing, agitation or churning, or treatment with detergents or bile salts such as deoxycholate. In the native state, the surface of the MFGM is not homogeneous; therefore, various washes and changes in the environmental conditions as well as mechanical agitation will affect the distribution of the various components. After disruption of the fat globules, the MFGM can be collected by centrifugal sedimentation (at high centrifugal forces, i.e., 100 000 *g*). Subfractions of MFGM can also be produced analytically using gradient centrifugation.

For commercial-scale isolation of MFGM material, products containing a high content of polar lipids, such as buttermilk or butter serum, provide a promising basis for further purification. Because of the absence of casein micelles, whey buttermilk seems to be a potential source for the isolation of MFGM fractions as well. However, the presence of neutral lipids is problematic, as polar lipids in dairy products are present mainly as MFGM fragments of lipoprotein particles and the neutral lipids are intimately associated with them. The separation of a polar lipid fraction of high purity is therefore challenging, especially without the use of solvents. Supercritical fluid extraction has been suggested recently as a means to separate the polar lipids from the neutral lipids in MFGM-rich isolates. Membrane filtration may also be a means to separate certain phospholipids, as their transmission in the permeate fraction has been reported. Renneting of heat-treated buttermilk has also been suggested as a way to fractionate milk phospholipids, as fat and phospholipids were recovered in the serum phase, while skim milk-derived proteins were collected in the curd.

The most significant challenge in obtaining a buttermilk-derived ingredient rich in MFGM is the separation of the caseins. In buttermilk, caseins are present in micellar form. Casein micelles, being colloidally similar in size to the MFGM fragments, are difficult to separate from the MFGM using size-based separations such as membrane filtration. With the addition of sodium citrate (i.e. 2%, w /v) to buttermilk, it is possible to disrupt the casein micelle structure; hence, MFGM fractions can be recovered by high-speed centrifugation or membrane filtration. With microfiltration, it is possible to obtain an MFGM concentrate containing approximately 60% (w/w) protein and 35% (w/w) lipid. However, membrane filtration can cause losses of the MFGM phospholipids, especially during the diafiltration steps, and further research is required on the optimization of the filtration conditions and impact of the source and processing history of the initial material. In addition, proteolysis has also been proposed as a means to disrupt the casein micelles and obtain an MFGM extract from buttermilk.

The Potential of Buttermilk and MFGM Fractions as Technological and/or Nutritional Value-Added Ingredients

There have been several studies on the nutritional and technological functionality of buttermilk and MFGM isolates. However, in the area of health effects, data are sometimes contradictory; the information needs to be evaluated carefully, as the method of isolation of the different components and the origin of the raw material greatly affect the properties of the final products. **Table 2** presents a summary of these properties.

Table 2 Technological and nutritional properties of buttermilk and MFGM fractions

Product/component	*Properties/function*
Technological value[a]	
Buttermilk	Emulsifying agent Flavor and texture improver in bakery products Protects against lipid peroxidation Reduces viscosity and prevents crystallization Improves cheese yield, moisture retention, flavor development and mouthfeel, body and meltability
MFGM	Emulsifying agent Production of liposomes
Nutritional value[b]	
Breast cancer type 1 or 2 susceptibility proteins (BRCA1, BRCA2)	Anticarcinogenic activities
Fatty acid-binding protein	Cell growth inhibition Anticancer factor
Sphingolipids and metabolites	Anticholesterolemic Neonatal gut maturation Suppression of gastrointestinal pathogens
Xanthine oxidase	Bactericidal agent
Butyrophilin	Suppression of multiple sclerosis
Phospholipids	Colon cancer inhibition Protects the human gastrointestinal tract against toxic attack Reduction of necrotizing enterocolitis Antidepressant Anticholesterolemic Anti-Alzheimer
MFGM hydrolysates	Antimicrobial activity against pathogens
Mucins and glycoproteins	Inhibition of *Helicobacter pylori* Protective effect against rotavirus infections

[a]Data compiled from Kanno *et al*. (1991), Corredig *et al*. (1998), Roesch *et al*. (2004), Mistry *et al*. (1996), and Thompson *et al*. (2006).
[b]Data compiled from Spitsberg (2005), Clare *et al*. (2008), Hirmo *et al*. (1998), Hancock *et al*. (2002), and Dewettinck *et al*. (2008).

Technological Value

It has been reported that buttermilk can be used as an ingredient because of its good emulsifying properties and the positive impact on flavor and texture. Buttermilk is used in baking and dairy industries and in the preparation of dry mixes, sauces, chips, and chocolate products. In the baking industry, buttermilk is used to improve the flavor and texture of bakery products. The addition of buttermilk powder to flour for bread-making leads to a greater loaf volume, increases water absorption capacity of the flour, and improves softening and development time of the bread dough. In addition, several isolates of buttermilk and MFGM, such as polar lipids, are used to ameliorate fat dispersion and staling in baked goods, as additives to chocolate to reduce viscosity and prevent crystallization, as wetting enhancers to improve the wetting properties of instant products, and as stabilizers of margarine to prevent spattering and browning.

Because of the amphiphilic nature of the MFGM, isolated MFGM has been referred to as a functional ingredient in emulsions. However, the MFGM isolates from industrial buttermilk show inferior emulsifying properties compared to whole buttermilk because of the contribution of the skim milk-derived components in the latter. Differences in the functional properties of various buttermilk samples are attributable to differences in the composition of the samples and differences in the ratio of MFGM components to skim milk proteins, or of proteins to phospholipids. Because whey buttermilk has a different composition, it exhibits higher emulsifying properties and lower foaming ability, possibly due to its higher ratio of phospholipids to proteins compared with commercial sweet or sour buttermilk samples. Also, when looking at a purified phospholipid fraction from buttermilk, studies show that liposomes produced from MFGM fractions by a microfluidization technique have different structural composition and properties than those prepared from soy- or egg-derived phospholipids. It has also been suggested that buttermilk may help stabilize food products against lipid peroxidation. Concentrations in the range of 0.1–0.2% were proven to be effective in delaying the propagation stage of lipid oxidation, although no significant differences from the control were noted during the onset of the reaction.

In the dairy industry, the addition of sweet buttermilk has been shown to improve cheese yield during pizza cheese-making. The cheese made with added buttermilk also showed a reduced amount of free oil. High levels of buttermilk, however, adversely affected the quality of pizza cheese. The potential use of MFGM fractions or ultrafiltered buttermilk retentate to change the structure and properties of various dairy products has also been studied. MFGM fractions prepared from pasteurized cream were retained better in the cheese curds than MFGM prepared from raw cream. When buttermilk ultrafiltrate or condensed buttermilk is used in cheese, usually levels of substitutions higher than 10% result in changes in the structure and quality of the cheese curds, including an increase in moisture retention. The heating process that the cream undergoes before butter-making promotes significant whey protein denaturation and therefore poor rennet coagulation properties of buttermilk are seen. The presence of whey protein aggregates is also the reason for the increase in cheese yield and moisture retention. An enhancement of flavor development and mouthfeel, body, and meltability has also been reported after the addition of buttermilk to reduced-fat cheese. Buttermilk is also used as a heat stability enhancer in dairy products, which results from its low calcium and β-lactoglobulin content and a high non-micellar casein concentration. Applications include improving the heat stability of reconstituted concentrated milk and whey powder (when added to milk before evaporation and drying). These observed effects widen the range of possible applications of buttermilk-based ingredients in dairy products.

In conclusion, the technological functionality of buttermilk can be attributed not only to the skim milk proteins, but also to the presence of MFGM fractions. However, studies on the functionality of the MFGM are still needed, as inconsistent results are often obtained, most likely because of the different treatments applied to and the variability of the sources of commercial buttermilk. Additional factors influencing the technological properties include the dairy source, the methods of MFGM isolation, and the temperature history of the product.

Nutritional Value

Although there is evidence of the nutritional functionality of the various components of the MFGM and buttermilk, the potential of the MFGM as a whole, as an ingredient beneficial to health, has not been elucidated. Controversy exists on the effect of MFGM components on health and disease. While some investigators have suggested that MFGM fractions have beneficial properties such as inhibiting the growth of cancer cells, lowering cholesterol adsorption, inhibiting pathogen adhesion to intestinal cells, or even antimicrobial properties, other researchers have reported adverse effects of buttermilk consumption in relation to certain human pathologies including coronary heart disease and autism. Notwithstanding, published epidemiological results on the latter area are contradictory and hence should be taken with caution.

On the positive effect of MFGM on health, glycosphingolipids stand out. These minor constituents of MFGM and their catabolites are involved in various biological functions such as cell–cell interactions, differentiation, proliferation, immune recognition, and transmembrane signaling, and also function as receptors for certain hormones. One study reported the marked intracolonic efficacy of MFGM in inhibiting intestinal β-glucuronidase in mice, suggesting its potential role in the treatment or prevention of colon cancer.

MFGM is rich in glycosylated proteins, and most of them are still not well understood. Their bioactivity, especially as physiological regulators, is a major area of current investigations. Milk glycoproteins derived from the MFGM have been linked to inhibition of pathogen adhesion in the gut. Sialic acid residues seem to be the principal cause for this bioactivity, because they confer a strong negative charge on epithelial cell surfaces, which translates into the prevention of wall to wall adherence and attachment of bacteria and pathogens to the gastrointestinal mucosa. It is known that the first stage of bacterial colonization is attachment and many pathogens use carbohydrate-binding proteins to attach to cells and begin the infection process; hence, mucins and other mucin-like proteins present in buttermilk and MFGM isolates may exert a positive effect on the prevention of infections. The inhibition of binding of enterotoxigenic *Escherichia coli* to the intestinal epithelial cells and guinea pig erythrocyte membrane, as well as the interference by mucin of the binding of *E. coli* to buccal mucosa, has been shown.

MFGM isolates were also proven effective in the inhibition of *Helicobacter pylori* infection in a BALB/cA mouse model and in the inhibition of hemagglutination and adhesion of *H. pylori* in cell monolayers. In this study, MFGM isolates were obtained directly from bovine buttermilk using ultrafiltration and diafiltration. Both defatted and non-defatted fractions caused similar effects, suggesting that the bioactivity was a result of the proteinaceous components of the MFGM. A similar effect has been suggested for rotavirus infections, which has been attributed to the glycoproteins PAS 6 and PAS 7.

Evidence is also accumulating on the positive effect of consumption of polar lipids and their human health-promoting properties. Phospholipids represent the most abundant species of polar lipids in the MFGM, and are known to affect beneficially diverse cell functions including absorption of nutrients, molecular transport, growth and development, stress response, regulation of the nervous system, and cancer development. In addition to their protective gastrointestinal functions, several antimicrobial functional properties of MFGM fractions have been proposed. Fractions of either raw or protease-digested MFGM were tested against several pathogens including *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica Typhimurium*, and *Bacillus cereus*, and their effects were compared to treatments with XO alone. It was found that these MFGM hydrolysates have variable antibacterial activity against all of the above bacteria, and that the effect was not necessarily attributable to enzymatic activity of XO. Similar results were obtained after testing different triglycerides and membrane lipids; it was concluded that milk fat sphingolipids and triglycerides, particularly those containing C10:0 and C12:0 fatty acids, may protect against food-borne gastroenteritis.

Finally, considering the many similarities between the components of the MFGM and that of intestinal cells, it has been suggested that two types of lipid-binding activity can be found: (1) that involving the nonpolar lipids, in which the concentration of lipids can affect the affinity of lactic acid bacteria, and (2) that involving the polar lipids, with which, regardless of composition, each strain of lactic bacteria shows specific binding activity. It has been suggested that there may be different degrees of lactic acid bacteria binding depending on the processing history and nature of the MFGM isolation method.

Conclusion

Buttermilk, the by-product of butter manufacture, is a complex mixture of components with important value not only technologically but also nutritionally. Isolates rich in MFGM can be obtained from buttermilk, but there are still important questions related to understanding the biological functions of the MFGM and its components. Its effect on human health is still under investigation. The question of whether MFGM plays a role in interactions between fat globules and in intestinal cells still remains unanswered. In addition, its nutritional significance in the light of its protective effect on the gastrointestinal tract is still unclear. However, it is also very important to note that the properties and composition of buttermilk and MFGM isolates are very sensitive to processing treatments and milk handling procedures; therefore, research is needed to understand if and when during processing biological functionality is lost, so that technologies and processes can be designed to scale up the production and isolation methods to optimize biological functionality.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Fat Replacers; Milk Fat-Based Spreads; Modified Butters; Properties and Analysis; The Product and Its Manufacture. **Cream**: Manufacture; Products. **Fermented Milks**: Buttermilk. **Mammary Gland**: Anatomy; Gene Networks Controlling Development and Involution; Growth, Development and Involution. **Milk Lipids**: Milk Fat Globule Membrane; Nutritional Significance; Phospholipids.

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Analytical Methods

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Fatty Acid Analysis

General Remarks

The fatty acid composition yields information about the relative proportions of the principal fatty acids in a product, giving clues about its origin and properties, and about the classes of fatty acids present (e.g., saturated fatty acids, or ω-3 fatty acids, for nutrition information panels). Fatty acids may also be determined individually in a product (e.g., linoleic acid in infant formulae).

Although the analytical procedures are mostly straightforward, care is needed if valid results are to be obtained. Milk fat contains a wide range of fatty acids, with relative molecular masses ranging from 88.1 for butyric acid (C4:0) to 340.6 for docosanoic acid (C22:0), esterified in triacylglycerols of widely varying melting points. This is a wider range than most common fats and oils and is the reason that milk fat analysis requires extra care. Fatty acid composition results are only as good as the lipid sample, and it is critical that the analyst obtains a sample that is truly representative of the product. The sample must be melted fully and thoroughly mixed before the test portion is removed.

For most purposes, an alkaline digestion method is recommended (Roese-Gottlieb or Mojonnier). Samples containing a significant proportion of phospholipids should be mixed with a sodium chloride solution prior to extraction. For total extraction of lipids from foods and plant and animal tissues, a chloroform/methanol mixture (2:1) can be used (Soxhlet, Folch, or Bligh and Dyer methods). Accelerated solvent extraction is also suitable, provided appropriate extraction conditions (solvent and temperature) are employed. The International Dairy Federation (ISO/IDF) has specified methods for the extraction of milk lipids from a range of dairy products. Acid digestion methods (Schmid-Bondzynski-Ratzlaff (SBR) or Gerber) are not suitable.

Methods

Gas chromatography (GC) of methyl esters is the usual method for determining the fatty acid composition. Fatty acid methyl esters (FAMEs) are formed by direct transesterification of the fat. If it is desirable to reduce the volatility of the short-chain fatty acids, then isopropyl esters or butyl esters can be used. Temperature programming of the gas chromatograph is required to achieve optimal separation of milk fat esters. Detection is by means of the flame ionization detector (FID), which is inexpensive, reliable, sensitive to hydrocarbons, and has a wide linear dynamic range. Gas chromatography–mass spectrometry (GC–MS) is also popular for the increased degree of certainty around identification of the fatty acids.

High-performance liquid chromatography (HPLC) has not been the method of choice, as capillary GC provides superior separation in a much shorter run-time. However, the availability of ultra-performance liquid chromatography (UPLC) and ultra-fast liquid chromatography (UFLC) instruments combined with new column technologies and highly sensitive tandem mass spectrometry detectors now makes liquid chromatography a viable alternative to GC.

Gas Chromatographic Analysis of Fatty Acids

International Organization for Standardization (ISO) and IDF have jointly published standard methods for the preparation of milk fat FAMEs, and determination of the composition by GC-FID. A method for the determination of specified ω-3 and ω-6 fatty acids has also been published. The other commonly used method in the food industry is the Association of Official Analytical Chemists International (AOAC) method 996.06, which determines total fat and saturated and unsaturated fatty acids in foods. Compared to the IDF methods, the AOAC method uses a different methylation reagent and a more polar column that is also significantly longer (100 m).

For preparation of the methyl ester derivatives, alkaline methylation is usually used (sodium methoxide or potassium hydroxide in methanol) and is suitable for trans esterification. Where free fatty acids or sphingomyelin-based fatty acids are to be detected, an acid catalyst (a methanol solution of boron trifluoride, sulfuric acid, or hydrochloric acid) is required. Boron trifluoride is known to generate artifacts, and under extended heating also leads to the degradation of conjugated linoleic acid (CLA) and the loss of butyric acid. Other reagents proposed include boron trichloride in methanol, tetramethylammonium hydroxide (TMAH), and acetyl chloride in methanol. All of the catalysts require neutralization after a certain time (6 min is often recommended) to prevent degradation of the esters formed.

Capillary GC columns give excellent separation of FAMEs, and there is no reason to use packed columns. On polyethylene glycol (PEG)-based phases such as WAX-type columns, fatty acids separate in the order of chain length and degree of unsaturation, with very little overlapping of peaks (**Figure 1**). More polar specialist phases based on cyanosilicones have been developed to provide optimal separation of the *cis* and *trans* isomers, and are used when determining *trans* fatty acids in milk fat, albeit with more overlaps of the different chain lengths. Free fatty acids can be separated underivatized on free fatty acid phase (FFAP) type columns, in which the PEG has been modified with nitroterephthalic acid to achieve greater polarity; however, this requires higher instrument temperatures for elution than for FAMEs.

On-column injection is preferred over split injection because the volatile short-chain esters can be lost disproportionately if split injection is used. In either case, an auto-injector is recommended to maximize the repeatability of injection.

FAME separations are usually predictable, and the major fatty acids can be identified easily by comparison of peak retention times with a standard chromatogram. In case of retention time shifts, relative retention times can be compared, as they change much less than the actual retention time. In cases of doubt, the FAME solution can be spiked with an appropriate concentration of standard FAME. Alternatively, the sample can be submitted for GC–MS, although it can be difficult to categorically distinguish two or more co-eluting fatty acids. More sophisticated techniques are required for charting the positions of double bonds (see section ‘Stereospecific analysis’).

Fatty acids are quantified using response factors to account for the differing response of short- and long-chain fatty acids in the FID. Most of the standard methods use empirical response factors based on a solution of injected FAME standards, although a strong case has been put for the validity of theoretical response factors, provided the detector has been optimized properly. Fatty acid profile results are often reported as mass percent of total fatty acids (normalization). When only selected fatty acids are to be determined, a known amount of internal standard is added prior to derivatization, and the fatty acids are quantified relative to the internal standard and reported on a product basis.

Evaluation of Results

While the composition of milk fat varies with species, diet, and lactation stage, certain features are typical. Butyric acid (C4:0) and CLA are present in the milk of cows, sheep, buffalo, and goats, and their absence means the fat is not that of a ruminant. Palmitic acid (C16:0) is almost always the most abundant, except in soft-fraction milk fats, where oleic acid (C18:1*cis*) predominates. A reasonable estimate of the identities of two fats mixed together can often be made, but may be impossible if the fats are similar in composition or there is only a small amount of one in the other. In these cases, analysis of the triacylglycerols is required (see next section).

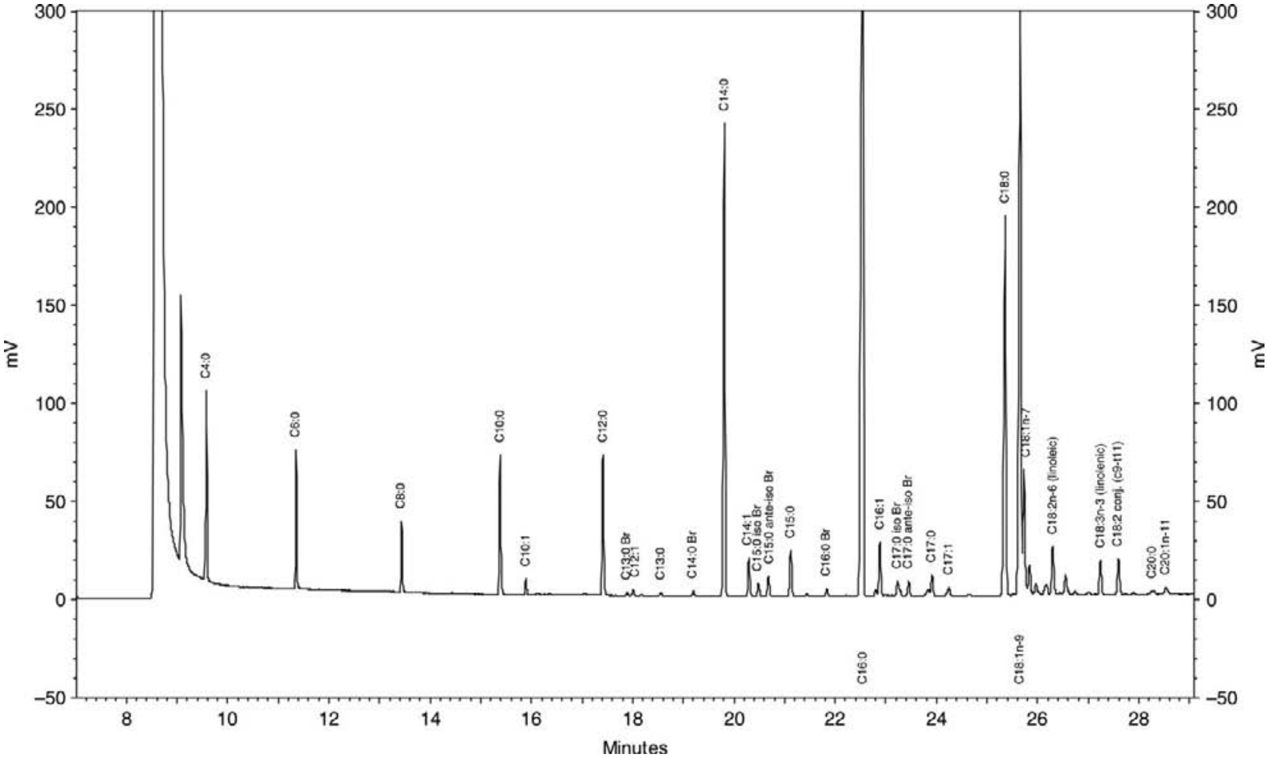

Figure 1 Bovine milk fat fatty acid methyl esters on an FFAP column, which is a modified PEG column.

Triacylglycerol Analysis

The properties of fats rely not only on the fatty acid constituents, but also on the way the fatty acids are arranged on the glycerol backbone. This produces triacylglycerols with a wide range of molecular weights and the accompanying wide range of physical properties (e.g., melting point). For the identification of fats and the overall properties of the fat, the analysis of the intact fats is very important.

The individual triacylglycerols can be categorized by their acyl carbon number (ACN or CN), which is the sum of the carbons of the three fatty acid constituents. For example, the triacylglycerols 16:0/16:0/16:0 and 18:0/16:0/14:0 both have ACN = 48.

Gas Chromatography

The analysis of triacylglycerols by GC requires high temperatures to volatilize the sample due to the approximately 3 times higher molecular weight compared to the individual fatty acids (350–400 °C for elution of milk fat) and is best carried out with on-column injection to avoid sample discrimination. Using short non-polar capillary columns, the triacylglycerols of milk fat can be separated simply by ACN (with no contribution from the different degrees of saturation). This provides a very simple trace (milk fat also has odd-numbered ACN, from the odd-numbered fatty acids) with which to compare the basic triacylglycerol composition of different milk fats, and especially for distinguishing samples of milk fat from other oils (**Figure 2**). Indeed this is the basis of an IDF method for the testing of adulteration of milk fat with other oils and fats (IDF 202:2010). The lack of influence of the degree of unsaturation becomes an advantage in this case as partially hydrogenated oils show the same peaks as their natural oil feedstocks (same ACNs), making determination and identity of the adulterant much simpler. Thus, the degree of hydrogenation of the adulterant is not a complicating issue. The basis of this method is the detection of samples that are outside the expected range for milk fat utilizing those peaks that differ most between the oil and milk fat. Note that in this method, the odd-numbered ACN peaks, if separated, are combined with their nearest lower even-numbered ACN peaks.

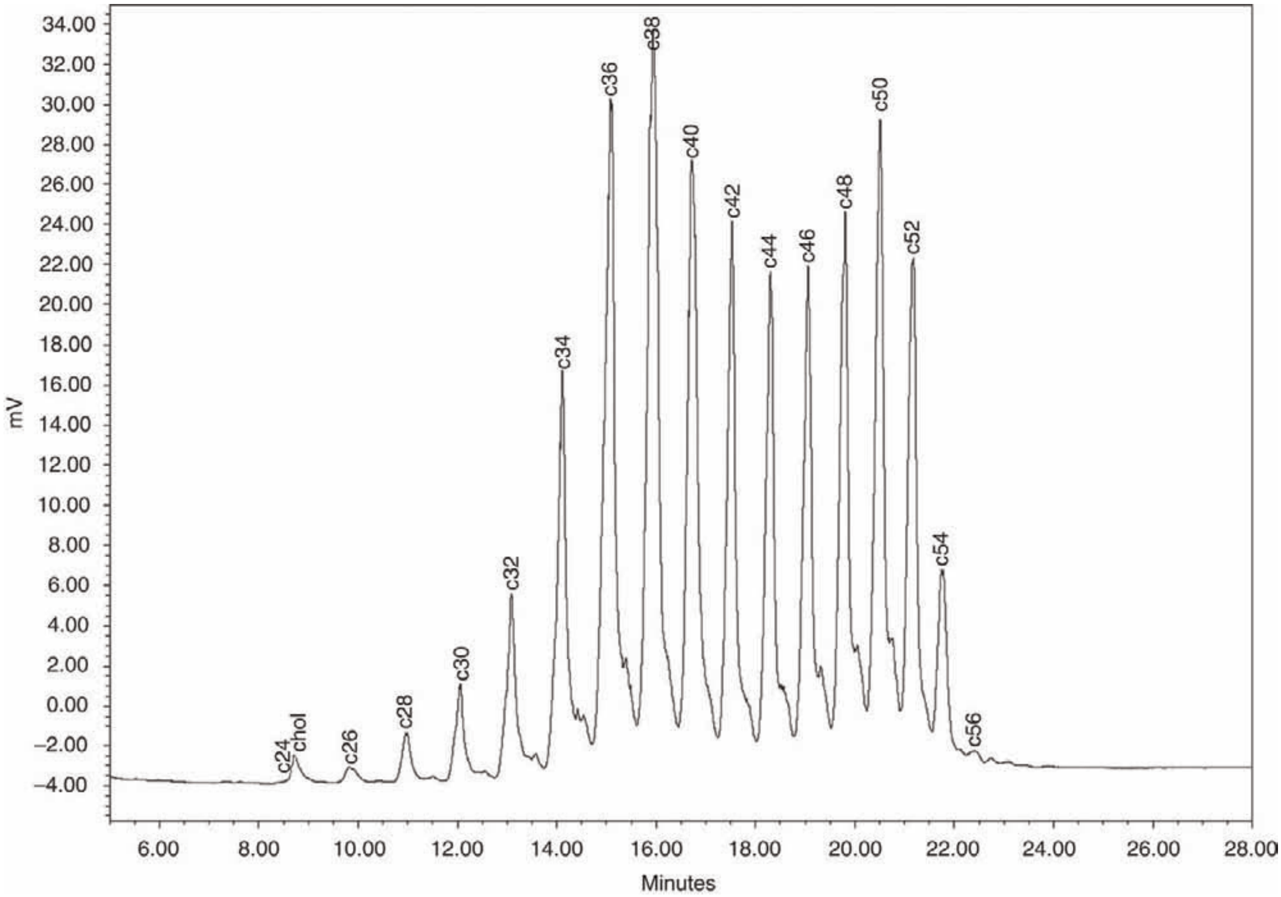

Figure 2 High-temperature gas chromatography of milk fat triacylglycerols, separated by acyl carbon numbers (ACNs) (van der Does and MacGibbon, personal communication). The small ACN 24 peak elutes just before the cholesterol peak.

With more polar columns, the single ACN peak splits due to the triacylglycerols with different numbers of double bonds having different interactions with the column and thereby eluting slightly differently. Realistically, because of the complexity of the triacylglycerols in milk fat, it is only when very polar columns are used that the separation becomes significant enough to show separate quantifiable peaks.

High-Performance Liquid Chromatography

Separation by reversed-phase HPLC (RP-HPLC) has been used in a similar manner to the very polar GC columns. The separation that occurs during RP-HPLC of triacylglycerols is based on both the chain length and the number of double bonds. A common expression that describes the influence of the two factors is

$$\mathrm{PN} = \mathrm{ACN} - (a' \times \mathrm{DB})$$

where PN is the partition number, ACN is the acyl carbon number, DB is the sum of the double bonds, and a' is the affinity factor of the double bond interaction. The affinity factor is often about 2, though it depends on the column and the solvent system. The double bond separation is reflected within each ACN group, so the trace shows a periodicity which greatly enhances identification. The elution order in RP-HPLC shows increasing ACN and decreasing number of double bonds, so for the same ACN, the species with the greatest number of double bonds elutes first. One study showed that this system could easily identify 61 different groups of triacylglycerols. In that case, a C18 column was used with a solvent system of acetonitrile and dichloromethane. However, even with that separation, there can be about 10 different triacylglycerols under the same peak, so the separation is by no means a complete separation of individual triacylglycerols (**Figure 3**).

Some work has been carried out on normal-phase HPLC, and in this case the elution order is decreasing ACN and increasing number of double bonds. This uses a silica column and a solvent system of hexane and hexane/methyl-*tert*-butyl ether/acetic acid. This method is complementary and in some cases shows better separation based on chain length within the ACN, but not as much by double bonds. It has been used for stereospecific determinations.

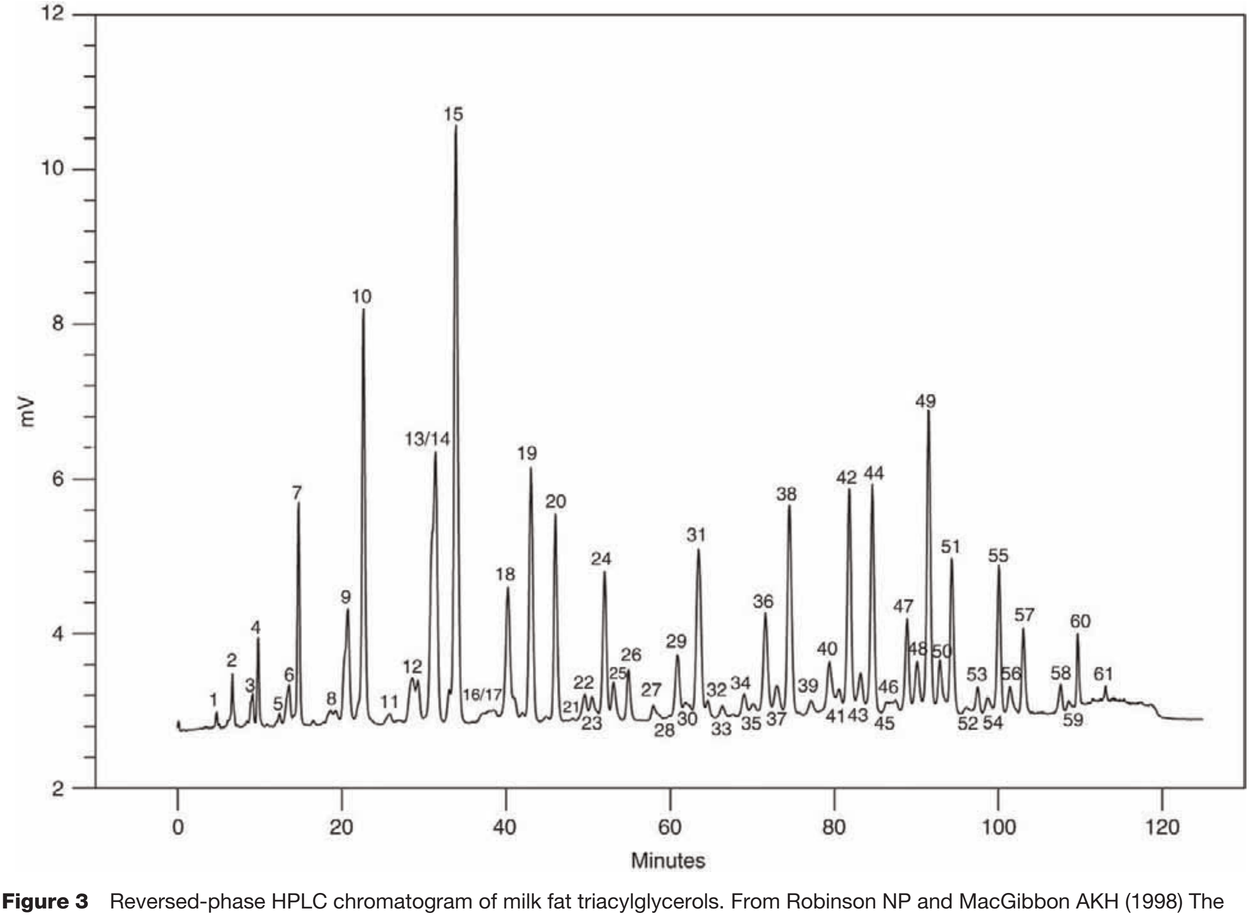

Figure 3 Reversed-phase HPLC chromatogram of milk fat triacylglycerols. From Robinson NP and MacGibbon AKH (1998) The composition of New Zealand milk fat triacylglycerols by reverse-phase high-performance liquid chromatography. *Journal of the American Oil Chemists' Society* 75: 993–999.

While HPLC allows great flexibility in separation conditions, for lipids it does have one disadvantage and that is in quantitation. Unlike the extremely linear response of the FID detector of GC, the HPLC detectors for lipids are less precise. For fats, the general UV response, refractive index, and more recently evaporative light scattering detection (ELSD) have been the means of observing the elution of lipids from the column. Particularly the ELSD can be very reliable; however, it does require calibration to ensure quantitation.

Mass Spectroscopy

The identification of triacylglycerols has been greatly enhanced by mass spectroscopy. This allows identification based on the molecular mass and can easily distinguish species on the basis of the number of double bonds (a decrease in mass of 2, compared to the saturated species for each double bond). The first mass spectral analyses were carried out using electron impact (EI) ionization; however, the introduction of electrospray ionization (ESI) to the analysis of lipids has rapidly increased the use of mass spectrometry in the area. ESI is a soft ionization technique that can result in little fragmentation, enabling the molecular ion to be observed, which is critical for the identification of the triacylglycerol molecule. The ions are usually $[\mathrm{M+NH_4}]^+$ or MH^+, although lithium ions have also been used. Further fragmentation of a particular molecular ion enables the identity of the fatty acid constituents to be determined (MS-MS). The ability to combine HPLC separation and MS has made this method extremely useful, although quantitation requires extra care as the response of the fatty acids differs. However, while the identity of the fatty acids may be determined, the position on the glycerol backbone is not so easily determined.

Stereospecific Analysis of Triacylglycerols

The position of the fatty acids on the glycerol backbone has an influence on the physical properties of the triacylglycerols (e.g., crystallization and melting point) as well as the nutritional properties (**Figure 4**). In digestion, pancreatic lipase attacks the *sn*-1 and *sn*-3 positions releasing the fatty acids while the fatty acid at the *sn*-2 position remains as a monoacylglycerol. Thus, depending on the position of the fatty acid, there are different impacts on absorption and subsequent metabolism (*sn*-2 fatty acid being largely retained in subsequent synthesis of the phospholipids and triacylglycerols). For example, in human milk, the palmitic acid tends to be bound at the *sn*-2 position and is absorbed by the infant as the more soluble monoacylglycerol, rather than the less soluble free palmitic acid, which would occur if it was on the other positions.

Triacylglycerol skeleton
A
B
C
sn-1
sn-2
sn-3

Figure 4 Fatty acid distribution on triacylglycerol.

Milk fat is unusually complex both in the number of fatty acids and in the stereochemistry of those fatty acids. Many oils exhibit regiospecificity (i.e., differences between *sn*-2 and *sn*-1,3); however, milk fat shows stereospecificity (i.e., differences between *sn*-1, *sn*-2, and *sn*-3) and so the full determination is more difficult. The specificity of *sn*-3 for C4:0 is an example of this difference between the *sn*-1 and *sn*-3 positions of milk fat.

Regiospecific Analysis

For regiospecific analysis, the traditional use of porcine pancreatic 1,3 lipase has proved effective and has been used in many studies. The 2-monoacylglycerol produced is separated and the fatty acid content is measured and the 1,3 mixture is determined from the original fatty acid content and the *sn*-2 fatty acid content.

$$sn\text{-}1 \text{ and } sn\text{-}3 = \frac{((3 \times \text{total}) - sn\text{-}2)}{2}$$

The other two positions cannot be differentiated and so have to be calculated as having the same fatty acid composition. This information is useful in digestive models and for some oils where the *sn*-1 and *sn*-3 compositions are similar.

In electrospray mass spectrometry, it has been observed that under certain conditions the response to further fragmentation of the molecular ion is regiospecific and that the different responses can be used to identify the *sn*-2 from the *sn*-1,3 position fatty acids. This is a huge advantage as it determines the regiospecificity for the single triacylglycerol molecular ion, rather than the bulk properties of the whole mixture.

Stereospecific Analysis

There is no lipase specific for only the 1 or 3 position and so stereospecific analysis requires a multi-step approach. Grignard reagent (EtMgBr) is used to produce 1,2 and 2,3 diacylglycerols. These are then reacted to form phospholipid-like molecules (with the phosphorus moiety at the *sn*-3 and *sn*-1 positions, respectively). Snake venom *sn*-2-specific phosphatase A2 reacts only with molecules that have the phosphorus moiety at the *sn*-3 position, hydrolyzing the *sn*-2 position fatty acid to produce only the lysophosphatide-like molecule with the remaining fatty acid retained at the *sn*-1 position. Also in the mixture are the free fatty acid from the *sn*-2 position, and the unreacted molecule with the fatty acids at the *sn*-2 and *sn*-3 positions and the phosphorus moiety at the *sn*-1 position. The molecules are separated and the fatty acids determined on a molar basis for the first two species; the last containing two fatty acids and so is not unique. The *sn*-3 fatty acids are determined by subtraction:

$$sn\text{-}3 = (3 \times \text{total}) - sn\text{-}1 - sn\text{-}2$$

Originally, Brockerhoff used phenyldichlorophosphate to form the phospholipid-like molecule; however, although the overall method has stayed remarkably similar, it is straightforward to form the phosphatidylcholines, a more natural substrate.

More recently, researchers have used chiral chromatography to determine the stereochemistry. An example is the use of a chiral derivatizing agent to form diacyl-*sn*-glycerol urethane derivatives from the diacylglycerols. These are separated and analyzed. The separation depends on the differentiation not being masked by wide variation in the fatty acid components and so has not yet been generally used for milk fat.

See also: **Milk Lipids**: Fatty Acids; Triacylglycerols.

Further Reading

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Rheological Properties and Their Modification

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Introduction

Milk fat exhibits unique rheological properties and is largely responsible for the consistency and textural attributes of butter. Milk fat and butter consistency are related to their complex composition and their viscoelastic nature. Strategies for modifying the rheological properties of milk fat are reviewed, including modifications based on compositional and processing factors. The composition of milk fat can be modified by methods such as blending, feed modification, fractionation, and interesterification. Processing factors that influence butter rheology include the type of process (batch vs. continuous churning), cream aging conditions, seeding, plasticizing (post-working), and storage conditions.

Rheological Properties of Milk Lipids

Composition and Melting Behavior

The rheological properties of milk lipids, including butter hardness and spreadability, are directly related to their complex composition. Milk fat contains dozens of different fatty acids, including very short-chain saturated, medium- and long-chain saturated, and long-chain polyunsaturated fatty acids. It also contains a considerable amount of *trans* isomers. The complex variety of fatty acids results in potentially thousands of different triacylglycerol species, each with its own melting point. The overall dropping point of milk fat is approximately 34 °C. However, because of its compositional complexity, milk fat actually has a very wide melting range, between −40 and 40 °C. This results in a fairly wide range of plasticity where both solid and liquid fat are present. **Figure 1** shows the solid fat content of milk fat as a function of temperature.

Saturated fatty acids have higher melting points than unsaturated fatty acids because their chains can pack in a more dense and ordered fashion in the solid state. Long-chain saturated triacylglycerols ($C > 42$) provide significant structural stability to milk fat. Similarly, the melting point of a *trans* fatty acid is higher than the corresponding *cis* isomer. The high proportion of short-chain saturates in milk fat is significant because these confer similar softening properties as the unsaturated fatty acids. The stereospecific distribution of the fatty acids within the triacylglycerols also influences the melting behavior.

Spreadability

Butter has notoriously poor spreadability at refrigeration temperature, and poor structural stability, including oil and moisture migration, at room temperature. Generally, a fat containing 20–40% solids exhibits good plasticity; thus, there is a limited temperature range over which desirable spreadability is achieved. **Figure 1** demonstrates the problem of poor spreadability typically encountered at refrigeration temperatures (5–10 °C). Within this range, the solid fat content is very high. **Figure 2** shows the effect of solid fat content on milk fat hardness where hardness is the force constant, a measure of the solid-like character of the material.

Spreadability is a term commonly used to describe butter texture and refers to how easily the fat can be spread on bread. Because spreadability is inversely related to hardness, some instrumental measure of hardness is often taken as an indicator of spreadability. Typically, hardness is determined by measuring the depth to which a cone placed on the surface of a butter sample will penetrate the sample in a given period of time. **Figure 3** shows the correlation between yield stress determined by cone penetrometry and spreadability evaluated by a sensory panel.

Seasonal Variability

Differences in texture between butters can be attributed to differences in composition. Winter butter is markedly harder than summer butter and often exhibits poor spreadability. This is partly related to compositional differences of individual fatty acids. For example, winter butter typically has a higher palmitic acid content and a lower oleic acid content than summer butter. Because the texture of milk fat is intimately linked to the overall degree of unsaturation, differences observed between summer and winter butter are most frequently attributed to differences in iodine value. Winter butter has a lower iodine value, about 30, than summer butter, which is typically 36. This seasonal variation is related to the fact that the polyunsaturated content of fresh green fodder consumed by ruminants during summer is higher

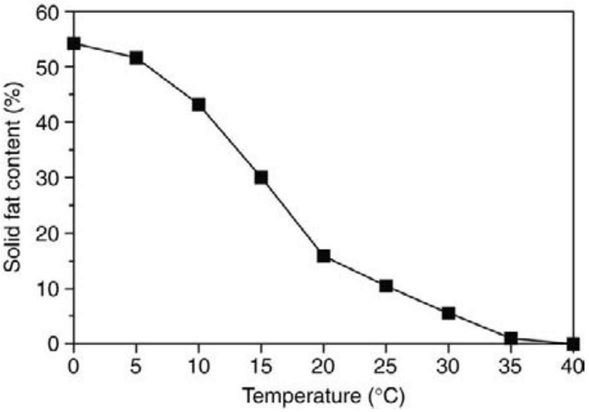

Figure 1 Solid fat content (%) vs. temperature (°C) for milk fat.

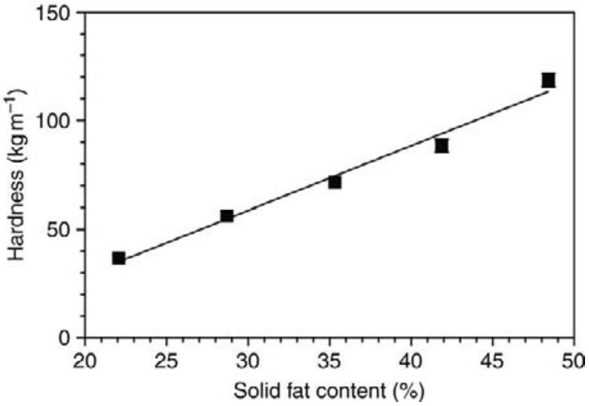

Figure 2 Hardness (kg m^{-1}) vs. solid fat content (%) determined by cone penetrometry for milk fat diluted with canola oil to achieve different solid fat contents.

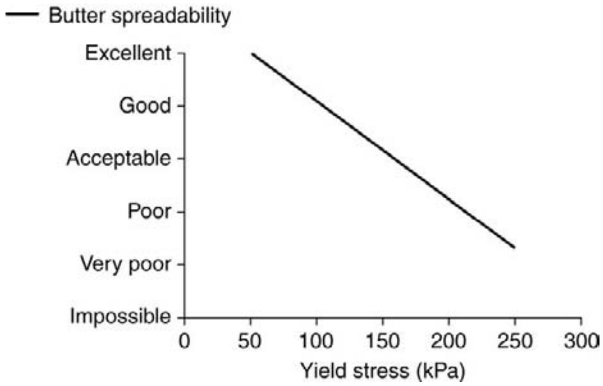

Figure 3 Butter spreadability determined by sensory panel vs. yield stress (kPa) determined by cone penetrometry.

than that of winter feed, which has undergone oxidation during storage. Seasonal variations have been related to specific triacylglycerol fractions present in the fat. Regional variations in butter firmness also exist and the stage of lactation can affect the textural attributes of butter.

Viscoelastic Nature of Butter

The fat crystals in milk fat and butter form three-dimensional networks and are closely associated with the continuous oil phase. As a result, these materials exhibit complex rheological behavior. Milk fat and butter display non-Newtonian plastic behavior and flow when the yield value is exceeded. Butter behaves as a pseudoplastic fluid because it exhibits shear-thinning. The typical flow behavior of semisolid milk fat is shown in **Figure 4**.

While the ratio of solid to liquid fat is the most important factor in determining butter consistency, the theories of viscoelasticity are useful in explaining the complex behavior. Over its melting range (between −40 and 40 °C), milk fat exhibits both solid- and liquid-like characteristics and behaves as a viscoelastic material. Four mechanical models used to describe the viscoelastic behavior of butter include Diener and Heldman's viscous Maxwell–Bingham model, the modified Bingham Body proposed by Elliott and Ganz, Tanaka's viscoplastic model, and the generalized yield-Maxwell model described by Awadhwal and Singh. **Figure 5** shows the frequency dependence of the storage and loss moduli of milk fat at low frequencies. Also, the low value of the loss tangent ($\tan\delta$), approximately 0.15, demonstrates the solid-like nature of milk fat at 5 °C.

The relationship between the storage modulus determined by small deformation oscillatory rheometry and hardness index determined by cone penetrometry for milk fat–canola oil blends is shown in **Figure 6**. Viscoelastic principles and testing are being used increasingly to characterize milk fat consistency and to explain the rheological properties of butter.

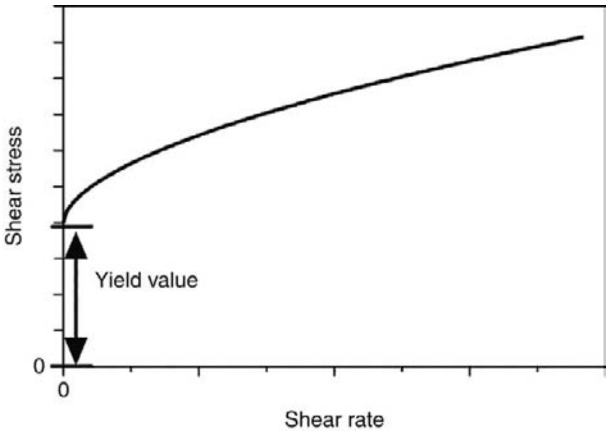

Figure 4 Pseudoplastic flow behavior of milk fat showing a yield value.

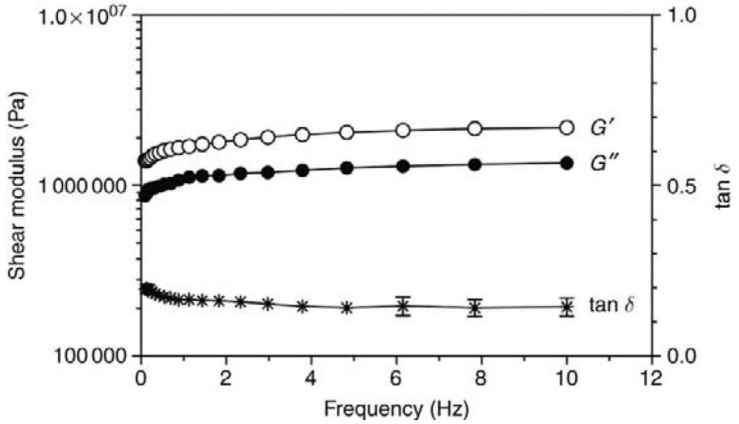

Figure 5 Frequency dependence of shear moduli (G′ and G″) and loss tangent (tan δ) for milk fat at 5 °C.

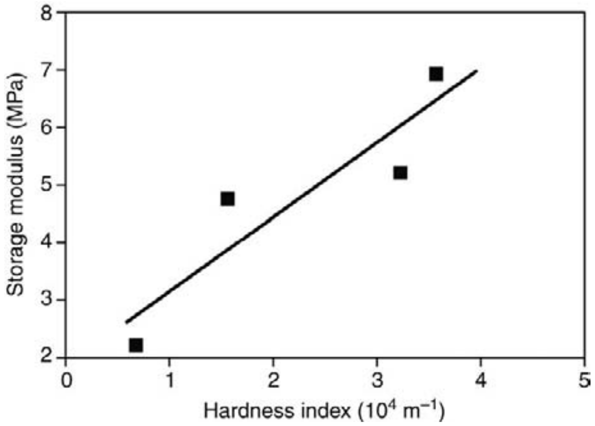

Figure 6 Storage modulus determined by small deformation oscillatory rheometry vs. hardness index (10^4 m^{-1}) determined by cone penetrometry for milk fat–canola oil blends at 5 °C.

Modification of Rheological Properties

Composition-Related Modifications

Blending and modification of feed unsaturation

Because poor butter texture has been associated with low iodine values, some attempts to improve its consistency have focused on increasing the iodine value of butter. Two ways by which this has been accomplished include blending with unsaturated oils and modifying feed to include more polyunsaturates. Blending milk fat with vegetable oils serves to increase the overall level of unsaturation and reduces the melting point. **Figure 7** shows the decrease in melting point and hardness index when canola oil is blended with milk fat.

Blending with vegetable oil improves cold temperature spreadability, but can have deleterious effects on stability at higher temperatures. Also, strict legal definitions of butter in some countries prohibit blending with non-dairy oils. Milk fat has also been blended with tripalmitin and used as an alternative to hard milk fat fractions in applications including edible films with moisture barrier properties.

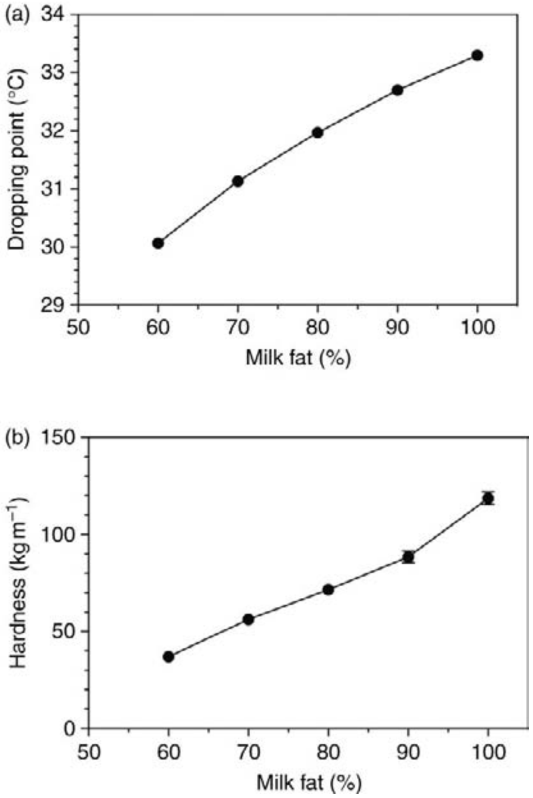

Figure 7 Melting point (°C) (a) and hardness (kg m^{-1}) (b) of milk fat–canola oil blends.

The iodine value of butter can be increased by changing the composition of animal feed to include more unsaturated triacylglycerols. Highly unsaturated vegetable oils can be encapsulated and included in a cow's diet. When the oils are encapsulated in formaldehyde-treated casein or consumed in the form of calcium salts, hydrogenation in the rumen is prevented. As a consequence, the fatty acyl chains are incorporated unaltered into the newly synthesized triacylglycerols. Modification of butter composition through animal feed, including for the purposes of improving texture, is a very active area of research. Similarly, butter texture can be manipulated for the production and sale of reduced-fat spreads by decreasing the typical 80% milk fat to 40% or less using non-fat milk water and including gelatin.

Fractionation

Fractionation can be used to modify the rheology of milk fat and can be accomplished by crystallizing the melted fat at a specific temperature and separating the crystals from the liquid oil by filtration or centrifugation. Crystallization in the presence of a solvent improves the separation of the crystalline mass from the oil; however, dry crystallization is preferred in food applications. Fractionation yields fractions of triacylglycerols that are chemically and physically distinct. Certain triacylglycerol fractions have been associated with butter hardness, as have individual triacylglycerols and fatty acids. Milk fat triacylglycerol fractions are typically distinguished from each other on the basis of their melting behavior. Accordingly, there are three main fractions of triacylglycerols: the low-melting, middle-melting, and high-melting fractions (LMF, MMF, and HMF, respectively). These fractions correspond to the three peaks observed for milk fat by differential scanning calorimetry. LMF is liquid at room temperature, owing to its higher content of long-chain unsaturated and short-chain saturated fatty acids. On the other hand, HMF is enriched in long-chain saturated acids and has a much lower content of long-chain unsaturated and short-chain saturated fatty acids.

Butter spreadability has been improved by fractionating milk fat and then recombining the fractions in different proportions. The addition of LMF to butter reduces its hardness by increasing the level of oleic acid and short-chain fatty acids. Alternatively, the addition of HMF leads to a firmer texture and reduced oiling-off and moisture migration at higher temperatures. In some regions, HMF and LMF butter are combined, achieving a very functional plastic spread. The milk fat fractions themselves have unique rheological properties. Because of its very solid nature and unique fatty acid composition, HMF has been used in puff pastries and as a partial cocoa butter substitute in confectionary products. Fractionation offers the possibility of improved butter texture and functionality, although the high costs associated are prohibitive on the largest scales.

Interesterification

Interesterification serves to change the behavior of milk fat by rearranging the fatty acids within and among the triacylglycerols. Both chemical and enzymatic interesterification with lipases cause randomization of the fatty acid distribution in the triacylglycerols. Both can improve the cold spreadability of butter, although the enzyme-catalyzed process is much more costly. **Figure 8** shows the changes in the melting profile of milk fat upon interesterification. The solid fat content at refrigeration temperatures is reduced, particularly in the enzymatically interesterified milk fat.

Milk fat interesterification causes a relative decrease in the C_{32}–C_{42} triacylglyerols and a relative increase in those triacylglycerols in the range of C_{44}–C_{54}. Further success has come from blending milk fat with canola oil and interesterifying the blends. **Figure 9** shows that interesterification of milk fat–canola oil blends leads to significant reductions in hardness. Unfortunately, the classic problem with interesterification of milk fat is a decrease in butter flavor intensity and the production of off-flavors.

Hydrogenation is used to alter the physical properties of fats by increasing their degree of saturation. Because this results in an overall increase in the firmness of the fat

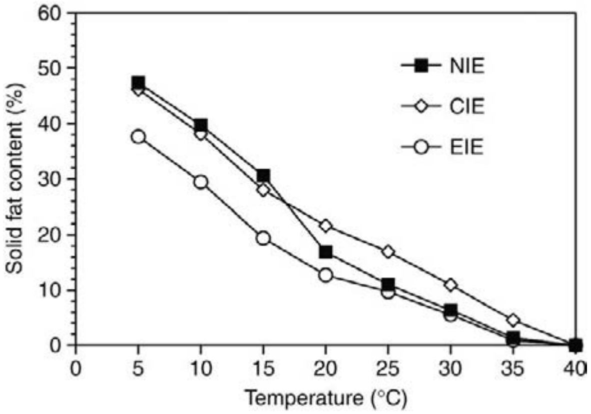

Figure 8 Solid fat content (%) vs. temperature (°C) of non-interesterified (NIE), chemically interesterified (CIE), and enzymatically interesterified (EIE) milk fat.

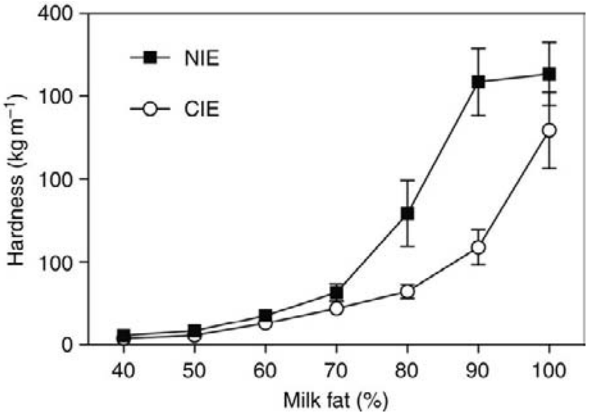

Figure 9 Hardness (kg m^{-1}) vs. solid fat content (%) of non-interesterified (NIE) and chemically interesterified (CIE) milk fat–canola oil blends.

and does not lead to improved cold temperature spreadability, it is rarely applied to milk fat.

Surfactants in butter and alterations in the moisture and air contents

The techniques discussed for modifying butter texture thus far have focused specifically on milk lipids. The rheological properties of butter can also be altered by adjusting the non-triacylglycerol components. Surfactants have been added in order to improve butter spreadability, although, depending on the surfactant, this can result in deleterious effects. Also, the effectiveness of this approach is debatable because surfactant effects seem to be overcome during the normal setting period of butter. Butter consistency can be modified by adjusting moisture content, although this can affect stability at higher temperatures. In addition, an increased water content increases the potential for microbial spoilage and raises concerns about legal standards of identity. Increasing the air content of butter also serves to reduce its hardness. Whipped butter, in which air or nitrogen is incorporated, can offer improved spreadability, although a loss of butter aroma can occur and, if proper processing conditions are not met, there can be issues with crumbly texture. Butter can be treated under reduced pressure in a vaculator. The result is a decrease in air content and butter that, interestingly, is harder, but reported to have improved organoleptic properties and a glossy appearance. This contradiction is explained by the fact that under pressure, oil, which is originally adsorbed onto air bubbles, becomes free and alters the flow properties of the butter.

Process-Related Modifications

Batch versus continuous buttermaking

The type of manufacturing process and processing parameters influence butter texture. The replacement of many batch buttermaking facilities with continuous operations saw a pronounced change in butter texture. The continuous Gold'n Flow process produces firmer butter than historical batch churning. **Figure 10** shows the higher shear stress failure associated with continuously produced butter over that which is batch churned.

The more rapid cooling rate employed in the continuous process is largely responsible for the differences observed in slowly and rapidly cooled butters. Slow cooling of milk fat typically results in reduced hardness. **Figure 11** shows the higher penetration depth found over time for slowly cooled milk fat compared to that which was rapidly cooled.

The effect of cooling rate has been explained on the basis of mixed crystal formation, differences in crystal structures, and polymorphism. The increased solid fat content associated with a fast cooling rate in milk fat accounts for some of the increased hardness. Milk fat contains a number of similar triacylglycerols, which compete for positions in the growing crystal lattice during crystallization and, upon rapid cooling, tend to form mixed crystals. During slow cooling, there is time for the most compatible triacylglycerols to become incorporated into the growing crystal. However, at a high cooling rate, some of the less compatible triacylglycerols are incorporated. Cooling rate also affects crystal size and homogeneity. **Figure 12** shows that rapid cooling of milk fat results in the formation of many, small crystals, while relatively larger crystals form under slower cooling conditions.

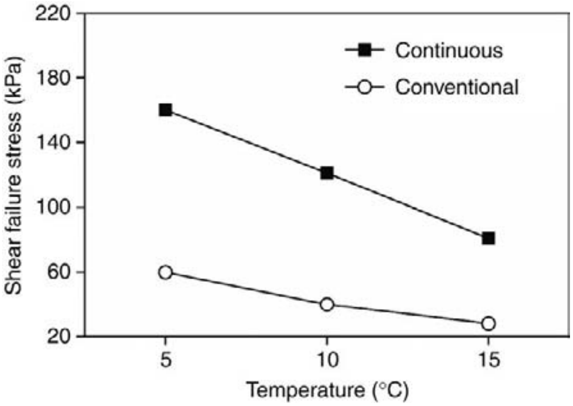

Figure 10 Shear stress failure (kPa) as a function of temperature (°C) for butters made by the conventional (batch churn) and continuous (Gold'n Flow) processes.

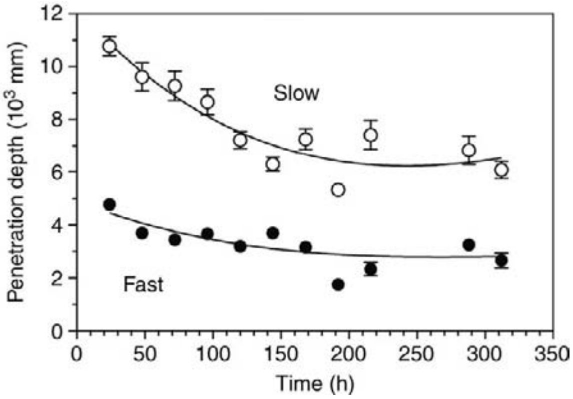

Figure 11 Penetration depth (10^3 mm) vs. time for slow (0.1 °C min^{-1}) and rapidly (5 °C min^{-1}) cooled milk fat at 5 °C.

It is postulated that rapidly cooled milk fat is firmer because the higher crystal surface area allows for more liquid oil to be tightly held by surface forces. Milk fat crystallization and the resulting microstructure are highly dependent on the degree of supercooling. Crystallization at higher temperatures results in a more spherulitic crystal microstructure, whereas at lower temperatures a more fine granular microstructure is observed. Slow cooling allows for more crystallization at the higher temperatures and can change the microstructure of the fat accordingly. Some attempts have also been made to explain the effects

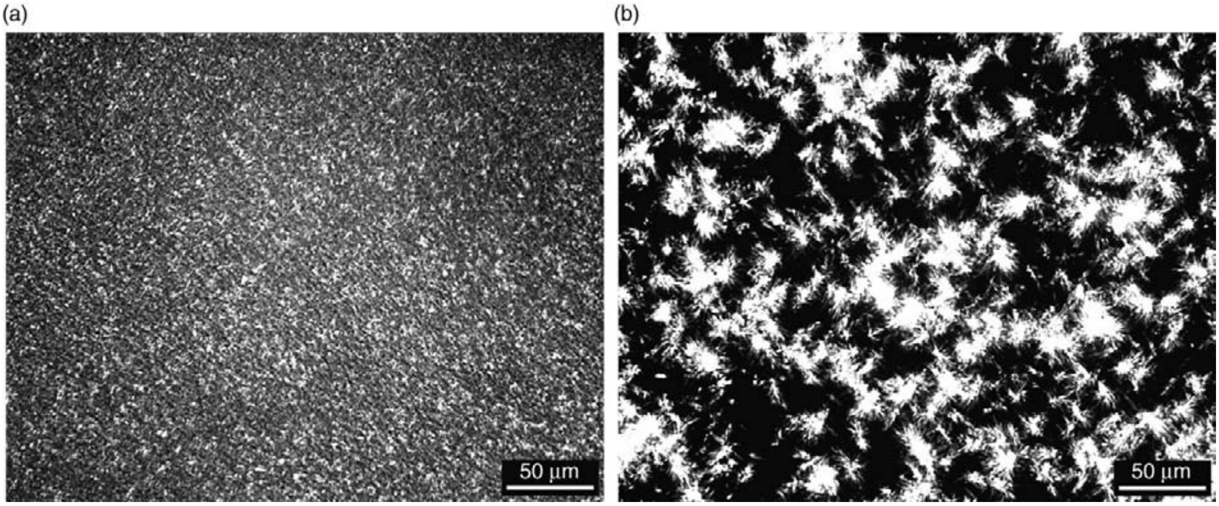

Figure 12 Polarized light micrographs of slow (0.1 °C min^{-1}) (a) and rapidly (5 °C min^{-1}) (b) cooled milk fat at 5 °C.

of cooling rate on polymorphism since polymorphism is affected by temperature conditions and is known to affect the macroscopic properties of several fats.

Cream aging, mechanical agitation, and seeding

The manufacture of continuously made butter typically involves aging the cream at a chilling temperature before manufacture. Prechilling leads to larger fat crystals and more free liquid fat, resulting in a high-quality butter. Cream ripening or tempering, as it is sometimes referred to, has been used industrially for decades and generally involves a cold–warm–cold treatment of the pasteurized cream. The ALNARP process for obtaining softer butter is one example of a commercially used ripening process.

Other processing parameters that can be controlled to improve the texture of butter include mechanical agitation of the cream and seeding with fat crystals early in the buttermaking process. Increased mechanical agitation promotes secondary nucleation and ultimately leads to more discrete fat crystals and a less firm fat at any given solid fat content. Seeding, or recycling, involves adding milk fat crystals to the cream during churning to seed the growth of larger, desirable crystals. While this can produce a softer fat, it unfortunately negatively affects the appearance of the product.

Plasticizing treatments

Commercial buttermaking operations include a mechanical treatment or plasticizing step after manufacture. This serves to soften the product. After mechanical working, butter exhibits thixotropic behavior in which there is a gradual recovery in its firmness. **Figure 13** shows the thixotropic hardening of butter after working.

Storage conditions

The storage temperature and the length of time for which butter is stored affect its firmness. At higher temperatures, the decrease in the amount of solid fat present results in a decrease in hardness. A phenomenon called setting, which is closely related to thixotropy, occurs especially in conventional butter after manufacture, even when stored at a constant temperature. Setting refers to the increase in the hardness of the butter over time and is thought to take place because of continued crystallization of some triacylglycerol species. Also, recrystallization of triacylglycerols, which melt because of heat introduced during working, occurs. The rate of change in hardness due to setting is most significant during the early time periods after manufacture.

Milk lipid rheology has been dominated by a desire to improve the cold spreadability of butter. Empirical tests can be used to measure butter consistency and often correlate well with sensory evaluated parameters like spreadability. However, milk fat and butter are complex systems. An understanding of the relationships between structure and rheological properties will help in achieving desired behaviors and textural attributes.

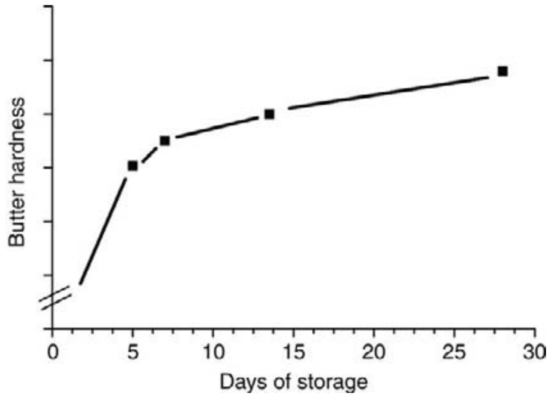

Figure 13 Thixotropic hardening of butter with storage time after working.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; The Product and Its Manufacture. **Feeds, Prediction of Energy and Proteins**: Feed Proteins. **Rheology of Liquid and Semi-Solid Milk Products**.

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Nutritional Significance

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Introduction

Lipid is the collective term for a diverse range of molecules which have in common the characteristic that they are insoluble in water but soluble in organic solvents, such as ether or chloroform. This range of molecules includes triglycerides (also known as triacylglycerols), other glycerides, phospholipids, sterols, fatty acids, fat-soluble vitamins, all of which have nutritional significance. The overwhelming majority of lipid molecules in milk (approximately 98%) are triglycerides, with small amounts of sterols and phospholipids as the next most important categories.

Lipids typically contribute approximately 40% of total energy in Western diets. This level has fallen slightly in recent decades in many countries due to the availability and promotion of low-fat and reduced-fat foods. Milk and milk products typically contribute about 20% of daily fat intake in Western countries, that is, approximately 8–10% of total energy intake.

Lipids are significant in the diet in a wide variety of ways. They contribute to the palatability of foods by influencing taste, texture, and mouthfeel. They provide a concentrated source of food energy ($9\,\mathrm{kcal\,g^{-1}}$ or $38\,\mathrm{kJ\,g^{-1}}$). Lipids, when deposited as body fat, help protect vital body organs and provide a store of energy for long-term exertion. Food lipids are a source of fat-soluble vitamins (A, D, E, K) and essential fatty acids (linoleic acid and α-linolenic acid).

Considerable research effort over many decades has focused on the health effects of food (including milk) lipids. Dietary lipid intakes have been related to the so-called 'diseases of affluence', such as coronary heart disease (CHD), stroke, and certain cancers. However, in discussing the putative role of dietary lipids, it is important to note the multifactorial etiology of these diseases, that is, there are other risk factors, such as cigarette smoking, hypertension, genetic factors, obesity, diabetes, and exercise level. Indeed, many other dietary factors apart from lipid intake are known to influence chronic disease risk, for example, the protective effects of high intakes of fruit and vegetables are well recognized. Thus, when discussing the health effects of milk lipids in isolation, it is important to emphasize that no food or food constituent, such as fat, should be considered healthy or unhealthy *per se*. All foods and food constituents are to be considered in the context of overall dietary intake, that is, one can discuss healthy/unhealthy diets but not individual foods or food constituents.

Metabolism of Lipids

Digestion

Lipids are a heterogeneous group of molecules which are soluble in nonpolar solvents. Digestion of lipids must take place in the intestinal tract which is an aqueous polar environment and subsequent lipid transport in the blood is also in an aqueous environment. However, as described below, the body has devised means for overcoming this problem.

Digestion of fat is initiated by the action of lingual lipase which is secreted in the mouth. However, the major site of fat digestion is the duodenum. The churning action of the stomach creates a crude oil-in-water emulsion of the fat prior to its entry into the duodenum. In the duodenum, the emulsification of fat is enhanced by the action of bile salts and phospholipids which are released from the gall bladder.

Pancreatic juice supplies lipase which catalyzes the hydrolysis of triglycerides to fatty acids. Fatty acids are cleaved from positions 1 and 3 of the triglycerides, resulting in 2-monoglycerides. Pancreatic lipase has a poor ability to cleave the fatty acid from position 2. However, a process known as acyl migration may occur and the fatty acid may move to position 1 and subsequently be cleaved by lipase. Additionally, the 2-monoglycerides can be absorbed directly without further hydrolysis.

Bile acids play a key role in facilitating the action of pancreatic lipase on triglycerides. The bile acid molecules have both hydrophobic and hydrophilic regions. The former region interacts with the oil surface of the fat droplets while the latter region interacts with the aqueous phase in the duodenum. The association of the bile acids with the oil droplets also results in a negative charge on the surface of the droplets. This negative charge causes pancreatic co-lipase to be attracted to the surface of the oil droplets. Thus, a complex of bile acids, pancreatic lipase, and co-lipase act at the oil–water interface to release fatty acids from the triglyceride molecules. A pancreatic cholesteryl hydrolase releases free fatty acids from any cholesteryl esters present in the duodenum. Phospholipase A cleaves fatty acids from phospholipids to form lysophospholipids.

It has been shown that short-chain fatty acids, for example, butyric acid which is commonly found in position 3 of milk fat triglycerides, are released more rapidly from triglycerides by lipase action than longer-chain fatty acids. However, due to the relatively long time that fat resides in the duodenum, breakdown of triglycerides to fatty acids is virtually complete.

However, malabsorption of fat and the appearance of significant amounts of fat in the feces (steatorrhea) may occur in diseased states if insufficient pancreatic lipase, colipase, and bile salts are secreted into the duodenum or if the intestinal mucosal cells become diseased due to severe bacterial infection or conditions such as coeliac disease.

Absorption and Transport of Fats in the Body

Fatty acids with fewer than 12 carbons are generally absorbed directly across the intestinal wall into the bloodstream where they are bound to plasma albumin and transported to the liver.

Long-chain fatty acids, monoglycerides, cholesterol, and lysophospholipids form micelles in the gut with bile acids. These are then absorbed by the enterocytes that line the gut. In the enterocytes, the lipid digestion products are reassembled into triglycerides, cholesterol esters, and phospholipids. These molecules are immiscible or poorly miscible with water. This is a major issue as these molecules must be transported by the blood (an aqueous environment) throughout the body. This problem is overcome by the association of absorbed lipid molecules with proteins to form lipoproteins. The proteins perform a dual role. Because of their amphiphilic nature, the proteins facilitate transport of lipids in the bloodstream. Second, the proteins (mainly a protein called apolipoprotein B-48, which is present in chylomicrons) allow the lipoprotein particles to be recognized by receptors on cell surfaces throughout the body, thus facilitating the controlled uptake of lipoprotein particles from the bloodstream. Chylomicrons are the lipoprotein particles that are assembled in the enterocytes following digestion and absorption of dietary fat from the gut. The chylomicrons enter the lymphatic system and subsequently the blood following their release from the enterocytes.

The blood enzyme, lipoprotein lipase, is involved in releasing free fatty acids from triglycerides in the chylomicrons. These fatty acids are taken up by tissues (e.g., for storage as triglycerides in adipose tissue or metabolism in muscle tissue) and the remainder of the chylomicron particles (remnants) may pass to the liver.

Triglycerides and cholesterol may also be synthesized directly in the liver rather than derived from the diet. The triglycerides synthesized in this manner are transported mainly in the bloodstream in lipoprotein particles called very low density lipoproteins (VLDL). Cholesterol is transported primarily in either low-density lipoproteins (LDL) or high-density lipoproteins (HDL). Thus, all lipoproteins contain proteins, triglycerides, phospholipids, cholesteryl esters, and cholesterol in varying amounts which account for the differences in their density.

Lipids and Obesity

A considerable body of evidence worldwide indicates that obesity is associated with increased risk of morbidity and mortality from chronic diseases such as CHD and certain cancers. Dietary guidelines worldwide advise individuals to maintain desirable body weight by controlling food intake and by undergoing regular aerobic exercise. It is also recognized that fat distribution in the body is important in predicting risk for chronic diseases. Central obesity, involving fat deposition around the waist and upper body, is a significant risk factor for CHD. Lower body obesity, involving fat deposition around the hips and thighs, is a much lower risk factor.

As noted earlier, the energy value (known as the Atwater factor) for triglycerides is about 9 kcal g^{-1} (38 kJ g^{-1}). This compares with approximately 4 kcal g^{-1} (17 kJ g^{-1}) for both carbohydrates and proteins. Thus, the more fat in the diet, the more energy-dense it is. This energy density, however, makes fat a more attractive energy store in the body.

The degree of digestibility of a fat influences the metabolizable energy available to the body. Most fats, including milk fat, are digested almost completely by healthy individuals. However, long-chain (over 18 carbons) saturated fatty acids are slightly less well digested and absorbed than shorter-chain fatty acids. On the other hand, medium- and short-chain fatty acids have lower metabolizable energy values as they undergo different pathways of metabolism from longer-chain fatty acids. These short- and medium-chain fatty acids are absorbed primarily into the portal blood and metabolized in the liver rather than stored in adipose tissue. Milk fat from ruminant animals contains significant amounts of short-chain fatty acids; about one-third of milk fat triglycerides contain a butyric acid residue.

A number of studies have addressed the issue of whether dairy consumption is inversely associated with reduced risk of the metabolic syndrome (obesity, high blood pressure, abnormal blood lipids). Pereira and colleagues reported a 70% decrease in risk of the metabolic syndrome over a 10-year period in individuals with higher levels of dairy product intakes.

Lipids and Coronary Heart Disease

CHD is the major cause of mortality in Western societies. CHD involves the inability of the coronary arteries to supply sufficient blood, and hence oxygen, to the heart muscle, a portion of which may then die. A heart attack (infarction) is a sudden event involving blockage (thrombosis) of a coronary artery by a blood clot. If significant atherosclerosis is present, that is, an accumulation of plaque deposit in the artery lining resulting in a narrowing of the arteries, the formation of a blood clot is likely to be more serious and often fatal.

The 'lipid hypothesis', that is, that the etiology of CHD is influenced by dietary fat intake, has been researched extensively for many decades. This hypothesis is that dietary fat, in particular saturated fat, results in an elevation of the blood cholesterol level, which over time results in atherosclerosis. Eventually, a coronary artery thrombosis may occur, resulting in a heart attack.

It is thought that atherosclerosis is initiated by damage to a blood vessel wall. This damage may result from factors such as the action of lipid free radicals, reactive oxygen species, infective agents, mechanical stress due to hypertension, and autoimmune reactions. Smooth muscle cells and immune system cells known as macrophages have been shown to accumulate at the site of blood vessel damage. Lipids, principally cholesteryl esters, begin to accumulate at the point of damage as a form of plaque. This atherosclerotic plaque tends to accumulate macrophages which become engorged with lipids, principally from low density lipoprotein (LDL). These lipid-engorged macrophages are known as 'foam cells'.

The ability of dietary fat intake to modulate blood lipid levels has been recognized for nearly a century. Landmark studies were conducted in the 1960s by two independent research groups led by Ancel Keys and Mark Hegsted which systematically evaluated the effects of different types of dietary lipids on blood lipid levels in human subjects maintained under strictly controlled metabolic ward conditions. The human subjects were fed a wide range of fats and oils of varying fatty acid composition. These studies led to the development of equations that predicted the changes that would occur in blood cholesterol level following changes in the intake of dietary saturated fatty acids (SFAs), polyunsaturated fatty acids (PUFAs), and cholesterol. Keys and Hegsted independently showed that SFAs were approximately twice as effective in raising blood cholesterol level as PUFAs. Dietary cholesterol intake had a relatively minor influence on blood cholesterol level.

These early investigators studied the effects of dietary fat on total blood cholesterol and did not discriminate between different lipoprotein fractions. It is now recognized that dietary parameters influence the levels of different lipoprotein fractions in different ways. Furthermore, it has been demonstrated clearly that the levels of different lipoprotein fractions are related to the risk of CHD in different ways. Elevated levels of LDL have been shown to be a major risk factor for CHD. Likewise, low levels of HDL are a risk factor for CHD.

Research findings indicate that dietary intake of different SFAs influences blood cholesterol level to different degrees. Short- and medium-chain fatty acids (4:0 to 10:0), which are found in ruminant milk fat, have little or no effect on blood cholesterol level; furthermore, stearic acid (18:0) has little effect on blood cholesterol. Thus, only lauric (12:0), myristic (14:0), and palmitic (16:0) acids have a significant effect on blood cholesterol and, in particular, LDL cholesterol level.

Monounsaturated fatty acids (MUFAs), in particular oleic acid (18:1), have been evaluated for their ability to influence blood cholesterol level. Data indicate that MUFAs, when substituted for SFAs, tend to reduce total blood cholesterol level and, in particular, again, LDL cholesterol. PUFAs of the *n*–6 family (linoleic, 18:2, being the most important in the diet) have been shown to reduce blood cholesterol level. On the other hand, PUFAs of the *n*–3 family (primarily α-linolenic, 18:3, from plant oils and eicosapentaenoic, 20:5, and docosahexaenoic, 22:6, from fish oils) tend to lower blood triglyceride level to a greater extent than the cholesterol level.

Many of the studies on the effects of fatty acids on blood cholesterol have been highly controlled experiments. Prudence is required in extrapolating the results of these studies to free-living populations where people eat a wide variety of fats as part of a mixed diet. Milk fat is hypercholesterolemic when evaluated in controlled experiments. However, epidemiological studies on free-living populations tend to indicate that intake of milk and milk products does not increase blood cholesterol level significantly. Indeed, studies of the Masai tribe in east Africa indicate that they have very low blood cholesterol levels on a traditional diet rich in milk (primarily fermented) and other animal products. It has been suggested that the Masai may have low blood cholesterol levels due to their low caloric intake and, perhaps, an inherited ability to regulate blood cholesterol level at a low value. Other researchers have suggested that milk contains an unknown cholesterol-lowering factor which counteracts the theoretical hypercholesterolemic effect of milk SFA. However, evidence for such a factor is scant.

Further work has investigated the possibility that cultured and culture-containing milk products may be hypocholesterolemic. There is some evidence that increased gut colonization by lactobacilli, whether by ingestion of culture-containing products or by manipulation of gut pH to favor lactobacilli, may modify cholesterol metabolism. However, the evidence, at best, suggests that lactobacilli are responsible for only small reductions in blood cholesterol.

Conjugated Linolenic Acid (CLA) in Milk Fat

Extensive hydrogenation of ingested PUFAs occurs in the rumen of ruminants, catalyzed by the diverse microflora present. A further effect observed in the rumen is the possible disruption of the original methylene-interrupted (unconjugated) sequence of *cis* double bonds. *Cis–trans* isomerization may occur and double bonds may migrate along the fatty acid chain. A *cis–trans* conjugated double bond sequence may evolve:

$$\mathrm{CH_3-(CH_2)_4-CH\underset{\mathit{cis}}{=}CH-CH_2-CH\underset{\mathit{cis}}{=}CH-(CH_2)_7}$$

$$\mathrm{COOH}(9\text{-}\mathit{cis},12\text{-}\mathit{cis}\text{-}18{:}2,\ \text{linolenic acid})$$

is converted to

$$\mathrm{CH_3-(CH_2)_5-CH\underset{\mathit{trans}}{=}CH-\ CH\underset{\mathit{cis}}{=}CH-(CH_2)_7COOH}$$

$$(9\text{-}\mathit{cis},11\text{-}\mathit{trans}\ 18{:}2,\ \text{conjugated linolenic acid, CLA})$$

9-*cis*,11-*trans*-CLA is the principal isomer found (about 90% of total CLA) in biological systems but other isomers of CLA have also been identified. Milk fat and dairy products have very high concentrations of CLA, which vary with season and stage of lactation. Cheese is the best dietary source of CLA, with reported levels of 0.6 mg g^{-1} fat in blue cheese, 1.9 mg g^{-1} fat in Parmesan, and up to 8.8 mg g^{-1} fat in processed cheeses containing whey protein concentrate. CLA is also found in meat from ruminant animals.

Over the last two decades, there has been significant research effort on the potential health effects of CLA ingestion. Data indicate that CLA is anticarcinogenic in animal models. It has been suggested that it may be partly responsible for the reduced risk of breast cancer that has been reported in women with high milk consumption. The mechanism by which CLA may exert an anticarcinogenic effect is unclear. It may act as an antioxidant, it may influence eicosanoid metabolism, and it may inhibit biosynthesis of proteins and/or polynucleotides that may be involved in the cancer process.

It has been clearly shown that dietary CLA is incorporated into body tissues, such as adipose tissue, human milk, and blood cells. For example, one study has shown that the consumption of 112 g Cheddar cheese per day for 4 weeks significantly increased the level of CLA in blood.

Lipids in Infant Nutrition

There is universal agreement that human milk is the most appropriate food for healthy human babies born at full term. However, as many mothers do not wish or are unable to breast feed, a need has arisen for infant formulae, most of which are based on cow's milk. The formulae try to mimic the composition of human milk to the greatest degree possible, although it is impossible to fully achieve this objective. The concentrations of proteins and salts (sodium) in cow's milk are too high for infants. The fatty acid composition of cow's milk fat also differs very significantly from human milk fat. Furthermore, human milk fat tends to contain slightly more cholesterol than cow's milk fat.

The level of short-chain fatty acids (4:0, 6:0, 8:0, and 10:0) is much higher and the level of long-chain saturated acids (14:0, 16:0, and 18:0) is significantly higher in cow's milk than in human milk fat. Conversely, the content of MUFAs (primarily 18:1 but also 16:1) is significantly higher in human milk fat. Furthermore, the level of PUFAs in human milk fat is considerably higher than in ruminant milk fat; the long-chain *n*–3 PUFAs, which are present at trace levels in cow's milk fat, are present at quite high levels in human milk fat.

Newborn human babies have a significant need for lipids for membrane synthesis, particularly in the brain and nervous system. High levels of arachidonic (20:4, *n*–6) and docosahexaenoic (22:6, *n*–3) are present in membrane lipids of the brain and nervous system. The levels of their respective precursors, linoleic (18:2, *n*–6) and α-linolenic (18:3, *n*–3), are much lower.

The optimum ratios of *n*–3 to *n*–6 fatty acids and of precursors (18:2 and 18:3) to products (20:4 and 22:6) in infant formulae have not yet been definitively elucidated. However, it has been suggested that long-chain *n*–3 fatty acids and long-chain *n*–6 fatty acids should provide 0.25–0.5% and 0.35–0.7% of energy, respectively. Furthermore, the need for *n*–6 linoleic and *n*–3 α-linolenic acids has been estimated to be 5–6 and 1% of energy, respectively. In the past, higher levels of linoleic acid have been used but there is now increasing recognition of the importance of *n*–3 fatty acids.

Conclusions

Milk lipids play a positive role in the diet as a source of energy, fat-soluble vitamins, and essential fatty acids. Milk lipids also contribute to the palatability of the diet. Concerns have been expressed regarding the hypercholesterolemic effect of dietary SFAs. Milk lipids from ruminant animals are rich in short-chain fatty acids which do not exert a hypercholesterolemic effect. Of the longer-chain fatty acids in milk fat, only 12:0, 14:0, and 16:0 exert a significant hypercholesterolemic effect in controlled feeding trials with human subjects. However, the evidence that milk intake in free-living populations is hypercholesterolemic is poor. There is some evidence that CLA present in milk fat may exert beneficial health effects. While a reduction in lipid intake, including milk lipids, may be beneficial for obese and overweight individuals, it is important to place a reduction of dietary fat intake for the general population in the context of the need to alter other practices such as smoking and exercise, and control of blood pressure. An optimum diet should emphasize moderation and variety. Milk and milk lipids can certainly play their part in such a diet.

See also: **Butter and Other Milk Fat Products**: Fat Replacers. **Dehydrated Dairy Products**: Infant Formulae. **Milk Lipids**: Conjugated Linoleic Acid. Cholesterol: Factors Determining Levels in Blood; Removal of Cholesterol from Dairy Products. **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake. **Vitamins**: Vitamin A; Vitamin E; Vitamin K; Vitamin B_{12}; Folates; Biotin (Vitamin B_7); Niacin; Pantothenic Acid; Vitamin B_6; Thiamine.

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Lipid Oxidation

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Introduction

Edible fats containing unsaturated molecules are susceptible to attack by molecular oxygen. This process is referred to as lipid oxidation and can give rise to undesirable volatile flavor compounds, potentially toxic oxidation products, and a general deterioration in the quality of the fat. Fat oxidation is influenced by a range of parameters including light exposure, temperature, presence of pro-oxidant metals, presence of antioxidant compounds, and the degree of unsaturation of the fat. The volatile flavor products of lipid oxidation include aldehydes, ketones, alcohols, esters, lactones, and hydrocarbons, of which the unsaturated aldehydes and ketones are primarily responsible for undesirable oxidized (rancid) flavors.

Lipid oxidation is a chain reaction involving initiation, propagation, and termination stages. Oxidation of unsaturated fatty acids results in the formation of odorless, tasteless intermediate products known as fatty acid hydroperoxides. These compounds are unstable and can degrade via a variety of pathways, ultimately yielding volatile flavor products. Inhibiting the progress of lipid oxidation in foods, including milk and milk products, is a key factor in maintaining quality and extending shelf life.

Milk is a complex biological matrix which contains many factors that can exert antioxidant and/or pro-oxidant effects. Subsequent processing and storage of milk may also exert a profound influence on the progress of lipid oxidation.

Mechanism of Lipid Oxidation

The fundamental principles of lipid oxidation were elucidated in the 1940s and are widely accepted. Oxidation of unsaturated edible fats (RH) is a free-radical process that involves initiation, propagation, and termination steps. These can be outlined as follows:

Initiation

$$\underset{\text{(fatty acid, free or esterified)}}{\mathrm{RH}} \rightarrow \underset{\text{(alkyl radical)}}{\mathrm{R^\bullet + H^\bullet}}$$

Propagation

$$\mathrm{R^\bullet + O_2} \rightarrow \underset{\text{(peroxy radical)}}{\mathrm{ROO^\bullet}}$$

$$\mathrm{ROO^\bullet + RH} \rightarrow \underset{\text{(hydroperoxide)}}{\mathrm{ROOH}} + \mathrm{R^\bullet}$$

$$\mathrm{ROOH} \rightarrow \underset{\text{(alkoxy radical)}}{\mathrm{RO^\bullet}} + \underset{\text{(hydroxy radical)}}{\mathrm{^\bullet OH}}$$

$$\mathrm{RO^\bullet + RH \rightarrow ROH + R^\bullet}$$

$$\mathrm{^\bullet OH + RH \rightarrow H_2O + R^\bullet}$$

$$\mathrm{RO^\bullet + ROOH \rightarrow ROH + ROO^\bullet}$$

$$\mathrm{ROOH} \rightarrow \text{Nonradical end products, for example, aldehydes}$$

Termination

$$\mathrm{R^\bullet + R^\bullet \rightarrow RR}$$

$$\mathrm{RO^\bullet + RO^\bullet \rightarrow ROOR}$$

$$\mathrm{RO^\bullet + R^\bullet \rightarrow ROR}$$

$$\mathrm{ROO^\bullet + R^\bullet \rightarrow ROOR}$$

$$\mathrm{ROO^\bullet + ROO^\bullet \rightarrow ROOR + O_2}$$

The initiation reaction generally involves removal of a hydrogen atom from the methylene (CH_2) group adjacent to a double bond in an unsaturated fat molecule. This generates the fatty acid radical (alkyl radical, $R^\bullet$). The formation of $R^\bullet$ from RH is influenced by light exposure, metal catalysts, heat, active oxygen species, and other factors. However, full understanding of the detailed mechanism of formation is not known.

The alkyl radical ($R^\bullet$) is extremely reactive and can combine with molecular oxygen to produce a peroxy radical ($ROO^\bullet$) which can combine with another unsaturated fatty acid molecule (RH) and generate the corresponding hydroperoxide (ROOH) and a new alkyl radical. Hydroperoxides are unstable and degrade to produce volatile, nonradical end products, many of which result in off-flavors. Alternatively, the hydroperoxides can degrade to produce an alkoxy radical ($RO^\bullet$) and a hydroxy radical ($^\bullet OH$), both of which are extremely reactive and can propagate the chain reaction by reacting with further RH molecules. The decomposition of hydroperoxides is commonly catalyzed by pro-oxidant metals, such as copper and iron.

The reaction ultimately terminates if the supply of RH molecules is exhausted, if O_2 becomes limiting, if radicals react with each other to generate nonradical end products as outlined earlier, or if radicals react with antioxidant molecules (see later).

Food lipids typically exhibit a lag phase (induction) during which the rate of lipid oxidation is low. However, as the autocatalytic chain reaction accelerates, hydroperoxides rapidly accumulate and reach a maximum, following which hydroperoxide decomposition reactions and the generation of volatile off-flavored (rancid) compounds become more important. The longer the induction period, the more stable the food to oxidation.

Oxidation Products and Off-Flavors in Milk

Milk is characterized as having a pleasing, slightly sweet taste with no unpleasant aftertaste. However, its bland taste makes it susceptible to a variety of flavor defects. Since milk fat contains many minor unsaturated fatty acids as well as major ones, very many carbonyl products may be produced during the autooxidation of milk fat. Thus, the overall flavor produced by autooxidation is the combination of many flavors imparted by individual volatile compounds present at minute concentrations. However, compounds such as *n*-hexanal, 2-octenal, 2-nonenal, 2,4-heptadienal, and 2,4-nonadienal have been reported in many studies as key compounds contributing to the oxidized off-flavor of milk fat. 2, 4-Decadienal and 1-octen-3-one have been reported also.

Flavor threshold values for carbonyl compounds are influenced by many factors, including the number of carbon atoms, number and location of unsaturated double bonds, isomeric form, and the nature of the food; carbonyl compounds have up to 100 times greater flavor potency in an aqueous medium (e.g., milk) than in oil (e.g., butteroil). Hence, off-flavors tend to be more noticeable at lower concentrations of carbonyl compounds in liquid milk than in butter.

Spontaneous Oxidation in Milk

Some samples of raw milk are more susceptible to oxidation than others. Milk which oxidizes spontaneously (spontaneous milk) is capable of developing oxidized flavor within 48 h of milking without the presence of contaminating iron or copper. Approximately 12–20% of raw milk samples exhibit this behavior. Susceptible milk does not oxidize spontaneously but develops oxidized flavors following contamination with iron or copper. Use of non-corrodible dairy equipment reduces the probability of oxidation in this category of milk samples.

Non-susceptible milk does not oxidize even in the presence of iron or copper.

Some cows consistently produce spontaneous milk, others occasionally, and others not at all. The phenomenon is influenced by heredity, stage of lactation, and feeding practices. Some research has suggested that the enzyme, xanthine oxidase, which is a major component of the milk fat globule membrane (MFGM), is involved in spontaneous oxidation. However, the research on this issue is equivocal. Other research suggests that the susceptibility of milk to spontaneous oxidation is related to its relative content and distribution of pro-oxidants and antioxidants. In general, milk from pasture-fed cows is less susceptible to oxidation than from ration-fed cows due to higher content of antioxidant tocopherols.

Factors Affecting Oxidation of Lipids in Milk and Milk Products

The rate and extent of lipid oxidation in milk and milk products is influenced by a range of parameters including oxygen, light, endogenous and exogenous metals, antioxidants such as ascorbic acid, tocopherols, carotenoids, thiols, proteins, and enzymes, MFGM, and storage temperature.

Oxygen

Oxygen is a requirement for oxidative deterioration of lipids. Oxygen has greater solubility in nonpolar than in polar solvents and, hence, is more soluble in liquid milk fat than in whole raw milk. A substantial percentage of the total oxygen in whole milk is present in the fat phase. Oxygen is excluded from the solid fat phase as it crystallizes. However, as the temperature of butter is reduced, oxygen excluded from the crystallized fat phase partitions into and saturates the liquid fat and aqueous phases of the butter. Thus, oxygen is available to react with the more unsaturated fat in the liquid phase and with phospholipids and pro-oxidants in the MFGM fragments. The maximum rate of hydroperoxide production in irradiated butter occurs at −20 °C, which may reflect an increased rate of chain terminations at higher temperatures and reduced propagation reactions at lower temperatures.

Removal of oxygen from liquid milk or its replacement by an inert gas, such as nitrogen, reduces the development of oxidized flavors. Similarly, the oxidative stability of whole milk powder can be enhanced by vacuum treatment or replacement of oxygen with an inert gas. Oxidized flavor is of minor importance in fermented dairy products such as cheese or yogurt. Several factors may be involved, such as depletion of the oxygen level by starter bacteria, the acidic pH of such products, or the formation of antioxidants by microorganisms.

Light

A considerable body of research data indicates that light is a very effective promoter of off-flavor development in milk and milk products. The extent of oxidation-induced off-flavor development is a function of the light wavelength, intensity, and duration of exposure. Light has been shown to penetrate milk to an appreciable depth.

Potential oxidation-induced off-flavors in butter may be reduced by light-barrier packaging, such as aluminum foil. Direct exposure to light is the principal factor affecting photooxidation of butter; temperature and duration of storage exert little effect on butter with subsensory levels of light-induced oxidation.

Metals

A wide variety of metal ions, including the pro-oxidant transition metal ions of copper and iron, are found in milk and milk products. Awareness of the role of metal ions in the oxidation of milk fat has existed for nearly a century. It has long been recognized that copper and iron are the principal metals involved. Both these metal ions occur naturally in milk but may also be present as contaminants. Copper has been reported at levels of 20–400 $\mu g\,l^{-1}$ and iron at levels of 100–900 $\mu g\,l^{-1}$.

These ions function as pro-oxidants primarily by decomposing hydroperoxides to generate new reaction chains. Either the oxidized or reduced metal ion can decompose hydroperoxides to allow the following catalytic cycle to occur:

$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + OH^- + RO^\bullet$$
$$M^{(n+1)} + ROOH \rightarrow M^{n+} + H^+ + ROO^\bullet$$

Thus, small quantities of metal ions can generate large numbers of reaction chains by cycling between the oxidized and reduced forms. The standard reduction potentials for iron and copper suggest that iron is a much stronger oxidizing agent than copper. However, copper in milk is more pro-oxidant than iron. This anomaly probably reflects differences between the interactions of the two metals with other milk constituents such as ascorbic acid, thiol groups, and serine phosphate residues which could modulate their pro-oxidant effects.

The endogenous copper in milk is derived via the bloodstream from the cow's feed. It has been reported that 10–35% of the indigenous copper and 20–47% of the indigenous iron in milk are associated with the MFGM. However, only 2–3% of added (exogenous) copper and virtually no added iron become associated with the MFGM. While indigenous copper and iron in milk are complexed with proteins and are nondialyzable at the normal pH of milk, added copper and iron are dialyzable to some extent. This suggests that added metals interact less with milk proteins than indigenous metals. It appears that the juxtaposition of a copper–protein complex with the phospholipids of the MFGM is an important factor in the development of oxidized flavor in liquid milk.

Antioxidants

Addition of antioxidant compounds is widely used in food products to inhibit the progress of lipid oxidation. However, addition of antioxidants to dairy products is prohibited in most countries. Thus, antioxidant effects in milk rely primarily on endogenous compounds.

Tocopherols

Tocopherols are effective antioxidants in lipid systems. While they may exert a pro-oxidant effect at high concentrations, this is unlikely to occur in milk which contains approximately 20 μg of α-tocopherol per gram of fat. The principal factor influencing the tocopherol content of milk is the feed of the cow which, in turn, is influenced by season. Summer milk produced on green pasture feed typically has a higher α-tocopherol content than winter milk produced on dry-lot feeding. Many studies have demonstrated the feasibility of increasing the α-tocopherol content of milk by supplementation of the feed with consequent increased resistance of the milk to spontaneous and copper-induced oxidation. However, only about 2% of ingested α-tocopherol is actually transferred to the milk and consequently the economics of direct supplementation of feed are unfavorable.

A significant correlation exists between the α-tocopherol content of milk fat and its oxidative stability. Tocopherol concentrations are at least threefold higher in lipids of the MFGM than in the core of the fat globule. Tocopherol concentration in the MFGM lipids shows a closer correlation with oxidative stability than the tocopherol content of butteroil. During storage of cream containing added ascorbic acid and copper, total destruction of tocopherols in the MFGM was observed compared to 30% destruction in the butteroil due to the proximity of the tocopherols in the MFGM to pro-oxidants and highly oxidizable phospholipids. Tocopherols have been reported to act as free-radical scavengers but have also been shown to quench active oxygen species such as singlet oxygen ($^1O_2$). Each tocopherol molecule can typically deactivate about 120 molecules of singlet oxygen before it is destroyed.

Ascorbic acid

Ascorbic acid is a very effective scavenger of alkoxy radicals ($RO^\bullet$). However, under certain circumstances it can exert a pro-oxidant effect. Concentrations of ascorbic acid above those typically found in milk (approximately 20 $mg\,l^{-1}$) provide antioxidant protection in milk. However, at the concentrations normally found in milk, ascorbic acid acts as a pro-oxidant. Ascorbic acid has been shown to be crucial to the development of oxidized flavor in cream. Cream washed free of ascorbic acid did not develop oxidized flavor over 3 days despite being contaminated with copper. It has been proposed that ascorbic acid reduces Cu^{2+} to Cu^{1+}, which in turn reduces molecular oxygen to hydrogen peroxide, which oxidizes lipids in the MFGM. Further work has shown that added copper did not promote oxidation in milk or butter depleted of ascorbic acid and that oxidation in ascorbic acid-free milk could be initiated by addition of ascorbic acid.

Carotenoids

The importance of singlet oxygen ($^1O_2$) as an initiator of oxidation has increased interest in prevention of singlet oxygen reactions by quenching to ground-state oxygen ($^3O_2$). Food constituents such as carotenoids and tocopherols have been reported to exert this effect. Quenchers must either have the ability to accept energy from the $^1O_2$ molecule (energy-transfer quenching) or have the ability to donate electrons to $^1O_2$ (charge-transfer quenching). Quenching of $^1O_2$ by carotenoids is an example of energy-transfer quenching whereas tocopherols, amines, and phenols have been shown to exert a charge-transfer quenching mechanism. β-Carotene is one of the most potent quenchers of $^1O_2$, with one molecule estimated to quench 250–1000 molecules of $^1O_2$. The rate of quenching is influenced by the number of conjugated double bonds present. Carotenoids with nine or more conjugated double bonds are efficient quenchers, whereas those with seven or less are not capable of accepting energy from $^1O_2$.

Thiols

Mild heat transfer (e.g., pasteurization) of milk increases its susceptibility to spontaneous, copper-induced, and photo-induced oxidation, probably due to the migration of copper to the cream phase of milk. However, many researchers have reported an inhibitory effect of high-heat treatment on the oxidative deterioration of milk and milk products. This effect has been associated with the activation of thiol groups primarily in the MFGM and the serum protein β-lactoglobulin. Thiol groups resulting from the heat treatment of milk exert an antioxidant effect probably as univalent reducing agents, peroxide decomposers, or metal ligands.

Proteins and enzymes

Caseins have been shown to possess significant antioxidant activity which may be related, in part, to their hydrophobic nature and orientation of potential antioxidant side chains of constituent amino acids at the lipid interface. Homogenized milk has been reported to be less susceptible to oxidation when milk fat droplets are resurfaced with casein. Caseins can also bind pro-oxidant metals to phosphoseryl residues. The major whey proteins have considerably less antioxidant properties than the caseins. Superoxide dismutase (SOD) has been detected in and isolated from milk but, apparently, it is present at insufficient levels to provide substantial antioxidant protection.

Milk Fat Globule Membrane

Milk fat exists primarily in globules surrounded by a complex membrane consisting of a mixture of unsaturated phospholipids, proteins, glycoproteins, and other minor constituents. The MFGM is a focal point for the oxidation of milk lipids due to the proximity of unsaturated phospholipids to various pro-oxidants in the lipoprotein matrix. Once oxidation is initiated in the MFGM, diffusion of the propagating chain reaction radicals into the more saturated fat globule core from the fat–plasma interface results in generalized oxidation of milk fat triglycerides. It has also been proposed that xanthine oxidase, a metalloprotein abundant in the MFGM, may also be partially responsible for the susceptibility of the membrane to lipid oxidation.

Cholesterol Oxidation

Cholesterol is an unsaturated alcohol and is susceptible to oxidation, with more than 60 known cholesterol oxidation products (COPs). The C7 atom of cholesterol is most susceptible to oxidation, giving rise to a series of A- and B-ring oxidation products. The C20 and C25 atoms in the side chain are also susceptible to oxidation. Humans are capable of absorbing COPs from the diet and there is ongoing research and debate regarding the health significance of COPs, particularly in relation to atherosclerosis.

The development of atherosclerotic plaque by the deposition of saturated cholesterol esters in arteries has been studied extensively for many decades. Many studies have shown, however, that cholesterol does not accumulate in the vascular endothelium until lesions occur. It has been proposed that COPs may be a cause of lesion formation, after which pure cholesterol and cholesterol esters accumulate as a secondary process, generating atherosclerosis.

COPs have not been detected in significant amounts in fresh, pasteurized, UHT, condensed, or skimmed milk. COPs have been reported in full-fat milk powders, baby foods, cheeses such as Parmesan, Cheddar, Blue, and Romano, butter and butteroil, and ghee.

While research and debate on the human health significance of COPs in foods continues, it would be prudent to optimize the processing, preservation, storage, and distribution of milk and milk products to minimize the formation of COPs.

Measurement of Lipid Oxidation

As low levels of oxidation can lead to off-flavors in milk and milk products, routine procedures to assess the extent of oxidation should be sensitive, reliable, and simple.

Measurement of hydroperoxides (peroxide value) is a classical method for quantifying lipid oxidation. A variety of procedures exist. One procedure used extensively to study lipid oxidation of milk fat involves the oxidation of ferrous to ferric iron by hydroperoxides in the fat in the presence of ammonium thiocyanate to produce ferric thiocyanate which can be quantified spectrophotometrically. Another commonly used procedure is based on the liberation of iodine from potassium iodide by hydroperoxides. A serious problem with procedures based on the determination of hydroperoxides is that they may not correlate well with the level of off-flavor in the products, particularly when oxidation has been extensive. During the course of lipid oxidation, peroxide values reach a peak and then decline.

Procedures using thiobarbituric acid (TBA) as an analytical reagent have also been used widely to monitor lipid oxidation in milk and milk products. The TBA procedures have been shown to correlate well with the intensity of oxidized flavor in liquid milk.

A variety of other chemical methods such as the anisidine value and the Kreis test may also be used to monitor lipid oxidation in milk fat.

Instrumental methods of detecting oxidized flavor volatiles include headspace analysis, especially using solid-phase microextraction (SPME) and solid-phase dynamic extraction (SPDE), combined with gas chromatography, with or without mass spectrometric detection.

See also: **Milk Lipids**: Lipolysis and Hydrolytic Rancidity.

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Lipolysis and Hydrolytic Rancidity

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Introduction

Lipolysis or hydrolysis of lipids can result in serious defects in milk and milk products and is a constant concern to the dairy industries of most countries. It is caused by the enzyme lipase, which hydrolyzes the triacylglycerols (triglycerides), the major constituents of milk fat. The products of this hydrolysis are free fatty acids (FFAs) and mono- and di-acylglycerols. The FFAs formed from milk fat, particularly the short-chain acids such as butyric, caprylic, and caproic, have strong flavors and can impart unpleasant flavors variously known as rancid, bitter, butyric, unclean, astringent, or 'lipase'. It should be noted that some of these terms are also applied to other flavor defects: rancid flavors can result from the oxidation of fats, bitter flavors from proteolysis, and unclean flavors from the growth of some bacteria. The flavor defect caused by lipolysis is sometimes referred to as 'hydrolytic rancidity', which should not be confused with 'oxidative rancidity', which results from the oxidation of fats.

Not all effects of lipolysis are undesirable. In some cheeses, lipolysis is important for the development of characteristic flavor. For example, lamb, kid, and calf lipases are used in the manufacture of Parmesan and Romano cheese to produce short-chain fatty acids that impart a piquant flavor, while FFAs produced by the lipases of the blue *Penicillium roqueforti* mold make a major contribution to the flavor of blue-vein cheese. Rennet pastes containing lipase also contribute to lipolysis in some cheese varieties. Lipases are also used in the production of various dairy flavorings used to enhance the flavor of some dairy products, chocolate, and other confectionery products. FFAs, particularly those of short chain length, have antimicrobial effects, and it has been suggested that these acids formed by lipolysis of fat in milk may contribute to the protection of the newborn against gut infections.

The other products of lipase action, the mono- and di-acylglycerols, have surface active properties that can affect some products. For example, they can cause milk to exhibit poor steam-foaming properties. Lack of foaming during the preparation of cappuccino coffee is a relatively common phenomenon, which can often be attributed to lipolysis in the milk.

The lipases that cause problems in milk and dairy products are of two main types – milk lipase, which occurs naturally in all raw milk, and bacterial lipases, which are produced by contaminating bacteria. Each of these lipases causes lipolysis under certain conditions and will be discussed separately below.

Another family of lipolytic enzymes that can affect milk and dairy products are the phospholipases. These hydrolyze phospholipids, which constitute a major part of the milk fat globule membrane (MFGM), and cause destabilization of the milk fat globule. The most well-documented defect caused by these enzymes is 'bitty cream', which results from damage to the membrane, causing partial coalescence of fat globules and the production of unsightly specks of fat on the surface of hot beverages. Fortunately, homogenization effectively disperses the specks and masks the defect in milk.

Lipolysis by the Milk Lipase System

The natural milk lipase is a lipoprotein lipase, which means that it is activated by lipoproteins, such as those in blood serum. (This can be easily demonstrated by adding some blood serum to milk and observing the lipolysis that results.) In milk, it appears to operate without the cofactors associated with blood lipoproteins, although there has been a conjecture that similar molecular entities may exist in milk and activate the enzyme. Milk lipase is inactivated by high-temperature short-time (HTST) pasteurization and hence does not cause lipolysis in pasteurized milk or products made from it. It is also inactivated to some extent by high-pressure treatment and pulsed, high-voltage electric field treatment, while high-pressure homogenization does not inactivate milk lipase but appears to activate it.

The fat portion of milk exists as small discrete globules, neatly enveloped in a biological membrane, the MFGM. The membrane facilitates dispersion of the fat in the aqueous phase of milk and also segregates the fat from the natural milk lipase, which exists in the aqueous phase. In fresh normal milk, this membrane is intact and is impermeable to the milk lipase. However, under certain circumstances, the integrity of the membrane can be disrupted, allowing contact between the lipase and its substrate, resulting in lipolysis. This can occur spontaneously in the milk on the farm or it can be induced during handling before pasteurization.

Spontaneous Lipolysis

Milk from some cows can undergo spontaneous lipolysis. It is initiated by cooling the milk to <10 °C soon after secretion from the cow and continues during refrigerated storage. Most of the lipolysis occurs in the first 12 h of storage. The milk from cows in late lactation and from poorly nourished cows is particularly susceptible to this type of lipolysis. In regions where cows are seasonally calved and late lactation corresponds to a time of year when natural feed is in short supply, spontaneous lipolysis can be a serious problem.

Individual cows vary considerably in the susceptibility of their milk to spontaneous lipolysis. In the author's experience, some cows never produce milk that spontaneously lipolyzes, while others (of the same breed and in the same herd) consistently produce such milk.

It is fortunate for the dairy industry that admixing the milk samples from these two types of cow inhibits, partially or completely, the spontaneous lipolysis that would occur in the 'spontaneous' milk alone. This phenomenon has been attributed to the presence of an inhibitor (or inhibitors) in 'normal' milk.

Spontaneous lipolysis has been attributed to a combination of three major factors: an imbalance of lipase 'activators' and lipase 'inhibitors'; susceptibility of the MFGM; and the amount of lipase. Each can affect the amount of lipolysis but the relative amounts of activators and inhibitors appear to be the most important factor. The nature of these components has not been properly determined, but the evidence suggests that both types of substances are present in milk and that spontaneous lipolysis occurs only when the balance shifts toward more of the activators and less of the inhibitors.

A necessary precondition for lipolysis is attachment of some of the lipase from the skim phase of milk to the MFGM. In milk that undergoes a high level of lipolysis, the amount of lipase redistributing to the cream phase from the skim phase can be a significant proportion of the total lipase present. The nature of this attachment is not well understood.

All raw milk contains sufficient lipase to cause lipolysis and produce a rancid off-flavor under favorable circumstances. Therefore, the amount of lipase in the milk is not normally a significant factor. However, where the activator–inhibitor balance and MFGM characteristics are similar, different levels of lipase can determine the final level of lipolysis. Similarly for the susceptibility of the MFGM, if the activator–inhibitor balance and lipase content are similar, the different levels of lipolysis can be attributed to differences in the membrane characteristics. The MFGM can become more permeable in late lactation, which may partially explain the increased susceptibility of late-lactation milk.

Milk from quarters of the udder with mastitis has a higher level of FFAs than milk from healthy quarters of the same cow after a period of storage. This may be due to a higher amount of lipolysis occurring during storage, but it is often due to a higher-than-normal FFA level in the milk as it leaves the cow. These fatty acids result from incomplete incorporation into triacylglycerols in the mammary gland. Their leakage into the milk is the result of disruption of the normal functioning of the mammary cell during mastitic infection; the more severe the mastitis (i.e., the higher the somatic cell count), the higher the FFA level.

Induced Lipolysis

This is usually associated with damage to the MFGM. It can occur through pumping, agitation (mechanical or air), or excessive mixing of air into the milk. In all of these cases, the incorporation of air is essential for disrupting the MFGM. Surface tension forces at the air–milk interface cause damage to the membrane. Hence, pumping induces little lipolysis unless air is simultaneously taken in through faulty seals, joints, and other parts. Similarly, on the farm, lipolysis can be induced in the milk-lines if excessive air is drawn in at the teat cups and surging occurs in the lines, particularly in vertical sections; the milk is particularly susceptible at this time because it is close to body temperature. Milking with automatic milking machines has been reported to lead to higher levels of lipolysis than when using manually operated machines.

The effect of agitation on lipase action can be easily demonstrated by placing some raw milk at about 30 °C in a blender and 'blending' it at high speed for a short time, say 30 s. Such milk will quickly develop a rancid flavor if left at room temperature.

One very effective way of initiating lipolysis is homogenization of raw milk. Lipolysis begins immediately and the milk quickly becomes rancid. This is avoided in practice in dairy processing by pasteurizing the milk either before or immediately after homogenization. Homogenization before batch pasteurization should not be performed at less than 60 °C to avoid excessive lipolysis during the time period between homogenization and pasteurization.

The fat in homogenized milk is an excellent substrate for lipase. Therefore, if raw milk is mixed with homogenized (pasteurized) milk, lipolysis results. This situation can occur in the industry and cause serious flavor problems in drinking milk. One situation where this has been encountered is when some homogenized milk is left over, for example, from a packaging run, and is returned to raw milk storage vessels for processing the following day. Therefore, constant vigilance is needed to ensure that such actions do not occur as their consequences are not immediately obvious to untrained factory personnel.

The result of mixing raw and homogenized milk can be simply illustrated by mixing equal quantities of raw and homogenized milk, leaving the mixture at about 30 °C, and observing the change in flavor over a time period of 2–3 h. This is also the easiest way to produce a typical 'lipase' flavor to demonstrate the defect. Milk with an FFA content up $6\,\mathrm{mmol\,l^{-1}}$ can be produced, and this can be mixed with unlipolyzed pasteurized milk (FFA $\sim 1\,\mathrm{mmol\,l^{-1}}$) to produce milk samples with a range of FFA levels. Most people detect the off-flavor when the FFA reaches $2\,\mathrm{mmol\,l^{-1}}$.

As in the case of spontaneous lipolysis, induced lipolysis usually involves attachment of the lipase to the MFGM. Under certain conditions of mechanical agitation, a substantial proportion of the total lipase activity associated with casein micelles in the skim phase can be transferred to the fat globules in the cream phase. Once attached to the fat globule membrane, the enzyme has enhanced heat stability and hence cream from milk that has been severely mechanically abused may retain some milk lipase activity after HTST pasteurization.

A unique way in which the milk lipase system can be activated is by 'temperature manipulation'. This involves cooling raw milk to a low temperature (≤10 °C), rewarming it to >20 °C, and then again cooling to ≤10 °C. 'Spontaneous' milk is more susceptible to this type of lipolysis induction. A situation where this can occur at the farm is when warm milk direct from the cow is mixed with refrigerated milk from a previous milking and the temperature reaches >20 °C.

Lipolysis by Bacterial Lipases

Lipolysis caused by bacterial lipases has become significant since the widespread introduction of cold storage of milk on farms. This change has caused the levels of lactic acid bacteria to decline and those of psychrotrophic bacteria to increase. During low-temperature storage, the proportion of psychrotrophic bacteria in the milk increases and they become dominant. Many of these produce extracellular lipases that can cause lipolysis. The principal bacteria concerned are pseudomonads, particularly *Pseudomonas fluorescens*. These are common bacteria in soil and water and are ever present in the farm and factory environments. Consequently, they constitute the dominant type of bacterial flora in both raw milk and pasteurized milk after a period of cold storage. They grow at low temperatures but the rate of growth of a particular strain does not correlate with the amount of lipase elaborated. In fact, some studies have shown that the slowest growing strains are the most prolific lipase producers.

One of the most important properties of these microbial lipases is their heat stability. Most retain at least some of their activity after pasteurization and even after ultra-high temperature (UHT) processing. This means that although the bacterial cells may be destroyed by the heat treatments used in dairy processing, their enzymes can remain active in the heat-treated product. Hence, a product may exhibit no defects immediately after manufacture but develop rancid off-flavors during storage. The longer the product is stored and the higher the temperature at which it is held, the greater the risk of lipolysis defects developing if the product contains small amounts of contaminating lipase.

In contrast to the natural milk lipase, most microbial lipases are not prevented from attacking the fat in milk fat globules by the MFGM. The reason for this difference is not completely understood. The practical significance of this is that milk fat globules do not have to be disrupted before these bacterial lipases can act on the fat.

Proper attention to time and temperature of storage of raw milk is required to prevent problems due to heat-stable bacterial lipases. In virtually all cases of such problems, failure to keep the temperature low or limit the time of storage has been identified as the cause. As a general rule, if milk is stored for more than 3 days, there is a risk of products made from it developing lipase-related defects. If during those 3 days the temperature is allowed to rise above 4 °C, the risk is increased.

One situation in which lipase problems have been encountered is when milk (or cream) is transferred between factories; in such situations, the time/temperature rules are often broken. Another situation is when milk is kept for extended periods of time before processing; this can occur over weekends or public holidays, at times of low milk production (and hence less frequent collection), or at times of adverse weather conditions causing transport delays between farm and factory.

Lipolytic Defects in Milk and Dairy Products

All milk products containing fat have the potential to suffer from lipolytic defects. However, it is important to understand the most likely cause(s) of the problem in different products so that procedures can be devised to minimize the risk. Once lipolysis has occurred to such an extent that a flavor defect is detectable, there are no processes available for eliminating the defect from the product.

Pasteurized Milk

Two major lipase-related problems are encountered in milk: rancid flavors and poor foaming capacity. Lipolysis is most commonly due to milk lipase, and may occur on the farm or in the factory before the milk is processed. If it occurs at the farm, it can be either spontaneous or induced. The former occurs most often when a large proportion of the cows are in late lactation and/or when high-quality feed is not available. A common cause of induced lipolysis is excessive air intake at the teat cup cluster. In the factory, it can be induced by excessive pumping (particularly with centrifugal pumps with faulty seals through which air is drawn into the milk causing foaming); homogenization of raw milk, without immediate pasteurization; and mixing of homogenized (pasteurized) milk and raw milk. Lipolysis caused by bacterial lipases is less likely in market milk as the bacterial count required to produce sufficient lipase to present problems in cold-stored milk over a relatively short time, $\geq 10^7\,\mathrm{cfu\,ml^{-1}}$, does not occur in raw milk and usually occurs only at the end of the shelf life of pasteurized milk. The end of the shelf life of pasteurized milk is characterized by off-flavors (bitter, sour, putrid, and oxidized), which tend to mask the rancid flavors that may also be present.

Foaming difficulties are significant in relation to making cappuccino coffee. When milk is foamed for making cappuccino coffee, steam is injected, with air, into the milk to create foam and to heat the milk to $\sim 70\,^{\circ}\mathrm{C}$. The volume of the foam plus milk should be about twice the original volume of the milk, and the foam should be stable for at least 10 min. However, at times, milk exhibits poor steam-foaming capacity and cannot be foamed enough to make acceptable cappuccino coffee. A major reason for the reduced steam-foaming capacity of milk is lipolysis; the greater the extent of lipolysis, the greater the depression of foaming. Milk with an FFA level of $\geq 2\,\mathrm{mmol\,l^{-1}}$ exhibits very poor foaming. The lack of foaming is related to the change in surface tension of the milk, but there is no evidence for a close relationship between surface tension and foaming behavior.

Ultra-High Temperature Milk

The most common cause of a rancid flavor in UHT milk is lipolysis by heat-stable bacterial lipases produced by psychrotrophic bacteria in the raw milk before heat treatment. Only trace quantities of lipase are required to cause an increase in FFA content during storage at room temperature until the use-by date, which can be 12 months after manufacture.

Butter and Anhydrous Milk Fat

Off-flavors in butter and anhydrous milk fat can be caused by lipolysis before or after manufacture. Off-flavors originating before manufacture are mostly due to milk lipase action and result in soapy, bitter, and 'back palate' taste sensations at manufacture. This is characterized by a high FFA content at manufacture. The FFAs present are generally longer in average chain length than those of the parent milk fat because the shorter-chain acids have some water solubility and are lost in the buttermilk and any washing steps during butter manufacture.

By contrast, post-manufacture lipolysis is caused by heat-stable bacterial lipase action and results in characteristically sharp, butyric, and 'front-palate' flavors. This is because all fatty acids are retained in the butter and contribute to the flavor. Gas chromatography of the FFAs can be used to observe their fatty acid profile. This allows the cause of the off-flavor to be determined. This type of lipolysis occurs more readily in butter because of its higher water content; however, it can occur in anhydrous milk fat through the small amount of water present, given that the product may be stored unrefrigerated for long periods of time before use.

Milk Powder

Residual bacterial lipases in milk and whey powders are a major concern. Since they usually act slowly in the product during storage, lipolysis may be detected months after manufacture and when used in the production of other foods such as recombined dairy products and confectionery. This can have serious economic ramifications.

Cheese

FFAs are an integral part of the typical flavor of most cheeses, especially the more highly flavored types. However, for most cheeses, excessive lipolysis results in a flavor defect.

As with butter, cheese can be affected by pre- or post-manufacture lipolysis. If the former occurs, the flavor defect is noted in the fresh cheese, but if the latter occurs, the defect develops during storage. It is unusual for serious problems to occur in cheese as a result of lipolysis in the milk; however, very serious problems can result from post-manufacture lipolysis, largely due to heat-stable bacterial lipases.

In cheese made from pasteurized milk, lipolysis by the native milk lipase does not occur after manufacture. Even when cheese is made from raw milk, the native milk lipase causes little lipolysis, if any, in the cheese.

Additives can be another cause of lipase action in cheese. Some additives, such as pepper, contain lipases that can cause soapy, rancid off-flavors.

Yogurt

Few problems are caused by lipase action in yogurt. This is due to a combination of factors including low pH (outside of the optimal pH range for most lipases of concern), low storage temperature, relatively short shelf life, and high normal flavor level. However, some additives used in yogurts have been known to cause a lipolytic defect through the action of their constituent lipases. For example, the lipase in passion fruit seeds can produce rancid flavors in some tropical fruit yogurts.

Analytical Methods

Free Fatty Acid Measurement

The most widely used measure of the extent of lipolysis in milk and dairy products is FFA content. The most commonly used methods are based on extraction–titration procedures in which the FFAs (and the fat) are extracted from the product and titrated with an alcoholic alkali solution. The extraction can be by a solvent system, such as isopropanol–petroleum ether or ethyl alcohol–diethyl ether, or by a procedure that isolates the fat from the product.

The fatty acids released from milk fat by lipases range from short-chain, water-soluble acids such as butyric acid (C4) to long-chain, water-insoluble acids such as oleic and stearic acid (C18). Extraction procedures seldom quantitatively extract all of these acids, so absolute FFA contents are usually not obtained. Procedures involving solvents extract more than do methods involving isolation of the fat, as the latter extract almost all the long-chain acids but a low proportion of the short-chain acids.

These two extraction methods also lead to different units for the FFA contents. For example, for milk, FFA values from the solvent extraction methods are reported as milliequivalents of acid per liter of milk or millimolar concentrations (mmol l^{-1}), while those from the fat extraction methods are reported as ADV (acid degree value) in milliequivalents of acid per 100 g of fat. An approximate conversion of one to the other can be made only if the fat content of the milk is known. Alternatively, a relationship between the two can be derived empirically.

Accurate procedures for determining the amount of each of the FFAs produced are now available using gas chromatography or high-performance liquid chromatography (HPLC). These methods are not generally used for routine dairy industry testing but they can provide useful information for troubleshooting and research applications. Gas chromatographic headspace analysis of short-chain fatty acids using solid-phase microextraction (SPME) has been shown to be suitable for milk. The electronic nose has been used successfully for detecting lipolysis in cheese.

Lipase Activity

Ideally, the dairy industry should have a simple, rapid, sensitive, and reliable test for low levels of lipase activity in milk and dairy products. If such activity could be measured before storage of products, only products free of activity can be stored for long periods.

Many different lipase assay procedures are available but to date no single method has been universally adopted for this purpose. Assays based on milk fat as substrate are intuitively ideal since it is the lipase action on milk fat during storage which the test is meant to predict. However, such tests are time consuming and/or not very sensitive. Consequently, methods using non-triglyceride substrates such as β-naphthyl, *p*-nitrophenyl, indoxyl, and umbelliferyl esters have been developed. The major advantage of these is that a colored or fluorescent product is formed, and it can be detected readily. These tests meet the criteria of simplicity, speed, and sensitivity, but most tests lack reliability because esterolytic enzymes that do not act on milk fat, as well as true lipases, are detected. Such tests are ideal screening tests and could be used in this way in the dairy industry.

Other available methods are based on the following: measurement of the decrease in turbidity of fat emulsions; enzymatic analysis of the FFAs; immunological detection of the lipase molecule; radiometric analysis of labeled fatty acids released from radioactive substrates; and chromatographic analysis of the lipolysis reaction products. Methods based on triacylglycerol substrates (such as milk fat or triolein) and a sensitive chromatographic analysis (either gas–liquid chromatography (GLC) or HPLC) of the released FFAs (and/or other products) are particularly attractive because they are sensitive and use natural substrates.

See also: **Cheese**: Accelerated Cheese Ripening; Cheese Flavor. **Enzymes Exogenous to Milk in Dairy Technology**: Lipases. **Enzymes Indigenous to Milk**: Lipases and Esterases.

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Cholesterol: Factors Determining Levels in Blood

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Introduction

Cardiovascular disease (CVD) is a broad term that refers to diseases of the blood vessels of the heart (coronary heart disease, CHD); brain (cerebrovascular disease, stroke); and the limbs (peripheral vascular disease). There are generally two main events that can lead to CVD, namely, atherosclerosis and thrombosis. Atherosclerosis refers to the thickening of the arterial wall and the gradual formation of a plaque that leads to narrowing of the arterial lumen through which blood flows, hence restricting blood flow. One of the major risk factors for CVD is a high cholesterol level in the blood. Therefore, it is important to keep the blood cholesterol level within the recommended guidelines ($<5.2\ \mathrm{mmol\,l^{-1}}$ or $200\ \mathrm{mg\,dl^{-1}}$).

Cholesterol is the principal sterol of animal tissues and is present in animal cell membranes as the free sterol and in blood in the form of cholesteryl esters (CEs). Cholesterol is essential for the body as it plays an important role in cell membranes (i.e., structural and fluidity roles) and in cell growth. It is also the precursor of bile salts, steroid hormones, and vitamin D. Cholesterol is produced in the liver (endogenously sourced) but can also be obtained from the diet (exogenous source). To facilitate intestinal absorption, dietary cholesterol is incorporated into mixed micelles in the gut, whereas biliary cholesterol is solubilized into micelles and phospholipid vesicles that then dissolve into the mixed micelles. Therefore, a mixture of endogenous and exogenous cholesterol is absorbed from the intestinal lumen. Free (unesterified) cholesterol is transferred to the mixed micelles, and once these particles are taken up by intestinal cells (enterocytes), cholesterol is re-esterified into CEs.

Before discussing the factors that influence blood cholesterol level, it is important to understand cholesterol transport and metabolism as well as the various components involved in altering blood cholesterol concentration.

The Importance of Lipoproteins

Cholesterol and phospholipids combine with protein carriers (apolipoproteins) to form lipoproteins. Regardless of the source, that is, endogenous or exogenous, cholesterol is transported around the body by lipoproteins in two forms: (1) free form, which is present largely in the hydrophilic outer layer of lipoproteins, and (2) esterified as CEs, which mainly reside in the lipoprotein hydrophobic core. Lipoproteins not only carry free cholesterol, but also CEs and triacylglycerol (TAG) molecules through the enterohepatic (intestine–liver) and hepato-peripheral (liver–body) cycles of cholesterol transport. TAGs are esters of fatty acids with glycerol and are often termed ‘dietary fat’. Therefore, lipoproteins have a core of neutral lipids (TAGs and CEs) surrounded by a single surface layer of polar lipids (phospholipids and free cholesterol) (**Table 1**). Apolipoproteins extend over the surface of lipoproteins.

The negative imbalance between atherogenic and atheroprotective lipoproteins is a strong modifiable risk factor for CVD. Consequently the levels, functions, and metabolism of lipoproteins and apolipoproteins have influential roles on cholesterol levels in the body. Five types of lipoproteins are involved in the transport of cholesterol, and each type has a specific function (**Table 2**). They are chylomicrons (CMs), very low-density lipoproteins (VLDLs), intermediary-density lipoproteins (IDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs). The general composition of the five lipoproteins is presented in **Table 3**. There are also five major classes of apolipoproteins (apoA–apoE), and as research progresses, subgroups of these apolipoproteins are being discovered, such as apoA-I and apoA-II. Apolipoproteins play important roles in determining the functions of the different lipoproteins (**Table 4**). They have a role in stability and also provide specificity on the lipoprotein molecules, which enables the lipoproteins to be recognized by and interact with specific cell surface receptors in different body tissues and organs. This, in turn, leads to the uptake of lipoproteins from the blood and the regulation of their metabolism. Some apolipoproteins are also involved in the functioning of lipoprotein enzymes such as lipoprotein lipase (LPL).

Chylomicrons (CMs)

When ‘packages’ of cholesterol, phospholipids, TAGs, and specific apolipoproteins leave the enterocytes, they are called chylomicrons (CMs). These lipoproteins are composed primarily of TAGs and transport dietary fat into the circulation (**Table 2**). The TAGs present in the core of CMs are stabilized by a coat of phospholipids and apolipoproteins (apoA and apoB-48). ApoB-48 in CMs is unique to enterocytes and identifies lipoproteins carrying dietary lipids. CMs are transported by the lymph system through

Table 1 Properties of lipoprotein particles

Lipoprotein	*Particle core*	*Particle surface*
Chylomicrons	TAG >> CE	PL > CH >> apoLP
Very low-density lipoproteins	TAG > CE	PL ~ CH >> apoLP
Low-density lipoproteins	CE > TAG	PL ~ CH >> apoLP
High-density lipoproteins	CE > TAG	PL > CH > apoLP

apoLP, apolipoprotein; CE, cholesteryl ester; CH, cholesterol; PL, phospholipid; TAG, triglyceride.

Table 2 Functions of lipoproteins

Lipoprotein	*Function*
CM	Transport exogenously sourced lipids from intestinal cells to the liver and peripheral tissues
$\mathrm{VLDL}_{\mathrm{INT}}$	Carry exogenously sourced lipids
$\mathrm{VLDL}_{\mathrm{LIVER}}$	Transport endogenously sourced lipids from the liver to the peripheral tissues
IDL	Breakdown product of VLDL-cholesterol
LDL	Carry cholesterol molecules to peripheral tissues and the liver
HDL	Remove cholesterol from tissues and transports cholesterol (CE) to the liver (reverse cholesterol transport) and other lipoproteins

CM, chylomicron; HDL, high-density lipoprotein; IDL, intermediary-density lipoproteins; INT intestinal sourced; LDL, low-density lipoprotein; LIVER, liver sourced; VLDL, very low-density lipoprotein.

Collated from Gurr MI (2000) Fats. In: Garrow LS, James W, and Ralph A (eds.) *Human Nutrition and Dietetics*, ch. 7, pp. 97–120. London: Harcourt Publishers Ltd.; Mann J and Skeaff M (2004) Lipids. In: Mann J and Truswell AS (eds.) *Essentials of Human Nutrition*, ch. 3, pp. 31–54. New York: Oxford University Press Inc.

Table 3 General composition of lipoproteins

Percentage (%)			*Lipids (%)*					
	Protein	*Lipid*	*TAG*	*PL*	*CE*	*Chol*	*FFA*	*Main apoLP*
CM	2	98	80–95	3–9	3	1		A-I, A-II, A-IV, B-48, CI-III, E
VLDL[a]	7–10	90	55–85	10–20	15	8	1	B-100, C-II, C-III, E
IDL	11	89	5–29	20–26	34	9	1	B-100, E
LDL	20–21	79	5–15	20–28	48	10	1	B-100
$\mathrm{HDL}_{\mathrm{INT}}$	33	68	16	43	31	10		A-I, A-II, CI-III, D, E
$\mathrm{HDL}_{\mathrm{LIVER}}$	50–57	43	5–13	20–46	29	6	6	

[a] $\mathrm{VLDL}_{\mathrm{INT}}$ contain apoB-48 and not apoB-100.

apoLP, apolipoprotein; CE, cholesteryl ester; Chol, free cholesterol; CM, chylomicron; FFA, free fatty acid; HDL, high-density lipoprotein; IDL, intermediary-density lipoprotein; LDL, low-density lipoprotein; PL, phospholipid; TAG, triacylglyceride; VLDL, very low-density lipoprotein .

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the thoracic duct, thus entering the jugular vein where these particles join the blood circulation. As CMs enter the bloodstream, they acquire apo-C and apo-E from HDL, which are then released into the blood and taken up by liver cells (hepatocytes). The first port of call for CMs is adipose tissue because CMs are recognized by their surface apoB-48. In adipose tissue, CMs are depleted of some TAGs by LPL. Therefore, the remnant particles, that is, the remaining CMs, contain less TAGs and more cholesterol. These remnants are taken up by apo-E receptors in the liver cells where cholesterol is used for bile salt formation, lipoprotein synthesis, and/or CE synthesis.

Very Low-Density Lipoproteins (VLDLs)

There are two types of VLDL, namely, those made in the small intestine ($\mathrm{VLDL}_{\mathrm{INT}}$) and others that are produced in the liver ($\mathrm{VLDL}_{\mathrm{LIVER}}$). $\mathrm{VLDL}_{\mathrm{INT}}$ carry exogenously sourced lipids, whereas $\mathrm{VLDL}_{\mathrm{LIVER}}$ transport mostly TAGs that have been synthesized in the liver. $\mathrm{VLDL}_{\mathrm{INT}}$

Table 4 Functions of apolipoproteins

Apolipoprotein	*Function*
apoA-I	Structural protein for HDL; activates lecithin-cholesterol acetyl transferase (LCAT)
apoA-II	Structural protein for HDL
apoA-IV	Activates lipoprotein lipase and LCAT
apoB-48	Formation of chylomicrons and VLDL_{INT}
apoB-100	Structural protein for VLDL, IDL, and LDL. Needed for assembly and secretion of VLDL
apoC-I	Activates LCAT
apoC-II	Involved with LDL activation and activity; activates lipoprotein lipase
apoC-III	Blocks apoE clearance of TAG-enriched lipoproteins and inhibits TG breakdown by lipoprotein lipase; inhibits lipoprotein lipase
apoD	Involved with CETP
apoE-2	Reduced affinity for LDL receptor
apoE-4	Enhanced affinity for LDL receptor

Collated from Kritchevsky D (2006) Cholesterol and other dietary sterols. In: Shils M, Shike M, Ross AC, Caballero B, and Cousins R (eds.) *Modern Nutrition in Health and Disease*, pp. 123–135. Philadelphia, USA: Lippincoot Williams and Wilkins; Link J, Rohatgi A, and de Lemos JA (2007) HDL cholesterol: Physiology, pathophysiology, and management. *Current Problems in Cardiology* 32: 268–314.

and CMs transport TAGs from the gut through lymph and blood to the liver, adipose tissue, and muscles. Apolipoproteins of VLDL_{INT} are different from those of $\text{VLDL}_{\text{LIVER}}$. Like CMs, VLDL_{INT} contain apoB-48, whereas $\text{VLDL}_{\text{LIVER}}$ possess apoB-100. $\text{VLDL}_{\text{LIVER}}$ secreted into the hepato-peripheral circulation have a core of CEs and TAGs with apoB-100, apo-C, and apo-E on their surfaces. VLDLs are degraded in a similar manner to CMs. VLDL remnants are normally called IDLs, and further degradation of these particles yields LDLs.

Low-Density Lipoproteins (LDLs)

LDLs are the primary carriers of cholesterol in blood because their main role is to deliver cholesterol to both peripheral and liver cells. Atherosclerosis develops when oxidized LDL-cholesterol is taken up by macrophages and deposited in atheromatous plaques that develop into atherosclerotic lesions. LDLs have apoB-100 as their sole protein constituent, which interacts with LDL receptors on target cell surfaces. The LDL-receptor complex is then taken up into the cell where LDLs are degraded and cholesterol is released. This, in turn, regulates the amount of cholesterol produced by the cell. Cholesterol taken up by peripheral cells redistributes to various cell membranes and also becomes involved in metabolic reactions. LDLs have CEs in the core and free cholesterol with phospholipids on the surface (**Table 1**).

High-Density Lipoproteins (HDLs)

A reduced level of HDLs in the blood has been associated with an increased risk of CHD and is as significant a risk factor as elevated blood levels of LDLs. HDL originates from the intestine (HDL_{INT}) and liver ($\text{HDL}_{\text{LIVER}}$), and each source provides different apolipoproteins that determine some of their functions. HDL particles have a core of CEs and TAGs with a surface composed of phospholipids, free cholesterol, and the apolipoproteins apoA-I and apoA-II. The main role of HDL is to carry cholesterol from peripheral cells to the liver, where it is degraded or repackaged. This process is known as reverse cholesterol transport. Cholesterol taken up by HDL is esterified by the enzyme lecithin cholesterol acyl transferase (LCAT), and the esterified cholesterol then moves to the core of HDL. LCAT, primarily activated by apoA-I, is secreted by the liver and has a high affinity for HDL. LCAT has two functions: (1) it converts cholesterol to its esterified form, which then migrates from the lipoprotein surface layer into the hydrophobic core, and subsequently (2) more cholesterol can attach to the HDL surface layer because surface cholesterol is esterified and moved to the core by LCAT. The migration from the surface to the core prevents re-uptake of cholesterol by the cells. HDLs then travel to the liver where CE transfer protein (CETP) is involved in exchanging the HDL core CEs for TAGs, thereby completing the process of reverse cholesterol transport. HDL-cholesterol is then broken down by the liver and eliminated as bile or converted into cholesterol-containing steroids. Therefore, efficient reverse cholesterol transport by HDL protects against the development of atherosclerosis.

Lipid Transfer Proteins

Exchange of core lipids from lipoprotein particles is mediated by lipid transfer proteins that are synthesized in the liver. They exchange lipids (TAGs, CEs, phospholipids) from one lipoprotein particle to another. For example, as mentioned earlier, CETP functions in the transfer of CEs and TAGs between lipoprotein subfractions, mostly VLDL, LDL, and HDL-cholesterol. This exchange results in the transfer of CEs from HDL to

VLDL and LDL, and TAGs from VLDL to HDL. In addition, $\mathrm{VLDL}_{\mathrm{INT}}$ and CMs exchange components with HDL particles during lymphatic transport and blood circulation. Apo-C and apo-E are transferred from HDL to VLDL and/or CMs, which is essential for the metabolism of CM particles. In turn, VLDL and CMs transfer phospholipids to HDL.

Factors that Affect Cholesterol Level

Several factors can influence the levels of lipoproteins, apolipoproteins, and hence cholesterol in the blood (**Table 5**). The effects on apolipoproteins are far more complex and unclear. Some determinants are modifiable, such as diet, smoking, body weight, and physical activity, whereas others are non-modifiable, including age and genetics. The amounts and proportions of lipoproteins in the blood respond to the influx of digestion products after the consumption of a meal. Several major dietary determinants of blood cholesterol levels have been identified, including dietary fat, cholesterol, fiber, protein, and alcohol consumption.

Dietary Fat and Fatty Acids

Dietary fats and cholesterol are the most important food components that alter blood lipoproteins. The main dietary approach for lowering LDL-cholesterol level and improving the LDL:HDL ratio is to replace cholesterol-raising fats by carbohydrate and/or unsaturated fatty acids. Recently, there has been a shift toward assessing the apoA-I/apoB ratio, which is also a reliable indicator of CHD risk.

Dietary cholesterol

There is considerable debate as to whether dietary cholesterol affects blood lipoprotein and/or cholesterol levels. Responses appear to vary considerably in individuals within and between studies. People can be either sensitive or resistant to cholesterol intake in the diet. Within the range of cholesterol intakes normally consumed, altering dietary cholesterol content seems to result in little change to circulating cholesterol level. Only when a high proportion of dietary fats are saturated (>15% of dietary energy) and cholesterol intake exceeds 300 mg per day is dietary cholesterol an important determinant of blood total- and LDL-cholesterol. An increase of 100 mg dietary cholesterol per day results in a 2–3 mg dl^{-1} increase in total cholesterol, of which approximately 70% is in the LDL fraction.

Saturated fatty acids (SFAs) and trans fatty acids

Individual SFAs have different effects on cholesterol level. High intakes of the SFAs myristic acid (14:0), palmitic acid (16:0), or lauric (12:0) acid elevate total- and

Table 5 Dietary determinants of lipoprotein and cholesterol levels in the blood

		Lipoprotein cholesterol levels		
Dietary factor	*Total cholesterol levels*	*VLDL*	*LDL*	*HDL*
Fats:				
Cholesterol[a]	+		+	
Trans fats	+		+	–
Saturated fat	+		+	–
Monounsaturated fat:				
Oleic acid			–	+
Elaidic acid			+	+
Polyunsaturated fat (PUFA):				
n-6 PUFA			+	–
n-3 PUFA		–		
High carbohydrate diet		+		
Fiber	–		–	
Soy protein			–	
Obesity		+	+	–
Weight loss		–	–	+
Exercise[b]		–	–	+
Smoking			+	–
Increasing age	+		+	

[a]Only when dietary saturated fat is greater than 15% of dietary energy and cholesterol intake exceeds 300 mg day^{-1}.
[b]dependent on the type of exercise.
+, increase in levels; –, reduction in levels.

LDL-cholesterol by suppressing LDL-receptor clearance of LDL by reducing LDL-receptor expression. This effect is thought to occur from a shift in the regulatory pool of liver cholesterol from the CE form to free cholesterol. Stearic acid (18:0) has not been shown to raise blood cholesterol, possibly due to the fact that it is converted rapidly to the monounsaturated fatty acid (MUFA), oleic acid. Trans fats are geometric isomers of cis-unsaturated fatty acids. They are more atherogenic than SFAs because trans fats not only raise LDL-cholesterol level, but also lower HDL-cholesterol level.

Mono- (MUFA) and Poly-unsaturated fatty acids (PUFA)

When dietary SFAs are substituted by cis-MUFA or n-6 PUFA, blood total- and LDL-cholesterol are lowered; however, epidemiological studies report conflicting data in relation to n-6 PUFA and thus fail to confirm a cardio-protective effect. PUFA are more effective than MUFA in relation to improving blood lipid profiles. Intakes of n-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA) lead to a reduction in blood VLDL level, but not LDL, which may be due to a reduction in the rate of synthesis of apoB. It has also been shown that LPL activity increases when 3 g n-3 PUFA is consumed on a daily basis for 6 weeks, thus resulting in an increase in CM clearance.

Plant sterols

Plant sterols function as structural components in plants by maintaining cell membrane structure and function, which is similar to the biological function of cholesterol in humans. A meta-analysis of 41 studies has shown that ingestion of 0.8–4.0 g plant sterols per day results in 10–15% reduction in LDL-cholesterol levels with the beneficial effect leveling out with higher doses (>2.5–3 g per day). Current recommendations are the daily consumption of 2–2.5 g per day in order to reduce blood cholesterol level. The exact mechanism by which plant sterols reduce serum cholesterol is not fully clear but it is generally accepted that there are three possible mechanisms: (1) competition between cholesterol and plant sterols for micellar solubilization, meaning that less cholesterol is absorbed through the intestinal wall; (2) co-precipitation of plant sterols with intestinal cholesterol, which results in the formation of insoluble particles that are then excreted *via* the feces; and/or (3) inhibition of cholesterol uptake due to competition at the absorptive site, that is, the brush border membrane.

Carbohydrates, Fiber, and Protein

A high carbohydrate diet is generally associated with an increase in VLDLs as a result of increased hepatic synthesis of TAGs. Soluble forms of dietary fiber (10–25 g per day), such as pectins and gums found in legumes and fruits, have been shown to reduce total- and LDL-cholesterol levels in the blood; however, the effect is not as significant as altering the nature of fat in the diet. Fiber may have at least three cholesterol-lowering mechanisms: (1) fiber may act as a bile acid sequestering agent; (2) fiber may reduce the rate of insulin rise by slowing carbohydrate absorption, thus slowing cholesterol synthesis; and/or (3) fiber may produce short-chain fatty acids that are absorbed by the portal circulation and inhibit cholesterol synthesis. Consumption of animal versus plant protein increases circulating cholesterol level.

Body Weight

There is a dose-dependent increase in cholesterol level with increasing body mass index (BMI), and there is also a strong link between increased BMI and low blood HDL. Obesity affects blood lipid and lipoprotein levels, which tends to cause elevated VLDL- and LDL-cholesterol concentrations in the blood as well as reduced HDL. Studies have shown that both cholesterol and TAG levels fall during weight loss. For instance, a meta-analysis of more than 70 studies observed that for every 4.5 kg of sustained weight loss, blood HDL increased by 2 mg dl^{-1}. VLDL levels tend to be low in lean individuals and those who are physically active.

Physical Activity

Lower blood TAG concentrations have been reported following regular physical activity. CM and VLDL-cholesterol have been shown to decrease after aerobic exercise, whereas LDL-cholesterol level remains unchanged. On the other hand, prolonged regular activity (>6 months) has been reported to lower LDL-cholesterol. Regular physical activity for longer than 12 weeks is more likely to increase HDL-cholesterol level in a dose-dependent manner. The mechanism(s) by which physical activity increases HDL is not fully understood. Exercise can increase apoA-I levels, whereas apoB changes usually correspond with LDL-cholesterol changes.

Alcohol and Smoking

An excessive intake of alcohol increases VLDL- and LDL-cholesterol levels in the blood. Although moderate consumption of certain alcoholic drinks, such as wine, has been shown to lower LDL-cholesterol and raise HDL-cholesterol levels, because of other health risks the benefit is not sufficient to recommend drinking alcohol. Smoking is one of the major risk factors of CHD, and suggested mechanisms include damage to the arterial wall, altered coagulation, and changes in lipid profile, especially LDL- and HDL-cholesterol levels. Smoking-induced HDL reduction is suggested to result from increases in CETP, decreases in LCAT activity, and apoA-I synthesis. Cessation of smoking has been shown to increase HDL levels, but with no changes in TAG and LDL levels.

Age and the Menopause

After 50, age becomes a predominant risk factor for CHD. A meta-analysis of 61 prospective observational studies characterized the age-specific associations of total cholesterol changes with CHD risk and mortality. As people age, there is a progressive increase in total- and LDL-cholesterol levels. Age has been reported to be an important independent predictor of LDL- and total-cholesterol in men, and total-cholesterol in women. It has been recommended that as an individual gets older, dietary intake should be adjusted for a more sedentary lifestyle in order to maintain a healthy blood lipid profile. Reducing calorie intake by about 20% in a nutritionally balanced diet over periods of 2–15 years has been shown to lower blood pressure as well as blood cholesterol and TAG levels.

HDL cholesterol tends to be higher in women than in men throughout their pre-menopausal state because estrogen elevates hepatic expression of LDL receptors and reduces abdominal LPL activity. However, post-menopausal women develop an unfavorable lipid profile and elevated total- and LDL-cholesterol levels in the blood due to the reduction in endogenous estrogen production

Genetics

Genetic variation of apolipoproteins and lipoproteins has significant effects on lipid and cholesterol metabolism, and as a result, CHD risk. For instance, individuals with the apoA-IV and apoE-2 genotypes have lower cholesterol levels and are less responsive (resistant responders) to dietary cholesterol. On the other hand, individuals with apoE-4 have higher levels of total-cholesterol, LDL-cholesterol, and TAG. They also show a noticeable blood cholesterol response to both dietary fatty acids and dietary cholesterol changes (sensitive responders).

There are several genetic disorders of lipid metabolism (**Table 6**), which are usually characterized by alterations in one or more lipoproteins. For instance, familial hypercholesterolemia is characterized by a marked rise in total- and LDL-cholesterol. Other disorders include familial combined hyperlipidemia, remnant hyperlipoproteinemia, familial hypertriglyceridemia, and common hypercholesterolemia. The disease sitosterolemia is a rare disorder associated with increased absorption of cholesterol and plant sterols from the intestine. Three major classes of LDL metabolism defects have been identified: (1) defects in apoB-100, (2) defects in the LDL receptor, and (3) defects in a binding protein that enables internalization of the LDL–LDL receptor complex. There are also three forms of abnormal HDL metabolism, namely, hyperlipidemia, dyslipidemia, and hypoalphalipoproteinemia. Additionally, there are other genetic contributors to abnormal HDL levels, such as mutations in LCAT, CETP, and HDL-associated apolipoproteins.

Table 6 Genetic conditions leading to abnormal cholesterol levels

Disorder	*Genetic defect*
Total- and LDL-cholesterol levels	
Familial hypercholesterolemia	Mutation in LDL receptor gene.
Familial defective apoB	Multiple mutations occurring in the ligand binding areas of apoB result in reduced binding to the LDL receptor.
Autosomal recessive hypercholesterolemia	Absence of the phosphotyrosine-binding domain protein leads to defective internalization of LDL bound to the LDL receptor.
Abetalipoproteinemia	Unable to produce a circulating form of apoB and as a result no CM, VLDL, or LDL appear in plasma. Fat and vitamin E malabsorption occurs.
Hypobetalipoproteinemia	Defect in the apoB gene.
HDL cholesterol levels	
CETP deficiency	Mutations in the CETP gene result in defective cholesterol transport.
Lipase polymorphisms	Reduces rate of conversion of VLDL to IDL and IDL to LDL.
LCAT deficiency	Absence of or reduced LCAT activity.
ApoA-I deficiency	Mutations in the apoA-I gene resulting in reduced levels of apoA-I.
Sitosterolemia	Also termed phytosterolemia. Caused by mutations in two genes encoding adjoining transporters namely ABCG5 and ABCG8, which result in excessive absorption of plant sterols and abnormal sterol metabolism.

Conclusion

As can be seen, a wide range of factors can influence cholesterol level in the blood, either directly or indirectly *via* alteration of lipoprotein and/or apolipoprotein levels and metabolism. Certain factors are modifiable, such as diet, smoking cessation, alcohol consumption, and physical activity. On the other hand, non-modifiable risk factors also exist, such as age and genetics. Hence, a multidisciplinary approach is needed to successfully achieve and maintain a healthy blood lipid profile.

See also: **Milk**: Milk in Human Health and Nutrition. **Milk Lipids**: Fatty Acids; General Characteristics; Nutritional Significance; Removal of Cholesterol from Dairy Products; Triacylglycerols.

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Removal of Cholesterol from Dairy Products

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Introduction

Cholesterol is a waxy, fat-like substance found in the cells of humans and animals. It is a key constituent of cell membranes and a precursor of bile acids, steroid hormones, and also vitamin D. It is located mainly in plasma membranes, lipoproteins, and fat deposits. The highest amounts of cholesterol are found in the brain, bone marrow, kidney, and liver. Cholesterol is a natural component of many food products, especially animal foods, but the amount varies widely from food to food. It is present in the milk of all mammals at modest concentrations, for example, 1 g of bovine milk fat generally contains about 2.6–3 mg cholesterol. During the commercial processing of milk, the cholesterol is carried over with the bulk fat to the product (**Table 1**). Plants, fruits, seeds, and leaf lipids, in addition to phytosterols, also contain cholesterol in very small quantities.

In the 1950s, an increased serum cholesterol concentration was related to the incidence of coronary heart disease and was therefore assessed to be an important risk factor. Therefore, cholesterol and saturated fats in foods came into focus as risk factors in medical research, now known as the 'diet–heart hypothesis'. The importance attached to dietary cholesterol as a possible risk factor in human hypercholesterolemia has led to certain foods being regarded as unhealthy. A restriction of the everyday intake of dietary cholesterol to 300 mg was suggested and the concentration of total cholesterol in serum was not to exceed 5.2 mmol l^{-1} (200 mg dl^{-1}).

Processes for the Removal of Cholesterol from Food

Fear of coronary heart diseases and the persuasive intervention of physicians led consumers to prefer low-cholesterol and cholesterol-reduced foods. As a natural consequence of this consumer preference, legislators such as the US Food and Drug Administration developed labeling guidelines for these products (per serving): cholesterol free, <2 mg cholesterol; low cholesterol, 20 mg or less cholesterol; and reduced cholesterol, >25% less cholesterol than a reference product. Many biological, physical, and chemical methods have become available for the development of cholesterol-reduced products.

Biological Processes

Microorganisms

In nature, there are a number of cholesterol-degrading bacterial species. *Nocardia*, *Rhodococcus*, and *Eubacterium* have been isolated from butter, bacon, pork fat, chicken fat, and soil. Of the approximately 120 bacterial strains isolated from these foods, as well as from cheese, beef fat, beef liver, and egg yolk, 19 isolates had cholesterol-degrading ability. The highest cholesterol-degrading ability was found in the isolate C-5 belonging to the genus *Kurthia*, where cholesterol was completely degraded via 4-cholesten-3-one into other compounds. A suspension culture of *Nocardia labegensis* appears to have potential for the biological degradation of sterols in animal and vegetable fats. Microcapsules containing cholesterol-degrading microorganisms may also be used to reduce the cholesterol levels in liquid foods. In experiments with humans, serum cholesterol concentrations can be lowered by oral administration of a coprostanol-producing microorganism.

Of the 99 strains of lactobacilli and 33 of enterococci, 10 were checked for their ability to reduce cholesterol in broth and cream. In broth, the cholesterol reduction was between 12 and 48% and in cream between 20 and 60%. For buttermaking, *Lactobacillus maltaromicus* AC3-16 and *Lactobacillus casei* ssp. *casei* AB16-65 were selected. The cholesterol reduction was dependent on the pH value but was not particularly successful. Processing with the first strain resulted in a cholesterol reduction of 0.1% at pH 4.46 and a reduction of 19.4% at pH 3.87. With the second strain, the results were 4.1% at pH 4.60 and 25.4% at pH 3.96.

The use of bacteria to lower cholesterol levels in foods raises some important issues. First, there is the question of bacterial inoculation of the food, which may be of great concern to the consumer. Second, some of the species used may be pathogenic; in fact, *Rhodococcus equi* has been

Table 1 Cholesterol content of milk and dairy products

Food	*Fat (g per 100 g)*	*Cholesterol (mg per 100 g)*
Cow's milk (pasteurized)	4.0	15
Skim milk (UHT)	0.1	0
Full milk powder	26.2	79
Cream (UHT)	34.9	101
Plain yogurt	3.6	11
Cheese		
Camembert	23.7	71
Emmental	33.8	101
Fresh cheese	18.5	54
Gorgonzola	31.2	94
Gruyère	32.0	96
Mascarpone	47.0	141
Parmesan	25.6	77
Quark, cream	15.6	52
Butter	82.3	241

Reproduced from SwissFIR – The official Swiss food information portal, http://www.swissfir.ethz.ch/, viewed on 17 November 2008.

identified as an important pathogen. Finally, there are the potentially harmful effects of the by-products produced after bioconversion of cholesterol. When cholesterol is reduced to coprostanol, for example, by cholesterol reductase from *Eubacterium*, this does not seem to be a problem as it is absorbed poorly or not at all. *Rhodococcus* strains catalyze the conversion of cholesterol to cholest-4-en-3-one, whose formation and disappearance differ depending on the strain used. In this case, the problem is more serious since oxidized cholesterol products are considered toxic and a potential health risk.

Enzymes

Various enzymes produced by different bacteria, principally cholesterol oxidase(s) and reductase(s), modify cholesterol. *Rhodococcus erythropolis* and *Streptomyces* spp. have cholesterol oxidase activity. These enzymes were shown to be of the intrinsic (membrane-bound) type. Extracellular enzymes have also been described in the broth filtrates of *Streptomyces violascens*, *Brevibacterium sterolicum*, *Streptoverticillium cholesterolicum*, and *Rhodococcus equi*. One strain of *Rhodococcus equi*, isolated from butter, produced more extracellular cholesterol oxidase than two other strains, isolated from butter and bacon. Cholesterol oxidase modifies cholesterol to cholest-5-en-3-one and then to the final product cholest-4-en-3-one. Other oxidized cholesterol products such as 7-keto- and 7-β-hydroxycholesterol could not be detected in milk incubated with *Pseudomonas fluorescens* cholesterol oxidase.

Physical Processes

Distillation and crystallization methods

Cholesterol is soluble in steam. Thus, steam-stripping technology can be used to remove large quantities from fat. This technique allows a reduction of up to 95% of cholesterol from anhydrous butterfat and fish oils. The procedure is quite simple. The fat is first deaerated, mixed with steam, heated, and then flash vaporized with subsequent removal of cholesterol by thin-film stripping in a counter-current of steam. The product is then cooled and stored in the absence of oxygen. The process consumes large amounts of energy, entails significant operating costs, and can lead to polymerization and oxidation of various components.

Short-path distillation and crystallization are processes that are used to fractionate fat. The first process consists of evaporation of fat into a substantially gas-free space. In the latter procedure, the fat is liquefied and is then cooled to specific crystallization temperatures. To crystallize different components that are subsequently separated from the liquid phase, diverse temperatures can be applied. Milk fat, a mixture of different triglycerides with varying physical properties, can be separated into fractions of different chemical composition and also different cholesterol levels.

Supercritical fluid extraction

Supercritical fluid extraction has been used for a long time in the food industry. It has been applied to delipidation of protein, deoiling of lecithin, isolation of important oils, fractionation of butterfat, decaffeination of coffee, oil extraction from oilseeds, removal of bitter aroma compounds from hops, extraction of spices, and removal of cholesterol from different foods. In this process, a product is treated with a gas, for example, carbon dioxide, of high density, low viscosity, and reduced surface tension under high pressure and at high temperatures. Carbon dioxide is used in the processing of natural products intended for human consumption because it has many advantages – it is easily removable, nontoxic, nonflammable, noncorrosive, nonoxidizing, and inexpensive; it is available in large quantities of high purity; and it has a relatively low critical temperature (+31 °C). Supercritical fluid extraction has the great advantage of low energy cost, high yield, superior quality (since a lower extraction temperature range is used), absence of potentially explosive or toxic solvents, and absence of toxic by-products. In general, no chemical solvent is left in the food with this technique. However, a substantial capital investment is required for the installation of the necessary equipment.

Chemical Processes

Solid–liquid extraction

Cholesterol can also be removed from liquid foods by selective solid-phase extraction using adsorbents such as activated charcoal, coated porous glass, ceramics, and plastics. Silica gel may also be used as an adsorbent. In addition, organic solvents or mixtures can be applied to remove cholesterol, but it should be noted that these solvents are mostly toxic and should not be used in foods. Other disadvantages of a process using an organic solvent are the extraction of other lipid-soluble components, denaturation of proteins, removal of flavor and nutritional components, relative nonselectivity as well as high costs.

Complex formation

β-Cyclodextrin, a cyclic oligosaccharide consisting of seven glucose units joined as α-$(1 \rightarrow 4)$ isomers, is nontoxic, edible, nonhygroscopic, chemically stable, and easily separable. It has a cavity at the center of the molecular arrangement and can thus form a stable insoluble inclusion complex with cholesterol. The effectiveness of cholesterol adsorption is dependent on the adsorbent concentration, stirring time, speed, temperature, and centrifugation conditions. Starch-containing products also seem to form stable complexes. All of these complexes are stable in aqueous solutions, which allow removal of cholesterol from the lipid phase. Incidentally, β-cyclodextrin can be fermented by human colonic flora. The safety aspect of residual β-cyclodextrin from egg was studied in rats in a subchronic toxicity study and indicated no toxicity. Extracted β-cyclodextrin can be recovered by heating or by the addition of increasing amounts of sodium chloride, but the recovery of β-cyclodextrin from dairy products is ineffective since considerable amounts are needed for removal of cholesterol, which leads to high costs. To overcome these problems, β-cyclodextrin can be cross-linked with adipic acid or immobilized on a solid support. A maximal level of β-cyclodextrin of 5 $\mathrm{mg\,kg^{-1}}$ per day in foods is recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). β-Cyclodextrin is 'generally recognized as safe' (GRAS) in the United States and a 'natural product' in Japan.

It is also possible to minimize the level of cholesterol in animal fat with an aqueous solution of bile salts and one or several glyceryl esters or with cyclic anhydrides such as succinic anhydride. The cholesterol-containing aqueous phase is then separated or the final product of the reaction with cyclic anhydrides is extracted with aqueous alkali. In another method, animal fat is brought into contact with phospholipid or anionic polysaccharides. The formed sterol-reduced fat is then removed from the aqueous mixture. Saponins such as quillaja powder (a commercial preparation of saponins from the bark of *Quillaja saponaria*), which are accepted for food processing and have received the GRAS list status in the United States, and also polymer-supported saponins such as digitonin (consisting of the aglycone digitogenin linked to a pentasaccharide) or tomatine (composed of a polycyclic steroidal secondary amine, tomatidine, and a tetrasaccharide) form insoluble cholesterol complexes, which can be removed by filtration or centrifugation. The polymers may be regenerated by benzene extraction, which restores the original cholesterol-binding capacity.

Use of the above Processes to Remove Cholesterol from Milk and Dairy Products

The processes described in world patents and the scientific literature relate mainly to the removal of cholesterol from fat of animal origin, especially from milk, egg yolk, and meat. The worldwide attempts to produce low-cholesterol products have generated many patents, indicating that this is of great economic interest. The preferred methods are complex formation with β-cyclodextrin, distillation, and extraction with supercritical carbon dioxide.

Milk

In milk treated with cholesterol oxidase, the cholesterol concentration was reduced by 50% within 10 h at 3 or 7 °C and by 78% within 3 h at 37 °C.

Cholesterol has been removed from milk and dairy products by a β-cyclodextrin-based process. Homogenized milk was treated with β-cyclodextrin to determine the optimum combination of five different factors (**Table 2**). Removal of cholesterol is mostly affected by factors such as β-cyclodextrin concentration, mixing temperature, mixing time, centrifugal forces, and centrifugation time. In various investigations, more than 90% of cholesterol was removed with cross-linked β-cyclodextrin.

Removal of cholesterol from homogenized milk by treatment with saponins is another option. This process was studied using response surface methodology. Using the optimum reaction time (30 min) and the optimum amounts of Celite (0.95%) and saponin (1.5%), the predicted removal of cholesterol by this method was estimated to be 73.4% compared to the experimental value of 73.7%. The use of β-cyclodextrin immobilized on glass beads led to a cholesterol reduction of only 41% in milk.

Milk Fat and Cream

Biological processes

The cholesterol content of anhydrous milk fat, incubated at 28 °C for 12 h with *Nocardia*, was reduced by 86–89%; in contrast, only 7% of the cholesterol was removed after incubation of cream with an extract of *Rhodococcus equi* for a period of 15 h.

Physical processes

After short-path distillation, the solid fraction contains cholesterol at a concentration of 0.2 $\mathrm{mg\,g^{-1}}$ fat compared

Table 2 Effect of various conditions on the removal of cholesterol from milk using β-cyclodextrin

β-Cyclodextrin		*Mixing temperature*		*Mixing time*		*Centrifugal force*		*Centrifugation time*	
(%)	*Cholesterol removed (%)*	*(°C)*	*Cholesterol removed (%)*	*(min)*	*Cholesterol removed (%)*	*(g)*	*Cholesterol removed (%)*	*(min)*	*Cholesterol removed (%)*
0.5	92.2^{a}	4	93.7^{a}	5	93.2^{a}	55	86.7^{a}	5	81.9^{a}
1.0	94.0^{a}	10	$94.6^{a,b}$	10	93.9^{a}	111	94.9^{b}	10	94.6^{a}
1.5	95.3^{a}	15	93.0^{a}	15	92.1^{a}	166	95.9^{b}	15	94.1^{a}
2.0	88.6^{b}	20	93.5^{a}	20	92.5^{a}	222	$91.5^{a,b}$	20	92.9^{a}
		25	95.2^{b}	25	90.3^{b}	178	87.3^{a}	25	88.3^{b}

Except where parameters were varied as above, experimental variables were β-cyclodextrin added, 1%; mixing speed, 800 rpm; mixing temperature, 10 °C; mixing time, 10 min; centrifugal force, 111 *g*; and centrifugation time, 10 min.
Means within a column with different superscript letters differ ($p < 0.05$).
Adapted from Lee DK, Ahn J, and Kwak HS (1999) Cholesterol removal from homogenized milk with β-cyclodextrin. *Journal of Dairy Science* 82: 2327–2330.

to 2.6 mg g^{-1} fat in native milk fat or 16.6 mg g^{-1} fat in the liquid fraction. After melt crystallization, the concentration of cholesterol in the liquid fraction (3.8 mg g^{-1} fat) was nearly twice as high as in the solid fraction (2.0 mg g^{-1} fat). This may be due to the higher affinity of cholesterol for the triglycerides of the liquid fraction or due to the purifying effect of crystallization.

Using increased pressure and different quantities of carbon dioxide, fractions were obtained in which the cholesterol content was reduced by up to 90%. At a pressure of 20 MPa at 80 °C, the cholesterol content in the liquid extract was 5.5 mg g^{-1}, in the solid extract 2.3 mg g^{-1}, and in the original fat 2.6 mg g^{-1}. When different pressure conditions were applied at 12, 15, and 20 MPa at 48 °C, the cholesterol was concentrated in the first of three extracts. Fractions in which the cholesterol content was reduced by up to 90% using increasing pressure and different quantities of carbon dioxide were obtained. In anhydrous milk fat, the selectivity is too low for direct extraction of cholesterol. By using an adsorbent, selective removal of the cholesterol can be achieved at 40 MPa, 70 °C, and a flow rate of 12.0 g min^{-1} using a multi-separation process. The use of silica gel with a particle size of 5–40 μm removes more cholesterol (97%) than silica gel with a particle size of 1–3 mm (18.2%) or calcium carbonate with a particle size of 50–100 μm (9.8%). Anhydrous milk fat was fractionated using supercritical carbon dioxide at a pressure of 32 MPa and a temperature of 52 °C in different fractions. By using an adsorption column with silica gel (particle size 15–45 μm) that was flushed with pure CO_2, removal of over 99% of cholesterol was achieved. In ewes' milk cream, extraction of cholesterol was effective at a plateau of 15 MPa with an extraction rate of 30% (40 °C) and 40% (50 °C).

Chemical processes

Application of the following reaction conditions to animal fats is recommended: refluxing at 130–140 °C with a mixture of cholesterol and succinic anhydride (1:3); incorporating 5% (by weight) acetic acid in the reaction mixture; and extraction of cholesteryl hemisuccinate with 5% aqueous sodium carbonate. A cholesterol reduction of 35–45% could be achieved and a reduction of 60–70% resulted if succinic anhydride was added to the reaction mixture in small amounts throughout the course of the reaction.

The optimum conditions for removal of cholesterol from cream were established by the use of response surface methods. β-Cyclodextrin concentration, stirring speed, and time were important factors. Although the reduction of cholesterol level varied, under optimal conditions 94% of the cholesterol in cream could be removed. Removal of cholesterol from cream of nearly the same level (92.7%) was attained with β-cyclodextrin cross-linked with adipic acid under the following optimal conditions: 10% of β-cyclodextrin mixed for 30 min at 1400 rpm at 40 °C (**Table 3**). When the cross-linked β-cyclodextrin was used up to 7 times, a removal of cholesterol of 89.4–91.5% resulted.

Butter and Butter Oil

Physical processes

The cholesterol content of supercritical carbon dioxide-extracted butter was reduced to less than one-quarter of the original concentration by passing the extract through a silica gel column. Fractions of butter oil obtained at a pressure of 17.2 and 20.7 MPa at 40 °C had lower cholesterol levels (1.07 and 1.76 mg g^{-1}, respectively) than those obtained at 10.3 and 13.8 MPa at 40 °C (2.96 and 2.78 mg g^{-1}, respectively).

It is also possible to simultaneously remove cholesterol and fractionate butter oil by extraction with supercritical carbon dioxide followed by adsorption on alumina, achieving a cholesterol removal efficiency of 96%. Supercritical extraction of butter oil can also be realized

Table 3 Effect of various conditions on removal of cholesterol from cream using cross-linked β-cyclodextrin

β-Cyclodextrin		*Mixing temperature*		*Mixing times*		*Mixing speed*	
(%)	*Cholesterol removed (%)*	*(°C)*	*Cholesterol removed (%)*	*(min)*	*Cholesterol removed (%)*	*(rpm)*	*Cholesterol removed (%)*
1	81.7^{a}	40	91.0^{a}	10	84.3^{a}	800	85.3^{a}
5	85.3^{b}	45	90.6^{a}	20	85.1^{a}	1000	87.5^{b}
10	90.7^{c}	50	88.7^{b}	30	91.2^{b}	1200	90.9^{c}
15	90.5^{c}	55	81.3^{c}	40	91.0^{b}	1400	92.7^{d}
20	90.0^{c}	60	85.0^{c}	50	90.6^{b}	1600	88.8^{b}

Except where parameters were varied as above, Other experimental variables were cross-linked β-cyclodextrin, 10%; mixing temperature, 40°C; mixing times, 30 min; mixing speed, 1400 rpm.

Means within a column with the same superscript letter are not significantly different ($p > 0.05$).

Adapted from Han EM, Kim SH, Ahn J, and Kwak HS (2007) Optimizing cholesterol removal from cream using β-cyclodextrin cross-linked with adipic acid. *International Journal of Dairy Technology* 60: 31–36.

using ethane as solvent and adsorption on alumina. The advantage of ethane is that it has a lower critical pressure than CO_2. The higher cost of ethane is compensated for by the lower consumption of ethane. Using the combined extraction/adsorption process at 40 °C and 17.2 MPa, the level of cholesterol in butter oil was reduced to 3% of that in the original oil.

Chemical processes

Cholesterol can be extracted from butter oil using a mixture of ethanol and propane or a cyclic anhydride. After mixing and feeding the extraction column, a mixture of ethanol and water was used as an extractant in a counter-current procedure at 30 °C and 1 MPa, resulting in the removal of about 90–95% of cholesterol. Multiple extractions with β-cyclodextrin allow the removal of up to 80% of the sterols from anhydrous butter. However, in cholesterol-reduced butter made from β-cyclodextrin-treated cream, the cholesterol concentration was reduced by 90%. The scores for hardness, elasticity, cohesiveness, and the color measurement value '*L*' were significantly increased and the color measurement value '*a*' decreased compared to control butter, whereas other parameters remained unchanged. The removal of cholesterol from butter oil using a combination of saponins and Celite was dependent on pH (75 and 56% at pH 4.5 and 7.0, respectively), the concentration of saponin solution (25 and 75% with 0.04 and 0.40 g ml^{-1}, respectively), the amount of Celite (75 and 54% with 250 and 50 mg g^{-1} butter oil, respectively), the temperature (83, 76, and 60% at 45, 55, and 65 °C, respectively), and the level of agitation (75% with low and median and 35% with high agitation), but not on the time of interaction (75% at 30 and 60 min). After repeated extractions with an aqueous solution of bile salts, the cholesterol content of anhydrous milk fat can be reduced by up to 97%.

Cheese

Cholesterol-reduced Cheddar, Camembert, and Blue cheeses were manufactured from milk treated with β-cyclodextrin or cross-linked β-cyclodextrin. In these cheeses, the cholesterol concentration was reduced by more than 90% compared to control cheeses. Cholesterol-reduced and control cheeses did not differ significantly in most of the examined parameters: short-chain free fatty acids, free fatty acids, and rheological and sensorial properties. Experiments to produce Mozzarella cheese from cholesterol-reduced milk fat showed a cholesterol reduction of 64%, and the cheese was found to be superior to the control with respect to appearance and flavor, but not to texture.

Removal of Cholesterol and Dairy Applications

The aim of removing cholesterol is to provide highly functional components, which can then be used as ingredients in foods or to produce reformulated products that have unchanged chemical composition, apart from the level of cholesterol. It is evident, however, that some functional properties of these components are changed and the different procedures used clearly have some effect on emulsion stability, foam-stabilizing ability, interfacial behavior, gelling characteristics, and other functional properties. The same is true for other low-fat, low-cholesterol components, which have many potential applications in new products. A butter oil with reduced cholesterol manufactured by removing the cholesterol using a steam-stripping process was recombined with skim milk, sweet buttermilk, and a butter-derived aqueous phase to manufacture 20% milk fat creams. These reformulated creams were quite similar to an oil-in-water emulsion typical of a natural homogenized cream; however, in terms of stability behavior, the incorporation of cholesterol-reduced butter oil into skim milk did not provide an acceptable result. In a processing plant with a capacity of 240 or 800 tonnes yr^{-1}, the fractionation of anhydrous milk fat by supercritical carbon dioxide was calculated to increase costs by US$ 0.75 or 0.34 per kg of product, respectively (results from 1993).

In 1990, according to taste panel evaluations and a preliminary market study, American consumers accepted low-fat milk containing cholesterol-reduced milk fat and a butter made from low-cholesterol milk fat. Nevertheless, conventional butter was significantly preferred by the panel. In a study with 33 healthy postmenopausal women and 10 students, butter, margarine, a dairy spread made from cholesterol-reduced milk fat, and a spread made from cholesterol-reduced lard mixed with vegetable oils were tested. Butter was preferred to the two spreads and margarine, and the flavor of the spreads was thought to be more similar to margarine than to butter.

Cholesterol-Reduced Food on the Market

The extensive published literature and numerous patents show that removal of cholesterol from food is economically and technically possible. While all foods of animal origin contain cholesterol and some contain relatively high concentrations, the focus has been on trying to reduce the level of cholesterol in milk fat and eggs. In the early 1990s, Corman and Entremont used β-cyclodextrin while Hoche employed a steam extraction process to produce cholesterol-reduced milk fat products. In the United States, the Omega Source Corporation modified a cholesterol-stripping technique for an industrial pilot plant, and in 1992 the NutraSweet Company developed a low-fat, low-cholesterol egg yolk ingredient (Eggcellent$^{\mathrm{TM}}$) using supercritical carbon dioxide technology. At about the same time, the American company Michael Foods introduced a low-cholesterol egg product to the American market, but with rather limited success. In 2008, Corman SA (Goé, Belgium) and Uelzena eG (Uelzen, Germany, acquired Hoche (Neunkirchen-Speikern) in 1998) produced cholesterol-reduced butter and butterfat on a commercial scale.

In western Europe also, the consumer response to low-cholesterol milk fat products was poor. When people are concerned about cholesterol, they prefer to eat low-fat products in general.

Removal of Cholesterol from Dairy Products – Scientifically Justified or Not?

During the last 10 years, the scientific interest in the topic 'Removal of cholesterol from dairy products' has faded more and more. Since then, removal of cholesterol from foods of animal origin is no longer justified for different reasons:

- The diet–heart hypothesis has never been proven.
- Cholesterol is not an essential food ingredient.
- Cholesterol intake from foods is 100–400 mg day^{-1}. In general, a limit value of 300 mg cholesterol per day per person is recommended. However, no persuasive arguments exist for this recommendation.
- With increasing dietary intake, the human body absorbs less cholesterol, because a homeostatic control mechanism exists.
- The human body itself synthesizes an enormous amount of cholesterol. One gram of cholesterol, from enterohepatic circulation across the blood, is eliminated per day in the bowel.
- Reduction of dietary cholesterol has practically no or only a small effect on the concentration of total serum cholesterol. According to a recent meta-analysis, a reduction of 100 mg of dietary cholesterol per day lowers the total serum cholesterol concentration by about 0.056 $\mathrm{mmol\,l}^{-1}$ ($=2.2\ \mathrm{mg\,dl}^{-1}$).

In a well-balanced diet, removal of cholesterol from animal-derived food is not scientifically justified, because dietary cholesterol has only a small effect on serum cholesterol levels and the human body itself synthesizes more cholesterol than it consumes. Therefore, further research on removal of cholesterol from food of animal origin is not necessary.

See also: **Milk Lipids**: Cholesterol: Factors Determining Levels in Blood; Nutritional Significance.

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http://en.uelzena.de – Uelzena eG.

MILK PROTEINS

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Introduction

Milk contains certainly more than 100 different proteins, usually classified into three nitrogen fractions: caseins (α_{s1}-, α_{s2}-, β-, and κ-casein), which are the most abundant (78%); serum or whey proteins (17%), which include α-lactalbumin, β-lactoglobulin, blood serum albumin (BSA), and immunoglobulins (Ig); and the nonprotein nitrogen (NPN) fraction (5%). Milk proteins also include milk fat globule membrane proteins and a large variety of enzymes (about 60) and hormones. In addition, the caseins and whey proteins are present as at least 28 identified genetic variants. Caseins also possess different degrees of glycosylation (κ-casein) and phosphorylation. They may also be partly hydrolyzed at milking, with the formation of breakdown products.

In this article, only the methods used routinely by the dairy industry or by research laboratories for quantitative and qualitative analysis of the proteins in raw milk are presented. For the particular aspects of milk protein analysis, such as amino acid analysis, sequencing, secondary and tertiary structure and proteolysis of dairy products, readers should refer to the relevant articles.

Among the methods currently used for milk protein analysis, we can distinguish two groups. The first group of methods is based essentially on the measurement of chemical or structural characteristics of the proteins, such as nitrogen, free NH_3^+ groups (terminal or basic amino acids), CO–NH peptide bonds, and aromatic amino acids (**Figure 1**). These methods are particularly suitable for the accurate determination of total proteins and the major nitrogen fractions in milk. They are used routinely for several purposes: animal breeding and feeding, milk payment, and control of the raw material before processing.

The second group of methods is based on physical and biological properties of the proteins, such as pH and salt solubility, electric charge at various pH values, molecular mass, hydrophobicity, secondary and tertiary structure, and antigenicity. These methods are used for the separation, identification, and possible quantification of individual proteins in research and for regulatory purposes.

[†] Deceased

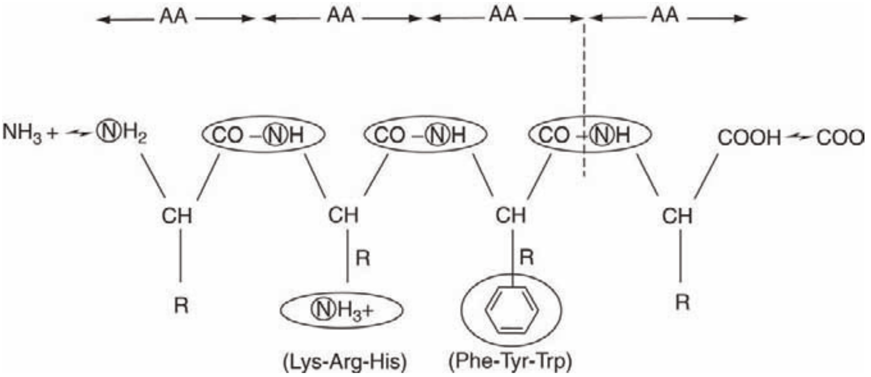

Figure 1 Reactive sites used for quantitative measurement of proteins. AA, amino acids.

General Criteria for Analytical Methods

When assessing the performance of methods of analysis, technical and economic criteria, and accuracy are taken into consideration. Accuracy represents the ability of a method to estimate the true value of an analyte with a known degree of uncertainty. It includes precision, trueness, limit of detection, and sensitivity, which have mathematical expressions, as well as specificity.

1. Precision corresponds to the random errors of a measurement. It is expressed as repeatability when measuring conditions are identical, and as reproducibility when measurements are made by different laboratories. Its mathematical expressions are the repeatability and the reproducibility standard deviation (s_r and s_R) or relative standard deviation (RSD_r and RSD_R). The maximum differences ($p = 0.95$) between two determinations performed under repeatability or reproducibility conditions are given by the repeatability and reproducibility limits (r or R).
2. Trueness is the systematic differences, or error, between the measured value (x) and the true or reference value (y). It is expressed as the residual standard deviation ($s_{y,x}$) of the regression equation, $y = bx + a$, and when the instrument is calibrated, by the mean bias (d_{x-y}) and the standard deviation (s_{x-y}) of the difference $x - y$ or by the standard error of prediction (SEP), which is equal to s_{x-y} when $y = x$.
3. Sensitivity is the ratio of the signal increase of the method corresponding to a unit increase in the analyte concentration.
4. The limit of detection is the lowest concentration that can be measured with a known degree of uncertainty.

Definition of Milk Proteins

Total proteins, caseins, whey proteins, and NPN, are complex chemical entities that can be defined only by their methods of analysis. These methods, which are based on the fractionation of proteins by the Aschaffenburg–Rowland method and then the measurement of nitrogen content by the Kjeldahl method, belong to type I or the defining methods of the Codex classification. Casein N corresponds to the N fraction that is insoluble in an acetic acid–acetate buffer at a pH of approximately 4.6; NPN corresponds to the fraction soluble in 12% trichloroacetic acid (TCA), and whey proteins N corresponds to the difference between non-casein N and NPN. The protein content is determined by multiplying N concentration by the conversion factor 6.38, which has been internationally accepted to give the true value for milk proteins. For applications, these definitions have two important consequences:

1. Because milk contains a significant and variable (3–8%) NPN fraction, it is advisable to distinguish between crude or total protein (total N × 6.38) and true protein ((total N − NPN) × 6.38).
2. The conversion factor 6.38 was established a century ago on the basis of the N content (15.6%) of purified acid-precipitated casein. Now, if we consider the primary structure of individual milk proteins, the best estimate for cow's milk total proteins would be 6.35 rather than 6.38, and would vary between 6.00 and 6.37 for individual proteins. When applying the Aschaffenburg–Rowland procedure, the correct coefficient for caseins, serum protein, and NPN would be 6.36, 6.28, and 3.60, respectively.

Analysis of Total Proteins

Three standardized methods are used routinely for quantitative analysis: nitrogen determination by the Kjeldahl or Dumas method, infrared spectroscopy, and the dye-binding method.

Nitrogen Determination

Kjeldahl method

The principle is to convert quantitatively all the organic (and inorganic) nitrogen into ammonium sulfate by digestion at 400–420 °C in a mixture of K_2SO_4/H_2SO_4 with a catalyst, and subsequently to estimate the nitrogen concentration by distilling the ammonium sulfate in the presence of NaOH and titrating the ammonia with standard acid.

To ensure accurate determination, two accuracy checks must be performed periodically:

- recovery ($\geq$98%) of a hard-to-digest compound, such as tryptophan or lysine-HCl, to check the digestion efficiency
- recovery ($\leq$99%) of a pure ammonium salt (sulfate or oxalate) standard to detect nitrogen loss

In addition, it should be kept in mind that milk compounds, particularly fat, consume acid during digestion. Consequently, with high-fat milk, the K_2SO_4 will become concentrated in the H_2SO_4 and will increase the boiling temperature, leading to nitrogen loss by pyrolytic decomposition of ammonia. Usually, crystallization of the mixture at the end of digestion indicates an excess of acid consumption and volatilization.

The Dumas and related methods

Instead of converting nitrogen into a titratable chemical compound, it is converted into gas after combustion at a high temperature (>1000 °C) in a furnace. After removing all interfering gases, the nitrogen is measured by thermal conductivity.

Instruments for this method should be calibrated with a pure compound with a constant nitrogen content (e.g., aspartic acid) or preferably against the Kjeldahl method. For ultra-high temperature (UHT) milk and fluid milk, repeatability and reproducibility limits are 0.015–0.080% and 0.041–0.093%, respectively.

Infrared Spectroscopy

Both the mid-infrared (MIR), from 2.5 to 25.0 μm (4000–400 cm^{-1}), and the near-infrared (NIR), from 0.7 to 2.5 μm (14 825–4000 cm^{-1}), regions are used to analyze milk. The fundamental vibrations of the molecules, which interact with infrared energy, occur mainly in the MIR, whereas absorption in NIR corresponds to harmonic (overtone) and combination frequencies of the fundamental vibrations. Compared with MIR, NIR absorption bands are broader and their intensities are weaker. For MIR analysis of milk, the strong absorption of water and a variable and significant light-scattering effect of the milk fat globules must be taken into consideration. To eliminate in part the influence of these two factors, a spectral subtraction of water is needed, and the instruments should have built-in homogenizers to reduce light scattering. In addition, a short-path length cuvette (37 μm) is used.

Mid-infrared analysis

An infrared spectrum of milk versus that of water shows several absorption bands that are specific to milk components (**Figure 2**). At 6.46 μm (1550 cm^{-1}), there is a peak corresponding to absorption of the amide II peptide bond; 30% of the peak originates from the C–N stretching vibration and 50% originates from the N–H bending deformation (**Figure 3**). In conventional filter instruments, fat, proteins, and lactose measurements are made at their specific wavelengths by reference to close-second reference wavelengths, corresponding to minimal

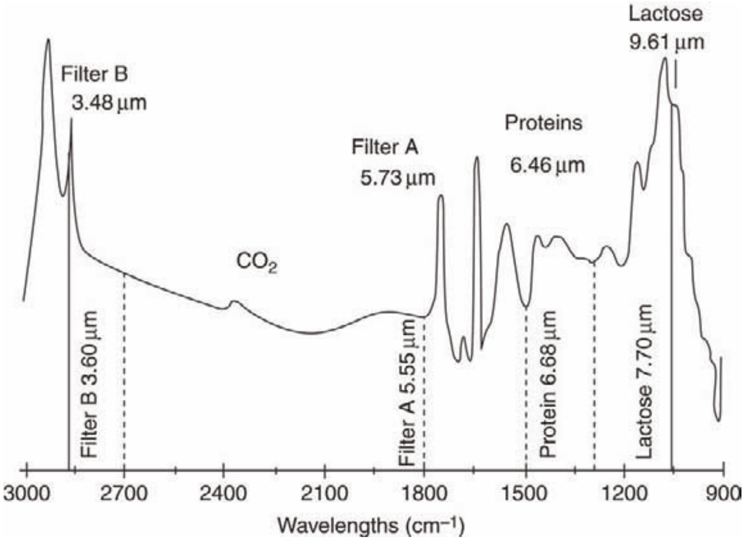

Figure 2 Mid-infrared spectrum obtained in the transmittance mode on a homogenized milk sample compared with water.

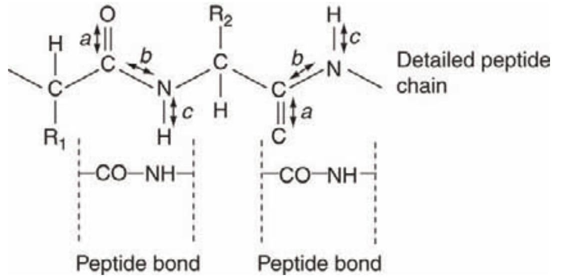

Figure 3 Mid-infrared absorption bands of the peptide bond. *a*, amide I (C=O stretch); *b*, amide II (C–N stretch); *c*, amide II (N–H bending); arrows, direction of the light-induced dipole; R_1, R_2, amino acid side chains.

absorbance. Even though proteins are the main absorbing components at 6.46 μm (1550 cm^{-1}), fat, lactose, phosphate, and organic acids with a free carboxylic group (COO^-) may interfere, provided their concentrations are naturally variable in milk and not highly correlated to the protein concentration. To eliminate fat and lactose interferences, intercorrection factors are calculated at each measuring wavelength during the calibration step, in order to obtain the corrected values for fat, protein, and lactose. In addition, with corrections automatically applied in modern milk analyzers, the variability of NPN (**Figure 4**(**a**)) and citric acid (**Figure 4**(**b**)) concentrations can explain most of the differences between infrared and Kjeldahl methods.

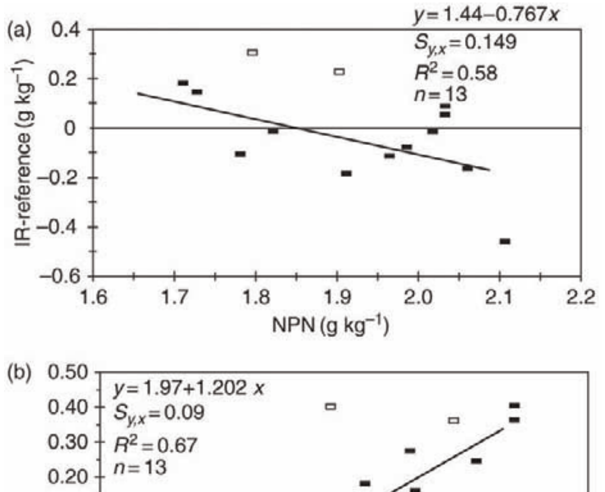

Figure 4 Influence of milk composition (medium molecular mass of fatty acids, non-protein nitrogen (NPN) concentration, citrate concentration) on the differences observed, on the determination of (a) total nitrogen and (b) protein content, between infrared (IR) filter instruments and the reference methods.

With Fourier transform infrared (FT-IR) spectroscopy, which provides the whole infrared spectrum in a few seconds, it is theoretically possible to include in the calibration all the known and unknown factors that may interfere in the measurement.

Near-infrared analysis

Although there are specific absorption bands of the polypetidic chains at about 1525 nm (first harmonic N–H stretching vibration) and 1600 nm (first harmonic-free N–H stretch/amide II combination), two wavebands near 2050 nm (N–H stretch/amide II combination) and 2180 nm (amide A/amide III or first harmonic amide I/amide III combination) are essentially used for milk analysis.

Except for the Infra Alyzer 400 Dairy (Brann and Luebbe, Delaware), which has a built-in homogenizer and a temperature-controlled sample cell, no NIR instrument has been specifically dedicated to the analysis of milk. Milk can be analyzed either in the transmittance or in the reflectance mode, and wavelength discrimination is obtained either with filters (Infra Alyzer) or with a grating (NIR system).

One of the most interesting features of NIR spectroscopy is the possibility of connecting a fiber-optic probe, to make an *in situ* measurement.

Calibration of NIR instruments follows similar principles to the calibration of MIR concerning the selection of milk samples. Multilinear regression, and more often partial least squares (PLS), or even neural networks are used. Often, preliminary treatment of the data, such as derivations, is necessary.

Because of the excellent performance of MIR milk analyzers, very few study results on the performance of NIR instruments for milk protein analysis are available. The repeatability standard deviation is about 0.2 g kg^{-1}, and the accuracy standard deviation is in the range of 0.3–0.8 g kg^{-1}.

Dye-Binding Methods

Dye-binding methods are based on the quantitative reaction between ionized groups of proteins and acid. The amido black method, which uses CI acid black I, is used routinely in different countries. In an acid buffer solution (pH 2.4), the positive charges on the terminal amino groups and the basic amino acid residues of proteins bind in a stoichiometric interaction with the negative sulfonic group of the dye, which must be in excess, to form an insoluble complex. In addition, hydrophobic interactions occur between free and bound dye. After centrifugation, the supernatant is measured spectrophotometrically at about 530 nm. As the quantity of dye bound per unit weight of protein varies with the different proteins, the trueness of the method is influenced by the proportions of the different proteins. Whey proteins bind approximately 27% more dye than caseins, and the NPN fraction does not bind dye.

Analysis of Major Nitrogen Fractions

The Aschaffenburg–Rowland procedure has recently been modified and standardized to measure directly caseins or true proteins with a single Kjeldahl determination instead of two determinations. Acid precipitation is made directly in the Kjeldahl flask, and the precipitated proteins are removed by filtration and analyzed. Caseins (or *para*-caseins) and whey proteins in milk can also be measured by infrared and the dye-binding methods. Because of dilution and the formation of free carboxylic groups with classical acid precipitation, the analysis of caseins by infrared is performed after separation of caseins by rennet coagulation or by precipitation with phosphoric acid, which does not have ionizable carboxylic groups. After calibration, the whey proteins are measured directly in the filtrate, and *para*-caseins or caseins are obtained by the difference between the total proteins in the milk and those in the whey. Attempts have also been made to measure the caseins in milk directly after appropriate calibration of the instrument. With the dye-binding method, only rennet coagulation is used.

Analytical Performances

Precision is a criterion common to all methods of analysis. **Table 1** gives the repeatability and reproducibility values obtained through international inter-laboratory studies for the reference methods. For the infrared and dye-binding methods, the repeatability limits are usually lower than 0.03 g 100 g^{-1} of protein; no reproducibility standard value can be given because the inter-laboratory variability is highly dependent on the procedure used to calibrate the instruments. Lower *R* values are expected when instruments are calibrated using common reference materials, as compared with calibrations made locally with the Kjeldahl method.

Trueness concerns only indirect methods of analysis, such as infrared and dye-binding. **Table 2** gives examples of trueness values obtained. Dairy laboratories nowadays

Table 1 Precision (International Dairy Federation standards) of reference methods for the determination of nitrogen (N) fractions in milk

N fraction	*Repeatability, r (g 100 g^{-1})*	*Reproducibility, R (g 100 g^{-1})*
Total N	0.0060	0.0077
True protein N (direct)	0.0038	0.0092
Casein N: direct	0.0064	0.0096
Casein N: indirect	0.0068	0.0113
Soluble N	0.0043	0.0072
Nonprotein nitrogen	0.0025	0.0052

Table 2 Examples of trueness values ($S_{y,x}$ or S_{y-x}) of infrared milk analyzers for the analysis of crude proteins, true proteins, and caseins of herd or individual cow's or goat's milk samples

				S_{x-y} (g kg^{-1})		
Instrument	*Analysis*	*Milk*	*n*	*Crude proteins*	*True proteins*	*Caseins*
Milko Scan 605	MIR (F)	Bulk	233	0.52	0.37	
		Individual	117	0.48		
Milko Scan 4000	MIR (F)	Herd	64		0.13	
		Individual	150		0.36	
Milko Scan 6000	FT-IR	Herd	55		0.15	
		Individual	112		0.37	
Milko Scan 104	MIR (F)	Individual Goat	81		0.35	0.25
Nicolet 740	FT-IR	Herd	73	0.38		0.46
Infra Alyzer	NIR (F)	Individual	20			0.18

MIR (F), mid-infrared; FT-IR, Fourier transform infrared; NIR (F), near-infrared.

Table 3 Influence of season on the exactness of measurement of crude and true protein of 163 bulk milks, with filter (Milko Scan 605) and Fourier transform-infrared (Nicolet 740) milk analyzers using single or multiple calibrations

		Milko scan 605		*Nicolet 740*	
Calibration		d_{x-y}	s_{x-y}	d_{x-y}	s_{x-y}
Single	Crude protein	−0.48	1.19	+0.10	0.53
	True protein	+0.35	1.21	+0.16	0.44
Multiple	Crude protein	0.00	0.48	0.00	0.46

use fully- or semi-automated dedicated instruments that can analyze up to 400 samples per hour. Repeatability standard deviation is always lower than $0.10\,\mathrm{g\,kg^{-1}}$. However, despite the fact that the entire spectral information is available, similar performances were obtained with filter and FT-IR instruments on a relatively homogeneous population of milk samples, or when calibrations were adjusted regularly to take into account seasonal variations in milk composition (**Table 3**). On the other hand, when a single calibration is used throughout the year, far better estimates for both crude protein and true protein are obtained with FT-IR than with a filter instrument (**Table 3**).

One of the most difficult aspects of analyzing milk proteins by infrared or amido black concerns calibration.

Analysis of Individual Proteins

Three techniques – electrophoresis, liquid chromatography, and immunochemistry – are well adapted for the identification of individual milk proteins. Nowadays, they also remain practically the only methods available for quantitative measurement of the individual proteins, even though none is entirely satisfactory and they are more appropriate for giving relative rather than absolute concentrations. Immunochemistry is particularly suitable for the detection and quantification of proteins at low concentration. The preparation of pure proteins that serve as references for identification and quantification is critical, because their secondary and tertiary structures may be modified and influence the measurement. Improvements in the chromatographic and electrophoretic protocols tend to achieve casein and whey protein separation simultaneously from skim milk. However, when this is not possible, the caseins and whey proteins can be separated preliminarily by precipitation of the caseins at pH 4.6 or by rennet action, ultrafiltration, or ultracentrifugation. For simplicity, examples of electrophoretic and chromatographic separation are limited to bovine milk.

Electrophoresis

Proteins can be separated using a vertical or horizontal slab gel system or within a capillary by various electrokinetic techniques, using the differences in one or a combination of the following: electric charge, isoelectric point, hydrophobicity, and mass ratio.

In slab gel electrophoresis, after migration, the proteins appear as more or less well-separated bands after being fixed by acid or alcohol precipitation and stained. The analytical performance of the quantitative measurements made by densitometry is greatly influenced by resolution between the bands, by the variability in the dye-binding capacity from one protein to another and from one run to another, and by the linearity and sensitivity of the dye, which increase dramatically in the following order: amido black < Coomassie blue < silver stains. The regression coefficient must be determined for each set of analytical conditions. The availability of ultrathin precast minigels with automated electrophoretic equipment, allowing shorter run times, has greatly improved the sensitivity, resolution, and speed of slab gel electrophoretic methods for milk proteins. In capillary electrophoresis, the eluting species pass in front of the spectrophotometer, and the intensity of absorption is registered immediately in return for correction of the velocity.

Polyacrylamide gel electrophoresis

In polyacrylamide gel electrophoresis (PAGE), proteins migrate mainly according to their charge-to-mass ratio. Alkaline conditions, where the proteins are negatively charged, are the most popular protocols for milk proteins. Caseins are better separated in the presence of urea, which dissociates intermolecular aggregates, and in the presence of a reducing agent (dithiothreitol (DTT)), which disrupts the disulfide bonds of α_{s2}- and κ-casein. In addition, the use of polyacrylamide in a gradient from 8 to 12.5% improves the simultaneous analysis of caseins and whey proteins, including some genetic variants and with different degrees of phosphorylation. However, γ_1- and γ_3-casein are not well resolved from κ-casein. The elution order in terms of increasing mobility is BSA, κ-casein and γ-casein, β-casein, β-lactoglobulin, α_{s2}-casein (two bands), α_{s1}-casein, and α-lactalbumin. Whey proteins appear as more diffused bands than the caseins, which affects their quantification.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Proteins are able to bind the anionic detergent sodium dodecyl sulfate (SDS) through hydrophobic interaction in the theoretical ratio $1.4\,\mathrm{g\,SDS\,g^{-1}}$ protein. Under these conditions, the charge-to-mass ratio is constant for each protein, which migrates according to its decreasing mass due to the sieving effect of the gel. However, the binding capacity of milk proteins differs noticeably from the theoretical value for each protein, which means that the molecular mass with reference to the protein standard of known molecular mass cannot be determined. Nevertheless, this method, together with a polyacrylamide gradient, has been used successfully to quantify in a single gel the main proteins in milk (**Figure 5**). Due to the shape of the bands and the somewhat poor resolution, quantification is more variable than for urea PAGE. Genetic variants are not resolved.

Isoelectrofocusing polyacrylamide gel electrophoresis

Separating proteins according to their isoelectric point appears to be the best method for identifying most of the non-silent genetic variants of the caseins (especially β-casein) and whey proteins. It is used routinely for the phenotyping of milks. The proteins migrate in a gel containing ampholytes until they meet the zone of the gel corresponding to their isoelectric pH, at which they precipitate. In combination with urea and a reducing agent, caseins are separated in the decreasing order of their isoelectric pH: γ-casein, κ-casein (A, B, C), α_{s2}-casein (A, D), β-casein (A^1, A^2, A^3, B, C), α_{s1}-casein (A, B, C). Whey proteins are separated without urea in the following order: α-lactalbumin B, β-lactoglobulin (A, B). This protocol allows the analysis of caseins and whey proteins together but with no differentiation of α-lactalbumin B

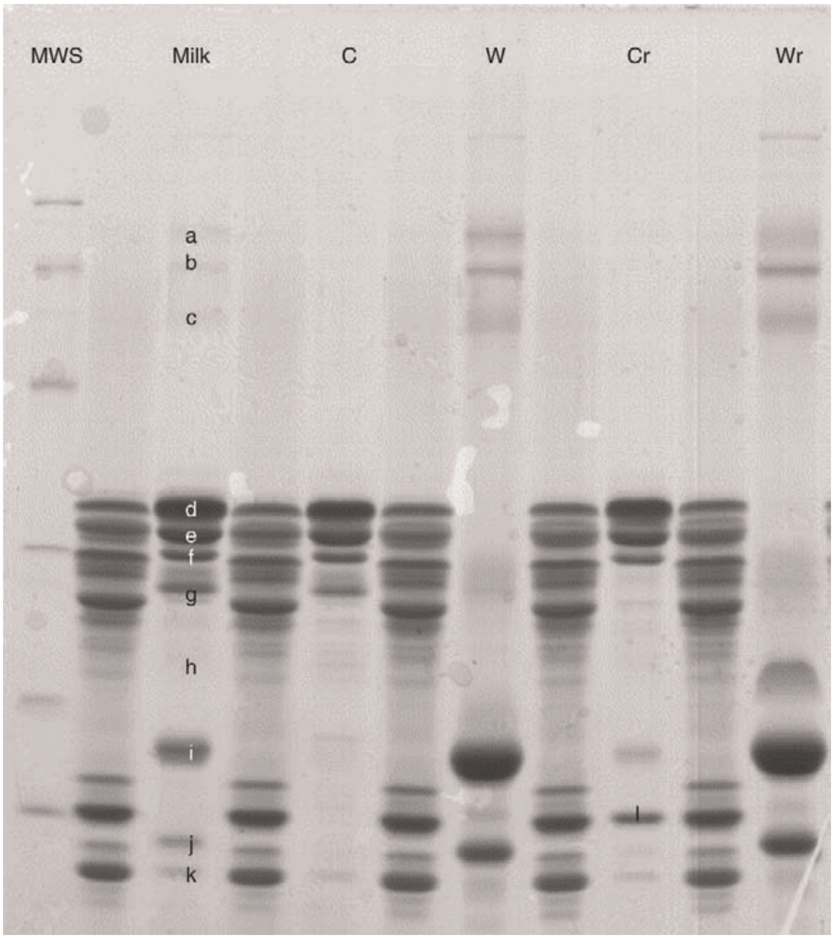

Figure 5 Bovine milk protein analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). MWS, molecular weight standard; C, caseins precipitated at pH 4.6; W, whey proteins soluble at pH 4.6; Cr, *para*-casein precipitated by rennet hydrolysis; Wr, whey proteins soluble after rennet hydrolysis; a, lactoferrin; b, serum albumin; c, immunoglobulin G; d, α_{s2}-casein; e, α_{s1}-casein; f, β-casein; g, κ-casein; h, γ_1-casein; i, β-lactoglobulin; j, α-lactalbumin; k, ($\gamma_2 + \gamma_3$)-casein; l, *para*-κ-casein.

and β-lactoglobulin A. Isoelectrofocusing (IEF) remains above all a qualitative method.

Two-dimensional electrophoresis

Using a combination of IEF (gel rod, first dimension) and PAGE (slab gel, second dimension), with or without SDS or urea, this high-resolution technique has been applied successfully to identify genetic polymorphism and to study specific protein fractions in complex mixtures. Due to the shape of the spots, a sophisticated image analyzer is needed for quantitation.

Capillary electrophoresis

In capillary electrophoresis (CE), elution is performed in a capillary instead of a slab gel. Resolution results from the combined effects of the presence and nature of a coating on the capillary and the characteristics of the eluting buffer (pH, ionic strength, modifiers), which determine the importance of the endoelectroosmotic flow and electrophoretic mobility of the molecule. Currently, the most competitive simultaneous separation of milk proteins is obtained with a hydrophilically coated capillary, an acidic citrate-eluting buffer containing urea, a reducing agent DTT, a polymeric additive methyl-2-hydroxyethyl cellulose (MHEC), and a sample buffer including Tris, DTT, 3-morpholinopropanesulfonic acid (MOPS) and urea at pH 8. Within 60 min, the proteins elute in the following order: α-lactalbumin, β-lactoglobulin, α_{s2}-casein (A, D), α_{s1}-casein (B, C), κ-casein, β-casein (A^1, A^2, A^3, B, C). The A and B variants of β-lactoglobulin and of κ-casein cannot be separated except when using highly dedicated protocols. Advantages of CE over slab gel electrophoretic methods include higher sensibility in terms of detection (fluorimetry, mass spectrometry), possible separation of uncharged silent variants or oppositely charged molecules (*para*-κ), greater automation, and smaller quantity of the sample. However, good reproducibility depends markedly on the sophistication of sample preparation and capillary cleaning protocol between injections.

Column Liquid Chromatography

Ion exchange and reverse-phase chromatography have been used extensively for the analytical separation of milk proteins. These techniques are complementary to gel filtration and hydrophobic interaction chromatography, which are particularly suitable for preparative analysis.

Ion-exchange chromatography

Separation of proteins by ion-exchange chromatography (IEC) is obtained because of their capacity to bind to a resin exchanger and to be separated with an NaCl gradient buffer. Milk proteins cannot be satisfactorily analyzed simultaneously. The Mono Q and TSK-DEAE-5PW anion exchange columns have been reported to separate the four caseins, and some of their genetic variants, and also according to their degree of phosphorylation. Separation of whey proteins (α-lactalbumin, serum albumin, β-lactoglobulin A and B) is performed on a Mono Q column. The cation exchange Mono S column is used mainly to separate minor proteins in milk or for some particular separation, such as β-casein A^1, A^2, and B variants.

Reverse-phase chromatography

This technique is based upon hydrophobic interaction between a stationary phase made of C_{18} (or C_8 or C_4)-bonded silica or polystyrene–divinylbenzene and the proteins in an aqueous solution of low ionic strength. Elution is obtained by increasing the hydrophobicity of the 0.01% trifluoroacetic acid (TFA) in the water mobile phase by increasing its content in acetonitrile. Until recently, an excellent separation of caseins could be obtained only in the absence of whey proteins. The caseins, dispersed in a citrate buffer containing urea and DTT, are separated in the following order: glycosylated κ-casein, κ-casein (A, B), α_{s2}-casein A, α_{s1}-casein (A, B + C, D), β-casein (C + A^1, A^2, A^3). With a recent modification of the sample buffer where urea was replaced by guanidine hydrochloride, simultaneous separation and quantification of caseins and whey proteins, including most of their genetic variants, were obtained. The latest developments include the use of two-dimensional (2D) chromatography, mass or derivative UV spectra, or dynamic surface tension detection.

Immunochemistry

In spite of their interesting features, such as high specificity and sensitivity, the performance of immunological methods is usually considered to be inadequate technically, commercially, and analytically, as compared with other techniques, for routine use for the quantitative analysis of milk proteins. For instance, their reproducibility ranges between 2 and 10% and, despite their theoretical specificity, their exactness is sometimes questionable. It has been shown, for example, for the measurement of immunoglobulin G, that systematic biases exist between methods, and that most discrepancies may occur with respect to the origin of antigens and antibodies used to construct the standard curves and to make measurements. On the other hand, these techniques are considered to be quite suitable for the analysis of individual proteins, either in the field of research or for clinical and regulatory purposes.

Practically all kinds of immunological technique, using either mono- or polyclonal antibodies, were developed to measure the proteins in milk (**Table 4**). Because milk is an extremely complex biological fluid, the development

Table 4 Examples of immunological methods used for quantitative measurement of proteins in milk

Performance					
Method	*Antibody*	*Proteins*	*Working range or limit of detection*	*Between-assay reproducibility (%)*	*Other applications*
MENI	pAb	α_s-Casein	4.0–34.3 g l^{-1}	1.9–3.4	
		κ-Casein	1.3–3.4 g l^{-1}	1.9–3.4	Whey curd
		β-Lg, α-La	0.6–2.0 g l^{-1}	2–5	
		IgG	>8 µg l^{-1}	2.3–10.0	
Competitive ELISA	mAb	β-Lg	0.03–2.0 ng ml^{-1}	<11	Heat treatment (semi-qualitative) cow in goat (0.03%)
Sandwich ELISA	pAb	β-Lg	>0.02 µg l^{-1}		Cow in human
Inhibition ELISA	mAb	α-La	10–500 ng ml^{-1}	11	Heat treatment
Competitive ELISA	pAb	α-La	20–1600 ng ml^{-1}	2.6–6.4	Heat treatment
RID	pAb	IgG_1	0.2–4 mg ml^{-1}	5.1	Semi-automated detection of abnormal milks
Sandwich ELISA	mAb	Plasmin Plasminogen	2–100 ng ml^{-1}	13	

MENI, microparticle-enhanced nephelometric immunoassay; ELISA, enzyme-linked immunosorbent assay; RID, radical immunodiffusion; pAb, polyclonal antibody; mAb, monoclonal antibody; β-Lg, β-lactoglobulin; α-La, α-lactalbumin; IgG, immunoglobulin G.

of accurate and easy-to-perform immunoassays has always been difficult.

Caseins

Because of their micellar structure, which interferes with the antigen–antibody reaction, and their great susceptibility to hydrolysis by indigenous proteases such as plasmin or cathepsin D, accurate and straight-forward quantitative measurement of caseins in milk is difficult to achieve. Microparticle-enhanced nephelometric immunoassay (MENI) has been applied to a large number of samples (1300 herd milks) for the measurement of α_{s1}-, β-, and κ-caseins. This technique has the advantage that no sample pre-treatment is required. However, it has several drawbacks, such as under-estimation of results, a high limit of detection (1 µg ml^{-1}), and interference of medium turbidity. More recently, an immunosensor allowing the simultaneous quantification of α_{s1}-, β-, and κ-caseins in milk has been developed. This assay consists of a two-step sandwich strategy, with two monoclonal antibodies directed against the N- and C-terminal extremities of each of the caseins, respectively. This strategy permits only intact caseins to be quantified, and not their degradation products. The technique is fast (10 min $sample^{-1}$), automated, and has been applied successfully to raw and drinking milks and cheeses.

Besides this method, very few other techniques have been developed. Rocket immunoelectrophoresis with a monospecific polyclonal antibody was used to quantify human β-casein but hydrolyzed β-casein was also recognized. A specific monoclonal antibody was produced and used to detect by radioimmunoassay as little as 0.3×10^{-4} mmol ml^{-1} of κ-casein in solution, but this method was not applied to milk.

Because of the significant differences in amino acid sequences, caseins (often β-casein) are excellent markers for the development of immunoassays (enzyme-linked immunoassay (ELISA) or immunoblotting) that allow the detection of usually below 1% of a milk added fraudulently to the milk of a different species, particularly cow's milk in goat's or ewe's milk.

Whey and minor proteins

In contrast to caseins, whey proteins have good antigenic properties, and are not usually hydrolyzed in raw milk. However, during heat treatment, they are susceptible to conformational modifications and to binding to other proteins, and therefore to antigen–antibody reaction changes.

In fact, advantage has been taken of this property to produce antibodies able to detect, for instance, native or denatured α-lactalbumin or β-lactoglobulin, and to develop immunoassays for the control of the intensity of the heat treatment of milk.

Since milk proteins are major food allergens, immunoassays allowing the detection of traces of these proteins in complex food matrices have seen important developments in recent years. Most of the commercially available kits correspond to ELISA-type techniques using monoclonal or, more usually, polyclonal antibodies as ligand. They allow a quick quantification of milk proteins in the milligram-per-kilogram range. Extraction of the proteins prior to detection may be required in processed food.

In relation to the presence of psychrotrophic bacteria in milk, particularly of *Pseudomonas* spp., which can produce heat-resistant proteinases responsible for the gelation of UHT milk, qualitative ELISA and MENI were developed to measure the caseinomacropeptide released from the hydrolysis of κ-casein.

Milk hormones, such as prolactin and somatotrophin, which can be present in milk, can also be measured by immunoassays (radioimmunoassay and sandwich ELISA).

Future Trends

Recently, progress has been made in the differential quantification of casein and whey proteins using infrared spectroscopy, and it is probable that separation of these fractions from other milk proteins will no longer be necessary for quantification. Therefore, it can be stated that infrared techniques will find growing applications in the dairy industry.

Immunological techniques will certainly be used more frequently in the near future, especially for applications where their sensitivity and specificity make them unavoidable (minor components, enzymes, hormones). Biosensors have been developed recently for the analysis of dairy products. They are defined as analytical tools or systems consisting of an immobilized biological material in intimate contact with a suitable transducer device that will convert the biochemical signal into a quantifiable electrical signal. Thus, they retain the specificity of biomolecules with the possibility of quantitative or qualitative detection of the biomolecule–analyte interaction by an appropriate, sensitive transducer. They are fast, reliable, sensitive, and sometimes automated. Some consist of electrodes that can make *in situ* measurements.

Finally, liquid chromatography is a powerful tool, especially when coupled with mass spectrometry, which allows the molecular mass of the molecules separated on the column to be determined. With this technique it has been possible to make major advances in the identification of proteins (including some genetic and post-translational variants) in milk and dairy products, interaction between them, and the effects of treatment (heat, photooxidation, etc.), and in understanding complex biochemical pathways such as proteolysis during cheese ripening. Capillary electrophoresis is now competing with liquid chromatography. It is now routinely used for the quantification of proteins (whey proteins in particular) in dairy products. But the challenge remains for both LC and CE to better recover proteins and to be able to quantify the modifications induced by milk treatments, heat in particular.

See also: **Analytical Methods**: Atomic Spectrometric Techniques; Chromatographic Methods; Electrophoresis; Immunochemical Methods; Infrared Spectroscopy in Dairy Analysis; Mass Spectrometric Methods; Nuclear Magnetic Resonance: Principles. **Milk Proteins**: Heterogeneity, Fractionation, and Isolation.

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Heterogeneity, Fractionation, and Isolation

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Introduction

Milk from approximately 150 of the estimated 4000 mammalian species has been analyzed with different degrees of detail and in all cases this secretion has been shown to contain a protein component that varies from 1% in human to >20% in rabbit milk. Berzelius in 1814 described the first method for the separation of casein, the major protein component of cow's milk. With the development of more sophisticated analytical techniques over the years, more than 200 types of protein have been characterized in bovine milk. The proteins in cow's milk are the most widely studied in terms of their isolation, characterization, structural properties, functions, and biosynthetic pathways. Hence, unless otherwise stated, the term 'milk proteins' in this article refers to the bovine species.

Milk protein is a very heterogeneous group of molecules, and for ease of description, could be classified into five main categories: caseins, whey proteins, milk fat globule membrane proteins, enzymes, and other miscellaneous minor proteins. Along with several factors that contribute to the heterogeneity of milk proteins, this article will focus on methods used for isolation, molecular structure, degree of post-translational modification, self-association and association between different types of protein, differences in the amounts and relative proportions of individual proteins, origin of the proteins, diversity of functions, and the presence of homologues across species. The heterogeneity of milk proteins is further complicated by the presence of genetic variants that have been identified in several species apart from the bovine. With the developments in molecular biology and the improvements in cloning techniques, it is possible to increase further the heterogeneity of milk proteins by site-directed mutagenesis, controlling the levels of expression of proteins indigenous to milk and of novel proteins that are foreign to the mammary gland.

The heterogeneity of milk proteins has contributed to difficulties associated with their fractionation and isolation, and as we acquire more information about the macromolecules involved, these problems are being overcome. For more than 50 years, it was thought that the casein fraction prepared by Hammarsten in 1883 was a pure protein. The application of moving boundary electrophoresis by Mellander in 1939 demonstrated the heterogeneity of the casein fraction with three electrophoretic components denoted as α-, β-, and γ-. Present-day knowledge confirms that each of the three components represents more than one protein. The primary structure and gene sequence of the four caseins (α_{s1}-, α_{s2}-, β-, and κ-) and three of the whey proteins (β-lactoglobulin, α-lactalbumin, and bovine serum albumin) are now established. The fractionation and isolation of protein components depend on the intrinsic physicochemical properties of the individual proteins. Because some of the proteins tend to self-associate or associate with other proteins, a denaturing reaction is required prior to fractionation. Techniques of ultracentrifugation, size-exclusion chromatography, ultrafiltration, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) could be adapted for the separation of certain proteins on the basis of molecular mass differences. Variability in net electrical charges; sensitivity to ions, for example, calcium; and solubility in the presence of denaturing agents, for example, urea could be exploited for the isolation of some proteins by precipitation with different concentrations of salt (ammonium sulfate, calcium chloride) and solutions of alcohol under different pH and temperature conditions. Based on the charge and ionic strength properties of the proteins, several chromatographic and electrophoretic procedures have been developed for their fractionation and isolation. The most appropriate procedures for the isolation of milk proteins will depend on the level of purity and the amount (analytical, preparative, industrial) of protein required.

For a better appreciation of this article, some familiarity with the nomenclature used for the milk protein system, as proposed by a committee of the American Dairy Science Association is recommended.

Classification and Nomenclature of Milk Proteins

When raw skim milk is adjusted to pH 4.6, a precipitate containing approximately 80% of the protein of cow's milk is formed. This precipitation procedure has been used as the basis for the classification of milk proteins into two main groups, with casein in the precipitate and whey protein (non-casein protein) in the supernatant. **Table 1** gives a summary of some of the characteristics of the four types of caseins and the four major proteins in the whey protein fraction. The primary structure (amino acid sequence) and the gene sequence of the four caseins,

Table 1 Characteristics of the major proteins in cow's milk

		Number of AA residues						
Protein	*Molecular mass[a] (Da)*	*Total*	*Pro*	*Cys*	*Number of PO_4*	*Presence of CH_2O*	*Concentration (g l^{-1})*	*Genetic variants detected*
α_{s1}-Casein	23 164	199	17	0	8	0	10	A,**B**,C,D,E,F,G,H
α_{s2}-Casein	25 388	207	10	2	10–13	0	2.6	**A**,B,C,D
β-Casein	23 983	209	35	0	5	0	9.3	A^1,**A^2**,A^3,B,C,D,E,F,G
κ-Casein	19 038	169	20	2	1	+	3.3	**A**,B,C,E,F^s,F^{l}G^S,G^E,H,I,J
β-Lactoglobulin	18 277	162	8	5	0	0	3.2	A,**B**,C,D,E,F,H,I,J
α-Lactalbumin	14 175	123	2	8	0	0	1.2	A,**B**,C
Serum albumin	66 267	582	28	35	0	0	0.4	
Immunoglobulin	143 000–1 030 000		8.4%	2.3%	-	+	0.8	

[a] Molecular mass are for the genetic variants in bold.

ranging in molecular mass from 19 038 for κ-casein to 25 388 for α_{s2}-casein, have been established. Among the major whey proteins, α-lactalbumin is the smallest (MW 14 175 Da) and the immunoglobulins are the largest (MW 143 000–1 030 000 Da). The complete amino acid sequence of β-lactoglobulin, α-lactalbumin, and serum albumin, with molecular masses of 18 277, 14 175, and 66 267 Da, respectively, are also known. Unlike β-lactoglobulin and α-lactalbumin, serum albumin and some of the immunoglobulins are not synthesized in the mammary gland. The immunoglobulins are extremely heterogeneous and their identity is based on immunochemical properties. Five classes of immunoglobulins (IgG, IgA, IgM, IgE, and IgD) have been identified in bovine milk. Their basic structure is similar to other immunoglobulins in having two heavy and two light polypeptide chains covalently linked by disulfide bonds. The molecular mass varies from 50 000 to 70 000 Da for the heavy chains depending on the type and about 25 000 Da for the light chains. In addition to differences in the organizational structure, there are also differences in amino acid sequences and carbohydrate groups present in the immunoglobulin molecules. More than 60 enzymes have been identified in milk and, as a group, they account for less than 1% of the total milk protein. Enzymes are heterogeneously distributed in milk, for example, catalase, lactoperoxidase, ribonuclease, and glucosaminidase are found mainly in the whey fraction while proteinases and lipase are associated with the casein micelles, and xanthine oxidase is found in the milk fat globule membrane. The fat globules of milk are stabilized by a complex membrane consisting of proteins and phospholipids; these proteins are designated as milk fat globule membrane proteins. The major proteins of the milk fat globule membrane were reviewed recently. Proteins that do not fall into the classification of caseins, major whey proteins, enzymes, and milk fat globule membrane proteins are categorized as miscellaneous minor proteins and include transferrin, lactoferrin, ceruloplasmin, lactollin, glycoprotein-a, kininogen, M-1 glycoprotein, epidermal growth factor, glycolactin, angiogenin, etc. A wide range of biological functions have been assigned to those minor proteins present at concentrations in the mg kg^{-1} range.

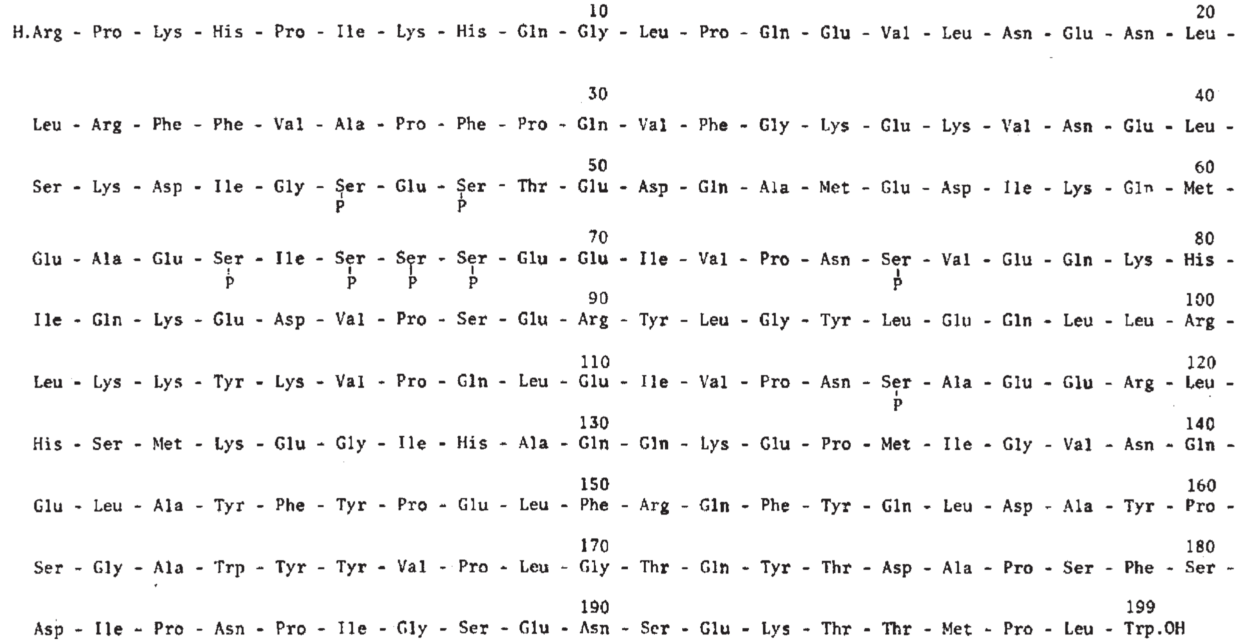
Heterogeneity of Milk Proteins

In addition to the high degree of heterogeneity that exists between the five different classes of milk proteins, there is also heterogeneity among proteins within each class. This is to be expected when one considers that milk proteins range from 10 000 to >1 000 000 Da in molecular mass and have different amino acid compositions and sequences that ultimately determine the structures and physicochemical properties of the molecules. The degree of post-translational modification (proteolysis, phosphorylation, glycosylation, formation of disulfide bridges) and the existence of genetic variants contribute further to the observed heterogeneity.

Caseins

Bovine milk contains four types of caseins denoted as α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein, each of which is the product of specific genes. However, PAGE of a whole casein preparation could resolve in excess of 20 protein bands. Several of the electrophoretic bands represent post-translational products of one of the four caseins (see below).

The caseins, which are synthesized in the mammary gland, are proteins containing ester-bound phosphate and due to their relatively high content of proline (see **Table 1**), they tend to have very little secondary structure. The primary structure of α_{s1}-casein, containing 199 amino acid residues, is shown in **Figure 1**. It has no cysteine residue and eight phosphates attached to serines. A minor



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                                                        10                                                          20
H.Arg - Pro - Lys - His - Pro - Ile - Lys - His - Gln - Gly - Leu - Pro - Gln - Glu - Val - Leu - Asn - Glu - Asn - Leu -

                                                        30                                                          40
  Leu - Arg - Phe - Phe - Val - Ala - Pro - Phe - Pro - Gln - Val - Phe - Gly - Lys - Glu - Lys - Val - Asn - Glu - Leu -

                                                        50                                                          60
  Ser - Lys - Asp - Ile - Gly - Ser - Glu - Ser - Thr - Glu - Asp - Gln - Ala - Met - Glu - Asp - Ile - Lys - Gln - Met -
                                 P           P
                                                        70                                                          80
  Glu - Ala - Glu - Ser - Ile - Ser - Ser - Ser - Glu - Glu - Ile - Val - Pro - Asn - Ser - Val - Glu - Gln - Lys - His -
                     P           P     P     P                                         P
                                                        90                                                          100
  Ile - Gln - Lys - Glu - Asp - Val - Pro - Ser - Glu - Arg - Tyr - Leu - Gly - Tyr - Leu - Glu - Gln - Leu - Leu - Arg -

                                                        110                                                         120
  Leu - Lys - Lys - Tyr - Lys - Val - Pro - Gln - Leu - Glu - Ile - Val - Pro - Asn - Ser - Ala - Glu - Glu - Arg - Leu -
                                                                                       P
                                                        130                                                         140
  His - Ser - Met - Lys - Glu - Gly - Ile - His - Ala - Gln - Gln - Lys - Glu - Pro - Met - Ile - Gly - Val - Asn - Gln -

                                                        150                                                         160
  Glu - Leu - Ala - Tyr - Phe - Tyr - Pro - Glu - Leu - Phe - Arg - Gln - Phe - Tyr - Gln - Leu - Asp - Ala - Tyr - Pro -

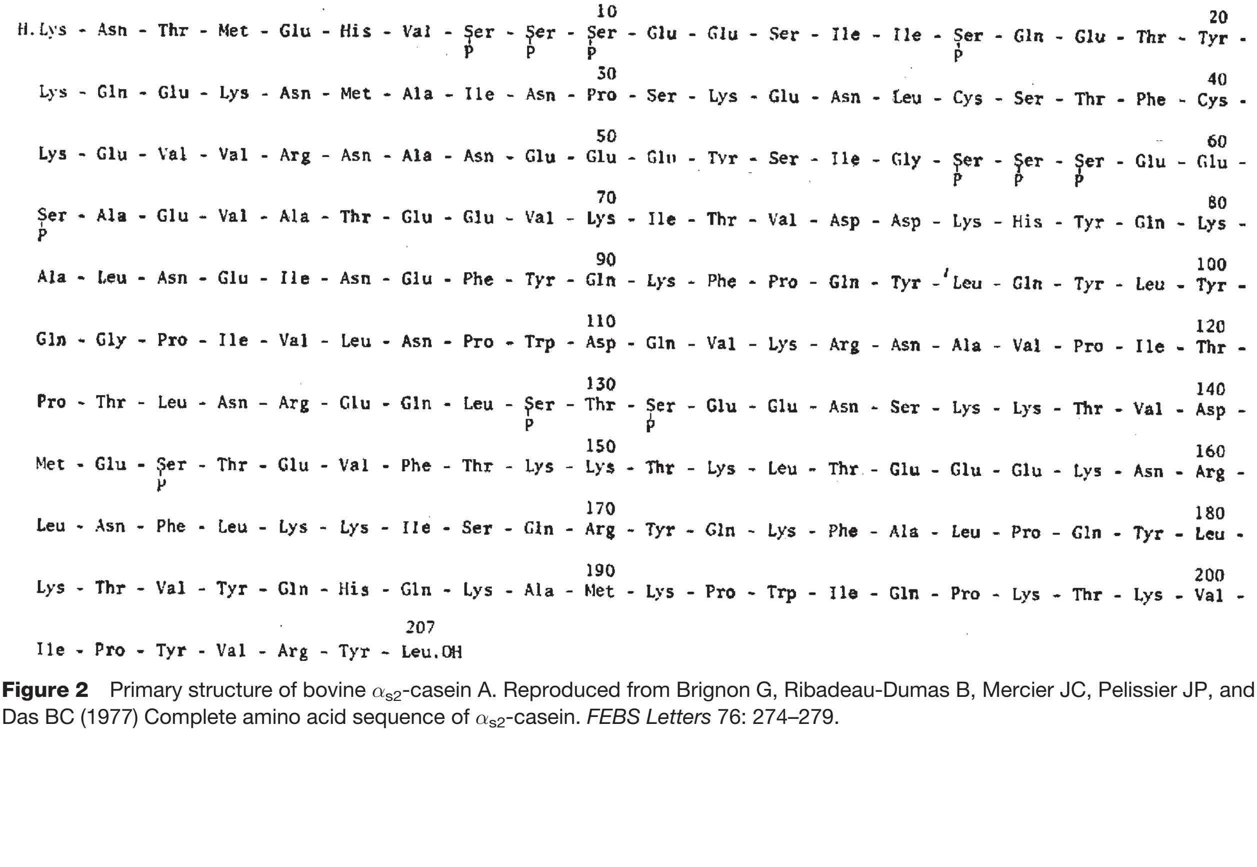
                                                        170                                                         180
  Ser - Gly - Ala - Trp - Tyr - Tyr - Val - Pro - Leu - Gly - Thr - Gln - Tyr - Thr - Asp - Ala - Pro - Ser - Phe - Ser -

                                                        190                                                         199
  Asp - Ile - Pro - Asn - Pro - Ile - Gly - Ser - Glu - Asn - Ser - Glu - Lys - Thr - Thr - Met - Pro - Leu - Trp.OH
```

Figure 1 Primary structure of bovine α_{s1}-casein B. Reproduced from Mercier JC, Grosclaude F, and Ribadeau-Dumas B (1971) Structure Primaire de la caséine α_{s1}-bovine. *European Journal of Biochemistry* 23: 41–51.

α_{s1}-casein with a faster electrophoretic mobility, denoted earlier as α_{s0}-casein, has been characterized and is slightly different from the former by having nine phosphorylated serines and hence contains one extra negative charge at alkaline pH. There are three hydrophobic regions, residues 1–44, 90–113, and 132–199. The sequence of residues 41–80 is very polar due to the presence of seven seryl phosphates, eight glutamates, and three aspartates.

As shown in **Figure 2**, α_{s2}-casein contains 207 amino acids. It has 10 prolines, more phosphoserines (see **Table 1**) and more lysines than the other caseins, and two cysteines at positions 36 and 40. Several forms of



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                                                        10                                                          20
H.Lys - Asn - Thr - Met - Glu - His - Val - Ser - Ser - Ser - Glu - Glu - Ser - Ile - Ile - Ser - Gln - Glu - Thr - Tyr -
                                             P     P     P                                   P
                                                        30                                                          40
  Lys - Gln - Glu - Lys - Asn - Met - Ala - Ile - Asn - Pro - Ser - Lys - Glu - Asn - Leu - Cys - Ser - Thr - Phe - Cys -

                                                        50                                                          60
  Lys - Glu - Val - Val - Arg - Asn - Ala - Asn - Glu - Glu - Glu - Tyr - Ser - Ile - Gly - Ser - Ser - Ser - Glu - Glu -
                                                                                             P     P     P
                                                        70                                                          80
  Ser - Ala - Glu - Val - Ala - Thr - Glu - Glu - Val - Lys - Ile - Thr - Val - Asp - Asp - Lys - His - Tyr - Gln - Lys -
   P
                                                        90                                                          100
  Ala - Leu - Asn - Glu - Ile - Asn - Glu - Phe - Tyr - Gln - Lys - Phe - Pro - Gln - Tyr - Leu - Gln - Tyr - Leu - Tyr -

                                                        110                                                         120
  Gln - Gly - Pro - Ile - Val - Leu - Asn - Pro - Trp - Asp - Gln - Val - Lys - Arg - Asn - Ala - Val - Pro - Ile - Thr -

                                                        130                                                         140
  Pro - Thr - Leu - Asn - Arg - Glu - Gln - Leu - Ser - Thr - Ser - Glu - Glu - Asn - Ser - Lys - Lys - Thr - Val - Asp -
                                                   P           P
                                                        150                                                         160
  Met - Glu - Ser - Thr - Glu - Val - Phe - Thr - Lys - Lys - Thr - Lys - Leu - Thr - Glu - Glu - Glu - Lys - Asn - Arg -
               P
                                                        170                                                         180
  Leu - Asn - Phe - Leu - Lys - Lys - Ile - Ser - Gln - Arg - Tyr - Gln - Lys - Phe - Ala - Leu - Pro - Gln - Tyr - Leu -

                                                        190                                                         200
  Lys - Thr - Val - Tyr - Gln - His - Gln - Lys - Ala - Met - Lys - Pro - Trp - Ile - Gln - Pro - Lys - Thr - Lys - Val -

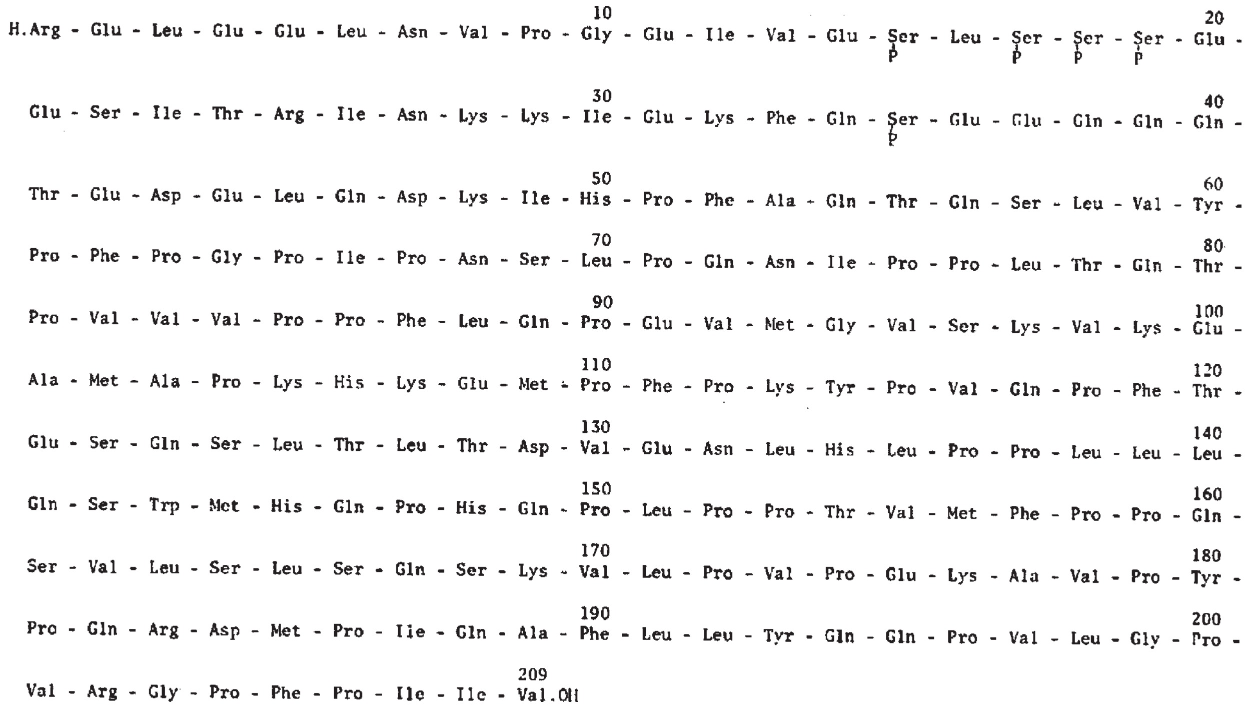
                                      207
  Ile - Pro - Tyr - Val - Arg - Tyr - Leu.OH
```

Figure 2 Primary structure of bovine α_{s2}-casein A. Reproduced from Brignon G, Ribadeau-Dumas B, Mercier JC, Pelissier JP, and Das BC (1977) Complete amino acid sequence of α_{s2}-casein. *FEBS Letters* 76: 274–279.

α_{s2}-casein are discernable by PAGE due to different degrees of phosphorylation, which range from 10 to 13 phosphate groups. These forms have been identified as α_{s2}-, α_{s3}-, α_{s4}-, α_{s5}-, and α_{s6}-casein (α_{s5}- is a dimer of α_{s3}- and α_{s4}-). The 13 phosphates (12 on serine, 1 on threonine) are located in three regions of the molecule: residues 7–31, 55–66, and 129–143. Among the caseins, α_{s2}-casein is the least hydrophobic with regions of hydrophobicity located at residues 90–120 and 160–207.

The sequence of the 209 amino acids in β-casein is shown in **Figure 3**. It is the most hydrophobic casein, it has no cysteine, and it has a high proportion of proline (35 residues), which has a profound effect on its structure. At milk pH, the N-terminal 21-residue segment is highly negatively charged while the rest of the molecule, which is very hydrophobic, has no net charge. The amphipathic nature of β-casein is the reason why it forms micellar aggregates in solution. Human β-casein has been shown to consist of six proteins with an identical amino acid sequence, but with 0–5 phosphate groups attached to serine residues. The γ-caseins, which have been known for a long time, are actually the hydrolytic products of β-casein, produced by the action of plasmin. γ-Caseins, corresponding to residues 29–209, 106–209, and 108–209 of β-casein, are present in the precipitate formed on isoelectric precipitation of whole casein at pH 4.6. Other fragments of β-casein (residues 1–28, 1–105, and 1–107) are found in the whey and they constitute part of a fraction formerly known as 'proteose peptone'.

κ-Casein is the only protein of the casein family that is glycosylated. The different degrees of glycosylation, as revealed by up to seven bands during PAGE, are related to the number of negative charges on the *N*-acetylneuraminic acid residues. The primary structure of the carbohydrate-free portion of the 169 amino acid-containing protein is shown in **Figure 4**. κ-Casein is the target for chymosin and it is the most extensively studied milk protein. It stabilizes casein micelles against precipitation by calcium and loses this protective role when the Phe_{105}–Met_{106} bond is cleaved by enzymes to form two peptides: para-κ-casein (residues 1–105), which is the hydrophobic portion and precipitates with the casein micelles, and caseinomacropeptide (residues 106–169), which remains in solution due to its high polarity and high content of negative charges. The glycosyl moieties bind to the caseinomacropeptide by O-glycosidic linkages with threonine at position 131, 133, 135, or 142. The two cysteine residues of κ-casein are located at positions 11 and 88, and the serine residue at position 149, and sometimes at position 127, is phosphorylated. The amphipathic character of κ-casein encourages it to from micelles in solution. Unlike other caseins, κ-casein does not bind calcium extensively and is not sensitive to precipitation by Ca^{2+}.



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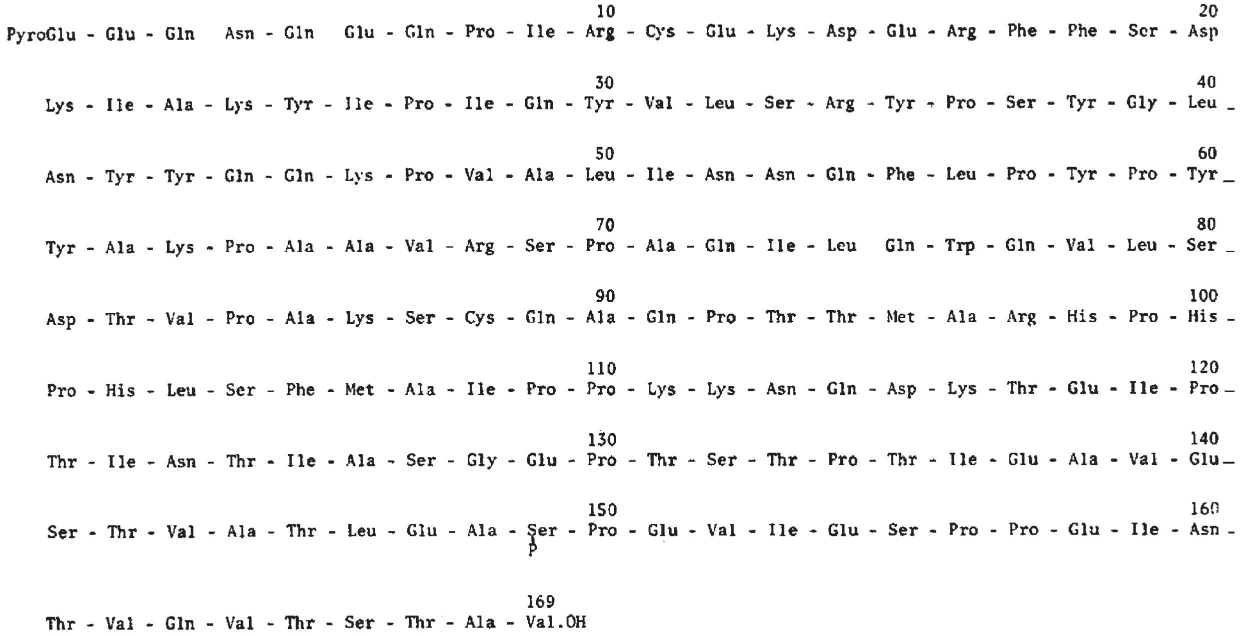
                                                            10                                                                      20
H.Arg - Glu - Leu - Glu - Glu - Leu - Asn - Val - Pro - Gly - Glu - Ile - Val - Glu - Ser - Leu - Ser - Ser - Ser - Glu -
                                                                                      P            P     P     P
                                                            30                                                                      40
Glu - Ser - Ile - Thr - Arg - Ile - Asn - Lys - Lys - Ile - Glu - Lys - Phe - Gln - Ser - Glu - Glu - Gln - Gln - Gln -
                                                                                    P
                                                            50                                                                      60
Thr - Glu - Asp - Glu - Leu - Gln - Asp - Lys - Ile - His - Pro - Phe - Ala - Gln - Thr - Gln - Ser - Leu - Val - Tyr -
                                                            70                                                                      80
Pro - Phe - Pro - Gly - Pro - Ile - Pro - Asn - Ser - Leu - Pro - Gln - Asn - Ile - Pro - Pro - Leu - Thr - Gln - Thr -
                                                            90                                                                     100
Pro - Val - Val - Val - Pro - Pro - Phe - Leu - Gln - Pro - Glu - Val - Met - Gly - Val - Ser - Lys - Val - Lys - Glu -
                                                           110                                                                     120
Ala - Met - Ala - Pro - Lys - His - Lys - Glu - Met - Pro - Phe - Pro - Lys - Tyr - Pro - Val - Gln - Pro - Phe - Thr -
                                                           130                                                                     140
Glu - Ser - Gln - Ser - Leu - Thr - Leu - Thr - Asp - Val - Glu - Asn - Leu - His - Leu - Pro - Pro - Leu - Leu - Leu -
                                                           150                                                                     160
Gln - Ser - Trp - Met - His - Gln - Pro - His - Gln - Pro - Leu - Pro - Pro - Thr - Val - Met - Phe - Pro - Pro - Gln -
                                                           170                                                                     180
Ser - Val - Leu - Ser - Leu - Ser - Gln - Ser - Lys - Val - Leu - Pro - Val - Pro - Glu - Lys - Ala - Val - Pro - Tyr -
                                                           190                                                                     200
Pro - Gln - Arg - Asp - Met - Pro - Ile - Gln - Ala - Phe - Leu - Leu - Tyr - Gln - Gln - Pro - Val - Leu - Gly - Pro -
                                                    209
Val - Arg - Gly - Pro - Phe - Pro - Ile - Ile - Val.OH
```

Figure 3 Primary structure of bovine β-casein A^2. Reproduced from Ribadeau-Dumas B, Brignon G, Grosclaude F, and Mercier JC (1972) Structure primaire de la caséine β bovine: séquence complète. *European Journal of Biochemistry* 25: 505–514.



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                                                     10                                                        20
PyroGlu - Glu - Gln   Asn - Gln   Glu - Gln - Pro - Ile - Arg - Cys - Glu - Lys - Asp - Glu - Arg - Phe - Phe - Ser - Asp

                                                     30                                                        40
   Lys - Ile - Ala - Lys - Tyr - Ile - Pro - Ile - Gln - Tyr - Val - Leu - Ser - Arg - Tyr - Pro - Ser - Tyr - Gly - Leu _

                                                     50                                                        60
   Asn - Tyr - Tyr - Gln - Gln - Lys - Pro - Val - Ala - Leu - Ile - Asn - Asn - Gln - Phe - Leu - Pro - Tyr - Pro - Tyr _

                                                     70                                                        80
   Tyr - Ala - Lys - Pro - Ala - Ala - Val - Arg - Ser - Pro - Ala - Gln - Ile - Leu   Gln - Trp - Gln - Val - Leu - Ser _

                                                     90                                                        100
   Asp - Thr - Val - Pro - Ala - Lys - Ser - Cys - Gln - Ala - Gln - Pro - Thr - Thr - Met - Ala - Arg - His - Pro - His -

                                                     110                                                       120
   Pro - His - Leu - Ser - Phe - Met - Ala - Ile - Pro - Pro - Lys - Lys - Asn - Gln - Asp - Lys - Thr - Glu - Ile - Pro -

                                                     130                                                       140
   Thr - Ile - Asn - Thr - Ile - Ala - Ser - Gly - Glu - Pro - Thr - Ser - Thr - Pro - Thr - Ile - Glu - Ala - Val - Glu -

                                                     150                                                       160
   Ser - Thr - Val - Ala - Thr - Leu - Glu - Ala - Ser - Pro - Glu - Val - Ile - Glu - Ser - Pro - Pro - Glu - Ile - Asn -
                                                   P

                                                     169
   Thr - Val - Gln - Val - Thr - Ser - Thr - Ala - Val.OH
```

Figure 4 Primary structure of bovine κ-casein B. Reproduced from Mercier JC, Brignon G, and Ribadeau-Dumas B (1973) Structure de la caséine κ B bovine: séquence complete. *European Journal of Biochemistry* 35: 222–235.

Heterogeneity of the caseins also arises from their interactions with one another and with other proteins and small ions. With the calcium phosphate present in milk, the caseins exist as micelles.

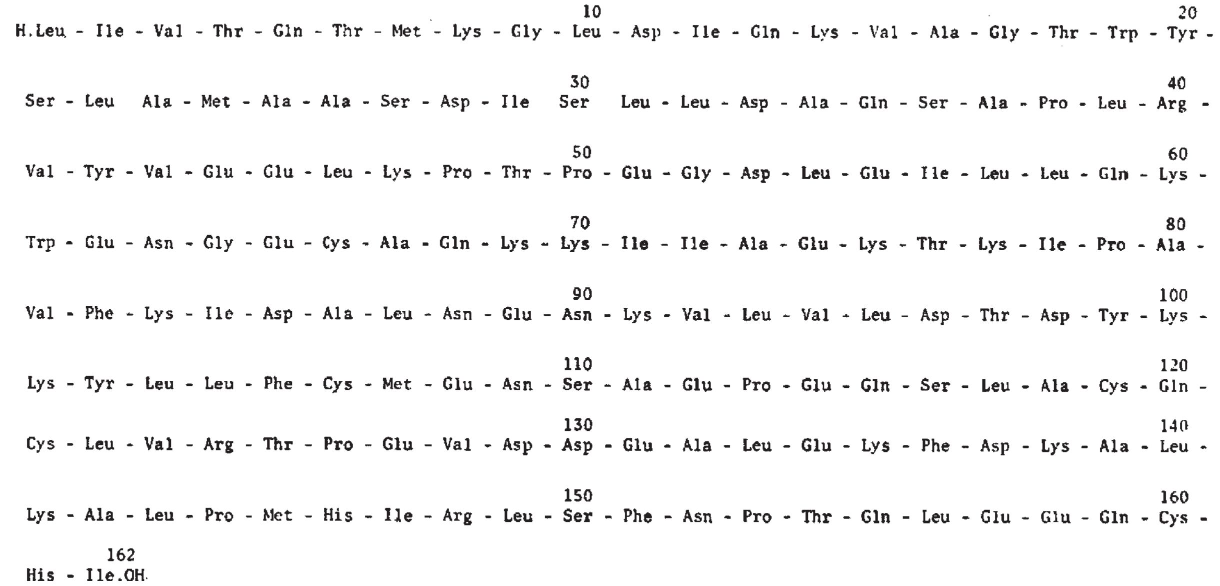
Whey Proteins

The whey protein fraction, which accounts for approximately 20% of total protein in bovine milk, comprises the non-casein proteins that remain soluble when caseins have been isoelectrically precipitated at pH 4.6. Whey proteins are even a more heterogeneous group of compounds than the caseins and share few common characteristics, other than being soluble under conditions that render the caseins insoluble. Unlike the caseins, which lack secondary structures, the whey proteins have more organized secondary and tertiary structures and most are globular proteins. Four major proteins, denoted as β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins, as shown in **Table 1**, account for >95% of the non-casein proteins. Both β-lactoglobulin and α-lactalbumin are synthesized in the mammary gland whereas serum albumin is transported to the mammary gland via the blood serum.

More than half of the whey protein is β-lactoglobulin; its primary structure of 162 amino acids is shown in **Figure 5**. It has five cysteine residues, capable of forming disulfide bonds between residues 66 and 160, 119 and 121, or 106 and 119. At pH 3 to 7, it self-associates to form dimers. α-Lactalbumin is the smallest of the major whey proteins, with a molecular mass of 14 175 Da. It plays a very important role in the biosynthesis of lactose by being the modifier in the lactose synthetase complex. The sequence of the 123 amino acids in α-lactalbumin is shown in **Figure 6**. All the eight cysteine residues in α-lactalbumin are connected by disulfide bridges between positions 6 and 120, 28 and 111, 61 and 77, and 73 and 91. Several studies indicate that α-lactalbumin binds calcium, phospholipids, and membranes. Minor forms of α-lactalbumin have been shown to contain a carbohydrate moiety consisting of mannose, galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid.

Milk serum albumin is physically and immunologically identical to blood plasma albumin. The sequence of the 582 amino acids in serum albumin is shown in **Figure 7**. All of the 35 cysteine residues (except Cys_{34}) form intra-chain disulfide bonds. The molecule is considered to have three major domains, each consisting of two large double loops and a small double loop, and assumes an ellipsoidal shape. The N-terminal region is more compact than the C-terminal region. There are different domains, varying in hydrophobicity, net charge, and ligand-binding sites. Isoelectric focusing patterns of serum albumin show evidence of considerable microheterogeneity of the protein.

The largest (MW >1 000 000 Da) and most heterogeneous of the major whey proteins belong to a group collectively known as the immunoglobulins, which have been classified as IgG, IgA, IgM, IgE, and IgD. They all exist either as monomers or as polymers of a basic unit made up of two light and two heavy chains, each containing about 200 and 450–600 amino acid residues,



```
                                              10                                                        20
H.Leu - Ile - Val - Thr - Gln - Thr - Met - Lys - Gly - Leu - Asp - Ile - Gln - Lys - Val - Ala - Gly - Thr - Trp - Tyr -

                                              30                                                        40
  Ser - Leu   Ala - Met - Ala - Ala - Ser - Asp - Ile   Ser   Leu - Leu - Asp - Ala - Gln - Ser - Ala - Pro - Leu - Arg -

                                              50                                                        60
  Val - Tyr - Val - Glu - Glu - Leu - Lys - Pro - Thr - Pro - Glu - Gly - Asp - Leu - Glu - Ile - Leu - Leu - Gln - Lys -

                                              70                                                        80
  Trp - Glu - Asn - Gly - Glu - Cys - Ala - Gln - Lys - Lys - Ile - Ile - Ala - Glu - Lys - Thr - Lys - Ile - Pro - Ala -

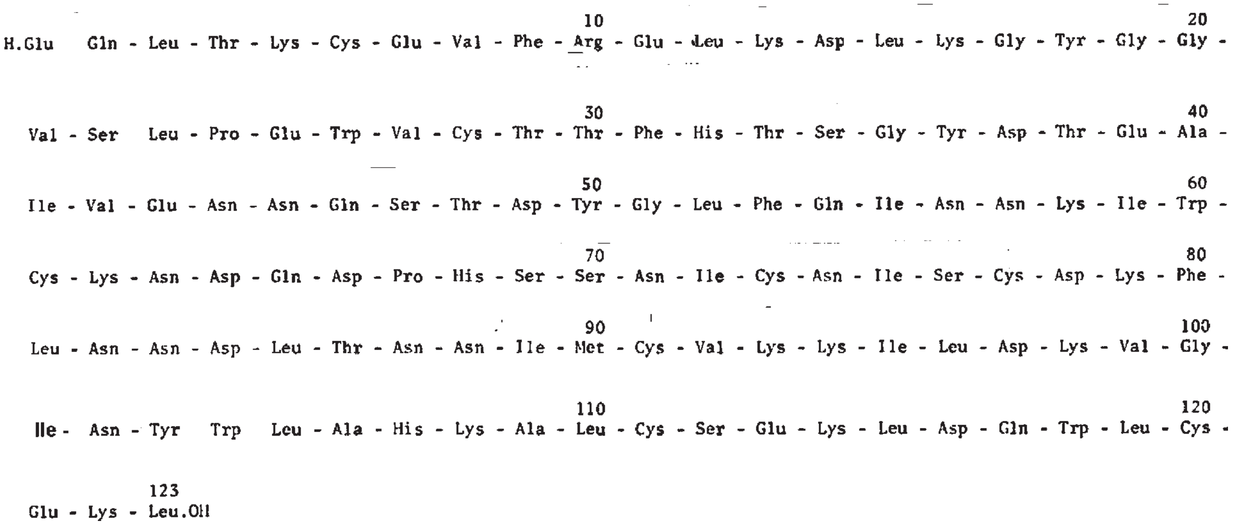
                                              90                                                        100
  Val - Phe - Lys - Ile - Asp - Ala - Leu - Asn - Glu - Asn - Lys - Val - Leu - Val - Leu - Asp - Thr - Asp - Tyr - Lys -

                                              110                                                       120
  Lys - Tyr - Leu - Leu - Phe - Cys - Met - Glu - Asn - Ser - Ala - Glu - Pro - Glu - Gln - Ser - Leu - Ala - Cys - Gln -
                                              130                                                       140
  Cys - Leu - Val - Arg - Thr - Pro - Glu - Val - Asp - Asp - Glu - Ala - Leu - Glu - Lys - Phe - Asp - Lys - Ala - Leu -

                                              150                                                       160
  Lys - Ala - Leu - Pro - Met - His - Ile - Arg - Leu - Ser - Phe - Asn - Pro - Thr - Gln - Leu - Glu - Glu - Gln - Cys -

         162
  His - Ile.OH
```

Figure 5 Primary structure of bovine β-lactoglobulin B. Reproduced Braunitzer G, Chen R, Schrank B, and Strangl A (1972) Automatische sequenzanalyse eines Proteins (β-lactoglobulin AB). *Hopper-Seyler's Zeitschrift für physiologische Chemie* 353: 832–834.



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                                              10                                                        20
H.Glu   Gln - Leu - Thr - Lys - Cys - Glu - Val - Phe - Arg - Glu - Leu - Lys - Asp - Leu - Lys - Gly - Tyr - Gly - Gly -

                                              30                                                        40
  Val - Ser   Leu - Pro - Glu - Trp - Val - Cys - Thr - Thr - Phe - His - Thr - Ser - Gly - Tyr - Asp - Thr - Glu - Ala -

                                              50                                                        60
  Ile - Val - Glu - Asn - Asn - Gln - Ser - Thr - Asp - Tyr - Gly - Leu - Phe - Gln - Ile - Asn - Asn - Lys - Ile - Trp -

                                              70                                                        80
  Cys - Lys - Asn - Asp - Gln - Asp - Pro - His - Ser - Ser - Asn - Ile - Cys - Asn - Ile - Ser - Cys - Asp - Lys - Phe -

                                              90                                                        100
  Leu - Asn - Asn - Asp - Leu - Thr - Asn - Asn - Ile - Met - Cys - Val - Lys - Lys - Ile - Leu - Asp - Lys - Val - Gly -

                                              110                                                       120
  Ile - Asn - Tyr   Trp   Leu - Ala - His - Lys - Ala - Leu - Cys - Ser - Glu - Lys - Leu - Asp - Gln - Trp - Leu - Cys -

              123
  Glu - Lys - Leu.OH
```

Figure 6 Primary structure of α-lactalbumin B. Reproduced with permission from Brew K, Castellino FJ, Vanaman TC, and Hill RL (1970) The complete amino acid sequence of α-lactalbumin. *Journal of Biological Chemistry* 245: 4570–4582.

respectively. The various classes of immunoglobulins found in milk are similar to immunoglobulins from other sources in terms of structure and functions. The N-terminal half (residues 1–115) of the light chain is known as the variable region because of its highly variable amino acid sequence. The C-terminal half is known as the constant region because the sequence of amino acids is not as variable. The heavy chains also contain a variable segment (residues 1–115) and a constant region (residues 310–500). The variable regions of the light and heavy chains are responsible for antigen binding, whereas functions of complement fixation, membrane transport, catabolism, and mediation of immediate-type hypersensitivity have been attributed to the constant region of the heavy chains.

Enzymes in Milk

While a large number of enzymes have been isolated from milk, only a relatively small number have been characterized in detail. It is not known whether some of the enzymes found in milk are truly indigenous or may have been introduced due to microbial contamination. The first reported enzyme in milk was lactoperoxidase

```
                                        10                                      20
Asp-Thr-His-Lys-Ser-Glu-Ile-Ala-His-Arg-Phe-Lys-Asp-Leu-Gly-Glu-Glu-His-Phe-Lys-
                                        30                                      40
Gly-Leu-Val-Leu-Ile-Ala-Phe-Ser-Gln-Tyr-Leu-Gln-Gln-Cys-Pro-Phe-Asp-Glu-His-Val-
                                        50                                      60
Lys-Leu-Val-Asn-Glu-Leu-Thr-Glu-Phe-Ala-Lys-Thr-Cys-Val-Ala-Asp-Glu-Ser-His-Ala -
                                        70                                      80
Gly-Cys-Glu-Lys-Ser-Leu-His-Thr-Leu-Phe-Gly-Asp-Glu-Leu-Cys-Lys-Val-Ala-Ser-Leu-
                                        90                                     100
Arg-Glu-Thr-Tyr-Gly-Asp-Met-Ala-Asp-Cys-Cys-Glu-Lys-Glu-Gln-Pro-Glu-Arg-Asn-Glu-
                                       110                                     120
Cys-Phe-Leu-Ser-His-Lys-Asp-Asp-Ser-Pro-Asp-Leu-Pro-Lys-Leu-Lys-Pro-Asp-Pro-Asn-
                                       130                                     140
Thr-Leu-Cys-Asp-Glu-Phe-Lys-Ala-Asp-Glu-Lys-Lys-Phe-Trp-Gly-Lys-Tyr-Leu-Tyr-Glu-
                                       150                                     160
Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe-Tyr-Ala-Pro-Glu-Leu-Leu-Tyr-Ala-Asn-Lys-Tyr-Asn-
                                       170                                     180
Gly-Val-Phe-Gln-Glu-Cys-Cys-Gln-Ala-Glu-Asp-Lys-Gly-Ala-Cys-Leu-Leu-Pro-Lys-Ile-
                                       190                                     200
Glu-Thr-Met-Arg-Glu-Lys-Val-Leu-Thr-Ser-Ser-Ala-Arg-Gln-Arg-Leu-Arg-Cys-Ala-Ser-
                                       210                                     220
Ile-Gln-Lys-Phe-Gly-Glu-Arg-Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg-Leu-Ser-Gln-Lys-
                                       230                                     240
Phe-Pro-Lys-Ala-Glu-Phe-Val-Glu-Val-Thr-Lys-Leu-Val-Thr-Asp-Leu-Thr-Lys-Val-His-
                                       250                                     260
Lys-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-Asp-Asp-Arg-Ala-Asp-Leu-Ala-Lys-
                                       270                                     280
Tyr-Ile-Cys-Asx-Asx-Glx-Asx-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu-Cys-Lys-Asp-Pro-Cys-
                                       290                                     300
Leu-Leu-Glu-Lys-Ser-His-Cys-Ile-Ala-Glu-Val-Glu-Lys-Asp-Ala-Ile-Pro-Glu-Asp-Leu-
                                       310                                     320
Pro-Pro-Leu-Thr-Ala-Asp-Phe-Ala-Glu-Asp-Lys-Asp-Val-Cys-Lys-Asn-Tyr-Gln-Glu-Ala-
                                       330                                     340
Lys-Asp-Ala-Phe-Leu-Gly-Ser-Phe-Leu-Tyr-Glu-Tyr-Ser-Arg-Arg-His-Pro-Glu-Tyr-Ala-
                                       350                                     360
Val-Ser-Val-Leu-Leu-Arg-Leu-Ala-Lys-Glu-Tyr-Glu-Ala-Thr-Leu-Glu-Glu-Cys-Cys-Ala-
                                       370                                     380
Lys-Asp-Asp-Pro-His-Ala-Cys-Tyr-Thr-Ser-Val-Phe-Asp-Lys-Leu-Lys-His-Leu-Val-Asp-
                                       390                                     400
Glu-Pro-Gln-Asn-Leu-Ile-Lys-Gln-Asn-Cys-Asp-Gln-Phe-Glu-Lys-Leu-Gly-Glu-Tyr-Gly-
                                       410                                     420
Phe-Gln-Asn-Ala-Leu-Ile-Val-Arg-Tyr-Thr-Arg-Lys-Val-Pro-Gln-Val-Ser-Thr-Pro-Thr-
                                       430                                     440
Leu-Val-Glu-Val-Ser-Arg-Ser-Leu-Gly-Lys-Val-Gly-Thr-Arg-Cys-Cys-Thr-Lys-Pro-Glu-
                                       450                                     460
Ser-Glu-Arg-Met-Pro-Cys-Thr-Glu-Asp-Tyr-Leu-Ser-Leu-Ile-Leu-Asn-Arg-Leu-Cys-Val-
                                       470                                     480
Leu-His-Glu-Lys-Thr-Pro-Val-Ser-Glu-Lys-Val-Thr-Lys-Cys-Cys-Thr-Glu-Ser-Leu-Val-
                                       490                                     500
Asn-Arg-Arg-Pro-Cys-Phe-Ser-Ala-Leu-Thr-Pro-Asp-Glu-Thr-Tyr-Val-Pro-Lys-Ala-Phe-
                                       510                                     520
Asp-Glu-Lys-Leu-Phe-Thr-Phe-His-Ala-Asp-Ile-Cys-Thr-Leu-Pro-Asp-Thr-Glu-Lys-Gln-
                                       530                                     540
Ile-Lys-Lys-Gln-Thr-Ala-Leu-Val-Glu-Leu-Leu-Lys-His-Lys-Pro-Lys-Ala-Thr-Glu-Glu-
                                       550                                     560
Gln-Leu-Lys-Thr-Val-Met-Glu-Asn-Phe-Val-Ala-Phe-Val-Asp-Lys-Cys-Cys-Ala-Ala-Asp-
                                       570                                     580
Asp-Lys-Glu-Ala-Cys-Phe-Ala-Val-Glu-Gly-Pro-Lys-Leu-Val-Val-Ser-Thr-Gln-Thr-Ala-
    582
Leu-Ala.OH
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Figure 7 Primary structure of bovine serum albumin. Reproduced with permission from Brown JR (1975) Structure of bovine serum albumin. *Federation Proceedings* 34: 591.

and to date over 60 enzymes have been reported as being indigenous to milk. In certain instances, the use of the prefix ‘lacto’ is confusing because it implies that these enzymes occur only in milk, for example, lactoperoxidase refers to a specific enzyme irrespective of its source. Nomenclature of the enzymes in milk does not pose any problems because the standard nomenclature and classification numbers are assigned. Milk enzymes are associated with different milk components, for example, catalase and ribonuclease are found in the whey protein fraction, proteinases are associated with the casein micelles, and xanthine oxidase is a major component of the fat globule membrane. Many enzymes, for example, catalase, are present predominantly in one component of milk, but are not confined exclusively to that component. This may be due to redistribution of the enzymes in the milk system. α-Lactalbumin, a major whey protein, may be classified as an enzyme because of its involvement in lactose synthesis.

Milk Fat Globule Membrane Proteins

A large number of proteins are present in milk at relatively low concentrations and are not isolated with either the casein or whey protein fractions but belong to a separate group known as milk fat globule membrane proteins. These proteins were traditionally classified on the basis of their relative mobility on SDS-PAGE. The protein bands were numbered consecutively from the point of sample application in order of increasing mobility. Confusion arose with regard to the naming of a specific protein because of inconsistencies in the use of the numbering system and different electrophoretic procedures used by various researchers. It has been recommended that the practice of identifying the milk fat globule membrane proteins by number be abandoned and be replaced by specific names as they are characterized by molecular cloning and sequencing techniques. For example, the term ‘butyrophilin’ is used to denote a protein that has been known under the following names: band IV/glycoprotein E, component IV, component V/glycoprotein 6, component 12/IV, Band 4/I, and CB5/PAS 5.

Minor Miscellaneous Proteins

A group of biologically significant milk proteins that do not belong to the above four groups of proteins are classified separately in this category. Two iron-binding proteins, transferrin and lactoferrin (formerly known as ‘red protein’), have been identified in milk. Lactoferrin is a nonheme glycoprotein consisting of a single polypeptide chain of 690 amino acid residues with a molecular mass of about 80 000 Da. Ceruloplasmin is a copper-binding protein that has been detected in milk, colostrum, and blood serum. Also present in milk are several proteins that bind other nutrients and components, such as folate, vitamin B_{12}, corticosteroids, and immunoglobulins. β_2-Microglobulin, also known as lactollin, is part of the histocompatibility complex. The 98 amino acids of β_2-microglobulin with a molecular mass of 11 636 Da have been sequenced. With the development of more sensitive analytical techniques, other proteins are being identified and added to the list of minor proteins. Recently, it was reported that a novel protein denoted as ‘glycolactin’ with a molecular mass of about 64 000 Da has been isolated from milk.

Variability in Protein Concentration

The large variations in the concentrations and relative proportions of proteins in milk could contribute to heterogeneity in properties and functionalities of the protein component. For the milk of over 150 species that have been analyzed, the data indicate that the total protein concentration varies from 1% in human to 24% in the white tail jackrabbit, and the casein-to-whey protein ratio varies from 0.25:1 in human milk to 6.3:1 in goat milk. In many instances, a limited number of samples have been analyzed for most of the species compared to cow’s milk and hence the values obtained are not reliable. Also, the nonbovine proteins have not been analyzed in great detail, as in the bovine milk. For the bovine species, the protein content and composition of the milk vary widely depending on genetics, health conditions, and a whole host of environmental factors, including nutrition of the animal, stage of lactation, age of cow, season, and geographical location. Genetic polymorphism of milk proteins (see below) also contributes to large variations in the concentration of certain milk proteins. An example of differences according to the breed of cow is demonstrated by comparing the average protein content of 3.42, 3.61, 3.58, 3.86, and 4.02% in the milk of Holstein, Brown Swiss, Ayrshire, Jersey, and Guernsey, respectively. In five regions of California, the milk protein concentration varies from 3.32 to 3.82%, with the caseins representing 76–78% of the total protein. Total protein, casein, and whey protein contents are higher during the winter and the early stage of lactation. Reports show that the ratio of casein to whey protein decreases as cows become older or with higher incidence of mastitis, and that in a herd of cows, parity number, month of test, stage of lactation, and mastitis had significant effects on the concentrations and relative proportions of individual caseins, β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins. Some minor proteins, for example, lactoferrin and transferrin, occur at higher concentrations in colostrum than in mature milk.

Homology of Milk Proteins

The wide range of profiles obtained when milk proteins from different species are subjected to PAGE on the same gel slab clearly demonstrates heterogeneity across species. In several instances, the nomenclature developed for bovine milk protein has been wrongly applied to the proteins of other species. This extension of name use is justified only if homologous proteins are solidly established based on structural and functional properties. Skim milk from all observed species has a chalky white or 'milky' appearance due to the scattering of light by casein micelles. Hence, it can be assumed that all milks contain a casein component. However, the four different types of casein are present in different proportions and in some cases, one or more of the caseins may be absent. There is evidence that human milk is devoid of α_{s1}- and α_{s2}-caseins, that is, the casein micelles are made up entirely of β-and κ-caseins. Caprine milk represents an interesting situation where the α_{s1}-Cn° allele is associated with no α_{s1}-casein in the milk. Milk from all the species analyzed (except the Californian sea lion) contains lactose and because α-lactalbumin is involved in the synthesis of lactose, one can assume that the presence of α-lactalbumin is universal. β-Lactoglobulin is the protein present at highest concentration in the whey from ruminant milks. Homologous β-lactoglobulin has been characterized in the milk of cows, goats, water buffaloes, and sheep. Apparently, milk from human, kangaroo, camel, guinea pig, rat, and mouse are devoid of β-lactoglobulin. There are indications that mare's milk contains two proteins that are very similar to bovine β-lactoglobulin and can be classified as β-lactoglobulin. A protein isolated from porcine milk is somewhat similar to the ruminant β-lactoglobulin in size and composition, although it lacks a free sulfhydryl group. A cysteine-rich protein denoted as whey acid protein (WAP) has been identified in the milk of rat and mouse, with 137 and 134 amino acid residues, respectively. This protein is not found in ruminant and human milk. Analogues of serum albumin and immunoglobulins are present in the milk of all species and are representatives of the proteins circulating in the blood for the species. It is not known whether all the milk fat globule membrane proteins and enzymes associated with milk are present in all species because those milks have not been analyzed as extensively as bovine milk. The activities of milk enzymes are species dependent. Ribonuclease activity is higher in cow's milk than in human milk whereas human milk has 1000 times more lysozyme activity than bovine milk. Lactoferrin has been identified in the milk of human, mouse, guinea pig, horse, pig, cow, and goat, but is absent in the milk of rabbit, rat, and dog.

Biological Roles of Milk Proteins

Milk is intended to be the sole source of food for the suckling newborn. Milk proteins are a heterogeneous group of compounds with a wide range of molecular structures and properties. Likewise, they serve many important biological roles, some of which have not yet been determined. As discussed earlier, milk from all species contains casein micelles. The caseins have a loose structure due to a high proportion of proline and hence they are very susceptible to hydrolysis by the digestive enzymes to yield a well-balanced mixture of amino acids for the nutrition of the neonate. Because the caseins are associated with high concentrations of calcium and phosphorus, the digestion of these proteins releases significant amounts of these minerals, which become available to the young animal.

α-Lactalbumin is also of universal occurrence and is required for the biosynthesis of lactose. According to the osmoregulatory theory, the production of milk depends on the presence of lactose.

No biological roles have yet been assigned to β-lactoglobulin although there are indications that it is a retinol-binding protein and it could also be involved in the activation of lipase. Serum albumin and the heterogeneous immunoglobulins are involved in protection against infection and convey passive immunity. The roles of many enzymes are not known. In general, their main significance seems to be the defense against intestinal infection. Lysozyme acts as a bactericidal agent by degrading the bacterial cell walls to enhance the activity of immune antibodies. Protein kinases are present in the milk fat globule membrane and catalyze the phosphorylation of other proteins, thus regulating the biological activities of the latter. Mammary-derived growth inhibitor appears to regulate cellular growth and function. The iron-containing proteins (lactoferrin and transferrin), folate-binding protein, and copper-binding protein serve to convey their prosthetic groups, which are essential for the nutrition in the growing sucklings. Lactoferrin also acts as a selective antibiotic because it chelates iron and renders it unavailable to the bacterial population in the gut, and in the mammary gland it may benefit the lactating cow by reducing mastitic infection.

Genetic Polymorphism of Milk Proteins

A very significant variability in milk proteins, leading to further heterogeneity, is due to genetic events leading to the occurrence of genetic variants. Since the discovery in 1955 of the presence of two forms of β-lactoglobulin, several groups around the world have been actively engaged in research covering various aspects of genetic polymorphism of milk proteins. Several electrophoretic and isoelectric focusing methods have been modified and adapted for the identification of genetic variants of milk proteins. At the latest count (see **Table 1**) 44 genetic variants have been identified among the four caseins, β-lactoglobulin, and α-lactalbumin and the number will undoubtedly rise as more sophisticated methods for detection are developed.

Genetic polymorphism of milk proteins is due to either substitutions or deletions of amino acids in the proteins as a consequence of mutations causing changes in base sequences in the genes. This type of polymorphism should be distinguished from that caused by post-translational modification such as the extent of phosphorylation and glycosylation of the molecule, as discussed previously. Among the 44 genetic variants presented in **Table 1**, two are due to the deletion of a segment in the α_{s1}-casein. Variants A and H of α_{s1}-casein differ from variant B by the absence of amino acid sequences 14–26 and 51–58, respectively. The deletion of residues 51–59 of α_{s2}-casein A leads to the occurrence of variant D. The remaining 41 genetic variants involve amino acid substitutions. For example, κ-casein A differs from κ-casein B by having a threonine at position 136 and an aspartic acid at position 148 instead of an isoleucine and an alanine at the two respective positions. The difference between variants A and B of β-lactoglobulin is due to two amino acid substitutions at positions 64 (glycine for aspartic acid) and 118 (alanine for valine). In both cases, the presence of an extra aspartic acid residue in the A variants renders the proteins more negatively charged and hence the A variants of κ-casein and β-lactoglobulin have faster electrophoretic mobilities under alkaline conditions than their B homologues.

The subject of genetic polymorphism of milk protein polymorphism has generated a lot of interest among several groups of researchers in different fields. Some of the genetic variants of milk proteins are associated with production, composition, and industrial properties of milk. Because the genetic variants are inherited according to the simple Mendelian mode of inheritance, it is possible to breed for specific variants of milk proteins. Such a scheme of selection is being practised in certain countries to favor higher frequencies of κ-casein B and β-lactoglobulin B in the dairy cattle population. The B variant of the two proteins is associated with higher levels of fat and casein in the milk, better coagulating properties on renneting, higher cheese yield, and quality. β-Lactoglobulin A milk has a higher content of protein, mainly due to increased production of β-lactoglobulin, but a lower concentration of casein than β-lactoglobulin B milk. At the κ-casein locus, the B variant is associated with higher concentrations of κ-casein, total casein, and total protein compared to variant A. These are only two examples that serve to illustrate how genetic polymorphism could contribute to variability in amounts and relative proportions of milk proteins.

Fractionation and Isolation

The choice of methods used for the isolation and fractionation of milk proteins will depend, to a large extent, on the nature of the starting material and their concentrations therein. Starting with skim milk, whole casein could be separated from whey proteins by coagulation at pH 4.6 or by enzymatic hydrolysis of caseins with rennet. The whole casein could then be further fractionated into α_{s1}-, α_{s2}-, β-, and κ-caseins. The whey fraction is a good source of β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, and glycomacropeptide (in case of enzymatic hydrolysis). Immunoglobulins and minor proteins such as lactoferrin could also be obtained from colostrum where they exist at much higher concentrations than in milk. Milk-derived bioactive peptides could be obtained by hydrolysis of the proteins with specific enzymes or by proteolysis from fermentation by specific bacteria. There is a whole range of starting materials for the production of bioactive peptides, including milk, whole casein, individual caseins, whey protein fractions, and individual whey proteins. Once produced, the bioactive peptides of interest need to be separated and purified from the mixture of hydrolysates. The bases for isolation and purification of those bioactive peptides are similar to those applied to proteins as discussed later.

The heterogeneous mixture of proteins in milk can be separated and characterized by various physicochemical methods. Comprehensive reviews on different procedures for the fractionation and isolation of milk proteins are available. Many of the procedures depend on the solubility differences and the isoelectric precipitation of the caseins. Some of the caseins are not precipitated at pH 4.6 while certain non-casein proteins may be coprecipitated with the caseins. There is no practical and absolutely clear-cut method for simply fractionating casein, and only casein, from non-casein proteins. Moving boundary electrophoresis was used to resolve whole casein into three fractions denoted as α-, β-, and γ-, none of which represented a pure protein. The interaction of casein with calcium ions was exploited by the use of $CaCl_2$ solution to precipitate a calcium-sensitive fraction, α_s-casein, which contained mainly α_{s1}-, α_{s2}-, and β-caseins from κ-casein, the fraction insensitive to precipitation by calcium. Milder conditions for the precipitation of the caseins by high salt concentration, for example, $(NH_4)_2$ SO_4, have been used. Not all the caseins precipitate and some immunoglobulins and glycoproteins may be included in the precipitate. In 1938, Rowland developed a chemical method to quantify the distribution of proteins in milk and was able to partition the protein as casein, lactalbumin (precipitated by saturation with $MgSO_4$), globulins, and proteose peptone. The rates of sedimentation of proteins from skim milk after dialysis against phosphate buffer were used as a method for the isolation of some proteins. Several casein components could be prepared starting from a 50% alcohol solution of casein in the presence of ammonium acetate, followed by precipitation by varying the pH, ionic strength, and temperature. The interactions between the caseins have hampered their fractionation. Dissociating agents such as urea and disulfide-reducing agent, for example, β-mercaptoethanol, are used routinely to permit better fractionation of the caseins. Individual caseins could be obtained starting with a whole casein solution in $6\,\mathrm{mol\,l^{-1}}$ urea and adding water to precipitate out the components. The α-casein complex is insoluble in 4.5 mol $\mathrm{l^{-1}}$ urea at pH 4.6–4.8 and contains mainly α_{s1}-, α_{s2}-, and κ-caseins. β-Casein is soluble in $3.3\,\mathrm{mol\,l^{-1}}$ urea, but insoluble in $1.7\,\mathrm{mol\,l^{-1}}$ urea. Urea fractionation of casein is not precise because some κ-casein remains in the urea solution with β-casein. In the method of Zittle and Custer, acid casein is dissolved in $6.6\,\mathrm{mol\,l^{-1}}$ urea and acidified with $3.5\,\mathrm{mol\,l^{-1}}$ H_2SO_4. On dilution of the mixture with water, a precipitate rich in α_{s1}- and β-caseins forms slowly. The supernatant is a good source of κ-casein.

The non-casein component obtained in Rowland's method of protein fractionation was resolved by moving boundary electrophoresis into immunoglobulins, α-lactalbumin, β-lactoglobulin, serum albumin, and proteose peptones. The complexity of the whey protein fraction was demonstrated over a century ago with the precipitation of a globulin fraction from an albumin fraction by saturation with $MgSO_4$. α-Lactalbumin and β-lactoglobulin were isolated later in crystalline form from the albumin fraction. Aschaffenburg and Drewry described a classical method for the isolation of β-lactoglobulin. The casein and fat from whole milk were precipitated out by the addition of Na_2SO_4 to a final concentration of 20% at 40 °C. By cooling the supernatant to 25 °C and adjusting the pH to 2.0, a precipitate containing α-lactalbumin was formed. β-Lactoglobulin was crystallized out after adjusting the pH to 6.0 and addition of more Na_2SO_4. Over the years, several methods for preparing crystalline β-lactoglobulin and α-lactalbumin by the use of other precipitating agents, such as trichloroacetic acid and $(NH_4)_2\ SO_4$, have been reported.

In most instances, the above procedures do not give a final product of high purity. Further fractionation and isolation steps involving different types of electrophoresis and chromatography are necessary to achieve the level of purity required. With electrophoretic methods, one could expect to obtain pure proteins in analytical amounts only. Considerably larger quantities of pure proteins may be obtained with the use of chromatographic methods. The development of membrane filtration has revolutionized the fractionation of milk components in the dairy industry.

Zone Electrophoresis

The proteins in milk have a wide range of isoelectric points and molecular mass; hence, they could be separated by different electrophoretic procedures, which could be adapted for the fractionation and isolation of milk proteins in microgram or nanogram amounts. Electrophoretic separations of the proteins have been performed on skim milk, whole casein, or whey protein samples. Among the various supporting media, including filter paper, cellulose acetate, starch, agarose, and polyacrylamide gels, that have been used for the electrophoresis of milk proteins, polyacrylamide is the most convenient and widely used. The concentrations of acrylamide and cross-linking reagent can be modified to prepare gels of different pore size in order to enhance separation based on molecular mass. Polyacrylamide gels can also be made with a concentration gradient of acrylamide. The inclusion of SDS in PAGE also resolves the proteins, according to their molecular mass. Denaturing agents, such as SDS and urea; chelating agents, for example, ethylenediaminetetraacetic acid (EDTA); and reducing agents, for example, mercaptoethanol or dithiothreitol, may be included in the gel to enhance separation of the proteins. A wide variety of gel and electrode buffers, differing in type, concentration, and pH, have been used for electrophoresis. Under alkaline conditions, and in the presence of urea, the mobility is in the following order: α_{s1}- > α_{s2}- > β- > κ-casein. Under acidic conditions, the genetic variants of β-casein migrate in the order $C > B > A^1 > A^2 > A^3$. The order for separation of major whey protein is: β-lactoglobulin > α-lactalbumin > serum albumin > immunoglobulins. Isoelectric focusing, which is considered as a modification of electrophoresis, has also been applied extensively for the resolution of milk proteins. The more recent literature abounds with the application of capillary electrophoresis for the resolution of milk proteins. This latter technique is more applicable for analytical work and may not be suitable for isolation and fractionation of milk proteins.

Chromatography

Different types of chromatography, based on electrical charge, molecular mass, or hydrophobic interactions, have been developed for the fractionation and isolation of milk proteins. Several review articles dealing with the chromatographic separation of milk proteins have been published.

Gel filtration (molecular sieving) chromatography has been used to fractionate and isolate proteins from skim milk, whole casein, or the whey protein fraction. The principle is based on the capacity of different proteins, depending on their molecular mass, to penetrate the pores of the gel stationary phase. Large proteins are excluded from the gel and are eluted rapidly. Chromatography of skim milk on a Sephadex G200 column with pH 7.0 phosphate buffer resolved three fractions containing mixtures of κ-, β-, and α_s-caseins followed by β-lactoglobulin and α-lactalbumin. Gel permeation chromatography is not suitable for fractionating the casein because they occur as micelles of different size. Removal of calcium results in dissociation of the micelles into large polydisperse aggregates containing various proportions of the different caseins. In the presence of urea and reducing agents, the caseins dissociate to monomeric forms, ranging in molecular mass from about 19 000 to 25 000 Da (see **Table 1**), which are too close for an effective separation. Whey proteins have been fractionated successfully into β-lactoglobulin, α-lactalbumin, serum albumin, and lactoferrin by gel filtration chromatography. The use of a Superose 12 column gives satisfactory fractionation of whey proteins in the order: immunoglobulins, serum albumin, β-lactoglobulin, and α-lactalbumin.

Chromatography on a hydroxyapatite column is interesting for the fractionation of caseins because the separation is based on phosphate content. As shown in **Table 1**, κ-casein has the least number (1) and α_{s2}-casein has the largest number (10–13) of phosphate residues. The most commonly used buffer is phosphate, pH 6.8, containing dithiotreitol and urea. The higher the net negative charge on a protein, the more tightly it is bound to the solid phase. Different proteins are separated by gradually increasing the phosphate concentration in the eluting buffer. Using such a method, whole casein can be fractionated into γ-casein, κ-casein with different degree of glycosylation, β-casein, and α_s-casein. The latter fraction indicates poor resolution of α_{s1}-casein from α_{s2}-casein.

Ion-exchange chromatography is by far the most commonly used chromatographic method for the fractionation of milk proteins. This method is based on binding the mixture of electrically charged proteins to the matrix and eluting the components with increasing salt concentration. Although several anion and cation exchangers have been used to fractionate milk proteins, the former is used more frequently. In both instances, NaCl provides counterions, with Na^+ as the cation and Cl^- as the anion. Fractionation of proteins in skim milk by ion-exchange chromatography is not as effective as from whole casein or whey protein preparation. The presence of urea and a reducing agent, such as 2-mercaptoethanol or dithiothreitol, is essential for the effective fractionation of the caseins. Satisfactory fractionation of whole casein into γ-, κ-, β-, α_{s2}-, and α_{s1}-caseins can be obtained with a diethylaminoethyl cellulose (DEAE-cellulose) column and eluting with a NaCl gradient in Tris buffer, pH 8.6, containing 6 mol l^{-1} urea. Many commercial ion exchangers have been used to fractionate the caseins, for example, DEAE-TSK-5PW, Mono Q HR 5/5, Protein Pak DEAE-15HR, and macro Q 50, to name just a few.

DEAE-cellulose chromatography gives satisfactory separation of the whey proteins with the fractionation of β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins. The amount of protein that can be fractionated by column chromatographic methods in the laboratory is relatively small, in the microgram to milligram range, and depends on the size of the column. To overcome this constraint, batch fractionation of whole casein on DEAE-cellulose has been achieved. This method involves suspending whole casein and DEAE-cellulose in 3.3 mol l^{-1} urea solution at pH 7.4 and desorbing the caseins by successive extractions with 0.00, 0.035, 0.085, 0.12, and 0.175 mol l^{-1} NaCl and filtration to recover the desired fractions. Starting with 20 g of whole casein, the recoveries are 2.2 g κ-casein, 6.58 g β-casein, 3.80 g α_{s2}-casein, and 4.89 g α_{s1}-casein. Semi-preparative fractionation of casein into pure κ-, β-, α_{s2}-, and α_{s1}-caseins is possible with high-performance liquid chromatography (HPLC) using a DEAE-15 HR anion exchanger with a NaCl gradient in Tris-urea buffer, pH 7.0. Commercially available cartridges containing anion exchangers, for example, diethyl (2-hydroxypropyl) quaternary amino cellulose, have been used successfully for the fractionation of individual caseins and whey proteins in gram quantities.

Although reversed-phase HPLC (RP-HPLC) and hydrophobic interaction HPLC (HI-HPLC) depend on the hydrophobic interactions between a stationary phase and the proteins to be fractionated, their applications are quite different. In RP-HPLC, adsorption occurs in an aqueous solution of low ionic strength and elution of proteins is obtained by increasing the hydrophobicity of the mobile phase. With HI-HPLC, adsorption of proteins is carried out in aqueous solution at high ionic strength and elution is achieved by reducing the ionic strength of the mobile phase. Whole casein and whey proteins can be fractionated on a phenyl hydrophobic interaction column. For whole casein, elution was done with 0.8–0.5 mol l^{-1}, pH 6.0, sodium phosphate buffer containing 3.75 mol l^{-1} urea. For whey proteins, fractionation is achieved with a 1.5–0.05 mol l^{-1} gradient of ammonium sulfate in 0.05 mol l^{-1} sodium phosphate, pH 7.0. Several studies have reported on the use of RP-HPLC to fractionate whole caseins. In a typical procedure, the whole casein is dissolved in pH 7 buffer containing urea and a reducing agent and applied to the column. The components are eluted by increasing the concentration of acetonitrile (hydrophobicity) linearly over time.

In addition to the fractionation of the caseins and the major whey proteins, all the above procedures have been used, as in other biochemical preparations, to isolate minor milk proteins, including enzymes, fat globule membrane proteins, and other miscellaneous proteins.

Industrial Fractionation

At the industrial level, proteins are fractionated for various purposes. The making of cheese is a form of protein fractionation where most of the casein, apart from the macropeptide portion of κ-casein, ends up in the curd and most of the whey proteins are in the whey. The whey can be further processed to give different protein-enriched products. Different combinations of temperature and heating time give rise to different types of cheeses, with the incorporation of varying amounts of whey protein, especially β-lactoglobulin. Heat-induced interaction between β-lactoglobulin and κ-casein alters the distribution of proteins in milk powders. Low-heat milk powders contain more soluble whey proteins and less β-lactoglobulin/κ-casein complexes. Heat, combined with pH adjustment, can be exploited to fractionate β-lactoglobulin and α-lactalbumin from whey. The manufacture of different types of whole casein is also considered as a method for protein fractionation. The whole casein can be further fractionated to yield individual caseins.

Development in membrane technology has provided different physical methods for the fractionation and isolation of milk proteins. Several review articles give details of the applications of membrane filtration in the dairy industry. In theory, the milk proteins can be separated according to their size in the following increasing order of molecular mass: α-lactalbumin < β-lactoglobulin < serum albumin < lactoferrin < immunoglobulins < casein micelles < milk fat globule membrane. Reverse osmosis requires pressures of 3500–10 000 kPa to remove water. It is conveniently used to concentrate the proteins in whey or skim milk. Ultrafiltration involves the use of membranes with a molecular cut-off range of 1–200 kDa and an operating pressure of 140–700 kPa. When applied to milk, it results in a retentate containing fat and proteins while water, minerals, and lactose pass through as the permeate. The objective of making cheese from ultrafiltered milk is to include the whey proteins in the finished product, and thereby increase yield. Microfiltration (membranes with a pore size of 0.2–2.0 μm; molecular cutoff >200 kDa) has contributed significantly in the industrial fractionation of milk proteins. Because the fractionation process is purely mechanical at a low temperature, there is no negative impact on the functionalities of the proteins. The differences between the size of casein micelles and the whey proteins are such that those two components can be fractionated easily by membrane filtration, with the whole casein being retained and the whey proteins passing through. Further fractionations can be achieved by application of other technologies. β-Casein can be isolated by microfiltration of calcium caseinate at 5 °C. Dilute solutions of sodium caseinate can be fractionated by ultrafiltration into a permeate rich in β-casein and a retentate rich in α_s-casein and κ-casein. A heat treatment and membrane separation process has been developed to separate β-lactoglobulin from whey. This involves the reversible aggregation of α-lactalbumin by heating at 55 °C for 30 min at a low pH and separation of the resulting aggregates consisting of α-lactalbumin and whey proteins other than β-lactoglobulin by microfiltration. The permeate is then processed by ultrafiltration in combination with diafiltration to yield purified β-lactoglobulin. The α-lactalbumin can be recovered from the retentate after solubilization at neutral pH, followed by ultrafiltration. Hydrophilic cellulose membranes with a large pore size have been used to fractionate whey proteins into α-lactalbumin + β-lactoglobulin and a higher-molecular-weight component containing bovine serum albumin, immunoglobulins, and lactoferrin. A protein fractionation robot coupled to an automated milking system has been used to demonstrate the concept of on-farm fraction of milk proteins. This system was adapted to isolate lactoferrin and lactoperoxidase using cation-exchange chromatography of raw milk.

Conclusions

With the progress being made in protein engineering and in molecular genetic techniques, further heterogeneity of milk proteins, as outlined above, is expected by modification of the milk protein genes and controlling the amounts of specific genes being expressed. Just a few examples will serve to illustrate this point. Breeding programmes are already in place to reduce the frequency of the α_{s1}-Cn° allele in dairy goats and to increase the frequency of the κ-casein B allele in bovine populations in order to increase the amount of casein in milk and improve its cheesemaking characteristics. The expression of α-lactalbumin, which is involved in lactose synthesis, could be controlled so that the lactose content of milk can be modified. The amount and type of κ-casein being expressed could be altered so as to improve micellar structure and the cheesemaking properties of the milk. The properties of β-lactoglobulin could be modified by altering its primary structure in such a way as to increase the degree of glycosylation or formation of disulfide bridges. In contrast to the above modifications of milk proteins that have direct implications for the dairy industry, proteins exogenous to the mammary gland have been successfully incorporated into milk: for example, the expression of β-lactoglobulin in mouse milk and of factor IX in ovine milk. The latter type of modification of milk proteins will be of great interest to pharmaceutical companies. Isolation and fractionation of milk proteins will still be based on the intrinsic physiochemical properties of the proteins. Different chromatographic techniques are more widely used for this purpose and in the future better solid matrices will be developed. On an industrial scale, membrane filtration techniques will play a major role in the isolation of milk proteins.

See also: **Enzymes Indigenous to Milk**: Lactoperoxidase; Lipases and Esterases; Other Enzymes; Phosphatases; Plasmin System in Milk;

Xanthine Oxidoreductase. **Mammals**. **Milk**: Human Milk. **Milk Proteins**: α-Lactalbumin; β-Lactoglobulin; Casein, Micellar Structure; Casein Nomenclature, Structure, and Association; Lactoferrin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins.

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Casein Nomenclature, Structure, and Association

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Introduction

The caseins of cows' milk are very important because of their nutritional and functional properties. For the newborn, they are readily metabolized and they carry substantial quantities of the important body-building elements calcium, magnesium, and phosphorus (as phosphate). In cheese, caseins provide the structural elements responsible for the textural and melting properties and, in some cases, its emulsification capacity. Their ready hydration and strong interactions with each other and with various other components make them valued ingredients in both food (e.g., emulsification mixes and nondairy creamers) and nonfood (e.g., glues and plastics) applications.

In the milk of most species, there are four gene products that, after post-translational modification, give rise to α_{S1}-, α_{S2}-, β-, κ-, and γ-caseins. In fresh milk, the caseins are present in the form of essentially spherical particles containing many protein molecules and amorphous calcium phosphate; these particles average 150 nm in diameter with sizes ranging from 15 to about 1000 nm in diameter. These particles are known as casein micelles. Apart from κ-casein, which is mostly found on or near the external surface of the micelle, the caseins appear to be more or less evenly distributed within the micelle.

Whole casein is isolated from milk by acidic destabilization of the micelle suspension or by size fractionation, and whole casein can be fractionated to give a number of pure caseins. Each casein has a sequence and properties different from those of the other caseins and, within each casein category, there are a number of separate protein species. This microheterogeneity is caused by varying levels of post-translational phosphorylation of serine (or threonine) and/or gylcosylation of threonine residues, by various mutational changes in the casein genes, by proteolysis by the indigenous milk enzymes, and by oxidation of cysteine to disulfide bonds.

In contrast to globular proteins, for example, β-lactoglobulin, the three-dimensional (3D) structures of the major caseins, α_{S1}-, α_{S2}-, β-, and κ-, cannot be determined precisely by X-ray crystallography, because they cannot be crystallized. Nuclear magnetic resonance (NMR) spectroscopy is limited because of concentration problems, and at the time of writing, many casein structural features are not amenable to NMR analysis. However, global estimates of structural features have been made by implications from the proteins and their peptides as determined by circular dichroic (CD), FTIR, Raman, and NMR spectroscopies, by biochemical and enzymatic modifications, and by association and physicochemical studies. Using these data, coupled with sequence-based secondary structural predictions, working, three-dimensional models have been produced, as shown in **Figure 1**.

An important feature of the caseins is their strong interactions with one another under physiological conditions and the marked effects that divalent cations have on their interaction and solubility at neutral pH. This characteristic is reflected in the high viscosity of casein solutions and in their ready ability to form foams and emulsions, and is critical to their functionality as ingredients in products.

Nomenclature

Initially, casein was considered to be a single entity, although as early as 1888 there was evidence that fractionation could give proteins with different properties. Sedimentation and electrophoretic studies on the solubilized proteins of milk in the late 1930s showed that there were a number of components, although the identity of these components was not particularly clear for another 30 years. Two important factors were the advent of high-resolution gel electrophoresis and the discovery that the caseins could be dissociated to non-interacting single polypeptides at near-neutral pH by a combination of disulfide-bond reduction and addition of urea to a concentration of 6 mol l^{-1}. Once separation and isolation of pure proteins were achieved, the primary sequence and post-translational modifications of each of the proteins were determined and a systematic nomenclature was devised and applied to the bovine caseins. Further research using these techniques showed that nearly all of the lower molecular weight components of the casein fraction are actually proteolytic fragments of the four parent caseins namely α_{S1}-, α_{S2}-, β-, and κ-casein. The caseins of other species have been classified by analogy with the bovine caseins, at either the amino acid or the nucleotide level.

The sequences of the four major bovine caseins and most of the various known genetic variations are

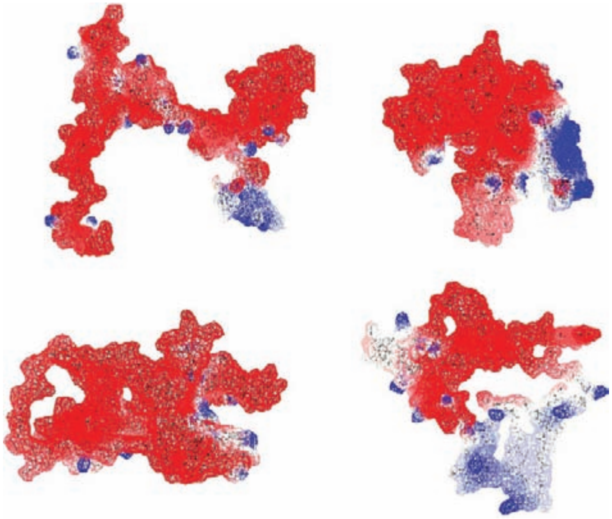

Figure 1 Three-dimensional molecular models of the caseins obtained from sequence-based secondary structural predictions and aligned with spectroscopic data. *Top*: *left* α_{S1}-casein, *right* α_{S2}-casein; *bottom: left* β-casein, *right* κ-casein. The models display a pseudo-charged surface potential with red being negative, light being neutral and/or hydrophobic, and blue positive. These models represent a working view of casein structure and are subject to change as experimentation progresses.

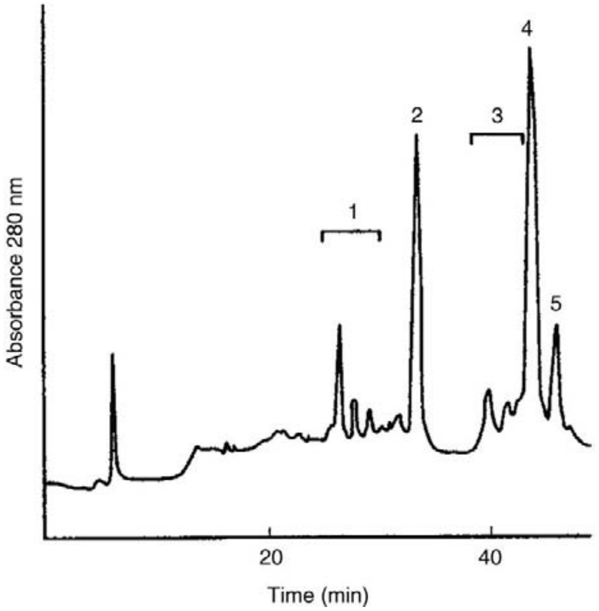

Figure 2 Diagram showing a typical analytical ion-exchange separation of the components of whole casein. The caseins were treated with dithioerythritol and then separated on a 7.5 × 75 mm column of TSK gel DEAE-5PW using a gradient of increasing NaCl concentration in a 0.02 mol l^{-1} Tris-HCl buffer, pH 8.0, and containing 4.5 mol l^{-1} urea. The regions and peaks identified as 1–5 are (1) the various κ-casein entities, (2) β-casein, (3) the variously phosphorylated α_{S2}-caseins, (4) α_{S1}-casein B-8P and (5) α_{S1}-casein B-9P. Adapted with permission from Creamer LK (2002) Milk Proteins Casein nomenclature, structure and association properties. In: *Encyclopedia of Dairy Sciences*, pp. 1895–1902. London: Elsevier Science.

discussed elsewhere (*see* **Milk Proteins**: Heterogeneity, Fractionation, and Isolation). The names for the caseins used therein are those recommended by the Nomenclature Committee of the American Dairy Science Association. The use of the latter nomenclature indicates the polypeptide sequence, that is, the genetic variant and the degree of phosphorylation, and, in the case of the γ-caseins, the particular fragment of β-casein; this nomenclature is generally restricted to definitions of a particular fraction that is used in that piece of work. A review of the various genetic variants of the caseins as well as their primary structures has been recently published by the above-noted committee. In general, the most common variants in the milk of Friesian cattle are α_{S1}-casein B, α_{S2}-casein A, β-casein A^2,and κ-casein A.

High-performance liquid chromatography (HPLC), either reversed phase (RP) or ion exchange, is also a good method for resolving the caseins; an example is shown in **Figure 2**. RP-HPLC relies on the total hydrophobicity of the caseins in the particular solvent and, to a lesser extent, on the net charge on the protein and the matrix. In ion-exchange chromatography, differences in net charge predominate and hydrophobic interactions between the solute and the matrix have only a minor effect. This is different for ion exchange or RP-HPLC of native globular proteins with a moderately rigid tertiary structure, where the surface character can be important. As with polyacrylamide gel electrophoresis (PAGE), it is necessary to minimize casein–casein interactions by modifying the solvent. Thus, it is common to include ethylene diamine tetra acetic acid (EDTA) in the solvent buffer to chelate the polyvalent cations, and the solvent often contains 4.5 mol l^{-1} urea or an organic solvent such as acetonitrile.

Casein Structure

The amino acid sequences of the major caseins (*see* **Milk Proteins**: Heterogeneity, Fractionation, and Isolation) have been known since the early 1970s, and it was immediately apparent that the sequences were quite dissimilar to those of the typical compactly folded globular proteins. In particular, most globular proteins have a mainly α-helical structure (e.g., bovine serum albumin) or a mainly β-sheet structure (e.g., β-lactoglobulin) and periodicities of particular amino acids and particular types of amino acids with hydrophobic and hydrophilic side chains. In addition, there are also regions with amino acids such as aspartic acid, proline, or tyrosine that favor various kinds of tight turns. Although the caseins have some of these features, there seems to be a lack of the large-scale periodicity seen in most globular or fibrous proteins. However, they have regions that are essentially acidic, basic, or neutral, and hydrophobic or hydrophilic, as seen when the primary sequence (*see* **Milk Proteins**: Heterogeneity, Fractionation, and Isolation) is displayed as a linear distribution of charge and hydrophobicity (**Figure 3**). The charges shown are for the proteins in solutions buffered at neutral pH with only monovalent cations present. **Figure 3** also shows the major plasmin cleavage sites for α_{S1}- and β-caseins and the major chymosin cleavage sites in α_{S1}- and κ-caseins. The other common feature of the secreted globular proteins, noted above, that is missing from the caseins is the presence of strategically placed cysteine residues which form intramolecular disulfide bonds that stabilize the tertiary structure of the protein; in contrast, κ-casein forms primarily intermolecular disulfide bonds with other κ-casein molecules. There are two important structures found in the caseins of all milks: the chymosin (or pepsin) cleavage site in κ-casein, which is critical for the proper coagulation of the casein micelles (*see* **Cheese**: Rennet-Induced Coagulation of Milk); and the phosphorylation sites, which are critical for the proper bonding of the caseins to the hydrated calcium phosphate entities present in the casein micelles.

All of the above information has led to the inclusion of the caseins in a new class of proteins which have been termed ‘intrinsically unstructured’ or ‘natively unfolded’. Previously, globular proteins such as those noted above were thought to fold or denature (unfold) in a concerted one-step process. The ‘New View’ of protein structure argues that the defined secondary structure leads to protein folding (or unfolding) in a multistep process. Uversky

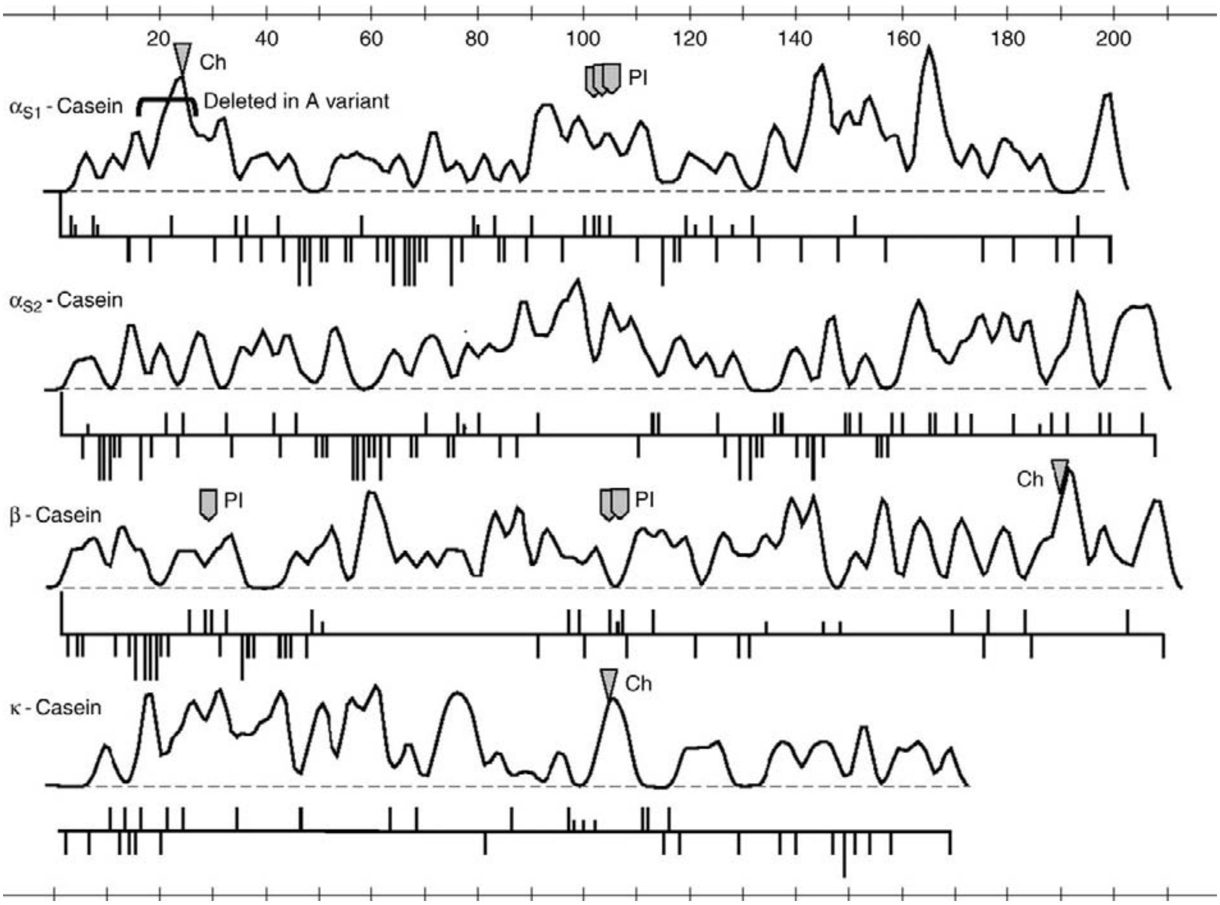

Figure 3 Plots of charge and hydrophobicity as a function of sequence position for the four major caseins, α_{S1}-casein B-8P, α_{S2}-casein A-11P, β-casein A^2-5P, and aglyco-κ-casein B-1P. The continuous curve for each protein is the distribution of smoothed hydrophobicity of the individual amino acids whereas the lines with vertical bars show the discrete charges for each amino acid in the sequence. The vertical bars below the horizontal lines show the phosphoserine (long bars), glutamic, and aspartic acid side chains and the C-terminal acid group. The bars above the horizontal line show the histidine (shorter bars), lysine, and arginine side chains. The charges on the terminal amino groups are also shown (note that κ-casein has a pyroglutamate terminus). The arrows indicate the most susceptible bonds, and Ch indicates chymosin and Pl indicates plasmin susceptibility. Adapted with permission from Creamer LK (2002) Milk Proteins Casein nomenclature, structure and association properties. In: *Encyclopedia of Dairy Sciences*, pp. 1895–1902. London: Elsevier Science.

has used a progressive nomenclature for this process with defined intermediate states ranging from unfolded coil to pre-molten globule to molten globule to natively folded protein. In this scheme the monomeric forms of α_{S1}-, α_{S2}- and β-casein are classified as natively unfolded pre-molten globules, whereas κ-casein is classified as a natively unfolded coil. However, upon self-association, three of the caseins become more compact, with β- and κ-casein classified as molten globules and α_{S2}-casein becomes a pre-molten globule. Interestingly α_{S1}-casein remains an unfolded coil. The 3D models in **Figure 1** demonstrate clearly that α_{S1}-casein is the least compact molecule.

It could be said, then, that the native states of the α_{S1}-, α_{S2}-, β-, and κ-caseins are the states in which they exist when fully immersed in the casein micelle (see **Milk Proteins**: Casein, Micellar Structure), in which most of the very acidic or very basic regions (**Figure 3**) are bound to hydrated calcium phosphate complexes and the hydrophobic regions of all the caseins are intermingled, with κ-casein molecules close to other κ-caseins and with the C-terminal (macro peptide) region of the κ-casein surrounded by the serum. The 3D models in **Figure 1** are more in accord with the structures of the caseins within the casein polymers rather than as isolated monomers.

α_{S1}-Casein

This major component of the caseins has the highest net negative charge in neutral pH buffer with only monovalent cations present and has a calculated hydrophobicity of 1170 kcal mol^{-1}. The hydrophobicity (**Figure 3**) is high near residues 25, 90–110, and 140–190. The last region is found between 10 and 7 o'clock in the 3D model of α_{S1}-casein (**Figure 1**) and close to the C-terminus. Clearly, α_{S1}-casein has a very acidic region between residues 38 and 78, which is responsible for the tight binding of calcium, and occurs at about 2 o'clock in the α_{S1}-casein model in **Figure 1**. In contrast, the N-terminal region (1–26 and 4 o'clock in the 3D model) is positively charged and could possibly bind inorganic phosphate.

Circular dichroism (CD) or Raman spectral analysis indicates that there is a low level of α-helix (~14%), an intermediate level of turn-like structure (24%), and a significant level of extended or β-sheet (~40%). Prediction methods indicate that likely positions for helix formation are near residues 60, 100, and 125. Interestingly, plasmin, which hydrolyzes bonds adjacent to lysine or arginine, cleaves this protein most rapidly at several sites (102–103, 103–104, and 105–106) in a predicted helical region found as the central link in the α_{S1} model in **Figure 1**. A major cleavage site for chymosin is bond 23–24, which is in a region predicted to have β-sheet propensity and is in a significantly hydrophobic region (**Figure 3**). Clearly, these regions are accessible to enzyme attack in the associated protein and must be sufficiently exposed to the solvent to allow the respective enzymes to form enzyme–substrate complexes.

Association studies of α_{S1}-casein at 20 °C have shown that there is a wide range of polymer sizes, from dimer to hexamer, which occurs in a progressive manner and which is dependent on pH and ionic strength. It is likely that the polymers are more or less rod-like. This has suggested that the hydrophobic regions near residues 20–25 and a region near the C-terminus could be alternatively involved in the polymers. However, recent work has shown that at 37 °C, the higher-order polymers are broken down and the protein is essentially a dimer under physiological conditions (**Table 1**). It has been shown that the dimerization involves residues 136–160 of each monomer (10 o'clock in the α_{S1}-casein model in **Figure 1**).

α_{S2}-Casein

This minor component is the least hydrophobic casein (1100 kcal mol^{-1}) and is the most highly and variably phosphorylated of the caseins. There are three phosphopeptide regions (5–18, 49–68, and 126–145) in the casein sequence (left side of the model of α_{S2}-casein in **Figure 1**) and a large central hydrophobic region (90–120) with very little charge (region of α_{S2}-casein protruding from the center of the model to 6 o'clock in **Figure 1**). There is a second large hydrophobic region (160–207) – but it has a number of positively charged residues – which represents the highest positively charged area for any casein (**Figure 3** and the right side of the α_{S2}-casein model in **Figure 1**).

Table 1 Weight average molecular weights of selected caseins and mixtures by analytical ultracentrifugation at 37 °C

Casein or mixture	*Wt. avg. molecular weight*	*Wt. avg. polymeric size*	*Rotor speed*
α_{S1}-Casein	56 000	Dimer	12 000
β-Casein	1 250 000	52 mer	3000
RCM κ-Casein	3 040 000	160 mer	3000
1.5 α_{S1}-: 1 RCM κ-	316 000	15 mer	3000
4 α_{S1}-: 1 RCM κ-	92 400	Tetramer	6000
4β-: 1 RCM κ-	1 010 000	43 mer	3000
1β-: 1α_{S1}-	213 000	Nonamer	3000
RCM whole casein	110 000	Hexamer	6000

All data were obtained at 37 °C, pH 6.75 in 25 mmol l^{-1} disodium piperazine-*N*,*N′*-bis(2-ethane sulfonic acid) with 80 mmol l^{-1} KCl to mimic milk salt conditions in the mammary gland in the absence of calcium; the rotor speeds were appropriate to the weight average molecular weight as previously described. RCM = reduced and carboxymethylated. Modified from Farrell HM, Jr., Malin EL, Brown EM, and Qi PX (2006) Casein micelle structure: What can be learned from structural biology? *Current Opinion in Colloid & Interface Science* 11: 135–147.

In milk, the majority (~90%) of the protein occurs with an internal disulfide bond between cysteine residues 36 and 40 forming a small loop in the structure. In addition, a small proportion of this protein exists as disulfide-bonded dimers as well as polymers with κ-casein.

CD and FTIR spectral analysis indicates that for α_{S2}-casein there is a relatively high level of α-helix (30–40%), an intermediate level of turn-like structure (~20%), and a similar level of extended or β-sheet (~20%). Prediction methods indicate that likely positions for helix formation are near both the acidic N-terminal and the basic C-terminal regions. This protein is readily hydrolyzed by plasmin at a number of sites, primarily in the afore-noted C-terminal region, so that at neutral pH these positively charged residues are primarily at the surface and could actively participate in the binding of inorganic phosphate.

Association studies at 20 °C show that monomeric α_{S2}-casein behaves in a very similar way to α_{S1}-casein except that there is a maximum association at an NaCl concentration of about 0.2 mol l^{-1}. However, at 37 °C this protein has been found to form elongated amyloid structures, perhaps through associations of the central hydrophobic core noted above.

β-Casein

This major casein component is the most hydrophobic (1335 kcal mol^{-1}) of the intact caseins and has the largest regions of high hydrophobicity (55–90 and 130–209) with a very acidic N-terminal region of 24 amino acids (**Figure 3**). The acidic N-terminal region is at the right top of the model in **Figure 1**. There are two regions where plasmin readily cleaves the protein (at bonds 28–29 and 105–106/107–108, respectively; 3 and 4 o'clock in the β-casein model in **Figure 1**). Cleavage at these sites by plasmin yields the fragments previously known as γ-caseins 1 and 2, respectively. β-Casein has one site which is particularly sensitive to chymosin (189–190/192–193; found at about 7 o'clock in the β-casein model in **Figure 1**). Loss of the C-terminal peptide (193–209) by chymosin cleavage substantially reduces the association–dissociation equilibrium. When the native protein is brought to higher temperatures, chymosin cleavage is much slower, presumably because access of the enzyme to the cleavage site is restricted by β-casein self-association. This leads to the unusual situation in which increasing the reaction temperature reduces the cleavage rate.

CD and FTIR spectral analysis indicates that at 6 °C, β-casein has a relatively low level of α-helix (~15%), an intermediate level of turn-like structure (~29%), and a similar level of extended or β-sheet (~30%) in dilute, low ionic strength, neutral solutions. However, there are significant increases in ellipticity at 220 nm when the solution temperature is increased from 6 to 37 °C. β-Casein is predicted to contain a significant amount (30%) of polyproline II structure, but these structures have been shown to occur at about 20% in β-casein by Raman spectroscopy FTIR and CD. Thus, it is possible that increases in both β-strand and polyproline II structures are responsible for this temperature-dependent structural change. It is also possible that, as the protein associates, incipient β-strands form interprotein H bonds that give rise to sheetlike structures.

The self-association of this protein is (detergent) micelle-like, and both ionic strength and temperature increase the quantity of polymer present (i.e., increased association constant) and the degree of association (n):

$$n\ \beta - \mathrm{CN} \leftrightarrow \beta - \mathrm{CN}_n$$

The number of monomer proteins in these micelle-like near-spherical polymers ranges from about 15 at 0.1 mol l^{-1} ionic strength at pH 7 and 20 °C to about 52 at 0.110 mol l^{-1} ionic strength at pH 6.7 and 37 °C (**Table 1**). The intrinsic volume of an individual β-casein within the polymer is significantly low and represents a molten globule-like state. Note that the 3D model for β-casein (**Figure 1**) represents the more compact form of the monomer within the polymer. The effect of temperature on intrinsic tryptophan and hydrophobic probe fluorescence both indicate that changing hydrophobicity or structure of the probe environment is an important consequence of association.

κ-Casein

This protein, which constitutes 10–12% of whole casein, plays a crucial role in stabilizing the casein micelles in milk and, after enzymatic cleavage, destabilizing the colloidal casein system. The enzymatic cleavage that brings about this transformation is important for the nutrition of the suckling young and for the production of many cheese varieties (*see* **Cheese**: Rennet-Induced Coagulation of Milk). This is achieved by the molecule having two distinctly different domains that can be separated by cleavage of a bond that is hydrolyzed by an acid protease at neutral pH. As seen in **Figure 3** (and the bottom portion of the κ-casein model in **Figure 1**), the N-terminal domain (residues 1–95) carries a net positive charge, is very hydrophobic, and interacts strongly with the other casein molecules. The C-terminal domain (residues 113–169) carries a net negative charge and contains a preponderance of polar residues (top portion of the κ-casein model in **Figure 1**); the overall hydrophobicity of the protein is 1205 kcal mol^{-1}. These two domains are joined by a peptide (residues 96–112; 10 o'clock in the κ-casein model in **Figure 1**) that carries a net positive charge, is generally predicted to be β-strand, is generally well conserved in most species, and contains a motif that is readily recognized by chymosin and rapidly and specifically cleaved to give a large positively charged hydrophobic peptide (para-κ-casein: κ-casein (f1–105)) and a smaller hydrophilic peptide (caseinomacropeptide (CMP), also called glycomacropeptide (GMP) or κ-casein (f106–169)). About half of the κ-casein molecules are post-translationally glycosylated with short oligosaccharide chains at one or more of the threonine sites 131, 133, 135, 136 (B variant only), and 142, and most of the κ-casein molecules are phosphorylated at Ser149.

CD and FTIR spectral analysis indicate that for κ-casein there is a relatively low level of α-helix (15%), an intermediate level of turn-like structure (~25%), and a higher level of extended or β-sheet (~30%). These structures appear to be thermostable.

As a reduced isolated protein, at 20 °C κ-casein self-associates in a manner similar to β-casein to a 600 000 Mw polymer. However, the reduced carboxymethylated (RCM) protein at 37 °C forms amyloid bodies which were first discovered in this casein. These amyloids have a soluble Mw of over 3 million (**Table 1**). The β-sheet structures at 6 o'clock in the model of κ-casein in **Figure 1** are thought to promote amyloid formation. In milk, κ-casein exists as a series of intermolecular disulfide bonded aggregates and this aggregation may well have occurred after the casein micelles have been assembled in the epithelial cells. Most of the properties of this protein are not affected by the state of aggregation, but about 10% of the cysteine residues in total are not disulfide bonded and may react with other proteins or participate in oxidation reduction reactions, for example, formation of complexes with β-lactoglobulin in heated products.

Casein–Casein Interactions

In addition to the studies on the interactions of individual casein species in solution, there have been a number of studies involving primarily binary mixtures. Such studies have shown that, in the absence of polyvalent cations and at 37 °C, α_{S1}- and κ-caseins associate most strongly, followed by α_{S1}- and β-caseins. As noted above, RCM-κ-casein forms high molecular weight amyloid structures. In brain diseases, amyloid structures form entanglements symptomatic of the disease. The Mw of these aggregates is greatly reduced by the presence of α_{S1}-casein at the ratio of 4:1 found in milk (**Table 1**). β-Casein has a much less effect on the amyloids as seen in **Table 1**. In addition, the high molecular weight polymers of β-casein are readily broken down by α_{S1}-casein (**Table 1**). In the New View of protein structure noted above, α_{S1}-casein can be considered to be a natively unfolded assembler in that it is able to break down aggregates of β- or κ-casein and lead to successful transit through the mammary secretory system prior to the addition of calcium in the Golgi apparatus. Recently, it has been shown that the amyloid bodies formed by α_{S2}-casein can also be broken down by α_{S1}-casein.

Sodium caseinate (whole casein in the absence divalent cations) is an excellent ingredient and finds many applications in food processing. This product can have somewhat varying properties depending on its method of preparation. The mixed associations of the individual caseins discussed above bring about the formation of a rather stable polymer with an average diameter of 18 nm and a Mw of about 280 000. Laboratory-prepared preparations of sodium caseinate can dissociate into monomers and small polymers at concentrations below 1%; note that at 0.1% the weight average Mw of RCM-whole casein is only 110 000 (**Table 1**).

Properties, such as viscosity, of casein solutions are concentration-dependent and a 15–20% casein solution is very viscous because of the associations of the molecules with one another and the absence of compact folded structures for any of the molecules, as discussed in the previous section. A number of emulsion and foaming studies have been done using either the individual pure proteins or various commercial whole proteins, for example, total milk protein (TMP) or milk protein concentrate (MPC) or whole casein materials.

Casein–Mineral Interaction

From the point of view of their functions in various dairy or industrial products, or in the casein micelles in milk, one of the very important characteristics of the caseins is the changes brought about by their binding of calcium. In the absence of calcium, the casein molecules, as described earlier, have separate mechanisms of self-association. Let us consider a simple system of α_{S1}-casein dissolved as described in **Table 1**. Increasing the concentration of calcium in such a casein solution gradually changes the system. Although there is little obvious visual change at low calcium concentrations (3 mmol l^{-1}), as viewed by sedimentation analysis, the α_{S1}-casein has bound several calcium ions and two peaks representing a tetramer and an octamer appear. However, once the number of calcium ions bound to the acidic (phosphoserine, glutamic, and aspartic acids) clusters (**Figure 3**) on the molecules have reduced the net charge (~6 mmol l^{-1}), the overall molecular interactions shift toward a lower net repulsion between the protein molecules, with an increase in the size of the casein aggregates. Once sufficient calcium has become associated with the α_{S1}-casein to reduce the net charge on the protein to almost zero, casein aggregates become very large and form a separate phase, which can precipitate, gel, or form colloidal suspensions. Similar precipitates occur for β- and α_{S2}-caseins but at lower and higher concentrations of calcium, which are related to their respective degrees of phosphorylation (**Figure 3**). Caseins are capable of binding almost all physiologically important divalent cations.

In contrast to the precipitation behavior described for α_{S1}-casein or α_{S2}- and β-caseins, κ-casein is affected little by moderate concentrations of calcium salts. However, when it is mixed with the other caseins, it interacts with them strongly and thus affects their behavior with minerals. When calcium is added to mixtures containing κ-casein, the calcium binds strongly to the phosphate-containing caseins, lowering the net charge of the complexes. However, instead of forming very large colloidal particles or precipitates of α_{S1}-, α_{S2}-, or β-casein, the κ-casein, strongly associated with these proteins, limits the size of the aggregates formed and generates colloidal particles, the size of which is determined by the proportion of κ-casein in the protein mixture. This characteristic is dependent on the structure of the hydrophilic C-terminal, 30% of the κ-casein molecule (*see* **Milk Proteins**: Casein, Micellar Structure).

In the presence of added orthophosphates, the behavior of the system becomes more complex because the inorganic phosphate competes with the protein phosphoserine-rich acidic clusters for the ionic calcium and forms complexes with the soluble calcium and these can react with casein molecules in preference to the simple calcium ions. In addition, inorganic phosphate binds to the positively charged areas of the caseins only in the presence of calcium. *In vitro* casein phosphopeptides react with inorganic calcium and phosphate to form nanoclusters of defined shape and size. Complexes of phosphopeptides and calcium do indeed enhance calcium uptake in experimental animals. However, for whole casein molecules, it must be noted that strong protein–protein interactions precede the binding of calcium and phosphate, which is not the case for phosphopeptides. All of these reactions and their products are very important for the behavior of the caseins and they not only lead to colloid formation but also affect many of the food and industrial uses of casein-containing products.

See also: **Cheese**: Rennet-Induced Coagulation of Milk. **Milk Proteins**: Casein, Micellar Structure; Heterogeneity, Fractionation, and Isolation.

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Casein, Micellar Structure

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Introduction

All mammals secrete milk for the nourishment and sustenance of their newborn offspring. As food for the suckling, this complex fluid is a blend of all the essential dietary constituents – lipids, proteins, carbohydrates, and minerals. These components are found in proportions generally specific to the nutritional and growth demands of each species. Milk composition therefore varies widely between species, and in some mammals this composition varies during lactation to meet specific developmental requirements. Between species, protein content can range from less than 10 g l^{-1} (human milk) to about 200 g l^{-1} (milk of some rabbits), with total calcium phosphate content varying in proportion. Delivering such levels of minerals and proteins to the offspring could present problems to the transport system (the mammary gland), but nature has neatly solved this by combining them in the casein micelle. Casein micelles are the colloidal aggregates of the caseins and mineral calcium phosphate. They have been found in the milk of all species that have been examined.

As a packaging system, these micelles convert milk into a free-flowing low-viscosity fluid and provide the means to transport the high levels of calcium and phosphate that would normally precipitate in the mammary gland in the absence of the caseins. Having converted the milk into a colloidally stable dispersion to overcome delivery problems, nature has also built into this casein micelle its own instability mechanism, which is necessary for efficient digestion of the nutritional material when ingested by the young. Thus, in the stomach of the calf, the enzyme chymosin hydrolyzes the κ-casein and induces destabilization of the micelle, forming a soft clot, which aids digestion and assimilation. The casein micelles are also destabilized by acidification, and in other species, the stomach acids achieve clot formation by this mechanism. Furthermore, these two destabilization pathways are exploited by man in cheese manufacture and in the production of fermented milk products, major preserved food sources for man and extensive outlets for dairy farm produce.

Central to our understanding of all these functional properties of the casein micelle is knowledge of the physicochemical properties of the different casein proteins. These dictate how the structural assembly, which we call the casein micelle, is built, and they govern the response of the micelle to destabilizing influences. Caseins and casein micelles have been studied extensively over a long period. Several different models for micellar assembly have emerged. Early primitive models, such as the submicelle model, have been discredited. Shortcomings in the more recent model of Holt have been identified, and it appears that fewest problems are encountered with the dual-binding model. This dual-binding model plausibly explains micelle assembly, and models observed micellar behavior in scenarios where application of earlier models was not even attempted. Furthermore, since its publication, more and more supporting evidence is emerging.

This article summarizes the pertinent physicochemical properties of the individual caseins, concentrating on those that govern their self-assembly and interactions. It briefly reviews the earlier micellar models and outlines the dual-binding model. The discussion is confined largely to bovine caseins because these have been most intensively studied, but, where possible, interspecies comparisons will be made throughout the text.

Casein Molecular Properties

Sequence Characteristics

Caseins constitute over 80% of the total protein in bovine milk, almost all of it aggregated with calcium phosphate in casein micelles. Originally defined as phosphoproteins precipitated from milk at pH 4.6, they are now defined by their sequence and their homology to the four distinct gene products constituting the primary members of the bovine group. Because the caseins utilize the same calcium-sequestering mechanism to regulate the calcium phosphate concentration of their environment, they have recently been identified as members of a wider family of secretory calcium-binding phosphoproteins descended from a common ancestor gene. These secretory phosphoproteins include enamel matrix proteins, dentine, salivary proteins, bone extracellular matrix proteins, and caseins, among others. The bovine caseins are designated α_{S1}-, β-, α_{S2}-, and κ-caseins. Sequences were first elucidated for all bovine members but cDNA and derived amino acid sequences are now available for many more individual caseins from a range of species.

Genetic variants have been detected for many of these sequences. The majority of these are amino acid interchanges at single sites or short deletions. The level of expression is also under genetic control. Null variants of α_{S1}-casein exist in the goat and the sheep, and that of β-casein also in the sheep. Since α_{S1}-casein is low in human milk also, there is the possibility that this too is the result of the existence of a null variant. There are also reports of some milk samples from Mongolian mares lacking κ-casein homologues. All such changes in sequence and casein composition have important consequences for the functional behavior of the casein proteins, although the major impact is often in the fine detail.

Two post-translational modifications of the newly synthesized casein molecules have a major impact on their physicochemical, functional, and assembly properties. These reactions are glycosylation and phosphorylation. In bovine milk, only κ-casein is found glycosylated. There are few details for other species. The reaction occurs at threonine residues, occasionally serine, in the hydrophilic C-terminal end of the κ-casein molecule. A great deal of heterogeneity is observed both in the numbers of residues reacted and in the lengths of the relatively short sugar chains attached, but all such reactions increase both the hydrodynamic bulk and the hydrophilicity of this end of the κ-casein molecule.

The second post-translational reaction is phosphorylation. All of the caseins are phosphorylated at serine, or rarely threonine, to varying extents. The phosphorylation reaction requires a unique sequence template, - Ser–X–A-, where X is any amino acid and A is Glu, SerP, or rarely Asp.

Because of the placing of the serine residues along the molecular sequences of α_{S1}-, α_{S2}-, and β-casein, most of the phosphorylated residues are found in clusters. Such clusters in the α_S- and β-caseins are highly conserved, and their numbers attest to the significance of the calcium phosphate requirement for post-natal growth in mammals. Thus, four of the five phosphorylated serine residues in bovine β-casein are found between positions 15 and 19, with the fifth at position 35. Four of the eight phosphorylated serines in bovine α_{S1}-casein are located between positions 64 and 68, with two more downstream at positions 46 and 48, and singlets upstream at positions 75 and 115. An additional serine in α_{S1}-casein is occasionally found phosphorylated at position 41, producing the protein previously called α_{S0}-casein but now designated as α_{S1}-casein-9P. α_{S2}-Casein can have a variable level of phosphorylation from 10 to 13 mol of P per mole of protein. The most abundant of these, α_{S2}-casein-11P, has three groupings of phosphorylated residues, one cluster of three from residues 8 to 10, four SerP spread as a group of three from 56 to 58 with the fourth member at 61, with the third cluster of two at positions 129 and 131. The remaining two single SerP residues of the total 11 are located at positions 16 and 135. The α_{S2}-casein molecule has two further potentially reactive sites at positions 31 and 135. Interestingly, one of the genetic variants of α_{S2}-casein, α_{S2}-casein D, results from the deletion of nine residues between 51 and 59 encompassing one of the phosphoseryl clusters. This should have a major influence on the physical and self-assembly properties of this protein. κ-Casein is unique among the caseins in the absence of phosphoseryl clusters. Most molecules of κ-casein contain only one phosphoserine residue, rarely two or three, and all are singlets located in the hydrophilic C-terminal region.

Bovine caseins are almost always fully phosphorylated to the level of their potential. At most, only one of the template serine sites is found without its expected phosphate residue in α_{S1}- and β-casein; most gaps are found with α_{S2}-casein, where the variability ranges from 10 to 13. This contrasts with the variations found in other species. In human milk, the level of phosphorylation of β-casein ranges from 0 to 5 mol of P per mole of casein. Similarly, wide heterogeneity in the level of phosphorylation is found in ovine and equine milk. The consequences of this for micelle assembly are considered later.

Molecular Structure in Solution

Secondary Structure

Attempts to predict the secondary structure of the caseins have met with little success. They are neither globular proteins, nor are they truly random-coil polymers. They have been described as rheomorphic proteins, indicating that the molecules adopt structures in solution dictated by the circumstances in which they find themselves; their structures ‘go with the flow’ but are also open and flexible. The caseins have little α-helical structure, no denaturation temperature, and high hydrodynamic volumes. Their open conformation allows easy access to proteolytic enzymes, facilitating rapid and extensive degradation. It has been suggested that some α-helical behavior exists on either side of the phosphate clusters, causing these centers to exist at the apex of a loop structure, but little consistency has been found in secondary structure predictions for other regions of these molecules.

Caseins as Amphiphilic Molecules: Adsorption and Self-Association

When categorized simply as nonpolar hydrophobic or polar/charged hydrophilic groups, the amino acids of the caseins show distinct segregation into different regions of the molecular chains.

Each casein provides its own pattern, and this has helped to rationalize the self-association behavior of caseins and the conformations they adopt on adsorption to a hydrophobic interface.

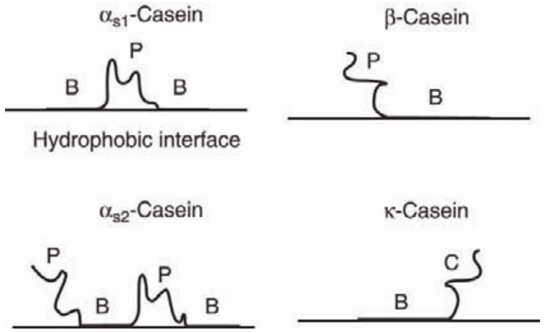

Figure 1 Part of the polymer network built up in the casein micelle according to the dual-binding model. Bridging of casein molecules across calcium phosphate nanoclusters, crosslinking of caseins via hydrophobic interaction sites and chain termination by k-casein are illustrated. B, hydrophobic regions; P, hydrophilic regions containing phosphoserine clusters.

Thus, in β-casein, there is a distinct grouping of charged hydrophilic residues, including the phosphoseryl cluster in the N-terminal region up to around residue 50, with a large number of hydrophobic amino acids in the remaining three-quarters of the protein chain from there to the C-terminus. Considerable experimental evidence has accumulated to suggest that it is this latter region that adsorbs strongly to hydrophobic oil or air interfaces with the N-terminal phosphorylated region extending out into the aqueous phase. The conformation of the adsorbed molecule predicted by self-consistent field (SCF) calculations reproduces this behavior (for schematic representation see **Figure 1**). This presents the hydrophobic region as a featureless block but the hydrophobic amino acids in this region also show grouping or clustering, which might suggest that hydrophobic interactions between such regions on different molecules might exhibit more of a velcro effect.

In α_{S1}-casein, the hydrophilic residues around the phosphoserines are in a more central position in the chain with bunching of hydrophobic residues earlier and later in the molecular sequence. Similar SCF calculations show that α_{S1}-casein adopts the loop structure also shown in **Figure 1** with a hydrophobic train of residues on either side of the loop, each train binding to the hydrophobic surface and the hydrophilic loop projecting out into the aqueous phase. Closer inspection of the α_{S1}-casein sequence reveals that the hydrophobic residues also exhibit clustering, allowing multiple weak interactions with hydrophobic surfaces. In an analogous fashion, such groupings of the hydrophobic and hydrophilic residues can be recognized in the sequences of both α_{S2}-casein and κ-casein. α_{S2}-Casein presents itself as having a hydrophilic N-terminal tail with a phosphoseryl cluster, followed by a hydrophobic train containing several hydrophobic clusters, a loop containing the major groupings of phosphoseryl residues, and then an extended hydrophobic C-terminal train broken up into several distinct hydrophobic clusters (**Figure 1**). κ-Casein has a hydrophobic N-terminal region leading up to the chymosin proteolysis site at Phe_{105}–Met_{106}. The C-terminal peptide cleaved off by chymosin is very hydrophilic since it also carries the glycosyl side chains on several of its threonyl residues, but, importantly, it has no phosphoseryl clusters.

Interaction properties: Self-association

Individual caseins in solution exhibit strong tendencies to self-associate, and the form of the structure adopted reflects both the distribution of hydrophobic/hydrophilic residues described earlier and the strictures applied by the demands of the highly charged phosphoseryl clusters wanting to be as spatially distant as possible from one another. Thus, β-casein, which resembles a detergent molecule with a hydrophilic head and a hydrophobic tail, forms detergent-like micelles, as in **Figure 2**, with the tails forming a central core and the highly charged hydrophilic heads sticking out into the aqueous phase, like the bristles of a hedgehog. Similarly, α_{S1}-casein self-associates in solution to form a wormlike chain

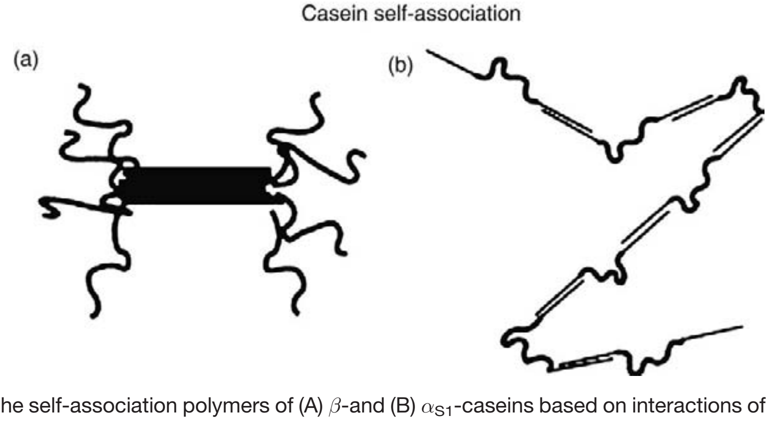

Figure 2 Structures of the self-association polymers of (A) β-and (B) α_{S1}-caseins based on interactions of their hydrophobic regions. Beta-casein forms detergent-like micelles, whereas aS1-casein molecules form a chain polymer.

polymer with the hydrophobic ends of different molecules interacting as depicted in **Figure 2**. κ-Casein also self-associates in solution, interacting via its hydrophobic N-terminal region, but here, polymer growth is also influenced by the intermolecular disulfide linkages produced by reaction of its cysteine residues.

For α_{S1}-casein and β-casein, it was demonstrated that the size of the α_{S1}-casein polymer or the β-casein micelle produced by self-association depends on pH and ionic strength, and also sensitively on temperature in the case of β-casein. Temperature is important for the strength of hydrophobic interaction but pH and ionic strength govern electrostatic repulsion and its range. The balance of these attractive and repulsive components in the overall interaction free energy equation thus controls aggregate size.

Interaction properties: Calcium binding and calcium-induced precipitation

This is also confirmed in the precipitation of the individual caseins induced by the addition of calcium chloride, a direct consequence of the calcium-binding properties of the molecules. The extent of calcium binding is directly related to the number of phosphoserine residues in the molecules. Thus, α_{S2}-, α_{S1}-, and β-casein have high binding capacities for calcium (and other divalent cations), and their order of sensitivity to calcium-induced precipitation reflects this capacity. κ-Casein with only one, occasionally two, phosphoserines binds little calcium, does not precipitate in its presence, and in mixtures with other caseins, restricts the growth of aggregates to colloidal dimensions. Mistakenly in the past, these aggregates have often been referred to as casein micelles. They contain no inorganic phosphate and are not casein micelles.

Precipitation is the direct result of the reduction on the negative charge of the protein molecule consequent on binding a number of calcium cations. The rate of calcium-induced precipitation can be quantitatively related to the square of the net protein charge, $Q = Z_0 + 2\nu$, where Z_0 is the protein charge carried at the pH and ionic strength of interest and ν is the number of Ca^{2+} bound under those solution conditions for the calcium ion concentration added. This relationship also holds when the charge on the protein is changed by chemical modification. Thus, converting a positively charged lysine residue into a neutral or even negatively charged residue increases the negative protein charge by one or two units and decreases the rate of calcium-induced precipitation accordingly. If the chemically modified residue becomes more hydrophobic, an enhancement of the rate of precipitation may also be observed, indicating that, as in self-association, attractive hydrophobic interactions also play a part in moderating the overall interaction potential governing the aggregation process.

If phosphate is introduced into the mix prior to the addition of calcium, another precipitation pathway is followed, with the phosphate playing an active role in this. This observation has important consequences for micellar assembly and formation.

Casein Micelle Properties

Almost all of the casein proteins present in bovine milk are incorporated into the casein micelles, together with a high proportion of the available calcium and inorganic phosphate. These micelles have average molecular weights of $\sim 10^8$ Da and mean diameters of ~ 100 nm (range 50–600 nm). The micelles are very open, highly hydrated structures with typical hydration values of 2–3 g $H_2O\,g^{-1}$ protein, depending on the method of measurement. The structure is not rigidly fixed but dynamic. The soluble casein level is strongly type and temperature dependent. Cooling from an udder temperature of 37 °C to refrigeration temperatures during storage brings about solubilization of a significant fraction of β-casein, some κ-casein, and much lower levels of α_{S1}- and α_{S2}-caseins. Raising the temperature back to 37 °C reverses this process. Almost complete disintegration of the micelles can be achieved either through the addition of a strong calcium sequestrant, such as ethylenediaminetetraacetic acid (EDTA), or through the addition of high concentrations of urea. Dissociation to molecular level is not achieved, however, and the dissociated species have average diameters on the order of 10–15 nm and are also of variable composition.

Micelle Size

Unless something is done to disintegrate or destabilize the casein micelle, average micelle size remains constant with the storage time of the milk. It does not change significantly on cooling or following mild heat treatments such as pasteurization. If an aggregation process forms the micelles, then some compositional factor must limit their growth to the size range observed.

Casein micelles can be fractionated from the initial wide size range found in milk by subjecting the milk to centrifugation steps of increasing speed and duration. The pellet may be resuspended in ultrafiltrate for sizing by dynamic light scattering and its composition and mineral content determined. The supernatant is used as the sample for the succeeding step. Largest micelles are found in the pellets produced by the slowest speed and shortest time combinations, the size decreasing as duration and speed increase.

Such experiments demonstrate that the proportion of α_{S1}- and α_{S2}-caseins is independent of micelle size and that κ-casein content is inversely proportional to micelle size. Changes in colloidal calcium phosphate content per mole of casein are consistent with the increasing content of κ-casein and the compensating decrease in β-casein fraction with decreasing micelle size. For a solid object such as a sphere, the surface-to-volume ratio is inversely proportional to size (radius), implying that the κ-casein component resides on the micelle surface, where it controls micellar surface area and hence micellar size. The results also imply that colloidal calcium phosphate and the other caseins are found primarily in the micellar core, contributing only to its volume.

Micellar Stability

The technological properties of milk are strongly influenced by the stability of the casein micelles. Deliberate destabilization of the casein micelles resulting in coagulation or gelation is exploited in the production of a range of dairy products, including all cheese varieties and fermented milk. Micellar destabilization can also be induced by addition of ethanol or any other organic solvent, or by extreme heat treatments, where its occurrence can create serious problems during processing (e.g., during the sterilization of concentrated milks) or storage (e.g., age gelation of ultra-high temperature (UHT)-treated milks).

Much research activity has centered on the curd formation reaction, which is the first stage in cheese-making. The κ-casein molecules that make up the outer surface of the micelle are thought to provide a steric stabilizing (hairy) layer, with their hydrophilic C-terminal peptides protruding into the aqueous phase. Curd formation is a direct consequence of the proteolysis of the κ-casein by chymosin, the casein being specifically cleaved at the Phe_{105}–Met_{106} bond, releasing the hydrophilic peptide and destabilizing the micelle. The gradual loss of peptide is accompanied by a decline in the micellar zeta potential from ~ -20 to ~ -10 mV. Before the aggregation events, the lower levels of κ-casein proteolysis are accompanied by a decrease in micellar hydrodynamic size as the hairy layer is shaved off enzymatically. Similar decreases in micellar size are seen at concentrations of ethanol too low to induce micellar destabilization, where the hairs are thought to collapse and fold in the nonsolvent mixture. Proton nuclear magnetic resonance (NMR) studies also indicate that the flexibility of the κ-casein hairs is diminished in the presence of ethanol.

Micellar Appearance

Viewing the casein micelle as a hairy sphere, sterically stabilized by its bristly coat, is adequate to explain many of the technological properties. Direct visualization by electron microscopy has provided a few options, some probably now recognized as artifacts of sample preparation. In early micrographs, the casein micelle strongly resembles a raspberry. This gave rise to the notion that the micelle is itself an assembly of subunits of defined structure, each with an estimated diameter of $\sim$15 nm. Neutron and X-ray scattering measurements can also be interpreted to support these notions. Transmission electron micrographs appearing in the literature in the last few years show the casein micelle as a diffuse structure with numerous small regions of high scattering density, akin to the appearance of currants in a bun. Interestingly, these currants disappear when pictures are taken at pH values around 5.5, their disappearance accompanied by the loss of the shoulder in the X-ray scattering profile apparent at high pH. It is now accepted that the regions of high density are calcium phosphate nanocrystals, spatially separated in the micelle by an average of $\sim$10–15 nm, which are solubilized and disappear when milk pH is reduced.

Micelle Models

There is no universally accepted model for the structure of the casein micelle. The major contenders are the subunit model, the Holt model, and the dual-binding model of Horne, introduced to overcome criticisms leveled at the first two.

Submicelle Model

Drawings representing the casein micelle as an agglomerate of spherical submicelles appear in all dairy science textbooks. It is suggested that the submicelles are linked together by calcium phosphate. To produce the κ-casein outer coating for the casein micelle, two different subpopulations of subunits must be created. Those rich in κ-casein provide the outer reaches of the structure, and those devoid of κ-casein form the micellar core. While this broadly satisfies the requirements of a picture of the micellar structure in terms of the largely discredited early electron micrographs, no mechanism has been proposed to account for the assembly of these subunits or to suggest a driving force for the creation of subunits of different composition. It is this latter failing – a failure to explain why κ-casein should associate with the other caseins to form the external submicelles but be totally excluded from the interior ones composed of the same caseins – that is the main criticism of the submicelle model, although other failings are apparent on deeper examination.

Holt Model

The Holt model suggests that the nodes for micellar assembly are calcium phosphate nanoclusters dispersed through the micelle. These interact with the phosphoserine clusters of the casein molecules. Because some of the individual caseins, α_{S1}- and α_{S2}-casein, have more than one such grouping, cross-linking and network formation are possible to give a microgel particle. Accepting the nanoclusters as providing regions of high scattering density, this model provides an explanation for the heterogeneity in appearance and scattering behavior of the micelle. Unfortunately, there is no inherent mechanism in this model that limits gel growth. It could continue until the milk was composed of a single giant micelle, a macrogel. There are vague suggestions of limitation by substrate exhaustion, and ring closure remains a possibility, but the chief criticism of this model is that it has no role for κ-casein. κ-Casein has no cluster of phosphoserines to bind with the nanoclusters, and consequently the Holt model has no explanation for the observed surface location of κ-casein in the natural micelle.

Dual-Binding Model for Micelle Assembly and Structure

In the dual-binding model, micellar assembly and growth take place by a polymerization process involving, as the name suggests, two distinct forms of bonding. These are cross-linking through hydrophobic regions of the caseins or through bridging across calcium phosphate nanoclusters. Central to the model is the concept that micellar integrity, and hence stability, is maintained by a localized excess of hydrophobic attraction over electrostatic repulsion. The individual casein molecules behave and interact as they did in their self-association equilibria described previously. Each casein molecule effectively functions as a block copolymer, as detailed in **Figure 1**, with each block possessing different and, possibly, multiple functionality for the cross-linking paths.

Thus, α_{S1}-casein can chain polymerize through the hydrophobic B blocks, through the hydrophobic clusters dispersed along this region, giving the observed wormlike chain of **Figure 2**. Further growth here is limited by the electrostatic repulsion of the hydrophilic regions, but in the casein micelle, the clusters of phosphoserine residues located therein form a template for the growth of calcium phosphate nanoclusters or for termination of a facet of their growth. Neutralization of the phosphoserine negative charges through incorporation into the calcium phosphate nanocluster reduces that electrostatic repulsion component and permits further molecules to enter and become hydrophobically linked to blocks B upstream or downstream of the nanocluster link, providing cross-links to other polymerization paths (**Figure 3**). Multiple protein binding into each nanocluster also exists, allowing a different network pathway to be built up. β-Casein, with only two blocks, the phosphoserine cluster (P) and the hydrophobic region (B), can form polymer links into the network through both and thus chain growth can extend through these. α_{S2}-Casein is envisaged in this model as having two of each block (**Figure 1**), two phosphoserine clusters, and two hydrophobic regions. It is only a small fraction of the total casein but, by being able to sustain growth through all its blocks, it is likely to be bound tightly into the network. κ-Casein is the most important of the caseins in this dual-binding model of micellar

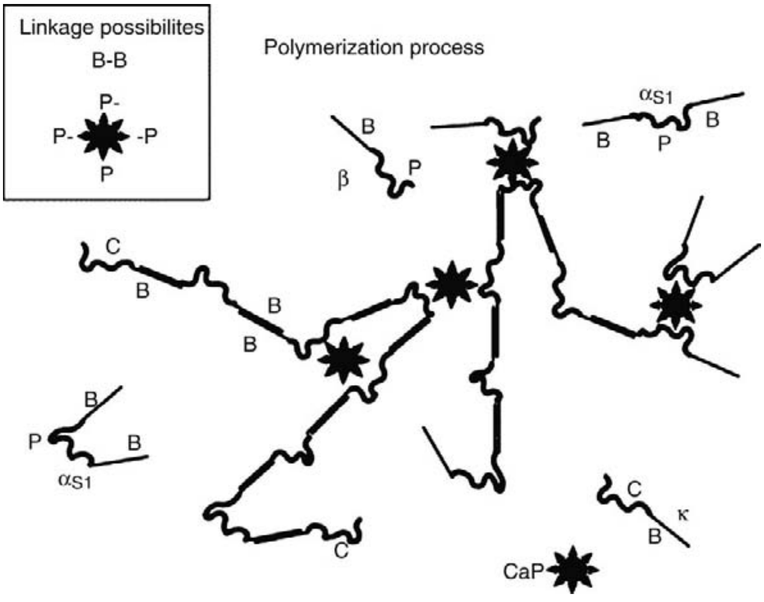

Figure 3 Schematic depiction of the conformations adopted by the caseins on adsorption at a hydrophobic interface. Those for α_{S1}-and β-caseins are based on calculated predictions from self-consistent ® eld theories; the diagrams for α_{S2}- and κ-caseins were developed by analogy. Hydrophobic regions (B) are represented by the bar structures, which do not imply any form of rigidity; hydrophilic regions containing phosphoserine clusters are denoted by P and the hydrophilic caseino-macropeptide of κ-casein by C.

assembly and structure. It can link into the growing chains through its hydrophobic N-terminal block, but its C-terminal block (denoted C in **Figure 1**) has no phosphoserine cluster and therefore cannot extend the polymer chain through a nanocluster link. Chain and network growth are therefore terminated here, leaving the casein micelle network with an outer layer of κ-casein molecules, a prime requirement for any structural model.

A major failing of the other micelle models is their lack of a plausible mechanism for assembly, growth, and more importantly termination of growth. All of these elements are in place in the dual-binding model. Furthermore, the concept of bonding occurring as a result of a localized excess of hydrophobic attractions over electrostatic repulsions successfully accommodates the response of the micelles to changes in pH, temperature, urea addition, or removal of calcium phosphate by sequestrants. Urea disrupts the hydrophobic bonds, bringing about micellar disintegration. Removal of calcium from the calcium phosphate nanocluster by EDTA addition restores the negative charge of the hydrophilic region and shifts the hydrophobic attraction/electrostatic repulsion balance in favor of repulsion and the micelle breaks up. Lowering the milk pH solubilizes the calcium phosphate, but the micellar negative charge is also titrated away, leaving the micelle largely intact. If the pH of that milk is restored by dialysis against milk at its natural pH, then the micelles of the treated milk again fall apart. Dropping the temperature decreases the level of hydrophobic attraction; any β-casein not linked through its phosphoserine cluster could then be released into the serum phase, behavior of the type shown by the breakup of the detergent-like micelles of β-casein (**Figure 2**) on lowering the solution temperature.

The dual-binding model also satisfies the appearance and scattering behavior shown by the native micelle. It reproduces the heterogeneity in structure required by X-ray and neutron scattering data. The dense calcium phosphate nanoclusters are seen as currants in the micellar 'bun' in recent electron micrographs, separated by a correlation length of some 10–15 nm estimated from X-ray scattering spectra. Also, loss of the currants from their micelle electron micrograph on lowering the pH and the parallel disappearance of the shoulder feature in the X-ray spectrum were demonstrated, while micelle integrity was maintained at this pH. The model can also accommodate the production of the raspberry-like appearance created artifactually in early micrographs, through the collapse of loosely linked more mobile regions onto those more densely cross-linked. Also, disintegration of the micelles into pieces by random breakage of bonds could lead to segments varying in composition. Those originating from surface regions would be rich in κ-casein, while those originating internally would lack κ-casein, but this is a one-way path. There is no requirement for these structures to be formed as a stage along the route to the creation of the casein micelle, as is necessary in the submicelle model.

Because the dual-binding model naturally provides a surface location for κ-casein in a growth-limiting role, it readily explains the destabilization of the micelle system on proteolysis of the κ-casein by chymosin and the loss of the steric-stabilizing layer. Indeed, once it is recognized that the proteolysis reaction affects only the surface regions and that internal micellar integrity is unaffected, the adhesive sphere model of rennet-induced gelation emerges as a special case of the dual-binding model. Limitations in the adhesive sphere aggregation model become apparent for acid-induced gelation because here there are effects on micelle integrity. These can, however, be accommodated very successfully within the framework of the dual-binding model. Similarly, the action of ethanol in collapsing the κ-casein hairs, which superficially follows the adhesive sphere model, requires the complexity of the dual-binding model when the nuances of this behavior are studied. Not enough is known about heat-induced destabilization of the micellar system, its aggregation pathway, and its chemistry, to test the ability of the dual-binding model to predict and reproduce such behavior. Dealing with the complexity of the many effects of heating milk remains a challenge for the future.

The dual-binding model imposes no specific requirements on the chemistry of the caseins at the amino acid level. It also has no demands for a defined secondary structure. The requirements are solely that the casein molecules be amphiphilic and that the majority possess a phosphoserine cluster. Major differences in composition between species can thus be tolerated with no loss of ability to assemble micelles from the proteins synthesized. In the milk of goats with the null variant of α_{S1}-casein, micelles are still found because the other caseins are present at a sufficient amount to construct the network. Again, in caprine milks with the α_{S1}-casein F variant with the deleted phosphoserine cluster sequence, micelles are again obtained. The model would predict that some of this α_{S1}-F would have a surface location. Although the levels of expression of this variant are low, measurements of casein composition variation with micelle size for milks with this protein indicate behavior consistent with such a location. Reports also exist of mare's milk that does not gel on treatment with chymosin. Such milk contains no κ-casein but still has micelles. Here, the sterically stabilizing entity is a dephosphorylated β-casein. Without a phosphoserine cluster, it cannot enter into chain polymerization through this link and hence fulfills the role of the missing κ-casein as a chain terminator. There is also the possibility that dephosphorylated β-casein could act in a similar fashion in human milk, where large numbers of small micelles are encountered. The dual-binding model also successfully accommodates the changing composition of marsupial milk and its partial disintegration in the presence of EDTA.

See also: **Cheese**: Rennet-Induced Coagulation of Milk. **Heat Treatment of Milk**: Heat Stability of Milk. **Milk Protein Products**: Functional Properties of Milk Proteins. **Milk Proteins**: Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; Interspecies Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity. **Milk Salts**: Interaction with Caseins.

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α-Lactalbumin

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Introduction

α-Lactalbumin is a major protein component of the whey fraction of bovine milk and was first isolated more than 70 years ago. Because of its relative abundance and ease of purification, α-lactalbumin served as a model for the early development of methods for investigating the chemical and biophysical properties of proteins. Although initially it had been considered to have only nutritional significance, in 1967–68 it was discovered that α-lactalbumin has a key function in the biosynthesis of lactose and the production of the aqueous phase of milk. Contemporaneous structural studies also revealed that bovine α-lactalbumin is similar in amino acid sequence and disulfide bond arrangements to type-c lysozymes from birds and mammals. α-Lactalbumin was the subject of an early exercise in homology-based modeling, which showed that its amino acid sequence is consistent with a close similarity in three-dimensional structure to lysozyme; this early model was surprisingly correct, but this was not confirmed until the first three-dimensional structure of an α-lactalbumin was determined in 1989. The common ancestry of the genes for α-lactalbumin, which is found only in mammals, and lysozymes, which are ubiquitous in eukaryotes, suggests that the α-lactalbumins developed through duplication of an ancestral lysozyme gene followed by mutational divergence that led to the development of a new protein function. This gene duplication event, which is believed to have been a key evolutionary step in the development of the unique ability of mammals to synthesize lactose and produce copious milk secretions, must have occurred early in mammalian evolution because the echidna, a representative of the egg-laying monotremes, expresses an α-lactalbumin in its milk, although it is present at relatively low concentrations; it is even possible that an early form of lactation developed in a premammalian synapsid. Unlike many of the type-c lysozymes, including the well-characterized human and chicken proteins, α-lactalbumin contains a tightly bound Ca^{2+}, which has been found to have a crucial role in its structure and function. At first, this appeared to be a unique property of α-lactalbumin, but further research has identified families of lysozymes that also contain a structural Ca^{2+} bound at the same site. The evolutionary relationship of α-lactalbumins and lysozymes is complicated by the existence of other lysozyme homologues that, like α-lactalbumin, have diverged functional properties.

Structure and Metal Binding

Bovine α-lactalbumin is synthesized as a pre-protein of 142 amino acids of which the first 19 form a secretion signal sequence, which is removed to produce the mature protein of 123 residues, which has a molecular weight of 14 128. Its polypeptide chain contains eight cysteines that are connected by four intramolecular disulfide bonds. A small fraction of bovine α-lactalbumin molecules are N-glycosylated at asparagine 71; this is a rare protein N-glycosylation site, which has the sequence Asn-Ile-Cys, as compared with the 'normal' N-glycosylation sequence Asn-X-Ser/Thr. The polypeptide chains of the mature α-lactalbumins from other mammalian species range in length from 121 to 140 amino acid residues. Their sequences differ in degrees that increase with the evolutionary separation of the species. The greatest difference may be between α-lactalbumins from eutherian mammals and those from monotremes, which have less than 50% identity in sequence.

A tightly bound Ca^{2+} is present in all α-lactalbumins that have been investigated; this metal ion is not required for its function in lactose biosynthesis, but has an important role in structure and stability. The calcium can be removed at low pH or using chelating agents to produce the apo-protein. The binding of Ca^{2+} to apo-α-lactalbumin is very tight under physiological conditions with a K_a in the order of $10^7\,\text{mol}\,l^{-1}$. At low ionic strength and physiological temperatures and pH, apo-α-lactalbumin has a molten globule structure, a state characterized by the presence of a large proportion of secondary structure but no fixed tertiary structure. A transition to the molten globule state of α-lactalbumin is promoted also by low pH, denaturants, and detergents. This form of α-lactalbumin is fairly compact and, unlike the native protein, can bind some hydrophobic dyes, such as

8-anilino-1-naphthalene sulfonic acid (ANS), and lipids. Stable partially folded states are relatively rare in proteins, although unfolded proteins and domains are not uncommon. Partially folded states can serve as models for intermediate states in protein folding processes, and the structure and stability of the α-lactalbumin molten globule state has been the subject of much research. As discussed below, this form of α-lactalbumin may have some biological and biomedical relevance.

At this time, X-ray crystallographic structures have been determined for the α-lactalbumins from cow, goat, human, baboon, and mouse, in different crystal forms and complexes with metals, and with bovine β-1,4-galactosyltransferase-1 and various ligands. These structures are similar to those of the related type-c lysozymes, having what is classified as an α plus β fold that consists of two lobes of structure, an α-helical lobe, and a β-sheet lobe that are separated by a cleft (**Figure 1**). The cleft corresponds to the open active-site cleft in the lysozymes that can accommodate an extended oligosaccharide substrate but, in α-lactalbumin, the cleft is closed at one end. The larger α-helical lobe is formed by the amino- and carboxyl-terminal sections of the polypeptide chain (residues 1–34 and 86–123), whereas the smaller β-lobe, which encompasses a small three-stranded antiparallel β-sheet, a short 3_{10} helix, and some irregular structure, is formed by residues 35–85 that represent the central section of the polypeptide chain.

The primary binding site for calcium ions (see **Figure 1**) is located in a loop of structure close to the junction between the two lobes, which has a high content of aspartic acid residues. The consensus amino acid sequence of this region in different α-lactalbumins is Lys^{79}-h-h-Asp-Asp-p-h-Asp-Asp^{88}, where h is a hydrophobic amino acid, and p, a polar amino acid such as Asp, Glu, or Asn. The Ca^{2+} coordinates with the carboxyl groups of aspartates 82, 87, and 88, the peptide carbonyl groups of Lys79 and residue 84, and two water molecules. The structures of calcium-binding lysozymes from echidna, horse, and dog have also been determined and show similar coordination fields. α-Lactalbumin can bind a second Ca^{2+} with lower affinity and an array of other metals including manganese (Mn^{2+}), zinc (Zn^{2+}), and lanthanides. The second Ca^{2+} binds at a site adjacent to the primary calcium-binding site (**Figure 1**), and Mn^{2+} appears to bind at a site similar to the binding site

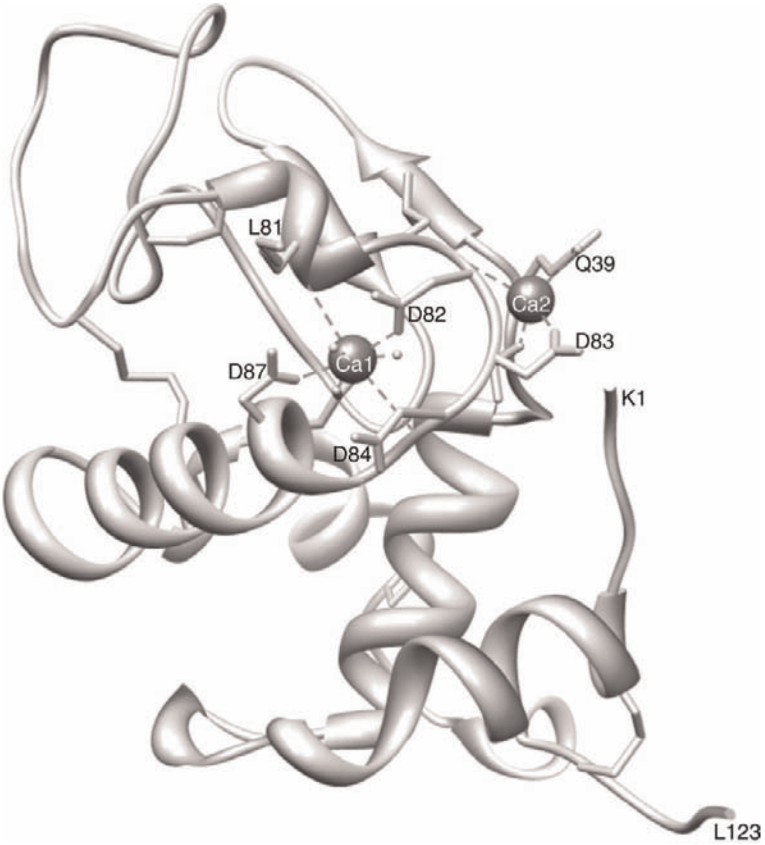

Figure 1 Ribbon structure of human α-lactalbumin complexed with two Ca^{2+}. The picture was generated from 1A4V.pdb using the graphics program CHIMERA. The side chains of residues that interact with the two Ca^{2+} are displayed and labeled together with the amino- and carboxy-terminal residues of the polypeptide chain.

of the second Ca^{2+}. Zn^{2+} promotes dimerization of α-lactalbumin by simultaneously interacting with two α-lactalbumin molecules. The full biological significance of Ca^{2+} binding to α-lactalbumin is not entirely clear. It is required for generating a functional α-lactalbumin molecule, because in its absence unfolded preparations of α-lactalbumin with reductively cleaved disulfide bonds are unable to fold to the native structure with appropriate pairing of cysteines in disulfide bonds, suggesting that it is required *in vivo* for the correct folding of α-lactalbumin in the endoplasmic reticulum following translation.

Apo-α-lactalbumin has a stable native structure at high ionic strength, and an X-ray crystallographic structure of bovine apo-α-lactalbumin has been determined. A comparison with the structure of the holo-protein shows that the overall structures are closely similar with only minor adjustments in the structure around the metal-binding site. However, a significant change was found on the opposite face of the protein that reflects an increased separation of the α-helical and β subdomains, probably as the result of a change initiated in the Ca-binding site that causes a rearrangement of α-helix 3. This change opens up the structure around Tyr103 and suggests that Ca^{2+}-binding may enhance folding by promoting the formation of long-range interactions between residues of the two subdomains – a possible rate-limiting step in the folding process.

Function in Lactose Synthesis

α-Lactalbumin acts as the regulatory protein of lactose synthase (EC 2.4.1.22), a role in which it modulates the affinity of the catalytic component UDP-galactose β-*N*-acetylglucosaminide β1,4-galactosyltransferase-1 (β4-galactosyltransferase-1; EC 2.4.1.38), for acceptor substrates through a reversible protein–protein interaction. Galactosyltransferase-1 is a member of a family of seven homologous galactosyltransferases (β4-galactosyltransferase-1 to β4-galactosyltransferase-7) found in mammals, which have somewhat overlapping specificities and vary in tissue distribution; this family of glycosyltransferases is ancient, being present in invertebrates as well as vertebrates. Like most mammalian glycosyltransferases that function in the processing of glycoproteins and glycolipids, β4-galactosyltransferase-1 encompasses a short N-terminal cytoplasmic domain, a transmembrane hydrophobic α-helix, a stem region, and a C-terminal catalytic domain. Forms of β4-galactosyltransferase-1 are expressed in most secretory cells including mammary epithelial cells, and it is an intrinsic component of the trans-Golgi membranes. In most cells, it catalyzes the transfer of galactose from UDP-galactose into a β-linkage with the 4-hydroxyl group of nonreducing terminal β-linked *N*-acetylglucosaminyl residues in glycan chains (**Figure 2**). Although β4-galactosyltransferase-1 can catalyze lactose synthesis (the transfer of galactose to free glucose) *in vitro*, it is ineffective as a catalyst for this reaction under physiological conditions because of its low affinity for glucose, reflected in a K_m of about 2 mol l^{-1}. α-Lactalbumin, which appears to be expressed only in the lactating mammary gland, binds reversibly with β4-galactosyltransferase-1 to form 1:1 complexes with a concomitant 1000-fold increase in its affinity for glucose. This interaction, which allows the efficient production of lactose at physiological concentrations of glucose, occurs in the lumen of the Golgi apparatus where β4-galactosyltransferase-1 is a relatively stationary component of the membranes. α-Lactalbumin is a secreted protein and becomes a transient soluble component of the contents of the Golgi lumen during its passage through the cell. This arrangement couples lactose synthesis with continued milk protein synthesis during lactation and results in the production of lactose in the lumen of the Golgi apparatus. As lactose cannot cross the Golgi membranes, and is a major osmole in milk, it also results in a flow of water into the Golgi lumen, which is important for the formation of the aqueous phase of milk. Lactose and α-lactalbumin are both secreted from lactating mammary epithelial cells into milk by packaging into secretory vacuoles whose contents are released from the cell by exocytosis. The importance of α-lactalbumin for lactation is highlighted by studies with transgenic animals. In mice, inactivation of the α-lactalbumin gene results in the production of thick milk, devoid of lactose, and an inability to sustain offspring, while replacement with a human α-lactalbumin gene restored lactose synthesis and milk production. Among the promising developments in this area is the observation that transgenic pigs that overexpress bovine α-lactalbumin show enhanced lactose levels in milk, increased milk production, and improved piglet growth rates.

The gene for β4-galactosyltransferase-1 is transcribed as two mRNA species by initiation at two different sites: a longer form associated with a general housekeeping promoter and a shorter form from a mammary-specific promoter. The utilization of the second promoter allows the synthesis of high levels of β4-galactosyltransferase-1 protein in the lactating mammary gland through enhanced transcription and as the result of the translation of a shorter polypeptide chain. Thus, the ability of most mammalian species to produce large quantities of lactose and sufficient milk to nurture their offspring is associated with adaptational changes in both proteins of the lactose synthase system.

Alternative Structures, Apoptosis, and Mammary Involution

The concept that proteins often have multiple functions is now broadly accepted, and research during the past decade has drawn attention to a biological activity in

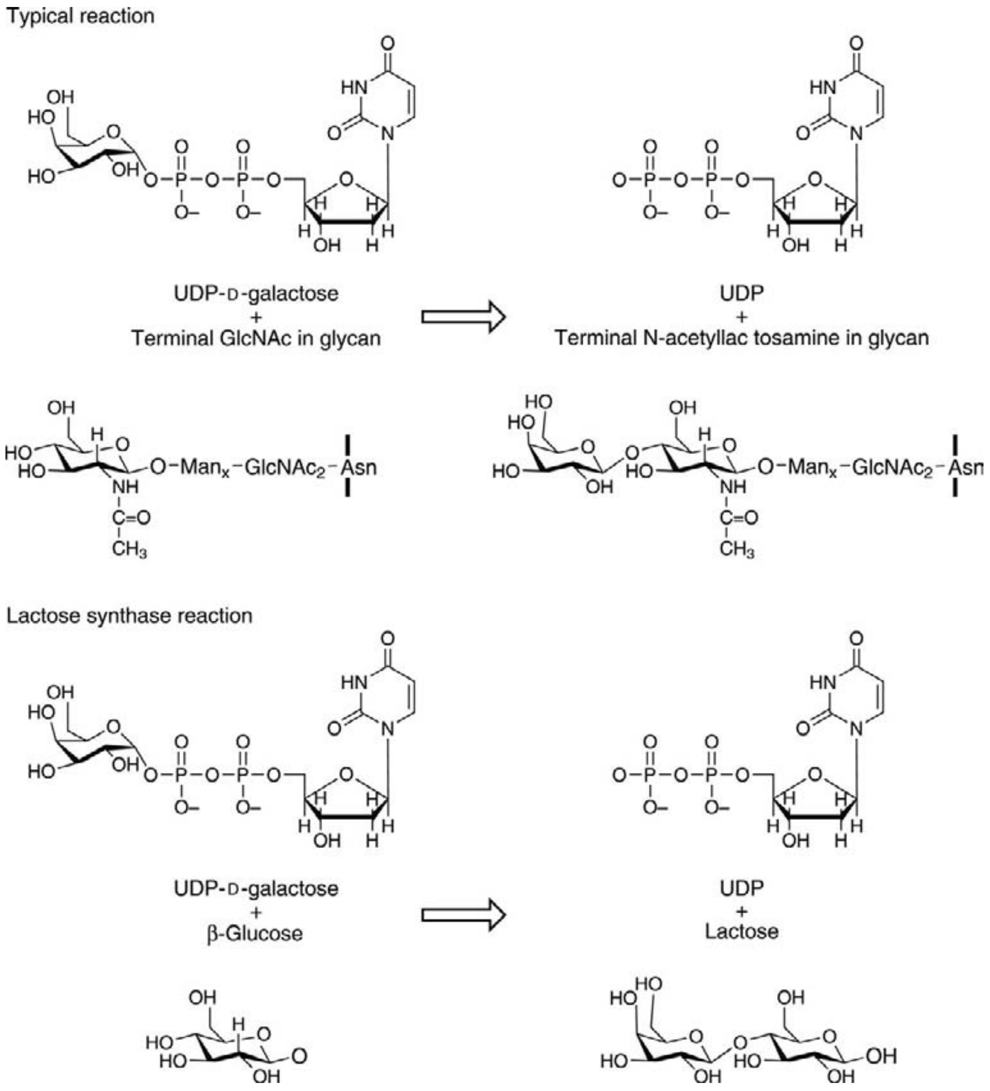

Figure 2 Reactions catalyzed by β4-galactosyltransferase-1, alone and lactose synthase.

α-lactalbumin that is distinct from its role in lactose biosynthesis. The partially folded molten globule state of α-lactalbumin, which has more exposed nonpolar side chains, has been the subject of many studies. Human milk was found to have apoptotic effects on a human lung cancer cell line, and this activity was found to be associated with a multimeric form of α-lactalbumin isolated from the casein fraction. Further work showed that this molecule can be generated from human apo-α-lactalbumin by chromatography on an ion-exchange column that has been conditioned with oleic acid. This form of α-lactalbumin, apparently a non-native state stabilized by complex formation with oleic acid, has been found to selectively induce apoptosis in tumor cells and has been designated HAMLET (Human α-lactalbumin made lethal to tumor cells). It has generated interest as a novel approach for cancer treatment. The mechanism through which it triggers apoptosis involves interaction with mitochondria to promote the release of cytochrome c, after transfer into the cell, followed by activation of caspases; it has also been shown to disrupt chromatin in the nucleus by binding to histones. HAMLET appears to have a more native structure than the molten globule state but differs from both native and molten globule forms. Although it can bind Ca^{2+}, like native α-lactalbumin, it differs in undergoing irreversible denaturation by heat or urea with the loss of its apoptotic activity.

Bovine α-lactalbumin has been reported to reduce the viability of bovine mammary epithelial cells and to induce apoptosis in Cape fur seal mammary cells. Involution of the mammary gland in most mammalian species generally occurs with the termination of suckling when milk accumulates in the ducts of the mammary gland; this appears to be regulated locally and, besides physical damage to tissue produced by increased pressure, it has been suggested that a factor or factors in the accumulated milk may trigger mammary gland involution following weaning. Recently, it has been proposed that α-lactalbumin may function as an inducer of involution based on its pro-apoptotic effects, as in the unusual case of the Cape fur seal. These otariid seals produce milk that is devoid of lactose but with high levels of protein and lipid. After giving birth, female fur seals develop a pattern of intensive suckling of their pups for 2–3 days on land with copious quantities of rich milk, followed by extended foraging trips lasting up to 23 days. In most mammals, a period of 23 days without milk removal would result in apoptosis and involution of the mammary glands, but this does not occur in the fur seal. In these animals, the α-lactalbumin gene is mutated so that little or no protein is produced. Also, the protein product is shorter than normal, containing only 104 amino acid residues so that it lacks amino acids that are essential for its activity in lactose synthesis and formation of a stable molten globule state. It has been proposed that these changes prevent apoptosis triggered by α-lactalbumin so that mammary function can be retained during the long intervals between suckling activities. These are interesting observations that suggest a possible regulatory role for α-lactalbumin in terminating lactation, in addition to its established function in initiating and maintaining lactose synthesis. However, the evidence is circumstantial and involves extrapolation from what may be a unique adaptation in one species to mammals in general.

Cetaceans (whales and dolphins) also produce milks that have high contents of lipid and proteins but low levels of lactose; their lactation time varies in length between 5 months and 3 years. Parts of the amino acid sequence of α-lactalbumin are known for many species in this order, and all differ from other known α-lactalbumins in having a unique unpaired cysteine at position 36. This could have a major effect on structure and/or activity but, at present, there are no reports regarding the properties of any α-lactalbumin from this group of mammals.

Molecular Basis of the Action of α-Lactalbumin in Lactose Synthesis

α-Lactalbumin interacts with β4-galactosyltransferase-1 only in the presence of substrates, forming 1:1 complexes in the presence of glucose, *N*-acetylglucosamine, or a combination of the metal cofactor, Mn^{2+}, and UDP-galactose (or other UDP-sugar). Synergistic binding of α-lactalbumin and glucose to the galactosyltransferase, reflected in mutual stabilization of complexes, results in a reduction of the K_m for glucose by about three orders of magnitude. Thus, the presence of α-lactalbumin changes glucose from being a very marginal substrate to a good substrate. In contrast, disaccharide substrates such as diacetyl chitobiose, or even a β-glycoside of GlcNAc, and α-lactalbumin bind in a mutually exclusive manner to galactosyltransferase-1; so α-lactalbumin is a competitive inhibitor of galactose transfer to such substrates. Mutational studies showed that Phe31, His32, Leu110, Gln117, and Trp118 are important residues in α-lactalbumin action, the first three of these having an effect on glucose binding while the latter two specifically stabilize the protein–protein association. X-ray crystallographic structures of various complexes of recombinant forms of mouse α-lactalbumin and the catalytic domain of bovine galactosyltransferase-1 have now been elucidated. The catalytic domain of β4-galactosyltransferase-1 has a GT-A fold, one of the two predominant classes of structure found among nucleotide-sugar utilizing glycosyltransferases. It consists of a central twisted β-sheet, containing both parallel and antiparallel strands, surrounded by α-helices. In the lactose synthase complex, 1310 $\mathring{A}^2$ of the combined surface area from the two proteins becomes buried in their interaction interface, amounting to 20% of the accessible surface in α-lactalbumin and 11% surface in β4-galactosyltransferase-1. From **Figure 3**, it can be seen that residues identified by mutagenesis as important for the action of α-lactalbumin in lactose synthesis, Phe31, His32, Met110, Gln117, and Trp118 (in the mouse variant Met replacing Leu110 of bovine α-lactalbumin), interact with a largely nonpolar region of β4-galactosyltransferase-1 comprising Phe280, Tyr286, Gln288, Tyr289, Phe360, and Ile363. The D helix, formed from residues 105 to 111 of mouse α-lactalbumin, interacts with a helix (residues 359–365) of β4-galactosyltransferase-1. Although the donor substrate (UDP-gal) stabilizes the complex between the two proteins, it does not interact directly with α-lactalbumin in the complex. Instead, the binding of UDP-galactose to β4-galactosyltransferase-1 induces a rearrangement of residues 345–365, including the formation of an α-helix by residues 359–365 and a reorientation of the side chain of Trp314. When *N*-acetylglucosamine binds an acceptor substrate, a hydrophobic pocket formed by Arg359, Phe360, and Ile363 interacts with the 2-acetamido group. Glucose has a hydroxy rather than acetamido at the 2-position, and in the complex of α-lactalbumin and glucose, α-lactalbumin binds to the hydrophobic pocket and interacts directly with glucose acceptor substrate by H-bond formation between the Nδ1 of the imidazole group of His32 and the C-1 and C-2 hydroxy groups of glucose. This substrate-induced structural change in β4-galactosyltransferase-1 is consistent with kinetic studies, which indicate that substrates bind to the enzyme in an obligatory ordered manner, donor substrate prior to acceptor, while the interaction of α-lactalbumin with the C-1 hydroxy group of glucose provides a molecular mechanism for the switch between synergistic binding of α-lactalbumin and acceptor in the case of monosaccharides and mutually exclusive binding in the case of oligosaccharide or glycoside acceptor substrates. α-Lactalbumin lowers the K_m values of other weak-binding acceptor substrates of galactosyltransferase-1, including

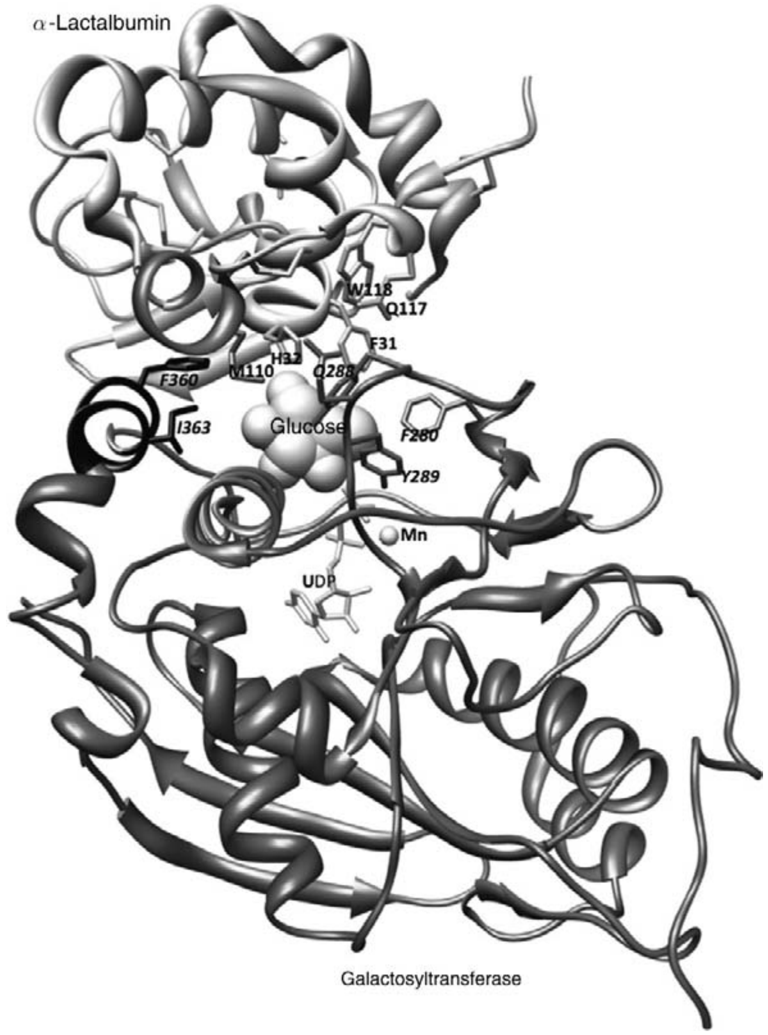

Figure 3 Ribbon structure of mouse α-lactalbumin in a complex with the catalytic domain of bovine β-4-galactosyltransferase-1 in the presence of glucose, Mn^{2+}, and UDP-*N*-acetylgalactosamine. The image was generated from 2FYD.pdb using CHIMERA. The side chains of residues from the two proteins that are involved in their interaction and in binding glucose are displayed and labeled, those from β-4-galactosyltransferase-1 are italicized. The helices from the two proteins that are important in the interaction are shaded more darkly than the rest of the structures.

xylose and cyclitols, and promotes the transfer of monosaccharides from marginal donor substrates such as UDP-glucose, presumably by stabilizing catalytically competent complexes of such substrates with β4-galactosyltransferase-1. Consequently, the α-lactalbumin–galactosyltransferase-1 system has practical applications in the enzymatic synthesis of novel glycans.

Conclusion

Despite about four decades of research following the discovery of the primary function of α-lactalbumin in lactose synthesis and the initial realization of its structural similarity to lysozyme, several questions remain regarding its function and properties. One significant issue discussed above is that of its possible role in triggering mammary involution and its connection with the partially folded molten globule state. A second related question is regarding the pro-apoptotic form of α-lactalbumin, HAMLET, which appears to have promise as an anticancer treatment. Modified proteins have proved to be effective treatments for other diseases, and it will be interesting to see if this novel ligand-stabilized misfolded protein can be translated into a clinical treatment.

See also: **Gamete and Embryo Technology**: Transgenic Animals. **Lactation**: Lactogenesis. **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose. **Milk**: Milk of Marine Mammals; Milk of Monotremes and Marsupials.

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β-Lactoglobulin

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Introduction

β-Lactoglobulin is present in the milk of most mammals and generally accounts for about 50% of the whey proteins in ruminants and about 10% of the total milk protein in cow's milk. The ratio of caseins to whey proteins is dependent on the species, with high casein to whey protein ratios being associated with the rapidly growing mammals, such as lambs and calves. These mammals are also those that are most commonly cultivated for their milk production.

β-Lactoglobulin is globular in nature with a well-defined three-dimensional structure. It has an α-helix along one side of a β-barrel and, in ruminant milk at least, exists as a dimer from pH 3 to 7. At about pH 7.5, the ruminant protein undergoes a well-defined change known as the Tanford transition. All β-lactoglobulins examined bind a range of small hydrophobic and amphiphilic molecules, and their structural similarity to other members of the large family of lipocalins, or extracellular lipid-binding proteins, indicates they could have a transport role for the newborn. Nevertheless, the biological function of this protein has not been clarified, and the absence of this protein from rat, mouse, hare, and human milk but presence in possum and baboon milk suggests that it may not be essential for many species.

Although cow's β-lactoglobulin exists in a large number of genetically determined forms (variants), it is generally present as either the A or the B variant. This variation is correlated with a number of milk characteristics, and milk processability and product function are clearly associated with different responses to heat treatments of the different variants.

Most of our knowledge of the characteristics and behavior of this protein has resulted from the extensive studies on the bovine protein, which is readily isolated in a pure state from milk by simple procedures, or can be purchased in a moderately pure state. This has led to its being used as one of the 'standard' proteins, along with serum albumin, ovalbumin, and α-lactalbumin, in many biophysical and biochemical studies.

As the major component of whey protein concentrates, it has also become a valuable food ingredient because of its combination of nutritional and functional advantages. As a result, the ways in which it unfolds and aggregates, particularly with other proteins, continue to be studied intensively.

Nomenclature

The proteins of milk were originally (pre-1890 or so) classified as either casein or whey proteins, and the precipitate from heated whey was called lactalbumin. This name continues to be used for a commercial product made by this process.

Some proteins of whey were found (about 1890) to be precipitated by saturating acid whey with magnesium sulfate or half-saturating it with ammonium sulfate. This fraction was called 'lactoglobulin', and the protein material that remained soluble was called 'lactalbumin'. Further research in the 1930s identified a crystallizable material in the 'lactalbumin' fraction, and two proteins were isolated and were called α-lactalbumin and β-lactoglobulin (β-lg). Later, bovine serum albumin was also found in this fraction.

Thus, there were two different materials called lactalbumin and another called α-lactalbumin. In the commercial and legislative area, there is the potential for confusion as to the composition and origin of some products and whether they are albumins or globulins.

Once the three-dimensional structure of β-lg had been determined, it became clear that the eight-strand β-barrel or calyx fold was very similar to that of serum retinol-binding protein. Since then, a large family of proteins called lipocalins has been identified with similar folds, and they rather specifically bind small hydrophobic or amphiphilic molecules. If the β-lg molecule were being named now, it might well be called 'milk fatty acid-binding protein' or 'milk lipocalin'.

In the 1950s, it was found that β-lgs exists in two forms, called the A and B variants, and the differences between these proteins were genetically determined. Since then, a number of rarer genetic variants have been identified (*see* **Milk Proteins**: Heterogeneity, Fractionation, and Isolation). It was also noted that β-lg A was present at higher concentrations in milk than β-lg B, and it is now recognized that β-lg A is expressed at about a 30% higher concentration than β-lg B in both homozygous and heterozygous cows. Control of expression level resides in the unexpressed upstream region of the gene. One important consequence is that the whey protein:casein ratio is higher in β-lg A milk and thus the results of experiments using milk from selected groups of cows may not extrapolate to the milk of another group or to bulk milk.

A number of genetic variants of β-lg have been identified in the milk of sheep and goats, and because the designation of A, B, and so forth, has been arbitrary, the relative differences between bovine B and C variants, for example, has no bearing on differences between sheep B and C variant structure or behavior.

Separation and Isolation

The proteins of whey or whey products can be separated readily using either native- or sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). With care, appropriate standards, and using amido-black dye, quantitative assessments can be made. Both ion exchange (**Figure 1**) or reversed-phase high-performance liquid chromatography (RP-HPLC) are useful for separation and for quantitation. Capillary electrophoresis and molecular sieves are becoming accepted methods also.

Traditionally, β-lg is prepared by fractional precipitation with $(NH_4)_2SO_4$ at a low pH, followed by repeated crystallization. Modern chromatographic methods have allowed it to be isolated by size exclusion and cation or anion exchange chromatography. One specific and rather unusual method is by affinity chromatography on a column of immobilized retinal, which is a ligand that is strongly and reversibly bound by β-lg.

A commonly used purification method involves separation of the fat and casein from fresh raw milk by centrifugation followed by acidification to about pH 4.5 at 35–40 °C. The clarified whey fraction is concentrated, adjusted to pH 2.5–3.0, and NaCl added to give a concentration of 7% (w/v) at about 20 °C. Most proteins except β-lg form a loose precipitate that can be separated from the β-lg-containing solution. The β-lg can be separated from the remaining whey by dialysis or by precipitation on increasing the NaCl concentration to 30%.

β-Lg can also be purchased from various sources. A typical SDS-PAGE comparison of a commercial product and a scrupulously prepared laboratory sample is shown in **Figure 2**. Clearly for precise work, it is better to purify the protein and not subject it to temperatures over 45 °C, freeze-drying, a pH above 6.5, denaturing solvents, or long-term storage. Any of these treatments can covalently modify or polymerize the protein. Even shaking solutions of β-lg can alter the protein.

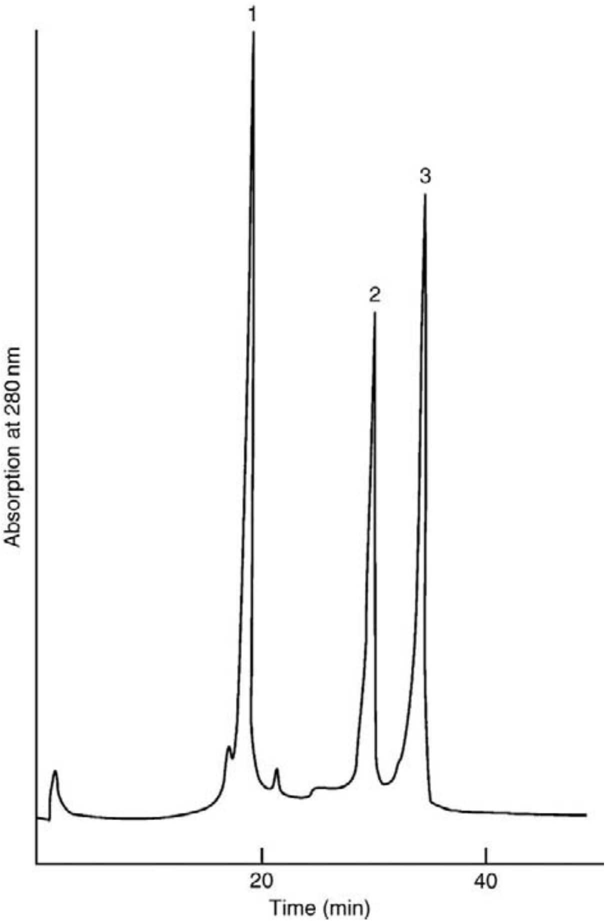

Figure 1 Typical analytical ion exchange separation of the components of whole acid whey that had been dialyzed against the starting buffer to remove orotic acid and other low molecular mass compounds. The column was a Mono Q HR5/5, and the buffer was 0.02 mol l $^{-1}$ piperazine-HCl, pH 6.0, and the proteins were eluted with 0 ± 0.4 mol l $^{-1}$ NaCl. The three major peaks were identified as (1) α-lactalbumin, (2) β-lactoglobulin B, and (3) β-lactoglobulin A.

Structure

Primary

The amino acid composition of β-lg was first reported in 1949, and refinements were made during the following decade. Partial sequences were published during the 1960s, and the complete sequence was published in 1972. Nevertheless, there was some controversy about the exact position of one of the disulfide bridges (i.e., whether it was between residues 106 and 119, between residues 106 and 121, or indeed a mixture of both). Early low-resolution X-ray structures were unhelpful, but

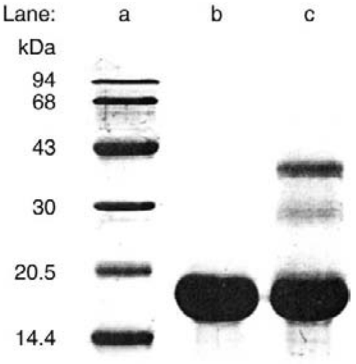

Figure 2 (a) SDS-PAGE patterns of molecular mass standards, (b) carefully purified β-lactoglobulin, and (c) commercial product. All samples were reduced with 2-mercaptoethanol. Whey was prepared from skim milk by acid precipitation of the casein, and the pH was raised to 7.2. β-Lactoglobulin was isolated by an adsorption-desorption step from diethylaminoethyl Sepharose-CL6B. This material was chromatographed on a size-exclusion column of Superdex 75BPG to give the pure sample (b). Reproduced with permission from de Jongh HHJ, Groenveld T, and de Groot J (2001) Mild isolation procedure discloses new protein structural properties of β-lactoglobulin. *Journal of Dairy Science* 84: 562–571.

high-resolution X-ray and NMR studies confirmed the chemical studies that suggested the disulfide bond was exclusively between Cys106 and Cys119 and that Cys121 was the free thiol. Nevertheless, this thiol is fairly unreactive in the native protein and is 'available' for reaction only after the protein had been unfolded in some way (e.g., raising the pH renders the thiol susceptible to heavy metal ions or in a solution containing at least 6 mol l^{-1} urea the reactivity is that of a free thiol). Heat treatment at near-neutral pH also exposes up to one thiol group per β-lg molecule.

Secondary

Estimates of the secondary structure of β-lg have been made since the 1960s using optical rotatory dispersion, circular dichroism, and infrared spectroscopy. On the basis of these results, β-lg was considered to have about 50% β-sheet and 10% α-helix. Attempts were also made to use predictive methods to identify the sites of the β-strands, helical regions, and various tight turns by applying structural probabilities (based on early X-ray structures of a range of mostly globular proteins) to the primary sequence. Once the medium-resolution X-ray structure was known, the predictions were found to be correct for the major helix and most of the β-strands, including all those in the major sheet. After heat treatments, the β-sheet content identified spectrally is essentially unchanged, but much of the α-helix content is lost. High pressure has similar effects, but exposure to chaotropic solvents reversibly reduces all of the secondary structures. More recently, NMR studies have revealed that some of the β-strand structure in the N-terminal half of the molecule can exist as α-helix that appears to be an essential intermediate in the folding pathway of the native structure.

Tertiary and Quaternary

Early studies showed that under conditions close to physiological, both β-lg A and B formed dimers reversibly and that dimerization was ionic strength-, pH-, temperature-, and protein concentration-dependent. β-Lg A also formed octamers at about pH 4.5 and around 4.5 °C.

Recently, high-resolution structures of β-lg have been obtained using both NMR and X-ray methods. Despite the considerable environmental differences (pH 2–3, monomeric protein and low ionic strength for the NMR and some rigidity in the crystals that were grown at pH 6.2–8.2 with either high (>3 mol l^{-1}) or low (<0.05) ionic strength), the folding of the polypeptide chains is very similar within the various structures and among the A, B, and C genetic variants. All show a stable β-sheet with an α-helix on one side and a second β-sheet on the other (**Figure 3**). Both X-ray and NMR show considerable main chain and side chain flexibility in the long loops.

Recent NMR work with the A34C mutation that produces a stable dimer at neutral pH not only confirmed that the core structure is stable and that dimerization does not cause large variations in the structure but also has allowed the refolding of the protein to be followed in some detail. Differences do exist, mostly in the external loops both within the various bovine crystal and NMR structures and, less surprisingly, between bovine, porcine, and reindeer crystal structures and also the equine NMR structure. Whereas bovine, ovine, and reindeer β-lg have very similar dimer structures involving an antiparallel arrangement of strand I, the dimer present in pig at pH 3.2 is quite distinct, does not utilize strand I, and involves a domain swap even though β strand I is H-bonded to strand A and is part of the major β-sheet for all three structures.

Variants

The structures of the A, B, and C variants of bovine β-lg have been analyzed by X-ray crystallography; the differences in the tertiary structures are minor. Closer examination and consideration of the energetics of the loss of native structure as a function of temperature (**Figure 4**) lead to the conclusion that Ala to Val (B → A) at position 118 changes the hydrophobicity within the cavity of β-lg, but the change of Gly to Asp (B → A) at position 64 alteres the charge on the CD loop as well as on the protein as a whole. This change could affect the stability of the Cys66-Cys160 disulfide bond.

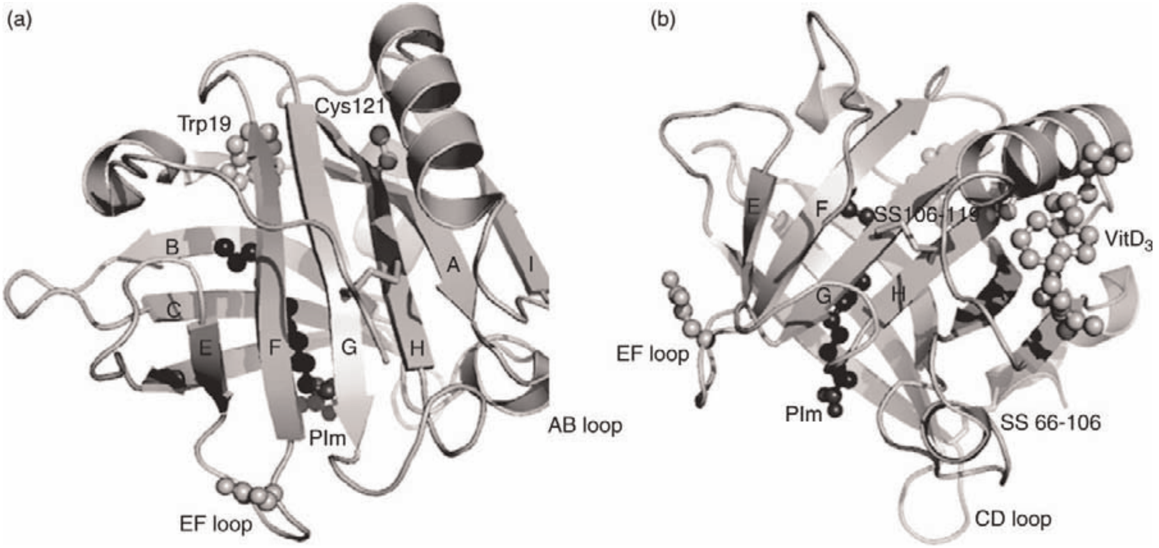

Figure 3 Cartoon representations of bovine β-lactoglobulin (variant A in crystal form Z containing the bound ligands), sodium palmitate (Plm; PDB:1gxa), and vitamin D_3 (VitD$_3$; transformed from PDB:2gj5) drawn using the program PyMol (W. L. Delano, The PyMOL Molecular Graphics System: DeLano Scientific, San Carlos, CA, 2002). The β-strands are labeled A–I. The CD and EF loops are labeled, and the side-chains of Trp19, which lies at the base of the hydrophobic cavity, Glu89 that triggers the Tanford transition, and the free Cys121 are also shown. (a) A view of the molecule from the 'top', with the entrance to the calyx behind the EF-loop which is in the open position. (b) A more end-on view of the calyx, showing the second binding site between the three-turn helix and strands A and I.

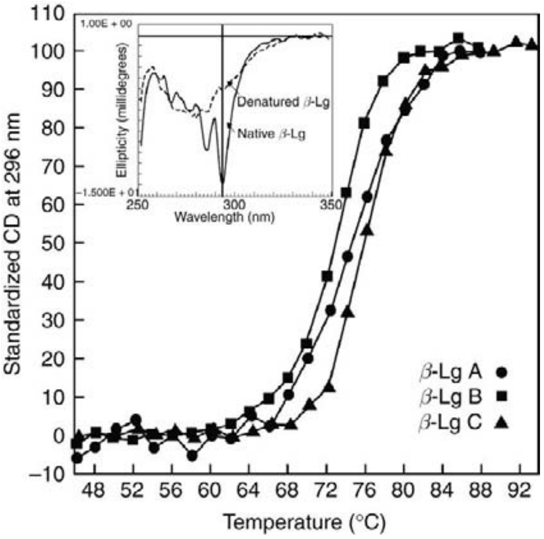

Figure 4 Effect of heating β-lactoglobulin A, B, and C on near-UV ellipticity. The three proteins (1.7 mg ml^{-1} in pH 6.7 phosphate buffer) were heated for 10 min at temperatures between 46 °C and 93 °C. The cooled samples were diluted to 1 mg ml^{-1} and scanned from 250 to 350 nm at 21 °C. The ellipticities at 293 nm were normalized prior to plotting. The inset shows the spectrum of an unheated β-lactoglobulin A sample and one that had been heated at 86 °C. Reproduced with permission from Manderson GA, Hardman MJ, and Creamer LK (1997) Spectroscopic examination of the heat induced changes in β-lactoglobulin A, B and C. In: *Milk Protein Polymorphism, Proceedings of the IDF Seminar in Palmerston North*, pp. 204–211. New Zealand. Brussels: International Dairy Federation SI 9702.

Tanford Transition

It was noticed that the titration of β-lg gave an anomalous result for one ionizable residue of the protein. It appeared that a glutamic or aspartic acid residue had a pK$_a$ of about pH 7.3 instead of pH 4.5–5.0, as expected. Structural analysis showed that Glu89 was H-bonded to Ser116 at pH 6.2 but not at pH 8.2. It was also found that loop EF was folded down onto the protein at pH values below about 7.5 as a consequence of this H-bond, and this prevented access of retinol and other ligands to the hydrophobic binding cavity. This structural change is also responsible for the pH-dependent changes in optical levorotation noted and possibly changes in the accessibility of the thiol group. Recent NMR data confirm this and propose that the mechanism of the transition is initiated by the deprotonation of Glu89 that allows a rearrangement of the H-bonding of Ile84, Asn90, and Glu108 in the hinge region of the EF-loop. This is then followed by the opening of the EF-loop permitting access to ligands like palmitate and retinol.

Ligand Binding

It has been known for a long time that the two major hydrophobic and amphipathic ligand binders in milk are serum albumin and β-lg. The binding characteristics of some β-lg-ligand complexes are shown in **Table 1**. The possibility that β-lg could be a useful drug carrier has been explored, and it has been speculated that β-lg could

Table 1 Selected ligand binding data for bovine β-lactoglobulin

Ligand	K_d *(mol l^{-1})*	*pH*	*Method*
Laurate	7.0×10^{-7}	7.0	Fl
Palmitate	1.0×10^{-7}	7.0	Fl
	1.5×10^{-6}	7.4	ED
Stearate	1.2×10^{-7}	7.0	Fl
SDS	3.2×10^{-6}		
cis-Parinaric acid	2.8×10^{-8}	7.0	Fl
Retinoic acid	2.0×10^{-7}	7.0	Fl
Retinol	1.5×10^{-8}	7.5	Fl
	2.0×10^{-8}	7.5	Fl
	3.6×10^{-8}	7.5	AC
	6.7×10^{-5}	7.2	ED
	3.8×10^{-8}	8.0	ED
Cholesterol	3.49×10^{-8}	7.0	Fl
Vitamin D_2	4.91×10^{-8}	7.0	Fl
Nonanone	4.1×10^{-4}	6.7	ED

Fl, fluorescence; ED, equilibrium dialysis; AC, affinity chromatography.

carry retinoids through the hostile stomach (abomasum) environment, or that β-lg is important in transporting fatty acids from the lipolysis site to the gut wall during milk digestion. It is now clear that fatty acids and retinoids, at least, are bound within the calyx (hydrophobic cavity) of native β-lg. There are other possible sites for ligands, such as 1,8-anilino-naphthalene sulfonate (ANS), which do not bind in the cavity but on the outer surface of the protein between the helix and strands A and I in the dimer interface. This site has also been found to bind vitamin D_3 in addition to its binding in the central calyx. Clearly, pure proteins are required for these studies because impurities such as serum albumin or denatured β-lg bind amphiphiles that the native protein does not.

Structure and Function of the β-Lactoglobulins in Milk

The polypeptide chain adopts a fold typical of the lipocalin family. This family is widely distributed and contains members with varied functions, though all are secreted from the cell and appear to be able to bind small, generally hydrophobic, molecules. The closest lipocalin member to β-lg is glycodelin found in the endometrium of human and baboon during the first trimester of pregnancy. High-resolution X-ray structures are now available for bovine, ovine (95% identical to the bovine protein), porcine (56% identity), and reindeer (94% identity) β-lgs, together with the NMR structures of bovine, porcine, and equine (69% identity) β-lgs and, for comparison, glycodelin (**Table 2**). In each case, the core structure of the protein is an eight-stranded, antiparallel β-sheet made up of two layers (β-strands A1–D and E–H, A2). Together, these form a conical binding cavity because the A strand is kinked significantly, forming sub-strands A1 and A2, around residue 22, allowing it to engage with strand H, and the general twist of the sheet strands also permits D and E to interact with one another (see **Figure 3**). In all cases, there is a three-turn α-helix after strand H and before a ninth strand, I. In the bovine, ovine, and reindeer proteins, strand I makes an antiparallel interaction with strand I of a second β-lg polypeptide to form part of the dimer interface at neutral pH. The helix is on the outer surface of the protein and located over, partially burying, the free thiol group of Cys121. Each β-strand is joined to the next one by a loop. Before strand A, in the loops AB (between strands A and B) and GH and in the C-terminal section, there are helical turns. Further, the extended loop AB makes contacts with the same loop from the other molecule of the dimer, thus strengthening the dimer interface. Loops BC, DE, and FG are at the ‘back’ of the molecule; that is, they are at the closed end of the central ligand-binding cavity (surrounding Trp19, **Figure 3**(**a**)). The loops joining the other ends of the strands, AB, CD, EF, and GH, are at the open end (near the foot of **Figure 3**(**a**), lower left of **Figure 3**(**b**)) and show signs of significant flexibility. In particular, the loop EF can adopt two independent positions depending upon the pH. At low pH, where the protein does not bind ligands readily, this loop forms a closure to the binding site whereas at high pH the loop is folded back, allowing ready access of the ligands to the hydrophobic cavity. Indeed, it is this loop that contains the anomalous carboxylate (Glu89) identified

Table 2 Percentage (%) sequence identities of selected β-lactoglobulins and glycodelin

	Sheep	*Reindeer*	*Pig*	*Horse*	*Cat*	*Dolphin*	*Baboon*	*Kangaroo*	*Glycodelin*
Cow B	95.1	95.1	64.4	57.4	61.1	71.3	50.0	30.1	42.6
Sheep B		92.6	64.4	57.4	61.7	68.5	51.3	32.0	43.2
Reindeer			64.8	58.0	62.3	73.5	51.8	34.0	44.4
Pig				52.5	62.3	62.3	48.7	29.1	39.5
Horse					71.6	58.6	55.6	26.8	53.1
Cat						60.5	56.8	32.7	51.2
Dolphin							54.9	29.6	44.4
Baboon								30.3	54.9
Kangaroo									27.2

by Tanford and responsible for the pH-dependent transition that bears his name. Also at low pH, protonation of Asp33 in loop AB weakens its interaction with Arg40 from the other subunit, thereby promotes dissociation. It is not clear why the A variant of bovine β-lg should form octamers (i.e., four dimers associating), but it is interesting that the pH of maximum association corresponds with the pK_a of a side-chain carboxyl group and that the A variant has an additional carboxyl at Asp64, the start of the CD-loop, that might facilitate a carboxyl-carboxylate association.

Neither porcine nor equine β-lg is a dimer at neutral pH although porcine β-lg dimerizes around pH 4, the pH at which the crystal structure was determined. Interestingly, the dimer interface is quite distinct from that observed in the bovine protein, involving an intertwining of the N-termini before strand A. Similarly, NMR studies of the equine protein in solution reveal a monomer at neutral pH. NMR studies on bovine β-lg have been carried out at around pH 2 because at this pH the protein is a monomer. The structures obtained are closely related to those observed by crystallography with the EF-loop in the 'closed' position. The folding of the protein has been observed by NMR techniques to involve the transient formation of a helical region involving β-strand A and, further, the upper and lower sheets (strands A1–D and E–H) fold essentially independently. Palmitate binding to β-lg has also been monitored by NMR and shown to mirror the Tanford transition in that binding is not observed below pH 6, whereas above that pH the ligand exhibits conformational variability at the carboxyl end. This is also in good agreement with the crystallographic results that show fatty acid or retinol bound within the central cavity. NMR structural data have also been obtained from the A34C mutant at neutral pH that are also in good agreement with the crystallographic studies. Recently, crystal structures that show the existence of a second binding site in the dimer interface region close to residues 136–148 have been obtained. ANS binds close to Arg148 in the loop between the end of the helix and the strand I while vitamin D_3 binds both in the same external region and also in the central cavity.

The physiological function of β-lg has been enigmatic with the consensus view being that it is a transporter of some sort. However, the wide selection of small hydrophobic molecules that bind does not provide any real clue to the identity of the physiological ligand(s). Fatty acids, principally palmitic acid, are detected bound to freshly isolated protein, but as porcine β-lg does not bind fatty acids, at least not at physiologic pHs, it is improbable that fatty acids are the true ligand. The species distribution also shows that the presence of β-lg in milk is widespread but not universal; there is no β-lg in human, rodent, or lagomorph milk. However, β-lg has been identified in the milk of the baboon and the macaque. Further, in cat, dog, and horse, for example, there are at least two distinct genetic loci, and pseudogenes have been identified in cow and goat. Estimated relationships between the known amino acid sequences of the available β-lgs, together with the most closely related sequence that emerges from the other lipocalins, indicate that the endometrial protein, glycodelin, appears to be a retinol-binding protein produced in significant quantities in the first trimester of pregnancy. It is possible therefore that β-lg is merely a convenient nutritional protein, in some cases with fortuitous additional properties, left over from the essential role provided by glycodelin in fetal development. One might expect to be able to identify pseudogenes for the protein in those species whose milk lacks β-lg.

Processing-Induced Structural Changes

During the processing of milk, a number of physical and chemical reactions can occur because of exposure of reactive groups, or because the higher energy (temperature or pressure) allows reactions to proceed faster. β-Lg is involved in many of these processes and has been studied extensively, particularly at the molecular level.

Intraprotein and Interprotein Reactions

When the environment of β-lg is altered, the structure of the protein changes. These changes are generally reversed when the previous environmental conditions are restored as long as the FGH triple-strand motif can re-form readily. An essential element of this re-formation is the retention of the Cys106-Cys119 disulfide bond. If either of these Cys residues becomes disulfide-bonded to a different residue, for example, this particular motif cannot re-form and hence the native structure that builds upon this stable motif cannot occur. This effect is probably common to perturbation by both heat and pressure, which change the energetic relationships that relate to the hydrophobic effect.

When β-lg is heated by itself at neutral pH at a temperature over 60 °C, it forms a series of polymers that are readily seen using PAGE analysis. These are formed by thiol-catalyzed disulfide bond interchange. In its simplest form, a free Cys residue interacts with a disulfide bond to bind itself to one of the partners, thereby freeing the other partner,

$$\begin{aligned} &R^1\text{-CysH} + R^2\text{-Cys=Cys-}R^3 \\ &\quad \leftrightarrow R^2\text{-CysH} + R^3\text{-Cys=Cys-}R^1, \end{aligned}$$

where R^1, R^2, and R^3 are the schematically shown different protein chains.

Thus, the native proteins containing intramolecular disulfide bonds are turned into a single large aggregate held together by intermolecular disulfide bonds. Early in the heat treatment of β-lg there are not only disulfide-bonded dimers and trimers but also monomers containing nonnative disulfide bonds (i.e., an internal Cys-disulfide exchange). There are also hydrophobically associated complexes of disulfide-bonded dimers and nonnative monomers.

The A, B, and C variants of β-lg denature to different extents for heat treatments at different temperatures. **Figure 4**, which shows how Trp61 is affected, is representative of other molecular parameters that demonstrate the same effects. The difference in response shown by β-lgs B and C is purely a change in the mid-point temperature, whereas the difference between β-lg A and either β-lg B or β-lg C also involves a change in the slope of the ellipticity (extent of denaturation) versus temperature. This conclusion explains the apparent disparity shown between different studies comparing the denaturation rates of β-lgs A and B.

Reactions involving structural change as a consequence of disulfide-bond interchange are responsible for the heat-induced gels made from whey protein isolates and concentrates, and also for protein fouling on heated surfaces in dairy plants. Reactions in milk often involve β-lg and κ-casein, whereas those in whey-derived material involve β-lg and α-lactalbumin (α-la). In each case, β-lg supplies the free thiol, but with κ-casein the free thiol can be either in the native protein or in various β-lg aggregates. With α-lactalbumin, the only reaction is between native β-lg and α-la. In this case, β-lg can also catalyze the formation of polymers of α-la.

H/D exchange at low pH/pD (where polymerization is very limited) has shown which parts of the tertiary structure are the most stable at low pH. As expected, the amide protons on the non-H-bonded peptide groups exchange rapidly at 37 °C. The various β-strands involved in β-sheets exchange over the temperature range 45–85 °C. At about 65 °C, the exchange of the helix protons occurs, and only at the highest temperature does exchange occur for the H-bonded residues in strands G and H. Thus, the stablest motif is that involving strands G and H, which encompasses the disulfide bond between residues 119 and 106.

At higher pH, reactions involving Cys and Lys residues can generate stable covalent interprotein and intraprotein lysinoalanine cross-links that can reduce protein digestibility.

Under certain conditions, β-lg is capable of self-assembling into long, thin fibrils. This occurs in a matter of weeks at room temperature in the presence of denaturants such as methanol, trifluoroethanol, or urea, or in a matter of hours during heating at low pH and low ionic strength. Fibrils typically have a diameter of approximately 5 nm, and can be as long as several micrometers. They are thought to have a 'cross-beta' structure similar to amyloid protein fibrils.

Many proteins are capable of self-associating into amyloid-like fibrils. Partial denaturation is a precursor to fibril formation, and hydrolysis is required in some cases, but a common mechanism of self-assembly has not been identified. Fibrillar aggregates of β-lg increase viscosity in a more efficient way than most other types of aggregates, and for this reason, β-lg fibrils are of interest as potential food ingredients and biomaterials.

Reaction with Lactose

The reactions of reducing sugars with the amino groups of proteins are well known as the Maillard reaction. The later products of this reaction are important in foods as they are major contributors to the flavor of caramel. One of the first steps in this reaction is the formation of β-lg-lactose adducts, and these can be observed as individual peaks in the mass spectra of such materials. When β-lg is heated in a lactose solution, the reaction is primarily with Lys47, but in a dried product, such as milk powder, there does not appear to be any specificity. The small reduction in the number of lysine residues does not adversely affect the nutritional value of the protein substantially. However, severe heat treatment or extended reaction times at moderate temperatures can cause extensive loss of lysine.

Hydrolysis

The allergenicity of β-lg, which is the most antigenic of the bovine milk proteins for humans, can be reduced by hydrolysis of the protein because the particular peptide sequences that react with the antibodies are cleaved into smaller fragments The epitopes on β-lg have been partially identified for the intact protein.

Bovine β-lg is readily hydrolyzed by proteolytic enzymes into peptides. The activity of the pancreatic protease, trypsin, has been studied extensively, and at the pH optimum of the enzyme (about 8.0), the variants are initially hydrolyzed in the order A > B > C.

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See also: **Milk Proteins**: Heterogeneity, Fractionation, and Isolation.

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Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins

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Introduction

The dynamics of the composition of the protein components of milk from the first lacteal secretion of colostrum through to the development of mature milk assist us in defining the functional significance of many constituent proteins. These secretions should be viewed more in a developmental context as an extension of the maternal influence over the development of the neonate in the face of the stimuli associated with the extrauterine environment than merely as a source of nutrient substrate. Analyses of the functional significance of the casein and whey protein components in the development of the neonate have now been superseded by the development of genomic and proteomic strategies, which are being used to expand our knowledge of the roles of these proteins and importantly their constituent peptides in developmental processes. However, there are many proteins and peptides that are not derived from the classical milk proteome but increase the functionality of colostrum and milk. Binding proteins associated with the transportation of nutrients, for example, not only act to deliver vitamins, minerals, and growth factors to their site of action, but may also exert their own biological activity through functional receptors.

The Role of Milk Proteins in the Developmental Programming of the Neonate

While the dominant class of proteins in milk is the casein family, comprising 80% of bovine milk proteins, the whey proteins and a large pool of proteins found in lower abundance provide a disproportionate contribution to the total bioactivity of the milk proteome. Interestingly, many of these are encrypted within the native amino acid sequences of the major protein constituents and some activities are duplicated within both casein and whey protein sequences.

A number of these proteins are now known to be multifunctional in the neonate. Many provide a specialized developmental input while at the same time boosting host defense mechanisms against pathogenic bacteria as the newborn develops its own immune defense system. Thus, the exposure of the developing neonate to the diversity of milk proteins and peptides provides a mechanism that regulates an animal's development. The most overt example of this potential resides within the large number of hormones and growth factors that have been identified in colostrum or milk. These include hormones of the hypothalamic-pituitary axis, thyroid and parathyroid hormones, gastrointestinal regulatory hormones as well as the classical growth factors. Although the provision of peptides as a source of amino acids to support the rapid increase in protein synthesis is a major role of milk proteins, we now recognize that the bioactivities residing within their sequences may be much more important in directing their development.

This article covers a number of minor proteins associated with the key biological functions of milk (**Table 1**).

Minor Milk Proteins and the Development and Control of the Vascular System

Several milk proteins play a role in angiogenesis while others regulate blood pressure and nutrient partitioning. Thus, the ingestion of colostrum and milk not only directs the vascularization of tissue but is also capable of influencing nutrient supply to tissues.

The Angiogenins

The angiogenins are monomeric proteins of approximately 14 kDa that belong to the pancreatic ribonuclease superfamily. Their name is derived from their ability to promote the development of the capillary blood system in tissues with the first discovered of these, human angiogenin, inducing neovascularization in a wide array of systems, including the chicken chorioallantoic membrane, rabbit cornea, and rabbit knee meniscus. The link between the ribonuclease activity and angiogenic capacity of these proteins seemed tenuous until site-directed mutagenesis studies of the catalytic domain of murine angiogenin 4 showed that ribonuclease activity was essential for angiogenic activity.

Table 1 Some properties of minor milk proteins

Protein	*Molecular weight (Da)*	*Source*	*Concentration in milk (mg l^{-1})*
Angiogenin 1	14 577[a]	Mammary gland	4–8 (bovine)
Angiogenin 2	14 522[a]	Mammary gland	0.15 (bovine)
Heparin affin regulatory protein	18 000[a]	Mammary gland	0.017–0.060(human milk–colostrum)
Kininogens	>68 000 and 16 000[a]	Blood	
β_2-Microglobulin	11 630[b]	Monocytes	9.5 (bovine)
Osteopontin	29 283[b]	Mammary gland	18 (bovine)
Proteose peptone 3	28 000[a]	Mammary gland	300 (bovine)
Folate-binding protein	25 720[a]	Mammary gland	8 (bovine)
Vitamin D-binding protein	52 000[a]	Blood	250 (colostrum)–6 (milk) (bovine)
Vitamin B$_{12}$-binding protein (haptocorrin)	Human – 68 000[a] Bovine – 44000[a]	Mammary gland	0.1–0.2 (bovine)
Riboflavin-binding protein	37 000[a]	Mammary gland	
Bovine serum albumin	66 433[b]	Blood	100–400 (bovine)
Muc 1	78 700–58 500[a]	Mammary gland	
Muc 15	130 000[a]	Mammary gland	

[a] Apparent molecular weight, glycosylated or phosphorylated.
[b] Calculated molecular weight.

More recently, these proteins have been associated with regulatory roles in the nervous system, with mutations being associated with familial as well as sporadic forms of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder characterized by selective destruction of motor neurons. Several genetic variants have been identified with varying function, while murine angiogenin 1 (ANG-1) is expressed in developing mouse nervous tissue and is associated with the neural differentiation of stem cells. Both ANG-1 and ANG-2 have been observed in bovine milk. Both have similar calculated molecular weights (14 577 and 14 522 Da, respectively), contain a similar number of amino acids (125 and 123 amino acids, respectively), and the conformation of each contains three disulfide bonds.

Bovine ANG-2 differs from ANG-1 in the presence of a single glycosylation site, which results in a reduction in its ribonuclease activity compared with ANG-1. While there is some homology between bovine ANG-1 and ANG-2 (57%), ANG-1 includes an integrin receptor recognition sequence, which may influence its role in angiogenesis.

Although we understand little of the function of bovine angiogenins, we now know that they are mainly responsible for the inhibitory effect of bovine milk on osteoclast-mediated bone resorption, and that they exert their activity by acting directly on the osteoclasts. They also induce the production of cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in human leukocytes.

Milk also delivers an antiangiogenic factor in the form of the pepsin-produced N-terminal fragment of iron- and heparin-binding lactoferrin, lactoferricin (LfcinB). LfcinB strongly inhibits both bovine fibroblast growth factor (bFGF)- and vascular endothelial growth factor (VEGF)165-induced angiogenesis by competing for binding sites on vascular endothelial cells.

Other Milk-Borne Proteins or Peptides That Regulate Vascular Function

Angiotensin-converting enzyme-inhibitory peptides

While the function of many angiogenic milk proteins is not well defined, the role of milk-derived peptides in inhibiting angiotensin-converting enzyme (ACE), responsible for the conversion of angiotensin 1 to the active angiotensin 2 and the degradation of the vasodilator bradykinin, is well established. Thirty-four ACE-inhibitory peptides have been identified in casein family sequences, while α-lactalbumin (α-La) and β-lactoglobulin contribute 18 similar functional sequences. Bovine serum albumin (BSA), lactoferrin, and β_2-microglobulin contribute a further five peptide sequences that may have a similar influence. The necessity for such a diversity of functional ACE-inhibitory sequences may be a result of the complexity of ACE bioactivity and its multiple sites of action. Its role as a central neuroregulator of vasopressin and neurotensin is consistent with a vasoregulatory function. ACE may also have roles in fertility by regulating gonadotropin-releasing hormone, and pain perception through substances P and K: all of these functions are associated with the control of lactation either directly or indirectly. ACE is also involved in hematopoiesis, the inflammatory response as well as tissue repair. Clearly, it is an important developmental molecule since the milk proteins have evolved multiple peptide systems to regulate its function in the growing neonate.

Heparin affin regulatory peptide

Heparin affin regulatory peptide (HARP), also known as pleiotrophin, heparin-binding growth-associated molecule, heparin-binding growth factor 8, and heparin-binding neurite-promoting factor, is a 136 amino acid (18 000 Da) growth factor displaying high affinity for heparin that is expressed in the mammary gland, brain, uterus, and prostate. It is expressed in both human colostrum and milk, with threefold higher concentrations being found in colostrum. It is present in the extracellular matrix of many tissues, interacting with heparin sulfate and dermatan/chondroitin sulfate glycosaminoglycans. It influences neuronal migration and epithelium–mesenchyme interactions during development.

Previously, HARP has been implicated in the control of angiogenesis and its effects are mimicked, at least in part, by synthetic peptides that correspond to its N and C termini. HARP is cleaved by plasmin, leading to the production of five peptides that correspond to distinct domains of the molecule. These peptides affect *in vivo* and *in vitro* angiogenesis and modulate the angiogenic activity of VEGF on human umbilical vein endothelial cells. This is associated with the role of this protein in regulating mitogenesis, cell motility, oncogenesis, differentiation, and synaptic plasticity: it promotes proliferation of human mammary and myoepithelial cell lines. It is also used as a tumor marker and importantly is elevated in response to proinflammatory cytokines.

Kininogen

Two kininogen molecules have been isolated from bovine milk, a high-molecular-weight form (greater than 68 000 Da) and a low-molecular-weight form (16 000–17 000 Da), which are different from those identified in bovine plasma. High-molecular-weight kininogen found in plasma (78 000 Da) is cleaved by the plasma protease, kallikrein, to release four fragments: heavy chain, bradykinin, fragment 1.2, and light chain. All of these fragments have been identified in bovine milk and the fragment 1.2 exhibits the ability to stimulate osteoblast proliferation. This fragment has been shown to be a multifunctional edible protein that promotes bone formation and inhibits bone resorption and its biological activity is resistant to pepsin.

Minor Milk Proteins and the Immune Defense System

The presence in colostrum and milk of a wide range of peptides and proteins that contribute to host defense mechanisms in the newborn is not surprising in view of the underdevelopment of the neonatal immune system. Many of these act as bacteriostatic molecules by preventing pathogens from interacting with the intestinal mucosa. While lactoferrin is recognized for its function as a high-affinity iron transporter, it is now considered one of a growing family of proteins that appear to have varying functions in different environments. Similarly, α-La is a calcium-binding milk protein, which plays a role in lactose synthetase activity; besides this function, both trypsin and chymosin hydrolysates of α-La have been shown to have bactericidal properties against Gram-positive organisms. Both of these proteins are not generally considered minor proteins of milk and thus are discussed elsewhere in this encyclopedia.

β_2-Microglobulin

β_2-Microglobulin has a calculated molecular mass of 11 630 Da and appears in bovine milk as a multimer but can also be found in other body fluids. Evidence from protease inhibitor treatment suggests that β_2-microglobulin is released as a product of the digestion of the cellular component of milk. β_2-Microglobulin has also been implicated in immune and histocompatability antigen mechanisms, but it is unclear what role this protein has, if any, in the mammary gland physiology of the cow or in the tissues of the neonate.

Osteopontin

Osteopontin is a secreted phosphoprotein first identified in the mineralized matrix of bovine bone; more recently, it has been detected in many tissues and fluids, including urine and milk.

Within the 262 amino acid sequence are a large number of serine or threonine residues that may be phosphorylated or glycosylated. The calculated molecular weight of the protein is 29 283 Da and includes a cell adhesion sequence (RGD) that may bind to integrins and facilitate its action.

Interestingly, this protein is upregulated in response to inflammation in a similar fashion to lactoferrin; it is also present in a 10-fold higher concentration in human milk than in bovine milk, as is lactoferrin. Evidence is now available to show that in milk this protein may act as a binding protein for the transport of lactoferrin, thereby enhance the effectiveness of this protein as a potent antimicrobial agent.

The importance of the interaction of minor milk proteins is further demonstrated through the anti-inflammatory actions of the milk fat globule-epidermal growth factor-8 protein (MFG-E8) by impeding the binding of cell surface integrins to osteopontin, which becomes activated during the gastrointestinal inflammatory disease, colitis. Osteopontin may also play a role in mammary involution as well as in casein synthesis. The expression of the gene encoding this protein, SPP1, is markedly upregulated in mammary tissue at involution, while suppression of the gene using small interfering RNA (siRNA) technology in bovine primary mammary epithelial cells decreases casein gene expression.

Proteose Peptone 3

Proteose peptone 3 is a phosphorylated glycoprotein with an apparent molecular mass of 28 000 Da composed of 135 amino acid residues isolated from the proteose peptone fraction of bovine milk. The peptide contains serine residues at positions 29, 34, 38, 40, and 46, all of which are phosphorylated. Two O-linked carbohydrate groups are located at Thr16 and Thr86, while one N-linked carbohydrate group is present at Asn77. Thr16 is only approximately 50% glycosylated. The amino sugar detected by the amino acid analyzer at Thr86 provides a linkage for galactosamine although glucosamine can also be found on this residue. In contrast, both glucosamine and galactosamine are found in the carbohydrate group linked to Asn77. This peptide is part of a family of glycoproteins, including glycomacropeptide, lactoferrin, and κ-casein, that may influence the immune response.

Transporter-Binding Proteins

A number of binding proteins responsible for the transport of key nutrients are also found in milk. Presumably, they serve a similar function by providing a mechanism for these important nutrients to be delivered to the neonate through the medium of colostrum and milk.

Folate-Binding Protein

Folate-binding protein is typically found in milk as a monomer exhibiting a molecular weight of approximately 25 720 Da. The 222 amino acid protein exhibits up to eight disulfide bonds and is glycosylated at two amino acids. It has a role in the sequestration of folate to facilitate cellular uptake including in the gastrointestinal tract. Folate has a critical role in embryonic development, with deficiencies associated with neural tube defects like *spina bifida*. Similar to other binding proteins, folate-binding protein in milk may regulate folate absorption in different parts of the gastrointestinal tract and sequester folate away from gastrointestinal microflora.

Vitamin D-Binding Protein

Vitamin D-binding protein (DBP), also known as group-specific protein, is found in the milk of several species and is likely to be derived from serum. The ~52 000 Da (human) protein of 458 amino acids is observed in the whey of colostrum and milk as well as serum although it is at a much higher concentration in serum and colostrum than in mature milk. These binding globulins from human, monkey, porcine, and bovine sources are of similar size although the bovine protein seems to lack a smaller binding protein. They may have a number of roles, including long-chain fatty acid binding as well as leukocyte chemotaxis. Other milk proteins have also been indicated in vitamin D transport in milk.

Vitamin B_{12}-Binding Protein

The importance of vitamin B_{12} (VitB_{12}; cobalamin) to ruminant species relates to the VitB_{12} dependence of the key hepatic pathway converting glucose to succinate. This vitamin and a number of its analogues, which are structural isoforms, are synthesized by bacteria. The ability of ruminant species to access VitB_{12} across the placenta or in milk or even retain the vitamin from direct injection is considered to be poor. VitB_{12} is obtained from dietary sources in nonruminant species. The human VitB_{12}-binding protein (haptocorrin) has a molecular weight of approximately 68 000 Da, 34% of which consists of carbohydrate, and seems to bind VitB_{12} to inhibit microbial uptake and thus may have a role in modulating the growth of gastrointestinal microflora.

Riboflavin-Binding Protein

Riboflavin-binding protein (Rfbp) is a protein of approximately 37 000 Da found in bovine milk (possibly sequestered from plasma) and it binds riboflavin. Its concentration in mammalian milk is higher than in plasma during lactation. The active carrier-mediated transport mechanism of riboflavin is regulated by the Ca^{2+}/calmodulin pathway, while there also appears to be a receptor-mediated endocytic component for riboflavin. The role of this carrier is most important in regulating the role of riboflavin in fetal development. Interestingly, the sequences of chicken Rfbp are 30% homologous with the sequences of chicken folate-binding protein, with eight of the nine disulfide bonds conserved between the two proteins, suggesting that the sequences for these important transport proteins have been conserved.

Other Minor Proteins of Milk

Bovine Serum Albumin

BSA is a protein found predominantly in the circulatory system of the cow but is also a constituent of the whey of bovine milk. BSA has a molecular weight of 66 433 Da (583 amino acids) and is believed to be found in milk due to a deterioration of the tight junctions between mammary epithelial cells, which restrict movement of molecules from the blood through to the secretary compartment of the bovine mammary gland. This observation is enhanced by the various reports of the identification of increased concentrations of BSA in bovine milk coinciding with mastitis and the corresponding immune/inflammatory response and the increased concentration of BSA in response to changes in milking frequency (e.g., from twice-a-day to once-a-day milking), which have been shown to affect the integrity of mammary epithelial cell tight junctions.

BSA in bovine milk has also been implicated in the allergic reactions in humans to bovine milk and has even been investigated as a possible cause for the stimulation of autoimmune disease leading to insulin-dependent diabetes mellitus in humans.

Mucins

Approximately 20 mucin proteins have been identified in humans and a variable number of glycosylated forms of the mucin core identified in bovine milk, with mucin 1 (Muc 1) and 15 (Muc 15) the best characterized. Bovine Muc 1 and 15 are present at higher concentrations in mature milk than in colostrum, yet the mucins are present in small quantities in bovine milk with Muc 15 representing only 0.08% of total protein in mature bovine milk.

Bovine Muc 1 is secreted by bovine mammary epithelial cells as a component of the milk fat globule membrane and may have a role in protecting epithelial surfaces from pathogens. A number of variants have been identified in bovine milk, with the longest being a protein of 460 amino acids and with a molecular weight of 78 700 Da and the shortest of approximately 140 amino acids and with a molecular weight of 50 400 Da. The protein has a carbohydrate content comprising 50–60% of its mass, is resistant to proteolysis, and may have some role in maintaining mammary structure as it lines the mammary ducts and as it is negatively charged may repel the opposite side of the duct to maintain the lumenal space.

Bovine Muc 15 is similarly highly glycosylated and is found in the milk fat globule membrane. Its carbohydrate content accounts for ~65% of its mass (~130 000 Da); Muc 15 is 307 amino acids in length.

The increasing complexity of the milk proteome demonstrates that this article cannot be exhaustive. Metal-binding proteins, prosaposin, and various other glycoproteins also are important contributors to the development and control of the immune and vascular systems of the neonate. Other peptides such as the lactocrine peptide, relaxin, appear to be involved in the process of gut closure. Doubtless, many additional members of this family of minor proteins will be identified in future as our understanding of these physiological systems develops.

See also: **Enzymes Indigenous to Milk**: Lactoperoxidase; Lipases and Esterases; Other Enzymes; Phosphatases; Plasmin System in Milk; Xanthine Oxidoreductase. **Lactation**: Galactopoiesis, Effect of Treatment with Bovine Somatotropin; Galactopoiesis, Effects of Hormones and Growth Factors; Galactopoiesis, Seasonal Effects; Induced Lactation; Lactogenesis. **Mammary Gland**: Anatomy; Gene Networks Controlling Development and Involution; Growth, Development and Involution. **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose; Milk Fat; Milk Protein; Secretion of Milk Constituents. **Milk**: Bovine Milk; Buffalo Milk; Camel Milk; Colostrum; Equid Milk; Goat Milk; Introduction; Human Milk; Milk in Human Health and Nutrition; Milk of Marine Mammals; Milk of Monotremes and Marsupials; Milks of Non-Dairy Mammals; Milks of Other Domesticated Mammals (Pigs, Yaks, Reindeer, etc.); Milk of Primates; Physical and Physico-Chemical Properties of Milk; Seasonal Effects on Processing Properties of Cows' Milk; Sheep Milk. **Milk Proteins**: Analytical Methods; Casein, Micellar Structure; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; Immunoglobulins; Inter-Species Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity; Lactoferrin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; Nutritional Quality of Milk Proteins; Proteomics; α-Lactalbumin; β-Lactoglobulin. **Nutrition and Health**: Diabetes Mellitus and Consumption of Milk and Dairy Products; Effects of Processing on Protein Quality of Milk and Milk Products; Galactosemia; Milk Allergy; Nutraceuticals from Milk; Nutrigenomics and Nutrigenetics; Nutritional and Health-Promoting Properties of Dairy Products: Bone Health; Nutritional and Oral Health-Promoting Properties of Dairy Products: Caries Prevention and Oral Health; Nutritional and Oral Health-Promoting Properties of Dairy Products: Colon Cancer Prevention; Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake; Nutritional and Health-Promoting Properties of Dairy Products: Fatty Acids of Milk and Cardiovascular Disease.

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Lactoferrin

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Introduction

Lactoferrin (LF), also referred to as lactotransferrin, is an iron-binding glycoprotein and was identified in cow's milk already in 1939 but was isolated only in the early 1960s. LF is closely related to serum transferrin, which also occurs in milk but in a lower amount. However, LF and transferrin are distinct molecules, are different gene products, and behave differently in biological systems. Also, affinity for iron is much stronger in LF than in transferrin. All mammals seem to be able to produce these iron-binding proteins in milk, although their concentrations vary between species. LF is synthesized in the epithelial cells of the mammary gland, but also in other exocrine glands, and consequently is found in all external secretions, for example, saliva, pancreatic fluid, tears, sweat, and semen. Also, LF is present at high concentrations in synovial fluid and neutrophils (**Table 1**). LF synthesis is tissue-specific and is considered to be hormonally regulated. LF present in plasma is predominantly derived from secondary granules of circulating neutrophils. Human milk is particularly rich in LF, the concentration ranging from <1 to 16 $\mathrm{g\,l^{-1}}$ in colostrum and about 1 $\mathrm{g\,l^{-1}}$ in mature milk. In bovine colostrum, the LF content is, on average, 1.5 $\mathrm{g\,l^{-1}}$, ranging from 0.2 to 5 $\mathrm{g\,l^{-1}}$ and decreases to about 0.1 $\mathrm{g\,l^{-1}}$ in mature milk. Very high concentrations (up to 50 $\mathrm{g\,l^{-1}}$) of LF are found in secretions of non-lactating human and bovine mammary glands. LF is also present in the milk of other species, for example, goat, horse, pig, mouse, and guinea pig, but is virtually absent in milk from the rabbit, rat, and dog. In these species, transferrin is the principal iron-binding protein. LF is promptly delivered by circulating neutrophils to sites of microbial invasion. In the blood of septic patients, in inflamed synovial fluid, or in mastitic milk, the LF content increases manifold due to activated and degranulating neutrophils. Thus, LF may have a protective role in the mammary gland. LF has a high affinity for iron, a property that is linked to the majority of LF's proposed biological activities. Many non-iron-related functions have been described, as well. Apart from antimicrobial effects, LF enhances the bioavailability of iron and inhibits iron-mediated oxidative reactions, regulates inflammatory reactions, and modulates the intestinal microflora and possibly bone growth. In view of these functions, LF is considered to play an important role in the natural nonspecific defense system of the body. The expanding knowledge about the multifunctional role of LF has enlarged its scope of applications from food preservation to infant formulae, health-promoting foods and supplements, health-care products, oral hygiene products, cosmetics, and animal feeds. Also, clinical trials to develop LF-based products for the treatment of different diseases, for example, some cancers and ulcers, are under way.

Structure and Biochemical Properties

LF is a glycoprotein and is composed of a single-chain polypeptide and carbohydrate moieties. Human LF contains 691 and bovine LF 689 amino acids per molecule; the degree of sequence homology is 69%. Shared antigenic determinants have been demonstrated for human, bovine, and pig LF. The molecular weight (MW) of human LF is between 77 and 82 kDa, depending on the attached carbohydrates, and the molecule contains 16 disulfide bonds. Bovine LF has an MW of 80–84 kDa depending on the degree of glycosylation and it differs from human LF by a few amino acids and the glycan side chain.

The molecular and spatial structures of human and bovine LF have been characterized in detail and the genes encoding these proteins have been cloned and sequenced. In humans, a single functional LF gene has been identified, although gene polymorphism may occur. In the cow, LF present in milk and neutrophils also appears to be the product of the same gene. Bovine milk LF is heterogeneous with two major glycosylation forms identified. It is not known if the differences in glycosylation affect biological functions of LF but fully glycosylated LF is more resistant to proteases, like trypsin, than a nonglycosylated LF molecule. The degree of glycosylation of LF does not, however, affect its iron-binding capacity.

Each LF molecule can bind two ferric ions ($\mathrm{Fe^{3+}}$) with the concomitant incorporation of bicarbonate ($\mathrm{HCO_3^{-}}$) or carbonate ($\mathrm{CO_3^{2-}}$) ion. The affinity of LF to bind iron is very high ($K_\mathrm{d} \sim 10^{-30}$), being about 300 times higher than that of transferrin, which has the iron-transporting function in serum. The LF-bound iron is strongly attached even at pH 3, while bound ferric ions dissociate from transferrin already at pH 5 or in the presence of strong iron scavengers, such as citrate ions. The level of iron saturation of LF is relatively low in human milk, about

Table 1 Concentration of lactoferrin in various biological secretions

Biological secretions	*Concentration (mg ml^{-1})*
Human	
Colostrum (1–5 days)	4.6 (0.6–16)
Milk (0.5–6 months)	1.5 (0.3–4.2)
(>6 months)	0.7 (0.1–3.5)
Tear fluid	2.2
Seminal plasma	0.4–1.9
Synovial fluid	0.01–0.08
Saliva	0.007–0.010
Bovine	
Colostrum	1.5 (0.2–5.2)
Milk	0.1 (0.07–0.12)

Adapted from Masson PL and Heremans JF (1966) Studies on lactoferrin, the iron-binding protein of secretions. *Protides of Biological Fluids* 14: 115–142; Korhonen H (1977) *Journal of the Scientific Agricultural Society of Finland* 49: 434–447; Prentise A (1995) Regional variations in the composition of human milk. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 115–221. San Diego, CA: Academic Press, Inc.

6–8%, compared to 20–30% for bovine LF. In its natural state, LF has a salmon-pink color, the intensity of which depends on the degree of iron saturation. Iron-depleted LF with less than 5% saturation is called apo-LF, whereas the iron-saturated form is referred to as holo-LF. In addition to iron, LF also binds other metal ions, like copper, cobalt, zinc, and manganese. Under physiological conditions (pH 7.5), LF is positively charged and binds strongly polyanions, such as heparin, bacterial lipopolysaccharides (LPSs), lysozyme, immunoglobulins (especially IgA), casein, and DNA. On the other hand, at a low pH, as in the stomach, LF is cleaved by pepsin into several polypeptides, some of which have strong antimicrobial and immune system-regulating properties. LF has a very high isoelectric point (pI). The theoretical pI value calculated for bovine and human LF is 9.4 and 9.5, respectively.

Technological Properties

The thermal stability of LF has been studied mainly in model systems using buffered aqueous solutions or when added to milk. According to many experimental studies, the standard pasteurization regimes (72 °C for 15 s) used in the dairy industry have practically no effect on LF structure, antibacterial activity, and bacterial interaction. Also, preheating at 70 °C for 3 min followed by ultra-high temperature (UHT) treatment (130 °C for 2 s) leads to only 3% loss in residual iron-binding capacity. UHT treatment, however, abolishes the ability of iron-saturated LF to bind to bacteria, as well as the bacteriostatic activity of apo-LF. In spray-drying of milk, a marginal loss of LF activity is observed. In the above heat treatments, apo-LF denatures faster than holo-LF. LF seems to protect unsaturated fatty acids against oxidation and may contribute to the extension of the shelf life of iron-enriched and high-fat dairy or plant-derived foods.

Biological Importance

The potential biological role of LF has been studied extensively over the last 40 years. Originally, LF's function was considered to be essentially an antimicrobial one, but later this glycoprotein has proven to be far more multifunctional. At present, the major known or speculated *in vivo* activities of LF in lacteal secretions are

1. defense against infections of the mammary gland and the gastrointestinal tract (antimicrobial activity, regulation of the immune system),
2. nutritional effects (bioavailability of iron, source of amino acids),
3. mitogenic and trophic activities on the intestinal mucosa and gastrointestinal tract-associated lymphoid tissue and on bone tissue, and
4. antineoplastic activity in the gastrointestinal tract.

These activities are summarized in **Table 2** and will be discussed in more detail below.

Antimicrobial and Antiviral Effects

The *in vitro* antibacterial, antifungal, and antiviral activities of LF are well demonstrated and documented. LF exerts its antimicrobial effects by different mechanisms, which can be divided into four main patterns:

1. iron sequestering in order to produce iron deprivation,
2. binding of LF or its cleavage products to membrane structures of microbes, leading to disruption of cellular metabolism,
3. protease-like antimicrobial activity toward some bacterial virulence factors, and
4. induction of apoptosis in host cells infected by some intracellular pathogenic bacteria.

The best known mechanism is mediated by binding of iron from the environment to apo-LF. Iron is an essential factor for the growth of many microorganisms and an important factor for the virulence of many pathogenic bacteria. In inflammatory sites, high amounts of activated neutrophils degranulate and release LF, leading to effective scavenging of iron. Iron deprivation leads to the inhibition of *in vitro* growth of a variety of bacteria and yeasts, for example, *Escherichia coli*, *Helicobacter pylori*, *Klebsiella*, *Salmonella*, *Proteus*, *Pseudomonas*, *Listeria*, *Bacillus*, *Streptococcus*, and *Candida albicans*. The iron deprivation-related bacteriostatic

Table 2 Potential biological effects of lactoferrin or lactoferrin-derived peptides

Activity	*Proposed mechanism*
Iron carrier	Increase in the solubility of iron and receptor-mediated uptake
Oxidation inhibition	Reduction in the formation of free iron-mediated free reactive oxygen species
Antibacterial	Inhibition of cell growth and spore germination by scavenging iron, bactericidal effect by membrane disruption, decrease of bacterial virulence by inhibition of adhesion, by disruption of bacterial plaques, and by protease-like activity on virulence factors
Antiviral	Prevention of virus attachment to host cells
Antifungal	Stimulation of immune functions
Anti-inflammatory	Binding of bacterial lipopolysaccharide, inhibition of phagocyte priming, reduction of proinflammatory cytokine production, stimulation of anti-inflammatory cytokine production, inhibition of complement classical pathway
Immunostimulating	Stimulation of the activity of immune cells, for example, natural killer cells, acceleration of T-cell maturation, stimulation of immunoglobulin production by B cells, stimulation of chemotaxis and phagocytosis by leukocytes
Anticarcinogenic	Stimulation of immune functions, stimulation of apoptosis, inhibition of angiogenesis

Adapted from Nuijens JH, van Berkel PHC, and Schanbacher FL (1996) Structure and biological actions of lactoferrin. *Journal of Mammary Gland Biology and Neoplasia* 1(3): 285–295; Baveye S, Elass E, Mazurier J, Spik G, and Legrand D (1999) Lactoferrin: A multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clinical Chemistry and Laboratory Medicine* 37(3): 281–286; Vorland LH (1999) Lactoferrin: A multifunctional glycoprotein. Review article. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 107: 971–981; Kruzel ML, Actor JK, Boldogh I, and Zimecki M (2007) Lactoferrin in health and disease. *Postepy Higieny i Medycyny Doświadczalnej* 61: 261–267; Weinberg ED (2003) The therapeutic potential of lactoferrin. *Expert Opinion on Investigational Drugs* 12(5): 841–851.

effect is most pronounced for *E. coli*, while some other bacterial strains are unaffected by this mode of action. By chelating iron, LF can in many cases block biofilm development by bacteria. Iron deprivation stimulates bacterial surface motility, causing the bacteria to wander across the surface instead of forming cell clusters and biofilms. The bacterial transition from a free-living form into biofilms can be devastating for the host, because biofilms protect bacteria effectively from the host defense mechanisms and antibiotics. LF is able to block biofilm development by *Pseudomonas aeruginosa* at concentrations below those that kill or prevent growth.

LF can have a direct bacteriostatic or bactericidal effect on Gram-negative bacteria by destabilizing their outer membrane, which results in the liberation of LPSs. Enhanced synergistic antibacterial action of LF is achieved in the presence of specific antibodies or lysozyme. Lysozyme and LF are present at high concentrations in neutrophil granules and at sites of inflammation. Lysozyme alone is not very effective against Gram-negative bacteria due to the LPS layer in the outer membrane but, by binding to the LPS layer, LF enables lysozyme to gain access to the peptidoglycan in the bacterial cell wall. The synergistic actions of LF and lysozyme are suppressed if LF is iron-saturated.

The addition of apo-LF to many other antimicrobial agents enhances their antimicrobial activity. The inhibitory concentrations of several antibiotics like vancomycin are lowered *in vitro* in the presence of LF.

On the other hand, some iron-requiring pathogenic microorganisms synthesize and secrete small iron-chelating molecules, siderophores, which bind ferric ions with a high affinity and transport them into cells through specific receptors. Some pathogens, such as *H. pylori*, *Neisseria* sp., *Treponema*, and *Shigella* sp., express specific outer cell membrane receptors for uptake of LF and may exploit iron (which is bound in LF) for promoting their growth and pathogenicity. These bacteria may benefit from the inflammatory reaction of the host.

Furthermore, partial hydrolysis of the LF molecule by heat or by pepsin results in the formation of an antibacterial peptide, known as lactoferricin (LFcin), which exerts a much stronger antimicrobial effect than the intact molecule. LFcin is derived from the N-terminal region of bovine (f17–41) or human (f1–47) LF. Recently, several other antibacterial peptides have been purified from the same region. Bovine LFcin consists of 25 amino acids, whereas human LFcin is composed of 47 amino acid residues, including a region homologous to bovine LFcin. Both LFcins have been shown to display variable antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria, fungi, molds, and protozoa. There is direct evidence for the generation of LFcin in human stomach after ingestion of bovine LF. LFcins are particularly bactericidal against enteropathogenic bacteria. Bovine LFcin has been found to be active against clinical isolates of enterohemorrhagic *E. coli* O157H:7 at concentrations significantly lower than those of the LF hydrolysate or LF itself. The mechanism of the action of LFcin is not known in detail but is probably similar to that of other cationic and amphipathic antibacterial peptides. The peptide binds to LPS in Gram-negative bacteria and to teichoic acid in Gram-positive bacteria through electrostatic interactions. In binding to the cytoplasmic membrane, LFcin kills susceptible microorganisms by causing depolarization and increase in permeability of the cell membrane, leading to disruption of the general energy metabolism and inhibition of macromolecular biosynthesis of microbial cell.

Also, protease-like antimicrobial activities toward some bacterial virulence factors have been found for LF. For example, human LF has been shown to reduce the invasiveness of an enteropathogenic *E. coli* strain by blocking proteins that contribute to adherence and cause hemolysis. Recently, bovine LF and LFcin have been shown under *in vitro* conditions to induce apoptosis, programmed cell death, in *Listeria monocytogenes*-infected THP-1 cells and in Caco-2 epithelial cells.

Experimental studies have shown that large amounts of orally administered bovine LF can, probably after cleavage of LF to LFcin, significantly reduce the counts of *H. pylori* colonizing the stomach. The glycans present in LF may bind to the bacterial adhesins, thus interfering with the attachment of this bacterium to the epithelial cells. LF and LFcin inhibit the growth of fungi and protozoa but the mechanism of action is not yet fully known. Bovine apo-LF and LFcin directly bind to *Candida* cells and are highly effective in disrupting the cell membrane. LF has been demonstrated to inhibit *in vitro* the multiplication of a wide spectrum of RNA and DNA viruses, such as human cytomegalovirus, human immunodeficiency virus, herpes simplex virus 1 and 2, human hepatitis C virus, and human poliovirus type 1, and also some naked viruses, for example, rotavirus. LF prevents the binding of several viruses to host cells by interaction with cell surface glycosaminoglycans and low-density lipoprotein receptors, which act as binding sites for some enveloped viruses. The antiviral activity does not involve iron binding. Apo-LF has been shown to be less effective than holo-LF and bovine LF to be more active against viruses than human LF.

There is increasing evidence suggesting that innate LF is actively involved in the prevention of certain microbial infections *in vivo*. Also, orally administered bovine LF may be beneficial in the prevention and treatment of various microbial infections in humans and farm animals. Many microbes isolated from mastitic milk are sensitive to bovine LF or bovine LFcin, whereas many pathogenic strains of *E. coli* and *Staphylococcus aureus* show resistance or can develop resistance to LF. However, bovine LF has a synergistic effect with penicillin G against several mastitic strains of *S. aureus*. Results of recent studies suggest that bovine LF added to penicillin G may offer an effective combination for the treatment of chronic infections caused by *S. aureus* strains showing high resistance to β-lactam antibiotics.

In newborn calves and piglets, LF-supplemented feeds have been proven to be beneficial in lowering the incidence of scouring. Feeding of bovine LF or LFcin to pathogen-free mice has been demonstrated to be effective in suppressing the growth of various intestinal bacteria, for example, clostridia, and bacterial translocation of *E. coli* from the intestine into other organs. Also, LF has been shown to protect mice against a lethal dose of *E. coli* in experimental infection. There is some evidence about the contribution of ingested LF against microbial infections in humans but further substantiation in clinical studies is required. Bottle-feeding of human infants with LF-supplemented infant formulae has been shown to reduce the number of coliform bacteria and simultaneously to increase the number of bifidobacteria in the feces. Promising preliminary results have been reported about the topical application of LF in the treatment of fungal diseases, such as tinea pedis and mucosal candidiasis. In human studies, the favorable effect of yogurt supplemented with bovine LF on rotavirus gastroenteritis and a similar effect of LF-containing tablets on chronic hepatitis C, rotaviral gastroenteritis, and *H. pylori* gastric infection have been reported. In dogs and cats, the therapeutic effect of LF-containing pet food on dermatitis has been demonstrated.

Modulation of the Immune System and Inflammatory Response

LF has the ability to bind to the surface of different immune cells, and receptors for LF have been found in several other cell types. It is likely that orally administered LF or peptides derived therefrom promote both local and systemic effects. Both stimulatory and inhibitory effects of LF on lymphocyte proliferation have been described. LF has been reported to induce *in vitro* maturation of T and B lymphocytes, to increase the activity of natural killer (NK) cells, and to enhance the phagocytic activity of neutrophils.

Orally administered bovine LF ($50\,\mathrm{mg\,day^{-1}}$) increased significantly the proportion of neutrophilic precursors in the peripheral blood of healthy human volunteers. Also, the same dosage reduced the spontaneous production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α by cultured peripheral blood mononuclear cells and enhanced chemotactic reactions of leukocytes. Furthermore, oral application of LF has been reported to increase leukocyte turnover and phagocytic activity and to enhance polymorphonuclear leukocyte (PMN) adherence to the endothelium in healthy humans. Recently, human recombinant LF has been demonstrated to induce the maturation of dendritic cells from human monocytes *in vitro*, suggesting that a key immunomodulative function of LF may be to link the innate immune response to an adaptive one by promoting dendritic cell maturation.

In mice, bovine LF has been shown to induce both the mucosal and systemic immune responses. Cell culture studies have demonstrated that LF influences the production of various cytokines that regulate the immune and inflammatory responses of the body. LF can suppress *in vitro* the release of pro-inflammatory cytokines IL-1, IL-6, and TNF from monocytes in response to activation by LPS. Furthermore, LF and LFcin stimulate the release of chemotactic cytokine IL-8 from human PMNs. It is postulated that LF can control the cytokine-induced pro-inflammatory cascade during the development of a systemic inflammatory response.

LF seems to regulate the inflammatory response by various mechanisms, which are still only partly known. In inflammatory sites, activated neutrophils release LF from secondary granules. The LF augments chemotactic activity of neutrophils and thus favors the rapid recruitment of PMN from blood to the inflammatory sites. The released LF binds to specific receptors on monocytes and capillary endothelial cells and inhibits priming reactions and the onset of production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, and stimulates the production of anti-inflammatory cytokines IL-4 and IL-10. LF also suppresses the progressive inflammatory cascade caused by bacterial endotoxin by binding bacterial LPS and preventing its interaction with leukocytes. LF also reduces the oxidative damage to host tissues due to the neutrophil oxidative burst reaction during inflammatory processes by effectively scavenging free iron from the immediate vicinity. In particular, LF seems to prevent peroxidation of cell membrane lipids. Furthermore, LF has been shown to inhibit the classical pathway of complement reaction. Experimental studies have demonstrated that feeding LF can protect against endotoxin lethal shock in germ-free piglets.

LF has been shown to be transported into the cell nucleus where it can bind DNA, suggesting that LF may regulate the phenotypic traits of the host. Recent studies suggest that LF and LF-derived peptides participate in the defense against the development and progression of tumors. Bovine LFcin is reported to be cytotoxic against a variety of human and rodent cancer cells *in vitro*. Human LF has been shown to suppress the growth of fibrosarcoma cells and to inhibit experimental metastasis of melanoma cells in mice. Bovine LF has been reported to reduce the development of neoplastic cells in the esophagus, colon, and lungs in rats treated with carcinogens. Bovine LF also had a synergistic effect in the prevention of chemically induced genotoxicity and development of carcinomas in hamsters. So far, relatively little is known about the mechanisms by which LF exerts its anticancer effect. Bovine LFcin is a potent inducer of apoptosis in several different human leukemia and carcinoma cell lines *in vitro*. Results from animal model studies suggest that both orally administered bovine LF and intraperitoneally administered bovine LF participate in the regulation of angiogenesis, inhibiting the growth of new capillaries essential for the progression and metastasis of solid tumors. The effect may be mediated by blocking the endothelial function and inducing the production of angiogenesis-inhibiting cytokines INF-γ and IL-18. LF has also been demonstrated to activate NK cells, PMNs, and lymphokine-activated killer cells. Thus, the antitumor activity may be mediated by the activating effect on cell-mediated cytotoxic immune reactions against neoplasia. A phase III clinical trial is in progress with the purpose of developing recombinant human LF-based pharmaceuticals for the treatment of non-small cell lung cancer.

Growth Factor-Like Effects

LF has been suggested to exert mitogenic and trophic effects in the gastrointestinal tract. It has been suggested that LF stimulates protein synthesis in endothelial cells, measured as thymidine incorporation into DNA. The potential regulatory role of LF in the intestinal maturation of the infant warrants, however, further research as the observed effects may have been of nonspecific character.

Both bovine LF and human LF have been reported to have an anabolic effect on bone tissue *in vivo* in a mouse model when injected locally into adult mice. In *in vitro* studies, bovine LF has been shown to stimulate osteoblast proliferation and differentiation, inhibit apoptosis of osteoblasts, stimulate chondrocyte proliferation, and inhibit development of osteoclasts. Thus, LF may also have a regulatory role in bone growth.

Nutritional Significance

LF is one of the major whey proteins in human milk and is also relatively abundant in bovine colostrum. From a nutritional point of view, LF is of interest as a dietary protein source of amino acids and for the bioavailability of iron.

LF has an amino acid composition that indicates a high nutritional value, but nutrition may not be the major role of LF, as it is known to be quite resistant to digestive enzymes. Although the amount of intact LF found in feces constitutes only about 10% of the amount ingested, it is thought to be supportive of a physiological role of LF in the gut of the infant. LF may exert its biological functions as undigested and partially digested forms in the gastrointestinal tract.

In toxicity studies using rat models with orally administered bovine LF and human LF as well as with purified recombinant human apo-LF and holo-LF, no adverse findings have been observed at a dose level of 1.8 g per day per kg of body weight for 13 weeks or with a diet containing 0.2% of bovine LF for 40 weeks.

The role of LF in the absorption of iron from milk is still controversial. The high bioavailability of iron from human milk and the discovery of LF receptors on intestinal brush border membranes of many species provide a basis for the assumption that LF promotes iron absorption in breast-fed infants. However, animal model studies with human LF and bovine LF and clinical studies on infants fed formula supplemented with bovine LF have failed to show any significant improvement in iron absorption. It has been suggested that the absorption capacity is affected by the highly species-specific receptors of LF. They may have an impact on the uptake of iron by endocytosis. Recently, orally administered bovine LF has been shown to increase hemoglobin and total iron concentration in the blood serum of pregnant women and to prevent the lowering of hemoglobin level in female long-distance runners during the training period without any side effects that are common with ferrous sulfate. In another study on young US women, iron was equally well absorbed from purified recombinant human LF as from ferrous sulfate. Thus, oral LF can influence iron homeostasis.

Application and Safety Aspects

Since the 1970s, several gel filtration and chromatographic methods have been developed for the isolation and purification of LF from colostral or cheese whey. Large-scale production techniques, based on cation-exchange resins, followed by gel filtration or ultrafiltration (UF) have been employed to isolate both LF and lactoperoxidase (LP) from cheese whey. Other methods developed for the isolation of LF include microporous membranes containing immobilized sulfonic acid, high-performance liquid chromatography (HPLC) coupled with cation exchanger, metal chelate interaction chromatography, monoclonal antibody immunoaffinity, and affinity chromatography using immobilized ferritin, heparin, DNA, or textile dyes.

The industrial large-scale production of bovine LF started in Belgium in 1985, but commercial exploitation of bovine LF has developed relatively slowly until recent years. Asian companies have utilized bovine LF already for more than 20 years as an ingredient of commercial foodstuffs, most of them being milk-based products, for example, infant formulae, yogurt, skim milk, and milk-type drinks. Also, bovine LF-containing food supplemental tablets, pet food, oral care products (mouthwash, gel, toothpaste, and chewing gum), and cosmetics have been launched on the markets in many countries. The US Food and Drug Administration (FDA) has approved the use of bovine LF (at not more than 2% by weight) as a spray to reduce microbial contamination on the surface of raw beef carcasses. The FDA has granted bovine LF a 'generally recognized as safe' (GRAS, GRN 67) status and this determination accounts for uses at defined levels in beef carcasses, subprimals, and finished cuts. Bovine LF and recombinant human LF are now commercially available for applications in preservatives, nutraceuticals, and pharmaceutical products. Toxicological studies in rats for 13 weeks with a daily oral administration of bovine LF and of recombinant human LF produced in transgenic cows have revealed that the level with no observed adverse effect was at least 2 g per kg of body weight per day.

See also: **Enzymes Indigenous to Milk:** Lactoperoxidase. **Milk:** Colostrum.

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Immunoglobulins

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Introduction

Immunoglobulins (Igs), also called antibodies, are proteins present in the milk and colostrum of all lactating species. The biological function of milk Igs is to give the offspring immunological protection against microbial pathogens and toxins and to protect the mammary gland against infections. To this end, the major mechanisms provided by milk and colostral Igs are augmenting phagocytosis and cell-mediated cytotoxicity reactions by leukocytes, agglutination of bacteria, neutralization of microbes and toxins, and activation of the complement system in milk. Igs are divided into classes having different physicochemical properties, structures, and biological activities. The major classes in bovine and human milk are IgA, IgG, and IgM.

The concentration of different Ig classes in milk and colostrum varies considerably according to species, breed, age, stage of lactation, and health status and is often different from that in blood (**Table 1**). For example, in human milk and colostrum, the IgA class comprises about 90% and in blood 15–20% of total Ig whereas in cow the IgG class is dominant in milk, colostrum, and blood (about 60–70, 80–90, and 90% of total Ig, respectively).

The transport of Igs from serum to milk is a selective process, favoring in most species homologous IgG. Specific receptors are involved in the process, enabling the characteristic concentration of Ig isotypes in milk and colostrum. In many species, the absorption of Igs from intestine is also selective and receptor mediated. However, in ruminants, for example, in the cow, the absorption of Igs takes place nonselectively during the first 12–36 h after birth of the offspring. Ruminant neonates are born virtually without Igs and the colostral Igs are essential for survival. Thus, in ruminants the colostrum contains remarkably higher amounts of Igs than the mature milk. Mature bovine milk contains only 1–2% of the Igs present in colostrum.

The Ig fraction of milk is used commercially as feed supplements and replacers of colostrum, mainly for neonatal calves and pigs, in order to prevent gastrointestinal infections. Increasing interest has recently been focused on the development of colostrum-based Ig products that contain specific antibodies for the prevention or treatment of infections in humans.

Structure and Functions of Immunoglobulins

Structure of Immunoglobulin Classes

The Ig classes in milk and colostrum of the cow are IgG, IgM, and IgA and of human, IgG, IgM, IgA, and IgD, respectively. The basic structure of all Igs is similar. They are composed of two identical light chains (molecular weight of each around 23 kDa) and two identical heavy chains, each of 53 kDa (**Table 2**). There are two types of light chains (κ and λ), differing in chain structure but having homologous amino acid sequences. The light chains contain a constant region (C_L) and variable region (V_L). The V_L region determines the immunological specificity. Light chains are attached to the heavy chains by a disulfide bond and the two heavy chains are held together by disulfide bonds near a hinge region. The hinge region gives the molecule flexibility needed in antibody–antigen interactions. The two identical antigen-binding sites needed in these interactions are formed by the N-terminal part of one heavy chain and the variable region of one light chain. The complete Ig, or ‘antibody’, molecule has a molecular mass that varies around 160 kDa. The bovine IgG molecule occurs predominantly in two subclasses: IgG_1 and IgG_2 (**Figure 1**(**a**)).

Monomeric IgM and IgA have a similar basic structure to IgG except for the addition of a C-terminal octapeptide to the heavy chains. IgA occurs as a monomer or dimer, the latter comprising two IgA molecules joined together by a J-chain and a secretory component. This complex is called secretory IgA (sIgA) and has a molecular weight of about 380 kDa. IgM consists of five subunits, similar to monomeric IgA, which are linked together in a circular mode by disulfide bonds and a J-chain; the molecular weight of pentameric IgM is approximately 900 kDa (**Figure 1**(**b**)).

Human sIgA consists of two identical monomers joined together by a 15 kDa J-chain and connected to secretory component, an 83 kDa epithelial glycoprotein. In human serum, the principal form is a monomer. Both human serum and milk IgM have a pentameric structure but, unlike the serum IgM, some of milk IgM is combined with the secretory component.

In humans the high diversity in structures of variable chains comprising the antigen-binding sites of Igs is

Table 1 Concentration of immunoglobulins in serum and mammary secretions of some species

		Concentration ($g\ l^{-1}$)		
Species	*Ig class*	*Serum*	*Colostrum*	*Milk*
Cow	IgG total	25.0	60 (20–200)	0.47 (0.15–0.8)
(*Bos taurus*)	IgG_1	14.0	15–180	0.35 (0.3–0.6)
	IgG_2	8–11	1–3	0.02–0.12
	IgA	0.4	3.5 (1–6)	0.05–0.14
	IgM	3.1	5 (3–9)	0.04–1.0
Human	IgG	13.5	0.4 (0.16–6.8)	0.04 (0.04–0.054)
	IgA	3.5	11.4 (6–40)	0.5 (0.26–1.8)
	IgM	1.5	0.3 (0.12–1.9)	0.03 (0.02–0.1)
	IgD	30[a]	0.36[a] (0.02–4.1)	
Pig	IgG	21.5	58.7	3.0
	IgA	1.8	10.7	7.7
	IgM	1.1	3.2	0.3
Horse	IgG total	21.9	113.4	0.39
	$IgG_{(T)}$	8.2	15.2	0.09
	IgA	1.5	10.7	0.48
	IgM	1.2	5.4	0.03

[a] mg l^{-1}

Data extracted from Hurley WL (2003) Immunoglobulins in mammary secretions. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 1: Proteins*, 3th edn., pp. 421–447. New York: Kluwer Academic; Plenum Publishers, Butler JE (1974) Immunoglobulins of the mammary secretions. In: Larson BL and Smith VR (eds.) *Lactation, Vol. III: Nutrition and Biochemistry of Milk/Maintenance*, pp. 217–255. San Diego, CA: Academic Press, Turner 1996, Harzer G and Haschke F (1989) In: Renner E (ed.) *Micronutrients in Milk and Milk Based Food Products*, pp. 125–237. Essex, England: Elsevier Publishers LTD, Brandtzaeg P (1983) The secretory immune system of lactating human mammary glands compared with other exocrine organs. *Annals of the New York Academy of Sciences* 409: 353–382 and Prentice A (1995) Regional variations in the composition of human milk. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 115–221. San Diego, CA: Academic Press, Inc.

Table 2 Properties of bovine immunoglobulins

	IgG_1	*IgG_2*	*IgA*	*sIgA*	*IgM*
Physico-chemical					
Molecular weight (kDa)	146–163	146–154	160	385–430	900
Heavy chain	56–59	54–59	61–63		62–76
Heavy-chain type	$\gamma1$	$\gamma2$	α		μ
Number of H- and L-chains	2	2	2	4	10
Structure	Monomer	Monomer	Monomer	Dimer	Pentamer
$S_{w,20}$	6.3–7.1 S	6.5–7.1 S	6.5–7 S	10.8–11 S	19 S
Carbohydrates (%)	2.8–3.1	2.6–3.0	6–10		10–12
Isoelectric point	5.5–6.8	7.5–8.3			
Immunological					
Opsonization	+++	+	0	0	0
Complement fixation	+++	+	0	0	++
Agglutination	+	+	++	++	+++

0 = No activity, + = low activity, ++ = moderate activity, +++ = strong activity. $S_{w,20}$ = sedimentation constant in Svedbergs units ($\times10^{-13}$ S).

Data extracted from Butler JE (1974) Immunoglobulins of the mammary secretions. In: Larson BL and Smith VR (eds.) *Lactation, Vol. III: Nutrition and Biochemistry of Milk/Maintenance*, pp. 217–255. San Diego, CA: Academic Press, Butler JE (1998) Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. *Revue Scientifique et Technique* 17(1): 43–70, Hurley WL (2003) Immunoglobulins in mammary secretions. In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 1: Proteins*, 3th edn., pp. 421–447. New York: Kluwer Academic; Plenum Publishers, Tewari UJ and Mukkur TKS (1975) Isolation and physico-chemical characterization of bovine serum and colostral immunoglobulin G (IgG) subclasses. *Immunochemistry* 12: 925–930, and Zeitlin L, Cone RA, Moench TR, and Whaley KJ (2000) Preventing infectious disease with passive immunization. *Microbes and Infections* 2: 701–708.

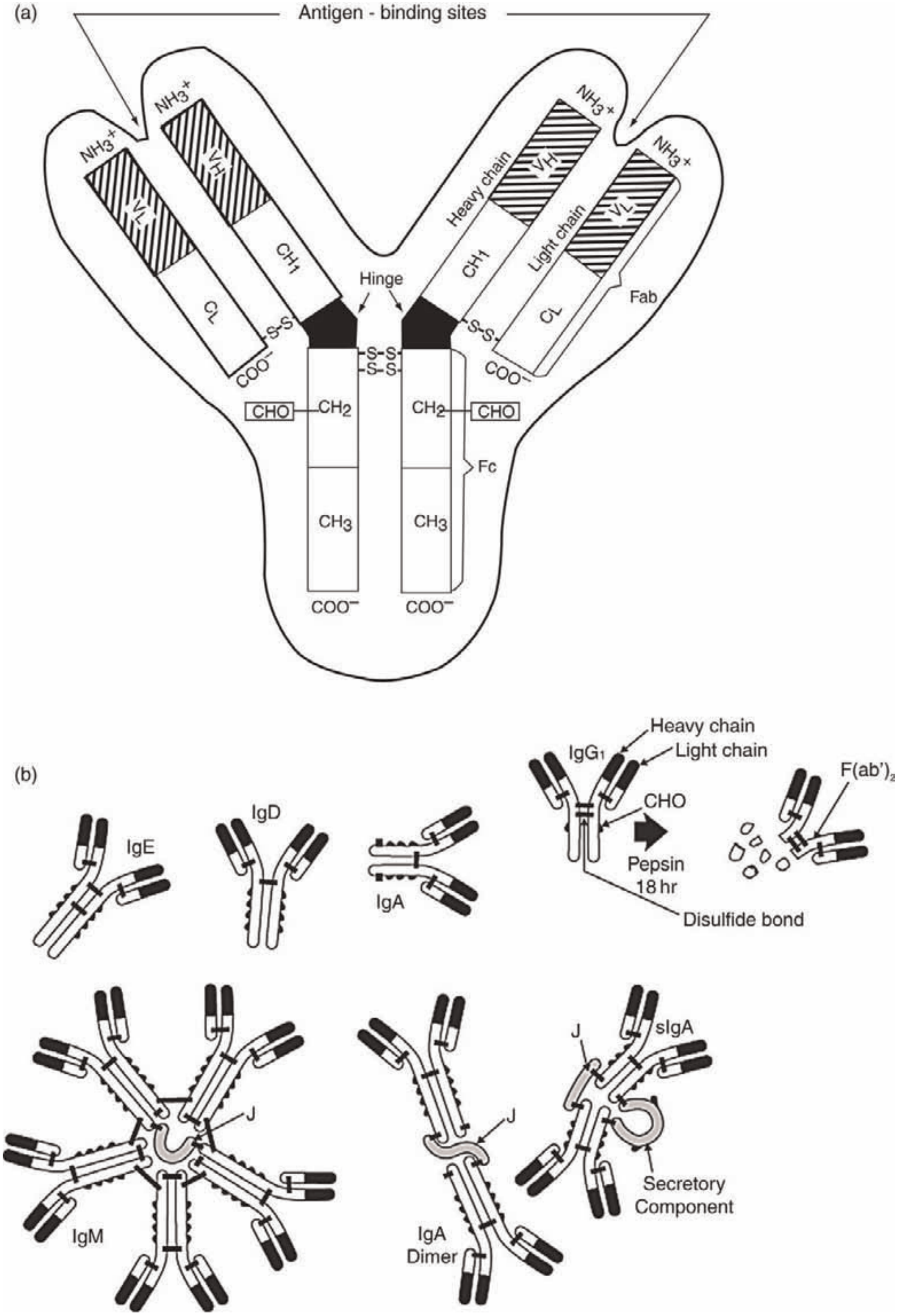
(a)
Antigen - binding sites
NH3+
NH3+
NH3+
NH3+
VL
VH
VH
VL
Heavy chain
Light chain
CH1
CH1
CL
CL
Hinge
Fab
S-S
S-S
-S-S-
-S-S-
COO−
COO−
CHO
CH2
CH2
CHO
Fc
CH3
CH3
COO−
COO−
(b)
IgE
IgD
IgA
IgG1
Heavy chain
Light chain
CHO
Pepsin
18 hr
Disulfide bond
F(ab')2
J
J
J
sIgA
IgM
IgA
Dimer
Secretory
Component

Figure 1 (a) Schematic diagram of a basic immunoglobulin. From Larson BL (1992) Immunoglobulins of the mammary secretions. In: Fox PF (ed.) *Advanced Dairy Chemistry, Vol. 1: Proteins*, pp. 231–254. London: Elsevier Science Publishers. (b) Structure of five classes of immunoglobulins. Adapted from Larson BL (1992) Immunoglobulins of the mammary secretions. In: Fox PF (ed.) *Advanced Dairy Chemistry, Vol. 1: Proteins*, pp. 231–254. London: Elsevier Science Publishers and from Butler JE (1974) In: Larson BL and Smith VR (eds.) *Lactation, Vol. III: Nutrition and Biochemistry of Milk/Maintenance*, pp. 217–255. San Diego, CA: Academic Press.

generated via rearrangements of a large number of divergent Ig gene segments which produce the genes encoding the variable regions of light and heavy chains, respectively. In contrast, cow and sheep having germline Ig gene pools of limited diversity employ alternative post-arrangement mechanisms for antibody diversification. Cow and sheep generate Ig diversity by non-antigen-dependent somatic hypermutations. The cow also has an exceptionally long complementary determining region 3 of the heavy-chain regions of IgM to compensate the lesser diversity of germline variable heavy-chain genes. However, the understanding of the processes that form bovine Ig repertoire is still incomplete.

Functions of Immunoglobulins

All Ig classes bind antigens and, in addition, exhibit one or more effector functions. While one part of an antibody (Fab) binds to antigen, other parts (mostly the Fc region) interact with other elements. In effect, the antibodies function as flexible adaptors linking various parts of the cellular and humoral immune system.

The prevention of the adhesion of microbes to surfaces like intestinal epithelial linings may be the most important mechanism of colostral and milk antibodies in protecting the host. The attachment of a pathogenic organism to the epithelial lining is the most critical step in the establishment of infection. The ability of Igs to agglutinate bacteria reduces the capability of bacteria to adhere to epithelial surfaces or to tooth enamel. Normal colostrum and milk are known to contain agglutinating Igs for a large number of pathogenic and non-pathogenic microorganisms. Igs can inhibit bacterial metabolism and reduce the production of harmful components, such as toxins, by blocking enzymes and receptors. This blocking may also reduce the pathogen's ability to produce structures needed in adherence to epithelia. Milk Igs, also partially degraded ones, can benefit the offspring by binding and inactivating toxins produced by pathogenic microbes and by neutralizing viruses. In clinical studies, colostral and milk Igs have been demonstrated to be able to protect the offspring against a variety of viral infections. Igs also augment the recognition and phagocytosis of bacteria by leukocytes (opsonization).

Leukocytes are an integral part of normal milk and colostrum and are of vital importance in defending the mammary gland against pathogens. Recent studies have shown that colostral leukocytes enhance the calf's defense against infection. The effector functions include also binding of Igs to leukocytes or to host tissues. In blood and tissues, the most important function of Igs is possibly the activation of complement-mediated bacteriolytic reactions, but the significance of this mechanism in milk remains obscure. Bovine and human colostra contain an active complement system participating in the immune defense of the udder (**Table 2**).

IgM antibodies, although produced in smaller amounts than IgG, are considerably more efficient than IgG with regard to most of the above activities, including complement fixation, opsonization, and agglutination of bacteria. IgM can prevent the migration of bacteria by binding to the flagellas used in movement. Bovine IgA, in contrast, does not fix complement or opsonize bacteria, but agglutinates antigens and neutralizes viruses and bacterial toxins. The main function of sIgA is to protect the mucosal barriers by binding viruses and bacteria, thus preventing them from attaching to mucosal epithelial cells. Mucosal IgA also contributes to the antigen presenting. The milk Igs have also been found to act synergistically with non-specific antimicrobial components of milk, such as lactoferrin and lysozyme, as well as the lactoperoxidase–thiocyanate–hydrogen peroxide system.

Concentrations of Immunoglobulins in Milk and Colostrum

Bovine colostrum contains substantially higher concentrations of Igs than mature milk. In bovine colostrum, Igs make up to 70–80% of the total protein content, whereas in mature milk, the Igs account for only 1–2% of the protein. The main change from colostrum to normal milk occurs in the first few days after parturition and continues at a reduced rate for approximately 5–7 days (see Colostrum). **Table 1** provides data on the concentrations of Igs in serum, colostrum, and milk of selected mammal species.

In the cow, the major Ig class is IgG. Its concentration at first milking *post-partum* ranges from 15 to 180 g l^{-1}, the mean being approximately 60 g l^{-1}. Thereafter, the IgG concentration falls sharply to about 1 g l^{-1} at 12th–14th milking. The concentration of the various bovine Igs in serum and lacteal secretions varies according to the breed, age, health status, and stage of lactation of the animal. The major IgG subclass of colostrum and milk is IgG_1, which accounts for about 50–80% of total Igs. The average concentrations of IgG_2, IgM, and IgA are relatively small compared to IgG_1 (see **Table 1**). Bovine colostrum contains about 1–6 g l^{-1} of IgA and mature milk 0.05–0.1 g l^{-1}. The concentration of IgM in bovine colostrum varies from 3 to 5 g l^{-1} and from 0.04 to 0.1 g l^{-1} in mature milk.

In serum, both IgG subclasses are present at about equal concentrations (IgG_1 11.2 g l^{-1}, IgG_2 9.2 g l^{-1}), while IgA and IgM occur at concentrations of about 0.4 and 3.1 g l^{-1}, respectively (see **Table 1**).

Cows in their first lactation produce significantly less (total yield about half or less) IgG than cows in later lactations due to both a lower IgG concentration and a smaller volume of colostrum.

In Indian buffalo (*Bos bubalus bubalis*), the concentrations of Igs in colostrum and milk are of the same order of magnitude as those of its close relative, the cow (*Bos taurus*). The IgG concentration in first colostrum and in transient milk has been reported to be about 50 and 150% higher, respectively, than in the cow. In buffalo, IgG_1 is the dominant Ig subclass and the content of IgG_2, IgA_1, IgA_2, and IgM is substantially smaller than that of IgG_1. IgG_1 and IgG_2 of buffalos and cows have very similar chemical and biological properties and are antigenically closely related.

In colostrum of the dromedary camel (*Camelus dromedarius*), the major Ig class is IgG but, unlike in bovines, IgG includes three subclasses IgG_1, IgG_2, and $IgG_{3.}$ IgA and IgM are present only in minor amounts. Camel Ig classes are antigenically very different from the bovine Igs and have several structural characteristics different from bovine Igs. In horse and pig colostrum, IgG is a major and IgA a minor component, but after transition of colostrum to mature milk, IgA becomes the major component (see **Table 1**). Also, in domestic cat, dog, and rat colostrum, IgG is the major class but after the transfer of Ig has ceased, IgA becomes dominant in the milk of these species. In human colostrum and mature milk, IgA is the dominant Ig class, comprising over 90% of all Igs, whereas IgG is the most abundant Ig in blood. The main form of human milk IgA is dimeric secretory IgA (sIgA), comprising over 90% of total IgA.

Transfer of Immunoglobulins

Main Pathways

Igs represent the mother's specific immune response to the antigens encountered in the past. This immunological protection is transferred from the mother to the offspring either *in utero* or via colostrum or milk. In the *in utero* pathway, Igs pass from the mother's blood into the fetus across the placenta or yolk sac. In transfer via colostrum or milk, the Igs, which are derived from blood or are synthesized in the mammary gland, are transported through mammary secretory cells into the colostrum or milk. The ingested Igs are transferred from the gut across intestinal cells into the blood of the offspring.

The eutherian mammals can be divided roughly into three groups according to their Ig transfer pathways. Species like human and rabbit have only placental transfer. Human infants are born with serum concentrations of IgG which equal those of their mothers. Most farm animals (pig, horse, cow, sheep, and goat) have no *in utero* transfer of Igs but an extensive lacteal transport. The newborns of these species are virtually agammaglobulinemic. Species like dog, mouse, and rat use both pathways. In marsupials, there is little evidence that maternal antibody transfer takes place across the placental barrier but several reports suggest that in the marsupial pouch, the young depends on passive immune protection from mother's milk for a long period after birth.

Transport Mechanisms

Transfer to milk

The epithelium sheet lining the mammary gland separates the milk space from the interstitial space. During the dry period, the epithelium is permeable to fairly large molecules, such as Igs, whereas during lactation (except during mastitis) this lining is impermeable to small molecules. Thus, a vast majority of IgG in bovine colostrum and milk is derived from blood plasma. The transport by cellular uptake involves binding of IgGs to Fcγ receptors in the basal membranes of the udder. The appearance and presence of specific high-affinity Fcγ receptors for IgG_1 and lower-affinity receptors for IgG_1 and IgG_2 on the mammary gland acinar epithelium corresponds to the amounts of these subclasses in colostrum and milk at the time of maximal IgG transport. The Fc receptor-mediated endocytosis is followed by vesicular transport and exocytosis at the apical membrane. In contrast to IgA transport, the receptor does not remain with the IgG in the milk.

The Igs are produced by B lymphocytes. Some Igs are carried on the surface of B lymphocytes where they act as receptors and the others are free in body fluids. Contact between B lymphocytes and antigen is needed to cause the B lymphocytes to develop into antibody-forming and secreting plasma cells. The ruminant mammary gland contains plasma cells adjacent to the site of transfer of Igs. These cells produce mainly IgA but IgM and IgG are also synthesized to a small extent. The transport of IgA appears to be similar in the mammary secretory cells to that present in other secretory tissues. Secretory component is synthesized in secretory cells and moved to the basolateral membrane as a receptor where it binds dimeric IgA with a J-chain synthesized in adjacent plasma cells. The complex is transported through the secretory cell to the apical membrane where sIgA is secreted by exocytosis and a segment of the secretory component is cleaved off. IgM is secreted similarly but a small amount of IgM of blood origin also appears in milk. IgA in bovine colostrum is derived partly from intramammary synthesis and partly from the blood.

Transfer from gastrointestinal tract to blood

The passage of ingested Igs across intestinal cells of the neonate can be specific or non-specific, depending on the species, and it occurs only for a certain period after birth. The gut of the suckling calf, pig, or foal is non-selective and open to the transport of a variety of macromolecules from 12 up to 36 h after birth, corresponding to the time of the highest IgG content of colostrum. The main Ig class of bovine colostrum is IgG_1, but the passive transport of other Ig classes, although in smaller amounts, is also considered important for the calf. In species which transfer the Igs both *in utero* and via colostrum after birth (e.g., rat and mouse), the absorption of ingested Igs from the intestine is selective. The $\mathrm{Fc}\gamma$ receptors on the enterocytes of the small intestine mediate selective binding and transcytosis of IgG until the day of weaning. Also in the human infant, the colostral Igs are absorbed in small quantities for a short period of 18–24 h after birth.

Importance of Colostral and Milk Immunoglobulins to the Offspring

Protection against Microbial Infections

Bovine colostral and milk antibodies represent the cow's immune response against a variety of microorganisms present in the cow's environment and food. Thus, Igs contribute to the natural antimicrobial properties of milk. Maternal milk of farm animals is well known to protect the newborn offspring against microbial pathogens.

Receiving colostrum within 12 h after birth is essential for the survival of newborn calves and piglets. Newborn calves and piglets which do not receive colostrum show a high mortality and poor weight gain during the first weeks of life. Calves deprived of colostrum or given colostrum of poor quality promptly suffer severe long scour episodes. To protect the calf against scouring, a newborn calf should receive a minimum of 2 l of first colostrum, containing about 100–120 g Igs, to achieve a normal serum IgG level of $10\,\mathrm{g\,l^{-1}}$. Twice as high mortality rates and higher odds of pneumonia have been observed in calves with $<10\,\mathrm{g\,l^{-1}}$ serum IgG, with tripled risk of pneumonia when the dam had clinical mastitis. The colostrum from the first two milkings is most important, since the concentration of Igs decreases steeply after the first milkings (*see* **Milk**: Colostrum and **Table 1**). A variety of colostral whey- and cheese whey-based antibody preparations have been used as feed supplements or colostrum substitutes for neonatal calves and pigs in order to prevent diarrheal diseases caused, for example, by rotaviruses and enterotoxigenic *Escherichia coli* strains.

In human colostrum, sIgA is predominant, the IgG being transported via placenta. Dimeric IgA is secreted by lymphocytes associated with mammary gland epithelia and is transferred by a polymeric Ig receptor to milk. The sites of the immune protection by Igs are restricted mainly to the gastrointestinal tract because the milk Igs apparently are not absorbed in significant quantities from the infant gut. Especially IgA antibodies appear to be particularly important during the first days *post-partum*, when the infant's own mucosal IgA production is deficient. Breast feeding has been shown to reduce the risk of intestinal infections, morbidity in gastroenteritis and diarrhea, otitis media, and pneumonia in suckling neonates. Breast milk also contains a variety of non-specific defense factors, which contribute to its antimicrobial effect. Milk IgA may contribute to the prevention of food allergies by blocking antigen entry at the intestinal surface of the infant. Low levels of total and specific IgA against food allergens are proposed to correlate adversely with the risk of developing allergy to these antigens.

Digestion of Antibodies

IgG, IgM, and IgA without secretory component, when ingested by humans, are normally degraded by proteases in the stomach and intestine into small peptides and amino acids which are subsequently absorbed. Proteolytic enzymes, pepsin, trypsin, chymotrypsin, carboxypeptidase, and elastase, initially degrade the antibodies to $\mathrm{F(ab')_2}$, Fab, and Fc fragments. Proteolytic degradation abolishes their agglutinating properties and dramatically decreases their functional affinity. However, $\mathrm{F(ab')_2}$ and Fab fragments from high-affinity antibodies retain some of the neutralizing and attachment-inhibiting activity of intact antibodies. The secretory form of milk IgA is relatively resistant to proteolytic digestive enzymes in the gastrointestinal tract due to the secretory piece but can be sensitive to a group of microbial enzymes. About 20–80% of sIgA present in human colostrum passes undegraded through the gut of the human infant. In contrast, the secretory component in IgM is not covalent and does not provide resistance against enzymatic cleavage.

In addition, bovine milk Igs, which have been subjected to proteolytic conditions of the human intestine, retain at least partially their immunological activity. Bovine colostral Igs are quite resistant to gastric acids but are degraded by proteases and are rather sensitive to trypsin. However, IgG_1 is an exception since it is rather resistant to trypsin. Bovine colostrum and mastitic milk contain a compound which inhibits trypsin activity. A part of bovine colostral IgG_1 contains *O*-glycans which increase their resistance to pepsin. From 10 to 30% of orally administered bovine Igs can be recovered from the stool of human infants and adults. These recovered Igs are in the form of $\mathrm{F(ab')_2}$, Fab, and Fc fragments. The survival of IgG in the ileum can be increased by encapsulation.

Utilization of Immunoglobulins

Technological Properties of Immunoglobulins

Milk Igs affect many dairy processes. Igs inhibit or retard the activity of renneting enzymes of bacterial origin. In normal milk, this is not noticed due to the relatively low Ig concentration but, if the Ig concentration in milk is increased, for example, by adding colostrum or if the milk is mastitic, renneting may be retarded. The antimicrobial properties of Igs may adversely affect fermentation processes. A slower fermentation by dairy starters is noted in colostrum and mastitic milk, which contain increased amounts of Igs. Also, high Ig concentrations may adversely affect the tests for antibiotic based on microbial growth, causing false positive results.

Igs contribute to cream formation by agglutinating fat globules, thus accelerating the ascent of cream to the surface. This phenomenon is attributed primarily to IgM which have been termed cryoglobulins or 'cold agglutinins'. The agglutination property of Igs is, however, abolished by heat treatment (pasteurization) and by mechanical agitation.

Among dairy technological processes, the properties of Igs are affected most by the thermal treatments. In high-temperature/short-time (HTST) pasteurization (72 °C/15 s) less than 30% of the Ig activity is lost, whereas ultra high temperature (UHT) treatment (138 °C/4 s) and evaporation processing destroy most of the specific immune activity of milk due to denaturation of Igs. A rapid heat inactivation of IgG starts at temperatures higher than 65 °C, and at 81 °C, as much as 90% of the virus-neutralizing capacity of Igs is lost in less than 2 min. On the other hand, heating high-quality colostrum at 60 °C for at least 120 min has no effect on mean IgG concentration or titer of neutralizing antibodies against bovine viral diarrhea virus type 1. A heat treatment of 60 °C for 30 min has been demonstrated to destroy viable *Mycobacterium bovis, Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella enteritidis* added to colostrum. Bovine IgG added to UHT-treated milk has been shown to retain its specific immune activity for several months. Also, Ig molecules seem to retain their specific activity well in milk powder, irrespective of the storage temperature.

Commercial Utilization of Immunoglobulins

Over the last 20 years, considerable progress has been made in isolation and enrichment techniques for Igs from bovine colostrum and cheese whey. Separation technologies, such as membrane filtration and chromatography, have been used alone or in combination. Using these methods, the recovery of Igs has varied from 40 to 70% of the level present in the starting material. Specific chromatographic techniques, such as immobilized metal chelate chromatography, immunoaffinity chromatography, and cation exchange chromatography, have been applied to improve the yield and purity of Ig preparations.

Commercial whey or colostral antibody preparations have been used for a long time as feed supplements or substitutes for farm animals in order to prevent gastrointestinal infections. They are used mainly for neonatal calves and pigs and have proven effective in preventing diarrheal diseases caused by, for example, rotaviruses and enterotoxigenic *E. coli* strains.

If the animal is immunized by a vaccination protocol against a certain pathogen (or antigenic structure derived from it) the specific antibody titer of colostrum or milk may increase up to 100–400 times of that in normal milk or colostrum. In field experiments, active immunization of the dam has improved the protection of the offspring against the pathogens used in the vaccine. Biotechnology industries have shown increasing interest in possibilities of raising specific antibodies in bovine colostrum by hyperimmunizing cows with vaccines made of human pathogenic microbes or their specific structures like toxins or other virulence factors. Numerous clinical studies have been carried in humans with the purpose of demonstrating the efficacy of hyperimmune colostral preparations in the prevention or treatment of infectious diseases. Promising results have been reported for the prevention of infections and also in treatment of diseases when infection is maintained through recurrent reattachment and reinfection inside the oral cavity or gastrointestinal tract. Encouraging results have also been obtained in clinical studies where toxins or inflammatory compounds are involved and have been neutralized by specific Igs (see **Table 3**).

A few immune milk preparations, for example, against rotavirus, are already on the market. The main limitation of the clinical use of bovine milk antibodies for humans is that they originate from a foreign species and thus can be used only orally against gastrointestinal pathogens. To overcome this limitation, trans-chromosomic cows have been cloned for producing human polyclonal Igs. However, several challenges, for example, expression of bovine antibody, remain to be overcome before large-scale production of human Igs for therapeutic purposes can begin. Bovine colostral or milk products from non-immunized cows that exploit IgG content have become expensive niche products worldwide on highly competitive markets for natural 'wellness' products.

Currently, the US Food and Drug Administration (FDA) has accepted the safety of hyperimmune milks on the basis of clinical studies which show no adverse health effects from these products.

Table 3 Examples of recent clinical human studies on the efficacy of bovine whey or colostral preparations

Microorganism used in immunization	*Target disease*	*Treatment regimen*	*Efficacy*	*References*
Five or one strains of *Escherichia coli*	Diarrhea	Once per day orally 0.5 g of IgG per kg of body weight, follow-up period for 6 months	Lower incidence of diarrhea and shorter duration of diarrhea episodes	Tawfeek *et al*. (2003)
No immunization	Upper respiratory tract infections	60 g of colostral protein daily for 8 weeks	Reduced significantly incidence of self-estimated symptoms of respiratory infections but no difference in duration	Brinkworth and Buckley (2003)
No immunization	Mild hypercholesterolemia	Orally 5 g of blood derived IgG daily for 3 or 6 weeks	Both total cholesterol and LDL levels decreased from baselines	Earnest *et al*. (2005)
No immunization	HIV-associated diarrhea	Orally for 4 weeks	Reduced diarrhea and increase in body weight, decrease in fatigue	Florén *et al*. (2006)
Clostridium difficile toxin and *C. difficile* whole cells	*C. difficile* diarrhea	Orally for 2 weeks as supportive treatment after antibiotic treatment	*C. difficile* toxins eradicated from 15 of 16 patients and no relapses in any patient during 11-month follow-up period	Van Dissel *et al*. (2005)
C. difficile whole cells	*C. difficile* diarrhea	Orally 1.6 g of Ig in 200 ml twice per day for 2 weeks	At day 14 after starting 89% of patients had responded positively to the treatment	Mattila *et al*. (2008)
C. difficile toxin and *C. difficile* whole cells	*C. difficile* diarrhea	Orally 5 g of whey powder for 2 weeks as supportive treatment after antibiotics	Clinical evidence for the safety for use in *C. difficile* patients	Young *et al*. (2007)
C. difficile toxin and *C. difficile* whole cells	*C. difficile* diarrhea	Orally 5 g of whey powder 3 times daily for 2 weeks for supportive treatment after antibiotics	Relapse in 10% of Ig-treated patients whereas relapse in 20–25% of control group	Numan *et al*. (2007)

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See also: **Milk:** Colostrum.

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Nutritional Quality of Milk Proteins

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Introduction

Dietary protein quality is related to the ability of a dietary protein source to achieve defined metabolic actions in relation to optimal health. Protein provides nitrogen and amino acids, and protein quality is usually related to the ability of protein to satisfy nitrogen and essential amino acid requirements for tissue growth, maintenance, pregnancy, lactation, and energy homeostasis. The corresponding dietary protein properties integrate amino acid composition, protein digestibility, amino acid bioavailability, and subsequent amino acid and protein metabolism. Milk represents a major dietary source for the young and adult humans, and cow milk proteins are considered to have a high nutritional quality. Milk protein is a complex mixture of different protein components present in different proportions in different mammalian species. Protein concentration is higher in bovine milk than in human milk (30–34 and 9–14 $\mathrm{g\,l^{-1}}$, respectively) (**Table 1**). Bovine milk nitrogen is constituted by a major casein fraction (78% of milk nitrogen), a whey protein fraction (17% of milk nitrogen), and a small non-protein fraction (5% of milk nitrogen). Whey protein is a mixture of soluble proteins with wide-ranging chemical, physical, and functional properties.

Milk Protein, Amino Acid Digestibility, and Nitrogen Retention

The first important aspect of protein quality is related to amino acid bioavailability, that is, the extent to which dietary protein is digested and absorbed in the gastrointestinal tract in the form of amino acids available for metabolic processes. Digestibility represents the proportion of dietary nitrogen and amino acid that is absorbed. It is usually determined from the difference between nitrogen intake and nitrogen losses. The two main difficulties with the measurement of true nitrogen digestibility is the presence of endogenous nitrogen secretion and that colon metabolism is not taken into account in quantifying the fecal digestibility. The presence of endogenous nitrogen secretions implies the need for discriminating between exogenous nitrogen (food) and endogenous nitrogen (secretions, desquamations, etc.). After their transit through the distal ileum, colonic bacteria metabolize most of the unabsorbed amino acids. As a consequence, the ileal rather than the fecal digestibility must be considered as a critical biological parameter for amino acid digestibility and availability.

Different methods have been proposed to determine ileal flux and both endogenous and exogenous amino acid absorption and losses. The determination of endogenous secretions in fecal or ileal nitrogen fractions may be achieved using a protein-free diet. Techniques based on stable isotopes allowed discriminating between dietary and endogenous nitrogen. It is possible to label with stable isotopes (^{15}N or ^{13}C) animal or vegetable protein intended for human consumption. These methods present the direct assessment advantage of true digestibility calculated from $[\mathrm{N_{ingested}} - (\mathrm{N_{feces}} - \mathrm{N_{fecal\ endogenous}})]/\mathrm{N_{ingested}}$. Analysis of milk protein digestion indicates a high true ileal digestibility of 95% in humans. For comparison, the true ileal digestibility for soy and rice protein is 91 and 88%, respectively (**Table 2**). Since metabolic availability of essential amino acids is a limiting step in protein quality, it is important to establish that nitrogen digestibility is a reliable reflection of the digestibility of amino acids, particularly the essential amino acids. The true ileal digestibility of some individual essential amino acids measured in humans for milk and soy protein indicated digestibilities in the range of 93% (Thr) to 96% (Val) for milk protein and 89% (Thr) to 95% (Phe, Lys) for soy protein (**Table 3**).

Another important marker of protein quality is related to nitrogen retention, that is, the extent to which absorbed dietary protein nitrogen is retained in tissues. The traditional approach for the determination of net nitrogen retention is derived from nitrogen balance measured in subjects after adaptation to different protein levels over several days. Diets containing poor-quality protein are associated with an increase in nitrogen losses owing to the inefficient utilization of indispensable amino acids. The nitrogen balance indicates the amount of nitrogen that is retained in the body according to the following formula:

$$\begin{aligned}\text{Nitrogen balance} = {} & \text{Ingested nitrogen} \\ & - \text{Excreted nitrogen (urine and feces)}\end{aligned}$$

Table 1 Protein composition of human milk and cow milk

Protein	*Human milk ($g\ l^{-1}$)*	*Cow milk ($g\ l^{-1}$)*
Total protein	9–14	32–34
Casein	3.7	26
Soluble proteins	6.9	6
Lactoferrin	1.5	0.2
Serum albumin	0.5	0.3
β-Lactoglobulin	-	3.2
α-Lactalbumin	3.6	1.2
Immunoglobulins	1–2	0.7
Peptone-proteose fraction		0.8

Table 2 Digestibility and protein digestibility corrected amino acids score (PDCAAS) values of dietary proteins in humans

Protein source	*True digestibility (%)*	*PDCAAS (%)*
Egg	97	>100
Milk, cheese	95	>100
Meat, fish	94	92
Rice	88	55
Wheat	86	54
Soy	91	91

Adapted from Agence Française de Sécurité Sanitaire des Alimentes (AFSSA) (2007) Apport en protéines: consommation, qualité, besoins et recommandations, 461 pp. *Report of the Working Group*. www.afssa.fr/Documents/NUT-Sy-ProteinesEN.pdf

Table 3 Amino acid digestibility in humans

True ileal digestibility (%)	*Milk protein*	*Soy protein isolate*
Total nitrogen	95	91
Amino acid nitrogen		
Thr	93	89
Val	96	92
Ile	95	93
Leu	95	93
Phe	96	95
Lys	95	95
His	95	92

From Gaudichon C, Bos C, Morens C, *et al*. (2002) Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. *Gastroenterology* 123(1): 50–59.

Table 4 Net post-prandial protein utilization values of dietary proteins in humans

Protein	*NPPU (%)*
Total milk proteins	78
Casein	71
Whey protein	65
Wheat protein	66
Soy protein	72
Pea protein	71

Adapted from Agence Française de Sécurité Sanitaire des Alimentes (AFSSA) (2007) Apport en protéines: consommation, qualité, besoins et recommandations, 461 pp. *Report of the Working Group*. www.afssa.fr/Documents/NUT-Sy-ProteinesEN.pdf

The net protein utilization (NPU) allows evaluation of the N ingested proportion that is retained in the body: $NPU = N_{retained}/N_{ingested}$. The biological value (BV) is the percentage of absorbed nitrogen retained: $BV = N_{retained}/N_{absorbed}$.

Similar to the digestibility, these values represent true or apparent nitrogen retention depending on whether the endogenous secretion is taken into account or not. Endogenous losses may be estimated either from a protein-free diet or by using stable isotope tracer methodologies. Thus, it follows that

$$NPU = \frac{\{N_{ingested} - [(N_{fecal} - N_{endogenous}) + (N_{urinary} - N_{endogenous\ urinary})]\}}{N_{ingested}}$$

where $NPU = BV \times$ digestibility.

Assessing the post-prandial utilization of dietary proteins is also a good marker of dietary proteins' nutritional quality, since it is known to influence protein synthesis and proteolysis. Different methods based on nitrogen digestibility and short-term nitrogen retention have been proposed for the determination of short-term utilization of dietary proteins. The net post-prandial protein utilization (NPPU) is calculated from true ileal digestibility and deamination of dietary protein measured during the 8 h following the ingestion of a standard meal by healthy human subjects. NPPU values of 78–80 and 70–72% were determined for milk protein and soy protein, respectively (**Table 4**). Taken together, these different data show that milk protein nitrogen and amino acid show high digestibility and retention in humans.

Milk Protein, Nitrogen, and Indispensable Amino Acid Requirements and the Protein Digestibility Corrected Amino Acids Score (PDCAAS)

The assessment of protein quality is related to the amino acid composition in conjunction with nitrogen and essential amino acid requirements. This approach needs to precisely quantify these requirements and to further

Table 5 Nutritional classification of amino acids

Strictly essential amino acids	Lysine, threonine
Other essential amino acids	Histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, valine
Conditionally essential amino acids (indispensable under specific pathological or physiological conditions)	Cysteine, tyrosine, taurine, glycine, arginine, glutamine, proline
Non-essential amino acids	Aspartic acid, asparagine, glutamic acid, alanine, serine

evaluate the ability of each dietary protein to satisfy these requirements under specific physiological and dietary conditions.

Protein requirement has been determined from nitrogen balance (difference between nitrogen intake and nitrogen losses) at a value of 0.83 g kg^{-1} d^{-1} in adults. Nine amino acids are considered essential for humans, including lysine, threonine, valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, and histidine (**Table 5**). Amino acid requirements are summarized in **Table 6**. Lysine requirement has received most attention, given its nutritional importance as the limiting amino acid in cereals. Threonine has been suggested to be the second rate-limiting amino acid in some dietary protein sources. Sulfur-containing amino acids, methionine and cysteine, are low in vegetables and abundant in cereals and animal protein. Tryptophan content in proteins is generally lower than that of many other amino acids. Its content is low in cereals, especially corn, and in some varieties it is the nutritionally limiting amino acid. Leucine is the most abundant amino acid in tissue and food proteins. Its non-protein functions remain unclear but it has been suggested that leucine is implicated in the control of protein synthesis and satiety. Compared to plant proteins, animal proteins are rich in essential amino acids. Milk protein presents a well-balanced amino acid composition and cow's milk is considered an excellent source of high-quality proteins (**Table 7**).

Table 6 Indispensable amino acid requirements in adults

	FAO/WHO/UNU 2007	
	mg kg^{-1} d^{-1}	*mg g^{-1} protein*[a]
Histidine	10	15
Isoleucine	20	30
Leucine	39	59
Lysine	30	45
Methionine + cysteine	15	22
Methionine	10	16
Cysteine	4	6
Phenylalanine + tyrosine	25	38
Threonine	15	23
Tryptophan	4	6
Valine	26	39
Total	184	277

[a] Mean protein requirement in adults: 0.66 g kg^{-1} d^{-1} of proteins.
Adapted from FAO/WHO/UNU (2007) Protein and amino acid requirements in human nutrition: Report of a joint WHO/FAO/UNU expert consultation. *World Health Organ Technical Report Series #935*. Geneva, Switzerland: FAO; WHO; UNU.

PDCAAS was introduced by the Food and Agriculture Organization of the World Health Organization (FAO/WHO) in 1991 and is the current internationally approved method for protein quality assessment. PDCAAS is based on the combination of an age-related amino acid reference pattern that represents human requirements and estimates of the digestibility of the protein. The amount of potentially limiting amino acids in the test protein is compared with their respective contents in the appropriate reference pattern, identifying the single most limiting amino acid that determines the amino acid score. The current consensus is that meeting the minimum requirements for lysine, methionine, and tryptophan, the most limiting amino acids in poor-quality proteins, determines the amino acid score and will lead to a plateau of nitrogen retention. At the plateau of nitrogen balance, any further increase in plasma amino acids would stimulate increased oxidation and elimination of the excess amino acids, implying that protein quality that exceeds requirements does not lead to any nutritional gain. This score is assumed to predict BV, or the anticipated ability of the absorbed test protein to fulfill human amino acid requirements. The score is then corrected for digestibility giving the PDCAAS value, which is assumed to predict NPU. Milk protein and, in general, animal proteins have a PDCAAS value >100% (**Table 2**). For adults, there are no limiting amino acids in milk proteins. Therefore they are often defined as reference proteins in this test.

Milk Protein and the Rate of Amino Acid Delivery

Digestion rate of dietary protein affects post-prandial protein metabolism and nitrogen retention. Indeed, the magnitude and the duration of changes in amino acid availability determine the anabolic effects of protein ingestion. Amino acids and insulin are two main factors regulating amino acid and protein metabolism after a meal. Typical meal ingestion leads to hyperaminoacidemia and hyperinsulinemia which are involved in the control of protein turnover in splanchnic and peripheral areas. Post-prandial hyperaminoacidemia is more important in splanchnic tissues because of adjoining splanchnic tissues position after absorption.

Table 7 Indispensable amino acid content of various dietary proteins (mg g^{-1} N)

Sources/AA	*Trp*	*Phe*	*Met*	*Lys*	*Thr*	*Leu*	*Ile*	*Val*	*Nb of limiting AA*
FAO/WHO	*90*	*180*	*140*	*270*	*180*	*300*	*270*	*270*	
Milk	90	310	150	490	290	630	400	440	0
Meat	*80*	260	150	510	280	490	320	330	1
Egg	110	330	190	420	330	560	360	450	0
Wheat	*80*	290	*100*	*170*	180	400	240	270	3
Maize	*50*	300	*130*	*190*	260	750	*250*	350	4
Rice	90	320	140	*220*	240	510	270	370	1
Pea	*50*	320	*80*	420	240	450	350	350	2
Soya	*80*	300	*80*	390	240	480	330	320	2

Nb, number. Adapted from FAO/WHO/UNU (2007) Protein and amino acid requirements in human nutrition: Report of a joint WHO/FAO/UNU expert consultation. *World Health Organ Technical Report Series #935*. Geneva, Switzerland: FAO; WHO; UNU.

It has been demonstrated that casein and whey proteins are digested differently in the intestinal lumen, since whey proteins are rapidly emptied from the stomach, mainly in an intact form, whereas caseins are slowly emptied as degraded products. Unlike casein, whey protein ingestion mediates large increases in post-meal peripheral aminoacidemia. The differences in amino acid delivery kinetics are responsible for different effects on whole-body protein metabolism in humans ingesting either casein or whey protein. Casein is considered a slow-digested protein and whey protein a fast-digested protein. Usually, total milk proteins are defined as protein mixtures which are slowly absorbed. Heat treatment or fermentation of milk can reduce the digestive transit of casein. Soy protein that has digestion rate similar to whey protein is defined as a fast-digested protein.

Recent studies have proven that leucine improves protein synthesis. The protein type can specifically influence post-meal aminoacidemia and it has been observed that leucine appears rapidly and transiently in the peripheral blood after whey protein ingestion whereas blood leucine level measured after casein ingestion is lower but lasts for a more prolonged period. Consequently, it has been demonstrated that in young adults whey protein improves peripheral protein synthesis but does not affect proteolysis whereas casein and total milk protein increase protein synthesis and inhibit proteolysis. A very fast delivery of dietary leucine cannot support the anabolic requirements throughout the post-prandial period. In young adult humans, ingestion of milk protein increases post-prandial nitrogen retention in the peripheral tissues, owing to its relation to a more favorable pattern of amino acids available for peripheral protein synthesis. As a consequence, in a young adult, a slowly digested protein like total milk protein and casein induces a higher postprandial utilization of dietary nitrogen than does a rapidly digested protein like whey or soy protein. However, the rate of amino acid delivery modulates protein turnover differently in young and elderly subjects; in elderly people, a fast delivery of amino acids and of leucine was demonstrated to be more efficient in improving post-prandial leucine and nitrogen balance than was the case with a slow-digested protein.

Conclusions

Animal proteins and particularly milk proteins have a high level of essential amino acids. Milk proteins represent a high-quality dietary protein source for protein nitrogen and essential amino acids requirements. As a consequence, a diet rich in milk protein efficiently supports nitrogen and essential amino acids requirements. Compared to plant proteins, milk proteins have a higher oro-ileal digestibility and demonstrate both a higher NPPU value and a higher PDCAAS value. In addition, milk proteins can play a functional role as a specific metabolic regulator of various physiological functions.

See also: **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Protein. **Milk Proteins**: Analytical Methods; Heterogeneity, Fractionation, and Isolation. **Milk Protein Products**: Functional Properties of Milk Proteins. **Whey Processing**: Utilization and Products.

Further Reading

Agence Française de Sécurité Sanitaire des Alimentes (AFSSA) (2007) Apport en protéines: consommation, qualité, besoins et recommandations, 461 pp. *Report of the Working Group*. www.afssa.fr/Documents/NUT-Sy-ProteinesEN.pdf

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Inter-Species Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity

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Introduction

Milk is a complete and complex food suited to the specific offspring's requirements for growth and development. This is the result of a long and slow adaptive evolutionary process which started 150 million years ago, far before domestication of ruminants, which took place (*c.*10 000 years ago) in the Fertile Crescent region.

There is increasing substantial evidence that milk contains many health-promoting compounds, impacting physiological functions or reducing disease risk. This statement is true for the main milk components, such as lipids, carbohydrates (including oligosaccharides), and proteins, as well as for minerals or vitamins. As far as milk proteins are concerned, numerous substantiated or potential bioactive protein components have been found and many others remain to be identified either as intact proteins or as derived peptides, encrypted in the sequence of milk proteins. This is probably one of the greatest challenges facing milk science in the immediate future to provide the food industry and consumers with the basis for health-promoting properties before their inclusion as ingredients into functional foods.

Milk proteins are found mostly in the aqueous phase, either in the soluble (whey proteins of which few are synthesized in the mammary epithelial cells while the large majority have a serum origin) or in the colloidal (caseins) state, but also in the lipid phase, associated with the milk fat globule membrane (MFGM).

It has been shown that caseins, which can account for more than 80% of milk proteins, have evolved rapidly and are highly divergent proteins across mammalian milks. However, it appears that although both copy number and sequence variation contribute to the diversity of milk proteins across species, milk and mammary genes are more highly conserved, on average, than other genes in the bovine genome. For a long time, we have believed that they were devoid of biological functions and were designed only to ensure amino acids supply and phosphate and calcium absorption. This is no longer true since we now know that peptides displaying proven biological activity are encrypted within caseins as well as whey proteins such as α-lactalbumin (LALBA), which can attain new functions by changing its three-dimensional (3D) structure.

Regarding the casein fraction, several genetic variants have been characterized so far in cow, ewe, and goat milks (**Table 1**). The past 20 years have seen remarkable progress in the understanding of milk protein genes' structure and function. Developments in molecular biology, genomics, and proteomics (mass spectrometry) have particularly highlighted how such genomic rearrangements contribute to changes in the milk protein gene complement of mammalian species and how genetic polymorphism is responsible for the extreme complexity and large variability (qualitative and quantitative) of the milk protein fraction, between, but also within, species. High conservation of MFGM protein-encoding genes between monotreme and placental mammal genomes strongly suggests that they are crucial for lipid secretion and that the secretory function was already established 150 million years ago.

Our purpose here is to provide the reader with an overview of our current knowledge of milk protein variability between species, at both the structural (amino acid sequence, post-translational modifications) and quantitative (ultimately absence) levels. Genetic polymorphisms when responsible for deep modifications will also be considered. Since a separate article covering the MFGM, including proteins, is given in this Encyclopedia, these proteins will not be considered here (*see* **Milk Lipids**: Milk Fat Globule Membrane).

Caseins

Caseins are phosphoproteins synthesized by the mammary epithelial cells (MECs) under multihormonal control as more or less large and stable particles, referred to as casein micelles and which appear like raspberries in electron micrographs. These spherical particles might be the result of aggregation of smaller discrete subunits or submicelles, cemented by a calcium phosphate salt (colloidal calcium phosphate). However, this casein micelle model remains a topic of discussion and controversial debates. Casein micelles are present in the milk of all mammals and have a statistically broad distribution in size, ranging in size between 60 and 600 nm. In bovine milk, the most thoroughly studied milk to date, casein micelles are made up of several casein molecules arising from the expression of four single-copy autosomal genes

Table 1 Genetic variants of milk proteins in the ruminant species: an overall picture of our present knowledge

	CSN1S1 ***(α_{S1}-casein)***	***CSN1S2*** ***(α_{S2}-casein)***	***CSN2*** ***(β-casein)***	***CSN3*** ***(κ-casein)***	***β-Lactoglobulin***	***α-Lactoglobulin***
Cattle	7 variants A–G (+H)	4 variants A–D	9 variants (+4) A^1, A^2, A^3, B to G (B2, A^4, A^3 Mong)	4 variants (+5) A, B, C/D, and E (F–J)	7 variants A–G	3 variants A–C
Goats	13 variants[a] (+ null alleles)	6 variants[b] (+1 null allele)	3 variants (+ null alleles)	13 Variants[c]	no variants characterized so far[d]	-
Sheep	5 variants[e] A–E	3 variants[e] A–C	2 variants[f]	2 variants[f]	3 variants A–C	2 variants A and B

[a] Bevilacqua C, Ferranti P, Garro G, et al. (2002) Interallelic recombinationis probably responsible for the occurrence of a new _s1-casein variant found in the goat species. *European Journal of Biochemistry* 269: 1293–1303.

[b] Sacchi P, Chessa S, Budelli E, et al (2005) Casein haplotype structure in five Italian goat breeds. *Journal of Dairy Science* 88(4):1561–1568.

[c] Prinzenberg EM, Gutscher K, Chessa S, Caroli A, and Erhards G (2005) Caprine kappa-casein (CSN3) polymorphisms: New developments in molecular knowledge. *Journal of Dairy Science* 88: 1490–1498.

[d] Ballester M, Sánchez A, and Folch JM (2005) Polymorphisms in the goat beta-lactoglobulin gene. *Journal of Dairy Research* 72: 379–384.

[e] Chianese L, Garro G, Mauriello R, Laezza P, Ferranti P, and Addeo F (1996) Occurrence of five α_{s1}-casein variants in ovine milk. *Journal of Dairy Research* 63: 49–59.

[f] Ceriotti G, Chessa S, Bolla P, *et al*. (2004) Single nucleotide polymorphisms in the ovine casein genes detected by polymerase chain reaction-single strand conformation polymorphism. *Journal of Dairy Science* 87: 2606–2613.

Data taken from Ng-Kwai-Hang KF and Grosclaude F (2003) In: Fox PF and McSweeney PLH (eds.) *Advanced Dairy Chemistry, Vol. 1: Proteins*, 3rd edn., Part B, pp. 739–816. New York: Kluwer Academic; Plenum Publishers.

which encode four distinct polypeptide chains, α_{s1}-, β-, α_{s2}-, and κ-caseins.

These four genes (five in some species, see thereafter), of which the structures are known in several species, are clustered (physically linked), in this order, on the same chromosome (six in cattle and goats, five in mouse, and four in human), whatever the species. Differences reported between species will be discussed briefly, since such genomic data provide clues that can plausibly improve our understanding of the mechanisms responsible for specific variations in the number and relative proportions of caseins as well as in their total concentration across mammalian milks. Caseins amount to nearly 80% (i.e., 25–28 g l^{-1}) of the whole protein in ruminants' milk, whereas in breast milk, the casein percentage does not exceed 50% (i.e., 5–8 g l^{-1}). Conversely, the casein content of some lagomorphs' milk can reach 200 g l^{-1} (**Figure 1**).

Besides this wide quantitative variability, it is worth noting that, between species, a high rate of sequence divergence generally occurs in orthologous gene products. Hence, the casein fraction of milk is a complex

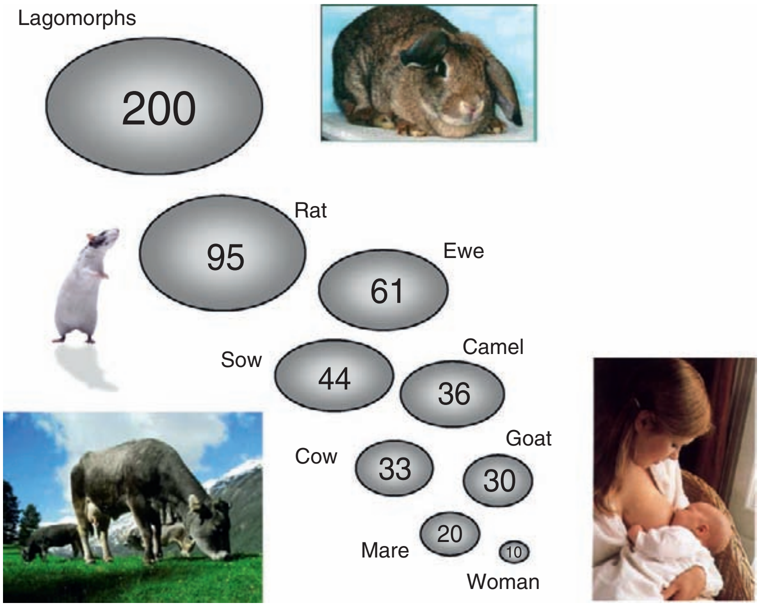


Figure 1 Concentration (g l^{-1}) of milk proteins in eight species.

and specific system which deserves to be considered in terms of diversity, particularly in light of the growing number of biologically active peptides derived from milk proteins, including caseins, identified during the past 20 years.

In addition, post-translational processing, such as phosphorylation, glycosylation, and limited proteolysis by plasmin, increases the heterogeneity of this system, which is complicated even more by the occurrence of genetic variants. The primary focus of this article will be interspecies comparisons in terms of quantitative and structural variability. Nevertheless, within-species variability will also be considered. Particular attention will be paid to discrete phosphorylation and exon-skipping events which contribute to protein diversity and evolution and very likely to specific micellar organization.

The Casein Gene Locus (*CSN*) and Quantitative Variability

Caseins are present in the milk of all mammals. However, their total concentration and their relative proportions are largely species dependent. The species studied so far produce more or less large quantities of milk, the protein content of which ranges between 10 and 200 g kg^{-1} (**Figure 1**). Breast milk shows one of the lowest protein content (10 g kg^{-1}), whereas that of rabbit milk is undoubtedly one of the highest (200 g kg^{-1}). Amongst dairy ruminants, with more than 50 g kg^{-1}, sheep milk shows the highest total protein content. Beyond this large variability in the milk protein content, there are large differences in the relative proportions of caseins between species (**Figure 2**). Thus, in human milk, β-casein is, by far, the main casein component. Conversely, α_{s1}-casein predominates in rabbit milk. Rat, porcine, and bovine milk contain approximately the same proportion of β- and α_{s1}-caseins. Today, human milk remains the only thoroughly studied milk in which α_{s2}-casein has not been found. The presence of two different α_{s2}-caseins has been detected recently in Equidae milk.

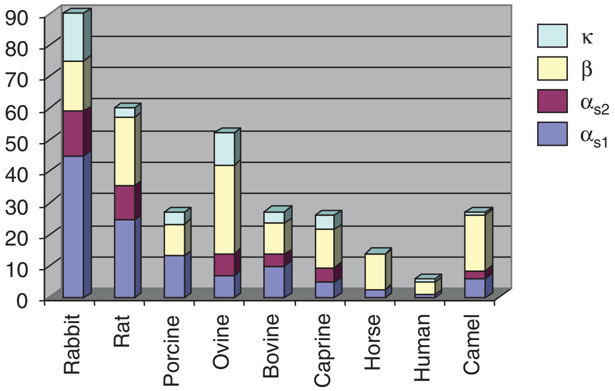


Figure 2 Comparative ratio of milk caseins for nine species. Numbers correspond to mean values from several data sets in the literature. The percentage of α_{s1}-casein given for sow's milk corresponds to α_s-caseins ($\alpha_{s1} + \alpha_{s2}$).

A comparative analysis of the casein locus organization (**Figure 3**) appears to indicate that it is highly conserved between species, even for ancestral mammals such as monotremes (platypus), in which casein genes are tightly clustered in the genome, as they are in placental mammals. The genomic organization of the platypus casein locus has been elucidated and compared with other mammalian genomes, including the marsupial opossum and several eutherians. Whereas the physical linkage of casein genes has been confirmed in platypus, a recent duplication of *CSN2* was observed in the monotreme lineage, as opposed to more ancient duplications of *CSN1S2* in the eutherian lineage, while marsupials possess only single copies of α- and β-casein-encoding genes. Another striking feature is the close proximity between *CSN1* and the main *CSN2*. The lineage-specific gene duplications that have occurred within the casein locus of monotremes and eutherians but not marsupials, which may have lost part of the ancestral casein locus, emphasize the independent selection of milk provision strategies to the young, most likely linked to different developmental strategies.

The four (or five) genes are confined to a 250–350 kb region on chromosome 6, in cattle and goats, and arranged in the order α_{s1}, β, α_{s2}, and κ. Loci encoding α_{s1}- and β-caseins were shown to be very close (between 10 and 20 kb apart) and convergently transcribed. These results were confirmed in cattle for which the genomic organization of the casein gene locus was determined. Despite some differences in the distance separating casein genes and their numbers, the overall organization of the locus is fairly conserved and the presence of dominant *cis*-acting regulatory elements, required for the high-level coordinate expression of the casein genes, is suspected in the α_{s1}/β-region.

Indeed, all four genes are coordinately expressed at high levels in a tissue- and stage-specific fashion. The three genes encoding the 'calcium-sensitive' caseins (α_{s1}, α_{s2}, and β), which are related through evolution, share common regulatory motifs in the proximal 5′-flanking region. Although the organization of the 5′-flanking region of the κ-casein gene is different, its expression pattern seems to be similar to that of the other casein genes. There is more and more evidence demonstrating that a common set of transcription factors is required, in most mammalian species, for the expression of milk protein genes. Mechanisms controlling milk protein gene expression, especially pertaining to the behavior of Stat5, in the cow are significantly different from those in the mouse. More precisely, the different organization of the hormone response regions of casein genes, to the binding of factors such as Stat5 and C/EBP, in different

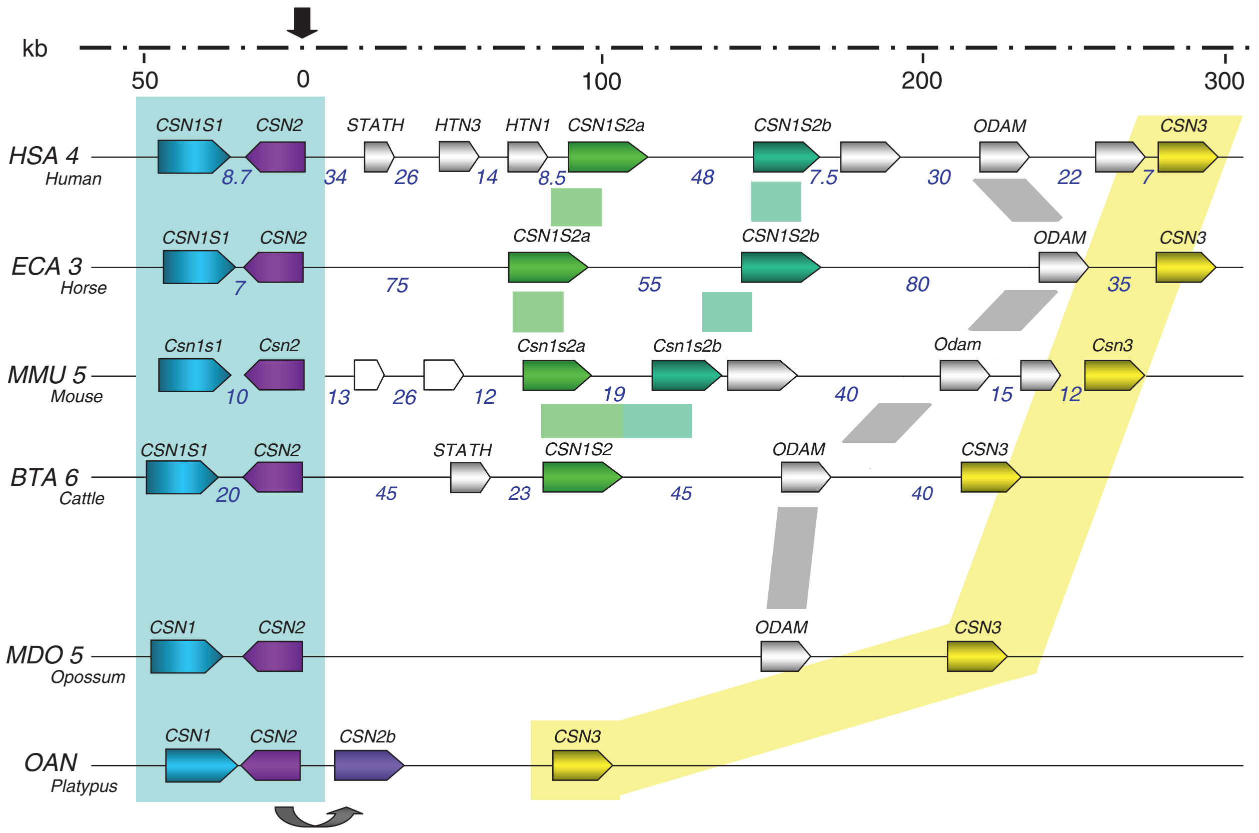


Figure 3 Evolution of the casein locus organization. The casein loci from platypus (*Ornithorhynchus anatinus*), opossum (*Monodelphis domestica*), cattle (*Bos taurus*), mouse (*Mus musculus*), horse (*Equus caballus*), and human (*Homo sapiens*) genomes are drawn approximately to scale in order to underline the expansion of this locus during the course of mammalian evolution. Genes are depicted by 'arrow boxes', giving the orientation of transcription. Blank boxes represent putative genes based on similarity, of which the expression remains to be demonstrated. Intergenic region sizes are given in kb. The human and horse loci have approximately the same size (320/330 kb), whereas the cattle locus (250 kb) is ~80 kb shorter. While the marsupial (opossum) locus is close in size to the cattle locus, on the other hand, the monotreme (platypus) locus is significantly smaller (less than 150 kb) with a duplication of *CSN2* (gray arrow) that occurred recently in this lineage. *STATH* (statherin) and *HTN3* and *HTN1* (histatins) are genes having a common origin and encoding salivary proteins that protect teeth by regulating the spontaneous precipitation of calcium phosphate salts on enamel surface. *ODAM* is a gene highly conserved across species encoding the odontogenic ameloblast-associated protein, a tooth-associated epithelia protein that probably plays a role in odontogenesis, possibly incorporated into the enamel matrix at the end of the mineralization process, but also conspicuous by its expression in several epithelial tissues. Adapted from Lefèvre CM, Sharp JA, and Nicholas KR (2009) Characterisation of monotreme caseins reveals lineage specific expansion of an ancestral casein locus in mammals. *Reproduction, Fertility and Development* 21: 1015–1027 and Warren WC, Hillier LW, Marshall Graves JA, *et al*. (2008) Genome analysis of the platypus reveals unique signatures of evolution. *Nature* 453(7192): 175–183, taking into account additional genomic information from the NCBI.

mammalian species, apparently does not result in fundamental differences in their responsiveness to lactogenic hormones, at least in transfected cell lines. The species-specific arrangement of transcription factor binding sites in the β-casein gene appears to be crucial for the strength and stage at which this gene is expressed in different species, including humans, rodents, and ruminants. For example, the bovine, but not the mouse, β-casein gene is strongly induced shortly before parturition. This difference in stage-specific expression was recapitulated in the expression of a bovine β-casein transgene (including 16 kb of 5′- and 8 kb of 3′-flanking regions) in transgenic mice, indicating that *cis*-acting sequences might be, at least in part, responsible for species-specific expression patterns.

Nevertheless, transcription is not the only level at which regulation of gene expression may occur. In the following, we will see that there are many other factors acting at the post-transcriptional level, including messenger RNA stability and processing as well as a translational regulation. The protein-coding regions of most vertebrate genes, including those encoding caseins in mammals, are split. Most eukaryotic messenger RNAs are thus transcribed as precursors (pre-messengers) containing intervening sequences (introns) which have to be removed to generate mature and functional mRNAs.

The process of intron removal, and exon joining (splicing), is a major function ensured, in the nucleus, by a large multicomponent (5 small nuclear RNAs and more than 50 proteins) complex, called spliceosome, assembled in a stepwise pathway. This accurate mechanism is governed by a set of rather strict rules to achieve high fidelity and efficiency in splicing. However, caseins spliced variants are widely spread across species. A dysfunction of this machinery may have dramatic biological consequences by modifying the message and accordingly the primary structure of the protein. This is well exemplified in mare's milk, in which a low-molecular-weight β-casein variant, showing a 132 amino acid residue internal deletion, has been characterized as arising from a cryptic splice site usage occurring within exon 7, during the course of primary transcript processing. Such deviant splicing behavior might be regulated by an intronic splicing enhancer, sometimes located far away from the splice site, as was shown for the gene encoding β-casein in mare mammary gland.

Primary Structure of Caseins: Comparison across Species

Since the elucidation of the primary structure of bovine α_{s1}-casein in the early 1970s, the complete amino acid or nucleotide (cDNA, gene) sequence of the four (five) caseins has been determined in a number of species (including humans, horses, ruminants, and rodents). Multiple alignments, which help to define functional domains, performed with sequences available today, can reach highly informative levels, provided that the structural intron/exon organization of the gene is taken into account. Indeed, in such a way, multiple alignments, in which gaps were introduced to maximize the alignment, reveal the conserved regions and also highlight their evolutionary pathways. This is particularly true for α_s-caseins, the genes of which are extremely split, comprising up to around 20 exons.

α_{s1}-Casein

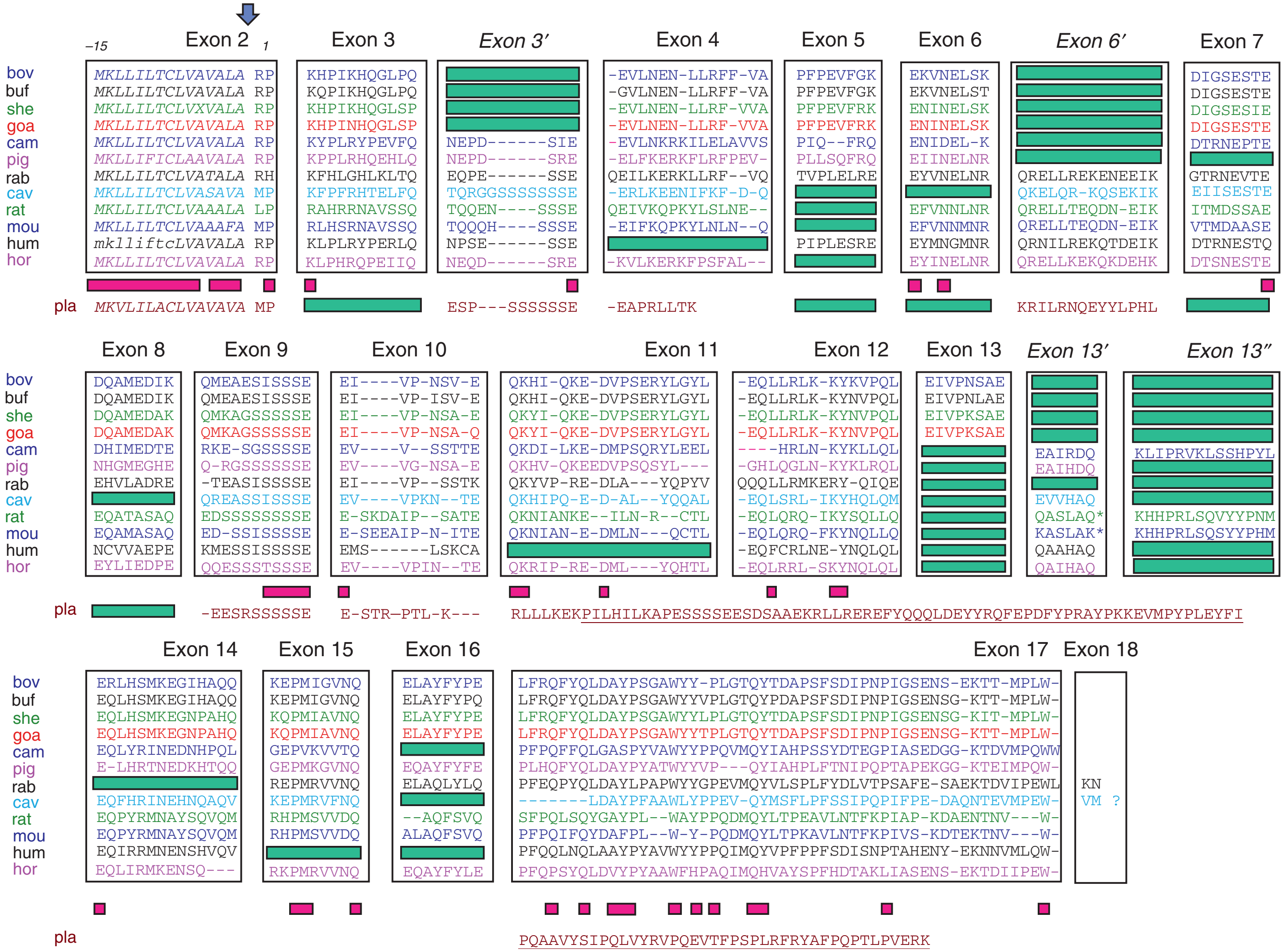
A multiple alignment of α_{s1}-casein from 12 species is presented in **Figure 4**. Even tuning the alignment, taking into account the exon modular splitting derived from the known gene structural organization, there are few, even short, segments of amino acid identity across the 12 species. Conversely, such a method of alignment immediately indicates the occurrence of insertion/deletion events. Exon skipping, first found in goat α_{s1}-casein, was shown to be responsible for such events and for the apparent relatively high structural divergence observed between α_{s1}-caseins from different species, as well as for its wide variability in size. α_{s1}-Casein ranges from 183 to 199 amino acid residues, in guinea pig and cattle, respectively, whereas, due to the insertion of a tandem-repeated hexapeptide sequence (QASLAQ), the protein is significantly larger (from 280 to about 300 amino acid residues) in mouse and rat. This sequence was shown to correspond to a short 'virtual exon' occurring within intron 13 of the bovine gene (*CSN1S1*) and surrounded by quite perfect consensus splice sequences. In addition, the same short sequence is recognized as an exon in the porcine α_{s1}-casein mRNA.

The three hydrophobic domains identified in the bovine molecule, spanning residues 1–44, 90–113, and 132–199, are more or less well conserved between species. The most highly conserved region, except the signal peptide, remains, however, the multiple phosporylation site, encoded by the 3′-end of exon 9. This SerP cluster is confined within a sequence (encoded by exons 7–10) carrying a high net negative charge (seven SerP, three Asp, and eight Glu, in bovine), at the natural pH of milk, while the remainder of the molecule is, under such conditions, essentially uncharged. These features are rather conserved in the 12 compared species, exemplified herein by the third hydrophobic domain (residues 132–199), corresponding to the 17th exon, probably being one of the most conserved parts of the molecule. α_{s1}-Casein does not contain a cysteinyl residue or cystine, except in rodents and humans, a feature which is usually found in α_{s2}-casein. In this connection, it is worth noting that, at least in human milk, in which the presence of α_{s2}-casein still remains to be demonstrated, α_{s1}-casein is capable of forming disulfide-linked heteromultimers with κ-casein, which contains only one cysteinyl residue.

β-Casein

With 209–217 residues, in cattle and pig, respectively, without a cysteinyl residue or cystine, this casein is the most hydrophobic of the four caseins. It is especially rich in proline and displays, in all species, an amphipathic structure with a single multiple phosphorylation site, located in the N-terminal part of the molecule. At the pH of milk, the extreme N-terminal part (30 last residues) is highly negatively charged (an average of 11 negative charges: 7 Glu and 4 SerP, in ruminants, concentrated in the 21 first N-terminal amino acid residues), whereas the rest of the peptide chain, which is highly hydrophobic, has no net charge. Such a feature explains the property of β-casein, which allows for micellar aggregates to be formed in solution.

Of the same 12 species, except the guinea pig, 11 are compared and aligned (**Figure 5**). There is no evidence in the extensive literature related to guinea pig caseins for the existence of β-casein. However, there is no irrefutable evidence for its absence from guinea pig milk. Again, as previously mentioned for α_{s1}-caseins, the conservation of the leader peptide is strikingly notable. Moreover, with ~80% homology between α_{s1}- and β-casein signal peptides, the evolutionary relationship between the genes encoding calcium-sensitive caseins is further substantiated. The close proximity of the main α- and


–15
Exon 2
1
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bov MKLLILTCLVAVALA RP | KHPIKHQGLPQ | 🟩 | -EVLNEN-LLRFF-VA | PFPEVFGK | EKVNELSK | 🟩 | DIGSESTE
buf MKLLILTCLVAVALA RP | KQPIKHQGLPQ | 🟩 | -GVLNEN-LLRFF-VA | PFPEVFGK | EKVNELST | 🟩 | DIGSESTE
she MKLLILTCLVXVALA RP | KHPIKHQGLSP | 🟩 | -EVLNEN-LLRF-VVA | PFPEVFRK | ENINELSK | 🟩 | DIGSESIE
goa MKLLILTCLVAVALA RP | KHPINHQGLSP | 🟩 | -EVLNEN-LLRF-VVA | PFPEVFRK | ENINELSK | 🟩 | DIGSESTE
cam MKLLILTCLVAVALA RP | KYPLRYPEVFQ | NEPD------SIE | -EVLNKRKILELAVVS | PIQ--FRQ | ENIDEL-K | 🟩 | DTRNEPTE
pig MKLLIFICLAAVALA RP | KPPLRHQEHLQ | NEPD------SRE | -ELFKERKFLRFPEV- | PLLSQFRQ | EIINELNR | 🟩 | 🟩
rab MKLLILTCLVATALA RH | KFHLGHLKLTQ | EQPE------SSE | QEILKERKLLRF--VQ | TVPLELRE | EYVNELNR | QRELLREKENEEIK | GTRNEVTE
cav MKLLILTCLVASAVA MP | KFPFRHTELFQ | TQRGGSSSSSSSE | -ERLKEENIFKF-D-Q | 🟩 | 🟩 | QKELQR-KQSEKIK | EIISESTE
rat MKLLILTCLVAAALA LP | RAHRRNAVSSQ | TQQEN----SSSE | QEIVKQPKYLSLNE-- | 🟩 | EFVNNLNR | QRELLTEQDN-EIK | ITMDSSAE
mou MKLLILTCLVAAAFA MP | RLHSRNAVSSQ | TQQQH----SSSE | -EIFKQPKYLNLN--Q | 🟩 | EFVNNMNR | QRELLTEQDN-EIK | VTMDAASE
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hor MKLLILTCLVAVALA RP | KLPHRQPEIIQ | NEQD------SRE | -KVLKERKFPSFAL-- | 🟩 | EYINELNR | QRELLKEKQKDEHK | DTSNESTE
pla MKVLILACLVAVAVA MP | 🟩 | ESP---SSSSSSE | -EAPRLLTK | 🟩 | 🟩 | KRILRNQEYYLPHL | 🟩
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bov DQAMEDIK | QMEAESISSSE | EI----VP-NSV-E | QKHI-QKE-DVPSERYLGYL | -EQLLRLK-KYKVPQL | EIVPNSAE | 🟩 | 🟩
buf DQAMEDIK | QMEAESISSSE | EI----VP-ISV-E | QKHI-QKE-DVPSERYLGYL | -EQLLRLK-KYNVPQL | EIVPNLAE | 🟩 | 🟩
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goa DQAMEDAK | QMKAGSSSSSE | EI----VP-NSA-Q | QKYI-QKE-DVPSERYLGYL | -EQLLRLK-KYNVPQL | EIVPKSAE | 🟩 | 🟩
cam DHIMEDTE | RKE-SGSSSSE | EV----V--SSTE | QKDI-LKE-DMPSQRYLEEL | ----HRLN-KYKLLQL | 🟩 | EAIRDQ | KLIPRVKLSSHPYL
pig NHGMEGHE | Q-RGSSSSSSE | EV----VG-NSA-E | QKHV-QKEEDVPSQSYL--- | -GHLQGLN-KYKLRQL | 🟩 | EAIHDQ | 🟩
rab EHVLADRE | -TEASISSSSE | EI----VP--SSTK | QKYVP-RE-DLA---YQPYV | QQQLLRMKERY-QIQE | 🟩 | 🟩 | 🟩
cav 🟩 | QREASSISSSE | EV----VPKN--TE | QKHIPQ-E-D-AL--YQQAL | -EQLSRL-IKYHQLQM | 🟩 | EVVHAQ | 🟩
rat EQATASAQ | EDSSSSSSSSE | E-SKDAIP--SATE | QKNIANKE--ILN-R--CTL | -EQLQRQ-IKYSQLLQ | 🟩 | QASLAQ* | KHHPRLSQVYYPNM
mou EQAMASAQ | ED-SSISSSSE | E-SEEAIP-N-ITE | QKNIAN-E-DMLN---QCTL | -EQLQRQ-FKYNQLLQ | 🟩 | KASLAK* | KHHPRLSQSYYPHM
hum NCVVAEPE | KMESSISSSSE | EMS------LSKCA | 🟩 | -EQFCRLNE-YNQLQL | 🟩 | QAAHAQ | 🟩
hor EYLIEDPE | QQESSSTSSSE | EV----VPIN--TE | QKRIP-RE-DML---YQHTL | -EQLRRL-SKYNQLQL | 🟩 | QAIHAQ | 🟩
pla 🟩 | -EESRSSSSE | E-STR—PTL-K--- | RLLLKEKPILHILKAPESSSSEESDSAAEKRLLREREFYQQQLDEYYRQFEPDFYPRAYPKKEVMPYPLEYFI
Exon 14
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bov ERLHSMKEGIHAQQ | KEPMIGVNQ | ELAYFYPE | LFRQFYQLDAYPSGAWYY-PLGTQYTDAPSFSDIPNPIGSENS-EKTT-MPLW-
buf EQLHSMKEGIHAQQ | KEPMIGVNQ | ELAYFYPQ | LFRQFYQLDAYPSGAWYYVPLGTQYPDAPSFSDIPNPIGSENSG-KTT-MPLW-
she EQLHSMKEGNPAHQ | KQPMIAVNQ | ELAYFYPE | LFRQFYQLDAYPSGAWYYLPLGTQYTDAPSFSDIPNPIGSENSG-KIT-MPLW-
goa EQLHSMKEGNPAHQ | KQPMIAVNQ | ELAYFYPE | LFRQFYQLDAYPSGAWYYTPLGTQYTDAPSFSDIPNPIGSENSG-KTT-MPLW-
cam EQLYRINEDNHPQL | GEPVKVVTQ | 🟩 | PFPQFFQLGASPYVAWYYPPQVMQYIAHPSSYDTEGPIASEDGG-KTDVMPQWW
pig E-LHRTNEDKHTQQ | GEPMKGVNQ | EQAYFYFE | PLHQFYQLDAYPYATWYYVP---QYIAHPLFTNIPQPTAPEKGG-KTEIMPQW-
rab 🟩 | REPMRVVNQ | ELAQLYLQ | PFEQPYQLDAYLPAPWYYGPEVMQYVLSPLFYDLVTPSAFE-SAEKTDVIPEWL | KN
cav EQFHRINEHNQAQV | KEPMRVFNQ | 🟩 | -------LDAYPFAAWLYPPEV-QYMSFLPFSSIPQPIFPE-DAQNTEVMPEW- | VM ?
rat EQPYRMNAYSQVQM | RHPMSVVDQ | --AQFSVQ | SFPQLSQYGAYPL--WAYPPQDMQYLTPEAVLNTFKPIAP-KDAENTNV---W-
mou EQPYRMNAYSQVQM | RHPMSVVDQ | ALAQFSVQ | PFPQIFQYDAFPL--W-Y-PQDMQYLTPKAVLNTFKPIVS-KDTEKTNV---W-
hum EQIRRMNENSHVQV | 🟩 | 🟩 | PFQQLNQLAAYPYAVWYYPPQIMQYVPFPPFSDISNPTAHENY-EKNNVMLQW-
hor EQLIRMKENSQ--- | RKPMRVVNQ | EQAYFYLE | PFQPSYQLDVYPYAAWFHPAQIMQHVAYSPFHDTAKLIASENS-EKTDIIPEW-
pla PQAAVYSIPQLVYRVPQEVTFPSPLRFRYAFPQPTLPVERK

Figure 4 Multiple alignment of the amino acid sequence of α_{s1}-casein from 12 eutherian species. Abbreviations and accession numbers are given in parentheses: cow (bov, M38641), water buffalo (buf, AJ005430), sheep (she, X03237), goat (goa, X59836), camel (cam, AJ012628), pig (pig, X54973), rabbit (rab, X13042), guinea pig (cav, X00938), rat (rat, J00710), mouse (mou, M36780), horse (hor, NM_001081883), and human (hum, X98084). Peptide sequences are split into blocks of amino acid residues to visualize the exonic modular structure of the protein as deduced from known splice junctions of the bovine, goat, and rabbit genes. Exon numbering (in bold) is that of the ruminants' genes. Additional exons are numbered with ′ and ″ (in italics). Large green boxes, within blocks, depict species-specific constitutively outspliced exons. Red boxes under exonic blocks identify highly conserved amino acid residues (>9/12) between species. Guinea pig α_{s1}-casein is the sequence of casein B. Italics correspond to signal peptides, of which the cleavage site is indicated by the vertical blue arrow. Dashes (–) are inserted gaps introduced to maximize the alignment. Star (∗) refers to the basic motifs of tandem hexapeptide repeats occurring in the rat and mouse α_{s1}-caseins. A monotreme (platypus: pla) CSN1 casein sequence (FJ548613) is also given for comparison. Underlined sequence represents part of the protein falling in an unresolved platypus genome sequence (Casein sequences in monotreme and platypus Lefèvre CM, Sharp JA, and Nicholas KR (2009) Characterisation of monotreme caseins reveals lineage specific expansion of an ancestral casein locus in mammals. *Reproduction, Fertility and Development* 21: 1015–1027).

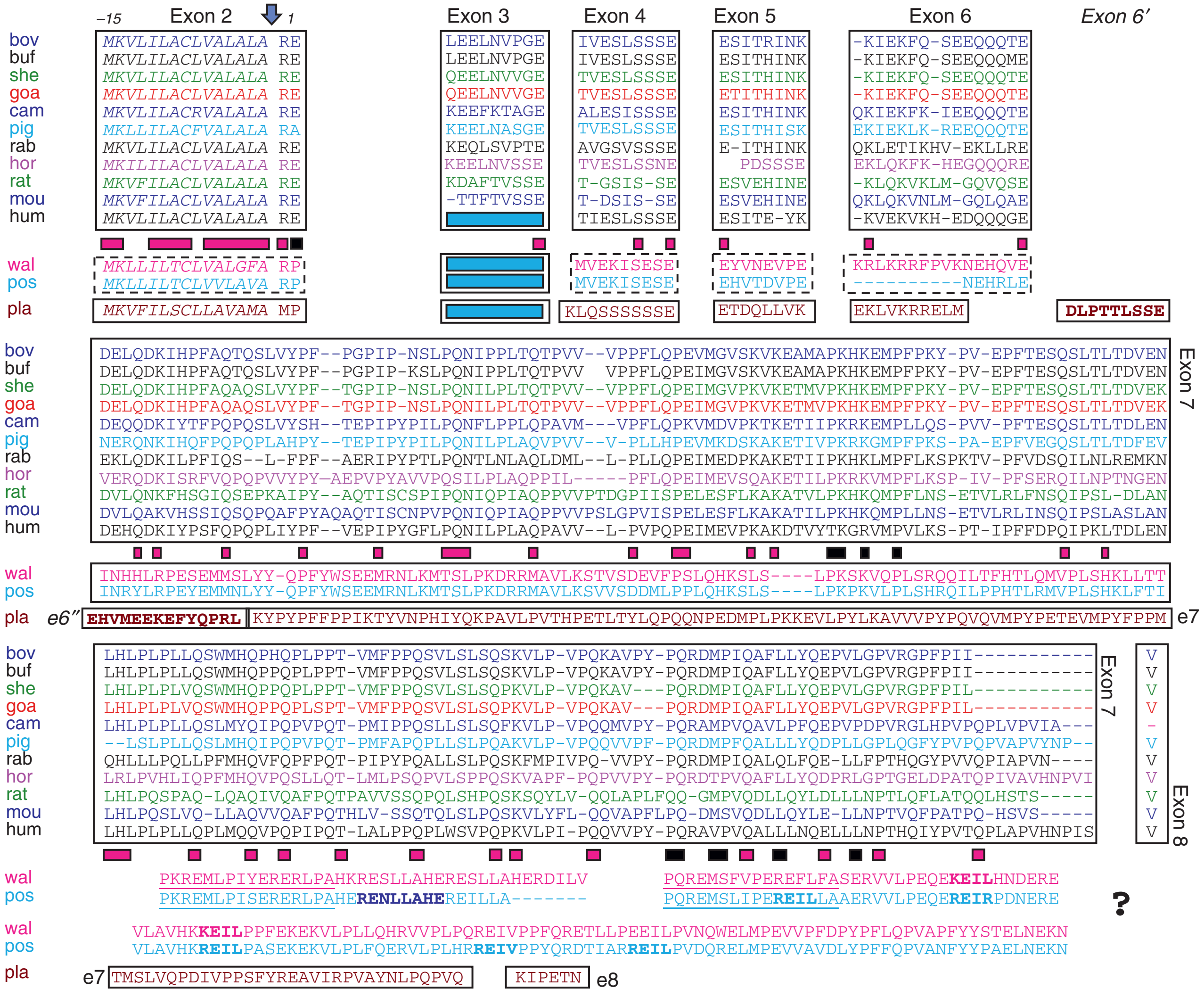

–15
Exon 2
1
Exon 3
Exon 4
Exon 5
Exon 6
Exon 6′
bov
buf
she
goa
cam
pig
rab
hor
rat
mou
hum
MKVLILACLVALALA RE
MKVLILACLVALALA RE
MKVLILACLVALALA RE
MKVLILACLVALALA RE
MKVLILACRVALALA RE
MKLLILACFVALALA RA
MKVLILACLVALALA RE
MKILILACLVALALA RE
MKVFILACLVALALA RE
MKVFILACLVALALA RE
MKVLILACLVALALA RE
LEELNVPGE
LEELNVPGE
QEELNVVGE
QEELNVVGE
KEEFKTAGE
KEELNASGE
KEQLSVPTE
KEELNVSSE
KDAFTVSSE
-TTFTVSSE
IVESLSSSE
IVESLSSSE
TVESLSSSE
TVESLSSSE
ALESISSSE
TVESLSSSE
AVGSVSSSE
TVESLSSNE
T-GSIS-SE
T-DSIS-SE
TIESLSSSE
ESITRINK
ESITHINK
ESITHINK
ETITHINK
ESITHINK
ESITHISK
E-ITHINK
PDSSSE
ESVEHINE
ESVEHINE
ESITE-YK
-KIEKFQ-SEEQQQTE
-KIEKFQ-SEEQQQME
-KIEKFQ-SEEQQQTE
-KIEKFQ-SEEQQQTE
QKIEKFK-IEEQQQTE
EKIEKLK-REEQQQTE
QKLETIKHV-EKLLRE
EKLQKFK-HEGQQQRE
-KLQKVKLM-GQVQSE
QKLQKVNLM-GQLQAE
-KVEKVKH-EDQQQGE
wal
pos
pla
MKLLILTCLVALGFA RP
MKLLILTCLVVLAVA RP
MKVFILSCLLAVAMA MP
MVEKISESE
MVEKISESE
KLQSSSSSE
EYVNEVPE
EHVTDVPE
ETDQLLVK
KRLKRRFPVKNEHQVE
----------NEHRLE
EKLVKRRELM
DLPTTLSSE
Exon 7
wal
pos
pla
e6″
EHVMEEKEFYQPRL
e7
Exon 7
Exon 8
e7
TMSLVQPDIVPPSFYREAVIRPVAYNLPQPVQ
KIPETN
e8
?

Figure 5 Multiple alignment of the amino acid sequence of β-casein from 11 eutherians. Abbreviations and accession numbers are given in parentheses: cow (bov, M15132), water buffalo (buf, AJ005165), sheep (she, X16482), goat (goa, AH001195), camel (cam, AJ012630), pig (pig, X54974), rabbit (rab, X13043), rat (rat, J00711), mouse (mou, X04490), human (hum, X17070), and horse (hor, NM_001081852, Q9GKK3 on Expasy UniProtKB). Two marsupials, tammar wallaby (wal, X54715) and possum (pos, AF128397), as well as a monotreme (platypus: pla, FJ548612) sequences are also given. Peptide sequences are split into blocks of amino acid residues to visualize the exonic modular structure of the protein as deduced from the known splice junctions of the rat, bovine, sheep, rabbit, and human genes. Exon numbering (in bold) is that of the ruminants' genes. Additional exons are numbered with ′ and ″ (in italics) for platypus sequence. Large blue boxes, within blocks, depict species-specific constitutively outspliced exons. Red and black boxes, between eutherian and marsupial sequences, identify highly conserved amino acid residues (>10/14) between species and anchoring points of marsupial sequences, respectively. Italics correspond to signal peptides, of which the cleavage site is indicated by the vertical blue arrow. Dashes (–) are inserted gaps introduced to maximize the alignment. Underlined and bold amino acids in the marsupial sequences depict duplications and the basic motif of the tandem octapeptide repeats found in marsupials, respectively.

β-caseins in monotremes strongly supports this statement. On the other hand, at the level of the mature proteins, the polyphosphorylated region (encoded by exon 4) is no longer the only region showing a clear conservation between species. Indeed, all along the large and mainly hydrophobic sequence, encoded by exon 7 (more than 160 amino acid residues), a rather high level of homology (30%) is observed. This ratio is quite good, given the number of sequences aligned, having a number of isolated amino acid residues (Q, P, K, and L) conserved. Furthermore, most of the substitutions tend to be conservative.

Albeit less frequently, probably owing to its less split genomic organization, exon skipping also occurs during the course of the processing of primary transcripts from β-casein-encoding gene (*CSN2*) in humans and horse. Despite apparent high dissimilarity with those of eutherian species, two marsupial (tammar wallaby and brushtail possum) sequences and one monotreme (platypus) sequence have been included in the alignment (**Figure 5**). Interestingly, this attempt strongly suggests again an outsplicing of exon 3, as reported for humans. Elsewhere, tammar β-casein and that isolated from the milk of the common brushtail possum, which are by far larger than the others (270 amino acid residues for the mature polypeptide chain vs. 209 in cattle), display a tandemly repeated (16 or 17 times) octapeptide sequence (RESLLAHE) in the C-terminal part of the molecule. This strongly supports the notion that the gene encoding β-casein may have grown through intragenic duplication, eventually coupled with (or followed by) changes of splice sites, before being subjected subsequently to duplications, giving rise to the cognate genes that have then evolved divergently.

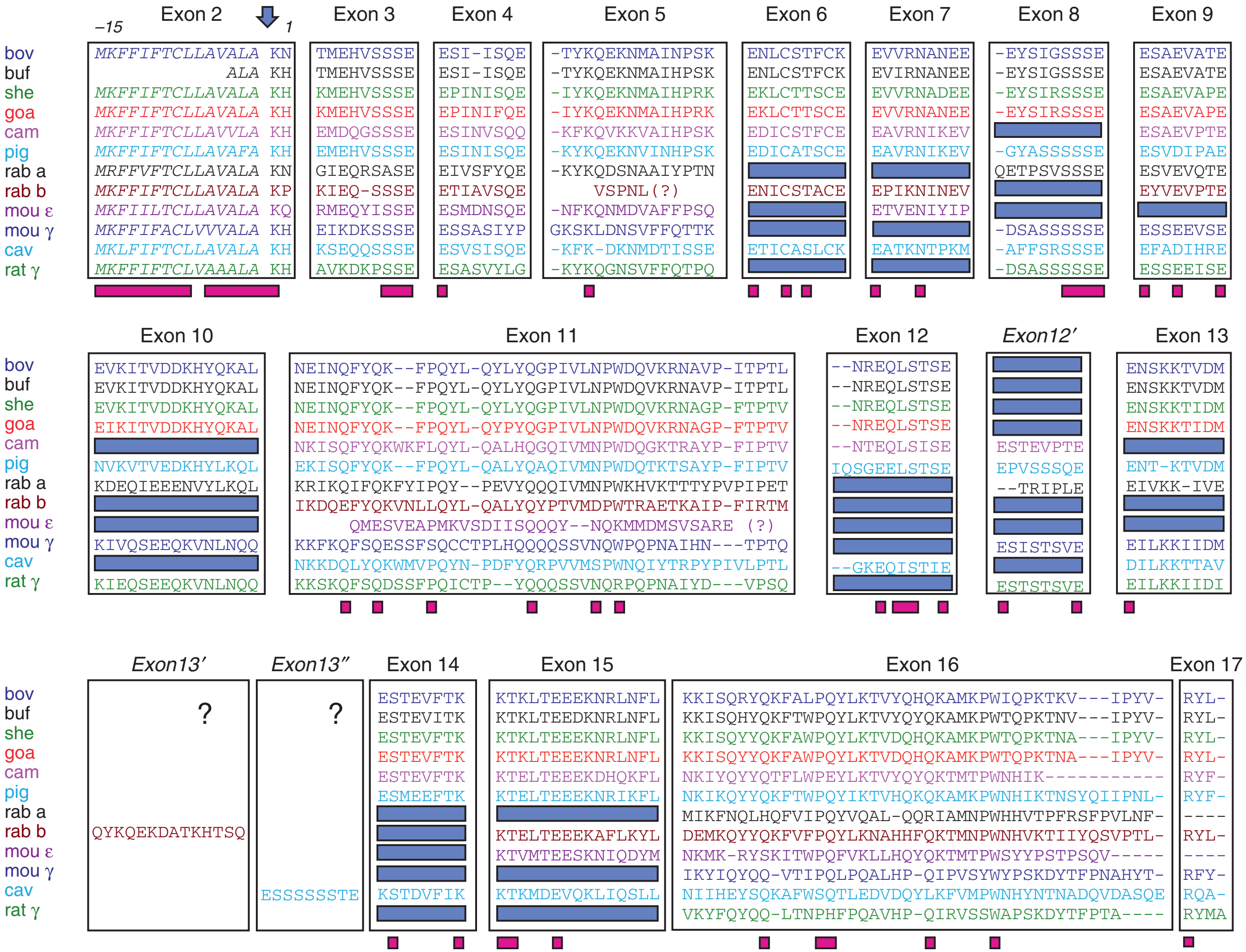
Considered together, these observations support the hypothesis of a presumed primitive and common ancestral gene resulting from the recruitment into a functional gene with a minimum of five exons: the first and the last corresponding to the 5′- and 3′-noncoding regions, the second encoding the signal peptide, the third a highly hydrophilic region including a multiple phosphorylation site, and the penultimate coding for a hydrophobic sequence required to ensure aggregation properties, which in turn is essential for casein micelle formation. Sequence similarities between the first exons of the genes encoding the three calcium-sensitive caseins confirm such an assumption.

α_{s2}-Casein

α_{s2}-Casein was the last bovine casein to be sequenced. Of the calcium-sensitive caseins, it is the most highly phosphorylated. α_{s2}-Casein occurs in milk in several forms and differs in the level of phosphorylation (10–13 phosphate groups/molecule). The peptide chain is 207 amino acid residues long and the phosphate groups are clustered in three regions of the molecule (7–31, 55–66, and 129–143). Peptide segments spanning residues 68–125 and the C-terminal part of the protein are predominantly hydrophobic. Given that human milk does not appear to contain α_{s2}-like casein and the equine sequence is still not available, amino acid sequence comparisons are restricted to only 10 eutherian species (**Figure 6**).

The structural organization of the α_{s2}-casein-encoding gene (*CSN1S2*), the longest of the four bovine casein-encoding genes, provides evidence that although it appears to be similar to that of the α_{s1}-casein-encoding gene (*CSN1S1*), sequence comparisons and the length of exons indicate that genes encoding α_{s2}- and β-caseins are more closely related to each other than to *CSN1S1*. However, analyses of interspecies relationships performed at the transcript level show that α_{s2}-caseins have diverged through extensive sequence rearrangements and a high level of nucleotide substitution. A tandem repeat was first detected in the amino acid sequence of bovine α_{s2}-casein. On the basis of the gene sequence, the large internal repeat was precisely extended to codons 43–124 and 125–204, which resulted in the formation of exons 12–16 by a duplication of exons 7–11. In addition, from both amino acid and nucleotide sequence comparisons, it is still evident that, with up to 60% similarity, exons 3–5 also arise from a duplication event of exons 8–10. Therefore, it can be hypothesized that this gene has been subjected to two successive duplications of a 5-exon module followed by the loss of one upstream (exon 7/12) and one downstream (exon 11/16) exon.

The same observation can be made for the other artiodactyls, camel, rat, and guinea pig sequences, while for the mouse and the rabbit the situation is not so clear and complicated by the existence of two (ϵ/δ and γ or α_{s2}-a and α_{s2}-b, respectively) α_{s2}-casein genes. In these species, the α_{s2}-like caseins are shorter than the others since they are 143, 184, 180, and 182 amino acid residues long, respectively, and all the duplicated sequences were apparently lost in the mouse ϵ/δ-casein. Thus, there are marked differences in size. Here, too, deleted segments, emphasized by multiple alignments based on the structure of the bovine gene, correspond to exon sequences outspliced from the mature messengers during the processing of the primary transcripts. Camel α_{s2}-casein, with 178 amino acid residues, lacks two internal stretches of 9 and 15 amino acid residues, very likely corresponding to exons 8 and 10. It now remains to demonstrate the presence, at the genomic level, of the relevant nucleotide sequence (encoding the missing peptide segment) and to characterize mutations responsible for this double exon skipping event, as had been done for the bovine α_{s2}-casein D variant. On the other hand, as previously mentioned for α_{s1}-casein, additional exons may also break out from intron sequences. The presence of an extra peptide sequence (IQSGEELST), between exons 11 and 12 in



	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
bov	*MKFFIFTCLLAVALA* KN	TMEHVSSSE	ESI-ISQE	-TYKQEKNMAINPSK	ENLCSTFCK	EVVRNANEE	-EYSIGSSSE	ESAEVATE
buf	*ALA* KH	TMEHVSSSE	ESI-ISQE	-TYKQEKNMAIHPSK	ENLCSTFCK	EVIRNANEE	-EYSIGSSSE	ESAEVATE
she	*MKFFIFTCLLAVALA* KH	KMEHVSSSE	EPINISQE	-IYKQEKNMAIHPRK	EKLCTTSCE	EVVRNADEE	-EYSIRSSSE	ESAEVAPE
goa	*MKFFIFTCLLAVALA* KH	KMEHVSSSE	EPINIFQE	-IYKQEKNMAIHPRK	EKLCTTSCE	EVVRNANEE	-EYSIRSSSE	ESAEVAPE
cam	*MKFFIFTCLLAVVLA* KH	EMDQGSSSE	ESINVSQQ	-KFKQVKKVAIHPSK	EDICSTFCE	EAVRNIKEV	▬	ESAEVPTE
pig	*MKFFIFTCLLAVAFA* KH	EMEHVSSSE	ESINISQE	-KYKQEKNVINHPSK	EDICATSCE	EAVRNIKEV	-GYASSSSE	ESVDIPAE
rab a	*MRFFVFTCLLAVALA* KN	GIEQRSASE	EIVSFYQE	-KYKQDSNAAIYPTN	▬	▬	QETPSVSSSE	ESVEVQTE
rab b	*MKFFIFTCLLAVALA* KP	KIEQ-SSSE	ETIAVSQE	VSPNL(?)	ENICSTACE	EPIKNINEV	▬	EYVEVPTE
mou ε	*MKFIILTCLLAVALA* KQ	RMEQYISSE	ESMDNSQE	-NFKQNMDVAFFPSQ	▬	ETVENIYIP	▬	▬
mou γ	*MKFFIFACLVVVALA* KH	EIKDKSSSE	ESSASIYP	GKSKLDNSVFFQTTK	▬	▬	-DSASSSSE	ESSEEVSE
cav	*MKLFIFTCLLAVALA* KH	KSEQQSSSE	ESVSISQE	-KFK-DKNMDTISSE	ETICASLCK	EATKNTPKM	-AFFSRSSSE	EFADIHRE
rat γ	*MKFFIFTCLVAAALA* KH	AVKDKPSSE	ESASVYLG	-KYKQGNSVFFQTPQ	▬	▬	-DSASSSSE	ESSEEISE

	Exon 10	Exon 11	Exon 12	*Exon12′*	Exon 13
bov	EVKITVDDKHYQKAL	NEINQFYQK--FPQYL-QYLYQGPIVLNPWDQVKRNAVP-ITPTL	--NREQLSTSE	▬	ENSKKTVDM
buf	EVKITVDDKHYQKAL	NEINQFYQK--FPQYL-QYLYQGPIVLNPWDQVKRNAVP-ITPTL	--NREQLSTSE	▬	ENSKKTVDM
she	EVKITVDDKHYQKAL	NEINQFYQK--FPQYL-QYLYQGPIVLNPWDQVKRNAGP-FTPTV	--NREQLSTSE	▬	ENSKKTIDM
goa	EIKITVDDKHYQKAL	NEINQFYQK--FPQYL-QYPYQGPIVLNPWDQVKRNAGP-FTPTV	--NREQLSTSE	▬	ENSKKTIDM
cam	▬	NKISQFYQKWKFLQYL-QALHQGQIVMNPWDQGKTRAYP-FIPTV	--NTEQLSISE	ESTEVPTE	▬
pig	NVKVTVEDKHYLKQL	EKISQFYQK--FPQYL-QALYQAQIVMNPWDQTKTSAYP-FIPTV	IQSGEELSTSE	EPVSSSQE	ENT-KTVDM
rab a	KDEQIEEENVYLKQL	KRIKQIFQKFYIPQY--PEVYQQQIVMNPWKHVKTTTYPVPIPET	▬	--TRIPLE	EIVKK-IVE
rab b	▬	IKDQEFYQKVNLLQYL-QALYQYPTVMDPWTRAETKAIP-FIRTM	▬	▬	▬
mou ε	▬	QMESVEAPMKVSDIISQQQY--NQKMMDMSVSARE (?)	▬	▬	▬
mou γ	KIVQSEEQKVNLNQQ	KKFKQFSQESSFSQCCTPLHQQQQSSVNQWPQPNAIHN---TPTQ	▬	ESISTSVE	EILKKIIDM
cav	▬	NKKDQLYQKWMVPQYN-PDFYQRPVVMSPWNQIYTRPYPIVLPTL	--GKEQISTIE	▬	DILKKTTAV
rat γ	KIEQSEEQKVNLNQQ	KKSKQFSQDSSFPQICTP--YQQQSSVNQRPQPNAIYD---VPSQ	▬	ESTSTSVE	EILKKIIDI

	Exon13′	*Exon13″*	Exon 14	Exon 15	Exon 16	Exon 17
bov	?	?	ESTEVFTK	KTKLTEEEKNRLNFL	KKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKV---IPYV-	RYL-
buf			ESTEVITK	KTKLTEEDKNRLNFL	KKISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTNV---IPYV-	RYL-
she			ESTEVFTK	KTKLTEEEKNRLNFL	KKISQYYQKFAWPQYLKTVDQHQKAMKPWTQPKTNA---IPYV-	RYL-
goa			ESTEVFTK	KTKLTEEEKNRLNFL	KKISQYYQKFAWPQYLKTVDQHQKAMKPWTQPKTNA---IPYV-	RYL-
cam			ESTEVFTK	KTELTEEEKDHQKFL	NKIYQYYQTFLWPEYLKTVYQYQKTMTPWNHIK-----------	RYF-
pig			ESMEEFTK	KTELTEEEKNRIKFL	NKIKQYYQKFTWPQYIKTVHQKQKAMKPWNHIKTNSYQIIPNL-	RYF-
rab a			▬	▬	MIKFNQLHQFVIPQYVQAL-QQRIAMNPWHHVTPFRSFPVLNF-	----
rab b	QYKQEKDATKHTSQ		▬	KTELTEEEKAFLKYL	DEMKQYYQKFVFPQYLKNAHHFQKTMNPWNHVKTIIYQSVPTL-	RYL-
mou ε			▬	KTVMTEESKNIQDYM	NKMK-RYSKITWPQFVKLLHQYQKTMTPWSYYPSTPSQV-----	----
mou γ			▬	▬	IKYIQYQQ-VTIPQLPQALHP-QIPVSYWYPSKDYTFPNAHYT-	RFY-
cav		ESSSSSSTE	KSTDVFIK	KTKMDEVQKLIQSLL	NIIHEYSQKAFWSQTLEDVDQYLKFVMPWNHYNTNADQVDASQE	RQA-
rat γ			▬	▬	VKYFQYQQ-LTNPHFPQAVHP-QIRVSSWAPSKDYTFPTA----	RYMA

Figure 6 Multiple alignment of the amino acid sequence of α_{s2}-casein from 10 mammalian species. Abbreviations and accession numbers are given in parentheses: cow (bov, M16644), water buffalo (buf, AJ005431), sheep (she, X03238), goat (goa, X65160), camel (cam, AJ012629), pig (pig, X54975), rabbit (rab a, X76907; rab b, X76909), mouse (mou ϵ, J00379; mou γ, D10215), guinea pig (cav, X00374), and rat (rat γ, J00712). Two sequences are given for rabbit (a and b) and mouse (δ/ϵ and γ). Sequences for the rat and guinea pig correspond to the γ-casein and casein A, respectively. Peptide sequences are split into blocks of amino acid residues to visualize the exonic modular structure of the protein, as deduced from the known splice junctions of the bovine gene. Additional exons are numbered with ′ and ″ (in italics). Large blue boxes, within blocks, depict species-specific constitutively outspliced exons. Red boxes identify highly conserved amino acid residues (>10/12) between sequences. Italics correspond to signal peptides, of which the cleavage site is indicated by the vertical blue arrow. Dashes (–) are inserted gaps introduced to maximize the alignment.

pig α_{s2}-casein, could be due to this kind of event. Similarly, one can anticipate the probable existence of an additional exon sequence within intron 14 of the gene encoding α_{s2}-casein b in the rabbit genome.

One of the main biochemical features of α_{s2}-casein, usually underlined and plausibly having an important functional role, is its ability to form disulfide bridges, owing to the presence of two close cysteinyl residues at positions 36 and 40, in the mature bovine peptide chain. These residues are encoded by exon 6, the sequence of which is rather well conserved, when present, with both cysteinyl residues at the same position. In contrast, in rat (γ), mouse (ϵ/δ and γ), and rabbit α_{s2}-casein, which lack this exon, both cysteinyl residues are obviously removed. Nevertheless, with a single and two contiguous cysteinyl residues in the middle of the peptide sequence encoded by exon 11 in rat and mouse γ-caseins, respectively, and since rat, mouse, and human α_{s1}-caseins contain at least one cysteinyl residue, there is no eutherian milk in which any α_s-casein is devoid of a cysteinyl residue.

κ-Casein

Glycosylated at various levels, κ-casein is highly heterogeneous, soluble in the presence of calcium and differing considerably in structure from the calcium-sensitive caseins. The functional duality of κ-casein, which is to interact hydrophobically with the other caseins and at the same time provide a hydrophilic and negatively charged surface on the micelle to stabilize the colloidal suspension, is strikingly reflected by its amphipathic primary structure. Its hydrophilic and flexible C-terminal part (caseinomacropeptide or CMP) is cleaved specifically by chymosin (between residues Phe_{105} and Met_{106}, in ruminants), thus leading to the destabilization of the micelle, to which the highly hydrophobic and insoluble N-terminal part (*para*-κ-casein) remains anchored. Two cysteine residues are found in the *para*-κ-casein region.

Although it belongs to the same chromosomal casein locus, the gene encoding κ-casein does not share any common structural organization scheme with the other casein genes. It is thought to be evolutionarily related to the γ-fibrinogen gene, which encodes a protein similarly involved in a clotting process (blood), following a limited proteolytic cleavage.

Interspecies comparison reveals that the κ-casein gene is identically organized in ruminants. The transcription unit invariably comprises five exons, three of which are small (65, 62, and 33 nucleotides in bovine), encoding the 5′-untranslated region (5′-UTR; exon 1) and the signal peptide (exons 2 and 3), which is longer (21 vs. 15 residues in the calcium-sensitive caseins). Therefore, the majority of the mature protein sequence (160 amino acid residues) is encoded by exon 4, while the last exon encodes the 3′-UTR.

Multiple alignments from the same 12 species, considered in this article, are shown in **Figure 7**. Gaps have been introduced to maximize similarity, taking into account comparisons performed at the nucleotide level of exon 4 for higher ruminants and extended to 21 species including cetaceans, hippo, deer, giraffe, tapir, and zebra.

Molecular Diversity of Caseins: Interspecies Variability

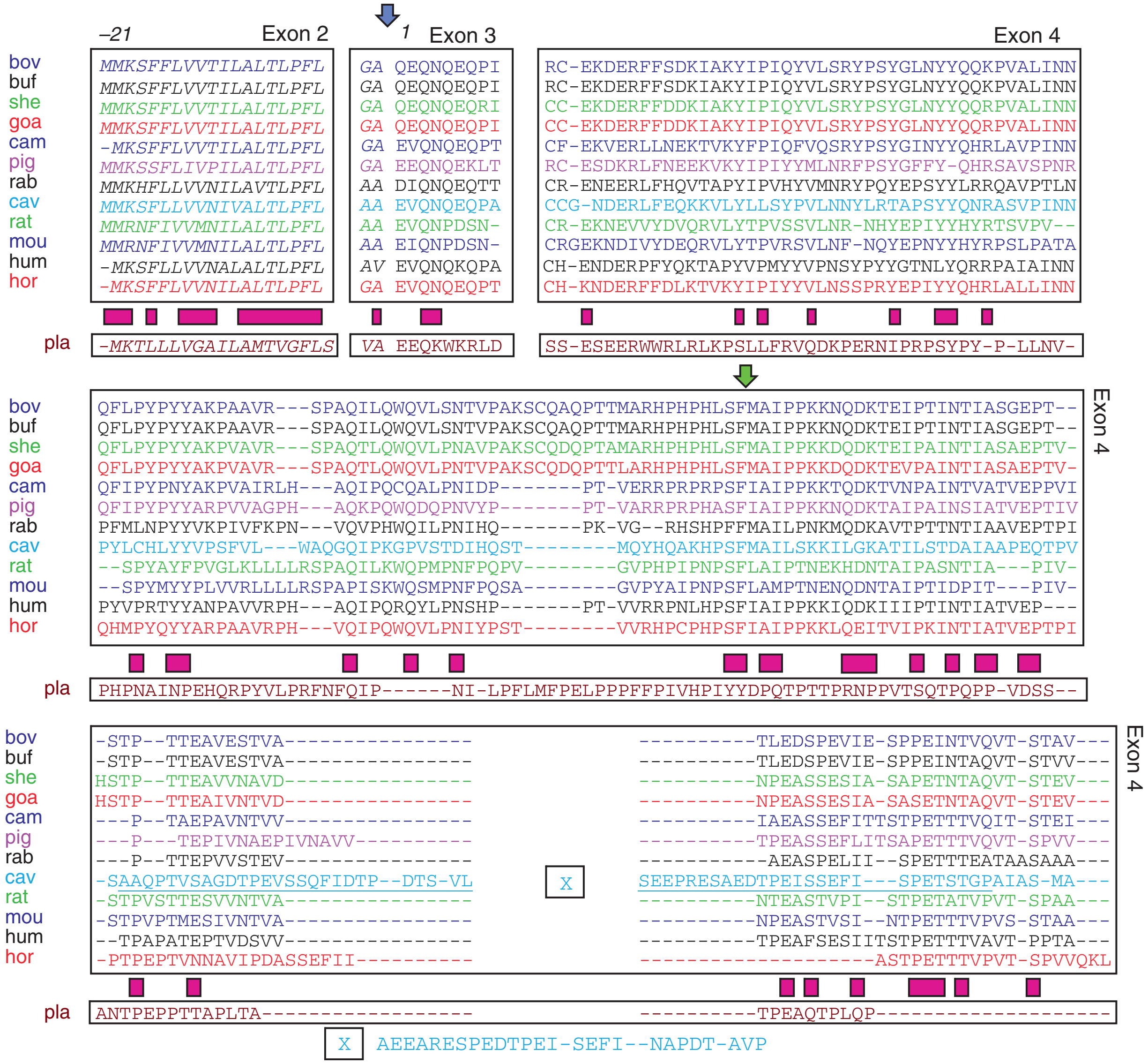
In addition to the differences in primary structure across species, examined above, which reflect changes at the genomic level within coding sequences and/or flanking sequences (splice site consensus sequences), further sources of variation occur at a post-transcriptional level. They affect mainly the processing of primary transcripts, and post-translational modifications such as phosphorylation (all caseins) and glycosylation (κ-casein). The extent of this variability and the complexity of the specific pattern within each species provide further criteria of distinctiveness between species.

Defect in the processing of primary transcripts: Splice variants

Two kinds of events may arise during the processing of primary transcripts, both leading to a shortening in the peptide chain length. The first event, referred to as cryptic splice site usage, primarily due to a ‘slippage’ of the splicing machinery, is induced by a ‘favorable’ junction sequence. As far as caseins are concerned, this defect in accuracy leads to the loss of the first codon (usually a CAG) of the 3′-exon. The second event, which is particularly well exemplified by small ruminants, gives rise to a stochastic alternative exon skipping (sometimes referred to as casual or non-allelic exon skipping). It is thought to be caused by weaknesses in the consensus sequences, either at the 5′- and/or 3′-splice junctions or at the branch point, or both.

Stochastic usage of cryptic splice sites

The casual deletion of a glutaminyl residue (Gln_{78} or Q_{78}), first detected in goat α_{s1}-casein, seems to be a rather frequent phenomenon, occurring in most of the species examined so far. This codon skipping, which is likely due to an erroneous 3′- cryptic splice site usage when exons 10 and 11 are joined, has been found in the four major ruminant species. It is worth noting that this kind of event may play a functional role in the structure and stability of casein micelles, since Q_{78} is located at the junction between the polar cluster of phosphoseryl residues and the hydrophobic domain of the protein. Interestingly, such a cryptic splice site activation does not only occur in ruminants. In humans, α_{s1}-casein transcript, in which exon 11 is lacking, a glutaminyl residue (Q_{37} in the mature protein), encoded by the first codon of exon



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        –21               Exon 2     1  Exon 3                                                 Exon 4
bov  MMKSFFLVVTILALTLPFL   GA QEQNQEQPI   RC-EKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINN
buf  MMKSFFLVVTILALTLPFL   GA QEQNQEQPI   RC-EKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINN
she  MMKSFFLVVTILALTLPFL   GA QEQNQEQRI   CC-EKDERFFDDKIAKYIPIQYVLSRYPSYGLNYYQQRPVALINN
goa  MMKSFFLVVTILALTLPFL   GA QEQNQEQPI   CC-EKDERFFDDKIAKYIPIQYVLSRYPSYGLNYYQQRPVALINN
cam  -MKSFFLVVTILALTLPFL   GA EVQNQEQPT   CF-EKVERLLNEKTVKYFPIQFVQSRYPSYGINYYQHRLAVPINN
pig  MMKSSFLIVPILALTLPFL   GA EEQNQEKLT   RC-ESDKRLFNEEKVKYIPIYYMLNRFPSYGFFY-QHRSAVSPNR
rab  MMKHFLLVVNILAVTLPFL   AA DIQNQEQTT   CR-ENEERLFHQVTAPYIPVHYVMNRYPQYEPSYYLRRQAVPTLN
cav  MMKSFLLVVNIVALTLPFL   AA EVQNQEQPA   CCG-NDERLFEQKKVLYLLSYPVLNNYLRTAPSYYQNRASVPINN
rat  MMRNFIVVMNILALTLPFL   AA EVQNPDSN-   CR-EKNEVVYDVQRVLYTPVSSVLNR-NHYEPIYYHYRTSVPV--
mou  MMRNFIVVMNILALTLPFL   AA EIQNPDSN-   CRGEKNDIVYDEQRVLYTPVRSVLNF-NQYEPNYYHYRPSLPATA
hum  -MKSFLLVVNALALTLPFL   AV EVQNQKQPA   CH-ENDERPFYQKTAPYVPMYYVPNSYPYYGTNLYQRRPAIAINN
hor  -MKSFFLVVNILALTLPFL   GA EVQNQEQPT   CH-KNDERFFDLKTVKYIPIYYVLNSSPRYEPIYYQHRLALLINN

pla  -MKTLLLVGAILAMTVGFLS  VA EEQKWKRLD   SS-ESEERWWRLRLKPSLLFRVQDKPERNIPRPSYPY-P-LLNV-

bov  QFLPYPYYAKPAAVR---SPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPT--   Exon 4
buf  QFLPYPYYAKPAAVR---SPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPT--
she  QFLPYPYYAKPVAVR---SPAQTLQWQVLPNAVPAKSCQDQPTAMARHPHPHLSFMAIPPKKDQDKTEIPAINTIASAEPTV-
goa  QFLPYPYYAKPVAVR---SPAQTLQWQVLPNTVPAKSCQDQPTTLARHPHPHLSFMAIPPKKDQDKTEVPAINTIASAEPTV-
cam  QFIPYPNYAKPVAIRLH---AQIPQCQALPNIDP-------PT-VERRPRPRPSFIAIPPKKTQDKTVNPAINTVATVEPPVI
pig  QFIPYPYYARPVVAGPH---AQKPQWQDQPNVYP-------PT-VARRPRPHASFIAIPPKKNQDKTAIPAINSIATVEPTIV
rab  PFMLNPYYVKPIVFKPN---VQVPHWQILPNIHQ-------PK-VG--RHSHPFFMAILPNKMQDKAVTPTTNTIAAVEPTPI
cav  PYLCHLYYVPSFVL---WAQGQIPKGPVSTDIHQST--------MQYHQAKHPSFMAILSKKILGKATILSTDAIAAPEQTPV
rat  --SPYAYFPVGLKLLLLRSPAQILKWQPMPNFPQPV--------GVPHPIPNPSFLAIPTNEKHDNTAIPASNTIA---PIV-
mou  --SPYMYYPLVVRLLLLRSPAPISKWQSMPNFPQSA--------GVPYAIPNPSFLAMPTNENQDNTAIPTIDPIT---PIV-
hum  PYVPRTYYANPAVVRPH---AQIPQRQYLPNSHP-------PT-VVRRPNLHPSFIAIPPKKIQDKIIIPTINTIATVEP---
hor  QHMPYQYYARPAAVRPH---VQIPQWQVLPNIYPST--------VVRHPCPHPSFIAIPPKKLQEITVIPKINTIATVEPTPI

pla  PHPNAINPEHQRPYVLPRFNFQIP------NI-LPFLMFPELPPPFFPIVHPIYYDPQTPTTPRNPPVTSQTPQPP-VDSS--

bov  -STP--TTEAVESTVA----------------          ----------TLEDSPEVIE-SPPEINTVQVT-STAV---   Exon 4
buf  -STP--TTEAVESTVA----------------          ----------TLEDSPEVIE-SPPEINTAQVT-STVV---
she  HSTP--TTEAVVNAVD----------------          ----------NPEASSESIA-SAPETNTAQVT-STEV---
goa  HSTP--TTEAIVNTVD----------------          ----------NPEASSESIA-SASETNTAQVT-STEV---
cam  ---P--TAEPAVNTVV----------------          ----------IAEASSEFITTSTPETTTVQIT-STEI---
pig  ---P---TEPIVNAEPIVNAVV----------          ----------TPEASSEFLITSAPETTTVQVT-SPVV---
rab  ---P--TTEPVVSTEV----------------          -----------AEASPELII--SPETTTEATAASAAA---
cav  -SAAQPTVSAGDTPEVSSQFIDTP--DTS-VL     X    SEEPRESAEDTPEISSEFI---SPETSTGPAIAS-MA---
rat  -STPVSTTESVVNTVA----------------          ----------NTEASTVPI--STPETATVPVT-SPAA---
mou  -STPVPTMESIVNTVA----------------          ----------NPEASTVSI--NTPETTTVPVS-STAA---
hum  --TPAPATEPTVDSVV----------------          ----------TPEAFSESIITSTPETTTVAVT-PPTA---
hor  -PTPEPTVNNAVIPDASSEFII----------          -------------------ASTPETTTVPVT-SPVVQKL

pla  ANTPEPPTTAPLTA------------------          ----------TPEAQTPLQP--------------------

                     X   AEEARESPEDTPEI-SEFI--NAPDT-AVP
```

Figure 7 Multiple alignment of the amino acid sequence of κ-casein from 12 mammalian species. Abbreviations and accession numbers are given in parentheses: cow (bov, M36641), water buffalo (buf, AJ011387), sheep (she, X51822), goat (goa, X60763), camel (cam, Y10082), pig (pig, X51977), rabbit (rab, Z18243), guinea pig (cav, X56020), rat (rat, K02598), horse (hor, NM_001081884), mouse (mou, M10114), and human (hum, M73628). Peptide sequences are split into blocks of amino acid residues to visualize the exonic modular structure of the protein, as deduced from the known splice junctions of the bovine, rabbit, and human genes. Red boxes identify highly conserved amino acid residues (>10/12) between species. Italics correspond to signal peptides, of which the cleavage site is indicated by the vertical blue arrow. Dashes (–) are inserted gaps introduced to maximize the alignment. The chymosin-sensitive bond is indicated by the green vertical arrow. X corresponds to the basic motif of the repeated sequence (underlined) in the guinea pig. The platypus (pla, FJ548626) sequence is also given, taking into account the structural organization of the gene (Casein sequences in monotreme and platypus Lefèvre CM, Sharp JA, and Nicholas KR (2009) Characterisation of monotreme caseins reveals lineage specific expansion of an ancestral casein locus in mammals. *Reproduction, Fertility and Development* 21: 1015–1027).

6′ (an additional exon found in the intron bridging exons 6 and 7), was also shown to be casually absent.

Examples of ‘Q’ insertion/deletion in protein, due to cryptic splice site usage, are well documented. It also occurs with other calcium-sensitive casein pre-messengers and for species other than humans and ruminants. It has been shown recently in rodents and horse. This loss in accuracy of the splicing machinery would be due to the nucleotide sequence at the intron–exon junction. The mechanism by which the 3′-splice site AG is accurately and efficiently identified involves a 5′-to-3′ scanning process. The first AG downstream from the branch point/polypyrimidine tract is preferentially selected. A second AG, competitive with the proximal one, can be used alternatively. Starting the exon sequence with a CAG (coding for a glutaminyl residue) would be a facilitating situation. The short size of the intron might be an enhancing factor. Indeed, introns 6 and 10, involved in human and ruminant genes encoding α_{s1}-casein, are 150 and 100 bp long, respectively.

'Species-specific' stochastic exon skipping

Structural characterization of caseins and/or analyses of relevant mRNA have enabled the identification, in the four ruminant species, of multiple forms of α_{s1}-casein. However, the extent of this heterogeneity depends on the species. While α_{s1}-casein phenotypes consist of a mixture of two forms (199 and 198 amino acid residues) in cattle and water buffalo, due to the alternative deletion of Q_{78}, there are, in sheep and goats, at least seven molecular forms that differ in their peptide chain length, regardless of genetic polymorphism. The main component corresponds to the 199-residue form initially described in goat milk. The others, in lower amounts, are shorter forms of α_{s1}-casein differing in deleted sequences (residues 110–117 and/or 141–148). Genomic and mRNA analyses demonstrated that these forms originated from exon-skipping events affecting exon 13 (encoding peptide 110–117) and/or exon 16 (encoding peptide 141–148) during the processing of the primary transcripts. Deletion of peptide 110–117, which contains four charged residues (SP_{115}, E_{110}, E_{117}, and K_{114}), and that of peptide 141–148, which contains only one (E_{141}), produce proteins with a different net charge.

The alternative splicing of exon 13 and/or 16 reported for small ruminant species has not been detected in cattle and water buffalo. Such differences in the processing of α_{s1}-casein pre-messengers in these closely related species are amazing and hard to explain, since none of the mutations (substitution or deletion) identified between small ruminants and cattle affects consensus splice sites. Long and short variants of α_{s1}-casein, which differ by the presence or absence of a stretch of eight amino acid residues encoded by exon 16, have been observed in camel milk also.

This phenomenon is clearly not restricted to ruminants. The existence of three α_{s1}-casein transcripts has been reported in the human mammary tissue. This heterogeneity is due to a differential splicing of exon 7 (bovine gene numbering) and to the usage of a cryptic splice site. Likewise, porcine α_{s1}-casein also shows such heterogeneity, with multiple forms differing by internal deletion. However, exons (12 and 13′, using the bovine gene numbering) showing such a casual alternative splicing are different from those reported for the other species.

Multiple forms arising from casual alternative splicing have been reported also in ovine α_{s2}-casein. Two non-allelic forms of α_{s2}-casein differing by an internal deletion of nine amino acid residues at positions 34–42 in the peptide chain have been found in ovine milk. Analysis of the products obtained by reverse transcription of mRNAs has shown a greater heterogeneity of α_{s2}-casein transcripts. In addition to the expected deletion of codons (34–42) affecting 30–40% of mRNA, another structural difference involving an internal stretch of 44 nucleotides in the 5′-UTR has been reported subsequently and shown to be caused by casual exon skipping.

Exon skipping is therefore to be considered as a frequent event, mainly in the case of α_{s1}- and α_{s2}-casein genes, for which the coding region is divided into so many short exons. However, since these pioneering works, a lot of additional examples have been found and the existence of intronic *cis*-element (intronic splicing enhancer) increasing the inclusion of 'weak' exons or influencing cryptic splice site usage has been reported in the equine β-casein gene. Do those deletions in calcium-sensitive caseins simply reflect the lack of accuracy of an intricate processing mechanism whenever mutations induce conformational modifications of pre-mRNA, preventing or enhancing the normal progress of events? Notwithstanding, these phenomena are mainly responsible for the great complexity of casein composition.

Genetic polymorphisms increase casein heterogeneity in peptide chain length

In addition to casual exon skipping, genetic polymorphism of milk proteins may sharply increase the heterogeneity of caseins in milk. Studies performed on goat milk have reported extensive genetic polymorphism of α_{s1}-casein, with at least 15 alleles at the goat α_{s1}-casein (*CSN1S1*) locus, distributed in seven different classes of protein variants, associated with four levels of expression. α_{s1}-Casein A, B, C, and E variants differ from each other in amino acid substitutions, while α_{s1}-casein variants F and G, associated with a low level of protein synthesis, are internally deleted. The establishment of the overall organization of the goat α_{s1}-casein gene (19 exons scattered along 17 kb), the characterization of allele F at the genomic level, as well as the analysis of their transcription products demonstrated that the internal deletion of 37 amino acid residues, occurring within variant F, arises from the outsplicing of three consecutive exons (9, 10, and 11), skipped *en bloc* during the processing of the primary transcripts. Furthermore, the α_{s1}*-CasF* allele was shown to yield multiple alternatively spliced transcripts, among which were transcripts lacking 24-nucleotide-long sequences encoded by exons 13 and 16. By comparison with the non-defective α_{s1}*-CasA, -CasB,* and *-CasC* alleles, a reduction in the amount of mRNA, due to mRNA decay and therefore accounting for a lower α_{s1}-casein content in milk, was observed. A single-point deletion in exon 12 of the α_{s1}-casein gene, leading to truncated proteins and hence a low content of α_{s1}-casein in the milk, has been described as being unique to the Norwegian goat population.

Likewise, a single-nucleotide deletion resulting in a premature stop codon is associated with a marked reduction in the amount and an extensive heterogeneity of transcripts from goat β-casein null allele in Créole and Pyrenean breeds. The occurrence of multiple and shorter β-*Cas0* transcripts has been shown. They differ from their full-length counterparts in large nucleotide stretches that are missing within exon 7. Four in-frame nonsense codons, due to a one-nucleotide deletion, were found in the β-*Cas0* allele and the cognate mRNA. Another β-casein null allele, identified in a Neapolitan goat breed, was shown to differ from the wild type by a transition C-T, affecting codon 157 in exon 7. The resulting premature termination codon is associated with a 10-fold decrease in β-casein mRNAs. However, data are lacking about the possible occurrence of multiple mRNAs.

As regards both α_{s1}-*CasF* (as well as a Norwegian *CSN1S1* allele) and β-*Cas0*, mutations are responsible for the existence of premature stop codons, associated with a decrease in the relevant level of transcripts and responsible for the presence of multiple forms of messengers, due to alternative splicing. Many reports have drawn attention to a possible relationship between nonsense codons and exon skipping. Indeed, some genes containing premature codons express alternatively spliced mRNA in which the exon containing the nonsense codon has been skipped. Among the hypotheses proposed to explain such a safeguard mechanism, one can mention 'nuclear scanning' which recognizes nonsense codons and then has an effect on exon definition. This raises the question: How is a normal termination codon (which does not usually mediate a reduction in the abundance of mRNA) distinguished from a premature stop codon? A rule has been proposed for the position of the termination codon. It must be located less than 50–55 nucleotides upstream from the 3′-most exon–exon junction. The normal termination codon in α_{s1}-casein, as well as in β-casein, transcripts is in part or fully encoded by the penultimate exon (exons 18 and 8, respectively) at 43 and 36 nucleotides upstream from the last exon–exon junction, respectively, thus conforming perfectly with the stated rule. Conversely, stop codons identified both with α_{s1}-*CasF* and the French and Italian β-*Cas0* alleles are located well beyond the 55-nucleotide limit. Therefore, they could be suspected to mediate mRNA decay and promote the occurrence of multiple forms of transcripts.

Differences in post-translational modifications

Our present knowledge of casein heterogeneity is rather advanced for a large number of species including cattle, goats, and even, until now, less thoroughly investigated species such as sheep, humans, or horse. With the growing resolving power of 2D electrophoretic techniques, the development of immunochemical procedures coupled with gel electrophoresis, and an increasing usage of mass spectrometry-based proteomics, we now have a clear vision of the complexity of the casein fraction in most of the species studied so far. Genetic polymorphisms remain one of the factors determining casein heterogeneity through alterations in electrical charge, molecular weight, and hydrophobicity of proteins. However, other factors such as post-translational modifications including phosphorylation and glycosylation (κ-casein) also contribute significantly. These factors will be examined below.

Phosphorylation

Phosphorylation of caseins is a post-translational event occurring in the Golgi apparatus and ensured by specific kinase(s) that recognize an amino acid triplet where the determinants are dicarboxylic residues (mainly Glu) or phosphoseryl residues. The occurrence of the tripeptide sequences Ser–X–Glu/SerP is a necessary but not a sufficient condition for phosphorylation of caseins to occur. Possible factors of constraint such as different intrinsic properties of both phosphate acceptor residues and acidic determinants, the characteristics of the local environment, secondary structure and steric hindrance, an insufficient available pool of kinase(s), etc., may explain incomplete phosphorylation.

Indeed, unlike milk from various ruminant species, human and equine milks display complex phosphorylation patterns. While bovine β- and α_{s1}-caseins exist predominantly as single phosphoforms, containing five and eight phosphate groups, respectively, equine and human β-caseins have variable phosphorylation levels with three to seven and zero to five phosphates, respectively. This notion, however, has to be considered cautiously, since in ewe's milk, α_{s1}-casein has been reported to exist as multiple phosphoforms with 7–11 phosphate groups. This is also true for β-casein (two to seven phosphates).

In **Table 2** is gathered our current knowledge on the phosphorylation level of the four main caseins from 12 mammals. The greatest amount of data refers to the four widely studied ruminant species while only limited information is currently available regarding the other species, with the exception for human and horse. Experimental data are compared to the theoretical number of sites expected on the basis of Mercier's rule.

Five variants of ovine α_{s1}-casein (A–E) have been described so far, associated with quantitative variation in casein content. The primary structure of three of them, A, C, and D (formerly called Welsh variant), has been determined. They differ from each other by few amino acid substitutions and the degree of phosphorylation. Differences between the three genetic variants, A, C, and D, are 'silent' substitutions affecting the degree of protein phosphorylation: variant C differs from variant

Table 2 Main structural features of the four (five*) caseins from 12 placental mammalian species

		Species											
Caseins	*Features*	*Cattle*	*Water buffalo*	*Sheep*	*Goat*	*Pig*	*Camel*	*Horse*	*Rat*	*Mouse**	*Guinea pig*	*Rabbit*	*Human*
CSN1S1	Mature protein (*n*)	199	199	199	199	191	215	197	187	187	179	197	170
	Signal peptide (*n*)	15	15	15	15	15	15	15	15	15	15	15	15
	Phosphorylation sites (*p*/e)	9/9	8/8	10/10	11/11	7/?	6/?	10/?	3/?	4/?	12/?	9/?	6/0–8
CSN2	Mature protein (*n*)	209	209	207	207	217	217	226	216	214	/	222	212
	Signal peptide (*n*)	15	15	15	15	15	15	15	15	15	/	15	15
	Phosphorylation sites (*p*/e)	6/5	5/5	6/6	6/6	5/?	4/?	9/7	11/?	9/?	/	4/?	6/6
CSN1S2	Mature protein (*n*)	207	207	208	208	220	178	/	163	128–169	209	166	/
	Signal peptide (*n*)	15	15	15	15	15	15	/	15	15–15	15	15	/
	Phosphorylation siters (*p*/e)	17/?	17/?	17/13	16/?	18/?	9/?	/	14/?	6/?–16/?	20/?	7/?	/
CSN3	Mature protein (*n*)	169	169	171	171	167	162	185	159	160	213	160	162
	Signal peptide (*n*)	21	21	21	21	21	20	21	21	21	21	21	20
	Phosphorylation sites (*p*/e)	5/3	5/3	5/3	6/3	?/?	?/?	2S + 6T/?	2/?	2/?	9/?	5/?	5/?

For each species, the number of amino acid residues of the mature chain and of the signal peptide and the number of phosphorylation sites (putative, p/effective, e) are indicated

A for the substitution Ser13-Pro, which determines the loss of a phosphate group at site 12 of the peptide chain, PSer12-Ser; a further substitution, PSer68-Asn, causes the disappearance of the phosphate group on both phosphorylated residues, Ser64 and Ser66, in variant D, which is widespread in Italian breeds.

As for other species, ovine α_{s2}-casein appears to be the most heterogeneous fraction because of its high degree of multiphosphorylation, with 9–12 phosphate groups. In sows' milk, polymorphism of α_{s2}-casein consisted of a fast-migrating band with a minor satellite band, which is absent from some samples, suggesting that this is determined by incomplete phosphorylation.

Camel caseins are less phosphorylated than bovine caseins. Six putative phosphorylation sites (Ser at positions 18, 68, and 70–73) have been identified in the camel α_{s1}-casein sequence, with a possible incomplete saturation. Although there are four predicted phosphorylation sites in the β-casein peptide sequence, from molecular mass measurements (matrix-assisted laser desorption ionization technique-mass spectrometry (MALDI-MS)) it was observed that the most frequent form has only three phosphate groups. One of the deletions that shortens camel α_{s2}-casein, likely due to the skipping of exon 8, is responsible for the loss of the phosphorylated serine cluster Ser56–Ser57–Ser58.

Two phosphorylation sites have been identified up to now (PSer151 and PSer168) out of five potentially phosphorylable sites (also including Ser127, Thr135, and Thr137) in the bovine κ-casein peptide chain. Using a proteomic approach (2D electrophoresis, combined with mass spectrometry) to analyze the casein fraction of milk from a single cow, homozygous for the B variant of κ-casein, 17 isoforms with different posttranslational modifications (PTMs) have been characterized and a previously unrecognized site (Thr166) that could be phosphorylated or glycosylated has been identified.

Glycosylation

Ovine κ-casein is O-glycosylated and Thr residues at positions 156, 158, and 159 have been proposed as putative glycosylation sites. The oligosaccharide units of ovine κ-casein contain both *N*-acetyl and *N*-glycolyl neuraminic acids at all stages of lactation. The disaccharide Galβ(1-3)GalNAc and the tetrasaccharide Galβ(1-3)[Galβ(1-4)GlcNAcβ(1-6)GalNAc] occur in mature ovine κ-casein, whereas defined tetra- and pentasaccharide structures are present in κ-casein from colostrum, indicating an evolution of the sugar moiety as a function of the time after parturition. In a comparative study, the caseinoglycopeptide from ewes' milk was shown to have greater antithrombotic activity than that of the cow.

The carbohydrate content of κ-caseinoglycopeptide is significantly higher in human milk (55%) than for bovine milk (10%). The monosaccharides Gal, GalNAc, and NeuAc are common to the κ-casein from both species, whereas Fuc and GlcNAc are specific for human κ-casein. An increasing number of oligosaccharide structures for human κ-caseinoglycopeptide are available. Among these oligosaccharides, GlcNAcβ(1-6)GalNac and GalNAcβ(1-4)GlcNAcβ(1-6)GalNAc represent novel types of core structures for mucin-type carbohydrate chains.

Although the primary structure of κ-casein from several species is available, glycosylation sites are essentially documented for bovine and human κ-caseins. Seryl residues were not glycosylated in either bovine or human κ-casein, whereas 9 out of 10 putative Thr-containing consensus sequences of human κ-casein are actually glycosylated. Potentially bovine κ-casein could have up to 12 NeuAc residues if all six glycosylation sites (only five in the B variant) were modified with the major tetrasaccharide, NeuAcα(2-3)Galβ(1-3)[NeuAcα(2-6)]GalNAc, identified. However, using 2D electrophoresis coupled with mass spectrometry, only fragment masses corresponding to a maximum of four NeuAc residues were observed in the multiply glycosylated forms. On the other hand, the core disaccharide, Galβ(1-3)GalNAc, to which the NeuAc residues are attached, appeared to be relatively stable, and fragment ions with up to three disaccharides were observed allowing the number of oligosaccharides attached to be determined.

Whey Proteins

A significant number of proteins synthesized or not in the mammary gland, with functions not fully understood, occur in the whey. We will discuss briefly hereafter variation across species taking into account quantitative as well as structural and biological aspects of selected major whey proteins.

Variation in the milk protein gene copy number potentially conributes to the diversity of milk protein composition. This is particularly well exemplified by the gene encoding β-lactoglobulin (*BLG*), which is one of the major whey proteins of ruminant species, apparently absent in humans, camels, rabbits, and rodents. Surprisingly, another major whey protein – the whey acidic protein or WAP – is frequently found instead of β-lactoglobulin (BLG). However, the presence of WAP in human milk has still to be demonstrated. On the other hand, both proteins are found in marsupial and monotreme milks (**Figure 8**). Up to now, swine is the only eutherian species for which WAP as well as BLG have been identified.

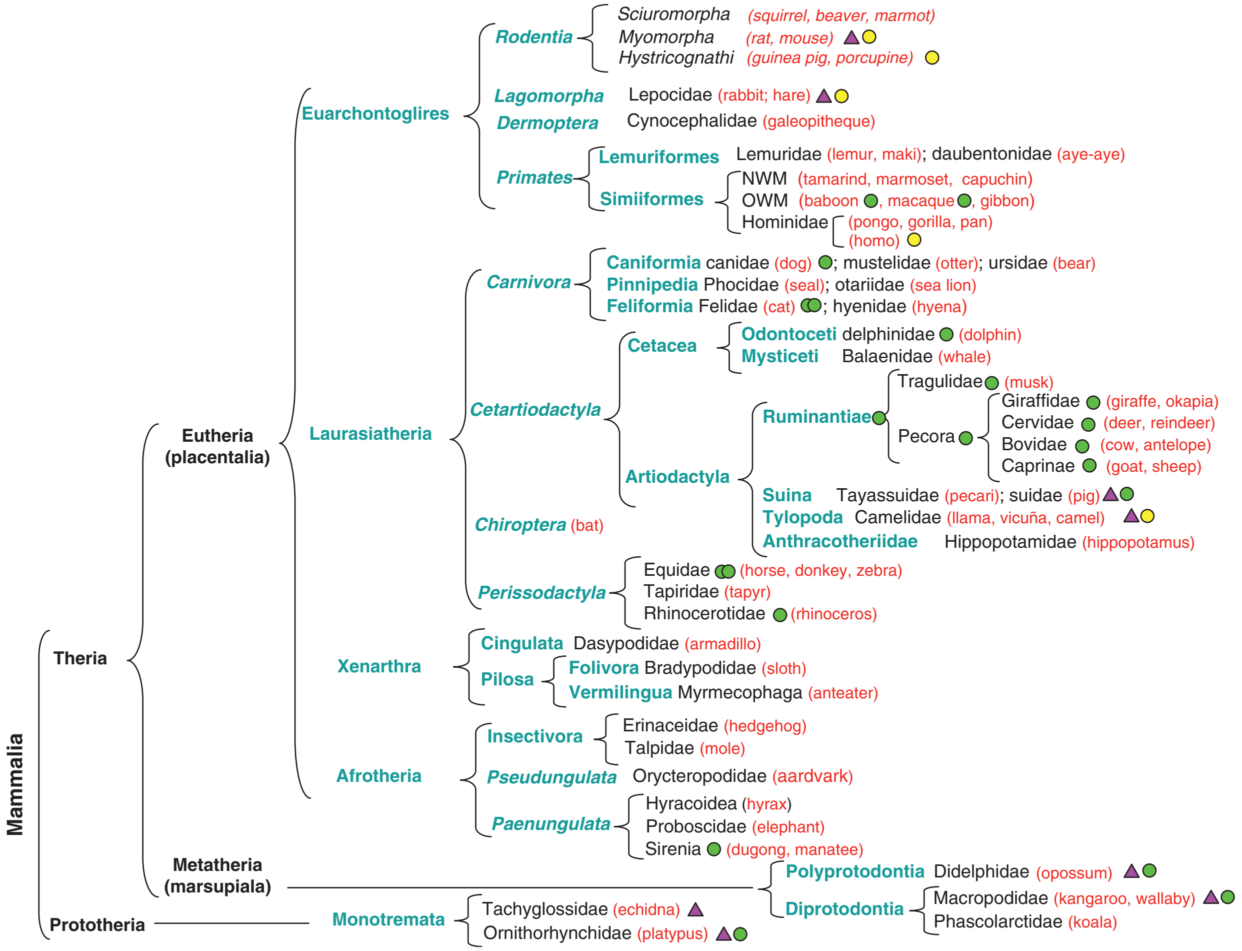
Mammalia
Theria
Prototheria
Eutheria (placentalia)
Metatheria (marsupiala)
Euarchontoglires
Laurasiatheria
Xenarthra
Afrotheria
Monotremata
Rodentia
Sciuromorpha (squirrel, beaver, marmot)
Myomorpha (rat, mouse) ▲○
Hystricognathi (guinea pig, porcupine) ○
Lagomorpha
Lepocidae (rabbit; hare) ▲○
Dermoptera
Cynocephalidae (galeopitheque)
Primates
Lemuriformes
Lemuridae (lemur, maki); daubentonidae (aye-aye)
Simiiformes
NWM (tamarind, marmoset, capuchin)
OWM (baboon ●, macaque ●, gibbon)
Hominidae
(pongo, gorilla, pan)
(homo) ○
Carnivora
Caniformia canidae (dog) ●; mustelidae (otter); ursidae (bear)
Pinnipedia Phocidae (seal); otariidae (sea lion)
Feliformia Felidae (cat) ●●; hyenidae (hyena)
Cetartiodactyla
Cetacea
Odontoceti delphinidae ● (dolphin)
Mysticeti Balaenidae (whale)
Artiodactyla
Ruminantiae ●
Tragulidae ● (musk)
Pecora ●
Giraffidae ● (giraffe, okapia)
Cervidae ● (deer, reindeer)
Bovidae ● (cow, antelope)
Caprinae ● (goat, sheep)
Suina Tayassuidae (pecari); suidae (pig) ▲●
Tylopoda Camelidae (llama, vicuña, camel) ▲○
Anthracotheriidae Hippopotamidae (hippopotamus)
Chiroptera (bat)
Perissodactyla
Equidae ●● (horse, donkey, zebra)
Tapiridae (tapyr)
Rhinocerotidae ● (rhinoceros)
Cingulata Dasypodidae (armadillo)
Pilosa
Folivora Bradypodidae (sloth)
Vermilingua Myrmecophaga (anteater)
Insectivora
Erinaceidae (hedgehog)
Talpidae (mole)
Pseudungulata Orycteropodidae (aardvark)
Paenungulata
Hyracoidea (hyrax)
Proboscidae (elephant)
Sirenia ● (dugong, manatee)
Polyprotodontia Didelphidae (opossum) ▲●
Diprotodontia
Macropodidae (kangaroo, wallaby) ▲●
Phascolarctidae (koala)
Tachyglossidae (echidna) ▲
Ornithorhynchidae (platypus) ▲●

Figure 8 Mammalian phylogeny and whey acidic protein (WAP)/β-lactoglobulin (BLG) presence in milk. Genus are given in red. NWM corresponds to New World monkeys, whereas OWM are related to Old World monkeys. ▲ indicates the presence of WAP in milk, ● marks the presence of BLG, while ●● indicate the occurrence of several copies of the gene encoding BLG in the genome. ○ marks the absence of BLG.

β-Lactoglobulin: A Singular and Enigmatic Whey Protein

β-Lactoglobulin (BLG) has been extensively studied across species and a comprehensive review was published few years ago. Despite relatively weak sequence homologies, multiple alignments reveal some striking features and short peptide sequences precisely conserved across species, including marsupials (**Figure 9**). The genomic organization of the gene encoding BLG is highly conserved across species, with seven exons, encompassing an ~5 kb genomic segment, located on chromosome 11 in cattle. With the release and assembly of the *Bos taurus* genome, it appeared that the gene encoding BLG is duplicated in cattle as it is in dog and horse genomes. The duplicated gene, first described in cattle as a pseudogene, shows similarities to *BLG-II* genes identified in the horse and cat. However, there is no evidence for its expression in the bovine mammary gland, thus being without any effect on the concentration of BLG in bovine milk. On the other hand, mutations within *BLG-I* gene, including its promoter region, seem to impact the BLG expression level.

The amino acid sequence and 3D structure of BLG show that this protein belongs to the widely diverse lipocalin superfamily, whose members share relatively low sequence similarity but have highly conserved exon/intron structure and 3D protein folding. Most of them bind small hydrophobic ligands and thus may act as specific transporters, as serum retinol-binding protein does. Bovine BLG binds a wide range of ligands, but this may not be the reason for its presence in milk. The structure and physicochemical properties of the protein have been reviewed recently. The apparent ability of the binding site to accommodate a wide range of ligands may point to a possible physiological function. However, by considering the lipocalin family, in general, and the species distribution of BLG in particular, some speculation can be made. It has been reported as being implicated in

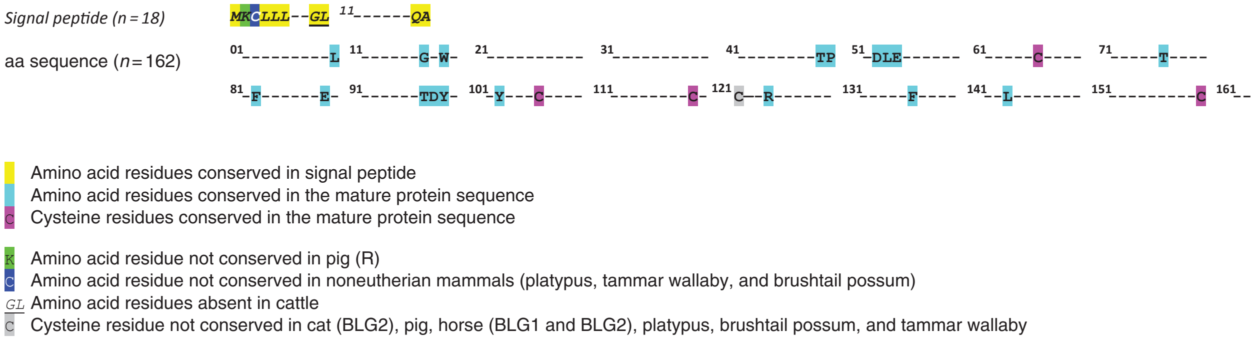
Signal peptide (n = 18)
MKCLLL--GL 11------QA
aa sequence (n = 162)
01---------L 11------G-W- 21---------- 31---------- 41--------TP 51-DLE------ 61-----C---- 71-----T----
81-F------E- 91------TDY- 101-Y---C---- 111--------C- 121C--R------ 131-----F---- 141--L------- 151---------C 161--
Amino acid residues conserved in signal peptide
Amino acid residues conserved in the mature protein sequence
C Cysteine residues conserved in the mature protein sequence
K Amino acid residue not conserved in pig (R)
C Amino acid residue not conserved in noneutherian mammals (platypus, tammar wallaby, and brushtail possum)
GL Amino acid residues absent in cattle
C Cysteine residue not conserved in cat (BLG2), pig, horse (BLG1 and BLG2), platypus, brushtail possum, and tammar wallaby

Figure 9 Amino acid residues conserved in β-lactoglobulin sequences of platypus, tammar wallaby, brushtail possum, horse, cattle, sheep, goat, and pig.

hydrophobic ligand transport and uptake, enzyme regulation, and the neonatal acquisition of passive immunity. However, these functions do not appear to be consistent between species. Sequence comparisons among members of the lipocalin family reveal that glycodelin (also known as PP14, placental protein 14, or progestagen-associated endometrial protein (PAEP)), found in the human endometrium during early pregnancy, is the most closely related to BLG. Although the function of glycodelin is not fully elucidated, it appears to have essential roles in regulating a uterine environment suitable for pregnancy and possibly to have effects on the immune system and/or to be involved in differentiation.

Twelve polymorphic variants of BLG are known in cattle (**Table 1**), but the most frequent two (A and B) were shown to be associated with differences in milk protein yield and composition. These variants differ by two amino acid substitutions in the polypeptide chain arising from two single-nucleotide substitutions in *BLG-I* gene: Asp64 (GAT)-Gly (G*G*T) and Val118 (GTC)-Ala (G*C*C). The latter T-C transition creates a *Hae*III restriction site, thus enabling a restriction fragment length polymorphism (RFLP) analysis at the *BLG* locus.

Quantitative effects of these common variants on milk composition and cheese-making properties have been reported. Allele B is associated with high casein and fat contents in cow's milk, while Holstein cows with BLG AA genotype were shown to produce milk containing more whey and total proteins than those of the other genotypes. This question and the impact of milk protein variants, including BLG, on milk composition have been studied extensively. Two studies dealing with this issue in cattle have been recently published. In addition, a higher expression of allele A has been described in heterozygous (AB) animals. This differential allelic expression has been explained by nucleotide differences in the promoter regions associated with these two alleles. Fourteen single-nucleotide polymorphisms (SNPs) were identified within the 5′-flanking region and two in the 5′-UTR (exon 1) of the bovine β-lactoglobulin gene. Some of them are located in potential binding sites for *trans*-acting factors or in the 5′-UTR. Sequences of 5′-flanking regions and *BLG* genotypes suggest that alleles A or B in the coding regions were connected with distinct promoter variants. Such intragenic haplotype associations may explain the observed differences in the effects of A or B variants of BLG on milk production traits particularly on BLG synthesis (A > B) in heterozygous cows.

By sequence analysis of the 5′-flanking regions of the main milk protein-encoding genes, altogether 65 variable sites have been revealed. Sixty were base substitutions and five were deletions/insertions. About 50% of the variable sites were located in potential protein binding sites. In cell culture tests, the investigated promoter variants led to different reporter gene expression. The promoter variant of the *BLG* A allele produced up to 3.5 times greater expression of a reporter gene than the promoter associated with the B allele. Differential expression was also shown using a reporter gene fused to bovine BLG A or B promoter variants in transiently transfected HC11 cells, the A variant driving more efficient expression than the B variant (57 vs. 43%).

More recently, Braunschweig and Leeb have shown the existence of an SNP (C-to-A transversion) 215 bp upstream the translation initiation site (g.-215C >A), segregating perfectly with a differential phenotypic expression of two *BLG* B alleles (B and B*). The sequence of the *BLG* B allele in the region of the mutation is highly conserved among four related ruminant species. The mutation site corresponds to a putative consensus-binding sequence for transcription factors c-Rel and Elk-1. These results support the hypothesis according to which sequence variation within the promoter of the *BLG* gene is probably one of the factors responsible for the differences in the protein content in milk.

Whey Acidic Protein

Whey acidic protein (WAP) has been identified in the milk of only a few mammalian species, including mouse, rat, rabbit, camel, pig, tammar wallaby, brushtail possum, echidna, and platypus, but it is absent in ruminant milks due to a frameshift mutation in the WAP encoding gene. The three ruminant WAP sequences reveal the same deletion of a single nucleotide at the end of the first exon when compared with the pig sequence. Due to the frameshift induced, the putative proteins encoded by these sequences do not harbor the features of a usual WAP protein with 2 four-disulfide core (4-DSC) domains, approximately 50 amino acids of which contain 8 cysteine residues in a conserved arrangement. Moreover, RT-PCR (Real Time-Polymerase Chain Reaction)experiments have shown that these sequences are not transcribed. This loss of functionality of the gene in ruminants raises the question of the biological role of the WAP.

The WAP proteins share limited amino acid sequence identities with the exception of these cysteine residues (at least one, usually two, 4-DSC domains in eutherian and even three in metatherian mammals) and positional conservation of several proline (P), glutamic acid (G), aspartic acid (D), and lysine (K) residues. Unlike the eutherian WAP sequences, marsupial WAP displays a conserved motif (KXGXCP) at the beginning of each 4-DSC domain (**Figure 10**). However, currently no functional significance has been ascribed for this motif, although it was thought to be important for correct folding of the protein. The presence of 4-DSC domain sequences on chromosome 20 (WFDC2 or HE4 protein) within the human genome raises the possibility (not yet demonstrated) that a secreted WAP may be present in human milk.

WFDC2/HE4 protein is a small secretory protein, shown to function as an antiproteinase (protease inhibitor) involved in the innate immune defense of multiple epithelia. The relevant gene is highly expressed in pulmonary epithelial cells and saliva and was also found to be expressed in some ovarian cancers and epididymis.

The organization of eutherian *WAP* genes is highly conserved and composed of four exons with exon 1 encoding the 5′-UTR, signal peptide, and first 8–10 amino acids of the mature protein. Exons 2 and 3 encode the two 4-DSC domains and exon 4 encodes the last 8–10 amino acids of the protein and the 3′-UTR. While the size of each exon remains rather conserved between species, intron size varies considerably. Exon 3, encoding 4-DSC domain II, has the higher degree of sequence conservation between species. It was proposed to be the primordial domain, with domain I likely to have arisen by intragenic duplication.

A third 4-DSC domain encoded by an additional exon has been identified in marsupial WAP as well as in platypus, whereas the *WAP* gene structure of echidna is different and closer to that of the *WFDC2* gene, with only two 4-DSC domains (**Figure 10**). It is possible that domain III of the marsupial WAP gene may be the ancestral gene, which was subsequently lost during evolution in eutherian species. It has been suggested that the evolution of the WAP gene in the mammalian lineage may be either through exon loss from an ancient ancestor or by rapid evolution via the process of exon shuffling.

Whereas eutherian WAP is expressed in the mammary gland throughout lactation, marsupial WAP is expressed only during mid–late lactation. This transient expression pattern in marsupials has to be correlated with a short gestation, giving birth to an immature young followed by a long lactation during which milk progressively changes in composition to suit the developing young requirements. This suggests that WAP may play a role in the development of the mammary gland or influence the development of the young. Interestingly, tammar mammary gland was shown to express a second WAP-like protein (WFDC2) at a high level during pregnancy, at a reduced level in early lactation, before disappearing into-midlate lactation. These different temporal expression patterns of WAP and WFDC2 suggest that they play complementary roles.

α-Lactalbumin

Among the main milk proteins, LALBA is so far the only one with an enzymatic activity. Together with β-1,4-galactosyltransferase it forms the lactose synthase complex which catalyses the formation of lactose from glucose and UDP-galactose, in the Golgi apparatus of mammary epithelial cells. LALBA was shown to be a calcium metalloprotein, in which the calcium ion has an unusual role in folding and structure. Most of the molecular structure and function is known and was reviewed by K. Brew. This protein has experienced a renewed interest with 'HAMLET' (human α-lactalbumin made lethal to tumor cells) a partially unfolded LALBA which aquires, after binding oleic acid, a tumoricidal function.

LALBA is present in the milk of almost every species of mammals, except some Otariidae (*Arctocephalus pusillus*: Cape fur seal) of which the milk is rich in fat (more than 20% long-chain fatty acids, mainly unsaturated) and devoid of lactose and LALBA. The female fur seal modulates its lactation by turning milk production 'on' and 'off' without regression and involution of the mammary gland. After undergoing a perinatal fast of 2–3-day suckling pups on shore, the mother leaves its young and the colony to forage at sea for 3 weeks to replenish body stores during which time the mammary gland remains active without initiating involution, demonstrating an apoptotic function for LALBA. This apoptotic potential, which is consistent with observations made on LALBA-deficient mice, has to be compared with the tumoricidal function of HAMLET.

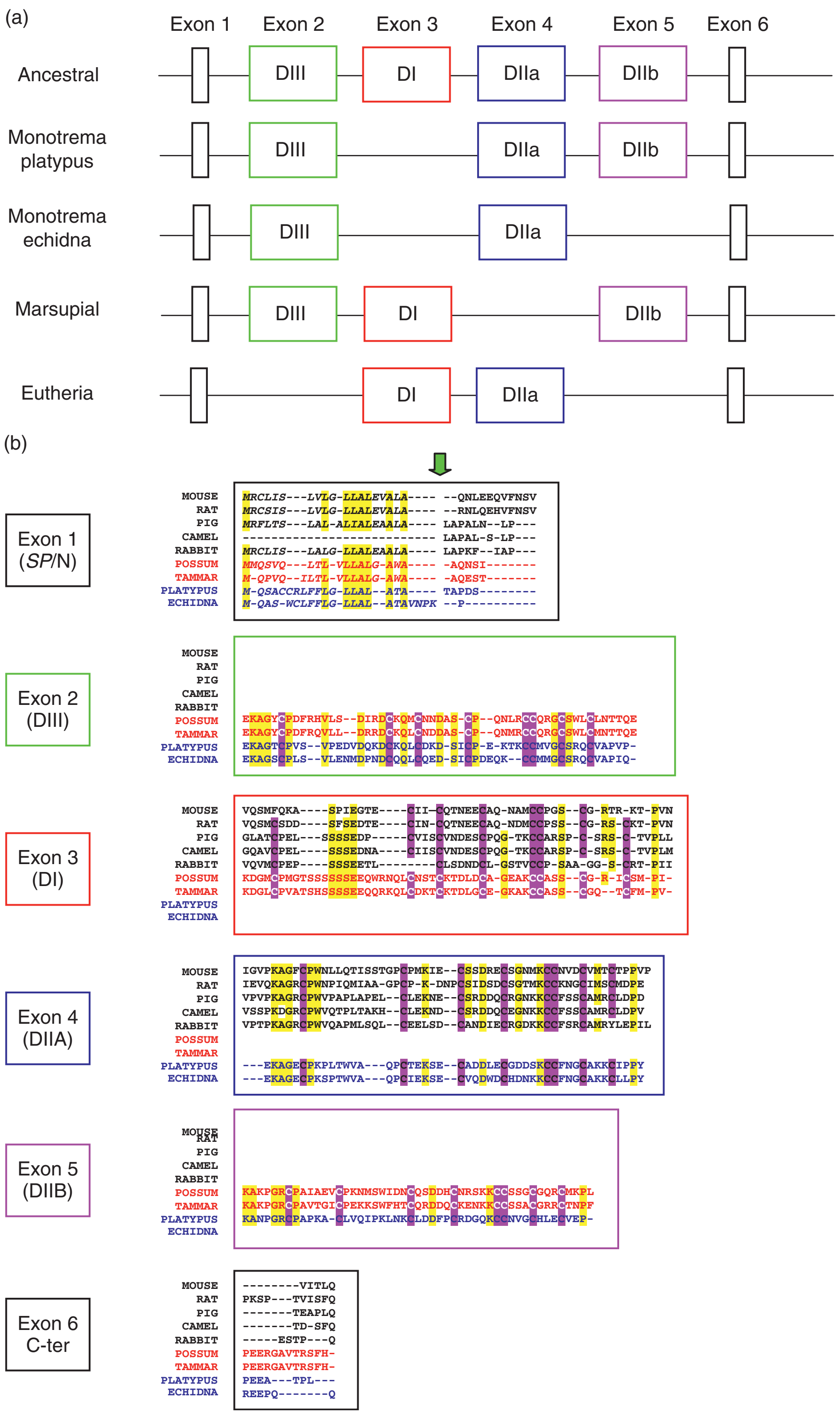
(a)
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
Ancestral
DIII
DI
DIIa
DIIb
Monotrema platypus
DIII
DIIa
DIIb
Monotrema echidna
DIII
DIIa
Marsupial
DIII
DI
DIIb
Eutheria
DI
DIIa
(b)
Exon 1 (SP/N)
MOUSE MRCLIS---LVLG-LLALEVALA---- --QNLEEQVFNSV
RAT MRCSIS---LVLG-LLALEVALA---- --RNLQEHVFNSV
PIG MRFLTS---LAL-ALIALEAALA---- LAPALN--LP---
CAMEL --------------------------- LAPAL-S-LP---
RABBIT MRCLIS---LALG-LLALEAALA---- LAPKF--IAP---
POSSUM MMQSVQ---LTL-VLLALG-AWA---- -AQNSI-------
TAMMAR M-QPVQ--ILTL-VLLALG-AWA---- -AQEST-------
PLATYPUS M-QSACCRLFFLG-LLAL--ATA---- TAPDS--------
ECHIDNA M-QAS-WCLFFLG-LLAL--ATAVNPK --P----------
Exon 2 (DIII)
MOUSE
RAT
PIG
CAMEL
RABBIT
POSSUM EKAGYCPDFRHVLS--DIRDCKQMCNNDAS-CP--QNLRCCQRGCSWLCLNTTQE
TAMMAR EKAGYCPDFRQVLL--DRRDCKQLCNDDAS-CP--QNMRCCQRGCSWLCMNTTQE
PLATYPUS EKAGTCPVS--VPEDVDQKDCKQLCDKD-SICP-E-KTKCCMVGCSRQCVAPVP-
ECHIDNA EKAGSCPLS--VLENMDPNDCQQLCQED-SICPDEQK--CCMMGCSRQCVAPIQ-
Exon 3 (DI)
MOUSE VQSMFQKA----SPIEGTE----CII—CQTNEECAQ-NAMCCPGS--CG-RTR-KT-PVN
RAT VQSMCSDD----SFSEDTE----CIN-CQTNEECAQ-NDMCCPSS--CG-RS-CKT-PVN
PIG GLATCPEL---SSSSEDP-----CVISCVNDESCPQG-TKCCARSP-C-SRS-C-TVPLL
CAMEL GQAVCPEL----SSSEDNA----CIISCVNDESCPQG-TKCCARSP-C-SRS-C-TVPLM
RABBIT VQVMCPEP----SSSEETL--------CLSDNDCL-GSTVCCP-SAA-GG-S-CRT-PII
POSSUM KDGMCPMGTSSSSSSEEQWRNQLCNSTCKTDLDCA-GEAKCCASS--CG-R-ICSM-PI-
TAMMAR KDGLCPVATSHSSSSEEQQRKQLCDKTCKTDLGCE-GKAKCCASS--CGQ--TCFM-PV-
PLATYPUS
ECHIDNA
Exon 4 (DIIA)
MOUSE IGVPKAGFCPWNLLQTISSTGPCPMKIE--CSSDRECSGNMKCCNVDCVMTCTPPVP
RAT IEVQKAGRCPWNPIQMIAA-GPCP-K-DNPCSIDSDCSGTMKCCKNGCIMSCMDPE
PIG VPVPKAGRCPWVPAPLAPEL--CLEKNE--CSRDDQCRGNKKCCFSSCAMRCLDPD
CAMEL VSSPKDGRCPWVQTPLTAKH--CLEKND--CSRDDQCEGNKKCCFSSCAMRCLDPV
RABBIT VPTPKAGRCPWVQAPMLSQL--CEELSD--CANDIECRGDKKCCFSRCAMRYLEPIL
POSSUM
TAMMAR
PLATYPUS ---EKAGECPKPLTWVA---QPCTEKSE--CADDLECGDDSKCCFNGCAKKCIPPY
ECHIDNA ---EKAGECPKSPTWVA---QPCIEKSE--CVQDWDCHDNKKCCFNGCAKKCLLPY
Exon 5 (DIIB)
MOUSE
RAT
PIG
CAMEL
RABBIT
POSSUM KAKPGRCPAIAEVCPKNMSWIDNCQSDDHCNRSKKCCSSGCGQRCMKPL
TAMMAR KAKPGRCPAVTGICPEKKSWFHTCQRDDQCKENKKCCSSACGRRCTNPF
PLATYPUS KANPGRCPAPKA-CLVQIPKLNKCLDDFPCRDGQKCCNVGCHLECVEP-
ECHIDNA
Exon 6 C-ter
MOUSE --------VITLQ
RAT PKSP---TVISFQ
PIG -------TEAPLQ
CAMEL -------TD-SFQ
RABBIT -----ESTP---Q
POSSUM PEERGAVTRSFH-
TAMMAR PEERGAVTRSFH-
PLATYPUS PEEA---TPL---
ECHIDNA REEPQ-------Q

Figure 10 Schematic representation of the structure and evolution of the whey acidic protein (WAP) gene. (a) The ancestral progenitor is depicted by six exons (boxes) numbered from 1 to 6. Colored boxes represent exons (2–5) encoding 4-DSC domains while shaded boxes represent exons encoding the signal peptide (*SP*) and the N-terminal part (N) of the mature protein (exon 1) and the C-terminal part (C-ter) of the protein (exon 6). In eutherian WAP, 4-DSC domains (DI and DIIa) are encoded by exons 3 and 4. The two 4-DSC domains of echidna WAP are encoded by exons 2 (DIII) and 4 (DIIa). Marsupial and platypus WAPs comprise three 4-DSC domains in different configurations: DIII–DI–DIIb and DIII–DIIa–DIIb, respectively. Evolution of *WAP* genes in mammalian species would have proceeded step by step by loss of exon to lead to the present *WAP* genes in monotremes (platypus and echidna) and marsupial and placental mammals. (b) Alignment of eutherian, marsupial, and monotreme WAP sequences shows conservation of protein structure. The 4-DSC domains represented by eight cysteine residues (C) are highlighted with a pink background in each domain (exon). Highly conserved residues are highlighted with a yellow background. Mouse (P01173), rat (P01174), pig (O46655), camel (P09837), and rabbit (P09412) for eutherian sequences have been aligned with brushtail possum (Q95JH3), tammar wallaby (Q9N0L8), platypus (A7J9L3), and echidna (A7J9L2) to maximize similarity within exons depicted by boxes for which the color code of (a) has been retained. Adapted from Sharp JA, Lefèvre C, and Nicholas KR (2007) Molecular evolution of monotreme and marsupial whey acidic protein genes. *Evolution and Development* 9: 378–392.

The gene structure and protein sequence are highly conserved across species, with more divergence in rodents than in primates. Because of its prominent role in milk synthesis, LALBA is considered a valuable genetic marker for milk production traits in cattle. However, LALBA appears rather weakly polymorphic in cattle (three variants) and sheep (two variants). After screening at the protein as well as the nucleotide level, few mutations have been found, mainly within the regulatory sequences of the LALBA-encoding gene.

Three SNPs, occurring at positions +15, +21, and +54 relative to the mRNA transcription start point, were identified in cattle within an approximately 2-kb fragment including 1952 bp of 5′-flanking region and 66 bp of the protein-coding region. The +15 and +21 variations occurred in the 5′-UTR of the mRNA, whereas the +54 polymorphism is a silent mutation in the signal peptide-coding region of the gene. SNP at position +15 (A/G) was shown to occur only in Holstein breed and to be associated with an increased milk yield. Cows with the A allele had higher milk yield, protein yield, and fat yield; the B allele was associated with a higher percentage of protein and fat. These data suggest that, although not located in the gene promoter, this SNP potentially alters the LALBA expression at the translational level and may be associated with differences in milk yield. In addition to SNP/+15, a second SNP (also a transition A-G), located at position −1689 from the transcription start point, was identified. The allele showing an adenine at position −1689 was designated as allele A, and that with a guanine at this position was designated as allele B. The −1689 and +15 polymorphisms were compared within the Holstein population to determine their linkage relationship. In this study, the +15 A variant was always linked to the variant A at −1689. These results suggest the existence of a haplotype A (+15A and −1689A) associated with higher milk, protein, and fat yields.

Lysozyme

Lysozyme is a bacteriocidal agent, structurally related to LALBA, sharing 40% similarity. Ranging in molecular mass between 14 and 18 kDa, this enzyme, also called β-1,4-*N*-acetylmuramidase, cleaves a glycosidic linkage in the peptidoglycan component of bacterial cell walls, resulting in a loss of cell wall integrity and in cell lysis. The concentration of lysozyme in milks varies from 1–3 mg l^{-1} in bovine milk to 400 (in human) and even 800 mg l^{-1} in mare's milk.

Lactoferrin

Lactoferrin (LTF) is of mammary origin and is found in the milk of most species. LTF is an iron-binding glycoprotein with a molecular mass around 80 000 Da, belonging to the transferrin family that is expressed and secreted by epithelial cells and found in the secondary granules of neutrophils from which it is released in infected tissues and blood during the inflammatory process. Initially described as an iron-binding molecule with bacteriostatic properties, LTF is now known to be a multifunctional or multitasking protein with multiple biological activities. It is a major component of the innate immune system of mammals. Its protective effects range from direct antimicrobial activities against a large panel of microorganisms, including bacteria, viruses, fungi, and parasites, to anti-inflammatory and anticancer activities. While iron chelation is central to some of its biological functions, other activities involve interactions of LTF with molecular and cellular components of both hosts and pathogens.

The internal structure of LTF is highly conserved, and is dedicated to binding iron. On the other hand, the external structure (its molecular surface) is much more variable across species, making it more difficult to identify functionally important sites. Recent work shows that the cationic N-terminus and associated lactoferricin domain on the N-lobe of LTF, in addition to its role in antibacterial activity and probable role in DNA binding, is also involved in complex formation with other proteins. Finally, it may be time to re-examine the importance of glycosylation, given the growing evidence that many pathogens depend on binding to glycans for pathogenesis.

The overall structural organization of the human, mouse, cattle, dog, and horse LTF genes is rather conserved, at least in terms of size and number of exons ($n = 17$). LTF is encoded by an approximately 30 kb gene (ranging in size between 23.5 kb in mice and 33.4 kb in cattle), located on chromosome 3 in human, 9 in mice, and 22 in cattle.

A total of 60 LTF nucleotide sequences with the complete coding sequence (CDS) and corresponding amino acids belonging to 11 species were recently analyzed, and differences within and across species studied. The length of the LTF cDNA with the complete CDS varies greatly, from 2055 to 2190 bp, due to deletion, insertion, and stop codon mutation, resulting in elongation. Observed genetic diversity was higher across species than within species, and *Sus scrofa* had more polymorphisms than any other species. Novel amino acid variation sites were detected within several species (8 in *Homo sapiens*, 6 in *Mus musculus*, 6 in *Capra hircus*, 10 in *Bos taurus*, and 20 in *S. scrofa*), illustrating functional variation.

Concluding Remarks

The past 10 years have seen a fantastic breakthrough in the knowledge of genome structure and organization. New insights and clues to better understand mechanistic details involved in the regulation and variability of gene expression have been provided already and are still expected. Data now available on the architecture of the casein locus in several species, including monotremes and marsupials, will contribute to our understanding of the mechanisms responsible for variations and heterogeneity in milk casein composition. However, it is perhaps in the functional field that we might progress significantly in the near future.

Development of instrumental techniques has played a determinant role in these breakthroughs, particularly in the field of post-translational modifications. Although we now know many of the details of casein structure, a number of questions remain unanswered in our understanding of the biogenesis of casein micelles. How caseins interact between themselves and with colloidal calcium phosphate? At what stage they are modified? How this process is influenced by (or influences) the cellular pathway of protein folding and assembly?

Although post-translational modifications and genetic polymorphisms were for a long time considered as the most potent factors capable of generating multiple protein products starting from a single gene, it is now obvious that alternative splicing is responsible for a considerable proportion of proteomic complexity in mammals. It is clear that this process must be intimately related to the great diversity and heterogeneity of caseins as well as to their evolution pathway. As frameshift mutations, which deeply change the nature of the message and/or lead to premature termination (linked to mRNA decay), such mechanisms, by promoting deletion or addition of protein domains through exon skipping or cryptic splice site usage, undoubtedly provide a real plasticity to gene information. With at least a total of 21 coding exons that can be, according to the species, constitutively included or skipped, the gene encoding α_{s1}-casein is one of the most impressive examples, in this regard.

Obviously, such a wide structural diversity is unlikely to be without consequences for the characteristics and the properties of casein micelles, particularly if one considers the possible unique function that seems to be played by α_{s1}-casein in the micelle assembly, transport, and secretion. In mammary epithelial cells of small ruminants, α_{s1}-casein appears to be a complex mixture of more or less internally deleted proteins. The occurrence of genetic polymorphisms disturbing the splicing machinery adds further to the complexity of the casein fraction. With up to 40 variants of α_{s1}-casein produced in the milk of a single goat, heterozygous A/F at the *CSN1S1* locus, the secretion pathway may be disturbed dramatically with an impact on milk composition and quality, including modifications in fat structure and composition, as well as in its susceptibility to lipolysis.

Notwithstanding, the growing number of casein genes displaying such complex patterns of splicing, thus increasing the coding capacity of genes, supports the notion that the extreme protein isoform diversity generated from a single gene can no longer be considered as an epiphenomenon. A parsimonious vision of this issue addresses the question of its biological meaning. Important new insights are expected in this field in the near future.

See also: **Milk Lipids**: Milk Fat Globule Membrane. **Milk Proteins**: Analytical Methods; Casein, Micellar Structure; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; Immunoglobulins; α-Lactalbumin; Lactoferrin; β-Lactoglobulin; Minor Proteins, Bovine Serum Albumin, Vitamin-binding Proteins; Nutritional Quality of Milk Proteins; Proteomics.

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Proteomics

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Introduction to Proteomics

Definition of 'Proteomics'

Proteomics is a term in the study of genetics that refers to all the proteins expressed by a genome; proteomics involves the identification of proteins and the determination of their role in physiological functions. It is a new science that focuses on the study of proteins: their roles, their structures, and their interactions. It involves a large-scale screening of proteins, their expression, and their modifications by using high-throughput approaches. The word 'proteome' is a combination of 'protein' and 'genome'. The proteome is the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system.

Proteomics is much more complicated than genomics, mostly because, whereas an organism's genome is more or less constant, the proteome differs from cell to cell. This is because distinct genes are expressed in distinct cell types, meaning that even the basic set of proteins that are produced in a cell needs to be determined.

'Proteomic analysis' (analytical protein chemistry), which corresponds to the characterization of proteins and their post-translational modifications, can be linked to 'expression proteomics' (differential display proteomics), which corresponds to the profiling of expressed proteins using quantitative methods.

Milk Proteomics

Milk from all species is dominated by the presence of just a few primary proteins. In bovine milk, these proteins are α_{s1}-casein, β-casein, κ-casein, α_{s2}-casein, β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), and bovine serum albumin (BSA), with relative abundances of approximately 30:30:10:12:10:4:1, respectively. Despite this, the milk proteome is still extremely complex. A great deal of this complexity is the consequence of abundant post-translational modifications (glycosylation, phosphorylation, proteolysis, protein interaction, and association) and the presence of numerous genetic variants of this limited list of proteins. An additional level of complexity arises from the many proteins of low abundance that require some form of enrichment to be visible against the background of abundant caseins and whey proteins.

In recent years, a number of articles have reported proteomic applications of high-resolution two-dimensional electrophoresis (2DE) of milk proteins from a limited number of species. Human and bovine milk dominate these articles, with other limited applications including analyses of goat, wallaby, and mouse milk.

The application of proteomic methodologies presents a unique opportunity for dairy protein science. The combination of high-resolution separation techniques and powerful yet versatile mass spectrometry (MS) analysis allows the acquisition of previously unattainable information. Although reported studies of milk proteomics are relatively limited, the area is growing at a rapid rate, with virtually all published studies in the area appearing over the past 5 years. In line with this increase in quantity, the studies completed are also becoming more sophisticated. Post-translational modification analysis and differential expression analysis are key areas within proteomics with great potential for dairy applications.

It is likely that 2DE techniques will continue to dominate proteomic analyses of milk for some time. With its high resolution, 2DE allows the separation of protein isoforms containing the slightest of differences, a key in the comprehensive analysis of individual milk samples. Pre-fractionation procedures, multiple separation techniques, and intelligent MS strategies are the keys to successful milk proteomics.

Two proteomic strategies for the analysis of global proteins have appeared in the last 10 years: 'gel-based' proteomics, the separation of proteins using 2DE followed by an MS identification of the proteins/spots obtained; and 'gel-free' proteomics, a first optional step, the separation of the proteins/peptides by liquid chromatography, followed by large-scale MS analysis of the proteins/peptides.

This article is focused on 'gel-based' proteomics (2DE coupled to MS), a mature and well-established technique, largely applied in biology and medicine, and a methodology that is becoming more and more attractive in food and agricultural sciences for studying the behavior of proteins in processed products.

Two-Dimensional Electrophoresis of Milk

Solubilization of Proteins

Milk and dairy products present a great advantage. They contain a large amount of protein; consequently, in most cases, there is no limitation of fresh material for a 2DE analysis. Nevertheless, it is essential to take care in selecting the buffer used to solubilize the proteins. For example, caseins are known to be highly hydrophobic and not easy to dissolve. The next paragraph focuses on 2DE-compatible compounds that are used to make the solubilization buffer.

A traditional solubilization buffer contains 7 or 9 mol l^{-1} urea, 0–2 mol l^{-1} thiourea (with a limitation of 9 mol l^{-1} urea + thiourea), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and 60 mmol l^{-1} dithiothreitol (DTT) as a reducing agent. For samples with DNA (with cells), 30 mmol l^{-1} spermine can be added to remove nucleic acids. Also, 0.05% (v/v) Triton X-100 can be added to improve protein solubility. The proposed solubilization buffer contains a saturated solution of urea and thiourea. These chemical compounds are neutral chaotropic agents, which means that they can help in the solubilization and denaturation of the proteins by removing non-covalent interactions (hydrogen and hydrophobic bonds). Urea is not completely stable in solution; it can be degraded to ammonium cyanate, especially at high temperatures. This cyanate can react with NH_2 (amino) groups of lysine and arginine and with cysteine, leading to isoelectric modifications of the proteins, which can be observed during isoelectric focusing (IEF). To prevent such artifacts, the urea buffers must not be heated above 35 °C.

CHAPS is a zwitterionic detergent that is able to cover hydrophobic parts of a protein, and is an excellent detergent for 2DE. It contains ionic charges, but the global net charge is zero. Because of the presence of charges, CHAPS has better solubilization power than nonionic detergents such as Triton X-100. As a complement to urea, CHAPS and Triton X-100 limit protein aggregation and aid in the extraction and solubilization of proteins.

DTT is used to solubilize proteins. It is a reducing agent, is able to break disulfide bonds within proteins (helps to unfold the tertiary structure), and can break disulfide bonds between different proteins, which are frequently observed after a heat treatment of milk proteins.

An aliquot of 100 μl of milk can be diluted (1/10, v/v) in 900 μl of solubilization buffer to obtain an approximately 3 mg ml^{-1} solution of protein, which is large enough for 2DE. Solubilization can be improved with gentle agitation at room temperature for a couple of hours. Before the first dimension of the 2DE, the protein solution should be clarified by ultracentrifugation (1 h at 100 000 ***g***) to remove insoluble material. The protein content of the solubilized samples can be estimated using a classical colorimetric method.

Isoelectric Focusing of Proteins: The First Dimension

Proteins are amphoteric molecules; they carry positive, negative, or zero net charge, depending on their amino acid composition. The net charge of a protein is the sum of all the negative and positive charges. The isoelectric point (pI) of a protein is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and are negatively charged at pH values above their pI. IEF is an electrophoretic separation method based on this specific biochemical characteristic of proteins.

Basically, the first dimension of the 2DE is achieved with a 'strip'. It is a dry gel that is formed by the polymerization of acrylamide monomers, linked by bisacrylamide with molecules of covalently linked immobilin. Immobilins are chemical components that are derived from acrylamide and have additional ionizable non-amphoteric functions. Immobilins of various p*K*a can create an immobilized pH gradient inside the acrylamide gel. Immobilin was developed by Righetti and Görg in the early 1990s and is now widely used in 2DE because the IEF gradient is very stable over time and in a high electric field, and shows good reproducibility and a large capacity for separation.

The strips of acrylamide gel are dried and cast on a plastic backing, 7–24 cm long. Prior to use, they are rehydrated in a solution containing a cocktail of carrier ampholytes to the corresponding pI range and with the correct amount of proteins in the solubilization buffer. In most cases, 50–250 μg of protein are sufficient for an analytical gel. The carrier ampholytes are amphoteric molecules with a high buffering capacity near their pI. Commercial carrier ampholyte mixtures, which comprise species with a pI spanning a specific pH range, help the proteins to move.

When an electric field is applied, the negatively charged molecules (proteins and ampholytes) move toward the anode and the positively charged molecules move toward the cathode. When the proteins are aligned according to their pI, the global net charge is zero and the protein is unable to move and is then focused. Focusing is achieved with a dedicated apparatus that is able to deliver up to 8000 or 10 000 V, but with a limitation in current intensity (50 μA maximum per strip) to reduce heat. The strips are usually first rehydrated without current for at least 5 h (passive rehydration), rehydrated with 50 V for 9 h (active rehydration), and then focused until 50–100 kV h^{-1}.

Equilibration of the Strips

The equilibration step is critical for 2DE. In this step, the strips are saturated with sodium dodecyl sulfate (SDS), an anionic detergent that can denature proteins and form a negatively charged protein–SDS complex. The amount of SDS bound to a protein is directly proportional to the mass of the protein. Thus, proteins that are completely covered by negative charges are separated on the basis of molecular mass.

The equilibration solution also contains buffer, with urea and glycerol. Equilibration of the strips is achieved in two steps: (1) with an equilibration solution containing DTT, to maintain a reducing environment; and (2) with an equilibration solution containing iodoacetamide, to alkylate reduced thiol groups, preventing their reoxidation during electrophoresis.

SDS-PAGE: The Second Dimension

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations, migration is determined not by the intrinsic electric charge of polypeptides but by their molecular weight. The SDS-denatured and reduced proteins are separated according to an apparent molecular weight, in comparison with a molecular weight marker. A linear relationship between the logarithm of the molecular weight and the distance of migration of the proteins can be used; it depends essentially on the percentage of polyacrylamide.

Equilibrated strips are embedded in 1% (w/v) low melting point agarose in Tris–glycine–SDS running buffer and with 0.01% bromophenol blue on the top of the second dimension acrylamide gel.

It is recommended that 1 or 2 W of current be applied during the first hour, followed by 15 mA per gel overnight, with the temperature regulated at 10 °C.

Gel Staining

When the bromophenol blue migration front reaches the bottom of the gel, the second dimension is finished and the acrylamide gel can be removed from the glass plates. The gel must firstly be immersed in a fixation solution containing acid (phosphoric acid or acetic acid) and alcohol (ethanol or methanol) as a function of the staining protocol selected. Different stains can be used, but with very different costs. Conventional 'visible' dyes are Coomassie blue, colloidal Coomassie blue, and silver nitrate, with quite different sensitivities: 50, 10, and 0.5 ng of detectable protein per spot, respectively. Commercially available fluorescent dyes such as Sypro Ruby, Flamingo, and Deep Purple have sensitivities of about 1 ng of detectable protein per spot. Fluorescent dyes have the advantage of a 2–4 log dynamic linear range but have the disadvantage of being more expensive. In comparison with fluorescent dyes, silver nitrate stain has a dynamic linear range of only 1.5 log, and is not recommended for a gel comparison study.

Image Analysis

Stained gels are scanned on a 'visible' or 'fluorescent' scanner according to the staining protocol selected. The image can then be imported to specific software to be analyzed and compared. For a comparison study, at least three repetitions of the same sample should be run; many migration artifacts can occur during 2DE and, to reduce such variability, a mean of several gels is essential. Software such as Image Master, Progenesis, PDQuest, and SameSpots can be used to detect spots and to compare the spot intensity between samples. Spots of interest, that is, spots specific to a sample or spots overexpressed under a condition/treatment, can be selected for further MS analysis.

Protein Identification by Mass Spectrometry

Preparative Gel

To identify the proteins within the spots of interest (according to the image analysis), a gel with a greater amount of protein, at least 200–800 μg, is prepared. In this case, the IEF step must be performed until at least 100 kV h^{-1}. The other steps of the 2DE are very similar to the previously described protocol. Colloidal Coomassie blue or fluorescent dyes are recommended for the staining of the preparative gel, because they have good compatibility with MS. In contrast, silver nitrate will give poor results. It should be noted that a specific spot picker robot, able to work with fluorescence, is essential when working with fluorescent dyes.

Stained spots are removed from the gel and digested with trypsin, and the solution of protein fragments is analyzed using MS.

MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS is the fastest and cheapest method of protein identification. Single protein species can be identified by peptide mass fingerprinting using this technology.

The proteins are first 'in-gel digested' with a highly specific endoproteinase such as trypsin, and the resulting peptides are extracted, mixed with a matrix, spotted on a target, and analyzed by MS. Peptides with different molecular weights (m/z ratio) are measured accurately by the mass spectrometer (accurate to at least 0.01 Da). A list of these peptide masses is produced to give a 'fingerprint', which is unique to each protein. Identification of a particular protein can then be achieved by submitting the peptide list to a protein-searching database. A probability is obtained and the protein identification is linked with a significant score.

Analysis of High-Abundance Proteins: An Example of Milk Protein Separation by 2DE and Identification by MALDI-TOF MS

The presence of numerous genetic variants and post-translational modifications makes the milk proteome extremely complex. Post-translational modifications, such as glycosylation, phosphorylation, disulfide bond formation, and proteolysis, have the potential to create a large number of different protein variants from a single gene product.

A typical 2DE gel of milk proteins is shown in **Figure 1**. Each spot on the gel represents a single protein isoform in milk. The protein isoforms are clearly very well resolved from each other, as they migrate to different positions based on their size and pI, and are subsequently visualized using a protein-specific stain (Coomassie blue). The resolution and the information obtained are much greater than would be achievable with a standard one-dimensional gel electrophoresis, as observed on the right-hand side of the gel.

This 2DE gel was prepared with 400 μg of milk proteins, using an 18 cm wide pI range (4−7) for the first dimension and a 20 cm large gel with 12% acrylamide for the second dimension. All the major milk proteins are presented on this 2D map. Spots for several caseins (α_{S1}-, α_{S2}-, β-, and κ-caseins), β-Lg, α-Lac, and BSA are observed.

Analysis of Low-Abundance Proteins

The analysis of low-abundance proteins from sub-cellular compartments is becoming increasingly common in cellular proteomics. Analysis of individual compartments not only provides information on protein localization, but also allows detection of protein populations otherwise not detectable in whole-cell proteomes.

The detection and identification of low-abundance proteins in milk is particularly problematic. Studies involving simple one-step preparations with positive matrix factorization (PMF) analysis often fail to detect and identify large proportions of this important group of milk proteins.

Precipitation of casein at pH 4.6, ultracentrifugation, and immunoabsorption can be used to remove most of the major milk proteins (caseins, β-Lg, α-Lac, and BSA). Analogous to this approach is the analysis of the proteins in the milk fat globule membrane (MFGM).

The MFGM proteins form a unique sub-category of milk proteins (approximately 2–4% of the total protein in human milk), the content of which is still largely

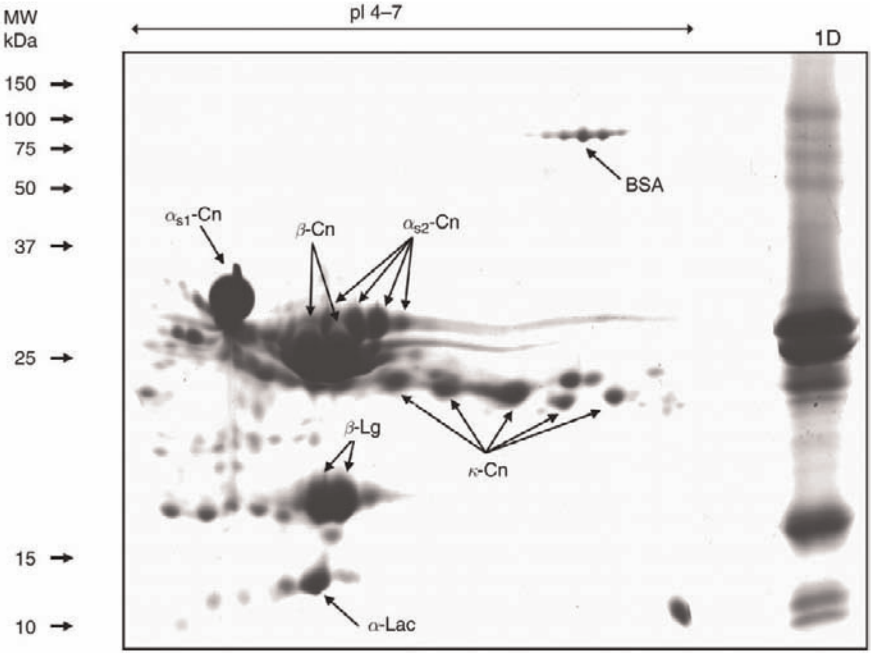

Figure 1 Two-dimensional electrophoresis map of proteins in bovine milk separated using an 18 cm pH 4−7 pI range for the first dimension and a 12% acrylamide gel for the second dimension. The most abundant spots as indicated by arrows were submitted for MS identification by MALDI-TOF peptide mass fingerprinting. BSA, bovine serum albumin; α_{s1}-Cn, α_{s1}-casein; α_{s2}-Cn, α_{s2}-casein; β-Cn, β-casein; κ-Cn, κ-casein; α-Lac, α-lactalbumin; β-Lg, β-lactoglobulin.

unknown. Many of the MFGM proteins are difficult to solubilize because they are highly hydrophobic, posing problems for their application to 2D-PAGE. The most productive strategies make use of a two-step procedure, with the first step using a detergent/reducing agent solution and the second step using an organic solvent extraction. Such strategies not only improve MFGM protein recovery, but also prepare the sample for optimum 2D-PAGE analysis. This strategy enhances the availability of MFGM proteins and reduces the amounts of several overabundant proteins, including the vast majority of caseins, allowing for more efficient detection of the MFGM proteins.

Post-translational Modification Analysis

Milk proteins are characterized by great heterogeneity and the presence of several molecular forms. These proteins are excellent examples of the complex heterogeneity that post-translational modifications can confer on proteins. They display significant levels of phosphorylation and glycosylation. Several post-translational modifications of milk proteins are well known: phosphorylation on serine residues of α_{S1}-casein (9 potential phosphorylation sites), α_{S2}-casein (12 potential phosphorylation sites), β-casein (5 potential phosphorylation sites), and κ-casein (2 potential phosphorylation sites); and six potential O-linked glycosylations on κ-casein.

These post-translational modifications induce phospho- and glycovariants, which are usually discernible on 2D-PAGE with noticeable pI and/or molecular weight modifications (**Figure 1**).

Conclusion

This article highlights proteomics as becoming a powerful tool for the characterization of milk and dairy products. The application of 2DE has proven to be of great use and, with the advent of MS techniques, new challenges have emerged. A merely descriptive view of milk proteins, aiming to provide information exclusively on their compositional complexity, has been replaced by a more complete view, which aims to also identify the proteins and to characterize them in terms of their structure, localization, modifications, interactions, biological activity, and functionality. Specific detection techniques that open new perspectives for the resolution of such research objectives are being developed. The application of proteomic methodologies presents a unique opportunity for dairy protein science. The combination of high-resolution separation techniques and powerful, yet versatile, MS analysis allows the acquisition of previously unattainable information. In the case of milk, the full characterization of milk from different species may reinforce the development of methodologies dedicated to the detection of milk adulteration. Techniques regarding protein polymorphism and post-translational modifications are being improved, which will facilitate the comprehension of structure–function relationships of caseins or whey proteins. Additionally, as the identification of minor milk proteins becomes an easier task, their potential biological roles and health implications may be postulated.

See also: **Analytical Methods**: Electrophoresis; Mass Spectrometric Methods. **Mammary Gland, Milk Biosynthesis and Secretion**: Milk Protein. **Milk**: Bovine Milk. **Milk Proteins**: Analytical Methods; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; α-Lactalbumin; β-Lactoglobulin.

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MILK PROTEIN PRODUCTS

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Membrane-Based Fractionation
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Milk Protein Concentrate

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Introduction

Milk protein concentrates (MPCs) could be loosely described as protein-enriched forms of skim milk that are usually processed and marketed in spray-dried form. They fall outside of the traditional classification of preserved and dried milk products, with preference being given to their description as functional dairy ingredients. MPCs owe their origin to the technological innovation that accompanied the application of developments in ultrafiltration (UF) in dairy processing. UF facilitates selective concentration of larger molecular size constituents such as milk proteins and, hence, enables the typical protein in dry matter content of skim milk powder (~35%) to be enriched, with the additional aid of diafiltration, to in excess of 85% protein in dry matter – the upper protein constraints being dictated by retained lipids and colloidal calcium phosphate (**Figure 1**). Given the wide band of protein concentration possible (40–85% protein), the hyphenated double-digit figure accompanying MPC usually denotes its protein content, for example, MPC-85 denoting 85% protein in dry matter (**Table 1**). At such levels of protein enrichment, high-protein MPCs come within the proximate compositional range of other milk protein ingredients such as caseins and caseinates. The term milk protein isolate (MPI) is used increasingly to distinguish a particular subgroup of MPCs that contain greater than 90% protein.

The retention of whey proteins as well as the native micellar state of the caseins distinguishes MPCs from caseinates – the latter having been isolated selectively under isoelectric pH conditions that also dissolve casein-associated colloidal calcium phosphate. Resolubilization of acid-precipitated curd using a choice of alkali (NaOH being the most common) is required to give a salt (e.g., Na)-solubilized, functional form of caseinate for use in food formulation. Consequently, there is considerable interest in the functional comparisons between these two groups of milk protein ingredients and the extent to which they may substitute for one another in product formulations. For example, studies using model whipped topping systems suggest that high-protein MPCs give emulsion and foaming properties comparable with those provided by sodium caseinate.

Proprietary technologies other than UF are used frequently to enrich protein content in skim milk-based concentrates so that the resulting products may also be described as MPCs. An investigation by the United States Trade Commission in the early 1990s concluded that such practices at the lower end of protein enrichment (typically MPC-42) did give rise to some trade distortion through displacement of regular skim milk powder markets, but recognized that innovation in functionality was a key driver behind technological developments in the manufacture of MPCs with higher protein contents.

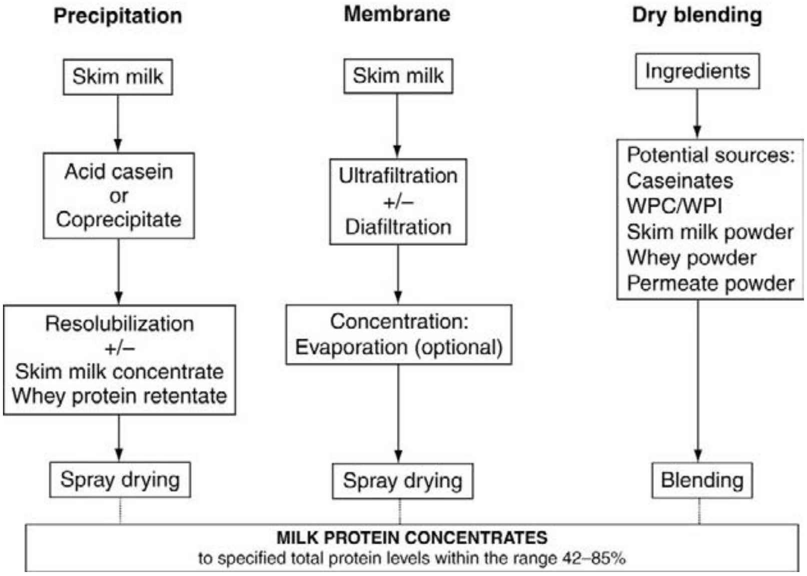
Precipitation
Skim milk
Acid casein
or
Coprecipitate
Resolubilization
+/–
Skim milk concentrate
Whey protein retentate
Spray drying
Membrane
Skim milk
Ultrafiltration
+/–
Diafiltration
Concentration:
Evaporation (optional)
Spray drying
Dry blending
Ingredients
Potential sources:
Caseinates
WPC/WPI
Skim milk powder
Whey powder
Permeate powder
Blending
MILK PROTEIN CONCENTRATES
to specified total protein levels within the range 42–85%

Figure 1 Processing options for the manufacture of milk protein concentrates (MPCs) based on membrane separation, protein precipitation-initiated processes, and ingredient dry blending. Note that only membrane separation is likely to retain the casein in its original micellar state. WPC, whey protein concentrate; WPI, whey protein isolate.

Table 1 Typical gross composition (%) of milk protein concentrates (MPCs) with protein concentrations ranging from 42 to 85%

Ingredient	*Moisture*	*Fat*	*Protein*	*Lactose*	*Ash*
MPC-40	3.5	1.0	42.0	46.0	7.5
MPC-70	4.2	1.4	70.0	16.2	8.2
MPC-75	5.0	1.5	75.0	10.9	7.6
MPC-80	3.9	1.8	80.0	4.1	7.4
MPC-85	4.9	1.6	85.0	1.0	7.1

Source: http://www.innovatewithdairy.com/Pages/MilkProteinConcentrateSpecSheet.aspx?Category=Ingredients&Filter=Milk%20protein%20concentrate

The antecedent in ingredient terms to MPCs would have been the so-called 'coprecipitates', that is, adapted casein coagulation processes that succeeded in coprecipitating whey proteins and caseins. These relatively crude processes relied on the use of added calcium chloride and/or heat treatment in order to entrap whey proteins during aggregation and precipitation. The resulting weaker curd was more difficult to recover and had poor functionality following subsequent drying. Although at the time they were regarded as nutritionally superior to caseinates due to the incorporated whey proteins, coprecipitates never gained significant market attention because of limited functionality. Concerted research efforts by Australian and New Zealand researchers during the 1970s led to some process refinements; however, market application never succeeded in getting beyond the use of coprecipitates in nutritionally fortified biscuits.

UF-Based Manufacture of MPC

The use of high-quality milk is emphasized for MPC manufacture given that relatively modest levels of heat treatments are used, particularly during the early processing stages (milk preheating, cream separation, skim milk pasteurization). Consequently, surviving microorganisms, spores, and any enzymes resulting from microbial activity will be retained by UF membranes and concentrated over the course of processing. UF processing at industrial level may be undertaken at either 10 or 50 °C, but the lower temperature is now generally preferred in order to limit microbial proliferation and enzymatic activity.

A typical manufacturing process for obtaining MPC involves UF of skim milk with or without the use of diafiltration. The resulting retentate, depending on the desired level of protein enrichment, is subjected to preheat treatment ideally in a steam infusion heater, evaporation in a one- or two-stage falling-film tubular evaporator, and spray-drying, preferably in a tall-form spray dryer, followed by 'after-drying' and cooling in an external fluid bed. Since the viscosity of the retentate increases as proteins are selectively enriched, the capacity of UF plants to maximize solid concentration is affected by a rapid decline in permeate flux across UF membranes; hence, additional thermal evaporation is necessary in order to increase retentate solids further so that the resulting spray-dried product has desired physical characteristics such as bulk density.

Effect of Processing Conditions on MPC Quality

Concentration and drying affect the quality of milk powders to varying degrees, mainly because of the exposure of highly concentrated constituents to the prevailing thermal conditions. Modern spray-drying approaches have evolved to minimize some negative effects through the use of multistage drying, and reduction of residence time in the primary drying zone. However, it is to be expected that the protein-enriched nature of MPC is likely to be even more challenged during drying given that the typical ionic environment provided by the soluble salts of milk has been depleted during membrane filtration. This may well be a factor associated with variability in the functionality of commercial MPCs on the market. A survey of MPCs from 10 different countries revealed wide variation in MPCs with respect to solubility, viscosity, foaming, and emulsification. A small number of high-protein MPCs gave emulsion and foaming properties in a model whipped-topping system comparable with those of sodium caseinate. Only four selected MPCs, with a protein content ranging from 82 to 86%, showed correlation between solubility and factors such as protein content, pH, and viscosity.

Properties of MPC-70 affected by spray dryer outlet temperature include moisture, water activity, bulk density, particle size of subsequently hydrated material, as well as rate of hydration. However, protein denaturation, foaming, and emulsion properties are unaffected by outlet temperature within the range of 65–90 °C. An increase in the inlet temperature (from 200 to 300 °C) causes an increase in the volume-weighted particle size *D*(4,3) and mean particle size *D*(0.5). The ease of dissociation of hydrated particles decreases significantly as the inlet temperature decreases. An increase of 4 °C in retentate heating temperature at 72 °C increases viscosity by 26% prior to spray drying MPC-80. A change to 76°C does not affect the volume-weighted particle size *D*(4,3) or mean particle size *D*(0.5), but results in increased surface-weighted particle size *D*(3,2) and decreased surface area. Milk powder insolubility is a particular challenge during spray drying because of thermal exposure of concentrated heat-labile components at the advanced stages of the process. MPC appears to be even more sensitive to heat at higher moisture contents than skim milk powder and the concomitant concentrations of lactose and minerals present also contribute to insolubility. Insoluble material consists of large particles (~100 μm) where the casein micelles are fused together by some form of protein–protein interactions. The amount of insoluble material in MPC powders increases with storage time at elevated temperatures and consists predominantly of α- and β-caseins. This material, formed largely by weak non-covalent (hydrophobic) protein–protein interactions, is dissociable under sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) conditions. Disulfide-linked protein aggregates (analyzable by two-dimensional (2D) SDS-PAGE) present in MPC powders consist of κ-casein, β-lactoglobulin, and some α_{s2}-casein, and may not always be sedimentable. While it is speculated that the insolubility of the MPC-85 is due to cross-linking of the proteins at the surface of the MPC-85 powder, further evidence is emerging to suggest that protein dissociation (particularly κ-casein) takes place from casein micelles at an advanced stage of MPC processing, and the subsequent aggregation of this associated protein along with β-lactoglobulin contributes to insolubility through the formation of a 'skin' on the surface of powder particles. However, other mechanisms, such as cross-linking of the proteins by hydrophobic and/or hydrogen bonding, are not ruled out. Lactosylation is also mentioned as a possible contributor, although it is not clear whether there is sufficient residual lactose present, particularly in high-protein MPCs, for this to happen. On the other hand, there is evidence to suggest that lactosylation gets underway in the early stages of milk processing.

Applications of MPCs

MPCs are being used increasingly as milk protein ingredients in food formulation, cheese, and health-related products.

Food Emulsions

The surface coverages of emulsions made with MPC-85 are approximately 10× greater than those prepared using sodium caseinate or whey protein concentrate although MPC-85 is less efficient as an emulsifier due to the more aggregated nature of its protein. However, emulsions made with the large casein aggregates found in MPC-85 and skim milk powder are more stable to creaming than those made with caseinate. It is believed that the protein aggregates in MPC may be too large to cause significant depletion flocculation, or alternatively, that they trigger a high degree of depletion flocculation and thus form very viscous emulsions, which will also reduce creaming. A comparison of emulsion properties in simple oil-in-water emulsions with those in the model coffee whitener formulation shows that the lower emulsifying ability of aggregated protein may be partially compensated by the addition of other ingredients.

Cheesemaking

Developments in UF technology in the early 1980s heralded new opportunities for pre-concentration of milk prior to cheesemaking. Greater productivity was anticipated since the classical separation duties associated with syneresis during cheesemaking could be supplanted by prior concentration using UF. Certain categories of cheeses, for example, cast and structured Feta, were readily adapted to the new technology. Greater problems, however, were encountered when adapting the traditional manufacturing processes of more established semi-hard cheese varieties. The formation of heavier milk coagulum during gelation and its subsequent handling demanded the construction of special handling equipment. The APV SiroCurd process was developed for the manufacture of Cheddar cheese from partially concentrated UF retentate. While the prospect of increased yield was an initial driving force behind commercialization of the technology, at the end of the day retained whey proteins gave rise to textural deficits and bitter flavors during ripening. As a result, the cheese manufacturing industry took a conservative view and continued to rely on traditional processes. In the meantime, UF milk retentates as they were initially known became firmly established in the market in the form of dried MPCs with a protein in dry matter content varying from 42 to 90%. Increasingly, these MPCs are finding their way back into cheesemaking for the purpose of protein standardization of cheese milk or as part of innovative processes for whey-less cheese manufacture.

Standardization of the protein content of cheese milk using MPC or similar protein-enriched sources has a beneficial effect on Cheddar cheese yield. An increase in milk protein from 3.3 to 4.0% improves milk fat and protein recovery in cheese, reduces cheese moisture, and increases the moisture-adjusted yield per 100 kg of milk, particularly when cheese milk protein is raised from 3.3 to 3.6% using MPC. Protein retention and cheese yield are also increased when MPC is used to fortify milk protein to 5.38% (Mozzarella) or 5.42% (Feta). Cheddar made from 4.56% protein milk and the Mozzarella and Feta show no defect in sensory characteristics when comparing fortified with control vats.

Addition of MPC also improves the yield of pizza cheese manufactured irrespective of whether culture-based fermentation or direct acidification is used. In the case of reduced-fat Cheddar cheese, improved recovery of total solids may double the yield and also contribute to less brothy and bitter scores. Lowering the setting pH of whole milk standardized with MPC-64 to pH 5.6–5.8 during the manufacture of low-moisture part-skim Mozzarella made by direct acidification improves meltability and produces a softer cheese.

Cheese functionality may also be modified by adapting MPC processing conditions, for example, partial depletion of β-casein using cold microfiltration of milk results in less firm cheese with a lower storage modulus and higher meltability and flow.

If early application of UF for pre-concentration of milk for cheesemaking was problematic, then it certainly would appear to have enthused researchers in the meantime to find solutions given the number of patents that are being filed steadily claiming innovation in the application of MPCs for whey-less cheese production. A whey-less process for production of natural Mozzarella cheese features the use of dry ingredients such as MPC and anhydrous milk fat in combination with whey protein concentrate, whey protein isolate, calcium caseinate, sodium caseinate, rennet casein, acid casein, and non-fat dry milk. White-brined cheeses with a protein content ranging from 12 to 17.0% but with similar total solids (approx. 40%) may be produced by direct recombination using MPC, skim milk powder, and anhydrous milk fat. Owing to the limited syneresis and the use of milk powders, considerable amounts of whey protein nitrogen (0.17–0.26%) and lactose (1.6–5.9%) were retained in the experimental cheese at the end of ripening. In addition, increasing protein content appears to hinder proteolysis.

The rehydration behavior of proteins prepared using typical MPC processes impacts on the quality of cheeses made subsequently. Calcium depletion by cation exchange during the preparation of MPC or MPI with >70% protein in dry matter enables cheese to be produced that is free from nuggets (thin protein gels of a different color from the cheese). The addition of at least one monovalent salt to MPI or MPC-70 achieves the same effect by enhancing solubility when dried and obviates the need for use of cation exchange treatment. Solubilization may also be improved by adjustment of the monovalent and divalent cation content of MPC during reconstitution.

Gel formation of MPCs containing 35–55% dry matter may be accelerated using defined high shear conditions so that the resulting gel structure is homogeneous and compact in order to achieve the final curd texture and consistency. The kinetics of rennet gel formation are readily favored when MPCs are reconstituted at high protein concentrations. Cast Feta cheese made from MPC-56 dispersions has more defined irregular structure characteristics and a harder texture compared with MPC-85 cheese, which possesses more void spaces, a sponge-like structure, and a very soft texture. Overall, satisfactory cast Feta cheese may be made from recombined MPC-56 and MPC-70, and also with MPC-85 to which lactose is added.

Heat treatment of recombined milk prepared from MPC results in a slower rate of increase in the storage modulus (G') of rennet-induced gels, a reduction in the gelation time, and a decrease in the yield force required to fracture gels. The extent of whey protein denaturation (as a result of heat treatment) is related to the decrease in the G' value of gels as well as the yield force. Rennet coagulation studies conducted on reconstituted non-fat dry milk solutions supplemented with MPC-82, prepared from milks subjected to either of two pasteurization conditions (73 and 85 °C, 17 s) at a protein concentration ranging from 4.65 to 6.25% by weight, showed that rennet clotting time was not significantly influenced by heat treatment, suggesting that whey protein denaturation (12 and 26%) in this instance is not a factor. There is a significant effect of retentate pH on rennet clotting time – samples with added MPC produced from pH 6 retentate have significantly higher levels of ionic calcium.

The total calcium present in MPC increases with increasing concentration of protein; however, the total calcium-to-protein ratio is lower in MPC-90 than in lower protein variants. The level of whey protein denaturation, the presence of κ-casein–whey protein aggregates in the supernatant after centrifugation, and the amount of caseins dissociated from the micelle increase as MPC protein concentration in the powder is raised. The total amount of casein macropeptide released is lower in samples from powders with a higher protein concentration than for MPC-56 or the skim milk control.

The gelation time of MPC dispersions (5% protein) is considerably lower and the gel modulus higher than those of reconstituted skim milk with similar protein concentration. When MPC dispersions are dialyzed against skim milk, a significant decrease in the gelation time and modulus occurs, with a complete loss of gelling functionality when MPC-90 is dispersed in water. This demonstrates that ionic equilibrium is central to the functionality of MPC.

Processed and Analogue Cheeses

A relaxation of the regulatory requirements governing minimum inclusion of natural cheese is accompanied by greater substitution with MPC in processed cheese formulations. Hence, research is currently addressing the relationships between processing conditions, particle size in MPC, emulsifying salt conditions, and final product characteristics in order to improve the understanding of the physicochemical relationships in the processed cheese systems. Emulsifying salts such as tetrasodium pyrophosphate (TSPP) induce gelation in reconstituted MPC dispersions when the added TSPP acts with calcium as a cross-linking agent between dispersed caseins and when the balance between (a reduced) electrostatic repulsion and (enhanced) attractive (hydrophobic) interactions becomes suitable for aggregation and eventual gelation of casein molecules.

A modified MPC, MPI, or micellar casein suitable for use as a complete or partial rennet casein replacer or extender in analogue Mozzarella and processed and imitation cheeses is the subject of an invention in which a high-protein retentate is prepared from pasteurized liquid skimmed milk, whole milk, MPC, or reconstituted milk feedstock and subjected to proteolytic breakdown using an enzyme, for example, chymosin, to modify the protein. The resultant cheeses may be used in frozen pizzas and other food products.

Spread-type processed cheese analogues with a fat in dry matter of ∼43% may be made by substituting up to 30% of rennet casein with MPC-85 powders. The inclusion of MPC increases firmness and sliceability, but decreases meltability. Increasing the NaCl concentration from 1.8 to 2.6% increases cheese meltability, but only slightly affects other physical properties.

Yogurt

MPC may be used as a replacer for skim milk concentrate, skim milk powder, and whey protein concentrate in low-fat set-type yogurt milks with comparable viscosity and syneresis index.

Ice Cream

Substituting the protein content of ice cream mix by up to 50% using MPC results in formulations with higher mix viscosity, larger amount of fat destabilization, narrower ice melting curves, and better shape retention. However, MPC does not result in significant modifications of the physical properties of ice cream when formulated on a constant protein basis with up to 50% replacement of protein supplied by skim milk powder. MPC usage in ice cream formulations may offer ice cream manufacturers an alternative source of milk solids non-fat, especially in mixes reduced in lactose or fat, where higher milk solids non-fat are needed to compensate for the reduced content of other milk constituents. There is also some suggestion that maltodextrin acts as an effective fat replacer in fat-free ice cream where MPC is used as a protein source.

Ice cream enriched with up to 60% more protein in its formulation may also be made by the addition of MPC without impairing texture. In fact, ice crystal size is favorably reduced at protein fortification levels up to 30%.

Spreads

Combinations of sodium caseinate and MPC-50 (3:1 ratio, respectively) perform more satisfactorily as emulsifying and stabilizing agents in model low-fat spreads provided that there is a minimum protein content of 11.7% in the aqueous phase. Viscosity control and protein solubility are judged to be more critical functional attributes than surface activity.

Coffee Cream and Whipping Cream

MPC addition may also be used to improve the keeping quality of coffee cream and whipping cream. For coffee cream, it is concluded that increasing milk protein content by 2–6 g kg^{-1} is sufficient to reduce the risk of cream separation. A complete replacement of carrageenan by MPC in whipping cream is not recommended, and it is recommended to use a combination of carrageenan and MPC in order to achieve optimal results.

Protein–Polysaccharide Interactions

The dilemma posed by the use of polysaccharides in dairy foods to increase viscosity and improve stability raises concerns about the potential risk of phase separation taking place. When milk protein ingredients (skim milk powder, MPC, whey protein isolate, and sodium caseinate) are combined with xanthan gum or κ-carrageenan in aqueous solutions, viscosity decreases markedly with increased shear rate. At low shear rates, the viscosity of the milk protein containing dispersions is higher than that of xanthan gum alone. Protein particles also appear to be more homogeneously suspended in solutions containing xanthan gum or κ-carrageenan.

Added pectin disrupts native intact casein micelles during acid-induced gelation of MPC, which is evident from a gradual change in shear modulus. On the other hand, a synergistic effect is obtained when pectin is added to dissociated casein at a later stage of acid gelation.

Nutritional and Therapeutic Products

'Clinically lactose-free formulae' for lactose-intolerant applications may be prepared using MPC. A formulation of an enzyme-treated easily digested MPC, named Unipro 50, containing 50% protein, 33% carbohydrate, 3% fat, minerals, and vitamins is aimed at therapeutic and sports applications. Solubility is improved by the addition of phosphatidylcholine and by granulation.

A patented immunization protocol that results in increased production of IgA in ruminant milk was accomplished by immunizing cows with a common pathogenic yeast, *Candida albicans*, resulting in the production of elevated levels of antigen-specific IgA antibodies in their milk. The resulting spray-dried MPC-80 reduces adherence of *C. albicans* cells in *in vitro* adherence assays. A special MPC prepared from the milk of hyperimmunized cows, previously shown to express anti-inflammatory and anti-arthritic activity in humans, has also been shown to be effective in the treatment of orthopedic disabilities of dogs.

A translucent milk-based drink may be obtained by passing a modified milk protein (selected from skim milk, MPC, or MPI) in the pH range 5.7–6.5 through a cation exchanger until the percentage transmission increases to at least 40%. The treated milk may be used as a base for a carbonated milk beverage that is nutritionally superior to conventional carbonated soft drinks. The beverages may contain vitamins, preservatives, thickeners, and flavor enhancers.

Selenium-enriched (1 mg kg^{-1}) MPC is superior to yeast Se in terms of Se bioavailability and capacity to suppress colonic oncogenesis. Suppression is believed to be a consequence of more effective enhancement of apoptotic deletion of DNA-damaged cells and the subsequent reduction in the frequency of K-ras mutations. Consequently, supplementation of Se via dairy products is a practical and effective approach to increasing human Se intake and preventing colorectal cancer.

See also: **Milk Protein Products**: Bioactive Peptides; Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Functional Properties of Milk Proteins; Membrane-Based Fractionation; Whey Protein Products.

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Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects

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Introduction

The industrial production of casein from bovine milk has been ongoing for most of the last century. Initially, the majority of bovine casein produced was used for technical (or non-food) applications, such as in glues. Since the 1960s, there has been a significant change from technical to edible uses of casein products, with caseins now regarded as high-esteem food ingredients used in a vast array of dairy products such as cheese analogues, yogurts, and powdered coffee creamers, while also being extensively used in nondairy food products such as beverages, bakery, and convenience foods, and in numerous nutritional, medical, and pharmaceutical applications. World production of casein products is currently estimated at 325 000 tonnes per annum; New Zealand and the European Union are the major producers of casein with Japan and the United States being the main end-user markets. This article describes the industrial processes used for production of casein ingredients, their compositional standards, and some regulatory aspects of the main casein products.

The Manufacture of Casein/Caseinate

A number of commercial casein-derived ingredients may be produced from bovine milk (acid casein, rennet casein, caseinates, and micellar casein). Good-quality raw milk (low bacterial count and low somatic cell count) is a prerequisite for producing high-quality edible casein ingredients. Bactofugation or microfiltration may be used to reduce both the bacterial load and spore count in milk, ensuring a casein product of high microbiological quality. For the production of casein products, the fat is removed from raw milk by centrifugal separation to yield skim milk which is then heat treated prior to manufacture of the casein. Casein is manufactured from skim milk to minimize possible flavor defects arising from deterioration of lipids in the dried casein products. Heat treatments can vary from thermization (65 °C for 15 s) to pasteurization (72 °C for 15 s) to ultraheat treatment of the milk (140 °C for up to 5 s).

Acid Casein

Acidification

When heat treated skim milk (pH ~6.6) is acidified, the calcium and inorganic phosphate are removed from the casein micelles, the net charge on the micelles decreases, and the micelles become less and less stable until the casein precipitates. Complete precipitation of the casein occurs at the isoelectric point, pH ~4.6. Precipitation can be achieved by acidification of the milk by one of the following processes:

1. Inoculation of milk with lactic acid-producing bacteria such as *Lactococcus lactis* subsp. *lactis* or *cremoris*. These bacteria, commonly known as starters, convert some of the lactose in the milk to lactic acid during the period of incubation (~16–18 h) at ~30 °C. This is the most commonly employed method of manufacture in New Zealand (**Figure 1**).
2. Chemical acidification which involves the direct addition of a dilute mineral acid (typically 1–2 mol l^{-1} HCl) or carbonic acid (CO_2 is added under pressure and combines with H_2O forming carbonic acid) into the milk at ~30 °C (**Figure 1**). Sulfuric acid is more commonly used in New Zealand.
3. Indirect acidification of skim milk by mixing milk (pH 6.6) at ~10 °C with sufficient cation exchange resin in the form of hydrogen to allow exchange of cations in the milk for the H^+, resulting in a milk acidified to pH ~2.0; separation of the acidified milk and resin and subsequent mixing of this acidified milk with untreated milk to achieve the desired pH. A number of alternative processes have also been patented in which skim milk is acidified by (1) addition of acidified whey or (2) electrodialysis. It is believed that these processes have relatively minor commercial significance.

Cooking/Acidulation

In the manufacture of acid casein, acidification of the milk is followed by heating of the mixture. Heating promotes agglomeration of the casein curd particles which subsequently synerese (shrink) to expel whey. At the same

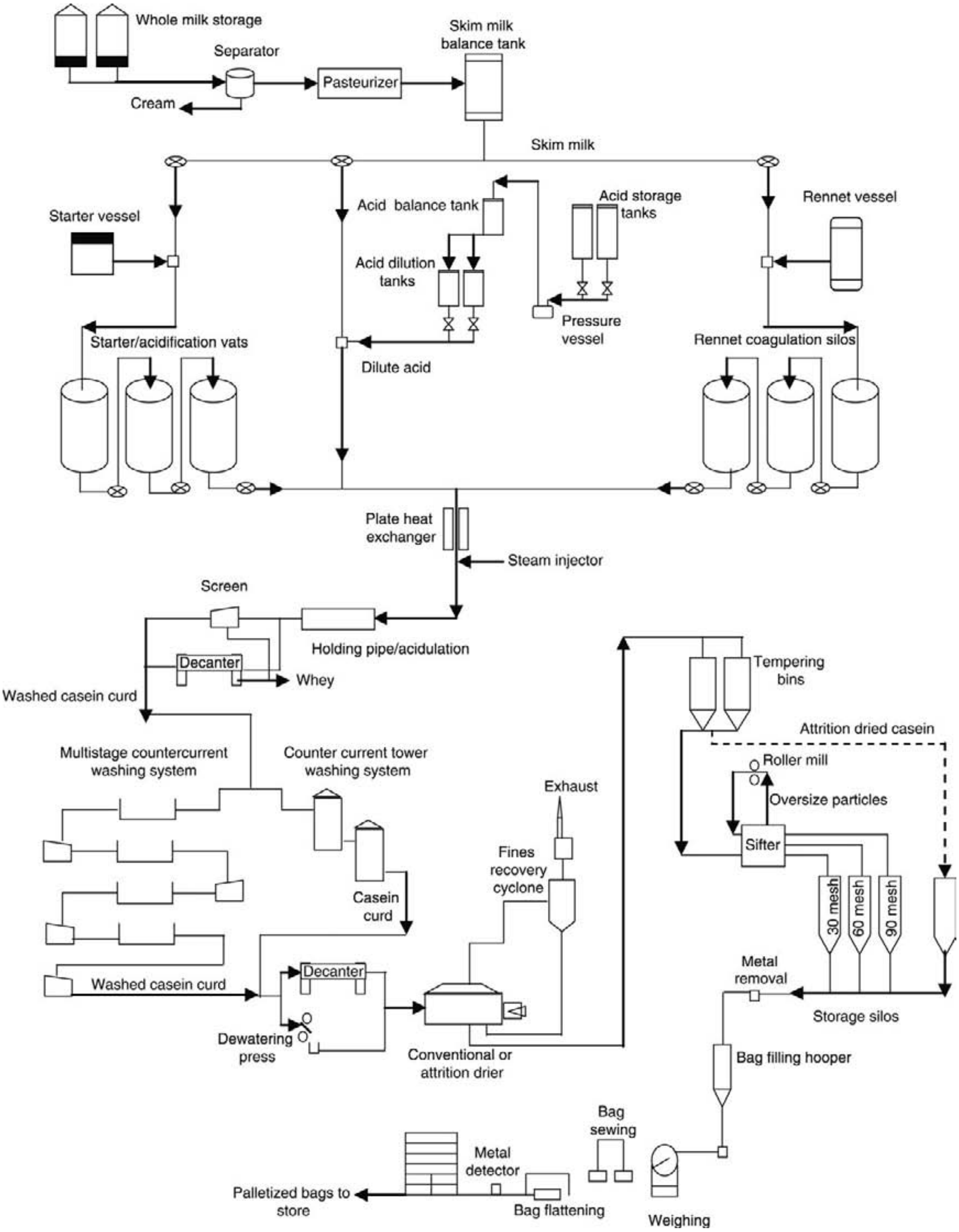
Whole milk storage
Separator
Cream
Pasteurizer
Skim milk balance tank
Skim milk
Starter vessel
Acid balance tank
Acid storage tanks
Rennet vessel
Acid dilution tanks
Pressure vessel
Starter/acidification vats
Dilute acid
Rennet coagulation silos
Plate heat exchanger
Steam injector
Screen
Holding pipe/acidulation
Decanter
Whey
Washed casein curd
Tempering bins
Attrition dried casein
Multistage countercurrent washing system
Counter current tower washing system
Roller mill
Oversize particles
Exhaust
Sifter
Fines recovery cyclone
Casein curd
30 mesh
60 mesh
90 mesh
Metal removal
Washed casein curd
Decanter
Storage silos
Dewatering press
Conventional or attrition drier
Bag filling hooper
Bag sewing
Metal detector
Palletized bags to store
Bag flattening
Weighing

Figure 1 Outline of the manufacturing steps involved in producing lactic acid casein, mineral acid casein, and rennet casein.

time, the curd becomes firmer and is able to withstand the mechanical processing that follows. Heating of the acidified milk is sometimes termed 'cooking' (as used in cheese manufacture) and may be carried out, usually in the temperature range 45–60 °C, by:

1. Direct injection of steam into the pipeline carrying the acidified milk.
2. Indirect heating by means of a heat exchanger.
3. A combination of both of the above – preheating through a heat exchanger, with steam injection to complete the heating process (**Figure 1**).

In each case, the cooked curd and whey are held in a 'cooking pipe' for a period of about 10–60 s before they emerge into an 'acidulation' vat. The curd and whey may remain here for a period varying from 30 s to about 15 min, during which time the curd is gently agitated in the whey until equilibrium (especially between the calcium in the curd and that in the whey) is attained. Alternative processes may employ a 'syneresis tube'; the cooked curd and whey are held in a large-diameter tube for a period of several minutes (also undergoing acidulation or equilibration).

Dewheying and Washing

Following acidulation, the curd is separated from the whey using vibratory, moving, or stationary inclined nylon or stainless-steel mesh screens, or polyester fabric screens in a cascade arrangement, or using mechanical devices such as a horizontal bowl centrifuge (decanter) or a roller press. A combination of screening and decanter dewheying can be used to reduce the hydraulic load on the decanter. After separation of the curd from the whey, the curd is washed with potable water to remove residual whey constituents such as lactose, salts, and whey proteins. The temperature of the wash water may be varied, depending upon particular requirements. Washing systems used include multistage countercurrent systems and countercurrent tower washing systems with wash cycle temperatures varying in the range of 35–75 °C (**Figure 1**). A gradient of purified wash water is normally used where the curd is washed in a counterflow to the direction of the curd, with the purest curd meeting the cleanest water. Sufficient washing of the curd is normally achieved using minimum volumes of wash water.

Dewatering

After washing, the curd is mechanically 'dewatered' to minimize the quantity of water to be removed during the subsequent drying. As drying is a relatively expensive operation, it is obviously worthwhile to remove as much water as possible from the curd before transferring the casein to the drier. The texture of the curd is affected by temperature; as the temperature of the wash water is increased, the curd releases more water during dewatering but becomes tougher and more plastic and is consequently harder to break up and dry. Therefore, it is necessary to regulate carefully the temperature at the end of washing to optimize the conflicting requirements of minimum water content and maximum friability of the curd. Equipment for dewatering casein curd consists of roller or belt presses or decanting centrifuges. Roller press, which has been in use for many years, is designed to reduce the moisture content of the curd to ~55%. Belt press will also reduce the moisture content to a similar level. Solid bowl decanters or screen bowl centrifuges are capable of reducing the moisture content of acid casein curd to ~45–55%.

Drying

In accordance with internationally recognized compositional standards for edible casein products, the casein curd is dried to a final moisture content of <12%. Drying of casein curd is commonly carried out using a horizontal vibrating fluid-bed drier. This drier has two or more perforated stainless steel decks (**Figure 1**). The combined effect of vibration of the decks and the flow of hot air (typical temperature range 75–115 °C) up through the holes in the decks causes the casein curd to become fluidized and materially helps in the removal of moisture from the particles. Most of the water is removed during the early stages of drying of the casein, as it is evaporated from the surface of the particle. The later stages of drying require the transfer of moisture from the centre to the surface of the particles, and this is a much slower process. Pneumatic-conveying ring driers and attrition driers are also widely used for drying casein. A pneumatic ring drier consists of a large stainless-steel ring-shaped duct through which high velocity, heated air, and moist, disintegrated casein curd are circulated continuously prior to being classified into dry and moist particles according to density differences using centrifugal forces; the dry casein particles are then collected and removed from the drier while the semidry casein is returned to the duct for further drying. The dry casein particles are then conventionally milled. Attrition drying involves grinding and drying in a single operation; the drier consist of a fast-revolving multichambered rotor and a stator with a serrated surface. Turbulence, vortices, and cavitation effects in the drier result in highly efficient grinding that pulverizes the casein curd into very small particles which are simultaneously dried in a hot air stream that flows through the drier with the curd to produce a dry product with an overall average particle size of ~100 μm (similar to spray dried casein). Attrition driers also employ internal classifiers, which grade the casein particles based on size.

Cooling, Tempering, Milling, Sifting, Blending, and Packing

Casein produced using fluid-bed or ring driers is warm and soft and unsuitable for immediate grinding in some types of mills, such as the roller mills. Consequently, the casein may be cooled and then transferred to 'tempering' bins where equilibration of moisture can occur in and between all the particles during a period of 8–24 h; this is often followed by blending to achieve a uniform moisture content.

The casein may then be ground and sieved, using multideck, gyrating screens, into various particle sizes, usually <600 μm. Very fine casein (e.g., <150 μm) is generally produced using pin mills. Blending after sifting is often used to achieve uniform particle size of the product.

Table 1 Typical composition and properties of commercial casein and caseinate products

	Acid casein	*Rennet casein*	*Sodium caseinate (Spray dried)*	*Micellar casein (Spray dried)*
Composition	*Amount per 100 g*			
Moisture (g)	11.4	11.4	4.5	4
Fat (g)	1.3	0.8	1	<2.0
Protein (g) dry basis ($N \times 6.38$)	85.4	79.9	92	86
Lactose (g)	0.1	0.1	0.1	<1.0
Ash (g)	1.8	7.8	3.6	7.2
Sodium (g)	<0.1	<0.1	1.3	<0.1
Calcium (g)	0.1	3	0.1	2-2.5
	Amount per kg			
Copper (mg)	2	2	2	2
Lead (mg)	<1	<1	<1	<1
Iron (mg)	5–20	5	5–10	5–10
Physical properties				
Color	Creamy white	Creamy white	Creamy white	Creamy white
Flavor	Bland, clean	Bland, clean	Bland, clean	Bland, clean
Solubility in water	Insoluble	Insoluble	Soluble	Soluble

Dried acid casein is usually classified using 30-, 60-, 90-, and 120-mesh sieves which correspond to mesh openings of 595, 250, 175 and 125 μm, respectively.

Casein is then packed into 25 kg multiwall paper bags with plastic liners and stored. A typical composition of acid casein is shown in **Table 1**.

Rennet Casein

Clotting of Skim Milk

In the manufacture of rennet casein, heat-treated skim milk at its natural pH and at ~30 °C (or lower) is mixed with a proteolytic enzyme preparation such as calf rennet or, increasingly, genetically engineered chymosin or rennet substitutes such as microbial (aspartic) proteinases in the approximate ratio (by volume) of 1:7500 and held for 20–40 min to induce clotting of the casein. If a lower temperature setting is used, renneting time must be correspondingly increased. It is also possible to reduce the quantity of rennet added under these conditions and consequently allow a longer time for the renneting action to occur. Nevertheless, renneting results in cleavage of the κ-casein on the surface of the micelles to form para-κ-casein and soluble glycomacropeptides; consequently, the modified casein micelles become susceptible to the calcium present in the serum phase of the milk and coagulate at temperatures above 20 °C.

Cooking

The usual technique for the cooking of rennet casein involves the injection of steam into a cooking line of coagulum pumped from a vat. However, the vat cooking technique (similar to that used in cheese manufacture) is also practiced in some countries. The cooking temperature employed in making rennet casein usually varies in the range of ~50–60 °C. Where indirect cooking of rennet casein is employed, a tubular heat exchanger may be used to cook the curd and whey to a temperature similar to that used in the ‘direct cook’ (steam injection) process. Other processing steps are similar to those described above for acid casein, and no acidulation step occurs after cooking of the rennet casein.

A typical composition of rennet casein is shown in **Table 1**. As no acid is added during the manufacture of rennet casein, the calcium and inorganic phosphate associated with the casein in the micelles are retained in the casein. Consequently, rennet casein has a much higher ash content (consisting of the oxides of calcium and phosphorus), and a correspondingly lower protein content, than acid casein (**Table 1**). Rennet casein is also devoid of caseinomacropeptide which is lost in the whey resulting in a reduction in yield by ~4%.

Caseinates

Acid casein is not soluble when redispersed in water but can be solubilized by the addition of alkali to produce caseinates. A number of caseinates are available which vary with respect to the method and the counter ion used for manufacture. All caseinates are substantially water soluble and are typically prepared as a solution of ~20% solids prior to spray drying. Roller-dried caseinates may be prepared from more concentrated solutions. It is also possible to prepare granular, partly soluble, or semidispersible forms of caseinate in which the casein and alkali have only been partly reacted. Conversion of casein to caseinate in the presence of a limited amount of water can also be achieved using extrusion techniques. Spray-dried sodium caseinate is the most common form of the water-soluble casein used in food applications. Sodium caseinate is prepared by mixing wet acid casein curd with water at 40 °C to a solid content of about 25% (or alternatively, dry acid casein in a slurry with water) prior to passing it through a colloid mill. NaOH (~2.5 mol l^{-1}) is mixed with the slurry to give a final pH of 6.6–6.8. The viscous slurry is then vigorously agitated and heated to ~75 °C in a series of vats to complete solubilization and then further heated to 95 °C in a plate heat exchanger. The pH of the solution is adjusted with NaOH, if necessary, to give a caseinate of the required pH; the viscosity of the solution is measured and controlled by addition of hot water, if necessary, ensuring efficient atomization, and the sodium caseinate solution is then spray dried (**Figure 2**). The dried powder dissolves completely in water to produce a viscous, sticky, straw-colored solution.

When Ca $(OH)_2$ is used as the base, calcium caseinate is produced, which gives a thin, opaque, white colloidal dispersion similar in appearance to milk, when dispersed in water. Other caseinates, such as those of potassium and ammonium, are similar in general properties to sodium caseinate. Magnesium caseinate has properties that are intermediate between those of sodium and calcium caseinates. However, relatively insignificant commercial quantities of these products are manufactured at the present time. A typical composition of sodium caseinate is shown in **Table 1**. On a moisture-free (dry) basis, it has a slightly lower protein content than the acid casein from which it is made, as a result of the sodium incorporated into it on reaction of the casein with sodium hydroxide (or other sodium alkali).

In the great majority of applications, in both edible and technical (non-food) uses, casein must first be made soluble before it can be employed in its final application. In the case of technical applications, most users dissolve the casein at their own factories. For edible applications, however, some users will themselves convert the dry casein to caseinate, whereas others tend to purchase the caseinate directly from the producer.

Micellar Casein

Micellar casein, generally referred to as phosphocaseinate, has compositional and physical characteristics similar to those of the native casein micelles in milk.

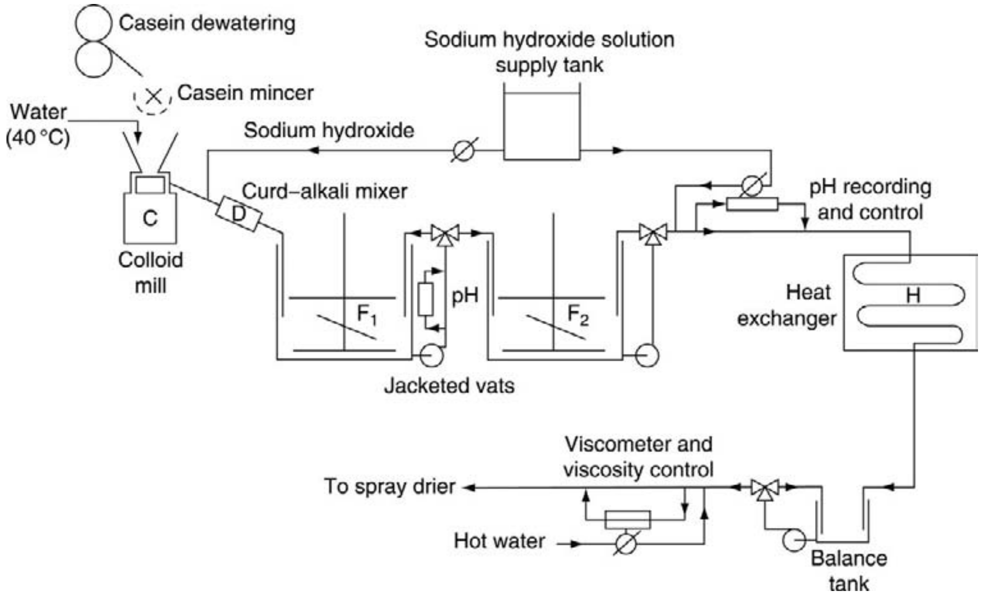

Figure 2 Suggested plant layout for dissolving casein in sodium caseinate manufacture. Dewatered casein curd is minced, mixed with water, and finely milled in a colloid mill (C). The curd–water slurry is then mixed (D) with dilute sodium hydroxide solution and transferred to the first of two jacketed dissolving vats (F_1) each equipped with an agitator. The sodium caseinate solution is subsequently pumped from the second dissolving vat (F_2) and extra alkali is added if necessary. The solution is heated by means of a tubular heat exchanger (H) and then pumped via a balance tank to the spray (or roller) drier for drying. The viscosity of the solution may be monitored and reduced, if necessary, by addition of hot water. Reproduced with permission from Southward CR (1985) Manufacture and applications of edible casein products. I. Manufacture and properties. *New Zealand Journal of Dairy Science and Technology* 20: 79–101.

In contrast to casein that requires the partial or full disruption of the casein micelle, micellar casein is manufactured by microfiltration at ~10 or 50 °C of heat-treated skimmed milk that has been standardized at pH 6.6 using a suitable membrane of pore size 0.1–1.0 μm that allows permeation of the serum proteins, soluble constituents, and low molecular weight nitrogenous compounds (peptides and free amino acids), whilst retaining the intact casein micelles. Other soluble permeable constituents are removed by ultrafiltration, which is often followed by diafiltration with water, and the retained solution (retentate) is spray dried. A typical composition of micellar casein is shown in **Table 1**.

Annual Production of Casein and Caseinate Products

The data in **Table 2** show the annual production of casein and caseinate products by the principal casein-manufacturing countries during the period 1994–2007. Major producers are New Zealand, France, and the Republic of Ireland.

New Zealand, like Australia and the Republic of Ireland, exports essentially all of its casein products, whereas the European countries engage in the import and export of casein products, some of which are used for domestic consumption.

Regulatory Aspects

Definition

From a regulatory and commercial perspective, casein is defined as a mixture of phosphoproteins obtained from cow's milk. The water-soluble salts of casein (produced by reacting acid casein with food-grade alkalis, neutralizing agents, enzymes, buffers, or sequestrants) are known as caseinates. Casein can be described as an off-white to cream colored, amorphous powder or granules without odor or taste, derived from the coagulum formed by treating skim milk with a food-grade acid (acid casein), enzyme (rennet casein), or other food-grade precipitating agent. It is very sparingly soluble in water and in nonpolar organic solvents, but is soluble in aqueous solutions of alkalis. It can be dissolved with some difficulty in acids. Thus, it can be considered amphoteric, forming salts with both acids and bases.

Compositional Standards, Specifications and Methods of Analysis of Casein Products

Standards for the composition, sensory and physical properties, and microbiological quality of casein products are shown in **Tables 3** and **4**. These standards have been issued by the European Union and the United States, and by international organizations such as the

Table 2 Annual production of casein and caseinates in selected countries (thousand t)

Country	*1994*	*1996*	*1998*	*2002*	*2004*	*2006*	*2007*
Australia[a]	4.6	6.2	9.0[b]	-[c]	-[c]	-[c]	-[c]
Denmark	11.9	12.7	-	-[c]	-[c]	-[c]	-[c]
France	26.4	35.4	38.3	37.6[d]	49.4[d]	36[d]	37.9[d]
Germany	8.4	12.4	12.9	-[c]	-[c]	34.3[e]	32.9[e]
Irish Republic	36	42.7	42	45[f]	52.1[f]	40[f]	47[f]
New Zealand[g]	79.4	79.2[h]	103.7[h]	109[i]	127[i]	148[i]	206[i]
Poland	3	2.2	6.9	3.4[j]	3.3[j]	0.1[j]	0.1[j]

[a] For 12 months ending 30 June.

[b] Data obtained from Australian Dairy Corporation Website (http://www.dairy.com.au/adc), 30 August 2000.

[c] Data not supplied.

[d] Data from Jennifer Huet, FILFrance.ALF. Dairy Association Française, personal communication, 18 March 2009.

[e] Data from Federal Bureau of Statistics, Federal Ministry for Agriculture, Central Market and Price Reporting Office, Association of Dairy Producers, Germany.

[f] Data obtained from the Irish Dairy Board website (http://www.idb.ie), 31 May 2009.

[g] For 12 months ending 31 May.

[h] Data from New Zealand Dairy Board Website (http://www.nzmilk.co.nz).

[i] Data obtained from the New Zealand Ministry of Agriculture and Forestry website (http://www.maf.govt.nz), 18 June 2009.

[j] Data from Andrzej Babuchowski (2009) Minister Councillor Representative of the Republic of Poland to the European Union, personal communication, 30 March 2009.

Unless otherwise indicated, data are from Richarts E (1997) National dairy markets. In: Wohlfarth M, Römer D, Wirges M, Osterberg S, and Sieger S (eds.) *ZMP Review – Dairy 1997: Germany – EU – World*, pp. 100, 101, 104, 105, 119, 129, 130. Bonn: Zentrale Markt- und Preisberichtstelle GmbH; Richarts E (1998) Länderspiegel. In: Römer D, Wohlfarth M, Wirges M, and Sieger S (eds.) *ZMP Bilanz – Milch 1998: Deutschland – EU – Weltmarkt*, pp. 164, 165, 168, 169, 183, 193, 194. Bonn: Zentrale Markt- und Preisberichtstelle GmbH; Richarts E (1999) National dairy markets. In: Wohlfarth M, Weiss D, and Kapahnke S (eds.) *ZMP Review – Dairy 1999; Germany – EU – World*, pp. 60, 101, 106, 120. Bonn: Zentrale Markt- und Preisberichtstelle GmbH; Richarts E (2000) Länderspiegel. In: Wohlfarth M, Weiss D, Wirges M, and Kapahnke D (eds.) *ZMP Marktbilanz – Milch 2000: Deutschland –EU–Weltmarket*, p. 149. Bonn: Zentrale Markt- und Preisberichtstelle GmbH; Anonymous (2000) Casein production. *Agra Europe – Preserved Milk*, No. 206, June, p. 7. London: Agra Europe (London), Ltd.

Table 3 The *Codex Alimentarius Standards* for the composition of edible casein products (Codex Stan 290-1995, Rev 1-2001)

Casein product	*Acid casein*	*Rennet casein*	*Caseinate*
Composition	*Amount per 100 g*		
Moisture (g)	12.0 max	12.0 max	8.0 max
Fat (g)	2.0 max	2.0 max	2.0 max
Protein (g) (nitrogen × 6.38), dry basis	90.0 min	84.0 min	88.0 min
Minimum content of casein in milk protein	95.0 min	95.0 min	95.0 min
Ash (g) (phosphorus fixed)	2.5 max	7.5 min	
Lactose (g)	1.0 max	1.0 max	1.0 max
Maximum pH			8.0
Free acid (ml 0.1 N NaOH)	0.27 max		
Physical properties			
Color	White to cream: free from lumps	White to cream: free from lumps	White to cream: free from lumps
Sediment	15.0 mg max in 10 g	22.5 mg max in 25 g	15 mg max in 25 g
Sensory properties			
Flavor and odor	Natural	Fresh odor	Bland, natural flavor and odor
Microbiological quality	*Amount per g*		
Standard plate count	30 000 max	30 000 max	10 000 max
Yeasts and molds	50 max	100 max	50 max
Thermophile count	5000 max	20000 max	5000 max
Staphylococci (β-hemolytic, coagulase positive)	Negative	Negative	Negative
	Amount per 0.1 g		
Coliforms	Negative	Negative	Negative
	Amount per 100 g		
Salmonella	*Negative*	Negative	*Negative*

Table 4 Composition, physical and sensory standards, and requirements for microbiological quality of edible grade caseins and caseinates

	Edible casein		*Edible caseinate*	
Casein product	*EU, extra (A) quality*[a]	*USA, extra grade*[b]	*EU, extra (A) quality*[a]	*USA, extra grade*[c]
Composition	*Amount per 100 g*			
Moisture (g)	12 max	10 max	6 max	12 max
Fat (g)	1.5 max	1.5 max	6.3 max	2.25 max
Protein (g) (nitrogen × 6.38), dry basis	95 min	95 max	92 min	84.0 min
Ash (g) (phosphorus fixed)	2.2 max	2.2 max	(fat + ash)	
Lactose (g)	0.2 max	1 max	0.20 max	2.0 max
	Amount per g			
Free acid (ml 0.1 N NaOH)	0.3 max	0.20 max		
Free alkali			None	None
	Amount per kg			
Copper (mg)	5 max	5 max	5 max	5 max
Lead (mg)	5 max	5 max	5 max	5 max
Iron (mg)	20 max	20 max	20 max	20 max
Physical properties				
Color		White to cream color: free from lumps	White to cream color: free from lumps	White to cream color: free from lumps
Sediment/scorched particles	15.0 mg max in 10 g	15 mg max in 25 g		22.5 mg max in 25 g
Sensory properties				
Flavor and odor	Natural	Bland, natural flavor and odor	Natural	Fresh odor
Microbiological quality	*Amount per g*			
Standard plate count	30 000 max	30 000 max	30 000 max	

(*Continued*)

Table 4 (Continued)

	Edible casein		*Edible caseinate*	
Casein product	*EU, extra (A) quality*[a]	*USA, extra grade*[b]	*EU, extra (A) quality*[a]	*USA, extra grade*[c]
Yeasts and molds	50 max	50 max	50 max	
Thermophile count	5000 max	5000 max	5000 max	
Staphylococci (β-hemolytic, coagulase positive)	Negative	Negative	Negative	
Coliforms	Negative	Negative	Negative	*Negative*
Salmonella	Negative	Negative	Negative	*Negative*

[a] EC: Council of the European Community (1983) Caseins and food grade caseinates. *Industrie Alimentari* 22(209): 768–770.
[b] USA: US Department of Agriculture (1968) US standards for grades of edible dry casein (acid). *Federal Register* 33(141): 10385.
[c] USA: National Academy of Sciences (1996) *Food Chemicals Codex* (Food and Nutrition Board, Institute of Medicine, National Academy of Sciences), pp. 92, 93, 155, 156 (as amended by the 1st supplement to the 4th edn., p. 115), 760, 761. Washington, DC: National Academy Press.

International Dairy Federation (IDF) and the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO). These organizations collaborated within the Codex Alimentarius Commission to produce the Codex Alimentarius internationally recognized standards and specifications for casein products (**Table 3**). In addition, most casein manufacturing and exporting companies have issued their own standards and specifications for casein products (**Table 4**), and many large users and buyers of casein products also have their own specifications. The majority of these standards and specifications are not published, but remain confidential to the buyers and sellers involved. Although the responsibility for the safety and suitability of casein products lies with the manufacturer, the code of hygienic practice for milk and milk products (CAC/RCP 57-2004) provides guidance to reach an appropriate level of public health protection for the product at all stages of production.

All standards and specifications must be related to standard methods of analysis. Official methods for the analysis of casein products are published by organizations such as the IDF and the Food Chemicals Codex, and by many individual countries' food regulatory authorities. Recommended methods of analysis and sampling of casein products are listed in Codex Standard 234-1999. In addition, nutritional evaluation

Table 5 Industrial safety properties of casein

Property	*Value/comment*
Chemical Abstracts Service registry number	9001-71-9
Boiling point/melting point	Dry casein does not melt or boil, but will char and decompose at sufficiently high temperature (e.g., >150 °C)
Reactivity with air	Nonreactive under normal conditions of storage (e.g., temperature 20-25 °C; relative humidity <70%)
Reactivity with water	Absorbs water and swells to about twice its dry volume. In this wet state, it can be degraded by bacteria and molds within a few days
Explosibility of dust	
Minimum ignition temperature	460 °C
Minimum ignition energy	60 mJ
'Lower limit of ignition'	45 g m^{-3}
Maximum rate of pressure rise	8500 kPa s^{-1}
Maximum explosion pressure	450 kPa
Density	
Bulk density of powder in air	0.5–0.6 g ml^{-1}
Water-free density of particles	1.36 g ml^{-1}
Health hazard information	
External (skin)	Very low risk. Not normally causing skin irritation, but has been known to produce itching and eczema on the skin, face, hands, and arms of persons sensitive to milk protein
Inhaled	Low risk. Not normally causing occupational respiratory disease but has been known to cause asthma in persons sensitive to milk protein

test procedures (for measurement of protein quality by means of rat bioassay and chemical methods) are published by the Association of Official Analytical Chemists (AOAC).

Health Aspects

The health aspects of casein and caseinates as food ingredients were reviewed in 1979 by the Federation of American Societies for Experimental Biology. It was concluded that there is no evidence to suspect a hazard from the use of casein or caseinates in food or packaging materials at current or expected future use levels. An assessment of caseinates as food additives has also been undertaken by the Codex Alimentarius Commission. That body has prescribed consumption of a food additive in terms of its acceptable daily intake (ADI). The ADI is defined as the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk (standard man = 60 kg). The ADI is expressed as milligrams of the additive per kilogram of body weight. The phrase "without appreciable health risk" is taken to mean "the practical certainty that injury will not result after a lifetime's exposure".

In 1983, the ADI for sodium caseinate was declared 'N.S.' (not specified), meaning that, in the opinion of the Joint FAO/WHO Expert Committee on Food Additives, the total daily intake of the substance, arising from its uses in accordance with good manufacturing practice, does not represent a hazard to health; for this reason, the establishment of an ADI is not deemed necessary.

Safety Aspects

Numerous standards (for instance, as Material Safety Data Sheets) have been issued for the safe handling of compounds and chemicals that are used industrially. Such safety aspects have also impacted casein because of its long history of use in technical, nonfood, uses, for instance in glues and adhesives, paper coatings, and plastics. A list of the properties of casein relevant in terms of its industrial safety is shown in **Table 5**.

See also: **Milk Proteins**: Casein, Micellar Structure; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation. **Milk Protein Products**: Functional Properties of Milk Proteins.

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Membrane-Based Fractionation

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Reverse Osmosis

Considerable pressures are required to reverse the transport phenomenon (osmosis) that prevails when water migrates across a semipermeable membrane in order to equilibrate the osmotic effect created by solutes present in the solution on the other side of barrier. The effect is demonstrated classically when water and sucrose solution are separated by a semipermeable membrane. Hence, whey with its relatively high content of lactose (~5.0%) was originally seen as an appropriate medium for the application of reverse osmosis (RO) technology as an economical means of dewatering.

In skim milk or whey, the osmotic pressure is around 0.7 MPa, a pressure that must be exceeded in RO before permeate (i.e., essentially water) will flow through the membrane. However, when milk or whey is concentrated by RO or evaporation to 25% total solids (commonly referred to as retentate), the osmotic pressure increases to 2.7–3.5 MPa. Thus, the osmotic effect of increasing lactose and mineral concentration in RO retentates limits both permeate flux and maximum concentration achievable. During the course of RO, component transport occurs by diffusion through the membrane, or essentially through the thin layer of the membrane which is semipermeable, and the porous remainder of the membrane is merely a support. The rate of transportation is proportional to the solubility of the component in the membrane and to its effective diffusivity. Factors diminishing the effective pressure difference during operation include the increasing concentration gradient near the membrane's surface as well as the likelihood of gel layer formation.

Nanofiltration

Like RO, from which it evolved, nanofiltration (NF) is also a pressure-driven membrane separation process which is capable of reversing the osmotic effects of liquids (**Figure 1**). In addition to dewatering/concentration, simultaneous transmission of ions represents a further bonus in certain processing applications, as NF membranes are also partially permeable to mineral ions and other low molecular weight constituents. NF membranes are usually fabricated as thin-film composites by forming, for example, a polyamide layer on a microporous polysulfone (PS) support to give surfaces rich in carboxylate groups.

At the time of its introduction to the dairy industry during the 1980s, a variety of terms were used to identify this membrane process, for example, Loose RO, Leaky RO, and a proprietary term Ultra-OSMOSIS®. NF is now the adopted nomenclature, as separation of solutes occurs in the nanometer (10^{-9} m) range. A size exclusion range of 10–100 Da suits the permeation of water, mineral ions, and low molecular weight nitrogenous compounds. However, some lactose is also lost.

NF membranes are highly selective to the permeation of monovalent ions (~55%), for example, Na^+ and K^+, but have a high rejection of cations (~90%) such as Ca^{2+} and Mg^{2+}. Ion removal is also susceptible to the Donnan effect, and this has been demonstrated by addition of citrate to increase the permeation of chloride in the case of acid casein whey.

Lactose losses of 1–2% demand the biological treatment of permeate before waste discharge. Ongoing efforts at improving the performance of NF membranes suggest that attempts at reducing lactose losses are likely to be at the expense of mineral permeability.

Ultrafiltration

Ultrafiltration (UF) is a membrane filtration process that separates components on the basis of molecular sieving. Macromolecular material such as protein (and fat if present) is readily recovered from solutions such as whey, while lower molecular weight material (lactose, minerals, other solutes, and water) permeate the membrane. UF operates at much lower pressures when compared to RO and NF, with transmembrane pressures of 70–170 kPa being typical.

The transition from earlier cellulose acetate membranes to polymeric membranes has played a significant role in providing the dairy industry with protein separation processes that are operationally robust and hygienic. A variety of polymers may be used, but the more commonly used membranes are constructed of either a PS or a polyethersulfone (PES) active layer deposited on a more porous PS support. Other polymers in use include polyvinylidene fluoride (PVDF) and

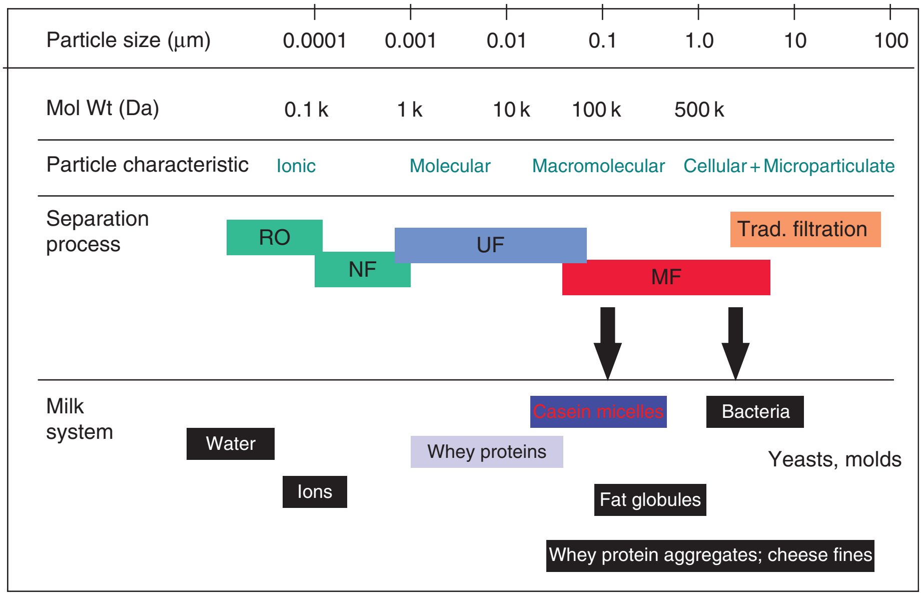
Particle size (μm)
0.0001
0.001
0.01
0.1
1.0
10
100
Mol Wt (Da)
0.1 k
1 k
10 k
100 k
500 k
Particle characteristic
Ionic
Molecular
Macromolecular
Cellular + Microparticulate
Separation process
RO
NF
UF
MF
Trad. filtration
Milk system
Water
Ions
Whey proteins
Casein micelles
Fat globules
Bacteria
Yeasts, molds
Whey protein aggregates; cheese fines

Figure 1 Overview of the membrane separation spectrum as applied to milk processing.

polyacrylonitrile (PAN). Modifications to the surface layer of membranes have also been suggested in order to improve performance with more difficult products (see 'Fouling'). Although identified more closely with microfiltration duties, membranes constructed of inorganic materials such as zirconium oxide and alumina are available in UF specification also.

Cross-Flow Microfiltration

Cross-flow microfiltration (cMF), as a pressure-driven membrane separation process, could be described as a more porous form of UF – instead of using a molecular weight cut-off criterion, cMF membranes are usually identified by their pore size cut-off in microns (μm). Fat, aggregated materials, bacteria, and somatic cells are usually retained during cMF, while protein passes through the more porous membrane. Fluid flow is usually tangential to the membrane surface, where its velocity is used to reduce deposit formation, hence the use of terms such as 'cross-flow MF' or 'tangential-flow MF'. The fouling tendency of organic cMF membranes when presented with protein solutions has limited their applications in dairy processes, although the UF-like behavior of a protein-fouled cMF membrane is occasionally exploited. Some of the more interesting, recent developments in the application of membrane processes have taken place, however, with cMF inorganic membranes.

Applications

Preconcentration/Partial Demineralization of Whey

Undoubtedly, the benefit of achieving a partial demineralization (40% max.) of whey during simultaneous preconcentration has been a major factor in the widespread adoption of NF technology, particularly when transporting over geographical distance, for instance, whey from regional cheese production units to large centralized processing plants. The opportunity to upgrade the quality of whey with a high ash content such as acid casein and fresh cheese (Quark, Cottage Cheese) is also appealing. The so-called 'white' whey released from cheese curd during the advanced stages of Cheddar manufacture contains a high level of added salt and may also be recovered using NF.

Permeate preconcentration to produce an enriched lactose concentrate may also be beneficial in some processes. Furthermore, the ion removal role of NF complements other dedicated demineralization processes such as electrodialysis (ED) and ion exchange (IE). The ultimate economic benefit of combining both processes may be specific to the individual circumstances of each case as it arises.

Whey Protein Concentrates and Isolates

Selective concentration of protein in whey by UF is used to produce whey protein concentrates (WPCs) with varying protein contents within the approximate range 20–80%. The upper limit is usually dictated by the residual fat content of the starting whey – fat too is preferentially concentrated along with protein. A commonly traded form of WPCs containing 35% protein (WPC-35) is usually aligned in gross compositional terms with skim milk powder. Thus, whey with its relatively low protein content (~0.7%) is initially concentrated by UF to yield a retentate containing 3–4% protein and ~9% dry matter. This retentate is concentrated further by evaporation before spray drying.

WPC with a higher protein content (e.g., 50–80%) can only be produced by the introduction of a diafiltration step in combination with UF; that is, water is introduced during UF to facilitate the removal of lactose and ionic constituents according as the retentate becomes more concentrated and permeate flux declines.

First-generation whey protein isolate (WPI) (protein content 90%+) products were produced by ion exchange treatment of whey and subsequent concentration of the eluate by UF. However, the later success of cMF using either a 0.1 or a 0.2 μm pore size ceramic membrane in removing the residual fat content of whey (*defatting of whey*) now enables the resulting WPCs prepared by UF to achieve a protein content of up to 90%. Either of the two approaches may be used: (1) separation of flocculated lipid material according to the 'thermocalcic process' by adjusting [Ca^{++}] to 0.03 mol l^{-1}, followed by a pH increase to 6.7–7.2 with heating to ~50°C; or more commonly, (b) separation of unflocculated lipid material by cMF of either unprocessed whey or whey protein retentates obtained following preconcentration by UF. Process optimization is aimed at minimizing protein retention while striving to attain maximum fat removal.

Such delipidation steps have the added benefit of reducing the microbial load in the resulting ingredients produced. This is further aided by the widely adopted 'cold filtration' processing of whey by UF – a technological advance that was greatly facilitated by the robustness, compactness, and cost effectiveness of spiral-wound membrane systems. Exacting microbiological specifications are expected when high protein WPCs and WPIs are incorporated into nutritional products and beverages.

Milk Protein Standardization

The efficiency of manufacturing processes for certain dairy products is improved by standardizing the protein content of the milk feed. Partial preconcentration of cheese milk using UF to increase milk protein content to 4.0–4.5% improves yield during Cheddar cheese manufacture. According to Codex Alimentarius standard Cx 207 for preserved milks, standardization of protein content to a minimum of 34 and 26% for skim milk powder and whole milk powder, respectively, is permissible using permeate produced by ultrafiltration of milk or other permitted ingredients, that is, where the natural milk protein level is in excess of the Codex minimum for milk powders. Conversely, should the naturally occurring protein level fall below the minimum, then the addition of milk protein retentate is permitted to raise its concentration.

Milk Protein Concentrate

Selective concentration of protein in skim milk by UF may be used to produce milk protein concentrates (MPCs) containing up to 80% protein, or more. A particular value of this approach is that the naturally occurring ratio of casein to whey protein (~80:20) is retained, and at the same time steps may be taken during processing to minimize changes to their native structure. The establishment of MPCs' unique functional and nutritional properties, for example, use in recombined dairy products, cheesemaking, and dietetic products, has expanded the markets for high-protein dairy ingredients, which were traditionally dominated by casein and caseinates. MPCs with protein contents of 90% or more are frequently described as milk protein isolates (MPI), and particular attention is given during UF processing to attain such a high degree of protein selectivity.

Native Phosphocasein

Casein in its native micellar form with a total protein content in excess of 80% may now be produced industrially by cMF. Technological developments based on ceramic membranes have been, particularly, to the fore in enabling selective concentration of micellar casein while permitting the permeation of whey proteins and lower molecular weight constituents. The resulting casein retentates may be concentrated further and dried to produce new functional protein ingredients. Also, the cMF 'whey' permeate arising from phosphocasein separation is of high quality and an ideal substrate for further advanced processing and fractionation options.

Skim Milk Powder With Enhanced Renneting Properties

To overcome the negative effect of thermal treatment on the cheesemaking properties of milk powders incurred during milk concentration and drying, a French patent for the manufacture of skim milk powder with superior renneting characteristics exploits the above principle of selective casein separation by cMF in combination with UF of the milk permeate arising from cMF so as to remove its whey protein content. The resulting protein-free permeate is recycled with the native phosphocasein-enriched retentate to reinstate the original skim composition, except for whey protein depletion. A high-quality

WPC is also obtained as a by-product (UF retentate stream) from the process.

Extending the Shelf Life of Retail Milk

The adoption by Tetra Laval of special hydrodynamic considerations – high cross-flow velocities and uniform trans-membrane pressure control (UTMP) – represented a major breakthrough in the successful application of ceramic cMF membranes for bacteria removal (Bactocatch®) from skim milk without the penalty of significant in-process fouling (**Figure 2**).

As a result, the shelf life of retail milk (the so-called liquid milk or market milk) may be increased from a typical 16 days to 42 days by a combination of cMF and thermal processes embodied in the Tetra Therm ESL™ system. It is claimed that the process replicates the sensory and chemical properties of pasteurized milk (72°C, 15 s) with the added benefit of achieving a significantly lower bacterial content. With bacteria reduction being in the order of 90–97% (1–2 log units) in normal pasteurized milk, the Tetra Therm ESL™ process improves this to 3–5 log units (i.e., 100–10 000 times better).

The process is based on dividing milk into its cream and skim milk phases so as to process each stream separately: skim milk is microfiltered using membranes (1.4 μm pore size or less) with high bacteria removal efficiency; that part of the skim milk which is retained by the MF membrane (retentate) is combined with the cream before heat treatment of the mixture at 120–130°C for 2–4 s. The final process steps, namely, mixing of all process streams, homogenization, and pasteurization, are performed in an enclosed system to minimize postpasteurization contamination.

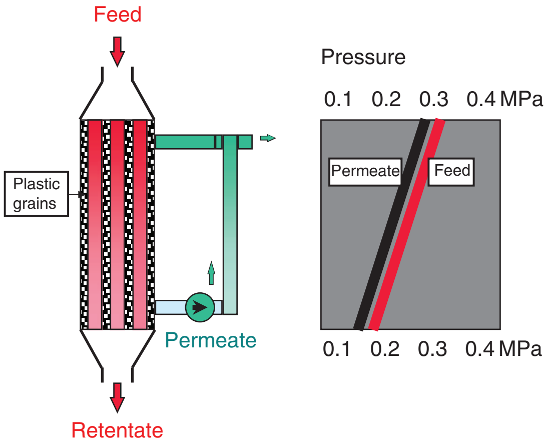

Figure 2 Tubular ceramic membrane module configured with permeate recirculation loop according to the principle of uniform trans-membrane pressure (UTMP) originally patented by Alfa Laval. Note the inclusion of plastic grains in the permeate channels. An alternative approach by APV for achieving a similar permeate pressure gradient is to surround the membrane elements with a contoured shroud within the module.

The claims for the Tetra Therm ESL™ system are based on milk with an initial bacterial contamination of 30 000 ml^{-1}, and may be improved further if the initial microbiological load in the raw milk is lower.

Designs

Membranes and Modules

The processing limitations (restricted temperature and pH tolerance) associated with cellulose acetate-based membranes originally used in the dairy industry were promptly surmounted through developments using polymeric materials. The evolution of different polymer types and their formation are reflected in the terminology now used to characterize various types of organic membranes.

Asymmetric structure

Asymmetric membranes are fabricated from the same polymer material throughout, except that the active separation layer is thin and relatively dense (0.1–0.25 μm) with defined pore size when compared to the porous (~100 μm) support. It is believed that rejection of different solutes is determined to a large extent by the pore size distribution of the skin layer, and that permeability is also influenced by the thickness of the skin layer rather than that of the whole membrane.

Skinless membranes

Skinless membranes are used mostly for microfiltration and consist of either isotropic or anisotropic microgels.

Composite membranes

Ultrathin films featuring the desired rejection properties are first formed and then laminated to a porous support membrane of high permeability and tensile strength. Frequently referred to as 'thin film composite' membranes, they are used mainly in RO and NF applications.

Organic membranes are usually assembled in modules of various configurations of defined filtration area, for example, tube, sheet, spiral-wound, and hollow fiber.

Tubular–organic

Tubular membranes are housed according to a shell-and-tube configuration. However, there is some variation in design detail between different manufacturers. For instance, a PCI-Memtech module may contain a bundle of 18 membrane tubes (12.5 mm diameter) – each supported by a perforated stainless-steel outer casing. The end cap design of a module permits parallel as well as serial and mixed flow modes of processed fluids. Other designs may not necessarily use casing supports for individual tubes within a bundle.

Tubular–inorganic

Ceramic microfiltration membranes produced by SCT (Société des Céramiques Techniques/Ceraver) consist of thin-walled channels of fine-grained ceramic (α-alumina) through which the product is fed. A typical element features 19 channels. Modules consisting of 1, 7, or 19 elements are mounted in parallel in stainless-steel housing. A surface layer of known pore size is supported by a coarser microporous support. In a typical industrial installation, two modules are assembled in series, forming a filter loop serviced by a single retentate circulation pump and one permeate circulation pump.

Metallic oxide surface layers (ZrO_2; TiO_2) on a carbon support are a key design feature of inorganic membranes (e.g., KERASEP® and CARBOSEP® by Novasep-Orelis, and INSIDE CéRAM® and Filtanium® by Tami-Industries). The KERASEP® range of multichannel ceramic membranes is constructed of monolithic Al_2O_3–TiO_2 material with internal diameter options of 6, 4.8, 3.5, and 2.2 mm in respect of units with number of channels 7, 8, 19, or 44; external diameter 25 mm; and length 1,178 mm.

Plate and frame

Plate-and-frame modules consist of a series of membrane disks mounted as a vertical stack or horizontally in a manner that is analogous to a plate heat exchanger configuration. Feed is pumped through very narrow channels that may be configured for parallel flow or as a combination of parallel and serial channels. Examples include the designs pioneered by DDS (*module* series 35...39) and Novasep-Orelis *Pleiade*® (UFP10, RP 2030; RP 2060; RP 2085).

A DDS module is usually divided into sections, in each of which the flow between pairs of membranes is in parallel. The sections are separated by a special membrane-support plate in which one hole is closed with a stop disc to reverse the direction of flow, giving serial flow between successive sections. In DDS module 38, the channel height is increased to 0.97 mm (from 0.72 mm).

Plate-and-frame designs were promoted, in particular, for concentration of milk to a high solids level during the preparation of 'pre-cheese', that is, milk retentate that was fermented and renneted before salting and packaging (e.g., Feta-type cheese), and coagulation and curd-handling (e.g., the so-called UF cheese or the UF versions of well-known cheese varieties) or for further concentration (scraped-surface evaporation) to produce cheese base (processed cheese-type applications). DDS module UF 37 enables whole milk retentates with a dry matter content of 48–49% to be obtained (compared to 38–40% for module 36).

Spiral-wound

A spiral-wound element may be obtained by wrapping two membrane sheets, separated by a supporting sheet and two mesh feed spacers, around a permeate collecting tube. A separate channel spacer allows permeate passing through the membrane to flow freely to the collection point at one end – achieved by sealing the assembled membrane along both sides and the other end. Channel spacers are also designed to promote turbulence, especially where low retentate velocities prevail. Anti-telescoping devices are fitted between the downstream ends of the membranes to limit the risk of membrane layer slippage into one another due to effects of fluid velocity.

Spiral-wound modules lend themselves to the design of very compact membrane separation systems, and thus contribute to reduced capital cost and installation requirements. Considerable progress over the years has been made in relation to the development of robust adhesives and improved sanitary features in module design. Wider spacers between membranes are also used to extend the range of UF applications to include concentration of more viscous products such as fermented milks, for example, fresh cheeses and cream cheeses. Well-established names of spiral-wound membranes in use by the dairy industry include, amongst others, Koch, Dow, and Novasep-Orelis – the latter now is offering their flat sheet *Pleiade*® membrane in spiral-wound configuration (PERSEP™). Koch's HFK-131 six-inch (refers to diameter) UF dairy spiral module (length 965 mm) is available with spacer thickness options of 0.7, 1.1, and 2.0 mm and respective membrane filtration areas of 19.5, 16.2, and 9.7 m^2. Dow FILMTEC® NF 45 series spiral-wound membranes figure prominently in whey nanofiltration applications.

Hollow fiber

Despite exhibiting some similarities, hollow-fiber membranes differ from their tubular counterparts in several respects. First, the membranes are narrower with diameters ranging from 0.5 to 2.7 mm. The active membrane surface is on the inside, whereas the outside of the fiber wall has a rougher structure and membrane support. The feed stream flows through the inside of these fibers; permeate is collected outside and removed at top of the tube. Back-flushing is possible by recirculating through the outer permeate connection to remove product deposits on the membrane surface.

Hollow-fiber modules contain bundles of between 45 and 3000 fiber-like membranes per cartridge. The fibers are oriented in parallel; all are embedded in a resin at their ends and enclosed in a permeate-collecting tube formed using epoxy.

Although efficient in terms of compact filtration area, this type of membrane has become less popular for dairy applications mainly because of hydraulic and fouling constraints associated with flow of viscous products through narrow-bore materials. Tami-Industries' introduction in

1999 of their Dahlia® series of ceramic membranes based on a 39 channel geometry with hydraulic diameter of 2.5 mm and filtration surface of 0.5 m^2 represents a close approximation of the hollow-fiber design in inorganic form.

Membrane Module Configuration

Most of the experimental evaluations of membrane applications are usually undertaken using either retentate recirculation (batch) or a feed-and-bleed (continuous) system in which a portion of the retentate with the desired solid concentration is bled from the plant at a rate that is consistent with the membrane's performance.

Industrial plants are usually configured to operate continuously on a multistage recycle basis (MSR) with limited recirculation (**Figure 3**). In this manner, the declining volume of the retentate due to concentration is handled in a separate stage where steps are taken to correct for the diminishing velocity and increasing viscosity.

Dynamic Membrane Systems

Dynamic membranes represent an emerging separation technology that may have potential uses in future dairy ingredient developments. Additional design features are incorporated that increase shear at the membrane surface, reduce fouling, and enhance permeate flux. Rotating disk filters may be configured with an impermeable disk rotating above a stationary membrane disk or with a stationary baffle next to a rotating membrane disk. Stationary membrane disk filters require less power input for given flow rates and rotational speeds, whereas rotating membrane disk filters produce a higher shear stress on the membrane surface which may reduce fouling. The PallSep™ (Pall Corporation) Vibrating Membrane Filter (VMF) utilizes vibrational energy generated by an AC motor that spins an eccentric mass (flat disc membrane pack) that is mounted near the edge of a seismic mass. When the rotational speed of the eccentric mass is increased, the filter stack oscillates in response to the seismic mass with a 180° phase lag. Microporous, UF, and NF membrane types may be employed. Another membrane vibration-based technology VSEP® (Vibratory Shear Enhanced Process) was created by New Logic Research of Emeryville, California, to eliminate membrane-blinding by vibrating its surface at an extremely high frequency.

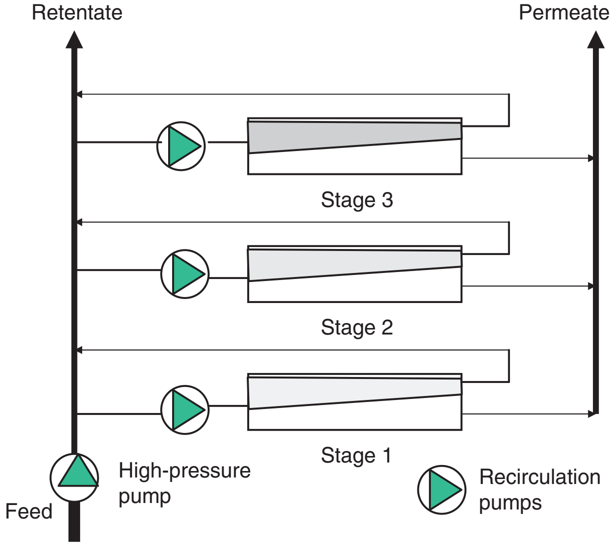

Figure 3 Outline of multistage membrane separation unit (three stages) showing separate recirculation loops.

Recent research suggests that such systems have the potential to selectively separate the two major whey protein fractions, namely, β-lactoglobulin and α-lactalbumin.

Low-Pressure, Immersed Membrane Technology

The dairy industry is a significant user of low-pressure, immersed membrane technology for waste-water treatment. Typically, modular cassettes containing MF/UF membrane fibers are immersed in the waste water being treated, where they rely on slight suction to the end of the fibers to draw water through their microscopic pores. With a nominal size of only 0.04 mm, the membranes prevent suspended solids, pathogens, and viruses from passing through. Automatically controlled cyclic aeration and intermittent back-pulsing are among the measures used to maintain the membrane fibers clean and operate at optimum efficiency. Conventional higher-pressure membrane filtration systems in MF or UF may also be applied.

Other Emerging Membrane Separation Systems

Although presently not widely adopted by the dairy industry, new membrane developments offer potential for advanced applications such as separation of target biomolecules. Among these are the use of charged UF membranes, UF in the presence of an electric field (electro-ultrafiltration), and membrane chromatography.

Membrane Performance

A typical membrane performance is characterized by a relatively rapid initial flux rate which drops off progressively over time – the rate of decline being dictated by the particular process (RO vs. NF vs. UF vs. cMF), applied pressures, membrane characteristics, module configuration, product characteristics, and the general state of the membrane following previous use and cleaning.

The permeate flux of water (expressed as $\mathrm{l\,m^{-2}\,h^{-1}}$) through a membrane is proportional to the applied transmembrane pressure (TMP) as described by D'Arcy's law:

$$\mathrm{J} = \frac{\mathrm{TMP}}{\mu_{\mathrm{p}} . R_{\mathrm{m}}}$$

where R_{m} is the membrane hydraulic resistance, μ_ρ is permeate viscosity, and TMP is the pressure drop between the retentate and permeate sides of a membrane at a particular point according to

$$\mathrm{TMP} = \frac{P_1 + P_2}{2} - P_3$$

where P_1, P_2, and P_3 are feed inlet, feed outlet, and permeate outlet pressures, respectively.

UF Processing Temperature

In general, most of the membrane separation processes are performed at temperatures in the range 40–50 °C. However, there has been a move in more recent years to perform whey UF under cold (<20 °C) conditions. The benefits of doing so include improved microbiological specifications through better control of thermoduric and spore-forming bacteria, and less alteration to protein structure. Extra filtration surface is required to compensate for reduced flux performance at lower operating temperatures but usually without undue cost penalty, because of technological advances especially in the mass production of spiral-wound membranes.

Fouling

The decline in permeate flux with time (or increase in transmembrane pressure in a constant-flux system) experienced in all pressure-driven membrane processes is attributed to product fouling. In fact, fouling would appear to be triggered by concentration polarization which occurs initially on start-up when a concentration gradient of retained components mediates the subsequent accumulation/concentration on the membrane surface (**Figure 4**). Generally, protein deposition and mineral (calcium phosphate) precipitation are major fouling agents during membrane processing of dairy products. Other product constituents such as lipid, or fat material, and peptides may contribute to a lesser extent.

Product fouling (R_{f}) adds another layer of resistance to that of the membrane (R_{m}), and the effectiveness of applied pressure is diminished by the increasing osmotic effects ($\Delta\Pi$) as macromolecular solutes become more concentrated. Hence, the following adaptation to D'Arcy's Law:

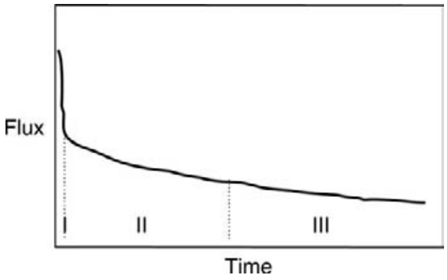

Figure 4 Conceptual diagram outlining fouling stages during UF at constant pressure: I concentration polarization; II membrane fouling; III multilayer formation via further particle deposition.

$$\mathrm{J} = \frac{\mathrm{TMP} - \Delta \prod}{\mu_{\mathrm{p}}(R_{\mathrm{m}} + R_{\mathrm{f}})}$$

In UF and RO, the general consensus is that a surface layer (also called dynamic membrane, gel layer, filter cake) forms on the membrane surface and dominates the subsequent behavior of the membrane. In cMF, both pore plugging and surface layer formation occur even with proteins considerably smaller than the pores. Indeed with protein-containing solutions such as milk or whey, the range of pore sizes where plugging occurs gets extended beyond that predicted from steric effects and severe pore plugging will eventually cause the formation of a surface layer. It is also clear that, once a surface layer has formed on a cMF membrane, its selectivity is dominated by the surface regardless of the original pore size of the membrane.

Since R_{f} is due to the combined resistance of various forms of fouling taking place, namely, adsorption (R_{a}), pore blocking (R_{p}), precipitation/gel layer formation (R_{g}), and concentration polarization (R_{cp}), a predominance of such factors may override performance as follows:

$$\mathrm{J} = \frac{\mathrm{TMP}}{R_{\mathrm{m}} + R_{\mathrm{a}} + R_{\mathrm{p}} + R_{\mathrm{g}} + R_{\mathrm{cp}}}$$

Concentration Polarization

Product concentration is more intense along the interface of a membrane as the driving pressure on the product side is opposed by the membrane's selectivity and the behavior of the rejected solutes. Concentration polarization is defined as an increase in concentration of a component in the boundary layer of the membrane as a result of its rejection. The phenomenon is characterized by a drop off in permeate flux through a membrane to a constant value irrespective of increasing TMP during RO or UF. Such fouling may become irreversible should the concentration of rejected component(s) increase to the point that deposition occurs on the membrane.

A number of models are used to describe concentration polarization. The 'film model' is considered to provide a reasonably good prediction of UF performance during protein processing. Here it is assumed that the concentration boundary layer resides within a thin laminar film at the membrane surface and that all mass transfer takes place by diffusion perpendicular to it. However, it does not apply that readily to cMF because small particles rejected by the membrane do not move away in a predictable manner.

In the 'gel model', gel formation at the membrane wall during UF occurs on reaching a limiting concentration, that is, when the polarized macromolecular layer becomes impermeable to other macromolecules. The gel layer, acting as a hydraulic barrier in series with the membrane, reduces permeate flux to the point that positive and negative mass transport forces are counterbalanced. Further increases in the transmembrane pressure result simply in the buildup of a thicker or denser cake of retained species with no benefit in terms of flux. Some caution is required when using the gel model, as its concentration and fluidity are known to deviate from predicted values depending on the prevailing hydrodynamic conditions.

The 'osmotic pressure model' is also used to describe the reversible changes in effective pressure or membrane resistance encountered in UF. Here, larger components (i.e., proteins) contribute to an exponential rise in osmotic pressure with increasing concentration. Thus, when protein is concentrated near the membrane surface, the rise in osmotic pressures may negate typical transmembrane pressures with consequences to permeate flux. It is suggested that the osmotic pressure model be applied only to solutes with a molecular weight less than 100 kD, and that gel layer limitation occurs more likely with high molecular weight solutes (>100 kD).

In practice, the influence of concentration polarization on permeate flux and to a certain extent on fouling may be minimized by improving the process hydrodynamics, for example, by increasing shear rate at the membrane surface using higher-velocity retentate circulation or other turbulence promoting measures.

Various membrane and module design approaches continue to be introduced with a view to improving overall plant performance. A patented rib design with similar-length flow channels on a flat membrane within a plate-and-frame-type module was developed to overcome uneven fluid distribution as a major contributor to fouling decay. The benefits of this new design include (1) pronounced improvement in the molecular selectivity of the membranes, (2) increased flux rate, (3) higher product concentration, and (4) easier cleanability and better flux recovery thereafter. The ISOFLUX® ceramic membrane introduced by Tami-Industries is characterized by a thickness gradient in the separating layer of the membrane surface to ensure constant permeate flow throughout the length of the module. The resulting improvement in separation performance is also accomplished with more favorable plant pumping requirements. It is claimed that a 4 log reduction in the bacteria load of skim milk may be achieved during MF using the ISOFLUX® ceramic membrane at a rate of 800 $\mathrm{l\,m^{-2}\,h^{-1}}$ at 50 °C for operating periods of up to 10 h.

Influence of Feed Parameters on Protein Fouling

Feed pH, ionic strength, calcium content, and degree of protein aggregation influence the nature of the fouling layer. As a consequence, its depth and porosity affect membrane selectivity and permeate flux.

During the processing of skim milk, the behavior of casein in response to pH change dominates membrane performance. From pH 6.0 to 5.6, there is a tendency for micelles to combine while retaining their individual shape. Fouling layer structure becomes increasingly compact with minimum interstitial pore space at pH 5.6. At pH 5.45–5.2, a different, more open, structure is apparent as the casein-forming chains link together. Below pH 5.2, the deposit consists of more or less individually clumped particles (demineralized casein micelles) which form an increasingly open structure.

In whey, calcium phosphate may give rise to severe fouling above pH 6.5 when the concentrations of both minerals (Ca and P) are high. Apart from its role in calcium phosphate precipitation, calcium generally promotes fouling either through protein–protein interactions in the boundary layer or through protein–membrane interactions. At lower pH, a decrease in whey flux at the isoelectric point is generally attributed to either decreased adsorption or deposition of protein or changes in the porosity of the deposited protein. Protein layer permeability is affected by ionic strength and ion valency: ion-binding increases protein size and, thus, its porosity when present in a deposited layer.

Protein Adsorption

In almost all instances of fouling, protein adsorption occurs on membrane surfaces. Both membrane hydrophobicity and charge influence protein adsorption. Such protein–membrane interactions are usually irreversible, and, because of their tightly bound nature, may be removed only by cleaning. Further increases in transmembrane pressure lead to further protein deposits on the membrane. This deposit, consisting of many protein multilayers, is not as strongly bound as the protein adsorbed directly to the membrane surface.

Changes in membrane hydrophobicity and charge difference between the membrane and protein may reduce the quantity of protein strongly adsorbed to the membrane surface. The availability of hydrophilic membranes represents a significant improvement, but no practical benefits have been obtained when using such polymer cMF membranes, thus leading to the conclusion that hydrophobicity and associated protein–membrane electrostatic interactions are not the only factors involved in membrane fouling.

The preferred view is that permeate flux is controlled more by the quantity of loosely deposited protein (fouling layer thickness) and the porosity of its multilayer structure, rather than by what is strongly bound to the membrane. These parameters are influenced by the properties of the product feed.

Calcium Phosphate Precipitation

Mineral-related fouling is experienced only when calcium phosphate precipitates during the filtration process. The precise conditions that lead to precipitation are not very well characterized, but factors such as high temperature, high pH, and high calcium concentration as well as the absence of protein, for example, permeate, all contribute to the likelihood of fouling. When operating conditions give rise to mineral precipitation, fouling is severe and is dominated by calcium phosphate with some protein complexation. In the absence of calcium phosphate precipitation, proteins are the main constituent of the fouling layer.

Fouling of Inorganic Membranes

The structural properties of inorganic membranes facilitate greater control of hydrodynamics such as turbulent flow and high wall shear stress as well as permit the use of permeate counter-pressure with or without permeate circulation. Inorganic membranes are used in cross-flow MF in the dairy industry.

UF and cMF membranes exhibit different fouling phenomena due to the diffused pore size distribution of the former and the differences in pore size range. UF separation results in surface fouling with the creation of a more or less homogeneous polarization layer of retained species (proteins). cMF separation/fractionation allows passage of proteinaceous material and particles through the membrane and its reactive porous support results in internal fouling such as adsorption which can eventually lead to pore narrowing. Moreover, a deposit of varying porosity of heterogeneously sized rejected particles, aggregates, and proteins is formed on the membrane's surface. The reduction of pore diameter or even complete pore blocking is the reason for loss of filtering areas during cMF processes, and thus for reduced permeability and increased rejection.

See also: **Liquid Milk Products**: Liquid Milk Products: Membrane-Processed Liquid Milk. **Milk Lipids:** Buttermilk and Milk Fat Globule Membrane Fractions. **Milk Proteins:** Heterogeneity, Fractionation, and Isolation. **Milk Protein Products**: Milk Protein Concentrate. **Whey Processing**: Demineralization; Utilization and Products.

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Whey Protein Products

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Introduction

Whey protein ingredients are protein-enriched fractions of whey that are designed for specific functional, nutritional, and nutraceutical applications. They range in protein content from 2.5 to over 90%. The non-protein components are water and various amounts of minerals, lactose, lipids, and possibly compounds produced by cheese starter bacteria. The functionality of whey protein ingredients depends on the composition of whey and interactions among compounds. A description of the different products, together with a discussion of processing and functional considerations, is given below.

Whey protein ingredients are added to foods for nutritional and functional reasons. For example, in products such as sports drinks, they provide high-quality protein that remains dispersed for the shelf life of the product. A functional requirement for such a product is heat stability, that is, at the desired pH and concentration the protein must remain dispersed and be at a target level of clarity after thermal processing and during storage also. Whey proteins also function in applications that require surface activity (emulsions and foams) and network formation (gels and edible films).

The majority of whey is processed into whey powder. The world production of whey powder has increased from 1.8 million tonnes in 1995 to more than 2.6 million tonnes in 2006. The production of whey and whey derivatives (sweet whey powder, demineralized whey, delactosed whey, whey protein concentrates (WPCs), whey protein isolates (WPIs), and lactose) in the United States and the European Union from 2003 to 2006 is listed in **Table 1**. The growth of dry whey production between 2003 and 2006 was 1–2%, approximately at the same rate as of milk volumes. Although the total dry whey production in the United States has not changed much since 1986, the amount of dry whey has shifted from animal consumption to human consumption, causing a decline in animal consumption from 14% of total production in 1986 to 5.4% in 2007. Similarly, 25% of WPCs was consumed by the feed sector in 1986 compared to 9% in 2007.

Whey derivatives are used as ingredients in a wide range of products. The derivatives having the lowest value are used predominantly by the feed industry. Derivatives with a higher value, such as WPCs and WPIs, are produced for the food, cosmetic, and pharmaceutical sectors. Recently, there has been a growing demand for whey powders and WPCs, predominantly from the food industry, resulting in an increase in their production. However, the market for value-added forms of whey, such as fractions and derivatives, is expected to have the largest growth, as much as 20% per year. The future growth is expected to be led by the industry's increasing focus on nutritional products, particularly in the dietary, sports, and clinical segments of the market. Lactose, used in food and pharmacy sectors, has been in high demand, and the production of lactose has grown 173% in the past 10 years. In 2007, in the US dairy industry, for human foods, 47.6% of dry whey was used while 27% was used as dry blends and 15.3% in the bakery industry. For WPCs, 42.4% was used by the dairy industry, while 23.4% was used in nutraceuticals, pharmaceuticals, and special dietary products, and 16.2% in infant formulae.

Whey is defined in the Unites States Code of Federal Regulations as

> . . . the liquid substance obtained by separating the coagulum from milk, cream, or skim milk in cheesemaking. Whey obtained from a procedure, in which a significant amount of lactose is converted to lactic acid, or from the curd formation by direct acidification of milk, is known as acid whey. Whey obtained from a procedure in which there is insignificant conversion of lactose to lactic acid is known as sweet whey. Sweet whey has a maximum titratable acidity of not more than 0.16 percent, calculated as lactic acid, and an alkalinity of ash of not more than 225 milliliters of 0.1 N hydrochloric acid per 100 grams. The acidity of whey, sweet or acid, may be adjusted by the addition of safe and suitable pH-adjusting ingredients.

Production of casein-based ingredients (caseins and caseinates) also provides a source of whey. The definition of whey can be modified to account for casein production by defining it as the liquid obtained after casein has been removed from milk. The components of whey are intimately associated with the process used to coagulate or remove casein. Cheesemaking practices that include the use

Table 1 Production (in tonnes) of whey and whey derivatives in the United States and the European Union according to USDA National Agricultural Statistics Service and USDA Foreign Agricultural Service

	2003		*2004*		*2005*		*2006*	
	US	*EU*	*US*	*EU*	*US*	*EU*	*US*	*EU*
Total dry whey	492 222	1 515 000	469 422	1 600 000	472 050	1 650 000	503 313	1 630 000
Reduced lactose and mineral whey	38 152	NR	38 507	NR	44 620	NR	41 547	NR
Demineralized whey	NR	150 000	NR	160 000	NR	170 000	NR	170 000
Delactosed whey	NR	110 000	NR	105 000	NR	100 000	NR	100 000
Total WPC	162 361	276 000	161 413	282 000	174 146	290 000	194 011	296 000
WPC 25–49.9	123 395	NR	123 266	NR	125 363	NR	134 928	NR
WPC 50–89.9	38 966	NR	38 147	NR	48 783	NR	59 083	NR
WPC >34	NR	108 000	NR	115 000	NR	118 000	NR	114 000
WPI	10 130	17 000	12 554	18 000	12 517	18 000	13 913	18 000
Lactose	278 495	360 000	301 921	370 000	323 854	380 000	335 049	390 000

NR, not reported; WPC, whey protein concentrate; WPI, whey protein isolate.

of chymosin to form the casein curd will release the glycomacropeptide (GMP) portion of κ-casein into the whey. Processes that coagulate casein by lowering the pH will shift the ionic equilibrium of the casein micelles and cause additional minerals to be in the whey. Fermentation bacteria can contribute polysaccharides, enzymes, and flavor compounds. These factors result in wide variations in the starting material used to make whey protein products.

Processing Methods

Whey contains around 0.6% protein and 93% water. WPC contains 25–80% protein, and WPI contains $\geq$90% protein. For every kilogram of WPI powder produced, one has to start with 150 kg of whey and remove at least 149 kg of water and solids by various processing methods. Pressure-driven membrane techniques used to produce WPCs and WPIs are reverse osmosis, ultrafiltration, microfiltration, and nanofiltration. Electrically driven membrane processes used are electrodialysis and electrodeionization. To date, the main adsorption technique employed has been ion exchange, but with the advent of new technologies, there are potential applications of affinity binding and other techniques that have historically been economically limiting. Thermal processing to inactivate microorganisms or increase processing efficiency is also used at various stages of processing. The net result is that whey protein ingredients can contain the same amount of protein, but vary widely, by design, in the amount of various non-protein compounds. Moreover, just measuring the amount and type of non-protein molecules presents an incomplete picture because we cannot account for molecules' interactions that are caused during processing. In many cases, it is the interactions among molecules that provide the functional properties rather than the amount of one or several components.

Over the past 15 years, whey-processing developments have focused on advanced liquid-handling techniques, maximization of quality and safety, and the introduction of sophisticated separation and fractionation methods. With the emphasis on cost-effectiveness and the retention of native protein functionality, specifically bioactivity, chromatographic techniques have helped pioneer cost-effective processes for whey protein isolation and fractionation at the commercial scale. Continuous separation (CSEP) chromatographic technology provides the benefits of traditional chromatography (specificity, reproducibility, and mildness), addresses the disadvantages (cost, throughput, flexibility, productivity, and complexity), and has facilitated the manufacture of unique WPIs (enriched in β-lactoglobulin and/or GMP), lactoferrin, and bioactive factors from whey. The development of membrane adsorber in membrane processing has allowed the manufacture of a wide range of specialized whey ingredients, including high-protein/peptide isolates and fractionated/purified bioactive proteins. Whey processing results in a large volume of lactose- and mineral-rich permeate. Advanced technology in the past 10 years has provided for a simple and efficient lactose hydrolysis and isolation at commercial scale, improving the overall cost-effectiveness in whey processing.

Concentrates

WPC is defined by the United State Code of Federal Regulations as

> . . . the substance obtained by the removal of sufficient nonprotein constituents from whey so that the finished dry product contains not less than 25 percent protein. Whey protein concentrate is produced by separation

> techniques such as precipitation, filtration, or dialysis. As with whey, whey protein concentrate can be used as a fluid, concentrate, or dry product form. The acidity of whey protein concentrate may be adjusted by the addition of safe and suitable pH-adjusting ingredients.

Most WPCs on the market contain either 34–35 or ~80% protein (**Table 2**). WPCs containing ~35% protein are used in the manufacture of yogurt, processed cheese, and infant formulae, and in various bakery applications. These products are also marketed for use in stews and sauces because of their thickening properties, as well as nutritional benefit. The addition of ~35% protein WPCs to luncheon meats and meat patties is another recommended application. The 51–54% lactose and high ash content distinguish this product from 80% protein WPCs. The performance of ~35% WPCs in food applications is based on the combined effects of protein, lactose, and minerals in these products.

There are some specialized whey protein products that have a protein content of 35–80%. Extruded WPC (50% protein) is another product used in meat applications or protein bars. The addition of an extruded WPC lends mouthfeel and texture to a product, while also providing a nutritional benefit. A WPC (53–60% protein) with a high level of butterfat (15–27%) is a product specifically geared to the manufacture of processed cheese. A WPC with added active yogurt cultures is marketed toward producers of frozen desserts and yogurt coatings, as it is claimed that it gives a cultured flavor to the product.

WPCs containing 80% protein are designed for applications in which proteins play a dominant functional role. Applications such as gelation, emulsification, and foam formation are often mentioned for these products. The low carbohydrate content of this type of WPC makes it an ideal ingredient for sports nutrition and weight management products. In many cases, they are designed to compete with other protein ingredients, such as those derived from egg white. One application for these ingredients is in meat products, where high gel strength and good water-binding properties are required.

Isolates

WPIs contain ≥90% protein and 4–6% water (**Table 2**). The remaining 4–6% of the ingredient is a combination of fat, lactose, and ash. Because of their high protein purity and solution clarity, WPIs are used extensively in nutritional supplements, sports and health drinks, and protein-fortified beverages. Ion exchange chromatography is one of the methods used in the manufacture of WPIs. It provides an additional level of selectivity above membrane processing because factors other than molecular size determine protein adsorption. Because of the high protein content, WPIs function as water-binding, gelling, emulsifying, and foaming agents. There are several ways in which processors can adjust the composition and functionality of these ingredients. For example, the amount of individual proteins can be varied. Some isolates contain GMP, while others do not. While it is possible to produce WPCs without GMP by using whey streams from processes that do not cleave κ-casein, the additional fractionation techniques used to produce WPIs also allow for selective removal of GMP from cheese whey. Additionally, pH can be altered prior to drying, creating a WPI with an acidic pH, primarily designed for use in acidic beverage applications. Proteins and other components of WPC and WPI are listed in **Table 3**.

Individual Protein Fractions

The main proteins found in WPCs and WPIs are β-lactoglobulin, α-lactalbumin, GMPs, bovine serum albumin, immunoglobulins, lactoferrin, and lactoperoxidase. Each of these proteins or group of proteins has been proven or implied to have unique functional, nutritional, or nutraceutical properties. Some putative nutraceutical activities are digestive function (β-lactoglobulin), prevention of dental cavities and satiety (GMP),

Table 2 Average composition and ranges of 35 and 80% protein WPCs and WPIs based on product specification sheets of nine companies

Ingredient	*Protein (%; dry wt.)*	*Moisture (%)*	*Lactose (%)*	*Fat (%)*	*Ash (%)*	*pH*
WPC 35[a]	35.3	3.7	52.3	3.3	5.8	6.2
WPC 35 range	34.0–36.4	2.9–4.0	47.0–56.0	2.5–4.0	1.6–8.0	4.9–7.0
WPC 80	78.7	4.3	4.9	6.4	4.0	6.4
WPC 80 range	72.9–82.8	3–5.2	0.15–7.4	1–10	2.5–11.0	3.2–7.2
WPI	90.9	4.8	1.5	0.9	2.7	6.0
WPI range	90–95	4–6	0.2–2.0	0.2–1.5	0.3–4.5	3.3–7.5

[a] Also made in a form that increases viscosity and has a low degree of gelation.
WPC, whey protein concentrate; WPI, whey protein isolate.

Table 3 Proteins and other components of WPI and WPC based on product specification sheets of two companies

Company	*β-Lactoglobulin*	*α-Lactalbumin*	*Glycomacropeptides*	*BSA*	*Immunoglobulins*	*Lactoferrin*	*Lactoperoxidase*
1 (WPC)	50–60	12–16	15–20	3–5	5–8	<1	NR
1 (WPC)	54.5	15.6	21	3.3	5.0	0.2	NR
2 (WPI)	43.8	15.2	20.3	1.2	3.4	NR	NR
2 (WPI)	69.2	14.2	1.6	3.3	2.1	NR	NR

All values are in percentages.
BSA, bovine serum albumin; NR, not reported; WPC, whey protein concentrate; WPI, whey protein isolate.

anticarcinogenicity, sleep enhancement (α-lactalbumin), antimicrobial immunity (lactoferrin and lactoperoxidase), and passive immunity (immunoglobulins).

α-Lactalbumin is rich in essential amino acids and is the principal protein in human milk. The high content of cysteine in α-lactalbumin has been found to improve the immune system, while a high level of tryptophan may help improve mood, sleep, and cognitive performance. α-Lactalbumin binds minerals, specifically calcium, magnesium, zinc, and cobalt. By being bound to a protein, these minerals are more readily delivered for absorption in the human body. The lack of β-lactoglobulin in human milk suggests that bovine whey protein products rich in α-lactalbumin and low in β-lactoglobulin would be more appropriate for infant formulae. Because of its nutritional and health benefits, α-lactalbumin is used in infant formulae, and health and performance products.

GMP has various unique characteristics compared to other whey proteins. The 'glyco' portion of its name refers to the saccharide groups that are attached to the peptide backbone of the molecule. The most important heterogeneous sugar chain related to GMP is *N*-acetylneuraminic acid, commonly known as sialic acid. Studies have shown that sialic acid may influence digestive function and promote the growth of bifidobacteria. Beneficial biological roles attributed to GMP or peptides derived from it also include stimulation of cholecystokinin (a hormone regulating energy and food intake) release from intestinal cells, inhibition of platelet aggregation, and reduction of dental caries. The glycosylation or the amount of sialic acid of GMP varies widely and is affected by the manufacturing process. Suggested applications of GMP include beverage, nutritional bar, dietary supplements, diet foods, and pharmaceutical products.

Lactoferrin was viewed originally as an iron-binding protein in milk with bacteriostatic properties, but its physiological roles are becoming increasingly evident. These include regulation of iron homeostasis, host defense against a broad range of microbial infections, antiinflammatory activity, regulation of cellular growth and differentiation, and protection against cancer development and metastasis. Lactoferrin is used in infant formulae, nutritional foods and supplements, sports nutrition supplements, and pharmaceutical products.

The composition of some whey protein fraction products is shown in **Table 4**. The low ash and fat contents in these products are similar to WPIs; however, the range among companies in the amount of non-protein components might be one point of differentiation between the products.

The proven and putative nutritional and nutraceutical properties of whey protein ingredients have led to the development of a number of products based on a single protein or an enriched amount of one or more proteins. The versatile functionality of whey proteins has also led to the development of whey ingredients with specific functionality. Examples of these products are WPIs or WPCs having higher heat stability, solubility, dispersibility, and improved gelation and water-holding properties. Specialty whey

Table 4 Composition of whey protein fractions based on product specification sheets of four companies

Company	*Protein type*	*Protein (%; dry wt.)*	*Moisture (%)*	*Fat (%)*	*Ash (%)*	*pH*
1	BSA	99.0	3.0	NR	2.0	6.8–7.2
2	Lactoferrin	97.0	5.0	0.5	2.0	>6.0
3	Lactoferrin	98.0	5.0	<0.1	1.0	6.0–7.0
4	Glycomacropeptide	82.5	7.0	1.0	7.0	7.0
3	Glycomacropeptide	80.0	5.0	0.5	NR	6.5
4	α-Lactalbumin	95.0	6.5	1.0	3.5	7.5

BSA, bovine serum albumin; NR, not reported.

Table 5 Specialty whey protein ingredients based on product specification sheets of four companies

Company	Function	Protein (%; dry wt.)	Moisture (%)	Fat (%)	Ash (%)	pH
1	Low viscosity	NR	NR	NR	NR	4.9–6.3
1	Medium viscosity	NR	NR	NR	NR	4.9
1	High viscosity	NR	NR	NR	NR	4.9–6.3
1	High in growth factors, immunoglobulins, and lactoferrin	73.5	4.0	15.0	3.5	6.5
1	Targets fat loss during weight management, partially hydrolyzed	84.0	5.0	1.0	8.0 (2% Ca)	7.0
1	High heat stability and solubility	90.0	4.0	1.0	7.0	7.5
1	Improved gelation and water holding	76.0	5.3	15.0	5.0	7.1
1	Improved gelation and water holding	35.4	4.0	4.0	7.0	6.8–7.2
2	α-LA-enriched WPC	78.0 (30% α-LA; 18% BLG)	4.0	12.0	3.0	NR
2	Strong gelling	NR	NR	NR	NR	NR
3	Hydrolysate of α-LA	88.0	6.0	4.0	2.0	6.1–6.8
4	Colostrum – increased intestinal health	55.0	5.0	1.0	2.6	6.7

α-LA, α-lactalbumin; BLG, β-lactoglobulin; NR, not reported; WPC, whey protein concentrate; WPI, whey protein isolate.

protein ingredients designed for specific applications are listed in **Table 5**.

Hydrolysates

Another way in which the nutritional and functional properties of whey protein ingredients can be altered is enzymatic hydrolysis. Peptides may be absorbed slightly better and more quickly than amino acids or whole protein. Because hydrolysis produces peptides with structures that are different from those in the intact protein, it is also used to reduce allergenicity. With better nutritional quality and less allergenicity, whey protein hydrolysates are currently used in infant formulae and enhanced-performance products. Different degrees of hydrolysis will impact flavor, molecular weight profile, and functional properties by different extents. Hydrolysis can improve foaming ability by increasing the amount of air incorporated into a foam (overrun) and can also increase stability. Likewise, hydrolysates can be used to increase emulsion stability. Hydrolysis can be used to change the conditions required for gelation, along with the physical properties of gels. Increased thermal stability is another application associated with whey protein hydrolysates. In this application, the hydrolysis process is designed to produce peptides that are less prone to aggregation when heat processed. Functional and allergenic properties of whey protein hydrolysates depend on the extent of hydrolysis. Generally, whey protein hydrolysates designed for nutritional applications such as sports nutrition drinks, nutritional bars, enteral formulae, and hypoallergenic infant formulae have a moderate-to-high degree of hydrolysis and a high content of short peptides. Lower levels of hydrolysis are associated with improved functionality in foaming, emulsification, and gelation. The composition of whey protein hydrolysates and the degree of hydrolysis of the products are shown in **Tables 6** and **7**, respectively.

Table 6 Composition of whey protein hydrolysates based on product specification sheets of four companies

Company	Protein (%; dry wt.)	Moisture (%)	Lactose (%)	Fat (%)	Ash (%)	pH
1	78.0	3.5	3.0	5.2	5.0	NR
1	75.0	3.5	3.0	5.5	4.5	NR
2	78.0	6.0	6.0	5.0	5.0	NR
3	90.0	5.0	1.0	1.0	6.0	6.5
4	90.0	5.5	1.0	1.0	6.0	8.5
4	94.0	5.5	1.0	1.0	5.0	7.5

NR, not reported.

Table 7 Peptide composition of whey protein hydrolysates based on product specification sheets of two companies

Company	*>20 000 Da*	*5000–20 000 Da*	*1000–5000 Da*	*<1000 Da*	*Degree of hydrolysis*
1	17	15.6	26.7	40.5	NR
1	9.5	6.6	12.9	70.8	NR
2	NR	NR	NR	NR	4.0
2	NR	NR	NR	NR	7.5

NR, not reported.

Whey Permeate

During ultrafiltration and diafiltration, whey proteins are retained by the filtering membrane, while substances of lower molecular weight, such as lactose and minerals, pass through the filter and become the permeate stream. Once moisture is removed from the liquid permeate, an off-white, free-flowing powder with a mild dairy flavor remains. After the protein (plus some lactose and minerals) is removed from this powder, the remaining collection of substances is called whey permeate or deproteinized whey. The composition of whey permeate varies by milk source, cheese type, and processing conditions, but its main ingredient is lactose. The typical composition of whey permeate is 65–85% lactose, 3–8% protein, 8–20% ash, <1.5% fat, and 3–5% moisture. Whey permeate can be used in a variety of processed foods and bakery applications to impart better browning characteristics to the finished product and to extend shelf life.

Conclusion

There has been a significant growth in the production and processing techniques of whey protein ingredients over the past 30 years. Manufacturers are finding more efficient ways to produce the products, while also making products for specific applications such as in nutraceuticals and cheese production, and for nutritional improvement. In the immediate future, it is likely that new whey protein products will be introduced, and existing products will continue to evolve in their production and uses.

See also: **Milk Protein Products**: Bioactive Peptides; Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Functional Properties of Milk Proteins; Membrane-Based Fractionation; Milk Protein Concentrate. **Whey Processing**: Demineralization; Utilization and Products.

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Bioactive Peptides

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Introduction

Peptides within the sequence of a parent protein can be released by enzymatic hydrolysis, for example, during gastrointestinal digestion or food processing. Bioactive peptides refer to different peptides of plant or animal origin that may have regulatory functions in the human systems beyond normal and adequate nutrition. Research in the field of peptides has intensified during the last two decades and has been reviewed extensively. Some of these peptides may act as regulatory compounds with a hormone-like activity. The activity is based on their amino acid composition and sequence. Bioactive peptides usually contain 3–20 amino acid residues per molecule. Peptides derived from milk proteins have been shown to possess multiple biological properties, including antimicrobial activity, modulation of digestive enzymes, nutrient absorption, immune responses, and opioid, antithrombotic, and antihypertensive activities (**Table 1**). Many milk protein-derived peptides have revealed multifunctional properties; that is, specific peptide sequences may exert two or more different biological activities. Due to their physiological and physicochemical versatility, milk-borne peptides are regarded as highly prominent ingredients for health-promoting, functional foods or pharmaceutical preparations. The majority of the studies have focused on the blood pressure-lowering effect of these peptides, showing a hypotensive effect in animals and humans. This article will discuss the current knowledge about milk protein-derived bioactive peptides and their potential application for promotion of human health.

Structures and Functions of Bioactive Peptides

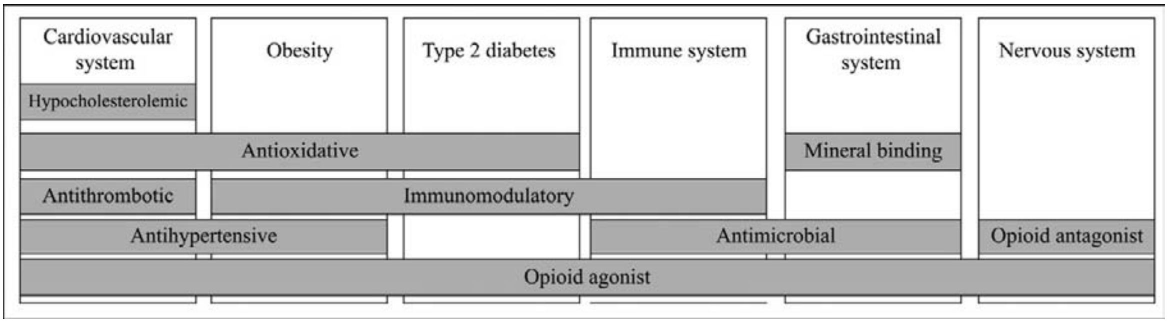
Table 2 presents examples of structural properties of milk protein-derived bioactive peptides, and **Table 3** lists some examples of peptide sequences with different bioactivities measured by *in vitro* cell cultures or *in vivo*.

Food-derived peptides with opioid activity were first recognized in the late 1970s and were termed 'exorphins' on the basis of their structural similarity to endogenous ligands (endorphins and enkephalins). Typical opioid peptides have the same N-terminal sequence, Tyr-Gly-Gly-Phe, and they exert activity by binding to specific receptors of the target cell, for example, the μ-receptor for emotional behavior and suppression of intestinal motility, the σ-receptor for emotional behavior, and the κ-receptor for sedation and food intake. The opioid peptides derived from a variety of precursor proteins are called 'atypical' opioid peptides, since they carry various amino acid sequences in their N-terminal region. Only the N-terminal tyrosine is conserved, and the presence of another aromatic amino acid at the third or fourth position forms an important structural motif that fits into the binding site of the opioid receptors. The major opioid milk peptides, β-casomorphins, are fragments of the β-casein sequence 60–70, and are characterized as μ-type ligands (**Table 3**). The α-casein exorphins were found to correspond to bovine α_{s1}-casein f(90–96), f(90–95), and f(91–96), and were σ-selective receptor ligands. Whey proteins contain opioid-like sequences in their primary structure, namely, α- and β-lactorphins. These peptides show low affinity for opioid receptors and are μ-type receptor ligands. Opioid activity has, furthermore, been found in fragments from bovine serum albumin (serorphin, f(399–404)).

Peptides from a peptic or tryptic digestion of bovine and human κ- and α_{s1}-caseins have been found to display opioid antagonistic properties. Various C-terminally methoxylated casoxins have been identified, for example, κ-casein f(33–38) (casoxin-6), f(34–38) (casoxin-5), and f(35–38) (casoxin-4). In addition, casoxin C (κ-casein f(25–34)), casoxin A (κ-casein f(35–41)), and casoxin B (κ-casein f(61–64)) showed opioid antagonistic activity, but only casoxin C's activity was comparable to that of the esterified casoxin-6 and casoxin-4. In general, the chemically modified peptides are more active than their nonmethylated derivatives. Casoxins show a preference for μ- and κ-receptors with a relatively low potency as compared to naloxone.

Angiotensin I-converting enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) plays a major role in the regulation of blood pressure. Within the renin–angiotensin system, ACE catalyzes the conversion of angiotensin I to angiotensin II, a hormone that results in vasoconstriction, and subsequently in an increase of blood pressure. In addition, ACE degrades bradykinin, which has vasodilatory properties. Thus, ACE inhibitors lower hypertension

Table 1 An overview of the beneficial effects attributed to bioactive peptides derived from milk proteins



Modified from Hartmann R and Meisel H (2007) Food-derived peptides with biological activity: From research to food applications. *Current Opinion in Biotechnology* 18: 163–169.

Table 2 Structural properties of bioactive peptides

Activity	*Structural elements*	*Remarks*
Opioid	Tyrosine at the N-terminus, coupled with the presence of another aromatic residue, e.g., Phe or tyrosine, in the third or fourth position	Fits into the binding site of opioid receptors
ACE inhibitory	Hydrophobic amino acids or positive charge of lysine or arginine at the C-terminus	Influence on the binding to ACE
Antithrombotic	Ile^{108}, lysine^{112}, and Asp^{115} residues of casoplatelins Sugar content	Important antithrombotic activity
Antioxidant	High amounts of His and hydrophobic amino acids	Peptides with a Pro-His-His sequence showed the greatest antioxidant activity among the tested peptides
Hypocholesterolemic	Low ratios of methionine–glycine and lysine–arginine in the dietary proteins. High amounts of hydrophobic amino acids	Hydrophobic amino acids can bind bile acids and thereby enhance fecal steroid excretion
Antiobesity	Peptide length	Influence on CCK-releasing activity
	Multiple arginine residues	Necessary condition for CCK release through direct binding to intestinal cells
Antimicrobial	Positively charged residues and hydrophobic region that contains tryptophan	Trp involved in membrane interactions

Modified from Erdmann K, Cheung BWY, and Schröder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry* 19: 643–654.

and are believed to prevent cardiovascular diseases. Since the first discovery of exogenous ACE inhibitors from snake venom, a great number of ACE inhibitory peptides have been isolated from the digests of various food proteins. The precise structure–activity specificity is not fully understood. ACE inhibitory peptides are generally short-chain peptides, carrying hydrophobic (aromatic or branched-chain) amino acid residues, like proline. For example, hypotensive peptides like Val-Pro-Pro and Ile-Pro-Pro can be released from precursor proteins (β- and κ-caseins) by the enzymes from *Lactobacillus helveticus*. Digestion with trypsin can liberate ACE inhibitory peptides from α_{s1}-casein (f(23–27), f(194–199)) and β-casein (f(43–68), f(177–183), f(191–202)). Also, whey proteins contain ACE inhibitory peptides; the highest activity has been found with peptides from β-lactoglobulin (β-Lg) (f(80–82), f(142–148)) and α-lactalbumin (α-la) (f(104–108)). The IC$_{50}$ value (inhibitor concentration leading to 50% inhibition) is used to estimate the effectiveness of different ACE inhibitory peptides. However, it is not always related to *in vivo* activity, since some peptides are susceptible to degradation or modification in the gut, vascular system, and the liver. Moreover, some long-chain candidate peptides can be degraded and new active fragments are generated by gastrointestinal enzymes.

Peptides that inhibit blood platelet aggregation and fibrinogen binding (γ-chain) to platelet surface receptors are encrypted within the sequence of bovine κ-casein, and are referred to as casoplatelins. Several casoplatelins have been reported to have an antithrombotic effect

Table 3 Examples of milk-derived bioactive peptide sequences and their effects in different models

Sequence	*Name*	*Fragment*	*Effect*	*Model*	*Release protease*
VPP/IPP	Lactotripeptides	β-Caseins and κ-caseins	ACE inhibitor	*In vitro*	Fermentation
			antihypertensive	Animal, humans	
FFVAP	α_{s1}-Casokinin-5	α_{s1}-cn f(23–27)	ACE inhibitor	*In vitro*	Proline endopeptidase
AVPYPQR	β-Casokinin-7	β-cn f(177–183)	ACE inhibitor	*In vitro*	Trypsin
WLAHK	Lactokinin	α-La f(104–108)	ACE inhibitor	*In vitro*	Trypsin
ALPMHIR	Lactokinin	β-Lg f(142–148)	ACE inhibitor	*In vitro*	Trypsin
IPA	Lactokinin	β-Lg f(78–80)	ACE inhibitor	*In vitro*	Proteinase K
			antihypertensive	Animal	
TTMPLW	α_{s1}-Immunocasokinin	α_{s1}-cn f(194–199)	ACE inhibitor	*In vitro*	Trypsin
			antihypertensive	Animal	
			Immunomodulatory	Cell culture	
YPFPGPI	β-Casomorphin-7	β-cn f(60–66)	Opioid agonist	*In vitro*	Trypsin
			ACE inhibitor	Cell culture	
			Immunomodulatory		
			Cytomodulatory		
RYLGYLE	α-Casein exorphin	α_{s1}-cn f(90–96)	Opioid agonist	*In vitro*	Pepsin
YGLF	α–Lactorphin	α-La f(50–53)	Opioid agonist and ACE inhibitor	*In vitro*	Pepsin
			antihypertensive	Animal	
YLLF	β-Lactorphin	β-Lg f(102–105)	Opioid agonist and ACE inhibitor	*In vitro*	Pepsin + trypsin
HIRL	β-Lactotensin	β-Lg f(146–149)	ACE inhibitor	*In vitro*	Chymotrypsin
			Smooth muscle stimulation	Animal	
			Antistress		
SRYPSY	Casoxin-6	κ-cn f(33–38)	Opioid antagonist	*In vitro*	Pepsin
RELEELNVPGEIVES*LS*S*S*EESITR	Caseinophosphopeptide	β-cn f(1–25)4P	Mineral binding	Animal, humans	Trypsin
			Immunomodulatory	Cell model	
			Cytomodulatory		
YFYPEL		α_{s1}-cn f(144–149)	Antioxidative activity	*In vitro*	Pepsin
MHIRL		β-Lg f(145–149)	Radical scavenging activity	*In vitro*	Corolase PP
YVEEL		β-Lg f(42–46)	Radical scavenging activity	*In vitro*	Corolase PP

(*Continued*)

Table 3 (Continued)

Sequence	*Name*	*Fragment*	*Effect*	*Model*	*Release protease*
IIAEK	Lactostatin	β-Lg f(71–75)	Hypocholesterolemic	*In vitro* Cell culture Animal	Trypsin
ALPMH	Lactostatin	β-Lg f(142–146)	Hypocholesterolemic	*In vitro* Cell culture	Trypsin
FKCRRWQWRMKK LGAPSITCVRRAF	Lactoferricin	Lf f(17–41)	Antimicrobial and immunomodulatory	*In vitro* Cell culture	Pepsin
RPKHPIKHQGLPEQ VLNENLLRF	Isracidin	α_{s1}-cn f(1–23)	Antimicrobial	*In vitro* Animal	Chymosin
VAGTWY		β-Lg f(15–20)	Antimicrobial	*In vitro*	Trypsin
VLVLDTDYK		β-Lg f(92–100)	Antimicrobial	*In vitro*	Trypsin
KTKLTEEEKNRLNFLKKISQRYQKFA LPQYLKTVYQHQK	Casocidin-I	α_{s2}-cn f(165–203)			Trypsin
MAIPPKKNQDK	Casoplatelin	κ-cn f(106–116)	Antithrombotic	*In vitro*, *ex vivo*	Trypsin

cn, casein; α-La, α-lactalbumin; β-Lg, β-Lactoglobulin.

in vitro and in guinea pigs after parenteral administration. κ-Casein f(106–116) inhibited ADP-induced platelet aggregation and combined with the fibrinogen receptors of blood platelets, consequently preventing fibrinogen binding with blood platelets. The two smaller tryptic peptides (κ-casein f(106–112) and f(113–116)) exerted an effect on platelet aggregation but did not inhibit fibrinogen binding.

Antioxidant properties that prevent enzymatic (lipoxygenase) and non-enzymatic peroxidation of essential fatty acids have been found in peptides derived from milk proteins. The identified peptides are encrypted in the sequences of α_{s1}-, β-, and κ-caseins, and β-Lg. The antioxidant activity has been attributed to the high amount of histidine and some hydrophobic amino acids.

Hypocholesterolemic effects have been reported for casein- and whey protein-derived peptides. The mechanism involved in these effects remains to be clarified. A β-Lg-derived peptide, Ile-Ile-Ala-Glu-Lys, termed lactostatin, decreases micellar solubility of cholesterol, which leads to reduced absorption of intestinal cholesterol. Lactostatin is also able to induce gene transcription of human 7α-hydroxylase, a cholesterol-metabolizing enzyme, resulting in a hypocholesterolemic effect.

Studies on the effects of peptides on immunomodulation have been conducted *in vitro* with cells of specific and non-specific immune systems. Immunomodulatory peptides can enhance immune cell functions, measured as lymphocyte proliferation, natural killer cell activity, antibody synthesis, and cytokine regulation. The casein-derived peptides, including α_{s1}-casein f(194–199) and β-casein f(63–68) and f(191–193), stimulate human and murine macrophages. Synthetic peptides (Tyr-Gly and Tyr-Gly-Gly) have been shown to enhance the proliferation of human peripheral blood lymphocytes. β-Casomorphin-7 and β-casokinin-10 suppress lymphocyte proliferation at low concentrations but are stimulatory at higher concentrations. β-Casein f(193–199) induces a significant proliferative response in rat lymphocytes. Bioactive peptides in yogurt preparations decrease cell proliferation with Caco-2-cells or IEC-6-cells. α_{s1}-Casein f(59–79) and β-casein f(1–25), having a phosphoserine-rich region, have been found to display mitogenic activity and enhance immunoglobulin production in mouse spleen cells. Lactoferrin-derived peptides have been reported to exert an influence in several cell types, for example, human monocytic and polymorphonuclear leukocyte cell lines, which are involved in the immune and inflammatory actions of the body.

The antimicrobial activity of milk is associated mainly with lactoferrin. Moreover, peptides released from lactoferrin by digestion with pepsin or by heat treatment at an acidic pH show higher antimicrobial potency than undigested lactoferrin. The identified antimicrobial peptides, named lactoferricins, originate from the N-terminal region of the molecule and have antimicrobial activity against various Gram-positive and Gram-negative bacteria, yeasts, and filamentous fungi. For example, lactoferricin B killed four clinical isolates of enterohemorrhagic *Escherichia coli* O157:H7 within 3 h at concentrations above 10 μg ml^{-1}. In addition, α_{s2}-casein f(165–203) (casocidin-I) purified from bovine milk hydrolyzed by a serine protease inhibits the growth of *E. coli* and *Staphylococcus carnosus*. The antimicrobial activity of lactoferricins and casocidin-I seems to be correlated with the net positive charge on the peptides. A distinctive feature is the high proportion and asymmetric clustering of basic amino acid residues. It is generally known that cationic amphipathic α-helical structures are related to antimicrobial activity by forming ion channels through membrane bilayers. Lactoferricins disrupt the normal membrane permeability of the cell, which is at least partly responsible for the antibacterial mechanism of lactoferrin-derived peptides. Peptides generated by enzymatic cleavage from α-La and β-Lg exhibit antimicrobial activity mostly against Gram-positive bacteria. The bactericidal peptides are negatively charged, which explains their weak effect against Gram-negative bacteria.

Tryptic digestion of casein proteins yields caseinophosphopeptides (CPPs) from the polar N-terminal region, which contain clusters of phosphorylated seryl residues. For example, α_{s1}-casein f(43–58), f(59–79), and f(43–79), α_{s2}-casein f(1–24) and f(46–70), and β-casein f(1–28), f(2–28), f(1–25), and f(33–48) have been isolated from the tryptic hydrolysate of whole casein. These phosphorylated clusters have been hypothesized to be responsible for the interaction between caseins and calcium phosphate that leads to the formation of casein micelles. CPPs retain the ability of whole casein to stabilize calcium and phosphate ions through the formation of complexes, thus enhancing their general bioavailability.

Possible Physiological Importance

The potential physiological role of bioactive peptides derived from milk proteins and the *in situ* formation of these peptides in the gastrointestinal tract are still largely unknown. It is known that di- and tripeptides are easily absorbed in the intestine. However, little is known about the absorption of larger-molecular-weight bioactive peptides in the intestine. In order to exert a beneficial effect, for example, an antihypertensive effect, the peptides have to be absorbed from the intestine and reach the target cells in the blood vessels in substantial concentrations. In fact, 6 h after absorption of fermented milk, the tripeptides (Val-Pro-Pro and Ile-Pro-Pro) in the milk were detected by HPLC in aortal tissues and ACE activity in the aorta was lower as compared to a control group that was given saline. The absorption and degradation of natural β-casomorphins and their analogues have been intensively studied. Studies have shown no intact transepithelial passage of β-casomorphins; therefore, it is generally concluded that the physiological influences are limited to the gastrointestinal tract. The situation is different in neonates, where passive transport across intestinal mucosal membranes occurs. This may lead to physiological responses such as an analgesic effect resulting in calmness and sleep in infants. The presence of caseinophosphopeptides and immunopeptides from casein in the intestinal lumen of mammals (rat, minipig, calf, human) or even absorption into plasma after ingestion of milk or a diet containing casein has already been shown. Most bioactive peptides are not absorbed in the intestinal tract. Hence, peptides may act either directly in the intestinal tract or via receptors and cell signaling in the gut.

Numerous studies in spontaneously hypertensive rats (SHRs) as well as in hypertensive human volunteers have been performed to determine the antihypertensive effect of milk protein-derived peptides. These studies demonstrated that several ACE inhibitory peptides significantly reduce blood pressure, after either intravenous or oral administration. The best-characterized ACE inhibitory peptides are Val-Pro-Pro and Ile-Pro-Pro, both found in fermented milk. About 20 studies on humans linking the consumption of products containing these two tripeptides with a significant reduction in both systolic blood pressure (SBP) and diastolic blood pressure (DBP) have been published. The maximum blood pressure reductions observed were $\cong$13 mmHg (SBP) and 8 mmHg (DBP) after active treatment as compared to placebo, and are likely to be reached after 8–12 weeks of treatment. Effective dosages of tripeptides range from 3.07 to 52.5 mg day^{-1}. *In vivo* comparative studies with captopril, a clinically used ACE inhibitor, have shown that ACE inhibitory peptides with an antihypertensive effect exhibit higher *in vivo* activity than that which could be expected from their *in vitro* activity. The mechanisms underlying this observation have not yet been identified. It has been suggested that food-derived peptides have higher tissue affinities and are subject to lower elimination. Besides ACE inhibition, other mechanisms, such as that of opioids, are responsible for the depressive effect as shown with α-lactorphin, providing further evidence for the multifunctional role of bioactive peptides. The release of vasodilatory substances, like NO and prostaglandin I_2, could also contribute to the blood pressure-lowering effects of various ACE inhibitory peptides.

The systems involved in the defense mechanism of the body are both varied and complex, and the main focus is on two peptide groups, namely, immunomodulatory and antimicrobial peptides. Only very limited information is available on the *in vivo* effects. The Tyr-Gly and Tyr-Gly-Gly peptides modulate the lymphokine production *in vitro*. Moreover, these peptides were used successfully for immunotherapy of human immunodeficiency virus infection. Furthermore, a study showed that oral administration of a commercial CPP preparation (consisting of α_{s2}-casein f(1–32) and β-casein f(1–28)) enhanced intestinal IgA levels in piglets. A hexapeptide (human β-casein f54–59) and a tripeptide Gly-Leu-Phe protected mice against *Klebsiella pneumoniae* injection after intravenous injection. An antimicrobial peptide α_{s1}-casein f(1–23), isracidin, has been shown to exhibit *in vivo* activity against *Staphylococcus aureus* and *Candida albicans* at concentrations comparable with those of known antibiotics. Moreover, bactericidal peptides may assist in protecting against microbial challenge, especially in the neonatal intestinal tract, and thus support the nonimmune defense of the gut. The immunostimulating activity may also have a direct effect on their resistance to bacterial and viral infections of adult humans.

Published data on the effect of CPPs in animal and human studies are contradictory. A rat model system indicated that CPPs increase passive calcium transport in the distal small intestine. In humans, small CPPs have been detected in the stomach and duodenum following ingestion of milk. However, no evidence has been supplied on the effectiveness of CPPs in increasing passive calcium absorption in humans. In a recent study, it was concluded that CPPs cannot enhance calcium absorption in the gut. Besides, CPPs can have anticariogenic properties, based on their ability to stabilize and localize calcium and phosphate ions at the tooth surface, promoting remineralization of enamel subsurface lesions.

It is generally accepted that protein is the most satiating macronutrient. Several studies speculate that peptides released from dietary proteins during digestion can initiate several satiety signals from the gut and thus prevent further food intake. Because these peptides act on the intestinal site, they do not need to be absorbed into the systemic circulation. It has been suggested that casein-derived peptides induce satiety by independent activation of both opioid and cholecystokinin receptors in rats. Moreover, casein- and whey protein-derived peptides appear to stimulate glucagon-like peptide-1 release. It remains to be determined which peptides are responsible for the abovementioned effects.

Production of Bioactive Peptides

A number of bioactive peptides mentioned in this article are released from host proteins by fermentation of milk, including cheese ripening. Several studies have demonstrated that *Lb. helveticus* strains are capable of releasing ACE inhibitory peptides, in particular the well-characterized tripeptides Val-Pro-Pro and Ile-Pro-Pro. The occurrence of bioactive peptides in cheese varieties has been reported in several studies, and their activity depends on the maturation of cheese. It seems that bioactive peptides liberated during cheese ripening can be further degraded into inactive fragments. Proline-specific peptidase has been found to contribute to the degradation of ACE inhibitory peptides during fermentation with an *Lb. helveticus* strain. Phosphopeptides are produced during cheese ripening by the activity of plasmin and the proteolytic enzymes derived from lactic acid bacteria. β-Casomorphins in cheeses have been found to be hydrolyzed by lactic acid bacterial enzymes. In fermented milk products, such as sour milk, yogurt, and quark, phosphopeptides, ACE inhibitory peptides, immunomodulatory peptides, and casomorphins have been found.

Another common way to produce bioactive peptides is enzymatic hydrolysis by a range of different proteolytic enzymes, such as alcalase, chymotrypsin, pancreatin, and pepsin as well as enzymes from bacterial and fungal sources. After hydrolysis, the peptides in hydrolysates have been fractionated and enriched using different methods, such as precipitation with salts or solvent, ultrafiltration, and chromatography. Application of an ultrafiltration membrane reactor, for continuous extraction of permeates enriched with bioactive fragments, has been described for the production of antithrombotic peptides. Membranes consisting of negatively charged materials have been used to desalt whey protein hydrolysates and to enrich cationic peptides having antibacterial properties. This technique provides new possibilities for enriching low-molecular-mass peptides and is easily upscaled.

Conclusions

There are plenty of reports on the bioactivity of milk protein-derived peptides that may be advantageous for human health. These effects include lowering of blood pressure and lipid levels. Evidence for the beneficial effects of bioactive peptides under the conditions of obesity has also been raised. Since cardiovascular disease is a significant public health problem worldwide, biologically active peptides may be of vital interest in maintaining a healthy population. Products that contain bioactive peptides are already on the market (**Table 4**). Until now, however, most of the claimed physiological effects of bioactive peptides have been observed *in vitro* or in animal model systems. So far, evidence for health effects exists only for a few peptides. The effect of fermented milk products, containing two tripeptides, on blood pressure has been scientifically proven in human trials.

Both casein- and whey protein-based hydrolysates exhibiting a high ACE inhibitory activity have been produced, a number of patents have been granted in this area, and such hydrolysates are now commercially available from dairy companies. Further progress in this area might be achieved by the use of predictive models and technological developments in large-scale continuous production and isolation of peptides. Studies on the interactions of bioactive peptides with other food components during processing and preparation and the effects of these interactions on their bioactivity are scarce. It cannot be excluded that processing may influence the final activity of peptides in real food systems

Table 4 Commercial dairy products/ingredients with health claims based on bioactive peptides

Brand name	*Type of product*	*Bioactive peptides*	*Health claims*	*Manufacturer*
Calpis	Sour milk	VPP, IPP	Reduction of blood pressure	Calpis Co., Japan
Evolus	Calcium-enriched fermented milk drink	VPP, IPP		Valio Oy, Finland
C12 peptide Casein DP	Casein hydrolysate	FFVAPFPEVFGK α_{s1}-casein f(23–34)		Kanebo Ltd., Japan DMV International, The Netherlands
Bio Zate	Hydrolyzed whey protein isolate	Whey protein-derived peptides		Davisco, USA
Bio PURE-GMP	Whey protein isolate	Glycomacropeptide κ-casein f(106–169)	Prevention of dental caries Antithrombotic, antimicrobial, and antiviral	Davisco, USA
Prodiet F200	Flavored milk drink Confectionery Capsules	α_{s1}-Casein f(91–100) YLGYLEQLLR	Reduces stress	Ingredia, France

since several peptides, such as ACE inhibitory peptides, have reactive amino acids in their active C-terminal zone (e.g., lysine, arginine).

Given the fact that milk protein-derived bioactive peptides are safe, they are good candidates for inclusion in healthy lifestyle changes to prevent or reduce diseases such as cardiovascular diseases, obesity, and stress.

See also: **Fermented Milks:** Health Effects of Fermented Milks. **Nutrition and Health:** Nutritional and Health-Promoting Properties of Dairy Products: Bone Health; Nutritional and Health-Promoting Properties of Dairy Products: Colon Cancer Prevention.

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Functional Properties of Milk Proteins

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Introduction

Owing to their nutritional importance and physicochemical properties, proteins are the main functional components in milk. A vast range of milk protein products, specifically designed for particular applications, is manufactured by the dairy industry. Many different grades and types of caseins and caseinates, whey protein concentrates and isolates, milk protein concentrates and isolates, and hydrolyzed proteins can be manufactured by altering processing steps during manufacture (see **Figure 1**). For successful marketing and utilization of milk protein products, it is important to understand the 'functional properties' of the product. In practical terms, the functional properties of proteins can be defined as those characteristics, except nutrition, that determine their satisfactory use in food products. These characteristics may include structure, appearance, texture, viscosity, and mouthfeel. Milk proteins possess functional properties that provide desirable textural or other attributes to the final product and for this reason have found numerous applications in traditional dairy products and in other foods. Some important functional properties for which milk proteins are added to food products and the relevant food systems are shown in **Table 1**.

The functional properties of proteins are governed by intrinsic properties; that is, their structural characteristics (e.g., size, charge, and surface hydrophobicity). These intrinsic properties are themselves affected by many extrinsic or environmental factors, such as pH, ionic strength, and temperature, and also by interactions between the proteins and other materials in the food system. The functional properties of milk protein products may be considered to be the consequence of the molecular structures and the interactions of milk proteins with other food components, such as fat, sugars, polysaccharides, salts, and flavor and aroma compounds. These interactions occur at two levels. First, the processing of milk into milk protein products alters the native structures and induces interactions of proteins that can exert a negative or positive effect on functional properties. Second, the milk proteins interact with other food components during the manufacture of prepared food products.

The functional properties of proteins may generally be classified into two main groups, hydrodynamic or hydration related, which includes water absorption, solubility, viscosity, and gelation. Functional properties such as emulsification, foaming, and film formation are related to the surface-active properties of proteins. Proteins usually exert several inter-dependent functional properties simultaneously in each food application. The functional properties of proteins vary with pH, temperature, ionic strength, and concentration of calcium and other polyvalent ions, sugars, and hydrocolloids, as well as with processing treatments.

Solubility

Protein–water and protein–protein interactions in aqueous systems are vitally important and control the dispersibility, viscosity, and solubility properties of proteins. A sensitive balance between repulsive and attractive intermolecular forces, which are, in turn, controlled by protein and water structures and affected by environmental factors, controls solubility. Generally, proteins are soluble in water when electrostatic and/or hydration repulsion between proteins is greater than the driving forces for hydrophobic interactions. Thus, the polar and ionizable groups of proteins largely confer water solubility.

The solubility of dried protein ingredients in aqueous systems over a range of pH, temperature, and protein concentration provides a good predictor of other functional properties, although this general rule may not always apply, particularly in the case of highly hydrolyzed milk proteins. Solubility is generally defined as the amount of protein in a sample that goes into solution or into colloidal dispersion under specified conditions and is not sedimented by low centrifugal forces. The most common methods for determining solubility involve preparation of a protein solution of known concentration (0.5–5.0%), centrifugation at a force ranging from 500 to 65 000 *g* for 5–40 min, and then protein analysis of the supernatant. The solubility of milk protein products depends on the chemical nature of the proteins in the product, processing treatments used, physical form of the powder, pH, temperature, and ionic concentration of the solution. In general, high-temperature treatments during the manufacturing process result in low solubility of the final product. Salts may enhance the solubility of protein up to a point and then reduce it. The pH affects

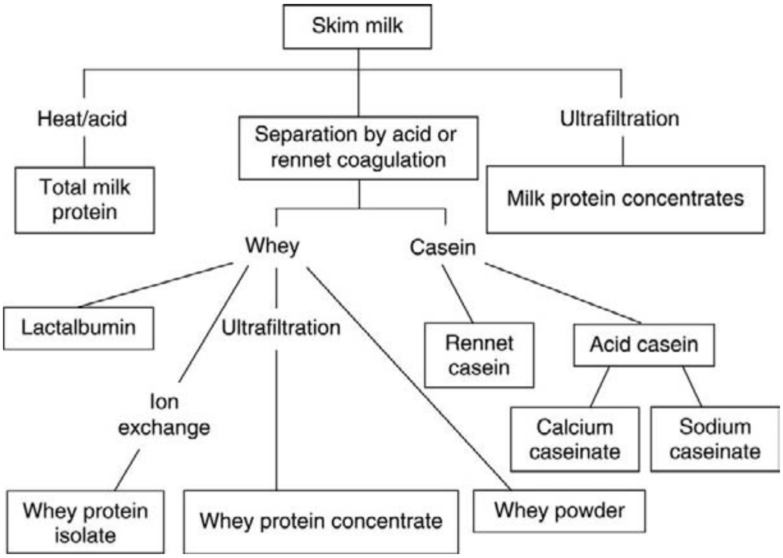

Figure 1 Functional protein products from milk.

Table 1 Functional properties of milk proteins in food systems

Functional property	*Food system*
Solubility	Beverages
Emulsification	Coffee whitener, cream liqueurs, salad dressings, desserts
Foaming	Whipped toppings, shakes, mousses, cakes, meringues
Water binding	Bread, meats, bars, custard, soups, sauces, cultured foods
Heat stability	UHT and retort processed beverages, soups and sauces, custard
Gelation	Meats, curds, cheese, surimi, yogurt
Acid stability	Acid beverages, fermented drinks

the charge and electrostatic interactions between protein molecules. Above and below the isoelectric point, proteins have a net negative or positive charge and enhanced solubility. At the isoelectric point, attractive forces predominate, causing protein molecules to associate, resulting in a loss of solubility.

Rennet and acid caseins, lactalbumin and coprecipitates are insoluble in water, and their food uses are limited to nutritive fortification or water binding. However, caseinates (Na, K, and NH_3) have excellent solubility at pH above 5.5; 10–15% sodium caseinate solutions can be readily prepared at pH 6.0–7.0. Calcium caseinate forms a coarse colloidal dispersion under these conditions. Caseinates are insoluble at the isoelectric pH (pH 4.0–5.0), but are also soluble at pH below 3.5, resulting in a U-shaped pH–solubility profile. Sodium forms of ‘conventional’ coprecipitates are less soluble than sodium caseinate, but the coprecipitates prepared from milk heated at alkaline pH values have solubility similar to that of caseinates. Enzymatic hydrolysis has been applied frequently to improve the solubility of caseinates in the isoelectric region.

Milk protein concentrates (MPCs), produced by ultrafiltration/diafiltration of milk, with up to 70% protein have good solubility at pH 7.0, but MPCs with a very high protein content (e.g., 85% protein) are generally known to have poor solubility upon reconstitution in water at 20 °C. The solubility improves at higher reconstitution temperatures but decreases with storage time at elevated temperatures. The major factor affecting the solubility behavior of MPC appears to be related to the rate of water transfer into the powder particle rather than to the thermal processes during manufacture. Various methods for manufacturing MPC with improved solubility in cold water have been proposed, involving the addition of a monovalent salt to the ultrafiltered retentate prior to drying or partial replacement (~30%) of the calcium content of ultrafiltered retentate by sodium ions.

Whey proteins show excellent solubility over the entire pH range, which enables them to be used in acid beverages. However, heat-induced denaturation renders them insoluble in the pH range 4–5. The level of denaturation and subsequent loss of solubility at pH 4.6 depends on heating temperature and time, whey pH, and ionic calcium concentration. Processing treatments used in the manufacture of whey protein concentrates (WPCs) and whey protein isolates (WPIs) may sometimes cause small amounts of denaturation, which tends to reduce their solubility. The solubility of whey proteins is generally not altered by ultrafiltration or spray drying, although pasteurization may cause up to 20% denaturation with a consequent loss of solubility.

In contrast to whey proteins, sodium caseinate is remarkably stable to heat treatments; a 5% solution at pH 7.0 can be heated at 110 °C for up to 8 h without loss of solubility. Calcium caseinate is less stable, and a 1% solution gels at 50–60 °C.

Viscosity

Milk protein products are widely used in a range of foods, such as soups, sauces, salad dressings, and dairy foods, to provide desirable viscosity and texture. Viscosity is related to quality attributes, such as physical appearance and mouthfeel, of food products. The viscosity of protein dispersions is governed by composition, size, shape, and charge of the protein molecules, which are influenced by environmental conditions, such as temperature, concentration, pH, ionic strength, and previous processing treatment history. The viscosity also reflects intermolecular interactions, resulting in the formation of weak and transient networks.

Different types of viscometers (e.g., 'Brookfield' viscometer, Ferranti-Shirley cone-and-plate viscosimeter, Haake coaxial cylinder viscosimeter, and Bohlin rheometer) have been used to study the viscosity of milk protein products. The values are usually expressed as relative or apparent viscosities.

The viscosity of sodium caseinate solutions increases exponentially with casein concentration; at concentrations >15%, caseinate solutions exhibit pseudoplasticity, which makes them difficult to process. In the temperature range 25–60 °C, the viscosity of a caseinate solution is logarithmically related to the reciprocal of the absolute temperature. Acidic casein solutions (pH 2.4–2.9) have higher viscosity than solution >pH 6 for the same protein concentration, and the solution viscosity decreases when the temperature is increased from 25 to 60 °C; at higher temperatures, the viscosity increases again. The apparent viscosity of sodium caseinate solutions is strongly dependent on pH with a minimum at pH 7.0. The addition of calcium causes considerable changes in the viscosity of sodium caseinate solutions, but the effect is dependent on pH, temperature, and protein concentration.

Calcium caseinate has a relatively low viscosity that decreases with increasing temperature and increases above pH 7.0. Solubilized coprecipitates tend to be more viscous than sodium caseinate.

Native whey protein solutions have very low viscosity compared with caseinates, and their viscosity decreases with increasing temperature between 30 and 65 °C. However, the viscosity increases at higher temperatures, due to denaturation and aggregation of the whey proteins. Solutions of WPC exhibit Newtonian behavior in the range 4–12% protein; at higher concentrations, more pseudoplastic behavior is observed.

Water Binding and Hydration

The ability of protein to hydrate and thus entrap or bind water is important in many foods, particularly when protein ingredients are used in viscous products, such as soups, custards, and doughs. The amount of water that can be retained by a protein matrix under a given set of conditions (expressed as g $H_2O\,g^{-1}$ dry protein) is referred to as its water-holding or water-binding capacity.

Water associates with protein in a number of ways. Bound water is not available as solvent, does not freeze, and has different physical properties from bulk water. This water is associated with proteins via hydrogen bonding to polar groups. Occasionally, non-polar groups are forced into water as a part of a specific protein structure. In addition, water may be held physically in capillaries within the product or trapped within the food structure by surface forces. In general, proteins that are completely soluble are less effective at water binding than those that are less soluble. An important aspect of protein hydration is the rate and extent of swelling.

Solution conditions, such as pH, ionic strength, and temperature, affect the hydration of proteins. Hydration capacity is minimal at the isoelectric point, where protein–protein interactions are favored over protein–water interactions. Low salt concentrations can also increase the hydration capacity of proteins.

Because a majority of the amino groups in globular proteins, such as whey proteins, are buried in the protein interior, the hydration capacity arises predominately from binding of water to amino acid residues on the protein surface. WPIs and WPCs are generally very soluble and thus do not bind large amounts of water in the native conformation. Hydration values determined for native individual whey proteins range from 0.32 to 0.60 g $H_2O\,g^{-1}$ protein. Heat treatment of the whey proteins causes them to unfold and to increase their water-binding capacity. Thus, most applications of whey proteins as water binders are in food systems, such as meat patties, sausages, breads, and cakes, that receive sufficient heat treatment to denature the proteins and increase the water-binding capacity. In yogurt and other similar products, whey proteins may be added to bind water that might be released during processing or storage. The rate and the extent of heating of these products are important variables in determining the effectiveness of water binding. They may be heated to a temperature that is high enough to denature the whey proteins without causing excessive aggregation and loss of water-holding capacity.

Casein micelles bind large quantities of water in their native state; values ranging from 2 to 4 g $H_2O\,g^{-1}$ protein have been reported. In contrast, sodium caseinate binds about 0.4 g $H_2O\,g^{-1}$ protein. A large amount of water in the micelles is largely due to entrapment of water in the micellar calcium phosphate–casein matrix and to binding by the hydrophilic surface of the micelles. It has been reported that acid casein, sodium caseinate, soluble low calcium coprecipitate, insoluble low calcium coprecipitate, and WPC absorbed 0.68, 2.50, 2.60, 0.75, and 0.65 g $H_2O\ g^{-1}$ protein, respectively.

Various techniques have been used to study water–protein interactions. These include sorption isotherms, nuclear magnetic resonance, dielectric dispersion, laser light scattering, X-ray scattering, intrinsic viscosity, and various spectroscopic techniques (e.g., infrared and Raman spectroscopy).

Emulsification

Milk proteins have excellent emulsifying properties and are therefore used in many food formulations as emulsifying agents. The manufacture of food emulsions, typically oil-in-water systems, is a highly energetic and dynamic process in which oil–water interfaces are created and are stabilized by the adsorption of surface-active materials. Caseins possess high surface hydrophobicity with a well-balanced distribution of hydrophilic and hydrophobic domains and possess a high degree of conformational flexibility, which allows them to interact strongly at the oil–water interface. Whey proteins also adsorb rapidly to, unfold and reorientate at the oil–water interface forming emulsions that are only slightly less stable than those formed with casein under the same conditions. The emulsifying ability of ‘aggregated’ milk protein products, such as MPC and calcium caseinate, is much lower than that of whey proteins or sodium caseinate (i.e., much higher concentrations of protein are required to make stable emulsions).

A wide variety of apparatus, including mixers, valve homogenizers, ultrasonic equipment, and several types of blenders, can be used to prepare emulsions. Two important fundamental properties, droplet size distribution and surface protein coverage, must be measured to characterize emulsions stabilized by proteins. The droplets in food emulsions are widely dispersed in size and may range from 0.1 to 10 μm in any one system. The state of the droplet size distribution reflects the emulsifying capacity of the proteins, the energy input during emulsion formation, as well as the effects of various factors (such as pH, temperature, ionic strength, and ratio of the two phases) on the surface activity of the proteins. In addition, the droplet size distribution influences markedly the properties of food emulsions, such as stability, viscosity, texture, and mouthfeel.

The surface protein coverage of emulsion droplets, usually expressed as milligrams of protein per unit area of the dispersed phase ($mg\ m^{-2}$), depends on the concentration and type of protein as well as the conditions used for emulsion formation. The factors that affect the surface protein coverage include protein concentration, volume of oil, energy input, state of protein aggregation, pH, ionic strength, temperature, and ionic calcium concentration. In emulsions formed with sodium caseinate or WPC, the surface protein concentration ($mg\ m^{-2}$) increases with an increase in protein concentration until it reaches a plateau value of about 2.0–3.0 $mg\ m^{-2}$. The surface protein concentration of emulsions formed with MPC is in the range 5–20 $mg\ m^{-2}$ depending on the protein concentration used in making the emulsions.

When caseinates or whey protein products are used as emulsifying agents, there is competition between individual proteins for adsorption onto the oil–water interface. A number of researchers have reported that β-casein is adsorbed in preference to α_{s1}-casein and other proteins in emulsions stabilized by a mixture of purified β- and α_{s1}-caseins, as it is the most surface active and hydrophobic. The preferential adsorption of the β-casein in sodium caseinate appears to be dependent on the concentration of protein used in making the emulsions. β-Casein is adsorbed in preference to α_{s1}-casein at low protein concentrations (<2.0%), whereas a larger amount of α_{s1}-casein than β-casein is present at the interface at high protein concentrations. When casein is in the highly aggregated form of casein particles, as in calcium caseinate or MPC, there is very little competitive adsorption and protein exchange. In these systems, the average surface composition is determined by the adsorption of protein aggregates of fixed composition.

It has been shown that preferential adsorption of caseinate over whey proteins occurs in emulsions made with caseinate and whey protein mixtures. No preferential adsorption between β-lactoglobulin and α-lactalbumin is observed. Once the adsorbed β-lactoglobulin molecules become partially unfolded and closely packed together at the interface, the slow intermolecular linking by disulfide bonds leads to the formation of a structured film, which continues to strengthen irreversibly with time.

The primary processes leading to instability of emulsions are creaming, flocculation, coalescence, and phase inversion. Creaming refers to the gravitational separation of emulsified droplets to form a densely packed phase without change in droplet size. Flocculation denotes the aggregation of droplets via interactions between adsorbed proteins. Although this generally modifies the physical properties of the emulsion, the particle size distribution remains unchanged and the ‘flocs’ can be dispersed readily as these interactions are usually weak. There are two kinds of flocculation, bridging flocculation and depletion flocculation, depending on the amount of protein available to cover the interface of oil/water droplets. Coalescence (i.e., an increase in droplet size) gradually results in separation of the oil and aqueous phases. Unlike creaming and flocculation, coalescence is always irreversible. Coalescence requires rupture of the stabilizing film at the oil–water interface, but this occurs only when the layer of continuous phase between the droplets has thinned to a certain critical thickness.

Emulsions stabilized by sufficient quantities of milk proteins are generally very stable to coalescence during prolonged storage, but they can be susceptible to different types of flocculation, which in turn leads to enhanced creaming or serum separation. When there is insufficient protein to fully cover the oil–water interface during homogenization, bridging flocculation and/or coalescence of droplets may occur during or immediately after emulsion formation. Bridging flocculation is commonly observed in emulsions formed with aggregated milk protein products, such as calcium caseinate or MPC, in which the droplets are bridged by casein aggregates or micelles. Optimum stability can generally be attained at protein concentrations high enough to allow full saturation coverage of the oil–water interface. However, at very high protein-to-oil ratios, the presence of excess, unabsorbed protein may lead to depletion flocculation in some emulsions. For example, sodium caseinate-based emulsions show depletion flocculation when the protein content is increased to above 3.0 wt.% in a 30% oil emulsion. This flocculation results in a marked decrease in creaming stability but is reversible by shearing or dilution with water. Emulsions formed with calcium caseinate, MPC, or whey proteins do not show depletion flocculation and are stable against flocculation, coalescence, and creaming for several weeks.

The methods used for evaluating the emulsifying properties of proteins can be separated into two categories. The first category, which includes the emulsifying capacity (EC) and the emulsifying activity index (EAI), provides direct information on emulsifying potential. The second category provides an estimation of the effects of proteins on the emulsion stability (ES). However, complete characterization of the emulsifying properties of proteins requires both of these approaches. In the EC test, the maximum amount of oil emulsified by a protein dispersion just prior to the inversion point is determined. The EAI is a rough estimate of the dispersed particle size of the emulsion, based on the interfacial area (calculated from turbidity) per unit of protein. The EAI measures the ability of the protein to disperse the oil phase. The ES can be evaluated by measuring the rate at which an emulsion creams or breaks. The rate of these changes can be measured by determining the distribution of oil droplets or by estimating the fat or water content in the upper or lower part of the emulsion. Other methods, measuring the dielectric constant of the upper part of the emulsion or the electrical conductivity of emulsions, have also been proposed. In addition, numerous methods for accelerating the separation process by centrifugation, heating, and so forth have been proposed to evaluate the long-term stability of emulsions. However, these methods should be used with caution because of the poor relationship between accelerated stability and storage stability.

Whipping and Foaming

As milk proteins are surface active, they have the ability to adsorb to the air–water interface during foam formation. Foams are most commonly formed by mechanically dispersing air into a solution containing surface-active agents. Essential for the formation of protein-based foams is a rapid diffusion of the protein to the air–water interface to reduce surface tension followed by partial unfolding of the protein. Further interactions between protein molecules at the interface lead to the formation of a cohesive film with a certain degree of elasticity, which stabilizes the foams. Milk proteins, in particular WPC and WPI, are capable of forming a cohesive structure surrounding the foam bubbles as well as providing excellent surfactant properties.

Foaming properties are commonly determined as foam volume (overrun) and foam stability. Caseinates generally give higher foam overruns but produce less stable foams than WPCs. The foaming properties are influenced by many factors, including protein concentration, level of denaturation, ionic strength, preheat treatment, and presence of lipids. Generally, overrun increases with protein concentration to a maximum value after which it decreases again; for WPC foams, this maximum is observed between 8 and 12% whey protein. Partial heat denaturation of the proteins improves the foaming characteristics of WPC, with the effect being greater on stability than on overrun. However, excessive denaturation and aggregation of the proteins have a detrimental effect on the foaming properties of WPCs. The presence of phospholipids and unsaturated fatty acids in WPCs can rupture the foam, as a result of their higher surface activity and their thinning effect on the protein film. Maximum overrun and foam stability have been observed at pH 4–5. This is attributed to the formation of a more cohesive protein film at the air–water interface, due to decreased electrostatic repulsions. Different ions vary in their effect on foaming properties. Partial hydrolysis of whey proteins with proteolytic enzymes increases the foam volume but reduces its stability. However, limited hydrolysis of WPC combined with heating at 55–70 °C gives excellent overrun and stability, provided the pH is between 7 and 8 before whipping.

Heat Stability

Heat stability, or lack of it, may be an important functional property in some food applications. Milk proteins vary in their susceptibility to heat-induced aggregation and precipitation. Casein micelles in milk or in MPC and aqueous solutions of sodium caseinate are remarkably heat stable; a 3% solution of sodium caseinate at pH 7 can be heated at 140 °C for 60 min, whereas skim milk is usually stable at 140 °C for about 20 min at pH 6.7. Calcium caseinate containing 1.5% calcium is not heat stable, with aggregation and coagulation occurring above 45 °C. Obviously, sodium caseinate or skim milk is very suitable for use in products where thermal stability is required, such as soups and sauces.

In contrast to caseins, whey proteins are readily denatured above 70 °C, followed by aggregation and precipitation. The susceptibility of whey proteins to heat denaturation is influenced by factors such as pH, total solids, ionic calcium concentration, protein concentration, and the presence of sugars and protein-modifying agents.

A common method for measurement of heat stability involves heating a protein solution of known concentration and pH in an oil bath for a given period of time at defined temperatures. Changes in free amino nitrogen, soluble ammonia, turbidity, and trichloroacetic acid (TCA)-soluble and pH 4.6-soluble amino nitrogen have been attributed to the effects of heating on protein solutions. The heat stability of a protein product can also be measured by assessing the ability of a reconstituted protein solution (5%) to withstand retort sterilization (121 °C, 16 min). However, a more appropriate approach is to measure the heat stability in a model food system simulating the final food application.

Gelation

Milk proteins have the ability to form rigid, heat-induced irreversible gels that hold water and fat and provide structural support. The ability of milk proteins to undergo gelation upon the addition of acid or rennet to milk is well known. Caseinates are rarely added to food products as gelling agents, but WPC and WPI products can provide gelation under a wide range of conditions.

The heat-induced gelation of whey proteins involves a series of steps, starting with the unfolding of protein molecules, followed by their aggregation in aqueous solution. A gel is formed when the extent of aggregation exceeds some critical level; a three-dimensional, self-supporting network that traps the solvent in the system is formed. When the extent of aggregation is below some critical minimum, soluble aggregates or a precipitate will form. Therefore, gel formation and the properties of the gel depend on the type and number of protein–protein interactions, which in turn are affected by variables such as type of protein, protein concentration, temperature, pH, ionic strength, and the presence of other ingredients, such as lactose.

The most outstanding microstructural feature of the gels formed from whey proteins is the presence of a homogeneous network of connected protein particles, usually referred to as 'fine-stranded' gels, or aggregates that form a three-dimensional matrix with the interstices filled by a liquid or aqueous solution, usually referred to as 'particulated' gels. β-Lactoglobulin, bovine serum albumin (BSA), and whey protein products are known to form either type of gel, depending on the pH and ionic strength.

Generally, heating a protein solution above the minimum denaturation temperature of the constituent proteins is required for gel formation. The strength of whey protein gels is affected by the concentration and purity of the protein. A protein concentration of 7.5% or greater is needed to form a strong gel from WPC at pH 7.0 upon heating for 10 min at 100 °C. Pure solutions of β-lactoglobulin and BSA can form gels at 5 and 4% protein, respectively, after heating for 15 min at 90 °C. As the protein concentration is increased, the number of potential interactions between molecules is enhanced, resulting in an increased gel strength, a reduced gelling time, and a finer gel network. When other factors are maintained, gel hardness increases with increasing heating temperature and time. Heating rate also affects the gelation process; slow heating allows the proteins enough time for unfolding and aggregation, resulting in much stronger gels.

Several investigators have found that opaque gels are formed on heating whey protein solutions at pH 4–6, whereas the gels are translucent above and below this pH range. Opaque gels have been described as soft and creamy, and they tend to lose water during compression. The transparent gels formed at pH below 4 are weak (low values for fracture stress) and brittle (low values for fracture strain), whereas those formed at pH above 6.0 are strong and rubbery, with high fracture stress and strain values. The variations in gel properties with pH are attributed to variations in electrostatic interactions and disulfide bonding.

It is well established that salts have a major effect on the properties of whey protein gels, especially at pH values far from the isoelectric point where the proteins carry a large net charge. Addition of NaCl or $CaCl_2$ to a dialyzed solution of WPC or WPI results in an increase in gel strength until maximum values are reached, and then the gel strength decreases at higher salt concentrations. A maximum in a gel property has generally been attributed to an optimum balance between protein–protein and protein–solvent interactions at a particular salt concentration. Calcium ions influence protein–protein interactions by shielding electrostatic repulsion and also by forming calcium bridges between protein molecules. In general, divalent calcium ions have a much greater effect on gel properties than monovalent sodium or potassium ions. This is due to the higher

Table 2 Key functional properties of different milk protein products

	Caseinates		*Whey protein products*		
	Na	*Ca*	*WPC*	*WPI*	*Milk protein concentrates*
Solubility	xxx	x	xxx	xxx	xx
Emulsification	xxx	x	xx	xx	x
Foaming	xxx	x	xx	xx	x
Water binding	xxx	x	x	x	x
Viscosity	xxx	x	x	x	x
Gelation			xxx	x	
Heat stability	xxx	x	x	x	x
Acid stability	x	x	xxx	xxx	x
Freeze–thaw stability	xxx	x	x	x	x

x, poor; xx, good; xxx, excellent.

binding affinity of calcium to specific binding sites, specific conformational changes, and intermolecular bridging.

Calcium caseinate solutions at protein concentrations greater than 15% form reversible gels upon heating to 50–60 °C. The gelation temperature increases with protein concentration from 15 to 20% and with pH in the range 5.2–6.0. Upon cooling, these gels are changed into viscous liquids.

The heat-induced gelation of protein can be described as the gelation time, as observed visually, or the gelation temperature, measured as the temperature at which it is possible to invert a tube containing the protein solution without movement of the contents. The rheological and textural properties of gels are determined using small, non-destructive strain techniques (i.e., sample deformation) and destructive techniques (i.e., sample rupture). In the first case, rheological characteristics using small, non-destructive strain allow a dynamic measurement of rheological transitions. Changes in rigidity or shear modulus (stress/strain), storage modulus (G'), and loss modulus (G') can be measured as a function of time or temperature.

Table 2 summarizes the important functional properties of a wide range of milk protein products. The relative importance of nine key functional properties to each product is also indicated.

See also: **Milk**: Physical and Physico-Chemical Properties of Milk. **Milk Protein Products**: Caseins and Caseinates, Industrial Production, Compositional Standards, Specifications, and Regulatory Aspects; Milk Protein Concentrate; Whey Protein Products. **Rheology of Liquid and Semi-Solid Milk Products**. **Whey Processing**: Utilization and products.

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MILK QUALITY AND UDDER HEALTH

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Test Methods and Standards

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Introduction

The quality of milk may be evaluated by measuring the parameters that indicate both its suitability for consumption or processing into dairy products and the health status of the cow or herd producing the milk. The principal parameter routinely used internationally in this context is the somatic cell count (SCC) per milliliter of milk, which enumerates epithelial cells shed from the udder tissue and white blood cells (WBCs) from the blood. Milk SCC results are generally expressed as the number of cells per milliliter of milk, or converted to a log value. It is one of the main indicators of udder health in lactating animals and, in many countries, it is used in animal health programs as a criterion for bulk milk payment schemes.

Different types of milk samples may be analyzed for SCC (or any other quality parameter), depending on the reason for testing:

1. milk from an individual quarter of a single cow;
2. mixed milk from all four quarters of an individual cow;
3. milk from a farm bulk tank, containing a mixture of milk from all the cows in the herd.

For a sample from an individual cow, SCC directly reflects the migration of WBCs into the milk (transfer across the blood/milk barrier) and therefore reflects the health status of the cow, and in particular the occurrence of mastitis. Bulk milk SCC therefore gives an indication of the overall average health of the milk-producing herd.

The quality of milk may also be assessed through measurement of the microbiological population, by determining either the total bacterial count (TBC) or the presence of specific types of bacteria. Bacteria in milk may originate from the udder, the environment, milk-handling equipment, and farm personnel (*see* **Milking and Handling of Raw Milk**: Milking Hygiene). The primary concern regarding the presence of bacteria in milk unquestionably relates to the health of the consumer; it was due to the presence of pathogenic bacteria such as *Mycobacterium tuberculosis* in milk that pasteurization of milk was introduced almost a century ago (*see* **Plant and Equipment**: Pasteurizers, Design and Operation). However, the presence of nonpathogenic spoilage bacteria in milk (such as *Pseudomonas* species) may also have profound implications for the keeping quality of the liquid milk or dairy products made from milk, highlighting again the importance of routine testing of the microbiological quality of milk. Milk TBC is usually applied as a payment or rejection standard in parallel with milk SCC. A partial list of the most significant bacteria in milk is given in **Table 1**.

In this article, the methods by which SCC, udder health, and indices of milk microbiological quality are evaluated will be reviewed. In the accompanying article (*see* **Milk Quality and Udder Health**: Effect on Processing Characteristics), the relationships between some of these parameters and milk processability, and the quality of dairy products, will be discussed.

Table 1 Bacteria that may be present in raw milk and their significance

Bacterium	*Origin*	*Significance*
Bacillus cereus	Feed, soil, dirt	Spoilage bacterium[a]
Brucella abortis	Diseased cow	May have disease links
Campylobacter jejuni	Dung, water	Food poisoning
Clostridium botulinum	Soil, water	Causes botulism
Clostridium perfringens	Soil, dung, water	Food poisoning
Corynebacterium bovis	Udder	No major problems
Coxiella burtenii	Diseased cattle	Causes Q fever in humans
Escherichia coli	Fecal contamination	Food poisoning/spoilage
Lactobacillus spp.	Milk handling	Spoilage (souring)
Lactococcus spp.	Milk handling	Spoilage (souring)
Listeria monocytogenes	Soil, dung, feed	Causes meningitis
Mycobacterium tuberculosis	Diseased cow	Human pathogen
Mycobacterium avium subsp. *paratuberculosis*	Cow	May cause Crohn's disease
Pseudomonas spp.	Milk handling	Spoilage bacterium
Salmonella spp.	Dung, water	Food poisoning
Staphylococcus aureus	Diseased cow, skin	Food poisoning
Streptococcus agalactiae	Udder, milking parlor	Food poisoning/spoilage
Streptococcus dysgalactiae	Udder, milking parlor	Food poisoning/spoilage
Streptococcus uberis	Udder, milking parlor	Food poisoning/spoilage

[a]May produce toxins at very high levels (but milk will spoil before this point).

A number of other factors that are known to affect milk quality, such as variations in the composition, freezing point, and free fatty acid content of milk, may be rooted in on-farm handling practices or may be linked to the status of the cow. Internationally, dairy processors may include such quality parameters when considering the acceptability or value of milk. Furthermore, testing schemes operated by processors may routinely analyze milk for the presence of undesirable additives or contaminants, such as antibiotics or cleaning and sanitizing agents, which would have both public health and product quality implications. However, in this article, only tests for udder health and microbiological quality will be discussed in detail.

Somatic Cells in Milk and Mastitis

Somatic cells in milk comprise epithelial cells shed from the udder tissue and WBCs from blood. The basic function of the WBC in the mammary gland is defense against bacterial invasion. In normal bovine milk from noninfected quarters, the SCC is typically $<100\,000\,\mathrm{ml}^{-1}$, of which ~50% are epithelial cells and the others are WBCs including ~15% macrophages, 10% lymphocytes, and 25% polymorphonuclear leukocytes (PMNs). In other dairy animals such as goats or sheep, SCC in noninfected halves is typically higher and can be ~150 000 to $300\,000\,\mathrm{ml}^{-1}$.

Upon entry of bacteria into the mammary gland, the response of the bovine immune system depends on the invading species. For example, infection by Gram-negative bacteria such as *Escherichia coli* typically results in a rapid and massive influx of somatic cells, mainly PMNs (such that SCC exceeds $1\,000\,000\,\mathrm{ml}^{-1}$, of which >90% are PMNs, within 6 h). In contrast, Gram-positive bacteria such as *Staphylococcus aureus* and coagulase-negative staphylococci can 'hide' for several days in the mammary gland and then develop into a clinical and/or chronic infection manifested by a fluctuating moderate influx of SCC between ~500 000 and $>1\,000\,000\,\mathrm{ml}^{-1}$. In the latter case, the number of PMNs is lower and the number of lymphocytes, mainly T-CD8^{+}, is higher.

Mastitic infections may be classified as either clinical or subclinical. The former is defined by the International Dairy Federation (IDF) as udder inflammation characterized by visible abnormalities in the milk and/or udder, and cases may be subclassified as mildly, moderately, or seriously severe. Cows with clinical mastitis may exhibit swelling of the udder and apparent pain or discomfort, and their milk may contain flakes or clots. Subclinical mastitis is defined as inflammation that is not visible and requires a diagnostic test for detection, such as measurement of SCC along with bacteriology, and is the most prevalent form of the disease (15–40 subclinical cases for every clinical case today). Chronic mastitis refers to cases where the infection is of long duration and may show periodic clinical symptoms.

In general, although milk SCC is an indication of the presence or absence of infection, any estimate of SCC should be supplemented by microbiological analysis to determine the true cause and nature of any indicated infection. Rapid diagnosis of subclinical mastitis presents a particular challenge and depends on the analysis of milk from individual cows or quarters. While subclinical mastitis can always be diagnosed by bacteriological methods, such approaches are slow, and hence a number of more rapid methods for early and rapid identification of subclinical infection have been developed, as will be discussed later.

Overall, the SCC of milk is an extremely important parameter of milk quality, as it reflects the health status of a cow (or a herd), and is known to be an indicator of the quality and processing properties of milk; hence, SCC is widely used as a payment parameter by dairy companies, as will be discussed later in this article.

Measurement of Milk Somatic Cell Count: Total Counting

The oldest method for enumerating somatic cells in milk is direct microscopic counting, often combined with methylene blue staining, which, although slow and labor-intensive, remains in many instances the reference method against which other methods are calibrated.

Several methods are being used for on-farm ('cow-side') estimation of SCC and thus for diagnosis of mastitis. In the California mastitis test (CMT), a small sample of milk (from an individual cow or a quarter) is mixed with an alkaline detergent on a special mixing device, and the extent of subsequent formation of a gel is ranked on a simple scale, which relates gel formation to the number of somatic cells in the milk. A slightly more complex variation of the CMT, known as the Wisconsin mastitis test (WMT), is intended as a rapid milk quality test on receipt at dairies, and involves the extent of gel formation being read as the volume of sediment in a calibrated tube.

Several instrumental systems for measuring bulk milk SCC, such as the Fossomatic, Coulter, or Bentley cell counters, are available. Such instruments give SCC in number of cells per milliliter of milk, and provide, when calibrated against microscopic counts, accurate, rapid, and precise measurement of SCC, but are too complex and expensive to be applied routinely on a farm. An online cell counter (OCC) has, however, been invented that may provide a precise cell count for every cow at every milking. Coulter counters use electric counting of formalin-fixed somatic cells passing through an aperture, while Fossomatic systems label the nuclei of somatic cells using ethidium bromide and then count the stained nuclei as they pass through a thin film under a high-energy light source. The Bentley Somacount system uses laser-based flow cytometry, again combined with ethidium bromide staining of DNA. The above methods are generally and routinely used for analysis of large numbers of samples in a dairy testing laboratory.

An indirect instrumental method that gives a measure of the health status of the cow is the electric conductivity measurement, which takes advantage of the influx of ions into the alveoli owing to the opening of the tight junctions when inflammation commences. This can form part of a computerized milking system in modern dairy farms, as manufacturers of modern computerized dairy milking parlors have developed devices to measure electrical conductivity during milkings and compare that conductivity value to the precedent value. Differences in values between milkings can signal to the milker that an unusual increase/decrease in this measure was noted, and the cow must then be checked by other means for the presence of mastitis. The system is used with all models of automatic milking systems.

Measurement of Milk Somatic Cell Count: Differential Counting

While measurement of total milk SCC has been recognized for many years as a very useful index of udder health, recently there has been an increased interest in differential measurement of somatic cell types in milk comprising the total count. The relative numbers of specific cell types, such as PMNs, macrophages, and lymphocytes, were first studied using light or electron microscopy. These are slow and laborious methods. To automate and hasten the cell-sorting process, some researchers in the 1980s used Coulter counter equipment to differentiate counted particles (cells) on the basis of size into groups, which can be enumerated separately. Most recent studies on differential SCC have used flow cytometry, where cells stained by mixing milk samples with a nuclear stain, such as acridine orange, pass singly through a detector cell in a narrow stream of liquid, and are differentiated based on fluorescence properties or cell size and shape into clusters, which can be directly related to different cell types. However, a more accurate method that differentiates between specific cell types involves using monoclonal antibodies targeting specific receptors on the cell surface. This method also allows information about the cell condition and function to be gathered.

Using flow cytometry, it has been confirmed that of the WBCs, macrophages and lymphocytes represent the majority of cells in milk from noninfected udder, while PMNs are the predominant cell types in mastitic milk. On this basis, it has been suggested that PMNs may be a useful tool for diagnosing mastitis, although the complexity and expense of the technique may preclude its routine use for large numbers of samples in dairy testing laboratories. However, flow cytometry undoubtedly offers advantages for a detailed study of the immunological status of the udder. In an alternative approach, monoclonal antibodies against PMNs have been used for the development of an enzyme-linked immunsorbent assay (ELISA) for the enumeration of the level of these cells in milk, and studies are performed to determine the distribution of PMNs in bulk tank and individual milk samples, and PMN levels are correlated with total SCC.

It must be noted that, as with SCC, differential milk SCC may also be affected by factors such as stage of lactation, stress, age of cow, and milking frequency, which must be considered when interpreting data.

Standards for Milk Somatic Cell Count

When considering milk SCC, it is essential to distinguish between the SCC for individual cows, the SCC of milk from individual quarters of the udder of the same cow, and the SCC of mixed milk in the farm bulk tank (bulk tank SCC (BTSCC)), which comprises a weight-averaged SCC of all cows with accepted milk in the herd. While the first two measurements relate directly to the health of the individual cow, the last is a function of the number of infected cows in the herd and the extent of withholding of high-SCC milk. It has been shown that there is a linear relationship between the level of infection in a herd and BTSCC. BTSCC is widely accepted as an indicator of mastitis control practices on farms, herd health and the suitability of milk for processing, but has limitations as a diagnostic tool for measure the cause and etiology of mastitis within a herd. Thus, in cases of high BTSCC, it is necessary to have data for each individual cow in the herd in order to understand and deal with the problem.

Some processors may apply penalties for exceeding certain SCC on even one occasion. However, dairy companies frequently calculate penalties or bonuses applied to individual milk suppliers on the basis of a geometric mean BTSCC over a fixed time period, such as 3 months. The advantages of using geometric means include normalization of data, reduced variance in raw data, which are not normally distributed, and less tendency to penalize single violations.

An SCC for an individual cow >200 000 ml^{-1} is generally regarded as indicating that an inflammatory response is either in progress or has occurred recently. Interpretation of SCC data for individual cows must, however, be tempered by the acceptance that SCC is a dynamic parameter that can vary considerably even in noninfected animals, requiring multiple SCC measurements before interpretations may be offered, and should be accompanied by a bacteriological examination of individual quarters. While an SCC >200 000 ml^{-1} is generally regarded as abnormal and milk with an SCC <100 000 ml^{-1} is generally regarded as normal, the status of cows with an SCC between these limits remains unclear. In some cases, SCC may be corrected to account for the age and breed of the cow, stage of lactation, and milk yield.

Table 2 Current international standards for maximum acceptable SCC

Country	*SCC limit (cells ml^{-1})*
European Union	400 000
New Zealand	400 000
Switzerland	350 000
Australia	400 000
Canada	500 000
United States of America	750 000

SCC, somatic cell count.

There is a lack of international consensus on the value of SCC above which bulk milk should be considered abnormal. EU Directive 92/46 sets the maximum allowable SCC for manufacturing milk at 400 000 ml^{-1} (for a geometric average over 3 months, with at least one BTSCC measurement per month), but the US Pasteurized Milk Ordinance (2003) states that milk with an SCC up to 750 000 ml^{-1} may be accepted. The US limit for SCC is currently the highest in major developed dairy countries (see **Table 2** for a comparison of international standards for SCC).

International debate on SCC limits frequently concerns the distinction between the perception of SCC as an index of milk quality or of milk safety. Unlike the general position of the European Union, BTSCC is not regarded in the United States as being directly linked to food safety, as consumers do not become ill from drinking high-SCC milk. High-SCC milk is nevertheless generally accepted as having an increased risk of the presence of pathogenic bacteria or toxic products. This acceptance drives policy toward reducing the acceptable BTSCC, which should thus reflect a lower incidence of mastitis, milk of better processing quality, and better farm hygiene. A high BTSCC may also suggest a risk of potential use of antibiotic treatment at the herd level, with concomitant human health implications due to the development of antibiotic resistance among human pathogens. Sometimes, BTSCC is also regarded as an index of herd welfare; Scandinavian countries tend to regard herds with a BTSCC >400 000 ml^{-1} as having poor welfare and being unhealthy overall. In general, it is likely that the trend in the future, driven by pressure from consumers and scientists alike, will be toward lower SCC limits, in particular in the United States.

In many countries today, the average SCCs are far below the legal maximum (e.g., in Norway, the mean BTSCC in 2000 was 142 000 ml^{-1}, or a geometric average of 112 000 ml^{-1}). Local milk payment schemes may be targeted toward financial incentives for the production of milk of considerably lower SCC, with penalties imposed when the milk exceeds certain SCC limits. Thus, there are obvious incentives for farmers to produce milk of lower SCC, including the fact that a reduced BTSCC is closely correlated with increased herd milk yield.

A potentially significant question is whether milk SCC can be too low, in terms of the biological function of the 'background' SCC in milk in immune surveillance and protection of the mammary gland and in initiating the cellular response that attracts huge numbers of PMNs into the mammary gland. For example, could animals selected for excessively low SCC ($<15\,000$ cells ml^{-1}) be, in the long term, less resistant to infection? While the key issue today is obviously ensuring low infection rates, this specific aspect of SCC control programs may nonetheless be relevant.

Incentives and the pressure to reduce BTSCC to 200 000 cells ml^{-1} or below have achieved a reduction in the frequency with which milk from mastitic cows is included in bulk milk. However, maintaining this level is expensive for the farmer, owing to the need to cull or treat udder infection. Moreover, it is not clear how further lowering of SCC will benefit the dairy industry. It is important to note that average SCC of 200 000 cells ml^{-1} by itself cannot reflect the health of each individual cow in the herd that is milked into the bulk, since such an average could be the outcome of cows with SCCs as low as 15 000 ml^{-1} and numerous cows with SCCs over a million. Therefore, it is of continuing importance to define new parameters that could influence the quality of the milk supplied to the dairy industry and assure elimination of milk from infected udders.

Measurement of Other Parameters Related to Udder Health

A number of other parameters indirectly related to SCC have been proposed as useful indices of udder health. The enzyme *N*-acetyl-β-D-glucosaminidase (NAGase), for example, has been implicated as an indicator of tissue damage during mastitis, as large quantities are secreted in the mammary gland during inflammation. While it has been suggested to have an antimicrobial effect, the exact physiological function of this enzyme in milk is unclear. NAGase levels in milk are typically highly correlated with SCC, and have been reported to be a better predictor of the extent of protein breakdown in milk than SCC, being more highly correlated with the activity of the indigenous proteolytic enzyme plasmin than SCC. Several rapid methods have been developed for measuring NAGase activity in milk. The alkaline milk proteinase plasmin has been of significant interest as an index of milk quality, both because its activity in milk is very sensitively affected by physiological factors such as mastitis and lactation, and because its hydrolysis of the caseins can have a direct impact on milk and dairy product quality (e.g., negatively affecting rennet coagulation and cheese yield). However, while there are a number of assays available for measuring plasmin activity, it has not been widely considered as a routine test for milk quality, perhaps due to the complex nature of the plasmin system and its effects on milk.

As mentioned above, flow cytometry could differentiate between the various types of somatic cells, and a number of studies have correlated the cell distribution with the cause of inflammation and milk quality. At present, this method is expensive and is used only for research purposes. However, implementation of one or two of these specific cell types into the automated cell counters may improve the quality of information gathered about the bulk milk.

The electrical conductivity (or alternatively electrical resistance) of milk has also been studied as an index of milk quality. This index has the advantage of being amenable to online measurement at the point of milking, allowing automated and immediate identification of problematic cows (both at subclinical and clinical stages) and rapid response (*see* **Mastities Therapy and Control**: Automated Online Detection of Abnormal Milk). A number of rapid automated sensors of milk conductivity have been described in the literature. Changes in milk conductivity reflect changes in the ionic content of milk, such as increases in the levels of sodium and chloride ions during mastitic infections. Increases in milk conductivity have been reported to occur earlier than clinical signs, allowing early diagnosis and rapid treatment of infection. Differences in milk conductivity between quarters of the same udder are particularly useful for detecting subclinical infection. Measurements of the conductivity of milk taken at different points during milking (e.g., foremilk, main milking, and stripping fractions) may provide further information on the magnitude and location of the infection within the quarter. The sensitivity and specificity of online diagnostic techniques may be improved by combining the online measurement of electrical conductivity of milk with other parameters that, alone, would not directly act as diagnostic parameters, such as milk yield and the temperature of the milk.

Other milk parameters that have been studied as potential indicators of changes in udder health include milk pH and levels of lactose, sodium, potassium, bovine serum albumin (BSA), lactate dehydrogenase, antimicrobial enzymes such as catalase and myeloperoxidase, and the proteinase inhibitor α_1-antitrypsin. Recently, acute-phase proteins such as haptoglobin and serum amyloid A, the levels of which are elevated in cases of physiological stress such as mastitis, have been proposed and studied as potential biomarkers of mastitis and raw milk quality.

Comparison of Methods for Evaluating Udder Health

An ideal test for udder health status must be unaffected by any factor other than the infection status of a particular quarter, preventing false-positive identifications. However, almost all parameters, with the exception of specific measurement of individual microorganisms, are affected by some factors other than infection status. For example, milk from older cows may have a higher SCC and NAGase activity, while SCC, NAGase, sodium, potassium, and lactose levels vary with the stage of lactation.

Several investigators have attempted to discriminate between diagnostic power and usefulness of various predictors of mastitis. A number of studies have concluded that SCC or log(SCC) is the best indicator of infected versus noninfected status, while NAGase activity discriminates between infections caused by major (streptococci, *S. aureus*; *E. coli*) or minor (coagulase-negative staphylococci, micrococci) pathogens. NAGase activity decreases much more rapidly than SCC after clinical mastitis. Monitoring BTSCC generally provides a useful indication of the incidence of mastitis on farms suffering outbreaks of contagious mastitis, which causes gross alterations to BTSCC, rather than farms with infections due to environmental pathogens, particularly in the case of subclinical infections, where BTSCC may not be severely affected.

In terms of relating milk SCC to suitability of the milk for production of dairy products such as cheese, some recent studies have suggested that different bacterial types causing mastitis can result in different responses in terms of leukocyte distribution, and differing effects on proteolysis in milk and subsequent coagulation properties. Studies of acute-phase protein levels in milk samples of different SCC have shown detectable levels of haptoglobin and serum amyloid A in milk samples of elevated SCC, and that samples with detectable levels of such proteins had reduced casein levels and evidence of increased proteolytic activity.

The optimum threshold SCC for differentiating between milk samples from infected and uninfected cows or herds has been reported to vary between herds, at different stages of lactation, and even from cow to cow, as individuals respond differently to mastitis pathogens. These factors are crucial for small ruminants, due to the variability of breeds, management, and particularly the seasonality of milk production, which result in increased SCC in the milk of healthy animals toward the end of lactation.

The correlation between SCC and the level of infected quarters is quite low (∼0.6), indicating that many other factors influence milk SCC. Thus, it has been suggested that application of a universal SCC threshold for diagnosis of mastitis may be flawed, as the background SCC varies sufficiently to reduce the sensitivity of diagnosis. However, it is likely that SCC will continue to be regarded as a primary indicator of milk quality, providing information of importance to the farmer, the processor, and the consumer, and that limits for acceptable milk SCC will continue to decrease in the future.

Determination of the Microbiological Quality of Milk

A rapid indication of the microbiological status of milk can be provided by simple tests such as dye reduction methods, where the presence of a high number of bacteria in a milk sample reduces a dye such as methylene blue or resazurin, resulting in a color change. Other rapid tests to determine indirectly the presence of bacteria in milk, through their fermentative activities, include testing the stability of a milk sample to boiling or mixing with alcohol solutions, and measurement of the titratable acidity of milk.

However, the preferred measure of overall bacterial quality of milk is the TBC (sometimes also known as standard plate count (SPC) or total viable colony count (TVC)) of milk, which is an accepted index of both milk hygienic standard and the shelf life and quality of products made therefrom. TBC may be determined by standard plating techniques on a suitable nutrient agar, although standard incubation times before counting can be as long as 3 days. Recent modifications to standard plate counting techniques include use of cylindrical tubes instead of dishes for incubation, spiral plate inoculation instruments, and of preprepared nutrient media in convenient disposable units (such as the Petrifilm system).

To obtain critical information on milk quality more quickly, rapid methods, such as the direct epifluorescent filter technique (DEFT) and automated Bactoscan instruments, have been adopted widely for analysis of large numbers of samples. Both these methods employ automated counting of bacteria whose nuclei have been stained with agents such as acridine orange; in the case of the DEFT system, bacteria are removed and concentrated from trypsin-treated milk by filtration, while the Bactoscan uses centrifugation to achieve this.

Metabolic products of bacterial cells in milk may also be measured, for example by determining the pyruvate content of milk or by determining the bacterial adenosine triphosphate (ATP) content through bioluminescence measurements.

A high milk TBC may result from poor teat hygiene, poor milking machine operation, inadequate milk refrigeration, or mastitis. In cases of mastitis caused by certain bacteria, such as *Streptococcus agalactiae*, *Streptococcus uberis*, or *E. coli*, high numbers of bacteria may be shed in milk, leading to high milk TBC. As with SCC, TBC may be measured either for bulk tank milk samples, as a quality index, or for individual cows or individual quarters, principally to aid in the diagnosis of causative agent in cases of mastitis. In the latter case, samples for analysis must be taken with great care, to avoid environmental contamination. EU and US legislation sets maximum acceptable TBC for milk of 100 000 ml^{-1}. In general, however, TBC is not used as an index of mastitis, as it can be influenced by many factors not directly related to udder health. For example, a large proportion of the bacteria in refrigerated milk will probably be psychrotrophic bacteria. However, measurement of the presence of specific types of bacteria can provide critical information for diagnosis of the type of mastitis and its causative agent, and hence for determining treatment strategies.

Specific bacteria in milk, such as individual mastitis pathogens, may be measured using specific microbiological media. This is particularly important for determining, for example, what type of antibiotic treatment should be applied to treat cases of mastitis. Either bulk samples containing mixed milk from all four quarters or, for more detailed diagnosis, individual quarter milk samples may be analyzed. Microbiological characterization of mastitis pathogens is complicated by the fact that many different causative organisms may be involved, with over 100 different species, subspecies, and biovars having been isolated from infected udders.

The presence of specific types of bacteria may be studied using specific DNA-based technologies, for example polymerase chain reaction (PCR) technology to detect genes from specific mastitis pathogens such as staphylococci or streptococci. PCR tests have also been used to semiquantitatively detect somatic cells in milk, through detection of eukaryotic ribosomal RNA sequences. For such applications, however, PCR tests may have to be modified due to inhibition of the polymerase reaction by substances found in milk.

ELISA methods have been developed for specific bacteria in milk, such as *S. aureus*. Immunological agglutination tests have been developed for a number of mastitis pathogens, and some cow-side tests for specific bacterial types, such as Gram-negative species, have also been developed.

Conclusion

The most common methods used to measure udder health, through indices of the level of either somatic or bacterial cells, are summarized in **Table 3**. In recent years, the trend, in terms of analytical techniques designed for operation in quality control laboratories in milk processing factories, has been toward development of highly automated methods capable of giving rapid and reliable information about the quality of large numbers of samples. However, there is undoubtedly a requirement for rapid simple tests that could be applied on the farm itself, both for diagnostic purposes and to ensure that quality control is applied right through the production and processing chain.

Table 3 Summary of methods for analysis of udder health

Somatic cell count and related properties
Direct microscopic cell counting (total or differential)
Electronic cell counting (e.g., Fossomatic, Bentley, Coulter counters)
Rapid cow-side tests (e.g., California mastitis test)
Rapid dairy tests (e.g., Wisconsin mastitis test)
Differential somatic cell counter (flow cytometry methods)
Differential cell counting (immunological)
Chemical/enzymatic indices (e.g., *N*-acetyl-β-D-glucosaminidase, bovine serum albumin, haptoglobin, serum amyloid A)
Total bacterial count or other microbiological parameters
Microscopic counts
Rapid indicator tests (e.g., dye reduction tests, titratable acidity, clot-on-boiling)
Standard plate counts
Automated bacterial counters (e.g., Bactoscan, epifluorescence methods)
DNA/polymerase chain reaction analysis
Specific culturing techniques for individual bacteria

See also: **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk. **Milk Quality and Udder Health**: Effect on Processing Characteristics. **Milking and Handling of Raw Milk**: Milking Hygiene. **Plant and Equipment**: Pasteurizers, Design and Operation.

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Effect on Processing Characteristics

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Introduction

The health of the udder can have a profound effect on the quality and processing characteristics of milk. The most widely used indicator of udder health is somatic cell count (SCC), a measure of the number of white blood cells, known as leukocytes, in milk. Many milk processing companies around the world have incorporated SCC as a key parameter in schemes that reward producers for producing milk of the highest quality.

An elevated SCC usually indicates the presence of mastitis. Mastitis is caused by pathogenic bacteria entering the mammary gland via the teat canal and multiplying within the udder sinuses or epithelia, or in the teat duct. This bacterial infection usually initiates a complex inflammatory reaction within the mammary gland as part of the cow's immune response. A key feature of this inflammation is an influx of leukocytes into the milk, which are sent to the site of infection to combat the invading bacteria.

Mammary inflammation during mastitis causes a range of physical, microbiological, and chemical changes in the milk. This includes changes to the chemical composition of milk, and because the different milk components have different functional properties, this leads to changes in the processing properties of the milk. Both subclinical mastitis (mastitic infection for which there are no clinical symptoms) and clinical mastitis can cause such changes.

Effects of Mastitis on Milk Yield

Mastitis reduces milk yield by 10–25% per cow. This decline in milk production is due to physical damage to the epithelial cells of the mammary gland, which limits the synthetic and secretory capacity of the gland. A reduction in the capacity of the mammary gland to synthesize and secrete lactose is important in this regard, given the role of lactose as the osmotic regulator of milk volume. A decline in milk yield can also result from blocked milk ducts or impairment of the blood supply of the precursors for milk production.

Effects of Mastitis on the Composition of Raw Milk

Mechanisms by Which Mastitis Affects Milk Composition

Changes to the chemical composition of milk associated with mastitis are shown in **Table 1**. These changes are brought about in three main ways:

1. Chemical mediators of inflammation (e.g., histamine, cytokines), along with toxins released by the invading bacteria, can damage the cells in the secretory tissue of the mammary gland. This leads to a reduction in the synthesis and secretion of most milk components synthesized in the udder.
2. Bacterial toxins can cause the junctions between the mammary epithelial cells to rupture, increasing the permeability of adjacent blood vessels. This allows leakage of blood constituents into milk, and of milk constituents into blood.
3. The activity of hydrolytic enzymes in milk is elevated during mastitis, leading to the postsecretory breakdown of milk constituents. These enzymes include those from bacteria and from the somatic cells themselves, but there is also an increase in the activity of the native milk proteinase, plasmin.

Milk Fat

The effect of mastitis on milk fat concentration is uncertain. A decline in milk fat concentrations during mammary infection is logical given the reduced synthetic and secretory ability of the mammary gland, and this is what some researchers have reported. It is possible, however, that in other studies the concentrating effect of a reduction in milk volume has offset any reduction in the synthesis and secretion of milk fat, resulting in either a negligible change or an increase in overall fat concentration.

Conditions within the infected mammary gland can increase the susceptibility of milk triacylglycerols to degradation by lipase, which leads to the release of free fatty acids. In turn, the presence of elevated quantities of free fatty acids can cause organoleptic defects, especially rancid flavors, in milk and dairy products. Free fatty acids also increase in milk during mastitis because of incomplete synthesis, as well as because of

Table 1 Effects of mastitis on milk composition

Lactose, fat and minerals	*Effect*	*Protein components*	*Effect*
Lactose	↓	Total protein	**?**
Fat	**?**	Total casein	↓
Free fatty acids	↑	Casein:total protein	↓
Na	↑	β-Casein	↓
K	↓	α_s-Casein	↓
Total Ca	**?**	κ-Casein	**?**
Cl	↑	γ-Casein	↑
		Whey protein	↑
		α-Lactalbumin	↓
		β-Lactoglobulin	↓
		Serum albumin	↑
		Immunoglobulin G	↑
		Lactoferrin	↑
		Transferrin	↑
		Plasmin	↑

↑ Concentration increases; ↓ Concentration decreases; ? Effect uncertain.
From Auldist MJ and Hubble IB (1998) Effects of mastitis on raw milk and dairy products. *Australian Journal of Dairy Technology* 53: 28–36.

increased lipolysis postsecretion (*see* **Milk Lipids**: Lipolysis and Hydrolytic Rancidity).

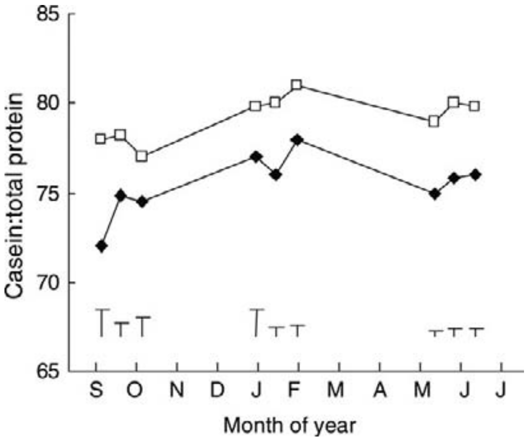

Figure 1 Casein:total protein ratios throughout the year for cows with somatic cell count –>300 000 cells ml^{-1} (◆) or <300 000 cells ml^{-1} (□). Vertical brackets represent standard errors of the means. From Auldist MJ, Coats S, Rogers GL, and McDowell GH (1995) Changes in the composition of milk from normal and mastitic dairy cows during the lactation cycle *Australian Journal of Experimental Agriculture* 35: 427–436.

Milk Protein

Reports on the effect of mastitis on concentrations of total protein are conflicting. Overall, mastitis usually causes a decrease in casein coupled with an increase in whey protein, producing a negligible change in total milk protein concentration. The direction of the change, if any, can be influenced by factors such as the severity and type of infection.

Decreased casein concentrations in high SCC milk are partly due to lower synthesis and secretion of casein, as a consequence of physical damage to the mammary epithelial cells. They are also due to postsecretory degradation of the different casein proteins, especially β-casein, by a range of proteinases originating from bacteria, leukocytes, or the blood.

Increased concentrations of whey protein during mastitis are partly due to an influx of serum proteins from the blood, through ruptured tight junctions between mammary epithelial cells. Such proteins include immunoglobulin G and bovine serum albumin, as well as the lesser proteins transferrin and α_2-macroglobulin. Lactoferrin, though synthesized in the mammary gland, also increases in milk during mastitis, an occurrence probably associated with its bacteriostatic function.

The net result of a reduction in casein and a concurrent increase in whey protein concentration during mastitis is a decline in casein as a percentage of total protein, or casein number (**Figure 1**). This has important implications for the manufacturing potential of the milk, particularly, but not exclusively, for cheese manufacture. It is also of concern to manufacturers who pay for milk using systems based largely on total protein concentration.

Plasmin

Milk with an elevated SCC can have increased quantities of many different proteolytic enzymes. A major proteinase is plasmin, which can rapidly cleave β-casein into γ-casein and smaller polypeptide fragments that then diffuse into the whey fraction. Thus, a decrease in total casein concentration during mastitis is often accompanied by low β-casein and high γ-casein, as proportions of total casein. Hydrolysis of α-casein also occurs during mastitis, although the breakdown products are unknown. The precise effects of mastitis on κ-casein concentrations are uncertain.

The caseinolytic capacity of plasmin is important for several reasons:

- The optimum temperature for plasmin activity is about the body temperature of the cow, which facilitates almost immediate postsecretory casein hydrolysis.
- The enzyme is heat stable, and thus able to survive high-temperature treatments during processing. This can shorten the shelf life of subsequent milk products.
- The inactive precursor of plasmin is plasminogen, present in milk and at up to 9 times the concentration of plasmin. Plasminogen is converted to plasmin by 'plasminogen activators', which may be secreted by somatic cells. Thus, plasmin concentration may increase during mastitis due to greater influx from the blood, as well as through increased conversion from plasminogen (*see* **Enzymes Indigenous to Milk**: Plasmin System in Milk).

Lactose

Mastitis causes a reduction in the lactose concentration in milk. The changes in lactose concentrations during mastitis are unlikely to be due to reduced synthesis and secretion at the cellular level because lactose is the osmotic regulator of milk volume. Thus, water is drawn into the cells only in sufficient quantities to maintain osmotic equilibrium (during mastitis, less lactose in secreted and so less water is drawn into the cells, resulting in less milk). It is more likely that lactose leaks out of milk following the breakdown of the epithelial barrier, as shown by elevated concentrations of lactose in the blood and urine of cows with mastitis.

Mineral Balance and Milk pH

The concentrations of many minerals are altered during mastitis (**Table 1**), and this change in the ionic environment leads to an increase in the conductivity of the milk. For example, Na and Cl, found in the blood in high quantities, leak into the milk and consequently their concentrations increase. Conversely, potassium, the most abundant mineral in milk from healthy cows, leaks into the interstitial fluid through the ruptured mammary epithelia during mastitis, and its concentration decreases.

Most Ca in milk is associated with micellar casein, and so mastitis generally results in a decline in total milk calcium concentrations as a result of reduced casein synthesis. No consistent effect of mastitis on the soluble and insoluble fractions of calcium has been reported.

Milk pH generally increases during mastitis, because of the movement of blood components into milk. These changes to the mineral balance and pH of milk can play important roles in determining the manufacturing quality of the milk, particularly for cheese manufacture.

Bacterial Contamination

An effect of mastitis on milk quality that is often overlooked is the contamination of bulk milk with the causative bacteria when milk from cows with mastitis is not excluded. Bacteria from cows with subclinical mastitis contribute to the total bacterial count of bulk milk, but usually not more than 10 000 cfu ml^{-1}. On the other hand, milk from cows with clinical mastitis can have a bacterial load of up to 10 000 000 cfu ml^{-1}, and admitting this milk to the bulk supply markedly increases total bacterial numbers. This bacterial contamination can lead to changes in milk composition via the action of hydrolytic enzymes released by the bacteria and, if the microorganisms are psychrotrophic, increases the likelihood of spoilage of milk and dairy products.

Effects of Mastitis on Dairy Products

Cheese

Most studies of the effects of mastitis on the yield and quality of dairy products have investigated cheddar cheese. Of these, most have demonstrated that mastitis causes changes to milk composition that are deleterious to the cheese-making process (**Table 2**). Cheese making

Table 2 Summary of reported product defects associated with elevated somatic cell count

Product	*Effects*
Cheese	Reduced yields and yield efficiencies Elevated moisture content Increased rennet clotting time Soft cheese and textural defects Higher loss of solids in whey Inferior organoleptic properties
UHT milk	Accelerated age gelation
Pasteurized fluid milk	Reduced shelf life Organoleptic defects
Cultured products	Increased coagulation time Inferior organoleptic properties
Butter	Extended churning times Reduced shelf life Inferior organoleptic properties
Milk powder	Altered heat stability Reduced shelf life
Cream	Altered whipping properties

Reproduced from Auldist MJ and Hubble IB (1998) Effects of mastitis on raw milk and dairy products. *Australian Journal of Dairy Technology* 53: 28–36.

involves the coagulation of milk from a colloidal dispersion to a network of aggregated casein micelles, forming a gel that entraps moisture and fat. This gel provides the means by which the fat and casein can be concentrated, recovered, and preserved in the form of a cheese curd. Thus, any changes to milk composition that interfere with this coagulation process can potentially affect cheese yield and/or quality.

An elevated SCC can cause low recoveries of milk fat and protein in cheese, and a decline in the yield of cheese per kilogram of milk. This is partly because of a decrease in casein as a percentage of total protein, since it is mostly casein that is incorporated into the curd while the whey is expelled during syneresis. It is also because a suboptimal ratio of casein to fat leads to less fat becoming entrapped in the curd. This loss of cheese yield and cheese yield efficiency is a key economic driver for companies to reduce the SCC of their bulk supply.

Mastitis causes increases in cheese moisture contents (**Figure 2**). This is a serious cheese quality defect, and can place the cheese outside product specifications if high SCC milk is used. The increase in cheese moisture that occurs as milk SCC increases is caused by a slow, weak coagulation due largely to alterations in milk protein composition and mineral balance, combined with increased milk pH. These changes in milk composition interfere with the expulsion of water from the curd during syneresis.

In turn, elevated cheese moisture contents usually cause decreases in curd firmness, leading to a deterioration of the organoleptic properties of cheese. Textural problems, specifically soft, pasty cheese, occur as a result of the high moisture content of the cheese. Flavor defects can also occur, especially during ripening, as a result of the increased activity of hydrolytic enzymes.

For example, in a study using milks obtained from commercial dairy farms during late lactation in Victoria, Australia, actual and moisture-adjusted cheese yields decreased (by 5 and 10%, respectively), as SCC changed from 252 000 to 1 463 000 cells ml^{-1} (**Table 3**). Concurrently, cheese moisture contents increased (8%), and textural defects were observed in the cheese from high SCC milk.

Similar declines in the yield of cottage cheese occur when high SCC milk is used, again due to a decrease in the recovery of milk solids in the final product. Additionally, there are similar elevations in cheese moisture contents and there are also increases in proteolysis during storage when high SCC milk is used compared those with low SCC milk (*see* **Cheese**: Overview).

Other Dairy Products

The influence of mastitis on the yield and quality of dairy products other than cheese has not been investigated nearly so extensively. Of those that have been reported, most have been detrimentally affected as SCC increased (**Table 2**).

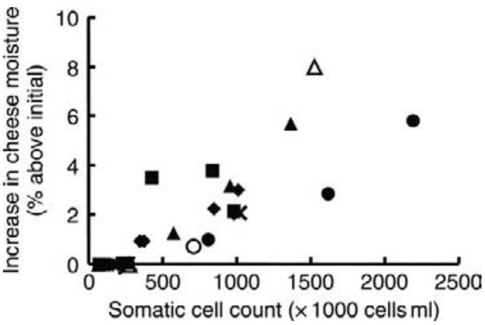

Figure 2 Effect of SCC on cheese moisture content. Data points represent the percentage increase in cheese moisture content above that for the milk with the lowest SCC used in each particular study. ■ Barbano DM, Rasmussen RR, and Lynch JM (1991) Influence of milk somatic cell count and milk age or cheese yield. *Journal of Dairy Science* 74: 369–388; ▲ Grandison AS and Ford GD (1986) Effects of variations in somatic cell count on the rennet coagulation properties of milk and on the yield, composition and quality of Cheddar cheese. *Journal of Dairy Research* 53: 645–655 (experiment 1); ● Grandison AS and Ford GD (1986) Effects of variations in somatic cell count on the rennet coagulation properties of milk and on the yield, composition and quality of Cheddar cheese. *Journal of Dairy Research* 53: 645–655 (experiment 2); △ Auldist MJ, Coats S, Sutherland BJ, Mayes JJ, McDowell GH, and Rogers GL (1996) Effects of somatic cell count and stage of lactation on raw milk composition and the yield and quality of Cheddar cheese. Auldist MJ, Coats S, Sutherland BJ, Mayes JJ, McDowell GH, and Rogers GL (1996) *Journal of Dairy Research* 63: 269–280 (late lactation); ○ Auldist MJ, Coats S, Sutherland BJ, Mayes JJ, McDowell GH, and Rogers GL (1996) *Journal of Dairy Research* 63:269–280 (early lactation); × Mitchell GE, Fedrick IA, and Rogers SA (1986) The relationship between somatic cell count, composition and manufacturing properties of bulk milk. 2. Cheddar cheese from farm bulk milk. *Australian Journal of Dairy Technology* 41: 12–14; ◆ Rogers SA and Mitchell GE (1994) The relationship between somatic cell count, composition and manufacturing properties of bulk milk. 6. Cheddar cheese and skim milk yogurt. *Australian Journal of Dairy Technology* 49: 70–74. (No studies reported decreases in cheese moisture content with increasing SCC).

For example, an elevated SCC in milk can cause an increase in the coagulation time of butter and yogurt cultures. The organoleptic quality of sweet cream butter made from high-SCC milk is also reduced, and the product deteriorates more rapidly during storage than butter made from low-SCC milk. A high SCC can also be associated with poor heat stability of full cream milk powder (*see* **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders).

Using high-SCC milk for the production of pasteurized fluid milk can decrease the shelf life of the product. This occurs via an increase in hydrolytic enzyme activity, which leads to organoleptic defects such as rancidity and bitterness (*see* **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products:

Table 3 Effects of somatic cell count (SCC) on cheese yield, coagulation times, and cheese moisture content

	Low SCC	*High SCC*
Milk SCC (×1000 cells ml^{-1})	252	1463
Cheese yield (kg 100 kg^{-1})	11.4	10.8
Moisture-adjusted cheese yield (kg 100 kg^{-1})	11.3	10.2
Coagulation time (min)	29.5	40.5
Cheese moisture (%)	36.0	38.9

Reproduced from Auldist MJ, Coats S, Sutherland BJ, Mayes JJ, McDowell GH, and Rogers GL (1996) Effects of somatic cell count and stage of lactation on raw milk composition and the yield and quality of Cheddar cheese. *Journal of Dairy Research* 63: 269–280.

Super-Pasteurized Milk (Extended Shelf-Life Milk); Liquid Milk Products: UHT Sterilized Milks).

Somatic Cell Count Threshold Affecting Dairy Products

It is difficult to associate a particular SCC with the onset of defects in dairy products. Some researchers have reported that SCC begins to affect product as it increases above 100 000 cells ml^{-1}, while others have suggested that the threshold is closer to 500 000 cells ml^{-1}.

This large variation in the SCC at which the manufacturing properties of milk are affected is probably due to a number of factors. Whether an elevated bulk milk SCC is due to milk from a small number of cows with extremely high SCC being included with milk from a predominantly healthy herd, or to large numbers of cows with low-level subclinical infections, probably contributes to variation in the effects of SCC on dairy products. Additionally, some pathogens affect milk composition in different ways, irrespective of SCC. Similarly, there is evidence that some types of somatic cells can have greater effects on milk composition than others (see below).

It is also likely that other factors such as nutritional status and stage of lactation could affect the magnitude of the impact of mastitis on milk composition and dairy products, possibly through an influence on the immune system of the cow.

Effects of Type of Somatic Cell

Somatic cells in milk are primarily white blood cells, or leukocytes. There are many different types, with the predominant being polymorphonuclear neutrophils (PMNs), macrophages, and lymphocytes. During an infection of the mammary gland, most somatic cells are likely to be PMNs as these cells migrate to the site of infection to perform their function of engulfing and digesting invading bacteria.

Research in Ireland demonstrated that the percentage of PMN in milk can vary between quarters, between cows, and between herds, and that the number of PMN present is correlated with the SCC of the milk. For example, the amount of PMN as a percentage of total leukocytes in bulk herd milks can vary from less than 20% to more than 70%, although the percentage is generally lower in low-SCC milks.

The above observations are important because the effect of PMN on milk composition may be more severe than for other types of cells. This could be a major contributing factor to the large variation in reports of the threshold at which SCC begins to affect the manufacturing potential of milk. It also implies that measuring both the number and the type of somatic cells may be more accurate than measuring only the number of cells, when assessing the manufacturing quality of milk.

Conclusions

Mastitis induces changes in milk composition via a reduction in the synthesis and secretion of milk components, postsecretory breakdown of milk components by hydrolytic enzymes, and 'leakage' of blood constituents into milk. Some of these changes are detrimental to the manufacturing properties of milk, particularly cheese manufacture. Using milk with high SCC for cheese making results in increased coagulation times, elevated cheese moisture contents, decreased cheese firmness, lower recoveries of milk solids in cheese, and reduced cheese yield and yield efficiency.

See also: **Cheese**: Overview. **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders. **Enzymes Indigenous to Milk**: Plasmin System in Milk. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Liquid Milk Products: UHT Sterilized Milks. **Milk Lipids**: Lipolysis and Hydrolytic Rancidity.

Further Reading

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MILK SALTS

Contents

Distribution and Analysis
Interaction with Caseins
Macroelements, Nutritional Significance
Trace Elements, Nutritional Significance

Distribution and Analysis

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Introduction

The salt fraction of milk consists of about 8–9 g of matter per liter of milk. It is composed of cations (calcium, magnesium, sodium, and potassium) and anions (inorganic phosphate, citrate, and chloride). These ions are more or less associated between themselves and with proteins. Depending on the type of ion, they are present in the aqueous phase or partially associated with casein molecules to form casein micelles (**Table 1**). The distribution of the ions and salts between aqueous and micellar phases is called salt equilibria.

Citrate is an organic molecule and not a mineral. However, as its contribution in the salt equilibria of milk and dairy products is considerable, it is necessary to take it into account in the salt fraction.

Soluble Salts and Micellar Calcium Phosphate – Milk Salt Equilibria

Table 2 shows the different ionic forms and salts present in milk. Their locations and forms of association are also indicated.

Salts in the Aqueous Phase

In the aqueous phase, ions are free or associated to form salts (**Figure 1** and **Table 3**).

Quantitatively, the interactions between cations and anions in the aqueous phase depend on the values of the different constants of association and the solubilities of the salts. Globally, calcium is ionic (Ca^{2+}) and associated with trivalent citrate Cit^{3-} and, in lesser degree, with inorganic phosphate (as a mixture of $H_2PO_4{}^-$ and HPO_4^{2-}) and chloride. The solubility of calcium phosphate salt is very low and consequently its concentration in the aqueous fraction is less than 1 mmol l^{-1}. Sodium and potassium are present mainly as free ions. A small part of them is associated with citrate, inorganic phosphate, and chloride to form salts. Thus, at pH 6.6–6.7 and by taking into account these different associations, the milk aqueous phase is supersaturated with calcium phosphate and its ionic strength is about 80 mmol l^{-1}.

The Micellar Calcium Phosphate

In the micellar phase, the organization of ions is complex, heterogeneous, and not well defined. The micellar calcium phosphate (MCP) can be described as a mixture of calcium caseinate (containing organic phosphate present in the phosphoseryl residues of caseins) and calcium–inorganic phosphate complex. Concerning the chemical nature of MCP, hydroxyapatite, brushite, and tricalcium phosphate were proposed. These different propositions were not in total agreement and the subject was always controversial. As it is difficult to observe *in situ* the MCP and to isolate it without changing its structure, some experiments are performed to prepare 'experimental MCP'. This preparation is carried out with the phosphopeptide 1–25 of β-casein in the presence of different milk salts, especially calcium and inorganic phosphate. The insoluble calcium phosphate in interaction with phosphopeptide is formed during a

Table 1 Ion concentrations (mmol l^{-1}) determined in cow milk. The aqueous fraction corresponds to a milk ultrafiltrate obtained with a membrane having a molecular weight cutoff of 10 000 Da. P_i: inorganic phosphate

Ions	*Total concentration*	*Aqueous concentration*	*Micellar concentration*
Ca	30.0	9.0	21.0
Mg	5.0	3.5	1.5
Na	22.0	21.5	0.5
K	35.0	34.3	0.7
P_i	21.0	11.0	10.0
Citrate	9.0	8.1	0.9
Cl	30.0	30.0	0.0

Table 2 Different ions and salts present in milk. P_o and P_i correspond to organic and inorganic phosphates, respectively. Concentrations of some ions and salts are indicated in **Tables 1** and **3**. (?) refers to a putative location

Ions	*Locations*	*Forms of association*
Ca	Aqueous phase	-Free -Associated with citrate, inorganic phosphate, and chloride -Associated with α-lactalbumin
	Micellar phase	-Associated with phosphoseryl residues -Associated with inorganic phosphate
Mg	Aqueous phase	-Free -Associated with citrate (?), inorganic phosphate (?), and chloride (?)
	Micellar phase	-Associated with phosphoseryl residues (?) -Associated with inorganic phosphate (?)
Na	Aqueous phase	-Free -Associated with anions
K	Aqueous phase	-Free -Associated with anions
P_o	Aqueous phase	-Esterified with small molecules (nucleotides, sugars)
	Micellar phase	-Esterified with casein (phosphoseryl residues)
P_i	Aqueous phase	-Free ($H_2PO_4{}^-$ $HPO_1{}^{2-}$) -Associated with calcium ($CaHPO_4$)
	Micellar phase	-Associated with micellar calcium, magnesium (?)
Citrate	Aqueous phase	-Free -Associated with calcium, magnesium, sodium, potassium (?)
	Micellar phase	-Associated with calcium (?)
Chloride	Aqueous phase	-Free -Associated with calcium, magnesium, sodium, potassium (?)

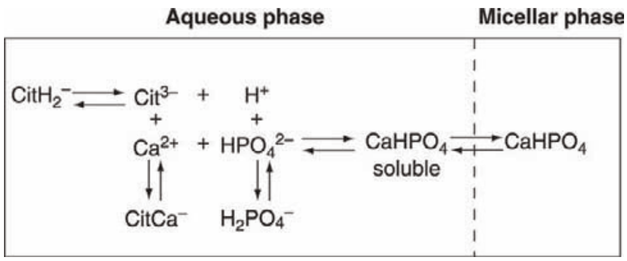

Figure 1 Phosphocalcic equilibria in the presence of citrate ions between aqueous and micellar phases at pH 6.75. In this figure, micellar calcium phosphate (MCP) was basically converted to $CaHPO_4$. Precisions concerning the composition of this MCP are reported in the text.

progressive increase of pH due to the enzymatic transformation of urea in ammonia. The nature, composition, and structure of this calcium phosphate, named nanoclusters, were studied in detail and it is claimed that the physicochemical properties of MCP and nanoclusters are relatively close. Chemically, X-ray absorption and infrared spectroscopy indicate that MCP is close to brushite (dicalcium phosphate). A modified dicalcium phosphate $Ca(HPO_4)_{0.7}(PO_4)_{0.2}$ was proposed after calculation of the apparent solubility product. In the model nanoclusters, it is reported that one casein chain containing four phosphoseryl residues (corresponding to the phosphopeptide 1–25 of β-casein) can bind 13.2 calcium ions. The precise

Table 3 Theoretical concentrations of ions and salts (mM) in the aqueous phase of milk at pH 6.75

Ions	*Free form*	*Ca^{2+}*	*Mg^{2+}*	*Na^{+}*	*K^{+}*
$H_2PO_4{}^-$	3.65	0.07	0.02	0.05	0.08
$HPO_4{}^{2-}$	3.67	0.60	0.67	0.51	0.69
$HCit^{2-}$	0.03	0.01	0.00	0.00	0.01
Cit^{3-}	0.17	6.04	1.79	0.02	0.03
Cl^-	27.8	0.24	0.09	0.34	0.58
Free ion	-	2.12	1.14	20.9	36.26

From Mekmene O, Le Graët Y, and Gaucheron F (2009) A model for predicting salt equilibria in milk and mineral-enriched milks. *Food Chemistry* 116: 233–239.

formula is $[Ca_{13.2}(P_i)_{6.5}Mg_{1.0}Cit_{1.3}SerP_4Cas]_{49}$. Physically and by using diffraction methods and high-resolution transmission electron microscopy, the MCP was determined as amorphous. By cryo-transmission electron microscopy and X-ray scattering, it is observed as small granules of high electron density with an estimated diameter of about 2.5 nm. In the different proposed structural models of the casein micelle, the MCP is an integral part of the casein micelle. Its presence probably contributes to the structure and stability of casein micelles. It is, at the same time, a cross-linking and a neutralizing agent of phosphoseryl residues. According to Holt, a typical micelle with an average radius of 108 nm, say, has a mass of 7.2×10^8 Da, corresponding to about 3000 polypeptide chains and, according to the model, contains 830 calcium phosphate nanoclusters. Caseins with more than one phosphate center per polypeptide chain are able, at least in principle, to cross-link the MCP particles and hence a substantial fraction of all the polypeptide chains.

Milk Salt Equilibria

Even without changes in the physico-chemical conditions, the milk salt equilibria are dynamic, and permanent exchanges of calcium and inorganic phosphate between aqueous and micellar phases exist. Depending on the location of calcium and inorganic phosphate ions in the casein micelles, three types of exchangeability are distinguished: slow, fast to exchange, and non-exchangeable. This last fraction could correspond to calcium and inorganic phosphate associated to the phosphoseryl residues of caseins.

Interspecies Comparison and Correlations between Various Salt Concentrations and Concentrations of Other Constituents

The comparison of the milk salt composition of several species (**Table 4**) to find different interrelationships between these ions is interesting to understand the behavior of this salt fraction.

Thus, it appears that the concentrations of calcium and citrate in the aqueous phase are positively correlated. On the other hand, the micellar concentrations of calcium, magnesium, and citrate are positively correlated with the concentration of MCP. The other correlations well known are between

- casein and calcium concentrations (positive);
- casein and lactose concentrations (negative);
- sodium and chloride (positive).

For milk of all species, the monovalent ions such as sodium, potassium, and chloride are mainly present in the aqueous phase.

Table 4 Total (T) and soluble (S) concentrations (mmol l^{-1}) of ions in the milk of different species. P_i (inorganic phosphate)

	Ca		*Mg*		*Na*		*K*		*P_i*		*Citrate*		*Cl*	
	T	*S*	*T*	*S*	*T*	*S*	*T*	*S*	*T*	*S*	*T*	*S*	*T*	*S*
Rat	80.4	2.5	8.8	1.0	38.3	38.3	43.6	43.6	54.2	12.2	0.06	0.04	36.1	36.1
Sow	104.1	7.1	9.6	1.9	14.4	14.4	31.4	31.4	51.2	3.3	8.4	3.0	28.7	28.7
Sheep	56.8	5.2	9.0	2.9	20.5	20.5	31.7	31.7	39.7	11.7	4.9	3.8	17.0	17.0
Goat	23.1	8.0	5.0	3.4	20.5	20.5	46.6	46.6	15.6	9.0	5.4	5.0	34.2	34.2
Cow	29.4	9.2	5.1	3.3	24.2	24.2	34.7	34.7	20.9	11.2	9.8	9.2	30.2	30.3
Mare	16.5	6.4	1.6	1.3	5.7	5.7	11.9	11.9	6.7	4.1	3.1	3.1	6.6	6.6
Human	7.8	5.0	1.1	1.0	5.0	5.0	16.5	16.5	2.5	1.9	2.2	2.7	6.2	6.2

From Holt C and Jenness R (1984) Interrelationships of constituents and partition of salts in milk samples from eight species. *Comparative Biochemistry and Physiology* 77A: 275–282.

All these positive or negative correlations are related to the mechanisms of formation and secretion of milk by the cells of mammary gland.

Variations in the Salt Equilibria as a Function of Physico-Chemical Conditions

The distribution of ions and salts between the aqueous and micellar phases are in equilibria (**Figure 1**). These equilibria depend on the physico-chemical conditions such as variations in pH (acidification or alkalinization), thermal treatments (increase or decrease in temperatures), and additions of different salts and chelatants (**Figure 2**). Theoretically, the modifications of salt equilibria are controlled by the solubility of the calcium phosphate salt in the aqueous phase. The consequences of these changes in the salt equilibria are modifications of the composition, structure, and stability of casein micelles.

Acidification

Acidification is a fundamental step in the manufacture of different dairy products like fermented milks, cheeses, and acid casein. Depending on the rate of acidification, casein molecules precipitate (fast acidification) or are organized to form a gel (slow acidification). During this pH decrease, the different acido-basic groups present in milk, like organic and inorganic phosphate, citrate, and carboxylic residues, bind the protons added or produced *in situ* by microorganisms. This binding of protons by these groups depends on their p*K*a values. The affinities of these different groups for protons are higher than the affinities for calcium ions. The decrease of pH is not linear because these groups, by binding protons, limit the pH decrease. This effect is named buffering capacity. During acidification, the MCP is dissolved (or solubilized) and the caseins are released into the aqueous phase. Up to pH 5.2, calcium and inorganic phosphate are transferred to the aqueous phase. The solubilization of one is strongly correlated to the second one. At pH 5.2, the totality of the inorganic phosphate initially present in the MCP is solubilized. This pH coincides with the maximal value of the buffering capacity of milk. At pH 5.2, a part of calcium is always associated to casein molecules. It is necessary to decrease the pH to 3.5 to observe its total solubilization. At this acid pH, calcium exists mainly as Ca^{2+} in the aqueous phase. The difference of pH required

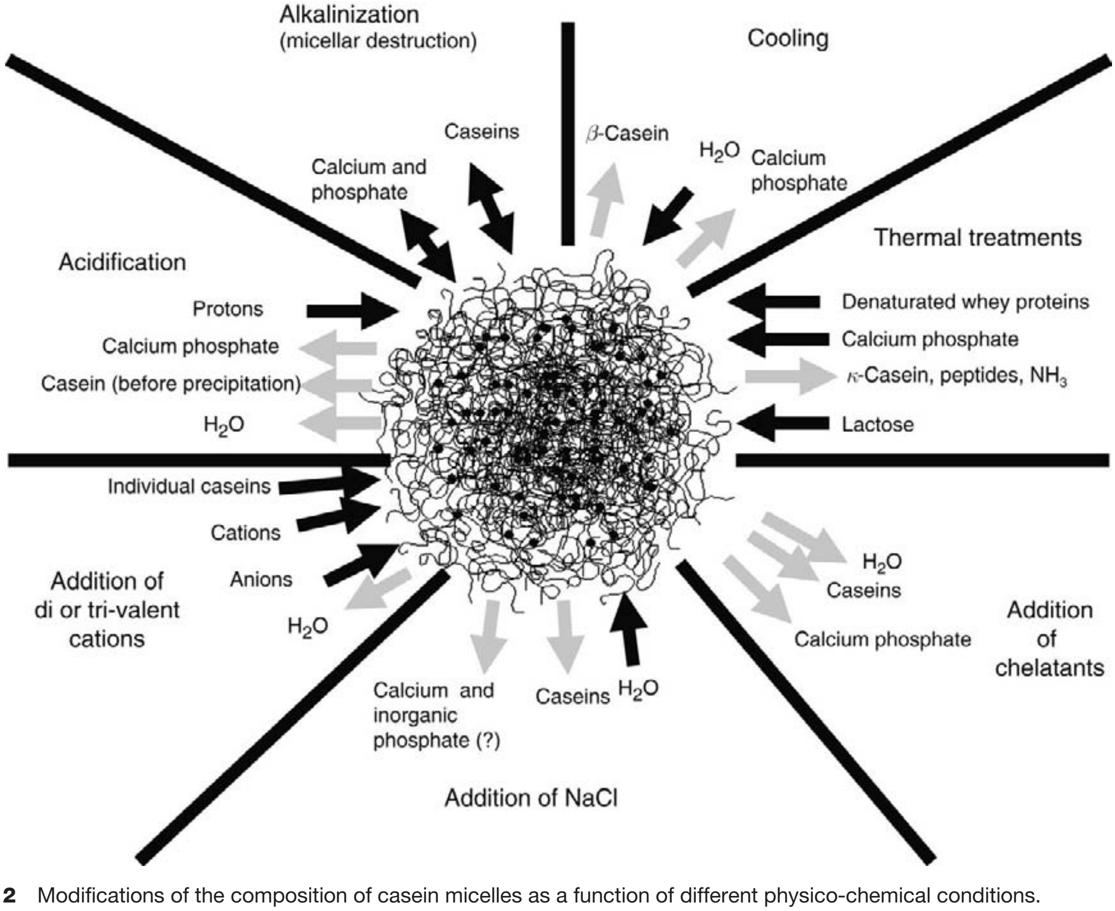
Alkalinization
(micellar destruction)
Cooling
Caseins
Calcium and phosphate
β-Casein
H₂O
Calcium phosphate
Thermal treatments
Acidification
Protons
Calcium phosphate
Casein (before precipitation)
H₂O
Denaturated whey proteins
Calcium phosphate
κ-Casein, peptides, NH₃
Lactose
Individual caseins
Cations
Anions
Addition of di or tri-valent cations
H₂O
H₂O
Caseins
Calcium phosphate
Addition of chelatants
Calcium and inorganic phosphate (?)
Caseins
H₂O
Addition of NaCl

Figure 2 Modifications of the composition of casein micelles as a function of different physico-chemical conditions.

to obtain the total solubilization of inorganic phosphate (pH 5.2) and calcium (pH 3.5) strengthens the hypothesis that calcium is associated in the casein micelles with inorganic phosphate and phosphoserine residues of casein molecules. During acidification, the anions associated to protons (e.g., chloride, nitrate, or sulfate in the case of acidification by hydrochloric, nitric, or sulfuric acids or lactate in the case of biological acidification) remain in the aqueous phase and contribute to the increase in its ionic strength.

These changes of salt equilibria are irreversible and the original MCP and casein micelles cannot be reconstructed after neutralization of the acidified milk.

Alkalinization

Alkalinization is not a common practice in the dairy sector. However, the consequences of this pH increase were studied to understand the milk salts equilibria. It is reported that alkalinization of milk induces an increase of viscosity and a decrease in its whiteness. The whiteness decreases from pH 8 and is minimal at pH 10. These modifications are related to changes in the structure and stability of casein micelles and especially of the salt fraction. From a mechanistic point of view, the pH increase induces modifications to the state of ionization of inorganic phosphate present in the aqueous phase and of amino groups of proteins. HPO_4^{2-} is transformed to PO_4^{3-}, which has a higher affinity for calcium compared to HPO_4^{2-}, and this triply ionized form reacts with calcium. Consequently, the calcium exclusively in interaction with the phosphoseryl residues of casein micelles is shifted toward the PO_4^{3-} to form calcium phosphate salt. As the calcium phosphate in the aqueous phase is saturated, the amounts of calcium and inorganic phosphate in the aqueous phase decrease, suggesting precipitation of this salt. The chemical and physical structures of the newly formed calcium phosphate are not well defined. The use of different microscopic studies has failed to observe and characterize these new salts of calcium phosphate. This result suggests a size of the order of a few nanometers. The initial calcium inorganic phosphate present in the micellar phase is not changed during alkalinization. The concentration of sodium from NaOH increases with the increase in pH. This ion remains in the aqueous phase and contributes to the increase in the ionic strength. In parallel to these changes in the salt equilibria, it is probable that the protein–protein and protein–water interactions are affected because the state of ionization of proteins is also affected by the increase in pH.

Thermal Treatments

Heat treatment is a common operation in the dairy industry. The most commonly used heat treatments are pasteurization (with low or high intensities), ultra high temperature (UHT) treatment, and sterilization. The aim of these treatments is to destroy pathogens to ensure a safe product. During heating, several biochemical reactions occur like denaturation of whey proteins, Maillard reactions, proteolysis, deamidation, destruction of some vitamins, and alterations of the salt equilibria. These modifications depend on the intensity of the heat treatment. Concerning the salt equilibria, it is known that when the milk temperature increases, the dissociation of inorganic phosphate present in the aqueous phase increases. Consequently, part of $H_2PO_4{}^-$ is transformed to HPO_4^{2-}, which has a higher affinity for calcium than $H_2PO_4{}^-$ and reacts with calcium to form a calcium phosphate salt. The aqueous phase being supersaturated in this salt, the concentrations in calcium and inorganic phosphate are reduced in this phase. The extent of these decreases depends on the intensity of the applied heat treatment. If the heat treatment is less than 95 °C for few minutes, the modifications of salt equilibria are reversible after cooling. If the heat treatment is severe (more than 100 °C for several minutes), alterations of the salt equilibria are irreversible. On the other hand, during intense heat treatments, casein molecules can be dephosphorylated. In these cases, the decreases of calcium and phosphate contents in the aqueous phase do not correspond to a simple precipitation of calcium phosphate. It is noteworthy that for severe heat treatment, some changes in the structure and composition of the MCP are observed. The newly formed phase is not clearly identified but it is related to the formation of crystalline β-tricalcium phosphate or hydroxyapatite.

Cooling

Inversely to the heat treatments, the dissociation of inorganic phosphate present in the aqueous phase decreases when the milk is cooled and part of HPO_4^{2-} is transformed into $H_2PO_4^-$ form. In this case, the solubility of calcium phosphate increases and the concentrations of calcium and inorganic phosphate increase in the aqueous phase, indicating that part of MCP (about 10%) is transferred to the aqueous phase. Simultaneously, β-casein is solubilized and the casein micelles become more hydrated. These changes are reversible and the previous partition may be re-established after re-warming.

Addition of Chelatants

It is known that the addition of chelatants of calcium like citrate, EDTA, oxalate, or cation-exchange resins to milk or to suspensions of casein micelles induces a decrease of lightness, an increase of viscosity, and an improvement of the heat stability. This last result is specially applied in the dairy sector. All these physico-chemical changes suggest a loss of the micellar structure. The explanations can be related to the modifications of the milk salt equilibria. Indeed, these different types of chelatants have high affinity for calcium present in the aqueous phase. Their affinity for calcium depends on their association constant. Consequent to this chelating action, the aqueous phase becomes less saturated in calcium phosphate and the salt equilibria are shifted from the micellar to the aqueous phase with increases of calcium and inorganic phosphate contents in the aqueous phase. At the same time, the pH of milk increases. This pH increase is probably due to a binding of free protons when the calcium is displaced from phosphate (organic or inorganic) toward the aqueous phase. The solubilization of calcium is positively correlated to those of inorganic phosphate, confirming the strong relation of both ions in the micellar structure. Indeed, these solubilizations are due to a disruption of the MCP causing a destruction of casein micelles.

Addition of NaCl

There are few industrial applications using the NaCl addition to milk but some scientific studies have been conducted to understand the changes of salt equilibria induced by NaCl addition. In these studies, the effects on the physico-chemistry of casein micelles were also studied. Globally, it is described that NaCl addition alters the salt equilibria and protein ion interactions and consequently the milk becomes more viscous. At the same time, the casein micelles become more hydrated suggesting some changes in the interactions between proteins and water molecules. At the same time, a reduction in pH is observed (a decrease of 0.1–0.3 pH units after addition of NaCl to more than 0.1 mol l^{-1}). At this level of NaCl, an increase in calcium concentrations in the aqueous phase is observed. From a mechanistic point of view, these changes are due to some exchanges between added sodium and (1) calcium bound to organic phosphate and (2) protons present in the casein micelles. To interpret this slight calcium solubilization, it is necessary to take into account the effect of the ionic strength which is increased. This increase reduces the activity coefficients and increases the dissociation of ion pairs. It is noteworthy that some authors report also a solubilization of inorganic phosphate and β-casein after NaCl addition.

Addition of Calcium

For nutritional and technological reasons, the calcium enrichment of milk and dairy products is often performed in the dairy industry. Depending on the physical and chemical nature of the calcium salts added, the salt equilibria are modified differently. If the salts added have low solubility like calcium phosphate, the salt equilibria and the casein micelles are not modified. If the calcium salts are soluble, like calcium chloride or calcium lactate, the physico-chemical characteristics of casein micelles are changed strongly. For example, it is reported that their zeta potential (casein micelles become less negative), hydration, and heat stability are reduced and the whiteness and turbidity of milk are increased. At the same time, the salt partitions between aqueous and micellar phases are altered. Thus, it is suggested that one part of the added calcium reacts with inorganic phosphate present in the aqueous phase, resulting in the formation of calcium phosphate with a release of protons, and the pH decreases (which can be regulated). Then, since the aqueous phase is saturated in this salt, the newly formed calcium phosphate is 'shifted' toward the casein micelles. The nature and the mode of association of this salt to the casein micelles are not known precisely. It is also important to note that part of the added calcium remains in the aqueous phase in the ionic form or associated to citrate molecules. The counter-ions of the added calcium salt (e.g., lactate or chloride) remain in the aqueous phase and increase the ionic strength. The intensity of these modifications depends on the concentration of the added salt, the eventual modification and regulation of the pH after calcium addition.

Analysis of Milk Salts

The determination of the total concentrations and the distribution of these different ions and salts between the aqueous and micellar phases of milk are often useful and interesting to control the biochemical and functional changes in dairy products during their manufacture. It is necessary to prepare the sample correctly and to use the correct analytical methods.

Preparation of Samples

Preparation for the determination of total ion content

Dry and wet ashing are the possible ways to destroy proteins, fat, and lactose before the determination of the ions contained in milk or dairy products. If the sample is milk, the micellar and aqueous phases are distinguished.

Dry ash is the material that remains after heating for several hours in the temperature range 400–800 °C. On the other hand, this mineralization induces the destruction of citrate and transforms organic phosphates (phosphate groups esterified to serine residues of caseins or to several small molecules) into inorganic phosphate. Chloride ions are volatilized if the dry ashing is performed at a temperature higher than 550 °C. After mineralization, the ash can be dissolved in dilute HCl or HNO_3 solution and one or several ions can be determined specifically. No specific reagents are required to do this dry ashing which can be applied to relatively large amounts of sample. Wet ashing corresponds to an acid digestion in a solution with the oxidation/destruction of organic matter. This preparation requires the use of various acid mixtures of HNO_3, H_2SO_4, and $HClO_4$ and is limited by the amounts of sample. However, to reduce the consumption of acids, it is possible to digest samples under high pressure in sealed and specific tubes. An alternative to obtain the total content is to perform an extraction with acid solutions. The acidification of milk to pH 3.0 causes the total transfer of calcium, magnesium, inorganic phosphate, and citrate from casein micelles to the aqueous phase and precipitates the caseins and whey proteins (if trichloroacetic acid is used). The determination of ions can be performed on the filtrate. It is noteworthy that during acid extraction, the organic phosphate corresponding to phosphoserine residues is not solubilized. To obtain this organic phosphate, enzymatic dephosphorylation by phosphatase is necessary.

Preparation for the determination of the concentration of ions in the aqueous phase

The aqueous phase can be recovered by ultrafiltration, ultracentrifugation, or rennet coagulation of milk.

For ultrafiltration, the molecular weight cut-off of the membrane should be close to 10 000 Da. Under this condition, the ions and salts present in the aqueous phase of milk pass through the membrane. The advantage of this preparation is that the aqueous phase obtained is free of protein. Ultrafiltration can be performed at a large range of temperature (between 4 and 50 °C) with a small volume of milk (2 ml minimum). However, as the filtration is frontal, a fouling occurs and can limit the recovery of a large volume of aqueous phase. Another disadvantage is that the units of filtration are often quite expensive. Depending on the supplier, some of them are for only one use and there is no problem of contaminating ions.

Typically, ultracentrifugation at 80 000 *g* for 2 h or 100 000 *g* for 1 h is performed to obtain the supernatant. In this case, the aqueous phase contains not only soluble ions and salts but also ions associated with serum proteins especially α-lactalbumin, which has in its structure one calcium ion per molecule. The concentration of this type of calcium corresponds to the concentration of α-lactalbumin in the aqueous phase of raw milk. The value is lower than 0.1 mmol l^{-1} which can be considered as negligible compared to the concentration of 10 mmol l^{-1} of calcium in the aqueous phase and to the sensitivity of the method used to determine the calcium concentration. This method requires an ultracentrifuge which is expensive. With this method, the use of ultracentrifugation tubes previously washed with acid solution and rinsed with demineralized water is obligatory.

For the recovery of the cheese whey, it is necessary to coagulate the milk by an enzyme like rennet, cut the gel, recover the whey, and filter it before the determination of minerals. This method is relatively rapid, simple, and not so expensive than ultrafiltration or ultracentrifugation. In this case, the aqueous phase obtained may contain serum proteins and casein micelles, which bind minerals, including calcium. Moreover, the enzymatic solutions used to coagulate the milk can contain sodium and chloride ions. This must be taken into account in the interpretation of results. It is noteworthy that the enzymatic action of rennet is very limited at low and high temperatures. At a low temperature (<20 °C), the activity is reduced, and at a high temperature (>50 °C), the enzyme can be denatured.

For connecting the concentrations determined in these different aqueous phases to the concentration existing in the milk, it is necessary to multiply the determined concentration by 0.96, which takes into account the excluded volume effect.

Analytical Determinations of Ions in Milk and Dairy Products

The concentrations of the main ions in milk are determined by different techniques which are classified according to their principles. Molecular absorption spectrometry, complexometric methods combined with titration, electrochemical methods (ion-selective electrode), atomic spectrometry, enzymatic methods, and separative methods (chromatography or capillary electrophoresis) are the most used and described (**Table 5**).

Table 5 Analytical methods for ion quantification in milk and dairy products. Depending on the sample preparation and the analytical method used, P can exist in different forms (**Table 2**)

	Ca	*Mg*	*Na*	*K*	*P*	*Cl*	*Citrate*
Atomic spectroscopic methods	x	x	x	x			
Titration	x	x				x	
Selective electrode	x		x			x	
Spectrophotometric method	x				x	x	x
Ionic chromatography	x	x	x	x	x	x	x
Capillary electrophoresis					x	x	x
Enzymatic method							x

Atomic spectroscopic methods

The atomic spectroscopic methods are rapid and accurate to determine the content of calcium, magnesium, sodium, and potassium in milk and dairy products. The best method is atomic absorption spectrometry but other techniques, like flame atomic absorption spectrometry, graphite furnace atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry, and inductively coupled plasma mass spectrometry, are available.

Titration

Calcium can be determined by its precipitation as calcium oxalate followed by titration of the excess of oxalate by a $KMnO_4$ solution. It is also possible to titrate calcium directly with the chelating agent, EDTA, in the presence of murexide that changes its color when it binds calcium. In a similar way, magnesium can also be determined by this method. In the case of a dairy mixture containing calcium and magnesium, the magnesium concentration is obtained as the difference between the two titrations, one with an indicator like murexide that measures calcium and the other with eriochrome black that detects both calcium and magnesium.

The method of Mohr is a classical method to determine chloride ions. It involves adding $AgNO_3$ to the dairy liquid to form a precipitate of AgCl. In dairy analyses, automatic equipments may be used to titrate automatically chloride ions with silver ions. When titration is complete, conductivity of the solution increases which can be sensed by electrodes causing the titration to stop. The instrument uses the elapsed titration time to calculate the chloride content.

Traditional methods for measuring sodium generally involve titration of chloride in the sample with silver nitrate with an indicator (potassium dichromate) for the end point. These methods are specific for chloride and indirectly measure sodium ion.

Ion-selective electrodes

They are used in conjunction with a reference electrode to form a complete electrochemical cell. The logarithm of the activity of a given ion in solution is correlated with the measured potential differences (ion-selective electrode vs. outer reference electrode potentials). Calcium, sodium, and chloride concentrations in milk and different dairy products (after adequate sample preparation) can be determined by these ion-selective electrodes.

Spectrophotometric methods

The calcium concentration can be measured in the presence of glyoxal-bis-(2-hydroxyanil), by forming a complex having an absorption maximum at 524 nm. This ion is also determined in the presence of *o*-cresolphthalein complexone and 2-amino-2-methylpropan-1-ol. The calcium–cresolphthalein complexone complex absorbs at 580 nm and pH 12.0.

The method of Briggs is a classical method to determine phosphorus concentrations. Under reducing conditions (presence of vitamin C or sulfite and hydroquinone), phosphorus reacts with molybdate (or vanadate) to form a blue complex, phosphomolybdate. This compound has a maximal absorption at 700 nm.

The content of citrate can be determined by measuring the absorbance of a product resulting from its reaction with pyridine in acetic anhydride. Another method for citrate determination is based on the complexation of Cu^{2+} by citrate. The complex formed absorbs at 280 nm. During enzymatic degradation of citrate in the presence of citrate lyase, malate dehydrogenase, lactate dehydrogenase, and NADH, H^+, the absorbance decrease of NADH, H^+ followed at 340 nm is proportional to citric acid concentration.

Ionic chromatography and capillary electrophoresis

The principal anions of milk and dairy products (chloride, inorganic phosphate, and citrate) can be separated by anion-exchange chromatography or by capillary electrophoresis. In both cases, the separation principle is based on differences in the charge-to-mass ratio of the ions analyzed. Their detected areas after elution are proportional to their concentration.

Theoretical Calculation of the Salt Equilibria

The different forms and location of ions and salts (**Tables 2** and **3**) in milk and dairy products are not all measurable experimentally and it is impossible, for example, to measure the amounts of calcium citrate and calcium phosphate in the aqueous phase. In this context, different research works were developed to calculate theoretically the salt equilibria. In the 1980s, calculation of the different ions and salts present only in the aqueous phase was described. Two studies proposed the theoretical calculation of the salt equilibria in milk by integrating the complexity of the micellar phase. The first study proposed by Holt in 2004 used a generalized empirical formula to calculate the composition of the nanoclusters containing calcium, magnesium, inorganic phosphate, and citrate in association with casein phosphorylated sequence named phosphate center. In addition, the mole fractions of the individual caseins not complexed to the calcium phosphate through one or more of their phosphate centers are included in the calculations. The calculated partition of salts between diffusible and non-diffusible fractions is well correlated with experimental values determined by other authors. The second model of calculation, proposed by Mekmene *et al.* in 2009, integrates

- affinities between cations (calcium, magnesium, potassium, and sodium) and anions (phosphoseryl residues and carboxylic groups of casein molecules, inorganic phosphate, chloride, and citrate) which contribute to the salt equilibria in milk and dairy products;
- pH;
- ionic strength of the aqueous phase; and
- solubility of calcium phosphate.

Good correlations between the calculated and experimental values are obtained. This second model offers the possibilities to calculate the distribution of ions and salts not only between aqueous and micellar phases in native milk but also under different physico-chemical conditions. These calculations constitute new and interesting tools to simulate salt equilibria in milk and dairy formulations containing different concentrations of minerals and caseins.

Conclusion

Research on the milk salt of milk has been conducted since the early 1900s and the knowledge concerning this fraction is gradually increasing. Today, and even if some questions are not totally resolved (chemical and physical nature of the MCP, its implication in the structure and stability of casein micelles), it can be considered that the salt composition and the salt equilibria are well described in native milk and also as a function of different physico-chemical conditions (pH and temperature variations, additions of supplementary salts). The use of different analytical methods to locate and quantify the different ions and salts present in milk and dairy products is probably a reason of this progress. This relatively good understanding of the milk salt equilibria is also confirmed by the good correlations found between the experimental determinations and values calculated theoretically.

See also: **Milk Salts**: Interaction with Caseins; Macroelements, Nutritional Significance; Trace Elements, Nutritional Significance.

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Interaction with Caseins

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Introduction and Definitions

The subjects of this article are the citrate (Cit), orthophosphate (P_i), and Cl^- salts of H^+, K^+, Na^+, Mg^{2+} and Ca^{2+}. These are the milk salts, though tradition also sanctions the use of the terms minerals or inorganic constituents for these components in spite of the fact that citrate salts are neither inorganic nor a mineral. The equilibria among these small ions cannot be considered in isolation because they interact strongly with other milk components, principally the Ca-sensitive caseins, β-, α_{S1}- and α_{S2}-caseins. For this reason, it is necessary to include some discussion of the structure of the colloidal particles in milk known as casein micelles.

Concentrations of the milk salts or individual ions are expressed here in units of mmol l^{-1} of skim milk, rather than in traditional units such as mg per 100 g milk because molar units form the basis for a thermodynamic treatment of the equilibria between the ions. Some aquatic mammals secrete milk with more than 50% fat so it is important to distinguish between whole and skim milk concentrations. Milk salts can be partitioned into diffusible and non-diffusible fractions by equilibrium dialysis or low pressure ultrafiltration across a membrane of suitable porosity. The concentrations in milk serum are denoted by a subscript s and non-diffusible or colloidal concentrations by a subscript c. Square brackets around a chemical symbol or anion abbreviation denote molar concentration and curly brackets, thermodynamic activity.

Secretory Mechanisms

Interrelationships of the Concentrations of the Monovalent Ions

Variations in the concentrations of the most osmotically important constituents of cows' milk show two different patterns of behavior. In healthy cows in mid lactation there is an inverse relation between lactose concentration, [L], and $[K^+]$ or $[K^+] + [Na^+]$ which is explained as a result of the drawing-in of water into milk to maintain a constant osmotic pressure during lactose synthesis. A notable result is the effect of insulin infusion of the goat that depresses lactose synthesis and reduces [L] in the milk while increasing [Casein]. A negative correlation of [L] and $[K^+]$ is found according to eqn [1] but $[Na^+]$ is unaffected.

$$[L] = 229.4 - 2.08(\pm 0.70)[K^+] \qquad [1]$$

On the other hand, at the end of lactation or during severe mastitic infection, the tendency is for $[Na^+]$ to be inversely related to [L] and $[K^+]$ and directly related to $[Cl^-]$. The observations can be explained as due to the mixing of the milk with an extracellular fluid rich in NaCl. Some examples of the two different patterns of interrelationships are shown in **Figure 1**.

Transcellular and Paracellular Routes of Secretion

The largely empirical knowledge of monovalent ion movements can be put into a wider physiological context of secretory mechanisms known as the transcellular and paracellular routes. According to a widely accepted scheme, $[K^+]$ and $[Na^+]$ will tend to attain electrochemical equilibrium across the apical membrane of the mammary secretory cell but in early or late lactation or during severe mastitic infection, the normally tight junctions between the mammary secretory cells become leaky and ions and other small molecules such as lactose can exchange with the NaCl-rich extracellular fluid. Since milk and blood have about the same osmolarity, Π, we can write:

$$\Pi = \text{constant} = \{L\} + \{Na^+\} + \{K^+\} + \{Cl^-\} + \cdots \text{smaller terms} \qquad [2]$$

so that $[K^+]$ is normally positively correlated with the other main ions and negatively correlated with [L], whereas, when the paracellular route overwhelms transcellular equilibration, $[K^+]$ becomes negatively correlated with $[Na^+]$ and $[Cl^-]$. Contrary to this theory, $[Na^+]$ and $[K^+]$ are uncorrelated with [L] in human milk but directly proportional to [Casein], which suggests that in high [L] milks, where serum salt concentrations are low, neutralization of net protein charge to achieve overall electrical neutrality becomes the dominant factor.

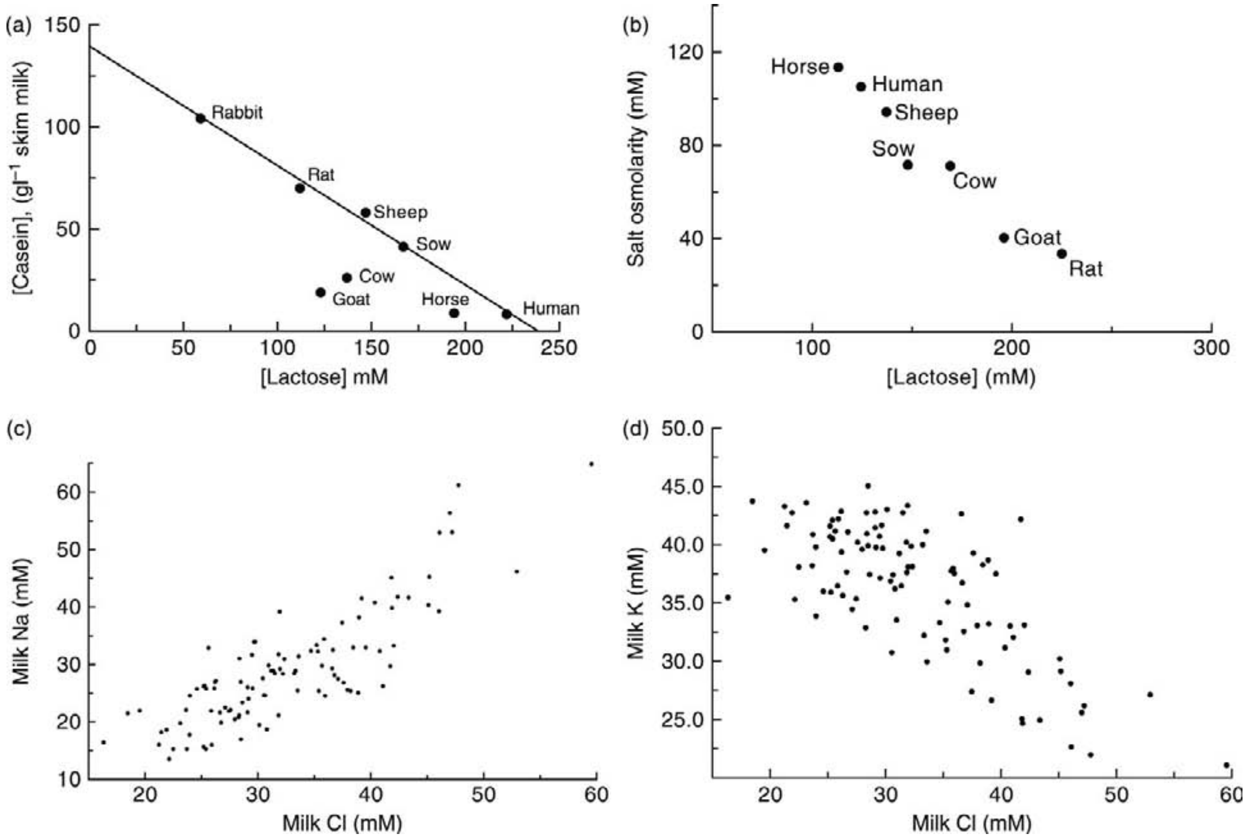

Figure 1 Direct and inverse correlations of the concentrations of lactose, casein and the monovalent ions. (a) Negative correlation of casein and lactose concentrations in the milks of eight species. (b) Negative correlation of calculated salt osmolarity with lactose concentration in milks of seven species. (c) Positive correlation of Na and Cl concentrations in the milk of individual Ayrshire cows at all stages of lactation. (d) Negative correlation of [K] with [Cl] in the milk of individual Ayrshire cows at all stages of lactation.

Lactose is synthesized in Golgi vesicles and transported to the apical membrane where exocytosis of the vesicle contents allows the aqueous phase of milk to accumulate in the alveolar lumen. This transcellular route is also followed by the great majority of the milk proteins and multivalent ions. Ca and P_i are concentrated in the vesicle by transport mechanisms that are far from clear and combine with caseins to form colloidal particles called casein micelles.

The sequestration of calcium phosphate by caseins is thought to provide a means by which this very insoluble material can be safely transported through the mammary gland, in high concentration in most species, without causing pathological calcification.

Vesicles appear to swell as they move toward the apical membrane. The swelling may be an osmotic dilution due, in eutherians, to lactose synthesis and the extent of swelling can be calculated from a simple theory which allows for a coupling of lactose synthesis to net anion transport through a coupling constant, ω. Assuming a constant concentration of casein in the unswollen vesicle, $[\mathrm{Casein}]_u$, the final concentration in milk is:

$$[\mathrm{Casein}] = \left(1-(1+\omega)\frac{[\mathrm{L}]}{\Pi}\right)[\mathrm{Casein}]_u \qquad [3]$$

which correctly predicts the negative correlation of [L] and [Casein] seen among 31 species. A maximum casein concentration of around $135\pm35\,\mathrm{g\,l^{-1}}$ is predicted.

Total Concentrations

Interspecific Variation

Our knowledge of the composition of milks is very sketchy. Of the 4000 or so extant species of mammals, the milk composition of only 200–300 has been reported in outline and only 30–40 have been sampled from three or more females. A nearly complete picture of the total and serum concentrations of the main salts is available for only about a dozen, common, domesticated or laboratory model species, and humans. Even in this small sample, the pattern of change from early to copious milk secretion is sufficiently diverse to make generalization dangerous. For example, a rise in citrate concentration seen in the early lacteal fluid of goats, cows, and women is the harbinger of copious milk

Table 1 Total concentrations (mmol l^{-1} skim milk) of salts in the milk of seven species

	Rat	*Sow*	*Sheep*	*Goat*	*Cow*	*Mare*	*Human*
Ca	80.5	49.6	56.8	23.1	30.1	16.5	7.3
Mg	8.8	4.1	9.0	5.0	5.1	1.6	1.4
P_i	54.2	24.4	39.7	15.6	20.9	6.7	2.2
Cit	0.06	8.4	4.9	5.4	9.8	3.1	2.2
Na	38.3	14.4	20.5	20.5	25.5	5.7	4.7
K	43.6	31.4	31.7	46.6	36.8	11.9	15.2
Cl	36.1	28.7	17.0	34.2	30.3	6.6	6.2
Casein[a]	2.97	1.75	2.46	0.80	1.11	0.35	0.41

[a] Assumed relative molecular mass of 23 600.

secretion but the rise occurs well before parturition in the horse and does not occur in either sows' or sheep milk. **Table 1** gives a summary of total concentrations of salts in seven species, representing a substantial part of the whole range of variation of composition in the few eutherian species that have been examined in sufficient detail.

The total milk concentrations are not arbitrarily determined because they are constrained by the secretory mechanism and physicochemical principles. In eutherians, [L] and [Casein] are negatively correlated because lactose synthesis draws water into the Golgi vesicle to dilute the proteins. [Casein], [Ca] and [P_i] are all positively correlated because they are largely present as a definite chemical substance, micellar calcium phosphate.

Variation in Cows' Milk

The variation in cows' milk is smaller than that seen among other species and occurs mainly in early and late lactation. In an individual, healthy cow throughout the greater part of lactation, milk salt composition is nearly constant, though there are consistent differences between animals. **Table 2** gives average values for total salt concentrations in the milk of Ayrshire cows in early, middle and late lactation and in milk from cows with sub clinical mastitis.

Table 2 Average values for pH and total salt concentrations in the skim milk of Ayrshire cows in early, middle and late lactation and from cows with sub-clinical mastitis[a]

	Early	*Middle*	*Late*	*Mastitic*
Ca	33.2	29.4	32.1	29.4
Mg	5.7	5.0	5.4	4.9
P_i	19.4	20.9	18.4	19.0
Citrate	9.8	9.1	8.5	8.8
Na	29.7	24.8	48.8	34.5
K	41.8	40.3	26.9	36.1
Cl	36.7	29.7	46.5	40.5
pH	6.53	6.73	6.98	6.87

[a] Recalculated data of White and Davies (1963) but with citrate values increased by 4%.

Serum Concentrations

Inter- and Intra-specific Differences

In cows' milk about one-third of the Ca, half the P_i, two-thirds of the Mg and over 90% of the Cit are in the milk serum but the proportions vary between species. However, in all species the K^+, Na^+, and Cl^- are nearly all in the serum. Total and serum [Cit] vary from virtually zero in the rat or mouse to more than 10 mmol l^{-1} in some ruminant species and the rabbit. In Scotland and some other countries, cows' milk citrate shows a seasonal variation associated with the 'spring flush' when the cows first go out to grass. The temporary depression by up to 1–2 mmol l^{-1} for about a month is probably directly related to steady state levels of citrate in the cytosol and the need for NADPH in fatty acid synthesis. **Table 3** gives some representative serum concentrations for seven species.

Interrelationships of the Serum Multivalent Ion Concentrations

There is a close positive correlation of $[\mathrm{Cit}]_s$ with $[\mathrm{Ca}]_s$ and generally also with $[\mathrm{Mg}]_s$, for variations among and within species, as shown in **Figure 2**.

The correlations of $[\mathrm{Ca}]_s$ with $[\mathrm{Cit}]_s$ shown in **Figure 2** are generally improved if $[\mathrm{Ca}]_s - [\mathrm{Ca}^{2+}]$ is plotted on the ordinate. The reason is that there is a pH dependence to $[\mathrm{Ca}^{2+}]$, caused by equilibria with the micellar calcium phosphate. The pH-related correlations for cows' milk are shown in **Figure 3**.

Multiple Ion Equilibria in the Serum

The multivalent ions and H^+ interact strongly in milk serum and this is why $[\mathrm{Ca}^{2+}]$ is normally less, and sometimes much less, than $[\mathrm{Ca}]_s$. The principal equilibria in cows' milk are the following:

$$\mathrm{Ca}^{2+} + \mathrm{Cit}^{3-} \rightleftharpoons \mathrm{CaCit}^- \qquad [4]$$

$$\mathrm{Mg}^{2+} + \mathrm{Cit}^{3-} \rightleftharpoons \mathrm{MgCit}^- \qquad [5]$$

Table 3 Serum concentrations of milk salts for seven species

	Rat	*Sow*	*Sheep*	*Goat*	*Cow*	*Mare*	*Human*
Ca	2.5	7.1	5.2	8.0	9.5	6.4	5.0
Mg	1.0	1.9	2.9	3.4	3.3	1.3	1.0
P_i	12.2	3.3	11.7	9.0	11.2	4.1	1.9
Citrate	0.04	3.0	3.8	5.0	9.2	3.1	2.7
Na	38.3	14.4	20.5	20.5	25.5	5.7	5.0
K	43.6	31.4	31.7	46.6	36.8	11.9	16.5
Cl	36.1	28.7	17.0	34.2	30.3	6.6	6.2
Lactose	112	167	147	123	137	222	194

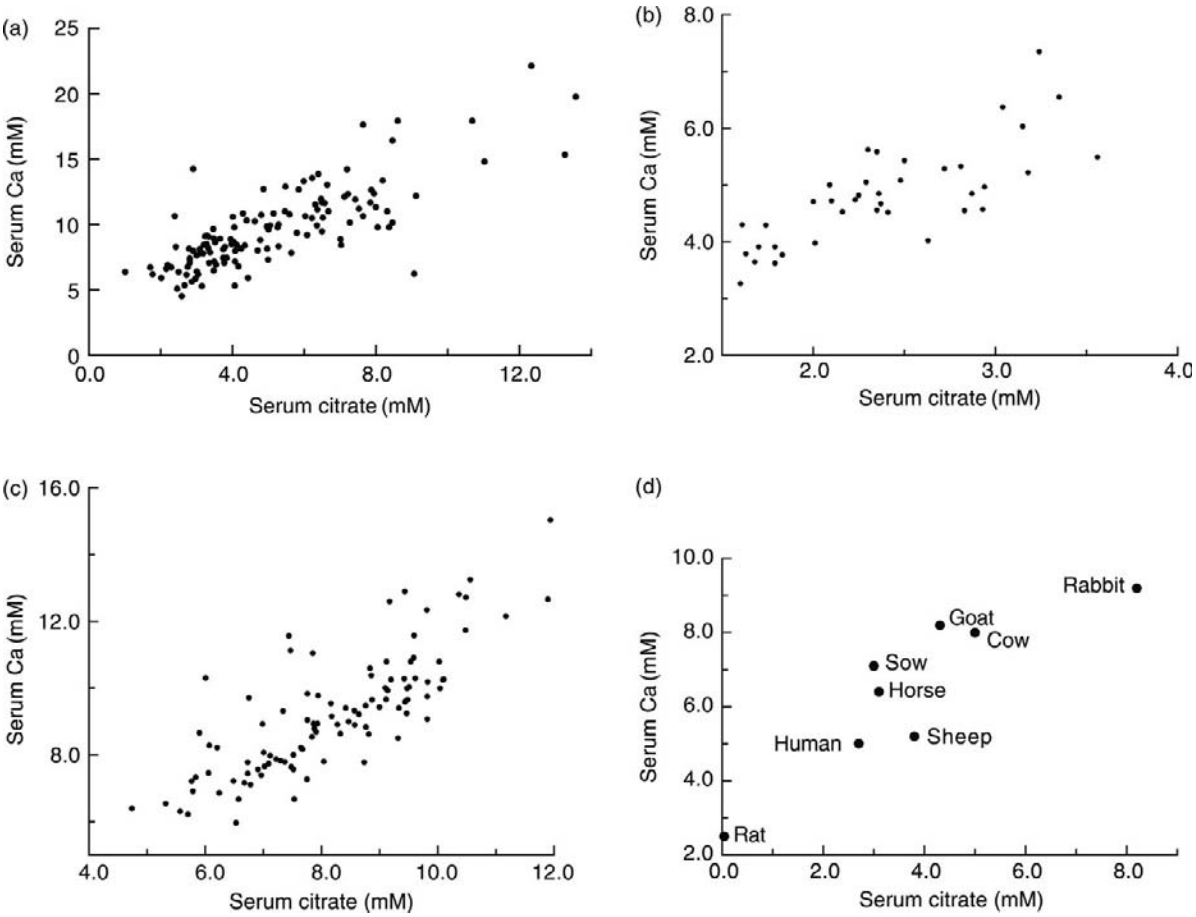

Figure 2 Close positive correlation of $[\mathrm{Cit}]_s$ with $[\mathrm{Ca}]_s$. (a) Natural variations in sows' milk and colostrum. (b) Natural variations in human milk in early, middle and late lactation. (c) Cows' milk in early, middle and late lactation. (d) Milk of eight species.

$$\mathrm{H}^+ + \mathrm{HPO}_4^{2-} \rightleftharpoons \mathrm{H_2PO}_4^- \qquad [6]$$

$$\mathrm{Ca}^{2+} + \mathrm{HPO}_4^{2-} \rightleftharpoons \mathrm{CaHPO}_4 \qquad [7]$$

The formation of a strong and soluble complex of the divalent cations with citrate is the reason for the positive correlations shown in **Figure 2**. It is possible to construct a full model of all the important ion equilibria in milk serum and, using appropriate ion activity coefficients, convert the derived activities to concentrations. **Table 4** summarizes one such calculation of the main ion species for a typical serum for cows in mid lactation.

The explanation for the correlations in **Figure 3** is quite involved but to a reasonable approximation can be obtained in the following way. The complex $\mathrm{CaHPO_4}$ is a weak one but of very limited solubility. Essentially, it goes on to form micellar calcium phosphate and the serum activities of the ions are constrained by a phase equilibrium condition:

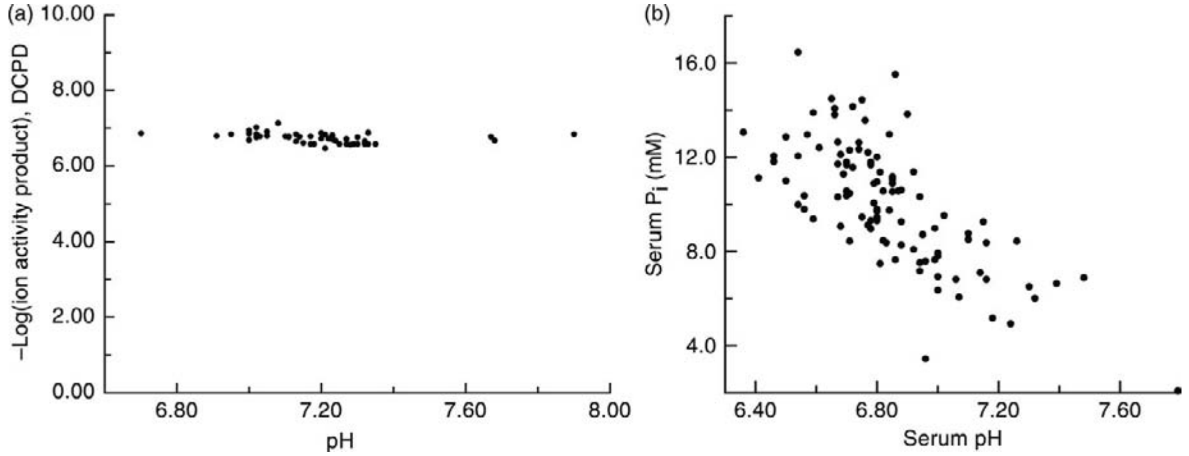

Figure 3 The pH related correlations of milk salt concentrations in cows' and human milk serum. (a) Apparently invariant ion activity product for a dicalcium phosphate salt in human milk sera. (b) Dependence of serum P_i concentration on pH in cows' milk.

Table 4 Summary calculation of the concentrations (mmol l^{-1}) of the main ion species in a typical milk diffusate[a] from cows in mid-lactation

		Cation complex			
Anion	*Free ion*	*Ca^{2+}*	*Mg^{2+}*	*Na^{+}*	*K^{+}*
Cit^{3-}	0.26	6.96	2.02	0.03	0.04
H_2PO^{4-}	7.50	0.07	0.04	0.10	0.18
HPO_4^{2-}	2.65	0.59	0.34	0.39	0.52
Cl^{-}	30.9	0.26	0.07	0.39	0.68
Free ion		2.00	0.81	20.92	36.29

[a] [Ca], 10.2; [Mg], 3.4; [Na], 22.0; [K], 38.0; [Cl], 32.3; [Cit], 9.4; [P_i], 12.4; [Glucose-1P], 2.6; [SO_4], 1.2; [CO_2], 0.44; [RCOOH], 3.1; pH, 6.70; Γ/2, 73 (calculated).

$$K_s = \{Ca^{2+}\} \times \{HPO_4^{2-}\} \qquad [8]$$

where K_s is an ion activity or solubility product. An invariant ion activity product of the form of eqn [8] is shown in **Figure 3(a)** for human milk. Similar relationships have been found in cows', goats', and sows' milk.

Since

$$[P_i]_s \approx [HPO_4^{2-}] + [H_2PO_4^{-}] \approx \{HPO_4^{2-}\} + \{H_2PO_4^{-}\} \qquad [9]$$

and

$$K_{H_2PO_4^-} = \frac{\{H_2PO_4^-\}}{\{HPO_4^{2-}\}\{H^+\}} \qquad [10]$$

the combination of eqns [8], [9], and [10] gives:

$$p\{Ca^{2+}\} \approx pK_s + p\left(1 + K_{H_2PO_4^-}\{H^+\}\right) - p[P_i]_s \qquad [11]$$

The precise form of the solubility relationship is more complex than eqn [8] but it is close enough to illustrate why, in eqn [11], natural variations in $[Ca^{2+}]$, pH and $[P_i]_s$ are interdependent, as shown in **Figures 3(a)** and **3(b)**.

Colloidal Concentrations

Interspecific Differences

Some values of colloidal concentrations for seven species can be readily derived by subtraction of the serum values in **Table 3** from the corresponding total concentrations in **Table 1**.

Interrelationships of the Concentrations of the Multivalent Ions

Both interspecific variations and intraspecific natural variations in the milk of cows, goats, and women show positive correlations of $[Ca]_c$, $[Mg]_c$, $[P_i]_c$, and [Casein] (**Figure 4**). Although comparably detailed data on the partition of milk salts are not available for many other species, the positive correlation between [Ca] and [P] in a number of other milks suggests that the micellar calcium phosphate is the predominant form of Ca in most milks, apart from primate, equine, and rhinoceros milks, where the casein concentration is quite small. The $[Ca]_c$, $[Mg]_c$, and $[P_i]_c$ also show positive correlations with intraspecific natural variations of $[Cit]_c$ in the cow and goat but not in womens' milk where the smaller concentrations give large errors in the determination of $[Cit]_c$. Moreover, inter-specific correlations of $[Cit]_c$ with any of the other colloidal components tend to be rather poor.

Some examples of the correlations of colloidal constituents both within and among species are shown in **Figure 4**.

Calcium Phosphate Nanoclusters and Micellar Calcium Phosphate

Calcium phosphate nanoclusters are nanometer-sized particles with a core of calcium phosphate and a shell of

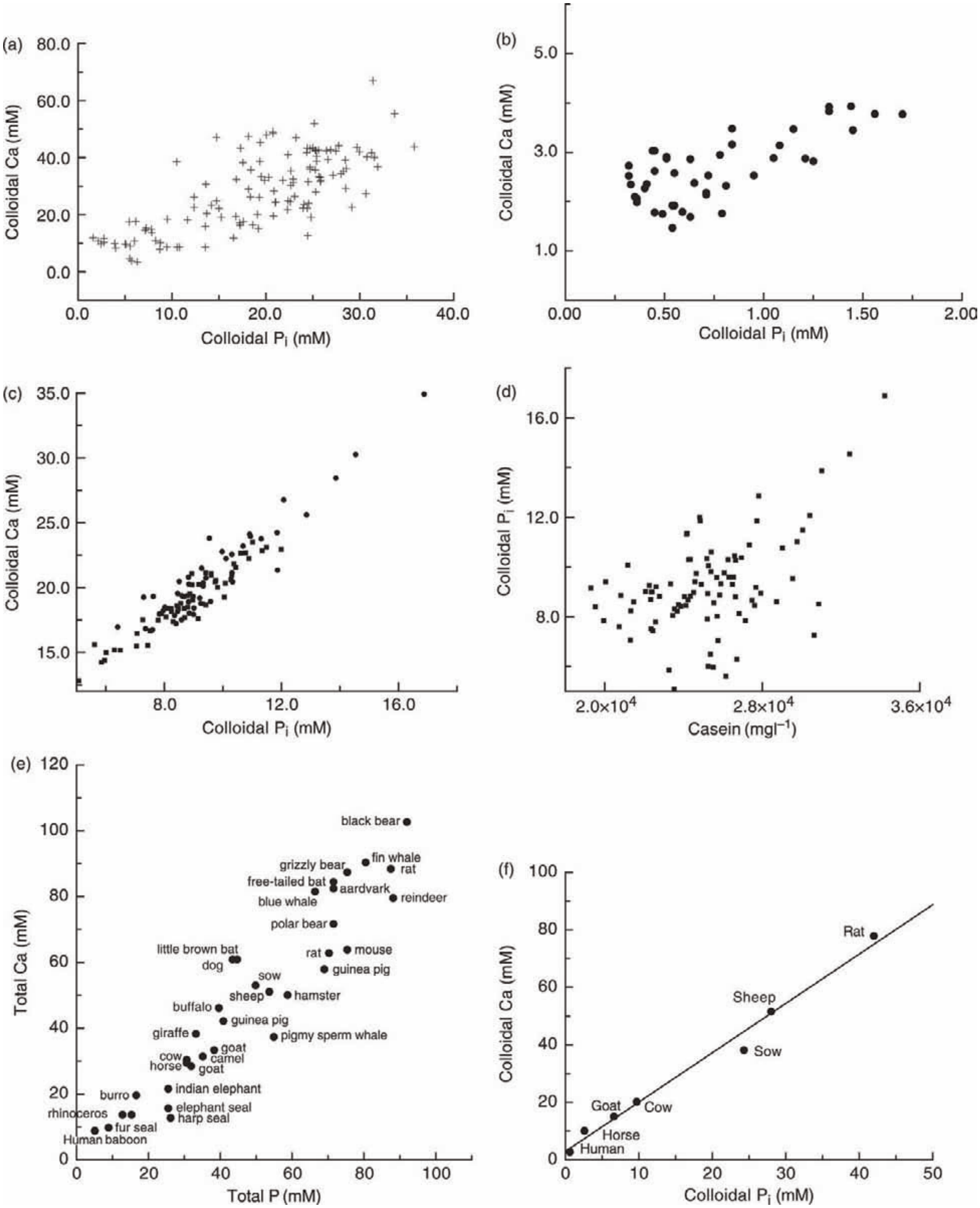

Figure 4 Some examples of the positive correlations of colloidal constituents, both within and between species. (a) $[Ca]_c$ and $[P_i]_c$ in sows' milk before, during, and up to 9 days after farrowing. (b) $[Ca]_c$ and $[P_i]_c$ in human milk. (c) $[Ca]_c$ and $[P_i]_c$ in cows' milk. (d) $[P_i]_c$ and [Casein] correlation in cows' milk. (e) Total Ca and P in 37 species. (f) $[Ca]_c$ and $[P_i]_c$ in seven species.

casein phosphopeptide. They form spontaneously from a solution containing 1.6 mmol l^{-1} phosphopeptide from the N-terminal region of β-casein, β-casein 4P (f1–25), together with milk salts at about the same concentrations as in cows' milk. The partition of salts in the nanocluster solution at pH 6.7, close to the natural pH of the milk, is essentially the same as in cows' milk and, in all other respects, the properties of the calcium phosphate in the nanoclusters closely approximate the known properties of the natural micellar calcium phosphate in cows' milk. The chemical formula of the nanoclusters is relatively invariant:

$$\left[\mathrm{Ca}_{13.2}(\mathrm{P_i})_{6.5}\mathrm{Mg}_{1.0}\mathrm{Cit}_{1.3}\mathrm{Casein}_{1.0}(\mathrm{SerP})_4\right]_{49} \qquad [12]$$

that is, there are 13.2 Ca ions per casein chain and the chain has 1 phosphate centre containing 4 phosphoserine (SerP) residues. A general formula for the composition of micellar calcium phosphate can be derived:

$$\left[\mathrm{Ca}_{13.2}(\mathrm{P_i})_{6.5}\mathrm{Mg}_{1.0}\mathrm{Cit}_{1.3}\mathrm{Casein}_{1/\bar{F}_b}(\mathrm{SerP})_s\right]_{49} \qquad [13]$$

where $\bar{F}_\mathrm{b}$ (≈1.6 in cows' milk) is the functionality or the average number of phosphate centers on Ca-sensitive caseins that are directly bound to the calcium phosphate through one or more of their phosphate centres. Equation [13] can be used to calculate the concentration of all the colloidal salts from a measurement of any one of them. The theoretical basis of the calculation derives from a simple model of casein micelle structure in which the micellar calcium phosphate is bound to a mol fraction, α, of all the phosphate centers in the caseins. The assumption is that micellar calcium phosphate has a defined and constant chemical formula as specified by the stoichiometric coefficients in eqn [13]. The concentration of unbound phosphate centers is then $(1-\alpha)\bar{F}_w[\mathrm{Casein}]$, where $\bar{F}_w$, (≈ 1.4 in cows' milk), is the number average functionality of whole casein. These unbound functionalities can then act as binding sites for small ions in competitive equilibria to give, principally, the isotherm functions $\bar{v}_\mathrm{Ca}$, $\bar{v}_\mathrm{Mg}$, and $\bar{v}_\mathrm{H}$ in units of mol ion per mol phosphate center. For example, the total colloidal concentrations of Ca and Mg are given by:

$$[\mathrm{Ca}]_\mathrm{c} = \bar{F}_\mathrm{w}[\mathrm{Casein}](13.2\alpha + (1-\alpha)\bar{v}_\mathrm{Ca} \times (\mathrm{pH}, \{\mathrm{Ca}^{2+}\}, \{\mathrm{Mg}^{2+}\})) \qquad [14]$$

$$[\mathrm{Mg}]_\mathrm{c} = \bar{F}_\mathrm{w}[\mathrm{Casein}](1.0\alpha + (1-\alpha)\bar{v}_\mathrm{Mg} \times (\mathrm{pH}, \{\mathrm{Ca}^{2+}\}, \{\mathrm{Mg}^{2+}\})) \qquad [15]$$

Within the range of natural variation, α is almost 1 so the term containing the binding isotherm is usually small enough to be neglected and the estimate of the colloidal concentration can be derived from the first term alone.

Such an analysis is shown in **Figure 5** for colloidal salts in the milks of seven species, where calculated values of $[\mathrm{Ca}]_\mathrm{c}$ and $[\mathrm{Mg}]_\mathrm{c}$ are compared with experiment.

As can be seen, the fitted lines for $[\mathrm{Ca}]_\mathrm{c}$ and $[\mathrm{Mg}]_\mathrm{c}$ have a slope close to unity and an intercept close to zero, as required by the theory. Not surprisingly colloidal citrate is poorly predicted by eqn [13], indicating that it is neither an invariant nor an essential component of the micellar calcium phosphate.

Conclusions

Milks are diverse in their composition, both quantitatively and qualitatively, and exhibit a multitude of patterns of change with respect to diet, season, and stage of lactation. Nevertheless, underlying the apparent complexity are patterns of change seen in the interrelationships of concentrations. Our understanding of the milk salts has advanced to the point where precise, physicochemical models give good predictions of the partition of salts in milk and account quantitatively for the interrelationships that occur as a result of natural variation.

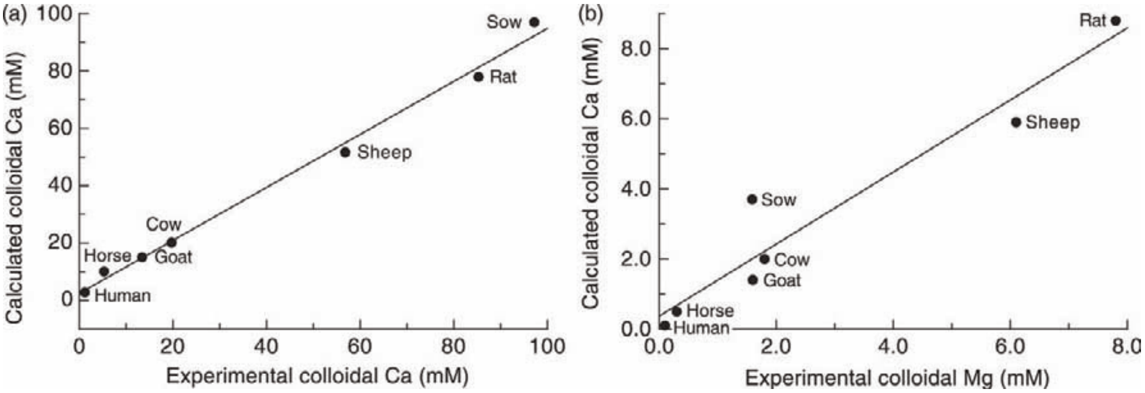

Figure 5 (a) Calculated and experimental values of $[\mathrm{Ca}]_\mathrm{c}$ for seven species. The regression equation shown has a slope of 0.924 ± 0.034 and intercept 2.4 ± 1.8 mM Ca. (b) Calculated and experimental values of $[\mathrm{Mg}]_\mathrm{c}$. The regression equation shown has a slope of 1.02 ± 0.13 and intercept 0.37 ± 0.49 mM Mg.

See also: **Analytical Methods**: Microscopy (Microstructure of Milk Constituents and Products). **Mammals**. **Milk Proteins**: Casein, Micellar Structure. **Milk Salts**: Distribution and Analysis.

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Macroelements, Nutritional Significance

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Introduction

In this article the term 'minerals' refers to those mineral elements that are of nutritional significance and, though the term may be chemically inaccurate, it is a commonly used and widely accepted terminology in the field of nutrition. Mineral elements occur in the body in a number of chemical forms, such as inorganic ions and salts, and constituents of organic molecules such as proteins, fats, carbohydrates, and nucleic acids. They serve a wide variety of essential physiological functions, ranging from structural components of body tissues to essential components of many enzymes and other biologically important molecules.

This article, in conjunction with another article within this Encyclopedia (*see* **Milk Salts**: Trace Elements, Nutritional Significance), outlines the nutritional roles, recommended intakes, and hazards of deficiency or excess of the 20 minerals that are considered to be nutritionally essential for humans, all of which occur in milk. In this article, 'milk' refers to cow's milk unless otherwise stated. The content, chemical form, bioavailability, and nutritional significance of these minerals in milk and dairy products are discussed.

The 20 minerals considered essential in the human diet are sodium, potassium, chloride, calcium, magnesium, phosphorus, iron, copper, zinc, manganese, selenium, iodine, chromium, cobalt, molybdenum, fluoride, arsenic, nickel, silicon, and boron. The essential minerals are sometimes classified into two groups: the macroelements (also known as the macrominerals) and the trace elements. The macrominerals (sodium, potassium, chloride, calcium, magnesium, and phosphorus) are present in the body in amounts larger than ~0.01% by weight, whereas the trace elements (the remaining 14 essential minerals) occur in the body at much lower levels and are required in the diet in amounts smaller than ~100 mg day^{-1}. The nutritional aspects of the former class of minerals will be discussed in this article, whereas the nutritional aspects of the latter class will be dealt with elsewhere (*see* **Milk Salts**: Trace Elements, Nutritional Significance).

Although the minerals are treated separately, it is important to realize that interactions of the minerals with each other, with other constituents of milk, and with other food constituents occur, and that such interactions are assuming an increasing importance in nutrition.

Content and Chemical Form of Macrominerals in Milk and Dairy Products

Mineral Content

The mineral content of milk is not constant but is influenced by a number of factors such as stage of lactation, nutritional status of the animal, and environmental and genetic factors. Reported values in the literature for the concentration of many minerals show a wide variation, which is due partly to these factors, but also partly to analytical errors and contamination during milk collection and from processing equipment and procedures. Representative values for the average macromineral content of milk are presented in **Table 1**.

Sodium, Potassium, Chloride

Particularly high levels of sodium occur in cow's colostrum, but the level decreases within a few days to the value shown in **Table 1**. The sodium content of milk is not influenced by dietary sodium intake within the normal range. Sodium concentration in milk tends to be higher at the end of lactation when milk yield is low. Removal of milk fat has little effect on milk sodium content. In contrast to most other minerals, potassium concentration in cow's colostrum is lower than that in mature milk, but increases to normal values within the first 2–3 days of lactation and is independent of potassium intake. The chloride concentration in milk decreases from higher levels in colostrum to lower levels in mature milk but increases sharply toward the end of lactation and is independent of dietary intake. The sodium, potassium, and chloride contents of other dairy products are shown in **Tables 2–5**. Levels of sodium (and also chloride) in cheese are dependent on the amount of added salt. Levels of potassium are no higher in cheese than in milk. In general, the concentration of macrominerals decreases as the fat concentration increases in milk and dairy products (**Table 3**).

Calcium, Phosphorus, Magnesium

The mean calcium and phosphorus contents of cow's milk are 1120 and 890 mg l^{-1}, respectively. In cow's milk, calcium concentration is slightly elevated in colostrum and at the end of lactation but varies little with feeding or

Table 1 Mean concentrations of macrominerals in cow's milk

Mineral	*Content (mg l^{-1})*
Sodium	430
Potassium	1550
Chloride	890
Calcium	1180
Phosphorus	930
Magnesium	110

Data from Food Standards Agency (2002) *McCance & Widdowson's. The Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

season. There is little variation in the phosphorus content of milk throughout lactation.

The mean concentration of magnesium in milk is 110 mg l^{-1} and is unaffected by fat removal. Magnesium concentration in colostrum is 2–3 times that in mature milk and decreases to the level in mature milk within the first 1–3 days of lactation, remaining relatively constant thereafter. The concentration of magnesium in milk is unaffected by dietary intake of magnesium.

The calcium, phosphorus, and magnesium contents of other dairy products are shown in **Tables 2–5**. Levels of calcium and phosphorus are generally highest in hard cheeses (Parmigiano, Gouda, Edam, and Cheddar), that is, up to 10 times that in milk, followed by mold cheeses (Brie, Stilton), that is, 4–5 times that in milk (**Table 5**). The lowest levels are found in cream and cottage cheese types. Levels of magnesium are ordered similar to calcium but only around 5 times higher in hard cheeses and 2–3 times higher in mold types.

Chemical Form of the Macrominerals

The chemical form of a mineral is important because it may influence intestinal absorption and utilization (the process of transport, cellular assimilation, and conversion into a biologically active form) and thus bioavailability.

Sodium, Potassium, Chloride

Sodium, potassium, and chloride are believed to be present in milk almost entirely as free ions. Practically all the sodium, potassium, and chloride in milk is absorbed in the

Table 2 Mean concentrations of macrominerals in concentrated milks

	Content (mg 100 g^{-1})			
Mineral	*Pasteurized skimmed*	*Dried skimmed*	*Evaporate (whole)*	*Condensed (whole)*
Sodium	43	550	180	140
Potassium	155	1590	360	360
Chloride	89	1070	250	230
Calcium	118	1280	290	290
Phosphorus	93	970	260	240
Magnesium	11	130	29	29

Data from Food Standards Agency (2002) *McCance & Widdowson's. The Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

Table 3 Mean concentrations of macrominerals in creams

	Content (mg 100 g^{-1})						
	Fresh cream				*Soured*	*Sterilized canned*	*UHT*
Mineral	*~10% fat*	*~20% fat*	*~35–48% fat*	*~60% fat*	*~20% fat*	*~25% fat*	*~32% fat*
Sodium	49	29	25	18	41	53	31
Potassium	120	104	86	55	110	110	107
Chloride	77	80	59	40	81	78	66
Calcium	99	80	58	37	93	86	54
Phosphorus	89	79	59	40	81	73	57
Magnesium	11	8	6	5	10	10	7

Data from Food Standards Agency (2002) *McCance & Widdowson's. The Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

Table 4 Mean concentrations of macrominerals in butter, yogurt, and dairy ice cream

	Content (mg 100 g^{-1})		
Mineral	*Butter (whole milk)*	*Yogurt*	*Dairy ice cream*
Sodium	606[a]	80	60
Potassium	27	280	174
Chloride	994[a]	170	110
Calcium	18	200	100
Phosphorus	23	170	91
Magnesium	2	19	12

[a]Unsalted butter contains 9 mg 100 g^{-1} sodium and 19 mg 100 g^{-1} chloride.
Data from Food Standards Agency (2002) *McCance & Widdowson's. The Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

Table 5 Mean concentrations of macrominerals in some cheese varieties

	Content (mg 100 g^{-1})								
Mineral	*Brie*	*Cheddar*	*Cream*	*Cottage*	*Edam*	*Feta*	*Gouda*	*Parmesan*	*Stilton*
Sodium	556	723	300	300	996	1440	925	756	788
Potassium	91	75	160	161	89	95	82	152	96
Chloride	900	1040	480	490	1570	2350	1440	1260	1230
Calcium	256	739	98	127	795	360	773	1025	326
Phosphorus	232	505	100	171	508	280	498	680	314
Magnesium	15	29	10	13	34	20	32	41	15

Data from Food Standards Agency (2002) *McCance & Widdowson's. The Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

gastrointestinal tract, although much of what is absorbed is not retained.

Calcium, Phosphorus, Magnesium

In cow's milk, 99% of the calcium is in the skim milk fraction, which explains why the calcium content is not affected by fat removal. Two-thirds of the total calcium occurs in colloidal form associated with the casein micelles, either as a calcium phosphate salt (about half of total milk calcium) or as calcium ions bound to phosphoserine residues (about one-sixth of total calcium); the remaining one-third is soluble. Ionized calcium in the soluble phase accounts for ~10% of the total calcium, and most of the remaining soluble calcium occurs as calcium citrate. A small amount of calcium (0.15%) is bound to α-lactalbumin.

Of the total phosphorus in cow's milk, 20% occurs as organic phosphate esterified to casein with the remainder occurring as inorganic phosphate. About 44% of the inorganic phosphate is associated with casein micelles as calcium phosphate and 56% is soluble, mainly as free phosphate ions.

In cow's milk, 98–100% of the magnesium is in the skim milk phase, where 65% of the magnesium is in a soluble form (40% as magnesium citrate, 7% as magnesium phosphate, and 16% as free magnesium ion), and the remainder is colloidal and is associated with the casein micelles (about one-half associated with colloidal calcium phosphate and the other half bound directly to phosphoserine residues in caseins).

Nutritional Significance of Macrominerals in Milk and Dairy Products

A simple and useful evaluation of the nutritional significance of macrominerals (as well as trace elements) in milk can be obtained by comparing the amounts of the different elements provided by 1 l of milk (**Table 1**) with the recommended daily intakes for these elements (**Table 6**). Another useful impression of the nutritional significance of milk and other dairy products can be obtained by computing, from data on dietary surveys, the actual contribution made by dairy products to the total intake of macrominerals and trace elements.

Sodium, Potassium, Chloride

Sodium is the principal cation in extracellular fluids and is the primary regulator of extracellular fluid volume. It is

Table 6 Recommended dietary intakes of selected macrominerals

		Minerals		
Category	*Age (years)*	*Calcium[a] (mg)*	*Magnesium[b] (mg)*	*Phosphorus[b] (mg)*
Infants	0.0–0.5	210	30[a]	100[a]
	0.5–1.0	270	75[a]	275[a]
Children	1–3	500	80	460
	4–8	800	130	500
	9–13	1300	240	1250
Males	14–18	1300	410	1250
	19–30	1000	400	700
	31–50	1000	420	700
	51–70	1200	420	700
	>70	1200	420	700
Females	14–18	1300	360	1250
	19–30	1000	310	700
	31–50	1000	320	700
	51–70	1200	320	700
	>70	1200	320	700
Pregnancy	≤18	1300	400	1250
	19–30	1000	350	700
	31–50	1000	360	700
Lactation	≤18	1300	360	1250
	19–30	1000	310	700
	31–50	1000	320	700

[a] US adequate intake values; from Institute of Medicine (1997) *Dietary Reference Intakes: Calcium, Magnesium, Phosphorus, Vitamin D, and Fluoride*. Washington, DC: National Academy Press.
[b] US recommended dietary allowance (RDA); from Institute of Medicine (1997) *Dietary Reference Intakes: Calcium, Magnesium, Phosphorus, Vitamin D, and Fluoride*. Washington, DC: National Academy Press.

important in the regulation of osmolarity, acid–base balance, and the membrane potential of cells, as well as in active transport across cell membranes. Chloride is the principal extracellular anion and is essential in the maintenance of fluid and electrolyte balance. Potassium is the principal intracellular cation, occurring in the cell at a concentration more than 30 times its concentration in extracellular fluid. Extracellular potassium contributes to the transmission of nerve impulses, to the control of skeletal muscle contraction, and to the maintenance of blood pressure.

Under normal circumstances, dietary deficiency of sodium, potassium, or chloride does not occur, but the body can be depleted of sodium and chloride under extreme conditions, for example, heavy perspiration, chronic diarrhea, or renal disease. Depletion of potassium can occur in the body under conditions where there are large alimentary or renal losses.

Minimum requirements for sodium, potassium, and chloride (e.g., 500, 2000, and 750 mg day^{-1}, respectively, in adults) have been established. Actual intakes of sodium and chloride are considerably higher than the minimum requirements in many populations and, given the evidence of a relationship of high salt intake to hypertension, it has been recommended that sodium intake be limited to 2.4 g day^{-1}. Similarly, considering the possible beneficial effect of potassium in hypertension, increasing potassium intake beyond the minimum requirement has been recommended.

Cow's milk contributes little to the dietary intake of sodium (7% in the United Kingdom), but some dairy products such as cheese and butter contain added salt and can be significant sources of sodium in some countries (e.g., ~13% of total sodium intake in the United Kingdom). It has been estimated that milk and dairy products provide 20% of total sodium and 24–29% of total potassium in the diet in Ireland and the United Kingdom.

The concentrations of sodium, potassium, and chloride in milk are of physiological importance in the feeding of the young infant, and clinical problems may arise if there is an excessive intake of these nutrients. The kidney of the young infant, as compared to that of the adult, has a limited capacity to concentrate solids, and the renal solute load exerts a major effect on water balance. Renal solute load is determined mainly by sodium, potassium, chloride, phosphorus, and protein (which gives rise to urea). Cow's milk has a much higher potential renal solute load (~300 mosmol l^{-1}) than human milk (~93 mosmol l^{-1}). The high renal solute load resulting from ingestion of cow's milk may be of relatively little significance in most circumstances because the kidney merely excretes a more concentrated urine. However, it does lead to a smaller margin of safety against dehydration, which can occur in conditions of diarrhea, fever, and low water intake, and for this reason it is recommended that the upper limit of potential renal solute load in formulae for young infants be ~220 mosmol l^{-1}. Recommended concentrations of sodium, potassium, and chloride in infant formulae are in the ranges 133–403, 534–1338, and 369–1360 mg l^{-1}, respectively. Cow's milk-based infant formulae currently in use fall comfortably within these guidelines.

Calcium

The adult human body contains ~1200 g of calcium, which amounts to ~1.5–2% of the body weight. Of this, 99% is found in bones and teeth, where it is present as calcium phosphate, providing strength and structure. The remaining 1%, found in extracellular fluids and intracellular structures and cell membranes, is responsible for a number of regulatory functions such as maintenance of normal heart beat, blood coagulation, hormone secretion, integrity of intracellular cement substances and membranes, nerve conduction, muscle contraction, and activation of enzymes.

Cow's milk and milk products, such as cheese and yogurt, are very good sources of dietary calcium. The contribution of dairy products to total calcium intake has been estimated as 75% in The Netherlands, 55% (dietary intake data) to 72% (food disappearance data) in the United States, 60% in the United Kingdom, and 52% in Ireland.

In the absence of milk and dairy products from the diet, calcium intakes in excess of 300 mg day^{-1} are difficult to achieve. This is far below the typical recommended intakes for calcium. For example, the recently established adequate intake (AI) values for calcium in the United States are 1000 mg for adults, 1200 mg for older adults, and 1300 mg for adolescents (**Table 6**). On this basis, it might be considered that consumption of dairy products is very important in order to achieve an adequate calcium intake. However, there is still considerable disagreement on human calcium requirements, and this is reflected in the wide variation in the recommended adult dietary intakes for calcium (400–1200 mg) that have been set by different authorities. The dietary reference value for calcium is currently under review in the United States, and the new recommendations will be released in Summer 2010.

In recent years, considerable attention has been focused on the bioavailability of calcium in milk. Evidence from studies on experimental animals suggests that essentially all of the calcium in human milk, cow's milk, and cow's milk-based infant formulae is potentially available for absorption in the gastrointestinal tract. In addition, it has often been suggested that the bioavailability of calcium is higher in human milk than in cow's milk or cow's milk-based infant formulae. This arises from the findings that calcium is absorbed more efficiently by human infants from human milk than from cow's milk or a cow's milk-based formula containing 738 mg Ca l^{-1}. However, the absolute amount of calcium absorbed is higher from cow's milk and formulae, and calcium absorption from cow's milk formulae with lower calcium contents (363–458 mg l^{-1}) is similar to that from human milk. Similarly, the efficiency of calcium absorption from human milk by weanling Rhesus monkeys has been reported as being significantly higher (72%) than from cow's milk-based infant formulae (45–53%), but the absolute absorption from human milk and formulae were similar due to the higher calcium concentration in the formulae. Moreover, bone mineralization in infants fed with cow's milk formula has been reported to be as good as, if not slightly better than, in infants fed with human milk.

Mean calcium absorption from cow's milk by healthy human adults has been variably reported in the range 21–45%. Calcium absorption has been reported as being similar from milk and from $CaCO_3$ by pregnant women at 36–47%, but calcium absorption from cow's milk by postmenopausal women has been variably reported in the range 5–41%. Calcium absorption from cow's milk by β-galactosidase-deficient subjects has been reported as being higher (36.2% of dose) as compared to a β-galactosidase-sufficient group (25.7%), and it has been suggested that this reflects lower habitual calcium intake owing to reduced milk consumption by the β-galactosidase-deficient subjects. It has been reported that calcium from various dairy products (including whole milk, chocolate milk, yogurt, Cheddar cheese, processed cheese) is absorbed equally well.

Thus, though it appears that all of the calcium in human milk, cow's milk, and cow's milk-based infant formulae is potentially available for absorption, the absolute amount of calcium absorbed by animals or humans is determined by physiological factors such as the efficiency of calcium absorption mechanisms in the gastrointestinal tract, which may be influenced by calcium needs, vitamin D status, and age, as well as by the calcium concentration in milk. In addition, some components of milk (lactose, phosphopeptides) may enhance calcium absorption.

There is strong evidence that lactose promotes intestinal absorption (particularly in the ileum) and body retention of calcium in rats. This effect of lactose is independent of vitamin D, but the mechanism by which it occurs remains unresolved. It has been suggested that undigested lactose reaching the ileum interacts with the brush border membrane, increasing its permeability to calcium. Lactose also appears to increase calcium absorption in human infants. For example, calcium absorption has been reported to be significantly higher from a soy-based infant formula containing lactose (48%) than from a similar formula in which the carbohydrate source was a mixture of starch hydrolysate and sucrose (33%). However, studies on the effect of lactose on calcium absorption by human adults have produced conflicting results. One study found no significant difference in calcium absorption from milk or lactose-hydrolyzed milk by either β-galactosidase-deficient or β-galactosidase-sufficient subjects, whereas another study reported that calcium absorption was similar from milk (21.4%) and lactose-free milk (lactose replaced by glucose) (26.8%) by healthy adult subjects, but lactose increased calcium absorption by β-galactosidase-deficient subjects. Overall, it is likely that lactose, at the level normally present in milk, does not have a significant effect on calcium absorption in healthy adults consuming normal diets. It is possible that lactose affects the non-saturable paracellular pathway for calcium absorption in the gut and its effect is most likely to occur in vitamin D deficiency, or when elevated levels of calcium are fed.

It has been suggested that phosphopeptides formed during the digestion of bovine caseins may be involved in promoting calcium absorption. Such phosphopeptides have the capacity to chelate calcium and to prevent the precipitation of calcium phosphate salts and may help to maintain a high concentration of soluble calcium in the intestinal lumen. There is evidence that phosphopeptides are present in the lumen of the small intestine of rats and pigs after a casein-containing meal; purified phosphopeptides have been shown to enhance the absorption of calcium in rats and chicks. The observation that calcium absorption in rats is enhanced by high-casein meals is consistent with this. However, the nutritional significance of these phosphopeptides in humans consuming milk remains unclear.

Age-related osteoporosis, a common bone disease and a major cause of disability in Western countries, is characterized by reduced bone density resulting in increased bone fragility and susceptibility to fracture. The condition is particularly common in elderly women, especially in Caucasians. It has been estimated that osteoporosis afflicts 10 million Americans (half of the women over 45 years of age and 90% of the women over 75 years of age).

Osteoporosis is a multifactorial disorder, but there is increasing evidence that inadequate calcium intake, particularly during early life, is a contributory factor. Adequate calcium intake is required in early life in order to develop maximum bone mass at maturity (during the third decade of life), and there is evidence that the amount of bone mass present at maturity is an important factor influencing fracture susceptibility in the elderly. Peak bone mass at maturity appears to be related to the intake of calcium during the years of bone mineralization. Inadequate dietary intake of calcium during the critical growth and building periods may result in failure to reach peak bone mass, causing osteopenia, osteoporosis, decreased skeletal integrity, and increased risk of fracture in later life. Many believe that the most promising nutritional approach to reduce the risk of osteoporosis in later life is to ensure a calcium intake that allows the development of each individual's genetically programmed peak bone mass during the formative years, that is, throughout childhood to the age of 25 years. In this regard, the prevalence of lower-than-recommended calcium intake among adolescent and young adult females in many countries, for example, in the United States and Europe, is of particular concern.

In recent years, evidence has been presented for a protective role of dietary calcium against hypertension, hypercholesterolemia, diabetes mellitus, and colon and rectal cancer. Dietary calcium has also been proposed to help regulate and maintain body weight. However, consensus on the role of calcium in these conditions is lacking and further research is required.

Phosphorus

Phosphorus is an essential nutrient for humans and serves a number of important biological functions. Phosphorus occurs as organic and inorganic phosphates in all body tissues and fluids; is an essential component of many biological molecules, including lipids, proteins, carbohydrates, and nucleic acids; and plays a central role in metabolism. As calcium phosphate, it is a major structural component of bones and teeth. Because almost all foods contain phosphorus, dietary phosphorus deficiency does not usually occur. The recently revised recommended dietary allowances (RDAs) for phosphorus are shown in **Table 6**.

Cow's milk and milk products, such as cheese and yogurt, are good dietary sources of phosphorus, and the contribution of milk and dairy products to total phosphorus intake has been reported as 30–45% in Western countries.

In the first few weeks of life, the infant's ability to regulate plasma calcium concentration is not fully developed, and hypocalcemia may result in neonatal tetany, which used to occur more frequently in artificially fed than in breast-fed infants. Excessive phosphorus intake contributes to this condition, and feeding unmodified cow's milk, which has a high content of phosphorus, increases serum phosphorus and lowers serum (ionized) calcium in the newborn infant. For this reason, it is recommended that the calcium-to-phosphorus ratio in artificial infant formulae be higher than that in cow's milk (~1.2:1) and more similar to that in human milk (~2.2:1).

In experimental animals, a high intake of phosphorus or a low calcium-to-phosphorus ratio in the diet could lead to bone loss. However, it is generally agreed that wide variations in phosphorus intake or in the calcium-to-phosphorus ratio in the diet do not adversely affect bone mass in adult humans.

Magnesium

Magnesium has an essential role in a wide variety of physiological processes, including protein and nucleic acid metabolism, neuromuscular transmission and muscle contraction, bone growth and metabolism, and regulation of blood pressure, and it acts as a cofactor for many enzymes.

There is little information from human studies on the bioavailability of magnesium from milk. Metabolic-balance studies in infants showed that 16–43% of magnesium is absorbed from cow's milk-based infant formulae and that lactose enhances the absorption of magnesium. Cow's milk and milk products contribute 16–21% of the total magnesium intake in Western countries.

Dietary deficiency of magnesium is uncommon except in conditions of severe malnutrition and certain disease states. There is evidence that many young adults, especially women, in various European countries and in the United States fail to achieve the recommended intakes of magnesium and this raises the issue of possible adverse effects of lower-than-recommended intakes. Scientists have attempted to demonstrate that suboptimal intake of magnesium (i.e., below the RDA, but not frank deficiency) is a contributor to the development of chronic maladies such as cardiovascular disease, hypertension, disorders of skeletal growth and osteoporosis, and diabetes mellitus. However, the results of studies in this area are ambiguous. The fact that the RDA for magnesium has been raised recently for most groups (**Table 6**) reflects nutrition scientists' belief that there is a negative consequence to suboptimal magnesium intake. Additional research is needed to fully justify this concern.

Conclusion

Even though the nutritional roles, requirements, and metabolism, and the quantitative relationship between dietary intakes and health for a number of macrominerals have been more clearly defined in recent years, there are still considerable deficiencies in our understanding of these issues, for example, the significance of calcium and magnesium in the etiology and treatment of osteoporosis and hypertension.

Reliable information on the content, and the principal factors affecting it, of most of the macrominerals in cow's milk is now available. The contribution of cow's milk and milk products to the diet in Western countries is significant for sodium, potassium, chloride, calcium, and phosphorus.

Sodium, potassium, and chloride are believed to be almost totally absorbed from milk and infant formulae. Though the bioavailability of calcium in milk has received considerable attention in recent years, there is little information on the bioavailability of magnesium in milk and infant formulae. In addition, there is a paucity of data regarding the bioavailability of macrominerals from other dairy products. Factors affecting the bioavailability of minerals are still poorly understood, and the role of possible enhancers (e.g., lactose, phosphopeptides) or inhibitors (e.g., proteins, calcium, phosphate) of macromineral absorption in milk and dairy products remains unclear. However, milk does not appear to contain substances that are strongly inhibitory to mineral absorption, such as phytate or polyphenols contained in foods from plants.

Finally, understanding the nutritional significance of macrominerals in milk and dairy products will also benefit from the improvement in our knowledge of fundamental aspects of minerals, such as their nutritional roles, requirements, and metabolism, and the quantitative relationship between dietary intakes and health.

See also: **Milk Salts**: Trace Elements, Nutritional Significance.

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Trace Elements, Nutritional Significance

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Introduction

There are 20 minerals that are considered to be nutritionally essential for humans, and these are sometimes classified into two groups: macroelements (also known as the macrominerals) and trace elements. The macrominerals (sodium, potassium, chloride, calcium, magnesium, and phosphorus) are present in the body in amounts larger than ~0.01% by weight, and the nutritional aspects of these minerals are dealt with elsewhere (*see* **Milk Salts**: Macroelements, Nutritional Significance). The trace elements (the remaining 14 essential minerals) occur in the body at much lower levels and are required in the diet in amounts smaller than ~100 mg day^{-1}. The nutritional aspects of these minerals will be discussed in this article.

The 14 trace elements considered essential in the human diet are iron, copper, zinc, manganese, selenium, iodine, chromium, cobalt, molybdenum, fluorine, arsenic, nickel, silicon, and boron. Although some of these, for example, arsenic, nickel, silicon, and boron, have not been shown to be essential for humans, they are essential for experimental animals and probably are also essential for humans. A number of other elements occur in milk, for example, lithium, bromine, aluminum, strontium, silver, lead, tin, vanadium, mercury, cadmium, rubidium, and cesium. These are not nutritionally essential and are not discussed here; moreover, many of them are toxic. However, their concentrations in milk are normally well below toxic levels.

This article outlines the nutritional roles, recommended intakes, and hazards of deficiency or excess of the 14 trace elements considered to be nutritionally essential for humans, all of which occur in milk. In this article, 'milk' refers to cow's milk unless otherwise stated. The content, chemical form, bioavailability, and nutritional significance of these trace elements in milk and dairy products are considered. In addition, in view of the widespread use of infant formulae based on cow's milk, some nutritional aspects of trace elements in these formulae are discussed.

Although the trace elements are treated separately, it is important to realize that interactions of trace elements with each other, with macrominerals, with other constituents of milk, and with other food constituents occur, and that such interactions are assuming an increasing importance in nutrition.

Content and Chemical Form of Trace Elements in Milk and Dairy Products

Mineral Content

The trace element content of milk is not constant but is influenced by a number of factors such as stage of lactation, nutritional status of the animal, and environmental and genetic factors. Reported values in the literature for the concentration of many trace elements show a wide variation, which is partly due to these factors, but also partly to analytical errors and contamination from milk collection and processing equipment and procedures. Representative values for the average trace element content of milk are presented in **Table 1**.

Iron, zinc, and copper

Although several investigations have reported lack of a developmental pattern for iron in milk, at least one study has reported that iron concentration decreases by 35–50% during the first 3 days of lactation and remains relatively constant thereafter. The iron content of milk is resistant to changes in dietary iron intake. Contact with metal containers can increase the concentration of iron in milk.

Mean zinc concentration in milk is 3.9 mg l^{-1}, but large variations in the zinc content of milk (2.0–6.0 mg l^{-1}) have been reported. There is a large decrease (50%) in the concentration of zinc in cow's colostrum during the first 3 days of lactation, with little change thereafter. Dietary zinc supplementation increases the zinc concentration in milk only slightly.

The concentration of copper in milk decreases by up to 50% during the first 3 days of lactation; it can be increased by dietary copper supplementation or by contact with metal containers and processing equipment.

Manganese, selenium, and iodine

The concentration of manganese is higher in colostrum (100–160 μg l^{-1}) than in mature milk (20–50 μg l^{-1}) and a decrease of over 50% has been reported to occur during the first 3 days of lactation. Oral supplementation of manganese to cows can increase the manganese content of milk, provided that large doses are administered over a long period.

Table 1 Mean concentrations of trace elements in cow's milk

Trace element	*Content (l^{-1})*
Iron (mg)	0.3
Zinc (mg)	4.0
Copper (mg)	0.09
Manganese (μg)	30
Iodine (μg)	100–770
Fluorine (μg)	20
Selenium (μg)	10
Cobalt (μg)	0.5
Chromium (μg)	2.0
Molybdenum (μg)	50
Nickel (μg)	26
Arsenic (μg)	20–60
Silicon (μg)	3000
Boron (μg)	500–1000

Adapted from Flynn A and Cashman K (1997) Nutritional aspects of minerals in bovine and human milks. In: Fox PF (ed.) *Advanced Dairy Chemistry*, Vol. 3, pp. 257–301.
London: Chapman & Hall and updated with data from Food Standards Agency (2002) *McCance & Widdowson's the Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

The mean selenium concentration in cow's milk samples from 15 countries was reported as 10 μg l^{-1} (range 3–40 μg l^{-1}). The concentration in milk depends on dietary intake, and in areas such as New Zealand and Finland, where the selenium content of the soil and plants is low, concentrations as low as 3–5 μg l^{-1} have been reported. It has been shown that the selenium content of milk increased linearly from ~30 to 55 μg l^{-1} when dietary selenium was increased from ~2 to 6 mg day^{-1}.

Summarized data from various countries on the iodine content of milk indicate mean values of 100–770 μg l^{-1} and a wide range of individual values from 20 to >4000 μg l^{-1}. The concentration of iodine in milk is influenced by season and is closely related to dietary intake, and feeding winter rations containing mineral supplements results in considerable increases in milk iodine. High concentrations of iodine in milk have been related to the addition of excessive amounts of ethylenediamine dihydriodide (EDDI) to dairy cow rations.

The use of iodophors for teat disinfection increases the iodine content in cow's milk. In addition, use of iodophors for the disinfection of containers, milking machines, and processing equipment can also cause contamination of milk with iodine. Standardization of mineral feed supplements and supervised and restricted use of iodophor disinfectants have been recommended as measures to reduce the iodine content of milk and milk products.

Chromium, cobalt, molybdenum, and fluorine

The mean concentrations of chromium and cobalt in milk are 2 μg l^{-1} (range 0.2–3.6 μg l^{-1}) and 0.5 μg l^{-1} (range 0.4–1.1 μg l^{-1}), respectively. Dietary supplementation with cobalt increases the concentration of cobalt in milk, but does not increase the vitamin B_{12} content of milk unless the diet is cobalt-deficient. The concentration of molybdenum in milk has been shown to be dependent on dietary intake, increasing over fivefold when cows were supplemented with ammonium molybdate. The mean fluorine content in milk is 20 μg l^{-1} (range 10–140 μg l^{-1}).

Arsenic, nickel, silicon, and boron

Reported concentrations of these elements in milk are presented in **Table 1**. Little is known about the influence of stage of lactation, maternal nutritional status, and environmental and genetic factors on the content of these trace elements in milk.

Trace Element Content of Dairy Products

The contents of selected trace elements in other dairy products are shown in **Tables 2–5**.

Chemical Form of the Trace Elements

The chemical form of a trace element is important because it may influence intestinal absorption and utilization (the process of transport, cellular assimilation, and conversion into a biologically active form), and thus bioavailability.

Table 2 Mean concentrations of selected trace elements in concentrated milks

	Content (per 100 g)			
Trace element	*Pasteurized skimmed*	*Dried skimmed*	*Evaporated (whole)*	*Condensed (whole)*
Iron (mg)	0.03	0.27	0.26	0.23
Copper (mg)	Trace	Trace	0.02	Trace
Zinc (mg)	0.4	4.0	0.9	1.0
Manganese (mg)	Trace	Trace	Trace	Trace
Selenium (μg)	1.0	11	3.0	3.0
Iodine (μg)	31	150	11	74

Food Standards Agency (2002) *McCance & Widdowson's the Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

Table 3 Mean concentrations of selected trace elements in creams

	Content (per 100 g)						
	Fresh cream				*Soured*	*Sterilized canned*	*UHT*
Trace element	*∼10% fat*	*∼20% fat*	*∼35–48% fat*	*∼60% fat*	*∼20% fat*	*∼25% fat*	*∼32% fat*
Iron (mg)	0.1	0.1	0.1	0.1	0.4	0.8	Trace
Copper (mg)	Trace	Trace	Trace	0.09	Trace	Trace	Trace
Zinc (mg)	0.3	0.2	0.2	0.2	0.5	1.1	0.2
Manganese (mg)	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Selenium (μg)	Trace	3.0	Trace	Trace	Trace	Trace	Trace

Data from Food Standards Agency (2002) *McCance & Widdowson's the Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

Table 4 Mean concentrations of selected trace elements in butter, yogurt, and dairy ice cream

	Content (per 100 g)		
Trace element	*Butter*	*Yogurt (whole milk)*	*Dairy ice cream*
Iron (mg)	Trace	0.1	Trace
Copper (mg)	0.01	Trace	Trace
Zinc (mg)	0.1	0.7	0.3
Manganese (mg)	Trace	Trace	Trace
Selenium (μg)	Trace	2.0	2.0
Iodine (μg)	38	63	32

Data from Food Standards Agency (2002) *McCance & Widdowson's the Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

Iron, zinc, and copper

In milk, 14% of the iron occurs in milk fat, where it is associated with the fat globule membrane. About 24% of the iron is bound to caseins, probably to the phosphoserine residues of caseins, whereas 29% is bound to whey proteins and 32% is associated with a low-molecular-weight fraction.

Most of the zinc in milk is in the skim milk fraction, with only 1–3% in the lipid fraction. Of the zinc in the skim milk fraction, over 95% is associated with the casein micelles, with a small proportion (5%) associated with a low-molecular-weight compound that has been identified as citrate. Within the casein micelles, one-third of the zinc is loosely bound to casein phosphoserine residues and two-thirds is more tightly bound to colloidal calcium phosphate. The distribution of copper in milk has been reported as 2% in the fat fraction, 8% bound to whey proteins, 44% to casein, and 47% in a low-molecular-weight fraction.

Manganese, selenium, and iodine

In cow's milk, manganese distribution is 67% bound to caseins, 1% to the milk fat globule membrane, 14% to whey proteins, and 18% to a low-molecular-weight fraction. About 12% of the selenium in milk has been estimated to be incorporated in the enzyme glutathione peroxidase (EC 1.11.1.9).

Most (80–90%) of the iodine in milk is in the inorganic form, mainly as iodide and is in the water-soluble fraction, and 5–13% is bound to proteins through either covalent bonds or loose physical associations, with less than 0.1% bound to fat.

Molybdenum, chromium, and fluorine

All the molybdenum in milk is considered to be associated with xanthine oxidase (EC 1.1.3.22). The chemical form of chromium in milk is unknown, although chromium in foods is generally in the trivalent state.

Table 5 Mean concentrations of selected trace elements in some cheese varieties

	Content (per 100 g)								
Trace element	*Brie*	*Cheddar*	*Cream*	*Cottage*	*Edam*	*Feta*	*Gouda*	*Parmesan*	*Stilton*
Fe (mg)	Trace	0.3	0.1	Trace	0.3	0.2	0.3	0.8	0.2
Cu (mg)	Trace	0.03	0.04	Trace	Trace	0.07	Trace	0.84	0.04
Zn (mg)	2.0	4.1	0.5	0.6	3.8	0.9	3.9	5.1	2.9
Mn (mg)	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Se (μg)	5.0	6.0	4.0	4.0	7.0	5.0	8.0	12.0	7.0

Data from Food Standards Agency (2002) *McCance & Widdowson's the Composition of Foods*, 6th edn. Cambridge: Royal Society of Chemistry.

About 46–64% of the fluorine in milk occurs as free fluorine ions, with the remainder bound to proteins.

Cobalt, arsenic, nickel, silicon, and boron

Little is known about the chemical form or distribution of these trace elements in milk and dairy products.

Nutritional Significance of Trace Elements in Milk and Dairy Products

A simple and useful evaluation of the nutritional significance of trace elements (as well as macrominerals) in milk can be obtained by comparing the amounts of the different elements provided by 1 l of milk (**Table 1**) with the recommended daily intakes for these elements (**Table 6**). During the last two decades, great progress has been made in our knowledge of the trace element nutrition of infants, particularly in the area of bioavailability.

Iron

Iron is an essential trace element that acts as a catalytic center for a broad spectrum of metabolic functions. Iron, as a component of heme in hemoglobin, myoglobin, cytochromes, and other proteins, plays an essential role in the transport, storage, and utilization of oxygen. It is also a cofactor for a number of enzymes. Deficiency of iron, resulting in anemia, afflicts about 30% of the world's population and occurs in Western countries as well as in developing countries.

The recently revised recommended daily allowances (RDAs) for iron in the United States are shown in **Table 6**. Milk and milk products are very poor sources of iron, and milk contributes little to the total iron intake.

Bioavailability of iron to the infant from human milk has been reported to be in the range 49–70%. Considerably lower absorption efficiency of iron from cow's milk by human infants has been reported, usually ~10–34%. To compensate for the relatively low bioavailability of iron in cow's milk, infant formulae are often supplemented with iron. Iron absorption by infants from cow's milk formulae containing 12 mg l^{-1} iron as ferrous sulfate is about 4–7%, but because of the much higher concentration of iron in such formulae, the absolute amount of iron absorbed is considerably larger than that from human milk.

The reason for the exceptionally high bioavailability of iron in human milk is not understood. It has been suggested that it may be related to the high concentration of lactoferrin (a glycoprotein in milk that can bind two ferric ions per molecule of protein) in human milk, but evidence for a role for lactoferrin in iron absorption is conflicting. Others have suggested that the apparent higher bioavailability of iron in human milk compared to cow's milk could be due to a number of possible factors, including a lower concentration of proteins, calcium, and phosphorus (potential inhibitors of absorption), and higher concentrations of lactose and ascorbate (enhancers of iron absorption) in human milk. However, studies on suckling rats, which have a very high capacity for iron absorption, have shown that a very high and similar proportion (90%) of iron is absorbed from human milk, cow's milk, and an iron-supplemented cow's milk-based formula, suggesting that there are no unabsorbable iron complexes in these milks. It is likely that the lower concentration of iron in human milk may be a contributory factor to the higher absorption efficiency of iron from human milk in infants, as iron absorption is subject to homeostatic control and small amounts are absorbed more efficiently than large amounts.

Iron deficiency is one of the most common nutritional deficiencies in infancy and childhood owing to rapid growth and marginal supply of iron in the diet. It has been suggested that the iron stores of a breast-fed infant 4–6 months of age may become compromised if they are not replenished from dietary sources, and dietary iron supplementation has been recommended at not later than 4 months of age for full-term infants and not later than 2 months of age for premature infants. Iron-fortified cow's milk-based formulae are effective in preventing iron deficiency, which may be partly attributable to the fact that ascorbate is also added to formulae at levels that markedly enhance the absorption of added iron.

Zinc

Zinc is essential for growth and development, sexual maturation, and wound healing, and it may also be involved in the normal functioning of the immune system and other physiological processes. It is a component of the hormone insulin and aids in the action of a number of hormones involved in reproduction, as well as being required for the synthesis of DNA, RNA, and proteins and as a cofactor for many enzymes involved in most major metabolic processes. Zinc deficiency in humans was first reported in the Middle East in the early 1960s, giving rise to dwarfism, impaired sexual development, and anemia. Mild deficiencies of zinc, although difficult to detect, have been shown to occur in Western countries, particularly in infants and young children, giving rise to low hair zinc levels, suboptimal growth, poor appetite, and impaired taste acuity.

The recently revised RDAs for zinc in the United States are shown in **Table 6**. Dairy products such as milk, cheese, and yogurt are moderately good sources of zinc, and it has been estimated that in Western countries milk and dairy products contribute between 19 and 31% of the total zinc intake.

Table 6 Recommended dietary intakes of selected trace elements

		Trace elements								
Category	*Age (years)*	*Iron (mg)*[a]	*Zinc (mg)*[a]	*Iodine (μg)*[a]	*Selenium (μg)*[a]	*Copper (mg)*[a]	*Manganese (mg)*[b]	*Fluorine (mg)*[b]	*Chromium (μg)*[b]	*Molybdenum (μg)*[a]
Infants	0.0–0.5	0.27[b]	2[b]	110[b]	15[b]	200[b]	0.003	0.01	0.2	2[b]
	0.5–1.0	11	3	130[b]	20[b]	220[b]	0.6	0.5	5.5	3[b]
Children	1–3	7	3	90	20	340	1.2	0.7	11	17
	4–6	10	5	90	30	440	1.5	1.0	15	22
	7–10	8–10	5–8	90–120	30–40	440–700	1.5–1.9	1.0–2.0	15–25	22–34
Males	11–14	8	8	120	40–55	700	1.9	2.0–3.0	25	34
	15–18	11	11	150	55	890	2.2	3.0	35	43
	19–24	8	11	150	55	900	2.3	4.0	35	45
	25–50	8	11	150	55	900	2.3	4.0	35	45
	50+	8	11	150	55	900	2.3	4.0	30	45
Females	11–14	8	8	120	55	700	1.6	2.0–3.0	21	34
	15–18	15	9	150	55	890	1.6	3.0	20	43
	19–24	18	8	150	55	900	1.8	3.0	25	45
	25–50	18	8	150	55	900	1.8	3.0	25	45
	50+	8	8	150	55	900	1.8	3.0	20	45
Pregnant		27	11–13	220	60	1000	2.0	3.0	29–30	50
Lactating	≤18	10	14	290	70	1300	2.6	3.0	44	50
	19–50	9	12	290	70	1300	2.6	3.0	45	50

[a] US recommended daily allowance; from Institute of Medicine (2000) *Dietary Reference Intakes: Vitamin C, Vitamin E, Selenium, and Carotenoids*. Washington, DC: National Academy Press, Institute of Medicine (2001) *Dietary Reference Intakes: Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington, DC: National Academy Press.

[b] US adequate intake values, from Institute of Medicine (1997) *Dietary Reference Intakes: Calcium, Magnesium, Phosphorus, Vitamin D, and Fluorine*. Washington, DC: National Academy Press, Institute of Medicine (2000) *Dietary Reference Intakes: Vitamin C, Vitamin E, Selenium, and Carotenoids*. Washington, DC: National Academy Press, Institute of Medicine (2001) *Dietary Reference Intakes: Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington, DC: National Academy Press.

A number of lines of evidence suggest that the bioavailability of zinc in human milk is higher than in cow's milk. Human milk (but not cow's milk) has a therapeutic value in the treatment of acrodermatitis enteropathica, a hereditary zinc malabsorption syndrome. The plasma zinc concentration of breast-fed infants has been reported to be significantly higher than that in infants fed a cow's milk-based formula containing 1.8 $\mathrm{mg\,l^{-1}}$ zinc. Studies on human adults using extrinsic labeling with ^{65}Zn with whole-body counting showed that zinc absorption from human milk (41 ± 9%) was significantly higher than from cow's milk (28 ± 15%) or cow's milk-based infant formulae (31 ± 7%). Short-term studies on human adults, pigs, and suckling rats have shown that zinc is absorbed more rapidly from human milk than from cow's milk or cow's milk-based infant formulae.

Two main theories have been proposed to explain the higher bioavailability of zinc from human milk compared with cow's milk:

1. Binding of a significant fraction of the zinc in human milk (but not in cow's milk) to a low-molecular-weight zinc-binding ligand (e.g., citrate) may enhance zinc absorption.
2. Binding of a large fraction of zinc in cow's milk to casein (present at about 10 times its concentration in human milk), which may result in the entrapment of zinc in casein curds formed in the stomach and thus render a significant proportion of the zinc unavailable for absorption.

However, studies on suckling rats, which have a high capacity for zinc absorption, showed that a very high and similar proportion of zinc (85–95%) was absorbed from human milk, cow's milk, and cow's milk-based infant formulae, suggesting that there are no unabsorbable forms of zinc in either human or cow's milk. It is likely that the lower concentration of zinc in human milk may be a contributory factor to the higher absorption efficiency of zinc from human milk by infants, as zinc absorption is subject to homeostatic control and small amounts are absorbed more efficiently than large amounts.

Copper

Copper is an essential element for a wide range of animal species. It is required for iron utilization and is a cofactor for enzymes involved in the metabolism of glucose and synthesis of hemoglobin, connective tissue, and phospholipids. Dietary deficiency of copper is uncommon except in conditions of severe malnutrition.

The recently established RDAs for copper in the United States are shown in **Table 6**. Milk and milk products are considered poor sources of copper, and cow's milk contributes little to the total dietary intake of copper.

Copper deficiency is rare in breast-fed infants, but has been reported in formula-fed infants in some countries. Feeding infant formulae containing 0.03 or 0.4 $\mathrm{mg\,l^{-1}}$ of copper has been shown to yield normal parameters of copper status similar to those of breast-fed infants.

Knowledge of copper absorption from milks is very limited. In a study on suckling rats, copper absorption was 83% from human milk, 76% from cow's milk, and 86–87% from cow's milk-based formulae, suggesting that there are no unabsorbable forms of copper in these milks.

Manganese

Manganese is an essential element for every animal species studied. It is a specific cofactor for glycosyltransferases that are involved in mucopolysaccharide synthesis and a nonspecific cofactor for a wide variety of other enzymes. There are two known manganese metalloenzymes: pyruvate carboxylase (EC 6.4.1.1) and superoxide dismutase (EC 1.15.1.1). Manganese is widely distributed in foods, and dietary deficiency is not known to occur in humans.

The recently established adequate intake values for manganese in the United States are shown in **Table 6**. Cow's milk is a poor source of manganese and contributes little (1–3% in Western countries) to the total dietary intake of this mineral.

Very little is known about the bioavailability of manganese in milks. One study reported that manganese absorption in healthy adults from human milk (8.2 ± 2.9%) was significantly higher than from cow's milk (2.4 ± 1.7%), whereas absorption from cow's milk-based infant formulae was 1.7–5.9%. However, the absolute amount of manganese absorbed from cow's milk and formulae was larger than from human milk due to the higher manganese concentrations in these milks. Studies with suckling rats showed that absorption of manganese from human milk (81%) was not significantly different than from cow's milk (89%) or cow's milk-based formulae (80%), suggesting that there are no unabsorbable forms of manganese in these milks.

Selenium

Selenium is an essential component of the enzyme glutathione peroxidase, which occurs in many human tissues where, together with vitamin E and the enzymes catalase (EC 1.11.1.6) and superoxide dismutase, it functions as an antioxidant, protecting cells against oxidative damage. In areas of China where the concentration of selenium in the soil is low, selenium deficiency causes Keshan disease, a cardiomyopathy that affects primarily young children and women of child-bearing age. Low selenium status has also been reported in New Zealand and Finland, countries where the concentration of selenium in the soil is also low.

The recently revised RDAs for selenium in the United States are shown in **Table 6**. The contribution of dairy products to daily dietary intake of selenium has been estimated as 5 μg (8% of the total intake) in the United Kingdom; 13 μg (10%) in the United States; 13 μg (21–26%) in Finland; and 11 μg (39%) in New Zealand.

Selenium intake of full-term infants fed on non-fortified, milk-based formulae has been shown to be near or below the RDA, whereas infants in the United States fed breast milk have a selenium intake meeting or exceeding the US RDA. Moreover, some studies have reported that the selenium status of milk-based formula-fed infants was lower than that of breast-fed infants. The finding that unsupplemented infant formulae have a lower selenium concentration than that in breast milk has meant that selenium is now being added to some infant formulae.

Iodine

Iodine is an essential component of the thyroid hormones thyroxine and triiodothyronine, which are important in controlling the rate of basal metabolism and in reproduction. Dietary deficiency of iodine causes enlargement of the thyroid gland and goiter, whereas a large excess of iodine in the diet reduces the uptake of iodine by this gland and also produces signs of thyroid deficiency (thyrotoxicosis). Worldwide, 1.6 billion people (30% of the world's population) are at risk of iodine deficiency. Of these, some 655 million have goiter. While the highest prevalence of iodine deficiency is in the less-developed regions of the world, it persists in parts of the industrialized world, too, in countries such as Germany and Luxembourg. Iodine is the only trace element for which there has been any suggestion of excessive amounts in cow's milk. Excessive addition of EDDI and use of iodophors for disinfection purposes have certainly contributed to the increased iodine content of milk in past years. However, there is evidence of a decline in the concentration of iodine in milk in the United States in recent years, although the situation in other countries is less clear.

The recently revised RDAs for iodine in the United States are shown in **Table 6**. The contribution of milk and dairy products to the daily dietary intake of iodine has been estimated as 37% of the total intake in the United Kingdom and only 6–7% in Germany.

Molybdenum

Molybdenum is an essential component of several enzymes, including xanthine oxidase, aldehyde oxidase (EC 1.2.3.1), and sulfite oxidase (EC 1.8.3.1), where it occurs in the prosthetic group molybopterin. It is not known whether the human requirement is for molybdenum *per se* or for molybopterin (or a precursor). Although molybdenum deficiency has been reported in a patient on long-term total parenteral nutrition therapy, dietary deficiency of molybdenum has not been observed in humans.

The recently established RDAs for molybdenum in the United States are shown in **Table 6**. Milk may contribute substantially to the intake of molybdenum (36% of total molybdenum intake).

Chromium, Cobalt, Fluorine, Arsenic, Nickel, Silicon, and Boron

Chromium is regarded as an essential nutrient for humans, and the earliest detectable effect of deficiency is an impairment of glucose tolerance. The only known function of cobalt in humans is its presence as an essential component of vitamin B12. Fluorine accumulates in the hard tissues of the body (bones and teeth), and although it is not strictly an essential element, it is regarded as beneficial for humans because of its protective role against dental caries.

The recently established adequate intake values for fluorine and chromium in the United States are shown in **Table 6**. As there is no evidence that the intake of cobalt is ever limiting in the human diet, no RDA is necessary.

There is substantial evidence to establish the essentiality of arsenic, nickel, silicon, and boron in animals, and it is likely that these trace elements are also essential for humans. However, the nutritional functions of these elements are still unclear and there are no reliable data on which to base estimates of human requirements.

Milk and dairy products do not contribute significantly to the intake of fluorine, arsenic, silicon, and boron. Milk may make a significant contribution to the intake of chromium (21%) and nickel (11%).

Conclusions

There is much less information on the nutritional aspects of some trace elements than those of others, and a considerable amount of current research is being carried out to clarify the roles of such minerals in nutrition.

Reliable information on the content of at least some of the trace elements in milk and the principal factors affecting it is now available. However, for the other trace elements there is still a wide variation in reported values in the literature, which is due, at least in part, to analytical difficulties. The contribution of cow's milk and milk products to the diet in Western countries is significant for zinc and iodine.

Although the chemical forms of the some of the trace elements (iron, zinc, copper, manganese) in milk are fairly well defined, the forms of many of the trace elements are unknown. Studies are required to characterize the chemical forms of a number of trace elements in milk.

Iodine is believed to be almost totally absorbed from milks and infant formulae. The bioavailability of some other trace elements (e.g., iron, zinc) in human milk appears to be very high (generally higher than in cow's milk), but the reason for this remains unclear. There is little information on the bioavailability of copper, manganese, selenium, fluorine, or other trace elements in milks, formulae, and dairy products. Factors affecting the bioavailability of minerals are still poorly understood and the role of possible enhancers (e.g., lactose, ascorbate, citrate, phosphopeptides, lactoferrin) or inhibitors (e.g., proteins, calcium, phosphate) of absorption of minerals from milks remains unclear. However, milk does not appear to contain substances that are strongly inhibitory to mineral absorption, such as phytate or polyphenols in foods from plants.

Finally, understanding the nutritional significance of trace elements in milk and dairy products will also benefit from the improvement in our knowledge of fundamental aspects of minerals, such as their nutritional roles, requirements, and metabolism, and the quantitative relationship between dietary intakes and health.

See also: **Feed Ingredients**: Feed Supplements: Macrominerals; Feed Supplements: Microminerals. **Milk Salts**: Macroelements, Nutritional Significance.

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MILKING MACHINES

Contents

Principles and Design
Robotic Milking

Principles and Design

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History

The oldest recorded evidence of domestication of the dairy cow appears to date from about 3100 BC. A relief frieze from Al-Ubaid, Iraqi Museum, Baghdad, provides verification of this. Since then, for a period of several hundred years, reliance on hand-milking has prevailed. The first record of an attempt to circumvent the sphincter muscle of the teat of the cow was in Egypt in about 400 BC. The first devices used were straws or tubes fashioned from quill feathers, which were introduced into the teat duct. Subsequently, mechanical milking was developed from three principles of milk extraction:

1. the use of a catheter inserted into the teat duct allowing the milk to drain from the udder
2. the use of the pressure principle to duplicate the technique of hand-milking
3. the use of vacuum (suction) to imitate the sucking action of the calf

The development of these principles did not follow in chronological order and there was much overlapping of these principles by the introduction of many devices. The major countries to actively develop these principles were the United Kingdom, Sweden, Australia, New Zealand, and the United States.

The Catheter Principle

The issue of mechanical milking next received attention in the early 1800s, when a small metal tube apparatus for insertion into the cow's teat was patented by Blurton in England in 1836 (**Figure 1**). In 1875, Sylvester A Smith of Letts, Iowa, patented a method of extracting milk from the udder of the cow, which consisted of inserting a tube into each teat; the tube was closed by a valve at the lower end. Milk ran into a pail when the simple sliding valve was opened (**Figure 2**). The idea of the milk tube persisted for many years and the Moreton automatic milker was advertised in England as late as 1916. The failure of this approach was no doubt due to the spread of udder diseases via the catheter and the injury to the sphincter muscle of the teat. It was reported that after using this treatment for a few months, milk dribbled from the teat continuously.

The Pressure Principle

Many devices were employed using the application of pressure on the teat in an attempt to duplicate hand-milking – plates, bars, belts, or rollers – and these were mechanically, hydraulically, or pneumatically driven. The Crees Lactator (**Figure 3**) is a notable example of this type of device patented in 1881. The Beyer and Rohde machine patented in 1886 (**Figure 4**) illustrates the complexity and crudeness of this method. In 1892, a Danish machine patented by Jens Neilson was another clumsy, complicated, hand-powered attempt at a pressure device (**Figure 5**).

The Ljungström and Dalén milking units were two similar machines using the pressure principle. Frederick and Birger Ljungström obtained a patent for their machine in 1902. Later, the Dalén machine, patented in 1908, utilized compressed air to drive pistons against the teat in a futile attempt to duplicate hand-milking (**Figure 6**). Two more pressure-type machines of a similar nature, the Omega, the forerunner of the Manus machine, appeared in 1909–10. Compared with

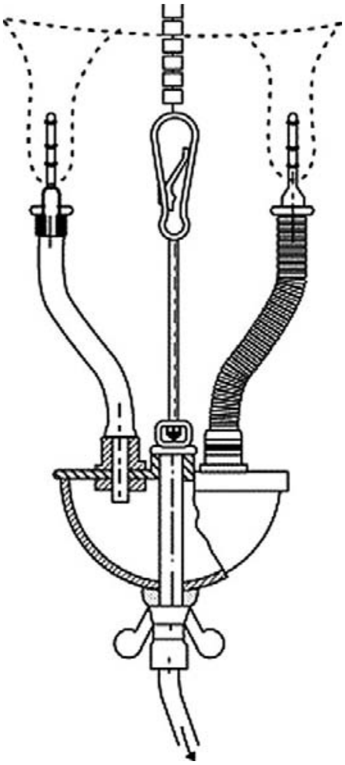

Figure 1 Catheter device patented by Blurton in England (1836).

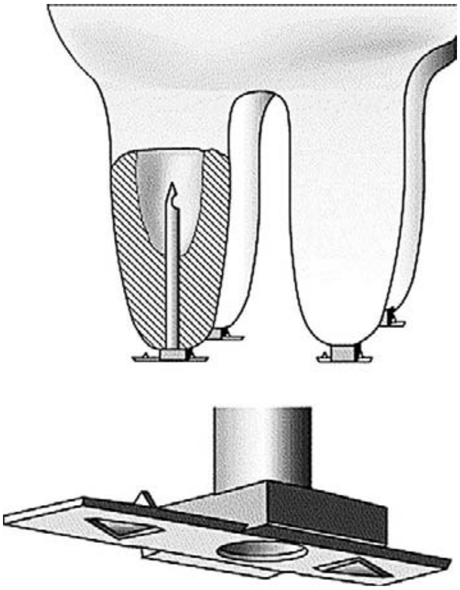

Figure 2 Milk tube with slide valve patented by Sylvester Smith, USA (1875).

hand-milking, these pressure-type machines milked at a slower rate, gave a lower milk yield, caused teat injuries, and required a high degree of operator skill. With such definitive conclusions, failure of this principle became inevitable.

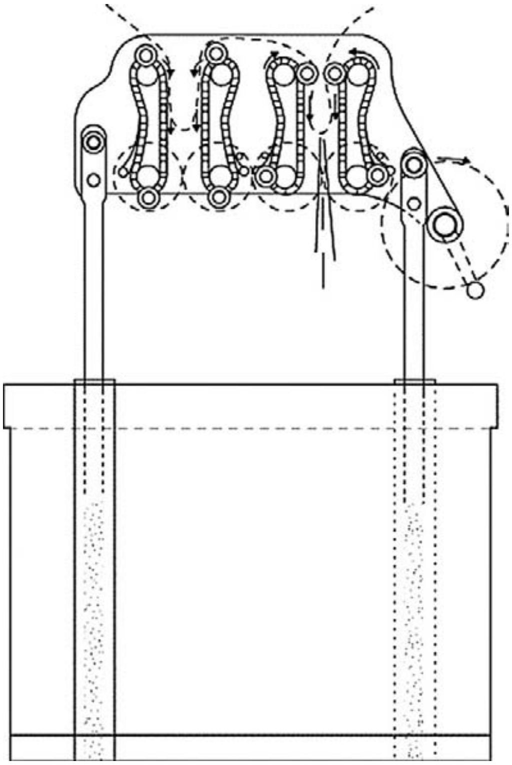

Figure 3 The Crees Lactator, patented in England (1881).

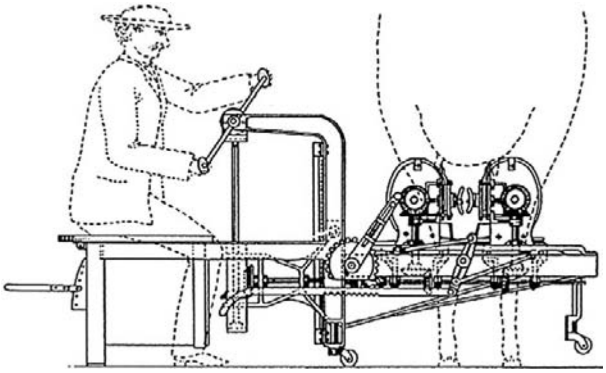

Figure 4 Complicated device by Beyer and Rohde, Wisconsin (1886).

The Vacuum Principle

In the meantime, there was increasing interest in the use of the vacuum principle. The initial idea of using a vacuum to extract milk from a cow is attributed to two British inventors, Hodges and Brockenden, in 1851. The L. O. Colvin hand-operated vacuum milker (United States) patented in 1860 utilized handles, which were moved back and forth by the operator to create a vacuum (**Figure 7**). Anna Baldwin of the United States made use

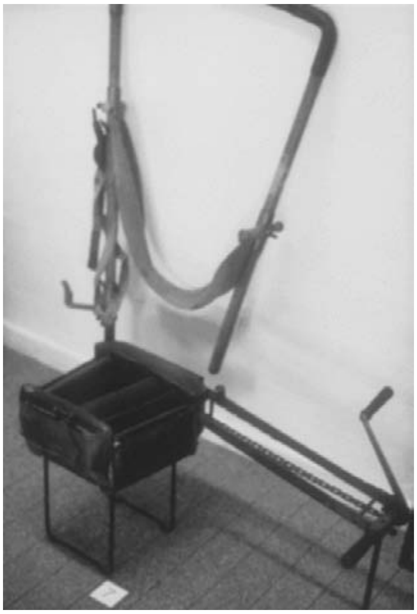

Figure 5 Hand-powered pressure device by Jens Neilson, Denmark (1892).

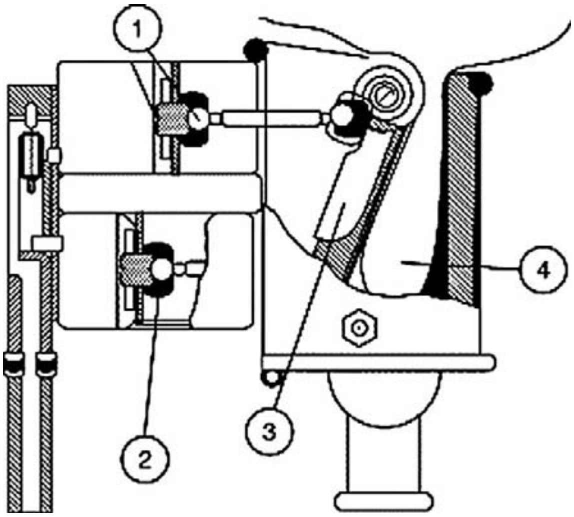

Figure 6 Compressed air-operated machine by Dalén (1908). Pistons 1 and 2 are driven by compressed air. The upper piston pushes forward the upper edge of the rubber plate (3), thereby closing the teat (4). Then the lower piston (2) presses the lower part of the rubber plate onto the teat and presses out the milk. The pistons retract together.

of the hand-operated water pump to create vacuum to milk (**Figure 8**). A foot-powered machine patented in Maryland, USA, in 1895 had limited success for several years (**Figure 9**). The Murchland teat-cup (**Figure 10**) had a rubber mouthpiece, which was later extended to form an open lining. Murchland also had the idea of piping the milk under vacuum to a central collecting point.

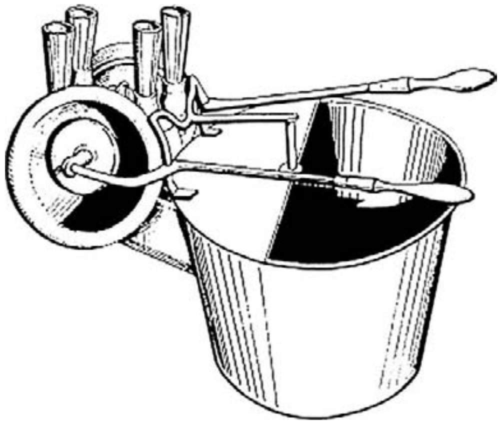

Figure 7 L. O. Colvin vacuum milker, USA (1860).

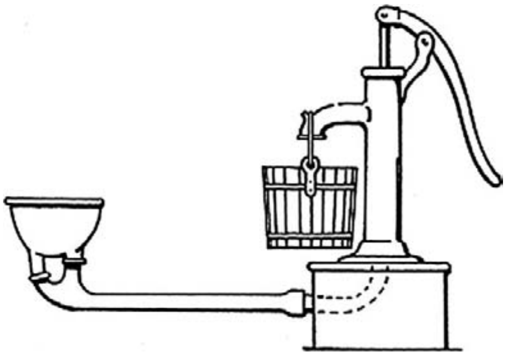

Figure 8 Utilization of a water pump to create vacuum by Anna Baldwin (USA, 1878).

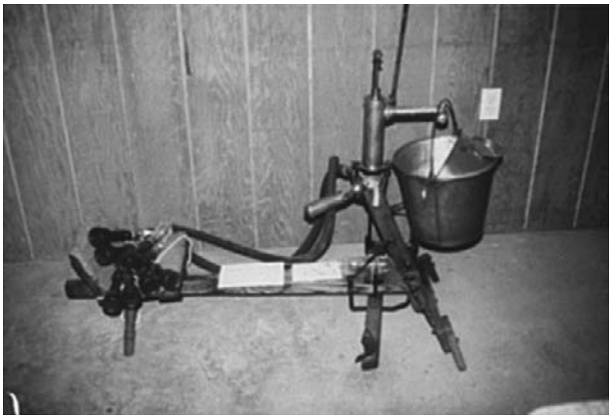

Figure 9 The foot-powered Mehring milker, Maryland (1895).

The first ‘pulsator’ was used by Dr. Shields of Glasgow (Thistle Mechanical Milking Machine Company). His idea was to relieve the stress on the teats of a constant vacuum by oscillating the vacuum on the teat. The single-chambered teat-cups were ‘pulsated’ at pressures between 50 and 15 kPa to form what was to become known as the ‘swinging vacuum’ machine.

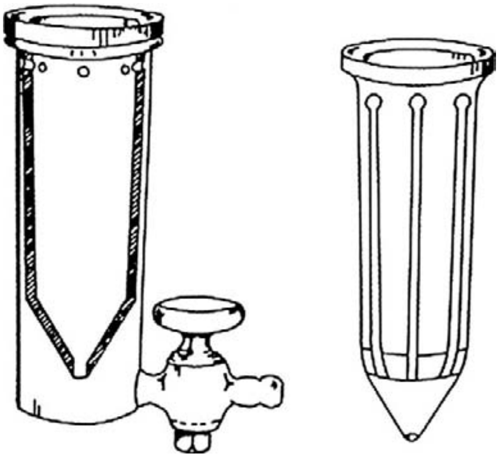

Figure 10 The Murchland teat-cup, Scotland (1889).

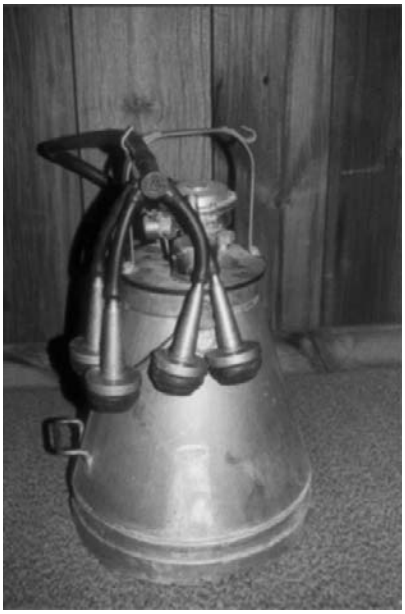

Figure 11 Burrell–Lawrence–Kennedy machine (1897). A US version of Lawrence–Kennedy machine, England).

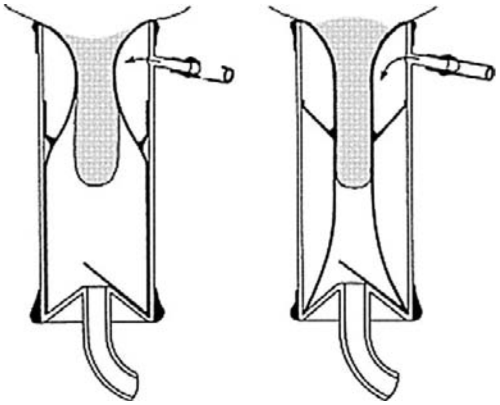

Figure 12 Double-chambered teat-cup by Struthers and Weir, Scotland (1892).

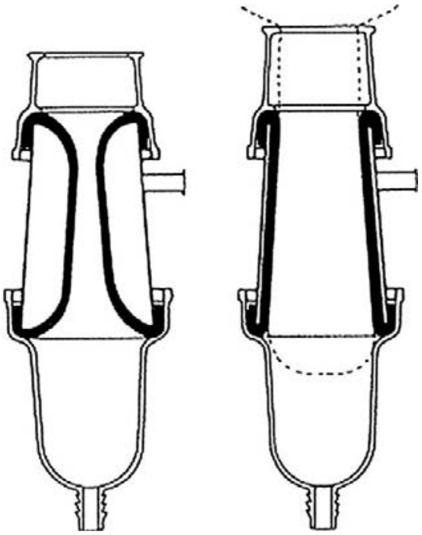

Figure 13 Double-chambered teat-cup by Harnett and Robinson, Australia (1893).

Patents for his ideas were recorded in 1895 and 1896. The Thistle Mechanical Milking Machine Company failed, but one of its directors, Robert Kennedy, joined up with a Glasgow engineer, William Lawrence, to produce the Lawrence–Kennedy machine in 1897. This machine was based on a single-chambered teat-cup with an intermittent vacuum (**Figure 11**). This machine is considered to be the first successful commercial milking machine.

Meanwhile, the first stages of the development of the double-chambered teat-cup were taking place. A patent was granted to Struthers and Weir of southwest Scotland in 1892 for a machine with a teat-cup made up of an outer shell with an inner liner of rubber (**Figure 12**). This advance does not appear to have had any commercial development. However, 1 year later, Harnett and Robinson, working independently in Australia, were granted a patent for a very similar invention (**Figure 13**). These devices were claimed to produce an intermittent pressure on the teat using a rubber diaphragm to 'counteract the influence of vacuum on the teat'. It is ironic that the Harnett patent included a device that 'automatically destroyed the vacuum in the tubes of the milking machines when the animal is sufficiently milked'. It took another 50 years before the principle of automatic cluster removers (ACRs) was incorporated into the design of milking machines.

In the same year (1902), an Australian dairy farmer by the name of Alexander Gillies patented a teat-cup

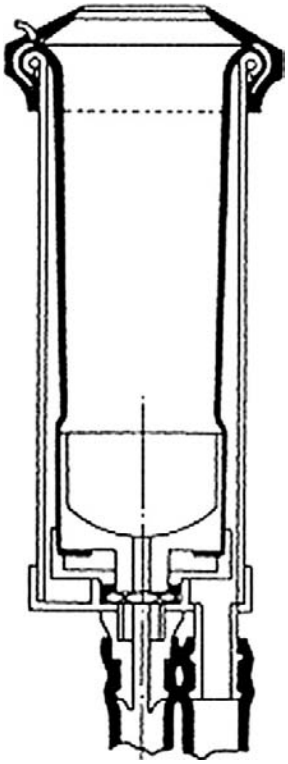

Figure 14 Teat-cup with a flexible mouthpiece by Alexander Gillies, Australia (1902).

consisting of a rigid rubber casing with a thin flexible lining (**Figure 14**). A second patent by Gillies in 1903 indicated that the assembly could be dismantled easily, along with an air bleed to move the milk more readily to the receiver. Thus, the basic principle(s) of the milking machine was born, and is used to this day.

Basic Milking Machine Function

The milking process consists of two phases of a pulsation cycle. Pulsation is defined by the International Standards Organization (ISO) as movement of the liner wall. The internal liner moves to an open position known as the milking phase and then to closure or the rest phase (**Figure 15**). A partial vacuum furnishes the energy to remove milk from the udder. At the interface of the teat and liner, the partial vacuum performs four basic functions:

1. The mouthpiece and barrel of the liner form a vacuum seal and friction on the teat, which offset the gravitational forces of the cluster weight.
2. The application of vacuum to the teat creates a pressure differential across the streak canal to remove milk from the teat during the milking phase of the pulsation cycle.

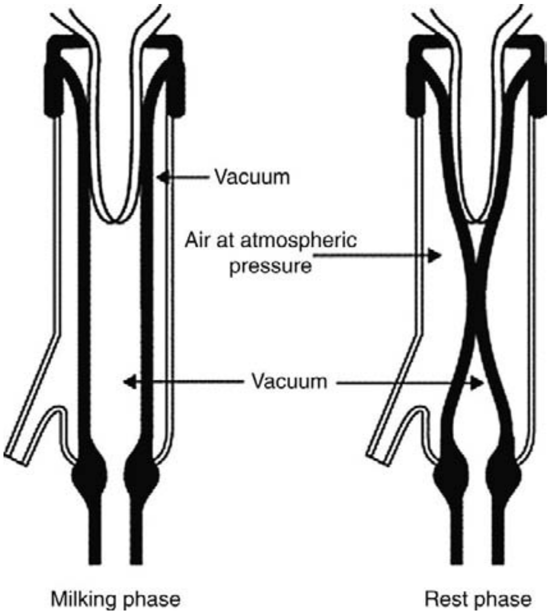

Figure 15 Basic operation of a milking machine showing liner in open and closed positions.

3. The partial internal vacuum within the liner closes the liner on the teat as atmospheric air is admitted between the liner and the shell. A compressive load is applied to the teat by the collapsing liner, which restores the streak canal to near its original diameter with each pulsation cycle so that milk can be removed effectively at the subsequent liner opening.
4. Liner opening is achieved by the application of a vacuum to the liner–shell chamber area.

In milking technology, vacuum is measured as gauge pressure expressed in kilopascal (kPa). Gauge pressure is referenced to atmospheric pressure, with vacuum expressed as a negative value relative to the atmosphere. The standard unit of force is the newton (N) and the standard unit of area is the square meter (m^2). Thus, pressure (P) is force per unit of area ($N\,m^{-2}$) and the derived unit is the pascal (Pa). The pressure unit used for milking is the kilopascal (kPa), which is equal to $1000\,N\,m^{-2}$ or 1000 Pa.

Vacuum has additional functions in machine-milking, such as transporting milk within tubes and pipes. In addition, it is the force used in cleaning complex piping systems without disassembly.

The ideal operating vacuum has not yet been established. The operating vacuum on the system varies among and within manufacturers. In general, the range tends to be between 40 and 50 kPa. Usually, some allowance is made for the height of the milk pipe above or below the cow platform, with increasing vacuum on high pipelines. In some cases, an allowance is made for the liner, depending on the collapse resistance of the liner. Operating vacuum influences milk flow rate, with yielding higher vacuum higher flow rate. Lower vacuum levels also increase the slip rate of most liners. As vacuum is increased, the postmilking strippings also increase, making the choice of operating vacuum a compromise. Excessively high vacuum (<50 kPa) has been shown to increase hyperkeratosis, or formation of callus-like tissue at the teat end, as does overmilking.

Vacuum Pumps

Electrical energy, which is normally used, is transformed to a pressure less than atmospheric pressure in a closed system by a vacuum pump. Until recently, the most popular type of vacuum pump has been the rotary vane. Its operation is depicted in **Figure 16**. The lobe-type pump is currently very popular (see **Figure 17**). The use of variable-frequency drive (VFD) systems in conjunction with vacuum pumps has resulted in energy savings and reduced noise levels. VFD turns the pump at the required rpm to equal the vacuum requirement. Regulation of milking vacuum is done electronically by the variable-speed drive; however, sometimes, a standard regulator is installed as an emergency measure in case of an electronic control failure.

The milking cluster airflow allowance is from 60 to 70 l min^{-1}, depending on pulsator rate and pulse tube dimension and length. This requirement consists of 20–30 l min^{-1} per milking cluster for the incremental component of effective reserve, pulsator consumption of 30 l min^{-1}, and cluster air admission of 10 l min^{-1}. The other major requirement variables during unit application and liner slips and falloffs. A falloff is the most severe usage requirement due to its sudden and large volume air admission. The minimum allowance for a falloff for each plant is 1000 l min^{-1}. An even greater allowance is indicated if the long milk tube of the cluster exceeds 16 mm in diameter. The cluster application requirement is then covered by the falloff allowance in the basic effective reserve. The final allowances for a newly installed system should allow for system leakage, pump wear, and regulation losses. Thus, the final allowances for the basic pump capacity are

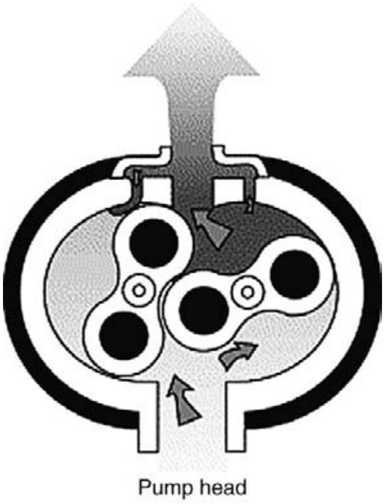

Figure 17 Lobe-type vacuum pump operation.

$$(1000 + 85n)\ \text{minl}^{-1}$$

where n is the number of milking clusters.

Milk Pump or Releaser

Milk pumps or mechanical releasers serve to remove milk from under vacuum to atmospheric conditions of a bulk tank. They are found in conjunction with the pipeline receiver or milk accumulator. Mechanical releasers have given way to milk pumps due to their low capacity. Milk pumps must be sized to the maximum flow rate of the total milk flow from the milking units and/or washing requirements. Liquid level controls are necessary to keep the milk pump from mixing air and milk, which causes an off-flavor in the milk. Recently, milk pumps have the option of being operated by VFDs. This is especially helpful when used in conjunction with a plate cooler or other type of inline cooling. The VFD improves cooling efficiency over start/stop operation of the milk pump by providing continuous milk flow at a lower flow rate than start/stop operation.

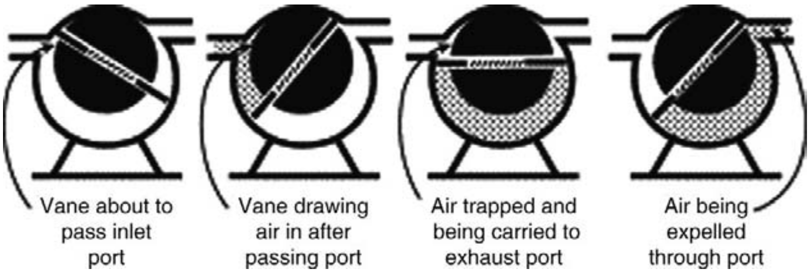
Vane about to pass inlet port
Vane drawing air in after passing port
Air trapped and being carried to exhaust port
Air being expelled through port

Figure 16 Vane-type vacuum pump operation.

Regulators

The regulator serves the function of maintaining a preset vacuum level by admitting excess air into the closed system. In the event that there is insufficient pump capacity to meet the vacuum usage, the vacuum level falls since the regulator only admits air in excess of the vacuum requirement.

There are many types of vacuum regulators. The early types of regulators were usually spring loaded or of the weighted type. The weighted types were lever-operated, deadweight, or deadweight compensated. Both the spring-loaded and weight-type units have been found to be flow rate sensitive, that is, the vacuum level changes as the airflow varies through the regulator opening. More recently, the type of regulator of choice has been the diaphragm type. This regulator maintains a more constant vacuum and is much less sensitive to vacuum change as airflow through the valve opening varies. It is generally accepted that constant vacuum (±2 kPa or less) is the desired operating condition for milking systems. A constant vacuum during milking is essential, since irregular vacuum fluctuations have been shown to increase the new infection rate of the udder.

The regulator controlling the VFD pumps is an electronic transducer. This device signals the frequency drive to change the rpm of the pump motor to meet the vacuum needs of the system. When installed and tuned properly, the VFD vacuum systems can maintain vacuum within the prescribed limits.

The Milking Cluster

The cluster consists of the liner, the shell, and the clawpiece or a manifold; the clawpiece connects the short milk tubes of the liners to a common long milk tube. The long milk tube is connected to a vacuum source. The clawpiece may also serve as a mounting device for the pulsator spreader. Each teat-cup has a short pulse tube connecting to the clawpiece spreader. The spreader connects to the long pulse tube(s), which is then connected to the pulsator. **Figure 18** shows the milking machine cluster.

The ideal capacity for the clawpiece has not been established; however, one study indicated that capacities of greater than 300 ml had no particular advantage. The clawpiece is equipped with an air vent to move milk away from the clawpiece, by breaking up the milk column. Air admission is normally 5–20 l min^{-1}. Basically, the air vent reduces the claw vacuum to a level lower than the source vacuum, thus moving the milk away from the clawpiece due to the difference in pressure (vacuum). The resulting admission of air increases the air-to-fluid ratio in a two-phase flow system and reduces hydrostatic head. Sometimes, the air venting is done in the short milk tubes. An air vent of proper size is an important consideration since inadequate venting may cause a substantial increase in claw vacuum in low-line or tunnel parlors due to hydrostatic head. Conversely, excessive air admission may adversely affect milk quality by activation of the free fatty acids of the milk, resulting in an off-flavored product.

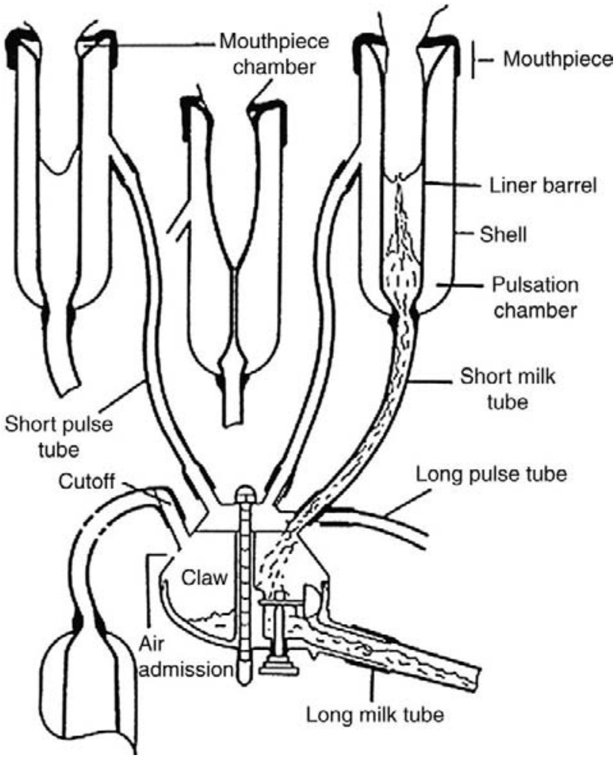

Figure 18 The milking machine cluster.

Automatic Cluster Removers

ACRs have become very popular on dairy farms. They serve to permit the operator to operate more units safely and prevent overmilking. Machine-on times can also be reduced, thereby resulting in faster throughput and reduced labor. Teat health may also be improved by reducing the number of liner closures on the teat. Excessive liner closures on the teat may exacerbate the condition of hyperkeratosis.

There are several means to determine the end point of milking, including the use of load cells, volumetric types, and mechanical and electronic flow rate sensors. Initially, the typical end point setting was when flow rate reached 0.23 kg min^{-1} and the delay time for removal was about 30 s. Recently, there is emphasis on increasing the flow rate setting and reducing the delay time for unit removal. Recent studies indicate that flow rate settings greater than 0.9 kg min^{-1} reduces milk yield. Unit removal set times may be as low as 3.0–5.0 s at the present time.

Shells

Teat-cup shells form a chamber around the barrel of the liner. This chamber is sealed at the top and bottom by the liner. The chamber can then be placed under vacuum or, alternatively, air admitted to this chamber to control liner wall movement or pulsation. The shell is fitted with a nipple in order to be connected to the spacer on the clawpiece with the short pulse tube. The location of the nipple is not important with respect to the influence upon the liner. Shells are normally constructed of stainless steel in a cylindrical form; however, some are molded in plastic. The vacuum changes in the shell–liner chamber are usually measured on the short pulse tube connecting the spacer and the shell. The resulting vacuum tracings are used to evaluate pulsator performance **Figure 19**.

Liners

The liner is a flexible tube made of natural, synthetic, or silicone rubber. The synthetic liners are currently the most popular option. The use of silicone is increasing in popularity and there is a decreasing use of natural rubber. However, most synthetic liners contain some natural rubber. There are many different liner designs in use. They are either molded or extruded into the desired shape for placement into the shell. They are normally under some tension in the shell to seal them at either end to prevent the entrance of moisture and soil. The tension also increases milking rate, but, if excessive, shortens liner life.

The classic opening and closing of the liner as shown in **Figure 15** is due to the imposed pressure differences across the wall of the liner as the pulsator operates. By definition, the movement of the liner wall is termed 'pulsation'. As the liner begins to close, this is known as the buckling pressure or the critical collapsing pressure difference (CCPD). Typical CCPD is 17–27 kPa, but liners with a barrel wall that is shaped other than round (oval, square, or triangular) will have a lower CCPD. Barrel wall thickness and tension affect the CCPD markedly, while rubber compounding has a relatively minor role in influencing CCPD.

A second measure of liner characteristics is its touch point pressure difference (TPPD), sometimes known as the 'inflection point'. This is the pressure difference at which the walls of the liner touch as the liner is in the process of closing. The liner continues to close beyond the touch point; however, measurement of the continuation of closure is not a repeatable measure. The major factor affecting the TPPD is the barrel wall thickness of the liner. TPPD may typically range from 30 to 45 kPa.

Liner slippage is related to an increase in the new infection rate of the udder. Operating vacuum influences liner slip, with higher vacuum having fewer slips. Liner slips are also influenced by udder shape, teat location, proper cluster adjustment, and teat preparation. Clusters applied to wet teats slip more frequently.

The mouthpiece chamber vacuum of the liner is quite variable but important with regard to milking characteristics. Wide-bore liners have a higher mouthpiece vacuum than narrow-bore liners. This is due to a combination of the teat seal on the liner barrel and air leakage of the mouthpiece of the liner. At the onset of milking and

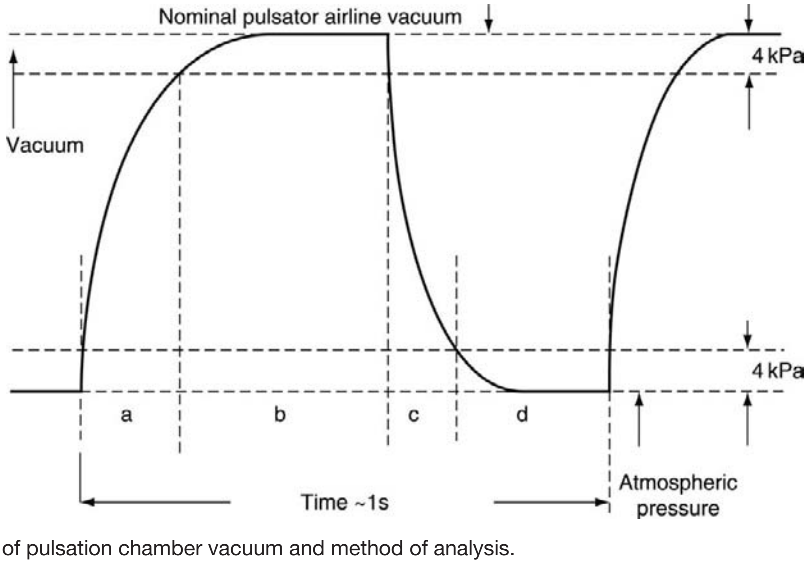

Figure 19 Waveform of pulsation chamber vacuum and method of analysis.

during milk flow, vacuum is relatively low and stable in the mouthpiece. As the teat milks out, vacuum generally increases and vacuum variability dramatically increases in relation to pulsator frequency. Teat penetration into the liner also influences mouthpiece chamber vacuum. Pulsator ratio has little effect on this characteristic.

High mouthpiece vacuum is associated with an increased frequency of tissue stress rings at the base of the teat, a greater risk of mastitis, increased discomfort to the cow, and a higher frequency of discolored teats. Mouthpiece vacuum is an excellent indicator of when the quarter has milked out due to the rather sudden increase in vacuum and vacuum variability.

Liner life is influenced by many factors such as rubber compounding, number of cow milkings, and number of washings. In the United States, liner life is usually recommended as 1000–1200 milkings, while in Europe, the typical recommendation is 2500 milkings. The difference is due to compounding requirements of regulatory agencies. A satisfactory method to determine the effective life of the liner remains an enigma to the industry, although peak milk flow rate appears to be the most sensitive indicator of liner age.

Pulsators

The purpose of the pulsator is to change the pressure conditions between the liner and the shell (pulse chamber) from atmospheric pressure to vacuum in an alternating manner (see **Figure 15**). The pulsator is basically an air valve, switching the pulse chamber pressure. By changing the vacuum to atmospheric pressure in the pulse chamber, the pulsator causes ‘pulsation’ or liner wall movement. The rate of change is known as the pulse rate. This rate is usually within the range of 50–60 min^{-1}, although greater ranges have existed from as low as 40 min^{-1} to as high as 180 min^{-1}. Generally, pulse rate has a small influence upon milking rate. The pulsator rate for small ruminants is usually higher than that for dairy cows, normally about 90 pulsations per minute.

Another pulsator characteristic is known as ratio. This term relates to the relationship between the milking phase of the pulsation cycle and the resting phase of the cycle. When the milking phase is equal to the resting phase, the ratio is 1:1 or 50% in the milking phase to 50% in the resting phase. Ratios as wide as 70% milking to 30% closed have been shown to be safe to the cows. The ratio has a definite influence on milking rate. Wider ratios milk faster; a 70:30 ratio reduced machine-on time by 1 min per cow compared to a 50:50 ratio in a US study. ISO specifies that the ‘b’ phase of the pulsation cycle shall be 30% or greater and that the ‘d’ phase shall not be less than 15% or 150 ms (see **Figure 19**).

Pulsation can be applied to the teats in different sequences. It is known as simultaneous pulsation when all four liners open and close at the same time (4 × 0). This is usually accomplished with a single long pulse tube and a four-way spreader at the clawpiece. When two liners are closed and the opposite two liners are open, it is known as alternating pulsation (2 × 2). Alternating pulsation can be applied in different patterns, including side to side of the four quarters, front to rear of the four quarters, and diagonal quarters. The last configuration is seldom used. A dual pulse tube is required for alternating pulsation. It is also possible to operate the milking unit sequentially, with each liner operated independently. Since sequential pulsation requires four pulse tubes, the flexibility of the milking unit is compromised, and this method is seldom employed.

Technically, pulsation is liner wall movement (ISO 3918). The method of defining the pulsator waveform is shown in **Figure 19**. While the liner wall generally follows the pressure changes in the pulse chamber, the waveform of the pulse chamber does not match the position of the liner. This relationship is shown in **Figure 20**.

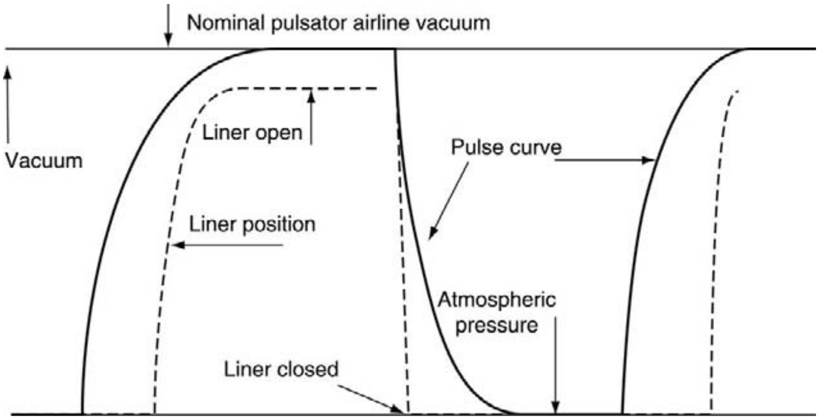

Figure 20 Waveform of pulsation chamber vacuum and the relative position of the liner wall.

Each liner design tends to have its own characteristic movement since it responds to the difference in pressure between the walls of the liner barrel.

Several types of pulsators are available. The pneumatic pulsator operates from the vacuum of the system, with each pulsator operating independently. While this type of pulsator does essentially the same function as an electronically controlled one, it is generally not quite as reliable due to fouling, temperature fluctuations, and humidity changes.

There are also several forms of electronic pulsators. Magnetic units may be centrally controlled by a make-and-break DC current. Some types of electric pulsators operate from a low-voltage AC current. Other types may be controlled from a master pulsator to a series of slave units located strategically in the milking area and operated by a pulse tube or pipe from the master. Whatever the type, they must perform the function of applying vacuum and atmospheric air to the pulse chamber. Pulsation failure has consistently been shown to increase the new infection rate of dairy cows.

Recent research indicates that a rapid pulsator 'c' phase decreases milking performance. The longer the milking times, the lower the peak and average flow. Yield in 2 min is lower while total yield is unchanged. Thus, a minimum 'c' phase of 12% (120 ms) is indicated. The effect appears to be due to the rate of liner wall closure. A fast moving liner (>120 ms) applies a greater pressure at a faster rate to the teat.

Supplementary Equipment

Milking machine components are generally assembled into a system on the dairy farm. The basic system may consist of a milking unit attached to a bucket with vacuum furnished from the vacuum pump via a vacuum piping system. Milk is then carried to a collecting point and ideally cooled prior to collection. Cooling is essential to prevent bacterial growth in the product.

Pipeline systems are more complex and have sanitary piping systems to transport milk from the cow to the collecting or cooling point. Sanitary piping systems are constructed of stainless steel or glass. Proper size in relation to degree of slope is given in **Table 1**. Piping systems are configured to be cleaned-in-place (CIP). Specialized cleaning compounds are circulated through the system after each use to prevent the accumulation of milk soils. Automatic cleaning controls are available to circulate solutions. Proper cleaning requires an adequate supply of potable hot water, and solution contact with all milk contact surfaces. Piping systems are cleaned most readily by a combination of air and cleaning solution in a slug formation completely around a circuit. Slug velocity should be kept between 6 and 10 ms, otherwise the slug may dissipate and no longer effectively contact the surfaces to be cleaned.

Table 1 Recommended milk pipeline size in relation to number of units per slope

Units per slope				
	Slope (%)			
Milkline diameter (mm)	*0.8*	*1.0*	*1.5*	*2.0*
50	2	3	4	5
64	6	6	9	10
75	11	13	16	20
98	27	30	38	45

Adapted from ASAE (American Society of Agricultural Engineers) S518.2. Jul 96

The pulsator side of the vacuum system may be constructed of iron pipe, stainless steel, or PVC. Pipe sizing should be adequate so that the difference in vacuum within the system does not exceed 2 kPa and the action of the pulsators does not influence vacuum by more than 2 kPa. Bucket systems should be provided with a means to clean vacuum supply pipes periodically.

A releaser or sanitary milk pump accomplishes circulation and removal of milk from under vacuum conditions. A liquid level control system or, more recently, VFD controls the rate of removal of fluid from the receiver. During milking, it is important to keep the milk level above the pump intake to prevent cavitation. Introduction of air by a starved milk pump leads to a serious flavor defect by the release of free fatty acids in the milk.

Milk recording of individual cows is possible with the use of recorder jars or milk meters. Glass recorder jars collect the milk yield of the cow at each milking and are calibrated to determine the amount of milk the cow has produced. Milk is released once each cow has been milked. Milk meters may be of the portable type or may be permanently installed. When permanently installed, electronic types of meter may collect the data and aid the dairy farmer in management decisions.

ACRs are popular adjuncts to milking systems that remove the milking units from the cow when a specified low milk flow condition is achieved. Typically, activation of the takeoff sequence is initiated when milk flow drops to 0.25–0.9 kg min^{-1}.

The most recent development in milking machines involves the use of robotic equipment to milk the cows on a voluntary basis without the attendance of an operator. The feasibility of using this equipment to milk cows has been amply demonstrated. The adoption rate and economic implications under a wide variety of conditions have not yet been established for this technology.

See also: **Animals that Produce Dairy Foods**: Reindeer. **Husbandry of Dairy Animals**: Goat: Milking Management; Sheep: Milking Management. **Mastitis Therapy and Control**: Management Control Options. **Milking and Handling of Raw Milk**: Milking Hygiene. **Milking Machines**: Robotic Milking. **Milking Parlors**.

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Robotic Milking

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Introduction

The main reason for starting the development of automatic milking systems (AM systems) was the need for improved labor efficacy due to the growing costs of labor in many dairy countries. Milking continues to be a time-consuming activity on many dairy farms, requiring about 25–35% of the annual labor demand, so milking contributes substantially to the costs of the farm enterprise. Apart from substituting manual labor by technology, robotic milking affects the whole farming operation. The farmer's presence at regular milking times is no longer required. The nature and organization of farm labor change such that manual labor is partly replaced by management and control activities. Regular visual checks of cow and udder health during milking are taken over by automated monitoring. Satisfactory cleaning of cows and teats, as well as milk analysis and separating abnormal milk, is required. An AM system is in use for 24 h a day, which requires a high reliability of the AM systems as well as the adaptation of cleaning and cooling systems. Permanent access to the milking system may require specific cow routing within the barn and is likely to affect the possibilities of grazing, although many AM farms are able to apply grazing. Reduced labor demand and better social circumstances for the dairy farmers are the attractive benefits of AM systems. Other potential benefits are improved animal health and well-being and increased milk yields.

Technical Aspects

The development of AM systems started with the development of equipment for automatic attachment of the teat cups. After the development of the milking machine, parlors, and automatic cluster removers, automatic attachment of the teat cups was the only missing step in the complete automation of the milking process. However, automatic milking requires more than automatic teat cup attachment. An AM system has to take over the 'eyes, hands, and partly brains' of the milker and therefore these systems have electronic cow identification, cleaning, and milking devices and computer-controlled sensors to control the milking process and to detect any abnormalities in both animals and milk.

An AM system has six main modules:

- milking stall,
- teat-cleaning system,
- teat detection system,
- robotic arm device for attaching the teat cups,
- control system including sensors and software, and
- milking machine.

The Milking Stall

AM systems can be divided into one- and multistall systems. The one-stall systems have a milking stall with an integrated milking machine and a robot device for attaching the teat cups. The multistall systems have up to five stalls and a mobile robot device, which moves from one stall to another to attach the teat cups. All AM systems are equipped with electronic ID reading systems to identify the cow's ID tag in order to decide if the cow has to be milked or not. AM systems have a concentrate dispenser to make the cow's visit to the system more attractive. Furthermore, the milking stall is equipped with a gate system to control cow traffic from barn to AM system and vice versa.

Teat-Cleaning System

The purpose of cleaning the teats is primarily to remove dirt and other particles that can contaminate the milk. Automatic cleaning is also necessary to meet (inter)national legislation and hygiene rules from the dairy industry. Research and practical experiences at conventional milking systems suggest that if udders and teats are clean, teat washing can be suspended without affecting milk quality. However, current AM systems do not have sensors to detect the amount of dirt at the teats, so the cleaning system should be based on more or less dirty teats. Efficient cleaning is of particular importance with high levels of spores in the environment of the cow. The teat-cleaning system should also aim at minimizing the risk of transferring udder pathogens from teat to teat or from cow to cow. To prevent residues in milk, teats should be free from disinfectants before attaching the teat cup.

There are several principles of teat cleaning with AM systems:

- sequential cleaning by brushes or rollers,
- cleaning with water in the same teat cup as used for milking, and
- cleaning by a separate 'teat cup-like' device.

Generally, plain water is used for the cleaning of the teats. Sometimes, the cleaning devices are flushed with plain water or disinfected between consecutive cleanings. The efficacy of the cleaning devices in cleaning the teats seems to be sufficient, although the results are not as good as manual cleaning by the herdsman in conventional milking systems. Extra attention to the hygiene conditions in the barn is essential to safeguard cleanliness of the udders. Besides cleaning the teats, automatic cleaning devices also stimulate the milk letdown process. Stimulation of the milk ejection reflex is necessary for efficient milking. The different teat cleaning methods do not differ with respect to the intensity of the milk ejection reflex; moreover, it is clear that the way of pretreatment in AM systems before each milking is very repeatable compared with conventional milking, which is seen as a positive effect.

Teat Detection System

The udder shape and teat position will differ substantially from cow to cow and besides breed and genetic merit are dependent on milk production, milking interval, deformation of the udder shape due to cows lying in the cubicle, and stage of lactation. Moreover, cows, although they are locked up, can move in the milking stall and so the position of the teats will change. AM systems have active teat detection systems to localize the four teats. This technically quite difficult process has been solved by using different techniques, like ultrasonic sound, laser techniques, and charge-coupled device (CCD) camera systems. All these techniques are used to find the position and place of the teats in reference to a fixed point at the robot arm. In fact, the system creates a three-dimensional view, so the robot arm knows where to attach the teat cup to the teat. The environment where these techniques have to operate is quite rough with moisture, dust, and manure, and special attention has to be paid to these circumstances to maintain good performance.

Robotic Arm

Different types of robot arms are used. Some robot arms imitate conventional milking by using an arm with a gripper, which picks up the teat cup from a storage rack at the side of the stall. The four teat cups are attached in succession. Some robot arms also take care of maintaining the position of the teat cups during milking and after milking for spraying and disinfection of the teats. Another system uses a robot arm with a fixed milk rack integrated with the robot arm. Each teat cup is attached separately, starting with the teat cups for the hind teats and finishing with the front teats. The robot arm is fixed to the milk stall, so each stall has its own robot arm.

In the multibox AM systems, a movable robot arm is used. In such a situation, the teat cups are positioned in a special teat cup holder close to the cow or the teat cups are mounted on a special milk rack. The robot arm moves from one stall to another, picks up the milk rack and teat cups, and starts the attachment process. After finishing the attachment, the milk rack stays in the same position and the robot arm is disconnected and can be moved to the next stall to start teat cup attachment for the next cow.

Control System and Sensors

AM systems need sensors to observe and to control the milking process as the milker is doing. AM systems therefore are equipped with a variety of sensors. These sensors are the 'eyes' of the AM system and their task is to monitor the technical functioning of the AM system, like cow identification, teat cup attachment, vacuum level, and start of the milk letdown process. The control unit in fact acts as the 'brains' of the AM system. In the case of deviations, the control system decides to cancel or to restart the milking process. Moreover, the control system takes care of an alert message to the end user. Modern AM systems are equipped with sensor technology to control the quality of the milking process, for example, check on abnormalities in the milk, milk yield, electrical conductivity (udder health), temperature of the milk, hormones in milk, feed intake, activity of the animal, and body weight of the animal. Sensors controlling milk quality like somatic cell counts (SCCs) and electrical conductivity are used to detect abnormal milk. If necessary, abnormal milk will be diverted automatically.

All measurements are automatically stored in the control system database and the accompanying management program is used to analyze these data and to control the settings and conditions for the cows to be milked. Attention lists and performance reports are presented to the farmer by screen or printer messages. In urgent cases like a breakdown or severe problems with a cow, the system immediately warns the herdsman by sending text messages to his mobile phone.

The Milking Machine

The milking machine is more or less similar to the milking systems in conventional milking parlors, except for the cluster. AM systems lack a milking cluster and are based on individual quarter milking. For each quarter, a teat cup, milk and pulse tube, and shutoff valve are used to control the milking of the individual quarter. The milk is kept separate till milk of the four quarters enters the central milk meter or the receiver. Although the basic technology is quite similar, in general milk tubes will be much longer than those applied in conventional milking, resulting in a considerable vacuum drop below the teat end during milking.

Barn Layout, Management, and Milk Production

Since AM systems largely depend on voluntary attendance, a well laid-out free stall barn is essential for a successful operation. Cows should have easy access to the milking stall and selection gates: long alleys, steps, and other obstructions should be avoided. A central location of the AM system in the barn minimizes walking distances of the cows. However, in many countries, regulations require that the AM system has to be located close to the milking room and accessible via a clean route. After visiting the milking system, the cow should have access to the feeding area. In 'forced cow traffic' systems, the cow has to pass the milking system in order to get access to the feeding area. In 'controlled-traffic' systems, one-way gates, with cow identification and selection capabilities, restrict cows from going directly to the feeding area only when the interval since the last milking exceeds the preset minimum. In 'free cow traffic' systems, access to the feeding area is unrestricted and only the concentrate fed in the AM system is used to attract cows. Grazing is also possible; however, long walking distances will negatively affect the visiting behavior of the cows to the AM system.

Changing over from a milking parlor to automatic milking will lead to big changes for both herdsman and cow and can cause stress to both. However, it can be concluded that cows in general adapt themselves quite easily to an AM system. Culling rate of cows, because they are not suitable for automatic milking, is reported to be less than 5%. These figures are comparable to those associated with the introduction of milking machines in the 1950s and milking parlors in the 1960s. More important is the introduction and learning period, during which cows should be handled quietly and consistently, to train them to adapt themselves to the new surrounding and milking system.

Automatic milking places emphasis on the cow's motivation to visit the AM system to be milked voluntarily. Cows can more or less decide themselves when to visit the AM system. The main underlying motive for a cow to visit the AM system is the supply of concentrate; therefore, all AM systems are equipped with concentrate dispensers. In the transition from conventional to automatic milking, cows have to learn to visit the AM system at other times than before. This needs special attention, and in the first days or week, human assistance will be necessary. Especially heifers with no history of conventional milking adapt themselves very easily to the conditions of automatic milking.

A large variation in milking intervals can be observed from cow to cow (**Figure 1**). In practice, the number of milkings per day varies from 2.5 to 3.0, but rather big differences in the milking intervals are reported from commercial farms. Cows that fail to visit the AM system voluntarily within the maximum set milking interval have to be fetched by the herdsman. These cows will not show any increase in yield or may even experience production losses. By changing the milking parameters of the AM system, it is quite easy to prevent cows from being milked at too low yields or too short intervals. But it is more difficult to prevent cows from being milked at long intervals. This means it will be necessary to manage the

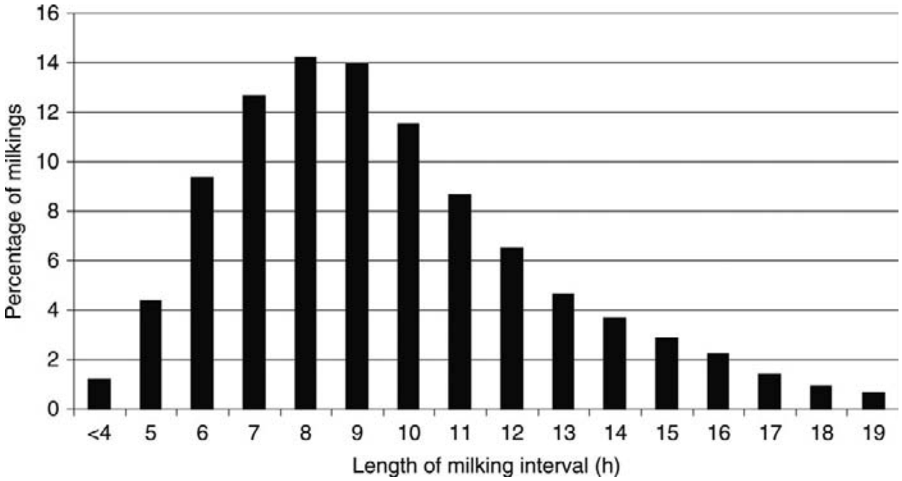

Figure 1 Frequency distribution of the intervals between milkings (2-year period, high-tech farm at Waiboerhoeve, The Netherlands).

intervals by fetching cows that have exceeded a maximum interval. Usually, this is done several times per day when other tasks such as cleaning and bedding have to be done.

The effect of automatic milking on labor requirement depends largely on the management approach, barn layout, and herd characteristics. On average, a 20–30% reduction in labor requirement is reported, but results show large variations. It is obvious that the character of the labor will change from manual work to managerial activities and observations of the cows and their behavior. Therefore, management is without doubt one of the key factors in the successful application of automatic milking.

Capacity

The capacity of an AM system is often expressed as the number of milkings per day. This number will largely depend on the configuration of the AM system, like the number of stalls and the use of selection gates, milking frequency, machine-on time, and herd size. Increasing the number of milkings per cow per day does not necessarily contribute to a high output capacity in kg of milk per day. This is due to the more or less fixed handling time of the AM system per milking and the decreasing amount of milk per milking. The challenge is to maximize the capacity of an AM system in terms of kg milk produced per day in order to minimize the costs per kg of milk produced.

Handling Time and Machine-On Time

A milking visit to the AM system consists of several activities. The cow walks to the AM system, is identified, and if it is allowed to be milked, the process starts. First the udder preparation and teat cleaning take place. The teat positions are detected and the four teat cups are attached. Then the milking process starts. After the teat cup takeoff, the teats are disinfected and the cow is allowed to leave the milking station. The milking visit ends when the entrance gate is opened again to allow the next cow to enter the AM system.

In fact, each milking visit can be divided into two main parts: the handling time of the AM system and the machine-on time covering the milking process. The handling time consists of the time for cow traffic (walking in and out of the system), identification, udder preparation, attachment of teat cups, and teat spraying. The handling time depends mainly on the AM system and on the time to attach the teat cups. Handling times between 2 and 4 min are reported from various studies. The machine-on time depends largely on the yield and flow rate of the individual cow. Between herds and between cows, the average flow rates will differ from 1.5 to 3 kg min^{-1} due to the yield per milking and genetic differences.

Daily Capacity

The maximum number of milkings per day and the capacity in kg per day can be calculated for the one-stall AM systems by using the handling time per milking visit, the machine-on time per visit, and the occupation rate of the AM system. For example, an occupation rate of 80% means that the AM system operates for 19.2 h day^{-1}. The remaining 4.8 h include time spent in rinsing and cleaning of the milking machine, refused milking visits, and the idle time, that is, the time spent by the AM system waiting for the next cow to be milked. In **Table 1** the results are presented for different yields per milking. Increasing the average yield per milking will result in less milkings, but in an increased capacity in kg per day. The results can also be used to predict the potential milking frequency of a herd. For example, 166 milkings per day divided by 60 cows in the herd will result in a milking frequency of 2.8 times per day.

The results show large differences in the number of milkings per day between the three flow rate groups (**Figure 2**). The minimum number of milkings will be 123 milkings per day for the low flow rate group at 15 kg yield per milking, and 230 milkings per day for the high flow rate group at an average yield of 7 kg per milking. Short intervals will result in a lower yield per milking and therefore an increased number of milkings per day. Increasing the yield per milking will result in less milkings per day, but in a higher daily production of the AM system. So milk flow rate and yield have a large impact on capacity in kg per day. By changing the milk criteria for

Table 1 Flow rate, machine-on time, number of milkings per day, and capacity in kg per day at an occupation rate of 80%

Yield (kg)	*7*	*9*	*11*	*13*	*15*
Flow rate (kg min^{-1})	2.27	2.37	2.47	2.58	2.68
Machine-on time (min)	3.08	3.79	4.45	5.04	5.60
Handling time (min)	2.50	2.50	2.50	2.50	2.50
Stay per milking visit (min)	5.58	6.29	6.95	7.54	8.10
kg per day	1444	1647	1825	1985	2134
Number of milkings per day	206	183	166	153	142

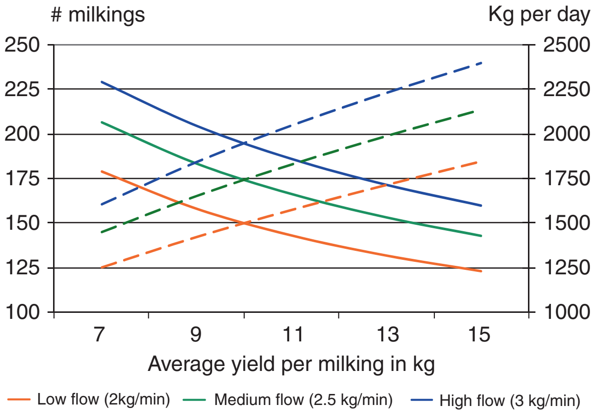

Figure 2 The calculated number of milkings per day and production per day at different yields and flow rates for a one-stall AM system.

Table 2 Global capacity in number of milkings per day for one- and multistall AM systems expressed in herd size

AM system	*Herd size (cows)*	*Number of milkings*
One stall	55–65	150–200
Multistall systems		
Two stalls	90–115	270–320
Three stalls	135–150	400–450
Four stalls	160–190	475–525

individual cows, the AM system can be optimized to realize a maximal capacity in kg per day. **Table 2** presents some global figures for the capacity of one- and multistall AM systems. Each extra stall will show a relatively smaller capacity due to extra walking distances and transport time of the robot arm.

Milk Quality

Milk quality is without doubt one of the most important aspects of milk production on modern dairy farms. Milk payment systems are based on milk quality, and consumers expect, besides food safety, a high quality level of the milk products they buy. Although automatic milking has more or less the same milking principles as conventional milking systems, there are some big differences. The AM system is in use for 24 h continuously. Visual control during the milking process is not possible, which implies both the milk and cleaning of teats. Cows will visit the AM system more or less voluntarily and this will result in a large variation in the milking frequency from cow to cow. All these aspects may influence the quality of the milk produced.

SCC and total bacterial count (TBC) are, respectively, measurements of the number of white blood cells and the total number of bacteria present in a milk sample. A high SCC might indicate reduced udder health due to mastitis (udder infection) and implies a lowered milk quality. The cleaning process and cleaning frequency of the milking equipment and the cooling of the milk seem to be the most important factors regarding the increase in TBC. In general, herds milked by AM systems consistently show slightly higher SCC and TBC values than conventionally milked herds, although differences are relatively small and far within the requirements of the dairy industry (**Table 3**).

Free Fatty Acids

It is generally known that the content of free fatty acids (FFA) in milk will increase with shorter milking intervals, all the more if the yield per milking is rather low. All studies with AM systems show a significant increase in FFA levels for AM systems. This increase cannot be explained solely by the shorter intervals, because the increase in FFAs with AM systems is even larger than with conventional milking parlors milking 3 times per day. Another explanation might be the increased air inlet by attachment of teat cups, during milking and at takeoff. Also the cooling process and a negative energy balance of the cow might play a role.

Economical Aspects

AM systems are more expensive than traditional milking systems. An AM system with two stalls requires an investment that is about 2 times greater than that

Table 3 Milk quality results for farms before and after the introduction of AM system

	Conventional milking		*Automatic milking*	
	Two times milking	*Three times milking*	*Before*	*After*
Bacterial count ($\times 1000\,\text{ml}^{-1}$)	8	8	8	12
Cell count ($\times 1000\,\text{ml}^{-1}$)	181	175	175	190
Freezing point (°C)	−0.520	−0.521	−0.521	−0.516
Free fatty acids (meq per 100 g fat)	0.44	0.54	0.41	0.59

From http://www.automaticmilking.nl;

required for a 2 × 8 automated herringbone milking parlor. These differences imply that total annual costs of milking with an AM system will be higher. However, more milk will be produced per cow and per herd with less labor than before. More milk means that the costs of milking per kg of milk will decrease. The same applies to the labor costs per kg milk. Theoretically, with an AM system more cows can be kept with the same labor force compared with the traditional milking system. But then also, additional investments have to be made for buildings, land, or feed, and for milk quota. On a farm with more than one full-time worker, the possibility exists to reduce labor input and thus costs. However, quite often, that does not happen and the time saved as a result of lower labor requirement will be used for personal activities: sports, family life, and other activities. Without doubt, milking with an AM system instead of a traditional milking parlor will have significant economical and social consequences.

Several simulation models are used to calculate the economical consequences, which will differ for each farm. The room for investment (RFI) model takes these differences into account. The RFI value equals the amount of money that can be invested in an AM system on a farm, without any change of the net return compared with the conventional milking system. The model accumulates the annual returns from increase in milk yield, annual savings in labor costs, and annual savings in not investing in the conventional milking parlor, and then divides this total by the annual costs of the AM system. The model is explained in **Table 4**. With this model, farm-specific factors and circumstances can be used to calculate the RFI value.

Table 4 Calculation of room for investment in €

Annual costs of conventional milking parlor	13 500
Labor saving using AM system	7750
Increase in milk yield	10 250
Total	31 500
Annual costs of AM system	25%
Room for investment	126 000

In **Figure 3**, the results of a combined sensitivity analysis are presented. The figure clearly shows that increase in milk yield and labor savings are essential factors regarding the economy of AM systems. The RFI value for the basic farm with 10% increase in milk yield, 10% labor saving, medium automated milking parlor, and 25% annual costs of the AM system amounts to € 134 000. The differences between the extremes are rather large, almost equal to the investment of a single-stall AM system. The lowest RFI value (0% yield increase, 0% labor saving) is € 83 000 and the largest value (15% yield increase, 30% labor saving) is € 194 000.

Economical results from commercial farms show that the use of AM systems in general is profitable, highly depending on the extra milk yield and labor savings. A Dutch case–control study of farms with AM systems and farms with conventional milking systems showed no differences in margin, although fixed costs for the AM farms were higher. AM farms saved 29% labor, and therefore when economical results were transformed to full-time equivalents (FTEs), AM farms in the case–control study had greater revenues, margins, and gross margins per FTE than the farms with conventional milking systems. So when deciding between investment in an AM system and in a conventional milking system, dairy farmers must weigh decreased labor needs for the AM system against the increased fixed costs of milking with an AM system. Therefore, in many cases, adoption of an AM system is for many dairy farmers a socioeconomic decision, rather than just a purely economic decision.

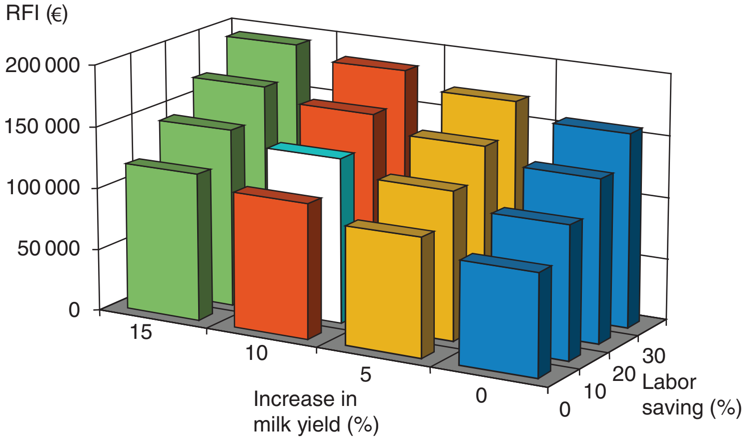

Figure 3 Room for investment (RFI) due to labor saving and increase in milk yield with annual costs for AM system of 25%.

See also: **Milking Machines:** Principles and Design.

Further Reading

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Relevant Websites

http://www.automaticmilking.nl – EU-Project Automatic milking; Implications of the introduction of automatic milking on dairy forms.

MILKING PARLORS

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Introduction

A milking parlor is part of a building where cows are milked on a dairy farm. Cows are brought to the milking parlor to be milked and are then returned to a feeding and/or resting area. Cows may also be milked in their housing area using bucket milkers or a pipeline milking system. Automatic, or robotic, milking systems usually operate without someone being present to supervise the milking process and have been in use for the past decade on commercial farms (see **Milking Machines**: Robotic Milking). The main advantages of a milking parlor when compared to milking cows in their housing area are that it increases labor efficiency, provides ergonomic advantages, and reduces the risk of injury, both traumatic and repetitive stress related. This article presents an overview of milking parlor design concepts. The major types and components of contemporary milking parlors and common forms of milking parlor automation are described. Milking parlor efficiency and hygiene are also discussed.

Design Considerations

The area where cows are milked (milking parlor) is usually part of a larger complex known as the milking center, which contains supporting structures and equipment for the parlor. A milking center typically contains the following use areas:

- Holding area – a pen for collecting cows before milking.
- Milking parlor – the location containing the milking equipment, milking stalls, cow platform, and operator's work area.
- Milk room – a room housing equipment for cooling and storing milk and for cleaning and sanitizing the milking and milk storage equipment.
- Utility room – a room that houses equipment such as vacuum pumps, refrigeration compressors, and water heaters.
- Supply area – a room or area for storing chemicals, drugs, towels, milk filters, and other supplies necessary for the milking operation.

As the size of the farm increases, it is common to incorporate other work areas and animal treatment areas into the milking center (*see* **Dairy Farm Layout and Design**: Building and Yard Design, Cool Climates; Building and Yard Design, Warm Climates). Milking centers may also contain the following additional use areas:

- Office – for record keeping and herd record storage. In some cases, office facilities for the entire farm operation are maintained at the milking parlor.
- Wash/drip pens – areas where the underside of a group of cows may be washed with floor-mounted sprinklers and then allowed to drip-dry before entering the holding area and/or milking area. Wash pens are used only in very dry climates.
- Herd health facilities – a central area for herd health needs on the farm to restrain animals for treatment or breeding, and to store veterinary and breeding supplies and equipment. Simple restraint facilities might be included in the milking parlor return lane if the milking parlor is not in use for extended periods.
- Hospital area – an area for isolation and convalescence of cows that cannot be kept with the milking herd.
- Worker comfort areas – areas for workers to wash, eat, relax, and so on.
- Maternity area – a housing area for cows ready for calving and a clean, isolated area for calving. In hot climates, the maternity area is usually separate from the milking parlor complex.
- Calf area – an area or room with hot and cold water for mixing calf rations. In hot climates, the calf area is usually separate from the milking parlor complex.
- Animal loading facilities – a ramp or dock for loading and unloading animal trucks or trailers.

The milking parlor and milking center house equipment for harvesting, cooling, and storing milk (*see* **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality) and must be designed to accommodate the special needs of these equipment (*see* **Milking Machine**: Principles and Design). The most important of these design criteria is that the milklines must be sloped toward a central receiver jar. Increasing the slope greatly increases the carrying capacity of milklines. All other pipelines in the milking system must also be sloped to drain point and generally conform to the slope of the milklines. The location of the milkline and dimensions of ancillary equipment, such as milk meters and pulsators, determine the required clearances between the floor of the operator's area and the cow platform. As a general rule, the total length of pipe and number of fittings in the milking machine should be kept to a minimum to reduce the cost of the system as well as improve milking and washing performance.

A well-designed milking parlor also allows easy and efficient disposal of waste milk and wastewater. Floors should be sloped with drains located to eliminate standing water that can create slippery or icy surfaces. The milking center is often the focus of activities on a dairy farm and the first stop for visitors to the farm. The location and layout of the milking parlor and milking center should facilitate desired activities and discourage entry into milking and milk handling areas except when necessary. Farmsteads should be arranged to minimize animals, vehicles, and people movement and interference. Seemingly minor design details, like floor plans that use a milk room for the main entrance to the milking center, can result in the accumulation of manure, debris, and extraneous materials.

Construction Methods

The various parts of the milking center require special construction materials and methods for ease of cleaning, moisture protection, ventilation, heating, fire protection, noise control, illumination, and so on. An understanding of the activities performed in the various use areas is required to specify the building materials and methods used for each.

Proper design, selection, and installation of electrical systems for milking parlors are crucial to using electricity safely and efficiently. Inferior wiring and equipment cause hazardous conditions for humans and livestock and often result in higher insurance premiums, increased maintenance costs, and greater risk of fire. Milking parlor equipment is washed regularly with chemicals, creating a wet and corrosive atmosphere. Corrosive gases, moisture, and dust hasten deterioration of electrical components. Wiring methods and materials that minimize this deterioration and maintain electrical safety and equipment function under these conditions are necessary for milking parlors. Consult relevant local codes and standards for the specific requirements for electrical equipment and materials suitable for use in the wet and corrosive environment found in milking parlors.

A basement can be built under the operator's area or milking areas to house milklines, pulsator airlines, milk meters, pulsators, receiver, and other equipment. This design can be used with herringbone, parallel, parabone, or side-open stall types. Milking equipment is protected from mechanical damage as well as excessive dirt and moisture. The operator's area is less cluttered, easier to keep clean, and quieter. The basement option generally has a higher building cost than a similar parlor without a basement.

Environmental Control

Environmental control is an important but often neglected component in milking parlor design and operation. Demands on the environmental control systems differ for the various areas of the milking center. Control of temperature, humidity, and odors can be accomplished through ventilation and heating and cooling systems that are properly designed, installed, and managed for each area. Ventilation systems must remove moisture, odors, and excessive heat from the milking parlor and holding area. Mechanical ventilation is typically used in parlors and milk rooms, while natural ventilation is most commonly used in holding areas. In cold regions, some type of heating system will be required in the milking parlor and offices. It is also common to have separate ventilation systems in the milking parlor for cold weather (to remove moisture and add heat) and hot weather (primarily to remove excess heat).

Parlor Types

Milking parlors are classified according to whether the cows are elevated above the person doing the milking (flat parlor vs elevated parlor), the type of stall used to confine cows during milking, and cow entry and exit methods. A description of the main parlor types follows.

Herringbone: In herringbone (or fishbone) parlors, cows stand on elevated platforms on either side and at an angle of about 45 ° to the edge of the operator's area (**Figure 1**). This orientation allows the operator access to the side of the udder for cow preparation and unit attachment. In larger parlors, the two rows of stalls may be arranged in a

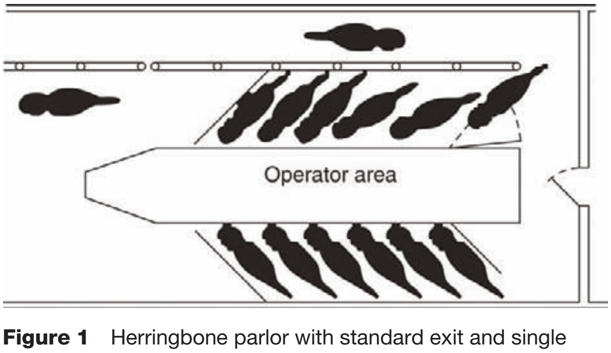

Figure 1 Herringbone parlor with standard exit and single return lane.

wedge or 'V' configuration, resulting in a wider operator area on the end away from the parlor animal entrance. This improves the visibility of units and cows from the other side of the operator area. Cows enter the milking stalls in groups according to the number of milking stalls on each side of the parlor. The rear portion of a herringbone stall is usually shaped in an 'S' pattern to position the rear end of the cow in close proximity to the milking unit. The front end of herringbone stalls can be stationary or fitted with indexing stalls (see definition in the next section) and can use either standard or rapid exit. In small herringbone parlors, it is common for cows on one side of the parlor to cross over to a common exit lane on the other side of the milking area. In larger parlors, an exit lane is provided for each side of the parlor (dual return).

Parallel: In parallel or side-by-side parlors, cows stand on elevated platforms at an angle of 90 ° to the operator area (**Figure 2**). Access to the udder for cow preparation and unit attachment is between the cows' rear legs. The cow platform in a parallel parlor is shorter but wider than that in a herringbone parlor. It is not possible to fit parallel stalls with arm-type cluster removers because of the limited access to the udder. Head chutes at the front end of the stall are used to position cows. Parallel stalls are commonly fitted with indexing front ends and rapid exit with dual return lanes. When compared to herringbone parlor types, the parallel configuration results in a shorter operator's area, which reduces the distance walked by operators.

Parabone: Parabone stalls place the cow at an angle of about 70 ° to the operator area (**Figure 3**). The entrance and exit features and positioning method of this stall design are similar to the herringbone type. The sharper cow angle makes the operator's area shorter, and units are commonly attached between the cow's back legs as in a parallel stall.

Side open or tandem: In side opening or tandem stalls, cows stand in an end-to-end configuration during milking, and units are attached from the side (**Figure 4**). This parlor type is less affected by variations in individual cow milking times than herringbone or parallel parlors as cows are moved one at a time rather than in groups. This parlor type also gives the maximum view of and access to cows by the operator during milking. It is ideally suited to situations in which individual cow care is done in the milking parlor. Entrance of cows may be automated.

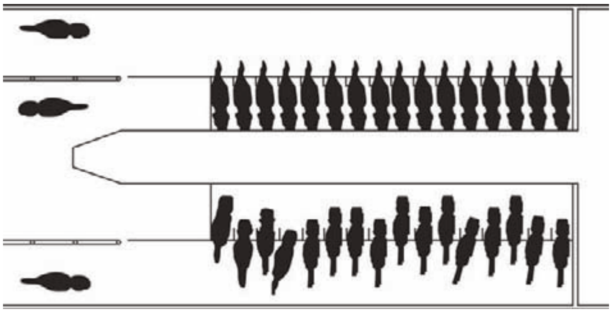

Figure 2 Parallel or side-by-side parlor with rapid exit and dual return lanes.

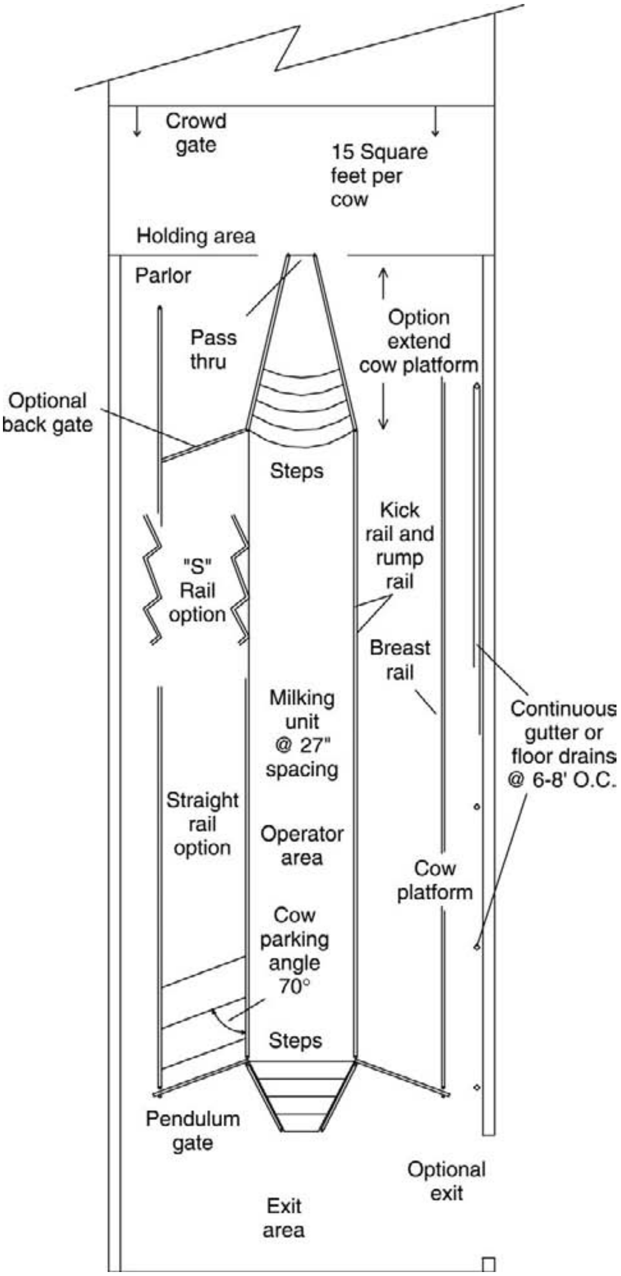
Crowd gate
15 Square feet per cow
Holding area
Parlor
Pass thru
Option extend cow platform
Optional back gate
Steps
Kick rail and rump rail
"S" Rail option
Breast rail
Milking unit @ 27" spacing
Continuous gutter or floor drains @ 6-8' O.C.
Straight rail option
Operator area
Cow platform
Cow parking angle 70°
Steps
Pendulum gate
Optional exit
Exit area

Figure 3 Parabone milking parlor with standard exit with fixed breast rail (bottom) and adjustable breast rail (top).

Rotary or carousel: In rotary (or carousel) parlors, cows walk onto a rotating milking platform one at a time (**Figure 5**). The cows move past an operator where cow preparation and unit attachment is performed. A second operator is positioned near the exit to remove milking units if detachers are not used and to apply postmilking

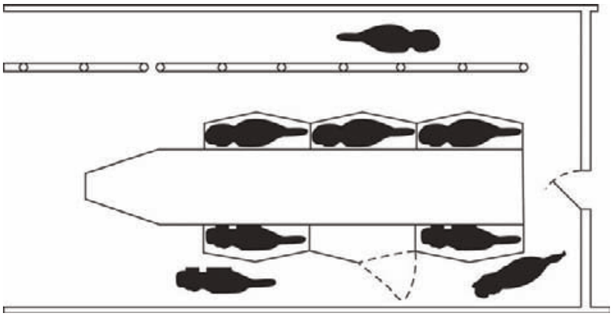

Figure 4 Side-open or tandem milking parlor with single return lane.

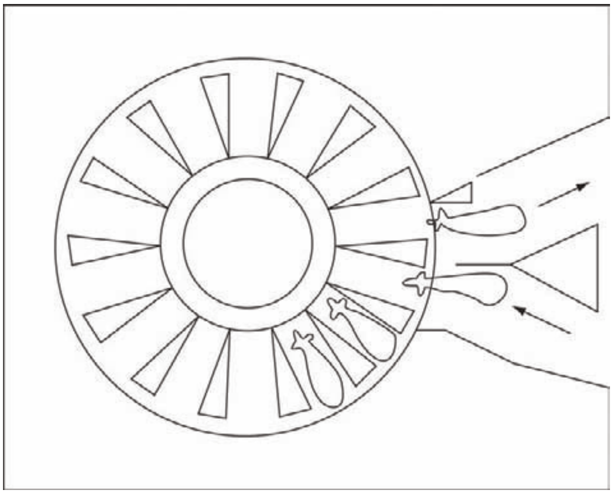

Figure 5 Rotary parlor with face-in cow platform and outside operator area.

teat sanitizer. A third operator may be employed to tend to unit slips and falloffs and other special cow needs during milking. The number of stalls in rotary parlors can range from 10 to 90. Although rotary parlors have gained popularity for large herds during the past decade, they still make up a relatively small percentage of parlors in the world.

Other Milking Parlor Design Elements and Support Equipment

Swing-over: The swing-over or swing configuration can be used on herringbone or parallel parlors but is most common with the parabone stall configuration (**Figure 6**). Stalls on either side of the operator's area share milking units. When milking is completed on one side of the parlor, the milking units are removed and 'swung' to the stall immediately across the operator's area. This configuration reduces the number of milking units required to reduce the initial cost of the parlor. Swing-over parlors usually milk slightly fewer cows per hour per stall but more cows per hour per milking unit compared to parlors with one milking unit per stall.

Flat parlors: In flat parlors, the milking area and the operator's area are at the same or similar elevation (**Figure 7**). Various stall designs are used, from simple stanchions in which the cow must back out of the stall after milking to more complicated gates that cows walk through after milking. Flat parlors can be inexpensively fitted into existing stanchion or tie-stall barns with highline milking equipment as a method to improve labor efficiency. They are not, however, as labor efficient or worker friendly as elevated parlors.

Indexing stalls: Both herringbone and parallel parlors may be fitted with movable or indexing stall fronts. The stall front is moved forward as cows enter to widen the stall area and facilitate rapid cow movement. When milking is ready to commence, the stall front is moved rearward to position the cows as close as possible to the milking unit and operator. This reduces the reaching distance required to prepare cows and attach units and also reduces cow movement during milking.

Entrance/exit gates: Entrance and exit gates to milking stalls may be manually operated or powered by pneumatic cylinders. Controls for both entrance and exit gates should be located at both ends of the operator's area and in intermediate locations in large parlors. The standard exit configuration for herringbone, parallel, and parabone stalls is a single exit gate located at the exit end of the stall row. In these configurations, cows walk out of the milking stalls in single file. Rapid exit stalls allow for all cows to exit the front end of each stall simultaneously. Methods used include raising, lowering, or rotating the entire stall front or opening a series of gates in front of each cow simultaneously. Rapid exit stalls require a wider exit area on the milking platform and a return lane on each side of the parlor. Rapid exit stalls improve performance in parlors with more than 8–10 stalls per side.

Automatic cluster removers (ACRs): ACRs or automatic detachers sense the end of milk flow and then shut off the vacuum to the claw and remove the milking unit from under the cow. ACRs improve labor efficiency primarily by removing the need for operators to observe the end of milking for each cow and, secondarily, by eliminating the detachment task, allowing one operator to handle more milking units. ACRs typically reduce milking time by reducing the amount of overmilking. With arm-type units, the milking unit is attached to a retractable arm that removes the unit from under the cow at completion of milking. With rope or chain ACRs, the milking unit is attached to the rope or chain. At completion of milking, the rope is retracted to remove the unit from the udder and lift it away from the cow. Arm-type units are more ergonomically friendly because they support the weight

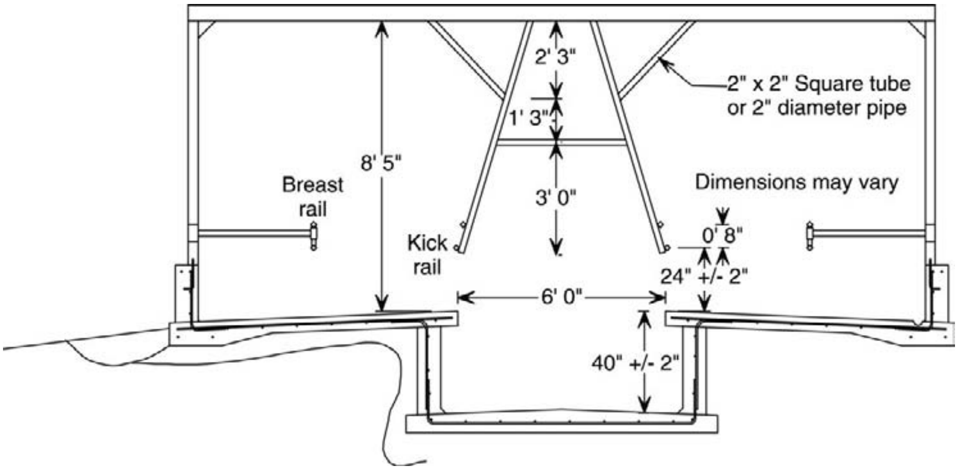

Figure 6 Cross section of a swing-over parlor.

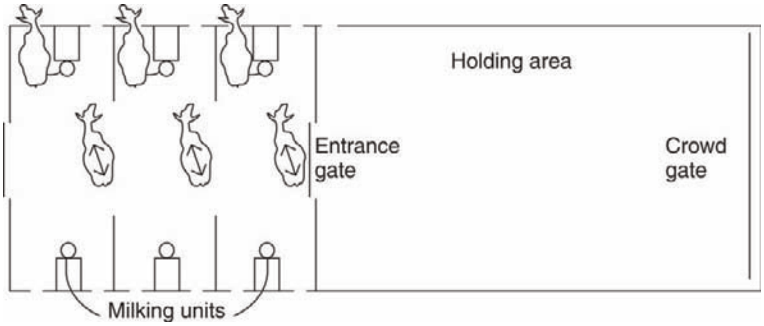

Figure 7 Flat parlor with back-out stalls.

of the cluster and hoses during unit attachment. They also offer superior support for the long milk and pulse tubes resulting in better balance of the milking unit on the udder, and prevent the milking unit from lying on the platform in the event of a cluster falloff.

Crowd gates: Power-driven crowd gates reduce the size of the holding pen as cows move into milking stalls. A bell or other signal device can be fitted to the crowd gate to alert cows to the movement of the gate. Crowd gates can also be fitted with controls to stop the drive unit automatically when the gate senses a cow. Some gates can be raised over a group of cows in the holding pen to return to their starting position, thus allowing a new group to be loaded in the holding pen while milking of the earlier group is being completed. Electrically charged wires on any crowd gate make cows nervous and are not recommended. The proper use of a crowd gate improves labor efficiency by eliminating the need for operators to leave the operator area during milking in order to encourage cows to enter the milking stalls.

Animal identification and data collection/records systems: Automatic animal identification systems read information from a transponder affixed to individual cows. Additional systems are available to automatically collect a variety of data for the identified animal, including milk yield, milking time, milk conductivity, activity level, and weight (*see* **Mastitis Therapy and Control**: Automated Online Delection of Abnormal Milk). These data are sent to a central collection point where they are organized, analyzed, and stored by a computer-based records system. Records systems help to identify animal health problems and determine reproductive status. Cow identification and performance data can also be used to automatically sort animals as they enter or leave parlors equipped with automatic sorting gates and pens and to control automatic animal feeding systems.

Work Routines and Labor Efficiency

A milking parlor is a tool that facilitates implementation of proper and consistent milking techniques (*see* **Milking and Handling of Raw Milk**: Milking Hygiene) while improving the efficiency, safety, and comfort of milking personnel. A worker-friendly facility will also help to attract and retain milking labor. The working posture in an elevated milking parlor should be as close as possible to an erect stance with arms and hands in a natural working position. The dimensions of the parlor and location of milking stations and stalls must be designed to accommodate good working postures. An adjustable height of the floor will make it possible for milkers of different heights to have a correct ergonomic position in the parlor. A primary reason for investing in a milking parlor is to improve labor efficiency. The milking facilities can comprise from 20 to 60% of the total capital investment in a dairy. Milking can make up more than half of the labor expended on a dairy farm. Proper planning, construction, and operation of the milking facilities can thus have a significant influence on the labor and economic efficiency and general work environment of a dairy enterprise. Parlor planning should always begin with a thorough economic analysis of the various options for the specific situation on each farm.

Labor efficiency in automated side-open, herringbone, and parallel milking parlors typically ranges from 40 to more than 80 cows milked per person per hour, depending primarily on the efficiency of work routines and tasks other than milking that may be performed in the parlor. These parlors typically are designed with 6–12 milking stalls per operator (3–6 stalls per side) when not automated and with 12–20 stalls per operator (6–10 stalls per side) when automated. The typical turnover rate ranges from 3 to 5 filling and emptying (turns) per hour for these parlor types, with larger parlors typically having a lower turnover rate. Rotary parlors typically range from 4 to 5 turns per hour with 2–4 operators.

The steps used in premilking udder preparation and the efficiency of work routine have a much larger effect on labor efficiency than does parlor type. Several recent analyses have shown that multiple small parallel and herringbone parlors are more labor efficient and have higher economic values than a single large parlor with the same number of milking stalls.

Hygiene

It is important to clean the exterior surfaces of milking and milk handling equipment to avoid contaminating milk during milk harvest and transport (*see* **Milking and Handling of Raw Milk**: Milking Hygiene). Parlor equipment should be cleaned after every milking and at least 3 times per day in large parlors milking around the clock. A milking parlor should be fitted with equipment to easily clean exterior surfaces of milking and milk handling equipment. This is typically accomplished with a combination of manual cleaning with a brush and cleaning solution and a rinse with water under medium or high pressure. The walls and floors are typically cleaned using a combination of manual scraping or brushing and then a rinse using hoses with water under medium or high pressure.

Some parlors are also fitted with devices to automatically clean the floor of the parlor and/or holding area during milking. This may be accomplished by periodic application of water under pressure or by a flush system in which a large volume of water is released to create a wave of water on floor surfaces that will carry manure and urine to a collection pit. Care must be taken in the design of flushing systems so that flush water does not contaminate milking units (*see* **Milking and Handling of Raw Milk**: Milking Hygiene).

See also: **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates. **Mastitis Therapy and Control**: Automated Online Detection of Abnormal Milk. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Milking Machines**: Principles and Design; Robotic Milking.

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MOLECULAR GENETICS AND DAIRY FOODS

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Introduction

Molecular genetics (MG) is a scientific discipline concerned with the structure and function of genes at the molecular level and encompasses the technique of genetic engineering, which can be defined as the direct manipulation of an organism's genome through the introduction of foreign DNA, or any artificial (not natural) modification that alters the structure and characteristics of the organism's genes. MG has enormous potential and will continue to contribute to the production of high-quality dairy products through advances such as the design of 'tailor-made' starter cultures used in the production of fermented dairy foods, for the selection of genetically elite animals (i.e., within the farm gate) and the generation of improved transgenic livestock (i.e., outside the farm gate). The objective of this overview is to briefly discuss the contribution of MG both within and outside the farm gate toward the production of dairy products, as well as discuss ongoing and future research in this discipline.

From Gene to Protein

Structure of DNA

DNA or deoxyribonucleic acid is the central molecule in almost all living organisms that serves as the carrier of genetic information. It is composed of chemically linked sequences of subunits; each subunit (collectively referred to as a nucleotide) contains a base molecule (adenine, guanine, thymine, or cytosine), a pentose sugar called deoxyribose (ribose in RNA), and a phosphate group. The double-stranded DNA helical molecule results from the pairing of adenine with thymine via two hydrogen bonds, and cytosine with guanine via three hydrogen bonds, while the sugar and phosphate group form the backbone of the molecule (**Figure 1**(**a**)). Three adjacent nucleotides encode for an amino acid, the building blocks of proteins (e.g., ATG codes for the amino acid methionine). The four nucleotides can be arranged in sets of three in 64 different ways, which is more than sufficient to code for the 20+ required amino acids. A gene is thus a segment of DNA containing a specific sequence of nucleotides that carries the information responsible for producing one particular protein. The simplicity of the structure of DNA makes it amenable to genetic engineering. Basically, the DNA strand can be cut with special enzymes called restriction endonucleases, which recognize short specific DNA sequences, and can be rejoined by the action of other enzymes called ligases, enabling the fusion of different DNA molecules. The polymerase chain reaction (PCR) enables the amplification or synthesis of several thousand copies of a desirable DNA sequence, which can also be fused with other DNA molecules. During the cloning process, the sequence of interest is ligated to a vector molecule, which is in essence an extra-chromosomal circular DNA molecule. These vectors are often derived from bacterial plasmids, which are naturally occurring extra-chromosomal DNA molecules but have been modified to harbor other genes to aid the cloning process.

Transcription and translation

In order to produce a protein, two processes must occur, namely transcription and translation (**Figure 1**(**b**)). During the process of transcription, messenger RNA (mRNA) is synthesized by the enzyme RNA polymerase, representing a single strand of the DNA. Transcription begins when the enzyme binds to a special sequence on the DNA called the promoter at the start of a gene and continues until it reaches a terminator sequence, generating a transcription unit (**Figure 1**(**b**)). A transcription unit may include more than one gene. Proteins/polypeptides are then generated from the mRNA by the process of translation whereby a ribosomal RNA (rRNA) moves along the mRNA strand while transfer RNAs (tRNAs) concomitantly deliver the correct amino acid to the rRNA for the growing polypeptide chain depending on the genetic code in the mRNA sequence.

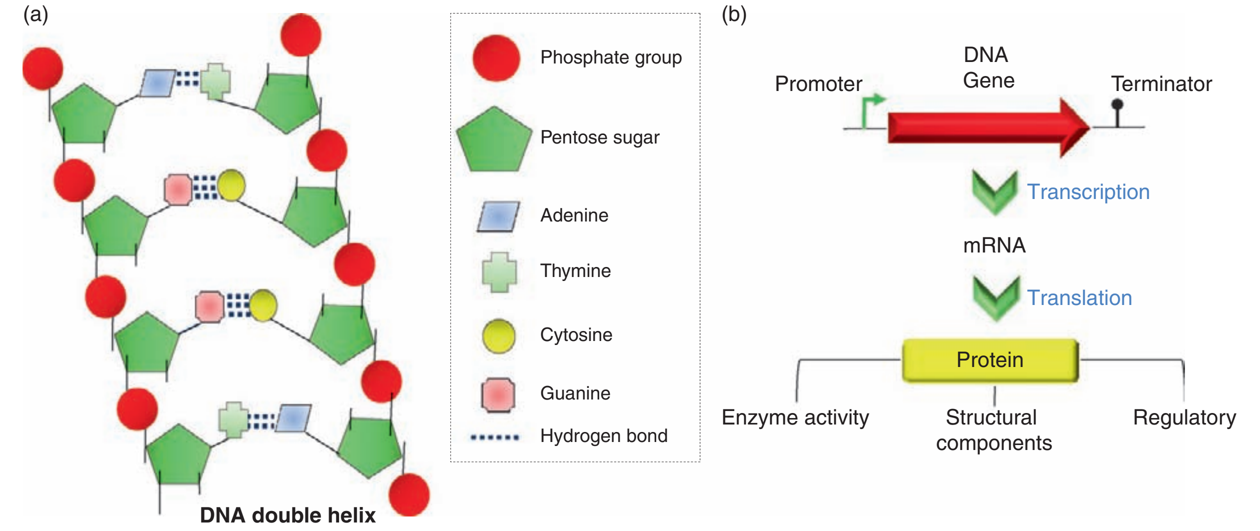

Figure 1 (a) Diagrammatic representation of the DNA double helix. In this diagram, the sequence of DNA is read as AGGT. (b) Sequence of events involved in transcription and translation. The gene is represented by the red arrow. The small green arrow before the gene represents the promoter sequence and the terminator sequence is represented by the black ball and stick at the end of the gene.

DNA Sequencing in the Twenty-First Century

A major advantage to MG has been the unprecedented upsurge in DNA sequencing in the last decade, from viruses and bacteria right through to mammals. The sequencing of the human genome was completed in May 2006 through the Human Genome Project (HGP). Indeed, a new proposal has now been launched called Genome 10K (G10K), which aims to obtain whole-genome sequence data for 10 000 vertebrate species. Projects of this nature are now becoming more feasible as new DNA sequencers promise ultra-fast and inexpensive delivery of DNA sequence information. With regard to the dairy industry, genome sequences are now available for a number of lactic acid bacteria (used in food manufacture and as probiotics) and their bacteriophages (bacterial viruses), many of which are fundamental to the dairy industry. In 2009, the first full genome sequence of a female Hereford cow, named L1 Dominette, was released by the Bovine Genome Sequencing Project, making her the first ever livestock animal to be completely sequenced. The vast quantity of sequencing data generated is handled and sorted by computer technology through the scientific discipline of bioinformatics. The benefits of an available genome sequence are vast in that it provides the entire genetic blueprint of an organism. With regard to livestock, available genome sequence data will lead to more accurate diagnosis of disease and to the production of healthier and more productive animals through animal transgenesis and strategic animal breeding programs, as discussed later. Genome sequences of dairy bacteria are providing information on the genes that encode fundamental traits such as production of flavor compounds, bacteriocin production, and resistance to bacteriophage infection, as well as providing genetic machinery for the development of improved genetic tools. Moreover, the availability of the genome sequence has pioneered the twenty-first-century science, systems biology, which aims to understand biological systems in their entirety through multidisciplinary approaches. It combines a range of modern technologies beginning with the annotated genome sequence and includes transcriptomics, the study of the complete set of transcription units in a cell under a particular set of conditions; proteomics, which involves the study of the protein complement produced by an organism; metabolomics, which describes the metabolite profile; and interactomics, which is the study of complex molecular interactions in cells, alongside mathematical modeling techniques to build models for biological interpretation and ultimately prediction of behavior.

Molecular Genetics Outside the Farm Gate

Gene Technology: Tailor-Made Cultures

MG has been used to generate ‘tailor-made’ bacterial cultures with many applications in the dairy food industry. For example, metabolic engineering has been applied to starter cultures to increase the production of various desirable flavor compounds. Diacetyl is an important flavor compound with the characteristic butter aroma and is produced by the dairy starter culture *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* via metabolism of citrate. Diacetyl production in *Lc. lactis* via metabolism of lactose (the main sugar in milk) instead of citrate was achieved either by disruption of the enzyme lactate dehydrogenase (LDH) or by over-production of NADH oxidase. High diacetyl production levels were achieved by combining the above strategy with the disruption of α-acetolactate decarboxylase. In another example, the carbon flux of *Lc. lactis* was rerouted toward the production of alanine, an amino acid with a sweet flavor, by expressing the *Bacillus sphaericus* alanine dehydrogenase gene. Moreover, expression of alanine dehydrogenase in an LDH-deficient strain permitted the production of alanine as the sole end product (homoalanine fermentation).

Bacteriophage infection of starter cultures during fermentation is a constant issue that can result in quality defects that affect the flavor, texture, and even safety of dairy products. A number of phage-resistance mechanisms have been identified and characterized, particularly in *Lc. lactis* (*see* **Lactic Acid Bacteria**: *Lactococcus lactis*). It has been possible to clone these phage-resistance mechanisms, mainly genes involved in restriction modification and abortive infection, into dairy starter cultures including *Lc. lactis* and *Streptococcus thermophilus*, producing superior cultures that are resistant to phage infection.

Bacteriocins are polypeptides produced ribosomally by bacteria and can have a bactericidal or bacteriostatic effect on other bacteria. The application of MG to the study of bacteriocins has resulted in the development of novel starter cultures with an inbuilt antimicrobial mechanism, thus endowing the fermented dairy product with an innate immunity to foodborne pathogens such as *Listeria monocytogenes*. One such example is nisin, a well-known bacteriocin produced by *Lc. lactis*. The genetic machinery encoding for nisin production has also been harnessed to develop specialized cloning vectors that enable controlled expression of the cloned gene. These vectors, termed the nisin-controlled expression (NICE) vectors, contain the promoter of the *nisA* gene, which encodes the bacteriocin nisin. In the natural situation, extracellular nisin acts as the signal for transcriptional activation of its own structural gene (*nisA*) and several other biosynthetic and immunity genes. The gene of interest is therefore cloned directly after the *nisA* promoter in the expression vector. The level of expression can be controlled by the amount of nisin used for induction and it has been reported that recombinant protein synthesis can reach levels up to 60% of the total protein. This system has been used for the efficient expression of many heterologous enzymes in *Lc. lacti*s. For example, the system has been exploited for the over-production of exopolysaccharides (EPSs) in a transgenic derivative of the probiotic strain *Lactobacillus paracasei* 338 (**Figure 2**). EPSs play a major role in the production of a number of dairy products such as yogurt, where their viscous nature enables them to contribute to texture, mouthfeel, taste perception, and stability of the final products, and in some cases EPSs have also been associated with beneficial health effects.

Lactococcus lactis has also been engineered to synthesize various therapeutic molecules for delivery to the gastrointestinal tract, many of which are already in advanced stages of preclinical and clinical development. For example, recombinant human interferon-β that exhibits antiviral activity was expressed in

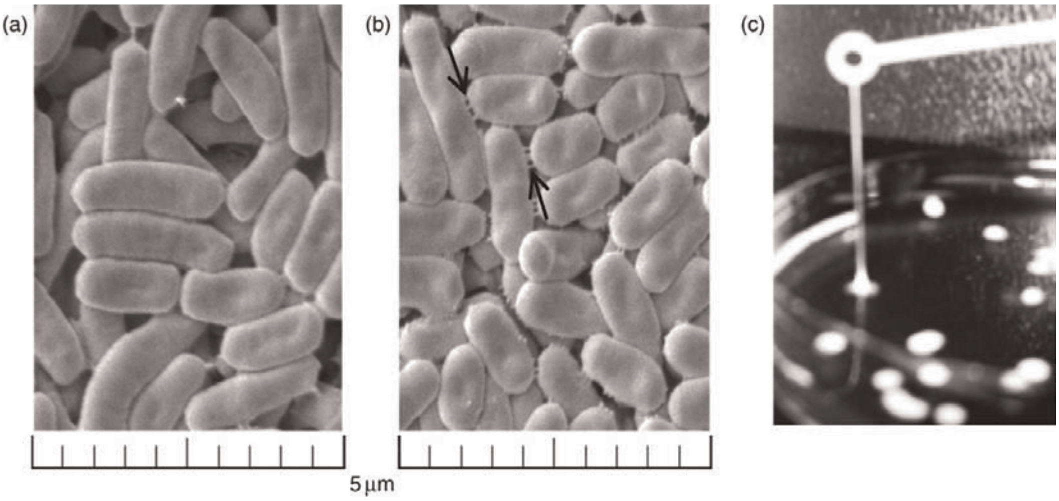

Figure 2 Scanning electron micrograph (5 μm) of *Lactobacillus paracasei* 338 (control) (a) and transgenic derivative *Lb. paracasei* 338 that contains the gene for producing exopolysaccharides (EPSs) (b) after growth in 7% glucose. Arrows indicate EPS production. (c) EPS production in transgenic derivative demonstrated by the loop touch test of a colony, where over-production of EPS causes a ropy phenotype. Adapted from Stack, H. M., Kearney, N., Stanton, C., Fitzgerald, G. F., Ross, R. P. (2010). Association of beta-glucan endogenous production with increased stress tolerance of intestinal lactobacilli.*Applied and Environmental Microbiology* 76: 500–507.

Lc. lactis. Another promising application for engineered *Lc. lactis* is its use as an antigen delivery vehicle for live mucosal vaccines. Indeed, a wealth of bioactive molecules from both microbial and eukaryotic backgrounds can be expressed in lactococci providing a food-grade vehicle for oral delivery of such compounds. Generation of dairy products using these genetically modified strains could create a whole new niche in the market; dairy foods with targeted medicinal benefits. Such foods would be distinctly separate from the highly popular functional foods, which aim to exert a positive effect on human health, most often linked to the presence of probiotic organisms or their metabolites.

Gene Technology: Transgenic Animals

One of the key breakthroughs of MG in dairy animals has been the ability to endow transgenic animals with traits that could not be achieved through standard breeding strategies. However, the time required to create transgenic livestock is approximately 5–7 years. A typical example has been the creation of transgenic dairy cows that express the antimicrobial protein lysostaphin in the mammary gland and consequently secrete the protein into milk. This protein is particularly active against the mastitis pathogen *Staphylococcus aureus*, and has been shown to protect the transgenic cattle in a dose-dependent manner. Mastitis is a disease of the mammary gland, affecting almost every dairy farmer globally at some stage and is estimated to cost $2 billion in losses annually in the United States alone. Robert J. Wall and coworkers from the US Department of Agriculture USDA, Maryland, who have pioneered this work, are also looking into the possibility of using other antimicrobial proteins with a broader spectrum of antimicrobial activity. Such strategies could also protect against other unwanted microbes, thus extending the shelf life of milk. The human antimicrobial protein lactoferrin has also been expressed in four transgenic cows at a concentration of up to $2\,\mathrm{mg\,ml^{-1}}$ milk. Human lactoferrin has demonstrated antibacterial, antifungal, and antiviral properties and could be important in defense against pathogens in infants.

Transgenic cows have also been created to produce milk that is more suitable for cheese manufacture. In this approach, the genes encoding β- and κ-caseins were cloned in cows, which resulted in the over-expression of both proteins in milk. Increased κ-casein has been linked to a reduction in micelle size and can improve the heat stability of micelles, all of which favor cheese production. Milk protein allergy is a common problem, particularly in children. Intolerance to the major protein allergen β-lactoglobulin has been addressed in transgenic cows through the inactivation of the β-lactoglobulin gene.

Public Concerns Regarding Genetically Modified Organisms

The US Food and Drug Administration (FDA) recently issued a formal risk assessment declaring food products from cloned cattle, pigs, and goats safe for human consumption, while the European Food Safety Authority (EFSA) issued a draft ‘Opinion’ with the same conclusion. While this may seem promising, cloned animals in essence do not contain genetically modified DNA and are thus different from transgenic animals. However, despite the efforts of the FDA and the EFSA, other groups such as the USDA and the European Group of Ethics do not agree for the moment that cloned animals should enter the food chain. Additional challenges clearly exist for the acceptance of transgenic animals. However, it is worth noting that recently a human-use anticoagulant (trade name ATryn) derived from the milk of genetically modified goats became the first approved pharmaceutical product having received FDA approval for use in patients with hereditary antithrombin deficiency. However, apart from the United States and Canada, the public view toward genetic manipulation is largely one of uncertainty. Those in favor of genetically modified organisms (GMOs) believe that the products of GMOs are generally safe and beneficial, and that the technologies used to generate them are well understood and can be managed and controlled by modern science. Those on the opposing side view this technology as unnatural and do not believe that perceived benefits outweigh the risks associated with food safety, animal welfare, as well as possible impacts on ecosystems and the environment, which cannot be controlled once the technology is introduced. It is worth noting, however, that the research field of biosafety was created recently to manage the ecological impact of GMOs. Concern is also expressed regarding the intent behind the research into genetic modification, particularly if the focus is on the creation of commercial products and maximization of corporate profit. Indeed, one of the major issues to date is that few if any genetically modified products exist that actually benefit the consumer directly. The availability of products such as foods that improve human health alongside studies evaluating the impact and safety of GMOs should play a significant role in increasing the acceptance of this technology.

Molecular Genetics within the Farm Gate

Exploitation of MG within the farm gate is through the identification of genetically elite parents of future generations. The interest in MG in animal breeding stemmed from the limitations of traditional, quantitative methods of genetic evaluation, which ignore the effect of individual genes but instead evaluate animals based on their entire complement of genes. Quantitative genetics is not without its weaknesses, in particular the long time period and resources required to obtain accurate estimates of the genetic merit of an animal. These estimates of genetic merit are generally termed estimated breeding values (EBVs) in animal breeding.

The potential in animal breeding of tools generated through advances in MG relies on the ability to identify the causative mutation giving rise to a difference in phenotype (i.e., the performance of the animal as observed in the field), or to identify a linked genetic marker, and to use this marker in a breeding program with the appropriate breeding goal. The end objective of this approach, as for any breeding program, is to effectively and efficiently disseminate the superior genetics into the commercial population. Possibly one of the greatest benefits of genomics in animal breeding is that genomic DNA is available from an animal at any age as well as from both genders. Knowledge of the 'ideal genotype' and the genotype of every animal can facilitate increased genetic gain through a combination of mainly altering accuracy of predicted EBVs and the time from birth to the realization of an accurate estimate of genetic merit.

Marker-Assisted Selection

One of the motivations for exploring the use of MG in animal breeding was that causative mutations, or linked genetic markers, could be included in a breeding program, an approach referred to as gene- or marker-assisted selection (MAS). The regions of the genome associated with performance are called quantitative trait loci (QTLs). Many different types of genetic markers exist but the markers of choice nowadays are single-nucleotide polymorphisms (SNPs). An SNP is a variation at a single-nucleotide position of the DNA sequence of individuals of the same species. The shift toward the widespread use of SNPs was to more readily facilitate automation and scoring of genotypes. Several SNPs have been shown in many studies to affect milk quality. For example, a dinucleotide polymorphism in the acyl CoA:diacylglycerol acyltransferase gene has been shown to be associated with milk fat and protein composition. Similarly, SNPs in the casein and lactoglobulin genes have been associated with milk quality.

Although MAS, using information generated from MG, theoretically has huge potential, such as the ability to increase the accuracy of predicted genetic merit at a very young age, it also has, like traditional quantitative approaches, its shortcomings. Disadvantages of MAS are (1) it is often difficult and resource-intensive to find the causative mutation or a genetic marker tightly linked to the causative mutation, (2) the QTL effects are often overestimated, and (3) the QTL(s) rarely explain all of the genetic variance and therefore polygenic or remaining genetic effects still need to be accounted for in the analysis.

Genomic Selection

One of the main shortcomings of MAS is that currently, and for the foreseeable future, identified QTLs are not likely to explain all the genetic variance in quantitative traits, that is, that multiple genes at different locations in the genome contribute to a particular phenotype. An alternative to identifying and selecting based on a limited number of QTLs is to select based on all genetic markers simultaneously. Genomic selection is based on the simultaneous selection for many thousands of genetic markers that densely cover the entire genome and is essentially a large-scale version of MAS made possible by the development of arrays with many thousands of SNPs for fast genotyping throughput. The success of genomic selection is based on exploitation of linkage, often called linkage disequilibrium, between the genetic markers and the causative mutation and this association is assumed to persist across the population. Therefore, dense marker coverage is vital to ensure that all causative mutations are tightly linked with a genetic marker.

The process of genomic selection follows two broad steps: (1) estimation of marker effects in a reference or training population and (2) prediction of EBVs of animals with no phenotypes. The first step is the most difficult since it requires the estimation of thousands of effects usually from a data set with considerably less records. Nevertheless, several statistical approaches are possible to estimate genomic EBVs using genome-wide dense marker maps. Genomic selection will soon become the method of genetic evaluations in most countries across a large proportion of agricultural species and MG was key to achieving this.

Future Requirements for Molecular Genetics in Animal Breeding and Food Processing

Explaining the 'missing heritability' will be arguably one of the most challenging areas for animal breeding in the future. Heritability is the ratio of genetic variance to phenotypic variance after excluding the variance attributable to systematic environmental effects. The missing heritability refers to the inability of large genome-wide association studies in species, including humans, to explain a large proportion of the genetic variation in a trait. One possible contributing factor to the missing heritability is that the expression of a phenotype may be due to rare alleles with large effects or indeed many common alleles with very small effects. Addressing the former may require deep sequencing of a large number of individuals for candidate genes, identified through systems biology.

Identifying common alleles with very small effects can be achieved through larger data sets in genome-wide association studies. Considerable data sets are required however to detect loci with small effects. A candidate gene approach may reduce the requirement for larger data sets. Genome-wide association studies, using currently available SNP array platforms, are unlikely to detect the causal mutation. However, by using bioinformatics, genes in the vicinity of the detected genetic markers or overrepresented biological pathways may be detected, which will aid in identifying putative candidate genes for further interrogation.

In terms of food processing, the accepted application of MG in dairy foods in the future will undoubtedly unveil a wealth of opportunities to increase their nutritional quality and safety, and as already mentioned may create a whole new role for dairy foods in areas such as medicine, making this a high-value sector. Moreover, as scientists unravel how nutrition influences metabolic pathways and homeostatic control of individuals through nutrigenomics, it is possible that MG may be used to produce dairy foods for personalized nutrition. Further progress supported by more cost-effective methodologies alongside systems biology approaches will undoubtedly lead to greater understanding of gene functionality and the effects of genetic manipulation whether in starter cultures or in transgenic livestock and should answer the many questions that still loom for both the public and regulatory authorities.

See also: **Lactic Acid Bacteria**: *Lactococcus lactis*.

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NUCLEOSIDES AND NUCLEOTIDES IN MILK

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Structural Aspects

Nucleosides are *N*-glycosides of pyrimidines and purines. The sugar moiety is either β-D-ribofuranose (ribonucleosides) or 2-deoxy-β-D-ribofuranose (2′-deoxyribonucleosides). The C1 carbon atom of the pentose is bound to N1 of a pyrimidine or N9 of a purine. The *N*-glycosidic linkage is in the β-configuration. Modified or rare nucleosides represent compounds with chemical modifications in the heterocyclic base and/or the sugar moieties. **Figure 1** shows the structures of nucleosides that occur in milk or that are otherwise of dietary interest. Only ribose forms are considered in this overview.

Nucleotides are *o*-phosphoric acid esters of nucleosides. The esterification may be present on position 2′, 3′, or 5′ in ribonucleosides and on 3′ or 5′ in 2′-deoxyribonucleotides. The most common site of esterification in naturally occurring nucleotides is the C5-hydroxyl group. Such compounds are called nucleoside-5′-phosphates (5′-nucleotides). This contribution discusses monophosphorylated 5′-nucleotides only. Nomenclature and usual abbreviations are as follows: adenosine (ado), 1-methyladenosine (m1ado), N6-methyladenosine (m6ado), N6-dimethyladenosine (m6,2ado), N6-(2-isopentenyl)-adenosine (ip6ado), N6-carbamoyl-L-threonyl-adenosine (t6ado), guanosine (guo), 1-methylguanosine (m1guo), 7-methylguanosine (m7guo), N2-methylguanosine (m2guo), N2-dimethylguanosine (m2,2guo), cytidine (cyd), inosine (ino), 1-methylinosine (m1ino), 7-methylinosine (m7ino), uridine (urd), pseudouridine (ψ), thymidine (thy), adenosine-5′-monophosphate (5′-AMP), cytidine-5′-monophosphate (5′-CMP), guanosine-5′-monophosphate (5′-GMP), inosine-5′-monophosphate (5′-IMP), uridine-5′-monophosphate (5′-UMP).

Compositional Aspects

Milk contains nucleotides and nucleosides as well as pyrimidines and purines as free bases in the micromolar concentration range. They are part of the nonprotein-nitrogen (NPN) fraction of milk. The concentrations of these compounds in the milk of several different mammalian species have been investigated. In bovine, ovine, and human milk, the concentrations of these compounds are highest directly after parturition, and most of the compounds generally tend to decrease as lactation or nursing proceeds. The composition and concentration patterns of the different compounds are specific for the milk of a given species. The fact that these species-specific patterns have not been eliminated by the evolutionary process implies that they are physiologically significant and important in the diet of the neonate. Owing to their potential as dietary modulators, there is currently great interest in the supplementation of infant formula with certain nucleotides. In view of these facts, it is surprising that nucleosides have become a topic of nutritional research only in the past 2–3 decades.

Nucleosides

Studies have been carried out on milk from cattle fed under controlled conditions. These studies show that the concentrations of the nucleosides in milk initially decrease during the colostral phase and remain constant at more mature stages of lactation. Thus, the concentrations of the common nucleosides (ado, cyd, guo, ino, and urd) are relatively constant after 3 weeks postpartum,

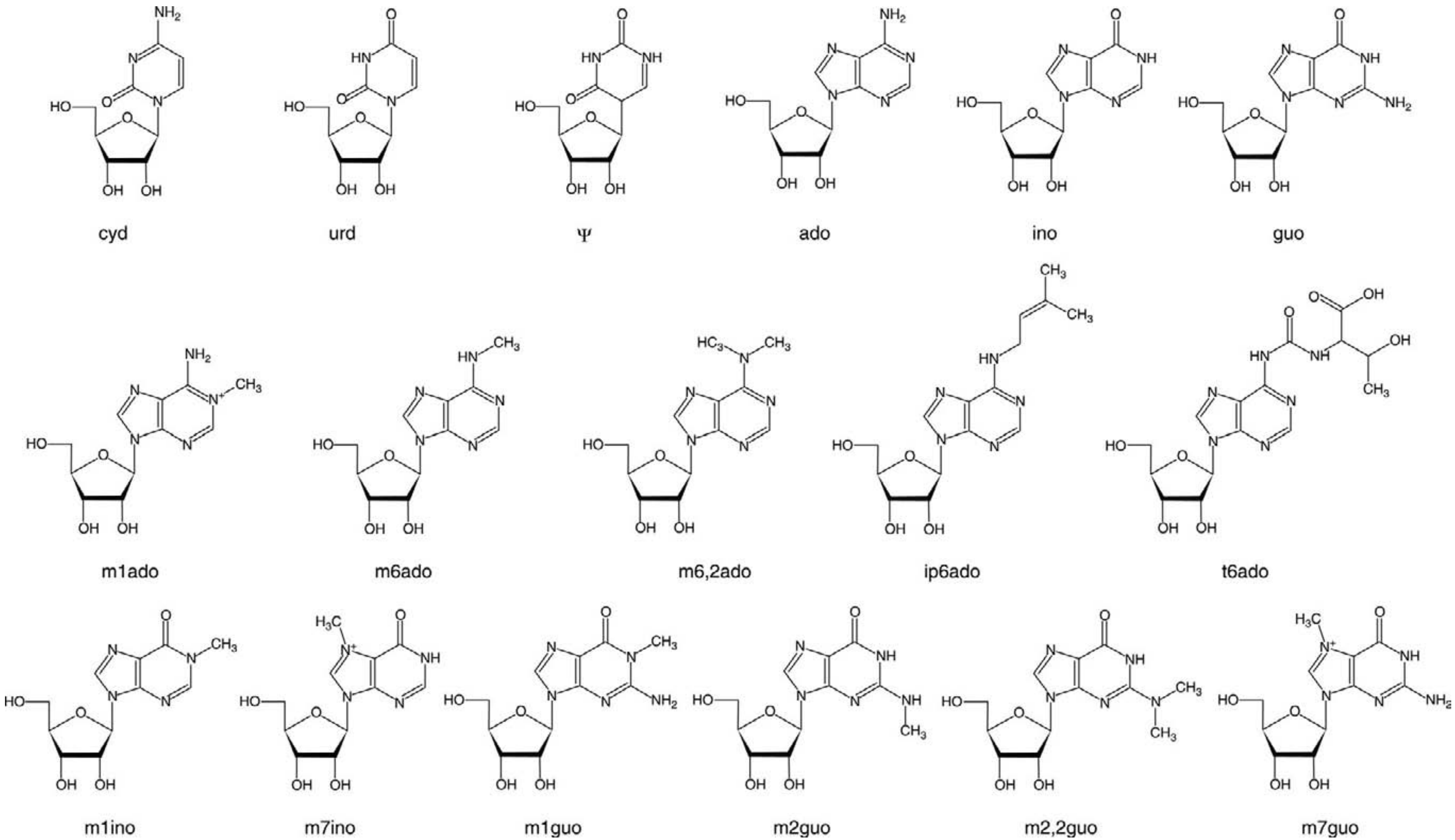
cyd
urd
Ψ
ado
ino
guo
m1ado
m6ado
m6,2ado
ip6ado
t6ado
m1ino
m7ino
m1guo
m2guo
m2,2guo
m7guo

Figure 1 Structures of nucleosides that occur in milk or that are otherwise of dietary interest.

Table 1 Nucleosides (μmol l^{-1}) in bovine colostrum and mature milk and in mature sheep and goat milk

	ado	*cyd*	*guo*	*ino*	*urd*	*m1ado*	*t6ado*
Bovine colostrum[a]	2.0	11.6	2.1	4.7	132.6	1.0	2.9
Bovine mature milk[a]	1.4	2.4	0.8	1.0	14.7	0.4	0.7
Sheep (mature milk)[b]	8.8	6.7	2.1	41.2	67.8	1.4	0.7
Goat (mature milk)[b]	2.4	8.8	2.9	60.6	76.3	0.9	0.6

[a] Adapted from Raezke KP and Schlimme E (1990) Ribonucleoside in Milch: Charakterisierung und Bestimmung des Konzentrationsprofils dieser minoren Komponenten über eine Laktationsperiode. *Zeitschrift für Naturforschung* 45c: 655–662. Each mean value is the arithmetic mean of determinations (three measurements each week in duplicate) in the first lactation week postpartum (bovine colostrum) as well as throughout the whole lactation period with the exception of the first 3 weeks postpartum (mature milk).

[b] Adapted from Martin D, Clawin-Rädecker I, Lorenzen PChr, Ziebarth M, and Barth K (2005) Ribonucleosid-Gehalte in Schaf- und Ziegenmilch. *Kieler Milchwirtschaftliche Forschungsberichte* 57(1): 21–32. Each mean value is the arithmetic mean of determinations (weekly in duplicate) throughout the whole lactation period with the exception of the colostrum.

All values rounded to one digit after the decimal point.

while the concentrations of the rare nucleosides m1ado and t6ado are constant after only 1 week postpartum. The distinct maxima observed in the graphic plots of concentration versus time postpartum for certain nucleotides are not observed for the corresponding nucleosides (**Table 1**). The sum of the concentrations of the pyrimidine nucleosides (17 μmol l^{-1}) is approximately fivefold greater than that of the purine nucleosides (about 3 μmol l^{-1}). The concentration of urd exceeds that of the other nucleosides by 1–2 orders of magnitude, probably reflecting the importance of uracil nucleotides (e.g., UDP-hexoses) for lactose biosynthesis in the mammary gland. The large, severalfold differences in the concentrations between colostrum and mature milk probably result from the greater metabolic activity in the mammary gland and the increased transfer rate of the nucleosides circulating in the blood through the blood–milk barrier in the first few days postpartum. In this period, colostrum contains relatively high concentrations of blood constituents. Therefore, postsecretory metabolic processes attributable to milk enzymes (e.g., alkaline phosphatase, adenosine deaminase), somatic cells, and microorganisms are stronger in this period.

The results in **Table 1** also support the reports of high contents (up to 300 μmol l^{-1}) of UDP-glucose and UDP-galactose during the colostral phase.

The raw milk of goats and sheep contains markedly greater concentrations of the unmodified nucleosides urd and ino than that of cows (**Table 1**). The species-specific patterns of nucleoside contents are particularly recognizable in the unmodified nucleoside contents and by calculation of nucleoside concentration ratios (unmodified and modified nucleosides). Thus, nucleoside determination is suitable for distinguishing raw milk from cow, sheep, and goat.

The nucleosides in human milk, however, differ both qualitatively and quantitatively from those in the milk of ruminants. **Table 2** shows the interindividual mean

Table 2 Nucleosides (μmol l^{-1}) in mature human milk

ado	*cyd*	*guo*	*urd*	*m1ado*	*t6ado*	*m1guo*	*ψ*	*References*
5.3	4.3	0.2	0.5	0.3	0.5	0.4	1.7	Topp *et al*. (1993)
1.6	4.4	0.3	6.9		No data published			Leach *et al*. (1995)
3.0	5.1	1.0	4.2	0.3	0.4	0.7	-	Schlimme and Schneehagen (1995)
Not Detected	5.4	Not Detected	4.9		No data published			Thorell *et al*. (1996)
10.0	79.0	8.0	37.0		No data published			Gill and Indyk (2007)

All values rounded to one digit after the decimal point.

Adapted from Topp H, Groß H, Heller-Schöch G, and Schöch G (1993) Determination of N6-threoninocarbonyladenosine, N2, N2-dimethylguanosine, pseudouridine and other ribonucleosides in human breast milk. *Nucleosides & Nucleotides* 12: 585–596; values reported for m2guo, m2,2guo, and m1ino were <0.1 μmol l^{-1} each; Leach JL, Baxter JH, Molitor BE, Ramstack MB, Ramstack MB, and Masor ML (1995) Total potentially available nucleosides of human milk by stage of lactation. *American Journal of Clinical Nutrition* 61: 1224–1230; Schlimme E and Schneehagen K (1995) Ribonucleosides in human milk – concentration profiles of these minor constituents as a function of the nursing time. *Zeitschrift für Naturforschung* 50c: 105–113; Thorell L, Sjöberg L-B, and Hernell O (1996) Nucleotides in human milk: Sources and metabolism by the newborn infant. *Pediatric Research* 40: 845–852; Gill BD and Indyk HE (2007) Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formula. *International Dairy Journal* 17: 596–605.

values of common and some rare nucleosides quantified during mature stages of lactation. The concentration profiles of the nucleosides tend to decrease steadily in the course of lactation.

Nucleotides

Table 3 presents the concentrations of some common nucleoside-5′-monophosphates in samples of raw bovine milk taken at different stages of lactation. Shortly after parturition (0–1 day), lower concentrations of most nucleotides are observed followed by a maximum 1–2 days postpartum. The concentrations then gradually decrease as lactation continues. The initial concentrations of 5′-AMP and 5′-CMP are reduced by half, while the levels of 5′-UMP decrease by two orders of magnitude. A similar pattern is also seen in the total amount of nucleotides, that is, including nucleoside-5′-mono-, di-, and triphosphates (ΣA_t, ΣC_t, ΣG_t, ΣU_t) as well as UDP-galactose and UDP-glucose. Two days postpartum, the concentrations (μmol l^{-1}) were $\Sigma A_t = 67.8$, $\Sigma C_t = 60.5$, $\Sigma G_t = 36.5$, $\Sigma U_t = 1495.0$, UDP-galactose = 320.2, and UDP-glucose = 362.3. Not shown in **Table 3** is orotic acid, which is the main nucleotide-related compound in bovine milk. Its concentration increases in the course of lactation by about one order of magnitude to a maximum of about 400 μmol l^{-1} 6 months after parturition.

The concentrations of common nucleotides exceed those of the corresponding nucleosides by 1–2 orders of magnitude. 5′-AMP and 5′-CMP are present in all milk samples from colostral to mature stages of lactation, while 5′-UMP has not been detected later than 3 weeks postpartum. The 5′-GMP content of bovine milk has been reported to be less than the limit of detection.

The reported concentrations of nucleotides in human milk vary within the range 4–70 mg l^{-1}, corresponding to about 10–200 μmol l^{-1}. At least part of the variation results from the different analytical procedures used. Concentrations in human milk at different stages of lactation are shown in **Table 4**. Most of the data show that cytosine and uracil nucleotides make up the largest fractions of the total nucleotide content, agreeing well with the pattern found for nucleosides shown in **Table 2**. Generally, the total nucleotide content of the milk of humans exceeds that of ruminants. On the other hand, orotic acid, which is present at relatively high concentrations in the milk of ruminants, is absent in human milk.

Table 3 Nucleotides (μmol l^{-1}) in bovine milk at different days postpartum

Days	*5′-AMP*	*5′-CMP*	*5′-GMP*	*5′-UMP*
0–1	39.7	31.9	n.d.	186.3
1–2	61.8	52.5	n.d.	390.0
30	27.5	33.2	n.d.	n.d.
60	20.3	18.9	n.d.	n.d.

n.d., not detectable.
Adapted from Gil A and Sanchez-Medina F (1981) Acid-soluble nucleotides of cow's, goat's and sheep's milks at different stages of lactation. *Journal of Dairy Research* 48: 35–44.

Table 4 Nucleosides, nucleotides, and *TPAN* in human milk

Heterocyclic base	*Nucleosides*[a]	*Nucleotides*[b]	*TPAN*[c]
Adenine	3	20	32
Cytosine	5	19	88
Guanine	1	3	31
Uracil	4	13	38

[a]Adapted from Schlimme E and Schneehagen K (1995) Ribonucleosides in human milk – concentration profiles of these minor constituents as a function of the nursing time. *Zeitschrift für Naturforschung* 50c: 105–113.
[b]Nucleoside-5′-monophosphates only (1 month postpartum); adapted from Gil A and Sanchez-Medina F (1982) Acid-soluble nucleotides of human milk at different stages of lactation. *Journal of Dairy Research* 49: 301–307.
[c]Adapted from Leach JL, Baxter JH, Molitor BE, Ramstack MB, and Masor ML (1995) Total potentially available nucleosides of human milk by stage of lactation. *American Journal of Clinical Nutrition* 61: 1224–1230.
Values (μmol l^{-1}) rounded to the nearest whole number.
TPAN, total potentially available nucleosides.

Although refined techniques of nucleoside and nucleotide analysis have been well established for many years, only a few studies report quantitative data on all the sources of nucleosides and nucleotides in body fluids, for example, nucleoproteins, nucleic acids, various derivatives including coenzymes all of which may contribute to the observed contents. Total amounts of nucleic acids (RNA, DNA) have been estimated so far to range from 190 to 580 mg l^{-1} for bovine and from 118 to 720 mg l^{-1} for human milk. The analytically accessible nucleoside content has been termed 'total potentially available nucleosides' (TPAN). The TPAN values for human milk shown in **Table 4** include the contents of nucleosides, nucleotides, and oligo- and polymeric ribonucleic acids as potential sources available in milk for the generation of these compounds by digestion and metabolism. As **Table 4** shows, including such potential sources increases the total concentration and alters the concentration ratios of the four nucleosides. The relatively small value of the ratio of adenine nucleotides initially present to the TPAN content of this nucleoside reflects above all the relevance of adenine nucleotides in bioenergetics and biosynthesis. Ino was detected in a few samples of human milk at concentrations one order of magnitude smaller than the

TPAN values of the other four nucleosides in **Table 4**. In most of the milk samples, however, ino was not detectable.

In a further study on the TPAN value in human milk, 160 samples were collected from 135 healthy, lactating women from different Asian countries at four stages of lactation. The average TPAN concentrations were 171.9 μmol l^{-1} in colostrum, 208.1 μmol l^{-1} in transitional milk, 221.6 μmol l^{-1} in early mature milk, and 210.6 μmol l^{-1} in late mature milk, with no notable geographical differences. The major sources of ribonucleosides were RNA (43.3% of TPAN) and free ribonucleotides (39.9% of TPAN). The sources of ribonucleosides and percentages of nucleotide bases are similar for all stages of lactation. For all stages after colostrum, more than 91% of the TPAN is present in the noncellular component. In addition, the study shows that the average TPAN level in Asian women is similar to that in European and American women, and free ribonucleotides in human milk represent less than half of the TPAN.

Functional Aspects

Biofunctional Properties

Biofunctional properties of food constituents are related to their dietary value and possibly to regulatory processes beyond basic nutritive functions. Although nucleosides and nucleotides are only minor components of milk and of many other foods, they are essential for cellular function and metabolism, and energy conservation and transfer. Furthermore, they are the monomeric precursors of nucleic acids.

Mammals meet their needs for nucleosides and nucleotides by a combination of *de novo* syntheses, reutilization of these compounds by the salvage pathway, and by dietary supply. Dietary nucleosides and nucleotides are ingested mainly as nucleoproteins, which are converted in the intestine into monomeric compounds. Nucleosides and nucleobases are transported across the plasma membrane by specific facilitated-diffusion carriers and/or sodium-dependent transporters.

In some tissues, for example, the intestinal mucosa, the salvage pathway is dominant because of a limited capacity for *de novo* synthesis. Therefore, the exogenous supply of nucleic acid-derived components is important for optimal function of such tissues. For example, when rat weanlings were fed a diet supplemented with nucleosides, both the formation of mucosal protein and DNA in the proximal segment of the gut and the length of the villi in this segment were greater than in a control rat group. Thus, nucleotides and related compounds have been recognized to be semiessential for rapidly growing tissues.

The following findings concerning food-related effects are of particular interest because they indicate possible therapeutic uses of these compounds especially for neonates:

- Ingestion of ino increases uptake of iron from the intestine of rats. Human milk therefore has been suggested as a factor in facilitating iron absorption in breast-fed infants.
- Dietary nucleotides may stimulate α-lipoprotein synthesis and thus be involved in lipid metabolism.
- A diet supplemented with nucleotides improved development (indicated by weight, body length, and head circumference) in a group of infants born small for gestational age. The authors attributed the observations to beneficial trophic effects of nucleotides on the intestinal mucosa.
- Clinical studies compared the effects on adults of RNA-supplemented and RNA-free enteral food. From these studies, it has been concluded that optimal patient outcome during a period of stress may depend on provision of dietary nucleotides. These data support the claim that dietary nucleotides are conditionally essential nutrients.
- The presence of specific nucleosides and/or nucleotides in human milk may be a factor in the immune response of breast-fed infants. These compounds are also postulated as growth factors for the neonate. A study of 370 full-term healthy infants reported that infant immunity is enhanced by diets of human milk and of infant formula supplemented with ribonucleotides at the same concentrations as in human milk. The development of the infants' immune systems for the first year of life was monitored by measuring the antibody response to the immunization vaccination. The study also registered the incidence of diarrhea and noted that this was less frequent in infants fed with nucleotide-fortified formula or human milk.

The following biochemical effects of nucleosides and nucleotides on human cell culture systems imply that these compounds have regulatory potential beyond their nutritive functions:

- Recent reports show the influence of nucleosides and nucleotides on liver regeneration *in vitro* and *in vivo.* DNA and RNA syntheses in hepatocytes as monolayer cultures were studied after the addition of ino, cyd, urd, thy, and 5′-GMP. The results confirm the importance of nucleosides and nucleotides in enhancing nucleic acid metabolism.
- The sucrase activity in the intestinal mucosa of early-weaned rats was enhanced when they were fed a nucleotide-supplemented diet of composition similar to human milk. *In vitro* studies showed that

culture media fortified with nucleotides enhanced the proliferation and maltase activity of cultures of small intestine crypt cells (ICE-6). The data suggest that nucleotide supplementation may enhance enterocyte proliferation and maturation *in vitro* and *in vivo*.

- The effects of added nucleosides on the *in vitro* proliferation and apoptosis of models of human cell cultures, namely peripheral blood lymphocytes (PBLs), promyelocytic leukemia cells (HL-60), and colon adenocarcinoma cells (Caco-2), have been investigated. Of the 16 nucleosides tested, the modified adenine ribonucleosides ip6ado and m6,2ado showed the strongest effects. Micromolar concentrations of these compounds inhibited proliferation of all three cell types and induced apoptosis in the PBL and HL-60 cultures. Ado, m1ado, m6ado, and m2,2guo (**Figure 1**) also markedly inhibited the proliferation of PBL and HL-60 cells. The same nucleosides, with the exception of m1ado, induced apoptosis in HL-60 cultures. Interestingly, the antiproliferative effect in HL-60 cells – and to a lesser extent the enhancement of apoptosis – increases with the degree of chemical modification of the adenine molecule.

Furthermore, ribonucleosides, in particular modified nucleosides, serve as valuable pathobiochemical marker molecules for cancer. In addition, chemically modified nucleosides have already found interesting applications as pharmaceutically active compounds in the treatment of different illnesses.

Infant Formulae – New Developments

Owing to their biofunctional properties, nucleotide supplements have been used in infant and follow-on formulae for many years in Japan, in some member states of the European Union as well as in some developing countries. As no harmful effects are known, the European Commission (EC) Directive 2006/141/EC (2006) permits the supplementation of infant and follow-on formulae with nucleotides according to the concentrations specified in **Figure 2**.

Supplementation of infant and follow-on formulae with nucleotides and/or nucleosides should be allowed up to levels present in human milk. Findings concerning the TPAN including all mono-, oligo-, and polymeric sources available in milk are given in **Table 4**.

The current EC Directive 2006/141/EC takes into account the contribution to the TPAN of milk of only the common, unmodified nucleosides and neglects the modified nucleosides and nucleotides that may be released by RNA degradation. Such modified or rare compounds may show regulatory effects but this has so far not been studied adequately.

Technofunctional Properties

The relatively constant concentration pattern of these minor constituents throughout lactation changes markedly on processing. As the changes depend on the processing conditions, the compounds are useful as chemical indicators allowing quantification of adulteration of milk and milk products, identification of different thermal treatments, and the characterization of differently processed products, for example, butter types. Furthermore, high-pressure treatment influences the nucleoside contents and the activities of the milk enzymes alkaline phosphatase and adenosine deaminase, which are of relevance to nucleosides.

Heat-Induced Changes

Heating raw milk under the conditions termed ‘thermization’ (e.g., 65 °C by 15 s) or ‘holder pasteurization’ (e.g., 30 min at 62 °C) causes the concentrations of cyd, guo, and ino to increase severalfold – up to 10-fold in the case of ino – and the ado concentration to decrease by 30%. The changes in milk nucleoside contents occurring during common dairy processes such as pasteurization, ultra-high temperature (UHT) treatment, and sterilization are mainly attributable to the increased catalytic activity of the milk enzymes adenosine deaminase and alkaline phosphatase during the heating-up phase. Stronger heating conditions with a sterilization value, F_0, exceeding 30 min, however, can cause hydrolysis of the phosphate groups of nucleotides, leading to an increase in the corresponding nucleoside concentration.

Thus, cyd, guo, and particularly ino can be used as chemical parameters for detecting thermization and, in particular, holder pasteurization. Unmodified nucleosides of milk seem to be unsuitable for characterizing heat treatment in the region of short-time pasteurization, high-temperature pasteurization, and UHT heating and sterilization.

The concentrations of modified, rare nucleosides such as t6ado and m1ado also vary only slightly throughout lactation. Typical values for t6ado and m1ado in raw bulk milk are 0.7 and 0.4 μmol l^{-1}, respectively. A further modified nucleoside, m6ado (**Figure 1**), is normally not detectable in fresh raw milk but forms in measurable concentrations when the milk is heated under certain conditions. Thus, on UHT treatment or sterilization of milk, it forms as a function of the initial m1ado concentration and of the temperature and holding time of the heat treatment. However, it is not detectable after heating to thermization, holder pasteurization, and short- and

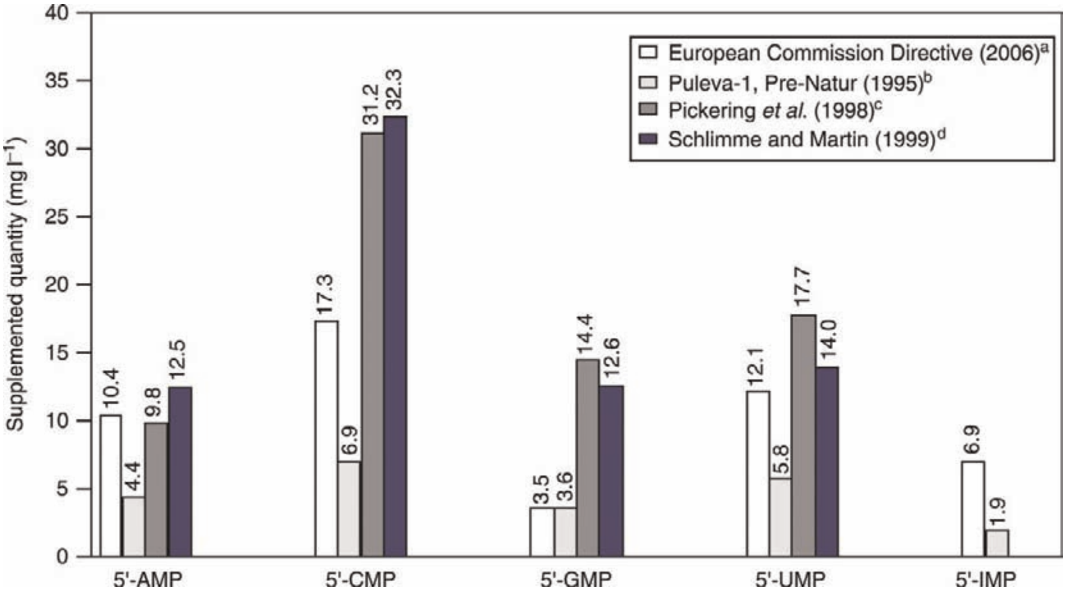

Figure 2 Comparison of the supplemented quantity of nucleotides (mg l^{-1}) in infant and follow-on formulae. The conversion to mg nucleotide per liter infant formula was calculated assuming that the infant formula is isocaloric with human milk (69 kcal 100 g^{-1} milk); nucleotide values are given as disodium salts. 5′-IMP, which was not used for nucleotide fortification of infant formula in the Pickering study (compare c), was also not recommended (compare d) because of its small contribution to the total potentially available nucleosides (compare d).

a. According to the European Commission Directive 2006/141/EC (22 December 2006), the following nucleotides (mg 100^{-1} kcal) may be added: 5′-AMP (1.5), 5′-CMP (2.5), 5′-GMP (0.5), 5′-IMP (1.0), and 5′-UMP (1.75); the total concentration of nucleotides must not exceed 5 mg 100^{-1} kcal.

b. Values adapted from Gil A and Uauy R (1995) Nucleotides and related compounds in human and bovine milk. In: Jensen RG (ed.) *Handbook of Milk Composition*, pp. 436–464. San Diego, CA: Academic Press; the conversion to mg l^{-1} was calculated assuming that the infant formula is isocaloric with human milk (69 kcal 100 g^{-1} milk).

c. Supplementation according to Pickering LK, Granoff DM, Erickson JR, *et al*. (1998) Modulation of the immune system by human milk and infant formula containing nucleotides. *Pediatrics* 101: 242–249.

d. The recommendation by Schlimme E and Martin D (1999) Nucleotid-Supplementierung von Säuglingsnahrung. *Kieler Milchwirtschaftliche Forschungsberichte* 51: 215–224 based on TPAN contents in human milk published by Leach JL, Baxter JH, Molitor BE, Ramstack MB, and Masor ML (1995) Total potentially available nucleosides of human milk by stage of lactation. *American Journal of Clinical Nutrition* 61: 1224–1230.

long-time pasteurization. The formation of m6ado is consistent with a Dimroth rearrangement from m1ado and follows first-order kinetics in the temperature range of 115–150 °C with holding times of 2–128 s. On stronger heating, however, the product decomposes so that depletion of m6ado competes with its formation, thus interfering with the study of its kinetics. However, the kinetics can still be readily analyzed under the conditions of temperature and holding times corresponding to milk sterilization values (F_0) ranging from 0.4 to 22 min. The kinetic parameters are well characterized in the temperature range of 110–150 °C (**Table 5**).

The z and Q_{10} values of 26.8 °C and 2.4, respectively, are similar to those determined for other thermally induced chemical reactions in milk. This confirms the suitability of the rearrangement of m1ado to m6ado as a chemical process parameter in the upper range of high-temperature pasteurization, the whole range of UHT heating, and in the range of sterilization normally applied in dairy technology.

Chemical Parameters for Identification of Butter Type

The German Butter Regulation in combination with the EU Regulation for Spread Standards defines three butter types: the two traditional products ‘cultured (ripened) cream’ butter and the ‘sweet cream’ butter and the novel ‘mildly soured’ butter. A declaration and quality control of the grading category of the butter types – defined for the first time in the German Butter Regulation of 16 December 1988 – is possible with a linear combination of at least three compositional parameters of the aqueous butter phase, namely, citric acid, lactic acid (sum of L- and D-isomers), and specific nucleosides (ado and urd). To aid the computer-assisted validation of analytical data,

Table 5 Thermal coefficients of the Dimroth rearrangement

Heating temperature *δ [°C]*	*D-values* *[s]*	*z-value* *[°C]*	*Q_{10}-value*
110	6069	26.8	2.4
114	3807		
115	3170		
119	2451		
135	608		
140	294		
140	318		
142	341		
150	247		

The *D*-value (decimal reduction value) is defined as the time necessary at a given temperature to reduce the reactant concentration to 90% of the initial value. The *D*-value is calculated according to $D = 2.303/k$ (s), where *k* is the reaction rate constant.
The *z*-value is defined as the increase in temperature in degree Celsius needed to increase the *D*-value 10-fold.
The Q_{10}-value is defined as the change in the reaction rate when the temperature is raised by 10 °C; Q_{10} is calculated according to $Q_{10} = 10^{10/z}$.
Adapted from Schlimme E, Ott FG, and Kiesner C (1994) Reaction kinetics of heat induced formation of N6-methyladenosine in milk. *International Dairy Journal* 4: 617–627; Martin D, Kiesner C, and Schlimme E (1997) Ribonucleoside: *Chemical parameters for controlling the heat treatment of milk.*

artificial neural networks have been successfully applied. The results of compositional analyses of butter samples were used as inputs for a three-layer, feed-forward, back-propagation network. A two data input network of the pH value and the citric acid content was satisfactory for the chemometric identification of most samples of the three butter types. However, a few butter samples were with respect to citric acid on the borderline between traditionally cultured and alternatively acidified (mildly soured) butter. The chemometric characterization of such samples requires an additional compositional parameter, for example, the urd content of the aqueous butter phase, thus creating a three parameter input network. This application of a neural network is the basis of the German standard DIN 10474 for the determination of butter types.

High-Pressure-Induced Changes

High-pressure treatment is an interesting technological process for preservation of food products. High-pressure treatment of raw milk induces the release of ribonucleosides, for example, for urd 11.50 ± 0.09 μmol l^{-1} at normal pressure and 50 °C and 15.12 ± 0.01 μmol l^{-1} at 500 MPa and 50 °C. In preheated milk, such as UHT milk, the changes are considerably smaller. The changes seem to be enzyme-controlled. High-pressure treatment (500 MPa at 50 °C) of raw milk causes a slight decrease in alkaline phosphatase activity, whereas adenosine deaminase is partially inactivated. The deaminase activity decreases from 673 ± 17 mU l^{-1} when heated to 50 °C at normal pressure to 318 ± 8 mU l^{-1} when pressure treated. High-pressure treatment of reduced-fat milk also induces a loss of enzyme activity. This activity reduction of approximately 53% is independent of the fat content. Due to the fact that adenosine deaminase converts ado to ino, the lower ino contents detected in samples treated by high pressure may be explained by a process-related reduction of adenosine deaminase activity.

Concluding Remarks

Nucleosides are *N*-glycosides of pyrimidines and purines; nucleotides are *o*-phosphoric esters thereof. Both groups of compounds belong to the bioactive substances in milk and colostrum. The concentration pattern is specific for the milk of a given species. The physiological capacity of these compounds in milk is given by the TPAN, that is, all potential sources available in milk for the generation of these compounds by digestion and metabolism. Especially in the early stages of life, the biofunctional properties of dietary nucleosides and nucleotides seem to be important in regulatory processes beyond basic nutritive functions. For example, they enhance antibody responses, contribute to iron absorption from the intestine, and are involved in lipid metabolism. Modified nucleosides, in particular, inhibit cell proliferation and activate apoptosis in human cell model systems. Owing to their biofunctional properties, nucleotides have been used as supplements in infant and follow-on formulae in Japan, in some member states of the EU as well as in some developing countries. As no harmful effects are known, the EC permits the supplementation of infant and follow-on formulae with nucleotides according to specified concentrations.

The relatively constant concentration pattern of nucleosides throughout lactation changes markedly on milk processing. Thus, these compounds, in particular the modified forms, are useful chemical indicators in dairy technology.

See also: **Dairy Education**: Dairy Technology. **Dairy Farm Management Systems**: Goats; Sheep. **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture. **Milk**: Human Milk.

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NUTRIENTS, DIGESTION AND ABSORPTION

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Fermentation in the Rumen

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Introduction

Nutrients consumed by weaned dairy cattle are subject to fermentation by microbes in their rumen or, more properly, their reticulorumen. This pregastric fermentation also affects the dynamics of digestion and absorption in subsequent segments of their digestive tract and, ultimately, the amount and pattern of nutrients available for metabolism.

The rumen microbial ecosystem in cattle fed typical diets is both open and complex, composed mainly of strictly anaerobic bacteria, archaea (single-celled microorganisms that have an evolutionary history and biochemistry that differ from bacteria), protozoa, and fungi. Symbiotic and other interactions of these microbes with each other and with the host animal are also nutritionally relevant. Anaerobic fermentation partially metabolizes nutrients consumed by the host, providing energy and nutrients needed by the microbes. Some end products of microbial metabolism (e.g., volatile fatty acids (VFAs) or short-chain fatty acids, primarily acetate, propionate, and butyrate, but not CO_2 and CH_4) and the microorganisms themselves then provide nutrients for the host. This relationship allows the host to capitalize on the ability of some ruminal microbes to digest plant cell wall carbohydrates (cellulose, hemicellulose, and pectin) otherwise unavailable to mammals. Additional benefits of pregastric fermentation are that the host animal can digest microbial protein passing from the reticulorumen, obtain vitamins synthesized by the microbes, and reduce the effects of some antinutrients (e.g., phytate and gossypol). The interaction of rumination with fermentation is also nutritionally important: chewing during rumination accelerates microbial colonization and fermentation, and as a result digesta particles become more fragile and susceptible to comminution during rumination.

Direct microscopic counts of bacteria in the rumen are 3- to 10-fold those of viable (i.e., culturable) bacteria. Recent data based on studies of rRNA confirm that many more species of bacteria are present in the rumen than have been cultured or identified to date. Some uncultivated organisms are abundant; however, isolated bacteria and archaea are able to perform most of the major transformations known to occur in the reticulorumen and provide a model of the ecosystem. At 1–5 μm in size, and assuming an individual cell volume of 1 μm^3 (or 10^{-9} μl), ruminal bacteria account for about 1% of fluid volume when present at $10^{10}\,ml^{-1}$ or 10% of fluid volume if counts reach $10^{11}\,ml^{-1}$. The larger ruminal protozoa vary in size from 20 to 200 μm and are normally present at concentrations of 10^4–$10^5\,ml^{-1}$; therefore, they can account for approximately half of the total microbial cell contents in the rumen. Counts of protozoa and bacteria in the rumen tend to be inversely related because the former prey on the latter. Anaerobic fungi are also part of this microbial ecosystem. Although they are difficult to quantify because of their complex life cycle, they have been estimated to contribute about 6% of total biomass.

A lactating, mature, 680-kg Holstein cow consuming a sample diet will provide the framework for this discussion of fermentation in the reticulorumen. It is assumed that the cow produced 45 kg of milk per day with 3.5% fat and 3.0% true protein; lived in a thermoneutral environment; and ate (on a dry matter basis) 7.08 kg of steam-flaked maize, 6.16 kg of immature legume hay, 5.61 kg of maize silage, 2.53 kg of whole cottonseed (with lint), 2.26 kg of sorghum-sudan silage, 1.41 kg of solvent-extracted soybean meal (48% crude protein), 0.98 kg of midmaturity grass (C3) hay, 0.87 kg of coastal Bermudagrass hay, 0.51 kg of a vitamin and mineral premix, 0.23 kg of ring-dried blood meal, 0.12 kg of sodium chloride, 0.09 kg of calcium carbonate, and 0.04 kg of monosodium phosphate (monohydrate) per day – a total of 27.9 kg of feed dry matter, 4.1% of body mass. The ration, as consumed, also included 18.9 kg of water and the cow drank an additional 115.6 kg of water per day; therefore, total water intake was 134.5 kg per day and total nutrient consumption was 162.4 kg per day. Ignoring contributions by saliva and scurf (sloughed epithelial cells from the digestive tract), the profile of nutrients entering the reticulorumen was then estimated using tabulated feed composition data (**Figure 1**). Nonnutritive lignin was accounted for; it and similar but unquantified compounds (e.g., tannins and acid-insoluble ash) are discussed separately. Once swallowed and upon entering the reticulorumen, nutrients become potential substrates for microbes to utilize; their various fates are now considered in turn.

Water

Considerably more water than dry matter is consumed by the lactating cow, 4.8 times as much in our example. Even more would have been consumed during periods of high environmental temperature. Although often an overlooked nutrient, water is clearly an extremely important one and consumption of such large amounts also impacts fermentation in the reticulorumen. Water is required for fermentation itself because the enzymatically catalyzed chemical reactions by which complex molecules in carbohydrates, protein, and fat are converted into assimilable forms, that is, hydrolysis, involve water.

A portion of imbibed water, perhaps 5–18%, bypasses the reticulorumen; however, the rest equilibrates with the 60–80 l of fluid normally present. Water in the

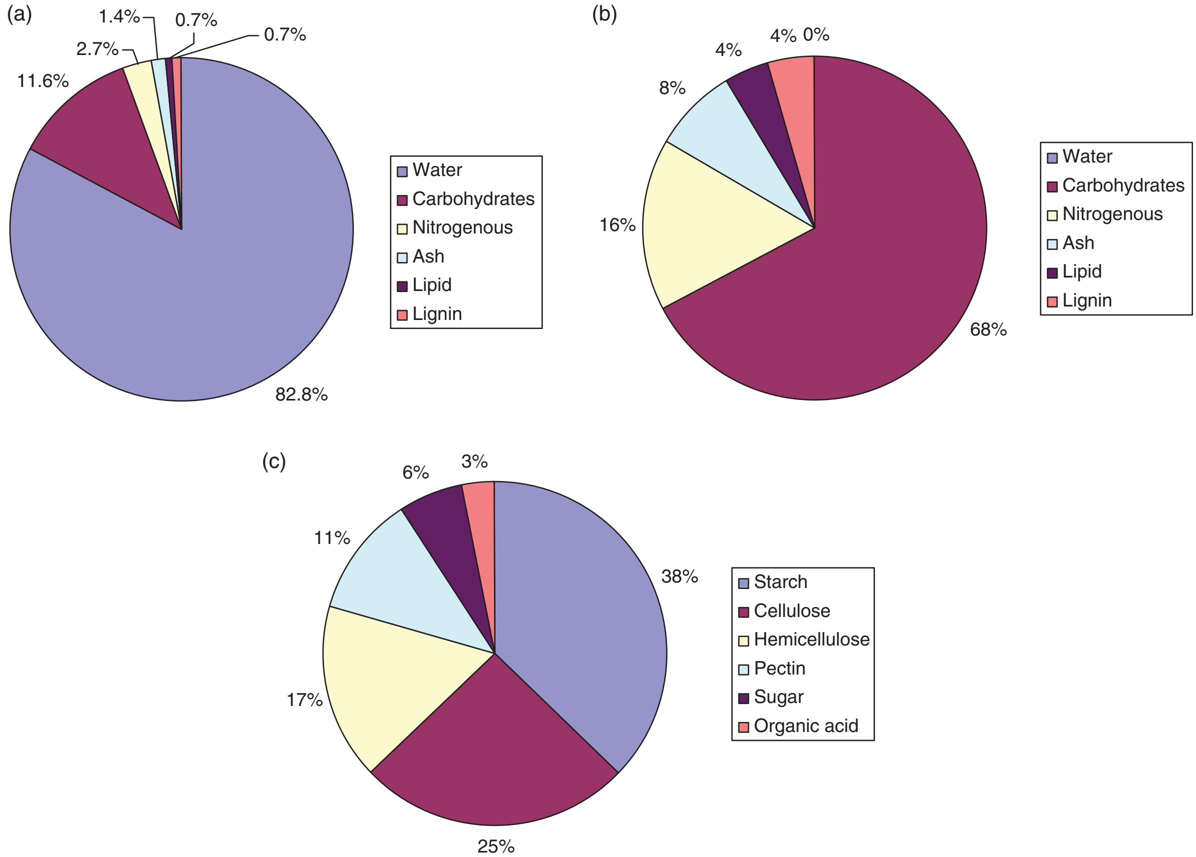

Figure 1 The profile of nutrients entering the reticulorumen: (a) nutrient consumption; (b) dry matter consumption; and (c) carbohydrate consumption.

reticulorumen (either drunk, from saliva, or in feed) also exchanges rapidly with body water; the half-life of water molecules in the reticulorumen is about 1 h. The fractional dilution rate in the reticulorumen is typically 10–17% h^{-1}; in our example, the value is likely toward the high end of this range. Unless a microbial species is continually reinoculated, it must multiply at least as quickly as the fractional dilution rate, attach to digesta particles having a slower fractional passage rate, or perhaps (if motile) sequester in a location having a slower turnover rate to avoid being removed from the reticulorumen. Microbes growing more quickly also grow more efficiently because a smaller proportion of their energy is utilized for maintenance.

Carbohydrates

The sample ration provided 18.8 kg day^{-1} of carbohydrates. The carbohydrate fraction of feeds is a complex mixture of monomers and polymers making up about 68% of dietary dry matter (**Figure 1**(**b**)). Carbohydrates can be fractionated, based on various analytic procedures related to chemical composition and rumen fermentability, into starches, cellulose, hemicellulose, pectins, sugars, and organic acids. Although various ruminal microbes are adapted to utilize all carbohydrate fractions, the dynamics of fermentation vary considerably. There are also known interactions between carbohydrate fractions; for example, too much or too rapid a fermentation of starch can cause ruminal acidosis that inhibits fiber (cellulose, hemicellulose, and pectin) digestion.

The overall scheme of carbohydrate fermentation by ruminal microbes is similar regardless of source (**Figure 2**). If not already in hexose form, carbohydrates are hydrolyzed and converted to hexose monomers. These are metabolized to pyruvate via Embden–Meyerhof glycolysis in bacteria and the Entner–Doudoroff pathway in archaea. Subsequent metabolism of pyruvate leads to the production of VFAs, CO_2, and CH_4. Methane is produced from CO_2 and hydrogen by the archaea (e.g., *Methanobrevibacter ruminantium*). Some methanogens are found attached to protozoa (exosymbionts) where they derive hydrogen directly from specialized protozoal organs (hydrogenosomes). The CH_4 cannot be utilized by the cow and represents a loss of 5–7% of the gross energy in her ration. That said, production of CH_4 does dispose of hydrogen and allow anaerobic fermentation to proceed. Although CH_4 production by a mature lactating cow consuming the sample ration would be nearly maximal in terms of liters per day, CH_4 production per kilogram of milk yield would be approaching its minimum; that is, the emission of 'anthropogenic' CH_4 per unit of human food produced is lower than it would be in cows producing less milk.

Starches

Fifty to ninety percent of the 7.0 kg day^{-1} of starch entering the rumen of the cow would be expected to be fermented in her reticulorumen, the large range indicating potential effects of many factors affecting starch degradation. The rate of starch fermentation varies with its source and processing. Starch in oats or wheat is degraded more quickly in the reticulorumen than starch in maize or sorghum; some of this difference is related to starch solubility and the ease with which proteins surrounding starch granules in the cereal's endosperm are fermented. Starch degradation rate is increased by cracking or grinding, and steam flaking. These processes increase relative surface area and disrupt starch granules. As with other nutrients, feed consumption affects the proportion of consumed starch fermented because it influences the fractional passage rate of digesta from the reticulorumen. This means that, for the cow fed the sample diet and eating 4% of her body mass in dry feed per day, starch digestion in the reticulorumen would likely be closer to 50% than 90% of that consumed.

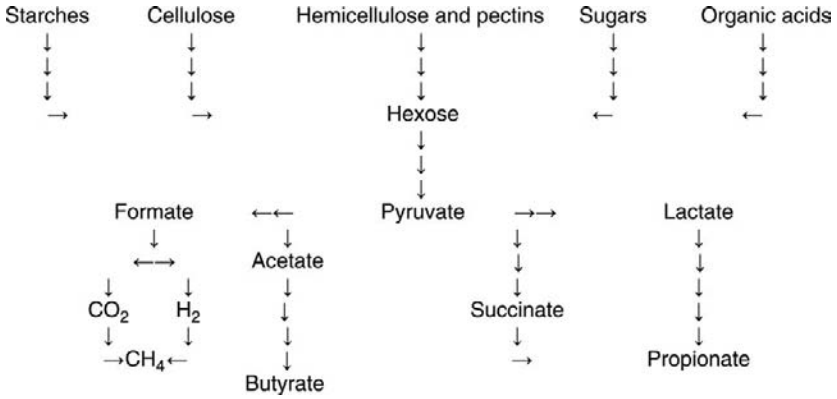

Figure 2 Overview of carbohydrate fermentation in the reticulorumen.

Predominant species of ruminal bacteria utilizing starch include *Butyrivibrio fibrisolvens*, *Ruminobacter amylophilus*, *Prevotella* sp., *Streptococcus bovis*, *Succinomonas amylolytica*, and some strains of *Selenomonas ruminantium.*

Cellulose

The sample ration provided 4.8 kg day^{-1} of cellulose to the reticulorumen and 30–60% of it would be hydrolyzed in the reticulorumen, albeit more slowly than starch. Among predominant ruminal bacteria able to use cellulose are *Fibrobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens*, and some strains of *B. fibrisolvens*.

Hemicellulose and Pectins

As with cellulose, 30–60% of the 3.2 kg day^{-1} of hemicellulose and 2.1 kg day^{-1} of pectins would be hydrolyzed in the reticulorumen. *Butyrivibrio fibrisolvens* and *Prevotella* sp. are predominant organisms using hemicellulose or pectins. *Lachnospira multiparus* is another predominant pectin fermenter.

Sugars

Fermentation of the 1.2 kg day^{-1} of sugars in the sample ration would be rapid; many ruminal microbes can utilize them. In cattle fed low-forage, high-grain diets, the half-lives of both sugars and starch in the reticulorumen are about 4 h. Predominant species of ruminal bacteria utilizing sugars include *B. fibrisolvens*, *S. ruminantium*, *S. bovis*, *Eubacterium ruminantium*, and *L. multiparus*.

Organic Acids

The 0.6 kg day^{-1} of organic acids provided by the sample ration, probably mostly citrate and malate, would be rapidly fermented in the reticulorumen. *Wolinella succinogenes* is a species that can use malate.

Nitrogenous Compounds

About 70% of the 4.5 kg day^{-1} of dietary protein and nonprotein N entering the reticulorumen of the cow fed the sample ration would be fermented there, leading to the production of microbial protein and ammonia. Considerable recycling of N can occur within the reticulorumen: between bacteria and protozoa, between one bacterium and another, and between the cow and the reticulorumen. Depending on the circumstances, these processes may be advantageous or disadvantageous to the nutrition of the cow. The advantages include microbial conversion of nonprotein N into protein that can then be digested to provide amino acids to the cow, conversion of poor-quality dietary proteins into higher-quality microbial protein, and utilization of salivary urea to provide protein to the cow when N intakes are low. The processes can be disadvantageous when degradation of dietary protein exceeds synthesis of microbial protein or when unprotected proteins of higher quality than microbial protein are fed. Defaunation (i.e., removal of protozoa from the reticulorumen) reduces N recycling; however, this has a potential downside. By ingesting starch granules, some protozoa (entodinomorphs) buffer its rate of fermentation and reduce the potential for lactic acidosis. A similar benefit can be provided by those protozoa (holotrichs) that compete directly with bacteria for soluble sugars.

Predominant rumen bacterial species utilizing protein, peptides, or amino acids are *Clostridium aminophilum*, *C. sticklandii*, *Megasphaera elsdenii*, *Peptostreptococcus anaerobius*, and *Prevotella* sp. *Clostridium aminophilum*, *C. sticklandii*, and *P. anaerobius* are all obligate amino acid fermenting bacteria; they cannot utilize carbohydrates for energy but deaminate amino acids much faster than other ruminal bacteria. Ammonia can be used by a majority of rumen bacteria for biosynthesis of amino acids and protein.

Minerals

The 2.2 kg day^{-1} of minerals in the sample ration can affect fermentation in the reticulorumen. Fermentation can also alter both the dynamics of mineral metabolism in the cow and the ultimate availability of some of these elements to the host.

Minerals soluble at the pH of the reticulorumen, normally 5.5–7.0, increase the osmolality of fluid in this compartment of the digestive tract and this can enhance the fractional dilution rate. Many of these elements (e.g., Cl, I, K, Mg, Na, S (in sulfide form), and Zn) can be absorbed from the reticulorumen. Carbonates also help buffer rumen pH and reduce the adverse effects of low pH (rapid fermentation) on fiber-degrading microbes. Provided that the ration contains adequate Co, fermentation in the reticulorumen supplies enough vitamin B_{12} (cobalamin) to meet requirements of the cow.

A three-way interaction of Cu, Mo, and S affects the availability of Cu in ruminants. Briefly, fermentation of Mo and S in the reticulorumen produces thiomolybdates. Copper reacts with thiomolybdates in the rumen to form highly insoluble complexes that are poorly absorbed. Thiomolybdates can also be absorbed by the cow, reducing the availability of systemic Cu.

Lipids

Most of the 1.2 kg day^{-1} of lipids ingested in the sample ration would undergo some form of transformation by fermentation in the reticulorumen. For example, dietary triacylglycerols (fats and oils, like those in cottonseed) would be normally hydrolyzed completely to free fatty acids and glycerol. The latter would be rapidly fermented to produce propionate, whereas 70–90% of the unsaturated fatty acids released in this process would be extensively biohydrogenated. A predominant bacterial species that hydrolyzes dietary triacylglycerols to free fatty acids and glycerol with little accumulation of mono- or diacylglycerols is *Anaerovibrio lipolytica.*

Fatty acids can interfere with fermentation in the reticulorumen both indirectly and directly. Their association with digesta particles may reduce microbial access to substrate. They can also directly inhibit microbial growth and metabolism.

Lignins

Although a portion of the 1.2 kg day^{-1} of lignin phenolics in the sample ration would be digested, absorbed, metabolized, and excreted in urine as aromatic acids, most would pass, unaltered, through the reticulorumen. Any delignification of plant cell walls would increase the availability of cellulose and hemicellulose in the reticulorumen.

See also: **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins; Fiber Digestion in Pasture-Based Cows; Small Intestine of Lactating Ruminants.

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Fiber Digestion in Pasture-Based Cows

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Introduction

When all ruminants are considered, pasture is the major feed consumed; however, for most dairy cows, grazed pasture is, in general, a less important feed than conserved forage and grain. Pasture-based dairying is becoming increasingly popular in industrialized nations because of the advantages it can offer in reduced input costs and perceived improvements in animal health and welfare.

There is, correspondingly, a large body of literature on the nutrition of pasture-fed cows. The traditional research emphasis, however, has been on remedying energy and protein deficiencies encountered relative to total mixed rations (TMRs).

In recent decades, there have been significant advances in both genetic improvement of pasture species and pasture management strategies, resulting in greater energy density and higher dry matter (DM) yield, particularly in temperate species such as perennial ryegrass (*Lolium perenne* L.). During the same period, there has been a steady increase in the knowledge of rumen function within TMR-feeding systems, and this has begun to reveal an important role for supplementary fiber; fiber supplementation has been demonstrated to improve rumen function and animal production when included in the ration above previously defined thresholds. These two distinct lines of pasture and rumen research have been drawn together in recent years, with attempts to extend to pasture systems the role demonstrated for fiber in TMR.

However, there are several reasons why the existing research on dietary fiber and rumen function in TMR systems should be extrapolated to grazing ruminants cautiously. A brief review of the history and development of our current understanding of fiber in ruminant diets assists in this explanation.

Fiber Assessment in Ruminant Nutrition

Fiber is considered to be that fraction of the diet contained in plant cell wall material and is not digested by endogenous mammalian enzymes; in general, it is generally accepted that this fraction is also either not digested or relatively slowly digested by the symbiotic microbial communities of the ruminant.

The original Weende assay technique used to assess the indigestible fraction of feeds, which was termed crude fiber (CF), was developed in the nineteenth century and remains a formally recognized method, although it is known to underestimate cell wall contents. To improve the determination of these cell wall contents, an alternative method was developed by Van Soest and colleagues in the United States Department of Agriculture. The method that they developed was known as the neutral detergent fiber (NDF), which includes all the plant material understood to compose 'fiber', broadly, lignin, cellulose, and hemicellulose. They then extended the classification to another fraction, acid detergent fiber (ADF), which represents the least digestible fiber content of lignin and cellulose only.

Role of Fiber in Rumen Function and 'Effective Fiber'

The role of fiber in the rumen environment has been intensively studied since the 1930s. Early research demonstrated that low levels of CF in the ration were associated with reduced rumen motility, including rumination, and lower milk fat content. This research led to further investigation of the mechanisms by which rumen motility and rumination were triggered, and it was demonstrated that reticulo-rumen epithelial contact, weight of rumen contents, rumen distension, and infusion of certain volatile fatty acids (VFAs) all altered rumen movement. In particular, it was demonstrated that firm particles of a critical size ('scabrous' material, or 'roughage') were effective in increasing rumination rates compared with 'softer' forage or concentrate diets.

With the increase in TMR feeding, the need to structure rations with appropriate 'roughage' suitable for producing satisfactory rumen environments directed research efforts toward alternative approaches to characterizing and measuring fiber content. The initial research was empirical, with fiber included from different sources and at different levels in the ration. The parameters measured were the time spent chewing, milk fat concentrations, and the ratio of acetate to propionate in rumen fluid. NDF began to be used as the standard classification of dietary fiber, rather than CF, because of the improved accuracy in determining total fiber content. From this research, the concept of 'effective' NDF (eNDF) or effective fiber was developed. This fraction is commonly defined as "NDF of a source and preparatory treatment that induces adequate chewing and therefore milk fat concentration", but it was limited in its usefulness because it was difficult to categorize feeds on their effect on animal production.

A method of classifying the particle size distribution of NDF in rations was developed to enhance the animal scientist's ability to define the appropriate effectiveness of ration fiber. This measure was termed 'physically effective' NDF (peNDF) and involved sieving feeds to determine particle size and comparing this against cow responses. This peNDF concept has been reported to satisfactorily predict cow responses to the feed.

Limitations of 'Effective Fiber' in Pasture

With the demonstration that the peNDF approach could satisfactorily assess the physically effective fiber content of TMR, there was considerable interest in application to pasture diets. However, several immediate difficulties arose. Determining the peNDF by the proportion of the diet that remains on a given sieve size is unsuitable for pregrazed pasture as it is generally presented to the ruminant intact, and is, therefore, of relatively large 'particle size'. For similar reasons, there is no reliable measure of the particle size distribution of pasture even immediately after mastication. Given that the physical separation of the ration was a key concept of peNDF quantification, to use the method for grazing cows it was necessary to ascribe to pastures an eNDF value by comparison with feeds for which peNDF could be measured. This could be done subjectively, on the basis of shared characteristics (e.g., suitably prepared silages) or on the basis of rumen pH and rumen passage rates of the feed. As an example of the latter approach, a meta-analysis study estimated the eNDF of a particular high-quality pasture to be in the range of 29–78%, with a mean of 43%.

This necessity for some extrapolative comparison with other feeds demonstrates that the critical difficulty for peNDF estimation of pasture is the unsuitability of particle size assessment in fresh uncut forages. A practical example of these difficulties is the poor prediction of ruminal pH of pasture-fed cows using models that more satisfactorily predict ruminal pH of cows fed a TMR, when energy density and other fermentation factors are broadly standardized. If the physical characteristics of pasture fiber cannot be determined accurately, it is evident that any further attempt to use them to guide ration development of pasture-fed cows needs to be approached cautiously.

In addition, it is important to note that the measurements used to determine the fiber content in pasture do not explain the corresponding rumen environment as satisfactorily as that of TMR-fed cows. This is because the current understanding of the actual mechanisms by which dietary fiber induces rumen motility and chewing remains incomplete. Physically effective fiber (peNDF) is considered to exert a positive effect in the rumen by altering chewing behavior (eating and rumination) and maintaining a physical separation between the rumen 'mat' and rumen fluid. However, both chewing times and the stratification of rumen contents in pasture-fed cows are very different to TMR-fed cows. In cows consuming a high proportion of pasture, rumen contents can be greater than 20% of live weight, and the rumen digesta effectively forms a single, relatively homogeneous mass with markedly reduced physical stratification or differentiation compared to that of cows fed TMRs, and greater rumen fill. In this environment, in contrast to rumens of TMR-fed cows, there is little effective separation of rumen contents.

The effect that differences in rumen content stratification have on rumination rates is not well understood; it is possible, however, that the reasons why high-peNDF feeds such as long chop straw are not as dominant an influence in inducing rumination in pasture-fed cows as in TMR-fed cows could be due to this basic difference in the rumen environment of grazing animals. Given the other rumen motility 'triggers' that have been identified (e.g., weight of rumen contents, rumen distension, VFA concentration), it may be that rumen epithelial contact with peNDF is not as critical a 'driver' of rumination in cows consuming a high proportion of pasture.

In addition, rumination may be a behavior as well as a physiological reflex, and little is understood about how rumination rates are affected by intake, or time available for grazing or loafing in pasture-fed cows. This is important because, in general, grazing animals have greater harvesting costs associated with a given energy intake; extended grazing periods may well reduce rumination time, not because of reduced rumen stimuli, but because free time that may have been spent resting and ruminating might be allocated to grazing. In contrast, if rumination is a behavior that is selected by the cow independent of direct rumen stimuli (e.g., in times of rest), there may be no advantage to increased rumination rates, above a physiologically necessary level, in improving the rumen environment or animal production. As the research to determine minimum chewing thresholds has been done with TMR-fed cows rather than pasture-fed cows, it is not possible to define what level of chewing activity is associated with adequate rumen function in pasture-based cows. Because of this, ascribing higher effective fiber content to certain pastures on the basis of higher rumination rates is, at present, outside the boundaries of the available research.

Research on the Effects of Supplementary Fiber in Pasture Grazing Cows

To date, the studies investigating the responses of pasture-based cows to supplementary fiber support the use of caution when directly extending the principles of effective fiber concepts developed under TMR feeding to pasture-based systems. The supplementation of cows grazing temperate pastures and legumes with additional fiber, in the form of cereal or grass straws, has not been demonstrated to alter the rumen environment in the manner an increase in peNDF has been demonstrated with TMR-fed cows.

A series of experiments in Australia, New Zealand, and Europe have examined the effect of supplementary straw fed to cows grazing ryegrass pastures with and without additional grain supplements. The Australian work used cows grazing good-quality ryegrass and legume pastures in different seasons and at different grain supplement levels to measure the changes in dry matter intake (DMI), pasture intake, milk yields, and various rumen function parameters, with the addition of supplementary straw up to 3 kg DM a day. No significant improvements in rumen pH, acetate-to-were propionate ratio, milk yield, or milk composition were demonstrated by the addition of supplementary straw. However, significant pasture substitution and reduced milk yields with increasing straw content were reported. Similarly, New Zealand work using a split herd trial design on ryegrass pastures reported that the provision of supplementary straw at 2 kg per cow daily was associated with a significant reduction in milk yield in that treatment group in spring.

Recent work at Lincoln University in New Zealand and by European researchers with real-time *in situ* assessment of rumen pH via indwelling sensors has noted that the addition of supplementary straw to high-production cows grazing ryegrass did not increase rumen pH across the diurnal cycle, did not significantly alter rumen VFA ratios, and did not increase rumination rates or milk yield and composition. This is despite the low rumen pH recorded in these grazing cows and, in the Lincoln University work, the relatively low NDF content of the pastures (320–380 $\mathrm{g\,kg^{-1}}$ DM). For example, in the Lincoln University work, the rumen pH recorded across approximately 500 days from cows grazing different pastures and during different seasons and has consistently presented with daily pH nadirs of between 5.0 and 5.5. Such pH nadirs appear to be common in these pasture grazing systems.

Overall, these studies have consistently reported no significant benefit in milk production or composition and no improvement in rumen pH or acetate-to-propionate ratios, while in some reports supplementary straw reduced pasture intake and milk production. Given the reports of measured rumen pH being commonly below 6.0 in pasture grazing cows of similar intakes, the consistent lack of a positive response to supplementary straw reported in these studies is revealing. It suggests that the rumen environment produced with high-quality pastures is less subject to influence by peNDF than the rumens of TMR-fed cows. It also suggests that the current understanding of the role of fiber in the nutrition of high-producing pasture-fed cows is incomplete; perhaps the central concepts of fiber nutrition in TMR-fed cows – the role in increasing chewing, particularly by rumination, through physical contact of the feed with the rumen – need to be reassessed in pasture-fed cows.

Conclusion

The role of fiber in the nutrition of pasture-fed cows is identifiably different from that of cows fed TMR, and attempts to directly extrapolate the existing research from the latter system to pasture-based cows have demonstrated the need for caution in doing so. The research undertaken to assess the response of pasture-based cows to supplementary straw has not demonstrated any significant advantage, and in some instances has reported notable disadvantages in milk production and intake. There is a need for further research specifically targeting the differences in the dominant mechanisms by which rumen motility and function are influenced in pasture grazing cows compared with TMR-fed cows.

See also: **Dairy Farm Management Systems**: Seasonal, Pasture-Based, Dairy Cow Breeds. **Diseases of Dairy Animals**: Non-Infectious Diseases: Acidosis/Laminitis. **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems; Lactation Rations in Cows on Grazing Systems; Systems Describing Nutritional Requirements of Dairy Cows. **Nutrients, Digestion and Absorption**: Fermentation in the Rumen.

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Small Intestine of Lactating Ruminants

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Introduction

The small intestine is the primary site of absorption of most major nutrients except the volatile fatty acids (VFAs) in ruminants. Long–chain fatty acids, amino acids, glucose, and most vitamins are absorbed only from the small intestine, while most minerals are absorbed there primarily. Because all these nutrients are vital for milk synthesis, with glucose and amino acids being potentially limiting nutrients, the small intestine plays a particularly important role for the lactating ruminant. Thus, quantification of the extent of digestion and amounts of nutrients absorbed from the small intestine is central to attempts to determine metabolic limits to milk production.

The principal functional difference between the small intestine of ruminants and that of nonruminants is that the substrates the former has to digest consist of those parts of the feed that have escaped digestion in the stomach together with the products of rumen microbial fermentation. Because lactating ruminants, particularly dairy cattle, have high rates of feed intake and thus passage of digesta, there is less time for digestion to occur in the stomach; therefore, more amounts of potentially fermentable feed enter the small intestine. Essentially all soluble carbohydrates and variable proportions of starch and fiber are removed by the time the digesta enter the duodenum. The amounts of protein and lipids entering the duodenum from the abomasum do not differ greatly from the amounts consumed, but they will have been extensively modified by microbial action.

Anatomy and Physiology

The small intestine constitutes about 80–85% of the total length of the intestines and is found to be 42–52 m long in lactating Friesian cows, while 26 m is quoted for sheep and goats. The small intestine of ruminants is anatomically and functionally very similar to that of nonruminant mammals. The duodenum is only about 1 m long in the cow and 60 cm in the sheep and goat. The duodenum is less convoluted than the jejunum (**Figures 1** and **2**(**a**)) and includes a proximal, ascending portion, which lies adjacent to the pancreas and liver, and a distal, descending portion, which leads to the duodenojejunal flexure (**Figures 2**(**a**) and **2**(**b**)). The jejunum and ileum constitute the remainder of the small intestine and cannot be easily distinguished from one another, although anatomically the ileum is the small intestine attached to the ileocecal fold (**Figure 2**(**a**)). The principal glands serving the small intestine are the pancreas and gallbladder, both of which secrete into the duodenum, and a series of glands in the gut wall. The principal enzymes secreted by the pancreas are amylase, trypsin, chymotrypsin, and lipase. Enzymes secreted by glands of the intestinal wall generally digest the incomplete breakdown products of pancreatic enzyme digestion into simpler monomers that can be absorbed.

Digesta enter the duodenum from the abomasum at an acidic pH of about 2.0–3.0, but this acidity is rapidly neutralized by the pancreatic and bile secretions. However, passage is also fast in the first part of the small intestine so that pH may not reach neutral values until the mid–jejunum. The digesta are very liquid, containing about 40 g DM kg^{-1}, though this varies from 20 to 60 g kg^{-1} between diets and between individual animals. Thus, the digesta flow through the proximal duodenum of a high–producing dairy cow may amount to 400 kg day^{-1}. During passage through the small intestine, a net uptake of fluids occurs so that at the terminal ileum the DM content will have risen to about 60–80 g kg^{-1}.

The flow of digesta into the duodenum varies over the day, though less so than in nonruminants due to the buffering effect of the rumen. In sheep it has been shown to increase shortly after a meal and then decline over a period of a few hours before gradually increasing, with the result that the flow to the duodenum in ruminants fed twice daily may be greatest overnight. There is very little information on digesta passage times through the small intestine of lactating ruminants. Passage is most rapid in the cranial part of the small intestine, and in sheep opaque markers have been shown to take only 20–40 min to pass from the duodenum to the proximal ileum but a further 2 h to reach the terminal ileum.

The intestines are supplied with blood via the celiac artery to the proximal duodenum and the cranial mesenteric artery to the rest of the small intestine and part of the colon. There is also a lymphatic system. In contrast to sheep and goats, the cranial mesenteric artery and vein of the small intestine of the cow include a collateral branch (**Figures 2**(**a**) and **2**(**b**)), which ensures adequate blood supply to the distal jejunum if contraction and convolution impede flow in the main branch. The portal–drained

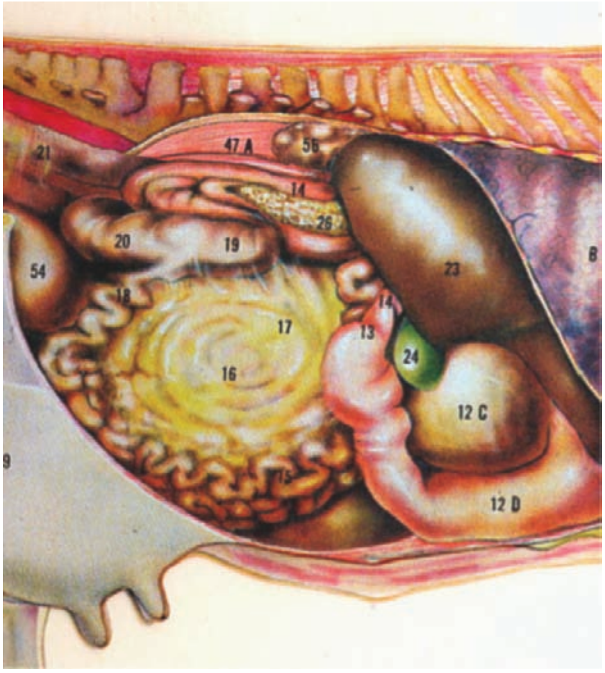

Figure 1 Position of the small intestine within the abdominal cavity of the dairy cow. Note the position of the abomasum (12D), pylorus (13), duodenum (14), jejunum (15), ileum (18), cecum (20), and colon (19). Also shown are the liver (23), gallbladder (24), and pancreas (26). Reproduced from a 'Transart' anatomy booklet published by Cyanamid of Great Britain, London, UK (1966).

viscera (PDV; entire gastrointestinal tract, pancreas, spleen, and mesenteric and omental fat) account for up to 25% of the total energy requirements of the lactating ruminant in part due to the high rate of protein turnover. Within the PDV, the tissues drained by the cranial mesenteric vein of cattle (largely the intestines and cecum) account for roughly half of the oxidative metabolism. Thus, the blood supply provides nutrients to maintain this activity as well as to remove nutrients absorbed from the digestive tract.

Various methods have been used in attempts to quantify digestion in the small intestine. Apparent digestion is the net difference between the amount of a nutrient entering the duodenum from the abomasum and the amount leaving the terminal ileum. In contrast, true digestion takes account of amounts secreted into the gut from glands, passing across the gut wall from the blood, or as cells being sloughed off the gut wall. Measurements of both true and apparent digestion depend on the use of cannulas inserted permanently into the proximal duodenum and terminal ileum.

Cannulation of the proximal duodenum has been a common research tool for studying digestion in all classes of ruminants and provides the basis for dividing the digestive tract into the stomach and intestines (usually, but inaccurately, referred to as the ruminal and postruminal tract). However, estimates of digestion in the small intestine additionally require cannulation of the terminal ileum to distinguish digestion in the small intestine from further fermentation in the cecum and large intestine. This presents greater surgical problems, and considerably fewer reports of the application of this technique in lactating ruminants have been published.

An alternative approach to quantifying digestion in the small intestine is to measure net uptake of glucose or amino acids into venous blood. Appreciable amounts of some amino acids and glucose are utilized by the PDV, so there is a significant difference between the amounts of these nutrients absorbed from the lumen of the gut and their net appearance in the portal vein. Glucose and

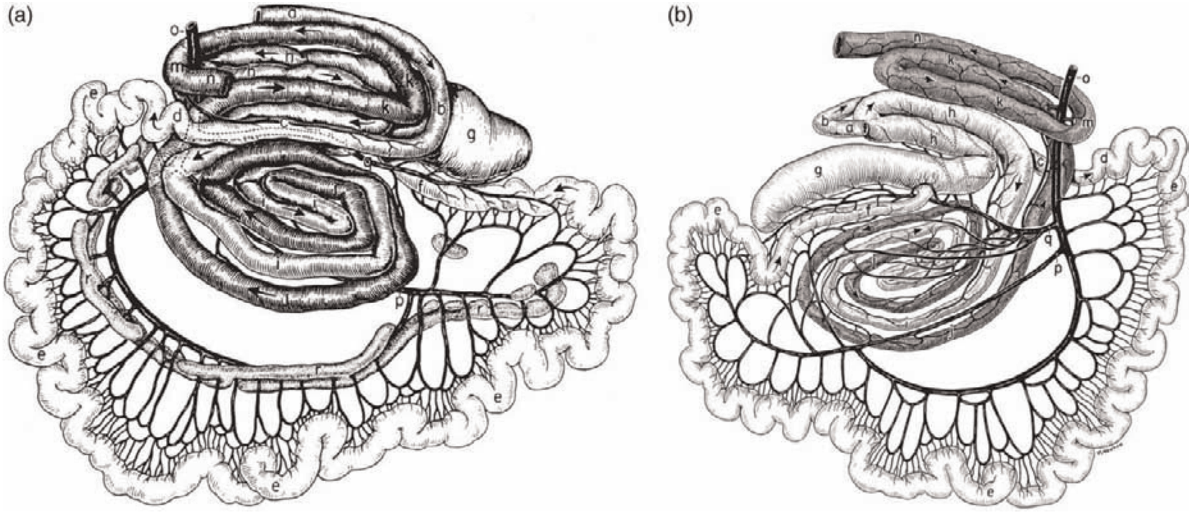

Figure 2 The intestine of the dairy cow: (a) left–side and (b) right–side view. Landmarks shown include the descending duodenum (a), duodenojejunal flexure (d), jejunum (e), ileum (f), cecum (g), cranial mesenteric artery (o) and collateral branch (p), and jejunal lymph nodes (r). From Habel RE (1992) *Guide to the Dissection of Domestic Ruminants*, pp. 56, 57. Ithaca, NY: R. E. Habel.

amino acids are used by the PDV both during their initial absorption from the gut, before reaching the mesenteric veins, and during their absorption from the arterial blood. Thus, the measurement of true rates of absorption can be obtained only by using isotopic labeling techniques. Though surgically difficult, much progress has been made in this area over the past 25 years.

Carbohydrates

The principal carbohydrates entering the duodenum from the abomasum are cellulose, from fiber, and starch. Essentially all the soluble sugars, oligosaccharides, and pectin from the feed are fermented to VFAs in the rumen.

Fiber is variably digested in the rumen, depending on various factors including its source and how it is processed, so only about 50–70% of the amount consumed reaches the duodenum. None of this is digestible by mammalian enzymes, so it is reasonable to assume that none will be digested in the small intestine. Although some fermentation may occur in the distal ileum, which could, in theory, allow limited fiber digestion proximal to the ileo–cecal valve, the amount is likely to be negligible and, for all practical purposes, all measured postruminal digestion of fiber can be assumed to occur in the hindgut.

Starch is by far the most important carbohydrate digested in the small intestine, as it provides the only exogenous source of glucose for the ruminant. The starch entering the duodenum is primarily the fraction of feed starch that has escaped fermentation in the rumen either because it passes through the rumen more rapidly or because it is more slowly fermented. The amount varies widely, being affected primarily by the amount and the chemical and physical form of starch in the diet, but also by the level of total diet intake through its effect on retention time in the rumen. Typically, wheat, barley, and cassava are rapidly fermented, while maize and sorghum are more slowly fermented. Starch supplies to the duodenum of lactating cows can exceed 5 kg day^{-1} when diets based on more slowly fermented starch sources such as ground maize or sorghum are fed, but this can be halved if the grain is flaked (steam–rolled) to make it more digestible. Wheat or barley in the ground or cracked form is rapidly fermented allowing only about 1 kg starch day^{-1} to reach the duodenum, but if it is fed as whole grains as in the case of mature whole–crop wheat, starch supplies to the small intestine can be enhanced to over 2 kg day^{-1} in cows but not in sheep or goats, which are more efficient at chewing small grains. A small proportion of duodenal starch, perhaps 5–10% of the total depending on the microbial population in the rumen, may be of protozoal origin.

Many estimates of postruminal digestion of starch (duodenum to feces) in lactating dairy cows have been published. Values for total postruminal digestion of maize starch average about 82% with little evidence of any change with increasing starch flow into the duodenum (**Figure 3(a)**). Of the various sources of starch, only sorghum is clearly less digestible than the average. A much greater difficulty is quantifying the contribution of the small intestine to this postruminal digestion. This is a key question as digestion within the small intestine is primarily, though not exclusively, by mammalian enzymes yielding glucose, whereas digestion in the large intestine is by fermentation yielding VFAs. A recent review of the published papers where duodenal–ileal digestion has been measured in lactating dairy cows showed that the small intestine contributed 88% of the total postruminal starch digestion (**Figure 3(b)**). The digestibility of duodenal starch in the small intestine averaged 69%, but with a wide variation associated with differences in starch sources and especially in grain processing. A lower mean value of 53% has been calculated for beef cattle.

The maximum capacity of the small intestine to digest starch has not been defined reliably in either beef or dairy cattle though the highest reported digestion in lactating cows is 3.4 kg day^{-1}. Digestive capacity is determined by the activity not only of pancreatic amylase, which breaks down starch to disaccharides, but also of intestinal

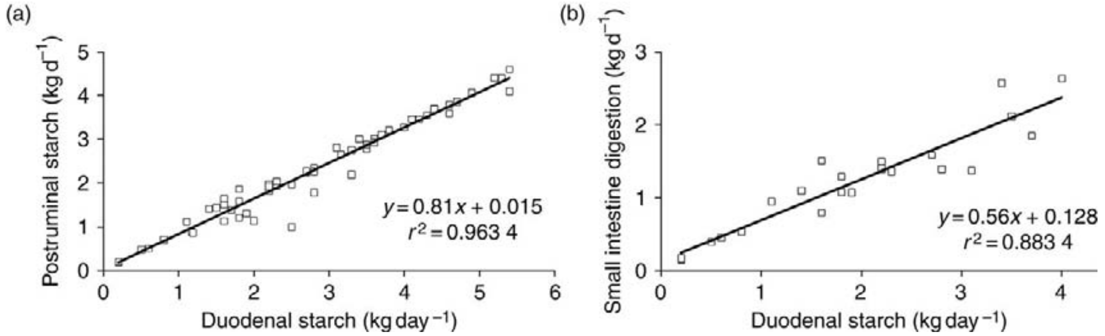

Figure 3 Relationship between duodenal starch flow and postruminal starch digestion (a) and small intestinal starch digestion (b) in lactating dairy cows.

disaccharidases (mainly maltase and isomaltase), which are needed to produce glucose. The buildup of oligosaccharides in the ileal digesta of steers suggests that both these enzyme groups may be limiting on occasion. Surprisingly, there is evidence from steers that amylase activity is greater on lucerne diets than on maize–based diets, which is contrary to the expectations that a mechanism might have evolved that would allow amylase secretion to increase in response to increased supplies of the substrate. However, recent studies suggest that protein and energy supplies have a more dominant role in determining pancreatic amylase secretion than starch supply *per se*.

A key question in relation to the lactating ruminant is how much of the apparently digested starch in the small intestine increases glucose supplies in the blood and so becomes available as a substrate for milk synthesis. In critical intraabomasal or intraduodenal infusion studies with steers, net recovery of apparently digested starch as glucose in the portal vein has been only about 35%, but this fails to account for incomplete digestion of starch. Higher recoveries of 38–57% have been found with glucose infusions, but even this still leaves large amounts of glucose unaccounted for. The problem arises from the fact that glucose is extensively metabolized by the PDV and the associated adipose tissues so that net absorption in lactating cows is often zero or even negative because metabolism by the PDV matches or exceeds the amount absorbed from the lumen of the gut.

Lipids

In ruminants the lipids entering the duodenum in the digesta have undergone fermentation in the rumen and hence have been extensively modified. The principal effect is to hydrolyze triglycerides to form free fatty acids (FAs) and then partially or completely hydrogenate the unsaturated FAs to form more saturated FAs via *trans*–conjugated dienes and monoenes (*see* **Nutrients, Digestion and Absorption**: Fermentation in the Rumen), some of which are associated with milk fat depression. In addition, some net microbial synthesis of lipid occurs but the extent is subject to debate. The total FAs entering the duodenum in the digesta may be higher or lower than the amounts consumed. In general, small increases of around 20% have been found with dairy cows given diets unsupplemented with dietary fat and containing around 20 g FA kg^{-1} DM intake compared with little change or small decreases of around 5–10% with diets supplemented with fat and containing about 60 g FA kg^{-1} DM.

There is no evidence of net FA absorption from the large intestine, so most of the net postruminal digestion of FAs as commonly measured between the proximal duodenum and the feces must be assumed to occur in the small intestine. Estimates of net absorption vary widely from 55 to 90% with most values ranging from 75 to 85% (**Table 1**). The reasons for the variability are uncertain, but clearly are not related to chain length or isomeric form. The efficiency of postruminal digestion has been found to decline with increasing dietary intake, with little effect noted in dairy cows with intakes of fat up to about 1000–1500 g day^{-1} but a declining efficiency observed above that threshold. Most studies of the mechanism of lipid digestion and absorption have been conducted in sheep and goats, but the main principles may be assumed to apply to dairy cows also. Small amounts of lipids enter the duodenum in the digesta as triglycerides, but most are in the form of FAs adsorbed onto particulate matter (feed particles and bacteria). These triglycerides are hydrolyzed by pancreatic lipases and phospholipases. Polyunsaturated FAs and lecithin from bile and pancreatic secretions are added to the lipid fraction of the digesta in the duodenum, the lecithin being hydrolyzed to lysolecithin by pancreatic enzymes in the jejunum. The FAs must be released from adsorption by the action of the bile salts and lysolecithin to form micelles, which is an essential prerequisite for absorption. The net effect is that the proportion of free FAs in the aqueous phase increases as

Table 1 Flow of fatty acids (FAs) through the digestive tract (g day^{-1}) and apparent postruminal digestibility (%) in dairy cows given high–and low–roughage diets

	High-roughage diet			*Low–roughage diet*		
Fatty acid	*Intake*	*Duodenum*	*Postruminal digestibility %*	*Intake*	*Duodenum*	*Postruminal digestibility %*
16:0	126	156	76.9	149	208	75.0
18:0	27	439	78.8	28	535	74.6
trans 18:1	0.3	61	85.1	0.1	120	84.3
cis 18:1	145	84	79.8	204	140	77.1
18:2	379	86	79.1	501	166	77.7
18:3	83	11	81.3	58	13	81.4
Total FAs	789	916	77.5	954	1288	75.6

Adapted from Kalscheur KF, Teter BB, Piperova LS, and Erdman RA (1997) Effect of dietary forage concentration and buffer addition on duodenal flow of *trans*–C18:1 fatty acids and milk fat production in dairy cows. *Journal of Dairy Science* 80: 2104–2114.

digesta pass through the jejunum to the ileum, and most of the absorption is thought to occur from the distal jejunum and ileum where the pH will have risen to about 7. Absorption is mainly into the lymph, but, with diets high in fat, some absorption of certain FAs into the portal vein may occur. FAs enter the lymph mainly in the form of very low–density lipoproteins in which form they are then transported via the thoracic lymph duct to the blood circulation and thence to the mammary gland and other tissues.

Differences between individual FAs in their absorptive efficiency are small though highly saturated FAs are less well absorbed. The data in **Table 1** show that the *trans*-18:1 FAs, which are produced during rumen fermentation of dietary unsaturated FAs, are absorbed as efficiently as *cis*–18:1 postruminally.

Energy

The current energy rationing systems, whether using net energy or metabolizable energy as the unit, are based on measurements of overall energy digestibility and metabolizability with no distinction between sites of digestion within the digestive tract. Thus, unlike protein rationing systems (see below), they do not depend on estimates or prediction of sites or products of energy digestion despite the importance of these factors in determining both the efficiency and the nature of energy utilization in lactating ruminants.

Most studies of sites of digestion in lactating ruminants have concentrated on quantifying the digestion of the major feed fractions and the products of that digestion, and few have measured these in energy units. Clearly, the contribution of the small intestine to the overall energy digestion will vary with a number of factors, in particular, the type of diet. A high–forage diet will result in a smaller proportion of the dietary energy being digested in the small intestine than, for instance, a diet containing a large amount of slowly fermentable starch or a high–fat diet. The published estimates, though small in number, suggest that, on average, the small intestine contributes about 30% of total apparent energy digestion with most diets in lactating dairy cows, but may reach 40% with diets high in maize grain.

Protein

Digestion of protein and absorption of amino acids in ruminants occur much the same way as in nonruminants, but, as for lipids, rumen fermentation markedly transforms the form and quality of the protein ingested before it reaches the duodenum. Fermentation has less of an effect on the quantity of protein passing through the stomach, but in general there is a slight gain in total N, which occurs as a consequence of endogenous N contributions. Depending on the nature of the protein and carbohydrate consumed and the rate of passage, digesta entering the small intestine include variable proportions of microbial, undigested feed and endogenous proteins, nucleic acids, peptides, and limited amounts of free amino acids. Most of the modern protein rationing schemes place much emphasis on the quantification of rumen N metabolism and prediction of the flow of microbial and undigested feed N in the digesta to the small intestine (*see* **Nutrients, Digestion and Absorption**: Fermentation in the Rumen). However, they usually rely on a single digestibility coefficient for predicting the uptake of amino acids from the small intestine despite the fact that this is such a vital step in the prediction of metabolizable protein supplies.

Protein digestion begins in the abomasum, where the acidic conditions solubilize proteins and activate pepsins that cleave specific peptide bonds. Peptide flow into the duodenum stimulates pancreatic enzyme secretion via the action of cholecystokinin. Pancreatic trypsin and chymotrypsin are active above pH 7; thus, as for lipids, further digestion is restricted until the pH of the chyme is effectively buffered by secretions of the pancreas and Brunner's glands within the duodenal mucosa, a process that may not occur until the chyme passes well into the jejunum. In the jejunum and ileum, peptides are reduced to smaller subunits and free amino acids by the action of carboxy–, di–, and aminopeptidases secreted by the mucosa. Free L–amino acids are absorbed into the epithelial cells by active transporters specific for groups of amino acids. These transporters also transport Na^+; thus, the maintenance of membrane potential requires a countertransport via Na–K–ATPase activity. Although amino acids compete for specific transporters, the absence of free amino acids in ileal digesta suggests that transporter activity is not limiting, even in lactating dairy cows. This is supported by the results of abomasal or duodenal infusion studies, which have rarely exceeded the digestive capacity of the entire postruminal digestive tract. Apparent amino acid digestibility in the small intestine ranges from 65 to 80% and appears to be unaffected by duodenal flow over a wide range (**Figures 4(a)** and (**b**)). Little difference has been observed between the digestibility of microbial protein and that of undigested feed proteins. More variability is observed in the digestibility of individual amino acids, tending to be as low as 50% for histidine and glycine and over 80% for arginine. Heat treatment of protein may reduce digestibility in the rumen, thus increasing rumen bypass, but also tends to reduce digestibility in the small intestine. Generally, the digestibility of specific proteins in the small intestine parallels their digestibility in the rumen.

Endogenous protein losses in the ileum must be accounted for when estimating true digestion of protein

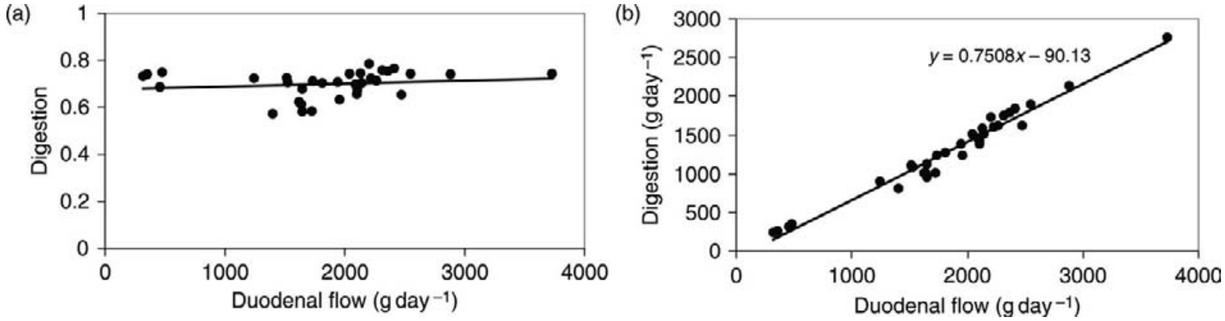

Figure 4 (a) Fractional digestion of total duodenal amino acids in the small intestine of lactating dairy cows. (b) Apparent digestion of total amino acids in the small intestine of lactating dairy cows. Data from Benchaar C, Vernay M, Bayourthe C, and Moncoulon R (1994) Effects of extrusion of whole horse beans on protein digestion and amino acid absorption in dairy cows. *Journal of Dairy Science* 77: 1360–1371; Reynolds CK, Harmon DL, and Cecava MJ (1994) Absorption and delivery of nutrients for milk protein synthesis by portal–drained viscera. *Journal of Dairy Science* 77: 2787–2808; Reynolds CK, unpublished.

and absorption of amino acids in the small intestine. In addition, endogenous protein losses are an important component of many systems for rationing protein to dairy cows. Measurements of isotopically labeled amino acid transfer from blood indicate that about 15% of protein flow in the ileum of dry and lactating cows is of endogenous origin. Like endogenous protein flow in the duodenum, this proportion does not vary with intake level such that total endogenous losses rise and fall with total protein flow and are not a fixed, basal amount as assumed in some systems used to calculate protein requirements.

In contrast to nonruminants, ruminants have an abundant secretion of ribonuclease from the Brunner's glands and are efficient at digesting purines, a source of both N and P, and purine digestibility in the small intestine averages about 80%. On average, nucleic acids account for about 20% of the nonammonia N flowing to the small intestine.

A considerable portion of amino acid absorption occurs via the transport of di–and tripeptides into intestinal epithelial cells. Historically, it has been presumed that these peptides were subsequently catabolized by membrane–bound peptidases or within the epithelium and absorbed into blood as free amino acids. Recent interest in the possibility that some peptides may be absorbed directly into the portal vein has not generated conclusive evidence that peptides represent a major route of absorption of digesta amino acids into the blood draining the small intestine.

As with the digestion of starch and the subsequent absorption of glucose, measurements of the net appearance of amino acids in the portal vein typically fail to totally account for amino acid disappearance from the lumen of the small intestine. This is often attributed to amino acid metabolism during absorption, as the intestinal mucosa has a high rate of protein turnover and preferentially uses some nonessential amino acids to meet energy requirements. However, this disparity between amino acid absorption from the small intestine and net appearance in the portal vein is also attributable to the use of amino acids from arterial blood to meet the amino acid requirements of other portal–drained tissues, such as the rumen and hindgut. In this regard, measurements of the net absorption of amino acids into the anterior mesenteric vein more closely equate with disappearance from the small intestine lumen, depending on the amino acid absorbed. In general, however, the PDV appears to meet its amino acid requirements largely from arterial blood, rather than exacting huge tolls during absorption across the small intestinal mucosa.

Minerals

Minerals are absorbed via passive diffusion or carrier–mediated transport mechanisms, which in many cases are highly regulated. while many minerals are absorbed by passive diffusion throughout the gastrointestinal tract, the small intestine is the predominant site of absorption for most of the macro–and microminerals. Exceptions include Mg, for which the primary site of absorption is the rumen and a considerable capacity for absorption is available in the colon. The rate of absorption of minerals entering the small intestine is affected by a variety of factors, including the molecular form of the mineral fed and interactions with other minerals within the digestive tract. For example, P in grain phytate is largely available to the cow through the actions of microbial phytase in the rumen, but any phytate bypassing the rumen as a consequence of the high rate of passage in lactating dairy cows would be as indigestible in the small intestine of the cow as in the pig or poultry. There are numerous interactions between minerals that affect their absorption, such as the inhibition of Mg absorption by K. Ca and P have antagonistic effects on each other's absorption when fed in excess, so diets are usually formulated to specific Ca:P ratios. Similarly, Ca can have antagonistic effects on the absorption of Zn and other divalent cations. Although many such interactions among minerals may occur, the true extent and importance of these in most production systems is not certain. However, most nutritionists would balance mineral concentrations in diets fed to avoid negative interactions.

Ca and P requirements for milk synthesis substantially increase their dietary requirements and alter the efficiency of their absorption from the small intestine. The absorption of Ca involves a Ca–binding protein in the enterocyte that is downregulated when Ca requirements are low, such as during the dry period. Losses of Ca in colostrum and the subsequent milk yield in early lactation dramatically deplete the blood Ca pool and plasma Ca levels, causing an increase in parathyroid hormone secretion. Parathyroid hormone has a variety of effects on Ca homeostasis that act to replete serum Ca levels, in particular the activation of vitamin D, which increases the absorptive capacity of the intestine for Ca. The absorption of other minerals such as P and Fe is also regulated, such that their net disappearance from the small intestine is reduced if their supply in the diet exceeds beyond requirements.

As for other nutrients, endogenous secretions of minerals occur throughout the digestive tract and influence the net absorption that occurs in the small intestine. In addition to minerals contained in sloughed cells and mucosal secretions, saliva, bile, and gastric and pancreatic juices all contain minerals; thus, there is a substantial endogenous secretion of many minerals into the duodenum. These endogenous minerals may be absorbed and thus recycled, as is the case for P, which is secreted in saliva at levels up to 5 times the amount consumed in feed. However, endogenous losses in feces also represent a substantial component of the maintenance requirement of the lactating dairy cow for many minerals.

See also: **Mammary Gland, Milk Biosynthesis and Secretion**: Lactose; Milk Fat; Milk Protein; Secretion of Milk Constituents. **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins; Fermentation in the Rumen.

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Absorption of Minerals and Vitamins

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Introduction

Minerals and vitamins differ from macronutrients in two important respects. First, absorptive processes vary enormously among micronutrients for a variety of reasons: contrasting properties of the micronutrient (vitamin solubility in water or fat; mineral ions that are positive or negatively charged); the capacity of the animal to up- or downregulate absorptive mechanisms depending on the balance between supply and demand; the need to provide protection from sporadic excesses of potentially toxic, essential ions and physicochemically similar nonessential ions. Second, the micronutrients themselves do not need to be broken down to simpler forms (i.e., digested) to be assimilated. However, the amounts of micronutrients presented for absorption and absorbed can be profoundly affected by digestive and physiological processes. Some micronutrients are present in feeds in complexes, which must be broken down before absorption can take place (e.g., P in the phytate molecule), while others interact with products of digestion to become more or less absorbable (Cu with sulfide). Fermentation in the fore stomachs greatly augments the supply of water-soluble vitamins (WSVs). The profuse flow of saliva induced by the ingestion of dry roughages can recycle minerals (e.g., P and Na), shifting the route of excretion from urine to feces and influencing the absorption of others (Mg). The stimulation of biliary secretion can have similar effects on Cu and Mn absorption. Simplistic techniques such as balance trials and repletion-slope ratio are widely used to measure absorption but may not fully characterize either the capacity of a feed to provide absorbable micronutrient or the capacity of the animal to absorb that which is absorbable. The individuality of micronutrient absorption may mean that average results from small experiments are not representative of wider populations. Our knowledge of mineral absorption is woefully inadequate for many micronutrients yet manipulation of micronutrient absorption by the animal or producer can determine the success and sustainability of milk production. The provision of organic mineral supplements has had a checkered history in that regard.

Major Minerals

Major minerals are distinguished from microminerals in being required in gram rather than milligram per kg dry matter (DM) concentrations while presenting relatively low risk of toxicity. The major challenge is to meet peak demands of late pregnancy and/or early lactation by complementing the obligatory mobilization of skeletal mineral reserves with a minimal supply of absorbed mineral and to provide an excess of mineral during late lactation and/or the dry period to replete the skeletal reserve. Optimization of absorption is preferable to permanent luxus supply because large additions of strongly ionized minerals can affect acid:base balance, prejudice the absorption of other minerals, and pollute the environment.

Calcium

Calcium is chiefly absorbed by facilitated diffusion across the small intestinal mucosa, the facilitator being a Ca-binding protein (CaBP), calbindin. CaBP is inducible by vitamin D_3, once the vitamin has been converted by twofold hydroxylation – first in the liver, then in the kidney – to a physiologically active form and bound to basally located mucosal receptors (vitamin D receptors (VDRs)) (see section 'Vitamin D'). However, studies with VDR-knockout mice show that Ca absorption (A_{Ca}) can continue by other pathways such as transcellular absorption and lysosomal transport. VDRs are induced by parathyroid hormone (PTH), which itself is induced by subnormal plasma Ca levels (hypocalcemia). Thus, Ca is absorbed according to the need, and absorptive efficiency will be low if the studied animals have low needs (i.e., are slow growing or nonlactating) or the feed is rich in Ca (alfalfa). CaBP is surprisingly nonspecific and its induction can increase the absorption of potentially toxic elements (PTEs) such as Pb and Al. The high-yielding dairy cow is unique in having a greatly increased Ca need once lactation commences. In the dairy sheep and goats, particularly those carrying more than one fetus, the need for Ca in late pregnancy is as great as that in early lactation. However, reported efficiencies of A_{Ca} in late pregnancy and early lactation are unexpectedly low. The most likely explanation is that obligatory mobilization of bone matrix to furnish the protein needs of offspring releases Ca into the bloodstream, lessening the perceived need. Adding more Ca to the diet either side of parturition will reduce the efficiency of A_{Ca} and can be counterproductive, turning off hormonal control mechanisms, which include the mobilization of Ca from bone by PTHs. The common disorder of dairy cows, milk fever, is a postparturient problem of inadequate bone Ca resorption rather than insufficient A_{Ca}. The incidence of milk fever is reduced by low ($<5\,g\,kg^{-1}$ DM) and raised by high dietary Ca ($>20\,g\,kg^{-1}$ DM), but treatment with massive oral doses of Ca can be successful because sufficient Ca is passively absorbed to alleviate hypocalcemia. Feeding low-Ca diets prior to late lactation to prime the hormonal mechanisms and enhance A_{Ca} is a high-risk preventive strategy and totally unsuited to species other than the dairy cow. Feeding 'acidified' or 'low-cation' diets around calving can reduce the incidence of milk fever in housed, high-yielding herds, but do so by improving bone Ca resorption rather than A_{Ca}.

There is no convincing evidence that Ca sources vary widely in availability for absorption and a high proportion (around two-thirds) is absorbable from most feeds. Reports of low A_{Ca} in feeds usually reflect low needs for one reason or another. It has been claimed that forages rich in oxalates provide poorly absorbable Ca, largely on the evidence of Ca oxalate crystals once detected in feces. However, rumen microbes adapt to oxalate-rich feeds by increasingly metabolizing oxalate and the animal should adapt to losses of unabsorbable Ca crystals by increasing the use made of absorbable Ca. Addition of fats to mineral-rich, concentrate diets may decrease A_{Ca} through the formation of insoluble soaps, but lipids naturally present in forages have yet to be shown to obstruct absorption. Phytate that escapes rumen degradation may form unabsorbable complexes with Ca.

Phosphorus

Although nutritionists have traditionally regarded Ca and P as inextricably linked, the link is established postabsorptively in bone and their absorption could hardly be more different. Phosphorus is primarily absorbed passively by diffusion down electrochemical gradients in the small intestine and the animal exerts little hormonal control over matters. The P absorption of ruminants is unique in two respects: first, copious secretion of P-rich saliva ensures that the diet is the minor source of P entering the rumen; second, most salivary and dietary P is sequestered in rumen microbial protein and microbial P is marginally less absorbable than inorganic phosphate (PO_4). Repeated cycling of P via saliva contributes to high efficiencies of absorption (A_P; 60–90%). Nevertheless, incomplete absorption of salivary P enables the feces to be the major route of excretion and therefore A_P cannot be measured in balance trials without correcting for endogenous loss. Grains contain most of their P in the form of mineral–phytate complexes but exposure to microbial phytases in the rumen during digestion can liberate nearly all of it as hydrolyzable phosphate (PO_4), 63% of the P in phytate-rich bran being absorbed by sheep. Lush herbage contains no phytate P and A_P can reach 90% in grazing beef cows. Phytate can increase microbial phytase activity by substrate induction but not if there is an abundant supply of PO_4. Surveys of fecal P in dairy farms in the United States found that 18% was present as phytate, about half the percentage present in the rations, suggesting that one-fifth of dietary phytate escaped degradation on average: degradation decreased as the levels of dietary PO_4 and Ca supplementation increased. Excessive dietary Ca (>5 g Ca per kg DM) can lower soluble P in the rumen at low dietary P levels and reduce A_P by about one-fifth in sheep on diets with dietary Ca:P ratios of >3.6. Direct measurement of duodenal flows of phytate P in cows given whole crop (wheat) silage showed that 75% phytate P escaped degradation and this was attributed to the passage of whole grains through the rumen. Thus, A_P will probably decline as the state of maturity of the ensiled crop advances, unless the grains are mechanically damaged. The only other important constraint on A_P might occur in the grazing animal exposed to infective nematode larvae on pasture either for the first time ('naive' offspring) or as immunosuppressed, periparturient dams. No studies have been made in cattle but infections of the small intestinal mucosa with *Trichostrongylus vitrinus* can almost halve A_P in sheep. The excretion of excess P in feces or urine is a major cause of pollution of surface waters, and the best way of reducing pollution is to stop overfeeding dairy herds with P and Ca and avoid feeding whole grains.

Magnesium

In ruminants, Mg is unusual among elements in being principally absorbed from the rumen by two mechanisms: one is active and the dominant force at lower Mg intakes, moving Mg against an electrochemical gradient across the mucosa; the other is passive and increases in importance as Mg intake increases. The active process is Na-dependent and inhibitable by K. Since the fecal endogenous loss of Mg is relatively small and constant, the apparent absorption of Mg (AA_{Mg}) recorded in balance trials is highly correlated with true absorption (A_{Mg}). Green forages contain sufficient K to reduce the overall efficiency of A_{Mg} substantially, the extent varying between diets and species to such an extent that the use of sheep to provide a model for A_{Mg} in cattle on unsupplemented natural diets is invalid. In sheep given fresh grass, AA_{Mg} falls from around 45 to 20% as the K level increases from 20 to $40\,g\,kg^{-1}$ DM; with mixed dry diets, A_{Mg} is only one-third as sensitive to K. In cattle, AA_{Mg} from grass is lower than in sheep at the same low K level (20% at 20 g K per kg DM), the difference narrowing as K increases. This may be due to a greater inhibitory effect of K in the bovine rumen and lower compensatory increases in A_{Mg} from the small and large intestines. In both sheep and cattle, AA_{Mg} is exceedingly poor from K-rich, dried grass (<15%). If the diet is deficient in Na, the compensatory increases in salivary and rumen K are sufficient to lower A_{Mg}. In sheep, Mg absorption from the rumen is also lowered by increases in pH and in rumen ammonia concentrations but the latter effect may be short-lived due to adaptation of the microflora. It is possible that sensitivity to both pH and ammonia may be less in pastured cattle. In general, health hazards related to inadequate A_{Mg} are largely confined to the period of transition from winter diets to spring grass, when all the factors mentioned above are at their most antagonistic, A_{Mg} is minimal, and hypomagnesemic tetany is caused. Provision of salt and sparing use of K fertilizers aid A_{Mg}. On mixed dry diets, A_{Mg} is usually >40% because all antagonisms are lessened and supplementation becomes unnecessary.

Sodium and Potassium

Na is actively absorbed through the activity of Na pumps in the intestinal mucosa. By contrast, K is absorbed passively from the rumen and is less regulated. The high solubility of Na and K inherent in and added to feeds ensures that efficiencies of absorption (A_{Na} and A_K) are close to 100% in healthy animals. When livestock become Na depleted, secretion of aldosterone causes a decrease in salivary Na and an increase in K. The resultant replacement of Na by K in the rumen delays the reduction in daily Na absorption and provides short-term protection from depletion. The principle threat to A_{Na} comes from damage to the absorptive mucosa by gut nematodes. Combined parasitism of the abomasum and small intestine is particularly harmful because the former increases the flux of Na into the gut lumen and the latter restricts reabsorption as well as absorption of dietary Na. The problem may be most critical in early lactation when immune resistance to parasitism is reduced and demand for Na is maximal due to high outputs in milk. High absorptive loads of K in forage-fed stock may weaken the homeostatic defenses against Na deprivation.

Sulfur

The absorption of S by ruminants is unusual in that the simpler of the three main forms in which it is absorbed, sulfide (S^{2-}) and sulfate (SO_4^{2-}), are virtually useless to the animal and only that reaching the small intestine as undegraded dietary protein, elaborated or incorporated into rumen microbial protein from degradable dietary S, is of nutritive value. The article dealing with amino acid absorption should be consulted (*see also* **Feed Ingredients**: Feed Supplements: Ruminally Protected Amino Acids). The focus here is on the factors that influence S^{2-} and $SO_4{}^{2-}$ absorption and what must be done to minimize absorption by these routes to optimize microbial – and hence milk – protein yield. Most of the S consumed, whatever the initial form, is rapidly degraded to S^{2-} during rumen fermentation and would pass by diffusion across the rumen wall if it were not partially captured by the microbes. Microbes require synchronous supplies of fermentable carbohydrate, ammonia, and soluble P to incorporate S into the S-amino acids of their cellular proteins. The aim must be to reduce the area under the postprandial rumen S^{2-} curve: this can be achieved by slowing the rate of food consumption and improving the degradabilities of dietary fibre and nitrogen. Provided these steps are taken, the provision of S as methionine analogs that are protected from rumen degradation is unlikely to improve S-amino acid absorption sufficiently to improve milk yield. Defaunation (i.e., suppression of protozoal populations) of the rumen improves the capture of dietary S in microbial protein, lowering rumen sulfide concentrations and raising the absorption of Cu, Mo, and Pb. Some S leaves the rumen in digesta as sulfate and is absorbed as such by an active transport mechanism in the ileum: together with material produced endogenously from the oxidation of S^{2-}, some SO_4 re-enters the rumen via saliva to begin the cycle again. Recycling is particularly important during the dry period and is quantitatively more important in species that do not continuously 'lose' S-amino acids to wool production (i.e., cattle > sheep). The S needs of dairy sheep and goats in terms of absorbed S-amino acids require investigation.

Trace Minerals

The prime determinants of the absorption of trace minerals are as diverse as their chemistries: for some (Cu), dietary composition and rumen fermentation dominate; for others, including Ca and Fe, the animal dominates by absorbing according to its need; and for those presented as anions (P, I, Se), absorption can be extensive and poorly regulated, although with P and Se the process is influenced by the intervention of rumen microbes. Because free cations are generally toxic, absorption has to involve complexation and carrier proteins to protect the absorptive mucosae. Most elements are absorbed by two processes: one is active, specific, and upregulated during deficiencies of the element; the other is nonspecific and downregulated when mineral intakes exceed the need. The latter involves divalent metal transporter-1 (DMT-1) and is responsible for a host of adverse interactions affecting mineral absorption. The form in which minerals occur in feeds is generally of little importance because rumen microbial activity is a great 'leveler': the notable exception is selenium, as described later. The forms of minerals in supplements appear to be relatively unimportant, beyond their purity. Superficially, it might appear that chelated or complexed trace element supplements would be immune to powerful antagonists in the gut, and early focus was placed on amino acid chelates because trace elements were thought to be absorbed principally as peptide complexes. However, the synthetic complex must have a higher affinity constant for the bound metal than any natural antagonist, remain stable in acid regions of the gut, and then release the mineral for normal absorption or metabolism. No organic mineral complex has yet been shown to possess such properties. Isolated production responses to organic minerals do not necessarily arise from superior availability: they may arise by chance (the number of trials conducted far outweigh the number whose results are published), through diminished rates of feed deterioration during storage, or through replacement of a subclinically toxic inorganic mineral being supplied in excess of need.

Chromium

The essentiality of Cr as an insulin potentiating factor is now generally accepted but the scope and need for manipulating A_{Cr} in dairy animals remain unclear. Supplements of organic Cr given at times of environmental or physiological stress (after transportation and prior to parturition) can have sustained effects on food intake and blood biochemistry without consistently improving health or productivity and may constitute pharmacological responses. Little is known about the forms in which Cr occurs in feeds or their fate in the rumen but inorganic Cr can be incorporated into glucose tolerance factor *in vivo.* The chelated form, Cr picolinate, is not degraded by rumen microbial activity – a rare quality among trace element chelates – and is probably absorbed intact but may not be readily utilized.

Copper

While the young are totally reliant on milk or milk substitutes, they absorb Cu with consistently high efficiency ($A_{Cu} > 80\%$), compensating for the low Cu concentration in milk; there is probably some decline with age as processes unique to the neonate (e.g., absorption of cuproproteins such as ceruloplasmin intact by endocytosis) wane. Weaning brings about a further abrupt decline because the intervention of anaerobic fermentation and S degradation creates a S^{2-}-rich environment in which any soluble Cu is rapidly converted to insoluble CuS, which passes unscathed through the acid abomasum and is excreted. Factors that promote S capture by microbes (e.g., defaunation) enhance A_{Cu} but ruminants rarely absorb >10% of the Cu they ingest. On S-rich green forages, A_{Cu} drops to below 3% and can reach <1% if pastures are underlain by alkaline, molybdeniferous soils that raise herbage Mo levels (>2 mg kg^{-1} DM). Molybdenum (Mo) enhances the antagonicity of S by allowing the formation of thiomolybdates ($MoO_{4-x}S_x$) in the rumen, which bind Cu irreversibly to the solid phase of the rumen contents when substitution of S for O is maximal (i.e., as tetrathiomolybdate (MoS_4)). High Fe intakes (from soil or supplements) can also reduce A_{Cu} substantially by an antagonism that is also partially S-dependent. Thus, it is dietary S, Mo, and Fe concentrations rather than Cu intake that determine the amount of Cu absorbed by ruminants. Furthermore, the risk of Cu deprivation is relatively high in all grazing livestock and low in housed stock because exposure to the antagonists is greater in the grazing situation. Use of defaunating agents increases A_{Cu} by reducing antagonism from sulfide. At low to moderate Cu intakes, absorbable Cu is taken up primarily by binding to a specific mucosal binding protein (Crt1) and at higher intakes by the nonspecific DMT-1. Attempts to bypass the rumen-based antagonisms by providing supplementary Cu in a 'protected' form, 'chelated' to specific or mixed peptides, have been unsuccessful. A Cu-glycinate has recently been shown to improve A_{Cu} on a diet high in Mo and S but the magnitude of the response suggests that A_{Cu} remained well below 10%. Threefold differences in A_{Cu} between 'meat' (Texel, high) and 'hill' (Scottish Blackface, low) breeds of sheep determine vulnerability to Cu toxicity and deficiency but corresponding data for dairy sheep and goats are limited: one comparison showed the East Friesland to retain Cu less efficiently than the Texel. Indirect evidence for differences between cattle breeds is emerging, although the differences are much smaller; comparisons of blood and liver Cu status in separate studies indicate that rankings in terms of AA_{Cu} may be Galician Blond < Holstein Friesian (HF), Jersey < HF, and Simmental < Aberdeen Angus.

Iron, Manganese, and Zinc

Iron, manganese, and zinc share the property of being absorbed according to the need, through the induction of receptor proteins in brush border and basement membranes in the intestinal mucosa. While some receptors are specific (e.g., hephaestin and ferriportin for Fe), DMT-1 can bind several essential and nonessential elements. Thus, excess Mn can reduce the efficiencies of Fe and Cu absorption (A_{Fe} and A_{Cu}) by downregulating DMT-1, which can facilitate the absorption of all three elements. By contrast, Fe deficiency can increase Pb uptake by upregulating DMT-1. Foraging livestock ingest large amounts of Fe and Mn as soil in both arid and cool temperate climates; although only a small fraction is absorbable, it may be sufficient in the case of Fe to cause subclinical hepatotoxicity and sufficient in the case of Mn to lower A_{Cu}. In the suckling or milk-fed young, A_{Fe} and A_{Mn} are high because demands are high and milk is low in Fe and Mn, but A_{Zn} may be relatively low because milk is rich in Zn (*c.* 35 and 48 mg Zn kg^{-1} DM in sheep and cattle, respectively). The normal abundance of Fe and Mn in the diet of the weaned animal ensures that only small proportions of ingested Fe and Mn (<10%) are generally absorbed but A_{Zn} can reach 70% from natural roughages; this is much higher than the level attainable in grain-fed monogastrics, because the major potential antagonist of absorption, phytate, is usually destroyed in the rumen. There may be partial escape of phytate when whole or finely milled grain is consumed by cattle, lowering A_{Zn}. There are no reported differences in A_{Zn} in feeds for ruminants in contrast to pigs and poultry, and the same probably applies to Fe and Mn. Chelated forms of Fe, Mn, and Zn are widely held to be substantially more 'bioavailable' than inorganic sources but production responses have generally been small and inconsistent. In the particular case of Zn, the provision of chelated Zn was claimed to stimulate replenishment of keratin in the teat canal in a way that inorganic Zn could not, but a recent study has failed to support the claim.

Iodine

Iodine, like se, is well absorbed but the two elements are absorbed by quite different mechanisms and at different sites. The abomasum provides the major site of absorption of I, possibly because the acid environment releases I, which is then passively absorbed. Iodine is retained rather than absorbed according to the need, and dietary excesses are removed in urine and milk. The fate of antagonists of I (goitrogens) in the rumen and their subsequent absorption and transfer to milk are arguably more important as causes of I deprivation but coverage is beyond the scope of this article.

Selenium

Most of the Se in feeds is present as selenomethionine (SeMet), which is absorbed by the pathway for methionine and enters the methionine pool: thereafter, some Se may continue to 'tag' the methionine, entering muscle or milk protein, for example. Rumen microorganisms are able to incorporate inorganic Se into SeMet. Selenite or selenate that escapes capture in the rumen is well absorbed in the small intestine and rapidly incorporated into the physiological transport forms as Se-cysteine. With grain, pasture, or inorganic Se providing moderate Se intakes (<0.3 mg kg^{-1} DM), AA_{Se} is around 65%. At higher Se intakes, that figure increases with SeMet as the source but decreases with inorganic sources, probably because more 'excess' SeMet escapes biliary secretion. Commercial pressures have spawned an illusion that provision of 'organic' rather than inorganic Se can benefit the cow and/or her calf but there is no evidence that it does. Selenium given as SeMet in Se-yeasts is less available for synthesis of selenoenzymes such as the important family of glutathione peroxidases (GPX). Supplementation of diets with SeMet certainly raises blood and tissue Se in cow and calf more than inorganic Se and also raises milk Se but these are all poor guides to functional availability to the animal and consumer of dairy products. Indeed, it is arguable that absorption of dietary SeMet is part of the problem rather than a panacea when it comes to Se-responsive diseases such as paralytic myoglobinuria and retained placenta. When increased muscular activity suddenly increases the need for antioxidants, of which GPX is one, Se that has 'tagged' or is tagging methionine may be 'unavailable for work'.

Cobalt

Co is an unusual micronutrient in three respects: first, rumen microbes incorporate Co into two physiologically active forms of vitamin B_{12}, methylcobalamin and adenosylcobalamin, that are essential to the host; second, these have to be complexed to intrinsic factor (IF), secreted in the abomasum, and facilitate absorption in the ileum, leaving vast quantities of B_{12} analogs, also produced in the rumen, to be harmlessly excreted in feces; third, rumen microbes have the same B_{12}-dependent biochemical pathways as the host, the one dependent on adenosylcobalamin working in reverse to promote the production of propionate from succinate. Rumen fermentation can therefore be affected by low-Co diets, succinate being produced at the expense of propionate. Host demand for adenosylcobalamin to process propionate may thus be reduced. For the first few weeks of life, IF is not produced and the young rely on the absorption of B_{12} bound to specific proteins in milk; this may be a device for denying potentially harmful gut pathogens a supply of the micronutrient that they also need. Once IF is induced, it acts as a carrier for vitamin released from binding to salivary R proteins by pancreatic proteases; this preliminary binding may stabilize B_{12} at the acid pH of the abomasum. The B_{12}–IF complex is absorbed by endocytosis and the B_{12} is transferred to a nonspecific carrier protein, cubulin, in the intestinal mucosa. The overall efficiency of conversion of ingested Co to absorbed vitamin B_{12} after weaning is poor (<15%), limited partly by the synthetic capacity of rumen microbes. Parasitism of the gastric or intestinal mucosa might be expected to limit vitamin B_{12} absorption but neither abomasal (*T. circumcincta)* or intestinal (*T. vitrinus*) parasitism in lambs accelerated the decline in plasma B_{12}, which commonly follows weaning, in one study.

Water-Soluble Vitamins

There is copious synthesis of essential WSVs during the course of rumen fermentation and they become available for absorption during the digestion of rumen microbial protein in the small intestine. The presence of complexed forms in feeds (e.g., of biotin, folacin, niacin, and riboflavin) should present no problems, since these are degraded in the rumen, unless there is heavy reliance on whole grain feeding in larger species. However, the potential methyl donors – dietary choline and its oxidation product, betaine – are degraded in the rumen. Possible benefits in milk production from the provision of rumen-protected choline and betaine to enhance methylation have been explored with inconsistent results, which may reflect the capacity of the animal to generate choline endogenously from methionine. Similarly, claims that supranutritional supplements of vitamin B_{12} and folate may increase milk yield, despite adequate dietary supplies of Co, by increasing methylation (folate facilitates the transfer of the methyl group from methylcobalamin) may only be justified if methionine is limiting. Vitamin C is converted to its reduced form in the rumen. Studies with monogastric animals indicate that some WSVs, such as riboflavin and niacin, can be absorbed by active, saturable, carrier-mediated processes, but in ruminants with a luxus endogenous supply, they are mostly absorbed well (>70%) by passive diffusion. Inefficiency of passive absorption may explain the low efficiencies of post-rumenal absorption of riboflavin and biotin (35 and 28%). Since WSVs pass readily into milk, the suckling is also presented with plentiful supplies of these nutrients in readily utilizable forms. Folate is strongly bound to proteins in milk, and in the neonate, these complexes may be directly absorbed, bypassing the normal receptor-mediated processes. Antinutritional factors that impair vitamin synthesis can occasionally cause problems. Rumenal outflow of thiamine can be reduced by 25% if sulfur intake is high. Provision of carbohydrate-rich rations encourages thiaminase production in the rumen, while the ingestion of certain weeds (e.g., bracken fern and horsetail) can introduce thiaminase. Thiaminases split the thiamine molecule, and the consequent absorption of the analogs produced and enhanced tissue needs for the vitamin conspire to produce a thiamine-responsive polioencephalomalacia. The disease, also known as cerebrocortical necrosis (CCN), can be a problem on molasses-based regimens. Relationships between vitamin intake and excretion in urine or secretion in milk could be used to measure the efficiency of absorption, which has received little study.

Fat-Soluble Vitamins

Little information has been published on fat-soluble vitamin (FSV) absorption (A_{FSV}) in ruminants, but in nonruminants, it was assumed to be influenced primarily by the quality and quantity of fat in the diet, absorption being better when the fat was unsaturated than saturated and poorer when fat was lacking. In ruminants, saturation of fat during rumen fermentation lessens the significance of fat quality, and any problems related to fat quantity are probably related to excess rather than deficiency. Thus A_{FSV} may fall with decreases in fat digestibility when tallow or Ca soaps are added to diets as energy supplements. However, A_{FSV} is far more complex. Absorption of carrier sterols is modulated by ATP-binding cassette (ABC) transporters, nuclear receptors, and intracellular trafficking proteins. Interactions between vitamin A, D, and E can arise because the modulators are themselves influenced by retinoic acid X-receptors, synthesized in the liver. Plant sterols have inhibitory effects on β-carotene and α-tocopherol absorption, which vary in magnitude, depending on whether they are esterified or not. In nonruminants, fat digestibility is impaired by pancreatic insufficiency, but parallels in ruminant nutrition have not been studied. In particular, Zn is required for the activity of phospholipase A2, needed for the reduction of fat globules to an absorbable size (chylomicrons). Severe Zn deprivation can thus reduce A_{FSV}. Reduced bile salt secretion from livers damaged by plant hepatotoxins or fluke (*Fasciola hepatica*), reduced acid secretion during abomasal parasitism, and damage to the intestinal mucosa by intestinal parasites may each impair absorption of all FSVs. In other respects, the individual vitamins are affected very differently by the processes of digestion and absorption.

Vitamin A

The major sources of vitamin A are provitamin A carotenoids that are partially released from feeds by proteolytic enzymes, lipases, and esterases in the stomach and small intestine. Carotenoids aggregate in lipid droplets and are absorbed inefficiently by passive diffusion and then partially cleaved by dioxygenases to retinaldehyde in the mucosal cells. Studies with monogastrics indicate that only 15% of ingested carotenoids end up in liver stores: although ruminants may be no better, the abundant supply of carotene in forages usually compensates for inefficient capture. Carotenoids differ in their potency, and shifts in isomer distribution may explain why carotene in alfalfa is more available than that in grass hay and why ensilage lowers availability. Any vitamin A added as a dietary supplement (i.e., retinol) is absorbed by facilitated diffusion, probably with high (*c.* 75%) efficiency, but it may lower the absorption of α-tocopherol.

Vitamin D

The natural source of vitamin D in plants is a provitamin, ergosterol, which is poorly absorbed and little is converted to ergocalciferol (D_2) in the tissues. Ruminants rely on UV irradiation to convert D_2 to cholecalciferol (D_3) in the skin while outdoors, but when housed for long periods they must receive supplements of $D_{3.}$ Vitamin D_3, like vitamins A and E, can be absorbed with high efficiency in fat micelles from the upper small intestine. Regulation of A_{Ca} (see section 'Calcium') depends partly on receptors for dihydroxy D_3 (VDR) in the gut but their number may decrease with age, making older females vulnerable to hypocalcemia when lactation commences.

Administration of analogs of vitamin D_3 by injection can override the mechanisms that regulate A_{Ca} but exposure to excess soluble Al may inhibit the response to D_3 by binding to calbindin (see section 'Calcium') if studies with broiler chicks are anything to go by.

Vitamin E

Unlike vitamins A and D, vitamin E occurs widely in feeds as a variety of tocopherols and tocotrienols, of which the D-α-tocopherol is the most prevalent and potent. Green and oily feeds are rich in tocopherol, and vitamin E status rises steadily while livestock graze lush pasture. The occurrence of vitamin E-responsive myopathies at turnout in spring is due to multifactorial increases in oxidative stress, which accelerate vitamin turnover, rather than poor absorption of the vitamin. Slow adaptation of rumen microbes to greatly increased supplies of polyunsaturated fatty acids (PUFAs) contributes to that stress. Vitamin E is more stable than vitamins A and D in the rumen, its kinetics after bolus administration being those of a soluble marker, and absorption occurs principally in the jejunum. Supplements of vitamin E are usually given as esters because they are relatively stable, but incomplete hydrolysis restricts their absorption, compared with tocopherol. The most widely used form, tocopherol acetate, is given a potency one-third less than that of α-tocopherol, while water-soluble esters such as the succinate are even less absorbable. Plasma α-tocopherol concentrations are commonly used to assess status but they can be poor indicators of vitamin E absorption when oxidative stress is high (e.g., when PUFA intakes are high) because turnover of the vitamin is greatly increased. Supplementation with vitamin E can enhance the absorption of vitamin A.

Vitamin K

Little is known about ruminant digestion and absorption of either the naturally occurring forms of vitamin K in plants, the phylloquinones (K_1), or synthetic forms such as menadione (K_3), rarely used as supplements. Of the FSVs, K alone is synthesized *de novo* by gut microbes and it is not included routinely in vitamin supplements.

See also: **Feed Ingredients**: Feed Supplements: Macrominerals; Feed Supplements: Microminerals; Feed Supplements: Organic-Chelated Minerals; Feed Supplements: Vitamins. **Nutrients, Digestion and Absorption**: Small Intestine of Lactating Ruminants.

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Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake

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Introduction

As early as 4000 BC, milk and dairy foods were valued by various societies as an important dietary staple because of their high nutritional quality. In the early twentieth century, as essential nutrients necessary for humans were being identified, scientists ascertained the nutrient content of milk and dairy foods. Detailed analyses revealed that milk and dairy products made from it are nutrient-rich foods that contain the essential nutrients and have these nutrients in appropriate concentrations. Thus, milk was recognized as an effective source of essential nutrients as part of a balanced diet. More recently, scientists have recognized that there are unique and complex structures that have emerged in milk through its evolution that provide a variety of health-promoting functions in humans beyond simple nutrition. This article provides an overview of the specific nutrients and bioactive compounds in milk and dairy foods, their nutritional contributions to Western diets, and their impact on human health. In addition, this article highlights emerging scientific discoveries and techniques that will allow scientists to delineate how milk and dairy foods affect human health.

Contribution of Milk and Dairy Foods to Nutrient Intake

During the first half of the twentieth century, scientists established the identities of all the essential nutrients. Chemists determined the precise molecular structures of all the essential nutrients, vitamins, minerals, amino acids and fatty acids; biochemists described their functions in cells; and nutritionists determined the amounts necessary in diets to prevent nutrient deficiency diseases. Foods were then analyzed for their nutrient content. As a direct result of the unique evolutionary role of milk in the complete nutritional support of mammalian infants, milk and dairy foods contain a uniquely complete package of essential nutrients. Including dairy products in human diets has been demonstrated to ensure adequate nutrient intakes across the life span. Together, milk, cheese, and yogurt are major contributors of calcium, potassium, phosphorus, protein, vitamin A, vitamin B_{12}, riboflavin, and niacin (as niacin equivalents). In addition, vitamin D-fortified milk and dairy foods are important dietary sources of vitamin D. Moreover, data from the Continuing Survey of Food Intakes by Individuals in the United States show that total milk and dairy intakes are associated with higher nutrient intakes in Americans. Because milk and dairy products supply substantial amounts of macro- and micronutrients in the diets of Americans, the 2010 *Dietary Guidelines Advisory Committee* reinforced the 2005 *Dietary Guidelines for Americans* recommendation to consume three servings of low-fat or fat-free milk or equivalent milk products daily (for individuals 9 years and older) as part of a healthy diet and lifestyle. In addition, official dietary recommendations in other countries recognize the importance of milk and other dairy foods in the diet to improve diet quality.

Macronutrients

Protein

Cow's milk is about 3.5% protein by weight, and milk and milk products contribute significantly to the protein available in the US food supply. Specifically, in 2005, milk and dairy foods (excluding butter) contributed approximately 18% of the protein available in the United States. Milk protein is a heterogeneous mixture of proteins and an excellent source of high-quality protein, containing all of the nine essential amino acids in varying amounts that humans require and in proportions resembling the distinct pattern of the various amino acids required in the diet. Of the total protein in milk, approximately 80% is casein and 20% is whey protein. As scientific research has begun to realize that dietary proteins exhibit biological activities beyond the simple provision of amino acids, milk proteins have been shown to be highly bioactive. Studies have shown that individual milk proteins exhibit a range of beneficial functions, such as stimulating muscle recovery after exercise, reducing the risk of dental caries, and inhibiting vasoconstriction.

Carbohydrates

Lactose is the principal carbohydrate in milk, accounting for about 4.8% of bovine milk by weight. In addition, milk contains minor quantities of glucose, galactose, and oligosaccharides. Milk and milk products contribute minimally to overall carbohydrate intake in the United States, accounting for only about 4.3% of the carbohydrates available in the US food supply.

The digestion of lactose in mammalian intestine requires the enzyme lactase. This enzyme is no longer produced in sufficient amounts after weaning in most mammals. Some individuals consuming large quantities of lactose experience symptoms resulting from the failure to digest lactose and this condition is called 'lactose intolerance'. In practice, this is defined as the inability to digest the amount of lactose in one quart (0.95 l) of milk without any unpleasant gastrointestinal symptoms. Remarkably, in human history, when milk was available in the diet due to the domestication of cows, individuals who contained distinct genetic mutations in their genomes that enabled them to continue to produce lactase throughout their lives were more likely to survive. As a result, about 25% of the world's population today is genetically able to digest lactose as adults. Additionally, research has demonstrated that individuals with lactose maldigestion can drink up to two cups of milk per day if milk is consumed with meals. Moreover, if an individual with lactose maldigestion continues to consume dairy foods on a regular basis, the body seems to adapt, resulting in fewer gastrointestinal symptoms. These results indicate that individuals without an active lactase enzyme are able to digest lactose due to the presence of specific bacteria within their intestines. In addition, other dairy foods, such as aged cheese and yogurts, with live and active cultures, are well tolerated by most individuals with lactose maldigestion. Therefore, by following simple dietary strategies, individuals consuming milk and other dairy foods regularly consume the nutrients without deleterious consequences due to lactose.

Advances in analytical chemistry and microbiology have allowed milk constituents with physiological benefits beyond simple nutrition to be identified. Oligosaccharides are a class of bioactive molecules that are composed of 3–10 monosaccharides covalently linked through glycosidic bonds. Interest in milk oligosaccharides began with the observation that human milk oligosaccharides stimulate the preferential growth of beneficial bacteria in the gastrointestinal tract. The benefits of these bacteria include a reduction in the incidence of pathogenic diseases by inhibiting the binding of pathogens to epithelial cells. Recently, approximately 40 free oligosaccharides were identified in cows' milk, with the majority of oligosaccharides containing a sialic acid. Research has suggested that sialic acids inhibit pathogen binding and enhance cognitive development. Similar results were obtained in a study that characterized free oligosaccharides in whey permeate. Interestingly, half of the oligosaccharides in whey permeate were identical to those found in human milk. Although this area of research is still emerging, preliminary data suggest that cows' milk could be a premier source of bioactive oligosaccharides with important biological functions.

Fat

The nutrition, appearance, texture, flavor, and satiability of milk and dairy foods are partly related to milk fat. The requirement for polyunsaturated fatty acids as essential fatty acids is quantitatively small in humans. The fatty acid composition of all mammalian milks is influenced by the mother's diet, tissue stores, and biosynthetic capacity of the mammary gland. Bovine milk is low in all unsaturated fats because of biohydrogenation of dietary fats within the rumen. Although milk is not typically rich in polyunsaturated fatty acids, it does contain adequate levels. The majority of lipids in milk are triglycerides, with lesser quantities of phospholipids, cholesterol, cholesterol esters, diacylglycerols, monoacylglycerols, and free fatty acids.

Observational data and human and animal studies have suggested that the consumption of diets high in saturated fatty acids elevates serum cholesterol. This relationship has been extended to examining populations consuming diets high in saturated fat, finding that saturated fat in the diet is positively associated with hypercholesterolemia (i.e., increased low-density lipoprotein (LDL)) in many cases, which is a risk factor for cardiovascular disease. However, large, retrospective epidemiological studies relating cardiovascular disease to milk consumption do not typically find evidence of a positive association between increased milk consumption and increased risk of heart disease. Ongoing research is attempting to find out the reason for this. In 2005, milk and dairy foods contributed approximately 8 and 16% of the total fat and saturated fat in the US diet, respectively. Although studies have shown that saturated fatty acids can raise blood total lipoprotein and LDL levels, individual fatty acids differ in their ability to raise blood cholesterol levels. For instance, milk fat contains a relatively high proportion of short- and medium-chain saturated fatty acids, which have been shown to have a neutral or even beneficial effect on blood LDL levels. Similarly, although the intake of industrially produced *trans* fatty acids is associated with increased cardiovascular disease risk, there is no scientific evidence that naturally occurring *trans* fatty acids in milk fat are atherogenic.

Emerging scientific evidence indicates that milk fat contains several unique fat-soluble components that may be beneficial to human health. For instance, milk fat is the richest natural dietary source of conjugated linoleic acid, which may reduce the risk of several types of cancer and cardiovascular disease and enhance immune competence. Additionally, dairy products are a good source of both sphingolipids (approximately 0.2–1.0% of the total lipids of milk) and the four-carbon short-chain fatty acid butyric acid, which have been shown in animal studies to protect against certain cancers.

Micronutrients

Vitamins

Milk and dairy foods are rich in several vitamins. Vitamin A is required for normal vision, reproduction, embryonic development, cell and tissue differentiation, and immune function. Both vitamin A and its precursors – carotenoids – are present in milk fat. One 8-oz (237 ml) serving of whole milk supplies about 258 international units (IU) of vitamin A. Because vitamin A is fat soluble, it is removed from milk and milk products when fat-free, low-fat, and reduced-fat milk or milk products are produced. Consequently, vitamin A must be added to the level in whole milk or fortified to 300 IU per 8-oz serving. However, most dairy processors in the United States fortify milk to a level of 500 IU of vitamin A per 8-oz serving. Overall, milk and milk products are an important dietary source of vitamin A, contributing approximately 11% of vitamin A in the US diet.

Vitamin D plays important roles in maintaining calcium homeostasis, insulin secretion, muscle function, immunity, and cellular differentiation. Vitamin D is present in unfortified milk, albeit at low levels (47–105 IU l^{-1}). In the United States, nearly all fluid milk is fortified with vitamin D, providing 100 IU per 8-oz serving. Therefore, vitamin D-fortified milk and milk products are a major dietary source of vitamin D in the United States. Although vitamins E and K are found in a wide variety of nutrient-rich foods, milk also contains low amounts of these fat-soluble vitamins. Vitamin E is a lipid-soluble antioxidant that helps maintain cell membrane integrity; vitamin K is required for blood clotting. One 8-oz serving of milk contains 0.06 mg per 100 g of vitamin E and 0.2 μg per 100 g of vitamin K.

In addition to the essential fat-soluble vitamins, milk and milk products are a significant source of B vitamins. Milk and milk products contain significant amounts of thiamine, a coenzyme required for numerous reactions of carbohydrate metabolism, contributing over 4% of the thiamine in the US diet. Consumption of three 8-oz servings of milk provides approximately 29 and 27% of the thiamine recommended for adult males and females in the United States, respectively. Milk and milk products are also excellent sources of riboflavin, which functions as a precursor for different coenzymes involved in the oxidation of glucose, fatty acids, and amino acids. Milk and milk products contribute 25% of the riboflavin to the US diet and three 8-oz servings of milk provide enough riboflavin to meet the US requirements for both adult males and females. Vitamin B_{12} is required for the maintenance of nerve tissue and red blood cell formation. Milk and milk products provide 0.44 μg of vitamin B_{12} per 100 g; therefore, three 8-oz servings will provide the recommended amount of B_{12} for most adults in the United States. Milk also contains variable amounts of niacin, vitamin B_6, folate, and ascorbic acid, contributing approximately 1.1, 6.8, 3.3, and 2.5% of each nutrient in the US diet, respectively.

Minerals

Milk and milk products are important dietary sources of essential minerals, especially calcium, magnesium, phosphorus, potassium, and zinc. Because the mineral content of milk is influenced by environmental factors and genetics and stage of lactation of the animal, the mineral content in milk may vary.

Milk and milk products are an excellent source of bioavailable calcium, providing nearly 300 mg per 8-oz serving. In fact, milk and milk products contribute more than 70% of the calcium in the US diet. Calcium is recognized as a key mineral in promoting and maintaining skeletal health. In addition, calcium is required for other important physiological functions, such as muscle contractions, blood coagulation, and myocardial function. Recently, Gao and coworkers modeled diets to determine the practical feasibility of meeting the recommended intake of calcium without the consumption of milk or milk products. The authors concluded that, although it was feasible to meet calcium recommendations by consuming calcium-fortified foods, adequate calcium intake cannot be met with a dairy-free diet while still meeting the recommendations for other macro- and micronutrients within a typical US dietary pattern.

Milk is also a good source of phosphorus, which plays a central role in metabolism and is a structural component of lipids, proteins, and carbohydrates. Milk and milk products contribute about 30% of the phosphorus in the US diet, with three 8-oz servings of milk supplying about 95% of the amount of phosphorus recommended in the United States. Magnesium is a cofactor for over 300 enzymes. Despite magnesium being found in a variety of nutrient-rich foods, especially those of vegetable origin, milk and milk products contribute nearly 14% of the magnesium in the US diet. Potassium is required for the transmission of nerve impulses and is involved in muscle contraction. Milk and milk products contribute about 16% of the potassium in the US diet, with three 8-oz servings of milk supplying about 55% of the 4700 mg of potassium per day recommended for persons aged 14 years and older. Moreover, according to the US Department of Agriculture national food intake surveys, milk is the top dietary source of potassium among US adults. To help meet potassium requirements, the 2005 Dietary Guideline Advisory Committee increased the amount of milk and milk products recommended to three cups of milk or milk equivalents per day for all persons aged 9 years and older.

Milk and milk products are rich in zinc, a trace mineral that is a constituent of about 100 enzymes. Milk and milk products supply 15% of the zinc available in the US diet. Furthermore, three 8-oz servings of milk provide about 27 and 37% of the recommended amount of zinc per day for adult males and females, respectively. Selenium is an integral component of the enzyme glutathione peroxidase, which is involved in the removal of reactive oxygen species. Although the selenium content of milk varies widely because of the selenium content of a cow's diet, three 8-oz servings of milk provide nearly 50% of the recommended amount of selenium per day for adults in the United States. In addition, milk and milk products contain insignificant amounts of other trace minerals, such as iron and iodine.

Beyond Nutrient Content: Milk as a Delivery System

From the first scientific breakthroughs discovering the principle of nutrient essentiality and the various molecules that are essential in the diets of humans, milk has been highly instructive. For example, the quantitative requirement for various nutrients ranges widely from submilligrams of particular vitamins to gram quantities of others. Assembling these various nutrients into a complete ensemble, each at their appropriate quantities, is vividly accomplished by milk. Furthermore, as scientists examined the essential nutrients, they recognized that most of the essential nutrients are poorly absorbable and readily form insoluble salts, crystals, or conjugates. Milk is conspicuous for its ability to contain all of the nutrients, each in complex forms that are able to ensure their absorption from the intestine. Calcium is largely present in milk complexed to phosphate within casein protein structures, making both of these nutrients bioavailable. Many essential nutrients are chemically reactive, and milk reveals mechanisms that simultaneously minimize reactivity and yet maintain bioavailability. Iron is highly reactive to a wide range of susceptible biomolecules. In milk, iron is complexed to proteins such as lactoferrin in which it is unreactive and yet bioavailable. Lipid-soluble vitamins are dissolved in milk lipids, enhancing their bioavailability. The difficulty in making all nutrients absorbable simultaneously is another challenge solved by milk in ways that scientists are still discovering. When saturated fatty acids are present in the intestine, they tend to bind calcium and reduce the bioavailability of both. In milk, the saturated fatty acids are generally present in the *sn*-2 position of the triglyceride. Because the digestive lipases do not release the *sn*-2 position fatty acid, it remains as a monoglyceride and is absorbed intact. Free fatty acids are not released, and calcium is highly absorbable due to this structural specificity.

Milk as an Intestinal Regulator

The basic view of nutrition is that nutrient availability is critical to the value of food as a carrier of essential nutrients. However, little research has examined whether nutrients should be absorbed rapidly and studies on milk are beginning to reveal principles that the most rapid absorption may not be the most nourishing. The net absorption of amino acids is slowed by the ability of caseins to form aggregates (gels) within the stomach. This has the net effect of slowing the absorption of amino acids, maintaining their concentrations in the bloodstream for a longer period.

Dietary constituents are not all digestible and absorbable. Their consumption has an interesting effect on human intestine, however, as they are available to large numbers of bacteria within the human intestine. Scientists are only beginning to address the complexity of these bacteria, their functions, and the consequences of their presence and activity. Germ-free animals provide some clues. Their intestines are poorly differentiated, their immune systems are naive, and their metabolic activities are inefficient. However, the simple absence of bacteria does not provide information as to how to alter bacteria in human intestines for benefit. Milk contains significant quantities of oligosaccharides that are neither digestible nor absorbable by humans. Nonetheless, their complex structures are digestible by a rare group of bacteria that predominate in the intestine of breast-fed infants. These data reveal a new function of diet – the guidance of a net beneficial microflora.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology; *Brevibacterium linens*, *Brevibacterium aurantiacum* and Other Smear Microorganisms; Lactic Acid Bacteria: An Overview; Probiotics, Applications in Dairy Products; *Propionibacterium* spp. **Fermented Milks**: Asian Fermented Milks; Buttermilk; Health Effects of Fermented Milks; Kefir; Koumiss; Middle Eastern Fermented Milks; Nordic Fermented Milks; Starter Cultures; Types and Standards of Identity; Yoghurt: Role of Starter Culture; Yoghurt: Types and Manufacture. **Milk Lipids**: Analytical Methods; Buttermilk and Milk Fat Globule Membrane Fractions; Cholesterol: Factors Determining Levels in Blood; Conjugated Linoleic Acid; Fat Globules in Milk; Fatty Acids; General Characteristics; Lipid Oxidation; Lipolysis and Hydrolytic Rancidity; Milk Fat Globule Membrane; Nutritional Significance; Phospholipids; Removal of Cholesterol From Dairy Products; Rheological Properties and their Modification; Triacylglycerols. **Milk Proteins**: Analytical Methods; Casein, Micellar Structure; Casein Nomenclature, Structure, and Association; Heterogeneity, Fractionation, and Isolation; Immunoglobulins; Interspecies Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity; Lactoferrin; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; Nutritional Quality of Milk Proteins; Proteomics; α-Lactalbumin; β-Lactoglobulin. **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins; Fermentation in the Rumen; Fiber Digestion in Pasture-Based Cows; Small Intestine of Lactating Ruminants. **Prebiotics**: Types; Functions. **Vitamins**: Biotin (Vitamin B_7); Folates; General Introduction; Niacin; Pantothenic Acid; Riboflavin; Thiamine; Vitamin A; Vitamin B_6; Vitamin B_{12}; Vitamin C; Vitamin D; Vitamin E; Vitamin K.

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Relevant Website

http://65.216.150.148/ifs/query.htm – MyPyramid.Gov. Steps to a healthier you. Food supply database. United States Department of Agriculture, Center for Nutrition Policy and Promotion.

Nutritional and Health-Promoting Properties of Dairy Products: Bone Health

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Introduction

The most common bone disease worldwide is osteoporosis, affecting several million people. Osteoporosis results in a reduced bone mass leading to enhanced bone fragility. Postmenopausal women and elderly male subjects are the most affected. The prevalence is higher in the white race than in blacks and Asians. The incidence of osteoporosis is still expanding owing to demographic changes that lead to an increase in the number of elderly people in the developed and also in the developing countries. Osteoporosis can be prevented by achieving maximal bone mass in young subjects compatible with their individual genetic background and by reducing the rate of bone loss in elderly subjects.

Bone Mass Changes during Lifetime

Bone mass increases until the third decade of life, and then gradually decreases (**Figure 1**). Bone health involves a complex interplay of genetics, mechanical forces, and hormonal and nutritional factors. According to the Utah paradigm of skeletal biology, muscle mass/strength is the most important force for bone health. Thus, the lower muscle mass of women as compared to men can also explain the lower bone mass observed in the female population.

In women, estrogens enhance bone density. Consequently, bone mineral density decreases in postmenopausal women. In the first 5 years after menopause 15% of the peak bone mass will be lost, and up to 30–50% will be lost before the age of 70, when menopause-related bone loss is completed. The bone loss rate associated with the process of aging is approximately 1% per year in both men and women. Therefore, having larger bones and spending it more parsimoniously, that is, reducing bone loss, delays reaching the bone density level at which fracture risk is high. Fracture incidence in individuals whose bone density is more than 1 SD above the mean is 50% lower at the age of 80 years.

Calcium and Bone Health

Calcium is considered to be an important nutrient for bone health. As more than 99% of the body's calcium stores are located in hard tissues like teeth and bones, a positive calcium balance is necessary to increase bone mineral density and, thus, bone strength. In contrast, a negative calcium balance always results in a loss of bone mineral density.

Very low calcium intake in children induces rickets, osteomalacia, and growth retardation. Infants who intake below 200 mg day^{-1} are susceptible to radiographic appearance of rickets. Clinical intervention trials provide evidence that increasing the dietary intake of calcium in pubertal girls above their habitual calcium intake of about 900 mg day^{-1} to the recommended intake of 1200–1300 mg day^{-1} is associated with positive effects on bone mineral accretion. However, mounting evidence from randomized clinical trials also suggests that the bone mass gained during childhood and adolescence through calcium supplementation is not retained postintervention. Thus, data indicate that an adequate calcium intake is necessary lifelong to preserve the positive effects of calcium on bone. The adequate daily calcium intake for middle-aged people is 1000 mg, and it is 1200 mg for people 51 years and older. The tolerable upper level of calcium intake is set at 2500 mg day^{-1}. Intakes above that level increase the risk of hypercalcemia and deposition of calcium in soft tissues. Randomized, prospective studies have demonstrated a 12% reduction in fracture risk by calcium supplements. The effects were more pronounced in subjects whose daily calcium intake was less than 700 mg. Moreover, the treatment effects were the best, when calcium doses of 1200 mg or more were used. However, controlled clinical trials in older men and women have also shown that daily calcium intakes close to and above the tolerable upper limit of intake increase the risk of cardiovascular events, indicating that the adequate daily intake level of calcium should not be exceeded by too high a margin. (*see* **Milk Salts**: Macroelements, Nutritional Significance).

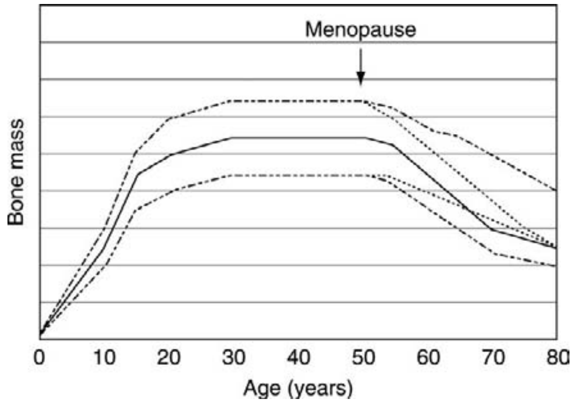

Figure 1 Changes in bone mass during lifetime. The solid line indicates the mean age-related change in bone mass. The dotted lines indicate the deviation in bone mass accretion during growth and early adulthood and the deviation of bone mass loss in elderly subjects.

Consumption of Dairy Foods in the Evolutionary Context

Dairy foods represent a distinct group in human nutrition and have a relatively high calcium content. Calcium in nondairy sources is less concentrated, making it difficult to meet the recommended dietary allowance of 1000–1300 mg day^{-1} for adolescent/adult subjects without concomitant consumption of dairy foods or supplements (**Table 1**). Cereal products and meat products are low in calcium content, but relatively high in energy content. Hence, without the consumption of dairy products, even an adequate energy intake can be associated with an insufficient calcium ingestion.

Dairy foods have not been part of the diets of adults for the most part of human evolution. In prehistoric times, a high daily calcium intake of approximately 1600–1800 mg was achieved primarily through ingestion of calcium-rich wild fruits and wild vegetables. Cereal grains were seldom consumed by hunters and gatherers, but these foods became a major part of the diet after the adoption of agriculture. In parallel to this adoption and to the domestication of dairy animals, selected populations began to use dairy products regularly well past the age of weaning. As a consequence, intestinal β-galactosidase activity remained high in the majority of these populations, for example, in white adults living in Central and Northern Europe. Hence, these people were able to consume milk without the occurrence of any gastrointestinal side effects like cramps, flatulence, and diarrhea.

Vitamin D promotes intestinal calcium absorption. In Europe there is a lack of ultraviolet rays in the sunlight during wintertime, resulting in an insufficient skin synthesis of vitamin D. The low vitamin D status during wintertime results in a concomitant low rate of calcium absorption. Nevertheless, lactose tolerance enabled the

Table 1 Calcium content and calcium–energy relations of some animal products, cereals, and wild plant foods

Food	*Calcium (mg/100 g)*	*Energy (KJ/100 g)*	*Ca/energy ratiomg (mg MJ^{-1})*	*rND*[a]
Animal products[b]				
Pork	3	443	6.8	0.05
Chicken	12	693	17.3	0.13
Beef	6	454	13.2	0.10
Salmon	13	842	15.4	0.11
Egg	56	646	87	0.64
Cereals[b]				
Wheat	38	1310	29	0.22
Rye	64	1245	51	0.38
Maize	15	1385	10.8	0.08
Oat	80	1478	54.1	0.40
Wild plant foods[c]				
Underground storage organs (*n* = 39)	122	395	1545	11.4
Fruits (*n* = 39)	84	492	452	3.35
Nuts (*n* = 9)	129	1310	415	3.07
Legumes (*n* = 14)	151	1371	556	4.12

[a] rND = relative nutrient density; ratio of the Ca density of a food (mg Ca MJ^{-1}) to the recommended Ca density (mg MJ^{-1}) of a distinct population group (here postmenopausal women, aged 51–65 years, 135 mg M J^{-1})
[b] Adapted from Souci SW, Fachmann W, and Kraut H (1994) *Food Composition and Nutrition Tables*. 5th revised and completed edn. Stuttgart: Medpharm GmbH Scientific Publishers.
[c] Adapted from Eaton SB and Nelson DA (1991) Calcium in evolutionary perspective. *American Journal of Clinical Nutrition* 45: 281S–287S.

European populations to increase the amount of absorbed calcium by simply increasing their oral calcium intake. Lactose tolerance could, thus, decrease the risk of rickets and female pelvis deformations in former times, which might have been important for the survival of the European population groups.

Calcium Content of Dairy Products

The calcium content of dairy products is listed in **Table 2**. Of all dairy products, cheeses, especially hard cheeses, have the highest calcium content, because of their low water content. One portion (30 g) of Emmental cheese, for example, delivers approximately 300 mg calcium, whereas the calcium content of one portion of fresh cheese (30 g) is only 24 mg. In the western diet, dairy products are the most important sources of calcium. Representative nutrition surveys have shown that approximately 60–70% of the daily calcium intake comes from these foods. The calcium/energy ratio of most dairy products is high and the relative calcium density is clearly above the recommended level of 1. Exceptions are fresh cheese and the nondairy product milk chocolate.

Milk and some foods with a high milk content are rich in lactose (**Table 3**). However, most cheeses

Table 3 Lactose content of dairy products

Food	*Lactose (g/100 g)*
Milk	
Cow milk (3.5% fat)	4.8
Skimmed milk	5.0
Milk-containing products	
Chocolate	9.5
Ice cream	6.7
Pudding	4.5
Dairy products (except cheese)	
Buttermilk	4.0
Yogurt (3.5% fat)	4.0
Cream (10% fat)	3.2
Cheese	
Fresh (min. 60% fat in dry matter)	2.6
Brie (50% fat in dry matter)	0
Limburger (40% fat in dry matter)	0
Camembert (45% fat in dry matter)	0
Edam (45% fat in dry matter)	0
Emmental (45% fat in dry matter)	0

Adapted from Souci SW, Fachmann W, and Kraut H (1994) *Food Composition and Nutrition Tables*. 5th revised and completed edn. Stuttgart: Medpharm GmbH Scientific Publishers and from Renner E and Renz-Schauen A (1996) *Nutrition – Composition Tables of Milk and Dairy Products*. Gießen: M. Drathen Publishers.

Table 2 Calcium content and calcium–energy relations of dairy products

Food	*Calcium (mg/100 g)*	*Energy (KJ/100 g)*	*Ca/energy ratio (mg MJ^{-1})*	*rND*[a]
Milk				
Cow milk (3.5% fat)	120	269	446	3.30
Skimmed milk	123	147	839	6.21
Goat milk	127	281	452	3.35
Milk-containing products				
Chocolate	214	2242	96	0.71
Ice cream	140	856	164	1.22
Pudding	100	393	255	1.88
Dairy products (except cheese)				
Buttermilk	109	157	694	5.14
Yogurt (3.5% fat)	120	293	410	3.04
Cream (10% fat)	101	510	198	1.47
Cheese				
Fresh (min. 60% fat in dry matter)	79	1407	56	0.42
Brie (50% fat in dry matter)	400	1430	280	2.07
Limburger (40% fat in dry matter)	534	1111	481	3.56
Camembert (45% fat in dry matter)	570	1185	481	3.56
Edam (45% fat in dry matter)	678	1469	461	3.41
Emmental (45% fat in dry matter)	1020	1593	640	4.74

[a] rND = relative nutrient density; ratio of the Ca density of a food (mg Ca MJ^{-1}) to the recommended Ca density (mg MJ^{-1}) of a distinct population group (here: postmenopausal women, aged 51–65 years, 135 mg MJ^{-1})
Adapted from Souci SW, Fachmann W, and Kraut H (1994) *Food Composition and Nutrition Tables*. 5th revised and completed edn. Stuttgart: Medpharm GmbH Scientific Publishers.

are lactose-free owing to the bacterial degradation of sugars during cheese ripening. Cheese is therefore well-tolerated even by people with lactose intolerance. Moreover, nonheated yogurt is an alternative source of calcium for people with lactose malabsorption, even though yogurt contains significant amounts of lactose. But yogurt also contains relatively high amounts of bacterial β-galactosidase and this enzyme can be reactivated in the human gastrointestinal tract to metabolize the lactose from yogurt.

Calcium Bioavailability from Dairy Products

Besides its high calcium content, milk has a good calcium bioavailability (~30%). A few nondairy foods, like spinach, sesame, kale, and certain mineral waters, also are rich in calcium content. However, though the calcium absorption from kale and from calcium-rich mineral water is comparable to that from milk, calcium bioavailability from sesame (783 mg Ca per 100 g) and from spinach (126 mg Ca per 100 g) is much lower than that from milk and clearly below 10%.

Contrary to earlier suggestions, lactose content of milk has no beneficial effect on intestinal calcium absorption. In lactose-tolerant subjects, lactose is rapidly digested in the upper small intestine indicating that no lactose-specific mechanism exists to enhance intestinal calcium uptake. Moreover, postprandial parathyroid hormone response, an indirect indicator of the amount of calcium absorbed, does not differ after ingestion of similar amounts of calcium from milk and from (lactose-free) ripened cheese.

The native vitamin D content of milk is low. However, in some regions of the world, for example, North America, fortification of milk with vitamin D is obligatory (10 μg per US quart (= ~0.95 l)). In other regions, for example, in Europe, such a measure is unusual or even prohibited. A better vitamin D nutrition may improve vitamin D status and may, thus, contribute to an improved intrinsic ability to absorb calcium. In particular, home-bound elderly people or elderly persons living in nursing homes often have an insufficient vitamin D status and a very low calcium intake (often below 500 mg day^{-1}). They might improve their calcium balance through a higher intake of (vitamin D-fortified) milk.

Calcium/Sodium Ratio of Different Dairy Products

The sodium content of dairy products can vary widely (**Table 4**). The differences are mostly due to the addition of sodium chloride during the technological process of cheese production. Sodium chloride has a calciuretic effect because of sodium–calcium exchange in the proximal renal tubule. An additional sodium intake of 1 g (43 mmol) has been associated with a renal calcium loss of 20–40 mg (0.5–1.0 mmol). If unremedied, the extra sodium would result in a skeletal loss of approximately 1% per year.

The calcium/sodium ratio of dairy foods varies from approximately 2.7 for milk to 0.21 for fresh cheese. Even in different cheeses, the calcium/sodium ratio can vary by a factor of 10. Young adults are able to adapt to a high sodium chloride intake owing to an increase in gastrointestinal calcium absorption and/or a decrease in

Table 4 Protein and mineral content of dairy foods

Food	*Sodium (mg/100 g)*	*Calcium/sodium ratio (mg mg^{-1})*	*Potassium (mg/100 g)*	*Protein (g/100g)*	*Calcium/protein ratio (mg:g)*
Milk					
Cow milk (3.5% fat)	45	2.67	141	3.34	35.9
Skimmed milk	53	2.32	150	3.50	35.1
Goat milk	42	3.02	181	3.69	34.4
Dairy products (except cheese)					
Buttermilk	57	1.91	147	3.50	31.1
Yogurt (3.5% fat)	48	2.50	157	3.88	30.9
Cream (10% fat)	40	2.52	132	3.10	32.6
Cheese					
Fresh (min. 60% fat in dry matter)	375	0.21	95	11.30	7.0
Brie (50% fat in dry matter)	1170	0.34	152	22.60	17.7
Limburger (40% fat in dry matter)	1300	0.41	128	22.40	23.8
Camembert (45% fat in dry matter)	975	0.59	110	21.00	27.1
Edam (45% fat in dry matter)	654	1.04	67	24.80	27.3
Emmental (45% fat in dry matter)	450	2.27	107	28.70	35.5

Adapted from Souci SW, Fachmann W and Kraut H (1994) *Food Composition and Nutrition Tables*. 5th revised and completed edn. Stuttgart: Medpharm GmbH Scientific Publishers.

endogenous fecal calcium loss. However, such a mechanism probably no longer exists in postmenopausal women and elderly people. Consequently, in postmenopausal women sodium chloride can induce a rise in bone resorption processes.

Calcium/Protein Ratio of Different Dairy Products

Protein intake is related to renal calcium excretion. To offset protein's calciuretic effect, calcium allowances have been recommended at a calcium/protein ratio (in mg:g) of 20:1. In milk this ratio is 36:1 and, thus, more favorable than the recommended value. In other dairy products, however, the calcium/protein ratio varies between 35:1 (Emmental cheese) and 7:1 (fresh cheese). Although a low calcium/protein ratio may increase the risk of a negative calcium balance, there is evidence that, at least in young subjects, the higher renal calcium loss during high protein intake can be compensated by an increase in intestinal calcium absorption rate.

Dairy protein consumption may also be beneficial to bone health. The protein value of dairy foods is high, and an adequate protein intake is important for maintaining muscle mass. Especially, elderly subjects are at an increased risk of inadequate protein intake. The Framingham Osteoporosis Study has demonstrated that elderly persons in the lowest quartile of protein intake, which is below the recommended daily protein intake, have the highest bone loss. Similar to the overall protein effect, a lower percentage of animal protein is related to bone loss at specific skeletal sites of increased osteoporotic fracture risk (spine and femoral sites).

Potassium Content of Dairy Products

Potassium appears to play an important role in protecting against calcium loss from the renal acid load of protein. The mammary gland is able to concentrate the potassium content against a high serum gradient. Frequent intake of certain dairy products like milk and yogurt can significantly contribute to the total daily potassium intake of 2–3 g. Epidemiologic data indicate a protective effect of potassium intake on bone density. Administration of potassium to postmenopausal women at significantly higher levels than the recommended protein intake has been found to decrease urinary excretion of the bone resorption marker hydroxyproline and increase serum osteocalcin, a marker of bone formation.

Is the Consumption of Dairy Products a Risk Factor for Osteoporosis?

Some cross-cultural comparisons have reported positive correlations between calcium intake and fracture risk. Moreover, some epidemiologic studies indicate a positive association between the intake of animal protein and the incidence of osteoporosis. In western diets, both calcium intake and protein intake are closely linked to the intake of dairy products. In some nonscientific publications, the relatively high consumption of dairy products in western societies has been made responsible for the high risk of osteoporosis. However, the cross-sectional nature of the above-mentioned data and the absence of controls for confounding factors such as genetic and lifestyle differences point toward a fluke association.

To obtain valid scientific data about the association between dairy consumption and bone health it is absolutely necessary to perform controlled clinical trials.

Lactose Intolerance and Bone Health

The impact of consumption of dairy products on bone health can be elucidated by examining the risk of osteoporosis in lactose absorbers and lactose malabsorbers of a population group. The prevalence of β-galactosidase deficiency in Central and Northern Europeans and their descendants ranges from 3 to 30% and rises up to approximately 50% in the subgroup of elderly subjects. As milk consumption can cause gastrointestinal symptoms in lactose malabsorbers, a marked reduction in milk consumption (and often also in the consumption of other dairy products) can be the consequence. White females with β-galactosidase deficiency have a mean lactose intake of 6–7 g day^{-1}, which is only one-third to one-half of the regular lactose intake of their lactose-tolerant counterparts. In parallel, dietary calcium intake also can get reduced by 50%. In clinical studies, a higher prevalence of lactose malabsorption has been found in osteoporotic than in non-osteoporotic women: prevalence of lactose malabsorption ranged from 26 to 65% in osteoporotic women and from 0 to 20% in nonosteoporotic women. However, lactose malabsorption is not the only reason for a spontaneous avoidance of milk and milk products. The true culprit for motivating the avoidance of lactose-related foods in the diet is not malabsorption as such, but gastrointestinal intolerance due to malabsorption of this carbohydrate. Lactose malabsorption, in fact, does not always lead to intolerance. Reasons for the variability of gastrointestinal symptoms among individual malabsorbers are the ability of the colon to absorb short-chain fatty acids derived from the bacterial fermentation of malabsorbed lactose; hydrogen consumption by colonic methanogenic bacteria and/or reduced colonic pH, which could lead to a significant decrease in the volume of colonic gas; and the possible induction of colonic bacterial lactase by lactose malabsorption. When lactose malabsorption is accompanied by symptoms of intolerance it represents a particular risk factor for osteoporosis.

Although yogurt and cheese are normally well-tolerated by subjects with lactose intolerance (see above), those who are not aware of this connection avoid these dairy products and are, thus, at an increased risk of inadequate dietary calcium intake. Approximately 50% of the subjects diagnosed with lactose malabsorption tolerate one single portion of 10 g lactose (equivalent to 250 ml milk) without sustaining gastrointestinal symptoms.

In the past, the association between β-galactosidase deficiency and osteoporosis has been questioned because African-Americans, among whom β-galactosidase deficiency occurs in approximately 90% of the adult population, have a lower incidence of osteoporosis than do whites. However, this discrepancy can be explained by the different genetic background, resulting in a higher initial bone mass, a resistance of bone to calciotropic hormones, and an altered bone remodeling, of African-Americans when compared to whites. These associations, however, are not an argument against the prevention of osteoporosis by a higher intake of dairy products in the white population.

Controlled Trials on the Effect of Dairy Products on Bone Health

Only randomized controlled trials can confirm or reject the hypothesis that adequate milk and dairy product consumption supports good bone health. Such a study design is mandatory, as epidemiological studies are not able to accurately assess dietary intake. Even a large sample size does not overcome that weakness of epidemiological studies. During the last decade, some interventional studies have been performed on pubertal/adolescent girls. These investigations have demonstrated a beneficial effect of dairy foods on bone health. No unfavorable results for this category have been published. Postintervention observations indicate that it is mandatory to continue with a lifelong high-dairy product diet to preserve the positive effects on bone health. The limited number of studies done so far do not allow the determination of the effect of dairy foods consumption on bone density. However, with an additional intake of 300 ml of milk per day it seems possible to increase the increment in bone mineral density during puberty by more than 10%. The beneficial effect may occur at least due to the higher dietary intakes of calcium, vitamin D, and protein.

In postmenopausal white women, diet supplementation by milk powder can result in cessation of bone loss at the hip. There is also evidence from controlled trials that school milk intervention and milk supplements, respectively, can increase bone mineral density in Chinese girls and young Japanese women, and in postmenopausal Chinese women. But increasing the consumption of dairy products alone seems to be less beneficial to postmenopausal bone mineral content than combining it with an additional restriction of dietary sodium chloride intake. It should be noted that no unfavorable results of interventional trials of dairy consumption on postmenopausal bone health have been reported.

Conclusions

There is evidence that adequate dairy foods consumption supports good bone health in adolescent girls and probably in postmenopausal women. Dairy products can contribute to adequate calcium and protein intake and, thus, to the maintenance of muscle and bone mass. However, some dairy products, particularly some processed cheese products, have a high sodium chloride and protein content relative to their calcium content. In postmenopausal women in particular, these nutrients can possibly adversely affect calcium metabolism. Accordingly, not all dairy products are equally good vehicles of calcium, and they may, hence, differ in their effect on bone mineralization.

There have been too few studies on white males and other ethnic groups, except Chinese females, for conclusions to be drawn about the effect of any dairy food on bone health among those groups.

See also: **Lactose and Oligosaccharides**: Lactose Intolerance. **Milk Salts**: Macroelements, Nutritional Significance. **Nutrients, Digestion and Absorption**: Absorption of Minerals and Vitamins.

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Nutritional and Health-Promoting Properties of Dairy Products: Colon Cancer Prevention

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Introduction

Worldwide, colorectal cancer ranks fourth in frequency of all cancers among men and third in women, with an estimated 1 million new cases being diagnosed annually around the globe. Though incidence rates are highest in the developed world – it is the second most common malignancy affecting humans in westernized cultures – they are increasing elsewhere, especially in Asia. Given that most instances of colorectal cancer arise from a benign polyp, screening and surveillance programs to detect polyps at a premalignant stage have been initiated in several countries and promise to reduce colorectal cancer incidence and mortality significantly. Parallel research into the genetics and molecular biology of this cancer has not only significantly enhanced our understanding of its pathogenesis, but also has provided some optimism for the future development of simpler, less-invasive diagnostic methods. Most colorectal cancers are sporadic, with only a small number (2–3%) arising in the context of genetic syndromes such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC, also known as the Lynch syndrome) or of preexisting diseases such as inflammatory bowel disease. A family history of one or more affected first-degree relatives has been obtained in 20% of cases. The sporadic form of the disease is now thought to represent the end result of a multistep genetic process (the adenoma–carcinoma sequence) involving mutations in a number of key genes that normally regulate cell growth and differentiation. A better understanding of the pathogenesis of the disease, including genotypic changes and phenotypic expression at different stages, provides the opportunity both for elucidating its mechanisms and for identifying the points at which reversal of the disease process might be possible with appropriate interventional, including dietary, strategies (**Figure 1**). Though our understanding of initiatory factors is poor, it is nevertheless clear that, along with aging (over 50% of cases occur among those over 50 years of age), diet can have a significant promotional or inhibitory effect on the disease. In some societies such as African nationals eating traditional diets the disease is virtually unheard of, whereas in some other societies it occurs with a prevalence of 30–50 cases per 100 000 population. Within some high-incidence societies a threefold difference in expression has been identified between urban and rural communities and between differing geographical climes with varying food–lifestyle preferences, for example, northern versus southern populations of India, Italy, and Sweden. The relevance of diet can also be readily deduced from the impact on colorectal cancer incidence of changes in dietary habits and, specifically, the effects of a move to a 'westernized' diet, whether occurring within a country (such as has happened in Japan) or to groups of individuals on migrating to the West.

With regard to diet and lifestyle, a number of factors such as the consumption of meat, animal fat, tobacco, and alcohol have been shown to increase risk, whereas high dietary intakes of fiber, fruit, and vegetables have appeared to be protective. Other features such as ethnic origin, physical inactivity, excess body weight, and central adiposity may also confer increased risk. It must be stressed, however, that the interpretation of studies on the relationships between diets or dietary components and colorectal cancer is fraught with difficulties. Confounding factors abound; those who eat more vegetables, for example, typically consume less meat, take more exercise, and are less likely to be obese; dissecting out the relative contributions of each of these factors in epidemiological studies and from dietary surveys is almost impossible. The issue of confounding factors must similarly be borne in mind when assessing the literature on the impact of dairy food consumption. A further complication in this instance is the theoretical possibility of different components of dairy products conferring diametrically opposite effects on cancer risk: calcium, for example, potentially reduces risk, while the high fat content of dairy foods may increase risk. Furthermore, colorectal cancer, though often treated in epidemiological studies as a single homogeneous entity, in reality comprises at least three distinct types: right-sided, left-sided, and rectal cancer, which not only demonstrate different clinical behaviors, but may have quite distinct molecular paths. It should come as no surprise, therefore, that though the study of dietary eating patterns and their influence on

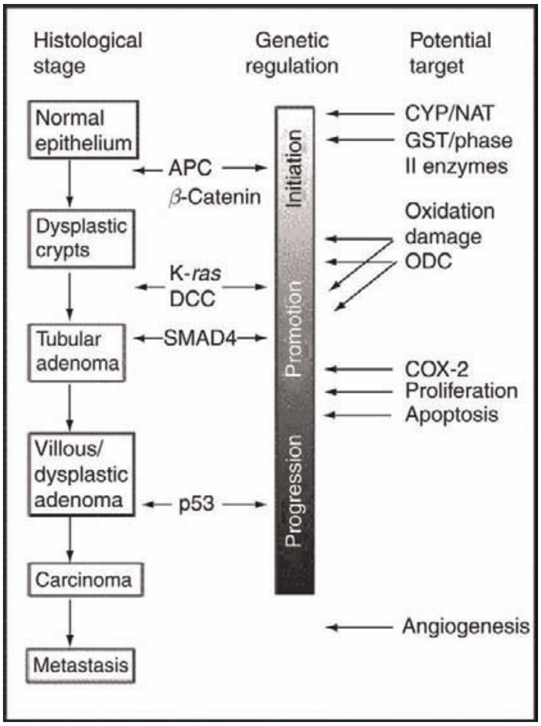

Figure 1 Multistep model of carcinogenesis with targets for chemoprevention. Significant genes involved in regulation at sequential stages in carcinogenesis include adenomatous polyposis coli (APC), K-*ras*, DCC, and p53. A number of inducible enzymes are involved at critical stages in promotion and/or protection. COX-2, cyclooxygenase-2; CYP, cytochrome P450; DCC, deleted in colorectal cancer; GST, glutathione *S*-transferase; NAT, *N*-acetyl transferase; ODC, ornithine decarboxylase. Reproduced with permission from Sharma RA, Manson MM, Gescher A, and Steward WP (2001) *European Journal of Cancer* 37: 12–22.

colon cancer risk has produced some detailed and comprehensive analyses of populations at risk, it has also led to some inconsistent results. For example, the 1997 report of epidemiological evidence by the World Cancer Research Fund and American Institute for Cancer Research came to the conclusion that "the evidence on the relationship between colon cancer and dairy products is inconsistent; no judgement is possible", adding the ambivalent statement "any increased risk may be due to fat, whereas decreased risk may be the consequence of vitamin D and calcium". The latter statement acknowledged the potential for protection, and that there was substantial support for several possible agents being effective in this respect. In examining the issue of dairy products, one must also be fully aware of dramatic variations in the consumption of milk and dairy products, as well as of the diversity of such products among countries and among regions within countries.

Some of the earliest case–control and epidemiological studies pointed to dairy foods such as milk, cheese, and/or other fermented dairy foods as being protective. The suggestion that dairy fat has a negative influence owing to its calories promoting cancer may be too simplistic, as there are a number of components in dairy fat (e.g., sphingolipid, vitamin D, conjugated linoleic acid (CLA), butyrate) that distinguish it from animal carcass fat and might be instrumental in protecting against cancer. There is significant evidence supporting the view that dairy products do not increase, but rather decrease risk. The presence of significant amounts of dairy food in the diet may also allow interactions or synergies with other major components of the diet, such as cereal grain foods. Commercial food processing is now an inevitable part of food preparation in western food cultures, and some significant changes may result, such as loss of nutritional value and production of reaction products (Maillard reaction products) that might bring about their own influences, such as antioxidant effects and/or altered digestibility. This may then influence the impact of such food preparations on large bowel health and colon cancer risk.

Studies of individual components of dairy foods and their influence on *in vitro* cancer cells and animal models of cancer have provided useful insights into possible mechanisms whereby they might reduce risk in humans; such studies have demonstrated anticancer effects of a variety of components in dairy products, including calcium, CLA, whey proteins, lactoferrin, α-lactalbumin, lactose, sphingolipids, and probiotics (in fermented milk products). However, translation of such experimental data to clinical impact has been more problematic and, in most instances, their relevance to man remains to be confirmed in carefully controlled dietary intervention studies. In examining the relationship between dairy foods and colon cancer, epidemiological and intervention studies will be discussed, along with what is known regarding the mechanisms of the effects of dairy foods and components on colon cancer expression.

Epidemiology

Studies spanning several decades have presented evidence that dairy foods, including fermented products, are associated with longevity and reduced risk of several cancers, including colon cancer. As long ago as 1933, a case–control study involving 462 cancer cases and matched controls in London, England, noted that cancer patients were more prone to have constipation, drank less milk, and ate less wholemeal bread and vegetables than controls. Milk and vegetable consumption was singled out as being worthy of further study. Since then, there have been case–control and/or cohort studies in India, Sweden, Finland, Russia, France, Italy, The Netherlands, Australia, and the United States, which have largely confirmed a protective effect for dairy foods against adenomas and/or colon cancer.

Three recent reviews have critically evaluated these data. Cho and colleagues pooled data from 10 cohort studies including a total of 534 536 individuals, among whom 4992 cases of colorectal cancer occurred over a 6- to 16-year follow-up period. Comparing the highest level of milk intake ($\geq 250\,\mathrm{g\,day^{-1}}$) with the lowest ($<70\,\mathrm{g\,day^{-1}}$) revealed a 15% reduction in distal colon and rectal cancers but not in proximal colon cancers. They went on to estimate that each $500\,\mathrm{g\,day^{-1}}$ increase in milk intake was associated with a 12% reduction in colorectal cancer incidence. Nonsignificant trends were observed for cheese and yogurt intake. Calcium was also protective, the highest level of intake being associated with a 14–22% reduction in colorectal cancer. In contrast, the protective effect for vitamin D was seen only when associated with the highest levels of calcium intake. Pufulete, reviewing the literature, also concluded that milk consumption is protective, but noted that this effect seemed greater for skim milk than for full-fat milk. She also contended that evidence for a protective effect of cheese, though suggestive, was inconclusive and that there was no apparent effect of fermented milk products. Huncharek and colleagues performed a meta-analysis of 26 335 cases from 60 observational studies and confirmed a significantly reduced relative risk (0.78) of colon cancer for high intakes of milk and dairy products. No protection was evident for rectal cancers. High calcium intake, in contrast, appeared protective for both distal colon and rectal cancer, and evidence for an independent effect of vitamin D was unimpressive. It should be noted, with regard to the latter, that dairy foods offer only modest amounts of dietary vitamin D (milk $30–40\,\mathrm{IU\,l^{-1}}$) unless fortified, as practiced in the United States. One recent study from Sweden deserves special attention; here, Larsson and colleagues prospectively examined relationships between high-fat dairy food consumption and CLA intake and colorectal cancer incidence among 60 708 women in the Swedish mammography cohort during an average of 14.8 years of follow-up. Comparing the highest intake of high-fat dairy foods ($\geq$4 servings per day) with the lowest intake (<1 serving per day) revealed a relative risk of 0.59 for colorectal cancer; this effect was greater for distal colon cancer (0.28) than for proximal colon (0.84) and rectal (0.62) cancers. These effects were independent of calcium intake. Protective effects of cheese, full-fat cultured milk, and butter, but not of whole milk, were also noted. The highest quartile of CLA intake was associated with a relative risk of 0.71 when compared with the lowest quartile of CLA intake. This study must be interpreted in the context of a population whose milk, cheese, and other milk product intake is considerably higher than average.

Intervention Studies

Given the long lead time associated with the development of colorectal cancer, intervention studies have used a number of surrogate measures to identify reduced cancer risk, with colonocyte proliferation and polyp recurrence being two of the most commonly used measures. Even though most cancers originate from adenomatous polyps, only 1–10% of polyps will progress to cancer. Other studies have examined fecal water cytotoxicity and/or genotoxicity, as well as other metabolic markers such as pH and the concentrations of secondary bile acids and butyrate, as possible biomarkers of risk. People at risk of developing sporadic polyp and colon cancer have regularly been enlisted for such intervention studies – that is, men and women aged 50 years and over, when incidence begins to increase dramatically.

One study examined the influence of removing dairy foods from the diet on risk markers in a group of healthy young male and female volunteers (**Table 1**). There was a 13% decrease of energy intake following the removal of dairy foods, mainly as a result of lower fat and protein ingestion, though carbohydrate and fiber remained constant. Calcium was lowered from 1488 to $372\,\mathrm{mg\,day^{-1}}$, which indicated that the dairy portion accounted for 75% of daily calcium intake.

Phosphate and vitamin D were lowered similarly. Fecal water was extracted from stools and cytotoxicity and genotoxicity measured using *in vitro* HT29 colon cancer cells and the comet assay. Cytotoxicity increased significantly in fecal water in the absence of dairy foods (seen as lower HT29 cell survival), whereas genotoxicity was not affected. This result pointed to dairy foods

Table 1 Human fecal water parameters in dairy-rich and dairy-free diet period

	Dairy product-rich	*Dairy product-free*	*Significance*
Wet weight of feces	130 ± 63	115 ± 50	ns
pH of fecal water	7.0 ± 0.5	6.7 ± 0.3	0.012
%HT29 cell survival	34 ± 28	20 ± 22	0.025
Caco-2 genotoxicity	6.1 ± 5.7	6.5 ± 5.9	ns

Eighteen healthy male and female volunteers were enrolled in a crossover design. Mean±SD $n = 18$.
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Table 2 Influence of calcium supplements on colorectal adenoma recurrence over a 4-year period of intervention in postpolypectomy subjects

Calcium supplemented daily for 4 years (1200 mg) placebo with 409 423 subjects with a recent history of adenomas – repeated colonoscopies

Adjusted risk ratio: adenoma recurrence was 0.81 (CI = 0.67–0.99, $p = 0.04$)

Adjusted ratio: average number of adenomas was 0.76 (CI = 0.6–0.96, $p = 0.02$)

Calcium supplementation is associated with a significant though moderate reduction in the risk of recurrence, independent of initial dietary fat or calcium intake

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providing significant protection, a result that was attributed to the presence of increased calcium and phosphate concentrations in the colon.

Calcium has been shown to reduce significantly the proliferation of colonocytes in the upper region of colonic crypts; this is another indicator of risk. A calcium polyp prevention study involving a randomized double-blind trial showed a 20–25% reduction in polyp recurrence over 4 years, associated with a daily supplement of calcium (1200 mg) as calcium carbonate. This result was independent of fat or calcium intake in the background diet (**Table 2**).

A large European randomized double-blind intervention trial study showed a slight but significant reduction in polyp recurrence with a daily ingestion of 2 g calcium as calcium gluconolactate and carbonate. This reduction was not seen in the other arm of the study, which examined the effect of a soluble psyllium fiber supplement of 3.5 g day^{-1}.

A recent Cochrane review, assessing these two studies, concluded that though calcium supplementation might contribute to a moderate degree to the prevention of colorectal cancer, there was no sufficient evidence to justify recommending calcium supplementation to prevent colorectal cancer. Intervention studies with vitamin D have been disappointing.

Mechanisms

Calcium and Vitamin D

The influence of calcium in the diet on colon function and cancer prevention has been investigated with animal modeling of a westernized diet, characterized by low calcium and vitamin D, high phosphate, high fat, and low fiber. The western diet is associated with an elevated epithelial cell proliferation and an increased tendency to develop aberrant crypts and tumors induced in rodents by 1,2-dimethylhydrazine or azoxymethane. Though the responsible mechanisms may be only partially understood, there is now good experimental evidence for significantly increased fecal water cytotoxicity associated with the diet, probably as a result of free fatty acids, secondary bile acids from microbiological activity, but possibly also resulting from genotoxic products of microbiological origin. The influence of these products is effectively diminished or eliminated in the presence of adequate calcium by neutralization and/or precipitation as innocuous soaps. A lowered pH may influence this process, through the fermentative production of short-chain fatty acids (**Figure 2**). Vitamin D assists with calcium uptake and utilization from the diet, and this has also been shown to affect cell function and diminish proliferation at the level of colonocytes in the large bowel (*see* **Milk Lipids:** Nutritional Significance; **Milk Salts**: Macroelements, Nutritional Significance; **Vitamins**: Vitamin D).

Probiotics

Probiotic bacteria have been shown to reduce the expression of tumorigenesis in animal models of colon cancer. Epidemiological evidence has been inconsistent in showing protection, even though there are some studies that showed clear benefits of fermented dairy foods, as mentioned previously. The main probiotic bacteria used in dairy foods have been strains of *Lactobacillus acidophilus* and bifidobacteria species, which are regularly added to fermented dairy foods like yogurt because of their perceived functional benefits to gastrointestinal health. Although a number of possible benefits in antimutagenic and anticarcinogenic terms have been reported, which have been demonstrated by *in vitro* and animal studies,

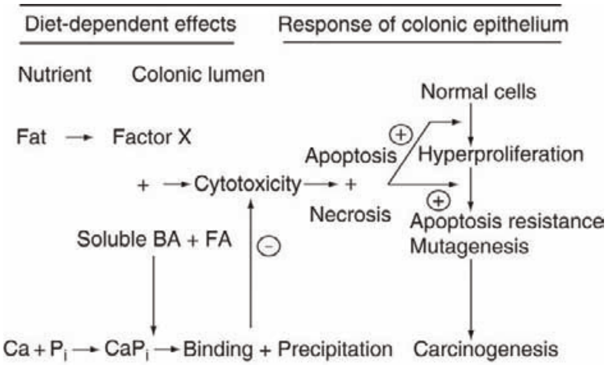

Figure 2 Proposed mechanism of the effects of dietary fat, calcium, and phosphate on colon carcinogenesis. BA, secondary bile acids; FA, fatty acids; CaP$_i$, insoluble amorphous calcium phosphate. Reproduced with permission from Van der Meer R, Lapre JA, Govers MAP, and Kleibeuker JH (1997) *Cancer Letters* 114: 75–83.

convincing evidence from human studies is lacking. On the other hand, benefits in the control of diarrhea, improved immune responsiveness, alleviation of constipation, and treatment of food allergies have been reported and appear to be promising potential applications (*see* **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products).

Dairy Proteins

Some epidemiological and experimental studies provide evidence supporting a protective role of dairy proteins relative to red meat, eggs, and some other alternative protein sources. Experimental investigation has been made possible by the availability of concentrated purified protein products (isolates and concentrates). Whey proteins, in particular, appear to offer significant protection in the rodent model of chemically induced colon cancer. Though the mechanisms of protection are still not well understood, the contribution of sulfur amino acids seems to be important. Lactoferrin and α-lactalbumin are some of the richest sources of cystine (disulfide bridge compounds) and in prior studies were associated with significantly increased hepatic glutathione, an important antioxidant and xenobiotic deactivating agent (**Figures 3** and **4**). It has a major role in protecting the host against toxic/mutagenic factors.

Fat levels in feces also appear to be significantly influenced by the protein source, and this effect may have reflected associations, such as saturated fat with red meat and soluble fiber components with soy protein in soybean meal. Dairy proteins have been identified in *in vitro* assay systems as being significantly antimutagenic and/or anticarcinogenic. Whether they can play a significant role in protection against colon cancer as part of a normal human diet is not yet established, but there is some evidence that they probably do (*see* **Milk Proteins**: Nutritional Quality of Milk Proteins).

Lactose and Lactulose

The possibility that lactose and/or lactulose might have a significant influence on colon cancer risk has been proposed and is an interesting possibility deserving further investigation. Lactose intolerance is not an uncommon condition in adult populations (prevalence in Australian adults may be somewhere in the range 4–20%) and could be having a significant influence on large-bowel health. It is capable of causing significant bloating and colic, and softening of the stool, owing to active fermentation in the hindgut. Lactulose can form during the heat processing of milk (e.g., ultra-heat-treated (UHT) milk 10–50 mg 100 ml^{-1}), and is commercially produced by enzymatic processing of lactose. Daily supplements of lactulose (e.g., 8–60 g day^{-1}) have been shown to reduce stool pH, transit time, and bile acid pool size (35%) and concentration (including deoxycholic acid, a cocarcinogenic secondary bile acid), and to increase stool size and frequency. Japanese studies have shown a major shift toward bifidobacteria in the stools of people ingesting 8 g lactulose day^{-1}. In many respects, lactose (in lactose maldigesters) and its derivatives, lactulose and lactitol, behave similarly to dietary fiber, providing an enzyme-resistant carbohydrate, which is fermented in the hindgut, possibly also altering the balance of bacterial flora in the process. Products of that fermentation could include short-chain fatty acids (including butyrate); other possible effects are reduced pH and bulking and other stool changes, which might act to reduce colon cancer risk. Feeding studies in rats colonized with human gut flora showed significantly reduced DNA damage (genotoxicity) by 1,2-dimethylhydrazine in their colonocytes as a

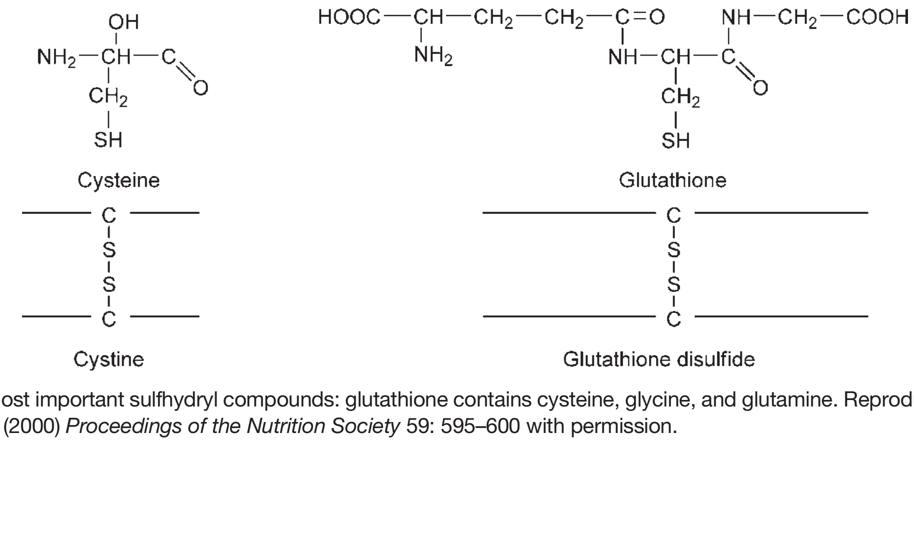

Figure 3 The most important sulfhydryl compounds: glutathione contains cysteine, glycine, and glutamine. Reproduced from Dröge W and Breitkreutz R (2000) *Proceedings of the Nutrition Society* 59: 595–600 with permission.

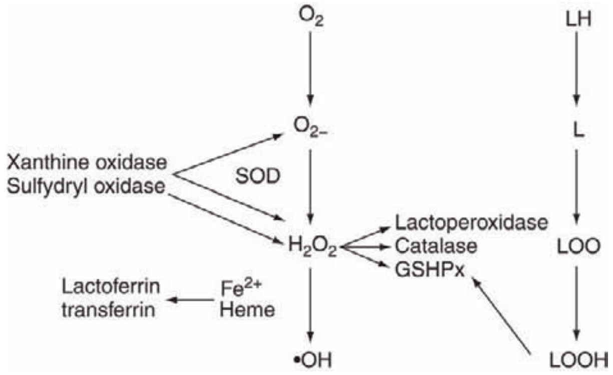

Figure 4 Proposed action of some pro- and antioxidant properties of milk. Reproduced with permission from Lindmark-Mansson H and Akesson B (2000) *British Journal of Nutrition* 84: S103–S110.

result of feeding 3% lactulose in place of sucrose in the diet. Increased lactobacilli were evident in the cecal flora of these rats, which may have provided some protection.

Sphingomyelin

The phospholipids and sphingolipids present in the fat component of milk act as a significant source of biologically active molecules, which may play an important role in signal transduction and cell regulation. Hydrolysis of sphingomyelin in the gut leads to the generation of phosphocholine and ceramide, a potent inducer of cell differentiation and programmed cell death (apoptosis). Sphingosine, a deacylated product of ceramide, is a potent inhibitor of protein kinase C, which otherwise induces significant cell proliferation and increases the risk of tumorigenesis. It competes with the inducers of this enzyme. These sphingolipids are released throughout the gastrointestinal tract during digestion and, given their antiproliferative effects, they have been referred to as tumor suppressor lipids. Along with significant amounts present in dairy fats/buttermilk, they are also found closely associated with whey protein isolates and concentrates. In chemically induced rodent colon cancer studies, sphingolipids have been shown to inhibit tumorigenesis, when present in concentrations of 0.025–0.1% sphingomyelin in a purified rodent diet.

Butyrate

The presence of significant amounts of the short-chain fatty acid butyrate in dairy fat derives from fermentation of carbohydrates in the cow's rumen and raises the possibility that it might provide some of its known antineoplastic and/or differentiating effects. There would appear to be rapid utilization of butyrate from the fat throughout the gastrointestinal tract epithelium, and hence it seems unlikely that it will reach the large intestine at least from this source. When provided in a stable form (e.g., tributyrin, a triglyceride with three butyrate molecules attached), significant systemic blood levels of butyrate were achieved, which have been associated with reduced cancer expression in a chemically induced model of breast cancer in rats. Butyrate, when present in the rat colon (direct infusions into the rat colon have been undertaken in amounts of about 400 mmol day^{-1} or by fermentative generation), leads to reduction of azoxymethane-induced aberrant crypt foci (early preneoplastic markers of cancer risk) adenomas and/or adenocarcinomas. However, when butyrate (as sodium butyrate) was included in the diet so as to deliver similar amounts, no effect was observed. At this stage, therefore, it remains speculative whether butyrate ingested through dairy products has any significant influence on colon health.

Conclusion

Some epidemiological studies have identified fermented and nonfermented dairy foods as providing significant protection against colon cancer. Though the interpretation of such data is complicated by the presence of many confounding factors, the effect of milk intake on distal colon cancer has been consistent. Though calcium, which in most diets is largely derived from milk and which is present in milk in a highly bioavailable form, has usually been considered the milk constituent most likely to confer this benefit, recent data point to the effect of milk and milk products independent of calcium.

Even though no data for intervention studies are available for milk and milk products *per se*, two intervention studies in adenoma-prone populations in Europe and the United States showed that calcium supplements in excess of 1 g day^{-1} produced a significant, though modest, reduction in adenomatous polyp recurrence rates.

Other components of dairy foods that have been shown to reduce mutagenicity or genotoxicity or diminish colon tumor formation in animal models of the disease include whey proteins, vitamin D, CLA, sphingolipids, lactulose, and butyrate. It is not possible currently to prioritize these components in terms of their potential contribution, to evaluate adequately their effects in combination, or to confirm their relevance in humans, but the fact that they occur together in dairy foods is supportive of their potential value as part of a cancer-preventing diet.

A number of possible mechanisms could be operating in cancer prevention, and this is a very productive area for ongoing research. It should in time help to confirm desirable dietary strategies, as well as to predict any modifications to dairy foods that are likely to be of additional benefit in this respect.

See also: **Bacteria, Beneficial**: Probiotics, Applications in Dairy Products. **Milk Lipids**: Conjugated Linoleic Acid;

Nutritional Significance. **Milk Proteins**: Nutritional Quality of Milk Proteins. **Milk Salts**: Macroelements, Nutritional Significance. **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake. **Vitamins**: Vitamin D.

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Nutritional and Health-Promoting Properties of Dairy Products: Fatty Acids of Milk and Cardiovascular Disease

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Introduction

Milk from domesticated animals has been used as a food item for more than 8000 years. Early in the twentieth century, public health bodies realized the nutritional benefits of milk and its products and recommended their consumption, especially for children and the elderly. However, during the past four decades, the nutritional image of milk and its products has suffered a decline because its content of saturated fatty acids (SFAs) is believed to increase serum cholesterol levels, which is considered a risk factor for coronary heart disease (CHD) and cardiovascular disease (CVD). Despite half-a-century of intensive research into prevention of CHD, multiple public health messages that largely advise reduction in SFA intake and lowering of serum cholesterol levels, and the production of 'heart-healthy' products by the food and pharmaceutical industries, CHD is still the leading cause of death in developed countries. This article examines, in a historical context, the strength of the evidence that associates SFA intake with the risk of CHD.

Early History

The introduction of penicillin in 1941, with other antibiotics following rapidly, resulted in infectious diseases, formally the leading cause of morbidity and mortality, being brought under control and as a result life expectancy increased. However, an alarming rise in the incidence of CHD was observed. At this time, the cause or causes of CHD were largely unknown. In 1948, the then National Heart Institute instituted the first longitudinal study to determine the risk factors for CHD among residents, aged 35–59 years, from the town of Framingham in Massachusetts. The first report from the Framingham study appeared in 1961 and noted three risk factors: elevated serum cholesterol levels, high blood pressure (BP), and an electrocardiography pattern of left ventricular hypertrophy.

Many later studies, including several large well-known prospective epidemiological studies conducted in different countries, also found a positive association between serum cholesterol levels and the risk of CHD. Subsequently, other important risk factors were established, such as cigarette smoking, obesity, lack of physical activity, diabetes, psychosocial issues, and elevated serum homocysteine levels. It is interesting to note that in the often-cited Seven Countries Study, 25-year follow-up data showed that serum cholesterol levels were linearly related to CHD mortality in all participating countries. However, the absolute levels of CHD mortality were strikingly different. At a serum cholesterol level of $5.2\,\mathrm{mmol\,l^{-1}}$, there was a fivefold greater mortality rate in Northern Europe than in Japan. These differences in mortality risk applied after adjusting for age, smoking, and systolic Blood Pressure (BP). This study suggests that there are other powerful, yet to be determined, risk factors for CHD in industrialized communities.

In 1954, before the first report from Framingham, Ahrens and colleagues from the Rockefeller Institute in New York reported the results of a carefully conducted metabolic ward study, which showed that when plant fats were substituted isocalorically for animal fats in men, there was a significant reduction in serum cholesterol level. This group later showed that the lowest serum cholesterol levels were seen when corn oil, safflower seed oil, or cottonseed oil constituted the sole dietary fat. Increased cholesterol levels were found with all other sole dietary fats with coconut oil and butterfat providing the highest levels. As a result of a number of studies with different types of oils and fats, Ancel Keys and colleagues produced a regression equation in 1957 that showed

$$\Delta\mathrm{Chol} = 2.74\Delta\mathrm{S} - 1.31\Delta\mathrm{P}$$

The equation showed that SFAs (S) had about twice the effect in raising serum cholesterol levels (ΔChol) as polyunsaturated fatty acids (PUFAs) (P) had in depressing it. Later, with more studies and improved data, other regression equations appeared. However, prediction equations are applicable to only large groups and not to individuals.

Not all SFAs raise serum cholesterol levels to the same degree. The short- and medium-chain length fatty acids C4:0, C6:0, C8:0, and C10:0 and some C12:0 that are present in milk fat are absorbed from the intestine, enter the portal circulation, and pass rapidly to the liver where they are oxidized. They are not incorporated into chylomicrons and have no serum cholesterol-elevating effect. The remainder of C12:0 together with C14:0 and C16:0 are hypercholesterolemic. C12:0 is possibly more potent than C14:0, which in turn is more potent than C16:0 in elevating total cholesterol levels. C18:0, like monounsaturated fatty acids (MUFAs), does not appear to influence serum cholesterol levels.

Associations between Saturated Fatty Acid Intake and the Risk of Coronary Heart Disease: The Epidemiological Evidence

Multiple studies have investigated the association between intake of SFAs and the risk of CHD.

Ecological Studies

Early epidemiological studies employed the international comparison or ecological format, where the apparent per capita intake of SFAs (or other items) is correlated with the death rates for a number of countries or regions. Most of these studies found that the intake of SFAs, dairy fat, animal fat, and total fat was strongly correlated with CHD death rates. However, other food items, such as protein, eggs, and sugar, and total energy often provided equally strong correlations as did other factors such as the number of TV and radio licenses, the number of registered motor vehicles, and national per capita income, factors that represent affluency. Ecological studies tell nothing about the diets of individuals who develop CHD and those who do not. Many other factors that may have a relationship to CHD can also differ between countries, which make the ecological format unsuitable for the determination of causality.

Case–Control Studies

Conducting within-country epidemiological studies can eliminate many of the disadvantages associated with the ecological format because the habits of the participants are more homogeneous. The simplest form is the case–control study where the diet and characteristics of patients with CHD are compared with that of control subjects free of the disease. Seven case–control studies were found. Four early studies analyzed the difference in mean daily intake of SFAs (in terms of $\mathrm{g\,day^{-1}}$ and as a percentage of daily caloric intake) between patients who had suffered a CHD incidence and representative control subjects who were free of the disease. The results are shown in **Table 1**. There were no statistically significant differences in the intake of SFAs between cases and control subjects. Three later studies used multiple logistic regression analyses to measure relative risk (RR) after adjustment for various confounders. Only one study, study 7 in **Table 2**, found a statistically significant association between SFA intake and the risk of CHD. None of the studies in **Tables 1** and **2** found a role for total fat, MUFAs, and PUFAs in the risk of CHD. Even so, the outcome of case–control studies can be influenced by bias because of failure to select appropriate control subjects, survivors of a heart attack failing to provide accurate data because of perceived gilt, and failure to include in the study individuals who died as a result of a heart attack.

Prospective (Cohort) Studies

Prospective or cohort studies are the preferred format for determining associations between dietary components and risk of CHD. Participants report their diet before the diagnosis of the disease and at a time closer to its initiation. Cases and control subjects are from the same community. Twenty-four publications on cohort studies that determined the associations between SFA intake and risk of CHD were found. Five of these publications were extended follow-ups of cohorts previously reported. Some studies presented results by age group, sex, area, and different CHD end points, which overall provided 33 data sets. **Table 3** lists 15 early studies that used a format where daily intake of energy, total fat, SFAs, and PUFAs in individuals with CHD was compared with the intake in those free of the disease. **Table 4** shows data from nine later studies that used multiple regression models to determine relative risk (RR) RRs of CHD with 95% confidence intervals (CIs) for the highest compared to the lowest intakes of total, saturated, monounsaturated, and polyunsaturated fats.

Only 5 of the 33 data sets show a statistically significant association between SFA intake and the risk of CHD. Studies 4 and 8 in **Table 3**, which are 6- and 10-year follow-up, respectively, from the Honolulu Heart Program found a positive association between percentage energy intakes of SFAs and risk of CHD, but not from absolute intake. In this cohort, there was also a positive association between PUFA intake and risk of CHD. There was also a positive association between percentage energy intakes, but not absolute intakes of SFAs and risk of CHD in the Ireland–Boston Diet Heart Study (study 10). A small Canadian study (study 17) found that increasing energy intake from saturated fat was significantly related to CHD mortality among 30–59 year olds (RR 1.11, 95% CI 1.04–1.18), but not among 60–79 year olds (RR 0.96, 0.88–1.03). Another small study from Britain found 100 g per week increase in saturated fat intake corresponded to an RR of 1.40 (1.09–1.79) in women. There was no increased risk for men (RR 1.00, 0.86–1.18). It is noteworthy that the largest three cohort studies, the Nurses' Health Study (study 22), the Health Professionals Follow-up Study (study 16), and the Alpha Tocopherol, Beta Carotene Cancer Prevention Study (study 19), did not find a positive association between saturated fat intake and CHD risk in fully adjusted models.

On the other hand, two cohort studies found a statistically significant negative association between SFA intake and the risk of CHD. Data from two Italian cohorts of the Seven Countries Study (study 12) showed that both the absolute and percentage energy intakes of SFAs were

Table 1 Comparison of mean daily intake of total fat and fatty acid classes, expressed as g day^{-1} and percentage of total calories (cal%), for control subjects (C) and heart disease patients (P) from four case–control studies

		SFAs				*PUFAs*				*Total fat*			
		(g day^{-1})		*(cal%)*		*(g day^{-1})*		*(cal%)*		*(g day^{-1})*		*(cal%)*	
Study (year)	*Cases*	*C*	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*	*P*	*C*	*P*
1. Zukel *et al*., North Dakota (1959)	162	58.5	58.9	18.9	18.7	-	-	-	-	140	141	45.3	44.8
2. Finegan *et al*., Ireland (1968)	100	71	74	18	19	14	14	4	4	154	160	39	41
3. Bassett *et al*., Hawaii (1969)													
Hawaiian	42	36.2	28.6	13.2	13.3	12.8	9.3	5.9	5.4	-	-	-	-
Japanese	68	29.9	21.6	11.1	10.7	13.6	10.0	6.3	6.3	-	-	-	-
4. Bolton-Smith *et al*., Scotland (1991)													
Men	68.4	40	42	16.3	16.4	12	12	4.8	4.8	91	95	37.0	37.4
Women	813	35	36	18.2	18.3	9	10	4.9	5.0	77	80	40.3	40.6

PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.

Table 2 RR with 95% CIs for the highest reported intake intervals vs. the lowest intervals of total, saturated, monounsaturated, and polyunsaturated fats for three case–control studies

Study (year)	*Cases*	*SFAs, RR (95% CI)*	*MUFAs, RR (95% CI)*	*PUFAs, RR (95% CI)*	*Total fat, RR (95% CI)*
5. Tzonou *et al.*, Greece (1993)	329	1.02 (0.41–2.54)	1.57 (0.54–4.51)	1.54 (0.78–3.01)	2.11 (0.87–5.17)
6. Suh *et al.*, Korea (2001)	108	1.0 (0.81–1.25)	0.94 (0.75–1.18)	0.97 (0.72–1.31)	1.08 (0.98–1.18)
7. Kabagambe *et al.*, Cobra-Rica (2003)	488	3.00 (1.54–5.84)	-	-	-

CI, confidence interval; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; RR, relative risk; SFAs, saturated fatty acids. References in Parodi (2009).

statistically higher in control subjects than in cases. In the Alpha Tocopherol, Beta Carotene Cancer Prevention Study (study 19), the RR of major CHD events and CHD death for the highest quintile of SFA intake compared to the lowest was 0.87 (0.73–1.03) and 0.73 (0.56–0.95), respectively. In this study, the so-called hypercholesterolemic fatty acids C12:0 to C16:0, which represented 62% of total SFA intake, were investigated separately. The RRs were 0.88 (0.74–1.04) and 0.74 (0.57–0.96) for total CHD events and CHD deaths, respectively. Data from the Nurses' Health Study were also used to determine associations between groups of SFAs and CHD risk, but no significant associations were found. For the highest versus lowest quintiles of C4:0 to C10:0, the RR was 1.00 (0.82–1.21); for C12:0 to C14:0, the RR was 1.05 (0.83–1.32); for C16:0, the RR was 1.03 (0.71–1.50); for C18:0, the RR was 1.16 (0.81–1.66); and for the sum of C12:0 to C18:0, the RR was 1.04 (0.72–1.48).

In only one cohort, the Nurses' Health Study, a statistically significant association between the intake of PUFAs and the risk of CHD was found. At 14-year (study 18) and 20-year (study 22) follow-up, the RRs for the highest versus lowest intake were 0.68 (0.53–0.88) and 0.75 (0.60–0.92), respectively. On the other hand, a statistically significant positive association between percentage energy intakes of PUFAs and risk of CHD was found in the Honolulu Heart Study at 6- and 10-year follow-up (studies 4 and 8) and in the Puerto Rico Heart Health Program for urban but not rural participants (study 5). Null associations were found in all the other studies.

It is interesting to note in **Table 3** that in all 12 cohorts the subjects free of CHD had a higher intake of energy than those who succumbed to the disease. This may be due to their higher rate of physical activity, which is considered a protective factor for CHD.

Autopsy Studies

Two studies were found that related the extent of atherosclerosis in the coronary arteries and aortas measured at autopsy with previous diet. Neither study found an association between SFA intake and degree of atherosclerosis.

Comment

The epidemiological studies provide no compelling evidence that SFA intake is associated with the risk of CHD, nor is there persuasive evidence that consumption of PUFAs will prevent the disease. However, epidemiological associations cannot be used to ascribe causality; for this, well-conducted randomized placebo-controlled clinical trials are required.

Randomized Controlled Trials

A randomized controlled trial (RCT) to determine if dietary SFAs are causally related to CHD would require isocaloric substitution of SFAs with unsaturated fatty acids without changes in total fat content, other dietary components, and lifestyle factors that could influence the risk of CHD. The study should be large enough to produce significant results, be blinded, and conducted for many years. These conditions are very difficult to obtain in practice, but a few studies have been conducted where SFA reduction was obtained by reduction in total fat intake, while others substituted animal fat in the diet by vegetable oils without undue difference in other factors. Six RCTs of diets with lowered SFA levels for CHD prevention are listed in **Table 5**. Because the various studies used different classifications for nonfatal CHD events, total and CHD mortality are used as end points. The Finnish Mental Hospitals Study is often cited as support for SFA and butter consumption as risk factors for CHD. This study is not included in **Table 5** because the design was unsound. The hospitals were randomized and not the inmates and therefore it was a cluster randomized trial with only two clusters. The inmates in the hospitals changed during the trial and there were differences for a number of CHD risk factors between hospitals. The hospital that fed the low-SFA, cholesterol-lowering diet also had a lower intake of trans fatty acids, now known to be more atherogenic than SFAs.

None of the estimates of risk for total and CHD mortality in **Table 5** attained statistical significance.

Table 3 Comparison of daily intake of energy, total fat, saturated fat, and polyunsaturated fat in individuals with CHD and those free of disease in 15 cohort studies

						Energy intake	*SFAs*		*PUFAs*		*Total fat*	
						(kcal)	*(g day^{-1})*	*(en%)*	*(g day^{-1})*	*(en%)*	*(g day^{-1})*	*(en%)*
Study (year)	*Cohort size*	*CHD events*	*End point*[a]	*Age (years)*	*Follow-up (years)*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*
1. Paul *et al*., Western Electric (1963)	1989	88	B	40–55	4.5	3082/3174	59/59	17.2/16.7	13.2/13.9	3.9/4.0	148/152	-
2. Medalie *et al*., Isreal (1973)	9764	427	B	40–60+	5	-	NS[b]	NS	NS	NS	NS	-
3. Morris *et al*., London Hospital (1977)	337	45	B	30–67	10–20	-	-	NS	-	NS	-	-
4. Yano *et al*., Hawaii (1978)	7705	179	B	45–68	6	2125/2290*	31/32	13/12*	16/15	7/6*	85/86	35/33*
5. Garcia-Palmieri *et al*., Puerto-Rico (1980)												
Urban	5798	213	B	45–64	6	2305/2413	36/37	13.6/13.5	17/16	6.7/5.9*	98/99	38.0/36.6*
Rural	2420	73	B	45–64	6	2241/2353	33/33	13.1/12.6	10/11	3.9/3.9	81/86	32.3/32.2
6. Gordon *et al*., Framingham (1981)	859	79	B	45–64	6	2488/2622	43/44	15.3/14.9	16/16	5.8/5.4	112/114	-
7. Shekelle *et al*., Western Electric (1981)	1900	-	A	40–55	19	-	NS	-	↓*	-	-	-
8. Mc Gee *et al*., Hawaii (1984)	7088	456	B	45–68	10	2229/2309*	31.7/31.9	12.7/12.3*	15.7/15.4	6.3/6.0*	86.4/86.3	34.7/33.2*
9. Kromhout *et al*., Zutphen (1984)	857	30	A	40–59	10	2792/3065*	54.6/59.8	17.7/17.6	18.3/20.1	5.9/5.9	-	-
10. Kushi *et al*., Ireland-Boston (1985)	1001	110	A	30–69	20	3208/3355	62/63	17.4/16.9[c]	9.3/10.1	2.6/2.7	-	39.4/38.5
11. Khaw and Barrett-Connor, South California (1987)												
Men	356	42	A	50–79	12	1997/2076	30.2/31.7	13.6/13.7	14.8/15.1	6.7/6.5	-	82.6/85.8
Women	503	23	A	50–79	12	1479/1589	21.6/24.4	13.1/13.8	11.9/12.2	7.2/6.9	-	59.5/66.5

(*Continued*)

Table 3 (Continued)

						Energy intake	SFAs		PUFAs		Total fat	
						(kcal)	*(g day^{-1})*	*(en%)*	*(g day^{-1})*	*(en%)*	*(g day^{-1})*	*(en%)*
Study (year)	*Cohort size*	*CHD events*	*End point*[a]	*Age (years)*	*Follow-up (years)*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*	*CHD/free*
12. Farchi *et al*., Italy (1989)	1536	58	A	45–64	15	2697/2900*	23.8/ 28.9*	8.0/9.0*	9.2/11.5*	3.2/3.7	76.9/90.8	26.0/28.5
13. Posner *et al*., Framingham (1991)	420	99	B	45–55	16	-/-	NS	NS	NS	NS	↑*	↑*
	393	114	B	56–65	16	-/-	NS	NS	NS	NS	NS	NS
14. Fehily *et al*., Caerphilly (1993)	2423	148	B	45–59	5	2179/2313*	72.1/ 76.1[d]	29.7/29.5	-	-	98.7/102	40.9/40.1
15. Goldbourt *et al*., Israel (1993)	10059	1098	A	40+	23	-	-	↓	-	↓	-	-

[a] A, CHD death; B, total CHD events.

[b] NS, not statistically significant.

[c] $p = 0.05$ after full adjustment.

[d] Animal fat.

CHD, coronary heart disease; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; ↓, inverse association; ↑, positive association.

Asterisk denotes statistical significance +, plus.

Table 4 RR and 95% CIs for CHD for the highest vs. the lowest reported intake levels of total, saturated, monounsaturated, and polyunsaturated fats from eight cohort studies

Study (year)	*Cohort size*	*CHD events*	*End point*[a]	*Age (years)*	*Follow-up (years)*	*Saturated fat, RR*[b] *(95% CI)*	*Monounsaturated fat, RR (95% CI)*	*Polyunsaturated fat, RR (95% CI)*	*Total fat, RR (95% CI)*
16. Ascherio *et al*., Health Professionals (1996)	43 757	734	B	40–75	6	0.96 (0.73–1.27)	-	1.04 (0.82–1.33)[c]	1.02 (0.78–1.34)
	43 757	229	A	40–75	6	1.72 (1.01–2.90)	-	1.28 (0.83–1.98)[c]	1.22 (0.75–2.0)
17. Esrey *et al*., Lipid Research Clinics (1996)	3925	52	A	30–59	12	1.11 (1.04–1.18)*	1.08 (1.01–1.16)*	0.99 (0.90–1.08)	1.04 (1.01–1.08)*
	621	40	A	60–79	12	0.96 (0.88–1.03)	1.00 (0.91–1.08)	1.00 (0.90–1.10)	0.99 (0.95–1.03)
18. Hu *et al*., Nurses Health (1997)	80 082	939	B	34–59	14	1.07 (0.77–1.48)	0.95 (0.64–1.39)	0.68 (0.53–0.88)*	1.04 (0.83–1.28)
19. Pietinen *et al*., Finnish (1997)	21 930	1339	B	50–69	6.1	0.87 (0.73–1.03)	0.82 (0.69–0.99)	1.11 (0.94–1.31)	0.87 (0.73–1.05)
	21930	581	A	50–69	6.1	0.73 (0.56–0.95)*	0.77 (0.59–1.00)	1.27 (1.00–1.61)*	0.85 (0.65–1.12)
20. Boniface and Tefft, Great Britain (2002) Men	1225	98	A	40–75	16	1.00 (0.86–1.18)	-	-	1.01 (0.93–1.10)
Women	1451	57	A	40–75	16	1.40 (1.09–1.79)*	-	-	1.19 (1.03–1.37)*
21. Jacobsen *et al*., Denmark (2004) Men	3686	228	B	30–71	16	1.03 (0.78–1.37)[d]	0.95 (0.65–1.40)[d]	0.80 (0.55–1.15)[d]	0.98 (0.87–1.10)[d]
Women	(♂+♀)	98	B	30–71	16	1.36 (0.98–1.88)[d]	1.01 (0.56–1.83)[d]	0.89 (0.50–1.57)[d]	1.12 (0.93–1.36)[d]
22. Oh *et al*., Nurses Health (2005) ♀	78 778	1766	B	30–59	20	0.97 (0.73–1.27)	0.82 (0.62–1.10)	0.75 (0.60–0.92)*	0.92 (0.77–1.09)
23. Leosdottir *et al*., Malmo (2005) Men	11 063	242	A	45–73	6.6	0.94 (0.58–1.53)	0.61 (0.36–1.03)	0.99 (0.65–1.53)	0.65 (0.45–0.94)*
Women	17 035	97	A	45–73	6.6	0.55 (0.26–1.17)	1.53 (0.65–3.64)	0.63 (0.33–1.22)	0.74 (0.40–1.36)
24. Tucker *et al*., Baltimore (2005)	501	71	A	34–80	18	1.04 (0.99–1.08)	-	-	-

[a] A, CHD deaths; B, total CHD events.
[b] Relative risk (RR), odds ratio (OR), or hazard ratio (HR). Data are for the fully adjusted multivariate model.
[c] Linoleic acid only.
[d] Risk associated with a 5% higher level of energy for the type of fat.
CHD, coronary heart disease; CI, confidence interval.
Asterisk denotes statistical significance.

Table 5 Outcomes of randomized control trials of low saturated fat diets and CHD prevention.

					Serum cholesterol		*Odds ratios (95% CI)*	
Study (year)	*Type*[a]	*N (diet / control)*	*Age range (years)*	*Follow-up (years)*	*Baseline (mmol l^{-1})*	*Difference*[b] *(%)*	*CHD death*	*Total mortality*
1. Research Committee UK (1965)	S	123/129	<65	3	6.8	7.9	0.89 (0.45–1.78)	0.87 (0.46–1.66)
2. Rose *et al*., London Hospitals (1965)	S	54/26	55.4	2	6.8	8.0	3.85 (0.46–32.5)	-
3. Research Committee UK (1968)	S	199/194	<60	2–6.8	7.0	10.2	0.97 (0.54–1.76)	0.88 (0.51–1.52
4. Dayton *et al*., Veterans (1969)	P + S	424/422	50–89	8	6.1	12.7	0.82 (0.53–1.26)	0.98 (0.76–1.25)
5. Woodhill *et al*., Sydney Heart (1976)	S	221/237	48.9	2–7	7.3	4.6	-	1.49 (0.89–2.51)
6. Frantz *et al*., Mannesoth (1989) ♂	S	2197/2196	<30 to >70	1.1	5.4	13.8	1.15 (0.72–1.82)	1.03 (0.82–1.30)
♀	S	2344/2320	<30 to >70	1.1	5.4	13.8	1.09 (0.59–2.00)	1.16 (0.87–1.53)

[a] S, secondary prevention trial; P, primary prevention trial.
[b] Difference in mean serum cholesterol between diet and control groups at the end of the trial.
CHD, coronary heart disease; CI, confidence interval.

One study was a primary prevention study, but lasted just over 1 year; in another study, participants were both free from and recovering from CHD at entry; the remaining studies were secondary prevention studies. Overall, it can be justifiably claimed that the patients were not representative of the general population, the numbers were too small, the duration of the trials was too short, and arteriosclerosis was too far advanced to benefit from dietary treatment. However, it is these studies, together with epidemiological studies and the serum total cholesterol-raising effect, that formed the basis for the demonization of saturated fat.

Multifactor Intervention Studies

Many multifactor intervention studies designed to reduce serum cholesterol levels were conducted. They variously reduced SFA intake in conjunction with reduced consumption of total fat and increased consumption of PUFAs and other beneficial dietary items, together with changes in lifestyle factors such as cessation of smoking and weight reduction. Some studies used medication to reduce BP and serum cholesterol levels. In general, apart from a benefit for a small proportion of patients at very high risk of death from CHD, cholesterol-lowering interventions failed to produce statistically significant reductions in CHD and total mortality until the advent of the statin drugs.

Serum Lipoproteins and Their Properties

Cholesterol is sparingly soluble in aqueous solution and is transported in blood attached to proteins, which are called apoproteins. Together with triglycerides and phospholipids, they form a lipoprotein complex. The lipoproteins are an extremely heterogeneous mixture with different chemical compositions, physical properties, and metabolic functions. Lipoproteins are crudely classified according to increasing density and decreasing size. There are five main classes: chylomicrons, very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs). As the density increases, the protein content increases whereas the triglyceride content decreases. Cholesterol content increases from about 8% in chylomicrons to about 48% in LDL, with about 20% in HDL. Each lipoprotein class has a distinctive apoprotein profile. Like serum total cholesterol, high levels of VLDL and LDL cholesterol are positively associated with the risk of CHD. On the other hand, high HDL cholesterol levels are negatively associated with the risk of CHD. The ratio of total to HDL cholesterol is considered a more powerful predictor of CHD than either value alone.

Apoproteins or apolipoproteins (apo) are designated by the letters A–E, with Roman numerals used for subspecies. Apoproteins have characteristic chain lengths and amino acid sequences and possess different physiological and biochemical properties. It is the composition of the apoproteins that determines the atherogenic properties, not the cholesterol. For example, positively charged regions of apoB of LDL interact with matrix proteins, including negatively charged proteoglycans (complex glycoproteins containing highly negatively charged carbohydrate chains) within the artery wall, an initiating event in atherosclerosis. Chylomicrons, VLDL, IDL, and LDL contain one molecule of apoB on their surfaces, but their cholesterol content varies and it is the apoB that reflects the number of atherogenic particles, although all particles may not be equally atherogenic. Serum apoB levels are considered superior to LDL cholesterol levels as a risk marker for CHD. ApoA is present on all HDL particles and is inversely related to the risk of CHD. The ratio apoB:apoA is the most important indicator of risk of CHD.

The major lipoprotein classes are themselves heterogeneous in size, density, composition, and properties. LDL size varies greatly among individuals and is genetically influenced. Small dense LDL particles are believed to be more atherogenic than larger more buoyant LDL particles. They are more easily taken up by the arterial wall, bind more tightly to arterial wall proteoglycans, and have greater oxidative susceptibility and reduced LDL receptor affinity. HDL is also a heterogeneous lipoprotein, which is metabolically dynamic with a number of subspecies that differ in apoprotein and lipid composition, size, and density and possess different physiological properties.

The Action of Saturated Fatty Acids on Lipoproteins

Early studies that provided a link between SFA intake and CHD, the so-called diet–heart or lipid hypothesis, depended on the measurement of serum total cholesterol levels, which is a crude measure of atherogenic potential. Although C12:0, C14:0, and C16:0 acids increase serum total and LDL cholesterol levels when substituted for carbohydrate or oleic acid, they concomitantly increase the level of antiatherogenic HDL cholesterol. The acids that increase LDL cholesterol the most likewise produce the greatest increase in HDL cholesterol. Regression models from a meta-analysis of 60 controlled dietary trials showed that C12:0 greatly increased total cholesterol, but most of its effect was on HDL cholesterol such that there was a decrease in the total:HDL cholesterol ratio. C14:0

and C16:0 acids had little effect on the ratio and C18:0 reduced the ratio slightly.

Lipoprotein(a) is an LDL-like particle where apolipoprotein(a) is linked to the C-terminal region of apoB of LDL. Its serum level is largely influenced by genetic factors and it may be more atherogenic than LDL. Normal lipid-lowering protocols do not reduce lipoprotein(a) levels and low-fat, high-carbohydrate diets increase its levels. However, SFAs, especially those present in milk fat, are known to reduce levels. Overall, when the effect of SFAs on all lipoproteins is considered, rather than just total and LDL cholesterol, they may well be atherogenically neutral.

Nondietary Causes of Hypercholesterolemia

There are many causes of hypercholesterolemia other than diet and SFA intake. Serum cholesterol levels increase with age, obesity, and in postmenopausal women due to cessation of estradiol production. Mental stress is an underemphasized cause of hypercholesterolemia and it is well documented that in students at exam time and accountants at peak work periods, serum cholesterol levels can rise substantially. A decline in cholesterol levels often occurs in metabolic ward patients, even those consuming a cholesterol-raising diet, as a result of reduced day-to-day stresses.

About 50% of the variation in serum cholesterol levels in the population can be explained by genetic polymorphisms in lipoproteins and related regulation factors. Examples are severe elevation in LDL cholesterol level that occurs when there is a mutation in the gene encoding the LDL receptor, which is responsible for the clearance of LDL cholesterol from the circulation. A defect in apoB, the ligand for binding LDL to its receptor, can also impair LDL clearance. There are many other points in the synthesis, secretion, and metabolism of lipoproteins where mutations in key elements can contribute to hypercholesterolemia

Even so, it is commonly cited that most people who succumb to CHD have normal or near-normal serum cholesterol levels. There is extensive overlap in distribution curves for total and LDL cholesterol levels in men with and without CHD. However, CHD patients with total cholesterol levels in the normal range mostly have other significant dyslipidemia. A study from Tufts University of men under 60 years of age with angiographically confirmed CHD found that more than half had a familial lipoprotein disorder. Only 12% had primary hypercholesterolemia, whereas the prevalence of HDL cholesterol and triglyceride abnormalities accounted for more than 50% of the lipoprotein abnormalities. For many years, public health bodies, nutritionists, and dietitians have recommended a high-carbohydrate, low-fat diet as a preventative measure for CHD. Whereas these diets may reduce serum LDL cholesterol levels, they may also decrease HDL cholesterol and increase serum triglyceride levels.

The Statin Drugs, Serum Cholesterol, and Coronary Heart Disease

The statin drugs are powerful hypolipidemic drugs. Depending on the class of statin and an individual's variability in response, they lower serum total cholesterol by 22–42%, LDL cholesterol by 27–55%, and triglycerides by 10–35% and increase HDL cholesterol by 4–8%. Statins act by competitively inhibiting 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step in cholesterol biosynthesis. Reduction in hepatic cholesterol levels results in an increased expression of LDL receptors to increase the uptake of serum LDL to maintain cholesterol homeostasis, which in turn lowers serum LDL cholesterol levels. Many studies have been conducted on primary and secondary prevention of CHD in clinical practice using statins and it is enthusiastically claimed that they reduce the risk of major coronary events by about 30%. These large reductions in serum total and LDL cholesterol levels with attendant CHD risk benefits are claimed to have justified the diet–heart hypothesis.

The Cholesterol Treatment Trialist's Collaborators recently conducted a meta-analysis of data from 90 056 participants in 14 randomized trials of statins. The mean duration of follow-up among survivors was 4.7 years. At 1 year, the weighed average difference in LDL cholesterol between controls and those on statin therapy was $1.09\,\mathrm{mmol\,l^{-1}}$. The RR for a $1.0\,\mathrm{mmol\,l^{-1}}$ LDL cholesterol reduction on nonfatal CHD, CHD death, and total mortality was 0.74 (0.70–0.79), 0.81 (0.75–0.87), and 0.88 (0.84–0.91), respectively. During the trial period, 6.2% of the control group suffered nonfatal CHD against 4.4% in the statin group. This translated to a 29% proportional reduction per $\mathrm{mmol\,l^{-1}}$ LDL cholesterol reduction. For CHD deaths and all-cause mortality, the corresponding figures are 4.4, 3.4, and 23% and 9.7, 8.5, and 12.4%. However, it is important to realize that the absolute risk reduction was small and on the order of 2% or less.

It is often suggested that reduction in LDL cholesterol by statins is the major, if not the only, mechanism for their beneficial effect in preventing CHD. However, there are a number of lines of evidence to suggest that this may not be the case and statins may possess cholesterol-independent properties (pleiotropic effects). Statins also reduce serum triglyceride levels and increase the levels of HDL cholesterol. To some extent they reduce homocysteine levels, and depending on the class they may also increase LDL particle size. There is also evidence that statins may reduce BP, lower the risk of developing diabetes, and improve left ventricular function. It is now generally accepted that inflammation plays an important role at all stages in the development and progression of atherosclerosis. Many lines of evidence from clinical and animal studies show that statins can modulate aspects of the inflammatory component of atherosclerosis. C-reactive protein (CRP) is a highly sensitive marker of systemic inflammation and is considered a stronger independent predictor of CHD than the LDL cholesterol level. A systematic review of 13 studies showed that statins reduced the concentration of CRP by 13–50% compared with placebo. This reduction was independent of their effect on serum lipid levels.

Vascular endothelial dysfunction is a strong and independent predictor of CVD. Vascular endothelial dysfunction was improved in patients taking a statin drug, but not in patients taking the cholesterol absorption inhibitor ezetimibe, even though both drugs lowered serum cholesterol levels to the same level. In addition to its role in the pathway to cholesterol synthesis, mevalonate can form activated isoprenes that condense to form geranylgeranyl and farnesyl pyrophosphates. These isoprenes bind (prenylate) a range of G proteins that transduce signals involved in inflammation. Squalestatin is an experimental statin that inhibits squalene synthase, thus preventing the synthesis of squalene downstream from mevalonate and the penultimate step in cholesterol synthesis. An animal study demonstrated that common statin drugs that inhibited mevalonate synthesis, but not squalestatin that inhibited squalene synthesis, produced antiinflammatory effects.

Given the small absolute reduction in CHD events after long-term treatment with statin drugs and their recorded pleiotropic effects on markers of CVD, their cholesterol-lowering effect cannot be used as evidence to support the diet–heart hypothesis. After all, the large reduction in LDL cholesterol levels may be considered as a surrogate measure of the efficiency of HMG-CoA reductase inhibition and its metabolic sequel.

Conclusion

The evidence presented in this article does not support a meaningful relationship between SFA intake and the risk of CVD. Even so, SFAs are not consumed as a single dietary entity, and in the case of milk and its products they are consumed along with considerable MUFAs and some PUFAs. The high calcium content of milk and dairy products can help lower BP, a major risk factor for CVD. BP levels may also be ameliorated by small digestion-resistant peptides encrypted in milk proteins. A recent meta-analysis of 15 prospective studies on milk and dairy consumption and the risk of CHD and stroke found that the RR for the highest level of consumption compared to low consumption levels was 0.84 (0.76–0.93) for CHD and 0.79 (0.75–0.82) for stroke.

At the time of the follow-up period for these studies, the milk consumed was almost entirely whole milk.

Meta-analyses of a smaller number of prospective studies found that the RRs for high versus low intakes of milk and dairy products and the incidence of type 2 diabetes and the metabolic syndrome, both important risk factors for CVD, were 0.92 (0.86–0.97) and 0.74 (0.64–0.84), respectively. By reducing the intake of milk fat for fear of CVD, individuals are deprived of a range of bioactive components, such as butyric acid, sphingolipids, and rumenic acid, certain branched-chain fatty acids, and fat-soluble vitamins with anticancer potential.

See also: **Milk Lipids**: Fatty Acids. **Milking and Handling of Raw Milk**: Influence on Free Fatty Acids.

Further Reading

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Nutritional and Oral Health-Promoting Properties of Dairy Products: Caries Prevention and Oral Health

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Introduction

Treatment of oral disease is extremely costly, and it is the fourth most expensive disease to treat in most industrialized countries. Oral diseases and disorders, although largely preventable, are ubiquitous and affect the vast majority of the world's population with varying degrees of severity. The available mechanisms of oral disease prevention are effective at the individual level and have also had success at the population level. However, although the severity of oral diseases has been reduced, they are still highly prevalent and there is scope for development of new approaches to prevention that are less reliant on behavioral change at the individual level.

Dairy products currently do not play a major role in dealing with oral diseases but progress is being made in the identification of dairy constituents and use of dairy products to promote oral health. A general overview of the prevalence of oral diseases precedes a summary of the pathogenesis of dental caries and periodontal disease. This is followed by consideration of research developments in the use of dairy constituents for the prevention of both diseases; other oral conditions are then considered.

Overview

Dental caries is the most common chronic disease of childhood and affects an estimated 95% of the world's population. Periodontal disease, a condition affecting the supporting structures of the tooth, affects an estimated 15% of the population and is most common in older age groups. The third condition, progressive tooth surface loss, although not a disease, is increasingly recognized as a potential challenge to the maintenance of a functioning dentition for life. The prevalence of this condition internationally is not well defined, but in two national surveys tooth wear into dentine was found to affect over one-third of 16- to 24-year-olds and approximately 90% of dentate 65+ year-olds in Ireland and the United Kingdom. Other oral conditions vary in frequency internationally. These include oral mucosal lesions including oral cancer, orodental trauma, denture stomatitis, candidiasis, noma (orofacial gangrene found in Africa and Asia), oral manifestations of systemic disease (e.g., HIV/AIDS), and congenital defects including orofacial clefts. These disorders add to the economic burden of oral disease with varying effect.

Microbes are a contributory factor in the development of the two most common of these conditions, caries and periodontal disease.

Dental Caries

Pathogenesis

Dental caries is a process that involves demineralization of apatite crystals in the tooth and the release of calcium, phosphate, and hydroxyl ions. It is a multifactorial disease and its development is determined by the interaction of three main factors over time (**Figure 1**), namely, host and teeth factors, microorganisms in dental plaque biofilm principally mutans streptococci and lactobacilli, and substrate, predominantly sucrose. Time is also an important factor because, even in the presence of the other three factors, the development of dental caries is a relatively slow process and clinically visible destruction of the enamel (cavitation) may take years to develop depending on age and surface type. The influence of the host is mediated by the saliva; saliva has powerful protective properties providing cleansing, lubrication, pellicle formation (provides a diffusion barrier), antimicrobial action to regulate oral bacteria, an ion reservoir for remineralization of enamel, and buffers to counteract acids in the mouth from bacteria and diet. Within the host, different teeth and tooth surfaces vary in their susceptibility to dental caries, with the majority of caries occurring on the biting and pit and fissure surfaces of the posterior teeth. The approximal surfaces of the posterior teeth are the next most commonly affected. Microorganisms are the second of the three factors necessary for caries to develop. Although many oral bacteria are acidogenic, the Gram-positive facultative anaerobe *Streptococcus mutans* is most frequently associated with the initiation of caries. It is well adapted as an early colonizer of enamel because of its ability to produce

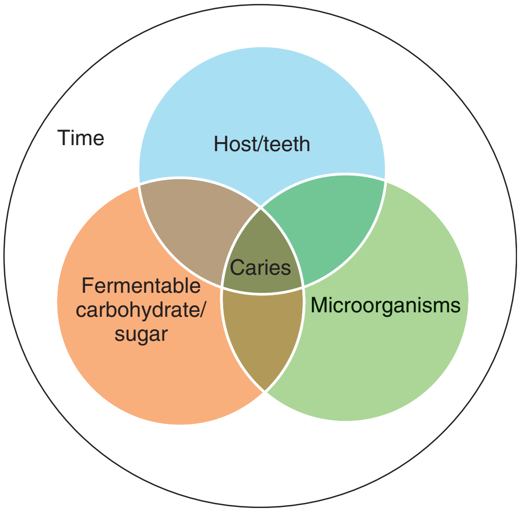

Figure 1 Occurrence of dental caries over time given the interaction of three essential factors.

extracellular dextran-based polysaccharides, which allows it to adhere to the smooth enamel surface. Thus the plaque biofilm starts to form and becomes colonized by other species. Once plaque is present, there is the capacity for dental caries to develop. The plaque biofilm produces acids as the bacteria metabolize fermentable carbohydrates. These acids can dissolve or demineralize the calcium phosphate mineral of the tooth enamel or dentin. Early enamel demineralization is not obvious to the naked eye. As it progresses, the demineralized surface looks white and chalky. If this process is not halted or reversed via remineralization, that is, redeposition of mineral via saliva, it eventually becomes an open cavity and the damage is irreversible.

The third factor is dietary sugars. Following ingestion of fermentable carbohydrate, cariogenic bacteria metabolize disaccharides such as sucrose to monosaccharides which they further metabolise producing lactic acid as a by-product. As a result, plaque pH falls below the critical pH, around pH 5.5, below which enamel demineralization occurs. Plaque pH falls almost immediately after sucrose ingestion and takes up to 40 min to recover; once it recovers, the enamel remineralizes again. Dilution and buffering by saliva play an important role in neutralizing plaque pH. The enamel remains intact so long as remineralization and demineralization are balanced.

Although dental caries is an almost ubiquitous disease, it has declined in severity since the 1960s, largely due to the widespread use of fluorides. The effect of fluoride is largely topical; teeth remineralize faster in the presence of fluoride, and fluoride also interferes with bacterial metabolism. Water fluoridation is the most effective and cost-effective public health measure for the control of dental caries. Currently, approximately 400 million people globally reside in communities served with water supplies containing added fluoride. The effectiveness of fluoride toothpastes is also now well established and it is estimated that over a billion people worldwide use them regularly. Other means of controlling dental caries include reduction in the frequency of consumption of foods and drinks sweetened with sugar and placement of resin sealants on the vulnerable pit and fissure surfaces.

Role of Dairy Products

The cariostatic effects of milk and cheese have been illustrated by animal studies, laboratory studies, and human observational and interventional studies since the 1950s. Some of the anticariogenic properties of milk may be attributable to its calcium, phosphate, casein, and lipid content. Postulated mechanisms involve buffering, salivary stimulation, reduction of bacterial adhesion, reduction of enamel demineralization, and/or promotion of remineralization by casein and ionizable Ca and P. The cariostatic properties of dairy products may result from a combination of the effects described in addition to the action of minor milk proteins and biologically active peptides in the protein fraction and/or that of naturally occurring probiotics.

Whole products

Traditionally milk and cheese are associated with healthy teeth and in the past have been marketed as being good for teeth. Milk, although it contains the natural sugar lactose, is not considered a cause of dental caries; lactose is known as one of the less cariogenic sugars. Early animal studies indicated that supplements of dairy products given with a cariogenic diet lowered the incidence of dental caries in rats. Epidemiological studies in recent years reported that children and adolescents with low incidence of dental caries drank more milk than those with high caries incidence. Frequency of cheese consumption has also been negatively associated with caries experience in 4-year-old children. A lower incidence of root surface caries development has been found in elderly people who eat cheese several times per week. The quantity of milk and milk products consumed has also been negatively associated with the number of root caries events during a 6-year study of 70-year-olds. Milk has even been recommended as a saliva substitute in patients with hyposalivation. The role of milk and dairy products in dental caries prevention has been the subject of a number of reviews. Following its expert consultation on Diet, Nutrition and the Prevention of Chronic Diseases (2003) the WHO/FAO report concluded that there was probable evidence that consumption of hard cheese decreased the risk of developing dental caries and possible evidence that consumption of milk reduced the risk of caries and dental erosion.

Although there have been numerous studies that have suggested a role for whole dairy products in the management of dental caries, an application to the European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies to deliver an opinion on the scientific substantiation of a health claim related to dairy foods (milk and cheese) and dental health in children received a negative judgment. The panel found that "on the basis of the data presented, a cause and effect relationship has not been established between the consumption of milk or cheese and dental health (prevention of dental caries) in children". The panel considered all studies presented for consideration to have too many weaknesses to allow definitive conclusions in 2008. Although one human interventional study was available for cheese, there was none for milk.

It appears that although dairy products have been identified as having cariostatic factors, the large volume required for a therapeutic effect limits their effectiveness in their natural state. Thus, identification and exploration of dairy derivatives with caries-protective or therapeutic properties for use as food additives or in oral health products has become a focus for researchers.

Derivatives

Dairy peptides

Much of the current research is based on the oral health-promoting potential of peptides and probiotics as they represent areas of interest in the rapidly growing functional food industry. Identification and isolation of the components of dairy products that are protective against dental caries could lead to an enhancement of the oral health benefits and functionality of dairy products or other products to which these components are added.

Cow's milk has two major groups of proteins – caseins (insoluble at pH 4.6), which represent ~80% of the total protein, and whey proteins (soluble in their native forms independent of pH), representing ~20% of total protein. Whey proteins include many minor dairy proteins such as enzymes, enzyme inhibitors, metal-binding proteins, vitamin-binding proteins, and growth factors. The bioactive properties of some of these minor proteins have been identified and they are used in nutraceutical products.

Extensive scientific evidence has illustrated the potential health benefits of biologically active peptides from dairy products. Some of the health-promoting effects are attributed to physiologically active peptides encrypted in, for example, milk and cheese protein molecules. These peptides are inactive within the sequence of the parent protein and can be released during gastrointestinal digestion or food processing. Bioactive peptides can also be produced from milk proteins through fermentation of milk, by starters employed in the manufacture of fermented milks or cheese. In fact, cheese in its natural form contains a huge reservoir of peptides; the technology for isolating these cheese peptides has been developed and future research will determine their bioactivity in the mouth.

One area that is the subject of ongoing research is the caries-preventive capacity of casein phosphopeptides and their use in oral health products and chewing gum. Casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) nanocomplexes, derived from bovine milk protein, casein, calcium, and phosphate, have been proposed as nontoxic, anticariogenic agents that could supplement the effects of fluoride. The suggested anticariogenic mechanism for CPP–ACP is the stabilization of calcium and phosphate as bioavailable ions and localization of calcium and phosphate ions into plaque producing a favorable concentration gradient, thus depressing enamel demineralization and enhancing remineralization. An alkaline, stable, and highly soluble CPP–ACP has been trademarked as Recaldent™ and has been commercialized in sugar-free gum and mints and in dental professional products (Tooth Mousse™).

A 2-year randomized clinical trial of the anticariogenic effect of sugar-free gum containing CPP–ACP nanocomplexes on approximal caries (caries on adjacent contacting tooth surfaces, between the teeth) among a fluoridated low caries risk population has been reported. The double-blind trial was carried out on Australian schoolchildren aged 11–13 years who were asked to chew their assigned sugar-free gum with (test gum ($n = 892$)) and without (control gum ($n = 857$)) 54 mg CPP–ACP per serving for 10 min, 3 times per day. Caries progression on approximal surfaces was estimated on digital radiographs taken at baseline and 24 months using an 8-point ordinal scale. Caries progressed on 5.4% of approximal surfaces in the CPP–ACP gum group compared with 6.5% of surfaces among the control group and there was a significant difference in the frequency distribution of caries transition scores along the 8-point ordinal scale (odds ratio (OR) = 0.82, $p = 0.03$). However, data on the effect of the product on caries on all surfaces among the two groups were not presented, but would be required to determine the overall efficacy of the product and the clinical relevance of the results.

Much of the earlier research on the anticariogenic effect of CPP–ACP was carried out using animal or laboratory-based studies or *in situ* studies using human volunteers who were asked to wear removable appliances containing blocks of artificially demineralized enamel. CPP–ACP has been reported to increase the level of calcium phosphate in plaque, to inhibit *in situ* demineralization of enamel, and to enhance *in situ* remineralization. Although most of the human studies had a small sample size (none exceeding 30) and were conducted under controlled conditions, the results of the Australian studies consistently show that products containing CPP–ACP produce greater subsurface remineralization compared to products with no CPP–ACP and that there is a dose–response effect, with higher doses producing greater remineralization. An independent study compared three calcium-containing chewing gums – two chewing gums (one with zinc citrate and one without) contained dicalcium phosphate, calcium gluconate, and calcium lactate, and one chewing gum contained CPP–ACP nanocomplexes – with a chewing gum lacking calcium. Conversely, this study found no significant difference in average remineralization or lesion depth between various calcium-containing compounds and controls; the authors concluded that the use of chewing gum offers no additional remineralizing benefit to buccal (cheek facing) tooth surfaces, even if the chewing gum contains calcium compounds. However, another study found that CPP–ACP was similar to dicalcium phosphate dehydrate in reducing lesion depth or lesion mineral content and that both products were significantly different from the control.

While the current evidence on the effectiveness of foods containing casein derivatives in remineralizing enamel under real life conditions is encouraging, the evidence is not universally accepted and further human trials are needed.

Fortified products

Milk is supplemented with many nutrients in several countries, i.e. Ca, Vitamin and D, I. Milk fluoridation programs are currently operating in six countries of the world (England, Bulgaria, Macedonia, Russia, Chile, Thailand). Studies on the clinical effectiveness of fluoridated milk in caries prevention have been carried out in several countries using different research methods. A Cochrane review concluded that there are insufficient studies with good quality evidence examining the effects of fluoridated milk in preventing dental caries. Of the two randomized controlled trials (RCTs) that satisfied the review's inclusion criteria, one used 7 ppm fluoride in 200 ml milk and the other used 2.5 ppm fluoride in 180–200 ml milk. These studies also varied in the delivery of fluoridated milk; one used a drinking cup and the other a straw. The results of the RCTs suggested that fluoridated milk was beneficial to schoolchildren, especially to their permanent dentition; however, more well-conducted research is necessary. Another review in 2007 suggested that fluoridation of milk can be recommended as a caries-preventive measure where the fluoride concentration in drinking water is suboptimal, caries experience in children is high, and there is an existing school milk program. It was recommended that the program should aim to provide fluoridated milk for at least 200 days per year and should commence before the children are 4 years of age.

Probiotics are added to food to promote alimentary health. The term probiotic is a relatively new word meaning 'for life' and it is currently used to name bacteria associated with beneficial effects for humans and animals. Probiotics have been isolated from dairy products and they have also been identified from other areas and delivered in dairy products. Both sources are considered here under the umbrella of probiotics and dairy products.

The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) have adopted a definition of probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host". Probiotics differ greatly; their mechanism of action is not clearly understood; they are likely to exert their effects through a variety of mechanisms including

1. competitive exclusion; prevention of colonization, cellular adhesion, and invasion by pathogenic organisms;
2. competition for essential nutrients;
3. direct antimicrobial activity, for example, production of bacteriocins; and
4. stimulation of the local and systemic host immune response.

The FAO and WHO suggest that the health benefits for which probiotics can be applied include conditions such as gastrointestinal infections, certain bowel disorders, allergy, and urogenital infections; they also point to emerging evidence to indicate that probiotics can be taken by otherwise healthy people as a means to prevent certain diseases and modulate host immunity.

The most commonly used probiotic agents are bacteria from the genera *Lactobacillus* and *Bifidobacterium*, which form part of the normal healthy intestinal microbiota.

Although the greatest body of probiotic research has focused on the colon, some progress has been made on the application of probiotics to promote oral health. One of the earliest papers on the use of probiotics for oral health was published in Russian in 1996. Since then, numerous probiotic strains have been investigated for their oral benefits. These include *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus rhamnosus* LC705, *Lactobacillus rhamnosus* LB21, *Lactobacillus casei*, and *Lactobacillus reuteri.*

LGG is one of the probiotics investigated for the prevention of dental caries. LGG is a strain of *L. rhamnosus* isolated from the intestinal tract of a healthy human being – GG represents the last names of two of the researchers who identified the probiotic bacterium, Sherwood Gorbach and Barry Goldin. It is particularly effective for the treatment of rotaviral diarrhea and it has also been shown to inhibit cariogenic mutans streptococci. It has also been studied for its effect on dental caries. Milk containing the probiotic LGG may have beneficial effects on children's dental health; LGG is unable to ferment sucrose or lactose, and this property increases its safety for oral use. A randomized double-blind placebo-controlled study ($n = 594$) to measure the effect of consumption of LGG in milk for a period of 7 months on dental caries and caries risk in 1- to 6-year-old Finnish children showed less dental caries in the LGG group and lower mutans streptococcus counts at the end of the study, especially in 3- to 4-year-old children. LGG reduced the risk of caries significantly ($\mathrm{OR} = 0.51$, $p = 0.004$), when controlled for age and gender.

LGG was combined with *L. rhamnosus* LC705 in cheese in another human intervention study in Finland. The study investigated the effect of daily consumption of 5×15 g of the probiotic cheese for 3 weeks on caries-associated salivary microbial counts in young adults. Altogether, 74 participants, who were 18- to 35-year-old subjects, completed this double-blind, randomized, placebo-controlled study. Stimulated salivary secretion rates, buffer capacity, and counts of salivary *S. mutans*, yeast, and lactobacilli were evaluated before and after the intervention and after a 3-week posttreatment period. Although there was no statistically significant difference between the groups in *S. mutans* counts after the intervention, at the 3-week posttreatment examination, these counts were significantly lower in the intervention group compared to the control group ($p = 0.05$). However, *S. mutans* counts decreased in 20% ($p = 0.01$) and yeast counts in 27% ($p = 0.005$) of all the subjects, regardless of the intervention group.

Combining probiotics with fluoride in daily milk drinks has provided encouraging results. Milk fortified with both *L. rhamnosus* LB21 (10^7 cfu ml^{-1}) and 2.5 mg fluoride per liter were found to have significant effects on caries development (prevented fraction 75%) and general health in Swedish 1- to 5-year-old children ($n = 248$) in a cluster-randomized, double-blind trial over 21 months. Children in the intervention group were served 150 ml of supplemented milk for lunch, while the control group received standard milk. Data were collected through clinical examinations and questionnaires. The mean baseline caries experience was 0.5 decayed, missing, or filled primary tooth surfaces (dmfs) in the intervention units and 0.6 in the control units and after 21 months 0.9 and 2.2 ($p < 0.05$). Children of the intervention units displayed 60% fewer days with antibiotic therapy (mean 1.9 vs. 4.7 days) and 50% less days with otitis media (0.5 vs. 1.0) ($p > 0.05$). No serious side effects were reported.

It may be beneficial to add bovine-specific antibodies against mutans streptococci to probiotic LGG-containing milk products. High titers of IgG antibodies against human cariogenic bacteria *S. mutans* and *S. sobrinus* were produced in bovine colostrum through immunization of pregnant cows with a multivalent vaccine. The purified immune product (IP) of this preparation had a number of anticariogenic properties, such as inhibition of streptococcal adherence to saliva-coated hydroxyapatite and inhibition of glucosyltransferase enzymes. IP added to LGG-fermented milk maintained its antibody activity against cariogenic streptococci during the expected shelf life of the product.

Lactobacillus casei has been evaluated for its caries-protective effects. A study in the early 1980s showed that rats monoinfected with *S. mutans* developed higher levels of caries than rats with greater than 1% *L. casei* in their plaque. *Lactobacillus casei* in a bioyogurt has been shown to inhibit oral streptococci. *Lactobacillus bulgaricus* in yogurt has also been shown to inhibit *S. mutans* when consumed twice daily for 8 weeks.

Lactobacillus reuteri has shown activity against *S. mutans*, when given in yogurt, water, or lozenge form, illustrating the potential for further development of foods containing this probiotic for the control of dental caries.

Current research on probiotics includes characterization of human oral lactobacilli and the selection of potential probiotic strains for oral health. Further strains have been identified that have antimicrobial properties against *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *S. mutans*. Thus, research continues to progress in this area, which in the future may have a major impact on the prevention and treatment of oral disease.

Other antimicrobial bacterial products have also been identified and purified from dairy products. An example with potential anticaries activity is lacticin 3147, which is a broad-spectrum lantibiotic produced by the food-grade strain *Lactococcus lactis* DPC3147. A powdered fermentate containing lacticin is effective in inhibiting a range of natural cariogenic *S. mutans* strains, and is effective in reducing this pathogen in human saliva. Further research on its effect on dental plaque biofilm is required to determine the potential of this powder as a nutritional additive for dairy products with the added benefits of preventing dental caries.

A small number of probiotics with potential for reducing caries risk by decreasing the level of *S. mutans* in the mouth have been identified and it is likely that this number will increase in the future as our knowledge and understanding of the area continues to improve. Use of probiotics in combination with other preventive agents has shown promise and is likely to be another avenue for future research. Probiotics may not replace conventional preventive approaches but the incorporation of strains with even slight oral health benefits in commonly consumed foods such as dairy products may provide a useful improvement in oral health at the population level.

Periodontal Diseases

Gingivitis that is initiated by microbial plaque is another very common oral disease affecting the vast majority of the population from time to time. Unlike dental caries, it is usually painless; the disease is characterized by inflamed gingivae, which may bleed on brushing or probing. Removal of plaque and maintenance of good oral hygiene results in resolution of the condition. The disease is modified by several factors, including smoking and hormonal changes such as those seen in puberty or pregnancy. Periodontitis is also initiated by microbial plaque and follows on from gingivitis, but occurs in a lesser proportion of the population than gingivitis. According to the WHO, 10–15% of the adult population worldwide suffer from advanced disease with deep periodontal pockets ($\geq$6 mm). Bacteria associated with periodontal diseases include Gram-negative anaerobic bacteria: among these, *Porphyromonas gingivalis*, *P. intermedia*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*), and *Treponema denticola* are strictly anaerobic and *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* and *Campylobacter rectus* are facultative/microaerobic organisms. Periodontal disease risk is influenced by genetic predisposition, the individual's immune and inflammatory response, and demographic and behavioral characteristics, such as gender, age, race, and smoking, as well as socioeconomic status. Local infection and inflammation of the periodontal ligament, the supporting structure that maintains the tooth in its bony socket, result in progressive destruction of both the ligament and the supporting alveolar bone, leading to various degrees of periodontal attachment loss in affected teeth. These changes are irreversible, but progress can be arrested by effective treatment. Prevention of the disease is centered on plaque control, which is also an important aspect of its treatment. Improving oral hygiene to ensure effective plaque control at a level consistent with disease prevention or treatment usually requires a behavioral change on the part of the affected person. Traditional educational interventions have been shown to be of little value in achieving long-term behavioral change, although there is tentative evidence from low-quality studies that psychological approaches to behavior management can improve oral hygiene-related behaviors. The development or identification of foods or products to help reduce either the quantity or virulence of plaque in the mouth could represent important progress in the management of periodontal diseases.

Role of Dairy Products

Whole products

The importance of minerals (and vitamins) in the prevention of periodontal disease has been investigated both directly and indirectly. The direct approach has involved the study of dietary intakes among populations with and without periodontal disease. While cross-sectional dietary analyses are not ideal in the study of this sporadic and chronic, usually slowly progressing disease, they can indicate where there are dietary components worthy of further research and can be useful for hypothesis formulation. One example is that of the third US National Health and Nutrition Examination Survey (NHANES III), a cross-sectional survey of 12 764 adults, which revealed that increased intake of dairy products was associated with a lower prevalence of periodontal disease. Individuals in the highest quintile of intake of dairy products were 20% less likely to have periodontitis than those in the lowest quintile. The inverse association between consumption of dairy products and periodontal disease was also reported in a cross-sectional study carried out in Japan. A study involving 942 adults showed that those eating $\geq$55 g lactic acid-containing foods per day had a significantly lower prevalence of deep probing depths (PDs) and severe clinical attachment loss (CAL) compared to those not eating these foods, after adjusting for confounding variables; the ORs for generalized deep PD and severe CAL were 0.40 (95% confidence interval (CI): 0.23–0.70) and 0.50 (95% CI: 0.29–0.87), respectively. These studies suggest a protective benefit of dairy products against periodontal disease, but further research is required to clarify this relationship and identify the specific protective factors.

The indirect approach looks at the relationship between bone mineral density (BMD) elsewhere in the body and periodontal disease. Besides exercise, a diet rich in calcium, phosphate, and vitamin D helps to protect against loss of BMD with advancing age. If periodontal disease is related to loss of BMD, then such foods could be considered to protect against osteoporosis as well as periodontal disease. Evidence for an association between BMD and alveolar bone loss has been reported, although some studies failed to find an association.

While there may be scope to fortify foods with vitamins and minerals to promote periodontal health, there is as yet insufficient evidence to support this development. However, recommending foods effective in improving general bone health and preventing osteopenia may be considered useful to promote alveolar bone health.

Derivatives

Compared to research on foods with caries-protective properties, there has been little progress in the development of foods with specific periodontal benefits. Development of probiotics in this area has focused on application of these products as therapeutic agents rather than as dairy food ingredients.

Other Conditions

Tooth surface loss describes the noncarious loss of tooth tissue, one of the causes of which is the consumption of acidic diets including acidic soft drinks. Milk does not have this problematic side effect.

There is some preliminary evidence that probiotic microorganisms can prevent or delay the onset of certain cancers outside the mouth (WHO/FAO, 2001). However, it is too early to make definitive clinical conclusions regarding the efficacy of probiotics in cancer prevention, and further research is required.

The use of probiotics for the control of *Candida albicans* has also been studied. *Candida* is the most frequently isolated yeast genus in the mouth, of which *C. albicans* is the most common species. An estimated 18% of the healthy and 40% of hospitalized people carry *C. albicans*; however, only a proportion of the population colonized by *C. albicans* develop candidiasis. A mixture of probiotics (LGG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS) delivered in 50 g cheese divided in two portions per day reduced the prevalence of oral *Candida* spp. and diminished the risk of hyposalivation and the feeling of dry mouth in an elderly population over a 16-week period. Over 16 weeks, probiotic intervention reduced the risk of high yeast counts by 75% and decreased the risk of hyposalivation by 56%; this outcome warrants further investigation.

Conclusion

Given the high prevalence of caries, gingivitis, and periodontal disease, the potential impact of new effective approaches to their prevention or treatment is considerable. Dairy products have been investigated for use in this respect for over 50 years. Further well-conducted RCTs are needed to determine the impact of whole unfortified dairy products such as milk and cheese on oral health. The enhancement of dairy products with single agents or combinations of agents can function to prevent or protect against a variety of oral conditions including dental caries, periodontal disease, tooth surface loss, and oral mucosal diseases including candidiasis. Derivatives of dairy products have demonstrated oral health-promoting activity and the potential exists to develop them as oral health-promoting additives for other foods or as therapeutic agents in oral health products.

Given the high prevalence of oral diseases and the cost of treating them, the development of even small preventive or therapeutic effects for commonly consumed dairy products has the potential to have an important impact on the burden of oral diseases at the population level.

See also: **Bacteria, Beneficial**: Lactic Acid Bacteria: An Overview; Probiotics, Applications in Dairy Products. **Cheese**: Public Health Aspects. **Biofilm Formation**. **Lactic Acid Bacteria**: *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus casei* Group. **Milk**: Milk in Human Health and Nutrition. **Milk Protein Products**: Bioactive Peptides.

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Milk Allergy

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Background

It is well known that human milk is the most appropriate food for infants, but when breast-feeding is not possible, milk from other species or infant formula milk is usually used as a substitute for human milk. This substitution can lead to some nutritional and immunological problems. One of these is milk protein allergy. In this context, the term milk allergy (MA) is focused on allergy caused by cow milk proteins because cow MA is one of the most common food allergies in children. Although most children outgrow MA by the age of 4 years, some retain the allergy for life. MA may occur in adults usually involving immediate allergic reactions or eczema. Several population-based studies in different countries have reported that the incidence of MA varies widely from 0.3 to 7.5%. MA occurs in some infants after ingestion of an amount of cow milk. In some cases, allergy to goat and sheep milk or to cheeses made from them has also been recognized.

Definition of Allergy

The word allergy means an altered or abnormal reaction. Such a reaction may occur when there is contact between a foreign protein, 'an allergen', and body tissues that are sensitive to it. The allergen may reach the tissues by direct contact with the skin or mucous membranes or through the bloodstream after absorption.

Types of Allergic Reactions

Allergic reactions have been classified into two types:

- The immediate reaction type in which the allergic manifestations occur within hours of the patient coming in contact with the allergen and often within seconds or minutes; in this form of allergy, skin tests are nearly always positive.
- The delayed reaction type in which manifestations may not appear for many hours or even for 2 or 3 days; in this type, skin tests are usually negative.

Milk Protein Allergy and Intolerance

MA is clinically an abnormal immunological reaction to milk proteins, which may be due to the interaction between one or more milk proteins and one or more immune mechanisms, and resulting in immediate immunoglobulin (Ig)E-mediated reactions. On the other side, reactions not involving the immune system are defined as milk protein intolerance (MPI). MPI produces a non-IgE antibody and is not detected by allergy blood tests. Meanwhile, it produces a range of symptoms very similar to MA symptoms, but can also include blood or mucus in the stool.

Clinical Manifestations of Milk Allergy

Symptoms of MA can appear immediately or start several hours or even days after the intake of moderate to large amounts of cow milk or its infant formula. A wide spectrum of clinical manifestations has been recorded with MA (**Figure 1**). Clinical symptoms involve immediate or delayed reactions operating separately or together. Immediate reactions are mainly IgE dependent, leading to cutaneous, intestinal, or respiratory symptoms and in some cases to anaphylactic reaction. Delayed reactions involve T-cell-dependent mechanisms and can be operative at both the skin and the intestinal level. The most frequent symptoms among the common manifestations of MA are gastrointestinal, which have been encountered in 50–75% of patients with MA. Respiratory and skin symptoms are also commonly involved in MA. These symptoms were recorded in 10–30 and 50% of patients with MA, respectively. Rhinitis is the most common respiratory manifestation of MA in some infants. Anaphylactic shock is a particularly serious symptom of MA. In some cases, death can result. Anaphylaxis was noted in 12% of patients with MA, but it was less commonly observed than most other symptoms.

Diagnosis of Milk Allergy

The clinical diagnosis of MA differs widely due to the multiplicity of symptoms. Diagnosis can be achieved by skin or blood tests. The positive blood or skin test is accomplished only with the immediate reactions

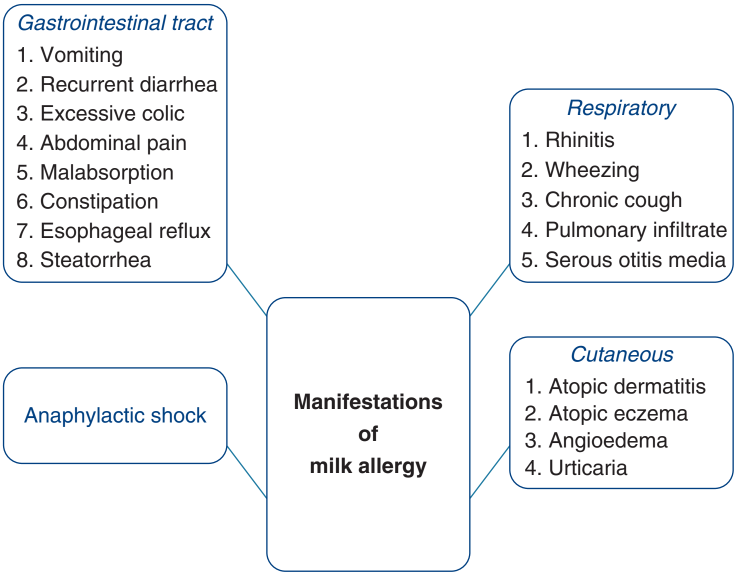
Gastrointestinal tract
1. Vomiting
2. Recurrent diarrhea
3. Excessive colic
4. Abdominal pain
5. Malabsorption
6. Constipation
7. Esophageal reflux
8. Steatorrhea
Respiratory
1. Rhinitis
2. Wheezing
3. Chronic cough
4. Pulmonary infiltrate
5. Serous otitis media
Anaphylactic shock
Manifestations of milk allergy
Cutaneous
1. Atopic dermatitis
2. Atopic eczema
3. Angioedema
4. Urticaria

Figure 1 Manifestations of milk allergy.

that develop after a few minutes because these tests detect IgE antibodies that are involved in the immediate type reaction. In the young child, about 60% of MA reactions are not of the immediate type but the delayed type (intolerant), and consequently are unlikely to give positive results with blood and skin tests. Different reliable diagnostic tests are shown in **Figure 2**.

Skin prick test (SPT) is especially accurate in the young child. The test is based on IgE being produced in patients when they are exposed to cow milk proteins. Once formed, IgE antibodies reside on the surface of mast cells present in the skin. Therefore, small drops of the suspected milk are placed on the forearm of patients to expose the mast cells present in the skin to the specific allergens (milk proteins). After 15 min, a wheal and flare reaction may appear, revealing that the patient is allergic to milk. Generally, the use of SPT for the diagnosis of MA cannot be considered reliable unless a strong reaction is noted. If it is desired to know to which particular protein the allergic individual is sensitive, then purified proteins must be used. Meanwhile, a 'patch' test may be more sensitive than SPT. There is no minimum age for SPT, which can be performed in babies and older children with useful results.

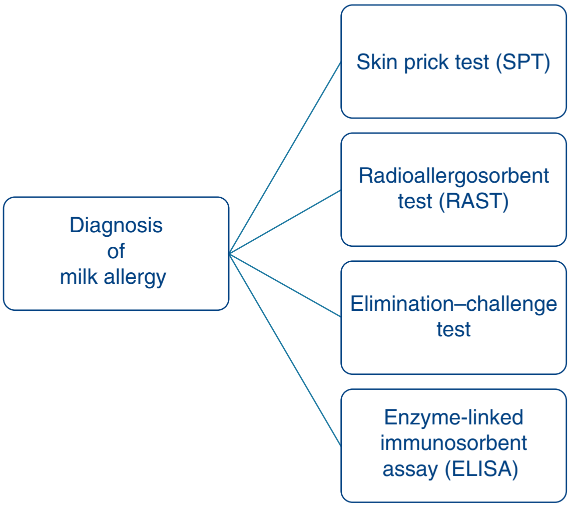
Diagnosis of milk allergy
Skin prick test (SPT)
Radioallergosorbent test (RAST)
Elimination–challenge test
Enzyme-linked immunosorbent assay (ELISA)

Figure 2 Methods for the diagnosis of milk allergy.

Radioallergosorbent test (RAST) and enzyme-linked immunosorbent assay (ELISA) are being used frequently to give more reliable results. Both measure the levels of IgE in the blood serum of the patient. In elimination–challenge test, allergy is confirmed if elimination of cow milk and products containing cow milk from the diet results in symptomatic improvement and if reintroduction of cow milk causes recurrence of symptoms.

Recently, a combination of assays was used for MA diagnosis, such as proliferation assay of peripheral blood mononuclear cells to cow milk, the quantitation of tumor necrosis factor-α (TNF-α; one of the mediators involved in adverse reactions to cow milk proteins), and serum-specific IgE determination. These assays are useful to identify MA among patients with immediate and nonimmediate adverse reactions, and they reduce the need for allergen challenges in young children.

Types of Milk Protein Allergens

Cow milk contains more than 20 protein allergens that can cause allergic reactions. The main proteins are caseins and whey proteins. Casein fractions (α_{s1}-casein, α_{s2}-casein, and

β-casein) and β-lactoglobulin (β-Lg) are the main allergens in cow milk. Allergic reactions to bovine serum albumin (BSA), IgG heavy chain, and α-lactalbumin (α-La) were also noted. Genetic polymorphisms of milk proteins play an important role in MA development. Goat milk with the α_{s2}-casein genotype caused less intestinal and systemic sensitization than goat milk with the α_{s1}-casein genotype in guinea pigs. This is very interesting and may have great potentials in selecting goat breeds for different casein genotypes, especially for α_{s2}-casein, which is not found in cow milk, and against α_{s1}-casein, which is dominant in cow milk. Allergic responses to lactoferrin and some cow milk enzymes have been detected in some patients with MA but none to mammalian lysozyme. The balance between casein and whey proteins in cow milk may determine its allergenicity. Allergenicity to goat and sheep milk caseins and cheese has been reported from some patients.

Infant Formula

Infant milk formula is an alternative cow milk substitute in which the protein is a hydrolyzed cow milk protein or goat milk protein. Casein, whey, or soy protein is hydrolyzed by proteolytic enzymes to develop a number of casein, whey, or soy protein hydrolysates. The products have been classified according to the degree of protein hydrolysis as extensively or partially hydrolyzed protein products. Casein hydrolysates have been used for almost 50 years, whereas whey hydrolysates are a more recent alternative. Both casein and whey hydrolysates appear to have a similar clinical tolerance. Generally, infant formulae can be classified into three categories:

Extensively hydrolyzed formula (EHF): This is a cow milk-based formula that has been treated with proteolytic enzymes. This formula often has a poor flavor and the taste may be bitter; however, it is recommended as a first alternative in children with MA before using other formulae. EHF is different from the partially hydrolyzed formula, since the latter is not indicated as a supplement for cow milk allergic children. In Italy, formulae made from goat milk are used and recommended by some physicians for feeding babies with MA.

Amino acid-based formula: This is another cow milk-based formula. It is necessary in around 10% of MA children who are allergic to EHF.

Soy formula: Soy formulae offer equivalent nutritional benefits to EHF but are more palatable. Soy formulae are not recommended for all cases of MA infants, since 17–47% of milk allergic infants can have adverse reactions to soy. However, around 53–83% of MA children can tolerate soy-based formula.

Alteration of MA

Different attempts have been made to reduce the allergenicity of cow milk proteins, and various technological processes have been applied in order to have better use of cow milk in infant formulae.

Heat treatment: Applications of prolonged heat have been made to modify the protein components of cow milk in an effort to reduce their allergenic potential. It was found that heating milk at 120 °C for 15 min did not affect the antigenicity of caseins from cow, buffalo, or goat milk. Heating bovine whey protein at 100 or 115 °C for 30 min resulted in no sensitization of guinea pigs or anaphylaxis. Heating of goat milk at 100 °C for 30 min resulted in alteration of BSA and IgG, whereas the antigenicity of α-La and β-Lg was not affected by heat treatment. Allergenicity of bovine β-Lg is affected by heat treatment, since rats immunized with native β-Lg had higher levels of total serum IgE than those immunized with heat-denatured β-Lg.

Enzymatic treatment: Another attempt to reduce allergenicity of milk proteins was by enzymatic treatment with a variety of enzymes. However, it was found that products resulting from enzymatic treatment have not had acceptable taste due to the development of bitterness and off-flavors, which are attributed to the liberation of peptides and amino acids from proteolysis. Meanwhile, the proteolytic digestion might itself generate new antigenic substances. It was reported that partial digestion of bovine milk with pepsin, or pepsin and trypsin resulted in peptides that belonged to β-Lg and which bound IgE from patient's sera with MA. The antigenicity of bovine β-Lg and α-La decreased by treatment with soybean trypsin inhibitor for 1 h, while BSA and Igs were more stable.

It was found that the effect of peptic or tryptic treatment on the allergenicity of goat milk proteins was different. Goat casein lost its own antigenicity by pepsin or trypsin treatment for 3 h, whereas goat α-La lost its whole antigenicity by pepsin but not by trypsin treatment. On the other hand, goat β-Lg antigenicity was not altered by either pepsin or trypsin treatment for 3 h.

Human Milk Proteins and Their Alternatives

Human milk proteins are different in their composition and structure from those of the milk of other species. It is known that the major whey proteins of bovine milk are β-Lg with 55% of total whey proteins, α-La with 20%, and BSA with 7%. These proteins differ in their types and ratios between goat, sheep, cow, camel, human, and buffalo milk. Human milk is free of β-Lg, one of the major allergens in cow milk, similar to camel milk, which also has no β-Lg. On the contrary, β-Lg is a major whey protein in cow, buffalo, sheep, and goat milk. Caseins in the milk of these species differ in fraction number and their charges, amino acid composition, and their peptide mappings. β-Casein is the major (70%) casein fraction in goat milk, while α_s is the minor one (30%), which is more like that of human casein and different from cow casein. β-Casein was more sensitive in goat and human casein to the action of pepsin than α_s-casein. The peptide mappings of goat and camel milk α-La and β-Lg are completely different from those of cow milk.

Several studies have evaluated the clinical use of milk from different animals such as cow, goat, camel, sheep, mare, donkey, and buffalo as alternatives to human milk. Some studies revealed that goat, donkey, and camel milk can be considered as proper alternatives to human milk due to hypoallergenic properties of their proteins. On the other hand, other studies have shown that the milk of goat, sheep, and buffalo cannot be useful in all cases as alternatives to human milk, because they can be as allergic as cow milk.

Milk Protein Cross-Reactivity

Cross-reactivity between milk allergens from different mammalian species and humans occurs when they share part of their amino acid sequence or when they have a similar capacity to bind specific antibodies due to their molecular structures. The cross-reactivity between milk proteins from different animal species has been studied. It was found that IgEs from sera of children allergic to cow milk are capable of recognizing most parts of milk proteins from sheep, goat, and buffalo. Weak cross-reactivity was observed with milk proteins from mares and donkeys, but none with camel milk. IgEs from a child allergic to sheep milk did not recognize any proteins of camel milk. Cross-reactivity between α_s-caseins from goat, sheep, and cow milk and their allergic potential were studied. In the three types of milk, α_s-casein was sharing more than 85% identical amino acids. When sera of allergic children to cow milk proteins were tested, significantly higher levels of IgE and IgG binding to goat and sheep α_s-casein were recorded, supporting a conclusion that goat and sheep α_s-caseins have an allergic potential and are not always suitable for the nutrition of cow milk allergic patients. Another study showed cross-reactivity between goat and human caseins, when anti-goat casein was used in immunoblotting technique. No cross-reaction between human α-La and antibodies against bovine α-La was detected. However, it was noted that human α-La is highly homologous to bovine α-La with 66% identity. Cross-reactivity between β-Lg and casein from cow and goat milk was detected by immunoblotting technique. It was reported that guinea pigs fed cow milk proteins and goat milk proteins with high α_{s1}-casein content developed high titers of anti-β-Lg, IgG1, with an important cross-reactivity between goat and cow β-Lg. A recent study showed that when specific antisera to camel milk proteins were applied in immunoblotting analysis, no immunological cross-reactivity between camel and cow milk proteins was found. Similar results were obtained when sera from some children allergic to cow milk were tested for the specificity of their IgE to camel milk casein or whey proteins (**Figure 3**). The study concluded that the absence of immunological similarity between camel and cow milk proteins can be considered an important criterion from the nutritional and clinical points of view, since camel milk may be suggested as a new protein source for the nutrition of children allergic to cow milk and can be used as such or in a modified form.

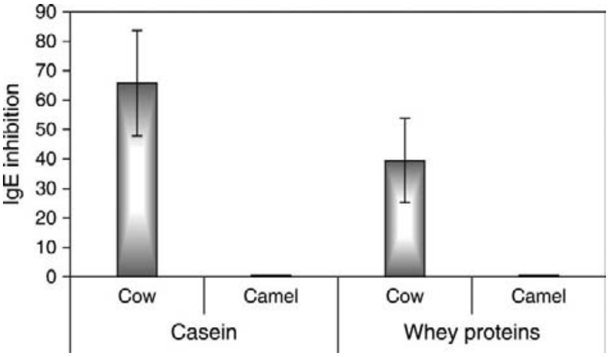

Figure 3 IgE-ELISA inhibition of cow and camel milk proteins. Reproduced from El-Agamy EI, Nawar M, Shamsia SM, Awad S, and Haenlein GFW (2009) Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Ruminant Research* 82: 1–6.

See also: **Analytical Methods**: Immunochemical Methods. **Animals that Produce Dairy Foods**: Camel. **Milk**: Bovine Milk; Buffalo Milk; Camel Milk; Goat Milk; Human Milk; Milk in Human Health and Nutrition; Sheep Milk. **Milk Proteins**: Casein Nomenclature, Structure, and Association; Immunoglobulins; α-Lactalbumin; β-Lactoglobulin. **Nutrition and Health**: Effects of Processing on Protein Quality of Milk and Milk Products.

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Diabetes Mellitus and Consumption of Milk and Dairy Products

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Introduction

Type 1 diabetes (formerly called insulin-dependent diabetes mellitus (IDDM)) is an autoimmune disease that presents as a loss of insulin production, often in younger members of the population. It is also known as juvenile-onset diabetes. Type 1 diabetes affects less than 1% of the population, although the incidence of the disease varies considerably between countries and ethnic groupings. The exact cause of type 1 diabetes is currently unknown; however, the disease is genetically linked: those individuals with alleles that predispose them to diabetes may get the disease, while those individuals without such alleles will not get it. It is believed that for individuals to develop diabetes they must be genetically predisposed to the disease and also be subjected to one or more environmental triggers. A number of triggers have been proposed, including viral and bacterial infections, exposure to chemicals, and the consumption of certain food types. Of the food types, special attention has been given to cows' milk because of its relatively early introduction into the diet and its role in infant formulae as a replacement for breast milk. There are studies that show an effect of cows' milk on the incidence of human diabetes, but a similar number of studies show that milk has no effect on the development of diabetes. The main evidence in support of an effect of the consumption of cows' milk on type 1 diabetes is epidemiological. Many of the major proteins in milk have been implicated as possible triggers of type 1 diabetes.

Type 2 diabetes (formerly called non-insulin-dependent diabetes mellitus (NIDDM)) is found at a much higher frequency than type 1 diabetes and affects over 5% of the population, although, as with type 1 diabetes, the incidence varies between countries and ethnic groupings. Unlike type 1 diabetes, which results from a problem with insulin production, type 2 diabetes is characterized by insulin resistance, or a problem with the way cells respond to insulin. In its early stages, type 2 diabetes can be managed through modifications of diet and exercise. Type 2 diabetes, like type 1 diabetes, is genetically correlated, but in contrast to type 1 diabetes the environmental factors that trigger type 2 diabetes are well known, with poor diet, a lack of exercise, and obesity promoting an alarming increase in the frequency of the disease. Approximately 90% of all cases of diabetes are type 2, with the frequency increasing in parallel with the increase in metabolic syndrome – a cluster of health risk markers including obesity, atherogenic lipoprotein profile, insulin resistance, and hypertension. Milk and dairy product consumption is inversely associated with insulin resistance and type 2 diabetes, although the underlying mechanism is yet to be established.

Type 1 Diabetes

Type 1 diabetes results from the progressive destruction of insulin-producing pancreatic β cells, which leads to the development of hypoinsulinemia and hyperglycemia. Although type 1 diabetes is genetically linked, identical twins are only ~40% concordant for the disease, suggesting that environmental factors are very important in triggering the disease. As cows' milk is a major component of infants' diets and is used almost universally as a replacement for breast milk in infant formulae, it has been scrutinized as a possible trigger of type 1 diabetes. An international meeting devoted to the subject of cows' milk and type 1 diabetes concluded in 1999 that there was nothing unique about cows' milk and that a number of different food types may be diabetogenic. Thus, the consumption of cows' milk may influence the development of type 1 diabetes in genetically susceptible humans, but many other foods could be expected to act in a similar manner. Despite the widespread acknowledgment that the induction of type 1 diabetes is multifactorial, the intense interest in cows' milk (the so-called 'cows' milk hypothesis') has overshadowed the other factors. It is possible that this intense attention paid to the effect of cows' milk on diabetes has led to a type 1 statistical error, leading to an overestimation of the effect.

Epidemiological Evidence for the Cows' Milk Hypothesis of Type 1 Diabetes

Between-country comparisons of the per caput consumption of milk and dairy products (inferred from national consumption data) and the incidence of type 1 diabetes have been proposed as evidence that milk is involved in some way in triggering this disease. Other evidence used to support the cows' milk hypothesis is based on comparisons of the incidence of type 1 diabetes in children who were breastfed for a prolonged period with that of children where infant formula was introduced into the diet before 3–6 months of age. In addition, some studies have reported higher levels of blood serum antibodies to certain milk proteins in children with type 1 diabetes than found in normal, age-matched children. The conclusions drawn from comparisons of breast-feeding with infant formula feeding have been challenged: a number of studies have been unable to find an effect of the early introduction of cows' milk on the induction of type 1 diabetes. Similarly, there is evidence that there are no specific differences between antibody levels to certain milk proteins in diabetics versus healthy controls. It should be noted that studies investigating antibodies to cows' milk need to take into account the possibility of diabetics being more immunosensitive than the control group. Meta-analyses of all the evidence for an effect of early introduction of cows' milk into the infant diet on type 1 diabetes found the effect to be statistically significant. However, and most importantly, epidemiological studies do not provide evidence for cause and effect and more specific mechanistic evidence is needed to establish whether cows' milk consumption is a factor in the induction of type 1 diabetes, or is merely correlated with some other as yet unidentified factor.

Animal Feeding Experiments and Type 1 Diabetes

Some animal model feeding studies have shown a significant effect of milk protein on the incidence of diabetes, whereas other studies have found that milk proteins have no influence on the occurrence of diabetes. If animal models of type 1 diabetes do represent good models of the human disease, then the timing of the introduction of milk protein into the diet and its interaction with other dietary components might be important in determining if it can influence the development of diabetes. The non-obese diabetic mouse (NOD mouse) and the biobreeding rat (BB rat) are the two animal models of choice for studying type 1 diabetes.

Some researchers have reported a high incidence of diabetes in NOD mice when they were fed a diet containing β-casein A1 but not when fed a diet containing β-casein A2. However, multinational interlaboratory studies using BB rats and NOD mice found that there was a significantly higher incidence of diabetes in rodents fed a cereal-based diet containing no milk components than in rodents fed diets containing milk components. With the exception of the cereal diet, individual laboratories did not produce consistent results. Results from animal feeding experiments indicate that even if milk proteins do contribute to the triggering of diabetes, it is likely that there would be little effect on the level of diabetes in the population if milk were to be removed from the diet, due to the consumption of other foods, particularly some cereals, that are equally likely to be triggers for diabetes.

Work with BB rats has shown that the stimulation of diabetes by diet is not a trigger-like process, but is cumulative and dose dependent. This finding is consistent with epidemiological studies on humans showing increased type 1 diabetes with increased per capita consumption of milk or per capita consumption of β-casein A1.

Evidence for the Involvement of Milk Proteins in Type 1 Diabetes

Of the proteins in milk suspected to be involved in the triggering of type 1 diabetes, bovine serum albumin (BSA), β-casein, and bovine insulin have received most attention.

Bovine serum albumin

The proposed link between BSA and type 1 diabetes is based on observations of elevated levels of anti-BSA antibodies in the blood of subjects with type 1 diabetes when compared with controls. It is proposed that, upon digestion, a peptide called ABBOS, which corresponds to BSA residues 126–144, is released and that the ABBOS peptide enters the bloodstream and causes the production of an immunoglobulin G (IgG) antibody that is specific for this peptide, but which also cross-reacts with a protein on the surface of the insulin-producing islet cells in the pancreas, targeting these cells for destruction. Through a process of molecular mimicry and autoimmunity, the insulin-producing capacity of the body is destroyed. However, the proposed relationship between BSA and type 1 diabetes has not been supported by the results from recent studies.

β-Casein

A specific T lymphocyte-mediated immune response to β-casein has been shown in diabetics, but not in normal humans. As with BSA, it has been proposed that exposure to cows' milk triggers anti-β-casein antibodies, which may cross-react with proteins on the surface of islet cells in the pancreas, leading to their destruction. However, other research showing that an elevated cellular immune response to β-casein was not specific to diabetics does not support this hypothesis.

There is epidemiological evidence that the consumption of β-casein variants with a histidine at position 67 (the A1-type genetic variants, which include A1 and B; also the minor variants C, F, and G) is highly correlated with type 1 diabetes. The consumption of other common variants of β-casein (A2 and A3) was not significantly correlated with type 1 diabetes. It is important to note that only 10 countries were included in the study and a number of unsubstantiated assumptions were made. The sample of cows phenotyped or genotyped for β-casein variants in each country was assumed to be representative of that country's milk supply, and the per capita consumption of milk protein (inferred from disappearance data) may not be representative of the consumption of milk protein by children within the countries examined. Many other countries were excluded from the study because of significant milk imports (which will distort derived consumption data), inadequate data on β-casein variants in the national dairy herd, or inadequate reliable data on diabetes incidence. Other evidence for an effect of the β-casein A1 variant comes from the animal feeding experiments described above.

Bovine insulin

Human insulin autoantibodies are generally observed to be present during the progression of an individual from a normal state to type 1 diabetes. Elevated levels of antibodies to bovine insulin have been observed in newly diagnosed patients suffering from type 1 diabetes. It is proposed that the presence of bovine insulin in infant formula and milk will stimulate the production of anti-bovine insulin IgG, which will then lead to immunization against human insulin. Evidence for such a mechanism is currently very limited and further research in this area is needed before this hypothesis can be critically assessed.

Possible Mechanisms Triggering Type 1 Diabetes

Molecular mimicry

Molecular mimicry has been proposed as a possible mechanism by which proteins present in milk induce the immune system to produce antibodies that are cross-reactive with either surface proteins on the insulin-producing cells or with human insulin produced by these cells.

Oral tolerance

Another mechanism may involve a breakdown in the oral tolerance mechanism. Oral tolerance usually results in the development of immune tolerance to antigens of dietary origin. It has been proposed that a breakdown in oral tolerance can be induced by the constituents of milk and other foods, or bacterial/viral infections. The proinflammatory immune response associated with the breakdown in oral tolerance could then make the digestive tract more permeable to food and other antigens. These antigens could in turn cause a breakdown in the self-recognition immune process, possibly through molecular mimicry.

Bioactive peptides

Milk proteins can release a number of bioactive peptides upon digestion. There is *in vitro* evidence that β-caseins A1 and B release a 7 amino acid bioactive peptide β-casomorphin-7 (BCM-7) in the presence of digestive enzymes, whereas β-casein variants A2 and A3 do not release this peptide. BCM-7 has been shown by various groups to have marked bioactivity, including immune modulation and opiate activity.

Selective release of BCM-7 from β-casein A1-type variants is believed to be due to a single amino acid change at position 67 in the 209 amino acid sequence of this protein. β-Casein A1-type variants have a histidine at this position and β-casein A2-type variants have a proline at this position. The presence of the proline causes a structure that cannot be hydrolyzed by digestive enzymes at the 66–67 peptide bond. Human β-casein has a proline at the equivalent position in the sequence, suggesting that BCM-7 is not released during the digestion of human milk.

BCM-7 has been observed to have a marked inhibitory effect on immune cell activity in NOD mice and extracted immune cells from NOD mice and prediabetic humans. In contrast, BCM-7 appeared to activate immune cell function in normal mice and normal humans. The administration of naloxone (a μ-receptor blocker) eliminated the effect of feeding β-casein A1 milk on the induction of diabetes in NOD mice. It is proposed that this is due to the blocking of the binding of BCM-7 to the μ-receptor on cells.

The role of β-casein and BCM-7 in the promotion of type 1 diabetes and other diseases is controversial. However, based on the current balance of scientific evidence, it is unlikely that β-casein or BCM-7 promotes the development of type 1 diabetes. It is also important to note that any cereal that contains gluten (including rye, wheat, and barley) is also capable of releasing exorphin peptides upon digestion, and that more NOD mice and BB rats developed diabetes when fed diets containing these cereals than when fed diets containing milk protein.

Camels' Milk and Type 1 Diabetes

There is some evidence to suggest that camels' milk may have a protective effect for type 1 diabetes. A small population study of an area in India where camels' milk is commonly consumed showed zero incidence of diabetes in camels' milk-consuming members ($n = 501$), while the incidence of diabetes in a control group consuming no milk was 5.5% ($n = 529$). Other studies have shown that consumption of camels' milk by type 1 diabetic subjects results in a decreased requirement for insulin. The mechanism of this effect is not known, but it has been proposed that the effect may be due to high levels of insulin in camels' milk and because camels' milk does not coagulate in the stomach and transits rapidly to the intestine, making intact insulin potentially available.

Absence of Breast Milk and Type 1 Diabetes

A mechanism based on the absence of breast milk rather than the presence of cows' milk has been proposed as a possible cause of type 1 diabetes. In such a mechanism, various factors, such as growth hormones and cytokines in human milk, would normally be required for the development of the systemic and/or gut immune system. When these factors are not present, the immune system does not develop as required, rendering genetically predisposed individuals susceptible to the development of IDDM. Currently, there is little evidence to support such a mechanism.

It is possible that elements of any or all of the proposed mechanisms could play a role in the induction of IDDM. It is also possible that there is nothing unique about cows' milk and that a number of different food types may be just as diabetogenic. Correlations with cows' milk may simply exist, as this is often the first food encountered after or in replacement of breast milk. They may also be found just because researchers are continually looking for them. Replacement of cows' milk with milk substitutes may thus have little influence on the development of IDDM in susceptible individuals.

Type 2 Diabetes

Metabolic syndrome is a serious international health problem and appears to be increasing in frequency. Metabolic syndrome is predictive of type 2 diabetes, as well as coronary heart disease. It is estimated that by 2025 there will be over 300 million people with type 2 diabetes, a doubling of the current level. Another disturbing trend is the increase in type 2 diabetes frequency in children, believed to result from poor diet and a lack of adequate exercise.

Diet and Type 2 Diabetes

Maintenance of a healthy weight through proper nutrition, exercise, and consequential energy balance is generally accepted as the best way to avoid type 2 diabetes. There is mounting evidence that the consumption of dairy products can help to prevent metabolic syndrome, insulin resistance, and type 2 diabetes. Combined data from four cross-sectional studies showed an inverse relationship between dairy intake and prevalence of type 2 diabetes when dairy foods were consumed at levels of between 3 and 4 serves per day (odds ratio 0.71; 95% confidence interval 0.57–0.89). A modest effect was observed in the meta-analysis of prospective studies. Recent observational studies have shown a variety of effects of dairy and milk consumption on type 2 diabetes, from a strong inverse relationship to no effect on incidence.

The mechanism by which milk and dairy products can provide a level of protection against type 2 diabetes is yet to be established. The calcium and vitamin D contents of milk and dairy products have been proposed as one of the mechanisms for the observed protective effects of milk and dairy products. Investigators have examined the effect of calcium and vitamin D on type 2 diabetes in studies focusing on supplement use, biochemical measures of calcium status, and dairy food intake (measured by serves eaten). Vitamin D is reported to have a supportive role in pancreatic β-cell function and extracellular calcium regulation. Calcium levels have been shown to have effects on insulin secretion and insulin action. The vitamin D and calcium contents of milk are the most discussed, but may not be the only mechanisms by which cows' milk and dairy products affect type 2 diabetes, and it is important not to focus only on the calcium and vitamin D contents of milk without consideration of a possible role of other components found in dairy products.

Other hypothesized mechanisms for the effect of dairy consumption on type 2 diabetes include

- magnesium as a cofactor for enzymes involved in glucose metabolism;
- the digestion of milk proteins, releasing insulinotropic peptides, amino acids, and incretin hormones;
- dairy fat contains medium-chain fatty acids which have a positive effect on insulin sensitivity;
- dairy foods have a relatively low glycemic load; and
- dairy proteins may enhance satiety, helping to maintain energy balance.

One study also found that the omega-3 fatty acids from milk were associated with a lower incidence of type 2 diabetes.

See also: **Milk Protein Products:** Bioactive Peptides. **Milk Proteins:** Casein Nomenclature, Structure, and Association; Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins; Nutritional Quality of Milk Proteins.

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Galactosemia

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Introduction

Galactose is an energy-providing nutrient and also a necessary basic substrate for the biosynthesis of many macromolecules in the body. Galactose is an important constituent of the complex polysaccharides, which are part of cell glycoconjugates, key elements of immunological determinants, hormones, cell membrane structures, endogenous lectins, and numerous other glycoproteins. In addition, galactose is incorporated into galactolipids, which are important structural elements of the central nervous system.

Metabolic pathways for galactose are important not only for the provision of these macromolecules but also to prevent the accumulation of galactose and galactose metabolites. Problems with galactose metabolism that result in galactosemia can cause a variety of clinical manifestations in humans.

Dietary Sources of Galactose

The principal dietary sources of galactose are milk and milk products, in which it occurs mainly as a component of lactose. Galactose can also be derived from lactose used as an extender in drugs.

Small amounts of galactose are present in cereals, fruits, legumes, nuts, organ meats, seeds, and vegetables in either the free form or bound in various glycosidic linkages and as a component of lipids. Considerable amounts of free galactose occur in some legumes (dried beans and peas), and bound galactose, in various glycosidic linkages, such as α-1,6, β-1,3, and β-1,4, and as a component of lipids, is ubiquitous in animals and plants. The bioavailability of these bound forms of galactose is unknown. Foods fermented by microorganisms for preparation or preservation purposes may contain free galactose.

Galactose Metabolism in Humans

Galactose is released (together with glucose) from lactose in the small intestine by the action of β-galactosidase (lactase) and both monosaccharides are absorbed by an active transport mechanism across the gut wall into the blood. The brush border of the human small intestine contains a sodium-dependent carrier, which is shared by glucose and galactose, and interaction between the carrier and a localized sodium pump allows the monosaccharides to be actively concentrated within the cell.

Hydrolysis of lactose by lactase is believed not to limit the rate of galactose absorption in lactose-tolerant humans. However, the absorption of both galactose and glucose is slower when ingested as lactose than when ingested as a mixture of monosaccharides. It is not clear why this should be so. In lactose-intolerant subjects, the absorption of galactose and glucose is much slower when ingested as lactose than when ingested as hydrolyzed lactose because lactose hydrolysis by intestinal lactase is rate-limiting.

The main route of metabolism of absorbed galactose is the Leloir pathway (**Figure 1**), which involves four enzymes and results in the overall conversion of galactose to glucose-1-phosphate. This metabolic pathway is critical for cellular energy production, modification of cellular macromolecules, for example, glycoproteins and glycolipids, and normal human development. UDP galactose is an important donor of galactose via galactosyl transferase reactions to form glycoproteins and glycolipids.

The liver is the primary site of the Leloir pathway but this pathway is also present in other tissues, including the kidney and erythrocytes. The congenital absence or inactivity of any of the first three enzymes of this pathway results in the clinical condition galactosemia.

There are three minor pathways for galactose metabolism in humans. The Isselbacher pathway involves conversion of galactose-1-P to UDP-galactose by the enzyme UDP-galactose pyrophosphorylase.

Reduction of galactose to the sugar alcohol galactitol is catalyzed by aldose reductase; this reaction is important in the development of cataracts in people suffering from galactosemia. Human tissues have only a limited capacity for further metabolism of galactitol.

Dehydrogenation of galactose produces galactonic acid, which can be detected in the urine of both galactosemic and nongalactosemic subjects after a galactose load.

Molecular cloning and characterization of all three human galactose metabolic genes have led to the identification of a number of mutations that result in three forms of galactosemia, which are caused by deficiency of the kinase (galactokinase (GALK)), the transferase (galactose-1-P:uridyl transferase (GALT)), or the epimerase (UDP-galactose-4 epimerase (GALE)). Of these, GALT deficiency, referred to as 'classical galactosemia', is the most widely studied. All forms occur as inborn

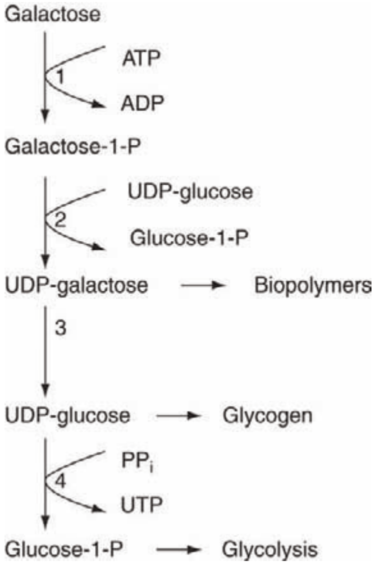

Figure 1 The Leloir pathway for galactose metabolism. 1, Galactokinase; 2, galactose-1-P:uridyl transferase; 3, UDP-galactose-4 epimerase; 4, UDP-glucose-pyrophosphorylase.

errors of galactose metabolism. Tolerance of galactose is reduced and the ingestion of galactose results in a chronically elevated blood galactose concentration with the subsequent accumulation and/or excretion of galactose and galactose metabolites. All conditions can be treated by means of a galactose-free diet.

Classical Galactosemia

Classical galactosemia is an autosomal recessive disorder due to a deficiency of GALT; ingested galactose can be phosphorylated to galactose-1-P but not metabolized further, and this results in the accumulation of galactose and galactose-1-P in tissues. GALT is immunologically intact although enzymatically defective; thus, a structural gene mutation is involved. At the cellular level, galactose-1-P interferes with the metabolism of glucose and the synthesis of glycoproteins and glycolipids and reduces the level of ATP in the cell.

Occurrence

The worldwide incidence of classical galactosemia is estimated at about 1:70 000. However, the incidence varies between countries – Norway, 1:96 000; Sweden, 1:81 000; Switzerland, 1:58 000; Germany, 1:40 000; United States, 1:62 000 – varying from 1:30 000 to 1:191 000 in different populations. In Japan and China, only a few cases have been detected, and in Japan, classical galactosemia is thought to be only one-twentieth as frequent as it is in Caucasian populations of the United States. A high incidence of 1 in 480 has been reported in the Traveller group (an endogamous group of commercial industrial nomads within the Irish population) in Ireland, compared to 1 in 30 000 among non-Traveller communities in Ireland.

Symptoms

Galactose-1-P accumulates in the newborn in various tissues when lactose or galactose is ingested. Galactosemic infants appear normal at birth but subsequent to milk ingestion they develop symptoms, including liver malfunction (hepatomegaly, jaundice), cataract, cerebral edema, mental retardation, renal damage, failure to thrive, and susceptibility to sepsis. Failure to thrive is the most common initial clinical symptom of galactosemia. Vomiting or diarrhea usually begins within a few days of milk ingestion. Jaundice of intrinsic liver disease may be accentuated by the severe hemolysis occurring in some patients. Cataracts have been observed within a few days of birth. There appears to be a high frequency of neonatal death due to *Escherichia coli* sepsis, with a fulminant course. Except for cataract (see later), the underlying pathophysiology of these effects is poorly understood.

Treatment

Early introduction of a galactose-free diet restores normal growth and can prevent permanent liver damage and halt (or sometimes reverse) cataract development. However, mental retardation is irreversible if a galactose-free diet is not introduced within 2–3 months.

While a galactose-restricted diet free of lactose is lifesaving in patients with GALT deficiency, it is now recognized that this does not prevent long-term complications such as developmental delay, mental disability and neurological syndromes, speech defects, poor growth, and, in females, ovarian failure. Most subjects have cognitive impairment.

The cause of the unsatisfactory outcome of seemingly good control of galactose intake and the disturbances in long-term development despite treatment is unclear. Possibilities include chronic intoxication by galactose metabolites, deficiency of galactose-containing glycoproteins and/or glycolipids as a result of an overrestrictive galactose-free diet, or alteration in the glycosylation process of glycoproteins and glycolipids.

Well-treated galactosemics retain a low level of red cell galactose-1-P, which is nevertheless much higher than the almost undetectable levels in normal subjects, and which increases if the patient departs from the prescribed diet. It is unknown how the galactosemic individual maintains this base level of galactose-1-P, whether from hidden galactose in the diet or through galactose biosynthesis from glucose, or both.

Lactose, found in dairy products and as an extender in drugs, has been considered to be the primary source of galactose in the diet. However, small amounts of galactose are present in organ meats and many plant foods, including cereals, fruits, legumes, nuts, and seeds. The role of free and bound galactose in these foods in the poor outcome seen in some patients with GALT deficiency is unknown. However, since galactose is widespread in foods, it is unlikely that the lactose restriction for patients with GALT deficiency ensures a galactose-free diet.

It is also possible that endogenous production of galactose may contribute to the base level of galactose-1-P. There is evidence that galactosemic patients, as well as normal subjects, synthesize gram quantities of galactose per day and it has been suggested that this may be an important factor in the pathogenesis of the complications of the brain and ovary in treated galactosemics.

Ovarian Failure

It has been found that the mammalian ovary is particularly susceptible to damage from the accumulation of galactose and galactose metabolites. Gonadal dysfunction, specifically hypergonadotropic hypogonadism with ovarian atrophy, in female galactosemics is an almost universal symptom and because of this, pregnancy is rare in women with classical galactosemia. The consequences range from failure of pubertal development, through primary amenorrhea or premature menopause. In contrast, male galactosemics have a relatively low risk of gonadal dysfunction.

Several candidate toxic states may be involved, including the galactose metabolites galactose-1-P and galactitol, and proposed mechanisms include interference with ovarian apoptosis and gonadotropin signaling. It has also been suggested that this complication may be at least partly prenatal in origin and studies in animal models suggest that ovarian dysfunction may have been caused *in utero*. Current dietary restrictions of galactose are inadequate to prevent ovarian failure.

Cataract

The mechanism by which cataract develops in subjects with galactosemia has been elucidated by studies in experimental rats. The primary cause of cataract is the chronic elevation of plasma galactose concentration resulting in a high concentration of galactose in the lens of the eye. Galactitol, formed in the lens from galactose by the action of aldose reductase, accumulates to high concentrations and this exerts a strong osmotic effect. Sugar alcohols do not diffuse readily through biological membranes, so the retention of galactitol within the lens leads to the imbibition of water to maintain osmotic equilibrium, which leads to lens fiber swelling, vacuole formation, and subsequent opacification. The process of sugar cataract formation in animals can be prevented by inhibiting aldose reductase. The development of galactose-induced cataract in galactosemic humans is thought to occur by the same mechanism as described for the rat.

Effects of Galactosemia *in utero*

There is direct evidence that in galactosemia due to GALT deficiency, galactose, galactose-1-P, and galactitol accumulate in the fetus by week 20 of gestation. However, the metabolic abnormality may develop earlier than this, since the key enzymes in galactose metabolism have been shown to be present in normal fetal liver from week 10 of gestation. There has been a report of increased galactitol in amniotic fluid obtained at week 10 of gestation, the outcome being that the baby is affected.

Cataract formation in the fetus is rare and is the only direct evidence that galactosemia may have harmful effects *in utero*. However, it has been concluded that the liver pathology seen in some infants who died in the neonatal period originated prenatally, and some studies have found that galactosemia is associated with reduced birth weight. Other observations, particularly those made from animal models, would suggest that ovarian dysfunction may have been caused *in utero*.

With regard to maternal galactose restriction, available biochemical evidence is insufficient to suggest any advantage to the fetus and galactitol accumulates *in utero* despite maternal galactose restriction.

Screening of Newborns

Methods for mass screening of newborns for galactosemia have been available since 1964. These are based mainly on measurements in blood of galactose, with or without galactose-1-P, and sometimes also including assay of GALT activity. Although galactosemia is rare, a number of countries (e.g., Germany, Switzerland, Canada, Norway, and some states in the United States) have included screening for galactosemia in their national screening programs, but many have not. Prenatal diagnosis for this disorder may be carried out by GALT assay in cultured amniotic fluid cells or in chorionic villus biopsies and by galactitol estimation in amniotic fluid supernatant.

One of the outcomes of screening programs has been the discovery of subjects with partial GALT deficiency who have mildly elevated blood galactose. These occur with a frequency about 10 times that of classical galactosemia (homozygotes with essentially total GALT deficiency) and are considered to be heterozygotes for classical galactosemia. Although there is no evidence of adverse effects of galactose consumption in these subjects, elimination of dietary galactose for at least the early months of life has been recommended as a prudent measure.

Genetics

After the cloning and sequencing of the GALT gene, more than 160 mutations have been described that have been associated with GALT deficiency. Q188R is the most common mutation in north European populations and those predominantly of European descent. K285N is much rarer but in some countries of eastcentral Europe it is the second most common mutation. In some populations of northern Europe and the white population in North America, these two mutations account for 70–80% of mutant chromosomes. Both mutations appear to be associated with a complete loss of enzyme activity and thus, a more severe biochemical phenotype. S135L is found almost exclusively in galactosemic individuals of African origin. In North America, this accounts for 62% of the alleles causing galactosemia in the black population and is associated with good outcomes.

The Duarte galactosemia variant is caused by a single amino acid substitution, N314D, which is found on both Duarte 1 and Duarte 2 alleles. Additional base changes that are different on each distinguish D1 from D2 alleles. Homozygosity for N314D reduces GALT activity to 50%. When either E203K or L218L (a neutral polymorphism arising from the 1721C → T transition (Los Angeles variant)) is present in *cis* with N314D, GALT activity reverts to normal.

Galactokinase-Deficient Galactosemia

GALK-deficient galactosemia, which occurs with a frequency of about 1 in 40 000, is characterized by cataracts that occur in the first or second decade of life but the subject is otherwise normal. In such patients, ingested galactose remains unphosphorylated and may be converted into galactitol, which causes damage to the lens fibers of the eye. The marked differences between GALK-deficient galactosemia and classical galactosemia in the severity and diversity of the symptoms and in the timescales within which they occur demonstrate that galactose-1-P is much more toxic than galactitol.

GALK deficiency is readily treated with a galactose-free diet and if this is started early in life, the only complication, cataracts, is avoided.

Epimerase-Deficient Galactosemia

Impairment of GALE results in epimerase-deficient galactosemia, an inborn error of metabolism with variable biochemical presentation and clinical outcomes reported to range from benign to severe. Molecular studies of the GALE loci from patients with GALE deficiency reveal significant allelic heterogeneity, raising the possibility that variable genotypes may constitute at least one factor contributing to the biochemical and clinical heterogeneity observed.

Inherited deficiencies of GALE have been associated with two distinct phenotypes. The vast majority of North American patients are clinically asymptomatic, are identified through newborn screening programs for classical galactosemia, and are of African-American descent. However, a severe, generalized form of GALE-deficient galactosemia has been described in a small number of subjects. The initial presentation is similar to classical galactosemia, including cataract, sepsis, and liver, kidney, and brain abnormalities. Despite treatment with a galactose-restricted diet, all have shown poor growth and moderate learning difficulties.

A single substitution mutation, V94M, is present in the homozygous state in all patients genotyped with the severe, generalized form of GALE-deficient galactosemia. Studies on the enzyme in a yeast model system support the hypothesis that elevated levels of galactose-1-P may underlie the observed toxicity.

Conclusion

Significant advances have been made in recent years in understanding the genetics of galactosemia. However, clinical galactosemia is a complex trait and the phenotypic expression is often not predictable. Except for cataract, the pathophysiology of galactosemia is poorly understood. Despite the concept that there may be continuous endogenous production of galactose in affected individuals, the treatment remains a diet as devoid of galactose as possible, within the confines of adequate nutrition for normal growth and development. However, it is now recognized that this does not prevent long-term complications and there is a need for new approaches to treatment, in combination with diet therapy, that could improve the outcome of patients with galactosemia.

See also: **Lactose and Oligosaccharides**: Lactose: Galacto-Oligosaccharides; Lactose Intolerance.

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Nutrigenomics and Nutrigenetics

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Introduction

In order to address the increasing incidence of many diet-related diseases, the role of diet and nutrition has been, and continues to be, studied extensively. To prevent the development of disease, nutrition research is investigating how nutrients and bioactive compounds can optimize and maintain physiological processes at the cellular, tissue, organ, and whole-body level. This involves a multitude of nutrient-related interactions at the gene, protein, and metabolic levels, and thus requires an understanding of how nutrients/bioactive food components act at the molecular level. The recognition that nutrients have the ability to interact and modulate molecular mechanisms underlying an organism's physiological functions has prompted a revolution in the field of nutrition with the emergence of the fields of nutrigenomics and nutrigenetics. These two fields, although with distinct approaches to elucidate the interaction between diet and genes, have a common ultimate goal, that is, to optimize health through the personalization of diet and to provide powerful approaches to unravel the complex relationship between nutrients/bioactive food components, genetic variation, and the biological system as a whole. This article will briefly overview the potential of these two fields for nutritional sciences.

Effect of Nutrition on Gene Expression

In the classic paradigm, genetic information encoded in the deoxyribonucleic acid (DNA) sequence of a given gene is first transcribed by ribonucleic acid (RNA) polymerases into messenger RNA (mRNA) and subsequently translated into proteins (**Figure 1**).

The transcription of a gene begins at the promoter region when double-stranded DNA is copied into mRNA. Transcription of a gene is not continuous or uninterrupted. Each gene contains DNA sequences that code for the amino acid sequence of the protein, which are called exons. The exons are interrupted by noncoding DNA sequences called introns, which are eventually spliced out during post-transcriptional processing. It is estimated that 80% of the influence of nutrients on the process of gene expression is at the level of transcription. Two types of factors regulate gene expression: *cis*-acting elements and *trans*-acting factors. *Cis*-acting elements do not encode proteins, but influence gene transcription by acting as binding sites for proteins that regulate gene transcription. These DNA sequences are usually organized in clusters located in the promoter region of the gene (i.e., TATA and CAAT boxes). *Trans*-factors, also known as DNA binding proteins, are proteins produced by other genes and include, for example, steroid hormone–receptor complexes, vitamin receptor proteins, and mineral–protein complexes. These *trans*-acting factors bind to specific DNA sequences in the promoter region of the target gene to promote gene transcription. Although the precise mechanisms by which *trans*-acting factors regulate gene expression have not been defined fully, it is thought that unfolding or folding of DNA may hide or expose regions of DNA.

A good example of how a nutrient impacts this step of the gene expression process is where vitamin D, in its active form, that is, 1,25-dihydroxyvitamin D, which interacts with the vitamin D receptor (VDR), stimulates the transcription of the gene encoding calbindin D9k – a cytosolic calcium-binding protein – in intestinal mucosal cells. Calbindin D9k facilitates the transepithelial transport of Ca^{2+}, thus enhancing efficiency of intestinal calcium absorption.

Once transcription has occurred, the resulting mRNAs are exported from the nucleus and form complexes with ribosomes, leading to the synthesis of polypeptides that form the given protein – this is translation. Three nucleotide bases within the mRNA molecule code for one amino acid and are referred to as codons. In some cases, multiple codons may code for and be translated into the same amino acid (e.g., the codons UGU and UGC both code for cysteine). The triplet AUG, known as the start codon (read as methionine), signals for commencement of translation from gene to protein. The subsequent codons are translated into amino acids, one at a time, to form polypeptides, which fold into complex and unique protein structures that are critical for normal body function. Nutrition can impact this step of the process by supplying amino acids and energy for the translational process. In some cases, the proteins require some post-translational modifications (chemical modifications) to become fully active. This post-translational modification of amino acids extends the range of functions of the protein by attaching to it other biochemical functional groups such as acetate, phosphate, and various lipids and carbohydrates, by changing the chemical nature of an amino

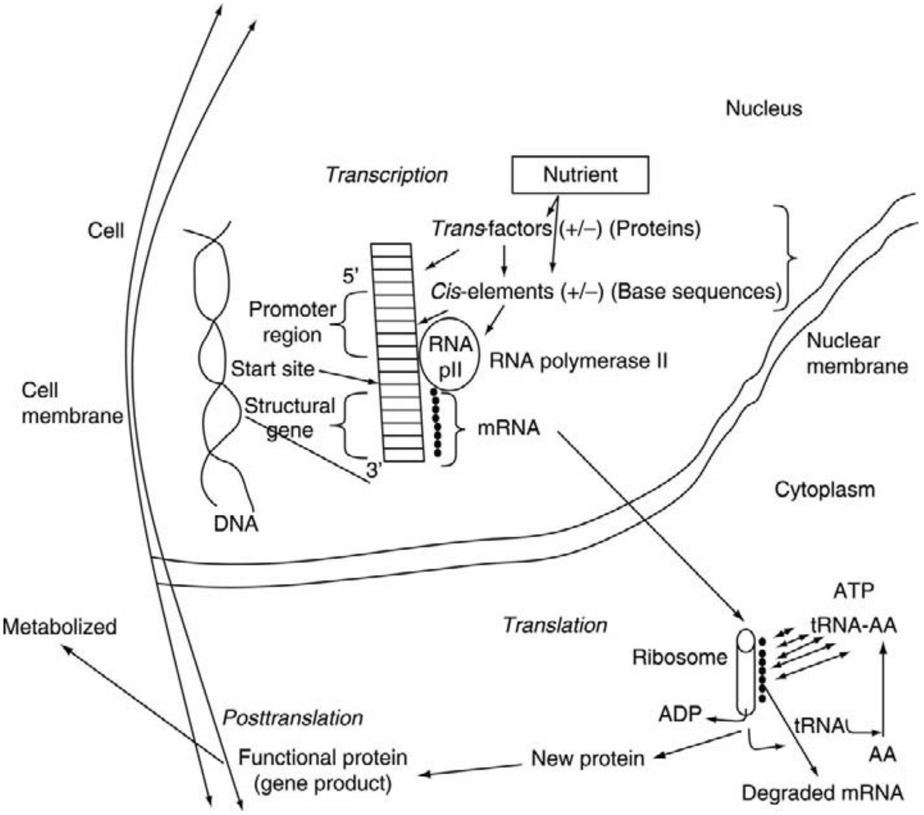
Nucleus
Transcription
Nutrient
Cell
Trans-factors (+/–) (Proteins)
5'
Cis-elements (+/–) (Base sequences)
Promoter region
RNA pII
Nuclear membrane
Start site
RNA polymerase II
Cell membrane
Structural gene
mRNA
3'
Cytoplasm
DNA
ATP
Metabolized
Translation
tRNA-AA
Ribosome
Posttranslation
ADP
tRNA
Functional protein (gene product)
New protein
AA
Degraded mRNA

Figure 1 Mechanism of gene expression.

acid (e.g., citrullination), or by making structural changes, such as by forming disulfide bridges. Again, nutrients may impact this step of the process. An example of where nutrition impacts this step of the gene expression process is how vitamin K plays an important role in the post-translational activation of several biologically active proteins involved in clotting and bone metabolism. Vitamin K is a cofactor for γ-carboxylase, which adds a carboxyl group to glutamate residues of certain proteins and by so doing increases their functionality.

The impact of nutrition on the various steps involved in the gene expression process has been researched for many years with traditional molecular biology approaches such as Northern blotting, polymerase chain reaction, and Western blotting. Use of the new 'omic' technologies in the nutrition field may provide even greater insights into the role of nutrition in human health.

Defining Nutrigenomics

This relatively new area of molecular nutrition within the wider nutrition field has brought with it an array of new terms. Nutritional genomics (which has led to the coined term nutrigenomics) can be defined as the study of the genome-wide influences of nutrition (or dietary components) on the transcriptome, proteome, and metabolome (defined below) of cells, tissues, or organisms at a given time.

- *Transcriptome*: The complete collection of gene transcripts (mRNAs) in a cell or a tissue at a given time (transcriptomics – the study of the complete collection of transcripts).
- *Proteome*: The complete collection of proteins detected in a cell or a tissue at a given time (proteomics – the study of the complete set of proteins).
- *Metabolome*: The full set of metabolites (which are intermediates and products of metabolism including hormones, signaling molecules, and primary and secondary metabolic intermediates) found within a biological tissue or fluid at a given time (metabolomics – the study of the complete collection of metabolites).

Thus, nutrigenomics aims to understand how nutrients and other bioactive food components affect gene expression and, in turn, how these changes influence protein expression, metabolism, and, ultimately, health outcomes.

These omic approaches produce vast amounts of data and there is a need to convert the data into some biological meaning. The field of bioinformatics has an instrumental role in this regard. By definition, bioinformatics is the application of information technology and computer science to the field of molecular biology. It entails the creation and advancement of databases, algorithms, computational and statistical techniques, and theory to solve formal and practical problems arising from the management and analysis of biological data. The primary goal of bioinformatics is to increase our understanding of biological processes.

Systems biology as a field tries to explore the complex interactions among biological systems. While currently there is no unifying consensus on how nutritional systems biology should be defined, some experts have described it as "covering all approaches targeted towards understanding the key processes that define nutrition in the context of regulation of transcription, protein synthesis and turnover, metabolism and genome stability in health and disease states at all levels of its complexity, to simulate these processes and to predict the outcome of any alteration (whether genetic or nutritional) of the system. Nutritional systems biology starts at the molecular and cellular levels and takes the regulatory processes onto the level of the different organs and the inter-organ flow of information, nutrients and metabolites to describe most comprehensively the organism response and the processes quantitatively in mathematical terms". More simply, nutritional systems biology tries to integrate information obtained from the different omic approaches to allow us to see or predict the impact of a nutrient/food component on physiology.

How Has Transcriptomics Advanced Nutrition Research

Transcriptomic methods such as microarray analysis (a technology that utilizes a microchip-based approach to analyze the expression of thousands of genes simultaneously) of samples from nutritionally relevant cell culture and animal models have led to the identification of many genes that are regulated at the mRNA level by exposure to different dietary components. The application of transcriptomics technology in human nutrition intervention studies, while being used only to a limited extent to date, has the potential to allow for genome-wide screening of the effects of diets or nutrients on physiological events and may possibly result in biomarker profiles indicative of positive or adverse changes. For example, in a human intervention study investigating the effects of a high-carbohydrate or a high-protein breakfast on satiety, van Erk and coworkers attempted to evaluate the potential of gene expression profiling in blood cells. The analysis showed that consumption of breakfast resulted in differentially expressed genes in blood leukocytes from healthy men: 317 genes for the high-carbohydrate breakfast and 919 genes for the high-protein breakfast. Bioinformatic analysis of the gene expression data showed that consumption of the high-carbohydrate breakfast resulted in significant changes in the expression of genes involved in glycogen metabolism, while consumption of the high-protein breakfast resulted in differential expression of genes involved in protein biosynthesis. Another recent study reported the post-prandial effects of intake of different fatty acids on gene expression profiles of peripheral blood mononuclear cells of healthy men participating in a dietary intervention study. Whole-genome gene expression profiles in the blood cells were examined before and 6 h after the intake of drinks containing polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs). The expression of genes involved in 'liver X receptor' signaling, which plays a major role in cholesterol transport, increased in response to dietary SFA and decreased in response to PUFA intake in the healthy men, suggesting that SFA intake may increase cholesterol efflux from cells, while conversely, PUFA intake may inhibit this efflux. These and other studies clearly show the value and potential of transcriptomic analysis of human tissue samples in human nutritional intervention studies.

How Have Proteomics and Metabolomics Advanced Nutrition Research

The proteome, unlike the genome, is dynamic and varies according to the cell type and functional state of the cell. Proteomic technologies, namely two-dimensional (2D) gel electrophoresis followed by liquid chromatography mass spectrometry (MS)/MS, can be used to identify changes in protein expression following an alteration in nutrient supply or environment. While proteomic analysis has been applied to nutrition research in relevant cell culture and animal models and has provided valuable new data, its application in human studies may be as, if not more, important to human nutrition research. Analysis of the serum or plasma proteome may provide further insights into the effects of nutrition on human health. Recently, this type of analysis was applied to plasma samples collected from adults in a human intervention trial. Folic acid supplementation for 12 weeks increased the expression of more than 60 proteins involved in metabolic pathways related to complement fixation, coagulation, and mineral transport, thus opening new avenues of investigation.

Metabolomics is one of the newer 'omics' to be applied to nutrition research, which aims to quantify (using MS and/or nuclear magnetic resonance spectroscopy) the temporary changes in cellular small-molecular-weight compounds (i.e., hormones, signaling molecules, and metabolic intermediates) in human biotissues (i.e., urine and plasma) in response to nutrients and/or bioactive food compounds. A recent study investigated the role of dietary phytochemicals in shaping human urinary metabolomic profiles. First-void urine samples were collected from healthy adults following a normal diet, a 2-day low-phytochemical diet, or a 2-day standard phytochemical diet. Metabolomic analysis showed acute changes in urinary metabolomic profiles after the consumption of dietary phytochemicals, illustrating the potential of detecting diet-induced changes. A second example is a recent intervention study that aimed to elucidate the metabolic changes in blood plasma following weight loss and fish oil supplementation. Two groups of women followed a 12-week weight loss program, followed by a 12-week weight maintenance period, and were randomized to fish or placebo oil capsules; a control group did not follow the weight loss program and were given placebo oil capsules. Lipid profiles changed dramatically upon fish oil intake and subtly across the two weight loss groups. While the fish oil supplementation increased the proportion of various phospholipid species, previously reported reductions in total triacylglycerides (TAGs) upon fish oil intake were shown to be driven by a reduction in a specific subset of the measured TAGs. This metabolomic study showed that the observed remodeling of TAGs may represent further beneficial effects of fish oil supplementation. The potential of metabolomic analysis may lie in better quantification of nutrient and nonnutrient intake as well as in the identification of biomarkers (distinct metabolite fingerprints) of relevance to nutrition-related disease.

Limitations of These ‘Omics’ Technologies

While novel omics technologies and bioinformatics tools continue to offer enormous potential to investigate the complex relationship between nutrition and the transcriptome/proteome/metabolome, it is also important to recognize their limitations. For example, a number of limitations are associated with microarray analysis. When human blood samples are used, the inter-individual human variation in gene expression profiles makes it difficult to identify gene expression signatures in response to nutrient intervention. This is why many of the studies use cross-over designs that use subjects as their own controls and thus minimize variability in gene expression profiles and optimize the chance of detecting changes in these profiles arising from dietary modulation. However, not all human intervention studies can be cross-over type in design. Furthermore, because nutrient interventions generally result in relatively small gene expression changes, it can be difficult to accurately facilitate data analysis and interpretation of the experimental results. Costs associated with microarray analysis are very high, and are often compounded by the need for several repeat analyses to obtain statistically accurate results. In terms of proteomic analysis, there are a number of limitations in 2D gel electrophoretic analysis, such as poor resolution for low abundant proteins as well as the inability to detect proteins with extreme properties (i.e., very acidic, very basic, very large, very small). Furthermore, 2D gel electrophoretic analysis has proven to be a costly and time-consuming method. Limitations in metabolomic research arise from the highly dynamic nature of the human metabolome, which makes it difficult to link metabolites back to specific genes and proteins; furthermore, it is difficult to isolate metabolic effects of food supplements, drugs, physical activity, and stress, among several other physical factors.

Defining Nutrigenetics

Nutritional genetics (which led to the coined term nutrigenetics) can be defined as the study of how genetic variation (different genotypes) among individuals may modify their physiological response to diet and how this ultimately influences parameters of health or disease.

Nutrigenetics is closely associated with ‘personalized nutrition’, an emerging concept that an individual’s diet can be tailored to their genetic makeup to optimize health status and prevent the onset of disease. This field has received significant impetus since the publication of the human genome in 2003. The human genome comprises more than 3 billion base pairs, encoding for approximately 30 000 genes and 100 000 functionally distinct proteins. Although human individuals are 99.9% genetically identical, most genes have a number of small sequence differences known as polymorphisms that occur (on average) approximately every 1500 base pairs. Single nucleotide polymorphisms (SNPs; the switching of one nucleotide to another) in genes can affect protein translation by changing the reading frame of that gene such that mRNA codes for a different amino acid. For example, if the codon for cysteine (UGU) has an SNP located at the third nucleotide to form UGC (a codon that also translates to cysteine), then the mutation is known as ‘silent’ and does not affect the resulting protein or its function. However, if the SNP occurs at the first nucleotide to form CGU, then the codon is translated to an alternative amino acid, arginine, that may or may not alter protein functionality. SNPs located on the first or second nucleotide in a codon commonly alter translation of amino acids, possibly impacting protein functionality.

Most nutrition-related diseases such as cardiovascular disease, metabolic syndrome, cancer, and osteoporosis, among others, are complex multigene disorders that are associated with the effects of multiple genes in combination with lifestyle and environmental factors. Several candidate genes have been investigated in terms of their involvement in these diseases (see **Table 1** for a list of possible candidate genes that have been suggested to have

Table 1 Candidate genes for osteoporosis

Candidate gene	*Physiological function*
Vitamin D receptor (VDR)	Calcium absorption; osteoblast/osteoclast activity
Estrogen receptor α	Osteoblast/osteoclast activity
Estrogen receptor β	Osteoblast/osteoclast activity
Collagen I A I	Matrix component
Transforming growth factor β-1	Osteoblast/osteoclast activity
Androgen receptor	Osteoblast function
Interleukin 6	Osteoblast activity
Apolipoprotein E	Vitamin K transport
Parathyroid hormone receptor	Calcium homeostasis; osteoblast/osteoclast activity
Calcitonin receptor	Osteoclast function
Peroxisome proliferator-activated receptor γ	Adiposite differentiation
Osteocalcin	Matrix component
Calcium sensing receptor	Regulation of calcium homeostasis
Methylenetetrahydrofolate reductase	Homocysteine metabolism
Metalloproteinase-1 gene	Matrix component

some role in the risk of osteoporosis). A candidate gene is a gene located in a chromosomal region suspected of being involved in the expression of a disease trait, with the protein product indicating the gene in question. A candidate gene can also be identified by association with the phenotype and by linkage analysis to a region of the genome. There have been many associational studies in molecular medicine and associated fields that link the presence of one or more SNPs within a candidate gene with the increased risk of a certain disease. Clearly, this has enormous potential in the area of molecular diagnosis.

In many ways, nutrigenetics tries to explore and exploit the interaction between genetic variation (SNPs) and nutrition. In recent times, several human dietary intervention studies have included a genotype dimension to their design. This allows them to additionally explore whether the response of a physiological/disease outcome to the dietary intervention is modulated by SNPs in candidate genes such that individuals with different genotypes respond differently. As an example in the area of diet and bone health, SNPs in the VDR gene were among the first to be studied widely as genetic risk factors for osteoporosis. However, an example of moving beyond molecular diagnosis and more toward using genotype information to inform treatment or nutrition intervention/advice, was demonstrated by Graafmans and coworkers, who reported an interaction between one such VDR polymorphism (defined by the *Bsm* I restriction endonuclease enzyme) and response of bone mineral density to vitamin D supplementation. The mean increase in bone mineral density in the vitamin D-supplemented group was higher in subjects with the '*BB*' and '*Bb*' VDR genotype compared with those with the '*bb*' VDR genotype. Another example is in the area of nutrition and cardiovascular health. A study reported a significant interaction between a polymorphism in the estrogen receptor-β (ERβ) gene (defined by the *Alu*I restriction endonuclease enzyme) and dietary isoflavone (IF) supplementation in terms of the response of C-reactive protein (CRP), a biomarker of cardiovascular disease risk, in postmenopausal women. *Alu*I ERβ genotype significantly modulated the response of CRP to dietary IF supplementation, such that women with the *AA* (ERβ) genotype responded to treatment with significantly lower CRP concentrations compared with women having the same genotype but in the placebo group, who experienced significantly elevated CRP concentrations following the 8-week intervention. Dietary IF supplementation had no significant effect on CRP levels in the *GG* or *GA* ERβ genotype groups, suggesting a beneficial cardio effect of dietary IF for postmenopausal women with the *AA* ERβ genotype.

In terms of the application and realization of a 'tailored/personalized nutrition' approach, that is, an individual's diet can be tailored to their genetic makeup to optimize health status and prevent the onset of disease, the associations in studies such as those mentioned above would need to be consistently shown to be the case, providing a solid evidence base. Often, unfortunately, this is not the case and may well be due to an overly simplistic view that one nutrient will interact with one gene in isolation to influence a biological process, whereas in reality there may be interactions of the gene in question with other genes in addition to its interaction with a nutritional and/or other environmental factor(s).

Conclusion

It is hoped that by building up the knowledge base, nutrigenomics and nutrigenetics will promote an increased understanding of how diet and nutrition influence metabolic pathways and physiological events, which will then be used to prevent the development of chronic diet-related diseases. Ultimately, the application of these fields in nutrition research could lead to the development of functional foods that will keep people healthy according to their individual needs. However, both fields are still at a relatively early stage and many of the associated technologies and study design parameters are still in the process of further development and/or refinement. Thus, while expectations are high, we need to monitor their success over the next decade to truly know their impact.

See also: **Molecular Genetics and Dairy Foods**.

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Nutraceuticals from Milk

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Introduction

Milk contains many components with physiological functions, such as proteins and peptides, fats, oligosaccharides, vitamins, and minerals. These different milk components could be used as nutraceuticals to promote good health and prevent disease. Milk fat is contained within the fat globule and surrounded by a membrane: the milk fat globule membrane (MFGM) (*see* **Milk Lipids**: Milk Fat Globule Membrane). This particular structure is composed of a core of triglycerides and a membrane consisting of phospholipids, cholesterol, proteins, and glycoproteins. Both the membrane and the core components possess antimicrobial functions. Major sphingolipids in milk (glycosphingolipids) may play a role in the numerous antiproliferative pathways associated with the suppression of carcinogenesis. Other lipid compounds, such as conjugated linoleic acid (CLA), have several biological activities, such as inhibiting cancer and atherosclerosis (*see* **Milk Lipids**: Conjugated Linoleic Acid). Lactose derivatives such as lactulose, lactitol, and oligosaccharides stimulate the growth of bifidobacteria (*see* **Lactose and Oligosaccharides**: Lactose: Galacto-oligosaccharides). Milk is a source of exogenous regulatory substances, including bioactive proteins and peptides with various biological properties. These biologically active milk proteins include lysozyme and lactoperoxidase (enzymes), immunoglobulins, transferrin, and lactoferrin, as well as various growth factors. Bioactive peptides, which can be released during gastrointestinal digestion, have also been identified in the casein and whey protein sequences and represent a new criterion when assessing the nutritional value of protein. A variety of activities are associated with milk-derived peptides (*see* **Milk Protein Products**: Bioactive Peptides): (1) in 1979, opioid agonistic peptides derived from milk protein were identified for the first time and, in 1986, peptides with antagonistic opioid activity were characterized; (2) in 1982, peptides with angiotensin-converting enzyme (ACE) inhibitory activity were discovered and these ACE inhibiting peptides were shown to have an antihypertensive effect; (3) peptides with fibrinogen-like sequences and thus having antithrombotic activity have been described; (4) numerous immunomodulating peptides are capable of increasing phagocytic activity and lymphocyte proliferation; (5) some peptides, including phosphopeptides, facilitate the absorption of minerals, especially calcium, magnesium, and iron; and (6) some peptides exhibit antimicrobial activities. It should be noted that many milk peptides possess multiple functional activities.

Gastrointestinal Digestion of Milk Protein-Derived Peptides

Milk proteins are subjected to digestion and absorption processes in the gastrointestinal tract. They can act within the intestinal tract or may exert their activities in various peripheral organs following absorption. In order to become active, bioactive peptides must be released either during gastrointestinal digestion or after their absorption. Gastrointestinal processes control the release of amino acids and nitrogen into the body and hence their metabolic utilization. The principal milk proteins are caseins and whey proteins. Bovine casein has four components: α_{S1}- and α_{S2}-casein, β-casein, and κ-casein. The main components of whey proteins are β-lactoglobulin and α-lactalbumin. The first step in protein digestion consists of acid secretion and pepsin hydrolysis in the stomach. Whey proteins are soluble and are emptied quickly into the duodenum, mainly in an intact form. In contrast, caseins are precipitated in the stomach and then slowly released into the duodenum in the form of degraded products. In the duodenum, these products are subjected to pancreatic enzymes. In the intestinal lumen, α-lactalbumin, β-lactoglobulin, and lactoferrin have been found in an intact form, unlike the caseins. The intestinal brush border contains several peptidases, and the major peptides are degraded into dipeptides and tripeptides. Some peptides are relatively resistant to these enzymes and may reach their body target intact. Proline-containing peptides are generally resistant to degradation by digestive enzymes.

Bioactive peptides may act directly on targets present in the gastrointestinal tract. Dipeptides and tripeptides can also cross the intestine and reach peripheral target sites. Other specific transport systems have been described, such as transcytosis, implicated in α-lactalbumin, β-lactoglobulin, and lactoferrin transport. These proteins have been found in an intact form in the blood. Peptides may also be released by enzymatic proteolysis in the body. Whereas these peptides are inactive in the native protein, the bioactive sequence is released in the body. It should be noted that these peptides can be produced by proteolytic bacterial species.

Control of Gastrointestinal Processes

The gastrointestinal processes involved in milk digestion control the release and absorption of milk peptide-associated minerals, which include calcium, magnesium, and iron. Some milk protein-derived peptides may have a specific regulating effect on gastrointestinal physiology and metabolism.

Absorption of Minerals

Casein occurs in milk in the form of micelles, which are carriers of phosphates and calcium, magnesium, and iron ions. Milk is an important source of calcium and it also contains lactose, which increases intestinal permeability of calcium. Some peptides have been isolated from different phosphorylated regions of α_{S1}-, α_{S2}-, and β-caseins, and are called caseinophosphopeptides (CPPs). They are capable of binding minerals, particularly calcium. Phosphopeptides may enhance calcium absorption by preventing the precipitation of insoluble calcium phosphate. These peptides are released during the gastrointestinal digestion of casein. They are particularly resistant to gastrointestinal and plasma proteolysis, and some of them have been found in feces. A few experiments have been carried out in pigs and rats; however, useful results were obtained only under certain experimental conditions, and mainly during investigations on the short-term calcium balance in growing animals. Phosphopeptides have exhibited positive effects on mineral solubility and absorbability and bone mineralization. This could be considered as beneficial to bone health in certain groups of population.

Regulation of Intestinal Motility and Metabolic Processes

The caseinomacropeptide (CMP), which represents the 64-amino acid (106–169) C-terminal fragment of bovine κ-casein, has been demonstrated to have a variety of biological activities, including the modulation of gastrointestinal motility and regulation of the release of gastrointestinal hormones, such as cholecystokinin (CCK). It has been shown to stimulate secretions by the pancreas. Opioid peptides have been identified in milk; they have morphine-like activity through their interaction with opioid receptors (μ-, δ-, and κ-type). Various biological effects have been investigated with respect to opioid receptors in the body. They are located in the nervous, endocrine, and immune systems and are also found in the gastrointestinal tract. Opioid peptides isolated from bovine β-casein (fragments 60–70) have been named β-casomorphins. They have been characterized as μ-type ligands. β-Casomorphins have also been found in human β-casein. Their role in the organism is related to their bioavailability. β-Casomorphins are relatively resistant to the action of gastric and pancreatic enzymes, but are hydrolyzed by intestinal brush border peptidases. β-Casomorphins are released after the ingestion of milk and exert their effects mainly in the gastrointestinal tract. If they are to interact with opioid receptors located on the intestinal surface, opioid peptides must be applied directly. They can also exert an antidiarrheal effect by stimulating electrolyte absorption and inhibiting intestinal motility. β-Casomorphins have also been reported to increase the release of somatostatin and a pancreatic polypeptide, and stimulate the release and formation of insulin, which is dependent on the blood glucose level. Other opioid peptides have been isolated from bovine α_{S1}-casein (residues 90–96); these are δ-selective receptor ligands. Some peptides have been found in whey proteins: α-lactorphin, β-lactorphin, and serorphin have been isolated from bovine and human α-lactalbumin (residues 50–53), β-lactoglobulin (residues 102–105), and serum albumin (residues 399–404), respectively. Opioid antagonists have been isolated from bovine and human κ-casein and named casoxins. Lactoferoxin has been identified in human lactoferrin, and some fragments of α_{S1}-casein possess similar activity. These fragments bind to the opioid receptors and exhibit an antagonistic action, which may be observed following the administration of naloxone.

Enhancing Body Defenses

Bovine milk and colostrum have various antimicrobial activities. The colostrum contains several immunoglobulins, which confer passive protection on neonates against a wide range of microbes. Milk contains immunological components, such as enzymes (lactoperoxidase), immunoglobulins, and growth factors, as well as certain antimicrobial peptides, which can modulate the immune system. These anti-infective components can be considered as functional foods because they enhance nonspecific body defenses.

Antimicrobial Activities

CMP has been found to prevent the adhesion of oral actinomyces and streptococci to erythrocytes. It can also inhibit the binding of cholera toxin to its receptor. CMP may contain 0, 1, or 2 mol mol^{-1} sialic acid. Because sialic acid residues are present on the surface of the target cells required for influenza virus infection, CMP has been found to inhibit the hemagglutination of influenza virus. In addition, human CMP has also been found to enhance the growth of the beneficial bacterium *Bifidobacterium infantis*.

Lactoferrin is an iron-binding glycoprotein in milk and is involved in host defenses against infection. Iron is essential to microbial growth. The antimicrobial activity of lactoferrin is due not only to iron chelation. It is able to induce the release of lipopolysaccharides from the bacterial membrane. By altering membrane permeability, bacterial susceptibility to lysozyme is increased. Lactoferrin has antimicrobial effects against bacteria such as *Escherichia coli*, *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Vibrio cholerae*, and certain yeasts, particularly *Candida albicans*. Lactoferricin, a fragment of 25 amino acid residues isolated by gastric pepsin cleavage of lactoferrin, is a more potent bactericidal substance than undigested lactoferrin. It contains several basic amino acid residues, and their asymmetric clustering generates an affinity for biological membranes. Lactoferricin may exert an antibacterial effect by increasing cell membrane permeability. It is highly effective against several pathogenic bacteria, including strains of *Escherichia*, *Listeria*, *Pseudomonas*, *Salmonella*, and *Campylobacter*, but not against beneficial bacteria such as *B. infantis*. This peptide is also effective against pathogenic yeasts such as *C. albicans*. A peptide of 47 amino acid residues, possessing similar activities, has been liberated from human lactoferrin.

Casocidin, which contains a high proportion of basic amino acids, has been isolated from α_{S2}-casein (residues 165–203). It inhibits bacteria such as *E. coli* and *Staphylococcus carnosus*.

Immunomodulating Effects

Some milk peptides have been found to act like immunopotentiating compounds. Peptides isolated during the tryptic and chymotryptic hydrolysis of α_{S1}- and β-caseins and α-lactalbumin enhance phagocytosis and modulate proliferation and differentiation of human peripheral blood lymphocytes. For example, an opioid peptide isolated from β-casein (residues 63–68) has been shown to stimulate the immune system and particularly phagocytosis. This example demonstrates that one peptide may have several biological activities. The mechanism by which peptides exert their immunomodulatory effect has not yet been defined, but it may be related to the opiate receptor. Two peptides isolated from human β-casein, a hexapeptide (Val-Gly-Pro-Ile-Pro-Tyr) and a tripeptide (Gly-Leu-Phe), were found to stimulate *in vitro* the phagocytic activity of sheep red blood cells by murine peritoneal macrophages and *in vivo* to prevent infection in mice after an intravenous injection of bacteria (*Klebsiella pneumoniae*).

Bovine and human lactoferrin inhibit lectin-stimulated lymphocyte blastogenesis. Human lactoferrin is able to differentiate CD4-/CD8-lymphocytes into CD4-helper T lymphocytes. It also has an effect on the release of cytokines in response to stimulation of monocytes by the lactoperoxidase system (LPS).

Isracidin is a fragment of α_{S1}-casein (residues 1–23). It inhibits the proliferation of *St. aureus* and *in vivo* protects mice from infection with *C. albicans* by stimulating phacocytosis and lymphocyte proliferation.

Following exposure to antigen, CMP decreases the immune response and especially the production of IgG-class antibodies by enhancing suppressor T lymphocytes.

In many cases, the immunomodulatory function of these milk peptides has been studied either *in vitro* or *in vivo*, and the studies have focused on specific components. In this field, it is important to investigate complex milk-derived products as well as individual milk proteins or peptides. Immunomodulating milk products are promising examples of health-promoting functional foods.

Putative Protective Effects on Chronic Diseases

Antihypertensive Effect

The angiotensin I-converting enzyme (ACEI) plays a key role in regulating the peripheral blood pressure. In the renin–angiotensin system, this enzyme converts angiotensin I into angiotensin II, which raises the blood pressure. Some milk peptides have been shown to inhibit this enzyme and lower the blood pressure. Such peptides have been identified mainly in α_{S1}-, β-, and κ-caseins. Some opioid peptides, such as α-lactorphin, have also been shown to possess an ACEI lowering activity. The antihypertensive effects of orally administered doses of Calpis sour milk or peptides (Val-Pro-Pro, Ile-Pro-Pro) were tested in spontaneously hypertensive rats (SHRs) and also in hypertensive patients. The systolic blood pressure had fallen significantly at 4 and 8 weeks after the beginning of ingestion. These effects on hypertension were mild in human volunteers. Most milk protein-derived peptides do not have an ACE inhibitory potential approaching that of captopril, one of the preparations commonly used to control blood pressure. However, as they are natural substances, they are not anticipated to produce the adverse effects associated with a synthetic preparation. Further clinical studies are necessary to demonstrate the efficacy and safety of these peptides as potential nutraceuticals (*see* **Fermented Milks**: Health Effects of Fermented Milks).

Antithrombotic Effect

A homology between the clotting of blood and milk has been described. Fibrinogen is essential for platelet aggregation. Some peptides from bovine κ-casein can act on platelet aggregation because of their structural similarity to the human fibrinogen γ-chain, particularly the (106–116) residue peptide. The latter inhibits the binding of fibrinogen and the aggregation of platelets treated with adenosine diphosphate (ADP). Shorter peptides also have antithrombotic activities. A peptide with a similar activity has been isolated from human lactoferrin (residues 39–42). These activities have been tested with *in vitro* models by evaluating ADP-induced platelet aggregation. After the ingestion of milk or yogurt, CMP, which contains an antithrombotic sequence, was detected in human plasma. After milk or milk product consumption, antithrombotic peptides may be found in the systemic circulation. The physiological consequences of their presence need to be demonstrated in humans.

Putative Anticarcinogenic Activity

Milk or its by-products may have beneficial activity with regard to the prevention of cancer. Recent epidemiological studies have suggested a relationship between the intake of milk components and a chemopreventive role in certain forms of cancer. However, research on the anticarcinogenic effects of milk components is difficult because of the different etiologies of cancer.

Many components have been studied in the context of cancer prevention, such as lactoferrin, dihydroxycholecalciferol (1,25$(OH)_2D_3$), calcium, phosphorus, lactic bacteria, butyrate, and CLA. Whey proteins also represent a source of anticarcinogenic components. Their putative anticancer activity has been studied extensively in animal models of tumorigenesis or during *in vitro* studies using tumor cell lines. In contrast to the lack of controlled clinical studies, the role of whey proteins in preventing cancer has been studied widely in rats. Their anticancer activity may be related to their high cysteine and glutamic acid contents, which serve as precursors of glutathione (GSH), a potent antioxidant whose release is enhanced during stress. Whey proteins may cause a depletion of GSH in cancer cells, thus slowing their replication, but not affecting normal tissues. A study performed on mice demonstrated that the incidence of colon cancer induced by dimethylhydrazine (DMH) was reduced in animals following the administration of whey proteins, because of enhanced tissue GSH concentrations. There is also some evidence from controlled clinical studies of the effectiveness of whey protein concentrate in limiting metastasis during anticancer therapy. Indeed, the beneficial effects of certain milk-derived components, such as lactoferrin or CLA, have been demonstrated in animals, although further studies under controlled clinical conditions are necessary. Other minor constituents, especially low-molecular-weight proteins and peptides, may also have anticancer properties, which need to be identified. Numerous clinical studies will be required to establish a clear relationship between consumption of dairy products and cancer prevention (*see* **Health Aspects of Dairy Products**: Nutritional and Health-Promoting Properties of Dairy Products: Colon Cancer Prevention).

Bone Resorption

Milk proteins have been shown to increase mineral absorption, and they can also act on bone resorption. Recent studies have shown that whey proteins contain active components that affect osteoclast-mediated bone resorption. Bone resorption and osteoclastic cell formation have been studied using an unfractionated bone-cell culturing system. The active components were isolated and their gut permeability was studied, and it was shown that they could be absorbed or transported via the intestine. Whey proteins could act on bone metabolism by suppressing osteoclast-mediated bone resorption and osteoclastic cell formation. Further investigations are necessary *in vivo* (*see* **Health Aspects of Dairy Products**: Nutritional and Health-Promoting Properties of Dairy Products: Bone Health).

Conclusion

Milk represents a source not only of essential amino acids and organic nitrogen but also of peptides with biological activities. Amino acid sequences with biological activities are well characterized in caseins and whey proteins. These peptides can be released by enzymatic degradation in the body and may be considered to act like exogenous metabolic modulators. The physiological importance of these peptides is the subject of extensive studies. They play a significant role in regulating gastrointestinal processes, such as intestinal motility, hormone release, and nutrient absorption. They can also act on the body defenses. They may be used for their health benefits in preventing chronic conditions, such as cardiovascular disease or cancer. On account of their different biological activities, these peptides represent some new and beneficial aspects in nutrition. However, further research is required, particularly in humans, to fully substantiate the role of these bioactive substances.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks. **Fermented Milks**: Health Effects of Fermented Milks. **Lactose and Oligosaccharides**: Lactose: Galacto-Oligosaccharides. **Milk Lipids**: Conjugated Linoleic Acid; Milk Fat Globule Membrane. **Milk Protein Products**: Bioactive Peptides. **Nutrition and Health**: Nutritional and Health-Promoting

Properties of Dairy Products: Bone Health; Nutritional and Health-Promoting Properties of Dairy Products: Colon Cancer Prevention; Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake.

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Effects of Processing on Protein Quality of Milk and Milk Products

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Overall Effects of Processing and Storage on Milk Proteins and Their Nutritional Significance

Besides being a source of dietary amino acids, milk proteins have many unique features. From a technological point of view, proteins affect the properties of dairy products more than any other constituent. Many specific proteins appear to be biologically active and to act as enzymes, antimicrobial or antioxidant agents, metal or vitamin binders, or growth factors. More recently, the physiological impact of some peptides encrypted in milk proteins has been demonstrated as well. For all the above reasons, milk proteins have been studied so extensively in terms of structure, function, and stability that, at present, these probably represent the best-characterized food protein system.

Processes adopted in food manufacturing have the primary purpose of obtaining safe, nutritious, stable, and tasty products. Heat treatment is involved in the processing of almost all milk products, and heating conditions may vary from mild thermization (about 65 °C for 15 s) to severe sterilization (e.g., 120 °C for 10–20 min). To some extent, storage of the finished product may be regarded as an additional heat treatment. In fact, some dairy products, like processed cheese or sterilized milk, are kept at room temperature on the shelves for months.

During heating and storage, milk proteins are subjected to a number of modifications involving the molecule itself or resulting from interactions with other milk constituents or non-dairy ingredients. Both the nature and extent of these modifications are temperature- and time-dependent, although other factors, like amino acid sequence and protein conformation, pH, and water activity (a_w), may play a relevant role. The most important non-enzymatic mechanisms involved are related to (1) denaturation, with conformational changes of the protein molecule and irreversible modification of the three-dimensional structure, and (2) covalent modifications, in which the side-chains of amino acids participate. Most of the time, these two types of mechanism occur in combination, as covalent changes may imply a change in protein conformation and vice versa, and both affect stability and technological properties of the protein fraction in heated milk products (**Table 1**). At the same time, these modifications could decrease the digestibility and nutritional value of milk proteins (*see* **Milk Proteins**: Nutritional Quality of Milk Proteins).

Protein Denaturation

Because caseins lack typical stable secondary and tertiary structures, they are very heat-stable molecules. Milk at its natural pH may withstand heating at 100 °C for hours without apparent changes in casein solubility (*see* **Heat Treatment of Milk**: Heat Stability of Milk). In contrast, whey proteins are typical globular proteins and, when exposed to high temperatures, undergo unfolding, with exposure of the reactive free thiol groups, and subsequent irreversible denaturation and aggregation reactions. Upon heating, whey proteins can either self-aggregate or form stable aggregates with casein, mainly involving disulfide bonds. Both κ-casein and α_{s2}-casein have cysteinyl residues and therefore both could form covalent aggregates with denatured whey proteins. However, unlike κ-casein, α_{s2}-casein is located mainly in the interior of the micelle and, therefore, is poorly reactive. Under the usual conditions of heat processing of milk, whey protein denaturation begins at about 65 °C, while soluble whey proteins are no longer detected in in-bottle sterilized milk. From the technological point of view, whey protein aggregation with casein in milk intended for cheesemaking progressively results in an increased rennet coagulation time and a weaker gel. On the contrary, in yogurt manufacturing, denatured whey protein molecules help in determining the target firmness of the gel and improving its water-holding capacity. Although heat treatments are generally assumed to impair protein quality, contradictory results are reported concerning digestibility of milk proteins in milk heated under different conditions. In addition, the different criteria and conditions used to determine protein digestibility make it difficult to compare data from different studies. The actual nutritional consequences of milk heat treatment have rarely been evaluated *in vivo* in humans. However, quite a good agreement exists on the finding that protein digestion is more rapid after UHT milk ingestion than it is with pasteurized milk, leading to an increased transfer of dietary nitrogen into plasma protein and urea. This effect could be explained by a softer coagulation occurring in UHT milk, likely due to the presence of denatured

Table 1 Main effects of various heat-induced non-enzymatic modifications on some milk protein characteristics

Modification	*Conformation*	*Chemical composition*
Amino acid racemization	Yes	No
Amino acid isomerization	Yes	Yes
Deamidation	Yes/no	Yes
Dephosphorylation	Yes	Yes
β-Elimination	Yes/no	Yes
Glycation	Yes/no	Yes
Cross-linking	Yes	Yes/no
Oxidation	Yes	Yes

whey proteins at the surface of casein micelles, and hence a higher susceptibility to enzymatic hydrolysis.

Maillard Reaction

Apart from the disruption and formation of disulfide bonds, leading to whey protein denaturation and casein–whey protein bonding, protein glycation via the Maillard reaction (MR), also known as non-enzymatic browning, is mainly involved in heat-induced covalent interactions in heated milk and milk products (*see* **Lactose and Oligosaccharides**: Maillard Reactions). The MR is usually described as divided into three stages: early, advanced, and final. The early stage involves the condensation reaction of the reducing moiety of lactose with an amine, mainly the ε-amino group of lysyl residues of casein and whey proteins. As a result, a Schiff's base forms and then rearranges to produce the more stable Amadori product lactulosyl lysine (LL). Other primary or secondary amines can react as well. The conjugation of lactose to milk proteins is greatly accelerated by heat, and it renders lysine biologically unavailable. Under severe heat treatments, LL further undergoes a complex series of condensation, rearrangement, and fragmentation reactions, forming a vast array of degradation products, predominantly poorly characterized and collectively named as advanced glycation end products (AGEs). Among these, N^{ε}-(carboxymethyl)lysine (CML) has been recognized as a biomarker of glycoxidative, lipoxidative, and oxidative stress in biological systems, as it may originate by different pathways, including oxidation of LL. As a result, the formation of CML in some dairy products, especially those dried, is not always closely related to the heat load applied in processing.

Many AGEs are unstable and may give rise to covalent intra- and inter-molecular cross-links involving other essential amino acids, thus affecting nutritional properties other than lysine bioavailability. During the final stage of the MR, which usually is not reached in dairy products, progressive polymerization into brown, water-insoluble melanoidins takes place. Their precursors, referred to as pre-melanoidins, can react with and destroy some vitamins and influence trace element metabolism. Direct nutritional effects of the MR on milk proteins are therefore mainly related to impairment of their bioavailability by derivatization of essential amino acids, such as lysine, arginine, histidine, and tryptophan, and, to a lesser extent, by cross-linking phenomenoa. In addition, it is possible that LL residues hinder the release of adjacent amino acids on digestion and therefore impair their utilization. Some of the molecules responsible for these phenomena will be considered later in this section. As a general remark, the biological role of exogenous food-derived AGEs is receiving much attention, given that the amount of MR products ingested daily with commonly consumed foods, such as heated milk, bakery products, and coffee, has been estimated to be 500–1200 mg of Amadori products and 25–75 mg of AGEs, which is much higher than the total amount of AGEs in plasma and tissues. Since a high proportion of the exogenous AGEs is absorbed and excreted via the kidneys, the contribution of AGEs and Amadori products in milk (based) products to plasma AGEs content could be greater in subjects with impaired renal function.

Other Modifications

A number of other non-enzymatic covalent cross-links of milk protein chains are formed starting from the heat-induced β-elimination reaction at cyst(e)ine or phosphoserine residues, which break down to form a dehydroalanyl (DHA) side chain. This reaction is enhanced when the pH of the protein microenvironment is increased. The subsequent reaction of DHA with adjacent nucleophilic lysyl, cysteinyl, or histidinyl side chains originates the xenobiotic cross-links lysinoalanine (LAL), lanthionine, or histidinoalanine, respectively (**Figure 1**).

Some essential amino acids are thus rendered non-bioavailable through this mechanism. It is generally accepted that *in vitro* digestibility of milk protein is adversely affected by heat and alkali treatment. This is primarily due to the formation of inter- and intra-molecular cross-links, which hinder access of proteolytic

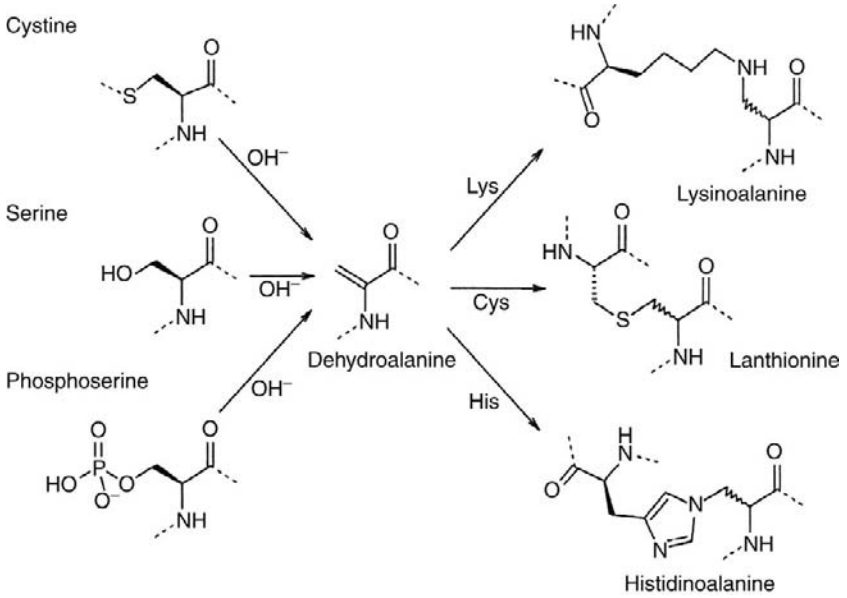

Figure 1 Formation of protein cross-links via β-elimination reactions.

enzymes. In addition, both lysyl and arginyl residues involved in cross-links are no longer recognized by specific proteases. It is still unclear to what extent dietary LAL ingested in heat-treated proteins is utilized by the human body. Urinary and fecal excretions in humans account for only about 60% of the intake, while lower recoveries were obtained with rats. The fact that rats exhibit renal lesions (cytomegaly) after consuming LAL-rich diets for several weeks has focused some toxicological interest on LAL; however, no similar effects have been observed in primates.

Furthermore, thermal dephosphorylation of phosphoserine residues of casein impairs the micelle's capacity to bind calcium and to produce bioactive caseinophosphopeptides, which, during the last decades, have been associated with some biological properties of caseins (*see* **Milk Protein Products**: Bioactive Peptides).

Formation of LAL is concurrent with racemization of amino acid residues in proteins; therefore, nutritional value and related changes resulting from the consumption of heat- and/or alkali-treated proteins arise as a consequence of both the chemical reactions. In addition, because D-isomers of some amino acids (alanine, valine, serine, leucine, isoleucine) are produced by various microorganisms used in the fermentation process, these are present in cultured milks or long-ripened cheeses independent of the heating conditions adopted in the manufacturing process. Owing to the wide species dependence of the nutritional utilization of various D-amino acids, a need exists to develop a better understanding of their specific role in human nutrition.

Amino acid deamidation refers to the hydrolysis of the side chain amides in asparagine and glutamine residues to form aspartic and glutamic acid, respectively. This phenomenon is responsible for the release of ammonia; nevertheless, only severe heat treatment in a restricted water environment can induce cleavage of the peptide chains yielding soluble peptides and increasing the non-protein nitrogen content of milk.

Evaluation of Chemical Markers as Indicators of Heat Damage of Processed Milk Products

The above-described chemical pathways have been studied extensively using simple model systems, comprising amines and reducing sugars, which allowed several newly formed molecules to be recognized and isolated. Nonetheless, the results obtained were not always directly transferable to more complex matrices such as milk and milk products. In recent years, the availability of advanced analytical techniques has allowed scientists to elucidate the heat-induced chemical pathways relevant to real milk products. On this basis, the quantification of some non-enzymatic, irreversible protein modifications has become a key criterion to assess the heat load applied during processing and, hence, to characterize the quality and nutritional value of the finished food. In this respect, the best applications concern the covalent interactions mediated by the MR. During the last few decades, in fact a variety of molecules have been studied, the formation of which is directly or indirectly consequent to the development of the MR in heated milk products. The best-known example is represented by LL, the formation of which is conveniently monitored by measuring the concentration

Table 2 Concentrations (mean ± standard deviation) of furosine, lysinoalanine, and blocked lysine in some dairy products

Type of product		*n*	*Furosine*	*Lysinoalanine*	*Blocked lysine*
			(mg 100 g^{-1} protein)		*(%)*
Liquid milk					
Raw		15	5.2 ± 0.5	<0.1	0.2 ± 0.02
Pasteurized		25	6.4 ± 0.8	<0.1	0.2 ± 0.03
UHT	Direct	55	94 ± 36	3.1 ± 2.5	3.5 ± 1.3
	Indirect	33	184 ± 39	8.6 ± 3.5	6.8 ± 1.4
In-can sterilized		5	319 ± 61	12 ± 1.5	11 ± 2.1
Dried products					
Skim milk powder		8	197 ± 63	0.5 ± 0.2	7.2 ± 2.2
Casein		14	18 ± 9.3	4.8 ± 3.9	0.7 ± 0.3
Caseinates		9	22 ± 3.4	14 ± 7.8	0.8 ± 0.1
Milk protein concentrates		10	80 ± 77	4.8 ± 2.7	3.0 ± 2.8
Cheese					
Processed		34	177 ± 163	4.1 ± 3.1	6.4 ± 5.7
Fresh		43	7.1 ± 1.2	<0.1	0.3 ± 0.04
Mozzarella imitations		29	15 ± 5.8	2.9 ± 1.8	0.6 ± 0.2
Infant formulae					
Powdered		5	779 ± 155	2.0 ± 1.4	25 ± 6.5
Liquid		15	324 ± 120	12 ± 5.3	12 ± 3.9
Yogurt		22	117 ± 49	2.6 ± 1.7	4.4 ± 1.7

UHT, ultra-high temperature sterilized.

of furosine (FUR), a derived molecule formed during acid hydrolysis. Due to the availability of a reliable, well-defined analytical method (i.e., International Standard ISO 18329:2004), the determination of FUR level has found wide application in the characterization of thermal treatments applied in the manufacture of different milk products, especially drinking milk (**Table 2**). Moreover, the concentration of this chemical marker for the lysine–lactose reaction may be used to calculate the percentage of blocked lysine in milk products.

A trace amount of FUR is found in raw milk, as non-enzymatic glycation of proteins also occurs *in vivo*. Mild heat treatments induce a limited increase in the extent of glycation, while the increase is sharp in sterilized milks, either UHT or in-can processed. Indirect heating via countercurrent flow heat exchangers results in a higher degree of protein modification than that occurring in direct heating via steam injection. However, indirect heating is, for economic reasons, the predominant process for UHT treatment. The innovative technology of high pasteurization by direct steam infusion could be very promising for the prolongation of shelf life of liquid milk products. However, a longer shelf life of liquid milk, as achieved by UHT treatment, is advisable in countries with predominantly high ambient temperatures and with a market structure that does not allow for constant refrigeration of the product. The level of FUR can be used for the classification of liquid milk and the evaluation of different heating processes and types of plant. At present, no upper limits are established to the severity of heat treatment, in terms of time/temperature conditions, for the different types of liquid milk. Consequently, unjustified abuse of heat could, in some cases, be used in milk processing, leading to enhanced levels of protein damage. This may be prevented by setting suitable upper limits for FUR in each class.

LAL is another very sensitive marker of heat treatment in milk proteins and also indicates the influence of alkaline pH during processing. As it is formed in the β-elimination reaction, it is particularly suitable for the evaluation of the heat load in milk products with a low lactose content. Under similar conditions of temperature, pH, and moisture, the formation of LAL is partly suppressed by lysine lactosylation in most of the dairy products, lactose being always present, although at different concentrations. In fact, while the lactose molecules are free to move and find lysine residues to react with, the DHA residues can react only with adjacent lysine residues. This partly explains why the ratio between the levels of FUR and LAL may vary widely but is always >1 (**Table 2**). The highest LAL concentrations are found in caseinates. In the industrial manufacturing process of these products, casein is acid-precipitated from milk, then solubilized with alkali, and dried. In caseinates, therefore, LAL level can reach tens of milligrams per 100 g of protein, while glycation is relatively low. In contrast, this molecule is not detectable in pasteurized milk and is present only at trace levels (<0.1 mg (100 g)$^{-1}$ protein) in genuine fresh cheeses, even in Mozzarella cheese which undergoes stretching in hot water.

The production of dried milk products represents the most convenient way of storing and shipping milk, as they are much more stable and reduced in weight and bulk with respect to the starting raw material. In milk powder production, the process includes a preheat treatment of liquid milk with the purpose of obtaining powders with different levels of denatured whey protein, the crucial parameter affecting the properties of the finished product. The subsequent evaporation and spray-drying steps do not denature whey protein significantly, the former being normally carried out at temperatures below 60 °C and the latter very rapid (*see* **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture). In contrast, these two steps promote extensive glycation of the protein component as is globally indicated by the high levels of FUR and conveniently evidenced by the separations obtained using HPLC and CZE techniques for whey protein and casein, respectively (**Figure 2**). The modern membrane filtration processes, for example, ultrafiltration and microfiltration, allow large-scale industrial production of a variety of dried milk protein products (casein and caseinates, milk protein concentrates, milk protein isolates, whey protein concentrates, etc.) having a protein content roughly ranging from 35 to 90% (*see* **Milk Protein Products**: Milk Protein Concentrate; Whey Protein Products). As a result, both technological and nutritional characteristics of these dairy powders may vary tremendously, and this aspect is of crucial relevance because they are treated mainly as food ingredients. As has already been mentioned, both type and extent of protein modifications occurring in these products (**Table 2**) depend on the lactose content and the pH conditions during processing at least as much as on thermal treatment. In addition, the stability of protein powders during storage is critically affected by the moisture content, more precisely by the a_w, and the storage temperature, the effects of these two

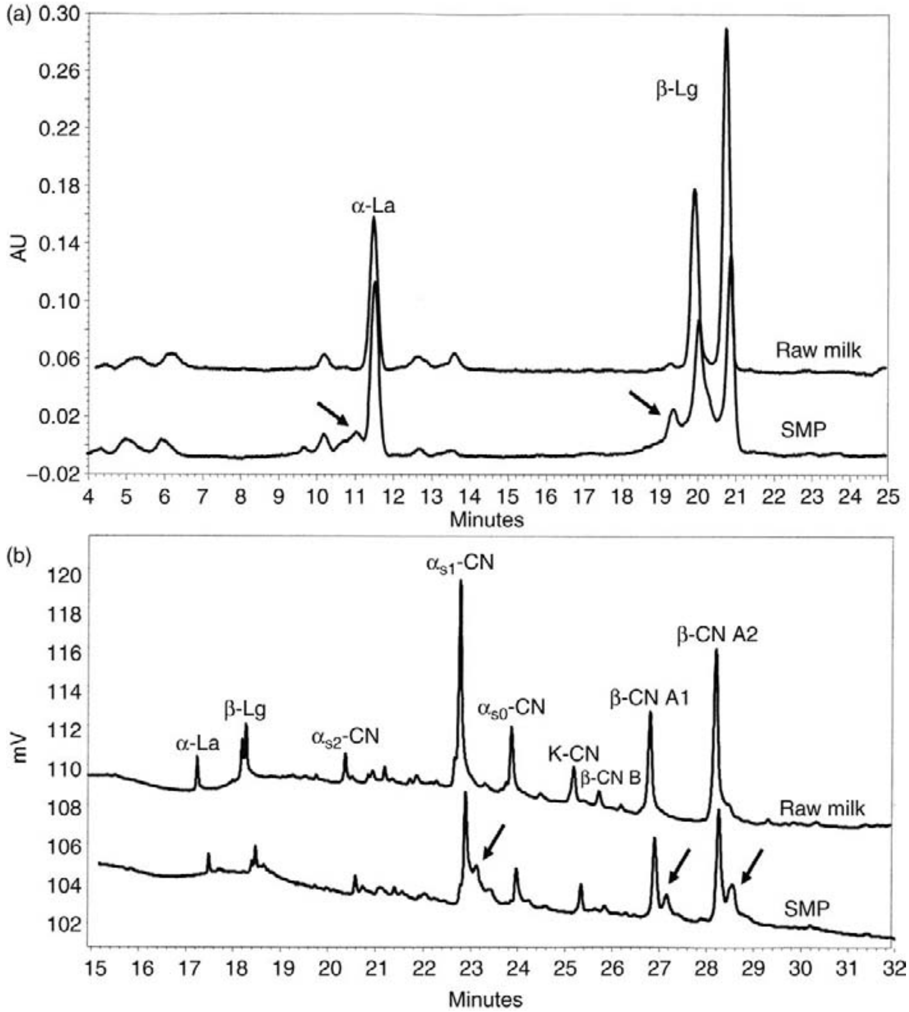

Figure 2 Separation of (a) soluble whey proteins by HPLC and (b) milk proteins by capillary electrophoresis of raw milk and reconstituted skim milk powder (SMP). Arrows: glycated forms.

factors being strictly dependent on each other. For example, for optimal preservation at 25 °C the a_w should be close to 0.2. Therefore, it is very important for the users of these ingredients in industrial food manufacturing to adopt methods for evaluating their keeping quality. This is particularly the case of food intended for infants and children, who have a high essential amino acid requirement. In fact, in infant formula manufacturing, besides the heat treatment applied, the use of processed ingredients (whey powder, caseinates, whey protein concentrates) in formulation may play a major role in promoting deep protein modifications in the finished product. As shown in **Table 2**, the highest values of FUR, and therefore the highest percentage of blocked lysine, can be reached in powdered infant formulae, whereas liquid UHT-treated formulae show levels of LAL comparable to those of in-bottle sterilized milk. In addition, storage under the conditions usually indicated by manufacturers (at room temperature for up to 12 and 24 months, for UHT and powdered products, respectively) promotes further protein modifications, as highlighted by the progressive accumulation of FUR and LAL shown in **Figure 3**. The evaluation of protein quality in infant formulae is of paramount importance since they may constitute the sole source of nutrition for newborns. In this regard, the substantial levels of both blocked lysine and molecular cross-link undoubtedly impair the nutritional quality of protein. Nevertheless, the LAL levels detected in infant formulae, even at the end of the shelf life, are far from those considered to be a health threat in rats (*see* **Dehydrated Dairy Products**: Infant Formulae).

Some milk-derived products may find application as ingredients in several food preparations and, therefore, undergo further processing during domestic cooking. This may have a great influence on quality-determining parameters like nutritional value and digestibility of protein components. In fact, FUR and LAL originate from the same complex series of reactions that produce the desirable flavor and color of baked and roasted foods. Therefore, to achieve an overall evaluation of the nutritional quality of protein in this kind of products, the effect of heat treatment applied during domestic cooking should be evaluated as well. Data reported in **Table 3** show the case of different types of cheese used as pizza toppings.

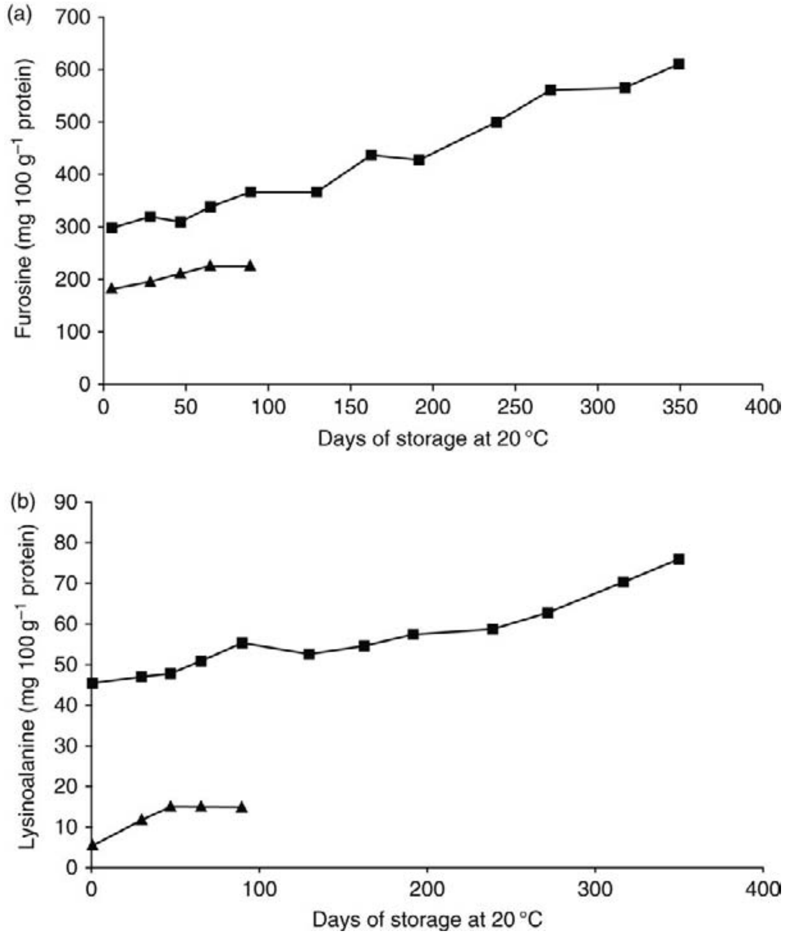

Figure 3 Accumulation of (a) furosine and (b) lysinoalanine in indirect UHT milk (▲) and in UHT liquid infant formula (■) during storage.

Table 3 Effect of domestic cooking in an electric oven on furosine and lysinoalanine levels in different types of cheese topped on pizza

	Furosine		*Lysinoalanine*	
	(mg 100 g^{-1} protein)			
Type of cheese	*Before cooking*	*After cooking*	*Before cooking*	*After cooking*
Mozzarella	7.4	50	0.1	0.5
Imitation Mozzarella	32	221	4.2	6.8
Processed	24	212	1.4	28

Following pizza baking at 200 °C for 20 min, the FUR content increased by a factor of 7–8 in all the topped cheeses, and in imitation Mozzarella and processed cheese it reached values characteristic of sterilized milk. The level of LAL showed a sharp increase in processed cheese only, probably because of its higher pH value. Both imitation Mozzarella and processed cheese, which are usually manufactured using dried dairy ingredients, like caseinates, milk protein concentrate (MPC), and whey powder, are often used in pizzas as a cheaper alternative to natural Mozzarella.

From the results presented above, it can be concluded that, for most milk and dairy products, a reliable assessment of type and extent of heat-induced covalent modifications of the protein components is feasible via the determination of FUR and LAL content. Nevertheless, when extremely severe thermal processes are adopted, the degradation of LL begins, with the consequent formation of either 1- or 3-deoxyosone (DG), depending on the reaction conditions, mainly the pH value of the environment. The unstable deoxyosones react readily with the side chain amino groups of the protein chain generating AGEs (**Table 4**) some of which have been identified in high-temperature-treated dairy products and proposed as additional indicators of the intensity of heat treatment and the type of the applied process.

The 3-DG-derived AGEs contain only the glucose moiety of the reacted lactose, as the β-1,4-glycosidic bond in lactose is broken during LL degradation. However, these compounds are generally present only in small amounts in heated milk and milk products, as this degradation pathway is not preferential for LL. As an example, protein-bound lysylpyrrolaldehyde (LPA) can form in the presence of lysine residues and it can be found in proteins of dairy products. Following alkaline or enzymatic hydrolysis, LPA can be quantified at microgram per 100 g protein amounts in sterilized milk and concentrated milks. Other 3-DG-derived AGEs are capable of cross-linking milk proteins; pentosidine (PTD), for example, is a product of cross-linking between residues of lysine and arginine. This molecule, although produced in significant amounts in model systems, was found only at low concentrations in UHT milk (0–7 μg (100 g^{-1}) protein), in-bottle sterilized milk (13–350 μg (100 g^{-1}) protein), and whey powder (0–8 μg (100 g^{-1}) protein). When LL degrades via the 1-DG pathway, the 1-4 glycosidic bond of lactose remains intact and, therefore, the whole carbon backbone of the disaccharide is still present in the derived AGEs. In this case, the rearrangement of 1-DG creates free molecules containing hetero rings (furans and pyrans). Galactosyl-β-pyranone (GAP) forms at heating conditions characteristic of milk sterilization. The level of GAP is well-correlated with the whole heat load of the process and therefore may allow direct UHT milk (0.1–1.5 mg l^{-1}) to be distinguished from indirect UHT milk (1.5–3 mg l^{-1}), and the latter from in-can sterilized milk (>6 mg l^{-1}). The level of GAP in UHT milk increases sharply when the proportion of milk recirculated in the plant is high or when milk is subjected to a second treatment. Upon prolonged heating or storage, GAP can dehydrate to form galactosyl-isomaltol (GAI), which has been detected in milk powders submitted to severe heating. Both GAP and GAI are soluble molecules and, therefore, their levels are not directly related to protein nutritional value. Nonetheless, they can further react with side chain amino groups, producing protein-bound xenobiotic molecules such as galactosyl-acetylpyrrole (GALP), pyridinium betaines, and furanones. Formation of GALP in casein–lactose model systems is promoted by heating conditions similar to those applied in industrial processes used for manufacturing processed cheese and infant formulae.

Table 4 Main AGEs deriving from lactulosyllysine degradation via deoxyosones

Via 3-deoxyosone	*Via 1-deoxyosone*
Lysylpyrrolaldehyde	Galactosyl-β-pyranone
Pentosidine	Galactosyl isomaltol
	Galactosyl-acetylpyrrole
	Pyridinium betaines
	Furanone

In conclusion, technological processes adopted in manufacturing liquid milk and other dairy products, mainly those involving high temperatures and extreme pH conditions, may affect the nutritional properties of protein components. Possible causes of protein damage include destruction of some essential amino acids, reduced digestibility owing to the formation of cross-linked amino acids, loss of phosphorus from casein, and adverse effects on mineral nutrition owing to chelation of trace elements. Although heat treatment is a necessary step in most cases, to meet the safety and shelf life requirements of the finished products, nutritional aspects could be disregarded by manufacturers, as they are not regulated. On this basis, operating conditions strictly following the GMP principles represent the only guarantee of avoiding abuse of heat load in milk processing. From this point of view, specific quality criteria based on chemical markers of heat load not only represent a tool for evaluating the overall quality of milk products but, with an adequate price structure, may also improve competitiveness among the producers. In the food industry, an increasing emphasis is today given to food products having higher nutritional value and, meanwhile, consumers are becoming more aware of the effects of diet on health. Both trends would be promoted by a better understanding of the effects of food manufacturing processes, mainly those requiring high temperatures, on the nutritional quality of proteins, a major component in many food products.

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See also: **Dehydrated Dairy Products**: Infant Formulae; Milk Powder: Types and Manufacture. **Heat Treatment of Milk**: Heat Stability of Milk. **Lactose and Oligosaccharides**: Maillard Reactions. **Milk Protein Products**: Bioactive Peptides; Milk Protein Concentrate; Whey Protein Products. **Milk Proteins**: Nutritional Quality of Milk Proteins.

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John W. Fuquay, Professor Emeritus of Dairy Science at Mississippi State University, served on the faculty there from 1969 to 1999. His areas of emphasis in teaching and research were environmental physiology and reproductive physiology. He received his BS and MS degrees from North Carolina State University and his PhD degree from Pennsylvania State University, all in the area of dairy science. After completing the PhD degree in 1969, he accepted a teaching and research position at Mississippi State University, where he progressed through the ranks from assistant professor to professor before retiring in 1999. Professor Fuquay served as Coordinator for the Graduate Program in Animal Physiology from 1986 to 1999. He was a Visiting Professor in the Animal Sciences Department, University of California-Davis in 1979 and in 1985–86.

Professor Fuquay was active in his professional society, The American Dairy Science Association. He was a member of the editorial board of *Journal of Dairy Science* for seven years, an editor for four years, and served as the first Editor-in-Chief for six years (1997–2002). For his professional contributions and service to the Association, Professor Fuquay was recognized as a Fellow in the American Dairy Science Association in 2001 and received the Association's Award of Honor in 2002. Other recognitions include the World Association of Animal Production Jean Boyazoglu Award in 2003, the Distinguished Dairy Science Alumnus Award from Pennsylvania State University in 2003, and several teaching and research awards from his university.

Professor Fuquay has participated in a variety of international activities. He has presented short courses and lectures as well as provided consultations in a number of countries, primarily in Asia and Latin America. In addition to his research publications, he is the coauthor of a textbook, *Applied Animal Reproduction* (Prentice Hall), that has been widely used by universities in the United States and internationally. The first edition was published in 1980 and the last (sixth) edition in 2004. In 2010, he published a memoir, *Musings of a Depression-Era Southern Farm Boy* (Vantage Press), which reflects on how the experience of growing up on a farm in the southern United States during the great depression instills one with an understanding of the importance of strong family bonds and a sound work ethic in meeting the challenges of the adult world.

Patrick F. Fox was Professor and Head of the Department of Food Chemistry at University College, Cork (UCC), Ireland, from 1969 to 1997; he retired in December 1997 and is now Emeritus Professor of Food Chemistry at UCC. Prof. Fox received his BSc degree in Dairy Science from UCC in 1959 and PhD degree in Food Chemistry from Cornell University in 1964. After postdoctoral periods in Biochemistry at Michigan State University and in Food Biochemistry at the University of California, Davis, he returned to Ireland in 1967 to take up a research position at the Dairy Products Research Centre at Moorepark before moving to UCC in 1969.

Prof. Fox's research has focused on the biochemistry of cheese, the heat stability of milk, physicochemical properties of milk proteins, and food enzymology. He has authored or coauthored about 520 research and review papers, and authored or edited 25 text books on Dairy Chemistry. He was one of the founding editors of the *International Dairy Journal.*

In recognition of his work, Prof. Fox has received the Research & Innovation Award of the (Irish) National Board for Science and Technology (1983), the Miles-Marschall Award of the American Dairy Science Association (1987), Medal of Honour, University of Helsinki (1991), the DSc degree of the National University of Ireland (1993), the Senior Medal for Agricultural & Food Chemistry of the Royal Society for Chemistry (2000), the ISI Highly Cited Award in Agricultural Science (2002), the International Dairy Federation Award (2002), Gold Medal of the UK Society of Dairy Technology (2007), and an autobiography published in Annual Review of Food Science & Technology (2011).

Prof. Fox has been invited to lecture in various countries around the world. He has served in various capacities with the International Dairy Federation, including President of Commission F (Science, Nutrition and Education) from 1980 to 1983.

Paul McSweeney is Professor of Food Chemistry in the School of Food and Nutritional Sciences, University College, Cork, Ireland (UCC). He graduated with a BSc degree in Food Science and Technology in 1990 and a PhD degree in Food Chemistry from UCC in 1993 and also has an MA in Ancient Classics. He worked for a year in the University of Wisconsin (1991–92) as part of his PhD and as a postdoctoral research scientist in UCC (1993–94). He was appointed to the academic staff of UCC in 1995. The overall theme of his research is dairy biochemistry with particular reference to factors affecting cheese flavor and proteolysis during cheese maturation including the role of non-starter lactic acid bacteria and smear microorganisms, the ripening of hybrid and non-Cheddar varieties, the specificity of proteinases on the caseins, proteolysis and lipolysis in cheese during ripening, and characterization of enzymes important to cheese ripening (proteinases, peptidases, amino acid catabolic enzymes). He is the coauthor or coeditor of eight books, including the third edition of *Cheese: Chemistry, Physics and Microbiology* (Amsterdam, 2004) and the *Advanced Dairy Chemistry Series* (New York, 2003, 2006, 2009), and has published numerous research papers and reviews. Prof. McSweeney is an experienced lecturer and researcher and has successfully managed research projects funded through the Food Industry Research Measure and its predecessors administered by the Irish Department of Agriculture and Food, the EU Framework Programmes, the US–Ireland Co-operative Programme in Agriculture/Food Science and Technology, and Bioresearch Ireland and Industry. He was awarded the Marschall Danisco International Dairy Science Award of the American Dairy Science Association in 2004 and in 2009 a higher doctorate (DSc) on published work by the National University of Ireland.

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GUIDE TO USE OF THE ENCYCLOPEDIA

STRUCTURE OF THE ENCYCLOPEDIA

The material in the Encyclopedia is arranged as a series of entries in alphabetical order. Some entries comprise a single article, whilst entries on more diverse subjects consist of several articles that deal with various aspects of the topic. In the latter case the articles are arranged in a logical sequence within an entry.

To help you realize the full potential of the material in the Encyclopedia we have provided three features to help you find the topic of your choice.

1. CONTENTS LISTS

Your first point of reference will probably be the contents list. The complete contents list appearing in each volume will provide you with both the volume number and the page number of the entry. On the opening page of an entry a contents list is provided so that the full details of the articles within the entry are immediately available.

Alternatively you may choose to browse through a volume using the alphabetical order of the entries as your guide. To assist you in identifying your location within the Encyclopedia a running headline indicates the current entry and the current article within that entry.

2. CROSS REFERENCES

All of the articles in the Encyclopedia have been extensively cross referenced. The cross references, which appear at the end of an article, have been provided at three levels:

i. To indicate if a topic is discussed in greater detail elsewhere.
ii. To draw the reader's attention to parallel discussions in other articles.
iii. To indicate material that broadens the discussion.

Example

The following list of cross references appear at the end of the entry entitled **Bacteria, Beneficial** | Lactic Acid Bacteria: An Overview

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; *Bifidobacterium* spp.: Morphology and Physiology. **Lactic Acid Bacteria**: Citrate Fermentation by Lactic Acid Bacteria; Lactic Acid Bacteria in Flavor Development; *Lactobacillus* spp.: General Characteristics; *Lactobacillus* spp.: *Lactobacillus acidophilus*; *Lactobacillus* spp.: *Lactobacillus casei* Group; *Lactobacillus* spp.: *Lactobacillus delbrueckii* Group; *Lactobacillus* spp.: *Lactobacillus helveticus*; *Lactobacillus* spp.: *Lactobacillus plantarum*; *Lactobacillus* spp.: Other Species; *Lactococcus lactis*; *Leuconostoc* spp.; *Pediococcus* spp.; Physiology and Stress Resistance; Proteolytic Systems; *Streptococcus thermophilus*; Taxonomy and Biodiversity. **Pathogens in Milk**: Enterobacteriaceae.

3. INDEX

The index will provide you with the volume number and page number of where the material is to be located, and the index entries differentiate between material that is a whole article, is part of an article, or is data presented in a table or figure. Detailed notes are provided on the opening page of the index.

4. COLOR PLATES

The color figures for each volume have been grouped together in a plate section. The location of this section is cited in the contents list. Color versions of black and white figures are cited in figure captions within individual articles.

5. CONTRIBUTORS

A full list of contributors appears at the beginning of each volume.

6. GLOSSARY

A glossary of terms used within the work is provided in Volume Four before the Index.

PREFACE

We are pleased to present the second edition of the *Encyclopedia of Dairy Sciences*. The first edition was published in 2003 by the Major Reference Works Division of Academic Press, now part of Elsevier Sciences, and it comprised 427 articles. The objective was to satisfy the need for an authoritative source of information for people involved in the integrated system of production, manufacture, and distribution of dairy foods. It was realized from the beginning that a program of revision would be needed to keep the Encyclopedia up to date. This goal has been met in the second edition through 503 articles, of which 121 are new articles and 382 are revised articles. We express appreciation to the Editorial Advisory Board for its role in evaluating articles for needed revision, reviewing new and revised articles, and for help in identifying new topics to be included along with appropriate authors. Likewise, we are grateful for the contributions of the many authors who have either revised their articles or prepared new articles.

The main topics related to milk production and dairy technology are addressed in addition to providing information on nutrition, public health, and dairy industry economics including aspects of trade in milk and dairy products. All species that produce milk for human consumption have been included in this work. Some of these species are of regional significance only, but they have been included because of the essential role that their milk plays in the nutrition of people inhabiting various regions of the world. A significant addition to the second edition is four introductory articles addressing the history of Dairy Science and Technology. A synopsis has been prepared for each article in the second edition and will appear with the online listing of the articles in this publication.

The primary aim of the Encyclopedia is to provide a complete resource for researchers, students, and practitioners involved in all aspects of the dairy sciences as well as those involved with economic and nutritional policy and members of the media. We have tried to do this with a writing style that is easily comprehended by persons who are not highly trained in the technical aspects of the Dairy Sciences. Users should be able to access information on topics that are peripheral to their areas of expertise.

We express appreciation to the staff of the Major Reference Works Division, responsible for this Encyclopedia, for their timely responsiveness to the needs of the editors and their essential administrative role in keeping this major reference work on-track toward a satisfactory completion within the desired time schedule. We remember Nancy Maragioglio, Senior Life Sciences Editor, who initiated the work and was ever responsive to queries by the editors, as well as Sera Relton, Esmond Collins, Milo Perkins, and Claire Byrne, Development Editors, and Charlotte (Charlie) Kent, Publishing Administrator, who kept things moving through their communication with editors, authors, and reviewers and who exhibited almost flawless administrative skills. Sera Relton was particularly helpful as she assisted us in moving through the final submission and review stages. Laura Jackson is recognized for her contributions as Production Manager of the Encyclopedia.

Special recognition is due to Ms Anne Cahalane, Senior Executive Assistant, School of Food & Nutritional Sciences, University College, Cork, whose stylized representation of a cow, a milk can, and a wedge of cheese adorns the cover of the first and second editions of the Encyclopedia of Dairy Sciences.

John W. Fuquay
Patrick F. Fox and
Paul L. H. McSweeney

FOREWORD

The cow is the foster mother of the human race. From the days of the ancient Hindoo to this time have the thoughts of men turned to this kindly and beneficent creature as one of the chief sustaining forces of human life.

William Dempster Hoard (1836-1918)
Former governor, state of Wisconsin, USA (1889-1891)
Founder of **Hoard's Dairyman** (1885)

We must never forget that milk and milk products are and will always be important sources of basic food nutrients for humans both young and old. The more scientific facts we can discover, understand, and apply related to producing, processing, and marketing milk and milk products, the better we will serve the nutritional needs of humanity throughout the world.

More than 2000 years ago Aristotle noted, *Everyone honors the wise and excellent.* We are indebted to those *wise* enough to conceptualize and envision the favorable global impact that is certain to follow by bringing together this exhaustive, rich collection of 503 pertinent articles written and reviewed by more than 700 world-renowned disciplinary experts representing 50 countries – persons each of whom bears the mark of *excellence.* Happily these timely topics are now recorded in four informative, important, engaging volumes. We thank, commend, and salute the prodigious efforts of the *wise* and *excellent* authors who generated, compiled, and put the spotlight on the useful information and data, and who now share them through their well-written articles.

One noteworthy value and enduring virtue of these articles is bringing into clear perspective the context of both the state-of-the-art and the future of dairy sciences. When the history and contributions of scholarly publications related to the all-important global dairy industry are recorded, the second edition of the *Encyclopedia of Dairy Sciences* will be cited often and with great respect and appreciation.

Fundamental to continued progress and success in the dairy industry have been the signal service, cooperation, and collective contributions of dedicated scientists, teachers, agricultural advisors/extension workers, and representatives of governments and industries. Additional exciting breakthroughs in applying new findings and developments in research and technology to the production and processing of milk are sure to follow as we move surefootedly through the twenty-first century. This continued growth and success will be aided immensely by the vast and extraordinarily useful knowledge base made available by the idea-rich, insightful authors, editorial advisory board members, editors, and publisher of the second edition of the *Encyclopedia of Dairy Sciences.*

Indeed, by perusing the comprehensive and authoritative articles of this greatly needed and monumental encyclopedia, readers will be made even more aware of the tremendous progress that has occurred in the basic and applied sciences underpinning the global dairy industry.

Ours is an internationally competitive and incredibly technological world. And unless talented, creative scientists continue to work together in researching and applying the most effective and economical ways and means of providing

an abundant, safe supply of milk and milk products for an ever-increasing world population, we will never reach our noble goal of adequately feeding all the earth's people.

May we utilize the comprehensive scientific knowledge base made available through this second edition of the *Encyclopedia of Dairy Sciences* as we pledge to realize advances in the health and well-being of the undernourished millions – including many who need and deserve to be rescued from the ugly grip of hunger – by increasing the availability of nature's most nearly perfect food – milk!

Pure milk from healthy animals is a luxury of the rich, whereas it ought to be the common food of the poor.

Mohandas Gandhi (1869-1948)
Indian nationalist leader

John R. Campbell

John R. Campbell, Ph.D., D.Sc. (Hon.)
President Emeritus and Professor of Animal Science
Oklahoma State University

Dean Emeritus and Professor Emeritus of Animal Sciences
College of Agriculture, University of Illinois

Professor Emeritus of Animal Sciences
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Past President, ADSA (1980-81)

April 2010

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O

OFFICE OF INTERNATIONAL EPIZOOTIES

Mission, Organization and Animal Health Code

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Introduction

The need for a global approach in the fight against animal diseases is now very clear. The World Organisation for Animal Health (which is also known by its historical acronym OIE (Office International des Epizooties)) is leading this fight worldwide. The OIE is the international standard-setting organization for animal disease control, the safety of international trade of animals and animal products, animal disease prevention, surveillance, control, and information, animal welfare, animal production, and food safety.

The purpose of this article is to describe the OIE and its efforts and importance in improving animal health in the world, thereby improving human health. In order to better understand the organization, its history, structure, mandate and activities, and its major publications such as the Animal Health Codes for Terrestrial and Aquatic Animals will be examined.

A Brief History of the OIE

In 1920, rinderpest, a devastating plague of cattle, was introduced to Belgium, through the port of Antwerp by zebu cattle that were en route by boat to Brazil from India. This was the impetus for an international conference to examine the animal health situation in the world, to discuss the exchange of animal health information, and to consider export health measures and disease control methods. This so-called ‘Paris Conference’ expressed the wish that an ‘international office of epizootics for the control of infectious animal diseases’ be set up in Paris. Thus, in 1924, more than 20 years before the creation of the United Nations, an agreement was signed by the veterinary authorities of 28 countries from Europe, North and South America, Africa, and Asia to establish the OIE in Paris – where it remains to this day.

In 1995, the World Trade Organisation (WTO) recognized the OIE as the international standard-setting organization for trade in animals and animal products under the agreement on the application of sanitary and phytosanitary (SPS) measures. The WTO’s SPS agreement states that “to harmonize sanitary and phytosanitary measures on as wide a basis as possible, Members shall base their sanitary or phytosanitary measures on international standards, guidelines or recommendations”. The agreement names the OIE as the relevant international standard-setting organization for animal health, including diseases transmissible to humans.

In May 2003, the representatives of all OIE members agreed to change the name of the organization from ‘Office International des Epizooties’ to ‘World Organisation for Animal Health’ but decided to keep its historical acronym ‘OIE’. As of 2009, the organization has 175 member countries and territories and more than 200 reference laboratories and collaborating centers. It has formal agreements with 35 international and regional organizations such as FAO, WHO, the World Bank, Codex Alimentarius, and non-governmental organizations representing producers and animal welfare groups.

The OIE’s financial resources are derived principally from regular annual contributions, backed up by voluntary contributions from members. The amount of the annual budget of the organization makes the OIE one of the most cost-efficient international organizations.

In 2004, the OIE established the World Animal Health and Welfare Fund, for the purpose of projects of international public utility relating to the control of animal diseases, including those transmissible to humans, and the promotion of animal welfare and animal production food safety. This effort was funded initially by international donors, including the World Bank, the United States Department of Agriculture, Switzerland, Japan, France, Canada, and Australia.

Structure of the OIE

The World Assembly of Delegates

The General Assembly of Delegates is the highest authority and the governing body of the OIE. It is comprised of one delegate per country, who is usually the chief veterinary officer (CVO), and is officially nominated by the Government of the member country. It meets every year at the annual general session in May in Paris.

The main functions of the General Assembly of Delegates are to adopt international standards in the field of animal health and the control of animal diseases; to elect the members of the governing bodies (President and Vice President of the general assembly, members of the council, members of the regional and specialist commissions); to appoint the Director General (by secret ballot); and to examine and approve the annual report of activities, the financial report of the Director General, the annual budget, and the strategic plans of the OIE.

Voting by delegates within the World Assembly of Delegates respects the democratic principle of ‘one country, one vote’. All resolutions voted by the World Assembly must be implemented by the Director General.

Council

The Council represents the World Assembly of Delegates during the interval between the assemblies. It meets at least twice a year to examine technical and administrative matters and, in particular, the working program and the proposed budget to be presented to the members. There are six elected members in addition to the President, Vice President, and past president of the World Assembly. The members are elected to reflect the regional balance.

Headquarters

The OIE headquarters is based in Paris, France. Under the authority of the Director General, the headquarters implements and coordinates disease information, and the scientific and administrative activities that the members have decided upon, as well as the World Fund for Animal Health and Welfare.

Furthermore, it provides the secretariat for the annual World Assembly of Delegates and for the meetings of the specialized commissions and other technical meetings. Assistance is also given by the headquarters to the secretariat of the OIE regional and technical conferences.

Regional Commissions

There are five regional commissions, for Africa; the Americas; Asia, the Far East, and Oceania; Europe; and the Middle East, whose objective is to promote cooperation and organize regional activities in the field of prevention and control of animal diseases and animal welfare promotion.

The President and three other members of each regional commission are elected by countries of each region for a 3-year term. A regional commission conference is organized once every 2 years in one of the countries of the region. These conferences are devoted mainly to technical items and to regional cooperation in the control of animal diseases.

Regional Representations

The OIE maintains representations in Africa, the Americas, Asia and the Pacific, eastern Europe, and the Middle East, and maintains close links with the relevant regional commissions.

The goal of these representations is mainly to provide regionally adapted capacity building programs to relevant policy makers of OIE members.

There are also currently sub-regional representations for the Southern African Development Community (SADC) in Botswana, in Tunis for northern Africa, in Brussels for western Europe, in Panama for Central America, and in Thailand for southeast Asia.

Specialized Commissions and Companion Groups and Supports

There are four specialized commissions. Their role is to use current scientific information to study the problems of epidemiology and the prevention and control of animal diseases, to develop and revise OIE’s international standards, and to address scientific and technical issues raised by member countries. The members of these commissions are elected at the World Assembly for a 3-year term.

Terrestrial Animal Health Standards Commission and Aquatic Animal Health Standards Commission (Code Commission)

Founded in 1960, these commissions are responsible for the preparation of standards adopted by members contained in the Terrestrial Animal Health Code and the Aquatic Animal Health Code (Terrestrial and Aquatic

Code) to ensure that they reflect current scientific information on the protection of international trade and surveillance methods for terrestrial and aquatic animal diseases. They work with internationally renowned specialists in *ad hoc* and permanent working groups to prepare proposed standards in light of advances in veterinary science. The Aquatic Animal Health Standards Commission is also responsible for the Manual of Diagnostic Tests and Vaccines for Aquatic Animals.

The Scientific Commission for Animal Diseases

The Scientific Commission for Animal Diseases, founded in 1946, assists in identifying strategies and measures for animal disease control. It also examines member country submissions for requests to be certified free of the four diseases for which the OIE can officially certify country freedom: foot-and-mouth disease, bovine spongiform encephalopathy, rinderpest, and contagious bovine pleuropneumonia.

The Biological Standards Commission

The Biological Standards Commission, founded in 1949, also referred to as the Laboratories Commission, is responsible for the preparation of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. It establishes standards for methods of diagnosing diseases of animals and for testing biological products, such as vaccines.

Permanent and ad hoc *working groups*

Comprising leading specialists from all OIE members, these expert groups are brought together to support specialist commissions for the preparation of draft standards and guidelines.

There are currently three permanent working groups: on wildlife disease, on animal production food safety, and on animal welfare.

Ad hoc groups are not permanent. Recently, they have been convened on a wide range of topics including biotechnology, brucellosis, communication, diseases of camels, epidemiology, and the evaluation of veterinary services (VSs).

OIE reference laboratories and collaborating centers

OIE reference laboratories are centers of expertise designated to pursue all the scientific and technical problems relating to a disease on the OIE list. The reference (leading) expert, responsible to the OIE and its member countries with regard to these issues, is an active researcher helping the reference laboratory to provide scientific and technical assistance and expert advice to the OIE and its member countries on diagnostics and topics linked to surveillance and control of the disease for which the reference laboratory is responsible. The laboratories also provide and coordinate scientific and technical studies in collaboration with other laboratories or relevant stakeholders.

By the end of 2009, the OIE had a global network of 187 reference laboratories with 161 experts covering 100 diseases/topics in 36 countries. The network brings together experts from many fields. This is an incalculable resource for the OIE headquarters and developed and developing countries, promoting research and encouraging development of laboratory standards. The laboratories provide members with confirmation of diagnostics, current methods for diagnosis, vaccine production, disease surveillance for animal diseases and zoonoses, and safe trade in animals and animal products.

OIE collaborating centers are centers of expertise in a specific designated sphere of competence relating to the management of general questions on animal health issues such as epidemiology, risk analysis, veterinary training, or validation of diagnostic tests. Twenty-nine collaborating centers are currently involved in the network covering 27 topics in 18 countries. In its designated field of competence, an OIE collaborating center provides its expertise internationally, and operates as a center of research, standardization, capacity building, and dissemination of techniques.

Laboratory twinning

Since a large majority of OIE member countries are developing countries and have variable scientific capacity or access to scientific expertise within their national veterinary laboratories, a project of laboratory twinning was developed, the main objective of which is to assist laboratories in developing or in-transition countries to build their capacity and scientific expertise with the eventual aim that some of them could become OIE reference laboratories in their own right. To apply this concept, a direct link between an existing OIE reference laboratory or collaborating center and another laboratory or institution in a developing or in-transition country is established on a strictly voluntary basis for exchange of scientific expertise and capacity building.

OIE Mandate

The core mandate of the OIE is to improve animal health in the world. Under this overarching mandate, the OIE is dedicated

- to guarantee the transparency of animal disease status worldwide,
- to collect, analyze, and disseminate veterinary scientific information,
- to provide expertise and promote international solidarity for the control of animal diseases,

- to guarantee the sanitary safety of world trade by developing sanitary rules for international trade in animals and animal products,
- to improve food safety from the farm to the abattoir,
- to develop standards for animal welfare, and
- to improve the legal framework and resources of national VSs.

Disease Information

Obligations of member countries

Information on the presence of disease is essential for controlling it. With the goal of minimizing the spread of disease comes the obligation to share information about disease outbreaks. Member countries of the OIE are therefore obligated to report disease outbreaks of the OIE-listed diseases, as well as any new relevant epidemiological event.

The OIE list of diseases: There are almost 100 OIE-listed diseases included in the first chapter of the Terrestrial Animal Health Code. The criteria used to determine whether a disease appears on the list are as follows:

- Is there international spread of disease, that is, has it spread internationally in the past, or is it currently affecting three or more countries?
- Does it have zoonotic potential, that is, can this animal disease affect people?
- If not, is it spreading in the native population with important morbidity (infecting a high percentage of animals) or mortality (killing an important percentage of the animals that are infected)?
- Is it an emerging disease with rapid spread or zoonotic potential?

A positive answer to any of these – international spread, zoonosis or high morbidity or mortality or an emerging disease – means that the disease is included on the OIE list.

Member countries are committed to report as follows. Immediate notification is required for the first occurrence of a listed disease or infection, the reoccurrence following a report, the first occurrence of a new strain of a pathogen, a sudden and unexpected increase in the morbidity, mortality, or distribution of a disease, or a change in the epidemiology of a disease. The immediate notification is to be by e-mail, fax, telephone, or telegraph. These are to be followed by weekly updates.

Members are further committed to semi-annual reports describing the situation regarding OIE-listed diseases in each country and annual reports, which also include information on diseases that are not on the OIE list and diseases of wildlife, the impact of zoonoses on the human population, animal population statistics, the structure of the VSs, national reference laboratories and the diagnostic tests they can perform, and, where appropriate, vaccine manufacturers and the vaccines they produce.

Tools for transparency

WAHIS is the World Animal Health Information System. It is the web interface that is available to member countries for disease notification, allowing countries to notify electronically in a rapid and simple manner. However, when the capacity for electronic reporting is not available, submission of paper reports is acceptable. Many countries have nominated a focal point for specific diseases, or species, whose responsibility is to report disease information to the OIE. This focal point receives specific training from the OIE.

WAHID – World Animal Health Information Database – is openly available on the OIE website. With the capacity to search by country or by disease, it provides a rapid, clear, and evident overview of the disease status of a country, the presence or absence of a disease, disease outbreaks or timelines, and the populations of animals in a country, even allowing a comparison of the animal health status of two countries.

The OIE publishes 'World Animal Health' every year, which is a compilation of all the information listed above. This publication is unique worldwide.

Disease tracking

OIE is also engaged in active search and verification of disease outbreaks. Seeking unofficial information from the reference laboratories, the regional representations, collaborating centers, internet resources, or the press, the OIE gathers information, analyzes it, and asks the member for verification where relevant. This is an extremely effective tool.

The OIE does not work alone. The Global Early Warning System for Animal Disease including Zoonoses (GLEWS) is a joint OIE/FAO/WHO initiative that synergistically builds on combining and coordinating the disease tracking and alert and response mechanisms of the three organizations. Through sharing of information on animal disease outbreaks and epidemiological analysis, the GLEWS initiative aims at improving global early warning as well as transparency among countries for controlling animal disease as well as zoonoses including food-borne diseases.

Veterinary Scientific Information

Reference laboratories, collaborating centers, and the four specialist commissions develop and gather scientific information on animal disease prevention and control methods, including zoonoses and food-borne diseases, and on animal welfare.

The OIE provides this information through various channels including

- global and regional scientific conferences,
- web site,
- The Bulletin,
- the yearly publication of the World Animal Health Situation,
- The Scientific and Technical Review, and
- other publications (handbooks).

The Bulletin is published 4 times yearly. Each issue is focused on a specific topic (e.g., animal welfare, wildlife diseases, or food safety). It also provides member countries with an update on current issues, on activities of headquarters and regional offices, and upcoming events and notifications of self-declarations of the disease status of member countries on a voluntary basis.

The World Animal Health is a yearly publication on the occurrences of animal disease throughout the world. It also contains information on the most important control, prevention, and prophylaxis measures adopted and the number of animals slaughtered, destroyed, or vaccinated. Figures on animal population are also provided. Other sections provide detailed information on human cases of the OIE-listed zoonotic diseases, veterinary personnel, national reference laboratories, and vaccine production. This publication is unique in the world.

The Scientific and Technical Review is a peer-reviewed journal that contains in-depth studies devoted to current scientific and technical developments in animal health and veterinary public health worldwide. The particular distinction of this publication lies in relating specialized research to practical problems encountered in safeguarding animal health and veterinary public health, an essential aspect for the improvement of animal production and the protection of public health. It appears 3 times per year.

Other technical publications include technical series on a variety of topics such as assessment and management of pain in animals, or epidemiology, and global or regional scientific conference proceedings.

International Solidarity

More than 120 members are developing countries or countries in transition. These countries often find it difficult to free themselves from epizootics, including zoonoses. This leaves a reservoir of pathogens that threatens the status of countries that have attained disease freedom, often at great expense.

The OIE influences the wealthier countries to help developing countries and offers its expertise and that of the networks as well as its own resources to help them meet the OIE standards. This results in a 'win-win' situation because the control of diseases in developing countries also results in reduction of poverty and increases food security, market access, and public health while contributing to help free countries safeguard their free status.

OIE offers to developing countries independent evaluation of their animal health policies and infrastructures, gap analysis, and donor opportunities if needed (*see* 'Strengthening Veterinary Services').

International Trade in Animal and Animal Products

As described above, the OIE is the international organization given the responsibility by the WTO for establishing standards in animal diseases and zoonoses. The standards contained in the Terrestrial and Aquatic Code are intended to prevent and control the spread of animal disease while avoiding unjustified sanitary barriers to the international trade of animals and animal products.

The OIE certifies countries free of four diseases (rinderpest, foot-and-mouth disease, contagious bovine pleuropneumonia, and bovine spongiform encephalopathy) according to specific guidelines by which a country can demonstrate that the disease is not present in its animal population. This allows importing countries to take decisions without having to control the situation in the exporting country directly in the field. For other diseases, such as avian influenza, there are specified criteria by which a country can certify itself as free from a disease.

The OIE can also play a role in mediating trade disputes between countries by offering a voluntary dispute settlement mechanism. This is a science-based approach to finding alternative solutions and resolving differences, as distinct from the legalistic approach used in the formal WTO system. The mechanism is voluntary and the agreement of both parties is needed before the OIE can initiate the process.

Animal Production Food safety

Preventing or eliminating hazards at their source, at the farm level, is clearly more effective than trying to detect and eliminate them downstream. A permanent working group on food safety was established in 2002, sharing membership with Codex Alimentarius, FAO, and WHO, to establish standards for food safety from the farm to the abattoir in order to eliminate hazards existing during production at the farm and prior to the slaughter of animals or the primary processing of animal products (meat, milk, eggs, etc.) that could pose a risk to consumers. This group is also working to prevent gaps and duplications between Codex Alimentarius and the OIE standards.

Veterinarians have an established role at the farm level. Under OIE guidelines, veterinarians working at the abattoir screen for diseases particularly during ante- and post-mortem inspection. They verify that animal welfare standards are met, and assure the humane slaughter. VSs are well placed as part of a multidisciplinary team of professionals, to work for the safe production of food, including dairy products from the farm to the fork.

As part of the effort to ensure the safety of food of animal origin, and indeed for the purpose of controlling animal disease outbreaks, there must be a reliable and effective way to trace the animal back to the farm of origin. This should be based on the identification of farms, individual animal identification, or identification of groups of homogeneous animals, the ability to track movement of animals, and a record-keeping system. Traceability has important implications for trade as well as for animal health, disease control, and food safety. Therefore, the OIE developed standards for animal traceability. In addition, the OIE organized a world conference in 2009 bringing together governments, international organizations, industry, and primary producers with the purpose of supporting the implementation of the relevant international standards for identification and traceability of live animals and facilitating the bridge of traceability between animals and animal food products globally.

Animal Welfare

Animal welfare was identified as a priority when OIE member countries mandated the organization to take the lead internationally on animal welfare and to elaborate recommendations and guidelines covering animal welfare practices. This is all the more relevant to the OIE since animal health is a key component of animal welfare. The Permanent Animal Welfare Working Group was inaugurated at the 70th World Assembly of Delegates in May 2002.

To date, the OIE has developed guidelines for the transport of animals by land, sea, and air, for the slaughter of animals, and for killing animals for disease control purposes. The next standards to be developed are on the control of stray dog populations, livestock production systems, and laboratory animal welfare.

To further progress on animal welfare standards, the OIE has held two global conferences, in 2004 and 2008, in order to promote the worldwide implementation of OIE animal welfare standards, to raise the profile of animal welfare, and to encourage veterinarians and VSs to take greater responsibility for animal welfare.

Strengthening Veterinary Services

In order to adequately implement OIE standards, a country requires a VS with adequate human, physical, and financial resources, technical authority and capacity, interaction with stakeholders, and access to markets. However, more than 120 of the 175 OIE members are developing countries where VSs may not always comply with international OIE standards on the quality and performance of VS. The OIE sees VSs as a global public good and their compliance with international standards as a priority for public investment. The OIE is therefore actively engaged in the evaluation and improvements of the capacities of national VS.

The process chosen by the OIE consists of the democratic adoption of quality standards contained in the Terrestrial Animal Health Code, and the creation of a tool to analyze the conformity of the countries to the standards.

The OIE's tool for the evaluation of the performance of VSs, the PVS tool, is designed to assist VSs to establish their current level of performance, to identify gaps and weaknesses regarding their ability to comply with standards described above, and to establish priorities and carry out strategic initiatives. This tool is the principal lever of the OIE to bring compliance with quality standards in the governance of VSs of all countries.

The PVS establishes a diagnostic. The OIE also offers a gap analysis in collaboration with FAO and various key funding agencies, permitting members to define detailed priorities for investment in order to be able to comply using their national budget and priorities and, if needed, soliciting aid from the international community.

For member countries requesting assistance with capacity building, the OIE also provides expertise and training for national senior officials, both to improve sanitary governance and to help prepare and implement animal disease control and eradication programs.

Standard Setting Procedures and Publication of International Animal Health Codes

The *Terrestrial Animal Health Code*, now in its 17th edition (2008), and the *Aquatic Animal Health Code*, in its 10th edition, are intended to assure the sanitary safety of international trade in terrestrial and aquatic animals and their products and to provide surveillance methods for important animal diseases. This is achieved through the detailing of health measures to be used by veterinary authorities to avoid the transfer of agents pathogenic to animals or humans, while avoiding unjustified sanitary barriers and while implementing surveillance of major diseases.

They are written in two sections: the first contains recommendations that apply to a wide range of topics, production sectors, and/or diseases (so-called 'horizontal standards') and the second contains recommendations on specific diseases (so-called 'vertical standards') including recommendations on agent inactivation and on surveillance and risk assessment.

The horizontal standards include most notably the general and ethical obligations for importing and exporting countries, methodologies for risk analysis, and the criteria by which diseases are included on the list and by which countries are to report disease outbreaks to the OIE. Other horizontal standards are animal welfare standards, veterinary public health measures such as the role of VSs in food safety, and the responsible use of antimicrobials in veterinary medicine.

The second section of the Codes consists of recommendations applicable to specific diseases on the OIE list. The diseases selected for inclusion in this list affect fish, shellfish, mammals, birds, or bees. These include diseases that are considered the most serious due to their potential for rapid spread beyond national borders, or for transmission to humans, as well as diseases that are less highly contagious but whose economic or health importance justifies their being taken into consideration in international trade. Each disease is dealt with in a separate chapter. Diseases are grouped as those that affect multiple species and those that affect a single species. There are more than 100 terrestrial and aquatic animal diseases for which standards have been developed.

These standards are related to the risks of transmission of the diseases or disease-causing agents linked to animal and animal products. But certainly it should be noted that certain products or commodities subjected to specific treatments may in fact pose no risk, no matter the sanitary status of the country.

The recommendations contained in the OIE Animal Health Codes are developed with the active participation of member countries, knowing that these will apply equally to themselves and to others. They are the fruit of a consensus of very senior veterinary authorities of member countries, thus accounting for their value and their very wide practical application.

Procedures for Updating the Codes

All standards are prepared and submitted by the elected specialized commissions to the World Assembly of National Delegates. They are adopted following the rule of 'one country – one vote'.

Other OIE Standards and Guidelines

While the Codes are highly important documents, it should be noted that they must be used with the companion documents, the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual)* and the *Manual of Diagnostic Tests and Vaccines for Aquatic Animals*, the reference standards for veterinary laboratories. Their purpose is to contribute to the international harmonization of methods for the surveillance and control of the most important animal diseases. Standards are described for laboratory diagnostic tests and the production and control of biological products (principally vaccines) for veterinary use across the globe, providing internationally agreed diagnostic laboratory methods. The Manuals set laboratory standards for all OIE-listed diseases as well as several other diseases of global importance. In particular, they specify those 'prescribed tests' that are recommended for use in health screening for international trade or movement of animals.

Other OIE Activities

Communication

In recognition of the centrality of communication for the VSs, which underpins everything they do, including disease surveillance, prevention, control and response, animal welfare, public health, and food safety, guidelines on communication useful for national VSs are being developed for inclusion in the Code.

Veterinary Education

In line with OIE's efforts to strengthen VSs is the recognition that veterinary education is basic to an effective VS. Society has placed increasing demands on veterinarians in the fields of food security, food safety, public health, and animal welfare. The OIE recognizes these, seeing them as integral to veterinary education. The harmonization and quality of veterinary curricula are a crucial component of sound national animal health systems.

A principal mandate of the regional representations and the subregional representations is to develop programs for capacity building in the members for the benefit of the delegates, and their national focal points, the people officially identified as national contacts with the OIE in specific areas.

As part of its multifaceted approach to improve VSs, the OIE has organized a meeting of all the world's veterinary schools with the aim of helping them incorporate into their curricula the concepts with the international public good principles expected from veterinary missions and activities.

Conclusion

A Global Public Good

Among the many challenges facing the world, the dramatic increase of human and domestic animal populations, globalization and the unprecedented movement of people and commodities worldwide, and the increasing encroachment on natural ecosystems are leading to increasing disease threats. As the interrelationships between animal and human health and the health of the ecosystem are better understood, it becomes clear that the consequence of an effective VS is a healthier animal and human population, less afflicted by zoonotic diseases, better nourished, and participating in an improved world economy. This entails prevention and control of emerging diseases at the human–animal interface. The concept of 'One World, One Health' has been developed jointly by the WHO, FAO, UNICEF, World Bank, and OIE, and accepted by most other international health organizations. It is based on more preventative actions, increased cooperation between VSs and public health authorities, and on strengthening emergency response capabilities while helping the poorest nations and strengthening animal and public health systems. The result should be a better capacity to respond to emerging disease situations. All countries must be prepared in face of these new disease threats and it is widely accepted that the work of the VSs is a global public good.

Since its inception in 1924, the OIE has been the global leader in animal disease prevention and control, has served as a focal point for international cooperation on animal health issues, has promoted global safe trade in animal products, has promoted transparency on the global situation of animal diseases including zoonoses, and has shared veterinary expertise among member countries.

With increasing trade, growing demand for foods of animal origin, growing disease threats, unprecedented movements of people and animals, and a changing climate, the role of the OIE has increasing importance. The larger vision of the OIE of contributing to improving public health, food safety and security, and the livelihoods of poor farming communities can only be achieved if governments agree to foster closer cooperation between all the sectors in the health system, to support VSs, and to share information.

Relevant Websites

http://www.oie.int – World Organisation for Animal Health.

ORGANIC DAIRY PRODUCTION

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History

In the early days of organic agriculture, products were sold at farmers' markets, cooperatives and directly from the farm. The definition of 'organic' and the actual methods for raising the products as organic varied from place to place and farm to farm. Gradually private and public institutions began emerging to set organic farming standards and provide third-party verification of label claims. Many producers turn to organic farming systems in order to take advantage of the high-value niche market and improve farm income, thereby enabling themselves to compete in today's vertically integrated agriculture system. Organic producers have an intense belief that their farming system is superior in its ability to care properly for the land and its finite resources. Today, organic production is well defined and has matured into a significant market segment.

Market Trends

According to the US Department of Agriculture, the amount of farmland managed under certified organic practices has expanded dramatically, as has consumer demand for organically grown food. In the United States, organic farming became one of the fas test-growing trends in agriculture during the 1990s. Certified organic cropland more than doubled from 1992 to 1997, and two organic livestock sectors – eggs and dairy – grew even faster. Organic foods are one of the top consumer trends today, accounting for more than US$7.8 billion in annual sales, and doubling every 4 years since 1990.

The Western Agricultural Economics Association published information on sales of organic milk in mainstream supermarkets showing a growth over the last 8 years, reaching US$75.7 million in 1999. Organic dairy products can be found in conventional supermarkets and natural-food stores across the United States and in the United Kingdom, where the demand for organic milk and dairy produce is now growing strongly. One leading retailer is predicting a 10-fold increase in its sales over the next 5 years. The Soil Association shows the United Kingdom organic food market currently growing at around 40% year^{-1} and by 2002 it is expected to top £1 billion.

The organic sector in Canada is small but growing rapidly. According to industry sources, farm cash receipts from this industry reached about Can$500 million in 1999, with an estimated retail value of Can$1 billion, including processed and nonprocessed products. Canadian organic retail sales growth is expected to rise from Can$0.7 billion in 1997 to Can$3.1 billion in 2005, which equates to an average growth of 20% annually. The industry anticipates that its market share will increase to 10% of the Canadian retail market by 2010.

Worldwide, growth in organic retail sales is between 20% and 30% per annum. At this rate, 30% of the land in Europe is predicted to be in organic production or in conversion to organic by 2010. In Europe, organic retail sales are estimated at approximately US$7.5 billion, in the United States at US$6.5 billion and in Japan at US$1.5 billion. It is predicted that, by 2005, the industry in Japan will hit the US$10 billion mark. Although still fledgling in Australia, the organic industry turns over US$250 million per year in retail sales of organic food and it exports approximately US$25–30 million worth of organic produce.

The Organic Products Exporters of NZ Inc. (OPENZ) was formed to encourage and support companies and organizations, which have an interest in the New Zealand organic export industry. OPENZ reported a significant increase in the value of organic exports to June 2000. Survey results showed that New Zealand certified organic exports reached over NZ$60 million for the year 1999–2000. This was an increase of 77% on the previous year's figure of NZ$34.08 million. The survey was administered by Trade New Zealand and received responses from 34 out of the 40 exporter members of OPENZ.

A report suggesting organic dairy products will become a profitable, long-term niche market worth up to NZ$200 million a year within a decade has convinced the Dairy Board to foster organic farming in New Zealand. New Zealand currently exports about NZ$60 million-worth of organic products, mostly fruit and vegetables. It has been estimated that the organic market will be unlikely to exceed 5% of the board's NZ$7.6 billion business, but it is a niche that the board wishes to exploit.

International Organic Standards Comparisons

There have been organic standards in the European Union (EU), and EU regulation 2092/91 has been in force since 1991. In many European countries, organic agriculture is known as ecological agriculture, reflecting the reliance on ecosystem management rather than external inputs. According to the Codex Alimentarius Commission:

Organic agriculture's increased momentum is due to consumer demand and to positive environmental impact. Many aspects of organic farming are important elements of a systems approach to sustainable food production, including in developing countries, both for domestic consumption and export.

The Committee on Food Labeling of the Codex Alimentarius Commission has developed *Guidelines for the Production, Processing, Labelling, and Marketing of Organically Produced Foods* (**Table 1**).

The International Federation of Organic Agriculture Movements (IFOAM) represents, internationally, the organic movement in parliamentary, administrative and policy-making forums. IFOAM has consultative status with the UNO and FAO. It sets and regularly revises the international *IFOAM Basic Standards of Organic Agriculture and Food Processing*, which are translated into 19 languages. IFOAM also operates the International Organic Accreditation Services, Inc. (IOAS), in order to administrate the IFOAM Accreditation Program to ensure equivalency of certification programs worldwide.

In the United Kingdom, the integrity of organic food is safeguarded by international legislation. Organic livestock production is regulated by EU Regulation 804/1999. The United Kingdom Register of Organic Food Standards (UKROFS) is the British control body for the organic sector. Food sold as organic anywhere in the EU must be certified as produced under an approved system authorized by an official inspection and certification service. The Soil Association is the best known of the UKROFS validating bodies.

Canadian organic dairy products have been widely distributed since 1995 and, in Canada, there is more information available on organic dairy farming, than on other types of livestock farming. The Canadian Organic Advisory Board Inc. (COAB) was established in 1992 as a national, nonprofit advisory body to represent the interests of organic production and certification groups across Canada. The Board is a vehicle for collaboration of stakeholders within the organic industry and, notably, agencies within the federal and provincial government that have been involved in the development of organic standards.

The Certified Organic Associations of British Columbia (COABC) works on a voluntary basis to maintain a credible set of organic production and processing standards. COABC ensures compliance with the standards by administrating the accreditation and auditing process in partnership with the British Columbia Ministry of Agriculture, Fisheries and Food (BCMAFF).

The United States signed into law the Organic Foods Production Act (OFPA) in 1990. The final rule implementing OFPA was published in the Federal Register in December of 2000. This final rule establishes the National Organic Program (NOP) under the direction of the Agricultural Marketing Service (AMS), an arm of the US Department of Agriculture. The goal of this national program is to facilitate domestic and international marketing of fresh and processed food that is organically produced and to assure consumers that such products meet consistent, uniform standards.

To ensure that access of New Zealand organic products into the EU is maintained, the Organic Products Exporters Group Inc. (OPEG) has requested that the Ministry of Agriculture and Forestry (MAF) Food establish an Official Organic Assurance Program for organic products exported to the EU. The objective of this program is to provide an official assurance to the EU that organic products exported from New Zealand comply with the requirements of Council Regulation 2092/91.

Japan's Ministry of Agriculture, Forestry and Fisheries (MAFF) have completed the development of their own national standard for organic production using the Codex *Guidelines for the Production, Processing, Labelling, and Marketing of Organically Produced Foods* as a base. The Japan standard covers plant products only and Japan's MAFF have advised that imported products labeled as organic will need to comply with the standard by April 2001.

A comparison of standards around the world shows that they are mostly consistent but do vary in a few areas. These areas are pasture requirements, percent of total feed which must be organic and the use of antibiotics.

Table 1 Standards for organic dairying

	International Federation of Organic Agricultural Movements	*Codex (1999 Draft only, not agreed upon)*	*Canada June 1999*	*Certified Organic Associations of British Columbia 1997*	*US Department of Agriculture NOP 2000*	*Soil Association UK 1998*
Living conditions as related to access to pasture or free range	Access to open air and/or grazing appropriate to type of animals and season	Herbivores must have access to pasture. May allow exceptions in certain circumstances	Environment suited to their needs that provides regular access to pasture, free-range open-air runways or other areas subject to weather and ground conditions	Free access to pastures, paddocks or runways. Access to grazing land 120 days of the year	Access to outdoors and direct sunlight. Access to pasture for ruminants. Allows temporary exemptions in case of certain circumstances or to protect soil	All stock must have access to pasture during grazing season unless specifically exempted
Conversion: dairy herds	Not less than 30 days 12 months organic feed, 90 days health and living conditions	Still under discussion	In accordance with the standards for at least 12 months	12 months incorporating all required practices. Replacements 90-day transition if certified livestock not available but must be heifers or 120-day dry-treated cows	12 months incorporating all required practices. New herd conversion, 80% organic feed for first 10 months	12 weeks
Feed	100% organically grown feed, with 50% coming from farm or produced within the region. If impossible, allowance for 15% of feed from nonorganic sources	Should be 100% organically grown. If operator can demonstrate such feed is not available, livestock will maintain status with 85% organic feed	100% from organic sources. May be 85% for ruminants in the short term only	Certified organic required, certified transitional feed is regulated	Certified organically produced feed and pasture required	Livestock systems should be planned to provide 100% in accordance with standards. Allowed 90% on a daily basis, or 85% dairy stock
Health care	Natural medicines and methods emphasized. Use of conventional veterinary medicines allowed when no alternatives are available	Use of veterinary drugs prohibited in absence of an illness. If no alternative permitted treatment or management, vaccinations and therapeutic uses permitted. Should not withhold necessary treatment to maintain organic status	Vaccination and use of veterinary drugs allowed only when disease cannot be combated by other means. Withholding of necessary treatments to maintain organic status is not permitted	Vaccinations allowed as appropriate to each bioregion. Withholding of necessary medical treatment that would disqualify organic status is prohibited	Vaccinations allowed. Administrations of medications in absence of illness prohibited. Withholding treatment to maintain organic status causing suffering or death shall be grounds for decertification	Use of veterinary medical products where no known problem exists prohibited. Medications must never be withheld where it will result in unnecessary suffering. Vaccines restricted to known disease risk that cannot be controlled by other means

(*Continued*)

Table 1 (Continued)

	International Federation of Organic Agricultural Movements	*Codex (1999 Draft only, not agreed upon)*	*Canada June 1999*	*Certified Organic Associations of British Columbia 1997*	*US Department of Agriculture NOP 2000*	*Soil Association UK 1998*
Use of antibiotics	When conventional veterinary medicines are used the withholding period shall be at least double	Withdrawal periods double that required by legislation. After 2005 antibiotics not allowed	If veterinary drugs used, withdrawal period at least double	Not permitted for slaughter animals. Allowed for breeding animals but not in a subtherapeutic manner. Use on animals in 3rd trimester or during lactating will disqualify offspring for slaughter. Milk to be withheld for 30 days or twice withdrawal period if longer	Not permitted	Permitted in clinical cases where no other remedy is effective. Withdrawal at least three times that permitted on product license and not less than 14 days
Use of parasiticides	When conventional veterinary medicines are used the withholding period shall be at least double	Withdrawal periods double that required by legislation	If veterinary drugs used withdrawal at least double	Not permitted for slaughter animals. Allowed for breeding herd use but use in 3rd trimester or during lactation disqualifies offspring as organic for slaughter purposes	Not permitted for slaughter stock. Allowed in breeder stock if sickness or infection present; routine use not allowed. Progeny can be sold as organic but not if used during 3rd trimester of gestation or during lactation. 90-day withdrawal or dairy animals	Permitted when used therapeutically when clinical symptoms appear. Restricted use on routine basis over a specific time period as part of the disease reduction program. Ivermectin-based products prohibited

Before the NOP rule was published, certification agency standards in the United States varied in regards to antibiotics. Some allowed none; other allowed their use with a 30–90-day withdrawal period. During this withdrawal period, the milk or milk products could not be sold as organic, and the meat could never be used as organic. Today under the NOP, antibiotics are never allowed on organic cattle. Under COABC regulations, cows can be brought back into the milking string after 30 days.

Though the particulars of organic livestock production may vary between nations, around the world the standards emphasize proactive health care, the principle of prevention versus treatment. Healthy cow care and early sick-cow recognition are crucial. By doing the utmost to control the animals' environment, and thereby prevent illness and lower stress, the animals remain healthier than similar cows where these preventative practices are not performed. Standards usually require access to the outdoors, fresh air, sunlight and shelter, and they recognize species-appropriate behavior and make allowances for it.

Using the NOP as a model, here is a glimpse into organic certification requirements:

The farmland itself must have no prohibited materials applied to it for at least 36 months before the harvest of organic crops. It must have distinct, defined boundaries and buffer zones such as runoff diversions to prevent the unintended application of a prohibited substance to the crop or contact with a prohibited substance applied to adjoining land that is not under organic management. The producer must select and implement tillage and cultivation practices that maintain or improve the physical, chemical and biological condition of soil and minimize soil erosion. The producer must manage crop nutrients and soil fertility through rotations, cover crops, and the application of plant and animal materials.

Milk or milk products must be from animals that have been under continuous organic management beginning no later than 1 year prior to the production of such products, except for the conversion of an entire, distinct herd to organic production. For the first 9 months of the year of conversion, the producer may provide the herd with a minimum of 80% feed that is either organic or produced from land included in the organic system plan and managed in compliance with organic crop requirements. During the final 3 months of the year of conversion, the producer must provide the herd with 100% organic feed.

The producer of an organic livestock operation must maintain records sufficient to preserve the identity of all organically managed livestock and all edible and nonedible organic livestock products produced on his or her operation.

The producer must not use animal drugs, including hormones, to promote growth in an animal or provide feed supplements or additives in amounts above those needed for adequate growth and health maintenance for the species at its specific stage of life.

The producer of an organic livestock operation must establish and maintain preventive animal health care practices. The producer must establish appropriate housing, pasture conditions and sanitation practices to minimize the occurrence and spread of diseases and parasites. Animals in an organic livestock operation must be maintained under conditions that provide for exercise, freedom of movement and reduction of stress appropriate to the species. Additionally, all physical alterations performed on animals in an organic livestock operation must be conducted to promote the animals' welfare and in a manner that minimizes stress and pain.

The producer of an organic livestock operation must administer vaccines and other veterinary biologics as needed to protect the well-being of animals in his or her care. When preventive practices and veterinary biologics are inadequate to prevent sickness, the producer may administer medications included on the National List of synthetic substances allowed for use in livestock operations. The producer may not administer synthetic parasiticides to breeder stock during the last third of gestation or during lactation if the progeny is to be sold, labeled, or represented as organically produced. After administering synthetic parasiticides to dairy stock, the producer must observe a 90-day withdrawal period before selling the milk or milk products produced from the treated animal as organically produced. Every use of a synthetic medication or parasiticides must be incorporated into the livestock operation's organic system plan subject to approval by the certifying agent.

The producer of an organic livestock operation must not treat an animal in that operation with antibiotics, any synthetic substance not included on the National List of synthetic substances allowed for use in livestock production, or any substance that contains a nonsynthetic substance included on the National List of nonsynthetic substances prohibited for use in organic livestock production. The producer must not administer any animal drug, other than vaccinations, in the absence of illness. The use of hormones for growth promotion is prohibited in organic livestock production, as is the use of synthetic parasiticides on a routine basis. The producer must not administer synthetic parasiticides to slaughter stock or administer any animal drug in violation of the Federal Food, Drug, and Cosmetic Act. The producer must not withhold medical treatment from a sick animal to maintain its organic status. All appropriate medications and treatments must be used to restore an animal to health when methods acceptable to organic production standards fail. Livestock that are treated with prohibited materials must be clearly identified and shall not be sold, labeled or represented as organic.

A livestock producer must document in his or her organic system plan the preventative measures he or she has in place to deter illness, the allowed practices he or she will employ if illness occurs, and his or her protocol for determining when a sick animal must receive a prohibited animal drug. These standards will not allow an organic system plan that envisions an acceptable level of chronic illness or proposes to deal with disease by sending infected animals to slaughter. The organic system plan must reflect a proactive approach to health management, drawing upon allowable practices and materials. Animals with conditions that do not respond to this approach must be treated appropriately and diverted to nonorganic markets.

The producer of an organic livestock operation must establish and maintain livestock living conditions for the animals under his or her care which accommodate the health and natural behavior of the livestock. The producer must provide access to the outdoors, shade, shelter, exercise areas, fresh air and direct sunlight suitable to the species, its stage of production, the climate, and the environment. This requirement includes access to pasture for ruminant animals. The producer must also provide appropriate clean, dry bedding, and, if the bedding is typically consumed by the species, it must comply with applicable organic feed requirements. The producer must provide shelter designed to allow for the natural maintenance, comfort level, and opportunity to exercise appropriate to the species. The shelter must also provide the temperature level, ventilation and air circulation suitable to the species and reduce the potential for livestock injury. The producer may provide temporary confinement of an animal because of inclement weather; the animal's stage of production; conditions under which the health, safety, or well-being of the animal could be jeopardized; or risk to soil or water quality. The producer of an organic livestock operation is required to manage manure in a manner that does not contribute to contamination of crops, soil or water by plant nutrients, heavy metals or pathogenic organisms and optimizes nutrient recycling.

Future of Organic Dairying

Nitrogen self-sufficiency through the use of legumes and biological nitrogen fixation, as well as effective recycling of organic materials, including crop residues and livestock manure, will positively affect the impact of the farming system on the wider environment. These practices, coupled with conservation of wildlife and natural habitats, are some of the many benefits of organic production practices.

Livestock manures are one of the most valued resources on an organic farm or ranch. Conservation of manure and its proper application are a key means of recycling nutrients, building soil and improving the sustainability of an organic operation. Ideally, manures for organic crop production are composted. However, uncomposted manures are allowed with restrictions. Raw, uncomposted livestock manures may not be applied to crops destined for human consumption unless incorporated into the soil a minimum of 120 days prior to harvest.

At the same time, water resources must be protected. Fertilizers and manures must be applied to prevent runoff and leaching, fields must be managed to prevent erosion, and 'catch crops' must be used where necessary to soak up excess nitrogen. Riparian zones must be stabilized and protected, natural wetlands must be maintained and protected, and waterways must be protected from livestock and livestock waste through the use of fencing and water tanks to prevent fouling natural streams.

Fliebach, Mader, Pfiffner, Dubois and Gunst recently released results of a 21-year field trial in Switzerland, comparing organic and nonorganic farming systems. The study shows dramatic differences in soil health. It was reported that there were more microorganisms (which play a role in soil fertility and delivering nutrients to roots) in the organically managed field than in the conventionally managed field.

Consumers have become increasingly aware of these environmental issues. The alleged liberal use of pesticides by farmers and the purported suffering of livestock have been highlighted in the press over recent years, and caused rising numbers of consumers to turn to organic products.

The organic sector is growing with sales projected to increase from $US1.31 billion in 1995 to $US4.37 billion in 2005. At present highest demand in the market is for single ingredients in the form of organic milk, cheese or yogurt. An increase in unit shipments of organic dairy desserts and organic ready-made meals is expected and should increase revenues over the rest of the forecast period.

See also: **Manure/Effluent Management**: Nutrient Recycling; Systems Design and Government Regulations. **Office of International Epizooties**: Mission, Organization and Animal Health Code. **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius; Trade in Milk and Dairy Products, International Standards: Harmonized Systems.

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P

PACKAGING

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Introduction

The packaging of food can be traced to ancient times although its early beginning was with crude materials. Early reports document wine being stored in animal skin and various liquids including water being packaged and stored in earthen vessels. Increases in the complexity of human civilization also saw the development and use of diverse types of packages made from varying types of materials. The Industrial Revolution, which started during the 1700s, also had an effect on food packaging because the new industrial workers demanded a more convenient manner for transporting and storing meals. Beginning in the 1950s and within modern times, the growth of the fast-food industry has significantly influenced the food-packaging industry. Examples of foods in this category include gravy preparations, dry cake mixes, boil-in-bag foods, and TV dinners. These developments created a demand for new types of packages. The packaging of fluid milk started prior to the 1950s when Gail Borden discovered and patented the process for condensed milk. This occurred in 1856 and was followed by the development of the glass milk bottle in 1884, the invention of the automatic bottle filler and capper in 1886, and the introduction of the first plastic-coated paper milk carton in 1932. Due to the properties of milk and dairy products, packaging is considered to be a critical step in the processing operations. The reason is that packaging is the last link in the processing chain. If the choice of packaging is inappropriate or its failure occurs during handling, transportation, and storage of milk products, the processing steps would be useless, even if they were properly executed. In an attempt to minimize food safety hazards associated with inadequately processed and/or packaged milk products, the Pasteurized Milk Ordinance (PMO) enacted by the US government mandates regulatory guidelines for the dairy industry. **Figure 1** shows a typical fluid milk filling room that complies with the PMO's requirements. The filling room is separated from other processing operations, and the equipment and facilities have the infrastructure to prevent any contamination of milk.

Purpose of Packaging of Dairy Products

The systems and requirements for packaging of dairy products are similar to those of other foods. Milk and dairy products are packed in various types of packaging materials depending on the specific product properties, processing conditions, storage, handling, and end use. The primary purposes of packaging are to preserve and protect dairy products against spoilage and harmful factors in the outside environment, to contain specific amounts of the product in units that are easy to handle during production, storage, transportation, and consumption, and to provide information about the product to the consumer and regulators. Important considerations about materials used to package dairy products are toxicity and compatibility with the product, resistance to impact, maintenance of sanitation, odor and light protection, tamper resistance, size specifications, shape and weight requirements, marketing appeal, printability, and cost. Examples of packaging types used for dairy products include glass and plastic bottles, gable-top and brick-type cartons, bags, pouches, two- and three-piece cans, aerosol containers, plastic tubs, and other containers.

Figure 1 Traditional milk filler room that meets the Pasteurized Milk Ordinance requirements to prevent milk contamination. Courtesy of Seiberling Inc.

Table 1 lists the common packaging materials, their properties, advantages, and disadvantages, and dairy products that are packaged in containers fabricated from them.

Packaging of Dairy Products

Fluid Milk

This is currently marketed as standardized low-fat milk with 0.5, 1.0, 1.5, or 2.0% fat content. It is also marketed as skim milk with <0.5% fat and as homogenized whole milk with a fat content of 3.25%. Milk flavor results mainly from proteins, lipids, and carbohydrates, and small amounts of other components. Depending on the way milk is handled, processed, and stored, its quality and flavor can deteriorate. This decline may be due to bacterial growth, enzymatic activity, and environmental factors such as oxygen and temperature. Additionally, milk is susceptible to the development of light-activated or light-induced off-flavors and vitamin A degradation. Therefore, the properties of the selected packaging materials are important if the quality and nutritional value of milk are to be maintained during storage.

Glass bottles were introduced in 1884 and were the first modern packaging material for fluid milk. An important property of glass that makes it suitable for milk and other foods in general is its fairly inert nature. As a result, it is not associated with the leaching of chemicals that might alter the taste of the product. Its impermeability makes it desirable for long-term storage of foods that are susceptible to volatile loss or the ingression of spoilage gases. Flint glass is transparent and allows product visibility. Due to its rigidity, glass maintains its shape and volume under vacuum or pressure. Glass is stable at high temperatures and has good consumer appeal. In spite of these advantages, glass is breakable and relatively heavy, and has a high manufacturing energy requirement and high cost of production.

Several types of laminated materials are used to package fluid milk. Single-use containers made of plastic/paper laminates (polyethylene (PE)-coated to paperboard) have been used widely because of their low cost, easy printability, and forming versatility. However, they have poor barrier properties against moisture and gases. Since this type of milk package is single use, only the carrying crates used to transport the unit containers require washing and reuse. Another type of laminated material that is used for milk packaging generally consists of seven separate layers that may include PE/adhesive/paperboard/adhesive/aluminum foil/adhesive/PE (or an ionomer). The heat-sealing material in this structure is the PE or ionomer that is bonded to the aluminum foil. The paperboard layer provides rigidity and strength while the aluminum is the oxygen barrier film. Packages made from this type of material are capable of extending the shelf life of the product at ambient temperatures if it is aseptically processed. In these materials, individual films are combined into a single structure to provide physical strength, resistance to puncture, machinability, sealability, environmental resistance, and barrier against moisture, oxygen, carbon dioxide, odor, and light.

High-density polyethylene (HDPE) blow-molded bottles are the single biggest types of milk package used in the United States. HDPE is easy to shape and mold into dispensers; it can be extruded into films to make pouches, or laminated with other materials such as aluminum foil, paper, and other packaging films. HDPE is a good barrier to moisture and is resistant to most solvents; however, it is a poor barrier to carbon dioxide and oxygen and offers little protection against light. To make HDPE opaque, colored resin or shrink-wrapping

Table 1 Properties of common materials used to fabricate packaging for dairy products

Material	*Water vapor transmission*[a]	*Gas permeability constant*[b]			*Advantages*	*Disadvantages*	*Products*
		O_2	*N_2*	*CO_2*			
Glass	Low	Low	Low	Low	Impermeable, easily recycled, inert	Heavy weight, breakable, high energy costs of manufacturing	Fresh milk, yogurt, cream
Metal	Low	Low	Low	Low	Excellent gas barrier, rigid, easily recycled	Requires coatings to prevent corrosion	Milk powder, condensed and evaporated milk
Films							
PVDC (Saron)	3.1	0.91	0.91	0.78	Excellent barrier to water vapor, gases, fatty and oily products	Produces toxic compounds when incinerated	Chesses, MAP[c]
Cellophane, nitrocellulose coated	4.7	0.07	0.07	0.07	Excellent clarity and sparkle, can be used in coatings and laminations	Not strong for heavy products	Cheeses
Polymer coated	7.8	0.03	0.03	0.03			
Plastics							
Low-density polyethylene	20.2	28.60	28.60	11.38	Very flexible, highly resistant to most solvents, good moisture barrier	Useless for rigid containers, poor barrier for gases	Liquid milk (pillow packs), condensed milk (squeeze bottles)
High-density polyethylene	4.7	7.15	7.15	3.25	Moderately flexible, stiffer, tasteless, odorless	Poor barrier for oxygen and other gases, softness, low softening point, poor clarity	Milk, yogurt, sour cream, ice cream
Polyvinyl chloride	62.0	9.75	9.75	-	Versatile material, compounded with a wide range of additives (plasticized)	Difficult to recycle, poor moisture barrier properties	Cheese, yogurt, MAP
Polyethylene terephthalate	Low	Low	Low	Low	High tensile strength, low gas and moisture permeability, high use-temperature range, high scuff resistance, excellent oil barrier	Lack of heat sealability	Milk
Paper							
Laminated papers	Low	Low	Low	Low	Laminated to aluminum foil and extrusion coated with PE offers barrier to moisture, flavor, and UV light	Poor barrier for gases	Butter
Coextruded laminated (aseptic)	Low	Low	Low	Low	Barrier against moisture, gas, odor, light, and UV light	Cost	Milk, milk powder, yogurt
Waxed papers	High	Low	Low	Low	Moisture barrier, low cost, good resistance, good heat-sealing characteristics	Difficult to keep folded	Tubs for ice cream or cream, butter

[a] g per m^2 · day at 38 °C and 90% RH.

[b] cc·mil per m^2 · day · atm at 25 °C and 50% RH (cm^3 per m^2 · day).

[c] MAP, modified atmosphere packaging; PE, polyethylene; PVDC, polyvinylidene dichloride.

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with a suitable label is used to protect against UV or fluorescent light in display cases.

Evaporated Milk

Evaporated milk is manufactured by a process of evaporation, concentration, homogenization, and sterilization of whole milk. The approximate composition of evaporated low-fat milk is 7.5–9.0% fat and 18–22% non-fat milk solids. In this process, the whole milk is exposed to an ultra-high temperature (UHT) process and then packaged aseptically. To prevent contact and reaction with the metal in tin cans, a protective coating is used. The base material in these cans is steel, but most cans are tin-plated, or coated with enamel, epoxy, or lacquers to prevent rusting or attack from the packaged product. Cans used to package milk are sealed by locking the curl on the lids to the flange on the body of the opened can. Once the seal is accomplished, it is called a double seam. Hermetically sealed cans are capable of safely storing evaporated milk for several months without refrigeration.

Sweetened Condensed Milk

Sweetened condensed milk is a product that has been concentrated by evaporation, to which sucrose is added to form an almost saturated sugar solution, after which it is canned. Concentration is usually done by evaporation, but reverse osmosis can also be used. The approximate composition of sweetened condensed milk is 8–9% fat, 20–22% non-fat milk solids, 10–11% lactose, 43–45% sucrose, and 25–27% moisture. This high sugar content helps to maintain the quality of the product during its shelf life. Normally, sweetened condensed milk is packaged in metal cans. In addition, sweetened condensed milk can be kept in the refrigerator for a brief period of time in order to prevent mold growth after it has been opened.

Properties of Coated Cans

Cylindrical cans are the most commonly used containers for condensed and evaporated milk. They are made from rigid steel or aluminum, and are capable of withstanding pasteurization and sterilization processes and still act as good gas and light barriers. Important characteristics of these containers are their design for easy opening, inexpensive costs, ease of processing on high-speed lines, and recyclability. There are several disadvantages associated with the use of metal cans for milk packaging. These include interactions between the metal and packaged products, and susceptibility of the empty container to damage, which could subsequently compromise the safety of the product.

Powder Milk

Non-fat milk powders and whole milk powders (WMPs) are made by evaporating fluid milk to dryness. Dried whole milk contains 26–40% fat and up to 5.0% moisture (by weight). This high fat concentration is a major cause of deterioration due to lipid oxidation during processing and storage. Thus, materials selected to package dried whole milk must limit photodeterioration in order to maintain the product's quality for extended shelf life (ESL).

Metal Cans

The use of three-piece tinplate cans was the traditional method for dried milk packaging for retail marketing. After filling but before sealing these containers, air is withdrawn from the powder and replaced by an inert gas such as nitrogen. This serves to reduce rancidity and extends the product shelf life. Metal cans are popular in many parts of the world due to their mechanical strength, ease of transport and handling, and the possibilities of reuse. Resealable cans usually have a pressure plug lid that provides a gas-tight seal. These types of cans usually have an aluminum foil diaphragm that is affixed to the underside of the sealing rim. This diaphragm provides an extra layer of protection to the unopened can.

Laminates

Within recent time, composite cans made from aluminum foil/polymer/paperboard laminates and pouches made from aluminum foil/polymer have been replacing metal cans for powdered milk packaging. Some of these laminated packages could be fabricated on form–fill–seal (FFS) machines. Gas flushing of the product is not uncommon prior to sealing the package. This is done by saturating the powder with inert gas (nitrogen) prior to filling the container. These materials have a light weight but have the disadvantage of not having the mechanical strength or durability of rigid metal containers. Also, they are difficult to recycle and sealing could be problematic for pouches if the dry powder contaminates the sealing area. The strength of a laminate is dependent upon the composition of the material. For instance, a laminate made from aluminum foil, paperboard, and low-density polyethylene (LDPE) has a lower burst strength than a similar laminate in which the paperboard is replaced by a PET film. These laminates could be mounted as roll-stock on FFS machines where they could be used to make gusseted or nongusseted pouches. Typically, a pouch used for dried milk packaging, and made on an FFS machine, would have a laminate made with a PET (17 μm)/LDPE (9 μm)/aluminum foil (9 μm)/LDPE (70 μm) structure.

Composite Cans

Composite cans are made by the spiral winding of paperboard strips. They can be produced with a wide variety of liners. Cans made from these materials have mechanical strength comparable with aluminum foil/LDPE/paper bags and metal cans. Composite packages have the added advantages of being lighter than metal cans and do not corrode under high-humidity conditions. Composite cans are filled in the same manner as metal cans but they cannot be cleaned with hot water. The material specifications for cans used to package whole or skim milk powders are 0.9 mm thick paperboard and 0.5 μm thick aluminum foil coating with a nitrocellulose lacquer to protect it from abrasion by the powder. An outer decorative label incorporating a fiber sealing material gives increased protection against moisture penetration. When compared with the filling of metal cans, the occluded air trapped in vacuoles within the milk powder is not removed during the gas flushing phase of fiber cans or laminate package filling. This means that the headspace oxygen in these composite packages is higher than in milk stored in metal cans. This has the potential to cause milk in composite packages to spoil faster. For ESL of milk power, residual oxygen concentrations should not exceed $0.02 - 0.03\,\mathrm{ml\,g^{-1}}$. A reduced oxygen environment could be created by conditioning the milk powder under vacuum for 24–48 h before gas flushing to remove the occluded air.

Ice Cream

The packaging of ice cream is often a complicated operation, especially if mixed flavors or exceptional shapes are required. Frozen dessert packages are designed to contain bulk product for the sale of dipped products such as ice cream cones or to package ice cream product into small containers for direct retail sale. The packaging steps may start in the hardening area to retain the shape of the portioned dessert. Practically all frozen desserts for bulk use are packaged in single-service containers made of paperboard or plastic. Some are packaged in reusable plastic containers, but the use of steel cans is limited. Packages for retail sale vary in size (US sizes vary from 100 ml to 1 l). The most common retail size package is the 250 ml carton. Typical metric package sizes are 100 ml, 250 ml, 500 ml, 1 l, 2 l, and 4 l with shapes that are rectangular, cylindrical (round), or conical (tapered cylinder). A relatively new shape called square/round (squround) is a modification of the conical container. The shape is rectangular, tapered, and rounded at the corners. Ice cream packaged in these containers is easier to scoop out when compared with ice cream in either the round or rectangular containers. Also, these squround containers have tighter seals and tend to fit better in the freezer compared with the round ones. The traditional rectangular 250 ml carton is made of plastic-coated paperboard. During the fabrication process, the label is printed on the carton, which is folded into a collapsed shape and then sealed on one side. Ice cream mixes are regularly packaged in polymeric cups or tubs, flexible plastic bags, or wax-coated paper. All types are distributed in either cartons or plastic shipping cases.

Cheese

Cheesemaking is a complex system with different reactions taking place during the manufacturing, maturation, and storage stages. In unpackaged cheese, quality and water loss depend on the chemical properties of the cheese and on the storage conditions. In packaged cheeses, quality and water loss depend not only on the storage conditions, but also on the permeability and protection provided by the packaging material. Factors that may be considered when selecting a package are the type of cheese and the resulting resistance to mechanical damage (hard or soft cheese); the presence of a specific microorganism; wholesale or retail packaging; permeability to water vapor, oxygen, CO_2, NH_3; and light; and labeling facilities.

Waxing can be applied to low-moisture cheese shortly after manufacturing. In the past, semi-hard cheese was often covered with paraffin wax. However, a latex emulsion is currently used to coat this type of cheese. Soft cheese is commonly wrapped in a three-layer film consisting of a wax/paper/varnish structure; in this material, the wax is in contact with the cheese. This combination results in a packaging material with low O_2 and water vapor transmission rates.

Fresh and cream cheeses are susceptible to photooxidation. Therefore, they require a packaging material that protects against transmitted light. Genuine vegetable parchment or grease-proof paper was frequently used to package fresh cheese but is currently being used for Petit Suisse. Paper coated with paraffin or polyvinyl chloride/polyvinylidene chloride (PVC/PVDC) copolymer is sometimes used in the form of a banderole. While a number of plastics have been introduced over the years, the standard material is polystyrene (PS), which is thermoformed on FFS machines. PS is also co-extruded or extrusion coated with PVC or PVC/PVDC co-polymer to improve its barrier properties. The coated PS is sometimes pigmented with TiO_2 to provide a better barrier against light.

Yogurt

Yogurt is a highly perishable product and packaging protects it during handling and helps to maintain its physicochemical, nutritional, and sensory characteristics. The package should also prevent loss of volatile flavors and/or the absorption of undesirable odors. Packaging for yogurt is classified into three main categories depending on the physical strength of the container.

Semi-rigid Containers

These types of containers are normally manufactured from plastic. The properties of different types of plastic materials that are important for yogurt packaging are water vapor, oxygen, and nitrogen transmission rates. Furthermore, yogurt containers must be acid resistant and prevent the loss of volatile flavors. Materials that are used to package yogurt are PE, polypropylene (PP), PS, PVC, and PVDC laminates.

Flexible Containers

These types of yogurt containers are mainly in the form of paperboard cartons and plastic pouches. Paper-based cartons are made from laminates (PE/aluminum foil/PE or PE/paper/aluminum foil/PE) and are used only to package dehydrated yogurt. An important property of flexible containers for yogurt packaging is permeability to gases and water vapor. Paperboard cartons had been used for yogurt packaging in the past. This type of container was susceptible to leakage. Consequently, its use as a yogurt container is limited despite improvements in its manufacture.

Rigid Containers

Glass bottles are still used in some countries (France and eastern Europe) to package yogurt. Although glass is an excellent packaging material, its high cost of manufacture and current market trends that favor single-use containers limit the use of glass for yogurt packaging. Other rigid containers that are used to package some yogurt-based products (e.g., dried yogurt) are metal cans.

Butter

Butter has a minimum milk fat content of 80–82%, total fat-free dry milk solids of 2%, and a maximum moisture content of 16%. Butter may also have some approved additives such as beta-carotene, sodium chloride, and cultures of harmless lactic acid-forming bacteria. Butter is very susceptible to light-induced flavors, as a result of its susceptibility to lipid oxidation. It is well known that fluorescent light and sunlight are the cause of oxidation of milk fats found in butter. Thus, the selection of an appropriate packaging material can significantly reduce the incidence of oxidation caused by light and the development of oxidized flavors.

Butter is normally wrapped in wet waxed, dry waxed, grease-proof, or vegetable parchment paper, or aluminum foil laminated with vegetable parchment or grease-proof paper. Dairy spreads are usually packaged in thermoformed PP or LDPE containers with lids of, for example, aluminum foil, PVC, or PE. Bulk butter is packed in 25 kg LDPE-lined paperboard cartons. For long-term storage, it is sometimes packaged in cans.

Several components of butter are known to influence its spoilage. These include free fatty acids, fat-soluble amino acids, and carotene, which promote autoxidation under the influence of light. Spoilage by microorganisms may cause several off-flavors (putrid, volatile acid, etc.). Therefore, the packaging material must not have a high permeability to water vapor because this could increase the risk of surface mold growth in areas where pockets of moisture could accumulate. Lipolysis also produces a soapy-rancid flavor and the degree of its formation depends on the light source, wavelength of the light, exposure time, distance from the light source, and carotene content of the butter. These conditions should be taken into account when selecting an appropriate packaging material.

Probiotic Dairy Foods

The selection of packaging materials to protect and assure the therapeutic activity of these foods is important for the commercialization of these products. It is also important that the level of oxygen within the package should be minimized in order to avoid toxicity and death of the microorganism and a resultant loss of product functionality. Glass bottles provide the best protection for probiotic products, with plastic being the second best alternative. Active and intelligent packaging is becoming increasingly important as a choice for these products. These packages function by (1) absorption of compounds that induce spoilage, (2) release of compounds that extend the shelf life of the product, and (3) monitoring of the shelf life.

Trends and New Concepts

Both the dairy and the wider food industries face similar challenges with respect to product packaging. Some of these challenges are related to the need to maintain efficiency and sustainability of the manufacturing process and to respond to current market trends. Food safety, governmental regulations, and the demand to use sustainable packaging are the issues that seem to be influencing the direction in which the industry is heading. The following are examples of current trends in packaging that are impacting the dairy industry.

The increased use of bio-based materials such as polylactic acid (PLA) to fabricate pouches has influenced packaging manufacturers to use ultrasonic sealing as the method of choice for these materials. The traditional method of heat-sealing used for PLA packages causes it to distort at the sealing areas. This is so because PLA shows poor heat stability. The use of ultrasonic sealing solves this problem because it is considered a cold-sealing technique. Containers made from PLA are compostable and/or biodegradable. Using PLA, a British company developed a milk bottle with a smart two-part system to aid its recyclability. The outer layer consists of recycled cardboard, which is lined with an inner sleeve of PLA made from corn starch. The outer layer protects the paperboard from becoming wet and soggy, but upon emptying, it decomposes in landfills.

The protection of milk, yogurt, and dairy beverages from the oxidative effects of light is still a concern because of its potential to reduce quality and lower the shelf life of the products. Lactra™ is an opaque white liquid technology that has been developed primarily for dairy packaging applications, and it is suitable for both single- and multilayered containers. This technique blocks the transmission of light at wavelengths of up to 550 nm while maintaining the aesthetics of the container. Lactra™ protects and extends the shelf life of dairy products packaged in PET and complies with the European Union (EU) and Food and Drug Administration (FDA) food contact regulations (**Figure 2**).

In another example, an aseptic flexible pouch developed in Europe was made with a polymeric film having calcium carbonate as two-fifths of its volume. This film is stronger and significantly lighter than currently used materials. This new aseptic pouch is sterilized with an electron beam emitter before shipment and this eliminates the need for chemicals and water sterilization before use.

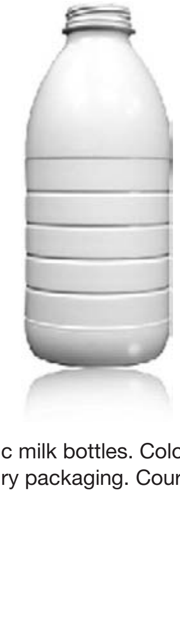

Figure 2 New plastic milk bottles. ColorMatrix's Lactra barrier protection for PET dairy packaging. Courtesy of color Matrix Europe Ltd.

Another recent development is a new aseptic PET bottle for shelf-stable UHT milk. The package is sterilized by a system that uses a dry decontamination technique in which vaporized hydrogen peroxide is applied to sterilize preforms just before they are blown into bottles. These bottles can be made of 20% less plastic because the blown bottle never has to withstand the rigors of conventional hydrogen peroxide sterilization and the thermal abuse associated with it. This dry decontamination provides additional energy-saving benefits because it virtually eliminates the use of a water rinse or hot air drying.

Active packaging is a relatively new technology for powdered milk. Active packaging (also called smart packaging) is the response of a package to a change in the internal or external environment. This response is designed to change the environment within the package and extend the shelf life of the product. As an example, an active package was created by the use of a plastic sachet made with PVC and PS materials. This system was designed to release α-tocopherol in the powdered milk at a controlled rate if the storage temperature exceeded a given threshold. Since α-tocopherol is an antioxidant, its controlled release reduces the potential for rancidity of the milk. In a second example of active packaging, a saturated salt solution was incorporated into the package of a cheese product. When this system absorbs oxygen, it adjusts the humidity within the package. This humidity control was accomplished by a two-way system that continually responded and adjusted to match the outside relative humidity (RH) by either adding or removing water to maintain a predetermined level of RH inside the package.

The FDA's approval of a linear aseptic filler for HDPE bottles for low-acid food and beverages is a significant recent event that has influenced food packaging. Consequently, the traditional paperboard and laminated box packages are being replaced by blow-molded plastic bottles. Products such as flavored milk shakes and coffee drinks that are packed in this linear aseptic filler can now be shipped at ambient temperatures with a 180-day shelf life. This feature is found in an ESL system that has a new sanitary valve without O-rings that does not come in contact with the bottle during filling. This eliminates the possibility of bacterial contamination. High speed is another feature found in two new fillers. One has 64 valves with 32-pocket starwheels. This filler is designed to fill plastic and glass bottles with a maximum diameter of 3.0′ (76 mm) at speeds of up to 800 bottles per minute. The other ESL high-speed filler is designed for half gallon and 2 l sizes at speeds of up to 120–140 cartons per minute,

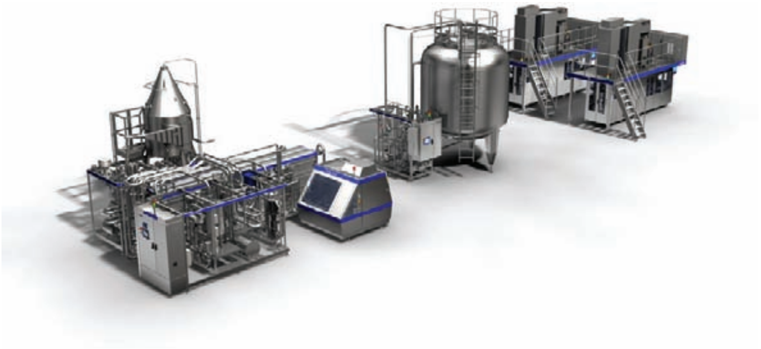

Figure 3 Tetra Lactenso production solutions are engineered to meet the product interacting areas of food safety, quality, efficiency, and sustainability. Courtesy of Tetra Pak.

depending on the carton size. An example of the new fillers is the aseptic system shown in **Figure 3**. The important features of the filler are the automated instrumentation, longer running time capabilities, shorter presterilization times, easy cleaning, and low maintenance.

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PATHOGENS IN MILK

Contents

Bacillus cereus
***Brucella* spp.**
***Campylobacter* spp.**
***Clostridium* spp.**
Coxiella burnetii
Escherichia coli
Enterobacteriaceae
***Enterobacter* spp.**
Listeria monocytogenes
***Mycobacterium* spp.**
***Salmonella* spp.**
***Shigella* spp.**
***Staphylococcus aureus* – Molecular**
***Staphylococcus aureus* – Dairy**
Yersinia enterocolitica

Bacillus cereus

A Christiansson, Swedish Dairy Association, Lund, Sweden

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Introduction

Bacillus cereus is an aerobic spore-forming bacterium, whose spores are commonly present at low levels in raw milk. In the 1960s and earlier, *B. cereus* was clearly a quality problem, due to coagulation (sweet curdling) of pasteurized milk and formation of flakes in cream when added to coffee (bitty cream). This was due to poorly cleansed equipment (e.g., milk cans) at the farm and in dairy factories and a lack of adequate refrigeration. Nowadays, these problems are rarely seen in countries where milk is kept at temperatures below 6 °C. However, when pasteurized milk is stored at higher temperatures, *B. cereus* may still be a limiting factor for the keeping quality. *Bacillus cereus* can produce several enterotoxins causing diarrhea and vomiting. There are few dairy-related cases, but milk and cream have been incriminated in both types of illnesses.

Characteristics

Morphology and Cultivation

Bacillus cereus is a Gram-positive, rod-shaped, motile bacterium with peritrichous flagella. The cells tend to grow in chains but may occur singly as well. The length of the bacterium varies between 3 and 5 μm and the diameter is more than 1 μm. Spores are oval or cylindrical, located centrally or paracentrally/subterminally, and do not distend the cell. A typical trait of the *B. cereus* group is the presence of storage granules of poly-*β*-hydroxybutyrate in the cytoplasm. These are easily seen by phase contrast microscopy. *Bacillus thuringiensis*, *B. mycoides*, *B. weihenstephanensis*, *B. pseudomycoides*, and *B. anthracis* have similar characteristics, except that *B. mycoides*, *B. pseudomycoides*, and *B. anthracis* are nonmotile. The species concept within the *B. cereus* group (which includes the genetically very closely related entities mentioned above) is still under debate. *Bacillus weihenstephanensis* and *B. mycoides* are better adapted to growth at low temperature than the other members within the *B. cereus* group. If not mentioned specifically in the text, '*B. cereus*' refers to the entire group (*B. cereus sensu lato*) except *B. anthracis*. *Bacillus cereus* forms colonies with typical appearance on agar media, generally with dull or frosted, grayish/whitish surface. *Bacillus mycoides* and *B. pseudomycoides* form rhizoid colonies. Widely used selective agar media for cultivation from food are mannitol egg yolk polymyxin agar (MYP) and polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA). The detection of *B. cereus* is based on the absence of mannitol fermentation and positive egg yolk reaction (lecithinase). *Bacillus cereus* can also be enumerated on blood agar with polymyxin added. Colonies with clear zones of hemolysis and a very sharp margin are a useful diagnostic feature. Strains producing emetic toxin have a narrow zone of hemolysis or none at all. The zone does not enlarge upon further incubation, which is the case with nonemetic isolates. A chromogenic selective plating agar (BCM, *B. cereus* group plating medium) that stains colonies expressing phosphatidylinositol-specific phospholipase C turquoise blue has proven to be a good alternative to standard media. It is not possible to differentiate between the species of the *B. cereus* group based on colony morphology only.

Physiology

Bacillus cereus is a versatile microorganism with respect to growth substrates. Most strains produce proteases that can degrade casein and gelatin and enzymes for starch hydrolysis. Enzymes such as lecithinase and sphingomyelinase for degradation of phospholipids and lipase, with activity against triglycerides, can also be produced. Sweet curdling of milk is due to a protease and bitty cream due to phospholipase activity. Several carbohydrates are utilized, for example, glucose, fructose, trehalose, *N*-acetylglucosamine, and maltose. Others are utilized by only certain strains, for example, salicin, cellobiose, inositol, and mannose. A majority of strains do not grow on lactose. Mannitol is generally not used. *Bacillus cereus* is in general Voges–Proskauer (VP) positive and utilizes citrate, but not urea. Most strains can reduce nitrate.

The minimum growth temperature differs among strains and is generally not lower than 5–6 °C, although a few strains have been shown to grow at 4 °C. Increased temperature from 6 to 9 °C markedly affects the growth rate among psychrotrophic (psychrotolerant) isolates (**Table 1**). Some strains have temperature minima as high as 10–15 °C. The optimum growth temperature is 30–37 °C and the maximum growth temperature is 37–50 °C. Strains producing emetic toxin do not grow below 10 °C and are able to grow at 48 °C. The psychrotolerant *B. weihenstephanensis* and *B. mycoides* are able to grow at 7 °C or below and do not grow at 43 °C. These species possess a cold-shock protein (CspA) that is detectable by PCR. Some strains that grow with a minimum temperature of 7 °C and above do not have *cspA* and are classified as *B. cereus* (*sensu stricto*).

The minimum pH for growth is 4.3–4.9 and the upper limit is 9.3. However, in the presence of organic acids, the minimum pH is higher, for example, pH 5.6 in 0.1 mol l^{-1} lactate. Although *B. cereus* grows best under aerobic conditions, anaerobic growth by fermentation of glucose or other carbohydrates or by anaerobic respiration with nitrate is possible. *Bacillus cereus* is able to grow in media with up to 7% NaCl if other conditions are optimum. Minimum water activity for growth is 0.92–0.95.

Table 1 Growth of *Bacillus cereus* (log cfu ml^{-1}) in pasteurized milk at various storage temperatures

Days of storage	*6 °C*	*7 °C*	*8 °C*	*9 °C*
1	-[a]	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	0.0	1.0
5	-	-	1.0	2.4
6	-	0.5	2.0	3.7
7	0.2	1.3	3.0	5.0
8	0.6	2.0	4.0	ND
9	1.0	2.8	5.0	ND
10	1.4	3.5	ND	ND

[a] Less than log 0.
ND, not done.
The values represent average data for pasteurized milk from 10 Swedish dairy plants in August. One milk package was collected from each plant and the milk from each package was divided aseptically into four aliquots, which were incubated in glass bottles in water baths with accurate temperature regulation (±0.1 °C).
Original data from Christiansson A.

Spores

Spores are formed on a variety of growth media under aerobic conditions, upon starvation. The presence of manganese and magnesium ions stimulates sporulation. Sporulation is a fairly lengthy and complicated process, occurring in the late logarithmic and early stationary phase of growth. Even under favorable conditions, sporulation may take up to 16–24 h to complete. Spores are never formed as a result of chilling if nutrients are available, that is, refrigeration of milk does not induce sporulation. For example, high levels of spores are not found in refrigerated pasteurized milk although the *B. cereus* counts may grow to 10^7 ml^{-1}. On the other hand, milk diluted 1:50 with water is still a good growth medium, but nutrients will be depleted after growth and spores are formed abundantly, particularly if the milk is present in thin layers. This is relevant to the cleaning situation in a dairy plant. The spores may germinate and grow out to vegetative cells again under favorable conditions. Germination is much faster than sporulation. The germination rate is highly temperature dependent and may occur within much less than an hour at favorable temperature. In milk, it is stimulated by high-temperature, short-time (HTST) pasteurization, that is, heat treatment. The spores become activated and substances that stimulate germination may be formed as a result of heat treatment. Increased pasteurization temperature in the range of 72–85 °C will lead to activation and germination of more spores. However, initiation of growth in refrigerated milk will occur only after a lag phase of several days.

The heat resistance of *B. cereus* spores is comparatively low. However, there is considerable variation in heat resistance among strains. Although not inactivated by HTST pasteurization, *B. cereus* spores are easily killed upon ultra-high temperature (UHT) treatment. Typical *D*-values at 100 °C are in the range of 0.3–10 min. For comparison, $D_{100\,°\mathrm{C}}$ for *Bacillus stearothermophilus* has been estimated to be approximately 3000 min. Strains producing emetic toxin produce spores that are among the most heat resistant within the *B. cereus* group. Generally, psychrotrophic strains tend to be less heat resistant than mesophilic strains, for example, with *D*-values of 2–9 min at 90 °C. Vegetative cells are easily killed by pasteurization.

Milk-Borne Illness

Bacillus cereus is a common contaminant in many food types, including milk, and a significant cause of foodborne illness worldwide. *Bacillus cereus* can cause diarrhea and/or vomiting when food (most often) containing large numbers of *B. cereus* is consumed. The symptoms are generally mild and transient, lasting no more than 24 h, generally without sequelae. Two types of outbreaks are known: diarrheal-type outbreak and emetic-type outbreak.

Diarrheal-Type Outbreak

The illness is characterized by a fairly long incubation period of 8–22 h. Watery diarrhea is very common, together with abdominal cramps, rectal spasms, and moderate nausea. Vomiting is rare. The duration of illness is generally 12–24 h. The delayed onset of symptoms indicates that illness is most likely due to growth of *B. cereus* in the small intestine, since the toxin(s) are very susceptible to inactivation by low pH and degradation by proteases. Preformed toxin in food will thus be inactivated in the stomach and ileum. Foods associated with diarrheal outbreaks generally contain high numbers of *B. cereus*, that is, 10^5–10^8 per gram food. Foods incriminated in diarrheal outbreaks include meat products, soups, vegetables, puddings, and milk products.

Emetic-Type Outbreak

The incubation period is short, that is, 0.5–5 h. The rapid onset of nausea and vomiting is due to a preformed toxin in the food. Abdominal cramps and diarrhea occur occasionally. Recovery is rapid, within 6–24 h. The level of *B. cereus* in incriminated food can vary between a few thousand and up to more than $5 \times 10^{10}\,\mathrm{g}^{-1}$, although it is generally high. Fried and cooked rice are typical foods frequently involved, but milk-borne cases are also known.

Toxins

The nature of the enterotoxins produced by *B. cereus* has remained elusive for decades. However, during the last 15 years, the knowledge about these toxins has increased considerably.

At least three types of enterotoxins capable of causing diarrhea have been identified. Two of these, hemolysin BL (HBL) and the nonhemolytic enterotoxin (NHE), are protein toxins consisting of three subunits each. All subunits are needed for full activity. Both toxins have been isolated from *B. cereus* strains involved in food poisoning. The third toxin, cytotoxin K (cytK), is a single protein toxin. CytK was involved in a rare foodborne outbreak, which caused the death of three persons, where the symptoms included bloody diarrhea. Additional toxins have been described but their involvement in foodborne illness is uncertain. The enterotoxin genes can be found in all species of the *B. cereus* group as judged by various PCR methods. Most strains are able to produce more than one toxin. *Nhe* genes are present in almost all strains, whereas *Hbl* and *cytK* genes can be found in approximately 50% of all strains, including strains in raw milk. However, *cytK* genes were not found in strains growing in pasteurized milk at refrigeration temperature. The toxin production potential (expression of the genes) varies considerably between strains and toxins. Strains involved in foodborne illness are generally more toxigenic than the average food or environmental isolate. They are often mesophilic, that is, they have a minimum growth temperature above 10 °C, but food poisoning strains growing at or above 7 °C are also known. However, strains belonging to *B. weihenstephanensis* and *B. mycoides* are generally less toxic than the other members of the *B. cereus* group. *Bacillus thuringiensis* and *B. cereus* (*sensu stricto*) have similar toxigenicity. From the point of food safety, there is therefore no need to differentiate between these two species as far as milk products are concerned. The toxins are heat labile and are considered to be inactivated by heating above 60 °C for 5 min. PCR primers have been published for detection of all enterotoxin subunits. However, the mere presence of the genes is not sufficient to judge the pathogenicity of *B. cereus*. Monoclonal antibodies have been developed for all subunits of NHE and HBL and can be used for evaluation of the toxin production potential. Cytotoxicity tests using, for example, Vero cells or Caco cells can be employed to assess the overall cytotoxicity of strains. NHE seems to be the most cytotoxic toxin followed by HBL and cytK.

The emetic toxin is a cyclic peptide, cereulide, which contains 12 modified amino acids and resembles the ionophore valinomycin. The molecular weight is

1.2 kDa. The toxin is quite heat resistant and cannot be destroyed even by heating at 121 °C for 1 h. Unlike the diarrheal toxins, the emetic toxin is encoded by a plasmid. The expression of the toxin varies strongly among strains and also depends on the composition of the food. The emetic toxin is a more serious health hazard than the diarrheal toxins and has been the cause of death in rare cases. Strains producing emetic toxin are rare in the dairy production chain (less than 1% of all isolates). Emetic strains do not grow in pasteurized milk that is kept refrigerated. A large number of cells (more than 5 log cfu g^{-1}) are needed for toxin production. Recently, a real-time PCR method with PCR primers for detection of genes has been developed. Furthermore, a detection method for the toxin, based on the motility of boar sperm, has become available and can be used for foodstuffs.

In addition to enterotoxins, several proteases, phospholipases, and hemolysins may have a role in the pathogenesis of *B. cereus*.

Outbreaks Related to Dairy Products

Outbreaks related to dairy products are rare. Some cases are presented in **Table 2**. Both diarrheal and emetic symptoms have been recorded. Consumption of (refrigerated) raw milk is never associated with illness, due to the low numbers of *B. cereus* present. Growth to high numbers is always necessary in order to cause food poisoning. From the table, it seems that young people and elderly may be at higher risk than the general population. Several factors may explain why milk-borne cases are few: Milk is generally kept at refrigeration temperature and growth of *B. cereus* is slow, thus the risk of exposure to high levels of bacteria is limited, although significant. In addition, sweet curdling often occurs when the product contains 10^6–10^7 *B. cereus* per ml of milk, with decreased risk of consumption. Psychrotrophic strains, in particular *B. weihenstephanensis*, will be enriched in pasteurized milk upon cold storage and these strains seem to be less toxigenic than *B. cereus* (*sensu stricto*). Psychrotrophic strains have a slower growth rate at the temperature of the human intestine (37 °C; close to their maximum temperature of growth) than mesophilic strains, which grow faster. They are therefore less likely to cause food poisoning. However, temperature abuse will increase the risk of illness. Highly toxigenic (mesophilic) strains have been found in raw milk and they may be important in other products such as milk powder.

Incidence in Dairy Products

Vegetative *B. cereus* cells are found in raw milk at <10 ml^{-1} to a few hundred per ml. These cells are killed by pasteurization. Spores are found from <10 l^{-1} to a few thousand per liter milk, that is, at much lower levels. There is a marked seasonal variation in psychrotrophic

Table 2 Outbreaks of milk-borne illness caused by *Bacillus cereus*

Product	*Year*	*Country*	*People ill*	*Symptoms*	*Analytical data*
Unpasteurized milk (heated and then kept at room temperature overnight)	1972	Romania	221 school children	Diarrhea and abdominal cramps after 8–11 h	20×10^6 *B. cereus* per ml in milk. *Bacillus cereus* found in children's feces
Cream, pasteurized	1975	England	Two 15-year-old girls	Vomiting after 8–10 h. One girl had diarrhea	5×10^6 *B. cereus* per gram in cream
Milk, pasteurized	1981	Denmark	1-year-old boy	Vomiting after 1.5 h, no diarrhea	2.6×10^6 *B. cereus* per ml in milk. Remaining milk was sweet curdled 1 h after consumption
Milk powder, infant formula	1981	Chile	35 neonate children	Diarrhea	*Bacillus cereus* found in stool cultures
Human breast milk	1981	India	Child, 6 months	Diarrhea, occasional vomiting	*Bacillus cereus* found in breast milk
Milk, pasteurized	1988	The Netherlands	42 elderly people	Nausea and vomiting after 2–14 h	0.4×10^6 *B. cereus* per ml in milk
Ultra-high temperature milk (process failure)	1991	Japan	201 people	Vomiting 95%, average after 5 h Diarrhea 55%	Milk distributed at room temperature

Compiled from Christiansson A (1992) The toxicology of *Bacillus cereus*. *International Dairy Federation Bulletin* 275: 30–35; Van Netten P, van de Moosdijk A, van Hoensel P, Mossel DAA, and Perales I (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology* 69: 73–79; Shinagawa K (1993) Serology and characterization of toxigenic *Bacillus cereus*. *Netherlands Milk and Dairy Journal* 47: 89–103; Cohen JV, Marmabio E, Lynch B, and Moreno A (1984) *Bacillus cereus* in food poisoning amid newborns. *Revista Chilena de Pediatrica* 55: 20–25.

spores, with the highest levels in summer and early autumn.

Milk and Cream

The number of *B. cereus* in pasteurized milk and cream depends on the quality of the raw milk, the process hygiene at the dairy plant, the storage temperature of the product, and age of the product at the sampling time. *Bacillus cereus* grows slowly at temperatures below 6 °C and will not be a quality problem, unless the sell-by date is set at several weeks. After 7 °C storage for 7 days, the incidence of *B. cereus* can typically vary between 5 and 90% (winter and summer) at <10 to 10^5 ml^{-1} (including differences in dairy hygiene). When stored below 5 °C, *B. cereus* is rarely detected unless there is a cleaning problem in the dairy plant.

Fermented Milks and Cheese

Bacillus cereus is rapidly inactivated in traditional yogurt manufacture as well as in the manufacture of fermented milk with lactococci. Some growth is possible within the first hours of fermentation. Multiplication in semihard cheese is likewise restricted to the first hours in the cheesemaking process. Inhibition occurs due to lactic acid at pH 5.6 but other inhibitors are also active. As the pH is lowered, vegetative *B. cereus* cells will die whereas spores that have not germinated may still be present. When present in these products, *B. cereus* seldom exceeds 100 g^{-1}.

Milk Powder

Bacillus cereus is frequently found in low numbers in milk powder and infant formula. The frequency of isolation varies between 30 and 100% of samples with origin worldwide. Under certain circumstances, there may be some opportunity for growth of *B. cereus* in the evaporation process. Most samples contain <10 cfu g^{-1} but samples with more than 10^3 cfu g^{-1} have been found. These are due to hygienic problems in the factory or due to raw milk with a high degree of contamination. High levels of *B. cereus* in infant formula may constitute a health risk. Regulation (EC) 1771/2007 on microbiological criteria for foodstuffs in the European Union defines process hygiene criteria for presumptive *B. cereus* in dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age. These are n (sample size) = 5, c (number of sample units giving values between m and M) = 1, acceptable limits (m) = 50 cfu g^{-1}, and unsatisfactory limits (M) = 500 cfu g^{-1}. In standard 1.6.1 of the Australia New Zealand Food Standards Code, which specifies microbiological standards for powdered infant formula products, the limits for *B. cereus* are $n = 5$, $c = 0$, and $m = 100$ cfu g^{-1}.

Source

At the Farm

Bacillus cereus is a ubiquitous microorganism. The spores are present in soil from 10^2 cfu g^{-1} and up to more than 10^5 cfu g^{-1}. Consequently, food products of plant origin frequently contain *B. cereus* spores. Soil is an important source of contamination for milk. There is a marked seasonal variation in the spore content of raw milk, with higher levels during the pasture period, when the teats of the cow may be contaminated with soil. Dirty teats that are not cleansed before milking are an important contamination source, particularly during wet weather. *Bacillus cereus* is able to grow and sporulate on insufficiently cleaned milking equipment, so equipment may be a secondary source of contamination. Used bedding material and feed may also contain spores of *B. cereus*.

In the Dairy Plant

There has been considerable disagreement whether the occurrence of *B. cereus* in dairy products is caused by recontamination of milk at the dairy plant or by contamination at the farm. To some extent, this was due to the inability to detect the low levels of spores in raw milk, whereas *B. cereus* was easily detected in pasteurized milk after storage. It is now generally agreed that the original contamination occurs at the farm from soil. The seasonal variation in the occurrence of *B. cereus* in dairy products, kept at temperatures above 6 °C, can to a large extent be explained by the increased contamination rate of the milk during the grazing period. However, additional contamination may occur from the dairy plant equipment. Since spores survive pasteurization, they will be present in the milk throughout the dairy process. Spores of *B. cereus* are very hydrophobic and will attach to surfaces of equipment, where they may germinate and form biofilms at sites that are difficult to clean. Several strain-typing methods (e.g., Random Amplified Polymorphic DNA-Polymerase Chain Rection analysis (RAPD-PCR), Amplified Fragment Length Polymorphism analysis (AFLP), riboprinting, Repetitive element sequence polymorphism-PCR analysis (rep-PCR), and pulsed field gel electrophoresis (PFGE)) have recently been applied to strains of *B. cereus*. These methods demonstrate a high discriminatory power and could be helpful in finding contamination sites in dairy plants. There is a very strong diversity among strains of *B. cereus* in raw milk. Recontamination of milk by *B. cereus* has been demonstrated in silo tanks, pasteurizers, milk pipelines

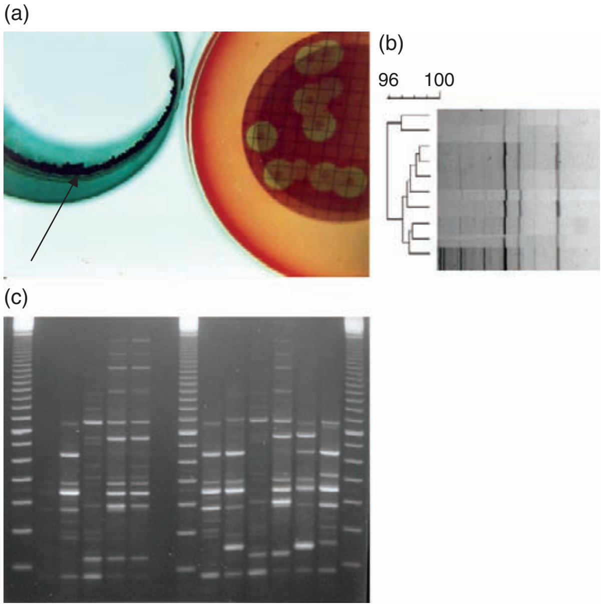

Figure 1 Examples of strain typing of *Bacillus cereus* isolates. (a) A milk stainless-steel pipeline with a very rough welded seam (arrow) was replaced at a dairy plant (to the left). Spores were recovered from the seam by rinsing with water and ultrasonication, collected by filtration, and then grown on blood agar plates (to the right). (b) All isolates showed the same RAPD fingerprint, which indicates that the welded seam was a source of recontamination. Similar fingerprints were found in pasteurized milk. (c) Examples of various RAPD fingerprints of strains from pasteurized milk. Lanes 1, 8, and 15 are molecular weight markers. A Christiansson, unpublished data.

with bad welding, and in packaging machines using RAPD-PCR (**Figure 1**). Automated ribotyping and rep-PCR have been used to identify surfaces of dairy equipment involved in recontamination of milk.

Control

The Farm

At the farm, measures to control *B. cereus* include careful teat cleansing before milking and proper cleaning and disinfection of the milking equipment. Since the teats become dirty with soil when the cows are outdoors during the grazing period, it is essential that they are clean before attaching the teat cups. During the indoor season, high levels of *B. cereus* spores may be found in used bedding material, if not replaced daily, and may contaminate the teats. The best cleansing routine includes the use of one moistened cloth per cow, followed by a dry paper towel. In addition, the milking equipment must be kept clean by careful cleaning after milking. Teat liners and other rubber material must be replaced regularly since aged rubber with cracks can harbor milk residues where *B. cereus* can propagate and sporulate.

The Dairy Plant

At the dairy factory, cleaning and maintenance is essential. Attention must be given to proper concentrations of cleaning agents, sufficiently high cleaning temperature (at least 75 °C for alkaline cleaning agents with 1–1.5% NaOH and at least 60–65 °C for acid cleaning agents with 0.6–0.9% nitric acid), and proper flow rates during cleaning, since spores are difficult to remove and to kill. *Bacillus cereus* is considerably more resistant in a biofilm with spores than in a planktonic state. The spores are not killed by hot water disinfection, but sodium hypochlorite at pH 6–7 is effective. Regular replacement of gaskets and other rubber parts is important.

In Dairy Products

The best control measure for *B. cereus* in pasteurized milk and cream is to keep a low storage temperature in the whole chain from the dairy plant to the customer. Below 5–6 °C, growth of most strains of *B. cereus* is insignificant. If the temperature is higher, the sell-by date must be shortened. Suitable time/temperature combinations may be found by storage tests. Seasonal variation, occurrence of recontamination at the dairy plant as well as possible moderate temperature abuse by the customer must be taken into consideration when choosing the recommended last consumption date of the products.

Milk powder is microbiologically stable and no growth of *B. cereus* can occur in the powder, although occurrence of contamination with *B. cereus* is frequent. However, milk powder is frequently used in infant formulae and in infant foods. When such powders are reconstituted, it is important that the product is consumed shortly after preparation unless it is not cooled to below 8 °C. Spores of *B. cereus* are able to germinate rapidly at the reconstitution temperature and will grow rapidly if the product is kept at room temperature. Young children may be more susceptible to toxins that may be produced than adults.

See also: **Analytical Methods**: DNA-Based Assays. **Biofilm Formation**. **Dehydrated Dairy Products**: Infant Formulae; Milk Powder: Types and Manufacture. **Heat Treatment of Milk**: Thermization of Milk. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: UHT Sterilized Milks; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Microorganisms Associated with Milk**. **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Plant and Equipment**: In-place Cleaning. **Psychrotrophic Bacteria**: Other Psychrotrophs.

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Brucella spp.

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Introduction

Brucella spp. are the causative agent of brucellosis, a zoonosis of worldwide importance. Human brucellosis is usually characterized by an intermittent influenza-like clinical pattern, which may be severe and may be followed by chronic, intermittent relapses. The main manifestations of animal brucellosis are reproductive failure, for example, abortion and birth of unthrifty offspring in the female, and orchitis and epididymitis in the male, and rarely arthritis. Persistent infection with shedding of *Brucella* in reproductive and mammary secretions is common.

Genetic and immunological evidence indicates that all members of the genus *Brucella* are closely related. However, due to relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, the classification of the genus includes nine species (**Table 1**).

Brucella abortus primarily infects cattle but may be transmitted to buffaloes, camels, deer, dogs, goats, horses, pigs, sheep, and humans. *Brucella melitensis* causes a highly contagious disease in sheep and goats although cattle and other species can be infected. It is the most important species in human infection. *Brucella suis* covers a wider host range than most other *Brucella* species. *Brucella canis* causes epididymo-orchitis in the male dog and abortion and metritis in the bitch. It has not been reported in other animals except humans. *Brucella ovis* is responsible for epididymitis in rams and occasionally infects ewes, but does not infect other animals or humans. *Brucella neotomae* is only known to infect the desert wood rat under natural conditions, and no other cases have been reported. In the last decade, isolations of previously unidentified species of *Brucella* have been reported from sea mammals (*B. ceti* and *B. pinnipedialis*). Finally, a new species, *Brucella microti*, was recently isolated in central Europe from the common vole (*Microtus arvalis*).

Characteristics

Morphology

Bacteria included in the genus *Brucella* are Gram-negative coccobacilli or short rods (0.6–1.5 μm × 0.5–0.7 μm) arranged singly and rarely in pairs or small groups. The morphology of *Brucella* spp. is fairly constant except in old cultures, where pleomorphic forms may occur. Brucellae are nonmotile and do not form spores, and flagella, pili, or true capsules are not produced. They usually do not show bipolar staining and resist decolorization by weak acids.

Culture and Growth Characteristics

Brucella spp. are aerobic, but many strains require an atmosphere containing 5–10% added CO_2 for growth (**Table 2**). The optimum pH for growth varies from 6.6 to 7.4, and culture media should be adequately buffered near pH 6.8 for optimal growth. The optimal growth temperature is 36–38 °C, but most strains can grow between 20 and 40 °C. Growth in liquid media favors dissociation of smooth-phase cultures to nonsmooth forms and is usually poor unless the culture is vigorously agitated. On suitable solid media, colonies are visible after 2 days of incubation. After 4 days of incubation, the colonies are round, 1–2 mm in diameter, with smooth margins, translucent, and a pale honey color when plates are viewed in the daylight through a transparent medium. When viewed from above, the colonies appear convex and pearly white. Later, the colonies become larger and slightly darker. Smooth *Brucella* spp. cultures have a tendency to undergo variation during growth, especially with subcultures, and dissociate to rough (R) forms, and sometimes mucoid (M) forms.

Biochemical Characteristics

The metabolism of *Brucella* spp. is oxidative, and cultures show no ability to acidify carbohydrate media in conventional tests. The *Brucella* species are catalase-positive and usually oxidase-positive (except otherwise stated in **Table 1**), and reduce nitrates to nitrites (except *B. ovis* and some *B. canis* strains). *Brucella suis* biovar 1, *B. neotomae* and biovars 1–4 and 9 of *B. abortus* produce H_2S from sulfur-containing amino acids (**Table 2**). Urease activity varies from fast to very slow. Indole is not produced from tryptophan, and acetylmethylcarbinol is not produced from glucose.

Antigenic Characteristics

All smooth *Brucella* spp. strains show complete cross-reaction with each other, but not with nonsmooth variants,

Table 1 Differential characteristics of species of the genus *Brucella*

			Lysis by phages[b]							
			Tb		*Wb*	*Iz_1*	*R/C*			
Species	*Colony morphology*[a]	*Serum requirement*	*RTD*[c]	*10^4*RTD	*RTD*	*RTD*	*RTD*	*Oxidase*	*Urease activity*	*Preferred host*
B. abortus	S	−[d]	+	+	+	+	−	+[e]	+[f]	Cattle and other Bovidae
B. suis	S	−	−	+	+[g]	+[g]	−	+	+[h]	Biovar 1: swine Biovar 2: swine, hare Biovar 3: swine Biovar 4: reindeer Biovar 5: wild rodents
B. melitensis	S	−	−	−	−[i]	+	−	+	+[j]	Sheep and goats
B. neotomae	S	−	−[k]	+	+	+	−	−	+[h]	Desert wood rat[l]
B. ovis	R	+	−	−	−	−	+	−	−	Rams
B. canis	R	−	−	−	−	−	+	+	+[h]	Dogs
B. ceti	S		+[m]		+[n]	+[o]	−	+	+	Cetaceans
B. pinnipedialis	S		+[m]		+[n]	+[o]	−	+	+	Pinnipeds
B. microti	S	−	−	+	+	+	−	+	+	Common vole

[a] Normally occurring phase: S, smooth; R, rough.

[b] Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1(Iz_1), and R/C.

[c] RTD: routine test dilution.

[d] *B. abortus* biovar 2 generally requires serum for growth on primary isolation.

[e] Some African isolates of *B. abortus* biovar 3 are negative.

[f] Intermediate rate, except strain 544 and some field strains that are negative.

[g] Some isolates of *B. suis* biovar 2 are not or partially lysed by phage Wb or $Iz_{1.}$

[h] Rapid rate.

[i] Some isolates are lysed by phage Wb.

[j] Slow rate, except some strains that are rapid.

[k] Minute plaques.

[l] *Neotoma lepida*.

[m] Some isolates are lysed by Tb.

[n] Most isolates are lysed by Wb.

[o] Most isolates are lysed by Iz.

Table 2 Differential *characteristics* of the biovars of *Brucella* species

				Growth on dyes[a]		*Agglutination with monospecific sera*		
Species	*Biovar*	*CO_2 requirement*	*H_2S production*	*Thionin*	*Basic fuchsin*	*A*	*M*	*R*
B. melitensis	1	−	−	+	+	−	+	−
	2	−	−	+	+	+	−	−
	3	−	−	+	+	+	+	−
B. abortus	1	+[b]	+	−	+	+	−	−
	2	+[b]	+	−	−	+	−	−
	3	+[b]	+	+	+	+	−	−
	4	+[b]	+	−	+[c]	−	+	−
	5	−	−	+	+	−	+	−
	6	−	−	+	+	+	−	−
	9	+ or −	+	+	+	−	+	−
B. suis	1	−	+	+	−[d]	+	−	−
	2	−	−	+	−	+	−	−
	3	−	−	+	+	+	−	−
	4	−	−	+	−[e]	+	+	−
	5	−	−	−	−	−	+	−
B. neotomae	−	−	+	−[f]	−	+	−	−
B. ovis	−	+	−	+	−[e]	−	−	+
B. canis	−	−	−	+	−[f]	−	−	+
B. ceti	−	−	−	+	+	+	−[e]	−
B. pinnipedialis	−	+	−	+	+	+	−[e]	−
B. microti	−	−	−	+	+	−	+	−

[a] Dye concentration in serum dextrose medium: $20\,\mu g\,ml^{-1}$.
[b] Usually positive on primary isolation.
[c] Some basic fuchsin-sensitive strains have been isolated.
[d] Some basic fuchsin-resistant strains have been isolated.
[e] Negative for most strains.
[f] Growth at a concentration of $10\,\mu g\,ml^{-1}$ thionin.

in agglutination tests with unabsorbed polyclonal antisera. Cross-reactions between nonsmooth strains can be demonstrated as well with unabsorbed anti-R sera. Lipopolysaccharide (LPS) comprises the major surface antigens of the corresponding colonial phase involved in agglutination. The S-LPS molecules carry the A and M antigens, which show different quantitative distribution among the smooth *Brucella* spp. strains (**Table 2**).

Serological cross-reactions have been reported between smooth brucellae and various other Gram-negative bacteria, and especially *Yersinia enterocolitica* O:9, which can induce significant levels of antibody cross-reacting with S-LPS *Brucella* spp. antigens in diagnostic tests.

Susceptibility to Phages

Over 40 phages have been reported to be specifically lytic for *Brucella* spp. Thus, lysis by specific phages is a useful test to confirm the identity of *Brucella* spp. and for speciation within the genus. The phages mainly used for *Brucella* spp. typing are Tbilisi (Tb), Weybridge (Wb), Izatnagar$_1$ (Iz$_1$), and R/C (**Table 1**).

Susceptibility to Dyes and Antibiotics

Susceptibility to the dyes thionin and basic fuchsin, which varies between biovars, is one of the routine typing tests of *Brucella* spp. (**Table 2**). On primary isolation, all brucellae are usually susceptible *in vitro* to gentamicin, rifampin, and tetracyclines. Most strains are also susceptible to ampicillin, chloramphenicol, cotrimoxazole, erythromycin, kanamycin, novobiocin, spectinomycin, and streptomycin, but variation in susceptibility may occur. *In vivo*, most strains are resistant at therapeutic concentrations to amphotericin B, bacitracin, β-lactamins, cephalosporins, clindamycin, cycloheximide, lincomycin, nalidixic acid, nystatin, polymyxin, and vancomycin.

Resistance and Survival

The ability of *Brucella* spp. to persist outside its mammalian host is relatively high as compared with most other non-spore-forming pathogenic bacteria, under suitable conditions. Thus, when conditions of pH, temperature, and light are favorable, that is, pH > 4, cool temperature, high humidity, and absence of direct sunlight, brucellae may retain infectivity for several months in aborted fetuses and fetal membranes, feces and liquid manure, water, wool, and hay, and on equipment and clothes. Brucellae are able to withstand drying particularly in the presence of extraneous organic material and will remain viable in dust and soil. Survival is prolonged at low temperatures, especially when freezing.

The persistence of brucellae in milk and dairy products is related to a variety of factors including the type and age of product, humidity level, temperature, changes in pH, moisture content, biological action of other bacteria present, and conditions of storage. The results of several studies are presented in **Table 3**. At low numbers in liquid media, brucellae are fairly heat-sensitive. Thus, dilute suspensions in milk are readily inactivated by pasteurization (high-temperature short-time or flash methods) or prolonged boiling (10 min).

Brucellae do not remain viable for prolonged periods in ripened fermented cheese. The optimal fermentation time to ensure safety is not known but is estimated at 3 months. However, in normally acidified soft cheese, the strictly lactic and short-time fermentation and drying increase the survival of *Brucella*. Pasteurization of milk or cream is the only means to ensure safety of these products.

Brucellae are fairly sensitive to ionizing radiation and are readily killed by normal sterilizing doses of gamma rays, under conditions that ensure complete exposure, especially in colostrum. In contrast to dairy products, the survival time of brucellae in meat is extremely short, due to acidic fermentation of the meat except in frozen carcasses where the organism can survive for many years. Therefore, meat consumption is less likely to be a source of infection. Direct contamination of abattoir workers and carcasses by milk and uterovaginal secretions is prevented by a proper and hygienic removal of mammary glands, reproductive organs, and lymph nodes, which are the most heavily contaminated organs.

Most commonly available disinfectants readily kill brucellae at normally recommended concentrations (phenol $10\,\mathrm{g\,l^{-1}}$, formaldehyde, xylene $1\,\mathrm{ml\,l^{-1}}$), except in the presence of organic matter or at low temperature, which drastically reduce their efficacy. Where possible, decontamination should be carried out by heat treatment, especially for surfaces. Diluted hypochlorite solutions, ethanol, iodophors, or isopropanol, and optimally substituted phenols, but not the alkyl quaternary ammonium, are effective for decontamination of exposed skin.

Animal Brucellosis

Brucellosis in Cattle

Brucella-infected cattle generally develop granulomatous inflammatory responses often located within lymphoid tissues and organs with a prominent reticuloendothelial component. There is a predilection for selected body sites

Table 3 Studies on *Brucella* survival time in dairy products

Product	*Species of Brucella*	*Survival time*	*Temperature (°C)*	*pH*
Milk	*B. abortus*	5–15 s	71.7	–
	B. abortus	<9 h	38	4.00
	B. abortus	24 h	25–37	–
	B. abortus	18 months	0	–
Fermented milk	*Brucella* spp.	>10 days	4	<4
Cream	*B. abortus*	6 weeks	4	–
	B. melitensis	4 weeks	4	–
Ice cream	*B. abortus*	30 days	0	–
Butter	*B. abortus*	142 days	8	–
Cheese				
Various	*B. abortus*	6–57 days	–	–
Various	*B. melitensis*	15–100 days	–	–
Feta	*B. melitensis*	4–16 days	–	–
Pecorino	*B. melitensis*	<90 days	–	–
Roquefort	*B. abortus and B. melitensis*	20–60 days	–	–
Camembert	*B. abortus*	<21 days	–	–
Erythrean	*B. melitensis*	44 days	–	–
Cheddar	*B. abortus*	6 months	–	–
White	*B. melitensis*	1–8 weeks	–	–
Whey	*B. abortus*	<4 days	17–24	4.3–5.9
	B. abortus	>6 days	5	5.4–5.9

From Davies G and Casey A (1973) The survival of *Brucella abortus* in milk and milk products. *British Veterinary Journal* 129: 345–353; Zúñiga Estrada A, MotadelaGarza L, Sánchez Mendoza M, Santos López EM, Filardo Kerstupp S, and López Merino A (2005) Survival of *Brucella abortus* in milk fermented with a yoghurt starter culture. *Revista Latinoamericana de Microbiología* 47: 88–91; Garin-Bastuji B and Verger JM (1994) *Brucella abortus* and *melitensis*. In: Hahn G (ed.) *The Significance of Pathogenic Microorganisms in Raw Milk*, pp. 167–185. Brussels: IDF.

such as reproductive organs, udder, and supramammary lymph nodes, and sometimes joints and synovial membranes. The localization and persistence of brucellae in these organs and tissues follow in the wake of a widespread distribution of *Brucella* during a generalized stage of infection. During this first stage of infection, the major clinical symptom is abortion but other signs due to a localization of brucellae may be observed (e.g., orchitis, epididymitis, hygroma, arthritis, metritis, subclinical mastitis). However, numerous animals develop self-limiting infections or they become asymptomatic latent carriers and potential excretors.

The second stage is characterized by the elimination of brucellae or, more frequently, by a persistent infection of the mammary glands and supramammary and genital lymph nodes, with a constant or intermittent shedding of the organisms in the milk and genital secretions. Animals generally abort once, from 5 to 8 months of gestation, but reinvasion of the uterus occurs in subsequent pregnancies through shedding of the microorganism in fluids and membranes. The pregnancy can also be full-term. Vaginal discharges after abortion or normal calving are the main source of contamination of congeners, other animal species, and man. The interherd spread of infection generally follows the movement or gathering of infected animals.

Persistent infection of mammary glands is associated with constant or intermittent shedding of the organisms in the milk in succeeding lactation periods and a drop in milk production estimated at 10%. The number of brucellae excreted in milk is relatively low and does not allow transmission through direct contact, except through the milker's hands.

In the male, localization in the reproductive organs generally results in brucellae being shed in the semen.

Congenital infection is of major epidemiological significance, since 2–20% of heifer calves born to infected cows may be persistently infected. Other calves fed with infected milk usually become infected, but most recover from these infections.

Brucellosis in Small Ruminants (Specific Features)

Brucella melitensis is the main causative agent of brucellosis in sheep and goats, but sporadic cases due to *B. abortus* have been observed. Pathologically and epidemiologically, *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle. The excretion from the vagina is more copious and prolonged than in the case of cows and last in goats for at least 2–3 months. In goats, about two-thirds of acute infections acquired naturally during pregnancy lead to infection of the udder and excretion of the organisms in the milk during the next lactation. Excretion may cease during a lactation period. Infection reduces milk production more drastically than in cattle.

Brucellosis in Other Species

In other milk-producing domestic ruminants (buffaloes, camels, reindeer, yaks), the risks associated with the shedding of brucellae in milk are comparable with those in cattle, sheep, and goats.

Human Brucellosis

Humans are accidental and almost always dead-end hosts of *Brucella* infections. The disease is primarily an occupational hazard in professionals who work with animals and their products, namely, veterinarians, farmers, laboratory technicians, abattoir workers, and others. The primary route of infection is through either direct or indirect contact on skin or mucous membranes. Another source is the ingestion of contaminated fresh dairy products. Humans are susceptible mainly to *B. abortus*, *B. melitensis*, and *B. suis*. *Brucella melitensis* and *B. suis* often give rise to the most severe form of infection. After an average 8- to 20-day (up to several months) incubation period, illness occurs in different forms. The asymptomatic form is frequent and mainly due to *B. abortus*, and is characterized by serologic evidence in persons with no symptoms consistent with brucellosis. The acute form is also common and symptoms include lassitude, headache, and muscular or joint pain, and drenching sweats, especially at night, are characteristic. The manifestations of brucellosis are sometimes most pronounced in or limited to a specific system or organ. Complication occurs in the course of acute infection, and localized brucellosis occurs in the absence of other signs of systemic illness (spondylitis and peripheral arthritis, especially of the hip, knee, and shoulder, epididymo-orchitis and thrombophlebitis). Nervous, genitourinary, hepatosplenic, and cardiovascular complications may be observed as well. Chronic brucellosis includes one or more of the signs described above and persists or recurs over a period of 6 months or more. Finally, *Brucella* dermatitis has traditionally been ascribed to allergy to brucellae. Brucellosis may be diagnosed on medical history but definitive diagnosis needs bacteriological and serological tests. Bacteriological studies consist essentially in blood cultures. However, cultural examinations are time-consuming, hazardous, and not sensitive, and cultural analysis must be performed in well-equipped laboratories with highly skilled personnel and are generally limited to hospital laboratories. Thus, diagnosis is frequently based on the detection of high or rising titers in serological tests such as serum agglutination test and the Rose Bengal test (RBT) as screening tests, and

Coombs' or complement fixation tests, or ELISA for confirmation.

Treatment of choice in acute brucellosis consists of antibiotic therapy. The best results are now achieved with rifampin (600–900 mg day^{-1}) combined with doxycycline (200 mg day^{-1}) given orally for at least 6–7 weeks. Treatment generally needs to be prolonged or repeated in persistent forms before a cure is achieved.

Diagnosis of Animal Brucellosis

In the absence of pathognomonic signs, the specific diagnosis of brucellosis can only be made on the basis of laboratory testing, especially in domestic animals.

Bacteriological Methods

There is no single test by which a bacterium can be identified as *Brucella* spp. A combination of growth characteristics, and serological and bacteriological methods is usually required.

Staining methods

Stamp's modification of the Ziehl–Neelsen method is the usual procedure for the examination of smears of organs or biological fluids. However, this method shows a low sensitivity on milk and dairy products where brucellae are often present at low numbers and interpretation is frequently impeded by the presence of fat globules. Furthermore, staining methods are not specific, and other organisms causing abortion, for example, *Chlamydophila abortus* (formerly *Chlamydia psittaci*) or *Coxiella burnetii*, are very difficult to differentiate from *Brucella* spp. organisms. The results, whether positive or negative, should be confirmed by culture.

Culture

Basal media

Direct isolation and culture of *Brucella* spp. are usually performed on solid media that enable the developing colonies to be isolated and recognized clearly, and limit the establishment of nonsmooth mutants and overgrowth of contaminants. However, the use of liquid media may be recommended for specimens where brucellae may be in small numbers. A wide range of commercial dehydrated basal media is available, for example, *Brucella* medium base, Tripcase or Trypticase soy agar, and Bacto™ tryptose. Addition of 2–5% bovine or equine serum is necessary for the growth of strains like *B. abortus* biovar 2, and many laboratories systematically add serum to the basal media, with excellent results. Other media such as serum dextrose agar or glycerol dextrose agar can be used satisfactorily. A nonselective, biphasic medium, known as the Castañeda medium, is recommended for the isolation of *Brucella* spp. from blood and other body fluids or milk, where enrichment culture is usually advised.

Selective media

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added in order to suppress growth of organisms other than *Brucella* spp. The selective medium most widely used is Farrell's medium, which is prepared by the addition of six antibiotics to a basal medium. A freeze-dried antibiotic supplement is available commercially. A selective biphasic medium made of the basal Castañeda medium with the addition of antibiotics to the liquid phase is sometimes recommended for isolation of *Brucella* spp. in milk.

These media allow the isolation of most strains of *Brucella* spp.; however, some strains of *B. melitensis* may be partially inhibited by bacitracin, included in the supplement. Sensitivity increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin's medium.

Collection and culture of specimens

Brucellosis is one of the most easily acquired laboratory infections; hence, safety precautions for sampling, and shipping, handling, and processing of the samples are extremely important, and work should only be carried out under level 3 containment (biosafety) conditions and by personnel adequately trained and made aware of the risks.

Milk

Samples of milk have to be collected aseptically after washing and drying of the whole udder and disinfection of the teats. It is essential that the samples contain milk from all quarters, and 10–20 ml of milk should be taken from each teat, avoiding contact of milk with the milker's hands. The first few streams are discarded and the sample is directly milked into a sterile vessel. Milk specimens should be cooled immediately after they are taken and sent to the laboratory by the most rapid route. If they are to spend more than 12 h in transit, they should be treated with boric acid (0.1%), or preferably frozen. On arrival at the laboratory, samples are frozen if they are not to be cultured immediately. Then, milk is centrifuged at 5–700 g for 15 min, and cream and deposits are spread on solid selective medium, separately or mixed. Brucellae are usually present in low numbers in bulk tank samples, and isolation from such specimens is very unlikely.

Dairy products

These materials are likely to contain small numbers of organisms, and enrichment culture is advised. Sampling methods are those classically recommended for bacteriological examination of dairy products and adapted to each sort of product. Specimens need to be carefully homogenized before culture, after they have been ground in a tissue grinder or macerated and pounded in a stomacher or an electric blender, with an appropriate volume of sterile phosphate-buffered saline. The superficial strata (rind and the underlying parts) and core of the product should be cultured. Brucellae grow, survive, or disappear more or less rapidly according to the local physicochemical conditions linked to specific process technologies, and their distribution among the different parts of the product varies. A previous inoculation into guinea pigs or mice may sometimes provide the only means of detecting the presence of *Brucella* spp., especially when the specimens are heavily contaminated or likely to contain a low number of brucellae. Spleen is then cultured and, if possible, a serum sample is subjected to specific tests.

Other specimens

The most valuable other specimens include aborted fetuses (stomach contents, spleen, and lung), fetal membranes, vaginal secretions, semen, and arthritis or hygroma fluids. On animal carcasses, the tissues preferred for culture are those of the reticuloendothelial system (i.e., head, mammary and genital lymph nodes, and spleen), the pregnant or early postparturient uterus, and the udder.

Identification and typing

Species identification is routinely based on lysis by phages and on simple biochemical tests (oxidase, urease, etc.). For *B. melitensis*, *B. abortus*, and *B. suis*, the identification at the biovar level is currently performed by four main tests: carbon dioxide requirement, production of hydrogen sulfide, dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M antisera.

The polymerase chain reaction (PCR), including the real-time format, based on selected sequences of the *Brucella* spp. genome, provides an additional means of *Brucella* detection and identification, which is unaffected by the colonial phase. A number of other methods including a multilocus sequencing scheme and several typing schemes based on the use of multiple locus VNTR analysis (MLVA) have recently been described, which can add useful epidemiological information allowing isolates to be differentiated to the species level or to be further subdivided at the subspecies level.

Serological Diagnosis

Diagnosis of *Brucella* spp. infection often has to be based on serological methods, in situations where bacteriological examination is not practicable. In routine veterinary tests, anti-*Brucella* antibodies are detected in serum and milk. The most widely used and recommended serum-testing procedures are (1) buffered *Brucella* antigen tests (BBAT), that is, card test and the RBT, or buffered plate agglutination test (BPAT), (2) complement fixation test (CFT), and (3) indirect ELISA tests. The milk ring test (MRT) or indirect ELISA performed on bulk tank samples have great usefulness for locating infected herds or flocks (the MRT is not usable in small ruminants). These tests are also of great interest to identify infected animals. The World Health Organization, the World Organization for Animal Health (OIE), the US Department of Agriculture, and the European Union have adopted specific recommendations for standardization of performance of the tests and interpretation of the results for all the different methods mentioned above. In small ruminants RBT and CFT are the most effective and the most widely used methods.

Allergic Tests

Delayed-type hypersensitivity reactions associated with cell-mediated immunity may be induced by either infection or immunization with living or adjuvant killed vaccines. Thus, a number of skin tests have been developed. Antigens free of S-LPS, such as Brucellin-INRA, should be preferred to crude preparations that interfere with serological diagnosis. Reactions are thus specific to the genus *Brucella*. Allergic skin test is now more and more widely used for nonvaccinated cattle, sheep, and goat herd surveillance, notably as a complementary test.

Control

Control, eradication, and prevention of brucellosis require the implementation of regional programs based on vaccination and/or test and slaughter of infected animals, and general nonspecific management practices and hygienic measures that reduce exposure potential. These measures would not be effective without health education, training, and mobilization of livestock owners and others engaged in animal production, and if animal identity is not well recorded and stock movements are not well controlled.

General Measures

General nonspecific control measures help to reduce the spread of infection. Field personnel should be aware of simple safety measures to prevent human contamination and passive intra- and interherd transmission. Isolation of females at parturition, and incineration or deep burying of nonliving products and fetal membranes are essential to limit the spread of infection. Contaminated materials and premises should be disinfected by heat treatment or by the use of the chemicals previously mentioned. All personnel handling contaminated material should wear disinfected or single-use protective clothing. Body surfaces that have been accidentally exposed to infection should be systematically washed and then decontaminated. Abattoir workers should take similar precautions, especially when handling udder and uterus, which should be systematically destroyed when infection is suspected.

In the laboratory, *Brucella* spp. present a very serious risk to workers handling heavily infected materials and cultures. Even when processing milk or dairy products risk exists but is lower. However, special safety precautions are not required for personnel engaged in routine serological diagnosis.

All personnel regularly exposed to infection should be kept under close clinical and serological surveillance. Currently no vaccine is efficient or safe enough to be recommended.

In infected areas, trade in fresh milk and dairy products should be strictly controlled and limited to officially declared brucellosis-free farms. The milk produced on infected farms should be heat treated whatever its commercial purpose.

Eradication by Test and Slaughter

Considering the low efficacy and the cost of antimicrobial chemotherapy in farm animals, test and slaughter of sero-positive animals is one of the two major forms of control and prevention of brucellosis. Such a strategy of eradication is justified on economic grounds when the prevalence rate of infected herds is 1% or below. The epidemiological surveillance of brucellosis-free herds is generally based on regular control by the use of bulk MRT (in cattle only) and/or individual serological testing. All susceptible animals should be permanently identified and movements of animals closely controlled. Eradication programs usually require an abortion notification and investigation scheme as well to detect infection. When positive results or abortions occur, safety measures should be undertaken and reactors or aborted females slaughtered. In some circumstances, for example, in free areas or in heavily infected herds, slaughter of the whole herd is advisable. Herd replacement should not subsequently occur and contaminated premises or pastures should not be used for animal housing or grazing, for 2–3 months.

Immunization

In high-prevalence areas or where the herds are large, or in extensive pastoral areas, it may be impossible to conduct the test-and-slaughter regime outlined above. Therefore, mass immunization is the only way to reduce the rate of infection. At present, the most widely used vaccines are the live attenuated vaccines S19 in cattle and Rev.1 in small ruminants. These vaccines have proved to be effective in reducing the number of abortions and also limiting the spread of infection. The RB51 vaccine, usable only in cattle, has become the official vaccine for the prevention of brucellosis in cattle in some countries. However, its efficacy as compared to the reference S19 vaccine remains controversial.

Vaccination cannot be expected to eradicate the disease from a herd. Furthermore, when used in adult animals, these vaccines induce long-term serological reactions and sometimes abortions. To reduce these reactions, immunization is generally restricted to young animals between the ages of 3 and 6 months and the conjunctival route is preferred to subcutaneous delivery. When the epidemiological situation improves, a combined scheme including immunization of young animals and test and slaughter of infected adults may be applied. Then, when the prevalence rate of infected herds becomes sufficiently low, test and slaughter as outlined above may be applied.

See also: **Diseases of Dairy Animals**: Infectious Diseases: Brucellosis. **Husbandry of Dairy Animals**: Sheep: Health Management. **Office of International Epizooties**: Mission, Organization and Animal Health Code.

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Campylobacter spp.

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Introduction to *Campylobacter* and Relevance to Milk

Introduction and History

Campylobacter spp. have been associated with disease in animals for many years. More recently, their role in human disease was established when they were isolated from stool samples of individuals with diarrhea using a direct technique developed by Skirrow in 1977. Thermotolerant species, in particular *Campylobacter jejuni* and to a lesser extent *Campylobacter coli*, have since emerged as major zoonotic human enteropathogens in most developed countries, being more frequently recovered from patients than salmonellae. These are predominantly transmitted to humans via the handling or consumption of contaminated foods of animal origin (particularly poultry meat and raw milk) or untreated surface water. Reported incidences of human campylobacteriosis tend to be single cases or small clusters, with larger outbreaks of disease seldom observed. The infective dose for *Campylobacter* is thought to be low, and clinical disease following infection varies from a mild self-limiting enterocolitis lasting 24 h to severe illness lasting in excess of 10 days. Sequelae to *Campylobacter* infection can manifest as meningitis, pneumonia, miscarriage, and a severe form of Guillain–Barré syndrome (GBS).

The epidemiology of *Campylobacter* spp. in livestock and wildlife, and contamination of foods of animal origin at harvest and postharvest phases of the food chain is highly complex with many data gaps remaining despite considerable research efforts made internationally over the last 30 years. Also due to the fastidious nature of these organisms, it is likely that much progress remains to be made in relation to optimal isolation and identification. Furthermore, with advances in molecular subtyping of zoonotic pathogens, it has become evident that considerable genotypic diversity in *C. jejuni* populations circulating in animals and foods of animal origin exists, making progress in understanding their epidemiology difficult.

It is likely that these organisms were first observed in 1886 by Theodor Escherich as nonculturable spiral-shaped bacteria. In 1913 McFadyean and Stockman isolated *Vibrio*-like organisms from aborted ovine fetuses, while in 1918 Smith observed spiral bacteria in aborted bovine fetuses and suggested that they belonged to the same *Vibrio* species as those recovered by McFadyean and Stockman 5 years earlier and proposed the name *Vibrio fetus*. In 1931, Jones and coworkers discovered a new *Vibrio* species in calves with dysentery and subsequently named the organism *Vibrio jejuni*. Another *Vibrio* species was isolated from pigs with diarrhea in 1944 by Doyle and was named as *Vibrio coli*.

In 1963, Sebald and Véron created a new genus, *Campylobacter*, and transferred *V. fetus* and *V. bubulus* to this new genus based on their DNA base composition, microaerophilic growth requirements, and nonfermenting metabolism. Subsequently in 1973, Véron and Chatelain published a taxonomic study of microaerophilic *Vibrio*-like organisms and suggested four species within the genus *Campylobacter*: *C. fetus*, *C. coli*, *C. jejuni*, and *C. sputorum*.

As the intestinal tracts of livestock are frequently colonized by *C. jejuni* and *C. coli*, including bovines, milk may be exposed to fecal contamination and hence to these enteropathogens during harvest. As a consequence, there are significant risks to public health associated with the consumption of contaminated milk, and the implementation of management interventions is required at both harvest and postharvest phases of the food chain.

Detection of *Campylobacter* in Milk

Growth Conditions

Campylobacter species are Gram-negative, curved, rod-shaped bacteria. They are motile by a single flagellum attached to either or both poles. They can become coccoid following prolonged culture or exposure to oxygen.

Campylobacter species have an optimal growth temperature range of 37–42 °C. They require microaerophilic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen). The optimum pH for growth is 6.5–7.5, and these organisms do not grow below pH 4.9. Survival at acid pH values is temperature dependent, but inactivation is rapid at pH values less than 4.0, especially above refrigeration temperatures.

Thermal Resistance

Campylobacter species are heat sensitive and the cells are destroyed at temperatures above 48 °C. *Z*-values for *C. jejuni* are in the range of 5.07–8.02 °C. A *Z*-value of 6.10 °C has been reported for *C. coli*. Continuous change in morphology from the spiral to coccoid form has been observed during heating. *Campylobacter* species are destroyed by commercial pasteurization treatments.

Susceptibility to Antibiotics

Campylobacter jejuni are generally susceptible to erythromycin and gentamicin. Resistance of *C. jejuni* and *C. coli* to other antibiotics varies between strains and has been reported at low rates for clindamycin, azithromycin, and meropenem. Resistance to ciprofloxacin, nalidixic acid, and particularly tetracycline is on the increase. *Campylobacter upsaliensis* and *C. fetus* have been reported as being resistant to nalidixic acid along with many strains of *C. jejuni* and *C. coli.* The use of antibiotic susceptibility for differentiating between strains is less reliable due to the increasing frequency and variability of resistance among *Campylobacter* species.

Biochemical Properties

Biochemical tests can further confirm and differentiate between different *Campylobacter* species; however, some biochemical properties are dependent on the age of culture and test conditions applied. Rapid catalase and cytochrome oxidase tests suggest presence of *C. jejuni*, *C. coli*, and *C. lari.* Only the two subspecies of *C. jejuni* possess the enzyme hippuricase (hippurate hydrolase) and can hydrolyze sodium hippurate to benzoic acid and glycine. *Campylobacter coli* and *C. jejuni* can also be differentiated based on polymorphism in a number of gene sequences including *ceuE*, the GTPase gene, the 16S rDNA gene, and the 23S rDNA gene. *Campylobacter* spp. do not utilize carbohydrates.

Serology

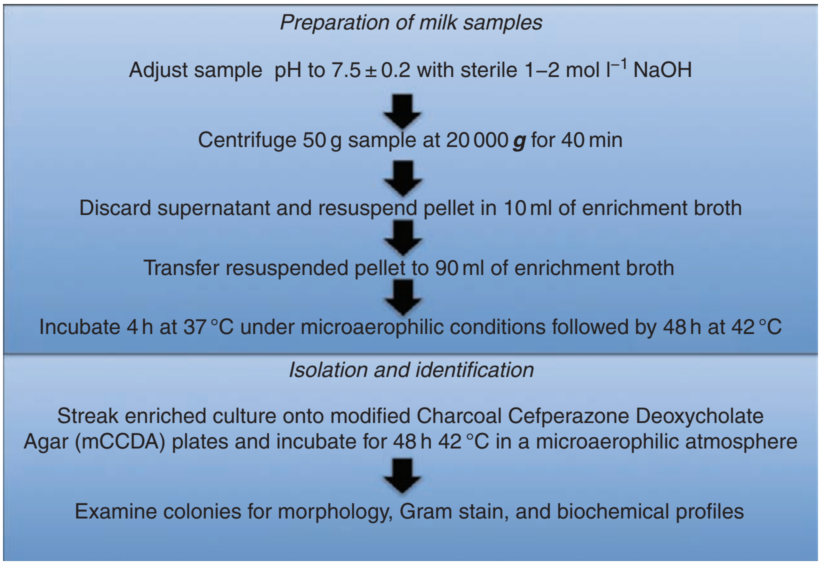
Penner (1980) and Lior (1982) described two methods of serotyping for *Campylobacter.* The Penner serotyping scheme involves detection of the heat-stable *O*-polysaccharide antigen by an indirect hemagglutination assay. The Lior serotyping scheme involves detection of the heat-labile flagellar and capsular antigens by a slide agglutination assay.

Campylobacter serology is poorly standardized and various crude bacterial antigen preparations have been used in the detection of *C. jejuni*-specific antibodies in patients suffering with GBS. The *Campylobacter* flagellar protein is the dominant immunogen recognized during *C. jejuni* infection in humans. Complement fixation assays (CFAs), immunoblotting, and enzyme-linked immunosorbent assays (ELISAs) have all been used for detecting these antibodies. Antigenic cross-reactivities, however, can result in low specificity of assays.

Isolation and Identification

The United States Food and Drug Administration (US-FDA)-recommended procedure for isolating *Campylobacter* from milk is extensively used and is outlined in **Figure 1**.

This particular method proposes the Bolton formulation as the enrichment broth, but others including Preston broth, Brucella broth, and Campy pack broth can also be used. Some procedures recommend that the initial



Preparation of milk samples

Adjust sample pH to 7.5 ± 0.2 with sterile 1–2 mol l^{-1} NaOH

Centrifuge 50 g sample at 20 000 ***g*** for 40 min

Discard supernatant and resuspend pellet in 10 ml of enrichment broth

Transfer resuspended pellet to 90 ml of enrichment broth

Incubate 4 h at 37 °C under microaerophilic conditions followed by 48 h at 42 °C

Isolation and identification

Streak enriched culture onto modified Charcoal Cefperazone Deoxycholate Agar (mCCDA) plates and incubate for 48 h 42 °C in a microaerophilic atmosphere

Examine colonies for morphology, Gram stain, and biochemical profiles

Figure 1 Procedure for isolation and identification of *Campylobacter* from milk. From US-FDA (2010) Bacteriological Analytical Manual. http://www.fda.gov (accessed 22 April).

enrichment be carried out at 37 °C for 4–6 h in the absence of selective supplements, in order to allow damaged cells to recover. Following incubation of selective plates, *Campylobacter* species appear as round- to irregular-shaped colonies with smooth edges. They can show thick translucent white growth to spreading, film-like transparent growth. Colonies are confirmed by Gram stain, catalase, oxidase, and other biochemical tests. This procedure can be lengthy, requiring 4–5 days to complete. Nucleic acid methods can be used and these are giving a more reliable and conclusive result. Such methods have been applied for the identification and quantification of *Campylobacter* in a wide range of food samples, including milk. They can be used to identify *Campylobacter* at the species level, which is often difficult and unreliable by traditional methods. In addition, samples with very low numbers of viable, but nonculturable cells may go undetected on agar media but may be detected by molecular methods.

Real-time PCR assays can be performed on enriched samples in as little as an hour with a detection limit of 1 cfu per PCR. An immunocapture PCR method that uses an avidin capture assay to detect PCR products also exists. This quantitative method does not require an enrichment step, requires 8 h to complete, and has a detection limit of 1 cfu ml^{-1}.

A rapid hybridization assay using a fluorescently labeled specific DNA probe can be applied for detecting *C. jejuni* at very low levels (3–10^5 cfu per PCR) in milk and poultry rinse samples. This method involves filtering samples through 0.22-μm hydrophobic grid membrane filters (HGMFs) and labeling with a 1475-bp chromagen-labeled DNA probe (pDT1720). Hybridized cells are subsequently detected in a colorimetric immunoassay. *Campylobacter jejuni* in food and clinical samples can also be detected by an immunomagnetic-hybridization technique. This method involves directing a monoclonal antibody against a specific outer membrane protein of *C. jejuni*. Captured *C. jejuni* cells are subjected to lysis by ultrasonication and the genomic DNA is reacted with a microtiter plate-immobilized RNA probe. Detection of the RNA–DNA hybrids formed in wells is then carried out using a monoclonal anti-RNA–DNA hybrid antibody. Overall, molecular-based methods are more specific, reliable, and faster at identifying *Campylobacter* at the species level compared with conventional identification methods.

Molecular Characterization of *Campylobacter*

Detection

Although food laboratories generally use conventional culture methods for the detection and identification of *Campylobacter*, more rapid alternative methods are available, including molecular methods. Alternative methods can be used as a screening tool to quickly assay a large number of samples and eliminate negatives at an early stage. Molecular methods can also be used to confirm the identity of a particular *Campylobacter* strain.

In addition to commercially available kits/assays, a number of publications report on the use of traditional PCR or, more recently, real-time PCR to screen for *Campylobacter*. A useful feature of nucleotide-based assays is the ability to speciate the strain.

Subtyping Approaches

Epidemiological investigations often benefit from rapid, reliable subtyping techniques. Several methods have been used to subtype *Campylobacter*. Subtyping methods based on analysis of the flagellin A gene include *fla*A restriction fragment length polymorphism (*fla*A-RFLP) and sequencing of a 321 bp short variable region (SVR). RFLP has sufficient discriminatory power to be considered a valuable epidemiological tool. However, DNA sequence based methods have advantages over RFLP. The *fla*ASVR typing protocol is useful for discriminating between even closely related *Campylobacter* strains and is widely employed because of its speed and simplicity.

Multilocus sequence typing (MLST) is a nucleotide sequencing technique that characterizes *Campylobacter* spp. based on the sequence heterogeneity present in seven housekeeping genes. These genes evolve slowly due to their critical role in central metabolism and are therefore particularly useful for the long-term analysis of diverse bacterial populations with weak clonal population structures. Sequence data determine the allelic profiles or sequence types (STs), which are then grouped into clonal complexes (CCs). MLST has confirmed the genetic diversity of *C. jejuni* and shown its population structure to be weakly clonal. MLST is a highly discriminatory typing system and is the current ‘gold standard’ for molecular typing of *Campylobacter*.

Whole-genome microarray analysis is a robust and sensitive method to determine genetic relatedness of bacterial populations. A whole-genome DNA microarray containing all 1654 genes from *C. jejuni* NCTC 11168 was constructed in 2001. Comparisons with 11 different strains identified 1300 core genes common to all, and 354 genes (21%) that were absent or highly divergent in at least 1 of the 11 samples. Core genes appear to encode proteins with a housekeeping function, while many of the remaining 21% encode surface-located structures including flagella, lipooligosaccharide, and the capsule. Whole-genome analysis offers the potential to identify genes encoding pathogenic factors and could prove useful as an epidemiological and/or diagnostic tool.

Antibiotic Resistance

Epidemiology of Resistance

Treatment of campylobacteriosis with antibiotics may be required in cases of immunocompromised patients or those showing no signs of improvement once the diagnosis has been made. Macrolides and fluoroquinolones are the antimicrobial agents currently chosen when therapeutic intervention is required. Resistance to erythromycin is low in most countries, and in general it remains the drug of choice. Fluoroquinolones such as ciprofloxacin may also be used, but resistance can be a problem in some regions. The frequency of fluoroquinolone resistance has been reported to be as high as 90% in Spain, Thailand, and Taiwan. Although lower rates of up to 45% have been reported elsewhere, current trends show that resistance is increasing. Antibiotic use in both animal production and human medicine can influence the development of antibiotic-resistant *Campylobacter*. Therefore, the emergence of antimicrobial resistance in enteric *Campylobacter* spp. due to the use of antimicrobial agents in husbandry is a matter of concern.

Resistance to Fluoroquinolones and Macrolides

Fluoroquinolones are a subgroup of quinolones, derived from nalidixic acid, and have a fluorine at the C-6 position of the quinolone structure. The targets of these agents are the type II topoisomerase DNA gyrase and topoisomerase IV. These enzymes are essential for DNA replication, recombination, and transcription. Inhibition appears to occur by interaction of the drug with complexes composed of DNA and either of these two target enzymes.

Resistance to fluoroquinolones is mainly due to chromosomal mutations in the *gyrA* and *gyrB* genes encoding the subunits of DNA gyrase, in genes that affect the expression of porin channels in the outer membrane, and in the genes encoding multidrug resistance efflux systems. Efflux was first postulated as a mechanism of multidrug resistance in *Campylobacter* in 1995. In 2002, a chromosomally encoded multidrug resistance–nodulation–cell division (RND) efflux system CmeABC was identified and characterized in *C. jejuni*, and later in *C. coli*.

Erythromycin is a protein synthesis inhibitor that binds to the ribosome causing dissociation of the peptidyl-tRNA, rather than blocking the peptidyltransferase activity as in the case of larger macrolides. In *C. jejuni* and *C. coli*, erythromycin resistance is chromosomally mediated and can be due to an alteration of the 23S rRNA gene, modification of the antibiotic, or efflux. Sequencing of the 23S rRNA genes from erythromycin-resistant *Campylobacter* spp. identified mutations that are associated with high-level resistance.

Methods to Determine Resistance

A lack of any agreed standardized approach for the testing of antimicrobial resistance in *Campylobacter* led to the issue being addressed by the Clinical and Laboratory Standards Institute (CLSI). Subsequently, an agar dilution reference method for *Campylobacter*, establishing quality control (QC) ranges for five agents, was introduced. In 2006, a new approved broth microdilution method, with QC ranges for 13 agents, was developed.

The aim of broth and agar dilution methods is to determine the lowest concentration of the antimicrobial compound (minimal inhibitory concentration (MIC)) that, under defined test conditions, inhibits the visible growth of *Campylobacter*.

Agar dilution involves the incorporation of different concentrations of the antimicrobial substance into an agar medium followed by the application of a standardized number of cells to the surface of the agar plate. Plates are read by observing the lowest drug concentration that inhibits visible bacterial growth.

The broth microdilution test is the standard method for determining levels of resistance to an antibiotic. Bacteria are inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent. Growth is assessed after incubation for a defined period of time and the MIC value is determined. The lowest concentration (highest dilution) of antibiotic preventing appearance of turbidity is recorded as the MIC.

The epsilometer test (E-test) has been found to compare favorably with agar dilution and broth microdilution methods, although economic reasons can prevent its use in routine laboratory screening. The E-test consists of a plastic strip containing a predefined gradient of antimicrobial compound concentrations. In this method, the strip is applied directly onto the surface of an inoculated agar plate. The drug on the strip then diffuses into the agar. Following incubation, an elliptical zone of inhibition is visible around the test strip. The point at which the zone edge intersects the plastic strip at a specific drug concentration is taken as the MIC.

Campylobacter and Disease

Clinical Disease

Experimental *C. jejuni* infections in humans have revealed that a low infectious dose of approximately 500 cells is sufficient to induce illness. Approximately 30% of patients experience an influenza-like prodrome of fever, headache, and myalgias lasting up to 24 h. The actual incubation period after ingestion is typically 2–5 days, but can range from 1 to 10 days. Symptoms include profuse diarrhea (often bloody), fever, and acute abdominal pain that may mimic appendicitis. Vomiting is uncommon, but nausea, headache, backache, and aching of the limbs may be experienced. The peak of the illness usually lasts for 24–48 h, with the symptoms resolving themselves within a week. The vast majority of patients suffering from *Campylobacter* enteritis do not require chemotherapy. Fluid and electrolyte replacement should enable a full recovery. Stools remain positive for several weeks, and minor relapses can occur. Approximately 1 in 1000 cases of *C. jejuni* infection develops GBS, an acute, autoimmune, polyradiculoneuropathy affecting the peripheral nervous system.

Pathogenesis

Bacterial gastroenteritis results from a complex set of interactions between the host and the invading organism. Consequently, the mechanisms by which *Campylobacter* spp. cause disease are not well understood. Pathogenesis will depend on the susceptibility of the patient as well as the virulence of the infecting strain. The role of several virulence factors has been studied and the mechanisms of pathogenicity are becoming clearer. Factors such as motility, chemotaxis, colonization, adherence, invasion, translocation, and toxin production, including the holotoxin cytolethal distending toxin (Cdt), have been implicated in the pathogenesis of *Campylobacter* enteritis.

Epidemiology of Outbreaks Associated with Milk

The main reservoir of *Campylobacter* is the gastrointestinal tract of warm-blooded animals, with studies describing the isolation of *Campylobacter* spp. from a broad range of animals. While Campylobacter spp. are commonly recovered from food animals, they have also been isolated from other vectors such as insects, wild birds, marine mammals, and both wild and domestic animals. Furthermore, these pathogens are also routinely isolated from environmental sources in close proximity to livestock and poultry farms. These sources include rivers, streams, lakes, sewage, drinking water, livestock and poultry farms, as well as slaughter line environments.

Transmission of *Campylobacter* to humans can occur through direct contact with infected animals or fecal matter and indirectly by the consumption of contaminated food or water. The primary source of foodborne campylobacteriosis seems to be from raw poultry meat through cross-contamination of ready-to-eat foods during food preparation, with the consumption of undercooked poultry being an alternate but less common source of infection.

Unpasteurized milk is commonly implicated in *Campylobacter* outbreaks. Cow's milk typically contains bacterial loads in the range of 10^3–10^4 cfu ml^{-1}. These numbers may represent bacteria on the lower end of the teat, organisms that have colonized the teat canal, and those from fecal contamination on the outside of the teat. The use of good hygiene practices when milking can significantly reduce the prevalence and levels of bacterial contamination in milk; these practices may involve thoroughly washing and drying udders before milking. However, the principal risk factor associated with milkborne gastrointestinal illnesses such as campylobacteriosis is insufficient or no heat treatment, with the consumption of unpasteurized milk and milk products a well-documented source of *Campylobacter* infection. Cases of campylobacteriosis associated with pasteurized milk have generally been linked with postpasteurization contamination or a failure in the pasteurization processing step.

Prevalence data on *Campylobacter* from 21 different countries showed that 30% (mean positives) of dairy cows sampled were positive for *Campylobacter* spp., with prevalence ranging from 6 to 64%. The same study also reported 3.2% (mean positives) of raw milk samples positive for *Campylobacter* spp., with prevalence ranging from 0 to 9.2%.

There is little evidence to suggest that *Campylobacter* poses a significant threat in fermented dairy products such as cheese and yogurt. The low prevalence of these pathogens in such products is due to their poor survival in acidic conditions. Other cases of campylobacteriosis outbreaks from milk have come from milk contaminated by bird pecking through the packaging.

Control of Milk Quality at Pre- and Postharvest Phases

Milk is an important nutritional component for humans in many societies throughout the world. It is a natural secretion of the mammary gland and is not a sterile commodity in this state. Milk derived from animals can contain pathogens and historically has been identified as a vehicle responsible for transmitting a significant burden of disease to humans in both developing and developed countries. Recent analysis of human outbreak data and other estimates suggest that milk is responsible for a small but substantial proportion of foodborne infectious disease (~2–9%) in Ireland and the United Kingdom. Interestingly, *Salmonella*, *Campylobacter*, and enterohemorrhagic *Escherichia coli* were the most frequently implicated agents in milkborne disease. Differences in the burden of human disease attributed to milk consumption are likely to vary widely due to regional or cultural differences, jurisdictional/legislative restrictions on the marketing of raw milk, and differences in consumer preferences for drinking raw milk or dairy products. In countries where the marketing of liquid raw milk is prohibited, consumption by families on dairy farms is still prevalent even in developed regions.

The subclinical carriage and intermittent shedding of *C. jejuni* and *C. coli* in bovines and other animal species from which milk is derived is a public health concern. Raw milk is frequently found to contain these significant *Campylobacter* species and is primarily indicative of fecal contamination during milking. In addition, less frequent reports of subclinical infection of mammary glands have also been observed, which can result in preharvest contamination of milk. The milking of animals is not a sterile process and contamination of milk with organisms of fecal origin is common and to an extent unavoidable even with the implementation of high standards of hygiene. Milk may also be contaminated with fecal organisms, including *Campylobacter* spp., at any stage postharvest, for example during bulk storage on-farm, transportation, processing, or postprocessing.

Integrated control and intervention is required along the milk production chain to manage risks and adequately protect public health. At farm level, good farming and husbandry practices are essential so that feed quality is controlled, waste is effectively managed, mastitis is prevented and managed in herds, and adequate standards of housing and stocking are maintained. In addition, drinking water should be of a potable standard and drinking troughs should be designed to minimize fecal contamination. Fecal soiling of hides, tails, and udders should be minimized as much as possible by, for example, adequate provision of clean bedding and prevention of enteritis within the herd. During harvesting of milk from cows, visibly contaminated teats should be cleaned and dried prior to attaching clusters. Milking equipment should be well maintained and adequate cleaning and sanitizing should be carried out between milking sessions using recommended cleaning agents, procedures, and a potable water supply. All milk should be pumped through a milk filter or ‘sock’ to remove any visible traces of fecal material or other suspended particulates, and milk should be cooled and stored appropriately before transportation to the processing plant.

The main public health intervention for liquid milk in developed countries is pasteurization, which involves the rapid heating of milk using plate heat exchangers to a minimum of 71.7 °C and holding at that temperature for 15 s. Once satisfactorily heated, the milk is then rapidly cooled again using plate heat exchangers, thus largely preserving the desired organoleptic properties of raw milk. Individual processing operations can vary this time–temperature treatment to achieve an equivalent reduction in bacterial populations. Pasteurization is sufficient to eliminate bacterial pathogens, including *C. jejuni* and *C. coli*, in milk. In terms of food safety assurance in a commercial processing facility, it is imperative that the pasteurization conditions are met consistently by monitoring time–temperature recording equipment, for example, thermographs, and the completion of routine microbiological testing to detect fecal indicator organisms (Enterobacteriaceae, *E. coli*). In addition, levels of the phosphatase enzyme, which is naturally found in raw milk, can be readily measured in pasteurized samples. This enzyme is relatively heat labile and is denatured at pasteurization temperatures; therefore, detection of active phosphatase indicates incomplete pasteurization or post-pasteurization contamination with raw milk.

Several studies have highlighted the risks of inadequate pasteurization or postprocess contamination of liquid milk associated with the transmission of *Campylobacter* spp. to humans. For example, an outbreak in the United Kingdom in which 110 people became ill (41 were confirmed to be infected with *C. jejuni*) was associated with the consumption of inadequately pasteurized milk from a local dairy. Other studies have highlighted the risk of equipment failure in the dairy plant resulting in the mixing of raw with pasteurized milk. The phenomenon of wild birds pecking the foil caps on milk bottles delivered to consumer’s doorsteps resulting in contamination of milk with *Campylobacter* spp. and infection in humans have also been well reported, again highlighting the risks associated with postprocess contamination and human health.

Although documented, the transmission of *Campylobacter* spp. to humans via the consumption of dairy products is rare and is considered to be a minor route of exposure to humans. The survival of *C. jejuni* in cheeses (including soft varieties) and fermented products such as yogurt is poor, most likely a result of the organisms intolerance to high salt concentrations and low pH, respectively. In soft cheeses artificially inoculated with 7 $\log_{10}$ cfu ml^{-1}, a 1 $\log_{10}$ reduction can be achieved within 4 h, the reduction increases to 3 $\log_{10}$ following 24 h storage and the organism is not detected after 336 h storage. For fermented dairy products with a pH of 4.2, the inoculated cells (7 $\log_{10}$ cfu ml^{-1}) did not survive beyond 3 days storage. These data suggest that such products manufactured from raw milk would not pose any substantial risk to consumers once products were sufficiently processed (e.g., to ensure adequate salt concentrations or pH levels were achieved) and matured/aged before being marketed for consumption.

Finally, in order to adequately and consistently protect public health, it is imperative that an integrated approach is taken based on the principles of hazard analysis critical control point (HACCP) systems at all stages of milk production (preharvest, harvest, and postharvest). This approach should be adapted by all stakeholders, including producers, processors, retailers, and legislators, so that increased levels of food safety assurance are achieved for liquid milk and dairy products.

Future Issues

The complex epidemiology of campylobacteriosis is widely acknowledged and the extensive dissemination of *Campylobacter* spp. within livestock and the environment has made its control difficult. In addition, molecular subtyping studies, using techniques such as pulsed-field gel electrophoresis, MLST, and others, have revealed the highly diverse nature of strains of this zoonotic agent in circulation in these animal and environmental reservoirs. Ongoing research is required in a number of areas to ascertain how this pathogen is introduced to livestock and to identify effective interventions to reduce its prevalence and potential transfer to raw milk and dairy products.

Further work is also required in relation to food attribution studies so that more accurate and detailed estimates of numbers of human cases of campylobacteriosis caused by the consumption of contaminated milk can be completed. Such data on the associated public health burden would provide valuable information for health professionals, the dairy industry, regulatory agencies, and other risk managers. It would also facilitate an impetus to communicate such risks to consumers of raw milk and other dairy products made from unpasteurized milk.

The standardization and dissemination of laboratory techniques used to detect, quantify, and characterize *Campylobacter* spp. in animals, foods of animal origin, and humans would also be beneficial, enabling the generation of more accurate and reproducible prevalence data and risk assessments.

In recent years, the prevalence of antimicrobial resistance among *Campylobacter* spp. of animal and food origin has increased. The corresponding antibiotic resistance profile of *Campylobacter* spp. implicated in human disease is similar. Antimicrobial therapy may be required in a minority of human campylobacteriosis cases where the drugs of choice are often macrolides and fluoroquinolones. Therefore, increased and multidrug resistance now represents a significant health risk where drug therapy is necessary. Strict licensing and prudent use of antibiotics in animal production and treatment are recommended. This strategy must be supported by the development and application of standardized methods to monitor antimicrobial resistance in *Campylobacter* populations, allowing reliable surveillance data to be generated on an ongoing basis, review of resistance trends and making interventions as required. This is an important component of public health protection, given the more recent hypotheses, for example, that infection in humans by quinolone-resistant strains of *C. jejuni* tends to cause more prolonged and severe symptoms. In addition, the development and promotion of herd health management programs on dairy farms should be encouraged so that a preventive approach to disease is taken, thus reducing the dependence on antimicrobials.

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Clostridium spp.

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Introduction

The interest in *Clostridium* spp. in milk products mainly pertains to two aspects, sanitary and technological. Of utmost importance is the presence of pathogenic clostridia in milk products because of their potentially harmful effects on human health: although cases of food-borne diseases attributed to clostridia in dairy products are rare (<70 cases in the last 10 years), some have even recently been reported, including a few related to commercial foods, which makes the risk more threatening due to the wide distribution such products can reach on the market. However, most of the *Clostridium* species relevant to dairy products are nonpathogenic to humans, yet their multiplication can cause deterioration and/or serious defects in the final products with consequent economic losses.

Clostridia are widely distributed in soil, dust, water, sediments, sewage, animal carcasses, and vegetation; healthy animals and humans normally carry low numbers of spores in their intestines ($\sim$100 g^{-1} feces). Contamination of feeds and especially silage, where clostridia can grow under certain conditions of water activity (a_w), pH, and temperature, contributes to the spread of spores. Raw milk can become contaminated with spores during the milking process, as a result of environmental cross-contamination. *Clostridium sporogenes*, *Cl. perfringens*, *Cl. butyricum*, *Cl. tyrobutyricum*, and *Cl. beijerinckii* are the most frequently encountered species, along with other rare species such as *Cl. botulinum*. Usually, processing of raw milk either kills the spores of clostridia or inhibits their multiplication in the dairies; however, depending upon the conditions applied and the characteristics of the strains, some can occasionally survive and grow.

This article will focus on *Clostridium* spp. commonly found in milk-based products and the control measures to be taken to ensure the wholesomeness and integrity of such widely consumed foods.

Morphology and Physiology

Clostridium spp.

The genus *Clostridium* comprises a heterogeneous group of microorganisms. They are Gram-positive, anaerobic, rod-shaped, and endospore-forming bacteria: the shape and length of the rods, and the position of the spores may vary considerably. Spores are very resistant to extreme chemical/physical conditions and therefore are widely distributed in terrestrial environments where they can germinate when conditions become favorable, that is, in low oxygen tension areas and in the presence of sufficient nutrients. The growth temperatures vary from 3.3 to 80°C, with an optimum between 25 and 40 °C for most clostridia.

Historically, the taxonomic definition of species within the genus has relied on the phenotypic characteristics displayed by the bacteria, such as metabolic activities, cultural conditions, chemical composition of the bacterial cell wall, and toxicity and pathogenicity. Some distinctive phenotypic features of the *Clostridium* spp. relevant to dairy products are given in **Table 1**.

Subsequent studies of the microbial genetic material allowed a more precise classification of species. Although the overall DNA base composition is of limited value for determining the relatedness of groups – the G + C content of members of the genus ranging from 22 to 50% – more recent phylogenetic studies based on similarities of 16S rRNA/DNA sequences have confirmed many of the preexisting species, while excluding others. Moreover, *Clostridium* spp. are distributed into 19 different clusters (I–XIX), the first of which essentially includes the clostridia most frequently found in milk and dairy products, often referred to as *Clostridium sensu stricto.*

Pathogenesis of *Clostridium* spp. Contaminating Dairy Foods

The pathogenic *Clostridium* spp. that can potentially spoil milk products are those associated with botulism (*Cl. botulinum* and neurotoxigenic *Cl. butyricum*), enterocolitis (*Cl. perfringens*), and possibly sudden infant death syndrome (SIDS) (*Cl. perfringens*, *Cl. botulinum*) and neonatal necrotizing enterocolitis (NE) (*Cl. butyricum*). Additionally, *Clostridium* spp. must be regarded as potential producers of harmful biogenic amines in cheese products.

The highly potent neurotoxin (lethal dose for humans: 1 ng kg^{-1} body weight) produced by *Cl. botulinum* is responsible for the serious neuroparalytic disease of botulism.

Table 1 Some metabolic and physiological features of clostridia associated with milk products

	Clostridium sporogenes	*Clostridium perfringens*	*Clostridium butyricum*	*Clostridium tyrobutyricum*	*Clostridium beijerinckii*	*Clostridium botulinum (proteolytic)*	*Clostridium botulinum (nonproteolytic)*
Fermentation of							
Glucose	v[a]	+	+	+	+	V	+
Lactose	–	+	–	–	+	–	–
Sucrose	–	+	+	+	+	–	+
Mannose	–	+	+	+	+	–	+
Digestion of							
Milk	d[b]	c[c]	c	–	c	d	c
Meat	d	d	–	–	–	d	–
Range of growth temperature (°C)	25–45 °C	20–37 °C	10–37 °C	10–37 °C	25–45 °C	10–40 °C	3.3–37 °C
D_{100} (min)[d]		0.2	2.3–2.5			25	<0.1
Minimum a_W		0.98				0.93	0.97
Minimum pH	5.7	6	4.6	4.6	4.6	4.6	5.0

[a] v, variable.
[b] d, digested (proteins are totally metabolized, with consequent clarification of the medium).
[c] c, clotted (proteins are partially metabolized and tend to aggregate, causing formation of ‘clots’ in the medium).
[d] D_{100} (min) = time in minutes required to inactivate 90% of the specific clostridial population at 100 °C.

Although seven serologically distinct botulinum toxins (A–G) exist, all have common protein structures and mode of action, consisting of zinc endopeptidase activity against specific protein targets of the presynaptic vesicles that contain the neurotransmitter acetylcholine. Cleavage of these proteins blocks the neurotransmitter release at the nerve terminal ends and ultimately produces the flaccid paralysis of botulism. Generally, one strain of *Cl. botulinum* produces a single type of neurotoxin: type A, B, and E botulinum neurotoxins account for nearly all of human botulism. Two forms of botulism may result from ingestion of food contaminated with either preformed toxin (food-borne botulism) or neurotoxigenic clostridial spores, which subsequently may colonize the intestine of the host – generally infants under 1 year of age and adults suffering from gastrointestinal distress – and synthesize the toxin *in vivo* (intestinal toxemia botulism).

Enterotoxin-producing *Cl. perfringens* type A is responsible for a form of mild enteritis. Classical symptoms include diarrhea, abdominal cramps, and, less frequently, nausea, vomiting, and fever, which follow shortly after ingestion of food contaminated with at least 10^8 *Cl. perfringens* cells per gram. The ingested microorganisms that succeed in surviving the high acidity of the stomach sporulate within the small bowel and release the enterotoxin by cell lysis into the intestinal lumen. Here, it rapidly binds the intestinal epithelial cells, causing cytotoxic effects by alterations to membrane permeability.

In addition, a role for *Cl. perfringens* type A in SIDS has been proposed, because many SIDS infants have symptoms of gastrointestinal infections prior to death, and *Cl. perfringens* and its enterotoxin are found in a significantly high number of feces from such infants.

Clostridium butyricum has been hypothesized as a causative agent of neonatal NE, a disease mostly affecting premature infants even though neonates with no predisposing factors may also be affected. Mucosal necrosis of the ileum and colon is the main symptom: although the exact etiology of the disease is still unknown, different bacteria are thought to cause the intestinal lesions, including *Cl. butyricum.* This microorganism is normally considered a harmless saprophyte: typically, it does not produce any toxin but an *in vitro* cytotoxic effect due to the production of butyrate has been demonstrated on various cells. Besides, unique strains of *Cl. butyricum* producing botulinum toxin type E have been implicated in intestinal toxemia botulism and food-borne botulism in Italy and China. These peculiar strains seem to have acquired the genetic information coding for botulinum toxin type E through DNA mobile vectors from an ancestral strain at some point during evolution.

Although the actual roles of *Cl. perfringens* type A and *Cl. botulinum* in SIDS, and *Cl. butyricum* in neonatal NE, are still questionable, it has been recommended to maintain the number of spores of *Clostridium* low in products intended for use by infants.

Finally, different *Clostridium* spp. commonly found in cheese products are decarboxylase-positive microorganisms and may thus contribute to the decarboxylation of free amino acids to biogenic amines, such as histamine from histidine. High levels of these substances may exert toxic effects, including dilatation of peripheral blood vessels resulting in hypotension, flushing, and headache, and induction of contraction of intestinal smooth muscle, which may be the cause of vomiting and diarrhea.

Significance of *Clostridium* spp. in Dairy Foods

Incidence of Clostridia in Milk and Dairy Products

Raw milk usually contains large numbers of bacteria. Of these bacteria, clostridia represent a minority, the contamination levels being around $10–10^2$ spores ml^{-1}. These levels can increase to $>10^3$ spores ml^{-1} when lactating animals are fed on heavily contaminated silage. Percentages of the different *Clostridium* spp. are variable, but *Cl. sporogenes*, *Cl. perfringens*, *Cl. butyricum*, and *Cl. tyrobutyricum* generally predominate, accounting for about 75% of the whole anaerobic spore-forming flora; other species of importance, such as *Cl. botulinum*, may be present at levels of less than 1 spore l^{-1}. The redox potential of raw milk does not allow for growth of clostridia in this medium.

Clostridia in dairy products mainly originate from the raw milk. They have been recovered from a variety of cheeses, cheese sauces and creams, pasteurized milk, powdered milk, sweetened condensed milk, yogurt, and ice cream: numbers generally do not exceed $10–10^2$ spores g^{-1}, reflecting those in the original milk even for species composition. The intrinsic properties of some dairy products, that is, pH, a_w, redox potential (E_h), nutrient availability, and microflora, can be compatible with the germination and multiplication of certain clostridial strains, especially when the extrinsic factors such as storage temperature and time are also suitable for growth (see section ‘Control’). The consequences of growth of clostridia in dairy foods are described below.

Technological Problems

Both texture and flavor quality of dairy products may be affected by growth and metabolism of clostridia. One of the major concerns in cheese manufacturing is the so-called ‘late blowing’ defect, consisting of formation of holes, which can crack the cheese, accompanied with undesirable off-flavors. It appears during the course of cheese ripening in a large variety of hard and semihard cheeses, including Parmesan, Grana Padano, Emmental, Gruyère, Gouda, and Provolone. Processed canned cheese and cheese spreads can also be affected.

Considerable amounts of CO_2 and H_2 are responsible for the appearance of holes and blowing of cheese, while butyric acid and minor organic volatile acids negatively affect the flavor and taste of cheese. Gas and acids in the cheese result from fermentation of lactate mainly by *Cl. tyrobutyricum* and, to a lesser extent, by other *Clostridium* spp. (*Cl. sporogenes*, *Cl. butyricum*, and *Cl. beijerinckii*).

The occurrence of 'late blowing' in cheese depends on different factors, including the number and species of clostridia originally contaminating the milk, the manufacturing process, and the characteristics of the final product. Silage is identified as the main source of milk contamination. When silage fermentation conditions are not prone to rapid decrease in pH and maintenance of uniformly anaerobic conditions, germination of clostridial spores and subsequent multiplication of vegetative cells can occur. In grass silage, clostridial numbers range from below the detection limit to a maximum of 6.89 $\log_{10}$ CFU g^{-1}, whereas for maize silage, clostridial counts range from below the detection limit to a maximum of 4.69 $\log_{10}$ CFU g^{-1}. Defective cheeses generally result from milk originally contaminated with at least 200–10^3 *Cl. tyrobutyricum* spores per liter. The growth of clostridia is critically influenced by ripening time and temperature, lactic acid concentration, salt concentration, pH values, fat content, and presence of other microorganisms. The shape, size, and structure of the cheese, including normal eye formation, can also play a significant role. The numbers of spores detected in defective cheeses vary from 10^4 to 10^7 g^{-1} for *Cl. tyrobutyricum*, and from 10^3 to 10^6 g^{-1} for *Cl. sporogenes*. Although the latter microorganism causes more frequent spoilage of milk, its growth requirements and predominantly proteolytic metabolism are the possible reasons why it is more rarely involved in the 'late blowing' of cheese.

Diseases Associated with Clostridia in Dairy Foods

Botulism

Dairy products have rarely been involved in outbreaks of food-borne botulism, probably because their intrinsic and extrinsic properties are generally unsuitable for growth of *Cl. botulinum*. However, some outbreaks have been reported, the more recent being listed in **Table 2**. Raw milk was the likely source of spores in all outbreak-related dairy foods, except for the Brie cheese, the commercial cheese sauce implicated in the 1993 US outbreak, the hazelnut yogurt, contaminated with botulinum spores and/or toxin from different sources (specifically, the straw used for cheese ripening; potato skins; and the hazelnut conserve added to the yogurt), formula milk powder, and *süzme* (condensed) yogurt buried under soil.

Numbers of *Cl. botulinum* spores naturally contaminating raw milk are very low (~1 spore l^{-1}); however, the preliminary steps used in dairy production, such as centrifugation and filtration, contribute to the concentrating of microbial spores to 10 or more per gram of cheese product, depending on the manufacturing procedures. Even low numbers of *Cl. botulinum* spores per gram of product can be harmful, if conditions are met for their growth and toxin production.

Some common features can be traced for all the outbreaks of food-borne botulism linked to consumption of dairy products. First, proteolytic strains of *Cl. botulinum* were always the causative agents: spores of these microorganisms are more heat- and acid-resistant than the nonproteolytic ones (**Table 1**). Second, both commercial and homemade milk products were made from pasteurized milk: the temperatures and times generally used for thermal treatment of these products are not sufficient to kill the proteolytic spores of *Cl. botulinum*, but they do eliminate viable cells of nonsporing organisms, which are potential competitors of *Cl. botulinum*, such as lactic acid bacteria, thus facilitating the growth of *Cl. botulinum* in the product. Finally, the intrinsic factors (pH, a_w, Eh) of the cheese products, as well as abused storage temperature/time, and packaging conditions in the case of commercial products, were suitable for the outgrowth of spores and consequent toxin production.

Although the source of *Cl. botulinum* spores is rarely detected in intestinal toxemia botulism, an infant formula milk powder contaminated with *Cl. botulinum* type B

Table 2 Most recent outbreaks of botulism caused by consumption of spoiled dairy products

Year	*Location*	*Food source*	*Botulinum toxin type*
1974	Argentina	Commercial cheese spread with onions	A
1974	France and Switzerland	Soft ripened cheese (Brie)	B
1978	France	Soft cheese	B
1989	UK	Hazelnut yogurt	B
1993	USA	Commercial cheese sauce	A
1996	Italy	Commercial soft-spread cheese	A
1997	Iran	Locally made cheese	A
2001	UK	Formula milk powder	B
2005	Turkey	Condensed yogurt	A

spores has very recently been associated with a case of infant botulism.

Finally, it must be noted that type E neurotoxigenic *Cl. butyricum* has not yet been involved in any case of botulism from consumption of contaminated dairy products. However, since *Cl. butyricum* is a frequent spoiler of milk, the risk posed by the neurotoxigenic variant of the species does exist, particularly since it has already been implicated in both food-borne botulism and intestinal toxemia botulism.

Clostridium perfringens *enteritis*

Although the food most frequently involved in *Cl. perfringens*-associated diarrhea is undercooked meat, other foods including dairy products have also been implicated in outbreaks. Precise epidemiological data are lacking because hospitalization is not necessary in most cases and sporadic cases remain unknown, as does the source of illness.

Histamine poisoning

Cheese is the second leading source of histamine poisoning, after fish. However, the problem is largely underestimated because of the mild symptoms; hence, the incidence is difficult to determine. Moreover, many species of bacteria can contribute to the production of amines such as histamine in cheese, and the main role played by each one can be hardly determined.

Safety of Milk and Dairy Products from Cattle Affected by Botulism

Since the recent marked increase in the reported incidence of suspected cattle botulism, the potential human health risk associated with consumption of meat or milk derived from animals from herds affected by botulism has been raised. Using current scientific evidence, the Food Standards Agency (FSA) recently considered the restrictions (movement of the cattle and meat and milk from affected herd into the food chain) previously adopted on unaffected animals within the flock to be overprecautionary. In addition, FSA recommends that in the absence of other signs, there should be no restrictions on milk or meat from healthy cattle, sheep, and goats from affected farms.

Detection and Enumeration of *Clostridium* spp.

Analyses for clostridial contamination are performed not only on defective cheeses and milk products or those implicated in disease outbreaks, but also on the milk intended for dairy production, as a significant preventive measure. Hence, methods of choice should be able to detect very high as well as very low numbers of spores; in addition, they should be rapid, specific, reproducible, and easy to perform. Although such methods are needed for routine assessment of clostridial contamination in milk and its derivative products, no standardized technique is yet available for this purpose.

In theory, isolation and enumeration should be equally suitable for both vegetative cells and spores of clostridia; in practice, killing of viable cells cannot be completely avoided, because all samples are generally subjected to a heating step before incubation, in order to eliminate vegetative cells of other microorganisms and stimulate spore germination. For this reason, only the total number of clostridial spores can be determined. Different heating temperature/time conditions can be applied, taking into account that spores of some species are less heat-resistant than others (**Table 1**), and they might be inactivated as well.

For adequately sensitive detection and enumeration of clostridia in fluid milk, concentration of microorganisms by membrane filtration or centrifugation may be required. Alternatively, direct incubation of the milk under anaerobic conditions after thermal treatment has been proposed. However, milk is not an ideal substrate for supporting growth of clostridia, due to the absence of any reducing agent, or because of the growth and metabolism of other thermal-resistant bacteria, resulting in a decrease in pH values to inhibitory levels. Certain *Clostridium* spp. that are not able to ferment lactose, specifically *Cl. butyricum* and *Cl. tyrobutyricum*, do not grow in milk unless their appropriate carbon sources – lactate and acetate – are added.

Because clostridia include organisms with very different cultural and metabolic characteristics, selective agents that equally favor all relevant species while inhibiting other microorganisms are hard to find. However, since sulfite reductase activity is a common property among clostridia, broth and agar media used for their detection and enumeration often depend on sulfite reduction as a key differential criterion. These media generally include sodium sulfite (<0.05%, because some *Clostridium* strains are sensitive to this substance) and an iron salt in their formulations. Reducing conditions are maintained by inclusion of cysteine or thioglycolate in the medium. Precipitation of dark iron sulfide in these media, or gas formation in lactate-containing media, is presumptive for clostridial growth, even if confirmation is desirable since a few other bacteria also reduce sulfite or ferment lactate.

Enumeration of clostridia may be achieved by both direct counting and most probable number (MPN) methods. Direct counting is carried out by streaking dilutions from the original product onto selective solid media, such as sulfite–polymixin–sulfadiazine (SPS) agar. Polymyxin is the only antibiotic that appears to be partially, but not entirely, selective for clostridia. Black colonies are enumerated after anaerobic incubation of plates at 30 or 37°C for up to 3 days. The MPN method consists of inoculating serial dilutions of the test food in three or five replicates of broth medium, such as differential reinforced clostridial medium (DRCM) or lactate RCM. After thermal treatment of the inoculated tubes and subsequent incubation under anaerobic conditions for 5–7 days, blackening or production of gas (depending on the medium used) is suggestive of clostridial growth. Numbers of clostridia are then extrapolated from appropriate tables. Although the MPN test is more sensitive in detecting low numbers of spores than the direct counting, because of the larger inoculum, it is less accurate because it assumes that blackening and production of gas are due to clostridia only, and it is also more time consuming.

Recently, several PCR assays (i.e., PCR-DGGE nested PCR, real-time PCR) for detection and quantification of cluster I clostridia, identified as the major causal agent of late blowing in cheese, have been reported.

Identification of *Clostridium* spp. may be necessary in some investigations: this relies on recognition of differential characteristics of strains, such as the metabolic or genetic properties. Biochemical tests including sugar fermentation patterns and enzymatic activities have proven useful for identification at the species level. Amplification through PCR of species-specific DNA sequences, such as those coding for toxins in toxigenic clostridia or those for nonconserved ribosomal RNA in other *Clostridium* spp., is also widely applied for early detection and identification of these microorganisms. For toxin-producing species, such as *Cl. botulinum* and *Cl. perfringens*, demonstration of toxicity is required in any case. Conventional methods for botulinum toxin detection involve the use of animals and specific antitoxins: many alternative *in vitro* tests, including enzyme-linked immunosorption assay (ELISA) tests and assays for the detection of the enzymatic activity of botulinum toxins, have been devised, some of the most recent ones approaching the sensitivity and specificity of the standard *in vivo* assay. *Clostridium perfringens* type A toxin is also commonly demonstrated by *in vivo* tests or by cytotoxicity in cultured cells: slide latex agglutination and ELISA tests are also applicable.

Control

Several control measures are effective in preventing or minimizing contamination of milk with clostridia and inhibiting their growth in dairy products.

Good Manufacturing Practices

Selection of high-quality raw milk with low levels of clostridial spores helps ensure that the final products are safe and wholesome. Special attention should be paid to the quality of the silage fed to animals, since preservation of crops by lactic acid fermentation and maintenance of anaerobic conditions may facilitate the growth of contaminating clostridia, particularly *Cl. tyrobutyricum* because of its ability to ferment lactic acid. Spores ingested by animals concentrate in the feces, which is one of the main sources of milk contamination.

In many countries, regulations specify requirements relating to the health and care of milk-producing animals and to the hygiene of milking, handling, and transportation. Raw milk intended for Parmesan and traditional Swiss-type cheese production is collected from cows not fed on silage.

Reduction of Spores in Raw Milk

High-speed centrifugation (bactofugation) removes most spore-forming organisms from milk and does not affect the composition, flavor, and nutritional value of milk: efficacy in reduction of spores is a function of initial contamination in the milk and the operating conditions.

Heat treatment is effective only at the temperature/time conditions of sterilization (>135 °C for 2–3 s), but sterilized milk is unsuitable for the manufacture of most dairy products. Pasteurization includes a variety of temperature/time combinations, but generally temperatures do not exceed 90 °C, which is destructive to vegetative cells but not to bacterial spores; besides, this thermal treatment might activate spore germination and outgrowth, especially in the absence of microbial competition, if intrinsic and extrinsic properties of the pasteurized processed milk product are permissive.

Prevention of Spore Outgrowth in Dairy Products

Depending on the type of milk product, milk product technology, and duration of storage or ripening, inhibition of clostridia may be achieved by use of chemical additives, biopreservation, refrigerated storage, a_w and pH reduction, and combination of preservative factors, according to the 'hurdle' concept.

Nitrates, hexamethylenetetramine (HMT), and polyphosphates are the chemicals more commonly used against clostridia in cheese technology. Nitrates are converted into the functional nitrites through the metabolic activity of other contaminating microorganisms in the product; small amounts of nitrites (10–50 ppm) exert inhibitory effects against clostridial spores, possibly by enhancing their heat sensitivity. However, there are concerns on the use of these substances, due to the production of carcinogenic and mutagenic nitrosamines by chemical and/or microbial transformation, and for this reason their use in cheesemaking is not allowed in some countries; in other countries, 10–30 g of nitrates per 100 l of milk is permitted for the manufacture of some hard cheeses, such as Gouda.

HMT is used in the production of the Italian hard cheese Provolone. HMT is a precursor of formaldehyde, since under acidic conditions it is decomposed to ammonia and formaldehyde. The latter compound inhibits spore development in cheese because it reduces the availability of certain macromolecules by combining with them. However, because of its high reactivity with nucleic acids also, its use is allowed only within certain limits, that is, 15–25 ppm in the milk at the beginning of the production; at the end of ripening, formaldehyde in the product must be <0.5 ppm, set as the upper limit for safe human consumption.

Polyphosphates have been demonstrated to control clostridial growth in pasteurized processed cheese spreads stored at refrigeration temperatures, but their mechanism of action is still unknown. In the European Union, the maximum level of total phosphate permitted in processed cheese and cheese spreads is 2%, even if lower quantities may be sufficient to completely inhibit clostridia.

Biopreservatives such as lysozyme and bacteriocins, especially nisin, also show some effectiveness against clostridia, thus representing valuable alternatives to the use of chemical additives in cheese. Egg white lysozyme, added '*quantum satis*', disrupts microbial vegetative forms and delays the germination of spores by cleavage of the bacterial cell wall. Some clostridial strains, however, are resistant to lysozyme. Its anticlostridial activity has been well demonstrated in hard cheeses, such as Grana Padano and Emmental. On the other hand, nisin – a bacteriocin produced by *Lactococcus lactis* – has been widely used to prevent clostridial germination in pasteurized processed and spread cheeses, with high moisture and reduced salt contents. Nisin is directly added during cheesemaking to levels up to 250 ppm, depending on a_w and pH of the product.

Lactic acid bacteria starter cultures used to ferment milk products such as yogurt consistently inhibit clostridia by lowering the pH of the food, and thus represent another means of biopreservation.

Low-temperature storage, pH and a_w reduction, and other factors, including many of the preservative substances listed above, often function in synergy to control clostridia in dairy products. A combination of techniques leads to establishment of multiple barriers ('hurdles') more restrictive than that achieved with individual factors. Refrigerated storage of dairy products, for instance, cannot prevent by itself the growth of psychrotrophic clostridia; also, any storage temperature abuse might be risky in the absence of other inhibitory agents. a_w and pH reduction has classically been applied for inhibiting growth of any microorganisms in foods: however, addition of salts and various acidulants to reach values that are inhibitory for most clostridia (**Table 1**) is possible only to a certain extent in some dairies, in order to obtain organoleptically acceptable products.

In conclusion, given the wide distribution of clostridia in nature, keeping the contamination level as low as possible or inactivating contaminating spores as early in processing as possible should be ensured through appropriate quality control procedures, specifically by proper application of HACCP (hazard analysis critical control point) programs.

See also: **Cheese**: Avoidance of Gas Blowing.

Further Reading

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Coxiella burnetii

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Characteristics

Coxiella burnetii is the etiological agent of the zoonosis Q fever, which was first described in Australia. It is a pleomorphic, Gram-negative bacterium with a size of 0.2–0.7 µm with obligate intracellular propagation and worldwide distribution.

Studies based on comparison of 16S rRNA encoding gene sequences demonstrate that this pathogen is most closely related to *Legionella* spp. and resides within the γ-subdivision of the Proteobacteria while members of the family Rickettsiaceae, the former family of *C. burnetii*, are grouped in the α-subdivision.

Genotyping of *C. burnetii* isolates revealed the heterogeneity of this organism. Pulsed-field gel electrophoresis (PFGE) of *Not*I-restricted DNA was the first method applied for differentiation of North American *C. burnetii* isolates, which could be classified into four groups. With the same technique, 80 *C. burnetii* isolates derived from animals and humans within Europe, the United States, Africa, and Asia could be distinguished in 16 additional restriction patterns. Genome sizes, calculated by summing up the *Not*I restriction fragment sizes, ranged from 1.6 to 2.4 Mbp. With the availability of whole genome sequences of *C. burnetii*, typing methods were developed based on markers consisting of variable numbers of tandem repeats (VNTRs). This method, termed multiple loci variable number of tandem repeat analysis (MLVA), was found to be highly discriminatory. Thus, out of 42 isolates investigated with MLVA, 36 different genotypes were identified.

In mammals, *C. burnetii* propagates in alveolar macrophages before disseminating and infecting other cells of the mononuclear phagocyte system, and infrequently fibroblasts or endothelial cells. *In vitro*, *C. burnetii* may be cultivated in a broad range of epithelial, fibroblast, and macrophage-like cell lines. *Coxiella burnetii* is internalized into host cells by microfilament-dependent, parasite-directed endocytosis after engaging the leukocyte response integrin a_vb_3 (phase 1) or the CR3 receptor (phases 1 and 2). The pathogen then resides and multiplies within the harsh and acidic environment (pH 4.8) of parasitophorous vacuoles resembling secondary lysosomes.

During multiplication, *C. burnetii* undergoes a unique developmental cycle with two cellular types termed small cell variants (SCVs) and large cell variants (LCVs). It has been suggested that metabolically dormant SCVs are phagocytosed by eukaryotic host cells. Conditions in the vacuole trigger vegetative differentiation of SCVs into metabolically active LCVs. Apart from size, SCVs and LCVs differ markedly in ultrastructure, antigenicity, and metabolic capability.

Furthermore, specific small, spore-like particles (SLPs) are described, which are supposed to be responsible for the high resistance of *C. burnetii* against environmental conditions. SCVs and SLPs are considered as extracellular survival stages. Viable organisms can be recovered after heating at 63 °C for 30 min, exposure to 10% salt solution for 180 days at room temperature, exposure to 0.5% formalin for 24 h, or sonication in distilled water for >30 min.

After serial cell culture passages, *C. burnetii* undergoes a phase variation. Virulent phase I coxiellae, synthesizing amphiphilic full-length lipopolysaccharides (LPSs), are replaced by low-virulent phase II variants producing truncated LPSs. The isolation of virulent phase I *C. burnetii* from specimen homogenates is accomplished by inoculation into immunocompetent hosts (e.g., guinea pigs). Subsequently, homogenates of infected spleens are passed serially in cell culture or yolk sacs of embryonated hen's eggs to generate less virulent phase II *C. burnetii.*

The size of the *C. burnetii* genome is less than 40% that of *Escherichia coli*, that is, about 1.2×10^9 Da or 2.0×10^6 bp. The G + C content is 43 mol%. Until now, genomes of five *C. burnetii* isolates have been sequenced completely. Progress in understanding the genetics of *C. burnetii* has been hampered because of the inability to genetically manipulate the organism. Intracellular dependence, slow growth rates, and the tendency to infect persistently rather than lyse host cells made application of known genetic techniques fairly difficult. Recently, *C. burnetii* was transformed after electroporation and the cloning and characterization of a mutant generated by transposon mutagenesis were reported. This may facilitate the study of the unique and exemplary biology and pathogenicity of *C. burnetii.*

Five different plasmid types (36–56 kb) and one plasmid-homologous sequence integrated into the chromosome may be distinguished, which comprise about 2% of the genome. Although their function is still cryptic, they are supposed to be of essential importance, since every isolate contains exactly one type of plasmid or plasmid-homologous sequence. In contrast to initial studies, *C. burnetii* plasmids are no longer considered to be responsible for either acute or chronic Q fever. Epidemiological studies confirm that host factors play a key role in the clinical outcome of the disease.

Symptoms

Acute Q fever in humans is most often described as influenza-like, self-limited, febrile illness, although the majority of *C. burnetii* infections are asymptomatic (~60%). After an incubation period of 2–6 weeks, high fever, rigor, profuse sweats, fatigue, severe headache with retroorbital pain, photophobia, nausea, general malaise, myalgia, and arthralgia are characteristic. In more severe cases, pneumonia or hepatitis can occur. Rare manifestations include encephalitis, pericarditis, and myocarditis. Illness normally lasts 1–6 weeks, but can show a prolonged course. In more than 10% of cases, especially after repeated exposure to the infective agent, acute disease fades to the mostly afebrile Q fever fatigue syndrome. It is characterized by fatigue, muscle aches and pains, night sweats, headaches, photophobia, sleep disorder, loss of libido, and weakness of short-term memory, and can last for more than 1 year.

Some patients, probably about 2%, develop chronic disease 1–20 years after the initial illness or exposure. The most frequent manifestation of chronic Q fever, particularly in patients with underlying valvulopathy, is endocarditis. Most cases involve the aortic valve and about 30% the mitral valve. Further occasional symptoms of chronic Q fever can be vasculitis, osteomyelitis, and granulomatous hepatitis, especially under immunocompromising conditions. In women, premature delivery or spontaneous abortion has also been reported.

Similar to humans, in animals the most common route of infection seems to be inhalation of aerosols. They, however, usually do not develop illness from *C. burnetii* infection. *Coxiella burnetii* infection does not induce respiratory pathology in any animal species. Furthermore, chronic infection with cardiac or hepatic localization does not occur in animals.

After infection, *C. burnetii* can be isolated from the organs of clinically healthy animals. The colonization of the female reproductive system and proliferation of *C. burnetii* in both uterus and mammary gland often lead to spontaneous abortion, stillbirth, or premature delivery, and shedding of *C. burnetii* by birth fluids and milk. Miscarriages due to *C. burnetii* are most frequently seen in small ruminants. In sheep, late abortions occur sporadically and rarely epidemically in as many as 60% of gravid ewes. In goats, abortion is predominantly seen 15 days before term. Flocks with an abortion history also show an increase of weak newborns. Although ovine and caprine *C. burnetii* infection is often described as transient and tends to spontaneous cure as in dogs, cats, and humans, some studies suggest that it has rather an enzootic character as in cattle where *C. burnetii* circulates from generation to generation.

In cattle, abortion and generally reduced fertility due to *C. burnetii* are described. The infection may cause subclinical mastitis with elevated somatic cell counts and might have an effect on coinfection with common mastitis pathogens. Manifest mastitis has rarely been seen but there are some descriptions in sheep and goats. In a mass outbreak in Bulgaria, sheep developed a progressive severe inflammation of the milk gland, with disappearance of the milk secretion, and general intoxication leading to death of some animals.

Coxiella burnetii in Milk

After initial infection and bacteremia, *C. burnetii* may be detected in the udder during successive lactations. As confirmed by real-time PCR, 10^1–10^4 cells ml^{-1} can be excreted with the milk. A common shedding pattern could not be identified. Shedding can be continuous, intermittent, sporadic, or even absent in infected animals. In a follow-up study comprising 7 concomitant milk samplings of 139 dairy cows of which 60% showed positive PCR results in feces, vaginal mucus, or milk samples, approximately 40% were detected as milk shedders. Two predominant milk shedding patterns, persistent (34.5%) and sporadic (51.7%), were found. Shedding was not restricted to the time following parturition and could not be linked to shedding by feces or vaginal mucus. Another PCR analysis of samples from 110 shedder cows revealed that 53.6% shed the *C. burnetii* via milk. Parallel detection in feces and/or vaginal mucus was possible in 11.9 and 25.4% of these, respectively; 62.7% shed only via milk. The study claims that excretion via milk might be more important in cows and goats than in sheep where fecal and vaginal excretion prevailed.

The serological status of an animal does not allow prediction of milk shedding. It seems, however, that animals with high titers in enzyme-linked immunosorbent assay (ELISA), immunofluorescent assays (IFAs), or complement fixation tests (CFTs) have a higher probability of being shedders or even heavy shedders spreading large amounts of particles.

In an infected herd, between 15 and 76% of the lactating animals were shown to excrete Coxiellae with milk. This wide margin is probably not only due to a regional clustering of *C. burnetii* infection but also due to the method of preparation of the milk samples and the sensitivity of the applied detection method. In a major PCR study testing the bulk tank milk of dairy herds from all over the United States over 3 years, more than 94% of the samples were tested positive.

Due to the high prevalence of *C. burnetii* in the milk of domestic ruminants, the zoonotic risk of oral infection is of special interest. Most reported Q fever cases can be assigned to airborne infections. When milk is suspected as the source, different probable modes of transmission occur at the same time. Therefore, distinguishing true oral infections and evaluating the risk potential of contaminated milk are difficult.

The efficiency of oral infection, that is, occurrence and proportion of associated illness, is doubted. In guinea pigs, experimental oral infection led to seroconversion in 8 of 25 animals and in 5 of these *C. burnetii* was found in internal organs. The manifestation of infection probably depends on the infective dose and virulence of the strain. Administration of high doses of corticoids favored the infection, whereas alternating the intestinal permeability by chemical or mechanical means had no influence upon antibody formation or distribution of the infective agent in the host. *Coxiella burnetii* was estimated to be less efficiently transmitted by the oral route than by aerosols. Effective infection in mice requires 10 000 times more Coxiellae by oral versus intraperitoneal administration.

In humans particularly, the ingestion of contaminated raw milk is suspected of causing Q fever. This is still a matter of controversy as no conclusive evidence proves the alimentary infection of humans. This issue gains relevance as there are efforts under way in the United Kingdom, Canada, and the United States to promote increased human consumption of fluid raw milk. Pasteurization is generally accepted as sufficient for inactivating *C. burnetii* in milk. Mandatory pasteurization parameters were adjusted to the heat stability of *C. burnetii* when it was recognized as the most heat-resistant organism of public health significance.

It is largely accepted that ingestion of *C. burnetii*-contaminated food can result in serological conversion, but cases of Q fever have been documented rarely. In addition, it is likely that seroconversion follows the ingestion of inactivated cells as well as of live cells.

A study undertaken among hundreds of individuals, living several miles away from dairy farms, revealed that persons consuming raw milk had a higher rate of seropositive reactions. In a group of prisoners, some showed seroconversion after ingestion of contaminated raw milk for more than half a year, but the majority of the inmates remained seronegative. In another study, a small number of volunteers did not develop symptoms or an immunologic response after drinking contaminated, unpasteurized milk.

Q fever was observed in creamery workers with practically no contact with the livestock but exposed to large amounts of contaminated milk. Unpasteurized goats' milk has been described as a potential source of infection during a Q fever outbreak among patients and staff of a psychiatric institution in southern France.

To summarize, the possibility of oral infection by *C. burnetii* via milk cannot be denied. Possibly a relatively high oral infective dose impedes outbreaks of Q fever. Single cases in not yet identified risk groups could easily be overlooked because of the flu-like character of the disease. Furthermore, it is unclear whether milk infection might evoke the chronic form of Q fever after years of incubation.

Reservoirs and Routes of Infection

Coxiella burnetii exhibits extreme geographical distribution and is endemic in every part of the world except for New Zealand and Antarctica. Its broad host range includes mammals, birds, and arthropods. However, the most common animal reservoirs are cattle, sheep, and goats, in which *C. burnetii* infections are far more frequent than generally expected. Studies in the 1960s and 1970s demonstrated that 10% of US dairy cows are constantly infected with an increasing seroprevalence among cattle. In herds with reported infertility problems, the proportion of infected animals may be up to 80%. Today, *C. burnetii* must be regarded enzootic in US dairy herds with more than 90% of bulk tank milk samples testing positive via PCR.

Infection cycles may occur among arthropods and mammals in nature, and numerous species of ticks are considered as reservoirs for *C. burnetii.* Ticks acquire the organism by bloodsucking and transmit it with their feces. However, various investigations suggest that only some species, like *Dermacentor reticulatus*, might be of importance as reservoirs.

Although infection cycles may occur among arthropods and mammals in nature, *C. burnetii* maintains an independent and more important airborne infection cycle among domestic livestock. Domestic ruminants shed the desiccation-resistant organism in urine, feces, and especially birth products, contaminating the environment. Parturient placentas of infected ruminants were shown to contain as many as 10^{12} cells g^{-1}. Dissemination of contaminated straw and manure or birth products rotting on pastures may cause the spread of the organism via dust especially in periods of dry and windy weather. The highly infectious particles (minimal infectious dose by aerosol transmission is one *Coxiella* cell per individual) can be spread by the wind over distances of several kilometers. In this way, subsequent Q fever occurs even in distant urban areas. In Europe, the majority of Q fever outbreaks can be assigned to airborne infections originating from birth products of sheep and goat flocks during lambing season.

Diagnostics

Prior to implementation of specific control measures, precise and reliable diagnosis of *C. burnetii* infection in animals and humans is required.

For the detection of *C. burnetii*, the most relevant techniques include isolation, immunodetection in tissue samples, and DNA amplification. Initially, *C. burnetii* was isolated by inoculation of specimen into laboratory animals (i.e., guinea pigs, mice) or embryonated hen's eggs. Nowadays, isolation and propagation are mainly carried out by means of conventional, permanent cell cultures. The recent development of a new medium, suitable for cultivation of *Coxiella* in the absence of host cells, will probably further simplify cultivation.

Since isolation of *C. burnetii* requires much time and experience, immunodetection of *C. burnetii* antigen in tissue samples, smears, or secretions is applied increasingly in routine diagnostics. To increase sensitivity and specificity of *C. burnetii* detection, DNA amplification methods were established. Several procedures have been described for PCR detection of *C. burnetii* in milk. Meanwhile, several real-time PCR assays were developed not only to detect but also to quantify *C. burnetii* cells in clinical specimens.

Despite improved methods for the detection of the pathogen, the diagnosis of Q fever and *C. burnetii* infections still relies mainly upon serology. Particularly, surveys with large numbers of specimens are most commonly performed serologically. Usually, IFA or CFT and ELISA techniques are applied. To summarize, CFT is very specific but lacks sensitivity; moreover, a prozone phenomenon is present with specimens of chronic cases that could lead to false negative results. ELISA tests in general are satisfactory for diagnosis. Nevertheless, for Q fever, diagnostic IFA is still the reference method, since it is both sensitive and highly specific. No cross-reactions with other organisms have been documented. Antibodies against *C. burnetii* can also be detected in milk by capillary tube agglutination tests.

For the diagnosis of Q fever, sera should be tested at different time points to demonstrate seroconversion. A fourfold increase in anti-*Coxiella* titers is considered to be characteristic of acute Q fever. Alternatively, antibody subclasses against *C. burnetii* should be differentiated and additionally phase I and phase II antigens may be applied in the test system. The results of serology are positive between 2 and 4 weeks after the onset of disease. In acute Q fever, IgM antibodies against phase II antigen rise prior to antiphase I IgM or IgG antibodies. IgM persists for 6–8 months and diagnosis can be made even when a single sample is IgM positive. In chronic Q fever, antiphase I antibodies predominate at very high levels. *Coxiella burnetii* serology in animals is predominantly carried out by CFT or ELISA.

Treatment

If Q fever is diagnosed early during illness, a regimen of tetracycline compounds especially doxycycline (200 mg day^{-1}) for 15–21 days is still recommended. Quinolones, like ofloxacin (600 mg day^{-1}) and pefloxacin (800 mg day^{-1}), were reported to be effective in Q fever pneumonia and Q fever meningoencephalitis. In prolonged Q fever, a combination of pefloxacin (800 mg day^{-1}) and rifampicin (1200 mg day^{-1}) applied for 15 days has been successful. In chronic Q fever (i.e., mainly endocarditis), tetracycline is usually combined with either rifampicin or quinolones and even chloroquine. However, since antimicrobial treatment ranges from 1 year to unlimited administration, valve replacement is often proposed for hemodynamic reasons.

So far, no satisfactory treatment scheme for coxiellosis exists in animals. Cattle and sheep, treated with tetracycline, continued to shed the organism. Nevertheless, a potential protective effect on pregnancies was not excluded.

Prevention

To prevent Q fever and coxiellosis, vaccination and hygiene management strategies must be applied. Despite great efforts to develop Q fever vaccines, only one vaccine, Q-Vax®, is commercially available and is restricted to Australia. The formalin-inactivated whole cell vaccine provides reliable protection. Unfortunately, it can induce mild to severe adverse reactions, especially when administered to individuals with prior exposure to the agent. Thus, strict prevaccination screening, for example, exclusion of persons with positive skin reactions, is necessary. Booster inoculations are problematic for the same reason.

In the area of veterinary medicine, two vaccines against coxiellosis of ruminants, Chlamyvax FQ® and Coxevac®, are commercially available. Chlamyvax FQ®, a whole cell vaccine containing inactivated *C. burnetii* phase II particles and a *Chlamydia* component, is solely approved in France. For Coxevac®, which contains inactivated phase I particles, European registration is in progress.

Generally, vaccines prepared with phase II particles are considered to be less protective. This was confirmed by a recent comparative study in which goats were challenged after vaccination. Only vaccination with Coxevac® led to a reduction of abortion incidence and shedding. The complete effectiveness of the vaccine against bacterial shedding in milk led the authors to suggest that vaccination might represent the microbiological quality assurance awaited by the producers of raw milk.

Possibly, the effectiveness of Coxevac® in small ruminants can be finally evaluated when results of obligatory vaccination programs for goat and sheep holdings in southern regions of the Netherlands, ordered to control a large Q fever outbreak, become available.

Coxevac® reduced the probability of susceptible (i.e., noninfected) dairy cattle becoming a shedder to one-fifth. This result was not seen if vaccinees were pregnant. Furthermore, Coxevac® failed to prevent or reduce *C.burnetii* shedding in cows that were infected prior to vaccination. Thus, vaccination with the phase I vaccine might protect noninfected herds. Nevertheless, infected herds need further hygienic and medical measures to reduce effectively the bacterial burden.

During Q fever outbreaks, predominantly originating from small ruminants, official recommendations regulate hygiene management measures in most countries. Following for example the recommendations of the German Robert Koch institute, sheep and goat herds must be kept at a distance of >500 m from inhabited areas. For disinfection of contaminated stables, solutions of 10–20% bleaching powder, 1% Lysol, or 5% hydrogen peroxide are recommended. Contaminated manure should be ploughed under after decontamination for 5 weeks with quicklime packages.

Special care has to be taken with the highly infectious birth products, which must be eliminated from stables or pastures immediately for proper disposal. After lambing, dams and offspring must be kept in closed stables for 2 weeks.

Shearing and storage of wool are restricted to closed rooms outside residential areas. Shearing personnel are obliged to wear dusk masks. If ticks are suspected as vectors, acaricides should be administered.

As discussed above, it is difficult to evaluate the risk originating from contaminated raw milk. Following the precautionary principle, pasteurization should be advised.

Meat, unlike udder and placental tissue, is not regarded as a source of infection. Eradication of *C. burnetii*-infected livestock is discussed periodically when Q fever outbreaks occur but cannot be recommended due to the high prevalence of coxiellosis in general, the environmental stability of the infective agent, and the unavoidable contact with wildlife reservoirs. Nevertheless, identification and culling of persistently heavy shedding individuals could reduce the bacterial burden.

Altogether, identification and proper disposal of material potentially contaminated with *C. burnetii* are the main objectives in Q fever prevention. Besides, physicians should be encouraged to include Q fever in their differential diagnosis to promote early and efficient therapy or control measures if infection occurs.

See also: **Analytical Methods**: DNA-based Assays. **Cheese**: Public Health Aspects. **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health. **Heat Treatment of Milk**: Thermization of Milk; Non-thermal Technologies: Introduction; **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Microorganisms Associated with Milk**.

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Escherichia coli

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Introduction

The genus *Escherichia* derives its name from Theodor Escherich, who first isolated this organism from feces in 1885. The type strain of the genus is *E. coli*, a common bacterium found in the gastrointestinal tract of humans and other vertebrates where most strains are nonpathogenic commensals. However, there are several groups of *E. coli* that can be pathogenic, causing a variety of diseases, some of which are fatal. The three clinical syndromes resulting from *E. coli* infection are urinary tract infections caused by uropathogenic *E. coli*, meningitis and septicemia resulting from necrotoxigenic *E. coli* infection, and diarrheal diseases. In food safety, the most important *E. coli* are those that cause diarrheal diseases. *E. coli* can also cause spoilage in dairy products, for example, blowing of cheese and ropiness in milk and cheese brines can be caused by *E. coli* growth.

General Characteristics

E. coli is a member of the family Enterobacteriaceae, which includes Gram-negative facultatively anaerobic rod-shaped bacteria (possessing both a fermentative and respiratory metabolism) and which do not produce the enzyme oxidase. *E. coli* cells are typically 1.1–1.5 μm wide, 2–6 μm long and occur as single straight rods. They can be either motile or nonmotile, and when motile produce lateral, rather than polar, flagella. In addition to flagella, many strains produce other appendages such as fimbriae or pili, which are proteinaceous structures (or appendages or fibers) that extend outward from the bacterial surface and play a role in attachment to surfaces including other cells or host tissues.

E. coli have strain-specific O lipopolysaccharide antigens on their cell wall (at least 181 O antigens are currently recognized) and flagella or H antigens if present (53 H types are recognized). There are also 80 different capsular polysaccharide (K) antigens. *E. coli* are serotyped based on the combination of O, H, and K antigens, although generally only the O and H types are listed, for example, *E. coli* O157:H7. Serotyping of *E. coli*, together with genome, virulence, and phage typing, is a useful epidemiological tool.

E. coli is closely related to *Shigella* spp., although *Shigella* tends to be less biochemically active than most strains of *E. coli*. *Shigella* and *E. coli* can be considered to be within a single genus based on genetic relatedness, but historically the two have remained separate to prevent confusion in medical diagnosis.

Pathogenic Types of *E. coli*

E. coli causing human diarrhea are assigned to groups based on their virulence properties and clinical manifestations.

Enterohemorrhagic *E. coli* and Shiga Toxigenic *E. coli*

The enterohemorrhagic *E. coli* (EHEC) group have a common ability to produce cytotoxins that are active on monkey kidney (Vero) tissue culture cells. These cytotoxins are mediated by genes carried by lysogenic bacteriophages and act in a similar manner by interfering with protein synthesis in eukaryotic cells. The toxins belong to two major antigenically distinct groups, Stx1 and Stx2, with various subgroups. Stx1 is similar to the Shiga toxin produced by *Shigella dysenteriae*; consequently, Shiga toxin-producing *E. coli* are referred to as both STEC (Shiga toxin-producing *E. coli*) and VTEC (verotoxigenic *E. coli*). EHEC is a term commonly used to refer to those STEC that cause enterohemorrhagic disease.

Since their recognition in the 1980s, EHEC have become the most notable of the enteropathogenic *E. coli* (EPEC) that are transmitted by food, including dairy products. They can cause illness in humans and diarrheal illness in young animals. Human EHEC infection can be asymptomatic or result in symptoms ranging from mild diarrhea to hemorrhagic colitis and life-threatening hemolytic–uremic syndrome (HUS). Strains causing hemorrhagic syndromes frequently, but not exclusively, carry accessory virulence factors to the Shiga toxins located on a chromosomal pathogenicity island, the locus for enterocyte effacement (LEE). The LEE encodes genes that lead to the formation of attaching and effacing lesions typically seen in the intestinal epithelium in both EHEC and EPEC (see below) infections. EHEC strains also frequently carry a unique 92-kb plasmid, pEHEC, that encodes several potential virulence factors, although none is considered essential in EHEC pathogenesis at this time. The pEHEC carries genes encoding an EHEC hemolysin that is used as a differential characteristic for putative EHEC in some agar isolation media.

There are many serotypes of STEC, although a limited number are commonly associated with HUS. The most common is the O157:H7 serotype and others include O26, O111, O45, and O103. Clinical isolates of these types often carry the same virulence determinants, for example, Stx1 and/or Stx2, the LEE, and pEHEC.

Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) produce the *E. coli* heat-labile (LT) and/or heat-stable (ST) toxins. ETEC are important causes of diarrhea among children in developing countries and of traveler's diarrhea. Symptoms include acute watery diarrhea that may be mild and of short duration and which in some cases is similar to cholera. ETEC strains are host specific, that is, some strains can cause diarrhea in young animals (e.g., calves, lambs, and piglets), while other strains will specifically infect only piglets, and others only humans.

The major virulence factors of ETEC are intestinal colonization factors, for example, fimbriae, and the enterotoxins. The host specificity of individual strains is determined by the type of colonization factors and fimbriae produced. The LTs are high-molecular-weight proteins similar to cholera toxin and consist of five enterocyte-binding B subunits and a biologically active A subunit. The internalized A subunit causes electrolyte imbalance and a net fluid loss to the gut lumen by activation of adenylate cyclase and accumulation of cyclic adenosine monophosphate. The STs are small polypeptides that act similarly through activation of guanylate cyclase and accumulation of cyclic guanosine monophosphate.

Enteropathogenic *E. coli*

EPEC are a major cause of acute or chronic enteritis in children in developing countries. EPEC typically produce attaching and effacing lesions on the intestinal epithelium. The genes encoding this activity are carried on a 60 MDa EPEC adherence factor plasmid and the LEE pathogenicity island, which is also present in many EHEC. The clinical symptoms are a consequence of the ensuing electrolyte loss and epithelial damage. There have been a limited number of outbreaks of EPEC disease linked to food- and waterborne transmission.

Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) invade the host intestinal epithelial cells where they multiply, causing cell destruction and an acute inflammatory response similar to shigellae. Patients may first develop watery diarrhea prior to onset of dysentery with a low volume of stools containing blood and mucus. Other symptoms are headache, fever, and cramping. Humans appear to be the main reservoir of EIEC, with little evidence to support EIEC carriage in animals or foods of animal origin. EIEC outbreaks are usually associated with water or food contaminated with human feces or person-to-person transmission. The incidence of the disease caused by EIEC is generally low in developed countries.

Enteroaggregative *E. coli* and Diffusely Adherent *E. coli*

Two other groups of diarrheagenic *E. coli* characteristically adhere to Hep-2 tissue cells. Those that adhere in an aggregative pattern resembling microcolonies are termed enteroaggregative *E. coli* (EAEC) and those producing a more diffuse adherence on the Hep-2 cell surface are known as diffusely adherent *E. coli* (DAEC). The mechanisms of pathogenesis of these groups are poorly understood, as is their epidemiology. Both groups are isolated from diarrheal cases in children in developing countries and there is limited evidence of foodborne outbreaks.

E. coli Outbreaks from Milk and Dairy Products

Milk and Cream

Raw milk has been implicated as the vehicle of sporadic cases and several outbreaks of EHEC-associated illness in North America, Europe, and the United Kingdom. While cows' milk is the most common source, goats' and sheep milk has been implicated in *E. coli* O157 infection. Outbreaks of EHEC infection have been associated sheep with raw milk and pasteurized milk contaminated postprocessing. In an outbreak of EHEC infection where nine children developed HUS in the United Kingdom, *E. coli* O157 was found in pipes and on a discarded rubber gasket from a milk-bottling machine, suggesting postpasteurization contamination. Postpasteurization contamination was also believed to have caused an outbreak of *E. coli* O104:H21 linked to pasteurized milk in the United States.

Cheese

Outbreaks of illness caused by EAEC, EIEC, ETEC, and EHEC have been attributed to the consumption of cheese. Cases of EAEC O92:H33 were associated with Pecorino cheese made from raw milk thought to be inadequately processed. An outbreak of EIEC O124 involved 387 people following consumption of Camembert cheese. Contamination was traced to river water used for cleaning processing equipment that had been filtered through a malfunctioning treatment system. ETEC serotype O27:H20 was isolated in an outbreak caused by the consumption of French Brie and Camembert that had been mishandled during shipping and distribution.

Cheese has been the attributed vehicle in several EHEC outbreaks. Outbreaks of *E. coli* O157:H7 have been attributed to a variety of cheeses such as farm-produced cheese, fresh cheese curds, a semihard Lancashire cheese, and a Gouda made from unpasteurized milk. Other serotypes have included EHEC O103:H2 contaminating soft cheese made from a mixture of unpasteurized cows' and goats' milks; O119 EHEC in fromage frais; and O26 EHEC from a raw milk Camembert cheese in France. The use of contaminated raw milk or post-processing contact with raw milk and subsequent survival of the EHEC during the cheesemaking process have been the major contributing factors.

Other Milk Products

An outbreak of *E. coli* O157:H7, which affected 16 people and caused 5 HUS cases among children, was linked to a live yogurt made on a farm from pasteurized milk. Postpasteurization contamination of milk was likely due to either inadequate cleaning or contamination from farmyard material. Ice cream contaminated with STEC O145:H28 and O26:H11 has been attributed to cases of HUS. Cases were guests at two birthday parties on the same farm where the ice cream was also made. STEC of the same serotypes were isolated from calves and the farm environment. There have been no documented cases of *E. coli* infection caused by dried or condensed dairy products, or by butter.

Incidence in Milk and Dairy Products

The primary source of *E. coli* on a farm is the animals and wildlife. Although EHEC and STEC are the most studied pathogenic *E. coli* in farm environments, other types (EAEC and EPEC) have been detected. Humans may be a significant source of other pathogenic types. Animal isolates of *E. coli* are dispersed via feces and can be found on hides and hair and in saliva. Fecal contamination is widely disseminated on dairy farms in soil, dust, non-potable water, and on pastures and dissemination is assisted by wildlife, for example, rodents, birds, and flies, and personal and farm equipment. Animal excretion of specific *E. coli* types may be intermittently varying with season and animal age, for example, EHEC O157:H7 may be carried by up to one-third of dairy herds and 4–10% of cattle within the herd may be excreting at any time.

Milk can be contaminated during collection indirectly via udders soiled with contaminated feces, soil, water, feed, and bedding, or directly if the animal has *E. coli* mastitis. Workers, either asymptomatic or ill, may excrete *E. coli* in their feces and poor personal hygiene practices can lead to contamination of dairy products and equipment. Equipment at farms and factories can become contaminated by raw milk, water, and soil, and contamination can be transferred from farm to factory via soil on bulk milk tankers and other transport vehicles.

Table 1 lists various studies on the incidence of *E. coli* in dairy products. Generally, dairy products produced from pasteurized milk and with good hygiene and sanitation protocols, as expected, are less often contaminated with *E. coli*.

Growth and Survival

Information on the growth and survival of *E. coli* in foods is generally not specific to the individual pathogenic types. Where this is available, this information is specified.

The optimal temperature for growth of *E. coli* is 35–40 °C, the minimum being 7–8 °C and the maximum about 46 °C. O157 EHEC have a more limited growth range and will not grow below 8 °C and grow poorly or not at all at 44–45.5 °C, the temperature often used in routine methods for detecting *E. coli*. The growth medium used can limit the ability of a particular strain to grow or produce gas at temperatures above the optimum.

E. coli is sensitive to heat and this depends on the composition, pH, and water activity (a_w) of the suspending medium or food matrix. The *D*-value or time required to reduce *E. coli* numbers by one log cycle in cows' milk at 58 °C (D_{58}) is 0.78 min, while in nonfat dry milk powder, the D_{58} value increases with the increasing percentage of milk solids. As the a_w decreases, the heat resistance can increase depending on the solute; for example, at an a_w of 0.98, sodium chloride has a greater effect than glucose. *E. coli* is somewhat more sensitive to heat than other enteric pathogens, for example, *Salmonella*, and will not survive adequate pasteurization. *E. coli* survives well in chilled and frozen foods. In pasteurized, unpasteurized, and skim milk, *E. coli* O157:H7 does not grow at 5 °C, although at 8 °C, 1–2 and 2–3 log increases may occur after 4 and 7 days, respectively. After initial freezing, there is normally a decrease in viable *E. coli*, and then the population can remain stable for periods greater than 12 months.

E. coli can grow at a pH between 4.4 and 10, the optimal being 6–7. The minimal pH allowing growth is influenced by the type of acidulant, the presence of inhibitory substances, for example, nitrite, temperature, and a_w. For example, strains of *E. coli* O157 will grow at 37 °C in a medium of pH 4.5 acidified with hydrochloric acid but

Table 1 A summary of some surveys of milk and milk products for *E. coli*

Product	*E. coli group*	*Number tested*	*Percent positive for E. coli*	*Country*	*References*
Raw milk	EPEC[a]	Unknown	19	India	Singh *et al*. (1970)
Milk products	Generic *E. coli*	5760	29	Europe	Otenhajmer *et al*. (1989)
Raw milk	Generic *E. coli*	1154	46	Europe	Otenhajmer *et al*. (1989)
Raw milk (bulk tank)	Generic *E. coli*	175	47	Trinidad	Adesiyun *et al*. (1997)
Raw milk (bulk tank)	*E. coli* O157:H7	268	0.8	United States of America	Murinda *et al*. (2002)
Raw milk	Toxigenic *E. coli*[b]	12	17	Zimbabwe	Gran *et al*. (2003)
Raw milk	*E. coli* O157:H7	930	34	Malaysia	Chye *et al*. (2004)
Raw milk	*E. coli* O157:H7	250	0.4	Hungary	Hucker *et al.* (2006)
Raw milk (milk filters)	*E. coli* O157:H7	536	3	Ireland	Murphy *et al*. (2005)
Naturally soured milk	Toxigenic *E. coli*[b]	21	33	Zimbabwe	Gran *et al*. (2003)
Cultured pasteurized milk	Toxigenic *E. coli*[b]	27	26	Zimbabwe	Gran *et al*. (2003)
Pasteurized milk	EPEC and ETEC	32	0	Brazil	Jakabi and Franco (1991)
Pasteurized milk and cream	Generic *E. coli*	430	6	Sweden	Lindberg *et al*. (1998)
Raw milk cheese (soft)	Toxigenic *E. coli*[b]	221	1.4	Spain	Quinto and Cepeda (1997)
Raw milk cheese (soft and semisoft)	*E. coli* O157:H7	153	0	Belgium	Vivegnis *et al*. (1999)
Raw milk cheese (soft, hard, unripened, and blue)	STEC	1039	3	France	Vernozy-Rozand *et al.* (2005)
Raw milk cheese (Castellano)	STEC	83	2	Spain	Caro and Garcia-Armesto (2007)
Raw milk cheeses (soft, semihard, and hard)	STEC	796	2	Switzerland	Stephan *et al.* (2008)
Camembert	Generic *E. coli*	102	3	Japan	Takeba *et al*. (1996)
Pasteurized milk soft cheese	Toxigenic *E. coli*[b]	75	0	Spain	Quinto and Cepeda (1997)
Butter (pasteurized and unpasteurized)	Generic *E. coli*	60	22	Italy	Bianchi and Geminiani (1994)
Ice cream	Generic *E. coli*	73	30	Turkey	Arslan *et al*. (1996)
Frozen yogurt	Generic *E. coli*	170	0	Spain	Lopez *et al*. (1997)
Cream confectionery	Generic *E. coli*	92	10	Italy	Simoni *et al*. (1983)

[a] Uncertain definition of enteropathogenic *E. coli* (EPEC).

[b] Enterotoxigenic *E. coli* (ETEC), Shiga toxigenic *E. coli* (STEC), and necrotoxigenic *E. coli* were isolated.

From Adesiyun AA, Webb LA, Romain H, and Kaminjolo JS (1997) *Journal of Food Protection* 60: 1174–1181; Arslan A, Gonulalan Z, Ates G, and Guven A (1996) *Turkish Journal of Veterinary and Animal Sciences* 20: 109–112; Bianchi E and Geminiani G (1994) *Industrie Alimentari* 33: 833–837; Caro I and Garcia-Armesto MR (2007) *International Journal of Food Microbiology* 116: 410–413; Chye FY, Abdullah A, and Ayob MK (2004) *Food Microbiology* 21: 535–541; Gran HM, Wetlesen A, Mutukumira AN, Rukure G, and Narvhus JA (2003) *Food Control* 14: 539–544; Hucker A, Mike-Schummel I, Unger A, and Varga L (2006) *Milchwissenschaft – Milk Science International* 61: 11–14; Jakabi M and de Franco BDGM (1991) *Ciencia e Tecnologia de Alimentos* 11: 170–181; Lindberg AM, Ljungh A, Ahrne S, Lofdahl S, and Molin G (1998) *International Journal of Food Microbiology* 39: 11–17; Lopez MC, Medina LM, Cordoba MG, and Jordano R (1997) *Alimentaria* 288: 39–45; Murinda SE, Nguyen LT, Ivey SJ, *et al*. (2002) *Journal of Food Protection* 65: 752–759; Murphy BP, Murphy M, Buckley JF, *et al*. (2005) *International Journal of Hygiene and Environmental Health* 208: 407–413; Otenhajmer I, Mijacevic Z, and Asanin R (1989) *Acta Veterinaria* 39: 127–136; Quinto EJ and Cepeda A (1997) *Letters in Applied Microbiology* 24: 291–295; Simoni F, Baldaccini G, and Bianucci P (1983) *Industrie Alimentari* 22: 337–341; Singh RS, Ranganathan B, and Laxminarayana H (1970) *International Dairy Congress, Sydney* 1E: 148; Stephan R, Schumacher S, Corti S, Krause G, Danuser J, and Beutin L (2008) *Journal of Dairy Science* 91: 2561–2565; Takeba K, Umeki F, Nakama A, Fujinuma K, and Kokubo Y (1996) *Annual Report of Tokyo Metropolitan Research Laboratory of Public Health* 47: 82–89; Vernozy-Rozand C, Montet MP, Berardin M, Bavai C, and Beutin L (2005) *Letters in Applied Microbiology* 41: 235–241; Vivegnis J, El-Lioui M, Leclercq A, Lambert B, and Decallonne J (1999) *Biotechnologie Agronomie Société et Environnement* 3: 159–164.

not with lactic acid. *E. coli* have several different regulatory systems that enable cells to adapt to and survive acid stress. Both acid-resistant and acid-sensitive strains have been found among nonpathogenic *E. coli*, and EIEC, EPEC, EHEC, and ETEC groups.

The minimum a_w allowing growth of *E. coli* is 0.95 (∼8% sodium chloride) and the optimal a_w is 0.995, with variations between strains. Combinations of parameters are often present as hurdles for controlling growth of *E. coli* in dairy products. For example, during cheese manufacture, low pH produced by starter cultures metabolizing lactose into lactic acid, low a_w developed with the addition of salt and during ripening, and temperature control inhibit *E. coli* growth. Curd cooking temperatures of 30–38 °C (for soft and semisoft cheeses) and 38–55 °C (for hard cheeses) can provide conditions suitable for the growth of *E. coli*; therefore, careful control of pH and a_w is essential to limit the growth of any pathogens present. In the initial stages of fermentation of soft and semisoft cheeses, any *E. coli* present (including pathogenic types) can grow until the pH drops, salt is added, and the temperature is reduced. However, it is possible for *E. coli* to survive beyond the ripening stages of some semihard, soft, and fresh cheeses. Zones of these intrinsic parameters are established during processing, for example, in soft and mold-ripened cheeses, and the behavior of any *E. coli* strain varies accordingly.

Control

Common factors causing outbreaks of *E. coli* disease due to dairy products include the use of contaminated raw milk, faulty pasteurization or failure of processing equipment, starter culture failure, and postpasteurization contamination. The application of a hazard analysis critical control point (HACCP)-based approach together with the prerequisites of good manufacturing practices (GMPs) and good hygiene practices (GHPs) is now required through the dairy food chain in many countries. Effective sanitation and cleaning of equipment at all processing steps and meticulous personal hygiene of food handlers are essential, whether on farm, during transport, or in factories. Equipment sanitation is important, as any residues of product on equipment or machinery may allow growth of *E. coli* contaminants at ambient temperatures, leading to subsequent product contamination. Use of potable water for cleaning and during manufacture is important and failures to adhere to this have resulted in outbreaks of *E. coli*. Effective monitoring at control points and corrective action in the event of a deviation are fundamental in this process.

Processing plants should be designed to ensure that contamination is not transferred from raw to final products. Movement of personnel and equipment between milk receival bays and processing areas should be prevented with similar separation of raw and pasteurized product areas. Specific control measures for particular products are discussed below.

Milk and Cream

As *E. coli* is part of the bacterial flora in feces of healthy dairy animals, contamination of raw milk can easily occur during collection. On-farm controls include the supply of safe feed and water, maintenance of animal health, and removal and rapid treatment of any animals with mastitis. GHPs on farm, including cleaning and disinfecting of udders before milking and effective cleaning of surfaces and equipment in the dairy parlor and on milk transport tankers, are essential to minimize contamination of the raw milk during collection and storage. Preventing environmental contamination of raw milk via dust and other particulate matter and ensuring personal cleanliness and hygiene are also important. Raw milk contains various antimicrobial factors that may restrict microbial growth (lysozyme, lactoferrin, lactoperoxidase, immunoglobulin); however, this alone is insufficient and chilling is also required. Chilling raw milk to below 4 °C within 2 h of collection restricts growth of *E. coli*, and regulations for milk storage are established in most countries.

Raw milk is potentially contaminated with *E. coli* and consumption of raw milk poses a threat to human health, particularly for young children, the elderly, and those who are immunocompromised. Heat treatment is the most common method for enhancing the safety of liquid milk. Any *E. coli* present in pasteurized milk is a consequence of ineffective pasteurization or postprocessing contamination. Pasteurization at 63 °C for 30 min and high-temperature, short-time (HTST) pasteurization at 72 °C for 15 s or equivalents are effective in reducing *E. coli* contamination to levels considered safe for human consumption. The effect of thermization varies depending on the temperature and time used. Low treatments (57 °C for 15 s) sometimes used in cheesemaking will have little or no effect in destroying *E. coli* compared with higher temperatures (62–65 °C for 15–20 s). Similar measures apply to cream, although the higher fat content can protect bacteria from the effects of heat, and higher temperature limits of these treatments are required. After pasteurization, considerable care is required to prevent recontamination, and pasteurized milk and cream are held chilled (preferably at less than 5 °C) to prevent growth if contamination occurs.

Critical controls include storage and pasteurization conditions (temperature and holding time), with prerequisites being effective cleaning and sanitation of all product contact surfaces, controlled airborne contamination, personal and environmental hygiene.

Cheese

The likelihood of *E. coli* contamination of cheese depends largely on the quality of the milk used and the performance criteria for the process. If milk is adequately pasteurized, the risk is minimal. When raw milk or low temperature thermised milk is used, there may be some loss of viability of *E. coli*; however, whether they survive or grow and their presence in the final product will depend on the combined effect of subsequent processing hurdles. Therefore, raw milk cheeses may pose similar threats to health as raw milk, depending on the method of cheese processing. The health risk has to be weighed up against cheese quality benefits when making raw milk cheese.

There is a diversity of cheese types and processes, each of which has control points with critical limits that have to be monitored. Use of starter cultures for rapid acidification contributes significantly to controlling pathogens; therefore, ensuring the activity of the starter culture is essential. Ensuring adequate temperature control during fermentation, cooking, and storage, and control of moisture and salt are required. In some countries, holding cheese made from raw milk for more than 60 days is required to ensure safety. This can no longer be relied on for control of some pathogenic *E. coli*, as O157:H7 strains have been found to survive, for example, in Cheddar cheese, beyond this time.

Fermented Milk Products

Production of fermented milk products, like cheese, relies principally on the production of organic acids from the activity of starter cultures. The resultant level of acid varies among products. Control of starter culture activity and the resultant pH is essential. This may need to be combined with other hurdles such as refrigeration in ensuring the safety of fermented products. Some strains of *E. coli* are capable of surviving at low pH and prevention of contamination of the product after pasteurization is essential. Fruits, nuts, and other flavorings added after pasteurization must be of high microbiological standard. Aseptic packaging of products is also important in reducing postprocessing contamination.

Ice Cream and Other Frozen Dairy Desserts

In addition to milk and other dairy products, ingredients such as sugar, eggs, fruits, nuts, flavors, and colors are used in the manufacture of ice cream and other frozen dairy products. These additional ingredients may introduce *E. coli* contamination, especially if added after pasteurization of the milk or dairy mix. Growth of *E. coli* in ice cream and frozen dairy desserts is limited by low a_w and storage temperature; however, growth of *E. coli* can occur if products are in the liquid state. Freezing may have little effect on the number of bacteria present; it is therefore important to maintain good hygiene and sanitation throughout manufacture. Control measures for ice cream and frozen dairy desserts include provision of raw ingredients of good microbiological quality (especially those that cannot be pasteurized), appropriate pasteurization or heat treatment, prevention of postpasteurization contamination, and storage at low temperatures. Attention to hygiene in serving frozen dairy products is important, as *E. coli* have been found contaminating ice cream and ice cream scoop water.

Dried and Concentrated Products

The heat treatment used in the manufacture of dried and concentrated dairy products is equivalent to, if not greater than, pasteurization and is sufficient to destroy viable *E. coli*. Such products, due to their low a_w, do not support the growth of *E. coli*. Prevention of postprocessing contamination is an important control measure in the manufacture of these dairy products.

Other Products

Butter has not been directly implicated as a source of human *E. coli* infections. Pasteurization of cream in the manufacture of commercial butter and the inhibitory qualities of the product ensure that it is unlikely to be a vehicle of *E. coli* infection. The addition of ingredients, for example, fresh herbs, may introduce contamination as described for frozen products (above).

See also: **Cheese**: Microbiology of Cheese; Raw Milk Cheeses. **Contaminants of Milk and Dairy Products**: Contamination Resulting from Farm and Dairy Practices; Environmental Contaminants. **Microorganisms Associated with Milk**. **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Risk Analysis**.

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Enterobacteriaceae

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Introduction

The family Enterobacteriaceae comprises a very large group of morphologically and physiologically similar bacteria. They are of great importance, as some of these organisms are involved in food spoilage, some are foodborne pathogens, and some are indicators of fecal contamination of food products.

The genera belonging to the Enterobacteriaceae family are often associated with intestinal infections, but can be found in almost all natural habitats. They are the causative agents of such diseases as meningitis, bacillary dysentery, and typhoid.

The most commonly encountered members of the Enterobacteriaceae in dairy products belong to 27 genera and include *Escherichia*, *Salmonella*, *Shigella*, *Yersinia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Edwardsiella*, *Erwinia*, *Morganella*, and *Providencia.* Several of the genera contain species that are psychrotrophic. Typically, Enterobacteriaceae can be isolated from 6% of pasteurized milk samples, and among the predominant species are *Hafnia alvei*, *Rahnella aquatilis*, and *Serratia liquefaciens.*

Morphology and Physiology

The Enterobacteriaceae are Gram-negative, non-spore-forming, non-acid-fast, straight rods (0.3–1.0 × 1.0–6.0 μm). They lack cytochrome oxidase and are referred to as oxidase negative. They are nonhalophilic facultative anaerobes, with optimal growth between 22 and 37 °C. Being chemoautotrophs (i.e., able to grow on simple organic carbon and nitrogen compounds), they have both respiratory and fermentative metabolism. The base composition of the DNA is 38–60 mol% GC.

An important distinguishing feature splitting the Enterobacteriaceae into two groups is the type of fermentation, either the mixed acid fermentation carried out by the *Escherichia*–*Salmonella*–*Shigella* group that is characterized by the production of acetate from pyruvate through acetyl-CoA, or the butanediol fermentation characteristic of the *Erwinia*–*Enterobacter*–*Serratia* group resulting in the formation of butanediol as the end product of fermentation.

All members of Enterobacteriaceae ferment glucose with acid production and reduce nitrates (NO_3 to NO_2 or all the way to N_2). Certain physiological groups of organisms may be recognized within the family Enterobacteriaceae. The most important of these are ‘coliforms’, which ferment lactose vigorously to acid and gas at 35–37 °C within 1 or 2 days. Most strains found in the genera *Escherichia*, *Enterobacter*, and *Klebsiella* fit the description of coliforms and are used as indicators of hygiene in food analysis. In addition, many strains of *Citrobacter* are also considered coliforms. Certain other enterics may ferment lactose, but minimal gas production and/or a lower temperature optimum for most of these organisms preclude them from being termed ‘coliforms’. In contrast, *Salmonella* and *Shigella*, which stand out as the major pathogens of the family, do not ferment lactose or sucrose. Therefore, inclusion of these sugars in plating media assists in their isolation, as nonfermenting colonies can be selected for further testing.

Another physiological group comprises the genus *Proteus* and its relatives, *Morganella* and *Providencia.* These organisms often appear on plating media used for the isolation of *Salmonella* and *Shigella* and may appear nonfermenting. A distinguishing characteristic of these organisms is their possession of the enzyme phenylalanine deaminase, for which a test can be easily carried out. Many organisms in this group also hydrolyze urea rapidly.

The members of Enterobacteriaceae are motile via peritrichous flagellae, with the exception of *Shigella* and *Klebsiella*, which are nonmotile. The Enterobacteriaceae also possess fimbriae (pili) and may have a capsule or slime layer. The cell wall is complex and the antigenic structure plays an important role for some species in epidemiology and classification.

Significance in Milk and Dairy Foods

Although milk may be contaminated by a number of routes like bovine feces, udder infection, milking personnel, and environmental sources, the most important with respect to Enterobacteriaceae is the contamination of milk by feces, which usually occurs at milking. Even with modern milking practices, it is impossible to entirely eliminate the possibility of contamination of milk. *Escherichia coli* and other Enterobacteriaceae have often been used as indicators of microbial quality and hygienic processing methods.

Milk

Consumption of raw milk is considered a risk factor for enteric infections including salmonellosis. The commercial distribution of raw milk in California has led to continuing outbreaks caused by *Salmonella* Dublin, while, in Scotland, the introduction of mandatory pasteurization eliminated the problem of milk-borne salmonellosis. However, there have been some outbreaks of salmonellosis caused by pasteurized milk, primarily due to poor plant design and incorrect operation of the pasteurizer. The largest salmonellosis outbreak where pasteurized milk was identified as the vehicle occurred in Chicago, USA in 1985, and involved 16 284 known cases, while the actual number of people affected might have been as high as 250 000. In another study of bulk tank milk in US dairies in 2004, coliforms were detected in 95% (818 of 860) of the samples. Twenty-two samples (2.6%) were found to be culture-positive for *Salmonella*, and eight serotypes identified were Montevideo, Newport, Muenster, Meleagridis, Cerro, Dublin, Anatum, and 9,12: nonmotile. A study conducted in downgraded Danish bulk tank milk during 2004 also reported the presence of coliforms in 20% of the samples.

Enterobacteriaceae were also found to be present in 212 (61.6%) goat's milk samples and 45 (71.4%) ewe's milk samples in ewe's bulk tank milk in Switzerland. *Campylobacter* spp. and *Salmonella* spp. were not isolated from any of the samples. However, 16.3% of goat's milk samples and 12.7% of ewe's milk samples were polymerase chain reaction-positive for shiga toxin-producing *E. coli*.

On the other hand, foodborne shigellosis almost invariably involves contamination by a food handler or from sewage-contaminated environmental sources such as water or soil. Thus, any dairy product that has been handled by a *Shigella*-infected person, and that is not to be heated directly before consumption, is a potential vehicle of foodborne shigellosis.

Although *Yersinia enterocolitica* is well established as an enteric pathogen, the number of cases that are associated with dairy foods remain small. Milk has been responsible for at least three large outbreaks of yersiniosis in the United States. In one such outbreak, chocolate milk was involved. The organism was introduced with the chocolate syrup, which was added after the milk was pasteurized. Pasteurized milk was linked to at least two other outbreaks among hospitalized children. The general consensus is, however, that the *Y. enterocolitica* strains isolated from milk belong to nonpathogenic serotypes.

As far as spoilage is concerned, coliforms cannot compete well at refrigeration temperatures and at a pH below 5.5, but *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* do grow well in refrigerated milk and are responsible for several milk defects that include significant proteolysis and lipolysis. These genera produce heat-stable proteases and lipases that have similar properties to those synthesized by *Pseudomonas* spp.

Dried Milk

Infant dried milk was the cause of a significant outbreak involving *Salmonella* Ealing in the United Kingdom during 1985. Insulation surrounding the drying chamber was found to be the source of contamination. A similar outbreak, involving infant formula, was reported in Australia, where the causative agent was *Salmonella* Bredeney. A survey conducted following these events suggested that *Salmonella* contamination in the environment of spray-drying plants was common.

Enterobacter sakazakii has been implicated in a rare but severe form of neonatal meningitis, with dried infant formula being implicated as the mode of transmission. The high mortality rate (40–80%) and the lack of information about this organism led to a study of the heat resistance of *E. sakazakii* in reconstituted dried infant formula. *Enterobacter sakazakii* strains (five clinical and five food isolates) were used to determine the heat resistance of this organism at 52, 54, 56, 58, and 60 °C in reconstituted dried infant formula and *D*-values of 54.8, 23.7, 10.3, 4.2, and 2.5 min were obtained for each temperature, respectively. The overall calculated *z*-value was 5.82 °C. In a comparison of the *D*-values of several members of the Enterobacteriaceae in dairy products, *E. sakazakii* was among the most thermotolerant organisms.

Cheese

Salmonella is normally destroyed or inactivated during fermentation of high-acid products (lactic acid *c.* 1%, pH value less than 4.55) such as yogurt and soft cheese. The degree of inactivation, however, was found to be less in cheese due to protection provided by casein and possibly fat. Also, *Salmonella* may grow in the curd of low-acid cheese (pH value greater than 4.95). As a small number of salmonellae may persist for significant periods, the practice of aging of raw milk cheese for 60 days at not less than 4.4 °C would not be an effective control measure.

Although cheese-borne salmonellosis is often associated with raw milk cheese, a large outbreak that occurred in Canada in 1984 was attributed to improper pasteurization. An estimated 10 000 people were affected, and the causative serovar was identified as *Salmonella* Typhimurium PT10.

Similarly, *Citrobacter freundii* in Camembert cheese was suspected in a diarrhea outbreak in Washington. In addition to being potential pathogens, Enterobacteriaceae can cause spoilage of low-acid cheeses. *Enterobacter* spp. have been shown to be involved in slimy curd spoilage of Cottage cheese and *Enterobacter aerogenes* is able to oxidize diacetyl to acetoin, a flavorless compound. This results in a cheese with a flat, bland taste.

A number of amines, such as tyramine and histamine, formed by members of the Enterobacteriaceae are toxic to humans. Cheeses, especially Swiss cheeses, can contain these biogenic amines. Amines were determined in the Emmental cheese milk, in cheese before brining, in cheese ripened for up to 49 days, and in cheese blocks or grated cheese stored for up to 5 months at 5 or 15 °C. Amine concentrations increased throughout cheesemaking, ripening, and storage. Histamine and tyramine dominated up to the end of ripening, and the concentration of putrescine and cadaverine increased during storage. Amine formation increased at higher storage temperatures, and was higher in grated than in intact cheese. Formation of biogenic amines in Emmental cheese was strongly influenced by the microflora, with high counts of Enterobacteriaceae being associated with high concentrations of putrescine and cadaverine.

Other Milk Products

Enterobacteriaceae infections have been occasionally associated with other dairy products like ice cream. In addition, cream-filled pastries are also known to constitute a foodborne disease problem. In a survey of 439 outbreaks in the United States associated with milk products, about 12.5% were attributed to *Salmonella*. Similarly, caprine raw colostrums were also found to be positive for Enterobacteriaceae.

A study conducted during the milk fermentation and preparation of buffalo's milk yogurt revealed that *S. enterica* Typhimurium PT8 had longer generation times in mixed cultures. *Lactobacillus bulgaricus* or its combination with *Streptococcus thermophilus* was found to be more inhibitory to the growth and survival of *Salmonella* than *Str. thermophilus* alone. In a recent study, *E. coli* 0157:H7 was found to survive in different yogurt products like yogurt drink, plain yogurt, and salted yogurt (yogurt native to Hatay, Turkey) at both 4 and 22 °C.

At least 2.13 $\log \text{cfu}\,\text{g}^{-1}$ of Enterobacteriaceae was reported in 'Kurut', a dairy product obtained by drying yogurt under the sun in rural areas of Turkey. Similarly, *Klebsiella*, *Escherichia*, and *Enterobacter* were found to be predominant among Enterobacteriaceae in Ethiopian traditional dairy products that included milk, butter, buttermilk, naturally fermented milks, and Cottage cheese.

Enumeration

Enterobacteriaceae are often isolated from fecal matter on agar containing lactose and a pH indicator. Colonies that ferment lactose would produce sufficient acid to cause a color shift in the indicator. For example, *E. coli* is a fermenter of lactose, while *Shigella*, *Salmonella*, and *Yersinia* are nonfermenters. Several commercial media, including ready-to-use petrifilms manufactured by 3M, are available for the detection of Enterobacteriaceae, and the International Dairy Federation has approved standard methods for their enumeration. Several tests relying on the detection of β-glucuronidase are also approved for the detection of *E. coli*, although not the enterohemorrhagic serovars.

A hydrophobic grid membrane filtration (HGMF) method for the enumeration of coliforms has also gained the status of an official method.

All Enterobacteriaceae isolates are identified biochemically using systems like API 20E manufactured by bioMérieux. Important serotypes can also be differentiated by their O (lipopolysaccharide), H (flagellar), and K (capsular) antigens.

Sources

Due to problems caused by recontamination of pasteurized milk with Gram-negative psychrotrophs, critical contamination sites for psychrotrophic Enterobacteriaceae were investigated. Milk samples were collected in three dairy plants at the silo tank; just before and just after the pasteurizer; from the buffer tank of pasteurized milk; just before the filling machine; and as filled and sealed consumer packages. There was a relatively high frequency of recontamination of refrigerated milk with psychrotrophic bacteria. Gram-negative bacteria were isolated from 40% of 87 packages, with pseudomonads being isolated from all contaminated packs, and Enterobacteriaceae from 9%. Recontamination occurred mainly during filling procedures, and it is considered that efforts to improve hygiene should be concentrated in this area.

Control

The vast majority of milk consumed in developed nations is cow's milk, although goat's milk is increasing in popularity in the United Kingdom and significant quantities of sheep's milk are consumed in Australia. In developing countries, sheep and goat may be of greater importance than cattle as sources of milk, while other sources of milk are buffalo, camels, and mares. The chemical composition of milk of different species varies considerably, but although this affects the organoleptic properties and, possibly, the development of the spoilage microflora, there is no evidence that the survival or growth of pathogenic microorganisms is significantly affected, except in camel milk.

Hazards from Enterobacteriaceae can be prevented by heating milk sufficiently to kill the bacteria, holding chilled milk and dairy products below 4.4 °C (40 °F), preventing postpasteurization cross-contamination, and prohibiting people who are ill from working in dairy food operations. The infective dose of virulent members of Enterobacteriaceae is dependent upon the particular strain, and it varies from only a few organisms to millions. For this reason, time/temperature abuse of food products may or may not result in illness.

Pasteurization is an essential process in providing milk that is free of hazardous microorganisms. Alternatively, ultra-high temperature (UHT) treatment wherein a temperature of 132 °C for not less than 1 s is employed is another popular process that has a good safety record. The microbiological problems in UHT milk are usually restricted to spoilage due to the heat-stable proteolytic enzymes alone. The same is true for milk products like cream and butter as well.

Thermal stress (curd cooking at 55 °C) during the production of Grana cheese was found to be only partially effective in the control of selected pathogens including *E. coli* 0157:H7 and *Salmonella* Typhimurium. Similarly, differences in pH values due to temperature-dependent whey retention accounted for the effect of cooking temperature on coliforms and fecal coliform counts during the manufacture and ripening of Manchego cheese prepared from ewe's milk. Although brine-salting had no effect on Enterobacteriaceae counts, a significant effect of salting time on Enterobacteriaceae and fecal coliform counts was detected. In addition to this, ripening temperature was the most important manufacturing variable with greatest influence on Enterobacteriaceae and fecal coliform counts during the whole curing period.

In concentrated milk preparation, the milk is heated to a high temperature prior to entry into the evaporator and this, together with operating temperatures in the first stages of the evaporator, would kill any vegetative pathogens present. Furthermore, the higher sugar levels in condensed milk lead to lowering of water activity, thereby preventing the growth of pathogenic organisms.

Dried milk may be made by either the roller process or the spray process. Due to the extensive heat treatment, the products generally do not pose any threat from the presence of Enterobacteriaceae in such products.

In Caprine colostrums, heat (56 °C for 60 min and 63 °C for 30 min) and high pressure (400 and 500 MPa for 10 min at 20 °C) were found to significantly reduce Enterobacteriaceae.

In addition to this, in the case of fermented milk products like hard and soft cheeses, yogurt, and several intermediate products, the heat treatment of milk and the controlled fermentation are important to prevent the growth of Enterobacteriaceae. Even in traditionally made yogurt and some unripened soft cheeses, the high acidity of the final product is sufficient to inactivate many pathogens including members of the Enterobacteriaceae. Moreover, these pathogenic contaminants are unable to grow in hard cheeses during ripening, and conditions of storage are intended to maximize the inactivation of any pathogen present. In the case of Ragusano cheese, presalting of curd (with 2% added salt) before stretching reduced the coliform counts in cheese by 1.41 log and also resulted in major reduction in early gas formation. Another approach is the addition of bacteriocin-producing adjunct cultures during cheesemaking that can help in inhibiting Enterobacteriaceae.

Prevention of recontamination is, however, the key factor in the safety of such products.

See also: **Cheese**: Mechanization of Cheesemaking. **Enzymes Indigenous to Milk**: Plasmin System in Milk. **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity. **Stress in Dairy Animals**: Heat Stress: Effects on Milk Production and Composition.

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Enterobacter spp.

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Introduction

Enterobacter species are widely dispersed in nature and exist in a diverse range of environments such as soil, water, households, food processing establishments, vegetation, and vertebrate and invertebrate hosts. *Enterobacter* are also increasingly recognized as potential human pathogens, especially as agents of nosocomial infections in the clinical environment. Currently, only *Enterobacter sakazakii* (*Cronobacter*) are considered foodborne pathogens and have been associated with cases of bacteremia, meningitis, and necrotizing enterocolitis (NEC) in infants. Powdered infant formula (PIF) has been identified as a major route of infection, and therefore these organisms are of particular concern to manufacturers of formula milk powders and milk derivatives that supply this industry.

Classification

The genus *Enterobacter* comprises a heterogeneous group of organisms in the Enterobacteriaceae family. They are Gram-negative, generally motile, oxidase-negative, nonsporing, flagellated, rod-shaped, facultative anaerobes. The genus *Enterobacter* was derived from members of the genus *Aerobacter* in 1960 when the motile and ornithine decarboxylase (ODC)-positive strains were separated as *Enterobacter* from the nonmotile and ODC-negative *Klebsiella* strains. Originally genera and species were defined based on phenotypic characteristics. The development of techniques such as DNA–DNA hybridization and nucleic acid sequence analysis has enabled the measurement of evolutionary distances and made it possible to determine more accurately the relationships between organisms. This has led to the proposed reclassification of several *Enterobacter* species and the transfer of *E. intermedius* to the genus *Kluyvera* as *K. intermedia*, *E. agglomerans* to the genus *Pantoea*, and *E. sakazakii* to the genus *Cronobacter* (**Table 1**). Species closely related to *E. cloacae*, such as *E. dissolvens*, *E. asburiae*, *E. hormaechei*, *E. kobei*, *E. ludwigii*, and *E. nimipressuralis*, are often referred to as belonging to the '*Enterobacter cloacae* complex'. All species within this complex are of clinical significance and are increasingly being implicated in cases of hospital-acquired infections. However no link has been established between ingestion of contaminated food and infection due to these species.

Enterobacter sakazakii, which was previously referred to as 'yellow-pigmented *E. cloacae*', has now been recognized as a group of multiple genomospecies and a novel genus, *Cronobacter*, has been proposed. *Cronobacter* consists of *C. sakazakii*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, and the as yet unnamed *Cronobacter* genomospecies 1. The genus *Cronobacter* is contaxic (synonymous) with *E. sakazakii*, and these organisms are known to cause serious invasive disease and are recognized as a particular problem for infection in neonates and, to a lesser extent, in older infants and immunocompromised adults. Neonatal infections have been epidemiologically linked to consumption of contaminated reconstituted PIF in neonatal intensive care units (NICUS).

Physiology

Growth

Enterobacter spp. generally grow well, aerobically and anaerobically, between 20 and 37 °C on nonselective laboratory media at neutral pH. The growth ranges of different species vary; *Cronobacter* can grow between 6 and 47 °C with a pH range of 4.5–10 at 37 °C. Most *Enterobacter* strains will grow and produce typical colonies on selective media for Enterobacteriaceae, such as Violet Red Bile Agar (containing glucose or lactose) and Hektoen or MacConkey agar. Some strains of *Enterobacter/Cronobacter* are reported to be unusually sensitive to antimicrobial agents commonly used in Enterobacteriaceae selective media, including antibiotics, brilliant green, crystal violet, bile salts, and sodium lauryl sulfate. *Cronobacter* are the only species for which specific isolation media have been developed.

Resistance

Water Activity

In general *Enterobacter* are not considered particularly resistant to low-water activity (a_w) environments. However, *Cronobacter* are noted for their ability to survive during desiccation and their persistence in dried formula for 1–2 years. High concentrations of osmolytes and humectants have been proposed as selective strategies. *Cronobacter* strains can grow in media containing up to 7% sodium chloride or 20% sucrose.

Table 1 Nomenclature of *Enterobacter* spp. and relevance to foodborne disease

Enterobacter *species*	*Taxonomic note*	*Clinical source*	*Food/environment sources*
E. aerogenes	*Klebsiella mobilis*	All sites	Ubiquitous (dairy)
E. agglomerans	*Pantoea agglomerans*[a]	All sites	Ubiquitous (dried food, PIF)
E. amnigenus	Biogroup 1 Biogroup 2	B, F, R, W	Milk, cream, Spanish pork sausage, plants (sugar beets)
E. asburiae[b]	*(Enteric group 17)*	AT, B, BF, F, L, R, U, W	Farm machinery, water, cucumber
E. cancerogenous	*E. taylorae*	AT, B, CSF, F, O, R, U, W	Trees, water, food
E. cloacae[b]	Subsp. *cloacae* Subsp. *dissolvens*	All sites	Ubiquitous (meat, dairy, infant food)
E. cowanii		B, P, SM, R, U, W	Food
E. dissolvens[b]	*E. cloacae* subsp. *dissolvens*	A, AS, B, S	Ubiquitous (plants)
E. gergoviae		AT, B, R, U, W	Water, cosmetics
E. helveticus		-	Infant food, fruit powder
E. hormaechei[b]	Enteric group 75	B, CSF, R, SM, W	Unknown (frog)
E. intermedius	*Kluyvera intermedia*[a]	BE, B, G, F, W	Water, soil
E. kobei[b]		A, B, R, F, U	Food
E. ludwigii[b]		B, R, S, F, U	Trees, plants
E. oryzae		-	Wild rice
E. nimipressuralis		-	Trees, plants
E. pyrinus		B, CSF	Trees, plants
E. radicincitans		B, CSF	Plants
E. pulveris			Fruit powder, infant food
E. sakazakii[c]	*Cronobacter* – 6 species[a]	A, AS, AT, B, CSF, F, O, R, S, SM, U, W	Ubiquitous (dried food, PIF, grains, cut meat)
E. turicensis		-	Fruit powder

[a] Current taxonomy.

[b] *Enterobacter cloacae* complex.

[c] Known to cause illness through ingestion of contaminated food.

- , no known clinical isolates; A, abdomen; AS, abscess; AT, anatomic tissue; B, blood; BE, bile; BF, body fluids; CSF, cerebrospinal fluid; F, feces; G, gallbladder; L, lochia; N, nose; O, bone; P, pus; R, respiratory tract; S, skin; SM, sputum; U, urine; W, wound.

pH

Cronobacter is classified as a moderately resistant member of the Enterobacteriaceae that can withstand transitory exposure to a pH of 3.0. It can survive for extended periods of time (>5 h) at a pH of 3.5.

Thermal Inactivation

Enterobacter do not survive pasteurization; however, there is a great diversity of thermotolerance among strains. Use of water at 70 °C has been recommended for reconstitution of powdered formula to inactivate possible *Cronobacter*/*Enterobacter* contaminants.

Antibiotic Susceptibility

Enterobacter species are naturally susceptible to a range of antibiotics. *Enterobacter hormaechei* is the species most susceptible to fosfomycin. Natural antibiotic resistance in *Enterobacter* spp. is similar to that of other Enterobacteriaceae. High-level cefoxitin resistance is also seen in species of the *E. cloacae* complex. Increasing prevalence of antibiotic resistance among *Enterobacter* spp. is an emerging problem worldwide. An increase in the use of antimicrobial agents within the hospital setting has led to the development of resistance to many β-lactam antibiotics via extended-spectrum β-lactamases (ESBLs), with ~25% of *Enterobacter* spp. being resistant to extended-spectrum cephalosporins. *Cronobacter* is notably more sensitive to antibiotics than other *Enterobacter* species.

Prevalence and Distribution

Environment and Host Reservoirs

Enterobacter are ubiquitous in the environment, and *E. cancerogenous*, *E. cloacae*, *E. asburiae*, and *Cronobacter* have been isolated from environmental and domestic water sources. Plants (including crops and trees), soil, and the microbial rhizosphere can harbor *Enterobacter* spp. They can be recovered from vertebrate and invertebrate hosts, and species of the *E. cloacae* complex can routinely be found in the feces of humans and animals. Although there are a few reports of *Cronobacter* isolates from fecal samples of asymptomatic colonized individuals, these organisms are not recognized as normal inhabitants of the human gut. In a farm environment, *Cronobacter* have been found in dried pellets of animal feed but not in cattle feces. *Cronobacter* strains were previously tested in a model of a bovine gut to establish whether they can survive a bovine digestive system. No survival of strains was recorded, which indicates that cattle may not be an important vector in the transmission of *Cronobacter*.

Food Contamination

Enterobacter species can be found on a wide range of foods. These include fresh, frozen, or powdered fruits and vegetables, legume products, tea, herbs, spices, animal feed (dried pellets), grains, nuts, seeds, flour, pasta, chocolate, beverages, and water. Other food sources include meat, fish, eggs, dairy products, and PIF. *Enterobacter cloacae* is a recognized contaminant of raw milk and dairy produce such as yogurt and cheese. Although they do not survive pasteurization, *Enterobacter* have been found in pasteurized milk and cream and in dried dairy products, possibly due to postprocess contamination.

PIF is used as an alternative to breast milk to provide nutritional requirements in addition to breast-feeding or where breast-feeding is not feasible. While ready-to-use liquid formula is marketed as sterile, the powdered form (including dried bovine milk and milk products) often contains bacterial spores and may contain low levels of pathogenic microorganisms. The most frequently isolated Enterobacteriaceae species from powdered milk products are *E. cloacae*, *Cronobacter*, *E. agglomerans* (*Pantoea*), *E. pulveris*, *E. helveticus*, and *Klebsiella pneumoniae.* Currently the recovery of *Cronobacter* from PIF is reported to be at approximately 2% of commercially available packages, and illness is reported to be at approximately six incidents per year. Therefore, the vast majority of contaminated formula consumed does not result in infection and the organisms have relatively low pathogenicity. Investigation of potential contamination routes in infant formula factories has indicated that raw milk and liquid ingredients are probably not the primary hazard. A diverse range of Enterobacteriaceae enter as contaminants in dry raw ingredients, via inefficient air filtration, water leaks, and human carriage. The majority of factory environment samples contaminated with *Cronobacter* appear to be found around spray dryer towers; this may be related to the relatively high desiccation resistance of *Cronobacter* as compared to other Enterobacteriaceae.

Foodborne Outbreaks

The earliest report of *Cronobacter* contamination in powdered milk was in 1950. The first case of neonatal illness attributed to *Cronobacter* occurred in London in 1958. The link between neonatal illness and PIF was first proposed in 1983. A number of outbreaks in NICUs have been epidemiologically linked to consumption of contaminated food, particularly reconstituted infant formula (**Table 2**).

In Iceland (1986), three neonatal cases shared identical biotypes, plasmid DNA profiles, and antibiograms with strains isolated from infant formula. In Belgium (1998), arbitrarily primed PCR typing partially confirmed strain similarity between isolates from milk and those from patients in an outbreak of necrotizing entercolitis. In the United States (2001), pulsed-field gel electrophoresis (PFGE) showed that *Cronobacter* isolates from opened and unopened containers of a nutritional supplement were indistinguishable from the isolate obtained from a neonate with meningitis. Finally, in France (2004), cases occurring in five hospitals were linked to the use of a hypoallergenic formula, which was subsequently recalled by the manufacturer. Investigation revealed failures in hospital practices with regard to the preparation, handling, and storage of feeding bottles. In some instances no direct link has been made to batches of PIF. In these cases extrinsic contamination of prepared formula and horizontal transmission from other infected/colonized hosts are the possible causes of infection. Multiple strains of *Cronobacter* have simultaneously been isolated from the

Table 2 Clinical cases caused by *Enterobacter* linked to milk

Region	*Countries*	*Cases (deaths)*	*Milk product suspected*	*Children (deaths)*	*Adult (deaths)*
Africa	Ethiopia	NS	NS	NS	0
Asia	China, India, Israel, Korea, Singapore, Thailand	13 (2)	5	9 (1)	4 (1)
Europe	Belgium, Czechoslovakia, Denmark, England, France, Germany, Greece, Iceland, Italy, Netherlands, Portugal, Scotland, Spain, Switzerland	85 (24)	54	76 (24)	9
North America	Canada, Mexico, USA	39 (6)	16	33 (3)	6 (3)
Oceania	New Zealand	5 (1)	55 (1)	0	
Total	Worldwide	142 (33)	80	123 (29)	19 (4)

NS, not stated.

same food containers, or single infected children, further complicating the epidemiology.

Clinical Relevance

Enterobacter spp. are increasingly recognized as opportunistic pathogens in a wide variety of settings and rarely cause disease in healthy individuals. They are common contaminants of hospital surfaces, medical supplements, catheters, and other medical and feeding equipment, as well as medical staff. In general, *Enterobacter* organisms are responsible for around 50% of nosocomial infections, mostly in immunocompromised patients, and can affect people of all ages. Community-acquired infections can occur through open wounds or severe crush injuries. *Enterobacter aerogenes* and the *E. cloacae* complex are the most frequently encountered *Enterobacter* species in clinical samples. Immunocompromised individuals are at particular risk of acquiring infection. *Enterobacter cloacae*, along with *E. aerogenes*, *E. hormaechei*, *E. gergoviae*, and *Cronobacter*, has been associated with infections in neonates. Risk factors in neonates include premature birth and low birth weight. Infants of normal gestational age and birth weight infected during the first month postpartum are more likely to develop meningitis, with high case fatality rates as compared to premature, low-birth-weight infants, who usually develop later onset of infections resulting in bacteremia. This may be due to sterile, parenteral initial feeding and administration of prophylactic antibiotics prior to exposure to contaminated nutritional products. *Cronobacter* infections are uncommon in adults and mainly comprise cases of wound infection, bacteremia, and aspiration pneumonia in elderly patients.

Necrotizing Enterocolitis

NEC is an inflammatory process of the small and large intestine. This condition is one of the most common causes of death in NICUs and the most relevant acquired intestinal complication during the neonatal period. NEC has an incidence of 2–5% in all preterm infants and up to 13% in those with low birth weight. The reported case fatality rates range between 0 and 20% in infants weighing >2500 g and between 10 and 55% in low-birth-weight infants (<1500 g). Initial symptoms include feeding intolerance, delayed gastric emptying, abdominal distension and/or tenderness, decreased bowel sounds, and bloody stools. NEC has no definitive known cause, and research suggests involvement of a combination of factors leading to intestinal damage, including intestinal mucosal immaturity/dysfunction, ischemia and/or reperfusion injury, the release of inflammatory mediators and bile acids, and downregulation of cellular growth factors. Empirical antibiotic use and intestinal bacteria are thought to be associated factors, though no common infectious agent has been identified. Decreased levels of bactericidal/permeability-increasing protein (BPI), particularly in premature low-birth-weight neonates, may be significant in cases where bacterial invasion is a contributing factor. BPI binds to lipopolysaccharides produced by Gram-negative organisms neutralizing the endotoxin and reducing intestinal wall disruption. NEC is rarely seen in infants prior to initiation of oral feeding. The use of infant formula as opposed to breast milk greatly increases the risk of NEC. Infant formula contaminated with *Cronobacter* has been associated with outbreaks of NEC.

Bloodstream Infections

Bacteremia is the presence of viable bacteria in the bloodstream, whether associated with active disease or not. When associated with the release of bacterial toxins into the circulation, bacteremia can elicit a vigorous immune response resulting in systemic inflammatory response syndrome. This is characterized by rapid breathing, low blood pressure, and fever, which may lead to multiple organ failure (septic shock). *Enterobacter* cause 3–9% of all bloodstream infections and up to 15% of bacteremia in the elderly. Case fatality rates for *Enterobacter* sepsis range from 20 to 50%. *Enterobacter cloacae*, followed by *E. aerogenes*, is the species most frequently isolated in *Enterobacter* bacteremia and is responsible for over 90% of all cases. *Enterobacter asburiae*, *E. cancerogenous*, *E. amnigenus*, and *Cronobacter* have also been reported to cause bacteremia in adults. *Enterobacter* are estimated to account for up to 8.7% of all bacteremia and approximately 20% of Gram-negative sepsis cases in children, with case fatality rates of 6–20%. Most infections are nosocomially acquired, with molecular fingerprint epidemiology suggesting that horizontal transmission plays a significant role. Risk factors associated with *Enterobacter* sepsis in children include parenteral nutrition and use of antibiotics and catheters. As with adults, *E. cloacae* and *E. aerogenes* are responsible for over 90% of cases. However, *Cronobacter* are notable for their association with cases in neonates and links to ingestion of contaminated infant food. *Enterobacter hormaechei* and *E. gergoviae* have also been responsible for outbreaks in NICUS.

Central Nervous System Infections

Enterobacter are rare aetiological agents of central nervous system (CNS) disease. Bacterial meningitis is a serious and frequently fatal infection of the protective membranes covering the brain and spinal cord. Infections can increase inner cranial pressure, requiring aspiration of fluid and drainage of cerebral infarction to prevent cerebral damage. Meningitis can lead to long-term complications in survivors, including mental and motor disabilities, convulsive disorders, hydrocephalus, and deafness. Common symptoms include headache, neck stiffness, fever, confusion/delirium, vomiting, and photophobia. These symptoms may not be obvious in young children, who may present with irritability and lethargy. *Enterobacter* spp. are estimated to cause 2.4–4.5% of meningitis cases in adults and up to 10.4% of cases in children. In adults the main species implicated are *E. cloacae* and *E. aerogenes* with a case fatality rate of 16–20%. However, in children *Cronobacter* are the most frequently reported species with case fatality rates of up to 80%. Ingestion of contaminated infant formula has been implicated in several outbreaks. Low birth weight, prematurity, length of hospital stay, invasive procedures, and overuse of antibiotics put infants at an increased risk. Patients with *Cronobacter* neonatal meningitis may suffer more severe outcomes than those with meningitis attributed to *E. cloacae* and other Gram-negative bacteria. *Cronobacter* causes cystic changes, abscesses, fluid collection, dilated ventricles, and infarctions. Cases of *Cronobacter* meningitis have been reported in children between the ages of 3 days and 4 years, with half of the cases occurring in the first week after birth and almost three-quarter during the first month. A retrospective study of 46 infants with *Cronobacter* infections indicated that meningitis was more prevalent in infants of normal gestational age and birth weight, with the onset of disease usually occurring within the first week following birth. In contrast, low-birth-weight, premature infants were more likely to develop bacteremia with no progression to CNS disease, and the age of onset was usually over 1 month. It is probable that *Cronobacter* has a developmental dependence on access to the CNS.

Other Infections

Enterobacter species cause up to 5% of nosocomial pneumonias, being a significant cause of ventilator-associated and early post-lung transplant pneumonia. They are also associated with other lower respiratory tract infections. Case fatality rates are particularly high in elderly patients. *Enterobacter* skin and soft-tissue infections include cellulitis, fasciitis, myositis, abscesses, and wound infections (including burns and crush injuries). *Enterobacter* species can infect wounds in any body site. Most are nosocomially acquired, with surgical complications and cephalosporin prophylaxis being associated with increased risk of infection. *Enterobacter* species that colonize the digestive tract may be isolated from intraabdominal sites following intestinal perforation or surgery and are responsible for approximately 10% of postsurgical peritonitis cases. A few examples of endocarditis due to *Enterobacter* have been reported. In most cases there was underlying cardiac disease, particularly mitral valve infection. *Enterobacter* cause up to 4% of nosocomial urinary tract infections (UTIs), usually associated with urinary catheters and/or prior antibiotic therapy. They are also occasionally associated with osteomyelitis and septic arthritis in adults and children. *Enterobacter* bone and joint infections can be difficult to cure, with relapses requiring additional treatment.

Isolation, Identification, and Subtyping

Detection Methods

There are no microbiological media specifically designed for the genus *Enterobacter*. These organisms grow well on nonselective laboratory media and can be easily cultured from clinical samples. All *Enterobacter* species ferment glucose, and the majority ferment sucrose (except *E. cancerogenous*) and lactose (except *E. cancerogenous* and *E. hormaechei*). Therefore most *Enterobacter* isolates will appear as typical Enterobacteriaceae colonies on selective media for this family, such as Violet Red Bile, MacConkey, or Hektoen Enteric agars. *Enterobacter* are not generally considered foodborne pathogens but contribute to the overall microbial presence in foods and, as members of the Enterobacteriaceae, are relevant as hygiene indicators in food production processes. The process hygiene criteria for pasteurized milk, milk/whey powder, and dried infant formula allow only very low levels of Enterobacteriaceae (less than 5 cfu ml^{-1}, and 10 and 0.01 cfu g^{-1}, respectively). The EC regulation for the detection of Enterobacteriaceae refers to ISO 21528-1:2004, which entails preenrichment in buffered peptone water (BPW), followed by selective enrichment in Enterobacteriaceae enrichment (EE) broth, streaking on Violet Red Bile Glucose (VRBG) agar, and confirmation of typical colonies based on negative oxidase activity and positive glucose fermentation.

Owing to their status as neonatal pathogens associated with infant formula, specific isolation methods have been developed for *Cronobacter*. Initially these were based on ISO 21528-1:2004 mentioned above, followed by culturing of Enterobacteriaceae isolates on nonselective media at a low temperature (25 °C) and biochemical confirmation of yellow-pigmented colonies. In recent years various fluorogenic and chromogenic media have been developed for the detection of *Cronobacter*. These are mainly based on detection of the enzyme α-glucosidase, which is constitutively expressed in *Cronobacter* spp. However, *E. helveticus*, *E. pulveris*, and *E. turicensis*, which can be found in the same ecological niches as *Cronobacter* (including dried food products and factory environments), can also produce presumptive colonies on these agars.

There is currently an ISO technical specification for the detection of *Cronobacter* in milk-based infant formula (ISO/TS 22964:2006). This entails preenrichment in BPW and selective enrichment in a modified lauryl sulfate tryptose (mLST) broth (incorporating 0.5 mol l^{-1} NaCl and 10 mg ml^{-1} vancomycin hydrochloride), followed by streaking on a chromogenic agar. It has been reported that some *Cronobacter* strains do not grow well in selective media commonly used for Gram-negative organisms, and a differential method, incorporating *Cronobacter* screening broth (10 g l^{-1} peptone, 3 g l^{-1} meat extract, 5 g l^{-1} NaCl, 0.04 g l^{-1} bromocresol purple, 10 g l^{-1} sucrose, and 10 mg l^{-1} vancomycin hydrochloride) in place of mLST, has been proposed for the EN ISO horizontal standard currently in development. Carbohydrate fermentation results in a color change from purple to yellow, and only positive (yellow) broths are streaked onto chromogenic media.

The US Food and Drug Administration (USFDA) proposes a method involving preenrichment, centrifugation, plating on two chromogenic agars (one containing a chromogenic β-D-cellobioside in addition to α-D-glucopyranoside), and a real-time PCR assay based on the *dna*G gene, which is a component of the macromolecular synthesis (MMS) operon.

Alternative methods for the detection of *Cronobacter* include the MATRIX® PSAK50 cationic paramagnetic particle capture technique and enzyme-linked immunoassays (Assurance® and TECRA HELIX®). Genetic-based assays include the BAX® and foodproof® Enterobacteriaceae plus *E. sakazakii* detection systems. The latter simultaneously qualitatively detect Enterobacteriaceae DNA and the presence of *Cronobacter*. Both DNA-FISH (Vermicon®) and PNA-FISH techniques have been developed for the detection of viable *Cronobacter* cells in infant formula.

Phenotypic Identification

The *Enterobacter* genus is heterogeneous and difficult to define using biochemical criteria. Owing to their close relationships, phenotypic identification of individual *Enterobacter* species can also be unreliable. To ensure the safety of infant formula and at the same time to reduce unnecessary disposal of product, it is important to identify between *Cronobacter* and *Enterobacter* species. *Cronobacter* can generally be distinguished from *Enterobacter* species based on hydrolysis of 5-bromo-4-chloro-3-indolyl-α-D-phosphate, ornithine decarboxylation, and use of the 2,3 butanediol fermentation pathway (determined by Methyl Red and Voges–Proskauer reactions). Individual *Cronobacter* species can be separated by their different phenotype profiles using fermentation of 1-0-methyl-alpha-D-glucopyranoside and dulcitol combined with production of indole and utilization of malonate (**Table 3**). The reliability of some biochemical confirmation galleries such as API 20 E has been questioned as species of the *E. cloacae* complex can be misidentified as *E. sakazakii* (*Cronobacter*). The ID 32 E kit has been proposed as a more accurate alternative, successfully confirming 90% of isolates with no false positive or false negative results. The use of yellow pigmentation as an identification for *Cronobacter* is unreliable and has been discontinued.

Molecular Identification

Oligonucleotide probes have been designed to detect the 16S and 23S rRNA gene sequences of the family Enterobacteriaceae; however, there are no molecular probes designed specifically to identify the genus *Enterobacter*. Multilocus sequence analysis (MLSA) using the *recN*, *rpoA*, and *thdF* genes can be used to extrapolate genetic similarities between the species of Enterobacteriaceae. The *rpoA* and *rpoB* gene sequences provide useful diagnostic tools to identify and differentiate species of this family and can be more discriminatory than 16S rRNA sequencing. A number of molecular approaches have been developed to identify *Cronobacter*. Targets for conventional PCR assays include the 16S rRNA gene, the *ompA* gene, the gene coding for the 1,6 α-glucosidase, and a gene encoding a zinc-containing metalloprotease. Real-time PCR assays target the 16S rRNA gene, the region located between the 16S and 23S rRNA genes, the region between the tRNA-glu and 23S rRNA genes, and the *dna*G gene in the MMS operon. The α-glucosidase and *dna*G gene-based methods have proved to be 100% sensitive and specific for *Cronobacter* using a broad panel of target as well as non-target strains.

Subtyping Methods

A number of approaches have been developed that can be used to characterize strains of *Enterobacter*, including antibiograms, biotyping, serogrouping, plasmid profiling, ribotyping, random amplification of polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), repetitive sequence-based PCR (rep-PCR), enterobacterial repetitive intergenic consensus (ERIC) PCR, amplified fragment length polymorphism (AFLP), and PFGE. Typing techniques are more discriminatory and reliable than classic techniques such as biotyping or antibiograms. Although there is frequent plasmid carriage within *Enterobacter* species, problems can arise due to plasmid instability and lack of extrachromosomal elements in some strains. PFGE has been used to investigate outbreaks in NICUs involving *E. cloacae*, *E.aerogenes*, *E. gergoviae*, and *Cronobacter* and is currently seen as the ‘gold standard’ for molecular subtyping of

Table 3 Differentiation of *Enterobacter* species

	Enterobacter sakazakii *(Cronobacter)*					*Other* Enterobacter												
Test	C. dublinensis	C. malonaticus	C. muytjensii	C. sakazakii	C. turicensis	E. aerogenes	E. cancerogenous	E. cloacae[a]	E. cowanii	E. gergoviae[b]	E. helveticus	E. hormaechei	E. ludwigii[c]	E. oryzae	E. pulveris	E. radicincitans	E. turicensis	
Oxidase	−					−												
Hydrolysis of 5-bromo-4-chloro-3-indolyl-α-D-phosphate	+					−	−	−	ND	−	+	−	ND	ND	+	ND	+	
Lysine decarboxylase	−					+	−	−	−	+	−	−	(−)	+	−	−	−	
Ornithine decarboxylase	+							+		+		+		−		+		− + + + − − −
Methyl Red	−					−	−	−	−	−	+	v	−	+	(+)	−	+	
Voges–Proskauer	+					+	+	+	+	+	−	+	+	+	(−)	+	−	
Indole production (Kovacs)	+	−	+	−	−	−	−	−	−	−	−	−	−	ND	−	−	−	
Malonate utilization	(−)	+	+	−	+	+	+	v	−	+	(+)	+	−	ND	−	+	+	
Yellow pigmentation	70−90% of strains; can be transient					−	−	−	v	−	+	−	−	−	+	−	+	
Acid from:																		
D-Arabitol	(−)	−	−	−	−	+	−	(−)	−	+	+	−	−	−	(+)	ND	+	
Dulcitol	−	−	+	−	+	−	−	(−)	+	−	+	(+)	−	+	v	ND	+	
α-Methyl-D-glucoside	+	+	−	+	+	+	−	(+)	−	−	−	(+)	+	+	−	−	−	
D-Sorbitol	−					+	−	+	+	−	−	−	+	+	−	+	−	
D-Sucrose	+					+	−	+	+	+	−	+	+	+	+	+	−	

[a]Similar percentages are obtained for *E. asburiae*, *E. amnigenus*, *E. dissolvens*, and *E. nimipressuralis* (with the exceptions that *E. asburiae* does not utilize malonate and *E. nimipressuralis* does not produce acid from D-sucrose).

[b]Similar percentages are obtained for *E. pyrinus*.

[c]Similar percentages are obtained for *E. kobei* except that some strains produce acid from dulcitol.

ND, no data; −, 0–10; (−), 11–20; v, 21–79; (+), 80–89; +, 90−100% of strains positive.

foodborne pathogens. The most common restriction enzymes used for *Enterobacter* are *Xba*I, *Spe*I, *Not*I, and *Sma*I. PFGE has been successfully used to analyze the dissemination of *Cronobacter* strains within infant formula and milk protein factories. A standard protocol for *Cronobacter* PFGE typing is currently being developed by the PulseNet International Program.

Prevention and Control

Manufacture

Owing to the high costs associated with product recalls, the risk associated with an unsafe product, and the impact of negative media attention, it is critical that *Enterobacter* and *Cronobacter* spp. are kept under control along the infant formula production chain. Hygienic standards within facilities are key to keeping contamination events to a minimum. Accumulation of food residues on structural surfaces can harbor potential pathogenic bacteria and act as continuous culture systems. Air is a potential source of contamination, and *Cronobacter* and *Enterobacter* spp. have been isolated from air filters. Contamination in infant formula production facilities has been previously found in the form of dust, water droplets, and airborne microorganisms. General handling of materials, spray drying, and milling and cleaning operations can create aerosols that disseminate through the plant. Molecular profiling of isolates from production facilities can identify the persistence of clonal strains and key contamination points within the manufacturing environment. This provides a basis to develop and implement continuous quality control and prevention strategies.

Contamination of milk powders by *Enterobacter* spp. may occasionally be due to failures in the pasteurization process or more likely due to postdrying contamination during mixing with other ingredients, packing, and filling. Over three-quarters of raw milk concentrate and 85% of the nonproduct processing line may be positive for Enterobacteriaceae. It is not possible with current technology to eliminate Enterobacteriaceae from a manufacturing plant but effective cleaning-in-place (CIP) strategies and limited passage of personnel into high-risk areas can act as control measures. Sampling protocols should be implemented to monitor plant hygiene and presence of potential pathogens. Reduction in Enterobacteriaceae levels in many factories has been achieved by implementing a policy of systematic dry cleaning when possible. It is critical to maintain a low-moisture environment postpasteurization, and the absence of water prevents proliferation and dissemination of microorganisms. PIF has a low water activity (∼0.2); therefore, this desiccated state limits the survival of many Gram-negative organisms. *Cronobacter* have been previously seen to be very resistant to osmotic stress and drying in comparison to other *Enterobacter* spp.

Ineffective cleaning of the processing equipment promotes the buildup of residues where the bacteria can grow and proliferate and subsequently contaminate the powder. Adhesion of microorganisms and the formation of biofilms are a concern in the food industry. Bacterial biofilms that are difficult to remove via CIP procedures can form within the equipment and be a constant source of contamination. They may compromise food quality and represent a significant public health risk. It is known that several groups of bacteria can attach to surfaces commonly found in the manufacturing environment, such as stainless steel and rubber. Bacterial within a biofilm may be more resistant to disinfectants and sanitizers when compared to bacteria in a planktonic state.

Consumers

Education of end users is an important factor in preventing foodborne illness due to contaminated infant foods. There is an ongoing debate as to the inclusion of warnings on packaging that powdered breast milk substitutes are not sterile products. The FAO/WHO recommends that powdered breast milk substitutes be reconstituted with water at 70 °C to reduce the risk posed by any contaminating *Enterobacter*.

In several outbreaks in hospital settings the feed preparation equipment has been found to be the likely source of contamination. Guidelines on preparation, storage, and feeding times have been issued to professionals to reduce the risk of further outbreaks in NICUs. These include the recommendation that sterile ready-to-use products be used whenever possible and appropriate hygiene and sterilization protocols be in place.

Conclusions and Future Perspectives

The WHO recommends exclusive breast-feeding for up to 6 months and then complementary feeding with continued breast-feeding for 2 years and above. Natural breast milk has been shown to enhance the neonatal gut barrier and protect against infection, diarrheal disease, and also NEC. PIF is used as an alternative to breast milk to provide nutritional requirements in addition to breast-feeding or where breast-feeding is not feasible. PIF constitutes over 80% of the infant formula used worldwide.

The focus to date has been on *Cronobacter* (*E. sakazakii*) in infant formula, with contamination by other *Enterobacter* being given less consideration. *Enterobacter* spp. have the ability to cause infections and have been previously seen to contaminate infant formula. The FAO/WHO categorizes *Cronobacter* and *Salmonella enterica* as Category A organisms with a clear evidence of causality, whereas Category B organisms (causality plausible, but not yet demonstrated) include *E. cloacae* and *E.* (*Pantoea*) *agglomerans*. For organisms that are regularly acquired by vertical transmission during birth or are endemic in the hospital environment, it is less likely that sufficient epidemiological evidence may be found to link cases of infection to powdered breast milk substitutes. However, measures implemented to control *Cronobacter* contamination also reduce the likelihood of the presence of other Enterobacteriaceae.

Understanding of the physiology and survival strategies of *Cronobacter* is an important step in the efforts to eliminate this bacterium from the critical food production environments.

Further Reading

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Listeria monocytogenes

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Introduction

Listeria monocytogenes, the causative agent of listeriosis in humans and animals, was first isolated by British researchers at Cambridge University in 1924 from the blood of infected rabbits. These animals exhibited a typical monocytosis, after which the bacterium was named. Although widely recognized as a cause of miscarriage in pregnant women and meningitis, encephalitis, and septicemia in newborn infants and immunocompromised adults, this organism did not emerge as a serious foodborne pathogen until 1985. Unlike most other foodborne illnesses, the outcome of listeric infections can be particularly devastating with a mortality rate of about 20%. During the 1980s, three major dairy-related outbreaks of listeriosis – two in the United States and one in Switzerland – were linked to consumption of pasteurized milk, Mexican-style cheese, and Vacherin Mont d'Or soft-ripened cheese, which together resulted in over 100 fatalities. These outbreaks, combined with a then presumed low oral infectious dose for susceptible individuals, prompted the United States to institute a policy of 'zero tolerance' for *L. monocytogenes* in all cooked/ready-to-eat foods, including dairy products. Since 1985, over 112 Class I recalls have been issued in the United States for *Listeria*-contaminated domestic and imported cheeses with an additional 58 Class I recalls involving unfermented dairy products, principally ice cream, at a financial cost exceeding $120 million. Although *L. monocytogenes* accounted for 13 of 18 (72%) dairy-related Class I recalls issued during 1994 and 1995, only 1 of 36 *Listeria*-related Class I recalls in 2000 involved a dairy product (domestically produced Cheddar cheese). Furthermore, the fact that only 29 *Listeria*-related recalls involving 27 cheeses and 2 other dairy products were issued since 2001 indicates that dairies in the United States and elsewhere have generally taken sufficient measures to minimize *Listeria* contamination within their processing facilities. However, in the United States the availability of Queso Fresco and other soft Mexican-style cheeses that have been illegally prepared from raw milk or have been illegally imported has become an increasing public health concern among the growing Hispanic population.

Characteristics of *Listeria* spp.

The genus *Listeria*, which is included among the coryneform bacteria, contains six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*; the first three species are β-hemolytic on laboratory media containing blood. *Listeria monocytogenes* is the only *Listeria* species that is of public health significance as a foodborne pathogen. *Listeria ivanovii*, widely recognized as pathogenic to domestic livestock, only rarely infects humans as is also true for *L. seeligeri*. *Listeria innocua*, the most commonly isolated species, is nonpathogenic as is also generally true for *L. welshimeri* and *L. grayi*.

Listeria monocytogenes is a Gram-positive, non-spore-forming, facultatively anaerobic, short, rod-shaped bacterium that occurs singly or in short chains. The organism is psychrotrophic, generally growing in nonselective laboratory media at temperatures between 1 and 45 °C with optimal growth occurring at 30–37 °C. However, *L. monocytogenes* strains reportedly can grow at temperatures as low as −0.1 °C in pasteurized milk during extended storage. At 4 °C, growth of *Listeria* is somewhat faster with generation times of 30–40 h. However, the growth rate triples (generation times of 10–13 h) when milk is held at mildly abusive temperatures (8 °C). When incubated at room temperature, broth cultures of *Listeria* exhibit a characteristic tumbling motility, which can be seen microscopically. Colonies on clear laboratory media are small, smooth, and bluish-gray when examined under obliquely transmitted light. Biochemically, all listeriae produce catalase and ferment glucose to acid without producing gas, whereas typical *L. monocytogenes* isolates ferment rhamnose but not xylose. All *Listeria* species hydrolyze aesculin, which leads to a characteristic blackening of commonly used *Listeria*-selective enrichment (e.g., Fraser broth) and plating media (e.g., modified Oxford agar) that contain aesculin and ferric ammonium citrate. In addition to a wide range of commercial biochemical-, antibody-, and DNA-based test kits for identifying *L. monocytogenes*, several chromogenic plating media have become available that can differentiate *L. monocytogenes* from other *Listeria* species.

Of particular concern to the dairy industry is the ability of *L. monocytogenes* to tolerate environmental extremes found in dairy processing facilities, grow at pH 4.3–10.0, grow in the presence of up to 10% NaCl (a_w 0.92), survive for several months in refrigerated 25.5% NaCl brine tanks, and develop limited tolerance to heat and acid. Based on somatic (O) and flagellar (H) antigens, 13 different *L. monocytogenes* serotypes have been identified. Serotype 4b has been most commonly associated with outbreaks of human illness followed by serotypes 1/2b and 1/2a, with most of the major dairy-related outbreaks traced to just a few strains of serotype 4b that have now been well characterized by various genetic typing methods including pulsed-field gel electrophoresis (PFGE) and ribotyping.

Symptoms of Listeriosis

Listeriosis, the disease caused by *L. monocytogenes*, is a relatively rare infection that most often occurs in three well-defined risk groups, namely pregnant women, newborn infants, and immunocompromised adults, with the last group including the elderly and individuals with predisposing conditions such as cancer, organ transplants, cirrhosis of the liver, and HIV/AIDS infections. Approximately 2500 cases occur annually in the United States, with 1–4 cases per 10^6 population reported in most developed countries. Unlike other more common foodborne illnesses caused by *Campylobacter* and *Salmonella*, listeriosis has a mortality rate of approximately 20%, making it among the deadliest of the foodborne diseases with an estimated annual cost of $2.33 billion in the United States. In addition to host susceptibility, development of listeriosis in humans is also affected by gastric acidity, inoculum size, and variations in virulence between different strains of *L. monocytogenes*. Despite repeated exposure of the general population to *Listeria* through the food supply, individuals not included in the three well-defined risk groups seldom develop invasive listeriosis, with healthy individuals rarely infected. However, a far less severe form of noninvasive listeriosis characterized by symptoms of gastroenteritis has also been described, including one well-documented outbreak traced to highly contaminated chocolate milk. Based on several recent risk assessments, ingestion of foods containing $>10^6$ organisms g^{-1} is now presumed to be responsible for the majority of invasive listeriosis cases among susceptible individuals.

Invasive listeriosis in immunocompromised adults frequently leads to meningitis, encephalitis, or septicemia. Symptoms that develop suddenly after an initial incubation period of 2–70 days include severe headache, dizziness, stiff neck or back, incoordination, and other disturbances of the central nervous system. Without proper antibiotic therapy, approximately 20% of those infected will die, with some survivors developing permanent neurological complications. In pregnant women, *L. monocytogenes* produces a mild flu-like illness characterized by sudden chills, fever, sore throat, headache, dizziness, lower back pain, discolored urine, and occasionally diarrhea. While expectant mothers almost invariably recover without complications, infection of the fetus can result in miscarriage, stillbirth, or premature delivery of an infant with perinatal septicemia – a severe infection of the respiratory, circulatory, and central nervous systems that can either terminate fatally or lead to permanent mental retardation.

Two factors, namely the growth of *L. monocytogenes* as an intracellular pathogen within macrophage cells of the spleen and liver and the inability of many antibiotics to effectively penetrate the blood–brain barrier, complicate treatment of listeric infections. Hence, a favorable prognosis depends on rapid diagnosis and appropriate antibiotic therapy, with oral administration of large doses of amoxicillin together with an aminoglycoside (e.g., gentamicin) for 2–4 weeks now the recommended treatment.

Outbreaks

The oral route for listeriosis was established from animal feeding studies conducted during the 1920s. However, evidence for *L. monocytogenes* as a human foodborne pathogen did not emerge until the 1950s when a sharp increase in stillbirths was observed among pregnant women in post–World War II Germany who consumed raw milk, sour milk, cream, and Cottage cheese (**Table 1**).

The eventual isolation of identical *L. monocytogenes* serotypes from a mastitic cow and stillborn twins whose

Table 1 Dairy-related listeriosis outbreaks involving 10 or more cases

Location	*Year*	*Number of cases*	*Product*
Halle, Germany	1949–57	~100	Raw milk, sour cream, cream, Cottage cheese
Massachusetts, USA	1983	49	Pasteurized milk
California, USA	1985	~300	Mexican-style cheese
Vaud, Switzerland	1983–87	122	Vacherin Mont d'Or cheese
Illinois, Michigan, Wisconsin, USA	1994	66	Chocolate milk
France	1995	33	Brie de Meaux cheese
France	1997	14	Pont l'Évêque cheese
Finland	1998–99	25	Butter
North Carolina, USA	2000	13	Mexican-style cheese
Quebec, Canada	2002	17	Raw milk cheese
Quebec, Canada	2008	22	Raw milk soft cheese

mother consumed the same raw milk before delivery confirmed raw milk as the source of infection. Despite the presence of *L. monocytogenes* in about 2.5–5% of the raw milk supply from the United States and most other developed countries, current pasteurization practices are sufficient to destroy *Listeria* in raw milk. Few additional cases have been traced to raw milk since most milk is now pasteurized before consumption to eliminate *Listeria* and other pathogens.

In 1981, the status of *L. monocytogenes* as a foodborne pathogen began to change following a major outbreak in the Maritime Provinces of Canada that was traced to consumption of contaminated coleslaw. Two years later, one particular brand of pasteurized milk in the United States was epidemiologically linked to 42 adult and 7 infant cases of listeriosis in Massachusetts. Fourteen patients died, giving a mortality rate of 29%. Inspection of the milk-processing facility failed to uncover any evidence of improper pasteurization or postpasteurization contamination. Although the dairy factory received milk from farms on which veterinarians diagnosed several cases of bovine listeriosis during the outbreak, *L. monocytogenes* was never recovered from the incriminated milk, which in turn raises questions concerning the role of pasteurized milk in this outbreak. In 1994, an unusual outbreak was reported in the United States in which consumption of pasteurized chocolate milk was directly traced to 66 cases of illness in Illinois, Wisconsin, and Michigan. Unlike previous outbreaks, symptoms of gastroenteritis predominated, with only four individuals requiring short-term hospitalization. Postpasteurization contamination of the chocolate milk followed by repeated episodes of temperature abuse allowed this atypical strain of *L. monocytogenes* serotype 1/2b to attain populations of 10^8–10^9 cfu ml^{-1} in the milk at the time of consumption. Except for one additional outbreak recently traced to consumption of butter in Finland, repeated attempts have generally failed to confirm culturally other unfermented dairy products, including fluid milk and ice cream, as vehicles of listeric infection.

Ingestion of *Listeria*-contaminated cheese has been more commonly linked to listeriosis, with seven major outbreaks and numerous sporadic cases having been reported. The first and largest of these outbreaks occurred in the United States in the Los Angeles area during the first half of 1985 and involved an estimated 300 cases. Consumption of California-made Jalisco brand Mexican-style cheese contaminated with *L. monocytogenes* serotype 4b was linked to 142 listeriosis cases in Los Angeles County alone, including 48 deaths (mortality rate of 34%). The contaminated cheese was subsequently recalled nationwide. Factory records indicated that raw milk may have been intentionally added to pasteurized milk used in cheesemaking. Although not isolated from the incoming raw milk supply, the epidemic strain was widespread in the factory environment, which also suggests ample opportunity for postpasteurization contamination.

In the second of these outbreaks, consumption of Vacherin Mont d'Or – a soft surface-ripened cheese – contaminated with *L. monocytogenes* serotype 4b was traced to 122 listeriosis cases in Switzerland from 1983 to 1987. Thirty-four patients died, giving a mortality rate of 28%. Two different epidemic-associated strains of *L. monocytogenes* serotype 4b were isolated from patients and the incriminated cheese as well as from the wooden shelves and brushes used in 40 different cheese ripening cellars. Surface samples from the cheese contained the epidemic strain at levels of 10^4–10^6 cfu g^{-1}, thus suggesting both contamination and growth of *L. monocytogenes* on the cheese surface during ripening. The outbreak ceased after installation of metal ripening shelves and thorough cleaning/sanitizing of the ripening rooms.

During the mid-1990s, two major dairy-related listeriosis outbreaks in France were traced to different varieties of soft surface-ripened cheese. In 1995, 20 individuals including 11 pregnant women developed listeric infections after consuming Brie de Meaux cheese that was prepared from raw milk. Unlike previous outbreaks, no geographic clustering was observed, with cases reported in 8 of 22 French regions. Isolates from patients were identical to those from the incriminated cheese, with this organism likely present in the raw milk used for cheesemaking. Two years later, 14 cases of listeriosis were linked to consumption of Pont l'Évêque cheese manufactured in Normandy. The implicated raw milk cheese contained *L. monocytogenes* serotype 4b at a level of >1000 cfu g^{-1}.

Improper manufacture of soft and semihard cheese is now responsible for the majority of both outbreak and sporadic cases that have been traced to dairy products, with consumption of such cheeses best avoided by high-risk individuals. One 2002 outbreak in Quebec, Canada, involving 17 cases of listeriosis (5 pregnant and 12 nonpregnant adults) was linked to consumption of commercially produced raw milk soft and semihard cheese that had undergone less than the legally required 60 days of aging. In late August and September of 2008, 22 cases of listeriosis (7 pregnant and 15 nonpregnant adults), including 1 adult fatality, 1 stillbirth, and 6 premature deliveries, were again traced to consumption of several soft French-style cheeses that were commercially produced from raw milk in Quebec, Canada.

In the United States, concerns surrounding consumption of soft cheese have generally focused on soft, unripened Mexican-style varieties that are frequently homemade and either illegally produced from raw milk in the United States or illegally imported from Mexico. In 2000, 13 cases of listeriosis including 11 perinatal infections and 5 stillbirths were traced to soft Mexican-style cheese that was locally prepared from raw milk and then sold either door-to-door or through small markets or street vendors to Mexican immigrants in North Carolina. Unlike the previous outbreaks, this epidemic strain of *L. monocytogenes* serotype 4b exhibited a rarely seen PFGE profile. Another similar but smaller outbreak in 2003 was also traced to raw milk Queso fresco cheese that was illegally produced in Texas. These outbreaks along with a growing number of sporadic cases among Hispanics have prompted renewed efforts to curtail the illegal importation, production, and sale of such raw milk cheeses in the United States.

Sources

Primary reservoirs for *Listeria* include soil, feces, water, and decaying vegetation. Consumption of aerobically spoiled and improperly fermented silage having a pH >4.5 has been routinely linked to listeriosis outbreaks in ruminant farm animals. Numerous wild and domestic animals, including cows, sheep, and goats, are susceptible to listeric infections, with large numbers of healthy asymptomatic carriers excreting high numbers of *L. monocytogenes* in their feces. Long-term survival of *Listeria* under adverse environmental conditions typically leads to further spread of this pathogen through the food chain. The hardy nature of this ubiquitous psychrotrophic food-borne pathogen, along with its ability to colonize, multiply, and persist in food production facilities for months or years, makes *L. monocytogenes* a major threat to manufacturers of dairy products as well as ready-to-eat meat and poultry products, smoked fish, prepared sandwiches, and delicatessen products, all of which have been frequently found to harbor *Listeria*. Being unable to survive pasteurization, this pathogen most often enters dairy products and other ready-to-eat foods as a postpasteurization contaminant. While most frequently isolated from floor drains, conveyor belts, and areas with condensate, *L. monocytogenes* has also been recovered from cheese vats and filling machines, which lends further support to this pathogen being a postpasteurization contaminant.

Incidence and Behavior in Milk and Dairy Products

Dairy cattle, sheep, and goats can intermittently shed *L. monocytogenes* in their milk at levels of up to 10^4 cfu ml^{-1} as a result of *Listeria*-related mastitis, encephalitis, or abortion. Milk from severely infected cows is unlikely to reach consumers due to a variety of overt symptoms, including excessive salivation, inability to eat or drink, impaired locomotion, and 'circling disease', all of which are related to disturbances of the central nervous system. However, mildly infected and apparently healthy animals can shed *L. monocytogenes* in their milk for many months and are thus of far greater public health concern. Composite results from numerous bulk tank surveys conducted since 1983 indicate that 2.5–5% of the North American and European raw milk supply can be expected to contain low levels (i.e., <10 cfu ml^{-1}) of *L. monocytogenes* at any given time. Hence, proper refrigeration is important, given several reports indicating that *L. monocytogenes* populations in naturally contaminated raw milk can increase 1000-fold after 4 and 10 days of storage at 10 and 4 °C, respectively.

Listeria monocytogenes is more thermally tolerant than most other non-spore-forming foodborne pathogens. However, current vat (63 °C for 30 min) and high-temperature–short-time pasteurization (72 °C for 15 s) practices will ensure total destruction of *L. monocytogenes*. Despite the ability of *L. monocytogenes* to attain populations of 10^6 cfu ml^{-1} in commercial skim milk, whole milk, chocolate milk, and whipping cream after 8 days of storage at 8 °C (a not uncommon temperature of home refrigerators), this organism has been rarely detected in pasteurized fluid milk products. While *L. monocytogenes* has been occasionally recovered from commercially produced butter with survival up to 70 days being reported in butter prepared from inoculated cream, this pathogen is a far more frequent postpasteurization contaminant of ice cream. Since May 1986, 47 of 58 *Listeria*-related Class I recalls issued in the United States for unfermented dairy products involved ice cream, ice cream novelties, and related frozen desserts containing very low levels of *L. monocytogenes*. Increased prevalence of this pathogen in frozen rather than fluid dairy products coincides with the relatively complex handling of such products, particularly ice cream novelties, during manufacture and packaging. However, given the presumed low levels of contamination, the inability of *Listeria* to grow in frozen dairy products, and the recall of over 3.1 million gallons of ice cream without incident, consumption of such products does not appear to pose a major public health threat.

As one might surmise from the aforementioned outbreaks, *L. monocytogenes* is a more frequent contaminant of cheese, most notably soft surface-ripened varieties such as Brie, Camembert, and certain Mexican-style varieties, which support growth of the organism during cheese ripening. Since 1986, 81 Class I recalls were issued in the United States for domestically produced cheese, principally Mexican-style cheese, contaminated with *L. monocytogenes*. During this same period, 31 imported cheeses, including French Brie, Danish Esrom, and Anari (goat's milk cheese from Cyprus), were also recalled. Results from extensive surveys suggest that about 1–5% of cheeses produced in Europe, primarily soft and semisoft varieties surface-ripened by mold or bacteria, may contain *L. monocytogenes*, with this pathogen seldom found in aged hard cheeses (e.g., Cheddar) or cheeses that undergo severe heat treatments during manufacture (e.g., Cottage, Mozzarella, Parmesan, Swiss, processed cheese).

Following the 1985 outbreak in California involving Mexican-style cheese, work was initiated to assess the behavior of *L. monocytogenes* during the manufacture and storage of yogurt, buttermilk, and a wide range of cheeses, with most of these studies describing the outcome of preparing these products from artificially contaminated pasteurized milk. *Listeria* populations generally increase <10-fold when milk is fermented with a traditional starter culture containing mesophilic or thermophilic lactic acid bacteria at an inoculum level of 1%, with growth ceasing at pH <5.2. In one of several studies that examined postpasteurization contamination, *L. monocytogenes* persisted an average of 3 weeks in refrigerated cultured buttermilk and yogurt inoculated to contain 10^3–10^4 cfu g^{-1} of *L. monocytogenes*. Regardless of the cheese variety manufactured, physical entrapment of *Listeria* in the curd during milk coagulation results in a 10-fold increase in numbers. Thereafter, the behavior of *Listeria* is dictated by the manufacturing steps for the particular cheese. The extent of acid development and curd cooking during cheesemaking along with pH, salt content, moisture content (water activity), and type/extent of ripening will determine the ultimate fate of *L. monocytogenes* in the final product. Growth of *Listeria* in cheese is primarily confined to soft/semisoft varieties ripened by mold (e.g., Brie, Camembert, Roquefort) or bacteria (e.g., French cheeses, Brick) and certain Mexican-style cheeses (Queso Fresco) with populations increasing to >10^6 cfu g^{-1} as the cheese attains a pH >6.0 during ripening (**Table 2**).

Although *L. monocytogenes* is generally unable to grow in cheeses having a pH <5.2, it can survive in many such cheeses for weeks or months and has even been recovered from experimentally produced 434-day-old Cheddar cheese. These findings raise legitimate concerns regarding the adequacy of the mandatory 60-day holding period at >1.7 °C to completely inactivate *L. monocytogenes* (and other pathogens) in Cheddar and other hard cheeses that can be legally prepared from raw milk or milk subjected to subpasteurized heat treatments. However, barring contamination during packaging, cheeses such as Cottage and Mozzarella that undergo severe heat treatments during manufacture should be free of *Listeria.*

Control

Given the widespread distribution of *Listeria* in the environment, control of *Listeria* must begin at the farm level with attention given to good animal husbandry practices, use of only high-quality feed/silage, hygienic milking practices, and proper refrigeration to minimize growth of the pathogen during bulk tank storage of milk. Current vat and high-temperature–short-time pasteurization practices are the only commercially practical means for destroying *L. monocytogenes* in raw milk. Thus, barring postpasteurization contamination, properly pasteurized fluid milk products will be free of *Listeria.* Well-designed sanitation programs that include weekly sampling for *Listeria* in problem areas within the factory are essential if the incidence of this pathogen is to be minimized in the production facility and in the finished product. Postpasteurization contamination most frequently occurs during extruding, filling, and packaging operations when the product is exposed to airborne contamination and

Table 2 Fate of *Listeria monocytogenes* in fermented dairy products as affected by composition

			pH		
Product	*% moisture*	*% NaCl in water phase*	*Initial*	*Final*	*Growth*
Fermented milks					
Buttermilk	–	–	4.2	4.4	–
Yogurt	–	–	4.1	4.1	–
Cheeses					
Blue	39	11.5	4.6	6.3	–
Brie/Camembert	55	4.7	4.6	7.5	+
Cheddar	37	4.6	5.1	5.1	–
Cottage	79	1.2	5.0	5.0	–
Feta	55	4.6	4.6	5.1	–
Mexican-style	51	4.0	6.2	6.2	+
Mozzarella	47	4.4	5.4	5.4	–
Parmesan	32	5.0	5.1	5.1	–
Ricotta	72	0.5	6.0	6.0	+
Swiss	33	2.7	5.5	5.5	–

difficult-to-clean food contact surfaces. Programs for reclaiming and reworking returned or expired product are also discouraged to minimize the chance of reintroducing temperature-abused products that may harbor higher numbers of *Listeria* into the processing facility.

See also: **Cheese**: Public Health Aspects. **Diseases of Dairy Animals**: Infectious Diseases: Listeriosis. **Mastitis Pathogens**: Environmental Pathogens.

Further Reading

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Mycobacterium spp.

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Introduction

The genus *Mycobacterium* is a member of the Actinobacteria. Mycobacteria are aerobic, acid-fast, nonmotile (with the exception of *Mycobacterium marinum*), nonspore forming bacilli (although recent findings indicate that some strains such as *M. marinum* may have the ability to form spores), which are widely distributed in the environment. There are over 100 different species of mycobacteria, most of which are nonpathogenic to humans and are found mainly in soil and water. However, tuberculosis and leprosy, two of the most notorious diseases known to man, are caused by members of this genus. While they are generally considered to be Gram positive, this is not strictly true. The unique cell envelope of mycobacteria contains a very high proportion of lipids that give the members of this genus unique characteristics such as resistance to acid, alcohol, and chlorination. It is the thick, waxy, lipid-rich cell wall that gives mycobacteria their characteristic pink/red color following acid-fast staining procedures (e.g., Ziehl-Neelson, see **Figure 1**). Although most mycobacteria live in soil, water (including chlorinated tap water supplies), and foodstuffs, some members of the genus are not free-living bacteria but are instead found living in animal hosts. These include *M. tuberculosis* and *M. bovis*, both of which are pathogens in both humans and cattle.

Mycobacteria consist of both fast- and slow-growing members, with the quicker-growing species usually being nonpathogenic saprophytes (feeding on dead or decaying organic matter) and the majority of the pathogenic strains belonging to the slower-growing species. Mycobacteria that form colonies visible to the naked eye on suitable substrates within a period of seven days are generally classified as fast-growing species (e.g., *M. smegmatis*), while species that take longer than this are considered to be slow growers (e.g., *M. tuberculosis*). From a clinical perspective, mycobacteria are classified into two groups: the *M. tuberculosis* complex (e.g., *M. tuberculosis*, *M. bovis*, *M. africanum*) and the nontuberculous mycobacteria (e.g., *M. avium*, *M. intracellulare*, *M. avium paratuberculosis*).

Mycobacterium tuberculosis Complex

The members of the *Mycobacterium tuberculosis* complex (MTC) (with the exception of strain BCG) are all pathogenic and capable of causing a deadly disease of the pulmonary system, tuberculosis, in a variety of hosts. Its members include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canetti*, *M. caprae*, and *M. bovis* Bacille Calmette-Guérin (BCG). Strains of the MTC are approximately 99.9% similar at a genetic level but there are distinct differences phenotypically with regard to the level of their pathogenicity. Approximately one-third of the world's population is infected with mycobacteria capable of causing tuberculosis, but most of these are asymptomatic and will never develop the disease. Nonetheless, tuberculosis is a leading global cause of death due to infectious disease. Although primarily a disease of the lungs, infections of the gastrointestinal tract are not uncommon. All members of this group are reportable to public health authorities, although many laboratories are not equipped to distinguish between the different species. As a result the exact number of tuberculosis cases caused by each species is not available.

Tuberculosis has been prevalent in human society since ancient times. Large-scale outbreaks such as the White Death have been recorded throughout history. With the development of antimycobacterial antibiotics, tuberculosis disease was thought to be under control. However, a resurgence in tuberculosis has been seen in recent times, especially among immunocompromised individuals, such as those affected by HIV (AIDS).

Mycobacterium bovis

The most commonly reported mycobacterial infection in cattle is caused by *M. bovis*. It causes bovine tuberculosis characterized by the development of granulomatous lesions in the lung tissue, lymph nodes, intestine, and other tissues. The primary hosts of *M. bovis* are cattle species, but it has the ability to infect a very wide range of mammalian hosts such as humans, deer, goats, pigs, dogs, cats, and badgers. *M. bovis* can cause tuberculosis in humans, with AIDS patients being the most at-risk group. Infection usually occurs through ingestion of contaminated milk, especially raw milk. Upon oral infection the bacterium can cause localized infections in the gut, and through infection of macrophages can establish infections of the lymph nodes and disseminate throughout the body, which can also lead to pulmonary disease. Symptoms of *M. bovis* infection include weight loss, fever, and general weakness. It is thought to have

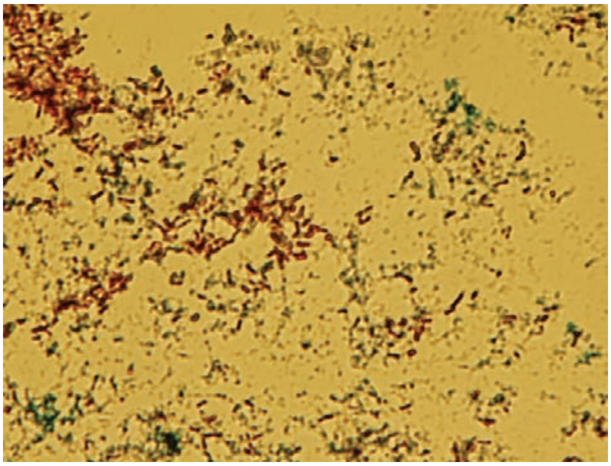

Figure 1 Acid-fast stain of *Mycobacterium avium paratuberculosis* (MAP). MAP cells appear stained red.

originally derived from an ancestral soil-dwelling saprophyte that lived in cow dung.

It has been estimated that *M. bovis* accounts for approximately 3.1% of all human tuberculosis disease with the vast majority being nonpulmonary infections. Infection is greatest in developing countries; *M. bovis* tuberculosis is quite rare in developed countries, accounting for less than 1% of infection.

Members of the MTC have a pattern of pathogenesis distinct from most other bacteria. Unless a host has an underdeveloped or compromised immune system the mycobacteria will not initially cause disease. Upon entering the body, bacteria encounter macrophages, cells of the immune system that engulf and kill bacteria. Mycobacteria can survive this process and replicate inside the macrophage. They can subsequently exist in a quiescent state for many years. This is referred to as latent infection. In 90% of the cases of infection with *M. tuberculosis*, the infection will not lead to disease in the host. However, in the remaining 10% of infected individuals the mycobacteria can reactivate and cause disease. This leads to active disease, and it is at this point that individuals in turn become infective. The reasons for reactivation of the mycobacterium are unknown, but it is more likely to occur in an individual who is malnourished or whose immune system has become impaired. This can be due to infection with the human immuno deficiency virus (HIV), which can cause a breakdown in the capability of the immune system to fight disease; old age; or the use of corticosteroids which inhibit the immune system. In the developed world mortality from tuberculosis is approximately 10% of those with active disease; in the developing world this percentage is much higher.

Mycobacterium tuberculosis

This bacterium is the etiological agent of most tuberculosis in humans. Although pulmonary infection is more common with *M. tuberculosis*, intestinal infection can also occur and is more common in immunocompromised hosts. Any part of the gastrointestinal tract can become infected. *M. tuberculosis* shows a strong tropism for lung tissue, and when injected intravenously it can translocate to the lungs and cause pulmonary infection. It is primarily associated with human disease, although it can also colonize cattle and a wide variety of other mammals. *M. tuberculosis* is thought to have evolved from *M. bovis*, gradually developing into a different species. Symptoms of intestinal infection include ulcers, thickening of the bowel wall, weight loss, diarrhea, and fatigue. Infection from dairy products usually occurs due to the consumption of contaminated, unpasteurized milk or cheese.

Mycobacterium africanum, M. microti, M. canetti, and *M. caprae*

M. africanum is commonly found in West African countries and, although it is rarely identified outside of this region, it has been detected in a small number of tuberculosis patients worldwide. Both humans and cattle are susceptible to colonization and infection. Its contribution to tuberculosis disease in the above regions is thought to be up to 50%. While its infectivity in humans is comparable to that of *M. tuberculosis*, the extent of its virulence is unknown. Again, it causes disease in a large number of HIV-positive patients. *M. microti* primarily infects small rodents, in which it causes tuberculosis. It has also been reported to be present in cattle. It very rarely causes disease in humans but has been reported both in immunocompetent and in immunosuppressed patients. *M. canetti* was first described in 1969. It grows more quickly than *M. tuberculosis* and forms smooth, shiny colonies. Infection in humans has only rarely been reported. Locations include the Horn of Africa, France, Madagascar, and French Polynesia. It is unknown if this species can infect cattle and cause infection through contaminated milk. *M. caprae* is the most recent addition to the MTC, and was only identified in 1999. It has been isolated from symptomatic cattle and very infrequently from humans.

Nontuberculous Mycobacteria

Nontuberculous mycobacteria (NTM) consist of species not part of the *M. tuberculosis* complex or *M. leprae.* They are also known as 'mycobacteria other than tuberculosis'

(MOTT). These bacteria are usually saprophytic but they can be opportunistic pathogens and can cause deadly infections in a susceptible host. Unlike in *M. tuberculosis* complex bacteria, person-to-person contact is not the primary source of infection. Infection normally occurs through contact with the bacteria from environmental sources via ingestion, inhalation, or through exposed wounds.

Detection of infections of humans by these bacteria has increased in recent years for several reasons. Better diagnostic and culturing techniques have allowed for better isolation of these bacteria from infected individuals; there has also been a rise in the numbers of susceptible hosts (such as individuals suffering from AIDS) which has led to this increased infection. In contrast to *M. tuberculosis* complex infections, NTM infections are not reportable to public health bodies, possibly resulting in an underestimation of the extent to which they cause disease.

NTM can cause both local infections and body-wide disease depending on the strain, host physiology, and point of entry. NTM infections were initially thought to cause disease only in the immunocompromised, but they are now also recognized as being capable of causing disease in immunocompetent hosts. They can cause a wide range of diseases, which can be pulmonary, intestinal, and disseminated throughout the host. NTM have been found in both raw milk and pasteurized samples. These include the following:

M. avium complex
M. avium paratuberculosis
M. ulcerans
M. fortuitum
M. gordonae
M. marinum
M. kansassii
M. scrofulaceum

Mycobacterium avium Complex

The *Mycobacterium avium* complex (MAC) consists of a group of closely related mycobacteria that are difficult to identify and differentiate. The members are very similar at a genetic level but they vary greatly in their host tropisms, disease phenotypes, and pathogenicity. The MAC consists of both saprophytes and opportunistic pathogens. Members of the MAC include *M. avium*, *M. avium avium*, *M. intracellulare*, *M. avium paratuberculosis*, *M. haemophilum*, and *M. avium hominis.*

Symptoms of a MAC infection can include the following:

- Fever
- Abdominal pain
- Weight loss
- Fatigue
- Diarrhea
- Night sweats

These mycobacteria are capable of survival and replication under a wide variety of environmental stresses which include large variations in temperature, low pH, and chlorination. Members of the MAC are slow-growing and usually nonpigmented. MAC bacteria are common in the environment and can cause infection in a susceptible host when inhaled or swallowed. Diarrhea and abdominal pain are associated with gastrointestinal involvement. Members of the MAC are pathogens of low virulence, but infections are common in immunocompromised individuals. In AIDS the risk of infection by these bacteria is inversely related to the CD4 count of a patient. Unlike other opportunistic infections in AIDS, infection by members of the MAC is not thought to be a result of the reactivation of a latent infection but rather a new infection. Infections by members of the MAC are difficult to eradicate with antibiotics and many are resistant to antituberculosis antibiotics.

Mycobacterium avium paratuberculosis

Mycobacterium avium paratuberculosis (MAP) was first described in 1895 and originally named Johne's bacillus. It is a member of the MAC and is very similar to the other members of this group. Unlike other members of MAC and NTM, it is a specific agent of intestinal inflammation in a wide variety of animals. It is the causative agent of Johne's disease, which is primarily a disease of ruminants but which can also be seen in some nonruminants such as birds, foxes, dogs, and some primates. It causes an intestinal infection that results in a chronic wasting disease that ultimately ends in the death of the animal. Infection usually occurs years before the animal displays symptoms of the disease. Chronic inflammation may emerge after a long latent period. This emergence of disease has been linked to stress on the animals and hormonal changes. Hundreds of millions of dollars are lost annually due to infection by MAP on account of decreased milk yields and culling of infected animals.

Like many mycobacteria, it is capable of surviving and replicating inside macrophages. The principal route of infection is through ingestion, with calves being at greatest risk. MAP can be passed from mother to calf in both the colostrum and milk, and *in utero* transmission of MAP has also been observed. MAP bacteria are also shed intermittently in high numbers in the feces of the infected cattle, contaminating bedding, pasture, and water sources. However MAP displays a strong tropism to the intestine and will cause intestinal inflammation if experimentally administered intravenously or subcutaneously. The primary symptoms of Johne's disease include diarrhea, weight loss, and decreased milk yield. Unlike all other mycobacteria, MAP requires the presence of mycobactin, an iron chelating agent, for growth in laboratory media.

Detection of the incidence of MAP in cattle herds has risen in the mid-twentieth century, which is possibly due to better laboratory cultivation techniques. Cattle displaying Johne's symptoms are usually culled to prevent further infection in the herd. Subclinically infected cattle are seen to secrete MAP in their milk, exposing the young with an underdeveloped immune system to the pathogen at an early age. The presence of MAP has been demonstrated in pasteurized retail milk, perhaps as a result of postpasteurization contamination. Some studies have shown a high degree of tolerance in MAP toward the pasteurization process, unlike in *M. tuberculosis* or *M. bovis*, which are effectively killed during standard high temperature short time (HTST) pasteurization. An increase in the hold time in HTST pasteurization has been shown to have a greater killing effect on MAP compared to an increase in temperature. MAP has also been shown to be capable of surviving in some cheeses for long periods in the environment. Amoebae have been shown to be a host in pastureland and allow for replication outside of a bovine host.

MAP has been linked to Crohn's disease, a gastrointestinal disorder in humans that is currently thought to be autoimmune in nature. Symptoms of Crohn's disease include weight loss, abdominal pain, diarrhea, and ileac obstruction. This link has proven to be controversial and no firm consensus has been reached on the matter. The link was first postulated in the early twentieth century, when the first comparisons were made between the pathology and symptoms of Crohn's and Johne's diseases (see **Table 1**). MAP strains have been detected and isolated from the blood, feces, and tissue biopsies of patients with Crohn's disease. MAP bacteria isolated in this way have been shown to cause Johne's disease in goats after experimental infection.

Table 1 Comparison of the major symptoms of bovine Johne's disease and Crohn's disease

	Johne's disease	*Crohn's disease*
Cause	MAP	Unknown
MAP DNA present	Yes	Frequently
MAP cultivated from biopsy	Yes	Intermittently
Diarrhea	Yes	Yes
Dormant periods	Yes	Yes
Weight loss	Yes	Yes
Lethargy	Yes	Yes
Extra GI complications	Yes	Yes
Intestinal blockage	No	Yes
Fever	No	Yes
Loss of appetite	No	Yes
Decreased milk production	Yes	N/A
Cramping and abdominal pain	N/A	Yes
Nausea and vomiting	N/A	Yes

GI, Gastro intestine; MAP, *Mycobacterium avium paratuberculosis*.

Other Nontuberculous *Mycobacterium* of Relevance to Dairy Production

A number of other NTMs are capable of causing skin infections and opportunistic wound infections. Several mycobacterial species have been reported to cause mastitis in cattle, including *M. chelonei*, *M. fortuitum*, *M. phlei*, and *M. smegmatis*. Infections of this nature in cattle can result in the pathogen contaminating the milk, leading to possible infection of young calves. The recently identified strain *M. avium silvaticum* has been suspected of causing chronic enteritis in calves, possibly being spread from cow to calf *via* contaminated milk. Other species of NTM have been isolated from raw milk products from cattle not suffering from any clinical infection. *M. flavescens*, *M. gordonae*, *M. abscessus*, *M. mucogenicum*, *M. marinum*, *M. terrae*, *M. kansassii, M. haemophilum*, and *M. agri* as well as several unidentified species have all been observed. Not much is known about how infectious these mycobacteria are when orally ingested by humans, but several have been shown to cause infections in immunocompromised individuals, especially transplant patients on immunosuppressing agents and those with AIDS. The NTM group has doubled in number since 1990, probably due to the increasing use of modern genetic techniques and improvements in mycobacterial culture techniques. Though the virulence of these organisms is generally considered very low, they can still play a role in infections of immunocompromised individuals through contaminated milk supplies.

Mycobacterial Contamination of Milk

Milk supplies can become contaminated at many stages of milk collection and processing. Cattle infected with pathogenic strains can secrete bacterium in milk itself. The bacterium can also be shed in huge numbers in the feces of the animal, leading to contamination of the external surface of the udder due to contact with the feces itself or with contaminated bedding. Washing of the udders before milking has been shown to decrease bacterial contamination; however, some contamination still does occur. Contamination can also take place postpasteurization. Some mycobacterial pathogens such as *M. tuberculosis* and *M. bovis* have been shown to be capable of establishing infections at very low doses. Most data pertains to pulmonary infection, however, and the numbers required to establish intestinal infection are unknown.

Infection within the Herd

Cattle can become naturally infected with mycobacteria through the respiratory or oral route. Infection via the respiratory system is the most common route in cattle, especially in the context of intense farming. Oral infection is more common in suckling calves. Cows can shed the bacterium in their colostrum and milk, or teats can become contaminated with feces, thus passing it on to calves. Apart from infection from inside the herd, a number of other avenues exist for the transmission of this disease.

Drinking water can easily become contaminated and lead to infection. Mycobacteria survive quite well in treated water supplies. Apart from oral infection, aerosols could also lead to respiratory infection.

Many mycobacterial species have been detected in the milk of the infected cattle, including *M. bovis* and MAP. Other sources of mycobacteria include the urine and feces of infected cattle. As a result bedding, pastures, and water can easily become contaminated. Shedding can be intermittent and occurs in sublinically infected cattle. The minimum dose of mycobacteria required to establish a lasing infection in cattle varies from species to species, and also depends on the route of infection and the health of the animal. Infected cattle are most likely to have been repeatedly exposed to a variety of doses.

Prevalence of Mycobacteria in Dairy Herds

Cattle are susceptible to colonization and infection by a wide variety of mycobacteria, *M. bovis* being the most commonly reported species. Prevalence varies depending on a number of factors, including the following:

- eradication programs
- farming policy
- wildlife reservoirs of mycobacterium
- geographical location

Eradication programs in industrialized countries have greatly reduced the prevalence of *M. bovis* infection in both animals and humans. This is a result of the monitoring programs that identify and eliminate the infected cattle, vaccination, and widespread pasteurization of milk. Farming policy can greatly impact *Mycobacterium* levels. Regulation of animal movement, animal identification systems, and coordination between agricultural and health authorities all play a part in the control of disease spread. Conversely a lack of these systems or breakdown in established systems can lead to an increased prevalence in the national herd of a country. As already mentioned, mycobacterial species have wide host ranges, and apart from infecting domesticated animals they can also infect wildlife. Other species can act as reservoirs of infection, reintroducing the bacterium to pasturelands and water supplies. Reported reservoirs of mycobacteria include badgers, possums, ferrets, wild deer, and assorted domesticated animals. Control measures have at times focused on this area as a means to control spread of mycobacterial disease. Culling of infected wildlife has been attempted in several species with varying degrees of success. Culling of badger populations has been shown to decrease levels of *M. bovis* in cattle inside the culling regions. However, in some instances this was seen to lead to an increased infection of cattle in adjacent regions.

Mycobacterial infection in humans due to contamination of dairy supplies has become less relevant in developed countries. With improvements in milk hygiene and mass pasteurization of dairy supplies, these countries have a vastly decreased mycobacterial load as compared to the prepasteurization era. These improvements in conjunction with vaccination, monitoring, and eradication programs for the tuberculosis complex bacterium have led to retail milk supplies that are safe from pathogens such as *M. bovis* and *M. tuberculosis*. A small percentage of the dairy in developed countries still involves making use of raw milk in the cheese-making process. Although the process itself can kill mycobacteria, this alone would not be sufficient to protect the consumer. If the process is coupled with effective monitoring and control programs the risk is greatly reduced. Even though the monetary cost of such surveillance is high, it is essential to safeguard human health and consumer confidence. This holds true for the majority of the NTM group of mycobacteria. However, the potential zoonotic capability of MAP continues to be a possible threat to the dairy industry. Its possible resistance to pasteurization process is a cause for concern should a link between MAP and Crohn's disease be proven. Even without the link to Crohn's disease, MAP still represents a substantial danger to economically important farmed animals through its presence in milk.

The main danger to human health as a result of mycobacterial contamination of milk supplies lies in the developing world where pasteurization is not practiced to as great an extent as is common in the developed world. Infections by mycobacteria of importance to human health are higher in cattle in these regions due to a lack of regulation in the industry coupled with a lack of effective monitoring and control programs. In the developed world the incidence of AIDS infection has remained low in the general population. However, this is not the case in the developing world, where the numbers of infected individuals have been seen to rise steadily. This, coupled with malnutrition, exacerbates the problem of mycobacterial disease in these countries.

See also: **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease; Infectious Diseases: Tuberculosis.

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Salmonella spp.

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Culture and Identification of *Salmonella* in Milk and Dairy Products

Different methods have been used for the isolation and identification of *Salmonella* bacteria in milk and dairy products. A standard cultural procedure is the ISO 6785:2001 method of the International Organization for Standardization whereby 25 ml of milk or milk product is preenriched in 225 ml of buffered peptone water (BPW), incubated for 16–20 h at 37 °C, after which 0.1 ml of the preenriched BPW is added to 9.9 ml of prewarmed Rappaport Vassiliadis (RV) selective enrichment broth and incubated at 42 °C for 20 ± 2 h and then plated onto selective agars for determination of the presence or absence of *Salmonella*. Presumptive *Salmonella* isolates are further confirmed by biochemical testing and serologically by typing with poly- and monospecific *Salmonella* somatic and flagellar antisera. Polymerase chain reaction (PCR)-based methods are increasingly used to detect the presence of DNA sequences, such as a part of the *invA* or *spaQ* gene (both required for invasion) located within the *Salmonella* Pathogenicity Island (SPI) 1 or the *ttrRSBCA* gene complex (required for tetrathionate respiration) located adjacent to the SPI 2. These genes are specific for nearly all *Salmonella* serovars. The cultural and identification methods employed have a significant impact on the percentage of samples that test positive for the presence of *Salmonella* or a DNA sequence thereof. An advantage of cultural methods is that an isolate is obtained that can be further examined for characteristics including the phage type (PT), antimicrobial susceptibility, the presence of plasmids, and the plasmid profile; the presence of virulence and other genes; and the pulsed field gel electrophoresis (PFGE) pattern. Determination of the PFGE pattern is commonly done in the case of outbreaks to determine if the isolates can be traced to the same source or sources and to direct the institution of control and preventative measures. An advantage of PCR-based methods is that they have attained a high level of sensitivity and specificity and often result in a higher percentage of samples that are identified as contaminated with *Salmonella* than the cultural methods.

Incidence of *Salmonella* in Milk and Dairy Products

Raw or Nonpasteurized Milk

Raw milk may contain a variety of food-borne pathogens including *Salmonella* spp., verotoxigenic *Escherichia coli* (VTEC), *Campylobacter* spp., *Listeria monocytogenes*, and *Yersinia* spp. Before pasteurization and the institution of eradication and control programs for brucellosis and tuberculosis, *Brucella* spp. and *Mycobacterium* spp. could also occasionally be isolated from raw milk.

Studies have been conducted to determine the incidence, prevalence, and occurrences of *Salmonella* bacteria in milk and dairy products. During a 2002 survey of 854 farms in 21 US states, *Salmonella* were isolated from 2.6% of the raw milk samples, whereas a real-time PCR indicated that *Salmonella* were present in 11.8% of the samples. In another study conducted in 2007 in 17 US states, 541 raw bulk tank milk samples and 523 in-line milk filters were collected and examined for the presence of *Salmonella* by a cultural method and by real-time PCR. *Salmonella* isolates were cultured from 6.7% of the milk samples and 19.9% of the milk filters, while PCR analysis indicated that *Salmonella* were present in 13.9% of the milk samples and 33.3% of the milk filters. Twenty-five *Salmonella enterica* subsp. *enterica* (hereafter denoted as *Salmonella*) serovars were identified; the most common serovars were *S.* Cerro (34 isolates), *S.* Kentucky (20), *S.* Muenster (14), *S.* Newport (10), *S.* Anatum (10), *S.* Montevideo (8), and *S.* Mbandaka (8). In a recent study in California, samples of bulk tank milk were taken at intervals of 2–3 months during a 29-month period and cultured for *Salmonella*. At each sampling period, 10–21% of the dairy farms were positive for *Salmonella*. The most commonly isolated *Salmonella* serovars in that study were *S.* Montevideo (33%), *S.* Typhimurium (14%), *S.* Dublin (13%), and *S.* Give (11%).

Pasteurization of milk is a process whereby the milk is heated at 72 °C for at least 15 s or at other temperature and time combinations. It is very effective in killing *Salmonella* and other pathogens in milk. Occasionally, pasteurized milk becomes contaminated as a result of faulty processing procedures. Investigation of a large outbreak of salmonellosis in the United States in 1984 showed that

2% low-fat pasteurized milk processed at the dairy plant in northern Illinois had become contaminated with *S.* Typhimurium. The likely cause of contamination was a cross-connection of a skim milk transfer line between tanks containing raw milk and a tank with pasteurized skim milk. Examination of the possible sources of contamination of the raw milk showed that 28 (5.5%) of pooled raw milk samples from 2786 milk producers in Wisconsin and Illinois contained *Salmonella.*

Certified Milk

Certified milk is raw nonpasteurized milk produced under conditions that comply with standards of sanitation. In California, outbreaks of salmonellosis due to *S.* Dublin occurred when people consumed certified milk.

Cheese

Soft cheeses or cheeses made from unpasteurized or insufficiently pasteurized milk may be contaminated with *Salmonella.* The largest food-borne outbreak of salmonellosis in Canada occurred during the spring and summer of 1984, affecting about 2700 people in the four Atlantic provinces (Newfoundland, New Brunswick, Prince Edward Island, and Nova Scotia) and in the more central province of Ontario, and the affected were note to have consumed Cheddar cheese contaminated with *S.* Typhimurium PT10. Production of the cheese was traced to a single plant on Prince Edward Island. Cheese was manufactured from either pasteurized (16 s at 73.8 °C) or heat-treated (16 s at 66.7 °C; not pasteurized) milk. The contamination level of the cheese was low. The level ranged from 0.36 to 9.3 *Salmonella* bacteria per 100 g of cheese made from the heat-treated milk, and it ranged from 0.36 to 4.3 *Salmonella* bacteria per 100 g of cheese manufactured from the pasteurized milk. Up to 60 samples per lot of cheese had to be examined to find a positive lot. Examination of raw milk samples from 327 farms on Prince Edward Island, Canada, supplying milk to the cheese processing plant showed that the bulk tank milk of one farm contained *S.* Typhimurium PT10. One of 24 cows in the herd, although clinically healthy, shed the same *S.* Typhimurium PT10 intermittently in the milk from one quarter of the udder during a 36-day observation period. Not all the milk used to produce the cheese was properly pasteurized since manual turning of an electronic flow diversion valve in the plant allowed some raw milk to flow into vats used for cheesemaking. The presence of alkaline phosphatase (a temperature-sensitive enzyme that is inactivated during pasteurization) in samples of cheese associated with human illness indicated that the milk had not been fully pasteurized. Examination of the isolates of *S.* Typhimurium by plasmid profiling and digestion of plasmid DNA with restriction endonucleases showed that two subgroups (I and II) of *S.* Typhimurium PT10 contaminated the cheese. *Salmonella* Typhimurium PT10 subgroup I was isolated from raw milk and cattle associated with the incriminated dairy, whereas subgroups I and II were recovered from employees at the dairy plant, from cheese obtained at the plant and in stores, and from consumers who became ill after consumption of the cheese. Investigation of the outbreak suggested that only a few *Salmonella* bacteria might cause infection in consumers. Studies on the fate of *S.* Typhimurium in the manufacturing and ripening of low-acid Cheddar cheese showed that after a rapid initial decline, the number of *Salmonella* remains the same and can survive refrigerated storage for more than 40 weeks. Similarly, during an outbreak of *S.* Newport in 2007 in northeastern Illinois, samples of 85 patients, a Mexican-style aged cheese (cotija) obtained at a local Hispanic grocery store, and raw milk from a bulk tank at a local dairy farm consumed by the patients tested positive for *S.* Newport. The isolates had indistinguishable PFGE patterns.

Raw goats' milk may also contain *Salmonella*, and the drinking of raw goats' milk and consumption of cheese made from raw goats' milk have resulted in several outbreaks of salmonellosis in humans. In a large outbreak in France in 1993, consumption of goats' milk cheese made from unpasteurized milk caused a large number of consumers to be infected with *S.* Paratyphi B. The organism was isolated from milk at the processing plant on 2 of 5 occasions and was found in the milk from only 1 of 40 farms that supplied the plant.

Dried Milk Products

Dried milk products are occasionally contaminated with *Salmonella.* In an outbreak of salmonellosis in infants in the United Kingdom, all infected infants had been fed a reconstituted dried milk product from one manufacturer. *Salmonella* Ealing was isolated from 4 of 267 sealed packets that were examined. Other outbreaks of salmonellosis due to *S.* Tennessee or *S.* Anatum have occurred in infants after consumption of powdered milk products and infant formula in England, Wales, Belgium, France, Canada, and the United States.

The prevalence of *Salmonella* serovars in dairy products is undoubtedly influenced by, but does not appear to entirely coincide with, the prevalence of *Salmonella* serovars causing infection or shedding in dairy cattle. The reason the two parameters are not in complete congruence with one another may lie in the fact that dairy products are commonly produced on a very large scale and contamination with a less common serovar may result in widespread outbreaks of salmonellosis. Also, the *Salmonella* serovar isolated from the milk or processed dairy product is whatever serovar happens to be present or has survived in the product when samples are taken for analysis, whereas differences among serovars in geographic distribution, host specificity, virulence, and infectious dose influence whether infection and shedding in cattle occur.

Sources

Excretion of *Salmonella* in the Milk

Excretion of *Salmonella* by cows in the milk occurs when the animal is febrile and experiencing an acute episode of salmonellosis. This happens most frequently during the *postpartum* period. Cows infected with the host-adapted *S.* Dublin may shed *Salmonella* bacteria with the milk, but shedding of *Salmonella* via this route has also occurred with a number of other *Salmonella* serovars including *S.* Typhimurium, *S.* Muenster, *S.* Give, *S.* Heidelberg, and *S.* Enteritidis. Occasionally, mastitis occurs in infected cows. Nonsymptomatic carrier animals may excrete *Salmonella* intermittently with the milk. *Salmonella* bacteria may be shed for prolonged periods of time from one quarter of the udder. In one study, *S.* Enteritidis PT8 was repeatedly isolated over a period of 7 months from the right hindquarter of the udder of a 5-year-old Holstein cow. The milk appeared normal at all times. In a study in the United Kingdom, 26 of 70 milk filters examined during a 4-year period (1983–86) tested positive for *S.* Typhimurium PT49a. At the end of the study period when milk samples were collected from 131 lactating cows, *Salmonella* bacteria were found in one milk sample. Milk samples taken 3 months later from 152 cows in milk at the same farm showed the same results; one milk sample cultured positive for *S.* Typhimurium. The affected cow shed *Salmonella* from one quarter of the udder.

The number of *Salmonella* bacteria shed in the milk may vary considerably. During an outbreak of *S.* Muenster infections in dairy cattle, one cow in midlactation in a herd of 35 cows shed *S.* Muenster at a rate of approximately 200 cfu ml^{-1}. The cow continued to shed the organism during the rest of the lactation period and at the next calving *S.* Muenster was again isolated. One of the quarters of the udder showed signs of clinical mastitis at calving. During the same outbreak affecting more than 200 herds, 3 other cows in 3 herds continued to shed *S.* Muenster in the milk, although the fecal samples were negative. Chronically infected carriers may shed 10–30 bacteria per ml of milk, but shedding of as many as 10^5 bacteria per ml of milk has been reported.

Contamination of Milk from Other Sources

Milk is most often contaminated after it leaves the cow by various means including fecal matter, contaminated equipment, dust, and other environmental sources. Adult cattle that recover from clinical disease may become active carriers and such apparently healthy animals may excrete *Salmonella* in large numbers in the feces for prolonged periods. Carrier animals are a major source of environmental contamination and infection of animals and humans. Cows excreting *Salmonella* may infect neighboring cows in a barn, which then in turn may excrete *Salmonella* in the milk and the feces. During an outbreak of salmonellosis in dairy cattle in Quebec, Canada, *S.* Give was isolated from the feces of cows with clinical salmonellosis and from the bulk tank milk of two herds. In one herd, the infection was so widespread that *S.* Give was isolated from the feces of 41% of the cows. Milk production was considerably reduced. Ten of 24 positive cows shed *Salmonella* in their feces for more than 6 weeks, and 5 of 23 cows for 11 weeks or longer. The only clinically affected cow had profuse diarrhea in its *postpartum* period. *Salmonella* Give was isolated from 2 of 5 cats that frequented the feeding alley in the barn. In a neighboring dairy herd, a febrile and diarrheic cow excreted *S.* Give in the feces, and a week later the two adjacent cows shed the same *Salmonella* serovar in the feces. The distribution of the infection appeared to be limited to the immediate environment of the clinically ill animal since feces from none of the other cows in the herd tested positive for *Salmonella*. One cow in this herd shed *S.* Give in the feces for at least 26 weeks.

Milking equipment may be contaminated with *Salmonella* before and during milking the cows. Dust, bedding, manure, other debris, and aerosols may be aspirated by the vacuum pump of the milking systems and contaminate the milk with *Salmonella* and other pathogens. Plant fibers and other particulate debris have been found on milk filters, suggesting that fecal matter, bedding, and other debris may have contaminated the milk. Farmers and farm workers may be infected and transfer *Salmonella* from themselves or from sources in the immediate environment to the milking equipment and the milk. *Salmonella*-infected cats and dogs on the farm may play a role in contaminating the milk. Direct or indirect contamination of the milk may occur when cows drink *Salmonella*-contaminated water or when the water contaminates the udder or the milking equipment. Farmers and their helpers may also contract the disease by direct transfer of *Salmonella* from infected cattle or calves via feces or saliva.

Cattle that drink from contaminated streams and creeks may ingest *Salmonella* and the water may contaminate the udder and teats. In the United Kingdom, cattle and calves grazing on pasture contaminated with human sewage became infected and *S.* Dublin, *S.* Typhimurium, and other *Salmonella* serovars were repeatedly isolated from milk filters.

Dried milk products may be contaminated with *Salmonella*. In one outbreak, the source of contamination was traced to a spray-dryer that had a hole in its inner lining, allowing contamination of the milk powder to occur.

Utensils used for cheesemaking may be contaminated with *Salmonella*. In an outbreak of salmonellosis caused by the consumption of cheese contaminated with *S.* Berta, the unpasteurized soft cheese was made by ripening the skim milk curds at room temperature in large buckets. One of the buckets had previously been used for the soaking of chicken carcasses before they were frozen for later consumption. *Salmonella* Berta was also isolated from the chicken carcasses.

Control Measures

There are many measures that can be taken to prevent salmonellosis. The most important public health control measure is undoubtedly pasteurization of the milk; if properly done, pasteurization is extremely effective in eliminating the risk of infection. Regulations under the US Public Health Service and a variety of state and local regulatory agencies requiring pasteurization of milk and a comprehensive set of sanitary procedures resulted in a decrease in the outbreaks of milk-borne illness from approximately 25% of all reported food-borne illness outbreaks in 1938 to less than 1% in 2008. Measures other than pasteurization include prevention of contamination, inhibition of bacterial growth, prevention of gastrointestinal infection in infants and adults by the consumption of fermented milk products, and control of the feeding of *Salmonella*-contaminated feeds to animals. The growth of *Salmonella* can be inhibited by lowering the pH of foods, by treating foods with organic acids or sodium chloride, and by refrigeration. Such preventative measures have been described in the literature (see 'Further Reading'). Vaccination of cattle is also used to prevent and control salmonellosis (*see* **Diseases of Dairy Animals**: Infectious Diseases: Salmonellosis).

Measures to curtail the drinking of unpasteurized milk have been highly successful in preventing infections. The implementation of legislation in 1983 in Scotland prohibiting the retail sale of untreated cows' milk effectively controlled the large general community outbreaks of milk-borne salmonellosis and *Campylobacter* enteritis. However, the drinking of raw milk by dairy farmers and their families and friends continues to be a common practice in many countries. It has caused numerous cases of salmonellosis among these groups. Dairy farmers and their families should be informed about the dangers of drinking of raw milk and the ensuing cases of illness and number of deaths due to infections with *Salmonella*, *Campylobacter*, *Listeria*, and VTEC. Such information can be provided by placing articles about milk-borne illnesses and measures to control such illnesses in dairy producers' magazines and other publications for farmers and their families.

The public at large, and especially chronically ill elderly patients and the parents of young children, should be cautioned against the drinking of raw milk, an increasingly popular 'health food'. Some consumers claim that drinking raw milk has health benefits including decreased risks for artherosclerosis, arthritis, and lactose intolerance. However, such claims are not supported by scientific evidence.

Large outbreaks in the United States and Canada of milk- and cheese-borne salmonellosis in humans occurred as a result of misdirecting the flow of raw milk into containers and vats intended to receive pasteurized milk. It is therefore imperative to maintain proper control of the flow of pasteurized milk and to maintain a strict separation of unpasteurized and pasteurized products in dairy processing plants.

Outbreaks of salmonellosis after consumption of reconstituted dried milk products prompted recommendations to manufacturers such as increased monitoring of the drying process so that defects in the process may be readily recognized and remedied, not to blend products with high counts of bacteria with batches with a low viable count, and not to keep raw milk and whey on-site at milk-drying plants.

The average number of milking cows per dairy worker has increased significantly during the last few decades in many countries and less time is available for attention to the hygiene of individual animals. In the process, the potential exposure of milk to contamination during production has also increased. Thus, strict hygienic measures before, during, and after milking the cows should be maintained. Such measures include a clean milking parlor, proper disinfecting and cleaning of the udder and teats, good maintenance, cleaning and disinfecting of milking equipment before and after use, and the institution of other measures to promote hygiene and prevent contamination of milk during production.

Good manufacturing practices must be maintained in order to produce cheese free from *Salmonella* contamination. Investigation of an outbreak of salmonellosis due to *S.* Heidelberg in Denver and Pueblo, CO, showed that the raw milk used at the dairy processing plant to make the cheese contained more than 3 million bacteria per ml. The raw milk was stored for 1–3 days in insulated but unrefrigerated holding tanks and the milk was filtered only after pasteurization, a violation of guidelines for pasteurization. Also, bacterial culturing and determination of the presence of phosphatase in the milk used to make the cheese were not carried out. The manufacturer was urged to take appropriate corrective measures.

Salmonellosis in Humans

Throughout the world, much of the milk consumed is still not pasteurized. In many countries, unpasteurized milk or certified milk is available for sale or can be obtained directly from the farm. In Canada, the sale of raw milk directly to consumers is prohibited. The distribution of raw milk is illegal also in Scotland. However, in England, registered producers are allowed to sell raw milk directly to the consumer. Although Food and Drug Administration (FDA) regulations in the United States require mandatory pasteurization of packaged milk and milk products for human consumption in interstate commerce, in 27 US states raw milk can still be bought directly from the farmer. Raw milk is sometimes available to the consumer through a 'cow share' purchasing program. The drinking of unpasteurized milk and the consumption of soft cheeses made from raw or improperly pasteurized milk or milk contaminated after the pasteurization process have resulted in numerous single case and small and large outbreaks of salmonellosis. Cases of food-borne outbreaks due to consumption of *Salmonella*-contaminated raw milk have been reported in many countries. The majority of milk-borne epidemics of salmonellosis in humans in the United Kingdom have been caused by *S.* Dublin and *S.* Typhimurium. These two *Salmonella* serovars have also been the most commonly isolated serovars from cattle in the western European countries, whereas in the United States and Canada *S.* Typhimurium has been the most frequently isolated serovar from bovine sources. Such outbreaks continue to occur. For example, the consumption of Mexican-style soft cheese made from raw milk caused several outbreaks of *S.* Typhimurium DT (definitive phage type) 104 infection in people in California and Washington State. In another recent outbreak of salmonellosis in Pennsylvania, 29 cases of diarrheal illness, 16 of them in children less than 7 years of age, occurred as a result of drinking raw milk or consuming a soft cheese (queso fresco) made from raw milk; all isolates had the same PFGE pattern (the DNA was digested using the *Xba*I restriction enzyme) and could be traced to the same dairy farm that held a state-issued permit to sell raw milk to customers.

Symptoms

The symptoms observed in cases of human salmonellosis are diarrhea, abdominal cramps, nausea, vomiting, fever, headache, and blood in the feces. The frequency and severity of the symptoms may vary. In the soft cheese-related outbreaks of infection with *S.* Typhimurium DT 104 mentioned above, diarrhea was observed in 100%, abdominal cramps in 93%, fever in 93%, bloody stools in 72%, and vomiting in 53% of cases, while 9% of patients were hospitalized. In California, during the period 1971–74, there were 79 cases of human salmonellosis due to *S.* Dublin; 74 of these 79 cases drank certified raw milk produced at a single large dairy farm. In 52 of the 79 cases, the organism was isolated from blood, urine, or deep tissue sites, demonstrating the invasiveness of the pathogen. Sixteen of the 79 patients died, of whom 13 had preexisting chronic debilitating diseases. In 1993, in France, consumption of a raw goats' milk cheese was associated with a *S.* Paratyphi B infection in 273 patients; in 240 patients the organism was isolated from the feces, in 15 from blood, in 14 from tissues, and in another 4 the site was unknown. Thirty-seven percent of the patients were hospitalized and one died. The largest single food-borne epidemic in the United States affected an estimated number of more than 160 000 persons who became ill as a result of *S.* Typhimurium infection from contaminated 2% low-fat milk produced by a dairy plant in Illinois in 1985. There were more than 16 000 culture-confirmed cases, 2777 patients were hospitalized, and 14 associated deaths occurred.

Patients infected and shedding *Salmonella* with the stool may experience sequelae to the primary infection such as extraintestinal salmonellosis and isolation of the *Salmonella* from the blood, from cases with septicemia, cystitis, and pyelonehritis, and from cases with abscesses and tissues. Sterile or reactive arthritis of the knees and occasionally of the ankles and other joints is not uncommon in patients who experienced a bout of diarrhea and from whom previously or concomitantly *S.* Typhimurium, *S.* Enteritidis, or other *Salmonella* serovars were isolated from the stool. Human leukocyte antigen (HLA) typing of such patients shows that they are usually B27 positive.

Susceptibility to *Salmonella* Infection and Severity of Illness

Susceptibility to, and severity of, *Salmonella* infection in humans depends on various factors including the dose and virulence of the pathogen for the human host, the type of food contaminated with *Salmonella* that was consumed, the age of the host, and factors known to affect the immune status of the host, such as infection with the human immunodeficiency virus (HIV), leukemia, and/or the use of immunosuppressive drugs. Other host-associated risk factors include diabetes, partial gastrectomy, and the low gastric acidity associated with these conditions. The very young and the elderly are most susceptible to the infection. The fat content of contaminated foods such as cheese may influence the dose required to cause an infection. *Salmonella* present in foods with a high fat content such as cheese may be trapped in hydrophobic lipid moieties and survive the acidic conditions of the stomach to subsequently attach to and invade the enterocytes lining the intestines. Cases in which humans became infected after ingestion of an estimated dose of 100–500 *S.* Heidelberg or, in another outbreak, less than 10 *S.* Typhimurium in Cheddar cheese, support this hypothesis.

See also: **Diseases of Dairy Animals**: Infectious Diseases: Salmonellosis.

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Shigella spp.

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Introduction

Dysentery is an intestinal disorder caused by microorganisms: bacteria, amoebas, or viruses. Bacillary dysentery or shigellosis refers to the disease caused by bacteria of the genus *Shigella.* This name was adopted in 1950 in honor of Kiyoshi Shiga, the scientist who first differentiated amoebic from bacterial dysentery, and who isolated *Bacillus dysenteriae* in 1898.

Shigella spp. are enteric bacteria essentially found in humans and primates, but they spread into the environment, especially into water and food, due to human activities. Milk and dairy products are susceptible to being contaminated by shigellae, causing thousands of outbreaks a year. Indeed, outbreaks are more and more common, due to adoption of new life styles. Consequently, dysentery is an emerging disease of worldwide public dimension that attracts the attention of food and health administrations in an attempt to minimize its societal impact. The following sections are devoted to the bacteriology of *Shigella* spp. and their occurrence in milk. Special attention is paid to pathogenesis, outbreaks, safety, and detection/identification methods.

Bacteriology

Shigellae (genus *Shigella*, family Enterobacteriaceae, according to Bergey's Manual, Section 5: "Facultatively Anaerobic Gram-Negative Rods") are straight rod-shaped, facultatively anaerobic, oxidase-negative bacteria that stain Gram-negative. Shigellae, unlike their closest relative *Escherichia coli*, are nonmotile, and do not ferment lactose or produce gas from glucose, nor reduce sulfate to hydrogen sulfide. However, enteroinvasive *E. coli* (EIEC), which also causes dysentery, is practically indistinguishable from shigellae. Infective dose, 1000-fold higher for EIEC than for shigellae, is perhaps one difference between these two pathovars. The four species of the genus *Shigella* are *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii*, and *Sh. sonnei.* A few serovars have been differentiated in each species, even though all serovars within each species share a specific O side chain in their lipopolysaccharide (LPS). This is the reason why *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii*, and *Sh. sonnei* are usually referred by epidemiologists as subgroups A, B, C, and D, respectively.

The natural hosts of shigellae are humans and monkeys, with the intestinal tract being their common habitat. However, these pathogens remain viable in the skin. Person-to-person transmission by the fecal/oral route is common, though sexual transmission is also possible. They are also viable in food, water, and beverages; consequently, these are additional modes of transmission. Special attention must be paid to asymptomatic carriers and flies, as both serve as silent transmission vehicles.

Pathogenesis

The main pathogenicity or virulence property of *Shigella* is cell invasion, which is responsible for most of the symptoms of dysentery. The invasion occurs mainly in the colon, though it can also be observed in the last part of the ileum. The process starts with the adhesion of pathogens to the surface of M cells of the follicle-associated epithelium. The host–pathogen contact causes internalization of the bacteria into vacuoles, in a process resembling macropinocytosis. After lyzing the vacuolar membrane, the pathogens escape to the cytoplasm, move to the edge of the nucleus, where they grow and form a microcolony. Cell-to-cell spreading occurs via bacteria-containing protrusions phagocyted by the adjacent cells. Spreading is not limited to the epithelial lining but proceeds to the lamina propria, where the bacteria are released and ingested by macrophages. The pathogens, rather than being killed by macrophages, escape the phagosome, induce release of interleukin-1β, and cause apoptotic death of the macrophages. The overall result is an inflammatory response that attracts polymorphonuclear leucocytes, which loosen the cellular junctions. Now, the pathogens at the colonic lumen are free to pass through the epithelial lining and to gain access to the basolateral surface. Then, more epithelial cells are invaded and the inflammatory response is amplified. This recurrent event results in the formation of colonic ulcers, a macroscopic hallmark of dysentery. The dysenteric stool is bloody and mucoid due to the infiltration of red blood cells and serum proteins.

Genetic Determinants of Virulence

Virulence is a multifactorial property in which both a nucleoid and a plasmid are involved. All pathogenic shigellae and EIEC bear a large plasmid ranging from 180 to 220 kb, called the virulence plasmid. This plasmid contains a pathogenicity island, needed for invasion and essentially composed of three operons: *ipa* (invasion plasmid antigens), *mxi* (membrane expression of invasion plasmid antigens), and *spa* (surface presentation of invasion plasmid antigens). While *mxi* and *spa* genes code for subunits of the type III secretion system, the *ipa* genes code for the effectors secreted by this system. Some other genes located in the virulence plasmid, such as *virG* (*icsA*), which codifies for an outer membrane protein, are also needed for invasion. The nucleoid-encoded genes, mainly responsible for the survival of the pathogen in the host, are also organized in a few pathogenicity islands. Enumerating all the nucleoid-encoded genes would require extensive discussion; but, due to its significance, the locus *stx* stands out, as it codifies for the shiga toxin in *Sh. dysenteriae* 1. This toxin is a potential inhibitor of mammalian protein synthesis and is responsible for the most severe pathogenic phenotypes. Recently, two new enterotoxins responsible for the clinical manifestation of dysentery have been characterized. Shigella enterotoxin 2 is codified in the virulence plasmid and is present in most shigellae isolates.

Disease Symptoms and Complications

Clinical manifestation of dysentery varies greatly from asymptomatic infections to fulminating dysentery, though mild diarrhea is also common. The severity of the disease depends not only on the person but also on the shigellae strain. For obvious reasons, immune-compromised or malnourished individuals and children are prone to suffer more severe infections. By species, *Sh. dysenteriae* and *Sh. flexnery* produce severe infections, while *Sh. sonnei* is more benign. The typical symptoms of dysentery are fever, permanent emission of bloody and mucopurulent stools that leads to dehydration, and intestinal discomfort characterized by cramps and tenesmus. Complications can cause death, due to septicemia and hypoglycemia, especially in children.

Reiter's syndrome is a common sequela of dysentery in individuals expressing the HLAB127 histocompatibility antigen. Hemolytic uremic syndrome, with renal failure, is not infrequent when infected with *Sh. dysenteriae* 1. Though very infrequent, ulcerative keratitis due to shigellae infection has also been reported.

Occurrence in Milk

Milk is a rich substance that supports bacterial growth; therefore, after contamination, bacterial counts increase within a wide range of time and temperatures. Consequently, the shelf life, quality, and safety of raw milk are determined by the types and load of bacteria. High loads of spoilage flora imply high enzymic activity of lipases and proteases, sometimes thermostable, that reduce the fat and protein content, consequently lowering the quality of raw milk. These enzymic activities have other undesirable organoleptic effects: bitterness, rancidity, and off-flavors. Pathogenic bacteria, such as *Shigella* spp., primarily affect food safety; hence, pathogen contamination must simply be avoided in milk.

Shigella spp. remain viable for at least 72 h at 4 °C or proliferate at 15–37 °C in raw milk. As the infective dose is very low – 10 to 100 bacteria are enough to trigger an infection – special care must be taken to avoid contamination of raw milk and dairy products by these pathogens. Occurrence of *Shigella* spp. is sometimes reported in milk, as in Sudan, where analysis of bulk tank cow milk indicates that 20% of the samples contain *Shigella* spp. in the range of 1×10^6 cfu ml^{-1}. Occurrence of *Shigella* spp. in dairy products is lower than in raw milk, due to the special properties of these foods, as discussed below.

Outbreaks

There are no accurate data available regarding the total number of dysentery outbreaks each year, but most likely millions of humans are affected worldwide. Estimates indicate 145 million cases per year, with mortality reaching between 0.5 and 1.5 million; children below 5 years of age are the main victims. Outbreaks take place worldwide, with the majority occurring in countries suffering from poor sanitation, hunger, wars, or catastrophes. There is also a general seasonal trend: in tropical areas the incidence is higher in the rainy season, due to flooding, while in subtropical areas the incidence is higher in summer, due to high temperature and drought.

Shigella flexneri and *Sh. dysenteriae* account for most cases of dysentery in developing countries. The former relates to the endemic disease, and the latter to epidemics. *Shigella sonnei* essentially affects well-developed countries. Finally, *Sh. boydii* rarely causes outbreaks except in the Indian subcontinent. An epidemiological transition from flexneri to sonnei is considered an indicator of economic development; such a transition should be seen in the near future in emerging economies such as India.

Milk and dairy products are suspected of being responsible for several dysentery outbreaks. Between 1998 and 2007, 83 foodborne pathogen-related outbreaks were publicly reported, of which 14.5% had been associated with dairy products. One of the recent outbreaks occurred in 2004 in Vilnius, Lithuania. Of the 41 cases detected, 36 were diagnosed as dysentery; 50% of the patients were children under 14 years of age, and 16% of the affected individuals were above 65 years.

As expected, all patients were positive for *Sh. sonnei.* Unpasteurized milk curd was the vehicle for transmission, and contamination of this product was due to handlers either in the dairy facility or in the market. Indeed, the same *Sh. sonnei* serotype was confirmed in patients, workers, and inhabitants of the dairy facility, and workers of the market. This case clearly shows the importance of microbiological safety measures needed in dairy production.

Microbiological Safety

There are several ways to reduce shigellae in raw milk, the most effective one being thermal treatment. Pasteurization and ultra-high temperature (UHT) treatment are very effective. For *Sh. dysenteriae*, the decimal reduction time (D value) in milk at 82.2 °C is 0.8 s, while in nutrient broth at 63 °C it is 5 min. Thus, the common thermal treatments applied to milk significantly reduce the total counts of *Shigella.*

Detection, Enumeration, and Identification Methods

Coliform bacteria are indicative of fecal contamination; therefore, a milk sample with a high coliform count is suspicious of harboring enteric pathogens also, such as *Shigella* or *Salmonella.* The prevalence of shigellae in milk is not negligible, and from time to time they appear in the dairy industry. When this occurs, it is important to monitor the possible sources of contamination in order to adopt control measures. Thus, it is worthy to review the traditional and molecular methods for detection and identification of shigellae.

There are no specific media for growing *Shigella* spp. Normally, the media used to cultivate these pathogens are of a broad bacterial spectrum, or designed either for enterobacteria or *Salmonella.* Typical enrichment media are Hajna (Gram-negative) broth, buffered-water peptone, selenite cystine broth, tetrathionate broth, and brilliant green bile glucose broth. As the counts of shigellae in milk are high, enrichment is not only unnecessary but contradictory, given that these pathogens outgrow in the presence of other enteric bacteria. If enrichment is needed, addition of novobiocin to the media greatly improves the recovery of *Shigella* spp. A successful strategy is to use this antibiotic along with a low carbohydrate concentration in the medium. Typical isolation (selective) media are Salmonella–Shigella agar, xylose lysine deoxycholate agar, deoxycholate citrate agar, its modified version deoxycholate lactose sucrose agar, eosin methylene blue agar, and tergitol-7 agar. Typical differential media are Hektoen enteric agar, MacConkey agar, and triple sugar iron agar slant. Chromogenic media have been developed, such as chromogenic Shigella plating medium (CSPM), which contains a proprietary mix of carbohydrates, selected pH indicators, and chromogens. Identification of *Shigella* spp. in CSPM is easy, since they produce white to clear colonies, while bacterial competitors produce colored colonies. Recovery of *Shigella* spp. from food in this medium is similar to that reported for MacConkey and Salmonella–Shigella agars; however, CSPM needs further evaluation with different shigellae serotypes. A single-tube screening test has also been developed for *Salmonella* and *Shigella.* This method, based on a four-layered semisolid medium, has 100% sensitivity (56 *Shigella* isolates tested) and 95% specificity (56 non-*Shigella* isolates tested).

After detection/isolation in culture media, the presumptive isolates must be further characterized by molecular tests, even if a differential medium had been used. The reason for the deeper characterization is that *Shigella* spp. are difficult to identify based solely on the abovementioned media. In addition, from a medical point of view, it is very important to determine which shigellae serotype is isolated.

Primary biochemical characterization of *Shigella* spp. involves four distinctive properties of these bacteria: (swarming) motility, production of H_2S, production of gas from glucose, and fermentation of lactose. These tests, which are carried out on agar media, are negative for most *Shigella* serovars. At this point, it must be mentioned that *Sh. sonnei* ferments lactose very slowly, and that *Sh. flexneri* 6, *Sh. boydii* 13 and 14, and *Sh. dysenteriae* 3 produce gas from glucose. Further biochemical characterization is also desirable though, once again, no specific tests for *Shigella* spp. are available. Test strips for bacterial identification, such as API20E® or similar tests, are very popular in diagnostic laboratories. These systems have two major drawbacks: they need expert interpretation and are time consuming. These concerns are overcome, at least in part, with automated systems such as Vitek®. The EPS (enteric pathogens) card of this system has been evaluated for enteric pathogens. The card gave a sensitivity of 99.5% for enteric pathogens, including several shiegellae serovars, and a specificity of 90.1% for nonpathogens. An automated system for the enumeration (based on the most-probable-number technique) and confirmation of enterobacteria, Tempo® EB (enterobacteria), has also been released; use of this system must be considered when fast results are required. Similar methods are becoming available in the market. More recently, a simple, fast, and inexpensive method specifically developed for *Shigella* spp. has been reported. It is a colorimetric method based on the detection of apyrase, a periplasmic enzyme necessary for intracellular spread of pathogen. The assay shows 96% sensitivity (over 23 isolates of *Shigella* and EIEC) and 80% specificity (over 34 isolates tested). Though the assay has been successfully applied on stools, it requires further validation and domains of application.

Immunological identification of *Shigella* spp. is based on surface antigens, such as LPS. This molecule is composed of three different regions: the lipid A, which anchors the LPS to the outer bacterial membrane, the core oligosaccharide, and the O-antigen. The latter, a polymer of repeating saccharide units, is strain specific, with all strains having the same O-antigen belonging to the same serotype. Serotype acknowledgment depends on various authors of different studies, but less than 46 seem to be acceptable. *Shigella boydii* can be subdivided in 20 serotypes, *Sh. dysenteriae* in 15, *Sh. flexneri* in 6, and *Sh. sonnei* in only 1 serotype. Interestingly, most of the *Sh. flexneri* serotypes share the same O-antigen basic structure. Different commercial kits for serotyping are available. Most of them use polyclonal sera raised in rabbit against reference strains, and slide/latex/tube agglutination, enzyme immunoassay, or immunomagnetic platforms for detection. The main drawback of serotyping is that sera can fail in detecting particular serotypes. In Bangladesh, for instance, 3% of isolates from patients with diarrhea have the typical biochemical properties of shigellae but they fail to be detected with the currently available sera. These are known as *Shigella*-like organisms, one of which has had a high prevalence over several years, and has been classified by molecular methods as a novel serovar of *Sh. dysenteriae*. Likewise, 180 isolates classified as *Shigella*-like organisms in France between 2000 and 2004 were recently identified by molecular methods as *Sh. boydii*. Several commercial kits for serotyping have been evaluated, and only one gave a performance lower than the conservative standard of 90% of accuracy.

Genetic identification is based on genotyping, that is, determining the identity of a strain based on its genetic material using a biological assay. The most popular and widely used method is the polymerase chain reaction (PCR), which consists of specific amplification of a small portion of the genetic material of an organism, such as bacteria, in a complex sample, such as food. Dozens of publications a year deal with PCR identification of *Shigella* spp. in food, as many as the genetic targets to be amplified, among them the loci *ial*, *virA*, or *ipaH*. A plethora of PCR-based assays are also popular, though their use in food is limited. Noteworthy is fingerprinting, based on the generation by PCR of a specific DNA pattern that serves to identify microorganisms. Restriction fragment length polymorphism (RFLP-PCR), amplified fragment length polymorphism (AFLP-PCR), enterobacterial repetitive intergenic consensus (ERIC-PCR), and repetitive extragenic palindromic sequence-based PCR (rep-PCR) are also notable. Recently, an automated system based on rep-PCR, called DIVERSILAB®, has been commercialized. This system allows rapid and specific detection of *Shigella* spp., though it has been applied in epidemiology only.

Before ending this section, identification methods based on a cell's ome, the units that define a cellular constituent or function, should be mentioned. Collectively these methods are called 'omics'. The most popular are transcriptomic, such as DNA (microarrays) chips; proteomic, such as mass spectrometry; and genomic, such as DNA sequencing. DNA sequencing is becoming very popular, since a few devices for fast, reliable, and cheap whole-genome sequencing have been developed in the last 5 years. Based on this high-throughput technology, new methods for identification are becoming available. An example is multiple loci sequence typing (MLST), an identification method halfway between genomics and genotyping. Conceived as a universal, portable, and definitive method, it consists in raising indexes of variation in multiple housekeeping genes of a particular organism, such as *Neisseria meningitidis*, for which the method was originally developed. A similar method, called variable-number tandem-repeat (VNTR) analysis (MLVA) has been applied to *Shigella* spp. A panel of 15 VNTRs has been released to detect *Shigella* spp., though the main drawback is that *E. coli* is also detected. More work in this area is needed to release a panel specific enough to detect only shigellae.

See also: **Analytical Methods**: DNA-based Assays; Microbiological. **Milking and Handling of Raw Milk**: Milking Hygiene. **Pathogens in Milk**: *Escherichia coli*; *Salmonella* spp. **Risk Analysis**.

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http://www.cdc.gov/FoodNet – Foodborne Diseases Active Surveillance Network.

http://www.icmsf.iit.edu/main/home.html – International Commission on Microbiological Specifications for Foods.

http://www.cdc.gov/mmwr – Morbidity and Mortality Weekly Report.

Staphylococcus aureus – Molecular

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Introduction

The genus *Staphylococcus* comprises more than 20 species, of which *S. aureus* is the most pathogenic. *Staphylococcus aureus* is a commensal of humans and being colonized with *S. aureus* is a risk factor for infection. *Staphylococcus aureus* can cause minor skin infections (abscesses, boils) but occasionally it can gain access to the bloodstream where it can cause serious invasive infections such as endocarditis, septic arthritis, and osteomyelitis. *Staphylococcus aureus* can also secrete potent enterotoxins that cause food poisoning if ingested. *Staphylococcus aureus* can colonize the udder of cattle from where it can infect the mammary gland epithelia causing mastitis. A lactating dairy cow with mastitis will excrete bacteria into milk. Bacterial cells will be killed by pasteurization but enterotoxins are heat resistant and if they are secreted into milk prior to heat treatment they will survive.

Molecular Typing Methods

A variety of molecular typing methods have been applied for studying collections of *S. aureus*. Most effort has gone into analysis of human clinical isolates, notably MRSA (methicillin-resistant *S. aureus*) strains. Some methods (pulsed field gel electrophoresis, random amplified polymorphic DNA) are highly discriminatory and are best applied to analysis of local outbreaks. Other methods are less discriminatory and are suited for studying the population as a whole. Multilocus sequence typing (MLST) has the advantage that it does not suffer from lack of reproducibility inherent in other methods. Segments of seven housekeeping genes are sequenced. Each unique gene sequence is given a numeric code (a bar code) and the unique combination given a sequence type (ST) number. A strain with a single nucleotide polymorphism in any of the seven genes is denoted a single locus variant. A collection of single locus variants are analyzed to form a clone with an ancestral ST in the center. The clone can be expanded to include double locus variants. A detailed analysis of human strains has been performed. Much less data are available for animal strains.

Population Structure

The population of *S. aureus* is described as being clonal. This means that discrete clones of the organism have emerged as a result of genetic divergence due to spontaneous mutations, with relatively minor contributions from gene transfer by recombination. Phylogenetic analysis by several methods agrees that the population is divided into two major groups. It is also evident that the carriage of *S. aureus* by domesticated animals (ruminants, rabbits) and chickens is the result of transmission from humans followed by a period of adaptation to the new host. The most widespread bovine clone diverged from ancestral human-associated clones by acquiring mutations, some of which have resulted in gene decay. Several genes important for human infection are no longer functional due to acquisition of mutations that result in truncation of the protein. Allelic variation occurs in genes encoding proteins involved in colonization, toxins, iron metabolism, and regulation, suggesting adaptation to the nonhuman host.

Superimposed upon the basic clonal population is the occasional emergence and rapid dissemination of new clones. This is exemplified by the recent emergence and spread of the virulent community-associated MRSA (CA-MRSA) strain of the USA300 PFGE type and the ST398 MRSA strain associated with intensively reared pigs.

Colonization: Bacterial and Host Factors

Staphylococcus aureus colonizes the moist squamous epithelium of the anterior nares of humans. This is regarded as the primary site of colonization. The presence of the bacterium on the skin is usually a consequence of nasal carriage. Nevertheless, temporary residence on the skin is a prerequisite for transmission from host to host. It is conceivable that the ability to adhere to keratinized epithelium is also important in colonization of the ruminant.

Recently, advances have been made in our understanding of bacterial and host factors that contribute to bacterial survival on the moist squamous epithelium of the nares and on skin of humans. *Staphylococcus aureus* permanently colonizes about 20% of the population and intermittently colonizes another 60%, whereas 20% of individuals are never colonized. This strongly suggests that host factors determine whether an individual is colonized or not. Individuals admitted to hospital who were permanently colonized developed bacteremia at a slightly higher rate (1.2%) than those who were not colonized (0.4%). Conversely, infected carriers had a lower mortality rate (8%) than infected noncarriers (32%). Carriers had higher levels of antibodies to certain virulence factors, which might have offered some protection when they acquired a bloodstream infection.

The ability to adhere to squamous cells *in vitro* is regarded as a correlate of proficiency in colonization of cornified epithelial tissue. Adherence is a multifactorial process involving teichoic acids and up to five different surface proteins, the best characterized of which are clumping factor B (ClfB) and iron-regulated surface determinant A (IsdA). Mutants defective in these proteins adhered poorly to isolated human squamous cells and colonized less effectively the nares of rodents, and in the case of ClfB, humans. The ligands recognized by these proteins are cytokeratin 10 and loricrin, which are major protein components of the corneocyte envelope. Polymorphisms in the glucocorticoid receptor gene contribute to the effectiveness of host immune responses and seem to determine in part the carriage status of the host. This indicates that colonization of the nares is controlled by host factors expressed in the nasal mucosal secretions. The ability to survive on skin is promoted by the ability of the bacteria to resist the bactericidal effects of skin lipids in sebum, the acidic pH, and cationic antimicrobial peptides.

Virulence Factors

Staphylococcus aureus expresses a diverse array of secreted and surface-associated proteins that promote virulence in experimental rodent models of infection and most likely do so in humans as well. Due to the unique environment of the mammary gland, it is likely that some of these factors are not relevant to the ruminant infection. Indeed several important factors in human strains are not expressed by a widespread bovine mastitis-causing strain of multilocus ST 151. The function of virulence factors can be categorized as (1) adherence to host cells or extracellular matrix proteins, (2) evasion of innate and induced immune responses, and (3) toxins that cause localized or systemic damage. Some factors are multifunctional and contribute to more than one category.

Adhesins

Staphylococcus aureus can elaborate on its cell surface up to 20 different proteins (Figure 1), some of which have been shown to bind to components of the extracellular matrix (fibrinogen, fibronectin, collagen, elastin) and thereby support bacterial adhesion to host tissue. At least four proteins (ClfA, ClfB, and fibronectin binding proteins A and B) bind to soluble fibrinogen and promote adhesion to immobilized fibrinogen and fibrin. However, each is multifunctional and can interact with other host proteins. ClfA, the dominant fibrinogen binding protein expressed by bacteria in stationary phase, also binds to and activates complement factor I. ClfB is expressed in exponential phase and binds to fibrinogen and to cytokeratin 10, the latter interaction being important in nasal colonization. Apart from binding to fibronectin, the eponymous fibronectin binding proteins A and B also bind to fibrinogen and elastin.

Protein A (Spa) is best known for its ability to bind to immunoglobulin (Ig)G at the Fc region, thereby inhibiting opsonophagocytosis. However, Spa is a multifunctional protein: it can also bind to (1) IgM and activate a subset of B cells, thereby exhibiting superantigen activity; (2) von Willebrand factor, contributing to thrombus formation and endocarditis; and (3) tumor necrosis factor receptor-1 on airway epithelial cells, thereby triggering inflammatory reactions that contribute to the pathogenesis of staphylococcal pneumonia.

When the genomes of *S. aureus* strains were sequenced, a number of novel putative surface proteins were identified. The serine aspartate repeat protein (SdrC, SdrD, and SasG) proteins have been shown to promote adhesion to desquamated epithelial cells and may contribute to nasal colonization. However, the ligand(s) involved are not known. Expression of SasG also promotes biofilm formation. The Isd proteins are expressed only in iron-limited conditions. They allow the acquisition of iron from hemoglobin. The IsdA protein also has important roles in promoting resistance to bactericidal lipids on skin, resistance to the bactericidal host protein lactoferrin (this could be important for bovine strains), and adhesion to desquamated epithelial cells. The IsdH protein also contributes to resistance to phagocytosis by promoting accelerated degradation of C3b by an as yet unidentified mechanism.

The fibronectin binding proteins are required for bacteria to become internalized by host cells that are not professional phagocytes. This is triggered by fibronectin acting as a bridge between bacteria and the $\alpha 5\beta 1$ integrin located on the surface of epithelial and endothelial cells. The ability of bacteria to invade mammary gland epithelial cells promoted by this mechanism could be important in the pathogenesis of mastitis.

Evasins

Evasion of innate immune responses of the host is crucial to the success of *S. aureus* as a commensal and pathogen. In humans, this centers around compromising the function

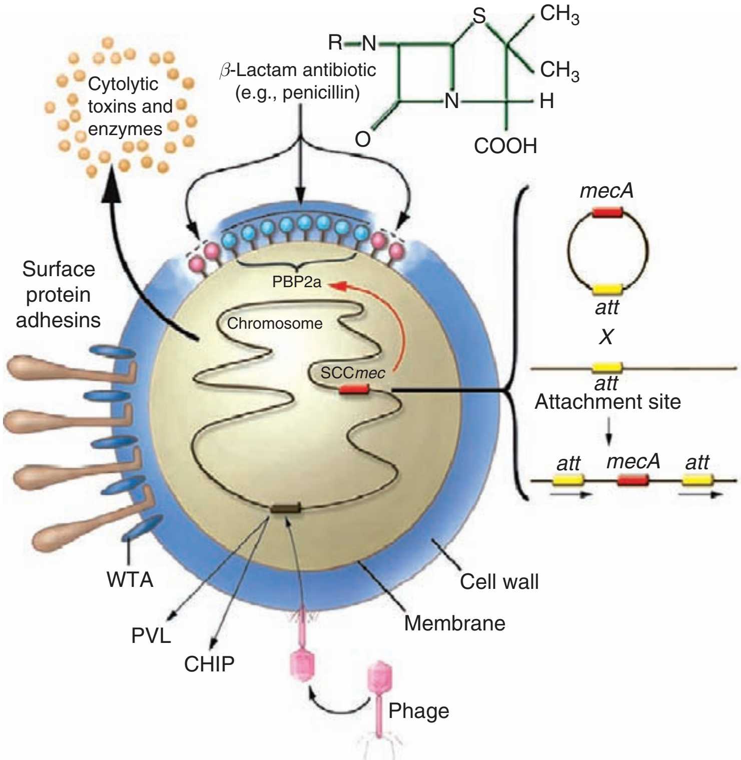

Figure 1 Schematic diagram illustrating how *Staphylococcus aureus* acquires resistance to methicillin and its ability to express different virulence factors. The bacterium expresses surface protein adhesins and wall teichoic acid (WTA), and also secretes many toxins and enzymes by activation of chromosomal genes. Adhesins and WTA have been implicated in nasal and skin colonization. Resistance to methicillin is acquired by insertion of a horizontally transferred DNA element called SCC*mec*. Five different SCC*mec* elements can integrate at the same site in the chromosome by a Campbell-type mechanism involving site-specific recombination. The *mecA* gene encodes a novel *β*-lactam-insensitive penicillin binding protein, PBP2a, which continues to synthesize new cell wall peptidoglycan even when the normal penicillin binding proteins are inhibited. Some virulence factors such as Panton and Valentine leucocidin (PVL) and the chemotaxis inhibitory protein, CHIP, are encoded by genes located on lysogenic bacteriophages. Reproduced from Foster TJ (2004) The *Staphylococcus aureus* 'superbug'. *Journal of Clinical Investigation* 114: 1693–1696.

of neutrophils, the first line of defense against invasion by *Staphylococcus*. Several small secreted proteins interfere with the migration of neutrophils from the blood vessels to the site of infection (**Figure 2**). The staphylococcal superantigen-like proteins SSL5 and SSL12 inhibit binding of neutrophils to inflamed endothelial cells in small blood vessels by blocking P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils from binding to P-selectin on endothelial cells. The extracellular adherence protein (Eap) (also called Map (MHC class II analog protein)) inhibits extravasation of neutrophils (diapedesis) by blocking leukocyte function-associated antigen LAF-1 from binding to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells. The chemotaxis inhibitory protein, CHIPS, inhibits neutrophil migration toward the focus of infection by blocking receptors for chemoattractants *N*-formyl peptide and complement C5a peptide.

Opsonophagocytosis of bacterial cells is important for efficient uptake and killing by neutrophils. Several secreted proteins prevent the efficient deposition of the complement opsonin C3b. The *Staphylococcus* complement inhibitor (SCIN) and the extracellular fibrinogen binding protein Efb (and the related protein relative Ecb) block complement C3b and C5a convertases.

Surface proteins contribute to reducing the efficiency of phagocytosis by enhancing the degradation of C3b. ClfA binds to and activates the complement regulator factor I, which results in enhanced degradation of the C3b opsonin. IsdH also promotes accelerated degradation of C3b. The capture of host plasminogen on the cell surface and its activation by staphylokinase destroys cell-bound IgG and C3b opsonins. Protein A binds to IgG by the Fc region, which then cannot engage in complement fixation or promote phagocytosis. Coating of the bacterial cells by plasma proteins (e.g., fibrinogen binding to clumping factor) and the presence of a polysaccharide capsule also contribute to reduced opsonophagocytosis.

If *S. aureus* is successfully engulfed by functional neutrophils or macrophages, bacteria can evade killing mechanisms in the phagosome. Expression of enzymes

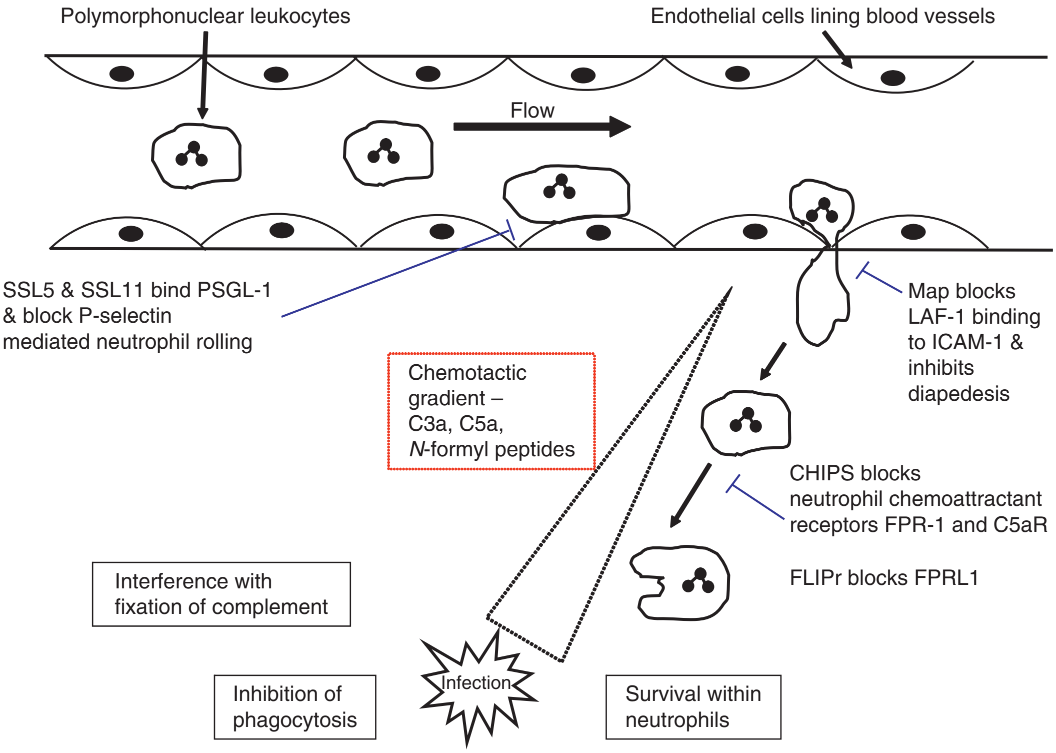
Polymorphonuclear leukocytes
Endothelial cells lining blood vessels
Flow
SSL5 & SSL11 bind PSGL-1 & block P-selectin mediated neutrophil rolling
Map blocks LAF-1 binding to ICAM-1 & inhibits diapedesis
Chemotactic gradient – C3a, C5a, *N*-formyl peptides
CHIPS blocks neutrophil chemoattractant receptors FPR-1 and C5aR
FLIPr blocks FPRL1
Interference with fixation of complement
Inhibition of phagocytosis
Infection
Survival within neutrophils

Figure 2 Migration of neutrophils from bloodstream to the site of infection summarizing the mechanisms of interference by *Staphylococcus aureus* secreted proteins. SSL5 and SSL11, staphylococcal superantigen-like proteins 5 and 11; PSGL-1, P-selectin glycoprotein ligand-1; Map, MHC class II analog protein; ICAM-1, intercellular adhesion molecule-1; LAF-1, leukocyte function-associated antigen-1; CHIPS, chemotaxis inhibitory protein of *S. aureus*; FPR-1, formyl peptide receptor 1; C5aR, receptor for complement C5a; FPRL1, FPR-like 1; FLIPr, FPR-like 1 inhibitory protein.

that modify cell surface teichoic acids and cytoplasmic membrane phosphatidyl glycerol by the addition of D-alanine and L-lysine, respectively, reduces the negative charge of the cell surface and reduces susceptibility to cationic peptides. Staphylokinase binds to and neutralizes antibacterial peptides. Cell wall peptidoglycan is modified to render it impervious to the bacteriolytic enzyme lysozyme. *Staphylococcus aureus* expresses catalase and superoxide dismutase, which counteract the reactive oxygen intermediates of the phagosome oxidative burst.

Toxins

Staphylococcus aureus expresses several membrane-damaging cytolytic toxins (α-, β-, γ-, and δ-toxins and several leucocidins) that cause damage to the membranes of host cells leading ultimately to lysis (**Figure 1**). At subinhibitory concentrations, damage to the integrity of the cells is manifested by expression of proinflammatory cytokines. The α-toxin is the archetypal β-barrel pore-forming toxin. It is composed of a heptamer, which assembles from monomers bound to the host cell membrane. The process of assembly results in a conformational change that creates the transmembrane β-barrel pore. The two-component γ-toxin and leucocidins (including the Panton and Valentine leucocidin (PVL)) form similar β-barrels composed of two subunits forming hexamers or heptamers. The PVL is particularly associated with the recently emerged CA-MRSA clones. The δ-toxin is a small cationic peptide with detergent-like properties. The β-toxin is a sphingomyelinase, which lyses cells with membranes that are rich in the lipid sphingomyelin. *Staphylococcus aureus* also expresses a number of small peptides with detergent-like properties called phenol-soluble modulins, which have potent cytolytic activity. Some toxins target a variety of host cells including erythrocytes, where the release of hemoglobin can provide a source of iron in the iron-restricted environment of the host. The leucocidins have a high affinity for neutrophils and thus compromise innate immune responses.

Secreted enzymes and stoichiometric activators also contribute to tissue damage and disease pathogenesis. *Staphylococcus aureus* expresses proteases, lipases, a nuclease, and a hyaluronidase. Staphylokinase binds to plasminogen in 1:1 stoichiometry and activates the latent activity of the host plasma protease plasmin, which degrades thrombin. Coagulase binds to and activates prothrombin, which conversely causes fibrinogen to form fibrin clots.

All strains of *S. aureus* express at least one of the major enterotoxins A–I (SEA–SEI), which are also potent superantigens. They also have the capacity to express several of the many newly described superantigen-like proteins. The enterotoxins have the ability to trigger emesis at low concentrations, but their importance to the pathogenesis of *S. aureus* infections lies in their ability to trigger T-cell activation. Toxic shock syndrome toxin-1 is another potent superantigen produced by both human and bovine strains of *S. aureus*. Superantigens trigger unregulated proliferation of T-helper cells by facilitating the binding of MHC class II molecules of antigen-presenting cells to T-cell receptors in a non-antigen-specific manner. This triggers a large proportion (2–20%) of T cells to become activated and to proliferate. This disrupts the normal immune response. Also, when high levels of toxins are expressed systemically, the massive release of cytokines causes toxic shock. In the rodent house musk shrew emesis model, enterotoxins induce 5-hydroxytryptamine (HT) release in the intestine, rather than in the brain, which is recognized by 5-HT(3) receptors on vagal afferent neurons and is essential for enterotoxin-stimulated emesis.

Bacteriophages

Any isolate of *S. aureus* will be lysogenic for one or more bacteriophages. The phages can contribute to the biology of the host bacterium in several ways. Upon induction of the lysogen, the phage capsid can incorporate fragments of chromosomal DNA or plasmids and introduce them by transduction into a new host. The pathogenicity island *S. aureus* pathogenicity island-1 (SAPI-1) that encodes toxic shock syndrome (TSST-1) in human strains is transduced at very high frequency by certain phages. Excision of the integrated element is actually stimulated by phage replication. A similar process might occur with a related pathogenicity island (PI) expressed by bovine strains. Some bacteriophages have incorporated genes that contribute to the virulence of the host. The genes are expressed when the phage integrates into the chromosome. CA-MRSA strains are commonly lysogenized with a phage that carries the genes encoding PVL (**Figure 1**). This could contribute to the enhanced virulence of CA-MRSA strains. About 60% of human *S. aureus* strains are lysogenized with a phage that carries an immune evasion gene cluster comprising genes encoding CHIPS, SCIN, staphylokinase (SAK), and SEA, or combinations thereof. The site of integration of the phage is within the coding sequence for the β-toxin, explaining why the majority of human strains do not express the toxin. In contrast, many bovine strains are β-hemolytic because carriage of this type of phage is less common. This could reflect an important role for β-toxin in promoting mastitis or the fact that the phage-encoded immune evasion proteins are specific for humans.

Biofilm

The formation of biofilm was first described in infections associated with indwelling medical devices caused by *S. epidermidis*, and later by *S. aureus*. However, it is now thought that the ability to form biofilm is important during *S. aureus* tissue infection. *In vitro* studies identified several stages in establishing a biofilm: (1) primary attachment; (2) cell–cell association; and (3) detachment and dispersal. Bacteria can bind to naked plastic or metal or to biomaterial that has been conditioned by host plasma proteins *in vivo*. The accumulation phase was originally thought to be due to the secretion of a polysaccharide intercellular adhesin PIA, but it is now recognized that cell surface proteins can also cause aggregation. Cells in a biofilm are in a semidormant state, which renders them much more resistant to antibiotics than planktonic cells. Furthermore, the physical structure of the biofilm prevents the immune system from functioning properly to eradicate the bacteria.

Small Colony Variants

Staphylococcus aureus is now regarded as a facultative intracellular pathogen, whereas previously it was thought to be exclusively extracellular. A subpopulation of *S. aureus* cells called small colony variants (SCVs) have an enhanced ability to enter into and survive within mammalian cells that are not normally phagocytic. SCVs are naturally occurring mutants defective in the biosynthesis of menadione, hemin, or thymidine. This results in reduced tricarboxylic acid metabolism. Phenotypically, SCV bacteria form colonies that are 10-fold smaller than normal colonies and which are nonpigmented and nonhemolytic. The fibronectin binding proteins that promote uptake into mammalian cells are expressed by SCV cells at higher levels than in wild-type bacteria. Once inside the host cell, the SCV can persist because it does not cause cell lysis or trigger apoptosis and the infected cells do not react by synthesizing proinflammatory molecules. Clinically, SCVs are associated with relapsing persistent infections such as osteomyelitis, some foreign body infections, and lung infections in cystic fibrosis patients. SCVs have not been associated with chronic mastitis in cattle, but this might be due to difficulties in the identification of the variants in the laboratory and their instability.

Antibiotic Resistance

The ability to treat *S. aureus* infections has been profoundly compromised by the development of resistance. Shortly after the introduction of penicillin in the late

1940s, penicillin-resistant bacteria emerged due to acquisition of the ability to express a β-lactamase that destroyed the antibiotic. The subsequent introduction of methicillin, a β-lactamase-resistant penicillin, was quickly followed by occurrence of methicillin resistance. This was due to acquisition of the *mecA* gene, which encodes a β-lactam-insensitive penicillin binding protein called PBP2a or PBP2′, which takes over cell wall biosynthesis in the presence of antibiotic (**Figure 1**). Methicillin resistance is now widespread in hospital strains and, very recently, highly virulent CA-MRSA strains have emerged and spread in the community at large. It should be emphasized that *mecA* confers resistance to all classes of β-lactam antibiotics including potent broad-spectrum cephalosporins, cephamycins, and monobactams.

In addition, *S. aureus* has developed resistance to all of the antibiotics that are used to treat staphylococcal infections (tetracycline, macrolides, aminoglycosides, quinolones). Wherever these antibiotics have been used to treat bovine mastitis, resistance has also been reported.

New antibiotics that are specifically targeted at MRSA have been licensed (Synercid, Zyvox, Cubicin) or are in development (a dehydrofolate reductase inhibitor called iclaprim, a fatty acid biosynthesis inhibitor called platensimycin, and a peptide deformylase inhibitor).

The rapid development of resistance is facilitated by horizontal transfer of resistance genes on mobile genetic elements or by mutation. In the case of methicillin resistance, the staphylococcal cassette chromosome (SCC) element can excise from the chromosome of the strain in which it is resident by site-specific recombination (**Figure 1**). It circularizes and can be transmitted to another strain by transduction or transformation. It then integrates into the chromosome at a specific site promoted by the element-encoded integrases. This process resembles the behavior of lysogenic bacteriophages. There are five major classes of SCC*mec* element that have transferred into many different strains to form distinct clones of MRSA. There is recent evidence of a specific MRSA type (ST398) infecting intensively reared farm animals, notably pigs. ST398 can be isolated from farm workers and human infections caused by this strain have been reported.

The majority of horizontally acquired antibiotic resistance is encoded by genes that are located on conventional plasmids and/or transposons. Resistance is caused by plasmid-encoded enzymes that modify the antibiotic binding site in the ribosome (macrolide lincosamide and streptogramin B resistance), by modification of the drug (aminoglycoside and chloramphenicol resistance), by specific drug efflux (tetracycline), or by displacement of the drug from its target (tetracycline). Resistance to fluoroquinolones occurs by stepwise acquisition of mutations that reduce the affinity of two topoisomerase enzymes, which are the inhibitory drug targets, and also by overexpression of the multidrug resistance efflux pump NorA, which reduces the intracellular drug concentration.

Vaccination

Recently, there have been several reports of the development and testing of subunit vaccines to combat *S. aureus* infections in humans. A polysaccharide vaccine comprising the two major serotypes of capsular polysaccharide 5 and 8 has progressed furthest but has shown limited success in human clinical trials. Successful vaccination of laboratory animals with recombinant surface protein antigens (e.g., ClfA and IsdB) has been reported recently. A combination of four of the best performing antigens (IsdA, IsdB, SdrD, SdrE) provided superior protection than each component alone. Vaccination to combat bovine mastitis using bacterins has achieved limited success in trials but has not been developed commercially. The poor performance is likely to be due to the nature of the immunogen, the method of administration, but particularly the difficulty of mounting an effective immune response in the lactating mammary gland.

See also: **Pathogens in Milk**: *Staphylococcus aureus* – Dairy.

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Staphylococcus aureus – Dairy

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Introduction

Enterotoxin-producing staphylococci, in particular *Staphylococcus aureus*, are probably the leading cause of foodborne illness throughout the world. The pathogenicity of *St. aureus* has been recognized for many years since this species causes mastitis and skin diseases in mammals or leads to foodborne intoxication. Milk and milk products can become contaminated if good hygiene practice (including mastitis control) is not exercised on farms and the milk is inadequately pasteurized or not heat-treated. After contamination with enterotoxigenic staphylococci food poisoning may happen when abundant growth and enterotoxin formation is encouraged during production and storage.

Staphylococcus aureus is classified in the family Micrococcaceae. The genus *Staphylococcus* is distinguishable from the morphologically similar members of the genus *Micrococcus* by biochemical methods such as the ability of growing anaerobically and demonstrating a fermentative metabolism. The two genera differ also in their DNA base, cell wall and enzyme composition.

The genus *Staphylococcus* has been divided into at least 27 species and seven subspecies. The major characteristics of the species *St. aureus* are coagulase and thermonuclease (TNase) production, but other coagulase- and TNase-positive species, such as *St. intermedius* and various strains of *St. hyicus*, are also described. Both species have been reported to produce enterotoxins at low levels.

Characteristics

Morphology and Culture

Staphylococcus aureus are Gram-positive, catalase-positive bacteria that do not produce endospores. They are 0.5–1.5 μm in diameter and divide in more than one plane to form irregular, three-dimensional clusters of cells. Colonies are smooth, raised, glistening and circular, and may reach a size of 4–6 mm in diameter on nonselective media. Colony pigmentation is influenced by growth conditions and varies from grey or grey–white with a yellowish tint, through yellow–orange to orange. In broth, growth changes from a uniform turbidity to a fine, easily suspended deposit.

Biochemistry and Factors influencing Growth

Nutritional requirements are moderate; organic nitrogen sources and group B vitamins are required for growth. *Staphylococcus aureus* is facultatively anaerobic, but grows best under aerobic conditions. Acid is produced aerobically and anaerobically from glucose, lactose, maltose and mannitol; under anaerobic conditions acid is produced from many other carbohydrates. Major end products of glucose metabolism are acetate and CO_2 (aerobic) and lactate (anaerobic). Catalase is produced by cells growing aerobically. *Staphylococcus aureus* is able to grow between 7 and 48 °C (optimum: 37 °C) and at pH values between 4 and 10 (optimum pH 6–7). *Staphylococcus aureus* tolerates lower a_w values than other bacteria (a_w minimum: 0.83–0.86). The microorganisms grow well at up to 10% NaCl and relatively slowly at 15%. Enterotoxins are produced during all phases of growth, but growth is possible at a wider range of environmental conditions than enterotoxin production. Most strains hydrolyse native animal proteins (e.g. caseins, gelatine, fibrin). Proteases, lipases and esterases are produced. Various lipids, Tweens and phospholipoproteins are hydrolysed with the release of fatty acids. Nearly all strains produce one or a combination of several haemolysins (α, β, δ and γ); β-haemolysin predominates in strains of animal origin. Some strains produce lecithinase.

Serology and Lysotyping

Due to the complex nature of the antigens, serotyping is not used often. However, together with phage typing, where the sensitivity to the lytic action of selected bacteriophages is tested, it is a useful tool in identifying the strain origin in epidemiological studies.

Resistance to Antibiotics

Antibiotic susceptibility and resistance are of eminent importance for all *St. aureus* induced illnesses. In

Table 1 Penicillin resistance of *Staphylococcus aureus* isolated from cow's milk

Reference	*Number of strains*	*Resistant (%)*
Untermann *et al*. (1973)	120	32.5
Schällibaum and Schären (1987)	250	33.3
Becker *et al*. (1989)	387	17.0
Adesiyun (1995)	250	23.6
Honkanen-Buzalski and Myllys (1996)	344 (1988)	31.8
	154 (1995)	50.7
Meaney and Flynn (1996)	182	65.9

Sources: Untermann F, Kusch D and Lupke H (1973) *Milchwissenschaft* 28: 686–688; Schällibaum M and Schären W (1987) *IDF Mastitis News* 12: 4; Becker H, Gang-Stiller K and Terplan G (1989) *Netherlands Milk and Dairy Journal* 43: 355–366; Adesiyun AA (1995) *Journal of Veterinary Medicine*, Series B 42: 129–139; Honkanen-Buzalski T and Myllys V (1996) *IDF Mastitis Newsletter* 21: 20–22; Meaney WJ and Flynn J (1996) *IDF Mastitis Newsletter* 21: 28.

connection with hospitalization, a widespread resistance against many antibiotics is reported. Concerning *St. aureus* causing mastitis in the udder of lactating cows, antibiotic resistance and susceptibility is most significant. Intramammary infusion of penicillin, which is successful in the treatment of streptococcal mastitis, is often ineffective against *St. aureus*. Staphylococcal mastitis has reached major importance today, due to the emergence of penicillin-resistant strains (**Table 1**). Data on the antibiotic resistance of *St. aureus* as a mastitic pathogen are gathered periodically from various countries by the International Dairy Federation.

Source

Infected Udder

Staphylococcus aureus is found on the skin, teats and mucous membranes of mammals. The infected mammary gland of cows and other animals used for milk production is the most important reservoir. From here the organisms spread to the udder skin, hands of milking personnel, udder cloth and bedding material, whereby transfer of the pathogens during milking is most important. When the udder is infected, *St. aureus* is excreted in the milk with high fluctuations in counts ranging from zero to 10^8 cfu ml^{-1}, but a level of 10^4 cfu ml^{-1} is usual. A characteristic of staphylococcal mastitis is its irregular shedding pattern that complicates diagnosis. Some cows never shed the organisms from the udder or do so infrequently; others shed the staphylococci intermittently at short intervals; and still others show a persistent shedding state, extended over several lactation periods. The frequency of the occurrence of *St. aureus* in milk is also related to age of the cow.

In contrast to reports on the frequency of enterotoxin producers in human strains, the findings with animal strains are very diverse and often apparently contradictory, depending on the source of strains, and the methods involved. The incidence of enterotoxigenic *St. aureus* from animal sources is generally lower than from strains in humans and may vary between 5% and 30%, with enterotoxin C predominating; the incidence seems to be higher in strains isolated from mastitic milk. In goats' milk, more than 30% isolates were enterotoxigenic.

Human Sources

Staphylococcus aureus is frequently found on the skin, nose, pharynx, axilla, umbilicus, perineum, gastrointestinal tract and urogenital tract of humans; the major reservoirs are the nails, skin and hair. The nose appears to be the principal site for multiplication. In various surveys, the incidence of human carriers ranged from 4% to 60%. The frequency of enterotoxigenic strains isolated from humans is high, varying between 40% and 60%; enterotoxin A (SEA) producers are most common. The organisms find their way into the food by hands (infected wounds, skin lesions) or by coughing and sneezing. In general, human contamination is the most important factor in staphylococcal food poisoning, because skin lesions are very common and often ignored by food handlers.

Environmental Sources

Originating from humans and animals, *St. aureus* is widespread in nature. The organisms have been isolated sporadically from soil, sand, marine and fresh water, sewage, plant surfaces and products, feeds, poultry and dairy products, and on the surfaces, dust, and air of inhabited areas. While the opportunities for environmental contamination of milk and dairy products are limited, some processes are still vulnerable, e.g. milk powder production.

Isolation and Identification

Numerous methods to isolate and identify staphylococci have appeared in the literature and are standardized by international and national organizations, e.g. International Organization for Standardization (ISO), Comité Européen de Normalisation (CEN), International Dairy Federation (IDF) and Association of Official Analytical Chemists (AOAC). For the bacteriological examination in mastitis control, nonselective media are used in most cases, where *St. aureus* is identified by its typical appearance on blood agar, Gram reaction and coagulase activity. For enumeration in foods, Baird–Parker agar (BPA) is recommended because of its high productivity, especially if foods containing stressed cells are analysed. However, the medium is not completely selective and reduction of tellurite and egg yolk reaction are poor diagnostic tools since competing microorganisms are also able to reduce tellurite and less than a half of the strains isolated from milk and dairy products yield a positive egg yolk reaction. Consequently, all black to grey colony types, irrespective of egg yolk reaction, must be examined for coagulase production. This lack of selectivity may lead to unreliable results in foods in which *St. aureus* is only a minor part of the total flora able to form colonies on the medium. Therefore ISO suggested rabbit plasma fibrinogen agar (RPFA) as an alternative. This medium allows the detection of coagulase directly on the plate due to the formation of fibrin haloes around the colonies, so that further confirmatory tests are not necessary. Plasma media with constant plasma quality are commercially available. For detection of low numbers ($<100\,g^{-1}$), enrichment procedures are used followed by streaking a loopful of the incubated media onto BPA or RPFA. Recommended enrichment media are tryptic soya broth containing 10% NaCl and 1% sodium pyruvate, Giolitti–Cantoni broth supplemented with 1% Tween 80 and liquid Baird–Parker medium. The two latter media are incubated anaerobically to enhance selectivity.

Identification of suspect colonies is done by the tube coagulase test using rabbit plasma EDTA. The production of thermonuclease may be used for screening purposes. As an alternative to the tube coagulase test, commercially available latex agglutination tests based on clumping factor and protein A detection are in use.

Detection of enterotoxins is of great importance in assessing a health hazard of a certain food. They are identified by their reactions with specific antibodies. To date, rapid and very sensitive methods for detecting the enterotoxins A–E based on sandwich enzyme-linked immunosorbent assay (ELISA) procedures are commercially available in test kit form which allows detection of 0.1–1 $ng\,g^{-1}$ of food. Compared to immunodiffusion assays, ELISA procedures are much more sensitive, but less specific and may give false positive results. For this reason, positive results, especially weakly positives, should be considered presumptive and confirmed by other tests. Reversed passive latex agglutination (RPLA) kits are also commercially available, but are less sensitive than ELISA and nonspecific agglutination is also possible. Due to their high specificity, immunodiffusion assays are still important for detecting strains of enterotoxigenic staphylococci. Recently, the polymerase chain reaction (PCR) has been introduced as a simple, highly sensitive and specific technique for the detection of enterotoxigenic strains.

Pathogenicity

Staphylococcus aureus and Infection

Staphylococcus aureus is able to cause acute infections in humans, which may be localized, e.g. pustular, or spread, e.g. septicaemia. These infections are mediated by a wide range of agressins and exotoxins that are chromosomal or plasmid mediated. Diseases due to specific protein toxins are known, e.g. toxic shock syndrome. In animals, *St. aureus* may cause pustular inflammations of the skin and other organs, of which mastitis is most important. Inflammation of the skin and tissues, especially the mammary gland, is treated by application of antibiotics, taking into account the antibiotic susceptibility and resistance properties of the pathogens involved.

Staphylococcus aureus and Intoxication

Food is implicated in staphylococcal disease in the case of enterotoxicosis. The agents responsible for staphylococcal food poisoning are a series of toxins described as enterotoxins because of their effects on the intestinal tract. They are labelled enterotoxins A (SEA), B (SEB), C1 (SEC1), C2 (SEC2), C3 (SEC3), D (SED) and E (SEE). Recently, the enterotoxins G, H and I were isolated and characterized, but only limited data about their characteristics exist to date.

Staphylococcal enterotoxins are single-chain, low molecular weight (26 000–29 000 Da), heat-stable proteins produced by many strains of *St. aureus*, but other coagulase-positive and even coagulase-negative staphylococci are also able to produce enterotoxins. Most food poisoning outbreaks involve enterotoxins A and D. Production of enterotoxins is strain-specific, but one strain may produce more than one toxin. The production of enterotoxins B and C is controlled by plasmids and occurs mainly at the end of the stationary phase whereas production of enterotoxins A, D and E is under chromosomal control and occurs throughout the logarithmic growth phase. Depending on temperature, pH, water activity, atmospheric conditions and the presence of other microorganisms, they can be produced in food when enterotoxigenic staphylococci are able to proliferate up to a level of at least 10^6 cfu g^{-1}. In most food poisoning outbreaks, counts of 10^8 cfu g^{-1} food and more, and enterotoxin concentrations of 1–5 μg g^{-1} food were detected. However, in sensitive individuals, 0.1–1 μg enterotoxin is sufficient to produce illness. Enterotoxins are resistant to proteolytic enzymes such as pepsin and trypsin; this makes it possible for them to pass through the digestive tract to the site of action.

The common symptoms of staphylococcal enterotoxicosis are nausea, retching, vomiting, abdominal cramps and diarrhoea and they usually develop within 1–6 h after ingestion of the enterotoxin- containing food. In severe cases, headache, muscular cramps, fever and drop of blood pressure may be observed. Deaths of children and elderly people have also been recorded, but they are rare. Recovery takes a few hours to approximately one day, so that a doctor is seldom consulted and consequently most cases are never reported. Effective treatment is not possible, primarily due to the short duration of the disease. In cases where excessive vomiting and diarrhoea occur, administration of fluids may be necessary to restore the salt balance.

Incidence in Milk and Dairy Products

Quarter Samples

Staphylococcus aureus is the most common pathogen causing both clinical and subclinical mastitis, not only in cows but also in ewes and goats. The organism is responsible for approximately 30% to 40% of all mastitis cases. The occurrence of *St. aureus* in quarter milk samples varies from 5% to 22%. In foremilk from subclinical cases, *St. aureus* counts vary from 30 to 380 000 cfu ml^{-1}, and in quarter milk from 210 to 78 000 cfu ml^{-1}.

Bulk Milk

The principal source of contamination of raw milk is the infected udder. For this reason, *St. aureus* is regularly found in bulk milk. However, the counts are related to the mastitis situation of the herd and may range from less than 10 to several thousands per ml milk with occasional counts of 10^5 ml^{-1}. In the EU, criteria for the *St. aureus* content in raw cows' milk intended for consumption without heating ($n=5$, $c=2$, $m=100$ ml^{-1}, $M=500$ ml^{-1}) and for the production of raw milk products ($n=5$, $c=2$, $m=500$ ml^{-1}, $M=2000$ ml^{-1}) have been established. In the criteria cited above, $n=$ number of samples analysed; $c=$ maximum allowable number of defective sample units; $m=$ the value of the characteristic separating good quality from marginal quality; $M=$ the value of the characteristic separating marginal quality from bad quality ($m<M$).

These standards can only be met when effective mastitis control programmes are incorporated on farms and the milk is stored at low temperatures before consumption and manufacture according to the cooling temperatures given in the EU Milk Hygiene Directive 92/46.

Dairy Products

Liquid products

In liquid milk, the organisms are eliminated by pasteurization. Nevertheless, if recontamination occurs or contaminated additives are added after pasteurization, pasteurized milk is an excellent medium for growth and enterotoxin production due to its optimal nutritional status, water activity, pH value, redox potential and the lack of competing flora. In general, fluid milk is less often involved in food poisoning outbreaks than dried milk or cheese.

Dried products

The technology of milk powder production includes several heating procedures, i.e. pasteurization, evaporation and the drying process itself. If contamination occurs, growth and enterotoxin formation during the production process is possible; during the storage of dried milk the organisms surviving the drying process slowly die. Poisoning may happen directly when the milk powder contains enterotoxin and indirectly when contaminating enterotoxigenic staphylococci are able to grow after reconstitution of the powder.

Fermented milk products

Fermented milk products may be considered as safe because the raw milk is heated at high temperatures. If recontamination occurs during manufacture the organisms are rapidly inactivated by amensalism and antagonism of the starters used. Actively growing lactic acid bacteria (LAB) inhibit the growth of staphylococci and also kill them off to some extent. If the fermented product itself is contaminated, the contaminants are not able to multiply due the acidic environment even when cooling is unsatisfactory.

Cheese

During cheese manufacture, the staphylococci in milk are physically concentrated approximately 10-fold in the curd. Therefore the *St. aureus* content in the young cheese is directly dependent on the *St. aureus* counts in milk. Further, the organisms are able to proliferate during the first 24 h (in the vat and during pressing/moulding) until the acids produced by the starters inhibit further growth. If abundant growth of enterotoxigenic strains is possible at this stage of production, enterotoxins will be formed. The magnitude of multiplication is strongly dependent on the amount, the activity and the type of the starters used. Growth and enterotoxin production is inhibited primarily due to the low pH and the formation of lactic acid, but during Gouda cheesemaking, inhibition was observed before the pH had dropped below 6.0 and it was concluded that the onset of inhibition of *St. aureus* may be due to the decrease in redox potential and the formation of antimicrobial compounds excreted by LAB. An increase of 1.5 to 3 log during the first 24 h of cheesemaking may be expected under normal conditions, but, in cheeses with suboptimal acidification, increases up to 5 log have been observed. Low acidification is caused by insufficient starter development due to inhibition of LAB by antibiotic residues in milk or by bacteriophage infection, but the acidification rate is also lowered when inadequate inocula were used or the starters are inactivated due to prolonged storage. In the production of Cheddar cheese, inhibition of LAB is also caused by addition of salt to the curd. A special case is the production of cooked hard cheeses made from raw milk (Emmental cheese, Gruyère type cheeses, extra hard cheeses), where the curd is cooked at temperatures between 50 and 57 °C. Due to these high cooking temperatures, the high temperatures on the press and the acidification with active thermophilic starters, *St. aureus* is usually inactivated during the first day of manufacture.

Although there tend to be large differences between cheese varieties, *St. aureus* counts decrease during ripening and storage of cheese manufactured with starters of normal activity. However, when acidification is suboptimal, the decline in numbers may not be apparent and less than 1 log cycle. In general, the higher the ripening temperature the higher is the decline in numbers, although the amount of salt present in the cheese may influence this. If enterotoxins were produced during manufacture, they may persist over years in the cheese.

Apart from the acidification rate at the beginning of manufacture, the *St. aureus* content of cheese is primarily dependent on the cheese variety (technology), the kind of milk used for manufacture (raw milk/heat treated milk) and the possibilities of contamination during the manufacturing process (open/closed system). In contrast to cheeses made from pasteurized milk, where *St. aureus* is usually not detected or present in low numbers, raw milk cheeses are a potential danger, unless contamination of milk is low and acidification is optimal. Therefore, the EU established criteria for cheeses made from raw milk ($n = 5$, $c = 2$, $m = 1000\,\mathrm{g}^{-1}$, $M = 10\,000\,\mathrm{g}^{-1}$), irrespective of the origin of the milk (cow, sheep, goat). If the maximum level M is exceeded, the cheese has to be tested for enterotoxins and also the enterotoxigenicity of the isolated coagulase-positive strains must be determined.

Control

General Hygiene

Contamination with *St. aureus* can be effectively controlled by good manufacturing practice (GMP). In recent years, the dairy industry was forced by international and national authorities to introduce the principles of Hazard Analysis Critical Control Point (HACCP) systems according to the EU Milk Hygiene Directive 92/46. This directive provides guidelines for the hygienic design and maintenance of buildings, services and equipment, and also for personal hygiene. Regular training of persons handling food is of eminent importance in preventing human contamination. Further, cleaning and disinfection schedules are necessary for eliminating staphylococci from surfaces. In the production of raw milk cheeses, it must be guaranteed that milk from mastitic cows is not used. This can be achieved by effective mastitis control programmes on farms including the culling of chronic *St. aureus* cows.

Cooling of milk

Since *St. aureus* fails to multiply at temperatures below 7 °C, cooling is the most effective means of preventing growth and enterotoxin production. When cheese is produced from raw milk, the milk has to be cooled immediately after production and extended storage must be avoided. In general, raw milk must be processed as soon as possible unless the milk is heat treated. Also preripening in the vat must be as short as possible to prevent undesired growth.

Heating of Milk

Pasteurization of milk (72 °C for 15 s) is effective in the elimination of *St. aureus*. However, even after applying this temperature/time combination, 0.38% of the staphylococci may survive, but no survivors were detected at 72 °C for 35 s. In cheesemaking thermization of milk (57–68 °C for at least 15 s) is also applied. The temperature/time combinations at these subpasteurization conditions do not guarantee elimination of *St. aureus* in every case. Thermal resistance is primarily dependent on the strain, the physiological state of the organisms and the medium. Decimal reduction times may vary between 0.43 and 8 min at 60 °C. Unlike the producer organism, staphylococcal enterotoxins are remarkably heat resistant, showing D-values of 3–8 min at 121 °C.

Interactions with other Microorganisms

Since *St. aureus* is a poor competitor, its growth is inhibited by common spoilage organisms. *Bacillus cereus*, *Proteus* *vulgaris*, *Escherichia coli*, *Enterobacter aerogenes* and *Achromobacter* spp. inhibit staphylococci by production of antibiotic substances, while *Serratia marcescens* and *Pseudomonas* spp. appeared to inhibit *St. aureus* by out-competing it for amino acids. In foods with reduced a_w (0.95 or less and salt concentrations of 5.5% and higher), growth of competing flora will be suppressed, whereas *St. aureus* is able to proliferate under these conditions. In such foods, a hazard can only be minimized by preventing contamination and storage at low temperatures.

In the production of fermented milk products, the strong antagonistic activity of LAB is most important. *Staphylococcus aureus* is inhibited by LAB due to low pH and redox potential, and the formation of acids (especially lactic acid) and antibiotic substances. In the manufacture of raw milk cheeses, sufficient acidification, especially in the early stages of cheesemaking, must be achieved by the use of a sufficient amount of an active starter. Measurement of cheese pH two hours after moulding gives information about the acidification rate.

Conclusions

The growing interest concerning the group of 'new emerging pathogens' (e.g. *Listeria monocytogenes*, *Campylobacter* spp., enterohaemorrhagic *E. coli*) has overshadowed the interest in *St. aureus*. Nevertheless, *St. aureus* remains one of the most important foodborne pathogens, therefore the knowledge of specific characteristics of this species, such as pathogenicity, and of its incidence in milk and dairy products is essential for finding effective ways to control this microorganism in food hygiene and food technology.

See also: **Mastitis Pathogens**: Environmental Pathogens. **Microorganisms Associated with Milk**.

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Yersinia enterocolitica

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Characteristics

Taxonomy

The genus *Yersinia* contains 15 species but only 3 are pathogenic to humans. *Yersinia pestis* is the cause of plague, *Y. pseudotuberculosis* is associated with intestinal infections and mesenteric lymphadenitis, and *Y. enterocolitica* is associated with foodborne intestinal infections. There are a number of species closely related to *Y. enterocolitica*, which were previously called 'atypical *Y. enterocolitica*' or '*Y. enterocolitica*-like organisms', but which have now been identified as separate species including *Y. intermedia*, *Y. kristenseniae*, *Y. frederikseniae*, *Y. mollaretii*, *Y. bercovieri*, *Y. aldovae*, and *Y. rohdei*. These species are widely distributed in the environment and can be isolated from food and water, and from human and animal feces, but there is no evidence linking them with human or animal disease.

Biotypes and Serotypes

Yersinia enterocolitica is a heterogeneous species that can be divided into a number of groups based on biochemical activity (biotypes) and lipopolysaccharide O-antigens (serotype). Six biotypes are described (**Table 1**). The strains associated with human disease are most commonly found in biotypes 1B, 2, 3, and 4. Biotype 1A strains were traditionally regarded as environmental but now there is evidence that some strains can cause human disease, and biotype 5 has been isolated only from animals and the environment.

At least 60 different serotypes are recognized, with O-antigens shared between *Y. enterocolitica* and its close relatives. Heat-labile flagella H antigens have been described but are not used in typing. There are strong associations between particular biotypes and serotypes (**Table 2**) and between bioserotypes, pathogenicity, and geographic distribution. Bioserotype 4, O:3 is the most common human pathogen and is now found worldwide. In northern Europe bioserotypes 2, O:9; 2, O:5,27; and 3, O:5,27 have also been important. However, since 2004 there has been a surge in O:8 cases in northern Europe. Biotype 1B strains (bioserotypes 1B, O:8; 1B, O:13a,13b; 1B, O:20; 1B, O:21), called North American strains, are more pathogenic than the European strains. Information on geographic distribution of bio/serotypes is given in **Table 2**.

Laboratory Identification

Morphology and Appearance in Cultures

Yersinia enterocolitica is a small Gram-negative rod. It is actively motile at 22–25 °C but not motile at 37 °C. It produces circular, smooth, low, convex colonies of 1–2 mm diameter with an entire or slightly crenulated edge after incubation at 37 °C for 24 h. On MacConkey agar, 1.5–2 mm translucent, pale non-lactose fermenting colonies are seen. Some environmental strains do ferment lactose and thus produce bright pink colonies on MacConkey agar. On cefsulodin–irgasan–novobiocin (CIN) agar *Y. enterocolitica* produces highly characteristic 1.5 mm diameter colonies with a dark pink center and a translucent border (bull's-eye) colonies.

Identification

The optimum temperature for growth of *Y. enterocolitica* is 28 °C, and if identification tests are incubated at 37 °C confusing results can be obtained. Commonly used identification tests are listed in **Table 3**. Identification kits such as API 20E (bioMérieux SA, France) kit system can be used but should be incubated at 30 °C. However, biochemical tests are not particularly reliable as non-pathogenic species can be misidentified as *Y. enterocolitica.*

Increasingly, molecular methods such as 16S rRNA sequencing are used for definitive identification. Molecular typing based on DNA–DNA hybridization and 16S rRNA sequencing can distinguish between biotype 1B (North American pathogenic mouse lethal strains) and the European strains. In addition, PCR for virulence genes such as the plasmid-encoded *vir*F and *yad*A, and the chromosomally encoded *ail*, *inv*, *yst*, and *rfb* genes can be used to distinguish *Y. enterocolitica* from its environmental relatives.

Physiological Properties

Yersinia enterocolitica is capable of growth at temperatures ranging from below 0 to 44 °C, although its optimum temperature range is 22–28 °C. It tolerates a pH range from 4.6 to 9.0 but prefers slightly alkaline conditions. The *D* values of *Y. enterocolitica* are approximately 2 min at 55 °C, 0.5 min at 60 °C, and 2 s at 65 °C. The *D* value for *Y. enterocolitica* in milk is 0.24–0.96 min at 62.8 °C.

Table 1 Biotypes of *Yersinia enterocolitica*

	Biotype					
Test	*1A*	*1B*	*2*	*3*	*4*	*5*
Lipase (tween esterase)	+	+	–	–	–	–
Esculin hydrolysis	+/–	–	–	–	–	–
Indole production	+	+	(+)	–	–	–
Acid from xylose	+	+	+	+	–	V
Acid from salicin	+/–	–	–	–	–	–
Acid from trehalose	+	+	+	+	+	–
Nitrate reduction	+	+	+	+	+	–
Pyrazinamidase	+	–	–	–	–	–
β-D-Glucosidase	+	–	–	–	–	–
Voges–Proskauer	+	+	+	+/–#	+	(+)
Proline peptidase	V	–	–	–*	–	–

*, biotype of serotype 0:3 found in Japan; #, some chinchilla isolates may be positive; +, positive; (+), delayed positive; –, negative; V, variable reaction.
Reprinted with permission from Barton MD and Robins-Browne RW (2002) *Yersinia enterocolitica*. In: Hocking A (ed.) *Foodborne Diseases of Public Health Significance*, 6th edn. Sydney, NSW: AIFST.

Table 2 Relationship between biotypes and serotypes in *Yersinia enterocolitica*

Species and biotype	*Serotype*	*Pathogenic in man or animals*	*Predominant distribution*
Y. enterocolitica			
Biotype 1A	O:4; O:5[a]; O:6[a]; O:6,3[a]; O:6,30[a]; O:6,31[a]; O:7,8[a]; O:7,13; O:10[a]; O:10,46[a]; O:14; O:16; O:17[a]; O:19[a]; O:21[a]; O:22; O:25; O:37; O:41,42[a]; O:46; O:57; NT[a]	Some serotypes possibly associated with gastroenteritis	Environment, water, human and animal feces – worldwide
Biotype 1B	O:8	Yes	Japan, USA, Europe
	O:13a,13b; O:20; O:21	Yes	USA
	O:4,32; O:16;O:18; O:25; O:41; O:42; NT	No	Environment
Biotype 2	O:8	Yes	Japan, USA, Europe
Biotype 3	O:1,2,3; O:3; O:5,27; O:47	Yes	Pigs – Europe, Australia
Biotype 4	O:3; O:15	Yes	Pigs – worldwide
Biotype 5	O:2,3	No – animals only	Europe

[a] Biotype 1A serotypes with reported epidemiological linkage to cases of gastroenteritis.

Table 3 Biochemical tests used in the identification of *Yersinia enterocolitica*

Test	
Catalase	+
Oxidase	–
Motility (25 °C)	+
Motility (37 °C)	–
Nitrate reduction	+
Fermentation of glucose	+
Fermentation of lactose	V
Fermentation of cellobiose	+
Fermentation of sucrose	+
Fermentation of trehalose	+
Urease	+
Citrate utilization	–
Triple sugar iron agar	Alkaline or acid slope, acid butt, negative gas, negative H_2S production
Lysine decarboxylase	–
Ornithine decarboxylase	+
Indole	V
Vogues–Proskauer (25 °C)	+

Serotyping and Biotyping

Serotyping and biotyping are useful indicators of the potential pathogenicity of isolates of *Y. enterocolitica.* Antisera to only a few serotypes (O:3, O:8, O:1,2, O:9, and O:5,27) are available commercially.

Phage Typing

Phage typing is carried out in a few specialized laboratories, and thus is not commonly used. Geographic distribution of phage types of bioserotype 4,O:3 is recognized, as is the association of particular phage types of this bioserotype with the development of postinfection sequelae. Phage typing has largely been replaced by molecular typing.

Molecular Typing

A number of molecular methods have been applied to the identification of *Y. enterocolitica* and differentiating

Table 4 Molecular identification and typing of *Yersinia enterocolitica*

Method	*Typability of strains*	*Reproducibility*	*Discriminatory power*	*Comment*
REAC	All	Poor	Moderate	Some serotype-specific patterns; useful for subtyping O:8 strains
Ribotyping	All	Good	Good	Differentiates species; useful for subtyping O:3 strains
16S–23S IGS typing	All	Good	Potentially useful	Only two studies – potential for subtyping
RAPD	All	Moderate	Moderate	Useful in epidemiological studies
PFGE	All	Good	Good	Best method to date for subtyping
MLST	All	Good	Good	Only one study to date – good inter- and intraspecies differentiation
AFLP	All	Good	Good	Only one study – strong alignment with biotype
16S DNA sequencing	All	Good	Good	Species identification – differentiation of pathogenic strains

16S–23S IGS, 16S–23S intergenic spacer typing; AFLP, amplified fragment length polymorphism; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; REAC, restriction endonuclease analysis of chromosome.

pathogenic from nonpathogenic strains (**Table 4**). Unfortunately, the high similarity between strains and the predominance of particular genotypes within bioserotypes limit the value of these methods.

Pathogenicity

Virulence determinants of *Y. enterocolitica* are found both on the chromosome and on a 70-kb virulence plasmid. In addition, the North American biotype 1B strains carry a chromosomal pathogenicity island associated with increased virulence for mice and humans.

Chromosomal virulence genes include *yst*, which encodes Yst, a heat-stable enterotoxin; *inv*, which encodes invasin, a surface protein important in the translocation of *Y. enterocolitica* across the intestinal epithelium and in the colonization of Peyer's patches in the mouse model of infection; and *ail* (found only in pathogenic strains), which encodes a membrane-associated protein that promotes attachment of the organism to eukaryotic cells, has a support role as a secondary invasion factor, and confers resistance to the action of serum complement. Numerous other chromosomal genes contribute to virulence, including a urease gene complex – pathogenic strains of *Y. enterocolitica* produce urease, which may facilitate survival of the organism in acidic environments such as the stomach.

The pYV plasmid of *Y. enterocolitica* encodes the Yop (*Yersinia* outer proteins) operon, which is an integrated virulence apparatus. Mouse studies have shown that strains lacking Yop are readily phagocytosed by macrophages and are unable to cause disease. The Yop operon is made up of at least 14 proteins, the majority of which have been shown to be essential for virulence. Another virulence gene carried on the pYV plasmid is *yad*A, which encodes YadA, an outer membrane protein expressed only at 37 °C and which is responsible for adhesion to intestinal epithelium, serum resistance, and resistance to killing by phagocytic cells.

Highly pathogenic biotype 1B strains carry a high-pathogenicity island (HPI), which is an iron uptake system that facilitates the growth of these strains under iron-depleted conditions at 37 °C.

Until recently, biotype 1A strains were regarded as nonpathogenic. They do not carry the pYV plasmid, but are occasionally isolated from humans with gastrointestinal diseases. Serotypes O:5, O:10, and O:6,30 have to date been associated with outbreaks of clinical disease.

The infectious dose of *Y. enterocolitica* is unknown but is estimated to be around 10^6–10^7 organisms.

Pathogenesis

After ingestion, pathogenic *Y. enterocolitica* pass through the intestinal tract to the ileum, where they bind to the M cells in Peyer's patches. This attachment is facilitated by YadA. The organisms penetrate the intestinal mucosa through M cells and colonize the Peyer's patches from where they can spread to the mesenteric lymph nodes through lymphatic vessels. This phase is facilitated by Yops. In rare cases *Y. enterocolitica* can spread through the bloodstream to other sites such as liver and spleen. YadA and Ail contribute to resistance to killing by serum complement.

Clinical Disease

Yersinia enterocolitica is an enteric pathogen, which in susceptible people can cause extraintestinal disease.

Enterocolitis is the most common manifestation and is seen principally in young children. Older children and adolescents may develop mesenteric lymphadenitis (mimicking appendicitis). Some cases are subclinical, and in the clinical cases the onset of clinical signs usually occurs within 24–48 h of ingestion of the organism. The illness usually lasts 1–3 days, with diarrhea seen in most cases plus fever, headache, and vomiting in many cases. Involvement of mesenteric lymph nodes leads to severe abdominal pain. The minimum infective dose for humans is not known, and the duration of excretion of the organism ranges from 2 weeks to more than 2 months.

Yersinia enterocolitica infection is normally self-limiting but long-term sequelae such as reactive arthritis, erythema nodosum, uveitis, glomerulonephritis, and myocarditis have been reported. Reactive arthritis is associated with the human leukocyte antigen HLA-B27 and certain strains of bioserotype 4, O:3 more common in Nordic countries. Patients with underlying iron metabolism disorders such as siderosis, thalassemia, or hemochromatosis are more susceptible to bacteremia and extraintestinal infections, and transfusion-related septicemia has been reported in patients receiving blood transfusions from asymptomatic blood donors.

Epidemiology and Ecology

Most cases of *Y. enterocolitica* infection are reported from the temperate areas of the world, for example, Scandinavia, northern Europe, and New Zealand. It is widely distributed in the environment and can be isolated from soil and water, and the intestinal tract of many vertebrates and invertebrates. The organism has minimal nutrient requirements at low temperatures and can remain metabolically active at very low temperatures. Most environmental strains belong to the nonpathogenic serotypes, but pathogenic serotypes O:8, O:9, and O:5,27 have been isolated from healthy pigs.

The pattern of distribution of serotypes has changed over the years. Originally, serotype O:3 was rare in the United States in comparison with serotype O:8, but in the late 1980s serotype O:3 overtook O:8 and is now the most common serotype worldwide. However, serotype O:8 causes more serious infections.

Sources of Infection

Infection with *Y. enterocolitica* has been linked most clearly to the handling and consumption of raw and undercooked pork, with the same serotypes (principally O:3, O:9, and O:5,27) and genotypes isolated from pigs and affected humans. *Yersinia enterocolitica* has often been isolated from foods, including animal products (beef, lamb, poultry, as well as pork), milk, shellfish, and crustaceans. However, most of the isolates belong to nonpathogenic serotypes.

Most infections with *Y. enterocolitica* occur sporadically, where the source of infection is hard to determine. A prospective case–control study in Norway suggested a strong linkage with consumption of raw or rare meat and untreated water. Vegetables and fresh herbs have also been incriminated.

Outbreaks of *Y. enterocolitica* have been reported in a number of countries including the United States, Canada, Japan, Finland, and Czechoslovakia. Incriminated sources include raw and pasteurized milk, chocolate milk, tofu, bean sprouts, and spring and stream water. In many of the outbreaks no source could be identified. Serotypes reported include O:3 (11 of 20 outbreaks); O:8; O:9; O:5,27; and O:13a, 13b. Person-to-person spread in family clusters and direct transmission from animals have been suspected.

Occurrence of *Yersinia enterocolitica* in Milk and Dairy Products

A small number of outbreaks associated with milk and dairy products have been reported from a number of countries (**Table 5**). No outbreaks have been reported in the literature since 2000. Some serotypes usually regarded as nonpathogenic are alleged to have been the cause of some of these outbreaks. Definitive proof, one way or the other, is difficult to obtain when nonpathogenic environmental strains of *Y. enterocolitica* are so widely distributed in the environment generally and in milk products specifically, as indicated in **Table 6**. In general, the microbiological evidence linking even the pathogenic serotypes with the outbreaks is not strong, and the causative linkage is based on epidemiological criteria from investigations such as case–control studies or analysis of food consumption histories. It is interesting to note that many of the outbreaks are associated with pasteurized milk, although *Y. enterocolitica* is quite heat-sensitive and readily destroyed by pasteurization at concentrations up to 10^5 organisms ml^{-1}. If the milk product is at fault, there must have been postpasteurization contamination. In several of the outbreaks there appears to have been the possibility of postprocessing contamination from pig-related contacts. *Yersinia enterocolitica* grows well in raw milk but there are few outbreaks linked to this source. The role of milk and dairy products in sporadic cases of *Y. enterocolitica* infection is not well studied.

There are a number of reports of isolation of *Y. enterocolitica* from raw and pasteurized milk, as indicated in **Table 5**, and some reports of isolation from milk products such as ice cream and yogurt. Most of

Table 5 Outbreaks of *Yersinia enterocolitica* infection associated epidemiologically with milk and dairy products

Product	*Number of human infections*	*Bioserovar*	*Number of isolates from product*	*Country (year)*
Raw milk	138	O:5,27	Different serotype	Canada (1976)
Chocolate milk	36	O:8	1	USA (1976)
Milk from powder	239	O:8	?	USA (1981)
Pasteurized milk	172	O:13; O:18[a]	Nil	USA (1984)
Pasteurized milk	19	1,O:10[a]; 1,O:6,30[a]	1 of each	UK (1990)
Pasteurized milk	11	O:3; O:6,30[a]	9 (O:6,30[a])	Australia (1991)
Raw buttermilk	25	4,O:3	Nil, 1 from well water	India (1997)
Pasteurized milk	10	O:8	1	USA (2000)

[a] Biotype 1A serotypes previously regarded as nonpathogenic.
?, no information provided.

Table 6 Isolation of *Yersinia enterocolitica* from milk and dairy products

	Serotype	*Countries*
Pathogenic	O:21; O:5,27; O:9	Canada, France, USA, Ireland, UK, Brazil, Bulgaria, Iran
Biotype 1A strains some of which may be pathogenic	O:4,32; O:4,33; O:5; O:6,30; O:6,60; O:7,8; O:14; O:15; O:18; O:34; O:41,42; O:46	Canada, Australia, France, USA, Italy, UK, Ireland, Brazil

the serotypes reported are those regarded as nonpathogenic, and there appear to be no reports of isolation of O:8 or O:3 or O:9 from raw milk, although there are a number of reports of isolation of O:5,27 (which includes both pathogenic and nonpathogenic strains). Many studies unfortunately do not report the serotypes isolated, and some do not differentiate between *Y. enterocolitica* and *Y. enterocolitica*-like species.

There are no reports of detection of *Y. enterocolitica* in ripened hard cheeses and only occasional reports of isolation of the organism from soft cheeses made from raw milk. However, experimental studies have shown that pathogenic serotypes can grow on outer and exposed surfaces of cheese such as Brie at 4, 8, and 20 °C, and in Cottage cheese. In most cases where serotyping is reported, the isolates from milk and milk products belong to the nonpathogenic serotypes of biotype 1A (e.g., serovars O:5; O:6,30; and O:7,8) or environmental stains in other biotypes. A number of studies where samples are spiked with microorganisms have reported that *Y. enterocolitica* does not survive as well as other enteric bacteria in fermented milk products such as yogurt.

Yersinia enterocolitica (serotypes not specified) have been isolated from dairy plant environmental samples and equipment in dairy factories.

Isolation and Detection of *Yersinia enterocolitica* in Food and Environmental Samples

The following are the factors that must be considered in approaching the isolation of *Y. enterocolitica* from food or environmental samples: first, the organism may be present in low numbers, so enrichment is required; second, the presence of the organism could be masked by the presence of large numbers of other bacteria, so a selective step is essential; third, nonpathogenic *Y. enterocolitica* and *Y. enterocolitica*-like species are likely to be present, so differentiation of pathogenic from nonpathogenic organisms is required. As a result, in recent years a combination of selective enrichment and molecular methods has been developed.

Conventional isolation procedures involve an enrichment procedure followed by plating onto selective media and confirmation of the identity of colonies that have typical colonial morphology. There is no single method of enrichment that will guarantee isolation of all pathogenic *Y. enterocolitica* serotypes, so the use of more than one approach is often recommended.

Enrichment approaches include

1. *Cold enrichment at 4 °C*: This approach takes advantage of the fact that *Y. enterocolitica* multiplies at 4 °C. Simple cold enrichment in phosphate-buffered saline

(PBS) or tryptone soy broth (TSB) or PBS supplemented with 1% sorbitol and 0.15% bile salts for up to 3 weeks can be used. The method is quite sensitive as *Y. enterocolitica* multiplies under these conditions while nonpsychrotrophic contaminating organisms die out. However, the method is too slow for routine use.

A modification of cold enrichment is to treat the enrichment broth with KOH, as *Y. enterocolitica* is quite tolerant of alkaline conditions. This is reported to be less effective than other enrichment methods.

2. *Single-step enrichment in various selective broths*: Selective broths used include modified Rappaport's medium (MRM) incubated at 22–25 °C for 2–4 days, irgasan–ticarcillin–potassium chlorate (ITC) medium incubated at 24 °C for 2–3 days, and modified selenite medium. Bioserotype 4, O:3 appears to grow better in MRM and ITC than do other bioserotypes (2, O:9; 2, O:5,27; and IB, O:8). Bile–oxalate–sorbose medium has been reported as suitable for serotype O:8.

3. *Two-step enrichment*: This method involves preenrichment in a nonselective medium such as PBS or TSB at temperatures between 4 and 25 °C for 24 h or longer, followed by inoculation into a selective broth such as MRM. This is reported to allow more sensitive strains of *Y. enterocolitica* to multiply in the nonselective medium so that a larger inoculum is exposed to the selective medium. A different strategy involving the use of Luria–Bertani–bile salts broth incubated for 24 h at 12 °C before the addition of irgasin and a further incubation for 48 h at 12 °C has been reported to be particularly successful in the isolation of serotypes O:8, O:5, O:27, and O:3 from spiked samples and in the isolation of bioserotype 4, O:3 from pig samples.

4. *Selective isolation media*: *Yersinia enterocolitica* grows readily on a range of media used for the isolation of other enteric organisms such as MacConkey agar, desoxycholate citrate agar, and salmonella–shigella agar. However organisms may be overlooked on such media, particularly if present in low numbers. CIN medium was developed specifically for the isolation of *Y. enterocolitica* and has the additional advantage that the organism produces the characteristic bull's-eye colonies on this medium. CIN is considered the most effective medium for the isolation of *Y. enterocolitica*.

A method for direct detection of virulence plasmid-carrying strains of *Y. enterocolitica* has been described, which involves subculture of Luria–Bertani–irgasan broths onto low-calcium Congo red brain heart infusion agar. Colonies of virulent (plasmid-bearing) *Y. enterocolitica* produce pinpoint red colonies on this medium.

Molecular Detection Methods

The traditional cultural methods for the detection of *Y. enterocolitica* have limitations, especially when attempting to detect the organism in food or environmental samples. It is clear that detection of *Y. enterocolitica* in such samples can be improved by the use of polymerase chain reaction (PCR) techniques. Target genes that have been used for PCR include *ail*, *yad*A, and *yst*. Generally speaking, chromosomally located target genes are preferred because the plasmid is unstable and easily lost during subculturing in the laboratory. Single, multiplex, and nested PCR methods have been reported, and, more recently, real-time PCR methods have been described. These have the advantage of greater specificity and require less time to complete than conventional PCR. However, there are some limitations and problems associated with the use of PCR. These include the presence of inhibitory substances in the sample, the inability to differentiate between live and dead organisms, and the low number of organisms in the sample. These problems can be overcome to some extent by using an enrichment step and applying the PCR to the incubated enrichment broth.

Prevention and Control

Pasteurization of milk and milk products is a key control measure. However, care has to be taken to ensure that postpasteurization contamination does not occur as *Y. enterocolitica* can survive and multiply in milk and on the surface of cheeses at refrigeration temperatures. Nonheat methods of treatment such as high-pressure cold pasteurization and pulsed electric field treatments may have a role to play, but their efficacy in killing *Y. enterocolitica* has not been evaluated. While in the past there may have been concerns about milk as a major source of *Y. enterocolitica* infections, it is now clear that most infections are directly or indirectly associated with pigs and pig products.

See also: **Analytical Methods**: DNA-Based Assays; Microbiological. **Cheese**: Public Health Aspects. **Contaminants of Milk and Dairy Products**: Environmental Contaminants; Contamination Resulting from Farm and Dairy Practices. **Microorganisms Associated with Milk**. **Psychrotrophic Bacteria**: Other Psychrotrophs.

Further Reading

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PLANT AND EQUIPMENT

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Process and Plant Design

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Introduction

The design of a dairy plant and its associated processes is often an open-ended problem with many possible solutions. Each design solution may have a unique impact on production capacity, process control, safety, environment, and economics. In conducting an engineering design project, several requirements relating to the following aspects must be met:

1. Product (raw materials and quality of the end product)
2. Process (plant capacity, equipment, controls, and cleaning)
3. Economics (cost of production)
4. Legal considerations (regulations relevant to process, equipment, and safety).

Although it is common to seek a compromise between these requirements for a specific application, careful consideration of these characteristics is essential to develop an optimal design. This requires an extensive literature search, knowledge of the food and its material properties, and a description of the equipment, including process flow diagrams. Numerous reference books are available for designing dairy processes and plants (see 'Further Reading'). Research journals such as *Australian Journal of Dairy Technology, Dairy Science & Technology, International Dairy Journal, Journal of Dairy Research,* and *Journal of Dairy Science*, patents, and technical literature available from manufacturers through the World Wide Web are also potential sources of information.

Process Flow Sheet

The design process begins with the preparation of a detailed flow sheet, which should clearly indicate the major plant equipment and the flow of product, utilities, and waste materials. Although a simple flow sheet is merely a sequence of processing equipment, it is often necessary to develop a detailed flow sheet with information such as the type of processing equipment, pipes, utility requirements, flow rates, and required instrumentation. It is not uncommon to develop separate flow sheets with more specific information for special purposes, such as for detailing all the control instrumentation. A 3D flow sheet is also generally used for a better visualization of the plant and equipment. A clean-in-place system should be included in the flow sheet for process equipment and pipelines. Each of these items must be labeled clearly. **Figure 1** shows the diagram of a multiproduct dairy plant and illustrates a simple flow sheet for a market milk process. This flow sheet may differ depending on different regulatory and marketing requirements. Although pasteurizing whole milk is a simple process line, complexities arise when manufacturing several types of milk products, such as whole milk, milk standardized to various fat contents, and creams with different fat levels.

After the flow sheet is completed, the next two steps include conducting mass and energy balances. A mass balance must be comprehensive enough to include all materials flowing through the plant and their composition. The energy balance is conducted to determine the energy requirements of all the operations identified on the flow sheet. After the mass and energy balances have been determined, the next step is to design each unit. At this stage, a preliminary economic appraisal should be conducted based on the data acquired for the mass/energy balances.

Equipment Selection

Selection of process equipment requires careful attention to equipment sizing, cost, efficiency, and ease of maintenance. A major emphasis is often placed on the capital as

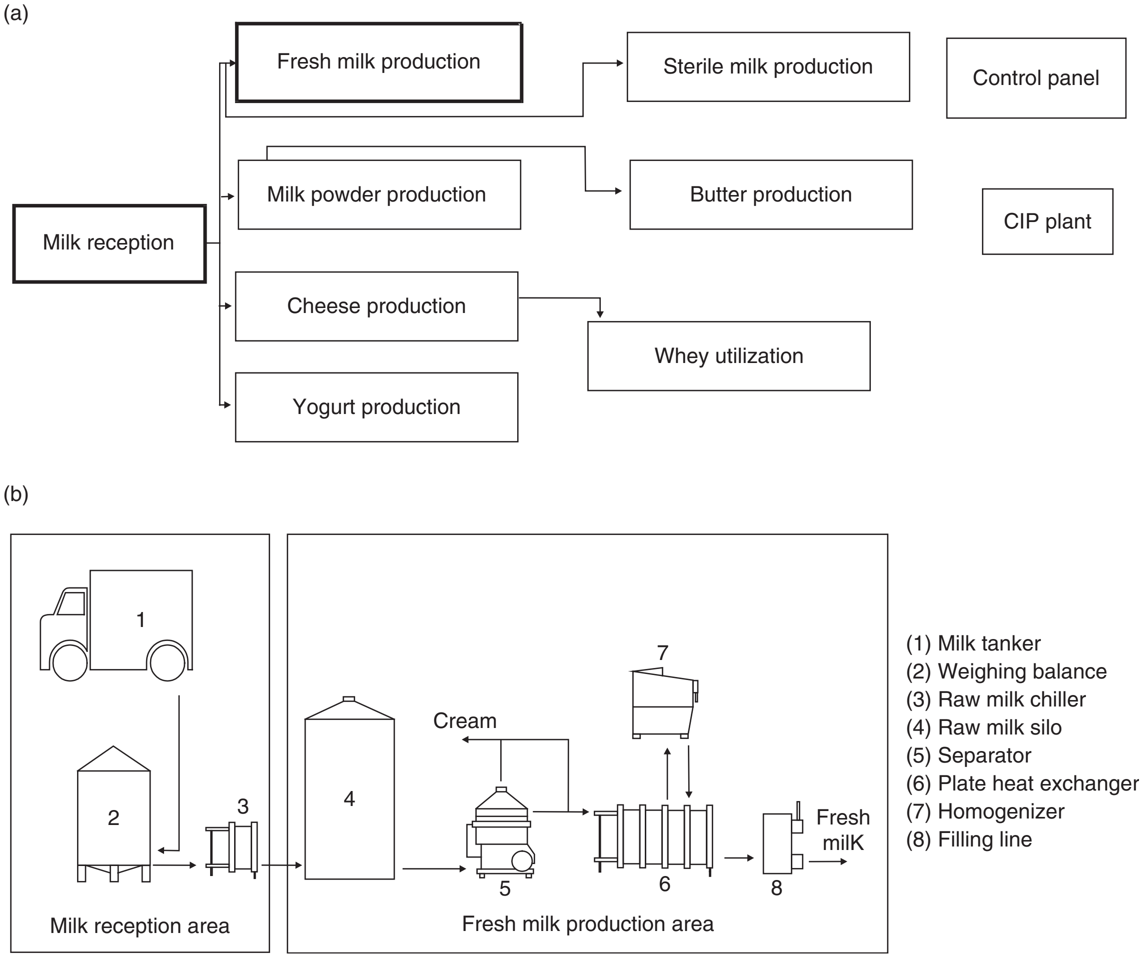
(a)
Fresh milk production
Sterile milk production
Control panel
Milk powder production
Butter production
CIP plant
Milk reception
Cheese production
Whey utilization
Yogurt production
(b)
1
2
3
4
Cream
5
6
7
8
Fresh milK
Milk reception area
Fresh milk production area
(1) Milk tanker
(2) Weighing balance
(3) Raw milk chiller
(4) Raw milk silo
(5) Separator
(6) Plate heat exchanger
(7) Homogenizer
(8) Filling line

Figure 1 (a) Schematic diagram of a multiproduct dairy plant. (b) A simple flow sheet for a market milk process. Adapted from Spreer E (1998) *Milk and Dairy Product Technology*. New York: Marcel Dekker.

well as operating costs, as these costs influence the profitability of the operation. In food and dairy processing, the ease of cleaning is an important consideration in selecting equipment.

Equipment Common to All Dairies

Tanks

Tanks of various sizes are essential in dairy processing. Sizes ranging from 100 to 100 000 l are commonly used. Typically, tanks serve two major functions: storage and processing.

Storage tanks (silo tanks) constructed of stainless steel range in size from 25 000 to 150 000 l. These storage tanks are often placed outdoors when indoor space is limited. The number and size of silo tanks used are determined by the quantity of milk processed per day and the number of different products manufactured. Furthermore, the frequency of processing days per week, as well as the number of processing hours per day, will determine the size or number of storage tanks. The size of silo tanks in the delivery area is determined by the expected schedule of raw milk delivery to the plant. If the plant is to be operated continuously, then a 7 h supply of raw material should be available. If milk is to be held for more than 8 h in a tank, then refrigerated or insulated tanks are required. In the process line, buffer tanks are used to hold milk for short durations. A general rule of thumb is to have a buffer capacity of a maximum of 1.5 h of normal operation.

Heat exchangers

Control of temperature is an essential feature of modern dairy plants. Processing steps require either heating or cooling, processes that are carried out using heat exchangers. For heating purposes, hot water or low-pressure steam is used; however, milk itself is used as a heating medium in a regenerative process. For cooling purposes, chilled water or glycol is used; again, milk is used as a cooling medium in a regenerative process. The three most common types of heat exchangers used in the dairy industry are the plate, tubular, and scraped-surface heat exchangers. Plate heat exchangers are best suited for the treatment of milk, the predominant product in the dairy industry. Heat exchanger design and performance are influenced by product flow rate, temperature and time requirements, cleanability issues, and the properties of the product undergoing heat treatment. Most texts on food engineering address the principles of heat transfer.

Pumps

Transport of liquids from one location to another in a dairy processing plant requires energy, which is provided by pumps. For handling milk and dairy products, the construction material used for pumps is typically stainless steel, food grade rubber, or plastic. The pump design should permit easy cleaning-in-place (CIP). The most common types of pumps used in milk and dairy processing include centrifugal, liquid-ring, and positive displacement pumps. Centrifugal pumps are well-suited for low-viscosity liquids, such as milk. When a liquid contains some amount of air, then liquid-ring pumps are suitable. These pumps are also used for CIP return solutions, which invariably contain air. For higher-viscosity products and those exhibiting non-Newtonian characteristics, such as cream, cultured milk products, and curds, positive displacement pumps are used. Piston pumps are used for metering purposes. A homogenizer is a type of piston pump that is used to break large fat globules into smaller ones (*see* **Homogenization of Milk**: Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers).

Pipes, fittings, and valves

Pipes are ubiquitous in a dairy plant; they are used for transporting low- and high-viscosity liquids and gases. Pipes convey not only dairy products but also all the utilities (steam, water, compressed gases) from one processing apparatus to another. The material of construction and design used for pipes depend on the product being conveyed. In designing pipelines for dairies, the important issues of concern include construction material, size, and contact surface, particularly around welded joints.

In dimensioning the pipelines, careful attention should be given to the selection of flow rates and velocities. Flow rates in fluid-handling systems are typically in the range of 100–30 000 l h^{-1}, with velocity being limited by the product characteristics. For example, the velocity of raw milk should not be greater than 1.5 m s^{-1} to avoid damage to the fat globules. For skim milk and buttermilk, the velocity is normally around 2 m s^{-1}. However, because cream has a high viscosity, its velocity would be less than 0.5 m s^{-1}. Utilities are pumped at much higher velocities: steam at around 30 m s^{-1} and water at 5 m s^{-1}.

A variety of fittings are used in the pipelines, such as elbows, tee connections, and connectors. These fittings match the diameter of pipelines and other process equipment. To control the direction and magnitude of flow, a variety of valves are used. Some examples are seating valve, check valve, air blow valve, and regulating valve.

Stirring devices

Stirring devices are used for equalizing temperature and/or concentration in a vessel. The design of stirrers depends on the viscosity of the product. For low-viscosity products (viscosity <1 Pa s), such as whole milk or whey, paddle, curved blade, propeller, or turbine impeller stirrers are used. For medium- to high-viscosity products (viscosity ≈0.5–5 Pa s), such as condensed milk, yogurt, or cream, cross rod, gate, or blade paddle stirrers are used. For high-viscosity products (viscosity >100 Pa s), such as quark or processed cheese, anchor, finger, or helical ribbon stirrers are used.

Centrifuges

Separating components in a liquid stream is an important process in dairy processing. For purposes of clarification centrifuges are used. Typical centrifuges used in the industry can separate particles of 4–5 μm diameter.

Homogenizers

In full-fat milk, the formation of a cream layer occurs during storage due, *inter alia*, to the large diameter of fat globules. However, if the size of these globules is reduced, creaming is minimized. Homogenizers are used for this process. The degree to which the products should be homogenized depends upon the expected storage time of milk.

Because the fat phase in milk must be in liquid state for homogenization to be effective, milk must be heated to a temperature in the range of 55–80 °C. A homogenization pressure in the range of 10–25 MPa is selected, depending on the product characteristics. Homogenization of cream with a fat content >12% is difficult owing to the lack of casein to stabilize the newly formed emulsion surface. For adequate levels of homogenization, 0.2 g of casein per gram of fat is necessary. Conventional homogenizers are triple-piston high-pressure pumps.

In milk processing, the homogenizer is located after the first regenerative section of a pasteurizer. In the production process of UHT milk, the homogenizer is located in the upstream section when an indirect heat exchanger is used, but on the downstream side when direct heat exchangers are used. When located on the downstream side, the homogenizer must maintain aseptic conditions, requiring special piston seals, packings, and aseptic dampers.

Special Equipment

Table 1 lists some of the special items of equipment required for dairy processing operations.

Materials

Construction materials that have direct contact with the product must be nontoxic and inert to foods. For hygienic reasons, surfaces that come in contact with dairy materials must be well-ground and polished, or cold-rolled. On the other hand, materials that do not make contact with foods, such as pipelines for utilities, must be corrosion-proof and remain stable. The surfaces of materials used for the construction of manufacturing process equipment must be well-ground and treated with lacquer or undergo a similar procedure.

Standards

Specifications for all equipment must be written down carefully. A number of standards are available for writing specifications, such as 3-A Sanitary Standards, 3-A Accepted Practices, International Dairy Federation Documents, and US Department of Agriculture publications. The US Department of Agriculture provides criteria, guidelines, and principles for the sanitary design and fabrication of dairy processing equipment, as well as a list of equipment that are rated as acceptable for use in dairy plants.

Process Control

Because processing equipment must have the capacity for trouble-free operation under variable conditions, control equipment is used to maintain desired liquid levels, flow rates, pressures, concentrations, pH levels, and temperatures. The control systems must be simple, rugged, and robust. Careful attention should be paid to maintenance requirements.

The level of automation in process control is determined by the type of processing equipment and its interaction with the instrumentation used for automation. The selection of instrumentation depends on how it will interact with the operator and with procedures that manage the information obtained from it, such as how errors will be corrected.

Typically, the automation systems use programmable logic controllers. These systems must be flexible, easy to expand, and reliable. These systems should include electronic solutions, with software for diagnostic tests. More recently, simulations have become an attractive part of the software.

Utilities

Dairy operations require a number of utilities such as electricity, steam, hot water, chilled water, compressed air, and refrigeration. Water consumption for a typical dairy plant is 1000–5000 l per 1000 l of milk. Water is transported in stainless-steel pipes with a diameter larger than 6.35 cm or in smaller galvanized-steel pipes. The pipeline system includes valves for shutoff and routing purposes, and pressure gauges. Often, dairy plants rely on their own water supply instead of using municipal water (*see* **Utilities and Effluent Treatment**: Water Supply).

For most heating applications, steam (140–150 °C) is the most commonly used heating medium in the dairy industry. Pipelines convey steam to the locations where it is required. Hot water systems are also used, but heated water must be kept under pressure to avoid boiling,

Table 1 Special equipment required in dairy processing

Product	*Common steps for the process*	*Basic equipment required*	*Comments*
Butter	Milk reception Preheating and pasteurization of skim milk Fat separation Cream pasteurization Vacuum deaeration Culture preparation Cream ripening Temperature treatment Churning Buttermilk collection Packaging	Plate heat exchanger Centrifuge Continuous buttermaking machine Packaging system	Capacities of continuous butter-making machines ≈200–5000 kg h $^{-1}$ butter from sour cream; 200–10 000 kg h $^{-1}$ butter from sweet cream.
Cheese[a]	Preparation of cheese milk Heat treatment Standardization Preripening process Protein coagulation Curd preparation and treatment Molding Pressing Salting Ripening Packaging	Cheese vat with tools for cheese manufacture Pressing and molding vat Brining system Ripening storeroom	Traditional and partially mechanized processes. Equipment is designed specifically for the type(s) of cheese to be manufactured. Nearly all operations are done manually. Completely mechanized processes. Capacities: 50–100 000 l day^{-1}; completely mechanized processes are economical with higher level of automation.
Ice cream	Preheating Formulation mixing Pasteurization/homogenization/cooling Aging Freezing Filling/extrusion/molding Hardening Cold storage	Mixing and processing tank Homogenizer Plate heat exchanger Aging tank Continuous freezer Filler	Rapid freezing process is used for the formation of small ice crystals. In a plant with a small capacity, dry ingredients are generally weighed and supplied to the mix tanks manually. Large-scale producers use automatic batching systems. In large production plants, a continuous flow is maintained using mix tanks with a volume corresponding to the hourly capacity of the pasteurizer.

Long-life products	Cleaning Fat content standardization Preheating and stabilization Cooling UHT[b] Homogenization Heat treatment Cooling Storage Filling	Balance tank Plate heat exchanger Nonaseptic homogenizer Aseptic tank Aseptic filling	To improve the texture and physical stability of certain products like cream, an aseptic downstream homogenizer is used.
Milk powder	Milk pretreatment Concentration Homogenization Drying Packaging	Milk concentrate tank Spray-dryer or drum dryer Cyclone Packaging system	Spray-dryers are used to manufacture milk products from concentrates. Drum drying. Typical specific steam consumption is 1.3–1.6 kg per kg of evaporated water, and 4.3–5.0 kg per kg of powder.
Yogurt	Pretreatment of milk: Standardization of levels of fat and dry matter Heat treatment Homogenization Stirred type yogurt:[c] Incubation Cooling Flavoring Filling Cold storage	Balance tank Plate heat exchanger Evaporator Homogenizer Incubation tanks Plate cooler Buffer tanks Packaging system	Yogurt is a thixotropic non-Newtonian fluid. Its viscosity decreases during processing. A proper optimization of the process is necessary to allow the viscocity to be fully regenerated and syneresis minimized.

[a] Cheese of various types is produced in several stages. Each type of cheese has its specific production formula. Some basic stages are shown here.

[b] UHT is the most common treatment for preserving liquid food products.

[c] Yogurt is typically classified as follows: set type (incubated and cooled in the package); stirred type (incubated in tanks and cooled before being packed); drinking type (similar to stirred type, but the coagulum is ‘broken down’ to a liquid before being packed); frozen type (incubated in tanks and frozen like ice cream); concentrated (incubated in tanks, concentrated, and cooled before being packed).

because most processing operations require water at around 100 °C. Typical steam consumption is around 185–200 kg of steam per 1000 l of raw milk.

Refrigeration systems are vital in dairy processing. At elevated and ambient temperatures (*see* **Utilities and Effluent Treatment**: Refrigeration), products are prone to microbial and enzymatic degradation. Therefore, rapid cooling to a safe temperature is often a necessity. Generally, the operating cost of the refrigeration system is important in the global cost estimation.

For automatic control purposes, especially in damp environments, pneumatically controlled automatic systems are preferred. These control systems require compressed air free from impurities. Compressed air is also used in some processing operations, such as agitation in storage tanks and emptying product from pipelines (*see* **Utilities and Effluent Treatment**: Compressed Air).

For an electric power demand of 300 kW, dairies take low-voltage supplies of 220–440 V. Many dairies rely on local generation of electricity in the event of power failures or sometimes for continuous operations (*see* **Utilities and Effluent Treatment**: Electricity). The typical electric power requirement for a dairy is 15–18 kW per 1000 l of processed raw milk.

Plant Layout

A plant layout includes the arrangements of processing, storage, and handling areas, as shown in **Figure 2**. The layout is developed after the process flow diagrams are completed, and the information is useful in determining construction and manufacturing costs. Several factors influence the plant layout, including the type and quantity of products to be manufactured, process and product controls, utilities, building specifications and code requirements, and waste handling and disposal.

Equipment should be arranged in such a manner as to minimize capital and operating costs. The actual location of process equipment must permit adequate space for cleaning and maintenance. Similarly, adequate aisle space must be provided for workers in conformity with any building codes. Future plant expansion should be considered.

Sanitary Design

In designing any food processing plant, emphasis on proper cleaning and disinfection of equipment and work space is paramount. Disinfection is carried out using chemicals mixed in water: about 1.7 kg of alkali and 0.6 kg of acid are required for each tonne of processed milk. Furthermore, steam is used for high-temperature sterilization. For optimum use of chemical agents, the nature of dirt, the surface characteristics, water quality, and the effectiveness of cleaning and disinfection agents must be clearly understood. According to the terminology used in cleaning, physical cleanliness is removal of all visible dirt from the surfaces; chemical cleanliness is removal of microscopic residues that can be detected by taste or smell; bacteriological cleanliness is obtained by disinfection; and sterile cleanliness is destruction of all microorganisms. In dairy cleaning operations, chemical and bacteriological cleanliness are achieved using chemical agents.

Cleaning-in-Place Systems

Modern dairy processing plants use CIP systems, which involve circulation of rinsing water and detergent

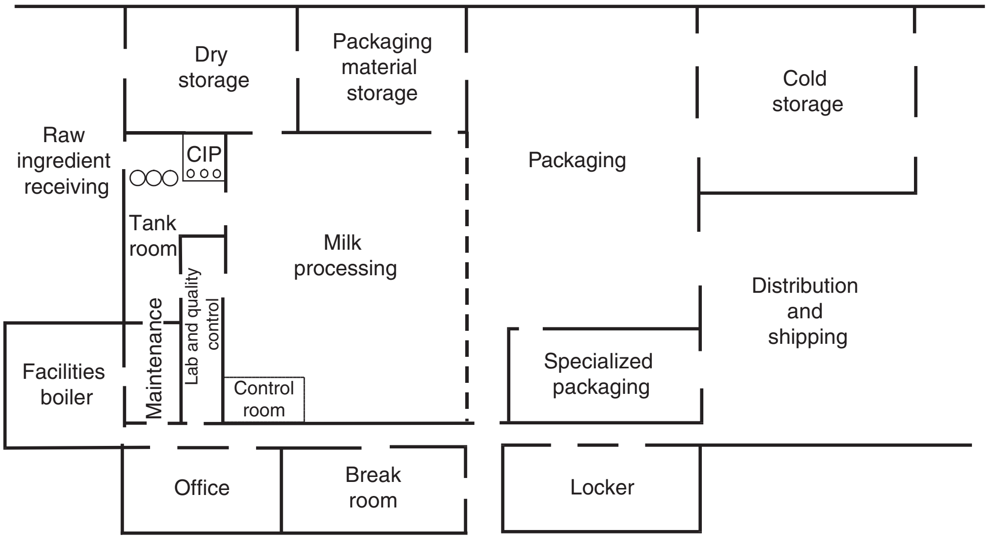

Figure 2 Dairy processing plant layout. Adapted from Hui YH (1993) *Dairy Science and Technology Handbook*. New York: VCH.

solutions through tanks, pipes, and other process equipment. Because the entire processing system is interconnected, there is no need to dismantle equipment for cleaning. An automated cleaning system requires that all surfaces be accessible to the detergent solution. If the detergent cannot get to any area, such as to the dead ends in a pipe, then the system fails. The installation of pipes and equipment must be done in a manner that promotes easy drainage of cleaning solutions. All components of the equipment that come into contact with chemical detergents must be able to withstand them. Depending on whether heated surfaces are involved in cleaning, a typical dairy CIP program would operate as follows.

A CIP program for a pasteurizer involves rinsing with warm water for about 10 min, circulation of an alkaline detergent solution (0.5–1.5%) for about 30 min at 75 °C, followed by rinsing out the alkaline detergent with warm water for about 5 min. Then nitric acid solution (0.5–1.5%) is circulated for about 20 min at 70 °C, followed by postrinsing with cold water and gradual cooling with cold water for about 8 min. With pipes and tanks, which may be considered as 'cold components', the process involves rinsing with warm water for 3 min, circulation of 0.5–1.5% alkaline detergent at 75 °C for about 10 min, and again rinsing with warm water for about 10 min. Disinfection with hot water at 90–95 °C for 5 min is carried out, which is then followed by gradual cooling with cold tap water for about 10 min.

After the process equipment is cleaned, it is sanitized to destroy residual yeasts, molds, bacteria, and bacterial spores. The surfaces must be thoroughly cleaned before sanitizing. Common chemicals used for sanitizing include chlorine compounds, iodophors, quaternary ammonium compounds, and acid-anionic surfactants.

Design Strategy and Optimization

In process design, optimization to minimize costs and maximize profitability plays a major role. A number of mathematical procedures are widely available to optimize manufacturing processes. These procedures have the following common elements: design variables, equality and nonequality constraints, feasible solutions, and objective function.

Process optimization is increasingly being used in the dairy industry. Typically, the optimization problem is to determine values of independent variables that result in an optimal value of a dependent variable. Commercially available software has made modeling and simulation more user-friendly. These models require reliable information on the physical and thermodynamic properties of foods, preferably in the form of computerized databases.

Cost data are also crucial in plant design. Typical data for processing plants manufacturing dairy products are shown in **Table 2**; this is for indicative purposes only, as differential inflation since 1987 will have changed the relative figures significantly.

Safety and Hazard Evaluation

In designing plants and processes, the safety of the operating personnel and people living in the vicinity of the plant is of major concern. A safe design involves appropriate steps to protect the life and health of the working population. In the United States, the Occupational Safety and Health Administration (OSHA) promulgates regulations regarding worker safety and health. In Europe, information can be found from EU agencies and national bodies.

Environmental Constraints

Since the 1970s, environmental protection has become a primary issue in many industrialized countries. This has resulted in the development of stricter environmental regulations and their enforcement. Dairy processing plants generate large volumes of organic waste, which must be properly discharged and treated. Treatment facilities for waste materials must be designed and constructed in collaboration with local agencies.

A large quantity of wastewater in a dairy plant contains milk components, particularly the water used for cleaning equipment and pipelines. Typically, 2 l of water is used for every liter of milk handled. Wastewater from employee washrooms, toilets, etc. amounts to 75 l day^{-1} per employee. A certain amount of wastewater results from cooling water used for cooling milk. Dairy wastewater is high in organic matter and hence must be diluted by a factor of 150 to 200 (*see* **Utilities and Effluent Treatment**: Design and Operation of Dairy Effluent Treatment Plants). As this would be extremely wasteful, dairies use treatment facilities, using either mechanical or biological means. The use of acids and alkaline detergents for cleaning equipment and pipelines results in the pH of wastewater ranging between 2 and 12. Typically, wastewater with a pH higher than 10 or lower than 6 must not be discharged into the sewage system. Effluents from the cleaning systems are usually collected and the pH carefully monitored and adjusted if necessary.

Another component of the dairy wastewater is whey. Recently, manufacturing processes have been developed to convert whey into economically attractive by-products such as whey and lactose powders. A variety of separation processes, such as ultrafiltration, reverse osmosis, and electrophoresis, in addition to enzymatic hydrolysis, are used for manufacturing whey products (*see* **Whey Processing**: Utilization and Products).

Table 2 Cost data referred to fixed capital and annual operating costs in US dollars

			Fixed capital cost (US$)			*Annual operating cost (US$ yr^{-1})*			
Plant	*Capacity ($\times 10^9$ g yr^{-1})*	*Operationg time (h yr^{-1})*	*Equipment*	*Mechanical and electrical*	*Civil*	*Materials*	*Utilities*	*Labor*	*Supplementary*
Mozzarella cheese plant	1875	3000	340 750	65 850	435 000	1 700 000	51 600	86 000	10 000
Ice cream plant	4000	2000	1 330 000	650 000	535 000	1 074 860	143 500	271 000	31 000
Milk powder plant	12 000	7200	2 700 000	715 000	585 000	13 210 000	1 088 000	98 000	160 000
Yogurt plant	25 000	3125	3 236 600	878 000	713 000	11 750 000	465 375	246 000	111 700

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It has become increasingly important for dairy plants to be certified by the ISO13000 series of norms that deal with environmental protection.

Computer-Aided Design

Several commercial computer-aided design packages are used in the chemical industry for simulation and design of processes. These simulators are valuable tools for flow sheeting, determining mass and energy balances, sizing the equipment, and analyzing performance. Although the majority of these packages are more suitable for the general chemical industry, some efforts have been made during the last decades to develop materials useful for the dairy and food industry.

See also: **Homogenization of Milk**: Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Utilities and Effluent Treatment**: Compressed Air; Design and Operation of Dairy Effluent Treatment Plants; Electricity; Refrigeration; Water Supply. **Whey Processing**: Utilization and Products.

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Materials and Finishes for Plant and Equipment

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Introduction

Hygienic design of processing equipment is a particular requirement for the dairy industry that in general does not apply to the bulk chemical area. The primary intention is to prevent the contamination of product materials during processing. Hygienic design is based on a combination of mechanical, process, and microbiological requirements. Two particular aspects of hygienic design are selection of appropriate materials of construction for equipment and specification of an acceptable surface finish for those materials. Hygienic equipment must be constructed of a durable material that will not compromise the purity of the product, is completely drainable, and has smooth surface finish. If equipment is of poor hygienic design, it will be difficult to clean and difficult to free from microorganisms, which may then survive and multiply in product residues in crevices. It should be noted that hygiene regulations for equipment handling dry product (such as powders) are more relaxed as dry products do not support the growth of microorganisms to the same extent as wet products.

There are a number of professional bodies that issue regulations concerning hygienic design. In the United States, the 3-A Sanitary Standards, Inc. (3-A SSI), is a nonprofit association representing equipment manufacturers, processors, regulatory sanitarians, and other public health professionals. This group has established a comprehensive inventory of 3-A Sanitary Standards and 3-A Accepted Practices now known around the world for dairy and food processing equipment and systems. The American Society of Mechanical Engineers (ASME) Bio-Processing Equipment (BPE) Code came into being in the United States in 1998, although it is primarily aimed at the biopharma and biotech sectors rather than the dairy sector. In Europe, the closest equivalent code is the EHEDG Regulations. The European Hygienic Equipment Design Group (EHEDG) is composed of representatives from research institutes, process equipment manufacturers, the food and biopharma industry, and legislative bodies. The objective is to provide specialist advice on hygienic and aseptic design. The EHEDG and both the 3-A and ASME BPE provide suggestions and recommendations but their standards are not mandatory.

Construction Materials

At the outset, it must be understood that when discussing the choice of acceptable materials and required surface finish for dairy plant and equipment, the focus is on product holding and processing equipment. In other words, it is equipment that has a product contact surface that is being examined (i.e., the insides of pipes and vessels). For equipment that does not come into direct contact with the dairy product, such as the pumps and pipes for service fluids (steam, water, and air), the conditions determining acceptable materials and surface finish are far less stringent. Likewise, the nonproduct-contact part of dairy processing equipment (such as legs and support features) is not subject to the same scrutiny.

In the dairy sector, conditions are in general less exacting than in the chemical industries. Processing temperatures (usually within the range −40 to 140°C) and usual operating pressure ranges (0–10 bar) are limited, and pH does not deviate widely from neutral (a pH of 2 or 3 being a minimum). However, dairy processing operations are unique in that the needs for sanitary design and operation are stringent. In all cases, product contact surfaces must withstand the aggressiveness of the product and processing (temperature, pH, flow velocity, roughness) and must meet special needs of resistance to wear and abrasion and corrosion resistance. The material of construction must also withstand the often greater aggressiveness of the chemicals used for cleaning and sanitation. As a general classification, most dairy products are slightly acidic, and detergents (especially caustic detergents) are alkaline. Sometimes though acids, including hydrochloric and nitric, are used for removing scale from dairy processing equipment and this must be taken into account in material selection. Regulations require that process equipment should be made from a durable, impermeable, and corrosion-resistant material to avoid the contamination of the product and be of such construction as to enable the equipment to be kept clean and disinfected. In Europe, the European Union issues regulations to the member states to this effect. Toxic substances, likely to endanger health (if consumed with the product) or taint the product, must not be used in the construction of equipment if contact with edible material can occur. Some materials are slightly soluble, nontoxic, and do not adversely affect the product. Tin and aluminum are in this category. Other materials are toxic and cannot be used for dairy contact surfaces such as lead (solder), zinc (present in galvanized steel), and iron compounds (mild steel). In principle, dairy processing equipment can be built from a wide variety of metals, ceramics, or polymers. However, as stainless steel (particularly the grades 304L and 316L) is so prevalent, it merits the majority of the subsequent discussion.

Stainless Steel

Introduction

Stainless steel is defined as an alloy of chromium and steel. It has the characteristic of greatly enhanced corrosion resistance over conventional lower alloy and carbon steels. This corrosion resistance derives from the addition of at least 12% chromium, although the most widely used grades contain about 18% of this element. Chromium on its own oxides quickly and produces a stable passive oxide film on exposed surfaces. This property also occurs when chromium is in a solid solution in iron becoming very marked as the amount exceeds 12% in low carbon steels. This corrosion-resistant film protects the underlying metal. This film can be up to 5 nm thick and contains varying amounts of Cr_2O_3. In an oxidizing media, any defect in the film that arises through abrasion is quickly repaired (i.e., the film is self-healing). However, it must always be remembered that no stainless steel is completely corrosion proof; oxygen is required to maintain passivity.

In terms of chemical composition, stainless steel contains the basic elements of steel (iron and carbon) plus chromium. Almost all stainless steels (with the exception of some martensitic steels) have low carbon contents, generally below 0.15%. The main reason is that under certain conditions (e.g., in the heat-affected zone of a weld), carbon can preferentially combine with chromium to form chromium-rich carbon deposits at grain boundaries. This depletes the prevailing chromium levels in this region of the material and makes corrosion possible. Chromium itself is the defining element of stainless steel. It is present in amounts from 12% (a minimum) to 30%, and the most widely used grades contain chromium in contents between 17 and 25%. There are two other elements that can be found in stainless steel, though not all grades of stainless steel contain them. Nickel is added to produce austenitic grades of stainless steel. These steels usually contain 9% nickel and have improved corrosion resistance especially against acids. Stainless steels with 18% chromium and 8% nickel are commonplace ('18/8 steels'). Finally, molybdenum can be added to improve further corrosion resistance in especially aggressive environments such as acids. It is added in amounts of 2% to the 18/8 steels.

Types and Classification

There are four main types of stainless steel microstructure: ferrite, austenite, martensite, and duplex. These are distinguished from each other by their chemical composition and consequent mechanical and corrosion-resistance properties. Furthermore, within each of these main groups, there is a huge variety of grades depending upon the number and amount of minor alloying elements present and the heat treatment that is carried out. Austenitic steels have excellent toughness (impact resistance) at low temperatures and they are the most readily weldable of the different stainless steel types, which is a very important consideration for a processing plant. One drawback is that the high nickel and chromium content make these alloys expensive.

Ferritic stainless steels are cheaper than the austenitic steels and have very good corrosion resistance. Their ductility and toughness is not comparable with that of the austenitic steels. Martensitic stainless steels can have carbon contents ranging from 0.1 to 1%. They have less corrosion resistance than the other steels. The high carbon content makes them very hard, and they are suitable for applications where wear and abrasion resistance is essential. Duplex stainless steels are a newer type of stainless steel with high-strength features. In this stainless steel, the internal structure is an equal mix of face-centered and body-centered crystal structures (austenitic and ferritic). Duplex stainless steels have better toughness than ferritic grades and much higher yield strengths than the austenitics.

Most of the above stainless steels are also available as L grades (e.g., 316L). The L designation means the carbon content in the steel is at a particularly low level, usually below 0.03%. This is to diminish further the possibility of chromium carbides being formed at grain boundaries and reducing the protection offered by chromium to the steel.

Corrosion

The very name 'stainless steel' can be misleading; stainless steels are protected by a passive oxide film, and defeat of this film is possible by certain corrosion mechanisms. There are many possible corrosion mechanisms: pitting corrosion, crevice corrosion, stress corrosion cracking, sulfide stress corrosion cracking, intergranular corrosion, galvanic corrosion, contact corrosion, microbial corrosion, and so forth. Stainless steel should be used for corrosion resistance where oxidizing conditions exist. This is to enable the oxide film to regenerate itself constantly. Furthermore, stainless steel is susceptible to corrosion caused by chlorine. Thus, corrosion of stainless steel is especially serious if accompanied by an absence of oxygen or presence of halogens or both. In such a situation, alternative materials of construction such as Hastelloy^{TM} or titanium should be specified.

Exposure to free chlorine in water droplets (e.g., sea air), brines, or cleaning fluids (hypochlorite bleaches) can cause pitting corrosion in stainless steel depending on factors such as concentration and temperature of the fluid. It is estimated that up to 90% of corrosion failures of stainless steel in the process industries are due to exposure to chloride-containing fluids. It should be mentioned here that chloride attack can lead to the formation of microscopic channels that cannot be detected by dye-penetration testing (only by radiographic methods), yet are significant as channels for bacterial ingress, for example in heat exchangers and water-jackets. Most cleaning solutions may only contain 20 ppm chlorine. However, if they are applied to the steel surface (in a pipe or vessel) and then allowed to dry off rather than being flushed away, the chlorine concentration can rise 10-fold. As a rule of thumb, stainless steel type 304 is liable to corrode when chlorine concentration rises above 200 ppm and type 316 when chlorine concentration is greater than 1000 ppm. The main problem with chlorine-containing cleaning agents is not the cleaning itself; it is not rinsing thoroughly with ordinary water so that chloride levels are allowed to rise to dangerous levels.

To maintain the corrosion resistance of stainless steel, a clean, dry surface must be exposed to oxygen (air) so that the passive layer is continually maintained. Poor plant and equipment design or layout can promote corrosion of stainless steel by preventing equipment walls coming in contact with air. When corrosion does occur, remedial action is possible by passivation of stainless steel. Passivation of stainless steel is carried out when the beginnings of rusting are first seen (say inside a liquid holding vessel or pipe network). First, the rust debris contaminant must be removed by rinsing with a 10% nitric acid solution. This is vital because if the rust debris is not removed, corrosion will recommence. Then air is allowed into the pipe and left for a few days (i.e., the pipe is taken out of commission and left idle). The oxygen in the air will regenerate the chromium oxide inert layer and restore corrosion resistance to the pipe.

Dairy Plant

In summary, stainless steel is the material most widely used for contact surfaces in dairy processing equipment. This metal offers resistance to corrosion, mechanical strength, hardness, and ease of fabrication (weldable). The preferred grades for general process fluid heating, storage, and distribution are AISI type 304 (Deutsches Institut für Normung (DIN) 1.4301) and 316 (DIN 1.4401). Type 316 is more expensive but offers greater corrosion resistance due to the inclusion of molybdenum. AISI 304 is nearly always used externally, or for the outer vessel jacket, as it is needed only for protecting the machine from the atmosphere, water, and any spilled liquids. For parts in contact with product, either type can be used though 316 is necessary for acidic products. In very acidic conditions, where the pH falls to 1, type 317 austenitic stainless steel is specified as it has a greater molybdenum content of 4%. For dry solid foods such as dairy powders, wear resistance is also needed in addition to corrosion resistance. The ferritic structural stainless steels offer good flow characteristics for hoppers, chutes, and conveyor lines. High loads on mechanical components or very abrasive conditions may result in the selection of the strong, hard martensitic stainless steels; for example type 410 and 420. These steels can find an application in knives and cutters and mixers and extruders. If chlorides are present, even type 316 is not suitable to prevent localized corrosion (where the oxide film is damaged), and materials such as AL-6XN (a molybdenum-containing super austenitic stainless steel) or titanium may be required. Alternatively in this situation, AISI 410 (DIN 1.4006) or AISI 329 (DIN 1.4406) can be employed.

Other Metals

In the rare cases where very extreme corrosion conditions are present, nickel alloys or titanium can be employed. Inconel 600 is a high-nickel alloy (three-quarters nickel) and offers excellent resistance across a broad range of corrosive conditions. It is unsurpassed in caustic conditions. It maintains high strength at elevated temperatures. Its main drawback is the high cost. Hastelloy C276 is a nickel–chromium–molybdenum alloy that has the broadest general resistance to corrosion compared with many other common alloys. It has good resistance to wet chlorine and strong oxidizers and can combat aggressive corrosion conditions that are combined with high temperature and high pressures.

Titanium, although very expensive, is sometimes used where extremely high corrosion resistance is required (e.g., to hold liquids containing chlorine ions). It is also used where a brine solution would attack stainless steel. Titanium is an excellent metal for use with oxidizing agents such as nitric acid and mixtures of nitric and hydrochloric acids. It also has good resistance to chloride ions. Inert to nitric acid, it gives acceptable corrosion rates of less than 0.05 mm yr^{-1} to sulfuric and hydrochloric acids but is attacked by concentrated sulfuric and hydrochloric acids (more than 1.25 mm yr^{-1}). Commercially pure titanium is available in several grades. Grade 2 is suitable for welding. Grade 4 contains traces of iron, has higher strength, but is not suitable for welding.

Aluminum and its alloys have the advantages of being light, easily fabricated, and of reasonably low cost. However, it is so susceptible to corrosion that it is probably safe only for special applications where it can be guaranteed not to encounter alkaline detergents used in cleaning in place (CIP) systems. The low mechanical strength of aluminum can be improved by alloying. Though slightly soluble in many foods, aluminum is not toxic. It may be used in food plants, but as the metal is attacked by both acids and alkalis, great care must be exercised. Aluminum is also attacked by nitric acid, which is a commonly used cleaning agent. As aluminum salts are colorless, tasteless, and claimed to be nontoxic, and the metal is cheap, light, easily cleaned, and has high thermal conductivity, use in food manufacture should be extensive. However, it can impart taints or off-tastes to certain foods. Milk 'churns' (cans) are made out of aluminum as its low weight compared with stainless steel is an advantage in handling these containers.

Copper and its alloys such as brass (copper–zinc), bronze (copper–tin), and Monel (copper–nickel) should not be used for dairy equipment. Copper alloys do offer ease of fabrication (very advantageous for pipe networks) and display durability in air and water. Alloying is necessary to overcome the lack of strength in pure copper. Copper and its alloys can oxidize food oils and fats and cause flavor and off-taste problems (even at concentration levels as low as 0.1 mg kg^{-1}) especially in food processing at higher temperatures (above 80 °C). It should be specifically avoided when handling high-fat foods. Copper is used where its excellent electrical and thermal conductivities are advantageous (e.g., electric conductors and cooling fins). It is also used in nonpotable water distribution circuits such as boiler feed water.

Finally, the undesirability of using galvanized steel, for example for motor covers or framework, should be emphasized as this is rapidly corroded by the typical alkaline detergents used in food premises.

Plastics and Rubbers

Plastic materials offer certain advantages (being cheap, easy to fabricate, and having low weight and good corrosion resistance) but suffer from low mechanical strength and poor temperature and fatigue resistance. Polymers such as polypropylene, Teflon, silicone, ethylene propylene diene monomer (EPDM), and so forth, can be used. In dairy processing equipment, they must be abrasion resistant and free from constituents that can migrate into foodstuffs especially if they are being used as seals in pipes and process vessels. Food-grade polymers must always be specified. Factors such as reactions with detergents and stability at sterilization temperatures must also be considered (so most polymers are appropriate only for lower temperatures). The relatively strong and heat-stable plastics (such as nylon and Teflon) are used in storage vessels, pipework, and pumps. Seals made from elastomeric materials are a common example of the use of plastic and rubbers in dairy processing equipment. Two issues are absorption of materials from the process liquid into the elastomer, which can produce swelling of it and extraction of components from the elastomer seal into the process liquid, which can cause contamination. The perfluoroelastomers behave well with regard to both these issues.

Surface Finish

Irrespective of the material chosen, the presence of pits, crevices, and any other surface defects on product contact surfaces are not allowable. These defects can act as harborage sites, trapping and protecting microorganisms from sterilization. Thus, product contact surfaces must be polished (usually by grinding and buffing) to ensure a low surface roughness. Generally speaking, the higher the degree of surface finish, the more cleanable is the surface. All manufactured surfaces will consist of a series of peaks and valleys, varying in both height and spacing. The main measurement parameter of this texture is designated R_a and is the arithmetic average value of the departure of the profile above and below an imaginary centerline throughout the sampling length. Surface quality is generally specified by this surface roughness number, R_a. Food product contact surfaces should have a surface finish of a maximum 0.8 μm R_a. Measurements of contamination levels on a surface after a cleaning regime indicate that the lower the R_a value, the smaller is the contamination level. There are four finishing techniques to reduce surface roughness:

1. Mechanical polishing: Aluminum oxide grit is glued to flat felt disks that are rotated at high speed while being traversed over the surface.
2. Abrasive blasting: The surface to be treated is blasted with high-speed glass beads.
3. Electropolishing: This process involves the electrochemical removal of a surface layer of material. It is the opposite of electroplating. The surface to be treated is dipped into a tank of electrolyte and connected to the positive pole of a current supply. Because peaks of the surface are removed preferentially, a leveling of the surface occurs.
4. Chemical finishing: Chemicals (pickling acids) are applied by brush and left on the surface for a few hours and then rinsed off.

Table 1 indicates the roughness level that can be achieved with each method.

Table 1 Surface treatments of stainless steel and the resulting surface roughness (R_a)

Treatment	*Grit number*	R_a (μm)
Cold rolling		0.2–0.5
Hot rolling		>4
Glass bead blasting		1
Pickling		0.5–1
Electropolishing		
Mechanical polishing	60	<3.5
	120	<1.1
	240	<0.5
	500	<0.2

The issue of acceptable surface roughness is particularly important for welds in equipment. The primary purpose of a weld is to provide a permanent joint of sufficient mechanical strength to meet all requirements. However, the weld must also satisfy hygienic requirements. Any excessively rough weld surface promotes adhesion of soil material and is difficult to clean. For a weld to be hygienic, it must have a smooth inner weld bead surface free of crevices, cracks, or pits that would harbor bacteria. Such welds permit sterilization of the equipment to be carried out satisfactorily. A typical series of finishes is the following:

- As-welded: flux chipped out and weld wire-brushed,
- Rough ground: welds are ground with coarse grit,
- Fine scurf: the ground weld scurfed with carborundum grit,
- Bright polished: polishing with/without aid of greases,
- Chemically pickled: application of pickling acids to remove weld scale.

Stainless steel, available as hot rolled plate, normally has a surface finish of greater than 5 μm R_a. High-quality Tungsten Inert Gas (TIG) welds will leave a finish of between 3 and 8 μm R_a. Thus, polishing is necessary to bring the roughness down to 0.8 μm R_a or less. A grit size of 150 is usually sufficient to give the required surface finish of 0.8 μm R_a.

See also: **Milking Machines**: Principles and Design. **Plant and Equipment**: Agitators in Milk Processing Plants; Centrifuges and Separators: Types and Design; Corrosion; Evaporators; Flow Equipment: Pumps; Flow Equipment: Valves; Heat Exchangers; In-Place Cleaning; Pasteurizers, Design and Operation; Process and Plant Design.

Relevant Websites

http://www.3-a.org/ – 3-A Sanitary Standards, Incorporated
http://www.asme.org/codes – ASME
http://ehedg.org – European Hygienic Equipment Design Group

Flow Equipment: Principles of Pump and Piping Calculations

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Principles of Calculation

Ideal Flow

Conservation of mechanical energy

The basic principle used to describe the flow of liquids in any type of installation is that the total mechanical energy of an element of the fluid at a given position, *A*, should be maintained while it flows, so that when it reaches another position, *B*, there may have been conversion of one form of energy to another, but the total should be the same. This analysis is applied at isothermal conditions, so that only mechanical energy needs to be considered (non-isothermal flow will not be addressed).

Three forms of energy are involved: pressure, potential energy and kinetic energy. The mathematical expression that translates this principle of conservation of mechanical energy is known as Bernoulli's equation (the differential form is Euler's equation), and for isothermal ideal flow of incompressible fluids (liquids), it can be written as:

$$\frac{P_a}{\rho \cdot g} + h_a + \frac{v_a^2}{2g} = \frac{P_b}{\rho \cdot g} + h_b + \frac{v_b^2}{2g} \qquad (1)$$

where P is pressure, h the height (vertical distance from the ground to the central axis of the pipe), v the average velocity and the subscripts a and b designate the values at locations A and B, respectively. ρ is the specific gravity of the liquid and g the acceleration due to gravity (9.8 ms^{-2}). The left-hand side is the sum of energy terms at location *A*, and the right-hand side at location *B*. This equation is written so that all terms have units of length (e.g. m), and are known as 'heads': the first term is the pressure head, the second is the potential head and the third is the kinetic head. **Figure 1** sketches a generic situation where all heads have changed from location *A* to *B*, in an example where the potential and kinetic heads have increased at the expense of the pressure head.

Conservation of momentum

In addition to Bernoulli's equation, it can also be stated that there should be a conservation of momentum. For steady flow, this principle reduces to a mathematical expression which simply states that the flow rate must be constant throughout the installation, otherwise there would be an accumulation of liquid. For incompressible fluids, the volumetric flow rate (Q) is used, which is equal to the average velocity of the fluid times the cross-sectional area of the pipe (A), and this leads to the so-called continuity equation:

$$v_a \cdot A_a = v_b \cdot A_b \qquad (2)$$

General design principles

Substituting eqn [2] in eqn [1] and with some basic manipulation, the expression that relates flow rate to the pressure difference between two locations (which is designated ΔP) is obtained:

$$\frac{\Delta P}{\rho \cdot g} = \Delta h + \frac{Q^2}{2g}\left(\frac{1}{A_a^2}\right) - \frac{1}{A_b^2} \qquad (3)$$

where $\Delta P = P_a - P_b$; $\Delta h = h_b - h_a$. It should be noted that, by definition, $Q = V_a \cdot A_a$ (or $Q = V_b \cdot A_b$) and that for cylindrical pipes (circular cross-section area), $A = (\pi \cdot D^2)/4$, D being the diameter of the pipe.

Equation [3] allows calculation of the pressure difference that must exist to ensure a given flow rate, or by reversing it, to calculate the flow rate that a given pressure difference will imply.

Nonideal Flow

Head losses

The analysis above implies that the fluid behaves ideally, i.e. there is no dissipation of mechanical energy. However, this is rarely true and fluids exhibit a resistance to flow which depends on their rheological properties. The concept of viscosity is familiar – a viscous fluid shows greater resistance to flow. Fluids are not rigidly structured materials and flow implies a continuous movement of molecules in relation to one another. Either through viscous shear energy dissipation or by molecular collisions, there is inevitably a loss of mechanical energy, dissipated in the form of heat. It is assumed that this is not so significant that the situation will cease to be isothermal – it is considered that the heat dissipated is transferred easily and lost to the outside and causes no significant rise in temperature. However, while flowing from *A* to *B*, part of the mechanical energy is lost. Equation [1] is therefore incorrect, and a term ($F_{A \rightarrow B}$) must be inserted to account for all head losses:

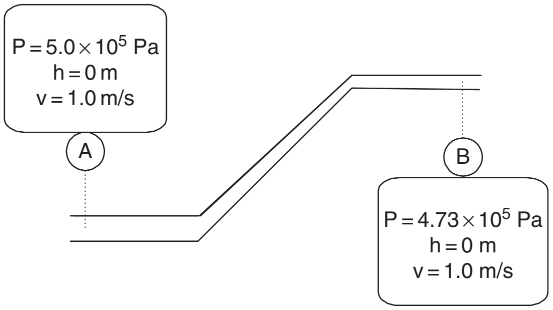

Figure 1 Conservation of mechanical energy in an ideal system.

$$\frac{P_a}{\rho \cdot g} + h_a + \frac{v_a^2}{2g} = \frac{P_b}{\rho \cdot g} + h_b + \frac{v_b^2}{2g} + F_{A-B} \qquad (4)$$

Hence, the problem can be solved in the same way as if the fluid was ideal, with a minor correction to eqn [3]:

$$\frac{\Delta P}{\rho \cdot g} = F_{A-B} + \Delta h + \frac{Q^2}{2g}\left(\frac{1}{A_a^2}\right) - \frac{1}{A_b^2} \qquad (5)$$

To deal with this new factor, it is necessary to know more about fluids and their flow behavior.

Viscosity and flow regimes

Reynolds performed a simple experiment to visualize how fluid molecules move in the flow of liquids. He injected a dye at a given point in a transparent tube section and noted how the color dispersed in the flow (**Figure 2**). He found that, for low flow rates, a smooth straight line developed. At different distances from the pipe wall, the velocity of this line was different. He concluded that, at low flow rates, the molecules move in layers and there is no collision between them, but there is friction between adjacent layers, as they move at different velocities (maximum at the centre of the tube, and approaching zero near the walls). He designated this situation as laminar flow.

However, when the flow rate is increased, a critical point is reached where turbulence starts to develop and the dye moves unpredictably along the tube. In this case, it is evident that there will be collisions between the molecules and the flow is turbulent. **Figure 2** shows how a single drop of dye solution might move along the tube – different drops will move along different paths, as turbulent patterns are unstable (time-dependent).

Figure 3 helps to visualize two layers of molecules moving adjacent to each other and at different velocities. Newton's law states that the shear stress (the force per unit cross-sectional area exerted tangentially to the layers, which is responsible for the velocity difference) should be proportional to the shear rate (the variation of the velocity per unit length), and that proportionality is the viscosity. Hence:

$$\sigma = \mu \frac{\mathrm{d}y}{\mathrm{d}y} \qquad (6)$$

where σ is the shear stress, μ the viscosity, $\mathrm{d}v/\mathrm{d}y$ the shear rate, v the velocity and y the height.

However, not all fluids obey this law, and those that do not are termed non-Newtonian. Water and milk are Newtonian fluids, while most viscous liquids are non-Newtonian.

It makes sense to think that the head losses due to viscous shear energy dissipation in laminar flow, where there are no collisions, should be proportional to the viscosity. This result has been verified empirically and later derived theoretically. The so-called Hagen–Poiseuille equation can be written as:

$$F = \frac{32 \cdot L \cdot v}{\rho \cdot g \cdot D^2}\mu \qquad (7)$$

where L is the length of pipe (distance from A to B) and v is the average velocity (it should be noted that the velocity of a specific layer varies with the distance from the wall), which is given simply by dividing the flow rate by the cross-sectional area.

On the other hand, when there is fully turbulent flow, with collisions between molecules causing the loss of so much energy that the effect of viscosity is irrelevant, the head losses should be proportional to the kinetic head. The so-called Darcy–Weisbach equation is applied in this case, stating that:

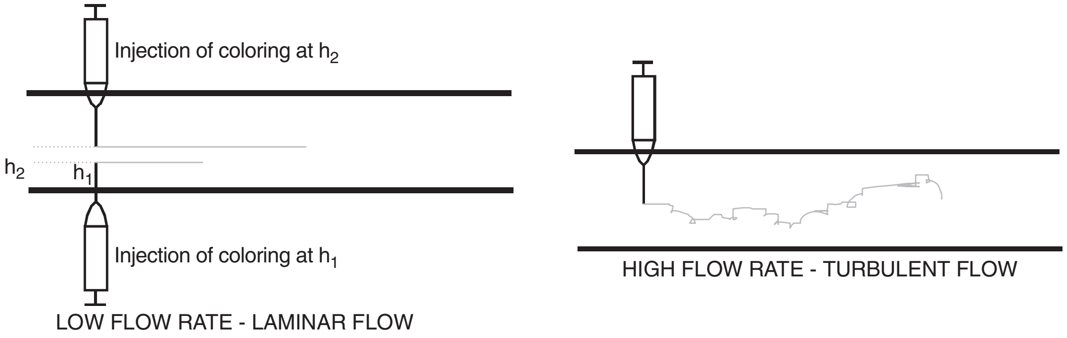
Injection of coloring at h2
h2
h1
Injection of coloring at h1
LOW FLOW RATE - LAMINAR FLOW
HIGH FLOW RATE - TURBULENT FLOW

Figure 2 Reynolds experiment and concepts of laminar and turbulent flow.

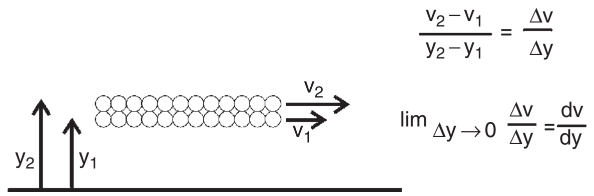
$\frac{v_2 - v_1}{y_2 - y_1} = \frac{\Delta v}{\Delta y}$
$\lim_{\Delta y \to 0} \frac{\Delta v}{\Delta y} = \frac{dv}{dy}$
v2
v1
y2
y1

Figure 3 Concept of viscosity as friction between adjacent layers of fluid moving at different velocities.

$$F = f \frac{L}{D}\frac{v^2}{2g} \tag{8}$$

where *f* is a friction factor that depends on the roughness of the pipe.

It has been found that the critical point when laminar flow ceases and eqn [7] is no longer valid occurs for all systems when the so-called Reynolds number reaches a value of 2000. The Reynolds number (*Re*) is the ratio between kinetic and viscous forces, and its application is based on the principle of dynamic similarity. It is:

$$Re = \frac{\rho \cdot v \cdot D}{\mu} \tag{9}$$

Note from eqns [7], [8] and [9] that it can be concluded that the Darcy–Weisbach equation can be used in laminar flow, provided *f* is considered as a value which varies with the Reynolds number, with $f = 64/Re$.

However, once laminar flow ceases, fully turbulent flow is not immediately established, where viscosity is irrelevant and eqn [8] applies. Between *Re* 2000 and 4000 there is an instability regime, where the flow can switch from laminar to turbulent, and above that there is a gradual change to fully turbulent flow as *Re* increases. This is well visualized by plotting the *f* value of the Darcy–Weisbach equation as a function of the Reynolds number, in what is known as the Moody diagram (**Figure 4**). In laminar flow, there is a straight line in a log-log graph ($f = 64/Re$). In fully developed turbulent flow, *f* is constant, and in between there are the instability and the transition regimes. The point at which fully turbulent flow is reached and *f* is constant depends on the roughness of the pipe wall. For very smooth tubes, which is the case of stainless steel pipes used in the hygienic design of food-processing installations, fully turbulent flow is not actually reached in regions of practical interest of *Re*. In transition flow, the Colebrook–White empirical equation can be used to estimate *f*:

$$\frac{1}{\sqrt{f}} = -2 \cdot \log\left(\frac{k}{3.7D} + \frac{2.51}{\mathrm{Re.}\sqrt{f}}\right) \tag{10}$$

where *k* is the roughness factor of the pipe (for perfectly smooth tubes, $k = 0$) and log is the decimal logarithmic.

Viscus shear energy dissipation

The effect of viscous shear energy dissipation and of collisions between molecules during flow due to turbulence are calculated using eqn [8], with *f* being given by eqn [10]. However, as this is an implicit equation for *f*, many people prefer to read the result graphically from Moody's diagram; a simple version is given in **Figure 4**.

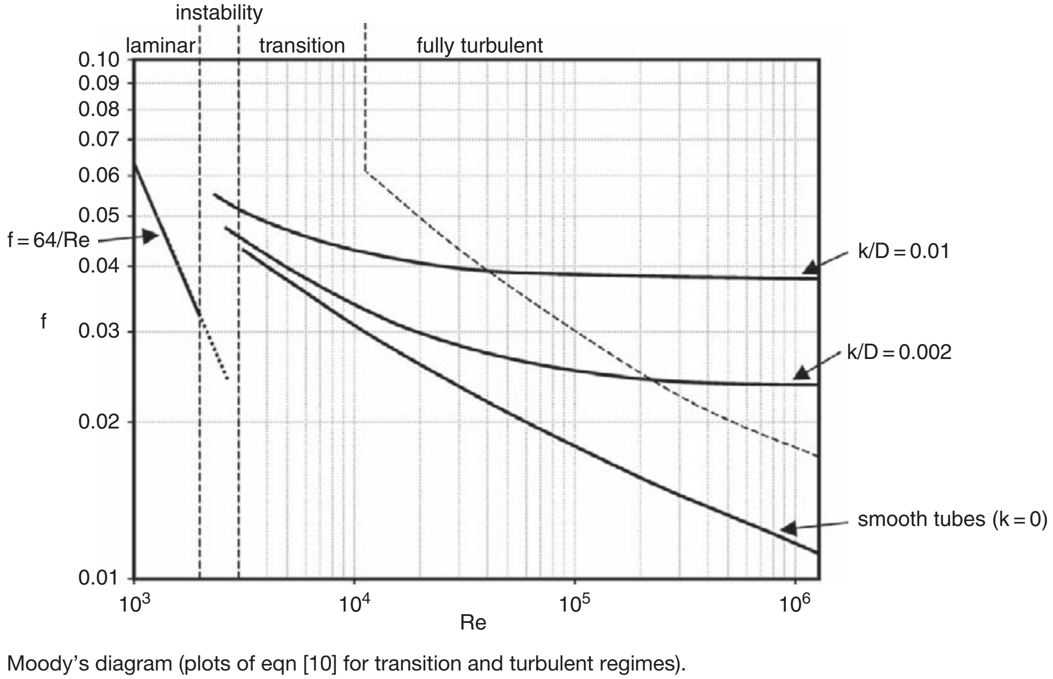

Figure 4 Moody's diagram (plots of eqn [10] for transition and turbulent regimes).

Localized head losses

It is evident that any geometric variation in the installation, such as bends, junctions and expansions, will cause collisions between molecules and the pipe walls, and between molecules themselves, resulting in turbulence. Whether the flow regime is laminar or turbulent prior to these geometric effects, there are kinetic losses due to collisions, and hence it is reasonable to assume that these head losses should be proportional to the kinetic head. The simple expression used is similar to eqn [8], but there is no need to individualize the length and diameter of these elements, and a simpler expression can be used:

$$F = K\frac{v^2}{2g} \tag{11}$$

where *K*, the so-called 'number of velocity head losses', must be estimated depending on the constriction. Various tables and graphs to estimate *K* for the various relevant elements that may exist in an installation can be found in the literature, such as bends (where *K* depends on the curvature), expansions and contractions (where *K* depends on whether the expansion/contraction is sudden or gradual and on the diameter before and after the element), junctions and flow dividers (where *K* depends on the type of junction), and valves (where *K* depends on the type of valve). Except at low Reynolds numbers in some cases, *K* is usually independent of *Re*, just like *f* in fully turbulent flow.

Calculation of head losses in an installation

When calculating the head losses in an installation, eqn [5] is applied and $F_{A\rightarrow B}$ estimated as the sum of all individual head losses: in each straight section of the pipes there is viscous shear energy dissipation given by eqn [8], and in each element that can cause localized head losses, eqn [11] is used with the corresponding *K*-value.

Net Positive Suction Head

Cavitation

When point *B* is located above point *A*, and with the added effect of head losses contributing to pressure decrease, Bernoulli's equation indicates that it is eventually possible to reach a pressure low enough to be at the boiling point of the liquid. This would result in the release of gas bubbles. From Bernoulli's equation, the points to check will be those with smaller cross-sectional area (higher velocity) and located higher in the installation. Gas bubbles are difficult to move, and this could result in severe loss of pumping efficiency. Moreover, the impact of gas bubbles collapsing against a surface moving at high speed is very destructive for centrifugal pumps (cavitation). Hence, the situation where point *B* is the entrance of a pump is of particular concern (**Figure 5**).

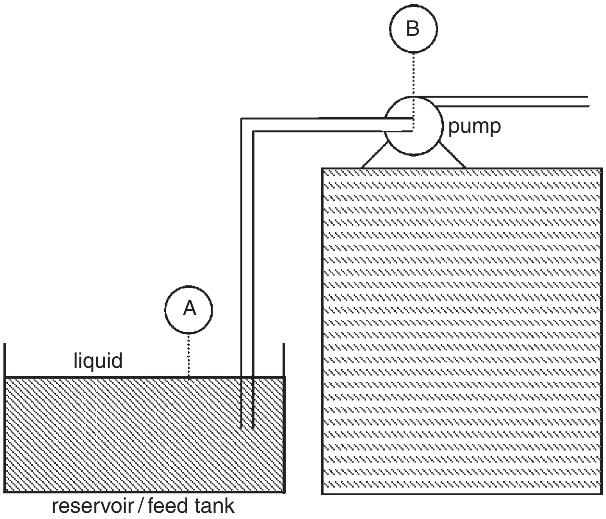

Figure 5 Pump in suction mode from lower reservoir.

Available Net Positive Suction Head

The net positive suction head (NPSH) in a given point is defined as the pressure head above the vapor pressure head, that is:

$$\mathrm{NPSH} = \frac{P}{\rho\cdot g} - \frac{P_V}{\rho\cdot g} \tag{12}$$

where *P* is the pressure of the liquid at that point and P_v is the vapor pressure (pressure at which the liquid is boiling for the system temperature – for instance, the vapor pressure of water at 100 °C is 1 atm, at 20 °C it is 0.025 atm).

The NPSH available ($\mathrm{NPSH_a}$) is the value of the NPSH at the entrance of the pump. It can be calculated by determining the pressure at point *B* from eqn [3], knowing the pressure at point *A*, and estimating all relevant head losses using the appropriate eqns [8] and [11]. Mathematically:

$$\mathrm{NPSH_a} = \frac{P_a}{\rho\cdot g} - \Delta h - F_{A\rightarrow B} - \frac{Q^2}{2g}\left(\frac{1}{A_b^2} - \frac{1}{A_a^2}\right) - \frac{P_V}{\rho\cdot g} \tag{13}$$

It is noted that if the cross-flow areas at *A* and *B* are the same, the kinetic flow rate term is zero.

Required Net Positive Suction Head

In principle, there would be no cavitation if $\mathrm{NPSH_a}$ is greater than zero. However, in every pump there will be head losses until the fluid starts to build up pressure – there are possibly geometric constrictions at the entrance, collisions with pump parts, etc. Usually, pump manufacturers will provide this information in terms of an NPSH required (NPSH_r), from bench scale tests. Basically, these are the head losses that occur at the pump entrance, until the liquid builds up pressure. Thus, the NPSH_r of the pump (or pumps being analyzed) must be checked to ensure that $\text{NPSH}_a > \text{NPSH}_r$. A safety margin of about 0.7–1 m is normally recommended.

It is noted that the NPSH_r, like all head losses, is obviously a function of the flow rate, and pump manuals usually provide the NPSH_r in the form of a graph, as a function of the flow rate or of the Reynolds number.

Pumping Efficiency and Power Requirements

Calculation of Pumping Requirements

Generically, a pump will be located somewhere between the entrance of an installation (piping line, piece of equipment) and the exit, and must promote a given flow rate. Applying Bernoulli's equation between the exit of the pump and the exit of the installation gives the pressure head that must exist at the pump exit to ensure the flow rate specified:

$$H_e = \frac{P_g}{\rho\cdot g} = \frac{P_{out}}{\rho\cdot g} + \Delta h + F_{e\rightarrow \text{out}} + \frac{Q^2}{2g}\left(\frac{1}{A^2_{\text{out}}} - \frac{1}{A^2_e}\right) \qquad (14)$$

where H_e is the pressure head at the exit of the pump, and the subscripts *e* and *out* indicate values at the exit of the pump and at the end of the installation, respectively. $F_{e\rightarrow \text{out}}$ is the total head loss from the pump onward.

Similarly, the pressure head at the pump inlet can be calculated by applying Bernoulli's equation between the entrance of the installation and the pump:

$$H_i = \frac{P_i}{\rho\cdot g} - \frac{P_{in}}{\rho\cdot g} - \Delta h - F_{\text{in}\rightarrow i} - \frac{Q^2}{2g}\left(\frac{1}{A^2_{\text{in}}} - \frac{1}{A^2_i}\right) \qquad (15)$$

Power, Energy and Calculation of Pumping Costs

Assuming the usual case of no variation in the potential and kinetic heads at the inlet and outlet of the pump (same cross-flow area and level height), the power provided by the pump to the liquid (W, energy per unit time) to ensure the flow rate, Q, is that required to raise the pressure head from H_i to H_e:

$$W = \rho\cdot g\cdot Q\cdot(H_e - H_i) \qquad (16)$$

To calculate the pumping energy costs, it must be noted that not all power used by the pump from the mains electrical supply will be delivered to the fluid, as there are various energy losses. The pump efficiency (η) is the ratio between the power delivered to the fluid (W) and that spent from the mains (W_{used}):

$$v = \frac{W}{W\text{used}} \qquad (17)$$

The pump efficiency depends on the type of pump and on the conditions of operation. Generally, there is an optimum efficiency for a given flow rate – operating above or below implies that the pump is under- or overdimensioned for the job, and the consequence is that the pumping efficiency is lower. Hence, engineers must select the pump dimensions according to the specific job, targeting the region of operation of the pump where η is maximum, as otherwise W_{used}, and hence pumping costs, are higher than they could be with an appropriate pump. They may also find that using more than one pump in parallel lines may be more efficient than a single, larger pump. Optimum efficiencies for centrifugal pumps are of the order of 80–90%. This subject is discussed in more detail elsewhere (*see* **Plant and Equipment**: Flow Equipment: Pumps).

Pressure Drop in Valves

In a certain way, valves are the opposite of pumps. They are the elements that cause a loss of pressure head. From eqn [14], as a valve increases, the head losses, Q, decrease. The head loss caused by a valve is generally described by eqn [11]. The value of K will depend on the type of valve and percentage opening, and is given by manufacturers (otherwise, estimates can be found in the literature for generic valves). Valves and pumps work together: pumps must have some overcapacity and have a valve next to it (the discharge valve) to control the pressure to the desired level. Otherwise, the flow would be subjected to fluctuations due to variations in atmospheric pressure and temperature. Discharge valves and surge valves may also be needed for start-up and/or shutdown procedures.

Care should be taken to ensure that the pressure head at the exit of the valve when it is in the lowest opening position (higher K) does not go below the vapor pressure, which can be checked with Bernoulli's equation, in a similar manner to NPSH_a calculations.

See also: **Plant and Equipment**: Flow Equipment: Pumps; Flow Equipment: Valves; Heat Exchangers; Pasteurizers, Design and Operation; Process and Plant Design.

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Flow Equipment: Pumps

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Introduction

Pumps are small pieces of equipment that promote the circulation of liquids between and through pieces of equipment. When selecting a pump, the main factors to consider are:

1. The viscosity of the fluid, and whether it is a clear fluid or contains solid particles in suspension.
2. The sensitivity of the fluid quality characteristics to shearing and viscous shear energy dissipation.
3. The pumping requirements (pressure, flow rate).
4. The hygienic requirements of the process (which are high for dairy products).
5. The cost of the pump and of its maintenance.

The main issues are to ensure that:

1. The mechanical action of the pump affects the fluid characteristics to the least extent possible.
2. The pump is operating close to its optimum point.
3. There is no cavitation (the pressure of the fluid cannot go below its vapor pressure anywhere in the pump).
4. The mechanical wear of the elements of the pump (moving parts, seals) is not extensive to the point of causing significant loss of pumping efficiency (a preventive maintenance program would be quite adequate for pumps).

General Classification

Pumps force liquids to flow in one of two ways, which gives rise to the major division between pumps: (1) centrifugal, (2) positive displacement.

Centrifugal pumps have a rotor that rotates at very high speed (thousands of rotations per minute) and accelerates the fluid. As the fluid leaves the rotor, it is suddenly forced to slow down, pressing against the fluid which is already there. By looking at Bernoulli's equation, it is evident that the sudden substantial loss of kinetic energy must correspond to a sudden increase in pressure (see eqn [4] in article **Plant and Equipment**: Flow Equipment: Principles of Pump and Piping Calculations). Those that provide particularly accurate flow rates are also called metering pumps. A centrifugal pump does not ensure either a specific flow rate or a specific pressure: both will depend on the piping system after the pump (level differences, head losses, etc.)

There is a wide variety of positive displacement pumps, but in general it can be said that they capture a small amount of fluid, cause it to compress in some way, and then deliver the compressed fluid at the outlet. As they deliver a fixed amount of fluid at specific regular intervals, these pumps generally assure a given flow rate, and pressure will be dictated by the piping system and can be estimated by applying Bernoulli's equation.

Centrifugal pumps will obviously cause a substantial amount of kinetic losses due to collisions of molecules with the rotating elements and walls, and between themselves. This can generate a substantial amount of heating and affect thermally sensitive fluids. In addition, the high shearing may also affect molecular networking. Therefore, fluids with delicate macromolecular structures (for instance, a milk coagulum) should not be run through centrifugal pumps. It also follows that very viscous fluids are not suitable, as heat generation would be substantial, and slip is also potentially high (which implies a low pumping efficiency). As a rule of thumb, centrifugal pumps are not generally recommended for fluids with 10 times the viscosity of water or higher. They are obviously not suitable for liquids containing solid particles either. Due to the high velocity of the moving elements, cavitation is a major problem, as it can wear the rotor blades quite extensively and very rapidly (the collapse of a gas bubble when impinging on a moving surface at high speed causes roughly the same type of destruction as the collision with a hard solid particle). In positive displacement pumps, the generation of gas bubbles affects mostly the pumping efficiency, and not so much the actual pump.

On the other hand, centrifugal pumps will deliver a steady flow rate. Positive displacement pumps are more prone to pulses and fluctuations in the flow rate, as the cycle capture–compress–deliver repeats itself. It can also be expected that centrifugal pumps will generally have lower pumping costs, as acceleration of a low-viscosity fluid is easy, and pressure is then generated by the fluid slowing down the energy costs are basically those of accelerating the fluid. Positive displacement pumps must supply the totality of pressure by direct mechanical means, and this can be expected to require generally higher energy levels. However, these are not general rules, as there are very different types of positive displacement pumps.

In general, it can also be said that the action of positive displacement pumps can be reversed (inlet and outlet may be swapped), which is totally impossible in centrifugal pumps.

Not all pumps will be reviewed in this article, only the more common types.

Centrifugal Pumps

General Design and Principles of Operation

Figure 1 shows a centrifugal pump, in front and side view. The fluid enters perpendicular to the axis of rotation of the rotor and will leave tangentially in relation to the rotor blades. As the fluid disperses from the center due to the action of the centrifugal force toward the walls of the rotor casing, it travels in the radial direction through the channel between the blades. It is also accelerated tangentially to the velocity of rotation of the blades. These are constructed in such a way that the distance between blades increases with the radius – therefore, this channel widens as the fluid moves toward the wall. As the continuity equation shows (see eqn [2] in article **Plant and Equipment**: Flow Equipment: Principles of Pump and Piping Calculations), the fluid pressure will therefore increase as its kinetic energy decreases, while moving radially between the blades. The fluid then joins the layer moving around the wall toward the exit, pushed by the centrifugal force, which obviously moves slower than the blades, and so the pressure increases again. This layer, close to the casing wall, may also have an increasing flow area as a result of an eccentricity of the axis of the rotor in relation to the center of the casing – again, the reason for this design is to cause a gradual slow-down and respective pressure increase. This eccentricity, and whether it exists or not, depends on the pump design. At the outlet, another slow-down will occur, as the fluid starts moving at the velocity corresponding to the flow rate through the piping system, and pressure reaches a maximum. The conversion of kinetic energy to pressure is therefore gradual, and it stands to reason that the more gradual it is, the higher the efficiency – a very sudden slow-down implies many collisions between molecules, hence loss of mechanical energy, dissipated in the form of heat. Some pumps have a static ring between the moving rotor and the casing (usually, with channels having the opposite obliquity to the rotor blades), which provides a further gradual step of conversion from kinetic energy to pressure. **Figure 2** illustrates this description schematically.

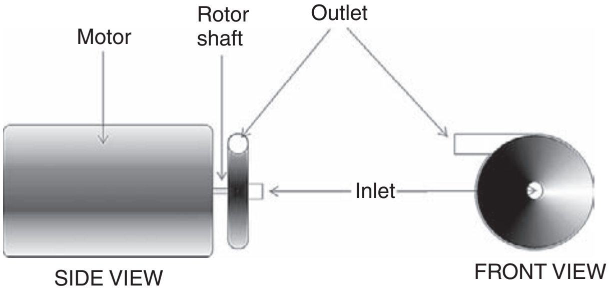

Figure 1 Front and side view of a centrifugal pump.

Hygienic Requirements

The most obvious hygienic requirement for a centrifugal pump is that it should be easy to disassemble, so that every part can be thoroughly cleaned easily. The blades should be rounded to avoid crevices, and therefore hygienic pumps generally have a lower number of blades (see **Figure 2**).

The pump motor releases much heat, as one would expect from a drive that generates velocities of the order of thousands of rotations per minute. Therefore, the motor casing of normal pumps is corrugated (the higher specific area promotes heat loss). However, this is not satisfactory for hygienic food processing, and food-grade pumps must have a smooth, stainless steel casing. This implies a pump that is built specifically for that, as other means of removing the heat generated by the motor must be improved; if a normal corrugated pump is covered by a smooth casing, it will run the risk of overheating.

Operating Points and Pumping Efficiency

Depending on the piping system to which the pump outlet is connected and on the rotor design and speed, a centrifugal pump will be able to deliver various flow

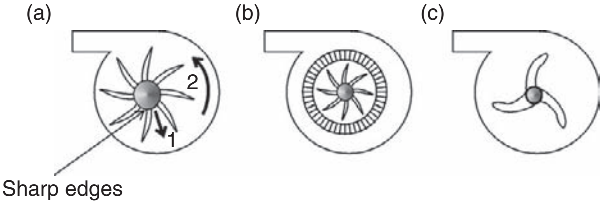

Figure 2 Rotors of centrifugal pumps. (A) Typical rotor without hygienic design considerations. Note path 1, where the fluid moves toward the wall pushed by the centrifugal force along a channel with increasing cross flow area, thereby increasing pressure; and path 2, where the fluid moves rotationally toward the axis, pushed by the centrifugal force, through a channel of increasing cross flow area due to the eccentricity of the rotor in relation to the casing; (B) rotor encased in a fixed channel distributor; (C) hygienically designed rotor, with smooth edges.

rates at various pressures. The pump characteristic curve indicates all possible operating points of a given pump. **Figure 3** shows a typical example. Pump manufacturers usually supply these graphs.

Pumping efficiency is not the same along these curves; it is normally maximum around the top right-hand corner and decreases as one approaches the limits of the curve. Therefore, a pump should ideally operate close to the flow rate and pressure combination that gives the maximum efficiency (at the expense of unnecessarily spending more power from the electrical mains to deliver the same amount of energy to the fluid). Lines of constant efficiency are usually drawn on these graphs (see **Figure 3**).

Checking the operating point of a pump in a given piping system is straightforward. Using Bernoulli's equation, the characteristic curve for the piping system can be constructed by calculating the pressure head at the outlet of a pump for various flow rates. An operating point for a given pump in a given piping system is the intersection of the two characteristic curves (pump and piping system). **Figure 3** shows a sketch of a situation where the maximum efficiency is achieved with the intermediate pump configuration, that actually delivers a slightly lower flow rate at a lower pressure than the configuration that gives the maximum flow rate. It should be noted that the characteristic curves of piping systems can be easily modified by operating valves, as they will increase head losses if partially closed. In the example of **Figure 3**, closing a flow rate control valve would increase head losses and the characteristic curve would be steeper. It would be possible to bring the piping system curve close to the maximum efficiency of the pump only if it would be possible to decrease the head losses.

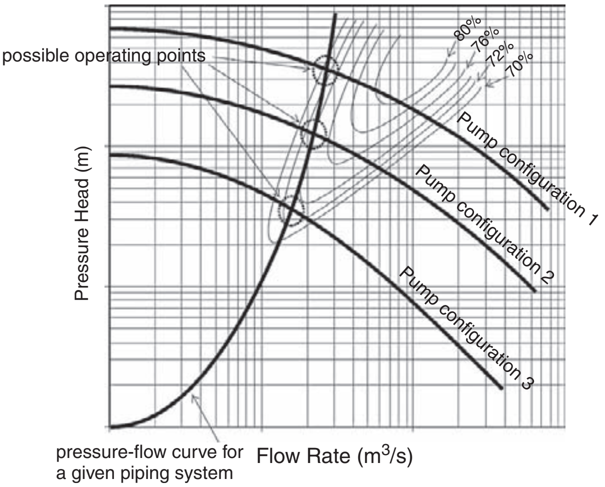

Figure 3 Typical characteristic curves for a pump in three possible configurations (different rotor design and/or speed), efficiencies and operating points.

Selection of Centrifugal Pumps

In order to select a centrifugal pump, one must know:

1. The net positive suction head (NPSH) available at the inlet of the pump (*see* **Plant and Equipment**: Flow Equipment: Principles of Pump and Piping Calculations).
2. The characteristics of the system (cross flow areas, level differences, total length of the piping system, localized head losses).

Item (1) is needed only for a verification: the NPSH available must be higher than the NPSH required by the pump chosen, in order to ensure that the vapor pressure is not reached inside the pump, which would cause cavitation and severe wear.

Item (2) is needed so as to apply Bernoulli's equation and determine the characteristic curve of the piping system, from where the operating point and efficiency of the pump can be calculated. The pump, and its operating variables, are chosen that give the maximum efficiency.

Manufacturers may provide graphs that indicate which of their makes/models are generally more suitable depending on pressure head and flow rate required. This helps to narrow choice to pumps that are designed for the type of demand in question. **Figure 4** shows a typical example.

Positive Displacement Pumps

Piston Pumps

General design and principles of operation

A typical piston pump, which operates according to a very straightforward principle, is shown in **Figure 5**. Fluid is admitted to a chamber through the inlet valve, as the piston moves back and sucks the fluid in. The

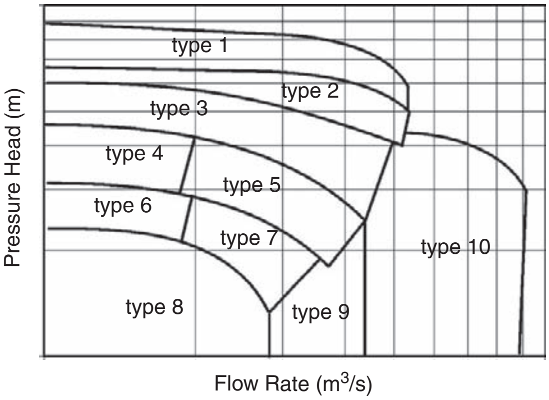

Figure 4 Typical chart for selection of the type of centrifugal pump better adapted to a given demand.

valve closes when the piston reaches its back position and then starts moving forward, compressing the fluid trapped in the chamber. When the specified pressure is reached (for incompressible fluids, this is obviously almost instantaneous), the outlet valve opens and the fluid is discharged, as the piston expels it while moving forward. When the piston reaches its forward position, the outlet valve closes, the inlet valve opens and the cycle begins again.

A piston pump will therefore deliver a flow rate equal to the volume of the chamber times the number of cycles per unit time. However, it is delivered only when the outlet valve is open, and nothing is delivered during the admission part of the cycle. In order to avoid a pulse in the flow, it is normal to use two chambers with the piston of each moving asynchronously, so that when one delivers the other admits and vice versa. Obviously, this can be neatly done by a dual chamber pump, with chambers placed back to back, so that the piston is actually the same. **Figure 6** sketches this design.

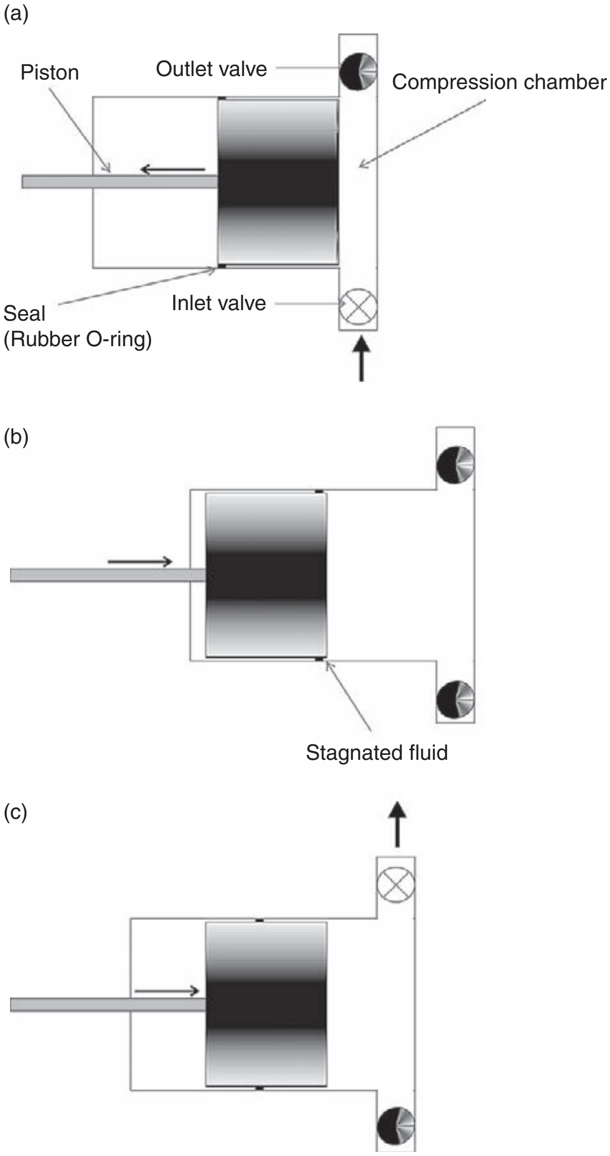

Figure 5 Piston pump. (A) Commencement of the admission stage of the cycle; (B) commencement of the compression stage; (C) during the delivery stage. White circles represent open valves and gray cones indicate closed valves.

Hygienic requirements: diaphragm pumps

Piston pumps are not very hygienic because fluid can be trapped in the space between the cylinder side and the chamber wall, at the back (see **Figure 5**). Diaphragm pumps were built to solve this problem. The piston is not in direct contact with the fluid, but pushes a rubber diaphragm, which fits totally in the chamber geometry. There are pumps commercially available that use compressed air to move the diaphragm.

Figure 7 sketches an example of a single-chamber diaphragm pump. For hygienic design, it would be ideal that the pump expels the totality of the fluid in order to avoid stagnated or dead volumes – the design in **Figure 7** shows an example which is particularly poor in this respect.

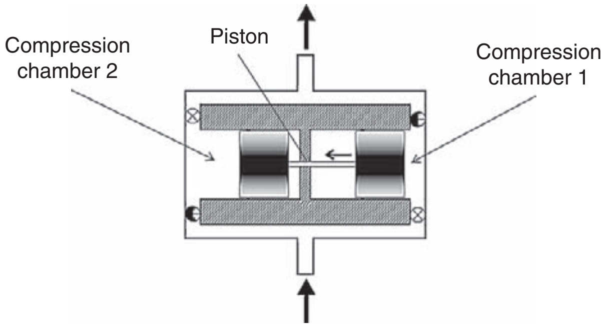

Figure 6 Twin-chamber piston pump. Chamber 1 is sucking fluid from the bottom and chamber 2 is delivering compressed fluid to the top. White circles represent open valves and gray cones indicate closed valves.

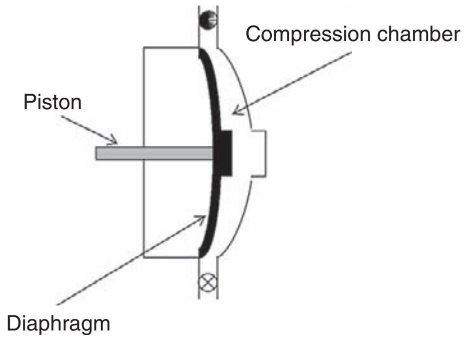

Figure 7 Diaphragm pump.

Rotary (Gear) Pumps

General design and principles of operation

Rotary pumps are also known as gear pumps. They have two rotors and dented wheels moving in such a way that the dents of one wheel fit snugly inside the other. Thus, the fluid is forced toward the outside wall and must move around. The fluid will be trapped inside chambers formed by two dents and the outside casing and is then forced out at the other side. **Figure 8** shows this principle of operation. Rotating speeds are about 100 times lower than those of centrifugal pumps.

These pumps are therefore more similar to centrifugal than to piston pumps in the sense that they assure a given flow rate rather than a given pressure: the flow rate is simply given by the volume between each pair of dents and the speed at which this volume is transferred to the other side. Pressure is then given by Bernoulli's equation, as once the flow rate is fixed, the piping system after the pump defines the pressure. Furthermore, while some compression occurs when the fluid is trapped inside the dents, most results from delivering the fluid to the other side and compressing it against the fluid which is already there, a similar situation to that in centrifugal pumps. However, this characteristic makes them the best metering pumps, with the flow rate being quite well controlled, as it is simply proportional to the rotating speed.

Hygienic requirements: lobular pumps

Rotary pumps such as that shown in **Figure 8** are not hygienic because the dents have sharp edges. Thus, lobular pumps have been developed, with well-rounded lobes, such as those shown in **Figure 9**. The sealing between the counterrotating rotors is obviously less good than in rotary pumps, as the rounded geometry implies fewer dents and therefore fewer points of contact for sealing at the center. A higher leakage flow between the high-pressure and low-pressure regions through the center is likely, compared to rotary pumps with more dents and more sealing points.

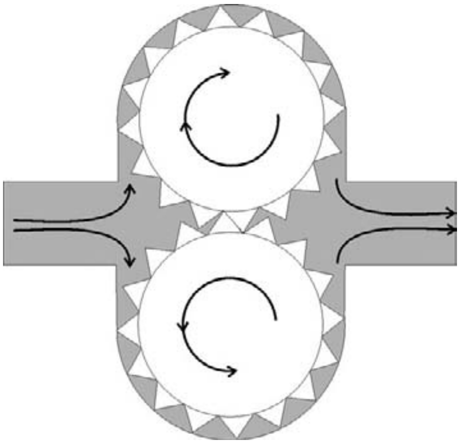

Figure 8 Rotary pump.

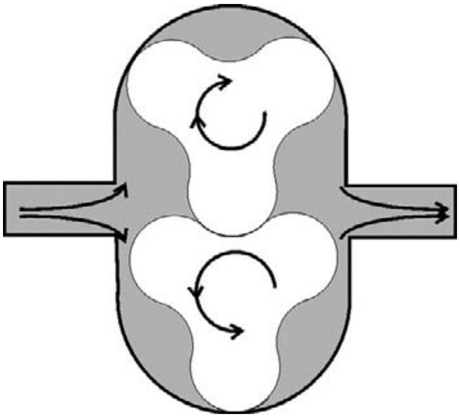

Figure 9 Lobular pump with three lobes per rotor.

Single-Rotor (Impeller) Pumps

Single-rotor pumps are also known as impeller pumps. They seem similar to rotary pumps, having only one rotor. However, their operation is quite different, as they can provide mechanical compression of the fluid in a much better way than rotary or lobular pumps. The blades are either made of rubber so that they can bend against the casing, or are made of metal, but have a spring at the base, so they can be pushed inward while moving against the casing. **Figure 10** shows both cases.

As the rotor moves, fluid is trapped between two blades. To improve compression, the casing may have a different curvature from the rotor axis, so that the space between them decreases. Therefore, the volume between two blades decreases while the rotor revolves, causing a compression (for relatively incompressible fluids, such as water and milk, this is not so important, while for viscous viscoelastic materials it would be helpful). On reaching the other side, the compressed fluid is expelled. In some pumps with rubber blades, the volume between blades is always the same, and the only compression results from the blades snapping at the inlet and squeezing at the outlet (see **Figure 10**).

Some fluid will certainly remain and proceed to a second turn – potentially, there could be a problem of stagnant fluid. The blades must therefore be designed in a way that helps the expulsion at the outlet, which is better achieved with rubber blades.

Progressing Cavity Pumps

Also known as monopumps, they have a curious design, with a rotating axis that looks like a twisted worm. It is not really a

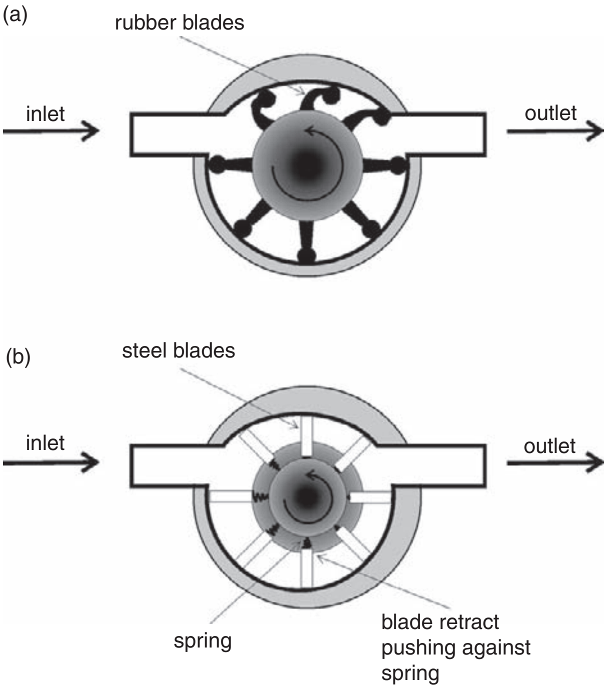

Figure 10 Single-rotor pumps. (A) Pump with rubber blades and no compression in the chambers between blades and outside casing wall; (B) pump with steel blades and with compression caused by decreasing volume between blades.

screw, it is more like a twisted bar which rotates against a rubber casing (the stator), that adapts to the movement of the rotor. The rubber casing has a grooved path of a different shape from the rotor curvature, so that at a given position there is a perfect match between grooves of the rubber casing and curved rotor, while a space exists between each two matching points (for instance, the rotor is a single helix and the casing is a double helix). As the rotor revolves, the points of contact appear to move forward. Hence, the fluid is trapped in a chamber formed by the rubber casing grooves and rotor, sealed at the matching points. The curvature of the rotor (and grooves) may show a decreasing amplitude, so as to reduce volume and hence cause compression (particularly helpful for viscoelastic materials). **Figure 11** shows this type of pump.

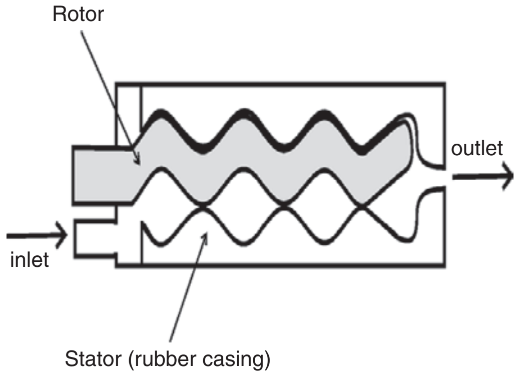

Figure 11 Progressing cavity pump.

These pumps have a good hygienic design, as all fluid is expelled, there is no need for valves, and there are only rounded edges and surfaces.

Peristaltic Pumps

Peristaltic pumps require a rubber tube through which the fluid flows. A wheel with rollers moves on top of the rubber tube, so that as the rollers move they squeeze the tube. This gentle squeeze causes a compression and therefore the fluid is trapped between two points of roller compression, forced to move forward and delivered at the outlet. Just like rotary pumps, peristaltic pumps basically deliver a fixed flow rate and most of the compression results from forcing the movement of the fluid. Hence, pressure will depend on the piping system, and it is the flow rate that is fixed by the pump operating characteristics. They can therefore provide good metering (flow rate accuracy and control). **Figure 12** shows this design.

These are obviously the most hygienic pumps of all, as there is no contact between pump parts and fluids. They are also the best for solid particles, as particles are not squeezed. The main limitation is that tubes must be relatively small, as it is not viable to compress very large tubes. Flow rate is therefore more limited than in other pumps. Pumping efficiency is also limited if very large pressures are required. In general, commercial peristaltic pumps can be found for flow rates up to 10 $m^3 h^{-1}$ and pressures up to 1.6 MPa.

Summary of Pump Selection Criteria

Table 1 provides an overview of the main selection criteria for pumps. In general practice, centrifugal pumps are used unless the fluid is too viscous, contains solid particles, or is too strongly affected by the high shearing and/or internal energy generation.

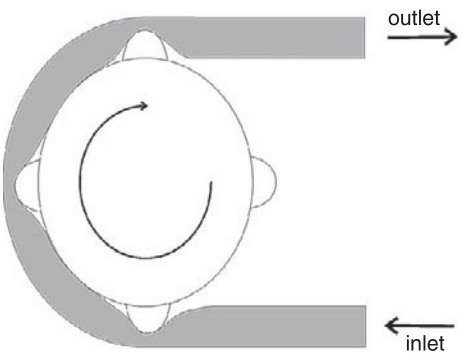

Figure 12 Peristaltic pump.

Table 1 Selection criteria for types of pumps

Type of pump	*Good for*	*Not good for*
Centrifugal	Low-viscosity fluids Clean fluids Steady flow rate	Viscous fluids Fluids containing solid particles Fluids sensitive to heat Fluids sensitive to shearing
Piston/diaphragm	Viscous fluids Clean fluids High pressures Precise pressure control	Fluids containing solid particles
Rotary/lobular	Viscous fluids Clean fluids Fluids sensitive to shearing Precise flow rate control	Fluids containing solid particles
Single-rotor	Viscous fluids Fluids with solid particles Fluids sensitive to shearing	High pressures
Mono	Viscous fluids Fluids sensitive to shearing	High pressures Fluids containing solid particles
Peristaltic	Viscous fluids Fluids with solid particles Fluids sensitive to shearing Flow rate control	High pressures High flow rates

See also: **Plant and Equipment**: Flow Equipment: Principles of Pump and Piping Calculations; Flow Equipment: Valves; Heat Exchangers; Process and Plant Design.

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Flow Equipment: Valves

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Introduction

Valves are devices for controlling the flow of fluids (liquids and gases), and in some cases granular or powdered materials. This article primarily discusses the valves used in product liquid lines in a dairy processing plant although many of the comments are equally applicable to the valves used for service fluids such as steam, refrigerant, compressed air, and process water, which will also be present in a dairy.

Broadly speaking, valves can be classified by their function as being either

1. on–off valves or
2. flow regulation valves.

On–off valves, which include shutoff, isolation, and changeover valves, have distinct positions, fully open or closed. These valves are used to stop flow or isolate part of a process. Their function is to minimize resistance to flow when fully open and to provide tight shutoff characteristics when fully closed.

Regulating valves, on the other hand, have a flow passage, the area of which can be changed gradually. Such valves are used to control flow rates and pressures at various points in the system. Flow control valves can be continuously adjusted from fully open to fully closed in order to govern the flow rate. Pressure control valves determine pressure downstream of the valve, that is, maintain it at some set value irrespective of the flow rate through the valve.

Valve Construction Principles

While a wide range of valves are used in industry, there are only a limited number of basic geometries by which an opening in a pipe can be opened or closed. Almost all valves can be classified as belonging to one of three fundamental designs or their variants. These are

- globe valve (**Figure 1**),
- butterfly valve (**Figure 2**), and
- gate valve (**Figure 3**).

The globe valve (also referred to as a seat valve) consists of a rigid valve body, weir, plug, and spindle. In the closed position, the plug rests on the valve seat in the weir. Linear upward motion of the spindle will raise the plug off the seat and open the valve. This is the most popular type of valve for dairy products.

The butterfly valve consists of a flat disc (equal in cross-sectional area to the bore of the pipe), a valve spindle, and an elastomeric or plastic seal ring. It operates on the principle of rotational rather than linear motion. In the closed position, the disc is face-on to the flow area and seals against the seal ring. Turning the spindle rotates the disc through a quarter turn (90°) and moves it to a side-on orientation with respect to the pipe bore, and thus allowing flow. This valve has low pressure drop characteristics.

The gate valve (sometimes known as the slide valve) like the globe valve operates on the principle of linear motion of a spindle. A flat disc is attached to the spindle, and in the closed position, it is fully extended and blocks the flow area of the pipe. Linear upward motion of the spindle will retract the gate into the valve body and permit fluid flow. This type of valve is not found in product lines in the dairy industry as it is unhygienic, although it is used for service fluids.

Valve Flow Design

Valve flow design applies the principles of fluid mechanics to determine the flow rate and pressure drop through the valve. Other issues that are of concern include valve flow characteristic and avoidance of water hammer.

Pressure Drop

All valves cause losses of head (i.e., pressure) in the system due to friction effects. In general, the smaller and more intricate the passage through which the fluid has to pass, the greater the pressure loss. Considering the flow of a

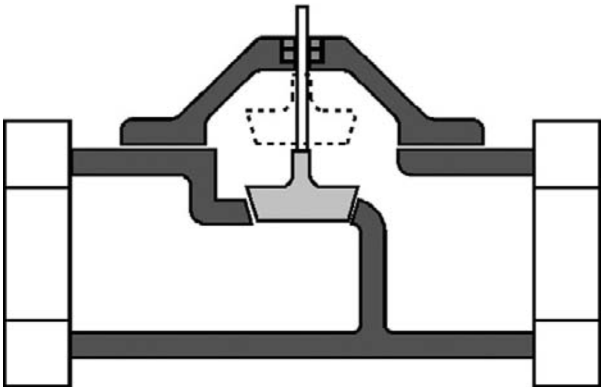

Figure 1 Globe valve.

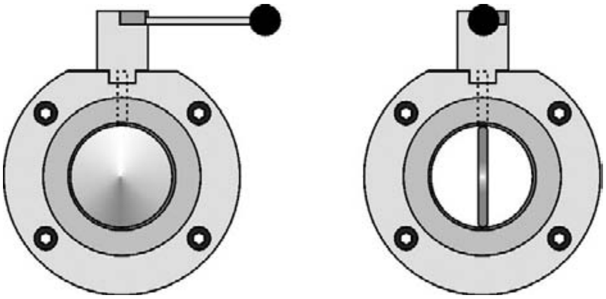

Figure 2 Butterfly valve.

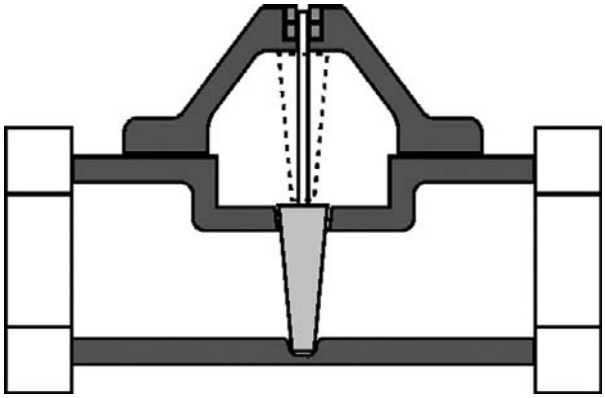

Figure 3 Gate valve.

turbulent and incompressible liquid (liquid milk for example), the pressure loss through a valve will be proportional to the square of the mean liquid velocity:

$$\Delta P = k\frac{\rho u^2}{2}$$

where ΔP is the pressure loss through the valve (Pa), ρ the density of the liquid (kg m^{-3}), u the mean fluid velocity (m s^{-1}), and k the friction factor (dimensionless).

Note that the mean fluid velocity refers to velocity through the adjacent piping (if piping of various diameters is used, this is taken to mean the velocity through the smallest diameter piping).

Using the continuity equation, the pressure drop can also be expressed in terms of the volumetric flow rate through the valve:

$$\Delta P = \frac{8k\rho Q^2}{\pi^2 D^2}$$

where Q is the volumetric flow rate (m^3 s^{-1}) and D the valve bore diameter (m).

Values of the friction factor k of the valve depend on the exact shape of the flow passage and are generally found from experiments. In some cases, the pressure loss through a valve is expressed in terms of an equivalent length of unobstructed straight piping in which an equal pressure loss would occur:

$$\Delta P = \frac{4fL_{\text{eq}}\rho u^2}{2D}$$

where f is the friction factor (dimensionless).

In the above equation, f, u, and D relate to the values associated with the adjacent straight piping and f is the Fanning friction factor, which is equivalent to 16 times the inverse of the Reynolds number in the pipeline where the pipeline flow is laminar. (In the US texts, the Darcy friction factor, which is 4 times larger than the Fanning friction factor, is often substituted for the $4f$ term in the above equation.)

Rather than this equivalent length being given as an absolute measurement in meters, it is more conventional to express it as an integer number, n, times the diameter:

$$L_{\text{eq}} = nD$$

Table 1 gives approximate values of the friction factor and equivalent lengths for a fully open globe valve and gate valve. It must be stressed that these figures are only rough estimates and many unstated assumptions (such as Reynolds number of flow, valve bore diameter, and pipe roughness) underlie them.

Valve Characteristic

An important design feature of modulating valves is the valve characteristic. This is the relation between the stem (spindle) position of the valve and the flow through the valve at a constant pressure drop. The valve characteristic determines how a control valve regulates the flow. Two

Table 1 Friction losses through valves

	k	*L/D*
Globe valve (wide open)	10	340
Gate valve (wide open)	0.2	13

characteristics must be evaluated for valve selection: the inherent and installed characteristics. The inherent flow characteristic is the relationship between valve stroke and the resulting fluid flow through the valve for the valve on its own. The installed flow characteristic is the actual relationship between valve stroke and flow when the valve is an element in a particular flow system. Hence the installed flow characteristic incorporates the inherent flow characteristic of the valve itself together with the flow characteristics of the whole line, that is, it depends upon the ratio of the pressure drop through the valve to the total pressure drop across the line and valve.

The inherent flow characteristic is the theoretical performance of the valve and is generally either linear or equal percentage. For a valve with a linear characteristic, the flow rate is linearly proportional to spindle position. For a valve with an equal-percentage characteristic, equal increments of spindle movement produce equal percentage changes in flow for a given pressure drop. An equal percentage valve may be combined with a centrifugal pump to provide a linear system. For the control system designer, the aim is to select a valve whose inherent flow characteristic gives an installed flow characteristic that makes the flow controllable over the whole range of valve movement, that is, stem position.

Water Hammer

Sudden closure of a valve can produce the phenomenon of water hammer in the pipe system. The name is a misnomer; this problem can occur with all liquids. Water hammer occurs when the abrupt deceleration of a liquid in a pipeline, caused by closing a valve too quickly, produces transient pressure shock waves in the liquid. These shock waves in turn can lead to appreciable and damaging levels of pipe vibration. Equipment such as process vessels and heat exchangers connected to the pipe may also be exposed to this vibration. Water hammer must be avoided in the design and selection of the pipe and valve system. Valves should be installed so that valve spring force and fluid pressure force act in opposite directions. Analysis of the phenomenon has been done in greater detail but fundamentally it involves imposing a minimum time limit on the duration of valve closure to avoid decelerating the liquid too rapidly.

Valve Hygienic Design

In a dairy, of all the pipe fittings in the product line, valves are probably the most technically complex. For hygiene reasons, all wetted metal parts of the valve are made of stainless steel. These are surfaces that intentionally or unintentionally (e.g., by splashing) come in contact with the product. Two main grades are used, AISI 304 and AISI 316. The valve body and all the fittings (actuator spindle, springs, flanges, bolts, operating levers, and wheels) will generally be made from 316 stainless steel.

Valve seals can be made from a variety of synthetic food grade rubbers; among the most common types are silicone rubber, butyl rubber, nitrile rubber, and EPDM (ethylene propylene diene monomer). The actual choice of rubber is determined by the demand of the particular application of the seal such as required mechanical properties (particularly compression set), temperature regime, and exposure to steam and cleaning/sterilizing agents.

Apart from functional performance, the most significant criterion in the selection of valves for milk processing plants is that they must be of a sanitary design and cleanable. Sanitary valves are designed according to the American 3A standards or other relevant hygienic design codes such as EHEDG (European Hygienic Equipment Design Group), Swedish, and German DIN standards.

Valves should be self-draining, free of dead spaces, and readily cleanable. They should protect the product from contamination, prevent product leakage, and not allow the ingress of microorganisms. For improved standards of hygiene and cleanability, valves should be connected to pipelines by butt-welding or by sanitary unions. Product contact surfaces should have a surface finish of 0.8 μm R_a or better, and be free of any pits, folds, or crevices.

A consequence of hygienic design considerations is that globe valves and butterfly valves are the most prevalent type of valves used in the dairy industry.

Valve Actuation

The choice is between manual, pneumatic, hydraulic, or electric drives to control valve position.

In general, manually operated valves are now found only in the smaller dairy plants. Automated valves have replaced them in large modern facilities although they are still used occasionally.

Pneumatic actuation is generally the first choice in the dairy industry with either a diaphragm or a piston actuator. Spent air can be discharged directly to atmosphere and there are no sparking risks. Because air pressures are generally low (in the region of 7 bar), there are upper limits on the forces that air systems can develop and there is no necessity to resort to impracticably large actuators. However, as valve opening/closing forces in dairy product lines are seldom of large magnitude compared to valve forces found in bulk chemical processing, this limitation is not of great significance.

If high valve opening/closing forces need to be generated, hydraulic actuation of the valve is suitable because of the very high oil pressures that can be generated. However, due to the possibility of contamination of the product with the oil, hydraulic actuation is very rare in the dairy industry. It can be found in applications where there is no possibility of contact with the product.

Electric solenoid actuation is not prevalent in the industry due to the fire/explosion risk from electric sparking. Also the damp conditions that can prevail in dairies can lead to problems with electrical connectors. They can be used, however, to control the pneumatic actuation circuits that in turn operate the main process and clean-in-place (CIP) valves.

Irrespective of the actuation mechanism chosen, valves can be arranged to be normally open or normally closed according to safe practice. Two arrangements are common:

- air closing/spring opening (a normally open valve, NO) and
- spring closing/air opening (a normally closed valve, NC).

Air opening/air closing arrangements are occasionally used.

In a system with pneumatic actuation, the usual configuration is that air under pressure is used to move the valve from its default (safe) state to the active state. If the pressurized air is removed, a compressed spring will automatically return the valve to the default state. Hence, occurrences such as electricity failure leading to a loss of compressed air need not have unwanted consequences. As an example, valves on outflow lines from silos or storage tanks will usually have the configuration of air to open and spring to close. This diminishes the possibility of loss of the product due to valve system failure.

Valves in Dairy Processing

The functions of valves in the dairy processing industry are summarized in **Table 2**.

The different types of valves used in dairy processing are described below.

Table 2 Dairy valve functions

	Valve type	*Function*
1	Product shutoff	Stop and start the flow of the product
2	Product changeover	Divert flow to another pipeline
3	Product separation	Separate two fluids, for example, product and detergent
4	Flow regulation	Adjust the pressure or rate of flow of the product
5	One-way flow	Ensure that the product flows in one direction only
6	Pressure relief	Control the maximum pressure of a fluid
7	Vacuum relief	Admit air to a vessel if vacuum exceeds a preset value
8	Constant pressure	Maintain the process liquid at a constant preset pressure

Globe Valve

The globe valve (also called seat valve or poppet valve) is operated by the movement of a valve stem, which raises or lowers a plug on to a valve seat. It can be used as a shutoff valve (**Figure 4**) or as a changeover or divert valve (**Figure 5**). When used as a shutoff valve in piping systems, it should close against the flow to reduce the possibility of water hammer. If used as a tank outlet valve, it should be installed so that static liquid pressure helps to keep the valve closed. Depending on system requirements, this valve is available with up to five ports.

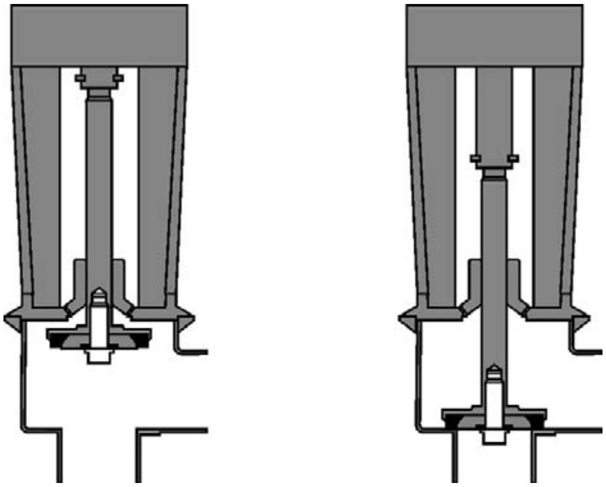

Figure 4 Seat valve – shutoff.

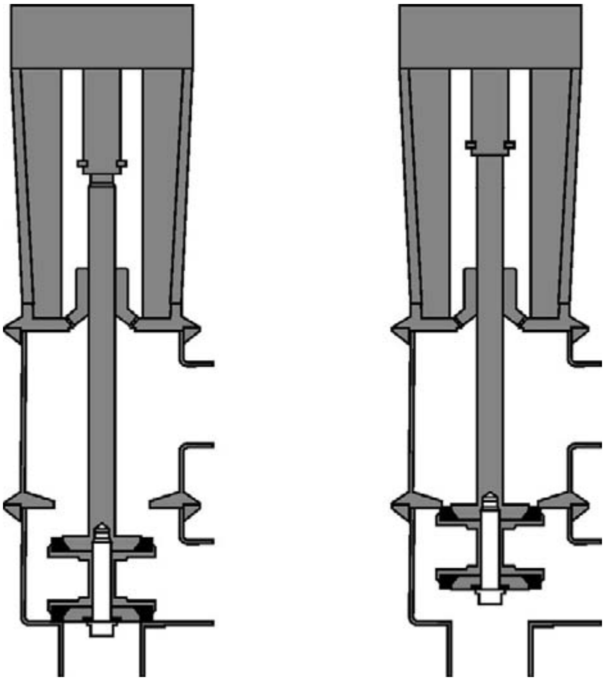

Figure 5 Seat valve – changeover.

Butterfly Valve

The butterfly valve (**Figure 6**) is designed primarily as a shutoff valve, but is sometimes used for flow regulation. When fully open, pressure drop is small, which makes the valve suitable for products that require delicate handling with minimum turbulence. It is also suitable for viscous products. The butterfly valve is available in manual or automated formats.

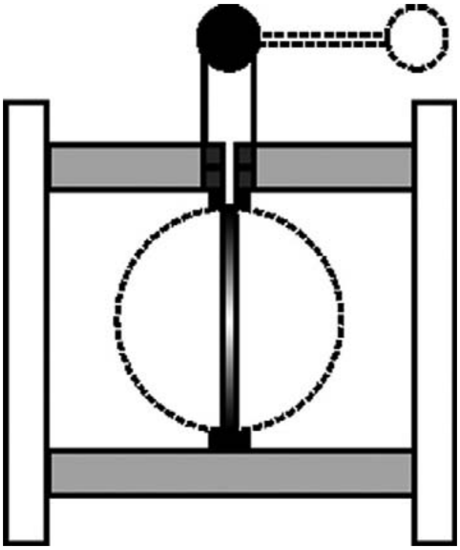

Figure 6 Butterfly valve.

Double-Seat Valve

Situations arise in the dairy industry in which two pipelines must be connected to allow the product to flow from one pipeline to the other, or separated to prevent mixing of two fluids such as product and detergent, and to ensure product integrity by directing any leaks to drain. This function can be achieved by use of three butterfly valves, three on–off globe valves, or one on–off and one changeover valve. It can also be achieved using one double-seat valve (**Figure 7**), which has two separate seals, one for each pipeline. Between the seals is a chamber, which is connected to atmosphere. Provision is made for in-place cleaning of this leakage chamber. To reduce the risk of a valve seat being lifted by surges in liquid pressure, this valve is available with a hydraulically balanced plug.

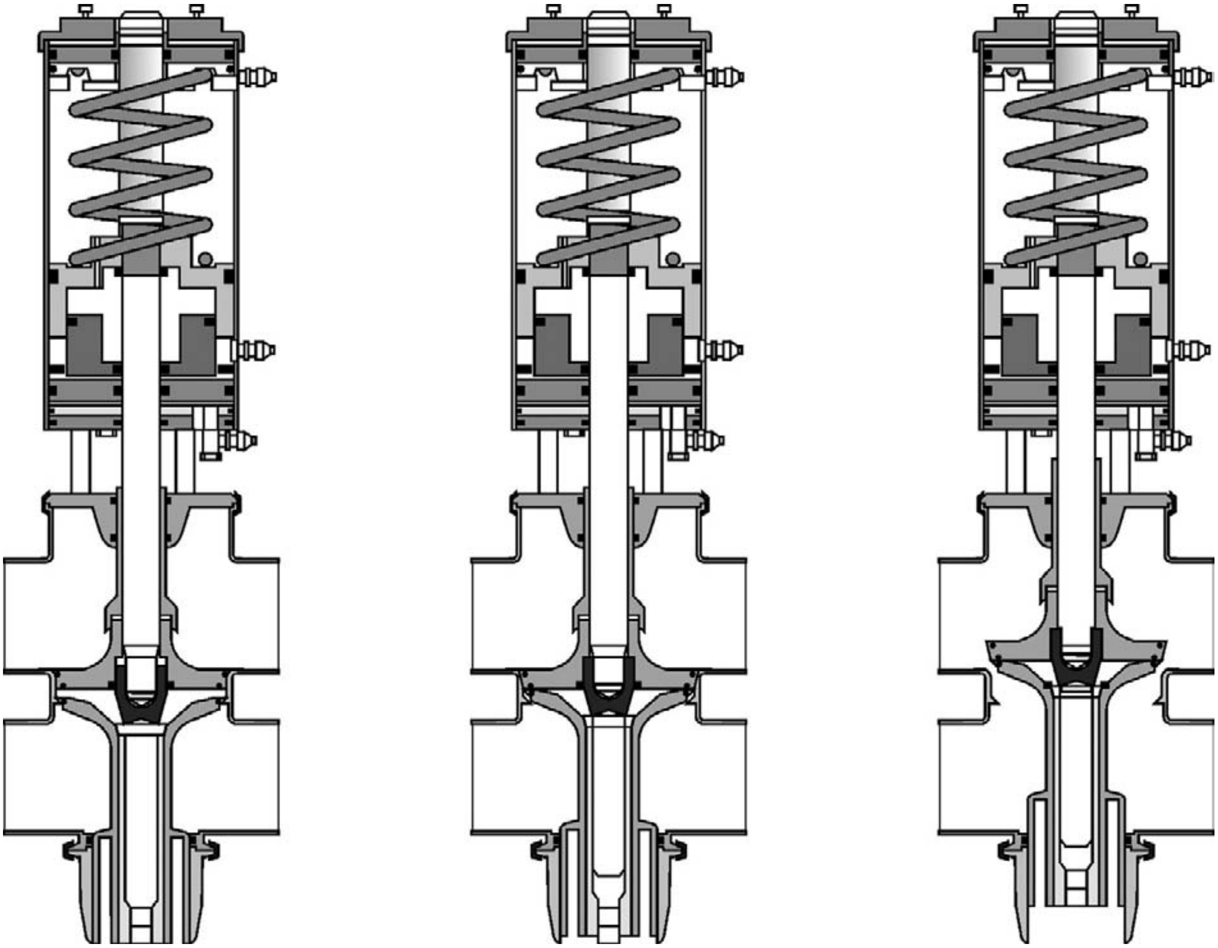

Figure 7 Double-seat valve.

Constant-Pressure Valve

In the constant-pressure valve (**Figure 8**), compressed air acting on a diaphragm modulates the flow in the system, in response to changes in product pressure. By this means, product pressure can be maintained constant, for example, for supply to an item of processing equipment.

Modulating Valve

The modulating valve (**Figure 9**), which is conceptually similar to the globe valve, is used to vary product flow rate. It operates by varying the position of the valve in the orifice in the flow weir and flow rate is reduced as the plug is lowered toward the valve seat. It may be operated in conjunction with a flow sensor, which detects product flow rate, and a controller, which adjusts the valve setting to the required flow rate.

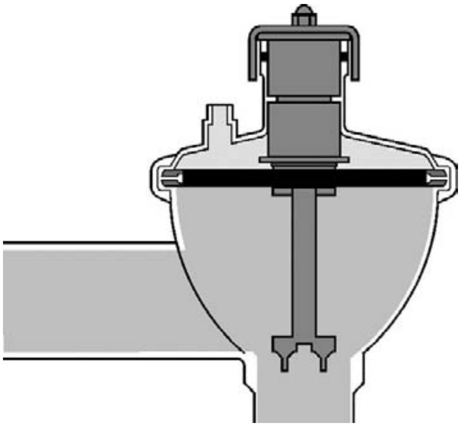

Figure 8 Constant-pressure valve.

One-Way Valve

The one-way valve or check valve (**Figure 10**) uses a spring-loaded seal to allow flow in one direction and to stop flow in the reverse direction. Fluid flow in the desired direction uses the momentum of the fluid to keep the valve open. If the direction of flow is erroneously reversed, the valve closes under the influence of the fluid and the spring, to prevent backflow.

Safety Valves

Pressure/vacuum relief valves (two possible configurations of a safety valve are shown in **Figure 11**) are used to ensure safe operation by limiting the maximum pressure/vacuum that can develop in a system. Pressure can be controlled by air pressure or by preset compression of a helical spring. Applications include the use of a pressure relief valve downstream of a positive displacement pump, to avoid damage in the event of a flow shutoff downstream. Since many safety valves are not air actuated, it is difficult to open them for CIP cleaning.

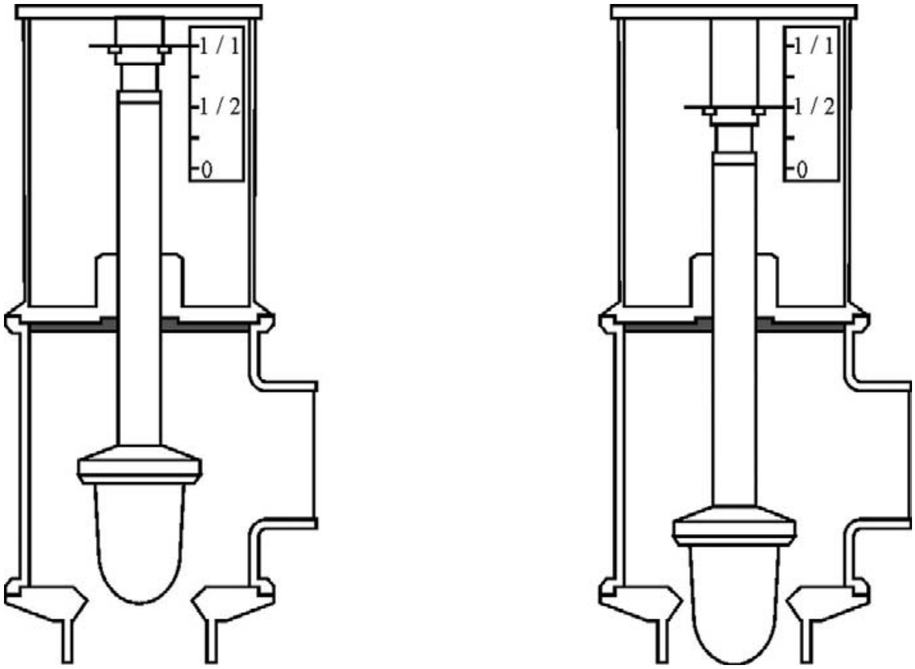

Figure 9 Modulating valve.

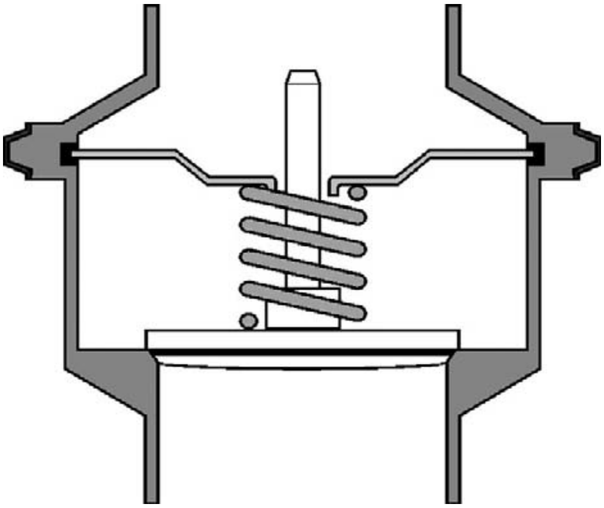

Figure 10 Check valve.

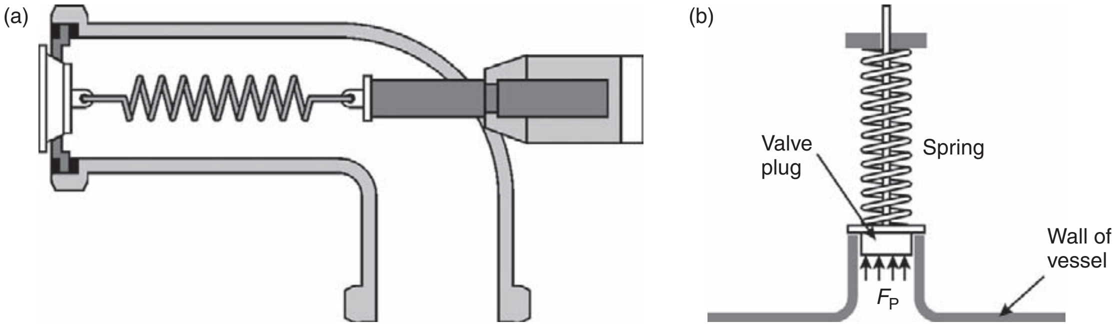

Figure 11 Safety valve.

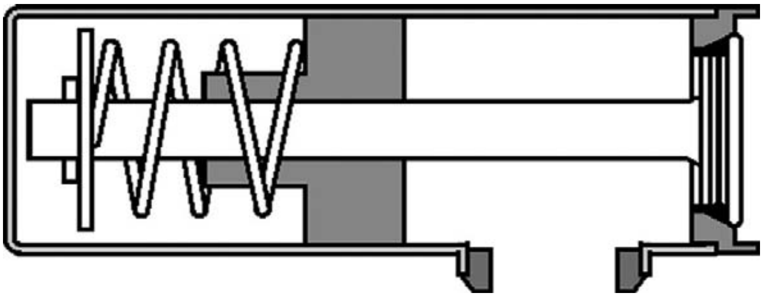

Figure 12 Air blow valve.

Air Blow Valve

Before in-place cleaning commences, the product is cleared from pipelines by use of compressed air introduced via an air blow valve (**Figure 12**).

Other valves used in the dairy industry include flow splitters, mixing valves, sampling valves, and plug cocks (for manual operation only).

See also: **Butter and Other Milk fat Products**: The Product and Its Manufacture. **Cream**: Manufacture. **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture. **Hazard Analysis and Critical Control Points**: Processing Plants. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Manufacture. **Milking Machines**: Principles and Design.
Plant and Equipment: Corrosion; Flow Equipment: Principles of Pump and Piping Calculations; Flow Equipment: Pumps; In-Place Cleaning; Instrumentation and Process Control: Process Control; Materials and Finishes for Plant and Equipment; Pasteurizers, Design and Operation; Process and Plant Design. **Utilities and**

Effluent Treatment: Compressed Air; Design and Operation of Dairy Effluent Treatment Plants; Heat Generation; Refrigeration; Water Supply.

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Agitators in Milk Processing Plants

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Introduction

There are many reasons for the agitation of milk and milk products, including

1. to maintain product uniformity,
2. to promote heat transfer, and
3. to disperse and dissolve solids in water.

However, excess agitation is to be avoided, as it can cause product damage by disrupting the fat globule membrane and exposing the fat to the lipase enzyme. Overagitation can also cause the formation of butter granules.

This article describes the types of agitators that are commonly used in the dairy industry. The mechanical and hygienic design of such systems is outlined. The issues involved in the selection and sizing of a particular agitation system are presented. Heat transfer in agitated vessels is explained, and finally an overview of the applications of agitation in milk processing is presented.

Types of Agitators

Several types of agitators are used in dairy processing and these can be broadly classified based on the viscosity of the product.

Propeller Agitators

The three-bladed marine impeller (**Figure 1(a)**) is a high-speed impeller (400–1750 rpm) with a small impeller-to-tank diameter ratio (0.2–0.3). It is used for agitating low-viscosity liquids (usually <1 Pa s) whereby the momentum generated by the impeller is easily transferred throughout the liquid. It produces an axial flow pattern (**Figure 1(b)**), which gives reasonable mixing and good suspension of particles.

Turbine Agitators

These impellers have an impeller-to-tank diameter ratio in the range of 0.2–0.5, and can be operated at high speeds if required. They are used for agitating low-viscosity liquids (usually <1 Pa s); however, they have also been applied for agitating liquids with viscosities as high as 50 Pa s. The basic turbine is a flat-blade design, and one of the most common turbines is the flat-blade impeller with six blades mounted on a disc (**Figure 2**), which is often used in fermentations. Turbine impellers can induce strong radial flows in addition to axial flows, which impart a much greater mixing capability than imparted by propeller agitators. Pitch-bladed turbines have blades set at an angle less than 90° from the horizontal (**Figure 3**) and are used in the dairy industry, for example, in lactose crystallization and yogurt manufacture. The smaller the angle, the milder the agitation, as less shear forces are exerted on the liquid and on any particle or droplet within the liquid.

Paddle Agitators

The basic paddle agitator resembles the basic turbine agitator except that it has a larger impeller-to-tank diameter ratio (>0.5 to <1) and rotates at low speeds, typically 10–150 rpm. Paddle agitators are used in the dairy industry for agitating medium-viscosity liquids (0.5–10 Pa s). Increasing the viscosity will more rapidly dampen the momentum transfer through the liquid, and thus greater contact between the impeller and the liquid is required, which results in larger-diameter impellers with greater contact area. There are many variants of the basic paddle, as illustrated in **Figure 4**, which give greater contact area. Some of these, such as the gate anchor agitator, can be used with high-viscosity liquids up to around 100 Pa s.

High-Shear Agitators

High-shear mixers are used for breaking up particles such as in powder reconstitution or emulsion droplet formation. Rotor–stator agitators (**Figure 5**) are commonly used, in which the product is drawn into a high-speed rotor, with a typical speed of 3000 rpm, positioned in a closely machined stator, in which the solids are subject to milling and intensive hydraulic shear. This may be done batchwise in a mixing vessel, or in-line for continuous blending. Powder-mixing systems are commercially available, incorporating powder hopper, venturi feeder, and in-line mixer.

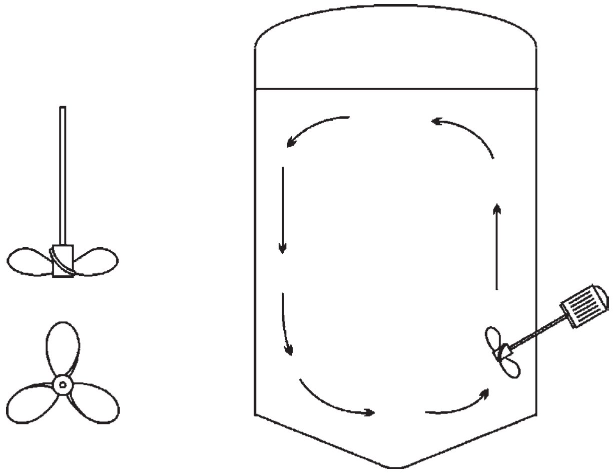

Figure 1 Three-bladed marine propeller showing typical flow pattern for side entry.

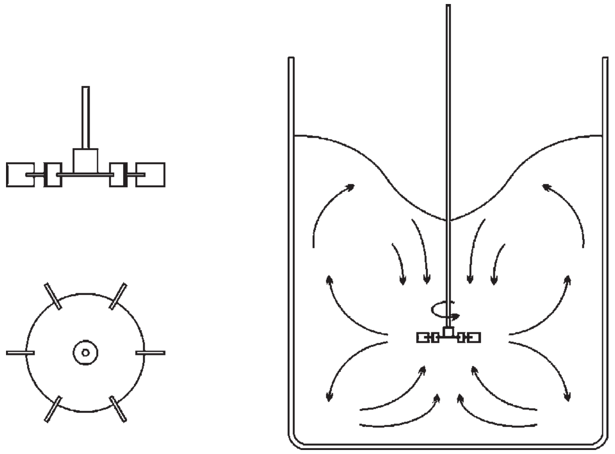

Figure 2 Six-bladed disc turbine impeller showing typical flow pattern for center axial entry.

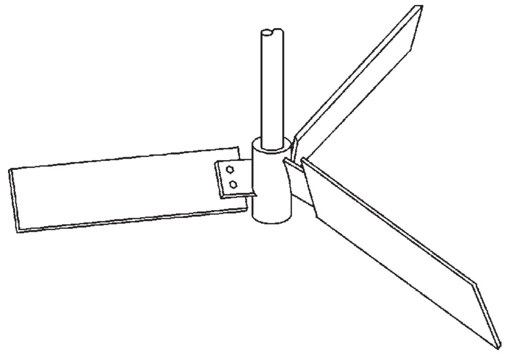

Figure 3 Pitched three-bladed impeller.

High-Viscosity Agitators

Helical and ribbon-type agitators are used for agitating very high-viscosity liquids (up to 1000 Pa s).

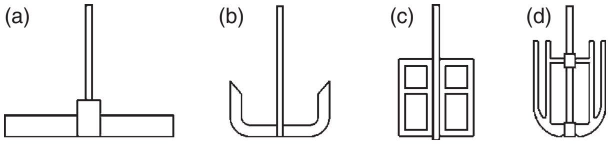

Figure 4 Paddle impellers: (a) basic paddle, (b) anchor, (c) gate, (d) anchor gate.

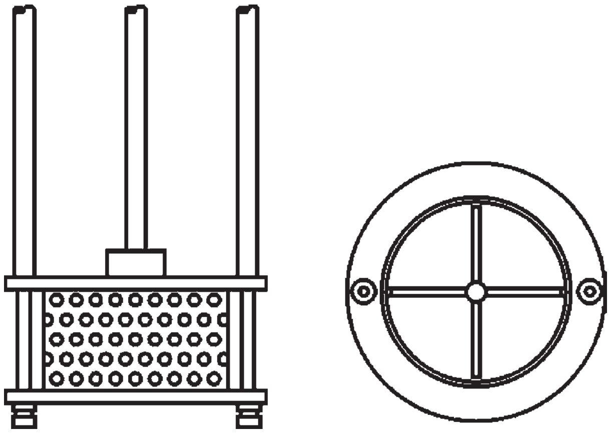

Figure 5 High-shear rotor–stator impeller.

Air Agitation

Milk in large storage tanks may be agitated by supplying compressed air near the base of the tank. The air bubbles rise, expand, and set up circulation currents in the milk. The air supply is regulated using a level detector, which matches the flow rate of air to the quantity of milk in the tank.

Mechanical Design

The agitation system consists of an electric motor transmitting power through a mechanical drive system. The drive system consists of a power transfer unit (gearbox or belt drive), shaft and impeller, bearings, and seals. **Figure 6** illustrates schematically the basic system configuration.

Electric Motor and Gearbox/Belt Drive

Electric motors rotate at an angular velocity (nominally 1500 or 3000 rpm) that is too high for the agitator impeller. Thus, a power transfer unit that allows a reduction in rotational velocity will be needed in the system, as in many cases impeller angular velocity will be less than 100 rpm. In the special case where a power transfer unit is not required, the system is referred to as direct drive. Two of the most common types of power transfer units are the gearbox and the belt drive. Gearboxes have the advantage of high torque transmission capability, high dimensional accuracy, and low friction losses, and they

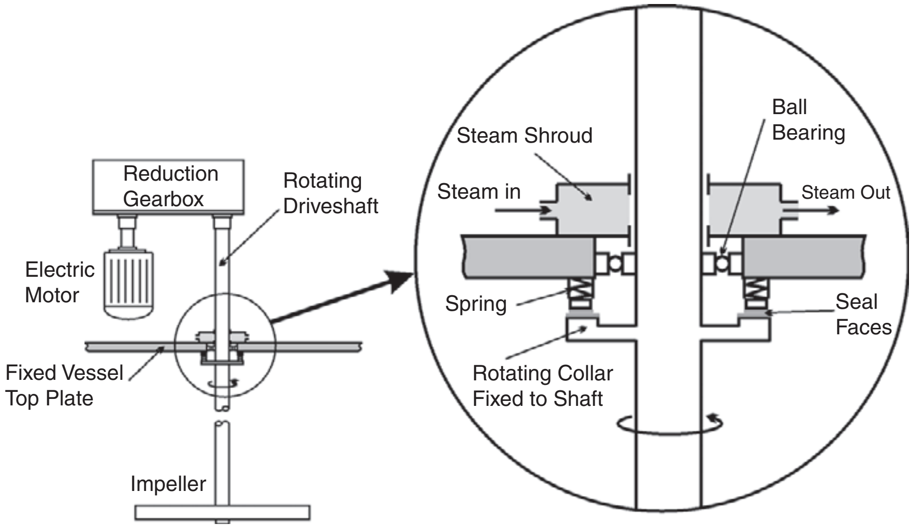

Figure 6 Drive system for top-mounted agitator.

predominate in this application. In many cases, the electric motor and gearbox will be supplied as a single unit. Belt drives are a flexible power transfer unit with the features of quiet running and good vibration damping properties. They are limited by (relatively low) operating temperatures and speeds and are less common in dairy agitation systems.

Drive Shaft

The drive shaft transmits mechanical power from the electric motor to the impeller. In tall tanks, it may be necessary to fit more than one impeller at different levels on the stirring shaft in order to obtain the required effect. The number of impellers required is approximately equal to the ratio of tank height to tank diameter. It will be necessary to support this shaft with bearings in order to minimize friction, wear, noise, deflection, and vibration. Bearings can be classified as journal (plain) bearings and rolling bearings, and it is the latter that are generally employed.

A variety of arrangements are possible to introduce the impeller shaft into the process vessel. The shaft can enter the vessel from the top (top mounted), from the bottom, or from the side. The shaft can be parallel, perpendicular, or inclined to the longitudinal axis of the vessel. Top-mounted vertical agitator drives are most common with the shaft either coaxial or eccentric (off-center) with respect to the vessel axis. Bottom or side entry may be advantageous if more space is needed on top for entry ports and manifolds, or if headspace is limited. The shaft can run the full length of the vessel and be supported by bearings at either end (top and bottom), or can terminate at the impeller and be supported by a bearing at the point of entry into the vessel.

Sealing

Satisfactory sealing of the rotating agitator shaft is essential to hygienic operation and yet difficult to achieve. As an example, in top-mounted systems, the dripping of lubricant oil from the gearbox into the tank contents is not acceptable even if a food-grade lubricant oil is specified. Any sealing arrangement must be able to resist the sterilizing temperatures applied. The most common type of dynamic seal is the axially loaded face seal (mechanical seal). Note that O rings should generally be avoided in food contact equipment.

A mechanical seal consists of a pair of rings: one stationary and one rotating with the shaft. They are spring-loaded together, and dynamic sealing takes place between the flat annular surfaces. Mechanical seals can be carbon rings (graphite) rubbing on carbon rings, or silicon carbide on silicon carbide. Silicon carbide is a ceramic material, is harder, and thus gives a longer seal life. For long-life and low-wear seals, tungsten carbide can be chosen. High-temperature, low-friction plastics such as Teflon (PTFE) are also employed. Mechanical seals with silicon carbide/carbon running surfaces are inert under normally encountered operating conditions and are considered safe in food processing. Steam barriers may also be employed to further guarantee containment.

Hygienic Design

Hygienic design of the agitator system involves consideration of issues such as materials of construction, cleanability, and surface finish. As food contact surfaces, which must remain inert, impervious, and durable, the shaft and impeller will in all likelihood be built from stainless steel. There are many grades of stainless steel but the austenitic grades AISI 304 and AISI 316 are the most common in dairy applications (*see* **Plant and Equipment**: Materials and Finishes for Plant and Equipment). Cleaning and sterilizing agents (caustic and chlorite bleaches) will generally be more dangerous to the steel than the product itself. It is for this reason that grade 316 may be preferred with its higher resistance to corrosion attack from chloride ions. This also highlights the need for careful water rinsing in the cleaning cycle to ensure that the concentration of chloride ions does not reach dangerous levels in inaccessible places such as any machine crevices.

The surface finish of the agitator affects the ability to clean, sanitize, and sterilize the shaft and impeller. Surface finish can be quantitatively measured by the value of the arithmetic mean roughness, Ra, number. Generally, such dairy product contact surfaces should have an Ra of less than 0.8 μm and such a finish is obtainable by mechanical- and electropolishing.

Agitator Selection and Sizing

Viscosity

Liquid viscosity is the resistance to flow of the liquid. Low-viscosity liquids show little resistance to flow and thus liquid momentum is easily transferred throughout the liquid and low power is required to agitate the liquid. On the other hand, high-viscosity liquids have a high resistance to flow, whereby viscous forces dampen liquid momentum transfer, and require higher power to agitate the liquid. In addition, many dairy liquids are non-Newtonian, and are mainly pseudoplastic, where viscosity decreases with increasing shear rates. This results in higher viscosities in regions of the liquid more remote from the impeller, which may lead to poor mixing in those regions. Some typical values for the viscosity of dairy liquids are presented in **Table 1** at specified temperatures, as viscosity is usually a strong function of temperature.

Agitator Selection

Selection of agitator type is determined by the viscosity of the liquid and the agitation job to be performed, whether it is mixing, heat transfer, particle dispersion, oxygen transfer, or a combination of these. Each agitator type has a range of viscosities where it performs best and an agitation duty that it is best suited to, as mentioned above. Equipment suppliers and process design companies have the experience and practical knowledge for selecting an appropriate agitator to perform a specified job.

Agitator Dimensions and Speed

Once an agitator type is selected, it then has to be sized in terms of its dimensions. This will depend on the size of the tank and the volume of the product to be processed. For a given impeller type, there are standard geometrical configurations for the impeller and tank, which give guidance to sizing the impeller. For most agitation applications with the exception of heat transfer, there is no well-developed mathematical analysis supported with property data that can be used for evaluating the effect of impeller speed and diameter on agitation performance. In these cases, it is necessary to evaluate on a small/pilot scale how impeller speed and diameter affect agitation performance and then scale up these results. Impeller tip speed is usually constrained in dairy processing because of its effect on product quality.

Agitator Power Requirement

There are many power curve correlations available in the literature for estimating the power requirement for specific impeller–tank configurations, and these are usually presented in the form of power number or function versus Reynolds number, as illustrated in **Figure 7**. Once the impeller speed and diameter, liquid density, and viscosity are known, then the Reynolds number can be calculated. The power number can be read from the power curve for the specific impeller–tank configuration, and the power requirement can be calculated from the power number or function. Separate curves are required for baffled and unbaffled systems due to possible vortex formation in unbaffled systems. As a general guide, the typical mixing power consumption varies from 0.2 kW m^{-3} for

Table 1 Typical viscosities of dairy liquids

Liquid	*Temperature (°C)*	*Viscosity (Pa s)*
Low viscosity		
Water	20	1×10^{-3}
Whole milk	20	2×10^{-3}
Skim-milk concentrate (33% solids)	25	13×10^{-3}
Medium viscosity		
Yogurt	10	1–4
Milk concentrate (48% solids)	20	1
Whey concentrate (65% solids)	10	5

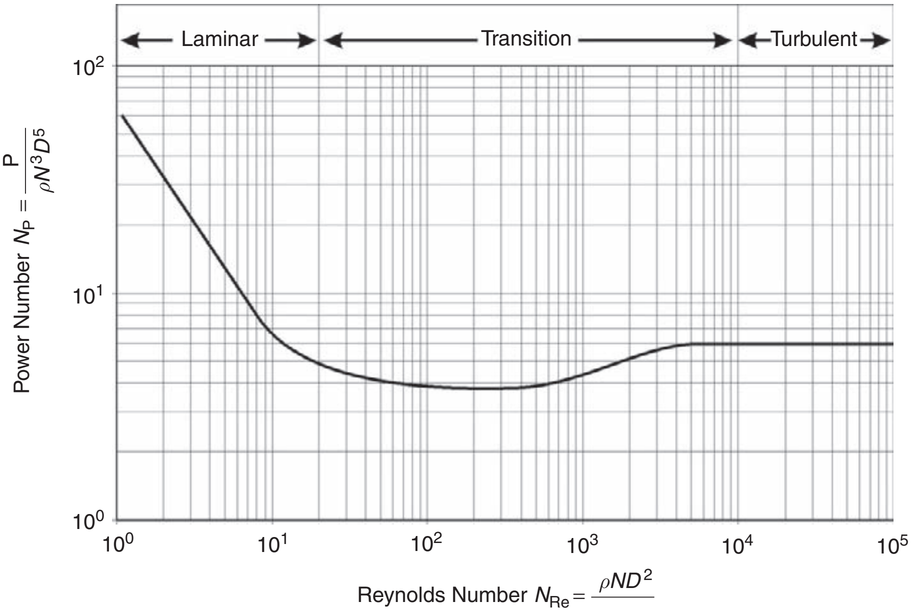

Figure 7 Power curve correlation for estimating agitator power requirement.

low-viscosity liquids up to 4 kW m^{-3} for blending materials that have the consistency of pastes and dough.

Vortexing

Tangential flow in the direction of rotation of the impeller can lead to vortex formation when agitating low-viscosity liquids by centrally mounted impellers in unbaffled tanks. Vortexing due to increasing rotational speed may lead to air entrainment by exposing the impeller to air. This can also produce large oscillating forces acting on the impeller shaft. Vortexing can be prevented by mounting the impeller off-center or using a horizontal side-entering impeller. There are cases where vortexing is advantageous, for example, powder reconstitution. When powder is added to the surface of water, the vortex can greatly improve powder sinkability by centrifugally spinning the powder into the water, and this becomes even more important as the solids content of the reconstituted mixture increases.

Heat Transfer in Agitated Vessels

Heat transfer coefficients for the heating or cooling of a liquid in a jacketed vessel may be calculated using the correlation

$$\mathrm{Nu} = a\mathrm{Re}^b\mathrm{Pr}^c\left(\frac{\mu}{\mu_\mathrm{W}}\right)^d$$

where

$\mathrm{Nu} = hD_\mathrm{T}/k$
$\mathrm{Re} = D_\mathrm{A}^2 N\rho/\mu$
$\mathrm{Pr} = C_p\mu/k$
h = heat transfer coefficient (product) (W m^{-2} K^{-1})
k = thermal conductivity (W m^{-1} K^{-1})
D_T = tank diameter (m)
D_A = agitator diameter (m)
N = rotational speed (s^{-1})
ρ = density (kg m^{-3})
C_p = specific heat (J kg^{-1} K^{-1})
μ = viscosity (Pa s)
μ_W = viscosity at wall temperature (Pa s)

Values of *a*, *b*, *c*, and *d* depend on system geometry. Heat transfer in a jacketed vessel may sometimes be problematic when dealing with medium- and high-viscosity liquids because of low heat transfer rates and formation of deposits on the tank wall. This is overcome by using paddles with small clearances between the impeller and the tank wall so that the blade surfaces sweep the wall of the tank, clearing away any deposits and preventing a stagnant layer at the wall surface.

Applications

On Farm

Following milking, milk is stored in refrigerated tanks pending milk collection and delivery to the dairy. Agitation is required for two purposes: to improve heat transfer while the milk is being cooled, and to avoid fat separation and facilitate milk sampling for analysis and payment. The typical agitation system used on farm is a paddle agitator, with an approximately 100 W motor geared down to about 30 rpm. The agitator will operate continuously during cooling and intermittently (e.g., 30 s/15 min) during storage.

Milk Intake

When milk is received at the dairy, it is stored as raw or pasteurized milk. Agitation is required at this stage to maintain uniform composition for downstream processing and inventory control. Typical agitation systems include (1) top-entry agitator with more than one pitched-blade impeller, (2) side-entry agitator, marine propeller, angled down from the horizontal, and (3) air agitation, which is less commonly used because incorporation of air in milk can lead to problems in heat exchangers and centrifuges. In modern installations, provision is made to inactivate the agitator if the product surface reaches the height of the agitator.

Cream Storage

Agitation of cream should be gentler than agitation of milk because of the greater possibility of product damage. The typical agitator used is the pitched-blade or paddle impeller, at a speed of 30–60 rpm.

Milk and Whey Concentrates

Milk concentrates are produced by evaporation for dehydration, or by the addition of solids for yogurt or ice cream manufacture. These products can be effectively agitated using a marine propeller or pitched-blade agitators at 200–400 rpm.

Powder Dispersion

The dispersion of powders in water is required for the manufacture of many dairy products such as ice cream, yogurt, dairy spreads, and dairy desserts. Complete dispersion of the solids in the aqueous phase is required, with no residual lumps and without air incorporation. Particular difficulty is encountered in dispersing stabilizers, for example, guar gum and locust bean gum, and emulsifiers, for example, mono/diglycerides. This is usually achieved using high-shear mixers.

Yogurt

Yogurt is characterized by a high solids content (about 20%) and a medium to high viscosity depending on whether the yogurt is stirred or set. Gentle agitation is required to avoid damage to product texture. This can be achieved by using a top-mounted agitator with large paddles and speeds of 20–60 rpm.

Processed Cheese

Processed cheese manufacture is characterized by high viscosity, dispersal of solids, and high heating rates. Agitation is achieved by anchor-type scraped-surface agitators, at speeds of approximately 100 rpm.

See also: **Butter and Other Milk Fat Products**: The Product and Its Manufacture. **Cream**: Manufacture. **Dehydrated Dairy Products**: Milk Powder: Types and Manufacture. **Hazard Analysis and Critical Control Points**: Processing Plants. **Ice Cream and Desserts**: Ice Cream and Frozen Desserts: Manufacture. **Milking Machines**: Principles and Design. **Plant and Equipment**: Corrosion; In-Place Cleaning; Materials and Finishes for Plant and Equipment; Process and Plant Design. **Utilities and Effluent Treatment**: Design and Operation of Dairy Effluent Treatment Plants.

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Centrifuges and Separators: Types and Design

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Introduction

Since ancient times it is known that milk tends to separate into cream and skim milk. In static systems this was just a matter of time. During the nineteenth century centrifugal separation was developed for milk skimming before it was introduced to other technologies.

Milk separators enabled a very quick skimming of the milk. The first machines were manually driven with a crank handle and mainly used on farms. For increasing the efficiency the original spiral channels were replaced by conical disks. The development of industrial dairies demanded higher capacities, and the machines were equipped with electric motors. Another important step forward was the introduction of self-discharging bowls in the 1970s, which enabled automatic cleaning or cleaning in place (CIP).

The Basics of Centrifugal Separation

Centrifugal seperation can be applied on two-phase systems composed of either solid particles dispersed in a liquid or liquid droplets emulsified in another liquid. The individual particles or droplets form a discontinuous phase in the continuous liquid phase. If the density of the particle or droplet is higher than that of the liquid, it will sink down (sedimentation), otherwise it will float up (creaming), as depicted in **Figure 1**.

The velocity of separation is described by Stokes' law:

$$V_\mathrm{s} = \frac{d^2 \cdot \Delta\rho \cdot g}{18 \cdot \eta}$$

where V_s is the velocity of sedimentation, d the diameter of particle or droplet, $\Delta\rho$ the difference of densities, g the acceleration due to gravity, and η the viscosity of the continuous phase.

This equation shows that the velocity of sedimentation is

- proportional to the difference of densities
- proportional to the square of the size of the particle or droplet
- inversely proportional to the viscosity of the continuous phase
- proportional to the acceleration due to gravity

Another important figure is the separating distance. In a slim and tall vat a particle or droplet has to travel a longer distance and needs more time to separate than in a wide flat pan of the same volume.

Density and particle or droplet size are product parameters that generally have to be taken as constant values. However, other parameters can be influenced to optimize the process.

- Usually increasing temperature reduces the viscosity and thus increases the sedimentation speed. Therefore, dairy separators are integrated in a pasteurizing process mostly at temperatures of 50–60 °C.
- Centrifugal acceleration in a separator is determined by the bowl diameter and the rotational speed. It can be 8000 times higher than the acceleration due to gravity.
- Separating distance can be minimized by the geometrical design of the flow path. In modern separators the space between the disks has been reduced to 0.3–0.6 mm.

The increase of the separation effect in a centrifuge is described by the acceleration factor ξ. This figure indicates the centrifugal acceleration as a multiple of the gravitational acceleration.

$$\xi = \frac{\omega^2 \cdot r}{g}$$

where ξ is the acceleration factor, ω the angular speed ($\omega = 2 \cdot \pi \cdot n \ \mathrm{rad\,min^{-1}} = \frac{2 \cdot \pi \cdot n}{60} \ \mathrm{rad\,s^{-1}}$, where n is the bowl speed in rpm), r the radius, and g the acceleration due to gravity (9.806 65 m s^{-2}).

As the acceleration factor is proportional to the radius of the centrifuge, the centrifugal acceleration is not constant but increases outwardly and decreases inwardly, as sketched in **Figure 2**.

In a static system the gravitational acceleration forces the heavy phase (i.e., solids) downward and the light phase (i.e., cream) upward. In a rotating centrifuge the heavy phase moves outward, and the light phase inward.

Separation in the Disk Stack

The feed flow is distributed to the rising channels and then split into a large number of parallel flows in between the conical disks. From here the liquid splits into

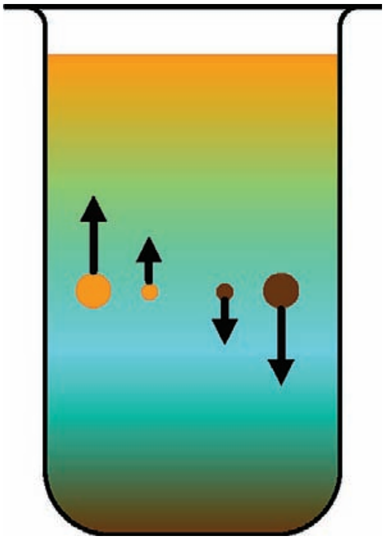

Figure 1 Creaming and sedimentation.

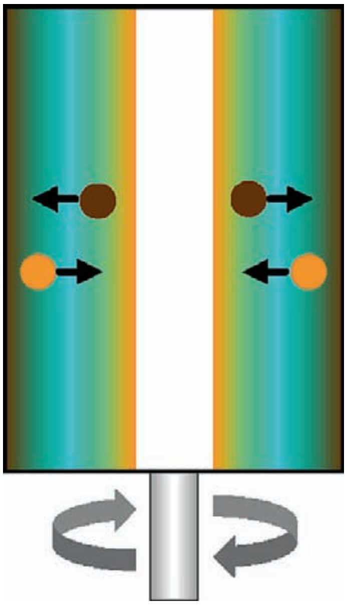

Figure 2 Centrifugation.

the light phase that flows inward and the heavy phase that flows outward.

A light fat droplet carried over to the heavy phase will move inward from the main stream until it hits the surface of the disk below. Here the wall speed of the heavy phase flow is close to zero, and the buoyant force is able to move the droplet inward against the main flow until it merges with the light phase. In the light phase the inverse process separates heavy particles and carries them back outward to the heavy phase, as depicted in **Figure 3**. **Figure 4** shows a typical disk stack.

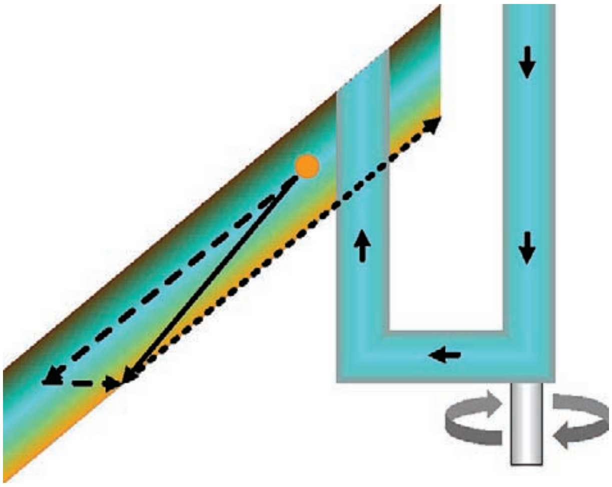

Figure 3 Separation in disk stack.

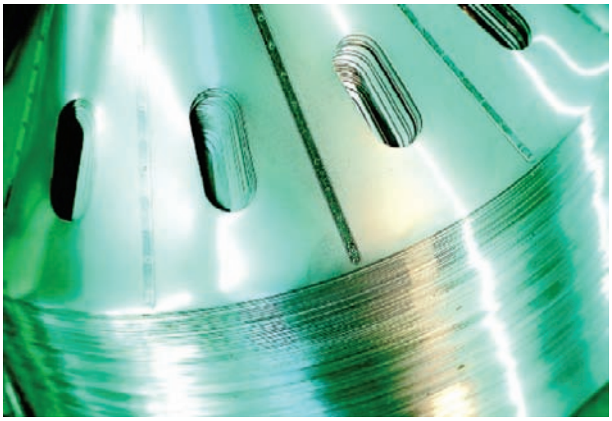

Figure 4 Separator disk stack.

Design Features

Separators

The most common type of centrifuge in dairy installations is the separator, which is a vertical centrifuge with a stack of conical disks. The size of the flow channels between the disks is defined by spacers welded onto the disks.

Phases

There are two main types of separators. Two-phase separators (**Figure 5**) provide a solid–liquid separation (clarification), whereas three-phase separators (**Figure 6**) deal mainly with liquid–liquid separation (skimming). As dairy products always contain a certain amount of solids, such as raw milk impurities or coagulated proteins, in skimming separators there is also a third phase of heavy solids.

With regard to the bowl components, the significant difference between two- and three-phase separators is the separating disk. In three-phase separators the heavy liquid

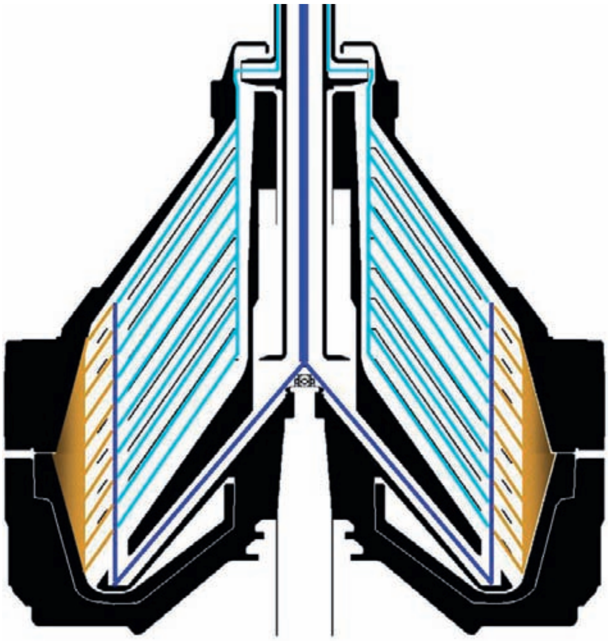

Figure 5 Two-phase bowl (clarification).

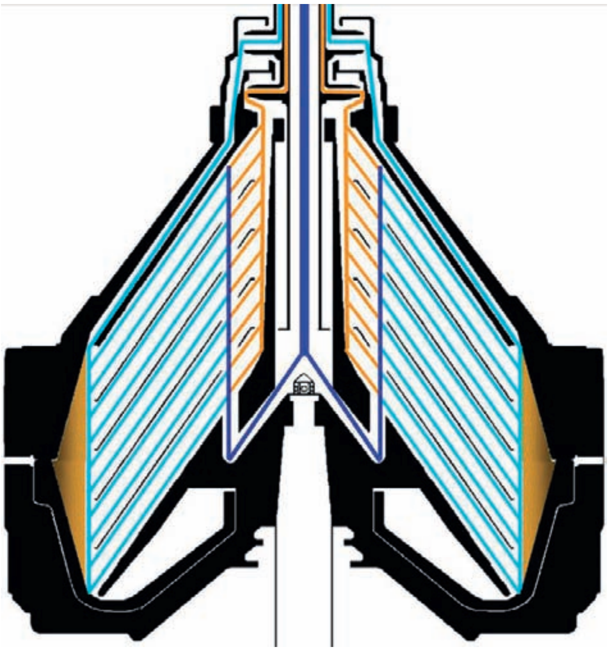

Figure 6 Three-phase bowl (skimming).

phase moves outward in the disk stack. It is redirected and flows inward on top of the separating disk to get to the discharge near the center of the bowl.

Sealing

Basically, there are two different types of sealing for the feed and discharge connections. Closed bowls use mechanical seals (hermetic sealing), whereas 'open' bowls are equipped with hydraulic sealings (hydrohermetic sealing). In terms of air intake there are no significant differences between the two types.

On open bowls the feed and the discharge are usually connected from the top. Due to the additional space requirement of the mechanical seals, the product supply to a closed bowl is preferably connected from below via a hollow spindle.

On hermetic separators the mechanical seals are fragile components, and they are prone to wear and tear.

An open-bowl separator generates the discharge pressures from the separator drive. On a closed-bowl separator the discharge pressure is largely created by the feed pump, so the machine drive can be smaller, but a bigger feed pump is required.

Discharge

In the original and the most simple design the liquid phases horizontally overflow from the bowl into collecting channels in the hood. The disadvantages of an open-discharge system are the generation of foam and the need for collecting vats for the discharged liquids. Nowadays, only very small separators feature this type of discharge.

Centripetal pumps are fixed devices that use the rotary speed of the liquids to create pressure and forward flow in a closed-discharge system. This system avoids foaming and air intake, and it enables the integration of the separator in a closed-pipe system.

The equivalent counterpart to a fixed centripetal pump in open bowls is a rotating impeller on a closed bowl.

For some applications, a continuous discharge of major quantities of solids is required, for example, for the production of fresh cheese. Nozzle bowls are equipped with a collar of calibrated nozzles on the circumference of the bowl. To avoid an accumulation of solids between the nozzles, the inside of the bowl has a special shape with conical pockets directing the sedimented solids to the nozzles.

Rising channels

In all cases the product is fed into a distributor that distributes it underneath the disk stack to a number of rising channels. The position (radius) of the rising channels determines the relation between the flow paths of the light and the heavy phases.

Channel positions close to the center result in long flow paths for the heavy phase and short flow paths for the light phase. Therefore, there is an improved removal of the light phase from the heavy phase, whereas the removal of the heavy phase from the light phase is less efficient. This is the typical configuration for skimming separators where the efficiency is defined by the residual fat content in the skimmed milk.

On the other hand, a clarifier needs long flow paths for the light phase to remove as much of the (heavy) solids as possible from the (light) liquid. Therefore, the rising channels are positioned further to the outside of the disk stack.

In some cases it is favorable to have no rising channels and the product rises completely outside of the disk stack.

Drive

The development of the drive system has proceeded from gear drive (**Figure 7**) via flat belt drive (**Figure 8**) to direct drive (**Figure 9**). With fewer changes of direction, the mechanical efficiency factor increases and the noise level reduces. The number of serviceable parts is reduced significantly as well.

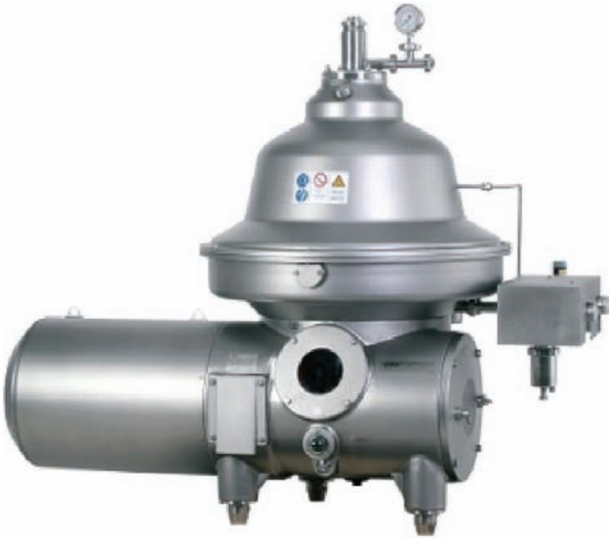

Figure 7 Gear drive separator.

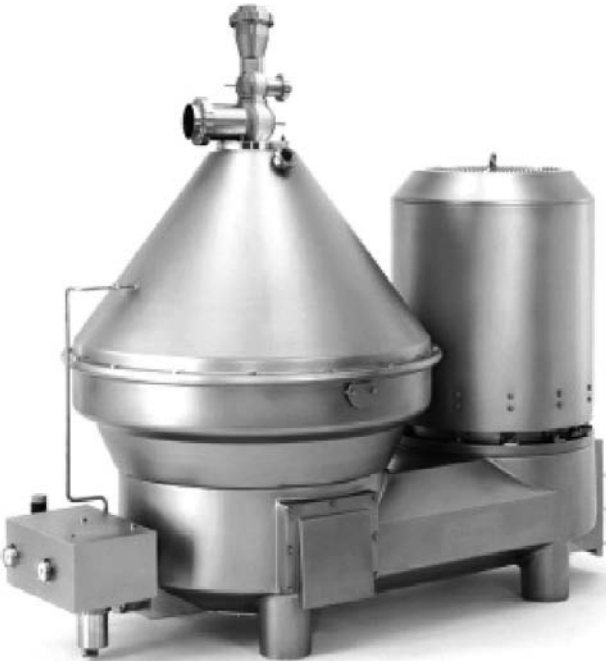

Figure 8 Belt drive separator.

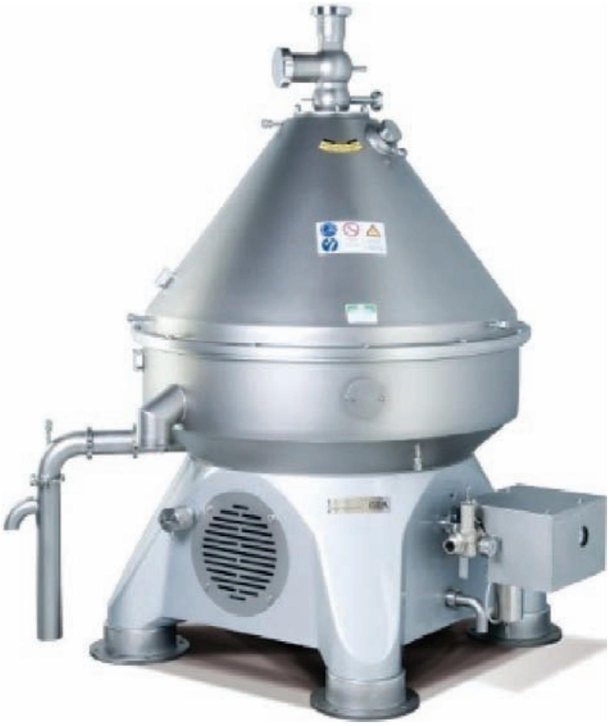

Figure 9 Direct drive separator.

A limiting factor for the rotary speed is the tensile strength of the bowl material. The admissible bowl speed depends on the bowl diameter and varies within a range of ~4800 rpm (>Ø 800 mm) to 11 000 rpm (<Ø 300 mm).

Hydraulic system

Until the 1970s all separators had solid wall bowls. They could be operated until the solids holding space was filled up with sediments. After production, they had to be completely dismantled and cleaned manually (‘takedown machines’).

One of the most important steps in the development of separators was the introduction of self-discharging bowls. A hydraulic system closes and opens the solids holding space at the bowl perimeter. During production this is used to perform regular discharges of the solids sediment. This enables long production runs without being limited by the solids load.

Furthermore, these machines are suitable for automatic cleaning or CIP. Discharges during the recirculation of cleaning solutions remove sedimented solids from the bowl, and at the same time the sudden high velocities in the disk stack effect a mechanical cleaning. Besides the cleaning of the machine itself, the integration of a separator in a CIP loop also provides for cleaning of the CIP solutions.

The discharged quantity can be controlled to achieve partial discharges during production and total discharges during CIP. A partial discharge ejects a quantity equivalent to approximately the volume of the solids holding space. This can be done without any interruption of the process.

A total discharge, however, ejects the complete contents of the bowl. The loss of rotating fluid means a loss of kinetic energy and slowing down of the rotary speed. In order to limit the drop of speed, the feed flow has to be closed during total discharges and has to be kept closed during a recovery time after the discharge.

Decanters

Unlike separators, decanters have a horizontal axis of rotation (**Figure 10**). The continuous discharge of sedimented solids is performed by a scroll rotating at a differential speed in relation to the bowl.

Generally, decanters are used for the removal of higher solid loads or to achieve a higher dry matter content in the discharged solids. In the dairy industry decanters are used only for a limited range of special applications.

Phases

Similar to separators, horizontal centrifuges are available for two-phase or three-phase separations. In dairy applications only two-phase decanters are used.

Discharge

The liquid discharge can be executed as an open discharge (**Figure 11**) or as a closed discharge with a centripetal pump (**Figure 12**). Depending on the further use of the liquid phase, both versions are used in dairies.

GEA
Westfalia Separator

Figure 10 Decanter.

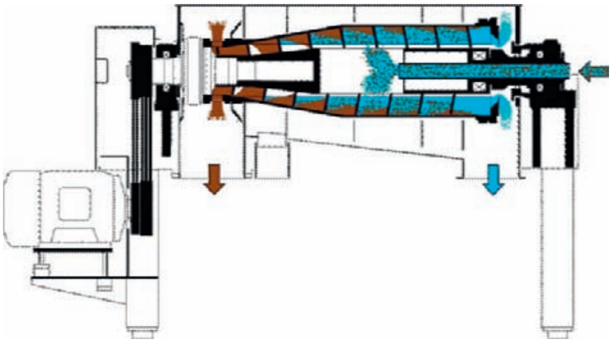

Figure 11 Decanter with open discharge.

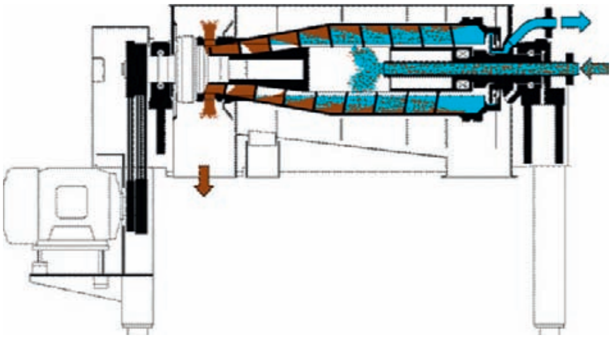

Figure 12 Decanter with centripetal pump.

Drive

A wide range of drive systems is available for decanters. The main classification is into fixed differential speed (selected by exchangeable pulleys, as shown in **Figure 13**) and variable differential speed (**Figure 14**).

The selection of the drive system is determined mainly by the solids load. If the quantity or quality of the solids is variable, the machine has to cope with it by a variable differential speed.

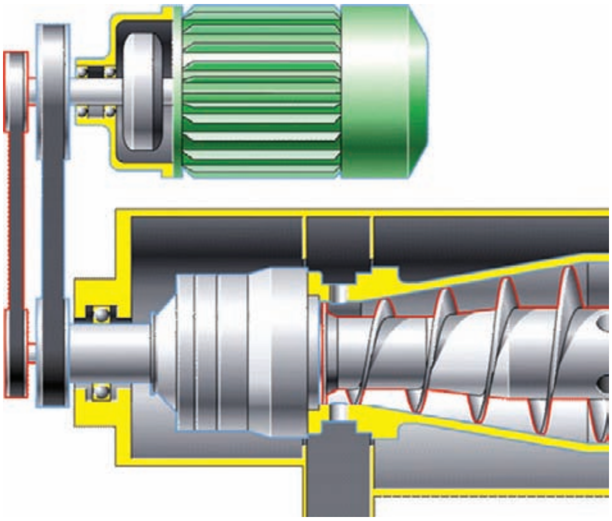

Figure 13 Decanter drive for fixed differential speed.

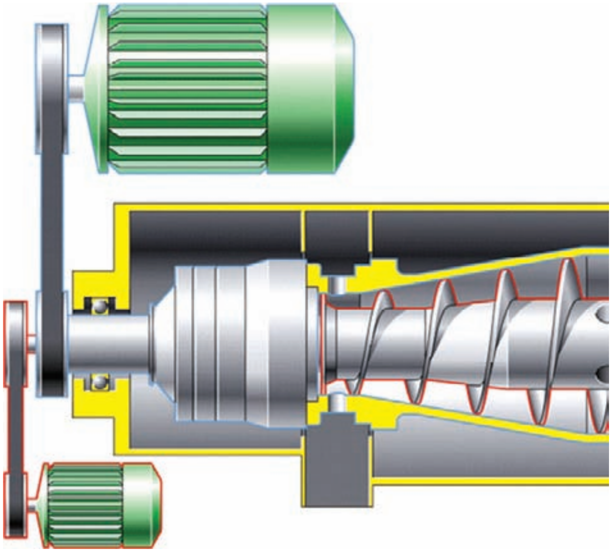

Figure 14 Decanter drive for variable differential speed.

Centrifuges for Dairy Applications

Skimming

Hot milk skimming

Milk skimming is still the most common and most important application for separators today. Worldwide, dairy legislation stipulates pasteurization of raw milk, and in all pasteurizers an important unit is the separator, which is integrated into the process at a temperature of 50–60 °C.

There are three modes of operation in milk skimming: full skimming (production of skimmed milk and cream), standardizing (production of standardized milk and cream), and back blending (production of whole milk). In all these modes, apart from skimming, the separator also cleans the milk from solid dirt.

According to the mass balance, the cream fat content is controlled by adjusting the cream flow rate:

$$\dot{V}_c = \dot{V}_r \cdot \frac{F_r - F_s}{F_c - F_s}$$

where $\dot{V}_c$ is the cream flow rate, $\dot{V}_r$ the raw milk feed flow rate, F_r the raw milk fat content, F_s the skim milk fat content, and F_c the cream fat content.

In the standardizing mode there is no requirement for a high skimming efficiency, so the skimming separator can be operated at a higher capacity than that is possible in the full-skimming mode.

Cold milk skimming

In some cases it is desirable to do the skimming at cold temperatures of 4–15 °C. Here, thermal changes are avoided and no heating energy is required at that stage of the process.

The skimming efficiency achieved is less than that achievable with hot milk skimming, and the milk fat is more crystalline than at hot temperatures. Therefore, the thickness of the flow paths has to be considerably larger. Also viscosities of the cream and skimmed milk are higher.

To increase efficiency and/or capacity, the temperature can be gently increased to ~20 °C.

Whey skimming

Compared to milk (4%), the fat content of whey (0.4%) is much lower. Whey originates mostly from cheesemaking, which is a batch operation; so the operating conditions cannot be kept constant as in a milk pasteurizing line.

Cheese whey always contains a certain amount of cheese fines. As a third phase, these particles tend to obstruct the separation process and can cause fouling of the disk surface. For a good skimming efficiency, a proper preclarification is indispensable. Best results are obtained with a clarifying separator rather than by screening.

Standardizing

Many dairy processes require standardized milk with a specified fat content. The blending of skimmed milk and cream can be done batchwise or continuously, applying different levels of automation.

For tank standardizing, the pasteurizer toggles between whole-milk and skimmed-milk production and both qualities are mixed in the right proportion in the pasteurized milk tank. This method is applied in manually operated plants.

Continuous standardizing is possible by splitting the cream flow and blending a part of the cream with the skimmed milk. Systems of different automation levels, from manual to fully automatic, are available.

Volumetric standardizing systems imply a constant raw milk fat content; they should be recalibrated after a raw milk tank change.

Modern high-end systems like the GEA Westfalia Separator ®standomat continuously measure the fat content of the cream, so that they can compensate for variations in the raw milk composition. The control unit can be equipped with additional features like a continuous density/protein measurement for fat in dry matter or fat/protein standardizing, or proportional dosing of extra cream or other additives.

The size of the cream discharge determines a maximum cream flow rate, which corresponds to a minimum cream fat content. The production of low-fat cream is possible with a cream dilution feature in the standardizing unit. **Figure 15** shows a typical standardizing unit.

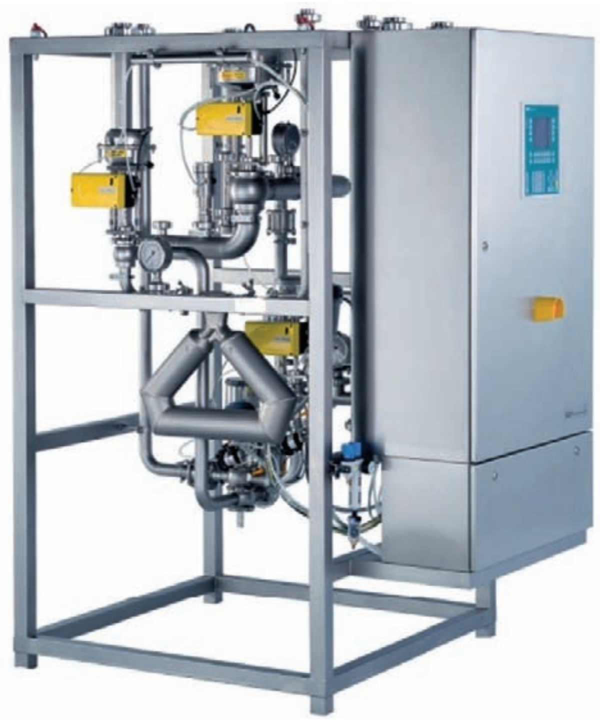

Figure 15 Standardizing unit.

Butter oil

For the production of butter oil or anhydrous milk fat (AMF) skimming-type separators are used in different ways.

- Cream concentration: The cream fat content is increased from 40 to ~80%.
- Oil concentration: After a phase inversion from oil-in-water to water-in-oil in a high-pressure homogenizer, the fat content can be further increased to over 99%.
- Oil polishing: Addition of water enables the elimination of concomitant substances.
- Serum skimming: This is the recovery of the remaining fat in the heavy phases discharged from the abovementioned stages.

Clarification

In the dairy industry clarifiers are mostly used for the cleaning of raw milk at an early stage of processing or for the clarification of whey before a whey skimming separator. Recovered cheese fines can be further dewheyed in a decanter and then be used for the production of processed cheese.

Bacteria Removal

Spore-forming bacteria include *Bacillus* (aerobic) and *Clostridium* (anaerobic) species. The spores of these species are dormant bodies that carry all the genetic material as is found in the vegetative form, but do not have an active metabolism. They are much more resistant against heat, dryness, and other negative ambient conditions than the vegetative form, so they act as a mean of survival during hard times. When the environmental conditions turn favorable, spores germinate to vegetative *Bacillus* or *Clostridium* cells.

The concentration of spores in milk varies with the conditions of feeding and milking. Wherever the cows are fed with silage in the winter season, there is an important peak in spore count during that time. Owing to the heat resistance of the spores, this imposes specific limitations on the quality of pasteurized milk. On the other hand, spores have a higher dry matter content and a higher density than vegetative cells, so they are easier to remove by centrifugal separation.

Removal of bacteria from liquid milk or from cheese milk is usually done in a pasteurizing line immediately before or immediately after the skimming separator. Besides the spores, vegetative bacterial cells and somatic cells from the cow's udder are also removed.

There are two different separation principles that can be applied. Most manufacturers use special clarifying separators to accumulate spores and heavy cells in the solids holding space before they are ejected from the bowl at regular intervals.

In contrast to this, GEA Westfalia Separator uses a skimming-type bowl where a small quantity of the heavy phase enriched with respect to bacteria is recirculated to the product feed. This heavy-phase flow acts as a carrier to promote the deposition of the spores and bacteria to the solids holding space and increases efficiency.

Fresh Cheese Production

Fresh cheese

Pasteurized cheese milk (skimmed or up to 3% fat) is coagulated by fermentation with or without the addition of rennet. Heating steps before and after coagulation serve to bond the whey proteins to the casein and to increase the yield of coagulated protein.

The coagulated protein is separated at 30–50 °C as heavy phase and continuously discharged via the nozzles on the perimeter of the bowl (**Figure 16**).

The discharged product quantity is a constant value; therefore, the dry matter of the product is controlled according to the mass balance by adjusting the feed flow.

Figure 17 shows a schematic diagram of a fresh cheese bowl.

Double-cream fresh cheese

Enriched cheese milk of 8–12% fat content is pasteurized and homogenized before it is coagulated by fermentation. The pretreatment aggregates the protein with the fat, so

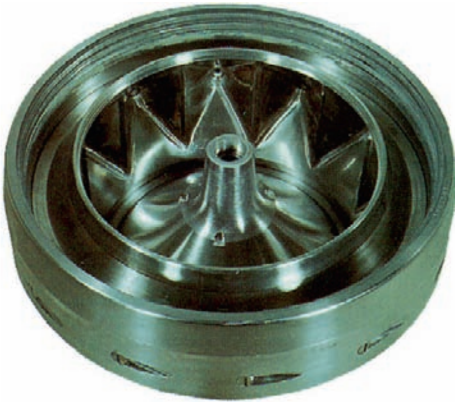

Figure 16 Inside shape of a nozzle bowl.

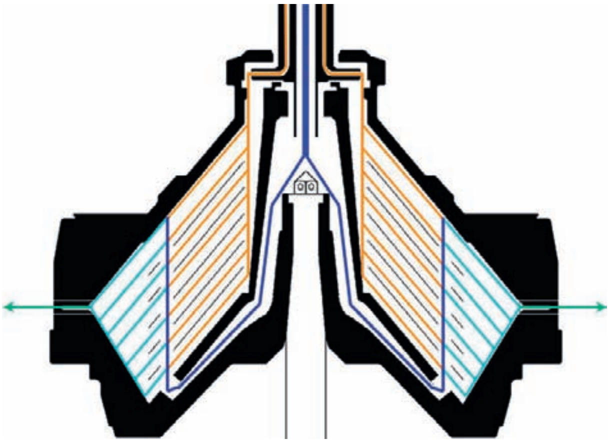

Figure 17 Bowl of fresh cheese separator.

the coagulate constitutes a lighter phase when compared to whey.

The separation is performed at ~80 °C in a specially designed skimming separator that can handle the high viscosity of the cheese.

The fat-in-dry matter content is determined by the properties of the cheese milk. The dry matter is controlled during separation by adjusting the discharged whey flow.

A schematic of a typical double-cream fresh cheese bowl is shown in **Figure 18**.

Decanter

Decanters are used in some dairy applications where high dry matter content of the solids phase is required:

- concentration of recovered cheese fines
- production of lactose
- production of casein
- production of high dry matter fresh cheese

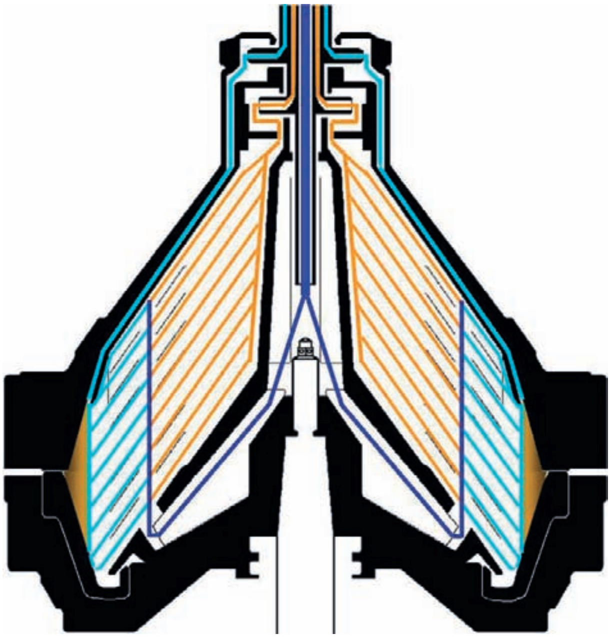

Figure 18 Bowl of double-cream fresh cheese separator.

Process Conditions

Satisfactory results are achieved with proper process conditions.

- The discharge pressure determines the (vertical) product level in the bowl. The adjustment of pressure to an appropriate value is fundamental for the functioning of a separator.
- The process control has to provide constant conditions with regard to temperatures, flow rates, and pressures.
- Any shearing during the pretreatment of the product tends to reduce the size of fragile particles or droplets and can reduce separation efficiency.
- An increased air content in the milk, owing to, for example, an inappropriate design of the balance tank, can change the flow pattern in the disk stack and reduce efficiency. However, a certain amount of released air has to be accepted if air-saturated milk is heated from storage temperature to separation temperature.

See also: **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry.

Further Reading

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Centrifuges and Separators: Applications in the Dairy Industry

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Introduction

Centrifugal separation is the mechanical fractionation of a fluid mixture of two or more immiscible phases of differing densities by the imposition of a centrifugal acceleration field. Density differences of not less than 3% are required for effective fractionation at respectable throughputs.

In the context of the dairy industry, the term centrifugal separation comprises centrifugation and cyclone separation. Centrifugation means separation in centrifuges with power-driven rotating bowls (rotating-boundary machines). Cyclone separation means separation in cyclone separators, in which the mixture to be fractionated, but no part of the machine, rotates; cyclones are stationary-boundary machines. In both cases, it is the rotation of the mixture that generates the centrifugal acceleration field required for fractionation.

In dairy processing, the following types of mixtures can successfully be separated centrifugally:

- Liquid–liquid (e.g., cream and skim milk)
- Liquid–solids (e.g., whey and casein curd)
- Gas–liquid (e.g., water vapor and heat concentrated milk)
- Gas–solids (e.g., dryer outlet air and milk powder)
- Liquid–liquid–solids (e.g., cream, skim milk, and suspended insoluble matter)

Rotation causes separation because, on a per unit volume basis, the higher the density of a component the greater is the centripetal (center-seeking) force that would have to be applied to prevent radial movement away from the axis of rotation. This is a consequence of Newton's first law of motion.

Centrifugal (center-fleeing) force, unlike centripetal force, is a fictitious force when, as is commonly the case, the Earth, rather than the rotating entity, is taken as the inertial frame of reference. However, it is a real force if the rotating entity itself (e.g., a centrifuge bowl) is taken as the inertial frame of reference. In a given case, centripetal force and centrifugal force are numerically equal, but opposite in sign. Centrifugal force is a concept useful in the analysis of a rotating system.

Consider the combined separation and clarification of whole milk in a centrifuge. In this context, whole milk can be considered to comprise milk plasma as a continuous phase and milk fat globules and insoluble particles (impurities) as dispersed phases. The centripetal force required to keep the whole of the centrifuge bowl contents rotating is supplied by the bowl wall. However, no centripetal force acts on the individual insoluble particles or fat globules, because these are free to move in the plasma. The insoluble particles, being denser than the milk plasma, accelerate towards the bowl wall, while the fat globules, being less dense than the milk plasma, are displaced (and decelerate) towards the bowl axis.

The following expression for the speed of movement of a dispersed particle (or globule) at a given radius can be derived by taking the rotating centrifuge bowl as the inertial frame of reference and then equating the centrifugal force to the sum of the two opposing forces – the buoyancy force (caused by the displacement by the particle of a volume of plasma equal to the particle's volume) and the drag force (caused by the movement of the particle through the plasma):

$$v = \frac{d^2 \Delta\rho \left(\frac{\pi N}{30}\right)^2 R}{18\mu} \qquad [1]$$

where v is the (radial) separating velocity (m s^{-1}), d the particle diameter (m), $\Delta\rho$ the density difference between the particle or fat globule and the continuous phase (plasma) (kg m^{-3}), N the bowl rotational speed (rpm), R the radial distance from the axis of rotation (m), and μ the viscosity of the continuous phase (plasma) (Pa s).

The net centrifugal force (centrifugal force minus buoyancy force) can be positive or negative, depending on whether the particle is denser or less dense than the continuous phase, but is independent of velocity. The drag force, on the other hand, is directly proportional to velocity (Stoke's law). Thus, the greater the density difference $\Delta\rho$ (eqn [1]), the greater the net centrifugal force and the higher the velocity at which this force comes into balance with the drag force. It is this phenomenon that makes centrifugal separation possible; phases differing in density, because they move radially at different velocities in the centrifugal acceleration field, inevitably become separated as the feed mixture flows through the centrifuge or cyclone separator.

$\Delta\rho$ and thus v are positive for the insoluble particles in milk (positive net centrifugal force), but negative for fat globules (negative net centrifugal force). The sign of velocity indicates the direction of radial movement during separation. As eqn [1] shows, the separating velocity, in either radial direction, increases with distance, *R*, from the bowl axis.

The dairy separation operations listed above could be achieved using gravitational sedimentation or filtration or both; eqn [1] applies to gravitational separation when the centrifugal acceleration term $\left(\frac{\pi N}{30}\right)^2 R$ is replaced by the gravitational acceleration, *g*. However, the rotational speed of a centrifuge bowl (which is limited only by the bowl's ability to withstand the centrifugal stress of its contents and its own mass) can be high enough to make centrifugal acceleration thousands of times greater than *g*, resulting in rapid separation.

Centrifugal separation is thus far superior to gravitational separation in terms of effectiveness and throughput, and is also superior in terms of controllability, hygienic operation, compactness, and ease of integration into continuous processing systems. In the case of centrifugation, these advantages outweigh the high capital cost and high power consumption of centrifuges.

Even though raw milk is routinely in-line filtered to remove impurities with large particle sizes, filtration is generally unsuitable for more demanding solids–liquid separation duties in the dairy industry owing to low efficiency, low throughput, hygiene risks, and other disadvantages. Filtration is, however, effective for solids–gas separation, and is used, for example, for recovering powder particles from dryer outlet air.

The main types of centrifugal separators used in the dairy industry are the disk bowl centrifuge, the decanter centrifuge, the scroll-screen centrifuge, and the cyclone separator. Applications of each of these are discussed in turn, with particular emphasis on separating conditions, significant features of machine design, separation control, and separating efficiency.

Applications of Disk Bowl Centrifuges

Whole Milk Separation

The classical, and still the most important and commonest, application of the disk bowl separator is the separation of whole milk into cream and skim milk. This operation is an early stage in the manufacture of most dairy products.

The development of the modern dairy industry began with the introduction of the centrifugal separator, first on individual farms and later at centralized creameries to which farmers brought their milk. Its use enabled the efficient concentration of milk fat, then considered the only milk component of significant value, into the cream stream while minimizing fat loss to the skim milk. The replacement of gravitational separation by centrifugal separation resulted in enormous increases in throughput and efficiency. The first hand-driven disk bowl separators began to be used on farms in 1893.

The capacity of the modern milk separator is around $60\,000\,\mathrm{l\,h^{-1}}$ for warm separation (52–55 °C) and $50\,000\,\mathrm{l\,h^{-1}}$ for cold separation (4–10 °C). Fine control of cream fat content is achieved by manual or automatic adjustment of the pressure balance across the separator's rotating bowl. Usually, the back pressure at the skim milk outlet is kept constant automatically, and cream fat content is controlled by manipulating the back pressure at the cream outlet either manually or automatically. Cream fat content can be adjusted to any desired value up to about 50% without compromising separating efficiency.

Separating efficiency, usually called skimming efficiency, is defined either as the percentage of the fat in the whole milk that is separated into the cream stream, or simply as the skim milk fat content. The former definition can be expressed as follows:

$$E = \left(1 - \frac{f_\mathrm{s}}{f_\mathrm{w}}\right) \times 100\% \qquad [2]$$

where E is the skimming efficiency, f_s the skim milk fat content (% w/w), and f_w the whole milk fat content (% w/w).

Equation [2] is derived from a fat mass balance across the separator that incorporates a pragmatic simplification based on the fact that the skim milk fat content is very much lower than that of the whole milk.

Separators designed for warm separation, and separators designed for cold separation operating at 20 °C, have efficiencies of about 99% (skim milk fat content = 0.05% w/w). In cold separation, efficiency drops with temperature to about 50% (skim milk fat content = 2% w/w) at 5 °C if the cold milk separator is operated at its full nominal throughput. The efficiency can be increased to about 94% or more by reducing throughput by half to increase the residence time of the milk in the bowl. This increase compensates for lower fat globule separating velocities, which are a consequence of $\Delta\rho$ decreasing and μ increasing with decreasing temperature (eqn [1]). It is not possible even in warm separation to recover fat globules of less than about 1 μm in diameter because of their very low separating velocities.

Separators designed for warm separation can be used for cold separation at temperatures as low as 20–25 °C, without an excessive drop in efficiency, provided creams of no more than 15% fat are required.

The bowls of dedicated cold milk separators are designed to cope with the very much higher cream viscosity, while avoiding churning of the cream and maximizing skimming efficiency. Cold separation, though not as efficient as warm separation, is useful in some circumstances, and is being more widely applied. Its advantages include better control of the product's microbiological quality, improved cream quality (e.g., improved whippability), improved quality of unpasteurized milk cheese, and greater flexibility in the processing of chilled milk on receipt from farms. Separators that can be used for either warm or cold separation are now available, allowing further improvements in flexibility.

Separating temperatures above 55 °C give negligible improvements in efficiency. Temperatures above 60 °C result in a loss of efficiency owing to the deposition of denatured whey protein on to the separator's disks (which disrupts the smooth two-way flow pattern in the interdisk spaces) and a drastic reduction in the tendency of fat globules to agglomerate; agglomeration is a useful tendency given the d^2 term in eqn [1], and should be preserved.

Skimming efficiency is adversely affected by a number of factors besides temperature. As fat globule separating velocity is directly proportional to the square of globule diameter (eqn [1]), pipelines should be designed to avoid damage to fat globules and fat globule agglomerates, and flow rates should be controlled by variable speed drives on pumps rather than by pump throttling. The natural decrease in fat globule size that occurs during lactation, and therefore during the dairy season in countries such as New Zealand and Ireland, can be compensated for by gradually decreasing separator throughput over time.

Whole milk to be separated should be deaerated, both to avoid fat globule damage and to avoid loss of efficiency caused directly by the presence of air in the separator itself. Air, being very much less dense than either skim milk or cream, is displaced rapidly to the inner edge of the disk stack, obstructing the flow of cream and in effect throttling the cream outlet. This causes an increase in the fat content and thus in the viscosity of the cream, increasing the throttling effect further. This vicious circle results in excessive fat loss to the skim milk.

Milk should ideally be separated when it is fresh, as prolonged storage results in changes in the fat globule membrane that increase fat globule density. This results in a decrease in $\Delta\rho$ (eqn [1]), in turn decreasing fat globule separating velocity and thus skimming efficiency.

Modern milk separators, which also act as clarifiers (see below) if the whole milk has not been purposely clarified prior to separation, are self-desludging. Sludge, comprising leukocytes, somatic cells, microbial cells and spores, blood and dirt particles, and some milk solids (mostly protein) are ejected at preset intervals. At least one separator manufacturer has introduced a separator bowl modification that almost eliminates loss of milk protein to the sludge. This increases protein yield and reduces sludge production, and reduces water usage by allowing longer intervals between desludges.

Equation [1] shows that separator bowl rotational speed, N, has a large influence on separating velocity, and thus on skimming efficiency; separators should be run at the bowl speed recommended by the manufacturer.

Protein streams or concentrated skim milk should not be blended with whole milk prior to separation as this results in an increase in milk plasma viscosity, and thus a reduction in skimming efficiency (eqn [1]). However, badly separated skim milk with an excessive fat content can be blended with the whole milk. This results in an improved overall fat recovery from the blend owing to agglomeration of smaller fat globules.

Partial Homogenization of Milk

So-called partial homogenization, mainly applied to retail milk, involves temporarily separating whole milk into skim milk and cream, homogenizing the cream, and then remixing the homogenized cream with the skim milk. Only the cream passes through the homogenizer, significantly reducing the homogenizer's power consumption, which is directly proportional to volumetric throughput; this is the purpose of the process. As stabilization of the homogenized cream emulsion depends on the presence of at least 0.2 g casein g^{-1} fat, the cream fat content should not exceed 12%. The economics of partial homogenization depend upon the cost of power, and the capital and operating costs of the centrifugal separator and homogenizer.

Milk and Cream Standardization

Milk and cream often require standardization of their fat and/or solids-not-fat (SNF) contents for retail sale or, in the case of milk, prior to the manufacture of products such as whole milk powder and cheese. In small-scale dairy processing, standardization can be carried out batchwise by blending whole milk with either cream or skim milk in appropriate proportions. In large-scale operations, so-called direct standardization is used.

The first step in direct standardization is centrifugal separation of the whole milk into skim milk and cream. A typical basic system works as follows: The back pressure at the skim milk outlet of the separator is kept constant automatically. The back pressure at the cream outlet is controlled automatically, using continuous in-line temperature-compensated sensing of cream density (inversely related to fat content) and cream flow rate, to give a fat-standardized cream. Part of the cream flow and the skim milk flow are remixed (by means of automatic flow rate ratio control using in-line measurements of cream and skim milk flow rates) in proportions that result in a reblended whole milk of the desired standardized fat content. Surplus cream leaves the system as fat-standardized cream. A variation of this system is one in which the fat contents of the standardized cream and the remixed standardized whole milk are controlled solely on the basis of in-line measurements of density. The accuracy of these systems is unaffected by variations in the flow rate, fat content, or temperature of the whole milk.

The capability of simple systems can be extended in a number of ways:

- In-line measurement of skim milk density allows fluctuations in the SNF content of the feed milk, which cause density fluctuations unrelated to fat content, to be automatically compensated for. It also allows the fat to SNF ratios of both milk and cream, as well as milk and cream fat contents, to be standardized.
- Separator size can be reduced by causing part of the feed whole milk flow to bypass the separator, and to be blended in-line with the remixed standardized cream and skim milk from the separator.
- Part of the skim milk flow from the separator can be made to bypass the skim milk–standardized cream remixing point and rejoin the remixed stream downstream of the homogenizer in the remix line, thus allowing partial homogenization as well as standardization.
- High-fat standardized milk can be produced by taking off part of the skim milk flow upstream of the cream–skim milk remixing point. Alternatively, cream from a separate source can be remixed with the skim milk along with standardized cream from the separator.
- Other additive streams, such as concentrated skim milk and whey permeate, can be added to the skim milk from the separator along with the standardized cream, depending on standardization requirements.

As skimming efficiency is of less importance in standardization than it is in separation *per se*, higher separator throughputs can be used in direct standardization systems.

Milk Clarification

Self-desludging disk bowl clarifiers are used to remove solid impurities, especially leukocytes and bacterial cells and spores, from (usually raw) whole milk. *Listeria* cells enveloped by leukocytes, and therefore heat resistant, are removed by clarification, while those not so enveloped are easily killed by subsequent pasteurization.

The clarifying efficiency of clarifiers is some 150% higher than that of separators, owing to the greater path length and thus longer residence time of the milk in the disk stack. Clarifiers operate at higher throughputs than separators do, and at low (<8 °C) or high temperatures (50–60 °C). Lower temperatures result in less loss of milk protein, while higher temperatures result in a greater reduction in total microbial count and allow complete removal of hair and other fibers, which are common contaminants of milk. The optimum temperature range is 50–55 °C. As a clarifier has only one outlet, and flow in the bowl is from the outside of the disk stack to the inside, no fat globule separation takes place. The sludge can amount to 0.05–0.1% of the milk volume, and contains 14–16% dry matter comprising nitrogen (6–8%), fat (0.25–0.35%), lactose (~4.7%), and nonmilk substances (1.5–3%).

Bacterial Clarification of Milk

When a substantial reduction of the bacterial contamination of milk is required, but without the use of severe heat treatment, purposely designed high rotational speed clarifiers (called ‘bactofuges’ by one separator manufacturer) are used for bacterial clarification prior to pasteurization.

Bacterial clarification of standardized cheese milk is carried out mainly to remove spores of a number of (anaerobic) *Clostridium* species, especially *Clostridium tyrobutyricum*, which otherwise cause late fermentation-induced gas production (late blowing) in semihard cheese. Their removal enables the use of spore-inhibiting substances such as nitrate to be greatly reduced or eliminated. The numbers of wild lactobacilli are also lowered, resulting in less competition for starter cultures in the manufacture of raw milk cheese. Clarification at 65 °C removes 98–99% of anaerobic spores and 95% of aerobic spores (e.g., those of *Bacillus cereus*). Clarification at 50 °C removes 90–92% of lactobacilli.

Bacterial clarification of cheese milk is particularly useful in Europe where cows are fed silage during winter; the spore content of milk is directly related to the extent of silage feeding and to the spore content of the silage.

Bacterial clarification of consumer milk prior to pasteurization can result in a useful extension, of 3–5 days, in pasteurized milk’s shelf life. It has become more important in regions such as northern Europe where consumers are purchasing pasteurized milk less frequently (but expect it to remain of high quality even after some days of storage) and distribution lines have become longer owing to increasing centralization of milk-processing facilities. Clarification at 50 °C reduces the total bacterial count by 86%. The removal of *B. cereus* spores is of particular significance in this application, as these spores survive pasteurization. The best results are achieved if the milk to be clarified is first separated into cream of 43% fat (to ensure a large density difference between cream and spores) and skim milk. Only the skim milk, which contains virtually all of the spores originally present in the feed milk, is clarified, before being standardized by cream remixing.

Bacterial clarification can also be applied to milk and whey for the purpose of improving the bacteriological quality of low-heat milk powders and whey protein concentrate powders, respectively; to milk to be made into fresh cheese to remove ascospores of the molds

Byssochlamys nivea and *Byssochlamys fulva*; and to skim milk destined for casein manufacture.

Two-stage bacterial clarification, using two machines in series, must be employed if one stage is inadequate.

The design of bacterial clarifiers varies. The simplest machines separate the milk into a clarified milk stream and either a bacteria-rich milk stream or a sludge stream. In a somewhat more complex design, the milk is separated into a clarified milk stream, a bacteria-rich milk stream, and a sludge stream. In the case of cheese milk, the sludge, the bacteria-rich stream, or both can be UHT sterilized and recombined with the clarified milk. This reduces or obviates loss of milk solids. In another design, used where the total quantity of bacteria-rich material removed from the milk must be minimized, a flow of partially clarified milk (3–5% of the feed flow rate) is continuously recycled from the outer part of the bowl to the bowl feed. This changes flow conditions in the bowl in a way that increases the efficiency of separation of the smallest bacteria, and reduces the amount of sludge produced. Sludge is the only stream removed from the milk in this design.

Cream Processing

The main products made from milk fat are butter and anhydrous milk fat (AMF). Centrifugal separation of whole milk to concentrate the fat in cream is the first significant processing step in each case.

Butter making

Butter can be made in four ways:

- traditional batch churning of 25–35% fat cream produced by a single separation step
- Fritz continuous churning of 40–41% fat cream, also produced by a single separation step
- continuous phase inversion of 75–82% fat cream. Milk is first separated to give 40% fat cream, which is then itself separated in a cream concentrator (a specialized disk bowl separator) to give cream of the required high fat content
- blending fresh AMF made using the direct-from-cream process (see below) with cream and salt, or with water, milk solids, and salt. The separation steps required in making AMF are described in the following section

Anhydrous milk fat manufacture

AMF can be made from cream or butter. The direct-from-cream process involves the following main separation operations using disk bowl separators: separation of whole milk to give 40% fat cream, concentration of this cream to $\geq$75% fat, concentration of the butteroil phase after phase inversion of the concentrated cream, and polishing of the butteroil phase after in-line wash water addition and perhaps neutralization of free fatty acids. Fat recovery is carried out by centrifugal separation of the heavy phase (secondary skim milk/buttermilk) from the cream concentrator. The heavy phase from the butteroil concentrator can be either recycled to the cream concentrator or centrifugally separated to yield β-serum (a phospholipid-rich heavy phase) and a light fat-containing phase (that is recycled).

In the manufacture of AMF from sweet cream butter, the melted butter is centrifugally concentrated and then polished in disk bowl separators prior to dehydration. If cultured butter is used, it may be necessary to separate the melted butter in a three-phase decanter centrifuge (see below) because of the butter's high solids content (which is mainly due to the generation of biomass during cream culturing). The decanter separates the melted butter into butteroil, buttermilk, and solids.

Buttermilk separation

Buttermilk has a fat content of 0.5–2.5%, which economically justifies separation to recover fat. As cream for buttermaking is pasteurized at temperatures high enough to denature whey proteins, it may be necessary to centrifugally clarify buttermilk prior to separation to avoid rapid fouling of the separator's disks. For separating cultured cream buttermilk, the feed rate to the separator should be about 50% of that for milk separation in the same machine, with frequent desludging if prior clarification has not been carried out. Separation is carried out at 40–45 °C for sweet buttermilk and at 35 °C for cultured cream buttermilk to avoid excessive protein precipitation. The cream produced contains about 25% fat.

Fresh Cheese

The large-scale production of fresh soft cheeses is made possible by the use of specialized disk bowl machines for separating curd and whey after coagulation. In the case of nonfat or low-fat cheeses made from fresh and recombined whole and skim milks, and buttermilk, the whey is discharged via a centripetal pump (paring disk), while the denser curd is discharged continuously via nozzles at the periphery of the bowl. In the case of double cream cheese, the whey and the less dense curd are both discharged via centripetal pumps.

Whey Processing

Cheese whey contain curd fines and fat, which can be removed by centrifugal clarification and separation. Such removal is essential where the whey is to be used as an ingredient of, for example, clear drink products, and where it is to be used as a feedstock for the manufacture of whey protein concentrate, whey powder, or lactose.

As the fines tend to block the disk spaces of a separator, it is necessary to clarify the whey prior to separation.

(One separator manufacturer used to offer a specialized clarifier separator whose disk stack had a lower preclarifying section and an upper separating section.)

Casein whey and casein wash water contain casein fines, which can be recovered with disk bowl clarifiers and recycled, thus increasing the casein yield.

Cheese fines recovered as sludge from whey clarifiers and separators can be put through a casein solubilization process. Undissolved denatured casein particles and other impurities are removed from the solution by centrifugal clarification.

It can be economically viable to separate whey with a fat content higher than about 0.045% during heat concentration. This is typically done between evaporator stages 2 and 3. Difficulties can be encountered owing to the precipitation of whey protein and adventitious homogenization of fat globules.

The dephospholipidation of whey, carried out in the production of whey protein concentrates with very low fat contents, involves treating the whey to cause the lipoproteins to flocculate and then removing the flocculates as sludge by means of centrifugal clarification. The clarifier used has a high rotational speed and nozzle discharge of the sludge, giving a simultaneous bacterial clarification effect. Dephospholipidation improves the performance of ultrafiltration plants and, as a significant proportion of the calcium phosphate in the whey is also separated, allows longer evaporator running times.

The fat content of whey protein concentrates made from whey that was itself separated can be reduced by 20–30% by centrifugal separation under optimal conditions. Separator throughput must be about half that of the machine when separating milk, because of the higher viscosity of retentate.

Whey can be purposely bacterially clarified, after the initial clarification to remove fines, to reduce the growth of bacteria in ultrafiltration plants, and to enable the production of whey powder suitable for use in baby food. Self-desludging nozzle discharge clarifiers are required in this case.

In the basic lactalbumin process, whey is first centrifugally clarified. It is then treated to cause denaturation and precipitation of whey proteins, which are centrifugally recovered as sludge from a second clarifier. This sludge is mixed with water, and the washed protein solids are recovered as sludge (lactalbumin) from a third clarifier. The dry solids content of the lactalbumin is increased from about 16 to 34–35% in a decanter centrifuge (see below) prior to drying.

Calcium phosphate is separated from whey permeate to avoid problems caused by precipitated calcium phosphate in evaporator plants and to minimize the salt content of products made from the permeate. Adjustment of pH to 6.6 and suitable heat treatment cause the formation of calcium phosphate flocculates, which can be separated as sludge using a self-desludging centrifugal clarifier. The sludge from cultured whey that is not pH adjusted is extremely compact and can be continuously discharged from the clarifier via nozzles.

Nozzle discharge disk bowl clarifiers are used to separate yeast from fermented serum in the production of ethanol from whey.

Other Applications of the Disk Bowl Separator

The use of centrifugal clarifiers to remove cheese solids from brine in the manufacture of brined cheese can sometimes be justified economically. Frequent cleaning in place is required because of the deposition of crystalline fat on the separator's disks at the low brine temperature, and the parts of the machine coming into contact with brine must be made of corrosion-resistant materials.

Applications of Decanter Centrifuges

Centrifugal decanters (also called conveyor bowl centrifuges) are used in solids–liquid separation duties where the solids concentration in the feed and the particle size of the solids are too high to allow the use of disk bowl separators, and where a high dry matter content is required in the separated solids phase. The principal dairy industry applications of the decanter occur in casein and lactose manufacture. These and other applications are described briefly below. The use of decanters in lactalbumin manufacture and in the manufacture of AMF from sour-cream butter has been mentioned above.

Casein

Decanters are commonly used to dewhey casein curd, the curd–whey mixture being first put through a dewheying screen to reduce the hydraulic load on the machine. After washing, the curd can be dewatered using decanters. In this operation, the aim is to obtain as dry a curd as possible prior to drying.

The scroll-screen (also called the worm-screen) centrifuge is used as an alternative to the decanter for dewatering. It consists of a perforated bowl of truncated-cone shape with an internal corotating helical-flight screw (like that in the decanter). The mixture to be separated is fed to the inside of the narrow end of the bowl, and the solids are conveyed to the wide end during separation. As it approaches the wide end, the layer of solids becomes looser owing to the increasing diameter, and efficient further washing can be achieved by means of water sprays.

Lactose

Decanters are used to separate crystals of so-called edible lactose from the crystal–mother liquor mixture resulting from the crystallization of whey or whey permeate. Two-stage decantation is used in which wash water is added to the solids phase from the first decanter. In the manufacture of refined lactose from edible lactose, decanters are used to recover the lactose after recrystallization.

Concentration of Cheese Fines

The fines in the sludge from disk bowl cheese whey clarifiers and separators can be concentrated to 30–50% dry matter by decanting the sludge after dilution to 8–12% dry matter with water and whey. The liquid phase is discharged by means of a centripetal pump.

Effluent

The sludge from the flotation and sedimentation steps in the treatment of dairy wastewaters can be concentrated in decanters before disposal, reducing disposal costs.

Decanter Operating Variables

Decanter operating variables are the radial location of the liquid discharge (which determines the thickness or depth of the layer of the separating mixture that rotates with the bowl), the rotational speed of the bowl, the differential speed (the difference between the rotational speeds of the bowl and the screw conveyor), and the feed rate. Liquid discharge at a larger radius gives a dryer solids phase, but increases fines losses, while discharge at a smaller radius has the opposite effects. Thus, in casein dewatering, for example, there must be a trade-off between the dryness of the curd and fines losses to the wash water, although, as mentioned previously, fines can be recovered from wash water (and whey) by means of disk bowl clarifiers.

The rotational speed of the bowl must be higher for smaller solids phase particles and/or for a smaller density difference between the particles and the liquid phase.

Differential speed must be matched to the rate at which solids are fed to the bowl, to avoid a solids buildup inside the bowl.

Feed rate must be matched to the bowl speed, a higher feed rate requiring a higher bowl speed to maintain separating efficiency. The lowest possible bowl speed should be used, to minimize machine wear and power consumption.

Cyclone Separators

Cyclone separators (used for solids–gas, solids–liquid, and gas–liquid separation tasks), in contrast to centrifuges, are mechanically simple; relatively cheap to purchase, install, and run; are compact compared with noncentrifugal (gravity) separators; and require little maintenance.

Cyclone separators are used in the dairy industry for

- gas–solids separation, mainly for separating product fines from the air leaving spray dryers and fluidized bed dryers, both to increase yield and to minimize air pollution. This is the commonest application of cyclones.
- gas–liquid separation, mainly for the in-line deaeration of milk (essential for maintaining the efficiency of downstream centrifugal separators), and for separating the concentrate leaving an effect of a multiple-effect evaporator from the water vapor (steam) evaporated from the product in the effect. In the latter application, clean separation prevents bubbles of steam being retained in the concentrate (ultimately diluting the concentrate when they condense on cooling) and carryover of concentrate into the steam side of the next effect, or into the condenser.
- solids–liquid separation, one application of which is separating solids from waste streams. Solids–liquid cyclone separators are called hydrocyclones.

Cyclone design is shown in **Figure 1**. The principles of design and operation can be understood by considering fines recovery from dryer air.

The cyclone is a vertical body with a cylindrical upper part and a conical lower part. The solids–air mixture enters tangentially at the top of the cylinder. The linear motion of the mixture is converted to rotational motion by the flow of the air around the curved wall of the cyclone; the cyclone wall provides the centripetal force required to make the air rotate.

As soon as the air starts rotating, the fines particles, which are free to move relative to the air, move toward the wall, as there is no centripetal force being applied to them to keep them in orbits about the cyclone's axis. When they hit the wall, they are separated from the air, and slide to the bottom of the cyclone under the influence of gravity. They are removed via a rotary valve.

The centrifugal effect is thus produced by the shape of the cyclone itself, the way the mixture to be separated enters the cyclone, and the velocity of the mixture (which is generated by a fan in the case of dryer air, or by a pump, or by a pressure difference).

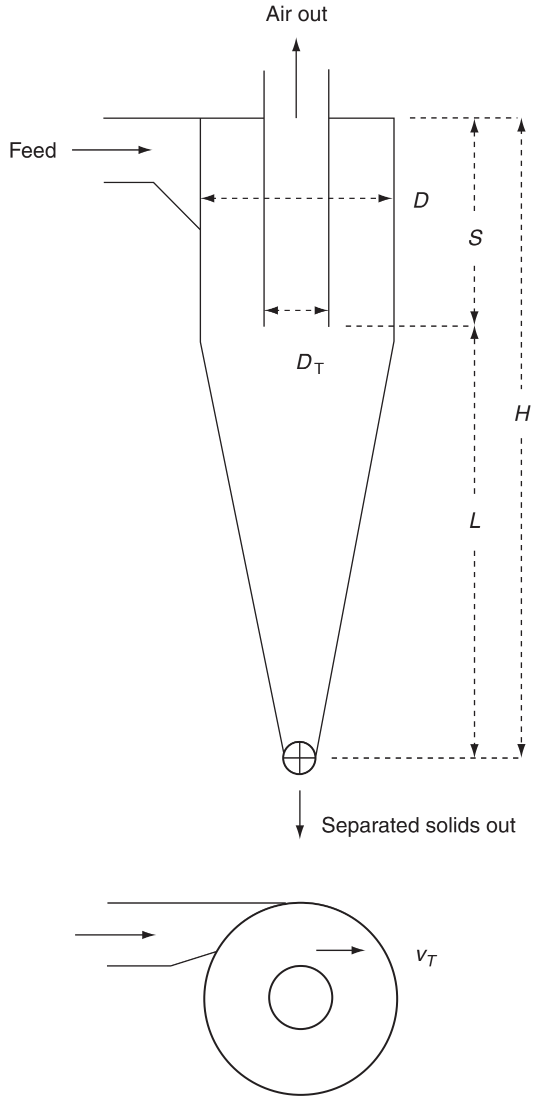

Figure 1 Elevation and plan views of a cyclone separator for recovering solids from an airstream.

Separation of the particles in the way described above is not instantaneous; a sufficiently long residence time is attained by the way the air flows through the cyclone. The air moves in a spiral path down the cylindrical part and then the conical part of the cyclone, then flows inward toward the cyclone's axis, and finally upward into a coaxial tube by which it leaves at the top of the cyclone.

Separating Efficiency

The solids (e.g., milk powder particles) in the solids–air mixture entering a cyclone invariably exhibit a particle size distribution; particle size ranges from very small to relatively large. Separating efficiency can be expressed in terms of the diameter of the limit particle, the smallest particle that can successfully be separated. A simplified analysis of the flow pattern inside the separator, which incorporates eqn [1], leads to the following expression for the limit diameter:

$$d_{\mathrm{L}} = \sqrt{\frac{18\,\eta\,Q}{v_{\mathrm{T}}^{2}(\rho_{\mathrm{particle}} - \rho_{\mathrm{air}})2\pi L}} \qquad [3]$$

where d_{L} is the limit diameter (m), η the viscosity of the continuous phase (Pa s), Q the volumetric feed rate ($\mathrm{m}^3\,\mathrm{s}^{-1}$), v_{T} the maximum tangential velocity of the particle ($\mathrm{m\,s}^{-1}$), ρ_{particle} the density of the particle ($\mathrm{kg\,m}^{-3}$), and ρ_{air} the density of the continuous phase ($\mathrm{kg\,m}^{-3}$).

The maximum tangential velocity occurs at the radial position $D_{\mathrm{T}}/2$ (**Figure 1**).

Equation [3] shows that it is possible to separate even very small particles in a cyclone if the entry velocity, v_{T}, is high; if the cyclone is long (tall); and if it is possible to distribute the flow of feed over a number of smaller cyclones rather than a big one (because d_{L} is directly related to volumetric flow rate, Q). Though the value of the recovered solids (e.g., milk powder fines) has to be balanced against the capital and operating costs of a battery of cyclones, air pollution control may be an overriding factor.

In practice, it is often found that the exhaust air contains a small percentage of particles with diameters larger than the limit diameter. The reason for this is probably that small particles impinge on larger ones and push them into the exhaust air.

On the other hand, a considerable proportion – often more than 50% – of particles with diameters lower than the limit value are successfully separated in the cyclone. These are particles that have reached the vicinity of the wall shortly after entering the cyclone. From there they are carried down and out by a secondary stream of air produced by frictional forces at the wall.

The following geometrical relationships (see **Figure 1**) have favorable effects on cyclone efficiency:

$$\frac{D}{D_{\mathrm{T}}} = 2 \text{ to } 25;\ \frac{H}{D_{\mathrm{T}}} = 7 \text{ to } 8;\ \frac{S}{D_{\mathrm{T}}} = 0.8 \text{ to } 1.3$$

As a general rule, the diameter of the exhaust pipe (D_{T}) should be as small as possible, and the entry velocity of the feed mixture as high as possible.

The separating efficiency of cyclones falls off dramatically in solids–air separation for particles below about 10 µm in diameter. For this reason, cyclone exhaust streams may need to be bag filtered to ensure that the emission standards aimed at protecting the environment are not exceeded. Cyclones may be entirely replaced by bag filters in milk powder manufacture.

Conclusion

It is clear from the foregoing that the modern dairy industry could not exist without centrifugal separation. Indeed, as intimated in the introduction, it could never have become established. Arguably, centrifugal separation is the most important unit operation in dairy processing, and the disk bowl centrifuge the single most important item of processing equipment.

Centrifuge manufacturers, who are extremely competitive, are continuously improving the performance of their already highly sophisticated machines, by clever design and control innovations. They also put much effort into designing and improving dairy processes in which their machines can be used. It does not seem that the limits of this advanced separation technology, and its application in the manufacture of dairy products, have yet been reached.

See also: **Cheese**: Avoidance of Gas Blowing. **Plant and Equipment**: Centrifuges and Separators: Types and Design. **Standardization of Fat and Protein Content**.

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Heat Exchangers

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Introduction

One of the most important requirements of modern dairying is to be able to control the temperature of products at every stage in the process. Heating and cooling are therefore very common operations in the dairy.

Heating

Milk is heated by a heating medium such as low-pressure steam (seldom used nowadays) or hot water, so that the temperature of the product rises and that of the water drops correspondingly (*see* **Utilities and Effluent Treatment**: Heat Generation).

Cooling

On arrival at the dairy, the milk is cooled to 5 °C or lower, to limit microbiological growth. After pasteurization, the milk is cooled to about 4 °C using chilled water, brine solution, or an alcohol solution such as propylene glycol.

Regenerative Heating and Cooling

In many processes, a product must first be heated and then cooled. During pasteurization, milk is heated from, perhaps, 4 °C to a pasteurization temperature of 72 °C, held at that temperature for 15 s, and then chilled again to 4 °C. The heat of the pasteurized milk is utilized to warm the cold milk. The process takes place in a heat exchanger and is called regenerative heat exchange or, more commonly, heat recovery. As much as 94–95% of the heat content of the pasteurized milk can be recycled.

Heat Transfer

Theory

Heat can be transferred in three ways: conduction, convection, and radiation.

1. Conduction means transfer of thermal energy through solid bodies or through layers of liquid at rest (without physical flow or mixing in the direction of heat transfer).
2. Convection occurs when fluids (gases or liquids) being heated or cooled develop internal mixing currents, either by the temperature-density effect itself (natural convection) or by mechanical agitation or turbulent flow (forced convection).
3. Radiation is the emission of heat from a body that has accumulated thermal energy. The thermal energy is converted to radiant energy, emitted from the body, and absorbed by other bodies, which it strikes. Almost all substances emit radiant energy.

Principles

Heat transfer in dairies occurs by convection and conduction. Two principles are used: direct and indirect heating.

Direct heating

Direct heating means that the heating medium is mixed with the product. This technique is used

- to heat water (steam is injected directly into the water and transfers heat to the water);
- to heat products such as curd in the manufacture of certain types of cheese (by mixing hot water with the curd); and
- to sterilize milk or milk products by steam injection or infusion of the product into steam.

The direct method of heat transfer is efficient for rapid heating. It offers certain advantages, especially in the production of long-life, ultraheat-treated (UHT) milk, where heating is followed by rapid flash-cooling. It does, however, involve mixing of the product with the heating medium, which necessitates certain steps in the subsequent process. It also makes strict demands on the quality of the steam. Direct heating is forbidden by law in some countries on the grounds that it introduces foreign matter into the product.

Indirect heating

Indirect heat transfer is therefore the most commonly used method in dairies. In this method, heat is transferred from the heating medium through a partition into the

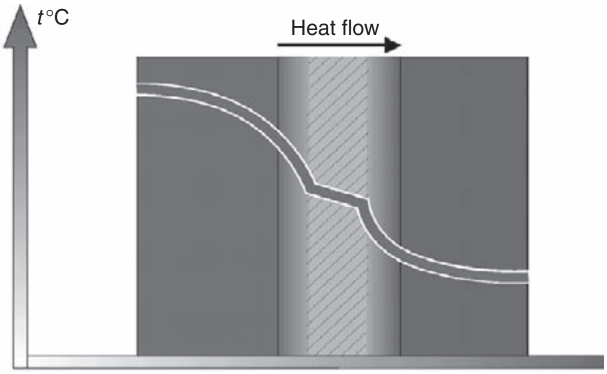

Figure 1 Heat transfer from a heating medium to a cold product on the other side of the partition. Courtesy of Tetra Pak.

product (**Figure 1**). In a plate heat exchanger, the plate is the partition. The velocity of the liquids is reduced by friction to almost zero at the boundary layer in contact with the partition. The velocity increases progressively and is the highest at the center of the channel. The temperature of the hot water is the highest in the middle of the channel. The closer the water is to the partition, the more it is cooled by the cold milk on the other side. Heat is transferred, by convection and conduction, to the boundary layer. Transfer from the boundary layer through the wall to the boundary layer on the other side is almost entirely by conduction, while further transfer to the milk in the central zone of the channel is accomplished by both conduction and convection.

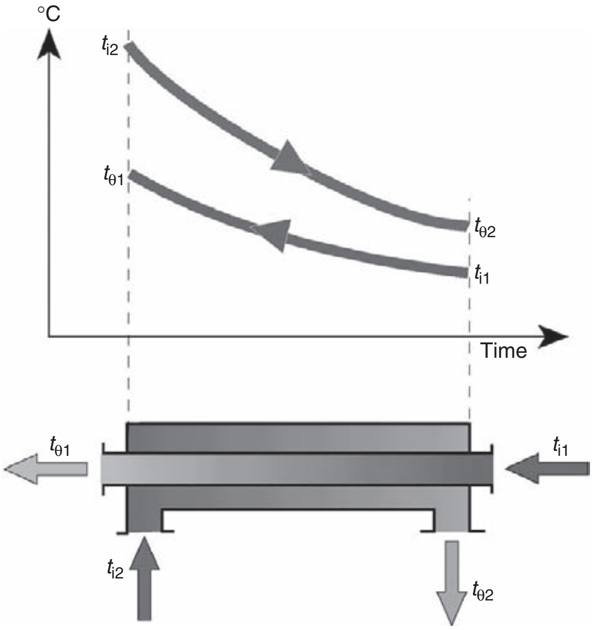

Figure 2 Temperature profiles for heat transfer in a heat exchanger with countercurrent flow. Courtesy of Tetra Pak.

The Heat Exchanger

The heat exchanger is used to transfer heat by the indirect method. The temperature profiles of the heating medium and milk in a typical tubular heat exchanger (THE) are shown in **Figure 2**.

Dimensioning Data for the Heat Exchanger

The size and configuration of the heat exchanger depend on many factors. The calculation is intricate and is normally done with the aid of a computer. The factors that must be considered are

- product flow rate
- physical properties of the liquids
- temperature program
- permitted pressure drop
- heat exchanger design
- cleanability requirements
- running time requirement

The formula for calculating the heat transfer area of the heat exchanger is

$$A = \frac{Q \times \rho \times c_p \times \Delta t}{\Delta t_m \times k} \tag{1}$$

where A is the required heat transfer area, Q the product flow rate, ρ the density of the product, c_p the specific heat of the product, Δt the temperature change of the product, Δt_m the logarithmic mean temperature difference (LMTD), and k the overall heat transfer coefficient.

Product flow rate

The flow rate Q is determined by the planned capacity of the dairy. Other factors being constant, the size of the heat exchanger is directly proportional to the flow rate.

Physical properties of the liquids

These include density, ρ, specific heat, c_p, and viscosity, μ. The values depend on product and temperature.

Temperature program

Several aspects of the operating temperature must be considered, including the change of temperatures, the differential temperature between the liquids, and the flow direction of the liquids.

Temperature change

Inlet and outlet temperatures of the product are determined by the preceding and subsequent process stages. The change of product temperature (Δt in the general formula above) can be expressed as (**Figure 2**)

$$\Delta t_1 = t_{o1} - t_{i1} \tag{2}$$

The inlet temperature for the service medium is determined by processing conditions. The temperature of the outgoing service medium can be calculated by an energy balance calculation. For a modern heat exchanger, the energy losses to the surrounding air can be neglected. Thus, the heat lost by the hot liquid is equal to the heat gained by the cold liquid. It can be expressed as

$$\underline{Q_1} \times \rho_1 \times c_{p1} \times \Delta t_1 = \underline{Q_2} \times \rho_2 \times c_{p2} \times \Delta t_2 \qquad (3)$$

Logarithmic mean temperature difference

There must be a difference in temperature between the two media for heat transfer to occur. The differential temperature is the driving force. For sensitive products there are, however, limits to how large a difference can be used. The differential temperature can vary through the heat exchanger. A mean value, LMTD or Δt_m, is used for calculation.

$$\Delta t_m = \frac{(t_{i2} - t_{o1}) - (t_{o2} - t_{i1})}{\ln((t_{i2} - t_{o1})/(t_{o2} - t_{i1}))} \qquad (4)$$

An important factor in determining the mean temperature differential is the direction of flow in the heat exchanger. There are two main options: countercurrent or cocurrent flow.

Countercurrent flow

The temperature difference between the two liquids is best utilized if they flow in opposite directions through the heat exchanger (**Figure 2**). The cold product then meets the cold heating medium at the inlet and a progressively warmer medium as it passes through the heat exchanger. During its passage, the product is gradually heated so that the temperature is always only a few degrees below that of the heating medium at the corresponding point. This type of arrangement is called countercurrent flow.

Cocurrent flow

With the opposite arrangement, that is, cocurrent flow (**Figure 3**), both liquids enter the heat exchanger from the same end and flow in the same direction. In cocurrent flow it is impossible to heat the product to a temperature higher than what would be obtained if the product and the heating medium were mixed. This limitation does not apply in countercurrent flow; the product can be heated to within 2–3 °C of the inlet temperature of the heating medium.

Overall heat transfer coefficient

This factor, *k*, is a measure of the efficiency of the heat transfer. It indicates how much heat passes through 1 m^2 of the partition per 1 °C of differential temperature. In the heat exchanger, *k* should be as high as possible. The heat transfer coefficient depends on the following:

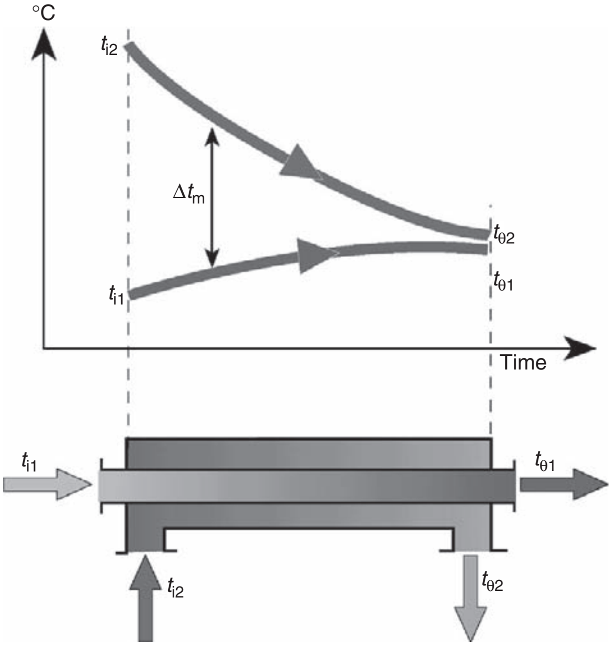

Figure 3 Temperature profiles for heat transfer in a heat exchanger with cocurrent flow. Courtesy of Tetra Pak.

- permitted pressure drops for the liquids
- viscosities of the liquids
- shape and thickness of the partition
- material of the partition
- presence of fouling matter

Permitted pressure drop

To increase the value of *k*, and improve heat transfer, it is possible to reduce the size of the channel through which the product flows. This reduces the distance over which heat must be transferred from the partition to the center of the channel. At the same time, however, the cross-sectional area of flow is reduced.

As a result, the flow velocity through the channel increases, which in turn makes the flow more turbulent and increases the pressure drop.

The greater the allowed pressure drops for the product and service medium, the more heat is transferred and the smaller the heat exchanger needed. Products sensitive to mechanical agitation (e.g., milk fat globules) may, however, be damaged by violent treatment. The product pressure before the heat exchanger must be increased to force the product through the narrower channels. It may then be necessary to install a booster pump. In some countries, installation of a booster pump is specified in legal requirements, basically to secure a higher pressure on the product side and thus prevent leakage of unpasteurized product into the pasteurized product.

Heat exchanger design

Viscosity of media

The viscosity of the product and the service medium are important determinants of the dimensions of the heat exchanger. A liquid with high viscosity develops less turbulence when it flows through the heat exchanger as compared to a product with lower viscosity. This means that a larger heat exchanger is needed, everything else being constant. Special attention must be paid to products with non-Newtonian flow behavior. For these products, the apparent viscosity depends not only on temperature but also on shear rate. A product that seems rather thick in a tank may flow much more readily when it is pumped through pipes or the heat exchanger.

Shape and thickness of the partition

The partition is often corrugated to create a more turbulent flow, which results in better heat transfer (**Figure 4**). The thickness is also important; the thinner the partition, the better the heat transfer. However, this must be balanced against the need for the partition to be strong enough to withstand the pressure of the liquids. Modern design and production techniques allow thinner partitions than were possible only a few years ago.

Material of the partition

For food processing, the normal material is stainless steel, which has fairly good heat transfer characteristics.

Presence of fouling matter

Most dairy products are sensitive to heating, which must therefore be done very carefully to avoid product damage. If the surface is too hot in relation to the product, there is a risk that proteins in the milk will coagulate and be deposited in a thin layer on the partitions. The differential temperature between the heating medium and the product should therefore be as small as possible, normally 2–3 °C above the pasteurization temperature. Heat must be transferred through the deposit, reducing the value of the overall heat transfer coefficient, *k*. The temperature differential between the heating medium and the product will then no longer be sufficient to transfer the same amount of heat

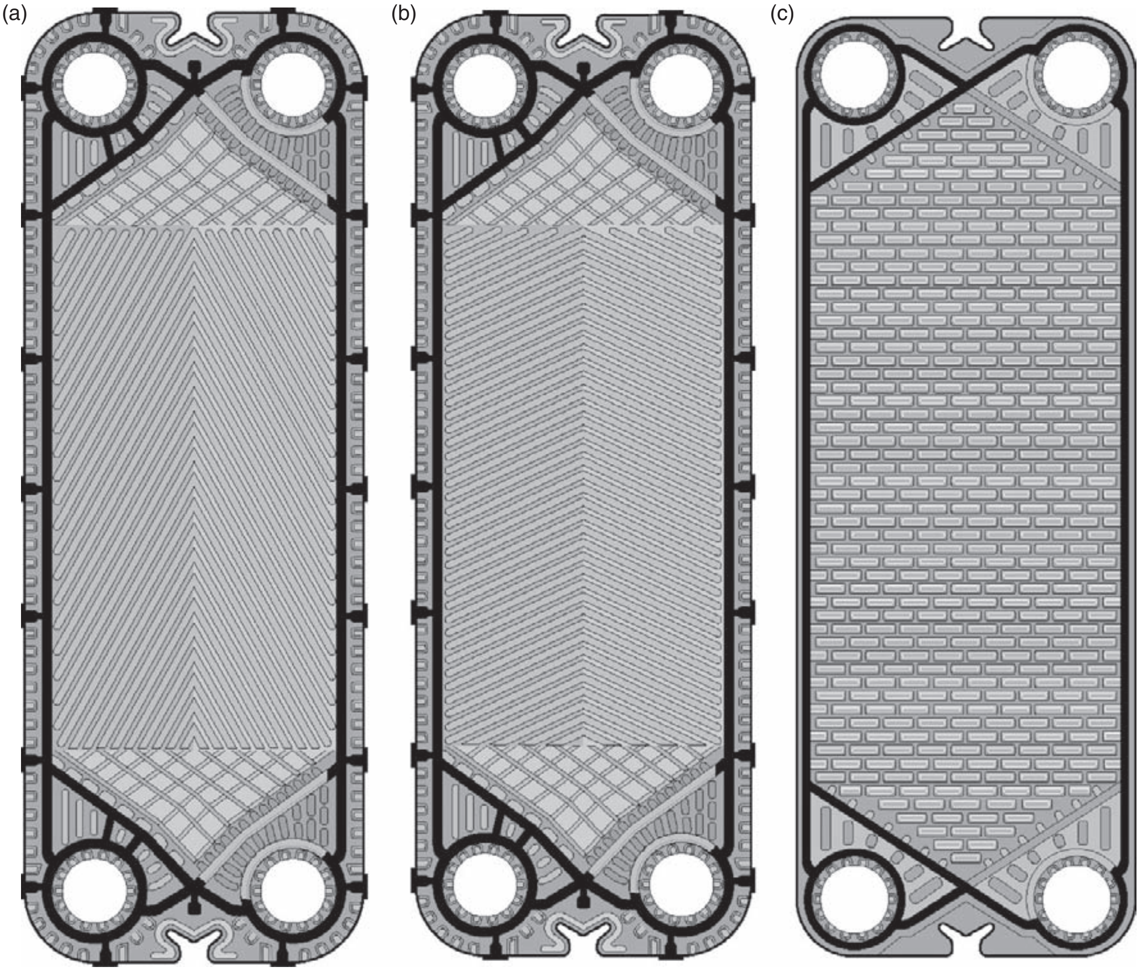

Figure 4 The shape of the partition in a plate heat exchanger may differ depending on the product to be treated and thermal efficiency requirements. (a) and (b) herringbone pattern; (c) washboard pattern. Courtesy of Tetra Pak.

as before, and the product outlet temperature will drop. This can be compensated for by increasing the temperature of the heating medium, but this also raises the temperature of the heat transfer surface so that more protein coagulates on the surface, the thickness of the crust increases, and the value of *k* drops still further.

The value of *k* is also affected by the flow rate through the heat exchanger. Increasing the flow rate makes the flow more turbulent and increases the value of *k*. Throttling the flow reduces the turbulence and hence also reduces the value of *k*. In modern pasteurizers and sterilizers the possibility of variable capacity is often included. In addition, multipurpose plants, that is, plants designed for the processing of various products in combination, are common today. As an example, a modern milk sterilizer for 10 000 l h^{-1} of whole milk may be capable of also processing milk-based vanilla pudding at 5000 l h^{-1}.

In calculating the heat transfer area, the sensitive nature of the product and the process demands must also be considered. Two such factors, not included in the formula, are the requirements for cleanability and running time.

Cleanability requirements

The heat exchanger in a dairy must be cleaned at the end of a production cycle, by circulating detergents. To achieve efficient cleaning, while designing the heat exchanger not only the temperature requirements, but also the cleaning requirements must be kept in mind. If some passages in the heat exchanger are very wide, that is, have several parallel channels, the turbulence during cleaning may not be enough to remove fouling deposits effectively. On the other hand, if some passages are very narrow, that is, have few parallel channels, the turbulence may be so high that the pressure drop will be very large. Such a high pressure drop may reduce the flow velocity of the cleaning solution, thereby reducing its effectiveness. The heat exchanger must therefore be designed for effective cleaning.

Running time requirement

Some fouling always occurs when milk products are heated to a temperature above 65 °C. Hence, the pasteurizer must be stopped periodically for cleaning. The length of the running time is difficult, not to say impossible, to predict, as it is determined by the degree of fouling, which depends on factors such as

- temperature difference between the product and the heating medium
- milk quality
- air content of the product
- pressure conditions in the heating section

It is especially important to keep the air content as low as possible. Excess air in the product will greatly contribute to increased fouling. Under certain conditions, the running time may also be limited by the growth of microorganisms in the downstream part of the regenerative section of the plate heat exchanger. This is, however, rare; when it occurs it is usually related to the pretreatment of the milk.

Hence it is important to allow for cleaning at regular intervals when making production plans for pasteurizers.

Regeneration

Utilizing the heat of a hot liquid, such as pasteurized milk, to preheat cold incoming milk is called regeneration. The cold milk also serves to cool the hot milk, thus economizing on water and energy. Regeneration efficiencies of up to 94–95% can be achieved in efficient modern pasteurization plants.

The percentage of regeneration is calculated as

$$R = \frac{(t_\mathrm{r} - t_\mathrm{i}) \times 100}{(t_\mathrm{p} - t_\mathrm{i})} \tag{5}$$

where R is the regenerative efficiency (%), t_r the milk temperature (°C) after regeneration, t_i the temperature (°C) of raw incoming milk, and t_p the pasteurization temperature (°C).

Holding

Correct heat treatment requires that the milk be held for a specified time at the pasteurization temperature. This is done in an external holding cell, which usually consists of a pipe arranged in a spiral or zigzag pattern and often covered by a metal shroud to protect operators against burns. The length of the pipe and the flow rate are calculated so as to provide the required holding time. Accurate control of the flow rate is essential to achieve the specified holding time. The holding time changes in inverse proportion to the flow rate.

Calculation of holding time

The appropriate tube length can be calculated when the hourly capacity and the inner diameter of the holding tube are known. As the velocity profile in the holding tube is not uniform, some milk molecules will move faster than the average. To ensure that even the fastest molecule is pasteurized sufficiently, an efficiency factor, η, must be used. This factor depends on the design of the holding tube, but is often in the range 0.8–0.9.

The length, L, of the holding cell is calculated as follows:

$$V = \frac{Q \times H}{3600 \times \eta}\ \mathrm{dm}^3 \tag{6}$$

$$L = \frac{V \times 4}{\pi \times d^2 \times 10}\ \mathrm{m} \qquad (7)$$

where Q is the flow rate at pasteurization ($\mathrm{l\,h^{-1}}$), H the holding time (s), L the length (m) of the holding tube, d the inner diameter (dm) of the holding tube, V the volume (l or $\mathrm{dm^3}$) of milk, and η the efficiency factor, dimensionless.

Types of Heat Exchangers

The following three types of heat exchangers are most widely used in the dairy industry:

- plate heat exchangers
- tubular heat exchangers
- scraped-surface heat exchangers

Plate heat exchangers

Most heat treatments of dairy products are carried out in plate heat exchangers. The plate heat exchanger (often abbreviated PHE) consists of a pack of stainless-steel plates clamped in a frame. The frame may contain several separate plate packs – sections – in which different stages of treatment, such as preheating, final heating, and cooling, take place. The heating medium is hot water and the cooling medium cold water, ice water, or propylene glycol, depending on the required product outlet temperature.

The plates are corrugated in a pattern designed for optimum heat transfer. Formerly, the so-called washboard pattern (**Figure 4(c)**) was predominant, but today the herringbone pattern is normally used for all types of plates, also within the dairy industry (**Figures 4(a)** and **4(b)**). The main advantages with the herringbone pattern are increased thermal efficiency and increased mechanical strength, the latter allowing the plates to be made thinner, which reduces the cost and weight of the plate. The increased thermal efficiency reduces the number of plates necessary, which in turn reduces the cost of the unit and also product hold-up volume.

The plate pack is compressed in the frame. Supporting points on the corrugations hold the plates apart so that narrow channels are formed between them. The liquids enter and leave the channels through holes in the corners of the plates. Varying patterns of open and blind holes route the liquids from one channel to the next. Gaskets around the edges of the plates and around the holes form the boundaries of the channels and prevent external leakage and internal mixing. **Figure 5** shows a typical arrangement.

Flow patterns

The arrangement of the plates is such that the product flows through alternate channels in the plate pack. The service (heating or cooling) medium is introduced at the other end of the section and passes through alternate plate channels. Each product channel consequently has service medium channels on both sides. For efficient heat transfer, the channels between the plates should be as narrow as possible, but both flow velocity and pressure drop will be high if a large volume of product must pass through these narrow channels. Neither of these effects is desirable and, to eliminate them, the passage of the product through the heat exchanger may be divided into a number of parallel flows. In **Figure 6**, the product flow is divided into two parallel flows, which change direction 4 times in the section. The channels for the heating medium are divided into four parallel flows, which change direction twice. This combination is written as 4 × 2/2 × 4, that is, the number of passes multiplied by the number of parallel flows for the product over the number of passes multiplied by the number of parallel flows for the service medium. This is called the grouping of the plates.

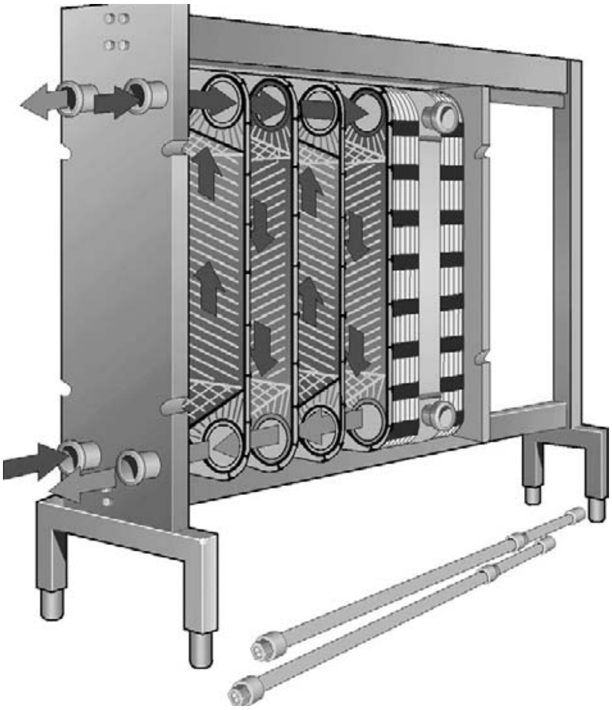

Figure 5 Principles of flow and heat transfer in a plate heat exchanger. Courtesy of Tetra Pak.

Tubular heat exchangers

THEs are today normally used for UHT treatment of dairy products. The THE (**Figure 7**), unlike plate heat exchangers, has no contact points in the product channel and can thus handle products with particles, the maximum particle size depending on the diameter of the tube. The THE can also run longer between cleanings than the plate heat exchanger in UHT treatment. From the standpoint of heat transfer, the THE is less efficient than the plate heat exchanger. THEs

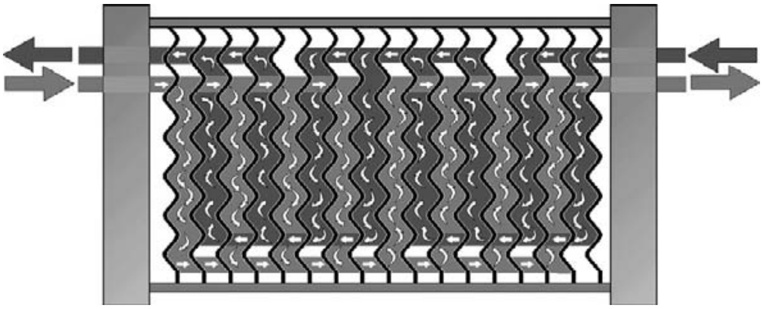

Figure 6 The system of parallel flow pattern for both product and heating/cooling medium channels. In this example the combination is written 4 × 2/2 × 4. Courtesy of Tetra Pak.

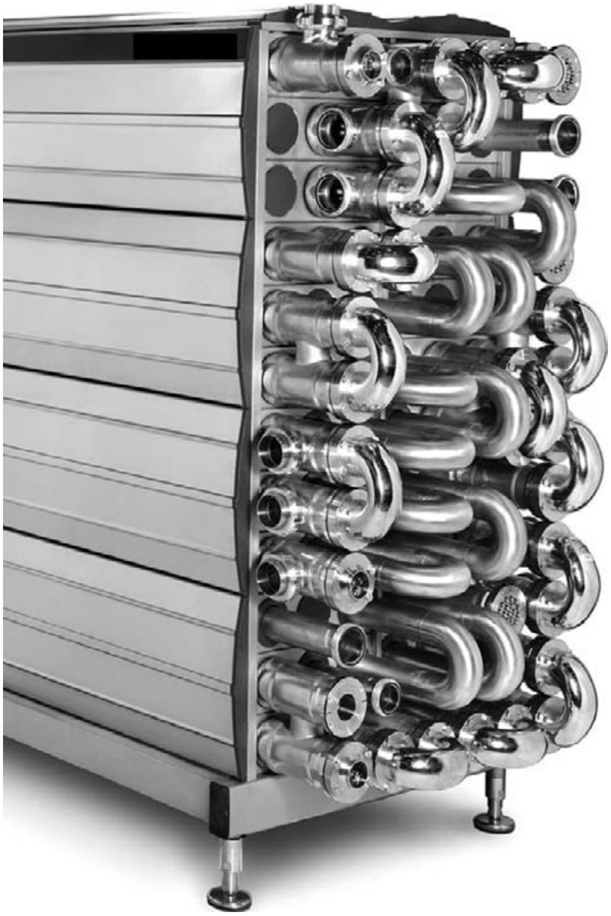

Figure 7 The tubular heat exchanger tubes are assembled in a compact unit. Courtesy of Tetra Pak.

are available in two fundamentally different types: multi/monotube and concentric tube.

Multi/monotubes

The multitube THE operates on the classic shell-and-tube principle, with the product flowing through a group of parallel tubes, and the service medium between and around the tubes. Turbulence for efficient heat transfer is created by helical corrugations on the tubes and shell. The heat transfer surface consists of a bundle of straight corrugated or smooth tubes welded into tube plates at both ends. The tube plates are in turn sealed against the outer shell by a double O-ring construction (floating design). This design allows the product tubes to be taken out of the shell by unscrewing the end bolts, and this makes the unit strippable for inspection. The floating design absorbs thermal expansion, and the product tube bundles in the shell can be changed, allowing different combinations to be used for different applications. The monotube is a version with only one inner tube, which will permit particles with a diameter up to 50 mm to pass. Multi/monotubes are well suited for processes operating at very high pressures and temperatures.

Concentric tubes

The heat transfer surface of a concentric THE consists of straight tubes of different diameters concentrically located on a common axis, connected by headers at both ends. The product flows in the gap between two concentric tubes and the service medium on both sides of these tubes. The floating design as described above for multi/monotubes is also applied to the concentric tubes. In addition, the multi/monotube inserts and the concentric tube inserts are interchangeable for maximum flexibility. The concentric tube is especially suited for processing viscous products, such as dessert puddings. Due to the thin product layer in the annular channel, very efficient heat transfer is achieved. Also, due to the single product channel design, the risk of maldistribution across parallel tubes for viscous products is eliminated.

Scraped-surface heat exchangers

The scraped-surface heat exchanger (**Figure 8**) is designed for heating and cooling viscous, sticky, or lumpy products, and for crystallization of products. The operating pressures on the product side are high, often as much as 4 MPa. All products that can be pumped can therefore be treated. The scraped-surface heat exchanger consists of a cylinder through which the

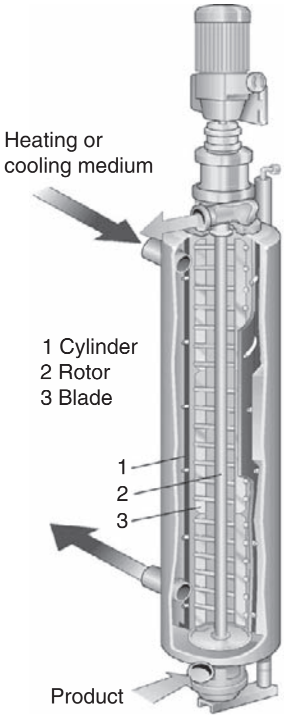

Figure 8 Vertical type of the scraped-surface heat exchanger. Courtesy of Tetra Pak.

product is pumped in countercurrent flow to the service medium in the surrounding jacket. Exchangeable rotors of various diameters, from 50.8 to 127 mm, and varying pin–blade configurations allow adaptation to different applications. Smaller-diameter rotors allow larger particles (up to 25 mm) to pass through the cylinder, while larger-diameter rotors result in shorter residence time and improved thermal performance. The product enters the vertical cylinder through the lower port and continuously flows upward through the cylinder. At process start-up, all the air is completely purged ahead of the product, allowing complete and uniform product coverage of the heating or cooling surface.

The rotating blades continually remove the product from the cylinder wall (**Figure 9**), to ensure uniform heat transfer to the product. In addition, the surface is kept free from deposits. The product exits the cylinder via the upper port. Product flow and rotor speed are varied to suit the properties of the product flowing through the cylinder. At shutdown, thanks to the vertical design, the product can be displaced by water with minimum intermixing, which helps assure product recovery at the end of every run. Following this, complete drainage facilitates cleaning-in-place (CIP) and product changeover. As mentioned above, the rotor and blades are exchangeable, an operation which is possible owing to the automatic hydraulic lift that facilitates raising and lowering of the rotor/blade assembly

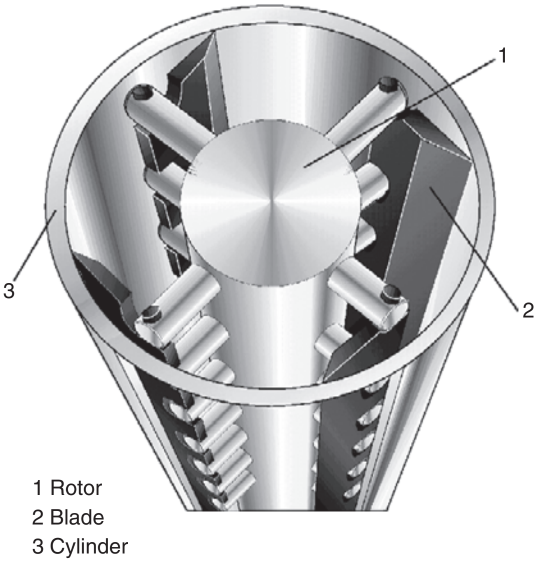

Figure 9 Section through a scraped-surface heat exchanger. Courtesy of Tetra Pak.

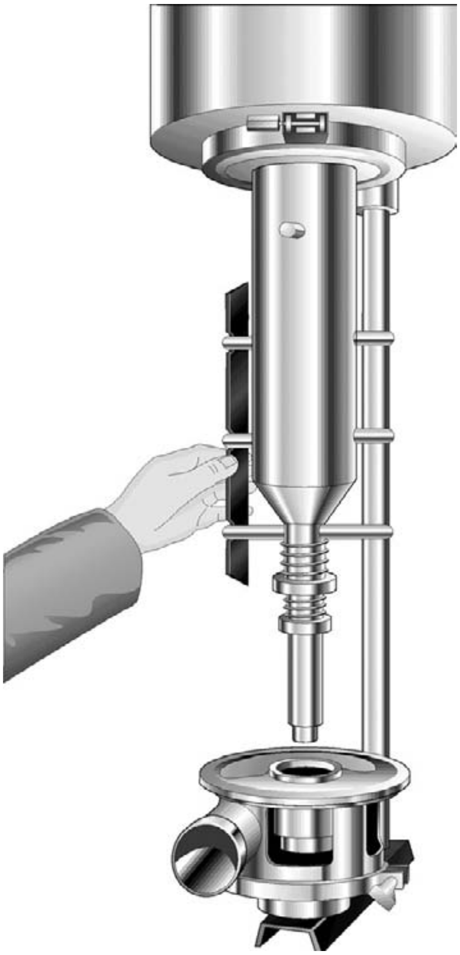

Figure 10 Removal of blades from the rotor assembly in lowered position. Courtesy of Tetra Pak.

(**Figure 10**). The typical products treated in the scraped-surface heat exchanger are ice cream and dairy spread, but fruit preparations, such as yogurt, are also treated. It is also used for fats and oils for crystallization of margarine and shortenings. The scraped-surface heat exchanger is also available in versions designed for aseptic processing. Two or more vertical-type scraped-surface heat exchangers can be linked in series or parallel to give a greater heat transfer surface depending on the processing capacity required.

See also: **Heat Treatment of Milk**: Sterilization of Milk and Other Products; Ultra-High Temperature Treatment (UHT): Heating Systems. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Liquid Milk Products: UHT Sterilized Milks. **Plant and Equipment**: Pasteurizers, Design and Operation; Process and Plant Design. **Utilities and Effluent Treatment**: Heat Generation.

Further Reading

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Pasteurizers, Design and Operation

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Introduction and Historical Development

Pasteurization is a heat treatment that inactivates vegetative pathogenic microorganisms so as to render the product safe for consumption if refrigerated, and also prolongs the shelf life of the product. Pasteurized products are not sterile and still harbor low numbers of nonpathogenic psychrotrophic bacteria, which eventually reduce the shelf life. The growth of thermophilic spore-forming bacteria, which survive the relatively mild heat treatment applied, in pasteurized milk is largely controlled by cold storage of the pasteurized milk. Thus, pasteurization is a process that has profound impacts on both the public health implications of milk consumption and the keeping quality of milk and dairy products.

The process is named after the French microbiologist Louis Pasteur, who discovered that wine could be preserved by inactivating bacteria by heating at a temperature below boiling. The principle was soon applied to milk, and the first systems for the commercial pasteurization of milk were introduced in about the last decade of the nineteenth century. Early systems relied on heating milk to about 63–65 °C and holding for about 30 min in batch heating units, followed by rapid cooling to less than 12 °C (i.e., low-temperature–long-time (LTLT) pasteurization). This process is still used by small-scale processors but, since about 1940, such systems have been largely superseded by high-throughput continuous-flow plate heat exchanger (PHE)-based pasteurizers, in which milk is heated to 72–74 °C and held for at least 15 s, in a process called high-temperature–short-time (HTST) pasteurization. This remains the most common design of industrial pasteurizers today, and will be the focus of this article. Public health aspects of pasteurization are discussed in **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects.

Heat Treatment Conditions Used for the Pasteurization of Milk

In 1980, the International Dairy Federation defined pasteurization as "a process applied to a product with the object of minimising possible health hazards arising from pathogenic micro-organisms associated with milk by heat treatment which is consistent with minimal chemical, physical and organoleptic changes in the product".

The time–temperature conditions selected for the pasteurization of milk were determined, primarily, from the knowledge of the sensitivity of pathogenic microorganisms in milk to heat and, secondarily, from the consideration of the negative impact of heat treatment on the flavor and perceived 'freshness' of milk. The most heat-resistant vegetative pathogen in milk was identified originally as *Mycobacterium tuberculosis* (*see* **Pathogens in Milk:** *Mycobacterium* spp.), and the conditions for LTLT pasteurization were defined to ensure inactivation of this bacterium. The pathogenic rickettsia, *Coxiella burnetii*, was subsequently recognized as being slightly more heat-resistant, and the need to inactive this species was a key factor in the development of HTST pasteurization conditions (*see* **Pathogens in Milk**: *Coxiella burnetii*). Heat treatments sufficient to thermally inactivate these bacteria kill all less resistant microorganisms, and thus pasteurization should render milk safe for consumption, at least in terms of vegetative bacteria.

In recent years, as a result of better understanding of the diversity of the microflora of raw milk and the thermal inactivation kinetics of various bacterial strains, there has been some discussion on modification of pasteurization conditions. For example, use of an extended holding time during HTST pasteurization (to 25–40 s) or a slightly higher pasteurization temperature has been suggested as being required to ensure inactivation of heat-stable strains of *Listeria monocytogenes*, *Escherichia coli*, and *Campylobacter* species (*see* **Liquid Milk Products**: Pasteurization of Liquid Milk Products: Principles, Public Health Aspects).

Also, in the last 15 years, there has been much debate on the heat sensitivity and public health significance of emerging pathogens in milk, particularly *Mycobacterium avium* subsp. *paratuberculosis* (*see* **Pathogens in Milk:** *Mycobacterium* spp.). This bacterium is the causative agent of Johne's disease, a bowel disease in cattle, and has been linked to a similar syndrome, Crohn's disease, in humans. Early experiments to determine its heat sensitivity were hampered by the immense difficulty in quantifying this bacterium in milk owing to its extremely slow growth rate in culture and its tendency to grow in clumps due to its rough waxy cell wall. Nevertheless, early studies on the thermal inactivation of this bacterium in milk suggested that conventional HTST treatments were not sufficient to ensure complete inactivation of *M. avium* subsp. *paratuberculosis*, leading to an extension of the holding time and/or use of a higher pasteurization temperature in many countries. However, more recent studies have suggested that, under the extremely turbulent flow conditions in a modern milk pasteurizer, protective clumps of this bacterium are disrupted, enhancing its heat sensitivity and permitting inactivation under conventional HTST conditions.

Principle of Operation of an HTST Pasteurizer

As stated above, in modern dairy processing plants, HTST pasteurization in continuous-flow PHEs is practiced almost exclusively, although on farms or small dairy plants, smaller batch units may still be used (*see* **Plant and Equipment**: Heat Exchangers). A typical PHE consists of a vertical stack of many stainless steel plates clamped in a frame. The frame may contain several groups of plates, known as sections, in which different stages of treatment, such as preheating, final heat treatment, and cooling, take place (Figure 1). The heating medium may be steam or hot water, and the cooling medium may be cold water, brine, glycol, or ice water, depending on the desired outlet temperature of the product. The capacity of a milk pasteurizer depends on the size and number of plates, and can be as high as 100 000 l h^{-1}.

The surface of the plates displays a corrugated pattern to increase turbulence in the liquid flowing over them and, thus, efficiency of heat transfer (Figure 2). The stainless steel plates are compressed together in the frame, but supporting points on the corrugations hold the plates apart so that thin, rectangular channels are formed between them. Gaskets (made from natural rubber or, more recently, from synthetic elastomers) around the edges of the plates and holes define the boundaries of the channels and prevent leakage. The liquids flowing in the PHE enter and leave these channels by openings at the corners of the plates, and varying patterns of open and blind openings route the liquid from one channel to the next.

The flow pattern in alternating plates is illustrated in Figure 3. Milk is introduced through a corner opening into a channel between two plates and flows vertically through the channel; it leaves through the opening at the opposite corner, and bypasses the next channel between plates. In parallel, the heating or cooling medium is introduced at the other end of the section and it passes through the alternating interplate channels in the same manner. Thus, milk has heating or cooling medium, in the respective sections, flowing countercurrent, on either side of it, but separated by the intervening plate wall.

For efficient heat transfer, the channels between the plates should be as narrow as possible; however, if a large

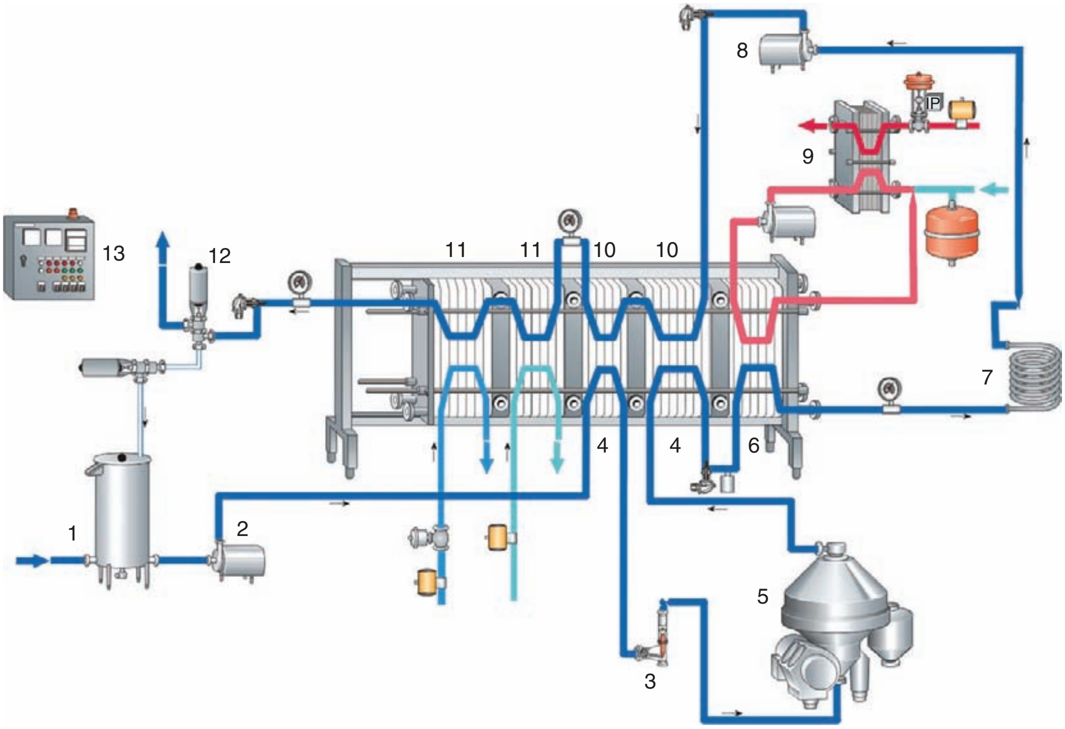

Figure 1 A complete pasteurizing plant, including (1) balance tank, (2) feed pump, (3) flow controller, (4) regenerative preheating sections, (5) centrifugal clarifier, (6) heating section, (7) holding tube, (8) booster pump, (9) hot water heating system, (10) regenerative cooling sections, (11) cooling sections, (12) flow diversion valve, and (13) control panel. Reproduced with permission from Tetra Pak A/B, Lund, Sweden.

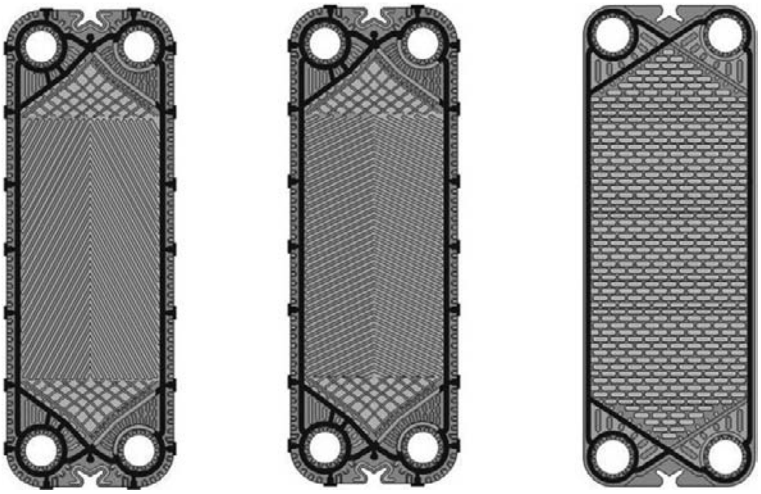

Figure 2 Shape and design of typical plate heat exchanger pasteurizer plates. Reproduced with permission from Tetra Pak A/B, Lund, Sweden.

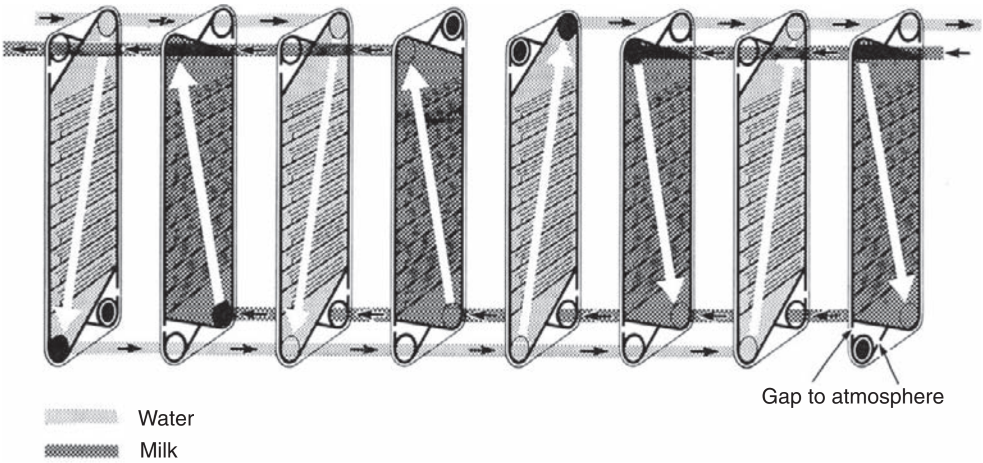

Figure 3 Principles of flow and heat transfer in the heating sections of a pasteurizer. Reproduced with permission from Harrison PC (1983) HTST pasteurising plant. In: Green E (ed.) *Pasteurising Plant Manual*, 3rd edn., pp. 7–36. Huntingdon, UK: Society of Dairy Technology.

volume of product has to pass through these narrow channels, both flow velocity and pressure drop will be high. Neither of these effects is desirable, and to minimize their significance the passage of product through the PHE may be divided into a number of parallel flows. One section of parallel flow is known as a pass, and passes in the PHE change the direction of flow a number of times. Within each section of the pasteurizer, there are many passes, that is, the direction of flow of product or heating/cooling medium is changed several times.

In a pasteurizer, cool raw milk is fed from a silo to a float hopper (balance tank) and pumped at a constant rate to the preheating, or regeneration, section of the pasteurizer, where it is heated to 68–70 °C. In this section, milk is heated by absorbing heat from outgoing pasteurized milk through the plates, with the medium being cooled simultaneously. Similarly, heat from the pasteurized milk is transferred to a cooling medium after reaching the pasteurization temperature. The use of pasteurized milk as a heating medium for incoming cold raw milk results in substantial energy savings, and is called heat regeneration. Raw milk is heated in this manner by pasteurized milk from about 6 °C to about 68–70 °C and the pasteurized milk is cooled to about 10 °C. The temperature of the milk thus preheated must be increased by only about 2–4 °C (depending on the pasteurization temperature set point) using steam or hot water in the final heating section, while in the cooling section the pasteurized milk must be cooled by only about 6 °C (from 10 to 4 °C). With a well-dimensioned regenerative section, it is possible to recover about 94% of the heat from the pasteurized milk.

Regenerative preheating of raw milk is usually divided over 2–3 sections of the PHE; milk between sections is thus at intermediate temperatures between 6 and 70 °C. Milk at around 50–55 °C is at the optimum temperature for treatments such as separation (fat removal and/or standardization; *see* **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry), clarification (removal of undesirable solids; *see* **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry), or homogenization (*see* **Homogenization of Milk**: Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers), as milk fat is in a liquid state. Incorporation of such processing steps into the pasteurization line avoids the need to heat milk separately to carry out these operations, making liquid milk processing more economical and reducing the chances of contamination of the milk. Thus, milk is typically routed out of the pasteurizer between regeneration sections for treatments such as homogenization or standardization, before being returned to the PHE for completion of the pasteurization cycle. Milk may, in theory, exit the regeneration section for such parallel processing while being either heated (raw milk) or cooled (pasteurized milk), but the former option is more common, as it avoids the risk of recontamination of pasteurized milk, which otherwise would have to be avoided by use of aseptic homogenization or separation systems. A complete process line for homogenized market milk is shown in Figure 4.

The heating medium in the final heating section is circulated by a pump from a hot water generator, the temperature of which is maintained at 2–7 °C above the set point by injecting steam via a steam-regulating valve, which is controlled by an automatic temperature controller (*see* **Utilities and Effluent Treatment**: Heat Generation). Both the pasteurization temperature and final milk temperature are generally logged on a chart recorder, which is mounted, along with the flow diversion valve (FDV) (see below) setting control, on a control panel.

Correct pasteurization requires that the milk be held for a specified time at the set pasteurization temperature. This can be achieved either in a specific section of plates within the PHE (holding section) or in an external holding tube. No heating or cooling should take place within the holding section or tube; the milk simply flows through a passage, the length of which is determined from the flow rate of the milk and the required residence (holding) time. The control of residence time may be undertaken with greater confidence in a tube than under the turbulent flow conditions encountered in flow between plates. As a fundamental principle, the minimum residence time of milk in the holding section should always exceed the residence time stipulated for the process. An external holding tube

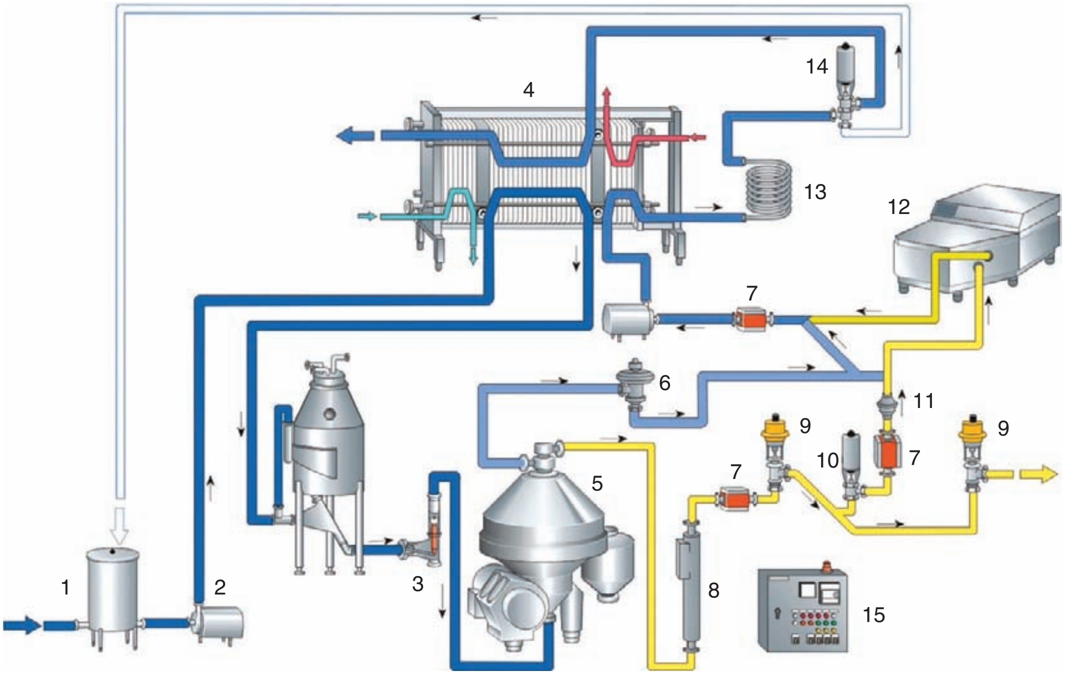

Figure 4 Production line for market milk with homogenization of cream, showing (1) balance tank, (2) feed pump, (3) flow controller, (4) plate heat exchanger, (5) centrifugal separator, (6) constant pressure valve, (7) flow transmitter, (8) density transmitter, (9) regulating valve, (10) shutoff valve, (11) check valve, (12) homogenizer, (13) holding tube, (14) flow diversion valve, and (15) control panel. Reproduced with permission from Tetra Pak A/B, Lund, Sweden.

typically consists of a length of piping arranged in a spiral or zigzag pattern, and should be insulated. Since the residence time in the holding cell is determined primarily by the flow rate, accurate flow regulation is essential, as changes in flow rate will directly and inversely affect holding time.

A resistance thermometer (thermistor) monitors the temperature of milk, generally at the point of exit from the holding section. Less commonly, the thermistor may be located at the start of the holding section. The latter position allows more time for the reaction to drop in temperature, ensuring that no underpasteurized milk can flow downstream for packaging, but does not account for the drop in temperature that can occur in an uninsulated external holding tube. The former position is thus more common, although a drop in temperature in the holding tube may be minimized by insulating the tube or by heating the milk slightly above the set pasteurization temperature in the heating section. However, the latter option implies overheating of milk, with concomitant potential alterations to flavor and properties.

The thermistor (which typically operates over the range of 60–80 °C) controls the action of a flow diversion device (FDD) or flow diversion valve (FDV), which is located downstream from the thermistor, either at the end of the holding section or, less commonly, after the cooling section of the pasteurizer. The plant shown in Figure 1 shows the FDV located in the latter position, while in Figure 4 the more common configuration is displayed. The function of the FDV is to prevent unpasteurized milk from reaching the packaging unit, by returning milk to the balance tank if the temperature determined by the thermistor drops below the target pasteurization temperature (sometimes referred to as the set point). The default, or fail-safe, position of the FDV of the pasteurizer is the closed or divert position, and the valve opens only if and when the temperature exceeds the set point. During operation, if the temperature of the milk, as measured by the thermistor, falls below the set point, a programmable logic controller (PLC) or similar device detects and records the decrease, often sounds an alarm for operators, and deactivates the FDV, causing the valve to close so that milk is diverted to the balance tank. The principle of operation of a pasteurizer's FDV is illustrated in Figure 5.

If the FDV deactivates and milk is returned to the balance tank, either the plant may stop, necessitating emptying, cleaning, and sanitizing before processing is recommenced, or the milk may be recirculated in the regeneration and cooling sections so as to allow restart when the set-point temperature is reached. In cases where the FDV is located at the outlet from the cooling section of the pasteurizer, the heat exchanger must be emptied, cleaned, and sanitized before restarting operation, if milk temperature decreases below the set point.

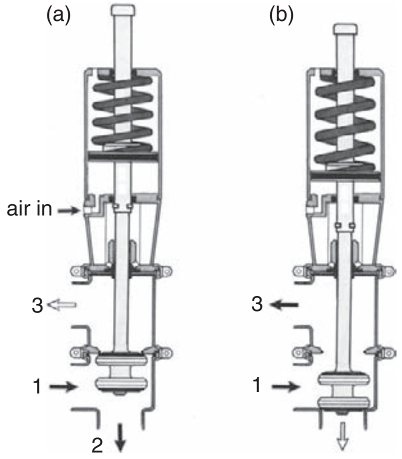

Figure 5 Principle of operation of a flow diversion valve of a high-temperature–short-time pasteurizer. Valves are shown in the (a) open (operating) and (b) divert positions. Indicated are (1) milk inlet from holding section or tube, (2) milk line to regenerative section, and (3) diverted milk line. Reproduced with permission from Tetra Pak A/B, Lund, Sweden.

There is always a possibility of fractures or pinholes occurring in pasteurizer plates, which could lead to recontamination of pasteurized milk. To guard against this possibility, a booster pump may be fitted to the pipeline as it leaves the holding section to increase the milk pressure slightly (by around 0.01–0.02 MPa); should a pinhole then occur, the direction of leakage would be of pasteurized milk into raw milk, thereby precluding postpasteurization contamination. A simpler means of protecting the pasteurized product is to increase the number of passes in the regeneration sections, thereby increasing the back pressure within the pasteurized milk section of the plant.

The chilled milk from the pasteurizer flows either to an intermediate storage tank or directly to a packaging unit. It is extremely important to avoid conditions that could introduce microorganisms into pasteurized milk during processing downstream from the pasteurizer; it has been acknowledged that postpasteurization contamination has in the past significantly influenced the shelf life and public health aspects of pasteurized milk.

The key operating parameters for an HTST pasteurizer are plant capacity, temperature program, regenerative efficiency, and the temperature of the heating and the cooling medium. During operation, the PHE may be controlled in a feedback loop by monitoring the temperature of the milk in the heating section, milk leaving the cooling section, the heating medium, or the cooling medium.

The shelf life of pasteurized milk is dependent on factors such as the microbial quality of the raw milk, the precise processing conditions used, postpasteurization contamination, and refrigeration temperature, but is generally around 10–16 days at 5 °C.

Pasteurization of Other Dairy Products

Similar processing conditions are generally used for the full range of fat-standardized liquid milk products. However, cream, which contains a higher level of fat, must be heated more severely (e.g., 80 °C for 3–5 s), as the fat acts as a thermal insulator, and increases the stability of bacteria (*see* **Cream**: Manufacture; Products). Ice cream and dessert mixes, which contain high levels of fat and sugar, are generally pasteurized at about 80 °C for 25 s. In some countries, milk or cream heated to around 80 °C for a few seconds may be referred to as being 'flash' pasteurized.

Testing and Maintaining Pasteurizers

The requirements for the successful operation of a pasteurization plant are shown in **Table 1**.

Pasteurizers should be checked routinely as follows:

- correct calibration of thermometers must be verified;
- the FDV must be shown to be in correct working order and functioning as intended; and
- heating plates should be inspected regularly for leaks.

It is recommended that basic parameters of pasteurizer operation, such as diversion temperature, recorder accuracy, and flow rate (determined by measuring the time to process a known quantity of milk), should be checked on a daily basis. The exterior of the plant should be inspected daily for leaks of all kinds and for general cleanliness. The FDV should be checked regularly for signs of wear or damage, which could impair its operating efficiency. Cleaning procedures for pasteurizers should be designed to prevent the growth of bacteria as biofilms (*see* **Biofilm Formation**), which have been shown to have the potential to proliferate on plate surfaces, but which can be removed by use of a cleaning cycle that includes exposure of surfaces to extremes of pH, as in cycled acid and caustic washes.

Methods for determining the integrity of PHEs and monitoring the occurrence of pinholes or cracks include differential electrolytic analysis and helium leak detection systems.

Testing for Milk Pasteurization

The efficiency of pasteurization is generally assessed by using an indicator enzyme, alkaline phosphatase, naturally present in raw milk (*see* **Enzymes Indigenous to Milk**: Phosphatases). This enzyme is almost completely inactivated by processing times and temperatures just higher than those required to kill *M. tuberculosis*, that is, under conditions required for pasteurization. There are simple tests to detect the residual activity of this enzyme in milk and, therefore, if milk has been heated sufficiently strongly to inactivate this enzyme, it is assumed to be free of

Table 1 Critical aspects of control of pasteurizer operation

Application of correct heating by the use of
Correct control of temperature of heating medium
Positive control of flow rate through holding tube
Correctly dimensioned holding tube
Flow diversion valve
Prevention of crosscontamination
Positive pressure between raw and pasteurized milk in regenerative section
Correct positioning of flow diversion valve
Routine inspection of seals for leaks
Cleanability
High-grade stainless steel used for fabrication
Welds, joins, and other parts of highest possible standard
All materials compatible with cleaning fluids
Limitation of heat damage
Minimize temperature difference between heating medium and milk
Minimize milk residence time in holding section
Ensure efficient cooling
Economic operation
Ensure efficient regeneration
Use a maximum heating surface-to-volume ratio

Adopted from Varnam AH and Sutherland JP (1994) Liquid milk and liquid milk products. In: *Milk and Milk Products*, pp. 42–102. London: Chapman and Hall.

surviving pathogenic bacteria. For many years, tests for the pasteurization of milk were generally based on the production of a colored product from an uncolored substrate by the enzyme (if active). In modern dairy testing laboratories, automated equipment, such as the Fluorophos™ system, is used to detect and quantify residual phosphatase activity in milk, where a small amount of milk is mixed in a small test tube with a substrate for alkaline phosphatase; if active enzyme is present, it hydrolyzes the substrate, yielding a fluorescent product, which is then measured by the instrument. Incubation, reading, and printing of results take place in a single small, compact unit, with results being produced within 1–3 min.

For cream, lactoperoxidase (an enzyme that is considerably more heat stable than alkaline phosphatase; *see* **Enzymes Indigenous to Milk**: Lactoperoxidase) is used as the indicator enzyme, rather than alkaline phosphatase, due to the requirements for use of a higher temperature in the pasteurization of cream. Lactoperoxidase activity may also be used to test for excessive heating during pasteurization of liquid milk, as HTST-pasteurized milk should be phosphatase-negative, but lactoperoxidase-positive.

Developments in Pasteurization Technology

The shelf life of pasteurized milk may be increased significantly by combining pasteurization with technologies that remove bacterial spores such as *Bacillus cereus*, which would otherwise grow, albeit slowly, during storage and spoil the milk. Approaches to spore removal before heat treatment generally involve either centrifugation (e.g., bactofugation) or microfiltration of skim milk, followed by mixing with strongly heat-treated cream, often followed by aseptic packaging (*see* **Milk Protein Products**: Membrane-Based Fractionation). Such extended shelf life (ESL) products have a considerably longer shelf life than pasteurized milk, but suffer significantly less undesirable nutritional or sensory effects than ultra-high temperature (UHT)-treated products. ESL-type processes may also be referred to as superpasteurization of milk (*see* **Liquid Milk Products**: Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk)).

See also: **Biofilm Formation**. **Cream**: Manufacture; Products. **Enzymes Indigenous to Milk**: Phosphatases. **Homogenization of Milk**: Principles and Mechanism of Homogenization, Effects and Assessment of Efficiency: Valve Homogenizers. **Liquid Milk Products**: Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk); Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Milk Protein Products**: Membrane-Based Fractionation. **Pathogens in Milk**: *Coxiella burnetii*; *Mycobacterium* spp. **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Heat Exchangers. **Utilities and Effluent Treatment**: Heat Generation.

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Evaporators

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Introduction

An evaporator is a device wherein evaporation occurs, that is, liquid is evaporated from a thin (low-density) feed material in order to produce a denser or thicker product (concentrate). The feed may be a solution, slurry, or suspension of solid materials in a liquid. The heat necessary for the phase change or vaporization is supplied through heat transfer across metallic surfaces by condensing steam. Thus, evaporation as a unit operation involves energy transfer (heat for vaporization and condensation), mass transfer (moisture removal), and fluid flow (feed and vapor flows).

In the dairy industry, evaporation is used for concentration of milk or whey. It is also used as a preliminary step to drying. Milk products intended for milk powder are normally concentrated from an initial solids content of 9–13% to a final concentration of 40–50% total solids before the product is pumped to the dryer.

Evaporation in the dairy industry involves boiling off part of the water from a solution. To do this, heat must be supplied. The products to be evaporated are normally heat-sensitive and can be destroyed by adding heat. To reduce this heat impact, evaporation is done under vacuum, sometimes at a temperature as low as 40 °C. At the same time, the evaporator should be designed for the shortest possible residence time. Most products can be concentrated with good results provided that the evaporator is designed for a low temperature and short holding time.

It requires a large amount of energy to boil off water from a solution. This energy is supplied as steam. To reduce the amount of steam needed, the evaporation station is normally designed as a multiple-effect evaporator. Two or more units operate at progressively lower pressures and thus with progressively lower boiling points. In such an arrangement, the vapor produced in the previous effect can be used as a heating medium in the following effect. The result is that the amount of steam needed is approximately equal to the total amount of water evaporated divided by the number of effects. Evaporators with up to seven effects are now used in the dairy industry.

Alternatively, electricity can be used as the energy source; in this case, an electrically powered compressor or fan is used to recompress the vapor leaving an effect to the pressure needed on the heating side.

Although evaporator plants generally work on the same principle, they differ in the details of their design. The tubes that form the partitions between steam and product can be either horizontal or vertical and the steam can be circulated either inside or outside the tubes. In most cases, the product circulates inside vertical tubes while steam is applied on the outside. The tubes can be replaced by plates, cassettes, or lamellae.

Operation

Processing Factors

The physical and chemical properties of the solution being concentrated and of the vapor being removed have a great effect on the type of evaporator used and on the following characteristics:

1. *Concentration of solutes in the liquid*: Usually, the liquid feed to an evaporator is relatively dilute, so its viscosity is low, similar to that of water, and relatively high heat-transfer coefficients are obtained. As evaporation proceeds, the solution may become very concentrated and quite viscous, causing the heat-transfer coefficient to drop markedly. Adequate circulation and/or turbulence must be present to prevent the coefficient from becoming too low.
2. *Solubility*: As solutions are heated and the concentration of the solutes increases, the solubility limit of the material in solution may be exceeded and crystals may form. This may limit the maximum concentration in solution which can be obtained by evaporation.
3. *Temperature sensitivity of materials*: Many products, especially foods and other biological materials, may be temperature-sensitive and degrade at higher temperatures or after prolonged heating. The amount of degradation is a function of temperature and length of time.
4. *Foaming or frothing*: In some cases, materials composed of caustic solutions, food products such as skim milk, and solutions of fatty acids form foam or froth during boiling. This foam accompanies the vapor phase and entrainment losses occur.

5. *Pressure and temperature*: The boiling point of the solution is related to the pressure of the system. The higher the operating pressure of the evaporator, the higher the temperature at boiling. Also, as the concentration of the dissolved material in solution is increased by evaporation, the boiling point increases. In order to keep the temperature low in heat-sensitive materials, it is often necessary to operate below atmospheric pressure, that is, under vacuum.
6. *Scale deposition and materials of construction*: Some solutions deposit solid materials, called scale or fouling, on the heating surfaces. These could be formed by decomposition products or due to insufficient solvent. The result is that the overall heat-transfer coefficient decreases and the evaporator must eventually be cleaned. The materials used in the construction of the evaporator are important to minimize corrosion.

Evaporator Types

There is no single type of evaporator that is satisfactory for all conditions or all kinds of materials. Heat-transfer properties, energy, and cost factors determine the choice between various types of evaporators for a particular application. Both tubular and plate-type steam-heated heat exchangers have been used. Major manufacturers of evaporators are APV Crepaco, Alfa-Laval, Cook Machinery, Dedert Corp., FMC, GEA Food and Process Systems Corp., Niro Evaporators, Signal Swenson Div., Tito Manzini, and Figi s.p.a.

Circulation evaporators

Horizontal tube natural circulation evaporator

The horizontal tube natural circulation evaporator is shown in **Figure 1**(**a**). The horizontal bundle of heating tubes is similar to the bundle of tubes in a heat exchanger. The steam enters the tubes where it condenses and the condensate leaves at the other end of the tubes. The boiling/liquid solution covers the tubes. The vapor leaves the liquid surface, often goes through some de-entraining device, such as a baffle, to prevent carryover of liquid droplets, and leaves from the top. This type is relatively cheap and is used for nonviscous liquids having a high heat-transfer coefficient and liquids that do not deposit scale. It is unsuitable for viscous liquids because circulation is poor.

Vertical-type natural circulation evaporator

In this type of evaporator, vertical rather than horizontal tubes are used; the liquid is inside the tubes and the steam condenses outside the tubes. Because of boiling and decreases in density, the liquid rises in the tubes by convection, as shown in **Figure 1**(**b**), and flows downward through a large central open space or downcomer. This natural circulation increases the heat-transfer coefficient. This type is often called the short-tube evaporator and is not used for viscous liquids.

Circulation evaporators can be used when a low degree of concentration is required or when small quantities of product are processed. In yogurt production, for example, evaporation is used to concentrate milk 1.1- to 1.25-fold, or from 13 to 14.5% or 16.5% solids content, respectively. This treatment simultaneously deaerates the product and removes off-flavors.

During the circulation evaporation process, the milk, heated to 90 °C, enters the vacuum chamber tangentially at a high velocity and forms a thin, rotating layer on the wall surface, as shown in **Figure 2**. As it swirls around the wall, some of the water is evaporated and the vapor is drawn off to a condenser. Air and other noncondensable gases are extracted from the condenser by a vacuum pump.

The product eventually loses velocity and falls to the inwardly curved bottom, where it is discharged. Part of the product is recirculated by a centrifugal pump to a heat exchanger for temperature adjustment and then to the vacuum chamber for further evaporation.

Long tube vertical-type evaporator

Because the heat transfer coefficient on the steam side is very high compared to that on the evaporating liquid side, high liquid velocities are desirable. In a long tube vertical-type evaporator, shown in **Figure 1**(**c**), the liquid is inside the tubes. The tubes are 3–10 m long and the formation of vapor bubbles inside the tubes causes a pumping action, giving quite high liquid velocities. Generally, the liquid passes through the tubes only once and is not recirculated. The contact time can be quite low in this type of evaporator. In some cases, as when the ratio of feed to evaporation rate is low, natural recirculation of the product through the evaporator is made possible by including a large pipe connection between the outlet concentrate line and the feed line. This is widely used for the production of condensed milk.

Falling film-type evaporator

A variation of the long tube type is the falling film evaporator, wherein the liquid is fed to the top of the tubes and flows down the walls as a thin film. Separation of vapor and liquid usually takes place at the bottom. This type of evaporator is widely used for concentrating heat-sensitive materials because the holdup time is very short (5–10 s or more) and the heat-transfer coefficients are high. It is also the type

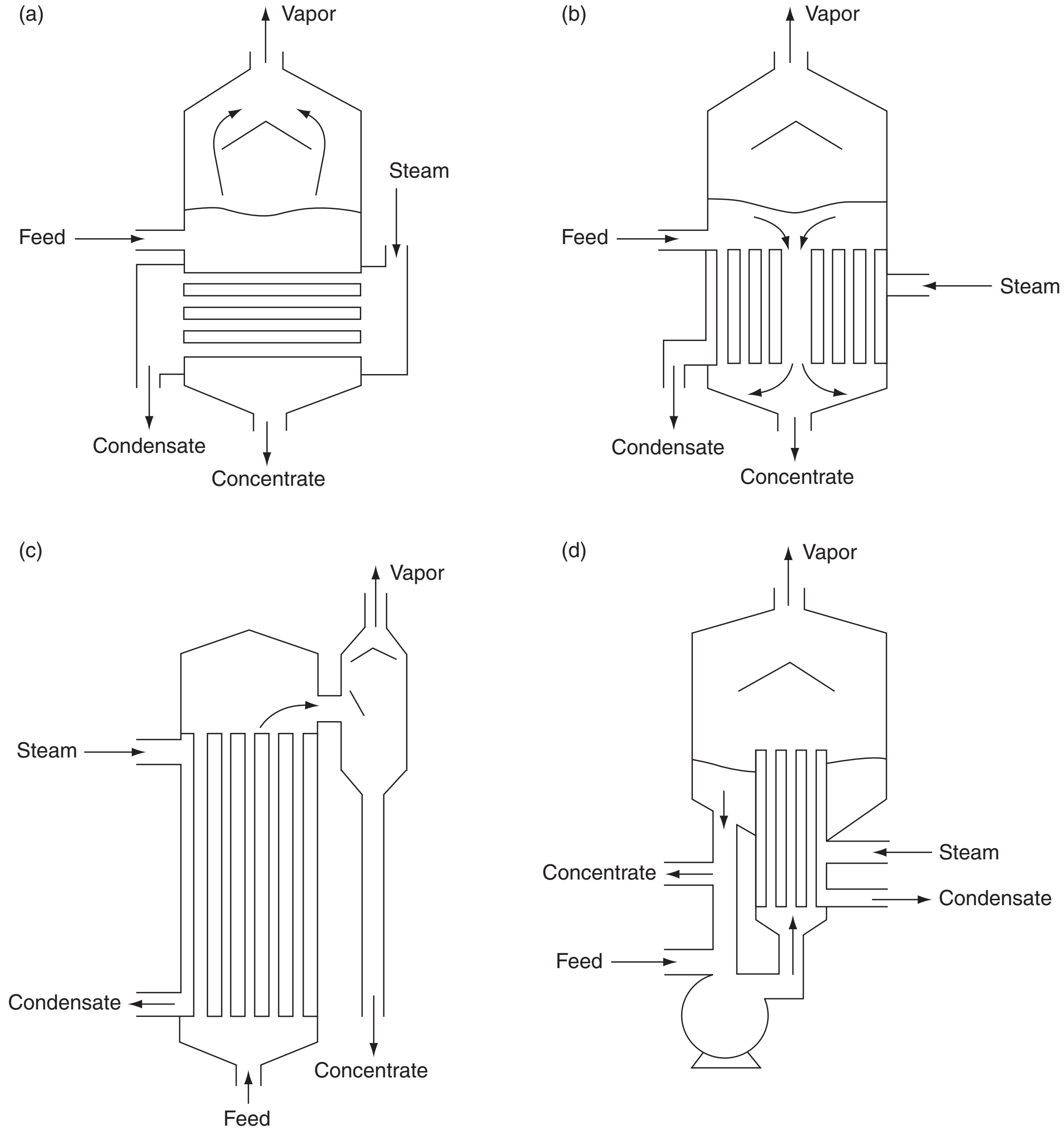
(a)
Vapor
Steam
Feed
Condensate
Concentrate
(b)
Vapor
Feed
Steam
Condensate
Concentrate
(c)
Vapor
Steam
Condensate
Concentrate
Feed
(d)
Vapor
Steam
Concentrate
Condensate
Feed

Figure 1 Types of evaporators: (a) horizontal tube type; (b) vertical tube type; (c) long tube vertical type; and (d) forced circulation type.

most often used in the dairy industry. The heating surface may consist of stainless-steel tubes or plates. The plates are stacked together, forming a pack with the product on one side of the plates and steam on the other.

Forced circulation-type evaporator

The liquid-film heat-transfer coefficient can be increased by pumping to cause forced circulation of the liquid inside the tubes. This could be done in the long tube vertical type shown in **Figure 1(c)** by including a pipe connection with a pump between the outlet concentrate line and the feed line. However, the vertical tubes are usually shorter in a forced circulation type (**Figure 1(d)**) than in the long tube type. In other cases, a separate and external horizontal heat exchanger is used. This type is very useful for viscous liquids.

Agitated film evaporator

In an evaporator, the main resistance to heat transfer is on the liquid side. One way to increase turbulence in this film, and hence the heat-transfer coefficient, is by mechanical agitation. This is done in a modified falling film evaporator with only a single large jacketed tube containing an internal agitator. This type of evaporator is very useful for highly viscous

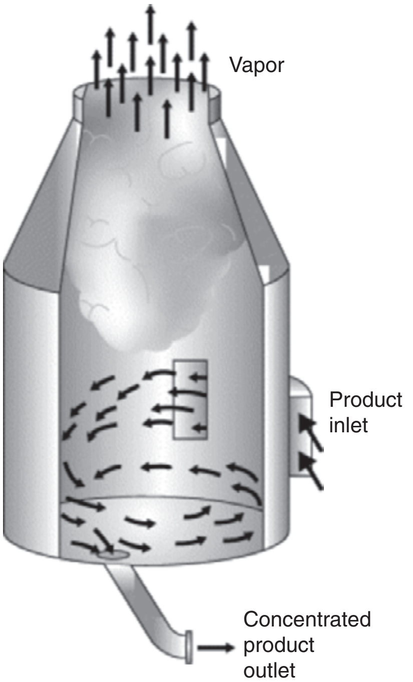

Figure 2 Product flow in a vacuum chamber. Courtesy of Tetra Pak, Sweden.

materials. However, it is expensive and of small capacity.

Modes of Operation of Evaporators

1. *Single-effect evaporators*: A simplified diagram of a single-stage (single-effect) evaporator is given in **Figure 3**. The feed enters at T_f and saturated steam enters the heat-exchange section at T_s. Condensed steam leaves as condensate or drip. Assuming that the solution in the evaporator is perfectly mixed, the concentrated product that exits the evaporator and the solution in the evaporator have the same temperature, which is T_1, because of thermal equilibrium with the boiling solution. The pressure is P_1, which is the vapor pressure of the solution at T_1. Single-effect evaporators are often used when the required capacity of operation is relatively small and/or the cost of steam is relatively cheap compared to the cost of the evaporator. However, for large-capacity operation, using more than one effect will markedly reduce steam costs.

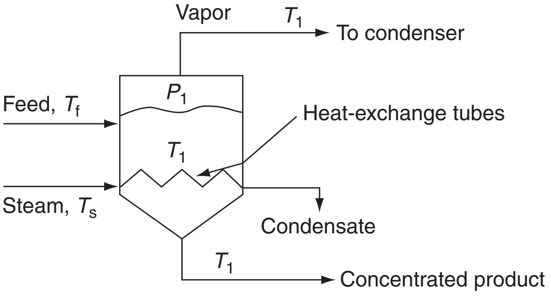

Figure 3 Simplified diagram of a single-effect evaporator.

2. *Forward-feed multiple-effect evaporators*: A single-effect evaporator, shown in **Figure 3**, is not energy-efficient because the latent heat of the vapor leaving is not used but discarded. However, much of this latent heat can be recovered and reused by employing a multiple-effect evaporation system, as shown in **Figure 4**. The first effect operates at a high enough temperature so that the evaporated water serves as the heating medium to the second effect. Here again, the evaporated water can be used as the heating medium to the third effect. Hence, the steam economy, which is kg vapor evaporated per kg steam used, is increased. This also approximately holds for a number of effects over three. However, this increased steam economy of a multiple-effect evaporator is gained at the expense of the original first cost of these evaporators. In forward-feed operation, as shown in **Figure 4**, the fresh feed is added to the first effect and flows to the next in the same direction as the vapor flow. This method of operation is used when the feed is hot or when the final concentrated product might be damaged at a high temperature. The boiling temperature decreases from effect to effect.
3. *Backward-feed multiple-effect evaporators*: In the backward-feed operation, shown in **Figure 5** for a triple-effect evaporator, the fresh feed enters the last and coldest effect and continues until the concentrated product leaves the first effect. This method of reverse feed is advantageous when the fresh feed is cold, because a smaller amount of liquid must be heated to the higher temperature in the second and first effects. However, liquid pumps are used in each effect, because the flow is from low to high pressure. This method is also used when the concentrated product is highly viscous. The high temperatures in the early effects reduce the viscosity and give reasonable heat-transfer coefficients.
4. *Parallel-feed multiple-effect evaporators*: Parallel feed in multiple-effect evaporators involves adding fresh feed and withdrawing concentrated product from each effect. The vapor from each effect is then used to heat the next effect. This method of operation is mainly used when the feed is almost saturated and the product is in the form of solid crystals, as in the evaporation of brine to make salt.

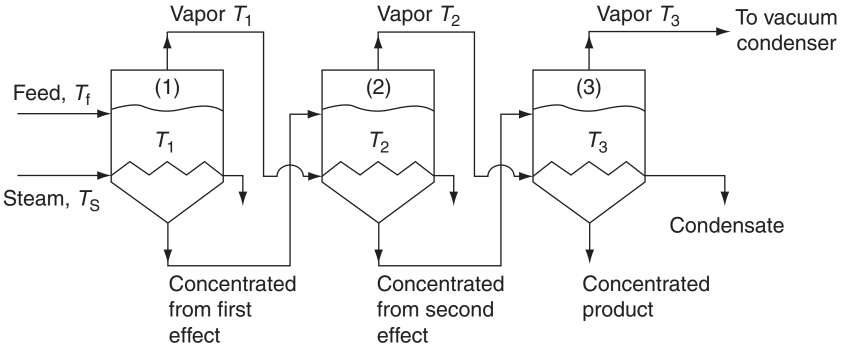
Vapor T_1
Vapor T_2
Vapor T_3
To vacuum condenser
Feed, T_f
Steam, T_S
(1)
(2)
(3)
T_1
T_2
T_3
Condensate
Concentrated from first effect
Concentrated from second effect
Concentrated product

Figure 4 Simplified diagram of a forward-feed triple-effect evaporator.

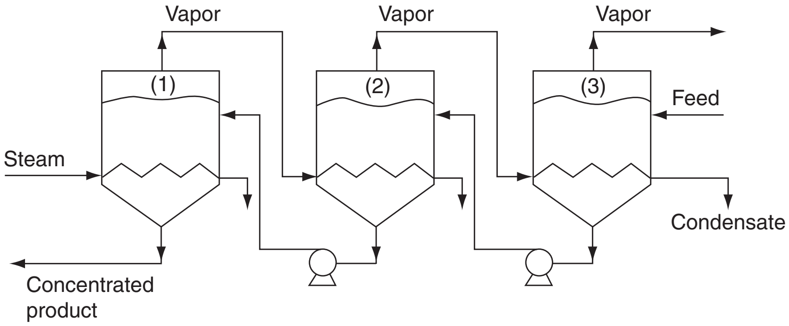
Vapor
Vapor
Vapor
(1)
(2)
(3)
Feed
Steam
Condensate
Concentrated product

Figure 5 Simplified diagram of a backward-feed triple-effect evaporator.

Heat and Material Balances for Evaporators

Single-effect evaporators

A single-effect evaporator is shown in **Figure 6**. The basic equation for solving for its capacity is

$$Q = UA\Delta T \tag{1}$$

where ΔT is the temperature difference between the condensing steam and the boiling liquid in the evaporator, Q is the heat flow, U the overall heat transfer co-efficient and A the contact surface area. In order to solve this equation, the value of Q must be determined by making a heat and material balance on the evaporator shown in **Figure 6**.

The feed to the evaporator is $\dot{m}_f$, having a solids content of w_f (mass fraction), temperature T_f, and enthalpy H_f. The concentrated liquid, $\dot{m}$, from the evaporator has a solids content of w, temperature T_1, and enthalpy H. The vapor $\dot{m}_v$ is given off as pure solvent having no solids, temperature T_1, and enthalpy H_v. Saturated steam entering is $\dot{m}_s$ and has temperature T_s and enthalpy H_s. The condensed steam leaving, $\dot{m}_s$, is usually assumed to be at T_s, the saturation temperature, with enthalpy H_c.

Total material balance:

$$\dot{m}_f = \dot{m}_v + \dot{m} \tag{2}$$

Material balance for the solute (solids):

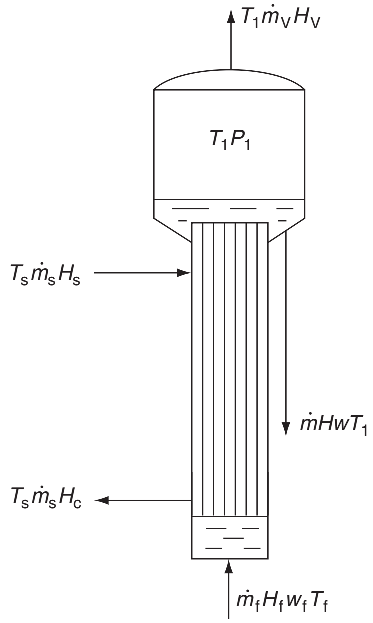
$T_1 \dot{m}_V H_V$
$T_1 P_1$
$T_s \dot{m}_s H_s$
$\dot{m} H w T_1$
$T_s \dot{m}_s H_c$
$\dot{m}_f H_f w_f T_f$

Figure 6 Simplified diagram of an evaporator.

$$\dot{m}_f w_f = \dot{m} w \tag{3}$$

Heat balance:

$$\dot{m}_f H_f + \dot{m}_s H_s = \dot{m} H + \dot{m}_v H_v + \dot{m}_s H_c \tag{4}$$

The heat Q transferred in the evaporator is

$$Q = \dot{m}_s(H_s - H_c) \quad (5)$$

Selection of Evaporators

Evaporator systems are major pieces of process equipment and are often purchased on a total responsibility basis. The purchaser's task is to define the process, quality, and mechanical limitations accurately to enable the vendor to engineer economical and suitable systems for the intended duty.

When selecting an evaporator, one should include the following considerations:

1. compare initial capital cost and operating cost
2. check to see that specifications have been met. Some important specification considerations are
   - operation capacity
   - product viscosity, suspended solids, and density
   - temperature–time profile as related to product quality
   - cooling water temperature
   - steam levels and requirements
   - materials of construction
   - condenser type
   - volatile recovery requirements
   - headroom requirements
   - any special requirements
3. Maintenance considerations
   - manual versus automatic control
   - reliability and simplicity of operation
   - sanitation, ease of cleaning, and cleaning cycle
   - safety requirements
4. Guarantees of after-sale services
   - start-up assistance
   - engineering expertise and response to problems
   - personnel training

Economics

Evaporation is one of the high capital cost and energy-intensive unit operations. During the era of low energy costs, the major selection criterion was based on the low initial equipment costs. Energy is by far the major cost factor in evaporator operations. As a result, more energy-efficient designs have gained favor for new installations.

Methods of Energy Savings

Considerable economic benefits can be gained by upgrading existing evaporator systems to reduce energy costs for evaporation. There are three general areas for modifying existing evaporators for energy saving:

1. *Fine-tuning existing evaporator*: These low-investment improvements do not change the basic evaporator layout.
2. *Modifying auxiliary hardware*: These moderate-investment changes normally can be authorized at the plant level.
3. *Major hardware modifications*: These high-investment changes normally require approval at the corporate level.

Thermocompression

The vapor evolved from the product can be compressed and used as a heating medium. This improves the thermal efficiency of the evaporator. A thermocompressor is used for this purpose. A single evaporator with a thermocompressor is as economical as a two-effect unit without one. Using thermocompression together with multiple-effect units optimizes thermal efficiency. The main components of an evaporator with thermal vapor recompression (TVR) are shown in **Figure 7(a)**.

Mechanical Vapor Compression

Unlike a thermocompressor, a mechanical vapor compression system draws all the vapor out of the evaporator and compresses it before returning it to the evaporator. The pressure increase is accomplished by the mechanical energy that drives the compressor. In mechanical vapor compression, the total amount of steam is circulated in the plant. This makes a high degree of heat recovery possible. A three-effect evaporator with mechanical vapor compression can reduce the operating costs by half compared to a seven-effect plant with a thermocompressor. The main components of an evaporator with mechanical vapor recompression (MVR) are shown in **Figure 7(b)**.

Evaporators in the Dairy Industry

Milk

Evaporators are used during the production of evaporated milk or as a step in the production of dairy powders. As shown in **Figure 8**, after preheating, the milk is evaporated under vacuum. It is of utmost importance that the evaporator works under optimal hygienic conditions, regardless of the fact that the product is sterilized afterward.

All modern milk evaporators are multiple-effect plants, and they almost always use either TVR or MVR.

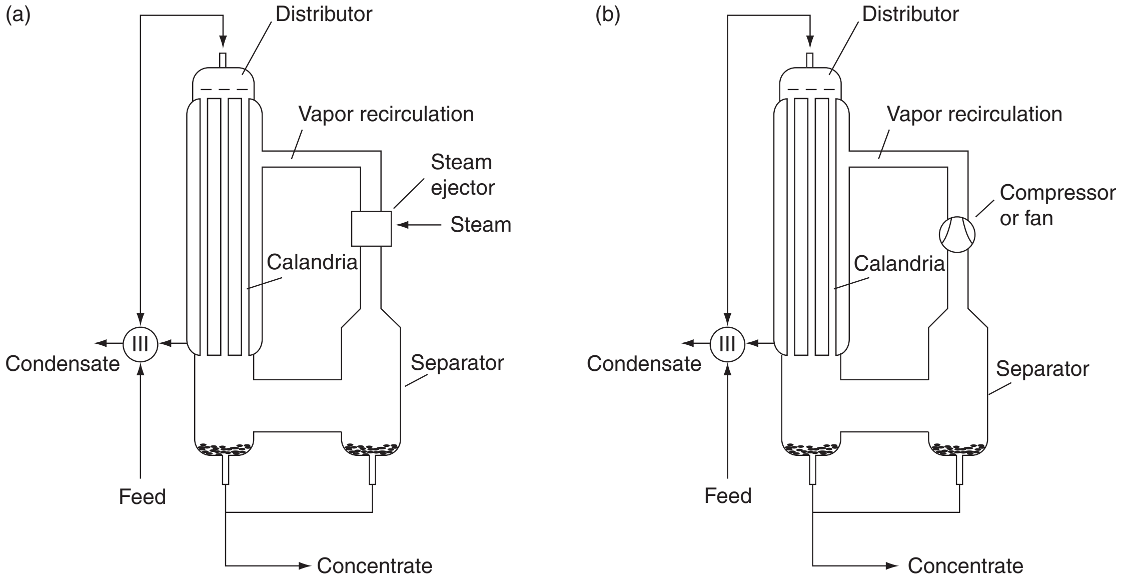
(a)
Distributor
Vapor recirculation
Steam ejector
Steam
Calandria
Condensate
Separator
Feed
Concentrate
(b)
Distributor
Vapor recirculation
Compressor or fan
Calandria
Condensate
Separator
Feed
Concentrate

Figure 7 Main components of vapor recompression evaporators: (a) thermal vapor recompression and (b) mechanical vapor recompression.

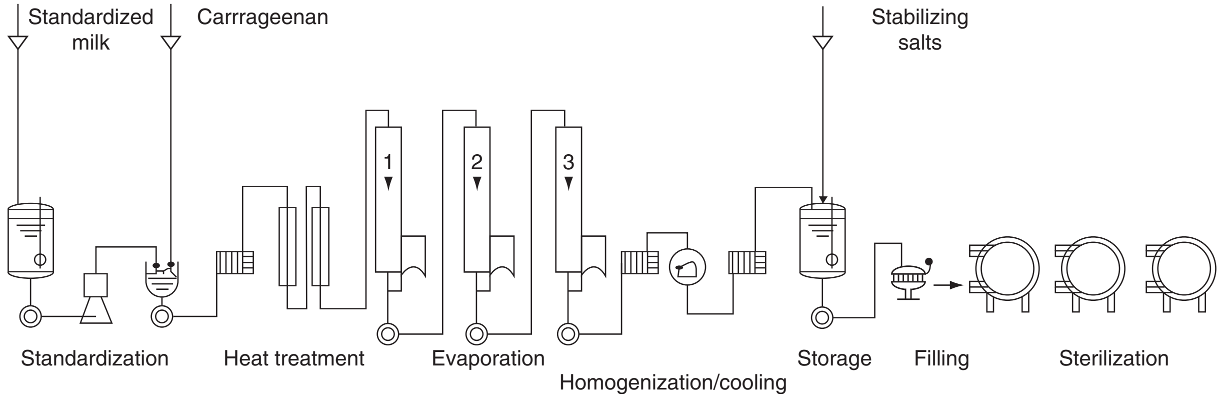
Standardized milk
Carrrageenan
Stabilizing salts
1
2
3
Standardization
Heat treatment
Evaporation
Homogenization/cooling
Storage
Filling
Sterilization

Figure 8 Basic flow diagram for the manufacture of evaporated milk.

As an example of a typical installation, consider a multiple-effect TVR unit. Prior to the milk entering the first effect, it is usually pasteurized.

Whey

The whey (typically 6% total solids) is normally evaporated, after pretreatment to remove fat (and/or cheese fines), to between 40 and 60% total solids. The choice of total solids in the final product depends on subsequent processing needs. If the whey concentrate has to be transported, 40% is preferred, to avoid crystallization taking place en route and causing unloading difficulties. Alternatively, when the whey is to be spray-dried (on-site), 60% is better. Both TVR and MVR falling

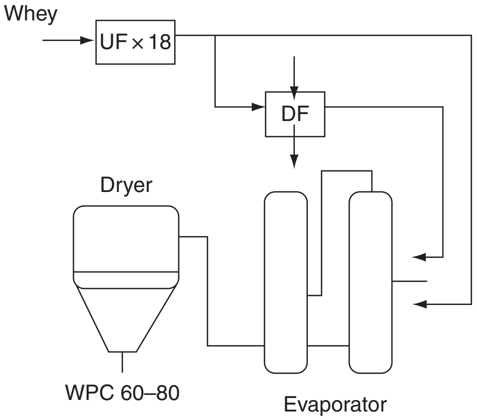
Whey
UF × 18
DF
Dryer
WPC 60–80
Evaporator

Figure 9 Production of whey protein concentrate (WPC). DF, diafiltration; UF, ultrafiltration.

film equipment can be used in this application as well as others, with the latter configuration usually giving better energy economy.

An advanced case of application is in the production of whey protein concentrate. As shown in **Figure 9**, membrane technology and evaporation are combined for protein recovery and the subsequent concentration of the protein solution.

See also: **Concentrated Dairy Products**: *Dulce de Leche*; Evaporated Milk; Sweetened Condensed Milk. **Plant and Equipment**: Corrosion; Flow Equipment: Pumps; Heat Exchangers; Process and Plant Design. **Whey Processing**: Utilization and Products.

Further Reading

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Milk Dryers: Drying Principles

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Introduction

Drying is defined as the unit operation in which volatile liquids are separated from solids by vaporization to yield (nearly) liquid-free products.

A number of different drying processes are in use in the dairy, food, chemical, and pharmaceutical industries, such as

- spray drying,
- fluid bed drying,
- drum drying,
- batch drying in trays,
- freeze drying,
- microwave drying, and
- superheated steam drying.

As the liquid to be removed in the dairy industry is invariably water, only water evaporation into atmospheric air is considered here, although the drying principles involved in the evaporation of nonaqueous, organic solvents into any controlled gaseous atmosphere are the same.

From the point of view of drying economy and the final product quality, the processes of major significance in milk powder manufacture are spray drying, fluid bed drying, and drum drying. Spray drying and fluid bed drying are most often used in combination and drum drying is only of limited use. Only spray drying and fluid bed drying processes will be discussed here.

Spray Drying

The basic principle of spray drying is exposure of a fine dispersion of droplets, created by means of atomization of pre-concentrated milk products, to a hot airstream. The small droplet sizes created, and hence a large total surface area, result in very fast evaporation of water at relatively low temperatures. Hence, heat damage to the product is minimized.

The spray drying process for milk drying comprises essentially five subprocess stages: (1) atomization of the feed, (2) mixing of spray and drying air, (3) evaporation, (4) separation of the product from the drying air, and (5) cooling of the powder. The last two points will be discussed elsewhere (*see* **Plant and Equipment**: Milk Dryers: Dryer Design).

Each of these process stages can be carried out in different ways, depending on the plant design, which in combination with operational conditions and other features, such as integrated and/or external fluid beds and destination of fine powder from powder separators, in turn determine the characteristics and properties of the final powder.

Atomization of the Feed

Atomization of the feed, that is, formation of a spray, is the characteristic feature of spray drying. The purpose of atomization is to create a large number of small-diameter droplets. Assuming a completely homogeneous spray, the total droplet surface area is inversely proportional to the droplet diameter, and the number of particles inversely proportional to the square of the droplet diameter. **Table 1** shows the number of droplets and the total surface area created by homogeneous atomization of 1 l of concentrate to different droplet sizes.

Rotary atomizers and pressure nozzles are in use in the milk powder industry, the former utilizing centrifugal energy and the latter pressure energy in the atomization process. The design of atomization devices is discussed elsewhere (*see* **Plant and Equipment**: Milk Dryers: Dryer Design).

Rotary atomizers (wheel atomization)

In rotary atomizers, the feed is accelerated to the applied peripheral speed of the atomizer wheel, which is typically in the range of 150–170 $\mathrm{m\,s^{-1}}$. The feed is introduced centrally around the atomizer shaft via a liquid distribution device. A number of different liquid distributor designs have been developed over the years, which emphasizes the importance of this component for optimum and trouble-free operation of the atomizer (absence of vibrations and product deposits).

The efficiency of atomization (droplet size) depends on a number of factors:

Table 1 Number of droplets and total surface area created by atomization of 1 l of feed

Mean droplet diameter (μm)	*Number of droplets per liter* ($\times 10^6$)	*Total surface area* (m^2)
500	15.3	12
250	122.2	24
100	1909.9	60
75	4527.1	80
50	15 278.9	120
10	1 909 859.3	600

- higher peripheral speed, vane height, and number of vanes decrease the mean droplet size; and
- higher rate, viscosity, and surface tension of the feed increase the mean droplet size.

The power consumption of a rotary atomizer is directly proportional to the feed rate and to the square of the peripheral speed of the wheel.

The main advantages of rotary atomizers are the following:

- very flexible with respect to feed rate and feed viscosity;
- higher feed solids concentrations can be handled, hence higher product capacity and better economy;
- no fouling or blockage problems;
- high-capacity units available (up to 200 tonnes h^{-1}, although not relevant in the milk powder industry);
- advantageous for abrasive, crystal-containing feeds like precrystallized whey or permeate; and
- different powder properties achievable with different wheel design.

Nozzle atomization

The nozzles used in spray drying are of the centrifugal pressure type, in which pressure energy is converted into kinetic energy of a thin liquid sheet with a partly rotational motion, which causes the spray pattern to be of the 'hollow cone' type.

Pressures in the range of 180–250 bar are used for most dairy products. However, pressures as high as 500–600 bar have been applied occasionally for highly viscous feeds, such as sodium caseinate.

The volumetric flow rate of a nozzle is directly proportional to the square root of the pressure:

$$Q = ANF\sqrt{p} \qquad (1)$$

where Q is the flow rate (m^3 h^{-1}), A the nozzle capacity factor for water, F the 'viscosity factor' (0.9 is used for most milk concentrates), p the nozzle pressure (bar), and N is the number of nozzles.

The effects of operating parameters on the efficiency of nozzle atomization (droplet size) are as follows:

1. higher capacity of nozzles, higher viscosity and surface tension of the feed, and larger orifice diameter (other parameters are constant) will increase the droplet size; and
2. higher pressure and wider spray angle will reduce the droplet size.

The power consumption of the high-pressure pump is directly proportional to the feed rate and nozzle pressure.

The main advantages of nozzle atomization are the following:

1. minimum aeration of the feed during atomization, hence virtually air-free particles and higher particle density. Typical particle densities of whole milk powder with different types of atomization are
   a. straight vane wheel atomization: 1.14 g cm^{-3},
   b. curved vane wheel atomization: 1.18 g cm^{-3},
   c. nozzle atomization: 1.23 g cm^{-3};
2. improved powder flowability;
3. possibility of individual directions of sprays from each nozzle in multinozzle installation, hence improved control of agglomeration; and
4. less fouling of dryers producing 'difficult' products.

The main disadvantages of nozzle atomization are the following:

1. inflexible to variation in throughput, as it affects the nozzle pressure and hence the atomization efficiency;
2. fairly low feed capacity per nozzle, ideally not more than 2000 kg h^{-1} and preferably lower;
3. multinozzle arrangements required for larger plants, resulting in more complicated plant start/stop procedures;
4. fouling with deposited 'milk stone' – particularly at higher feed temperatures – causing a gradual increase in nozzle pressure at a constant throughput; and
5. wear of orifice and swirl chamber/core grooves limiting the lifetime of the components. Depending on the operating conditions and product type, the nozzle insert parts (made of tungsten carbide) should be renewed after 400–800 h of operation.

Mixing of Spray and Drying Air

The air disperser and the atomizing device are the most vital components in a spray dryer. It has been metaphorically claimed that they are 'the lungs and the heart' of a spray dryer. Consequently, the air disperser design has to be in unison with the atomization device and the desired airflow pattern in the drying chamber. Air disperser design and airflow patterns in different types of dryers are discussed elsewhere (*see* **Plant and Equipment**: Milk Dryers: Dryer Design).

In recent years, the development of computerized fluid dynamics (CFD) software and the emergence of required powerful computers have provided a new tool for the study of airflow, temperature profile, and particle paths in spray dryers. Particularly, the newer three-dimensional (3D) CFD software combined with the specific drying characteristics of the product in question has proven to be a very powerful tool in the design of dryers and in troubleshooting. **Figure 1** shows examples of 3D CFD simulations.

Evaporation

Similar to the drying air, the product to be dried (the feed) undergoes dramatic changes in its physical properties during the drying process. Before entering into a more detailed discussion of the actual evaporation process, the definition of certain terms is required.

Properties of air/water vapor mixture

Partial water vapor pressure

Partial water vapor pressure is the pressure the water vapor would exhibit if existing alone in the same volume and at the same temperature. The sum of the partial pressures of all gaseous components is equal to the total pressure of the mixture. The relation between partial pressure, p_w (Pa), absolute humidity, x (kg kg^{-1} water vapor in dry air), and total air pressure, p_{total} (Pa), is

$$p_w = x\frac{p_{\text{total}}}{x + 0.622} \tag{2}$$

Saturated water vapor pressure

Saturated water vapor pressure is the maximum vapor pressure at a given temperature exhibited by air saturated with water vapor. The extended Antoine equation describes the relation between saturated water vapor pressure (Pa) and temperature (°C):

$$P_{\text{sat}} = \exp\Big(72.55 - \frac{7206.7}{T + 273.15} - 7.1385 \times \ln(T + 273.15) + 0.000\,004\,046 \times (T + 273.15)^2\Big) \tag{3}$$

where T is the temperature in °C.

Relative humidity

Relative humidity (RH) is the water vapor content relative to the content at saturation at the same temperature, expressed as %:

$$\text{RH} = \frac{p_w}{p_{\text{sat}}} \times 100 \tag{4}$$

Properties of the humid air can be shown in a humidity chart (or psychrometric chart, Mollier diagram, I-X diagram) (**Figure 2**).

In the diagram, which is valid only for an atmospheric pressure of 1013 hPa, the water vapor content in g kg^{-1} dry air is plotted on the abscissa and the temperature on the ordinate. The parallel, sloped lines are curves of enthalpy in kcal kg^{-1} dry air and finally the curved lines show the RH. By knowing two of the

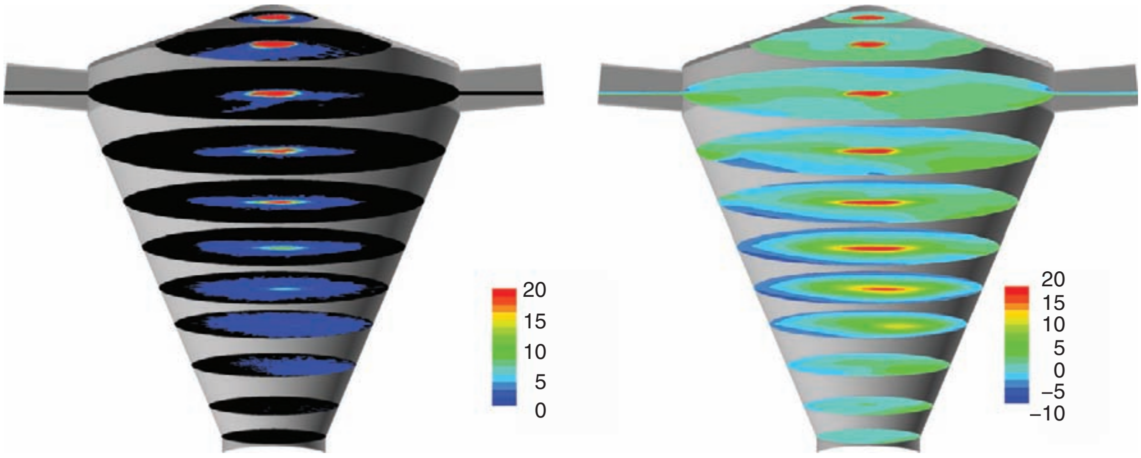

Figure 1 Three-dimensional computerized fluid dynamics simulations of an MSD™ dryer. Left: Axial air velocities (m s^{-1}); right: evaporation rate (g m^{-3} s^{-1}).

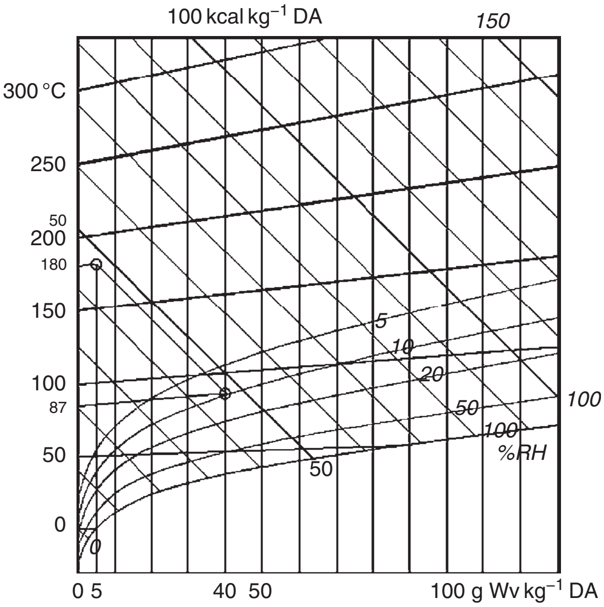

Figure 2 Humidity chart showing a spray drying process. Ambient air with 5 g moisture per kg dry air is heated to 180 °C. It is assumed that adiabatic drying to a relative humidity (RH) of 10% in the exhaust air, which is assumed to give the required final product moisture content, results in an outlet temperature of 87 °C. Wv, water vapor; DA, dry air.

four parameters (for instance, the temperature and RH, which are easily measured), the other two can be found from the diagram.

Common drying terms

The moisture in the feed is present as (1) unbound moisture or (2) bound moisture. The physicochemical properties of the product determine the relation between the two types. Bound moisture is bound to the solids by bonds of different strength. Water of crystallization in α-lactose monohydrate, ionic bonds in minerals and proteins, and hydrogen bonds in proteins and lactose are examples of different bond strengths. Bound water exerts a water vapor pressure lower than that of pure water. A product containing bound water is said to be hygroscopic. In a nonhygroscopic material, all water is unbound.

Equilibrium moisture is the moisture content of the product when it is in equilibrium with the partial water vapor pressure in the surrounding air. Free moisture is the moisture in excess of the equilibrium moisture. A curve showing the relation between moisture content and the RH in the surrounding air at constant temperature is called a sorption isotherm.

Figure 3 shows graphically the relationships between the different terms in an ideal case. In practical spray drying, the equilibrium moisture content is never reached due to variations in temperature and humidity inside the drying chamber and limited residence time.

Properties of the feed

Moisture content and solids content

The moisture content of the feed is most often expressed as % (w/w). The solids content equals 100−moisture content. However, in drying theory, moisture content on dry basis (DB), which is the weight of moisture per unit weight of solids, is often used:

$$\text{moisture content (DB)} = \frac{\text{moisture content (g)}}{\text{solids content (g)}} \tag{5}$$

Water activity (equilibrium relative humidity)

The water activity expresses the moisture content of a product as the RH of the surrounding air with which the product is in equilibrium:

$$a_{\mathrm{w}} = \frac{p_{\mathrm{w}}}{p_{\mathrm{sat}}} \tag{6}$$

Drying

The drying rate and the droplet temperature during drying depend on whether bound or unbound moisture is being evaporated. As long as unbound moisture is available at the droplet surface, the partial water vapor pressure in the interface between particle and air will be near the saturated vapor pressure at the droplet temperature, the drying will proceed at a near-constant rate, and the droplet temperature will be near the wet-bulb temperature. At the critical moisture content, the diffusion of moisture inside the particles is no longer sufficient to maintain saturated conditions on the particle surface. The result is a gradual decrease in vapor pressure and drying rate and a concomitant increase in the droplet temperature. These drying characteristics are shown in **Figure 4**.

It can be seen that the product temperature never reaches the outlet temperature (T-out) from the dryer and that the residual moisture content is higher than the equilibrium moisture content for the reasons mentioned above.

Changes in the state of drying air

The humidity chart is also useful in illustrating the changes that the air undergoes during the spray drying process. **Figure 2** shows an example where the ambient air with an absolute humidity of 5 g kg^{-1} is heated to 180°C. If a change of state of the air during drying takes place without any heat exchange with the surroundings, the enthalpy of the system will not change and hence follow a line parallel to the enthalpy lines, and the change is said to be adiabatic. The drying process shown is assumed to be adiabatic. However, in a real situation in a spray dryer, this is not quite the case. Heat will be added to the system with the warm concentrate ($T \neq 0$°C), but on the other hand, heat is also removed from the system

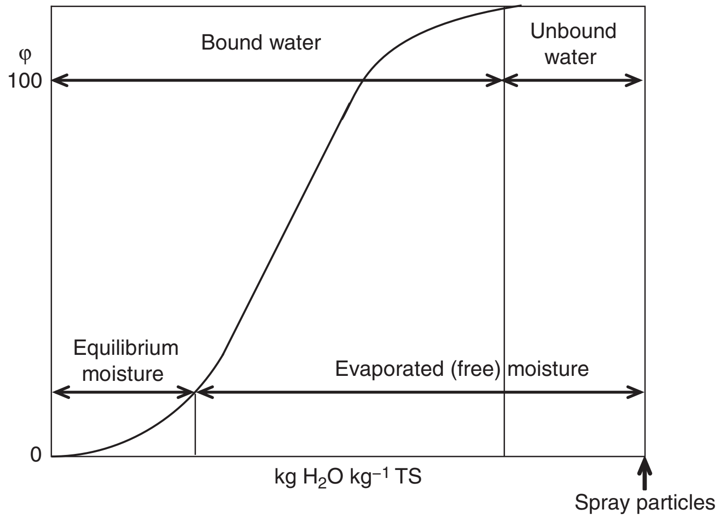

Figure 3 Sorption isotherm and common drying terms.

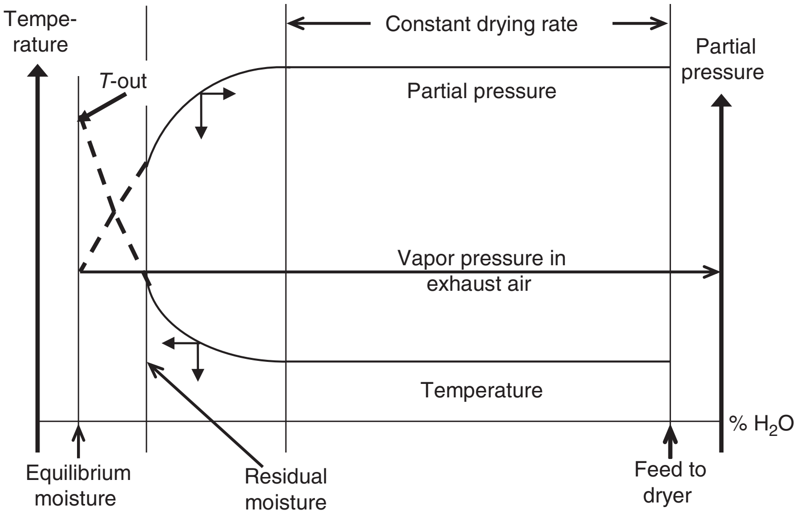

Figure 4 Temperature and vapor pressur vs. moisture content during drying.

by transmission loss and with the warm powder leaving the dryer. It is assumed that an RH in the exhaust air of 10% results in the required powder moisture content, which corresponds to a *T*-out of 87°C.

Any additional airflow to the dryer, such as air disperser and atomizer cooling air or integrated fluid bed air, will also have an effect, but for the situation in a conventional dryer, the adiabatic change as shown in **Figure 2** is a reasonable approximation.

If the drying air rate of a plant is known, the evaporative capacity can be estimated under any given drying conditions (ambient humidity, inlet and outlet temperature). In the example in **Figure 2**, the ambient humidity is 5 g kg^{-1} dry air and the outlet air humidity is 40 g kg^{-1}, that is, the evaporation is 35 g kg^{-1} dry air, which multiplied by the air rate yields the evaporative capacity of the plant. Likewise, the heat input required to increase the temperature from ambient, say 20°C, to the inlet temperature (*T*-in) can be read. The enthalpy of air at 20°C and absolute moisture content of 5 g kg^{-1} dry air is approximately 8 kcal kg^{-1} dry air. After heating to 180°C, the enthalpy is approximately 47 kcal kg^{-1} dry air, that is, a heat input of $47-8=39$ kcal kg^{-1} dry air is required. It can further be calculated that the specific heat consumption is $35/39 \times 1000 \sim 1115$ kcal kg^{-1} water evaporation.

Change of state of droplets

When pure water is dried, the water droplets will reach the wet-bulb temperature in the initial stage of drying. However, the presence of dissolved and/or dispersed solids in the droplets causes the water activity (a_w) of the drying product to decrease as drying proceeds. The driving force in the drying process is the difference between the partial vapor pressure in the droplet/air interface and the partial vapor pressure in the surrounding air or (roughly) between a_w of the product and the RH of the drying air. Any decrease in a_w of the particles or increase in RH of the drying air will reduce the driving force.

The relation between a_w (or equilibrium RH) and moisture content at constant temperature is called a sorption isotherm. As hysteresis effects are quite common, it is important to differentiate between absorption and desorption isotherms in connection with drying. A typical shape of a sorption isotherm is shown in **Figure 3**.

In practice, the minimum outlet temperature from a spray dryer (the highest RH of the outlet air), and the highest capacity and best drying economy without getting excessive deposits, depends mainly on the corresponding a_w. If the particle and gas residence time were infinite, equilibrium between the product and drying air would be reached. However, this is obviously not the case and the outlet air RH must be kept well below the product a_w to achieve the desired powder moisture content and achieve trouble-free dryer operation. In **Figure 2**, it can be seen that the RH at a given absolute humidity is highly dependent on temperature, so the lower RH is achieved by operating at increased outlet temperatures.

The outlet temperature is a very important process parameter, and to cope with smaller changes in other key parameters and still maintain constant product moisture content, the following guideline can be given:

$$\Delta T\text{-out} = \frac{\Delta T\text{-in}}{10} + \Delta\%\text{TS} + \frac{\Delta x\text{-amb}}{2.8} - K\Delta \text{H}_2\text{O} \quad (7)$$

whereΔT-out is the required change in outlet temperature, ΔT-in is the change in inlet temperature, Δ%TS is the change in percent total solids in the concentrate, Δx-amb is the change in ambient humidity in g kg^{-1} dry air, K is a product-dependent factor, which is about 5 for skimmed milk powder and 6 for whole milk powder, and ΔH_2O is the change in powder moisture content. It can be seen that an increase in T-in of 10°C, an increase in %TS of 1%, and an increase in absolute humidity of $2.8\,\text{g kg}^{-1}$ should be compensated for with an increased T-out of 1°C.

Fluid Bed Drying

A fluid bed is basically a box, divided by a perforated air distributor plate into a lower clean air plenum section and an upper product section. Different types of fluid beds are used:

- back-mix or plug-flow fluid beds;
- stationary or vibrated fluid beds; and
- external fluid beds or fluid beds integrated in the drying chamber.

Stationary plug-flow beds are used for products that are easily fluidizable at the inlet conditions of the bed. On the other hand, back-mix beds are used for products that are not directly fluidizable, but may be so when mixed and conditioned with the (partly) dry powder already present in the fluid bed.

Fluid bed design and technology will be discussed elsewhere (*see* **Plant and Equipment**: Milk Dryers: Dryer Design).

Originally, the external, vibrating plug-flow fluid beds were implemented for cooling purposes only, but the advantages of using them for the drying of the last amount of moisture as well were soon realized. When the drying takes place in one stage in the drying chamber, the outlet temperature has to be kept fairly high to maintain the required driving force to achieve the desired final moisture content during the fairly short residence time (10–30 s) in the drying chamber. The residence time in fluid beds is typically in the range of 10–20 min, thus allowing the time-dependent diffusion of moisture to the particle surfaces to take place.

Stickiness and Glass Transition

Powder buildup on drying chamber walls is a well-known phenomenon, caused by certain product properties described with terms such as stickiness or thermoplasticity.

Thermoplasticity is a very descriptive term that implies that the product plasticizes at elevated temperatures. It is well known that increased powder moisture increases the stickiness of the products. The relation between powder moisture and T-out, influenced by other drying parameters as well, is shown in eqn [7]. It shows that increased T-in or %TS – changes that increase the plant capacity – will result in increased powder moisture content and potentially in powder buildup if not compensated for by T-out. Increased ambient air humidity will have the same effect. However, T-out can only be increased within limits, partly for product quality reasons, but also because the powder particles become sticky at a certain T-out due to thermoplastic behavior, even though the moisture content may be quite low. This phenomenon is shown in **Figure 5**. It should be emphasized that the sticking curve is a product property that depends on product composition, whereas the T-out–moisture relation (and hence T-particle–moisture relation)

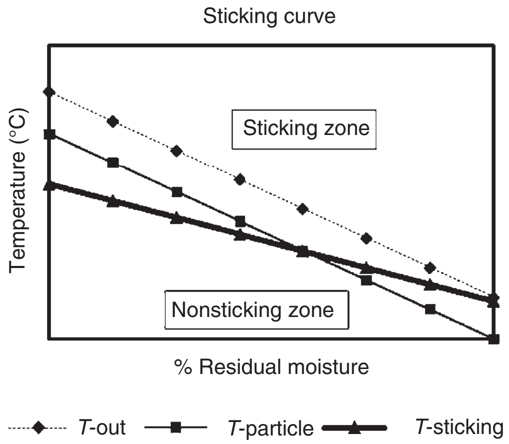

Figure 5 Empirical sticking curve. Relationship between moisture content, outlet air temperature, particle temperature, and the sticking point temperature.

depends on product properties (composition) as well as drying conditions (*T*-in, %TS, etc.).

The sticking curve approach described above is a rather empirical one. The use of the glass transition concept in the last decade has formed a more theoretical basis for understanding the phenomena.

A glass is an amorphous, high-viscous liquid in a non-equilibrium state, exhibiting the mechanical properties of a solid, but with structural characteristics of a liquid, that is, contrary to the crystalline state, a glass is without any ordered molecular arrangement.

Due to the non-equilibrium state, a glass is thermodynamically unstable and can undergo phase transitions. The glass transition in amorphous systems is a reversible change in the physical state from a mechanically solid glass to a viscoelastic, rubbery state, which takes place at the characteristic glass transition temperature, T_g. T_g of a product depends on the product composition and in particular on the presence of plasticizers, of which water is very potent, that is the T_g declines drastically at increased moisture content.

In spray-dried milk powders, the lactose is usually in an amorphous state because the drying took place so fast that there was no time for the molecular ordering required for crystallization of the lactose. Hence milk powders exhibit glass transition. Although the glass transition temperature of a milk powder is not identical to the 'sticking temperature', defined as the temperature at which powder buildup in dryers may occur, there is still a relation between T_g and the sticking temperature. Indications are that the 'sticking' temperature is about 20–25°C above the T_g.

Glass transition is accompanied by measurable physical changes in viscosity, heat capacity, and other properties. The change in heat capacity can be measured by differential scanning calorimetry.

Models describing T_g in binary systems (one component and a plasticizer) are available. The Gordon–Taylor model [8] has been used extensively, being a generalization of the Couchman–Karasz equation [9].

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \tag{8}$$

$$T_g = \frac{w_1 \Delta C_{p1} T_{g1} + w_2 \Delta C_{p2} T_{g2}}{w_1 + w_2 \Delta C_{p2}} \tag{9}$$

where w_i is the weight fraction of component i, T_{gi} the glass transition temperature of component i, k is the softening constant, and ΔC_{pi} the specific heat change of component i across the glass transition.

Table 2 shows the glass transition temperature of some food components. It can be seen that lower molecular weight carbohydrates exhibit lower T_g (monosaccharides < disaccharides, and the higher the DE (dextrose equivalent) of maltodextrins, the lower the T_g). The high T_g of starch and low-DE maltodextrins explains why these components are good 'carriers' for more difficult components in spray drying. The extremely low T_g of lactic acid also explains the difficulties of drying acid whey.

Although the relation between a_w of milk powders and T_g is essentially sigmoidal, it has been found to be almost linear in the a_w range of 0.2–0.65,

$$T_g = -143.6 a_w + 77.8 \tag{10}$$

Knowledge of the parameters in eqn [7] for a given product and drying process together with knowledge of the corresponding desorption isotherm and the glass transition temperature as a function of a_w can be very helpful in defining optimized but still safe drying conditions.

Table 2 Glass transition temperature of different food components

Component	T_g *(°C)*
Fructose	5
Glucose	31
Galactose	32
Sucrose	62
Maltose	87
Lactose	101
Maltodextrin DE 36 (MW ~550)	100
Maltodextrin DE 25 (MW ~720)	121
Maltodextrin DE 20 (MW ~900)	141
Maltodextrin DE 10 (MW ~1800)	160
Maltodextrin DE 5 (MW ~3600)	188
Starch	243
Lactic acid	−60
Water (amorphous)	−135

MW, Molecular Weight, g mol^{-1}.

See also: **Plant and Equipment**: Milk Dryers: Dryer Design.

Further Reading

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Milk Dryers: Dryer Design

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Introduction

This article focuses on spray dryers. Although other means of drying are possible, this is by far the most common in the dairy industry.

By definition, spray drying is the transformation of a product from a fluid state into a dried form by spraying the liquid feed into a hot drying medium. The feed can be a solution, a suspension, or a paste, depending on the characteristics of the dairy product to be dried. The dried product is a powder consisting of single particles or agglomerates, all depending on the chemical composition and physical properties of the feed as well as on dryer design and operation.

Drying Principles

A spray dryer operates in the following way:

The feed is pumped from the product feed tank to the atomizing device that is situated in the air disperser at the top of the drying chamber. The drying air is drawn from the atmosphere via a filter by a supply fan and is passed through the air heater to the air disperser. As the atomized droplets meet the hot air, evaporation takes place cooling the air at the same time. After the drying of the atomized feed in the chamber, the majority of the dried product falls to the bottom for further processing. The fines, which are the particles with a small diameter, will remain entrained in the air. Therefore, the air has to pass through powder collectors like cyclones or bag filters. The air passes from the powder collector to the atmosphere via the exhaust fan. The two fractions of powder are collected, for example, in a pneumatic system for conveying and cooling. After separation in a cyclone, the powder is bagged off.

A conventional spray dryer consists of the following main components (**Figure 1**):

1. Drying chamber
2. Hot air system and air distribution
3. Feed system
4. Atomizing device
5. Powder separation system
6. Pneumatic conveying and cooling system
7. Integrated fluid bed
8. Fluid bed after-dryer/cooler

Drying Chamber

Various designs of the drying chamber are available on the market. The most common one is a cylindrical chamber with a cone of 40–60°, enabling the powder to leave the chamber by gravity. The chamber is also found with a flat bottom in which case a scraper or suction device is needed for removing the powder fraction from the chamber. Horizontal box-type drying chambers are also used, and they, too, operate with a forced (i.e., scraper or screw) powder removal system (**Figure 2**).

Generally, it can be concluded that chambers with a cone for gravity discharge of the powder give the best flexibility for adapting various drying processes like integrated fluid beds or belts to the plant and therefore offer the greatest possibility for drying different products. The tendency in modern designs of drying chamber is to avoid any object inside the chamber that can obstruct the air flow.

In the chamber of the TALL FORM™, the emphasis has been put on designing a plant with a laminar air flow and a special air outlet system, where the diameter of the cone is bigger than the diameter of the cylindrical part thus forming a ring duct termed 'bustle'. This minimizes the cyclone fraction by the low velocity of the exhaust air. This chamber is especially suited for infant milk formulae or protein products dried from low-solid content feed.

The drying chamber should always be equipped with inspection doors and overpressure vents to withstand a pressure of 1.6 mbar(g). Other safety equipment such as fire extinguishing equipment in the form of water or steam nozzles is always standard in a modern dryer.

Drying chambers are usually insulated, either with removable air-filled sandwich panels (see **Figure 3**) or with 80–100 mm mineral wool covered with a stainless-steel plate. The advantage of the removable panels is that inspection for cracks in the chamber wall is possible. Furthermore, the risk of having wet insulation material, which can foster bacterial development or cold spots on the chamber wall, is eliminated.

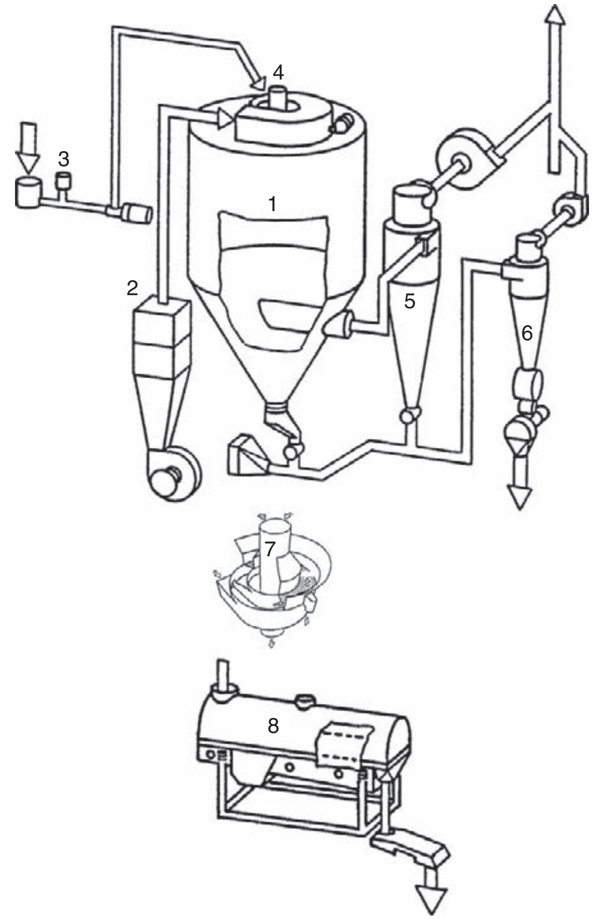

Figure 1 Spray drying plant. 1, Drying chamber; 2, Hot air system and air distribution; 3, Feed system; 4, Atomizing device; 5, Powder separation system; 6, Pneumatic conveying and cooling system; 7, Integrated fluid bed; 8, Fluid bed after-dryer/cooler.

Integrated Static Fluid Bed

In an attempt to improve the drying efficiency, a static fluid bed is integrated in the drying chamber. The secondary drying air, typically 25% of the main drying air, is introduced into a plenum chamber below a perforated plate, through which the drying air is distributed. This type of dryer can be operated in such a way that the primary particles reach a moisture level higher than that obtained by using the VIBRO-FLUIDIZER™.

A specially designed and patented perforated plate, the BUBBLE PLATE™ (see **Figure 4**), provides an air–powder mixture that ensures optimal drying without attrition and powder penetration into the clean-air plenum. Furthermore, the BUBBLE PLATE™ has a more sanitary finish than the other types of perforated plates.

The static fluid bed is available in two configurations:

- Ring-formed fluid bed (compact drying chamber)
- Circular fluid bed (multistage drying (MSD) chamber)

Ring-formed fluid bed (compact drying chamber)

The ring-formed back-mix bed is placed at the bottom of a conventional chamber cone around the exhaust duct placed in the center. The powder is discharged continuously from the static fluid bed by overflowing an adjustable powder weir, thus maintaining a certain level of fluidized powder. When the powder leaves the drying chamber it may be cooled in a pneumatic conveying or VIBRO-FLUIDIZER™ system. The resulting powder will consist of single particles.

For fat-containing products, cooling should be done in a vibrating fluid bed that is also used when agglomerated powders are produced. In this case, the cyclone fraction is returned to the atomizer device for agglomeration (see **Figure 5**).

Circular fluid bed (multistage drying (MSD™) chamber)

To improve the dryer efficiency and the powder properties even further, the multistage dryer MSD™ has been designed (see **Figure 6**).

The dryer operates with three drying stages, each adapted to the moisture content prevailing during the drying process. In the preliminary drying stage, the concentrate is atomized by co-current nozzles or a rotary atomizer placed in the hot-drying air duct. Air enters the dryer vertically through the air disperser, ensuring optimal mixing of the atomized droplets with the drying air. The particles reach a moisture content of 6–15%, depending upon the type of product. The fluid bed is supplied with air at a sufficient velocity and temperature for the second-stage drying. The drying air from the preliminary drying stage and the integrated fluid bed leaves the chamber from the top passing through powder separators.

This type of dryer offers a perfect choice if the aim is to produce an agglomerated product. Owing to the velocity of the primary drying air, a venturi is formed around the atomizing device, thus sucking in secondary air with powder entrained so that agglomeration is facilitated, that is, attrition between the primary spray particles and the fines powder.

When the powder has reached a certain moisture content it is discharged via a rotary valve into a VIBRO-FLUIDIZER™ for the final drying and subsequent cooling.

The powder exhibits a coarse powder structure originating from the natural agglomeration taking place in the chamber.

Hot Air System and Air Distribution

Air Filtration System

Until a few years ago, no special requirements were placed on filtration of the process air for the spray drying process. Today, however, very strict requirements are presented by local authorities to ensure a cleaner

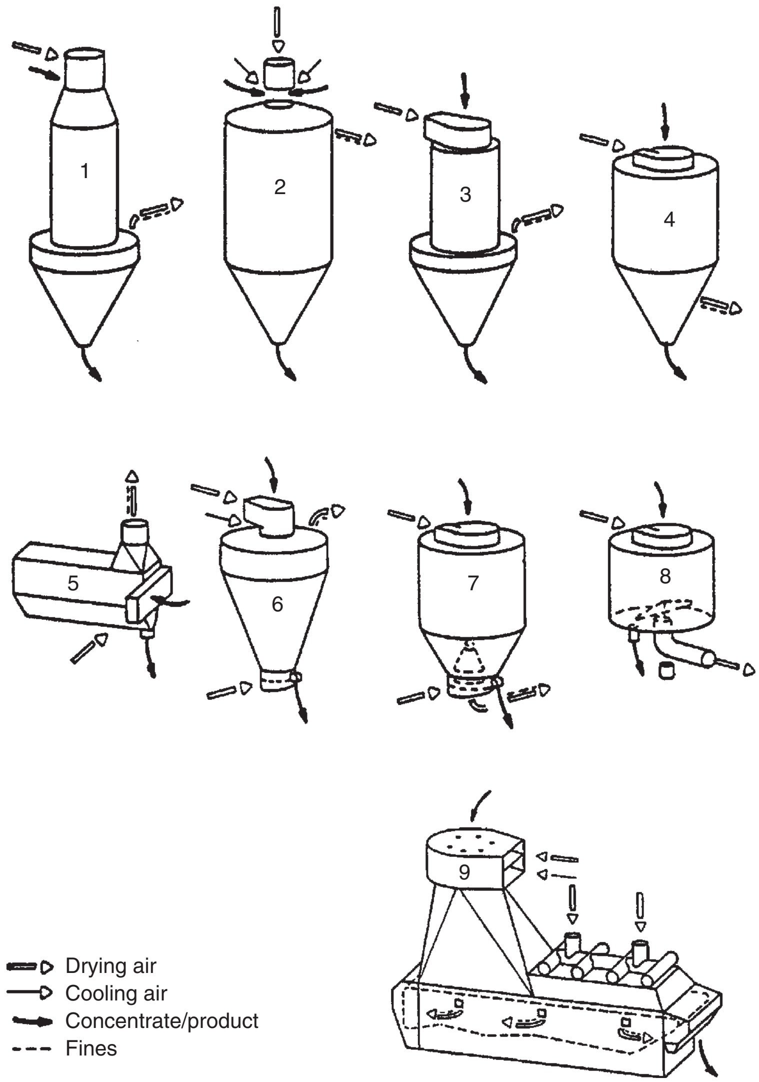

Figure 2 Different types of drying chambers. 1, TALL FORM™ dryer type seen in Japan. It is equipped with a low-velocity air disperser, and, although it is used for milk, it is not very suitable for this product; 2, TALL FORM™ dryer with high-temperature primary drying air. Secondary air is sucked into the drying chamber during the drying operation. The 'mix' air temperature is similar to that of a normal spray dryer; 3, Conventional TALL FORM DRYER™ chamber used predominantly for baby food and protein products; 4, Conventional drying chamber with conical bottom; 5, Box dryer for one-stage drying only – poor economy and normally seen only in the United States; 6, Multistage MSD™ drying chamber with integrated fluid bed; 7, Conventional COMPACT™ drying chamber with integrated fluid bed; 8, Flat-bottom spray drying chamber for one-stage drying only. No longer seen on new installations; 9, FILTERMAT® drying chamber in a special design for very sticky products.

operation and a higher level of food safety. Common for the different standards are as follows:

- The air should be prefiltered and supplied by a separate fan to the fan/filter/heater room, which must be under pressure to avoid the entry of unfiltered air (**Figure 7**). As an alternative to a fan room, complete ducting of all air flows is fully acceptable.
- Filtration degree and filter position depend on the final temperature of the process air as follows:
  - For air to be heated above 120 °C only coarse filtration up to 90% (filter class EU7/F7) is needed. The filter should be placed on the pressure side of the fan.
  - For air to be heated below 120 °C or not heated at all, the filtration must be 95% (filter class EU/F9) or above, and the filter must be placed after the heater/cooler. Some countries and companies have even stricter requirements demanding a filtration of up to 99.995% (filter class EU13-14/ H13-14).

Air Heating System

The drying air can be heated in different ways: indirect (steam/oil/gas/hot oil/electricity) or direct (gas).

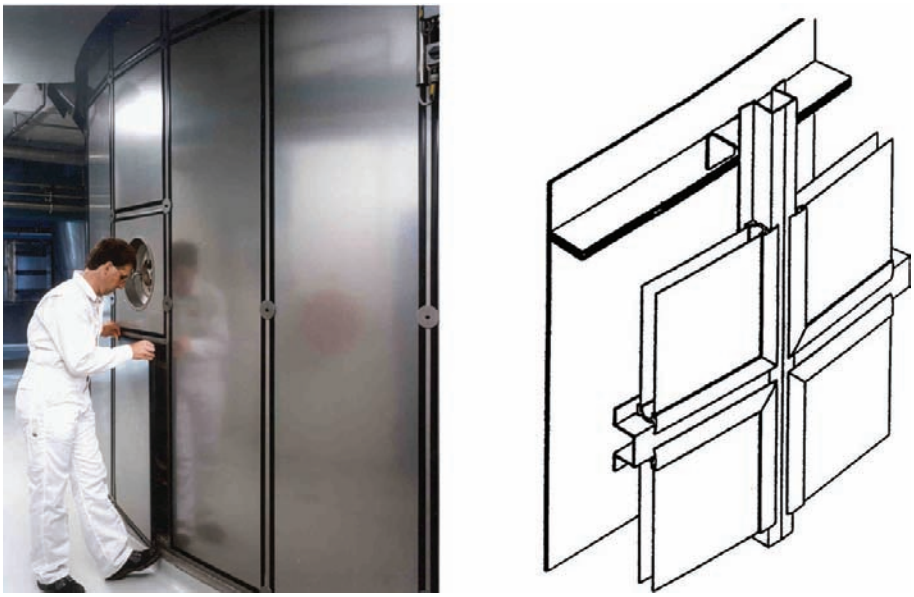

Figure 3 Removable insulation panels for spray drying chambers.

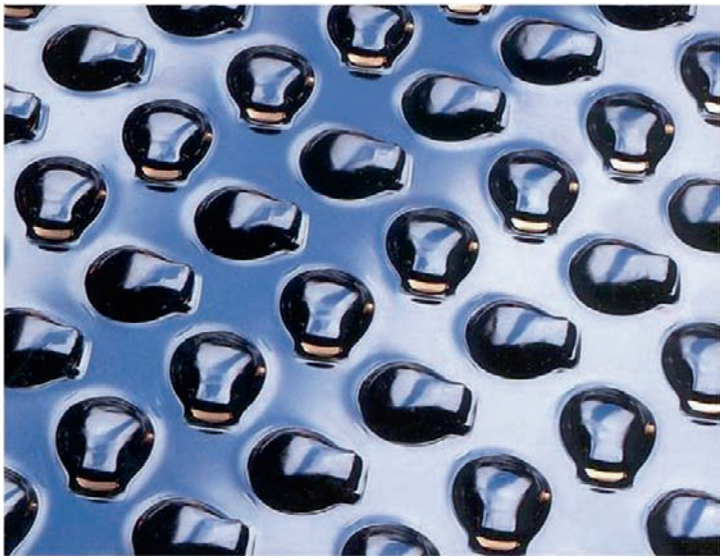

Figure 4 BUBBLE PLATE™.

Indirect heating

A steam heater is a simple radiator. The temperature to be obtained depends on the steam pressure available. The air heater consists of rows of finned tubes housed in an insulated metal case. The heat load is calculated from the quantity and specific heat of the air. The heater size depends on the heat transfer properties of the tubes and fins and is usually about $50\,\mathrm{kcal}\,{}^{\circ}\mathrm{C}^{-1}\,\mathrm{h}^{-1}\,\mathrm{m}^{-3}$ for an air velocity of $5\,\mathrm{m\,s}^{-1}$. To avoid corrosion of the tubes in the air heater, use of stainless-steel tubes is recommended.

In indirect oil and gas heaters, drying air and combustion gases have separate flow passages. The combustion gases pass through galvanized tubes, which act as the heat transfer surface for the drying air. The combustion chamber is made of heat-resistant steel. Heaters of this type will have an efficiency of about 85% in the range of 175–250 °C (see **Figure 8**).

Hot oil liquid phase air heaters are used either alone or to boost the inlet drying air temperature when the steam pressure is not high enough. The heater system consists of a heater, which can be gas- or oil-fired, and an air heat exchanger. Between these two components, a special food-grade oil or heat transfer fluid, which does not crack at high temperatures, is circulated at high speed.

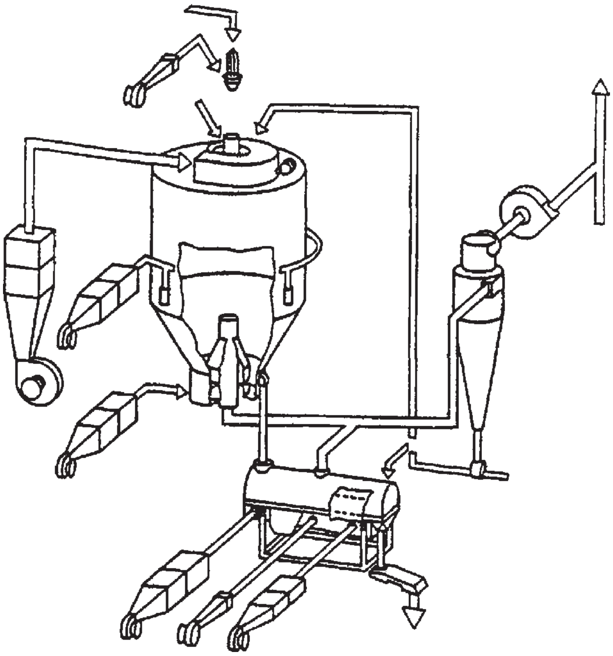

Figure 5 Compact spray dryer with VIBRO-FLUIDIZER™ as agglomerator/instantizer (CDI).

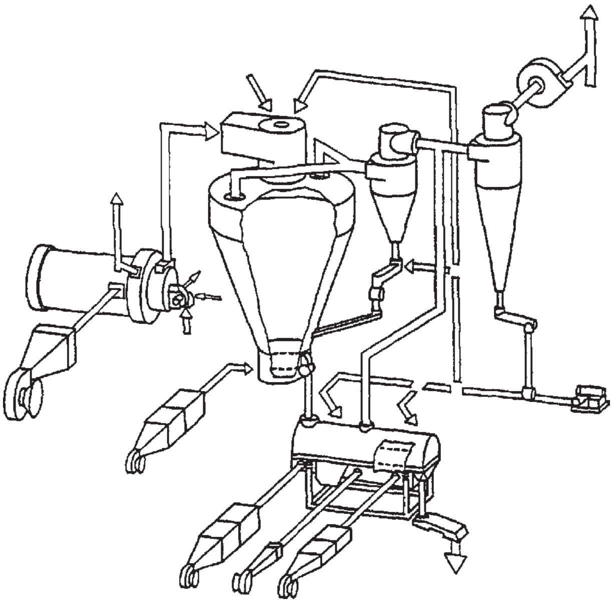

Figure 6 Multistage spray dryer (MSD).

The main advantage of a hot oil liquid phase heater is that it is an open, pressureless system.

Electrical air heaters have for many years been used mainly for laboratory and pilot plant spray dryers. This heater has low investment costs, but previously the operation costs were considered to be too high for commercial production. However, as the price of electricity in certain parts of the world can be very low during off-peak periods, it is becoming more common to use electrical heaters as boosters instead of, for example, hot oil liquid phase air heaters.

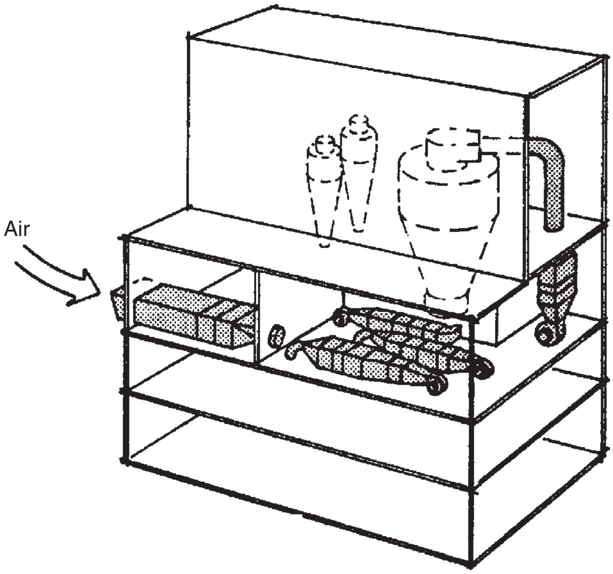

Figure 7 Filtration of process air.

Direct heating

Direct gas heaters are used only when the combustion gas can be allowed to come into contact with the product. They are, therefore, not common in the dairy industry. Direct gas heaters are inexpensive, they have a high efficiency, and the obtainable temperature can be as high as 2000 °C. When a plant is designed with an air heater with direct combustion, it is necessary to calculate the amount of vapor resulting from the combustion (44 mg kg^{-1} dry air °C^{-1}), as this will increase the humidity of the drying air. The outlet temperature has therefore to be increased to compensate for this increase in humidity and to maintain the relative humidity.

The heater system can be designed with separate heaters for each consumption point or with fewer heaters, of which some supply two or more consumption points. By mixing warm air from the main air heater with cold air, the entire dryer can be run with only one heater.

Air Distribution System

Drying-air distribution is one of the most vital functions in a spray dryer. There are various systems depending on the plant design and the type of product.

The most common system is where the air disperser is situated on top of the dryer ceiling, and the atomizing device is placed in the middle of the air disperser, thus ensuring an optimal mixing of the air and the atomized droplets. In cylindrical vertical dryers, the whole ceiling may be perforated, thus creating a plug-flow air stream – numerous nozzles are situated in the perforated plate to

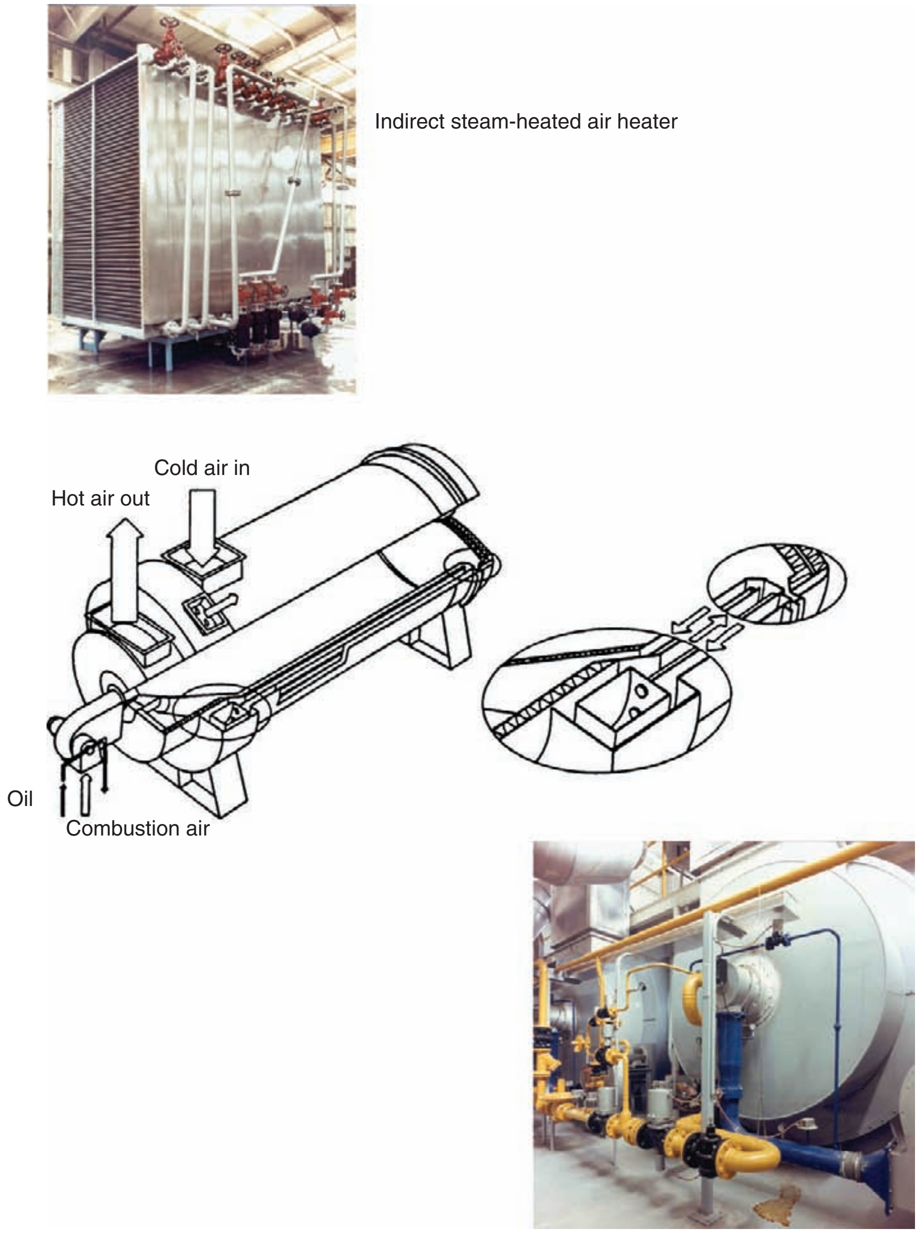
Indirect steam-heated air heater
Cold air in
Hot air out
Oil
Combustion air

Figure 8 Indirect air heaters.

ensure that the air is cooled by the concentrate. This system, however, operates with a low air velocity, and it makes fines return complicated. It is therefore not suitable for all dairy products. It should be noted that an air disperser should have the ability to guide the air and the atomized droplets in the right direction to avoid deposits in the drying chamber.

Two different types of air dispersers are currently used in spray dryers for food and dairy products:

Rotary air stream

The air enters tangentially into a spiral-shaped distributor housing (see **Figure 9**), from where the drying air is

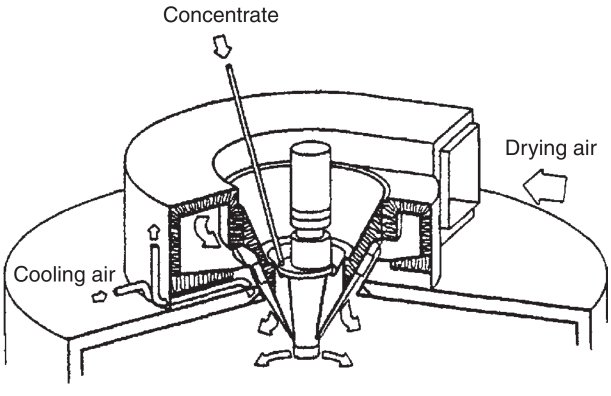
Concentrate
Drying air
Cooling air

Figure 9 Ceiling air disperser with adjustable guide vanes.

led radially and downward over a set of guide vanes provided for adjustment of air rotation. This type of air disperser is used for rotary atomizers and nozzle atomizers placed in the center of the air disperser and is used in conventional drying chambers.

Plug flow air stream

The air enters radially through one side (see **Figure 10**) and is distributed through a specially designed air guiding arrangement, which ensures a uniform air flow pattern in the entire air disperser area. This enables a very precise, laminar, high-velocity plug flow, which is required in TALL FORM™ dryers or multistage dryers.

This type of air disperser is used for nozzle atomizers only, and has excellent possibilities for nozzle position adjustment and thereby the adjustment of agglomeration structure.

Feed System

The feed system (see **Figure 11**) is the link between the evaporator and the spray dryer, and comprises the following:

1. Feed tanks
2. Water tank
3. Concentrate pump
4. Preheating system
5. Filter
6. Homogenizer/high-pressure pump
7. Feed line, including return line for cleaning-in-place (CIP)

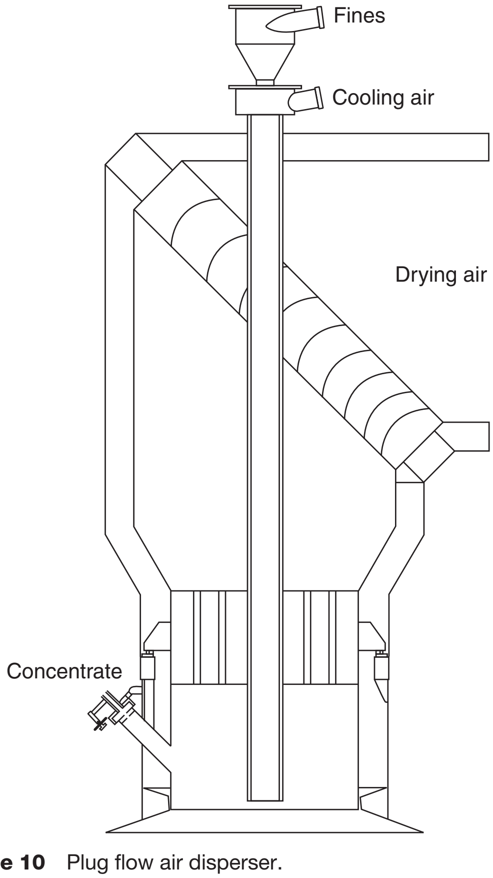

Figure 10 Plug flow air disperser.

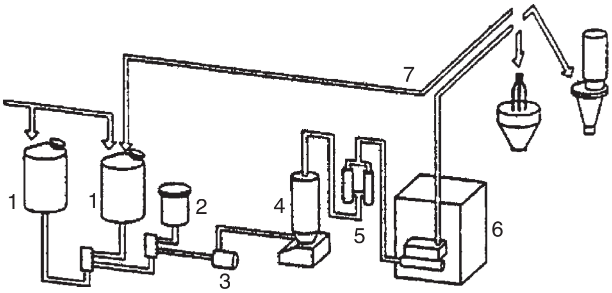

Figure 11 Feed system. 1, Feed tanks; 2, Water tank; 3, Concentrate pump; 4, Preheating system; 5, Filter; 6, Homogenizer/high-pressure pump; 7, Feed line, including return line for CIP.

Feed Tanks

If feed tanks are used, the use of two tanks is recommended so as to change from one to the other at least once an hour to avoid the risk of bacteria growth. One is therefore in use while the other one is being cleaned. The size of each tank should correspond to 15–30 min of the feed capacity of the dryer. The feed tanks are very often omitted, and the last-stage evaporator is designed as a buffer tank under vacuum. The evaporator then operates as a 'slave' to the dryer, because the level switches in the evaporator buffer tank control the inlet feed to the evaporator.

Water Tank

The water tank is used during the start and stop of the plant, and during the run if there is a sudden shortage of concentrate. It is used only when the evaporator is used as a buffer tank. As an alternative, a direct water supply to the feed line is often used.

Concentrate Pump

If a rotary atomizer is used, the most common feed pump is the mono type, as it has lower energy consumption and can handle concentrates of high viscosity. In plants equipped with homogenizers for the production of whole milk powder, the homogenizer is used as a feed pump. In plants equipped with high-pressure nozzles, a high-pressure pump is used – often combined with a homogenizer.

Preheating System

Preheating of the concentrate to a higher temperature than that coming from the evaporator is advantageous, not only from a bacteriological point of view. It also produces a decrease in viscosity, which together with the applied calories results in a capacity increase of the spray dryer and an improved solubility of the powder produced.

The heating can be either indirect or direct. Indirect preheaters may be of the following types:

1. Spiral-tube heat exchanger
2. Plate heat exchanger
3. Scraped-surface heat exchanger

Spiral-tube heat exchanger

The spiral-tube heater (see **Figure 12**), often with corrugated tubes, is able to heat a concentrate with high solid content to a higher temperature without frequent scaling and cleaning owing to high product velocity and a low ΔT throughout the heater. Furthermore, this type of heater has no moving parts; hence, maintenance costs are minimized.

Plate heat exchanger

A plate heat exchanger system is inexpensive, but if the concentrate should be heated to >70 °C, if the solid content is >46%, or if a 20 h run is aimed at, it is necessary to have two interchangeable heaters allowing one to be cleaned while the other is being used. Steam or warm water can be used as the heating medium.

Scraped-surface heat exchanger

In the scraped-surface heater, the heat transfer surface is continuously scraped off by a fast-rotating scraper made of food-grade synthetic material to avoid any product adherence. The scraped-surface heater is especially suitable for products with high solid content and when high temperatures are required. They can operate continuously for 20 h and are cleaned together with the remaining feed system. The disadvantages are high cost of maintenance as well as big variation in holding time.

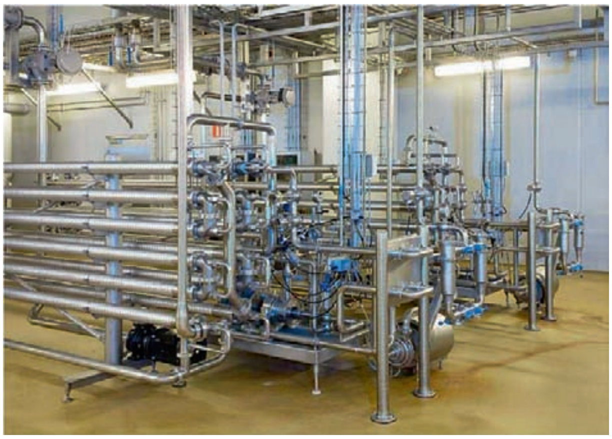

Figure 12 Spiral-tube heat exchanger.

Direct preheaters may be of the following types:

- Direct steam injection (DSI)
- Lenient steam injection (LSI)

Direct steam injection

In the DSI unit, steam is introduced into the milk concentrate via a nozzle, producing relatively big steam bubbles resulting in a superheating of some parts of the concentrate, which leads to protein denaturation.

Lenient steam injection

In the LSI unit, steam is mixed into the concentrate by a dynamic mixer. Very small steam bubbles are created, and superheating/denaturation is avoided. Therefore, a much higher steam pressure can be used. The LSI unit can be used in combination with the spiral-tube heat exchanger if temperatures above 80 °C are required in the concentrate.

Filter

An in-line filter is always incorporated in the feed system after the heater to avoid lumps, etc., passing to the atomizing device.

Homogenizer/High-Pressure Pump

If whole milk powder is to be produced, it is recommended that a homogenizer be incorporated to reduce the free-fat content in the final powder. A two-stage homogenizer is preferred; the first stage is operated at 50–100 bar g, and the second stage at 25–50 bar g.

Usually the homogenizer and feed pump are combined in one unit. If nozzle atomization is used, then a higher pressure (up to 250 bar g for the nozzles + 150 bar g for homogenizing) is required, and a combined homogenizer/high-pressure pump is chosen.

Temperatures of ≥80 °C are needed to produce a whole milk powder with a good coffee stability. In view of calcium phosphate precipitation – which is abrasive – the pistons should be made of a ceramic material.

Feed Line

The feed pipe should be of stainless steel and, of course, of the high-pressure type if atomization is to be carried out by means of nozzles. The dimensions of the pipe should be such that the feed velocity is $\geq 1.5\,\mathrm{m\,s^{-1}}$. In a feed system, a return pipe should also be included for the cleaning solution, so that the entire equipment can be cleaned thoroughly.

Atomizing Device

The aim of atomizing the concentrate is to provide a very large surface from which evaporation can take place. The smaller the droplets, the bigger the surface and the easier the evaporation; and thus a better thermal efficiency of the dryer is obtained. The ideal from the point of view of drying would be a spray of drops of the same size, which would mean that the drying time for all particles would be the same to obtain an equal moisture content.

As mentioned previously, air distribution and atomization are the factors key to the successful utilization of the spray dryer. Atomization is directly responsible for many distinctive advantages offered by spray drying: First, the very short drying time of the particles; second, a very short particle retention time in the hot atmosphere and a low particle temperature (wet bulb temperature); and, finally, the transformation of the liquid feed into a powder with long-storage stability ready for packing and transport.

In summary, the primary functions of atomization are

- to create a high surface-to-mass ratio resulting in a high evaporation rate
- to create particles of the desired shape, size, and density

To comply with these requirements many atomization techniques have been used in spray dryers. However, the most common ones can be summarized as follows:

- pressure nozzles using pressure forces
- two-fluid nozzles using kinetic forces
- rotating discs using centrifugal forces

Pressure Nozzle Atomization

The basic function of pressure nozzles is to convert the pressure energy supplied by the high-pressure pump into kinetic energy in the form of a thin film, the stability of which is determined by the properties of the liquid such as viscosity, surface tension, density, and quantity per unit of time, and by the medium into which the liquid is sprayed.

Most of the commercially available pressure nozzles are designed with a swirl chamber giving the liquid a rotation, so that it will leave the orifice as a hollow cone (see **Figure 13**). Capacity can usually be assumed to be directly proportional to the square root of pressure retain:

$$\text{Capacity } (\text{kg h}^{-1}) = K \times \sqrt{P}$$

As a rule of thumb, higher viscosity, liquid density, and surface tension, and lower pressure will result in bigger particles. Typically, a feed rate of 1000–1500 kg h^{-1} per nozzle is used in industrial dryers. The advantages when using high-pressure nozzles are as follows:

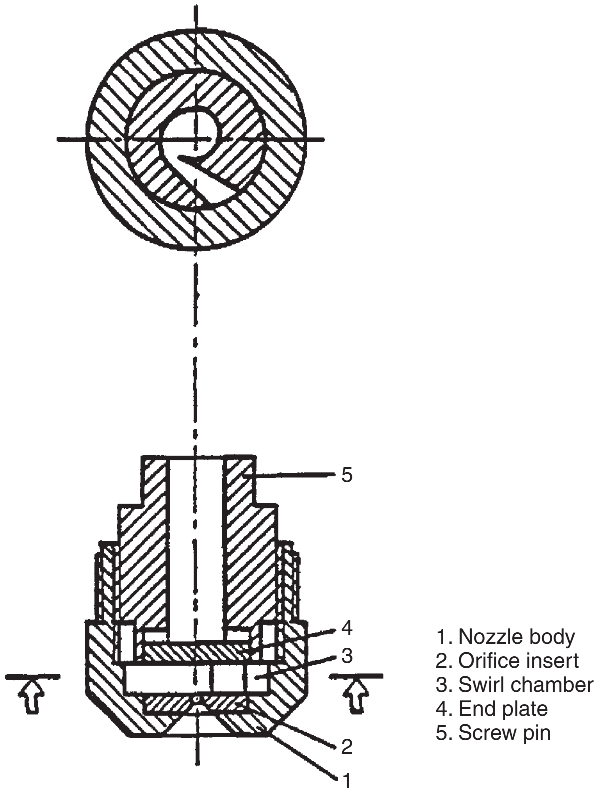

Figure 13 High-pressure nozzle ‘Delavan’.

- Powder with a low level of occluded air
- Powder with a high bulk density
- Improved flowability, especially for whole milk
- Tendency to form less deposits in the drying chamber when difficult products are produced
- Ability to produce big particles

Two-fluid nozzle or pneumatic atomization

The energy available for atomization in two-fluid atomizers is independent of liquid flow and pressure. The necessary energy (kinetic) is supplied by compressed air. Two-fluid atomization is the only successful nozzle method for producing very small particles, especially from highly viscous liquids. It is not normally used in the drying of milk products. However, it is often used in secondary systems such as lecithin application to the powder.

Rotary atomization

In rotary atomizers the liquid is accelerated continuously to the wheel’s edge by the centrifugal forces produced by the rotation of the wheel. The liquid is distributed centrally, then extended over the wheel surface in a thin sheet and discharged at a high speed at the periphery of

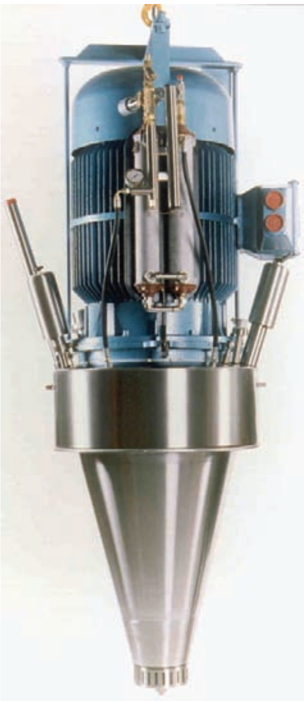

Figure 14 Rotary atomizer with direct drive.

the wheel. The degree of atomization depends upon peripheral speed, properties of the liquid, and feed rate (**Figure 14**).

To select an optimal atomizer wheel, the following factors should be taken into consideration:

Liquid feed rate

Droplet size varies directly with feed rate at a constant wheel speed and will increase with increased feed rate.

Peripheral speed

The peripheral speed depends on the diameter of the wheel and the wheel speed, and is calculated as follows:

$$V_\mathrm{p} = \frac{\pi \times D \times N}{1000 \times 60}$$

where V_p is the peripheral speed (m s^{-1}), D the diameter of the wheel (mm), and N the speed of the wheel (rpm).

Peripheral speed is widely accepted as the main variable for the adjustment of droplet size. However, it has been shown that droplet size does not necessarily remain constant when equal peripheral speeds are produced in wheel designs of various diameter–speed combinations, as there is a tendency for bigger wheels to produce bigger particles, all other things being equal. However, in the choice of wheel diameter, one should rather look at the reliability of the atomizer, as the differences in spray characteristics are negligible.

Viscosity of the liquid

Droplet size varies directly with viscosity, and bigger particles are obtained when the viscosity of the feed is higher. To ensure an optimal atomization, the viscosity is therefore normally kept as low and as constant as possible, often by heating the concentrate prior to atomization. Regarding droplet size distribution, it becomes broader with increased viscosity – an effect sometimes used when bulk density of the powder is to be increased.

Rotary atomizer has been known and used in the dairy industry for many years; its main advantages are as follows:

- Flexibility in throughput
- Ability to handle large quantities
- Ability to handle highly viscous concentrates
- Different wheel designs giving different powder characteristics
- Ability to handle products containing crystals
- Ability to handle higher solid content in the feed; therefore, better economy

To decide whether to use a pressure nozzle or rotary wheel is therefore a question of achieving the demanded properties of the final dried product, given the properties of the feed.

Powder Separation System

As the drying air leaving the chamber will contain a small proportion of the powder (10–30%), it is necessary to clean it by separating the powder particles. This powder fraction is usually referred to as the ‘fines’, as they normally represent the smallest particles.

The most widely used separators in the milk powder industry are

- Cyclone
- Bag filter
- CIP-able bag filter
- Wet scrubber
- Combinations of the above

Cyclone

Cyclone has some obvious advantages, such as high efficiency, if constructed properly. It is easily maintained, as there are no moving parts. Furthermore, it is easy to clean if the construction is with a fully welded center cyclone.

The operation theory is based on a vortex motion where the centrifugal force is acting on each particle and therefore causes the particle to move away from the cyclone axis toward the inner cyclone wall. However, the movement in the radial direction is the result of two opposing forces where the centrifugal force acts to move

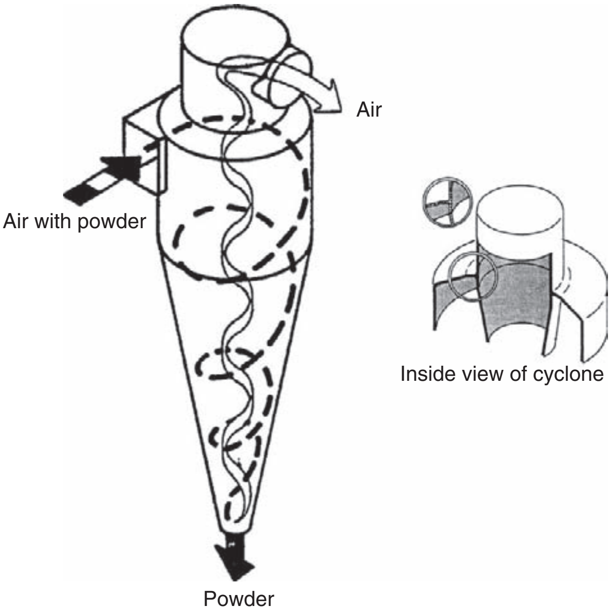

Figure 15 Cyclone.

the particle to the wall, while the drag force of the air acts to carry the particles into the axis. As the centrifugal force is predominant, separation takes place.

Powder and air pass tangentially into the cyclone at equal velocities. The mixture swirls in a spiral form down to the base of the cyclone separating the powder out to the cyclone wall. Powder leaves the bottom of the cyclone via a locking device. The clean air spirals upward along the central axis of the cyclone and leaves the cyclone at the top (see **Figure 15**).

The centrifugal force that each particle is exposed to is given by the following equation:

$$C = \frac{m \times V_t^2}{r}$$

where C is the centrifugal force, m the mass of the particle, V_t the tangential air velocity, and r the radial distance to the wall from any given point.

From this equation it can be concluded that the higher the particle mass, the better the efficiency. Also, the shorter distance the particle has to travel, the better the efficiency; that is, the closer the particle is to the wall, the better the efficiency, because the velocity is the highest and the radial distance is short.

However, time is required for the particles to travel to the cyclone wall, so a sufficient air residence time should be taken into consideration when designing a cyclone.

From the above equation, it is evident that small cyclones (diameter <1 m) will have the highest efficiency, a fact that is generally accepted.

However, as the big-tonnage dryers in operation in dairy industry today would require many cyclones, a compromise is sought with bigger sizes at the expense of high efficiency. Thus, the cyclones have become bigger and bigger and are now constructed with diameters of up to 4.0 m.

When designing a cyclone, various key factors should be taken into account to obtain the highest efficiency. This is achieved if

$$\frac{\text{cyclone diameter}}{\text{exit duct diameter}} \approx 3$$

$$\frac{\text{cyclone height}}{\text{exit duct diameter}} \approx 10$$

Increased air throughput (velocity V_t) and increased pressure drop will also increase the efficiency, but the energy requirement will also increase simultaneously, so in general the upper limit is 175–200 mm WG for skim milk powder. For whole milk, 140–160 mm WG is the maximum so that deposits and final blocking can be avoided.

To determine a cyclone's efficiency, the following terms have to be defined:

- critical particle diameter
- cut size
- overall cyclone efficiency

Critical particle diameter is defined as the particle size that will be completely removed from the air flow (100% collection efficiency). However, as there is no sharply defined point where a particle size is 100% separated or 100% lost, the critical particle diameter is not very valuable.

Cut size is defined as the size for which 50% collection is obtained and is a much more useful parameter for stating the efficiency of cyclones. To determine a cyclone's cut size, grade efficiency curves are constructed by systematically operating the cyclone with a uniform particle size dust (see **Figure 16**).

Overall cyclone efficiency is the one obtained when handling a product of definite size distribution. Knowing the grade efficiency curve of the cyclone and the particle size distribution of the powder passing to the cyclone, the overall efficiency can be calculated, that is, the powder loss can be predicted.

Another method for determining cyclone efficiency is by a simple powder loss measurement at the exit of the cyclone. A very small fraction of the outgoing air is passed through a high-efficiency minicyclone or through microdust filters. The amount of powder collected is directly proportional to the powder loss, which will mainly be a result of

- Feed with low solid content or feed containing air
- High outlet air temperature
- Low particle density (e.g., as a result of the above)
- Leaking product outlet on account of old-fashioned nonadjustable rotary valves

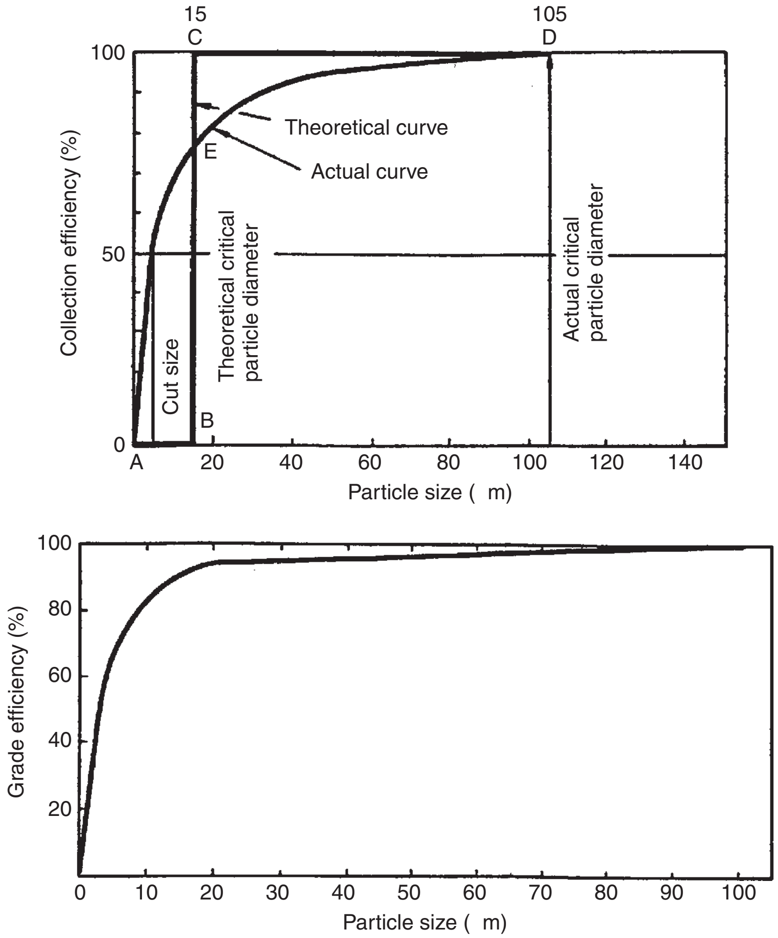

Figure 16 Critical particle diameter vs. grade efficiency curves for a cyclone.

- Blocked cyclone
- Changes in drying parameters resulting in a decrease in mean particle size

Average powder loss from a normal, high-efficient cyclone should not exceed 250 mg Nm^{-3} when spray drying skim milk.

Bag Filters

However, local authorities in general conclude that 250 mg Nm^{-3} is too high, thus requiring a final cleaning of the air. This is usually done by using bag filters consisting of numerous bags or filters arranged in such a way that all bags receive almost equal quantities of air. The direction of the air is from the outside, through the filter material, to the inner part of the bag from where the cleaned air enters an exhaust manifold. With a correct selection of filter material high efficiencies can be achieved, and collection of 1 μm particles has been reported by manufacturers. The collected powder is automatically shaken off by blowing compressed air through the filter bags from the inner side. This powder is collected at the bottom via a rotary valve (see **Figure 17**).

Wet Scrubbers

The wet scrubber is based on the venturi scrubber principle. The droplet separator is designed according to the well-known cyclone principles, however, with a modified outlet, resulting in a minimum liquid level, thereby minimizing bacterial growth, and a design ensuring deaeration, thus avoiding foam building.

The principle of venturi wet scrubbers is as follows (see **Figure 18**). The outlet air from the spray dryer containing powder particles is accelerated to a high velocity in the venturi inlet, where the liquid also is injected through full-cone nozzles. Due to the different velocities of the air/particles and the liquid droplets, they will collide, and the powder will dissolve in the liquid droplets. Passing through the subsequent diffuser this process will continue simultaneously with a certain pressure recovery of the air–droplet mix.

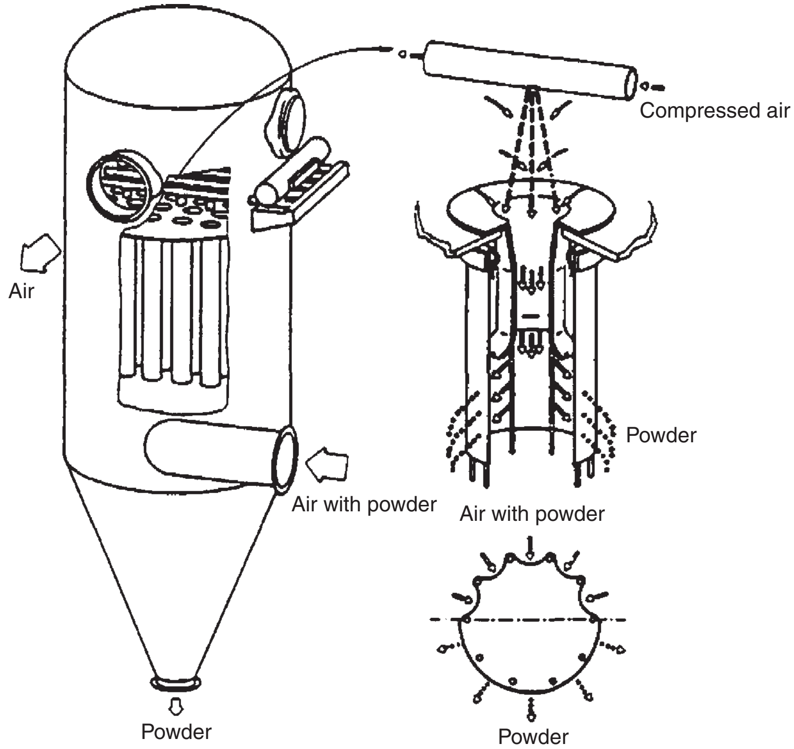

Figure 17 Bag filter.

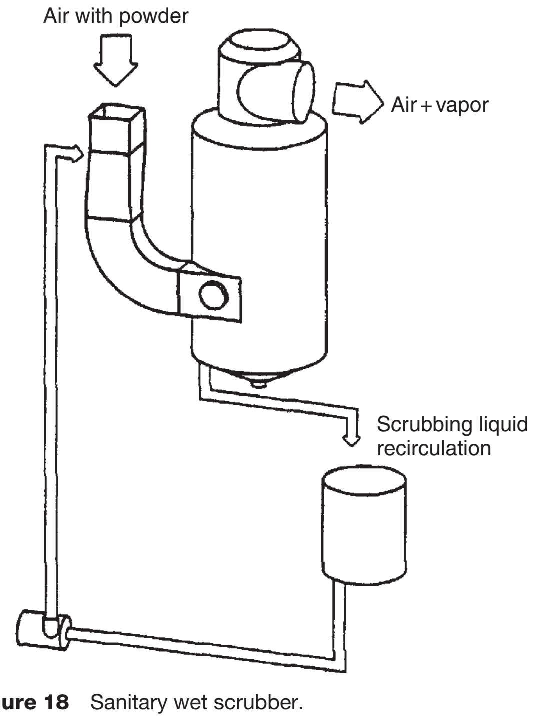

Figure 18 Sanitary wet scrubber.

Passing through the separator, the air and the liquid are separated. The air leaves through the central duct and the liquid through the bottom outlet for further processing or recycling depending on the system selected.

Recirculation of water

According to the above description of the principle, water is recycled by means of a centrifugal pump. The flow is controlled by a valve. The level is kept constant in the separator by a tank with an adjustable float simultaneously ensuring addition of water to make up for the evaporation taking place in the scrubber. The evaporation takes place owing to the high temperature of the air from the dryer, which is 90–95 °C for example, being cooled to the wet-bulb temperature (45–50 °C), at the same time evaporating the water (see **Figure 19**).

As the temperature of the water continues to be around 40–45 °C, bacterial growth must be expected after some time, and a CIP-able system is therefore recommended. The scrubbing liquid is used as animal feed.

CIP-Able Bag Filters

Common for all powder separators is the pressure drop across the cyclones, bag filters/scrubbers, or combinations thereof. In a continued effort to comply with the authority's demand for reduced powder emission and the powder producer's demand for lower energy consumption figures and reduced space requirements, an optimized powder recovery system has been developed – the CIP-able bag filter – which replaces the cyclones as well as the bag filter.

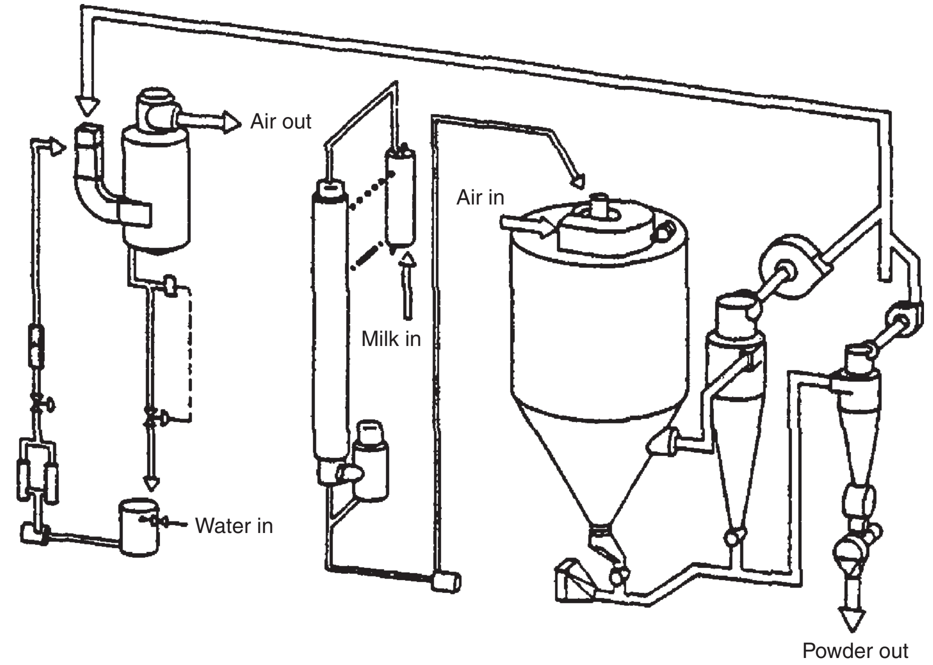

Figure 19 Wet scrubber recycled with water.

Based on almost 10 years of research, development, and testing of a CIP-able bag filter by GEA Niro, the SANICIP™ filter has reached a point where it is setting the standard for almost all dryers.

The SANICIP™ bag filter

The SANICIP™ bag filter (see **Figure 20**) is of the reverse-jet type. It consists of a cylindrical bag housing with a spiral-shaped air inlet, a clean-air plenum on top, and a conical bottom with fluidized powder discharge. During operation, the product collected on the outside of the filter material is removed by a compressed-air jet stream from the inside of each bag. The bags are clean-blown individually, resulting in a very even discharge of the powder.

The air supply system for the fluidizing bottom has a multiple purpose: During production, the cone of the bag house is first heated by the warm air, which subsequently is used for fluidizing the powder in the bottom. This ensures an even powder flow out of the bag house. During standstill, the air is used for the heating of the cone alone and is in a closed loop.

The filter bags are made from a special three-layer gradient polyester material, which is heat-treated to give a special dust-releasing surface. Each bag is supported on a stainless-steel cage and is easily dismountable.

In the SANICIP™ filter, a special reverse-jet air nozzle positioned above each bag (see **Figure 21**) is

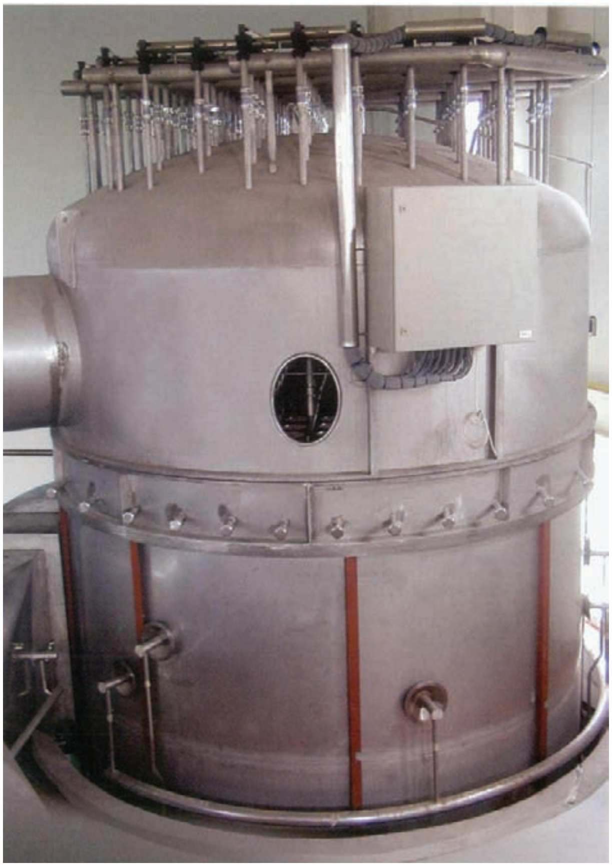

Figure 20 SANICIP™ CIP-able bag filter.

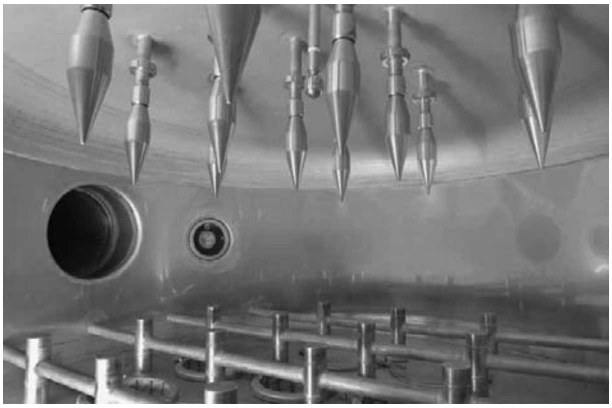

Figure 21 Reverse-jet air nozzle.

used. Compressed air is blown into the bag through this nozzle. A jet is formed that draws into the bag air from the clean-air plenum as well, thereby saving compressed air.

The CIP system of the bag house is divided into the following main items:

1. The internal bag CIP system cleans the bag from the inside toward the powder side (outside). Clean water is injected into the inside of the bag through the reverse-jet nozzle and the water is atomized by compressed air. Powder that has penetrated into the bag material is forced out toward the powder side by the water spray. No recirculation of water in this step.
2. The clean-air plenum CIP cleans the clean-air plenum of the bag filter above the hole plate. No recirculation of water in this step.
3. The hole plate CIP cleans the bottom side of the hole plate and the snap ring area of the bag using a specially designed nozzle, also with a dual purpose: During the process, the nozzle is purged with compressed air to keep the hole plate free of deposits, thereby avoiding discoloring/denaturation. The water is recirculated.
4. The shell CIP is performed by means of standard retractable CIP nozzles. The water is recirculated.

Normal acid and caustic are used as CIP agents. The CIP is followed by bag drying. Estimated time for complete CIP and dry out is 10 h.

Advantages of the SANICIP™ filter as follows:

- Low pressure loss across the bag filter and, thus, across the entire exhaust system; that is, reduced energy consumption and noise emission
- Designed for optimum air-to-cloth ratio and powder load (owing to one bag being blown at a time)
- Higher yield from raw materials owing to the absence of second-grade products
- Designs with 4 or 6 m bags to suit specific building requirements
- Reduced space requirements for new installations
- Easy replacement of cyclones for retrofits without major building changes
- Short dry-out time, as compared with other CIP-able bag filters

The pros and cons of all the above-mentioned powder recovery tools are listed in **Table 1**.

Final Drying and Cooling of Powder

Pneumatic Conveying and Cooling System

A pneumatic conveying system is established when powder has to be conveyed from one place to another. The conveying medium is air, and the quantity is determined by the product. Products with a high fat content require more air (5 times the powder) than that required by skimmed milk (4 times the powder). It is, however, not recommended that powders with

Table 1 Comparison of powder separators

	Cyclone	*Cyclone + bag filter*	*Cyclone + wet scrubber*	*SANICIP^{TM}*
Emission	20–400 mg Nm^{-3}	5–20 mg Nm^{-3}	max. 20 mg Nm^{-3}	5–20 mg Nm^{-3}
Pressure loss – exhaust system (including ducts, etc.)	280 mm WG	340 mm WG	340 mm WG	170 mm WG
Auxiliaries	None	Compressed air	Liquid circulating system	Compressed air
Cleaning	Suitable for CIP	Difficult	Suitable for CIP	Suitable for CIP
Hygroscopic products	Insensitive	Sensitive	Insensitive	Insensitive
Use of separated product	First grade	First and second grade	Not recommended	First grade
Maintenance	Minimal	Servicing of compressed air system and change of bags	Minimal	Servicing of compressed air system and change of bags
Sanitary conditions	Good	Relatively good	Less good	Good

a fat content higher than 30% be conveyed, as blocking may occur in the ducts.

Air at any temperature may be used, and the powder temperature will naturally follow the air temperature. If hot air is used there will be a drying effect. This will, however, be minimal, as the residence time is short (air velocities of 20 m s^{-1}).

A pneumatic conveying system is inexpensive and can handle large quantities of powder, but it will destroy any agglomerates, resulting in a powder with maximum bulk density. The powder is separated from the conveying air in a cyclone.

Fluid Bed After-Dryer/Cooler

In modern dryers, pneumatic conveying and cooling systems are replaced by a VIBRO-FLUIDIZER™, which is designed also as an after-dryer, that is, drying is divided into two or more steps. The first step is done in the spray drying chamber transforming the liquid into powder particles and evaporating the main portion of water. The subsequent drying is done in a fluid bed (see **Figure 22**). The fluid bed drying technology has proved especially suitable, as the residence time in the fluid bed is so long that the moisture from the center of the particle can reach the surface from where evaporation takes place.

The VIBRO-FLUIDIZER™ is a horizontal box divided into an upper and a lower section by a perforated plate welded to the side wall of the box (see **Figure 23**). For drying, or alternatively cooling, warm or cold air is introduced into the air plenum chamber, which is distributed evenly over the whole area of the perforated plate. The perforation and amount of air are determined by the air velocity necessary for the fluidizing of the powder; however, special care must be taken to avoid attrition of agglomerates.

The temperature and area are determined according to the required evaporation duty. The hole size in the perforated plate is chosen to give an air velocity high enough to fluidize the powder on the plate. The air velocity should be so high that the fines powder becomes airborne and leaves the fluid bed with the air, and is returned to the atomizing zone for agglomeration.

The fluid bed can also be designed as a static back-mix bed integrated in the drying chamber.

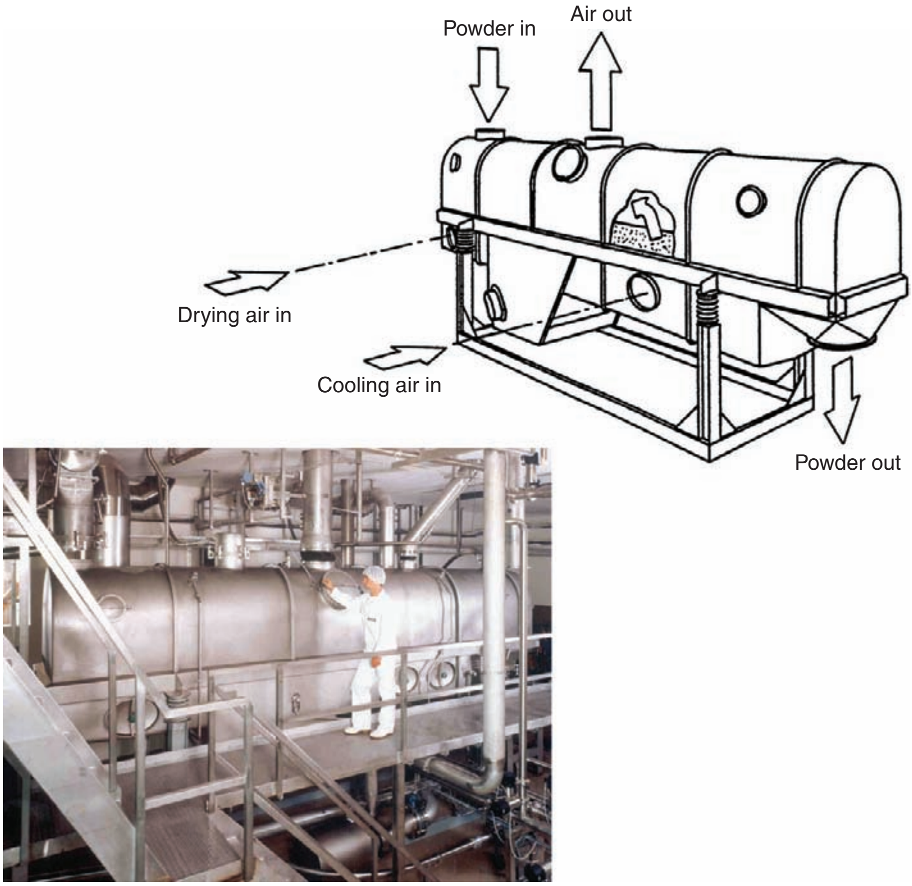

Figure 22 VIBRO-FLUIDIZER™.

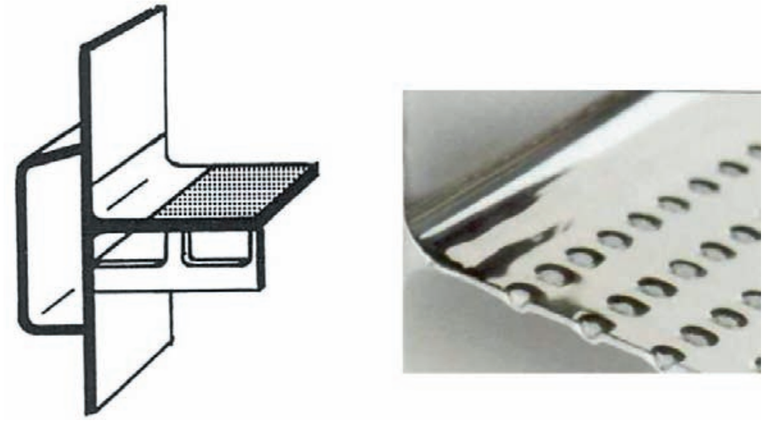

Figure 23 Construction detail of a sanitary VIBRO-FLUIDIZER™.

Fines Return System

Agglomeration means getting smaller particles to adhere to each other to form a powder consisting of bigger conglomerates/agglomerates, which are essential for easy reconstitution in water. By means of a fines return system, the cyclone fraction(s) is(are) conveyed back to the atomizer mist, the static fluid bed, or the VIBRO-FLUIDIZER™, depending on the required degree of agglomeration.

Fines return systems consist of the following:

- High-pressure blowers (the quantity of air is dependent on the amount of fines – typically, 1 kg of air can convey 3–5 kg of powder)
- Blow-through valves (devices to discharge powder from cyclones and/or bag filters into the conveying line)
- Conveying line/diverter valves to convey the fines powder to the desired destination – typically a 76–102 mm (3–4 inch) pipe
- Fines introduction to the atomization zone

The aim is to bring the fines as close as possible to the atomizer wheel. In modern dryers, fines are introduced from above through the air disperser (FRAD system) via four fines pipes situated just above the atomizer cloud. Deflector plates at the end of each fines pipe ensure a correct introduction and distribution of the fines (see **Figure 24**).

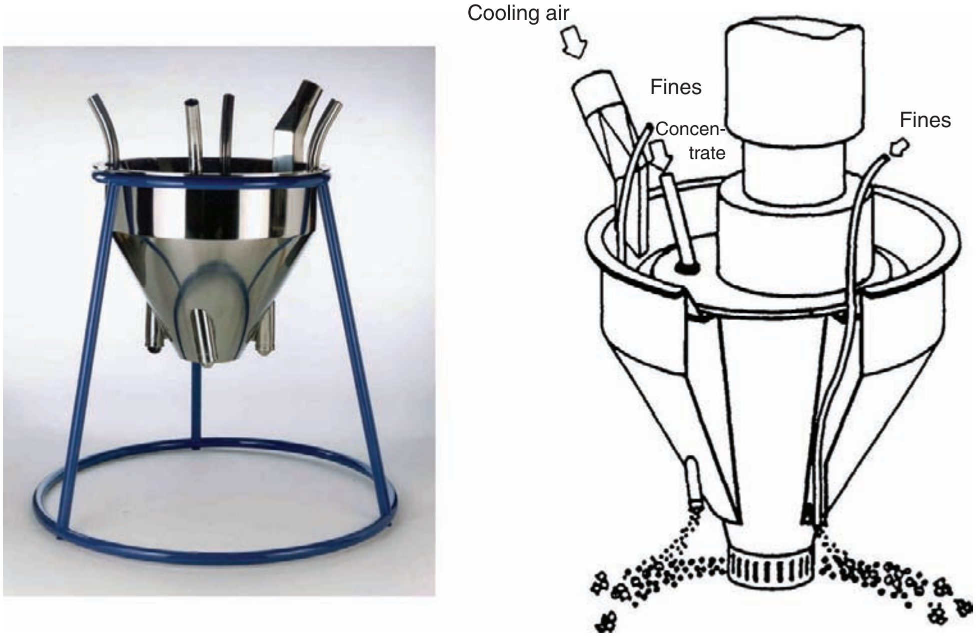

Figure 24 Fines return for rotary atomizer FRAD.

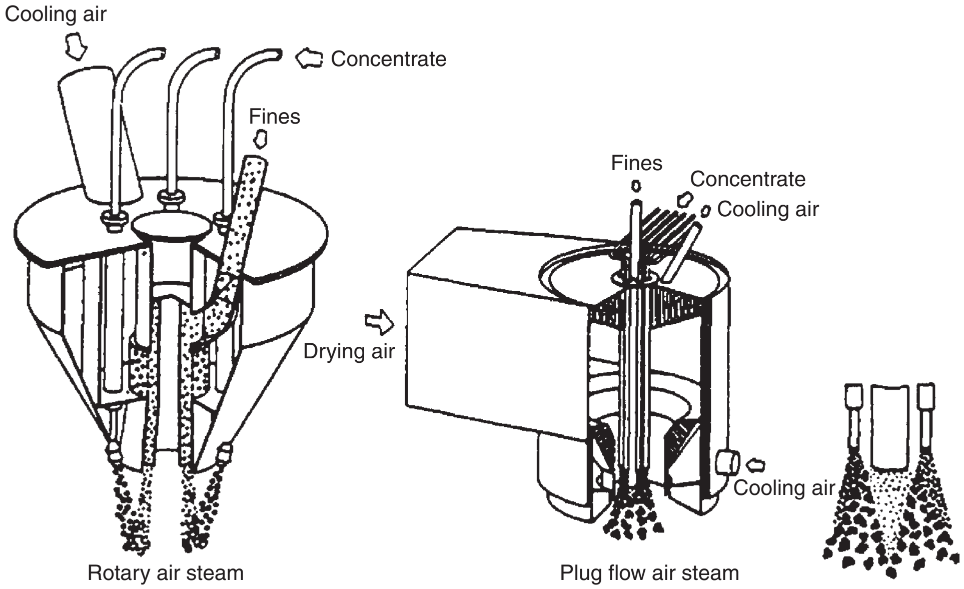

Figure 25 Fines return for nozzle atomizer.

For nozzle atomization, the fines return is an integral part of the nozzle unit with the fines duct in the center surrounded by nozzles at the periphery (see **Figure 25**), provided the dryer is designed for rotary air flow or is with vertical air flow.

Conclusion

Spray drying plants are designed today to fulfill many requirements, including low energy consumption, high final-product quality, reduced space requirements, and a high degree of environmental protection – a challenge taken up by the designers and suppliers of the dryers.

See also: **Analytical Methods**: Sampling; Sensory Evaluation. **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders; Milk Powder: Types and Manufacture; Infant Formulae. **Milk Protein Products**: Milk Protein Concentrate; Whey Protein Products. **Plant and Equipment**: Evaporators; Milk Dryers: Drying Principles. **Rheology of Liquid and Semi-Solid Milk Products**.

Further Reading

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Instrumentation and Process Control: Instrumentation

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Introduction

Industrial evolution in the second half of the twentieth century was influenced mostly by four types of interrelated factors: progress in digital technology, advances in science, evolution of societal requirements and demands and, particularly over the last 30 years, evolution of business concepts.

Developments in digital technology and in systems theory led to major progress in sensor and information technology and a revolution in the availability of distributed control systems and open software applications. New concepts, particularly knowledge-based measurement and advanced control methodologies, are slowly but steadily being brought into the practice of process operation, performing online and in real time.

Societal and economic factors have driven evolution in the same direction. The increasing concern for health, safety and sustainability issues, market quality requirements, economic pressure and the evolution of company strategy from local to global business concepts – the so-called knowledge economy concepts – all together reflected on plant investment decisions, favoring process automation for cleaner and safer operation, higher product quality and improved process efficiency and productivity.

Discussing plant automation from a technical point of view means a discussion on instrumentation, control system configurations, data communications and theoretical control structures.

This article deals with instrumentation, addressing in particular both basic and advanced concepts concerning sensors and the issue of how to integrate local hardware for automatic control, usually dispersed throughout the plant.

Basics of Plant Automation

There are well-established methodological steps for the design and implementation of a control structure: (1) designing and implementing an appropriate, flexible, control configuration; (2) performing first-level data acquisition and process monitoring, including record keeping and first-level alarms; (3) performing data interpretation (implementing second-level process monitoring); and (4) designing and implementing optimization and control solutions.

In plant-wide distributed control configurations, as illustrated in **Figure 1**, the backbone of transmission is all digital. Communication protocols (some open, some proprietary) ensure data transmission for centralized data interpretation, for monitoring and for plant scale optimization. Control at sector and unit level is usually performed locally. Local-level signal transmission has for many years been analog only; initially, pneumatic signals and later electric (current or voltage) signals. However, more and more, information also flows digitally between (smart) sensors and controllers.

At a local level, process control system instrumentation includes: (1) sensors for measurement of process variables; (2) controllers, for implementing a proper (digital) control structure (e.g. proportional-integral derivative (PID), predictive or adaptive controller, etc.); (3) final control elements for manipulation of process inputs; and (4) support devices such as general signal conditioners of both input and output signals, electric (V/I and I/V) transducers, electric-to-pneumatic transducers and hold elements.

The range of instrumentation and control systems is very large today. Nowadays, industrial users are available through the internet information sites of instrumentation manufacturers with very detailed information on all types of instrumentation and control systems configurations, including data communications, related to plant automation.

A relevant management decision in the automation of older plants – and most fall into this category – is how to step from existing local control solutions to integrated distributed control. Investment in a complete new solution is high and the tendency is to try to adopt a solution that makes use of available equipment. This is often hard to achieve and leads to a final configuration that mixes too

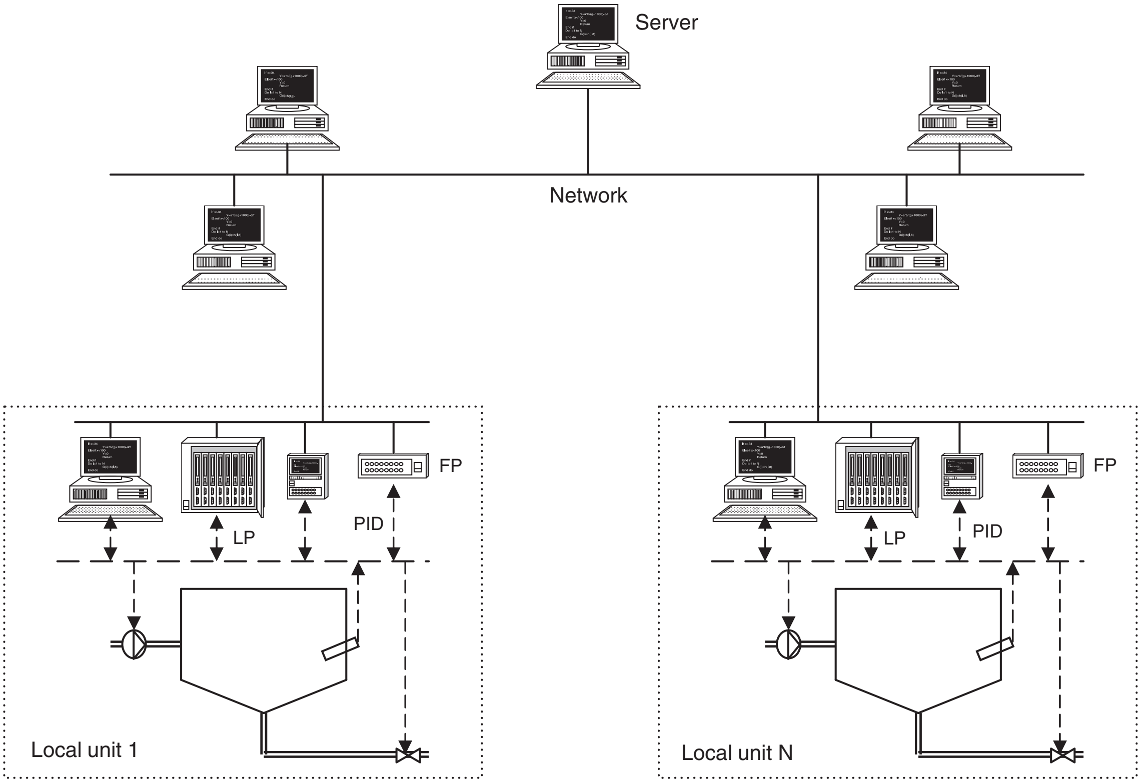
Server
Network
FP
LP
PID
FP
LP
PID
Local unit 1
Local unit N

Figure 1 State-of-the-art instrumentation for process control: devices, signal transmission and information flow. Continuous line-Network line (e.g. Fieldbus protocol/devices); Dashed line-Analog line (0–10 V, 4–20 mA) or Digital serial line (RS232, RS485); PID-Proportional-Integral Derivative; LP-Local Process; FP-FieldPoints.

many different control equipment suppliers, with the related costs of operation and maintenance.

Measurement Instrumentation

A key limitation to the application of process control is the lack of appropriate sensors for many process variables. This is so in dairy processes and therefore measuring techniques for online use in the dairy industry are subject to considerable research and manufacturing interest. Basic concepts and the main measurement techniques are reviewed in this section.

Basic Characteristics of Sensors

A sensor is composed of a sensing element and a transmitter, as indicated schematically in **Figure 2**. The sensor output, whether analog or digital, should be a standard signal suitable to be supplied to a controller.

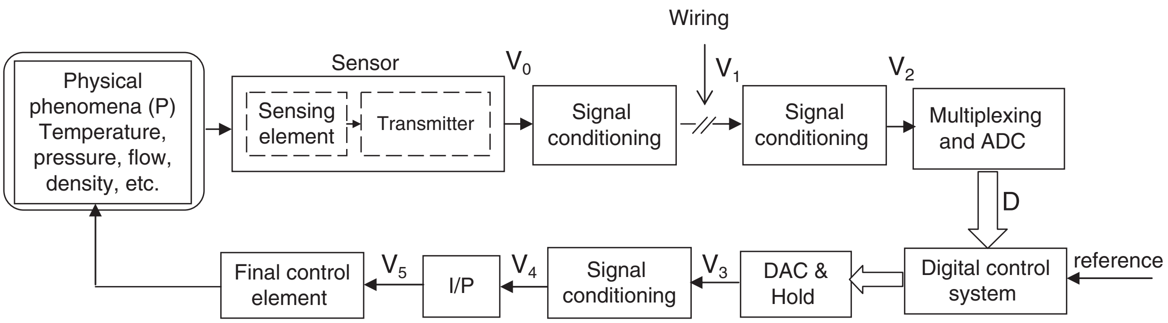
Wiring
Sensor
V_0
V_1
V_2
Physical phenomena (P) Temperature, pressure, flow, density, etc.
Sensing element
Transmitter
Signal conditioning
Signal conditioning
Multiplexing and ADC
D
Final control element
V_5
I/P
V_4
Signal conditioning
V_3
DAC & Hold
Digital control system
reference

Figure 2 Schematic representation of stages in a digital control system.

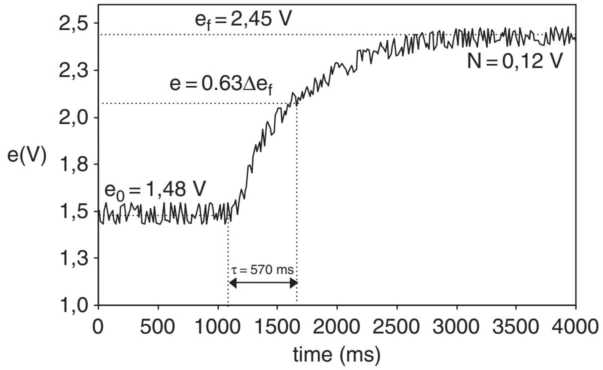

Figure 3 Time response of a pH electrode to a step change in pH (4–7).

Most transmitters respond rapidly. When the sensor element response is also fast, then measurement dynamics can be neglected in view of modeling process dynamics. Such a case is indicated in **Figure 3**, which shows the time response of a pH electrode to a step change in pH from 4 to 7. The characteristic first-order time constant for the sensor dynamics (the time required for the response change to reach 63% of the step change) is in the order of milliseconds ($\tau \cong 570$ ms). There are, however, cases where the measurement dynamics, particularly time lag, may be significant and ignoring it can lead to control difficulties.

Sensors can exhibit linear or non-linear behavior. This is related to and expressed by the relation between the variation of the property value and that of the transmitted signal. Sources of non-linearity usually lie on the sensing element. For a linear sensor, the gain for a given calibration is constant and equal to the ratio between the set span and the range of the sensor output. Nowadays, with digital data acquisition, transducer non-linearities are easily incorporated in the data interpretation software and cause no practical difficulties.

Specialized Sensors/Measurement Systems

The most important properties of dairy processes subject to measurement that reflect both process operation and product quality are classified as objective or subjective. Examples of the former are pH, temperature, flow rates, pressure and level. Sensors for online measurement of such properties have been available for a long time. Properties such as taste, flavor, color and consistency are considered to be subjective and difficult to measure.

Commercial sensors applied in the dairy industry are subject to several quality constraints, such as sanitary, safety and environmental requirements. A trend in new sensor design technologies is the increasing integration of the sensing elements into silicon chip micro-circuits. These new measurement devices directly incorporate all circuitry needed to self-compensate for environmental changes and yield an output that is suitably amplified for transmission to standard electronic controllers. These sensors offer the advantage of small size, reduced price and practically no mechanical parts to wear out.

Next, measurement techniques and instrumentation for the most important objective properties in the dairy industry are considered.

pH

pH measurements are of paramount importance for quality control in milk fermentation and related processes. For example, inadequate pH can be a cause of excess free whey and excess or inadequate tartness in fermented products; pH changes are related to the viscoelastic properties of yogurt and they are also correlated to the physiological state of bacteria in the lactic acid fermentation. Also, the final pH value is normally a feature of the final product: fermentation converts lactose to lactic acid, causing a drop in pH to a value in the range of 4.25–4.5; rapid cooling at the correct level of lactic acid then stops bacterial action.

pH electrodes can be in direct contact with food, if they meet sanitary requirements. In general terms, pH measurements, particularly in conjunction with electrical conductivity measurements, constitute an important means for continuous, real-time process monitoring.

Temperature

In industrial applications, the main measuring devices for low temperatures (below 250 °C) are based either on thermoelectric effects (thermocouples) or on resistance changes (e.g. platinum resistance temperature devices (RTD), thermistors). Measurement characteristics, particularly sensitivity and the degree of (non)linearity, favor the use of platinum RTD; the most widely used is the so-called PT100 device.

Thermal processing is a key stage in most dairy process operations. As an example, during pasteurization of yogurt the temperature is raised to 85–90 °C to destroy undesirable microorganisms and denature the whey to improve viscosity and prevent syneresis. Then, the product is cooled to 40–45 °C so that the mix is ready for inoculation. Also, freezing and refrigeration are used to prevent the growth of unwanted microorganisms. In general, these operations and process units require a reliable and accurate temperature-monitoring and control system.

Level, density or interface level

Liquid height, density or interface level between two liquids can be measured either by differential pressure (d/p cells) sensors or by measurement of buoyancy force on a displacer suspended in a liquid. An electronic transmitter converts the output of the sensing element to an appropriate analog or digital output signal.

Nowadays, instrumentation companies offer the dairy industry a variety of special transmitters for level monitoring and control applications in inventory tanks and clean-in-place (CIP) vessels.

Pressure

In most process operations, particularly when thermal processing is required, pressure regulation is one of the essential control loops. Pressure sensors are thus among the most commonly used on-line sensors. D/p cell transmitters for either gauge or absolute pressure are the solution which is generally adopted.

Thermal conductivity

Thermal conductivity expresses the ability of a substance to conduct heat. The most common thermal conductivity probes consist of an assembly of an electric heated wire and a temperature measurement system (based on thermocouples or thermistors) from where heat fluxes are measured and heat conduction is inferred.

Thermal conductivity measurements find wide application in dairy chemistry and biochemistry and in food process engineering. The measurement of thermal conductivity by line heat source probes may be used for in-line determination of the coagulation time of milk for cheese curd and yogurt production and may be helpful for automating these processes, aiming at maximizing the yield of cheese and yogurt. Coagulation time may be detected by the sharp increase in the temperature difference between probe temperature and initial milk temperature.

Electrical conductivity

Electrical conductivity (*G*) expresses the ability of a substance or medium to conduct electricity. It is employed for quality control and further finds on-line use in identifying features of fermentation states. For example, electrical conductivity measurements allow the urease activity and the acidification activity in lactic acid fermentations to be distinguished. For example, electrical conductivity measurements allow the urease activity and the acidification activity in lactic acid fermentations to be distinguished.

In a dairy plant where many fermentations are performed, either simultaneously or sequentially, the real-time prediction of fermentation completion time is very important for scheduling raw material supply and energy utilization. Data-driven models (e.g. neural network-based) relating characteristic properties of the fermentation state (model outputs) to pH and electrical conductivity (model inputs) can be firstly identified (trained) and subsequently used to monitor the fermentation process and to predict fermentation times.

Viscosity

The main items of equipment for viscosity measurement are process viscometers. Shear viscosity for Newtonian fluids can be measured by a capillary flow viscometer. Cone and plate viscometers are suitable to determine the shear viscosity of time-independent non-Newtonian fluids. From the changes in viscosity, the stages of aggregation and gel formation can be described and calculated. The viscosity can finally be used as an objective measure for the control of coagulation processes.

In industrial operations involving slurries, pulps, grease or other similar media, consistency, rather than viscosity, is measured by rotation and oscillation rheometers. These devices allow, for example, the continuous online monitoring of the transition of milk fluid into a viscoelastic gel structure.

NIR spectroscopy

Infrared and near-infrared (NIR) spectroscopy can be used to measure the levels of water, fat and protein in liquid milk and related products on-line (and *ex situ*). This is a commonly used technique for quality control, but may also be used for on-line closed-loop control.

Optical density

Optical density (OD) is commonly used to measure biomass concentration on-line and *ex situ.* The measurement principle is based on the individual or combined use of measurements of transmission, reflection or scattering of light. The interpretation of the signal is complex, but it is normally linearly correlated to biomass concentration in diluted solutions.

Final Control Elements

Final control elements (FCE) are devices, driven by controller signals, used to manipulate process control variables. In most cases control actions consist of adjusting flow rates of process input or output streams (solid, liquid or gas) or cooling and heating fluids. The most widely used FCE are flow regulator valves.

Designing a valve involves taking decisions on valve size, choice of body material, choice of type of valve (signal-to-close or signal-to-open) and choice of flow versus aperture characteristics (essentially linear or equal percentage valves).

Valve sizing (computing the *Cv* parameter) and the choice of valve flow characteristics require consideration of hydrodynamic aspects, particularly pressure drops along the piping. The choice of material depends on the properties (corrosive, slurry, etc.) of the process fluid. For the dairy industry, stainless-steel valves are most common. The decision on working with normally open or normally closed valves derives directly from answering the safety question of how the valve should stay in an emergency due to energy failure.

Regulator valves are typically driven by a pneumatic signal (range 3–15 psig or 0.02–0.1 MPa). Signals are normally transmitted to the FCE as analog current signals (4–20 mA), being converted locally by a current-to-pneumatic transducer. Alternatively, a step motor, driven by a digital signal from the controller, can actuate valves.

On–off valves normally use electrical or pneumatic actuators. They are used mainly for sequential control and during start-up and shutdown procedures. Other final control elements, namely displacement devices and pumps, are increasingly digitally actuated by step motors.

Digital Control Equipment

Digital control instrumentation represents the new standard and indeed has introduced a major change of mindset with respect to control system structures and solutions, with new procedures concerning communications and calibration routines. Digital control instruments offer the computing power required to implement advanced model-based monitoring and control algorithms (software sensors and predictive control) at local level and in real time. They enable the implementation of plant-wide monitoring, optimization and control solutions through the available distributed control architecture.

Distributed Control Systems

A distributed control system (**Figure 1**) is simply an arrangement whereby control devices and computer processing power are distributed through a network instead of being centralized.

The main devices in a distributed control system are essentially microprocessor-based sub-systems such as programmable logic controllers (PLC), smart sensors, supervisory and engineering stations and other input–output (I/O) devices (e.g. FieldPoints, device integrators, etc.). All devices in the network must be integrated with proper hardware and software for communications.

PLC are today's industrial standard for local digital control. They are reliable special-purpose computers for control in the industrial environment, consisting of a set of I/O modules and a programmable central processing unit. They can perform analog-to-digital (AD) and digital-to-analog (DA) conversion and have special-purpose digital I/O ports (PLCs were originally designed mainly for event control). PLC normally use proprietary programming languages.

Smart sensors are devices that through their digital system can be connected to the network, communicating bidirectionally with the other devices. In particular, they accept remote commands for remote calibration.

General-purpose computers (PC, workstations) may be interconnected in the control system network to carry out inferential measurement procedures, high-level data analysis, supervisory duties or more sophisticated dedicated control tasks.

FieldPoints are modular I/O devices that connect a bank of analog and/or digital I/O modules to an industrial network, being able to perform AD/DA signal conversion.

The key feature of a distributed control system is thus that the measurement and control tasks are distributed out into the field. Integrated hierarchical control configurations may be built where high-level tasks, like process supervision and optimization, are fully integrated with the low-level data acquisition and local control tasks.

Communication Standards

Industrial communications refer to the networking hardware and software, together with the respective communications protocol.

A number of industrial network standards, designed to meet different application requirements, are available today: Ethernet, DeviceNet, Foundation Fieldbus, PROFIBUS and controller area network (CAN). Details of these industrial network standards can be found in **Table 1**.

Some specify low-level sensor–controller–actuator communication protocols (like CAN and DeviceNet), whereas others are specially oriented for distributed control systems in the process industries (Fieldbus and PROFIBUS). They differ on communications bus specifications, on velocity of data transfer, on communications protocol used and on the communications model. For distributed control systems in large factories, Fieldbus and PROFIBUS are the two most important standards.

Ethernet is the most widely used local area network (LAN) technology. An Ethernet LAN may use coaxial cable, special grades of twisted pair wiring, or fiber optic cable. 'Bus' and 'star' wiring configurations are supported. Ethernet devices compete for access to the network using a protocol called carrier sense multiple access with collision detection (CSMA/CD). Ethernet conforms to the IEEE 802.3 specifications and runs commonly under the high-level transfer communication protocol–internet protocol (TCP–IP) (although many others are possible). The Fieldbus standard has adopted a second alternative for the physical layer that is based on Ethernet, thus providing a solution for factory-to-factory communication.

Basics of Analog-to-Digital and Digital-to-Analog Signal Conversion

All digital control systems contain a data acquisition interface that performs AD/DA conversion. Such tasks are commonly performed by standard computers with AD and DA cards, by FieldPoint modules or by PLC.

It is worth examining basic aspects of the data acquisition and control problem, namely the flow of information from the 'process property' to the 'binary word in the

Table 1 Industrial communications standards

Standard	*Description*	*General features*	*Application areas*
DeviceNet	Low-level network designed to connect industrial devices (sensors, actuators) to higher-level devices (controllers)	Powered bus consisting of two separate twisted-pair cables Built on CAN protocol Producer–consumer model for data transfer	Mainly manufacturing industries
Foundation Fieldbus	Digital network standard designed specially for distributed process control; expected to substitute for the 4–20 mA analog standard	H1-powered 31.25 kb s^{-1} bus (standards ISA S50.02-1992; IEC 61158-2) or high-speed 10/100 Mb s^{-1} Ethernet (HSE) Fieldbus (communication) protocol (IEC 1158-2)	Mainly process industries
PROFIBUS DP/FMS/ PA	Family of communication standards. Leading open Fieldbus system in Europe; PA is mainly used in the process industries	DP and FMS: RS485 serial line with baud rates up to 12 Mb s^{-1} PA: Fieldbus standard (IEC 1158-2)	Manufacturing and process automation
Ethernet	EtherNet is an industrial standard that defines only the physical layer. Some industrial standards are built on top of Ethernet	• Coaxial cable with BNC connectors or telephone wiring with RJ45 connectors or fiber-optic cable (10–1000 Mb s^{-1}) (standard IEEE 802.3) • Ethernet does not define itself as the communication protocol. It runs commonly under the TCP–IP	Mainly in LANs for PC-to-PC communication.
CAN	Designed originally for in-vehicle automotive communications	CANbus (serial bus) with CANbus communication protocol	Also for process industries

CAN, controller area network; LAN, local area network; PA, process automation; DP, decentralized periphery; FMS, Fieldbus message specifications; BNC, bayonet nut connector; TCP–IP, transfer communication protocol–internet protocol.

computer' and the feedback from the 'binary decision variable' to final control element.

Data acquisition

The data acquisition chain is represented schematically in **Figure 2**. The design stage of the data acquisition system should start with a qualitative analysis, addressing the following main considerations:

- The sensor should be chosen with the objective of maximizing sensitivity for the desired measurement interval. This means that calibration should be such that the sensor measurement span should match the measurement interval and be mapped into the full range of the output signal.
- Industrial analog-to-digital conversions (ADCs) are nowadays standard. A 12-bit AD converter is generally sufficient. The input range is not a problem, assuming that the required signal conditioners are available.
- Signal conditioning should be such that the sensor output signal V_0 is transduced into a signal V_2 that exhibits the same range as that of the ADC. This situation maximizes the overall resolution of the acquisition chain.

Still at the design stage, the quantitative analysis must be performed in steps from the process to the computer:

- The sensor normally delivers an analog electrical signal (V_0), which should be a known function of the process property, *P*. Assuming, for simplicity, a linear relationship, eqn [1] holds:

$$V_0 = k_0\rho + z_0 \qquad (1)$$

- This signal will generally undergo some form of conditioning (transduction, amplification, attenuation, etc.), after the sensor and before the ADC, depending mainly on aspects related to compatibility and range of transmission signals (V_1 and V_2). These types of transformations can usually be adequately expressed by linear relationships of the form:

$$V_1 = k_1 V_0 + z_1 \qquad (2)$$

$$V_2 = k_2 V_1 + z_2 \qquad (3)$$

- Considering an *n*-bit ADC, with an input range [$V_{\min}$, $V_{\max}$], the digital word *D*, corresponding to V_2, is given by:

$$D = \mathrm{INT}\left[\frac{V_2 - V_{\min}}{V_{\max} - V_{\min}} 2^n\right] \qquad (4)$$

The corresponding discretization error is given by $\pm 0.5(\mathrm{V}_{\max} - \mathrm{V}_{\min})/2^n$. For a 12-bit converter this error is well inside industrial requirements, generally lower than all other errors in the chain.

- For the choice of the sampling time (*t*) in the implementation, a simple rule of thumb is often used in industrial applications, adopting a sampling time value of about one-tenth of the process characteristic time constant.The implementation stage of local signal acquisition corresponds to effectively programming the multiplexing, ADC, data reading (binary value *D*) and data decoding (getting the property *P* from the binary value *D*).
- For each scanning (multiplexing and data reading) of input channels, performed at every time interval *t*, programming of data decoding is performed by successively computing the values from the binary word *D* to the property *P*, by solving eqns [4], [3], [2] and [1] with respect to V_2, V_1, V_0 and *P*, respectively.

$$V_2 = V_{\min} + (D + 0.5)\frac{V_{\max} - V_{\min}}{2^n} \qquad (5)$$

$$V_1 = (V_2 - z_2)/k_2 \qquad (6)$$

$$V_0 = (V_1 - z_1)/k_1 \qquad (7)$$

$$P = (V_0 - z_0)/k_0 \qquad (8)$$

- At this stage, process values are available in the data acquisition application for all types of desired actions, namely data interpretation, data plotting, bookkeeping, analysis of alarms and related actions, computing of control actions and output of control commands.

Control action

It is outside the scope of this article to analyze control algorithms. Assuming that a control decision has been taken, given by a binary word *C*, such a command is transmitted from the control system to the final control element through an elementary chain including the digital-to-analog conversion (DAC) (with a hold element), and, in the more general case where the final control element is a valve, a signal conditioner, a power buffer amplifier and a current-to-pneumatic signal transducer (**Figure 2**). For design purposes, assuming that the DAC is set for a voltage output, eqn [9] represents the DA conversion, where *n* is the number of conversion bits of the DAC, V_{ref} is the reference voltage (corresponding to an output interval of $0 - V_{\max}$, with $V_{\max} \cong V_{\text{ref}}$):

$$V_3 = V_{\text{ref}^*} C/2^n \qquad (9)$$

The industrial standard for DA converters is normally of 12 bit. Most common ranges of output signals are 0–10 V or 4–20 mA.

Finally, referring back to signal conditioning, care must be taken to ensure that wiring is correct, that instruments and source grounds are of good equivalent level (a comment that also applies to wiring in AD lines) and that appropriate buffer amplifiers are used to protect control devices from high currents.

Advanced Topics

Two topics closely related to the state of the art in both information technology and systems theory are now discussed: how to make measurable what is not so; and how to integrate information and manage large-scale systems.

Software Sensors

In many cases, key process variables and characteristic parameters, namely kinetic and transport parameters, are not available directly on-line and in real time, either because they are really not measurable or simply because measurements may be expensive and/or unreliable.

Software sensors are algorithms for the on-line computation of those state variables and parameters that are not measurable in real time, from more easily accessible (accurate and inexpensive) related measurements. The concept is closely related to those of inferential measuring and of state observers, widely discussed in the specialized systems engineering literature. The design and implementation of software sensors provide in some cases a suitable answer to cope with the lack of instrumental sensors. It may require the computational power of a dedicated computer in the distributed control network, a requirement that nowadays represents no technical or economical problem.

A software sensor relies on a process (sub-system) model that establishes the relationship between measured and estimated properties. Hence, the key for success (or failure) of a software sensor is the availability of knowledge/information about the relationship between measured and unmeasured properties, i.e. the accuracy and robustness of the underlying model. With respect to and in the context of dairy industries, the kinetics in fermentation tanks are the most difficult part of the process to model. Traditionally, designing adaptive observation/estimation algorithms, of which the most frequently reported technique is the extended Kalman filter, circumvents the problem. This type of method requires a number of simplifying assumptions that are not always acceptable. More recently, knowledge-based software sensors have been reported. They rely on artificial intelligence (AI) models like artificial neural networks (ANN) and fuzzy or hybrid neuro-fuzzy models, combined with mechanistic expressions of process behavior. Though models reported in the literature are excellent in their ability to predict based on minimum information, the requirements of both know-how and computational power for implementation are still substantial. This hinders its industrial application on a wide scale, in the short term.

Measurement of Subjective Properties

Properties such as taste, flavor, and consistency are subjective properties that are extremely important for process operation in food industries. At present operators use their human senses, i.e. smell/aroma, feel, taste, as a gauge for the acceptability of products. Measurement techniques for subjective properties are reported to be under intensive investigations. The electronic nose concept is maybe the best-known example. The electronic nose mimics the human nose to detect specific aromas and smell. This technology is commercially available today. Image analysis is now a well-developed technique. Properties such as material visual aspect can be inferred from these techniques, providing new information for further and new automatic processing.

Factory-to-Factory Automation

As a final point, with the internet, it has become clear that the information flow scheme of **Figure 1** in an automated factory may move down the hierarchy. As this technology becomes reliable and secure, we may speak of a factory-to-factory (worldwide) automated information flow and distributed processing. Whilst this does not represent a state-of-routine automation solution, it represents a real management tool in the context of prevailing business concepts.

Conclusions

Basic measurement concepts and control instrumentation applied in the dairy industry have been reviewed in this article. The intention was to provide an insight to the main principles and characteristics of classical and more advanced sensor devices and control equipment.

The measurement mechanisms considered range from the commercially available sensors for important objective properties of dairy processes to more sophisticated model-based software sensors and new technological solutions for monitoring subjective properties in a variety of food industries.

The discussion of digital control system instrumentation is also focused on core items such as final control elements, distributed control systems with respective communications standards and signal conversion required for data acquisition.

Though the topics considered are far from being a complete overview of all research and industrial developments in this area, the article provides structured information on the main instrumentation aspects routinely implemented or potentially applicable in the dairy industry.

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See also: **Hazard Analysis and Critical Control Points**: Processing Plants. **Plant and Equipment**: Flow Equipment: Valves; Instrumentation and Process Control: Process Control; Process and Plant Design.

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Instrumentation and Process Control: Process Control

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Introduction – Dairy Industry, Aims, Trends

Dairy processing industries worldwide have undergone rationalization, with a trend toward fewer but larger plants specialized in a limited range of products and operated by fewer people. Plants producing market milk and products with short shelf-life, such as yogurts, creams, and soft cheeses, tend to be located on the fringe of urban centers close to consumer markets. Plants manufacturing items with longer shelf-life, such as butter, milk powders, cheese, and whey powders, tend to be located in rural areas closer to the milk supply.

Basic dairy processes have changed little in the past decade. However, changing global needs and the pressure of globalization create contemporary challenges related to rapid development of new, high-value-added products, improvements of production efficiency, and more energy-efficient, cost-saving, and environmentally friendly processes. Issues such as pollution prevention and reduction of waste loads and product losses are critical for the dairy industry. To achieve these requirements, an integrated smart production policy is expected based on a holistic plant-wide representation of the production chain from process control level, through plant management, to corporate management. Modern dairy technology consists of a few hierarchical stages:

Enterprise resource planning (ERP), which refers to a system that sits at the top of an integrated dairy infrastructure to manage the demands of business, in terms of functions such as sales and distribution, accounting, materials management, asset management, and plant maintenance.

Manufacturing execution (ME) refers to a system that centers on the product as it moves through the plant.

Supervisory control and data acquisition (SCADA) is a system that provides the operator and other users with access to direct regulatory process control, such as programmable logic controllers (PLCs). The SCADA system works in real time and provides graphical status displays and process monitoring. Though the PLCs form the basis of most traditional process control systems in the dairy industry, the use of more sophisticated control systems, information technology (IT)-based modern control equipment, and new control algorithms are solutions implemented by an increasing number of companies. For example, specialized processes such as ultrafiltration and modern drying processes have increased the opportunity for the recovery of milk solids that were formerly discharged.

ME provides a bridge between ERP systems and real-time control supplied by PLC and SCADA systems, for functions such as batch process control and production scheduling.

Batch and Semibatch Process Operation

Batch or fed-batch mode of operation is a typical production scheme for a large group of dairy processes. It is related to the formulation of control problems in terms of the economic or performance objective at the end of the process; for example, milk powder production quality is evaluated by the total quantity and concentration of solids at the end of the process. The main challenge of batch production is the large batch-to-batch variation of the final characteristics. This lack of process repeatability is an inherent consequence of the variability of raw materials and uncontrolled parameters and not necessarily due to improper control policies, though the latter would greatly enhance the problem. Over the past 10 years, a number of control methods have been researched to cope with process constraints and different objectives derived from economic or environmental considerations with the objective to drive the process to its optimal state of profit maximization and cost minimization. Some of the most typical control paradigms and new model-based control trends are presented in the rest of this article.

Classical Closed-Loop Process Control – Established Control Engineering Practice

Any control system in which the output is monitored (measured), compared (subtracted) with the reference (desired) input, and the difference (the error) used to actuate the controller until the output equals the

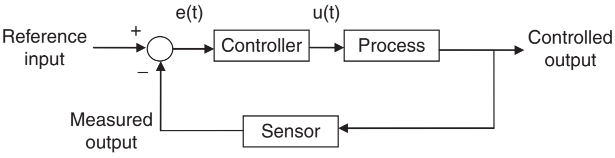

Figure 1 Closed-loop process control.

reference is called a closed-loop or feedback control system (see **Figure 1**).

The controller design consists of computing the PID parameters K_p, K_I, K_d such that the closed-loop performance and stability requirements are achieved. Each parameter is related to one of the terms (proportional, integral, or derivative) of the control law.

Proportional action. The magnitude of the contribution of the proportional term to the overall control action is determined by proportional gain, K_p. A high proportional gain results in a large change in the output for a given change in the error. If the proportional gain is too high, the system can become unstable. In contrast, a small gain results in a small output response to a large input error and a less responsive (or sensitive) controller. In the absence of disturbances, pure proportional control will not settle at its target value but will retain a steady-state error that is a function of the proportional gain and the process gain.

Integral action. The contribution of the integral term is proportional to the error magnitude and error duration. Summing the instantaneous errors over time (integrating the error) gives the accumulated offset. The magnitude of the contribution of the integral term to the overall control action is determined by the integral gain, K_I. The integral action (when added to the proportional action – PI controller) accelerates the movement of the process toward the setpoint and eliminates the residual steady-state error that occurs with a proportional-only controller. However, because the integral term responds to accumulated errors from the past, it increases oscillations and overshoots of the setpoint.

Derivative action. The rate of change of the process error is calculated by determining the slope of the error over time (its first derivative over time) and multiplying this rate of change by the derivative gain, K_d. The magnitude of the contribution of the derivative action to the overall control action is termed the derivative gain, K_d. The derivative action slows the rate of change of the controller output and thus decreases oscillations and overshoots, an effect that is most noticeable close to the controller setpoint. However, differentiation of a signal amplifies noise, and thus this term in the controller is highly sensitive to noise in the error term and can cause a process to become unstable if the noise and the derivative gain are sufficiently large.

Statistical Process Control

Statistical process control (SPC) is one of the most powerful tools for process improvement. Although SPC is new to the dairy industry, it is not really a new approach. It has been used successfully in manufacturing businesses for more than 60 years. SPC is a set of analytical tools of which the control chart is one of the most important. Control charts are helpful in signaling that a change has occurred. The fundamental concept of control charts is to distinguish between inherent random variation and real changes in output, quality, or measured performance. SPC methods can be used to signal emerging problems and to evaluate the positive or negative impact of a change in a management practice or the implementation of a new product. Next, some of the most commonly used online multivariate SPC techniques are presented briefly.

Empirical Linear Techniques

Batch process modeling and monitoring has been always a challenging problem in dairy engineering due to the presence of nonlinear behavior and serial correlation, correlated and/or collinear data, varying batch lengths, and multiproduct production. Current state of the art empirical techniques include the bilinear approaches of multiway principal component analysis (MPCA), the multiway partial least squares (MPLS), and the trilinear methodologies of parallel factor analysis (PARAFAC) and PARAFAC II. Although the above bilinear and trilinear techniques have been applied successfully to batch processes, they experience a number of limitations. For example, they do not incorporate the process dynamics, and with the exception of PARAFAC II, the duration of the batches is assumed to be constant. Moreover, for online monitoring, it is required that the whole batch trajectory is known or is predictable. This requirement results in certain assumptions being made to in-fill the unknown future values of the batch trajectory. Finally, all these techniques are linear and to a greater or lesser extent fail to capture the nonlinear nature of a batch process. Alternative approaches that overcome the issues of data in-filling and unequal batches are presented next.

Moving Window Principal Component Analysis

In moving window principal component analysis (MWPCA), typically, measurements from a batch process are arranged in a three-dimensional matrix **X** ($NB \times NV \times NT$) where NB, NV, and NT are the number of batches, variables, and time instants (**Figure 2**(**a**)). The three-dimensional matrix **X** can be transformed to a bidimensional matrix by unfolding over the batch dimension ($NB \times (NV \times NT)$), as shown in **Figure 2**.

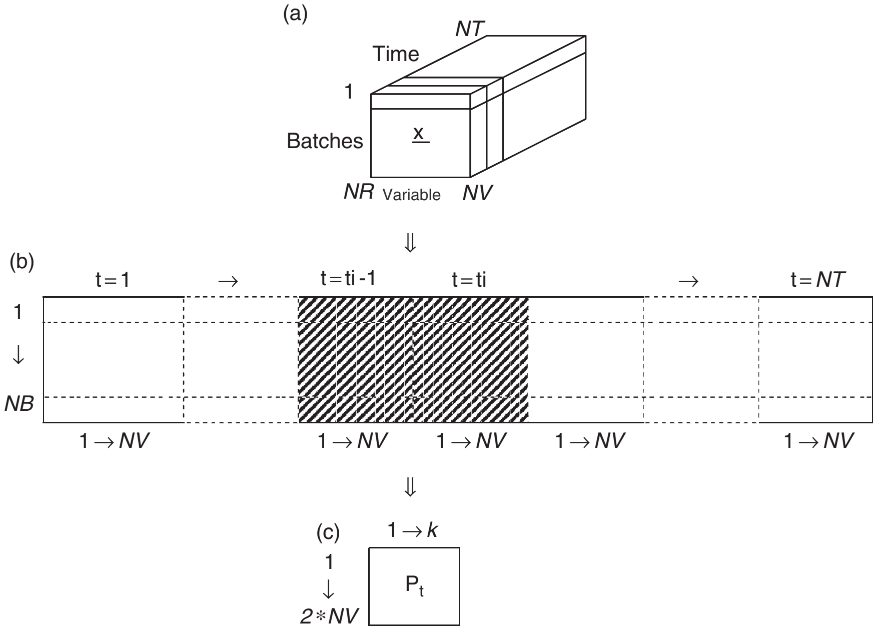

Figure 2 Moving window principal component analysis (MWPCA).

A scaling is usually applied to the unfolded matrix **X** before an ordinary principal components analysis (PCA). The mean of each column of **X** is subtracted from each data element of this column. This way of mean centering is very important as it results in the removal of the main nonlinear component in the data. Furthermore, by scaling the variables in each column of **X**, the differences in the measurement units between variables can be handled to allow equal weight to be given to each variable at each time interval. A PCA model is then developed on a moving window of data. Having selected the length of the moving window (L), MWPCA then develops $NT{-}L+1$ PCA models for each time interval by decomposing the ($NB \times NV$) matrix **X** into a systematic and noisy part:

$$\mathbf{X} = \mathbf{T}_k\mathbf{P}_k^T + \mathbf{E} \qquad [1]$$

where $\mathbf{T}_k$ and $\mathbf{P}_k$ are the matrices of the k retained principal component scores and loading respectively, and **E** is the matrix of the residuals. The number of the retained principal components, k, is usually determined by the means of cross-validation. For the application exemplified, the order of the moving window was selected to be $L = 2$. For each PCA model, the loading matrix $\mathbf{P}_k$ is stored. Having performed a PCA analysis, a set of online monitoring tools can then be developed. Typically, these tools are Hotelling's T^2 and squared prediction error (SPE) control charts. For instance, considering that a new batch $\mathbf{x}_{\text{new}}$ is to be monitored, the Hotelling's T^2 is calculated using the k retained PCA scores by:

$$\mathbf{t}_k = \mathbf{x}_{\text{new}}\mathbf{P}_k \qquad [2]$$

$$T^2 = \mathbf{t}_t\mathbf{S}_t^{-1}\mathbf{t}_t^T \qquad [3]$$

where $\mathbf{t}_k$ are the k retained PCA scores and **S** is their covariance matrix. The SPE is then calculated as follows:

$$\text{SPE} = \mathbf{e}_t\mathbf{e}_t^T \qquad [4]$$

$$\mathbf{e} = \mathbf{x}_{\text{new}}\left(\mathbf{I}-\mathbf{P}_k\mathbf{P}_k^T\right) \qquad [5]$$

Batch Dynamic Principal Component Analysis

The MWPCA approach does not capture the dynamic behavior within a batch process. The batch dynamic principal component analysis (BDPCA) is an alternative method that uses lagged variables to incorporate process dynamics. More specifically in BDPCA, each batch is isolated from the others (see **Figure 3**(**a**)). A matrix $\mathbf{X}_{iv}$ ($NT \times NV$) is formed for each iv batch. Then each of the NV variables is lagged d times resulting in a lagged $\mathbf{X}_{iv}$ $[(NT - d) \times (NV \cdot (d+1))]$ matrix (see **Figure 3**(**b**)). The covariance matrix of the lagged $\mathbf{X}_{iv}$ matrix, $\mathbf{S}_{iv}$, is then calculated (see **Figure 3**(**c**)). The procedure is repeated for all NB batches, resulting in NB $\mathbf{S}_{iv}$ covariance matrices. The elements in each of the $\mathbf{S}_{iv}$ matrices are a measure of the dynamic relationship between variables in batch iv. Having calculated these dynamic correlations for all NB batches, an average covariance matrix, $\mathbf{S}_{\text{avg}}$, is then calculated based on the NB $\mathbf{S}_{iv}$ covariance matrices (see **Figure 3**(**d**)):

$$\mathbf{S}_{\text{avg}} = \frac{(NT-d-1)\sum_{iv=1}^{NB}\mathbf{S}_{iv}}{NV(NT-d)} \qquad [6]$$

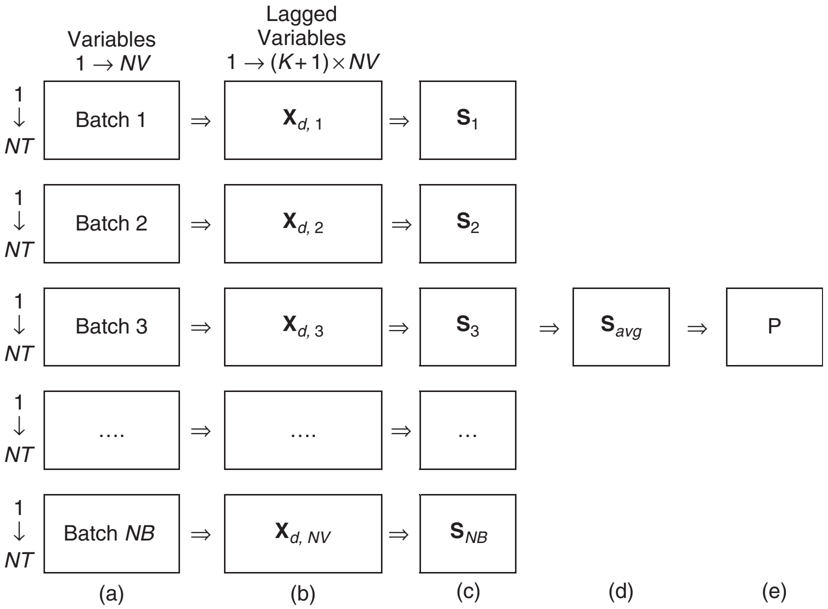
Variables
1 → NV
Lagged
Variables
1 → (K+1)×NV
1
↓
NT
Batch 1
⇒
X_{d, 1}
⇒
S_1
Batch 2
X_{d, 2}
S_2
Batch 3
X_{d, 3}
S_3
S_avg
P
....
...
Batch NB
X_{d, NV}
S_NB
(a)
(b)
(c)
(d)
(e)

Figure 3 Batch dynamic principal component analysis (BDPCA).

The average covariance matrix, $\mathbf{S}_{\text{avg}}$, expresses the average dynamic relationships between the process measurements. A PCA model is then developed based on $\mathbf{S}_{\text{avg}}$ (see **Figure 3**(**e**)). The resulting BDPCA model is finally used to calculate the T^2 and SPE statistics for monitoring purposes.

Batch Observation Level

The batch observation level (BOL) method considers the problem with unequal batch lengths. In BOL, the original three-way data is unfolded over the variable's dimension (see **Figure 4**(**a**)). A dummy y-variable that can be a time index or a batch maturity index is then specified. Data are scaled and matrix X is transformed into a systematic and noisy part as in eqn [1]. Partial least squares (PLS) analysis is then performed between the unfolded matrix **X** and the dummy **y** vector:

$$\mathbf{y} = \mathbf{T}_k\mathbf{c} + \mathbf{f} \qquad [7]$$

where **c** is the regression vector of **y** onto the PLS scores $\mathbf{T}_k$ (see **Figure 4**(**b**)) and **f** are the PLS model residuals. The number of PLS latent variables to be retained are selected as those that provide an adequate description of both the **X** and **y** spaces. For setting up an online monitoring scheme, the PLS scores retained are then rearranged over the batch dimension resulting in an $[NB \times (NT{\cdot}k)]$ matrix (see **Figure 4**(**c**)) and their mean and standard deviation calculated for each sample point and stored (see **Figure 4**(**d**)). In an online situation,

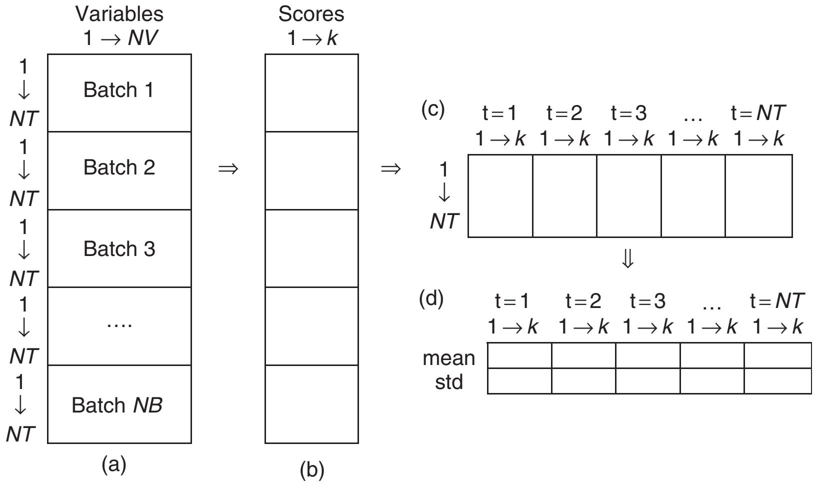
Variables
1 → NV
Scores
1 → k
1
↓
NT
Batch 1
Batch 2
Batch 3
....
Batch NB
⇒
(c)
t=1 t=2 t=3 ... t=NT
1 → k 1 → k 1 → k 1 → k 1 → k
⇓
(d)
mean
std
(a)
(b)

Figure 4 Batch observation level (BOL).

when a new sample is obtained, the scores are calculated initially and then scaled using the mean and the standard deviation of the corresponding sample point. These scaled scores are plotted against their control limits in univariate score plot charts. Similar to the previous approaches, T^2 and SPE charts can also be constructed.

Time-Varying State Space Modeling

Time-varying state space (TVSS) modeling is an alternative approach for batch process modeling and monitoring with the following state space model:

$$\mathbf{t}_{t+1} = \mathbf{C}_t \mathbf{t}_t + \mathbf{w}_t \qquad [8]$$

$$\mathbf{y}_t = \mathbf{H}_t \mathbf{t}_t + \mathbf{e}_t \qquad [9]$$

where **t** is the system states, **y** is the available process measurements, and **w** and **e** are the state and output residuals with covariance matrices **Q** and **R,** respectively. Finally, **C** and **H** are the state space model matrices, which are assumed to be time-varying as they aim to describe a nonstationary process. To develop the model, the data are initially unfolded and scaled as in MWPCA. The procedure to compute the TVSS matrices **C** and **H** then proceeds through the identification of the system states. For a time interval $t = k$, the past and the future of the system are defined as shown in **Figure 5**(**a**). The past (***p***) of the process is associated with the past process measurements of all batches at time *k* up to a specific lag (in **Figure 5**(**a**), the time lag, *K*, was set to a value of two):

$$\mathbf{p}_t = \left[\mathbf{y}_{t-1}\ \ \mathbf{y}_{t-2} \cdots \mathbf{y}_{t-K}\right]^T \qquad [10]$$

The future (***f***) of the process is the current and future process measurements of all batches (in **Figure 5**(**a**), the future horizon, *L*, is set up to a value of one):

$$\mathbf{f}_t = \left[\mathbf{y}_t\ \ \mathbf{y}_{t+1} \cdots \mathbf{y}_{t+L}\right]^T \qquad [11]$$

Now, by applying any one of either PLS, principal component regression (PCR), or canonical variate analysis (CVA) between the past (eqn [10]) and the future (eqn [11]) of the process, new latent variables that provide a reliable approximation of the true system states can be calculated. PCR scores capture the variability between process measurements, while PLS and CVA latent variables are those linear combinations of the past that include the information required to predict process future. The result of applying either a PCR, PLS, or CVA analysis is a weighting matrix $\mathbf{J}_t$, which is used to identify the system states through the past vector $\mathbf{p}_t$:

$$\mathbf{t}_t = \mathbf{J}_t \mathbf{p}_t \qquad [12]$$

Once the system states have been identified, the state space matrices can be computed using a least squares solution.

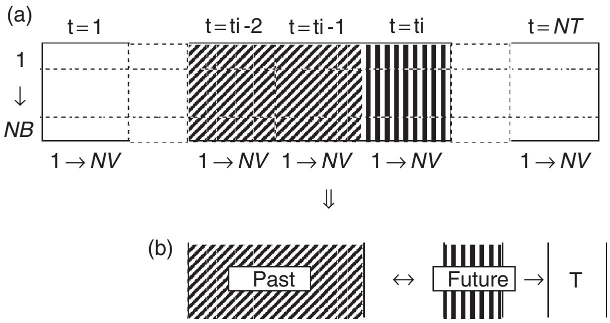

Figure 5 Time-varying state space model (TVSS).

Intelligent Control

The process modeling and monitoring techniques discussed in the section ‘Statistical Process Control’ are the first step toward developing modern computer-based control systems able to collect a large amount of process operational data, store it in databases, and display it to the operator. The next step is the subsequent decision making that still relies mainly on the human operators; however, new concepts and methodologies for automatic analysis and developing a computationally intelligent control (IC) are slowly being introduced in dairy manufacturing. IC deals with the application of data mining, machine learning, and knowledge discovery paradigms, artificial intelligence, expert systems, fuzzy logic, and neural networks for controlling complex physical processes that are difficult to control using conventional methods. The main modules of an IC system are discussed below.

Perception Subsystem

Information from the plant and the environment is collected and processed into a form suitable for perception. Basic elements of a perception subsystem are

- Sensor arrays – provide raw plant and environmental data
- Signal processing – transforms data into information and knowledge
- Data fusion and pattern recognition – uses multidimensional and varying nature data spaces to extract underlying patterns describing the plant and the environment

Cognition Subsystem

In an IC framework, cognition is concerned with the decision-making process under conditions of uncertainty. Basic activities of a cognition subsystem are

- Reasoning – using knowledge-based algorithms and fuzzy logic
- Strategic planning – using adaptive search and genetic algorithms for optimum policy evaluation and path prediction
- Learning – using adaptive supervised (teacher supported) or unsupervised (self) learning paradigms

The Actuator Subsystem

The actuators operate using signals from the cognition subsystem to drive the plant to some desired state. In the event of actuator/sensor failure, the IC system has to be able to reconfigure its control strategy.

Though milk production is a traditional dairy process for which conventional controllers have been used intensively, IC appears to be quite plausible for such processes. If the temperature drifts during processing, safety can be compromised or the product flavor or texture might be ruined. To perform the delicate balancing act that dairy products demand, plant operators need complete control, including the ability to know precisely what is happening at every moment and to perform urgent changes on time. It is not easy, especially when the product is hidden from sight in tanks and pipes more than 90% of the time. Companies that process the same products day in and day out have experience on their side, but those that change frequently have to be very agile and adaptable. In this sense, fuzzy and model predictive controllers are very good examples for building IC systems that are well accepted generally in the process industry.

Fuzzy Logic Control System

The basic structure of a fuzzy logic control system (FLCS) is shown in **Figure 6**.

Fuzzification Module

Fuzzification is a process of mapping the input variables to the fuzzy logic controller (FLC) into a set of membership functions, known as fuzzy sets. Fuzzy set theory provides a means for representing uncertainty. In general, probability theory is the primary tool for analyzing uncertainty and assumes that uncertainty is a random process. However, not all uncertainty is random, and fuzzy set theory is used to model the kind of uncertainty associated with imprecision, vagueness, and lack of information.

Conventional set theory distinguishes elements that are members or not members of a set, with very clear, crisp boundaries between them. For example, temperatures between 20 °C and 30 °C belong to the crisp set 'medium temperature', and all temperatures between these boundaries have a membership value of one ($\mu = 1$). The central concept of fuzzy set theory is that the membership function μ can have a value between 0 and 1. The shape of the membership function is also known as the universe of discourse. Among the most typical fuzzy set shapes are symmetrical triangles, trapezoids, and Gaussian or bell-shaped curves. Each set is given a linguistic label to identify it; for example, positive big (PB), positive small (PS), about zero (Z), negative big (NB), negative small (NS). The size of the universe of discourse depends on the range of the variable and the number of the sets. The number and shape of fuzzy sets are a trade-off between precision of control action and real-time computational complexity.

Fuzzy Rulebase Module

The fuzzy rulebase consists of a set of antecedent-consequent linguistic rules of the form

$$\text{Example: OR IF } e \text{ is PS AND } ce \text{ is NS THEN } u \text{ is PS} \qquad [13]$$

where e is the error (process reference – measured value), ce is the rate of change of the error $ce = \dfrac{\mathrm{d}e(t)}{\mathrm{d}t}$, and the objective of the FLC (see **Figure 6**) is to minimize e and ce.

In this example, the FLC input variables are e and ce, the fuzzy logic controller (FLC) output variable is u, and

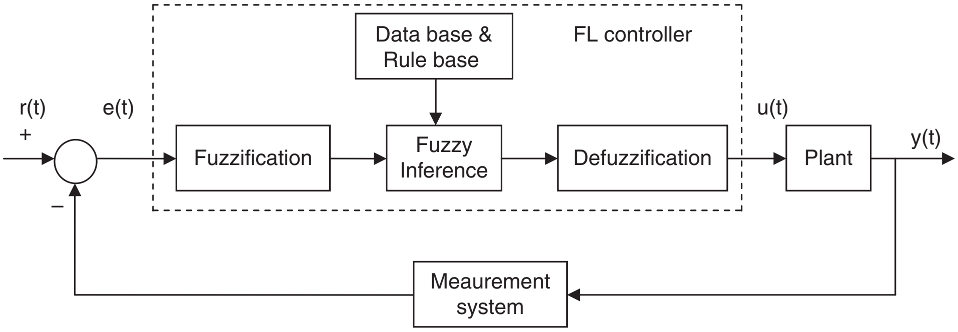

Figure 6 Fuzzy logic (FL) control system.

they all have values defined as fuzzy sets. The fuzzy conditional statement eqn [13] is often called a Mamdani-type rule after Mamdani who first used it to control a steam plant. The rule base is constructed using *a priori* knowledge from the following sources:

- Physical laws that govern the plant dynamics.
- Data from other controllers.
- Imprecise heuristic knowledge obtained from experienced plant operators and experts.

Fuzzy Inference Module

Fuzzy inference is the process of mapping membership values from the input variable(s) through the rulebase to the output variable(s).

Example (continued): If at a certain moment the membership values of the input variables are

$$\mu_{PS}(e) = a,\ \mu_{NS}(ce) = b \qquad [14]$$

based on fuzzy set operations and the fuzzy rule eqn [13], the membership value of the control action u is computed as

$$\mu_{PS}(u) = \max[\min(\mu_{PS}(e), \mu_{NS}(ce))] \qquad [15]$$

Equation [15] is referred to as the max-min inference process or max-min fuzzy reasoning.

Defuzzification Module

Defuzzification is the process of mapping from a set of inferred fuzzy control signals belonging to fuzzy sets to a nonfuzzy (crisp) control signal. The center of area is the most well-known defuzzification technique, which can be expressed as

$$u\,(\text{crisp}) = \frac{\sum_{i=1}^{n} C(A\mu(u_i))A\mu(u_i)}{\sum_{i=1}^{n} A\mu(u_i)} \qquad [16]$$

where $A\mu(u_i)$ is the area of the ith membership function computed at the previous step (the fuzzy inference), and $C(A\mu(u_i))$ is the center of the respective area.

Model-Based Predictive Control

Modeling Approaches

Process modeling strategies can be divided into the following main streams:

- The analytical approach (*white-box models*) is concerned with building the so-called mechanistic model, also known as first-principles model, which is a set of mathematical expressions that reflect the dynamic (differential equations) or static (algebraic equations) behavior of the modeled plant. The mechanistic model is a result of extensive, specially designed experiments and domain knowledge on the physical laws that govern the process at hand. Analytical modeling is time and resource consuming, but it has the main advantage of permitting good generalizations and scale-up.
- Data-driven alternatives (*black-box models*) are based on data mining and machine learning techniques and aim at extracting process knowledge from databases collected during the normal operation of the plant. The development of data-driven models usually takes less time and resources; however, their generalization outside the data space used to build the model is poor. One of the most common black-box models is the artificial neural network (ANN) paradigm, which will be described in more detail later.
- Hybrid modeling (*gray-box model)* is a combination of the two previous approaches and is also known as knowledge-based hybrid modeling (KBHM). KBHM offers a reasonable compromise between the extensive efforts to obtain a fully parameterized structure and the poor generalization of the data-driven models.

Process modeling for the dairy industry is strongly influenced by the recent trends in building data-based or KBHM models. For example, fouling, the unwanted formation of deposits on heated surfaces, is a major unsolved problem in the dairy industry. A direct consequence of fouling is reduction in the processing efficiency, because the material deposited disturbs both the fluid flow and the heat transfer, which in turn may impair product quality. Additionally, the deposit removal shortens the running time between cleaning cycles and thus increases the costs. As a result, daily cleaning is a common practice in the dairy industry and is necessary for hygienic and product quality requirements. The additional annual costs in the dairy industry caused by fouling are estimated at US$260 million per year. A relevant model of fouling would be a powerful tool for the development of strategies to avoid or reduce this unwanted process. However, due to its very complex nature, milk fouling is only partially understood, and its modeling is a challenging issue. A KBHM model combining parameterized equations (for fluid flow, heat and mass transfer) with qualitative knowledge in fuzzy logic form (for protein and salt deposition) was developed a few years ago. The model describes the fouling behavior with regard to the temperature and the pressure drop in a time-dependent manner, and it is not restricted to a certain dairy product or plant configuration. KBHM was successfully used for testing technological improvements in the heat treatment of milk in tubular heat exchangers.

Model Predictive Control – General Formulation

The term model predictive control (MPC) does not refer to a particular control method; instead, it corresponds with a general control approach. The MPC concept, introduced in the late 1970s, has evolved to a mature level and has become an attractive control strategy implemented in a variety of process industries and in the dairy industry in particular. The main difference between the MPC configurations is the model used to predict the future behavior of the process or the implemented optimization procedure. First, the MPC based on linear models gained popularity as an industrial alternative to PID control, and later nonlinear cases such as tubular heat exchangers and drying processes were reported as successfully MPC-controlled processes. MPC is an optimization-based multivariable constrained control technique that uses a dynamic model, from the types described in the previous section, for process output predictions.

At each sampling time, the model is updated on the basis of new measurements and state variables estimates. Then, the open-loop optimal manipulated variable moves are computed over a finite (predefined) prediction horizon with respect to some performance index, and the manipulated variables for the subsequent prediction horizon are implemented. The prediction horizon is shifted or shrunk by usually one sampling time into the future, and the previous steps are repeated.

Artificial Neural Networks

Over the past 20 years, ANNs became a well-established methodology not only as a reliable classifier with countless applications but also as a data-driven modeling framework. The remarkable success of the ANN approach is in great part due to the following features:

1. ANNs are universal approximators. It has been proved that any continuous nonlinear function can be approximated arbitrarily well over a compact set by a multilayer ANNs, which consists of one or more hidden layers.
2. Learning and adaptation. The intelligence of ANNs comes from their generalization ability with respect to unknown data. Online adaptation of the weights is possible.
3. Multivariable systems. ANNs may have many inputs and outputs, which makes it easy to model multivariable systems.

ANNs have been applied to design robust neural controllers with guaranteed stability and reference tracking. The neural control problem can be approached in a direct or indirect control design framework. Direct ANN control means that the controller has an ANN structure, whereas in the indirect ANN control scheme, first an ANN is used to model the process to be controlled, and this model is then used in a more conventional controller design. The implementation of the first approach is simple, but the design and the tuning are rather challenging. The indirect design is very flexible, the model is typically trained in advance, and the controller is designed online. Moreover, the ANNs appear to be rather convenient numerical models when dealing with nonlinear systems or in general with systems for which data are available but little is known on the physical mechanisms that determine their dynamics.

The most popular ANN structures for modeling reasons are feedforward networks (FFNNs) and recurrent networks (RNNs). The RNNs are most suitable for dynamic system modeling, due to the memory introduced by the recurrent (delayed and/or fed back) signals (see **Figure 7**). Normally, the RNN has two vector inputs (*r* and *p*) formed by past values of the process input and the network output, respectively. A linear activation function is often located at the output node (layer 2), and S-shape functions are usually the hidden nodes (layer 1).

ANN Error Tolerant Model Predictive Control

MPC controllers proved to be the most promising alternative to the traditional PID control that has the potential to overcome the problem of the lack of repeatability and related product recycling or loss increase. It has been applied successfully in batch dairy processes assuming linear process dynamics. However, online execution of MPC with predictions running on a large number of empirical and analytical nonlinear algebraic differential equations (the process model) make this alternative computationally more demanding or even

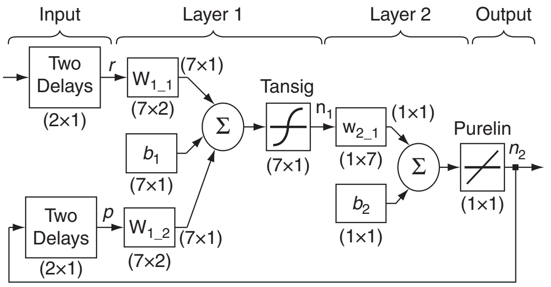

Figure 7 Recurrent neural network (RNN) architecture.

unfeasible for dairy processes with fast nonlinear dynamics. Even for processes where the standard control approach (e.g., ladder logic) is not the best solution, the implementation of MPC is impeded due to high computational costs.

A recent modification of the classical MPC, termed ANN Error Tolerant (ET) MPC, reduces considerably the average duration of each optimization step and makes the MPC computationally more efficient and attractive for industrial applications (see **Figure 8**). The ANN model is integrated into the controller, and the optimization procedure is executed only when the error is above a predefined value.

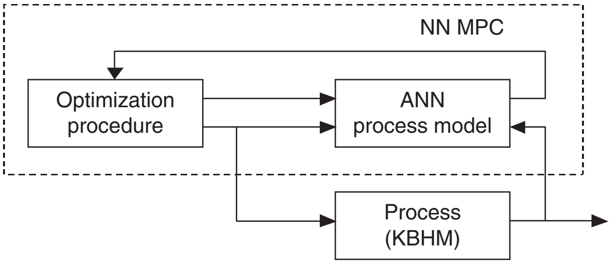

Figure 8 ANN-based model predictive control (MPC).

The discretized version of the modified performance index is

$$u(t+k) = \begin{cases} u\text{:}\left\{ \min\limits_{[u(t+k),u(t+k+1,\ldots u(t+H_c))]} \quad F = \lambda_1 \sum_{k=1}^{H_p} (e(t+k))^2 - \lambda_2 \sum_{k=1}^{H_c} (\Delta u(t+k))^2, \text{if } E_{\sum} > \alpha \right. \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad , \alpha \in R^+ \\ u^* \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad , \text{if } E_{\sum} < \alpha \end{cases} \qquad [17]$$

where $E_{\sum} = \frac{1}{H_p}\sum_{k=1}^{H_p} \left| e\,(t+k) \right|, e(t+k) = ref\,(t+k) - y_p(t+k), \Delta u(t+k) = u(t+k-1) - u(t+k-2)$, is the prediction model response. The prediction horizon H_p is the number of time steps over which the prediction errors are minimized, and the control horizon H_c is the number of time steps over which the control increments are minimized. $u\,(t+k),\ u\,(t+k+1), \ldots u\,(t+H_c)$ are tentative values of the future control signal, which are limited by $u_{\min}$ and $u_{\max}$. The controller is denoted as an ET MPC formulation because the optimization is performed only when the error function $E_{\sum}$ is bigger than a predefined real positive value α. To reduce the computational burden when the error is less than α, the control action is equal to u^*, which is the last value of u, computed before the error enters the α strip. Note that $E_{\sum}$ in eqn [17] is defined as the mean value of the future errors, between the predicted output and its reference along the next H_p steps.

Conclusion

In new integrated dairy plants, each process is carried out in multiple phases, and there exists strong nonlinear and dynamic effects between the variables. Therefore, modern process control systems have usually a hierarchical architecture including decentralized controllers, remote input and output (I/O) modules, fieldbus systems, local area networks (LANs), and smart sensors and other devices. The huge amount of information flows are stored and used online or off-line for executing the IC alternatives like FLC, ANN MPC, or SPC, all methods described in this article.

Interested readers are advised to consult not only the references in the 'Further Reading' section but also to follow publications in the *Journal of Food Engineering*, *Journal of Biotechnology and Bioengineering*, and the *International Dairy Journal*.

Acknowledgment

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See also: **Plant and Equipment:** Instrumentation and Process Control: Instrumentation.

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Robots

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Introduction

Robots are electromechanical devices that perform repetitive operations otherwise carried out by humans. In a modern dairy factory, human handling is rare in processing equipment; from liquid milk to packaged product, there are few, if any, manual operations. Even packaging lines have been highly automated, and operators in a modern dairy plant are needed mostly to load/unload some machines, check the controls, and react to production problems. The interest in robots in dairy lies mostly in the two extremes of the process: (1) milking and herd management; (2) final palletizing and stock management.

Robots rarely look like humans, as they only need to have the parts that perform the specific action. In essence, an industrial robot must have sensors to detect positions, shapes, and forms, a program that allows it to identify what it will be handling, moving parts with grippers for grabbing and handling, and other sensors directing the moving parts to place what it is handling in the correct position. They may also have valves, pumps, and other devices, depending on the nature of the action they need to perform. Robots must be programmable, so that the actions can be set up by the operator with the required flexibility. This is the main reason why robots are a solution where automation is otherwise not possible. Usually, a period of training of the program will be necessary, so the accuracy of the action can be adjusted to the inputs from the sensors.

Dairy offers a good example of the difference between automation and robotics. The latter implies the former, but not the reverse. It is possible to milk a cow using automated systems that collect the milk and even detach the cups from the teats automatically, once they detect that milking should cease. However, it takes a human to place that automated milking device on a cow, because individual animals have their own size and anatomy and teats may be affected by some illness or defect and cannot be handled as if they were all the same. Furthermore, mastitis or any problem with teats or the udder needs to be assessed, and individual animals may need individual attention in feeding or medication. Using robots means that humans are no longer needed for these actions. Simple automation can work only for standardized situations, while robotics can provide the flexibility that the human analysis gives, by mimicking what that analysis does.

Robots can have several advantages. The most obvious are that they can have much higher productivity and consistency than humans, and that they minimize labor costs. In the food industry, in general, they also have the advantage of high hygienic conditions and ease of sanitizing, compared to humans, therefore ensuring more aseptic conditions. Whether they are a valuable investment or not depends, therefore, on their costs versus labor costs, productivity gains, and lower quality rejection costs. There are other benefits that may accrue in some specific cases, and milking is one of those.

Milking Robots

Milking robots have been one of the most popular applications of robotics in agriculture. The first farm to implement such a system in Europe did so in 1992; the first installation in North America was in 1999 (Canada). Interest in the subject rose steadily in the 1990s. In 1997, *Computers and Electronics in Agriculture* devoted a special issue to robotic milking. In 2000, the European Union commissioned a project on the introduction of automatic milking on dairy farms, under its R&D Framework Programme, which also produced substantial information and analysis (completed in 2003, but the website is still accessible). The Future Dairy project run in Australia also provides comprehensive information and details to assist farmers in evaluating the interest these systems may have for them. In 2006, there were over 4000 farms worldwide reported to use robotic milking.

The need to lower farming costs, and particularly labor costs, in developed countries is the most obvious driver for investment. Lack of human resources for farming is also a growing problem in these countries, as the average age of farmers continues to increase, and less people are willing to work as salaried peasants. Robots that facilitate strenuous farm activities have also been claimed to permit farmers to work to a later retirement age, and to be able to take care of themselves better (e.g., cows need to be milked daily even if the farmer needs to be in hospital for a couple of days). Labor issues are therefore a primary reason for a dairy farmer to decide investing in robotic milking, but it should be noted that in farming that is more than just costs. Furthermore, a fully automated milking system (AMS) has other advantages:

1. The increased sanitization and hygienic conditions from the absence of humans minimize cross-contamination of mastitis and health problems in general (not only between animals, but also between animals and humans), which improves well-being and minimizes health costs.
2. Milking can take place at any time throughout the day, therefore avoiding that animals may need to wait for long times, thus improving their general well-being.
3. Animals can be milked more than the conventional 2 times daily. It has been found that increases in milk production of 5 up to 25% typically result from increasing milking from 2 times daily to 3 times daily.
4. Each animal can set the regime that suits it best, instead of a 'one-size-fits-all' scheduling of conventional milking (with robotic milking, the animal decides when to be milked). As a result, well-being and production are improved.
5. Feed regimes can be individualized. Typically, animals are attracted to the milking robot by feeding, and so milking robots can also control the feed and nutrient intake, as well as any medication needed.
6. Milking robots can also detect illnesses, such as mastitis, and presence of blood or contaminants in the milk with sensors, besides knowing if the specific animal is going through some medication regime, and automatically divert contaminated milk, thus allowing for an easy management of health-related issues in milk collection.

Milking robots cannot be confused with automatic milking systems operated by people, where the animal traffic is controlled by farmers, the animals are placed in position, and automated milking cups are then placed by farmers on the teats for automatic pumping and collection in vats, such as in a rotary dairy parlor. Relatively new dairies may have quite some automation already, such as in-shed computer-managed feeding, automatic cup removal (ACR), teat spraying, and drafting. In fact, one could consider that the only new element of the robot is automatic cup attachment (ACA), so why not add just that one element in an already fairly automated process? Unfortunately, retrofitting a rotary dairy for robotics is not feasible, and in general, any previous automations are made redundant if a farmer invests in milking robots.

Typically, robotic milking goes together with free animal traffic, and the animal is motivated to go to the robot, rather than being pushed or forced in some way. Feeding is the usual attraction. As an animal approaches an available robot, its entrance gate opens, the animal goes in to feed, and the gate then closes. The robot detects the individual animal from its collar or tag (if fitted with a relevant device, such as a small radio frequency tag), and may therefore dispense a tailored feed (with eventual medication or supplementation needed). The sensors detect the position and size of the animal, and activate an electromechanical arm that reaches under the udder and puts the automated milking cups in place. Teats are brushed first and cups attached one at a time. The robot will also have sensors to detect the flow rate of milk, so it controls the milking amount (thus avoiding overmilking), as well as color, conductivity, and presence of blood. It can therefore divert the milk collection in case of contamination. It may also decide not to collect milk from one of the cups and hence not handle a teat, if there is any reason for the system to do so, such as a health issue. The cups are removed in sequence; as the milk flow rate falls below a given threshold, it removes the quarter teat cup, and so on, until all quarters are milked. Milk collection management by quarter instead of overall udder mix is one of the advantages of AMS, especially if one of the teats has any particular problem. A teat spray is then applied to improve hygiene. All information regarding each individual animal is stored in the system. The animal is released through the exit gate, and the entry gate can then open for the next one. Animal traffic management can be designed to optimize the performance.

New animals are trained relatively fast (2–6 milking days have been reported in the literature, with heifers being trained more easily than cows), and adjust their daily cycles to distribute themselves regularly throughout the whole day (and night). Studies showed that cows visit robots an average of 3–4 times a day, and that the number of cows that do not attend a robot for milking over a day, for no apparent reason (thus called 'lazy cows'), can reach a figure as high as 10%. This was found to relate quite strongly to the palatability of the pelleted concentrate added to the feed, so feed composition plays an important role in motivating the animals to the robot.

Ideally, the barn itself should be designed with the implementation of the robots and animal traffic in mind, rather than retrofitting. The design of the infrastructure, farm layout, laneways, ramp, automatic control gates, and strip grazing layouts are very important. Both management and facilities need to be redesigned to integrate AMS successfully. It is not surprising that the herd manager will spend more time servicing the equipment and reading and interpreting the information collected in the system about each individual animal than in actual attending to the animals themselves. The data collected by the sensors will detect estrus, as well as mastitis and other illnesses, and the farmer can plan medication and feeding in the robot, so the health care seems to take place by proxy. On the other hand, the farmer has more time to devote to analyzing the detailed information, which allows for more informed and speedy decisions to maximize efficiency and profitability.

The economics of robotic milking are not straightforward. Some analyses indicate that the financial benefits are clearer for smaller producers, while others point to a break-even level of investment that is about twice that of a conventional parlor milking system and hence a greater financial clout than that of small farms. Cost factors vary from year to year and location to location, and which benefits are more important to individual farmers also vary substantially, depending on various factors such as size of herd, type of management, lifestyle, age, and availability of human resources on the farm. Each farm therefore needs to consider its specific situation to evaluate the feasibility of implementing AMS properly. There may be various reasons for robotic milking to be attractive to one farmer and not to the neighbor.

It should be noted that milking robot costs are not just investment. Running costs of a milking robot include maintenance of a sophisticated system and electrical power supply. Typically, a robot will require between 15 and 25 kW per tonne of milk, while being capable of harvesting 2–2.5 tonnes of milk per day.

The most important factor to bear in mind is the need for a strategic approach to the whole milking and farming process. The use of electronic tagging and automated gates permits a full herd management approach based on the individuality of each animal, thus including health management to the detail of medication and feeding of individuals, which maximizes the well-being and the benefits. Personalized feeding may prove financially more beneficial than saving labor costs, as the former account typically for 45–50% of the dairy farm costs, compared to 8–15% for labor (approximately half the labor costs are due to milking). The better hygienic handling and more precise health management can also lower health costs. Robotic milking is not only a cost analysis, as gains need to be factored as well, namely the increased milk production resulting from more efficient health and feed management and more frequent milking.

The main manufacturers of milking robots are Lely and DeLaval. The former has been on the market for longer and is the market leader. The latter reported selling its 5000th unit in April 2009. Supplier support and training has been considered crucial and therefore a good working relationship between farmers and manufacturers is essential.

Palletizing Robots

Once individual containers of any dairy product are bundled in secondary packages (carton boxes, or simple plastic wrapping of individual containers, as with many liquid milk cartons), these must be piled on palettes at the end of the packaging line. These will be stored in warehouses and later loaded onto trucks for sale. The palettes are moved around with forklifts, and often piled on several layers in warehouses. All these operations can be robotized.

Figures 1–3 show a robotic palletizing solution for 1 l containers. Robots are fed from two fully automated packaging lines for the 1 l containers that begin by forming them out of reels of the packaging material, and fill them volumetrically in one machine. Both lines can then send them individually to the next room, or group them in packs of 10 (or 12) with plastic wrapping in another machine. Conveyors then transport the individual 1 l containers or the packs of 10 or 12 in separate lines from the packaging to the palletizing room, one conveyor line for each robot. **Figure 1** shows one of the conveyors delivering the packs of 10 or 12 to robot 1 for palletizing. This robot moves a double gripper that can pick up and then place two packs at a time on one of two palettes. This is the most usual type of palletizing robot, which can also handle other types of boxes, such as yogurt and butter containers. Note in **Figure 1** that the packs of 10 were moved so they are not aligned, and that 3 were placed at the base. This will not be a problem for the robot, as its sensors allow it to know exactly where the packs are, when to close the gripper, and to what strength, so that two packs are picked up and placed gently in place.

Figure 2 shows the whole palletizing room, and gives a different view of this first robot. It is placing two packs of 10 packages of 1 l at a time on two palettes. In order to improve stability, the stacking patterns are changed from layer to layer. The robot stacks the layers on the palettes

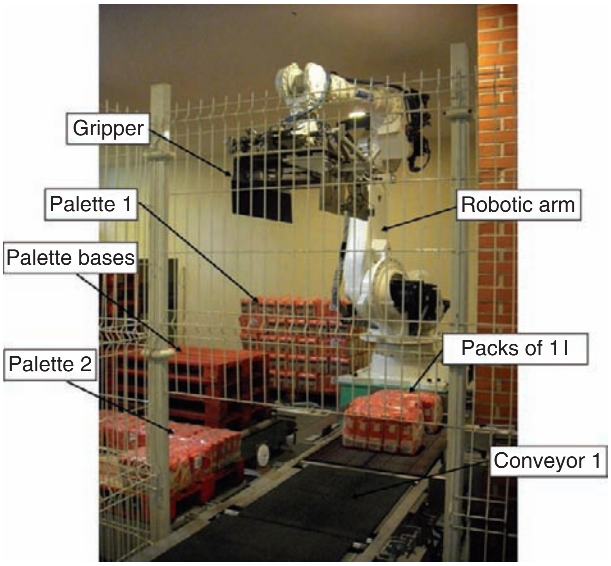

Figure 1 Palletizing robot with a double gripper. It is picking up two packs of ten 1 l containers at a time and stacking them with varying patterns on two palettes, alternately. The palette (wooden) bases are moved by the robot itself from the stack at the commencement of each palletizing. A conveyor brings the packs to the robot from the packaging room. Photographed by the author, courtesy of Ernesto Morgado S.A.

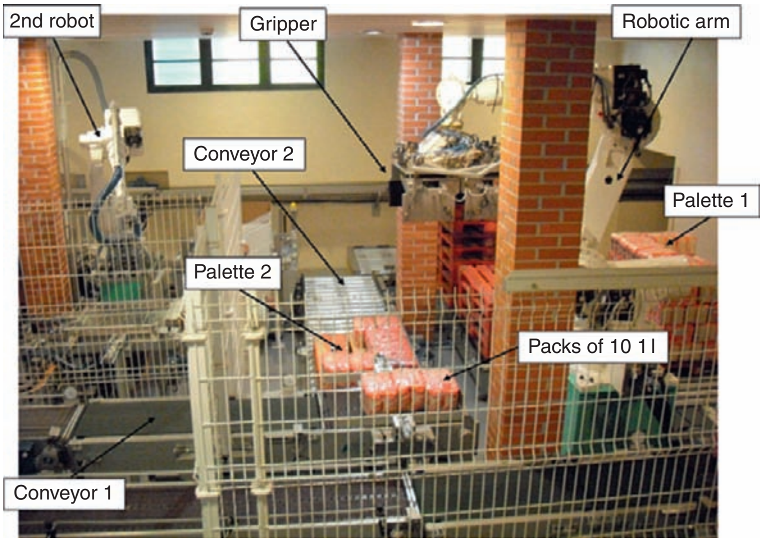

Figure 2 Example of a robotic palletizing room implemented in a tight space. The robot in **Figure 1** is on the right, behind a column. One of the roller conveyors that slide palettes away when ready can be seen at the center. A train line then transports the palettes to the next room. A second robot is on the top left of the figure. Each of the robots in this room is handling over 1000 containers per hour, a rate that is actually controlled by the rate at which conveyors move packs and packages rather than by rate constraints of the robot itself, and could thus be increased further to about 2500 per hour. Photographed by the author, courtesy of Ernesto Morgado S.A.

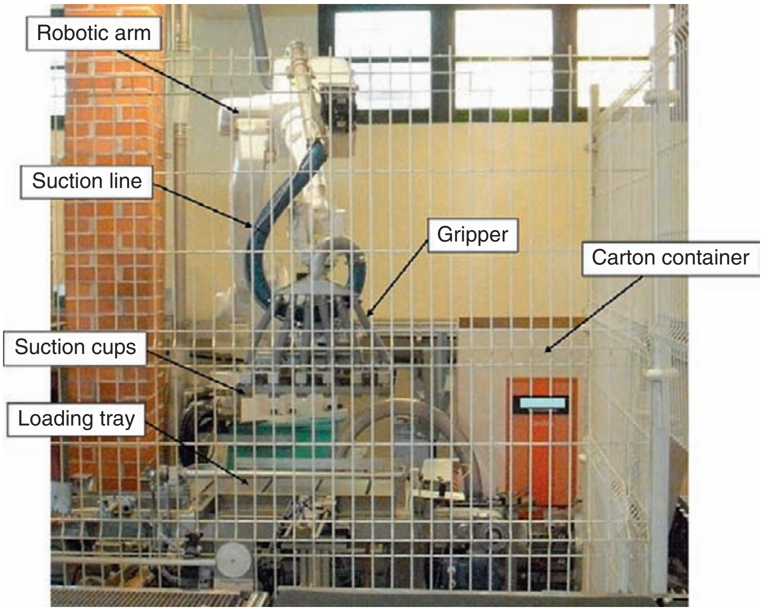

Figure 3 Palletizing robot with suction cups. One-liter packages are transported by a conveyor from the packaging room and accumulated as a 6 × 8 layer of 1 l packs at the bottom plate. The gripper plate, with 48 suction cups, picks up the layer and stacks it on the carton container seen on the right of the picture. When ready, it is slided out to the train line by a roller conveyor, and then moved to the next room. Photographed by the author, courtesy of Ernesto Morgado S.A.

so they will be ready to move on alternately (when one is ready, the other is about half done). When ready, each palette slides with roller conveyors to a train line (near the wall) that will carry the palettes to another room, where they will be automatically wrapped with plastic for extra rigidity, and are then ready for forklifts to carry them to the warehouse. A second robot is also seen on the left of **Figure 2**. Note also in this example how robots can be operated in a very tight space, and work around the constraints of an awkward building. Each of the robots in this example is handling over thousand 1 l containers per hour, working at less than 50% of its maximum achievable rate (this is because in these lines the rates are constrained by the rates of the packaging machines).

Achieving these productivities with human handling would require a much bigger room.

The second robot can be seen in more detail in **Figure 3**. It has a different gripping system, so it can handle a whole layer composed of individual 1 l packages into a big carton container. This robot uses a plate with suction cups to pick up a 6 × 8 layer of individual 1 l packages from the bottom plate, which is fed by the other conveyor coming from the packaging room. These two robotic solutions reflect the client requirements of this particular company. Some clients wish to have packs of 10 or 12 to place on shelves at the point of sale; others prefer to receive a big carton container that is placed like that at the shop floor.

From the beginning of forming the 1 l containers to the fully wrapped palettes, the only human intervention in this case is placing the reels of packaging material in the packaging machines and the base of the palettes in the robot stack. In addition to increased productivity and reliability, this has eliminated human intervention, which can be strenuous and prone to employee absence for health reasons, such as 'bad back'.

There are many manufacturers of such systems, such as Robomatic, Robotworx, Kuka, and Yaskawa (Motoman make).

The example shown has no more robotic solution, so the forklifts to handle the palettes are still operated by humans. It is however possible to robotize that step also and actually run an entire automated warehouse, with robotic vehicles forklifting and moving the palettes to the warehouse, placing them in locations that its own management program defines, and retrieve them when necessary also according to the store management program of the automated system. Instead of small vehicles, it is also possible to operate the system with hoists and cranes moving around the ceiling, although this would be rather expensive for handling palettes the size of those in **Figures 1–3**.

A robotic warehouse for palettes is rarer, though, because the economic advantages are less significant than those of palletizing. Replacing one or two persons operating forklifts (which is not a strenuous task) by an expensive robotic system likely results in investment costs that are difficult to recoup. Automated warehouses can be found in retailing, where a high turnover of small items is needed, or in assembly lines using a large amount of small components (as in electronic manufacturing).

Some manufacturers who offer these solutions include RMT Robotics and Kiva Systems.

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Relevant Websites

http://www.automaticmilking.nl – EU-Project Automatic Milking. Website of the EU integrated project on automatic milking. It contains several articles and project results.

http://www.lely.com and www.delaval.com – Lely and DeLaval. Websites of the main manufacturers of milking robots. They contain several illustrative pictures of their equipment.

http://www.roboticdairy.com – Robotic Dairy. Website of an Australian dairy farm. It has four live cameras at different parts of the farm where the operations can be seen in real time.

http://www.futuredairy.com.au – Website of an Australian Project. It covers more than robotic milking, and also contains several articles and analysis on this subject.

Corrosion

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Introduction

Corrosion is the deterioration of metals by an oxidation–reduction reaction, usually with loss of the metal to solution. Metals are generally found in their natural state in the Earth as metal oxides or ores. Most metals are more thermodynamically stable as oxides rather than as pure metals, with perhaps the exception of the noble metals, for example, gold and platinum. Mining and refining a metal is therefore an energy-intensive process, that is, there is an input of energy, and corrosion can be seen as the return of the metal to a more thermodynamically stable state. Corrosion causes the loss of billions of dollars per annum, and in industrial nations constitutes a large fraction of the gross national product. These losses are due to replacement of materials and labor costs, as well as plant downtime. Losses due to leaking or contamination of product and heat transfer problems are also significant. Conservation of natural resources is enhanced by the prevention of corrosion. In order to understand corrosion, one needs to be familiar with the basic principles of thermodynamics and electrochemistry.

Thermodynamics and Electrochemistry

The change in the Gibbs free energy, ΔG, of a reaction indicates whether or not the reaction will proceed. A reaction is said to be spontaneous if ΔG is negative. At constant temperature and pressure, the maximum amount of work, ω_{max}, a system can perform is given by the Gibbs free energy, that is, $\Delta_r G = \omega_{\text{max}}$. The Gibbs free energy of a reaction under non-standard conditions can be related to the equilibrium quotient, Q, by the following equation:

$$\Delta_r G = \Delta_r G^o + RT \ln Q \qquad [1]$$

where $Q = \Pi_j a_j^{\upsilon j}$ and υ is the stoichiometric number of the species j. Terms that make up the equilibrium quotient include the activities of metal ions, protons, and gases. In an electric cell, work is due to transfer of charge in the form of electrons across an electrochemical potential, E, between two electrodes. Therefore, the product of charge and potential results in work:

$$\Delta_r G = \omega_{e\ \text{max}} = \upsilon FE \qquad [2]$$

The Gibbs free energy can be related to electrochemical potential using the Nernst equation:

$$E = E^0 - \frac{RT}{\upsilon F} lnQ \qquad [3]$$

This equilibrium relates potential to standard potential and the equilibrium under non-standard conditions.

Standard Reduction Potential

Electrochemical reactions involve the transfer of electrons resulting in a change of oxidation state:

$$Cu^{2+}(aq) + Zn \rightarrow Cu + Zn^{2+}(aq) \qquad [I]$$

Reaction [I] can be broken down into a combination of two half-cell components, one for reduction (IIa) and one for oxidation (IIb):

$$Cu^{2+}(aq) + 2e^- \rightarrow Cu \qquad [IIa]$$

$$Zn \rightarrow Zn^{2+}(aq) + 2e^- \qquad [IIb]$$

Half-cell reactions occur at specific potentials. The overall potential of a reaction is the difference between the individual half-cell potentials. However, it is not possible to measure the absolute potential of an electrochemical process; so it is necessary to define a standard half-cell reaction relative to which the potential of all others is measured. For this purpose, by convention, a hydrogen electrode, also known as the standard hydrogen electrode (SHE), was chosen and arbitrarily assigned a value of 0 V:

$$H^+(aq) + e^- \rightarrow \frac{1}{2}H_2(g),\ E(H^+, H_2) = 0V \qquad [4]$$

The standard reduction potential of a test electrode is defined by whether it is oxidized or reduced when connected to a standard hydrogen electrode. If electrons flow from the hydrogen electrode to the test electrode, causing reduction of the test electrode, the measured potential is positive. The standard reduction potential is negative if the electrons flow in the opposite direction, that is, from the test electrode to the hydrogen electrode, thus oxidizing the test electrode. Using the standard hydrogen electrode as reference, tables have been constructed for all other electrodes, the reduction potential of which is either positive or negative relative to hydrogen. A partial list is given in **Table 1**.

The same principle applies to any two electrodes when constructing an electrochemical cell. The electrode with the lower standard reduction potential is the

Table 1 Standard reduction potentials for selected half-cells

Electrode	E° *V*
$Au^+ + e^- \rightarrow Au$	+1.83
$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$	+1.23
$Ag^+ + e^- \rightarrow Ag$	+0.80
$Fe^{3+} + e^- \rightarrow Fe^{2+}$	+0.77
$Hg_2Cl_2 + 2e^- \rightarrow 2Hg + 2Cl^-$	+0.27
$Cu^{2+} + 2e^- \rightarrow Cu$	+0.34
$Co^{2+} + 2e^- \rightarrow Co$	−0.28
$2H^+ + 2e^- \rightarrow H_2$	0.00
$Fe^{3+} + 3e^- \rightarrow Fe$	−0.04
$Fe^{2+} + 2e^- \rightarrow Fe$	−0.44
$Zn^{2+} + 2e^- \rightarrow Zn$	−0.76
$2H_2O + 2e^- \rightarrow H_2 + 2OH^-$	−0.83
$Al^{3+} + 3e^- \rightarrow Al$	−1.68
$Mg^{2+} + 2e^- \rightarrow Mg$	−2.37

anode, where oxidation occurs, and that with the higher potential is the cathode, where reduction occurs. The difference in the standard reduction potentials of the two electrodes, that is, cathode potential minus anode potential, is the overall cell potential. Using **Table 1** the potential of the cell in eqn [I] is found to be 1.1 V and when substituted into eqn [2], gives a negative Gibbs free energy. The current generated by this cell arises from the spontaneous flow of electrons from the zinc anode to the copper electrode. The flow of electrons from copper to zinc is a non-spontaneous process and requires external energy, as in the recharging of a battery by the electric mains.

Thermodynamics of Corrosion

The basic concepts of electrochemistry and half-cells can also be applied to corrosion. In fact, corrosion is the combination of a metal electrode (IIIa) with a water/oxygen electrode (IIIb). In this section, iron and various grades of stainless steel, due to their extensive use in the dairy industry, will be used to illustrate thermodynamic and kinetic data regarding corrosion:

$$2Fe(s) \rightarrow 2Fe^{2+}(aq) + 4e^- \qquad [IIIa]$$

$$2H_2O(l) + O_2(g) + 4e^- \rightarrow 4OH^-(aq) \qquad [IIIb]$$

$$2Fe(s) + 2H_2O(l) + O_2(g) \rightarrow 2Fe(OH)_2(s) \qquad [IIIc]$$

V, VI. or VII, therefore as in the previous example, the reaction can be broken down into its individual half-cell components and their corresponding standard reduction potentials as in **Table 1**:

$$Fe^{2+}(aq) + 2e^- \rightarrow Fe(s), E = -0.44V \qquad [IV]$$

The exact form of the water/oxygen electrode half-cell depends on the chemical environment, which can be acidic or alkaline.

In acidic solution:

$$2H^+(aq) + 2e^- \rightarrow H_2(g), E = 0V \qquad [V]$$

$$4H^+(aq) + O_2 + 4e^- \rightarrow 2H_2O(l), E = +1.23V \qquad [VI]$$

In alkaline solution:

$$2H_2O(1) + O_2 + e^- \rightarrow 4OH^-(aq), E = +0.40V \qquad [VII]$$

As shown in the previous section, when two electrodes are combined, the electrode with the lower standard reduction potential is the anode and undergoes oxidation. The electrode with the higher standard reduction potential is the cathode and is reduced. Since iron has a lower standard potential (IV) than the half-cells , V, VI or VII, therefore it undergoes oxidation when in contact with water/oxygen. This leads to an overall positive potential and a negative Gibbs free energy, showing that oxidation, that is, corrosion of iron, is a thermodynamically favorable process.

A common method for illustrating the thermodynamic data of a metal involves the use of a Pourbaix diagram, which is shown for iron in **Figure 1(a)**. It shows the most stable species of iron as a function of potential and pH. The activities of iron and other ions in solution determine at which pH and potential the transitions between different metal phases occur. Water is stable between the lines **a** and **b** (reactions [V] and [VI], respectively). A potential below line **a** causes the reduction of water to hydrogen, while above line **b**, water is oxidized and oxygen is evolved. However, the activity of protons in solution is pH dependent. Substitution of **a** or **b** into the Nernst equation [3] allows potential to be expressed as a function of proton activity or, more conveniently, as a function of pH:

$$E = 0.00 - 0.059 \text{ pH} \qquad [5]$$

$$E = 1.23 - 0.059 \text{ pH} \qquad [6]$$

The potential for the oxidation and reduction of water, lines **a** and **b**, respectively, decreases by 59 mV for every increase of 1 pH unit. In the Pourbaix diagram of iron (**Figure 1(a)**), the horizontal lines represent equilibrium reactions that are independent of pH, while the vertical lines represent reactions that do not involve the transfer of electrons. Diagonal lines are reactions that involve electron transfer and are pH dependent. The exact position of these lines is determined by the concentration of iron ions or by the presence of other ions in solution. All these reactions can be substituted into the Nernst equation [3] to derive expressions relating potential to standard potential, pH, and ion concentration. For a more complete description of the phase diagrams of

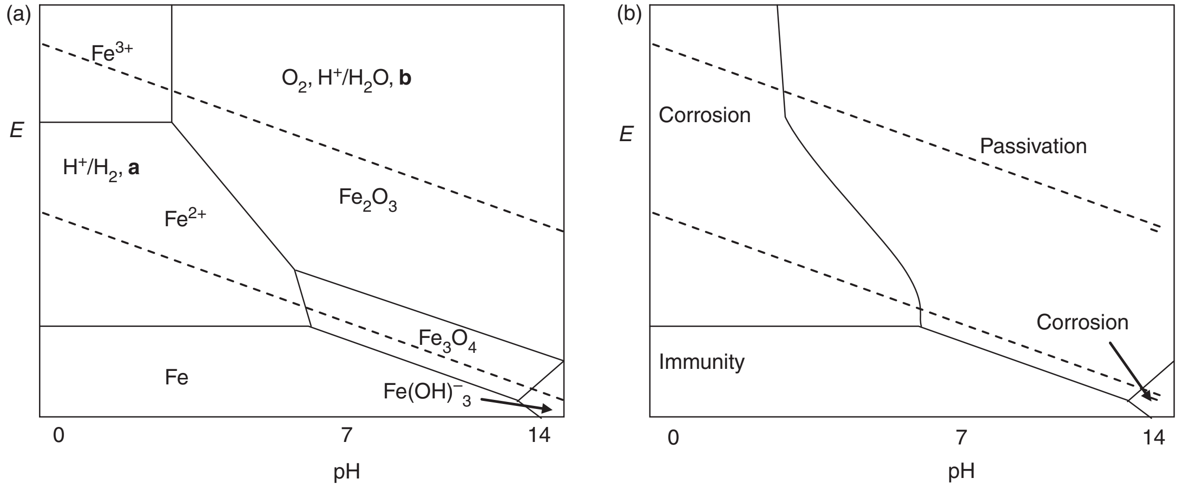

Figure 1 (a) Pourbaix diagram for most stable species of iron in pure water. (b) Pourbaix diagram showing regions of immunity, passivation, and corrosion.

iron, as well as many other electrodes, the reader is referred to the Further Reading section.

Figure 1(a) shows that at low pH and potential, iron is stable. Increasing the potential at low pH results in hydrated Fe^{2+} and Fe^{3+} and so corrosion occurs. At high pH, iron exists as oxides or hydroxides, with the oxidation number increasing with potential. If the oxide is soluble, further corrosion occurs. However, if the iron oxide is insoluble in water, a thin layer of oxide builds up on the surface. This oxide then protects iron from further oxidation and the metal is said to be 'passivated'. A similar effect is seen in the formation of green copper oxide, for example, on rooftops where the initial oxidation of copper forms a protective film and prevents further oxidation. **Figure 1(b)** shows the regions of immunity, corrosion, and passivation for iron.

Kinetics of Corrosion

Potential measurements and Pourbaix diagrams indicate whether or not corrosion is thermodynamically favorable. However, thermodynamic data provide no information about the rate of corrosion. Therefore, it is necessary to develop kinetic methods to measure the rate of corrosion. Kinetic information in electrochemistry is usually represented by a Tafel plot. When an electrode is at equilibrium, oxidation and reduction currents of equal magnitude but opposing direction result in zero net current. This equilibrium current is known as the exchange current, i_0, and the potential at equilibrium is E_{eq}. In order to measure i_0, a positive or negative polarizing current is applied resulting in an overpotential, η. Overpotential is defined as the deviation of potential from E_{eq}, that is, $\eta = E_{eq} - E$. At high overpotential, a plot of $\ln|i|$ as a function of η is linear(see **Figure 2**). Extrapolation back to E_{eq} gives the value of exchange current:

$$ln|i| = lni_0 + \frac{\alpha nF\eta}{RT} \qquad [7]$$

The slope gives information about the symmetry, α, of the reaction, which relates the reactant and the product to the activated complex along the reaction coordinate. A value of 0.5 means that the structure of the activation complex resembles that of the reactant and product equally.

Corrosion is a two-electrode system at equilibrium. The combination of two electrodes results in a mixed potential, known as corrosion potential, E_{corr}. Corrosion current, I_{corr}, is equal to the absolute values of the opposing oxidation and reduction currents. When the Tafel

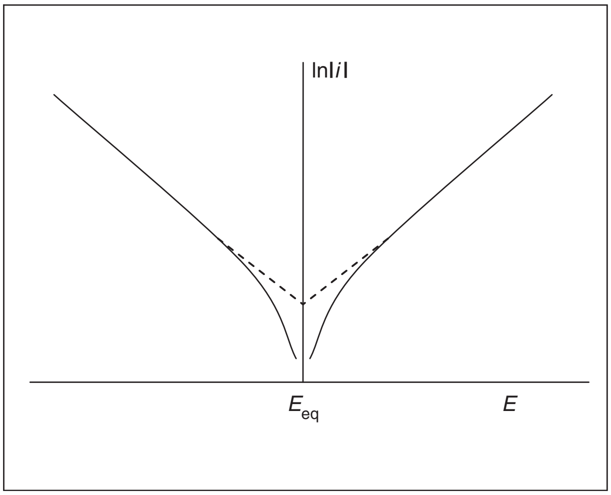

Figure 2 Tafel plot for the measurement of exchange current, i_0. Transfer coefficient, α, is measured from the slope and has a typical value of 0.5.

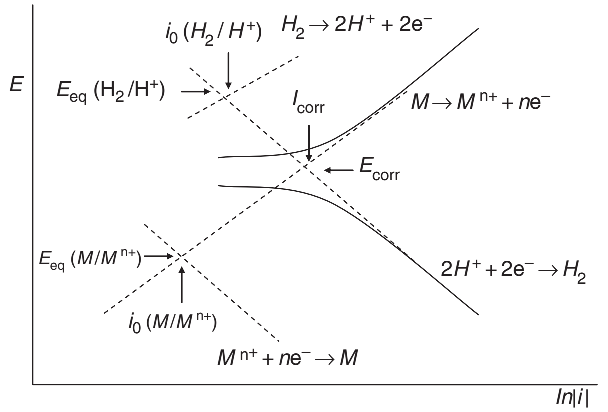

Figure 3 Tafel plot for corrosion of a metal in water showing equilibrium potential, E_{eq}, and exchange current, i_0, of hydrogen and metal electrodes. Extrapolation of both slopes yields corrosion potential, E_{corr}, and corrosion current, I_{corr}.

plots for both hydrogen and metal are combined (see **Figure 3**), the point of intersection gives the corrosion potential and corrosion current. The exchange current and equilibrium potential for the individual hydrogen and metal electrodes are also shown in the figure.

Stern and Geary derived a simplified version of the Tafel relationship, using empirical values of b, which does not require knowledge of the surface:

$$I_{corr} = \frac{1}{2.3}\frac{b_a - |b_c|}{b_a + b_c}\frac{I_{appl}}{\Delta E} \qquad [8]$$

where $b = 2.3RT/\alpha nF$. When the potential is varied by not more than ±10 mV of E_{corr}, it varies linearly with applied current, I_{appl}. The slope, I_{appl}/E, is known as polarization conductance, K_{corr}. The rate of corrosion, R_{corr}, can be related to the corrosion current by Faraday's law:

$$R_{corr} = \frac{I_{corr} Mt}{nF\sigma A} \qquad [9]$$

where M is the molecular mass ($\mathrm{g\,mol^{-1}}$), σ the density ($\mathrm{g\,cm^{-1}}$), A the area ($\mathrm{cm^2}$), and n the number of electrons transferred. Integration of this equation for $t = 1$ year gives the more common expression for corrosion, $\mathrm{cm\,yr^{-1}}$.

Properties and Types of Steel

Alloying of iron is an effective approach to increasing mechanical strength and resistance against corrosion. Stainless steel is a chromium–iron–nickel alloy, with a low carbon content, which has good mechanical properties and is resistant to corrosion. There are three different types of steel, martensitic, ferritic, and austenitic, which differ in their crystal structure (*see* article **Plant and Equipment**: Materials and Finishes for Plant and Equipment). The most popular steel in use in austenitic stainless steel, which contains 18% chromium and 8% nickel and is more commonly known as 18-8 austenitic steel. The carbon content is kept low (0.08%) to ensure that chromium, which is necessary for the prevention of corrosion, is not precipitated as chromium carbide.

Types of Corrosion

There are many types of corrosion. A plant engineer should know the rate of corrosion but should also be aware of the various modes of attack. This information has to be taken into account for plant design. Corrosion of iron or steel is always a result of exposure to air and water. The time span for corrosion can vary dramatically, from hours to years. The amount of damage also depends on the exact form of the corrosion process, which can be influenced by the chemical environment, mechanical stresses, or metallurgical properties. The exact reason for corrosion can be due to any one or a combination of these factors.

Pitting and Crevice Corrosion

Uniform corrosion of a metal results in an easily measurable loss of metal. However, metal surfaces are rarely homogeneous and are usually covered with a protective layer of oxide or hydroxide. Pitting and crevice corrosion are two forms of localized corrosion where anodic and cathodic areas, that is, regions where oxidation and reduction occur, develop on the metal surface. The anodic area undergoes severe corrosion, whereas the cathodic area is unaffected.

Environments containing aggressive species often result in the formation of deep pits on the surface of the metal. This is known as pitting corrosion. The most common cause of pitting is the chloride ion, which is able to penetrate the porous oxide layer (see **Figure 4**). Pitting generally occurs locally, due to variations in the structure and thickness of the oxide film or due to surface defects, rather than over the entire surface. Chlorides compete with oxygen for adsorption onto metal sites. Once adsorbed on the metal, chloride ions encourage hydration of metal ions and removal from the surface rather than the buildup of a passive oxide layer. Due to a difference in oxygen concentration, a large potential gradient of up to 0.5 V builds up between the anodic area within the developing pit and the cathodic bulk steel, which allows for a considerable current flow between the anodic and cathodic sites. More highly mobile chloride is then attracted into the pitting site in order to balance the charge built up by iron ions. Iron chloride undergoes hydrolysis, further decreasing pH,

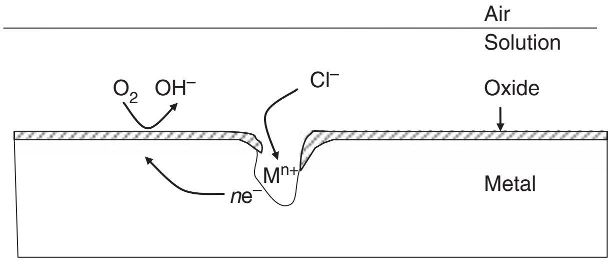

Figure 4 Schematic illustration of pitting corrosion. Pitting corrosion occurs when the protective oxide layer is breached by chloride. It is an example of localized corrosion on a metal surface, which arises from concentration gradients.

and so pitting corrosion continues in an autocatalytic manner. Therefore, corrosion continues in areas where pitting has been initiated rather than starting at new sites. In this way, the pit can grow very quickly. A Pourbaix diagram for iron modified for a chloride solution shows a region where pitting occurs in addition to areas of passivity, immunity, and corrosion (see **Figure 5**).

Crevice corrosion occurs in areas where movement of electrolyte is likely to be confined, such as between bolts, washers, or gaskets (see **Figure 6**). The electrolyte within the crevice is stagnant and has different oxygen and metal ion concentrations from bulk surface metal causing a potential gradient. Metal ions released within the crevice cause the local pH to be lowered by up to 6 units. As in pitting corrosion, chloride ions migrate into the crevice to balance the charge. As a result, passivity within the crevice is broken and the potential gradient is increased further, accelerating the rate of corrosion. Another cause of crevice corrosion arises if the protective oxide layer is breached due to a scratch, thereby exposing the metal. A potential is again built up between the two sites and crevice corrosion is initiated.

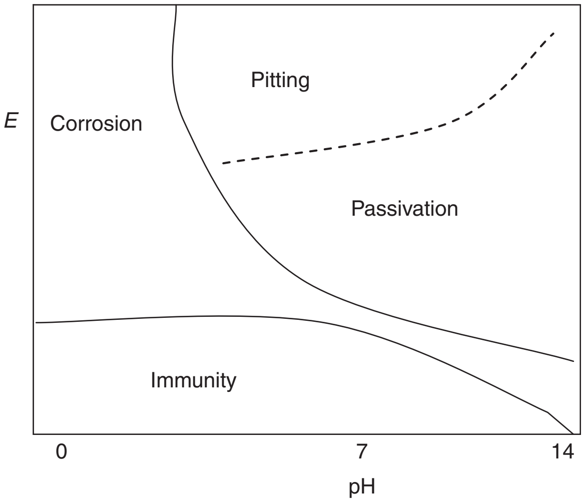

Figure 5 Pourbaix diagram for iron in salt solution showing four regions: immunity, passivation, corrosion, and pitting.

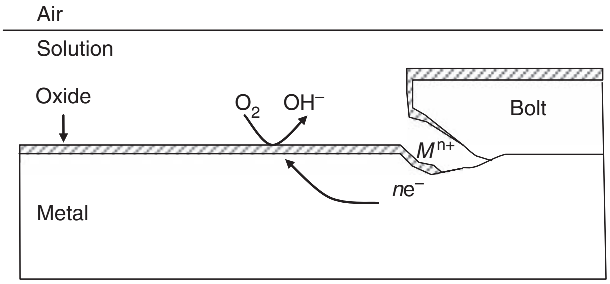

Figure 6 Schematic illustration of crevice corrosion. Crevice corrosion occurs when the electrolyte is stagnant, such as under a bolt. Differing electrolyte composition within the crevice compared to the bulk results in a potential difference and localized corrosion.

The main difference between pitting and crevice corrosion is that pitting starts only when a critical pitting potential is reached, as shown in **Figure 5**. Crevice corrosion depends on whether passivity due to the oxide layer can be breached within the crevice and can also proceed with ions other than chloride, such as sulfates, nitrates, or acetates.

Intergranular Corrosion

Intergranular corrosion is a localized attack at metal grain boundaries, which form during the cooling process in steel production where several nucleation points for crystal growth exist. The orientation of each crystal is random and so when two crystals meet they are often out of phase and a grain boundary is formed. The Gibbs free energy at the boundary is higher than that of the bulk metal and the boundary is preferentially corroded. It is thought that the intergranular corrosion results from improper, or sensitizing, heat treatments of the metal during production or welding. Sensitizing heat treatment of stainless steel depletes the grain boundary of chromium, which precipitates as chromium carbide; chromium is necessary for corrosion protection. Corrosion of this type penetrates the whole boundary, reducing mechanical strength and resulting in failure, even though actual loss of metal is low.

Stress Corrosion Cracking

Stress corrosion cracking results from tensile or residual stress on a metal in a corrosive environment. Without the corrosive environment, the stress would not induce cracking. Stress corrosion cracking can be initiated at metal discontinuities, pits, or intergranular boundaries. Whether propagation of the crack is intergranular or transgranular depends on the exact chemical environment and pretreatment of the metal.

Corrosion Fatigue

When subjected to a repeated alternating stress, a metal will fail after a number of cycles; this is known as metal fatigue. In a corrosive environment, the number of cycles required to cause failure is reduced; this is known as corrosion fatigue and is usually generally transgranular and branched. The damage caused by corrosion fatigue is greater than the sum of the individual effects of corrosion and fatigue due to branching.

Cavitation

Cavitation occurs under conditions of rapid fluid velocity, where repetitive high- and low-pressure areas are developed and bubbles are formed, which then collapse at the metal–liquid interface. The metal becomes deeply pitted due to mechanical damage and chemical removal of the protective oxide film. Cavitation often occurs on rotors and turbine blades. This is a physical process, not chemical, and it is therefore a form of erosion rather than corrosion proper. It is appropriate, however, to list it as well.

Galvanic/Bimetallic Corrosion

Galvanic corrosion occurs when two metals separated by an electrolyte are in close contact. The metal with a lower standard reduction potential is oxidized, whereas the other is reduced. A common source of galvanic corrosion are bolts used to attach a fixture to steel; corrosion then occurs at the joint. Another cause is the welding together of two different grades of steel.

Cathodic protection refers to the preferential oxidation of the metal with a lower standard reduction potential over another when two different metals or alloys are in contact with oxygen; the second of the two metals remains unaffected by corrosion. This approach to protection against corrosion is a common practice on ships where a zinc block, which has a lower standard potential than steel, is attached to the hull and is preferentially corroded by seawater while the steel hull remains uncorroded.

Environmental Factors Affecting Corrosion

The availability of oxygen is critical for the corrosion process and its availability is affected by a number of interrelated factors. This is the basis of corrosion prevention methods, such as painting, where oxygen availability is suppressed. Steady-state corrosion is dependent on the diffusion rate of oxygen to the metal surface. Therefore, at low oxygen concentrations, corrosion is proportional to the concentration of oxygen. However, above a certain critical concentration, corrosion decreases with increasing oxygen level due to oxide passivation of steel. The critical concentration is increased by increasing salt concentration and/or temperature but is reduced by increasing fluid velocity and pH.

Since corrosion is controlled by diffusion of oxygen, its rate increases with temperature. However, at high temperatures, oxygen availability is reduced and the rate of corrosion decreases. Moving liquids supply oxygen to the surface and hence increase the rate of corrosion. At higher flow rates, partial passivity occurs. However, at very high flow rates, mechanical removal of the oxide layer or the prevention of passive oxide layer formation increases the rate of corrosion. Also, in the presence of chlorides, as in brines, oxide layers are not formed and corrosion will continue to increase with flow rate. Increasing the NaCl concentration reduces the solubility of oxygen. However, corrosion increases to a maximum at 3% NaCl, due to Cl^-, and then declines; this is due to breaching in the passive oxide layer.

See also: **Plant and Equipment**: Materials and Finishes for Plant and Equipment.

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Continuous Process Improvement and Optimization

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Introduction

Origin of the Concept

'Continuous process improvement' is a term that emerged from the studies undertaken in American business science on the manufacturing strategies used by the Japanese industries. As such, it is often known by its Japanese term 'kaizen'. There are many other terms that became popular and are related to managerial and operational strategies from Japanese practice, of which continuous process improvement is one element, such as quality engineering, lean manufacturing, value engineering, manufacturing excellence, and world-class manufacturing, and even six sigma can be related to it. Six sigma is discussed further in the article **Plant and Equipment**: Quality Engineering. The methodologies developed for continuous process improvement are now regarded as crucial for ensuring competitiveness in a global market, and as such, have been permeating across all sectors of the manufacturing industry.

It is worthwhile to understand the origins of the concept. Following World War II, Japanese industry was devastated. In the 1950s, products made were of generally poor quality and the lack of economies of scale and of investment capacity seemed to condemn Japanese industry to a secondary role for a long time, compared to the paradigms of the day, the US industry. However, by the 1970s, American companies began recognizing that the Japanese products were of superior quality and lower price, beating them in their own markets. One of the industrial sectors more strongly affected by the giant leaps of quality and efficiency of the Japanese industry was car manufacturing. It was obvious by then that some time during the 1950s and 1960s Japanese manufacturing was able to find methods to produce high-quality products at a cheaper rate, even though it did not avail (at that time) of the economies of scale that the 'Detroit giants' (Ford, Chrysler, and General Motors) had. As the Toyota Motor Company was at the forefront of these developments, and it practiced a notable open philosophy about itself, it became the most widely studied by American business science researchers. Toyota was not the only company developing these approaches in Japan, nor the creator of all the methodologies used, but was one of the pioneers, and became the best known. In a sense, the fact that various people analyzed various aspects of what was later called the 'Toyota Production System' (TPS) and then added their own perspectives and interpretations has ultimately led to the emergence of several 'westernized' concepts, such as just-in-time, total quality management, continuous process improvement, and lean manufacturing. However, the Japanese, and Toyota in particular, would not consider any of these concepts as an independent methodology, but as part of a whole.

The first studies of TPS brought to global attention a completely different way of organizing production, with just-in-time stock management, a production pull system rather than push system, and a revolutionary approach to the role and intervention of operators in the process, which in itself sparked a new philosophy about teamwork. Studies of the TPS became known as the first paradox (how can quality be achieved with low cost when there are few economies of scale?) after another influential work addressed what it called the second Toyota paradox, which deals with the new product development approach of Toyota (how can a new product development cycle be faster and cheaper by delaying decisions as much as possible?). Another landmark in the dissemination of these practices was the seminal work on lean manufacturing, The Machine That Changed the World.

From Car Manufacturing to Dairy

There is a very important conclusion to bear in mind from the historical backgrounds of continuous process improvement and all the TPS-related paraphernalia of modern management best practices and fads: they were not developed and implemented by Toyota, the biggest car manufacturer in the world (that is not what Toyota was then). They were developed and implemented by Toyota, a small company producing low-quality products at a high cost because it had no economies of scale, and therefore had very little financial capacity for investments. What it had was the belief that it could do better, that it could solve the paradox 'high quality and low cost even without economies of scale'. Toyota did not develop its famous TPS by hiring expensive consultants to design optimum solutions by applying best-in-class principles. It had no financial or human resources to do so. It started with months of observation of what happened in the reality of the factory floor, what operators really did and how. Then, considering what was the best in class, how could those efficiencies be emulated? And finally, finding out that they could actually be bettered. It is very informative to know what really happened: Liker provides a historical account that is well worth a read.

The relevance of this to modern industries is obvious, as emulating means that abstract principles can be applied across industrial sectors, and there is none that has been immune to the application of the principles of continuous process improvement, lean manufacturing, six sigma, and other improvement programs (see for instance, the list of main clients of the Kaizen Institute – one of the many consultancy groups providing services in this area – at its website). For the dairy industry, see the cover story by Markgraf on an American dairy.

It is however crucial to bear in mind the main lesson of Toyota's origins: if deploying continuous process improvement (or any associated principles) requires an expensive engagement of external experts who will propose an optimum solution from their analysis, then the proposition negates the philosophy right from the start. Continuous process improvement (1) must be a transformation that comes from within (albeit guidance by external experts facilitates commencement, cuts corners, and can thus save time and money), (2) must be ardently desired by the most senior management, and (3) should require few, if any, costs.

Continuous Process Improvement or Process Optimization?

As the name suggests, continuous process improvement uses methodologies to improve a manufacturing process, so the first question should be what is the target of improvement – costs? productivity? quality? This is a methodology to improve what? The answer is simple: everything. That is one of the reasons for using the word 'paradox' in relation to the TPS: How can everything be made better, when reality usually requires compromises and trade-offs? How can costs be reduced and quality improved? The second feature of the term is the word 'continuous': process improvement is a never-ending journey, the process is to be made better all the time, one improvement after another, because optimum is an ideal, and as such, unreachable.

Semantics, therefore, show that the concept implies an incremental approach (one step at a time), where no improvement is too small (summing enough small improvements leads to a big improvement). This, therefore, reveals a fundamental difference between continuous process improvement and the 'western' counterpart, process optimization. The practice in the United States (and Europe) had been to design an optimum process. This implies defining what the optimum target is, often accepting that the multicriteria nature of 'optimum' will require some compromise or trade-off, and from that conceptual definition of what the optimum should be, the process is then designed in one single go to reach that optimum, using sophisticated methods. As the design is complex, it needs advanced knowledge, so the designer is an expert in designing, does so expensively, and is unlikely to be engaged in the actual daily operation of that design. On the other hand, the operators are typically not involved in the design, and are supposed to do exactly as told and exactly as planned in the design. This is, of course, a gross generalization, but the underlying nature of the approach can lead to such extreme dichotomy between the optimum abstract ideal of the design and the practical reality on the factory floor.

Therefore, continuous process improvement (kaizen) experts often like to boast of the improvements they were able to extract out of what was supposedly an 'optimum' process. Notwithstanding, process optimization methodologies are not to be discarded, some kaizen engineering design methods over rely on simplified approaches, and 'western' engineering design has developed some more consistent and comprehensive ones. From a practical point of view, of course, semantics are irrelevant, and what matters is the efficacy of the methods deployed to improve competitiveness.

Operational Improvements

The first step for improving a manufacturing process is to analyze its operations, from procurement to sales, and not only on the factory floor. Starting with purchase orders (when are ingredients purchased and how, where are they stored and for how long?) and ending with sales (manufacture-to-stock, or manufacture-to-order?) means that stock management strategies are an integral part of process improvement. In relation to the manufacturing process itself, the whole sequence of operations needs to be considered, including not only the actual processing functions, but also its associated functions, such as quality control. In essence, a factory organizes a series of operations, generically, buy → make → test → sell (not necessarily linearly). The first core principle of kaizen is to analyze the operations from the point of view of the flow of the product itself, which already brings in a difference from 'western' conventions, where processes tend to be described from the managerial perspective.

In order for a process to be improved, this combination of operations must be the most efficient. Redesigning the entire set can be associated to the concept of business process reengineering (BPR), a management concept that gave mixed results and was therefore more popular in the 1990s than it is today. BPR considers the 'western' approach: that an optimum can be designed from scratch by best principles applications, using sophisticated methods if necessary, and isolated from real practice if need be. A BPR proposes a giant leap to an ideal world. It is not a useless concept, and it can give very good results (and has in reported literature), but obviously, the more reality deviates from ideality, the greater the chance that the giant leap may be toward a big hole. Literature also reports negative experiences of companies with BPR. Incremental approaches therefore have this big advantage: they may be more modest but will always lead to a better situation, and over several incremental steps, the gains will become significant. Critics will contest that incremental solutions may not explore areas of the solution space beyond convention, while advocates prefer to explore without excessive risk in the explorations, and point to practical end results that are equally enviable. It is noted that BPR often shows up associated to the introduction of novel solutions from information technologies (IT) to reorganize business processes. This is a special case of BPR, where it comes associated with the digestion of new IT procedures by company and staff, which in itself (with BPR or not) has its own success and failure factors. Introducing IT may facilitate process improvement, or it may not, and it is not a concern here. IT is a tool; it should be recognized that in fact IT always facilitates kaizen from a conceptual (theoretical) perspective – but how will it work in practice? kaizen in itself is not about a better business concept, it is about a better business reality.

Continuous process improvement will suggest starting more modestly than reinventing from scratch, by analyzing all operations and apply what is also known to some extent as value engineering. Of all the business operations involved in manufacturing, some add value to the product and thus to the end client, but some do not. They may well be necessary for some reason, or they would not be there, but if they do not add value, then they are a waste from the point of view of the product and of the client. The Japanese word for wasteful activity ('muda') is often cited in this context. A better process will be one where waste is nonexistent, so these operations should, ideally, be eliminated. Therefore, it is logical to start improving a process by eliminating non-value operations (why spend time and resources improving those that are best eliminated altogether?).

This analysis is not as straightforward as it looks, because company executives and operators are not used to think from the point of view of the client or of the product. A typical example is quality control: from the point of view of the product or the client, it is a wasteful operation. This might seem an excessive comment, but a more detailed analysis shows the philosophy at play here. Quality control is a problem of the manufacturing company, not of the client. The client expects that quality, indeed, he paid for it. If the company is not able to deliver that quality, that is the company's problem that becomes a client's waste. From the point of view of the product, being stored somewhere waiting for the results of a quality control test is a waste of time. The product should have quality unless the process is faulty. An improved process should not be faulty, so the product should have quality all the time. This would lead to the concept of quality by design, which is discussed in detail in the article **Plant and Equipment**: Quality Engineering.

A second principle is that the flow of the product must be continuous. From entering the raw materials storehouse to leaving for sale, the product (ingredients/components, etc.) must not stop; if it does, it is lying idle, and so there is a waste. Hence, storage is a waste, and so the concept of just-in-time, one of the first that was immediately grasped for its financial advantages. The best way to ensure this continuous flow is to pull the product from the end, that is, the last operation sends an order to the before last, and so forth, so the product is pulled from the end. A pull operation (request for a product or part needed) was originally sent with a card in Toyota, or 'kanban' in Japanese, a term that endures as synonym of just-in-time operations by a production pull strategy.

A good starting point for analysis of the improvements that can be made by eliminating wasteful operations (and hence become 'lean') is to apply the lean questionnaire developed by MIT (LESAT – Lean Self Assessment Tool), available for free at the website of the Lean Advancement Initiative of MIT (this site contains plenty of other free tools and studies under the 'products' menu). It may help to give a different perspective on what can be classified as waste, and how much waste there is in a normal business operation when it is analyzed from the perspective of the value to the client, or of the product flow.

Waste is not confined to operations that should be eliminated because they do not add value; TPS deals with two other types of waste. An obvious waste comes from product variability and consequent loss of quality, which is addressed in the article **Plant and Equipment**: Quality Engineering, and often referred to by its Japanese term 'mura'. The third is waste resulting from overcomplexity ('muri' in Japanese). This means that every operation and sequence of operations should be as simple and direct as possible. Standardization of simple operations and their straightforward combination are the ideal basis to eliminate this type of waste and hence maximize productivity. In some industrial sectors where operations involve manual handling, eliminating muri requires appropriate ergonomic design of the workplace and workstations (the operation should be easy and simple to perform, thus minimizing energy, effort, and time).

Process Engineering Improvements

General Approach

The operational and managerial implications of kaizen leave a highly flexible, efficient, and productive factory floor, alongside an entire lean supply chain. Operations on the factory floor involve a series of equipment units where the product components are progressively incorporated and turned into the final product. Each of these operations must now be improved.

In the context of a process industry such as dairy, the raw materials undergo transformations toward a product of a different nature (e.g., fermentation in yogurts, coagulation in cheese) or that hinder microbial activity to provide shelf life (e.g., ultra-high temperature (UHT) processing, drying). These operations are controlled by a number of variables or control factors, such as temperatures, flow rates, amounts of ingredients, and time of operation. The company decides on the settings (values) of these factors from its knowledge of the system, characteristics of the equipment, recipes, and other things. Each of these control factors can be set at any value within a range of physical interest, and there is an infinite number of combinations of settings of these factors that result in a final product, although not all combinations are possible. Some, however, result in products that are better than others, or in processes that are cheaper, or more productive, or use less energy, and so on (for instance, the objective of reaching a given microbial lethality in the thermal treatment of liquid milk can be achieved equally at a higher or lower temperature, with the processing time being longer the lower the temperature, but the quality characteristics of the product, such as nutrient retention and taste, are better at higher temperature-short time than at low temperature-long time). In this context, process improvement will deploy methods to find a combination of settings of the control factors that will result in a better performance.

It may be possible to estimate better combinations of these settings by developing mathematical models that mimic the operation of the equipment by applying first principle equations (laws of conservation of mass, energy and momentum, thermodynamics, kinetics, heat and mass transfer, etc.). However, such models will need to make assumptions that may or may not be good images of reality, and may also be too complex. The continuous process improvement concept prefers to 'let the system speak by itself', and obtain process data from which to infer improvements. Process optimization does the same, only that it assumes that a single giant leap is possible, and that the absolute optimum is identifiable. Kaizen is prepared to move stepwise.

The need for process data to infer directions of improvement is an issue in some industrial sectors, such as dairy. If the equipment operates with large quantities of product at any given time, and the business margins are small, there is little room to perform tests with the equipment, each of which could cost many kilograms of product that run the risk of being unsuitable for sale (when the test happens to try settings of the control factors that do not lead to a suitable product). However, process data can also be collected simply from the historical records of the process. This has the huge disadvantages of providing data that have not been planned, with many data points around the same settings, with uncontrolled variabilities, and a scan of the solution space decided by chance and the inaccuracies of the control systems. However, it is 'free' data and may reveal useful information about the system, if handled properly.

Design of Experiments

Kaizen recommends an experimental plan, and as such it starts by giving great importance to the planning of the tests. It is obvious that if each test involves the actual process and equipment, the most information needs to be obtained from the least amount of data, and therefore, it is not surprising that the area of statistics dealing with design of experiments (DoE) must be brought in.

The most widely used approach in Japan is due to Genichi Taguchi, and is generally known as the Taguchi method of robust engineering design. The method seeks to achieve improvement of performance and also consistency of performance, and as such it is the foundation of quality engineering too, discussed in the article **Plant and Equipment**: Quality Engineering. In the context of performance improvement or optimization, it is noted that in order to minimize experimental requirements and take as much information as possible from the data, Taguchi chose designs based on orthogonal arrays (aka Latin squares). They are usually designated as L-4, L-8, L-9, L-12, L-16, L-27, and so on, where L stands for 'Latin squares', and the number indicates the number of rows of the array, which is also the number of tests that needs to be performed. When consistency of performance is to be considered as well, the whole set must be repeated a number of times, and therefore, using arrays with as few rows as possible is important. Each array will allow testing a number of control factors, which depends on the array, with one factor associated to a column of the array. That column will contain the settings to be used for the factor. Some arrays have only two different settings (two-level design), others have three (three-level design), and other commonly used arrays have four by combining columns of two-level factors (for instance, M-16 or L-16M refers to the L-16 array modified, which tests four different settings of factors, but it can be used for much less factors than the original L-16). There are also some mixed level designs. **Table 1** shows an example, the L-8 array, which can be used to test up to seven factors with only two settings used for each.

These experimental designs are very good for limiting the number of tests that need to be performed, but come at a cost: the design generates an intricate set of confoundings that may be difficult to separate. 'Confounding' is the statistical name given to a combination of terms or factors in the design that results in their effect being indistinguishable. It does not mean that the confounded factors are confounded by nature, it is a consequence of the experimental design that their impact is confounded (pooled together). For instance, if a design tests a system at 20 and 30 °C, and also considers a flow rate of 1 or $2\,\mathrm{l\,s^{-1}}$, but in all tests performed the flow rate of $1\,\mathrm{l\,s^{-1}}$ was always used with 20 °C and the flow rate of $2\,\mathrm{l\,s^{-1}}$ was used only with 30 °C, then when analyzing the data it is not possible to know if the differences were due to (1) the temperature increase, with flow rate change being irrelevant, or (2) the flow rate increase, with the change in temperature being irrelevant, or (3) both changes. Orthogonal arrays do not produce confoundings between factors, but they do between the effects of factors and the effects of their interactions.

An 'interaction' is a basic feature of nonlinearity in a system. It means that the way that a factor influences a system depends on the actual value of another factor. For instance, if increasing the flow rate has no effect at lower temperature but is important at higher temperature, there is an interaction between flow rate and temperature.

Table 1 L-8 orthogonal array

Test no.	*1*	*2*	*3*	*4*	*5*	*6*	*7*
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	21	2	1	2	
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

Control factors are assigned to columns, and the rows indicate the settings of each factor for each of the eight tests that need to be performed. Each factor is tested with only one of two settings. Designs should be replicated, if possible.

For this reason, 'western' statisticians prefer other experimental designs that provide a better differentiation between different effects, such as the 'central composite design'. This design uses five settings for each factor, and tests some combinations of those settings that conform to a particularly well-balanced view of the solution space, which minimizes error regions of mathematical models then used to interpret the data. However, it requires a large amount of data than the orthogonal arrays.

Whatever DoE is chosen, it is noted that it can be considered as a plan for tests that will be done (the ideal scenario), or as a filter to be used to collect data from historical records in order to ensure minimum bias of the analysis of those data (even if only a small subset of data are thus collected; this is preferable to overweighing parts of the solution space).

Stepwise Approach

The Taguchi method advocates a comprehensive approach including meetings, brain-storming, and teamwork, that is not detailed in this text, but it is noted that the first step must be to consider how many factors may influence the system. Usually, this analysis compiles a large number of factors that may be interesting to analyze. If the system should 'speak for itself', then one should not curtail the list by rational thought; instead, it is better to rely on Vilfredo Pareto's famous principle, the 80/20 rule, and use a first experimental plan to zoom in on the 20% of factors that may be causing 80% of the consequences. This is also where analyzing historical records could be helpful, and provide 'free' information. A simple two-level orthogonal array design can be used for this purpose. An L-8 can test up to 7 factors with 8 runs, an L-16 allows one to consider up to 15 factors with only 16 runs, and an L-32 would allow for up to 31 factors with just 32 runs (can, and should, be repeated), and so on, so that the selection can be done quite efficiently.

It is noted that the consequences of the confoundings of this design are that some factors that may be considered important in the data analysis could actually be negligible (and this would be because one of the confounded interactive effects was relevant), but if a factor is judged to be negligible, then it is, and so are all effects confounded with it. That means that nothing of importance is lost by analyzing in this way which factors are more crucial for improving the performance.

There are possibilities for overlooking important issues with these two-level designs, though: if a factor has an influence that shows a maximum or minimum of its average impact on the performance, it is possible that by a stroke of bad luck the averages at the two extremes used for the settings (low and high, 1 and 2) are similar, and the data analysis will then infer that the factor was negligible, while data from a design with three levels would conclude otherwise. Continuous process improvement is however prepared to be incremental – there will always be time to go back into more analysis and improvements.

Once two, three, or at most four control factors are chosen for being the most crucial ones, then a design with more levels (three, four, or a central composite design) can be used and the solution space explored in more detail to reveal regions of improved performance.

Data Analysis: Identifying Crucial Factors

The Taguchi method does not need to fit mathematical models to the data obtained with the orthogonal array design. Instead, it applies an analysis of variance (ANOVA), a statistical method that quantifies the variability of the data collected by its variance, and then determines how much of it can be explained by the fact that each of the factors changed its settings. If the amount of data is sufficient to have enough degrees of freedom with the DoE used, a significance test can also be applied to ascertain which factors have a statistically significant effect. Results can be shown in the form of a pie chart, which gives a very good image of what is more important and what might be neglected in a first approach.

A typical result from ANOVA, with the most relevant outcomes in the form of a table and pie chart for a situation where the relevance of seven factors was tested with an L-8, is shown in **Figure 1** (case study regarding a particle coating process). In this example, the design was replicated (2 runs for each condition, totaling 16 data points), which gives enough degrees of freedom to test for significance. The pie chart shows not only the relative importance of each factor, but also how much of the variability of the data is unexplained. The unexplained amount of the variance may be due to (1) the relevance of interactions and the consequences of the intricate confounding of the design, (2) the influence of other factors that were not controlled and not considered in the design, and (3) the natural variability, or white noise, which may come from variability in control factors, in the characteristics of the materials or process, and also of the method of analysis of the performance, which is similar to random experimental error. The example shown suggests that changing the settings of three of the factors accounts for a large proportion of the changes that can be achieved in the performance.

Data Analysis: Identifying Settings for Improved Performance with the Taguchi Method

The estimate of which is the combination of settings of the control factors that gives the best performance is done in the Taguchi method by simply choosing for each factor the setting that had the best average performance. This has the advantage of not relying on any model fitting, and the disadvantages are that it will suggest settings for all factors from only among those that were used in the experimental design (even if the best combination is not one of those tested), and that it does not account for interactive effects. It is possible to include a correction for the effect of some interactive effects if the DoE has enough degrees of freedom for that, but it is a matter of interpretation which interactive effects are being tested because of the intricate nature of the confoundings. Taguchi recommends the calculation of a severity index to at least evaluate the relative potential importance of all interactions between pairs of control factors, but the extent to which this effectively gives unique results is not clear. Furthermore, when searching for a region of optimum performance, the designs usually have at least three (if not more) settings, and in this case it is virtually impossible to properly account for any interaction, unless one is effectively using a full factorial design (e.g., only two factors in an L-9). Therefore, the best combination of settings obtained by the Taguchi method assumes in practice that interactions between factors are negligible. Taguchi recommends validation tests for the new settings, of course.

Figure 2 gives an example of the graphs showing the averages of the data for each setting of each factor, known as the 'means plots', for a system where three factors were changed with four levels, according to a modified L-16. The maximum performance in that case is suggested for the combination of settings 3-4-2 of the three factors. The estimated performance of the system for that combination of settings is 99.08, obtained simply by adding to the global average of the data (81.29 in that case) the incremental benefit of choosing the respective setting of each factor, as per the means plots. Estimating performance in this way may lead to physically inconsistent values in some cases (for instance, performance above 100% or losses below 0%), which can be improved by a logarithmic transformation, such as the omega transformation.

Data Analysis: Identifying Settings for Improved Performance with Response Surface Method

An alternative that has been widely applied in Europe and the United States is the response surface method (RSM; or response surface analysis (RSA)). It can be applied to any design, including the central composite design, and is based on postulating a mathematical model to describe the influence of the factors (and interactions) on the performance. It has the advantage that designs such as the central composite design that have much less confounding issues can be handled, but

ANOVA table for data

FACTORS	SS	df	VARIENCE	F
Torque	0.000 798	1	0.000 798	0.739 589
Rotational speed	0.001 58	1	0.001 58	1.464 292
Temperature	9.51E-05	1	9.51E-05	0.088 097
Oil viscosity	0.008 145	1	0.008 145	**7.548 277**
Drip feed rate	0.037 733	1	0.037 733	**34.96 838**
Drip concentration	0.021 098	1	0.021 098	**19.551 75**
Coating	0.037 153	1	0.037 153	**34.430 41**
Error	0.008 633	8	0.001 079	
Total	0.115 234	15	0.007 682	

Total no. of data points:	16	F-limit: 3.457 919 Ne: 2

(a)

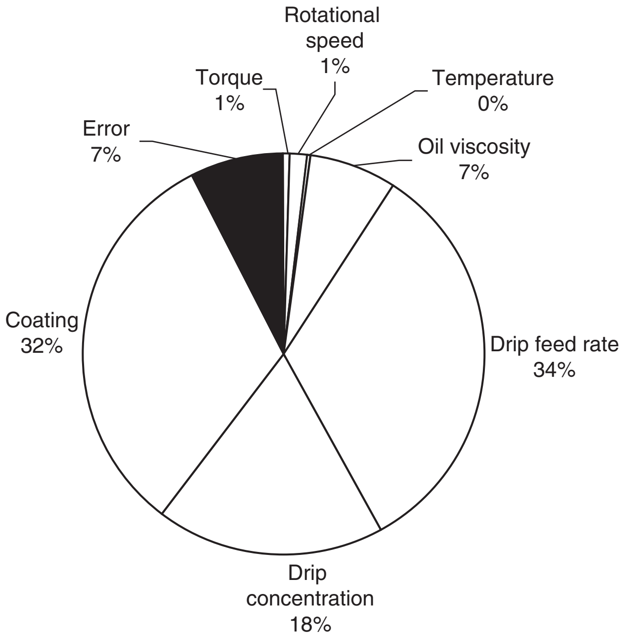

Figure 1 Example of an analysis of variance (ANOVA) table (a) and pie chart of the corrected sums of squares (b) for a system potentially influenced by seven factors, tested with an L-8 design replicated once. Factors in bold in the ANOVA table have statistical significance at a 90% confidence level. Ne is the effective number of data points, which is equal to the actual number of data points divided by 1+ the sum of degrees of freedom of the factors used to produce the estimate. The table and graph were produced in MS Excel.

the disadvantage is that the results will assume the validity of the mathematical model, and so the lack of fit is added to the overall amount of unexplained variance. The simplest model is a quadratic multifactorial polynomial, that is, the sum of linear, interactive, and quadratic terms. A linear term is proportional to the value of the factor, an interactive term is proportional to the product of the value of one factor and the other, and a quadratic term is proportional to the square of the value of the factor. All values must be normalized between maximum and minimum, which is called 'coding' (numerically, −1 and 1 are common, but can also be 0 and 1, or 0 and 100%). While being useful in terms of all parameters of the model having a very clear meaning, it assumes parabolic curves for all effects, which tends to suggest points of minimum or maximum that do not really exist.

Once a model is fitted to the data and its goodness of fit accepted, it can be used to pinpoint the location of the best combination of settings (searching for the point of maximum or minimum within the constraints of the solution space). The goodness of fit is typically quantified by the coefficient of determination (designated R^2), which quantifies the percentage of the variance of the data that is explained by the model, and values over 90% are usually desired.

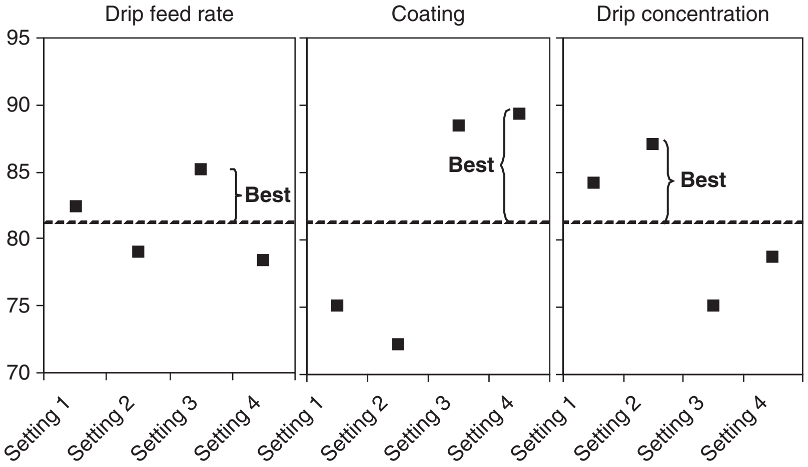

Figure 2 Example of means plots of the performance of a system affected by three factors changed with four settings each, according to a modified L-16 array. The dotted line indicates the average of all data. For each factor, the data are divided in four subsets according to the setting of that factor and the dots show the four averages. Calculations and plots were produced in MS Excel.

There are two useful plots, Pareto charts and surface plots. The Pareto chart represents the standardized effects as horizontal bars, and as such the limit of statistical significance can also be represented as a vertical line. The bigger the bar, the more important the effect. Standardized effects are the effects divided by the standard errors, and when using a model they are also equal to the parameters of the model divided by their confidence intervals. An example is shown in **Figure 3** (case study of loss of an active ingredient in a pasteurization process, as affected by flow rate, inlet temperature, and length of holding section). Surface plots can only be made for a pair of factors at a time, and they can be represented in three dimension (3D) or two dimension (2D) (**Figure 4**) to help visualizing the regions of improved performance, as predicted by the model.

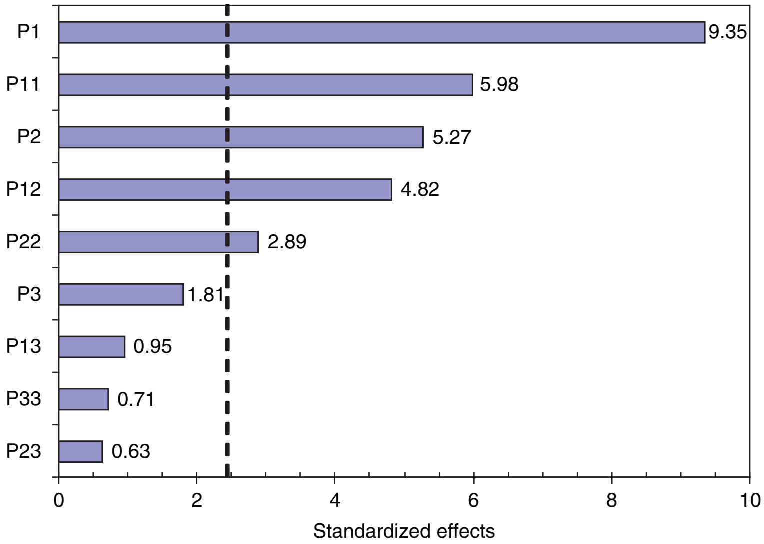

Figure 3 Example of a Pareto chart for a system affected by three factors, which was tested with a central composite design, fitted with a quadratic model. The vertical line represents the limit of significance at a 95% confidence level. The notation 1, 2, or 3 refers to the linear effects (linear terms of the model), 11, 22, and 33 to the quadratic effects, and 12, 13, and 23 to the interactive effects, where 1, 2, and 3 are the generic names of the factors. In this example, factor 3 and all its interactions are not statistically significant. Factor 1 is the most important, and its effect is strongly nonlinear, showing a significant curvature (quadratic effect very relevant) and an influence affected by the settings of factor 2 (interactive effect 1 × 2 significant). In this case study, the factors were inlet temperature, flow rate, and length of holding section in a pasteurizer. The calculations and graph were produced in MS Excel.

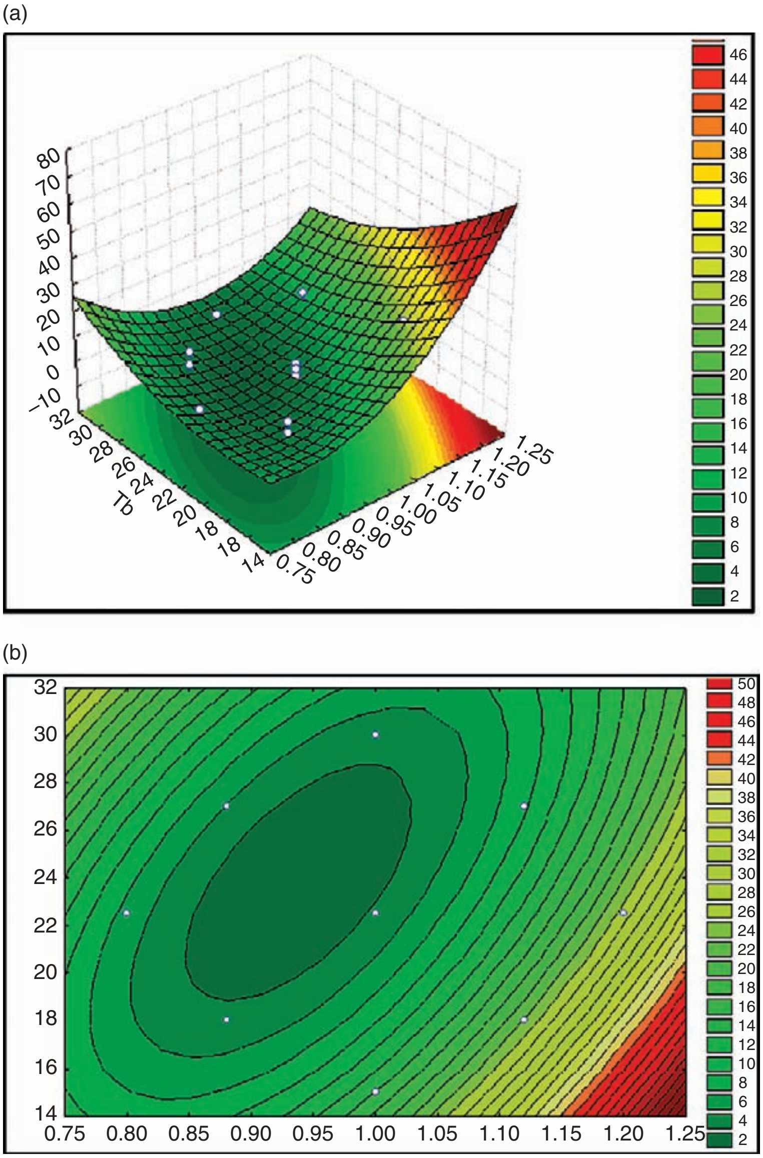

Figure 4 (see color plate 89) Examples of three-dimensional (a) and two-dimensional (b) plots of the influence of two factors on the performance of a system predicted by a model (other influential factors are kept at a constant setting). In the example, optimum performance means minimum losses of a valuable active ingredient, and the two factors shown are inlet temperature and flow rate. The plots were made using the Statistica software (Statsoft).

See also: **Plant and Equipment**: Quality Engineering.

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Quality Engineering

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Quality Engineering and Quality by Design

Engineering is the application of scientific and empirical knowledge to create a new good or service. To 'engineer' something means to design what it should be, how it should be made, and how the process of making it should operate. 'Quality engineering' therefore means that the quality of a product (or service) can be engineered in its production process itself, so that it becomes a characteristic inherent to the product every time it is manufactured in the process. Quality not being a characteristic that is measured, but a consequence of the design of the process and of the way that it operates, leads to the term 'quality by design' (QbD) alternatively used to express this concept.

Conventional quality control systems act on the end result: products are tested for quality and compared to the desired specifications. Non-conformity of a product to the specifications results in a waste: the product must be discarded or reprocessed, depending on whether something can be done to ensure conformity. Quality control is therefore a waste-generating activity. On the other hand, having no quality control can result in a worse consequence, from simple loss of market image to more serious impact, such as that resulting from inadequately processed foods unsafe for consumption. Quality control therefore implies a lack of trust on the capacity of the process to perform consistently and provide quality products all the time. As the process will certainly have been developed properly, this means that it is subjected to variability in its conditions and/or materials it uses, and that variability may result in non-conformities. Not knowing how these sources of variability can present themselves and what can be done to compensate for them, the only option is to check the result at the end, and pray that it is on target most of the time.

Achieving QbD, therefore, implies one of two things: (1) eliminate all sources of variability, controlling obsessively everything so that there is no variation and the system repeats itself exactly the same, time after time; (2) analyze the variabilities of the system inputs, know what their impact will be, and operate the system so that it dampens those variabilities to oscillations within conformity. The first case requires good control systems, and the second needs good process intelligence.

The first concept would be hopeless in most food industries because of the natural variability of biological materials: for instance, in dairy, the composition of milk varies throughout the year, with the lactating cycles of the animals. However, there are sectors where such variability in raw materials does not occur, and one could then envisage that tight control systems would suffice. One should consider, though, whether the cost of such tight control would be an acceptable proposition. In the pharmaceutical industry, there has been a growing deployment of process analytical technologies (PATs), one feature of which may be that more sophisticated sensors measure better aspects of the process so that it may ensure better consistency. Some companies have acquired mass spectrometers for applications such as these. However, some simple calculations should be considered: How much does a mass spectrometer cost to buy and to run? What is the value of the quality gains from the better control? How long does it take for the investment to be recovered? In the pharmaceutical industry, it may well be that the product margins are such that expensive equipment is paid for in some years; in the case of the dairy industry, those margins might point to a few centuries. This has led to a much less interest of the food industry in general for PAT, as deployed in the pharmaceutical sector, which is a pity, because in fact PAT does not necessarily imply that very expensive analytical technologies are used to monitor the process and enable a proactive approach to quality control.

It is therefore useful to focus on the second option described above, go back to basics, and work out a better system by using process intelligence to achieve QbD, independent of whether acquiring that intelligence requires expensive analytical technologies or not.

The Principles of the Taguchi Method

The pioneering work of Genichi Taguchi was a very influential basis of the modern QbD approach. Appointed to lead the Electronic Communications Laboratory (Nippon Telephone and Telegraph Co.) of Japan in 1950, he was faced with ensuring consistently good communications on resources devastated by war. The problem required solutions to be found fast and there were few resources to help and little money to invest. Taguchi spent the best part of the next 12 years developing methods to improve quality and reliability. One of the companies that took up his concepts early on was Toyota, and through it and the Toyota Production System (*see* **Plant and Equipment**: Continuous Process Improvement and Optimization), the Taguchi method began permeating US and then European industrial practice in the late 1980s. That such a story is at the origin of QbD is very relevant, as it shows that the essence of the system is all about being able to solve problems without throwing money at them.

There are two important concepts at the basis: differentiating between control factors and noise factors, and the loss of quality function.

The performance of a system is influenced by factors that are controlled by the operator, probably within tight margins, such as temperatures, flow rates, and pressures. It is, however, also influenced by factors that are not controlled, and these may vary more or less significantly depending on random or non-random effects. For instance, milk composition varies throughout the year and also from producer to producer, as it is influenced by animal feed too, not to mention the genetics of the animals. Although control factors may have a bigger influence on the performance, as they are controlled tightly, the variability of performance may be due more to noise factors than to improper control of the control factors. Therefore, more sophisticated control systems for the control factors may be almost pointless. First and foremost, the sources of variability, and how they influence product variability, must be understood. For instance, a pasteurizer may be set to work at 92 °C, and the control system does not give better than ±1 °C. On the other hand, the pasteurizer was designed considering that ambient temperature was a given constant figure (e.g., 15 °C). However, depending on the location, this may vary during the year from anywhere between −20 and 40 °C. Even though ambient temperature in itself may be less important than the operating temperature for the design of the equipment, in the operation of the pasteurizer its variability may cause more fluctuations than that of the process temperature. However, it is obvious that controlling ambient temperature tightly is a preposterous idea, so controlling variability may not be an option, and the only possibility may be to find a way to live with it.

As an engineer, Taguchi knew that changing the settings of a system influences its inertia and so the way that it amplifies, or dampens, the input oscillations. Therefore, one should look for the settings of the control factors that lead to a system that dampens the input oscillations as much as possible: hence the term robust design. A system has a robust design when it performs consistently in spite of the variability of the inputs.

The concept of loss of quality function was another original contribution of Taguchi. Previously, the norm was to consider that a product was either acceptable (within the range of specifications defined) or not acceptable, so there was full acceptance or full loss of a product. Taguchi considered that every time that the product is not exactly on target, there is a loss, which is bigger the further the result is from the target. Taguchi used a simple parabolic function to quantify the loss of quality as the quality indicator deviates from the target value. In fact, if quality is below expectations, there is loss to the client, which over time becomes loss to the company in terms of market image, market share, and so on. If quality is above expectations, there is loss of opportunity for the company, which could have made use of that better quality to get a better price or greater market share. Furthermore, if that higher quality is presented to a client once, that client will then expect the same higher quality next time, and therefore there can be a greater loss of quality by underperforming to expectations the next time. Therefore, the quality loss function should be minimized, and the process should be steered to the quality indicator value that can be delivered more consistently. This means that one would even prefer a business where the product has a lower average quality but that can be delivered consistently, to one with a greater variability even though on average it may be better. In the long term, the second case is going to pile up client dissatisfaction for one reason or another and hurt the business. It can be argued that the evolution of the world wine markets between French producers and New World producers offers a case in point.

In order to improve both average quality and its consistency, Taguchi defined the signal-to-noise ratio (S/N) as the objective of an optimization approach: search for the combination of settings of the control factors that gives the maximum S/N. S/N integrates the two objectives: best average quality and best quality consistency. There are three mathematical definitions of S/N, depending on the type of problem, known as 'bigger is best' (the higher the average of the quality indicator, the better), 'smaller is best' (the lower the average of the quality indicator, the better), and 'nominal is best' (the closest the average of the quality indicator is to a nominal target, the better). In essence, the Taguchi method for robust engineering design consists in deploying an experimental plan and statistical data analysis procedure to identify the combination of settings of the control factors that maximizes S/N (for the methods that Taguchi selected for experimental design and data analysis, *see* **Plant and Equipment**: Continuous Process Improvement and Optimization).

The Taguchi method does not consist solely of applying statistics to plan and then analyzing a set of tests, but it considers the entire framework of operation, teamwork, and other things. See articles in Further Reading for more information.

It is noted that once the 'process intelligence' has been gathered in terms of availing of a simple model that relates input variability, control factor settings, and output variability (S/N), it is possible to develop a proactive approach to process control (also known as feed forward; *see* **Plant and Equipment**: Instrumentation and Process Control: Process Control). By measuring the input factors and their variability (even if they are not controlled, as noise factors), the model can predict what the outcome will be, and hence correct the settings of the control factors to ensure the robustness of the operation (*see* **Plant and Equipment**: Instrumentation and Process Control: Process Control).

Recently, Charteris advocated the use of experimental design methods and the Taguchi method in particular for competitive quality systems in the food, and specially dairy, industry. The increasingly extensive application of the method in various companies across industrial sectors is its greatest selling point.

Theoretically, the Taguchi approach has been contested, mostly for two reasons: it relies on orthogonal arrays in the experimental design, which leads to intricate confoundings (*see* **Plant and Equipment**: Continuous Process Improvement and Optimization), and the S/N ratio concept integrates average and standard deviation, and therefore model predictions bundle them together. It may also be noted that in practice maximizing S/N assumes no interactions between factors (*see* **Plant and Equipment**: Continuous Process Improvement and Optimization). However, by giving more and due importance to variability and repeatability, it often leads to good results from a limited amount of data.

Statistical Process Control

As Taguchi was beginning his work in Japan, 'western' statisticians were also assisting industry to develop methods for improving quality consistency. That a proactive approach to quality control was needed was also concluded early. The process should be monitored at several key moments, the variability must be addressed then, and actions must be taken immediately, as needed. The concepts of statistical process control (SPC) were thus developed (for more details, *see* **Plant and Equipment**: Instrumentation and Process Control: Process Control). In this case, the type of variability is categorized between common and special causes. The former are identified primarily due to the randomness of variability, while the latter are pinpointed by a clear pattern in the data that cannot be due to chance.

The original practice involved using process charts to identify the sources of variability and progressively eliminate or squeeze them, which an enthusiast of the Taguchi method might regard as too laborious and hence not pro-active enough. It also has the obvious disadvantage of relying on process data, which means that the range of values considered for the control factors may be too small and the data excessively biased.

SPC is basically very good for ensuring that the oscillations in a given process are minimized, but is not really designed to establish best conditions of operation that offer a more robust operation. It may also lead to the over-zealousness of controlling factors with increasingly expensive methods even if such control results in savings that do not justify the costs. Quality engineers deploying SPC may need to resist the temptation for expensive applications of information technologies (IT) or PAT, unless the benefits are clear upfront. For further details, see articles in Further Reading.

Six Sigma

The best practice of modern manufacturing industries is generally regarded to be the six-sigma system, although there is some controversy on this. Its name reveals its primary focus on maximizing consistency and thus eliminating waste, as sigma is the Greek letter used in statistics to denote the standard deviation, a quantitative measure of the spread of a series of data. If the data are normally distributed, then the band defined by the average $\pm 6\sigma$ contains 99.999 998 1% of the data. Bringing some element of reality of process operations, the six-sigma creators discounted 1.5σ from this, saying that over time a process becomes more 'sloppy' than the original design by this much, that is, a process is said to be 6σ when the range of average $\pm 4.5\sigma$ is within the specification range: thus, one would be out of spec only 3.4 times in one million (99.999 66% of the data of a normal distribution within $\pm 4.5\sigma$). In practice, it really means that the process is designed/operated to deliver consistency all the time, or as sometimes stated in business science literature, 'first time right, every time right'.

It is generally accepted that the origin of the system lies in Motorola in the early 1980s, and that it was particularly disseminated by Jack Welch, a charismatic former CEO of General Electric (who famously stated that 'variation is evil'). In broader terms, six sigma results from giving an American managerial approach and organization to projects aimed at eliminating variation that apply much of the concepts and methods of Japanese manufacturing, with the Taguchi concepts being particularly eminent. Six sigma is a method, or work system, not a tool, and therefore it collects and deploys whatever tools can assist to achieve its objective better. Just like the Taguchi method, it involves a comprehensive approach including teamwork, planning, and other things, and it is data driven, that is, it relies on obtaining and analyzing actual process data. In that respect, six sigma is more an American version of the Taguchi approach than the Taguchi method is a part of six sigma. There is some confusion in the literature as to what is a tool and what is a system, and often the Taguchi method is taken as the distillation of its statistical approaches – that, however, is only a part of the whole.

Just like Taguchi did, six sigma picks up the tools it finds more useful. Among these are the tools used in the Taguchi method (e.g., design of experiment (DoE), analysis of variance (ANOVA), S/N), but many others as well, such as SPC and lean analysis. When lean thinking is added, it is often denoted as the lean six-sigma approach. As it incorporates both operational and process improvements, it is a proper approach to achieve manufacturing excellence in an organized and systematic manner.

Critics contest its novelty, as almost everything it uses existed before, and so are the aspects of its implementation. For someone experienced in the Taguchi method, it also seems to be overcomplex, and could lead to the deployment of different approaches that yield the same end result, with no particular benefit in the redundancy.

It is common to summarize the overall approach with acronyms: a project to achieve optimum consistency in an existing process applies a series of steps known broadly as DMAIC (define, measure, analyze, improve, and control), while a project geared at developing a new process or product is composed of a series of steps represented by DMADV (define, measure, analyze, design and validate, or verify). The latter is also known as DFSS, from design for six sigma, that is, design the new process or product so that it will deliver a six-sigma consistency, which is therefore the same as QbD.

See also: **Plant and Equipment:** Continuous Process Improvement and Optimization; Instrumentation and Process Control: Process Control.

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Safety Analysis and Risk Assessment

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Importance of Plant Safety in the Dairy Industry

Safety is important in both milk production and dairy processing facilities. Both people and animals can be exposed to a diverse range of hazards. These need to be identified and managed. As an example, people who handle chemicals, antibiotics, vaccines, and veterinary drugs need to be familiar not only with their benefits but also with their potentially hazardous properties. To ensure that these substances are handled safely, the use and distribution of Material Safety Data Sheets (MSDSs) to all involved personnel are a prerequisite.

Chemicals, fertilizers, and pesticides can cause environmental damage and possible contamination problems if improperly used or incorrectly handled. Contamination and pollution can also be caused by incorrect handling and storage of manure and dairy wastes. In some instances, asbestos insulation may be present in older farm buildings, which carries the risk of mesothelioma and necessitates a careful and organized program for removal of such insulation by qualified asbestos removal contractors. Dust can also become a health hazard and, if combustible, could lead to fire and/or explosion.

Typical job safety hazards are also posed by effluent ponds where drowning can occur and with electrical equipment, often of a temporary and makeshift nature, where electrocution through use of ungrounded electrical equipment, in an aqueous environment, can occur. The use of unguarded prime movers, the temporary removal of guards, and the failure to isolate machinery during maintenance are all sources of hazard and risk: the use of lockout/tagout procedures is essential if maintenance of equipment with moving parts is involved. Also, personnel who work at heights may risk falling and those who engage in manual lifting may sustain injuries.

The dairy industry, when compared with many other industries, is not normally associated with high-risk activities. However, large dairy facilities may use a number of hazardous materials that can still pose high risks to both plant personnel and the neighboring communities. As an example, the following substances can present significant risk:

1. Anhydrous ammonia which may be used in refrigeration systems. Release of anhydrous ammonia to the environment, which is normally stored as a liquid under pressure, can result in a highly toxic subcooled aerosol mist that can hug the ground until it heats up and disperses. Such releases can have quite devastating effects in the path of release.
2. Liquid chlorine which may be stored under pressure and normally used for sanitization purposes. Chlorine gas is heavier than air and is highly toxic: it can cause death even in relatively small amounts.
3. Propane which is stored as a liquefied gas in bullets under pressure. Propane may be used as a heating medium for boilers and heating systems. A number of dangerous incidents have arisen as a result of propane bullets becoming overheated, due to external fires impinging on them and causing Boiling Liquid Expanding Vapor Explosions (BLEVEs).

Overall, many different types of hazards are often present at a dairy facility and the failure to be aware of these can lead to accidents and injuries. It therefore follows that potential hazards, in the first instance, need to be formally identified in order for them to be managed.

The key steps in safety analysis are (1) the systematic identification of hazards that are present, whether obvious or latent, (2) the prioritization of hazards using a risk matrix approach, and (3) the management of hazards through the introduction of risk control or risk mitigation measures. Usually, these three steps are adequate for the majority of in-plant safety issues.

Where greater issues that could pose significant risks not only to in-plant personnel but also to the neighboring communities exist, it may be necessary, in addition, to perform a Quantitative Risk Assessment (QRA).

Formal Safety Analysis

A formal safety analysis can be performed using a number of alternative methodologies. These are known collectively as Process Hazards Analysis (PHA). Typically, they proceed as shown in the following six steps:

Step 1: Collect engineering drawings such as Process Flow Diagrams (PFDs), Piping and Instrument Diagrams (P&IDs), and documents that specify the nature and operation of the facility in question. In addition, MSDS for all hazardous substances stored or used at the facility should be available for the analysis.

Step 2: Break down the facility into definable and specific subunits that provide a common function. This is a form of itemizing and is sometimes known as creating 'nodes'.
Step 3: List questions for each subunit or node that can explore and investigate design or operational deviations that might reveal undesirable or possibly hazardous conditions outside the normal range.
Step 4: For each question listed in Step 3, record the causes for the deviations and the consequences that might occur or result from such deviations. In addition, the safeguards that may already be in place to either prevent the cause of the deviation or to protect or mitigate the consequences should be recorded. Furthermore, any recommendations should be considered to reduce the risk by providing additional safeguards that are not currently available.
Step 5: Proceed throughout the facility so that all subunits or nodes are covered.
Step 6: Prepare a detailed report that includes both documentation on the facility as well as details of the PHA.

When this stage is reached, it is possible to create a risk management plan to determine what needs to be done to reduce the risk in order to make the facility safer. Also, when the PHA is being undertaken, it is often best to assemble a team of personnel responsible for the design and operation of the facility so that all possible concerns are systematically investigated.

There are four different types of PHA that are typically used:

1. Hazard and Operability (HAZOP) analysis, where deviations are created by applying guidewords such as High, Low, Reverse, As Well As, Part Of, and Other Than to properties such as Flow, Level, Pressure, Temperature, Concentration, and pH. This is a popular methodology within the process industries but is really applicable only where the systems involve flowing fluids, often on a continuous basis. HAZOP may also be applied to batch operations where the guidewords also include Sooner and Later to account for time aspects.
2. 'What if. . .' analysis, where deviations are created by listing questions that pose design and/or operational problems. This is a relatively simple technique that, although less structured than HAZOP, is applicable to almost any facility or part of any facility regardless of design, function, or operation. Furthermore, it is easy to learn and apply.
3. Failure Mode and Effects Analysis (FMEA), where items of equipment are broken down into components. For each component, the deviations are different possible failure modes for that component. The consequences are the effects of the various types of failure that are identified. FMEA is normally recommended for analyzing equipment failures as opposed to fluid system failures. It can typically be applied to prime movers, instrumentation, and control equipment, and, in addition, is a useful method for improving reliability through the identification of possible failure modes.
4. Checklist analysis, where a list of questions and concerns is created from either preexisting data or information or based upon previous experience. This technique is less structured than the other three methods described above but is useful prior to commissioning a new system where there are concerns over any residual issues that might have been created or overlooked by construction teams.

In addition to these above four methodologies, 'What if . . .' analysis and Checklist analysis are often combined as 'What if/Checklist'. This ensures that an adequate list of questions (i.e., possible deviations) is created. Furthermore, when applying these types of methodologies to occupational safety, the form of analysis used is Job Safety Analysis (JSA) where people's jobs are specifically analyzed for hazards.

All of the above methods are oriented toward the identification of potential hazards and hazardous situations, whether obvious or latent. These analyses normally identify single jeopardy hazards as opposed to double or multiple jeopardy situations. From the standpoint of likelihood, single jeopardy is the most likely and double or multiple jeopardy is far more unlikely.

Definition of Risk

While the identification of hazards, including potential hazards, is of paramount importance, the quantification of risk can, in some instances, be desirable. Risk is a measure of the importance of hazards posed and is a function of both the severity of the hazard and the likelihood, or frequency, of the hazard occurring. The technical definition of risk is the product of the consequence, that is, the severity of the hazard and the frequency, that is, the likelihood of the hazard ever occurring. In other words

$$\text{Risk }(R) = \text{Consequence }(C) \times \text{Frequency }(F)$$

The consequence may be expressed as the chance of mortality, as financial damage that may be incurred or the loss of revenue that may result from an incident. Typically, when consequence is expressed in terms of mortality or, more specifically, in terms of the probability of fatality, the values provided in **Table 1** can be useful.

The frequency is often expressed as the number of times per annum that the incident may occur. Hence, for example, if the incident were likely to occur once every year or so, this would be deemed as 'very likely' but if it occurred only once in a 1000 years it would be deemed as 'very unlikely'. It can seem difficult to envision individual risk levels without comparison to known risks experienced on a day-to-day basis. **Table 2** provides this comparison.

Table 1 Value of fatality probability and associated effects

Value of fatality probability	*Associated effects*
1.0	Effects would be fatal for any individual exposed to the hazard
0.1	With 10 persons exposed to the hazard there would be one fatality
0.01	With 100 persons exposed to the hazard there would be one fatality
0.001	With 1000 persons exposed to the hazard there would be one fatality

Table 2 Example of comparative mortality statistics

Hazard	*Total number of deaths*	*Individual chance of death per year*
Heart disease	757 075	3.4×10^{-3}
Cancer	351 055	1.6×10^{-3}
Work accidents	13 400	1.5×10^{-4}
All accidents	105 000	4.8×10^{-4}
Motor vehicles	46 200	2.1×10^{-4}
Homicides	20 465	9.3×10^{-5}
Falls	16 300	7.4×10^{-5}
Drowning	8100	3.7×10^{-5}
Fires, burns	6500	3.0×10^{-5}
Poisoning by solids or liquids	3800	1.7×10^{-5}
Suffocation, ingested objects	2900	1.3×10^{-5}
Firearms, sporting	2400	1.1×10^{-5}
Railroads	1989	9.0×10^{-6}
Civil aviation	1757	8.0×10^{-6}
Water transport	1725	7.8×10^{-6}
Poisoning by gases	1700	7.7×10^{-6}
Pleasure boating	1446	6.6×10^{-6}
Lightning	124	5.6×10^{-7}
Hurricanes	93	4.1×10^{-7}
Tornadoes	91	4.1×10^{-7}
Bites and stings	48	2.2×10^{-7}

Data on Mortality Statistics for USA, 1974, and revised, 2000: Chemical Manufacturer's Association.

In Figure 1, an example of a risk matrix is shown that can be used in conjunction with a PHA to enable members of a risk analysis team to assess first-order estimates of risk associated with an activity or item of plant. When values are in excess of 10^{-3} deaths per annum then some additional risk control measures or additional risk mitigation should be considered.

Every activity normally carries some level of risk, however small it may be, and most people accept this as part of the day-to-day reality of life. Zero risk for an activity is not usually possible unless the activity ceases altogether.

Risk Assessment

When incidents involving hazardous materials could cause significant in-plant damage and also threaten neighboring communities, it may be in order to consider performing a QRA for the facility. The following steps are usually undertaken:

Step 1: From the PHA already performed and by applying the risk matrix methodology, determine which incidents are high-enough risk to warrant full risk quantification and assessment.

Step 2: Model the consequence of the incident as a function of distance from the hazard source. This is done using mathematical models as typically listed in Table 3.

Step 3: Model the frequency of the potential incidents. A variety of methods can be used as shown in Table 4.

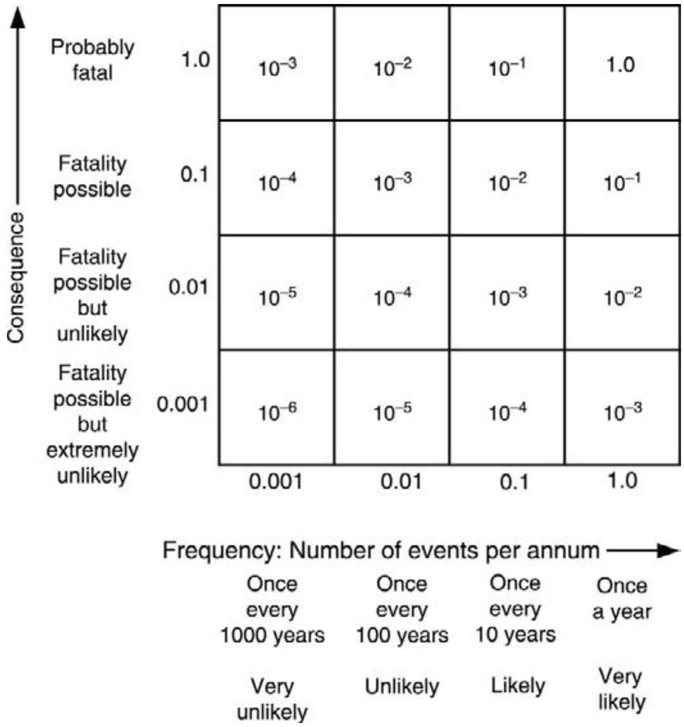
Step 4: Having numerically determined both the consequences, in terms of probability of death per occurrence and frequency of occurrence in times per annum per event, individual risk can be computed as

$$R_i = C_i \times F_i$$

where R_i = individual risk, in deaths per annum for a single event, *i*; C_i = probability of death, dimensionless from the consequence of event, *i*; and F_i = frequency of single event, expressed in events per annum.

The idea behind individual risk is the risk that might be posed to an individual who is in the vicinity and exposed to the hazard in question.

Individual risk is also a function of distance from the hazard, which, although not affecting the frequency,



Consequence		0.001	0.01	0.1	1.0
Probably fatal	1.0	10^{-3}	10^{-2}	10^{-1}	1.0
Fatality possible	0.1	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Fatality possible but unlikely	0.01	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Fatality possible but extremely unlikely	0.001	10^{-6}	10^{-5}	10^{-4}	10^{-3}

Figure 1 Matrix showing individual risk in deaths per annum.

Table 3 Listing of consequence models used for risk assessment

Hazard	*Consequence model*	*Effects and modeling*
Fire	Pool fire Jet fire Fireball Flash fire	Thermal radiation generated by fire can cause burns that can be lethal and models can predict exposure levels as a function of distance from source. Probit analysis can predict probability of death as a result of radiation dosage and exposure time.
Explosion	Vapor Cloud Explosion (VCE) Missiles and shrapnel generation Boiling Liquid Expanding Vapor Explosion (BLEVE)	Overpressure and momentum forces can result in lung and ear-drum damage and cause the collapse of buildings leading to death. Probit analysis can predict likely effects and probability of death as a function of overpressure. Simple models for missiles and shrapnel generation exist that indicate likely size, number, and range. BLEVEs are also modeled as fireballs.
Toxic vapor releases	Vapor dispersion	Vapor dispersion and dilution occur in the atmosphere and can be modeled as a function of distance from the source of the release for both neutrally buoyant and dense gases taking into account meteorological conditions, wind direction, and wind speeds. Probit analysis for specific gases can predict probability of death with dosage and time elapsed.

could increase the consequence if the exposure to the hazard is increased. Since there is often more than one source of risk, it is also relevant to consider total or integrated risk.

Thus, the overall integrated risk, considering n events and each event having its own specific consequences and frequency, can be expressed as

$$R_{\text{overall}} = \sum_{i=1}^{n} (C_i \times F_i)$$

where R_{overall} = integrated individual risk, in deaths per annum for n events in total.

Step 5: Determine the basis for benchmarking or judging what levels of risk can be considered as acceptable. In judging individual risk criteria, the criteria shown in Table 5 may be considered.

Societal risk, as opposed to individual risk, represents the integrated risk that may be posed to a group or multiple individuals located within a specific area or zone. Societal risk may thus be expressed mathematically as

$$R_{\text{societal}} = N \sum_{i=1}^{n} (C_i \times F_i)$$

Table 4 Modeling of frequency or likelihood of incidents

Method	*Basis*	*Data used*
Fault trees	Fault trees model events using a top-down approach to show top event as the undesirable event and all contributory factors and subevents that lead to the top event.	Available failure rate data, reliability data, and contingent factors analysis where no data are readily available.
Event trees	Proceeds from left to right starting with initial cause of incident and considers optional outcomes that can be assigned probabilities of occurring or failing to occur. A range of possible final outcomes is the end result.	Original causal event frequency is assigned based on failure rates of piping, gaskets, etc. Subsequent events likelihood use assigned probabilities based on available data, best judgments, and estimates.
Historical data	Use of available databases to provide frequencies of specific types of events.	Recorded data from same, similar facilities and recorded data available on a global basis.

Table 5 Risk tolerance criteria

Individual risk level in deaths/annum	*Tolerance level*
$>1 \times 10^{-3}$	Exceeding 1×10^{-3} deaths per annum is deemed intolerable
1×10^{-3}	Should not exceed 1×10^{-3} deaths per annum maximum for workers provided that As Low As Reasonably Practicable (ALARP) measures are in place
1×10^{-4}	Should not exceed 1×10^{-4} deaths per annum maximum for the public provided that ALARP measures are in place
$\leq 10^{-6}$	Individual risk criteria are broadly acceptable at 1×10^{-6} deaths per annum

Data from ALARP – Guidance on as low as reasonably practicable decisions in COMAH: Health & Safety Executive (HSE), UK.

where N = total number of persons exposed to the individual total risk and where risks vary depending on the numbers in different communities and their locations:

$$R_{\text{societal overall}} = R_{\text{societal overall},1} + R_{\text{societal overall},2} + R_{\text{societal overall},3} + \cdots$$

Although societal risk may be evaluated, the acceptability of criteria is more complex than that of individual risk. FN curves may be used for societal risk where the ordinate represents the cumulative frequency distribution of N or more fatalities and the abscissa represents the consequence (N fatalities). Although there are published data on FN curves, their acceptability has not been widely adopted. Currently of greater interest is the ALARP (As Low As Reasonably Practicable) criteria. This recognizes three regions. The first of these is the 'unacceptable region' where the activity is of such a high risk as to render it unacceptable. The second region is the 'broadly acceptable region' where the activity has a very low risk and no further measures are needed for risk reduction. The third region is the 'tolerable region' where the level of risk falls between 'unacceptable' and 'broadly acceptable' and has been reduced to the lowest level of risk as considered to be practicable.

Step 6: Apply risk management principles. Risk may be managed once the hazards have been identified. If the QRA route has been undertaken, then the calculated overall risk, R_{overall} or $R_{\text{societal overall}}$ should be compared to what may be deemed as tolerable. Depending on the level of tolerable risk, the decision to accept the risk or take remedial action(s) must be made. If the level of risk is within accepted margins, then no further action may be necessary. If the level of risk is excessive, then actions requiring remediation and costing plant modifications, procedural changes, as well as emergency response planning may be needed.

Risk Reduction and Risk Mitigation

Risk reduction is possible only if hazards are identified and then measures are taken to reduce these risks. Although it may be possible to reduce the risk to a level that is considered acceptable, it is rarely possible to eliminate risk altogether. When facilities are designed originally, there may be good opportunities to introduce design features that can minimize risk whereas if safety is addressed as an issue only late in the design it may be extremely costly to incorporate such features.

Safety in design is sometimes considered in terms of 'active' and 'passive' safety features. A passive safety feature requires no form of activation or initiation for the feature to be protective. For example, increased distance and spacing is valuable as a passive safety feature as would also improved road access, dikes around tanks containing flammable materials, and reduced inventories of hazardous materials. An active safety feature would be instruments, controls and automated trips, and most safety devices operated electrically, electronically, hydraulically, pneumatically, or even mechanically. Although both active and passive safety features are needed at a facility, passive safety features are much more dependable. On existing facilities, it may be difficult or impossible to add passive safety features and very often only additional active safety features can be incorporated where risk reduction is required.

Qualitative Risk Analysis versus Quantitative Risk Assessment

Risk may be analyzed either qualitatively or quantitatively. For the majority of cases where no significant risk is posed to adjoining communities, the qualitative analysis should be adequate. However, where the potential for encroachment by new housing communities is an issue, the quantitative assessment has merit for establishing recommended buffer zones around the facility.

It is often difficult to say where an assessment of risks ends and risk control begins or to assess risks without making a number of assumptions; at best, a risk assessment is an order of magnitude estimate and is directional as opposed to being absolute. Unless inputs and assumptions are very similar, the repeatability of risk assessment is hard to achieve.

Risk assessment is a tool for extrapolating from statistical, engineering, and scientific data, a value that people will accept as an estimate of the risk attached to a particular facility. There are many techniques for risk estimation, tailored to different applications that cover a wide range of different disciplines, such as toxicology, engineering, statistics, economics, and demography.

The true value of risk assessment, through the QRA, lies mainly in comparing overall risk levels both before and after risk remediation is incorporated. For example, suppose an overall level of individual risk is determined to be 10^{-4} deaths per annum for a facility and that after remediation it is reduced to 10^{-6} deaths per annum. This means that remediation has made the facility 100 times safer. This improvement may be considered to be more important than trying to determine exact levels of risk.

Risk Assessment and Emergency Response Planning

A QRA can identify situations where an Emergency Response Plan (ERP) or buffer zones or restrictions should be considered. The output of an analysis, of risk versus distance from the hazard source, can indicate hazard zones.

Depending on these findings, a detailed ERP may be needed. This will also address the Emergency Response Planning Guideline (ERPG) levels 1, 2, and 3 distances.

The distance to the ERPG-3 level corresponds to the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for 1 h without experiencing or developing life-threatening health effects. The distance to the ERPG-2 level corresponds to the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for 1 h without experiencing irreversible or other serious health effects or symptoms that could impair their abilities to take protective action. The distance to the ERPG-1 level corresponds to the maximum airborne concentration below which it is believed that nearly all individuals could be exposed for 1 h without experiencing other than mild transient adverse health effects or perceiving a clearly objectionable odor.

See also: **Risk Analysis**.

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In-Place Cleaning

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Introduction

Cleaning in place (CIP) is a vital discipline within the modern food, dairy, and beverage processing industry. Dairy and beverage have tended to lead the way due to the major products being liquid and the process equipment lending itself to CIP. However, many food or pharmaceutical operations now incorporate CIP and the technology is therefore much more common.

In the 1990 edition of the Society of Dairy Technology (SDT) *Manual Cleaning In Place*, CIP was defined as "The cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. The process involves the jetting or spraying of surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity." This was taken from the National Dairymens Association (NDA) Chemical Safety Code, 1985, and while the NDA has been superseded, their definition of CIP is still felt to be quite appropriate.

Practice in the Dairy Industry

The modern dairy plant, be it for liquid milk or the multitude of other dairy products, will have at least two CIP sets at its heart; it is generally accepted as best practice that raw and finished product should be segregated to avoid cross-contamination. The raw milk CIP set will be responsible for cleaning the raw milk silos and associated milk intake pipe-work along with any in-line coolers and filters including transfer lines to the pasteurizer. It is at this point that the segregation between raw and finished (pasteurized) products is maintained. In many cases the pasteurizer with its associated items of processing equipment such as homogenizer, separator, and standardization unit will be cleaned together. The cleaning operation can be single-stage or two-stage, but the principle of single use remains. In a few sites, a partial recovery system may be used. There are benefits and drawbacks in each type of system and these will be discussed in more detail later. The finished milk CIP set will clean all items of plant that are used to store, process, and pack finished or pasteurized product. It is vital that this cleaning equipment be maintained to the highest standards in order to ensure good plant hygiene and to avoid product contamination, either physical or microbiological, that would have an adverse effect on final product quality or shelf-life.

Outline of a CIP System

The main stages of CIP are similar to any other standard cleaning routine: removal of gross debris (product purge); pre-rinse; detergent (normally acid- or caustic-based); intermediate rinse; second detergent if applicable; intermediate rinse; disinfectant; and final rinse with potable water. The diluted detergents are generally stored in tanks as part of the CIP unit or CIP set and will be built up into a fully operational CIP set with valves, manifolds, and interconnecting pipe-work, including an automated control system.

The design of the CIP set will depend on the duty required. Other considerations such as available space and budget constraints do influence the design but making compromises at the design stage is not recommended, as poor CIP performance can have a significant impact on product quality. **Figure 1** shows a four-tank partial recovery system with a single channel or CIP route operation. On larger sets, there can be five or six separate channels linked by common inlet and outlet manifolds. Other configurations are possible, with or without a rinse recovery tank. It is quite unusual to find recovered disinfectant storage tanks as these require very close management and can easily become contaminated leading to potentially serious consequences; hence, the tank denoted as 'utility' in **Figure 1** when used for disinfection is likely to be of single use.

Most CIP sets have some degree of automation, the most basic being a set of timers to open and close automatic valves in a particular programmed sequence at specific times. More sophisticated sets incorporate significant levels of field instrumentation with sensors, usually mounted in-line to monitor flow, temperature, pressure, conductivity, turbidity, etc. Control of detergent concentration is usually automated and the most common configuration is a control conductivity probe situated in a recirculation loop to ensure good mixing when extra detergent is added to the solution.

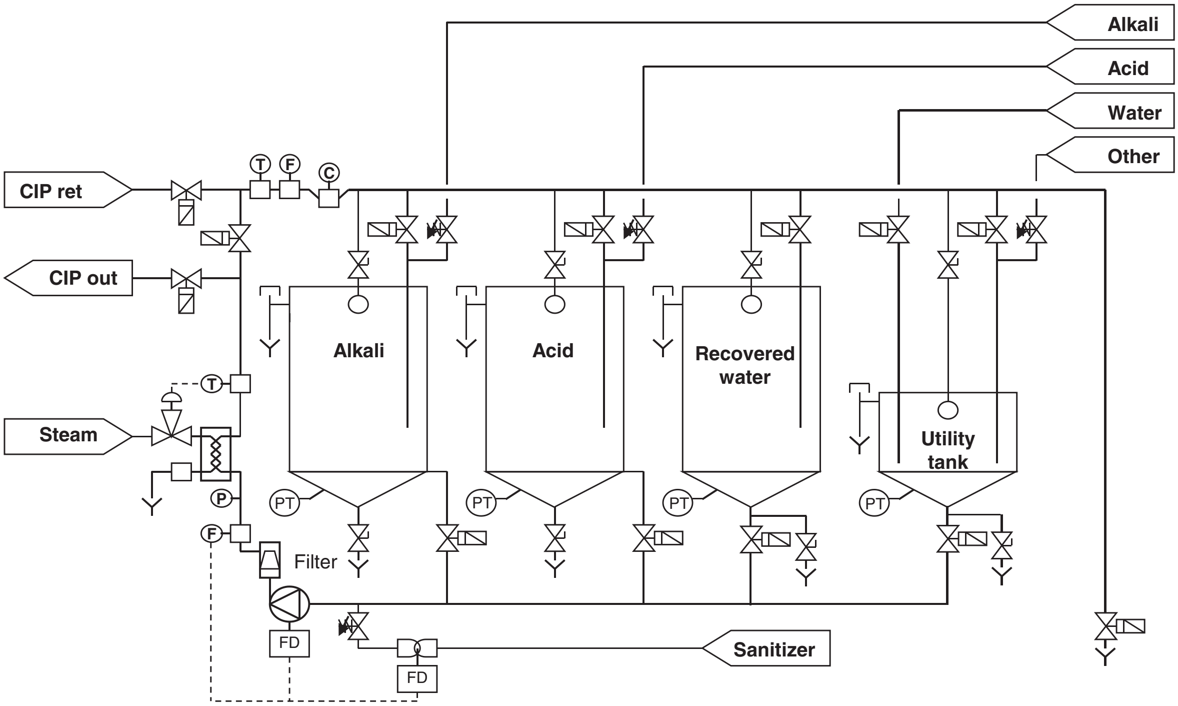

Figure 1 Single-channel, four-tank partial recovery CIP system.

Detergents and Disinfectants

In the dairy industry, the most common type of detergent is a caustic soda-based product, quite often containing a blend of sequestrants, surfactants, and other additives to assist with the cleaning task. The detergent also needs to be compatible with the prevailing water hardness conditions in order to prevent scale deposition, especially during rinsing. The selection of the correct detergent is a specialist activity and needs to take into account factors such as materials of construction, soiling type and levels, and product safety. The effectiveness of the caustic soda-based material is heavily influenced by the specific blend of additives and these are designed to remove dairy soils such as fat, protein, and more complex molecules and structures that are created by the process or simply by heat such as calcium carbonate. In certain circumstances, acidic detergents are used; these are often based on phosphoric or nitric acid or blends of the two and are found to be effective at removing inorganic deposits in dairy processing plants.

Disinfectant solutions can generally be divided into oxidizing and non-oxidizing products, the former being more common for CIP use as they tend to be more efficacious and have a lower tendency to foaming that can lead to rinsing difficulties. The traditional dairy disinfectant was sodium hypochlorite, a very cost-effective product for CIP disinfection but with the major drawback for dairy CIP of being corrosive to stainless steel. It is now more common to utilize an equilibrium mixture of hydrogen peroxide and acetic acid – *peracetic acid* – and this is commercially available, often supplied at 5 or 15% activity.

Application in Dairy Equipment

The four main types of equipment encountered in a typical dairy situation are pipelines, vessels, fillers, and cheesemaking equipment. These are all normally cleaned using CIP and it is important to ensure that each is cleaned in the correct manner, for example, to clean a pipe effectively, turbulent flow should be achieved. As a generally accepted 'rule of thumb' the flow rate required to achieve turbulent flow and therefore provide optimal cleaning is around 1.8 $\mathrm{m\,s^{-1}}$.

Fillers and complex items such as cheesemaking equipment will require purpose-built cleaning and spray systems installed within the plant to ensure good coverage. In some cases, there is a requirement to clean internal surfaces via CIP and also to include external surfaces, such as on a liquid milk filler, and utilize a specific, permanently installed foam cleaning system.

All tanks and process vessels will include a spray device of some description. Traditionally this was a simple spray ball, which is now being superseded by the use of rotating spray heads that provide a much more effective clean and have the added benefit of lower water consumption.

See also: **Biogenic Amines**. **Hazard Analysis and Critical Control Points**: Processing Plants. **Utilities and Effluent Treatment**: Design and Operation of Dairy Effluent Treatment Plants.

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POLICY SCHEMES AND TRADE IN DAIRY PRODUCTS

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Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy

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Introduction

Agricultural support is a very important element in agricultural policy in many countries. Agricultural support is basically an instrument to meet the overall objectives of the agricultural policy – objectives set by society. There are a great number of instruments and ways of intervention in agricultural policy and they have different functions and consequences. Often, price mechanisms are used as support instruments, while direct income support is used in other cases. Choice of support system is of major importance and may have far-reaching consequences.

Objectives and Instruments in Agricultural Policy

Intervention through agricultural policy is a very important phenomenon in the agricultural sector in many countries. Often, the intervention takes place through the market, and the aim is to improve or stabilize the economic conditions. Intervention itself is not an objective, but it is an instrument to achieve the overall objectives and aims set by society.

Before the examination of the different instruments, it will be valuable to expose the underlying factors that legitimize those instruments, including support and price policy, of agricultural policy. There is a close correlation between the objectives and the instruments in agricultural policy.

Basically, society has set up a number of objectives, which lay down guidelines and directions for the development of agricultural policy. These objectives, which to a large degree are similar from country to country, explain and set the grounds for the instruments in agricultural policy.

There are a number of common features in the objectives that are found in agricultural policy in developed countries. In general, agricultural policy in developed countries aims at improving

- income in agriculture,
- income distribution among farmers,
- productivity in agriculture,
- efficiency in the processing and marketing chain,
- supply and price stability,
- demographic situation,
- environmental status, and
- export, employment, production, added value, and so on.

Many different types of instruments can be used to achieve the given objectives, and it is a very complicated relationship. Some instruments can be used to achieve several different objectives, whereas other instruments benefit the achievement of some and limit the achievement of others. Finally, important differences with respect to financing, effect on production and trade, transparency, and other elements are observed.

The instruments in agricultural policy can be divided into different groups:

Price Support

Support in the form of higher market prices than, for example, on the world market.

Deficiency Payments

Transfers from taxpayers to farmers corresponding to the production multiplied by the difference between the world market price and a given target price on the domestic market.

Support Coupled to Input Factors

- Area premiums
- Headage premiums
- Financial support
- Other supports to reduce costs

Direct Support Coupled with Other Factors

- Extensification
- Protection of landscape
- Support to enhance structural change
- Economic development in rural areas

Support Fully Decoupled from Production

- Compensation for the effects of drought and other calamities
- Income support, lump sum payments
- Early retirement schemes

Furthermore, one finds a number of other instruments that should not directly be used to achieve the objectives, but should be used to reduce supply and/or costs related to agricultural policy. Quotas and set-aside are examples of such instruments.

Price support and deficiency payments are the most important instruments in the agricultural policy of industrialized countries and account for about 75% of the total agricultural support.

High and Low Price Systems

Market price support and deficiency payments are two very important instruments in agricultural policy; however, they belong to two different support regimes or support systems. Market price support operates in the so-called high price system and is financed by consumers, while deficiency payments operate in the so-called low price system and are financed by taxpayers.

In the high price system, support is given mainly by means of import regulations, etc., which ensure a relatively high domestic price. In the low price system, support is given by means of direct support, while market prices are left undistorted at, or close to, world market level.

The two different support systems have very different implications for agricultural production, financing, markets, and aspects; still, there is an income transfer to agriculture in both systems in the short run.

The balance between market price support and direct payments varies greatly from country to country (**Figure 1**).

In countries like the United States, Ukraine, and Australia, agricultural support is granted mainly as direct payments financed by taxpayers, while market price policy, financed mainly by consumers, is predominant in countries like Japan, Korea, and Russia.

Figure 1 also shows the total level of agricultural support. Agricultural support includes transfers from consumers and taxpayers to agricultural producers arising from policy measures that support agriculture – producer support estimate (PSE). PSE is here measured as a percentage of gross farm income including support. The figure illustrates that countries like Iceland, Japan, Korea, Norway, and Switzerland have a high agricultural support level. On the other hand, countries like New Zealand, Chile, Brazil, South Africa, and Ukraine have an almost liberalized agriculture.

During the recent decades, agricultural support has changed significantly. The level of support has decreased – protectionism has weakened and liberalization has strengthened. At the same time, the composition of agricultural support has changed significantly. Consumer-financed market price support has decreased, and taxpayer-financed direct support has increased (**Figure 2**).

Structure and Function

As shown in **Figure 1**, countries like Korea and Japan use mainly the high price system in agricultural policy. In this system, support to farmers is given through high market prices maintained by different instruments like import tariffs (variable or fixed) or other import restrictions, export subsidies, and so on. These instruments ensure an artificially high price level compared to the price level that would result from the interaction of supply and demand in an undistorted market.

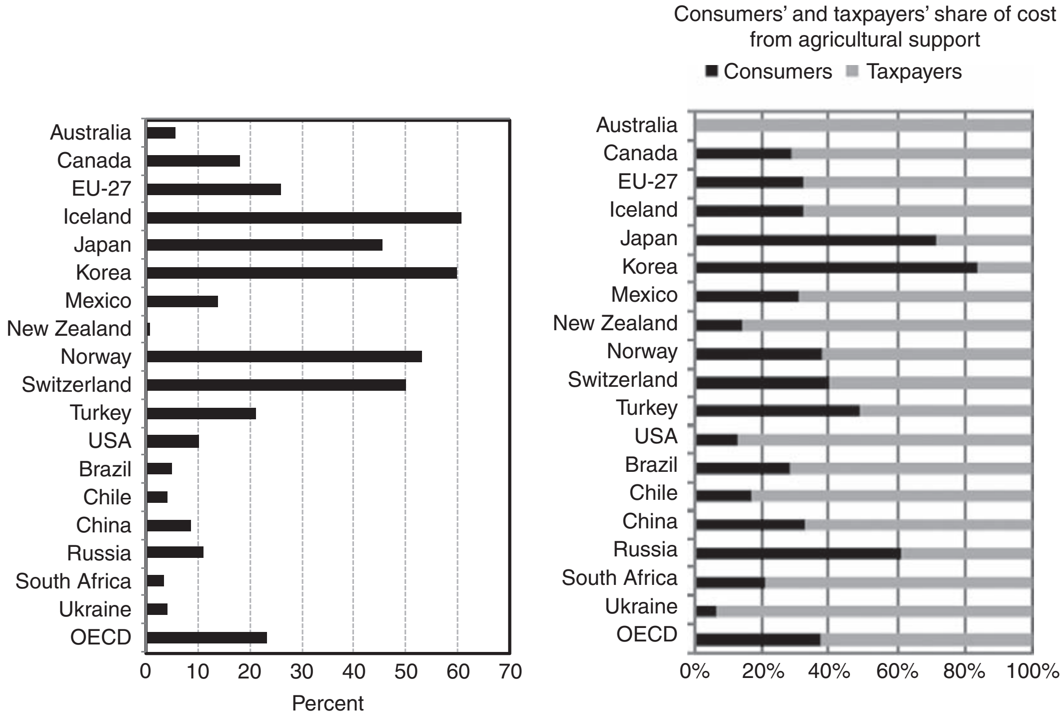

Figure 1 Level and composition of agricultural support (2007). From OECD (2009) producer and consumer support estimates, OECD database 1986–2008.

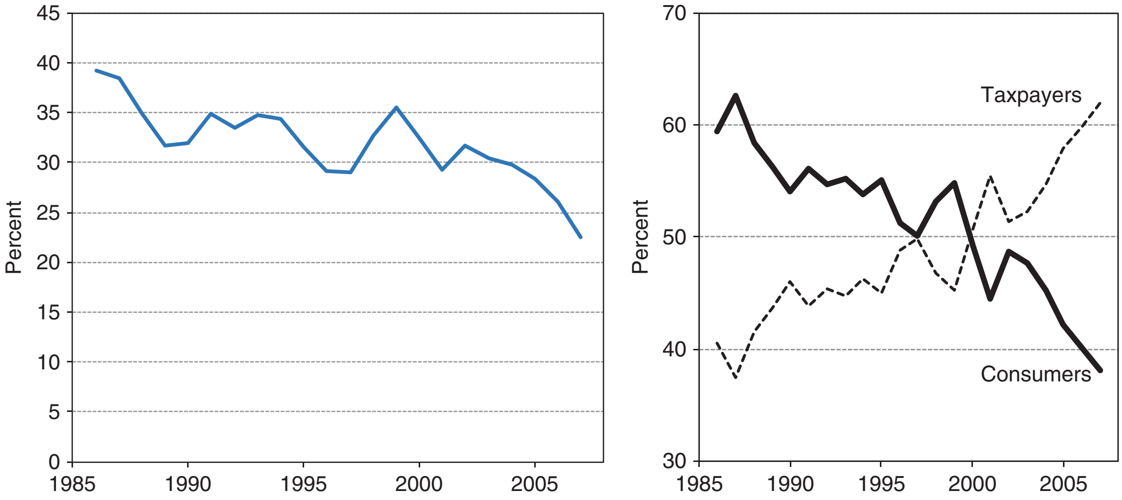

Figure 2 Level and composition of agricultural support in OECD (1986–2007). From OECD (2009) producer and consumer support estimates, OECD database 1986–2008.

Support in high price systems is financed by consumers through high consumer prices. Depending on the self-sufficiency rate, public costs and income are also affected. If the country is a net importer, the country will receive a revenue from the import tariff. On the contrary, a net exporting country will have to pay export subsidies to ensure the price level on the domestic market.

The low price system has for decades been the predominant support system in the agricultural policy in the United States. As a result of the recent reforms of the Common Agricultural Policy (CAP) in the European

Union and as a result of more focus on decoupled support in the World Trade Organization (WTO) negotiations, the European Union has moved toward more low price support and less high price support. Low price support system is now the most important support system in the European Union.

In low price systems, market prices are more or less unaffected, and farmers receive prices which in principle correspond to world market prices. Instead, market support payments are given directly to the farmers.

These payments can be coupled with production or they can be fully decoupled. Coupled support means that a farmer will receive a payment corresponding to the production multiplied by the difference between the world market price and a given target price on the domestic market. In this case, there is no major difference between a high and a low price system from a farmer's point of view.

If support is more or less decoupled from production, the economic transfer to farmers may have an element of income or social aid. Support can be coupled with the agricultural area or the number of animals belonging to the farm. In this case, support is still decoupled from production.

Low price systems are financed by the public budget, indicating that the taxpayers finance this kind of agricultural support in the end.

High and low price systems may have different modifications, individual structures, and so on. The income transfer can have various nuances giving different consequences in each case. However, the general structure of high and low price systems is shown in **Figure 3**.

It does not make sense – *a priori* – to determine whether one system is superior to the other. Support level is independent of the support system, and both systems have advantages and disadvantages. Therefore, it is necessary to compare these *pros* and *cons* with the objectives in the agricultural policy. It is evident that the choice of high and low price systems may have profound consequences within and outside the agricultural sector.

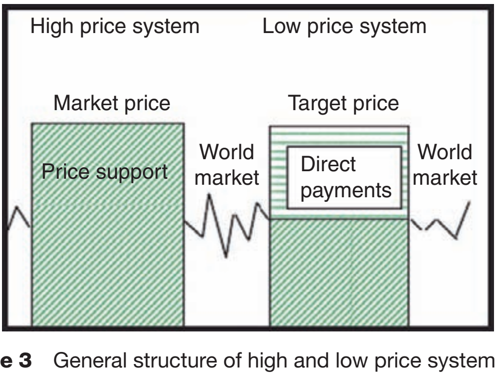

Figure 3 General structure of high and low price systems.

Consequences of High and Low Price Systems

Conditions and Competition in the (Processing) Food Industry

High price systems necessitate border protection of commodities traded internationally. This means that the border protection must comprise processed commodities and not the basic agricultural raw materials. This is the case for milk where border protection must cover the processed and traded goods like butter, cheese, and condensed milk.

In this way, the high price system will influence a major part of the food industry and not only the primary agricultural sector. This must be seen in relation to the fact that it is normally only the conditions in the primary agricultural sector that should be improved through the agricultural policy.

Especially if the food industry is very concentrated and having great market power, the farmers may not achieve the intended advantages of the high price system.

In other fields, a high price policy can be negative for the food industry. At first, the raw materials of the food industry will become more expensive, and unless this cost increase will be fully compensated through other systems, it will lead to worsening of the competitive power.

Such distortions of competition conditions in the food industry will not occur in a low price system. Here, a free world market price exists and is created by supply and demand without market intervention, and the food industry will automatically adjust correspondingly to the international comparative advantage of the sector.

Competition Conditions in the Agricultural Sector

Another problem with the high price system is that it is often difficult to grant the same subsidies to all products. It is most difficult to implement a uniform subsidy if it is a question about high and low processed products, and also, for some products there is only import protection and for others there may also be supply restrictions.

Furthermore, a general price increase for all agricultural products of, for instance, 10% will primarily benefit the crop production whereas gains for animal production will be lower. The explanation is that a major part of the production factors in animal farming consist of crop production, and in this way a general price increase will not have total trenchancy in these production areas.

For all industrial countries, there is a clear negative correlation between the grade of self-sufficiency and agricultural subsidy. This means, the higher the grade of self-sufficiency, the lower the agricultural subsidy.

It is characteristic that some countries often reduce the subsidy level for the products where the degree of self-sufficiency increases considerably to over 100%. With an increasing net export, the nationally financed agricultural subsidy increases, and therefore there will be a distinct incentive to reduce the subsidy. Apart from this, the self-sufficiency objective determines that one in particular protects the products where the grade of self-sufficiency is low.

In both cases, there are signs that one in particular protects the products that the agricultural trade already has poor possibilities to produce. On the other hand, the subsidy for the products that have the best natural conditions will be relatively low. It is assumed that a relatively high degree of self-sufficiency all in all is a sign of a comparative advantage.

Seen in a global perspective, the subsidy is highest in countries where the degree of self-sufficiency is low. Seen in a national perspective, it applies correspondingly that the subsidy is relatively the highest on products where the grade of self-sufficiency is relatively low. Both factors are part of a blurring of the comparative advantages and the result is welfare economic losses.

In the low price system, it can also be difficult to grant the same subsidy to all products; however, it is less complicated than in the high price system. One explanation is that the subsidy is granted directly and past the processing sector and in this way the real agricultural subsidy in the individual production areas is easier to calculate.

Apart from that, it is not seen in a low price system that price subsidy for one product has the effect as extra costs in another product area.

On the other hand, in a low price system, it can be very difficult to distribute a 'fair' subsidy independent of production among the farmers. Historic, structural, or social criteria are often necessary; however, they are rarely logical and they can be very static and not least they can be very difficult to control.

The Composition of Consumption

The composition of consumption is also affected by the choice between the high and low price systems. In the high price system, the consumers will, through higher food prices, primarily finance the agricultural policy. This means that food prices will increase compared to other products, and in this way the consumption of food will decrease compared to the consumption of other products. The result will be that the consumers' purchasing power will decrease.

At first, the consumers' loss as a result of the high price policy can seem great, approximately 25% of the agricultural production value is subsidy, and for the European Union 33% of the subsidy is consumer financed.

It should also be considered that the above-mentioned costs are calculated with the actual world market prices as reference basis. After both a one-sided and general liberalization, these prices will increase and thereby the consumers' gains will be smaller than the actual costs calculations show.

Even though the prices of agricultural products in a high price system are forced high, it will have far from full trenchancy on the food prices. This is due to the fact that only approximately 25–30% of the consumer price on food in highly developed countries traces back to the agricultural trade. The rest of the costs are wages in the processing industry, transport costs, and so on, and these costs are really independent of the subsidy level in the primary production.

Income Distribution in the Society

The choice between the high and low price systems will also influence the income distribution in the society.

A high price system, which will cause an increase in the food prices, will after all be the largest burden to the lowest income groups in the society. People with low incomes use a relatively large part of their earnings on food, which means that an increase on these products will limit their consumption possibilities relatively much. Higher prices on food and other necessary products as a result of political or economical measures will in this way have the same effect as a degressive tax.

On the other hand, the low price system builds on low prices to producers as well as consumers and that is why this form of protection will be the cheapest solution for the part of the population that have the lowest incomes. The financing of public expenses for income support, supplementary payments, and other supports is normally done by means of income tax, which in most cases is progressive. Contrary to the high price system, the costs of the agricultural policy in this case will be placed on citizens with higher incomes.

The State Expenses

High and low price systems have a significant impact on public costs and expenses. Market intervention often implies economic support, taxes, levies, and revenues, which means that public expenses will be affected.

For a net import country, the revenue of the state will at first increase by imposing a high price system based upon import tax. The state receives customs receipts, and at the domestic market the consumers finance the price subsidy to the agricultural sector.

On the other hand, there can be large costs for the state finances with the low price system where direct support to the farmers is a major instrument.

Finally, any intervention and protection measure will have a negative impact on resource allocation and economic welfare in society. Different measures have different consequences, but in general, coupled price support tends to be the most distorting measure imposing the highest loss of economic welfare in society.

The change in agricultural policy during the last decades in OECD countries – decreasing support and increasing role of taxpayer cost – has reduced the total cost, but the taxpayer cost has increased in relative and nominal terms.

Direct or Indirect Subsidy

The choice between the high and low price systems can also be of great importance as to how direct the subsidy systems are. In a high price system, the agricultural subsidy is given 'through the market', and therefore the subsidy is more indirect and invisible. In a low price system, where by means of tax collection the money is directly transferred to the agricultural sector, the transfer is much more obvious.

The low price system contains in this way a very direct subsidy to the farmer; however, the effect on international trade is more indirect and invisible. However, in most cases, the effect is the same for agricultural trade, and therefore it is only a pedagogical and comprehension problem. Still, it is certain that a low price subsidy is so visible that there will be a natural pressure from the surroundings (the taxpayers) to reduce the subsidy.

Production and Productivity Development

The choice between the high and low price systems can also be of great importance to agricultural production and productivity.

The high price system gives, at first, the farmers better sales prices, thus better terms of trade, and it will undoubtedly stimulate production. The size of the productivity increase will depend on the size of the supply elasticity. In general, the agricultural production responds relatively weakly to price changes. In the long term – and especially in case of price increases – it is characteristic that the agricultural production to a great extent adjusts itself to the changed price relations.

Normally, productivity will be improved through structural and political instruments, where through research, development, education, advising, and other means one can make the production more rational. However, the high price policy will also be able to affect productivity. On the one hand, there will be an incentive to increase production in relation to, for instance, the acreage effort. In this way, there will be an increased yield, and also the yield of the livestock production will increase. This will increase productivity.

On the other hand, the agricultural policy will also attract input factors which under normal conditions would be used in other sectors or which would not be used at all. For instance, poor soil will be cultivated and this will reduce the average yield. This will also be the case for other input factors, for instance, fertilizers, pesticides, capital, and labor.

The low price system has in principle the same consequences for production and productivity development provided that it concerns fully production-coupled subsidies. If, on the other hand, the payments to the farmers are partly or fully decoupled from the size of production, the consequences are crucially different. A totally independent income subsidy means that the farmers receive a relatively low price for their products, and that they have no incentives to increase production. It is only economically optimal to increase production as long as the marginal earnings are larger than the marginal costs, and this point is reached relatively fast with the low market prices.

At the same time, the income subsidy is assigned to the farmer regardless of the size of production, which means that production does not increase considerably.

However, it must be expected that even a decoupled production income subsidy in a low price system can seem encouraging to production. All agro-political measures will affect the resource allocation in the society, and in this way an income subsidy will maintain resources in the agricultural sector. In this way also the production will be affected to a larger or smaller extent.

Decoupled income subsidy will very much limit production development in the agricultural sector. Farmers will not be sufficiently urged (or forced) to introduce new technology or new production methods. At the same time, the more efficient farmers do not benefit sufficiently from the extra effort or risk which they undertake. The high and low price systems can, in this way, have different consequences for production and productivity development in the agricultural sector. One cannot in advance say that one consequence is better than the other.

Market Price Subsidy

The market price subsidy – where the market price is kept higher than on the world market – is still a common subsidy measure in the agricultural policies of the Western world (**Figure 1**). Among others, the European Union has through decades used the market price subsidy as an important instrument in the agricultural policy.

The use of the market price subsidy in a high price system demands, naturally, a considerable regulation of the markets. To secure the high price level, the markets are more or less isolated from the surrounding world, as

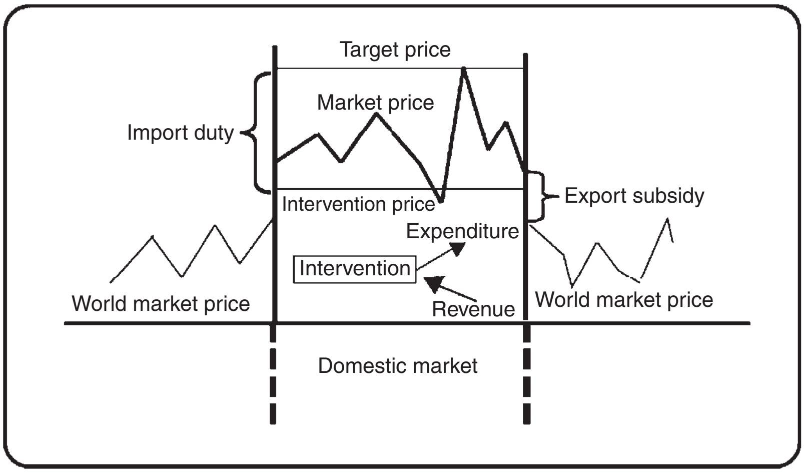

Figure 4 Instruments in a market price system (high price system).

free import or export will make the system collapse. Furthermore, there can be a need for public buying (intervention) or export support, dependent on the degree of self-sufficiency.

There are different types of market price subsidies but the most important one is a price system, where the state in different ways is adjusting the market with the purpose to ensure that the farmers on the market itself are able to obtain the aimed prices. This type of market systems can be schematically illustrated as shown in **Figure 4**.

The target price is the price aimed at for the producers to obtain on the market. The intervention price forms a safety net for the price formation on the market as the product can be sold within the European Union at this price. The actual market price will often be between the target price and the intervention price. If the market price levels drop below the intervention price, some suppliers begin to sell to intervention. This will reduce the supply on the market as the bought-up products will be stocked. This will normally lead to recovery of the market price. The intervention price and the intervention system are, in other words, a central part of the internal regulation of a high price market. However, intervention alone is not enough to secure the price, as there must also be a regulation by import and export.

When importing, an import duty is collected, which, in principle, is the difference between the price on the world market and the threshold price. In principle, it can be both a variable and a firm import duty. If the import duty varies, it can continuously be changed according to the world market price, and it therefore increases when the world market price is low and vice versa. In this way, the variable import duty can be a part of securing a constant price level on the internal market.

Previously, the variable import duty was often used, but as a result of the WTO agreements a gradual change in the tariffs has taken place. This means that the import barriers have changed to more firm tariff rates. When exporting, an export restitution (subsidy) is paid, which in principle is the difference between the price on the world market and domestic market price.

In the European Union, the market price subsidy works in such a way that the EU farmers have secure higher prices than on the world market. This is still the case for some products where reforms have not yet changed the original support system. This is naturally especially true for products where the market price subsidy is the most important measure and where the subsidy level is high. This applies for, among others, milk, whereas the market price subsidy on the cereal area has decreased considerably as a result of reforms in the EU agricultural policy (**Figure 5**).

Future Developments

Several conditions will influence the future development with regard to the agro-political instruments. The choice between the high and low price systems must not be seen from an economic and social point of view alone. The international negotiations in WTO are also of great importance. The explanation is that the high and low price policy influences the international trade in different ways.

First, it is important that the consequences for the size of production are different. All influences on the size of the production will directly influence the foreign trade, as for instance an increase in the production will make the import decrease or the export increase. In this way, these trade-influencing instruments are made objects of negotiations in, for instance, WTO.

As mentioned before, all agro-political measures will always influence the resource allocation and production,

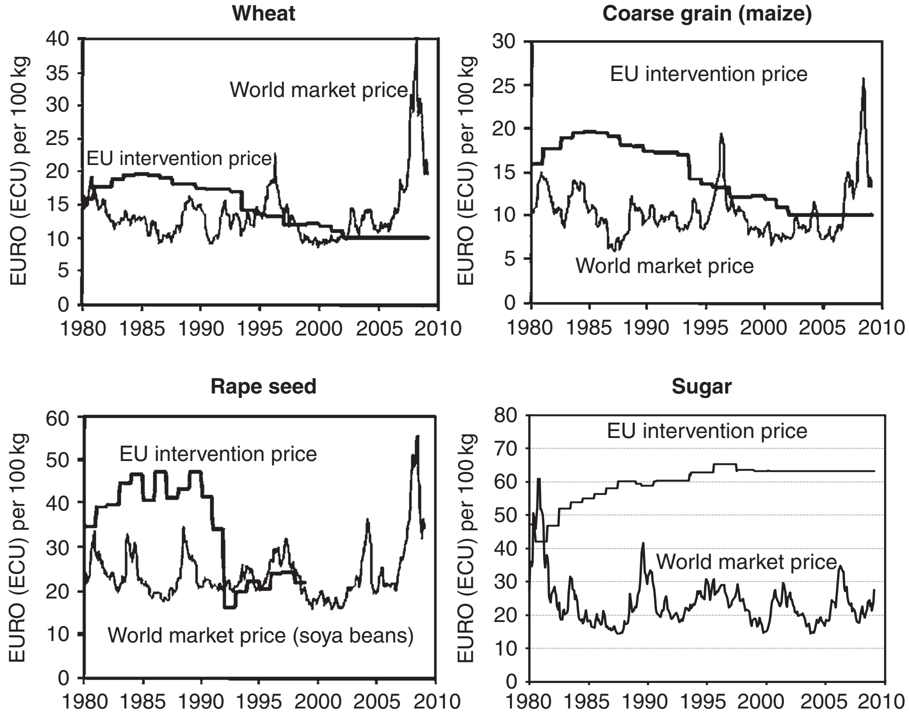

Figure 5 EU market prices and world market prices for agricultural products. In general, the intervention price system in the European Union has stopped or is diminishing.

and with this, also the foreign trade to a larger or smaller extent.

Still, in the international trade negotiations, there is talk about 'non-trade distortions', which are instruments with no influence on the trade. It is implied that some agro-political instruments have a less harmful influence on the international trade and that they in this way are more or less legitimate to use.

Second, it is important that in a high price system one is forced to introduce trade barriers, which in a very obvious way illustrate protection. The trade barriers can of course be of the same magnitude in a low price system, but here the trade protection is less transparent. Politically seen, the relationship to the trade partners can therefore favor the low price system.

The use of import duty, import tax, and especially export subsidy is normally necessary in a high price system, but they very clearly state that one wishes to protect the domestic producers against the surrounding world. This is probably also one of the explanations why the EU agricultural policy was so heavily attacked during the WTO negotiation rounds.

It is certain that the WTO rounds were a defeat for the high price system and a victory for the low price system. This fact must be seen in spite of the fact that the low price system does not necessarily create more free trade or greater economical welfare than the high price system.

On the other hand, the results of the WTO rounds, until now, mean that more countries in the future will be prompted to operate an agricultural policy based upon the low price system.

Also, more independent experts argue for a gradual change from the high price system to the low price system. The arguments are, for instance, that the subsidy rates will be more transparent and sometimes more trading neutral as well. Also, the instruments and the subsidy level in a low price system can easily be gradually removed and even completely replaced by pure social support arrangements.

See also: **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: European Union's Common Agricultural Policy; Agricultural Policy Schemes: Other Systems; Agricultural Policy Schemes: United States' Agricultural System.

Further Reading

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Agricultural Policy Schemes: European Union's Common Agricultural Policy

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Background

The Common Agricultural Policy (CAP) was established on the basis of the Treaty of Rome, with effect from 1 January 1958. Article 39 stipulates five fundamental objectives:

1. to increase agricultural productivity by stimulating technical progress and ensuring the rational development of agricultural production and the optimum utilization of factors of production, in particular labor;
2. to ensure a fair standard of living for the farming population, in particular by increasing the earnings of the persons engaged in agriculture;
3. to stabilize markets;
4. to assure the availability of food supplies; and
5. to ensure that supplies reach consumers at reasonable prices.

In the following years, the CAP gradually firmed up. It was based initially on the idea of a dual agricultural policy, consisting of structural measures on the one hand and price and market-related measures on the other hand. Eventually, the price and market policy became the overall dominating element of the CAP.

The price and market system comprises all the major agricultural products, including milk. In the original form, the policy was based on the following principles:

- free movement of goods within the European Union and common prices for the same good;
- common preferences in relation to third countries (common import duty system); and
- common financial responsibility for market and price policies of the European Community Fund via the European Agricultural Guidance and Guarantee Fund (EAGGF).

These principles were adopted at the Stresa Conference in 1958 and meant that the politically fixed prices became the central element of the CAP and the annual price negotiations of the EU Council of Ministers, which took place in April, started attracting great interest.

Up to the implementation of the General Agreement on Tariffs and Trade (GATT) in 1995, three prices of the principal products were fixed at the price negotiations: target prices, intervention prices, and threshold prices. The target price was the price aimed at in the market, but with no guarantee for the producers. The intervention prices for butter and skim milk powder, however, formed the safety net of the price formation in the market, as at worst the products – with various modifications – could be sold to the EU Commission at this price. As dairy produce consists mainly of fat and protein, the safety net really covers all products. Originally, the threshold price was the lowest acceptable import price for third-country products. The threshold price was used to calculate the variable import taxes, which, in principle, formed the difference between the world market price and the threshold price. However, the GATT agreement signed in 1994 meant that import taxes were frozen on 30 June 1995, which is why threshold prices are no longer fixed.

The fourth fundamental principle of the EU price and market system is export refunds. Refunds are paid on exports and in principle they form the difference between world market prices and the EU market price. The size of the fixed refunds is the same for all EU Member States, but may be differentiated by destination, if special conditions apply.

In connection with the reform of the EU CAP in 1992 (the MacSharry reform), far-reaching changes in price and market policies were introduced, particularly regarding cereals and beef. However, in the milk sector, the old system still applied (**Figure 1**) as the proposed reform was unacceptable to the EU Council of Ministers. Thus, there were no major changes in the milk regime until the implementation of the CAP Reform in 2003, as discussed later.

Financing of the CAP

The CAP system was financed by the EAGGF, which is divided into a guarantee section, financing the price and market policies, and a development section, financing the structural policies. From the start of the European Union, the CAP consumed by far the largest

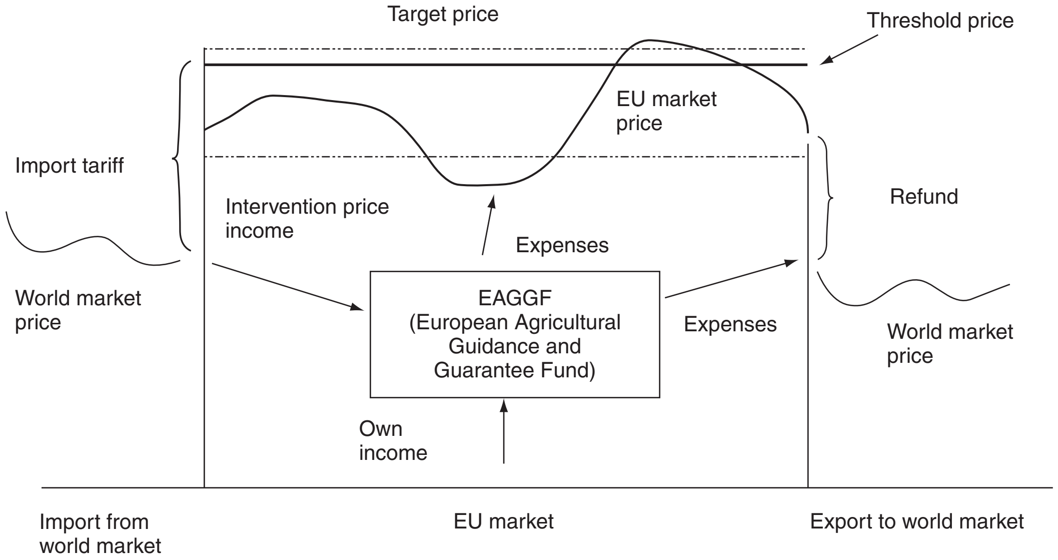

Figure 1 The EU market scheme.

share of the total EU budget. Relatively speaking, the expenses for the agricultural policies have been declining gradually since the 1970s, and now total about 40% of the total EU budget. This decline must be seen in the context of other EU policies and a growing request to stabilize farm expenses. Concurrent with restrictions on farm expenditure, larger funds have gradually been transferred to finance the development of other structural funds, with particular emphasis in recent years on energy, the environment, and new Member States. The EU revenues are based on

- contributions based on gross national income,
- contributions from all Member States on a value-added tax (VAT) basis,
- customs receipts, and
- various production levies.

The Price and Intervention Scheme for Milk and Dairy Products

The EU basic regulation on milk and dairy products was finally adopted in 1968 (EEC 804/68). The institutional prices for milk and dairy products were fixed for a whole dairy year, running from 1 July to 30 June. The target price was fixed for milk, with 3.7% fat carriage paid at any processing factory. The intervention prices were fixed for skim milk powder and butter and formed a safety net under the milk prices. In this way, the main ingredients, protein and fat, were safeguarded and stored in a form that could be controlled by intervention buying. While intervention prices continue to be fixed as in the past, the levels are now considerably lower following CAP Reform as discussed later. Up to 1987, the Intervention Boards of the individual Member States were obliged to purchase any product for sale at the fixed intervention price. Subsequently, various modifications have been made.

The System prior to CAP Reform 2003

Skim milk powder and skim milk (EC 1255/99 art. 7, 11, and 12)

During the winter season, from 1 September to 28 February, intervention with regard to skim milk powder is suspended. From 1 March to 31 August, intervention may be suspended; however, private storage of skim milk powder may be subsidized. Only first-class produce meeting the set requirements on age and packaging may be the subject of intervention. As of the market year 1995–96, a minimum protein content of 30% in skim milk powder for intervention was introduced. At a protein content of 34% and above, maximum subsidy is paid, whereas contents between 30 and 34% have 1.75% deducted from the intervention price for each percentage point below 34%. Products subject to intervention, which cannot be sold on normal market terms, may be subject to special stock disposal measures and sold at reduced prices. As for skim milk powder, its use in mixed feedstuffs for calves is subsidized (the most important scheme) as well as its use in mixed feedstuffs for pigs and poultry. Skim milk for processing into casein and caseinates is also subsidized. These products are used as the primary material for processing of various industrial products and foodstuffs, such as processed cheese. Subsidies for casein and caseinates are a production subsidy, as distinct from the price subsidy schemes.

The intervention system for butter (EC 1255 art. 6 and 13)

From 1987, the intervention system for butter has been a tender procedure. Tenders are submitted every 2 weeks, as the EU Commission fixes a maximum buying-in price. All bids below this price are purchased. Since 1987, the buying-in price has been steady at 90% of the formally fixed intervention price. Like skim milk powder, butter for intervention must meet certain requirements on quality, age, and packaging. When the market situation allows, subsidized butter pursuant to regulations is remarketed on terms that do not damage the competitive position of butter in the market. Butter subject to intervention is remarketed within the European Union under the special scheme for sale of butter at reduced prices, for use in the food industry and for the manufacture of pastry products, ice cream, and other foodstuffs. Analogous to the sale of subsidized butter for food manufacturers, similar subsidies are paid for the use of fresh butter and cream in the food industry. Butter for social institutions and hospitals is also subsidized, as well as for the armed forces. To safeguard the normal market supply and price of butter during winter months, private storage of butter and cream is financially supported. The storage period, fixed by the EU Commission, usually starts on 1 April and ends on 15 August. The stock disposal period is from 16 August to 28 February the following year. The storage period must be a minimum of 4 months.

The intervention system for cheese (EC 1255/99 art. 8)

In addition to the general intervention schemes for butter and skim milk powder, private storage of the cheese types Grana Padano, Parmigiano Reggiano, and Provolone may be subsidized in Italy. The special scheme was established as the production of these particular cheese types is a staple element of the Italian dairy industry.

Subsidies for sale of liquid milk (EC 1255 art. 14)

To stimulate liquid milk consumption, the European Union contributes to the implementation of the Member States' special aid schemes to supply milk and selected dairy products for schoolchildren at reduced prices.

The Import System for Milk and Dairy Products

Before the introduction of the GATT/World Trade Organization (WTO) agreement on 1 July 1995, third-country imports were subject to variable import levies. Now these levies are tariffed, that is, converted into a fixed tariff rate, payable in Euro per tonne or as a percentage of the import price. Pursuant to the agreement, the rates have been reduced by an average of 36% compared to the basic period from 1986 to 1988. Moreover, the GATT/WTO agreement imposes minimum import access quotas at reduced tariff rates, equal to 5% of consumption in the basic period. In addition, the European Union is obliged to give access to butter from New Zealand at a special low rate. This amount represents the average amount exported annually to the United Kingdom by New Zealand under bilateral agreements during the GATT/WTO basic period. Further to GATT/WTO obligations, the European Union has entered a number of bilateral agreements aimed at facilitating market access on a mutual basis. For instance, there are special quotas for the United States, Canada, Norway, Switzerland, South Africa, and others.

Export Schemes for Milk and Dairy Products

As a matter of principle, the European Union subsidizes most dairy products for export to balance the price gap between the European Union and the world market, except when this price gap disappears as in the 2007/2008 period. Non-Annex I products are subsidized as well; these are processed products containing agricultural produce, such as cereals, sugar, eggs, and milk. After the implementation of the GATT/WTO agreement, the refund system was somewhat restricted. Compared to the basic period of 1986–90, subsidized exports were reduced by 21%, in parallel with a 36% reduction of the refund budgets. The budget restrictions apply only to non-Annex I products. In order to ensure that the restrictions are met, all exports qualifying for refunds are subject to presentation of an export license, prefixing the refund. Export licenses are limited to the permitted quantity, which implies that it is a scarce commodity in times of great demand. The limited opportunity to use refunds means that export refunds for cheese no longer exist for a number of destinations. This generally applies to areas such as the United States, Canada, Australia, Switzerland, and Norway. In other areas, refunds for only selected products have been abolished.

The Milk Quota Scheme

As a result of the increasing imbalance between production and demand, the milk quota scheme was introduced in 1984. The purpose of Article 39 of the Treaty of Rome had long been accomplished and the choice was between a reduction of prices and limiting production. Production was chosen and the measures proved effective in limiting surplus production. Each Member State was allocated a national quota (reference quantity) for the quota year 1984–85, which as a rule equaled the total national milk production in 1981 plus 1%. Ireland, Italy, and Northern

Ireland got a somewhat larger quantity. The Member States were allowed certain latitude to implement the quota scheme in one of two ways, either as direct sales quotas or as dairy quotas. Under the direct sales quota scheme, the national reference quantity was reallocated to individual milk producers. Under the dairy quota scheme, the quota was reallocated to the dairies, which subsequently had to fix quotas for individual producers. In the event of milk production in excess of the quota, a superlevy was collected, totaling 115% of the target price. Regardless of the choice of management scheme, the producers who exceed their quota must pay the superlevy. The dairy quota scheme provides the option to use a net principle, allowing the underuse of quota by some producers to be converted into a deduction for producers who have exceeded their quota. In this way, the quota is fully utilized and the payment of a superlevy reduced.

Agenda 2000

Following nearly 2 years of discussion, the EU Heads of State finally made the decision to reform the EU CAP, entitled Agenda 2000, at the summit meeting in Berlin in March 1999. Agenda 2000 also embraced the budgetary framework of the European Union for the period 2000–06 and the plans for enlargement by the inclusion of central and east European countries as well as a reform of the structural policy.

The fundamental element of the agricultural reform was a reduction of refunds for the most essential agricultural products, as opposed to extended financial aid to producers by premium schemes, only partly related to production. For agricultural produce and beef, the 1992 reform was further expanded, whereas in the case of the milk and dairy sector it is a profound breach of previous policies.

The purpose of the reform was to

- improve the competitiveness of EU agriculture on both domestic and external markets,
- facilitate the progressive integration of new Member States,
- prepare the European Union for the next WTO round,
- ensure continuously stable farm incomes, and
- integrate environmental goals into the CAP.

The original intention was to implement the reform of the dairy sector in the period 2000–03. However, the final agreement between the Heads of State in Berlin postponed the implementation to 2005–08. The principal elements of the reform that was agreed were as follows:

1. A total 15% reduction in intervention prices for butter and skim milk powder, in three stages from 2005–06 to 2007–08.
2. To compensate for the price cut, milk producers were to be allocated a direct payment per tonne milk quota, fixed at €5.75 in 2005, €11.49 in 2006, and €17.24 in 2007.
3. In addition, each Member State would receive financial support by the so-called ‘national envelopes’, which may be allocated according to nationally determined criteria.
4. The total quantity eligible for direct payments in each Member State would be equal to the sum of all individual reference quantities for the 12-month period 1999–2000.
5. A total increase of milk quotas of 2.8 Million tonne (2.4%): in the years 2000–01 to 2001–02, the national quotas were increased for Spain (10%), Italy (6%), Northern Ireland and Ireland (3%) as well as Greece (11%). The increase for the remaining countries was to be 1.5% in the years 2005–06 to 2007–08.
6. The milk quota scheme would continue up to 2008. In 2003, a ‘mid-term review’ would be initiated.

CAP Reform (Mid-Term Review) 2003

In 2002, the Mid-Term Review of Agenda 2000 commenced. It concluded in June 2003 with a fundamental reform which provided for the decoupling of direct payments from production in the case of livestock production, milk production, and arable crops, with partial decoupling options for Member States that did not wish to decouple fully. Direct payments (coupled or decoupled) were made conditional on compliance by farmers with a range of food safety, environmental, and animal welfare measures.

With regard to the dairy sector specifically, the most important elements were as follows:

1. An asymmetric reduction in intervention price: 25% for butter (from €328.20 to 246.39 per 100 kg) and 15% (from €205.52 to 174.69 per 100 kg) for skim milk powder. The reduction was brought forward to 2004–05, with the butter price reduction spread over 4 years (7, 7, 7, and 4%) and the skim milk powder price reduction in three equal annual steps.
2. Partial compensation for the intervention price cut for dairy farmers: a direct payment of €24.49 per 100 kg of quota and a supplementary payment per Member State equivalent to approximately €11 per 100 kg. Such compensation is paid for the total of national quota as at 1999/2000. Originally, the coupled payments had been programmed in Agenda 2000 at a lower level. The payments were to be decoupled at the latest in 2007.
3. Discouragement of butter intervention: by introducing the possibility to open a tender for intervention buying-in after 30 000 tonnes at fixed prices have been bought in.
4. Expiration of production quotas on 1 April 2015.
5. Postponement by 1 year of the gradual quota increase of 1.5% in three steps of 0.5% for 11 Member States, as

already foreseen in Agenda 2000. The increase corresponds to 1.4 million tonnes of milk.
6. Reduction of the superlevy: in four steps from €35.63 per 100 kg in 2003/2004 to €27.83 per 100 kg from 2007/2008 onward.

Coinciding with the start of the dairy reform in 2004, 10 new Member States joined the European Union. This increased the EU base quota by 18.5 million tonnes and added 80 million consumers. Furthermore, in accordance with the accession agreements, a restructuring reserve of 0.67 million tonnes was established for eight of the new Member States. This additional reserve was added to their national quotas on 1 April 2006. In 2007, a further two new Member States with a total quota of 4 million tonnes joined the European Union, bringing the total amount of quota for the EU-27 to 142 million tonnes. Thus, by 1 April 2008, further to 103 million consumers, 24.5 million tonnes of additional quota will have been added to the EU total since 2003.

The aim of the 2003 dairy reform was to increase competitiveness and market orientation. It was intended that by reducing the guaranteed price for butter and SMP, these products would be less attractive to produce and this would give the industry an incentive to produce more value-added products like cheese and fresh dairy products. Increasing the quota at the same time would encourage additional production, facilitate restructuring of the sector, and encourage entrance into the sector of young farmers.

It will be recalled that the European Commission's proposal for the CAP Reform 2003 was to increase quota by 2% on top of the 1.5% increase already agreed in Agenda 2000. In the June 2003 compromise, however, the Council declared that "No additional quota increase in 2007 and 2008 will be decided now. The Commission will present a market outlook report once the reform is fully implemented on the basis of which a decision will be taken."

CAP Health Check 2008

As part of CAP Reform 2003, a mid-term review of policy was completed in 2008, which became known as the CAP Health Check. Agreement was reached among farm ministers in November 2008. The main points related to dairying were as follows:

1. Five annual milk quota increases of 1% each with effect from April 2009, prior to total abolition of the quota system as from 1 April 2015. As is now traditional, when it comes to milk quota, Italy will receive a special derogation that allows it to increase its quota by the full 5% in the first year.
2. The rate of modulation (shifting funds from direct aids to rural development aids) will be raised from 5% at present to 10% by 2012. The increase will be made gradually: 7% in 2009, 8% in 2010, and 9% in 2011. The progressive modulation concept has been watered down; only recipients of more than €300 000 will face a higher modulation rate: 4 percentage points higher than the standard rate. The resulting money will be allocated for 'new challenges' – climate change, energy, biodiversity, and water management – but it will also have to fund 'accompanying measures' for the dairy sector.

Conclusion

The EU CAP is and will remain a fundamental basis of EU cooperation. Financial problems, disputes about GATT/WTO principles as well as problems regarding the enlargement of the European Union have permanently placed reforms of the agricultural system on the EU political agenda. For the first time, the market scheme for milk and dairy products underwent fundamental change in 2003 and further changes may be anticipated in the decade ahead, particularly when milk quotas are finally abolished in April 2015.

See also: **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: Other Systems; Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy; Agricultural Policy Schemes: United States' Agricultural System; Trade in Milk and Dairy Products, International Standards: World Trade Organization.

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Agricultural Policy Schemes: United States' Agricultural System

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Introduction

An active agricultural commodity policy was developed in the United States in the 1930s in response to economic conditions of the Great Depression. Major commodity programs for grains, oilseeds, cotton, peanuts, sugar, tobacco, and dairy that are in place today have their origins in the programs that began nearly 70 years ago.

Dairy policy in the United States comprises the following major components:

1. Border measures that create import barriers for most dairy products and export subsidies for a few manufactured dairy products.
2. Federal and state marketing orders that regulate milk prices at the processor and farm levels.
3. Government purchases of manufactured dairy products to support the farm price of milk.
4. Income support to dairy farmers through deficiency payments.

Federal, state, and local governments also have longstanding food safety and sanitation regulations for milk and dairy products. In addition, there are myriads of more recent environmental, land use zoning, labor, and other regulations or incentives that influence the dairy industry.

This article provides an overview of the key elements of US dairy policy, and provides some statistics to illustrate the economic effects of these programs.

Border Measures for Dairy Products

Trade barriers for many dairy products have limited US imports of these products to less than 5% of US consumption (**Table 1**). Import barriers have traditionally kept the domestic price of dairy products above the price for traded products in world markets, although the gap has narrowed recently (**Figure 1**). By insulating the domestic dairy economy from foreign supplies of dairy products, the import barriers also make possible the key domestic elements of the dairy program – milk marketing order pricing rules and the price support program (described in the following sections).

As a part of the Uruguay Round trade agreement that took effect on 1 July 1995, a system of absolute import quotas gave way to a system of tariff rate quotas (TRQs) that set a relatively low tariff on imports up to a determined quantity (the quota) and a relatively high tariff on over-quota quantities. Although the quantity of access expanded with the Uruguay Round agreement, the second-tier tariffs applied to over-quota imports remain prohibitively high; therefore, for the present, the effects of the TRQs remain the same as the absolute quotas that were replaced, although at expanded import quantities. Imports of fluid milk, cream, butter, cheese, milk powders, and many other dairy products are subject to TRQs. For those products subject to TRQs, imports accounted for 5% or less of domestic consumption, but for other products, including casein, milk protein concentrate, and some cheeses, imports are not restricted. Not surprisingly, imports of partly or fully unrestricted dairy products represent the bulk of US dairy imports – caseins, milk protein concentrates, and cheeses represented nearly 80% of total import value in 2009. Overall, the United States imports more than $2 billion worth of dairy products each year, and is a substantial importer as well as exporter in the world dairy market.

In addition to limiting import access to the domestic market for most dairy products, the US government continues to provide small amounts of direct financial subsidy for US exporters of dairy products. Subsidized exports, along with donations to domestic food programs and international food aid, have long been used to dispose of stocks of dairy products acquired under the price support program. Subsidized exports have been considered a market for US dairy products that does not disrupt domestic commercial sales. In addition to the disposal of government stocks, the Dairy Export Incentive Program (DEIP) has provided explicit price subsidies for commercial dairy product exports since the 1980s. Commodities eligible for DEIP (and annual Uruguay Round WTO maximum subsidized export volumes) are skim milk powder (68 000 tonnes), butter (21 000 tonnes), and cheese (3000 tonnes). With high world market prices for dairy products in recent years, DEIP subsidies have been infrequent. But very low world prices in 2009 triggered DEIP subsidization of 37 200 tonnes of milk powder, 17 400 tonnes of butter, and 1800 tonnes of cheese.

Table 1 US production, trade, and consumption of select dairy products, 2006–09

	2006	*2007*	*2008*	*2009*
All cheese				
US production (1000 tonnes)	4320	4433	4505	4583
US exports (1000 tonnes)	71	100	131	108
US imports (1000 tonnes)	206	198	170	162
Consumption[a] (1000 tonnes)	4500	4545	4600	4682
Imports/consumption (%)	4.6	4.4	3.7	3.5
Butter				
US production (1000 tonnes)	657	695	746	711
US exports (1000 tonnes)	11	41	91	29
US imports (1000 tonnes)	32	29	16	19
Consumption[a] (1000 tonnes)	651	687	778	697
Imports/consumption (%)	4.9	4.2	2.1	2.7
Skim milk powder/nonfat dry milk				
US production (1000 tonnes)	676	669	845	775
US exports (1000 tonnes)	287	258	391	258
US imports (1000 tonnes)	2	2	1	1
Consumption[a] (1000 tonnes)	397	386	399	518
Imports/consumption (%)	0.5	0.5	0.2	0.2

[a] Due to storage, consumption does not equal production plus imports minus exports. Government intervention purchases are not included in consumption. Data compiled from US Department of Agriculture, National Agricultural Statistics Service and Foreign Agricultural Service.

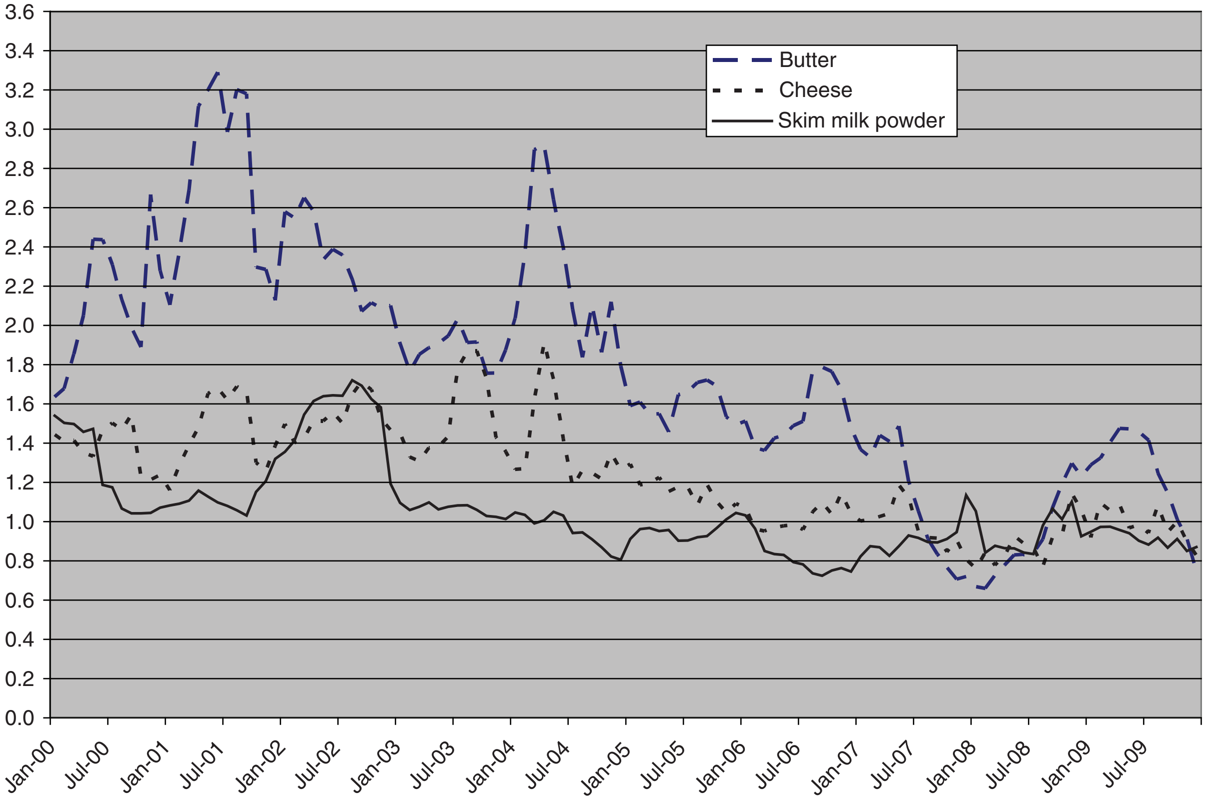

Figure 1 Ratio of US market price to Oceania export price for primary dairy export products, 2000–09. Data from the US Department of Agriculture as reported at http://future.aae.wisc.edu/tab/prices.htm#13.

Regional Milk Marketing Orders

The pricing of nearly all of the milk produced in the United States is regulated by milk marketing orders. In 2010, 10 federal marketing orders regulated the sale of about two-thirds of all milk produced in the country. California, which operates its own marketing order, regulates the sale of another 20% of the country's milk. Some of the remainder is regulated by other state marketing orders (Maine, Montana, Nevada, Virginia) and some (notably Idaho with 6% of US milk production) is not regulated by any marketing order.

State and municipal governments set separate sanitary standards for milk that may be used in fluid products and milk that may be used only in manufactured dairy products. Grade A milk is milk that meets sanitary standards for use in fluid products. Of all milk produced in the United States, 99% is grade A. Grade B milk is eligible for use in only manufactured dairy products and is not regulated by milk marketing orders.

Both federal and California milk marketing orders use price discrimination to raise the average price received by producers, setting minimum prices that processors must pay for grade A milk according to its end use (classified pricing). Federal orders distinguish between four end-use classes: fluid products, fresh and frozen products, hard cheeses, and butter and dry milk powder. Each month, federal orders set the minimum prices for milk used in cheese and milk used in butter and dry milk according to formulae that take into account the wholesale prices of these products. The minimum prices for milk used in fluid products (Class I) and soft and frozen products (Class II) are set as a specified differential over the manufacturing-use minimum prices. The differential for Class II is the same across all federal orders, but Class I differentials vary by order.

Although the details of the Federal Milk Marketing Order (FMMO) pricing rules have changed over time, the key element of price discrimination remains; the minimum price for milk used in fluid products is set at a premium over the minimum price set for milk used in manufactured dairy products. The California state order distinguishes among five end-use classes, and uses similar formulae to set minimum prices for each class.

In addition, each federal marketing order administers a revenue-sharing or 'pooling' scheme that distributes revenues from relatively high-priced Class I milk across all grade A milk. Each month, each federal order pools revenues from all end-use classes and announces a uniform, order-wide average price to individual farmers delivering milk to that order, regardless of how any individual producer's milk was actually used. The weighted average or pool price in any order depends not only on the class prices but also on the utilization rates of milk in the various end-use classes, which also vary from order to order.

California's revenue-sharing scheme differs from that used in the federal system. In California, a quota program determines how milk revenues from the various end-use classes are distributed among producers. The milk quota program in California does not restrict production or marketing. Rather, for each 100 kg of milk quota owned by an individual producer, the producer receives a fixed payment of $3.75 from the statewide pool of total milk revenues in a month. The remainder of total regulated milk revenues (i.e., what is left over after subtracting total quota payments) is distributed uniformly among all producers in the same way as federal orders. Overall, quotas cover about 22% of all the milk produced in the state.

To the extent they raise the average price of milk above what it would be in their absence, both federal and state milk marketing orders encourage milk production. By setting relatively high prices for milk used in fluid products, marketing orders reduce sales of fluid milk. As a result, marketing orders encourage production of manufactured dairy products such as cheese, butter, and milk powder.

Each marketing order regulates milk within a geographically defined marketing area. **Figure 2** is a map of the 10 federal marketing areas. The relationship of prices among federal orders is determined, in part, by the formulae used to set minimum prices in each order. By formula, the minimum prices for milk used in manufactured dairy products are the same across orders. However, the fluid differentials, and thus the minimum price for milk in fluid uses, are different for each order. Differentials range from a high of $13.23 per 100 kg in parts of Florida, to a low of $3.53 per 100 kg in parts of the Upper Midwest. **Table 2** lists the fluid differentials, Class I milk prices, and pool prices that were in effect for the 10 federal orders in 2009.

In order to maintain different minimum prices in each marketing order, regulations are in place to discourage the transport of milk across regions. Milk transported freely across marketing order borders would undermine the maintenance of separate fluid milk markets in different orders. Regulations on inter-order milk shipments ensure that there is little economic advantage to arbitrage across prices in different orders. Because marketing orders create separate fluid milk markets in different regions, the benefits and costs of milk marketing orders vary regionally.

Federal Price Supports for Dairy Industry

As early as 1935, the federal government was purchasing manufactured dairy products in order to support the farm price of milk. The Agricultural Act of 1949 required the

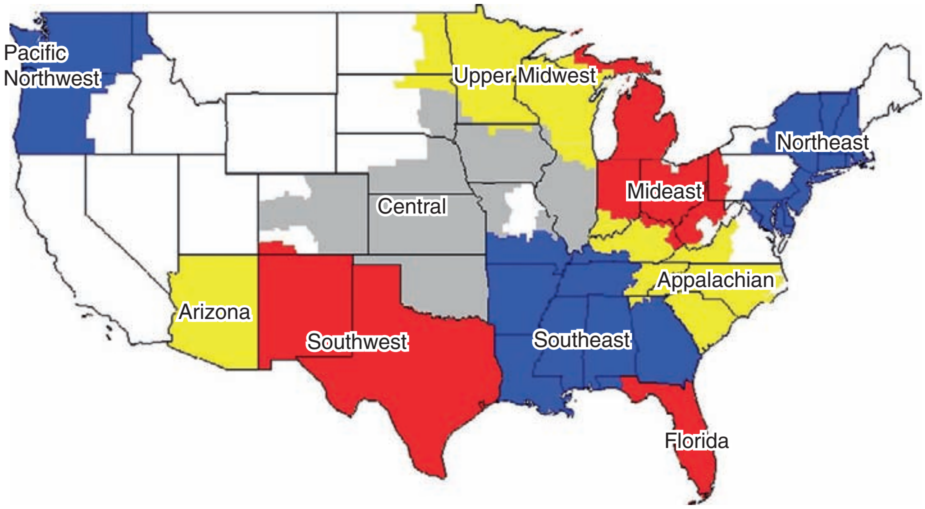

Figure 2 Map of the Federal Milk Marketing Order areas as of 1 January 2010. Differences in shading merely serve to differentiate between marketing areas. Reproduced from US Department of Agriculture, Agricultural Marketing Service, Dairy Programs.

Table 2 Federal Milk Marketing Order annual average prices,[a] 2009

Marketing area[a]	*Class I differential ($ per 100 kg)*	*Class I milk price ($ per 100 kg)*	*Pool price*[b] *($ per 100 kg)*
Northeast (Boston)	7.17	32.48	28.62
Appalachian (Charlotte)	7.50	32.81	30.87
Southeast (Atlanta)	8.38	33.69	31.38
Florida (Tampa)	11.91	37.22	35.61
Mideast (Cleveland)	4.41	29.72	26.66
Upper Midwest (Chicago)	3.97	29.28	25.51
Central (Kansas City)	4.41	29.72	25.75
Southwest (Dallas)	6.62	31.93	28.05
Arizona (Phoenix)	5.18	30.50	26.61
Pacific Northwest (Seattle)	4.19	29.50	25.91
Weighted average	6.34	31.66	27.41

[a] Prices quoted at 'principal pricing points' (in parentheses) within each marketing area.
[b] Pool price is the market-wide weighted average of all minimum end-use class prices. Data reproduced from US Department of Agriculture, Agricultural Marketing Service, Dairy Programs.

US Department of Agriculture (USDA) to continue to support the farm price of milk. Since that time, the USDA has purchased butter, non-fat dry milk, and cheese from processors at administratively determined intervention prices calculated to help ensure that the farm prices of manufacturing milk remain above the legislated support price. In 2008, the dairy price support program was modified to remove the requirement that USDA support a specific milk price, but intervention prices for eligible dairy products remained the same. The name of the support program was changed from the 'milk' price support program to the 'dairy product' price support program.

Table 3 lists the support price for milk and the government purchase prices for eligible dairy products from 2000 through 2009. Note that, on average, market prices exceeded intervention prices, but occasionally fell far enough below during the year to trigger government purchases. Annual purchases of butter, cheese, and non-fat dry milk are shown in **Table 4**.

Since 1990, dairy price supports have played a minor role and government purchases have been relatively small compared to the 1980s. The 1996 FAIR Act lowered dairy price supports by 33 cents per 100 kg to $21.83 per 100 kg through 1999, at which time the program was scheduled to be terminated. However, the price support program was extended and subsequently reinstated in omnibus farm legislation passed in 2002.

While the support price program plays a potentially important role in flooring prices for manufactured dairy products, the current intervention prices provide only the lowest of safety nets. Moreover, added costs of selling to the government (nonstandard packaging, mandatory federal inspection) mean that market prices for cheese often

Table 3 US market prices and US Department of Agriculture price support and purchase prices, 2000–09

	Milk		*Butter*		*Cheese*		*Nonfat dry milk*	
	Support	*Class III price*[a]	*Support*	*Market*[b]	*Support*	*Market*[b]	*Support*	*Market*[c]
Year	*($ per 100 kg)*		*($ per kg)*					
2000	21.83	21.48	1.45	2.76	2.45	2.53	2.23	2.30
2001	21.83	28.89	1.70	3.86	2.49	3.17	2.08	2.27
2002	21.83	22.97	1.96	2.31	2.49	2.61	1.95	2.08
2003	21.83	25.18	2.32	2.58	2.49	2.90	1.76	1.85
2004	21.83	33.94	2.32	4.05	2.49	3.64	1.76	1.96
2005	21.83	30.97	2.32	3.39	2.49	3.29	1.76	2.15
2006	21.83	26.21	2.32	2.73	2.49	2.73	1.76	2.04
2007	21.83	39.78	2.32	3.10	2.49	3.88	1.76	4.03
2008	NA	38.45	2.32	3.38	2.49	4.09	1.76	3.00
2009	NA	25.04	2.32	2.82	2.59	2.86	1.83	2.26

[a] Federal Milk Marketing Order price for milk used to make cheese.
[b] Chicago Mercantile cash market prices. Market and support prices for cheese price are for Cheddar in 40-pound (18.14 kg) blocks.
[c] Wholesale price for western high-heat non-fat dry milk.

Table 4 US government net purchases of dairy products

	Butter	*Cheese*	*Non-fat dry milk*
Year	*(tonnes)*		
2000	4017	12 711	328 994
2001	0	1749	224 878
2002	0	7179	372 683
2003	13 182	18 712	301 185
2004	−2971	2692	47 816
2005	0	−907	−36 281
2006	0	0	−31 293
2007	0	0	−12 245
2008	0	0	52 154
2009	4535	13 605	62 585

Negative values denote net sales from government stocks. Values include DEIP subsidies in the form of product.

fall below the intervention price without triggering government sales. This further diminishes the ability of the program to provide a price floor.

Direct Deficiency Payments – The Milk Income Loss Contract Program

The 2002 US 'Farm Bill' introduced another dairy subsidy scheme in the form of the Milk Income Loss Contract (MILC) program. MILC is a target price-deficiency payment program that makes payments to all dairy farmers (subject to a production cap) in any month when milk prices fall below a target level. The initial base target price was $37.35 per 100 kg in reference to the Class I milk price announced for Boston. If the Boston Class I price in any month fell below $37.35, then all US milk producers were eligible to receive 45% of the difference.

An annual cap on the amount of milk per farm eligible for payment was initially set at 1.1 million kg, which represented the annual production of about 120 cows. The payment rate was reduced from 45 to 34% on 1 October 2005, but reinstated at 45% in the 2008 Farm Bill. That legislation also provided for an upward adjustment in the MILC target price if dairy feed costs exceed a base level and raised the annual farm production cap to 1.35 million kg.

MILC payments since the retroactive inception of the program through 2009 are shown in **Figure 3**. Payments closely follow milk prices. No payments were made during 2007 and 2008. The crash in milk prices in 2009 combined with a higher target price from the feed price adjuster led to record high payments during the first half of the year.

The MILC program has been controversial among producers, mainly because the production cap favors regions with a smaller average herd size. Large western dairies can exceed the production cap in a single month. Their owners argue that they have been forced to bear the brunt of adjusting milk supply to low prices because MILC payments insulate smaller producers.

Final Remarks

In the United States, the federal government and several state governments directly and indirectly subsidize milk producers and regulate dairy prices. These programs stimulate additional milk output, raise the price of beverage milk, and shift income from taxpayers and consumers to the dairy industry. Economic research has documented that costs to taxpayers and consumers are significantly larger than gains to producers as a group, but of course, any individual producer gains much more than the system costs a typical dairy consumer or taxpayer.

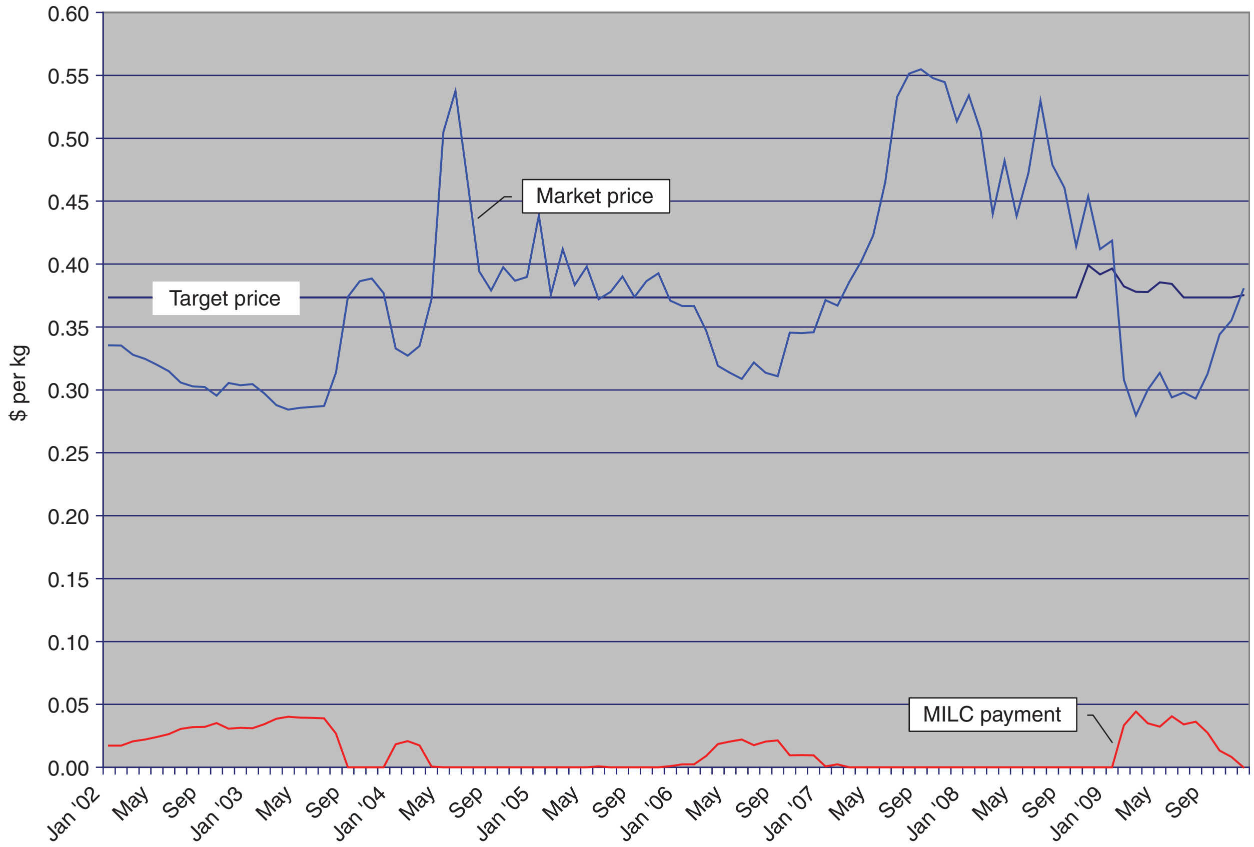

Figure 3 Milk Income Loss Contract (MILC) payments. Reproduced from US Department of Agriculture as reported at http://future.aae.wisc.edu/milc.html.

See also: **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: European Union's Common Agricultural Policy; Agricultural Policy Schemes: Other Systems; Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy.

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Agricultural Policy Schemes: Other Systems

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Introduction

Milk producers in many countries benefit from government interventions that increase the prices they receive for their raw milk production. In some countries, a system of milk production quota was established to control the growth of surplus production while maintaining the market price support. Below are reviewed the current dairy policies in Canada, Japan, Australia, and New Zealand. The review illustrates the very different policy approaches and the degree of dairy policy interventions.

The Organisation for Economic Co-operation and Development (OECD) collects and analyzes information regarding the level of support provided to producers through agricultural policies and calculates some measures of the monetary transfers caused by such policies – the producer support estimate (PSE). Since 2005, the total PSE is no longer broken down for individual commodities, which reflects the gradual shift (in many countries) away from direct commodity-linked supports. **Figure 1** shows the change in %PSE for selected countries and the OECD average during the periods 1986–88 and 2002–04. The %PSE expresses the monetary value of the support as a share of gross farm receipts. A notable feature of the %PSE for milk is the reduction in support since the early 1990s, although the reduction varies considerably among countries.

Since 2005, a new commodity-based indicator is calculated by the OECD, the so-called single commodity transfers (SCTs), which shows the annual monetary value of gross transfers from policies linked to the production of a single commodity such that the producer must produce the designated commodity in order to receive the transfer. For the countries reviewed below, the change from the commodity-specific PSE to the SCT indicator does not make much difference for New Zealand and Australia, where milk producers are supported either very little or not at all, while in Canada and Japan, a majority of the support (more then 90%) has been based on market price support (MPS), which continues to be reported in SCTs. The evolution of the SCT for milk production in the reviewed countries is illustrated in **Figure 2** for the period 1986–2008.

Canada

Background

Since the first dominion dairy commissioner was appointed in 1890, the Canadian Federal Government has played an active role in policymaking for the dairy sector. A milk supply management system was introduced in the early 1970s, and it remains the cornerstone of Canada's current dairy policy. Import restrictions at the border and milk production quotas are the main instruments allowing for a high level of support to the dairy sector, which continues to be the most heavily supported sector within Canada's agriculture. In 2004, around 35% of all support to Canadian agriculture (as measured by PSE) went to the dairy sector. **Figure 1** also illustrates that Canada's support to dairy farmers is higher than that in OECD countries on average. When considering the SCT, 33% of the total SCT was attributable to the dairy sector in 2008. **Figure 2** shows the relatively stable levels of SCT support, which declined notably after 2006 as international reference prices soared. (In 2009, the SCT level increased significantly following the dramatic price fall on the international dairy markets which has not been transmitted to the domestic market in Canada. The preliminary figures estimated by the OECD indicate an increase of % SCT to a level of 60%.)

Canada and Its Milk Supply Management System

The milk supply management system, introduced in the early 1970s, is the essence of Canada's dairy policy. The system is governed by the Canadian Milk Supply Management Committee (CMSMC). The committee is responsible for policy determination and supervision of the national milk marketing plan. The CMSMC annually sets a national production target, the market sharing quota

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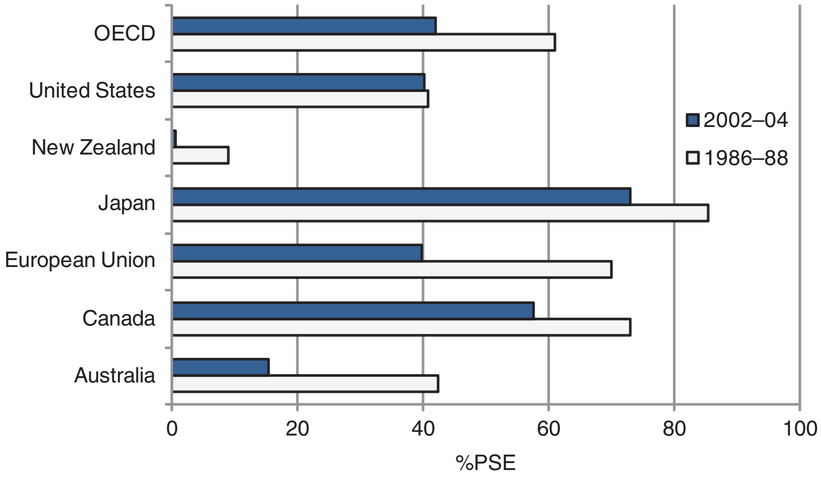

Figure 1 Producer support estimates (PSEs) for milk in various countries. Source: OECD.

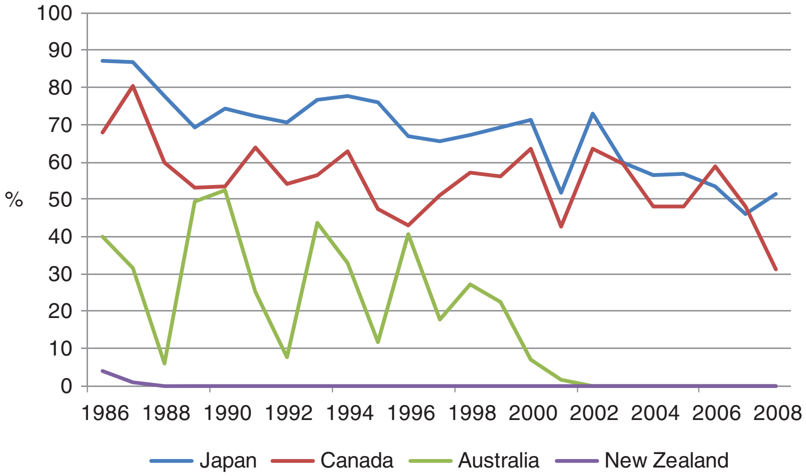

Figure 2 Single commodity transfers (SCTs) for milk over the period 1986–2008 in various countries. Source: OECD.

(MSQ), for industrial milk. The MSQ is set with the goal to achieve a domestic market balance in terms of butterfat, and is assigned to provinces largely on the basis of historical shares. In addition to the MSQ, each province controls its own production quota for fluid milk, and the entire milk quota – industrial and fluid together – is allocated to producers.

For the supply management to be effective there is a need to continue the policies of substantial border measures. In other words, a quota system allows a domestic market to be managed only if that market is isolated from external sources of supply. Under the 1994 Uruguay Round Agreement on Agriculture (URAA), the diverse forms of trade measures were converted to tariffs, and market access for sensitive products was provided through a system of tariff rate quotas (TRQs). Under the TRQ system, exporting countries have access to the Canadian dairy market to the tune of 5% of the domestic consumption. For most products, the final access quota quantities remain below 1000 tonnes (i.e., 1 kt), but are 3.2 kt for butter and dry whey, 20.4 kt for cheese, and 64.5 kt for fluid milk. While the in-quota duties are generally low, tariff rates applicable to over-quota imports are prohibitively high, ranging from 202% for skim milk powder to 246% for cheese and 299% for butter.

A strict control of supplies from domestic production and imports allows prices paid to producers to be supported at levels marginally above the costs of production. The Canadian Dairy Commission annually reviews and establishes a target price for industrial milk. This target price is supported by market intervention for butter and skim milk powder (SMP) at support prices set similarly on an annual basis. The support prices have been increasing steadily over the last decades. In 2009, the support prices for butter and SMP were raised to Can$7102.4 and

Can$6178.3 per tonne, respectively. For a comparison, the respective prices in 2000 stood at 5540.7 and Can$4684.2 per tonne, which translates to a 28 and 32% increase in the support prices over the 2000–09 period. (Over the same period, 2000–09, in the European Union the butter and SMP intervention prices declined by 25 and 15%, respectively. Using the October 2009 Can$/Euro exchange rate of 0.65, the butter and SMP support prices of Canada in 2009 would amount to €4611 and €4011 per tonne, respectively. The corresponding intervention prices for butter and SMP in the European Union were €2462 and €1764 per tonne, respectively.)

In 1995, a supplementary scheme was introduced that provided for the pricing of five classes of milk by virtue of a new permit system. The change allowed dairy processors to purchase surplus milk (over the quota milk) at a discount rate, determined by the government, for the production of dairy products for exports. The United States, joined by New Zealand, claimed that this in fact constituted an export subsidy, that it was in violation of Canada's commitments under the Uruguay Round, and requested investigation by a World Trade Organization (WTO) compliance panel. In December 2002, the WTO confirmed that Canada's approach to the export of dairy products constituted an export subsidy. On 9 May 2003, Canada announced that it had entered into an agreement with the United States and New Zealand, and eliminated the subsidies that violated the WTO rules.

The supply management system in Canada is sometimes used as an example of a functioning stable system that enables milk producers to receive good prices. Supply management might be considered the second best option in a narrow sense as it alleviates surplus accumulation resulting from a high market price support, but it is unlikely to be the long-term solution in the face of rapid technological and structural developments throughout the world. The problems linked to supply management include the inefficiencies that the system may create, the costs that it imposes on consumers, the difficulties and costs of administration that may arise for governments, the difficulty in setting the quota at a level that would match consumption, the vested interests that it generates (quota rent), and importantly the need to continue the policies of high border measures.

Japan

Background

Japanese domestic dairy policy focuses mainly on supporting milk destined for the production of dairy products, which procures a lower price than drinking milk and is subject to international competition. Up until 2001, government support was provided mainly through three programs: price support, voluntary program to limit supply, and import tariff rate quotas. Price support was administered via a system of deficiency payments on manufacturing-milk introduced in 1966. The deficiency payments, defined as the difference between a guaranteed price and the price paid by dairy plants for milk used in processing (standard transaction price), were limited to an annually fixed quota volume and to designated products such as butter, SMP, and condensed milk. A voluntary production quota for liquid milk was initiated in 1979 in an effort to regulate shipments from Hokkaido – the low-cost production region with over 80% of milk used for manufacturing – to higher-cost production regions. This quota is determined by the Central Council of Dairy Cooperatives and allocated between prefectural cooperatives, who in turn allocate a quota to each farmer. The allocation between prefectures takes into account production of the previous year and planned production for the coming year. Since the voluntary production planning system has no legal binding power, some 5% of dairy farmers choose to operate outside the production guidelines. As measured by the PSE, total transfers to dairy farmers relative to total gross receipts amounted to 73% in the period 2002–04. The support has declined from the levels calculated in the mid-1980s although the Japanese dairy sector remains among the most heavily supported in the world.

A Change in Dairy Domestic Policy in 2001

In April 2001, a policy change was introduced to deal with the rigidity of the guaranteed price system, increased budgetary cost, and the additional pressures to reduce domestic support resulting from Japan's commitments under the Uruguay Round. The policy abolished the guaranteed price and the standard transaction price along with the deficiency payment scheme, and a new direct-payment program was introduced instead; but the annually determined manufacturing-grade milk quotas were kept in place. The move away from a market-intervention system to a payment-based system was designed to improve the market orientation of dairy farms.

Producers' direct payments were to be set annually in light of the unit rate paid in the previous year and the changes in cost of raw milk production. In 2001, in order to ensure a smooth adjustment to the new policy, the direct payments were set equal to deficiency payments of 2000 at ¥10.3 per kg. The amount of manufacturing-milk eligible for payment was set at 2.27 mt. The evolution of deficiency payments and, from 2001, direct payments together with the eligible quantities is illustrated in **Figure 3**. The move to a more market-oriented system was compensated for by the introduction of emergency measures to protect farmers from

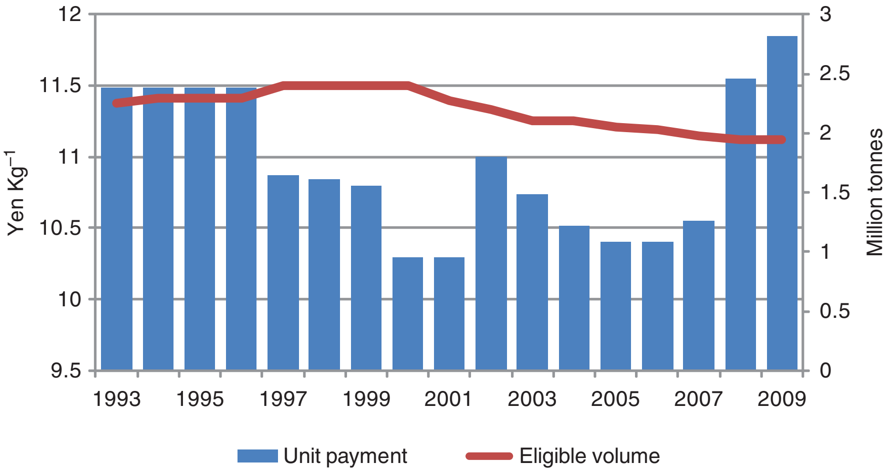

Figure 3 The evolution of deficiency and direct payments for milk together with eligible quantities, in Japan. Source: MAFF, Japan. Deficiency payments until 2000; direct payments afterward.

unforeseen fluctuations in the price of manufacturing milk. If the average market price falls, then 80% of the difference between the average market price and the base price (the average transaction price during the previous 3 years) is to be compensated from an income stabilization fund to which producers and the state contribute at the ratio of 1:3. An additional system of direct payments was introduced to provide incentives for environmental conservation under the land using-type dairy farming promotion project and the direct payment system in hilly and mountainous districts.

Japan manages imports of dairy products under TRQs by import licensing and state trading. Quantities under import license are allocated by the Ministry of Agriculture, Forestry and Fisheries (MAFF) to private importers based on historical records. The quota access at preferential tariffs for SMP and butter are set at 116 and 1.9 kt, respectively. Typically these quotas remain significantly underfilled. The in-quota *ad valorem* tariffs are set at 16, 24, and 35% levels for SMP, whole milk powder, and butter respectively. In addition to in-quota tariffs, the government of Japan or its sales agents are able to charge the so-called markup, which can amount to 392, 413, and 594% for SMP, whole milk powder, and butter respectively.

The tariffs for out-of-quota imports are set prohibitively high at 210, 316, and 733% levels for SMP, whole milk powder, and butter, respectively (Agricultural Market Access Database (AMAD)). Cheese imports to Japan are not subject to quota. There are various import tariffs for cheese depending on the use of the product, but the average rate is about 31.2%.

Although the policy change toward direct payments is in the right direction, the change is relatively small given the design of the policy. The support remains very high and continues to be paid by consumers. The primary reason for the very modest impact of the new policy is the lack of changes to dairy trade policy, which keeps Japan's dairy industry highly protected from cheaper imports. Due to very high border measures, the majority of support still falls under the market price support category. In 2008, the market price support stood at 93% of all SCTs.

Australia

Background

With the Kerin plan in 1986 and the Crean plan in 1992, Australia began a reform process of dairy policies including a gradual reduction in support and a planned elimination of support for manufacturing milk by 1 July 2000. In 1995, a redesigned plan was introduced to ensure that Australia complied with its WTO commitments on export subsidies under the Uruguay Round. The domestic market support (DMS) scheme was restructured so as to ensure that support was provided independently of export sales. In addition to this reform of support policies for manufacturing milk, a regulatory reform process for market milk was initiated in 1995, stipulating that in each state only farm gate price controls would remain in place by January 1999. In July 1999, a review of market milk regulations in Victoria concluded that there was no net public benefit from retaining farm gate price controls. An industry restructuring plan was developed to avoid possible interstate price wars and an industry collapse, which was implemented on 1 July 2000. Following the industry deregulation, support to the Australian dairy sector has declined dramatically and, measured by the PSE, the value of transfers to the dairy industry relative to gross farm receipts fell to 15% in the period 2002–04 (**Figure 1**). As measured by the SCT, after the deregulation, the direct market support to dairy industry has dropped to zero (**Figure 2**).

Policy Reform of the Dairy Industry in 2000 and beyond

A new policy reform package was introduced on 1 July 2000, which removed simultaneously the DMS scheme and fresh milk regulations, and allowed the market to determine milk prices. At the same time, a structural adjustment package was introduced through the Dairy Industry Adjustment Act to help producers to cope with the adjustment to lower prices or to choose to leave the industry. The adjustment package was to be funded by a levy of 11 cents (A$) per liter on all domestic sales of fresh milk for 8 years until the package would be fully funded.

The individual adjustment programs were called the dairy industry adjustment package, the dairy structural adjustment program, the dairy exit program, and the dairy regional assistance program. Dairy farmers were eligible for dairy structural adjustment program assistance and received a fixed quarterly payment over 8 years, with payments being based on milk production in 1998–99, and subject to income tax. Producers could also opt to leave the dairy industry altogether, and receive an exit payment of up to A$45 000 tax-free under the dairy exit program. The conditions attached to the program prevented the farmers from reentering the industry at a later date. Finally, the dairy regional assistance program was intended to assist dairy-dependent communities in generating alternative employment opportunities and to deal with any social dislocation from deregulation.

After the reform, the dairy industry has become fully exposed to world market conditions and emerged as a globally cost-competitive industry. However, following the deregulation, the dairy sector not only had to absorb the reform adjustment pressures but also had to cope with a series of severe droughts that resulted in increased herd contraction. Cow inventories in Australia increased in 2008/09 for the first time in 7 years, however contracted again in 2009/10 and the future industry growth remains sensitive to availability and management of water supply. To address these issues, in 2008 and 2009, the Australian government has strengthened the water policy reforms and environmental programs, and also announced an initiative, Australia's Farming Future, to help the industry through research and information to manage the impact of climate changes. The Australian Government is also committed to implementing the emissions trading scheme in 2011, which can be expected to impact the dairy sector. Nevertheless, at the same time, a program concentrating on reducing emissions from livestock has been initiated focusing on research into alternative feeds to reduce methane production or genetic approaches to developing low-emitting animals.

The industry does not use export subsidies to increase its market share of dairy products, although it has a strong focus on export sales. In 2009, the Australian government has announced a reform of the system of export quota allocations to the United States and the European Union. Under tariff rate quotas, certain amounts of Australian dairy products can be exported into the United States and the European Union at reduced or zero tariffs. The old system of distributing fixed shares of quota based on historical entitlements is replaced by a system in which exporters receive a share of quota based on 3-year rolling averages of export performance.

Australia has also entered into a number of free trade agreements (FTAs) that have been or are in the process of negotiation. The trade barriers between Australia and New Zealand were fully removed in the Closer Economic Relations Trade Agreement, which came into effect already on 1 January 1983. Australia has FTAs also with Singapore, Thailand, the United States, and Chile. Most recently, the negotiations between ASEAN, Australia, and New Zealand for a free trade agreement (AANZFTA) were concluded on 28 August 2008, and the agreement was signed on 27 February 2009. This was the largest FTA Australia signed to that date. A separate agreement is being negotiated with Malaysia, and there are plans to negotiate an FTA with Indonesia. FTA agreements are also being pursued with China, Japan, and the Gulf Cooperation Council.

New Zealand

Background

Prior to 1984, the support to farmers in New Zealand went as high as 40% of the farmers' income. Domestic farm support policies were scaled down dramatically during the 1980s with input subsidies eliminated in 1984 and government involvement in calculation of product prices withdrawn in 1988. The main dairy policy issue following the deregulation of 1984 was related to the export monopoly of the New Zealand Dairy Board (NZDB) and the potential for indirect subsidization of dairy exports. This potential existed as Section 27 of the Dairy Board Act allowed for pooling of revenues from domestic and export markets and, thus, cross-subsidization of lower revenue from export sales by higher revenue from domestic sales. This section was abolished in 1998. The dairy industry is one the most important elements of the New Zealand economy accounting for about one quarter of New Zealand's total export earnings. Measured by the PSE, total transfers to dairy farmers relative to gross farm receipts were close to zero in 2002–04 (**Figure 1**). As measured by the SCT, the direct market support to the dairy industry has been zero for more than 20 years (**Figure 2**).

Dairy Industry Restructuring Act

On 9 April 2001, the New Zealand Government passed the Dairy Industry Restructuring Act (DIRA), which agreed to the formation of a large cooperative Fonterra, originally called GlobalCo, through the merger of most of New Zealand's cooperative dairy processing companies. The act also stipulated that following the merger, the new company will absorb the activities of the NZDB. The NZDB, established through the Dairy Board Act in 1961, controlled the marketing of all export dairy products and was the largest exporter in New Zealand. An amendment to the Dairy Board Act in 1996 brought the status of NZDB closer to that of a company in which milk processing cooperatives own shares in proportion to their milk deliveries. Nevertheless, the NZDB remained subject to criticism for its export monopoly power. Given the trade agreements and its export power, the NZDB was able to extract quota rents from dairy exports (e.g., to the EU), which were then passed back to individual producers thus increasing the farmers' marginal returns. The DIRA ended the statutory export monopoly of NZDB and established 11 regulated dairy export markets. On a transitional basis, the act provided Fonterra with exclusive export access to these markets for a fixed period of time. In 2007 the New Zealand Government reviewed the regulated export market established in the DIRA and started the deregulation process to be concluded over the period 2007–10.

The DIRA also provided for a regulation that aimed to protect firms from Fonterra's monopoly pricing. The provision required that 5% of Fonterra's milk be made available at a predetermined price to other independent processors to allow for a level playing field. The review of this regulation conducted in 2008 concluded that independent processors were able to procure milk at a lower price than what Fonterra paid to its farmers. A new mechanism was proposed under the Dairy Industry Restructuring Bill; this bill (in reading as of October 2009) proposes that the margin be charged from the 2010/2011 dairy season and also provides for raw milk to be allocated through auctions in later years.

Government policy in New Zealand affects the dairy industry mainly via policy measures addressing agri–environmental issues. For example, the dairying and clean streams accord, which was agreed between Fonterra and the New Zealand Government in 2003, aims to achieve clean water, including streams, rivers, lakes, and ground water, and wetlands in dairying areas. Moreover, in September 2007, the government released a comprehensive statement on climate change and a range of initiatives across all sectors, including the Emissions Trading Scheme (ETS). Agriculture is likely to be part of the scheme from 2013.

New Zealand is the largest exporter of butter, SMP, and whole milk powder, and the second largest exporter of cheese in the world. New Zealand achieves this position without relying on production or market subsidies and without protecting the domestic market from overseas competition. In the absence of progress in lowering trade barriers on a multilateral level, New Zealand is seeking trade agreements on a bilateral basis. Apart from New Zealand's free trade agreement with Australia (already in place since 1983), more recent FTAs were concluded with Singapore, Thailand, the Trans-Pacific Partnership (involving Singapore, Brunei, and Chile), China, and ASEAN (signed in February 2009). Negotiations are currently underway with Malaysia, Hong Kong, and the Gulf Co-operation Council (Saudi Arabia, UAE, Oman, Qatar, Bahrain, and Kuwait). Negotiations are also due to commence for the enlargement of the Trans-Pacific Partnership and bilateral FTAs with Korea and with India.

See also: **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy; Agricultural Policy Schemes: European Union's Common Agricultural Policy.

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Codex Alimentarius

C Heggum, Danish Dairy Board, Aarhus, Denmark

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Introduction

Codex Alimentarius is Latin for food code and refers today to the international food code established under the United Nations. It is a collection of internationally adopted food standards that constitute a global reference point for national food legislators and control agencies, the international food trade, and food handlers and consumers. The code has a great impact on the approach to food quality management throughout the world.

The code is being developed by the Codex Alimentarius Commission (CAC), which is an international organization run jointly by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). One hundred and seventy-eight individual countries are members of the CAC (end of 2008). In addition, about 160 other international intergovernmental and international non-governmental organizations contribute to the work. The CAC's objective is to establish standards, codes of practices, guidelines, and recommendations concerning foods aimed at protecting consumer's health, ensure fair practices in trade, and facilitate international trade.

With the establishment of the World Trade Organization (WTO), the Codex Alimentarius has gained importance due to the fact that two WTO Agreements (the Agreement on the Applications of Sanitary and Phytosanitary Measures (SPS) and the Agreement on Technical Barriers to Trade (TBT)) refer to Codex Alimentarius texts as being the reference for dispute settlements and for application by national legislation.

The Establishment

The Need for Harmonization of National Food Regulations

The need for international food regulation has developed with international trade. Quality standards for individual commodities have been known since ancient times, but the first general food laws were established in the 1800s.

At the beginning of the 1900s, the establishment of international food standards began; it was of growing concern to food traders worldwide that the national standards and regulations developing independently (and sometimes spontaneously) in individual countries started to create trade barriers. As a response to this development, trade associations that were formed as a reaction to such barriers pressured governments to harmonize their various food standards so as to facilitate trade in safe foods of a defined quality. One of the earliest such associations was the International Dairy Federation (IDF), founded in 1903.

After World War II, there was heightened international concern about the direction being taken in the field of food regulation. Countries were acting independently and there was little, if any, consultation among them with a view to harmonization. In addition, and as a reaction to the food supply situation during the 1940s, many attempts were made to protect and support domestic food production. Food regulations in different countries were conflicting and contradictory, in particular with regard to nomenclature, and much legislation was not based on scientific knowledge.

Many efforts have been made to harmonize national food regulations. In the dairy field, the IDF developed during the 1950s a vast number of codes and standards intended to be applied by the dairy sector and to form the basis of national legislation. These efforts resulted in two initiatives being taken at the government level:

- the establishment of the 'Stresa Convention' – a multilateral agreement between a number of European countries that governed the naming and composition of a number of individual cheese varieties (*see* **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products);
- the establishment of the Joint FAO/WHO Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products (the 'Milk Committee') – a worldwide committee established to develop international identity standards for milk products, most of them prepared by IDF, within the framework of the Code of Principles Concerning Milk and Milk Products (*see* **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products).

Catalyzed by the success of the Milk Committee, the 1961 FAO Conference decided to establish the Codex Alimentarius. The CAC was established in 1962 to govern the work. Due to the importance of the role of WHO in all health aspects of food, the WHO joined in 1963.

Since its founding, many food standards, codes of hygienic and technological practice, and maximum residue limits (MRLs) for food contaminants have been established by Codex Alimentarius (**Figure 1**).

The Codex Alimentarius

204 food standards for commodities

47 codes of hygienic or technological practice

2930 maximum limits for 218 evaluated pesticides

1112 maximum levels for 292 evaluated food additives

441 maximum residue levels for 49 evaluated veterinary drugs

Guidelines for 12 contaminants

Figure 1 Standards, codes, and recommendations established by Codex Alimentarius.

The Milk Committee, since its establishment in 1958, was integrated into the Codex Alimentarius system. However, its rules and procedures were different from those of Codex Alimentarius for many years. As late as 1993, the Milk Committee was replaced by the 'Codex Committee for Milk and Milk Products' and the rules and procedures were aligned with those applicable for the rest of the Codex system.

The Scientific Basis

The Codex Alimentarius is science based. Experts and specialists in a wide range of disciplines contribute to every aspect of the code to ensure that its standards withstand the most rigorous scientific scrutiny.

Much work is carried out in the form of collaborative studies between individual scientists, laboratories, institutes and universities, and joint FAO/WHO expert committees and consultations.

The membership of expert consultations is of critical importance. The credibility and acceptability of any conclusions and recommendations depend to a very large degree on the impartiality, scientific skill, and overall competence of the members who formulate them. For this reason, care is taken in the selection of experts invited to participate. Those selected must be preeminent in their specialty, have the highest respect of their scientific peers, and be impartial and objective in their judgment. They are appointed in their own personal right – not as government representatives or as spokespeople for organizations.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Joint FAO/WHO Meetings on Pesticide Residues (JMPR), and the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) have generated a large amount of scientifically based food data.

JMPR was established in 1963 with the task of recommending MRLs for pesticide and environmental contaminants in specific food products. JMPR members are independent scientists who are expert in aspects of pesticides and environmental chemicals and their residues. There is close cooperation between JMPR and the Codex Committee on Pesticide Residues (CCPR). CCPR identifies substances requiring priority evaluation. After JMPR evaluation, CCPR discusses the recommended MRLs and, if they are acceptable, forwards them to the Commission for adoption as Codex MRLs.

JECFA was established in 1955 with the task of considering chemical, toxicological, and other aspects of contaminants and residues of veterinary drugs in foods for human consumption. JECFA provides the Commission and other Codex bodies with expert advice relating to food additives, contaminants, and residues of veterinary drugs. The Codex Committee on Food Additives (CCFA), the Codex Committee on Contaminants in Foods (CCCF), and the Codex Committee on Residues of Veterinary Drugs in Foods (CCVDF) identify food additives, contaminants, and veterinary drug residues that should receive priority evaluation and refer them to JECFA for assessment before incorporating them into Codex standards.

JEMRA was established in 2000 in response to the increasing need for risk-based scientific advice and information and tools to undertake microbiological risk assessment. The objectives of JEMRA are the development and optimization of the utility of microbiological risk assessment (MRA) as a tool to inform actions and decisions aimed at improving food safety and to make it equally available to both developing and developed countries.

Purpose and Organization

The task of creating a food code is immense. Food standards need to mirror the dynamic environment in which they will be applied. Product development, changes in trade and consumption patterns, consumer perception, and scientific progress make creation and review of food standards virtually endless.

Furthermore, it requires more effort and resources to create standards that, on the one hand, aim at protecting consumers and ensuring fair practices in the sale of food and, on the other hand, facilitating trade. The need to involve scientific experts from consumers' organizations, expertise from production and processing industries, and food control administrators and traders is obvious.

The finalization of food standards and their compilation into a code that is credible and authoritative requires extensive consultation followed up by confirmation of final results and, sometimes, a compromise to satisfy divergent but scientifically sound views.

The above comprehensive process makes developments slow. On the other hand, once a standard has been adopted, it reflects worldwide consensus and is therefore useful in practice.

Objectives of Codex

The objective of Codex is to conduct the Joint FAO/WHO Food Standards Programme, the purpose of which is

(a) to protect the health of the consumers;
(b) to ensure fair practices in the food trade;
(c) to promote coordination of all food standards work undertaken by international governmental and non-governmental organizations;
(d) to determine priorities and to initiate and guide the preparation of draft standards through and with the aid of appropriate organizations; and
(e) to finalize food standards and to publish them in a Codex Alimentarius as either regional or worldwide standards.

Structure of Codex

The CAC is the supreme body of Codex Alimentarius. It meets every year, alternately at FAO headquarters in Rome and at WHO headquarters in Geneva. Plenary sessions are attended by as many as 800 people. Representation at sessions is on a country basis. Senior officials appointed by their governments lead national delegations. Delegations may, and often do, include representatives of industry, consumers' organizations, and academic institutes. A number of international governmental and non-governmental organizations also attend in an observer capacity. Although they are 'observers', the tradition of the CAC allows such organizations to put forward their points of view at every stage except in the final decision, which is the exclusive prerogative of Member Governments.

The Commission is empowered to establish three kinds of subsidiary bodies:

1. Codex Committees, which prepare draft standards for submission to the Commission. These are classed as either General Subject Committees or Commodity Committees.

   The work of the General Subject Committees has relevance for all foods and applies across the board to all commodities. Therefore, these are sometimes referred to as 'horizontal committees'. They develop all-embracing concepts and principles applying to foods in general, specific foods, or groups of foods, endorse or review relevant provisions in Codex commodity standards, and, based on the advice of expert scientific bodies, develop major recommendations pertaining to consumers' health and safety. Currently, there are 10 General Subject Committees (see **Figure 2**).

   Commodity Committees have responsibility for developing standards for specific foods or classes of food. In order to distinguish them from the horizontal committees and recognize their exclusive responsibilities, they are often referred to as 'vertical committees'. Currently, there are 11 Commodity Committees (see **Figure 2**).
2. Coordinating Committees, through which regions or groups of countries coordinate food standards activities in the region, including the development of regional standards. Coordinating Committees play an invaluable role in ensuring that the work of the Commission is responsive to regional interests and to the concerns of developing countries. Currently, there are six Coordinating Committees (see **Figure 2**).
3. *Ad Hoc* International Governmental Task Forces are established to consider closely defined issues within a time-limited period. Currently, three such Task Forces are operating (see **Figure 2**).

Each worldwide committee and task force is hosted by a member country, which is chiefly responsible for the maintenance and administration cost of the committee and for providing its chairperson. Regional committees have no standing host countries; the Chair is elected at meetings.

The Codex Step Procedure

Typically, the Codex work program consists of revision of older, outdated texts and the development of new texts. The latter is usually initiated with the consideration of discussion papers.

Once a standard, a code, or a guideline is identified as a potential subject for work, it will be developed in three phases, normally comprising eight formal steps (**Figure 3**):

- the decision to initiate work (constitutes step 1), resulting in the adding of the subject to the work program and allocation of the responsibility to prepare a Proposed Draft Codex Standard, a Proposed Draft Codex Code of Practice, a Proposed Draft Codex Guideline, or another proposed draft Codex recommendation;
- the process of furnishing a draft text (constitutes steps 2–5), resulting in preparing an almost finalized draft, which is adopted as a Draft Codex Standard, a Draft

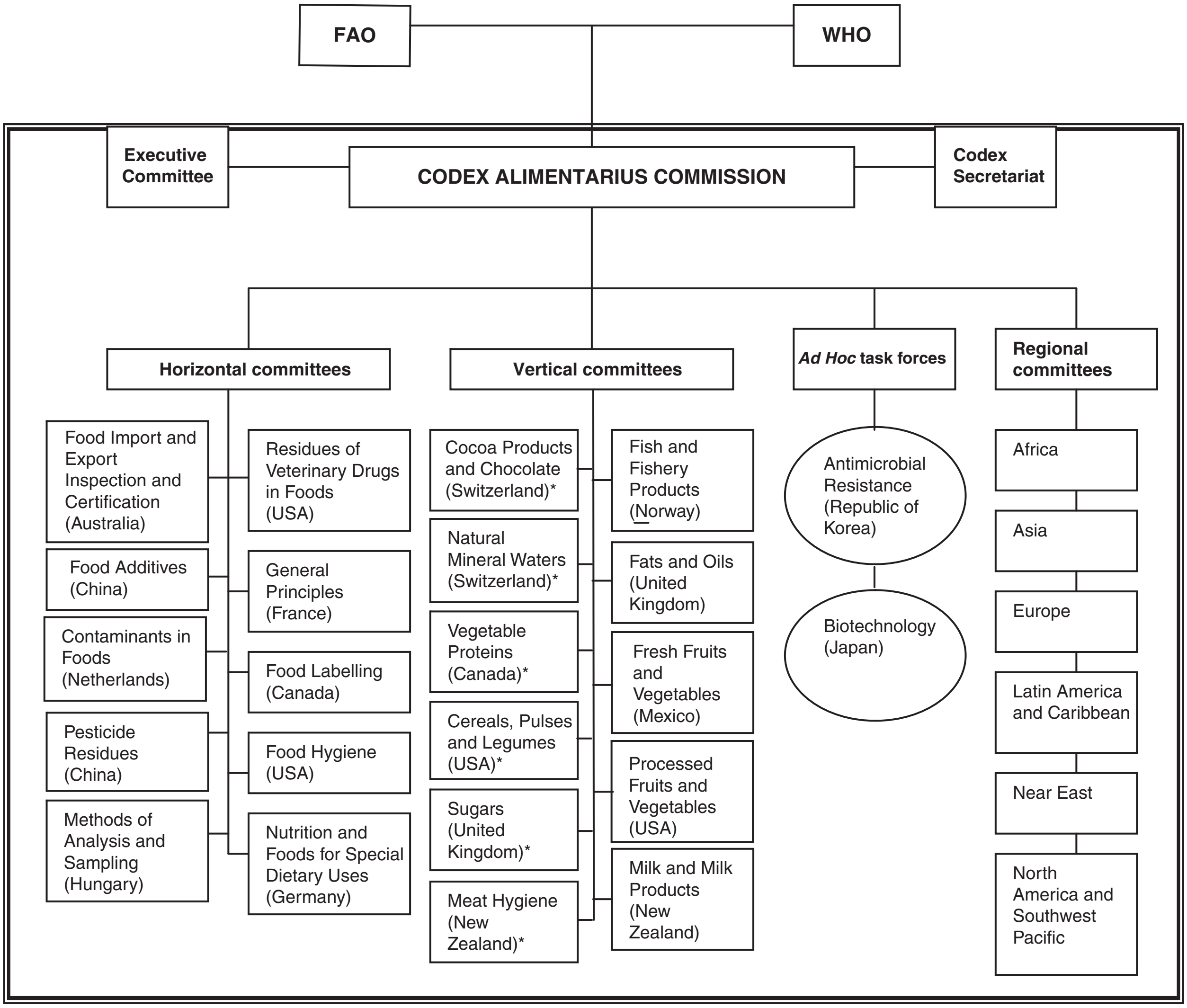

FAO
WHO
Executive Committee
CODEX ALIMENTARIUS COMMISSION
Codex Secretariat
Horizontal committees
Vertical committees
Ad Hoc task forces
Regional committees
Food Import and Export Inspection and Certification (Australia)
Food Additives (China)
Contaminants in Foods (Netherlands)
Pesticide Residues (China)
Methods of Analysis and Sampling (Hungary)
Residues of Veterinary Drugs in Foods (USA)
General Principles (France)
Food Labelling (Canada)
Food Hygiene (USA)
Nutrition and Foods for Special Dietary Uses (Germany)
Cocoa Products and Chocolate (Switzerland)*
Natural Mineral Waters (Switzerland)*
Vegetable Proteins (Canada)*
Cereals, Pulses and Legumes (USA)*
Sugars (United Kingdom)*
Meat Hygiene (New Zealand)*
Fish and Fishery Products (Norway)
Fats and Oils (United Kingdom)
Fresh Fruits and Vegetables (Mexico)
Processed Fruits and Vegetables (USA)
Milk and Milk Products (New Zealand)
Antimicrobial Resistance (Republic of Korea)
Biotechnology (Japan)
Africa
Asia
Europe
Latin America and Caribbean
Near East
North America and Southwest Pacific

Figure 2 Organization of the Codex Alimentarius Commission. *-Adjourned sine die.

Codex Code of Practice, a Draft Codex Guideline, or another draft Codex recommendation; and

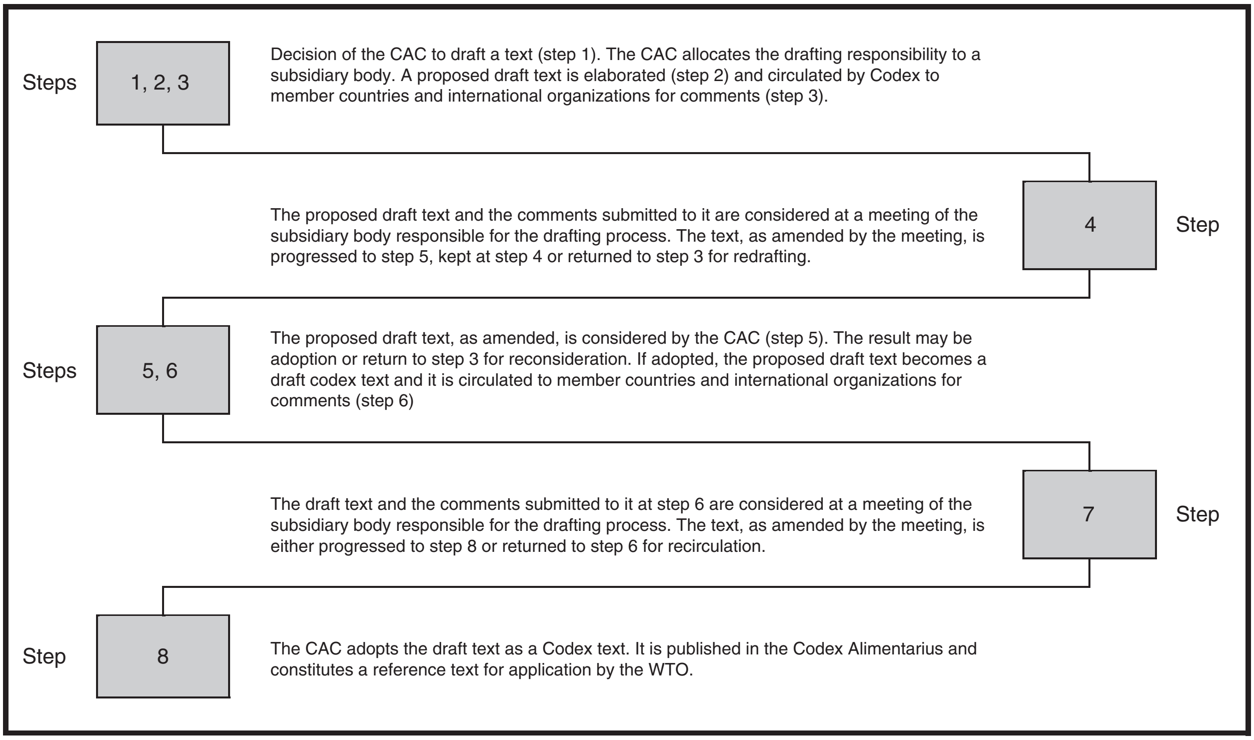
- the process of finalizing a text (constitutes steps 6–8), resulting in a Codex Standard, a Codex Code of Practice, a Codex Guideline, or another Codex recommendation.

Initiation of New Work

A proposal for a standard or another text can be submitted by any member country or any of the interested international organizations registered. In the dairy field, most proposals derive from the work of the IDF.

Prior to the approval of new work a project document that details the purpose and scope, its relevance, main aspects to be covered, need for any expert scientific advice, and timeline for completion is required. Furthermore, an assessment of the formal criteria for the establishment of work priorities is needed. For commodity standards, these are

1. consumer protection from the point of view of health and fraudulent practices;
2. volume of production and consumption in individual countries and volume and pattern of trade between countries;
3. diversification of national legislation and apparent resultant impediments to international trade;
4. international or regional market potential;
5. amenability of the commodity to standardization;
6. coverage of the main consumer protection and trade issues by existing general standards;



Decision of the CAC to draft a text (step 1). The CAC allocates the drafting responsibility to a subsidiary body. A proposed draft text is elaborated (step 2) and circulated by Codex to member countries and international organizations for comments (step 3).

The proposed draft text and the comments submitted to it are considered at a meeting of the subsidiary body responsible for the drafting process. The text, as amended by the meeting, is progressed to step 5, kept at step 4 or returned to step 3 for redrafting.

The proposed draft text, as amended, is considered by the CAC (step 5). The result may be adoption or return to step 3 for reconsideration. If adopted, the proposed draft text becomes a draft codex text and it is circulated to member countries and international organizations for comments (step 6)

The draft text and the comments submitted to it at step 6 are considered at a meeting of the subsidiary body responsible for the drafting process. The text, as amended by the meeting, is either progressed to step 8 or returned to step 6 for recirculation.

The CAC adopts the draft text as a Codex text. It is published in the Codex Alimentarius and constitutes a reference text for application by the WTO.

Figure 3 Standard elaboration procedure of Codex texts.

7. number of commodities that would need separate standards indicating whether raw, semiprocessed, or processed; and
8. work already undertaken by other international organizations in this field.

The first drafting is normally assigned to the Codex Secretariat, a Member Government, an *ad hoc* working group established among interested Member Governments and organizations, or, in the case of dairy standards, the IDF. Commodity standards follow a uniform format. The structure of codes of practices and guidelines varies according to the nature of the content and the traditions within the Codex Committee where the draft is developed.

A draft Codex text is sent to governments and international organizations a number of times in a stepwise procedure, which, if completed satisfactorily, results in the draft becoming a ‘Codex standard’. In an accelerated procedure, the number of steps required for the development of a standard varies from a maximum of eight to a minimum of five. In many cases, steps are repeated. Most standards take 8 years to develop.

Revision of Codex Standards

The Commission and its subsidiary bodies are committed to revision of Codex standards and related texts as necessary to ensure that they are consistent with and reflect current scientific knowledge. The procedure for revision follows the same procedure as used for the initial preparation of new standards.

The Commission can, however, decide to omit any other step or steps of the procedure where an amendment proposed by a Codex Committee is either editorial in nature or consequential to provisions in similar standards adopted.

Application and Role of Codex Texts

The Codex Alimentarius has relevance to the international food trade and also to domestic legislation and regulation. With respect to the ever-increasing global market, in particular, the advantages of having universally uniform food standards are self-evident. Texts developed by the CAC are applied in the following contexts:

1. as reference texts for the SPS and TBT Agreements of the WTO;
2. as reference texts and/or foundation for national legislation, regional regulation, and trade agreements; and
3. as reference texts for commercial trading parties.

SPS and TBT Agreements of the WTO

Both the SPS and TBT Agreements acknowledge the importance of harmonizing standards internationally so as to minimize or eliminate the risk of national legislation and regulation becoming barriers to trade.

The SPS Agreement

In its pursuance of harmonization, the SPS Agreement has identified and chosen the standards, guidelines, and recommendations established by the CAC for food additives, residues of veterinary drugs and pesticides, contaminants, methods for analysis and sampling, and codes and guidelines of hygienic practice. In other words, Codex texts are considered scientifically justified and have become an integral part of the legal framework as accepted benchmarks against which national measures and regulations are evaluated (**Figure 4**).

Codex texts have already been used as the benchmark in international trade disputes, and it is expected that they will be used increasingly in this regard.

As a consequence, interest in Codex activities and participation at the meetings of governments and international inter- and non-governmental organizations has increased.

There are no obligations for a government to use Codex standards, only an encouragement. However, as Codex texts by definition are recognized as being scientifically based, a government need not perform a formal risk assessment (*see* **Risk Analysis**) if a Codex recommendation is followed. Thereby, a government can save considerable resources. Otherwise, a government can be faced with the SPS requirement to defend and justify according to scientific evidence every deviating detail in their national legislation.

This possibility to choose Codex standards provides the opportunity for countries with economic limitations to follow Codex recommendations and simultaneously comply with the SPS requirements. In countries where the necessary resources are available, additional risk assessment might be carried out in order to justify desired deviations from Codex. A government can only implement stricter food safety requirements than those recommended by Codex, and if justified. This means that Codex recommendations relating to food safety serve as minimum provisions.

The TBT Agreement

The basic principle of the TBT Agreement is that any measure should be enforced only if a so-called legitimate objective exists (e.g., prevention of deceptive practices, meet quality and performance requirements). The legitimate objective should be transparent in order to avoid disguised protection of domestic production and to avoid arbitrary decisions.

The TBT Agreement does not make reference to any particular international organization to be used as the 'benchmark'. With respect to food, however, it is generally recognized that Codex Alimentarius serves this function (**Figure 5**).

It is relatively easy for a government to justify that a particular part of an international standard, for example, a

The SPS Agreement

The Agreement on the Application of Sanitary and Phytosanitary Measures acknowledges that governments have the right to take sanitary and phytosanitary measures necessary for the protection of human health. However, the SPS Agreement requires them to apply those measures only to the extent required to protect human health. It does not permit Member Governments to discriminate by applying different requirements to different countries where the same or similar conditions prevail, unless there is sufficient scientific justification for doing so.

Article 2.2 of the SPS Agreement states:

"Members shall ensure that any sanitary and phytosanitary measure is applied only to the extent necessary to protect human, animal or plant life or health, is based on scientific principles and is not maintained without sufficient scientific evidence ..."

Article 3.1 of the SPS Agreement states:

"To harmonize sanitary and phytosanitary measures on as wide a basis as possible, Members shall base their sanitary and phytosanitary measures on international standards, guidelines or recommendations, where they exist, except as otherwise provided for in this Agreement."

Figure 4 The Agreement on the Applications of Sanitary and Phytosanitary Measures (SPS).

The TBT Agreement

The Agreement on technical barriers to trade seeks to ensure that technical regulations and standards, including packaging, marking, and labeling requirements and analytical procedures for assessing conformity with technical regulations and standards do not create unnecessary obstacles to trade.

Article 2.4 of the TBT Agreement states

"Where technical regulations are required and relevant international standards exist or their completion is imminent, Members shall use them, or the relevant parts of them, as a basis for their technical regulations except when such international standards or relevant parts would be an ineffective or inappropriate means for the fulfilment of the legitimate objective pursued, for instance because of fundamental climatic or geographical factors or fundamental technical problems."

Article 2.5 of the TBT Agreement states

".... Whenever a technical regulation is prepared, adopted or applied for one of the legitimate objectives......., and is in accordance with relevant international standards, it shall be rebuttably presumed not to create an unnecessary obstacle to international trade."

Article 2.6 of the TBT Agreement states

"With a view to harmonizing technical regulations on as wide a basis as possible, Members shall play a full part, within the limits of their resources, in the preparation by appropriate international standardizing bodies of international standards for products for which they have either adopted, or expect to adopt, technical regulations."

Figure 5 The Agreement on Technical Barriers to Trade (TBT).

labeling requirement, is not appropriate in a particular country or that additional requirements are needed locally. It is allowed to deviate from Codex TBT-related provisions in both stricter and less strict directions, if an appropriate legitimate objective exists. Codex regulations, which are not aiming at protecting human health, are therefore merely to be seen as guidelines.

Codex standards may not be the only body that provides reference provisions, as objectives of the TBT Agreement are broader than those of Codex Alimentarius.

National Legislation and Regulation

The harmonization of food standards is generally viewed as a prerequisite to the protection of consumer health as well as allowing the fullest possible facilitation of international trade. For that reason, both SPS and TBT Agreements encourage the international harmonization of food standards.

While the growing world interest in all Codex activities clearly indicates global acceptance of the Codex philosophy – embracing harmonization, consumer protection, and facilitation of international trade – in practice, it is difficult for many countries to accept Codex standards in the statutory sense. Differing legal formats and administrative systems, varying political systems, and sometimes the influence of national attitudes and concepts of sovereign rights impede the progress of harmonization and deter the acceptance of Codex standards.

Most countries have, however, responded by introducing long-overdue or reviewed/aligned existing food legislation and Codex-based national standards and by establishing or strengthening food control agencies.

Regional Regulation and Trade Agreements

The Uruguay Round Agreements provide groups of member countries the opportunity to enter into trade agreements among themselves for the purpose of liberalizing trade. So far, three such agreements have been established, all of them having adopted measures consistent with the principles embraced by the SPS and TBT Agreements and which relate to Codex standards:

- NAFTA (North American Free Trade Agreement between Canada, the United States, and Mexico) includes two ancillary agreements dealing with sanitary and phytosanitary measures and technical barriers to trade. With regard to SPS measures, Codex standards are cited as basic requirements to be met by the three member countries in terms of health and safety aspects of food products.
- The Food Commission of MERCOSUR (Treaty of Asunción establishing the Southern Common Market between Argentina, Brazil, Paraguay, and Uruguay)

recommends a range of Codex standards for adoption by member countries and is using other Codex standards as points of reference in continuing deliberations.

- APEC (Asia–Pacific Economic Cooperation between 18 Asian and Pacific countries) has drafted a Mutual Recognition Arrangement on Conformity Assessment of Foods and Food Products. This calls for consistency with SPS and TBT requirements as well as with Codex standards.

In addition, the EU (European Union) directives and regulations frequently refer to the Codex Alimentarius as the basis for their requirements.

Commercial Trade

Besides providing the reference for national legislation, the texts established by the CAC, in particular the commodity standards, are frequently used as references in commercial trade, independent of legislation. Such reference points may be used for price setting and for specifying any deviations agreed.

Codex Texts Relevant for Dairy Production and Trade

The Codex Alimentarius includes general texts applicable across-the board to all foods (general standards, codes of hygienic practices), other codes of practices (technology, control), and commodity-specific texts (commodity standards, commodity-specific codes of practices).

Milk Product Standards

When Codex decided to adjourn the old Milk Committee in 1993 and replace it with the Codex Committee on Milk and Milk Products, it was agreed that the file of standards established earlier needed extensive revision. This task has almost been accomplished. Updated versions of the milk product standards are as follows (*see* **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products):

- butter (2006);
- dairy fat spreads (2008);
- milk fat products (including butter oil, anhydrous milk fat, and ghee) (2006);
- cream and prepared creams;
- cheese (2008);
- unripened cheese, including fresh cheese (2001);
- cheese in brine (1999);
- individual cheese varieties (Mozzarella, Cheddar, Danbo, Edam, Gouda, Havarti, Samsø, Emmental, Tilsiter, Saint-Paulin, Provolone, Cottage Cheese, Coulommiers, Cream Cheese, Camembert, Brie, Extra hard Grating) (2008);
- whey cheese (2006);
- fermented milks (2008);
- evaporated milks (1999);
- sweetened condensed milks (1999);
- milk powders and cream powders (1999);
- edible casein products (2001);
- whey powders (2006); and
- lactose (now part of the standards for sugars) (1999).

Revisions have not yet been finalized for named variety processed cheese, processed cheese, and processed cheese preparations. The nonrevised standards date back to the 1960s and 1970s but are still valid as references.

Other Commodity Standards

From a dairy perspective, international standards for non-dairy foods that contain significant dairy ingredients and/or are intended to replace dairy products in consumption patterns are of interest. The most important of these commodities regulated by Codex are

- standards for chocolate, cocoa butter, coconut milk and three standards covering various blends of preserved skimmed milk and vegetable fat;
- standards for infant formula, follow-up formula, canned baby foods, and processed cereal-based foods for infants and young children; and
- vegetable protein products and soy protein products.

Food Hygiene

Food hygiene constitutes the cornerstone in Codex food safety activities. Microbiological hazards constitute the greatest risk for human health. In recent years, focus has been on the development of appropriate tools to assess and manage risks associated with the intake of microbiological hazards through food. New metrics have been identified and described, but they are not implemented as yet. Risk managers including Codex continue using general and specifically targeted good hygienic practices supplemented by the HACCP (Hazard Analysis and Critical Central Point) approach. Of particular interest to the dairy sector are the following Codex hygiene texts that build on well-established concepts:

- Code of Practice of General Principles for Food Hygiene (2003);
- The HACCP System and Guidelines for its Application (1997);

- Principles for the Establishment and Application of Microbiological Criteria (1997);
- Guidelines on the Application of General Principles of Food Hygiene to the Control of *Listeria monocytogenes* in Ready-to-Eat Foods (2007); and
- Code of Hygienic Practice for Powdered Formulae for Infants and Young Children (2008).

Other texts that introduce more risk-based approaches for food hygiene management include

- Code of Hygienic Practice for Milk and Milk Products (2004);
- Principles and Guidelines for the Conduct of Microbiological Risk Management (2007); and
- Guideline for the Validation of Food Safety Control Measures (2008).

From a dairy perspective, it is also worth noting the existence of the Codex Guidelines for the Preservation of Raw Milk by the Lactoperoxidase System from 1991. The guidelines for this system apply only where the infrastructure does not provide facilities for refrigeration.

Food Labeling

Labeling constitutes a significant part of most food regulations. A number of standards and guidelines for the labeling of foods, primarily prepackaged foods, have been developed. These standards and guidelines have gained widespread use worldwide and are implemented in the national legislation of most countries. For dairy products, the most relevant are

- General Standard for the Labelling of Prepackaged Foods (2008);
- General Guidelines on Claims (1991);
- Guidelines on Nutrition Labelling (2006); and
- Guidelines for Use of Nutrition and Health Claims (2008).

Among the issues currently being considered are issues related to nutrition labeling and labeling aspects of foods derived from modern biotechnology.

The General Standard for the Use of Dairy Terms is especially of interest from the dairy point of view. It provides guidance on where and how to use terms in the labeling and marketing of foods (*see* **Labeling of Dairy Products**).

Food Additives

Codex addresses food additives in horizontal texts as well as in commodity standards. Immense resources have been allocated to the establishment of a comprehensive General Standard for Food Additives. This standard is kept under constant review (*see* **Additives in Dairy Foods:** Consumer Perceptions of Additives in Dairy Products; Emulsifiers; Legislation; Safety; Types and Functions of Additives in Dairy Products).

Contaminants

Codex addresses contaminants in horizontal texts as well as in commodity standards. A comprehensive General Standard for Contaminants and Toxins in Food is the most important. This standard is kept under constant review. In addition, the Code of Practice for the Reduction of Aflatoxin B_1 in Raw Materials and Supplementary Feeding Stuffs for Milk-Producing Animals (1997) and a Code of Practice for the Prevention and Reduction of Dioxin and Dioxin-like PCB Contamination in Food and Feeds (2006) are of interest to the dairy sector.

Control of contaminant levels is also addressed in the HACCP System and Guidelines for its Application (*see* **Contaminants of Milk and Dairy Products**: Environmental Contaminants; Contamination Resulting from Farm and Dairy Practices; Nitrates and Nitrites as Contaminants).

Residues of Veterinary Drugs in Foods

Within the veterinary field, Codex has established a database on MRLs for individual foods and categories of foods, including those that specifically relate to milk. Also of specific relevance to the dairy sector are the Guidelines for the Establishment of Regulatory Programme for the Control of Veterinary Drugs in Food and a Code of Practice for Control of the Use of Veterinary Drugs (1993) and a Code of Practice to Minimize and Contain Antimicrobial Resistance.

Pesticide Residues

Codex has established a huge database on recommended maximum limits for individual foods and categories of foods.

Food Import and Export Inspection and Certification Systems

The objective of Codex to facilitate the free movement of foods is also pursued through the establishment of recommendations for food control and inspection agencies. For the dairy sector, the most relevant texts developed include

- Principles for Food Import and Export Certification (1995);
- Guidelines for Design, Production, Issuance and Use of Generic Official Certificates (2007);
- Guidelines for Food Import Control Systems (2006);
- Principles for Traceability/Product Tracing as a Tool Within a Food Inspection and Certification System (2006);

- Model Export Certificate for Milk and Milk Products (2008, amended 2010); and
- Guidelines for the Judgement of Equivalence of Sanitary Measures Associated with Food Inspection and Certification Systems (2008).

Methods of Analysis and Sampling

In principle, any criteria specified in an established Codex text need to be followed up by the identification of appropriate and validated methods of sampling and analysis. Codex frequently reviews and updates an inventory of endorsed methods (Analysis of Milk and Dairy Products).

See also: **Additives in Dairy Foods**: Consumer Perceptions of Additives in Dairy Products; Emulsifiers; Legislation; Safety; Types and Functions of Additives in Dairy Products. **Contaminants of Milk and Dairy Products**: Contamination Resulting from Farm and Dairy Practices; Environmental Contaminants; Nitrates and Nitrites as Contaminants. **Labeling of Dairy Products**. **Policy Schemes and Trade in Dairy Products**: Standards of Identity of Milk and Milk Products. **Risk Analysis**.

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Standards of Identity of Milk and Milk Products

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Introduction

When compared to other food sectors, the dairy sector has a distinct tradition of regulating production and trade through identity standards. This tradition derives primarily from the cooperative structure of the sector prevailing in the late 1800s and the beginning of the 1900s, and was introduced mainly by exporting countries in support of their trade activities and by countries with a significant domestic competition.

Since the mid 1980s, food legislation in general has been subject to substantial changes. General (horizontal) regulation supersedes commodity (vertical) legislation. This change is caused mainly by changed budgetary priorities at national governments level and by the entire focus on food safety issues. The general trend is that vertical legislation decreases, and specific dairy legislation even disappears in some countries.

Dairy legislation itself has changed as well. A few decades ago, dairy legislation was, in many countries, characterized by a positive approach (i.e., what is not specified is not permitted), the most significant result being a vast number of identity standards. Today, most dairy legislation has been aligned with the approach used for other food sectors, where everything considered safe and suitable is allowed in principle, and governed primarily by informative labeling. Identity standards fit into the latter approach by providing the specific conditions for referring to a regulated name without by themselves enforcing general restrictions.

The dairy trade needs some degree of international regulation to ensure fair practices and to minimize barriers to trade. For the enforcement of the WTO trade agreements, the international trade further needs a sound reference to enable monitoring of whether import restrictions are justified, and if not, to provide the tool for pursuing the goal of avoiding technical barriers to trade.

This article focuses mainly on the Codex standards of identity, as these have the greatest potential for widespread use.

Role of Identity Standards

Identity standards serve a multifunctional role as follows:

1. *As legislative reference texts*

   Nationally, such reference texts may be needed to facilitate understanding and management of other regulatory means (e.g., safety limits, additive provisions, inspection practices, certification, statistics, monitoring import/export quotas)

   Internationally, reference texts are intended to

   - enable the WTO system to solve trade disputes (Codex standards);
   - assist governments (and other parties concerned) in the elaboration and implementation of national food legislation in an appropriate manner; and
   - guide international trade.

2. *As facilitators of trade*

   International harmonization is a prerequisite for free movement of products, which can be achieved only through international cooperation. The result of harmonization is, as per definition, the minimization of barriers to international trade. The establishment of proper reference texts is a prerequisite for harmonization.

   If trade is to be facilitated, it is necessary to ensure that such reference texts are kept up-to-date with respect to technological, scientific, and practical knowledge. Otherwise, they may become obstacles to trade.

3. *As promoters of fair trade practices*

   The most important objective of an identity standard for milk products is to promote fair trade practices through specifying detailed conditions for the use of specific names reserved for well-defined milk products.

   International standards also assist in protecting against misleading presentations and practices, for example, a false description of the nature of the product in question.

   However, achieving international consensus is difficult, mainly because those involved in the process are also commercially competing on the world market. On the other hand, if no difficulties existed, there would be no commercial need for attempting harmonization!

   With regard to milk and milk products, the most important international text for supporting this objective is the Codex General Standard for the Use of Dairy Terms which reserves, with a few exemptions, names and terms related to dairy products for milk and milk products. Many dairy countries worldwide have established similar regulations.

Identity standards normally do not aim specifically at protecting public health, as this objective is typically regulated in more general legislative texts (e.g., Codes of Practices, hygiene rules, MRLs).

Standard Setting

National Standards

Several countries do, despite of the general change in the approach to food regulation, retain a significant number of specific identity standards for milk products in their national legislation. This is particularly the case in countries where the dairy industry has played and still plays a significant role in the national economy and retention is generally supported by the domestic dairy industries. On the other hand, very few new identity standards for milk products are being established.

Examples of dairy nations that retain a significant number of identity standards for milk products include Canada, Denmark, France, Germany, Greece, Gulf countries, Italy, Japan, MERCUSOR countries (Argentina, Brazil, Uruguay, and Paraguay), South Africa, Spain, Switzerland, and the United States. Certain countries, mainly European, have, in addition to generic identity standards, established systems for reserving certain product names as protected geographical designations.

In general, only the basic and the most significant milk products are regulated today by identity standards. Typically, national legislation that includes identity standards for milk products, retain such for drinking milk, traditional fermented milks characterized by specific microorganisms, individual cheese varieties, milk powders, and butter. The cheese varieties most commonly regulated by national identity standards are Brie, Camembert, Cheddar, Cottage Cheese, Cream Cheese, Danbo, Edam, Emmental, Gouda, Mozzarella, Parmesan, Provolone, and Tilsiter.

Regional Standards

The Stresa Convention

The first international standard-setting body within the dairy sector was the so-called Stresa Convention, named after the Italian city Stresa. The Stresa Convention, adopted in 1951, lays down a number of standards for individual cheeses and stipulates regulations for the use of these product names. The International Dairy Federation (IDF) played a significant role in its establishment. Originally, eight European countries ratified the Convention. Several of these have now left. For this reason, and since the standards regulated by the Convention have hardly been up-dated since their establishment, the Stresa Convention today plays an insignificant role. However, the principles contained therein have been adopted in other cheese regulations at national, regional, and international levels (**Figure 1**).

EU standards

With the establishment of the European Common Market, a number of regional identity standards have been developed, particularly during the 1970s and 1980s. Identity standards have been established for preserved milk products (milk powder, evaporated milks, sweetened condensed milks), edible casein products, butter, including reduced fat butters, and drinking milk. The EU standards are mandatory in all EU member states. At the beginning of the 1990s, the strategy for establishing regional standards was abandoned and replaced by a system of registering protected designations of origin and certificated products of specific character.

Gulf standards

The Gulf Cooperation Council (GCC), established in 1981, develops Gulf standards relating to foodstuffs, intended for adoption by its member states (Saudi Arabia, Kuwait, Bahrain, Qatar, Oman, and the United Arab Emirates). Their influence has become increasingly significant.

Most dairy products are currently regulated by the GCC identity standards: raw milk (cow, goat, and

Reservation of four cheese names to be used only by the country in which the names were first developed (Annex A): Rquefort (France), Gorgonzola (Italy), Parmiggiano Romano (Italy), and Pecorino Romano (Italy).

Mutual permission to use 30 cheese names on domestic and international markets, on the labels and by reference anywhere, provided adherence to the identity standards subordinated (Annex B):

Cheese name:	*Origin of name:*
Danablu, Danbo, Elbo, Fynbo, Havarti, Maribo, Mycella, Samsø, and Tybo	Denmark
Brie, Camembert and Saint Paulin	France
Gruyere	France and Switzerland
Asiago, Caciocavallo, Fiore Sardo, Fontina, Provolone	Italy
Edam, Frisian, Gouda, Leyden	The Netherlands
Gudbrandsdalsost, Nøkkelost	Norway
Ädelost, Herregaardsost, Svecia	Sweden
Emmental, Sbrinz	Switzerland

Figure 1 Main elements of the Stresa Convention.

camel), drinking milks (pasteurized, sterilized, UHT, flavored), preserved milk products (dried milk, evaporated milk, sweetened condensed milk, lactose, and caseinates), fermented milk including yogurt (with and without heat treatment after fermentation), cream, butter, milk fat products (anhydrous milk fat, butteroil, and ghee (samn)), processed cheese (including spreadable), cheese, whey cheese, and a number of individual cheese varieties, which besides all varieties standardized by Codex Alimentarius also include Feta, Domiati, Gruyere, Hallom, Kashkaval, and Ras. These standards are subsequently adopted by the GCC member states.

GMC standards

In 1991, the MERCUSOR (Treaty for the Organization of a Southern Common Market) was established with four countries participating (Argentina, Brazil, Uruguay, and Paraguay). To facilitate trade, harmonized food legislation is being developed by the Common Market Group (GMC). GMC standards are mandatory in all MERCUSOR member states. Identity standards for the following milk products have been established: butter, butteroil, dairy cream, dairy cream for industrial use, UHT milk, fluid milk for industrial use, powdered milk, *dulche de leche*, food caseinates, food casein, cheese, grated cheese, processed cheese, powdered cheese, and the individual cheese varieties Danbo, Tilsit, Mozzarella, Minas Frescal, Tybo, Cottage Cheese, Tandil, Pategras, Sandwich, and Prato.

International Standards

Codex Alimentarius

The elaboration of international milk product standards was initiated several years prior to the establishment of Codex Alimentarius. In 1958, on the initiative of the IDF, the FAO established the Joint Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products. The establishment of this 'Milk Committee' showed the need for international cooperation in the area of food and triggered the establishment of Codex 4 years later.

The Milk Committee was active until 1990, during which period 52 international milk product standards and several other texts related to milk products were developed. However, as the running of the Milk Committee was financed by the FAO, financial priorities resulted in difficulties that hampered the continuation of the work. The Committee was very close to being adjourned at the beginning of the 1990s.

In the light of the elaboration of the new WTO trade agreements, however, a FAO/WHO Conference held in 1991 highly recommended the revision of all commodity standards developed by Codex. This was the main reason for establishing the new Codex committee for Milk and Milk Products in 1993. The new committee has almost completed its work after a thorough revision of all the existing standards as well as elaborating new standards for additional milk products. When the work is fully completed in 2010, approximately 34 specific identity standards for various milk products will have been described.

Codex standards are intended as recommendations for adoption by member states and for use by the commercial trade. However, they also serve as international references for application in trade disputes brought to the WTO for settlement (*see* **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius).

World Customs Organization

The World Customs Organization (WCO) is an independent, inter-governmental organization, the purpose of which is to enhance the effectiveness and efficiency of customs administration on a worldwide basis. The most successful instrument developed by the WCO is the Harmonized Commodity Description and Coding System (Harmonized System).

The Harmonized System (HS) is a multipurpose international product nomenclature covering about 5000 commodity groups. Each commodity group is identified by a unique six-digit code and is defined in such a way as to facilitate uniform classification. In many cases, explanatory notes provide identity descriptions of individual commodities.

More than 177 countries use the system as a basis for determining customs tariffs and for the collection of international trade statistics. Other uses include the following: the basis for rules of origin; the collection of internal taxes; the basis for trade negotiations (e.g., the WTO schedule for tariff concessions), transport tariffs and statistics, and the monitoring of controlled goods (such as hazardous wastes, narcotics, and chemical weapons).

The majority of dairy products are found in Chapter 4 of the Harmonized System. However, Chapter 4 does not cover lactose (Chapter 17), ice cream, and dairy spreads (Chapter 21) or albumins, including concentrates of two or more whey proteins (Chapter 35).

The work of the WCO has become increasingly important as world trade in dairy products continues to grow. In particular, product definitions and classification within the Harmonized System greatly influence decisions in product development and marketing.

In addition, the Harmonized System is incorporated by individual governments into domestic operating procedures for a variety of purposes. Among these are internal taxes, monitoring of controlled goods, freight tariffs, transport statistics, price monitoring, quota controls, and more.

Product definitions developed by the WCO in support of the HS do not necessarily correspond with the definitions established in support of food legislation. The individual definitions serve different purposes and are usually developed independently from each other. Consequently, a product labeled with a name in accordance with the food regulation in force may not be classified accordingly by the HS and *vice versa.*

Codex Milk Product Standards

Key Prerequisites for Establishing a Codex Milk Product Standard

Consumer protection from the point of view of health and fraudulent practices is one of the key objectives of Codex activities. As health protection alone does not justify the establishment of a commodity standard as such, Codex commodity standards primarily aim at protecting the consumers from fraudulent practices and ensuring fair trade practices. Public health protection is covered by other horizontal and commodity-specific Codex texts concerning hygiene, additives, and contaminants.

Whether a risk of fraudulent practice exists depends on the following:

- The degree of consumer's recognition of the product (or designation), expressed as the number of countries in which the product is manufactured and consumed;
- The needs of importing-countries to require specific labeling to ensure that adequate consumer information is provided with regard to the nature of the product; and
- Existing differences in national definitions of the product.

An estimate of the global production may be needed to ensure that drafting resources are not wasted on products that are insignificant in international trade. Production and trade statistics are important tools for evaluating whether an international standard will be justified.

Information on the number of countries involved in the trade of the product in question is used for guiding the decision. Local trade between a smaller group of countries (e.g., within one trading block) is not a sufficient justification.

It is apparent that the number of countries having established national identity standards and/or industry standards governing the use of a certain product name constitutes a potential for trade problems. Significant deviation between such standards may alone trigger a need to harmonize at international level (**Figure 2**).

Principal Contents of Codex Milk Product Standards

Codex milk product standards aim at describing the nature (identity) of the products by addressing the essential characteristics associated with them. The areas covered by such standards are as follows:

- Identity characteristics (common understanding of the meaning of a product name including an end-product description, principal method(s) of manufacture, compositional requirements, and ingredients)
- Food additives that are safe and technologically justified
- Labeling provisions that are needed in addition to general labeling rules and/or that are considered necessary for the correct application of a general labeling principle.

This approach is based on the almost completed revision of the milk product standards developed in the 1960s and 1970s. The former versions contained primarily provisions that aimed at ensuring technical product quality and product identity.

Elements that are not justified as essential for the identity of the product cannot be addressed in the standards. Where found appropriate, such material can be provided as information on usual patterns of production in appendices to the standards. The content of an appendix (1) is intended to be applied by commercial trade parties (where found useful), (2) need not be implemented in national legislation, and (3) is not intended to be used by the WTO as reference material. Typical information provided in appendices indicates quality limits on nonhazardous contaminants (e.g., copper); technical quality guidelines; characteristic flavor and taste; nonessential sizes and shapes; and nonessential manufacturing practices.

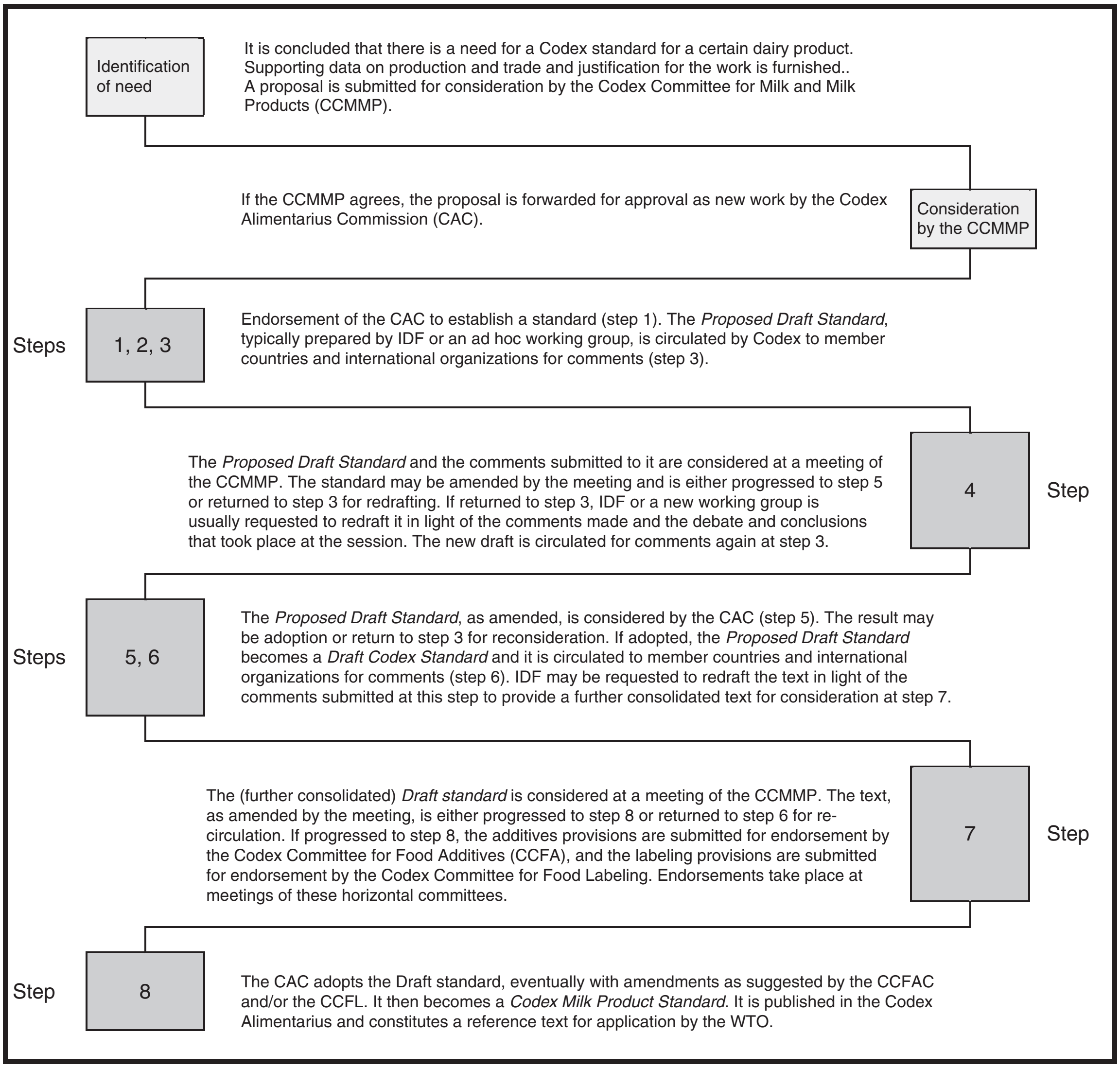
In addition to the principal contents, food safety and general labeling issues are addressed by the Codex milk product standards, mainly by identifying and referring to other relevant Codex texts applicable to the product (e.g., contaminants, hygiene, labeling, and methods of sampling and analysis).

General Approach to Codex Milk Product Standards

Scope

The Codex milk product standards apply both to retail products sold directly to the consumer and to non-retail products intended for further processing (e.g., as ingredients in other milk products and foods, or for processing into processed cheese, cutting/slicing, drying, fermentation, etc.).

Most of the standards do not address addition of non-dairy ingredients intended to provide specific non-dairy flavors (such as fruit preparations, meat, vegetables, spices, and sweeteners). The provisions governing such additions are located in the Codex General Standard for the Use of Dairy Terms (GSUDT). According to the GSUDT, such products are identified as 'composite milk products' and



It is concluded that there is a need for a Codex standard for a certain dairy product. Supporting data on production and trade and justification for the work is furnished.. A proposal is submitted for consideration by the Codex Committee for Milk and Milk Products (CCMMP).

If the CCMMP agrees, the proposal is forwarded for approval as new work by the Codex Alimentarius Commission (CAC).

Endorsement of the CAC to establish a standard (step 1). The *Proposed Draft Standard*, typically prepared by IDF or an ad hoc working group, is circulated by Codex to member countries and international organizations for comments (step 3).

The *Proposed Draft Standard* and the comments submitted to it are considered at a meeting of the CCMMP. The standard may be amended by the meeting and is either progressed to step 5 or returned to step 3 for redrafting. If returned to step 3, IDF or a new working group is usually requested to redraft it in light of the comments made and the debate and conclusions that took place at the session. The new draft is circulated for comments again at step 3.

The *Proposed Draft Standard*, as amended, is considered by the CAC (step 5). The result may be adoption or return to step 3 for reconsideration. If adopted, the *Proposed Draft Standard* becomes a *Draft Codex Standard* and it is circulated to member countries and international organizations for comments (step 6). IDF may be requested to redraft the text in light of the comments submitted at this step to provide a further consolidated text for consideration at step 7.

The (further consolidated) *Draft standard* is considered at a meeting of the CCMMP. The text, as amended by the meeting, is either progressed to step 8 or returned to step 6 for re-circulation. If progressed to step 8, the additives provisions are submitted for endorsement by the Codex Committee for Food Additives (CCFA), and the labeling provisions are submitted for endorsement by the Codex Committee for Food Labeling. Endorsements take place at meetings of these horizontal committees.

The CAC adopts the Draft standard, eventually with amendments as suggested by the CCFAC and/or the CCFL. It then becomes a *Codex Milk Product Standard*. It is published in the Codex Alimentarius and constitutes a reference text for application by the WTO.

Figure 2 Typical process of elaboration of a Codex milk product standard.

the milk product names can be used in combined designations of composite milk products, provided that the added nondairy ingredients are not intended to replace any milk constituent(s), in whole or in part.

Principal method of manufacture

Milk product designations often originate from descriptive terms that refer to the way in which they are manufactured, for instance, milk powder, evaporated milk, fermented milk, and so on. As a natural consequence, the principal method of manufacturing is reflected in the identity description of many milk products. This approach is often much simpler than attempting to describe all end-product characteristics in full detail.

One of the general principles in the WTO Trade Agreements is the principle of equivalence. Since methods of manufacture applied are not static, and because identity standards should not, unless specifically justified, constitute an obstacle for technological development, most Codex standards for milk products include appropriate wording that, in addition to the principal method of manufacturing, provides for alternative technologies that achieve an equivalent outcome.

Raw materials

The general approach is that all milk products derived from any milking animal species can be used as raw material for milk products. This means that milk products can be processed from milk derived from cows, goats, sheep, buffaloes, yaks, camels, reindeer, and so on. Corresponding provisions address whether the dairy species need be labeled (*see* **Labeling of Dairy Products**). However, many individual cheese varieties (but not all) are characterized by the origin of the milk (texture, color, flavor) wherefore restrictions on type of milk may be imposed.

Further, in general, any milk constituents and milk products, including intermediate products, can be used, including those needed for reconstitution and recombination, as long as the compositional criteria and other characteristics of the products are met. There are a few exemptions from this approach (e.g., milk powders).

Composition

The minimum and/or maximum requirements for the composition of end products constitute a core part of any identity standards. For all milk product standards established by Codex, such criteria comprise milk fat, and additional criteria as necessary according to the nature and characteristics of the product in question (see **Figure 3**).

The compositional sections of the standards typically specify the reference composition and, in addition, the limits and conditions for modifying the composition of the reference product, by specifying, for example, the range of composition permitted. Therefore, some compositional criteria are absolute (minima or maxima).

The Codex General Standard for the Use of Dairy Terms states that products that have been modified in composition beyond the composition of the reference product identified in the relevant standard are only allowed if the following principles are adhered to:

- That a qualifier that clearly describes the compositional modification made is placed in association with the name of the product
- That the modification is achieved only by the addition and/or withdrawal of milk constituents
- That such modification does not alter the basic identity of the product and is within the limitations identified in the Codex identity standard concerned.

See **Figure 4** for examples of modifications covered.

In some cases, compositional modification is not desirable, for instance, protein contents below the minima specified and fat reductions below the absolute minima, where such are specified (**Figure 4**).

Food additives

A positive list of justified and permitted groups of additives and/or individual additives is provided. Technological justification for each functional group of additives and for each additive, with a numerical ADI-value listed, is a prerequisite for acceptance by Codex. The IDF provides the information necessary for this purpose.

Labeling

The labeling section makes cross-reference to generally applicable labeling standards and lays down additional labeling provisions as well as practical guidance on the application of general labeling principles (*see* **Labeling of Dairy Products**).

Supporting methods of sampling and analysis

Any criteria specified in a Codex standard are to be supported by appropriate analytical methods for verifying compliance. Recognized methods are published in the Codex Alimentarius.

Milk product	*Milk fat*	*Milk protein*	*Moisture or dry matter*	*Solids-not-fat*	*Lactose*	*Ash*	*Other*
Creams	√						
Fermented milks	√	√					Acidity, microorganisms flavoring ingredients
Butter	√		√	√			
Dairy fat spreads	√			√			
Butteroil	√		√				
Evaporated milks	√	√	√				
Sweetened condensed milks	√	√		√			
Milk and cream powders	√	√	√				
Cheese							Ratio of whey protein to casein
Individual cheese varieties	√		√				Variety specific
Caseins	√	√	√		√	√	Casein, free acid
Caseinates	√	√	√		√		pH, casein
Whey powders	√	√	√		√	√	pH

Figure 3 Components regulated by compositional requirements in certain Codex milk product standards.

Fat-modified products such as *high fat*, *reduced fat* (*light*), *low fat*, or *skimmed/nonfat,*
Purified products such as *demineralized whey powder, lactose-free milk powder,* and *cholesterol-free butter*;
Fortified products such as *vitaminized milk powder* and *calcium enriched yogurt*;
Compositionally altered products such as *fractionated butter.*

Figure 4 Examples of compositionally modified milk products.

The Individual Codex Milk Product Standards

Drinking milk

Codex has not established, nor does it intend to establish, an identity standard for drinking milk. Although manufactured and marketed in most countries worldwide, drinking milk is primarily sold domestically. Hence, due to significant local differences in consumer perception, attempts to harmonize this area would probably fail.

However, some general principles governing drinking milk have been provided in the Codex GSUDT. These principles are targeted national regulations in this area. The GSUDT states that, in general, drinking milk that is modified in composition by the addition and/or withdrawal of milk constituents may be identified with a name using the term ‘milk’, provided that a clear description of the modification to which the milk has been subjected is given in close proximity to the name.

However, fat and protein adjustment may be permitted without such description only if

- the milk is sold where such adjustment is permitted in the country of retail sale;
- the minimum and maximum limits of fat and/or protein content (as the case may be) of the adjusted milk are specified in the legislation of the country of retail sale (the protein content shall be within the limits of natural variation within that country); and
- the adjustment methods permitted by the legislation of the country of retail sale are used. Such methods shall include only the addition and/or withdrawal of milk constituents without altering the whey protein-to-casein ratio (as achieved by traditional ultrafiltration technology).

Creams

The standard for cream addresses ‘cream’ (bulk) and ‘prepared creams’ intended for direct consumption. The standards include a definition of cream and descriptions of recombined and reconstituted cream, respectively. A number of specific consumer products are described, including prepackaged liquid cream, whipping cream, whipped cream, cream packed under pressure, thickened cream, and fermented cream.

The standard characterizes creams as milk products comparatively rich in fat (absolute minimum of 10% milk fat), in the form of an emulsion of fat in skimmed milk, obtained by physical separation from milk or by recombination/reconstitution of specified raw materials.

Fermented milks

The standard characterizes fermented milks as milk products obtained by fermentation of milk by the action of specific microorganisms and resulting in a reduction of pH with or without achieving coagulation. These specific microorganisms shall be viable, active, and abundant in the product. In addition, the standard includes specific categories of fermented milks that are additionally characterized by specific microorganism(s) used for the fermentation, which organisms during product shelf life are present in numbers exceeding 10^7 cfu g^{-1} (plain part of the end product). Specific categories will include yogurt, kefir, acidophilus milk, and kumys.

The products may be heat treated after fermentation, in which case the name shall be ‘heat treated fermented milk’. Reference to the specific names defined by minimum bacterial counts is obviously not relevant in these products.

The standard aims also at including fermented milks modified in composition by concentration of the protein to minimum 5.6% – identified as ‘concentrated fermented milks’.

This standard (being the only one at that) includes flavored products (i.e., plain fermented milk to which other foods/ingredients, such as fruit, sugar/sweetener, or cereals, have been added to obtain a characteristic nondairy flavor).

Butter and milk fat products

Three milk product standards for yellow milk fats exist: one for butter, one for dairy fat spreads, and a third for milk fat products (covering the names ‘milk fat’, ‘anhydrous milk fat’, ‘butteroil’, ‘anhydrous butteroil’, and ‘ghee’).

Butter is, according to the Codex standard, characterized by being a fatty milk product, principally in the form of an emulsion of the water-in-oil type, with minimum 80% milk fat, maximum 16% moisture, and maximum 2% milk solids-not-fat. These compositional criteria are specified as absolute. Unlike many national standards, no upper milk fat limit is specified. Instead, it is stated that fat contents above 95% trigger the use of a qualifier in association with the term ‘butter’; such a qualifier can be, for instance, ‘cooking’.

Dairy fat spreads are conceptionally butter with lowered fat contents above 10% and where milk fat constitutes at least two-thirds of the dry matter.

The milk fat products covered are characterized as fatty milk products obtained by means of processes that result in almost total removal of water and nonfat solids. For ghee, restrictions on milk ingredients as raw material are specified, and the product is further characterized by having a special flavor and physical structure, without further details being provided, however.

Preserved milk products

Standards for three categories of traditional preserved milk products have been established: for evaporated milks, for sweetened condensed milks, and for milk powders and cream powder.

In common, these products are characterized by being obtained by the partial removal (to various degrees) of water from milk (or cream) by the use of heat or other processes leading to the same composition and characteristics. Milk-based raw materials are restricted.

Sweetened condensed milk is further characterized by being preserved with the addition of sugar (sucrose alone or sucrose in combination with other sugars).

The adjustment of fat and/or protein is specifically addressed in all three standards, and can be done provided that it is achieved only by addition/withdrawal of milk permeate, milk retentate, and lactose, and provided that the whey protein-to-casein ratio of the milk subjected to adjustment is not altered.

Common to all three standards is also that the whole range of fat content is covered by a classification system and that the minimum protein content should be 34% of milk solids-not-fat. Corresponding criteria for milk solids-not-fat content are established for the liquid products, whereas a maximum moisture content of 5% is specified for the powders. These compositional criteria are specified as absolute.

Cheese

A general standard for Cheese supplemented by subordinated group standards for cheese in brine and unripened cheeses, respectively, has been established.

Cheese is primarily characterized according to the way it is manufactured. Cheese is the ripened or unripened, soft or firm, hard or extra hard, product obtained by

(a) coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from such coagulation, while respecting the principle that cheesemaking results in a concentration of milk protein (in particular, the casein portion), and that consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made; and/or

(b) processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end product with similar physical, chemical, and organoleptic characteristics as the product defined under (a).

Part (a) reflects the traditional manufacturing method which is still the dominating process used worldwide, and part (b) allows for other processing techniques such as recombination, reconstitution, protein standardization, and membrane filtration in general are covered by subparagraphs. Products complying either to part (a) or to part (b) are equivalent, that is, they are not to be distinguished in any way. Today, the cheese manufacturing process is a combination of (a) and (b).

The standard does not specify any compositional criterion but that (1) the protein content is distinctly higher than that of the milk used and that (2) the whey protein-to-casein ratio does not exceed that of the milk used. If the whey protein-to-casein ratio is higher, then the product is to be categorized as 'whey cheese'.

Cheese is generally classified according to principal ripening and firmness as follows:

Classification according to principal ripening

- Ripened cheese, which is cheese not ready for consumption shortly after manufacture but must be held for such time, at such temperature, and under such other conditions as will result in the necessary biochemical and physical changes characterizing the cheese in question;
- Mold ripened cheese, which is ripened cheese in which the ripening has been accomplished primarily by the development of characteristic mold growth throughout the interior and/or on the surface of the cheese;
- Cheese in Brine, which is ripened cheese that has been ripened and preserved in brine until delivered to, or prepacked for, the consumer; and
- Unripened cheese, which is cheese ready for consumption shortly after manufacture.

Classification according to firmness

- Soft cheese, which is cheese with a content of moisture on fat-free basis above 67%;
- Firm (or semihard) cheese, which is cheese with a content of moisture on fat-free basis between 54 and 69%;
- Hard cheese, which is cheese with a content of moisture on fat-free basis between 49 and 56%; and
- Extra hard cheese, which is cheese with a content of moisture on fat-free basis below 51%.

The general cheese standard applies to all cheeses, including individual varieties of cheese.

Individual cheese varieties

Subordinate to the general cheese standard, 17 standards for individual cheese varieties exist: Cheddar, Danbo, Edam, Gouda, Havarti, Samsø, Emmental, Tilsiter, Saint-Paulin, Provolone, Cottage Cheese, Coulommiers, Cream Cheese, Camembert, Brie, Extra Hard Grating, and Mozzarella.

Raw materials for the manufacture of these individual cheese varieties are restricted to milk and milk products derived from cows and buffalos. However, in the case of Extra Hard Grating, the dairy species permitted are goats, ewes, and cows (but not buffaloes), and in the case of Cream Cheese no restrictions in this regard apply. Further, each individual variety will, in addition to the general characteristics applicable to cheeses, be characterized by end-product description including classification according to principal ripening and firmness, as well as color, texture, structure, and ripening characteristics, where appropriate. In certain cases, characteristic elements of the manufacturing methods and dimensions are specified as well.

Compositional specifications are unique for each variety and include limitations for compositional modification of the fat content.

Specific milk constituents

Two milk product standards regulate whey powders and edible casein products, respectively. A group standard for sugars includes lactose.

Whey powders are characterized as being milk products obtained by drying whey or acid whey, where whey means the fluid milk product obtained during the manufacture of cheese, casein, or similar products by separation from the curd after coagulation of milk and/or of products obtained from milk. Reference to 'whey' without qualification indicates that coagulation is obtained through the action of rennet-type enzymes, while using the term 'acid whey' means that the coagulation is obtained by acidification.

Whey powders are further characterized by a reference lactose content, minimum milk protein content, and maximum content of moisture and ash. For whey powder and acid whey powder, the values of these criteria differ slightly. The two powders are principally distinguished by pH. In addition, the standard includes specific reference to demineralization and neutralization as being acceptable compositional modifications.

Edible casein products are characterized as being milk products obtained by separating, washing, and drying the coagulum of skimmed milk and/or of other products obtained from milk. The type of coagulation (acid precipitation or enzymatic coagulation) determines whether the product is classified as acid casein or rennet casein. Differentiation is further supported by specification of ash content (acid casein max. 2.5% and rennet casein min. 7.5% ash; both figures include P_2O_5). No additives are permitted (enzymes are categorized in milk product standards as 'ingredients').

Edible caseinate is characterized as being the milk product obtained by the action of edible casein or edible casein curd with neutralizing agents, followed by drying. Caseinates may be manufactured by the use of acidity regulators, including specific neutralizing agents, emulsifiers, bulking agents, and anticaking agents.

All casein products are further characterized by criteria for protein content, casein in total milk protein, moisture content, and milk fat content. All compositional criteria specified are absolute.

Lactose is characterized as a natural constituent of milk normally obtained from whey with an anhydrous lactose content of not less than 99.0% on a dry basis. Lactose may be anhydrous or may contain one molecule of water of crystallization, or may be a mixture of both forms. No additives are permitted.

See also: **Labeling of Dairy Products**. **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius.

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Trade in Milk and Dairy Products, International Standards: Harmonized Systems

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The Historical Basis

In the aftermath of World War II, several organizations were established to secure, facilitate, and harmonize trade. The General Agreement on Tariffs and Trade (GATT), now the World Trade Organization, is the best known, but other areas have developed their own rules and objectives.

In September 1947, the 13 governments represented on the Committee for European Economic Co-operation agreed to set up a study group on the establishment of one or more European Customs Unions based on the principles of GATT. In 1948, the study group, established in Brussels, set up two committees, an Economic Committee and a Customs Committee.

The Economic Committee later became the Organization for Economic Cooperation and Development, and the Customs Committee became the Customs Co-operation Council (CCC), created on 15 December 1950, when the convention was signed in Brussels. It took 2 more years before the first session was held on 26 January 1953, a date that 30 years later became known as International Customs Day.

Customs Co-Operation Council or the World Customs Organization

The aim of this organization is to study all questions relating to customs cooperation and the technical and the economic aspects of customs systems to attain the highest possible degree of harmony and uniformity. This work is done through conventions like the Kyoto Convention on Simplification and Harmonization of Customs Procedures.

On the operational level, the work is carried out in technical committees, of which the Harmonized System Committee is central for the topic of this article.

In October 1994, the CCC adopted the working name World Customs Organization (WCO), thus mirroring the growing international significance of the organization and the development in world trade.

Nomenclature and Classification

With the growing world trade after World War II, the need for an internationally recognized common nomenclature became more and more obvious. This was discussed in CCC, and the Brussels Convention of 15 December 1950, on Nomenclature for the Classification of Goods in Customs Tariffs laid down the basics. It came into force on 11 September 1959, and was known as the Brussels Tariff Nomenclature (BTN). In 1974, it was renamed Customs Co-operation Council Nomenclature (CCCN).

The CCCN was divided into 1241 headings, 96 chapters, and 21 sections, each heading thus being identified by two groups of two digits, one for the chapter and the second for the position in the chapter. The CCCN served only one objective, tariffs, and was purely numerical.

At the same time, there were other classifications serving other purposes, the best known of which is the Standard International Trade Classification (SITC) for statistical purposes.

During the 1950s and 1960s, great effort was made to correlate these two nomenclatures, but it was still evident that there was a great need for international harmonization, especially concerning commodity description and coding.

Harmonized System Committee

In 1970, a study group was set up to prepare a Harmonized Commodity Description and Coding system that would handle classification in relation to tariffs, statistics, transport, and production, thus being a multipurpose international products nomenclature. The result of this work was the creation in 1973 of the Harmonized System Committee, which would prepare the Harmonized System (HS) on the background of the CCCN. This resulted in the Harmonized System Convention that entered into force on 1 January 1988. Since that day, an ever-increasing number of countries adhere to this system. According to the WCO, by August 2000 more than 177 countries were using the HS, and 98 countries were contracting parties.

According to Article 3 of the convention, contracting parties are obliged to ensure that their import customs tariffs and statistical nomenclature for imports and exports are in conformity with the six-digit HS. They are also obliged to make public their import and export statistics at or beyond that level (if at all).

The Harmonized System Committee is responsible for the development of the HS and takes care of dispute settlement whenever two contracting parties cannot agree on the nomenclature code for a specific product, a situation that can have substantial economic consequences for the involved parties.

The committee meets twice a year to discuss these problems and reach an agreement, eventually by voting on the subject. If one of the contracting parties does not agree with the decision taken by the committee, the party can transfer it to the council.

This means that the main tasks of the Harmonized System Committee are to propose amendments to the convention for updating the HS every 4–6 years and to prepare explanatory notes and classification opinions and other advice as guides to the interpretation of the HS and recommendations to secure uniformity in the interpretation and application of the system.

Harmonized System

The HS itself is thus a six-digit products classification system used by most countries in the world to collect tariffs and produce statistics. Some countries still have alternative systems for some uses, like the SIC codes for domestic production in the United States, but more and more the HS is being used for all purposes and by international organizations like the Organisation for Economic Co-operation and Development (OECD) and the World Trade Organization.

According to the HS, all goods can be classified in 21 sections but, for more precise classification, sections are divided into chapters. The HS consists of 97 chapters, of which Chapter 77 is reserved for future use. Furthermore, Chapters 98 and 99 are reserved for special uses by adhering countries.

To describe a chapter, two digits are always used (e.g., 04 for the chapter containing dairy products and 35 for the chapter containing casein, albumins, and others). The subdivision of chapters is done either by material (e.g., 02 – meat, 03 – fish) or by degree of manufacture or processing (e.g., 01 – live animals, 02 – meat and edible meat offals).

Titles in chapters are only guidelines; therefore two-digit chapters are not sufficient. Thus, to clarify and underline the differences between products in the same chapter, most of the chapters are divided into headings (four digits) and even subheadings (six digits), each level adding another pair of two digits to a total of six digits. In this way, the system lists more than 5000 product lines.

The HS as mentioned is dynamic. The latest amendments were decided at the 43rd Session of the Harmonized System Committee in March 2009. They were adopted by the WCO Council at its annual session in June 2009, and the recommendation is now being promulgated under the provisions of Article 16 of the Harmonized System Convention. This implies that HS contracting parties have 6 months during which they can object to a recommended amendment. The amendments will enter into force on 1 January 2012.

The Council Recommendation of 26 June 2009, with the HS2012 amendments is the fifth to amend the HS, though it is only the fourth recommendation to make major amendments to the HS since the WCO Council approved the Harmonized System Convention.

The main reasons for the latest set of 221 amendments are new environmental and social issues and the use of the HS as the standard for classifying and coding goods of specific importance to food security and early warning data falling within the ambit of the Food Security Information for Action Program of the Food and Agriculture Organization (FAO) of the United Nations.

Dairy Products in the Harmonized System

Let us take a closer look at the way dairy products are classified.

This happens in the first section of the HS, Section I – Live Animal and Animal Products. This section is divided into five chapters:

01. Live animals
02. Meat and edible meat offal
03. Fish, crustaceans, and other aquatic invertebrates
04. Dairy produce, birds' eggs, natural honey, edible products of animal origin, not elsewhere specified or included
05. Products of animal origin, not elsewhere specified or included

This is followed by the section on vegetable products.

In itself, the definition of Chapter 04 tells us that we have to be more specific to find the right code for a product. We therefore have to look at the headings:

04. Dairy produce, birds' eggs, natural honey, edible products of animal origin, not elsewhere specified or included
04.01. Milk and cream, not concentrated nor containing added sugar or other sweetening matter
04.02. Milk and cream, concentrated or containing added sugar or other sweetening matter

04.03. Buttermilk, curdled milk and cream, yogurt, kefir and other fermented or acidified milk and cream, whether or not concentrated or containing added sugar or other sweetening matter or flavored or containing added fruit, nuts, or cocoa
04.04. Whey, whether or not concentrated or containing added sugar or other sweetening matter; products consisting of natural milk constituents, whether or not containing added sugar or other sweetening matter, not elsewhere specified or included
04.05. Butter and other fats and oils derived from milk; dairy spreads
04.06. Cheese and curd
04.07. Birds' eggs in shell, fresh, preserved, or cooked
04.08. Birds' eggs, not in shell, and egg yolks, fresh dried, cooked by steaming or by boiling in water, molded, frozen, or otherwise preserved, whether or not containing added sugar or other sweetening matter
04.09. Natural honey
04.10. Edible products of animal origin, not elsewhere specified or included

This did bring us one step further, but it did not solve all our problems, as it is evident that, for example, the fat content of different milk powders would qualify for different tariff rates. We therefore have to be even more specific and add a level of subheadings before we can define the right tariff nomenclature code within the HS.

04. Dairy produce, birds' eggs, natural honey, edible products of animal origin, not elsewhere specified or included
04.01. Milk and cream, not concentrated nor containing added sugar or other sweetening matter
04.01.10 – Of a fat content, by weight, not exceeding 1%
04.01.20 – Of a fat content, by weight, exceeding 1%, but not exceeding 6%
04.01.30 – Of a fat content, by weight, exceeding 6%.

As per 1 January 2012, this last subheading will be replaced by the following:

04.01.40 – Of a fat content, by weight, exceeding 6% but not exceeding 10%
04.01.50 – Of a fat content, by weight, exceeding 10%
04.02. Milk and cream, concentrated or containing added sugar or other sweetening matter
04.02.10 – In powder, granules or other solid forms, of a fat content, by weight, not exceeding 1.5%
– In powder, granules or other solid forms, of a fat content, by weight, exceeding 1.5%
04.02.21 – – Not containing added sugar or other sweetening matter
04.02.29 – – Other
– Other
04.02.91 – – Not containing added sugar or other sweetening matter
04.02.99 – – Other
04.03. Buttermilk, curdled milk and cream, yogurt, kefir and other fermented or acidified milk and cream, whether or not concentrated or containing added sugar or other sweetening matter or flavored or containing added fruit, nuts, or cocoa
04.03.10 – Yogurt
04.03.90 – Other
04.04. Whey, whether or not concentrated or containing added sugar or other sweetening matter; products consisting of natural milk constituents, whether or not containing added sugar or other sweetening matter, not elsewhere specified or included
04.04.10 – Whey and modified whey, whether or not containing added sugar or other sweetening matter
04.04.90 – Other
04.05. Butter and other fats and oils derived from milk; dairy spreads
04.05.10 – Butter
04.05.20 – Dairy spreads
04.05.90 – Other
04.06. Cheese and curd
04.06.10 – Fresh cheese, including whey cheese, and curd
04.06.20 – Grated or powdered cheese, of all kinds
04.06.30 – Processed cheese, not grated or powdered
04.06.40 – Blue-veined cheese
04.06.90 – Other cheese

As exports of dairy products are done by a very limited number of important players, most of the countries using the HS for tariff purposes are importing countries satisfied with this level of specification, as tariff rates in general are rather uniform between these large groups of products. Some countries importing, exporting, and producing a large variety of dairy products do, however, use their right to supplement the six-digit HS codes with their own further subcodes. One example is the US subdivision of Chapter 04.06.20, grated and powdered cheese, into 38 10-digit subheadings according to cheese types, milk used, and tariff quotas.

Classification Principles

To classify a given dairy product under the correct HS nomenclature position, one must both consider some general principles and make reference to the explanatory notes given by the Harmonized System Committee. Formally, the descriptions in sections, chapters, and headings cannot be used to classify a product. One must go to the specific subheading texts.

As the HS is not very specific, this will solve most of the problems. However, when mixing products from different chapters, this may result in a totally different code under a third chapter. If, for example, butterfat is mixed with vegetable oil to make a mixed dairy spread, it will be classified in Chapter 21. This classification is independent of the production process. If vegetable fat is added to a dairy product, according to the explanatory notes it cannot be classified under Chapter 04. In principle, there are then two possible alternatives, Chapter 19 and Chapter 21. A product that needs to be distinguished from a normal dairy product falling under Subheadings 04.01–04.04 will fall under Chapter 19 according to the explanatory notes. This, however, is not the case for dairy spreads or cheeses where the milk fat part has been totally or partly replaced by vegetable fat. In this case, the final products will need to be distinguished from products falling under Subheadings 04.05 and 04.06 of the dairy chapter. Such products according to the explanatory notes must be classified under Chapter 21. In this case, more specific descriptions should come before more general descriptions.

This, however, does not mean that one cannot add anything from outside the chapter. If butter or cheese is mixed with small quantities of spices like garlic or cumin, it retains the character of butter or cheese and shall be classified as such.

These questions are very delicate as they could be used in an attempt to circumvent high tariff rates or tariff quotas for some products by adding rather neutral or very small quantities of substances that would place the final product in a different chapter with no tariff or unrestricted access. Therefore, this is an important part of the explanatory notes defined by the Harmonized System Committee.

Mixing products from within the same chapter is a different matter. If Blue cheese were mixed with processed cheese, the point of departure would be a general rule giving the nomenclature code of the substance with the highest position, in this case, the position of the processed cheese. If the two substances are identifiable, the principle would be to choose the position that makes up more than half of the quantity. However, if one of the substances has a very specific character, like a dominating taste, one should consider using the code for this substance even if it represents less than half of the quantity.

The principle of the highest code also applies within a chapter; this means that if a product fits into a subheading, it cannot be placed under a subheading that comes later under the same heading. If, for example, fresh cheeses like Mozzarella, be it the Italian type or the American variety, are grated, the resulting product remains under Subheading 04.06.10 as a fresh cheese and does not fall under Subheading 04.06.20 as a grated cheese.

This, however, does not mean that a fresh cheese cannot be processed with melting salt and classified as a processed cheese under Subheading 04.06.30, as this production process substantially alters the cheese used as raw material, so that this can no longer be identified as such.

Classification Examples

With these principles in mind, let us take a closer look at some dairy products and their classification. The author must stress that this relies on his personal opinions and experience and does in no way commit the WCO or the Harmonized System Committee. The official opinions of these bodies are to be found in the relevant texts issued by the WCO. In view of the developments in modern technology, the WCO does also have a website on the Internet (www.wcoomd.org). On this site, HS classification decisions can be found. The decisions will be published as soon as they are approved by the council under the provisions of Article 8.2 of the Harmonized System Convention. This will be about 2 months after the meetings of the Harmonized System Committee at which the decision is taken. The details published will include a complete description of goods, six-digit HS classification, and the legal basis for the classification decision.

On the website the user will also find useful information on contracting parties, HS amendments, and amendments to the explanatory notes and a compendium of classification opinions.

Heading 04.01

Under Heading 04.01 we find normal fresh drinking milk and cream, with no added sugar. However, we also find Ultra High Temperature (UHT) processed milk and sterilized cream, as these products are in no way concentrated or condensed, and thus cannot fall under Heading 04.02. If any flavor (e.g., chocolate) is added, this milk-based drink no longer falls under Heading 04.01 but under Heading 22.02.

Heading 04.02

Take some of the fresh milk or heat-treated products falling under Heading 04.01 and add some sugar, and you have a 04.02.99 product.

However, most of the products under Heading 04.02 are either milk powders or condensed milk, which in relation to the HS does not represent big problems. One should, however, bear in mind that lactose in a milk powder is not a sweetening agent. If lactose is added to a milk powder, it will, however, change position to Heading 04.04.90 (see below under Heading 04.04).

Heading 04.03

All fermented milk products fall under this heading, be it in liquid or solid form. This means that both buttermilk and buttermilk powder will fall under Subheading 04.03.90 in the HS.

Heading 04.04

All whey products, except whey butter, will fall under Heading 04.04.10 – as long as the total protein content by weight calculated on the dry matter does not exceed 80%. If it does exceed 80%, it falls under Heading 35.02, which is where you will have to classify whey protein concentrates.

In modern dairy production, a lot of products no longer contain the milk constituents in their natural composition. Lactose, whey, permeates, and other constituents are added or deducted. It is even permitted to add small quantities of non-dairy ingredients. These products fall under Heading 04.04.90. This does not mean that all milk powders fall under this heading if their composition is changed, this happens only if they no longer respect the normal natural composition. This problem will have less impact in the future because of the Codex Alimentarius Commission decision to allow protein standardization to a minimum of 34% in milk powders, as this will set a norm for the natural composition and avoid inferior products to be sold as 04.02 products.

Heading 04.05

Under this general heading 04.05.10 we find butter, also produced from whey or if recombined. Dairy spreads under 04.05.20 must not contain other fats than butterfat. These products would fall under Heading 21.06 if the butter is mixed with or replaced by vegetable fat. We also find butter oil in this chapter under Heading 04.05.90.

Heading 04.06

Here we find all kinds of cheese. As the HS is not very specific, the classification does not represent big problems. We have seen above that all fresh cheeses, also frozen or vacuum-packed Mozzarella, grated or not, will always fall under the Subheading 04.06.10.

Subheading 04.06.20 is meant mainly for cheese powder and Subheading 04.06.30 for processed cheese. Here, as was the problem with protein standardization before the Codex decision, the problem is not one of classification but of definition.

Another point worth mentioning is the fact that all Blue cheeses fall under Subheading 04.06.40, not only the tasty types like Roquefort and Danish Blue Cheese, but also very mild types and types with mixtures of blue and white mold. The decisive point is the presence of blue mold.

Combined Nomenclature

According to Article 3 of the Harmonized System Convention, any contracting party is allowed to establish in its customs tariffs or its statistical nomenclatures subdivisions classifying goods beyond the six-digit level of the HS, provided no changes are made to the HS level.

In the European Union, the introduction of the Harmonized System on 1 January 1988, was taken as an opportunity to modernize the European Union nomenclature system. Until that point, customers used the Common Customs Tariff while the statistical instrument was the NIMEXE statistical nomenclature. As per 1 January 1988, the Combined Nomenclature (CN) replaced these two nomenclatures.

The CN is, as it should be, based on the HS, but to form the CN one group of two more digits is added to create further subheadings covering some 10 800 eight-digit product groups compared with the approximately 5000 product groups of the HS. For certain import arrangements, the European Union supplements the CN code with a further 2-digit code giving the 10-digit European Union integrated tariff with 14,000 positions.

Dairy Products

As shown above, Chapter 04 of the HS contains in total 6 four-digit headings and 20 six-digit subheadings covering dairy products. These are also found in the CN, but at the eight-digit subheading level we now find 153 dairy positions (2009).

Within Headings 04.01 and 04.02, the subdivisions mainly take care of packet sizes. In Heading 04.03, the subdivision is made by fat content or sweetening of the product. In Heading 04.04, both fat and protein content and sweetening account for the subdivision. In Heading 04.05, products are at the CN level sorted out by some quality parameters: natural butter, recombined butter, or whey butter, and for the dairy spreads also the fat content. Finally, in Heading 04.06 for cheeses, the subdivision takes care of all the European Union specific varieties.

Classifying a product according to the CN is thus more complicated than according to the HS but follows broadly the same principles as mentioned above. Only in this case, the explanatory notes of the HS supplemented with the same notes from the European Union competent authorities on the CN become even more important.

Going through all these subheadings is beyond the scope of this article; one example, however, could be shown. As mentioned earlier, the United States is subdividing for import tariff purposes the HS heading 04.06.20, grated or powdered cheese of all kinds, into 38 ten-digit subheadings.

In the CN, the European Union is only subdividing this heading into 2 eight-digit subheadings:

04.06.20.10	Glarus herb cheese (known as Schabziger), made from skimmed milk and mixed with finely ground herbs
04.06.20.90	Other

Refund Nomenclature

At the European Union level, one further addition has been made to the CN to make up the Refund Nomenclature (RN). The RN is composed of the eight digits of the CN plus a four-digit extension, thus in total 12 digits. The purpose of the RN is to differentiate the export subsidy according to the real content of milk or milk substances in the final product. During recent years, substantial changes have been made to the RN to exclude positions with no products traded or no subsidy granted. If such a product with no subsidy is exported (e.g., Roquefort cheese), it shall be classified under the relevant CN heading, in this case 04.06.40.10.

If we turn once more to the example of the cheese powder chapter, in the RN we find the following subdivision:

Ex 04.06.20	–	Grated or powdered cheese, of all kinds
Ex 04.06.20.90	– –	Other
04.06.20.90.9100	– – –	Cheeses produced from whey
	– – –	Other
	– – – –	Of a fat content, by weight, exceeding 20%, of a lactose content, by weight, less than 5%, and of a dry matter content, by weight:
04.06.20.90.9913	– – – – –	Of 60% or more, but less than 80%
04.06.20.90.9915	– – – – –	Of 80% or more, but less than 85%
04.06.20.90.9917	– – – – –	Of 85% or more, but less than 95%
04.06.20.90.9919	– – – – –	Of 95% or more
04.06.20.90.9990	– – – –	Other

The logic here shows us that while the Schabziger cheese must be mentioned in the CN for import purposes – as it is produced in Switzerland – it is not needed in the RN as refunds are granted only on products of European Union origin.

Knowing the right position of a product in the RN is of very substantial economic importance to the exporter, as this decides his export subsidy, the refund. The same can be said of an importer's interest to have the right CN code on imports. It is therefore possible, according to regulation 450/2008 Article 20, to obtain a binding tariff information (BTI) or a binding origin information (BOI) from the customs authorities. With this information, an individual can be sure to use the right classification of his goods, thus knowing his rights or obligations.

Conclusion

The implementation in international trade of the HS has been of great help to customs officials as well as trading companies all over the world. Problems arising from different interpretations are discussed in a common forum, thus giving more stringent replies. For trading partners, this has led to a higher degree of knowledge of the economic conditions that apply to a specific trade action. The result of this is a general increase in world trade and thus, according to normal economic reasoning, in general welfare worldwide.

See also: **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius; Trade in Milk and Dairy Products, International Standards: World Trade Organization; World Trade Organization and Other Factors Shaping the Dairy Industry in the Future.

Relevant Website

On the WCO homepage (www.wcoomd.org), the relevant conventions can be read:

- ○ Convention establishing a Customs Co-operation Council – signed in Brussels on 15 December, 1950; entered into force on 4 November 1952. Fifteen pages.
- ○ Convention on the Harmonized Commodity Description and Coding System – entered into force 1 January 1988. Including list of countries, territories, or customs or economic unions applying the HS. 13 pages.
- ○ International Convention on the Simplification and Harmonization of Customs Procedures (Kyoto Convention) – entered into force 25 September 1974; revised version June 1999. 17 pages.

On the European Union homepage (http://ec.europa.eu/), the relevant regulations can be read:

- ○ European Union Combined Nomenclature as expressed in the Annex I to Council Regulation (EEC) 2658/87 last amended by Commission Regulation (EC) No. 948/2009 of 30 September 2009, Official Journal L 287, 31/10/2009, 897 pages.
- ○ European Unions Refund Nomenclature as expresseds in Commission Regulation (EC) No. 1298/2009 of 18 December 2009, replacing the Annex to Regulation

(EEC) No. 3846/87 establishing an agricultural product nomenclature for export refunds, Official Journal L 343, 31/12/2009, 40 pages.

○ Council Regulation (EEC) No. 450/2008 of 22 April 2008, laying down the Community Customs Code (Modernized Customs Code), Official Journal L 145, 04/06/2008, 64 pages.

http://ec.europa.eu/taxation_customs/customs/procedural_aspects/general/community_code/index_en.htm

Trade in Milk and Dairy Products, International Standards: World Trade Organization

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Introduction

The World Trade Organization (WTO) is the successor to the former General Agreement on Tariffs and Trade (GATT). The GATT organization was formed in 1948 after World War II as one of three legs in the international economic system; the other legs are the International Monetary Fund (IMF) and the World Bank. The GATT agreement was signed by 23 countries in 1947. In 1995, 123 countries in the multilateral trading system transformed the former GATT into the WTO. Membership by January 2002 is 144. A number of countries are negotiating for membership, amongst them Russia, the only major country of the international economy that is not already a member. China became a member in 2001.

Until 1995 the trading system was organized as an agreement, but with the WTO entering into force, the system now consists of a fully fledged international organization with rights and obligations attached to its members. The WTO has its head office in Geneva, Switzerland. The director-general is the former New Zealand minister Mike Moore who took office in 1999.

The core functions of the WTO are to ensure a non-discriminating, smooth, predictable, and free trade between member countries. At the heart of the organization are the following activities:

- administering WTO trade agreements
- forum for trade negotiations
- handling trade disputes
- monitoring national trade policies
- technical assistance and training for developing countries
- cooperation with other international organizations.

Both GATT and WTO evolved around trade negotiation rounds. So far there have been eight rounds of negotiations within GATT (**Table 1**). In 2001, negotiations were in progress on whether to start a new all-encompassing trade round in the WTO or to divide the negotiations into separate subject areas.

During the course of time the GATT and now the WTO have increased their agenda according to the developments in the surrounding society. The WTO now covers a wide number of different areas of trade under the three main headings GATT (trade in goods), GATS (trade in services) and TRIPS (trade in intellectual property rights). Within the areas of GATT and GATS there are a number of extra agreements, some of which are listed in **Table 2**.

The decision-making process in the WTO is fundamentally built upon consensus. The top decision-making body is the Ministerial Conference, which consists of the Member Countries' foreign ministers. This conference convenes at a minimum every second year. On a daily basis, the General Council is the central decision-making forum. All the Member Countries are represented in the Council by their ambassadors or a counterpart. The General Council also acts as Dispute Settlements Body and as Trade Policy Review Body.

Principles of the WTO

A number of fundamental principles govern the general agreements and the relations between Member Countries. At the core of these principles is antidiscrimination, amongst trading partners and between domestic and foreign producers of goods. The most important principles are as follows.

Most Favored Nation Status

The Most Favored Nation (MFN) clause stipulates that favors such as easier market access (lower duties) given to one country's produce should be multilateralized and therefore applicable to all member countries. All Member Countries should by this principle be entitled to equal treatment and equal access. The major exception from this rule is free trade areas, customs, economic and political unions that can apply for and under certain conditions be granted exemption.

National Treatment Clause

The National Treatment Clause stipulates that imports and domestic or local produce should be equally treated

Table 1 GATT trade rounds

Year	*Name of the round and place*
1947	Geneva
1949	Annecy
1951	Torquay
1956	Geneva
1960–61	Dillon Round (Geneva)
1964–67	Kennedy Round (Geneva)
1973–79	Tokyo Round (Geneva)
1986–94	Uruguay Round (Geneva)

Source: www.wto.org

Table 2 Agreements within the areas of GATT and GATS

The 'extra' goods agreements (under GATT)	*The GATS annexes (under GATS)*
Agriculture	Movement of natural persons
Health regulations for farm products	Air transport
Textiles and clothing	Financial services
Product standards	Shipping
Investment measures	Telecommunication
Antidumping measures	
Customs valuation methods	
Preshipment inspection	
Rule of origin	
Import licensing	
Subsidies and countermeasures	
Safeguards	

Source: http://www.wto.org

upon market entrance of the import products. Duties and import taxes are not regulated by this principle. This rule applies to goods, services, trademarks, copyrights and patents. In practice the National Treatment Clause undermines the possibility of governments giving preferences to nationally produced products over imports and is an important instrument in increasing the transparency of the nontariff barriers to trade.

Least-Developed Countries

More than 100 of the WTO member countries are developing countries. Within the WTO system there is special treatment for these countries with regard to their potential to fulfill their obligations according to the agreements, rules and regulations. For the group of least-developed countries, there are broad exemptions from the obligations. WTO has set up a system in order to help developing countries to gain knowledge and understanding of the organization and particularly to pass on experience with the trade negotiation system.

Dispute Settlement Body

With the creation of the WTO, the trading system established a much more comprehensive dispute settlement system than was the case under the GATT, led by the Dispute Settlement Body (DSB). This dispute settlement system is based on the rule of law and with the strengthened possibilities of enforcement, the WTO has increased its regulatory and arbitrating powers. The Dispute Settlement Body is one of the cornerstones in the transformation from an international agreement to an international organization.

Arbitration between members is a vital task for the WTO in order to secure the adherence by members to different agreements and rules under the WTO, but also to obtain the support of the members for the policies for the development of the international trade regime. The Dispute Settlement Body mainly facilitates the process by which Member Countries obtain legal guidance and counseling to solve bilateral disputes concerning trade issues under coverage of the different agreements under the auspices of WTO. A trade dispute taken to the WTO follows a predetermined structural process with set deadlines and obligations to implement the rulings. This allows for the development of jurisprudence and case law precedent within the WTO, and creates a much more predictable trading system.

The different stages of a WTO case are shown in **Figure 1**. A panel makes first rulings in the WTO; their report is approved unless there is a consensus against this. The panel ruling can on legal grounds be taken to appeal to the appellate body. The appellate body's decision is final, unless there is a consensus against the decision. This procedure implies that no single country or party to a dispute can veto a final decision and by that hinder a ruling against its interest. Member Countries are compelled to implement rulings of the WTO or to pay compensation to cover the opponent's losses resulting from the illegal trade practice. The WTO may also allow for retaliation and introduction of sanctions, if a party to a case does not intend to follow the final ruling.

Non-tariff Barriers

In the early days of GATT the focus was on reducing tariffs and import duties in order to enhance trade and reduce protectionism. This goal has largely been achieved for industrial produce during the course of the numerous trade rounds. Tariffs within trade in industrial products have been reduced from approximately 40% to less than 5% in the period from the establishment of GATT to the current implementation of the Uruguay Round. This tariff reduction process is only in an early stage with respect to agricultural and food products.

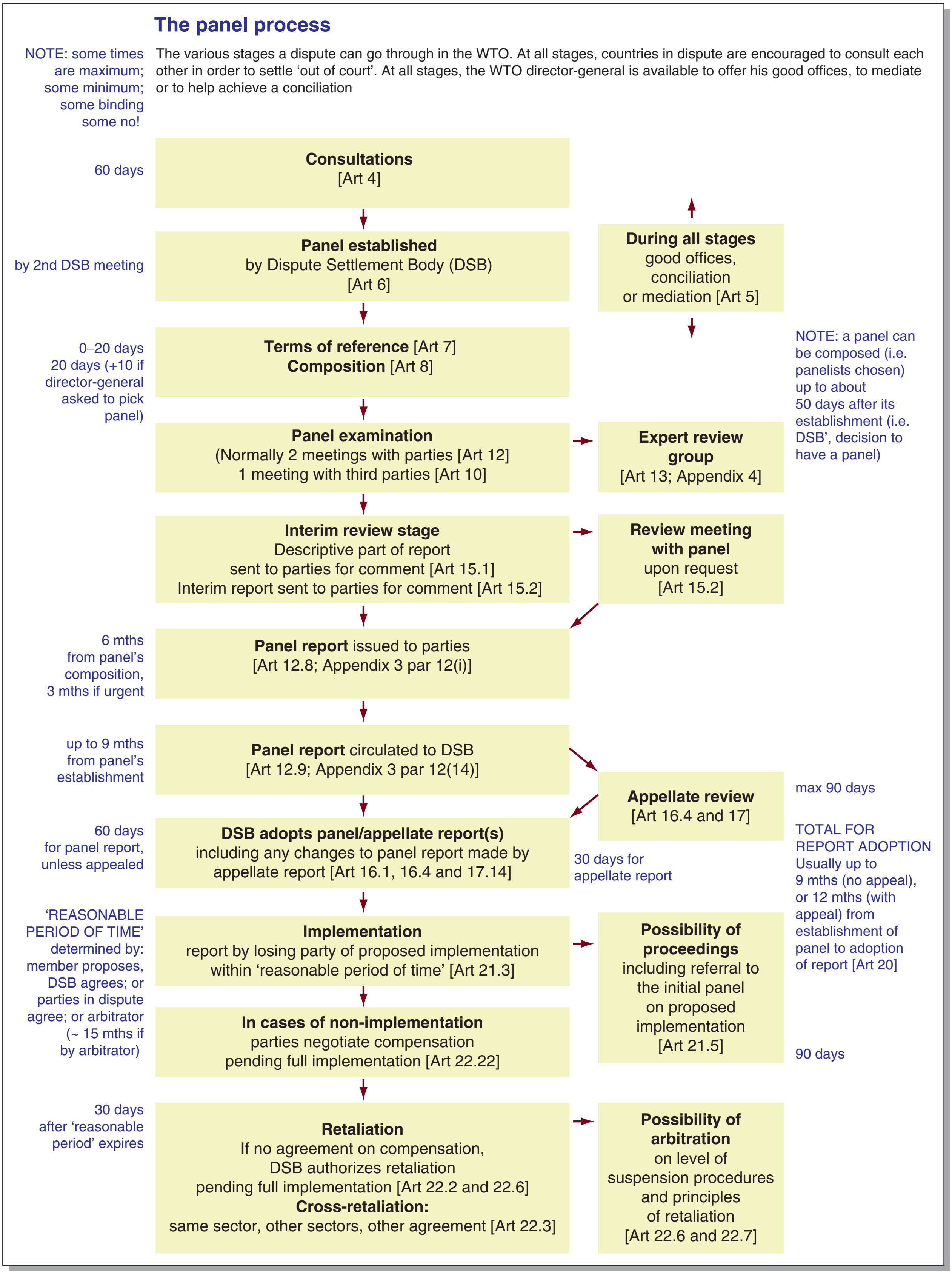
The panel process
NOTE: some times are maximum; some minimum; some binding some no!
The various stages a dispute can go through in the WTO. At all stages, countries in dispute are encouraged to consult each other in order to settle 'out of court'. At all stages, the WTO director-general is available to offer his good offices, to mediate or to help achieve a conciliation
60 days
Consultations [Art 4]
by 2nd DSB meeting
Panel established by Dispute Settlement Body (DSB) [Art 6]
During all stages good offices, conciliation or mediation [Art 5]
0–20 days
20 days (+10 if director-general asked to pick panel)
Terms of reference [Art 7]
Composition [Art 8]
NOTE: a panel can be composed (i.e. panelists chosen) up to about 50 days after its establishment (i.e. DSB', decision to have a panel)
Panel examination (Normally 2 meetings with parties [Art 12] 1 meeting with third parties [Art 10]
Expert review group [Art 13; Appendix 4]
Interim review stage
Descriptive part of report sent to parties for comment [Art 15.1]
Interim report sent to parties for comment [Art 15.2]
Review meeting with panel upon request [Art 15.2]
6 mths from panel's composition, 3 mths if urgent
Panel report issued to parties [Art 12.8; Appendix 3 par 12(i)]
up to 9 mths from panel's establishment
Panel report circulated to DSB [Art 12.9; Appendix 3 par 12(14)]
Appellate review [Art 16.4 and 17]
max 90 days
60 days for panel report, unless appealed
DSB adopts panel/appellate report(s) including any changes to panel report made by appellate report [Art 16.1, 16.4 and 17.14]
30 days for appellate report
TOTAL FOR REPORT ADOPTION Usually up to 9 mths (no appeal), or 12 mths (with appeal) from establishment of panel to adoption of report [Art 20]
'REASONABLE PERIOD OF TIME' determined by: member proposes, DSB agrees; or parties in dispute agree; or arbitrator (~ 15 mths if by arbitrator)
Implementation report by losing party of proposed implementation within 'reasonable period of time' [Art 21.3]
Possibility of proceedings including referral to the initial panel on proposed implementation [Art 21.5]
In cases of non-implementation parties negotiate compensation pending full implementation [Art 22.22]
90 days
30 days after 'reasonable period' expires
Retaliation
If no agreement on compensation, DSB authorizes retaliation pending full implementation [Art 22.2 and 22.6]
Cross-retaliation:
same sector, other sectors, other agreement [Art 22.3]
Possibility of arbitration on level of suspension procedures and principles of retaliation [Art 22.6 and 22.7]

Figure 1 Flowchart of the dispute settlement process in the WTO. (Reproduced with permission from www.wto.org.)

As duties have diminished, the non-tariff barriers (NTB) have attracted increasing attention as they turn out to be as trade-distorting as flat rate tariffs. Non-tariff barriers consist of a number of different rules, regulations, standards, technical issues, administrative and bureaucratic procedures and other market-related obstacles that exporters meet while trying to gain access to a certain market. The WTO tries to highlight this area with a policy of transparency and information, but also with restrictions on the use of nontariff barriers.

Agreement on Technical Barriers to Trade

The Agreement on Technical Barriers to Trade (TBT) is closely tied to the above-mentioned non-tariff barriers. This agreement aims at creating a free flow of international trade by regulating the general use of technical standards in a protectionist way. The agreement installs the principle that technical regulations and standards concerning packaging, marking and labeling, and procedures for assessment of conformity with technical regulations and standards should be used in a nondiscriminative way, both in respect to different trading partners and with regards to domestic versus import products.

The agreement encourages the use of internationally recognized standards in national and local laws. At the same time member countries have to enhance transparency and public access with regard to applied and upcoming standards.

Agreement on Sanitary and Phytosanitary Standards

The Agreement on the Application of Sanitary and Phytosanitary Standards (SPS) defines and gives opportunities for Member Countries to adopt measures necessary to protect human, animal and plant life and health and at the same time regulates the use of these measures in order to avoid their discriminative use as barriers to trade.

In the SPS agreement the scientific principle prevails. Sanitary and phytosanitary measures restricting trade should at all times have a scientific foundation that is widely recognized. Concurrently, countries are to treat different rules and standards alike, if their ultimate effect is the same for the protection of the human, animal and plant life and health. The Codex Alimentarius is recognized in the SPS agreement as a standard-setting reference point in this area and in the case of disputes among member countries. The objective is to harmonize the basis for sanitary and phytosanitary measures and meanwhile to create transparency and access to information concerning different rules and demands within this area.

Agricultural Agreement

With the finalization of the Uruguay Round of trade negotiations agricultural trade fully came under the regulation of the WTO. The Agricultural Agreement is the first real attempt to achieve a common understanding of the trade mechanisms for agricultural and food products and the Agreement implements a number of regulations which in the last half of the 1990s and at the turn of the century set fundamental boundaries and rules for governance and policy-making within agriculture worldwide (**Table 3**).

The major elements of the Agreement fall under three headings:

1. Export subsidies and competition.
2. Market access/imports.
3. Internal/domestic support.

Together with the three central parts of the Agricultural Agreement, the Sanitary and Phytosanitary Agreement

Table 3 The outline of the support reductions in the agricultural agreement

The main demands for reduced agricultural supports 1995 to 2001		
Exports with support	*Imports*	*Internal support*
21% reduction in export quantities subject to support	All non-tariff barriers converted to tariffs	20% reduction in all trade distorting internal supports based on the AMS calculation
36% reduction in budgetary outlays for export subsidies	Average 36% reduction in tariffs including converted non-tariff barriers, at minimum 15% reduction pre product line	Base period: 1986–88
Base period: 1986–90	Minimum access at reduced tariff rates of 3% increasing to 5% of domestic consumption Base period: 1986–88	

Source: http://www.wto.org

(see above) constitutes an attempt to formulate an overall framework for agricultural trade and by that the domestic policies concerning production and regulation of food supply.

Within the WTO there are a number of different views concerning liberalizing versus protecting agricultural production and trade. The most vocal groupings in the agricultural negotiations are the United States, the EU and the Cairns Group, a number of food exporting countries with Australia and New Zealand at the forefront. The developing countries are with respect to their interests mainly divided into two main groups, net food exporters and net food importers.

A number of very important trade disputes concern trade in agricultural produce, such as bananas, hormone-treated beef or milk quotas, to mention just a few. These WTO cases between different Member Countries have been some of the most stringent tests to the dispute settlement system within the WTO. Agricultural and consumer-related issues have proved to be the most politicized cases in the short history of the Dispute Settlement Body of the WTO. But they are also acknowledged as the core challenge to the sustainability of the system and to the Member Countries' genuine support and acceptance of the WTO rules and governance.

Blair House Accord

The Blair House Accord between the EU and the United States is named after the presidential official guesthouse in Washington DC where the Agricultural Agreement was finalized. This agreement, reached in November 1992, was a breakthrough in the ongoing Uruguay Round of negotiations in GATT. The accord laid the foundations for the current trade liberalization within agriculture and also facilitated an ending to the very long and at times antagonistic negotiations for a general conclusion to the trade round.

The Blair House Accord sets the basic reduction factors both for exports and internal support measured in quantities and budgetary outlays. The accord also sets a 6-year implementation period and the reference periods. Alongside this, the EU and the United States agreed on solving a number of outstanding trade disputes particularly the one on oilseeds. The agricultural breakthrough in the GATT system is widely recognized to rest on the Blair House Accord and hence on the hard-won compromises and common understanding between the EU and the United States. The EU has a mandate to negotiate for all Member Countries of the European Union, which act as one within the WTO.

In December 1993, one year after the Blair House Accord, the final GATT agreement was reached and it was signed in Marrakesh in Morocco in April 1994. The Agricultural Agreement entered into force in 1995 running to 2001, with a peace clause extending it to 2003. The Agreement stipulated that further negotiations were to start in the WTO by 1999 in order to prepare for a new agreement. These negotiations are currently (2002) in process with all the parties outlining their specific interests and suggestions for a new agenda and ultimately an agreement.

Peace Clause

It was envisaged in the Blair House Accord that a new agreement might not be reached among the trading partners to take over from the Uruguay Round agreement exactly in 2001. Therefore it was decided to extend the results of the final stage of the implementation – the so-called year 6 (2000/2001) – onward to 2003 to provide some time for negotiations on a new agreement. From 2001 to 2003 no further developments in the levels of subsidies, import tariffs and internal support are anticipated unless a new accord is in place. At the same time, the agreed reductions will not be reversed.

The situation for the agreement on agriculture is, however, uncertain if a new deal is not in place by 2003. One option is to agree to extend the peace clause; however, a potential conflict is possible.

Traffic-Light Model – GATT Boxes and Decoupled Support

One of the main objectives of the Agricultural Agreement is to reduce support levels in general; however, support is divided into more and less trade-distorting types. The aim of the Agreement is to target the most distorting support forms which directly influence the international markets for agricultural products, and hence the competition situation between different agricultural producers.

Aggregated Measurement of Support

The Total Aggregated Measurement of Support (AMS) is a calculation of the total amount of support given to agricultural producers in one country, except for domestic support not subject to reductions, because of their nondistorting or decoupled nature. The AMS calculation is used to facilitate comparisons between countries and to equalize different types of support in order to obtain reductions in all types of distorting supports.

Traffic-light model

In popular terms the different types of agricultural support are labeled with different colors: red, amber, green – and blue. The red support forms should be stopped immediately, the amber ones should be phased out and the green ones can be left, as they are not seen as directly distorting. This rule-of-thumb makes for the name 'traffic-light model'. The different colors also give names to the box scheme, which is another way of describing the different support forms: amber, blue and green boxes.

Export subsidies

The export subsidies used by various countries fall into the amber box and must be reduced according to the agreement by 21% for supported export quantities and by 36% in total budgetary outlays. The reduction had to be linear over the implementation period, reaching the final commitments in Year 6, 2001. Developing countries must reduce their support by 14% in quantities and 24% in budgetary outlays with an implementation period of 10 years. Least-developed countries are exempt from this obligation. Member Countries have to keep account of the use of export subsidies for the individual product categories.

Decoupled support and blue box subsidies

In order to encompass the American deficiency payments and the EU animal and area premiums of the mid-1990s, the blue color was introduced. Blue color support forms are semi-decoupled, whereas the green ones are decoupled altogether.

The term 'decoupled' is used to describe the situation where the size and amount of support are not linked to the actual form and size of the agricultural production, as opposed to price support that is directly linked to the production output. Green box types of support are within the areas of research, disease control, government services and many more. Direct income support also falls in this category, as long as it is independent of the production. Semi-decoupled support can take the form of direct payments under production limiting programs with fixed references – area, yield or animal numbers.

The blue box supports are not targeted by reduction demands because of their limited trade-distorting nature. The introduction of categorized support forms has allowed for a dynamic agricultural policy reform process in different countries. The major agricultural production and trading countries have undergone a number of reforms since the GATT agreement, all directly related to the WTO policies, in order to enhance competitive powers and allow for a more fair trade with agricultural produce. In most WTO member countries development of agricultural policy instruments is under way in order to avoid potential disputes.

Imports and Market Access

The import rules in the Agreement specify that tariffs have to be reduced by an average of 36%, with a minimum 15% per product line from 1995 to 2001. Prior to the reduction all non-tariff barriers have to be converted into tariff equivalents to enhance transparency as to the actual level of trade restrictions. This conversion is termed tariffication. It is the overall tariffs (duties, levies, quantitative restrictions, non-tariff barriers and so forth – old and new ones) that are to be reduced by 36%. The tariff rates are listed in the country list for each country with initial and bound rates. Developing countries are only obliged to reduce tariffs by 24%, and they have an extended implementation period, to 2004. Least-developed countries are exempt from the agreement to reduce tariffs.

Minimum import access

In order to create some immediate effect for market access it was decided to create a certain minimum access to a preferential tariff far below the general and even reduced tariffs. This minimum quota is set in relation to the consumption in the base period 1986–88. At the beginning of the implementation in 1995 the quota was 3% of base period consumption for the respective products. This amount has gradually increased to 5% at the end of the 6-year implementation period in 2000/2001. Current access opportunities are to be maintained and supplemented with the above-mentioned minimum access in case the actual imports are below the 3–5%.

Trade in Dairy Products

It was widely believed that the WTO agreement would increase the international trade in agricultural products, including dairy produce. The actual development does indicate that the total trade is developing; however, what is more noticeable is the change in the trading patterns. **Table 4** shows the shift from an EU-dominated international trade in dairy products to a situation where Oceania is the predominant exporter. This situation is reflected in different claims and demands in respect of the ongoing negotiations on a WTO II agreement. It was also expected that the price for dairy products would be affected in an upward trend by the Agricultural Agreement and the increased trade, but this element is harder to deduce from the statistics. However, some signs in the market do indicate a general upward trend in prices for dairy products.

Table 4 The development in world market shares for dairy products

World market share (%)	*1990*	*2000*
EU	51	39
USA	13	2
New Zealand	27	41
Australia	9	18

Source: Danish Dairy Board.

See also: **Office of International Epizooties**: Mission, Organization and Animal Health Code. **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: European Union's Common Agricultural Policy; Agricultural Policy Schemes: Other Systems; Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy; Agricultural Policy Schemes: United States' Agricultural System; Trade in Milk and Dairy Products, International Standards: Harmonized Systems.

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World Trade Organization and Other Factors Shaping the Dairy Industry in the Future

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Introduction

How would dairy industry look like after the conclusion of WTO (World Trade Organization) Doha negotiations? Is there going to be a final deal? Is there a need for a Doha deal?

Starting with the last question, the answer is 'yes'. In my view it is not necessary to discuss here the importance of the Doha round and the advantages of a multilateral approach. Bilateral and not even regional agreements cannot match the multilateral system. Issues related to standards or dispute settlements are just two examples that need multilateral consideration. The value of WTO trade negotiations that seek broader consensus has also been underlined in empirical studies. For example, an OECD (Organisation for Economic Co-operation and Development) study pointed to the logical fact that the domestic supply adjustment for dairy sector can be expected to be the highest if a country reforms its dairy policy unilaterally. As more countries join the reform process, adjustments become smaller and would be least in the case of a multilateral reform.

As there is a need for a multilateral framework to govern global trade, it could be expected that there will be a final deal. However, in the aftermath of the global economic crises, the question mark after the word 'When' is not getting smaller.

The answer to the first question is even more complex. It is always difficult to predict how the world would look like after a specific event, and it is not easy to isolate the impact of that particular event from other factors influencing the evolution of the world. In the last decade the dairy industry has been going through remarkably dynamic changes worldwide, becoming truly global in scope. Would this have happened even without the WTO Uruguay Round Agreement on Agriculture? Or, was globalization in fact propelled by numerous border measures protecting domestic markets that provided incentives for foreign companies to circumvent national border measures through investment in protected markets?

A Doha deal could be expected to have an impact especially on those domestic markets that have relatively high market price support measures in place. But the actual impact depends on the global market situation and the way countries organize support to agriculture. Moreover, the forces of globalization, underpinned by economic growth, urbanization, and technology transfers, will likely continue to shape the dairy industry, be it pre- or post-Doha. This article will discuss not only concerns related to Doha but also other issues that are likely to have an impact on dairy markets in the near future.

Doha Round in the Context of WTO Negotiations

Established on 1 January 1995, the WTO replaced the GATT (the General Agreement on Tariffs and Trade) as the legal and institutional foundation of the multilateral trading system of its member countries. Although GATT has been providing the multilateral rules governing much of the global trading system since 1948, these rules were largely ineffective in disciplining key aspects of agricultural trade. The Uruguay Round was the first trade round that considered agricultural issues, and it became a turning point in the reform of the agricultural trade system. The Uruguay Round Agriculture Agreement (URAA) has accomplished the development and implementation of a framework to address barriers and distortions to trade in three major policy domains: market access, domestic support, and export subsidies. The diverse forms of trade measures were converted under the URAA to tariffs. Market access for sensitive products was provided through a system of tariff rate quotas (TRQs). The use of export subsidies was reduced by the agreement, while domestic policies that affect production and trade of agricultural products were constrained by a set of rules and bindings. The reduction in domestic support was implemented through a commitment to reduce the total aggregate measurement of support (AMS) for each country.

The URAA numerical targets for each policy domain in agriculture are summarized in **Table 1**. The table indicates that in the developed countries the total AMS support was scheduled to be reduced by 20% over 6 years, while in the developing countries the total AMS

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Table 1 Uruguay round numerical targets for agriculture

	Developed countries	*Developing countries*
	6 years: 1995–2000	*10 years: 1995–2004*
Tariffs		
Average cut for all agricultural products	−36%	−24%
Minimum cut per product	−15%	−10%
Domestic support		
Total AMS cuts for sector (base period: 1986–88)	−20%	−13%
Exports		
Value of subsidies	−36%	−24%
Subsidized quantities (base period: 1986–90)	−21%	−14%

Source: WTO.

support was scheduled to be reduced by 13% over 10 years. Moreover, the agreement required a reduction of tariffs by 36%, on average, for developed countries, and by 24% for developing countries. It is evident from the table that although the Uruguay Round achieved a historic change, the actual level of agricultural support was reduced only moderately.

Nevertheless, the achievement of the Uruguay Round should be seen in the establishment of rules for agricultural support and as a start of the process of long-term objective of substantial reduction in support and protection. This process was to continue in the new round of negotiations. At the Fourth Ministerial Conference in Doha, Qatar, in November 2001, WTO member governments agreed to launch a new round – the so-called Doha round – of negotiations. The round embarked on a very complex agenda with a focus on developing countries, trade facilitation, competition rules, environment, investment, liberalization of trade in services, and liberalization of trade in agriculture, which is likely the single most difficult item.

The Doha Ministerial Declaration has set several key dates for the negotiations. The modalities for countries' commitments were expected by 31 March 2003, and countries' comprehensive draft commitments by the fifth ministerial conference in September 2003 in Cancún, Mexico, with the overall deadline set for 1 January 2005. However, after a failed – the so-called – Harbinson proposal in March 2003, the fifth ministerial conference in September 2003 ended up in a stalemate. Ten months after the Cancún deadlock, an agreement on a framework for the modalities on agriculture was reached. The so-called July Framework gave a clearer shape to the modalities for the next phase of the negotiations.

The 2004 July Framework Agreement was a starting point toward a draft of the detailed modalities to be agreed at the WTO's sixth ministerial conference held in Hong Kong, from 13 to 18 December 2005. Rather limited progress was made in reaching an agreement on precise numerical formulae or targets for liberalizing agricultural trade, the original aim of the Hong Kong Ministerial. Perhaps the most tangible outcome of the Hong Kong Ministerial was the continuing support to eliminate all forms of export subsidies, and disciplines on measures with equivalent effects. The next deadline for reaching agreement on modalities was set for 1 August 2006. As no agreement could be reached, the negotiations had been suspended at the General Council meeting on 27–28 July 2006.

In 2007, the negotiations were resumed and a new set of modalities were circulated in July and August 2007. A series of working documents followed, as well as a series of revised draft modalities on 8 February and 19 May 2008, respectively. A revision of the previous versions was circulated on 10 July 2008. But the meeting of ministers in Geneva over 21–30 July did not reach an agreement. The disagreement occurred over the special safeguard mechanism (SSM) for agricultural products in developing countries. Safeguard mechanisms are used to restrict imports in special circumstances such as a sudden surge in imports. The unresolved issue was the size of the trigger allowing to invoke a special safeguard measure. In particular, the disagreement was related to the situation where the SSM raises tariffs above the commitments made by the countries in the 1986–94 Uruguay Round – the pre-Doha Round bound rates.

Some commentators viewed the July 2008 failure more as a collapse of negotiations; others noted the progress on issues other than the SSM. It is difficult to estimate what would happen if the questions of the SSM were resolved. In his report to the trade negotiations committee, the chairman of the special session of the committee on agriculture, Ambassador Crawford Falconer, noted that despite the fact that members were prepared to accept compromises it could not be taken for granted that even with the SSM agreement the rest could have fallen into place. There are still unresolved questions related to new tariff quota

Table 2 Overview of GATT and WTO negotiation rounds

Name	*Start*	*Duration*	*Countries*	*Subjects covered*
Geneva	April 1947	7 months	23	Tariffs
Annecy	April 1949	5 months	13	Tariffs
Torquay	September 1950	8 months	38	Tariffs
Geneva II	January 1956	5 months	26	Tariffs, admission of Japan
Dillon	September 1960	11 months	26	Tariffs
Kennedy	May 1964	37 months	62	Tariffs, antidumping
Tokyo	September 1973	74 months	102	Tariffs, nontariff measures, framework agreements
Uruguay	September 1986	87 months	123	Tariffs, nontariff measures, rules, services, intellectual property, dispute settlement, textiles, agriculture, creation of WTO, and so on
Doha	November 2001	?	141 (November 2001)	Tariffs, nontariff measures, agriculture, labor standards, environment, competition, investment, transparency, patents, and so on
		?	153 (September 2008)	

Adopted from Neary JP (2004) Europe on the road to Doha: Towards a new global trade round? *CESifo Economic Studies* 50(2): 319–332.
? – not yet known.

creation, tariff simplification, and issues related to cotton. These could be the other deal-breakers. Nevertheless, it was clear from the outset that the agenda is ambitious and that a lot of time and work would be needed to successfully conclude the round. Moreover, the time spent so far on negotiations cannot be considered excessive, despite the slow progress.

Table 2 summarizes the time-bound efforts of the previous GATT and WTO rounds.

Implications of the Doha Round for the Dairy Sector

The Doha round has not fundamentally changed the rules as agreed by the URAA, but larger reductions (or even elimination) have been considered. Although the actual modalities have not been agreed to and are subject to change, the key factors so far could be described as follows: (1) Elimination of export subsidies in all forms (already agreed in July 2004 Framework) and improved disciplines on all export measures whose effects are equivalent to those of export subsidies. (2) Depending on the base of Overall Trade-Distorting Domestic Support (OTDS), a reduction is envisaged in the range of 50–85% using a tiered formula; the framework also widened the range of support that would be disciplined in the Doha round; Blue Box supports are to be capped at no more than 5% of the value of a country's agricultural production, in the 1995–2000 period. (3) Tariffs shall be reduced using a tiered formula that requires deeper cuts for higher tariffs.

The least developed countries would not be required to make any reduction on domestic support and tariffs. The negotiated framework also provides a number of flexibilities intended to meet specific concerns of individual or groups of countries. These flexibilities, which are in fact numerous exceptions – such as the designation of sensitive products and special products for developing countries, special agricultural safeguard measures – have substantially increased the complexity of the negotiations and introduced doubts about the effectiveness and success of tariff cuts.

What would be the implication of the Doha round for the dairy sector? Dairy markets have traditionally been among the more distorted, and even after the full implementation of the URAA, dairy trade continued to be among the most protected agricultural sectors with high average bound tariffs, low minimum access requirements, a number of special safeguard provisions, complex systems of tariff-rate quotas (TRQs), and large domestic support and export subsidies and other export support measures. In almost all instances, tariffs on dairy products are above the country average for all agri-food products and are among the highest on agricultural products (**Table 3**). Thus, it could be expected that, after a Doha deal, countries with high tariffs would need to reduce them considerably, but, as noted above, dairy products could be listed as sensitive products falling under the exemption category.

Under the current legislation it has been possible to avoid reductions in dairy AMS by adjusting the AMS amounts in other sectors. Such compensation mechanisms are limited in the Doha framework, which specifies product-specific AMS limits and envisages capping of the product-specific funding. The product-specific AMS limits for all developed country WTO Members other than the United States shall be the average of the product-specific AMS during the Uruguay Round implementation period (1995–2000) as notified to the Committee on Agriculture. For the United States, the

Table 3 Average (scheduled) tariffs in 2000 for main commodities

	In-quota	*Out-of-quota*
Coarse grains	100	217.8
Wheat	73.2	184.4
Rice	15	197.5
Sugar	15.8	126.7
Beef	36.3	166.9
Pig meat	55.5	180.2
Poultry	39	171.7
Sheep	30.9	153.3
Butter	48.3	369.5
Cheese	31.8	121.1
Skim milk powder	48.1	191.6
Whole milk powder	79.5	260.7
Whey powder	37.8	545.7
Average of all commodities	52.67	184.18

From OECD (2002) *Agriculture and Trade Liberalisation: Extending the Uruguay Round Agreement*. Directorate for Food, Agriculture and Fisheries, Committee for Agriculture. Paris: OECD.
Average tariff was calculated as an unweighted average of each tariff line for the following countries: Argentina, Australia, Canada, European Union, Hungary, Japan, Korea, Mexico, New Zealand, Poland, United States, Iceland, Norway, and Switzerland.

product-specific AMS limits shall be the resultant of applying proportionately the average product-specific AMS in the 1995–2004 period to the average product-specific total AMS support for the Uruguay Round implementation period (1995–2000) as notified to the Committee on Agriculture.

It is not necessary to go through all the details of modalities as these can be found in the original WTO document. However, an important point needs to be made here about the overall process. The WTO negotiations are not about eliminating agricultural policies but about shifting to more effective policies. Hence, countries will be able to implement or keep policies that do not distort trade. It follows that the green box is likely to remain in existence and act as a home for measures that are decoupled from production as far as possible and targeted at specific objectives and beneficiaries. (The green box support measures include those deemed to distort trade only minimally, or not at all, such as some forms of direct payments to producers, decoupled income support, and government financial support for income insurance and income safety net programs.)

The simulation results of studies that have analyzed the impact of further trade reforms on the global dairy sector have indicated that, following the reforms, world dairy prices would be lifted while supply would shift toward more efficient areas, although there would not be any significant change in total world milk production. It is however difficult to extrapolate these results in the context of recent developments as the agricultural markets have undergone dramatic changes. This is especially true of the dairy sector, which has been going through rapid structural changes for some time with several countries also embarking on policy reforms. As a consequence, international dairy markets have slowly shifted from a supply-driven paradigm distorted by price-depressing policies and serving as an outlet for excess supplies, to a more demand-driven paradigm, responsive to market signals and changing consumer preferences. The very recent events on the markets have further refocused attention on food security, availability, and safety. These profound market changes, some of which are believed to be structural and long-term in nature, will have a significant impact on the dairy sector, be it pre- or post-Doha. In order to get a better grasp on global dairy prospects it is essential to understand this shift.

Global Market Changes and Prospects

The prices of nearly all agricultural commodities have risen sharply in the 2007–08 period but the prices of dairy products were the first to start the climb. International dairy prices recorded strong gains in the first half of 2007, already peaking in the second half of that year well ahead of similar developments for other commodities (**Figure 1**). On a year-over-year basis between 2006 and 2007, world butter prices increased by 66% and cheese prices rose by 50%, while prices of milk powders soared by more than 90%.

The OECD–FAO Agricultural Outlook report expects that all dairy prices will weaken over the short-term as demand softens and supply, with some lag, reacts to the strong price incentives. Nevertheless, dairy prices in real terms are expected to average 20–30% higher as compared to those of the last decade. The prices in real terms are also expected to resume a modest declining trend, albeit from a much higher level than in the past (**Figure 2**). However, it is important to note that these projections are conditional on the underlying macroeconomic assumptions, most notably solid economic growths and high crude oil prices. **Figure 2**, which depicts butter and skim milk powder (SMP) prices in real terms, also illustrates two additional points. First, when viewed from the perspective of last couple of decades, the recent spike of prices in real terms is much less impressive, certainly for butter. Second, over the last several decades the value of milk components on the international market changed considerably and shifted toward non-fat solids away from fat. (The steady decline in milk fat value on world markets could be, to some extent, attributed to the policy decision in heavily protected countries to tilt butter/SMP support prices in favor of butter, which has favored the production of fat for which demand has been stagnating.)

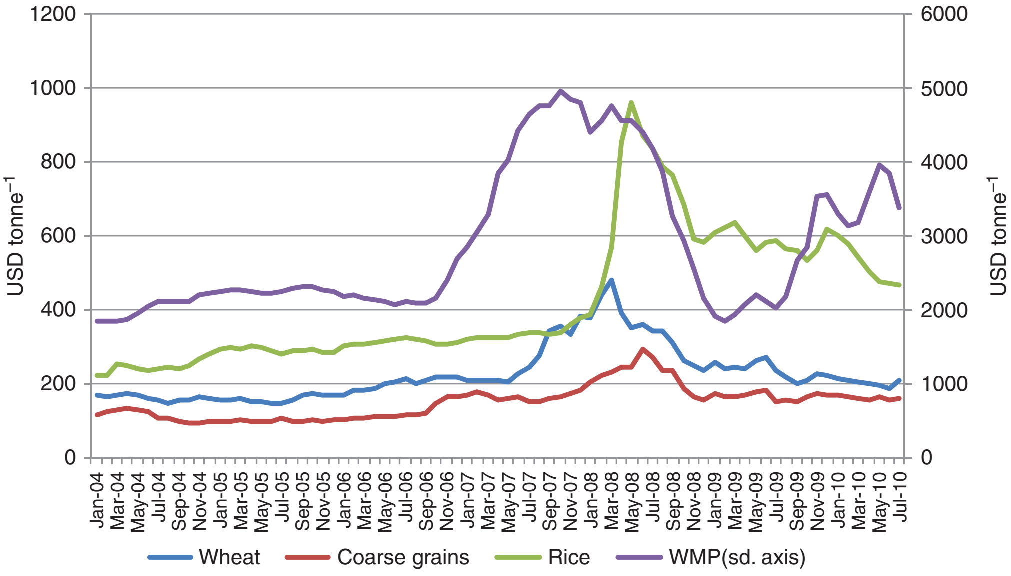

Figure 1 International monthly commodity prices. Source: OECD–FAO.

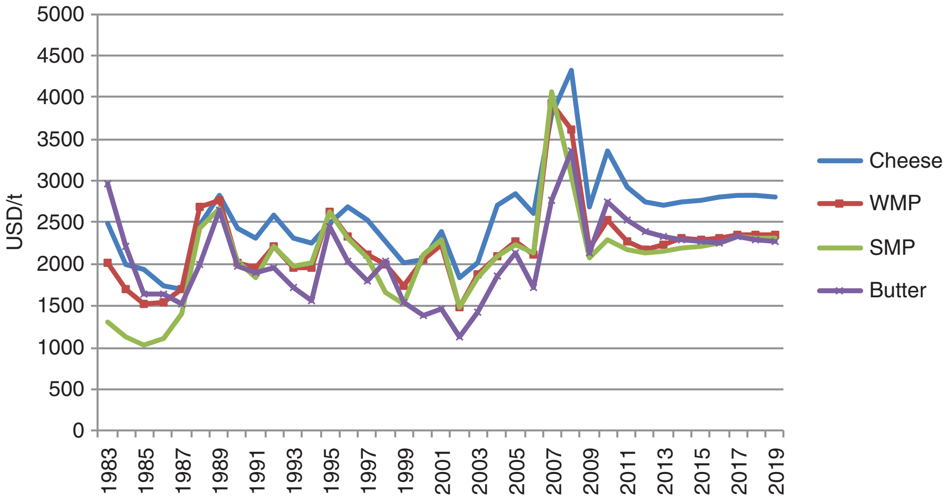

Figure 2 Historical and projected international prices (in real terms) of SMP and butter. Source: OECD.

The reasons for expectations of a higher plateau for global dairy prices merit a short consideration. Some of the factors behind the higher prices are related to more structural shifts while others could be considered as transitory. For example, a more permanent change in the markets relates to changes in demand patterns particularly in developing countries where consumers are switching to a more protein-based diet fueled by urbanization, westernization, and growth in per caput income. Consumption of milk and dairy products is rising nearly everywhere, exhibiting the highest growth rates among agricultural food commodities. The rise is particularly marked in rapidly growing economies of the Pacific Rim where an expanding middle-class population is consuming more sophisticated processed foods. Over the medium term, in developing countries, demand growth is expected for all dairy products with whole milk powder (WMP) consumption showing the strongest growth, followed by butter. Nevertheless, OECD countries continue to dominate cheese consumption and maintain their three-quarter share of the world total (**Figure 3**).

Another important development that slowly changed the usual picture was the decline in the importance of intervention products on world markets, particularly from the European Union and the United States. While in the period 2002–03 the share of EU and US SMP stocks alone amounted to half of the global exports of SMP, in the period 2005–07 this share dropped to below 10%, reducing the buffer against shocks in supply and demand (**Figure 4**). The lower stock levels have added to market and price volatility, and there is a strong nonlinear relationship of changes in stock levels to the changes in price. When ending stocks are sizable, large

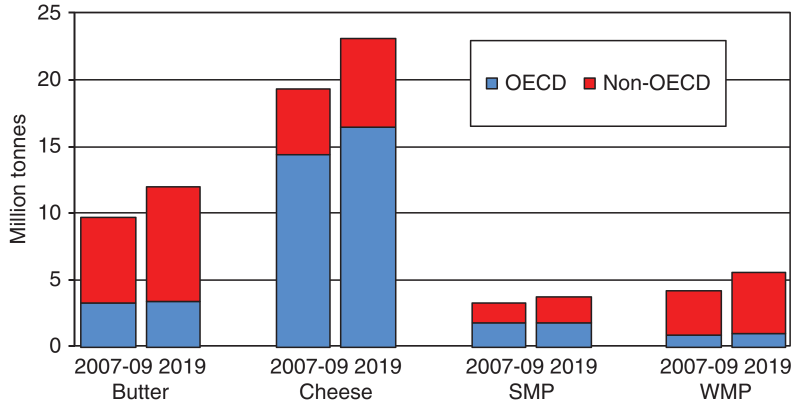

Figure 3 Outlook for dairy product consumption. Source: OECD.

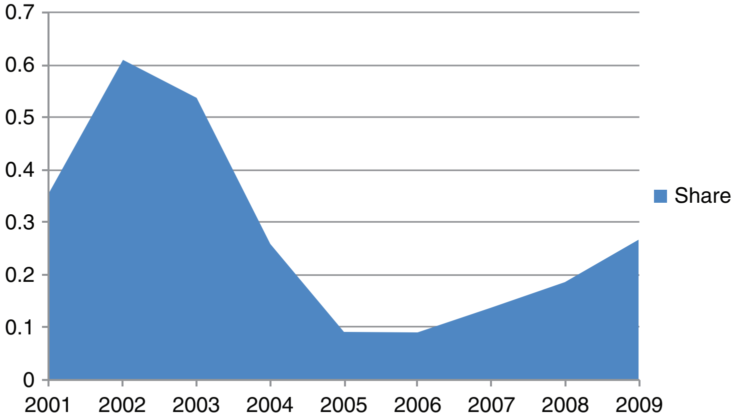

Figure 4 Ratio of EU and US stocks of SMP to global exports. Source: OECD.

changes in stock levels may be needed to change prices by a small amount. When stock levels are low, very small changes in stock levels can be associated with major price swings.

The tight situation on the market in 2007 was further aggravated by the decline in milk production in Australia and Argentina. Production everywhere was hindered by a strongly increasing cost of production – rising oil and feed prices. Oil, energy, and feed prices are critically important factors in the increased production costs for milk, and a higher plateau of these prices seems to be a more permanent factor that could keep prices above past average levels. The situation on the dairy markets was also to some extent exacerbated by policy decisions in certain countries to tax or ban exports. These *ad hoc* actions should be considered temporary, and introducing policies that create distortions and that undermine appropriate market responses should be avoided in the future.

Finally, an important factor, not unique to the dairy sector, has been the depreciation of the US dollar. Stronger currencies *vis-à-vis* the US dollar mitigated the producer price gains in local currencies and at the same time facilitated higher demand by importers, thus driving world prices higher in terms of US dollars. In general terms, when the dollar is weak, commodity prices tend to be higher, and when the dollar is strong, commodity prices tend to be lower (**Figure 5**).

Nevertheless, in discussing the prospects for the global dairy markets it is important to keep in mind that the most certain thing about the future is that it is uncertain. Weather, economic conditions, and the evolution of policies (induced by Doha deal or not) remain among the key factors influencing the dairy market's future, with a considerable uncertainty about them. For example, a slowdown in economic growth would moderate international prices. A severe drought in any important dairy-producing region could have a critical impact on the sector in any given year, pushing prices higher. These factors are uncertainties that to some extent form an integral part of the dairy markets. What are the other factors that will likely shape the dairy markets in the near future – be it pre- or post-Doha? Although the choice for the discussion might be subjective, it seems

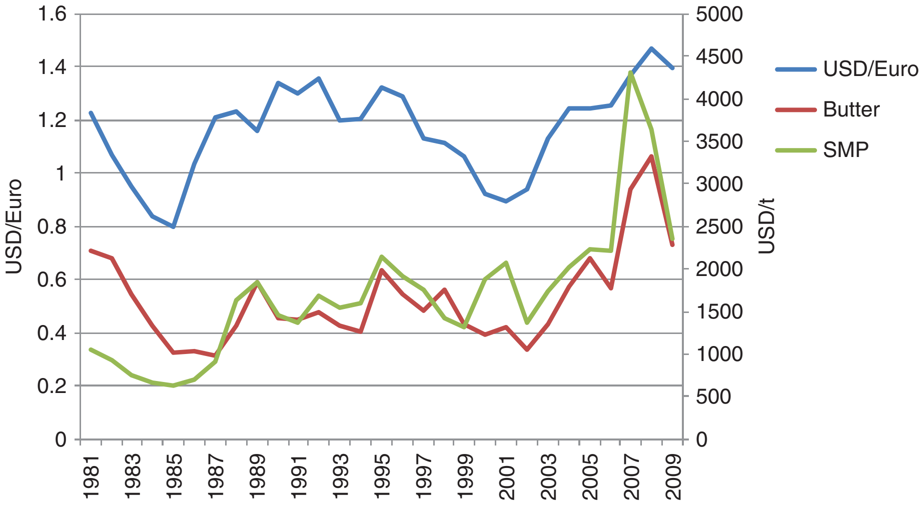

Figure 5 Exchange rate and global prices (in nominal terms). Source: OECD.

to me that the shifts in the markets will be reinforced by the following mutually inclusive factors: continued globalization, technological progress, and vertical integration of food supply chains.

Globalization

It is evident that the dairy industry is becoming more global in scope. It could be expected that globalization will continue to reshape the dairy markets in the future and will be fueled by the processes of economic growth, urbanization, technology transfer, and a convergence in consumption patterns. Moreover, the ability to expand, reduce costs, and secure milk supplies will remain additional drivers behind industry consolidation, and the development of discount stores and private labels will quite probably put additional pressure on milk processors, forcing them to look for further cuts in costs. Mergers, strategic alliances, joint ventures with foreign partners, and direct foreign investment and acquisitions will be the main vehicles of the structural change.

Competition among dairy firms in well-established developed markets is set to intensify with the focus turning to health and convenience, and increased penetration of foodservice, catering, and restaurant sectors. As a result, many firms will try to enter growing but less established markets in developing countries to source milk and dairy products from multiple locations while the original domestic market will become less relevant. This is also linked to the continued expansion of supermarkets, which will likely contribute to the further weakening of the position of local dairy firms but will help to spread international brands. Thus, for international dairy companies this means that a branded product can be promoted in multiple markets, but perpetuation of local products and catering for local tastes and preferences are to remain important.

Vertical Integration of Food Supply Chains

Globalization of the dairy industry goes hand in hand with the expansion of supermarkets and transformation of the agri-food sectors in general. It could be expected that the transition from independent markets toward much more tightly aligned food supply or value chains will continue. The profound changes in the global food system are marked by (1) increase of trade in food, (2) rapid rise of economic concentration of supermarkets, (3) shift to centralized procurement via distribution centers – from spot market procurement toward dedicated wholesalers and direct purchase from growers or grower associations, (4) creation of a multiplicity of private standards, often built on top of public standards, (5) rise of third-party certification of food production, (6) development of new technologies, biotechnologies, and process control throughout the entire chain, (7) shift toward nonprice competition among supermarket chains, (8) greater differentiation of food products by class, and (9) the development of new forms of contractual relationships between supplies and buyers. This transition has also been (and will be) accompanied by an increasing use of contracts. The results of several studies indicate an increased use of contracts in most agricultural sectors and dairy is not an exception. However, an increased use of contracts together with a rising upstream concentration in the supply chain could create concerns about the impact of this form of supply chain governance on farmers and issues related to market transparency and market power.

The growing market power in the supply chain might have an impact not only on farmers but also on established dairy companies. The growing power of retailers, and in particular the developments of retailers' private labels, will increasingly represent a challenge for established brands of dairy companies, forcing these firms to continuously innovate.

However, the overriding concern for the future seems to be the capability to assure the quality and safety of food. The importance of consumer confidence in the dairy supply chain is ever more evident following one of the largest food safety crises in recent years which spiraled as a result of milk adulteration with melamine in China. The toxic industrial chemical melamine has been added to milk to make it appear higher in protein. A number of babies died and thousands fell ill after drinking melamine-contaminated milk formula. It could be expected that this incidence will irreversibly change dairy markets and the way the milk and dairy production process is monitored and tested. The share of milk and dairy products in consumers' diets may suffer, so the dairy industry needs to remain proactive and innovative in order to maintain the image of a safe and healthy product. The substitution effect was evident in the recent melamine scare when Chinese consumers sought available alternatives, mainly soy milk. In fact, Starbucks in China announced that all milk used at its cafes in China will be soy-based for the near future. In fact, Starbucks in China announced after the incidence that all milk used at its cafes in China would be soya-based.

Technological Progress

Technology has been changing the dairy sector for decades, and only a true pessimist would say that the progress has arrived at the end of the road. Given the higher commodity price situation, more attention has been and can be expected to be paid to food production. It follows that private and public money flowing to research and development activities and agricultural extension services could push the technology frontier further. Productivity gains stimulated by increased automation of the production process, improved feed efficiency, improved health and longevity of cattle, and the ability to improve productivity via GM technology could be some of the alternatives.

In the milk-processing sector, the availability of ultrafiltration technology has enabled the development of milk component-based markets for milk solids with increasingly broad applications. This trend is expected to continue. Similarly, the growth of demand for dairy products as ingredients in other food products (particularly in pizzas, hamburgers, sandwiches, etc.) will continue.

The future will also see a myriad of new dairy-based products. Recent years have already witnessed product developments such as new functional foods; cosmeceutical, nutraceutical, and pharmaceutical products; and new beverages such as omega 3- and calcium-fortified milk. A promising recent development is also the introduction of a new lactose-free dairy drink produced by a special filtration process that removes half of the milk lactose. Lactose-free milk could be an important factor, driving higher milk consumption, particularly in Asia, where more than half of the population is believed to have some form of lactose intolerance. Finally, in the context of consumer-driven food safety and health concerns, traceability becomes the norm, and this will require the adoption of new technologies and tests for the analysis of residues.

Conclusions

In the context of the current global economic situation and the lack of progress in the Doha round negotiations, it is difficult to guesstimate when the final deal of the Doha round could be sealed. It could indeed be expected that the final modalities will impact future dairy markets. However, it is important to keep in mind that the Doha process is not about eliminating agricultural policies but about increasing transparency, fairness, and efficiency of agricultural trade. It follows that adjustment pressure, following the Doha round deal, on producers in developed countries that support domestic agriculture might not be excessive, although those domestic markets that have relatively high market price support measures in place will likely be affected more. But, the actual impact depends on the global market situation and the way countries organize future support to agriculture.

Many support policies decoupled from production are already in place and one could expect further shifts toward more effective policies better targeted at specific objectives, possibly including disadvantaged producers or production zones, and delivery of social benefits related to environmental and regional concerns. In fact, good agricultural policies do not need to depend on the WTO negotiations. Moreover, even without a Doha deal it seems that it would become increasingly difficult for governments to continue domestic support based on trade barriers for the traditional dairy products in the face of the rapidly evolving trade in new dairy product and dairy ingredient markets, and globalization of the dairy markets in general.

For example, the emergence of a sophisticated ultrafiltration process and expanding markets for milk ingredients have already diminished, to some extent, the importance of increasingly dated dairy support policies. Moreover, the process of globalization is crossing traditional trade barriers and is changing the structure of dairy markets, which have already started to be much more responsive to market signals and changing consumer preferences. Globalization of the dairy sector is set to continue, and international dairy companies will continue to penetrate the less developed markets to satisfy both local and growing export demand. The investments of multinational dairy processing firms will influence the development of new products and the transfer of technologies that improve market size and reach. The investments of multinational dairy processing firms will influence the development of new products and the transfer of technologies that improve market size and reach. These developments will however need to respect environmental considerations. It could be expected that increasingly important factors which are to influence dairy industry in the future are deterioration of natural grasslands, limited water resources and water pollution.

The attention on food security and safety will persist. Safety of milk and dairy products could be expected to become an overriding requirement for producers and dairy supply chain in the future. A related issue for the future dairy sector is the ability of milk and dairy products to keep a good-and-safe image in a broader sense. The industry will need to remain proactive and innovative in order to maintain the share of milk and dairy products in consumers' diets. Nevertheless, milk is a very unique product that contains very unique components, and it is difficult to see how the dairy industry would not be able to profit from this natural advantage.

See also: **Policy Schemes and Trade in Dairy Products**: Agricultural Policy Schemes: European Union's Common Agricultural Policy; Agricultural Policy Schemes: Other Systems; Agricultural Policy Schemes: Price and Support Systems in Agricultural Policy; Agricultural Policy Schemes: United States' Agricultural System; Codex Alimentarius; Standards of Identity of Milk and Milk Products; Trade in Milk and Dairy Products, International Standards: Harmonized Systems; Trade in Milk and Dairy Products, International Standards: World Trade Organization.

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PREBIOTICS

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Types

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Introduction

The human gastrointestinal tract becomes inhabited by a huge number of microbes immediately after birth. It has been calculated that in adults this complex open ecosystem, or the intestinal microflora formed in the colon (cecum to proximal and distal colons to rectum), is composed of some 10^{14} bacteria of hundreds of different species. The microflora as a whole or individual microbes are likely to play pivotal roles in the development of normal gut functions and maturation of mucosal immune system, and in the prevention and/or stimulation of intestinal disorders. The composition and metabolic activity of the gut microflora are influenced by various environmental factors, namely diet, age, stress, health status, and medication. Among all, dietary carbohydrates are the predominant carbon and energy sources for the gut microbes, and hence affect the growth of individual bacterial species in the colon.

It has been calculated that ~20–60 g of dietary carbohydrates ingested escapes digestion by human digestive enzymes daily, and they become substrates for fermentation in the colon. Among these nonabsorbed carbohydrates, 5–35 g is resistant starch, 10–25 g is nonstarch polysaccharides, and 2–8 g is nondigestible oligosaccharides (for terminology, see below). The diversity and amounts of these carbohydrates influence the composition and metabolic activity of the colonic bacterial ecosystem, which in turn strongly affect human health. The term 'prebiotics' was introduced by Gibson and Roberfroid in 1995 to describe the "nondigestible food ingredients that beneficially affect the host health by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon". A characteristic underlying the prebiotic concept is to increase 'indigenous' beneficial bacteria in the gut by virtue of feeding specific substrates that are preferentially utilized by these bacteria, in contrast to that of 'probiotics', which are themselves beneficial bacteria and therefore should directly influence the composition and metabolism of the gut microflora. In the past two decades a substantial number of food ingredients that definitely or possibly exert prebiotic effects have been described. The vast majority of them so far are short-chain carbohydrates that are not absorbed or are poorly digested by human enzymes, and are often called nondigestible oligosaccharides (NDO). In addition, recent extensive studies have revealed that certain nondigestible polysaccharides, which are often referred to as dietary fiber, exert health benefits through being fermented by a limited number of colonic bacteria into short-chain fatty acids, and so are capable of being prebiotics.

In this article, the current knowledge of prebiotics especially from a technological and biochemical point of view is summarized.

What Are Prebiotic Effects?

The principal effect of prebiotics is to improve the balance of the gut microflora by increasing the numbers of beneficial bacteria and decreasing those of potentially harmful bacteria, as defined above. This is achieved by the preferential utilization of prebiotic carbohydrates by beneficial bacteria such as bifidobacteria and lactobacilli. As a consequence of the alteration of the gut microflora composition, the metabolic activity and the systemic effects of the gut microflora will influence the host health status. In general, toxin production, intestinal putrefaction leading to production of harmful and carcinogenic substances, unbalanced immune response, (opportunistic) infection, and the overgrowth of harmful bacteria are suppressed, and the enterocyte activity, bowel movement, and the mucosal immune system are optimized.

It is widely accepted that lactobacilli and bifidobacteria are typically beneficial for human health. Primary fermentation products of bifidobacteria and lactobacilli from dietary carbohydrates are acetic and lactic acids. However, the final colonic fermentation products from dietary carbohydrates are short-chain acids (SCA) (mainly acetic, propionic, and butyric), some other organic acids (lactic and succinic), and gases (H_2, CO_2, and CH_4). The proportion of each organic acid in the cecal and fecal contents depends on the composition of the gut microflora and the amounts and forms of supplied carbohydrates. Main players of these diverse fermentation processes are bacteria of clostridia and *Eubacterium* clusters, which not only directly utilize dietary carbohydrates but also further ferment acetic acid and lactic acid to butyric acid and propionic acid, although the detailed characters of these bacterial groups are not well known yet.

There would be some other prebiotic effects based on the production of SCFA and other organic acids, for instance, improvement of mineral absorption in the colon, activation of colonocytes, acidification of cecal and fecal contents, or improvement of lipid metabolism.

Classification and Terminology of Dietary Carbohydrates

Carbohydrates are classified into monosaccharides, disaccharides, oligosaccharides, and polysaccharides based on the number of monosaccharide units contained in them (which is often referred to as degree of polymerization or DP) (**Table 1**). Oligosaccharides are defined as carbohydrates with a DP from 2 to ~10, according to the IUB–IUPAC nomenclature. However, some authorities recommend using the term disaccharides for those having two monosaccharide units (DP = 2). In fact, most disaccharides fit into simple digestible sugars, while there are some disaccharides that resemble longer oligosaccharides in physiological and biochemical characteristics; for example, they are poorly digested and absorbed in the small intestine, but fermented thoroughly in the colon like a sort of non-digestible oligosaccharides, as will be described later. It is difficult to find chemical, structural, and physiological reasons to fix the definition for oligosaccharides. In this article, oligosaccharides with a DP from 2 to ~10 that are not digested by mammalian digestive enzymes in the small intestine are defined as NDO.

The sources of NDO are diverse. Some are isolated from natural origins (human milk oligosaccharides (HMO), soybean oligosaccharides (SOS), levan-type fructans, etc.) and some can be enzymatically or chemically produced from polysaccharides (maltooligosaccharides and isomaltooligosaccharides (for both, α-glucooligosaccharides), oligofructose, xylooligosaccharides, chitin oligosaccharides, etc.) or from mono- and disaccharides (galactooligosaccharides (GOS), fructooligosaccharides (FOS), lactosucrose, gentiooligosaccharides, etc.).

Polysaccharides occur naturally and have more complex structures and lengths than oligosaccharides, and can be classified into starch (α-glucans) and nonstarch polysaccharides (NSP) (**Table 1**). In both classes, there are soluble and insoluble polysaccharides. Of the carbohydrates in foods 80–90% are starch consisting of α-1,4-linked amylose and α-1,4- and α-1,6-linked amylopectin, which are both principally hydrolyzed by human α-amylase. However, the physical structure and DP of starch are diverse, and thus not all starch molecules consumed are hydrolyzed by human digestive enzymes and absorbed in the upper small intestine. The undigested starch entering the colon is called resistant starch (RS) and provides colonic bacteria with a carbon and energy source. On the other hand, NSP are the sum of non-α-glucans and β-glycans mainly found in plant cell walls such as cellulose, hemicellulose, xylan, arabinoxylan, mannan, pectin, and lignin. Some NSP are intracellular polysaccharides of plant cells such as gums, mucilage, and inulin. All these NSP are poorly digested and absorbed in the small intestine, and hence enter the colon undigested and are readily fermented by colonic bacteria to various extents. Different bacterial groups consume different polysaccharides; bacteroides mainly utilize amylose, amylopectin, and pullulan, which are all α-glucan-type RS, while various anaerobic clostridial species utilize NSP.

The term 'dietary fiber' was originally defined by Trowell, in 1972, as remnants of plant cells remaining after digestion in the mammalian gastrointestinal tract. Nowadays a number of other indigestible carbohydrates of plant and animal origin have been proposed and considered for inclusion in the group of dietary fiber. Hence it is most likely that dietary fiber will be defined as food carbohydrate polymers that are not hydrolyzed by the mammalian digestive enzymes in the small intestine and includes NSP, RS, and NDO.

Among these, NDO occupy the most important position in prebiotic substances as they have been extensively studied and shown to be specific substrates for a limited number of colonic bacteria, especially bifidobacteria and/or lactobacilli, in colonic fermentation, and are more readily assimilated in the colon than NSP and RS. Therefore, NDO's exertion of the prebiotic effects could be quicker and more tangible than others' after they enter

Table 1 Classification of dietary carbohydrates

Carbohydrate classes (DP)	*Subclasses*	*Examples*	*Fate in the gastrointestinal tract*
Monosaccharides (1)	Sugar	Glucose, fructose, galactose	Absorbed in the small intestine Glucose gives rapid glycemic response
	Sugar alcohol	Sorbitol, xylitol, mannitol	Absorbed in the small intestine
Disaccharides (2)	Digestible sugar	Sucrose, maltose, trehalose, (lactose)[a]	Absorbed in the small intestine Digestible by endogenous hydrolyzing enzymes Rapid glycemic response
	Nondigestible disaccharides	(Lactose),[a] lactulose	Not absorbed Nondigestible, but fermented in the large intestine
	Sugar alcohol	Maltitol, lactitol	Poorly digested and absorbed in the small intestine Partly or fully fermented in the large intestine
Oligosaccharides (2–10)	α-Glucans	Maltooligosaccharides (isomaltooligosaccharides)[b]	Digestible but partly undigested in the small intestine and give rapid glycemic response
	Nondigestible oligosaccharides	Soybean oligosaccharides Galactooligosaccharides Fructooligosaccharides	Nondigestible Fermented in the large intestine No glycemic response
	Human milk oligosaccharides	Fucosyllactose Sialyllactose Lacto-*N*-tetraose, etc.	Nondigestible Partly fermented in the large intestine
Polysaccharides (>10)	Starch (α-glucans)	Amylose, amylopectin, pullulan	Digested and absorbed in the small intestine Rapid glycemic response
	Resistant starch (α-glucans)		Digestible but undigested in the small intestine Fermented in the large intestine
	Nonstarch polysaccharides (β-glycans)	Cellulose, hemicellulose, inulin, guar gum	Nondigestible Partly fermented in the large intestine

[a] Lactose may be both a digestible and nondigestible sugar.

[b] Isomaltooligosaccharides are less susceptible to digestive enzymes in the small intestine.

the colon. The other reasons are that they are easy to handle in food processing and have organoleptic characteristics as mild sweeteners, both of which are important causes for NDO to be widely spread as commercial products.

Disaccharides

A certain class of disaccharides characterized with a β-linkage between the two units could have a role as prebiotics. This class of disaccharides includes lactose, lactulose, lactitol, and several by-products from various oligosaccharide production processes (**Figure 1(a)**). These disaccharides are poorly or very slowly hydrolyzed by human digestive enzymes. Human studies, as well as *in vitro* growth analyses, published so far have shown their possible prebiotic effect particularly on stool habits.

Lactose

Lactose (4-β-D-galactopyranosyl-D-glucopyranose or β-D-Gal*p*-(1 → 4)-D-Glc*p* or Galβ1-4Glc) is the primary sugar of mammalian milk. Human milk contains ~7% lactose, and cow's milk ~4.8%. Even though lactose is the major carbon source for suckling infants, hydrolysis of lactose is rather a rate-limiting step. In addition, the lactase (β-galactosidase) activity in the small intestine gradually declines as an infant grows: According to a survey of Pima Indians in the United States, lactose malabsorption was seen in 40% by age 3–4 years, 71% by age 4–5 years, and almost 100% by age 8 years. The onset and degree of lactase deficiency are not homogenous among individuals and races. While most Caucasians in Northern European countries, Australia and New Zealand, and North America retain lactase activity, most Africans, Asians, and Native Americans are nonpersistent.

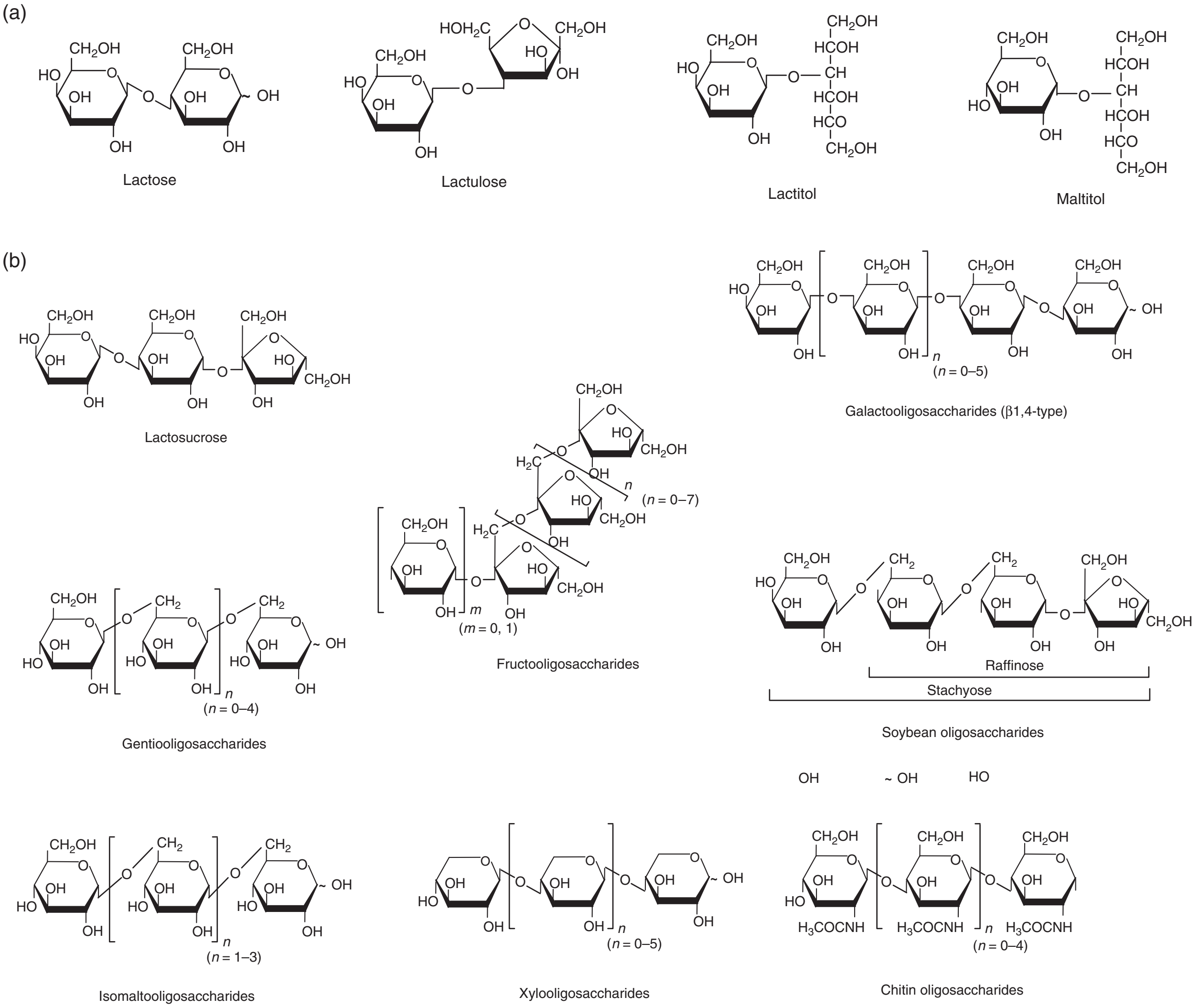
(a)
Lactose
Lactulose
Lactitol
Maltitol
(b)
Lactosucrose
(n = 0–5)
Galactooligosaccharides (β1,4-type)
(n = 0–7)
(m = 0, 1)
Fructooligosaccharides
(n = 0–4)
Gentiooligosaccharides
Raffinose
Stachyose
Soybean oligosaccharides
OH
~ OH
HO
(n = 1–3)
Isomaltooligosaccharides
(n = 0–5)
Xylooligosaccharides
(n = 0–4)
Chitin oligosaccharides

Figure 1 Chemical structure of major prebiotic disaccharides (a) and oligosaccharides (b). Vertical bars without any formula at the tips of angles indicate a hydroxyl group. Hydrogen atoms on the main frames are not indicated.

Therefore, a substantial number of people are lactose malabsorbers. In these populations lactose behaves like nondigestible carbohydrates especially in lactose maldigesters. While the adverse effects of lactose are extensively studied, few reports concerning the effect on gut microflora are available. Although lactose is utilized preferentially by bifidobacteria and lactobacilli in *in vitro* fermentation, additional human studies are still needed to elucidate its prebiotic effect.

Lactulose

Lactulose (Galβ1-4Fru) is a synthetic disaccharide produced from lactose by chemical isomerization under alkaline conditions in the presence of sodium hydroxide and boric acid. Lactulose also naturally appears in heat-treated cow's and human milks. The generation of lactulose during lactose preparation was first reported in 1930. In the history of searching growth-promoting factors for bifidobacteria (bifidus factors) in human milk, Petuely described lactulose as a bifidus factor in 1957. Since then there are a large number of reports published about the effects of lactulose on the gut microflora, stool habits, SCA production, fecal enzyme activity, gut physiology, and so on. Lactulose is not hydrolyzed by human digestive enzymes and is preferentially utilized by bifidobacteria and lactobacilli as well as by bacteroides and some strains of clostridia and Gram-positive cocci inhabiting the human gut. According to these extensive studies, lactulose is now widely used not only as a food additive but also as a drug for constipation, hepatic encephalopathy, and *Salmonella* infection worldwide.

Sugar Alcohol (Polyol)

Sugar alcohols are derivatives of mono- and disaccharides obtained by reducing a hexose moiety. Major sugar alcohols available and of commercial interest are the monosaccharide polyols sorbitol, mannitol, and xylitol, and the disaccharide polyols lactitol (Galβ1-4-sorbitol) and maltitol (Glcα1-4-sorbitol). In recent years, most of these sugar alcohols have been developed and widely applied for commercial use as noncariogenic sweeteners. They all have less sweetness than sucrose and lower calorific values than normal sugars. While the monosaccharide polyols are all efficiently absorbed from the small intestine, the digestion and thus the absorption of disaccharide polyols, namely, lactitol and maltitol, are much less than those of their parent disaccharides, lactose and maltose, respectively. The average rates of hydrolysis of lactitol and maltitol in human small intestine were shown to be ~1.5 and 10% of those of lactose and maltose, respectively. These disaccharide polyols are good substrates for fermentation with colonic bacteria. Lactitol exerts quite similar effects to lactulose in clinical situations and is also used as a drug for treatment of chronic constipation and hepatic encephalopathy, although it may show a colonic fermentation profile different from that of lactulose.

Oligosaccharides

The main and the most important constituents of prebiotics are NDO. Research and application of NDO have attracted much attention since the 1990s as the importance and the role of the gut microflora in human health are becoming more and more documented. As an intrinsic characteristic of NDO, they must escape hydrolysis by human digestive enzymes and be fermented by a limited number of colonic bacteria. Human pancreatic and intestinal digestive enzymes include those hydrolyzing the α-glycosidic bonds of various monosaccharide moieties like glucose, galactose, and fructose, except for lactase (β-galactosidase) (EC 3.2.1.108), which mainly hydrolyzes the β-linkage of lactose, whereas many colonic bacteria produce a variety of carbohydrate-hydrolyzing enzymes that act on oligo- and polysaccharides with β-glycosidic bonds. Therefore, the principal NDO are β-glycans (**Table 2** and **Figure 1(b)**). The calorific values of most NDO are about half of those of digestible sugars, namely ~2 kcal g^{-1}, which are calculated from energy values available by utilization of organic acids produced in the colon after fermentation. Major NDO that are commercially available or of physiological importance are listed in **Table 2**.

Production of Nondigestible Oligosaccharides

There are three ways to produce NDO: partial hydrolysis of polysaccharides, synthesis by transglycosylation from mono- and disaccharides, and extraction of naturally occurring oligosaccharides. **Figure 2** shows the fundamental processes of industrial production of the former two types of NDO. In the industrial transglycosylation process, a high concentration of a substrate solution of mono- or disaccharides at more than 40–50% is used, and more than 50 up to 70% of the crude products are the target oligosaccharides after enzyme reaction. On the other hand, when polysaccharides are used as substrates, oligosaccharides are obtained either by direct endoglycosidase treatment (fructooligosaccharides, xylooligosaccharides, chitin oligosaccharides) or by liquefaction with complete glycolysis followed by transglycosylation (isomaltooligosaccharides, pannose oligomers). The substrates and enzymes used in the production process are indicated in **Table 2**.

Table 2 Nondigestible oligosaccharides and their structural features

Name of NDO	*Structures*	*Sources*	*Methods of preparation*
Lactulose	β-D-Gal-(1 → 4)-D-Fru	Lactose	Isomerization in alkali
Galactooligosaccharides	[β-D-Gal-(1 → 4)]$_n$-D-Glc ($n = 2$ to ∼6) or[β-D-Gal-(1 → 6)]$_n$-β-D-Gal-(1 → 4)-D-Glc ($n = 2$ to ∼5)	Lactose	Enzymatic transgalactosylation with β-galactosidase (EC 3.2.1.23)
Fructooligosaccharides	α-D-Glc-[(1 → 2)-β-D-Fru]$_n$ ($n = 2$–4)	Sucrose	Enzymatic transfructosylation with β-fructosylfuranosidase (EC 3.2.1.26)
Inulin-type fructans	α-D-Glc-(1 ↔ 2)-β-D-Fru-[(1 → 2)-β-D-Fru]$_n$ ($n = 1$ to ∼8)D-Fru-[(1 → 2)-β-D-Fru]$_n$ ($n = 2$ to ∼9)	Inulin	Partial enzymatic hydrolysis with inulinase (EC 3.2.1.7)
Raffinose	α-D-Gal-(1 → 6)-α-D-Glc-(1 ↔ 2)-β-D-Fru	Sugar beet	Natural product
Soybean oligosaccharides	[α-D-Gal-(1 → 6)]$_n$-α-D-Glc-(1 ↔ 2)-β-D-Fru ($n = 1$, 2)	Soybean extract	Extraction
Xylooligosaccharides	β-D-Xyl-[(1 → 4)-D-Xyl]$_n$ ($n = 1$ to ∼6)	Xylan	Partial enzymatic hydrolysis with xylanase (EC 3.2.1.8)
Human milk oligosaccharides	α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 4)-D-Glc, α-NeuAc-(2 → 3)-β-D-Gal-(1 → 4)-D-Glc, β-D-Gal-β-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-D-Glc, etc.	Human milk	Natural products
Chitin oligosaccharides	β-D-GlcNAc-[(1 → 4)-D-GlcNAc]$_n$ ($n = 1$ to ∼5)	Chitin	Enzymatic hydrolysis with chitinase (EC 3.2.1.14) or acid hydrolysis
Lactosucrose	β-D-Gal-(1 → 4)-α-D-Glc-(1 ↔ 2)-β-D-Fru	Lactose, sucrose	Enzymatic transfructosylation with β-fructosyltransferase (EC 2.4.1.9)
Isomaltooligosaccharides (α-glucooligosaccharides)	α-D-Glc-[(1 → 6)-D-Glc]$_n$ ($n = 1$ to ∼3)	Starch	Enzymatic hydrolysis followed by enzymatic transglucosylation with transglucosidase (EC 3.2.1.70)

Gal, galactose; Fru, fructose; Glc, glucose; Xyl, xylose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; NeuAc, sialic acid.

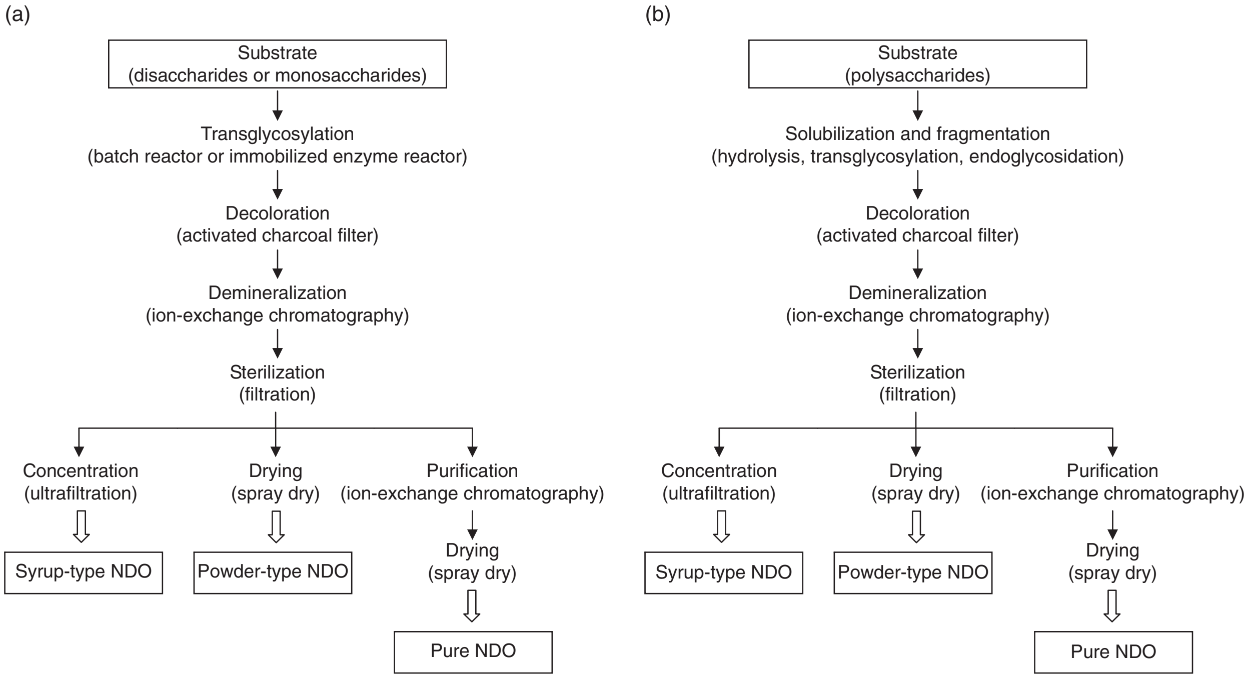
(a)
Substrate
(disaccharides or monosaccharides)
Transglycosylation
(batch reactor or immobilized enzyme reactor)
Decoloration
(activated charcoal filter)
Demineralization
(ion-exchange chromatography)
Sterilization
(filtration)
Concentration
(ultrafiltration)
Drying
(spray dry)
Purification
(ion-exchange chromatography)
Syrup-type NDO
Powder-type NDO
Drying
(spray dry)
Pure NDO
(b)
Substrate
(polysaccharides)
Solubilization and fragmentation
(hydrolysis, transglycosylation, endoglycosidation)
Decoloration
(activated charcoal filter)
Demineralization
(ion-exchange chromatography)
Sterilization
(filtration)
Concentration
(ultrafiltration)
Drying
(spray dry)
Purification
(ion-exchange chromatography)
Syrup-type NDO
Powder-type NDO
Drying
(spray dry)
Pure NDO

Figure 2 Flowcharts of the synthesis of NDO by transglycosylation using mono- and disaccharides (a) or by hydrolysis/fragmentation of polysaccharides (b).

Galactooligosaccharides

GOS are industrially produced from lactose by enzymatic transgalactosylation. β-Galactosidase (EC 3.2.1.23) of various origins such as *Bacillus circulans*, *Aspergillus oryzae*, and *Cryptococcus laurentii* is used for the industrial production of GOS. The enzymes from *Bifidobacterium* strains also have this transgalactosylation activity. The enzyme reaction basically proceeds by the addition of the galactose moiety to the nonreducing end of lactose and transgalactosylated oligomers, resulting in the production of tri-, tetra-, and pentasaccharides, traces of multioligosaccharides, and some disaccharide by-products with different β-glycosidic bonds. The products have mainly β1,4- or β1,6-glycosidic bonds between the added galactose moieties (DP from 3 to ~6) and a glucose unit at the reducing end of the molecule. GOS are physically stable in various conditions; they were not degraded at 160 °C at a neutral pH or at 120 °C at pH 3 for 10 min.

GOS are not digested by human digestive enzymes at all, but are readily fermented in the colon. From extensive studies of the utilization of GOS, it has been revealed that *Bifidobacterium* and *Bacteroides* strains predominantly grow utilizing GOS as the sole carbon source. The prebiotic effect of GOS was first demonstrated by the pioneering work of Tanaka and colleagues in 1983, which showed an increase in indigenous bifidobacteria and a decrease in Bacteroidaceae after a daily intake of 10 g GOS for 2 weeks, in a human study. This was further confirmed by additional human studies. The potential of GOS to improve defecation in subjects with a tendency toward constipation, to reduce harmful enzyme activities, to reduce the incidence of cancer, to stimulate bone mineralization, and to reduce the production of secondary bile acids in feces has been documented in human and/or animal studies.

Fructooligosaccharides

There are two different ways to produce FOS: one is to partially hydrolyze fructose polymers of plant origin, and the other is to transfer the fructose moiety onto sucrose. Fructose polymers occur naturally in a number of vegetables and fruits as β-2,1-linked inulin or β-2,6-linked levan. Inulin is mainly used for the production of FOS having a DP of 2 to ~10 by partial enzymatic hydrolysis using endoinulinase. This type of FOS, sometimes called inulin-type oligofructose, is a mixture of $\mathrm{Glc}\alpha 1\text{-}2\beta\mathrm{Fru}[1\text{-}2\beta\mathrm{Fru}]_n$ ($n = 1$ to ~8) and $\mathrm{Fru}[1\text{-}2\beta\mathrm{Fru}]_n$ ($n = 1$ to ~9). FOS are also industrially produced from sucrose by enzymatic transfructosylation using *Aspergillus niger* β-fructosylfuranosidase (EC 3.2.1.26). This type of FOS has a DP of 3–5 including an α-1,2-linked glucose residue at the terminal of each molecule, and thus is nonreducing. The stability of FOS at neutral pH is as high as that of sucrose, and FOS do not degrade up to 150 °C. However, FOS are less stable in acidic conditions; when boiled at pH 3, most FOS molecules degrade into smaller molecules within 15 min.

Table 3 Utilization of NDO by various intestinal bacteria

	Glucose	*Lactose*	*Lactulose*	*GOS*	*FOS*	*SOS*[a]	*XOS*	*COS*	*Lactosucrose*
Bifidobacterium adolescentis	+++	+++	+++	+++	+++	++ ∼ +++	+++	++	+++
Bifidobacterium bifidum	+++	+++	+++	+++	−	−	−	++	−
Bifidobacterium breve	+++	+++	+++	+++	++	+++	−	++	+++
Bifidobacterium infantis	+++	+++	+++	+++	+++	+++	+ ∼ +++	+	+++
Bifidobacterium longum	+++	+++	+++	+++	+++	+++	++ ∼ +++	++	+++
Lactobacillus acidophilus	+++	+++	+++	++	++	− ∼ +	−	+	
Lactobacillus casei	+++	++	+++	−	−	−	−	++	−
Lactobacillus gasseri	+++	+++	+++	−		+ ∼ ++			
Lactobacillus salivarius	+++	+++	+++	−	+++	++ ∼ +++	− ∼ +	++	−
Bacteroides distasonis	+++	+++	+++	+++	+	+ ∼ +++	++		+++
Bacteroides fragilis	+++	+++	+++	+++	+++	+ ∼ ++	− ∼ +		+++
Bacteroides ovatus	+++	+++	+++	−	+++	+++	+ ∼ ++		
Bacteroides thetaiotaomicron	+ ∼ +++	+++	+++		+++	− ∼ +	++		+++
Bacteroides vulgatus	+++	+++	+++	+++	+++	+ ∼ ++	− ∼ +		+++
Mitsuokella multiacidus	+++	+++	+++	−		++ ∼ +++			
Rikenella microfusus	+++	++	−	−		−			
Megamonas hypermegas	+++	+++	+++	+++		+++			
Clostridium butyricum	+++	++	++ ∼ +++	−	+++	+ ∼ ++	−		++
Clostridium difficile	+++	−	−	−	−	−	−	+	−
Clostridium innocuum	+++	−	−	−		−			
Clostridium perfringens	+++	+++	++ ∼ +++	−	−	− ∼ +	−	++	+++
Clostridium ramosum	+++	+++	++ ∼ +++	−		++		−	
Eubacterium aerofaciens	+++	+++	+++	−		− ∼ +	−		
Eubacterium limosum	+++	+	+ ∼ ++	−	−	− ∼ +	−		
Peptostreptococcus anaerobius	+++	+++	+++	−	−	−	−		
Peptostreptococcus prevotii	+++	−	−	−		+++	−		
Peptostreptococcus productus	+++	+++	+++	−		+++	++		
Propionibacterium acnes	+++	++	++	−	−	−	−		
Fusobacterium varium	+++	−	+	−	−	− ∼ +	−		
Veillonella alcarescens ssp. *dispar*	+++	−	−	−	−	−			
Megaphaera elsdenii	+++	−	−	−	−	−			
−*Enterococcus fecalis* ssp. *fecalis*	+++	−	++	−	−	− ∼ ++	−		−
Enterococcus faecium	+++	−	++	−		++			
Escherichia coli	+++	++	+ ∼ +++	−	−	−	−	−	−

[a] The data include raffinose.

Indication for the growth of bacteria: +++, same as that on glucose; ++, less than that on glucose; +, slight growth; −, no growth; no symbol, no data available.

Adapted from Hayakawa Y and Committee for New Materials of Foods (eds.) (1998) *New Knowledge of Oligosaccharides*. Tokyo (in Japanese): Food Chem. Newspaper Co Ltd.

Bifidogenic effect and other prebiotic effects of FOS such as lowering pH in the colon, reducing potentially harmful bacteria, reducing putrefactive substances, and improving stool habit have been demonstrated in human studies. In addition, beneficial effects of FOS on lipid metabolism and mineral absorption have been suggested in animal studies. While the effect on calcium absorption was further confirmed in several human studies, evidence of beneficial effects on lipid metabolism in humans is not conclusive.

Raffinose, stachyose, and soybean oligosaccharides

SOS can be readily isolated from soybean extract and constitute raffinose (Galα1-6Glcα1-2βFru) and stachyose (Galα1-6-raffinose) as oligosaccharides, as well as sucrose, glucose, and fructose. The contents of raffinose and stachyose in SOS are usually $\sim$8 and 24%, respectively, whereas the content of sucrose plus glucose and fructose is $\sim$55%. Pure raffinose is also commercially produced from beet syrup.

Raffinose and stachyose (Figure 1(b)) are not digested in the human upper intestine, but are readily fermented by colonic bacteria, and so are NDO. Most strains of *Bifidobacterium* species except *B. bifidum* can grow in a medium containing SOS, raffinose, or stachyose as the sole carbon source, since they usually express α-galactosidase activity, which can hydrolyze SOS. The bifidogenic effect of SOS and raffinose has been shown in human studies, where the minimum effective dose could be as low as 0.5 g oligosaccharides equivalent day^{-1}. In addition, SOS have the potential to reduce fecal levels of ammonia, *p*-cresol, and indole, to reduce harmful enzyme activities, and to alleviate constipation at the dose of 1 g oligosaccharides equivalent day^{-1}.

Xylooligosaccharides

Xylooligosaccharides (XOS) are β-1,4-linked xylose oligomers with a DP of 3 to $\sim$8, and industrially produced exclusively in Japan from xylan by partial enzymatic hydrolysis using endoxylanase (EC 3.2.1.8). Xylan, a sort of hemicellulose, is usually found in plant cell walls in conjunction with cellulose and pectin. For the commercial production of XOS, plant materials containing large amounts of xylan such as bagasse and cottonseeds are used.

XOS is fermented by limited types of colonic bacteria such as *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides vulgatus*, and *Peptostreptococcus products*. Bifidogenic effect *in vivo* has been shown in a couple of human studies where the effective minimal dose of XOS was as low as 0.4 g day^{-1}. Alleviation of constipation and stimulation of mineral absorption were also documented in human and rat studies, respectively.

Chitin Oligosaccharides

Chitin oligosaccharides (COS) are *N*-acetylglucosamine (GlcNAc) oligomers (DP = 2–6) with β-1,4-linkages, and are produced from chitin derived from crabs and shrimps by partial acid hydrolysis in a hydrochloride solution. COS can be produced by enzymatic hydrolysis of chitin using bacterial chitinase (EC 3.2.1.14) as well.

In addition to the beneficial effects on the gut microflora, attention has been paid to COS due to their immunomodulatory and antimicrobial activities, although the mechanisms behind these effects are mostly obscure.

Human Milk Oligosaccharides

A French pediatrician, Tissier, observed more than a century ago that bifidobacteria were predominant microbes in the feces of breast-fed infants but not of formula-fed infants, and had an idea that this bifidus flora played a role in the reduced incidence of infection in breast-fed infants. A number of possible substances as bifidogenic factors in human milk have been proposed and utilized to maintain the bifidus flora in formula-fed infants since then. Examples of substances that had been tried include lactose, *N*-acetylglucosamine-containing oligosaccharides, whey proteins, and vitamins, none of which, however, provided any evidence of modulation of the host gut microflora so far.

The composition of HMO is complex. A variety of neutral and acidic oligosaccharides are found in human milk and colostrum. Although the major carbohydrate component of human milk is lactose, the total HMO levels reach up to 20% of the total carbohydrates, or over 12 g l^{-1} in mature milk and 22 g l^{-1} in colostrum, depending on individuals and the stages when the milk/colostrum is collected. The core carbohydrates in HMO are lactose and lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glu), which are usually fucosylated and/or sialylated at nonreducing ends and other sites. Genomic and molecular biology studies have revealed that strains of *Bifidobacterium longum* ssp. *longum*, *B. longum* ssp. *infantis*, *B. bifidum*, and *B. breve* have various combinations of genes coding for enzymes responsible for the utilization of HMO, while HMO are not digested and absorbed in human small intestine. Therefore, HMO are most likely to be the bifidogenic factor in human milk. A certain group of HMO share the structural motifs with the cell surface glycoconjugates (glycoproteins or glycolipids) and mucins to which pathogens adhere at an initial step of infection. Thus, HMO could also be important as absorbers of pathogens to prevent infection during breast-feeding.

A practical method to produce lacto-*N*-biose, one of the active bifidobacteria-specific carbon sources derived from lacto-*N*-tetraose, from sucrose and *N*-acetylglucosamine with the enzymes sucrose phosphorylase (EC 2.4.1.7), UDP-glucose-hexose-1-phosphate uridylyltransferase (EC 2.7.7.12), UDP-glucose 4-epimerase (EC 5.1.3.2), and lacto-*N*-biose phosphorylase (EC 2.4.1.211) in large quantities has been established. However, it is still to be confirmed what components in HMO are and to what extent the HMO are responsible for the predominant growth of bifidobacteria in breast-fed infants.

Other Oligosaccharides

Other commercially available oligosaccharides are malto-oligosaccharides (α-1,4-linked D-glucose oligomers) and isomaltooligosaccharides (α-1,6-linked D-glucose oligomers), both of which are often called α-glucooligosaccharides as well, palatinose oligomers (oligomers of 2–4 palatinose (Glcα1-6Fru) units), α-glucosylsucrose (coupling sugar), lactosucrose, nigerooligosaccharides (α-1,4-linked D-glucose oligomers with an α-1,3-linked D-glucose at the nonreducing end), gentiooligosaccharides (β-1,6-linked D-glucose oligomers), chitosanoligosaccharides (β-1,4-linked D-glucosamine oligomers), and so on. Although they have not been necessarily intended to be used as prebiotic food additives, but rather to be used as alternative sweeteners with low calorific values and a low sweetness, evidence is accumulating that some of them have bifidogenic activity. In addition, oligosaccharide fractions obtained from partially hydrolyzed NSP such as guar gum, acacia gum, and wheat bran are possible prebiotic agents.

Polysaccharides

Recent studies on dietary polysaccharides have revealed that a variety of dietary polysaccharides have physiological roles, which include significant fermentability by colonic bacteria leading to the production of SCA, influence on colonic microflora, stimulation of mineral absorption, and so on. While numerous studies have shown an increase in SCA production in the colon after the ingestion of RS, NSP, or even starch polysaccharides, an increasing number of studies have shown the effect of dietary polysaccharides on the gut microflora and other physiological parameters in humans or animals. In this section some examples of these polysaccharides are described.

Fructans

Fructan is the general name of soluble polysaccharides in which one or more fructosyl–fructose links constitute the majority of glycosidic linkages. Two types of fructans have been identified: one is inulin, which is mainly of plant origin and has a β2,1-linkage between fructosyl residues, and the other is levan, which is mainly produced by fungi and bacteria and has a β2,6-linkage. Both types of fructan are neither digested by hydrolases of human origin nor absorbed in the intestines. Inulin has been manufactured, and thus has been studied extensively, and is of industrial importance. The biosynthesis of inulin in plant cells involves two enzymes: sucrose-sucrose fructosyltransferase (EC 2.4.1.99) leading to the formation of 1-kestose (Glcα1-2βFru1-2βFru) followed by chain elongation by fructan-fructan fructosyltransferase (EC 2.4.1.100) leading to the formation of inulin (β-D-fructofranan). A number of plants contain fructans as storage carbohydrates, some of which we eat as vegetables and fruits; examples are onion, garlic, asparagus, artichoke, chicory, and bananas. Among all, the root of chicory and the tuber of Jerusalem artichoke are the exclusive, if not the only, materials utilized for the industrial production of inulin because of the simplicity of extraction and purification. While native chicory inulin has a DP of 3–70 (average, ~35), native inulin preparation from Jerusalem artichoke has a DP of 2 (sucrose) to 15 (average, ~7).

Inulin shows physiological properties as dietary carbohydrates similarly to the shorter FOS as evidenced in a number of studies published so far. Inulin has a bifidogenic effect like that of FOS, although inulin itself is not fermented by bifidobacteria in *in vitro* cultivation. This could be because the natural inulin preparation contains shorter oligomers like FOS in addition to longer polymers or because the fructosyl–fructose linkage in polymers may be labile in acidic conditions in the stomach or be digested to oligomers by other colonic bacteria, thereby being preferentially utilized by bifidobacteria. Its bifidogenic effect and fecal bulking with increased water content have been observed in human studies as well as in those using human fecal batch cultures or pure bacterial cultures.

Other preliminary physiological effects of fructans reported so far are improved bowel habit, reduced putrefactive fermentation in the large intestine, improved calcium and magnesium absorption, and reduced total serum lipids and cholesterol. All these predictive health effects still await further confirmation in well-designed human trials.

Resistant starch

RS is a generic term for starches that escape hydrolysis by human digestive enzymes and absorption in the upper gastrointestinal tract. Starches are storage carbohydrates of plant cells consisting of linear α1,4-D-glucan chains (amylose) and those having some additional α1,6-linkages (amylopectin). During food processing, extensive treatment of starches leads to breakages and conformational changes of starches to various forms, depending on the source and due the amylose-to-amylopectin ratio, resulting in the generation of retrograded starch with a crystalline structure. In addition, there is another type of RS that just escapes digestion in the small intestine due to its physical structure and due to other materials surrounding the starch. Englyst and colleagues, in 1992, classified RS into three groups: retrograded starch, physically indigestible starch, and RS granules.

The bifidogenic effect of RS was first suggested by a feeding study on rats, followed by synbiotic (both probiotic and prebiotic) application to pigs, in which concurrent feeding of high-amylose maize starch and bifidobacteria resulted in higher fecal excretion of bifidobacteria. However, this effect needs to be confirmed by human studies.

Other polysaccharides

It has been reported that germinated barley foodstuff (GBF), which contains low-lignified hemicellulose and cellulose, has the ability to increase the number of bifidobacteria in the gut microflora and to produce more butyrate in the colonic contents. This could be explained as the production of butyrate by the coordinated action of bifidobacteria and *Eubacterium*, because the batch culture with both *B. longum* and *Eubacterium limosum* strains in a medium with GBF as the sole carbon source resulted in the accumulation of butyrate in the medium, whereas no or little butyrate was detected in the single cultures. This indicates that cellulose and hemicellulose, which cousitute a major part of NSP, may have the bifidogenic effect through an indirect supply of fermentable oligosaccharides for bifidobacteria, which are generated by partial breakage of the polysaccharides by *Eubacterium*. As such, bifidogenic and/or prebiotic effects of various indigestible polysaccharides could be substantiated in the future, as the research in this field will proceed.

Conclusions and Prospects

After establishment of the definition of prebiotics in conjunction with that of probiotics, scientific research on food components and additives that influence the composition and function of human gut microflora in health and disease has been accelerated. While the ‘bifidogenic’ effect is recognized as a fundamental for prebiotics, other effects of oligo- and polysaccharides on the host health have been described. However, as it has been defined and revised later, the term ‘prebiotics’ should be used for the food components that have the ability to change the composition and/or activity of the intestinal microflora to healthier states.

Nowadays more than 10 different di- and oligosaccharides are considered as prebiotic agents due to their bifidogenic effect, and the number is still increasing as research and development on dietary carbohydrates proceeds. In addition, the health benefits of prebiotics or dietary carbohydrates are extended wider than previously expected. This is typically explained by the effect on mineral absorption, which may be attributed to the increased production of SCA or to the acidification of the intestinal contents, although the precise mechanism underlying the effect is still to be confirmed. However, there are substantial reports that also describe the stimulating effect of dietary polysaccharides (dietary fiber) on mineral absorption. As such, while a number of different prebiotics with health benefits have been developed, which are composed of a variety of monosaccharide units, with different linkages and lengths, and have different physicochemical properties, the mechanism of action and the outcome of the effects could be similar to each other’s. In this respect, we may need some common biomarkers and analysis procedures with which sound scientific evaluation of each prebiotic agent can be made.

See also: **Prebiotics**: Functions.

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Functions

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Introduction

At the end of the nineteenth century, H. Tissier discovered a huge number of specific bacteria, bifidobacteria, in the feces of breast-fed infants. It was believed after his discovery and from the subsequent studies that the bifidobacteria-dominated microflora, called 'bifidus flora', played an important role in the reduction of infectious diseases in breast-fed infants. In the same line of evidence E. Metchnikoff speculated in his book *The Prolongation of Life* published in 1907 that fermented milk with lactic acid-producing bacteria could have a role in keeping the intestines healthy, thus leading to longevity. In addition, there was accumulating evidence indicating that the healthy gut microflora helps the animal to resist infections, which was hence called 'colonization resistance' or 'competitive exclusion'. All these observations in conjunction with practical applications of certain bacteria to treat infections in humans and animals generated an idea that certain beneficial bacteria can modulate the gut microflora to maintain a healthy balance of the flora, thus relieving the adverse effects of disturbed gut microflora and keeping the host animal healthy. R. Fuller proposed in 1989 to call these beneficial bacteria 'probiotics', and lactic acid-producing bacteria, namely, lactobacilli and bifidobacteria, have been recognized so far as typical probiotics.

Considering the determinants of the composition of the gut microflora, in turn, dietary components of our daily life strongly affect the growth and metabolism of the gut microbes; especially, dietary carbohydrates are the major energy and carbon source for the bacteria inhabiting the colon. Although the colonic bacteria as a whole have glycolytic activities against a variety of carbohydrates consisting of different monosaccharide units with different linkages and different lengths, the individual bacterial species/strains have a different set of enzymes with different substrate specificity. Therefore, the diversity of dietary carbohydrates has a strong influence on the composition of the gut microflora. As scientific evidence showing the effects of various carbohydrates on the composition of microflora accumulates, it has been recognized that certain carbohydrates can stimulate the growth of beneficial bacteria such as lactobacilli and bifidobacteria, and can provide the host with health benefits. Based on these observations, G. Gibson and M. Roberfroid proposed in 1995 the term 'prebiotic' for a food component that beneficially modulates the gut microflora to improve host health. As the research on prebiotics proceeds through the years, the health-promoting effects of prebiotics seem to be wider than initially expected. There have been published reports on a wide variety of dietary carbohydrates with various health benefits. The overall fate of prebiotics in the large intestine and their health benefits are briefly illustrated in **Figure 1**. In this article the possible health effects of prebiotics are described.

Definition of Prebiotics

A prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host health by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon". As prerequisites of a prebiotic agent, the food ingredient must be neither degraded nor absorbed in the upper intestinal tract and be a selective substrate for a limited number of indigenous beneficial bacteria; thus it alters the balance of the gut microflora in favor of a healthier composition. While carbohydrates, proteins, lipids, and other minor components of foods like vitamins and minerals that are supplied unabsorbed through the small intestine could be candidates for prebiotics, only nondigestible carbohydrates of different monosaccharide units with a rather short chain length are recognized and established so far as prebiotic agents.

Among dietary carbohydrates, nondigestible oligosaccharides (NDO) including some disaccharides are the main prebiotics, which can either be extracted from natural plants and animals or be manufactured by enzymatic or chemical reactions (for a review, *see* **Prebiotics**: Types). Nowadays, a substantial number of NDO with different structures and lengths are commercially available. In addition, fewer differences between the effects of prebiotics and those of indigestible dietary polysaccharides (or dietary fiber) are detected now than in the past, as the research on both NDO and dietary fiber advance. Especially the production of short-chain fatty acids (SCFA) from these compounds and the subsequent physiological effects in response to SCFA are probably common for prebiotics and dietary fibers. In addition, certain indigestible polysaccharides were reported to be bifidogenic, thus being able to be prebiotics. As a conclusion, the bifidogenic effect is a key criterion to

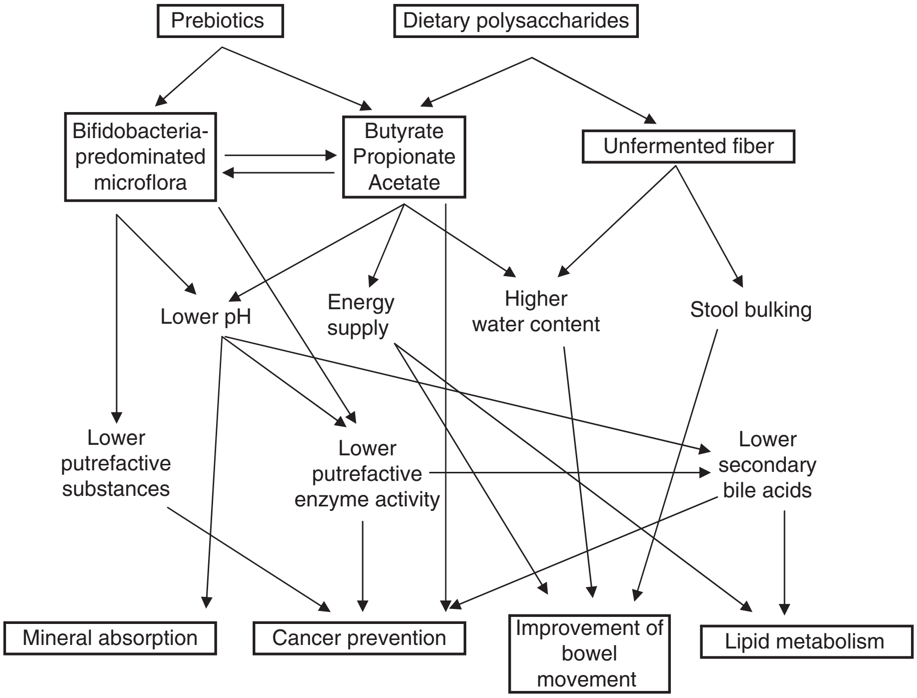
Prebiotics
Dietary polysaccharides
Bifidobacteria-predominated microflora
Butyrate Propionate Acetate
Unfermented fiber
Lower pH
Energy supply
Higher water content
Stool bulking
Lower putrefactive substances
Lower putrefactive enzyme activity
Lower secondary bile acids
Mineral absorption
Cancer prevention
Improvement of bowel movement
Lipid metabolism

Figure 1 Flow of metabolism of indigestible carbohydrates in the large intestine and their proposed effects.

distinguish prebiotics from other fibers. However, it is conceivable that this definition may be open for revision in the future.

Composition of the Human Gut Microflora and Health

The human gastrointestinal (GI) tract, especially from cecum to rectum, is heavily colonized by microbes, reaching $10^{12}\,\mathrm{g}^{-1}$ contents or 10^{14} in total, with more than 200 species in a person or probably a total of 500–1000 species. This implies that about half of the solid content of the colon or feces is bacteria. After birth, the composition of the gut microflora of a newborn undergoes changes in response to factors such as changes of diet, health status, stress, age, and medication. The fetal GI tract is sterile, but the colonization initiates during birth. It is well understood that during the period of feeding with mother's milk, the composition of the gut microflora of the infant is rather simple and that the predominant microbes are bifidobacteria. At the weaning period, there is a drastic change in the composition of the gut microflora from the infant type to the adult type, which is characterized by a more complex composition with increased contents of *Bacteroides* and clostridia, and a wider variety of different species. These commensal bacteria, about 70% of which are still uncultivable or unidentified, not only interact with the host at the mucosal surface but also constitute a complex metabolic machinery that provides the host with a variety of metabolites.

The most numerous microbes isolated from the adult colon are obligate anaerobes such as *Bacteroides* (10^{10}–$10^{11}\,\mathrm{g}^{-1}$ wet feces), *Eubacterium* (10^{10}–$10^{10.5}\,\mathrm{g}^{-1}$), *Bifidobacterium* ($10^{9}$–$10^{10.5}\,\mathrm{g}^{-1}$), *Peptostreptococcus* (10^{9}–$10^{10}\,\mathrm{g}^{-1}$), and *Clostridium* (10^{9}–$10^{10}\,\mathrm{g}^{-1}$). Facultative anaerobes such as Enterobacteriaceae, *Enterococcus*, and *Lactobacillus* are also indigenous but less numerous (10^{5}–$10^{8}\,\mathrm{g}^{-1}$). Some aerobes such as *Bacillus*, *Staphylococcus*, *Pseudomonas*, and yeasts are occasionally isolated at very low levels (10^{3}–$10^{5}\,\mathrm{g}^{-1}$), and are thought to be transient passengers.

While the composition and the activity of the gut microflora of healthy persons are relatively stable in individuals, they could be easily disturbed by environmental changes such as illness, medication, stress, and a drastic change of the diet. For instance, antibiotic treatment often causes complete disruption of the composition of the gut microflora and severe diarrhea, and parenteral nutrition also disturbs the gut microflora as well as the gut functions.

The intestinal microflora is a complex open ecosystem where the inhabiting microbes in the highly dense microbial community interact with each other and with the host animal, thus constituting the front line of defense against infection or modulating the host mucosal immune system. This could generate a barrier function, which is often called 'colonization resistance' or 'competitive exclusion', against pathogenic agents. Based on numerous studies, it has been observed that there are beneficial as well as potentially harmful microbes inhabiting the human GI tract. Lactic acid-producing bacteria like *Lactobacillus* and *Bifidobacterium* are considered beneficial as they produce acids (lactic acid and acetic acid) that contribute to maintain an acidic condition within the intestines, produce vitamins, and potentially modulate the host immune system, whereas some species of *Bacteroides*, *Clostridium*, Enterobacteriaceae, and yeasts are potentially harmful as they produce toxins and putrefactive substances, and sometimes become opportunistic infectious agents. Furthermore recent studies have provided indications that some indigenous bacteria and yeasts could have roles in initiating and activating intestinal inflammatory responses. Therefore it is widely accepted that the homeostasis and improvement of the healthy gut microflora in the direction of increasing beneficial bacteria and decreasing potentially harmful bacteria are definitely valuable to keep the host healthy.

Fermentation of Prebiotic Carbohydrates in the Large Intestine

Production of Short-Chain Fatty Acids

It has been estimated that among the dietary carbohydrates a person consumes everyday, about 20–60 g escape hydrolysis by the intestinal digestive enzymes and become substrates for fermentation in the colon: 5–35 g are resistant starch (RS), 10–25 g nonstarch polysaccharides (NSP), 2–10 g unabsorbed mono- and disaccharides, and 2–8 g NDO. The pathway of carbohydrate metabolism in the colon is schematically illustrated in **Figure 2**. Most colonic bacteria have a variety of glycolytic enzymes with different substrate specificity. The major products of microbial fermentation of these carbohydrates in the large intestine are SCFA (mainly acetic, propionic, and butyric acids) and gases (H_2, CO_2, and CH_4). It is difficult to precisely measure the profile of fermentation of dietary carbohydrates in the colon, especially in human, because most SCFA produced are rapidly absorbed at the site of production. Many researchers have tried to estimate the amounts and available energy values of SCFA by using animal models, isotope-labeled substrates, and *in vitro* fermentation system, or by indirect calculations. The overall stoichiometry for the hydrolysis of dietary carbohydrates in the intestines can be drawn from G. Liversy and M. Elia's calculation using the following equation:

$$\begin{aligned}58C_6H_{12}O_6 + 36H_2O \rightarrow\ & 60CH_3COOH + 24CH_3CH_2COOH \\ & + 16\,CH_3(CH_2)_2COOH + 92CO_2 \\ & + 256[H^+]\end{aligned}$$

where H^+ will be further accepted by another H^+ or some other molecule, and the total yield of SCFA from 100 g of carbohydrates is calculated to be 64 g. This value is almost the same as that obtained from *in vitro* fermentation of starch using a human fecal sample. However, the fermentability and the molar ratio of acetic, propionic, and butyric acids produced from carbohydrate substrates vary considerably. Thus the yield of SCFA from mixed carbohydrates is usually between 30 and 50%.

In contrast, the contribution of prebiotics in the production of SCFA is different. Considering the predominant utilization of prebiotics by bifidobacteria and/or lactobacilli where the contribution of bifidobacteria is much more than that of lactobacilli due to their numerical advantage, the fermentation profile of prebiotics in the colon will be shifted to a bifidobacteria-driven one. The stoichiometric equation for the hydrolysis of hexoses by bifidobacteria is as follows:

$$2C_6H_{12}O_6 \rightarrow 3CH_3COOH + 2CH_3CHOHCOOH$$

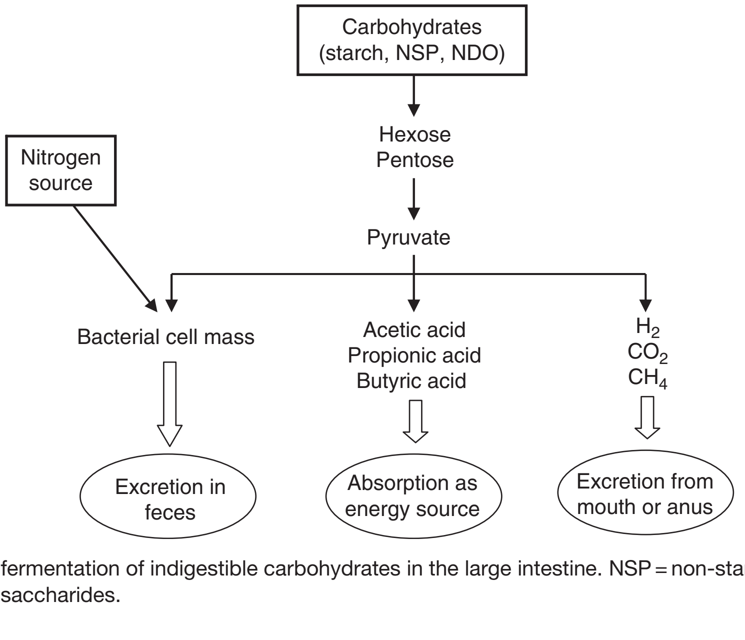

Figure 2 The pathway of fermentation of indigestible carbohydrates in the large intestine. NSP = non-starch polysaccharides, NDO = non-digestable oligosaccharides.

where the available energy value remaining as SCFA (acetic acid) is about 50%, since absorption of lactate in the colon is very slow. In this reaction, gases are not produced as primary fermentation products, although other colonic bacteria such as *Megasphaera elsdenii*, *Eubacterium hallii*, and *Anaerostipes caccae* can further metabolize lactate into butyrate plus hydrogen and carbon dioxide gases. The fermentation pattern by lactobacilli is somewhat different. There are two types of lactobacilli, homofermentative and heterofermentative, which produce only lactate and a mixture of lactate, acetate, and ethanol, respectively, from carbohydrates. The contribution of these lactic fermentations in the physiology of the lower small intestine could be substantial, because lactobacilli are the major inhabitants of that region, although that in the large intestine is probably negligible.

Nutritional Values

The SCFA produced in the fermentation process are rapidly absorbed from the mucosal surface of the large intestine and used as energy source in various ways. The calorific value of indigestible and fermentable carbohydrates has been a subject of debate due to the difficulty of direct measurement. Considering that the calorific values of digestible carbohydrates absorbed from the small intestine are 4 kcal g^{-1} (16.8 kJ g^{-1}), the yield of SCFA from indigestible carbohydrates that are completely fermented in the colon is approximately 60%, and the efficiency of availability of SCFA in the colon is about 0.85. The practical calorific values of indigestible but easily fermentable carbohydrates like NDO are calculated to be approximately 2 kcal g^{-1} (8.4 kJ g^{-1}) for most of these compounds.

The fates of SCFA in the body are distinctive: butyrate is exclusively utilized by the colonocytes, which derive 60–70% of their energy from butyrate; acetate is probably metabolized by skeletal and cardiac muscles and brain, is always detected in the bloodstream at the basal level of 50 μmol l^{-1}, and rises to 100–300 μmol l^{-1} after meals containing indigestible carbohydrates. Acetate is also utilized for the synthesis of long-chain fatty acids, glutamine, glutamate, and so on. Propionate is a major glucose precursor in ruminants, but the fate of propionate in man is much less known. Probably it is also a substrate for hepatic gluconeogenesis in man, and its effect on the lipid metabolism has been proposed.

Modulation of the Gut Microflora

A prerequisite characteristic of a prebiotic substance is to be utilized by a limited number of beneficial colonic bacteria. A fermentability profile of a variety of NDO by different colonic bacteria *in vitro* has revealed that most strains of the genus *Bifidobacterium* can utilize these carbohydrates efficiently, while the fermentation of NDO by other major genera inhabiting the colon such as *Bacteroides*, *Clostridium*, *Eubacterium*, and *Peptostreptococcus* is limited (for details, *see* **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; Probiotics, Applications in Dairy Products. **Prebiotics**: Types). In fact, from the cell extracts of bifidobacteria grown in a medium with glucose as a sole carbon source, hydrolyzing activities against α-glucosyl, β-glucosyl, β-galactosyl, and β-fucosyl bonds have been detected. This is not necessarily achieved by different enzymes. A couple of β-galactosidases (EC 3.2.1.23) and β-glucosidases (EC 3.2.1.21) with diverse substrate specificity have been identified in various strains of bifidobacteria of human origin. In contrast, animal strains of bifidobacteria analyzed so far except for *Bifidobacterium animalis* produce fewer glycolytic enzymes, the phenomenon being possibly associated with the presence of complex oligosaccharides in human milk. The production of the enzyme β-fructosylfuranosidase (EC 3.2.1.26) responsible for the digestion of fructooligosaccharides (FOS) is induced by FOS in the medium. All these characteristics of bifidobacteria enable the identification of prebiotics as bifidogenic substances.

Numerous studies have been conducted to substantiate the bifidogenic effect of prebiotic preparations. Petuely discovered in 1957 that lactulose produced from lactose has a bifidogenic effect in formula-fed infants. Supplementation of lactulose in the cow milk-based formula causes the formation of the so-called bifidus flora and a reduction of fecal pH in formula-fed infants. It also reduces the production and absorption of ammonia in the colon, and hence is approved as a medicine for constipation as well as hepatic encephalopathy. The first oligosaccharide found to exert a bifidogenic effect was galactooligosaccharide (GOS). Tanaka et al. demonstrated that after 1 week of GOS intake at doses of 3–10 g day^{-1}, the fecal bifidobacteria increased in a dose-dependent manner, which often accompanies the change of the composition of the gut microflora from a Bacteroidaceae-predominant to a bifidobacteria-predominant one and/or a decrease in Bacteroidaceae. This is also the case for FOS, lactosucrose, xylooligosaccharides (XOS), sucrosylooligosaccharides (SOS), gentiooligosaccharides, and isomaltooligosaccharides (or α-GOS).

The generation of bifidus flora in breast-fed infants has been confirmed in recent studies with both the authentic plating method and the molecular method, indicating that human milk oligosaccharides (HMO) or certain components in human milk act as prebiotic substances.

Effective doses of prebiotics to exert the bifidogenic effect in man have been estimated to be approximately 3–10 g day^{-1} for most NDO. However, XOS has been reported to show the effect at a dose less than 1 g day^{-1}, and NDO having α-glycosidic linkages like isomaltooligosaccharides usually need more than 10 g day^{-1} to show the effect similar to that of β-linked NDO due to their partial digestibility in the upper intestine.

Physiological Effects

Improvement of Stool Frequency

Beneficial effects of intake of NDO on the nature of feces have been shown in several human studies. NDO improve both the frequency and the consistency of defecation after habitual intake. For example, administration of GOS at a dose of 2.5–5 g day^{-1} for 1 week led to a significant increase in the frequency of defecation in a group of women in a double-blind placebo-controlled trial. The effect was more apparent for subjects who tended to be constipated. The mechanism behind this effect is not precisely known yet. However the intake of prebiotics in addition to normal diet results in an increase in acids especially acetic acid and lactic acid due to the predominant utilization of the substrate GOS by bifidobacteria. In addition, it has been recognized that in some cases succinic acid also accumulates in the cecal and colonic contents after ingestion of GOS in rats with humanized gut microflora and in humans. Unlike the SCFA that are rapidly absorbed from the mucosal surface of the large intestine, lactic acid and succinic acid are less efficiently absorbed, and thus contribute to the decline of the pH of the colon and to the increase in fecal water content. All these alterations of the physiology of the colon could have a role in improving the stool frequency.

In contrast, it has been observed that FOS exert an effect to prevent traveler's diarrhea, which is probably due to the stabilization of bifidobacteria- and lactobacilli-predominated healthy gut microflora.

Reduction of Putrefaction

The colonic microflora is an agent for the metabolism of various substrates. It provides the host with a variety of enzymes, and the metabolic activity of the gut microflora is estimated to be as high as that of the liver. While dietary carbohydrates are fermented into SCFA and gases, proteins and amino acids that reach the colon are fermented into SCFA as well as branched-chain fatty acids, isobutyrate, isovalerate, and 2-methylbutyrate arising from valine, leucine, and isoleucine, respectively. In addition, proteolysis followed by amino acid catabolism causes an accumulation of ammonia, phenolic compounds, amines, and sulfur compounds, which are all putrefactive substances. These substances are absorbed into the bloodstream, and, either directly or after further metabolization in the liver, show detrimental effects on human health. In a study with rats approximately 40% of blood ammonia was derived from the intestine. It has been demonstrated that the amount of urea in rat urine, the final product of ammonia metabolism, is significantly lower in germ-free rats than in normal rats. In a human study with healthy volunteers, the ingestion of GOS not only reduced the fecal ammonia concentration significantly, but also reduced other putrefactive products such as phenol, *p*-cresol, and indole in the urine. The reduction of blood ammonia concentration by GOS was further confirmed in an additional study using hyperammonemia patients. Lactulose and lactitol have been approved as medicines for hepatic encephalopathy, because they are effective for reducing blood ammonia level of the patients probably due to their effect on intestinal putrefaction. Therefore it is concluded that NDO such as GOS and lactulose can modulate the gut microflora to reduce its putrefactive metabolic activity.

Colon Cancer Prevention

Several bacterial enzymes such as β-glucuronidase, β-glucosidase, and nitroreductase derived from the gut microflora can activate precarcinogens to proximal carcinogens. For instance, bile salts secreted from the liver are converted to secondary bile acids by the enzyme β-glucuronidase derived from bacteria, and the resultant products are potential promoters of colon carcinogenesis. In a couple of studies with human volunteers, a daily intake of GOS at a dose of 10 or 15 g significantly reduced the fecal β-glucuronidase activity.

In model systems using rats and chemical carcinogens, there are a few reports that have analyzed the suppressive effect of prebiotics on the development of cancer. In a model that monitored the development of colorectal cancer induced by 1,2-dimethylhydrazine (DMH) in rats, fully fermentable GOS appeared to be highly protective, while poorly fermentable cellulose was not effective. In another model, the effect of dietary carbohydrates including FOS and inulin on the development of aberrant crypt foci (ACF), which are recognized as early preneoplastic lesions in the colon, caused by the treatment with azoxymethane (AOM) was analyzed. The formation of AOM-induced ACF was significantly reduced by treatment with inulin, FOS, pectin, or coffee fiber (rich in arabinogalactan). The increase in butyrate concentration in the colon was suggested to be an effective change for the reduction of ACF formation in the colon, because only carbohydrates that resulted in the production of large amounts of butyrate reduced AOM-induced ACF formation. This supposed effect of butyrate on the suppression of cancer development could be explained in part by the inhibitory effect of butyrate on the proliferation of cells including colon tumor cells. In addition, FOS and inulin showed enhanced apoptotic effect in the distal colon of rats after treatment with DMH, where inulin was more effective than FOS. SCFA – acetate, propionate, and butyrate – can in fact induce apoptosis in colorectal tumor cell lines at a concentration of 0.5 mmol l^{-1}, where butyrate is the most effective agent. All these results imply that SCFA, especially butyrate, produced by the colonic fermentation of dietary carbohydrates may have a role in protecting against the development of cancer in the colon by inducing apoptosis in the injured cells or proliferating cells. However, the enhanced frequency of apoptosis may also imply that the colonic cells become more susceptible to the carcinogen by the increased butyrate concentration or that the activation of the procarcinogen was stimulated by the higher concentration of butyrate. There also appears a conflicting view that fully fermentable carbohydrates such as inulin, as compared with wheat bran, may enhance colon carcinogenesis in the distal colon based on the increased PKC activity and PKC $\beta 2$ level in response to increased diacylglycerol in the colon in rats fed with a high-fat diet with inulin. Thus, the suppressive effect of prebiotics and dietary carbohydrates on colon carcinogenesis is still inconclusive.

Immune Modulation

There have been very few reports suggesting modulation of the immune system by prebiotics and dietary carbohydrates. It is not likely that prebiotics directly impact the body's immune system; however, the improvement of the intestinal environment could enhance the immune system. As described above, prebiotics and dietary carbohydrate supplementation may reduce the development of colonic neoplasia. In general, cells having neoplastic lesions are excluded through the body's immunological defense mechanism. The fact that the supplementation of FOS, inulin, or GOS in the diet resulted in the reduction of colon carcinogenesis by chemical carcinogens in rats suggests that the immune system involved in this process may be activated by these substances. Stimulation by FOS and inulin of apoptosis of colonocytes induced by treatment with a chemical carcinogen supports this idea. The question remains whether or not, and if so how, prebiotics and dietary carbohydrates directly or indirectly affect the immune system.

An alleviation of the symptoms of atopic dermatitis in infants by raffinose has been reported. It could be mediated by the improvement of the colonic microflora; especially a reduction of *Candida* level in the gut microflora is supposed to be effective in alleviating the symptoms. However, this should be confirmed by additional sound scientific clinical studies.

Stimulation of Mineral Absorption

Evidence is accumulating showing the enhancing effect of NDO on the absorption of minerals including calcium, magnesium, iron, and zinc. An increasing interest is focused especially on the intake of calcium because of its role in preventing osteoporosis. From animal and human studies, it has been shown that indigestible polysaccharides, NDO, and other carbohydrate compounds stimulate mineral absorption, and that the major site of action for absorption of minerals is the large intestine. This implies that the large intestine has a significant capacity to absorb minerals.

Among NDO, inulin, FOS, GOS, lactulose, isomaltooligosaccharides, and raffinose have been demonstrated to stimulate mineral absorption in animal models. In normal rats, it has been shown that the enhancement of calcium and magnesium absorption was totally dependent on the reduction of cecal pH due to the enhanced production of SCFA by colonic bacteria, since simultaneous addition of neomycin with GOS did not show increased calcium and magnesium absorption. Increased bone mineralization and increased bone strength were also shown by using GOS, FOS, and lactulose in ovariectomized rat models.

Unlike the numerous animal studies, however, human studies with clear positive effects of NDO on mineral absorption are not yet available. Ingestion of 15 g day^{-1} of inulin, FOS, or GOS for 3 weeks did not affect mineral (calcium and iron) absorption significantly in 12 healthy young men. Feeding of a larger dose of inulin (40 g day^{-1}) significantly increased calcium absorption in nine healthy men. More recently by using postmenopausal women as subjects, it has been shown that feeding of 10 g day^{-1} of FOS significantly increased magnesium absorption. Since there have not been many human trials regarding the effect of NDO or dietary carbohydrates on mineral absorption and bone mineralization, more studies are clearly needed.

Modulation of Lipid Metabolism

The possibility that prebiotics may have some effects on blood lipid metabolism is an attractive idea. In rats it has been demonstrated that feeding FOS significantly reduced triglycerides in very low-density lipoprotein (VLDL), which is likely to be due to a reduction of lipogenesis in the liver. Regulation of hepatic cholesterol synthesis by SCFA and precipitation of bile acids due to deconjugation and acidification in the intestines have been proposed as likely mechanisms of this effect. However, only very slight effects have been observed in human studies using healthy volunteers, diabetic patients, or hypercholesterolemic persons, which are not yet conclusive. It is still an open question whether or not prebiotics can exert beneficial effects on lipid metabolism and cholesterol lowering.

Conclusion and Perspectives

Our daily diet is at the base of our healthy life. We eat a variety of foods derived from animals and plants every day, which basically include all the necessary components of nutrients we need. However, the balance of nutrients is not always sufficient to keep our health. Especially the modern dietary habits often cause the shortage of certain nutrient factors. A typical example is that of formula-fed infants who consume cows' milk-based foods prior to weaning. It has been demonstrated scientifically that cow's milk-based formula needs supplementation to fulfill completely the baby's needs to prevent infection and to keep the infant's gut microflora healthy. As the role of the gut microflora in human health is recognized more, the importance of management of the intestinal microflora will receive more attention.

In the last two decades, a variety of dietary carbohydrates that fulfill the criteria for prebiotics have been developed and have become commercially available. At the same time numerous studies have been conducted in animal models and in humans to show different health effects of prebiotics, thus increasing their health claims. However, we must recognize what the true health benefits of the prebiotics are, and which ones are supported by sound scientific evidence. Unfortunately not every health effect of each of the prebiotics described in this article is sufficiently substantiated in human clinical studies, not even the principal effects of certain established prebiotics. However, these putative effects may counteract any additional physiological effects of prebiotics, and the elucidation of the mechanisms underlying the effects may add a new insight into the field of nutrition.

Our knowledge of the composition and the function of the gut microflora is still limited. Recently established molecular identification techniques for bacteria have revealed that a number of bacteria even predominant in the intestinal microflora are still uncultivable. Although a prebiotic is required to be bifidogenic, the influence of the particular prebiotic on the fate of other bacteria is not precisely known. Considering the more than 200 or even 400 different bacteria inhabiting the colon, there may be other beneficial as well as harmful bacteria that preferentially utilize the prebiotic in the flora. Individual prebiotics are probably utilized by different sets of bacteria, thereby influencing the gut microflora and physiology in different ways. However, most physiological effects of prebiotics described so far are almost all possessed by every prebiotic uniformly. It may be very difficult to distinguish one prebiotic from another in their physiological characteristics, but when this will be achieved, the era of prebiotics will come.

See also: **Bacteria, Beneficial**: *Bifidobacterium* spp.: Applications in Fermented Milks; Probiotics, Applications in Dairy Products. **Prebiotics**: Types.

Further Reading

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PSYCHROTROPHIC BACTERIA

Contents
Arthrobacter spp.
Pseudomonas spp.
Other Psychrotrophs

Arthrobacter spp.

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Introduction

The genus *Arthrobacter* belongs to the ecologically and industrially important class Actinobacteria, family Micrococcaceae, which includes microorganisms that live in soil, subterranean cave silts, sea, glacier silts, sewage, water sludge, aerial surfaces of plants, vegetables, fish, and various animal species. In the environment, they are often the most numerous bacteria because of their versatility. Some species are psychrophilic and psychrotrophic, can use a wide range of organic substrates as sole or principal sources of carbon and energy, and do not require vitamins or other organic growth factors. Aromatic compounds can also be utilized. Arthrobacters are widespread in nature and they readily contaminate raw food, milk and milk products, meat and meat products, and fish and fish products. In food, arthrobacters may be recognized as indicators of sanitation or hygiene quality, or as contaminants of no particular importance. However, they may also grow and be involved in spoilage or ripening of food products. Some *Arthrobacter* strains have been isolated from human sources and consequently are considered to be opportunistic pathogenic microorganisms.

The taxonomy of the genus *Arthrobacter* has been redefined many times. Great difficulty has been encountered in identifying and classifying *Arthrobacter* and related coryneforms such as *Brevibacterium*, *Caseobacterium*, *Cellulomonas*, *Corynebacterium*, *Curtobacterium*, and *Microbacterium*. For these reasons, sometimes many isolates have been identified as arthrobacters or 'arthrobacter-like' simply because they showed the rod–coccus growth cycle (**Figure 1**) and staining reactions that are characteristic of the genus.

Strains of *Arthrobacter* can be readily recognized in different environments by their morphological properties, although they cannot easily be distinguished from closely related coryneform genera.

The development of taxonomy for the genus *Arthrobacter* is as follows:

1928 – isolation from soil;
1933–38 – similar microorganisms confirmed in soil;
1957 – genus included in the family of Corynebacteriaceae;
1986 – two groups distinguished: *A. globiformis*/*A. citreus* and *A. nicotianae*.

Taxonomy

The genus *Arthrobacter* is closely related to *Aureobacterium*, *Caseobacterium*, *Cellulomonas*, *Corynebacterium*, *Curtobacterium*, and *Microbacterium* and is more distantly related to *Brevibacterium*. Phylogenetically, it is a member of the high-GC Actinomycetes, and *Arthrobacter* species could not be separated from members of the genus *Micrococcus*. These are Gram-positive eubacteria. Only minor differences enable the genus to be distinguished from

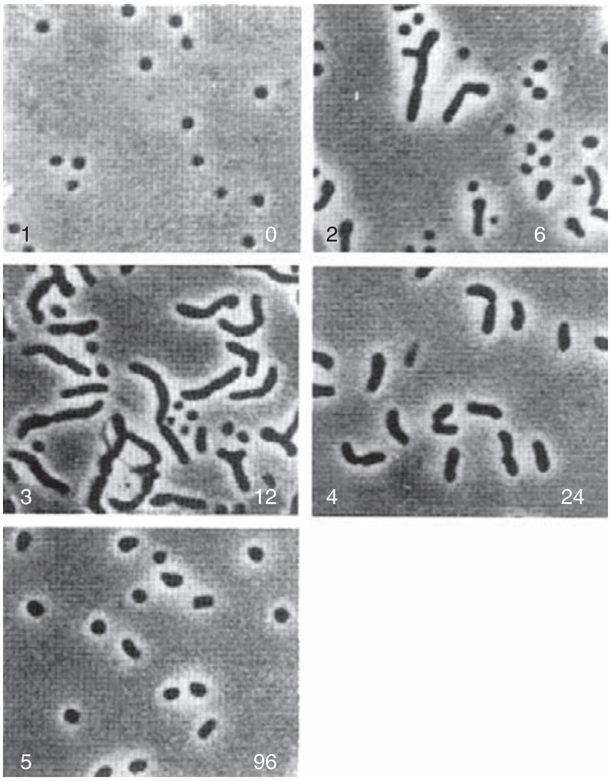

Figure 1 Growth cycles of *Arthrobacter globiformis* AC 166 starting from coccoid stage, on a rich medium at 25 °C. Reproduced from Crombach WHJ (1974) Morphology and physiology of coryneform bacteria. *Antonie van Leeuwenhoek* 40: 361–376, with kind permission from Kluwer Academic Publishers.

coryneform bacteria. Several tests must be employed simultaneously to classify and identify *Arthrobacter* spp. and avoid confusion with closely related genera.

Molecular and chemotaxonomic techniques are important for the characterization of coryneforms and *Arthrobacter* spp. because traditional methods based on morphological and physiological features only are insufficient to describe their biodiversity. In recent years, many methods have been developed to classify and identify arthrobacters. Traditional approaches are still used, but must be applied in conjunction with chemotaxonomic and molecular techniques, as the modern taxonomy of bacteria requires a multiphase classification strategy. The following approaches are the most recent and widely used approaches to *Arthrobacter* taxonomy:

1. Deoxyribonucleic acid (DNA) base composition and DNA–DNA homologies (DNA hybridization).
2. Analysis of particular cell constituents such as peptidoglycans, fatty acids, phospholipids (diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylinositol), and glycolipids, and analysis for the presence or absence of teichoic acids. Numerous studies of the cellular fatty acid composition of coryneform bacteria have been used for their classification. In many cases, acyl types allow for a more precise characterization. Recently, the identification of cellular fatty acids has enabled the classification into four groups of coryneform bacteria belonging to the genera *Arthrobacter*, *Brevibacterium*, *Caseobacterium*, *Caseobacter*, *Cellulomonas*, *Corynebacterium*, and *Curtobacterium*.
3. Isoprenoid quinone analysis. It evaluates the presence of dehydrogenated menaquinones with 8 (MK-8), 9 (MK-9), or 10 (MK-10) isoprene units. This is another new method of characterization, but its application has given rise to several problems in distinguishing species and genera.
4. PCR analysis. It is a new method of identifying and classifying *Arthrobacter* based on the analysis of 16S rRNA by polymerase chain reaction (PCR) and various electrophoresis techniques, such as temperature-gradient gel electrophoresis (TGGE) or denaturing-gradient gel electrophoresis (DGGE). Comparative TGGE is considered more useful in taxonomic studies of coryneform soil bacteria because a high number of strains from the principal species of the genera *Aeromicrobium*, *Agromyces*, *Arthrobacter*, *Aureobacterium*, *Cellulomonas*, *Curtobacterium*, *Nocardioides*, and *Terrabacter* can be tested and characterized. In addition, positive results obtained by comparative TGGE can be confirmed by whole-cell fatty acid methyl ester analysis. Finally, PCR amplification of 16S rRNA gene (rDNA analysis), followed by sequencing, allows the identification of new species of *Arthrobacter* (**Figure 2**).
5. Biochemical and physiological characteristics. The phenotypic characteristics do not enable differentiation of *Arthrobacter* species.

The multiphase approach has led to the discovery of new species such as *A. rhombi* sp. nov. (**Figure 3**), isolated from the Greenland halibut (*Reinhardtius hippoglossoides*), in addition to *A. albus* sp. nov. and *A. luteolus* sp. nov., both isolated from human clinical specimens. It is only by simultaneous employment of several methods (biochemical characteristics, DNA G + C content, wall murein composition and structure, 16S rRNA gene sequence) that researchers can identify and classify old and new species of *Arthrobacter* with certainty, and distinguish them from closely related genera.

Morphological and Physiological Characteristics

The genus *Arthrobacter* includes a group of microorganisms with a rod–coccus growth cycle. Initially, the microorganisms grow as rods in a simple medium during the log phase, subsequently becoming shorter in the stationary phase and taking on the appearance of large cocci. In aged cultures, cells may have entirely coccoid conformations, but mixed rod–coccus types are often seen. When aged cells are transferred into fresh broths, they

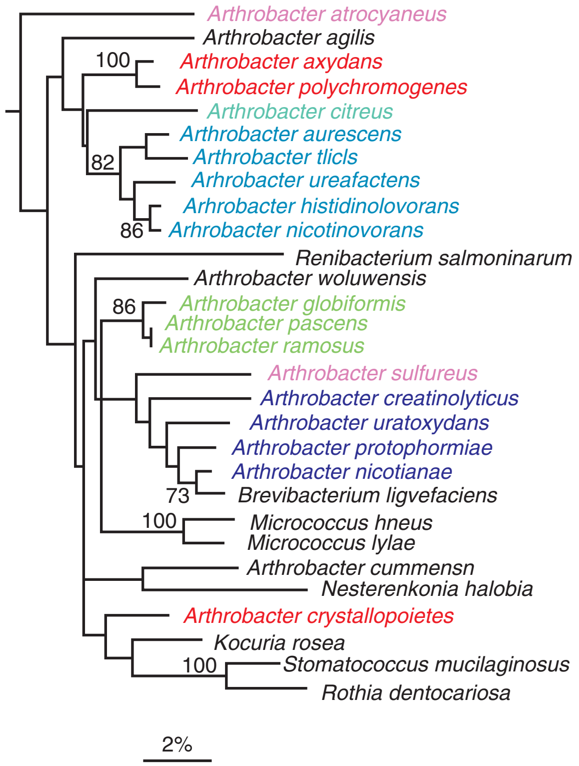
Arthrobacter atrocyaneus
Arthrobacter agilis
100
Arthrobacter axydans
Arthrobacter polychromogenes
Arthrobacter citreus
Arthrobacter aurescens
Arthrobacter tlicls
82
Arthrobacter ureafactens
Arhrobacter histidinolovorans
86
Arhrobacter nicotinovorans
Renibacterium salmoninarum
Arthrobacter woluwensis
86
Arthrobacter globiformis
Arthrobacter pascens
Arthrobacter ramosus
Arthrobacter sulfureus
Arthrobacter creatinolyticus
Arthrobacter uratoxydans
Arthrobacter protophormiae
Arthrobacter nicotianae
73
Brevibacterium ligvefaciens
100
Micrococcus hneus
Micrococcus lylae
Arthrobacter cummensn
Nesterenkonia halobia
Arthrobacter crystallopoietes
Kocuria rosea
100
Stomatococcus mucilaginosus
Rothia dentocariosa
2%

Figure 2 Phylogenetic relatedness among authentic species of the genus *Arthrobacter*, which have been subgrouped based on the results of 16S rDNA analysis. Organisms the names of which are displayed in the same color exhibit the same peptidoglycan structure. Numbers within the dendrogram indicate the percentages of occurrence of the branching order in 500 bootstrapped trees (only values of 70 and above are shown). Sequences of the species of the genera of the family Micrococcaceae served as root. The scale bar represents 2 nucleotide substitutions per 100 nucleotides. Reproduced from Stackebrandt E and Schumann P (2006) Introduction to the taxonomy of Actinobacteria. In: Dworkin M, Falkow S, Rosembreg E, Schleifer CK, and Stackebrandt E (eds.) *The Prokaryotes: A Handbook on the Biology of Bacteria*, 3rd edn., Vol. 3, pp. 297–321. New York: Springer, with kind permission from Springer (New York, USA) and the authors.

become irregular rods, sometimes rudimentarily branched and arranged in V-shaped formations. Cell size is variable and the diameter can be anywhere between 0.6 and 1.2 μm.

This life cycle is observed in nonselective media, especially if seeded with food. Many studies have demonstrated that the life cycle matures within 24 h when the cells are isolated from mixed broth food and grown at 25 °C.

Both conformations are Gram-positive, but on aging, they may be rapidly decolorized and appear Gram-negative. The cell wall mureins contain L-lysine as the main dibasic amino acid.

All *Arthrobacter* strains are non-acid-fast and non-spore-forming. The rods are nonmotile or occasionally motile. They are obligate aerobes, catalase-positive and oxidase-negative, but a few soil and sea strains have been recognized as oxidase-positive.

A large number of arthrobacters are mesophilic, with optimum temperature of 20–30 °C. However, some strains are psychrophilic or psychrotrophic and may grow at 4–6 °C, or even at close to −5 °C (*A. glacialis*) or 0 °C, in some cases. Psychrophilic strains, usually isolated from soil or sea, are characterized by an optimum temperature of 20 °C. Mesophilic strains can be adapted to grow at 6 °C. Finally, a few strains can grow at 37 °C. Temperature seems to have a significant influence on the life cycle. At 25 °C, rod–coccus transformation takes place faster than at 15 °C. This effect does not seem to be restricted to *A. globiformis*. In contrast, pigment production appears to be unaffected by temperature. Orange, yellow, and pale red pigments may occur in *Arthrobacter*. Pigment development seems to depend on various factors such as the strains involved, exposure to light or dark, the growth medium, and the presence or absence of salt in the medium. Nevertheless, many *Arthrobacter* strains are not pigmented. The cells are rapidly killed by heating at 63 °C for 30 min in skimmed milk or in some other nonselective broths.

All *Arthrobacter* strains are chemoorganotrophic and strictly aerobic. Metabolism of carbohydrates and other carbon sources is exclusively respiratory and never fermentative. The most widespread strains of *Arthrobacter*, especially those from soil, can utilize glucose, saccharose, glycerol, acetate, and citrate. Numerous studies have demonstrated that *Arthrobacter* strains may utilize more than 90 different carbon sources. Furthermore, *Arthrobacter* strains seem to have no particular nutritional requirements. Only a few species require biotin, B-vitamins, amino acids, and a siderophore. Many arthrobacters are able to utilize as nitrogen sources either ammonium nitrogen salts or a mix of ammonium nitrogen salts and a single amino acid. In general, it seems that only strains isolated from cheese, sea fish, or food require organic nitrogen. *Arthrobacter* strains are not inhibited by 3–5% NaCl at pH >6. In contrast, growth slows down at pH <6. Acidic substrates inhibit growth, since pH is a selective factor. The salt tolerance of *Arthrobacter* strains enables them to grow in salty food such as cheeses and meat products, a parameter that may be used as a selective factor in isolation media.

Because of their great nutritional versatility, arthrobacters are frequently isolated from substrates with usual and unusual organic compounds, and have become important in bioremediation and environmental fields. It is known that some arthrobacters are able to dehalogenate 4-chloro-, 4-fluoro-, and 4-bromobenzoate, and to desulfurize heterocyclic organosulfur compounds. These properties mean they can be utilized as starters for the microbial degradation of haloaromatics or other carbon sources in the environment.

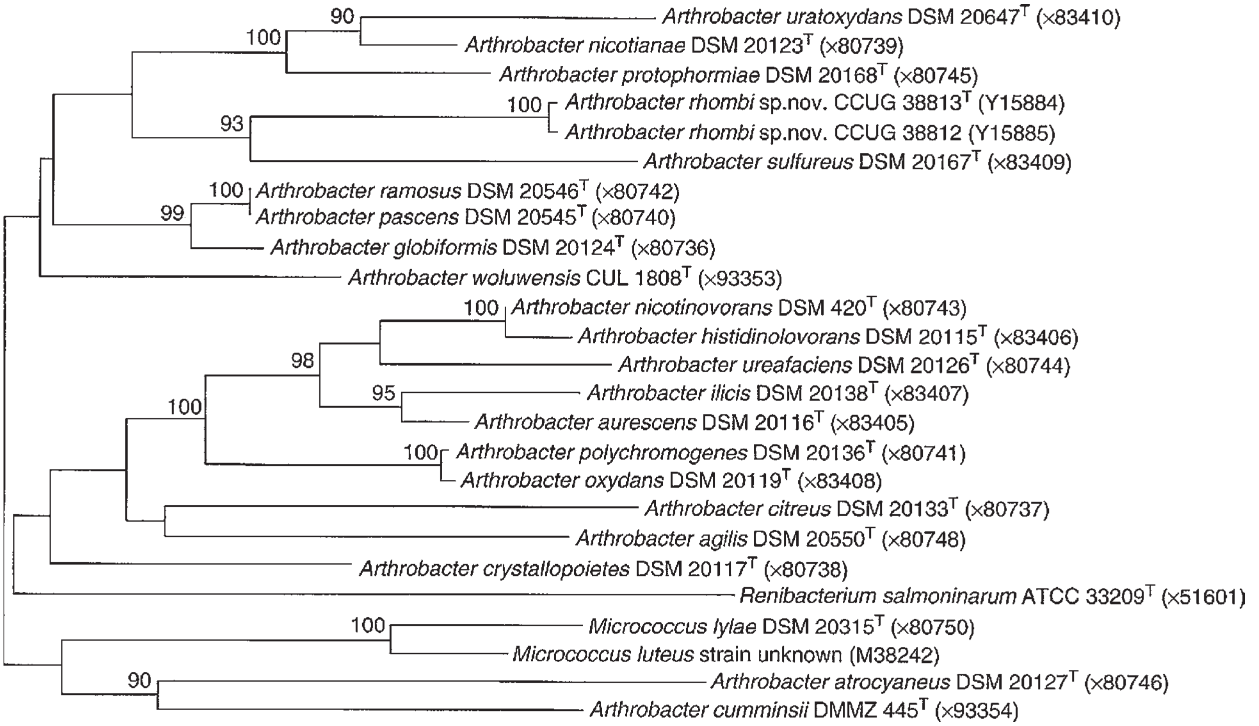
90
100
Arthrobacter uratoxydans DSM 20647T (x83410)
Arthrobacter nicotianae DSM 20123T (x80739)
Arthrobacter protophormiae DSM 20168T (x80745)
100
Arthrobacter rhombi sp.nov. CCUG 38813T (Y15884)
93
Arthrobacter rhombi sp.nov. CCUG 38812 (Y15885)
Arthrobacter sulfureus DSM 20167T (x83409)
100
Arthrobacter ramosus DSM 20546T (x80742)
99
Arthrobacter pascens DSM 20545T (x80740)
Arthrobacter globiformis DSM 20124T (x80736)
Arthrobacter woluwensis CUL 1808T (x93353)
100
Arthrobacter nicotinovorans DSM 420T (x80743)
Arthrobacter histidinolovorans DSM 20115T (x83406)
98
Arthrobacter ureafaciens DSM 20126T (x80744)
95
Arthrobacter ilicis DSM 20138T (x83407)
100
Arthrobacter aurescens DSM 20116T (x83405)
100
Arthrobacter polychromogenes DSM 20136T (x80741)
Arthrobacter oxydans DSM 20119T (x83408)
Arthrobacter citreus DSM 20133T (x80737)
Arthrobacter agilis DSM 20550T (x80748)
Arthrobacter crystallopoietes DSM 20117T (x80738)
Renibacterium salmoninarum ATCC 33209T (x51601)
100
Micrococcus lylae DSM 20315T (x80750)
Micrococcus luteus strain unknown (M38242)
90
Arthrobacter atrocyaneus DSM 20127T (x80746)
Arthrobacter cumminsii DMMZ 445T (x93354)

Figure 3 Unrooted tree showing the phylogenetic relationship of *Arthrobacter rhombi* sp. nov. within the genus *Arthrobacter*. The tree constructed using the neighbor-joining method was based on a comparison of approximately 1320 nucleotides. Bootstrap values, expressed as a percentage of 200 replications, are given at the branching points. Bar, 1% sequence divergence. With the exception of *A. rhombi* CCGU 38812, all other strains are type strains; accession numbers are given in parentheses. Reproduced with permission from Osorio CR, Barja JL, Hutson RA, and Collins MD (1999) *Arthrobacter rhombi* sp. nov., isolated from Greenland halibut (*Reinhardtius hippoglossoides*). *International Journal of Systematic Bacteriology* 49(3): 1217–1220.

Hydrolytic activities may also occur in *Arthrobacter* strains that hydrolyze gelatin, casein, and lipids, but not starch and cellulose. In particular, hydrolytic activities are marked in strains isolated from soil and cheeses; *Arthrobacter* strains are the major components of smear microflora of cheeses. Extracellular esterases, proteases, and proline iminopeptidases from *A. nicotianae* strains isolated from smear cheeses have been purified and characterized. The properties of these enzymes demonstrate that arthrobacters may play an important role in casein hydrolysis of smear-ripened cheese.

The characteristics of a chitinase enzyme of arthrobacters have also been studied. The aim was to propose an industrial application in the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc), which is one of the most common polysaccharides found in nature.

Arthrobacter Species

Arthrobacter agilis, *A. albus*, *A. atrocyaneus*, *A. aurescens*, *A. citreus*, *A. creatinolyticus*, *A. crystallopoietes*, *A. cumminsii*, *A. globiformis*, *A. histidinolovorans*, *A. ilicis*, *A. luteolus*, *A. nicotianae*, *A. nicotinovorans*, *A. oxydans*, *A. pascens*, *A. polychromogenes*, *A. protophormiae*, *A. ramosus*, *A. rhombi*, *A. sulfureus*, *A. ureafaciens*, *A. uratoxydans*, and *A. woluwensis* are all included in the genus.

The number of species identified may rise or fall. The taxonomy of the genus *Arthrobacter* and related genera has been redefined many times. It is well known that some strains isolated from cheese, and originally recognized as arthrobacters, have now been identified as *Brevibacterium* or coryneforms, and vice versa.

Almost all species have been isolated from soil, sea, vegetation, pure or sewage water, water sludge, glacier silts, and other natural environments where they play an active role in the degradation of various carbon sources. Recently, new species have been isolated from soil of cold areas, from the marine environment, and monuments. The main new species include *A. ardleyensis*, *A. castelli*, *A. defluvii*, *A. flavus*, *A. gandavensis*, *A. gangotriensis*, *A. humicola*, *A. kerguelensis*, *A. koreensis*, *A. monumenti*, *A. oryzae*, *A. parietis*, *A. pigmenti*, *A. psychrolactophilus*, *A. psychrophenolicus*, *A. radiotolerans*, *A. simplex*, *A. soli*, *A. stackebrandtii*, *A. subterraneus*, *A. russicus*, *A. roseus*, *A. tecti*, *A. terregens*, and *A. tumbae*.

Arthrobacter cumminsii, *A. woluwensis*, *A. creatinolyticus*, *A. luteolus* nov. sp., and *A. albus* nov. sp. are considered opportunistic pathogens because they have been isolated only from human clinical specimens such as skin, urine, and blood culture. Their role in diseases is not proven and their pathological significance has not yet been assessed.

Finally, some *Arthrobacter* species, such as *A. aurescens*, *A. arilaitensis*, *A. bergerei*, *A. globiformis*, *A. variabilis*, *A. citreus*, *A. uratoxydans*, *A. protophormiae*, and *A. nicotianae*, may also be found in food, especially cheeses.

Isolation of *Arthrobacter*

Arthrobacters are usually isolated from different environments by nonselective media, such as plate count agar. Especially where they do not represent the main species, an enrichment culture, containing a variety of organic substrates as sole carbon and energy sources in mineral salts solution, could be used. However, in order to achieve good results in isolation, the media must contain a sufficient amount of organic compounds and mineral constituents. Poor nutriments inhibit the growth of *Arthrobacter*.

To improve the isolation, sometimes antibiotics (nystatin 50 μg ml^{-1}, cycloheximide 50 μg ml^{-1}) have to be added to the medium in order to suppress fungal growth. The Winogradsky standard salt solution is recommended for their isolation from soil. A mix of soil extract with added yeast extracts, salts, glucose, and peptone is also often used. A selective medium, containing Trypticase soy agar, yeast extract, NaCl, cycloheximide, and methyl red, has been suggested. Cycloheximide (0.01%) inhibits fungal growth, a 2% concentration of NaCl inhibits the majority of *Streptomyces*, *Nocardia*, and Gram-negative bacteria, and methyl red (150 μg ml^{-1}) acts against other Gram-positive bacteria (bacilli and micrococci). The pH of the medium must be between 5.0 and 8.5, and the presence of Trypticase soy and yeast extract improves the growth of arthrobacters.

The cultures can be preserved in soil extract agar or in plate count agar for more than 2 months at 20 °C, or for several years if they are frozen on glass beads at −70 °C. Lyophilization is suitable for long-term storage.

Arthrobacter in Milk and Dairy Products

Arthrobacters contaminate meat and meat products, fish and fish product, fruit, vegetables, and milk and dairy products. Since they are obligate aerobes, arthrobacters grow mainly on the food surface and may result in spoilage, ripening, and colored smear or slime. Growth occurs when the storage temperature is 4–30 °C, the pH is over 5.5, and the redox potential is positive. The organic sources of food do not constitute a limiting factor for these microorganisms, which display great nutritional versatility. Antagonistic flora, including lactic acid bacteria, Enterobacteria, *Pseudomonas*, Micrococcaceae, and so on, may represent a potential limit. The competition of other microorganisms and the change in pH brought about by the production of lactic acid or other organic acids can inhibit or reduce the growth of arthrobacters. In addition, arthrobacters are rarely found in high concentration in food and never constitute the predominant flora. Where they can rapidly develop on food surfaces, arthrobacters produce colored smears, but they are always present in coculture with coryneforms, micrococci, staphylococci, and yeasts.

Arthrobacters may occasionally be isolated from the surfaces of refrigerated fruits, vegetables, and meat and meat products (such as sausages, cooked hams, and air-dried hams). The significance of their presence in these foods has not yet been clearly defined and, in some cases, more detailed investigations are required. Arthrobacters are always present in cocultures with strains of lactic acid bacteria, coryneforms, Micrococcaceae, *Pseudomonas*, *Brochothrix thermosphacta*, *Microbacterium*, *Moraxella*, *Acinetobacter*, and other psychrotrophic bacteria. On the above-mentioned food products, arthrobacters constitute an insignificant contamination whose growth depends on antagonistic microflora and on the presence of oxygen. Arthrobacters are more frequently isolated from milk and cheese. Several studies have demonstrated that soil- or environment-derived arthrobacters readily contaminate raw milk, where they may survive even if heated at 72 °C for 15 s. In milk, *Corynebacterium* and *Arthrobacter* strains are recognized as indicators of sanitation or hygiene quality. High numbers of both groups in pasteurized milk are never associated with spoilage, although they do indicate bad sanitation. In fact, *Arthrobacter* strains are usually considered contaminants of no particular significance. In cheese, they are responsible for either spoilage or ripening.

Arthrobacter aurescens, in coculture with *Zymomonas mobilis*, produces an abnormal yellow discoloration in yogurts and red-orange streaks on the surface of sliced Gorgonzola cheese. Other species, together with strains of *Brevibacterium*, *Rhodococcus*, *Micrococcus*, *Staphylococcus*, and *Corynebacterium*, and some yeasts such as *Debaryomyces hansenii*, *Galactomyces geotrichum*, *Kluyveromyces marxianus*, and *Pichia membranifaciens* play an important role in determining the characteristics and flavor of smear surface-ripened cheeses. Brie de Meaux, Epoisses, Germain, Pont l'Évêque, Reblochon, Saint-Nectaire, Tomme de Savoie, Fourme d'Ambert, Pur Brebis, Morbier, Cantal, Comté, Fromage fermier, Fromage montagne, Domiati, Ras, Edam, Gouda, Gruyère, Taleggio, Quartirolo, Limburger, Livarot, Rocamadour, Weinkäse, Harzer, Munster, Saint-Paulin, Appenzeller, Brick, Trappist, and Tilsiter are the best-known and most popular smear surface-ripened cheeses. *Arthrobacter nicotianae*, *A. globiformis*, and *A. arilaitensis* sp. nov. seem to be the main strains involved in aroma and color production in red smear cheeses. The ripening process begins with the growth of yeasts, which metabolize lactic acid previously synthesized by lactic acid bacteria, causing the pH to increase at the cheese surface. The pH alteration and the presence of growth factors from yeast autolysis stimulate the growth of a mixed population including *A. globiformis*, *A. variabilis*, *A. citreus*, *Brevibacterium linens*, and *Brevibacterium ammoniagenes*. This remarkable microflora grows mainly on the cheese surface since it is strictly aerobic, producing colored smears and low-molecular-weight compounds that are responsible for the typical aroma of the product. The growth occurs within the first 2 weeks of ripening and then stops until the time of consumption. Thereafter, the aromatic compounds spread from the surface into the cheese, a process that is necessary for the development of the characteristic flavor and taste of the product. More specifically, *Arthrobacter* and *Brevibacterium* strains cause casein and lactate breakdown, changes in pH, and the production of ammonia, methanethiol, sulfides, dimethyl disulfide, *S*-methylthioesters (*S*-methylthioacetate, *S*-metylthiobutyrate, *S*-methylthiopropionate, and *S*-methylthioisovalerate), other volatile sulfur compounds, and low-molecular-weight nitrogen compounds. The volatile sulfur compounds are products of L-methionine catabolism. The enzymes involved seem to be L-methionine demethiolase, L-methionine aminotransferase, and α-keto-γ-methyl-thiobutyric acid demethiolase. The microorganisms produce orange and reddish brown pigments, owing to their enzymatic activity on casein and amino acids. The proteinases of *Arthrobacter* spp. may play a significant role in the ripening of smear surface-ripened cheeses. Most notably, proline iminopeptidase seems to have a fundamental activity.

Some *Arthrobacter* species and related genera may also produce antilisterial compounds, an activity that has been demonstrated on solid media. The presence and growth of undesirable microorganisms, such as *Listeria monocytogenes*, on the surface of smeared cheeses is a severe problem for the dairy industry. Various studies are under way with the goal of selecting strains that are capable of carrying out antagonistic activities on cheese surfaces against pathogenic microorganisms. No starter culture with both ripening and antilisterial activity has been produced yet.

Because of high consumer demand for innovative, well-aged, territory-specific tastes, there is likely to be an increasingly widespread use of flavor-enhancing bacteria and yeast strains with a proteolytic enzyme activity. Arthrobacters are being more and more often proposed in cocultures, and also as part of starters, for cheese production. Starters including *Staphylococcus* and *Arthrobacter* strains, *B. linens*, and *D. hansenii* have been tested to improve the quality of experimental Tilsit, Raclette, and Kùssnachter cheeses. Relevant data demonstrate that the starters rapidly predominated on the surfaces and were able to reproduce the characteristic taste, flavor, and color of red smear cheeses. However, in the case of Raclette and Kùssnachter cheeses, it seems that *Arthrobacter* spp. do not necessarily need to be a component of the starter culture, while *B. linens* and *D. hansenii* are essential to produce the typical aroma and the red smear. Conversely, the presence of arthrobacters in Tilsit cheese seems to be necessary for both aroma and color.

Conclusion

The *Arthrobacter* genus is extensive and includes a large number of species that are widespread in nature. Because of their nutritional versatility, arthrobacters are commonly isolated from soil, sewage, food, and several other environments by using nonselective and simple media such as plate count agar. However, selective media and broths are also proposed. Strains with a rod–coccus growth cycle, which are strictly aerobic, and which have lysine as the cell wall diamino acid may be presumably identified as arthrobacters. The genus has great importance in environmental and industrial applications, as different strains are often used in bioremediation and in the degradation of heterocyclic organosulfur compounds, haloaromatics, and other carbon sources (herbicides, pesticides) from the environment. Most notably, *A. phenanthrenivorans* can break down phenanthrene, *A. nitroguajacolicus* breaks down 4-nitroguaiacol, and *A. defluvii* degrades 4-chlorophenol. Some *Arthrobacter* species may have an important role in phytohormone production, in dinitrogen fixation, and in lysing yeast and mold cells that trigger plant diseases. By chitinase production, some strains can be used as a biological control of *Fusarium* diseases. Moreover, other *Arthrobacter* strains are important commercially because they are used to produce glutamic acid, α-ketoglutaric acid, and riboflavin. Recently, some *Arthrobacter* strains have been successfully used as probiotic bacteria to preserve shrimp post-larvae from pathogens such as *Vibrio parahaemolyticus* and *Vibrio nereis*. Starter cultures supplemented with *Arthrobacter* spp. are used to improve the quality of alkaline noodles, a typical food of eastern Asia made with wheat flour, water, sodium chloride, and alkaline salts (sodium and potassium carbonates, bicarbonate, phosphates). The aim is to increase the degree of yellowness of the noodles and prevent the formation of organic acids, which cause a decrease in pH.

Arthrobacters also contaminate food, where they may have no significant role or else cause spoilage. Some *Arthrobacter* strains largely contribute to the production of the typical taste, flavor, and color of smear cheeses.

See also: **Analytical Methods**: DNA-Based Assays. **Bacteria, Beneficial**: Brevibacterium Linens, Brevibacterium Aurantiacum and Other Smear Microorganisms. **Cheese**: Secondary Cultures; Smear-Ripened Cheeses. **Microorganisms Associated with Milk**.

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Pseudomonas spp.

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Morphology and Characteristics

Pseudomonads are Gram-negative, straight or curved rods, which are motile by polar flagella. They are aerobic and their metabolism is never fermentative. They are catalase-positive and the majority of species are oxidase-positive. *Pseudomonas fluorescens* is found predominantly in soil and water and it produces a diffusible fluorescent pigment, pyoverdin. The taxonomy of the genus *Pseudomonas* is complex and there is extensive genetic heterogeneity among its members.

Sources of *Pseudomonas* spp. in Milk

Contamination from Milking and Storage Equipment

Significant contamination of milk by pseudomonads occurs due to inadequately sanitized surfaces of milking and milk storage equipment. The organisms grow in milk residues present in crevices, joints, rubber gaskets, and dead ends of badly cleaned milking plants. Although many different bacterial types can be introduced into milk from milk mineral deposits present in milking equipment, the most important of these are the Gram-negative psychrotrophs, which predominate among the microflora that adhere to stainless-steel milk transfer pipelines.

Variations in cleaning regimes and levels of contamination from farm to farm lead to differences in the microflora found on milking equipment. The only effective way to limit the introduction of bacteria into the milk supply during milking is to ensure adequate cleaning and disinfection of all the equipment. The effectiveness of sanitation depends to a large extent on the design of the plant and on other factors such as the hardness of the water supply.

Generally, farm bulk tanks do not contribute greatly to the bacterial load of raw milk as they are easy to clean and, consequently, have a much lower bacterial content than the milk pipeline. However, ancillary equipment such as agitators, dipsticks, outlet plugs, and cocks can be difficult to clean and these may be a possible source of contamination. Any residual bacteria present have the potential for growth during storage. Milk may be collected from farms on alternate days, or even longer in some instances. Thus, at collection, part of the milk in the bulk tank may be 48 h old or more. Although alternate-day collection may have little effect on the bacteriological quality of milk rapidly cooled to 4 °C or below before addition to the tank, the growth potential of the raw milk microflora is significantly affected. Thus, milk collected on alternate days will contain a greater number of bacteria that are entering the exponential phase of growth when the milk arrives at the processing site, and the amount of time that this milk can be subsequently stored will be reduced. For example, it has been shown that *Pseudomonas* spp. isolated from milk that had been stored at 7 °C for 3 days grew 10 times faster at 7 °C, had 1000-fold more proteolytic activity, and were 280-fold more lipolytic than pseudomonads isolated from freshly drawn milk. However, the *Pseudomonas* counts in the milk increased by only about 1 log cycle during the storage period.

Contamination during Transportation and Storage at the Processing Facility

Adequate cleaning of any dairy equipment used for the collection, transport, and storage of refrigerated raw milk must be performed to prevent fouling with milk film, which can support growth of bacteria that then become a source of contamination to subsequent batches of milk. Milk is usually transported in insulated tanks or in refrigerated tankers, and may be transferred to larger vehicles for longer journeys. During transportation, the main cause of increased bacterial count is inadequately cleaned vehicles and growth of bacteria already present in the milk. The latter is dependent on the milk temperature and journey time. A twofold increase in count is common during transportation of milk from the farm to the processing site and this is due primarily to the growth of psychrotrophic bacteria, including pseudomonads. Critical sites in the milk tanker for cleaning have been identified as the air separator, the milk meter, the milk sieve, and the suction hose, and factors that contribute to inadequate cleaning include blockage of the cleaning-in-place (CIP) spray system and low water pressure and flow rate. These can lead to buildup of milk stone on the inner surface of the tanker.

Changes in dairy industry practices such as the introduction of a 5-day working week, and milk shortages at certain times of the year due to the adoption of quota systems, have led to milk being stored for longer times before processing. Thus, the temperature at which milk is stored becomes critical. It has been recommended that milk is cooled to, and maintained at, 3 °C on receipt at the processing plant before storage. The average psychrotrophic, aerobic bacterial count of silo milk at several dairies in southwest Scotland was 1.3×10^5 cfu ml^{-1}. The majority of bacteria present were pseudomonads (70.2%) but Enterobacteriaceae (7.7%), Gram-positive bacteria (6.9%), and other Gram-negative, rod-shaped organisms were also isolated. When the milks were stored for a further 48 h at 6 °C, the psychrotroph count increased by 2 log cycles to 1.3×10^7 cfu ml^{-1}. Psychrotrophic growth patterns were independent of whether milks were selected according to their initial counts, and whether they were stored in large air-agitated silos or small, paddle-agitated vats. Growth rates were highest during filling of the silos, due possibly to temperature fluctuation, and final bacterial numbers were dependent on initial counts and storage time, with the latter being the most significant factor affecting milk quality.

It has been shown that raw milk stored at low temperatures is spoilt exclusively by Gram-negative bacteria. In earlier studies where identification was primarily performed by phenotypic analysis, *Pseudomonas fluorescens* biovar I (32.1% of isolates), *Ps. fragi* (29.6%), *Ps. lundensis* (19.8%), and *Ps. fluorescens* biovar III (17.3%) were the most commonly isolated. However, more recent studies conducted in Belgium using molecular methods such as Repetitive sequence-based Polymerase Chain Reaction (REP-PCR) and 16S RNA sequencing identified *Ps. lundensis* and *Ps. fragi* as the most common proteolytic psychrotrophs isolated from milk and these two species accounted for 53% of proteolytic strains isolated. The authors of this study concluded that there was an overestimation of the prevalence of *Ps. fluorescens* in previous studies due to problems with the taxonomy of the genus leading to inappropriate identification methods being used.

There seems to be little difference between the types of spoilage microorganisms associated with bovine milk and ovine and caprine milks.

Postpasteurization Contamination

Although Gram-negative, psychrotrophic bacteria present in raw milk do not survive pasteurization, these organisms are commonly isolated from pasteurized milk and cream, again with *Pseudomonas* spp. being the most frequently encountered. Thus, the shelf life of pasteurized products is limited by postpasteurization contamination. The microflora of short shelf life milks (i.e., <5 days at 4–6 °C) comprise almost entirely of *Pseudomonas* spp. (approximately 90% of isolates), while those with shelf lives >10 days have a higher proportion of other types of microorganisms. In the latter milks, *Pseudomonas* spp. account for about 70% of isolates. More recent work using sequence analysis of denaturing gradient gel electrophoresis DGGE fragments to investigate the microbial ecology of pasteurized milk stored at 4 °C revealed high diversity among *Pseudomonas* spp. in the milk samples. *Pseudomonas putida* and *Ps. migulae* grew to high numbers during storage and *Ps. fluorescens* and *Ps. fragi* were also found.

A variety of sources of contamination by pseudomonads exist in the processing plant. However, personnel and air probably contribute little to the contamination of pasteurized fluid milk products by these organisms. It has been shown that *Pseudomonas* spp. are able to form biofilms. A likely cause of postpasteurization contamination is shedding of bacteria from biofilms formed on gaskets in pasteurized milk pipelines. Electron microscopy has been used to show that *Pseudomonas* biofilms can develop on the sides of gaskets, despite operation of CIP systems, and it is well accepted that sanitizer efficacy is greatly reduced against bacteria growing in biofilms.

There is substantial evidence that the filling operation has the greatest influence on the potential shelf life of pasteurized milk. Low levels of psychrotrophic bacteria (10–500 cfu l^{-1}) can be introduced into the product at this stage and these can have a profound effect on shelf life. Improvements to the design of fillers have resulted in dramatic effects on the keeping quality of milk. Aseptically packaged milk can be kept for about 48 days when stored at 3 °C.

Consequences of Growth of Pseudomonads in Raw Milk

In addition to being able to grow rapidly in refrigerated milk, psychrotrophs produce extracellular enzymes that can degrade milk components. Although most psychrotrophs present in milk are not heat resistant, in many cases the extracellular enzymes that they produce can survive pasteurization (70–80 °C) and even UHTs (120–140 °C). Both proteases and lipases produced by psychrotrophs, representative of a number of genera, retained 60–70% of their activity after heating at 77 °C for 17 s and about 30–40% of their activity remained after UHT treatment at 140 °C for 5 s. Recent work has shown that proteolytic activity of bacterial origin in milk has a *D*-value at 75 °C of about 360 min and a *z*-value of about 29 °C. The heat stability of these enzymes is influenced by milk proteins, which exert a stabilizing effect. Not all the pseudomonads found in milk are capable of synthesizing these enzymes and, even in those that do, the levels produced are affected by many factors, including growth phase, nutrient supply (such as iron availability), phase variation, and environmental conditions, including temperature and oxygen tension. In a Belgian study, the incidence of proteolytic psychrotrophs was lower in milks collected in winter than in summer but the strains isolated in winter exhibited greater proteolytic activity than their summer counterparts. It has also been demonstrated that the enzymatic activity of pseudomonads and hence their spoilage potential can be predicted by their ribotype.

Extracellular Enzymes

Psychrotrophic bacteria in milk produce many types of extracellular enzymes, including proteases, lipases, phospholipases, exopeptidases, and glycosidases. Proteases and lipases have been better characterized and are thus better understood.

Proteases

Most of the proteases isolated from pseudomonads are metalloenzymes, containing 1 zinc atom and up to 16 calcium atoms per molecule. There are reports of variable content of zinc and calcium, owing to the diversity of proteases from psychrotrophs. Analysis of the protease-encoding gene, *aprX*, showed that pseudomonads expressed proteases with a high degree of heterogeneity. Some strains of *Pseudomonas* have been found to produce more than one type of protease and strain-to-strain variability is common. Most proteases have milk-clotting activity, and are readily able to degrade κ-, α_{s1}-, and β-casein, yet have low activity on nondenatured whey proteins. Isoelectric pH values of proteases from *Ps. fluorescens* vary from 5.1 to 8.8. The pH optima of proteases fall into two broad categories: neutral proteases with a pH optimum of pH 7 and alkaline proteases with optima at pH 8–9. The molecular weights of most proteases range from 40 to 50 kDa. Temperature optima range from 30 to 50 °C; in all cases, activity decreases sharply at temperatures above the optimum but all proteases for which data are available retained activity at 4 °C. *Pseudomonas fluorescens* proteases are extremely heat stable but most are unstable around 60 °C. Stability data at high temperatures are not reported for all proteases. This is very important from a spoilage perspective because the presence of proteases following commercial pasteurization (72 °C for 12–15 s) and UHT processing (135–148 °C for 2–5 s) can lead to quality defects.

Lipases and Phospholipases

Lipolytic degradation of milk is not as predominant as proteolytic degradation. Lipases of *Ps. fluorescens* typically form aggregates with lipids, or lipase–polysaccharide complexes. It is generally thought that *Pseudomonas* produces only one lipase; however, a second lipase has been isolated from *Ps. fluorescens. Pseudomonas* spp. typically produce a lipase that is active on milk fat. Lipases from *Pseudomonas* spp. can actively hydrolyze a variety of natural oils and synthetic triglycerides ranging from tributyrin to triolein. One of the most important enzymes produced by *Pseudomonas* is lecithinase, a phospholipase, which is able to hydrolyze the protective membrane of milk lipid. The membrane, composed primarily of lecithin, maintains the globular structure of milk lipid. Hydrolysis of the membrane can lead to fat aggregation and render milk lipid susceptible to the action of native milk lipases. Although lipases from *Pseudomonas* share one or more of the same epitopes, they are structurally diverse, which severely limits the ability to design DNA- or antibody-based probes for their detection. The optimal pH range for lipases from *Ps. fluorescens* is 7–8; however, activity is maintained from pH 5 to 11. Reported temperature optima range from 22 to 55 °C, and activity has been found at −29 °C. The pH and temperature optima reported are dependent on the substrate type used for their determination. The molecular weights of most lipases range from 32 to 633 kDa.

Lipases appear to be less thermostable than proteases; however, activity has been found at temperatures as high as 130 °C. Although strain-to-strain variation prevents generalizing on the thermostability of lipases from *Pseudomonas* spp., there are several reports indicating that lipases produced by *Ps. fluorescens* can survive commercial pasteurization and UHT processing and can affect the keeping quality of dairy products.

Regulation of Extracellular Enzymes

A variety of factors, including quorum sensing, growth phase, and environmental and nutritional factors, are involved in the regulation of synthesis of extracellular enzymes. For example, pH, temperature, oxygen tension, adenosine triphosphate pools, presence of ions, organic nutrients, triglycerides, and many more have been found to influence enzyme synthesis. Surprisingly, the regulation of synthesis of extracellular enzymes by pseudomonads is not unequivocally established, and probably involves a variety of regulatory mechanisms acting in concert. This fact further highlights the complexity and diversity of *Pseudomonas* extracellular enzymes. Understanding the complex mechanisms that direct enzyme synthesis will provide strategies to target for control. For example, it has been shown that disruption of quorum sensing results in a decreased production of protease by *Ps. fluorescens*; however, synthesis of the enzyme is not entirely prevented. Heat-based processing methods that are currently employed to curb enzyme-related spoilage are also detrimental toward product quality.

One factor responsible for regulation of extracellular enzymes is the phase of growth. Related to this is a growing body of research on cell-to-cell communication termed quorum sensing, dealing specifically with coordinated timing of cellular events according to cell density.

Pseudomonads, like other Gram-negative bacteria, produce an autoinducer, an acyl homoserine lactone derivative. This autoinducer is produced throughout the growth cycle, but it is only when its concentration reaches a threshold level toward the end of the log phase and early stationary phase that it is able to regulate the expression of several genes, including, in part, those responsible for the synthesis of extracellular enzymes of *Ps. fluorescens*. It has been shown that mutants of *Ps. fluorescens* that are incapable of producing extracellular proteinase are also incapable of synthesizing the autoinducer molecule.

Significance in Milk and Dairy Products

In general, the overall keeping quality of milk (pasteurized and UHT) and other dairy products is mainly limited by the action of proteases and lipases. When approximately 10^6 psychrotrophs per ml are present in raw milk before UHT heat treatment, gelation of the product will occur in less than 20 weeks of storage. Psychrotroph counts in the raw milk of about 10^7 cfu ml^{-1} will result in gelation after storage of the UHT milk for 2–10 weeks, accompanied by a gradual development of 'lack of freshness' and bitter flavors. Pasteurized milk having previously supported growth of psychrotrophs to levels of 5.5 log cfu ml^{-1} has been described as having an inferior flavor. However, defects in pasteurized milk are not as prevalent as in other dairy products, which may be due to the short period of storage of raw milk prior to processing. The development of flavor defects is dependent on the strain of *Pseudomonas* and fat content of the milk. Cheese can be affected by both proteases and lipases, with the resultant reduction of yield and flavor defects (such as soapiness, rancidity, and bitterness). It has been suggested that the slight adverse effects of proteases on cheese quality might be due to their removal in whey. However, lipases appear to be retained in cheese, and, for this reason, lipid-associated flavor defects predominate rather than protease-related reduction in cheese yields, although the presence of free fatty acids resulting from controlled lipolysis of milk fat is necessary for the characteristic development of the cheese flavor. Excessive lipolysis of cheese typically results in off-flavors, usually associated with psychrotroph counts in raw milk of about 10^7–10^8 cfu ml^{-1}. Butter can also undergo considerable hydrolytic rancidity from heat-resistant lipases. The result is the production of rancid, putrid flavors due primarily to the growth of *Pseudomonas* spp. in water droplets. By virtue of its high fat content and the tendency of lipase to partition preferentially into the cream phase of milk, cream is very susceptible to lipases from psychrotrophs. Flavor defects are the most common complaint resulting from the growth of lipolytic psychrotrophs in cream. Yogurt and cultured dairy products have been described as having unacceptable flavor scores and bitter, unclean, or fruity flavors when made from milk having high levels of psychrotrophs. *Pseudomonas* spp. are also associated with spoilage of Cottage cheese and they are able to oxidize diacetyl to acetoin, a flavorless compound. This results in a product with a bland taste.

It is clear that the growth and presence of psychrotrophs in dairy products prior to heat processing can adversely affect the quality of the finished products. Postpasteurization contamination at very low levels by *Pseudomonas* spp., however, is more detrimental to the keeping quality of pasteurized milk at 4–8 °C than the number of psychrotrophic bacteria in raw milk.

Control of *Pseudomonas* spp. and Related Enzymes

There is general agreement that it is very difficult to prevent contamination of raw milk by *Pseudomonas* spp., probably due to their ubiquitous nature. Therefore, approaches involving control of extracellular enzyme production seem to be a more promising solution than methods for prevention. Examples of the wide range of methods currently proposed to control psychrotrophic bacteria and/or their extracellular enzymes include thermization (a heat process of 60–66 °C for 5–20 s), additives (CO_2, nitrogen), high-pressure treatment, modified atmosphere storage of raw milk, microbial antagonism, activation of the lactoperoxidase system in milk, addition of enzyme inhibitors, addition of bacteriocin-producing lactic acid bacteria, and low-temperature inactivation of enzymes, to name a few. Only one of the aforementioned processes (low-temperature inactivation of enzymes) specifically targets enzyme synthesis. Most practices currently employed in the industry focus on elimination of the bacteria by heat processing.

Enumeration

Two general approaches are used to enumerate psychrotrophic microorganisms. Both apply to enumerating *Pseudomonas* spp. from dairy products. The traditional method selects specifically for psychrotrophs by incubation of test samples at 7 °C for 7 days. Due to the lengthy incubation, it is not very suitable as a quality assurance measure in the dairy processing industry. A second, and more rapid, approach to enumerating psychrotrophs involves incubation at 21 °C for 25 h. Most colonies appearing on agar plates using this method will be psychrotrophs since their optimum growth temperatures are typically between 20 and 22 °C. The second method is more rapid and correlates very well with the traditional 7-day incubation at 7 °C.

Pseudomonas spp. grow well on nonselective media (e.g., plate count agar, tryptic soy agar, Luria-Bertani agar, blood agar, MacConkey agar, eosine methylene blue agar). There exist a variety of media for specifically targeting fluorescent pseudomonads. These media enhance production of the typical green fluorescent pigment, pyoverdin, and therefore allow for easy identification of colonies on agar plates exposed to ultraviolet light. Examples of media found effective for identifying fluorescent pseudomonads include medium B and various media containing penicillin G, novobiocin, and cycloheximide.

Considerable interference from other Gram-negative bacteria is encountered when attempting to isolate *Pseudomonas* spp. on a nonselective medium. One promising selective medium has been developed based on heart infusion agar with addition of the selective agents cephaloridine, fucidin, and cetrimide. This medium effectively suppresses growth of Gram-positive bacteria and inhibits growth of other Gram-negative bacteria. Following inoculation and spread plating, the agar is incubated at 25 °C for 48 h.

A polymerase chain reaction assay for protease-producing pseudomonads, which targets the *aprX* gene, has been proposed but, due to gene heterogeneity, it failed to detect *Ps. lundensis* strains.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, aerobic rod, and is an opportunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft-tissue infections, bacteremia, and a variety of systemic infections, particularly in victims of severe burns and in cancer and AIDS patients who are immunosuppressed. *Pseudomonas aeruginosa* may cause mastitis. The organism can be isolated from soil and water. *Pseudomonas aeruginosa* isolates may produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. *Pseudomonas aeruginosa* produces two types of soluble pigments, pyocyanin and (fluorescent) pyoverdin. The latter is produced abundantly in media of low iron content, and could function in iron metabolism in the bacterium. *Pseudomonas aeruginosa* is primarily a nosocomial pathogen.

The organism has an optimal growth temperature of 37 °C but is unable to grow at 4 °C. This makes *Ps. aeruginosa* an uncommon contaminant of refrigerated milk.

See also: **Milking and Handling of Raw Milk**: Effect of Storage and Transport on Milk Quality; Milking Hygiene. **Psychrotrophic Bacteria**: *Arthrobacter* spp.; Other Psychrotrophs.

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Other Psychrotrophs

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General Considerations

Several species of Gram-negative and Gram-positive bacteria which have the capacity to spoil milk and other short shelf-life dairy products and can grow at or below 7 °C (**Table 1**) have been isolated from raw milk and freshly pasteurized milk and cream.

Compiled from: Craven HM and Macauley BJ (1992) *Australian Journal of Dairy Technology* 47: 38–45; Cromie SJ *et al.* (1989) *Australian Journal of Dairy Technology* 44: 74–77; Griffiths MW and Phillips JD (1990) *Journal of the Society of Dairy Technology* 43: 62–66; Suhren G (1989) In: McKellar RC (ed.) *Enzymes of Psychrotrophs in Raw Food*, pp. 3–34. Boca Raton: CRC Press; Ternström A *et al.* (1993) *Journal of Applied Bacteriology* 75: 25–34.

Commercial high-temperature, short-time (HTST) pasteurization (72 °C for 15 s) of raw milk of reasonable microbial quality essentially eliminates heat-labile psychrotrophic pseudomonads. Laboratory-pasteurized or pasteurized and aseptically packed milk and cream are free from postpasteurization contaminants but after prolonged cold storage become microbiologically spoiled, even at 1 °C (**Table 2**). Spoilage of such milk is mainly caused by sporeforming psychrotrophic and aerobic *Bacillus* spp. Unless aseptically drawn, good-quality raw milk contains after milking several thousand bacteria per milliliter, half of which are usually coryneform bacteria and *Micrococcus* spp. Psychrotrophic *Pseudomonas* spp. and other heat-labile Gram-negative psychrotrophs, such as *Flavobacterium*, *Alcaligenes* and *Chromobacterium*, are usually reported to be minor components of the microflora of good-quality fresh raw milk. Corynebacteria, *Micrococcus*, *Arthrobacter* and *Streptococcus* are frequently both psychrotrophic and thermoduric. Many of these species, along with spores of *Bacillus* and *Clostridium* spp., survive HTST pasteurization. The total number of bacteria that survive HTST pasteurization is often higher than 1000 ml^{-1}. The number of spores in raw milk is very variable but rarely exceeds more than 2% of the total microflora. The psychrotrophic *Bacillus* spp. may represent only a small part of total spore count. Frequently, the number of spores of psychrotrophic *Bacillus* spp. in freshly pasteurized milk is so low that it can be determined only by the most probable number (MPN) technique. Spores of *Clostridium* spp. occur in markedly lower numbers than spores of *Bacillus* spp.

The method recommended for the enumeration of total psychrotrophic count is the same as standard plate count except that plates are incubated at 7 °C for 10 days. Short methods with counts after 25 h based on incubation at an elevated temperature have also been developed. A way to obtain the proportion of bacteria present in milk as spores is to conduct viable count of a sample in which the vegetative cells have been killed by heat. Crystal violet or preparations of other selective inhibitors have been used to prevent Gram-positive bacterial growth. Procedures that include biotyping (examination of profile of biochemicals utilized by the cells) have been widely used for identification of dairy isolates of psychrotrophic nonpseudomonads. Many of the isolates have only been identified at the genus level. Computer-aided biotyping using miniaturized commercial kits has been introduced for identification of both Gram-positive and Gram-negative bacteria, including many nonpseudomonad psychrotrophs. Enumeration, detection and typing of *Bacillus cereus* group isolates from milk have been extensively studied. Biotyping, serotyping, phage typing, nucleic acid-based techniques, immunological methods, electrophoresis of proteins, gas chromatographic analysis of cellular fatty acids and pyrolysis have been used for the identification and/or typing of these microorganisms. Randomly amplified polymorphic DNA procedure which includes polymerase chain reaction (RAPD–PCR) has been used for subtyping of *Bac. cereus*, *Bac. licheniformis* and *Bac. thuringensis*. RAPD–PCR also allowed the tracing of contamination routes of *Bac. cereus* in dairy processing.

Growth at Refrigeration Temperatures

Compared with *Pseudomonas* spp., available data on the effect of temperature on the growth rate and properties of enzymes produced by other psychrotrophs are limited. Based on the comparison of generation times, it can be generalized that *Pseudomonas* spp. are more potent psychrotrophs than other genera (**Table 1**). However, minimum growth temperatures (T_{min}) calculated from the Ratkowsky 'square root' equation indicate that *Alcaligenes* spp. and *Chromobacterium* spp. are as likely to be psychrotrophic as *Pseudomonas* spp. (**Table 3**).

[†] Deceased.

Table 1 Generation time of psychrotrophic bacteria in heat-treated milk and frequency of their isolation from raw or pasteurized contaminated milk and cream

			Frequency of isolation (%)		
	Generation time (h) at			*From spoiled pasteurized milk or cream stored at*	
	3–5 °C	*7–9 °C*	*From cold stored raw milk*	*4–5 °C*	*7 °C*
Gram-negative bacteria	—	—	—	90	89
Acinetobacter spp.	14.4	—	nd–6	—	—
Alcaligenes spp.	—	13	nd–1.5	0.4	1.3
Achromobacter spp.	9.0	5.5	nd–2	—	—
Enterobacteriaceae	10.3	—	8–15	1	1
Flavobacterium spp.	—	14.1	1–14	2	0.8
Pseudomonas spp.	6.5–8	3.5–4	30–86	45–90	50–83
Gram-positive bacteria	—	—	—	5–70	10–55
Bacillus cereus	—	7–48	nd–10	—	—
Bacillus circulans	—	5–12	—	—	—
Bacillus polymyxa	—	22 (at 6 °C)	—	—	—
Bacillus spp.	—	—	—	5–70	10–55
Micrococcus spp.[a]	26.2	20.9	—	—	—
Psychrotrophic yeasts	—	—	—	—	0.7

[a] Some can also be Gram-negative. nd, not detected by plate count.

Table 2 Effect of temperature on the shelf-life and growth of thermoduric psychrotrophs in aseptically packed high-temperature, short-time (HTST) pasteurized milk

Temperature (°C)	*Shelf-life (days) terminated due to flavor defects*	*Count (cfu ml^{-1}) at the time of spoilage*	*Days to reach a count of 10^7 cfu ml^{-1}*
1	75	5×10^7	51
3	52	7×10^7	34
7	28	6×10^7	20
12	11	2×10^7	10

Adapted from Dommett (1992).

Both the lag time and the generation time of psychrotrophic *Bacillus* spp. vary considerably. *Bacillus circulans* can grow at 1 °C and has been found in independent studies to have a shorter lag time and generation time than six other species of psychrotrophic *Bacillus* spp. Psychrotrophic and thermoduric psychrotrophs may be simply variants of mesophilic organisms, which have adapted to grow at low temperatures.

The growth rates and lag times in ultra-heat treated (UHT) milk and pasteurized milk or single and double cream are similar for Gram-negative psychrotrophs as well as *Bacillus* spp. $T_{\min}$ for different bacterial cultures are similar in milk and other media. Spores of psychrotrophic *Bacillus* spp. show maximum germination activity at 15 °C, with a possible second maximum peak at 5 °C. Increasing the temperature of HTST pasteurization of milk may actually have an adverse effect on its keeping quality because temperatures higher than 72 °C may induce the germination of spores.

Spores of *Bacillus* spp. may germinate and grow rapidly during the first hours of incubation of fermented milks until the pH decreases to a value which is inhibitory to these organisms. Psychrotrophic yeasts and moulds can spoil fermented milks.

Psychrotrophic Pathogens

Listeria monocytogenes, *Yersinia enterocolitica*, *Bacillus cereus* and probably enterohemorrhagic *Escherichia coli* (*see* **Pathogens in Milk**: *Bacillus cereus*; *Escherichia coli*; *Listeria monocytogenes*; *Yersinia enterocolitica*) are psychrotrophic pathogens that can grow at or below 8 °C, but

Table 3 Theoretical minimum temperature (T_{min})a for selected bacteria

Organism	*T_{min} (K)*
*Acinetobacter*b spp. 55	266.6–267.0
Aeromonas hydrophila 1383	269.2–269.4
Alcaligenes faecalis G2/7	258.7–259.0
Bacillus cereus (four strains)	272.9–278.7
Bacillus circulans (two strains)	262.7–266.1
Bacillus lentus MRM 305	265.8–267.0
Bacillus polymyxa MRM 304	270–272.6
Chromobacterium spp. 12	260.2–263.7
Citrobacter freundii 1197	268.4–269.8
Coliforms (two strains)	264–274
Coryneforms (two strains)	275.8–278.5
Enterobacter agglomerans 1498	266.6–267.7
Micrococcus spp. (two strains)	273.6–273.7
Pseudomonas spp. (five species)	260.2–269.8
Serratia spp. (two species)	266.3–274.2
Streptococcus faecalis NCIB 775	273.6–273.8

[a] Calculated from Ratkowsky 'square root' equation: $\sqrt{r} = b(T - T_{min})$, where $\sqrt{r}$ is the square root of the growth rate constant, T is temperature (K) and T_{min} (or T_0) is theoretical (conceptual) temperature of no metabolic significance (conceptual minimum growth temperature).
[b] For single strains, data are from growth in skim and full-fat milk.
Compiled from: Phillips JD and Griffiths MW (1987) *Food Microbiology* 4: 173–185 and Ratkowsky DA *et al*. (1982) *Journal of Bacteriology* 149: 1–5.

only *Bac. cereus* is a significant determinant of shelf-life of pasteurized milk or fresh dairy products. Proteolytic psychrotrophs may stimulate the growth of pathogens in milk.

Incidence of Genera Other than *Pseudomonas* in Raw and Pasteurized Milk

Psychrotrophs that Survive Pasteurization

The incidence of aerobic sporeforming bacteria in milk is highly seasonal. Studies in several countries have shown the highest number of spores during late summer and early autumn. Soiling of the udder during grazing may be responsible for the high count of sporeforming bacteria.

Frequently, the total number of spores reported in raw milk is in the range 1–160 ml^{-1}. Occasionally, high numbers (>10^4 ml^{-1}) of spores or vegetative cells of *Bacillus* spp. have been reported. Both *Bac. cereus* group, *Bac. licheniformis* and *Bac. coagulans*, are the species of sporeforming bacteria predominantly in fresh raw milk. The number of spores in freshly pasteurized milk varies widely between dairy plants and on a daily basis in the same dairy plant.

The spoilage potential of thermoduric/sporeforming psychrotrophs has been well demonstrated by incubating milk or cream free from postpasteurization contaminants and therefore free from *Pseudomonas* spp. and other heat-labile microorganisms. Spoilage may occur at 3 °C after approximately 7 weeks of storage (**Table 2**). The shelf-life of pasteurized, noncontaminated milk is approximately three times longer at 7–10 °C than the shelf-life of commercially produced milk, which becomes contaminated after pasteurization and stored at 3–5 °C (**Table 4**). Coryneform bacteria dominate freshly pasteurized cream or milk (**Table 5**). Very often, only 0.05–1 spore of psychrotrophic *Bacillus* spp. is detected in freshly pasteurized milk or cream by the MPN technique. Vegetative cells of *Bacillus* spp. can probably be detected on a selective medium such as polymyxin–egg-yolk–mannitol–bromothymol-blue agar in every package of freshly pasteurized milk which has been preincubated at room temperature. Raw milk has been shown to be a markedly more important source of psychrotrophic spores than postpasteurization contamination. *Bacillus circulans* occurs in both raw and freshly pasteurized milk at lower numbers than *Bac. cereus*. However, data from different sources show that *Bac. circulans* dominates spoiled milk or cream, free from postpasteurization contaminants, stored at 3–7 °C. At 12 °C, a significant proportion of bacteria at the time of spoilage are *Bac. cereus*, coryneform bacteria, micrococci and streptococci (**Table 5**).

Table 4 Effect of postpasteurization contamination (PPC) on shelf-life and psychrotrophic count of pasteurized milk or cream. Postpasteurization recontamination was avoided using aseptic packing or laboratory pasteurization

Effect on shelf-life			*Effect on psychrotroph count*		
	Shelf-life (days) of milk or cream			*Count (cfu ml^{-1}) after storage at 6 °C*	
Storage at	*PPC-free*	*Contaminated*	*Storage of cream for*	*PPC-free*	*Contaminated*
3–5 °C	49–28	11–6	6 days	4×10^3	7×10^5
7–10 °C	35–20	7–5	13 days	7×10^5	5×10^7

Adapted from: Muir, 1996a; Muir, 1996b and Muir, 1996c and Stepaniak, L., 1991. Factors affecting quality and possibilities of predicting shelf-life of pasteurized and ultra-high temperature heated milks. *Italian Journal of Food Science* 3, pp. 11–26. Stepaniak (1991).

Table 5 Microbial population patterns (% occurrence among isolated bacteria) of aseptically packed fresh and cold stored cream and cold stored concentrated milk

	Fresh cream, pasteurized at		*Cream, pasteurized at 72 °C for 15 s, by the end of shelf-life at*			*Concentrated 1:2 milk, pasteurized at 72 °C for 15 s, by the end of shelf-life at*		
	72 °C for 15 s	*80 °C for 15 s*	*3 °C*	*7 °C*	*12 °C*	*3 °C*	*7 °C*	*12 °C*
Bacillus circulans	nd	nd	100	50	18	90	95	65
Bacillus cereus	nd	2	nd	2	30	10	5	35
Other *Bacillus* spp.	3	5	nd	nd	5	nd	nd	nd
Streptococci	5	nd	nd	2	15	nd	nd	nd
Micrococci	22	3	nd	5	15	nd	nd	nd
Coryneforms	70	90	nd	41	17	nd	nd	nd

nd, not detected by plate count.
Adapted from Dommett (1992).

Incidence of Psychrotrophs in Commercially Pasteurized Nonaseptically Packed Milk

Postpasteurization contamination of commercially pasteurized, nonaseptically packed milk is unavoidable. Recontamination in modern dairy plants can be as low as one bacterial cell per liter, and frequently is in the range of the number of psychrotrophic *Bacillus* spp. spores in freshly pasteurized milk. Major contaminants are Gram-negative rods, especially *Pseudomonas* spp., which are the most significant bacteria determining shelf-life of nonaseptically packed milk stored at 7 °C or lower. The generation time and the lag time of microflora in pasteurized milks with an initial psychrotrophic count $<10\,\mathrm{l}^{-1}$ are markedly longer than in milk containing 100 psychrotrophs ml^{-1} after pasteurization. Milk spoiled by coliforms at 7 °C has also been reported occasionally. Pasteurized milk containing 1–100 coliforms ml^{-1} after preincubation at 21 °C is much more frequently unacceptable after 10 days of storage at 7 °C than milk in which coliforms have not been detected. In one study, at 10–12 °C, *Bacillus* spp. and Gram-positive cocci each comprised 32–35% of the total spoilage microflora. The plate count of postpasteurization contaminated milk preincubated at 21 °C with a mixture of crystal violet, nisin and penicillin has been used to estimate, within about 48 h, if milk will have satisfactory keeping quality at 6 °C. The test correlates well with a standard method, which requires 5 days' incubation at 6 °C. The shelf-life of pasteurized milk, which was free from postpasteurization contaminants, was predictable from the storage time of raw milk.

Wide daily fluctuations in the number, growth rate and germination properties of psychrotrophic thermoduric microorganisms and a variable level of postpasteurization contamination have been observed in pasteurized milk from the same processor. Large differences have also been observed between processors. Due to these variations, the correlation between shelf-life of cold stored pasteurized milk and the total count of microorganisms in raw milk is usually poor. A good index for predicting the shelf-life of contaminated pasteurized milk stored at different temperatures is the average $T_{\min}$ of its microflora, the value of which is variable and is influenced by the contribution of thermoduric and heat-labile psychrotrophs.

Product Defects Caused by Psychrotrophs and Their Enzymes

Milk usually shows flavor defects when the total count of *Bacillus* spp. and/or *Pseudomonas* spp. is 2×10^7–5×10^7 cfu ml^{-1}. Flavor defects occur in pasteurized, aseptically packed milk spoiled by thermoduric and sporeforming bacteria when the number of these microorganisms is $>10^7$ cfu ml^{-1} (**Table 2**). Occasionally, flavor defects are noted at a population of *Bacillus* spp. $<10^7$ cfu ml^{-1}. Extracellular enzymes and volatile metabolites have been detected in milk incubated with psychrotrophic *Pseudomonas*, *Alcaligenes*, *Bacillus* or with the indigenous flora, at populations around 10^6 cfu ml^{-1}. The threshold number for some psychrotrophic yeasts required to cause sensory changes in quark varies from 2.4×10^4 to 4.2×10^6 cfu g^{-1}. The pattern of volatile compounds analysed by gas chromatography or an electronic nose may be characteristic for spoilage organisms and may permit their identification.

The production of heat-stable enzymes at low temperatures is not limited to the genus *Pseudomonas*. At least 5% of extracellular proteolytic activity survives in cell-free supernatants from one or more species of *Bacillus*, *Enterobacter*, *Serratia*, *Alcaligenes*, *Flavobacterium* and *Achromobacter* after exposure to 140 °C for 5 s. Lipases with similar heat stability are secreted by *Bacillus*, *Enterobacter*, *Serratia*, *Citrobacter*, *Moraxella* and *Achromobacter*. More than 50% of the lipolytic or proteolytic activity of enzymes secreted by psychrotrophs other than pseudomonads can survive HTST pasteurization. For comparison, the D-value for *Flavobacterium* cells determined at 70 °C is 0.05 s while the D-value for spores of several *Bacillus* spp. was between 16.5 and 20.5 min at 85 °C. Some strains or species of *Bacillus*, *Flavobacterium*, *Alcaligenes* and *Aeromonas* also produce heat-stable phospholipase.

The secretion of heat-labile proteinases and lipases or phospholipases by strains of the above and other psychrotrophic genera have also been reported. Some strains were only proteolytic or only lipolytic. Some species (**Table 6**) secrete more than one proteinase. Low-temperature treatment at 55 °C, which effectively inactivates proteinases and lipases secreted by *Pseudomonas* spp., causes little reduction in the activity of proteinases and lipases from other genera of psychrotrophs.

Proteinases of *Pseudomonas* spp., as well as those of other psychrotrophs (both Gram-negative and Gram-positive), hydrolyze the major casein fractions with different specificity. Limited proteolysis produces an unclean flavor while advanced proteolysis causes gelation and sweet curdling of milk. Sweet curdling is associated with proteinase from *Bacillus* spp.

Some *Bacillus* spp. isolated from milk can ferment lactose. Flavor defects caused by different *Bacillus* spp. grown in milk at 7 °C have been described as fruity, sour, yeasty, gassy or unclean. The lipolytic activity of thermoduric psychrotrophs produces rancid and fruity off-flavors in milk products. Lipases from different genera show differences in specificity toward various triglycerides. Phospholipases, proteinases and glycosidases from *Pseudomonas*, *Citrobacter* and *Enterobacter* may act synergistically in damaging the milk fat globule membrane. The aggregation of fat globules, which produces bitty cream, has been linked to the specific activity of phospholipase from *Bac. cereus*. The defect is not caused by Gram-negative psychrotrophs and can be substantially reduced if cream is stored below 5 °C. Some psychrotrophic nonpseudomonad Gram-negative bacteria may be responsible for the reduction of diacetyl in buttermilk.

Spoilage of sterile products by heat-stable enzymes from *Pseudomonas* spp. has been studied extensively. However, very little is known about the significance of heat-stable enzymes of genera other than *Pseudomonas*, isolated from cold-stored milk, to the spoilage of UHT-sterilized milk. Enzymes and other metabolites accumulated during the postpasteurization growth of psychrotrophs are likely to be mainly responsible for shortening the shelf-life of pasteurized milk and cream.

Thermoduric Psychrotrophs and Extended Shelf-Life Dairy Products

Ongoing centralization of the dairy industry, resulting in the formation of large dairy plants, extends supply lines, while the new habit of consumers to shop less frequently calls for pasteurized milk and other cold-stored perishable dairy products with improved keeping quality. During storage and distribution, dairy products may be exposed to increases in temperature. It is therefore desirable that the products have built-in 'quality buffers' to minimize

Table 6 Some properties of proteinases (Pr), lipases (Li) and phospholipases (Ph) from psychrotrophs other than *Pseudomonas* spp.

	Molecular mass[b] *(kDa)*			*Optimal temperature*[a]			*Optimal pH*[a]			*Inhibitors*		
Microorganism	*Pr*	*Li*	*Ph*	*Pr*	*Li*	*Ph*	*Pr*	*Li*	*Ph*	*Pr*	*Li*	*Ph*
Alcaligenes spp.	—	—	—	—	50	—	—	9	—	—	—	—
Achromobacter spp.	27	—	—	50	37	—	6.2	7	—	S	—	—
	20	—	—	66	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	8.5	—	—	—	—	—
Acinetobacter spp.	102	33	25–35	37	—	—	7.6	8.5	—	M, C	—	M
Alteromonas spp.	63	—	—	70	—	—	9.3	—	—	M	—	—
Bacillus coagulans	420	—	—	—	—	—	7.5	—	—	M	—	—
Bacillus spp.	26, 34	—	20, 23	—	—	50	6, 7.5	—	6.6, 8.0	M	—	M
Chromobacterium spp.	—	—	—	—	—	—	—	6.5	—	—	M	—
Pr 1	75	—	—	—	—	—	5.3	—	—	—	—	—
Pr 2	62	—	—	45	—	—	6.2	—	—	M	—	—
Pr 3	65	—	—	—	—	—	6.2	—	—	—	—	—
Enterobacter spp.	—	—	—	41	—	—	8.0	—	—	M	—	—
Flavobacterium spp.	13	—	—	42, 50	—	—	7, 10	—	—	S	—	—
Micrococcus spp.	29, 38	25, 250	—	25, 60	8.5, 10	—	7, 10	—	—	M, S	M	—
Vibrio spp.	70	—	—	—	—	—	6, 7.5	—	—	—	—	—

[a] Maximum and minimum values are given when more than one strain was studied.

[b] Values for molecular mass are determined by gel permeation chromatography.

M, inhibitors of metalloenzymes; S, inhibitors of serine-type enzymes; C, inhibitors of cysteine-type enzymes.

Compiled from: Fox PF *et al*. (1989) In: McKellar RC (ed.) *Enzymes of Psychrotrophs in Raw Food*, pp. 57–120. Boca Raton: CRC Press.

spoilage at elevated temperatures. In the United States, extended shelf-life (ESL) (*see* **Liquid Milk Products**: Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk)) products have become an acronym that refers to the total process which encompasses everything from raw product quality to final distribution. The International Dairy Federation has suggested a target shelf-life of 25–45 days for ESL products. With the improved control of postpasteurization contamination, psychrotrophic sporeforming bacteria have emerged as important causes of spoilage of both normal and ESL milk. A large portion of microflora at the time of spoilage is often represented by Gram-positive psychrotrophs. Apart from airborne contamination and contamination from the udder, inadequately cleaned milking equipment, pipelines, farm bulk tanks and product lines in dairy plants may be important sources of spores and thermoduric microorganisms. ESL milk with reduced or zero postpasteurization contamination may often be spoiled by *Bacillus* spp. only.

Elopak and APV have developed the Pure-Lac™ system that combines heating of milk at 130–140 °C for less than 2 s and instant cooling in a special steam infusion chamber with a packing system, which may reduce or completely eliminate postpasteurization contamination. The heating system substantially reduces the number of spores but does not denature much more whey proteins and does not cause more pronounced sensory changes than normal HTST pasteurization. Milk processed by this system is, according to European Union directive, termed as pasteurized at high temperature. Airborne contamination is avoided by surrounding the system with air filtered to remove bacteria and spores. Contamination from the package is eliminated by its sterilization using a solution of hydrogen peroxide at low concentration and UV light. Milk produced by the Pure-Lac system may have a shelf-life of up to 45 days at 10 °C.

Activation of the lactoperoxidase system, treatment with carbon dioxide, addition of bacteriocin-producing lactic acid bacteria and bacteriocin or antimicrobial peptides derived from lactoferrin may inhibit the growth of all types of psychrotrophs in cold stored milk. Microfiltration (*see* MEMBRANE SEPARATION) and centrifugation (bactofugation) markedly reduce the number of spores and psychrotrophic bacteria in milk. These techniques, combined with reduction or elimination of postpasteurization contamination, may assure the production of ESL milk and cream of the requisite keeping quality.

Contamination with spores of *Bacillus* spp. and their enzymes produced in raw milk will be increasingly significant for the keeping quality of ESL products. The development of a rapid method for the detection of contamination with psychrotrophic spores or enzymes from thermoduric psychrotrophs is warranted.

See also: **Bacteriocins**. **Heat Treatment of Milk**: Ultra-High Temperature Treatment (UHT): Aseptic Packaging; Ultra-High Temperature Treatment (UHT): Heating Systems. **Liquid Milk Products**: Liquid Milk Products: Super-Pasteurized Milk (Extended Shelf-Life Milk). **Microorganisms Associated with Milk**. **Milk Protein Products**: Membrane-Based Fractionation. **Pathogens in Milk**: *Bacillus cereus*; *Escherichia coli*; *Listeria monocytogenes*; *Yersinia enterocolitica*.

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REPLACEMENT MANAGEMENT IN CATTLE

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Growth Standards and Nutrient Requirements

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Introduction

The goal of a dairy heifer-rearing program is to provide a regimen that will enable the heifer to develop her full lactation potential at the desired age and at a minimum expense. The optimum age and size at first calving are heavily dependent upon the breed and the genetics for body size of the animal. With increased popularity of crossbreeding of dairy animals, defining optimum body size has become more challenging. Calving heifers at too young or too old an age is associated with reduced production. Studies from a population of US Holsteins found the optimum age of first calving to occur between 22 and 24 months of age. However, for earlier-maturing breeds, such as Jerseys, the optimum age for first calving may be 1 or 2 months lower. Similarly, the optimum age for larger breeds may be higher by 30–60 days. Optimum body size must be defined relative to parturition. Suggestions for desired body weights based upon US genetics of representative breeds are shown in **Table 1**. This table is not intended to be representative of breeds throughout the world but serves to demonstrate the expected changes in body weight relative to parturition. It should also be noted that genetics within breed has a large impact on body size. As an example, within the US population it is estimated that genetics accounts for approximately 35 kg of the variation in body weight at first calving.

In addition to body weight, height at the withers or hips has been used as an indicator of growth. More recently, hip heights have become the more preferred measure of height of the animal as measurements are more repeatable and less influenced by the position of the animal's head during measurement. Genetics is the major determinant of an animal's potential for growth in height. However, the feeding program has considerable influence on the animal's ability to achieve its genetically predetermined potential for growth. As an example, **Table 2** shows median, 75th, and 95th percentiles for weight and height of a national sample of US Holsteins. Note the variation observed in height in this population.

Ideally, body composition data would provide valuable information, which might be a better indicator of future lactation yield than body weight alone. One would expect heifers with greater lean tissue growth and moderate levels of body fat content to produce the most milk. Unfortunately, the noninvasive techniques utilizing urea space, ultrasound, and electrical conductivity have all proven to provide information of limited value in measuring composition in dairy heifers, partly due to low body fat content of dairy

Table 1 Desired body weights (kg) at first calving

	Time of measurement		
Breed	*Before calving*	*Postcalving*	*Peak lactation*
Ayrshire	563	509	486
Brown Swiss	625	563	536
Guernsey	534	477	459
Holstein	636	572	545
Milking Shorthorn	591	532	509
Jersey	409	368	352

Adapted from Hoffman PC (2003) Heifer fundamentals. In: PC Hoffman and R Plourd (eds.) *Raising Dairy Replacements*, ch. 6, pp. 53–56. Ames, IA: Midwest Planning Service.

heifers as compared to the beef breeds. The only reliable method to measure body composition is to sacrifice the animal and measure body fat directly. This technique is costly and eliminates the ability to obtain later milk production information.

Rate of Gain

Measurements of body size are not adequate to describe the influence of growth on performance and productive life of the dairy animal. The rate of gain in weight and height, and timing of growth must be considered. About 50% of the total gain in height occurs during the first 6 months of life, 25% from 7 to 12 months and the remaining 25% during the last 12 months. The proportion of body weight to wither height increases linearly and the increase in wither height as a proportion of height at maturity is greatest during the first 6 months. This shows that the young heifer has a propensity for rapid growth if properly nourished. Before proceeding further, it is beneficial to review the biology of heifer and mammary growth. At birth, the mammary gland is rudimentary with both the gland and teat cisterns evident and ducts present close to the gland cistern. From birth to 3 months of age, the gland grows at a rate similar to the rest of the body. However, beginning at approximately 3 months of age, the gland begins an allometric growth phase, where the mammary gland grows at a much faster rate than the rest of the body. This phase continues until puberty when once again the gland assumes the same rate of growth as the rest of the body. Just prior to parturition, the mammary gland once again grows at a rate exceeding the rest of the body. The timing of these shifts in differentiation and growth vary with breed and the hormonal events triggering them have not been fully elucidated. The influence of feeding programs and rates of growth during the first 2 years of life has been the subject of much research. There are indications that a higher rate of growth (over 800 g day^{-1} for large breed heifers) prior to weaning during the milk feeding period has a positive influence on mammary development. Studies have shown that the milk-fed calf is capable of rates of growth exceeding 1.5 kg day^{-1}. Using the test day model, Cornell researchers can attribute major positive differences in first lactation milk yield to the rate of gain during the preweaning period of life. However, there is a large body of evidence that indicates that rapid growth (over 950 g day^{-1} for large breeds and less for smaller breeds) after weaning and before puberty may have a negative

Table 2 Median, 75th, and 95th percentile weights and heights indicated for Holstein heifers in the National Animal Health Monitoring System (NAHMS) project

	Weight (kg)			*Height (cm)*		
Age (months)	*Median*	*75th*	*95th*	*Median*	*75th*	*95th*
1	54	62	65	79	84	84
3	96	106	129	89	91	96
5	141	154	187	96	99	102
7	192	213	246	104	109	114
9	241	271	320	109	114	119
11	290	324	353	117	119	124
13	331	368	415	119	124	129
15	383	423	485	124	129	135
17	423	466	541	127	132	137
19	458	494	581	129	134	137
21	494	541	624	132	137	142
23	522	581	645	135	137	145
24	532	591	702	135	140	145

From Heinrichs AJ and Lammers B (1998) *Monitoring Dairy Heifer Growth*. University Park, PA: Pennsylvania State University Cooperative Extension.

impact on mammary development. Heifers fed for more rapid rates of gain postweaning exhibit depressed levels of growth hormone and increased growth of the mammary fat pad. However, the influence of diet on growth of mammary parenchyma is less clear with some studies indicating minimal influence on growth of the secretory tissue. The critical rates of gain that may influence mammary development vary with breed as studies have shown that apparent decreased mammary development occurs at lower rates of gain in Jerseys and Red Danish heifers as compared to Friesians.

Rate of gain can also be considered in a stair-step fashion. In this scenario, heifers are reared in alternating periods of slow and rapid rates of gain during the second and third trimesters of gestation, respectively. During the restricted phase, energy is fed to 70% of requirements and during the rapid phase at 130% of National Research Council (NRC) requirements for protein and energy. The rapid phase of growth during the third trimester corresponds to a period of robust mammary parenchymal growth. Growth responses and milk production of control and stair-step-reared animals are shown in **Table 3**.

Table 3 Performance of Holstein dairy heifers reared at a steady rate of gain or in a stair-step manner

Item	*Control*	*Stair-step*
Initial body wt. (kg)	281	278
Final body wt. (kg)	553	575
Daily gain (kg day^{-1})	0.68	0.98**
DM intake (kg day^{-1})	9.3	7.5*
Overall efficiency		
Energy (g Mcal^{-1})	32.6	57.9*
Protein (%)	54.2	96.5*
Composition of mammary tissue		
DNA (mg g^{-1})	4.3	5.6*
RNA (mg g^{-1})	14.5	29.7*
Lipid (mg g^{-1})	730.7	628.3*

$^{*}P < 0.05$
$^{**}P < 0.01$
Control = 750 g average daily gain (ADG).
Stair-step = alternating requirements at 70 and 130% of NRC requirements.
From Park CS (2000) Personal Communication.

In the stair-step management scheme, heifers grow more efficiently and the composition of the mammary gland reveals more secretory cell numbers and cell activity and less lipid as compared to heifers growing at a uniform rate. The stair-step rearing program shows great promise although it requires a departure from conventional thinking and it may present challenges to implement under practical feeding situations on many growing operations.

The challenge of adopting uniform growth standards is that dairy breeds vary widely in their genetic capacity for growth and mature size. The adoption of the targeted growth system recognizes that nutrient requirements are linked to an animal's rate of gain and current body size relative to mature size. In the 2001 Dairy NRC, target weights for breeding and calving are based upon percentages of mature size. Puberty usually occurs when heifers have reached 45–50% of mature size. Heifers should be pregnant by 55% of mature size and calve by 82% of mature size. **Table 4** demonstrates the breeding and calving weight targets for heifers of differing mature size based upon desired age at first calving. Requirements are predicted based upon the expected mature weight of the animal being fed, the desired age at first calving, the current weight of the heifer, and the chemical composition of the feeds and dry matter intake (DMI) of the diet.

Nutrient Requirements for Growing Dairy Heifers

Previous predictions for DMI and nutrient requirements for energy and protein have been expressed on a tabular basis. However, more recent research and the development of nutrition models for growth and production have led to the use of equations to predict intake and nutrient requirements based upon a variety of readily available animal, environmental, and dietary measures. The 2001 Nutrient Requirements of Dairy Cattle utilizes the following equation, based upon beef cattle data to predict intake:

Table 4 Application of the targeted growth system under different management scenarios based upon expected mature weight, current weight at 4 months, and targeted calving at 22–24 months of age

Mature body weight (Kg)	*Age at first calving (months)*	*Current age (months)*	*Current weight (kg)*	*Target postcalving weight (kg)*	*Target breeding weight (kg)*	*Target age at breeding (months)*	*Target growth rate 4 months calving (kg day^{-1})*
450	22	4	100	375	250	13	0.50
650	22	4	125	525	350	13	0.73
800	24	4	160	675	450	15	0.85

$$\text{DMI (kg day}^{-1}) = \text{Body weight}^{0.75} \times \frac{(0.2435 \times \text{NE}_\text{M} - 0.0466 \times \text{NE}_\text{M}{}^2 - 0.1128)}{\text{NE}_\text{M}}$$

where NE_M is net energy maintenance

However, another equation using over 5000 observations of DMI in Holstein heifers from various US locations was developed to predict intake that accounted for over 59% of the variation in daily DMI. The simplified model was DMI $(\text{kg day}^{-1}) = -29.86 + (-0.54\text{E-}05 \times \text{body weight}) + (0.157 \times \text{metabolic body weight}) + (2.090 \times \text{daily gain}) + (-0.118\ \text{daily gain}^2) + (0.730 \times \text{TDN}$ (total digestible nutrients)) $+ (-0.005 \times \text{TDN}^2) + (-0.001 \times \text{body weight} \times \text{daily gain}) + (-0.019 \times \text{TDN} \times \text{daily gain})$. This equation was subsequently verified with data sets representing multiple locations across the United States and found to be equally successful in predicting intake. This equation is most successful in predicting intake of heifers weighing 100–400 kg.

Energy requirements for maintenance are based on Mcal of net energy required to support basal metabolism with adjustments for physical activity and temperature regulation. A dynamic model adopted by the 2001 NRC considers body size, temperature, relative humidity, and hair coat condition in estimating energy requirements for maintenance. Determination of the nutrient requirements for growth of the dairy heifer is based upon targeted growth concept, which is used to estimate body composition at a given stage of growth. Body composition at birth and maturity is relatively predictable. However, younger animals have the capacity to deposit more lean tissue per unit of gain. As animals mature they deposit more fat than lean tissue per unit of gain. This principle is utilized to estimate nutrient requirements regardless of the breed. Since a gram of fat contains more calories than a gram of lean tissue, nutrient requirements for energy and protein will change as the animals reach maturity. As body weight increases, the energy content of gain increases and protein content of gain decreases because more energy is deposited as fat. Also, as the rate of gain increases, energy content of gain increases (more fat deposition) and protein content of gain decreases. As animals increase in weight, protein required does not decrease as rapidly as expected because the efficiency of protein absorption decreases.

The revised nutrient recommendations consider protein requirements of the animal in light of rumen microbial protein synthesis as well as dietary protein, which escapes degradation in the rumen to yield an estimate of metabolizable protein available to support growth and maintenance. The new models developed to estimate amino acid requirements have frequently resulted in recommendations for feeding lower levels of protein in the diet of older animals. Feeding higher levels of protein in diets of growing heifers is not recommended because as dietary protein levels increase the amount excreted via the urine and feces increases. This is not only economically inefficient, but also contributes to environmental pollution. Increasingly, the nutritionist is challenged to provide the amount of nutrients that foster optimal, economical growth with minimal detrimental influence on the environment.

Mineral requirements are estimated based upon the factorial method using revised estimates of bioavailability of mineral sources. Vitamins are typically supplemented in the diet without regard to amounts supplied by other dietary ingredients with the exception of fresh forage, which supplies abundant amounts of vitamins.

Examples of suggested ration specifications for large breed (Holstein) and small breed (Jersey) replacements are shown in **Tables 5** and **6**. These tables were

Table 5 Nutrient requirements for small breed heifers at 600 g gain day^{-1}

		Heifer weight (kg)				
Item	*Unit*	*100*	*150*	*200*	*250*	*300*
Intake	kg day^{-1}	3.1	4.2	5.2	6.1	7.0
Energy						
TDN	% of DM	62.9	62.9	62.9	62.9	62.9
ME	Mcal day^{-1}	7.0	9.5	11.8	14.0	16.0
Protein						
CP	% of DM	16.3	13.9	12.6	11.8	11.3
RUP	% of DM	6.7	4.3	3.0	2.2	1.7
RDP	% of DM	9.6	9.6	9.6	9.6	9.6

CP, crude protein.
DM, dry matter.
ME, metabolizable energy.
RUP, rumen undegradable protein. It refers to rumen 'bypass' protein.
RDP, rumen degradable protein.
Adapted from National Research Council (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy Press.

Table 6 Nutrient requirements for large breed heifers (mature weight = 650 kg) at 800 g gain day^{-1}

		Heifer weight (kg)				
Item	*Unit*	*150*	*200*	*250*	*350*	*400*
Intake	kg day^{-1}	4.2	5.2	6.2	7.9	8.8
Energy						
TDN	% of DM	63.4	63.4	63.4	63.4	63.4
ME	Mcal day^{-1}	9.6	11.9	14.1	18.2	20.1
Protein						
CP	% of DM	15.9	14.2	13.1	11.7	11.3
RUP	% of DM	6.2	4.5	3.4	2.0	1.6
RDP	% of DM	9.7	9.7	9.7	9.7	9.7

CP, crude protein.
DM, day matter.
ME, metabolizable energy.
RUP, rumen undegradable protein. It refers to rumen 'bypass' protein.
RDP, rumen degradable protein.
Adapted from National Research Council (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy Press.

developed using the dynamic models utilized by the 2001 NRC for Holstein heifers gaining 800 g day^{-1} and Jersey heifers gaining 600 g day^{-1}.

Conclusions

1. The concept of targeted growth should be practiced when determining growth standards. This enables one to establish desired rates of gain based upon the expected mature weight of the animal.
2. Rate of gain during the first 2 years influences mature weight and height.
   a. During the first 6–9 months of life, large breed heifers should be fed to gain between 750 and 900 g of gain per day and small breed heifers should gain approximately 500–700 g per day.
   b. After puberty, rate of gain is not as critical as long as desired height and weight goals after calving are reached.
3. Growth standards can be applied to dairy heifers regardless of breeding based upon expected mature size. Stated in this fashion:
   a. Calves should double their birth weight by 56 days of age.
   b. Puberty can be expected to occur when heifers have achieved approximately 45–50% of mature weight.
   c. When heifers attain 55–65% of mature weight they should be bred.
   d. At first calving, heifers should have attained 82–85% of mature weight.
   e. Body weights at second and third calvings should be 92 and 96% of mature weight, respectively.
4. Desired rates of gain can be calculated by comparing the heifer's current weight and age with the desired weights at the benchmarks described previously.
5. Target calving ages are 22–24 months for large breed heifers and 22–23 months for small breed heifers.
6. Nutrient levels in the diet must be adjusted according to environment and the ability of the animal to withstand heat and cold stress.

See also: **Replacement Management in Cattle**: Growth Diets.

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Pre-Ruminant Diets and Weaning Practices

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Introduction

The newborn calf is essentially a monogastric animal requiring highly digestible nutrients in the liquid diet, especially during the first month of life. During this time, the young calf has limited development of enzyme systems to digest ingredients supplying protein and energy. This dictates that the calf be fed protein and energy sources closely resembling those contained in milk products which will supply needed amino acids and energy from fat and lactose. However, within weeks, as the calf begins consumption of starch-containing grains, rumen microflora develops, which produces volatile fatty acids that stimulate preferential development of the rumen epithelium. Successful nutritional management of the calf is indicated when the following goals are met:

- Eighty-five percent of calves born on the farm achieve a serum immunoglobulin G (IgG) $>10\,\mathrm{mg\,ml^{-1}}$ or serum protein $>5.5\,\mathrm{g\,dl^{-1}}$ between 1 and 7 days of age.
- Ninety percent of calves double their birth weight by 56 days of age.
- Less than 30% of calves are treated for disease during the first 30 days.

Key factors to consider in the preruminant feeding program include

- colostrum management
- provision of high-quality liquid diets fed in sufficient quantity to encourage biologically normal growth and immunity
- weaning at a reasonably early age
- provision of adequate amounts of high-quality water and calf starter grain
- provision of a favorable rearing environment

Colostrum Management

Consumption of adequate quantities of high-quality colostrum early in life is the single most important factor determining health and survival of the calf. The first milk produced by the dam is a rich source of immunoglobulins (Igs), nutrients, and immune cells. As shown in **Table 1**, the level of Igs declines rapidly over the first three milkings.

Fortunately, the intestine of the newborn calf is able to absorb these Igs, unchanged for the first 6–24 h of life. Successful absorption is achieved by the consumption of 100–200 g of Ig as soon as possible after birth. Although the level of Ig is variable, good-quality colostrum contains approximately $50\,\mathrm{g\,Ig\,l^{-1}}$. The period of time that the calf is able to absorb colostrum Ig is also quite variable. It may cease within 6 h of birth, but generally ceases within 24 h. Reasons for cessation of absorption are unclear, but research indicates that the presence of large quantities of bacteria in the intestine may inhibit absorption of Ig. Feeding contaminated colostrum or a dirty calving environment may accelerate bacterial colonization of the intestine and impair Ig absorption. Thus, successful immunity transfer occurs when the calf consumes 3–4 l of 'clean' colostrum within the first 12 h of life. Colostrum may also be involved in the establishment of cellular immunity in the calf. Additional feedings of colostrum beyond the first day result in improved growth of the intestinal epithelium as well as improved absorptive capacity.

Liquid Diets for Calves

Many alternatives exist for feeding the neonatal calf after the first day. They include whole saleable milk, waste milk unsuitable for human consumption, and milk replacer. Milk replacers vary widely in nutrient content and ingredient composition. Before selecting an alternative, one should consider the benefits and risks of each alternative feeding program for calves.

Whole milk usually fosters the best growth of the calf as it contains high levels of protein (24% on a dry matter (DM) basis) and fat (28.7% on a DM basis) to support calf growth. In addition, it is likely that these nutrients are more highly digestible than those from other sources. However, in nearly all cases, whole milk is more expensive and there is a risk of transmission of disease to calves by feeding raw milk.

On most dairies, there is a supply of waste milk from cows that have calved within the past 3 days or from cows that have been treated with antibiotics. Generally, waste milk contains similar levels of protein, fat, and lactose as whole milk. However, fat levels can vary from 2.0 to more than 4% and protein from 2.5 to 4.0%. Field studies with dairies utilizing waste milk for calves indicate that the

Table 1 Summary of the composition of colostrum, transition milk, and normal milk

	Milking number			
Parameter	*1*	*2*	*3*	*Milk*
Specific gravity[a]	1.056	1.040	1.035	1.032
Solids (%)[a]	23.9	17.9	14.1	12.9
Protein (%)[a]	14.0	8.4	5.1	3.1
Casein (%)[a]	4.8	4.3	3.8	2.5
Immunoglobulin (mg ml^{-1})[a]	48.0	25.0	15.0	0.6
Fat (%)[a]	6.7	5.4	3.9	3.7
Lactose (%)[a]	2.7	3.9	4.4	5.0
Vitamin A (μg dl^{-1})	233–400[b]	190[a]	113[a]	34[a]–38[b]
Vitamin E (μg g^{-1} fat)[c]	45–206			

1, Colosrum; 2 and 3, transition milk.

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supply of waste milk varies from 2.5 to 9 kg per calf per day. On many dairies, the supply of waste milk is adequate to meet the needs of less than 50% of the calves prior to weaning. Therefore, feeding programs based upon use of waste milk must make accommodations for supplementation with additional milk solids when the supply of nutrients is inadequate. In addition to challenges with varying supply and nutrient content, waste milk frequently contains antibiotic residues (in the United States) and microorganisms that are potentially harmful to the calf.

Fortunately, systems have been developed to effectively pasteurize either whole milk or waste milk, which renders waste milk less likely to cause disease in calves. Successful pasteurization occurs when milk is heated to a sufficient temperature for an adequate time period to destroy 98% of the microorganisms present. Most systems used for pasteurizing waste milk can be described as being batch or high-temperature short-time (HTST) units. Batch systems (**Figure 1**) are typically simpler and more suitable for feeding smaller number of calves (<100).

Batch systems heat a 'batch' of milk to a temperature of 62 °C for 30 min. The HTST units (**Figure 2**) utilize either heat exchange plates or tubes to heat milk more quickly to 72 °C for 15 s. These systems are more expensive but their operation and cleaning can be more easily automated. Either system can be successful if properly installed, maintained, and operated. Successful pasteurization is generally indicated when the aerobic plate count has been reduced to 20 000 cfu ml^{-1} or less. Pasteurization does not sterilize milk but reduces bacterial numbers by 98%. Therefore, it is important that waste milk be handled with the same care as that which is sold for human consumption. If the aerobic plate count prior to pasteurization exceeds 2 000 000 cfu ml^{-1}, then the goal of <20 000 cfu ml^{-1} after pasteurization may not be achieved. It is also important that all vessels receiving waste milk after pasteurization be thoroughly cleaned

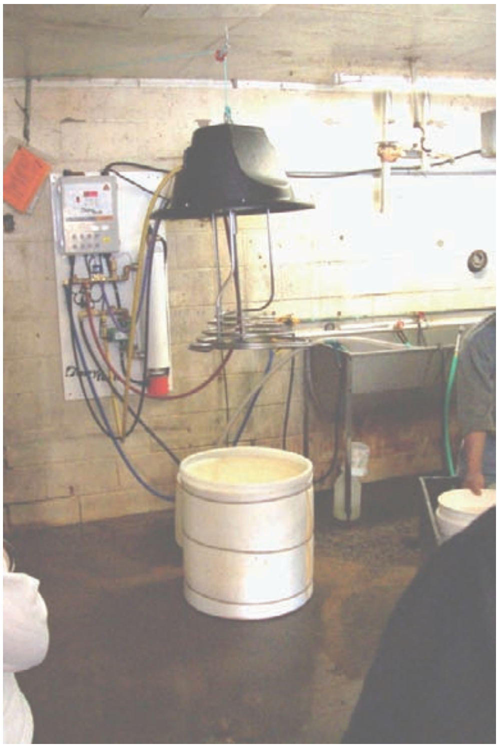

Figure 1 Example of a batch pasteurizer system (Dairy Tech, Windsor, CO).

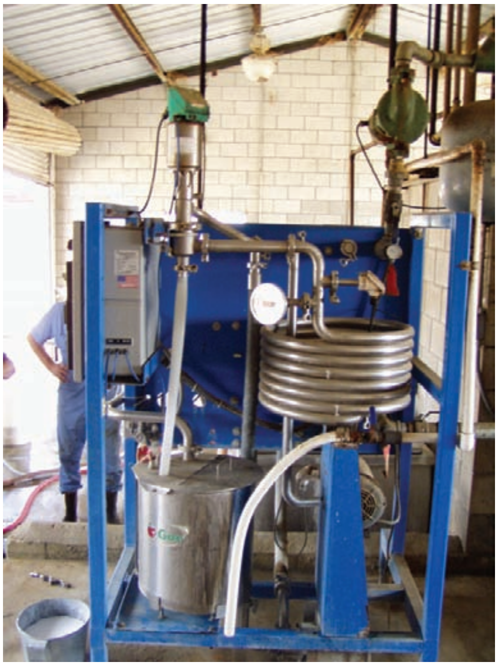

Figure 2 Example of high-temperature short-time pasteurizer (Goodnature, Orchard Park, NY).

and sanitized as microbial growth of pasteurized milk is very rapid when placed in unclean tanks or feeding bottles.

Adoption of a pasteurized waste milk-feeding program requires institution of stringent quality control programs to ensure that pasteurization has occurred and to account for variations in supply and nutrient content of waste milk. Samples of waste milk obtained after the last calf has been fed should contain less than 100 000 cfu ml^{-1}. Milk should also be tested for total solids and percentage of fat and protein. Additional milk solids should be added if waste milk contains less than 12.5% solids. Frequency of testing is determined by number of calves fed, but should be no less than once per month.

Milk Replacers

Milk replacers enable dairy producers to market more saleable milk. The objective of the formulation of a milk replacer is to provide nutrition to the calf that results in nearly equal growth and performance as that achieved with whole milk at less cost and with greater biosecurity. Typically, milk replacers are based upon the use of whey proteins, which are by-products of cheese manufacturing. The different whey products used in milk replacers include dried whey, delactosed whey, and whey protein concentrate, which contain progressively higher concentrations of protein. In an effort to further reduce costs, soy, wheat, and plasma proteins have been used as partial substitutes for milk proteins. Soy is treated to concentrate the proteins and reduce undesirable antigenic properties that can cause an allergic reaction in the intestine of the young calf. Milk replacers based upon milk proteins are recommended for calves less than 1 month of age due to their higher digestibility as compared to vegetable proteins. When vegetable proteins are utilized, it is recommended that they substitute for less than 50% of the milk proteins. The acceptability of various ingredients as sources of protein in milk replacers is shown in **Table 2**.

In the United States and most part of the world, lard and tallow are the primary sources of fat used in milk replacers. Due to concern over bovine spongiform encephalopathy (BSE), palm and coconut oil are used as a replacement for animal fats in EU countries. These oils appear to be of high digestibility and are well tolerated by the calf. Lactose is the primary carbohydrate used in milk replacers as it is of the lowest cost and is most efficiently digested by the calf.

Milk replacers are marketed as dry powders that contain 10–25% fat and 20–28% protein. In the United States, whole milk on average contains approximately 3.6% fat and 3.1% protein, which on a dry powder basis would equal 28% fat and 24% protein.

Liquid-Feeding Programs

There has been a substantial shift in the concept of liquid-feeding management of the dairy calf over the past 10 years. Traditional liquid-feeding programs for calves sought to minimize rearing expenses and encourage early development of the rumen to enable early weaning of the calf. A substantial body of work has demonstrated

Table 2 Ingredients used as sources of protein for milk replacers

Recommended	*Acceptable*	*Marginal*	*Not acceptable*
Dried whey protein concentrate	Soy protein isolate	Soy flour	Meat solubles
Dried skim milk	Protein-modified soy flour		Fish protein concentrate
Casein	Soy protein concentrate		Wheat flour
Dried whey	Animal plasma		
Dried whey product	Wheat gluten or isolate		

Bovine Alliance on Management and Nutrition Information Sheet (2008) A guide to calf milk replacers.

that feeding approximately 454 g of solids per day containing 20% protein and 10–20% fat appeared to be the best compromise between desired growth, calf performance, and encouragement of early consumption of dry calf starter grains. Under this traditional model, the calf grew modestly, especially during the first 2–3 weeks of age (<200 g day^{-1}), and began consuming dry grains by the end of the second week. Some rearing programs resulted in consumption of sufficient dry feeds to enable weaning by the end of the fourth week of life or earlier. Although it achieved a low daily feed cost, this practice ignored the influence of body size, environment, and ingredients utilized in the diet on growth and development as well as health of the preweaned calf. The concept of limit-feeding calves liquid diets is a departure from the approach of diet formulation used for older dairy animals, which considers requirements for maintenance, body size and expected growth, or other performance measures such as milk production. Rather the goal with the traditional limit-fed program in preweaned calves is to reduce nutrient intake early in life to stimulate premature intake of dry feeds and reduce cost.

Research over the past 10 years has resulted in the determination of nutrient requirements for body weight gain as well as the composition of gain in the growing calf. Using all-milk protein sources it appears that daily protein accretion in the calf plateaus at 28% crude protein (CP) on a DM basis, which is also similar to the protein content of whole milk. These studies have also shown that body fat accretion decreases with increasing protein in the diet and that the protein content of body weight gain increases with increasing protein intake of the diet. Energy intake is derived from lactose, fat, and to a lesser extent from protein. Most milk replacer diets contain 10–20% fat, considerably below that found in milk solids. Research has shown that growth rate is not appreciably influenced by differences in the proportion of energy provided as lactose or fat. However, as the fat intake increases, the accretion of body fat increases. When diets are formulated with regard to nutrient requirements for growth as well as maintenance, the amounts of solids recommended to be fed to the preweaned calf are considerably higher than 454 g day^{-1} common to traditional systems.

Nutrient requirements can be partitioned into requirements for maintenance and growth. Maintenance requirements are a function of age, body size, environmental temperature and exposure, and disease status. The thermoneutral zone for young adapted calves appears to be 15–25 °C. This means that the calf would require additional energy for maintenance when temperatures exceed this zone. Smaller, younger animals would require proportionately more energy to meet energy requirements than older, larger animals because smaller animals have proportionately more body surface area and thus lose body heat more readily. Therefore, during cold weather, both solids intake and fat content of the diet should be increased. **Table 3** demonstrates the influence of environmental temperature on maintenance requirements of calves of different body sizes. Note the increase in maintenance requirements and the amount of milk solids required to meet maintenance needs as temperature decreases.

The most recent edition of the *Nutrient Requirements of Dairy Cattle* has used a dynamic approach in determining nutrient requirements of the dairy calf that considers body size as well as temperature. **Table 4** demonstrates the influence of environment and composition of the diet upon expected growth of a 55 kg calf.

Table 4 demonstrates that limit feeding a 20:20 milk replacer during cold weather has a detrimental influence on calf growth with possible repercussions on health if body fat stores are depleted. Additional research has demonstrated that maintenance requirements of small-breed calves such as Jerseys may be at least 20% higher than the maintenance requirements of large-breed calves due to increased heat loss from the greater surface area of smaller calves as compared to larger calves. As a result,

Table 3 Influence of environmental temperature and body size on megacalories of net energy maintenance and gram of whole milk required to meet maintenance requirements of calves of different body sizes

	Environmental temperature (°C)		
Body weight (kg)	*15*	*0*	*−15*
30	1.25 Mcal 270 g	1.7 Mcal 370 g	2.14 Mcal 460 g
50	1.83 Mcal 400 g	2.49 Mcal 554 g	3.14 Mcal 800 g
70	2.35 Mcal 510 g	3.12 Mcal 690 g	4.04 Mcal 870 g

Adapted from National Research Council (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy Press.

Table 4 Expected body weight gain of 55 kg calf fed whole milk or two different milk replacers under different environmental temperatures

		20 °C		*0 °C*	
Liquid	*per day*	*Energy allowable gain*	*Protein allowable gain*	*Energy allowable gain*	*Protein allowable gain*
Whole milk	4 l	213 g	345 g	Weight loss	Weight loss
Whole milk	9 l	1110 g	990 g	852 g	990 g
20% protein:20% fat milk replacer	4 l at 12.5% DM	45 g	218 g	Weight loss	Weight loss
28% protein:20% fat milk replacer	6.7 l at 17% DM	1010 g	1150 g	739 g	1150 g

Adapted from National Research Council (2001) *Nutrient Requirements of Dairy Cattle*, 7th revised edn. Washington, DC: National Academy Press.

in milk replacer powders, fat levels of 25% may be warranted during cold stress experienced by small-breed calves.

There is a growing body of evidence that restricting calves to consumption of 454 g of milk solids per day may not be advisable. A recent study in the United States revealed that mortality from birth to weaning exceeded 8% in limit-fed calves while that of more liberally fed beef calves was less than 3%. These surveys of US dairy operations indicated that over 40% of calves were considered to be ill or unthrifty and required medication prior to weaning. A predisposition to illness could be caused by feeding calves at or below maintenance. In one study, calves fed 4 l of pasteurized waste milk daily experienced higher daily gains than calves fed a similar volume of milk replacer containing 20% CP and 20% fat reconstituted to 12.5% solids. In addition to better growth, the calves fed pasteurized waste milk experience lower levels of morbidity (12 vs. 32%) and mortality (2.3 vs. 11.6%). Differences in mortality and morbidity were more striking when comparing the influence of season; calves fed milk replacer experienced 21% mortality and 52% morbidity as compared to 2.8% mortality and 20.4% morbidity for calves fed whole milk during the winter season. Improved performance and health of calves fed pasteurized waste milk could be attributed to increased intake of protein and fat as compared to calves fed the milk replacer.

Growth rate of the calf prior to weaning may have a profound effect upon mammary development. Calves fed a milk replacer containing 28.5% protein and 15% fat for an average daily gain of 666 g had 32% more mammary parenchymal mass and 47% more parenchymal DNA than calves fed a 20% protein and 20% fat milk replacer for an average daily gain of 379 g at 8 weeks of age. Several studies have demonstrated that calves fed more liberally during the preweaning period produced more milk per day during first and later lactations than limit-fed calves receiving 454 g of milk replacer or an equivalent amount of solids from whole milk. The more liberal feeding programs have been commonly referred to as 'intensive' or 'accelerated' while 'more biologically normal' might be a better terminology as the more liberal feeding program more closely resembles what takes place when the calf is allowed to nurse the dam. Additional responses to more biologically normal feeding rates include indications for improved immune function. Insufficiencies of protein or energy are known to impair health and immune system function in other species. Nutritional insufficiency is likely to be much more problematic in smaller, younger, limit-fed calves during cold or heat stress when maintenance requirements are known to be higher.

More liberal feeding programs result in increased lean tissue growth and, when whole milk or milk replacers contain 20% or more fat, increased body fat deposition. In the young calf (less than 3 weeks of age), additional reserves of body fat may be a desirable trait. When calves become ill, intake is frequently decreased. Concurrently, an illness of infectious origin generally results in increased body temperature and increased energy requirements. If young calves are fed whole milk or milk replacers with higher protein and fat at higher intakes, the extra body fat may assist them in recovering from disease.

Practical Management of Milk-Feeding Programs

Limit Fed

There are numerous studies indicating that calves can be successfully reared when liquid feeding is limited to 4 l of milk or milk replacer containing the equivalent of 454 g of milk solids with 20% protein and 20% fat. The following management strategies increase chances for a positive outcome with this program. The environment needs to be optimal to minimize both cold and heat stress, which increase nutrient requirements for maintenance. During cold weather (<15 °C), the proportion of solids should be increased to provide additional energy and protein. Avoid the use of vegetable proteins for the first 3 weeks of life due to their lower digestibility. Outside of the EU countries, plasma proteins are an acceptable, lower cost partial substitute for milk protein. Due to the lower intake of milk solids, there is a stronger incentive for calves to consume dry starter feeds at an earlier age and there is a trend for early weaning. The greatest limitation to limit-feeding systems is that cost per pound of gain may be higher as significantly more nutrients are required for maintenance with less remaining to support growth. During cold and heat stress, there may be a higher risk of morbidity and mortality.

Intensive, Accelerated, or Biologically More Normal

Liquid-feeding programs that involve feeding 680–1150 g of milk solids (5.5–7.5 l) per day more closely resemble what takes place when the calves are allowed to nurse their dams. Consequently, their growth and feed efficiencies are more similar to other young farm animals such as piglets or lambs. It is essential that milk-derived proteins be used throughout the liquid-feeding period as a higher intake of less-digestible vegetable proteins may increase the risk of diarrhea. During the first week of life, intake of milk is generally limited to 4 l for small-breed calves and 6 l for large-breed calves. Milk replacers usually contain 26–28% CP and 16–25% fat. Higher fat levels are recommended for small-breed calves, especially during times of cold stress, which can amount to 6 months of the year when ambient temperature is less than 15 °C. Milk replacer powders are reconstituted by the addition of warm water to a final solid level of 12.5–17%. The higher level of solids enables use of 3 l nipple bottles for twice-daily feeding. Once calves reach full feed of 5.5–7.5 l day^{-1}, they are maintained at this level until weaning. The feed cost per kilogram of gain may be less with this program as nutritional requirements for maintenance comprise less of the daily nutrient intake leaving more nutrients to support body weight gain. The limitation of this feeding system is that the rearing cost per day is significantly higher. Intake of dry calf starter is delayed by several weeks, but once the calves begin consuming calf starter they generally compensate due to their larger body size at a given age as compared to limit-fed calves. Calf feeders will note that feces from more liberally fed calves are looser and should not be confused with increased diarrhea.

Calf Starters for Preweaned Dairy Calves

Calf starters should be formulated and delivered to the calf to encourage intake early in the calf's life. As they are replacing nutrients provided by the liquid diet, the ingredients should be highly palatable and digestible and encourage development of the rumen epithelium and musculature. Historically, producers believed that early consumption of hay was critical to rumen development. However, research has shown that the consumption of grains high in starch is responsible for stimulating exponential growth of rumen epithelium. Consumption of hay prior to weaning is not recommended as it is much lower in energy than grains and will reduce energy intake and possibly delay weaning. In addition, calves frequently waste large portions of the hay that is offered. Ground hay can be included in calf starter grain mixtures in limited amounts (5–10%) provided it is high in energy and protein and low in fiber. Unfortunately, difficulty in handling such ground hay and grain mixtures precludes them from being used in most commercial calf starter mixtures.

Calf starters typically contain 18–24% CP on a DM basis. The National Research Council (NRC) recommends 20% CP (DM basis) for calf starter feeds. When calves are limit-fed a 20% CP:20% fat milk replacer, little improvement is observed when higher CP% calf starters are fed. However, calves managed under a more liberal milk-feeding program using whole milk or higher protein milk replacers appear to adjust better to the diet postweaning with higher feed efficiency when a calf starter with up to 24% CP is fed. This is logical when one considers that the calf has received a higher protein liquid feed during the preweaning period. Little benefit accrues from prolonged feeding of higher-protein calf starter mixtures (>20% CP) beyond the first month after weaning.

Ingredient composition of calf starter mixtures influences palatability and digestibility. Soybean meal (48% CP) and corn appear to be the most reliable protein and energy sources. It is not uncommon to include crimped oats owing to their palatability. However, oats are lower in energy and starch content and frequently a more expensive ingredient. Various forms of corn have been used in calf starter grains. Research comparing whole, dry-rolled, roasted-rolled, and steam-flaked corn in calf starter mixtures resulted in no difference in weight gains, but feed efficiency and dry feed intake postweaning favored starter grain mixtures containing whole or dry-rolled corn. Molasses is commonly added to calf starter grains at 2–5% to improve palatability and reduce dustiness. Higher levels have been shown to reduce gain and solid feed intake. Molasses has a tendency to deteriorate during storage and causes clumping of the mixture thereby making handling of the starter difficult.

The addition of nonforage fiber sources such as beet pulp, soy hulls, wheat midds, corn gluten feed, and cottonseed hulls to calf starter diets at low inclusion rates (12–20%) is common. In most cases, feed intake is either not affected or increased only slightly. Since these feedstuffs contain less energy as corn or other high-starch grains, they commonly result in reduced feed efficiency. Improved gains when high-fiber starters are fed can be attributed to greater rumen fill and not necessarily improved lean tissue growth. If ground hay or nonforage fiber sources are included, they should be limited to 5–10% of the final mixture.

Fat is occasionally added to calf starter mixtures to reduce dustiness. Addition to calf starters at levels exceeding 3.5% of the mixture reduces intake and live weight gains.

Consistent improvement in live weight gains of calves has been observed when anticoccidial compounds such as decoquinate, lasalocid, or monensin are added to calf starter grains. They aid in controlling some types of coccidia. Lasalocid and monensin have also been shown to improve feed efficiency through an influence on rumen fermentation. The use of microbial probiotics and yeasts has resulted in benefits in some trials, but responses have been highly variable and less conclusive than with lasalocid or monensin.

Water Management

Provision of ample supplies of fresh, clean water to calves is essential to successful early consumption of calf starter and early weaning regardless of the liquid-feeding program. Water should be provided in a bucket separate from that used for feeding milk. The water bucket should be located a sufficient distance away from the calf starter bucket so that the calf cannot dampen the starter with water nor foul the water with calf starter.

Successful Weaning

Calves can usually be weaned successfully when they are consuming at least 1.5–2.0 kg of dry calf starter grain per day. Exceptions can be made during extremely cold weather when liquid feeding may be prolonged for a week or two longer. Weaning usually occurs at around 6–8 weeks of age. Calves can be encouraged to increase consumption of dry feeds in several ways. Maintaining a constant rate of liquid feeding from birth means that the liquid diet provides progressively less of their daily nutrient requirements as the calves grow larger. During the fifth or sixth week, the amount of nutrients obtained from the liquid diet can be reduced by feeding once daily, or in the case of milk replacers, maintaining liquid intake but providing half of the amount of milk powder in the same volume of water. The latter examples are commonly used and are very successful in stimulating dry calf starter intake and weaning. Calves should be maintained in their current environment until 1–2 weeks after weaning to minimize social stress. The highest quality dry hay has be to introduced 2–3 weeks after weaning.

Conclusion

Calves can be reared successfully with either limit-feeding or biologically more normal systems. However, the most important factor influencing success is obtaining adequate immunity from colostrum. This is achieved by feeding 4 l of colostrum containing at least 50 mg IgG ml^{-1} within the first 6–12 h of life. Whole milk, pasteurized waste milk, and milk replacers with higher levels of protein and energy can support gains of 500 g or more per day and promote excellent health and development. Early consumption of a digestible and palatable calf starter grain is essential regardless of the liquid-feeding program. Weaning is achieved when starter intake reaches 1.5–2 kg day^{-1}.

See also: **Husbandry of Dairy Animals**: Goat: Replacement Management; Sheep: Replacement Management.

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Growth Diets

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Introduction

Dairy heifers should be fed and managed to achieve 55% of their mature weight at first breeding and 82–85% of their mature weight at first calving. These principles enable the establishment of growth goals regardless of breed. In addition, heifers should have a body condition score after calving of 3.5 and be free of disease. Probably, no factor other than the feeding program influences achievement of these goals. The feeding program also comprises 50–70% of the cost of rearing heifers to first calving. Therefore, profitable heifer-rearing enterprises must concentrate on economical feeding programs.

The purpose of this article is not to provide a 'how to' description of heifer-feeding programs because available feed resources and facilities vary to a great extent. This article will focus on a discussion of decision-making areas with considerable importance to the financial success of the heifer-feeding programs. Managing the feeding program of the heifer enterprise is not unlike that of any other decision made on the farm. Good heifer managers maximize benefits, control expenses, and manage risk well. This article will concentrate on the major goals the heifer grower needs to achieve during the rearing period:

1. successful weaning from liquid diets to forage- and concentrate-based feeding programs with minimal stress and loss of weight, and transition to group housing management systems;
2. controlling the rate of gain during the prepubertal period to enable early breeding (12–13 months) while assuring desired mammary development;
3. sustained growth after breeding and optimization of economy of feeding; and
4. preparing the heifer for eventual calving.

The development of the feeding program should consider that approximately 50% of the total gain in height occurs during the first 6 months of life with 25% occurring from 7 to 12 months and the remaining 25% during the 12 months before calving. Feed cost is generally lowest per unit of gain during the first 6 months of life and then increases at a decreasing rate during the remaining 18 months. The proportion of body weight to wither height increases linearly and the increase in wither height as a proportion of total height is greatest during the first 6 months. This demonstrates that assuring adequate growth during the first 6 months is critical to success in growing the dairy heifer and is where nutrition and management must be optimal. Poor growth prior to puberty cannot be compensated for later in the rearing period. After puberty and when the heifer has attained early growth goals, opportunities for considerable economy of feeding exist. Profitable heifer management requires labor-efficient systems and the ability to evaluate ration dry matter intake (DMI) and animal performance. Periodic weighing of heifers with comparison to established growth goals is critical to achieving desired performance.

Heifer-feeding programs vary widely depending on the environment and available forage resources. In tropical and more temperate areas of the world, pasture-based systems are more popular because they provide nutrients at the lowest cost and promote excellent animal health. In other areas where land resources are more valuable, or the length of the grazing season is limited, confinement systems may be more conducive to economical heifer growth. In many areas of the world, a combination of systems where heifers are raised on pasture during the warmer months and moved to confinement during the winter is commonly practiced. Basic principles of nutritional management using pasture-based and confinement systems will be discussed.

Managing the Transition Calf

This stage refers to the time between 2 weeks prior to and 2 weeks after weaning. Success during this phase is dependent upon excellent housing and health programs. Regardless of the rearing system, it is assumed that the calf is consuming adequate amounts of a high-energy calf starter grain prior to weaning. When preweaned calves have been housed individually, it is recommended that calves be placed into small groups of 4–6 calves several weeks after weaning to allow them to adjust to competition for feed. However, when the environment is optimal, the author has observed newly weaned calves reared in groups of 20–30 with great success. Under this scenario, ventilation is outstanding, bedded pens are cleaned frequently, and calf starter grain is fed often to keep the feed fresh. Calves that have been fed their liquid diet in groups from mob or robotic feeders adapt to weaning quite well as there is no social stress associated with adapting to the group housing and feeding environment. Calf starter grains can vary widely, but should be highly palatable and digestible,

Table 1 Desired nutrient levels in a calf starter grain

Nutrient	*Amount recommended*
Crude protein (% of DM)	18.0–22.0
Fat (% of DM)	3.0
TDNs (% of DM)	80
Metabolizable energy (Mcal kg^{-1} DM)	3.1
Calcium (% of DM)	0.60
Phosphorus (% of DM)	0.40
Vitamin A (IU kg^{-1})	2200
Vitamin D (IU kg^{-1})	300
Vitamin E (IU kg^{-1})	24

DM, dry matter; TDNs, total digestible nutrients.

with nutrient concentrations as shown **Table 1**. Under more intensive liquid-feeding programs for calves, protein levels may be increased to 22% of dry matter (DM).

For calves that have been housed individually prior to weaning, Morrill suggests three transition pens after weaning. The first pen has 4–8 calves with 2.8 m^2 per calf and a starter the same as they had been receiving prior to weaning. Two weeks later, the calves are moved to a larger pen with more calves and with the same starter and about 15% chopped alfalfa hay added to the ration. In the next transition pen, the amount of chopped alfalfa is increased to 20% of the mixture. By the time the calves are moved to the last transition pen, they can be switched to a more economical grower concentrate mixture, with examples shown in **Table 2**.

Table 2 Examples of calf starter grain mixtures

Feed	*Grower 1*	*Grower 2*
Cracked corn	63.8	53.23
Rolled oats	9.9	
Rolled barley		20.48
Molasses	3.5	2.97
Soybean meal	20.4	
Canola meal		21.33
Limestone	1.2	1.08
Dicalcium phosphate	0.27	
Salt	0.18	0.18
Trace mineral mix	0.09	0.09
Vitamin E mix	0.09	0.09
Vitamin ADE mix	0.07	0.07
Additives	0.50	0.46

Values are percentage of total mixture on an as-fed basis.
Composition of trace mineral mixture (%): Co, 0.01; Cu, 1; Fe, 5; I, 0.06; Mn, 4; Se, 0.03; Zn, 4.
Composition of vitamin ADE mixture, per kg: A, 44 000 KIU; D, 990 KIU; E, 17 600 IU.
Vitamin E supplement contains 44 000 IU kg^{-1}.
Grower grain mixture should contain coccidiostat or other additive as desired.
Reproduced from Morrill J (1999) *Proceedings of the Third Conference of the Professional Dairy Heifer Growers Association*, p. 26. Minneapolis, MN. Savoy, IL: Professional Dairy Heifer Growers Association.

Workers at North Carolina State University have successfully developed a self-fed calf starter grain utilizing cottonseed hulls to limit intake. Calves are offered the starter from birth through 4–6 months of age. Cottonseed hulls represent a uniform, consistent source of more slowly digested fiber that helps limit intake in the older heifers. Critical to success of the system is maintaining a reliable source of clean cottonseed hulls, keeping the self-feeders clean, and providing plenty of water. Nutrient content of these starter mixtures on a DM basis is 16–18% crude protein (CP), 76% total digestible nutrients (TDNs), 0.66% calcium, and 0.42% phosphorus. The ingredient composition is 493 kg ground corn, 300 kg cottonseed hulls, 184 kg soybean meal, 9 kg calcitic limestone, 5 kg tricalcium phosphate, and 5 kg white salt, ionophore, and a vitamin–trace mineral mixture. Although apparently successful, these starters are lower in energy and require higher intakes to meet the energy requirements for acceptable growth.

Managing Growth of the Heifer from Weaning to Breeding

Once the heifer has been successfully weaned and has transitioned to group housing, control of rearing rate is of primary concern. If one assumes that a goal of age at first calving of 22–24 months is desirable, breeding should be initiated at 12–13 months. Animals must be of adequate weight and body condition to accomplish this objective. **Table 3** demonstrates the challenges faced in feeding the large-breed heifer prior to breeding. It also demonstrates the difficulty in attaining ages at first calving below 20 months. Based upon these assumptions, it is assumed that rations for the large-breed heifers should foster a gain of 750–900 g day^{-1}. Smaller breed heifers require gains of 500–650 g day^{-1}. Excessive gains may increase the risk for problems with mammary development, particularly if protein is limiting in the ration.

Pasture-based systems

In many areas of the world, the greatest forage asset is the availability of abundant, low-cost land suitable for pastures. The best example for pasture systems exists in

Table 3 ADG necessary to achieve suggested 360 kg weight at breeding and postcalving body weight of 570 kg at varying ages

Age at calving goal (months)	20	22	24
Age at breeding goal (months)	*11*	*13*	*15*
ADG birth–breeding (g)	953	800	680
ADG breeding–calving (g)	884	884	884

Assumes 39 kg birth weight, 45.4 kg loss of weight at calving, and 280 days of gestation.
ADG, average daily gain.

Ireland, New Zealand, southern Chile, and Argentina, and similar climes that have cool temperatures with frequent, moderate rainfall during a large portion of the year. Less desirable examples of grazing systems are range management systems in arid areas, which require extensive land bases exceeding 20 hectares per animal and they support little more than maintenance nutrient requirements. In many areas of the world, the role of pasture in heifer-feeding systems is probably in between these extremes. It is important to evaluate realistically the advantages and liabilities of pasture systems and determine how best to optimize their benefits and minimize the risk of their shortcomings.

The biggest challenge faced in the pasture system is reliably estimating the carrying capacity of the land. If land is to be utilized for pasture, its use during the year must be maximized to provide the best compromise of yield of animal growth and forage nutrient yield. **Figure 1** shows the effect of grazing pressure on production per animal and per acre. Note that optimal growth per head is not the same as optimal growth per acre. Optimum growth per animal occurs at a point less than optimum for land utilization. When grazing pressure is low to medium, heifers graze on only the best quality forage. When pasture output is optimized at higher stocking rates, heifers are forced to consume some of the less nutritious forage and animal performance declines. This represents a challenge for the heifer grower or dairy producer.

Extremes in stocking rate are undesirable. Long periods of low grazing pressure lead to a loss of legumes in a stand and increased growth of weeds and less desirable species. Long periods of high grazing pressure result in temporary or long-term decreases in forage production as nutrient reserves of desirable forage species are depleted and plants become less dominant in the sward. For optimum grazing, one should maintain available forage at approximately 1122–1685 kg DM per hectare. This is the equivalent of a 7–10 cm high stand of bluegrass/white clover or 15–20 cm of tall grasses and legumes.

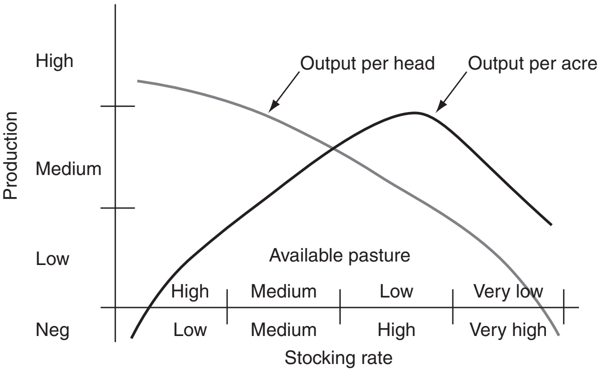

Figure 1 Effect of grazing pressure on production per animal and per acre. Reproduced from Hall MH (2009–2010) *The Agronomy Guide*. University Park, PA: Department of Crop and Soil Sciences, The Pennsylvania State University.

Continuous or rotational grazing?

Historically, continuous grazing has been the most popular grazing system since it is simple and requires little labor. Grazing pressure is adjusted by adding or subtracting animals or temporarily fencing off areas for hay harvest. However, continuous grazing is a land extensive system, and low production of gain per hectare makes it inefficient. In contrast, rotational grazing can dramatically increase animal performance and forage DM yield per hectare. In heifer feeding systems, intensive rotational grazing systems are probably not as important. Improvements in nutrient quality of forage accrued from more daily movement of fences or shifting heifers to new paddocks daily do not offset the labor and fencing expense and convenient provision of water. Many heifer growing systems include some combination of both continuous and rotational systems. A continuously grazed paddock may be used to house animals during the winter or during periods of drought to enable other areas to recover forage growth. Other paddocks are designed to enable movement of fences on less frequent interval of 3–14 days.

Supplemental nutrition of pasture systems

For many areas of the world, dairy heifers cannot be reared without significant nutrient supplementation during some portion of the year. The nutrient variation of grazed forage species represents one of the greatest challenges of grazing, particularly for animals less than 1 year of age. In the more temperate areas of the world, protein concentrations of pasture can vary from 6% to levels exceeding 20%, while the growing dairy heifer may require levels ranging from 12 to 18%. Similarly, energy values will range from those similar to corn silage to levels that more nearly resemble straw. Although heifer rearing regimes might tolerate some variation in growth, in today's economic climate it is important that heifers calve at an early age and with desired body size and condition.

When forage availability is adequate, but quality is lacking, provision of supplemental energy and protein through concentrate feeding is advised. At other times such as winter months or during severe drought, heifers require supplementation with both forages and concentrates. It is beyond the scope of this article to address adequately the supplemental nutrient needs of pasture-reared heifers.

Pasture-reared heifers require more energy than those in confinement due to their increased activity and exposure to environmental conditions, particularly during the winter. Research at Virginia Tech has shown that confinement-reared heifers require 12–25% less energy than indicated by National Research Council (NRC). They are also less influenced by severe cold and wet weather. Therefore, managers of pasture-reared heifers must make adjustments in nutritional strategies when environmental conditions are less than perfect. Factors such as cold weather, nonthermal resting areas, wind, rain, and snow increase demand for energy for maintenance thereby reducing that available for growth. Environmental conditions for heifers raised without housing may become so severe that it is not possible to maintain sufficient growth, even with substantial energy and protein supplementation. Supplementation of rations with energy must be based upon observed growth of heifers during inclement weather. Similarly, supplementation can often be reduced at considerable savings when pasture growth and environment are optimal.

The greatest challenge of pasture systems lies in the establishment of pastures that enable maximal grazing throughout the season and provisions for supplementation when pasture nutrients are insufficient to promote desired growth. Depending upon the climate and resources, successful growers utilize a mixture of swards containing cool and warm season perennial grasses and legumes.

Young calves readily adapt to pasture systems as they provide ample opportunity for exercise, excellent air quality, and when pasture growth is young and rapid, a plentiful supply of energy, protein, mineral, and vitamins. However, it is important to note that calves weighing less than 150 kg require nutrients at least as high as that for the lactating cow (>16% CP and >2 Mcal of metabolizable energy (ME) per kg of DMI). An additional consideration for younger calves is their susceptibility to parasitism. Aggressive parasite control programs are recommended because the young animals have not developed sufficient immunity more common in animals in their second grazing season. By 300 kg body weight, concentration of nutrients is less important as adequate intake (7 kg DM per day) of forage providing 12% protein and 2 Mcal ME per kg DMI is adequate for 700 g of gain per day. It is uncommon to formulate rations for grazing dairy heifers. Rather the manager assesses pasture quality and availability, and heifer growth to determine if supplemental nutrition is needed.

Confinement rearing systems

Young heifers, less than 150 kg body weight, require diets of high forage quality to enhance rumen function and promote economical growth. Forages must be free of mold and spoilage to ensure adequate ration intake. Fermented feeds and a wide variety of by-product feeds are readily accepted by heifers more than 150 kg body weight. Forage quality, as defined by nutrient content, becomes less important for the heifer over 150 kg body weight as intake is usually not the limiting factor in nutrition. Professional heifer growers have been especially aggressive in seeking ways to provide nutrients at the lowest possible costs. This strategy requires the grower to 'think outside the box' when it comes to selecting ration components. Rations are presented to demonstrate the possibilities for utilization of by-product feeds. They are based upon those used by several large heifer growers in Colorado and Texas as well as conventional rations used in heifer feeding trials at Virginia Tech.

Each ration was evaluated using the Cornell Penn Minor (CPM) program to determine expected gains based on ME and metabolizable protein (MP). Ration I relied heavily on by-products from vegetable processing, wet brewer grains, and low-cost alfalfa silage, which were of insufficient quality for lactating dairy cattle or export. Frequent weighing of heifers revealed growth of 800 g day^{-1}, while the CPM model indicated that ME supplied by the ration should provide for only 690 g of gain per day and sufficient MP for 1.17 kg of gain per day. Ration II used an exceptional array of by-product feeds. Alfalfa was of lower quality, as was the whole cottonseed. Outdated dairy products (ice cream, cottage cheese, yogurt, and other products) were also used as available in this ration. In comparison to ration I, this ration contained an abundance of protein of a very degradable nature. Sufficient ME and MP were present to support gains in excess of 1 kg day^{-1}, which supported observations on the feedlot of rapid gains, and heavy body condition. Ration III represents the traditional ration fed to dairy heifers in Virginia. MP and ME were present in sufficient amounts to support daily gains in excess of 900 g. These rations demonstrate the ability of heifers to grow at rates that support early calving at recommended body sizes at very low ration costs. The greatest limitation involved in the successful use of by-product feeds is personal prejudices and preconceived ideas of what will be successful. Once it has been determined that by-products contain no harmful substances and that product quality is predictable, many by-products serve as economical ingredients for heifer rations (**Table 4**).

Feeding the Breeding Age Heifer and Bred Heifer

Growers should not need to increase nutrient levels for the breeding age heifer, since she should already be on a high plane of nutrition promoting 750–900 g of average daily gain (ADG) for large-breed heifers or 500–650 g of ADG for small-breed heifers. Once the heifer is bred, it is important to maintain these growth rates, although it is possible to tolerate some variation as long as goals for growth are attained at calving. As shown earlier, attaining a breeding weight of 363 kg at 13 months of age requires an ADG exceeding 800 g. However, attaining

Table 4 Example rations for growing a 225 kg heifer at an average daily gain of 815 g

	kg DM	*CP (%)*	*RUP (% of CP)*	*TDNs (%)*	*ME (Mcal lb^{-1})*	*NDF (%)*	*Cost (\$ day^{-1})*
Ration I	6.0	11.2	45.4	66	2.2	46	0.72
Ground wheat straw, 1.45 kg; wet brewers' grain, 2.9 kg; carrots, 1.8 kg; wet beet pulp, 1.8 kg; corn screenings, 1.1 kg; alfalfa silage, 3.6 kg; Rumensin™ was included in the mix.							
Ration II	5.85	17.2	29.4	68	2.6	35.91	74
Cotton gin trash, 730 g; rolled corn, 1.27 kg; distillers' grains, 454 g; whole cottonseed, 363 g; alfalfa hay, 1 kg; wheat midds, 1.63 kg; cottonseed meal, 363 g; sorghum silage, 1.27 kg; waste dairy products, 1.82 kg.							
Ration III	13.6	12.0	33.9	67	2.44	41.3	90
Corn silage, 6.82 kg; soybean meal, 454 g; ground shelled corn, 1.6 kg; orchard grass hay, 2.27 kg.							

All rations were fed as total mixed rations for *ad libitum* intake, with prevailing feed prices as of August 2009.
DM, dry matter; CP, crude protein; RUP, rumen-undegradable protein; TDNs, total digestible nutrients; ME, metabolizable energy; NDF, neutral detergent fiber.

the postcalving weight of 570 kg requires heifers to continue to gain 750–900 g day^{-1}. Opportunities to utilize by-product feeds in total mixed rations continue. These heifers can tolerate variations in gain, and rapid compensatory growth prior to calving is well tolerated provided that heifers do not become overconditioned.

Feeding Management Considerations

Grouping heifers is a challenging issue to resolve, as it is a compromise between facilities, labor, and nutrient efficiency. Heifers should be placed in as many groups as is efficient from a labor standpoint. Suggested grouping are transition heifers, 4–8 months, 9–12 months, breeding-age heifers, and pregnant heifers. If possible, place heifers in groups within a range of 50 kg. Group heifers by size and body condition, paying close attention to note heifers significantly older within a group that may need to be culled. When multiple breeds are present in a herd, it is important to remember that smaller breeds mature more rapidly than larger breeds. If heifers are grouped by size, smaller breed heifers such as Jerseys will frequently become overconditioned. It is recommended that Jerseys and other earlier-maturing breeds are housed with slightly larger and older large-breed heifers. Provision of sufficient feed bunk space is an important consideration in such situations.

Factors influencing growth and feed efficiency of dairy heifers

A common misconception regarding dairy heifer nutrition is that published nutrient requirements provide sufficient nutrients to assure the stated rate of gain under a wide variety of environments. One must remember that these recommendations are based on the assumption that replacement heifers are clean, dry, fed *ad libitum*, free of disease and parasites, unbred, and raised at moderate temperatures. A survey of Wisconsin dairy herds showed that much of the variation in gains could be attributed to environment rather than feeding programs. Net energy maintenance requirements were 12–24% higher for fall/spring and winter as compared to summer. Failure to adjust for these added nutrient needs could decrease ADG by 90–180 g or more. Cold stress is especially problematic for smaller heifers or when the animal has lost insulating capacity of its coat due to excess mud or moisture.

Temperature has an influence on DMI. However, it was found that although temperature had a statistically significant influence on intake, it is of less importance than for lactating dairy cattle. In heifers, intake does not increase appreciably unless the temperature is less than –10 °C for more than several days. Likewise, heifers are not as prone to experiencing a meaningful depression in daily DMI during hot weather as they delay eating during the day and consume the majority of their ration during the cooler hours of the evening.

Housing type has a strong influence on growth and feed efficiency. Heifers housed in well-designed confinement systems are not subjected to wind, rain, snow, or solar radiation. Nutrient expenditures for exercise are also reduced compared to pasture or more open housing systems. Several studies at Virginia Tech conducted in a counter-sloped heifer barn have demonstrated that heifers reared in housing systems with a resting area of ~4.2 m^2 per head had 10–20% higher feed efficiency than expected according to published nutrient requirements. This is attributed to lower maintenance costs and less exercise. Similarly, housing can have a dramatic influence on animal performance in heifers changing from confinement systems to systems that are more extensive, such as might happen when heifers reared in confinement during the winter are moved to a pasture system. Research at Virginia Tech has shown that when Holstein heifers reared in a counter-sloped heifer barn with 4.2 m^2 or less per heifer were moved to a pasture system, they lost between 500 and 1000 g day^{-1} for the first 30 days. This was primarily due to increased activity of the heifers. This experience has demonstrated the need for transition housing under these circumstances and the need for substantial increases in energy in the diet during the transition.

The latest version of *Nutrient Requirements of Dairy Cattle* (2001) has included adjustments for environmental conditions in its estimates of nutrient requirements for growth. In addition to expected growth, the user is requested to enter estimates for previous temperature, coat condition, hair depth, and evidence of heat stress and nighttime cooling. These factors are considered when estimating maintenance requirements for the growing heifer and represent a significant improvement. In addition, the program may be used for grazing animals as it includes distance animals are expected to walk and the topography of the land being grazed in estimating energy requirements.

Unlike the high-producing dairy cow, the nutrient requirements of the growing heifer can be met at less than the animal's intake capacity. Recent research has demonstrated that feeding dairy heifers at less than *ad libitum* intake results in reduced fecal output and improvements in feed efficiency. Diets are formulated to provide adequate nutrients for the desired rate of gain at 80–90% of *ad libitum* intake using higher-quality forages and/or more-concentrate-type ingredients. Since these diets are typically consumed within a relatively short period of time, the limit-fed systems require that feeding facilities have sufficient feed bunk space for all animals to eat simultaneously and that the bedding used is not edible. In contrast, the advantages of balancing rations for *ad libitum* intake are that less expensive by-products and high-fiber feeds can be utilized to reduce ration cost. Growers and producers believe that it also encourages body development, but research has not confirmed this anecdotal observation. The economic advantages of either system depend upon ingredient costs and existence of facilities with sufficient feed bunk space. A significant negative side effect of limit feeding is that heifers become bored quickly and will readily consume fences and housing facilities if they are constructed of wood.

Probably one of the most important components of the heifer-feeding program is the implementation of a system to weigh and measure heifers on a routine basis. For the lactating herd, the dairy herd improvement (DHI) program has provided a valuable decision-making tool for herd management. Similarly, heifer weights and heights are essential to successful heifer growing systems. Scales should be electronic with facilities to enable weighing animals easily with minimal stress to the animal or grower. Such management information is necessary if the grower is to respond in a timely manner to the environmental and health-related factors that might impair heifer growth or lead to overfattening.

An excellent example of the effectiveness of routine body weight monitoring is the management system of the New Zealand Grazing Company that has contract raised over 300 000 heifers on pasture-grazing systems. All heifers are weighed (monthly up to 10 months of age and subsequently bimonthly) by a technician using electronic scales. Using an internet management system, the company is able to analyze the performance of each heifer compared to predetermined benchmarks, which means that the data are quickly translated into management information for the grower and enables a meaningful report to the owner of the heifers. This has enabled the New Zealand Grazing Company to guarantee performance of heifers and build a business that raises 5000–10 000 heifers annually. By 2009, the New Zealand dairy industry has widely accepted the principle of regular monitoring and reporting heifer growth performance, and consequently most dairy farmers outsource their dairy replacement growing allowing increased profitability from their dairy herd.

Feeding programs for heifers must first achieve the ultimate goal of providing an animal capable of expressing her genetic potential at a reasonable age. Current research indicates that this is somewhere between 22 and 24 months of age and a body weight of 550 kg after calving for Holsteins and 350 kg after calving for Jerseys. Future research may yield ways in which age at calving may be reduced without significant risk to mammary development. At the present time, average ages of first calving below 22 months are not advisable for large-breed heifers. The second requirement for success involves aggressively seeking out low-cost ingredients, which will enable attainment of growth goals. Profitable heifer-growing operations will thrive in locations adjacent to sources of by-products or low-cost pasture, which will enable economical feeding programs. The third requirement for success involves monitoring body weights of the growing heifers. Facilities must be incorporated into heifer management system that enable weighing and measuring animals.

Conclusion

Heifer feeding management requires a different mindset than feeding cows. Heifer performance is not monitored well enough and we are not sure of the effects of heifer management decisions on the heifer's ability to lactate.

1. The importance of forage quality declines in importance as the heifer ages. Significant opportunities for economy exist for growers willing to consider unusual by-product feeds and forages of insufficient quality to use in the lactating herd rations.
2. Transition to group housing requires well-designed facilities that permit easy accommodation of outliers from the average.
3. Control of the rate of gain from weaning to onset of puberty is critical. Too much energy and too rapid a rate of gain enhance the onset of puberty, but at the risk of impaired mammary development. Increasing protein avoids overfattening to a point, but still may not result in normal udder growth. Too little energy and protein or poor environmental conditions reduce gains and delay breeding and calving, resulting in significant increases in rearing costs.
4. Bred heifers offer significant opportunities for economizing feeding as nutrient density of rations is more moderate.
5. Feeding systems should be labor efficient, enable monitoring of intake and growth of heifers, and permit documentation of rearing expenses.

See also: **Replacement Management in Cattle:** Breeding Standards and Pregnancy Management; Growth Standards and Nutrient Requirements; Health Management; Pre-Ruminant Diets and Weaning Practices.

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Breeding Standards and Pregnancy Management

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Value of Replacements

Heifers give birth to approximately 25–33% of all calves born on a dairy. This percentage can be increased by applying sex-biased semen from a number of superior sires, although conception rates are generally low, reaching about 60–80% of that achieved with conventional semen. Consequently, when using superior proven sires, heifers should represent the most advanced genetics in the herd. The genetic merit of artificial insemination (AI)-sired calves from heifers should be superior to that of AI-sired calves from older cows. Rearing and breeding of replacement heifers is critical to survival of the dairy farm because it represents 15–20% of total farm costs. Age at first calving is the single most important variable influencing the costs of raising heifers. Age at first calving could be defined as total days on feed since birth and is a function of the rate at which breeding weight (age) and conception are achieved. Once pregnancy is established, total days on feed become fixed. Costs associated with age at first calving include feed, labor, housing, interest on investment, breeding and veterinary health, and death loss. To reduce the costs associated with rearing heifers, one must reduce age at first calving or reduce feed costs because they represent approximately 60% of the total rearing costs. Reducing age at first calving is more easily achieved than saving on low-cost feeds given the lack of universal availability of inexpensive feeds to most producers. In a recent survey, the average cost to raise a home-grown heifer was US$100 more than that required to raise the heifer on a custom heifer-rearing operation.

Age at First Calving

Lifetime milk yield, 305-day lactation yields, and lifetime profit of replacement heifers are maximized when heifers calve for the first time between 23 and 24 months of age. An evaluation of 6 million US dairy cow records from 1960 to 1982, however, found no appreciable change in calving age for any of six dairy breeds. Mean ages (months) at first calving for 1960 and 1982 were as follows: Ayrshire, 28.4, 28.6; Brown Swiss, 28.2, 27.8; Guernsey, 27.6, 27.4; Holstein, 27.3, 27.8; Milking Shorthorn, 27.7, 27.8; and Jersey, 26.0, 25.9 months, respectively. Since 1980, however, age at first calving has decreased to current mean age ranging from 24 months for Jerseys to 28 months for Ayrshires, with the other dairy breeds falling in between these extremes. Holsteins had the smallest standard deviations for age at first calving (21.2–24.8 months) of any breed.

Puberty in heifers is dependent on many factors including, but not limited to, breed, age, and body weight. Nonetheless, age at puberty is generally not considered to be a limiting factor for age at first conception and thus age at first calving. Most dairy breeds achieve puberty by 11–12 months of age or sooner as long as they are fed according to the minimum standards suggested by the National Research Council for energy, protein, minerals, and vitamins.

Heifers less than 1 year of age should be fed to maximize growth without achieving excess body condition. Increased nutrient intake and average daily gain from 4 to 10 months of age improved feed efficiency and increased structural growth rates with a small increase in body condition of heifers by 10 months of age.

Feeding a high-energy diet for a short duration (3 or 6 weeks beginning at 11 weeks of age) altered body growth and fat deposition in a time-dependent linear manner consistent with feeding a high-energy diet for a long duration (12 weeks).

Feeding prepubertal heifers a high-energy diet for a longer duration resulted in a linear decrease in both the percentage of mammary epithelial cells that were proliferating and the mass of fat-free mammary parenchyma per unit of carcass. High-energy feeding and excessive prepubertal body gain hastened puberty and reduced the first and later lactation performance attributable to decreased mammary epithelial cell proliferation in areas of active ductal expansion.

Because feeding heifers a high-energy diet will likely reduce mammary parenchymal mass at puberty, controlling the rate of body weight gain is likely a key to reducing mammary tissue loss resulting from excess body condition. Feeding dairy heifers a high-concentrate (75%) diet (beginning at 125 kg of body weight and continued for 245 days) did not affect most structural growth characteristics and puberty attainment, and equaled or improved 150-day milk and milk component yield after calving compared with heifers fed a high-forage (75%) diet as long as both diets were fed for equal average daily gains. Little biological rationale exists opposing the use of high-concentrate rations for dairy heifers, provided the daily gain is controlled and feed ingredients can be used to maintain a healthy rumen environment. Heifers on pasture have increased maintenance requirements, and depending on the nutrient quality of the pasture, less pasture nutrients may be available to support growth. If heifers have adequate high-quality forage, supplementing concentrates may not be necessary, particularly in pasture-based dairy systems. Therefore, monitoring and supplementing diets of pastured heifers before anticipated breeding occurs may prevent their underdevelopment before first breeding.

Attempts to reduce age at first calving much less than the recommended 23–24 months should be avoided. Unless grown adequately, heifers calving at younger ages (<22 months) are more likely to experience dystocia and are subsequently 3–4 times more likely to have a retained placenta, metritis, reduced reproductive efficiency, and are likely to be culled from the herd. In addition, first lactation milk yields may be compromised.

Reproductive Cycle and Breeding Standards

Research indicates that breeding for milk yield is more important than breeding for size because genes that control body size seem to be independent of those for milk yield. Dairy heifers reach puberty as indicated by the regular occurrence of estrus. The period of estrus and a new (or first) estrous cycle usually begins when heifers first stand to be mounted from the rear by another heifer. This period is about 10–18 h in duration and begins each new estrous cycle (day 0 of the cycle). About 90% of cycling heifers show a slightly bloody discharge (bloody tail or metestrual bleeding) from the vulva 1–2 days after estrus whether or not they were inseminated or conceived. This bloody discharge is a sign that they were in estrus.

The estrous cycle is about 21 days in duration and normally ranges from 18 to 24 days. The cycle consists of four stages: estrus (estrogen is the dominant hormone and initiates mating behavior), metestrus (time of ovulation and early corpus luteum development), diestrus (progesterone is the dominant hormone as the corpus luteum grows and matures), and proestrus (decreasing progesterone, increasing estrogen, and final follicular maturation). Cycles shorter than 18 days may occur in heifers after they experience their first estrus at puberty. The estrous cycle is cyclical because in the absence of fertile mating or AI during estrus, estrus will recur in approximately 3 weeks.

During normal estrous cycles in heifers, follicles grow in either two or three wave-like patterns, with the majority of heifers exhibiting three waves (**Figure 1**). Follicular waves are induced by increased follicle-stimulating hormone (FSH) secretion. The largest or dominant follicle of the third wave generally matures during proestrus because of increased pulse secretion of luteinizing hormone (LH) and secretes estrogen to induce estrus. A preovulatory surge of LH secretion then causes ovulation. Ovulation occurs approximately 24–32 h after the beginning of estrus and subsequently the oocyte (egg) is released into the oviduct. At the site of ovulation, the ruptured follicle transforms into a corpus luteum. The corpus luteum produces progesterone necessary to prepare the uterus for a potential pregnancy. In the absence of a viable conceptus about days 16–17 of the 21-day cycle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is secreted by the uterus to cause death or regression of the corpus luteum (known

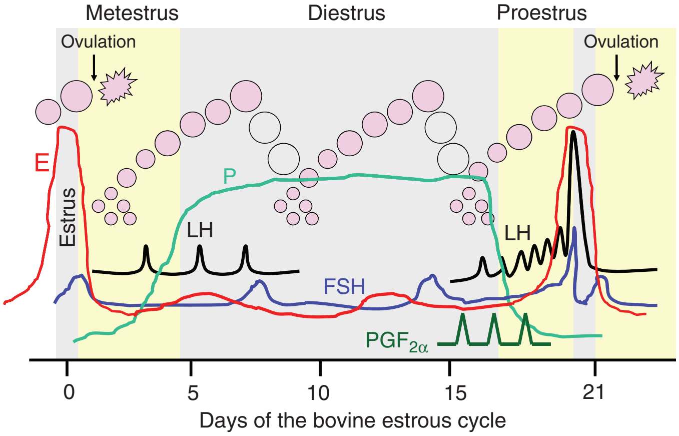

Figure 1 Characteristics of the bovine estrous cycle. Four stages of the cycle are illustrated (estrus, metestrus, diestrus, and proestrus) in addition to various hormonal changes that occur. Three follicular waves are illustrated (repeating patterns of circles) with the third wave producing the ovulatory follicle. Large dominant follicles of the first two waves become atretic and never ovulate during a normal cycle. E, estradiol or estrogen; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P, progesterone; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

as luteolysis). Diestrus ends as luteolysis is initiated and proestrus begins. Otherwise, the conceptus secretes a pregnancy signal (interferon-τ) to preserve the corpus luteum to allow pregnancy to continue. As the corpus luteum regresses in the absence of pregnancy, the dominant follicle continues to mature and will ovulate just after the heifer goes out of estrus, producing the egg that potentially will form a new conceptus upon fertilization. Thus, the cyclic nature of the estrous cycle continues, only to be interrupted by pregnancy.

The recommended age to begin a breeding program with heifers is about 12–14 months, provided the heifers are adequately grown and cycling. Growth rates are important to reach targeted body weights and frame sizes (skeletal growth measured by wither height) by breeding age as well as expected calving at 22–24 months of age (**Table 1**). Breeding body weight as a percentage of first postcalving body weight should be in the range of 60–65% for most breeds. Hence recommended median body weights and median wither heights at first insemination of replacement heifers are as follows: Ayrshire (318–340 kg and 117–122 cm); Brown Swiss (340–363 kg and 122–130 cm); Guernsey (318–340 kg and 117–124 cm); Holstein (340–363 kg and 122–127 cm); Jersey (238–261 kg and 109–114 cm); and Milking Shorthorn (340–363 kg and 117–122 cm), respectively. If heifers are inseminated too young or before adequate growth occurs, their first lactation yields will be compromised.

Furthermore, overconditioned (fat) heifers do not reproduce well and will not produce milk to their genetic potential. Waiting to inseminate heifers when older than 14–15 months negatively affects their lifetime milk production. It is important that heifers calve at or near 2 years of age, which reduces their rearing costs and also results in milk production at an earlier age.

Proper feeding management for adequate growth is necessary to ensure puberty has occurred before breeding age. Adequate growth is even more critical for seasonal breeding systems because the window of opportunity for breeding and hence timely calving is limited. It is needless to say that growth rates of heifers on pasture should be similar to those raised in confinement. Thus, raising cattle on pasture necessitates management decisions about grazing and forage systems and supplementation regimens to support proper growth of dairy heifers.

Collectively, in addition to age, body weight of heifers at breeding and immediately before or after calving plays a role in their subsequent lactational performance. Thus, age at first calving is considered to be less than 24 months and postcalving body weights of at least 82% of mature weight is recommended for all breeds. In seasonal breeding dairy systems, calving at the beginning of the calving season is more critical to survivability and economic return than age at first calving.

Management of Breeding

Age at first breeding and at first calving may be managed more precisely through the combined management of estrous cycle before AI. Well-managed heifers exhibit greater conception rates than lactating cows, resulting in lesser costs per pregnancy generated and per replacement heifer produced. Therefore, the most effective management strategy to increase genetic progress and maximize profitability on a dairy is to use estrus or ovulation synchronization before AI. This is particularly critical in seasonal, pasture-based dairy systems that require heifers to calve at the beginning of the herd-calving season when forage supplies are optimal. Seasonal breeding requires an efficient and effective use of labor and other resources

Table 1 Recommended median body weights (kg) for dairy heifers by age (months) and by breed

	Breed					
Age	*Aryshire*	*Brown Swiss*	*Guernsey*	*Holstein*	*Jersey*	*Milking Shorthorn*
2	90	85	70	85	55	90
4	130	120	120	125	100	135
6	165	195	170	175	125	180
8	210	250	210	220	160	225
10	225	310	260	265	195	275
12	290	340	295	310	220	320
14	330	380	335	350	250	370
16	375	450	385	395	280	410
18	410	465	410	445	305	450
20	440	495	445	475	330	490
22	485	550	485	515	350	520
24	510	560	500	530	370	545

Adapted from Heinrichs J and Lammers B (1998) *Monitoring Dairy Heifer Growth*. Accessed http://das.psu.edu/dairy/pdf-dairy/ud006.pdf.

related to detection of estrus, breeding program, and calving at specific times of the year.

Before 1980, a few or no hormonal products were available to synchronize estrus and ovulation in heifers. Therefore, breeding of heifers entirely depended on visually detecting estrus before AI. Today, various products include orally active (feed additive) or intravaginally placed progestins, gonadotropin-releasing hormone (GnRH), and $PGF_{2\alpha}$. Managing the estrous cycle to the convenience of the breeder is now possible even in large heifer developer operations where replacements are raised on contract for individual dairy producers or are raised for sale to other producers.

Progestins

Feeding melengestrol acetate (MGA: 0.5 mg per heifer per day) for 14 days synchronizes estrus (see (1) in **Figure 2**). Depending on the stage of the estrous cycle in which any heifer begins the MGA feeding period, a few may have a functional corpus luteum after 14 days of feeding. Most heifers show estrus within 2–6 days after withdrawing MGA from the feed. This estrus is quite infertile in those heifers that began MGA feeding after day 10 of the cycle. Because the identity of the less fertile heifers is unknown, this first estrus after MGA withdrawal is passed over and heifers are given an injection of $PGF_{2\alpha}$ 17–19 days after MGA withdrawal. Insemination of heifers based on detected estrus usually occurs between 2 and 5 days after $PGF_{2\alpha}$ administration. It is possible to time inseminate any noninseminated heifers at 72 h after $PGF_{2\alpha}$ administration but conception rates will be approximately 60–75% of those achieved based on observed estrus.

Progesterone Inserts

Insertion of a progesterone-impregnated intravaginal insert (progesterone-releasing intravaginal device (PRID) or controlled internal drug release (CIDR) insert) in addition to $PGF_{2\alpha}$ effectively synchronizes estrus in a short-term, 5- or 7-day period (see (2) in **Figure 2**). $PGF_{2\alpha}$ lyses any functional corpus luteum when injected at removal of the insert. Generally, inseminations occur after detected estrus during a 2- to 5-day period after its removal.

In an attempt to further control estrus and ovulation, ovulation can be synchronized by inducing synchronous emergence of a new follicular wave in the presence of high blood concentrations of progesterone. This method is similar to the previous method (2); however, the progesterone insert is placed intravaginally in conjunction with a first injection of GnRH, and $PGF_{2\alpha}$ is injected with removal of the insert 5 or 7 days later. If timed AI (TAI) is desired, a second injection of GnRH should be administered 48–56 h after $PGF_{2\alpha}$ injection and insert removal, with AI occurring 12–20 h later.

Prostaglandin $F_{2\alpha}$

A simpler and less expensive method (see (3) in **Figure 2**) includes detection of estrus during 6 days and then inseminating any estrual heifers according to the signs of estrus. On the seventh day, $PGF_{2\alpha}$ is injected into any noninseminated heifer to induce luteolysis and estrus for subsequent insemination. The success of this method depends on the accuracy and efficiency of visual detection of estrus.

A more complicated method involves administering two injections of $PGF_{2\alpha}$ 14 days apart. One can inseminate only estrus-detected heifers after the second of two injections (see (4) in **Figure 2**) or inseminate after both injections (see (5) in **Figure 2**) and reduce the number of second injections to all noninseminated heifers. Timing of inseminations without regard to detected estrus at 72–80 h after $PGF_{2\alpha}$ produces lesser conception rates than those made after detected estrus.

Prostaglandin $F_{2\alpha}$ + Gonadotropin-Releasing Hormone

Another technique (see (6) in **Figure 2**) combines injection of GnRH to induce the release of FSH and LH plus injection of $PGF_{2\alpha}$ 7 days later followed by visual detection of estrus and AI. The GnRH injection in some heifers better controls follicular development and synchronizes it with luteolysis that follows $PGF_{2\alpha}$. About 10% of heifers show estrus within 24 h of $PGF_{2\alpha}$, and therefore, for optimal results, detection of estrus should begin 24–48 h before $PGF_{2\alpha}$.

An alternative (see (7) in **Figure 2**) to the previous method allows for a single TAI after the injection of $PGF_{2\alpha}$. This protocol (i.e., Ovsynch) synchronizes ovulation (rather than estrus), thereby allowing for AI at a fixed time without detection of estrus. One gives a second injection of GnRH to all heifers at about 48–56 h after $PGF_{2\alpha}$ and then inseminates about 12–20 h later without regard to detected estrus. Of course, if estrus is observed before $PGF_{2\alpha}$ or the second GnRH injection, one may inseminate the heifer based on visual signs and discontinue the remainder of the injections.

It is apparent that in all systematic breeding programs, the conception rate at first AI will not reach 100%. First-service conception rates should range from 50 to 70% in heifers. Therefore, some heifers will need additional inseminations in order to become

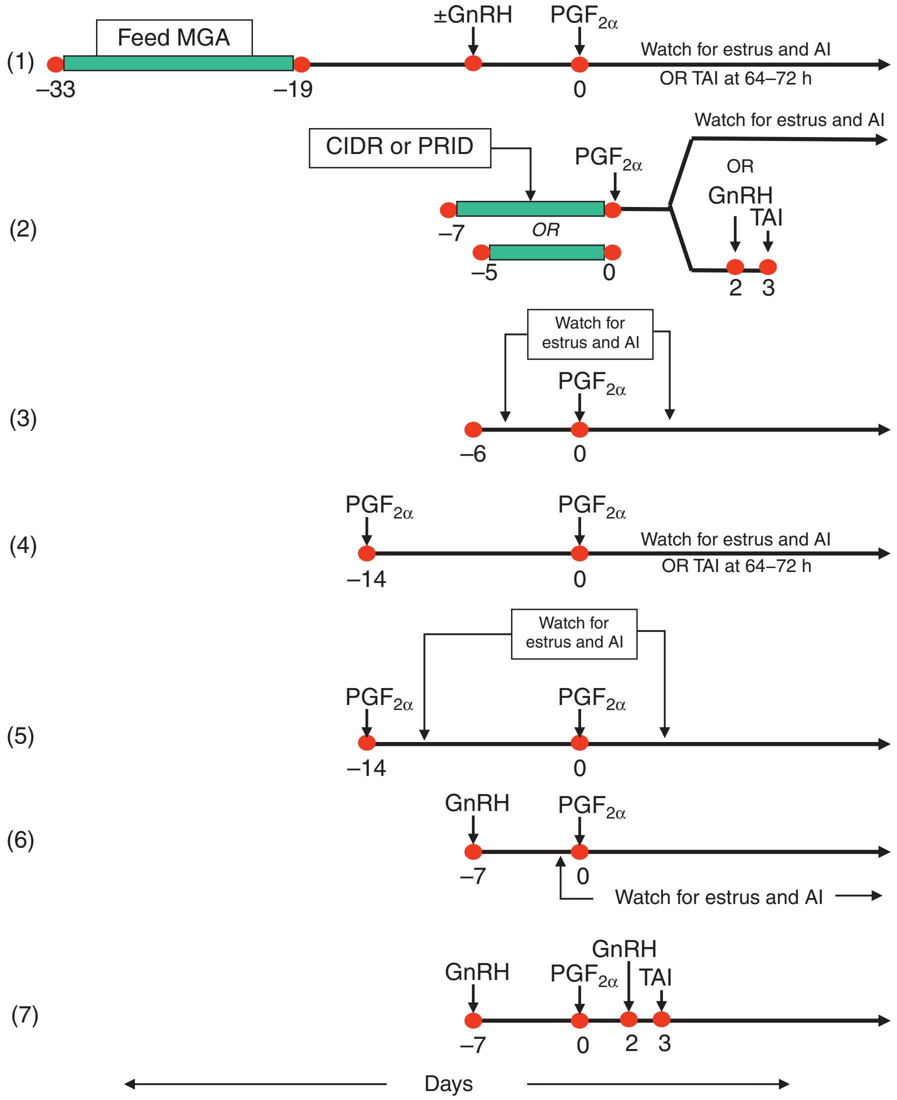
(1)
Feed MGA
±GnRH
$PGF_{2\alpha}$
Watch for estrus and AI
OR TAI at 64–72 h
–33
–19
0
(2)
CIDR or PRID
$PGF_{2\alpha}$
Watch for estrus and AI
OR
GnRH
TAI
–7
OR
–5
0
2
3
(3)
Watch for estrus and AI
$PGF_{2\alpha}$
–6
0
(4)
$PGF_{2\alpha}$
$PGF_{2\alpha}$
Watch for estrus and AI
OR TAI at 64–72 h
–14
0
(5)
Watch for estrus and AI
$PGF_{2\alpha}$
$PGF_{2\alpha}$
–14
0
(6)
GnRH
$PGF_{2\alpha}$
–7
0
Watch for estrus and AI
(7)
GnRH
GnRH
$PGF_{2\alpha}$
TAI
–7
0
2
3
Days

Figure 2 Seven programs for synchronization of estrus or ovulation are illustrated for dairy heifer replacements. (1) Feeding of melengestrol acetate (MGA) for 14 days and passing over the estrus expressed upon MGA withdrawal followed by an injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) given 17–19 days after MGA. AI, artificial insemination; GnRH, gonadotropin-releasing hormone; TAI, timed AI. (2) Intravaginal insertion of a progesterone-releasing insert (progesterone-releasing intravaginal device (PRID) or controlled internal drug release (CIDR) insert) for 5 or 7 days with $PGF_{2\alpha}$ injection administered at insert removal; or injection of GnRH at insert placement, injection of $PGF_{2\alpha}$ at insert removal, followed by second injection of GnRH given 48–56 h after $PGF_{2\alpha}$ with one TAI 12–20 h later. (3) Visual detection of estrus for 6 days before injecting all noninseminated heifers with $PGF_{2\alpha}$ on the seventh day. (4) Two injections of $PGF_{2\alpha}$ given 14 days apart with inseminations occurring after the second injection or (5) inseminate after either injection. (6) An injection of GnRH 7 days before an injection of $PGF_{2\alpha}$. (7) Same as (6) but a second injection of GnRH is given 48–56 h after $PGF_{2\alpha}$ with one TAI 12–20 h later.

pregnant. Producers should pay close attention 18–24 days after AI to detect heifers that return to estrus. All of these breeding programs only synchronize estrus or ovulation for the first AI. Subsequent estrous periods, however, are fairly well synchronized in those heifers that fail to conceive to the first AI. Estrus detection aids such as tail chalk or tail paint, heat mount detectors, or more sophisticated electronic devices can be used to detect estrus before insemination. Because heifers tend to display very pronounced signs of estrus, they can be easily detected by consistent twice-daily visual observations. About 5% of heifers eventually fail to conceive for various reasons and must be culled.

Early detection of pregnancy allows identification of those heifers that are not pregnant so that prompt reinsemination can occur. Pregnancy can be accurately determined by transrectal ultrasonography as early as day 28 after insemination or by transrectal palpation of the uterine contents by days 35–40. The other available tool is a blood test to measure the presence of a pregnancy-specific protein (pregnancy-specific protein B or other pregnancy-associated glycoproteins (PAGs)). These PAGs are produced by the conceptus and can accurately determine pregnancy status as early as 18–30 days after breeding. The objective of this diagnosis is to find nonpregnant heifers so that they can be treated promptly to induce a new fertile estrus. Treatments utilized on nonpregnant heifers can include any of those short-term hormonal methods described previously. Historically, when transrectal palpation occurs, the heifer is given an injection of $PGF_{2\alpha}$ when a functional corpus luteum is palpated and subsequent behavior is monitored for visual signs of estrus. When synchronization of estrus is performed before first AI, the day of estrus is generally known when palpation occurs. If palpation occurs at 35 days after AI, one is generally correct in assuming that an estrus was not detected at 20–21 days post-AI; hence the heifer is on cycle days 14–15 when pregnancy diagnosis occurs. This is an ideal time to give $PGF_{2\alpha}$ to induce a fertile estrus. Given similar costs of palpation and a $PGF_{2\alpha}$ injection, any nonpregnant heifer is generally given $PGF_{2\alpha}$ and observed for subsequent signs of estrus. Injections of $PGF_{2\alpha}$ cause abortions in pregnant heifers, so caution is warranted.

Management of Pregnancy

Once pregnant, replacements should not be forgotten and allocated to pasture or other areas without observation. Some embryonic and fetal losses occur after conception. It is recommended to reconfirm pregnancy by 90–100 days of pregnancy to preclude maintaining an open replacement until she is found not pregnant at her projected calving date.

Pregnant heifers should continue to grow at rates recommended by the National Research Council to achieve adequate body weight and size by calving time. Depending on the breed, ration formulations to attain daily gains of 0.50–0.80 kg are usually adequate to achieve desired first-calving weights.

Regardless of when first breeding begins in heifers, once pregnant, heifers should be fed to calve in good body condition. Without adequate body reserves of fat, it becomes very difficult to achieve good first-lactation milk yields. Which heifers will use body reserves to maximize milk yield is difficult to predict. Those that consume greater amounts of dry matter tend to be those that produce more milk.

Precautions should be taken to prevent disease and injury to gestating heifers. Adequate shade during hot months of the year prevents low birth weights and subsequent poor milk yield and reproductive performance after calving. Furthermore, adequate shelter to eliminate windchill during cold months minimizes frostbite to teats when udder and tissue edema occur during late gestation.

Immunizations before first breeding and again between 50 and 60 days before expected parturition for prevention of infectious bovine rhinotracheitis (IBR), parainfluenza (PI_3), bovine viral diarrhea (BVD), bovine respiratory syncytial virus (BRSV), clostridial spp., leptospirosis (five way), and calf scour pathogens (*Escherichia coli*, *Clostridium perfringens* (type C), rotavirus, and coronavirus) are recommended. Reimmunization against calf scour pathogens is recommended at 3 weeks precalving. Depending on the location of heifers (pasture vs. concrete confinement facilities), dewormers (to prevent gastrointestinal roundworms) should be administered at or near calving.

Parturition

In addition to milk yield, milk components, and physical type traits, sire selection for heifers is critical for what occurs at calving. It is recommended to choose calving-ease sires for heifers. The United States Department of Agriculture (USDA) produces sire summaries with calving-ease information on all sires whose semen is available for purchase from semen-producing organizations or bull studs. The information reported is the percentage of difficult births in heifers as reported by observers attending calvings. Calving difficulty scores assigned at calving range from 1 to 5 (1 = no assistance; 2 = slight problem; 3 = needed assistance; 4 = considerable force; 5 = extreme difficulty). The calving-ease percentage reported for each sire is based on the percentage of births in heifers when the calving difficulty scores were 4 or 5. In the Holstein breed, the average calving-ease percentage is about 9%. For heifers, one should use sires with calving-ease percentages that are less than average while not compromising selection for excellent production traits of sires. Preventing calving difficulty or dystocia is important. Dystocia may predispose heifers to placental retention, metritis, breeding inefficiency, and greater culling rates.

Most births do not require assistance (82%) and rushing delivery can injure the dam; however, waiting too long, after the water breaks, may deprive the calf of sufficient oxygen and cause death. Heifers should be observed frequently as due dates approach because some heifers may need assistance at calving. Observation of calving can reduce the number of stillborn calves and increase survival rates. The calf is born with no protection from disease or infection; hence, every effort must be made to limit its exposure to pathogens. Passive immunity is bestowed on the newborn by ingesting colostral milk antibodies shortly after birth.

Calving areas should be clean, dry, well lit, draft free, free of hazards, provide good footing, and spacious to allow the heifer to move about and position herself without pinning herself against an obstruction in the calving area in which the calf has no room to be delivered. When assistance is required for a large calf, twins, or breech births, arms, hands, and instruments should be sanitized. Plenty of lubricant should be used. When a calf jack or obstetrical chains are used, one should only pull when the abdominal muscles of the mother contract, therefore working with her contractions. Applying too much force can injure the heifer and damage the calf. Heifers and calves that experience difficulty need extra attention.

The absolutely most important measure and end point of successful reproduction is survival of the calf at birth and at various intervals thereafter. Immediately after birth, proper management of the newborn is critical to its survival. This includes high-quality colostrum (IgG concentrations >60 mg ml^{-1}) feeding immediately after birth, navel-dipping (7% tincture of iodine) to prevent navel invasion of microorganisms, immunizations, and other treatments. Delay in colostrum feeding can significantly diminish or preclude immunoglobulin absorption through the gut. Moreover, calves should be immediately identified for good record keeping. It is extremely important to disinfect calving pens between calvings. All contaminated bedding should be removed and the surface cleaned with a disinfectant.

Conclusion

Because replacement heifers represent the future genetic investment of any dairy herd, their management is critical to herd survival and longevity. Associated costs and investments in replacements are significant at 15–20% of all farm costs. Timeliness of establishing pregnancy can be significantly improved by using various hormonal schemes to program the estrous cycle to facilitate the use of AI and ensure a greater proportion of heifers calve by 23–24 months of age. The key to efficient reproduction is proper growth and body weight by calving time. Sire selection should emphasize production traits and calving ease to maintain good production but facilitate fewer problems at first parturition. Because heifers are more fertile than their lactating counterparts, the best available proven sires should be used with a much greater cost–benefit ratio. Perhaps, the most effective management strategy to increase genetic progress and maximize profitability on a dairy is to use synchronization of estrus and ovulation before AI in all dairy heifers. Furthermore, using gender-selected semen in replacement heifers available from several of the AI companies is the most cost-efficient application of this new technology because of the greater fertility in heifers than in lactating dairy cows. However, one can expect a reduction in fertility associated with gender-selected semen of 60–80% of that achieved with conventional semen, with the realization of 85–90% of the resulting calves to be females.

See also: **Replacement Management in Cattle**: Growth Diets; Growth Standards and Nutrient Requirements; Health Management; Pre-Ruminant Diets and Weaning Practices. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Estrous Cycles: Puberty.

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Health Management

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Introduction

Replacements are the future of the dairy industry. Focusing on improving health management of replacements will yield tremendous returns through decreased losses of animals with the greatest genetic potential on the dairy, decreased costs of medication, improved growth rates, improved feed efficiency and earlier entry into the milking herd.

Precalving and Calving Management

Health management of dairy replacements begins before the replacements are born. Several factors, such as nutrition of lactating and dry cows, vaccinations of lactating and dry cows, length of dry periods, cleanliness of the calving environment and disease status of the dams, will ultimately affect disease resistance and health of replacements. It is important to note, however, that cows that are overfed tend to have difficulty calving because of being excessively overconditioned. Cows that are underfed, which results in mineral or vitamin deficiencies or lack of body condition, may produce inferior and low-volume colostrum. They also may experience difficulty calving. Protein deficiency in cows during the dry period may lead to low birth weights, low metabolic rates and poor vigor of calves, resulting in poor survivability. Some research also indicates that inadequate protein and energy nutrition of the dam results in poor absorption of immunoglobulins from colostrum by the calf. Cows that lose condition during the dry period are also at greater risk of experiencing calving difficulty. Calves that experience difficult births require more time before being able to stand, experience an increase in the time to voluntary suckling and have a decreased ability to absorb immunoglobulins. All these problems result in decreased transfer of passive immunity from the dam to the calf and increased risk of disease in calves. As the degree of calving difficulty increases, the risk of mortality for calves increases. Proper nutrition of dairy cows during lactation and the dry period will help decrease disease risks for replacements.

Vaccinations of the dams will also impact disease resistance of dairy replacements. Proper vaccination of the dairy herd will increase the concentration of antibodies (immunoglobulins specific for diseases) in colostrum. Dams may be vaccinated during the dry period against pathogens that are common causes of diarrhea in calves, such as *Escherichia coli*, rotavirus and coronavirus. Vaccination of the dams increases the concentration of antibodies against these pathogens in colostrum, thus providing increased protection for calves, resulting in decreased incidence or duration of diarrhea. Vaccination of the dams during the dry period is more effective for prevention of disease in calves than vaccination of calves at an early age. The immune system of neonatal calves is unable to respond quickly to a vaccination or an infection because the immune system of the newborn is immature at birth. Both numbers and effectiveness of antibody-producing cells are lower in calves at birth than in adult cattle. Therefore, it is important for calves to obtain antibodies against common diseases of calves by consumption of colostrum rather than from an attempt to vaccinate calves at an early age. Vaccination of the dams against pneumonia may also help to decrease the incidence or severity of this disease in replacements.

Another important factor that may affect the health of replacements is the length of the dry period of the dam. A dry period that is too short, i.e. less than 6 weeks, may not provide enough time for involution of the mammary gland and preparation for the next lactation. Cows with shortened dry periods produce small quantities of colostrum that may also have low concentrations of immunoglobulins. It is important for health of replacements, therefore, that cows have at least a 6-week dry period for production of high-quality colostrum.

Management of the calving environment has a tremendous impact on the health of replacements. It is important for calves to be born in a clean, dry environment. Wet, sloppy stalls provide a perfect environment for growth of bacteria. Calving on a grass lot may be the best alternative when the climate is dry and mild. If a maternity barn is used, it is important to clean stalls thoroughly between calvings to prevent transfer of disease. Maternity stalls should only be used for calving and never for housing sick cows. Maternity pens and sick pens should be kept in separate facilities in order to prevent transfer of disease to highly vulnerable neonates and periparturient dairy cows. It is also important for the cows to be as clean as possible at calving in order to prevent calves from contracting disease organisms when suckling or attempting to suckle their dams. Preferably, calves should be separated from dams prior to suckling in order to prevent the calf from ingesting pathogens present on the legs, belly, flanks or udder of the cow as the calf attempts to nurse. Separating the calf from the dam and feeding colostrum by bottle also ensures adequate intake of colostrum for transfer of passive immunity from the dam.

Finally, it is important to know the disease status of cows prior to calving. Diseases such as Johne's disease, bovine viral diarrhea (BVD), and bovine leucosis virus (BLV) may be passed *in utero* or through colostrum. Calves should only be fed colostrum from cows known to be free of these diseases. It is important, therefore, to maintain a supply of frozen, high-quality colostrum from cows free of such diseases.

Care of Young Calves

The importance for baby calves of adequate consumption of immunoglobulins from colostrum has been reviewed elsewhere (*see* **Replacement Management in Cattle**: Pre-Ruminant Diets and Weaning Practices). Mortality resulting from lack of consumption of adequate amounts of immunoglobulins is commonly greater than 35% and has been reported to be as high as 60%. Others have indicated a 74-fold increased risk of mortality when calves do not consume colostrum. Along with economic losses from high mortality rates as a result of lack of colostrum consumption, there are also increased costs associated with increased medication and poor feed efficiency. Transfer of passive immunity (absorption of immunoglobulins from colostrum) can be determined using commercial kits that measure immunoglobulins in the blood. For adequate protection of calves, blood immunoglobulin concentrations should be at least $10\,\mathrm{mg\,ml^{-1}}$. Serum protein concentrations in calves are also highly correlated with the concentration of immunoglobulins in blood and can be used to determine adequate transfer of passive immunity. A hand-held refractometer can be used to measure serum protein; levels greater than $5.0\,\mathrm{g}\ 100\,\mathrm{ml^{-1}}$ by 24 h of age indicate adequate consumption of colostrum. The use of colostrum substitutes and replacers may help improve disease resistance in calves when high-quality colostrum is not available.

The most prevalent health problem of calves on most farms in the United States is diarrhea. Organisms such as *Cryptosporidium parvum*, rotavirus and coronavirus that cause diarrhea will not respond to antibiotic treatment. For cryptosporidiosis, the only means of prevention is sanitation, which includes controlling flies. For rotavirus and coronavirus, the most effective prevention is vaccination of the dam to increase antibodies in the colostrum against these organisms. Other organisms, such as *E. coli* and *Salmonella* sp., may be resistant to many of the commonly used antibiotics. Producers often give antibiotics to calves during episodes of diarrhea in order to prevent secondary infections; however, this practice often does more damage than good, killing beneficial gut microflora and damaging the gut lining. The first step in caring for calves with diarrhea is to provide fluids for hydration and electrolytes for mineral loss, while continuing to provide milk for protein and energy. An electrolyte solution can be fed from 20 min to 2 h after each feeding of milk or milk replacer until feces return to normal. Secondly, the organism causing diarrhea should be identified to determine whether antibiotic treatment is needed.

Pneumonia is the second most prevalent health problem of replacements, especially for replacements raised indoors. Research has shown that calves raised in individual hutches (plastic, fiberglass or wooden structures providing individual housing) perform very well and have fewer health problems, especially pneumonia, than calves raised in closed buildings. Open-front housing for older heifers should also help prevent pneumonia. Adequate, draught-free ventilation is important for prevention of pneumonia. Hutches, pastures and open-front housing for replacements provide optimal ventilation. In addition, hutches can be moved from location to location, giving producers the opportunity easily to remove old bedding and to break disease cycles. No matter what type of housing is used for replacements, cleanliness, dry bedding and adequate ventilation are essential to decrease incidence of disease.

Another important factor for controlling disease in replacements is grouping of heifers. Most producers in the United States house young calves individually. In other areas, housing calves in groups and using mob-feeders is an efficient method of rearing calves during the liquid feeding phase. After weaning, calves should be housed in small groups of 10 or fewer until they have successfully made the transition from liquid feed to dry feed and the transition from individual housing to competing for food. Additionally, by housing in small groups (rather than mixing large groups of animals at one time), producers can limit the exposure of calves to disease organisms and match calves more closely by size. As calves age, they can be housed in increasingly larger groups; however, animals should be grouped so there is not more than 50 kg difference in size of animals up to 6 months and not more than 90 kg difference in size for older animals.

Biosecurity

All dairy producers must actively institute biosecurity measures to prevent introduction of disease into the herd and to minimize spread of disease within the herd. For replacements, it is extremely important to prevent exposure of younger animals to older animals that may have Johne's disease. Exposure is not limited merely to animal-to-animal contact, but also includes articles of transmission, such as manure on hands, clothing and boots of workers, manure from older animals on equipment for feeding and handling replacements, or water that has been contaminated by older animals. In addition, flies can transfer diseases from older to younger animals. Producers must determine whether to have a closed herd or to allow introduction of new animals to the farm. If new animals are brought to a farm, the producer should work closely with a veterinary surgeon to determine which vaccinations animals should receive prior to coming to the farm. Once new animals arrive on the farm, or animals return to the farm from contract-growers or exhibitions, they should be quarantined for at least 30 days. This will allow time to determine if the new animals are likely to become ill and to allow the new animals to be exposed more slowly to any disease organisms currently on the farm.

Other potential sources of disease entry into replacements are visitors, vehicles removing dead animals, feed-delivery vehicles, wild and domestic animals, and birds. Within the herd of replacements, diseases can be transferred by using needles on more than one animal or using the same glove to palpate more than one animal. Producers must identify potential sources for transfer of disease-causing organisms within the herd and from outside the herd and institute a management plan to control them.

Digestive Disorders

Digestive disorders can occur in dairy replacements, resulting in problems such as acidosis and overeating diarrhea. Overeating diarrhea is found in replacements during the liquid feeding phase and may be prevalent in systems using accelerated feeding programs. This form of diarrhea can be treated by decreasing the amount of dry matter offered to calves in the liquid diet until the consistency of the feces returns to normal. Care should be taken to determine whether increased fluidity of the feces is caused by overeating or by disease organisms. If caused by disease organisms, treatment should include administration of an electrolyte solution and may require use of antibiotics.

Acidosis can occur in replacements if they consume large amounts of grains. Forages comprise the basis for diets for replacements after 3 months of age. Animals that gain access to fields of maize or bags of feed by accident will often suffer acidosis leading to laminitis (founder) or even death. Animals that are affected will generally have severe diarrhea. They can be treated by withholding grain until feces return to normal, followed by gradual reintroduction of grain into the diet.

Internal Parasites

Several types of internal parasites are found in dairy replacements. Perhaps the most common problem is coccidiosis. Coccidiosis causes diarrhea, which may be severe, resulting in weight loss, dehydration and anemia. Animals can be treated with a coccidiostat, such as amprolium, for severe coccidiosis. Coccidiostats such as decoquinate or lasalocid may be included in grain rations or even in milk replacers to help control coccidiosis.

Another common internal parasite of calves is *Cryptosporidium parvum.* This organism causes diarrhea in young calves at 7–10 days of age that lasts approximately a week. There are no cures for cryptosporidiosis and no means of prevention other than sanitation to decrease the pathogen load. Treatment involves electrolyte solutions along with continued milk feeding.

Replacement animals are very vulnerable to internal parasites (especially worms) during their first grazing season. Deworming of heifers yields economic returns in growth rates and feed efficiency. Producers should consult their veterinary surgeon to determine the most effective method of treating internal parasites both to decrease the parasite load in the animals and to prevent shedding of eggs onto pastures. Depending on geographical location, different deworming strategies are needed to control internal parasite populations. Producers should be aware that cold temperatures cause larvae to undergo arrest, even when ingested into the host. During this arrested stage, the larvae are resistant to most deworming agents.

External Parasites

Many external parasites, including various species of flies, affect health and growth of replacements. Several species of blood-sucking flies affect replacements. Horn-flies can be a major problem for cattle. They can cause substantial blood loss, transmit diseases including mastitis to replacements and decrease growth rates. Use of forced back rubs is probably the most effective method of decreasing populations of horn-flies. Additionally, removal of manure, which is the major breeding habitat for horn-flies, helps decrease populations. Another type of fly, the stable-fly, breeds in wet feed. Severe infestations of stable-flies can cause up to a 50% decrease in milk production. Counts of 25 flies per animal cause economically important losses in milk production and growth. Removal of waste feed from under feed troughs and other areas to decrease breeding areas is the most important mechanism for control. Horse-flies and deer-flies are also blood-sucking flies and may be responsible for spread of several diseases but are impractical to control.

Common house-flies are not blood-sucking insects but feed on muzzles, eyes and open wounds. They can be contaminated with more than 30 viruses and 175 bacteria, as well as other disease-causing organisms. The main form of control for common house-flies is sanitation and removal of breeding material because many house-flies are resistant to insecticide sprays. Cattle grubs are another parasite common in North America. The main damage to cattle is caused by the migration of the grubs through host tissues and production of cysts on the animals' backs. Growth rates can be adversely affected with infestations of cattle grubs. Appropriate insecticide treatment will kill grubs; however, care must be taken not to administer insecticides when large numbers of grubs may have accumulated in the spinal canal. Killing of large numbers of grubs at once can lead to anaphylaxis in cattle. Other external parasites that may affect dairy cattle include fleas, lice, ticks and mites. Itchiness and formation of scabs should be examined by a veterinary surgeon who can prescribe appropriate forms of treatment.

Vaccinations

Many disease occurrences can be prevented or at least minimized by appropriate vaccination programs. The program that is appropriate, however, will vary from region to region, and even farm to farm. Establishment of a vaccination program requires knowledge of diseases prevalent in the area, history of diseases on the farm, history of diseases in the herd, vaccinations used previously in the herd and an assessment of the risk of contracting economically important diseases based on management of the herd (open or closed). Producers should, therefore, consult their veterinary surgeon to develop a vaccination program appropriate for their animals, management and location.

Timing of vaccinations is important for replacement animals. If the colostrum consumed by the calf contained antibodies against the disease organism present in the vaccine, the vaccine will not generate a sufficient immune response in the replacement animal. Maternal antibodies obtained from colostrum may be present up to 6 months of age, preventing an adequate response to vaccinations. It may be beneficial to wait until 6 months of age or greater for many initial vaccinations in calves in order to avoid interference from maternal antibodies. Additionally, many vaccines are not effective in neonatal calves because their immune system is not sufficiently developed to generate a protective response.

Common mistakes made in vaccination programs are lack of booster vaccinations at the appropriate time and lack of frequent vaccinations. If the vaccination protocol calls for an initial vaccination followed by a booster vaccination within 2 to 3 weeks, maximum protection will not be achieved without the booster vaccination. Essentially, the money spent for the first injection is wasted. The second problem, lack of frequent vaccinations, is seen especially with leptospiral vaccines. Leptospiral vaccines should be administered every 6 months to achieve adequate protection. It is also important for heifers to start receiving leptospiral vaccinations at 6 months of age so that they have received two vaccinations by the time they are used for breeding.

Conclusions

Health management of replacements requires attention to many different areas, ranging from nutrition and management of late lactation and dry cows to vaccinations of replacements. Health management of replacements is an area that is often overlooked because producers do not see an immediate return on their efforts and prefer to spend their time improving management of the milking herd. For health management of replacements, however, the old saying that “an ounce of prevention is worth a pound of cure” really holds true.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Diseases of Dairy Animals**: Infectious Diseases: Johne's Disease; Infectious Diseases: Leptospirosis; Non-Infectious Diseases: Acidosis/Laminitis. **Feeds, Ration Formulation**: Dry Period Rations in Cattle; Lactation Rations for Dairy Cattle on Dry Lot Systems. **Milk**: Colostrum. **Replacement Management in Cattle**: Growth Diets; Pre-Ruminant Diets and Weaning Practices.

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REPRODUCTION, EVENTS AND MANAGEMENT

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Estrous Cycles: Puberty

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Introduction

The term puberty is derived from the Latin word *pubescere*, which is translated to mean 'becoming covered with hair'. Puberty originally referred to the appearance of body hair at the time of sexual maturation in humans. Today, puberty refers collectively to all of the physiological, morphological and behavioral changes that occur in animals as their gonads undergo the transition from the infantile to adult condition. The so-called onset of puberty refers to the time at which an individual gains the capacity to reproduce. Onset of puberty is the result of a series of developmental events that occur within the reproductive endocrine system. This article focuses on sexual development in females with emphasis on the dairy heifer.

Characteristics and Importance

Heifers are said to have attained puberty when they first exhibit estrus, followed by a viable ovulation and an estrous cycle of normal length (18–25 days). First estrus is not accompanied by ovulation. Rather, it is followed by luteinization of an ovarian follicle and a short estrous cycle (<18 days). An accurate determination of puberty onset in heifers requires not only estrus detection, but also examination of the ovaries and/or assessment of circulating concentrations of progesterone to confirm the presence of a corpus luteum.

The average age at which heifers attain puberty is 11 months of age. However, average age at puberty varies greatly, ranging from approximately 8 to 24 months (**Table 1**). In general dairy heifers (e.g. Holstein) attain puberty earlier than beef heifers (e.g. Angus, Hereford). It is not unusual for Holstein heifers to attain puberty at 8–9 months of age, whereas English breeds of beef cattle attain puberty at 10–14 months of age. It is important to emphasize that age at puberty is profoundly influenced by plane of nutrition. For example, in an early study done at Cornell University, Holstein heifers fed a low, medium or high plane of nutrition exhibited first estrus at 20.2, 11.2 and 9.2 months of age, respectively.

Age at puberty is an extremely important economic trait in production systems where reproductive

Table 1 Differences in age at puberty among various breeds of cattle

Breed	*Age*
(months)	
Jersey	8
Guernsey	11
Holstein	11
Ayrshire	13
Beef (European)	10–15
Zebu	17–27
Water buffalo	15–36

efficiency is a primary goal. One of the greatest costs in dairy production is feed. Generally, feeding animals that are not productive reduces production efficiency. In most intensive dairy production systems, producers strive to have heifers calve by 24 months of age and produce one calf each year thereafter. In order to accomplish this a heifer must become pregnant by 14 months of age, have sufficient body size for pregnancy and dystocia-free parturition and sufficient body energy reserves to return to oestrus following calving. An understanding of mechanisms controlling onset of puberty should facilitate management of heifers to optimize production efficiency.

Review of the Reproductive Endocrinology of the Female

The major anatomical components of the reproductive endocrine system of females include the hypothalamus, anterior pituitary gland, ovaries and uterus. These are endocrine organs and therefore produce hormones which permit communication among the various reproductive tissues. To understand the physiological mechanisms governing onset of puberty it is necessary to understand how the hypothalamus, anterior pituitary gland and ovaries interact in an endocrine fashion (**Figure 1**). Discussions of the role of the uterus in reproduction are included in other articles.

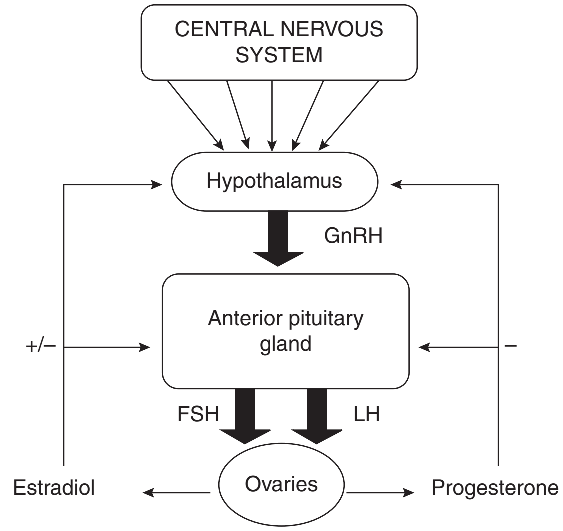

Figure 1 Endocrine interactions within the hypothalamic–pituitary–ovarian system. Various inputs conveying information about the external and internal environments converge on the hypothalamus. The hypothalamus transduces these neuronal signals into an endocrine signal, i.e. the pattern of GnRH secretion. GnRH regulates release of FSH and LH from the anterior pituitary gland. These gonadotropins regulate ovarian function which includes the synthesis and secretion of the steroid hormones estradiol and progesterone. These steroids feed back to regulate gonadotropin secretion. In prepubertal heifers, low concentrations of estradiol exert a negative feedback effect on pulsatile release of LH. Estradiol also inhibits release of FSH. High concentrations of estradiol (i.e. those present during estrus) induce a preovulatory surge of LH. Progesterone is the major negative feedback hormone controlling LH secretion in the adult female.

The central nervous system, which includes the hypothalamus, plays a pivotal role in regulation of reproductive function. Higher centers of the brain receive neuronal inputs from a variety of sensing systems that monitor internal and external environments (e.g. ambient temperature, day length, nutritional status). Information carried by these systems is integrated in the hypothalamus and transduced into a neuroendocrine signal. A small number of neurons in the hypothalamus produce gonadotropin releasing hormone (GnRH), a decapeptide which is secreted into capillaries located in the median eminence of the hypothalamus. GnRH enters these capillaries and is carried to the anterior pituitary gland via portal vessels, which drain into another capillary bed. The major action of GnRH is to stimulate release of gonadotropins, i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH is secreted in a pulsatile manner and has been shown to cause the pulsatile release of LH and possibly FSH. The pulsatile pattern of LH release can be demonstrated by measuring circulating concentrations of LH at frequent intervals for several hours. The pulsatile release of GnRH has been documented by measuring concentrations of this hormone in blood collected from the pituitary portal vessels. During pulsatile secretion concentrations of a hormone increase abruptly within minutes. This increase is followed by a slower decrease, which reflects the clearance of the hormone from circulation. Pulsatile secretion is typically characterized by quantifying the frequency of pulses (number of pulses in a given period of time) and the amplitude of pulses (difference between the peak of a pulse and the previous nadir). **Figure 2** illustrates pulsatile LH secretion.

The gonadotropins, LH and FSH, are released into the general circulation by gonadotropic cells in the anterior

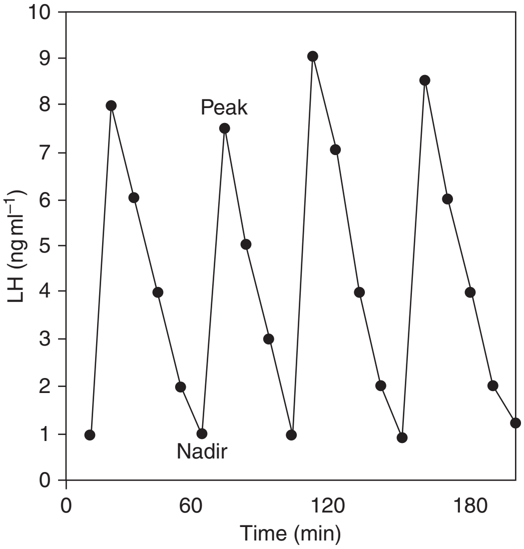

Figure 2 Example of pulsatile patterns of LH in a series of blood samples collected at 10-min intervals for 180 min. Each pulse is characterized by a rapid increase in concentrations, followed by a slower decrease. Pulsatile release of LH is typically characterized by quantifying the pulse frequency (number of peaks per unit time) and average pulse amplitude (difference between the peak concentration and preceding nadir).

pituitary gland. These hormones regulate ovarian function. It is generally accepted that the pulsatile release of LH is necessary for the initiation and maintenance of ovarian activity. With respect to onset of puberty, LH plays important roles. First, a high-frequency mode of LH secretion (>1 pulse h^{-1}) is necessary for follicle maturation. Second, an LH surge, which accompanies estrus, is necessary for ovulation and formation of the corpus luteum. The role of FSH in onset of puberty is not well defined. It is generally accepted that FSH is important in stimulating early follicle development (prior to maturation to the preovulatory stage).

The gonadotropins regulate production of ovarian steroid hormones, which in turn regulate gonadotropin release, uterine function, sexual behavior and expression of secondary sex traits. LH and FSH act together to regulate production of estradiol by granulosa cells of ovarian follicles, whereas LH alone appears to govern progesterone synthesis by the corpus luteum.

An appreciation for the regulation of gonadotropin secretion by ovarian steroids is critical to understanding sexual maturation (**Figure 1**). In prepubertal heifers, oestradiol is the major negative feedback regulator of LH secretion. In postpubertal females, progesterone is responsible for most of the inhibitory effects of the ovary on LH secretion. Estradiol inhibits LH secretion by suppressing pituitary responsiveness to GnRH, and by inhibiting release of GnRH by the hypothalamus. Progesterone appears to act at the level of the hypothalamus to inhibit GnRH release. Little is known about the regulation of FSH secretion during the prepubertal period. Estradiol and inhibin, a peptide hormone produced by granulosa cells of follicles, appear to regulate FSH release via feedback inhibition.

Whereas low levels of estradiol inhibit pulsatile LH secretion in prepubertal heifers, high levels of this steroid (i.e. those present during estrus) facilitate LH secretion. The high levels of estradiol produced by the preovulatory follicle induce an LH surge, which results from enhanced pituitary response to GnRH, as well as an increase in GnRH release.

Development of the Hypothalamic–Pituitary–Ovarian System

An understanding of the mechanisms controlling sexual maturation in heifers has been gained by systematically examining the development of the reproductive endocrine axis. The experimental approach has been to identify the physiological and/or anatomical component(s) that are rate-limiting in terms of the timing of puberty onset.

Hypothalamus and Anterior Pituitary Gland

It is generally accepted that the hypothalamic–pituitary axis and the capacity of this system to synthesize and secrete GnRH and gonadotropins is fully competent long before onset of puberty. The hypothalamic–pituitary portal vascular system appears to be developed and functional prior to birth. GnRH secretion has not been measured directly in heifers. However, bull calves exhibit GnRH pulses in portal blood by 2 weeks of age. In heifers and bull calves, pulses of LH appear in the peripheral circulation by 2 weeks of age. Collectively, these results support the idea that the neuroendocrine mechanisms controlling the pulsatile release of GnRH/LH are competent by 3–5 weeks of age. This casts doubt on the notion that development of this system is the rate-limiting step in onset of puberty.

Ovaries

The ovaries of heifers appear to be functionally competent long before the onset of puberty. Vesicular follicles are present by 2–4 weeks of age, and reach a peak in average size by 12–16 weeks of age before declining to an average size that varies little until the peripubertal period. These follicles will respond to exogenous gonadotropins. Treatment with FSH and LH induces ovulation in heifers by 1 month of age, but number of induced ovulations increases between 1 and 5 months of age.

Between birth and puberty follicles grow in waves, meaning that at regular intervals, dominant follicles emerge from pools of growing follicles, and then regress before reaching the preovulatory stage. The size of the dominant follicle increases with age; the largest dominant follicles are found during the peripubertal period.

The dominant follicles of the late prepubertal period produce sufficient estradiol to induce estrous behavior and LH surges. The first of these estrous periods are not necessarily followed by ovulation and a normal estrous cycle. Peripubertal heifers may exhibit oestrus in the absence of a subsequent estrous cycle. First ovulation is normally preceded by one or two LH surges followed by elevations in progesterone that are lower in magnitude and shorter in duration than those expressed during a normal luteal phase. These short luteal phases are attributed to ovarian follicles that become luteinized, but do not ovulate.

Ovarian Control of Gonadotrophin Secretion

As mentioned previously, the ovaries regulate gonadotropin secretion via steroid hormones. Ovulation is induced by a surge of LH which is caused by elevated concentrations of estradiol. This important positive feedback system appears to be fully developed prior to onset of puberty. In beef heifers, injections of estradiol induce preovulatory-like surges of LH in prepubertal heifers as early as 5 months of age. This suggests that onset of puberty may be limited more by development of events leading up to the LH surge (i.e. elevated levels of estradiol) than by development of the capacity to produce an LH surge. Although dominant, vesicular follicles appear in waves throughout the prepubertal period, they do not produce enough estradiol to induce an LH surge. At this point it is important to reiterate that both the ability of follicles to respond to gonadotropins as well as the ability to elicit an LH surge appear to be fully developed months before onset of puberty. Therefore, the rate-limiting step in sexual maturation appears to be appearance of the endocrine signal that stimulates follicle development to the preovulatory stage. This signal appears to be the high-frequency mode of LH secretion.

Examination of LH pulse patterns between birth and puberty reveals that LH pulse frequency increases between birth and 3–5 months of age, but then declines and remains relatively low until a few weeks prior to onset of puberty. The frequency of LH pulses before this peripubertal increase in LH secretion is much lower than that of the follicular phase of the estrous cycle, when LH pulses occur with a frequency of approximately once per hour. This observation begs an important question: why does LH pulse frequency remain low even though the mechanisms necessary for the pulsatile release of LH appear to be fully developed by one month of age?

Pulsatile secretion of LH is regulated by the negative feedback actions of ovarian steroids. In heifers a rise in LH concentrations following ovariectomy has been demonstrated as early as 1 month of age, suggesting that ovarian negative feedback is present at this age. Estradiol appears to be the major negative feedback regulator of LH secretion before the onset of puberty. In ovariectomized heifers, physiological doses of this steroid lower concentrations of LH to preovariectomy levels.

The transient increase in pulsatile LH secretion between 3 and 5 months of age may reflect development of the estradiol–LH negative feedback loop. Although the estradiol negative feedback system may be present by 1 month of age, it may not be expressed at this time due to extremely low levels of estradiol. The increase in LH concentrations during the first several months of age may be attributed to ovarian estradiol reaching a threshold for inhibiting LH secretion.

By 3–5 months of age, the ovaries produce enough estradiol to suppress LH secretion. However, the ability of estradiol to suppress LH release changes with advancing age in prepubertal heifers. Physiological doses of estradiol suppress concentrations of LH in ovariectomized heifers until the age at which ovary-intact heifers attain puberty (**Figure 3**). At this time estradiol becomes less effective in suppressing LH pulses, i.e. animals appear to escape estradiol negative feedback.

Little is known about the cellular and molecular mechanisms regulating the negative feedback actions of estradiol. It seems likely that such changes are attributed

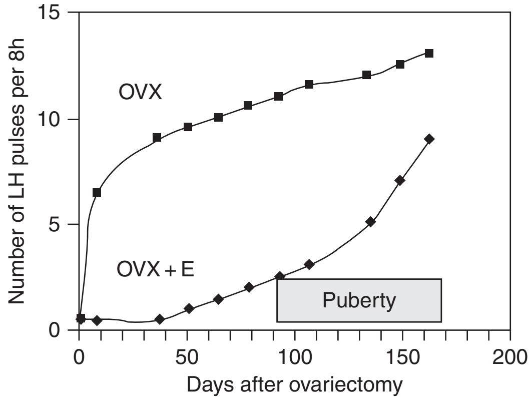

Figure 3 Circulating concentrations of LH in prepubertal beef heifers that were either ovariectomized (OVX) or ovariectomized and treated chronically with physiological concentrations of estradiol (OVX + E). Ovariectomy results in an immediate increase in LH due to removal of ovarian negative feedback. Replacement with estradiol prevents the postovariectomy increase in LH suggesting that this steroid is the major negative feedback hormone regulating LH secretion in prepubertal heifers. Eventually heifers become less sensitive to this negative feedback and LH concentrations increase. This escape from negative feedback coincides with the onset of puberty in ovary-intact animals. (Based on data from Day *et al*., 1984.)

to changes in GnRH neurons and/or neurons that regulate GnRH neurons. There is evidence to support the idea that changes in response to estradiol negative feedback reflect changes in number of estradiol receptor in neurons that regulate secretory activity of GnRH neurons.

Theory of Puberty Onset

The observations discussed above serve as the basis for the widely accepted theory of puberty onset summarized in **Figure 4**. The central claim of this theory is that a cascade of endocrine events is required for ovulation, i.e. a high-frequency mode of LH secretion which stimulates growth of a dominant follicle to the preovulatory stage, which results in production of enough estradiol to induce estrus and a preovulatory surge of LH. The prepubertal heifer has the capacity to express this cascade by 5–6 months of age, but does not due to a high sensitivity to estradiol negative feedback which maintains a low-frequency mode of LH secretion. Onset of puberty can occur only after the hypothalamic–pituitary axis escapes estradiol negative feedback, or when this negative feedback effect is overridden by administering exogenous GnRH or LH.

It is unclear whether the decrease in sensitivity to estradiol negative feedback is strictly a developmental event. Recent evidence suggests that the timing of this change in sensitivity may be more a function of metabolic status than one of age. Restriction of dietary energy delays onset of puberty, apparently by delaying escape from estradiol negative feedback. Moreover, precocious puberty in heifers appears to be related to favorable energy status and high growth rates. Preliminary studies suggest that in beef heifers, high-energy intake early in life (birth to 6 months of age) induces transient but cyclic luteal function, suggesting that the estradiol negative feedback system may have been prematurely inactivated. Additional studies are required to test this hypothesis. Nevertheless, these observations reinforce the idea that the hypothalamic–pituitary–ovarian system is functionally mature by 5–6 months of age.

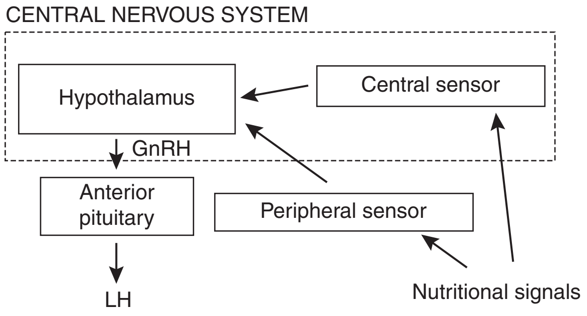

Figure 4 Summary of the prevailing working hypothesis explaining how nutrition influences timing of puberty onset. According to this hypothesis, various blood-borne signals reflecting nutritional status (e.g., metabolites, metabolic hormones) are detected by sensors in the peripheral and/or central nervous systems. These sensors trigger various neuropathways that converge on the hypothalamus, where they are transduced into signals that regulate pulsatile secretion of GnRH and LH (e.g., response to estradiol negative feedback). In this way, nutritional status influences the timing of the increase in LH pulse frequency that is critical for onset of puberty.

Timing of Puberty Onset

Although the endocrine events leading to onset of puberty have been well characterized in heifers, it remains unclear why puberty occurs at a particular age in individual animals. It seems reasonable that timing of puberty onset is a function of genetic and environmental factors, as well as genetic×environment interactions. Although breed type has been shown to influence age at puberty, there has been no attempt to understand these effects in terms of the endocrine mechanisms controlling puberty onset. In other words, we know little about the effects of breed on the endocrine mechanisms timing onset of puberty.

Of the studies that examine effects of environment on sexual development most have focused on the effects of nutrition. A few studies examined the effects of season on sexual development.

Nutrition, Growth and Onset of Puberty

Research in the 1950s and early 1960s established that age at puberty is inversely related to growth rate in dairy heifers. This was later documented in beef heifers. Most studies dealing with growth and puberty focus on the effects of plane of nutrition on age at puberty. For example, beef heifers fed to sustain an average daily gain of 0.23 kg reached puberty later than those fed to sustain an average daily gain of 0.82 kg. In one study, postweaning growth rates were inversely related to age at puberty in heifers fed to gain an average of 0.2 kg day^{-1}, but not in heifers fed to gain 0.4 kg day^{-1}. This lead to the hypothesis that growth rate has little effect on sexual development above a certain critical body weight, or that puberty onset occurs at a critical body weight. However, the fact that heifers maintained on different planes of nutrition typically attain puberty at different body weights casts doubt on this 'critical body weight hypothesis'. Nevertheless, it is clear that sexual development is somehow linked to growth and/or nutritional status.

Mechanisms Linking Onset of Puberty and Nutrition

It is beyond the scope of this article to review the voluminous research concerning the possible endocrine mechanisms linking nutrition and sexual development. However, it is generally agreed that nutrition influences sexual development via signals that influence the pulsatile release of LH. A working hypothesis illustrating how nutritional status might influence sexual development is summarized in **Figure 5**. Using this framework, there appear to be four major areas of focus for research in this area: nutritional signals, peripheral and central systems that sense these signals, neuropathways that transduce these nutritional signals into neuroendocrine signals, and GnRH/LH secretion. In cattle attention has focused primarily on mechanisms controlling LH secretion. In beef heifers, it has been demonstrated that a delay in onset of puberty caused by undernutrition is accompanied by a delay in escape from estradiol negative feedback. Moreover, underfeeding induces a decrease in LH pulse frequency and cessation of estrous cycles in sexually mature heifers suggesting that the effects of nutrition on reproductive activity are not confined to sexual development. Although it can be concluded that undernutrition suppresses the pulsatile secretion of LH in heifers, we know very little about the signals that reflect nutritional status or how these signals are detected and transduced into signals that influence LH secretion.

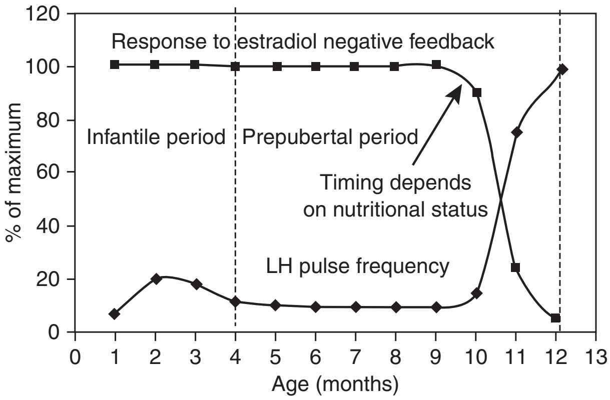

Figure 5 Summary of the prevailing theory explaining control of puberty onset in the heifer. During the early infantile period pulsatile LH secretion increases slightly due to limited follicle development and minimal estradiol secretion. This promotes vesicular follicle development and estradiol secretion. Due to a high sensitivity of the hypothalamic–pituitary axis to the negative feedback actions of estradiol, pulsatile LH secretion decreases and remains low throughout the prepubertal period. The end of the prepubertal period is heralded by a decrease in response to estradiol negative feedback, and a resultant increase in LH pulse frequency. The timing of this escape from negative feedback is dependent on nutritional status; heifers on high planes of nutrition escape at an earlier age than those on low planes of nutrition. The increase in LH pulse frequency is critical to onset of puberty because it stimulates follicle development to the preovulatory stage, thereby inducing estrus and a preovulatory surge of LH.

Season and Sexual Development

Sexual development in seasonal breeders

Seasonal breeders exhibit a well-defined anestrous period each year. Short-day breeders such as sheep and goats exhibit regular estrous cycles in late summer, when day length is decreasing, and enter a period of anestrus in early spring, when day length is increasing. Extensive research has supported the theory that in these species, photoperiod regulates reproductive activity by affecting the pulsatile release of LH. During the anestrous season, LH pulse frequency is suppressed due to enhanced responsiveness to estradiol negative feedback, as well as a steroid-independent effect on GnRH secretion. This inhibition is lifted during periods of short day lengths. The transition from the anestrous to the breeding season resembles the transition from the prepubertal to pubertal state, i.e. increased frequency of LH pulses, maturation of a dominant follicle, increased production of estradiol, onset of estrus and induction of an LH surge.

In temperate climates, lambs are typically born in late winter and early spring and attain puberty in late summer and early autumn. As in heifers, puberty in ewe lambs is the result of an escape from estradiol negative feedback which allows pulsatile LH secretion to increase. As might be expected, season influences onset of puberty in seasonal breeders. For example, ewe lambs born during the autumn attain puberty at 10–12 months of age (during the subsequent autumn), whereas lambs born in the spring reach puberty at 5–6 months of age (during the autumn). Although it is beyond the scope of this article to describe details of how season influences onset of puberty in sheep, a few general comments seem appropriate. First, it seems clear that photoperiod is the major mediator of the effect of season on puberty onset in sheep. In order for puberty to occur at a normal age (5–6 months of age), lambs must experience several months of long day lengths before exposure to short days. Second, the delay in puberty onset experienced by autumn-born lambs is associated with low LH pulse frequency, due to high sensitivity to oestradiol negative feedback. Third, the pineal gland and its pattern of melatonin secretion appear to be important in mediating the effects of day length on sexual development.

Effects of season on sexual development in cattle

Cattle are not seasonal breeders. Nonpregnant heifers and cows will exhibit regular estrous cycles throughout the year. However, there is evidence to suggest that season influences sexual development in heifers. Early studies described an effect of season of birth on age at puberty in dairy heifers. Heifers born during the spring and summer were younger at first estrus than those born during autumn and winter. Similar observations were reported for beef heifers; heifers born in the spring were younger at puberty than those born in the winter. Examination of year-round reproductive records in Wisconsin revealed that the winter environment appears to delay onset of puberty in autumn-born heifers. Autumn-born heifers that failed to attain puberty before the subsequent winter did not attain puberty until the subsequent spring and summer. It appears that both season of the first 6 months of life and season of the second 6 months of life influence sexual development. Heifers born near the time of the autumnal equinox (23 September) were younger at puberty than those born near the time of the vernal equinox (21 March). In both autumn- and spring-born heifers, exposure to spring–summer conditions (i.e. increasing day length and temperature) resulted in an earlier onset of puberty compared to autumn–winter conditions (i.e. decreasing day length and temperature).

The environmental variable(s) responsible for the seasonal effects on puberty in cattle remain(s) unidentified. Artificially lengthening photoperiod (16L:8D) during the winter months hastens onset of puberty in heifers and bull calves suggesting that day length may be important, as it is in seasonal breeders.

Few studies have addressed the physiological mechanisms whereby season influences sexual development in heifers. Based on the importance of pulsatile LH secretion in regulating onset of puberty, it is reasonable to hypothesize that seasonal effects on sexual development are somehow mediated by changes in LH patterns. However, support for this hypothesis remains elusive.

Summary

Puberty in dairy heifers is a gradual process involving maturation of the endocrine mechanisms controlling ovarian development. Although puberty does not occur until 8–14 months of age, the hypothalamic–pituitary–ovarian system appears to be functionally competent as early as 5–6 months of age. By this time the estradiol–LH negative feedback loop is established and secretion of estradiol by ovarian follicles is sufficient to maintain pulsatile LH secretion in a low-frequency mode. As long as this mode of LH secretion is sustained, follicle development will not progress to the preovulatory stage. When the hypothalamic–pituitary axis escapes this inhibitory effect of estradiol, pulsatile LH secretion enters the high-frequency mode, allowing a dominant follicle to mature and release enough estradiol to induce estrus and a preovulatory surge of LH. It is likely that both genetic and environmental factors influence timing of puberty onset in cattle. We understand very little about genetic variation in the physiological mechanisms that regulate onset of puberty. While the effects of nutrition on puberty onset clearly involve effects on pulsatile LH secretion, the mechanisms mediating the effects of other environmental factors such as season remain unclear.

See also: **Husbandry of Dairy Animals**: Sheep: Reproductive Management. **Replacement Management in Cattle**: Growth Standards and Nutrient Requirements. **Reproduction, Events and Management**: Estrous Cycles: Characteristics; Estrous Cycles: Seasonal Breeders.

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Estrous Cycles: Characteristics

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Introduction

The estrous cycle represents the cyclical nature of ovarian activity that facilitates female animals to go from a period of reproductive receptivity to nonreceptivity ultimately allowing the establishment of pregnancy following mating. The normal estrous cycle in cattle is 18 to 24 days. The cycle consists of two discrete phases: the luteal phase (14 to 18 days) and the follicular phase (4 to 6 days). The luteal phase is the period following ovulation when the corpus luteum (CL) is formed, while the follicular phase is the period following the demise of the corpus luteum (luteolysis) until ovulation. During the follicular phase final maturation and ovulation of the ovulatory follicle occurs which leads to the release of an oocyte (the female gamete) into the oviduct allowing the potential for fertilization.

Commencement of regular estrous cycles in cattle occurs at the time of puberty. Heifers reach puberty between 6 and 24 months of age typically at 50% of mature body weight. At the onset of puberty, the first estrous cycle tends to be of a short duration (3 to 12 days) and follows a silent ovulation (that is not associated with expression of behavioral estrus). Estrous cycles cease during pregnancy due to the prolonged presence of elevated progesterone from the CL. Following parturition estrous cycles recommence after a variable period of anestrus and anovulation. Similar to the transition from prepuberty to puberty, the resumption of ovarian cycles postpartum is usually associated with a silent ovulation followed by a short cycle (*see* **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity).

Behavioral Changes throughout the Estrous Cycle

Estrous behavior in cattle is closely related with time of ovulation and allows successful mating to occur prior to ovulation to maximize the chances of conception. During estrus, heifers and cows will stand to be mounted by herd mates or a bull, if present. Estrus is expressed at 18- to 24-day intervals and lasts for 8 to 24 h. Estrus is caused by the secretion of the proestrus estradiol surge from the preovulatory dominant follicle.

The estrous cycle may be divided into diestrus, proestrus, estrus and metestrus. During diestrus, cyclic animals will not show signs of estrous behavior. During the preovulatory period there is a peak in butting activity and attempted mounting without standing. During estrus heifers will stand to be mounted and also show increased mounting activity. Levels of investigatory behavior (including sniffing, rubbing, licking, chin-resting and orientation) occurs at similar intensities both before, during and after the period of standing to be mounted. Standing to be mounted is considered to be the definitive sign of estrus in cattle. A number of other qualitative signs of estrus have been described including: mounting of other cows, ruffling of the rump hair or abrasion of the rump skin, vulval relaxation and moistness, estrous mucus appearing from the vulva, sensitivity to palpation of the rump and general restlessness; however, none of these signs are as definitive as standing to be mounted. Cows or heifers that are in proestrus, estrus or metestrus simultaneously within a herd usually form sexually active groups that will include a bull if present (*see* **Reproduction, Events and Management**: Mating Management: Detection of Estrus).

Follicular Growth and Development

The growth, development and maturation of ovarian follicles is a fundamental process for effective reproduction in farm animals. Primordial follicles are established in the ovary during embryonic development. A fixed number of primordial follicles are established during fetal development. During the lifetime of the female, primary follicles enter a growing pool. Initial stages of follicle growth occur independently of gonadotrophic hormones; antral follicles then become responsive to and subsequently dependent on follicle stimulating hormone (FSH). The pattern of follicle growth in cattle has been clearly characterized with the use of transrectal ovarian ultrasonography. Several detailed studies have demonstrated that there are either two, three or occasionally four waves of follicle growth during the normal estrous cycle of cattle. During each wave of follicle growth, a cohort of two to five follicles emerges to grow beyond 4 mm in diameter to medium (5–9 mm) size classes (emergence) (**Figure 1**). From the pool of medium follicles that emerge a single follicle is selected to become the dominant follicle (selection). Selection is a hypothetical physiological process whereby 'excess' follicles are reduced to the ovulatory quota. This determines the species-specific ovulation rate in females thereby playing a major role in determining the number of offspring born per pregnancy. The selected dominant follicle continues to grow in size, while other follicles in the cohort undergo atresia. Dominance is a process that enables the 'selected' follicle to suppress further growth of other follicles, escape initial atresia and continue to grow until either atresia (during the luteal phase) or ovulation (during the follicular phase). The preovulatory dominant follicle undergoes a period of final maturation, following CL regression and increased luteinizing hormone (LH) pulse frequency, and it then ovulates. After ovulation, a CL is formed which secretes progesterone throughout the luteal phase of the cycle.

The number of follicular waves during the estrous cycle is affected by breed and management of animals. In dairy cows two waves of follicular growth per estrous cycle are more common, while in beef heifers up to 70% of ovarian cycles consist of three follicular waves. During the transition from prepuberty to puberty and from anestrus to resumption of ovarian cycles in postpartum cows, the short ovarian cycle that typically occurs is associated with a single follicular wave.

Endocrine Regulation of Ovarian Function

Ovarian activity throughout the estrous cycle is controlled by the hypothalamic–pituitary–ovarian–uterine axis (**Figure 2**). Gonadotropin releasing hormone (GnRH) is secreted in a pulsatile manner by neurons of the hypothalamus into the hypophyseal portal blood system. It is transported to the anterior pituitary where it controls the secretion of LH and FSH, collectively known as the gonadotropins. Pulsatile secretion of GnRH from the hypothalamus is virtually 100% coincident with pulsatile secretion of LH from the anterior pituitary.

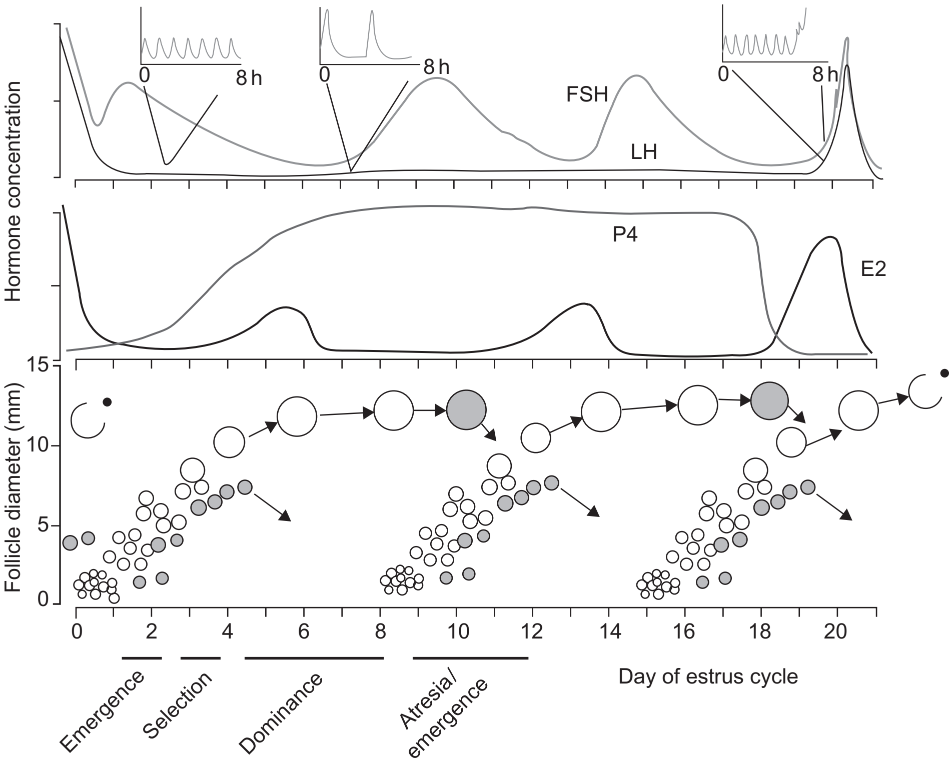

Figure 1 Schematic depiction of the pattern of secretion of follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P4) and estradiol (E2); and the pattern of growth of ovarian follicles during the estrous cycle in cattle. Each wave of follicular growth is preceded by a transient rise in FSH concentrations. Healthy growing follicles are shown as open circles, atretic follicles are shaded. A surge in LH and FSH concentrations occurs at the onset of estrus and induces ovulation. The pattern of secretion of LH pulses during an 8-h window early in the luteal phase (high frequency, low amplitude), the mid-luteal phase (low frequency, low amplitude) and the follicular phase (high frequency, building to the surge) is indicated in the insets in the top panel.

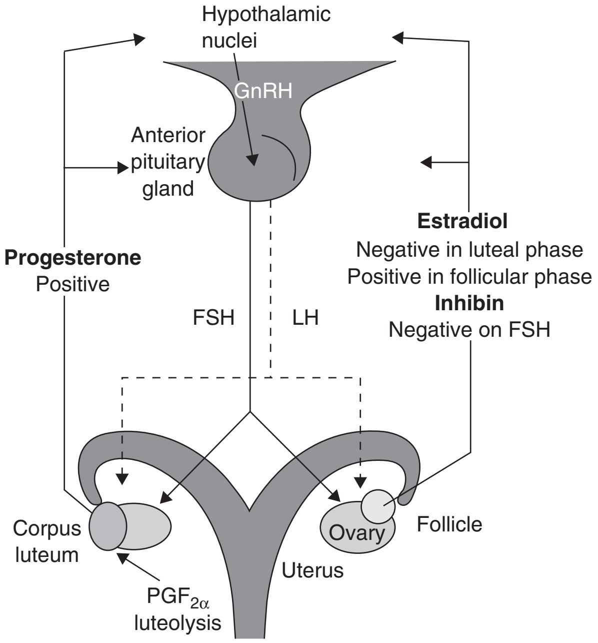

Figure 2 Schematic diagram depicting the endocrine feedback mechanisms of the hypothalamic–pituitary–ovarian–uterine axis regulating the estrous cycle in cows. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are both secreted from the anterior pituitary gland. LH pulses are tightly associated with pulses of gonadotropin releasing hormone (GnRH) from the hypothalamus. GnRH and hence LH pulse frequency are regulated in a negative feedback mechanism by estradiol and progesterone during the luteal phase, while in the follicular phase there is a positive feedback of estradiol (in the absence of progesterone) from the preovulatory follicle inducing the GnRH, LH and FSH surges. Secretion of FSH requires GnRH pulses, but is regulated by feedback mechanisms of estradiol and inhibins.

Secretion of FSH is also regulated by GnRH and other endocrine and paracrine mechanisms. The gonadotropins when secreted from the anterior pituitary enter general circulation and have their major biological effect at the gonads. In heifers, receptors for LH have been located in the CL, the theca cells of healthy growing follicles and the granulosa cells of large growing follicles ($\geq$8 mm in diameter). Receptors for FSH are present in the granulosa cells of healthy antral follicles.

Follicle stimulating hormone is the main hormone that stimulates follicular growth. Follicle stimulating hormone concentrations are related to follicular growth throughout the estrous cycle in heifers. Associated with each new wave of follicular development, FSH concentrations increase as emergence occurs (**Figure 1**). This transient rise in FSH concentrations occurs over a period of 1 to 2 days during emergence of each new wave of follicle growth. Thus, in a typical three-wave estrous cycle, recurrent FSH rises occur on days 0.5 to 1.5, 9 to 11, and 13.5 to 15, whereas in a two-wave estrous cycle only the first two recurrent FSH rises occur. During all physiological states where follicle waves occur (that is, cyclic cattle, postpartum cows during anestrus, and prepubertal heifers), associated transient increases in FSH concentrations coincide with follicle wave emergence. The process of selection of the dominant follicle occurs during a period when FSH returns to nadir concentrations. The physiological mechanisms that allow one follicle to become selected and continue growing while other follicles in the cohort undergo atresia in, the presence of declining FSH concentrations remain to be elucidated. However, early survival factors have been identified and they include development of LH receptors by the granulosa cell layer allowing a switch away from FSH dependency toward LH dependency and an increase in the amounts of bioavailable insulin-like growth factor-I (IGF-I). The increase in bioavailable IGF-I is thought to be mediated through a decrease in insulin-like growth factor binding proteins. Final maturation and either atresia or ovulation of a selected dominant follicle is then dependent on the prevailing LH pulse secretory pattern.

The precise pattern of pulsatile LH during each wave of follicle growth has been less clearly described. However, during the early luteal phase low amplitude and high frequency (20–30 pulses 24 h^{-1}) LH pulses occur, in the mid-luteal period LH pulses are high amplitude and low frequency (6–8 pulses 24 h^{-1}) and the preovulatory surge occurs on day 18/19 with high frequency and high amplitude pulses occuring during the surge (**Figure 1**). The LH pulse frequency is at a minimum during the mid-luteal phase (days 7–13 of the estrous cycle; 2.7 to 3.4 pulses per 12 h window); LH pulse amplitude increases from early (0.5 ng ml^{-1}) to mid-luteal phase (1.04 to 1.3 ng ml^{-1} on days 8–11); subsequently decreases to 0.7 to 0.8 ng ml^{-1} on days 12–14; and recovers to about 1.0 ng ml^{-1} from days 15–19.

There also appear to be subtle changes in pulsatile secretion of LH during the different phases of a follicular wave. LH pulse frequency decreases between day 5 (first day of dominance; 7.5 ± 0.4 pulses 12 h^{-1}) and day 8 (end of growth phase of the first dominant follicle; 5.7 ± 0.8 pulses 12 h^{-1}) while LH pulse amplitude increases between day 5 (0.45 ± 0.04 ng ml^{-1}) and day 11 (loss of dominance of the first wave dominant follicle; 1.1 ± 0.2 ng ml^{-1}). Thus, there are good characterizations of the pattern of LH secretion during the estrous cycle. However, the relationship of LH pulse pattern to the stage of the follicle wave has only been characterized for the first wave, so the precise role of LH in controlling follicular dynamics throughout the entire estrous cycle remains somewhat unclear. Nonetheless, it is hypothesized that LH pulse frequency decreases once a follicle is selected to become dominant, with an associated increase in LH pulse amplitude; an increase in LH pulse frequency and a decrease in amplitude occurs when a nonovulatory dominant follicle undergoes atresia or both frequency and amplitude increase as a dominant follicle proceeds to ovulate during the follicular phase. Further evidence for the role of increased LH pulse frequency at later stages of follicular development is provided by the fact that LH receptors are acquired by granulosa cells of growing 'selected' follicles once they reach 8 mm in diameter. This is further supported by the fact that dominant follicles may be maintained for prolonged periods of time following artifical induction of luteal phases using low levels of progesterone (in the absence of endogenous CL) during which an increase in LH pulse frequency occurs. The pattern of secretion of LH at the time when a dominant follicle is selected is responsible for determination of the fate of that dominant follicle. Luteal phase LH pulse frequencies allow dominant follicles to turn over and undergo atresia; whereas, follicular phase LH pulse frequencies are associated with dominant follicles that ovulate.

Ovarian steroid secretion is also controlled by LH and FSH. Estradiol is secreted predominantly by the physiologically active dominant follicle. Cyclical changes in estradiol that correspond to the periods of growth of each follicular wave have been reported. Estradiol concentrations in the utero-ovarian vein increase during the follicular phase reaching a peak coincident with the LH surge. This is followed by a second increase in estradiol 3–7 days after the LH surge and a mid-luteal increase in estradiol (days 13–15 of the estrous cycle). These increases in estradiol in the utero-ovarian vein coincide with the first and second dominant follicles that develop during the estrous cycle. Furthermore, estradiol concentrations in plasma collected from the posterior vena cava are pulsatile, throughout the estrous cycle, with each pulse of estradiol following a LH pulse. Androgen secretion occurs in the thecal cells and appears to be controlled by LH in both sheep and cattle. In the granulosa cells, androgens are converted to testosterone which is aromatized to estradiol-17β under the influence of FSH. Serum estradiol concentrations are elevated 1–2 days prior to the LH surge reaching a peak of 9.7 pg ml^{-1}. This increase in estradiol in the follicular phase acts in a positive feedback mechanism (**Figures 1** and **2**) to induce the LH (and FSH) surge required for ovulation.

Once the corpus luteum is formed after ovulation, progesterone secretion from the CL increases and remains elevated throughout the luteal phase of the cycle. Both estradiol and progesterone act in a feedback mechanism to control gonadotropin secretion. Estradiol and high progesterone concentrations during the luteal phase of the cycle act to decrease pulsatile secretion of the gonadotropins. In the presence of low progesterone concentrations during the follicular phase, GnRH and LH pulse frequency increase ultimately reaching a GnRH and LH (and FSH) surge required for ovulation.

A further hormone family termed inhibins, secreted by the granulosa cells of follicles, is also implicated in the control of gonadotropin secretion. Inhibin has been defined as a hormone which selectively suppresses the synthesis and secretion of FSH. Inhibin is a dimeric glycoprotein consisting of two subunits, termed α and β, linked by disulfide bridges. Both subunits are secreted as large precursor molecules and can occur in many sizes and combinations. For example, >160, 108, 95, 78, 57, 47, 32 and 29 kDa forms of inhibin have been isolated. However, bioactive inhibin is usually defined as being the 32 kDa form of the α–β dimer. There is a lot of confusion as to the exact mechanisms by which all the inhibin forms control and affect follicular growth. Evidence exists that inhibins have a local as well as a systemic role in controlling follicular growth.

Corpus Luteum Function

The CL forms from the collapsed preovulatory follicle after ovulation. In the cow, the weight and progesterone content of the CL increase rapidly between days 3 and 12 of the estrous cycle and remain relatively constant until day 16. The main function of the CL in cattle is to secrete progesterone. In cattle progesterone and its metabolite δ-pregnene-20β-ol-3-one are the major progestagens secreted by the CL. Progesterone is secreted both from CL formed during normal estrous cycles and pregnancy. During pregnancy, progesterone plays a similar role in decreasing gonadotropin secretion and prevention of behavioural estrus as occurs during the luteal phase of the estrous cycle.

Luteinizing hormone is the major luteotropic hormone in cattle and is responsible for stimulating luteinization of the theca and granulosa cells of the preovulatory follicle into luteal cells. Luteal cells may be classified into small and large cell types both of which secrete progesterone. Small bovine luteal cells appear to secrete progesterone in response to LH stimulation, while large bovine luteal cells produce greater amounts of progesterone under basal conditions and are generally insensitive to exogenous LH stimulation.

Luteolysis of the Corpus Luteum

Prostaglandin $F_{2\alpha}$ (PG) is secreted by the uterus in cows. It is the major luteolytic hormone in ruminants. During the late luteal phase of a normal cycle PG is secreted in a pulsatile pattern from the uterus, but during the equivalent period of early pregnancy, pulsatile PG secretion is attenuated. The presence of an embryo prevents luteolysis by suppressing the ability of the uterus to release PG in a pulsatile manner rather than reducing the ability of the uterus to synthesize PG. This pulsatile secretion of PG induces luteolysis. Uterine PG reaches the CL through a local mechanism of countercurrent exchange between the uterine vein and the ovarian artery (**Figure 3**).

Oxytocin plays an integral role in induction of PG release required for luteolysis. It stimulates the uterine endometrium to secrete PG *in vitro* and *in vivo*. Oxytocin along with estradiol potentiates the release of PG from the uterus; estradiol from the ovulatory dominant follicle appears to stimulate the development or formation of oxytocin receptors in the uterus and enables increased binding of oxytocin, which in turn stimulates PG synthesis from the nonpregnant uterus. Pulsatile PG secretion in ruminants is associated with pulsatile oxytocin secretion. Pulses of oxytocin occur concurrently with PG during luteolysis and most of this oxytocin appears to be luteal in origin. Uterine PG and luteal oxytocin comprise a positive feedback loop, and PG can stimulate luteal oxytocin secretion. The trigger for luteolytic pulses of PG to be secreted from the uterus has not been identified but oxytocin from the posterior pituitary has been suggested by some authors to be involved.

In species such as cattle that require PG for luteolysis, oxytocin can only stimulate the uterus to secrete pulsatile PG during the late luteal phase. Thus, one or more components of pulsatile PG release must be absent in the uterine endometrium during the nonresponsive stage of the estrous cycle. Endometrial oxytocin receptors only increase approaching the time of luteolysis and their absence is thus a likely candidate for limiting the responsive period for PG secretion. The control mechanisms of induction of oxytocin receptors in the uterine endometrium and the secretion of PG appear to involve the steroid hormones progesterone and estradiol. In ovariectomized ewes or cows, oxytocin will only stimulate PG secretion after the animal has been exposed for 7–10 days to luteal phase progesterone concentrations. Some of the processes that are required to supply precursors for PG secretion appear to be progesterone-dependent, such as accumulation of lipid droplets and triglycerides in the uterine endometrium. Estradiol administration during the mid-luteal phase of the cycle will induce premature CL regression. In sheep, acute treatment with estradiol on days 9 and 10 of the cycle induces an increase in endometrial oxytocin receptors within 12–24 h and premature luteal regression. Thus, progesterone and estradiol both appear to be necessary in the development of uterine oxytocin receptors.

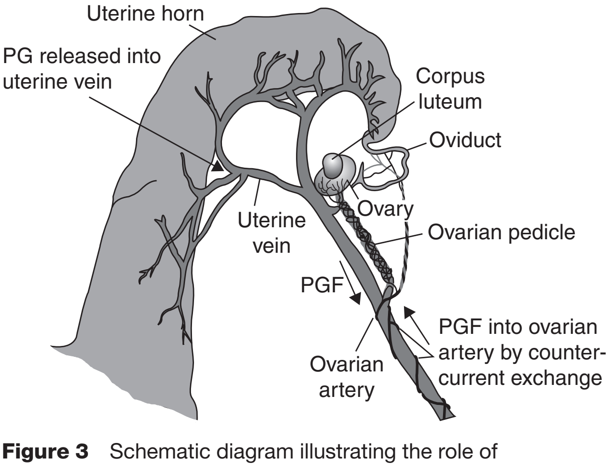

Figure 3 Schematic diagram illustrating the role of prostaglandin $F_{2\alpha}$ (PG) in controlling luteolysis. Prostaglandin released from the uterine endometrium into the uterine vein is picked up by the ovarian artery through countercurrent exchange and is delivered back to the CL as a local mechanism where it causes luteolysis. (Adapted and reprinted from CD-ROM *Learning Reproduction in Farm Animals*, with permission of Rodney D. Geisert, Oklahoma State University.)

Estradiol appears to have the additional role of enhancing secretion of oxytocin from the posterior pituitary and infusion of low doses of estradiol to ovariectomized ewes results in pulsatile secretion of oxytocin from the posterior pituitary. Thus, the mechanism of luteolysis appears to be initially triggered by increased estradiol from the dominant follicle that increases oxytocin pulse frequency from the posterior pituitary, which in turn will stimulate PG secretion from the uterus during the late luteal phase, i.e. when oxytocin receptors are present on the endometrium. The initial secretion of PG stimulates luteal oxytocin secretion which in turn stimulates further PG secretion from the uterus and causes the uterus to become temporarily refractory to oxytocin for 6–8 h, thus establishing a pulsatile pattern of uterine PG secretion at 6–8 h intervals.

In cattle and sheep, the presence of an embryo appears to inhibit the formation of oxytocin receptors in the endometrium, thereby preventing pulsatile release of PG required for luteolysis. The presence of certain proteins from the developing embryo in the uterus, e.g. trophoblast protein-1 (interferon-τ), prolongs the estrous cycle, presumably because they inhibit pulsatile release of PG by some mechanism. Interferon-τ is now recognized as the key mediator of maternal recognition of pregnancy in cattle.

Conclusions

It is concluded that the estrous cycle in cattle is typically 18–24 days in duration, with estrous behavior being expressed for an 8- to 24-h period during the late follicular phase. During the normal estrous cycle there are typically two to three and occasionally four waves of follicular growth each involving a period of emergence and selection followed by either atresia or ovulation of the dominant follicle. The gonadotropin hormones FSH and LH are the main regulators of folliculogenesis and steroidogenesis with LH being the major luteotropic hormone. LH pulse frequency is the major determinant affecting the ultimate fate of a selected dominant follicle. Pulsatile prostaglandin $F_{2\alpha}$ of uterine origin is the main hormonal signal that induces luteolysis of the corpus luteum and the switch from the luteal to the follicular phase.

See also: **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity; Estrous Cycles: Puberty; Mating Management: Detection of Estrus.

Further Reading

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Estrous Cycles: Postpartum Cyclicity

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Introduction

In dairy cows, the interval from calving to resumption of ovulatory ovarian cycles has increased and fertility has decreased over the past half century as average milk production per cow has increased more than threefold. Understanding the physiological processes that regulate ovarian follicular development and ovulation is a necessary prerequisite for shortening the interval to first ovulation, increasing fertility, and optimizing reproductive management in modern dairy operations.

During pregnancy, cows have a corpus luteum (CL) and do not ovulate additional follicles. Nevertheless, follicular growth in waves continues throughout pregnancy. Wave activity decreases as pregnancy progresses and dominant follicles are less prominent. Coincident with the reduction in follicular wave activity during pregnancy is a reduction in the pulsatile release of circulating luteinizing hormone (LH) in blood. Following parturition, ovarian follicular growth and ovulatory follicular cycles must be reinitiated if the subsequent pregnancy is to be established within a reasonable amount of time. The mechanisms associated with the resumption of ovulatory waves postpartum are complex and there is a large variation in the interval from parturition to first ovulation in lactating dairy cows. This article will review the timing of postpartum ovarian cyclicity in dairy cows and the mechanisms associated with normal and abnormal ovarian cycles during the postpartum period. The cellular and molecular mechanisms regulating ovarian follicular development and ovulation as influenced by body condition and energy balance will be discussed.

Ovarian Follicular Dynamics during Estrous Cycles in Cattle

The characteristics of estrous cycles in cattle have been reviewed (*see* **Reproduction, Events and Management**: Estrous Cycles: Characteristics). However, a brief summary here is pertinent to the discussion of postpartum ovarian activity. The dynamic nature of ovarian follicular growth in cattle was unknown for a considerable period of time. A typical estrous cycle in cattle has two or three waves of follicular growth. The initiation of each follicular wave (recruitment) is characterized by the growth of a cohort (usually 2–6) of small follicles from approximately 2–4 to $\geq$5 mm in diameter. The initiation of each wave of follicular growth is preceded by a transient increase in circulating follicle-stimulating hormone (FSH). The recruited follicles continue their growth to approximately 7–8 mm in diameter. At this time, one follicle is typically selected (selection) to continue to grow to ovulatory size (14–18 mm in diameter; dominant follicle). The remaining subordinate follicles undergo atresia. If the cow is in the luteal phase of the estrous cycle, the dominant follicle maintains its maximum size for 3–6 days, but undergoes atresia and another wave of follicular growth is initiated. The dominant follicle in the second or third wave will ovulate if luteal regression occurs during the growing phase.

Postpartum Follicular Growth

Gonadotropins

At parturition, concentrations of LH, but not FSH, are low. In particular, mRNA expression of the β subunit of LH is low, and there is little LH in the pituitary or in blood circulation. With increasing time following parturition, synthesis and release of the gonadotropins increase. There is little release of LH following gonadotropin-releasing hormone (GnRH) injection for the first week postpartum, but the mean concentration of LH and the pulsatile release of LH increase thereafter, and a GnRH-induced release of LH capable of inducing ovulation is possible within 2 weeks postpartum. Similar to initiation of follicular waves during normal estrous cycles, a transient increase in FSH precedes initiation of waves of follicular growth postpartum. The first wave of follicular growth is usually initiated within 5–10 days postpartum. Growth of follicles to approximately 9 mm in diameter follows the stimulation of the transient increase of FSH. Growth beyond this size is dependent upon additional stimulation with LH in addition to the FSH. When adequate concentrations of LH are present, follicles continue their growth to ovulatory size.

Follicular Growth

Waves of follicular growth following calving are similar to those seen in normal estrous cycles and are usually initiated within 10–14 days. With each wave of follicular growth, a cohort of follicles is recruited to grow above

5 mm in diameter and the follicles continue their growth to about 7–8 mm in diameter, whereupon one follicle is selected for further growth. The selected follicle of the first follicular wave follows one of three fates: (1) it continues its growth to ovulatory size and ovulates (cyclic cows); (2) it grows to various sizes but not ovulatory size, stops growth, and undergoes atresia, following which a new wave of follicular growth is initiated (anovulatory anestrous cows); or (3) it surpasses ovulatory size and develops into an ovarian follicular cyst (cystic cows).

Postpartum Ovulatory Follicles

Postpartum follicles that ovulate probably do so through the stimulatory effects of normal levels and secretory patterns of FSH and LH. Dairy cows that ovulate the first wave dominant follicle postpartum are generally in good body condition and are not experiencing extreme negative energy balance. Cows in poor body condition, in extreme negative energy balance, or with metabolic or infectious diseases at calving often have delayed initiation of follicular waves, multiple follicular waves before first ovulation, and extended intervals to first postpartum ovulation.

Postpartum Anovulatory Follicles

In some cows, the first follicular wave postpartum does not end in ovulation, but instead ends in atresia. In cows that do not ovulate the first wave dominant follicle that occurs following parturition, initiation of follicular growth may occur at the same or at a later time than those that ovulate the first wave dominant follicle. Recurrent follicular waves usually occur in postpartum cows that do not ovulate the dominant follicle (**Figure 1**). While the processes of recruitment, selection, dominance, and atresia of follicles in anovulatory waves are generally similar to those observed in ovulatory waves, some differences have been noted. Anovulatory follicular waves are usually longer than ovulatory waves, and the maximum size of the selected follicle is usually smaller. The growth rate from recruitment (5 mm in diameter) to selection (7–8 mm in diameter) and to maximum size of the anovulatory dominant follicle is usually similar to that of the ovulatory follicle. Instead of reaching ovulatory size (16 mm in diameter), however, the anovulatory follicle usually stops growing when it is 10–14 mm in diameter. The smaller-sized follicles produce less estradiol than the larger ovulatory follicles, and the amount of estradiol produced is likely less than the threshold amount needed to induce the preovulatory LH surge. The reason that anovulatory follicles stop growing at the smaller size and have decreased synthesis and release of estradiol may be because of decreased LH secretion or decreased responsiveness of follicles to LH support. These mechanisms will be discussed in the following sections.

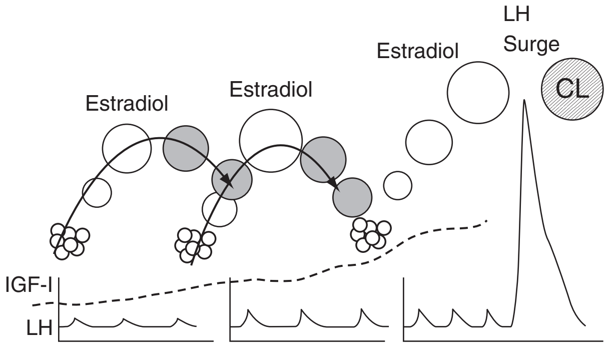

Figure 1 Development of ovulatory follicles in postpartum cows. Ovarian follicles grow in waves during the postpartum period. Successive waves produce larger dominant follicles that secrete greater amounts of estradiol. The luteinizing hormone (LH) surge is induced and the corpus luteum (CL) is formed when estradiol concentrations reach a threshold level. Blood concentrations of insulin-like growth factor I (IGF-I) (dashed line) and LH (pulses) increase during the postpartum period from basal levels (first few days postpartum) to greater levels (3–4 weeks postpartum). The postpartum increase in LH and IGF-I depends on the nutrition and body condition of the cow. The LH and IGF-I synergistically promote follicular growth and development.

In some dairy cows, the initiation of follicular waves is delayed for extended periods of time after parturition. Cows that do not initiate follicular waves postpartum are usually in extremely poor body condition at calving or have severe metabolic or infectious diseases that cause excessive loss of body condition and poor health. In general, any deleterious postpartum health problem increases the interval to initiation of follicular waves and ovulation, and decreases fertility (**Table 1**) .

Mechanisms Associated with Ovulatory and Nonovulatory Postpartum Follicles

Initiation of postpartum ovarian cyclicity is related to body condition at calving and to negative energy balance following calving, which affects the change in body condition during the postpartum period. Cows should be in good

Table 1 Influence of health status on reproductive performance of dairy cows

	Healthy cows (n = 38)	*Cows with major health problems (n = 26)*
Days to first estrus	37.7	51.2
Days to first insemination	58.7	68.4
Days to pregnancy	71.9	84.1
Cumulative pregnancy rate (%)	88.9	63.2

Adapted from Barton, *et al*. (1996) *Journal of Dairy Science* 79: 2225–2236.

body condition at calving (body condition score (BCS) should be 3–3.5 on a 5-point scale, where 5 is obese and 1 is very thin). High-producing dairy cows are usually in a negative energy balance following parturition because there is a dramatic change in energy requirements to meet the energy demands of lactation. There is an immediate shift in metabolism from nutrient partitioning toward body reserves and fetal mass to one of nutrient mobilization of energy and protein stores to meet the demands of lactation. Energy and feed intake are not maximized in lactating dairy cows for a few weeks following parturition. It is during this time that negative energy balance reaches its maximum, and the resumption of postpartum cyclicity is dependent upon the severity of the negative energy balance and body condition of the cow. The effects of various nutritional and environmental factors on postpartum cyclicity are described below.

Body Condition

Attaining the optimum BCS of 3.0–3.5 during the dry period is important for reestablishment of ovarian cyclicity during the postpartum period. Cows that are overly fat (BCS near 5) have a decreased appetite following parturition compared with more ideally conditioned cows (BCS 3.0–3.5) and, thus, are in a greater negative energy balance and have a much larger change (decrease) in BCS. Cows that have increasingly larger negative changes in BCS during the postpartum period take a longer time to achieve first ovulation postpartum, and fertility is decreased.

In addition, some cows calve with less than optimum body condition. Cows calving with BCSs of 1.5 or less have a longer interval to first ovulation than those that calve in better condition. Cows in very poor body condition do not have energy stores available to mobilize for maintenance and lactation requirements. It has long been known that the body diverts energy from reproductive processes when conditions do not meet the needs for maternal survival. Cows that experience the greatest change in body condition after parturition take longer to achieve ovulation than those that have less change in body condition, and conception rates of cows with a greater BCS loss are lower than those of cows with less BCS loss postpartum. Similarly, cows in a lesser negative energy balance or in a positive energy balance have greater conception rates than those with a more negative energy balance.

Energy Balance

As stated previously, high-producing dairy cows are in a negative energy balance following parturition. The negative energy balance increases for a period of time as milk production increases faster than feed intake. When the amount of negative energy balance decreases, events leading up to the first ovulation begin. Ovulation of a dominant follicle postpartum is dependent upon stimulation through increasing mean concentration and pulsatile release of LH (**Figure 1**). Pulsatile release of LH is greater in cows that ovulate a dominant follicle in the postpartum period than in those that have recurrent follicular waves, and the size of the ovulatory dominant follicle is usually larger than the nonovulatory dominant follicles. In most cases, follicles that fail to ovulate do not reach full ovulatory size. Low energy availability decreases LH pulsatility and, thus, follicular stimulation and estradiol synthesis are less. Also the responsiveness of follicles to LH may be decreased because of lower concentrations of circulating insulin-like growth factor I (IGF-I) (**Figure 2**). The concentrations of circulating IGF-I are directly correlated with energy status. Cows in a greater negative energy balance have lower concentrations of circulating IGF-I. The IGF-I concentrations are greater in cows that are ovulating.

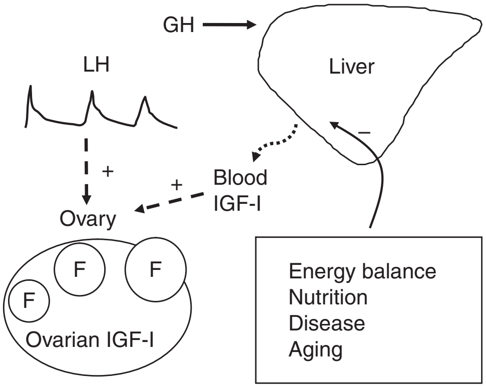

Figure 2 Control of follicular growth by luteinizing hormone (LH) and insulin-like growth factor I (IGF-I) secretion in postpartum cows. At parturition, pulses of LH and concentrations of IGF-I in blood are low. Blood growth hormone (GH) concentrations are elevated and GH drives nutrient partitioning for milk synthesis. Most of the IGF-I in blood is derived from the liver and is released in response to GH. In early postpartum cows, the GH–IGF-I axis is uncoupled so that high concentrations of GH do not lead to elevated blood IGF-I. Negative energy balance, undernutrition, disease, and aging increase the amount of uncoupling and reduce IGF-I synthesized and secreted from the liver. The GH–IGF-I axis is recoupled postpartum so that the synthesis and secretion of IGF-I into the blood are increased. Pulses of LH increase postpartum and stimulate follicular growth. The greatest LH pulsatility is found in cows with better body condition and less negative energy balance. The effects of LH on the ovary are synergistic with IGF-I in blood. Greater IGF-I and more LH pulsatility postpartum act in a synergistic manner to increase the growth and development of ovarian follicles. Factors such as negative energy balance may decrease IGF-I by preventing the recoupling of the GH–IGF-I axis. Negative energy balance may also inhibit LH pulses. Collectively, low IGF-I and low LH pulsatility may not provide adequate stimulation for the development of a preovulatory follicle.

Abnormalities of the Puerperium

Cows must be healthy for efficient postpartum reproduction. Diseased cows are less fertile and the effects of disease on reproductive performance are greater than any other factor. Both metabolic and reproductive diseases and disorders can negatively affect reproduction. Cows with metabolic and reproductive diseases are usually in poorer general health, lose a greater amount of body condition, and experience delayed postpartum ovarian cyclicity. Thus, cows with abnormal puerperium require additional time to establish ovarian follicular waves and develop ovulatory follicles. In addition, fertility is lower in affected cows than in cows with no abnormalities during the periparturient period. Metabolic disturbances and diseases include dystocia, retained placenta, metritis, pyometra, milk fever, ketosis, acidosis, mastitis, laminitis, brucellosis, and tuberculosis. Several studies have demonstrated the relationship between health and reproduction. For example, healthy cows in one study had a shorter interval to first estrus, shorter days to first insemination, shorter days to pregnancy, and fewer services per conception than cows that needed veterinary assistance for postpartum health problems (**Table 2**). Mastitis also has a major effect on reproduction in postpartum dairy cows. Cows that develop clinical mastitis have delayed intervals to first insemination, greater services per conception, and greater days open. A regular herd health program for veterinary care can prevent many deleterious effects of postpartum disease on reproduction.

Ovarian Follicular Cysts (Cysts)

The third fate of follicles during the postpartum period is the development of cysts. Cysts have been classified as anovulatory ovarian follicular structures of at least 2.5 cm in diameter that persist in the absence of a CL for a period of at least 10 days. This definition may no longer be accurate due to the dynamics of follicular growth and the dynamics outlined in previous sections. Nonetheless, the cystic condition is a serious cause of reproductive inefficiency in dairy cattle because cows are infertile as long as the condition persists. It is estimated that 10% of dairy cows develop cysts annually.

Characteristics of Cysts

Follicular dynamics in cows with cysts has similarities to follicular dynamics in normal cows. Development of cysts begins when a cohort of follicles less than 4 mm is recruited to grow beyond 5 mm in diameter. At approximately 7–8 mm in diameter, one follicle (sometimes more than one) is selected for continued growth to become dominant over the other follicles as occurs in normal ovulatory follicles. The growth phase to ovulatory size is similar to ovulatory follicles. However, instead of ovulating, the follicle destined to become a cyst continues to grow for several more days and becomes enlarged and is anovulatory. In some cases, more than one follicle continue growth and codominant or multidominant cysts are formed. The size of the cysts may be slightly less than in the classical definition when more than one cystic structure develops.

Earlier research suggested that cysts persisted for considerable periods of time if left untreated. More recently, studies using ultrasonography or charcoal marking have shown that cysts do not always persist as previously thought. Three fates of cysts have been shown to occur:

1. Persistence, as originally thought. The percentage of cysts that are persistent is approximately 15–20%. Some cysts persist for longer than 60 days, remain dominant, and inhibit follicular growth during this time.
2. Cyst turnover, whereby the original cyst loses dominance, and a new wave of follicular growth is initiated. One (or more) follicle is selected to become dominant from the cohort of new follicles that are recruited, and develops into a new cyst. Cysts that have lost dominance have morphological and endocrine characteristics of atretic follicles. This condition may continue for repeated waves of cyst development, and the anovulatory condition, but not a single cyst, persists. The period of the waves of cyst growth may be similar in length to normal follicular waves, but is, on

Table 2 Effects of problems occurring during lactation on reproductive traits

Item	*Average days to postpartum breeding*	*Average calving interval (days)*	*Average services/ conception*
No problem	86	395	1.8
Metritis	99	433	2.3
Cystic ovaries	107	447	2.1
Retained placenta	92	419	2.0
Anestrus	141	480	2.2
Aborted	80	402	2.4

Data are from 2352 observations in dairy herds (HA Garverick, unpublished data).

the average, twice as long. Cyst turnover is prevalent in most cases (60–65%) of cows with cysts.

3. Turnover with initiation of a new wave of follicular growth whereby the cow self-corrects and ovulates a new dominant follicle. Approximately 20% of cows with cysts self-correct the condition.

Etiology of Cysts

A number of factors including endocrine imbalances, heredity, milk production, and seasonality have been associated with the development of cysts. Endocrine imbalances include various abnormalities of the hypothalamic–hypophyseal–ovarian axis. There is an increase in mean circulating and pulse amplitude secretion of LH during cyst development, and pulse frequency is similar to that observed in a normal follicular phase (**Figure 3**). Both mean concentration and pulse amplitude of LH are nearly twice as high in cows with cysts compared with cows that ovulate. However, there is an absence of the preovulatory LH surge in cows with cysts. Cysts may produce more estradiol than ovulatory follicles, but the LH response to estradiol is absent in cows with cysts. There is no difference in pituitary concentrations of FSH and LH in cows with cysts and cows that ovulate. The concentration of GnRH in the hypothalamus is lower in cows with cysts, but the GnRH content of the median eminence is greater in cows with cysts. Thus, the GnRH content of the median eminence is likely released and caused increased secretion of basal LH.

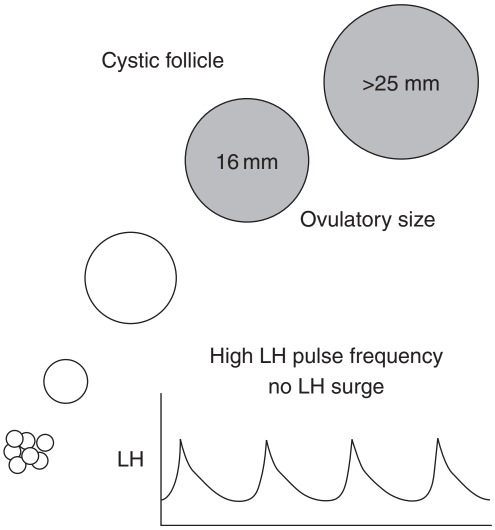

Figure 3 Formation of ovarian follicular cysts in cows. Cysts form when postpartum luteinizing hormone (LH) secretion is highly pulsatile. A large follicle develops on the ovary and secretes estradiol. Estradiol secretion, however, fails to trigger an LH surge and the cow becomes cystic because the follicle does not ovulate.

Heredity has also been associated with cyst development. However, the estimation of heritability has been difficult because of confounding factors such as nutrition, body condition, and milk production. Increased milk production has also been associated with cyst development. However, it is unclear whether increased milk production produced cysts or whether cows produced more milk because they were cystic and were not pregnant for a longer period of time. Season of the year has also been associated with the development of cysts. However, there are reports that did not find a relationship between cysts and the level of milk production or seasonality.

Numerous miscellaneous factors have been linked to cyst development. These include estrogen content of forages, which is consistent with the altered response of the cows with cysts to estradiol as previously mentioned. Abnormal reproductive and metabolic events during the postpartum period have also been linked to development of cysts, suggesting that the increased stress associated with these events contributes to cyst development.

Diagnosis and Treatment of Cysts

Classical diagnosis of cysts was based on behavioral symptoms of intense sexual desire or nymphomania. Cows exhibiting such behavior exhibited estrus for extended periods of time, sometimes with repeated periods between times of no estrual activity. However, it is now clear that most cows with cysts do not exhibit estrous activity (anestrous). Diagnosis of cysts is usually based upon finding a follicular structure of 2.5 cm in diameter or larger following a single examination via manual palpation per rectum or ovarian ultrasonography. While diagnosis based upon the single examination is efficacious for producers, the diagnosis may not be accurate for several reasons. First, size alone is not an absolute criterion because size is influenced by stage of development, which is difficult to know with one examination. Second, there are often large follicles on the ovaries of cows during a normal estrous cycle as previously described. Thus, the dynamic nature of follicular and cyst growth complicates diagnosis. Third, some CLs developing during the first 5–7 days following estrus exhibit characteristics similar to cysts when diagnosis is by manual palpation. Luteal structures during this period are often soft and contain fluid-filled structures that rupture during manual palpation. Diagnosis with ultrasonography greatly reduces this type of diagnostic error.

Cysts are typically treated with GnRH to induce an endogenous LH release, or treated with human chorionic gonadotropin (hCG) that has LH-like activity. In both cases, successful treatment is based upon luteinization of the cystic structure and subsequent production of progesterone in response to the GnRH-induced LH release or the exogenous hCG. Success rates are about 80% with these treatments. Response of the cystic structure is dependent upon its state at treatment. Responding cysts are those that are 'healthy' in that theca and granulosa cells appear morphologically intact and are producing estradiol. More recently, progesterone implants have been used successfully to treat cysts. Treatment with progesterone must raise blood levels of progesterone to high enough levels to mimic luteal phase concentrations. Success of the aforementioned treatments is dependent upon increased concentrations of circulating progesterone that restore the sensitivity of the hypothalamic–pituitary axis to estradiol. With these treatments, concentrations of LH are inhibited, the cyst(s) undergo atresia, and a new follicular wave follows that results in selection of an ovulatory dominant follicle that secretes estradiol. The sensitivity of the hypothalamic–pituitary axis to estradiol is restored and an LH surge and ovulation of the dominant follicle occur.

Conclusion

Initiation of ovulatory ovarian cycles following parturition is a prerequisite for the establishment of pregnancy within an opportune time interval. Following parturition, waves of ovarian follicular growth are usually established within 10–14 days. There are three fates of the first wave dominant follicles. The selected follicle may (1) continue its growth to normal ovulatory size and ovulate, (2) grow to less than ovulatory size, fail to ovulate, and undergo atresia, or (3) surpass ovulatory size and develop into an ovarian follicular cyst. Body condition at calving, negative energy balance, and disease determine the fate of ovarian follicles postpartum (ovulation, anovulation, or cystic) by affecting the endocrinology of the postpartum cow (LH, FSH, and IGF-I secretion).

See also: **Reproduction, Events and Management**: Estrous Cycles: Characteristics.

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Estrous Cycles: Seasonal Breeders

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Introduction

The effects of seasonal influences on reproduction are directly linked to milk yield, as the production of offspring is required for initiation of lactation, and is important in relation to targeting milk markets. For example, in India and Pakistan, water buffaloes calve primarily between July and December, which can result in excess milk production in the winter months and milk shortages during the summer months. Similarly, dairy sheep producers are particularly cognisant of the effects of seasonality on lambing intervals of ewes and on an ewe's lifetime lactation yield in light of the strict seasonal nature of some sheep breeds used in intensive dairy sheep operations. While an understanding of seasonality is critical from a production standpoint, from an evolutionary perspective seasonal breeding has evolved as an adaptation to ensure a favorable reproductive rate and the survival of offspring in regions with great variations in climatic, nutritional, and/or other adverse environmental conditions. The reasoning behind the evolution of these strategies in the context of agriculturally important livestock species will be explored herein, in addition to the physiological basis for seasonality and how it might be controlled in a production setting. For comparative purposes, the seasonal nature of several multipurpose livestock species will be indicated, with emphasis on those used worldwide for the production of milk and milk by-products.

Strategies and Theories for the Seasonal Regulation of Reproduction

Some species do not exhibit seasonal cycles in reproductive activity (nonseasonal breeders), while others may display 'clusters' of reproductive cycles that occur only during a certain season of the year (seasonal breeders). Of the seasonal breeding species, some exhibit reproductive cycles during the short day lengths (autumn; e.g., sheep and goats), while others exhibit reproductive cycles only during the long day lengths (spring; e.g., mare). Patterns of seasonal breeding range from species that have one period of estrus (receptivity and mating) each year (monoestrus), to species that exhibit a series of estrous cycles limited to a portion of the year (seasonally polyestrus). True seasonal breeding patterns are inherent in some species (e.g., ewe, doe, and mare), while other species may show seasonal patterns due to environmental or other overriding influences. Of these, climatic, nutritional, and behavioral effects on seasonal cycles will be described below. However, one should be cognisant of the fact that seasonality is a coordinated effort in which multiple exogenous (environmental) and endogenous (hormonal) rhythms may be involved in the integration of seasonal reproductive cycles.

Climatic

Climatic events that regulate seasonal reproductive processes encompass primarily the effects of photoperiod and temperature, with climatic variables including drought and precipitation influencing reproduction as related to nutrient availability (nutritional considerations will be described in greater detail below). Of these, photoperiod is seen as a primary mechanism driving the endogenous circannual rhythm that synchronizes mating and birthing seasons. The effects of photoperiodism (photic cues) are translated into reproductive effects via hormonal mediators (e.g., melatonin), which augment or suppress endocrine and neuroendocrine pathways critical to reproductive processes (see 'Endocrine and Neuroendocrine Regulation of Reproduction in Seasonal Breeders'). The influence of photoperiod on the reproductive system was first observed in relation to the timing of puberty for lambs born at different times of the year. Specifically, spring-born lambs attain puberty in about 30 weeks of age, which they reach during the breeding season, while fall-born lambs reach the pubertal age of 30 weeks during the nonbreeding season, and thus do not exhibit reproductive cycles until the following breeding season at nearly 1 year of age. Such dramatic effects illustrate the prevailing and central role that photoperiod plays in the reproductive lives of strict seasonal breeders.

In conjunction with or independently from photoperiod, which changes incrementally during the year, alterations in ambient temperatures (high and low) can contribute to shifts in seasonal breeding activities as well. For dairy cattle bred by natural or artificial means in the southern United States and elsewhere, it has been observed that fertility is lowest in late summer when ambient temperature and humidity are high, and is often followed by a slow recovery time thereafter producing a lag effect into the fall. To this end, heat stress in cattle has been the primary focus of research, which has illustrated how severe thermal stress can suppress reproductive processes, negatively impacting livestock production operations – particularly in dairy cattle. Summer heat stress has been shown to lower semen quality in bulls, alter hormonal secretory patterns, suppress estrous behavior, reduce conception rates, and negatively affect embryo quality and survival. Moreover, heat stress during late gestation can adversely affect fetal growth in cattle and sheep, with summer-born calves and lambs being generally smaller than winter-born calves and lambs, and an increased incidence of stillbirths has been observed in swine.

Low temperatures (i.e., cold stress) at the time of birth also have implications relative to offspring survival (e.g., *Bos indicus* calves are more susceptible to cold stress than *Bos taurus* calves), yet in relation to mating success the effects of cold stress are less well characterized in livestock than the effects of heat stress. This is due to the confounding effects of food intake, which increases during colder periods to facilitate metabolic heat production (i.e., maintenance of thermoneutrality) as part of an adaptive response to low environmental temperatures. Nevertheless, appropriate adaptation periods to low environmental temperatures are required to alleviate both endocrine and exocrine effects on reproductive processes in some breeds. For example, in Brahman bulls translocated to the northern United States, reduced concentrations of testosterone and decreased semen quality have been observed during the winter months (0 to −10 °C).

When considering both heat and cold stress together, it seems reasonable that seasonal breeding strategies would have evolved, in part, to coordinate breeding periods for an increased probability of mating success and postparturient neonatal survival in relation to annual changes in environmental temperatures.

Nutritional

Late pregnancy, birth, and lactation require energy and nutrient demands above and beyond what is required for normal body maintenance. This means that conception must occur months earlier in relation to other environmental cues (e.g., photoperiod) to achieve a birthing season that will coincide with the season of greatest abundance in nutrient quantity and quality. When energy and protein, for example, become limiting, a number of reproductive processes may be affected, resulting in delayed puberty, suppressed estrus and ovulation, reduced conception rates, an increased incidence of fetal resorption, and premature or weak offspring. As evident from these examples, the seasonal suppression of puberty or estrous cycles due to nutritional deficiencies could delay transitions into seasonal breeding periods, while deficiencies later in the year may influence survival of the fetus late in pregnancy or the neonate postpartum. Conversely, an abundance of nutrients may permit animals to exhibit reproductive cycles earlier in the season, and adequate nutritional reserves during pregnancy and lactation would directly benefit offspring survival postpartum. Food in this regard has been described as having a 'proximate' and 'ultimate' action in relation to seasonal breeding. Specifically, the quantity of nutrients available can have an immediate beneficial or detrimental effect on an animal's reproduction (a proximate action), and the fact that the presence of nutrient resources can vary seasonally in an animal's environment is an important factor in the evolution of that animal's reproductive strategy (an ultimate action). The tendency toward seasonal breeding begins to manifest primarily in regions where nutrient availability varies somewhat, with increases in the severity of this variation leading to stricter and stricter breeding and birthing seasons out of an apparent necessity for nutrient resources. This is, of course, superimposed on other climatic events (photoperiod, temperature, precipitation, etc.) that can influence growing seasons and plant vigor.

Social/Behavioral

Reproductive processes can be dramatically affected by social cues relayed from one individual to another of the same or opposite sex. This can occur via pheromones, which are chemical substances liberated from one animal (through urine or other secretions) and received by another (olfactory cues), or through visual or tactile cues associated with complex mating rituals. These behavioral or chemical cues have a priming effect that can alter hormonal secretions (primarily luteinizing hormone (LH) and prolactin from the pituitary) and advance the breeding season. In sheep, this is often referred to as the 'ram effect', as ram exposure can increase LH pulsatility in the ewe and elicit signs of estrus in the female that would not normally be present in the absence of a ram. Furthermore, introduction of a ram into an ewe flock during the transition from anestrus to estrus can in turn result in a high degree of synchrony in first mating. A similar reaction is achieved in wapiti (North American elk) and red deer, in which vocalizations from the stag during the early rut can hasten seasonal reproductive cyclicity in females. While in today's livestock management systems such cues have become less vital from a mating strategy standpoint, for animals in the wild with varying social structures (e.g., dominance hierarchies, single-sex groups) and a need to locate receptive mates, such behavioral cues are critical to mating success and genetic survival. While social interactions are not a singular cause for initiating or terminating seasonal reproductive events in most species, they can greatly augment the degree to which an animal responds to other environmental cues regulating seasonal processes.

Endocrine and Neuroendocrine Regulation of Reproduction in Seasonal Breeders

Seasonal breeders are often referred to as undergoing an annual puberty, in that reproductive cycles must begin anew following a period of anestrus (i.e., no estrus or the absence of heat cycles). The principal mechanism responsible for transitions into and out of periods of sexual activity in strict seasonal breeders is mediated by the retinal-hypothalamo-pituitary pathway (**Figure 1**). Specifically, photic cues detected by the sensory neurons in the retina of the eye are transmitted via the suprachiasmatic nucleus of the hypothalamus to excitatory cervical ganglia that can alter the release of the hormone melatonin from the pineal gland and, in turn, influence hypothalamic gonadotropin-releasing hormone (GnRH) and pituitary LH release. The pineal gland is located posterior to the hypothalamus between the hemispheres of the brain, and increased sympathetic activity induced by darkness increases the secretion rate of its primary hormone, melatonin. Exactly how melatonin acts singularly or in a coordinated fashion with other hormones (e.g., norepinephrine) to influence reproductive processes is still unresolved. Nevertheless, in short-day breeders (e.g., sheep and deer), cyclic activity that occurs during the short photoperiods (longer nights) of fall and winter is characterized by greater melatonin release and an active hypothalamic GnRH neurosecretory system, while the long photoperiods (short nights) of

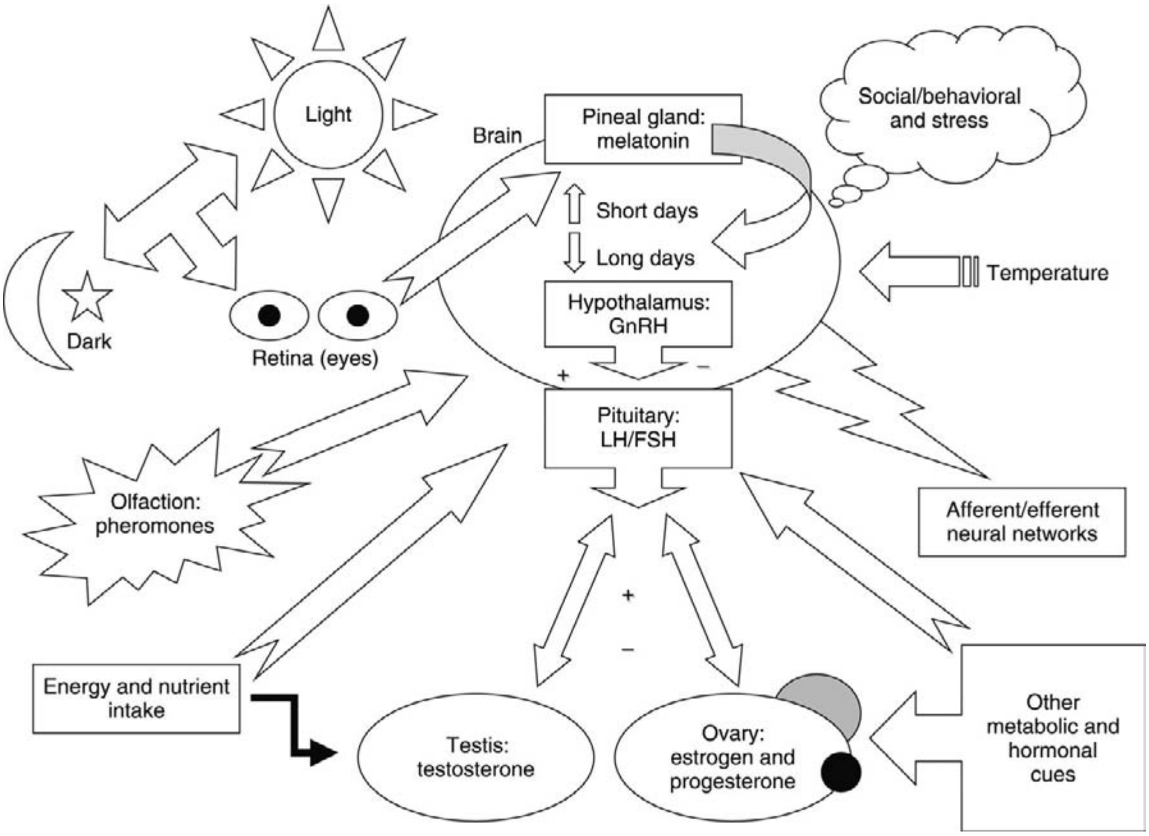
Light
Brain
Pineal gland: melatonin
Social/behavioral and stress
Short days
Long days
Temperature
Dark
Retina (eyes)
Hypothalamus: GnRH
+
–
Pituitary: LH/FSH
Afferent/efferent neural networks
Olfaction: pheromones
+
–
Energy and nutrient intake
Testis: testosterone
Ovary: estrogen and progesterone
Other metabolic and hormonal cues

Figure 1 Integration of environmental cues on endocrine and neuroendocrine pathways regulating seasonal reproduction. A multitude of exogenous environmental factors and endogenous hormonal cues coordinate the seasonal reproductive cycle in short- (e.g., sheep) and long- (e.g., horse) day breeders. Central to seasonal regulation is the role of photoperiod (light:dark cycles), which changes annually (see **Figure 2c**). To this end, photic cues are relayed via the retino-hypothalamo-pituitary pathway using melatonin as a primary regulator of GnRH and LH/FSH release either directly or indirectly by mediating changes in the sensitivity of the hypothalamus and pituitary to the negative feedback effects of gonadal steroids. It has been said that seasonal breeders undergo an annual puberty, with spermatogenesis and estrous cycles beginning anew each breeding season as they make the transition from sexual inactivity into periods of reproductive competence and back again. While strict seasonal breeders are driven principally by photoperiodic cues, other species may exhibit seasonal cycles directed by a variety of climatic, nutritional, and/or behavioral indicators. FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

spring and summer are characterized by lower melatonin release, and thus periods of reproductive dormancy. In long-day breeders like the horse, the relationship between melatonin and season remains the same (high during short days and low during long days), but the effects on the reproductive axis are opposite to that seen in short-day breeders (i.e., for the mare, reproductive dormancy during fall and winter and reproductive competence during spring and summer).

The role of melatonin in coordinating the seasonal regulation of reproduction has been demonstrated in studies where melatonin has been administered to short-day breeders during long days (thus, increasing melatonin and mimicking the effects of short days) to induce gonadal recrudescence. In sheep, the switch from the breeding season to anestrus, or vice versa, is associated with marked changes in hypothalamic GnRH pulse frequency, which has downstream effects on pituitary LH release, gonadal steroid hormone production, and gamete development. There is conclusive evidence that changes in photoperiod can specifically alter the communicative relationship between the gonads and higher brain centers. Indeed, during seasonal transitions in and out of the breeding season in response to changes in photoperiod, there are marked changes in the sensitivity of hypothalamus (GnRH release) and pituitary (LH release) to the negative feedback effects of gonadal steroid hormones in both males and females. These coordinated activities influence not only gonadal activity, but also related behavioral processes and secondary sex characteristics.

Opposite to melatonin, pituitary secretion of prolactin is high when melatonin is low (long days) and vice versa. While prolactin has been implicated as being inhibitory to reproductive function in some short-day breeders and induced (reflex) ovulators (i.e., species that require stimulation of the vagina and/or cervix for ovulation to occur), there is strong evidence that fluctuations in prolactin are not related to the seasonality of mating; however, an endogenous rhythm of prolactin is noted in the ram and stag. Changes in prolactin have been linked specifically to secondary seasonal characteristics, such as coat growth and molt in red deer stags, suggesting that seasonal changes in prolactin may be more related to alterations in temperature. This notion is supported by studies of blind bulls and steers that have shown that photoperiod does not affect prolactin secretion, implicating temperature as the dominant environmental cue regulating prolactin. Thus while photoperiod has been described as being central to the regulation of seasonality, multiple factors can positively or negatively affect the sensitivity and integration of the endocrine and neuroendocrine systems in mediating seasonal cycles and seasonal reproductive transitions (**Figure 1**).

Artificial Manipulation of Seasonal Breeders

Advancement or prolongation of the breeding season in seasonal breeders used in livestock production has been desired in some areas to coordinate breeding seasons with other management-related events (e.g., forage availability). An example specific to the equine racing industry is that foals are routinely assigned a universal birth date of 1 January (northern hemisphere) regardless of when they are born, making advancement of the breeding season beneficial to achieve the birth of foals as early in the year as possible. At present, the primary means to alter circannual rhythms in strict seasonal breeders includes manipulation of photoperiod through exposure of animals to alternate light cycles, and the use of pharmacological (hormonal) means. While these methods will be highlighted in more detail, it should be noted that a variety of other management-related alterations have similarly resulted in a dampening of seasonal influences for some species. For example, seasonal fluctuations in libido and semen quality, estrous activity, and conception rates have been overcome in dairy cattle and water buffalo by providing cooling facilities during heat stress. Additionally, other management practices including early weaning (beef cattle and small ruminants) and increased nutrition (e.g., flushing) have also aided in facilitating early returns to estrus to override any seasonal influences on reproductive function. Finally, behavioral influences should also not be overlooked, as the exposure of ewes to a ram prior to the breeding season can induce early cyclicity (the ‘ram effect’).

Artificial Manipulation of Light–Dark Cycles

The artificial manipulation of light–dark cycles is achieved primarily by altering housing strategies. This can be accomplished by blocking natural light from entering stalls or barns for specified periods of time at the beginning and end of each day, or through controlled lighting. In a long-day breeder like the mare, changes in photoperiod to mimic long days (16 h of light:8 h of darkness) during the nonbreeding season (short days) will stimulate reproductive function in anestrous mares. Conversely, periods of seasonal anestrus in the ewe, a short-day breeder, can be altered by changing day length during long days to mimic short days (8 h of light:16 h of darkness). While dairy cattle are not traditionally considered as seasonal breeders, supplemental lighting (16–18 h of light:6–8 h of darkness) has been shown to boost milk production through increased secretion of insulin-like growth factor-1 (IGF-1), which acts on the mammary gland, and concomitant decreases in melatonin secretion. How IGF-1 is regulated in response to reduced concentrations of melatonin when day length increases remains unclear. To this end, the manipulation of photoperiod to alter melatonin release may have numerous implications in relation to the reproductive and lactational abilities of animals sensitive to photoperiodic cues.

Pharmacological Control: Exogenous Melatonin and Other Hormonal Means

Pharmacological control of seasonal breeding has been achieved in a number of species, and has been implemented as part of routine production management strategies in some areas. As described previously, increasing concentrations of melatonin of pineal origin during short days arouse dormant reproductive processes in short-day breeders. Thus melatonin implants have been inserted in ewes for periods of 30–40 days to advance the breeding season for matings in spring or early summer. However, one problem with using these types of manipulations (melatonin and changes in photoperiod) is that animals can become refractory (i.e., temporarily unresponsive) after a period of time to the stimulatory effects of melatonin and light, thus limiting their use. Moreover, use of melatonin for advancement of the breeding season may not be effective in all seasonal breeding species. For example, in the female goat, melatonin administration has been less successful than in sheep in advancing the breeding season. In addition to melatonin, a whole host of other hormones have been utilized to stimulate reproductive activity during the nonbreeding season or as methods for prolonging the breeding season itself. Specifically, hormonal treatments have included equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), crude pituitary extracts, GnRH, progesterone, and prostaglandins. In camels, GnRH treatment can stimulate sexual activity in males during the nonbreeding season, while in other induced ovulators such as the llama and alpaca, hCG and GnRH have been used to induce ovulation outside of normal periods of sexual receptivity. Taking management strategies a step further, combinations of artificial lighting and pharmacological approaches have also been used with some success. For example, in the mare, initiation of a 16-h photoperiod for 60 days prior to treatment with altrenogest (a synthetic progestin) for 12 days followed by the administration of hCG on day 2 of estrus has been reported to be an effective regime for induction of estrus and ovulation in the mare early in the year outside of the normal breeding period. Such lighting and hormonal combinations and other clinical therapies abound for short-day and long-day breeders alike, with the intent to modify periods of sexual activity to meet management constraints, whether environment- (e.g., temperature or forage availability) or industry- (e.g., the equine racing industry) related.

Seasonal Breeding in Domesticated and Semidomesticated Multipurpose (Meat and Dairy) Livestock

While not all of the livestock species described herein are used in typical dairy production operations (e.g., swine), for comparative purposes the seasonal nature of reproductive cycles, whether endogenously generated or due to exogenous environmental cues, will be discussed briefly for each species indicated. As described previously, photoperiod, temperature, and a multitude of other climatic and/or nutritional factors are the driving forces regulating the timing of breeding and birthing seasons in most species.

Domestic Cattle: Temperate (*Bos taurus*) and Tropical (*Bos indicus*)

Cattle are nonseasonal and polyestrous, yet the onset of puberty and postpartum reproduction are often stimulated by exposure to long days. Moreover, seasonal trends emerge in some climates due to adverse environmental conditions. Specifically, heat stress is often implicated as the cause of reduced reproductive performance, particularly in temperate, European-type cattle (*B. taurus*). Summer heat stress conditions can lower semen quality in the bull, reduce fertilization rates, affect embryo quality and viability, and result in an overall decrease in conception rates. Of all temperate breeds, dairy cattle (e.g., Holstein) in particular show marked seasonal fluctuations in reproductive function due to the effects of environmental heat stress and to meet the metabolic demands of lactation. In the southern United States, cows calving in spring and summer have reduced reproductive performance, with milk production depressed for cows that calve in summer and fall. During heat stress, dairy cows exhibit shorter less intense periods of estrus (as much as 6–8 h less) and a reduced frequency of mounting activity than during cooler seasons. These effects contribute to a lower estrus detection rate, an increased number of artificial breeding services per conception, and an overall decrease in conception rates in production dairy operations during the summer months. Nevertheless, through the implementation of housing strategies to offset environmental extremes (e.g., cooling with fans and sprinklers during heat stress), seasonal influences on fertility in cattle can be markedly reduced. In contrast to heat stress, cold stress has not been implicated in causing seasonal depressions in fertility in cattle, with the exception of when tropically adapted cattle (Zebu; *B. indicus*) have been translocated to colder environments without appropriate periods for adaptation. To this end, Zebu cattle exhibit the greatest entrainment to seasonal cycles (primarily temperature-related) than all other breeds, with the frequency of estrus, ovulation, and conception rates being higher during the summer months than during the winter months in equatorial Africa.

Water Buffalo (*Bubalus bubalus*)

Water buffaloes are polyestrous and can breed year-round. However, peaks in calving during the year do occur, which indicates possible seasonal effects on fertility that are most likely the result of temperature, photoperiod, and nutritional interactions. The impact of these effects is seen in buffaloes that calve in winter and spring and do not exhibit postpartum estrous cycles as early as cows that calve in summer and fall. Those calving in winter and spring would be returning to reproductive function during the summer months when high temperatures, increased photoperiods, and elevated prolactin levels might prolong periods of anestrus. Moreover, high temperatures (heat stress) may contribute to reduced sexual activity of male buffaloes during this time. In tropical regions where water buffaloes are maintained, conception rates have been observed to be greatest 2–4 months following the peak in the rainy season. This coincides with cooler temperatures and increasing forage availability.

Yak (*Bos grunniens*)

Yaks are considered seasonally polyestrous in their native environments, although female yak showing only a single estrus in a season, even if mating and conception does not occur, is not uncommon. The driving force affecting the onset and end of the breeding season is primarily climatic factors including forage availability and location (latitude). When ambient temperatures and vegetation increase as the winter thaw progresses, females will show an increase in body condition and weight gain, which can initiate cyclicity. The breeding season begins in June and reaches its peak in July and August (in China and upper Mongolia), when temperatures are highest and forage availability is maximal. Estrous activity decreases in frequency and generally stops around November. As the yak is usually found at high altitude, it has been observed that at lower elevations of 1400 m breeding seasons begin earlier (late May), while at higher elevations of 2700 m breeding seasons tend to begin later (late June).

Sheep (*Ovis aries*) and Goats (*Capra hircus*)

In temperate regions, both sheep and goats are seasonally polyestrous. Seasonality is driven by photoperiodism with increased estrous activity during decreasing day lengths. In tropical regions, sheep and goats tend to breed throughout the year. Nevertheless, even in tropical environments, feed restriction (dry seasons) and high temperatures may cause a suppression of sexual activity, but when rainy seasons resume sexual activity increases. Genetics plays a large role in the breeding seasons of sheep and goats, with some breeds showing longer breeding seasons (sheep: Dorset, Merino, and Rambouillet; goats: Anglo-Nubian) than others with more restrictive breeding seasons (sheep: Southdown, Shropshire, and Hampshire; goats: Toggenburg, Saanen, and French Alpine). Unlike the female, the male is less restrictive with respect to seasonal breeding activity, although sexual activity is greatest in the fall in response to decreasing day length when the secretion of testosterone, testis size, and testicular spermatogenesis increase. As such, melatonin interacts with the neuroendocrine axis mediating the sensitivity of higher brain centers (hypothalamus and pituitary) to the negative feedback effects of steroid hormones in both sheep and goats (see general model depicted in **Figure 1**).

Deer (Cervidae species)

The majority of deer are classified as seasonal breeders that are polyestrous, short-day breeders. Nevertheless, 19 species of the 40 or more existing deer species are found in equatorial regions (between 20° N and 20° S latitude) and exhibit nonseasonal breeding capabilities, although peaks in breeding and fawning seasons differ markedly among tropical deer species – some of which inhabit the same ecosystem. In white-tailed deer (*Odocoileus virginianus*), for example, the distribution of this species extends from 55° N to 18° S latitude, with white-tailed deer in temperate regions breeding strictly seasonally, while those in tropical climates (Caribbean, Central America, and northern part of South America) breeding year-round. In addition to short-day and nonseasonal breeders, one species, Peré David's deer (*Elaphurus davidianus*), has an advanced onset of the breeding season, which begins mid-summer (3–4 months before typical short-day breeders) when day lengths are long, melatonin is low, and prolactin levels are high. Thus, some have referred to the Peré David's deer as a long-day breeder. While photoperiod is the primary factor that mediates seasonal cycles in most deer, an inherent rhythmicity in metabolism, antler and coat (pelage) growth, and hormonal cycles is evident when photoperiods are altered dramatically or eliminated completely. This suggests entrainment of reproductive and metabolic cycles to an endogenous rhythm that is overlaid on the influence of photoperiodic cues. Even in tropical species of deer (e.g., axis deer; *Axis axis*), circannual cycles of antler growth, testis size, and body weights are observed, yet estrous cycles, testicular spermatogenesis, breeding, and fawning occur throughout the year. For comparative purposes, the year-round estrous cycle activity of a strict seasonal breeder

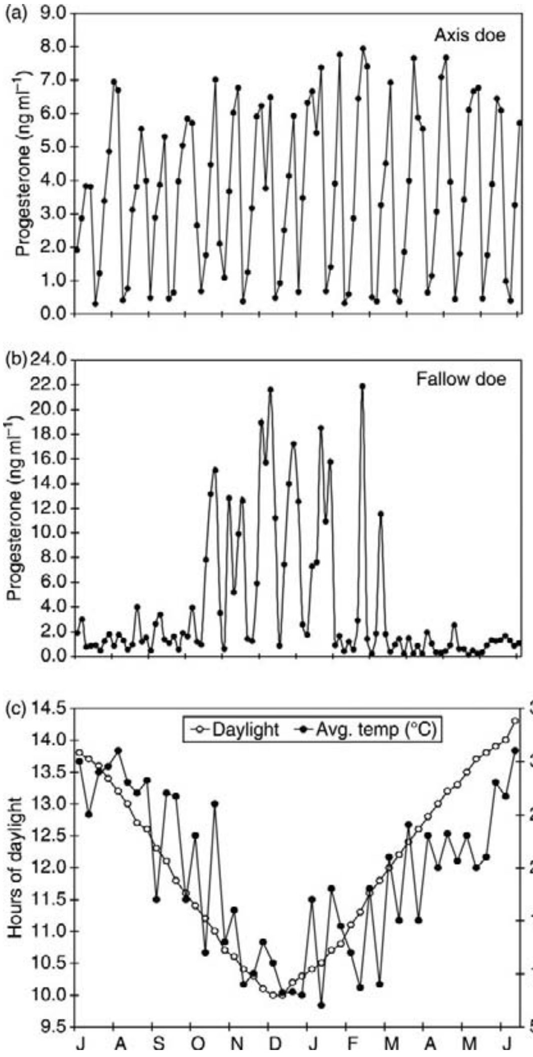

Figure 2 Serum concentrations of progesterone depicting the estrous cycles of nonseasonal (axis deer (a)) and seasonal (fallow deer (b)) species of farmed deer in relation to photoperiod and ambient temperature (c). The axis deer is a tropical deer species that is a nonseasonal breeder and exhibits continuous estrous cycles irrespective of changes in photoperiod (a). Note the presence of ~18 estrous cycles throughout the 346-day sampling period for the axis doe depicted here (serum samples for progesterone analysis were collected twice weekly). In contrast, the seasonally polyestrous fallow doe shown in panel b exhibits only 6–7 estrous cycles annually, which are restricted to the short photoperiods between October and March. Both deer were maintained at the same location at the Texas Agricultural Experiment Station in Overton, Texas (32°16′N; ST Willard and RD Randel, unpublished data).

(e.g., fallow deer (*Dama dama*)) and a nonseasonal breeder (e.g., axis deer) is shown in **Figure 2** for axis and fallow does maintained at the same location in Texas. Irrespective of the nonseasonal nature of some deer species, seasonal peaks in the frequency of breeding and fawning are still noted in tropical deer, and are attributed to climatic and/or nutritional cues to achieve fawning during periods that would favor fawn survival. This is true for all deer, as evidenced by the fact that late-born fawns show lower survival rates due to a shortened lactation (early weaning), low birthweights, and winter death loss, while early-born fawns exhibit similarly low birthweights and may succumb to summer thermal stressors. Thus a window of opportunity exists for fawns born neither too late nor too early, which would favor their survival. For reindeer (*Rangifer tarandus*) in the arctic, highly variable rates of fertility are noted with reproductive success intimately linked to body fat reserves and the timing of births directed toward snow melt and the emergence of new plant growth. As in sheep and goats, reproductive cyclicity in deer is driven by the myriad of hormonal interactions that occur between melatonin, prolactin, GnRH, gonadotropins, and gonadal steroid hormones in response to photoperiodic, metabolic, and/or other exogenous and endogenous cues.

Camel (*Camelus dromedarius*)

Female camels are typically seasonally polyestrous and are induced ovulators. Decreasing day length appears to be the stimulus for reproduction in most regions, although in equatorial locations when adequate rainfall and nutrients are available year-round breeding can occur. It is well documented that by providing sufficient food, nutrition may override the effects of photoperiod and increase sexual receptivity in the female. Male camels also exhibit seasonal sexual activity, with higher concentrations of testosterone and increased spermatogenesis during cooler months. Nevertheless, spermatogenesis can continue throughout the year in the male, reaching a peak during the rutting period. Increased prolactin (hyperprolactinemia) during the nonbreeding season has been suggested as the cause of reduced fertility and sexual activity via prolactin-induced inhibition of gonadotropin (FSH and LH) secretion, although more research in this area is needed.

Llama (*Lama glama*) and Alpaca (*Lama pacos*)

Like camels, llamas and alpacas are induced ovulators that show rhythmic patterns of follicular development and periods of sexual receptivity. In their native highlands of Peru, llamas and alpacas exhibit seasonal sexual cycles from December to March, which are the warmer summer months for this region. However, in these wild settings, males and females are generally together throughout the year, and when females are separated from males in production management settings and pair-mated monthly, they will exhibit sexual activity year-round. Therefore, seasonal breeding can be directly influenced by the continuous contact of females with males, versus when males and females are managed separately. Ovulation rates, fertilization rates, and embryo survival do not appear to be affected by the season in which breeding occurs under these management circumstances. Male llama and alpaca produce fertile ejaculates throughout the year, although the quality of the ejaculate is directly affected by season and nutrient availability, with higher testosterone and greater spermatogenesis during the spring and summer months (the peak breeding periods). The degree to which other environmental cues (visual, olfactory, etc.) may influence reproductive processes in the llama or alpaca is unclear. However, like camels, food availability may directly influence the existence of nonseasonal breeding activity. In North America, llama births are observed throughout the year, reaching a peak during the warmer months of June–November.

Horse (*Equus caballus*)

Mares are seasonally polyestrous, long-day breeders. However, considerable variation exists among breeds and with respect to location (latitude). Some mares exhibit a strict breeding season accompanied by estrus and ovulation, while others may have a defined fertile breeding period preceding and followed by a transitory period in which estrous cycles are present but may not be accompanied by ovulation. Still others may show continuous, year-round periods of estrus and ovulation such as for those mares maintained in equatorial regions. It is well accepted that photoperiod is the primary regulatory mechanism controlling seasonal breeding in the mare as mediated by the hormonal interactions of melatonin, prolactin, GnRH, FSH, LH, and estradiol. Unlike the mare, the stallion does not show as defined a breeding season, with fertile ejaculates capable of being collected throughout the year. Nonetheless, seasonal differences in reaction time, mounting frequency, testis size, semen volume, numbers of spermatozoa, and other semen quality characteristics are observed in the stallion, decreasing during short photoperiods and increasing during long photoperiods.

Swine (*Sus domesticus*)

Swine are polyestrous and nonseasonal. Nevertheless, fertility (mean litter size) declines sharply when photoperiods are long and temperatures become elevated (summer months). In addition to effects on litter size, increased ambient temperatures can cause sperm output and the motility of spermatozoa in the boar to decrease, which undoubtedly contribute to a decrease in fertilization rate and subsequently litter size. Unlike dairy cattle, which show a decrease in the duration of estrus during the hotter summer months, periods of estrus in sows and gilts are longest during summer and shortest in winter. Today, any traces of seasonality in swine breeds have been, for the most part, completely controlled or eliminated due to the enclosed housing environments within which most swine are currently maintained.

See also: **Stress in Dairy Animals:** Heat Stress: Effects on Milk Production and Composition; Heat Stress: Effects on Reproduction.

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Control of Estrous Cycles: Synchronization of Estrus

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Introduction

Techniques for synchronizing the onset of estrus in dairy cattle and other species have been available since the 1960s. Research effort to improve the performance of estrus synchronization programs continues to this day and more work is required to make estrus synchronization more acceptable on the farm. The reasons for using estrus control vary between breeds and species and between different farming systems. In dairy cattle, estrus synchronization is often used to aid mating management by improving the efficiency and accuracy of detection of estrus (heat) or by reducing the labor requirement for estrus detection. Estrus synchronization is especially useful in small herds with year-round calving where there may be only one animal in estrus at any given time or in animals, such as nulliparous heifers on pasture, that cannot be easily accessed for estrus detection. Estrus synchronization can also be used to increase the percentage of cattle conceiving within a defined period of time. This is achieved by improving the efficiency and accuracy of estrus detection and by allowing most eligible cattle in a herd to be bred within a few days rather than spread over an entire estrus cycle. Another important usage of estrus synchronization in dairy cattle is to synchronize recipient animals for embryo transfer so that the desired number of recipients at the appropriate stage of the estrous cycle are available. There are other likely benefits from estrus synchronization in dairy cattle, such as facilitating the adoption of treatments or management practices that must be implemented at a specific stage of pregnancy or lactation.

A successful estrus synchronization program should have the following features:

- easy implementation with minimal distress to animals;
- applicability to animals of different physiological status, for example, cyclic versus noncyclic;
- precise onset of estrus to minimize or eliminate estrus detection;
- no detrimental effect on reproductive performance compared with that achieved under the current system;
- cost-effectiveness relative to the objectives to be achieved.

This article discusses the techniques available to synchronize dairy cattle for breeding after detection of estrus. Techniques for synchronizing ovulation so that breeding can be carried out at a fixed time after treatment without estrus detection are discussed elsewhere (*See* **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Ovulation and Insemination). Ovulation synchronization removes the need for estrus detection and is well suited for production systems where accurate estrus detection is difficult and costly. However, a successful ovulation synchronization program usually involves synchronization of follicular wave development, which requires additional treatments with hormones. There may be semen wastage associated with ovulation synchronization because not all treated animals ovulate around the time of artificial insemination (AI).

Principles of Estrus Control

During each bovine estrus cycle, there are characteristic changes in sexual behaviors, follicular development, and the circulating profiles of several reproductive hormones (*see* **Reproduction, Events and Management**: Estrous Cycles: Characteristics). Among all the reproductive hormones, progesterone can be considered as the main ‘orchestrator’ of events during an estrus cycle. During the luteal phase of the estrus cycle when circulating progesterone concentration is high, no estrous behavior or ovulation occurs. The decrease in circulating progesterone concentration after the onset of luteolysis relieves the reproductive system from the negative feedback control of progesterone and, consequently, estrous behavior and ovulation ensue within a few days. Therefore, estrus control mainly involves manipulating the circulating concentrations of progesterone. This can be achieved either by artificially prolonging the luteal phase of the estrus cycle using exogenous progesterone or a synthetic progestogen or by shortening the luteal phase using prostaglandin $F_{2\alpha}$ (PG) or one of its analogues, or by a combination of both mechanisms.

Estrus Control Using Progestogens

For a group of cattle at random stages of the estrus cycle, treatment with progestogen for more than 14 days will produce a synchronized onset of estrus within 2–3 days after cessation of the progestogen treatment. The progestogen treatment prevents cows whose corpora lutea (CLs) undergo spontaneous luteolysis during the treatment period (cows on day 5 or later of the estrus cycle at treatment initiation) from showing estrous behavior and ovulating until after the end of treatment. For animals in the early stage of the estrus cycle (days 0–4), the progestogen treatment either prevents the formation of CLs or shortens the life span of freshly formed CLs.

Early studies on estrus synchronization with progestogens involved daily injection of sufficient amounts of progesterone or the feeding of orally active and highly potent progestogens, such as melengestrol acetate (MGA). The development of progestogen application devices, including the norgestomet ear implant, the progesterone-releasing intravaginal device (PRID), and the controlled internal drug release (CIDR) device, has facilitated the use of progestogens for estrus synchronization in dairy cattle. However, a common adverse effect of estrus synchronization with progestogen alone is the reduction in conception rate at the synchronized estrus. The longer the duration of progestogen treatment, the better the synchrony, but the lower the conception rate. It is now known through studies of follicular dynamics using ultrasonography that the reduction in conception rate at the synchronized estrus is due to the development of persistent dominant follicles. The doses of progestogens administered in these programs, while effective in suppressing estrus and ovulation, are ineffective in suppressing the development of persistent dominant follicles in the absence of a functional CLs. Oocytes from persistent dominant follicles can be fertilized, but the resulting embryos have reduced developmental capability. As a result, estrus synchronization programs using progestogens alone are no longer widely used commercially.

Estrus Control Using Prostaglandin

The luteolytic property of PG and its analogues was discovered in the early 1970s. This was followed by numerous studies on the use of PG for estrus synchronization. Several PG products are licensed for use on dairy cattle and there does not appear to be significant differences among products in their efficacy for estrus synchronization. A single injection of PG will reliably induce regression of the CLs between days 7 and 18 of the estrus cycle. Regression of the CLs results in a drop in circulating progesterone concentration. This allows the normal sequence of physiological and endocrinological events associated with the follicular phase to proceed, leading to estrus and ovulation. However, PG is not effective at all stages of the estrus cycle. It has no consequence if administered after the spontaneous onset of luteolysis. The developing CLs before day 5 of the estrus cycle are not responsive to PG and young CLs (days 5 and 6) are less responsive to PG than mature CLs. Consequently, about two-thirds of cows treated with a single injection of PG can theoretically show estrus over a 3-day period. Estrous response to PG injections can be lower in *Bos indicus* than in *Bos taurus*, which may be related to poor estrus expression in *B. indicus* rather than luteal responsiveness to PG.

Several approaches have been developed to circumvent the problem that not all cows in a group will respond to a single PG treatment (**Figure 1**). The most widely used approach is to administer two injections of PG 11–14 days apart (**Figure 1**, program (1)). This treatment program ensures that all animals are at a stage of the estrus cycle when they are capable of responding to the second PG treatment, irrespective of whether they have responded to the first PG treatment or not. A variation of the above program is to treat all cattle with PG and breed on detection of estrus, followed by retreatment 11 days later of those that have not responded to the first PG (**Figure 1**, program (2)). A third approach is to mate cows for 5–7 days on detection of estrus and then to treat with PG animals that have not been mated (**Figure 1**, program (3)). Another approach is to treat only cows that are identified, using rectal palpation, ultrasonography, or progesterone measurement, to have responsive CLs. This approach is not often used systematically at the herd level because of errors in, and costs associated with, identifying cows with responsive CLs.

Initially, it was hoped that the double-PG program would result in a synchronized onset of estrus that was precise enough for a single fixed-time insemination to achieve an acceptable conception rate. Such a hope has never been realized because the interval from PG injection to onset of estrus is influenced, among other things, by the developmental stage of the dominant follicle at the time of PG treatment. In nonlactating heifers, estrous response rate

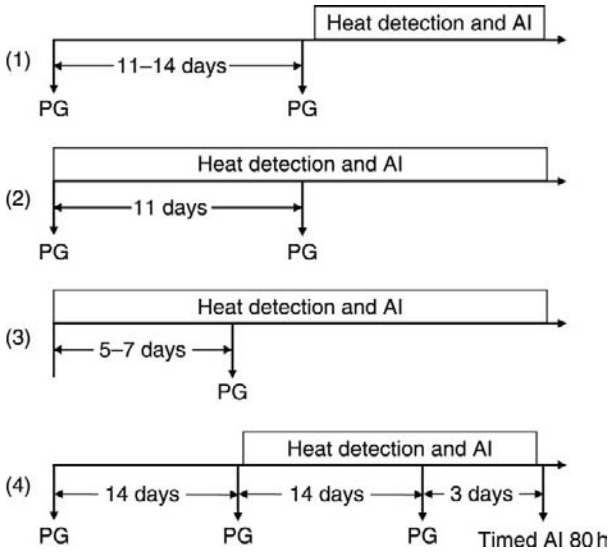

Figure 1 Diagrammatic illustration of various estrus synchronization programs involving $PGF_{2\alpha}$ or its analogues (PG). AI, artificial insemination; h, hour.

Table 1 Estrous response rate (%) of cyclic lactating cows synchronized with two injections of PG 14 days apart and the effect of progesterone supplementation for 5 days before the second PG

Day after second PG	*No_progesterone ($n = 572$)*	*Progesterone ($n = 605$)*
Day 2	7.9	9.9
Day 3	40.6	30.9[a]
Day 4	14.9	25.1[b]
Day 5	9.6	12.1
Day 6	7.3	8.1
Day 7	2.5	3.5
Total over 6 days	82.9	89.6[a]

[a] $p < 0.01$ compared with the corresponding value for cows in the no_progesterone group.
[b] $p < 0.001$ compared with the corresponding value for cows in the no_progesterone group.
PG, prostaglandin $F_{2\alpha}$.
From Xu ZZ, Burton LJ, and Macmillan KL (1997) Reproductive performance of lactating dairy cows following estrus synchronization regimens with $PGF_{2\alpha}$ and progesterone. *Theriogenology* 47: 687–701.

within 7 days after the second PG treatment can exceed 90% with a large peak response (up to 60%) between 48 and 72 h. By contrast, estrous response of lactating dairy cows to the double-PG treatment is inconsistent and less precise compared with that of heifers (**Table 1**). The percentage of cows that are noncyclic at the time of PG treatment can affect the estrous response. Stage of the estrus cycle at the time of PG treatment can also affect estrous response, with advanced stages of the luteal phase being associated with increased estrous response rate (**Table 2**). Thus, a 14-day interval between the two PG treatments will result in a higher estrous response rate compared with an 11-day interval due to an increase in the proportion of animals in the middle to late stages of the luteal phase. Progesterone supplementation for 5 days before the second PG injection can affect the pattern of onset of estrus and increase the estrous response rate (**Table 1**). The increase in estrous response rate mainly occurs in cows in the early to middle stages of the luteal phase (**Table 2**).

Many studies have found that conception rate after PG treatment is normal or even improved compared with nonsynchronized animals. Some of the improvement in conception rate could be due to improved estrus detection accuracy in synchronized cows. In herds with high estrus detection efficiency and accuracy, estrus synchronization with PG can reduce conception rate. The reduction in conception rate mainly occurs in cows in the early to middle stages of the estrus cycle at the time of the second PG injection. Therefore, it is not the PG treatment *per se* that reduces conception rate, rather the reduction is probably caused by shortening of the luteal phase, thus reducing the total amount of progesterone the reproductive system is exposed to before ovulation. The reduction in conception rate can be largely eliminated by supplementing progesterone for 5 days before the second PG injection (**Table 2**).

In some countries, PG is the only drug that is licensed for estrus synchronization in lactating dairy cows. Systematic breeding programs based on PG have been developed. Targeted breeding is a systematic breeding program that is advocated by the manufacturer of Lutalyse, Pharmacia-Upjohn (now a division of Pfizer) (**Figure 1**, program (4)). It consists of three PG injections at 14-day intervals. For simplicity of implementation in nonseasonal herds, PG is usually administered on 1 day of the week to all cows that qualify in that week. To get more cows bred early, the first set-up injection of PG could be given to cows that are between 7 and 14 days before the end of the voluntary waiting period. Cows are not mated after the first set-up injection. The set-up injection ensures that cows will be in a stage of the estrus cycle when the CLs are responsive to the second PG injection. The second PG injection is administered 14 days later and cows are mated after detection of estrus. Those that have not been detected in estrus after the second PG treatment are given a third PG injection 14 days later, and the cycle can continue. For the targeted breeding program, a fixed-time insemination is carried out at 80 h after the third PG injection on cows that have not displayed estrus by that time. Alternatively,

Table 2 Effects of stage of the estrous cycle at second PG and progesterone supplementation for 5 days before second PG on ORR (%) and CR (%) of postpartum lactating cows synchronized with two injections of PG 14 days apart

	No_progesterone		*Progesterone*	
Stage of estrous cycle	*ORR*	*CR*	*ORR*	*CR*
Days 5–9	75.9	52.3	86.8[a]	64.8[b]
Days 10–13	85.5	59.3	91.5[a]	66.2
Days 14–19	93.1	71.3	94.3	71.4

[a] $p < 0.05$ compared with the corresponding value for cows in the no_progesterone group.
[b] $p < 0.1$ compared with the corresponding value for cows in the no_progesterone group.
CR, conception rate; ORR, estrous response rate; PG, prostaglandin $F_{2\alpha}$.
From Xu ZZ, Burton LJ, and Macmillan KL (1997) Reproductive performance of lactating dairy cows following estrus synchronization regimens with $PGF_{2\alpha}$ and progesterone. *Theriogenology* 47: 687–701.

the first PG injection in the targeted breeding program could be given to those cows that have just passed the voluntary waiting period and cows are mated after detection of estrus. This treatment program will be cheaper due to the reduced total number of PG injections, but the interval from calving to first AI will be longer because only two-thirds of the cows can potentially respond to the first PG injection.

Combination Treatment Programs

To improve the precision of synchrony and to reduce the adverse effects on fertility, various treatment programs involving a combination of two or more drugs have been developed. Some of the popular combination programs for synchronizing estrus are discussed here.

Progestogen and Prostaglandin

Progestogen in combination with PG is a widely used combination treatment program for estrus synchronization. It usually involves a short period (commonly 6–8 days) of progestogen treatment with a PG injection 1–2 days before or at the time of termination of the progestogen treatment (**Figure 2**, program (5)). This combination program ensures that, at the time of PG injection, animals either do not have functional CLs or have CLs that are responsive to PG. Most treated animals (85% or more) will show estrous behaviors between 2 and 5 days after the end of treatment. The time and the magnitude of the peak estrous response are affected by when PG is administered relative to the termination of progestogen treatment. If PG is injected 1–2 days before the end of progestogen treatment, peak estrous response occurs on the second day after progestogen treatment, whereas it occurs on the third day if PG is injected at termination of progestogen treatment. Conception rate to inseminations at the synchronized estrus has been reported to be normal or slightly reduced, but the reduction is generally less compared with the situation in which long-term progestogen treatment is used for synchronization. Persistent dominant follicles can still develop in some animals that are in the late stage of the estrus cycle at the start of progestogen treatment.

Another combination program involving progestogen and prostaglandin is to presynchronize animals with a long-term (e.g., 14 days) progestogen treatment, followed by an injection of PG during the late luteal phase of the synchronized cycle (e.g., 17–18 days after the end of the progestogen treatment) (**Figure 2**, program (6)). This program takes advantage of the ability of a long-term progestogen treatment to precisely synchronize estrus and the high estrous response and normal fertility when PG is administered in the late luteal phase (**Table 2**). However, this program requires that progestogen treatment be initiated more than 30 days before the start of breeding when many postpartum cows may have not resumed ovarian cyclicity. Therefore, this program may not be well suited for postpartum lactating dairy cows.

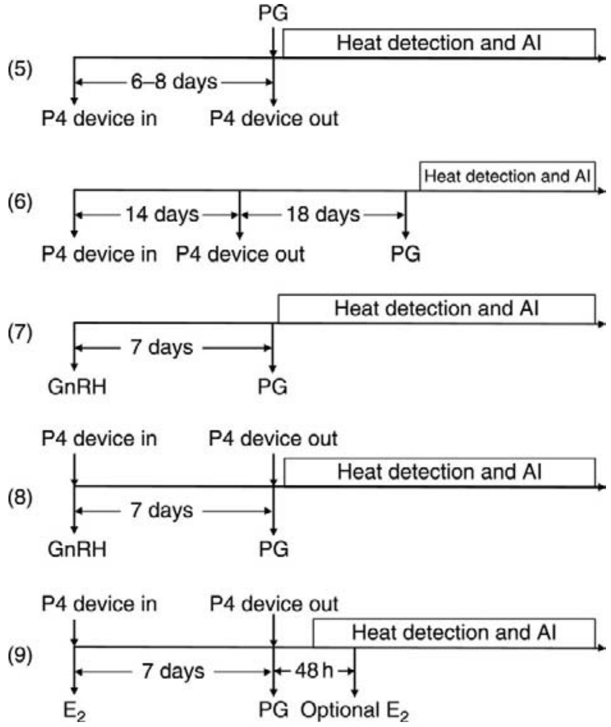

Figure 2 Diagrammatic illustration of the various estrus synchronization programs involving a combination of two or more hormones. PG, prostaglandin $F_{2\alpha}$ or its analogue; GnRH, gonadotropin-releasing hormone; P4, progesterone or synthetic progestogen; E_2, estradiol-17β or its derivatives; AI, artificial insemination.

Estrogen and Progestogen

Studies have shown that an injection of progestogen and estrogen at the start of an estrus synchronization program using progestogen can reduce the duration of progestogen treatment from >14 to 9 days. This has formed the basis for the Synchro-Mate-B (SMB) treatment for estrus synchronization in beef cattle and dairy heifers. The SMB program involves a 9-day treatment with an ear implant containing 6 mg of norgestomet plus an injection of 5 mg of estradiol valerate and 3 mg norgestomet at the time of implant insertion. The injection of estradiol valerate and norgestomet serves two functions. First, the injection causes regression of large antral follicles and initiation of a new follicular wave 4–5 days after treatment so that a newly developed dominant follicle is available for ovulation after the 9-day treatment program. Second, the injection, presumably the estradiol valerate in the injection, causes premature luteolysis of CLs during the treatment period, irrespective of the stage of the estrus cycle at the start of

SMB treatment. Estrogen induces luteolysis by stimulating PG secretion from the uterus. In addition, progestogen treatment during metestrus can also prevent normal CLs development in some animals. Estrous response following SMB treatment is typically greater than 90% and fixed-time insemination between 48 and 54 h after implant removal can achieve acceptable reproductive performance. However, SMB has been withdrawn from the market.

Gonadotropin-Releasing Hormone and Prostaglandin

Studies, mainly in North America, have shown that treatment with gonadotropin-releasing hormone (GnRH) followed 7 days later by PG can be used for estrus synchronization (**Figure 2**, program (7)). The dose of GnRH used in this program is typically half of that recommended for treating follicular cysts. The GnRH treatment induces ovulation of existing dominant follicles and the formation of new or accessory CLs. This prevents most animals in the late luteal and follicular phases of the estrus cycle from showing estrus before PG injection. However, between 5 and 10% of treated cows can still show estrus between the GnRH and PG treatment, thus reducing the effectiveness of this program for estrous synchronization. Nevertheless, some studies have shown similar or improved reproductive performance after this program compared with the double-PG program.

Progestogen, gonadotropin-releasing hormone, and Prostaglandin

Studies in New Zealand and Ireland have investigated synchronization programs that incorporate progesterone treatment between GnRH and PG injections (**Figure 2**, program (8)). A CIDR device is inserted at the time of GnRH injection and removed at PG injection. The progesterone treatment prevents estrus and ovulation before PG injection and may also improve conception rate at the synchronized estrus by increasing circulating progesterone concentrations in cows without a functional CLs. An estrous response rate of greater than 90% has been obtained and the conception rate at the synchronized estrus is similar to that of nonsynchronized herd mates inseminated at detected estrus.

Estrogen, Progestogen, and Prostaglandin

Estrogen has been used at the beginning of combination treatment programs involving progestogen and PG (**Figure 2**, program (9)). The purpose of this estrogen treatment is to regress dominant follicles that are present at the time of estrogen treatment so that freshly developed dominant follicles are ready to ovulate after the program. A gelatin capsule containing 10 mg of estradiol benzoate is developed for intravaginal use together with the CIDR device or PRID. Injection of 5 mg of estradiol-17β or 2 mg of estradiol benzoate has also been found to improve the precision of synchrony and conception rate compared with no estrogen treatment. Estrogen has also been used after the end of progestogen and PG treatment to increase the precision of onset of estrus. An injection of 1 mg of estradiol benzoate 48 h after the end of progesterone and PG treatment can significantly increase the percentage of cows showing estrus between 48 and 72 h (85 vs. 57%).

However, in 2006, the European Union banned the use of estradiol and its derivatives for estrus synchronization in food-producing animals. This has led other countries, such as New Zealand and Australia, to adopt the EU Directive and ban the use of estrogens for estrus synchronization. Consequently, the use of GnRH at the beginning of a progesterone–PG program to increase the precision of onset of estrus and/or after the progesterone–PG program to synchronize ovulation has gained popularity.

Treatment of Noncyclic Cows

In a group of cows eligible for breeding, some may be noncyclic. The problem of noncyclic cows at the start of the breeding season is particularly severe in high-producing dairy cows or in seasonal dairy cows grazing on pasture. Therefore, an effective estrus synchronization program should also be able to induce estrus and ovulation in noncyclic cows.

In New Zealand, a popular program for treating noncyclic cows involves progesterone treatment in the form of an intravaginal CIDR device for 6–7 days, followed by an injection of 1 mg of estradiol benzoate either 24 or 48 h after CIDR removal. An estrous response rate of close to 90% and conception rate of around 35% have been obtained with this treatment program. However, this program does not work for cyclic cows and special effort is therefore needed to separate cyclic from noncyclic cows.

Recent studies have shown that a combination program involving GnRH, progesterone, PG, and estradiol may be used to synchronize all cows in a herd regardless of their cyclic status. Cows are treated with a CIDR device for 7 days, along with GnRH at CIDR insertion and PG at CIDR removal. At 48 h after CIDR removal, 1 mg of estradiol benzoate is injected to cows that have not been detected in estrus by that time. This program has been tested for treating noncyclic cows due to nutritional stress and has been found to result in better estrous response (93 vs. 89%) and conception rate (47 vs. 29%) than the program based on progesterone and estradiol benzoate only. The GnRH and PG used in this program could also be beneficial for treating noncyclic cows due to ovarian cysts and uterine infection.

Following the ban on the use of estrogens in food-producing animals, the available programs for both cyclic and noncyclic cows in New Zealand have been changed to Ovsynch-type programs involving a combination of GnRH and PG, with or without progesterone in the form of the CIDR device. A second GnRH injection around 56 h after PG and fixed-time insemination at 72 h may improve pregnancy rate compared with insemination after detected estrus.

Practical Considerations

Despite the aforementioned advantages from using estrus synchronization, it remains a major challenge to make this technique widely accepted by commercial dairy producers. Costs of drugs and labor for implementing an estrus synchronization program are among the first things considered by dairy farmers when deciding whether to use estrus synchronization. Estrus detection can be more difficult in a large group of synchronized cattle because so many animals are in estrus at the same time and it is difficult to identify those that are in genuine estrus. This problem can be solved by developing synchronization programs that allow fixed-time insemination. Herd managers need to have good organizational skills and some technical knowledge of the program in order to implement a successful estrus synchronization program. Good communication and cooperation among herd managers, veterinarians, and AI technicians are essential. Good record keeping is also important because the use of PG on pregnant animals will lead to abortion.

The Future

The challenge for future research on estrus synchronization will be to increase the estrous response rate, to improve the precision of synchrony, and at the same time to maintain and increase conception rate at the synchronized estrus. A reduction in conception rate at the synchronized estrus is a common feature following most estrus synchronization programs. Although many studies have reported improved reproductive performance after estrus synchronization compared with nonsynchronized control animals, most of this increase is probably due to improvement in the efficiency and accuracy of estrus detection and not due to improved fertility. The objective is to achieve a conception rate that equals the conception rate to inseminations at correctly detected natural estrus or to natural mating. It is likely that achieving this objective will require the use of multiple drugs. Therefore, the other challenge for research is to develop smart drug delivery systems that simplify the implementation of estrus synchronization programs. Furthermore, the development of semen products that allow sperm to survive for several days in the female reproductive tract will eliminate the need for tight synchrony. Finally, the challenge will be even greater to develop estrus synchronization systems that can effectively synchronize the onset of estrus in those animals that return after a synchronized insemination.

See also: **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Estrous Cycles: Characteristics; Mating Management: Detection of Estrus.

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Control of Estrous Cycles: Synchronization of Ovulation and Insemination

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Introduction

Intensive genetic selection for milk production without attention to reproductive performance has contributed to an inverse relationship between milk production and reproduction. Inclusion of productive life and daughter pregnancy rate and, more recently, the availability of sire conception rate, as a measure of phenotypic service-sire fertility, appear to have reduced the rate of decline in fertility in the United States. Reproductive management of the lactating dairy cow has been a challenge because of poor expression of estrus and low fertility to insemination at a detected estrus. The duration of estrus is reduced as milk production increases, and the frequency of double ovulations and subsequent occurrence of twins is also increased in cows with high levels of milk production at the time of the breeding period. The high-producing dairy cow of today expresses estrus for approximately 7 h during which time an average of 6.5 standing events take place with an accumulative period of standing of 20 s (i.e., 3 s per standing event).

Pregnancy rate over a 21-day period for the national herd of dairy cows in the United States is approximately 16.2%. The component parts of pregnancy rate are the rate of estrus detection and conception rate. Technology is available for systems to detect estrus accurately, but a major issue is that lactating dairy cows do not display strong symptoms of estrus. Expression of estrus has been affected adversely by high milk production and associated metabolism of hormones, as well as housing facilities (e.g., concrete floors) that reduce the cow's willingness to be sexually active. An additional challenge is the high occurrence of nonovulatory dairy cows that either have reoccurring follicle waves without ovulation or develop ovarian cysts.

A major advance in reproductive management that has addressed how to improve pregnancy rate has been the development of timed artificial insemination (TAI) programs based on the development of systems to control or program optimal development of ovarian follicles, induce ovulation, and develop a corpus luteum (CL) capable of supporting pregnancy. The component pharmaceutical agents available to the dairy industry in many countries for use with dairy cattle are gonadotropin-releasing hormone (GnRH), luteolytic prostaglandins, and intravaginal progesterone (using a controlled intravaginal drug release (CIDR) insert, or similar device). These are pharmaceuticals that mimic the actions of the cow's endogenous hormones, are physiological, and pose no health hazard to the cow. The original TAI protocol is the Ovsynch procedure. This protocol has been in use for approximately 12 years. During this period, both basic and applied research has led to major advancements in optimizing the system. As a consequence, pregnancy responses have increased, the system has been extended to resynchronization of nonpregnant cows, and programs have been developed for TAI in dairy heifers. The dynamics of various cow factors such as body condition score, parity, and health status in the transitional-periparturient period have been shown to influence pregnancy rates in the controlled breeding program. The present objective is to update major advancements that will increase reproductive performance in controlled breeding in dairy cattle.

Lactating Dairy Cattle

It is essential that producers and veterinarians understand the physiological reasons why certain components of the reproductive management program are able to improve reproductive performance or conversely why a misunderstanding of the program can lead to catastrophic pregnancy results. No one reproductive breeding program is practical and economically optimal for all dairy production units due to differences in available facilities, size of the unit, labor that places reproduction as a high priority, and a functionally dynamic record system.

Optimizing Stage of the Estrous Cycle at the Onset of Ovsynch

The original Ovsynch program involved two injections of GnRH administered 7 days before and 48 h after an injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and cows were inseminated 16–20 h after the second injection of GnRH. If TAI in the Ovsynch protocol is performed at the same time as the second GnRH injection, then the protocol is referred to as Co-synch.

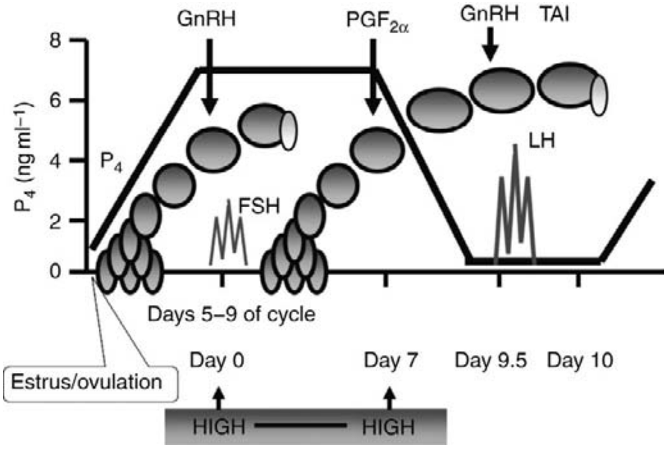


Figure 1 Follicle dynamics and hormonal responses to the Ovsynch protocol. FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; P$_4$, Progesterone; TAI, timed artificial insemination.

Optimization of stage of the estrous cycle (i.e., days 5–9) at the onset of the Ovsynch protocol is important to achieve a subsequent synchronized ovulation at the second GnRH preceding the TAI (**Figure 1**). Programming the stage of the estrous cycle at the time the Ovsynch protocol is implemented (e.g., days 5–9 of estrous cycle) ensures there is progesterone availability throughout the period between the first injection of GnRH and injection of PGF$_{2\alpha}$, and that there is a CL to respond to the luteolytic injection of PGF$_{2\alpha}$ (**Figure 1**). The continual exposure to progesterone is important for sequentially programming the brain, oviduct, and uterus with the appropriate changes in hormones, receptors, and secretions leading to an induced ovulation, fertilization, and development of an embryo capable of maintaining a pregnancy with minimal embryonic and fetal losses. Programming the start of the Ovsynch protocol to occur between days 5 and 9 of the estrous cycle increases the probability that the first injection of GnRH will induce ovulation of the first wave follicle and recruitment of a new follicle wave (**Figure 1**), which upon induction of ovulation in response to the second GnRH increases the probability of producing a viable oocyte for fertilization and a robust CL. Indeed ovulation of the first follicle wave results in the presence of both the original CL and an accessory CL, induced by the GnRH injection, which are responsive to the injection of PGF$_{2\alpha}$.

The Ovsynch protocol preceded by a PGF$_{2\alpha}$ presynchronization program (Presynch–Ovsynch) has become the nucleus program for reproductive management in the industry. Successful use of such a program is highly dependent upon obtaining good compliance in implementing all component parts of the protocol. The original Presynch–Ovsynch program entailed two injections of PGF$_{2\alpha}$ given 14 days apart with the Ovsynch protocol initiated 12 days after the second injection of PGF$_{2\alpha}$ for presynchronization (**Figure 2**). This system increased pregnancy rates compared to Ovsynch alone for

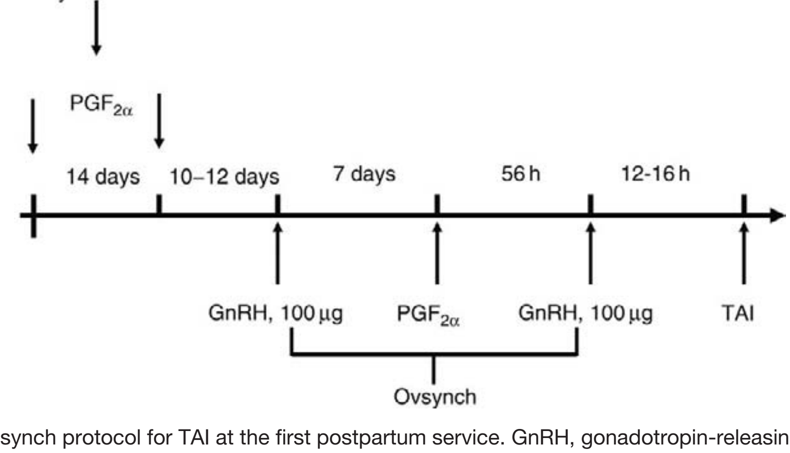


Figure 2 Presynch/Ovsynch protocol for TAI at the first postpartum service. GnRH, gonadotropin-releasing hormone; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; TAI, timed artificial insemination.

Table 1 Pregnancy rates for lactating dairy cows receiving various reproductive management systems for timed insemination

Treatment (n)	*Control (n)*	*Treatment (Percent pregnant to AI)*	*Control (Percent pregnant to AI)*	*References*
Presynch-12d/Ovsynch (269)	Ovsynch (274)	48.3	36.9	Moreira *et al*. (2001)
Presynch-12d/Ovsynch (304)	Ovsynch (310)	46.8	37.5	El-Zarkouny *et al*. (2004)
11-Day Presynch (410)	14-Day Presynch (412)	40.5	33.5	Galvao *et al*. (2007)
33-Day Resynch (180)	26-Day Resynch (189)	39.4	28.6	Sterry *et al*. (2006)
38-Day Resynch (GnRH (357)/CIDR (316))	38-Day Resynch (386)	33.6/31.3	24.6	Dewey *et al*. (2009)

AI, artificial insemination; CIDR, controlled intravaginal drug release; GnRH, gonadotropin-releasing hormone.

the reasons outlined above, when the Ovsynch protocol is initiated in early diestrus (**Table 1**). Dairy producers were keen to extend the period when Ovsynch was initiated to a 14-day interval such that four of the five sequential hormonal injections would be given on the same day of week. Field experiences indicate that 60% of detected estruses occur on days 3–6 after the second injection of $PGF_{2\alpha}$ of presynchronization. A recent study indicated that an 11-day interval after presynchronization (i.e., cows would be predominately 5–8 days of the estrous cycle) is better than a 14-day interval to begin the TAI protocol. The overall ovulation rate in response to the first injection of GnRH was greater for an 11-day than a 14-day interval (62 vs. 44.7%). This was attributed to GnRH being given at 11 days when the first wave follicle will ovulate whereas the 14-day interval increased the proportion of cows injected early in the second follicle wave at a time the follicle was developed insufficiently to ovulate in response to GnRH. The latter follicle would continue to develop and be slightly more aged and/or dominant compared to the newly recruited follicle from the day 11 injection interval for GnRH. Indeed pregnancy per TAI was 6.6% greater for the 11-day interval (40.1 vs. 33.5% at day 38 after TAI; **Table 1**). Thus, subtle changes in presynchronization protocols can cause substantial increases in pregnancy rate, and the optimal period to start the Ovsynch protocol is 10–12 days after the second $PGF_{2\alpha}$ injection of presynchronization (**Figure 2**).

Interval from $PGF_{2\alpha}$ to Ovulatory Injection of GnRH and Timing of AI

It has been well documented that cows should be inseminated 8–16 h after the onset of estrus for an optimal conception rate. The preovulatory surge of luteinizing hormone (LH) occurs very close to the onset of estrus with ovulation occurring approximately 28 h after the LH surge. It is important to recognize that the second injection of GnRH of an Ovsynch program is analogous to the onset of estrus since an LH surge is induced immediately. Indeed maximal rate of pregnancies per artificial insemination (AI) was achieved when a timed insemination was made at 16 h after the injection of GnRH. In contrast, percent pregnant to AI was decreased when inseminations were made at the time of GnRH injection or 28 h later. Producers often favor the convenience of carrying out a TAI at the time of GnRH injection (i.e., referred to as a Co-synch program) to reduce the number of times cows need to be held up. Alternatively, some producers prefer to perform TAI on the following day at approximately 24–28 h after the GnRH injection for convenience. Either option likely will reduce percent pregnant to AI. The importance of the correct timing is indicated by a study completed at the University of Wisconsin. All cows were presynchronized with two injections of $PGF_{2\alpha,}$ and the Ovsynch protocol was started 11 days later. The optimal timing program was to inject GnRH 56 h after the injection of $PGF_{2\alpha}$ and inseminate the cows 16 h after the injection of GnRH, which was 72 h after the injection of $PGF_{2\alpha}$ (see **Figure 2**). Percent pregnant to AI was 36.1% compared to Co-synch 48 h (26.7%) and to 72 h (27.3%) programs. The last two programs injected GnRH and TAI concurrently at 48 or 72 h, respectively. Clearly, subtle changes in the timing of the GnRH injection and time of insemination result in substantial differences in percent pregnant to AI responses. If a Co-synch program is to be followed, one needs to understand the physiology of the injection sequence so that functionally active ovarian follicles are at an optimal stage analogous to a follicle in the close periestrus period when GnRH/TAI is performed.

Resynchronization of Nonpregnant Cows Following First Service

A reproductive management challenge following first service is to reinseminate cows that did not conceive as quickly as possible. The same principles to optimize the Presynch–Ovsynch program are applicable to development of a resynchronization program. However, a resynchronization system is somewhat constrained in that programming nonpregnant cows to ovulate must be done in a manner that will not interfere with cows that are pregnant to first service. Thus, accurate identification of nonpregnant and pregnant cows is important, and timing of the diagnosis is dependent upon the technology applied (i.e., rectal palpation at 35–42 days, ultrasound diagnosis at 30–32 days, blood pregnancy test at 27–30 days (measurement of PAG)). To some degree, there is a natural presynchronization of nonpregnant cows because those detected in estrus have a median return to estrus interval of 22 days in which 64.3% show estrus within 17–24 days after first service. Thus, initiation of Ovsynch at 30 days after first service would mean most cows would be at approximately day 8 of the cycle. GnRH injection would induce ovulation of a first wave follicle and initiate recruitment of a new follicular wave under a high progesterone environment. At 37 days after first service, a decision can be made to inject $PGF_{2\alpha}$ in cows diagnosed nonpregnant (e.g., rectal palpation). These cows would then be injected with GnRH and TAI at 56 and 72 h after the $PGF_{2\alpha}$ injection, respectively.

Several days after first service (days 19, 26, and 33) have been examined to begin resynchronization of nonpregnant cows with Ovsynch. Starting resynchronization on day 33 resulted in the highest pregnancy rate for the second service. Ultrasound technology was used for detection of nonpregnant cows at day 26 or day 33 after first service for the day 19–26 and 33 resynchronization groups, respectively. Hypothetically, the timing of GnRH at day 26 would tend to target the majority of cows too early in their follicle wave (i.e., day 4 of the wave) to induce follicle turnover, whereas at day 33 they would be ovulating potential first or second wave follicles and a sustained progesterone environment would be present for cows potentially returning to estrus between 17 and 24 days after first service. Experimental results clearly document that fertility was increased for the day 33 resynchronization group (i.e., 33.7%) compared to the day 19 and 26 groups (27.1 and 26.6%, respectively). The benefit of the day 33 resynchronization on pregnancy per TAI compared to the day 26 resynchronization group was repeated (39.4 vs. 28.6%; **Table 1**) with the benefit most apparent in primiparous cows. In the latter study, insertion of a CIDR insert in cows without a CL improved pregnancy rate per TAI in the multiparous cows to a level comparable to that of primiparous with or without a CL.

An alternative resynchronization strategy is a more conventional system based solely on pregnancy diagnosis per rectal palpation at day 38 (**Figure 3**). In this scenario, an Ovsynch 72 h Co-synch (GnRH, 7 days later PGF, and 72 h later GnRH and TAI) was initiated at day 38 after first service in three groups of nonpregnant cows (Group 1: control, GnRH; Group 2: received a GnRH injection on day 31 at 7 days before pregnancy diagnosis; Group 3: received a CIDR insert on day 38 that was removed at the time of $PGF_{2\alpha}$ injection; **Figure 3**). Pregnancy rate per TAI was greater and tended to be greater for GnRH/Group 2 (33.6%) and Group 3/CIDR (31.3%) cows than Group 1 (24.6%) cows (**Table 1**). It is likely that presynchronization with a single injection of GnRH at day 31 programmed a new follicle wave and increased the occurrence of a CL at the beginning of the Ovsynch 72 h Co-synch protocol. Insertion of a CIDR insert likely improved the synchronization of ovulation associated with the 72 h Co-synch response because it held ovarian follicles from ovulating prematurely in cows that were in late diestrus at the time the Ovsynch 72 h program was started.

It is clear that several alternatives are available for resynchronization of lactating dairy cows. With the acquisition of new technology for a cow side diagnosis of nonpregnant cows early after insemination (e.g., 27, 28, or 30 days), it will be possible to implement even earlier resynchronization systems for TAI within 3 days (e.g., day

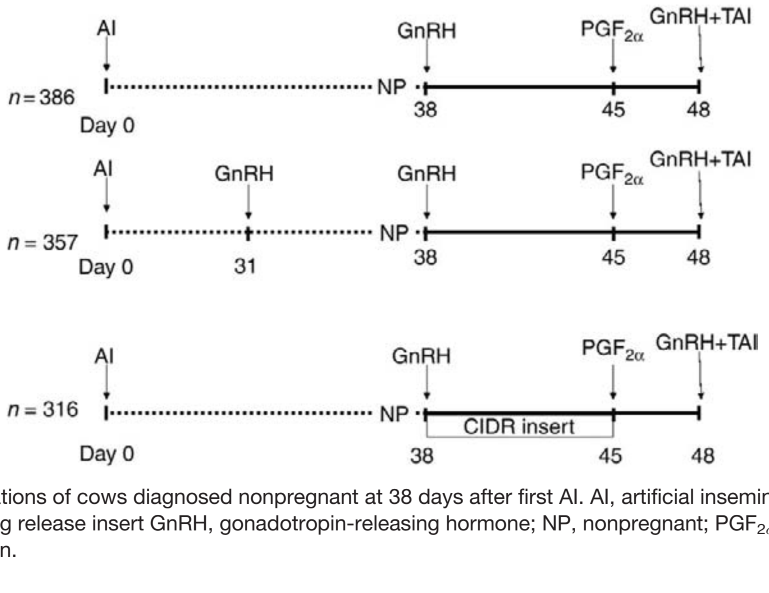


Figure 3 Resynchronizations of cows diagnosed nonpregnant at 38 days after first AI. AI, artificial insemination; CIDR insert, controlled intravaginal drug release insert GnRH, gonadotropin-releasing hormone; NP, nonpregnant; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TAI, timed artificial insemination.

30, 31, or 33) after the diagnosis of a nonpregnancy. This would offer a reduction in reinsemination interval of 9.5–17 days compared to the promising systems described above.

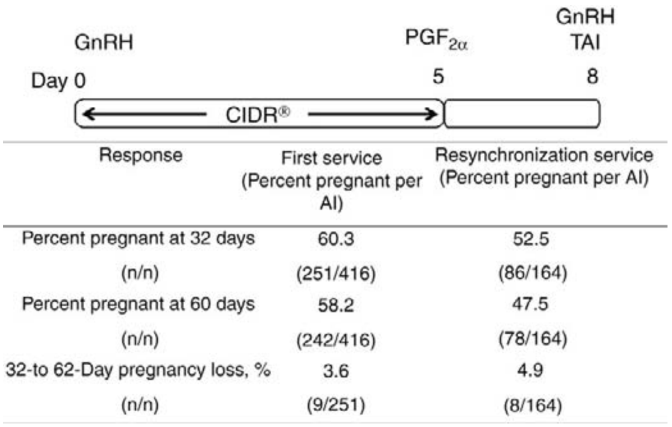
Timed Artificial Insemination for Dairy Heifers

A major limitation for using AI in dairy replacement heifers is time and effort associated with daily estrus detection. Unfortunately, however, the Ovsynch has resulted in unacceptable pregnancy rates of approximately 38% in dairy heifers (**Table 2**). Compared to lactating cows, heifers have a faster rate of follicular growth and a higher frequency of three wave follicular cycles. Consequently, approximately 57% of the cycle is comprised of times when follicles are unresponsive to the first injection of GnRH. Furthermore, rapid turnover and growth of follicles lead to asynchrony with heifers expressing estrus during different stages of the protocol prior to the second ovulatory injection of GnRH for TAI. Investigators at Ohio State University working with beef cattle deduced that an increase in percent pregnant per TAI could be achieved by insertion of a CIDR insert at the time of GnRH injection and, after 5 days, withdrawing the CIDR insert and injecting $PGF_{2\alpha}$. An extended proestrus period was achieved by allowing a 3-day interval from the time of $PGF_{2\alpha}$ injection to the time of GnRH injection and a concurrent TAI. The entire protocol takes only 8 days to be accomplished. Since the interval from the first GnRH injection and CIDR insertion to $PGF_{2\alpha}$ injection is 5 days, a second injection of $PGF_{2\alpha}$ was given ~12 h after the first $PGF_{2\alpha}$. This program in beef cows resulted in a higher pregnancy rate compared to a 7-day Ovsynch with a CIDR in which the second GnRH and TAI occurred at 60 h. This program has been further modified for use in dairy heifers in which only a single injection of $PGF_{2\alpha}$ is given at the time of CIDR removal (5-day CIDR Co-synch 72 h with one injection of $PGF_{2\alpha}$; **Figure 4**). Pregnancy rates to first and second services at 32 days after TAI were 60.3 and 52.5%, respectively (**Figure 4**). Essentially, 81% (337/416) of the heifers were pregnant after two programmed TAIs following a 5-day CIDR Co-synch 72 h with one injection of $PGF_{2\alpha}$. This is an efficient reproductive management program that successfully synchronizes ovulation for TAI and reduces labor costs associated with estrus detection. Indeed all of the TAI procedures described in **Table 2** incorporated some degree of estrus

Table 2 TAI in dairy heifers

Protocol	*n*	*Percent pregnant to AI*	*References*
Ovsynch	187	45.5	Schmitt *et al*. (1996)
Ovsynch	77	35.1	Pursley *et al*. (1997)
Ovsynch	113	42.5	Stevenson *et al*. (2000)
6-Day Co-synch 48 h	175	34.3	Rivera *et al*. (2004)
6-Day Co-synch 48 h	95	29.5	Rivera *et al*. (2005)
6-Day Co-synch 48 h + CIDR	94	31.9	Rivera *et al*. (2005)
6-Day Co-synch 48 h	82	45.1	Rivera *et al*. (2006)
Overall	823	38.3	

AI, artificial insemination; TAI, timed artificial insemination.



Response	First service (Percent pregnant per AI)	Resynchronization service (Percent pregnant per AI)
Percent pregnant at 32 days	60.3	52.5
(n/n)	(251/416)	(86/164)
Percent pregnant at 60 days	58.2	47.5
(n/n)	(242/416)	(78/164)
32-to 62-Day pregnancy loss, %	3.6	4.9
(n/n)	(9/251)	(8/164)

Figure 4 Pregnancy rate of dairy heifers to a 5-day CIDR Co-synch 72 h with one injection of $PGF_{2\alpha}$. AI, artificial insemination; CIDR, controlled intravaginal drug release; GnRH, gonadotropin-releasing hormone; $PGF_{2\alpha}$, prostaglandin F2α; TAI, timed artificial insemination.

detection that is not necessary with the present program. The concept of a 5-day interval between GnRH and $PGF_{2\alpha}$ (i.e., with or without a CIDR insert) and a subsequent 3-day proestrus period (i.e., 72 h Co-synch) warrants investigation in lactating dairy cows.

Conclusion

Tremendous advances have been made in improving milk production, but have in turn resulted in an overall decline in reproductive efficiency for the dairy industry. Problems associated with the cow include inability to properly express estrus and altered hormonal profiles resulting in low conception rates and increased early embryonic death. Coordinated systems of reproductive management offer means to improve herd reproductive performance, and major advances have been made for synchronization of ovulation in both lactating dairy cows and dairy heifers. Such systems are predicated on a greater understanding of the factors controlling follicle development, ovulation, and CL development. The programs as described require the producer, veterinarian, and reproductive management staff to understand the programs and make an effort to obtain a high level of compliance. The platforms used for controlling first service and resynchronized subsequent services in cows that do not conceive provide valuable platforms to implement new technology such as the use of sexed semen, embryo transfer, and cow-side chemical diagnosis of nonpregnant cows. Functional and efficient computer record programs are essential to implement such reproductive management programs. For the research scientist, the reproductive management systems provide the infrastructure to quantify the effects of nutritional, health, and physiological interventions on pregnancy rate. With the advent of new technologies to precisely manipulate reproductive function in lactating dairy cows, dairy producers are presented with a new opportunity. Coordination of management strategies to maximize both milk production and reproductive performance may optimize the economical return of dairy herds, and allow the industry to take complete advantage of the genetic potential to improve milk production through AI.

See also: **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Estrous Cycles: Characteristics; Estrous Cycles: Postpartum Cyclicity; Estrous Cycles: Puberty; Estrous Cycles: Seasonal Breeders; Mating Management: Artificial Insemination, Utilization; Mating Management: Detection of Estrus; Mating Management: Fertility; Pregnancy: Characteristics; Pregnancy: Parturition; Pregnancy: Periparturient Disorders; Pregnancy: Physiology.

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Mating Management: Detection of Estrus

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Introduction

Estrus detection is one of the key components in fertility management programs on dairy farms. Excellent reproductive performance can be defined as the ability to consistently have 90% or more of the cows in a herd conceive and maintain pregnancies in a timely, economically justified manner. On the other hand, low detection rate is strongly associated with poor fertility, long calving interval, intensive replacement of heifers, and reduced genetic progress, resulting in heavy economic losses. Maintaining a consistently high-performing reproductive program requires a substantial investment in management, labor, and other costs, such as semen and pharmaceuticals. The goal of an estrus detection program should be to identify estrus positively and accurately in all cycling cows and to identify all of the cows that are not cycling or are expressing irregular cyclicity. The ultimate goal should be to predict the time of ovulation, thus allowing for insemination that will maximize the opportunity for conception.

Dairy farming is one of the most intensive technologically integrated systems in the world of production agriculture. In general, the market sorts out which technologies offer a competitive advantage and which do not. Most of the aids that have been developed are not reliable or sensitive enough to relieve the farmer from frequent visual observation of the herd. Furthermore, none of the technologies is appropriate for every farm. New approaches are being developed to provide automated systems for estrus detection using remote-sensing technology, but the development of these new tools is in its infancy.

Behavioral Characteristics of Estrus

The expression of estrous behavior is brought about by high systemic concentrations of estradiol-17β produced by the preovulatory follicle, which stimulates behavior coincident with the ovulatory surge of luteinizing hormone. Traditionally, a cow that 'stands' to be mounted is the most definitive behavioral sign of estrus, in particular when reproductive management is based only on artificial insemination (AI). However, 'standing' cannot be the only distinguishing sign of estrus. This is particularly true for silent ovulation (i.e., ovulation without the expression of estrous behavior), which occurs mostly during the postpartum period. Occasionally, pregnant cows exhibit signs of estrus, particularly during middle to late gestation. Also, cows with ovarian follicular cysts may have a hormonal milieu that leads to estrous behavior similar to that of cyclic cows in estrus. Therefore, estrous behaviors other than 'standing' are crucial for accurate identification of estrus coincident with ovulation.

Secondary signs of estrus include attempting to mount other cows, clear mucus discharge from the vulva, swelling and reddening of the vulva from increased blood flow, bellowing, restlessness, trailing other cows, chin resting, sniffing the genitalia of other cows, and lip curling. These signs may serve as clues that cows are near estrus so that they can then be observed more intensely for 'standing' behavior, but cannot be used as a practical predictor of ovulation. Other characteristics are reduction in food intake followed by reduced milk production during estrus, but these are not overt in all animals.

The time of the day and duration of observation are the most important factors for a high detection rate. Traditional management of visual estrus detection is based on a twice-a-day detection regime or observation (20 min each) 3 times a day while taking into account the aforementioned primary and secondary signs of estrous characteristics. Observations are mostly performed in coincidence with each milking. Additional observations are recommended during periods of high activity, such as feeding time or while going to and coming back from the milking parlor. Nevertheless, estrus detection rate in dairy cows is low (<50%) because of limited observation times and the fact that high-yielding dairy cows express shorter duration of estrus.

Automated Systems to Detect Physical Activity

Various approaches have been examined to detect the accurate time of estrus. It includes monitoring of electrical resistance, vaginal and core body temperatures, and progesterone level in plasma or milk, but none of them have yet been implemented in practice. Determination of the onset of estrus is only possible with continuous monitoring for behavioral activity. Based on this concept, few techniques have been developed. The ideal aid should provide continuous surveillance (24 h day^{-1}) of cows, and accurate and automatic identification of the cows in estrus.

Time-Lapse Video Recording

Video recording allows continuous monitoring of behavioral activity. Investigations revealed that cows spent considerably more time walking when in estrus, and less time resting and eating, than when they were not in estrus. Studies with advanced video equipment tested the hourly distribution of onset of estrus. Pooled 6-h intervals demonstrated that the highest frequency of onset of estrus occurs at 1200–1800 and 0600–1200 h in primiparous and multiparous groups, respectively. For a large herd, more cameras are needed because of the resolution of the equipment. Recognition of cows can sometimes be problematic. In addition, the time needed to watch video tapes makes this method not very practical. Time-lapse video recording is mostly used for research rather than for estrus detection in farms.

Pedometer

A pedometer is an electronic device attached to the cow's leg or its neck collar, which measures cow activity (number of steps) in a 24-h day. The potentially useful field application of pedometry is based on the recognition that female mammals display a predictable increase in physical activity when in estrus. Guernsey cows equipped with mechanically activated pedometers were characterized as exhibiting 218% higher physical activity during estrus than during late diestrus and proestrus or during metestrus. Similarly, pedometer has been found to be a practical tool for estrus detection in dairy cows with an average 393% increase in activity at the time of estrus, or approximately 4 times the activity of cows not in estrus when housed in a freestall barn.

Individual cows differ significantly in the amount of activity expressed under the same conditions. A modified pedometer was designed to internally compare the activity change during a specific time interval to the five previous morning or afternoon activity recordings. This modification was implemented to account for the individual activity. **Figure 1** represents a typical activity graph for a complete lactation.

The aim of most reported applications of pedometers was to improve the rates of estrus detection. It was claimed that 70–80% of cows in estrus are detected by pedometer measurements. Pedometry systems that in addition to efficient and accurate estrus identification allow identification of its onset would increase the usefulness of such technology in animal breeding. However, most current pedometry systems do not use real-time data transfer, thus requiring the activity information to be retrieved by an interrogation device. Since retrieval of activity measurements can be performed 2 or 3 times daily, usually at milking, the effectiveness of determining the timing of insemination is not optimal.

An active technology for estrus identification and activity measurements in dairy cows and buffalo was

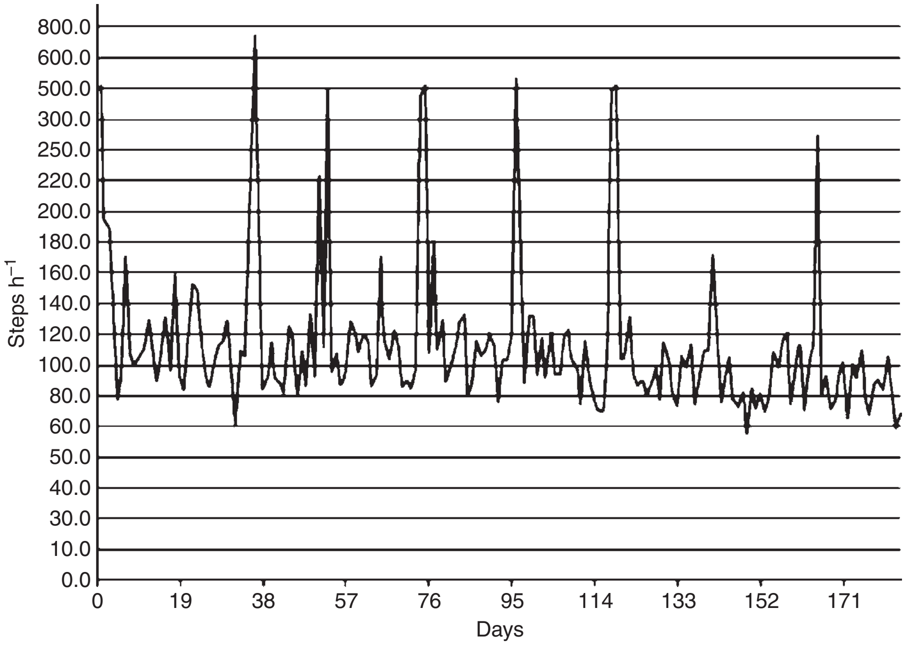

Figure 1 Typical activity graph for the first 175 days of lactation. Reproduced with permission from Nebel RL, Altemose DL, Munkittrick TW, Sprecher DJ, and McGilliard ML (1989) Comparisons of eight on-farm milk progesterone tests. *Theriogenology* 31: 753–764.

developed in the late 1990s and is already being successfully operated (SCR Engineers Ltd., Israel). The technology is based on an integrated neck tag that includes an acceleration sensor, microprocessor, and memory, which enable the recording of a general activity index, which is stored separately in the tag's memory for every 2-h period. This separate recording enables monitoring the cow's activity on a time axis with great accuracy regardless of the time intervals between tag readings. The tag can store data for up to 24 h. The identification unit ('Heatime') is based on an infrared communication method that enables reliable communication. A recent study reported reliability of estrus detection in Holstein heifers with 100% efficiency and 83% accuracy at 1.4 times the threshold value for a 7-day reference period by using a novel radiotelemetric leg pedometer (Gyuho, Comtec, Miyazaki, Japan). The pedometer, powered by a replaceable lithium battery, automatically counted the number of signals generated in 1-h intervals using a pendulum switch (MK-060; Yamasa Tokei, Japan) and temporarily stored the data, which were then read by telemetric receiver (reading ability of 150 m distance) and transferred to a desktop computer.

Pedometry systems that allow prediction of ovulation in addition to efficient and accurate estrus identification will increase the usefulness of such technologies in animal breeding. A study examining the relationship between estrous behavioral parameters, pedometer recordings, and time of ovulation revealed that the increase in the number of steps preceding ovulation, as recorded by the pedometer, rather than other behavioral signs of estrus, could be used to detect estrus and to predict time of ovulation fairly accurately. Cows were equipped with pedometers (Nedap Agri B.V., Groenlo, The Netherlands) that stored number of steps in a 2-h period. Ovaries were examined rectally using an ultrasound scanner. The average interval between onset of pedometer estrus and ovulation was 29.3 ± 3.9 h and ovulation occurred 19.4 ± 4.4 h after the end of pedometer estrus. The interval between onset of pedometer estrus and time of ovulation did not differ between primiparous and multiparous cows. However, the interval between end of pedometer estrus and time of ovulation was shorter for primiparous cows (16.9 ± 3.0 h) than for multiparous cows (20.6 ± 4.5 h), most likely due to the longer duration of estrus in the former. Since the optimal time of AI was found to be 24–12 h before ovulation, based on the above data, inseminations should be performed 5–17 h after the onset of increased activity. Despite the large variation in times from pedometer estrus to ovulation, the pedometer appears to be a promising tool for practical prediction of ovulation as long as the threshold for increased activity is based on the cows' individual activity patterns, and hence could be a tool for improving fertilization rate.

The growing use of computerized dairy management systems and the accurate recording of cow activity by the pedometer system have created new opportunities for the use of cow locomotors as a means of improving the management of both beef and dairy herds. This approach was recently developed by a group of Israeli scientists. Data from serial independent field studies demonstrated that activity data provided by a pedometer system (S.A.E. Tzacham Afikim®, Israel) can serve as a predictor of health problems or stress. Significant differences between pedometric activity of sick and healthy cows were reported in the same herd. It was found that during ketosis, left displaced abomasums, and digestive disorders, walking activity declined several days before the decline in milk production or diagnosis by the herd practitioner. This approach is highly relevant for the early detection of lameness. Clinical lameness causes considerable financial losses for the dairy farm, mostly due to decreased production and delayed estrus and conception. Examination of the pedometer's efficacy at predicting lameness earlier than the appearance of its clinical signs revealed that 46% of the lame cows showed a reduction in pedometric activity (5% or more compared to the average activity recorded through the previous 10 days), 7–10 days prior to the appearance of clinical signs. Given the vast opportunities to improve management routines via the detection of behavior variables, a new behavior sensor has been recently developed by S.A.E. (Tzacham Afikim®), which, in addition to activity (number of steps) for estrus detection, also measures cumulative lying time and lying bouts. For example, this sensor enables the identification of behavioral changes that could indicate calving within 24 h.

Pressure-Sensing Radiotelemetric System

Radiofrequency data communications is the base technology employed by the commercially available pressure-sensing radiotelemetric HeatWatch® system (DDx Inc., Denver, CO, USA) shown in **Figure 2**. A radiotelemetric device attached to each cow consists of a miniaturized radiowave transmitter, powered by a lithium 3-V battery and linked to a pressure sensor enclosed in a hard plastic case that is 5.3 × 8.1 cm and 1.8 cm in height. Each device is secured in a water-resistant pouch, attached to a saddle-shaped nylon mesh patch that is glued with contact-type adhesive to the hair caudal to the sacral region. Activation of the pressure sensor by weight of a mounting herdmate for a minimum of 2 s produces a radiowave transmission (0.4 km range). Transmitted data consist of sensor identification, date (month, day, and year), time (hour and minute), and duration of sensor activation (seconds). Transmitted signals are sent to a microcomputer via a fixed radio antenna. The remote signal receiver should

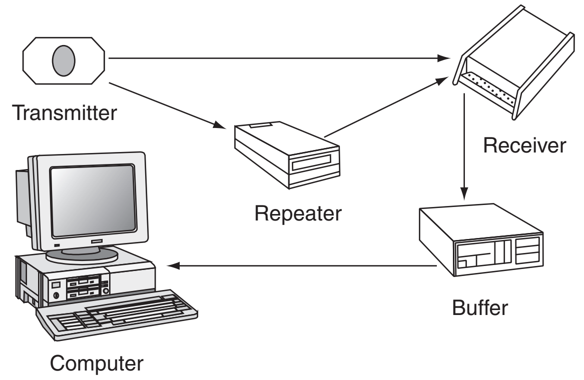

Figure 2 Configuration of the radiotelemetric HeatWatch® system.

be centrally located on each farm to maximize transmission area and situated to minimize transmission interference. Transmitted data from a remote receiver are chronologically stored in a buffer external to the microcomputer and transferred to the microcomputer upon software request. The software generates both fixed management reports and individual cow files, which can be viewed or printed.

Using the radiotelemetric system to monitor mounting activity and ultrasonography to determine the time of ovulation, a significant positive relationship between duration of estrus and time of ovulation was reported. A prolonged duration of mounting activity was associated with an extended interval from first mount to ovulation. However, this relationship existed over a relatively brief time interval (25 ± 34 h); therefore, differences in the duration of estrus would have limited importance in the timing of AI and did not have a significant effect on conception rate.

Duration of estrus, defined as the time interval from first to last standing event recorded by the radiotelemetric system, averaged 7.1 ± 5.4 h for 2055 estrous periods. The duration of estrus varies greatly not only among cows in the same herd but also among different studies (**Table 1**). Differences in age, herd size, management conditions, frequency of observation, and definition of onset of estrus may account for most of the variation in the duration of estrus among studies.

Factors That Affect Estrous Behavior

Most of the older studies report a mean duration of estrus around 18 h; however, recent reports indicate a decrease in 'standing heat', to as low as 37% with shorter periods of around 13 h and, in some cases, as short as 4 h. While not clear enough, various physiological or management factors and health-related problems should also be taken into account.

High Milk Yield

An increased level of milk production has been reported to inversely affect the duration and intensity of estrus and has been related to decreased estradiol concentration in the serum. Since estrogen acts by inducing estrus, both reduced estradiol production and metabolic clearance of estradiol related to high production could account for the inverse correlation between milk level and physical expression of estrus.

Postpartum Period

The postpartum period is critical for efficient reproduction. The occurrence of silent ovulation during the postpartum period is common for the first ovulation following calving. Without progesterone priming, ovulation will occur without clearly expressed behavior. Silent estrus is also related to the extent and duration of negative energy balance and loss of body condition during the transition period or approximately the first 30 days postpartum. Documentation of silent ovulation has been based on endocrine assay and visual observation, including video recording, combined with additional information provided by techniques such as rectal palpation, the use of marker animals, animal activity, and milk–temperature measurements. A recent study using radiotelemetry (HeatWatch® System) that allowed continuous monitoring of cow mounting activity to identify mount acceptance by estral animals and frequent blood collection for progesterone analysis characterized 22% of all ovulations during the postpartum period as being silent ovulations. If visual observations for the detection of

Table 1 Characteristics of estrus and conception rates (least square means ± standard error) for Holstein and Jersey cows and heifers continuously monitored by a rump-mounted pressure-sensitive radiotelemetric system

	Estrous periods (n)	*Standing events (n)*	*Estrous duration (h)*	*Conception rate (%)*
Cows				
Holstein	845	8.8 ± 1.7	7.2 ± 0.31	47 ± 1.5
Jersey	410	123 ± 1.4	8.8 ± 0.37	53 ± 1.1
Helfers				
Holstein	355	23.6 ± 1.3	10.7 ± 0.39	66 ± 1.11
Jersey	166	38.8 ± 1.2	125 ± 0.51	56 ± 0.9

estrus were the only criteria for determination, 62% of all ovulations during the postpartum period would have been classified as silent ovulations. However, many ovulations were associated with fewer mounts accepted by the estral cow and were of shorter duration than mounts accepted at the time of subsequent ovulation.

Circadian Rhythm

Conflicting data concerning diurnal and nocturnal estral activities are found in the literature. While the factors underlying these discrepancies are probably complex, direct or indirect effects on estral expression may be predominant. If estral activities are light-mediated, then suppression of estrus expression would be expected during the nocturnal period. Using the radiotelemetric system to monitor mounting activity in pasture-fed cows, the onset of estrus and total mounting activity were found to be equally distributed throughout the day when grouped into 6-h periods. However, individual hourly variation did occur, with the greatest number of first mounts or onset of estrus occurring between 1200 and 1500 and between 2100 and 2300 h. Total mounting activity did not parallel the hourly distribution of onset of estrus and was more evenly distributed throughout the day, despite a trend toward more mounting activity in the afternoon.

Management Problems

The simultaneous presence of other animals in estrus affords the opportunity of sharing estrous behavior. As a consequence, behavioral signs of estrus involving interactions between cows, such as standing heat, are affected by the number of cows in estrus at any given time. Total herd size as well as milking herd size is an important factor in mating management. A minimum of two cows near or at estrus is required to identify clear estrous behavior. Having few cows in estrus might have an amplifying effect on mounting activity. Small herds are characterized by a low number of mounts per cow in estrus and lack of standing activity, most likely due to the absence of cow–cow interactions.

When housed in comfort stalls, cows were about 2.76 times more active during estrus, indicating that the type of housing influences the magnitude of the change in physical activity. The type of floor can also influence mounting behavior. For example, slippery, wet, or concrete floors are not conducive to mounting, while a dirt floor or thick straw bedding is preferred. Studies in heifers have shown that detection indices by pedometer systems (Gyuho, Comtec, Miyazaki, Japan) were influenced by the location of pedometer attachment (neck or leg) and rearing condition (grazing, paddock, tie stole) with a high value for leg pedometer in paddock rearing condition.

Seasonal variation in ambient temperature, photoperiod, and humidity can influence estrous behavior. Heat stress lengthens the estrous cycle and decreases the duration and the intensity of estrus. Estrus can be 20–30% longer in temperate or moderate climates than in very hot or cold weather. Combinations of different detection systems, including modern cooling systems, have been reported as a better strategy than the use of a single system in order to increase estrus detection and conception rates. When more than one system was used, a higher conception rate was obtained under summer conditions. Therefore, this strategy should be included in dairy herd management programs.

It should be noted, however, that stresses other than heat stress can promote a decline in normal forms of estrous behavior. Different daily activities such as walking to the milking parlor, cleaning the resting area, or number of feedings can also affect estrous behavior. Physical discomfort or social stress caused by moving cows from their original groups can result in altered behavior and production performance.

Estrus Detection and Prediction of Ovulation and Time of Insemination

Several studies have examined the optimal time for insemination relative to the onset of estrus. In one study, each of 17 farms selected a different interval to inseminate cows identified in estrus during the previous 24 h. Pregnancy status was determined by data for return to

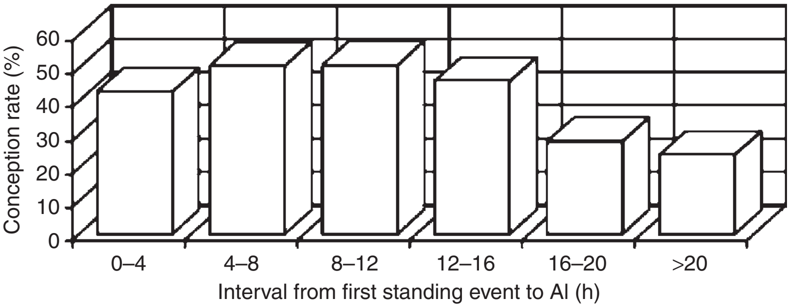

Figure 3 Percentage of pregnant cows by 4-h intervals relative to timing of artificial insemination (AI) from first standing event detected by the radiotelemetric HeatWatch® system across 17 herds and 2661 inseminations.

estrus and palpation of the uterus 35–75 days following insemination. The bar graph shown in **Figure 3** represents the proportion of pregnant cows for each 4-h interval from first standing event to insemination. Inseminations performed between 4 and 12 h following onset of estrus achieved a conception rate of approximately 50 versus 30% for inseminations performed after 16 h from onset (**Figure 3**). From previous studies, near-optimal conception rates would be expected for cows submitted for insemination 12–18 h after detection of estrus. Mathematical modeling to predict the optimal time for AI using activity pedometers and visual signs of estrus estimated 11.8 h from onset as the optimal time for AI, which coincides with the approximate midpoint of the 5 ± 16 h optimum using the HeatWatch® System.

Since the chance of fertilization strongly depends on the interval from insemination to ovulation, it is reasonable that insemination time be based on time of ovulation rather than detection of estrus. Pedometer activity systems and pressure sensing to monitor mounting activity appear to be promising tools for predicting ovulation and hence could serve for the improvement of fertilization rate. Use of these systems showed that ovulation takes place 22–32 h after the first increase in activity. Since the optimal insemination time is 24–12 h before ovulation, the optimal time of insemination becomes 4 ± 17 h after the increase in locomotive activity or 0–12 h following the first standing event associated with the onset of estrous behavior.

Conclusion

Remote-sensing systems for the detection of estrus are expected to be more efficient but not necessarily more accurate than visual observation. Differences in housing and environmental conditions, in addition to labor, cost, and efficacy, have resulted in variable acceptance of remote-sensing technologies. Detection efficiency and accuracy can be improved by the simultaneous use of more than one technology. Combining technologies for simultaneous measurements of several physiological events associated specifically with the onset of estrus and ovulation time should provide more accurate predictions of the optimal time for insemination. Ultimately, herd management must interpret the information gathered by these technologies and judge whether and when to inseminate the identified cows based on visual inspection.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Replacement Management in Cattle**: Breeding Standards and Pregnancy Management. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Estrous Cycles: Characteristics; Mating Management: Artificial Insemination, Utilization; Mating Management: Fertility.

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Mating Management: Artificial Insemination, Utilization

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Introduction

Artificial insemination (AI) of cattle represents the most successful sophisticated program of animal breeding ever implemented to improve the quality, productivity, and reproductive health of dairy cattle and other farm animals. This article, in conjunction with other articles on AI (*see* **Gamete and Embryo Technology**: Artificial Insemination), provides an overview of the advanced technology developed, the facilities and management required, and the genetic improvement in cattle as a result of the use of AI during the past half century. Other species are considered briefly. Recent advances in genomics, computerized mating programs, gender sorting of semen, and cloning relevant to AI programs are considered.

Components of a Successful Artificial Insemination Program

The key to any successful program is capable, well-trained, and dedicated people. Expertise represented by the array of people in AI encompasses geneticists to select bulls, expert bull handlers, semen collectors and laboratory technicians, highly trained field staff, skilled inseminators, and superior farm managers. All require appropriate facilities and equipment to conduct a high-quality program. The two major factors responsible for the success of AI are (1) improved reproductive health, particularly through the control of venereal diseases, and (2) genetic improvement in productivity and a reduction in lethal genes.

All of the components of AI and their relationship can be quantified by two simple equations. A sire's genetic contribution will depend upon its genetic superiority and the number of progeny produced:

$$\text{genetic impact per sire} = (\text{genetic superiority of the sire}) \times (\text{number of progeny per sire}) \quad (1)$$

The physiology and management that impact on the number of progeny produced per sire are represented by the equation

$$\text{number of progey per sire per year} = \frac{(\text{number of sperm harvested per sire})}{(\text{number of sperm inseminated per cow})} \times (\text{fraction of the semen used for insemination}) \times (\text{fertility : \% of inseminations producing progeny}) \quad (2)$$

All of the improvements in harvesting sperm from the bull, preserving sperm with minimal loss, and skillfully placing the right number of sperm in the well-managed cow at the proper time will affect the number of progeny.

Landmarks in the Development of Artificial Insemination Facilities

Early in the development of AI for livestock breeding, facilities were very limited, with a modified existing barn to house bulls, an area equipped to serve as a simple laboratory, and a semen collection chute, often outdoors. Originally, developed around a liquid semen program, regionally sited bull studs were needed. Following the introduction of semen cryopreservation, nationally sited centers were developed. With sophisticated facilities allowing assured health surveillance, international trade became possible. Semen cryopreservation required special equipment for processing, packaging, storing, and transportation of semen. The equipment developed for bull sperm provides the basis for the worldwide cryopreservation of biologics today (*see* **Gamete and Embryo Technology**: Artificial Insemination). Modern facilities that were better, larger, and more expensive emerged. Many small bull studs were merged for efficiency, as climate-controlled bull barns, herd health surveillance and isolation facilities, special semen collection areas, animal clinic areas, and modern laboratory facilities were built by consolidated larger AI organizations. Today in the field, supervisors and reproductive specialists help AI technicians and herdsmen to manage the on-farm AI program.

Frozen Semen in the Field

Equipment and techniques were developed for handling semen stored in liquid nitrogen at −196 °C in the laboratory and moving it safely to the field for each inseminator's storage unit. Today, inseminating technicians are given special training to handle frozen semen into and out of the liquid nitrogen tank, and to retrieve the proper breeding unit for insemination without exposing the remaining units in storage. Proper handling of frozen semen and maintenance of an unbroken chain of cryogenic temperature (less than −130 °C) are important. Otherwise, carefully prepared high-quality sperm could be damaged with resultant lowering of fertility. Proper thaw and insemination methods are discussed later in this article. Inseminations today are performed by both professional inseminators and herdsmen who buy semen from a producer. As herd size has increased, many on-farm inseminators have gained proficiency by inseminating several hundred cows, one or more times.

Farm Facilities and Detection of Estrus

Upon adoption of AI, a very important task faced by dairy farmers is accurate detection of estrus, so that cows could be inseminated at the proper time. Extensive efforts are made to ensure that all users of AI implement sound programs for estrus detection. This includes proper identification of each animal (highly visible cow ID), turning out cows in stanchions, and watching of cows for estrus for about 30 min each morning and evening. Some herdsmen manage this program better than others. Many aids for detection of estrus have been developed. Several will be listed here because poor detection of estrus is the largest single cause of prolonged, uneconomic calving intervals (*see* **Reproduction, Events and Management**: Mating Management: Detection of Estrus).

Estrus detection aids include using surgically altered bulls that could not mate with animals, but could mount and roll colored paint on the rump of animals that stood when they were mounted. Alternatively, a colored crayon or especially brittle paint could be used to stripe the tail-head of any animals due to be inseminated. This stripe is smudged or the paint broken up when that cow is mounted. Different types of pressure-sensitive patches, which are easily attached to the rump, were developed that become more visible when pressed hard by a mounting animal. One version uses small digital radio transmitters incorporating a pressure switch in the tailhead patch. The transmitter monitors mounting activity and transmits the data (cow ID, date and time of mount, duration of the mount) to the herd computer. Electronic probes to measure changes in electrical resistance of cervical mucus are also effective in revealing changes at estrus. Pedometers of various types record walking activity of the animal, indicated on a mechanical component of the pedometer. More advanced types transmit activity electronically, along with the cow identification, to a receiver.

To develop effective estrus detection programs and evaluate their efficiency, it was necessary to monitor the estrous cycle of cows. This became possible with the discovery that the cyclic hormone progesterone could be measured in milk. The milk progesterone followed a similar pattern as blood progesterone, so simply collecting small samples of milk 2 or 3 times per week for progesterone determination permitted the cyclic activity of each cow to be tracked. In addition, this monitoring enabled one to determine missed heats, plus cows that were inseminated at the wrong stage of the estrous cycle or when pregnant. Studies in Israel and at Cornell University have shown that the use of some of these technological aids, plus training of the farm managers and the inseminators, can minimize mating at the incorrect time, and thereby maintain an optimal calving interval. Heifers often are housed in open areas with bulls. However, they should be managed to use AI because more genetic progress is made when they are inseminated with conventional or sexed semen from genetically superior bulls.

Procedures for Artificial Insemination

Effective care needs to be taken to prevent temperature fluctuations of thawed semen, abrupt cooling of semen (cold shock), and semen or supplies from sun exposure or warming beyond body temperature. Loading the AI gun should take place in a protected area, free of extreme temperatures and close to the cryostorage unit. All AI should be completed within 15 min of straw thawing. The number of straws thawed at one time is dependent on the quantity that can be used within 15 min. In large herds, teams of inseminators work together to prepare AI guns, and perform AI.

Unless specified otherwise, semen should be 'warm-water thawed'. Some organizations produce semen as straws processed by alternative methods that permit straws to be 'pocket thawed' as well as 'warm-water thawed'. Upon retrieval from the cryostorage unit, straws that require warm-water thawing are thawed in water (~0.5 l) in an insulated vessel at 33–35 °C for a minimum of 40 s. A maximum of four straws at a time can be thawed in the vessel. This prevents the water temperature from cooling to temperatures below specifications. Straws should be agitated slightly to ensure uniform thawing.

Straws that are 'pocket thawed' are immediately placed in a folded paper towel for protection following retrieval from the cryostorage unit. After this preparation, straw and towel are placed into a thermally protected pocket. A minimum of 2–3 min of thawing time within the pocket is provided before preparing the AI guns. For retrieval of multiple straws, straws are placed into separate towels to ensure uniform thawing. Straw forceps should be used at all times to prevent contact between bare fingers and the straw.

If needed, the temperature of the AI gun is tempered by friction and prior placement within the inseminator's coveralls. Before sliding the thawed straw into the barrel of the AI gun, the temperature should be checked by touch, as a subjective method to avoid temperature extremes. The loaded gun is covered with a paper towel for insulation while cutting the straw end. A sheath is slid over the loaded gun and is secured. The loaded gun is then placed within a clean breeding glove, providing an added layer of thermal protection, and is finally tucked inside the coveralls.

The following is a conventional rectovaginal insemination procedure. Generally, the left arm, covered with a disposable glove and lubricated with mineral oil, enters the rectum. Through the thin tract layers, the cervix is located and grasped. Folded paper toweling is used to spread the vulva, allowing clean entrance of the gun into the vestibule of the vagina. Care is taken to gently manipulate the cervical rings of the lumen over the end of the gun until the gun tip reaches just past the anterior cervical ring. Location of the gun tip in the uterine body is determined with an extremely light touch through the uterine body wall. When satisfied with the position, the outside end of the AI gun is braced on the opposing arm and the plunger is pushed to deliver semen to the uterine body in 3–5 s. The gun tip is not extended past the internal uterine bifurcation to avoid the possibility of any injury to the uterine mucosal tissue. Cows that have been bred once, but then exhibit estrus behavior again should be rebred with caution as an abortion can be initiated in a pregnant female. Therefore, the AI gun should be passed completely through the cervix only if the tract is felt to be typical for a female in normal estrus.

Use of Computerized Mating Programs in Artificial Insemination

In dairies that utilize computerized mating programs, each cow in a herd is examined and scored in a linear trait evaluation format. Cows are compared to their contemporaries for each trait and the differences are weighted by the heritability of the specific trait. Deviations from contemporaries are adjusted for the herd's genetic level. Pedigree information is also included. When a mating is planned, the cow's complete phenotypic evaluation linear score is viewed and from computer files of sire information, potential mating sires are selected, emphasizing corrections to worst faults for maximum genetic progress.

A very important benefit of using computerized mating program is that it is possible to control and restrict the level of inbreeding in the herd. A mating program will ensure that no member of a three-generation pedigree is duplicated in the mating. This is expected to maintain inbreeding below 6.25%. Furthermore, mating programs can be set up so that embryo losses that result from harmful gene interactions can be avoided. Cattle that carry the same lethal recessive gene should not be mated.

Artificial Insemination in Estrus-synchronized Cattle

Several programs have been developed to synchronize estrus and ovulation so that a group of cows can be inseminated at a fixed time. These programs involve the injection of prostaglandin F2α or analogues to regress the corpus luteum (the source of progesterone), and an injection of gonadotropin-releasing hormone (GnRH) to stimulate ovulation (*see* **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination).

In viewing 2008–09 data (500 000+ inseminations) from a company providing extensive professional AI service in US herds, AI to synchronized cattle approximates 27% of total inseminations; however, in many herds, AI to synchronized cattle accounts for 80% of inseminations. Of these AIs, Cosynch and Ovsynch accounted for 25 and 75%, respectively. Differences in fertility (as unadjusted nonreturn rate means) between the two synchronization protocols were essentially zero. An inspection of adjusted fertility rates revealed that AI to synchronized cattle approaches, but does not equal, AI to nonsynchronized cattle (−1.5% for probability of conception). There is an interaction of bulls and synchronization. That is, while bulls can be ranked with respect to overall fertility, bulls will rerank in a different order when semen is used on synchronized cattle.

Use and Efficiency of Artificial Insemination in the United States

The changes in dairy cow numbers and the use of AI in the United States are summarized in **Table 1**. Milk production per cow has tripled since 1925 while the number of cows has been reduced by two-thirds. Consequently, the many fewer cows today produce as much milk nationally as was produced in 1925. This production meets demands as milk consumption per capita has decreased.

Table 1 Dairy and beef cow numbers and the percentage inseminated artificially in the United States

	Dairy cows[a]		*Beef cows*	
Year	*Number*	*Artificially inseminated (%)*	*Number*	*Artificially inseminated (%)*
1925	25 000 000	0	10 000 000	0
1950	21 500 000	12	15 000 000	<1
1975	12 000 000	57	45 000 000	4
2000	9 000 000	65	34 000 000	10

[a] Dairy cow numbers do not include heifers; a lower percentage of heifers is inseminated artificially. Data indicate trends only, as precise data are not available for all time periods, particularly with many inseminations by on-farm personnel not reported.

This massive increase in the efficiency of milk production, with fewer cows and 99% fewer bulls used through AI, has saved 25 million tonnes of maize otherwise needed for feed. Along with other improvements in management, it has allowed many producers to become so efficient that they have survived the increased costs of doing business.

Economic and organizational efficiencies have resulted in the reduction of the number of AI organizations worldwide. In the United States, there were about 100 AI organizations in the 1940s. Most of these organizations have merged or closed. Today, six AI organizations supply most of the semen for dairy cattle AI.

Extent of Artificial Insemination Worldwide

In **Table 1** it is shown that about 65% of the cows in the United States are enrolled in an AI program. In many European countries, use of AI is as high as, or higher than, 65%. In Czechoslovakia and Hungary, more than 90% of the dairy cows are artificially inseminated, and in Denmark, Israel, and Japan the proportion of cows impregnated by AI is essentially 100%. The various countries comprising the former USSR also use AI extensively.

Most countries rely essentially 100% on frozen semen for cattle. Countries with large dairy cow populations are listed in **Table 2**. In New Zealand, a majority of cows are inseminated during 2–3 months so that cows will calve and initiate lactation during the excellent pasture season. During this short time, mostly liquid semen is used to meet demands, with 2 000 000 sperm per cow giving acceptable fertility. This permits a few top sires to be scheduled to meet the market demands at low costs of semen processing. Frozen semen is banked at other times, with 20 000 000 sperm per breeding unit, providing more than is necessary to compensate for freezing damage.

Sire and Selection Programs and Genetic Progress

The modern progeny test system as a useful genetic program for identifying superior sires for extensive use in AI was developed simultaneously by Henderson at Cornell and by Rendel and Robertson in Scotland. A young bull for progeny testing is produced by inseminating a genetically elite dam with semen from an elite sire. The young bull enters the stud through its isolation facility, enters the production herd, matures and produces semen for progeny testing at about 12–15 months. This semen is used in AI to produce daughters, whose subsequent records for production traits contribute to the production proof of the bull. Bulls being progeny tested are returned to service with a progeny test proof at about 4–5 years of age.

The genetic progress, along with levels of milk production, is illustrated in **Figure 1**. Little genetic progress was made before 1955, but by then a few bulls proven in AI through the progeny testing scheme outlined above were available. Progress has been rapid since 1975 when many tested bulls were available. There is no indication yet that a peak is being reached.

Effect of Genomic Evaluations on Artificial Insemination Utilization

Genomic information in cattle is increasing rapidly, aided partly by cross-species homologies. Geneticists working with cooperating AI companies used DNA derived from a pooled frozen library of semen from generations of dairy bulls and the resulting daughters' milk records. They identified DNA segments associated with phenotypic traits, and this genomics approach, with the application of microarray identification of single nucleotide polymorphisms (SNPs), marker-assisted selection, and other reproductive technologies, has increased the intensity of selection and shortened generation intervals with more

Table 2 Use of frozen semen in countries reporting ≥1 500 000 cows artificially inseminated

Country[a]	*Number of dairy and beef cows artificially inseminated*	*Use of frozen semen (%)*	*Number of sperm per cow* $(\times 10^6)$[b]
Australia	1 600 000	100	20
Brazil	2 860 000	100	40
Canada	1 500 000	100	15
China	10 000 000	100	
France	4 800 000	90	20
Germany	5 600 000	98	15
Italy	2 450 000	100	18
Japan	1 600 000	99	20
Korea	1 600 000	94	30
Netherlands	1 650 000	100	20
New Zealand	3 800 000	37[b]	20
Spain	1 800 000	95	30
United Kingdom	2 600 000	100	20
United States	10 500 000	100	20

[a] Data from the former USSR were not available, but historically it has been a leader in numbers.

[b] For bulls in high demand, the insemination dose may be reduced to 10 million sperm, and with the liquid semen program (63%) in New Zealand only 2 million sperm are used per insemination.

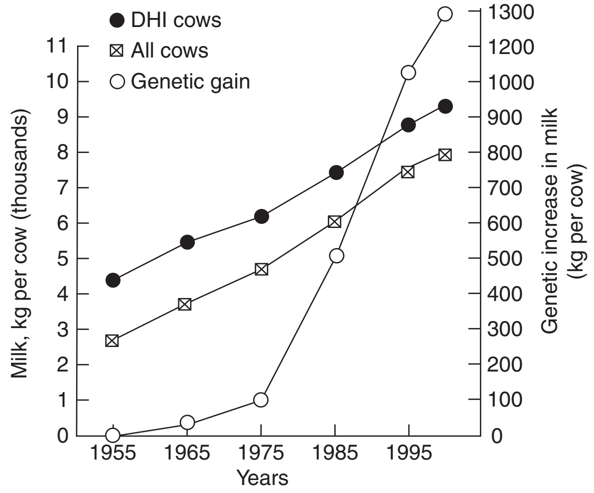

Figure 1 Changes in milk production per cow in all herds and those on test in dairy herd improvement (DHI) in the United States, along with the estimated genetic improvement, primarily as a result of superior sires used in AI.

rapid progress in genetics (*see* **Genetics**: Selection: Concepts). Positive identification of a variety of alleles present in newborn animals will assist in selecting those with the greatest promise for transmission of genes associated with desired traits.

In the United States, more than 30 000 cattle, primarily Holsteins, have been genotyped since December 2007 for important production traits such as milk, fat, protein, somatic cell score (SCS), and daughter pregnancy rate (DPR). While genomic evaluations have a lower reliability, many outstanding bulls have been identified with genomic evaluations, reflecting several years of added genetic progress. In 2009, young Holstein bulls with genomic-only evaluations began to be utilized for breeding purposes. Due to lower reliability, the pattern of AI usage is changing to limit the amount of semen used from an individual bull and to utilize semen from much larger groups of bulls. In 2009, a large proportion of semen being utilized for AI in the United States is from bulls with genomic-only evaluations.

Selection and Mating Based upon Multiple Traits

Selection of Mating Sires Based on Production-Associated Evaluations

Much information is collected on sires proven in AI in addition to the milk, butterfat, and protein production of their daughters. Daughters are checked for traits such as body size, leg conformation, mammary gland size, udder support and teat placement, and milking speed. This information is published so that those who wish to select semen from sires that produce daughters with particular strengths besides high milk yield can do so. Bulls that produce small calves are identified, and may be selected to minimize calving difficulties when small heifers are inseminated. The broad scope of possibilities among frozen semen available from many bulls through AI gives the producers almost limitless opportunities to plan a program that meets individual desires. However, when selection is made for more than one trait, the genetic gain for each trait is reduced. The most profitable program for commercial dairy producers is to put major emphasis on milk yield.

Selection of Mating Sires Based on Fertility-Associated Evaluations

Fertility of the bull is important, as high reproductive efficiency is a major factor in the success of the dairy enterprise. Bulls with low fertility are in limited demand, even when genetic merit is high. Semen from low-fertility bulls is seldom used to inseminate repeat breeder cows. AI companies with large numbers of professional inseminator staff can utilize breeding receipt and farm records to provide sophisticated fertility evaluations. For many companies, it is more difficult to obtain such records as the proportion of semen sales directly to farms has grown. National evaluations for sire fertility exist in many countries. From the USDA's use of milk test records, estimations of sire fertility, as sire conception rate (SCR), are available. SCR results are based on confirmed pregnancies. Important factors that are adjusted include year, herd differences, parity, service number, location, season, milk yield, and sire age. Selection of a mating sire based on its DPR is also possible. A calculated DPR includes calving interval and days open in the calculation. Predicted transmitting abilities (PTAs) for cow fertility traits have high reliabilities only after hundreds of daughters are recorded.

Advanced Reproductive Technologies in Artificial Insemination

Multiple Ovulation and Embryo Transfer

Multiple ovulation and embryo transfer (MOET) herds are an important source (oocytes, sperm) for elite genetics used in cattle AI. With the introduction of genomic evaluations, newborn cattle of either gender produced in MOET herds are quickly evaluated for their genomic estimated production superiority. While conventional AI provides genetic material from superior males, MOET provides a greater influx of genes into the population from superior females.

A genetic evaluation based on MOET-derived cattle is possible. Full brother–sister embryos are produced and transferred. As full brothers and sisters are born at the same time, full sisters can be raised in a sib test to provide contemporary production information for the sires. This decreases the generation interval compared with the progeny test system, although the comparative information is a little less reliable than the progeny test. By harvesting oocytes from juvenile females and fertilizing them *in vitro* to produce embryos, the generation interval can be further reduced (*see* **Gamete and Embryo Technology**: Multiple Ovulation and Embryo Transfer). With MOET programs, animals are usually raised in a central facility. This makes it possible to record multiple production information and related traits. For example, body and udder conformation and milking speed can be recorded. Such a facility could also use sexed sperm to produce embryos of the desired sex by *in vitro* fertilization.

Embryos produced by any method can presently be sexed and in the future can be tested with DNA analysis, allowing genomic evaluation (*see* **Gamete and Embryo Technology**: Sexed Offspring). Also, embryos can be split quite easily with 50% more progeny per donor cow. All these techniques can be combined, and each one adds a small increment to the rate of genetic progress. However, the greatest single component of genetic gain is the use of AI-proven sires.

Artificial Insemination with Sexed (Gender-Sorted) Semen

The ability to use sexed semen has been an important development in AI. With ample replacement cattle now available, it is viewed as allowing the dairy herds to obtain their heifers from their best maternal lines, increase calving ease, close their herds if desired for biosecurity (growing from within the herd), and provide an ability to absorb increased culling rates, allowing disposal of problem cows. Sexed semen, however, is expected to have a lower fertility rate (*see* **Gamete and Embryo Technology**: Sexed Offspring). For this reason, best results are obtained when breeding heifers exhibiting standing heat. Research is ongoing on whether sorted semen can be economically used in cows. Two types of products are available for AI: 90% gender purity and 75% gender purity. While sorting purity can be achieved for either gender, in the dairy industry it is overwhelmingly desired toward producing female offspring.

In 2008, 14% of all USDA-AIPL reported heifer breedings in the United States were with sexed semen AI. During the period from 2006 to 2008, AI with conventional semen in heifers and cows achieved a 56 and 30% conception rate, respectively, while AI with sexed semen achieved 39 and 25%, respectively. In viewing current US data gathered in large commercial herds (188 700+ total professional AIs), AI with sexed semen accounted for 16% of AI and in some herds 100% of heifers are bred with sorted semen. In these herds, use of sorted semen, after adjustments for interactions, was found to reduce the probability of conception by 12.7%. Bulls ranked by overall AI fertility may rerank differently when their semen is sorted.

Cloned Sires in Artificial Insemination

Currently, there are a few cases where semen for AI is currently being processed from sires produced as clones from deceased genetically evaluated individuals. The genetic evaluation of the original sire is utilized as the evaluation for the clone. Currently, cloning is inefficient and expensive. If bulls with rare or unique genes are selected and cloned, the resulting clones can be used to inexpensively disseminate desired genes by AI. Tissue appropriate as a source of cloning could be preserved by cryopreservation for use in cloning.

Artificial Insemination in Beef Cattle and Buffaloes

AI and embryo transfer played an important role following limited importation of several European breeds of beef cattle into North America. In some breeds, AI usage was as high as 75% initially, but with expansion it has decreased. The overall estimated usage of AI is shown in **Table 1**.

Beef bulls produce approximately the same number of sperm as dairy bulls. The management of beef sires is similar to that of dairy sires, but owners should not let beef bulls get fat, as sperm output and quality are depressed. Testis size and quality should be measured on all sires. The quality of frozen beef semen varies more than the quality of semen from dairy bulls, probably because much of the frozen beef semen is custom collected, with less culling of bulls for semen quality.

The major management difference between dairy and beef is in management of the females. Beef cows and heifers for AI must be confined. Animals should be in good condition. Cows suckling calves, and with poor body condition, have low pregnancy rates. High pregnancy rates in beef cattle are much more important than in dairy cattle, as females with no progeny contribute nothing economically to the beef enterprise.

Buffaloes are a major source of meat, milk, and work in many Asian countries where 95% of the world's buffaloes exist. Leading countries include India, Pakistan, China, Nepal, and Thailand. Significant numbers are also found in Romania, Egypt, and Italy (*see* **Animals that Produce Dairy Foods**: Water Buffalo). Reliable statistics on the percentages of AI use and results are not available.

Use of Artificial Insemination in Species other than Cattle

A summary of the technical feasibility of using AI in several species is given in **Table 3**. The fastest growing use of AI is in swine. Sows are usually confined; they are easily inseminated. Large breeding and production farms can capitalize on inseminating many more sows with semen from superior boars than through natural mating. In China, 10 000 000 sows are inseminated annually, with an estimated 50 000 000 inseminated worldwide. With liquid semen, pregnancy rates and litter sizes are normal. Freezing boar sperm depresses fertility.

The poultry industry also depends essentially 100% upon AI in the industrial breeding flocks to produce hatching eggs for turkeys, broilers, and egg-laying hens. Semen is easily collected. Many caged females per male can be inseminated rapidly with fresh semen once a week with high fertility. This permits selection of the best males to produce the next generation of poultry.

Tens of millions of sheep and goats are artificially inseminated worldwide, particularly in Russia, Bulgaria, Romania, China, several other Asian countries, and South America. In western Europe, France is the leading country, where 850 000 sheep are inseminated with liquid semen and 580 000 goats with frozen semen. In Australia, 200 000 ewes are artificially inseminated, particularly in flocks producing rams for sale. The number inseminated

Table 3 Technical feasibility of using AI in species other than cattle

	Fertility of semen		
Species[a]	*Liquid*	*Frozen*	*Any limitations*
Sheep	Good	Poor[b]	Problem with large ranges. Low value per ewe, so AI costs must be very low
Goats	Good	Fair	Detection of estrus in small herds. Insemination is more difficult. Lack of available semen
Swine	Good	Fair	Supply of liquid semen and rapid transportation is required. Improved detection of estrus in gilts. Cost per sow must be kept low, but this is competitive
Horses	Good	Fair	Long estrus. Multiple inseminations needed. Frozen semen conception rates are reduced. Breed restrictions
Turkeys, chickens	Good	Poor	No limitation in breeding flocks. Use of fresh semen is the method of choice

[a] Note that cattle have an advantage over other species in number of progeny possible per tested sire per year: cattle, 50 000; sheep and goats, 5000; swine, 2000; horses, 750. The successful development of a frozen semen bank is especially advantageous in seasonal breeders such as sheep, goats, and horses.

[b] Frozen semen can be used successfully provided a laparoscope is used with intrauterine inseminations.

AI, artificial insemination.

with frozen semen has been limited by low fertility, unless intrauterine insemination with the aid of a laparoscope is used.

Investigation of AI in the early 1900s was directed toward the horse, but declined as the horse populations decreased on farms. In the United States, horse AI has increased to about 300 000 mares inseminated annually, as several breed associations have revised breed codes to permit registration of foals produced by AI. Lighting is often used to alter the seasonal breeding of mares.

The smallest animals where AI is used extensively are laboratory rabbits and foxes. The latter are raised for the fur trade in northern Europe. Deer farming is extensive in a few countries. There are many examples of selective AI used for endangered species in zoos where few males are available, or if females refuse to accept the male.

See also: **Animals that Produce Dairy Foods**: Water Buffalo. **Gamete and Embryo Technology**: Artificial Insemination; Cloning; Multiple Ovulation and Embryo Transfer; Sexed Offspring; Transgenic Animals. **Genetics**: Selection: Concepts. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Mating Management: Detection of Estrus.

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Mating Management: Fertility

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Introduction

In dairy cows, reproduction has two major functions: to induce the onset of lactation and to provide replacement animals for the current generation of cows. Consequently, reproductive efficiency is a major factor affecting production and economic efficiency. Over the past four decades, milk yield per cow has increased significantly through genetic selection and improved management. Not withstanding these improvements, there has been a steady decline in reproductive performance of dairy cows coincident with the improvement in yield. The focus of this article is to review the components that determine reproductive efficiency of dairy cows, and to investigate how best to use the new information that has emerged over the last few decades to improve the reproductive management of a herd and consequently its production efficiency.

Reproductive Targets

Although it is frequently argued that milk production efficiency is at its highest when cows reproduce once every 365 days, the optimal reproduction function that maximizes profit is dependent on numerous factors, including production system, level of milk production, milk prices, and feed cost, among others. Consequently, it is impossible to give a set of specific targets applicable to all systems of production. However, the three measures presented in the following section (see also **Table 1**) are useful as initial measures of reproductive performance in seasonal and all-year-round calving herds. Using only one measure of reproductive efficiency can be misleading and can mask other important inefficiencies.

Components of Reproductive Efficiency

Even though there are numerous factors that affect the reproductive performance of individual cows and consequently herd reproductive performance, these factors can be categorized under the following three broad headings:

1. The interval from calving to resumption of ovulation and regular estrous cycles
2. Estrous detection efficiency and submission rate
3. Conception rate following service

The Interval from Calving to Resumption of Ovulation and Regular Estrous Cycles

Generally, about 80% of dairy cows will have ovulated within 28 days of calving and about 10% of cows will not have commenced ovulation by 42 days postcalving. In dairy cows, the main cause of anestrum is prolonged negative energy balance (NEB), which results in low LH pulse frequency, decreased concentration of insulin-like growth factor-I (IGF-I), low estradiol production, and ultimately the failure of the dominant follicle (DF) to ovulate.

The number of ovulatory estrous cycles preceding insemination has been shown to beneficially influence subsequent conception rate. Consequently, it is desirable that dairy cows resume ovulation in the first 4 weeks after calving. Following delivery of the calf and fetal membranes, there is a decline in the plasma concentrations of progesterone and estradiol and a corresponding removal of the negative feedback effects of these steroids, particularly estradiol, on gonadotropin synthesis and secretion. Follicle-stimulating hormone (FSH) secretion commences during the first week postpartum, and this stimulates the commencement of ovarian follicle growth and the appearance of a DF on the ovary at about days 12–16 postpartum. The fate of this follicle in terms of whether it ovulates or undergoes atresia appears to be related to whether it produces estradiol, which in turn appears to be associated with exposure to an adequate LH pulse frequency and concentration of IGF-I.

NEB in early lactation does not affect the follicle population or the timing of recommencement of DF growth but does affect the ovulatory fate of the first DF. However, energy balance (EB) in early lactation, rather than milk yield *per se*, appears to be the more important factor affecting resumption of ovulation postcalving, and dry matter intake (DMI) is a more important determinant of EB than milk yield. Positive association between EB in early lactation and the interval from calving to resumption of estrous cycles has been recorded. There is increasing evidence that IGF-I is a potential mediator of nutritional effects on fertility. Increased plasma concentrations of IGF-I during the first 2 weeks postpartum are associated with early resumption of ovulation. It has been demonstrated that feeding dairy cows an insulin-promoting diet increases plasma concentrations of insulin and also shortens the interval from calving to first postpartum

Table 1 Targets for seasonal and all-year-round calving herds

Variable	*Seasonal calving herds*	*All-year-round calving herds*
Calving interval	365 days	<420
Culling for infertility	<5%	<10%
Compactness of calving	80% calved in 60 days	NA

ovulation in both high- and low-genetic merit dairy cows. Insulin is a potent stimulator of follicle differentiation and steroidogenesis, promotes DF differentiation, and enhances responsiveness to LH and in turn increases estradiol secretion leading to a preovulatory LH surge and ultimately the ovulation of the DF. NEB also causes a decrease in circulating concentrations of IGF-binding proteins (IGFBPs). Because the IGFBPs transport and increase the half-life of IGFs, low blood concentrations of IGFBPs brought about by NEB would therefore limit the availability of IGFs to target cells in the follicle and hence limit their ability to synergize with pituitary gonadotropins to stimulate cell proliferation and steroidogenesis and ultimately ovulation (**Figure 1**).

An objective with dairy cows in early lactation is to achieve a high DMI, as this would be expected not only to hasten the onset of estrous cycles postcalving but also to increase conception rates (see later) and shorten calving-to-conception intervals. Increasing dietary intake is restricted by the requirement for inclusion of fiber in the diet to maintain rumen function as well as by the variability in voluntary feed intake by cows during this period.

Improving Heat Detection

The single most important factor affecting heat detection efficiency is the ability of those responsible for checking for heat to fully understand the signs of heat and their commitment to heat detection for as long as is planned to use artificial insemination (AI). About 10% of the reasons for failure to detect heats can be attributed to cow problems, and 90%, to 'management' problems. The latter

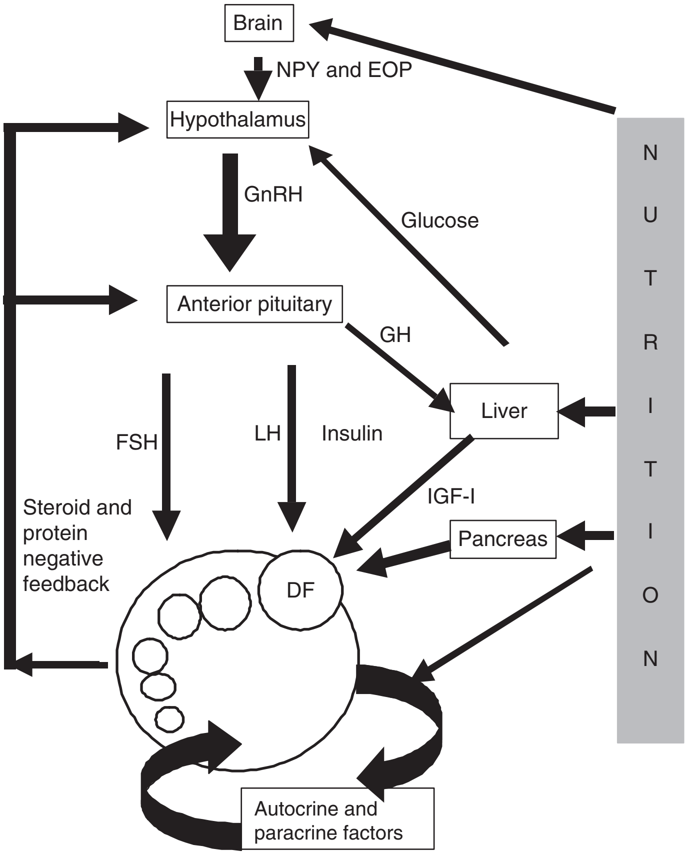

Figure 1 Possible mechanisms by which nutrition could affect ovarian follicular function. EOP, endogenous opioid peptides; NPY, neuropeptide Y. Reproduced with permission from Diskin MG, Mackey DR, Roche JF, and Sreenan JM (2003) Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Animal Reproduction Science* 78: 345–370.

would include too few observations per day for checking for heat activity, too little time spent observing the cows, and/or observing the cows at the wrong time or in the wrong place, such as feeding time or in the collecting yard at milking time. Another major reason for failure to detect heat is that those involved in heat detection do not understand the signs of heat.

Records

Individual animal records are an essential part of good breeding management. All animals must be clearly and permanently identified by one of several methods, such as plastic ear tags, neckbands, or freeze branding. Whichever system is preferred, it is essential that the animal number be clearly legible from a reasonable distance. Breeding records should include (1) animal number, (2) calving date, and other information relevant to calving, (3) prebreeding heat dates, (4) first and repeat service dates, sire used on each date, and inseminator code, (5) date and result of pregnancy diagnosis, and (6) date of expected calving. Good records are not only part of good farm management practice, but also the first essential step in all infertility investigations.

Monitoring submission rate

Submission rate is calculated as the proportion of cows calved at the beginning of the breeding season that are intended for rebreeding and submitted for insemination. A submission rate of at least 80% of the eligible cows during a 21-day period is desirable. Submission rate, which is easily calculated, is an excellent measure of heat detection rate and should be calculated at the end of the first 21-day period of the breeding season. A submission rate of less than 80% indicates a problem with heat detection, and diagnosis of this problem at an early stage allows corrective action to be taken before much of the breeding period has elapsed.

Technological Aids to Improve Heat Detection

The low to moderate heat detection efficiencies achieved on most farms reflect the difficulty of detecting heat in cows. Consequently, it has been and is the goal of many animal science programs to develop more objective systems to overcome some of the problems of heat detection. An ideal system for detecting estrus should have the following characteristics: (1) continuous surveillance of the cow; (2) accurate and automatic identification of the cow in estrus; (3) operation for the productive lifetime of the cow; (4) minimal labor requirements; and (5) high accuracy and efficiency (95%) for identifying the appropriate physiological events that correlate with estrus or ovulation or both. A number of aids and technologies, inexpensive or expensive, are available to meet some, but not all, of these criteria. In any case, use of the various technologies to identify the symptoms associated with estrus, ovulation, or both will require the judgment of the herd management, based on common husbandry experience, to verify whether or not the cow seems to be in estrus.

Tail-painting

Research from a number of laboratories has shown that applying paint or chalk to the tailhead of cows is effective in indicating standing activity. When such tail-painted cows are mounted from the rear some or all of the chalk or paint is rubbed off indicating that the painted cows possibly stood in estrus while being mounted by a herd mate. When combined with early-morning and late-evening observations, checks for paint loss at milking times should result in a heat detection rate of close to 90%.

Vasectomized bulls with chin ball marking harness

Active vasectomized teaser or detector bulls are useful in identifying cows either coming into or on heat. Vasectomy should be carried out 40–60 days prior to introduction to the herd. Many small- to medium-size dairy herds in Ireland are now finding that teaser bulls are particularly useful after the first 3 weeks of the breeding season when fewer cows are in heat each day and when the level of heat-related activity in the herd is reduced as more cows become pregnant. However, considerable variation in libido exists among bulls, and they require the same management as full bulls without conferring any of the advantages. As an alternative to vasectomized bulls, cows or heifers treated with testosterone or estradiol can be useful in detecting cows in estrus.

Pressure-activated heat mount detectors

These devices, including the ones marketed as Kamars, Bovine Beacon, and Mate Master, are affixed to the tailhead of the cow and change color when pressure is applied by the weight of the mounting animal. Reported efficiencies of heat detection using such heat mount detectors vary from 56 to 94%, whereas the accuracy of heat detection is reported to vary from 36 to 80%. The relatively low accuracy of heat detection, combined with the difficulties in keeping the devices affixed to the tailhead, limits the potential of this approach.

Pedometers

Estrus in cattle is accompanied by increased physical activity. Cows in heat do 2–4 times more walking than a nonestrous cow. Pedometers can be attached to the leg of the cow to measure the amount of her activity over a unit time-span. Early pedometer-aided heat detection systems operated with a reported heat detection efficiency of 60–100% and with an accuracy in the range of 22–100%. The low level of accuracy was related to a high proportion of false-positives and to technical problems that led to breakage, malfunction, or loss of the pedometers. New improved pedometric technology has now led to improved information storage systems; improved analytical capabilities to allow comparison of current with previous physical activity; incorporation of internal power supply to operate the electronics; and the development of self-contained devices to interrogate the pedometers in milking parlor and relay and store the information in a personal computer. Some systems have an inbuilt alert system, such as a bleeper or flashing light, which alerts the farmer when a cow is deemed to be in heat. A number of pedometric systems are commercially available in the United States and Europe. Even though scientific information on their operating efficiencies is not yet available, these systems would appear to have significant commercial potential particularly when cows are housed.

Radiotelemetric devices

The primary sign of heat is standing to be mounted. A number of research laboratories have attempted to develop pressure-sensitive devices that measure such standing activity. Such a system (HeatWatch II; CowChips Manalapan, NJ, USA) is currently commercially available in the United States and in a number of other countries. This system involves the location of a pressure-sensitive battery-powered transmitter on the cow's tailhead, which, when activated by the mounting cow, emits a radio signal, which is picked up by either a receiver or a repeater and relayed to a buffer and ultimately to a personal computer where the information is digitized and stored. The time, date, and duration of each mount along with the identity of each cow are recorded. From this information, the time of heat onset is calculated. The HeatWatch software generates management reports and individual cow reports that can be viewed or printed. HeatWatch classifies a standing heat as a cow having three standing events in a 4-h period. A cow with fewer standing events is recorded as a 'suspect heat', and such a cow should be checked for secondary signs of heat prior to deciding to inseminate her. Periodically during the day, the farmer checks the computer for a listing of the cows in heat. The data available suggest that HeatWatch operates with both an efficiency and an accuracy of almost 100% in detecting cows in heat.

Heat detection patches

Recently, a number of scratch card type patches have come on the market, including Estrus Alert® and ESTROTECT™. These are affixed to the cow's tailhead. Friction from mounting activity rubs off the silver coating to reveal a bright-colored patch underneath. These devices show significant potential to improve submission rates in dairy herds.

The consistent drawbacks with all of these systems that require the fixing of a device such as a kamar, beacon, or transponder to the tailhead of a cow are the significant amount of effort required to maintain the devices on the cows and the high loss rates. Similarly, tail-paint or chalking must be reapplied to cows at 7–10 day intervals – again requiring handling and time.

Conception Rate

This is the third major factor affecting reproductive efficiency. The main factors implicated in causing conception failure or embryo death are normally categorized as those of genetic, physiological, endocrine, and environmental origin.

Fertilization rate and early embryo loss rates in Cattle

Based on published data, there is little evidence to suggest that fertilization rates are likely to be different in the modern high-producing cow as compared with lower-producing cows or heifers particularly under temperate climatic conditions. When adequate numbers of spermatozoa are used from bulls of high fertility and cows are correctly inseminated during or shortly after the end of standing estrus, fertilization rates approaching 90% should be expected. Although fertilization rate is apparently similar in high- and moderate-producing cows and is unlikely to be affected by whether the cows are on pasture or high-input total mixed ration (TMR) diets, the average calving rate to a single service, nevertheless, is significantly lower in high-producing cows than in either low-producing cows or heifers. An embryonic and fetal mortality rate (excluding fertilization failure) of ~40% is calculated for moderate-producing cows based on a fertilization rate of 90% and an average calving rate of ~55%, with an estimated 70–80% of the loss being sustained between day 8 and 16 after insemination. The comparative figure for high-producing dairy cows, based on a fertilization rate of 90% and a calving rate of 40%, would be 56%.

Pattern of early embryo loss

Based on published literature, there is some evidence that the pattern of early embryo death in the modern high-producing cow may be different from that observed in heifers and lower-yielding dairy cows. The extent of early embryo loss appears to be larger in the modern high-producing dairy cows, with a much higher proportion of the embryos dying before day 7 following insemination. The expected outcome of 100 inseminations of British-Friesian and Holstein-Friesian cows is summarized in **Figure 2**. Because fertilization rate is close to 100%, conception failure is almost synonymous with embryo and fetal loss.

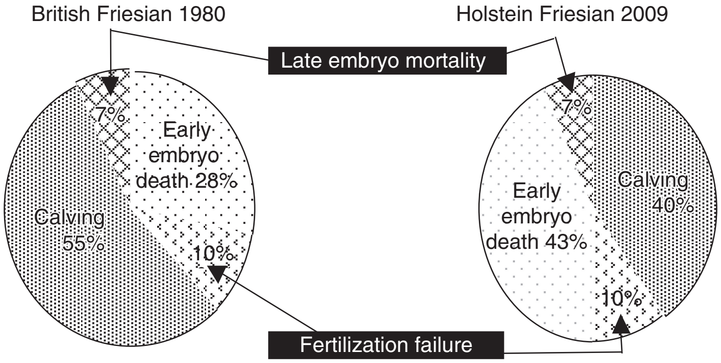

Figure 2 Reproductive outcomes in British-Friesian vs. Holstein-Friesian cows.

With the advent of ultrasound scanning, it has been comparatively easier to accurately establish the extent and timing of late embryo/fetal mortality. A recent study by the Teagasc laboratory, Galway, Ireland, quantified the extent and pattern of embryo/fetal loss from days 28 to 84 of gestation in 1046 lactating dairy cows and 162 dairy heifers managed on pasture-based systems of milk production. The overall embryo/fetal loss rates between days 28 and 84 of gestation were similar for cows (7.2%) producing on average 7247 kg of milk and heifers (6.1%), and the pattern of loss over this period was also similar for cows and heifers. Almost half (47.5%) of the total recorded loss occurred between days 28 and 42 of gestation. There was no significant association between the level of milk production or milk energy output measured up to day 120 of lactation, milk fat concentration, milk protein concentration, or milk lactose concentration and the late embryo/fetal loss rate. The extent and pattern of embryo/fetal loss were not related to either the cow's or the cow sire's genetic merit. The author does acknowledge that the extent of late embryo/fetal mortality recorded in the Irish pasture-based studies is much lower than that reported for some US-based studies. However, a clear explanation for the reported differences is not apparent but may be related to the level of milk production, ambient temperature, and/or the breeding of cows following various Ovsynch-based protocols in the United States.

Progesterone during the cycle immediately prior to insemination and embryo survival rate

Data from a recent study conducted by the Teagasc laboratory, Galway, Ireland, clearly show that there is a positive linear association between the concentrations of progesterone on the day of PGF-2α-induced luteolysis and the subsequent embryo survival rate (**Figure 3**).

Following a literature review, it was concluded that the most probable effect of low concentrations of progesterone in the cycle preceding estrus on subsequent embryo survival rate is preterm oocyte maturation, which subsequently compromises its ability to continue normal embryo development after its fertilization.

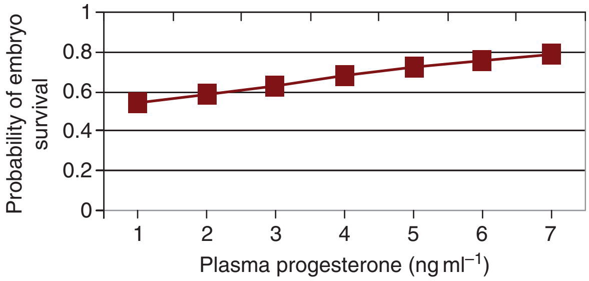

Figure 3 Relationship between plasma concentrations of progesterone on day of induced luteolysis and subsequent embryo survival rate.

Post insemination progesterone and embryo survival rate

Recent studies by the Teagasc laboratory, Galway, Ireland, that have employed logistic regression techniques to model the relationship between the binomially distributed dependent variable (conception/embryo survival rate; yes or no) and the continuously distributed independent variable (progesterone) have established a relationship between circulating progesterone and embryo survival rate. In a study by Stronge *et al.* (**Figure 4**) there was a positive linear relationship between milk concentrations of progesterone on days 5, 6, and 7 post insemination and the embryo survival rate, and a quadratic relationship between the rate of change in concentrations of progesterone between days 4 and 7 and the embryo survival rate. Further analysis of this data set reveals that 75, 72, and 56% of dairy cows had concentrations of progesterone that were optimal for conception on days 5, 6, and 7 post insemination, respectively. There is evidence that progesterone supplementation of dairy cows having low endogenous

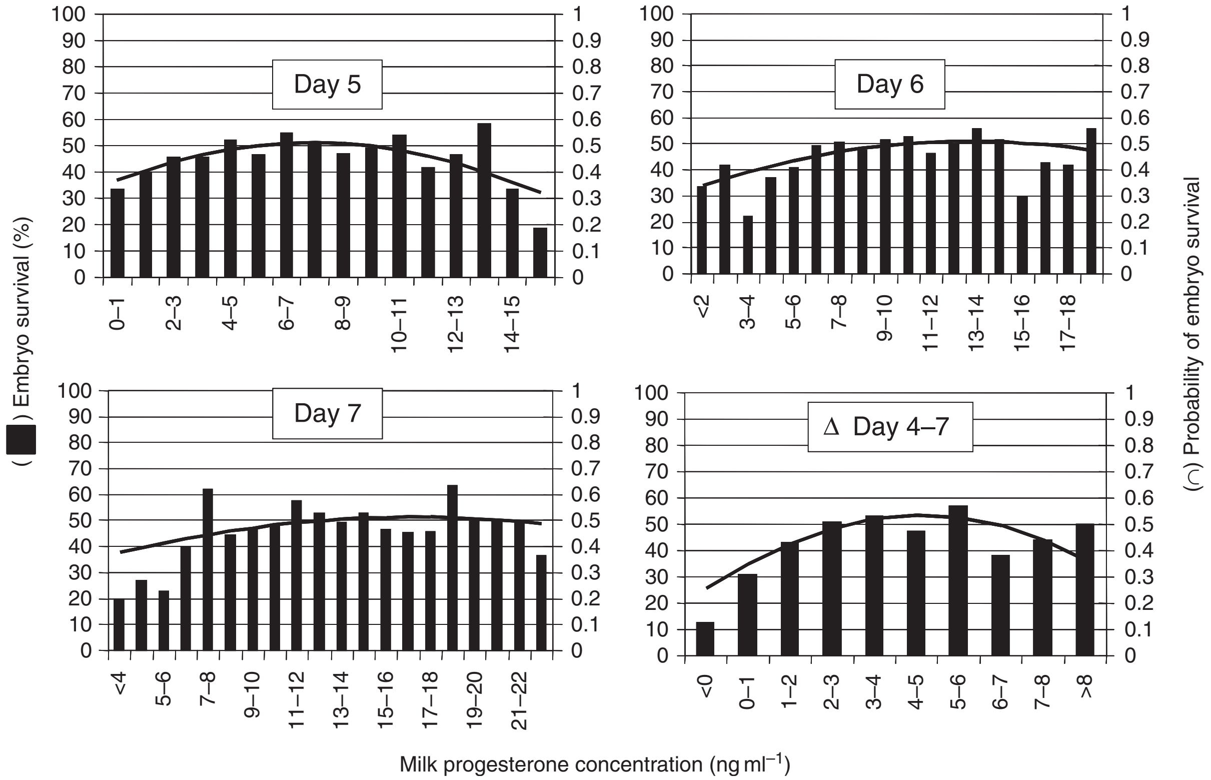

Figure 4 Relationship between milk concentrations of progesterone on day 5, 6, and 7 after AI and subsequent embryo survival rate in lactating dairy cows. Reproduced with permission from Stronge AJH, Sreenan JM, Diskin MG, Mee JF, Kenny DA, and Morris DG (2005) Post-insemination milk progesterone concentration and embryo survival in dairy cows. *Theriogenology* 64: 1212–1224.

concentrations of progesterone, and consequently at risk of suffering embryo death, will have improved embryo survival rates.

A series of studies with dairy cows at the University of Wisconsin have shown that peripheral concentrations of both progesterone and estradiol are lowered by increased plane of feed intake owing to increased metabolic clearance rate (MCR) of the steroids, which is related to liver blood flow (LBF). From these studies, it would appear that LBF is elevated in high-producing lactating dairy cows and this in turn would result in a lowering of peripheral concentrations of progesterone thus increasing the risk of embryo death. The reduced progesterone effect may retard the growth and development rate of the embryo by hampering uterine secretion of proteins and growth factors essential for early embryo development. Interferon-τ, the embryonic signal required for the maintenance of the corpus luteum and the estalishment of pregnancy, has also been shown to be positively correlated with progesterone. Uterine expression of the mRNA for progesterone receptor and estradiol receptor and of the retinol-binding protein mRNA are all sensitive to changes in peripheral concentrations of progesterone during the first week after AI.

Nutrition–Energy Balance

Over the past three decades, intensive genetic selection for milk yield has increased the differences between feed intake potential and milk yield potential. This has resulted in dairy cows that have a greater predisposition for mobilizing body reserves and for NEB. It is also clear that even under optimal grazing conditions total DMI is lower than when cows are fed maize-based TMR diets. A pasture DMI of ~3.4–3.6% of body weight has been recorded for early-lactation cows grazing high-quality pasture compared to 3.9–4.2% of body weight for cows fed a nutritionally balanced TMR. From this, it is clear that under optimal grazing conditions the actual DMI of cows is significantly lower than the cows' potential intake, and this is likely to have implications for EB status and subsequent fertility in early lactation, particularly for cows with a high genetic potential for milk production.

Energy balance during the early postpartum period and subsequent conception rate

The relationships between EB, DMI, and peripheral concentrations of IGF-I measured during the first 28 days of lactation and subsequent conception rate have recently been explored in a number of Teagasc studies. All three variables were positively associated with first-service

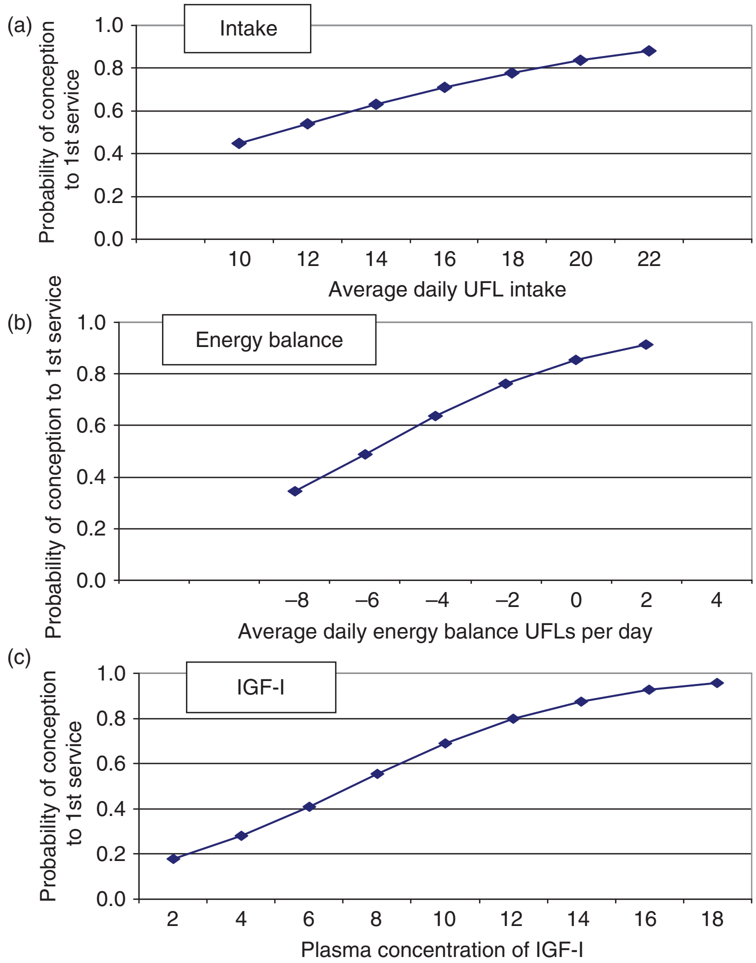

Figure 5 Relationships between (a) average daily intake (UFL day^{-1}), (b) average daily EB (UFL day^{-1}), and (c) plasma concentration of IGF-I during the first 28 days of lactation and probability of conception rate to first service in dairy cows. Reproduced with permission from Patton J, Kenny DA, Mee JF, *et al*. (2006) Effect of milking frequency and diet on milk production, energy balance, and reproduction in dairy cows. *Journal of Dairy Science* 89: 1478–1487.

conception rate, and the results are presented in **Figure 5**. This is a particularly interesting observation and suggests that there may be long-term carryover effects of nutrition/EB on conception rate. It has also been hypothesized that follicles exposed to adverse conditions such as a negative EB during their initial stages of growth would have impaired development resulting in the production of inferior quality oocytes and dysfunctional corpora lutea. The results of this study strongly emphasize the importance of maximizing feed intake and minimizing NEB in the immediate postcalving period.

Energy balance at around the time of insemination and subsequent conception rate

It is clear that DMI is lower for cows grazing pastures than for cows fed maize-based TMR diets. Supplementation of dairy cows at pasture with concentrates increases the total DMI, but its effects on conception rate are equivocal. Following a review of a number of experiments that examined the effects of supplementation on conception rate, Diskin *et al.* (2008) concluded that supplementation had little effect on conception rate but that withdrawal of the supplementation during the breeding period may be counterproductive to conception rate. Only a small proportion of the additional feed intake achieved by concentrate supplementation is partitioned toward an improvement in EB, with >80% supporting increased milk production. This clearly highlights the difficulty that improving the EB of the modern dairy cow presents at this stage of lactation when grazed grass is the predominant component of the diet. Based on the Wisconsin study, it is reasonable to hypothesize that the increased milk production resulting from concentrate supplementation may well be associated with a further increase in hepatic blood flow resulting in increased metabolism of progesterone and consequently in lowering of the peripheral concentrations of progesterone, thus predisposing cows to greater risk of embryo death.

Effect of sudden reductions in feed intake on conception rate

Studies by the Teagasc laboratory, Galway, Ireland, show that sudden reductions in DMI at around the time of insemination adversely affect embryo survival in heifers. When energy intake was reduced from a high level of twice their maintenance requirement to 0.8 times maintenance for 2 weeks immediately after AI, embryo survival rate in heifers was consistently <40%. When heifers were either provided with a constant level of feed intake or changed from a low to a higher level of feed intake, embryo survival was consistently high at 65–71%. In one study where heifers were used, there was no indication of any association between energy intake and systemic progesterone concentration.

Unlike the situation in sheep and pigs, there was no change in systemic progesterone following either an increase or a reduction in energy intake. Changes in progesterone metabolism may have been balanced by changes in progesterone production.

Protein nutrition and conception rate

Dairy cows at pasture frequently ingest high quantities of protein, often with a high proportion of the ingested protein being rapidly degradable in the rumen. The effects of high intakes of crude protein on conception rate are equivocal. For example, US data have shown that high-protein intake reduces uterine pH, which has been hypothesized to have a detrimental effect on either the gametes or the developing embryo. High-protein diets elevate plasma urea nitrogen (PUN) levels. PUN in excess of $19\,\mathrm{mg\,dl^{-1}}$ has been associated with a 20% depression in conception rate in dairy cows. However, in an extensive range of studies by the Teagasc laboratory, Galway, Ireland, using beef heifers in positive EB, no effect of a high crude-protein intake on conception in heifers was recorded, irrespective of whether the crude protein was derived from highly nitrogen-fertilized grazed grass or from added urea to a silage-based diet. Furthermore, a retrospective analysis of the data failed to record any association between peripheral concentrations of urea and embryo survival, notwithstanding peripheral concentrations of urea having been elevated up to $25\,\mathrm{mmol\,l^{-1}}$. It is concluded that elevated peripheral concentrations of urea *per se* are not detrimental to embryo survival. However, it needs to be clarified whether the observed adverse effects of urea on embryo survival are dependent on the energy status of the animal.

Insemination technique

The reported effects of the site of placement of semen within the uterus on conception rate are equivocal. In a recent study by the Teagasc laboratory, Galway, Ireland, involving 3546 dairy cows in 51 herds and 8 inseminators, Diskin *et al.* (2005) recorded a significant effect ($P < 0.02$) of insemination site and inseminator on conception rate. For some inseminators, there was a significant increase (up to + percentage points) on conception rates following cornual insemination, whereas for others there was no effect. A retrospective analysis of all the data showed that there was an inverse relationship between the improvement in conception rate and conception rate following uterine body insemination. The largest improvements in conception rates were recorded by inseminators with the lowest conception rate following body insemination. These results suggest that conception rates could be improved for individual inseminators by adopting the practice of placing half of the inseminate beyond the curvature of each uterine horn as opposed to body insemination, which is the normal practice. It is clear from many studies that placement of the inseminate in the cervix results in significantly lower conception rates. Therefore, it is critical to at least ensure that the inseminate is placed in uterine body, and for skilled and experienced inseminators, it would appear beneficial to place half of the inseminate in each uterine horn.

Time of insemination

Results from a recent large-scale study that utilized the HeatWatch system to detect the onset of standing heat concluded that conception rates were optimum when dairy cows were inseminated 4–16 h after heat onset. Insemination later than 16 h after heat onset results in significantly lower conception rates. However, in most instances the time of heat onset is not accurately determined, and in such situations once-daily AI for cows observed in standing heat is equally effective as inseminating cows in accordance with the long-established a.m.–p.m. guidelines.

Calving difficulty

Calving difficulty, besides affecting calf and cow mortality and the milk yield, also decreases cow rebreeding performance. Teagasc data clearly show that as the severity of calving difficulty increases, conception rate to the first and to all services combined also decreases (**Figure 6**). This reduction in conception rate is owing to the abnormalities directly arising from calving difficulty, including delayed uterine involution and increased uterine infection, damage to the reproductive tract, and the development of uterine and ovarian adhesions. Furthermore, the interval to first heat is often extended after a difficult calving. For optimal reproductive performance, calving difficulty must be minimized.

Two factors that greatly influence the incidence of calving difficulty are the age of the cow and the breed of the sire. The incidence of calving difficulty is 4–8 times higher in first-calving heifers than in mature cows and about twice as high in second calvers as in mature cows. The breed of the sire and indeed the individual sire within a breed should be carefully selected for use on

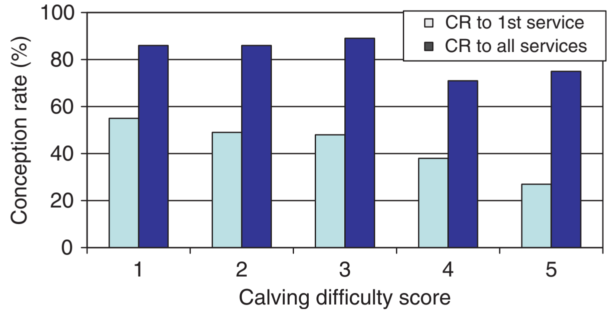

Figure 6 Relationship between calving difficulty score and conception rate to first services and to all services combined. Difficulty ranges from score 1 (unassisted birth) to score 5 (severe difficulty requiring mechanical extraction of the calf).

heifers and on young cows to minimize the risk of calving difficulty and therefore of subsequent infertility.

In some countries, there is a practice of breeding late-calving dairy cows to beef sires. The combined effect of the longer gestation and the increased incidence of calving difficulty makes it even more difficult to achieve a 365-day calving interval in such cows. The wisdom of this practice, especially if the objective is to optimize reproductive performance, is therefore questionable.

Bull fertility

Bull reproductive performance is influenced by several factors including testicular development, semen quality, libido, mating ability, and physical soundness. On farms using natural service, the level of bull fertility can have a major impact on pregnancy rate and calving spread. Published data suggest that up to 5% of bulls in natural service may be completely infertile and that a further 30% may be subfertile. Unfortunately, if a bull is infertile, it is not usually discovered until at least one repeat interval has elapsed since joining the herd.

Although a veterinary examination combined with a semen evaluation 1 month before the start of the breeding season will help to identify the majority of infertile bulls, it will not identify subfertile bulls. Furthermore, it should be realized that a bull may not remain fertile for all of his working life or even throughout a single mating season. For example, a bull that is ill with a raised temperature for a number of days may have a period of temporary infertility ~40–60 days later. Similarly, injury to the penis, sheath, or prepuce, though not necessarily affecting mounting behavior, can prevent mating. Therefore, the bull should be observed regularly for serving ability, and all mating dates recorded. Such recording will help identify infertile or subfertile bulls at an early stage.

AI versus natural service

AI is often criticized on the grounds that conception rate is lower than when following natural service. Apparent improvement in conception rate often arises following the introduction of a bull. This apparent improvement is likely to be because of cows being mated at a longer postpartum interval and/or because of inaccuracies in heat detection being eliminated. Where heat detection is accurate, and when insemination is timed and carried out correctly, conception rate is similar following either AI or natural service.

Relative Importance of Heat Detection Efficiency and Conception Rate

Once estrous cycles have resumed postcalving, then it is the product of heat detection efficiency and conception rate that determines the overall herd reproductive efficiency (**Table 2**).

The clear message from **Table 2** is that low or relatively low conception rates can be compensated for by improving heat detection efficiency. Practical and easily adoptable technologies are available to improved heat detection efficiency.

Conclusion

It is well established that reproductive performance is critically important, particularly in seasonally calving herds, to maintain compact calving close to the onset of the grazing season. Even though the modern high-genetic merit Holstein-type dairy cow selected solely for milk production is biologically more efficient at converting forage, irrespective of source, to milk, their sustainability in predominately pasture-based systems of production is questionable given their low fertility. Therefore, it is important to develop appropriate supplementation strategies, probably beginning before parturition, to improve fertility in dairy cows on pasture-based systems of production. In the medium to long term it should be possible to develop more balanced breeding strategies with greater emphasis on fertility- and feed intake-related traits, which are critically important to pasture-based systems of milk

Table 2 The effect of different heat detection (submission) and conception rates on the percentage of the herd that is pregnant at 90 days after onset of breeding season

		Conception rate (%)			
		60	*50*	*40*	*30*
Heat detection rate (%)	*90*	96	91	83	71
	70	89	82	73	61
	50	76	68	59	48
	40	67	59	50	40

production. It is clear that sufficient genetic variability exists within the Holstein breed for important fertility and feed intake traits. Alternatively, strains of cows derived from more balanced breeding objectives, such as the New Zealand Friesian, or alternative dairy breeds such as the Jersey or Norwegian Red could also be utilized in such production systems.

It is now becoming increasingly clear that EB during the immediate postcalving period affects both the onset of estrous cycles postcalving and the subsequent conception rates. Development of feeding strategies that increase intake without proportionally increasing milk yield, thereby improving EB, is important. Paying more attention to other factors that are predominately under management control, particularly heat detection, can significantly offset some consequences of inherently low fertility traits that exist with the modern dairy cow. Improving heat detection efficiency by 12–15% has the equivalent effect of increasing conception rate by 10 percentage points. Based on numerous published reports, it can be concluded that there is scope in most herds to improve heat detection efficiency by at least 15 percentage points by adopting well-described practices. Conception rate is affected by a range of both cow and management-related factors. Producers should ensure that cows presented for insemination are in heat and are properly inseminated with high-fertility semen. Sudden reductions in feed intake during the breeding season should be avoided.

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Pregnancy: Characteristics

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Introduction

Milk, like all consumable products obtained from domestic animals, is acquired through exploitation of reproductive processes. Therefore, dairying will be efficient only if females regularly produce offspring. Both sexes must be involved for the initiation of pregnancy but, with the ejaculation of viable spermatozoa into either a natural or artificial vagina, the male's role is finished. In contrast, the female's role has just begun. The mammalian embryo is referred to as a zygote from fertilization through the early cleavage divisions, a morula when a spherical cluster of cells has formed, and a blastocyst once it consists of a hollow sphere and the two cell lineages that will give rise to the embryo proper and the placental membranes. Once organs have formed, the developing offspring is referred to as a fetus. At any stage, it can be referred to as a conceptus, the product of conception. For the purposes of this article, these terms may be used interchangeably.

As gestation progresses, the maternal systems are modified to provide an environment that supports development. The process includes remodeling of uterine structure and function to provide nutrients for growth plus suppression of maternal immune responses so that the potentially antigenic conceptus is not rejected. The following text deals with the establishment, progression, duration, and detection of pregnancy. Although most of the world's milk comes from *Bos taurus* or *Bos indicus* cows, information on other species used for dairying will be included when available.

In the Beginning

Preattachment Development: Zygote to Expanded Blastocyst

Embryos pass through the first few cleavage stages as they travel down the oviduct. All development up to the mid-blastocyst stage occurs while the embryo is confined within its zona pellucida (**Figures 1**(**a**)–**1**(**d**)). Because zona-enclosed embryos do not grow larger, as the number of blastomeres doubles at each cleavage their individual size is halved. In cattle, sheep, and goats, embryos enter the uterus 3–5 days after fertilization, typically at the 8- to 16-cell stage.

In the sheep, if two oocytes are released from the same ovary and no ovulation occurs from the other ovary, one embryo commonly migrates down the ipsilateral horn of the uterus, passes through the uterine body, and develops in the contralateral horn (i.e., on the opposite side). In contrast, migration between uterine horns in cattle is rare. If there are two ovulations from the same ovary, both embryos will remain and develop in the adjacent uterine horn. It was thought that uterine capacity might be limiting, such that twins gestated in separate horns would be more likely to survive to term. However, studies have found this to be a problem only with nulliparous heifers. Twinning in cattle, regardless of uterine position, is associated with substantially higher rates of calf mortality, dystocia, and metabolic problems. An additional problem associated with twins in cattle is the possible fusion between the placental membranes of the two conceptuses. This situation leads to exchange of cells and signaling molecules between fetuses. If one fetus is male and the other female, the male will be normal but the female will have abnormal sexual differentiation. Most heifer calves that develop as cotwins with males become sterile freemartins characterized by apparently female external genitalia with variable asexual or phenotypically male internal genitalia. Although some aspects of this centuries-old phenomenon remain a mystery, studies of freemartins in cattle have made fundamental contributions to our understanding of sexual differentiation and immunology. In fact, the groundbreaking work by Medawar (cowinner of Nobel Prize, 1960) on naturally acquired immunological tolerance was initiated by studying transplantation reactions in freemartins.

Soon after entering the uterus, the embryo passes through the morula and to the blastocyst stage (**Figures 1**(**e**) and **1**(**f**)). Fluid accumulates between some of the cells and these spaces unite to form the blastocoel, a fluid-filled cavity. A cluster of cells give rise to the inner cell mass, which will form the embryo proper, while the remainder surrounding the cavity form the trophectoderm, the precursor of the placental membranes. Embryos

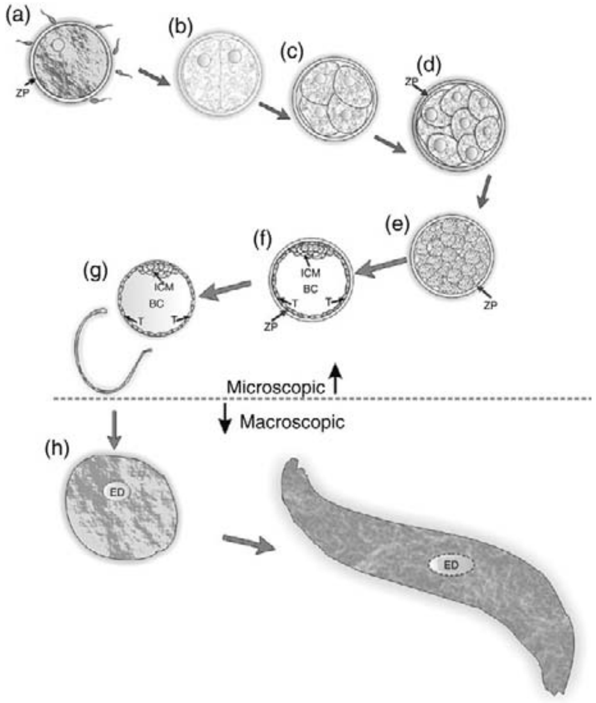

Figure 1 Development of the conceptus from fertilization to elongation of the chorionic vesicle. (a) Fertilization, the union of spermatozoon and oocyte to form the zygote or embryo; ZP, zona pellucida. (b) First cleavage, occurring about 24 h after fertilization, producing an embryo with two cells or blastomeres still contained within the zona pellucida. (c) Second cleavage, usually within 12 h of first cleavage, producing four blastomeres. (d) Third cleavage, within a few more hours, resulting in eight blastomeres within the zona pellucida. (Blastomere division is not synchronized exactly, so zygotes with intermediate numbers may be observed.) (e) Subsequent cleavages produce the morula with 16–64 blastomeres, still within the zona pellucida. (f) Continued cleavage results in a blastocyst with a distinct inner cell mass (ICM) and a layer of trophectodermal cells (T) surrounding a fluid-filled central blastocoelomic cavity (BC). (g) Outward pressure from the growing blastocyst ruptures the zona pellucida (hatching), so the conceptus can now increase in size. (h) The conceptus grows rapidly into and through the spherical blastocyst stage. At this time, it is a fluid-filled sac with a distinct embryonic disc (ED) on the surface. (i) In most ungulate species, the spherical blastocyst elongates quickly into a filamentous form that grows to fill much of the uterine lumen. The embryonic disc remains as a distinct spot on the surface of this chorionic vesicle. It is the trophectoderm or outer cell layer that interacts with the endometrium to form the placenta.

intended for transfer are typically collected from the uterus at the morula or blastocyst stage.

Early in the second week of gestation, the zona pellucida ruptures and the blastocyst is freed or hatched (**Figure 1**(**g**)). Cell division continues and, since the blastocyst is no longer confined within the zona, it can now increase in size. By 10 days, the inner cell mass forms an embryonic disc located as a discrete structure at the surface of a hollow sphere (**Figure 1**(**h**)). The original cells forming the inner cell mass and trophectoderm constitute the ectoderm, the first of the three primary germ layers. A sheet of flattened cells, the endoderm, proliferates from the inner cell mass during the second week of gestation and develops into a single cell layer closely associated with the inner surface of the trophectoderm. This proliferation progresses until the endoderm lines the entire inner surface, forming a two-layered or bilaminar membrane surrounding the blastocoel. Shortly after the bilaminar blastocyst forms, further differentiation within the inner cell mass initiates production of mesodermal cells beneath the disc. The mesoderm or the third germ layer proliferates and spreads between the ectodermal and endodermal cells resulting in a trilaminar blastocyst. During subsequent development, the three germ layers interact to produce tissues and organs within the developing embryo/fetus plus the various embryonic membranes.

The hatched blastocysts of domestic ruminants remain in the spherical stage (**Figure 1**(**h**)) for several days. The trophectoderm, which has now become the outer embryonic membrane or chorion, extends to form an oval shape and quickly stretches into an elongated, sausage casing-like, filamentous structure (**Figure 1**(**i**)). Much of this initial increase in length is accomplished through cellular remodeling into greatly stretched configurations rather than by cell proliferation. The embryonic disc begins to develop a neural fold and somites, giving rise to a primitive embryo that is enveloped by an infolding of the rapidly growing membranes. Late in the second or third week of gestation, the filamentous blastocyst, also called a chorionic vesicle, assumes a fixed position within the uterus, stretches to a substantial length, and begins to fuse with the uterine lining.

Formation of Embryonic Membranes

As a blastocyst elongates and the embryo forms, several other membranes develop. A yolk sac grows out from the middle section of the embryo and proliferates within the chorionic vesicle. The yolk sac quickly develops an extensive vascular bed and extends toward, but never reaches, the chorionic tips. Both the chorion and yolk sac synthesize peptides, proteins, and steroid hormones, some of which serve as signals from the conceptus to the dam, coordinating maternal responses to embryonic requirements. In addition, the yolk sac produces the first embryonic blood cells and serum proteins. The primordial germ cells, which subsequently migrate to the genital ridge and transform this region into a gonad, also originate in the yolk sac. Once the liver and other organs develop and assume their functions, the yolk sac regresses.

Another embryonic membrane, the allantois, develops from the hind region of the embryo late in the third week of gestation. It begins as a small sac but soon becomes filled with fluid and expands. In most species, the allantois displaces the yolk sac and, within a few days, lines most of the chorionic vesicle. The allantoic and chorionic membranes fuse to form the chorioallantois. This combined membrane rapidly develops an extensive blood vascular system that is connected directly with the embryonic circulation via the umbilical cord. In most mammals, the chorioallantoic membrane constitutes the outermost layer of the embryo/fetal unit that attaches to the uterine lining forming the placenta.

As the yolk sac and allantois are developing, the differentiating embryonic disc sinks below the surface of the chorionic vesicle. The embryo is then surrounded or enveloped by infolding chorion that fuses in the middle to form a separate sac, the amnion. This compartment is also filled with fluid and intimately surrounds the embryo/fetus to provide a stable physical environment for growth and a cushion against mechanical shock. Embryos implant in the distal third of the ipsilateral uterine horn, and the chorionic vesicle expands into the contralateral horn. In camelid species, embryos tend to implant in the left horn even though ovulation can occur on either side, with membranes expanding into the opposite horn.

Development of the Fetus

As the embryonic membranes organize, the ectodermal, mesodermal, and endodermal components of the inner cell mass differentiate and begin forming specific organs. Early in gestation, the embryonic disc region organizes through the primitive streak, neural fold, and somite stages. Around the time when placental attachment commences, the heart forms and vascularization of the embryo and associated membranes begins. At this stage, a head and body region become recognizable but bear little resemblance to the final product. The individual organs, limb buds, and other features appear within a very few weeks, heralding the transition from an embryo to a fetus.

During the initial stages of development, there is almost no increase in uterine size. Once the attachment of the chorion to the uterine epithelium is well under way, fluids begin to accumulate in the chorioallantoic and amniotic space and the uterus enlarges (**Figures 2(a)** and **2(b)**). It is this fluid that accounts for much of the increase in size through midgestation. In the later stages, growth of the fetus accelerates so that by term it will account for about half of the total conceptus-uterine weight. In late gestation, the fetal fluids probably account for another quarter, with the placenta and uterus constituting the remainder.

Nutrition of the Conceptus

Mammalian oocytes have sufficient stores of yolk material to provide nutrients to support embryonic development through the first few cleavage divisions. However, once cell numbers begin to increase through the morula and blastocyst stages, the embryo must obtain and metabolize materials from the mother. There are two possible ways for the embryo to obtain nutrients. First, material secreted by the epithelial lining of the endometrium (inner layer of uterus) is absorbed by the outer surface of the chorionic vesicle. This mode of transfer, known as histotrophic (histiotrophic) nutrition, is carried out by a uterine lining of tall, columnar cells loosely associated with the fetally derived layer. Uptake is facilitated by areolae, which are pockets of columnar trophoblast cells overlying

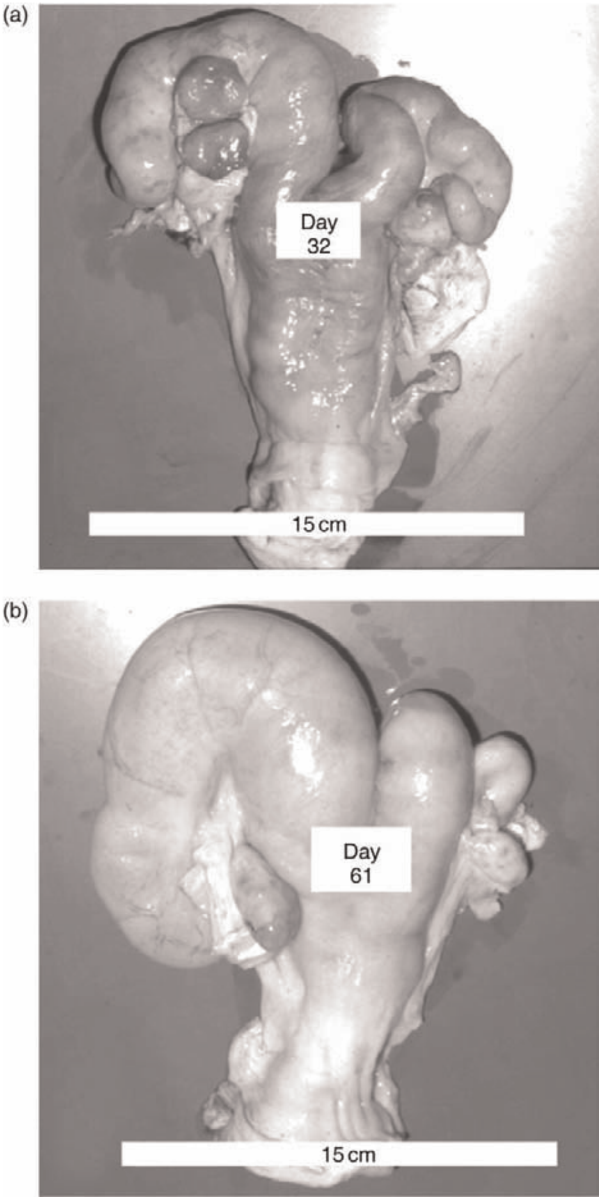

Figure 2 (a) Bovine uterus, day 32 of gestation. The embryo/fetus is located near the middle of the slightly enlarged left (ipsilateral) uterine horn. The right (contralateral) horn is just beginning to enlarge. The corpus luteum on the left ovary is cut open. (b) Bovine uterus, day 61 of gestation. The fetus is located in the substantially enlarged left uterine horn. By this stage, the contralateral horn is also obviously distended.

the mouths of uterine glands. In the second route, material diffuses between maternal and fetal circulations through various tissue layers. This mode of nutrient transfer, hemotrophic nutrition, requires a well-vascularized fetal layer and a thinning of the layers separating maternal and fetal vasculature. The placenta provides this situation.

All mammalian embryos are dependent on histotrophic nutrition up to the blastocyst stage. These stages are considered to be anaerobic, such that embryos require a supply of nutrients but minimal levels of oxygen. Subsequently, the relative importance of histotrophic nutrition and the timing of the switch to hemotrophic nutrition are highly variable among species. In humans, the period of histotrophic exchange is believed to be very brief, since the embryo invades and implants within the uterus quickly (10 days after fertilization) and the placenta is formed relatively early in pregnancy. In contrast, in all ungulates (hoofed animals), attachment to the uterus is delayed and the placenta is formed later in gestation. Because histotrophic transfer is important throughout the entire gestation, conceptuses in ungulate species typically undergo considerable expansion prior to attachment to occupy a larger surface area of the uterus.

Placentation

In all mammals, placentation begins when the embryonic vesicle assumes a fixed position within the uterus (apposition), followed by adhesion between chorion and uterine epithelium and the interdigitation of finger-like projections (microvilli) that develop on apposed maternal and embryonic cells. In many mammalian species, attachment is followed by destruction of uterine epithelium, and invasion of endometrium by embryonic cells (e.g., true implantation). However, placentation in ungulates is characterized by simple attachment to the uterine epithelium with little or no destruction of maternal tissues. In essence, the ungulate placenta forms as a result of interaction between the outermost membrane of the embryonic vesicle, the chorion, and the epithelial lining of the uterus. Attachment begins on days 18–19 in cows and goats, and on days 14–15 in sheep. The intimate association between chorion and endometrium constitutes the mature placenta in camelids.

In true ruminants (e.g., cattle, goats, sheep), the most obvious features of the placenta are placentomes, which consist of caruncles – raised round or oval areas on the inner uterine lining – interacting with cotyledonary areas of the chorioallantoic membrane (**Figure 3**(**a**)). Individual caruncles are composed of dense connective tissue covered by simple columnar epithelium. A distinctive feature of the caruncular endometrium is its lack of glands; the openings of uterine glands are found only on the intercaruncular surface. Although caruncles do not come into play until pregnancy, they begin to develop during fetal life, and are easily visible in the inner surface of a prepubertal calf uterus (**Figure 3**(**b**)). The number of caruncles is highly variable both between and within species, with estimates of 70–142 in cows, 160–180 in goats, and 60–150 in sheep.

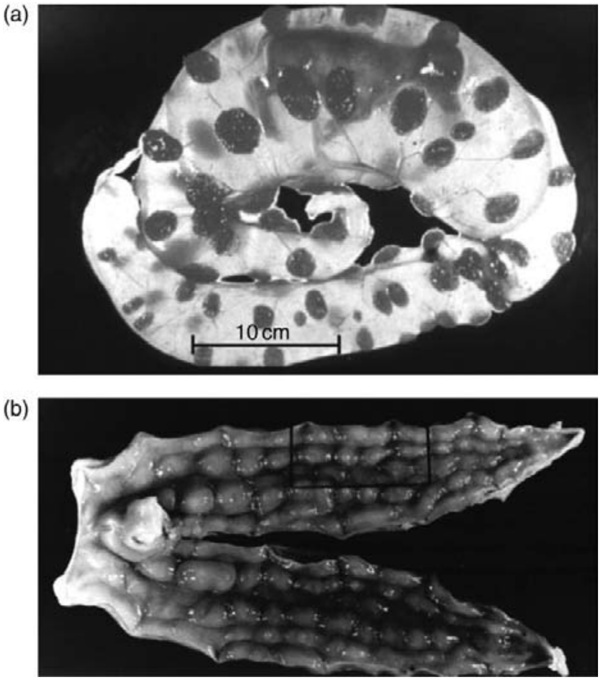

Figure 3 (a) Bovine fetus within the placental membranes showing conspicuous cotyledons at the surface. Note that the largest cotyledons are nearest to the fetus. The gestational age is approximately 6.5 months. (b) Inner endometrial surface of the uterus from a 6-month-old calf. Note the four rows of caruncles in each uterine horn. Modified with permission from Atkinson BA, King GJ, and Amoroso EC (1984) Development of the caruncular and intercaruncular regions in the bovine endometrium. *Biology of Reproduction* 30: 765, Figure 2(a).

In both intercaruncular and caruncular regions, apposition and subsequent attachment of the chorion and uterine epithelium commence near the embryonic disc. The union of maternal and fetal surfaces is consolidated by ridging or folding of the interface, which follows almost immediately after attachment begins. In the cow, physical union between these tissues extends over the entire mucosa by day 27. Attachment at placentomes is reinforced by the penetration of chorionic villi into crypts that form within the endometrium (~day 33 in the cow). The folding of the maternal–fetal interface together with the progressive branching of the villi and deepening of the maternal crypts provide a vast surface area for exchange.

The placentomes are such obvious features that the ruminant placenta is classified as a cotyledonary placenta. This terminology infers that definitive placentation involves only the placentomes. However, interplacentomal regions are also involved in exchange. Indeed, placentomal and interplacentomal regions are specialized for hemotrophic and histotrophic nutrition, respectively. The caruncles, with their thin, closely applied covering of trophoblast and extensive vascular bed, are well suited for hemotrophic exchange of gases and small molecules.

The glandular interplacentomal regions with their areolar-gland complexes are well suited for histotrophic transfer, and are believed to be primarily involved in the exchange of large molecules. Histotrophic nutrition is of greater significance in ungulates than in any other placental group.

Gestation Length

The duration of gestation in mammals is controlled almost entirely by genotype with major differences between species and minor variations among breeds or strains. The age of the dam, sex of the fetus, or season of birth may have slight effects, but these are small in comparison to the interspecies variation. The ranges of gestation lengths for various domestic animals used for milk production are given in **Table 1**.

In cattle, birth weight is correlated positively with gestation length since the longer a fetus remains *in utero*, the larger it grows. Also, the heavier male calves are usually carried slightly longer than female calves. Equine gestation length is somewhat longer and more variable than that in the ruminant dairy animals. The interval for mares may range from just over 300 days to almost 1 year, but most parturitions probably occur from 330 to 350 days. The differences between breeds and season of foaling are significant. Gestation is longer than normal in mares carrying mules. With the wide ranges reported for the various camelids, one might question how accurately mating was recorded or whether a number of offspring were born prematurely.

Table 1 Gestation lengths for species of animals that might be used for dairying

Species	*Gestation length (days)*
Cow – *Bos taurus*	278–290
Cow – *Bos indicus*	285–295
Water buffalo – *Bubalus bubalis*	302–317
Goat – *Capra hircus*	142–154
Sheep – *Ovis aries*	143–153
Horse – *Equus caballus*	330–350
Arabian camel – *Camelus dromedarius*	345–395
Asian camel – *Camelus bactrianus*	370–440
Llama – *Lama guanicoë glama*	320–345
Alpaca – *Lama guanicoë pacos*	325–366
Reindeer – *Rangifer tarandus*	210–240
Yak – *Bos grunniens*	250–270

Pregnancy Diagnosis

The main hurdles to successful pregnancy are breeding the female to a fertile male when she is in estrus, conception, and the resulting conceptus attaching to the uterus and developing a placenta. The majority of pregnancy failures are early in gestation (first 2 months in cattle). Thus, mating even during estrus is not a guarantee that females will complete gestation. Because of this uncertainty, managers of both intensive and extensive livestock production units may seek assurance that mated or male-exposed animals are truly pregnant.

The ideal pregnancy test would be sensitive enough to detect pregnancy early to allow timely rebreeding of the animal. It would be specific, in that it correctly distinguishes nonpregnant from pregnant animals. Pregnancy tests should be inexpensive and simple to conduct under field conditions. The procedure itself should have no negative impact on pregnancy, involving minimal physical manipulation of the pregnant uterus or stress to the pregnant female. At this point, the pregnancy test for dairy animals that meets all these criteria does not exist. An example of an ideal indicator is the chorionic gonadotropin of higher primates (e.g., human chorionic gonadotropin (hCG) in humans), which can be measured in blood and urine as early as 1 week after conception. Unfortunately, analogous molecules have not yet been identified in dairy species.

Detecting Nonpregnancy by Return to Estrus

Although the occasional cow may show weak estrus-like signs during pregnancy, female domestic animals rarely exhibit any signs of sexual behavior at one cycle interval after a fertile mating. Thus, for animals mated on known dates, demonstration of sexual receptivity when the next estrus is due indicates no conception from the previous service. Conversely, the absence of sexual behavior is a strong evidence for pregnancy. When hand mating is practiced, cows, ewes, or does can be tested with males just before and during the period when the next cycle should occur. Animals that show estrus can then be remated or treated hormonally. However, one caveat is that in many dairy herds relying on visual observation of estrus for mating via artificial insemination, estrus detection efficiency may be less than 50%. In such cases, a presumptive diagnosis of pregnancy based on missed estrus cannot be very accurate.

Direct Methods of Pregnancy Detection

Ballottement

The increasing size of fetal membranes and associated fluids within the uterus eventually produce abdominal distension. The physical enlargement usually becomes obvious during the later stages of gestation and is too late to be of much practical use for management decisions. Palpation of the firm fetal mass through the body wall may be attempted in ewes or does accustomed to being handled enough to relax their abdominal muscles. In cows, it is frequently possible to detect the presence of mid- or late gestation fetuses by ballottement. The open palm or fist is placed against the right flank just above the stifle joint, pressed firmly into the body wall with a short rapid movement, and then held in this position. The pressure wave produced by this movement shifts the fetus within its fluid-filled sac away, then back toward the hand. Once the fetal mass reaches a substantial size, it can be detected as a rebounding bump. Right horn pregnancies are easily detected by ballottement during the last trimester. However, the presence of the rumen between the uterus and left flank often hides fetuses developing on that side. In some instances, left-horn pregnancies may be detected by right-side ballottement.

Rectal palpation

Rectal palpation is an extremely useful procedure for pregnancy diagnosis in larger animals such as cattle. The bovine rectum is quite distensible and elastic allowing considerable mobility by the palpating arm and thus complete examination of the reproductive tract. Very early pregnancies can be diagnosed by detecting the very slight bulge of the embryo within its amnion before any obvious swelling of the gravid horn occurs. It is also possible to detect twins by this method. In the fifth or sixth week of gestation, the bovine conceptus enlarges within the uterus and the embryonic membranes thicken, so they can be felt through the uterine wall. The tactile sensation generated as the uterine wall is gently released between thumb and fingers provides the membrane slip test for pregnancy. Later, actual placentomes and fetal parts may be palpated. Around midgestation, the weight of the fetus and placental fluids pull the uterus forward into the abdominal cavity, so it cannot be reached by palpation. At this stage, it is still possible to diagnose pregnancy through examination of the middle uterine arteries and detection of the characteristic fremitus or dynamically vibrating pulse to the gravid uterine horn. By late gestation, the limbs and head are again within reach.

Although learning to diagnose pregnancy by rectal examination is not beyond the ability of most people, some expert instruction and considerable practice is necessary to develop this skill. Careless or rough handling, particularly in early gestation, can induce abortions. Thus, the procedure should be left to experienced professionals and even they must be very careful when examining animals before 50 days.

Ultrasonography

Several types of ultrasound units are commercially available for pregnancy diagnosis. The most sophisticated and accurate are real-time B-mode ultrasound models equipped with rectal or surface probes. Although these units are relatively expensive, ultrasound examinations are an integral part of reproductive management programs for a substantial number of dairy practitioners. Experienced technicians can detect pregnancy in cows by the third or fourth week of gestation. Some herd health programs also include a follow-up examination a few weeks later to determine fetal sex. Simpler Doppler or amplitude-depth (A-mode) ultrasound units are also available. The Doppler principle is based on detection of movement – blood flow in uterine or umbilical arteries, fetal heartbeat. A-mode ultrasound detects a fluid-filled uterus, which can occur in situations other than pregnancy. Although these less expensive units cannot be used as early in gestation, or with the same accuracy, as real-time units, they can be reliable in experienced hands.

Reports on the reliability of ultrasound for early pregnancy diagnosis in sheep and goats are variable. Using rectal probes in research settings, pregnancy in sheep and goats can be consistently diagnosed by days 20–25. However, in the field, it is generally recommended to scan after day 45 and even later (70–90 days) for information about number of fetuses. While the sensitivity and accuracy of ultrasound methods in all species vary with the skill level of the technician and type of equipment, the results in goats and sheep are influenced by additional factors such as animal position (for rectal) and whether wool is shaved (abdominal probes in sheep). Although rectal probes have the advantage of earlier detection, they may result in rectal damage in small ruminants.

Indirect Methods of Pregnancy Detection

Indirect methods for pregnancy diagnosis are based on measurements of substances in blood or milk. Antibody-based methods, either radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs), have been developed for detection of these molecules. Because RIA uses radioactively labeled tracers that require expensive detection equipment, the emphasis is now on development of ELISAs that are based on changes in color.

Despite many decades of research, options for early pregnancy markers in dairy ruminants are still limited. Elevated concentration of progesterone, a steroid hormone produced by the ovaries, in blood or milk at the time of next expected estrus is the best established biochemical test for pregnancy in dairy animals. Unfortunately, progesterone levels are elevated during both the luteal phase of the nonpregnant cycle and pregnancy, so this test must be interpreted carefully. A more recently developed biochemical test is based on concentrations of pregnancy-associated glycoproteins (PAGs) in maternal blood. Although the functions of PAGs in pregnancy are not clear, the fact that they are produced by the placenta suggests that they would make an ideal marker for pregnancy. The limitations of these two approaches will be discussed in more detail below.

Milk progesterone concentrations as indicators of nonpregnancy

There are a number of variations but generally milk samples are collected from cows 20–24 days after service. In concept this same method is available in goats and sheep, but blood rather than milk is often used. If the female is pregnant, progesterone concentrations should be elevated. In contrast, luteolysis will occur in cows that were serviced without conception and progesterone concentrations should be very low. The test is very accurate for nonpregnancy but not so reliable for a positive diagnosis. For example, if a female is inseminated not at estrus but during the luteal phase and a sample taken 3 weeks later, she will be in her next luteal phase and could be incorrectly identified as pregnant. An animal with a prolonged luteal phase would also produce a false-positive result. With careful interpretation, milk progesterone tests are a valuable management aid for herdsmen with good estrus detection practices. Researchers in Denmark have developed a mathematical model capable of processing raw progesterone values from milk collected by a robotic milking system into a palatable form, providing the biological interpretation. These innovations, particularly when combined with in-line measurement technology, will be of great interest to operators of large herds.

Pregnancy-associated glycoproteins in plasma as indicators of pregnancy

The PAGs, including pregnancy-specific protein B (PSPB), are a large family of glycoproteins produced by binucleate cells within the chorion of the ruminant placenta. These unusual cells, which constitute up to 20% of the trophectodermal cells, are present only during pregnancy. However, detectable levels of PAGs have been reported in a proportion of virgin heifers, unbred cows, and even bulls, suggesting an alternate source. For this reason, the cutoff value to discriminate between pregnant and nonpregnant animals must be carefully considered. Another drawback of PAGs is that, typical of heavily glycosylated proteins, they have a long half-life in the circulation. Plasma concentrations rise to very high levels in dairy cows during the last few days of gestation, which carry over into the postpartum period. Residual levels of PAGs that would be considered diagnostic of pregnancy can still be detected up to 3 months postpartum, which limits the use of PAGs for early pregnancy detection. A third drawback of PAGs is that they are not secreted into milk to any appreciable extent, which necessitates collection of a blood sample.

Development of assays for pregnancy-associated glycoproteins

RIAs utilizing antisera from rabbits immunized with purified or semipurified preparations of bovine cotyledons have been described in the scientific literature beginning in the late 1980s. These assays have also been used to detect PAGs in sheep and goats, using bovine standards and tracer. More recently, RIAs using antisera raised against preparations of ovine and caprine placentomes have been developed. Curiously, when used for screening of cattle, these heterologous RIAs measured higher concentrations than homologous assays using all bovine reagents. Indeed, an RIA using a mixture of rabbit antisera produced with cow, sheep, and goat PAGs as immunogens found greater differences in PAG concentrations in pregnant versus nonpregnant cattle.

ELISAs do not require expensive detection equipment or radioactivity, and can be modified to provide qualitative 'cow-side' kits for on-farm use. A test based on an ELISA developed at the University of Idaho has been commercially available since 2005 (BioPRYN™, BioTracking, Moscow, ID, USA). This test requires that a blood sample be sent to a testing lab. A 'sandwich' type of ELISA has been developed at the University of Missouri, Columbia, using a mixture of monoclonal antibodies to trap PAG in the wells and a polyclonal antiserum to detect the bound PAG. The goal was to develop an assay that detected the PAG subset that was expressed during early pregnancy rather than the late gestation forms that persist into the postpartum period. The ELISA outperformed the RIAs in this regard, with the vast majority of cows with undetectable levels by 8 weeks postpartum. A cow-side version of this test is currently under development and may eventually be available for commercial use.

One of the challenges in developing these assays is that PAGs are a diverse family of molecules rather than a single molecule. As gestation progresses, differentially glycosylated forms of particular PAGs and even different PAG molecules are expressed. Detection of these different forms is highly dependent on the assay system. PAG secretion may even be affected by parity and level of milk production. Developing robust immunological assays for these molecules clearly presents a different challenge than for a molecule like progesterone, which is biologically identical across all mammals.

Management Considerations in Development of Pregnancy Diagnosis Strategies

Features of currently available methods for early pregnancy diagnosis in cattle, goats, and sheep are summarized in **Table 2**. However, dairy producers must recognize that there is one inherent problem that will continue to be associated with even the most reliable pregnancy test. No matter how accurate the procedure, the results are valid only for the time the sample was

Table 2 Methods for early pregnancy diagnosis in common dairy ruminants

Method	*Species*	*Stage*	*Comments*
Nonreturn to estrus	Cow Goat Sheep	Days 20–24 Days 20–24 Days 16–19	Best to assess nonpregnancy; requires knowledge of breeding dates and behavioral observation
Progesterone (milk or blood)	Cow Goat Sheep	Days 20–24 Days 20–24 Days 16–19	Best to assess nonpregnancy; requires knowledge of breeding dates
Rectal palpation	Cow	After day 35[a]	Some risk to developing fetus; requires considerable expertise
Ultrasound	Cow, rectal Goat and sheep, rectal Abdominal	After day 28 After day 25 After day 35	Use of rectal probes in small ruminants is associated with risk of rectal damage; thus, animal restraint system must be factored into decision to use this approach
PAG/PSPB (blood)	Cow Goat and sheep	After day 30 After day 24	At present, no animal-side test available, so samples must be shipped to testing laboratory

[a] Pregnancy can be diagnosed in heifers as early as days 28–30, but rectal palpation at this early stage is not recommended due to the risk of damage of the very fragile conceptus.

PAG, pregnancy-associated glycoprotein; PSPB, pregnancy-specific protein B.

collected or the physical examination was conducted. A substantial number of pregnancies are lost during the first trimester, so any early pregnancy diagnosis should be reconfirmed at a later date. False positives in early assessments and early embryo losses will be detected at a follow-up check. But false negatives (animals incorrectly identified as nonpregnant) are potentially disastrous, in that prostaglandins used to induce estrus for rebreeding would cause abortion in pregnant animals.

See also: **Gamete and Embryo Technology**: Artificial Insemination; Multiple Ovulation and Embryo Transfer. **Husbandry of Dairy Animals**: Goat: Reproductive Management; Sheep: Reproductive Management. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Mating Management: Fertility; Pregnancy: Parturition; Pregnancy: Physiology.

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Pregnancy: Physiology

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Physiology

Pregnancy is a key event in the life of a dairy animal because the hormonal processes that are played out during this period include those that prepare the mammary gland for lactation following parturition. Indeed, perturbations in the placenta, the source of many mammogenic hormones, can conceivably lead to a reduction in the magnitude of milk synthesis in the subsequent lactation. For pregnancy to succeed, the conceptus must be able to successfully progress through a series of preprogrammed developmental steps that transform it from an undifferentiated one-cell organism into a neonatal animal capable of life outside of the womb. Accordingly, the maternal system must be made to serve the nutritional, immunological, and physiological needs of the conceptus. Not surprisingly, then, there is a close coordination of maternal and conceptus physiology that is achieved by the exchange of information between mother and offspring through chemical signals.

The complexity of pregnancy makes it susceptible to failure and the newly formed conceptus has only a fair chance of completing development. In many populations of lactating dairy cows, especially those that are intensively managed for high milk production, only about 15–30% of cows that are inseminated carry a fetus to term. Pregnancy failure is most prevalent early in development but can occur throughout gestation (**Figure 1**). Historical data indicate that pregnancy rate per conception in dairy cattle declined through the last 30–40 years or so of the twentieth century in most developed countries. The reasons for this decline are unclear but likely include the consequences of increased milk yield, changes in animal housing, and increased inbreeding. Understanding the physiology of the processes involved in pregnancy, then, offers the opportunity to increase reproductive function and improve the efficiency of milk production.

Unfortunately, a diverse and not always coherent system has arisen for naming organisms during the period of prenatal life. In this article, the term conceptus will be used to refer to all of the products of conception (the embryo or fetus and its associated extraembryonic membranes). Until differentiation of placental tissues, the term embryo can be considered analogous to conceptus. Thereafter, embryo refers to the part of the conceptus that will give rise to the neonate. Once the embryo has undergone sufficient differentiation to be reasonably identifiable as a member of the species, it is referred to as a fetus. This transition, which is somewhat arbitrary, occurs at about day 45 of gestation in cattle and day 34 in sheep.

Preimplantation Period

Pregnancy is initiated at the moment of conception when the newly ovulated oocyte is fertilized by one of the spermatozoa resident in the oviduct following mating. The early preimplantation embryo is distinct from most other cells in that the embryonic genome is largely repressed for the first several cleavage divisions. Rather, the embryo relies on mRNA formed during oocyte growth for direction of the bulk of protein synthesis. In the cow and sheep, major activation of the embryonic genome occurs at the 8- to 16-cell stage. There is limited transcription before these stages, however, and heat shock can induce transcription of genes for heat shock protein 70 as early as the two-cell stage. Activation of the embryonic genome is initiated following the loss of maternally derived mRNA, which is in turn caused by the loss of proteins such as MSY2 that mask mRNA.

A schematic illustration of events in early pregnancy is presented in **Figure 2** (*see* **Reproduction, Events and Management**: Pregnancy: Characteristics). Early stages of development involve a series of cleavage divisions whereby cell number of the embryo increases while size of individual cells (called blastomeres) decreases. Early cells of the embryo are totipotent – a new individual can be derived from a single blastomere appropriately transferred to an empty zona pellucida (the protein coat surrounding the embryo). Differentiation of the preimplantation embryo is first initiated at the morula stage of development (5–6 days after ovulation in cattle and 3–4 days in sheep), which is characterized by a loss in the ability to view individual cell membranes. Morulae are formed through a process called compaction in which embryonic size decreases somewhat and cells become more tightly associated. The underlying basis for compaction is the formation of intercellular junctional complexes between blastomeres at the basolateral regions of the cells. These tight junctions limit fluid movement between cells and lead to the polarization of blastomeres into apical

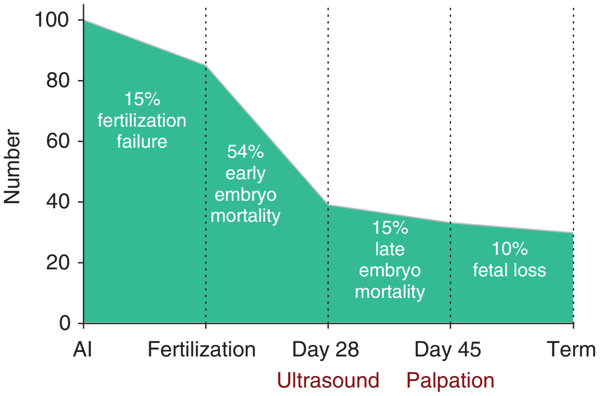

Figure 1 Pregnancy loss throughout gestation in a hypothetical herd of 100 lactating dairy cows with a calving rate of 30%. The *y*-axis represents the number of cows. Note that fertilization occurs only in about 85% of inseminated cows. Following fertilization, large numbers of embryos die before pregnancy diagnosis by ultrasound at day 28. Other losses occur between days 28 and 45 (about 15% of pregnancies), when pregnancy is diagnosed by rectal palpation, and day 45 and term (about 10% of pregnancies present at day 45). AI, artificial insemination. The figure is reproduced with permission from Hansen PJ (2007) Hidden factors affecting fertility. *WCDS Advances in Dairy Technology* 19: 339–349.

regions in contact with the external environment of the embryo and basolateral regions located near the interior of the embryo. The blastocyst is formed from the morula when fluid accumulates in the embryo to form a blastocoelic cavity. Embryos progress from the morula stage to the blastocyst stage at about days 6–8 after ovulation in cattle and days 6–7 in sheep. At the same time, two distinct cell layers can be identified – the trophectoderm, which subsequently gives rise to the extraembryonic membranes, and the inner cell mass, which gives rise to the fetus. Two processes have been implicated in the formation of the blastocoel (**Figure 3**). The outer layer of blastomeres develop Na^+/K^+ ATPase pumps on the basolateral surface that use movement of ions to direct water into the interior of the embryo. In addition, water channel molecules called aquaporins allow direct movement of water across blastomeres.

Emergence of the blastocyst from the zona pellucida follows blastocyst formation. This process, called hatching (at about days 9–10 postovulation in cattle and days 7–8 in sheep), is caused by a combination of proteinases from the

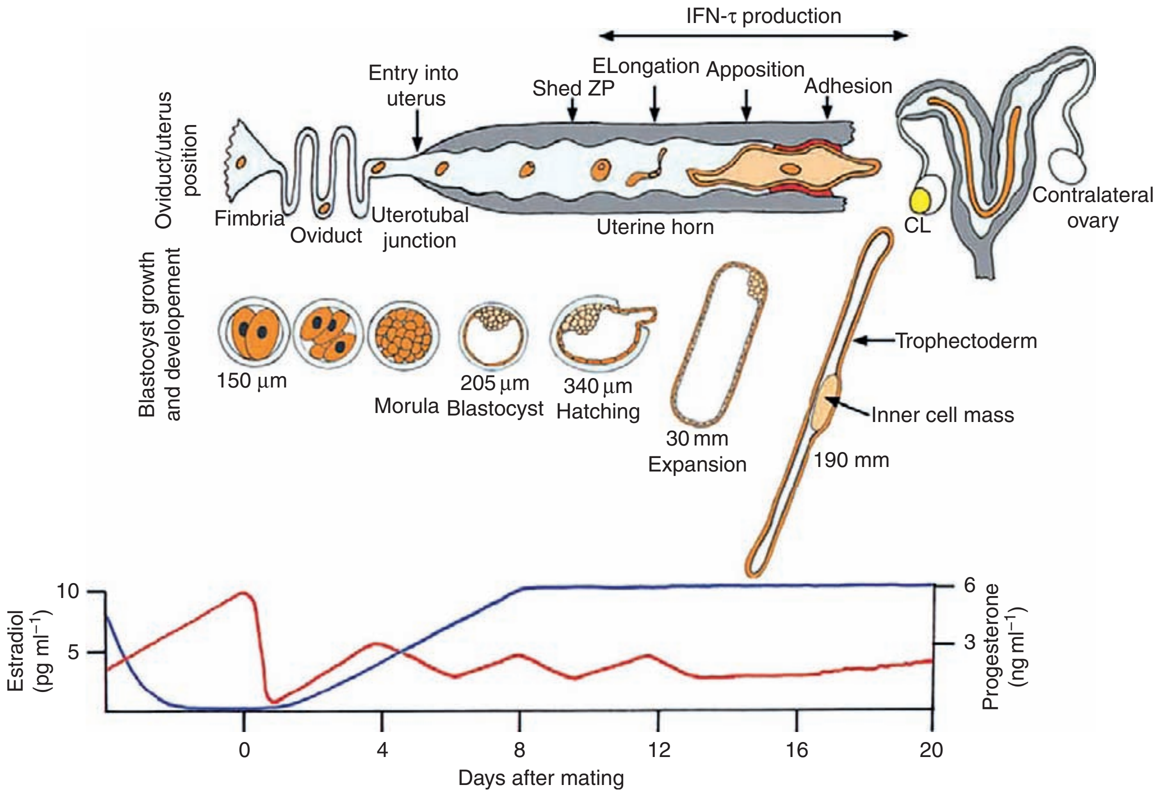
IFN-τ production
Oviduct/uterus position
Entry into uterus
Shed ZP
ELongation
Apposition
Adhesion
Fimbria
Oviduct
Uterotubal junction
Uterine horn
CL
Contralateral ovary
Blastocyst growth and developement
150 μm
Morula
205 μm
Blastocyst
340 μm
Hatching
30 mm
Expansion
190 mm
Trophectoderm
Inner cell mass
Estradiol (pg ml^{-1})
10
5
6
3
Progesterone (ng ml^{-1})
0
4
8
12
16
20
Days after mating

Figure 2 Schematic diagram of events during early pregnancy in the sheep. Events in the cow are similar except that timing relative to days after mating differs slightly. For example, interferon-τ (IFN-τ) secretion starts between days 15 and 17 of pregnancy and the earliest attachments to the uterus are at day 18 of pregnancy. In the bottom panel, progesterone concentrations are represented by blue lines. CL, corpus luteum; ZP, zona pellucida. The figure is reproduced with permission from Spencer TE, Johnson GA, Bazer FW, Burghardt RC, and Palmarini M (2007) Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. *Reproduction, Fertility and Development* 19: 65–78. Copyright IETS 2007. Published by CSIRO PUBLISHING, Melbourne, Australia – http://www.publish.csiro.au/nid/45/issue/3364.htm.

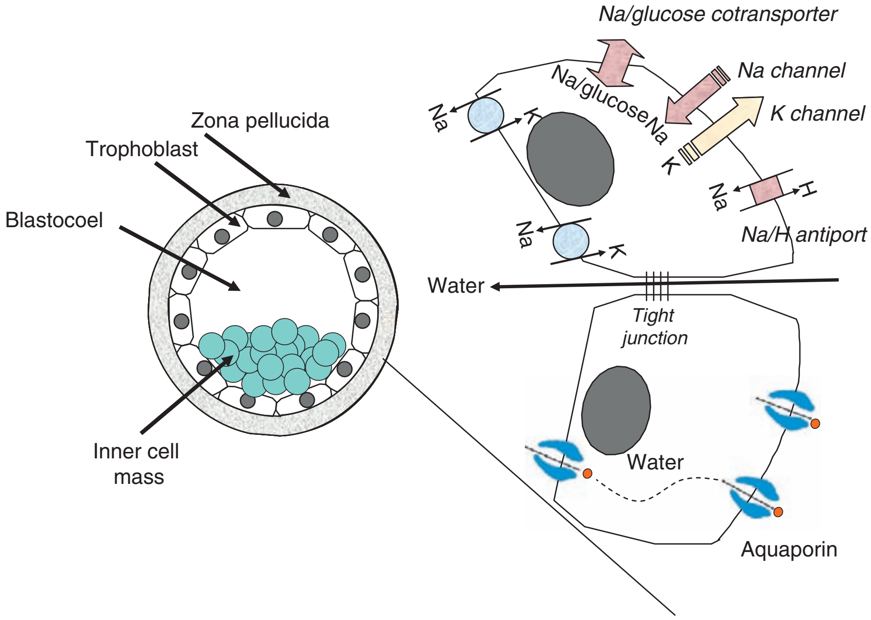

Figure 3 Blastocoel formation in embryos. Formation of a central, fluid-filled cavity is a central event in development, which results in morphological differentiation of the embryo into inner cell mass (from which the fetus will arise) and trophectoderm (from which the extraembryonic membranes arise). Two processes have been implicated in the formation of the blastocoel. The first involves pumping of sodium. The outer layer of blastomeres develop Na^+/K^+ ATPase pumps on the basolateral surface of the cell membrane that pump sodium out of the cell. Other specialized proteins on the apical side of the cell allow sodium to enter the cell and remove potassium that is pumped into the cell. Water flows between cells and into the central cavity of the embryo to maintain osmolarity. The second process involves channel proteins called aquaporins that allow direct movement of water across blastomeres. These proteins are present on the apical and basolateral surfaces of the plasma membrane of trophectoderm cells.

embryo and uterine environment as well as the physical forces applied to the zona pellucida as the blastocoel undergoes periodic contractions and expansions. The process of expansion and contraction is dependent upon embryonic synthesis of prostaglandin E_2. Hatching is followed by a process initiated at about day 14 or 15 in cows and day 11 in sheep whereby the trophoblast rapidly expands to transform the embryo into a long filamentous structure. Expansion is rapid – while the day 13 cow embryo is 3 mm in diameter, it reaches a size of 25 cm by day 17 and begins to grow into the opposite uterine horn by day 18. The regulation of the process of trophoblast expansion, which is caused largely by increased cell division, is unknown. However, it is likely that the process requires a uterine signal since the process cannot be mimicked *in vitro*. One purpose of elongation is to ensure that the conceptus covers much of the endometrium so that secretory molecules regulating endometrial function (particularly those for inhibition of prostaglandin synthesis) are distributed widely throughout the uterine horn in which the embryo resides.

Ruminant embryos spend a considerable period of time (16 and 18 days in sheep and cattle, respectively) unattached to the epithelial lining of the reproductive tract. Before attachment, the embryo is dependent upon secretions from the oviduct and endometrium for nutritional support. The embryo has only a limited ability to utilize glucose as an energy source before compaction and glucose utilization can potentially be inhibitory to embryo development. During this period, pyruvate, lactate, and amino acids are the primary energy source. Many specific amino acids are present in high concentration in the oviduct and these not only serve as an energy source but also help regulate osmotic pressure and pH in the embryo and inhibit deleterious actions of glucose. Beginning at compaction, which represents the first major energy-expensive event in development, glucose uptake increases and glucose becomes the preferred energy substrate.

As is apparent from the successful development of embryos produced by *in vitro* fertilization, there is no absolute requirement of the embryo for macromolecules specific to the reproductive tract environment. However, *in vitro*-produced embryos have aberrant biochemical and molecular properties compared to embryos produced *in vivo* and it is likely that macromolecules in oviductal and uterine fluid regulate early embryonic development.

Many of the components of oviductal and uterine fluid are derived from transudation from the blood. In addition, both the oviduct and endometrium secrete specific molecules into the lumen of the reproductive tract. Secretion of many of these locally produced factors is under the control of progesterone or estrogen. Some molecules, such as oviductal secretory protein-1 and osteopontin, may play a role in fertilization. Many are regulatory molecules that mediate the complex communication between the cells of the reproductive tract and embryo. For example, growth factors and other regulatory molecules produced by cells of the oviduct and endometrium include insulin-like growth factors I and II, basic fibroblast growth factor (bFGF), activin, and leukemia-inhibitory factor. Similarly, the embryo produces epidermal growth factor and transforming growth factor-α. The reproductive tract also produces proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1 (TIMP-1) that likely prevent degradation of the lining of the female reproductive tract by embryonic proteinases and thereby block implantation of the embryo into the underlying stroma.

Most embryonic mortality occurs during the preimplantation period, that is, before day 20 of pregnancy (see **Figure 1**). Numerous causes of embryonic loss have been identified. These include chromosomal abnormalities, inheritance of specific alleles causing embryonic or fetal death (e.g., allelic variants in fibroblast growth factor 2, STAT5A, and uridine-5′-monophosphate in cattle), reduced progesterone concentrations or premature luteolysis in the dam, and defects in the process of development (e.g., inadequate trophoblast development and reduced secretion of interferon-τ). Oocytes damaged during follicular development can give rise to embryos with reduced developmental competence. Environmental influences that increase embryonic mortality include provision of feed with high levels of rumen-degradable protein, various microbial or viral diseases, and heat stress.

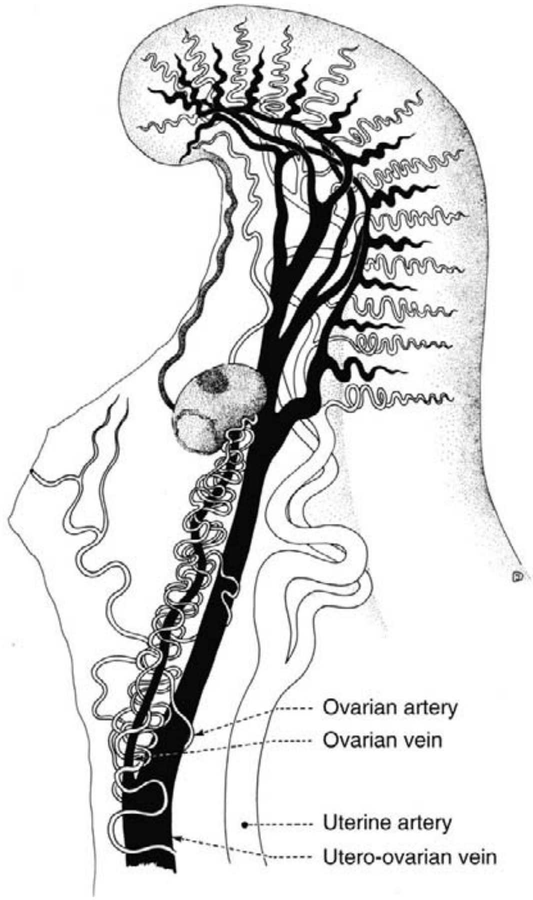

Figure 4 Arrangement of the uterine and ovarian vasculature of the sheep. Note that the utero-ovarian vein, which carries blood draining the uterus, is in very close apposition to the highly coiled ovarian artery carrying blood to the ovary. The close anatomical relationship of these two vessels facilitates the countercurrent movement of prostaglandin $F_{2\alpha}$ from the uterus to the ovary during luteolysis. Figure reproduced with permission from Flood PF (1991) The development of the conceptus and its relationship to the uterus. In: Cupps PT (ed.) *Reproduction in Domestic Animals*, 4th edn., pp. 315–360. San Diego, CA: Academic Press.

Maintenance of the Corpus Luteum

Among the changes in maternal physiological function necessary for the maintenance of the corpus luteum is the rescue of the corpus luteum from luteolysis. In the absence of pregnancy, the corpus luteum regresses beginning around days 15–16 in cattle and days 13–14 in sheep as a result of release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) produced by endometrial epithelial cells. Prostaglandin is rapidly metabolized in the lung and other organs; the prostaglandin responsible for luteolysis arrives at the corpus luteum via a local pathway involving movement out of the utero-ovarian vein and into the closely apposed ovarian artery (**Figure 4**). Some prostaglandin may also reach the ovary through the lymphatic system. $PGF_{2\alpha}$ causes luteolysis by stimulating local luteal synthesis of cytokines, endothelin-1, and nitric oxide, which in turn initiate functional (loss of progesterone secretion) and structural luteolysis (loss of luteal cells by apoptosis). Endothelin-1, for example, decreases progesterone production by luteal cells while stimulating additional luteal production of $PGF_{2\alpha}$.

As shown in **Figure 5**, the embryo blocks luteolysis by inhibiting $PGF_{2\alpha}$ synthesis. The embryo first acts to inhibit luteolysis between days 15 and 17 of pregnancy in cow and days 12 and 14 in sheep. Removal of the embryo before this time does not affect luteal life span but the life span of the corpus luteum is extended if the embryo is removed afterward. The most important of the antiluteolytic signals produced by the embryo, at least during the initial rescue of the corpus luteum, is a molecule called interferon-τ. This molecule is a member of the class I interferon family, which also includes

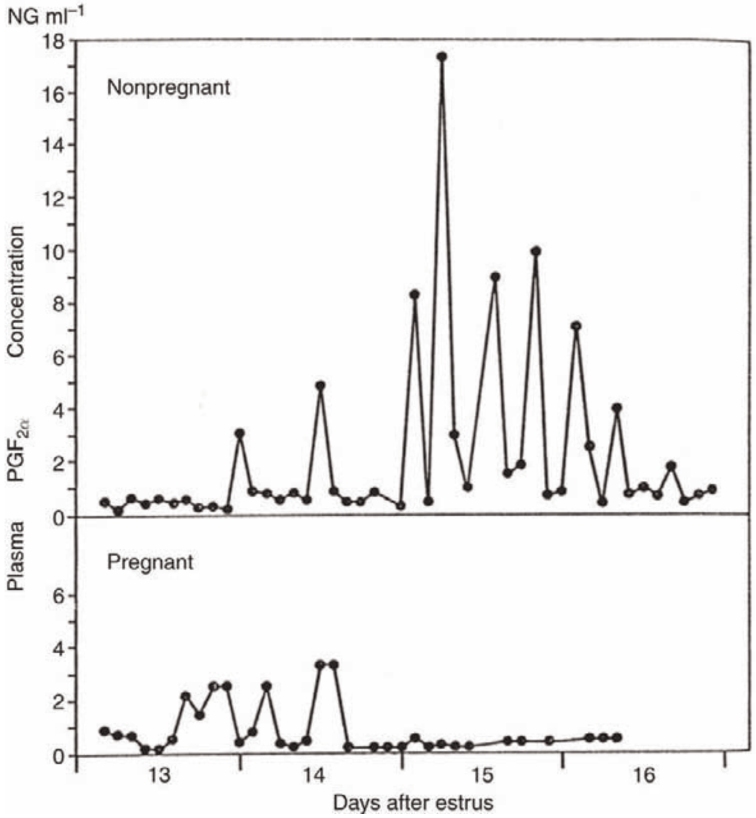

Figure 5 Concentrations of prostaglandin F$_{2\alpha}$ in utero-ovarian plasma of nonpregnant and pregnant ewes during the time of expected luteolysis. Note the large pulses of prostaglandin F$_{2\alpha}$ in the nonpregnant ewe that are responsible for luteolysis. These pulses are attenuated in the pregnant ewe. Figure is reproduced from Barcikowski B, Carlson JC, Wilson L, and McCracken JA (1974) The effect of endogenous and exogenous estradiol-17β on the release of prostaglandin F2α from the ovine uterus. *Endocrinology* 95: 1340–1349 with permission of the Endocrine Society.

interferon-α, β, and ω. Interferon-τ arose in evolution in ruminants via gene duplication about 36 million years ago. While interferon-τ is similar in many ways to other class I interferons (including binding to class I interferon receptor, signal transduction, antiviral, and immunosuppressive activity), it differs from these interferons in the fact that gene expression is under the control of a trophoblast-specific promoter and expression is not strongly induced by virus.

The pattern of expression of interferon-τ gene is distinct. The protein can be detected in low amounts when the embryo undergoes blastocyst formation. There is then a massive upregulation of gene expression coincident with impending luteolysis so that interferon-τ becomes the major secretory product of the conceptus from around days 15–20 in cattle and days 13–17 in the sheep. Gene expression is then rapidly turned off so that secretion is low by 25 days in cows and 21 days in sheep.

Interferon-τ acts on endometrial epithelial cells to inhibit PGF$_{2\alpha}$ secretion. Proposed mechanisms by which interferon-τ inhibits prostaglandin secretion are illustrated in **Figure 6**. Endometrial PGF$_{2\alpha}$ synthesis is caused by oxytocin-induced activation of cyclooxygenase-2, the rate-limiting step in the conversion of arachidonic acid to PGF$_{2\alpha}$. Responsiveness of the endometrium to oxytocin is dependent upon estradiol-induced synthesis of oxytocin receptors. There is evidence to suggest that interferon-τ acts to inhibit expression of oxytocin receptor gene. Inhibition of oxytocin receptors may be due to inhibition of gene expression of estrogen receptors to make the endometrium unresponsive to estradiol induction of oxytocin receptors. This seems to be the case for sheep. In the cow, oxytocin receptors can be inhibited independent of the changes in estrogen receptor. In addition, interferon-τ increases the fatty acid linoleic acid, at least in cattle, and this molecule acts as an inhibitor of cyclooxygenase.

The corpus luteum also becomes more resistant to destruction by PGF$_{2\alpha}$ in early pregnancy. This could be due to interferon-τ because small amounts of the molecule leave the uterus, due to the actions of another prostaglandin, PGE$_2$, that is produced by uterine endometrium, or due to another unknown molecule. Later in pregnancy, some unidentified molecule is important for

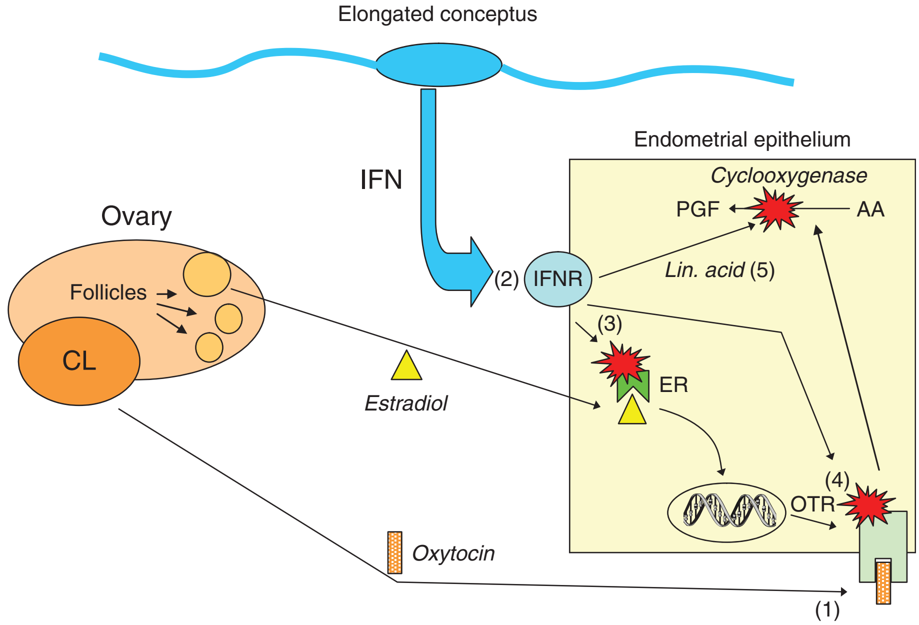

Figure 6 Proposed mechanism by which interferon-τ (IFN-τ) inhibits estradiol- and oxytocin-driven induction of luteolytic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release from endometrium. Luteolytic pulses of $PGF_{2\alpha}$ are driven by the release of oxytocin from the corpus luteum (CL) and hypothalamus (1). Oxytocin acts through receptors (OTR) that are synthesized under the control of estradiol to activate the synthesis of $PGF_{2\alpha}$ from arachidonic acid (AA) by cyclooxygenase. IFN-τ acts through binding to its receptor (IFNR) (2) to cause several changes that lead to inhibition of $PGF_{2\alpha}$ synthesis. Foremost is a reduction in the synthesis of OTR, caused by inhibition of the estrogen receptor (ER) (3), as in the sheep, or by direct inhibition (4), as occurs in the cow. The reduction in OTR makes the endometrium unresponsive to oxytocin. In addition, IFN-τ increases intracellular concentrations of linoleic acid (Lin. acid), at least in the cow, and this fatty acid acts to inhibit cyclooxygenase (5).

luteal maintenance. This is so because administration of interferon-τ to nonpregnant animals extends luteal life span for a limited time that is much shorter than the period during which the corpus luteum of pregnancy persists. For example, intrauterine infusion of interferon-τ extended interestrous interval from an average of 23 days in controls to 32 days in cattle and from 16 days to 32 days in sheep.

Role of Progesterone

While progesterone is indispensable for pregnancy in all mammals, the major sources of progesterone vary between species. In both cattle and sheep, progesterone is produced by the placenta, especially the fetal cotyledon. In cattle, however, placental secretion of progesterone is low for most of pregnancy, possibly because of the presence of an endogenous inhibitor of steroidogenesis. Removal of the corpus luteum before day 200 of pregnancy leads to pregnancy loss. Placental progesterone synthesis rises at the end of pregnancy. Pregnancy can continue if the corpus luteum is removed after day 200 although premature parturition is common. In the sheep, in contrast, the placenta becomes a sufficient source of progesterone earlier in pregnancy, by day 50 of gestation.

Progesterone maintains pregnancy by exerting a variety of actions on the uterus. One target is the myometrium – progesterone maintains the quiescence of this muscular layer of the uterus by causing hyperpolarization of myometrial cells. *In vitro*, progesterone causes increased proliferation of endometrial stromal cells in cattle without affecting epithelial cells. *In vivo*, however, progesterone causes some increase in the proliferation of luminal endometrial epithelial cells in sheep. It is likely that progesterone's effects on the epithelium are mediated by growth factors such as hepatocyte growth factor that are secreted by the stroma.

Progesterone increases expression of multiple genes in the endometrial lining of the uterus. Use of DNA microarray technology has led to the identification of 140 genes whose expression is higher during the luteal phase of the estrous cycle. Some progesterone-induced genes encode for proteins involved in conceptus nutrition. For example, retinol binding protein, which binds retinol and presumably transports it to the conceptus, is a progesterone-induced endometrial secretory protein in cattle. It is also likely that the actions of progesterone are required for attachment of the conceptus to the endometrium and for subsequent placentation. Among the genes induced in the endometrium by progesterone is osteopontin, which is a glycoprotein component of the extracellular matrix that promotes cell–cell attachment by binding to cell surface integrins. Osteopontin may have an important role in mediating attachment of the trophoblast to the maternal endometrium. Other genes induced by progesterone regulate angiogenesis to increase vascular blood supply to the placenta. Among the genes identified as being upregulated during the luteal phase in cattle were several involved in angiogenesis including angiotensinogen, hypoxia-inducible factor 2-α, and ephrin-A1.

Exogenous administration of progesterone early in pregnancy hastens the growth of the embryo. Certain cases of infertility may be due to inadequate progesterone secretion because correlations have been made in cattle between the concentrations of progesterone in the blood early in pregnancy (days 4–5) and embryo development, amounts of interferon-τ in the uterus at day 16 of pregnancy, and pregnancy rate following insemination. The lactating dairy cow, which represents an infertile animal type, often experiences reduced circulating concentrations of ovarian steroids.

Placental Function

Placentation is a gradual process in ruminants (*see* **Reproduction, Events and Management**: Pregnancy: Characteristics). Invasion of trophoblast cells into the uterine endometrium is limited and the basement membrane of the luminal epithelium of the endometrium remains intact. Attachment to the endometrium is a gradual process that starts with apposition of conceptus and endometrium, adhesion of the two tissues mediated by interdigitation of microvilli, invasion of trophoblast binucleate cells into the endometrial epithelium, and fusion of binucleate cells with endometrial epithelial cells to form a syncytium. In the cow, the first attachments are seen at day 18 and binucleate cell invasion is initiated at ∼day 20. In sheep, the conceptus is adhered to the endometrium by day 16. The adhesion process is facilitated by cell adhesion molecules on the surface of the trophoblast (e.g., integrin) and molecules secreted by the endometrium such as galectin 15 and osteopontin.

The anatomy of the definitive placenta, which has differentiated by about day 37 of pregnancy in the cow and day 30 in the sheep, is described elsewhere (*see* **Reproduction, Events and Management**: Pregnancy: Characteristics). Most growth in the placenta is completed by midgestation. Growth occurs at a much slower rate after this time in cattle and ceases in sheep. The placenta serves as the sole organ for chemical exchange between fetus and mother. All of the water, oxygen, and nutrients necessary to support life and growth of the developing fetus move through the placenta. In addition, the placenta serves as the organ by which fetal waste products including carbon dioxide and by-products of metabolism are removed to the mother for eventual elimination via the maternal respiratory, renal, and digestive systems. In addition to its transport role in fetal respiration, nutrition, and electrolyte balance, the placenta is also an endocrine organ. The hormones it produces alter maternal function to support pregnancy, lead to development of the mammary gland in anticipation of parturition, and participate in the process leading to parturition. Other regulatory molecules secreted by the placenta probably act locally on the uterus to optimize maternal function during pregnancy.

The mechanism for transport across the placenta varies depending upon the specific chemical. Some molecules such as oxygen, carbon dioxide, free fatty acids, and some electrolytes passively diffuse across the placenta. The glucose transports GLUT1 and GLUT3 are involved in the transport of glucose. Amino acids are also actively transported a cross the placenta. Fatty acids are poorly transported across the ruminant placenta. As a result, despite being major sources of energy for the adult, fatty acids contribute in only a minor way to the energy needs of the fetus. Movement of some other molecules poorly soluble in water is facilitated by carrier molecules secreted by uterine glands. For example, retinol is carried by retinol binding protein and iron by transferrin.

Most of the chemical exchange between mother and fetus occurs at the placentomes, which are formed from the union of specialized protrusions of the chorioallantois called cotyledons with corresponding structures on the maternal endometrium called caruncles (*see* **Reproduction, Events and Management**: Pregnancy: Characteristics). As a result, about 94% of fetal blood flowing to the sheep placenta is distributed to the cotyledons and 84% of maternal blood distributed to the uterus is distributed to the caruncles. Nonetheless, some placental exchange occurs in the interplacentomal regions also. In this region, there is a close contact between maternal and fetal epithelia. Moreover, maternal endometrial glands are in the interplacentomal regions and their secretions are released into the fetal–maternal interface.

Placental efficiency can be estimated by determining the mass of fetal tissue that a given unit of placenta can support. By this measure, the ruminant placenta is a relatively efficient organ. The ratio of fetal to placental tissue is 13:1 in cattle and 10:1 in sheep and goats. In contrast, each gram of human placenta can support only about 6 g of fetus. The efficiency of the placenta is due largely to the architecture of the maternal blood vessels supplying the placenta relative to the fetal vessels. In ruminants, maternal and fetal blood vessels are arranged so that most blood flow is in a countercurrent or cross-current direction (**Figure 7**).

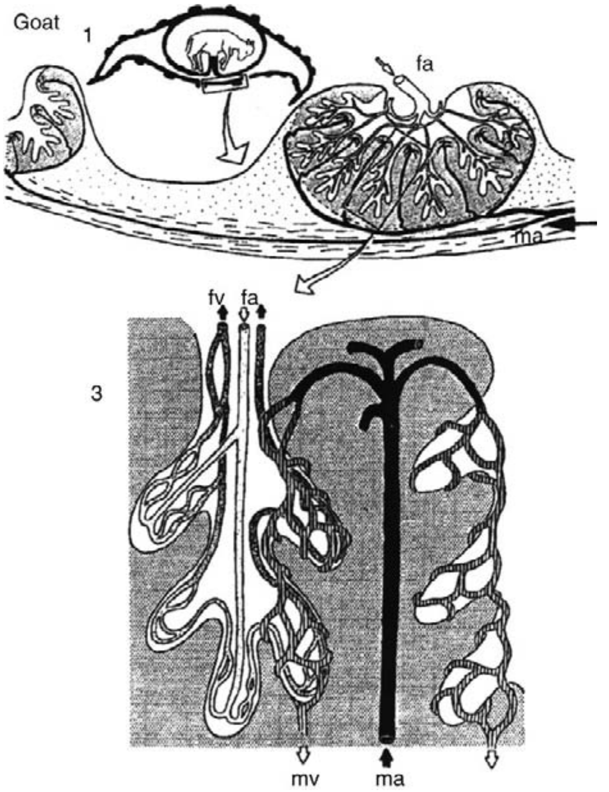

Figure 7 Overview of the ruminant placenta. The placenta of the ruminant is characterized by an outer placental membrane formed from the fusion of the chorion and allantois (1). Knob-like projections of the chorion called cotyledons fuse with complementary structures on the maternal endometrium called caruncles to form placentomes. A single placentome is shown in (2) where fetal tissue is white and maternal tissue is stippled. Note the chorionic villi that project into the crypts in the maternal caruncles. As shown in (3), fetal vessels in chorionic villi are in close apposition to the corresponding maternal vessels in the caruncular septa. Basket-like maternal capillaries surround fetal venous capillaries. This arrangement of vessels allows for very efficient exchange of materials between mother and fetus because the pattern of flow is crosscurrent and countercurrent. fa, fetal artery; ma, maternal artery; mv, maternal vein. The figure, which was modified from Leiser R, Krebs C, Ebert B, and Dantzer V (1997) Placental vascular corrosion cast studies: A comparison between ruminants and humans. *Microscopy Research and Technique* 38: 76–87 and is reproduced with the permission of the publisher, is based on an earlier figure from Dantzer V, Leiser R, Kaufmann P, and Luckhardt M (1988) Comparative morphological aspects of placental vascularization. *Trophoblast Research* 3: 221–244.

Placental blood flow increases during pregnancy to match the increased demands of the fetus. Increased blood flow is due to vasodilation and angiogenesis. During late pregnancy, endothelial cells in the uterine artery increase synthesis of two vasodilators, prostacyclin and nitric oxide. Angiogenesis is at least in part regulated by growth factors such as vascular endothelial growth factor (VEGF) and bFGF produced by the placenta. These molecules, as well as others such as angiotensin II, also likely contribute to vasodilation by participating in the regulation of synthesis of prostacyclin and nitric oxide.

The placenta is an endocrine organ that produces hormones whose synthesis occurs uniquely in the placenta as well as those also produced by other organs. Among the former is placental lactogen, which is a member of the prolactin/growth hormone family. Synthesis occurs by the binucleate cells that migrate from the trophoblast into the uterine endometrium. Concentrations of placental lactogen in the blood increase as gestation progresses. Placental lactogen is believed to play two roles – increase mammary development in anticipation of the birth of the neonate and act as a metabolic hormone to redirect maternal metabolism to provide nutrients for the fetus.

The role of the placenta in progesterone production has already been noted. In addition, the ruminant placenta produces estrogens, androgens, and prostaglandins in increasing amounts toward late pregnancy. Estrone synthesis predominates over estradiol-17β synthesis and most of the circulating estrogens exist in conjugated, inactive form (e.g., estrone sulfate). Estrogen plays at least two roles during late pregnancy. With progesterone, estrogen stimulates growth of the mammary gland. Estrogen also plays a role in parturition by counteracting the effect of progesterone on uterine contractility (causing depolarization of myometrial cells so that the uterine capacity for contractions is increased) and by acting on the fetus to increase adrenocorticotropin, the hormone that acts as the central regulator of the timing of parturition. Unlike some species, ruminant placentas do not produce a chorionic gonadotropin or relaxin.

Another secretory product of the placenta is a group of proteins called the pregnancy-associated glycoproteins (PAGs). These proteins, which are members of the aspartic proteinase family, are very unusual in that they are encoded by a large number of genes (perhaps as many as 100). Some PAGs are produced predominately in binucleate cells, while others are produced throughout the chorionic epithelium. It is not clear whether the PAGs are hormones or what function they serve during pregnancy. However, PAG genes have undergone rapid evolution, suggesting that they play an important role in pregnancy. Radioimmunoassay of PAGs (originally called pregnancy-specific protein B) has been utilized as the basis for a pregnancy test in cattle. The long half-life of PAGs in the blood after parturition has limited the usefulness of PAG-based pregnancy tests, however.

Maternal stress can lead to a disruption in placental function and compromised fetal development. Since hormones produced by the placenta are involved in mammogenesis, disruptions in placental function can conceivably alter subsequent milk yield. One example is heat stress, which causes a redistribution of blood from the internal organs (including the placenta) to the skin to

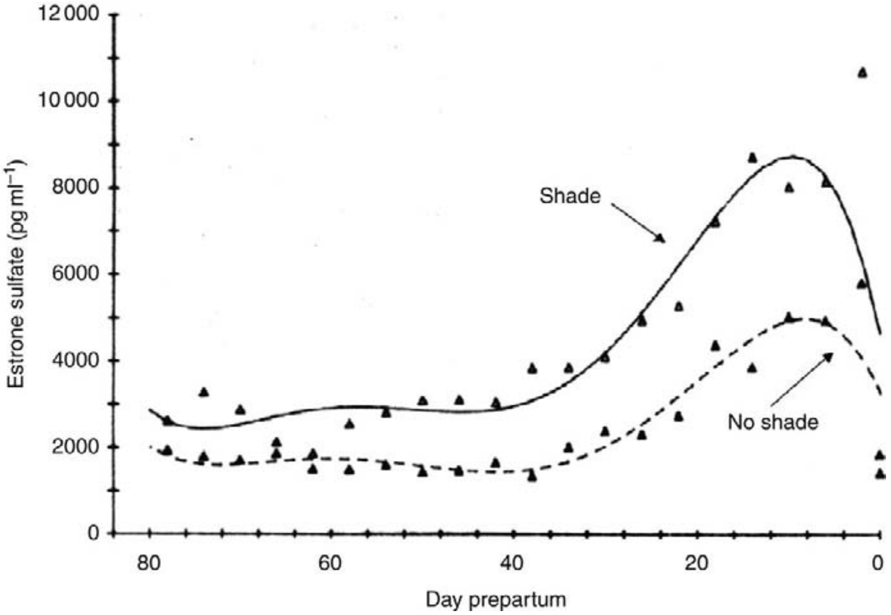

Figure 8 Effect of heat stress on plasma concentrations of estrone sulfate in pregnant cows. Cows during the summer in Florida were housed with or without shade. Note the reduction in estrone sulfate concentrations in cows housed without shade. This reduction represents reduced secretion of estrone sulfate from the placenta. The figure, which is reproduced with the permission of the publisher, was modified slightly from Collier RJ, Doelger SG, Head HH, Thatcher WW, and Wilcox CJ (1982) Effects of heat stress during pregnancy on maternal hormone concentrations, calf birth weight and postpartum milk yield of Holstein cows. *Journal of Animal Science* 54: 309–319.

increase heat loss to the environment. The resultant decrease in placental perfusion leads to depressed placental growth accompanied by reduced fetal growth and placental hormone secretion (see **Figure 8**). There is evidence in cattle that failure to cool cows during late parturition can compromise subsequent milk yield. Undernutrition can also have complex effects on placental function. The placenta can adjust somewhat to a reduction in circulating glucose concentrations by increasing the synthesis of glucose transporter GLUT3. However, severe glucose deficiency can lead to reduced placental uptake of glucose and fetal growth retardation. In fat ewes, underfeeding during early and mid-pregnancy can actually lead to increased placental growth while overfeeding during the same period can sometimes reduce placental growth. Nutritional deprivation during fetal life can also have consequences such as altered cardiovascular function that persist into adult life.

Immunology of Pregnancy

The conceptus inherits transplantation antigens from its father that could potentially lead to activation of tissue rejection responses against the conceptus by the mother. However, fetal gene expression in the placenta is altered to minimize the antigenicity of the tissue that is in direct contact with maternal tissues. Tissue graft rejection responses are typically mounted by the host against two classes of proteins called major histocompatibility complex (MHC) antigens. The expression of MHC antigens on the trophoblast is limited. This absence of antigenicity on the placental surface is likely a major cause of survival of the conceptus as a foreign tissue. In sheep, both class I and class II MHC are absent from the surface of the placenta. The same is true in cattle except that there is some expression of MHC class I antigen on the interplacentomal parts of the chorionic epithelium, especially during late pregnancy. Most of these trophoblast MHC class I molecules represent 'nonclassical' MHC proteins that have limited genetic polymorphism and which may regulate populations of lymphocytes called natural killer cells that ordinarily kill cells not expressing MHC class I molecules.

The conceptus and uterus secrete molecules that regulate the function of maternal lymphocytes. Early in pregnancy, interferon-τ secreted by the trophoblast of the cow and sheep causes upregulation of a host of immune response genes in the endometrium. Later in pregnancy, molecules that inhibit immune responses predominate. Progesterone is one of the major systemic regulatory molecules produced during pregnancy and it regulates uterine immune function. In fact, progesterone can promote survival of skin grafts and xenografts in the sheep uterus. Progesterone can directly inhibit lymphocyte function at micromolar concentrations. However, it is likely that concentrations of progesterone at the fetal–maternal interface do not reach these high concentrations until after placental progesterone synthesis becomes sufficient to maintain pregnancy (i.e., day 50 of gestation in sheep and day 200 in cattle). Rather, it is believed that progesterone regulates uterine immune function primarily by inducing endometrial secretion of molecules that block lymphocyte proliferation. In the ewe, this activity has been ascribed to a progesterone-induced protein of the serpin superfamily of serine protease inhibitors called ovine uterine serpin (also called SERPINA14).

Despite the prevalence of immunosuppressive molecules in the uterine environment, some leukocyte populations accumulate in the endometrium during pregnancy and may become activated to play roles in immune regulation, placental growth, parturition, or host defense against uterine pathogens. One such population of cells is the macrophage. Another, at least in the sheep, is a type of lymphocyte called the $\gamma\delta$-T lymphocyte. These granulated cells, which increase in both number and granularity after about day 50 of pregnancy, have been identified as $\gamma\delta$-T lymphocytes. The cells express perforin and may function as lytic cells to facilitate parturition or to lyse virally infected cells. They may also secrete cytokines that promote placental growth. There is some limited evidence that expulsion of the placenta is facilitated if the mother and conceptus have dissimilar MHC class I haplotypes. Perhaps, macrophages, $\gamma\delta$-T cells, or other leukocyte populations are involved in the placental expulsion process.

See also: **Reproduction, Events and Management**: Pregnancy: Characteristics.

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Pregnancy: Parturition

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Introduction

Parturition in animals, after a normal gestation, is a unique physiological process that signifies the termination of pregnancy and the beginning of extrauterine life of the newborn (neonate). Once parturition is initiated, it must proceed to completion and, as such, is difficult to interrupt or delay. It is a timed event that requires the fetus to have attained a stage of maturity that will enable it to survive extrauterine life. Parturition involves the orchestration of a complex series of physiological events that require the rotation of the fetus into the birth position, which is accompanied by a cascade of endocrine changes that terminates with a successful delivery. When the fetus is in the birth position, the temporal alignment of these endocrine changes is paramount in determining the timing of delivery. Although many of the hormonal cues as well as physiological and anatomical changes that precede delivery are similar among domestic animals, each species seems to have developed its own temporal relationship among events that govern parturition. A great deal of research has been undertaken over the past 25 years, particularly on the endocrine parameters of the fetal hypothalamic–pituitary–adrenal (HPA) axis and endocrine contribution of the fetal membranes, to understand better the mechanisms associated with parturition in humans and domestic animals alike. With the exception of the mouse, the sheep has been the most popular animal model of study and data from this model have contributed significantly to this field of reproductive biology. Parturition in domestic animals is initiated by the fetus but can be postponed temporarily, as in the case of the mare, when disturbed. Feeding can also alter the exact timing of delivery in sheep and cattle. It is now very apparent that both the mother and the fetus contribute significantly to the events associated with parturition and the importance of their individual contributions will be addressed in this article.

Duration of Pregnancy

In the western hemisphere the most common dairy breeds of cattle are of European origin. However, other domestic animals, including the goat, sheep and, to a lesser degree, the horse, are used for the harvesting of milk for production of dairy products. Observations of these domestic breeds have shown that duration of gestation is genetically determined (*see* **Reproduction, Events and Management**: Pregnancy: Characteristics). However, factors that can modify length of gestation include age and parity of the dam, sex and size, normality and abnormality, and genotype of the fetus, twinning (in cow and mare) and an array of environmental factors (**Figure 1**).

The average duration of pregnancy in dairy breeds of cattle is approximately 280 days but there are considerable differences between breeds (**Table 1**). The yak, although not a common species, is an important related species of cattle found in the harsher climates of Asia where it is used as a source of meat and milk. In this species, duration of pregnancy is much shorter (258 days) and this is reflected in the lower birth weight of calves (bull and heifer calves, 14.2 and 12.9 kg, respectively). The camel, an important beast of burden and source of meat and milk in desert environments, displays species variation in duration of gestation. The African dromedary has a gestation length of 385 days (~13 months) while the Bactrian camel has a gestation length ranging from 370 to 440 days. There is also a breed-dependent difference in the duration of pregnancy in the horse (pony, light and draft breeds, 330–345 days), sheep (domestic breeds, 144–152 days) and goat (domestic breeds, 145–151 days). Genotype is apparent when cows are crossed with certain breeds of sires.

Crossbreeding may result in an increase in length of gestation. Embryo transfer studies have clearly established that the breed of the embryo determines length of gestation (i.e. embryos from breeds with shorter gestation transferred to recipients with longer gestation and vice versa). Gender has also been cited as a factor, with bull calves having a slightly longer gestation (1–2 days) than heifers of the same breed. Size and/or weight of calf may contribute to a longer gestation (**Table 1**), a factor that may be influenced by season and/or level of nutrition of the dam. Twinning is thought to occur in 1–2% of cattle and is more frequently observed in multiparous or older cows. In sheep, the genotype of the fetus accounts for approximately two-thirds of the variation in length of gestation; male lambs and singletons are carried longer and gestation lengths increase with age of the dam.

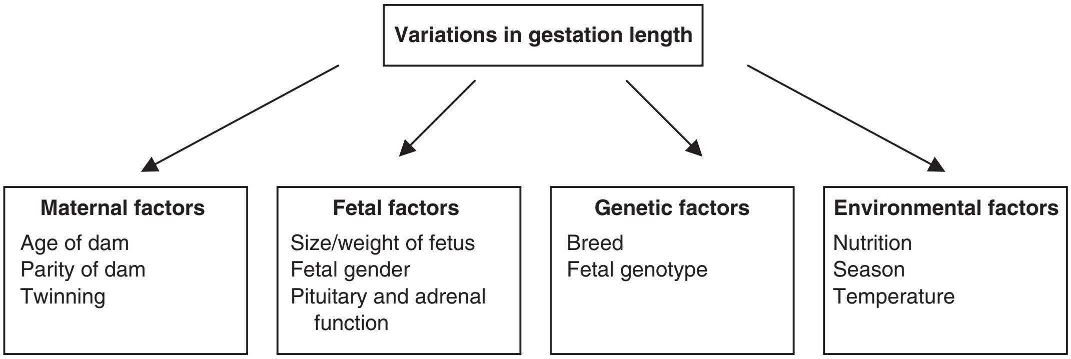

Figure 1 Schematic representation of factors that may influence length of gestation in the cow, goat, sheep and horse. (Reproduced with permission from Jainudeen and Hafez (2000).)

Table 1 Gestation length and birth weights of different breeds of cattle

Breed	*Average length of gestation in days (range)*	*Average birth weight (kg)*
Aberdeen Angus	280 (273–283)	28.0
Ayrshire[a]	279 (277–284)	34.0
Brown Swiss[a]	286 (285–287)	43.5
Charolais	287 (285–288)	43.5
Friesian–Holstein[a]	279 (272–284)	41.0
Guernsey[a]	284 (281–286)	30.0
Hereford	286 (280–289)	32.0
Jersey[a]	280 (277–284)	24.5
Milking Shorthorn[a]	283	?
Simmental	288 (285–291)	43.0
South Devon	287 (286–287)	44.5
Yak	258 (226–283)	13.0

[a] Common dairy breeds.
Compiled from Noakes (1997)

Presentation of Fetus in Birth Position

As parturition approaches, the fetus must rotate into the birth position. For most of gestation, the fetus usually rests on its back with its feet directed upward. After rotation into the birth position, the fetus rests on its abdomen or thorax with its nose resting between the forefeet as they extend toward the uterine aspect of the cervix (**Figure 2**). Correct positioning of the fetus allows for parturition to proceed more easily while malpresentation of the fetus predisposes to a complicated delivery that may, in severe cases, lead to the loss of both fetus and dam. This is true for the cow, sheep, horse and goat. Abnormal presentation of the fetus occurs in 5% of dairy cows and is observed more frequently with twin pregnancies. Such abnormal presentations may range from one leg to both legs and/or head reflected back to severe breech positions, including tail presentation toward the uterine aspect of the cervix (**Figure 3**).

Fetal Hypothalamic–Pituitary–Adrenal Axis

The initiation of parturition is associated with a spontaneous increase in activity of the fetal HPA, and is completed by a complex interaction of endocrine, neural and mechanical events. The discovery in the 1960s that hypophysectomy of the fetal sheep abolished initiation of parturition in the ewe demonstrated that the fetus played a fundamental role in the process of parturition. Since it is essential that the fetus is born at a time when it is capable of surviving extrauterine life, it is fitting that it plays a role in determining the time of parturition. It is now well established that the fetus initiates parturition and that together the fetal and maternal endocrine environments orchestrate the process of delivery. What drives the activation and maturation of the peripartuerient fetal HPA axis is unclear. Several lines of evidence have suggested that the increase in activity is a programmed event, and that the rapid growth of

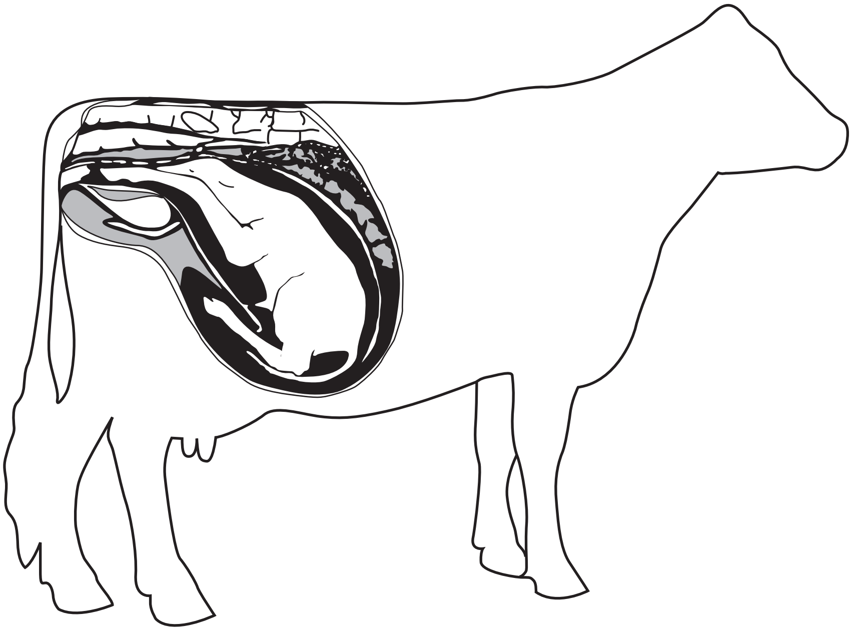

Figure 2 Normal birth position in cows assumed before labor. After rotation into the birth position, the fetus rests on its abdomen or thorax with its nose resting between its forefeet as they extend forward toward the uterine aspect of the cervix. The horse, sheep and goat adopt a similar birth position. (Adapted from Salisbury GW, Van Demark NL and Lodge JR (1978) *Physiology of Reproduction and Artificial Insemination in Cattle*, 2nd edn, San Francisco: WH Freeman.)

the fetus in the final weeks of gestation causes the release of endocrine signals from the placenta, leading to the maturation of key organ systems in the fetus, including the HPA axis, and terminating with delivery (**Figure 4**).

Research data suggest that the increase in fetal HPA axis activity at the end of gestation is associated with the increased secretion of estrogen by the placenta. Chronic increases in estradiol and androstenedione, another placental hormone, can advance the day of parturition in the sheep and other species. Others have promoted the hypothesis that release of prostaglandin E_2 (PGE_2) by the placenta facilitates the maturation of the HPA axis, resulting in increased secretion of adrenal corticotrophic hormone (ACTH) and cortisol. The rapid growth of the fetus toward the end of gestation is thought to be a genetically programmed event stimulating both fetal and maternal sources of PGE_2 that facilitate the maturation of the fetus and preparation of the mother for delivery. In the latter stages of gestation, the pituitary source of ACTH is from corticotrophic-releasing hormone (CRH)-sensitive cells that act on the adrenals via type II receptors to increase fetal cortisol production. Placental PGE_2 overrides the negative-feedback effects of cortisol on CRH release, thus enhancing fetal adrenal cortisol production. Cortisol enhances prostaglandin endoperoxide H synthase-2 (PGHS-2) expression, leading to an increase in PGE_2 production that in turn promotes C17α hydroxylase and cytochrome P-450$_{c17}$ enzyme activity in the placenta. This elevated enzyme activity increases the amount of C19 steroids for conversion to estrogen, resulting in increased contraction-associated protein (CAP) gene expression (oxytocin and prostaglandin receptors, Na^+ and Ca^{2+} ion channels, gap junction protein connexin-43). The functional expression of these CAP genes leads to increased spontaneous activity of the myometrium and enhanced responsiveness to oxytocin and prostaglandin. In addition to these changes, the coincidental increase in the estrogen-to-progesterone ratio promotes production of both oxytocin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The increase in estrogen synthesis toward the end of pregnancy is a common phenomenon among mammalian species but strategies associated with its production may vary.

In the bovine, parturition is initiated by endocrine changes originating from the fetus when it reaches a critical size and stage of maturation. The fetal adrenals become increasingly responsive to ACTH secreted by the fetal pituitary. In turn, there is a corresponding increase in the rate of cortisol secretion from the fetal adrenals that induces placental trophoblast 17α-hydroxylase and 17,20-lyase activity. This activity increases the metabolism of progesterone to estrogen, thereby increasing the maternal estrogen-to-progesterone ratio. During pregnancy, progesterone maintains the quiescent state of the uterus that facilitates the growth and development of the fetus. In the cow, the onset of

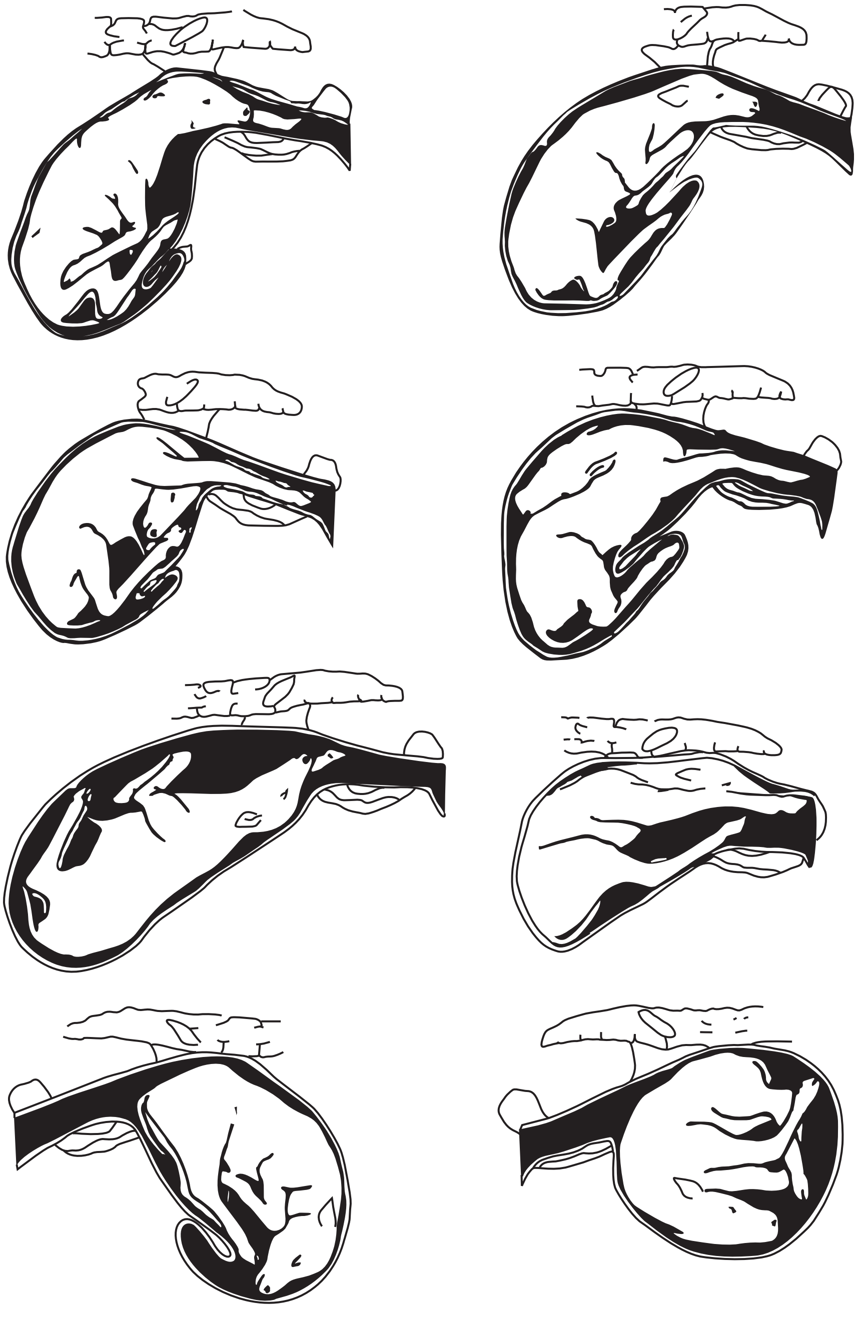

Figure 3 Diagrammatic representation of abnormal birth positions that may be observed in cattle. (Adapted from US Department of Agriculture (1942) *Diseases of Cattle*.)

labor is associated with a switch from a state of progesterone domination to one of enhanced estrogen influence. The decline in circulating progesterone, often referred to as progesterone 'withdrawal', together with elevated circulating concentrations of estrogen, stimulates increased prostaglandin production, formation of myometrial gap junctions, sensitization of the uterus to uterotonic agents (prostaglandins, oxytocin), cervical ripening and the commencement of labor. Cortisol is thought to increase prostaglandin synthesis during labor primarily due to an increase in PGHS-2 (inducible cyclooxygenase, COX-2 gene product) expression and function in uterine and intrauterine tissue. The activity of prostaglandin endoperoxidase synthase in the placenta (cotyledons) increases and mirrors the changes in ACTH concentrations seen in fetal plasma at this time. The prostaglandin cascade is also activated by proinflammatory cytokines (i.e. interleukin-1β) and these effects are modulated by antiinflammatory cytokines (i.e. interleukin-10). Oxytocin, a potent

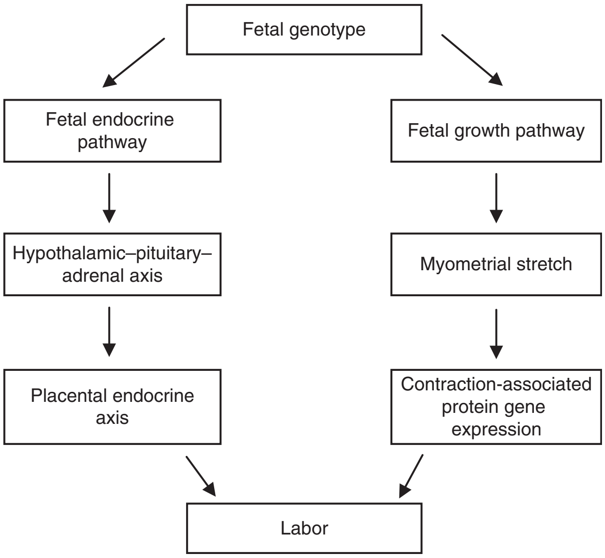

Figure 4 Schematic representation of factors associated with the onset of events involved in labor in domestic animals.

uterotonic stimulant, is expressed in intrauterine tissue in late pregnancy and stimulates myometrial contractility and fetal expulsion.

Maternal Endocrine Changes and Myometrial Activity

Progesterone is the primary hormone responsible for the maintenance of pregnancy. In the pig, goat and cow the presence of functional corpora lutea is essential for the production of progesterone and pregnancy maintenance, while in the ewe and the horse the placenta is the primary source after the luteoplacental shift has occurred. This shift in source of progesterone is completed by approximately day 55 and day 150 of pregnancy in the ewe and mare, respectively. Removal of the ovaries before this date will terminate the pregnancy.

Parturition cannot proceed without uterine contraction. At term, there is a dramatic increase in uterine myometrial activity. This activity is facilitated by spontaneous changes in the secretion of estrogen and progesterone at the end of gestation. During pregnancy, progesterone maintains uterine quiescence by hyperpolarizing the myometrial smooth muscle cells. As the rate of production and concentrations of estrogens increases relative to progestagens, the myometrial cells begin to depolarize and increase in contractile activity. This activity is augmented by the release of prostaglandins. In most mammalian species, the increase in estrogen toward the end of gestation seems to be a common theme and the placenta seems to be the source of the estrogen responsible for the increased myometrial activity. However, the strategy in the production of estrogen differs among species. At the end of gestation, the sheep placenta, under the influence of increased fetal cortisol levels, acquires the ability to induce 17α -hydroxylase enzyme activity, which enables the synthesis of placental estrogen. Thus, the maturation of the fetal HPA axis is critical for the initiation of parturition in ruminants.

In the horse, the source of estrogen is the fetoplacental unit because the equine placenta alone does not possess an inducible 17α-hydroxylase enzyme system. In this instance, the steroid precursors, for example, dihydroepiandrosterone (DHEA), necessary for estrogen synthesis, are derived from the fetal horse gonads. Gonadectomy of the fetal horse results in the interruption of initiation of parturition in this species. Humans and other primates are similar to the horse in that estrogen synthesis is also dependent on a functional fetal–placental unit. However, the source of steroid precursors (i.e. DHEA) is the fetal adrenals since the placenta in these species lacks the cytochrome $P\text{-}450_{C17}$ enzyme system to produce estrogen from cholesterol.

Parturition depends on the coordinated rhythmic contraction of the uterine smooth muscle (myometrium) and on involuntary contraction of abdominal muscles. Throughout most of pregnancy the myometrium is nonexcitable and relatively unresponsive to uterotonic agents (prostaglandins, oxytocin). This relative quiescent state of the uterus is due to progesterone or the progesterone 'block'. Under the influence of progesterone, myometrial activity and coordination of contractions and responsiveness to uterotonic agents are diminished. However, the onset of labor is linked to a transformation in the pattern of contractile activity of the myometrium. The myometrium becomes highly responsive to prostaglandins (PGE_2 and $PGF_{2\alpha}$) and oxytocin once the progesterone block has been removed. This increased capacity of the myometrium to develop contractions is referred to as myometrial activation, and once activated it can respond to paracrine and endocrine stimulation from increasing concentrations of uterotonic agents. This concomitant activation and stimulation enable the myometrium to generate intense, high-amplitude and high-frequency contractions that are necessary to dilate the cervix and facilitate fetal expulsion. Also, fetal growth leading to increased tension on the uterine wall and stretching of the myometrium can augment CAP gene expression. It has been suggested that two parallel pathways are initiated by signals within uterine tissues that lead to the development of intense and synchronous myometrial contractions that facilitate dilation of the cervix and accommodate delivery of the fetus (**Figure 5**).

During pregnancy and in the face of elevated progesterone, fetal growth stimulates myometrial stretch and hypertrophy which in turn reduces myometrial tension. At term, the increase in the estrogen-to-progesterone ratio terminates the uterine growth response, while increased fetal growth enhances myometrial tension, CAP gene expression and initiation of labor. The rise in estrogen not only increases myometrial activity but also enhances the synthesis and release of prostaglandins and oxytocin during labor. Myometrial smooth muscle contraction is dependent on an increase in intracellular free calcium, which causes formation of a calcium–calmodulin complex. This complex binds to and activates myosin kinase to interact with actin to cause contraction of the smooth muscle fibers. Oxytocin and $PGF_{2\alpha}$ influence smooth muscle activity by regulating intracellular concentrations of Ca^{2+}. Prostaglandins increase free calcium by liberating Ca^{2+} from intracellular binding sites while oxytocin has a direct effect on the rate of Ca^{2+} influx and also lowers the threshold for initiation of action potential activity. Oxytocin is not effective on myometrial activity until uterine receptors are expressed. This is an estrogen-dependent event in sheep. Similar patterns of myometrial activity exist before parturition in the sheep, goat and cow. Recent information also suggests that the fetal membranes in the sheep and human contribute to the enzyme pathways that facilitate and promote endocrine (prostaglandin) synthesis at parturition.

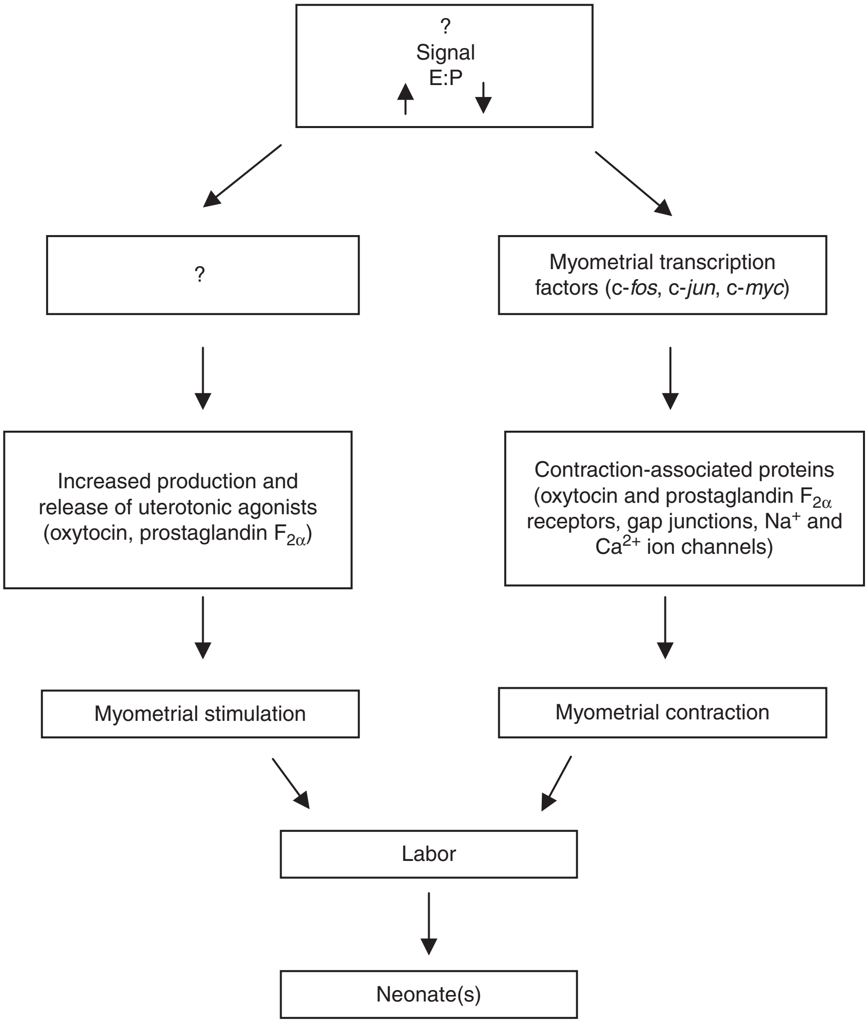
?
Signal
E:P
↑ ↓
?
Myometrial transcription factors (c-*fos*, c-*jun*, c-*myc*)
Increased production and release of uterotonic agonists (oxytocin, prostaglandin $F_{2\alpha}$)
Contraction-associated proteins (oxytocin and prostaglandin $F_{2\alpha}$ receptors, gap junctions, Na^+ and Ca^{2+} ion channels)
Myometrial stimulation
Myometrial contraction
Labor
Neonate(s)

Figure 5 Proposed scheme for the initiation of parturition. E:P, estrogen-to-progesterone ratio. At term, the uterine environment switches from a progesterone-dominated to an estrogen-dominated environment. The consequence of this shift is the activation of parallel pathways that involve a complex series of molecular, cellular and biochemical events within uterine tissues terminating with the expulsion of the fetus and fetal membranes. (Adapted from Lye SJ (1996) Initiation of parturition. *Animal Reproduction Science* 42: 495–503.)

Progesterone may regulate myometrial activity by downregulating the synthesis of oxytocin receptors, prostaglandin receptors and gap junction proteins, as well as inhibiting pituitary oxytocin release and suppressing prostaglandin production. Progesterone blocks high-amplitude myometrial cell contractions but low-amplitude, high-frequency contractions persist during pregnancy. However, there are other endocrine factors that regulate myometrial activity. For example, relaxin, a small polypeptide hormone, is capable of inhibiting spontaneous myometrial contractions. Although a bovine relaxin gene and protein have not been identified, there is immunohistochemical evidence for the presence of the protein hormone in the corpora lutea of pregnant cows but little or no activity was found in the placenta. Heterologous radioimmunoassay studies have detected relaxin in plasma of pregnant cows between days 5 and 1 prepartum and this therefore suggests a role for relaxin in preparation of the birth canal for delivery, as in other domestic species. A relaxin-like factor (RLF) gene and protein is expressed by the bovine corpus luteum of pregnancy; however, its role in the corpus luteum is unclear. Moreover, the identification of a third relaxin gene in the human (H3) and the mouse (M3) may prove to be the active relaxin gene in the bovine, although such a gene has yet to be identified in this species. Whether similar expression of RLF and/or a third relaxin gene occurs in the bovine placenta has yet to be determined, and implicating a role during delivery for RLF protein would be speculative. A recent but all-important discovery in the relaxin field is the activation of orphan receptors (heterotrimeric guanine nucleotide-binding protein (G-protein)-coupled receptors) by relaxin via an adenosine 3′,5′-monophosphate-dependent pathway. This discovery will undoubtedly lead to new studies that will enhance our understanding of the role relaxin plays in parturition. Other inhibitory uterotonins, including prostacyclin and nitric oxide, have an inhibitory effect similar to relaxin. Progesterone, in association with relaxin, nitric oxide and prostacyclin, suppresses spontaneous and stimulated myometrial activity, thus preventing the spontaneous expulsion of the fetus.

In summary, parturition is thought to be a linear biochemical and physiological process beginning with fetal maturation, leading to an increase in fetal cortisol, which initiates synthesis and release of uterotonic agents that intensify uterine myometrial contractile activity, leading to delivery. The endocrine events associated with the periparturient period are complex, and summarized in **Figures 5** and **6**. Enhanced myometrial activity and delivery at term result from the dual activity of increased uterine activation as characterized by increased connexin-43 (gap junction proteins), oxytocin and prostaglandin receptor expression, along with increased concentrations of oxytocin and prostaglandins.

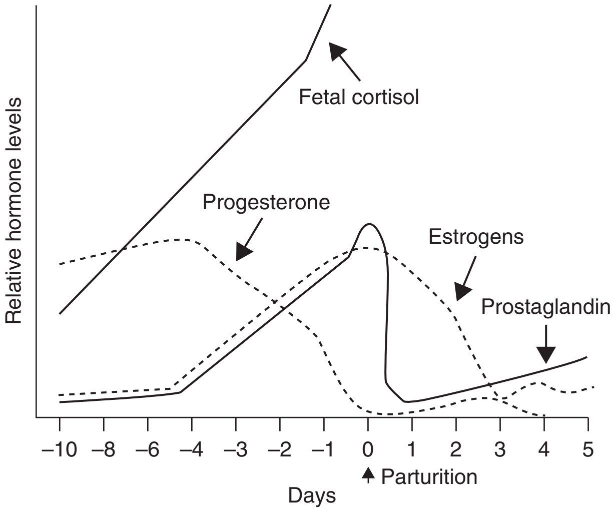

Figure 6 Relative hormone profiles in the cow during the periparturient period. (Adapted from Senger PL (1997) *Pathways to Pregnancy and Parturition*, p. 243. Pullman: Current Conceptions.)

Cervical Ripening and Expulsion of the Fetus

During pregnancy in mammals, the cervix is a relatively firm and inextensible structure that protects the fetus from the external environment and prevents spontaneous delivery. This firmness is due to the highly organized arrangement of collagen fibers in dense parallel bands. As term approaches, the cervix begins to soften and the collagen fibers become dispersed and randomly oriented within an increased proteoglycan extracelluar matrix, accompanied by an increase in the uptake of water. Remodelling of cervical tissue is necessary in order to accommodate normal expulsion of the fetus and fetal membranes at term. This softening or remodeling and increased distensibility of the cervix are regulated hormonally. Several hormones, including estrogen, relaxin, prostaglandin and oxytocin, have been implicated in the stimulation of cervical distensibility. Unlike in the mare and ewe, the ovaries of the cow are essential for the latter stages of pregnancy maintenance and thus may be the primary source of hormones for initiating cervical ripening. Relaxin is an important hormone of pregnancy in many species and is known to facilitate tissue remodelling of the reproductive tract, including the cervix. The effects of relaxin on cervical ripening are particularly important in the mare and the sow since failure of relaxin to facilitate these events can result in increased incidence of dystocia and stillbirths, as is the case in the pig. Interestingly, the placenta is the source of relaxin during pregnancy in the mare, while the corpora lutea of pregnancy are the principal source in the sow. Although the evidence of a bovine relaxin is sketchy, there are sufficient physiological data to support the presence of such a hormone in this species. The biochemistry of cervical ripening is a complex process that involves the upregulation of proteolytic factors such as matrix metalloproteinases and the corresponding tissue inhibitors of matrix metalloproteinases. Also, the increased expression of gap junction proteins (connexin-26, -32 and -43) is thought to be important in this process. Connexins are gap junction proteins that belong to an evolutionarily conserved, multigene family of channel-forming proteins which are important for cell–cell communication and are hormonally regulated.

In cattle, the ratio of fetal birth weight to maternal weight is high (approximately 11% and may be higher when crossbreeding is practiced), and the pelvic cavity expands rapidly during the last 4 days prepartum to facilitate delivery of the calf. Little is known concerning secretion of relaxin in the cow; however, there is reason to suspect that relaxin may loosen the pelvic ligaments and facilitate softening of the cervix. Treatment of cows with highly purified porcine relaxin a week before their due date resulted in a rapid increase in pelvic height and width similar to the elevation of the tail head seen in prepartum cows. When porcine relaxin was placed in the cervical os of beef heifers 5–7 days before the anticipated day of delivery, a premature increase in the dilation of the cervix occurred within 8–12 h of relaxin treatment. In addition, this relaxin-induced cervical ripening was observed to reduce the incidence of dystocia in the treated heifers. In general, little is known about the hormonal regulation of cervical ripening or distensibility in cattle at parturition. Most of our knowledge of the changes that occur in the cervix before delivery is derived from studies performed in pigs and rodents.

Stages of Labor

Labor has been described as occurring in three stages; great variations in the duration of these stages exists among species (**Table 2**). Stage I involves the initiation of labor by the fetus and the resultant dilation of the cervix. Uterine contractions begin to occur in a more rhythmic fashion and continue until the cervix is fully dilated and continuous with the vagina. This period is accompanied by increased restlessness of the dam accompanied by elevated body temperature, pulse and respiratory rates. This is more evident in the horse where the mare may break out in a sweat. Changes in the fetal position and posture will also occur at this stage.

Stage II is associated with fetal expulsion. During this stage of labor the dam may assume recumbency and commence straining, which results in the rupture of the allantochorion and release of chorionic fluid from the vulva. Moments later, the amnion appears as a whitish fluid-filled bag at the vulva. This is followed by the appearance of the forelimbs and nose of the fetus and is accompanied by strong uterine and abdominal contractions. The expulsion and delivery of the fetus follow rupture of the amnion. This stage of labour is the shortest and is associated with the commencement of strong uterine and abdominal contractions and completion of cervical dilation. The cervix remains in a dilated state until the termination of the delivery of the fetus. In sheep and goats, twin births are usually more rapid than single births, but the interval between the delivery of twins may vary from minutes to a couple of hours.

Stage III is the final stage of labor and is associated with the expulsion of the fetal membranes. During this stage of labor the uterine contractions begin to decrease in frequency and amplitude. For successful expulsion of the placenta to occur, loosening of the chorionic villi from the maternal endometrial crypts must take place. The spasmodic contraction of the uterine wall, which abates following the inversion and expulsion of the fetal membranes, facilitates this process. However, the interval between birth of the fetus and expulsion of the fetal membranes varies among species. In the horse, the placenta is shed within 30–60 min after delivery compared to the 6–12 h it typically takes in the cow (**Table 2**).

Table 2 Average duration of the three stages of labor in different domestic species

	Stages of labor (h)		
Animal	*I: Dilation of cervix*	*II: Expulsion of fetus(es)*	*III: Expulsion of fetal membranes*
Cow, buffalo	2–6	0.5–1.0	6–12
Ewe	2–6	0.5–2	0.5–8
Goat	?	?	?
Mare	1–4	0.2–0.5	1
Sow	2–12	2.5–3	1–4

Compiled from Jainudeen and Hafez (2000).

Complications (Dystocia and Retained Placentas)

Complications associated with parturition can impact negatively on postpartum cows in terms of their reproductive efficiency and milk productivity (*see* **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity; Pregnancy: Periparturient disorders). Dystocia and retained placenta are the most common problems associated with parturition in dairy cattle and are probably linked to a number of interrelated variables. Dystocia is defined as prolonged or difficult delivery and can be due to either fetal or maternal abnormalities. Fetal dystocia refers to an abnormality of the fetus that may lead to incorrect birth positioning while maternal dystocia refers to abnormality of or physical problem of the mother. Such abnormalities of the mother may include poor pelvic confirmation, incomplete ripening of the cervical canal and immaturity. The most common causes of dystocia in cattle include the incompatibility of size of the fetus and the mother's pelvis and the malpresentation, position or posture of the fetus *in utero*. In beef cattle, size and birth weight of calves have been determined to be the most common factor contributing to dystocia. Very large calves also tend to have higher perinatal mortality. Other factors that contribute to dystocia are uterine inertia, uterine torsion and failure of the cervix to dilate.

In primiparous or first-calf heifers, stricture of the vulva can also be problematic and may lead to dystocia and stillbirth. In addition, the economic need to place heifers into the milking parlor at an earlier age has led to the practice of breeding heifers at under 15 months of age, increasing the risk of dystocia in first-calf heifers. In the yak, the incidence of dystocia is low (3.3%) and twinning is rare (0.5%). In high-yielding dairy cows, reduced blood calcium concentrations at time of parturition can be problematic and increase the risk of dystocia in these animals. This condition is associated with milk fever and the incidence is on the rise as dairy producers genetically select for high-yielding cows. Milk fever is a metabolic disorder of calcium homeostasis and decreased blood calcium reduces the availability of Ca^{2+} for uterine smooth muscle contraction and, in so doing, increases the risk of a difficult delivery and/or retained placenta (*see* **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever). Feeding anionic salts to near-term cows, which mobilizes bone reservoirs of calcium, thereby elevating blood Ca^{2+} concentrations and availability for uterine muscle tissue, can alleviate this condition. Treatment with opiate antagonists (e.g. naloxone) in low doses has recently been shown to be a safe method to offset the adverse effects of milk fever in cows and thus reduce muscular complications at delivery (dystocia and retained placenta).

Table 3 Recommended weight and age of dairy heifers at time of mating, by breed

Breed	*Minimum weight range (kg)*	*Minimum age (months)*
Ayrshire	308–318	14–15
Brown Swiss	374–397	14–15
Friesian–Holstein	374–397	14–15
Guernsey	308–318	14–15
Jersey	263–272	14–15
Milking Shorthorn	308–354	14–15

Compiled from Gillespie JR (1998) *Animal Science*, p. 948. New York: Delmar.

Dystocia in horses is also a serious problem and if proper assistance is not given can put both the mare and foal at risk. The incidence of dystocia in horses is breed-dependent, with a rate of 4% in light breeds (thoroughbred, standardbred, walking horse, saddle horse) but increases to 8% in pony breeds (Shetlands, Connemara), with an incidence of 10% in the heavy draft breeds (Shire, Belgian). The causes of dystocia in horses are many but the most common appear to be reflected head and neck and/or forelimbs when the foal is in anterior presentation. Other contributing factors include deformed limbs, oversized fetus and hydrocephalus, being more frequently observed in pony breeds.

Dystocia may also affect the future reproductive performance of the cow and is often associated with a delayed postpartum estrus, extended calving interval and an overall reduction in fertility (*see* **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity). Implementation of two simple rules of thumb may help reduce the incidence of dystocia and resulting stillbirths in a herd. The first is to avoid breeding young heifers when below their optimum breeding weight (**Table 3**) and the second is to take advantage of the selection of easy-calving bulls that have been identified by breed associations to inseminate heifers. Dairy heifers should attain at least 60% of the mature adult body weight for the breed before being mated. European and Israeli dairy producers have taken advantage of genetic information provided by their dairy breed associations on control of dystocia due to feto-pelvic incompatibility, resulting in approximately a 60% and 40% reduction in the incidence of dystocia and stillbirths, respectively. The bottom line is to select carefully ease-of-calving sires, giving due consideration to maternal traits to avoid producing progeny that may be small of stature as adults and thus confound the dystocia problem. It is important to remember the genetic (breed) and environmental (size, pelvic dimensions, uterine environment) factors that the dam contributes to a pregnancy and the impact they have at delivery. Studies have shown that fetal genotype is the primary factor controlling birth weight in crossbred breeding, but that the maternal uterine environment (size, number of placentomes) could promote or markedly limit the full genetic growth potential of the calf *in utero.*

Implications of hormonal imbalances have been correlated with dystocia. Scientists have looked at blood concentrations of prolactin, progesterone and the various estrogens (estrone, estradiol-17α and -17β) for an endocrine association with the incidence of dystocia in dairy cows. Estrogen concentrations were markedly depressed in animals experiencing dystocia but in most cases there seemed to be an accompanying anatomical incompatibility. The possibility of other endocrine factors known to be important in parturition, including adrenal corticosteroids, uterine prostaglandins, oxytocin and relaxin release, cannot be overlooked.

Placental dystocia refers to the retention or difficult delivery of the placenta and is more commonly referred to as retained placenta or retention of fetal membranes. It is the failure of the chorionic villi (cotyledons) to separate from the caruncles of the uterine endometrial wall and be expelled within a specific timeframe (**Table 2**). A placenta is usually classified as retained if the cow has not shed the placenta within 24 h of delivery. Retained placentas increase the risk of uterine infections (metritis), predispose the cow to toxemia, diminish milk production and markedly reduce the reproductive efficiency of the animal (*see* **Reproduction, Events and Management**: Estrous Cycles: Postpartum Cyclicity). The incidence of retained placentas in cattle is thought to be 5–15% of the deliveries in healthy herds. In herds where the incidence runs higher than 15%, there is usually an underlying cause contributing to the problem. For example, poor nutrition and lack of essential micronutrients such as vitamin A or selenium can cause a higher than normal incidence of retained placentas in a herd. Also, cases of retained placentas are more frequent after difficult deliveries, induction of parturition, premature deliveries and twins; first-calf heifers are at higher risk than multiparous cows. This may be true in cases where the calving age of heifers is less than 24 months. Retained placenta in the horse is regarded as the most common postpartum problem and may be associated with the failure to shed part or all of the allantochorionic membranes with or without the amniotic membrane. The time it takes to pass the placenta in this species is critical. Although varying times have been given, failure of a mare to pass the placenta within 2 h postpartum is regarded as a retained placenta. As in the cow, failure to shed the placenta is associated with failure of the microvilli (microcotyledons) to separate from the endometrial crypts. Failure to pass the placenta in a timely manner usually predisposes the animal to uterine infection and increases the potential for poor postpartum reproductive efficiency. This is especially problematic in horses and dairy cows.

See also: **Diseases of Dairy Animals**: Non-Infectious Diseases: Milk Fever. **Replacement Management in Cattle**: Breeding Standards and Pregnancy Management; Health Management. **Reproduction, Events and Management**: Pregnancy: Characteristics; Pregnancy: Periparturient disorders; Pregnancy: Physiology; Estrous Cycles: Postpartum Cyclicity.

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Pregnancy: Periparturient Disorders

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Introduction

The majority of diseases that affect dairy cows occur during the peripartum time, which is defined as parturition, initiation of lactation and the early postpartum period. Parturition and the onset of lactation impose tremendous physiological challenges that may predispose the dairy cow to physiological and infectious disorders. These disorders include parturient paresis (milk fever), calving difficulty (dystocia), retained fetal membranes (fetal membranes retained beyond 24h postpartum), metritis (infection and infammation of the uterus), fatty liver (pathological 2310 PREGNANCY/ Periparturient Disorders fat accumulation of the liver), ketosis (excessive ketone body production) and displacement of abomasum (displacement of the abomasum to the left side of the abdominal cavity). These disorders result in signifcant economic losses to dairy producers by reducing reproductive performance and milk yield during the subsequent lactation, cost of treatments and culling.

Dynamics of the Dry Period

When dairy cows are 7 months pregnant milking should be discontinued to allow them a 2 month rest period (dry period). The dry period allows lacteal tissue involution and regeneration, which prepares cows for the next lactation. It is generally perceived that during the dry period, the pregnant nonlactating dairy cow is in a quiescent metabolic state. This is an incorrect assumption since the late pregnant cow undergoes a series of complex metabolic changes as parturition approaches. These changes are related to growth of the calf, reduction of feed intake during the end of gestation and the initiation of lactation. Collectively, these events increase the energy and calcium demands during parturition and the initi-ation of lactation. If difficulties occur in making the necessary peripartal energy and calcium adjustments, metabolic disorders such as fatty liver, ketosis and parturient paresis may occur (**Figure 1**).

Fetal Growth

Fetal growth occurs exponentially, with over 60% of total fetal growth occurring during the last 2 months of gestation. Maximal growth occurs during the last 6-8 weeks of gestation. This fetal and placental growth results in a nutritional burden of pregnancy to the cow during the dry period. Therefore, inadequate nutrition at this time can result in loss of maternal body reserves in order to sustain fetal and placental development. These losses in body reserves or fat mobilization cause an increase in blood of certain type of fats, called plasma nonesterifed fatty acids (NEFA), which predispose the cow to hepatic fat accumulation or fatty liver.

Parturition

Parturition occurs as a result of a complex series of hormonal changes in the dam that are initiated by the fetus (**Figure 1**). When the size of the fetus reaches a certain relation to the mother's size, corticoidal hormones increase gradually from 3 weeks to 4 days prepartum. This increase in fetal corticoidal hormones during the last month of gestation is responsible for the conversion of placental progesterone and pregnenolone to estrogenic hormones. One week prepartum, in response to the increase in estrogen production, a fatty acid hormone, prostaglandin F2a is released from the uterus. This increase in prostaglandin results in lysis of the corpus luteum of pregnancy and is accompanied by a decline in luteal progesterone. Similar hormonal changes, are also common in other domesticated species and in women.

Lactation

Initiation of lactation occurs in concert with the hormones that are involved in the initiation of parturition, which are: an increase in estrogen, a reduction in progesterone and an increase in corticoids. Alteration in these hormones also induces changes in a series of other regulatory hormones (thyroid hormones, insulin, glucagon, growth hormone,

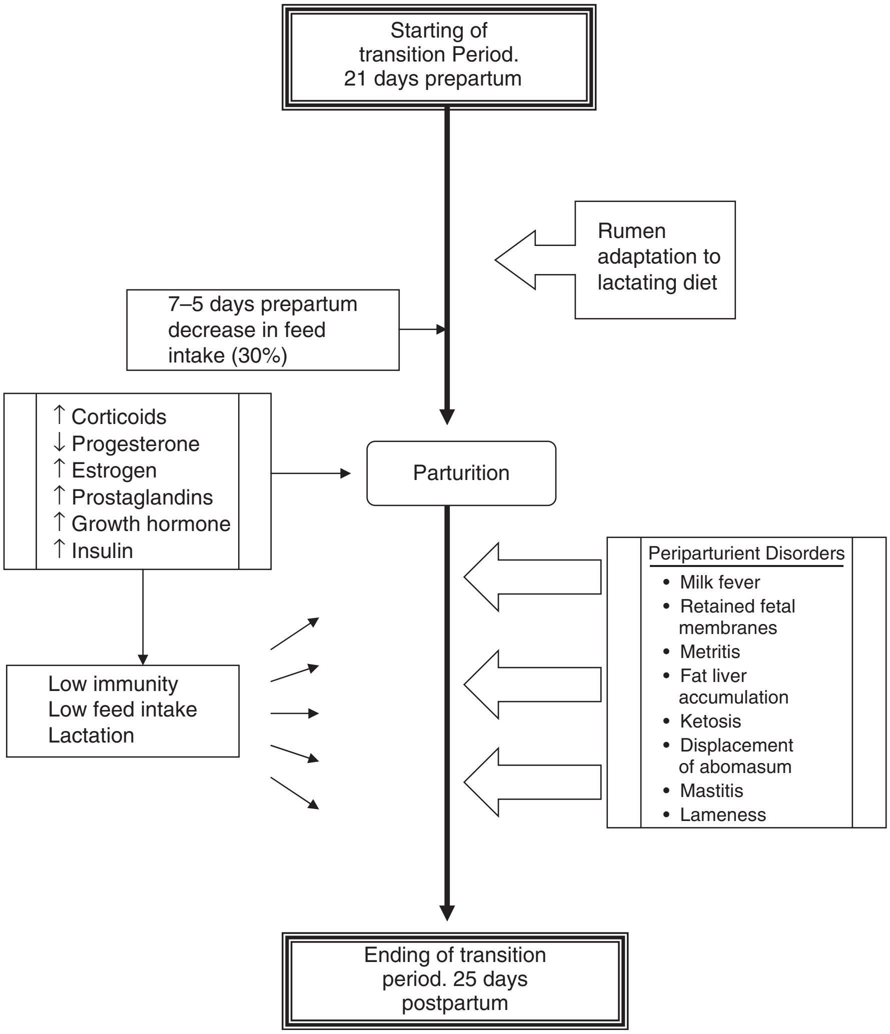
Starting of
transition Period.
21 days prepartum
Rumen
adaptation to
lactating diet
7–5 days prepartum
decrease in feed
intake (30%)
↑ Corticoids
↓ Progesterone
↑ Estrogen
↑ Prostaglandins
↑ Growth hormone
↑ Insulin
Parturition
Periparturient Disorders
• Milk fever
• Retained fetal membranes
• Metritis
• Fat liver accumulation
• Ketosis
• Displacement of abomasum
• Mastitis
• Lameness
Low immunity
Low feed intake
Lactation
Ending of transition
period. 25 days
postpartum

Figure 1 Dynamics of the transition period in dairy cows.

placental lactogen, prolactin) that promote the production of glucose and ketone bodies and the mobilization of fatty acids. These complex hormonal and metabolic alterations shift the priorities of the cow from tissue and fetal gain to mammary development and copious milk production.

Feed Intake

Feed intake decreases 5 to 7 days prior to parturition. This reduction can reach up to 30% under normal feeding management practices. Depression of feed intake before parturition has been considered a major factor in the development of the fatty liver complex. Accumulation of fat in the liver occurs during periods of elevated fat mobilization, especially in cows with excessive fat body reserves at parturition. During the last 2 weeks prepartum, NEFA increases twofold in blood. This increase in plasma NEFA concentration prepartum has been attributed to excessive fat mobilization that provides energy for the growing fetus/maternal tissues, during the period of feed intake depression and the endocrine events associated with parturition.

Energy Status, Body Condition Score and Reproductive Performance

Dairy cows reach peak milk production around 8 to 10 weeks post calving. However, feed intake increases slowly after parturition lagging 2 weeks behind the peak of milk production. This lag explains the adipose reserve mobilization experienced by cows, with a typical weight loss during the first one-third of lactation. Because energy output (milk yield) exceeds energy intake via feed consumption, dairy cows undergo a period of negative energy balance in early lactation (**Figure 2**). Any periparturient disorder that predisposes the cow to reduce feed consumption will exacerbate

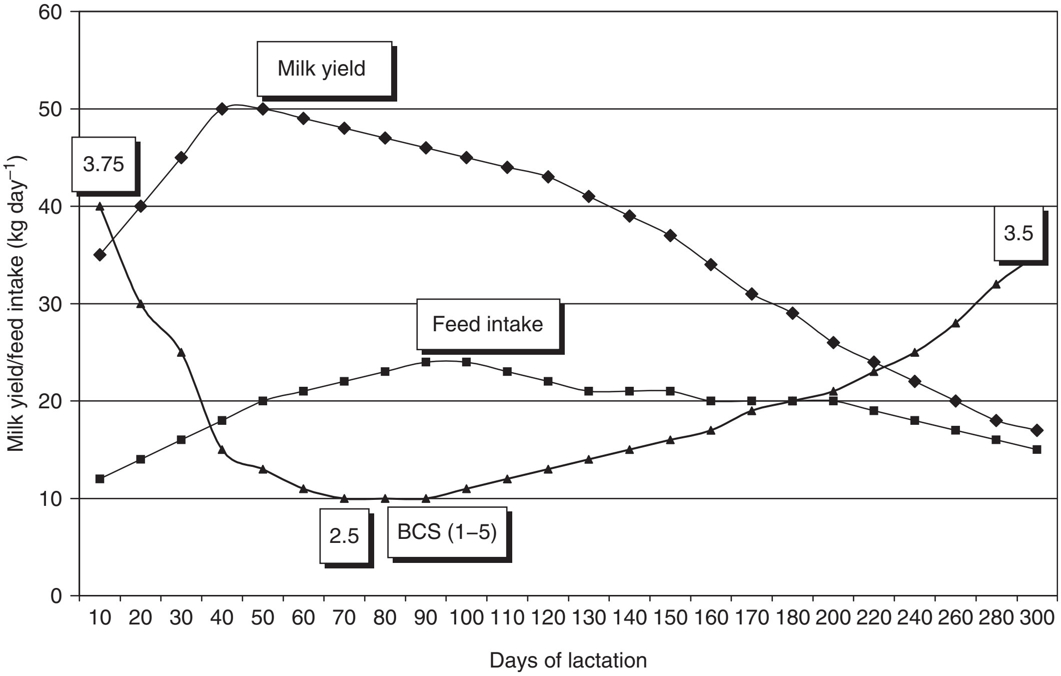

Figure 2 Milk production, feed intake and body condition score (BCS) in a typical dairy lactating cow.

the negative energy status already present during the postpartum period.

The energy status of the postpartum cow has an effect on integrated reproductive activity during early lactation. Cows losing weight may experience a delay in the resumption of cyclicity. A return toward a positive energy status appears to be important in the initiation of cyclicity. The marked deficit in early energy status for the anestrus cow exerts a negative carryover effect on conception.

Body reserves can be measured through a visual evaluation of different anatomical points of the cow. This methodology called body condition scoring (BCS) has been validated through different studies using scales from 1 to 5 or 1 to 9, being 1 a very thin cow and 5 or 9 a very fat cow. Body condition score has been related with the magnitude and severity of negative energy balance. Cows that lose more than 0.5 to 1.0 BCS (scale 1-5) during the frst 60 days postpartum have been shown to have lower conception rates to first insemination. A score of 3.25 to 3.75 at calving is recommended with no more than a 0.5 point loss of body condition during the frst 60 days postpartum (**Table 1**).

Table 1 Target body condition scores (BCS scale 1-5)

Stage	*Ideal BCS*	*Range*
Dry-off (around 7 month of pregnancy	3.5	3.25–3.75
Calving	3.5	3.25–3.75
Early lactation	3	2.5–3.25
Mid-lactation	3.25	2.75–3.25
Late lactation	3.5	3.0–3.5
Growing Heifers	3	2.75–3.25
Heifers at calving	3.5	3.25–3.5

Cows that are overconditioned at calving (>3.75) experience less feed intake, more fat mobilization and are at high risk of experiencing calving-related disorders. Consequently, BCS should be monitored specially during the last one-third of lactation and the dry period. Losses of more than 0.25 units should be avoided during the entire dry period. Changing body condition through dietary manipulations requires some strategic planning and careful consideration. Underconditioned cows (thin animals) should be allowed to recover condition during the late lactation period because during this time lactating cows are more efficient in restoring body condition than during the dry period. In addition, the dry period may be too short to fully recover condition needed prior to calving. Cows should not lose weight during the dry period, as the cow must gain 0.45 to 0.68 kg day-1 simply to meet the needs of the rapidly developing fetus.

Periparturient Disorders Milk Fever (Parturient Paresis)

During calving or shortly thereafter, the fall of calcium levels in blood (hypocalcemia) is inevitable in the dairy cow and is characterized by a blood calcium concentration

<1.88 mmol l-1(<7.5 mg dl-1). Hypocalcemia develops as a result of the sudden drain of calcium to colostrum at the onset of lactation, resulting in a tremendous challenge to cows' ability to maintain normal calcium levels. At parturition, 30 g or more of calcium must be replenished into the calcium pool each day to maintain the normal calcium levels. Clinical hypocalcemia or milk fever develops when blood calcium concentration is below 7.5 mg dl-1. A cow affected with milk fever may present with nervousness, staggering but usually becomes recumbent and is unable to rise (paresis). If blood calcium concentration is not restored quickly death occurs. Ten to fifty percent of cows may develop low calcium levels without symptoms of milk fever (subclinical hypocalcemia) up to 10 days postpartum. Hypocalcemia may affect organs that have smooth muscle function such as the uterus, rumen and the abomasum. Hypocalcemia is a significant risk factor for calving difficulty, retained fetal membranes, metritis, uterine eversion or prolapse, displacement of abomasum and clinical ketosis. These disorders have been associated with no estrus or heat expression (anestrus), cyst in the ovaries and metritis, which negatively affect the reproductive performance of dairy cows.

Retained Fetal Membranes (Retained Placenta)

Retained fetal membranes (RFM) is defined as the lack of detachment of fetal membranes (cotyledons) from the maternal membranes (caruncles) within the first 12-24 h after calving. The incidence of retained fetal membranes may range from 1.3% to 39.2%. The cost of a case of RFM ranges between US$106 and $285. Some risk factors for RFM are hypocalcemia, calving difficulty, parity, abnormal gestation length, season and sire of the calf. Retained fetal membranes have been the major factor predisposing cattle to metritis. About 20-25% of cows affected by RFM may develop moderate to severe metritis.

Metritis (Puerperal Metritis, Toxic Metritis)

Metritis (infection of the uterus) is one of the most frequent disorders affecting dairy cows during the postpartum period. Metritis is a major cause of economic losses to the cattle industry. The condition is characterized by an abnormal uterine discharge, with local or systemic symptomatology. Systemic or toxic metritis is characterized by a foul-smelling, watery uterine discharge usually accompanied by a severe drop in milk production and a fever, and it may be life threatening. The total cost to producers for each lactating dairy cow with a uterine infection has been estimated in US$106. Some predisposing factors for metritis are calving difficulty, retained fetal membranes, twins or stillbirths, fat cows at calving and metabolic disorders.

Ketosis and Liver Fat Accumulation (Fatty Liver)

Ketosis is defined as a metabolic disorder characterized by high levels of ketone bodies (-OH-butyrate, acetoacetate and acetone) affecting dairy cows in the period from parturition to 6 weeks postpartum. There are two types of ketosis, primary or secondary. A cow with primary clinical ketosis has a decreased appetite and elevated serum, milk, urine or breath ketones in the absence of another concurrent disease. Ketosis may be clinical or subclinical. Subclinical ketosis is defined as a preclinical stage characterized by an elevated ketone body level but not clinical signs, such as loss of appetite, hard feces, or dullness; however reproductive responses might be affected. The incidence of ketosis may range from 1.3% to 18.3%. Economic losses from ketosis have been estimated to total approximately US$145 per case.

Feed intake starts to decrease precipitously, and the reduction could reach as high as 30% on day 1 or 2 before calving and does not recover until 1 to 2 days after calving. Glucose availability is an important factor in the pathogenesis of clinical ketosis and liver fat accumulation. When glucose availability is low, ketone bodies formation occurs; when glucose is high, fat deposition is favored. Elevation of plasma fatty acids starts prior to feed intake depression, on day 5 before parturition. Liver fat infiltration does not occur until the concentration of plasma fatty acids are maximized on day 1 after calving. Liver fat accumulation can occur very rapidly. Within 48 h, hepatic fat levels can rise up to 25%, under conditions of extreme fat mobilization.

Left Displacement of Abomasum (LDA)

In left displacement of the abomasum, the abomasum slides under the rumen and dorsally along the left body wall. The result is a partial impairment of abomasal outflow, leading to abomasal gas accumulation, electrolyte pooling with subsequent systemic alterations, and depressed gastrointestinal motility and appetite. Left displacement of abomasum occurs most commonly 2 weeks prepartum to 8 weeks postpartum.

The incidence of LDA may range between 0.3% and 6.3%. This digestive disorder may occur in cows from first lactation and later. Cows with displacement of the abomasum experience severe losses of milk yield and body weight. They are more likely to have another disease than healthy

Table 2 Summary of periparturient disorders in dairy cattle

Disorder	*Incidence (%)*	*Risk factors*	*Cost per case*	*Prevention*
Hypocalcemia	0.03–22	• Parity • Milk yield • Breed	$335	Acidic (Anionic) diets prepartum
Retained fetal membranes (RFM)	1.3–39.2	• Parity Hypocalcemia • Calving difficulty • Twins	$285	Adequate feeding and obstetrical management
Metritis	2.2–37.3	• Hypocalcemia • RFM • Calving difficulty • Ketosis	$106	Avoiding predisposing factors Adequate treatments for RFM
Ketosis	1.3–18.3	• Hypocalcemia • RFM • Displacement of abomasum • Parity • Metritis • Mastitis • Milk Yield • Fat cows	$145	Avoiding risk factors Good feeding management
Displacement of abomasum	0.3–6.3	• Ketosis • RFM • Metritis • Mastitis • Hypocalcemia	$340	Good feeding management Avoiding risk factors

cows and thus are more likely to be culled than normal cows. Left displacement of abomasum is a multifactorial disease. Hypocalcemia, calving difficulty, ketosis, excessive body condition score at calving and incorrect feeding management practices are risk factors for displacement of abomasum. Prevention and Management of Periparturient Disorders

The most common periparturient disorders of dairy cattle are summarized in **Table 2**. Prevention of these disorders is best accomplished by providing a well-balanced diet to prepartum cows. Enhancing calcium availability during the time of parturition is crucial in preventing milk fever. This may be achieved by feeding a diet low in potassium and selecting feedstuffs with higher chlorine and sulfur contents (anionic diets). These diets induce a mild metabolic acidosis, which increases calcium levels in blood. Thus, the effectiveness of these diets can be tested monitoring the acidity of urine (urine pH). Too much acidity or alkalinity of the urine indicates that the cows are not responding to the anionic diets. Furthermore, prevention of hypocalcemia with oral calcium products have been recommended. These products are administered near calving, as supportive therapy in the management of clinical hypocalcemia in cattle. The most common oral products are calcium chloride and calcium propionate. However, under adequate management of acid diets and low incidence of hypocalcemia, oral products as prevention may not be necessary. Intravenous calcium products are only recommended for treatment purposes.

In order to prevent liver fat accumulation and ketosis, the key is to minimize the increase in fat mobilization from adipose tissue as parturition approaches. This can be accomplished by ensuring that the ration contains the required amount of energy and maintenance of maximum feed intake during the prepartum period (**Table 3**). Vitamins and minerals should be fed to dry cows at the

Table 3 Example of anionic diet for close-up[1] prepartum dairy cow[1]

Nutrient	*Close-up dry cows*[2]
Feed Intake (dry matter basis) kg/day	10.1
NEL,[3] Mcal/kg	1.58
Crude protein(CP), %	12.4
UIP,[4] %	4.5
Acid detergent fiber (ADF), %	21.8
Neutral detergent fiber (NDF), %	37.2
NFC,[5]%	41.6
Calcium, %	0.98
Phosphorus, %	0.37
Magnesium, %	0.38
Potassium, %	1.32
Sulfur, %	0.31
Sodium, %	0.15
Chlorine,%	0.89

[1] Table 14-11, NRC (2001)

[2] Dry transition cows 21 days before expected calving

[3] NEL = Net energy for lactation.

[4] UIP = Undegradable intake protein.

[5] NFC = Nonfiber carbohydrate, calculated as 100-CF-CP-NDF-Ash.

Table 4 Example trace mineral and vitamin diet for close-up prepartum dairy cow[1]

Nutrient	*Close-up dry cows*[2]
Cobalt, ppm[3]	0.11
Copper, ppm	13
Iodine, ppm	0.4
Iron, ppm	13
Manganese, ppm	18
Selenium, ppm	0.3
Zinc, ppm	22
Vitamin A, IU/kg[4]	7300
Vitamin D, IU/kg	1824
Vitamin E, IU/kg	132

[1] Table 14-11, NRC (2001)
[2] Dry transition cows 21 days before expected calving
[3] Parts per million
[4] International Units/kg

National Research Council recommendations as shown in **Table 4**.

Good feeding management not only includes the formulation of the diet, but also feed trough space in order to allow maximal feed intake per cow, adequate shade and good quality water and a comfortable environment where cows can lie down. Cows during the prepartum period need to adapt to the new diet, when the cow commences lactation. This challenge requires gradual changes through the prepartum until the early postpartum period and is the art of feeding transition cows.

Conclusions

During the postpartum period dairy cows are at a higher risk of developing metabolic diseases that impair milk production and reproductive performance. Many of these diseases are a result of improper nutritional management during the dry period. Because dry cows do not contribute to the economics of the dairy farm, many producers ignore these animals and their nutritional needs are compromised. We have to convince producers that the dry period is a preparatory phase for the next lactation, and that dry cows must be considered an investment for the next lactation.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction; Measurement Techniques and Data Processing. **Diseases of Dairy Animals**: Non-Infectious Diseases: Displaced Abomasum; Non-Infectious Diseases: Ketosis; Non-Infectious Diseases: Milk Fever. **Feed Ingredients**: Feed Supplements: Anionic Salts. **Feeds, Ration Formulation**: Dry Period Rations in Cattle. **Mammary Gland**: Growth, Development and involution. **Reproduction, Events and Management**: Pregnancy: Parturition.

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RHEOLOGY OF LIQUID AND SEMI-SOLID MILK PRODUCTS

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Introduction

The rheological behavior of milk is, in a general sense, typical of a semi-dilute emulsion/suspension. However, the complex composition and microstructure of milk result in complexities in behavior not exhibited by simple systems of this kind. These complexities become more pronounced when milk is subjected to such treatments as concentration (of fat or of total solids), fractionation, homogenization, heating, renneting, and acidification during its conversion to dairy products.

In this article, quantitative descriptions (mathematical models) of the rheological properties of milk, cream, concentrated milks and creams, sweetened condensed milk, ice cream mix, yogurts, buttermilk, and fresh cheeses are presented. The models are of three kinds: those that relate rheological behavior to composition and microstructure, those that quantify objective properties related to texture (texture itself must be measured subjectively), and phenomenological models useful for process engineering design and other technological purposes.

Milks and Creams

The rheological behavior of milk and cream is in accord with that of emulsions and suspensions in general. Milk and cream can exhibit Newtonian or non-Newtonian behavior depending on composition, temperature, prior treatment, and measurement conditions.

Fresh skim milk, whole milk, and cream can for almost all practical purposes be treated as Newtonian liquids under the following conditions: fat content <40% (w/w), temperature >40 °C (milk fat completely molten; no cold agglutination of fat globules), and moderate to high shear rates. Rheological behavior is completely characterized by a temperature-dependent coefficient of viscosity, defined by Newton's law of viscosity:

$$\tau = \eta\ \dot{\gamma}\ \text{(Pa)} \qquad [1]$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), and η is the coefficient of viscosity (Pa s). The term 'coefficient of viscosity' strictly denotes the proportionality constant in eqn [1]. The term 'viscosity' is a more general one, meaning the extent to which a fluid resists being sheared. The distinction between the two is irrelevant for Newtonian liquids, though not for non-Newtonian liquids.

Representative values, at 20 °C, of the coefficient of viscosity of whole milk and fractions derived therefrom, measured under conditions where Newtonian behavior exists, are given in **Table 1**.

Several points can be made on the basis of these data. First, lactose, the major low-molecular-weight milk component, and even the whey proteins influence viscosity to a relatively small extent. Second, the fat content has a major influence. Third, the (micellar) casein content has by far the greatest influence. The viscosity and the rheological behavior of milk (and the semi-liquid products made from it) depend largely upon the state and concentration of the fat and casein, and thus on factors that affect these.

The viscosity of whole and skim milk, for conditions under which Newtonian behavior occurs, can be modeled with Eilers's semi-empirical equation:

$$\eta = \eta_0 \left(1 + \frac{1.25\Sigma(\phi_i)}{1 - \Sigma(\phi_i)/\phi_{\max}}\right)^2 \qquad [2]$$

where η is the coefficient of viscosity of the milk product (Pa s); η_0 is the coefficient of viscosity of the portion of the product consisting of water and low-molecular-weight substances other than lactose (Pa s); ϕ_i is the volume fraction of a dispersed component with a particle size at least an order of magnitude greater than the size of the water molecule; $\Sigma(\phi_i) = \phi_{\text{fat}} + \phi_{\text{cas}} + \phi_{\text{wp}} + \phi_{\text{L}}$, where fat represents milk fat, cas is casein, wp whey proteins, and L lactose; and $\phi_{\max}$ is the assumed value of $\Sigma(\phi_i)$ for maximum packing of all dispersed particles; $\phi_{\max}$ depends on the overall size distribution of the dispersed particles and on particle shapes (though not on particle size *per se*).

The volume fraction of an individual component is given by

$$\phi_i = V_i c_{v,i} \qquad [3]$$

where V_i is the voluminosity of component i ($m^3\,kg^{-1}$ of dry component) and $c_{v,i}$ is the volume concentration of the component in the product ($kg\,m^{-3}$ of product). Representative values of voluminosity are given in **Table 2**. Voluminosity and volume fraction pertain to

Table 1 Representative values of the coefficient of viscosity, at 20 °C, of whole milk and fractions derived from it

Milk fraction	*Viscosity (mPa s)*
Whole milk	2.13
Skim milk (≅ whole milk minus fat globules)	1.79
Rennet whey (≅ skim milk minus casein micelles)	1.25
5% Lactose solution (≅ whey minus whey proteins)	1.15
Water (≅ whole milk minus all dissolved and dispersed components)	1.00

From Jenness R and Patton S (1959) *Principles of Dairy Chemistry*. London: Chapman & Hall.

Table 2 Representative values of the voluminosities of milk components

Component	*Voluminosity*
Fat globules	$\sim 1.11 \times 10^{-3}$ m^3 kg^{-1} of lipid in fat globules
Casein	$\sim 3.9 \times 10^{-3}$ m^3 kg^{-1} of dry casein
Whey proteins	$\sim 1.5 \times 10^{-3}$ m^3 kg^{-1} of dry protein
Lactose	$\sim 1.0 \times 10^{-3}$ m^3 kg^{-1} of lactose

From Walstra P and Jenness R (1984) *Dairy Chemistry and Physics*. New York: Wiley.

hydrodynamic volume, and thus account for particle shape and water of hydration as well as volume *per se.*

Equation [2], for $\Sigma(\phi_i) \rightarrow 0$, reduces to Einstein's equation for the viscosity of a very dilute solution of hard spheres:

$$\eta = \eta_0(1 + 2.5\phi) \qquad [4]$$

Einstein's equation assumes no particle–particle interaction; Eilers's equation accounts for both the presence of the dispersed phase(s) and the hydrodynamic interaction between particles during flow. Eilers's equation does not, however, account for mutual attraction/repulsion between particles.

Eilers developed his equation at a time when viscometric techniques were too unsophisticated to detect non-Newtonian behavior at low shear rates. The equation is useful for predicting the effects on viscosity of variations in the composition and concentration of milks and creams. Viscosity increases with %TS (total solids), but, for a given %TS, is inversely related to %fat content (F) because of the lower voluminosity of fat when compared with that of casein in particular. When $\Sigma(\phi_i)$ exceeds 0.6 (which corresponds to $\eta/\eta_0 \approx 10$), viscosity increases steeply with $\Sigma(\phi_i)$, and the rheological behavior becomes non-Newtonian.

The volume fraction of the casein (ϕ_{cas}) in milk is the main determinant of $\Sigma(\phi_i)$, and thus of viscosity. Large differences in viscosity between different lots of milk are almost certainly attributable to differences in ϕ_{cas}, which depends, in turn, upon such factors as the concentration of colloidal calcium phosphate, Ca^{2+} activity, and pH. The viscosity of milk and cream increases with age owing partly to changes in ionic equilibria. The viscosity of milk increases slightly with increasing pH, perhaps owing to swelling of the casein micelles. Conversely, a small decrease in pH reduces viscosity, whereas a large decrease causes micelle aggregation.

Temperature and Thermal History

Viscosity is inversely related to temperature *per se.* Cooling of milk results in an increase in viscosity, which is partly the result of an increase in η_0 and partly the result of a sharp increase in V_{cas} and thus in ϕ_{cas}. Furthermore, some caseins, especially β-casein, dissociate from the micelles at low temperatures; this contributes to an increase in viscosity because dissociated proteins have higher hydrodynamic volumes.

Heating of skim milk or whole milk to 65 °C, followed by rapid cooling, results in a temporary decrease in viscosity (measured at a lower temperature) because of the increased association of β-casein with the micelles that would have occurred at the higher temperature; viscosity recovers exponentially with time as some β-casein gradually dissociates again at the lower temperature.

Warming of milk to temperatures above ambient causes viscosity to decrease, because η_0 decreases and ϕ_{cas} decreases moderately. The change in ϕ_{cas} is reflected by a decrease in the ratio $\eta_{\text{skim}}/\eta_{\text{whey}}$ when temperature is increased from 5 to 30 °C. The decrease is less marked at temperatures >30 °C.

During dynamic heating and cooling (e.g., 15 ↔ 80 °C), the viscosity of milk can vary depending on both temperature and temperature history; plots of viscosity versus temperature can display hysteresis. The hysteretic pattern for fresh milk itself depends on temperature history, and this effect is attributable to the reversible melting and crystallization behavior of the milk triglycerides. Different hysteretic patterns are found with aged milk; these are considered to reflect the decreases in the heat stability of proteins with age.

The viscosity of whole milk increases with age. The rate of increase is inversely related to the temperature of measurement. Pasteurization, a relatively mild heat treatment, results in no noticeable change in the rheological properties of whole milk.

Non-Newtonian Behavior

Non-Newtonian behavior manifests itself in raw whole milks and creams under conditions that favor cold agglutination of fat globules (temperatures of <40 °C and low shear rate). Shear thinning is the predominant rheological behavior. Aggregates of fat globules contain trapped interstitial plasma, and thus together have a high effective volume fraction, especially at low shear rates. As shear rate is increased, shearing forces cause aggregates to become more regular in shape or to break down. The volume fraction and consequently the apparent viscosity then decrease. Apparent viscosity, which is thus shear rate dependent, is defined

$$\eta_{\mathrm{app}} = \frac{\tau}{\dot{\gamma}} \qquad [5]$$

As shearing forces become larger compared with the attractive forces holding the globules together, successive increases in shear rate have smaller and smaller effects on apparent viscosity. At sufficiently high shear rates, the behavior becomes Newtonian. At high fat contents, non-Newtonian behavior is more pronounced and persists to higher shear rates.

At very low shear rates ($\dot{\gamma} \leq 30\,\mathrm{s}^{-1}$), at ambient temperature, the shear rate dependence of the viscosity of model emulsions made by mixing pasteurized cream with pasteurized skim milk is due only to the skim milk. Skim milk exhibits shear-thinning behavior at shear rates $<250\,\mathrm{s}^{-1}$ and temperatures $<30\,°\mathrm{C}$.

Lower temperatures enhance cold agglutination. This increases both the apparent viscosity and the non-Newtonian behavior, the latter persisting to higher shear rates. Separation of milk at temperatures $\geq 40\,°\mathrm{C}$ gives cream in which cold agglutination is largely reduced owing to the loss of agglutinin to the skim milk. Conversely, cold separation enhances cold agglutination. Thus, the separation conditions influence the rheology of cream. Cold agglutination in raw cream, and the presence of homogenization clusters in homogenized cream, can result in time-dependent shear thinning (called thixotropy if recovery of viscosity occurs on resting after shearing).

The apparent viscosity of raw milk at infinite shear rate ($\eta_{\dot{\gamma}=\infty}$), at temperatures $<40\,°\mathrm{C}$, can be related to ϕ_{fat} by the following equation:

$$\eta_{\dot{\gamma}=\infty} = \eta_{\mathrm{skim}}(1 + 3.25\phi_{\mathrm{fat}}) \qquad [6]$$

Evaluation of apparent viscosity at $\dot{\gamma} = \infty$, by extrapolation, allows the effects of fat content to be separated from the effects of shear rate.

The Cross equation, which relates apparent viscosity to shear rate, has been found to describe exactly the pseudoplastic nature of raw cream:

$$\eta_{\mathrm{app}} = \eta_{\dot{\gamma}=\infty} + \frac{\eta_{\dot{\gamma}=0} - \eta_{\dot{\gamma}=\infty}}{1 + b\dot{\gamma}^{m}} \qquad [7]$$

where η_{app} is the apparent viscosity at $\dot{\gamma}\,\mathrm{s}^{-1}$; $\eta_{\dot{\gamma}=0}$ is the apparent viscosity at $\dot{\gamma} = 0$, found by extrapolation; and b and m are constants. The factors $\eta_{\dot{\gamma}=0}$, $\eta_{\dot{\gamma}=\infty}$, b, and m are each dependent on temperature and fat content. The applicability of the Cross equation implies that a state of equilibrium exists during flow between the size and number of fat globule aggregates and shear rate.

Shear thickening can be observed in high-fat creams; shearing induces partial coalescence of globules, thereby increasing the effective ϕ_{fat} by entrapment of plasma. Cream is viscoelastic; its rheological behavior may be represented by the generalized Maxwell body (a mechanical analog), the relaxation time of which is nearly independent of fat content. Cream, a concentrated emulsion, can in fact exhibit most of the possible types of non-Newtonian behavior depending on conditions and treatment. The viscosity of milks and creams depends on technological treatments as well as on composition, concentration, and shear rate.

Effects of Technological Treatments

Homogenization

Homogenization of milk and cream results in an increase in viscosity measured at high shear rates. The increase is inversely related to fat globule size. At low shear rates and ambient temperature, the viscosity of homogenized milk is lower than that of raw milk. This is possibly a consequence of the destruction of agglutinin by homogenization. Homogenization of cream reduces deviation from Newtonian behavior, possibly for the same reason.

Heat treatment, renneting, acidification

The effects of heat treatment, renneting, and acidification on the viscosity of milk, under conditions where coagulation has not (yet) occurred, can be modeled using the so-called adhesive hard sphere (AHS) theory.

It has been shown that skim milk behaves rheologically as a semi-dilute colloidal suspension of hard spheres, the casein micelles, in a medium comprising all of the other milk components. For such a suspension, the viscosity (strictly speaking the coefficient of viscosity at $\dot{\gamma} \rightarrow 0$) is given by the following theoretical equation (which is a modification of Einstein's equation [4]):

$$\eta = \eta_0(1 + 2.5\phi + k_{\mathrm{H}}\phi^2) \qquad [8]$$

where k_{H} (the Huggins constant) accounts for hydrodynamic interactions. For $\phi \leq 0.2$ (semi-dilute dispersion), in the absence of mutual attraction/repulsion between the micelles (nonadhesive hard spheres), k_{H} is equal to 5.913. Viscosity is then given by

$$\eta = \eta_0(1 + 2.5\phi + 5.913\phi^2) \qquad [9]$$

Untreated skim milk closely follows this equation.

A series development of the semi-empirical Eilers's equation (with $\phi_{\mathrm{max}} = 0.74$) gives a similar expression:

$$\eta = \eta_0(1 + 2.5\phi + 4.94\phi^2 + 8.78\phi^3 + \cdots) \qquad [10]$$

Both eqn [9] and eqn [10] reduce to Einstein's equation when ϕ is small enough to preclude interactions of any kind between the suspended spheres.

The Huggins constant is given by the following equation:

$$k_{\mathrm{H}} = 5.913 + \frac{1.9}{\tau_{\mathrm{B}}} \qquad [11]$$

where τ_{B} is the Baxter interaction parameter, which is a measure of adhesive interactions between the spheres (stickiness). It is related to the second osmotic virial coefficient, B_2, a property of the casein micelle suspension:

$$\frac{B_2}{V_{\mathrm{HS}}} = 4 - \frac{1}{\tau_{\mathrm{B}}} \qquad [12]$$

where V_{HS} (=$4/3\pi a^2$) is the volume of a hard sphere of radius *a*.

For nonadhesive hard spheres (no mutual attraction) $\tau_{\mathrm{B}} = +\infty$, and $k_{\mathrm{H}} = \mathrm{constant} = 5.913$, as indicated above (eqn [9]). When mutual attraction does exist (adhesive hard spheres), τ_{B} has a positive finite value. Then, $k_{\mathrm{H}} > 5.913$, and viscosity has a value larger than that predicted by eqn [9]. The adhesive hard-sphere theory is embodied in eqns [8], [11], and [12].

Heat treatment of skim milk severe enough to cause denaturation of β-lactoglobulin and its subsequent association with the casein micelles results in an increase in ϕ and a decrease in τ_{B}, the latter change indicating the development of mutual attraction between the casein micelles. This results in a viscosity increase larger than that predicted by either eqn [9] or eqn [10].

A computer-based model utilizing both the AHS theory and the denaturation kinetics of β-lactoglobulin has been developed that allows the prediction of the viscosity of skim milk resulting from any combination of heating temperature and heating time. The model shows that viscosity not only depends on the extent of denaturation of β-lactoglobulin but, for a given denaturation, also is higher for a higher heating temperature or a higher heating rate.

The addition of chymosin to milk at the start of renneting initiates two sequential changes. First, there is a fall in viscosity caused by the enzyme cutting κ-casein hairs from the surfaces of the casein micelles, with a consequent reduction in ϕ_{cas}. Second, the loss of the stabilizing κ-casein layer causes the micelles to become mutually attractive (adhesive hard spheres). This leads to an increasingly rapid rise in viscosity, which eventually culminates in micelle flocculation. These two effects together are qualitatively and quantitatively modeled well by the AHS theory. The theory can be used to predict coagulation time, defined as the time, after chymosin addition, at which the viscosity recovers its initial value after its temporary fall.

Acidification of milk, for example, that resulting from starter bacterial activity during yogurt manufacture, causes micelle flocculation (eventually leading to coagulation) owing to a loss of the steric stabilization of micelles. Loss of stabilization is due to the loss of the extended conformation of the κ-casein hairs on the micelle surfaces, and their eventual collapse. The AHS theory accurately quantifies the relationship between viscosity (increasing) and pH (decreasing) during acidification.

The AHS theory has been applied mainly to normal skim milk. The effects of other variables such as fat content and calcium concentration on the applicability of the theory are apparently yet to be investigated.

Storage

The viscosity of ultra-high temperature (UHT) sterilized milk can increase gradually during storage. This phenomenon, age thickening, can eventually lead to gelation. It is thought to be the result of the gradual release, from the casein micelles, of β-lactoglobulin–κ-casein complexes formed during the high-temperature heat treatment, and the subsequent cross-linking of the complexes to form a gel network.

Technologically Useful Relationships for Predicting the Rheological Properties of Milks and Creams

Successful attempts have been made to establish empirical relationships useful for technological and engineering purposes between the viscosity of Newtonian-fluid milk products and their temperature and composition. A number of these relationships, the development of which ignored any observed (slight) non-Newtonian behavior, are given in **Table 3**.

In the case of non-Newtonian milks and creams, phenomenological relationships between shear stress and shear rate have been developed for practical purposes. (These contrast with expressions for viscosity at zero shear rate, which are valuable for relating rheological behavior to structure and composition in a fundamental way.)

The shear-thinning behavior of raw whole milk in the shear-rate range 1–1500 s^{-1} can be modeled well by the power-law (Ostwald–de Waele) equation:

$$\tau = k\dot{\gamma}^{n} \qquad [13]$$

Typical values of *k* and *n* for raw milk (3.35% fat) at 25 °C are 6.14×10^{-3} Pa s and 0.798, respectively. Rheological behavior becomes essentially Newtonian at shear rates $>800\ \mathrm{s}^{-1}$.

The shear-thinning behaviors of pasteurized skim milk, whole milk, and cream at low shear rates and

Table 3 Technologically useful relationships between the viscosity of Newtonian milk products, and temperature and composition

Product specifications	*Relationship*
Milk, 8–28 %TS, 0.07–7.4% fat, Fat to solids-not-fat ratio: 0.01–0.4 0–80 °C	$\log\eta = 0.249 - 1.3\times10^{-2}\theta + 5.2\times10^{-5}\theta^2 + (2.549\times10^{-2} - 9.8\times10^{-5}\theta + 4\times10^{-7}\theta^2)(\%TS) + (5.43\times10^{-4} - 1.39\times10^{-5}\theta + 1.117\times10^{-7}\theta^2)(\%TS)^2$
Milk, 0.03–15% fat, 70–135 °C	$\ln\eta = 3.92\times10^{-5}\theta^2 - 1.951\times10^{-2}\theta + 0.666 + F(-9.53\times10^{-6}\theta^2 + 1.674\times10^{-3}\theta - 4.37\times10^{-2}) + F^2(9.75\times10^{-7}\theta^2 - 1.739\times10^{-4}\theta + 9.83\times10^{-3})$
Milk of normal composition, 25 °C	$\eta = 0.96 + 0.058F + 0.156P$
Milk and cream, 0.1–30% fat, 0–30 °C	$\ln\eta = \left(\dfrac{2731.5}{(273+\theta)}\right) + 0.1F - 8.9$
Milk and cream, 0–40% fat, 40–80 °C	$\log\eta = A(F + F^{5/3}) + \log\eta_0$ where $A = 1.2876 + 11.07\times10^{-4}\theta$, and $\eta_0 = 0.7687\left(\dfrac{10^3}{273+\theta}\right) - 2.437$

η and η_0 = viscosity in mPa s, θ = temperature in °C, %*TS* = % total solids, *F* = % fat, and *P* = % protein.

temperatures <40 °C, can be modeled satisfactorily by the Bingham equation:

$$\tau = \eta_{\mathrm{pl}}\dot{\gamma} + \tau_0 \qquad [14]$$

Values of η_{pl} (the plastic viscosity) for skim milk and whole milk (3.5% fat) at 25 °C are 1.53×10^{-3} and 1.81×10^{-3} Pa s, respectively. The corresponding values of τ_0 (the yield stress) are 1.98×10^{-2} and 4.6×10^{-2} Pa. For cream of 38% fat, at 25 °C, the values of η_{pl} and τ_0 are 9.44×10^{-3} Pa s and 0.381 Pa, respectively.

Raw cream of 60% fat has been found to obey the Bingham equation at constant temperature in the range 15–80 °C. When subjected to dynamic heating and cooling in this temperature range, the rheological behavior of the same cream is modeled better by the power-law equation [13]. The values of *k* and *n* at a given temperature depends on thermal history, a phenomenon attributed to the interaction between heating/cooling rates and the rates of melting and crystallization of milk triacylglycerols.

Cream of 40–55% fat, at 5–20 °C, has been shown to obey the Herschel–Bulkley equation:

$$\tau = k\dot{\gamma}^n + \tau_0 \qquad [15]$$

This equation is a generalized form of the Bingham equation [14]. Values of *n* have not been reported. (The power-law equation [13] and Newton's equation [1], as well as the Bingham equation, are special cases of eqn [15].)

As stated above, the Cross equation [7] accurately models the shear dependence of the apparent viscosity of cream.The numerical values of the parameters in these phenomenological equations depend on composition, concentration, and temperature.

Non-Newtonian Behavior in Concentrated Milks and Creams

When milk is concentrated by heat evaporation or by membrane processes (ultrafiltration, reverse osmosis), $\Sigma(\phi_i)$ increases because of the concentration effect *per se* and because particle–particle interactions (especially micelle–micelle interactions) increase owing to smaller interparticle distances. These interactions lead to aggregate formation. The effects of aggregate formation on rheological behavior are essentially the same as the effects of concentrating only the fat globules, as in cream: apparent viscosity increases; shear thinning becomes more pronounced; and deviation from Newtonian behavior persists to higher shear rates. Time-dependent shear thinning appears at a sufficiently high concentration or after a sufficiently long storage time (at concentrations and temperatures above certain minima) during which structure development occurs. Such structure development, and the consequent steady viscosity increase, is known as age thickening.

Rheologically, there are no fundamental differences between concentrates produced by heat, by reverse osmosis, or by ultrafiltration, or between whole milk and cream concentrates on the one hand and skim milk concentrates on the other. Rather, the differences are of degree. For given conditions, non-Newtonian behavior tends to appear at lower %TS in ultrafiltration skim milk concentrates than in evaporated skim milk (because of the preferential concentration of proteins in the former), and at lower %TS in skim milk concentrates than in whole milk concentrates (because the proportion of protein in the dry solids is higher in the former). For the same reasons, viscosity at a given %TS is higher in ultrafiltration skim milk concentrates than in evaporated skim milk, and in skim milk concentrates than in whole milk concentrates.

The viscosity of any concentrate under given conditions of %TS, shear rate, temperature, dry solids composition, time after preparation, and other variables such as preheat treatment and pH is directly related to $\Sigma(\phi_i)$. This is discussed first below. Then, phenomenological relationships that have been found useful in describing the non-Newtonian behavior of concentrates as a function of shear rate are reviewed. Last, the phenomenological description of age thickening is presented.

Dependence of concentrate viscosity on total volume fraction

It has been shown that the apparent viscosity (at a specified shear rate) of heat-concentrated whole and skim milks is weakly related to %TS but closely related to total volume fraction calculated by

$$\Sigma(\phi_i) = \phi_{\text{cas}} + \phi_{\text{nwp}} + \phi_{\text{dwp}} + \phi_{\text{fat}} \qquad [16]$$

where ϕ_{nwp} and ϕ_{dwp} are the volume fractions of native and denatured whey proteins, respectively; the effect on the whey proteins of preheating the milk prior to concentrating is thus automatically taken into account.

Volume fractions (ϕ_i) of individual components were calculated by

$$\phi_i = \phi_{i,\text{ milk}} \times \frac{\text{TS}_{\text{conc}}}{\text{TS}_{\text{milk}}} \times \frac{\rho_{\text{conc}}}{\rho_{\text{milk}}} \qquad [17]$$

where ρ is the density (kg m^{-3}) and the subscripts 'milk' and 'conc' refer to the milk prior to evaporation and the concentrated milk, respectively.

Concentrates are shear thinning; $\eta_{\text{app},\dot{\gamma}=\infty}$ of freshly prepared heat concentrates (no age thickening) is closely predicted by Eilers's equation [2]. ϕ_{max} in the equation, for heat concentrated skim milk, has been shown to be 0.79, by extrapolation of heat gelation time versus ϕ_{protein} curves to gelation time = 0. The same value has been found to be satisfactory for whole milk concentrates.

η_{o} in Eilers's equation may be calculated for heat-evaporated and reverse-osmosis milks by

$$\eta_0 = \eta_{\text{water}} + (\Delta\eta_s + \Delta\eta_{\text{L}s})\left(\frac{(\%\text{TS})_{\text{conc}}}{(\%\text{TS})_{\text{unconc}}}\right) \qquad [18]$$

$\Delta\eta_s = \eta_{\text{water + salts}} - \eta_{\text{water}} = 0.02\ \eta_{\text{water}}$ and $\Delta\eta_{\text{Ls}} = \eta_{\text{water + 5\%lactose}} - \eta_{\text{water}}$. For ultrafiltration concentrated milks, $\eta_0 = \eta_{\text{permeate}}$. Temperature dependence of viscosity can be allowed for by evaluating $\Delta\eta_{\text{s}}$, $\Delta\eta_{\text{Ls}}$, and η_{permeate} at the required temperature using literature data or direct measurement.

The apparent viscosity, the rate of age thickening, and the degree of shear thinning of milk concentrates made by evaporation all increase with both time and %TS. At elevated holding temperatures, for example, 50 °C, even $\eta_{\dot{\gamma}=0}$ increases slightly with holding time (at a given %TS), suggesting a permanent change in concentrate structure that cannot be reversed by shearing.

The change in the apparent viscosity with time during age thickening can be predicted by a modified Eilers's equation:

$$\eta_{\text{app}} = \eta_0\left(1 + \frac{1.25\Sigma(\phi_i)(1 + t.k_{\Sigma(\phi_i)}/\Sigma(\phi_i))}{1 - \Sigma(\phi_i)(1 + t.k_{\Sigma(\phi_i)}/\Sigma(\phi_i))\phi_{\text{max}})}\right)^2 \qquad [19]$$

where t is the time, $\Sigma(\phi_i) = \Sigma(\phi_i)$ at t = 0, and $k_{\Sigma(\phi_i)} = \text{d}(\Sigma(\phi_i))/\text{d}t$. For a given shear rate and temperature, $k_{\Sigma(\phi_i)}/\Sigma(\phi_i)$, the so-called shear-thickening constant, is independent of %TS. The rate of age thickening is somewhat lower for whole milk concentrates than for skim milk concentrates because the fat in the former, while contributing to the overall volume fraction, is inert with respect to the age-thickening process.

Phenomenological rheological relationships for concentrated milks and creams

As %TS increases, the rheological behavior of freshly prepared milk concentrates changes from Newtonian to time-independent shear thinning to time-dependent shear thinning.

Under conditions where time dependence is absent, flow behavior changes, as %TS is increased, from Newtonian (eqn [1]) to shear thinning with no yield stress (power-law behavior; eqn [13]) to shear thinning with a yield stress (Bingham plastic behavior (eqn [14]) or Herschel–Bulkley behavior (eqn [15])).

In fact, because (as pointed out above) the Newtonian, power-law, and Bingham equations are all special cases of the Herschel–Bulkley equation, this last equation can be said to describe adequately the flow curves of time-independent milk concentrates and the instantaneous flow curves of time-dependent concentrates, under all conditions of temperature, shear rate, and other variables. When τ_0 = zero and $n = 1$ in the Herschel–Bulkley equation, $k = \eta$, the Newtonian coefficient of viscosity.

For a given type of concentrate, the constants n and k of the power-law equation [13] tend to be inversely related to one another in ways that are independent of %TS, temperature, and other factors. This reflects the fact that structure development in concentrates that results in greater deviation from Newtonian behavior (lower n) also results in increased viscosity (higher k).

The constant k can often be related directly to $\Sigma(\phi_i)$. For example, a unique direct relationship between $\log_{10} k$ (at

20 °C) and concentrate $\Sigma(\phi_i)$ has been demonstrated for ultrafiltration whole milk and cream concentrates. This relationship is linear up to $\Sigma(\phi_i) = 0.5$, and also linear, but with a higher slope, for $\Sigma(\phi_i) > 0.5$. For $\Sigma(\phi_i) < 0.5$, concentrates are Newtonian and the relationship is

$$\log_{10} k = \log\eta = 3.82\Sigma(\phi_i) - 3.35 \qquad [20]$$

where the units of k and η are Pa s^n and Pa s (i.e., $n = 1$), respectively.

Shear-thinning behavior exists at $\Sigma(\phi_i) > 0.5$. This critical value of ϕ is close to the theoretical maximum value of 0.52 that could exist in an ideal monodisperse suspension in which the suspended particles are in contact with one another but able to move in straight lines in the direction of flow. The dependence of k (at 20 °C) on $\Sigma(\phi_i)$ at $\phi > 0.5$ is higher than that expressed by eqn [20] and has been found to be

$$\log_{10} k = 10.15\Sigma(\phi_i) - 6.51 \qquad [21]$$

Fresh skim milk heat concentrated 6.22-fold was found to obey the Bingham equation [14]. Yield stress was found to be proportional to $(\phi_{\text{cas}})^{4.1}$ at $\phi_{\text{cas}} > 0.3$. The value of the exponent is close to the value of 3.85 exhibited by weakly flocculated latex suspensions, implying that concentrated milks are themselves weakly flocculated suspensions.

Phenomenological description of time-dependent behavior

Milk concentrates can exhibit two forms of time-dependent rheological behavior: time-dependent shear thinning (a decrease in viscosity with time at constant shear rate) and age thickening (an increase in viscosity with storage time). These can be directly related: age thickening is caused by gradual structure development, which at a certain point starts to result in time-dependent shear-thinning behavior – the result of the time-dependent breakdown of the developed structure when the age-thickened concentrate is sheared.

When concentrates age thicken, the Herschel–Bulkley equation *per se* or the Bingham equation has been found to describe adequately the instantaneous flow curves.

The rate of viscosity increase during age thickening can be quantified in terms of the rate of evolution with time of the flow equation constants or the rate of increase with time in apparent viscosity, which at a given time is a function of these constants.

Sweetened Condensed Milk and *Dulce De Leche*

Sweetened condensed milk (SCM) is essentially a high-viscosity suspension of lactose crystals, fat globules, casein, and whey proteins in a saturated solution of lactose and sucrose. Its rheological behavior is complex.

SCM exhibits age thickening, which occurs faster at higher storage temperatures. The structure development is thought to involve not only casein micelles but also the whey proteins (especially if these have suffered a high degree of denaturation during milk preheating) and the fat globules. The viscosity and the rate of age thickening of recombined SCM are significantly influenced by the conditions used during milk powder manufacture.

SCM exhibits time-dependent shear thinning. Recovery of structure (and consequently of viscosity) at ambient temperatures is highly retarded by the still-high viscosity that exists after shearing.

The rheological properties of shear-thinned SCM can be characterized by the power-law equation [13] or the Herschel–Bulkley equation [15], depending on composition and processing conditions. Deviation from Newtonian behavior is slight immediately after manufacture, but increases with time during storage.

The viscoelastic properties of SCM have been described by means of a generalized Maxwell model that incorporates yield stresses.

Dulce de leche is an Argentinean dairy product similar to SCM in composition but with even more complex rheological properties. There are three types: standard, low calorie, and confectionery. The last contains a hydrocolloid thickener. Like SCM, *dulce de leche* is a time-dependent shear-thinning material. The properties of shear-thinned samples can be modeled by the power-law equation [13], the Herschel–Bulkley equation [15] or the Casson equation, depending mainly on composition. The Casson equation is

$$\sqrt{\tau} = \sqrt{\eta_{\text{Ca}}}\sqrt{\dot{\gamma}} + \sqrt{\tau_{0,\,\text{Ca}}} \qquad [22]$$

where η_{Ca} and $\tau_{0,\,\text{Ca}}$ are the Casson viscosity and the Casson yield stress, respectively.

The Herschel–Bulkley and the Casson equations appear to be equally useful models for samples that possess a yield stress.

Dulce de leche, like SCM, is viscoelastic, as indicated by the stress overshoot that occurs just after the start of a constant shear rate experiment. Thereafter, the shear stress declines with time as the structure is broken down by shear. The entire shear stress–time data can be modeled well with a generalized form of the Bird–Leider equation:

$$\tau = k\dot{\gamma}^n\left[1 + (b\dot{\gamma}t - 1)\sum_{j=1}^{j} w_j \exp\left(-\frac{t}{\delta_j n}\right)\right] \qquad [23]$$

where k and n are the power-law equation constants, b and δ are fitting parameters that model the time dependence, and w_j is a weighting factor. At long times, this equation converges to the power-law equation [13], which describes the equilibrium flow behavior of many foods.

At times post the maximum (overshoot) stress, the decay of shear stress during shear thinning can be modeled with the Weltman equation:

$$\tau = A - B\ln t \qquad [24]$$

where A is the maximum (overshoot) shear stress, B is the coefficient of time-dependent breakdown, and t is the time.

Oscillatory rheometric frequency sweep measurements, at strains within the linear elastic region, of the dynamic elastic modulus G' and the loss modulus G'' (where $G' + iG'' = G^*$, the complex elastic modulus) reveal differences in structural characteristics among the three types of *dulce de leche*. The behavior of all three approaches that of concentrated solutions, especially when presheared. The confectionery type, when unsheared, possesses characteristics intermediate between those of a concentrated solution and a gel, and may thus be termed a weak gel.

Ice Cream Mix

Unfrozen ice cream mix is a dispersion of milk fat and vegetable fat in an aqueous phase containing non-fat milk solids, carbohydrate sweeteners, and a stabilizer (usually a hydrocolloid). The mix commonly contains, in addition, an added emulsifier. The total fat concentration is about 10% (w/w). Ice cream mix is thus essentially a dilute oil-in-water emulsion and in this respect is similar to cream. However, viscosity and rheological behavior are more variable, as they depend on both mix formulation and processing conditions.

Ice cream mixes are time-dependent shear-thinning liquids that can exhibit significant viscosity recovery after subjection to high shear. Shear thinning and viscosity recovery can be attributed partly to deflocculation and flocculation, respectively, of fat globules (to the surfaces of which the micellar casein is attached) and to changes with time in the crystalline state of the fat.

Age thickening of unsheared mix has been attributed to progressive flocculation of fat globules.

Mixes exhibit viscoelastic behavior typical of weak gels, the gel network being the result of interactions involving fat globules, and the proteins and polysaccharides present in the aqueous continuous phase.

Ice cream mix flow curves can generally be described adequately by the power-law equation [13]. The values of the constants k and n in the equation are largely influenced by the presence, type, and concentration of the hydrocolloid stabilizer and by processing treatments such as homogenization.

Melted ice cream has a much weaker structure, and consequently a lower viscosity and a lower extent of deviation from Newtonian behavior, than had the original mix. This is a consequence of the continuation, on melting, of the fat globule coalescence that takes place during the initial stages of whipping and freezing. The rheological properties of the melt are important with respect to the subjective assessment of ice cream on the palate.

Yogurt

Two basic types of yogurt are made commercially: set yogurt and stirred yogurt. Yogurt rheology is complex. Yogurt exhibits viscoelastic behavior (especially the set type) and highly time-dependent shear thinning in flow. The rheological behavior of the final product depends on the concentration, composition, and pretreatment of the milk (especially heat treatment), starter culture and incubation conditions, and post-incubation shearing (stirred type). Measurement of rheological properties is useful in objectively investigating, characterizing, and predicting the effects of these variables on the nature of the final product, especially its texture.

Heat Treatment

The development of desirable final rheological properties is highly dependent on there being sufficient interaction between, and flocculation of, the casein micelles during incubation, so that a satisfactory coagulum forms; heat treatment of the milk must be severe enough to cause extensive denaturation of β-lactoglobulin, some denaturation of α-lactalbumin, and the interaction of both of these whey proteins with the micelles. The effect of heat treatment depends on whether it is carried out before or after homogenization.

Fermentation

Though acid production by the starter microorganisms is the main cause of micelle flocculation and consequent structure development, the starter strain can be chosen to influence the rheological properties. In particular, strains that produce exopolysaccharides can be used to increase yogurt viscosity and decrease susceptibility to syneresis. Final rheological properties are dependent both on strain and on incubation conditions (time and temperature).

Post-Fermentation Shearing

The rheological properties of stirred yogurt are greatly influenced by the shearing to which the yogurt is subjected during post-fermentation stirring, cooling, and packaging. The shear history and consequent structure breakdown are related directly to equipment geometry and to process conditions such as temperature and flow rate. The final product is a strongly shear thinning, viscous liquid.

Solid-like and Viscoelastic Behavior

The solid-like behavior of yogurt (especially set yogurt) can be determined very simply by measuring the force required to push a probe into the product under standard conditions. The method is empirical, but simple, rapid, and inexpensive. The result obtained is some function of the elastic, viscous, and breakdown properties of the product. The results can be correlated to texture, and can be used to investigate the relative effects of changes in processing conditions.

Objective measurements of the texture-related rheological properties of set-type yogurts can be made using the technique of texture profile analysis, which involves force–distance measurements during compression/decompression tests in suitable rheometers. When measurements are to be made on intact yogurt gels in well-defined rheometer geometries, either during or at the end of fermentation, the fermentation process must, of course, be carried out in the rheometer itself.

Fundamental viscoelastic properties of both set and stirred yogurts have been determined using dynamic oscillatory rheometry and stress relaxation measurements. The mechanical spectra (dynamic elastic moduli versus frequency) of the set and the stirred yogurts are similar, with the moduli of the former being 8–10 times higher than those of the latter.

In one study (of a stirred yogurt) dynamic measurements showed that at low strain ($\gamma < 3\%$) linear viscoelastic behavior existed: the rigidity modulus G' and the loss modulus G'' were constant and independent of strain. For $3\% < \gamma < 32\%$, there existed a non-linear region where G' started to decrease but G'' remained fairly constant, indicating a partial breakdown of the elastic structure. At $\gamma = 32\%$, G'' became larger than G', indicating a change from a predominantly solid-like to a predominantly liquid-like behavior. For $\gamma > 32\%$, G'' became increasingly larger than G' as the strain was increased. Pre-shearing and then resting resulted in the same behavior, but the values of the moduli in the linear region were ~20% lower, indicating a temporary or perhaps permanent loss of structure. Within the linear region, values of G' measured dynamically were in good agreement with the values of the relaxation modulus measured in stress relaxation experiments. (Critical values of strain such as those given above would be expected to vary with yogurt and the method of measurement.)

Flow Behavior

Rheological behavior in continuous shear can be determined either by empirical tests, in which no attempt is made to generate fundamental shear stress–shear rate data, or by tests in which such data are generated and then modeled phenomenologically.

The rheological characterization of a yogurt by means of a standardized empirical measurement (e.g., the apparent viscosity at a specified spindle rotational speed in a rotary viscometer, or the area of the hysteresis loop on a plot of torque versus rotational speed obtained with the same type of viscometer) can be useful when the manufacturing process itself is highly standardized; in such a process the rheological character of the final product may be predictable from a simple measurement of this kind.

Stirred yogurt, in particular, is amenable to more fundamental characterization by means of experiments, usually in rotary viscometers, in which instrument-independent values of shear stress and shear rate can be measured. Three approaches are commonly used: the generation of hysteresis loops by increasing and then decreasing the applied shear rate; the measurement of the shear stress as a function of time at constant shear rate; and the measurement of the effect on viscosity of time and shear rate at constant shear stress.

Flow curves and hysteresis loops

Hysteresis in up–down flow curves is a consequence of the highly time-dependent shear-thinning behavior of yogurt. As the recovery of viscosity (i.e., of shear damaged structure) on post-shear resting is usually slight, this behavior has been called ‘irreversible thixotropy’.

Individual flow curves (up, down, or equilibrium) have been modeled with the power-law equation [13], the Bingham equation [14], the Herschel–Bulkley equation [15], and the Casson equation [22].

The Herschel–Bulkley equation has been found particularly useful for characterizing yogurt flow curves. However, one comparative study found that over the complete range of shear rates involved, the following hyperbolic relationship, inspired by the well-known Michaelis–Menten equation, modeled flow curves more accurately:

$$\tau = \frac{Q\dot{\gamma}}{(R + \dot{\gamma})} + \tau_0 \qquad [25]$$

$(Q + \tau_0)$ is the hypothetical asymptotic value of shear stress, τ, at infinite shear rate, and R is the shear rate at $\tau = \tau_0 + Q/2$.

Values of the parameters in these phenomenological models are highly dependent on the experimental procedure used to obtain the up–down flow curves, as is the area enclosed by the curves. This area is proportional to the degree of time-dependent behavior.

The modeling of thixotropic loops can be useful in product formulation. For example, it is found that the addition of pectin to stirred yogurt increases both viscosity and shear stability, whereas the addition of fruit concentrate increases viscosity in a less shear-stable way. Rheological measurements allow rheological properties to be manipulated purposely by the addition of these two ingredients in appropriate proportions.

Constant shear rate experiments

If the initial viscosity of a yogurt (the viscosity at zero time of shearing) can be modeled by a phenomenological equation such as the power-law or the Herschel–Bulkley equation, the decay of shear stress (and thus apparent viscosity) with time at constant shear rate can be modeled by incorporating into the equation a time-dependent structure parameter, λ. The Herschel–Bulkley equation, for example, is then written as

$$\tau = \lambda(k\dot{\gamma}^n + \tau_0) \qquad [26]$$

The value of λ ranges from unity at the start of shearing ($\lambda_0 = 1$, corresponding to $\eta_{\text{app, 0}}$) to an equilibrium value, λ_e, after prolonged shearing that is less than unity and corresponds to an equilibrium viscosity, $\eta_{\text{app, e}}$.

The rate of change of λ with time is given by the following *b*th-order kinetic equation:

$$-\frac{\mathrm{d}\lambda}{\mathrm{d}t} = K(\lambda - \lambda_e)^b \mathit{for}\lambda > \lambda_e \qquad [27]$$

where

$$K = a\dot{\gamma}^d \qquad [28]$$

K is a rate constant, and *b*, *a*, and *d* are empirical constants.

A definition of λ is obtained by combining eqns [5] and [26]:

$$\lambda = \frac{\eta_{\text{app}}\dot{\gamma}}{(\tau_0 + k\dot{\gamma}^n)} \qquad [29]$$

At equilibrium,

$$\lambda_e = \frac{\eta_{\text{app, e}}\dot{\gamma}}{(\tau_0 + k\dot{\gamma}^n)} \qquad [30]$$

By integrating eqn [27] and incorporating the definitions represented by eqns [26], [29], and [30], the dependence of apparent viscosity on time at constant shear rate can be expressed as

$$\begin{aligned}\eta = \Big\{ &\left[\eta_{\text{app, 0}} - \left(\tau_0\dot{\gamma}^{-1} + k\dot{\gamma}^{n-1}\right)\right]^{1-b} \\ &- Kt(1-b)\left(\tau_0\dot{\gamma}^{-1} + k\dot{\gamma}^{n-1}\right)^{1-b}\Big\}^{1/(1-b)} \\ &+ \left(\tau_0\dot{\gamma}^{-1} + k\dot{\gamma}^{n-1}\right) \qquad [31]\end{aligned}$$

Analogous equations have been developed for yogurt by starting with the power law or with an exponential model modified as in eqn [26]:

$$\tau = \lambda k\dot{\gamma}^n \qquad [32]$$

$$\tau = \lambda\left(\tau_0 + \dot{\gamma}\sum_{p=1}^{\infty}\eta_{\text{app}, p}\exp(-t_p\dot{\gamma})\right) \qquad [33]$$

In a study of a commercial stirred yogurt, the decrease in shear stress with time at constant shear rate was modeled in a somewhat different way by means of the Weltman equation [24], modified as follows:

$$\tau = A - B\left[\log\left(\frac{t}{t_{\max}}\right)\right] \quad \text{for } t \geq t_{\max} \qquad [34]$$

where $t_{\max}$ is the time (e.g., 12 s from the start of a 60 min shearing time) at which τ is at a maximum (the overshoot shear stress); thus $A = \tau_{\max}$. The value of τ at zero time, which cannot be expressed by eqn [34], is obtained by a separate linear graphical procedure. This initial shear stress and the parameters *A* and *B* in eqn [34] are quantitatively related to shear rate and temperature.

Equation [31] and its analogs, and eqn [34], are currently the best quantitative descriptions of the time-dependent shear-thinning properties of both stirred and set yogurts.

The Cross equation [7] has been used successfully to model the shear rate dependence of the equilibrium apparent viscosity of yogurt. It has been shown that a vane viscometer gives the same results as that of a bob-in-cup viscometer. An advantage of the former instrument is that it enables measurements to be carried out on yogurt containing fruit pieces.

After fermentation, yogurt must be transported to filling equipment and dispensed into the final containers. The effects of pumping yogurt through pipework and filling heads on the viscosity of the final filled product, which is closely related to texture, can be predicted by a procedure based on straightforward rheological measurements made under controlled shear rate conditions that simulate process conditions. By measuring changes in apparent viscosity over a sequence of shear rates consistent with those existing in different stages of the real process – shear rates being applied for periods of time consistent with yogurt residence times in these stages – the end-of-process viscosity can be predicted. The extent of structural recovery in the filled containers can also be predicted by simple oscillatory tests conducted on the unsheared and sheared yogurt.

Constant shear stress experiments

Processing effects can be predicted by an alternative approach, as follows. The time-dependent shear thinning of yogurt can be described by means of a plot of shear rate against time at constant shear stress (measurements being made using a controlled-stress rheometer). The typical plot shows a steep slope initially, which then decreases with time to a constant positive non-zero value. It has been shown, for yogurt that has been sheared (e.g., in processing and filling operations post-fermentation), that the straight line part of the plot can be predicted from the straight-line part of a similar plot determined for an unsheared (unprocessed) sample. This is done by offsetting the latter plot in a negative time direction by a period that can be related to the work done on the yogurt per unit volume during processing. This approach, which allows prediction of the effect of process handling on the rheological properties of the yogurt as packaged, was demonstrated to be accurate in the case where the 'process' was flow through a straight round pipe (a relatively simple system). It can in principle be applied to (more complex) real processing systems that are amenable to the fluid mechanical analysis required.

Cultured Buttermilk

An Irish study showed that eqn [31] could be used to model the time dependence, at 5 °C, of cultured buttermilk made directly from skim milk. In this particular case, it was found that $b=2$ in eqn [27], and that the dependence of K in the equation on shear rate was relatively slight. The latter finding allowed eqn [27] to be integrated, using an average value of K, to give an equation for shear stress in terms of only shear rate and time:

$$\tau = \left[\lambda_e + \frac{1}{(1/\lambda_0\,\lambda_e) + K\ \ t}\right](\tau_0 + k\dot{\gamma}^n) \qquad [35]$$

Numerical values of the variables in eqn [35], for the particular buttermilk investigated, were as follows:

$$\tau = \left[0.449 + \frac{1}{1.815 + 0.0051t}\right]\left(0.4 + 1.362\dot{\gamma}^{0.385}\right) \qquad [36]$$

An American study found that both 'modern' cultured buttermilk (made directly from low-fat milk, as in the Irish study) and 'traditional' cultured buttermilk (made from cultured cream) were time independent at 25 °C and could be characterized rheologically by the power-law equation [13]; at 10 °C, the modern buttermilk exhibited time-dependent behavior.

It is appropriate to mention here that eqn [31] (with $b=2$) has been found to describe adequately the time-dependent shear thinning of a thickened cream (containing 35% fat, and gelatin as stabilizer). Clearly, this approach to modeling such behavior is especially useful for dairy products.

Fresh Cheeses

Low-total solids fresh cheeses have structures similar to that of stirred yogurt. However, the wider ranges of composition and processing technique lead to a correspondingly wider variation in rheological behavior.

Some are shear thinning, but time independent. Flow curves can be modeled by the power-law equation [13], or by the following expression for apparent viscosity:

$$\log\ \eta_{\text{app}} = \log\ \eta_{\theta=\infty,1} + B\log\dot{\gamma} + \frac{E}{RT} \qquad [37]$$

where $\eta_{\theta=\infty,1}$ is the hypothetical apparent viscosity (Pa s) at a temperature (θ °C) of infinity and a shearing time of 1 s; E is the activation energy of flow (J mol^{-1}), R is the gas constant (8.314 J K^{-1} mol^{-1}), T is the absolute temperature (K), and B is a constant.

Some fresh cheeses exhibit time-dependent shear thinning. For a constant D the fall in apparent viscosity with time at constant shear rate has been modeled by the following equation:

$$\log\ \eta_{\text{app}} = \log\ \eta_{\theta=\infty,1} + \frac{E}{RT} + D\log t \qquad [38]$$

For such cheeses at 1 °C, the relationship between apparent viscosity and time in experiments where the shear rate was oscillated linearly between 0 and 4.5 s^{-1} at a frequency of 0.05 Hz has been modeled for the shear rate range 1–4.5 s^{-1} by the equation

$$\eta_{\text{app}} = \frac{1}{\dot{\gamma}}\left[\tau_{t=\infty} + (\tau_{t=} - \tau_{t=\infty})\ \exp\left(-\frac{t}{P}\right)\right] \qquad [39]$$

where P is the time required for η_{app} to become equal to $(1/\dot{\gamma})\ (\tau_{t=\infty} + (\tau_{t=0} - \tau_{t=\infty})/\text{e})$.

At any given time, the phenomenological relationship between shear stress and shear rate could be modeled well by the Bingham equation [14] for shear rates between 1 and 4.5 s^{-1}. Over the whole shear rate range of 0–4.5 s^{-1} the data could be modeled by the power law equation [13] or the Herschel–Bulkley equation [15] or the Casson equation [22]. The power law was the poorest (though still reasonably good) model. The best model was found to be a modified Bingham equation:

$$\tau = \tau_0 + \eta_{\text{pl}}\dot{\gamma} - \frac{C}{\dot{\gamma} - \dot{\gamma}_0} \qquad [40]$$

$C/\dot{\gamma}$ is the difference between the shear stress value calculated using the Bingham equation and the value found by fitting the experimental results with eqn [40]. $C/\dot{\gamma}_0$ is the value of $C/\dot{\gamma}$ at $\dot{\gamma} = 0$. (The unit of C is Pa s^{-1}.)

Fresh cheeses are viscoelastic. Such behavior can be investigated conveniently by measuring the dynamic rigidity and loss moduli (G' and G'', respectively) using oscillatory rheometry. Such measurements indicate, for example, that a soft cheese similar to Mozzarella behaves in a way typical of a weak viscoelastic gel. In contrast, for double cream cheese, the frequency dependence of G' suggests viscoelastic behavior dominated by a network, whereas the frequency dependence of G'' is similar to that observed for a nonchemically cross-linked polymer. Measurement of the dynamic moduli is a sensitive way of investigating and interpreting the effects of making changes to cheese milk composition and cheesemaking conditions.

The Bird–Leider equation (see eqn [23]) has been found to model the combined viscoelastic and time-dependent properties of whipped cream cheese, but only moderately well; this is perhaps owing to the absence of yield stress as a variable in this equation, which is one of the limitations of the equation. In spite of this, the equation has been found to be satisfactory for modeling the sensory property 'thickness' of whipped cream cheese when shear rate in the equation is replaced by an expression for shear rate in the mouth that incorporates thickness and other relevant variables.

It has been possible to model the stress relaxation properties of commercial fresh cheeses using the Avrami equation:

$$\tau = \tau_{t=0} - (\tau_{t=0} - \tau_{t=\infty})\exp\left[-\left(\frac{t}{R_1}\right)^n\right] \qquad [41]$$

where n = exponent of time and R_1 = relaxation time after 1 s of stress relaxation.

The time-dependent shear thinning, at constant shear rate, of spreadable cheeses made from whole milk pre-concentrated by ultrafiltration has been modeled by eqn [31] with $b = 2$. At a given shear rate, the constant K in eqn [31] changed from one value (K') during the early part of the shearing time to a lower value (K''),which then stayed constant during the remainder of the shearing time. K' decreased with increasing shear rate. K'' was about half the value of K'. These findings may be evidence for macroscopic shear-induced breakdown initially, followed by slower breakdown at a finer structural level.

Empirical rheological measurements made by uniaxially compressing or penetrating the cheese sample can give data of practical usefulness.

Conclusion

In spite of the complexity of milk and the semi-solid products made from it, there has been considerable success in quantitatively elucidating the interrelationships between rheological properties and microstructure, and in providing quantitative descriptions of rheological behavior useful in dairy process design and control.

These is no doubt that future advances in rheology theory, in computing, and, perhaps especially, in the sophistication of rheometers will lead to a deeper understanding.

See also: **Cheese**: Cheese Rheology. **Concentrated Dairy Products**: *Dulce de Leche*; Evaporated Milk; Sweetened Condensed Milk. **Fermented Milks**: Buttermilk; Yoghurt: Types and Manufacture. **Ice Cream and Desserts**: Dairy Desserts. **Milk**: Physical and Physico-Chemical Properties of Milk. **Milk Lipids**: Rheological Properties and Their Modification.

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RISK ANALYSIS

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Introduction

Risk analysis is a formalized scientifically based approach that is recognized by the World Trade Organization as a tool for addressing food safety issues and which shall establish food safety regulations. When carried out correctly, risk analysis provides a tool for the identification, assessment, management, and communication of risk.

In the area of food safety, risk analysis approaches have been applied for many years to assess and manage chemical food hazards (e.g., food additives, veterinary drugs, and pesticides). More recently, risk analysis techniques have been established for addressing microbiological food risks.

The application of risk analysis techniques is an emerging discipline. This article attempts to provide an overview of the concept of risk analysis, the individual steps of risk analysis, and the application of risk analysis.

Purpose and Role

Traditionally, the approach to food safety control, both by the food industry and by public authorities, has been technical, *ad hoc*, and mainly reactive, based upon utilization of experience obtained from many years of exposure to various hazards, taking into account local practices, traditions, and technological possibilities. This approach has proven to be insufficient to ensure public health and fair international trade in foods.

Analyses of major food safety problems that have occurred through the last decades demonstrate that these are, most of the time, the consequence of organizational deficiencies, reflecting defective global organizations for controlling food safety.

Risk analysis provides an opportunity to address these organizational difficulties by systematic integration of scientific understanding of the risks involved and the legitimization of decisions taken.

The rationale for utilizing a formal risk analysis approach is multiple:

1. To assist in the control of the multiple foodborne risks in a proactive and cost-effective way.
   The multiplicity of food safety comprises
   a. potential microbiological foodborne risks (for instance, from 'classical' salmonellosis to emerging pathologies due to protozoa or viruses);
   b. chemical/toxicological risks, such as naturally and environmentally occurring toxicants and residues of chemicals and drugs used;
   c. newly emerging areas of concern, such as allergenicity, antimicrobial resistance, genetic engineering, and nanotechnology.
2. To support national food safety regulation by providing a sound, science-based, systematic and target-focused tool that secondarily facilitates fair international trade. The Sanitary and Phytosanitary (SPS) Agreement of the World Trade Organization has established the tenet that "members shall assure that their sanitary and phytosanitary measures are based on an assessment, as appropriate to the circumstances, of the risk to human, animal or plant life or health, taking into account risk assessment techniques developed by relevant international organizations" (Article 5.1). Codex Alimentarius has established international principles and guidelines for risk analysis.
3. To address the increase in the social unacceptability of food risks.

As food becomes objectively safer, the remaining and occasional risks are even less tolerated by the public at large, a trend that is enhanced by the general public feeling increasingly alien to food safety control activities (decisions are perceived to be mainly the affair of the food industry and/or the public agencies having jurisdiction).

The application of the risk analysis concept has, however, also some disadvantages when used to support national food legislation. Many legislative measures are multifunctional as they address public health issues as well as other issues such as wholesomeness/suitability of foods, environmental protection, and basic animal welfare. Therefore, the challenge for legislators is to achieve sufficient transparency in the objective(s) of reasoning for any such measure to avoid confusion.

Risk analysis is usually described as a process consisting of three elements: risk assessment, risk management, and risk communication. It is a decision-oriented process and making decisions is a managerial activity (**Box 1**).

Box 1 Hazards and risks

The meaning of the terms hazards and risks is often confused. This is due mainly to translation problems as, in many languages, these terms are directly translated into the same word.

Although often synonymous in everyday life, they have taken different meanings in the technical language used in risk analysis.

A hazard is a biological, chemical, or physical agent in, or a condition of, food with the potential to cause an adverse health effect (Codex Alimentarius).

A risk is the function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard in food (Codex Alimentarius). In more common words, a risk is the likelihood and severity of a failure causing deaths or illnesses among consumers.

For instance, the probability of humans being affected by the survival of a pathogenic microorganism during pasteurization is the risk while the microorganism itself is the hazard.

Risk analysis should not be confused with the similar term hazard analysis, the latter constituting the assessment steps of the hazard analysis and critical control point (HACCP) system. Although hazard analysis contains some of the same steps as does risk analysis, it is principally limited to a single type of food from a single processing line (or from the specific food chain it has been following). Hazard analyses of two processing lines/food chains are independent and may result in different outcomes (HACCP plans), as the objective is to ensure the safety of the specific food lots resulting from the specific process. Risk analysis is principally broader in scope, typically addressing one or more food types (e.g., a whole food category) from multiple manufacturers/food chains, the objective being to protect the health of the population.

Risk Assessment

Risk assessment is the scientific part of risk analysis that is initiated and commissioned by risk managers. The purpose is to estimate the severity and likelihood of harm from exposure to a certain hazard by furnishing all scientific data relevant for the evaluation. The output might, for example, be an estimate of the annual rate of illness per 100 000 inhabitants or an estimate of the rate of human illness per eating occurrence.

The scientific data needed are both qualitative and quantitative. They concern the nature and sources of the hazard, how it affects human health, and how it behaves under various conditions. In addition, scientifically based information on the potential exposure of humans is needed.

The risk assessment process comprises four steps: hazard identification, exposure assessment, hazard characterization, and risk characterization. The information is passed to the risk managers to assist them in continuing the risk management process.

Codex Alimentarius has established general principles for the conduct of risk assessments.

Hazard Identification

Hazard identification is predominately a qualitative process, the purpose of which is to identify the hazards of concern associated with food.

Hazards can be identified from relevant data sources. Information on hazards can be obtained from scientific literature, from databases such as those in the food industry and government agencies, and through expert elicitation/consultation. Relevant information includes data in areas such as clinical studies, epidemiological studies and surveillance, laboratory animal studies, investigations of the characteristics of the hazards, the interaction between hazards and their environment through the food chain, and studies on analogous hazards and situations.

Exposure Assessment

The purpose of exposure assessment is to obtain a quantitative assessment of the actual or anticipated human exposure to a food hazard. It is normally based upon realistic exposure scenarios, including the potential extent of food contamination, and on actual dietary information. Susceptible and high-risk population groups with regard to acute, chronic (including long-term), cumulative, and/or combined adverse health effects should also be brought into consideration.

Typical factors considered include the following:

1. the frequency and level of contamination of food over time, which are influenced by
   a. the characteristics of the hazard,
   b. the nature/ecology of the food,
   c. the initial contamination of the raw material,
   d. the level of process controls,
   e. the methods of processing, packaging, distribution, and storage of the foods,
2. patterns of consumption, which relate to socioeconomic and cultural backgrounds, ethnicity, seasonality, age differences (population demographics), regional differences, and consumer preferences and behavior.

In practice, exposure assessment of foods can be qualitatively categorized according to (1) the likelihood that the foodstuff will or will not be contaminated at its source and (2) whether or not the level of the hazards in the food can increase over time, taking into account the potential for abusive handling.

Hazard Characterization

The purpose of hazard characterization is to provide a qualitative or quantitative description of the severity and duration of adverse effects that may result from the ingestion of contaminated food. The level of the hazard that causes an adverse health effect (dose–response assessment) should be estimated if such data are obtainable.

Several important factors that are considered in hazard characterization relate both to the hazard itself and to the human host.

Factors related to the hazard:

- potential of the hazard to replicate;
- virulence and infectivity of the hazard;
- impact of interactions between the host and the environment;
- potential for transfer of genetic material (e.g., antibiotic resistance, virulence factors);
- potential for spread through secondary and tertiary transmission;
- incubation period (clinical symptoms can be delayed substantially following exposure);
- potential for changed pathogenicity due to the attributes of a food, for example, fat content.

Factors related to the host:

- genetic factors;
- increased susceptibility due to the breakdown of physiological barriers;
- individual host susceptibility characteristics such as age, health and medication status, concurrent infections, immune status, and previous exposure history;
- population characteristics such as population immunity and population behavior, and persistence of the organism in the population.

Risk Characterization

Risk characterization represents the integration of the results of hazard identification, hazard characterization, and exposure assessment, the purpose being to provide qualitative or quantitative estimates of the likelihood and severity of the adverse effects, which could occur in a given population. The data may permit only a qualitative estimate of risk.

The degree of confidence in the final estimation of risk depends on the variability, uncertainty, and assumptions made in all previous steps.

Variability represents heterogeneity within biological systems and populations, while uncertainty represents a lack of precise knowledge associated either with the data themselves or with the choice of model. Variability and uncertainty arise at all steps of the risk assessment process:

- Hazard identification, where uncertainty or variability may arise because of (1) misclassification of the agent, (2) the potential unreliability of the screening method used to identify the hazard, or (3) problems in extrapolating the information provided by the screening test for predicting human hazards.
- Hazard characterization, where uncertainty and variability arise when extrapolating from high to low doses and from one species to another and when considering varying sensitivities within human populations. When models are used, additional uncertainty as to whether they represent actual biological processes is introduced. For instance, the transfer of data from animal studies into estimates relating to humans involves uncertainties. For this reason, a 100-fold safety factor is often applied to account for likely inter-species differences in susceptibility.
- Exposure assessment, where many uncertainties arise due to the lack of detailed data on, for example, the level of the agent in food products and the frequency, duration, and magnitude of human intake of food products, and changes in the concentration of the chemical or microbiological agent during storage, processing, and preparation of the food product. There is also a great deal of variability in dietary habits.
- Risk characterization, where uncertainty and variability arise because of the uncertainties and variability involved in its constituent steps and in the model used for constructing the distribution of individual or population risk.

Risk Assessment of Chemical Hazards

Chemical risk assessment in one form or another has been applied to the evaluation of various chemical hazards in foods for many years. Assessment of food additives and that of contaminants fundamentally differ because food additives, which are generally of low toxicity, are deliberately added to food, whereas contaminants are unavoidable and generally demonstrate greater potential toxicity. Food additives can be controlled easily, while the elimination of contaminants from foods incurs cost, such as reduction in food availability and/or affordability.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) and JMPR (Joint FAO/WHO Meeting on Pesticide Residues) carry out risk assessments of the following substances:

- food additives, resulting in the establishment of acceptable daily intakes (ADIs);
- food contaminants and naturally occurring toxicants, resulting in the establishment of provisional maximum tolerable daily intakes (PMTDIs) or provisional maximum tolerable weekly intakes (PMTWIs) where no observed effect can be identified; in other cases, other outcomes are provided;
- veterinary drug residues, leading to the establishment of ADIs and, taking into account good practices, the establishment of maximum residue levels (MRLs) in target animal tissues, milk, and egg;

- pesticide residues, resulting in the establishment of ADIs and acute reference doses and, taking into account good practices, the establishment of MRLs in foods.

The approach is somewhat different from risk assessment, but does have the advantage of preventing problems associated with deciding on an acceptable level of risk.

The acceptable or tolerable intake is an indication of both the magnitude and the duration of acceptable intake. The ADI usually represents an acceptable average daily intake for the life span of an individual. Tolerable intakes for contaminants should be compared with intake surveys of appropriate duration. In cases where no threshold is thought to exist, such as aflatoxins, JECFA does not allocate tolerable intake values but recommends that the level of contaminant in food be reduced to as low as reasonably achievable (ALARA). The ALARA level is regarded as the concentration of a substance that cannot be eliminated from a food without having to discard that food or severely compromising the availability of major food supplies.

Microbiological Risk Assessment

The risk assessment process was originally developed for chemicals. Extending the practice to microbial pathogens poses significant difficulties. Therefore, most microbial risk assessments currently have a qualitative base. However, in recent years, the interest in qualitative approaches to microbial food safety has increased dramatically and quantitative models for specific pathogen/food combinations are developed in many circles.

One difficulty relates to the fact that microbial pathogens can multiply as food moves from the farm to the table, making intake assessment very difficult. In addition, many data gaps exist, limiting the precision necessary for quantitative risk assessments. For example, little information is available to accurately estimate the relationship between the quantity of a biological agent and the frequency and magnitude of adverse human health effects, particularly for susceptible populations.

Microbial pathogens multiply and die, and the biological interactions are complex. The contamination levels of the raw material entering the food chain dictate the character of the initial microflora, but this can be modified markedly by subsequent events. Additionally, there are marked differences in the virulence and pathogenicity of animal and environmental strains for humans, and the individual interactions of host and pathogen are very variable.

Factors to consider for exposure assessment include the frequency and level of contamination of raw materials, possibilities of post- and cross-contamination, and the level of contamination of the food during the shelf life of the product. The characteristics of the pathogenic agent, the microbiological ecology of the food, the level of basic hygiene, the level of sanitation and process controls, and the methods of processing, packaging, distribution, and storage of the foods impact these factors.

Microbial pathogen levels may be kept low, for example, by proper time/temperature controls during food processing, but they can increase substantially with abuse conditions. Therefore, exposure assessment should include different scenarios describing the pathways from production to consumption and should be constructed to predict the range of possible exposures.

Construction of scenario trees for all steps from production and processing through to intended end-uses of a food describes the pathway for exposure, and targeted research is often required to accumulate appropriate microbiological data. Predictive modeling plays an important role in this respect. Unfortunately, dose–response data to service the hazard characterization component are currently very limited.

Because of the wide variability inherent in much microbial data, Monte Carlo simulation modeling is being used increasingly to generate probabilistic risk estimates that are biologically realistic.

FAO and WHO have recently established an expert body system, similar to JECFA and JMPR, the tasks of which are to carry out microbiological risk assessments.

Physical Risk Assessment

Risk assessment of physical hazards can be achieved readily. The characteristics of the hazards do not usually change once they have been introduced to the food, and adverse health effects can usually be subjected to simple ranking systems to generate estimates of risk.

Risk Management

Risk management is a continuing process and constitutes the managerial and political part of risk analysis. It concerns the transfer of the results of risk assessment into actions in accordance with established political priorities. Risk management sets priorities, commissions risk assessments, and implements, monitors, and reviews the chosen strategies and options.

The risk management process comprises four steps: risk evaluation, risk management options assessment, implementation, and monitoring and review.

Risk Evaluation

The initial part of the risk management process sets the stage for risk assessment, and evaluates the outcome of the risk assessment process, which should result in a risk estimate.

Risk profiling

A risk profile is developed when a new food safety problem has been identified or if surveillance information shows an unacceptable increase/level of a disease or a hazard. The food safety problem and its context are described briefly, including the size and nature of the problem, available data, type of foods involved, main sources, the values expected to be placed at risk (e.g., human health, economic concerns), stakeholders perceptions, distribution of risks and benefits, and what immediate action(s) may be necessary, including whether a risk assessment should be carried out.

Goal setting/acceptable level of protection

To guide the rest of the decision-making process, the goals for the risk management activity need to be identified as early as possible. However, the results of a subsequent risk assessment process and subsequent steps of risk management may identify needs to modify or redefine the goals. One management goal can be to establish relevant risk-based metrics such as food safety objectives (FSOs) and performance objectives (POs).

Any goal should be related to the acceptable level of protection – defined by the SPS Agreement as the level of protection deemed appropriate by the member state to protect human life within its territory and could, for instance, be expressed as the acceptable number of cases of a particular foodborne disease per million inhabitants. Usually, when no significant food-related public health problem exists, the acceptable level of protection is the level obtained from the sanitary measures already practiced.

Decisions on acceptable levels of protection should be determined primarily by human health considerations, but other factors may legitimately be taken into account, for example, technological feasibility and economic/political/social concerns (**Box 2**).

Different approaches to acceptable levels include

- ‘zero-risk’ policies, for example, *de minimus*, ADI;
- risk-balancing policies, for example, cost–benefit, ALARA;
- risk threshold policies, for example, specified levels of risk deemed acceptable;
- risk comparison policies, for example, comparison between sources, precedence;
- procedural approaches, for example, negotiation, consensus building.

Risk assessment policy

Risk assessment policy setting serves to protect the essential scientific independence and integrity of the risk assessment. It provides guidelines for value judgments and policy choices that may be needed at specific decision points in the risk assessment process and addresses how to ensure transparency, clarity, and consistency in outcome and how to deal with uncertainties (e.g., application of safety factors) (**Box 3**).

Box 2 The SPS Agreement

The key point in the Sanitary and Phytosanitary (SPS) Agreement is that any sanitary measure has to be based on science. A government cannot restrict trade or maintain a restriction against scientific evidence. Science can, of course, be misused. Therefore, the Agreement also specifies that the scientific approach applicable is the scientific assessment principles and evaluation procedures established by international organizations such as Codex Alimentarius.

The SPS Agreement stipulates government’s rights to decide what they regard as the appropriate level of protection, or in other words, the right to decide on the acceptable level of risk that should be valid on their territory. Therefore, the level of protection may differ between countries, but it shall be determined using harmonized risk analysis procedures. Therefore, the importance of transparency in the risk assessments carried out is obvious. A government must be able to show which factors it has considered and what have been the results of its consideration. This is to ensure that potential differences between the regulations of two countries (e.g., differing maximum limits) are not due to differences in scientific evidence but only due to differences in the politically decided acceptance levels.

Box 3 Examples of risk assessment policies at specific decision points in chemical risk assessment

- Reliance on animal models to establish potential human effects.
- Using body weight scaling for inter-species comparison.
- Assuming that absorption in animals is approximately the same.
- Using a 100-fold safety factor to account for likely inter- and intraspecies differences in susceptibility, with guidelines for deviations that are permitted in specified situations.

Commissioning of risk assessments

Commissioning of the risk assessment process is a risk management activity that aims at ensuring that the needs of the risk managers are addressed and that resources are used in the most effective way. Typically, it includes clear statements of purpose and scope of the assessment addressing the risk management goals.

Consideration of the result of risk assessment

When the results of the risk assessment are available, a risk estimate is established. Risk estimates should take into account variability, uncertainties, and assumptions made during the risk assessment process.

Risk Management Options Assessment

Risk management option assessment typically includes four steps:

- identification of available management options;
- selection of the preferred management option, including consideration of an appropriate level of protection (ALOP; see above);
- evaluation of the impact of the preferred management option on other factors;
- final management decision targeted at appropriate stages throughout the food chain.

Identification of available options

Risk management options include consideration of all general and hazard-specific measures.

General risk management measures

- developing or encouraging the development of good agricultural practices (GAPs), good veterinary practices (GVPs), good hygienic practices (GHPs), and good manufacturing practices (GMPs);
- developing, or encouraging the development of, guidelines for the establishment of hazard analysis and critical control point (HACCP) systems, and establishing public inspection schemes and audit procedures;
- setting up approval and certification procedures;
- promulgating awareness and developing educational and training programs for consumers and industry.

Measures targeted at the individual commodities/hazards

- setting risk-based targets for benchmarking purposes, for example, FSOs (or POs at the end of shelf life) for a particular food safety hazard, leaving flexibility to the industry to select appropriate control measures to meet the target;
- setting maximum limits (e.g., MRLs, microbiological criteria) in support of testing procedures intended to determine the presence (or levels) of specific hazards;
- establishing specific control measures required to be used by industries that do not have the means to establish appropriate measures themselves or that adopt such control measures;
- tailoring frequencies of testing and monitoring for each commodity/hazard;
- requiring export/import certificates.

Selection of options

The SPS Agreement states that sanitary measures must not be more trade restrictive than required to achieve the ALOP, taking into account technical and economic feasibility (Article 5.6). A measure would be more trade restrictive than required if another equivalent and reasonably achievable measure is significantly less restrictive.

The outcome of the risk management process for a specific hazard will differ in various societies, due to natural or cultural differences. Such difference can be scientifically justified, for instance in relation to the exposure situations in different countries. Differences from the contaminant levels recommended by Codex Alimentarius may, for instance, be justified where the average body weight differs and where relatively little average consumption permits higher threshold levels in a particular food.

Also, the prevalence of various foodborne pathogens in the food chain and variation in foodborne disease patterns may justify different risk management outcome.

In selecting the preferred option, the consequences of impact on other factors should be estimated, such as

- impact on consumption patterns (e.g., nutritional consequences of restricting food availability),
- possible introduction of substitute risks (i.e., increasing another risk by reducing a risk, for instance increasing microbial risks when not allowing a preservative),
- impact on public acceptability of measures that intervene in cultural patterns and traditions (e.g., requiring that cheeses be made from pasteurized milk).

Examples of socioeconomic and technological factors may be taken into account and these could, for instance, result in the best management option being

- control at the source rather than later in the food chain,
- regulation through detailed GMP rules rather than, for example, mandatory HACCP systems,
- food safety verification through end product testing rather than reliance on HACCP systems (for instance, where the origin of the food is unknown),
- reliance on labeling, the effectiveness of which has been subject to validation.

Use of risk-based targets

An important element in risk management is the responsibility of competent authorities to specify the level of control that food businesses should achieve, typically in the form of food safety metrics, such as microbiological criteria (for analytical testing purposes), process criteria (e.g., pasteurization temperature), and product criteria (e.g., water content of milk powder). New metrics have been developed by Codex Alimentarius (see **Box 4**), which are related more directly to public health outcomes through risk assessment processes.

FSOs assist in making the ALOP operational, that is, by translating the health risk into food-related targets that can be used readily by food business operations to design their food safety management systems appropriate to location and role within the particular food chain. Conceptually, the FSOs can be viewed as the consumers'

Box 4 The three new risk-based food safety targets

- Food safety objective (FSO), defined as the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP).
- Performance objective (PO), defined as the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to an FSO or ALOP, as applicable.
- Performance criterion is the effect in the frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a PO or an FSO.

maximum level of exposure to a hazard that achieves the ALOP. As such, FSOs articulate the overall performances expected of a food chain in order to reach a stated or implied public health goal.

POs assist in making the FSOs operational throughout a particular food chain. A PO is a means by which a particular control measure combination can be shown to contribute to achieving an FSO. The PO describes the expected outcome of the food chain up to the point of its application.

A performance criterion (PC) is an expression of the target change in the frequency and/or concentration of a hazard in a food that must be obtained by the application of one or more control measures. PC can be expressed, for instance, in terms of a desired reduction (or acceptable increase) in the concentration of a hazard in the course of a particular control measure.

Equivalence

Differences in food safety programs inevitably exist between countries. Therefore, determination of the equivalence in the sanitary measures applied in importing and exporting countries is becoming a priority issue in the international trade. The SPS Agreement requires that sanitary measures of other countries are accepted as equivalent, even if they differ from their own or others, if the exporting country objectively demonstrates that its measures achieve the ALOP established by the importing country (Article 4.1).

The quantitatively expressed risk-based metrics, FSOs and POs, are also intended to be applied as a means to demonstrate whether different processing lines, plants, or food chains can achieve equivalent outcomes.

Risk Communication

Risk communication is the third component of risk analysis, and is a central and integral part of effective food safety management.

Every stage of risk management should rely on an exchange of information and opinions about risk between risk managers, risk assessors, and all other stakeholders concerned about or affected by the problem and the risk management decision. Risk communication and involvement of stakeholders are crucial for open, transparent, and effective decisions. Communication of correct and updated risk assessment information to the food manufacturers is also crucial for obtaining correct hazard analyses and designs of HACCP programs.

Risk communication aids in considering the different, and at times conflicting, interpretations about the nature and magnitude of the risk; it offers an opportunity to bridge gaps in understanding, language, values, and perceptions; it ensures that public values are considered; it generates better accepted and more readily implemented risk management decisions. In brief, it supports democratic decision making. Poor risk communication will almost always increase conflict and distrust over risk management decisions.

See also: **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health; Processing Plants. **Policy Schemes and Trade in Dairy Products**: Codex Alimentarius; Trade in Milk and Dairy Products, International Standards: World Trade Organization.

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RODENTS, BIRDS, AND INSECTS

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Introduction

Basic pest control in the dairy processing plant is an essential prerequisite for a food safety program. Unfortunately, many processors have never established a systematic, organized pest control program. This article outlines the pests of concern and steps that the dairy processors can take to start a pest control program. All dairy processing facilities, regardless of size, must maintain a hygienic facility, and this requires a sanitation program. One section of the sanitation program should address the exclusion/control of pests: rodents, birds, and invertebrates. Current good manufacturing practices (cGMPs) specified in the Code of Federal Regulations (CFR) clearly state that pests cannot be present in the food-processing environment. To prevent infestation, the processor must take a proactive approach to stopping these pests from threatening the safety and quality of the product.

The pest control program is both a stand-alone program and a prerequisite program under hazard analysis and critical control points (HACCP). Most small food plants must decide whether to maintain a pest control program themselves or contract the program to a pest control company. There are positive and negative aspects of each approach. **Table 1** illustrates key differences between plant operated programs and contract pest control programs.

Many small dairy processing facilities hire a pest control company because the processor lacks the personnel and expertise to run such a program. Such pest control contractors must be reputable and have proper training and experience. A pest control contractor must provide records and reports to the processor, verifying that the program is effective and operating successfully. This verification is usually done through visual inspection for pests and/or evidence of pests in the plant or product. The processor must maintain these records with the plant's hazard analysis records to prove that the contracted pest control program is effective. The verification records should include evidence of contractor training and certification to apply pesticides in a food manufacturing environment.

Many processors choose to develop and maintain a pest control program themselves. An effective program can be developed in-house if the processor understands how to control pests. The program should consist of several written sections and include the following items:

1. *Pest control procedures*: The activities performed to control each type of pest. The written procedures should be detailed and include frequency of action.
2. *Recordkeeping*: The documentation of each performed activity. The records must be accurate, up-to-date, and include inspection for evidence of pests in each plant area.
3. *Responsible individuals*: The person(s) who are responsible for performing pest control procedures and recordkeeping and the supervisor who is responsible for signing off on reviewed records.
4. *Deviation*: Evidence of a pest problem is a subjective determination that requires expertise. For example, periodically finding a cockroach under a waste bin may be accepted as evidence of a possible problem, while finding many cockroaches would be a deviation, that is, when an allowable limit has been exceeded.
5. *Corrective measures*: Written action steps in the plan that will be performed if there is a deviation from the pest control program. Often, they may include increasing control procedures, retraining of employees, cleaning up the area, and other measures.
6. *Verification and validation*: Written scientific evidence that the procedures are effective in controlling pests. This material is often available from chemical, trap, and pest control equipment makers. Also, verification is documentation of visual inspection for evidence of pests.

Common Pests in Dairy Plants and Ways to Control Them

Potential pests in the dairy plant range in size from mammals and birds down to barely visible invertebrates. While problems can arise from almost any animal seeking to take advantage of the food available, including stray dogs and cats, the most common sources of pests are discussed below.

Rodents

Rodents include rats and mice. They must be controlled in and around a food plant because they carry and transmit disease and because they can cause significant economic loss by damaging food containers, contaminating food with their droppings, and consuming food. Two

Table 1 Major differences between internal and external pest control programs

	Plant operated program	*Contracted program*
Upfront costLower	Higher	
Processor time commitment	High – at least one dedicated employee	Minimal – need to periodically meet the contractor
Pest control expertise	Usually low	High
Recordkeeping	Plant supported	Contractor maintained
Equipment and chemicals	Plant must obtain	Contractor provided
Validation materials	Processor obtains/develops	Contractor provided
Overall benefits	Must be evaluated by the processor, taking into account all the factors necessary to develop and maintain the program	

major species of rats are found in and around human habitation: the gray or Norway rat (*Rattus norvegicus*) and the black or roof rat (*Rattus rattus*). The house mouse (*Mus musculus domesticus*) is the common mouse species prevalent around human population in the United States and Europe. Both rats and mice reproduce rapidly, with rats having 20 offspring per year and mice up to 35 young per year. Both mammals are primarily nocturnal, but they leave behind several signs of infestation. Signs of rodents are as follows:

1. *Droppings*: Fecal matter is a sign of the presence of rodents, and the quantity can indicate the extent of infestation.
2. *Visual sightings*: Seeing rats or mice often indicates a serious and probably well-established infestation, but most experts believe that visual sightings are the least reliable indicators.
3. *Noises:* Shrill squeaks, gnawing sounds, and scurrying sounds could be caused by rodents.
4. *Smudge marks*: Rodents emit oily lipid material from their fur and leave greasy smudges at entry points and frequent travel paths. Rat smudge marks are often more noticeable than those left by mice.
5. *Tracks*: Coating the area around suspected entry points and travel ways with talc, chalk, or flour can detect tracks and tail marks to identify locations for bait station or trap placement.
6. *Gnawing*: Both rats and mice chew and gnaw materials, which is a sure sign of the presence of rodents. Rats and mice are known to gnaw the insulation of electrical wires, causing fire hazards. Mice are known to cause extensive damage to insulation materials.
7. *Urine stains*: Both rats and mice leave urine stains, which can be detected with long-wavelength UV light as a yellow-to-blue fluorescent spot.

Elimination of harborage is the most effective way to control rodents. This includes removing all general clutter from the food plant and storage areas to eliminate rodent hiding places. Maintain an open, well-kept perimeter around the processing plant to discourage rodent activity; a bare concrete or bitumen surface is ideal. Next, food and water sources must be eliminated. This would include environmental management to reduce or eliminate free water and food sources. Third, rodents must be denied entry into the food plant. This would include filling all structural cracks, screening fan and vent openings, and installing drain covers to prevent rodent entry. It has been shown that a mouse can squeeze through a 6 mm gap, for instance under a poorly fitting door. Mice and black rats are also exceptional climbers, which means openings should be located and closed at all levels in the facility.

Next, a physical control system should be included. Physical control systems would include strategically placed poisons, glue boards, bait boxes, ultrasonic devices, and traps, described in more detail below.

1. *Toxic baits and concentrates*: Primary types are the anticoagulant baits; they are relatively safe to use, inexpensive, and effective. Single- and multidose anticoagulant products are available, as are products with active ingredients other than anticoagulants. Prebaiting with similar nonpoisonous bait may be effective if the rodents exhibit bait shyness. Regular rotation of brands and formulations of baits may also deter bait shyness. Poisons may be administered in bait blocks, liquid baits, pelletized baits, or treated grain.
2. *Toxic tracking powders*: Tracking powders are designed to kill the rodents when they groom themselves. These powders are placed along rodent travel ways or in burrows.
3. *Trapping*: Traps are a safe and effective method of eradication, especially for mice and roof rats. Rodents, especially rats, can become trap shy. Glue traps are also effective, and they may trap cockroaches as well.
4. *Ultrasonic devices*: There is controversy about the effectiveness of using ultrasounds (above 30 000 Hz) to repel rodents. Some devices alter the wavelength and direction of sound, and most are somewhat effective when placed at openings to food plants. Most rodents become used to ultrasonic devices on exposure.

Rodenticides can be used in food-processing plants when placed in secure/tamper-proof stations and restricted to areas where food is not processed (warehouse, storage and service spaces, utility rooms/closets, offices, etc.).

Normally, three perimeters are established for physical control measures. First, bait stations are positioned at the perimeter of the processing plant fence. Second, the outside wall of the plant should be spotted with bait boxes placed directly against the wall, with entry holes to the boxes parallel to the wall. Boxes should be locked and chained to prevent tampering. Finally, a third perimeter of traps inside the plant should be concentrated at areas of high rodent density and near entrances to the plant. All bait stations should be numbered and inspected once each week. Traps should be inspected daily. The density of bait placements may need to be adjusted upward during the fall when a large number of rodents seek winter harborage inside buildings. Norway rats most often build their nests below ground and may be effectively controlled by baits placed directly in their burrows, if that can be done without risk of exposure to nontarget animals or tampering. Roof rats present special control problems since they typically nest in overhead areas. Solid and liquid baits should be placed in attics or above drop ceilings. Block baits or traps should be attached to rafters, joists, and sills in open overhead spaces.

Birds

Several species of birds harbor disease and pose a risk to food plant hygiene. The most common species involved are pigeons (*Columba livia*), sparrows (*Passer domesticus*), and starlings (*Sturnus vulgaris*).

Birds pose a threat to the food processor by carrying disease-causing microorganisms, by contaminating product areas with excreta and feathers, or by carrying external parasites such as mites. The most common microorganisms spread by birds are *Salmonella* spp. Up to 50% of house sparrows were found to contain these microorganisms. *Campylobacter jejuni* has also been commonly isolated from wild birds.

The best and most effective means of controlling birds is to eliminate nesting and feeding sites on the building(s) and in the vicinity. This includes initial construction of window, door, and ledge areas to prevent roosting and nesting. Birds are difficult to eradicate once they frequent a dairy. Once a bird problem develops, an effort should be made to scare and deter birds from roosting areas.

There are a number of common bird repellent methods:

1. *Scaring devices*: Decoys of natural predators, such as owls and hawks, have been used to scare birds but often become ineffective after birds learn to ignore them.
2. *Sticky pastes*: Pastes can be applied to roosting areas to entangle birds and frighten them away.
3. *Electrical wires*: Wires that emit a shock to roosting birds can be effective but are difficult to maintain and costly to operate.
4. *Netting*: Placing netting or chicken wire over nesting sites such as trusses on a loading dock can be very effective. This has been used extensively to prevent pigeons from roosting on older commercial buildings where there are plenty of wide ledges, and on monuments and federal buildings in Washington, DC.
5. *Entry barriers*: Devices designed to block entry to a building, such as automatic doors, vertical plastic strips, and even high-velocity air curtains, are available. A double barrier system is often needed as some birds, for example, robins, can learn to get around strips by hitching a lift on a forklift truck.
6. *Needle strips*: Needle strips are applied to ledges, rooflines, and other roosting points. They have been shown to be very effective if installed correctly.

Traps can effectively remove bird pests. Starlings are the most easily trapped bird pests. Traps can become expensive, because they must be examined regularly so that accidentally trapped nontarget species are not destroyed. (Bats, for instance, are very heavily protected under European law.)

Baiting and poisoning of birds is debatable, and highly contentious. This method is usually a last resort when other means of control have failed. Poisons are indiscriminate, having the potential to harm desirable species of birds as well as pest birds. It is recommended that only professional pest control applicators use toxicants for bird pests. Several chemical control agents are commercially available, and Avitrol is one of the most commonly used chemical. There is a fumigant formulation that is available for use in warehouse areas. As with all chemicals, one should follow the manufacturer's instructions on application and use.

Insects

Insects are the most common source of invertebrate infestation problems and may be divided into those that essentially crawl, for example, cockroaches, and those whose adult forms normally fly.

Cockroaches

There is no insect, other than the housefly, that is more easily recognized and detested than the cockroach. Cockroaches have been shown to transmit diseases including those caused by pathogenic foodborne bacteria such as *Salmonella* spp., *Vibrio cholerae*, and *Staphylococcus aureus* by carrying these in their gut and also on the exterior surface of the body.

Each species has specific habitat preferences, although any species could be found in a food plant building. A good way to detect cockroaches is to enter a darkened production or storage area, turn on the lights, and quickly look for cockroaches scurrying back into hiding. Cockroaches may also be found by inspecting inside the electrical junction boxes, receptacles, and control panels, or by looking behind objects and in floor drains. Glue traps are often a good monitoring device; some come equipped with a pheromone attractant. The use of flushing gases (a number of pyrethroid aerosol products are very good for this purpose) is a common method of driving them out in the open. These materials are so highly repellent that a single squirt into a suspect crack or crevice can cause the cockroaches to come out into the light. Also, look for droppings and egg cases, which indicate their presence.

Control of cockroaches starts with the elimination of debris (especially cardboard boxes that could harbor the insects or their egg cases) and elimination of their harborage. This is done by sealing and filling cracks and crevices and maintaining a sealed, smooth surface throughout the plant in production and nonproduction areas. Seal junction boxes and trunking, receptacles, and control panels. Seal openings around conduits and pipes where they pass through walls and ceilings. Inspect shipments (packaging, ingredients, etc.) and reject infested shipments. Chemical control requires that European Community (EC)- or United States Environmental Protection Agency (USEPA)-permitted insecticides be used in the food plant. These products are generally formulated as sprays, aerosols, or dusts. Dry powders and dusts, such as boric acid and insecticide powders, take advantage of the cockroaches' habit of preening themselves. It is important to understand that no pesticide can be used in a food-processing plant unless the statutory authority, for example, the EC or EPA, has approved such use.

Flying insects

The most common flying insects are the housefly and fruit fly. A single housefly has been estimated to carry up to 3.6 million bacteria. Flies transmit disease by spending part of their life in direct contact with or in close contact with fecal matter or decaying material. Flies must liquefy their food before ingestion, so they secrete salvia (often called vomitus) onto surfaces. Flyspecks are dried vomitus and fecal material. The movement of flies from unwholesome sources to fresh food products, processing equipment, and other surfaces provides many opportunities to transmit disease-causing bacteria. The common housefly is a known carrier of diseases and pathogens, including *Listeria* spp. and *Salmonella* spp. It has been estimated that in a 6-month period, a pair of houseflies and their offspring would total 191 000 000 000 000 000 000 if all survived.

Removal and elimination of breeding sites is a key to fly control. This primarily involves avoiding the availability of garbage. Garbage must be kept away from doors in sealed/enclosed containers and removed frequently; in addition, waste disposal areas must be regularly cleaned and properly maintained. Next, flies must be excluded from entering the food-processing facility. This includes using air curtains (air screens) and/or doors that close automatically.

Electrocution traps with blue fluorescent light traps are effective in reducing flying insects, including flies. One drawback of these electrocution traps is that they can literally cause the fly to explode, throwing an aerosol of fly matter into the air. As these particles can drift down some distance from the trap, it is best to place these traps away from food-handling areas, or well removed from food-handling surfaces in these areas. Blue-lighted sticky traps, baited jug traps and strips, or sticky ribbons are a safer alternative in these areas. Dead flies should be removed from traps at regular intervals. Catch basins of electrocution traps or jug traps, and strips should be cleaned daily. Sticky devices should be replaced at least once a week. Other commercial methods utilize insecticidal sprays or fogs to suppress flies, but exclusion should be the main line of defense.

Pests of Stored Products

These are primarily invertebrates that use the food as both nourishment and a habitat. They are usually small insects that infest and destroy foods during all stages of their life cycles. This group includes beetles, weevils, borers, and moths. Stored product pests are not generally associated with disease, as are cockroaches and flies, but they are considered a major food contaminant. As a group, they prefer dry products such as cereal grains and flours, but other foods such as nuts and dried fruits may be infested, as well.

Weevils infest stored grain and cause economic losses worldwide. The life cycle of most weevils is 4–5 months, and they can infest nearly every cereal grain. Flour moths lay their eggs in flour or meal, where the larva destroys the product, and they are important grain pests. A variety of beetles can infest foods and food ingredients. These include grain beetles, flour beetles, and others.

The purchase of quality, pest-free grain and food ingredients is the first step in the prevention of stored product pests. Regular cleaning of storage areas and processing equipment to remove ingredient spills and accumulated dust is also important. Fumigation of empty storage bins with phosphine, ethylene oxide, or carbonyl sulfide is an effective control option for stored product pests, subject to statutory approval in the country of operation. Similarly, fumigation may be used in facilities when processing operations are over for the day.

Traditional cheesemakers and affineurs (those who mature bought-in cheeses) can have significant problems with cheese mites, which feed on the rind of cheeses that are not encapsulated in barrier films or wax.

Integrated Pest Management

Integrated pest management (IPM) is a strategy to manage pest populations safely and economically through a well-balanced combination of control practices. The small processor should begin a pest control program by determining which activities will best control each pest most effectively.

1. *Inspection (monitoring)*: Thorough inspection of the entire plant by an expert to objectively identify pest problems is recommended. A written analysis should be provided, with details on problem areas within the plant. Inspections should be conducted at a predetermined frequency. For a small processor, it may be cost-effective to hire a pest management specialist
2. *Physical control*: A standard of cleanliness must be established, with direct accountability for cleaning. This includes all areas inside and around the outside of the facility. Exclusion practices combined with routine inspection and repair restrict the ability of pests to enter and move from place to place in the plant. Some examples of these practices would be proper landscaping, adequate door seals, no entrances from outside directly into the processing area, and proper placement of dumpsters.
3. *Mechanical control*: These are nonchemical means that stop pests or prevent infestations, such as the sticky traps, electronic fly traps, needle strips mentioned earlier. Storage insects can often be controlled by temporarily raising or lowering ingredient temperatures or by reducing the moisture content to levels at which they cannot grow.
4. *Chemical control*: IPM does not eliminate the need for pesticides, and they should be used when necessary. Only qualified personnel should apply pesticides. Application of restricted-use pesticides requires certification, and it may be practical to hire a professional exterminator.

Once the methods of control have been chosen, they must be written down in a concise program with specific instructions, frequency of monitoring, responsible persons, monitoring activities, and reassessment. The program should be available for viewing by government inspectors. Many small processors completely contract out the pest control program to private exterminators who provide all procedures, monitoring, and documentation.

Verification of the Pest Control Program

All food safety programs (HACCP, recall, sanitation, pest control) must have validation documentation that demonstrates that the instituted procedures are effective. For example, if the pest control program calls for air curtains on the loading dock door, is this measure effective at preventing incoming insects? Often, manufacturers of pest control products provide documentation of the effectiveness of their products. This is evidence that the procedures in the program are effective. Most importantly, the pest controls must also be verified by evaluating data collected on pest numbers and frequencies and by visual checks of the processing plant.

Action Steps for Dairy Processors

- Read and obtain a copy of regulations pertaining to pest control in dairy plants.
- Decide whether to create an internal program or hire a private company.
- Assess current pest control procedures and define areas that need correction or addition.
- Document the pest control program in concise form with required procedures, recordkeeping materials, verification materials, frequency, and so on.
- List the individuals responsible for each aspect of the program.
- If needed, contact pest control experts who can evaluate your plan for completeness and effectiveness.

See also: **Contaminants of Milk and Dairy Products**: Environmental Contaminants. **Hazard Analysis and Critical Control Points**: HACCP Total Quality Management and Dairy Herd Health. **Plant and Equipment**: Process and Plant Design; Safety Analysis and Risk Assessment. **Risk Analysis**.

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STANDARDIZATION OF FAT AND PROTEIN CONTENT

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Introduction

Milk of all mammals (including human milk as well as milk of cows, buffaloes, goats, and sheep) contains, in addition to water, four major types of components: fat, protein, carbohydrate, and minerals. As the cow (genus *Bos*) is by far the most important provider of the raw material used by the dairy industry worldwide, the following discussion focuses on cows' milk only, even though most of the information would be applicable to other milk types as well.

From an economic standpoint, milk fat has been considered the most valuable component of milk used for manufacturing dairy products for the regular consumers' market. Indeed, the economic value of milk was (and generally continues to be) directly related to the fat content. Thus, altering the fat content in market milk and other dairy products has been accepted as a widespread practice used by dairy processors for decades. In recent years, the value of milk protein has increased significantly, mainly due to the steadily increasing popularity of cheese (a milk protein-based product) as a major component of Western-style diets. The advances of modern dairy technology make it now possible to manipulate the protein content of fluid milk in a manner similar to that used for fat standardization. Although the technological approaches in the two cases are somewhat different, the reasons for the need to control the content of the two nutritionally as well as economically important milk components in various dairy products (destined for direct consumption or for further industrial use) are similar. The two aspects of the component standardization in milk and dairy products can be compared, focusing on the compositional, technological, nutritional, and economical determinants of the two processes.

Milk Composition and Properties Relevant to Component Standardization

It is well known that the fat content of raw milk fluctuates rather widely, depending on the breed, feeding practices, geographical location, and many other factors. The average fat content of cows' milk is approximately 4% with the range for this value being given in various textbooks as 2.5–5.5% or wider. Less well recognized is the fact that the average protein content of milk (3.3–3.4%) also shows relatively wide natural variations (2.8–4.2%) in international comparisons, with narrower limits (~± 0.5% or less) reported for individual countries. **Table 1** illustrates the fluctuation of the protein content of cows' milk in several main dairy producing countries.

Clearly, the wide fluctuations in the contents of the two most valuable components of milk can result in significant nutritional as well as economical and regulatory consequences when no standardization is practiced. The original aim of fat standardization was to divert some of the fat from the 'rich' raw milk to the production of cream and butter, considered premium dairy products. At present, however, fat standardization has assumed another important role, that of providing consumers with dairy products perceived by many to be 'healthier' because of their low fat content. Most modern dairy products are labeled (some are legally required to be) as to their fat content; thus, accurate fat standardization processes have become an integral part of the modern dairy technology.

In contrast to the well-established standardization of fat, protein standardization is still an issue being debated in international dairy circles. The technology enabling the online, continuous protein standardization has not been available until relatively recently, and this is why protein content standardization of milk is not practiced

Table 1 Protein content in cows' milk in different countries

Country	*Protein content (g per 100 g)*
Canada	2.75–4.09
Spain	2.80–3.25
Ireland	2.85–3.60
UK	2.96–3.54
Finland	3.11–3.40
Germany	3.26–3.48
New Zealand	3.16–4.22
World (min–max range)	2.75–4.22

Adapted with permission from Rattray W and Jelen P (1996) Protein standardization of milk and dairy products: A review. *Trends in Food Science and Technology* 7: 227–234.

widely, and, in general, only rarely approved by the various regulatory bodies in individual countries or states (e.g., some states in Australia).

Technological approaches to both fat and protein standardization are based on the forms in which these components are present in milk and on the physical properties of the system. The milk fat globules, present in the raw milk in diameters of approximately 0.2–10 μm, have a much lower density (~920 kg m^{-3}) than that of the aqueous fat-free fraction of milk (density ~1035 kg m^{-3}). Consequently, separation of the milk fat from the aqueous phase can be easily accomplished by centrifugation based on the density differential. In contrast, the milk proteins are distributed in the nonfat portion of the milk as individual molecules or as molecular clusters (containing up to 10^4 molecules in the case of casein micelles but only as little as 2–8 molecules in the case of whey proteins). Although the size of the largest micelles (~300 nm) is approaching that of the smallest milk fat globules, the molecular mass of even the smallest free whey protein molecules (~14 000 Da) is still much larger than that of the next molecular species on the milk component spectrum, lactose (molecular mass 342 Da). Differential molecular sizes are the basis for fractionating aqueous solutions using the 'molecular sieve' principle of membrane processes. Specifically, the molecular sizes of milk proteins are ideal for the use of ultrafiltration (UF) as the method of choice for separation of milk proteins from other components of the fat-free aqueous phase of milk. As a result, the protein content in skim milk can be standardized either upward by removing a portion of the protein-free UF milk permeate, or, more typically, downward by admixing excess protein-free UF permeate to regular skim milk. In theory, UF does not affect the ratios of the individual water-soluble molecules capable of permeating the UF membrane; thus, the upward or downward standardization of the protein content by adding or removing relatively small amounts of the UF permeate should not affect the lactose or the mineral compositions of the final product (*see* **Milk Protein Products**: Membrane-Based Fractionation). However, as discussed below, this theoretical assumption does not always hold perfectly.

Technological Principles of Fat Standardization

Centrifugal separation is a straightforward process. Raw milk entering the continuous dairy centrifuge is subjected to a centrifugal force that causes the heavier nonfat aqueous milk phase to migrate toward the perimeter of the centrifuge bowl and the lighter fat globules are concentrated in the volume toward the center of the bowl. To facilitate this movement, the bowl is filled with a multitude of conical disks. The separation occurring along these disks produces two fractions: the fat-free skim milk of greater density and the more concentrated emulsion of the fat globules in a smaller volume of the nonfat aqueous milk fraction, resulting in the production of cream. Depending on the running conditions of the separator, the fat content of the cream can be adjusted within wide variations of the required final fat content. Because the process does not affect the condition of the fat globules, the final cream structure is the same as in the original milk, that is, cream is still an oil-in-water emulsion (*see* **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Centrifuges and Separators: Types and Design).

The production of the various low-fat liquid milk products demanded by the market is typically accomplished by reblending some of the cream into the skim milk produced as the high-volume stream emerging from the continuous centrifuge. The reblending may occur online with modern centrifugation equipment, or it can be accomplished in a storage tank by mixing known volumes of skim milk and cream of known composition.

Nutritional and sensory quality consequences of the fat standardization have been well documented in the literature and accepted by the market. As milk fat is the principal carrier of delicate dairy flavors, the taste of low-fat or fat-free liquid milk products is more bland and watery. The main nutritional effect of fat downstandardization is lower food energy value and lower content of saturated fat, features considered as positive by many consumers today. However, as some fatty acids are nutritionally indispensable especially for children and adolescents, the exclusive consumption of low-fat and especially skim milk products by these age groups without other adequate sources of the indispensable fatty acids may be a questionable nutritional practice. Downstandardization of fat in other dairy products may have either positive or negative effects. Dried dairy products containing fat are known to be prone to undesirable oxidation and other chemical changes, resulting in a potential for major quality impairment. On the contrary, reducing the fat content of standard cheeses can lead to major textural and flavor defects in comparison to full-fat products. In other classes of products (e.g., some fermented dairy foods such as yogurt or sour cream, as well as ice cream or dairy desserts), the removal of fat may be compensated for by using suitable fat mimetics, resulting in very minor quality alterations. Because the technology of fat manipulation does not affect other milk components (with the exception of minute quantities of components associated with the milk fat globule membrane), the nutritional consequences are primarily limited to the effects of the presence (or absence) of the fat itself.

Technological Principles of Protein Standardization

Similar to the fat standardization procedure, protein standardization may be accomplished online or in a batch mode using the UF membrane process. Typically, the milk is first skimmed, and the standardization of the protein content is then carried out on the skim milk. As shown in **Figure 1**, the UF plant, similar to the centrifuge in fat standardization, produces two streams: protein-free permeate (analogous to fat-free skim milk) and protein-enriched retentate (akin to the cream in the fat content manipulation process). Depending on the aim of the protein standardization process, one of the streams is then blended in varying ratios with regular fluid milk, which could then undergo additional fat standardization if desired. In a batch arrangement of this process, the permeate obtained by an UF process would be accumulated in a storage tank and then reblended with a batch of skim – or fat-adjusted – milk in a separate blending facility. In industrial practice, the permeate for protein downstandardization of fluid market milk could come from a separate department or factory, most often from a fresh cheese manufacturing plant, as the use of UF for pretreatment of the cheese milk before the cheese manufacturing process is practiced widely in some countries. Such practice could result in additional technological complications; as the transportation of the unconcentrated permeate is costly, a preconcentration could be used but this may result in precipitation of some mineral complexes.

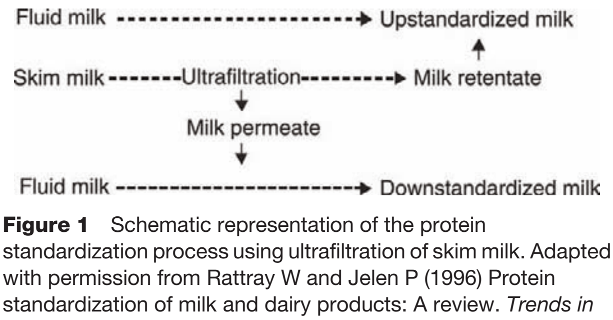

Figure 1 Schematic representation of the protein standardization process using ultrafiltration of skim milk. Adapted with permission from Rattray W and Jelen P (1996) Protein standardization of milk and dairy products: A review. *Trends in Food Science and Technology* 7: 227–234.

In some cases, upstandardization of the protein content may be desired, either to increase the nutritional value of a special product, or to bring the protein content of regular milk to a legal minimum, should such a minimum exist or be agreed upon. Present European Union (EU) legislation specifies that cows' milk shall contain not less that 2.8% w/v total protein (N × 6.38). It is thus conceivable that in some instances of low protein content, upstandardization could be required, especially if there were to be an international agreement on a minimum protein content in fluid milk (a value 3.0% has been proposed at various international discussions). Again, the UF offers the simplest alternative for such upstandardization; in this case, it is the retentate that is used directly as the upward standardized product (e.g., skim milk with increased protein content) or is blended with incoming unstandardized fluid milk (**Figure 1**).

In addition to the direct use of membrane processes, several technological alternatives to the UF-based protein standardization are now practiced by the industry. These include addition of skim milk powder to regular milk to increase the amount of protein and calcium in the market milk (thus also increasing the contents of lactose and other components), or addition of powdered lactose for protein downstandardization as approved by the Codex Alimentarius specifically for adjustments of protein content in milk powders. These two alternatives, together with the UF approach, do not violate the main principle governing the protein standardization considerations – that of maintaining the casein:whey protein ratio of the original milk unchanged. For this reason, using powdered whey or whey protein products for down- or upstandardization of market milk would not be permissible.

Sensory, Nutritional, and Technological Properties of Protein-Standardized Products

In contrast to the well-established sensory effects of milk fat, dairy proteins (both casein and whey proteins) have been shown to have a minimal impact on the flavor of fluid milk. Evidence in the literature confirms that protein standardization within rather wide limits (1.5–6.4% for whole milk, 2.4–6.5% for skim milk) by using UF milk permeate had no effects on the sensory properties of such standardized milk, which was indistinguishable from the nonstandardized controls. Investigations with other types of UF permeates (e.g., from acidified milk or cheese whey) showed that the acidity itself was not detrimental within relatively wide protein standardization limits (2.8–3.4%), but the use of permeates from fermentation-acidified products imparted major sensory changes in virtually all cases.

Using other membrane processes (e.g., reverse osmosis) for upstandardization of the protein content produced a noticeable salty-sweet sensation with as little as 0.3% total solids (TSs) (i.e., ~0.1% protein) adjustment. Similarly, addition of as little as 0.5% lactose to fluid milk was shown to result in a detectable increase in sweetness. Addition of some powdered lactose to the protein-standardized fluid milk to increase the low TSs content could be considered in the case that a minimum TSs in protein-standardized fluid milk products is required. The TS content of typical UF milk permeates is approximately 5.2–5.4% TS; thus downstandardization of protein in fluid milk with these permeates could be considered as adulteration, similar to the addition of water. Use of freezing-point measurement as a control technique for adulteration of raw milk with water is widely established; the same method could not be used to detect any 'dilution effects' of protein standardization of regular market milk.

Considering the relatively wide natural variations in the protein content in milk (**Table 1**), the nutritional consequences of the slightly lower protein content in the downstandardized milk should be considered of a very minor importance. However, in contrast to the fat standardization, the UF technique used for protein manipulation can have an effect on other nutritionally important milk components, especially calcium. Because of the association of most of the milk calcium with casein micelles under normal milk pH conditions (~6.6), the calcium content in the milk UF permeate (~270 mg kg^{-1}) is much lower than in milk (~1150 mg kg^{-1}); using this permeate for protein downstandardization will thus lower the calcium content of such milk. Published calculations show than within the rather narrow limits of the protein standardization being considered (where less than 10% of the final product milk solids would come from the permeate) the changes in some of the affected micronutrients (in addition to calcium, also zinc and vitamin B_{12}) will be minimal, certainly lower than resulting from natural variability. However, unlike lowering of the fat content, manipulation of the protein content could be viewed negatively by consumers, especially if accompanied by decreases in contents of other valuable nutrients, no matter how small.

Use of permeates from UF processing of milk to downstandardize protein in dried skim milk can have small but technologically important effects in such powders. As the profile of the mineral fraction of the standardized milk may be slightly different and the mineral content slightly increased, this may affect the buffering capacity and ionic strength of the reconstituted milk, resulting in needs for small adjustments in established processes where such milk may be used (e.g., in recombination or evaporation). Also, the total protein profile of the standardized product will be slightly altered by the additional nonprotein-nitrogen content of the UF permeate.

Regulatory Aspects and Current Status of Protein and Fat Standardization

Although fat standardization and production of low-fat – or even fat-free – dairy products have been the accepted practice for decades, protein standardization is still an issue hotly debated in the regulatory bodies such as Codex Alimentarius, EU, and national agencies. Protein downstandardization in concentrated and dried dairy products has been approved by the Codex Alimentarius; however, similar practice is generally unacceptable for regular market milk. Use of protein downstandardization for specific unregulated products such as UHT milk may have been practiced in the past without labeling the products as protein standardized; such practice is now expressly forbidden in the EU. Published research indicated no unusual sensory quality or technological problems in UHT products that were downstandardized with UF milk permeate to 3.2–2.6% protein.

Fluid milk products with a higher-than-normal protein content are now quite common in various countries; their market positioning is as being nutritionally and/or sensorically superior to the corresponding regular products. In California, skim milk or low-fat milk is routinely fortified with skim milk powder to improve the sensory properties; this practice also increases the protein content without the products being labeled as protein standardized. In Australia and other countries, fluid milk products with increased calcium content are being promoted. Such products can be obtained easily by subjecting the regular milk to limited UF, which increases not only the calcium but also the protein content.

Protein adjustment (i.e., *de facto* standardization) in other dairy products and in milk used as a raw material for manufacture of products such as cheese or yogurt is being practiced widely, mostly as upstandardization. The main reasons are technological, increasing the output of specific equipment (e.g., in cheesemaking) or improving the technological and/or sensory properties of final products (e.g., gelling properties of yogurt, overrun control of ice cream). Downstandardization of protein content in certain products (e.g., sour milk, buttermilk) could lower the sometimes undesirable high viscosity of such products, although no published reports of such practices seem to exist. Fat adjustment in these products is a well-established practice with no controversial consequences.

Conclusions and Future Prospects

The international dairy community is currently divided on the issue of general protein standardization of milk. Although there are no technological barriers and no real nutritional issues of major consequence (other than the general distrust of the consumers if such a practice were to become common), the major issues concerning widespread downstandardization of protein are economical. It has been calculated that if all fluid milk produced by the EU, the United States, Australia, and New Zealand were to be standardized to 3% protein, the additional amount of protein that would be available for processing into other dairy products (principally cheese) would be over 170 000 tonnes annually, corresponding to more than 500 000 tonnes of cheese. With this extra cheese production, the market could experience similar distortions as used to be common in the days of butter surpluses, resulting in fact from the downstandardization of fat in dairy products. The milk production aspects (with negative consequences for the farmers) could be also severely impacted by protein adjustments, as would be the general credibility of the dairy industry in the eyes of consumers.

Downstandardization of milk fat in many dairy products, combined with the nutritionally unfavorable image that butter has been saddled with in recent times on one side, and the continuing practice of rewarding producers for high fat content in their milk on the other, has resulted in difficulties for the dairy industry still searching for new uses for butterfat. Similarly, large surpluses of UF milk permeate, resulting from modern cheese manufacturing practices, are becoming a major industrial problem. Use of at least some of this permeate for slight adjustment of the protein content in milk and other dairy products is a tempting and technologically fully justifiable option. Some consumer research indicates that if several types of fluid milk products with varying protein content (and correspondingly adjusted prices) were to be available on the market, the downstandardization of protein content might be viewed favorably. It can be expected that protein standardization of milk and dairy products will remain one of the highly controversial issues for the dairy industry for the foreseeable future.

See also: **Milk Proteins**: Heterogeneity, Fractionation, and Isolation. **Milk Protein Products**: Membrane-Based Fractionation. **Milk Salts**: Macroelements, Nutritional Significance. **Plant and Equipment**: Centrifuges and Separators: Applications in the Dairy Industry; Centrifuges and Separators: Types and Design.

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STRESS IN DAIRY ANIMALS

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Cold Stress: Effects on Nutritional Requirements, Health and Performance

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Introduction

Environmental stresses, which include temperature, humidity and wind speed, can alter both nutrient utilization and animal performance. The effects of heat stress have received the majority of attention in research studies. The impact of cold stress on nutrient metabolism and animal performance in ruminant animals has received minimal research attention, the majority of investigations being conducted with beef cattle. The quantity of data relating to the effects of cold stress on dairy cattle is very limited. A primary reason for this dearth of information is the housing systems utilized in dairy production systems. Typically, dairy cattle are housed in barns that minimize the impact of environmental temperature fluctuations on the animal. Pasture systems are used in dairy production systems in many parts of the world. However, these systems are usually limited to the warmer months of the year. It is rare to find dairy cattle housed outside during the winter months in most dairy areas. The result is that very few dairy cattle are actually exposed to cold stress conditions. Dairy replacement heifers may be housed outside during the winter months and could be exposed to cold stress in some situations.

There are a number of terms that need to be defined prior to examining the influence of cold stress. These terms include:

- thermoneutral zone (TMZ), i.e. the range of environmental temperatures where normal body temperature is maintained and heat production is at the basal level
- lower critical temperature (LCT), i.e. the environmental temperature at which an animal needs to increase metabolic heat production to maintain body temperature.

There are a number of factors that determine the LCT for a specific animal. These factors include body surface area (SA), heat production by the animal (HE), retained energy (RE), external insulation (EI), internal insulation (TI), evaporative losses and respiratory losses. The quantity of subcutaneous fat and thickness of the skin are the main factors that determine TI. The primary factors that influence EI are hair coat depth, wind, precipitation and mud on the hair. Thus, there will be a specific LCT for an animal as the above factors vary. The LCT for dairy heifers may range from 10 °C to −20 °C. The LCT for lactating dairy cows may range from −20 °C to −50 °C. The lower LCT range for lactating dairy cows is mainly due to the HE of the animal (**Table 1**).

Heat Loss

There are four primary ways in which animals can lose heat to the environment. These are:

Table 1 Estimated lower critical temperatures for dairy animals (°C)[a,b]

Animal type	*Body weight (kg)*	*Average daily gain (kg)*	*Milk (kg)*	*LCT–A*[c]	*LCT–B*[d]
Heifer	220	0.8	–	−6.7	4.8
Heifer	440	0.8	–	−19.4	−9.8
Dry cow	700	–	–	−9.3	−3.8
Milking cow	635	–	45	−35	−24
Milking cow	635	–	25	−21	−13

[a] Heifer LCTs estimated using the 2001 Dairy–NRC program.
[b] Dry and milking cow LCTs estimated using the Cornell Net Carbohydrate and Protein System (CNCPS) model, version 4.
[c] Situation A: hair depth, 1.28 cm; wind speed, 1 kph; hair coat, clean and dry; ambient temperature, 20 °C.
[d] Situation B: hair depth, 1.28 cm; wind speed, 10 kph; hair coat, wet and matted; ambient temperature, 20 °C.

1. Evaporation. The body heat is used to evaporate water mainly from the skin and hair.
2. Conduction. This is heat loss that occurs as result of direct contact by the animal with items such as bedding or stall surfaces.
3. Radiation. This is the transfer of heat through the air from a warm object.
4. Convection. This loss occurs when air passes over the body. Exposure to wind increases the heat lost by this mechanism.

Metabolic and Physiological Adaptations

A number of adjustments take place in animals exposed to cold stress. These can be grouped into the following categories:

1. Increase in response to cold stress:
   - rumination
   - gastrointestinal tract motility
   - rate of passage of feed in both the rumen and total tract
   - liquid passage rate in the rumen
   - protein flow to the small intestine
   - basal metabolic rate
   - whole body oxygen consumption
   - cardiac output
   - circulating epinephrine, cortisol and growth hormone levels
   - increased lipolysis, gluconeogenesis and glycogenolysis
   - increased hepatic glucose output
   - increased hepatic uptake of glucose precursors (propionate, glycerol).
2. Decrease in response to cold stress:
   - rumen volume
   - *in vivo* and *in situ* cell wall digestion
   - diet dry matter digestibility
   - insulin response to a glucose infusion
   - temperature of skin, ears, legs and other extremities.

Nutrient Requirements

Research literature has very little data on the effect of cold stress on nutrient requirements. The primary change is an increase in the maintenance energy requirement as environmental temperature decreases. **Table 2** contains metabolizable energy (ME) requirements for dairy animals at varying temperatures. These requirements are

Table 2 Maintenance ME multipliers for animals at varying environmental temperatures[a,b]

Temperature (°C)	*Heifer*[c] *(220 kg)*	*Heifer*[d] *(440 kg)*	*Dry cow*[e] *(700 kg)*	*Lactating cow*[f] *(635 kg)*
20	1.0	1.0	1.0	1.0
10	1.05	1.05	1.05	1.05
0	1.13	1.13	1.13	1.13
−10	1.24	1.23	1.23	1.23
−20	1.57	1.36	1.37	1.37
−30	2.00	1.61	1.64	1.52

[a] Simulations carried out with the Cornell Net Carbohydrate and Protein System (CNCPS) model, version 4.
[b] Hair depth, 1.28 cm; wind speed, 1 kph; hair coat, clean and dry.
[c] Base maintenance ME requirement, 7.9 Mcal day^{-1} (33 MJ day^{-1}).
[d] Base maintenance ME requirement, 13.8 Mcal day^{-1} (57.7 MJ day^{-1}).
[e] Base maintenance ME requirement, 20.2 Mcal day^{-1} (84.5 MJ day^{-1}).
[f] Base maintenance ME requirement, 17.1 Mcal day^{-1} (71.5 MJ day^{-1}).

listed as a multiple of the base maintenance ME requirement for an animal with no stress. The effect of cold stress on requirements for protein, minerals and vitamins has not been defined.

Ration Formulation Programs

The challenge for nutritionists is how to account for and incorporate the effects of environmental conditions on nutrient requirements, feed utilization and projected animal performance. The majority of the available ration formulation programs do not contain equations to assist in making these adjustments. There are at least three ration formulation programs that can account for some of these factors: the 2001 Dairy-NRC, CPM Dairy and the Cornell Net Carbohydrate and Protein System (CNCPS) models. All of these programs adjust maintenance requirements for changes in environmental temperature (*see* **Feeds, Ration Formulation**: Models in Nutritional Management; Lactation Rations for Dairy Cattle on Dry Lot Systems). None of these programs adjusts feed passage rate or feed digestibility in response to cold stress.

Milk-Fed Calves

The LCT for newborn calves is estimated to be 10 °C; this LCT is for a calf housed under dry conditions with no wind. This temperature decreases to about 0 °C by the time the calf is 1 month old. If the calf is subject to wind, precipitation or a wet hair coat, the LCT could be considerably higher.

The 2001 Dairy-NRC program was used to estimate the impact of environmental temperature on the ME maintenance requirement and predicted energy allowable gain. A milk replacer containing 20% fat and 20% crude protein was used. The feeding rate was 0.6 kg dry matter (DM) day^{-1}. The results of this simulation are given in **Table 3**.

Table 3 Impact of environmental temperature on the ME maintenance requirement and predicted energy allowable gain[a,b]

	ME for maintenance		
Temperature (°C)	*Mcal*	*MJ*	*Energy allowable gain (kg day^{-1})*
20	1.74	7.28	0.41
10	2.21	9.24	0.26
0	2.68	11.21	0.09
−10	3.23	13.51	Weight loss

[a] For a dairy calf with a body weight of 45 kg.

[b] Feeding 0.6 kg day^{-1} of dry milk replacer.

A number of options exist to increase energy intake to maintain average daily gain as the temperature decreases. One option would be to feed a higher quantity of the milk replacer powder each day. This would require about 17% more DM from the milk replacer as the environmental temperature decreases from 20 °C to 10 °C. A second option would be to formulate a milk replacer with a higher fat content. A third option would be to add a fat supplement to the currently used milk replacer. The addition of 0.1 kg of tallow would provide adequate supplemental energy to maintain average daily gain as the temperature dropped from 20 °C to 10 °C.

It is important to remember that the above calculations are relevant only for calves in a clean, dry and draught-free environment. These calves are assumed to have a clean, dry hair coat. If calves are exposed to more severe environmental conditions, the maintenance requirement would increase and the projected average daily gain would decrease. One option that has become available in the United States is the use of calf blankets. These coats keep the hair coat dry and decrease the heat loss to the environment from the calf. This would increase the proportion of the daily energy intake available to support weight gain. It is also important to have water available to the calves and to keep it from freezing in cold weather.

Replacement Heifers

Environmental conditions will alter both the dry matter intake (DMI) (**Table 4**) and growth rates (**Table 5**) of dairy heifers. **Table 5** contains model predicted energy allowable gains for dairy heifers exposed to three environmental temperatures, and the effects of wind speed, hair depth and coat condition. The same diet is fed in these examples. However, DMI was predicted to increase at colder temperatures. This would result in an increase in daily energy intake. Note that energy allowable gain decreases by more than 50% in the most severe environmental conditions used in this example. These differences in average daily gain reflect the combined effects of temperature, wind speed and hair coat condition.

Dry Cows

The maintenance requirement of dry cows increases as the environmental temperature decreases (**Table 2**). The data in this table assumes a dry cow with a clean, dry hair coat, a hair depth of 1.28 cm and a wind speed of 1 kph. Cows with mud on the hair coat, a different hair depth or subjected to higher wind speeds would have changes in both the ME maintenance requirement and the predicted LCT.

Table 4 Predicted daily dry matter intake (kg day^{-1})[a,b]

Temperature (°C)	*Heifer (220 kg)*[c]	*Heifer (440 kg)*[d]	*Dry cow (700 kg)*[e]	*Milking cow (635 kg)*[f]
20	5.7	11.0	13.9	23.2
10	5.8	11.3	14.1	23.5
0	6.0	11.5	14.6	24.3
−10	6.3	11.8	15.4	25.6
−20	6.7	12.7	16.4	27.3

[a] Heifer dry matter intakes predicted using the 2001 Dairy–NRC program.
[b] Dry cow and milking cow dry matter intakes predicted using the Cornell Net Carbohydrate and Protein System (CNCPS) model, version 4.0.
[c] Average daily gain = 1.0 kg day^{-1} at 20 °C.
[d] Average daily gain = 0.9 kg day^{-1} at 20 °C.
[e] 240 days pregnant.
[f] Milk production = 40 kg day^{-1}.

Table 5 Predicted energy allowable gain and lower critical temperature (LCT) for Holstein heifers[a,b]

Temperature (°C)	*Wind speed (kph)*	*Hair depth (cm)*	*Coat*[c]	*DMI (kg)*	*Average daily gain (kg)*	*ME*[d] *(Mcal day^{-1})*	*LCT (°C)*
20	1	1.28	1	11.3	0.88	14.3	−18.9
20	10	2.54	3	11.3	0.88	14.3	−5.8
0	1	1.28	1	11.8	0.82	16.1	−22.7
0	10	2.54	3	11.8	0.82	16.1	−8.7
−20	1	1.28	1	13.3	0.77	19.5	−31.2
−20	1	2.54	1	13.3	0.77	19.5	−41.6
−20	10	1.28	3	13.3	0.41	24.1	−10.1
−20	10	2.54	3	13.3	0.62	21.5	−15.3

[a] 15-month-old heifer, 440 kg body weight.
[b] Simulations carried out with the Cornell Net Carbohydrate and Protein System (CNCPS) model, version 4.
[c] Coat condition: 1, clean and dry; 3, mud on lower body.
[d] ME maintenance requirement.

Milking Cows

A series of simulations were run using both the 2001 Dairy-NRC program and the CNCPS 4.0 programs (**Table 4**). A mature cow weighing 635 kg was used. The food ration contained 2.66 Mcal of ME kg^{-1} of DM (11.1 MJ of ME kg^{-1}). Clean, dry hair coats with a hair depth of either 1.28 or 2.54 cm were used. Environmental temperatures ranging from 20 °C to −30 °C were used with wind speeds of 1 or 10 kph. The maintenance ME requirement changed with decreasing environmental temperature as indicated in **Table 1**. There was also an increase in the predicted DMI as temperature decreased. The predicted DMI for a cow exposed to an environmental temperature of −30 °C was 17% higher than a cow in a 20 °C environment. There was no change in the predicted daily milk production of 30 kg day^{-1} as environmental temperature decreased. Previous reviews have indicated a potential for depressed milk production when environmental temperatures are less than −10 °C to 15 °C. However, as indicated earlier, no adjustments in either rate of passage or feed digestibility are included in these ration formulation programs.

One concern for dairy cattle exposed to cold temperatures is the potential for frostbite or frozen teats. One approach to minimize the risk of frozen teats is to make sure that teats are dry after milking before cows return to the cold environment. A second option is to use some specialized teat dips that contain emollients. Commercial ointments or salves could also be applied to the teats if evidence of chapping becomes visible.

Summary

There are a number of metabolic and physiological adaptations that occur in animals exposed to cold environmental temperatures. The basal metabolic rate and maintenance energy requirements increase. Dry matter intake also increases while diet digestibility decreases. There are a number of factors that alter the effects of cold temperatures on animals. These include wind, hair depth and hair coat condition. An animal with a clean, dry hair coat has a lower LCT at the same environmental temperature than an animal with a wet, matted hair coat. This emphasizes the value of a clean, dry environment for animals exposed to low environmental temperatures.

In most dairy production systems, dairy cattle are rarely exposed to environmental temperatures lower than 0 °C because of the housing systems used. This temperature is higher than the LCT for milking cows. However, the LCT is higher for calves and heifers versus cows (**Table 1**). Cows can withstand a colder outside environmental temperature than either heifers or calves. In some cases, replacement heifers may be housed outdoors for the winter months. In this situation, the LCT could be used as an index of the need for a windbreak or housing. This would be true as the environmental temperature approaches or is lower than the LCT. A simple windbreak, increased DMI and a clean, dry hair coat can minimize the decrease in ADG in dairy heifers housed outdoors in the winter months.

Management practices have the ability to counteract many of the effects of cold stress on dairy animals. A key component is to keep animals in a clean, dry environment with minimal wind. Maintaining a clean, dry hair coat will also help to decrease the impact of cold stress on dairy animals. One key adjustment to make on the nutrition side is to ensure that animals can consume additional DMI to compensate for the higher maintenance energy requirements of animals exposed to cold stress conditions.

See also: **Feeds, Ration Formulation**: Lactation Rations for Dairy Cattle on Dry Lot Systems; Models in Nutritional Management; Systems Describing Nutritional Requirements of Dairy Cows. **Stress in Dairy Animals**: Cold Stress: Management Considerations; Heat Stress: Effects on Milk Production and Composition.

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Cold Stress: Management Considerations

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Introduction

Providing environments for dairy calves, heifers and cows that permit them to grow, mature, reproduce and maintain health is a primary goal in housing design. While climate in a particular geographic area influences design choice, the barn environment finally provided for the animal must positively influence the animal's health, welfare and productivity. In winter, outdoor winter air is used to provide the ventilation necessary for barns or other enclosures. Since dairy animals adapt well to cold climates, maintaining indoor air temperature equal to or slightly above outdoor air temperature is quite tolerable to the housed animals. Coincidentally, providing the ventilation rate necessary to maintain this minimum temperature difference leads to good air quality. Protecting the animal from extreme drafts, providing dry lying places that contribute to a dry, fluffy, erect haircoat, meeting the nutritional needs of the animal and allowing the animal sufficient freedom of movement are essential.

Ventilation

Ventilation permeates all aspects of the animal environment. Most often associated with respiratory health of animals, ventilation – directly and indirectly – impacts many other aspects of health as well. Good ventilation in the stall area of the lactating cow helps to keep bedding dry, a factor favoring good udder health. Good ventilation along alleys helps to keep walking surfaces dry, contributing to healthy feet. Good ventilation may lead to greater productivity, dry matter intake during hot weather may be maintained as a result of good air movement and more comfortable cows in the area of the feed manger. A comfortable, well-ventilated stall area encourages animals to lie down, an important contribution to many aspects of animal health.

Ventilation is bringing outside air into a barn where it collects moisture, heat and other contaminants, all produced by the animals, then exhausting it to the outside. Ventilation is an air exchange process – contaminated air inside the barn is exchanged for fresh outside air. To determine ventilation rates, we focus on the moisture content of the air, measured by relative humidity. But moisture is only one aspect. Ventilation removes other undesirable contaminants as well.

Air Quality

Animal health and disease are influenced by air quality. Air quality, in turn, is related to ventilation and the positive result of removing contaminants from the air. Empirical observations and field trials suggest that the aerosol spread of pathogens between animals and the influence of air pollutants on pulmonary defense mechanisms are important, especially to respiratory health. Excess moisture, gases and other contaminants in the air are considered to be problematic as well. But the exact relationship among these factors is not fully understood.

The term air quality is not easily defined. With respect to animal spaces, good air quality generally implies that the characteristics of ambient air bear no harmful effects on the animals in the space. Actually, the ambient air itself is not at issue – the contaminants in the air are the source of concern. Ambient air, in itself, begins as a mixture of clean, dry air (a mixture of gases, chiefly nitrogen and oxygen) and varying amounts of water vapor. When moisture in the air in an animal space is considered to be a problem, it is actually the concentration of moisture in the air above some arbitrary level that is of concern. Moisture is considered to be an air contaminant above some concentration. Other contaminants may include pathogens, harmful gases, dust and undesirable odors. But it is the concentration of a contaminant above some predetermined level that is considered a threat to animal health.

Dilution Effect of Ventilation

Ventilation is truly a process of dilution. Air moved through a barn actually serves to dilute the inside air and, very importantly, to dilute all of its components. Dilution reduces concentrations of moisture and heat. Dilution reduces concentrations of airborne disease organisms, harmful gases and dust, and undesirable odors as well. One air change can theoretically reduce the concentrations of air pollutants by 63.2%.

Conversely, concentrations of air pollutants increase when ventilation is lacking. For example, a 7.8-fold increase in airborne bacteria was observed when the outlet ventilation openings in a 15-stall dairy barn were closed. Odors increase also and, in fact, can be an important indicator of the poor air quality that results from underventilation. Terms like 'barny', 'close' and 'stuffy' are sometimes used to describe the environment inside a barn that is suffering from underventilation and poor air quality.

Indeed, concentrations of gases are related to ventilation. In an Alberta study of air quality in six freestall barns, average ventilation rates could, in fact, be estimated by measuring building carbon dioxide concentrations.

When ventilation is reduced below the recommended level – usually in a misguided effort to warm the barn using animal heat – less moisture is removed. Sometimes the consequences of the resulting moisture build-up and lack of proper ventilation are masked by: (1) insulating the barn, (2) using a greenhouse effect, (3) providing supplemental heat, or (4) dehumidifying the inside air. For example, adding heat to the air reduces relative humidity, without the need for air exchange. So it is possible to have substantial quantities of moisture added to the air and, if accompanied by heating of the air, have the relative humidity remain in an acceptable range. Thus, air quality may be deemed to be satisfactory if relative humidity is the only measure of air quality. But even though excess moisture may not be apparent, the reduced dilution does indeed result in increased concentrations of airborne disease organisms, harmful gases and dust, and undesirable odors. If these increases are ignored, animal health problems are inevitable.

Essential to maintaining a healthy animal is providing an environment that does not needlessly stress or challenge the animal (*see* **Stress in Dairy Animals**: Management Induced Stress in Dairy Cattle: Effects on Reproduction). Maintaining good air quality is a fundamental aspect of that healthy environment with ventilation providing the key. But air quality is more than just measuring relative humidity. Through ventilation the air inside the barn is continually diluted, assuring that the air the animal breathes has low concentrations of all contaminants that threaten the animal's health. If sufficient ventilation is provided for moisture control during the winter, the undesirable effects of other airborne pathogens and noxious gases apparently are minimized.

The Air–Water Vapor Relationship

Because ventilation involves moisture in the air, relative humidity – a measure of the concentration of moisture in the air – is of primary interest. Understanding the relationship between relative humidity and ventilation is helpful to comprehending the ventilation process itself.

Relative humidity expresses, as a percentage, the relationship between the amount of moisture present in a quantity of air and the amount of moisture the air is capable of holding. For example, if the air in a room is at 40% relative humidity, 40% of the moisture-holding capacity of the air is occupied (**Figure 1**). From the standpoint of ventilation and the need to pick up moisture, 60% of the air's moisture-holding capacity remains. If no air exchange occurs, the air can take on moisture only until its relative humidity reaches 100%. Thus, ventilation requires continuous air exchange and the rate of exchange must be sufficient to maintain relative humidity below 100%. A range of 60% to 75% is preferred for animals.

The relative humidity of a quantity of air varies with the air temperature. This fact is useful in controlling the ventilation process (**Figure 2**). As moist air is warmed, its moisture-holding capacity increases markedly. Although

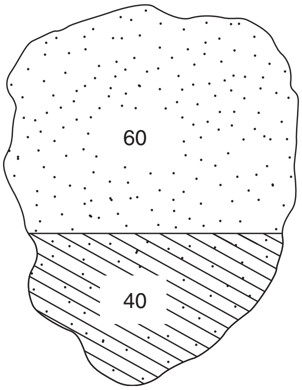

Figure 1 An illustrated quantity of air at 21 °C and 40% relative humidity. Forty percent of the air's moisture holding capacity is occupied; 60% remains.

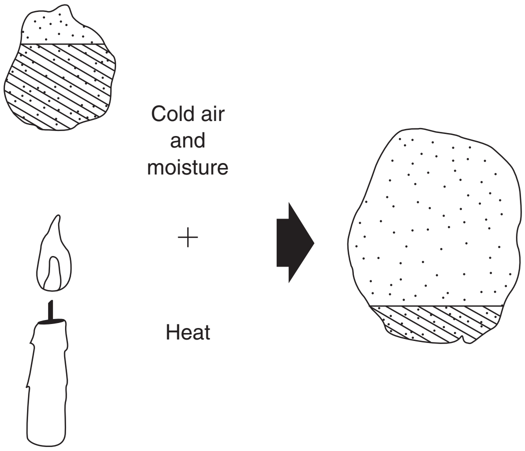

Figure 2 Heating a quantity of air and raising its temperature reduces its relative humidity and increases its moisture-holding capacity; the change in relative humidity is dependent on the temperature rise.

the air expands as it is warmed, its moisture content, expressed in absolute terms, does not change; relative humidity therefore drops. This warmed air of lower relative humidity becomes more able to pick up moisture.

For example, if typical outside winter air at −18 °C and 80% relative humidity is used for ventilation (**Figure 3**), this air will not be very useful for picking up additional moisture and certainly not for achieving a relative humidity of less than 80%. But if this air is used for ventilating a warm calf barn kept at 7 °C, the air will be heated to 7 °C as it mixes with inside air and passes through the barn, reducing its relative humidity to about 10%. From the standpoint of ventilation for moisture control, heating the air increases its moisture-holding capacity. What remains is to carefully balance the calves' moisture production rate, the rate at which heat must be added to the air, and the necessary ventilation rate so the resultant relative humidity in the calf barn is controlled at 60–75% and the desired temperature is maintained. This is the basis for determining the minimum continuous ventilation rate for moisture control.

If calves are to be kept in a warm barn, say 10 °C, supplemental heat must be provided. Calves simply do not produce enough heat to properly warm the amount of ventilation air that is required to remove the moisture they produce. On the other hand, mature cows do produce substantial amounts of heat in relation to their moisture production. This heat, when used to warm the ventilation air required for moisture removal, allows stanchion and tie stall dairy barns to be kept above freezing in the winter. But there is a limit to the temperature rise that cow heat can impart to the ventilating air. In a well-insulated tie stall barn, filled to capacity with cows, the maximum temperature rise of the ventilating air that can be obtained from cow heat alone is of the order of 20 K. This means that ventilation can proceed without interruption until outdoor temperature falls below about −20 °C. Beyond that, the indoor barn temperature would fall below freezing and water lines would freeze, a condition that is avoided by installing a safety thermostat on the fan providing the minimum continuous ventilation.

In a cold barn, the temperature rise of the ventilating air is limited to 3–6 K. Even so, the dependence of relative humidity on temperature is beneficial. For example, if the −18 °C, 80% relative humidity outdoor air is warmed by only 5.5 K, its relative humidity decreases to less than 50%. Thus even this cold air, warmed just slightly, is useful for picking up moisture. Moreover, the heat produced by the animals themselves can cause this slight temperature rise. Care must be taken, however, to assure sufficient air movement, preferably by natural means, to limit the temperature rise to 3–6 K. This is the basis for cold housing, a preferred alternative for all dairy animals.

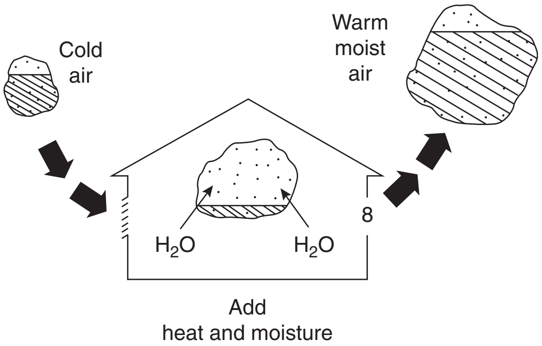

Figure 3 Cold outside air with a high relative humidity can pick up large quantities of moisture in a barn if the air is heated. Even a slight temperature rise of 5.5 °C to 8.3 °C, as can occur in cold dairy barns, is useful.

Minimum Continuous Winter Ventilation

A minimum rate of ventilation is required for removing animal moisture from barns in the winter regardless of outside temperature. This applies to barns designed to be warm barns and cold barns alike in winter. In addition, the minimum ventilation should be continuous. Continuous dilution of inside air acts to maintain concentrations of moisture and contaminants in the air at minimal levels. The minimum rate depends on outside weather design conditions, number and type of animals in the barn, age and size of animals, and whether the barn is intended to be cold or warm.

With mechanical ventilation, the capacity of the fan chosen to supply the minimum continuous rate should closely match the rate of ventilation that is calculated for a particular housing situation. Mechanical ventilation is most often associated with warm housing where ventilation must have a high degree of control.

With natural ventilation, minimum ventilation is less controlled. Depending upon thermal and wind forces, natural ventilation is most often associated with cold housing. As will be described in later sections, deciding between warm and cold housing is critical to the design and subsequent management of the ventilation system. Understanding the differences between the two types of environments is especially important as related to the need for maintaining the minimum continuous ventilation for winter in either situation that is in the best interest of animal comfort and health.

Barn Categories and Winter Temperatures

Barn environments are often categorized according to temperatures maintained in the barn in winter. The particular environment, based on desired indoor temperature, is the first determination in ventilation system design.

In a cold barn, indoor temperatures fluctuate with outdoor temperatures. Ventilation maintains indoor temperatures within 3–6 K of outdoor temperatures in winter. Usually, the barn is not insulated and ventilation is largely unregulated, except to adjust for seasonal changes. Adequate air exchange during cold weather to assure moisture control eliminates the need for insulation.

Warm barns are well insulated and, by necessity, have a well-controlled ventilation system. These barns are designed to provide a relatively uniform environment throughout the winter. Tie stall barns, where indoor temperatures are to be maintained at least above freezing, remain the principal example of this type of housing for dairy animals. The key to success is ventilation that is regulated in order to compensate for changing outside climatic conditions.

Some barns do not fit into either the warm or cold category. An in-between barn or modified environment barn usually has indoor temperatures in winter above freezing, even though it is substantially colder outdoors. Minimum insulation (perhaps only under the roof) reduces evidence of condensation. Recommended ventilation features include open ridge, eaves and side walls. Unfortunately, ventilation openings may be closed or blocked during extreme weather to keep manure from freezing and for other reasons. This practice can result in inside temperatures rising 10–20 K above the outside temperature, significantly higher than the 3–6 K temperature difference limit considered acceptable for cold barns. This alone can create problems because of excess moisture build-up and a high relative humidity. But, even more seriously, openings that may remain closed or blocked after severe weather conditions have passed restrict airflow during less severe conditions. The result of this mismanagement is underventilation and poor environmental conditions. A properly designed and managed in-between barn is more like a warm barn, in terms of both design and operation. Thus, to avoid problems, the design and management of in-between barns should follow the guidelines for a warm barn.

Consequences of Mismanaged Ventilation in Winter

Underventilation in winter is one of the most serious threats to air quality and to the environment of animals. Both improper design and improper management of the ventilation system may be reasons that wintertime ventilation is lacking, compromising animal health. Problems are most likely during colder seasons of the year, especially during rainy weather and times of warmer days coupled with cold nights.

Underventilation occurs after ventilation is adjusted for the worst case – severe winter weather – and is not readjusted to allow increased ventilation when milder winter weather appears. Cold barns with manually controlled natural ventilation can be a particular problem. Perhaps ventilation openings are closed in anticipation of a windy, cold, blustery night, but are not opened the next day when, although the temperature may still be cold, the wind subsides and the sun shines. Very simply, reduced wind reduces ventilation, reducing air exchange and reducing the positive effects of dilution.

In a cold barn, the inside temperature is maintained no more than 3–6 K above the outside temperature in winter. Usually this is accomplished through largely unregulated natural ventilation with appropriately sized ridge and eaves openings along with adjustments in door and wall openings.

The danger of mismanaging ventilation in a cold barn in winter increases when insulation is installed under the roof. Insulation suggests that the barn is something other than a cold barn, that an available option is to close or block ventilation openings during extreme weather conditions to restrict ventilation. The most serious deficiency associated with this approach is the lack of automatic control to restore ventilation rates when extreme weather has passed. The consequences of this mismanagement are even more serious if high-producing cows are in the barn because of their higher rates of heat and moisture output.

Condensation on the underside of the roof of a cold enclosed barn may, in fact, be considered a management tool, a signal to the farmer that underventilation is present. Condensation is a sign that additional ventilation is needed for a higher rate of air exchange to reduce moisture build-up. The presence of insulation can take away this important indicator resulting in a potentially unfavorable environment.

If a barn is to be insulated, it must be designed, constructed and operated more like a warm barn than a cold barn. Adequate insulation, a high level of management and well-regulated ventilation are necessary ingredients.

Barn Ventilation – Construction and Management

In general, a cold barn with natural ventilation has these minimum characteristics: (1) no insulation, (2) open ridge and eaves, and (3) side walls and end walls that open. Providing an open ridge along with open eaves has long been recognized as a means of utilizing a stack effect to cause air exchange, especially for controlling moisture in winter. Indoor temperatures are expected to be a few degrees warmer than outdoor air temperature due to the heat being given off by the animals being housed. Current recommendations call for providing a ridge opening of 5 cm per 3 m of barn width and equivalent open area divided between the two eaves. Raised ridge caps are to be avoided. Their performance has been unpredictable due to local wind patterns, often channeling winds into the structure, thereby increasing the entry of wind-blown snow and rain.

Very importantly, the combination of the open ridge and eaves should be viewed as the sole source of ventilation only during the most severe winter weather – during periods when temperatures reach the lowest levels or times when especially windy, stormy conditions are present. During all other times in winter, additional ventilation must be provided. Typically, doorways are left open for this purpose, or portions of the end wall sections of the gable roof may be left uncovered, or side walls away from prevailing winter winds may be left open. Then, as temperatures rise into spring and summer, side walls and end walls are opened fully. As a general rule, too much ventilation is preferred over too little. In winter, air movement through the barn should be sufficient to maintain inside temperature within 3–6 K of outside temperature.

Natural ventilation has been used successfully for warm housing. Such barns are temperature controlled in cold weather and guidelines for construction and for sizing chimney and side wall openings must be followed carefully. Moreover, if ventilation openings are not operated automatically, frequent attention to management of the ventilation system is essential to avoiding underventilation and associated consequences. Except for higher initial and operating costs, a well-controlled mechanical ventilation system is desirable for a warm barn in order to minimize risks associated with the reduced rate of air dilution that accompanies warm housing.

Other Factors Related to Cow Comfort

Besides ventilation, other aspects of housing influence cow comfort. A reasonably clean, dry, resilient bed upon which to lie down helps maintain a dry erect haircoat for the small calf in cold weather as well as providing the larger cow with a clean, comfortable place to lie down for rest and to relieve stress on feet and legs. An animal will lie down for longer periods of time and more total hours per day when provided a dry, comfortable lying place. The outcome is longer resting time, less time for the animal to be standing in wet manure and more drying time for wet feet, especially important to a mature cow.

Bedded areas, including bedded manure packs, are common in cold climates for housing groups of young animals and dry cows. Bedded pens that are frequently cleaned are recommended for calving as well. However, in most areas today, when barns are provided for lactating cows, these barns are equipped with freestalls.

Cleanliness and comfort are two basis prerequisites that must be satisfied in freestall design and construction. Cleanliness relates to clean, dry conditions, especially a clean, dry stall bed in the vicinity of the udder. Comfort means a comfortable bed to allow the cow to lie comfortably in the stall along with roomy dimensions that make it easy for the cow to enter the stall, lie down, rise and exit. In simplest terms, the main purpose of a freestall is to reduce exposure of the teat ends to mastitis-causing organisms. What follows is to make the stall so appealing that a cow chooses no other place but a freestall to lie down 10–14 h day^{-1}.

Adequate supplies of feed and water, besides being of suitable quality, must be easily accessed as a result of proper feed manger and waterer space and design. Providing a minimum manger space of 60 cm per cow is recommended. Waterers should provide a space for every 15–20 cows in a group. Two waterer locations per group are recommended.

Skid-resistant walking surfaces reduce injuries, improve movement to feed, water and resting areas and enhance estrus detection. As in all aspects of facilities design, a compromise must be reached. On the one hand, the surface should provide enough texture so as to keep the animal from skidding. On the other hand, the surface cannot be so aggressive as to injure the underside of the foot of the cow. Grooves in concrete surfaces, up to 1 cm deep and 1 to 1.5 cm in wide, spaced 8–9 cm in on centre, are an acceptable method.

Summary

Proper ventilation system design and management is essential to providing an environment that allows dairy animals to grow, mature, reproduce and maintain health during cold weather. Stalls and beds, access to feed and water and walking surfaces must be considered also. Dairy farm profitability relies on sound management and quality animal environment. Both factors depend on the buildings and equipment on the dairy farm. Understanding the sometimes complex interactions involved, such as between cow comfort and barn design or between grouping strategies and the management plan, is essential when building new facilities as well as remodelling existing ones.

See also: **Reproduction, Events and Management**: Estrous Cycles: Characteristics; Estrous Cycles: Postpartum Cyclicity. **Stress in Dairy Animals**: Cold Stress: Effects on Nutritional Requirements, Health and Performance; Heat Stress: Effects on Milk Production and Composition; Heat Stress: Effects on Reproduction; Management Induced Stress in Dairy Cattle: Effects on Reproduction.

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Heat Stress: Effects on Milk Production and Composition

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Heat Stress Defined by Environment and Cow Response Measurements

The most productive dairy breeds predominantly used around the world were developed in temperate climes, and are most productive between the temperatures of 5 and 15 °C. As temperatures increase from 15 to 25 °C, cows experience a small degree of loss in production. However, as temperatures exceed 25 °C, dramatic reductions in milk production can occur. As a result, 25 °C is usually considered the upper critical temperature for lactating dairy cows.

In addition to ambient temperature, relative humidity should be considered when assessing the heat stress effect of the environment on dairy cows. The temperature–humidity index (THI) is a single value combining the air temperature and humidity. It has been developed to better define the environmental conditions under which productivity and the well-being of animals are likely to be compromised. This THI is also called the 'discomfort index'. It can be calculated for outside cattle using the following equation:

$$\begin{aligned}\mathrm{THI} = {} & (1.8 \times \text{dry bulb temperature } (\mathrm{C}^{\circ}) \\ & +32)-((0.55-0.0055 \times \% \text{ relative humidity}) \\ & \times(1.8 \times \text{dry bulb temperature } -26))\end{aligned}$$

Traditionally, the THI system has been used to describe the expected negative effects of a hot environment on milk production. Milking dairy cows are considered to experience no stress when THI is <72, mild stress when THI is between 73 and 77, significant stress when THI is between 78 and 88, severe stress when THI is between 89 and 99, and possible death when THI is >99. However, recent research conducted at the University of Arizona indicates that milk production by today's higher producing dairy cows is negatively affected starting at a THI less than 72. In eight separate studies involving 100 Holstein cows housed in environmental chambers and producing at least 35 kg of milk, production of milk was reduced when the daily minimum THI was 65 or when the daily average THI was 68 for more than 17 h per day. For example, when the minimum temperature settles at 19.6 °C and 50% humidity (THI = 68), effects of heat stress will start taking a toll. These guidelines may shift somewhat depending on the degree of air movement and direct solar radiation. Current research suggests the need to adjust the THI equation to place more weight on humidity for hot, humid areas and more weight on ambient temperature for semiarid regions in order to make THI a more accurate tool for predicting heat stress. This is based upon examination of the effects of heat stress on first-parity milk yield by cows managed in the semiarid region of Phoenix, AZ ($n = 81\,889$ cows) and in the hot, humid region of Athens, GA ($n = 12\,473$ cows). Yet recent work from Florida indicates that temperature alone had the greatest influence on predicting comfort of dairy cows managed under subtropical conditions.

Although THI is a useful tool to assess the degree of heat stress potential on the cow, her own responses to the hot weather are a more true indicator of her degree of heat stress. Increased breathing and sweating are classified as evaporative cooling techniques and are the primary means of dissipating excess heat under heat stress conditions. A rise in body temperature indicates the cow's inability to completely dissipate her heat load. **Figure 1** plots body temperatures and respiration rates collected from lactating dairy cows (<13.6 kg day^{-1} of milk) housed in chambers at eight different ambient temperatures. Points in each curve at which the measured responses made a dramatic shift upward were at ~60 respirations per minute and at ~39.2 °C rectal temperatures. In 2009, Arizona scientists reported a linear relationship between respiration rates and rectal temperatures of higher producing dairy cows expressed as $y = 0.028x + 37.438$. Based on this equation, a rectal temperature of 39.2 °C would be accompanied by 63 respirations per minute, values that support **Figure 1**. These values may be used as reference points to identify cows on the verge of experiencing significant heat stress: points at which the cow will heat up exponentially if exposed to increasing temperature and humidity.

Heat Stress Effects on Cow Performance

Studies that have been conducted to evaluate the effects of heat stress on cow performance have utilized three main approaches. Animal performance comparisons have been made between (1) cows milking during cool and hot seasons, (2) cows managed with and without methods of heat stress abatement such as shade, fans, and sprinklers, and (3) cows managed in environmentally controlled chambers.

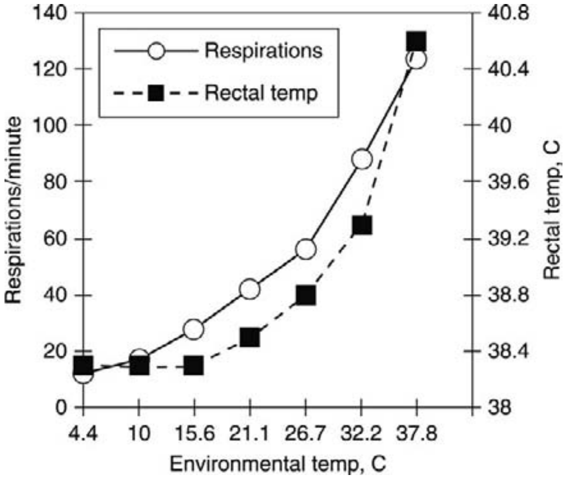

Figure 1 Effect of environmental temperature on respiration rates and rectal temperatures of lactating dairy cows. From Regan WM and Richardson GA (1938) Reaction of the dairy cow to changes in environmental temperature. *Journal of Dairy Science* 21: 73–79.

The Prepartum Period

Reducing the effects of heat stress during a cow's nonlactating, pregnant stage of life has shown benefits. Florida and Israeli workers provided either little or significant relief from heat stress conditions to cows during their last 60 days of pregnancy. Upon calving, both groups were managed the same. Cows provided relief from thermal stress gave birth to heavier calves (~3 kg) and produced more milk during the next lactation. Animals exposed to heat stress conditions likely consume less dry matter (DM) (evidenced by greater plasma nonsterified fatty acids (NEFA)), which may limit fetal growth in the last trimester, leading to smaller birth weights of calves and lower milk production. In addition, uptake of glucose and amino acids by the fetus is reduced under heat stress conditions, which contributes to reduced birth weights of calves according to research conducted at Colorado State University.

Cooling during the close-up dry period also has improved colostrum quality. Holstein heifers were exposed to (1) a THI of 65 or (2) a THI of 82 from 09.00 to 20.00 h and a THI of 76 from 21.00 to 08.00 h during the last 3 weeks before calving and 36 h postcalving. Concentrations of immunoglobulins G and A were lower in the first four milkings of heifers exposed to greater THI.

The Postpartum Period – Milk Production and Dry Matter Intake

The production of heat by a cow increases when she is in the metabolic state of lactation. This is due to (1) an increase in feed intake, which increases the heat of fermentation and nutrient metabolism, and (2) the output of a daily renewable product (milk) whose synthesis generates heat within the body. Both sources of metabolic heat must be dissipated from the body; this is more difficult when the environment has a high THI.

At the upper critical temperature of 25.5 °C, the cow often begins to eat less feed. Elevations in body temperature may signal the hypothalamus to reduce voluntary intake. As the temperature increases, the amount of energy expended by the cow to maintain homeothermy is increased. It is estimated to be 20% greater at 35 °C compared to that at 20 °C. In order to cover this additional energy cost, the intake of DM must increase. However, during hot weather, DM intake decreases. Therefore, the energy status of the cow is challenged due to greater energy costs required to maintain homeothermy and less energy consumption. Thus, it is not surprising that milk production goes down because availability of energy for lactation is less. However, the decrease in DM intake may only account for 35–50% of the decrease in milk production. Arizona research suggests that heat-stressed cows partition more nutrients toward other tissues and less toward the mammary gland, contributing to reduced milk production.

A review of 12 studies from the scientific literature indicates a simultaneous drop in feed intake and milk production as cows experience heat stress. As rectal body or milk temperatures increased from an average of 38.8 to 39.9 °C, average DM intake decreased from 18.4 to 15.6 kg day^{-1} and average milk production decreased from 22.4 to 19.2 kg day^{-1}. For the most part, these cows are considered 'lower producing cows'. To examine whether higher producing cows are more sensitive than lower producing cows to thermal stress, the studies were divided into two groups. Three studies in which cows produced an average of >30 kg milk day^{-1} under thermoneutral conditions were grouped together. A second group of nine studies was composed of cows that produced an average of <25 kg milk day^{-1} at thermoneutral conditions. As the average body temperature of 'lower' producing cows increased from 38.9 to 39.9 °C, average intake of DM decreased from 17.4 to 15.0 kg day^{-1} and average milk production decreased from 19.0 to 16.3 kg day^{-1}. Milk production decreased 2.7 kg day^{-1} per 1 °C increase in body temperature. As the average temperature of 'higher' producing cows increased from 38.5 to 39.8 °C, average intake of DM decreased from 21.3 to 17.5 kg day^{-1} and average milk production decreased from 32.6 to 27.9 kg day^{-1}. Milk production decreased 3.6 kg day^{-1} per 1 °C increase in body temperature. Based upon limited information, it appears that heat stress is more detrimental to cows of greater milk production. When Holstein cows (summary of 8 studies involving 100 cows) were housed in environmental chambers to control THI, milk production decreased linearly by 2.1 kg day^{-1} for every 1 °C increase in body temperature between 38 and 41 °C.

Other studies have implied this same principle. As temperature and humidity rose, milk production decreased to a greater extent for Holstein cows than for Jersey and Brown Swiss breeds. Cows in the early part of lactation experienced a greater depression in feed intake than cows in midlactation during the summer season. The use of bovine somatotropin (bST) as a lactational promotant can increase body temperature as a result of increased milk production. Cows injected with 20 mg day^{-1} of bST were reported to increase their milk production by ~4 kg day^{-1} and milk temperature was increased between 0.4 and 0.5 °C. As cows are managed to produce more milk, we should be equally dedicated to providing them the means to cope with the additional heat load that comes with this additional milk during hot weather.

In a 10-year study, involving 100 purebred Holstein cows in their first lactation in Maryland, cows calving in January and February produced 17% more milk than cows calving in July and August. Major differences in performance took place in the first 50 days of lactation. Environmental conditions, mainly temperature, had a negative effect on appetite. Cows that calved in July and August consumed 14% less feed and produced 21% less milk than cows that calved in winter. As a result, loss of body weight was greater for summer calvers compared to winter calvers.

Cows can inadvertently increase the heat load on themselves by consuming much of their ration prior to the hottest part of the day. Peak heat production by the cow occurs about 3–4 h after eating. Therefore, the rising body temperature due to feed intake may coincide with the rising ambient temperature, thus increasing the maximum heat load on the cow. Cows fed in the evening cooled down more quickly after exposure to elevated THI in environmental chambers than cows fed in the morning. The pattern of eating also differs during cool and hot conditions. Under hot conditions, cows often eat more frequent meals of smaller size resulting in a lower intake per day, yet they experience a greater increase in body temperature.

Cows may attempt to eat more feed at night than during the day. Under conditions when the THI still exceeded the upper critical THI of 72, Florida workers reported that the amount of feed consumed at night did not compensate for the greatly depressed intake during the day. The ability of a cow to cope with heat stress may be dependent upon the extent to which she can cool down at night. Very warm evenings (i.e., lack of night cooling) can prevent cows from making up the DM intake they lost during the day. Daily intake of DM began decreasing when the minimum environmental temperature was above 19 °C for lactating Holstein cows. Scientists in Arizona documented that milk production of Holstein cows suffered when THI failed to drop below a THI of 65. Loss of milk averaged 2.2 kg per cow per day for cows producing at least 35 kg day^{-1} at thermoneutral temperatures. Jersey cows in Arizona were able to maintain milk production until nighttime THI failed to drop below 75 and then production dropped 2.8 kg day^{-1}.

Sometimes, it is the intermittent heat waves that are more dangerous than the regular episodic thermal stress. In 1997, more than 100 feedlot cattle died due to extreme THI over a 4-day period. It was the third such heat wave in 3 weeks. What made the third heat wave so lethal may have been an increased DM intake of animals just prior to the third event. Relatively cool weather had come in right after the second heat wave so the animals had compensated for their reduced intake during the second heat wave by eating much more feed. Greater gut fill during the third heat wave increased their metabolic heat load on top of the environmental heat load, which may have prevented the animals from dissipating the heat needed to survive.

Without question, water is the nutrient of greatest importance in hot weather. Cows drink more water under heat stress conditions. This undoubtedly aids in cooling the body core of the cow. In addition, the cow loses additional water from the skin and lungs as she works to minimize her increased body temperature. Intake of drinking water by lactating cows increased 29% (16.8 kg day^{-1}), and loss of water via the skin and respiration increased 59 and 50%, respectively, when ambient temperature increased from 18 to 30 °C (**Table 1**). Any factor that may inhibit cows from

Table 1 Intake and excretion routes of water by lactating cows at two environmental temperatures

Measurement	*18 °C*	*30 °C*	*Percentage difference*
Water drank (kg day^{-1})	57.9	74.7	29.0
Feed water (kg day^{-1})	1.6	1.4	−14.3
Urine volume (kg day^{-1})	11.1	12.8	15.0
Fecal water (kg day^{-1})	17.9	12.0	−33.0
Evaporation			
Surface (g m^{-2} h^{-1})	94.6	150.6	59.3
Respiration (g m^{-2} h^{-1})	60.6	90.7	50.0

Reproduced with permission from Collier RJ, Beede DK, Thatcher WW, Israel LA, and Wilcox CJ (1982) Influences of environment and its modification on dairy animal health and production. *Journal of Dairy Science* 65: 2213–2227.

drinking must be eliminated. If water intake is restricted in hot weather, the drop in milk production will be precipitous. The cows will become hotter than normal, and DM intake will decrease to a greater extent as intake of DM and water are closely linked. Providing cool, clean water in *ad libitum* amounts is simply good management.

The Postpartum Period – Milk Composition

Milk fat concentration

In 6 of 14 scientific studies, milk fat percentage decreased significantly (4.05 vs. 3.58%) when heat stress conditions were intensified. All other studies reported no change in milk fat percentage as rectal temperatures increased. An additional study involving nearly 23 000 observations on Florida dairy farms examined the relationships between many variables, including milk composition and environmental temperature. As temperatures increased from 9.4 to 36.1 °C, the authors reported that milk fat concentration dropped from 3.85 to 3.31% and milk protein dropped from 3.42 to 2.98%. Milk constituents were not influenced by the degree of relative humidity. The effect of heat stress on milk fat content appears to be influenced by the forage-to-concentrate ratio of the diet. Fat concentration of milk from cows fed a 35% hay diet was lower during hot conditions compared to thermoneutral conditions (3.69 vs. 3.90%). However, milk fat concentration was unchanged by heat stress when cows were fed a diet containing 65% forage. In a separate study, cows were fed diets of 16, 17.9, 19.4, and 21.2% acid detergent fiber during warm (THI between 64 and 77) and hot weather conditions (THI between 72 and 84) (**Table 2**). Milk fat percentage was unchanged by the diet during the period of warm temperatures. However, under greater heat stress conditions, milk fat content decreased linearly as the fiber content of the diet decreased. The authors speculated that a slight acidosis was corrected with the higher fiber diet, resulting in improved ruminal efficiency and digestion.

It appears that cows subjected to heat stress may be at increased risk to ruminal acidosis. It appears that heat-stressed cows can have lower ruminal fluid pH, less ruminating activity, low milk fat percentage, and reduced buffering capability by the saliva. Fistulated Holstein cows were kept at either 18 °C and 50% relative humidity or 29 °C and 85% relative humidity for 5 weeks. Ruminal fluid was measured 12 times postfeeding for pH and lactic acid concentration. Cows kept in the hotter environment had lower ruminal pH (~5.8 vs. ~6.3) and greater lactic acid (~1.9 vs. ~0.45 meq l^{-1}). In another study, mean ruminal fluid pH (24 h average) was lower (6.53 vs. 6.66) for Holstein cows denied access to shade. Fecal pH was lower (5.92 vs. 6.08) for unshaded compared to shaded lactating cows.

A predisposition to acidosis during heat stress is indicated by the report that elevated environmental temperatures negatively affected ruminal contractions. The number of ruminal contractions decreased from 2.4 to 1.7 per minute when lactating cows were not provided shade (rectal temperatures of 38.7 vs. 39.6 °C). Missouri workers kept ruminally fistulated Holstein cows at an ambient temperature of 38 or 18 °C for 5 days (rectal temperatures of 40.9 vs. 38.4 °C). The hotter cows had less rigorous ruminal contractions as evidenced by a 50% reduction in the average amplitude. The frequency of contractions (2.2 vs. 1.7 per minute) closely followed the pattern reported earlier but was not significant. A lower DM intake by cows at the higher temperatures in the Missouri study was not responsible for the difference in rumen movement because DM intakes were equalized by placing uneaten feed into the rumen via fistula. A decreased number or intensity of ruminal contractions due to heat stress may have a negative effect on saliva production, thereby reducing the buffering activity in the rumen, resulting in a lower ruminal pH. This decreased activity of the rumen musculature of heat-stressed cows may be related to a reduced concentration of volatile fatty acids (VFAs) in

Table 2 Effect of heat stress on milk production (kg day^{-1}) and milk fat percentage of cows fed diets differing in ADF concentrations

	Milk production (kg day^{-1})		*Milk fat percentage*	
Percentage ADF of diet	*Warm temperatures*[a, b]	*Hot temperatures*[c, d]	*Warm temperatures*[a]	*Hot temperatures*[b, c]
16.0	32.3	24.6	3.24	3.21
17.9	32.6	25.8	3.49	3.28
19.4	31.4	26.4	3.58	3.50
21.2	28.9	22.7	3.62	3.69

[a] Minimum and maximum values of THI were 64 and 77, respectively.
[b] Linear effect.
[c] Minimum and maximum values of THI were 72 and 84, respectively.
[d] Quadratic effect.
ADF, acid detergent fiber.
Reproduced with permission from West JW, Hill GM, Fernandez FM, Mandebvu P, and Mullinix BG (1999) Nutritional strategies for managing the heat-stressed dairy cow. *Journal of Dairy Science* 82: 2455–2465.

the rumen. That ruminal VFAs may play an essential role in stimulating rumen motility by influencing the neural receptors in the rumen wall may partially explain the reduced motility noted under heat stress conditions. When ruminally fistulated Holstein cows were kept at either 18.2 or 37.7 °C, the concentration of VFAs decreased by more than 50% in heat-stressed cows despite maintaining equal intake of DM through placement of uneaten feed into the rumen.

Respiratory alkalosis is an additional physiological mechanism operating during heat stress conditions that may contribute to ruminal acidosis and thereby lower the fat content of milk. As cows become heat stressed and respiration rates increase, CO_2 is eliminated from the lungs faster than it is produced. This results in decreased blood CO_2. In an attempt to keep the CO_2-to-bicarbonate (HCO_3^-). ratio constant in the blood, the kidney excretes more HCO_3^-. With more CO_2 leaving from the lungs and more bicarbonate leaving in the urine, bicarbonate concentration in the blood drops, H^+ concentration in the blood drops as H^+ is used to produce more CO_2, and blood pH becomes more alkaline (termed respiratory alkalosis) (**Figure 2**). This drop may, in turn, reduce the bicarbonate concentration in saliva, thus reducing the buffering activity in the rumen and increasing the risk of ruminal acidosis.

A last factor that may contribute to lower milk fat content is the habit of cows to selectively consume concentrates and minimize intake of forages to a greater degree in hot compared to cooler weather, thus predisposing them to acidosis and lower milk fat content.

Milk protein concentration

Lactating cows exposed to heat stress conditions can experience lowered protein concentration in milk. In 5 of 10 studies examining heat stress effects on animal performance, milk protein concentration was significantly reduced by an average of 0.18 units. The solids-not-fat content was reduced due to heat stress in three other studies by 0.2–0.4 units. These decreases in milk protein percentage due to heat stress may be due to lowered microbial protein synthesis in the rumen and lowered protein intake due to lowered intake of DM.

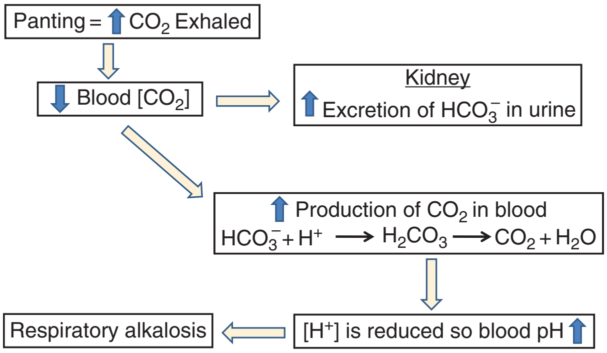
Panting = ↑ CO_2 Exhaled
↓ Blood [CO_2]
Kidney
↑ Excretion of HCO_3^- in urine
↑ Production of CO_2 in blood
$HCO_3^- + H^+ \longrightarrow H_2CO_3 \longrightarrow CO_2 + H_2O$
[H^+] is reduced so blood pH ↑
Respiratory alkalosis

Figure 2 Metabolic responses to heat stress work to maintain equilibrium of CO_2 and HCO_3^- in bloodstream.

Milk somatic cell count

In five of six studies, somatic cell counts (SCCs) were higher in the milk of cows exposed to greater heat stress and two of these five studies reported significant increases. The number of white blood cells in plasma was reduced 16% in cows exposed to heat stress conditions using environmental chambers. The immune system of the cow is likely under greater strain during heat stress conditions and therefore the cow is less able to manage subclinical mammary infections.

Other milk constituents

The fatty acid profile of milk fat is changed as cows are exposed to greater heat stress conditions. The concentrations of the shorter chain fatty acids (C_6–C_{12}) were reduced in the milk of cows housed in chambers kept at 29.4 °C and 70% relative humidity compared to those housed in chambers kept at 18.3 °C and 50% relative humidity. This is possibly because of lower production of VFAs in the rumen due to lowered feed intake. The proportion of $C_{18:1}$ in milk fat was also decreased whereas the proportion of $C_{18:0}$ was increased in hotter conditions. This is thought to be due to lower feed intake that results in slower passage of feed from the rumen, thus allowing a greater degree of biohydrogenation of dietary polyunsaturated fats.

The potassium content of milk decreased from 2005 to 1853 ppm when cows were denied access to shade but sodium content was unchanged (521 vs. 543 ppm). Unlike man, the primary electrolyte lost in skin secretions of cattle is K (K_2CO_3 and $KHCO_3$) rather than Na. As heat stress conditions intensify, the production of secretions by the skin and the concentration of K in the secretions increase resulting in an exponential loss of K. The decreased potassium in milk may reflect a conserving mechanism by the cow for prioritized secretion of potassium from the skin.

When exposed to heat stress conditions, milk concentrations of growth hormone increased 38% and prolactin increased 85%. Prolactin may be involved in helping the heat-stressed cow meet increased requirements of water and electrolytes.

Conclusion

Lactating dairy cows begin to experience seriously the negative effects of heat stress when rectal temperatures exceed 39.2 °C. A respiration rate of >60 per minute is an indication that stress due to heat and humidity is becoming excessive. Pregnant cows provided relief from thermal stress during the nonlactating period will have heavier calves at parturition, show improved colostrum quality, and will produce more milk postpartum. Intake of feed is reduced as cows attempt to maintain homeothermy in thermal stress conditions. Intake was reduced 15% in a review of 12 scientific studies as rectal temperatures increased from 38.8 to 39.9 °C. As a result, production of milk was reduced 14% as well. Higher producing cows as well as all cows in their first 50 days of lactation are most sensitive to heat stress. Management of feed and water intake is important in helping cows cope with conditions of thermal stress. Cows exposed to significant heat stress are more susceptible to ruminal acidosis. Ruminal pH may be lowered because of reduced buffering of the rumen and a reduction in the number and intensity of ruminal contractions. Percentages of milk fat and protein are often lowered (∼0.5 and 0.2 percentage units, respectively) during the times of heat stress and during the summer season. The decrease in milk fat percentage may be aggravated by feeding diets that contain borderline concentrations of fiber. There is an increase in SCC, growth hormone, and prolactin and a decrease in potassium content in the milk of cows exposed to greater heat stress.

See also: **Stress in Dairy Animals**: Cold Stress: Effects on Nutritional Requirements, Health and Performance; Cold Stress: Management Considerations; Heat Stress: Effects on Reproduction; Management Induced Stress in Dairy Cattle: Effects on Reproduction.

Further Reading

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Heat Stress: Effects on Reproduction

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Introduction

Mammals are homeotherms and regulate heat production and heat loss to maintain a constant and high body temperature. It becomes difficult for mammals to successfully regulate body temperature when heat production increases (such as during vigorous exercise or associated with lactation) or when the environment limits the degree of metabolic heat loss from the animal. Heat stress represents the combination of high air temperature, intense solar radiation, and high humidity that together reduce the flow of heat from the animal so that body temperature increases. The condition of elevated body temperature is known as hyperthermia. Biological and productive functions are compromised by heat stress because the physiological adjustments animals undergo to regulate body temperature can have other deleterious effects on the animal and because hyperthermia itself can alter cellular function. One example of a physiological adjustment to heat stress that can compromise biological function is a reduction in feed intake. About half of the decrease in milk yield in dairy cows exposed to heat stress is caused by reduced appetite and feed intake. An example of alterations in cellular function caused by hyperthermia is the embryonic death that occurs when preimplantation embryos are exposed to temperatures above normal body temperature.

All mammals are susceptible to heat stress but lactating cows are particularly so. The large amounts of heat produced as a result of lactation make it difficult for the lactating female to regulate its body temperature. Indeed air temperatures as low as 25–29 °C can cause hyperthermia in lactating dairy cows. As a result, heat stress has greater effects on body temperature and reproduction in lactating animals than in nonlactating animals (**Figure 1**). In addition, cows producing more milk experience a greater decline in reproductive function during heat stress than do cows producing less milk.

There are two consequences of the relationship between milk yield and heat stress. First, lactating dairy cows intensely managed to produce large amounts of milk are susceptible to heat stress in most temperature regions of the world and not just in regions considered to have a hot climate. Indeed a reduction in fertility in summer has been reported in Alberta, Canada. Second, further increases in milk yield, due to genetic improvement or changes in management, are likely to make cows more susceptible to heat stress in the future. This, coupled with the possibility for global climate change, are likely to increase the economic losses and reduce animal comfort associated with heat stress.

Actions of Heat Stress on the Female

Expression of Estrus

Reduced intensity of estrus limits the use of artificial insemination (AI) and other reproductive management techniques dependent upon visualization of estrus (*see* **Reproduction, Events and Management**: Mating Management: Detection of Estrus). The reduction in estrus behavior involves reduced mounting activity and shorter periods of estrus. In a Virginia study, Holsteins in estrus during the summer had about half the number of mounts of cows in estrus during the winter. In another study, the duration of estrus was 20 h for cows maintained in cooled conditions compared to 11 and 14 h for heat-stressed cows in temperature control chambers or the natural summer environment, respectively. As a result of a reduction in behavioral estrus, the proportion of cows not detected in estrus can rise to 80% in summer, as has been reported in Florida. Lack of detected estrus in summer is a result of suboptimal estrus behavior rather than because cows become acyclic in the summer. Once established, cyclicity is generally unaffected by heat stress.

Fertility

Even if lactating cows are detected in estrus, the probability that pregnancy will be established and maintained after mating is low during heat stress. Depending upon the degree of heat stress, first-service conception rates in lactating dairy cows exposed to heat stress can be 10% or less as compared to rates of 25–40% in cows not exposed to heat stress. Fertility is also reduced by heat stress in other mammals used for milk production, including sheep.

The reduction in fertility caused by heat stress is due to a combination of reduced fertilization rate and increased embryonic mortality. Changes in oocyte function are responsible for some of the infertility. The early embryo is very susceptible to elevated maternal

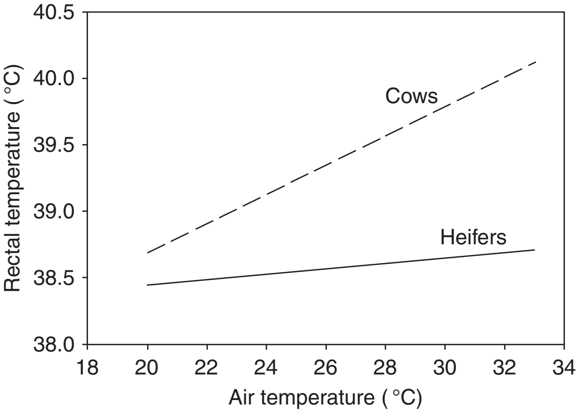

Figure 1 The relationship between air temperature and rectal temperature for nonlactating heifers and lactating cows. Data were collected in Wisconsin and are from Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, and Wiltbank MC (2002) Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. The figure was prepared by PJ Hansen and is reproduced with permission of Intervet.

temperature, but resistance increases as development proceeds so that by the morula stage (day 5 after estrus) a single day of heat stress has no effect on embryonic survival.

As illustrated in **Figure 2**, there are three windows in the reproductive life cycle of cows where heat stress results in reduced pregnancy rate after mating. The first is the period before ovulation. The oocyte that ovulates coincident with estrus is located in a follicle that initiated growth 90–110 days previously, and it is possible that heat stress during some or all of this period of follicular growth can lead to ovulation of an oocyte with reduced competence for fertilization and development. It has been demonstrated experimentally that heat stress 20–26 days before estrus alters follicular function. Studies in Israel indicate that there is a lag in restoration of fertility in autumn and that the length of this lag can be shortened by removing follicles that experienced growth during summer. The second window is the periovulatory period. Experimental heat stress during this time did not decrease fertilization rate, but the ability of the embryos formed after fertilization to develop normally was reduced. The third window is the period of embryonic development. As mentioned above, embryos are initially sensitive to heat stress but become resistant by the morula stage. The reason why embryos acquire thermotolerance during development is not known for certain but probably involves decreased free radical formation and acquisition of biochemical mechanisms to stabilize cells exposed to elevated temperature (e.g., antioxidants such as glutathione and heat shock protein 70).

Although embryos become resistant to heat stress by the morula stage, heat stress can cause some embryonic

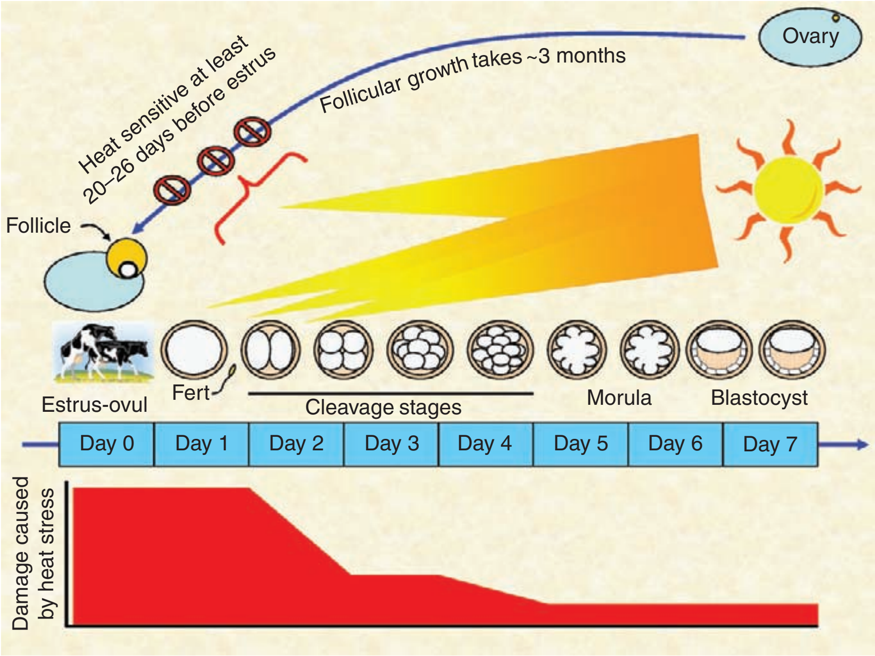

Figure 2 (see color plate 94) Changing sensitivity of the cow to heat stress. The follicle takes about 3 months to complete growth. Heat stress can disrupt follicular function at least as early as 20–26 days before estrus. Heat stress can also disrupt the oocyte on the day of estrus and inhibit development of the early embryo. By day 3 after breeding, however, heat stress has little effect on embryonic survival, and the morula and blastocyst present beginning at about day 5 after breeding are resistant to elevated temperature. The figure was prepared by PJ Hansen and is reproduced with permission of Intervet.

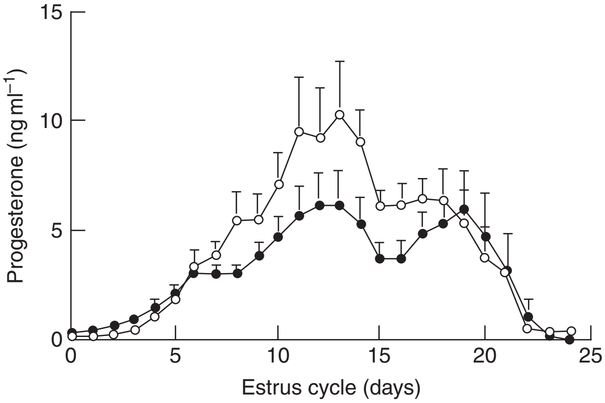

Figure 3 Average daily serum concentration of progesterone during spring (○) and summer (●). Reproduced with permission from Howell JL, Fuquay JW, and Smith AE (1994) Corpus luteum growth and function in lactating Holstein cows during spring and summer. *Journal of Dairy Science* 77: 735–739.

loss later in pregnancy. Effects of heat stress that can contribute to pregnancy loss at the morula stage or after are a reduction in circulating concentrations of progesterone (**Figure 3**), reduced uterine blood flow, and disruptions in the process by which the embryo antiluteolytic signal, interferon-τ, causes reduced synthesis and release of prostaglandin $F_{2\alpha}$ by the uterus (*see* **Reproduction, Events and Management**: Pregnancy: Characteristics; Pregnancy: Physiology).

Fetal Growth

Heat stress during the latter half of pregnancy has been implicated in intrauterine growth retardation, resulting in smaller birth weights (**Table 1**). Intra-uterine growth retardation has been seen in sheep, especially those carrying twins, and in cattle. One consequence of intra-uterine growth retardation caused by heat stress is increased neonatal mortality, especially in sheep.

Table 1 Spring lambing performance of ewes maintained in three environmental conditions during the last third of pregnancy

	Ewe treatment		
Item	*Range control*	*Restricted feed*	*Hot room*
Number of ewes pregnant	15	15	14
Ewe condition[a]	2.0 ± 0.0	2.1 ± 0.1	2.0 ± 0.0
Ewe disposition[b]	2.0 ± 0.0	2.0 ± 0.1	2.0 ± 0.0
Lamb %, live[c]	167 ± 19	173 ± 15	100 ± 26[c]
Lamb %, total	173 ± 18	173 ± 15	150 ± 23
Average birth weight, live (kg)	4.69 ± 0.26	4.16 ± 0.16	3.26 ± 0.31[d]
Average birth weight, total (kg)	4.57 ± 0.26	4.16 ± 0.16	3.18 ± 0.22[d]
Lamb condition[e]	1.9 ± 0.2	2.0 ± 0.1	1.5 ± 0.3[d]

[a] 1, fat; 2, good; 3, poor.
[b] 1, wild; 2, calm.
[c] Lambs ÷ pregnant ewes × 100.
[d] 0, dead; 1, weak; 2, average; 3, strong.
[e] $p < 0.01$ after the removal of sex and multiple birth effects.

Data are from Brown DE, Harrison PC, Hinds FC, Lewis JA, and Wallace MH (1977) Heat stress effects on fetal development during late gestation in the ewe. *Journal of Animal Science* 44: 442–446.

Some effects of heat stress on placental function represent redistribution of blood to the periphery and reduced perfusion of the placental vascular bed. However, placental blood flow per gram of fetus was similar both in the heat-stressed and in the control ewes in one study, and it may be that increased vascular resistance in the placenta caused by aberrant angiogenesis is more important. In addition, placental capacity to transport glucose using the glucose transporter GLUT8 can be reduced by heat stress.

Another consequence of heat stress during gestation is a decrease in milk yield after calving. This effect presumably reflects alterations in secretion of placental hormones by the thermally compromised placenta.

Actions of Heat Stress on the Male

Semen Quality

Summer conditions in most regions of the world are severe enough to lower semen quality. In one study, ambient temperatures above 27 °C for as little as 6 h day^{-1} were sufficient to lower semen quality in *Bos taurus* bulls. Effects of heat stress include a reduction in sperm concentration and progressive motility as well as an increase in sperm with abnormal morphology (**Table 2** and **Figure 4**). The reason why germ cells are damaged by heat stress is not completely understood but increased oxidative stress in the testis is one cause. Spermatocytes, spermatids, and, to a lesser extent, B spermatogonia are the germ cells most sensitive to heat

Table 2 Mean semen characteristics of breeds of bulls before, during, and after heat stress

	Initial motility			*Abnormal spermatozoa*		*Spermatozoan concentration*	
Breed[a]	*Period*[b,c]	*%*	*% of initial*	*%*	*% of initial*	*10^6 ml^{-1}*	*% of initial*
Holstein	1	53	100	9	100	1194	100
	2	45	85	13	144	1303	112
	3	34	64	53	589	537	48
	4	42	79	29	467	460	39
Red Sindhi × Holstein	1	48	100	11	100	1239	100
	2	33	69	21	191	2150	174
	3	46	96	38	345	1734	140
	4	43	90	23	209	903	73
Brown Swiss	1	48	100	16	100	935	100
	2	35	73	21	131	1322	141
	3	23	48	33	206	476	51
	4	43	90	20	125	523	56
Red Sindhi × Brown Swiss	1	49	100	16	100	1126	100
	2	40	82	12	75	1291	115
	3	48	98	22	138	852	76
	4	51	104	18	113	1062	94

[a] Crossbred–purebred × period of interaction significant ($p < 0.05$) for all variables.

[b] 1, three weeks prior to heat stress (initial); 2, one week of heat stress; 3, one–three weeks after heat stress; 4, seven–nine weeks after heat stress.

[c] Period effects significant ($p < 0.01$) for all variables.

Data are from Johnston JE, Naelapaa H, and Frye Jr. JB (1963) Physiological responses of Holstein, Brown Swiss and Red Sindhi crossbred bulls exposed to high temperatures and humidities. *Journal of Animal Science* 22: 432–436.

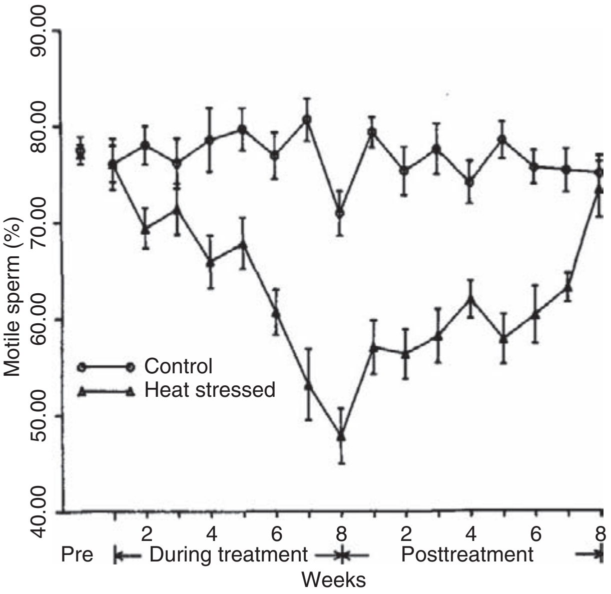

Figure 4 Time course of effects of heat stress on motility of ejaculated spermatozoa in bulls. Control bulls were maintained in a chamber at 23 + 1 °C for 16 weeks. Heat-stressed bulls were maintained for 8 weeks in a chamber that was at 31 + 1 °C for 8 h and 35 + 1 °C for 16 h each day. Thereafter, heat-stressed bulls were returned to an environment of 23 + 1 °C for 8 weeks. Reproduced with permission from Meyerhoeffer DC, Wettemann RP, Coleman SW, and Wells ME (1985) Reproductive criteria of beef bulls during and after exposure to increased ambient temperature. *Journal of Animal Science* 60: 352–357.

stress. One consequence is that semen characteristics are not immediately affected by heat stress because damaged spermatogenic cells do not enter ejaculates immediately. Similarly, elimination of heat stress does not lead to improved sperm quality until the damaged germ cells have completed spermatogenesis. In the bull, for example, where spermatogenesis takes about 61 days, semen characteristics change about 2 weeks after heat stress and do not return to normal until up to 8 weeks following the end of heat stress.

Libido

It is reasonable to expect that heat stress would decrease libido but the experimental evidence to support this idea does not exist. In addition, changes in testosterone secretion in response to heat stress are transient, with an initial decrease in testosterone secretion after heat stress reversed within 2 weeks.

Management to Reduce the Effects of Heat Stress

Environmental Modifications to Reduce the Magnitude of Heat Stress

As shown in **Figure 5**, a wide range of housing systems have been developed to reduce the magnitude of heat

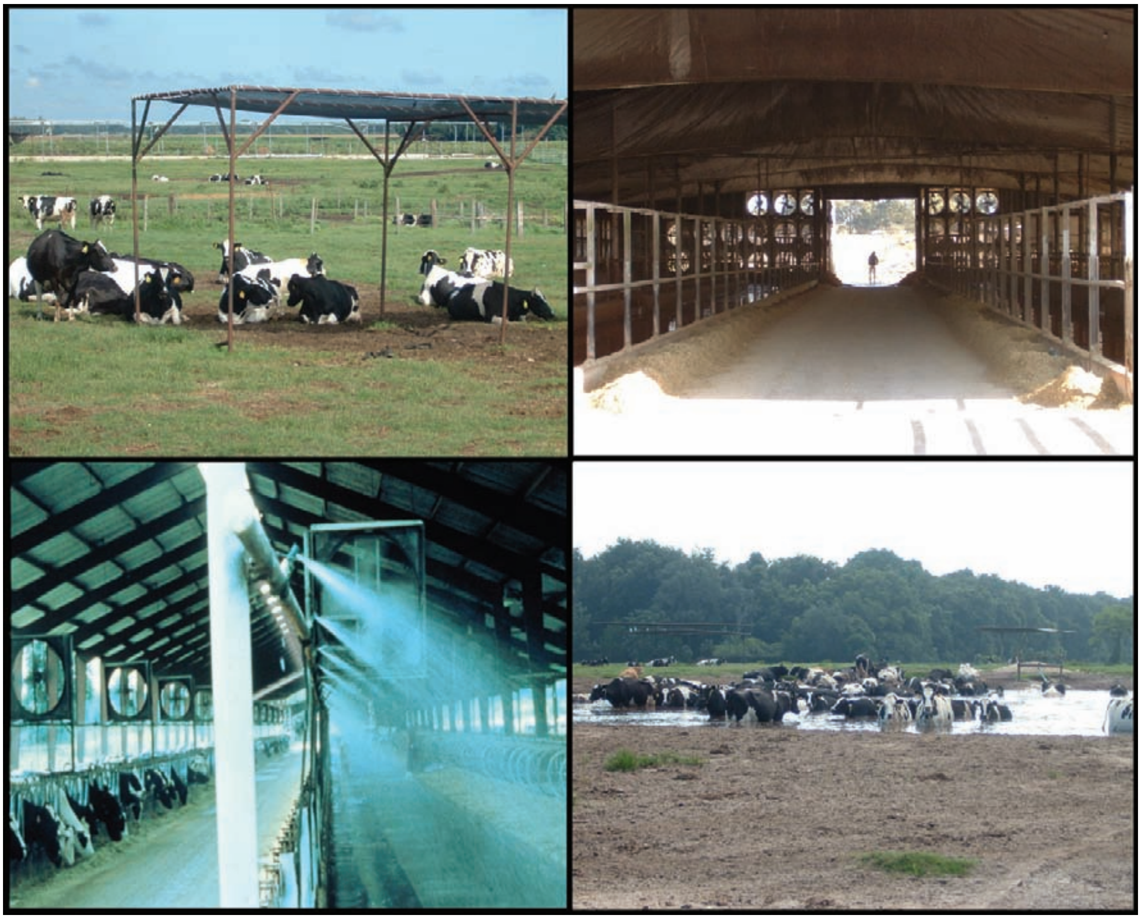

Figure 5 (see color plate 90) Some housing systems to cool dairy cattle during heat stress. The shade structure shown in the upper left panel is insufficient for adequate cooling in lactating cows. The photograph in the lower left shows a barn with sprinklers and fans installed over the feeding area. Such a system can provide significant cooling but often reproduction is still compromised during heat stress. A barn with tunnel ventilation is shown in the upper right photograph. Tunnel ventilation can be an effective system for cooling cows but is also expensive to construct. The photograph in the lower right panel shows cows cooling themselves in a man-made cooling pond. Reproduced from *Horizons* with permission of Genex Cooperative (2006) 12(3): 12–13.

stress faced by lactating animals (*see* **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates). Provision of fans, sprinklers or misters, shade, and cooling ponds can reduce the magnitude of heat stress affecting the cow, improve the animal's ability to regulate its body temperature, and enhance milk yield. There can be reproductive benefits also. For example, in a Mississippi study, fan cooling of shaded, lactating Holsteins that were synchronized with two injections of prostaglandin $F_{2\alpha}$ resulted in 71% exhibiting estrus, compared to 33% for shaded cows without fan cooling. Under on-farm conditions, however, seasonal variation in reproductive function persists in most herds even when environmental modifications are present. Recently, the so-called tunnel ventilation barns and cross-ventilation barns have received much attention in popular press as effective structures for cooling cows. In these low-ridged barns, fans are located at one end of the barn to create a wind tunnel-like effect. Evaporative cooling is by means of misters or evaporative pads through which air is drawn by the fans. A comprehensive study to evaluate reproductive function in tunnel ventilation or cross-ventilation barns has not yet been conducted.

Overcoming Poor Detection of Estrus

Detection of estrus during heat stress can be improved by various methods, including application of paint to the tailhead, commercial mount detectors, and radiotelemetric pressure transducers, such as the HeatWatch^{TM} system. The advent of hormonal programs to synchronize ovulation for fixed-time insemination can eliminate the need for estrus detection during heat stress (*see* **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Ovulation and Insemination). In a study in Florida, Holsteins subjected to synchronized ovulation with timed insemination for

Table 3 Seasonal variation in pregnancy rate in dairies in Florida and Georgia as affected by mating system

	Natural service (%)	*Natural + AI (%)*	*AI (%)*
Summer	9.8	9.3	8.1
Winter	18.0	17.8	17.9

From De Vries A, Steenholdt C, and Risco CA. (2005) Pregnancy rates and milk production in natural service and artificially inseminated dairy herds in Florida and Georgia. *Journal of Dairy Science* 88: 948–956. Pregnancy rate was the proportion of cows that became pregnant out of those eligible to be bred in a 21-day period. Herds were designated natural service if 90–100% of cows were bred with bulls, natural + AI if the proportion of matings with bulls was from 11 to 89%, and AI if 0–10% of breedings were with bulls. Season × mating system, $p < 0.001$.

the first insemination had more pregnancies (16.6%) at 90 days postpartum than did controls (9.8%). In another Florida study, pregnancy rates at 90 days were 34.3% for cows given the synchronized ovulation with timed insemination treatment compared to 14.3% in controls. The improvement in the proportion of cows pregnant through the use of a timed AI program is the result of inseminating more cows and not of fertility to service, which remains compromised by heat stress.

Some producers utilize natural service to reduce the requirement for estrus detection and AI. A study in Florida indicates that the pregnancy rate (defined as the percentage of cows that become pregnant out of those eligible to be bred in a 21-day period) in summer was slightly higher for herds using bulls or a mix of bulls and AI than for herds using AI alone (**Table 3**). In winter, pregnancy rates were similar for all three types of herds. Loss of genetic progress and increased feed costs associated with natural service may negate the economic advantage of the higher pregnancy rates achieved with use of bulls.

Improving Embryo Survival

One important cause of damage to gametes and the embryo due to heat stress is increased production of free radical species. There is some evidence that feeding the antioxidant β-carotene can improve fertility in dairy cows exposed to heat stress; further studies are required to verify this effect. A variety of hormonal treatments has also been used in attempts to improve fertility in lactating dairy cows exposed to heat stress (*see* **Reproduction, Events and Management**: Mating Management: Fertility). Among these treatments are bovine somatotropin, human chorionic gonadotropin, and gonadotropin-releasing hormone. Unfortunately, no hormonal treatment that can consistently improve embryo survival has been identified. It is probable that damage to the oocyte before ovulation caused by heat stress leads to a reduction in the oocyte's competence to be fertilized or to develop into an embryo and that this damage is not reversible by hormonal treatments given at or after breeding.

As mentioned previously, restoration of fertility after the end of the hot season is gradual because follicles that were damaged early in development need to complete folliculogenesis and be cleared from the ovary by atresia or ovulation. Research in Israel indicates that restoration of fertility in autumn can be hastened by ablation of follicles on the ovary by increasing follicular turnover through bovine somatotropin or follicle-stimulating hormone treatment, or by physical ablation of follicles.

Use of Embryo Transfer to Bypass Effects of Heat Stress on the Oocyte and Embryo

From an examination of **Figure 2**, it is apparent that embryos that reach the blastocyst stage at day 7–8 after estrus have already passed through the most thermosensitive periods of development. It follows, therefore, that pregnancy rate for embryo transfer during summer would be higher than pregnancy rate for AI. Indeed, this is often the case, as illustrated in a large-scale study in Brazil in which lactating cows were either inseminated or subjected to embryo transfer. In cool weather, fertility was the same for both groups. However, the decline in fertility in summer seen in cows bred by AI did not occur for cows subjected to embryo transfer (**Figure 6**).

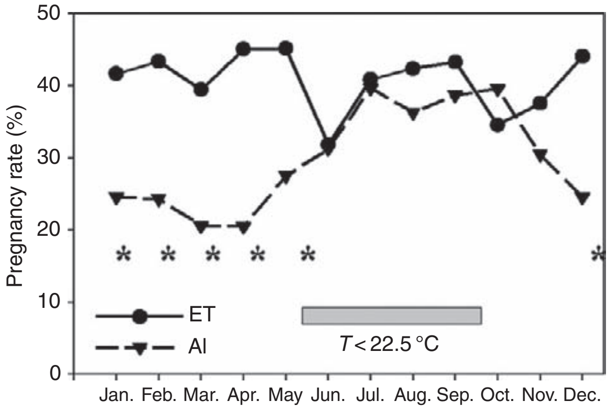

Figure 6 Differences in pregnancy rate between embryo transfer recipients and inseminated animals in lactating cows in Brazil. Lactating Holstein cows either were inseminated or received a fresh or frozen-thawed embryo produced by superovulation. Data are either the number pregnant/number inseminated or number pregnant/number receiving an embryo. Months in which the average ambient air temperature was less than 22.5 °C are shown with the gray bar. Note that embryo transfer increased pregnancy rate in hot weather but not in cool weather. Data are redrawn from Rodrigues CA, Ayres H, Reis EL, Nichi M, Bo GA, and Baruselli PS (2004) Artificial insemination and embryo transfer pregnancy rates in high production Holsetin breedings under tropical conditions. In: *Proceedings of the 15th International Congress on Animal Reproduction*, vol.2, p.396. Porto Seguro, Brazil (abstract) with permission.

In fact, embryo transfer represents the only consistent method for improving pregnancy success in heat-stressed cows. Estrus detection is not a limiting factor in the utilization of embryo transfer during heat stress because embryos can be transferred at a fixed time when ovulations are programmed using hormonal regimens developed for timed AI. One limitation is the high cost of embryo production, especially for embryos produced by superovulation or *in vitro* after harvesting oocytes via transvaginal ultrasound-guided follicular aspiration (*see* **Gamete and Embryo Technology**: *In Vitro* Fertilization; Multiple Ovulation and Embryo Transfer). The most inexpensive embryo is one produced by *in vitro* fertilization using oocytes recovered from abattoir material. The genetic merit of cows sent to slaughter is only slightly lower than average, and embryos can be produced inexpensively using semen from bulls of high genetic merit because one straw of semen can fertilize 100 or more oocytes.

Genetic Selection

As illustrated in **Table 2**, there are distinct breed differences in the ability to regulate body temperature during heat stress. In cattle, *Bos indicus* are better able to regulate body temperature during heat stress than *B. taurus*, which evolved in temperate climates. Under conditions where feed availability, housing, and veterinary care are low, *B. indicus* dairy breeds or their crosses with *B. taurus* may produce a greater economic return than the breeds of *B. taurus* selected for milk yield. In most cases, however, European dairy breeds outperform *B. indicus* or *B. indicus* cross-breeds, even under situations where heat stress is severe.

Even among European dairy breeds, however, there are genes that control the regulation of body temperature and selection for body temperature regulation is possible (*see* **Genetics**: Selection: Concepts). One gene that has been demonstrated to control body temperature is the ‘slick-hair’ gene. Introduced into Holsteins from Senepol cattle, cows with the slick-hair gene have short hair coats, increased capacity for sweating, and lower body temperatures during heat stress.

There is some evidence for genes controlling cellular resistance to elevated temperature. Thus, preimplantation embryos from two thermally adapted breeds, the Brahman and Romosinuano, are more resistant to disruption in development when exposed to elevated culture temperature than embryos from the Angus, Holstein, or Angus × Holstein. Identification of the genes responsible for these breed differences could lead to their introduction into thermosensitive breeds.

Conclusions

Lactating animals are among the mammals most sensitive to heat stress because of the metabolic demands of lactation. The result is reduced intensity of behavioral estrus, low fertility, and compromised fetal growth. In males, too, heat stress can affect reproduction with the most noticeable effects being compromised sperm output and increased sperm abnormalities. Prevention of heat stress effects on reproduction is difficult but the magnitude of effects can be reduced by provision of housing that contains shade, sprinklers or misters, and fans, and by using reproductive management tools such as timed AI and embryo transfer.

See also: **Dairy Farm Layout and Design**: Building and Yard Design, Warm Climates. **Gamete and Embryo Technology**: Artificial Insemination; *In Vitro* Fertilization; Multiple Ovulation and Embryo Transfer. **Genetics**: Selection: Concepts. **Reproduction, Events and Management**: Control of Estrous Cycles: Synchronization of Estrus; Control of Estrous Cycles: Synchronization of Ovulation and Insemination; Mating Management: Artificial Insemination, Utilization; Mating Management: Detection of Estrus; Mating Management: Fertility; Pregnancy: Characteristics; Pregnancy: Physiology.

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Management Induced Stress in Dairy Cattle: Effects on Reproduction

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Introduction

The correct definition of stress is important in any discussion of dairy reproduction. A *stress* or *stressor* is a force external to a system which acts to displace the system. A stress condition can be quantified and applied equally across animals. A *strain* is the animal's response to stress (the magnitude of the displacement). The strain often represents a cost to the individual animal and the level of strain can vary from animal to animal. Stressors assume a variety of forms including environmental, physical, physiological, and psychological stressors. Environmental stress caused by heat and humidity in the summer is common in dairy cattle and its effects are discussed elsewhere in this encyclopedia. Physical stress involves an acute injury that causes a dramatic physiological response, for example, when a cow sustains a life-threatening injury involving hemorrhage. Physical stress is uncommon in dairy cattle. Physiological stress is common in dairy cattle and includes high milk yield and disease. Psychological stress is also common in dairy cattle. The common forms of psychological stress include social interactions with other farm animals and people.

There are many examples of stress and the associated strain on dairy reproduction. For example, when dairy cows have high milk production (a stress), their reproductive efficiency will decrease (a strain that is caused by the high milk production). In this example, the stress is applied equally across animals but the strain will vary because some high-producing cows remain reproductively healthy whereas others become infertile.

Endocrine Pathways Associated with Stress and Reproduction

It is impossible to understand the mechanisms linking stress and reproduction without a cursory knowledge of the pituitary gland and the endocrine mechanisms controlling pituitary function. The pituitary is an endocrine gland that synthesizes and secretes hormones that control many aspects of animal physiology. The major hormones synthesized and secreted by the pituitary are growth hormone, prolactin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), adrenocorticotrophic hormone (ACTH), and thyroid-stimulating hormone (TSH). Hormones of the pituitary play major roles in coordinating growth, reproduction, lactation, and metabolism of animals. They are also involved in the stress response. The pituitary is positioned below a specialized region of the brain called the hypothalamus. Neurons within the hypothalamus synthesize releasing factors that control pituitary hormone synthesis and secretion. Releasing factors are secreted into the median eminence, a region of the hypothalamus that contains the terminals of hypothalamic neurons as well as a capillary bed. Releasing factors enter the capillaries and travel from the median eminence to the pituitary via portal vessels. The portal vessels lead to a second capillary bed within the pituitary. The system of two capillary beds and the connecting blood vessels is called the hypothalamic-pituitary portal system. Binding of releasing factors to endocrine cells within the pituitary causes the secretion of pituitary hormones. The hypothalamus also synthesizes and secretes hormones that inhibit the secretion of hormones by the pituitary. The balance between stimulatory and inhibitory factors determines the level of secretion for individual pituitary hormones.

The Hypothalamic-Pituitary-Gonadal (HPG) Axis

The hypothalamic releasing factor responsible for reproduction is gonadotropin-releasing hormone (GnRH). GnRH is released in pulses from the hypothalamus and the pulsatile release of GnRH causes the pulsatile release of LH from pituitary cells called gonadotrophs. The release of FSH from gonadotrophs is also under the control of GnRH but FSH release is less pulsatile because other hormones modify FSH release. Pulses of LH in blood stimulate ovarian follicular growth. A massive release of LH into blood (the LH surge) causes ovulation. FSH will also stimulate follicular growth but in most instances LH and not FSH is the limiting factor that controls reproductive processes in dairy cattle. Collectively, the endocrine system controlling reproduction is called the HPG axis (**Figure 1**).

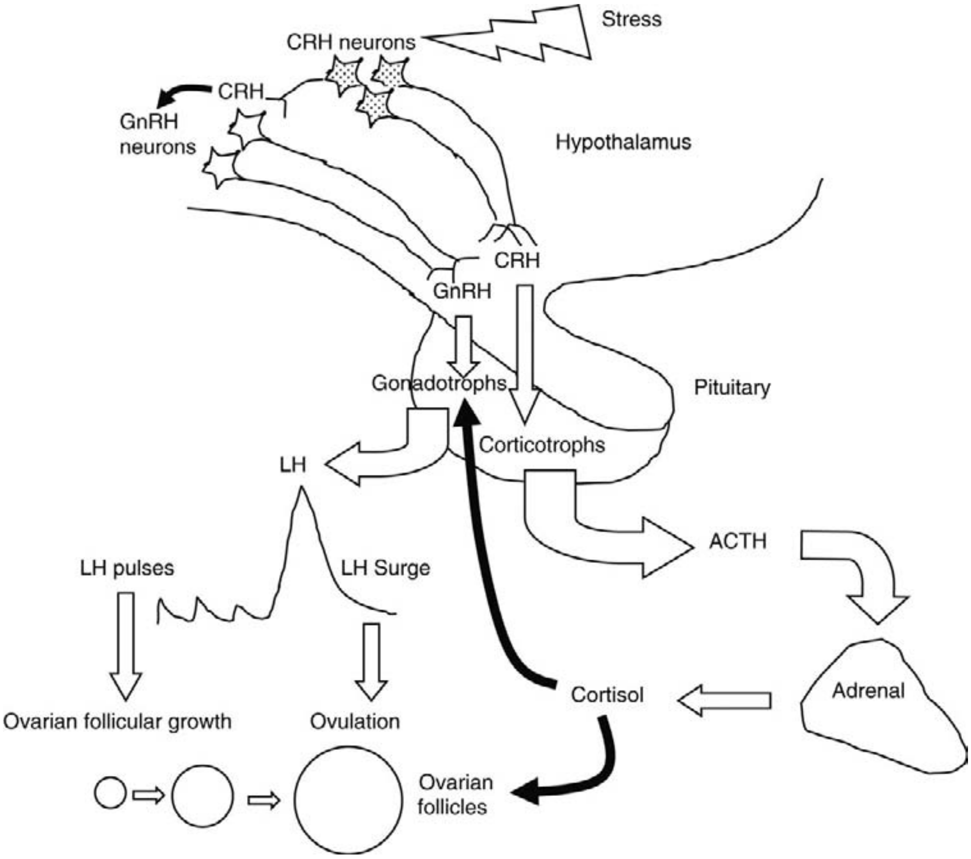

Figure 1 The interaction between the hypothalamic-pituitary-gonadal (HPG) axis and the hypothalamic-pituitary-adrenal (HPA) axis in animals. Neurons within the hypothalamus synthesize and secrete pulses of GnRH. The GnRH travels through the portal vessels into the pituitary where it causes the secretion of LH from pituitary gonadotrophs. Pulses of LH cause follicular growth. A high blood concentration of estradiol from the preovulatory follicle triggers an LH surge that causes ovulation. When the HPA axis is activated, CRH inhibits the activity of GnRH neurons and blocks GnRH release. Thus, follicular growth and ovulation are inhibited. Cortisol is also inhibitory to the reproduction because cortisol decreases the responsiveness of the gonadotrophs to GnRH and decreases the responsiveness of ovarian follicles to LH. Heavy black arrows show pathways for HPG inhibition by an activated HPA axis. The diagramed mechanisms are still under investigation and do not apply to all stresses that affect reproduction.

The Hypothalamic-Pituitary-Adrenal (HPA) Axis

A second endocrine axis called the HPA axis is activated in response to stress. The magnitude of HPA axis activation is not an accurate reflection of the degree of stress. Furthermore, not all stressors activate the HPA axis. Nevertheless, there is evidence that the reproductive system can be affected through the HPA axis. Therefore, the HPA axis will be presented here.

Neurons within the hypothalamus secrete corticotropin-releasing hormone (CRH) into the median eminence. The CRH travels through the hypothalamic-pituitary portal system and causes the release of ACTH from pituitary corticotroph cells. A second hypothalamic hormone, arginine vasopressin (AVP), can increase the activity of CRH on corticotrophs. ACTH travels through the blood and causes the adrenal gland to synthesize and secrete glucocorticoids. The primary glucocorticoid in dairy cattle is cortisol. Glucocorticoids are initially permissive to the stress response and improve an animal's ability to cope with stress. After their initial permissive effects, glucocorticoids are suppressive to the stress response and may play an important role in limiting the potentially damaging effects of stress-activated defense mechanisms.

Control of Reproductive Processes by the HPG axis

LH is a critical hormone for the attainment of puberty in heifers and the resumption of normal estrous cycles in postpartum cows. In heifers, ovarian follicles grow and develop throughout the prepubertal period. Follicles grow in cycles (known as follicular waves) and achieve progressively larger sizes until they reach physiological maturity and trigger estrus and ovulation (puberty). Following ovulation, cells within the follicle differentiate and form the corpus luteum (CL) that secretes the hormone progesterone that is required for pregnancy. The process is similar (but not identical) in postpartum cows. The maternal ovary is relatively inactive during pregnancy. Ovarian follicles grow and develop but follicles do not ovulate (i.e., release an oocyte for fertilization). This makes physiological sense because ovulation and the potential to establish pregnancy are futile in animals that are already pregnant. Shortly after calving, however, the ovary resumes the cyclical process of ovarian follicular growth. Ovarian follicular development within the first 2 weeks after calving can lead to ovulation (i.e., a follicle grows to ovulatory size and ovulates with subsequent CL formation). However, alternative ovarian follicular development may also occur. These alternative processes represent the strain caused by the stress of pregnancy, calving, disease, and lactation. The first possibility is anestrus. Anestrus occurs when ovarian follicles resume the cyclical process of development but fail to grow to an ovulatory size and fail to ovulate. A short period of postpartum anestrus (3–4 weeks) is normal for dairy cattle. Longer periods of postpartum anestrus lead to infertility because estrus is not expressed and cows do not ovulate. The second possibility is a follicular cyst. Follicles grow to an abnormally large size (a cyst) and fail to ovulate. Estrus may occur with cysts but cysts fail to ovulate resulting in infertility.

In heifers, the frequency of LH pulses increases before puberty and stimulates maturation of preovulatory follicles. The same is true for postpartum cows; greater frequency of LH pulses leads to maturation of preovulatory follicles. Preovulatory follicles secrete large quantities of estradiol that cause the hypothalamus to release a surge of GnRH. The estradiol-dependent release of GnRH causes the LH surge. The LH surge is necessary for ovulation and formation of the CL. Stress can cause a strain on reproduction by slowing the pulsatile release of LH or blocking the LH surge.

Mechanisms Linking the HPG and HPA Axes

Tentative links have been made between the HPG and the HPA axes. These links remain under scientific investigation and will require additional research. As mentioned above, not all stressors affect the HPA axis. Thus, stress-induced changes in the HPG axis that affect reproduction are not always caused by activation of the HPA axis.

The stressors that cause activation of the HPA axis may cause infertility by affecting LH (**Figure 1**). Some CRH neurons within the hypothalamus terminate on the cell bodies of GnRH neurons. When CRH neurons are stimulated and release CRH, GnRH release from GnRH neurons may be blocked. The inhibition of GnRH neurons decreases the pulsatile LH release and prevents the LH surge. Decreased LH pulsatility leads to a decrease in follicular growth that delays the onset of puberty in heifers and delays the onset of estrous cycles in postpartum cows. Blocking the LH surge prevents ovulation. The increase in cortisol that occurs in response to the increase in ACTH may also affect the endocrine physiology of GnRH and LH. Cortisol inhibits the pituitary release of LH in response to GnRH. Furthermore, cortisol may decrease responsiveness of ovarian follicles to LH. Therefore, GnRH release (through a CRH-mediated mechanism), the release of LH in response to GnRH (through a cortisol-mediated mechanism), and the response of ovarian cells to LH (through a cortisol-mediated mechanism) may all be inhibited when the HPA axis is activated by stress.

Stressors that affect ovarian function in dairy cattle commonly do so by interfering with the normal process of LH release. Not all stressors, however, affect reproduction through the HPA axis. For example, undernutrition in dairy cattle (a stress) will cause a decrease in the frequency of LH pulses (a strain). The decrease in frequency of LH pulses delays puberty in prepubertal animals because LH secretion is inadequate and follicles fail to mature to a preovulatory size. Likewise, in postpartum cows, undernutrition or severe weight loss shortly after calving will decrease the frequency of LH pulses and cause anestrus. The exact mechanisms through which undernutrition slows the frequency of LH pulses are poorly understood but a variety of mechanisms that are acting independently from the HPA axis are probably involved (see later).

Common Stressors and Their Effects on Reproduction in Dairy Cattle

Physiological Stressors

Nutrition

Underfeeding energy or protein or failing to supply adequate amounts of essential vitamins and minerals are stressors that affect reproduction in dairy cattle. Inadequate vitamin and mineral nutrition in dairy cattle is an uncommon stressor that will not be reviewed here. The stress of mineral and vitamin deficiency will have dramatic effects on health and reproductive function of the animal but effects will vary depending on the specific deficiency.

Underfeeding energy is an important nutritional stressor because the pulsatility of LH depends on caloric intake. Postpartum cows are fed *ad libitum* but nonetheless may be ‘undernourished’ because they are producing large quantities of milk and cannot consume enough feed to meet their energy requirements. They reside, therefore, in a state of negative energy balance for several weeks postpartum (see later). Cattle that are in negative energy balance fail to achieve normal LH pulsatility. In prepubertal heifers, the onset of puberty is caused by an increase in frequency of LH pulses. Undernutrition in heifers delays onset of puberty by delaying the age when the frequency of LH pulses increases. In postpartum cows, negative energy balance delays the onset of ovarian activity after calving through a similar mechanism. Cows that are in negative energy balance fail to resume normal LH secretory pulsatility and do not ovulate. These cows are termed 'anovulatory' (failing to ovulate) or 'anestrus' (failing to express estrus). Although the psychological stress of a short-term fast may activate the HPA axis, effects of chronic undernutrition on the HPG axis are probably independent of the HPA axis. Mechanisms linking undernutrition or negative energy balance to LH pulsatility are still obscure but may involve metabolic hormones like insulin, leptin, and insulin-like growth factors that are closely tied to nutrition and adipose tissue mass of the animal. Brain hormones like opiod peptides and neuropeptide Y also play a central role in regulating activity of GnRH neurons in response to changes in nutritional status.

Once heifers are pubertal or once dairy cows initiate regular postpartum estrous cycles, the effects of undernutrition or negative energy balance are slightly different. Several weeks of severe undernutrition are required to force cattle back into the prepubertal state or back into anestrus (cows). Therefore, even when they are undernourished, cattle will temporarily (several estrous cycles) continue to express estrus and ovulate. Conception rate, however, is decreased in undernourished cattle. The decrease in conception rate may be caused by a decrease in progesterone synthesis by the CL of undernourished animals.

Protein intake also affects reproductive efficiency. Underfeeding protein results in slow growth in heifers and low milk production in lactating cows. Underfeeding protein can therefore delay onset of puberty and may affect interval to estrous cyclicity in postpartum cows. Overfeeding protein is the more common stress in lactating dairy cows. Diets high in crude protein are fed during early lactation so that milk production is increased. Lush springtime grasses can also have high protein content. The stress of high protein feeding causes a strain on reproductive efficiency. Blood concentrations of ammonia and urea are increased. The increase in ammonia and urea does not delay the time of postpartum cyclicity. However, increased ammonia and urea cause a change in uterine pH that alters uterine secretions. These secretory and pH changes create a toxic uterine environment and cause early embryonic death and infertility.

High milk yield

Dairy cattle have been genetically selected for greater milk production. Currently, the best dairy cows are capable of producing over 30 000 kg of milk in a single lactation. The ability of dairy cattle to produce large quantities of milk depends on their ability to partition nutrients and body reserves toward milk production and away from other biological processes. In early lactation, the energy requirements for milk production increase rapidly and are greatest during peak lactation (4–6 weeks after calving). The ability of cows to consume feed, however, lags behind their ability to produce milk (**Figure 2**). Therefore, early postpartum cows must mobilize nutrients stored in the body (primarily adipose tissue) to support milk production during early lactation. Cows that cannot consume adequate feed are in 'negative energy balance' because their intake energy is less than the energy required for milk production and maintenance. By selecting for

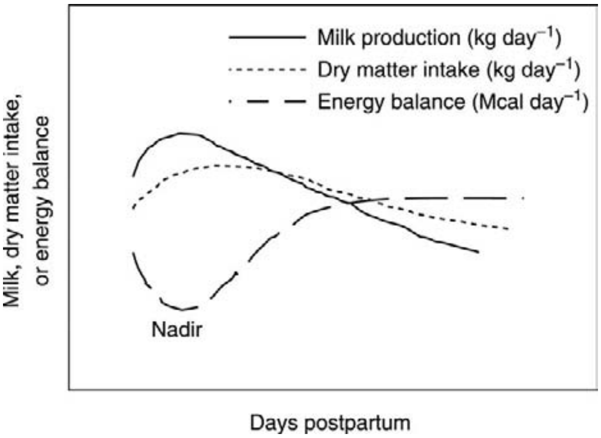

Figure 2 Conceptual diagram of changes in milk production, dry matter intake, and energy balance in postpartum dairy cattle. Energy balance is negative in early postpartum dairy cattle because milk production increases faster than dry matter intake. Cows reach their energy balance nadir within 1–2 weeks postpartum.

greater milk production we have created cows that will enter negative energy balance and mobilize adipose tissue to support high milk production during early lactation. We have also created cows that are larger and have the body capacity to consume large quantities of feed.

Negative energy balance is a stress that creates a strain on reproduction in dairy cows. Cows in more negative energy balance initiate estrous cycles later postpartum and are more likely to develop cystic ovarian follicles. Anestrus in negative energy balance cows is caused by reduced LH pulsatility. Cows in negative energy balance have fewer LH pulses than cows in positive energy balance. The mechanisms linking energy balance to LH pulsatility may be similar to those that link undernutrition to LH pulsatility.

Once the cow becomes cyclic, the strain that high milk production places on fertility is much less. Several large epidemiological studies suggest that high-producing dairy cows are only slightly less fertile than low-producing dairy cows. Postpartum cows with a low body condition score (indicative of inadequate fat mass), however, are susceptible to infertility. The low body condition score during lactation is usually caused by extreme negative energy balance shortly after calving. High-producing as well as low-producing cows are susceptible to infertility caused by low body condition.

Disease

The effects of increased milk production or rbST on reproductive performance are relatively minor compared to the effects of disease on reproduction (**Figure 3**). Diseases that place a strain on reproduction include metabolic diseases (ketosis and fatty liver), uterine and mammary infections (mastitis, metritis, and pyometra), and periparturient disorders (dystocia and retained placenta). The strain is manifested as either anestrus (secondary to a reduction in feed intake and excessive body condition loss), cystic ovarian follicles, or a decrease in conception rate following insemination.

The mechanisms through which diseases affect reproductive processes vary. Dystocia predisposes cattle to retained placenta and uterine infection (metritis and pyometra). Uterine infections lead to a uterine environment that is not conducive to early embryonic development. Some forms of mastitis generate endotoxins that can activate the HPA axis. Activation of the HPA axis inhibits the reproductive axis by inhibiting the activity of GnRH neurons (see earlier). It is also possible for cytokines (hormones released from activated immune cells) to block GnRH release. The decrease in activity of GnRH neurons reduces LH secretion and can block follicular growth and ovulation. Acute mammary infections can also increase release of prostaglandin $F_{2\alpha}$ from the uterus, which in pregnant cows is especially problematic because prostaglandin $F_{2\alpha}$ can regress the CL and cause abortion. This may explain why dairy cows that contract mastitis within the first 21 days after insemination have a decrease in conception rate.

Metabolic diseases (ketosis and fatty liver) are risk factors for infertility in dairy cattle but mechanisms through which their effects are exerted are less clear. Ketosis and fatty liver are sometimes secondary to a displaced abomasum, an inversion of the intestinal tract that blocks normal digestion and decreases feed intake. The drop in feed intake creates excessive mobilization of fatty acids, an excessive loss in body condition, accumulation of fatty acids in liver, and excess metabolism of fatty acids to ketones (ketosis). Displaced abomasum, fatty liver, and ketosis typically occur in early postpartum cows so that the strain on reproduction is initially manifested as anestrus. However, there can be carryover effects of these diseases on conception rates during the breeding period, especially in cows that lose considerable body condition.

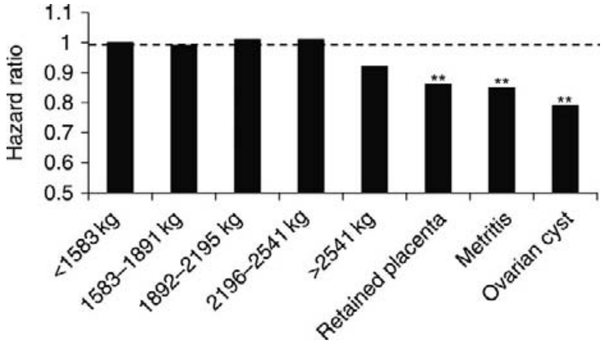

Figure 3 Effect of milk yield (first 60-day cumulative yield in kilogram) and disease on hazard ratio for conception in 13 307 New York State Holstein cows. The hazard ratio is the relative risk of conception. A hazard ratio of 1.0 equates to a neutral effect (dashed line). Hazard ratios less than 1 indicate reduced likelihood of conception (i.e., cows experiencing a disease with a hazard ratio of 0.86 are 14% less likely to conceive compared to a healthy cow). $^{**}p < 0.01$. From Gröhn YT and Rajala-Schultz PJ (2000) Epidemiology of reproductive performance in dairy cows. *Animal Reproduction Science* 60–61: 605–614.

Housing and facilities

The consolidation of the dairy industry into larger farms with more cows per farm has necessitated movement of dairy cattle into confinement housing. Confinement housing is different from traditional grass pastures because cows are kept in large barns with concrete floors and open stalls (free stall barns) or in large corrals with concrete or dirt floors. Convenience and productivity of cows in confinement housing has forced the move from grass pastures. Moving cows into confinement housing and managing cows in larger herds are stressors that increase risk of mammary and uterine infections. Increases in mammary and uterine infections create a strain on reproductive efficiency (described earlier). Managing cows in large groups can also cause psychological stress (see later).

In addition to disease stress, the stress of concrete floors also creates a strain on reproduction by decreasing estrous activity. Dairymen use mounting behavior to determine when a cow is in estrus and when cows should be inseminated. Duration of estrus, mounting activity, and standing activity are greater on dirt than on concrete. Therefore it is difficult to appropriately time insemination in cows that are housed on concrete floors because it is more difficult to determine when cows are in estrus. Concrete floors also predispose cows to lameness. Thus, poor expression of estrus may be secondary to lameness (a strain) caused by the stress of concrete floors on the feet and legs of cattle.

Psychological Stressors

Transportation

Transportation of cattle in a vehicle (trucking) causes immediate activation of the HPA axis. Activation of the HPA axis can potentially inhibit the HPG axis through mechanisms described earlier but the effect is only temporary. Transportation stress is uncommon in lactating dairy cows because dairy cows are generally not trucked during lactation. Heifers may be purchased and trucked from other locations prior to breeding but there is no evidence that trucking has any long-term effect on reproduction in cattle.

Social interactions with cattle

Cattle are social animals that live in groups with a dominance hierarchy. Mixing groups of cattle inevitability leads to decreased productivity because cows must spend time and energy to reestablish the dominance structure within the group. A recent study of dairy cows showed that dairy cows losing social status during the breeding period had a longer interval from calving to conception and required more inseminations per conception (**Figure 4**). The mechanisms linking changes in social status to reproductive efficiency are not clear but may involve activation of the HPA axis and subsequent inhibition of the HPG axis in animals that are subjected to aggression from other cows.

Establishment of a dominance hierarchy depends on the ability of cattle to recognize one another. Cows may have difficulty recognizing one another when they are penned in large groups. The optimum group size for maintaining a stable social order may be as small as 50–60 cows. Therefore, cows in large dairy herds may be especially susceptible to psychological stress caused by an unstable social structure.

Isolation

Social interactions create stress in cattle (see earlier) but isolation from the herd also creates a stress. Isolation is a stress that activates the HPA axis. Although the effects of isolation on reproduction are unknown, activation of the HPA axis during isolation can potentially affect the HPG axis. Therefore, isolation could potentially create a strain on reproduction in dairy cows.

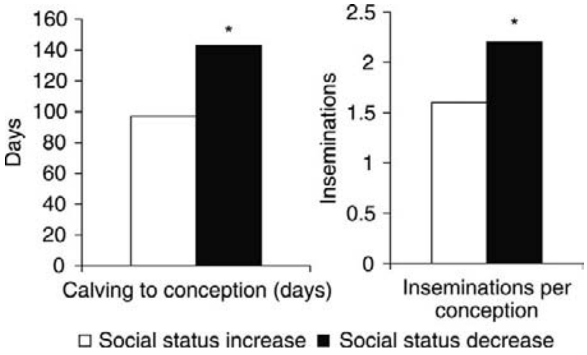

Figure 4 Calving-to-conception interval and inseminations per conception in dairy cows with increasing or decreasing social status. *$p < 0.05$. From Dobson H and Smith RF (2000) What is stress, and how does it affect reproduction. *Animal Reproduction Science* 60–61: 742–752.

Human–cow interaction

Dairy cattle can recognize individual people and have better performance when handled by gentle people compared with aggressive people. For example, cows milked in the presence of an aggressive handler had 70% more residual milk left in the udder following milking. The effects on milk yield may be directly related to a stress response. Cows have an increase in heart rate during milking when an aggressive handler is present. The increase in heart rate is suggestive of epinephrine release and psychological stress caused by the aggressive person. To our knowledge, no studies have evaluated the effects of aggressive versus gentle handling on conception rate. However, there are clearly differences in conception rates across individuals that perform artificial insemination. Part or all of the conception rate differences could be related to handling of the animals before and during insemination.

Conclusions

Stress is an everyday part of the lives of dairy cattle. Physiological and psychological stressors generally inhibit the HPG axis and cause a decrease in LH release. The decrease in LH release slows follicular growth and prevents ovulation. Some stressors activate the HPA axis and affect LH through a HPA-dependent mechanism. Other stressors (i.e., negative energy balance or undernutrition) have similar effects on LH but act through pathways that do not involve the HPA axis. A certain amount of management-induced stress is unavoidable in dairy farms. The best reproductive performance, however, will be achieved when management-induced stress is minimized so that the HPG axis can function at its maximal level.

See also: **Body Condition**: Effects on Health, Milk Production, and Reproduction. **Reproduction, Events and Management**: Mating Management: Fertility; Estrous Cycles: Characteristics; Estrous Cycles: Postpartum Cyclicity. **Stress in Dairy Animals**: Heat Stress: Effects on Reproduction. **Welfare of Animals, Political and Management Issues**.

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UTILITIES AND EFFLUENT TREATMENT

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Water Supply
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Design and Operation of Dairy Effluent Treatment Plants
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Water Supply

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Introduction

Water is an important utility in the dairy Industry; its uses are numerous and diverse, ranging from product ingredient to cleaning agent.

The utilization of water in the dairy industry can be divided into processes in which water has direct contact with the product, and those in which water has indirect contact with the product.

To comply with EU legislation, water that comes into direct contact with the product (e.g., steam injection of milk in the production of UHT milk, or when used as a cleaning or rinsing agent) must be of 'drinking water' quality as defined by EU legislation (Council Directive 98/83/EC on the quality of water intended for human consumption).

For water that has only indirect contact with the product, for example, when used as a coolant, the EU legislation has not stipulated any water quality requirements. However, in many dairy plants drinking water is used as the coolant to ensure that accidental leaks of cooling water into the product will not cause contamination.

Some special applications such as readymade infant formula require water of higher specification than that of 'drinking water'. Such applications are beyond the scope of this article.

This article examines drinking water generation and distribution in the dairy industry, and also outlines treatment processes required for boiler and cooling waters.

Generating Drinking Water for the Dairy Industry

Water of EU-defined 'drinking water' quality required in the dairy industry may be delivered to the facility from a municipal water distribution system, or it may be generated onsite using natural water sources such as groundwater or river water.

Drinking Water Sourced from Municipal Water Systems

Should the water be supplied to the site via a municipal drinking water system, filters are typically installed at the factory boundary to ensure that the intake of water is free from particulate contamination, which might have been picked up en route. Finer-rated filters may be installed closer to delivery points of supplies that feed particularly sensitive processes. EN13445 or similar standards may provide guidance on the selection of filters for drinking water systems (DWSs).

Water from Natural Sources

Untreated water from natural sources used for DW generation onsite may contain

- dissolved natural minerals, gases, and possibly some other chemicals
- insoluble debris – vegetable and mineral
- microorganisms – living or dead.

Some of these constituents may make the water unsuitable for use in specific applications in the dairy industry (see **Table 1**). The aim of water treatment is simply to modify these constituents to render the water suitable for a desired application. The following are some water treatment processes routinely employed in the production of drinking water:

Table 1 Specific problems caused by common constituents of water

Suspended solids	Settlement to form deposits on equipment and piping Facilitation of the growth of microorganisms
Iron and manganese	Fouling and staining of equipment and piping
Microorganisms	Microbiological contamination of equipment surfaces Secondary contamination of products or potable supply
Organic compounds	Promotion of growth of microorganisms Imparting of taste or odor to product Staining of equipment and piping
Calcium and magnesium	Scale formation when water is heated or evaporated Increase in detergency requirement (and reduced performance)
Dissolved gases (O_2 and CO_2)	Increased corrosion of pipework, etc.
Other dissolved materials	Contamination of product
Total dissolved solids (TDS)	Increased blowdown requirement in boiler and cooling systems

Removal of suspended solids and turbidity

Suspended solids and turbidity are typically removed by coagulation, flocculation, and settlement, followed by filtration. Chemical coagulants exploit the electrostatic charges on suspended particles, encouraging coalescence to form larger particles. Flocculants provide a chemical bridging between these particles to form yet larger and heavier flocs that may be removed by sedimentation. After flocculation the suspended solids are allowed to settle out. The clear supernatant liquid is removed and filtered.

The process of filtration can be achieved by a number of methods, although the traditional method of sand filtration is still the most common. The water is allowed to percolate through appropriately graded sand and suspended materials become trapped within the bed. A backwash process is used to clean and regenerate the sand filters periodically.

For filtering smaller amounts of water, disposable or washable cartridge-type filters may be suitable. There are two standard types of cartridge: the depth type (where the solids are trapped within the cartridge itself) and the pleated filter type (which depends on the surface layer to perform the filter duty, similar to filter papers used in the laboratory). Most filter elements are disposable, though washable types are available. Cartridge filters are rated either absolute or nominal, depending on the percentage of suspended solids of a particular size held back by the filter. Water can be filtered up to 5 nm using present technology but, in general, the finer the filtered particle specification, the more expensive the element.

Bag filters offer a variation on the cartridge filter and can normally hold a larger quantity of filtered materials between filter changes. The water is fed into the centre of the bag and forced to flow outward through the bag to service. Filtered material trapped within the bag is removed periodically by manual or automatic procedures. Many systems depend on periodic disposal of the bag.

The limiting factors for the sizing of filters are the maximum flow rate required, the concentration of suspended solids present, and the desired treated water quality.

Removal of Organic Compounds

Organic materials appear occasionally in groundwaters and commonly in surface- and postprocessing waters. The high costs of both water extraction and effluent treatment have led to a renewed interest in techniques for the recovery and reuse of postprocessing waters, for example, the second condensate from milk evaporators, for reuse as process water.

One method for removing organics in this scenario is dosage of the condensate to a high level with an oxidizing agent. Chlorination at 6–10 mg l^{-1} or ozonation at 0.1–1.0 mg l^{-1} per 1 mg l^{-1} of organic material present would suffice. The contact time required depends on the oxidant used. The treated water is then passed through a carbon filter that destroys residual oxidizing agent and removes excess and oxidized organics by absorption into the carbon matrix. Carbon filters require periodic backwashing and the activated medium requires replacement from time to time. This process combines microbiological disinfection with organic removal.

Ultrafiltration is also used to remove organic contaminants from water. These filters comprise molecular pore sizes of 5–10 nm and are used in a cross-flow configuration. Water is split into two streams by the ultrafiltration element. The reject stream, containing the bulk of the solid materials, is diverted to the drain while the product water or permeate becomes the treated water stream. The cross-flow configuration allows the process stream waste constantly and tangentially to wash the upstream surface of the membranes, thereby reducing the risk of fouling.

Removal of hardness

Scale formation and degradation of detergents due to the presence of calcium and magnesium salts are widely experienced problems in the dairy industry. The most common method of removing hardness salts is by base-exchange softening. Typically, the water is passed through an ion-exchange resin, held in a pressure vessel fitted with some type of control valve system, usually automatic. Base exchange removes the hardness salts by adsorbing calcium and magnesium ions on to the resin in exchange for the ionic equivalent in sodium ions. Sodium salts rarely form scale. In time the resin will become saturated with the adsorbed hardness metal ions, and regeneration is effected by passing a concentrated sodium solution (NaCl brine) through the resin bed. This solution displaces calcium and magnesium ions and leaves a sodium-rich resin recharged for the next service period. Concentrated hardness salts are washed to the drain during regeneration. The process involves exchange of metals and does not lead to a reduction in the overall dissolved solids.

Water softeners should be sized to maximum flow rate, total hardness of the influent, and the required hardness of the effluent, thus determining the quantity of resin and the control-valve design.

Reduction in dissolved solids concentration

It is sometimes desirable to reduce the total dissolved solids (TDS) in the water supply. Two processes in the dairy industry requiring low TDS are waters used for boiler makeup or in the manufacture of cream liqueurs. Two systems are commonly in use.

Ion exchange

Ion-exchange systems typically use two resin beds, housed in separate pressure vessels in series. The first stage contains a cation exchange resin that will exchange positively charged metal ions for hydrogen ions. The cation exchanger is normally regenerated (while offline) using hydrochloric acid. The water from the cation exchanger is fed to the anion exchanger unit, which exchanges the negatively charged anionic salts (e.g., chloride, bicarbonate) for hydroxide ions. The anion exchanger is regenerated using a caustic (sodium hydroxide) solution. The resultant hydrogen and hydroxyl ions react to form water. The major factors affecting the size of deionization equipment are flow rate and the quantity of dissolved solids in the raw water.

Reverse osmosis

The second method of TDS reduction in common use is reverse osmosis, which uses a membrane system similar to that described for ultrafiltration. However, this membrane has a pore size of 0.1–1.0 nm. By applying upstream pressure higher than the natural osmotic pressure of the feed water, water can be forced to flow through the membrane. This effectively allows the dissolved materials to be concentrated in the reject stream and produces a purer permeate stream with a low dissolved-solid content. Reverse osmosis is particularly cost-effective for waters with very high TDS. Typically, chemical pretreatment is required.

Removal of microbial contamination

Disinfection of water is the process of destroying or inactivating the microorganisms present in the water and thereby providing drinking water fit for human consumption.

Disinfection of water entering a water distribution system can be relatively easily achieved; however, the subsequent maintenance of the water distribution system itself to maintain water quality within the distribution system offers more challenges. Both will be considered together in the following section.

Disinfection and Sanitization of DWSs

Depending on the source of the water, conditions of use, and magnitude and extent of microbiological contamination of the water, in addition to microbiological contamination of the distribution surfaces in the form of a biofilm, disinfection may be needed on a continuous basis or occasionally as shock short-term sanitization (see **Table 2**) should the microbial contamination in the DWSs exceed some threshold.

Table 2 Typical concentrations and application times for shock sanitization of some chemicals

Chemical	*Normal conc. (mg l^{-1})*	*Min. conc. (mg l^{-1})*	*Application time (hours)*
Hypochlorite	50 as Chlorine	10 as Chlorine	8–12
Chlordioxide	5–10 as Chlorine	50 as Chlorine	8–12
Hydrogen peroxide	150	150	24

If the presence of a potentially contaminating biofilm is factored in and understood, then the management of the quality of the DWS becomes easier to control (*see* **Biofilm Formation**).

In low-nutrient DWSs, bacteria can only grow and multiply in the so-called biofilms attached to surfaces; planktonic, or free-floating, bacterial cells released from the biofilm are important for dissemination only.

These planktonic bacterial cells are easier to enumerate and destroy than the bacterial cells attached to a surface existing in a community as a biofilm. Because of the structure of the biofilm, with its protective polysaccharide coating and altered phenotype cells, bacteria in biofilms are greatly protected from chemical attack from biocides, drying, and mechanical removal. Bacteria in biofilms may be up to 500 times more resistant to chlorine than in their planktonic state, well exceeding shock concentration limits.

In practice biofilm activity can only be suppressed below a certain level, but not completely reduced. Therefore, only disinfection, not sterilization, of DWS can be achieved in practice.

There are three commonly used techniques for the destruction of microorganisms in water. The advantages and disadvantages of these are summarized in **Table 3**

Oxidizing Agents

Oxidizing agents in common use are chlorine and its compounds (e.g., calcium hypochloride, sodium hypochloride), ozone, and chlorine dioxide (bromine normally in cooling systems). All oxidizing agents require an adequate contact time to disinfect properly. Stronger oxidizing agents require lower concentrations and shorter contact times. The water may be treated before the

Table 3 Advantages and disadvantages of most commonly used disinfection methods for water supply

Chlorination	
Advantages	*Disadvantages*
Very effective; proven in practice	May give the water a chlorine taste
Provides residual disinfectant	Turbidity may reduce the effectiveness of chlorine
Residual easy to measure	May require high concentrations of chlorine to kill certain bacteria and viruses
Chlorine readily available at reasonable cost	Special storage and handling requirements for gaseous chlorine are required
Can be used for multiple water problems (bacteria, iron, etc.)	May cause corrosion of system components
Appropriate as both primary and secondary disinfectant	
Ozone	
Advantages	*Disadvantages*
Very effective being a strong oxidant, proven in practice	Must be generated onsite, as ozone is unstable. Ozone generator is more complex than chlorination generator
Effective against virtually all bacteria and viruses	Relatively high cost
	May cause corrosion of system components
	May require a secondary disinfectant (chlorine), as ozone does not maintain adequate disinfection residual
Ultraviolet light	
Advantages	*Disadvantages*
No chemical added to the water. Does not change taste or odor of water nor causes corrosion of system components	Relatively high capital cost
Kills bacteria and viruses almost immediately	High electrical demand
Simple operation and maintenance for high-quality water	Local operation only, may require a secondary disinfectant, as there is no disinfection residual
Handling and storage of chemicals is not required	Requires pretreatment of surface water as water must be clear
	Requires frequent cleaning and installation of new UV lamps

storage tanks or by installing an inline contact pressure vessel after the dosing point.

Ozone is a replacement for chlorine in some applications. As a strong oxidizing agent that does not form trihalogenated methanes (THMs), it is particularly suited for disinfecting waters with a high content of organics or colorants. Ozone naturally decomposes rapidly to oxygen, and for this reason does not provide the extended postprotection offered by other oxidizing compounds. Water treated with ozone may be chlorinated to a low concentration to provide postprotection.

All of the above chemicals should be dosed according to the volume of water to be treated, and in continuous processes the chemical flow rate needs to be set in proportion to the water flow rate so that the required concentration is reached. Chemical concentration is often monitored by an online analyzer to allow for automatic control of the chemical flow rate.

Ultraviolet Irradiation

Ultraviolet (UV) radiation used to control microorganisms has some advantages over chemical methods, which entail inconvenient and potentially dangerous handling. Chemicals may also leave undesirable residual products in the water. The major disadvantage of UV systems lies in the failure to provide postprotection. There is, then, the possibility of water becoming re-infected after treatment. Ideally, UV systems should be used as close to the point of use as possible. Sizing to the particular flow rate is critically important, as is matching to the UV transmittance of the pretreated water.

Nonoxidizing Biocides

Nonoxidizing biocides are usually microbiologically toxic organics and find maximum application in the treatment of cooling- and chilled-water systems. Typically, two chemicals are used alternatively to prevent the development of resistant strains.

Typically, DWS designed to the latest design standards, for example, EN 806, should not require disinfection. However, should it become necessary, the system should first be surveyed for problem areas, which should be rectified before disinfection to avoid rapid reoccurrence of microbial contamination. Typically, chemical disinfection is used. Disinfection of DWS itself is best applied by specialist companies.

As a guideline as to when it may be necessary to sanitize a DWS, the Austrian standard ÖN B5019 may be helpful. **Table 4** gives some details.

Guidelines for DWS Design and Operation

Drinking water is a valuable resource needing protection from contamination during its distribution from the source to the tap. Therefore, the water must be distributed in a suitable manner preventing contamination. The DWSs designed to the latest standards, for example, EN806, for distributions systems within facilities are not likely to exhibit premature aging (corrosion) or excessive microbial growth.

The design and maintenance of drinking water generation and distribution systems requires specialized knowledge. However, some general guidelines can be given as follows:

1. *Do not Oversize* To avoid the formation of local corrosion nests and excessive microbial contamination in stagnation and areas of low flow, drinking water distribution systems should not be oversized. Correct sizing of hot-water storage is essential to avoid temperature gradients in storage tanks, which may encourage bacteria to grow in some areas of the tank.
2. *Avoid Dead Legs* Dead legs or rarely used pipe sections where water may remain stagnant for long periods should be avoided. This is particularly important for hot-water systems, as in the stagnant sections the normal hot-water temperature may not be able to be maintained, which in turn may lead to excessive microbial proliferation. In addition, dead legs may reduce the service life of the pipes due to corrosion.
3. *Positive Pressure* Maintain a positive pressure in the DWS to prevent inward entry of contamination.
4. *Insulate Pipes* To impede microbial growth the temperature of the water in the cold-water pipes should not exceed 20 °C. Insulation of the cold water pipes which

Table 4 When is sanitization necessary according to Austrian standard ÖN B5019

Legionella cbu/100 ml	*Normal conc. (mg l^{-1})cfu/l*	*Assessment*	*Action*
>10 000	>100 000	Very high concentration	Stop using system for certain applications, i.e. showers
1001–10 000	10 001–100 000	High concentration	Sanitization is required
101–1000	1001–10 000	Medium concentration	Sanitization may be required
10–100	100–1000	Low concentration	Sanitization may be required
<10	<100	Low concentration	No sanitization necessary
Nondetectable	Nondetectable	Legionella not detectable	None

run close to hot services (steam, condensate, hot water, etc.) may be necessary.

5. *Material of Construction* Many different materials from plastics (PVC, PP, PE, etc.) to steels (ductile iron, galvanized steel, stainless steel) are used to convey drinking water. While no material of construction can be considered ideal, all materials used for a DWS should be approved for use by an authorized body.

A major problem with construction materials is corrosion. Corrosion is a term used to describe deterioration of materials over time. Corrosion may contaminate the DWS with corrosion products, reduce the throughput of the pipes, and lead to a foul taste and to other problems (see **Plant and Equipment**: Corrosion). Before these symptoms are detected, corrosion may have remained undetected for a long time. The root cause of corrosion in DWS may have many sources: galvanic, microbial influenced, pitting, and crevicing, to name but a few.

6. *Backflow Prevention* Backflow from non-DWS into the drinking water distribution system may contaminate the drinking water. Therefore all drinking water standards stipulate backflow preventers.

The incoming water from the municipal water distribution system must be protected from contamination by a suitable backflow preventer (see EN 1717). Water taps are generally protected from backflow by an air gap of a suitable length (>50 mm). Firewater, boiler water, wastewater, and other services not directly related to a DWS must also be segregated by backflow preventers from the DWS (see EN 1717).

7. *Construction, Pressure Testing, and Documentation* Install pipes in a hygienic manner to avoid contamination of the pipes, valves, and other DWS components during the construction.

Pressure tests should be carried out using either drinking water or oil-free air.

Construction debris should be removed using a drinking water rinse.

The documentation should as a minimum include all relevant P&IDs, layouts, maintenance records, and certificates on the materials of construction.

8. *Regular Inspection and Maintenance* Regular inspection and maintenance will help to maintain the availability of the DWS for the lifetime of the plant.

Boiler Water and Cooling Water Treatment

Treatment of makeup water for boilers frequently requires special consideration and is therefore often separated from that of the main water supply.

Boiler water influent is known as feed water and needs to have minimal dissolved solids and hardness, and low corrosive qualities. As a general rule the higher the operating pressure of the boiler, the purer the boiler feed water must be; for details, see standards such as EN 12953.

Evaporative cooling systems should also be fitted with automatic blowdown systems for TDS control. Cooling towers are normally dosed with biocides and either a scale or a corrosion inhibitor.

Chilled-water systems are typically treated with a corrosion inhibitor only, though microbiological protection may be required in some instances.

Appendix

A List of Some European Standards

EN 806 Parts 1–5: On the conveying of water for human consumption.

EN 1717: Protection against pollution of potable water in water installations and general requirements of devices to prevent pollution by backflow.

EN 12953-10 Shell boilers – Part 10: Requirements for feedwater and boiler water quality.

EN ISO 19458: Water quality – Sampling for microbiological analysis.

prEN 14897: Water conditioning equipment inside buildings – Disinfection devices using ultraviolet radiation – Requirements for performances, safety, and testing.

EN 13445 Part 1–3: Water conditioning equipment inside buildings – Mechanical filters.

EN 14812 Water conditioning equipment inside buildings. Chemical dosing systems. Preset dosing systems. Requirements for performance, safety, and testing.

ÖNORM B5019 (Austrian standard) Hygienic aspects of planning, construction, operation, maintenance, surveillance, and rehabilitation of central heating installations for drinking water (in German).

See also: **Plant and Equipment**: Corrosion; Instrumentation and Process Control: Process Control; Process and Plant Design; **Utilities and Effluent Treatment**: Dairy Plant Effluents; Design and Operation of Dairy Effluent Treatment Plants.

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Heat Generation

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Introduction

Heat generation is a very broad subject and has many applications. These may be classified according to the amount of power involved, e.g. high power values are transferred in power plants where the expansion of water vapor in turbines generates electricity. Low powers are frequent in industrial applications.

In the dairy industry, heat generation is a vital service, because many of the processes require heat and vapor. Some examples include pasteurizing milk by heating it to a temperature of about 72 °C or above, heating milk with hot water in a heat exchanger, cooking coagulated milk and producing hot water for washing, all of which are processes in the production of cheese and in the manufacture of concentrated, dried or sterilized milk products.

Water is abundant in nature and is the preferred working fluid for heat transfer because of its thermodynamic properties.

Properties of Water and Steam

The thermodynamic properties of water have been determined experimentally for equilibrium states. A thermodynamic equilibrium state is reached when mechanical, thermal, chemical and phase equilibrium exists. It is characterized by two independent properties.

Consider a constant mass of liquid water at pressure $P_1 = 1\,\text{kPa}$ and temperature $T_1 = 20\,°\text{C}$ (state 1) contained in a piston-cylinder arrangement (**Figure 1**).

Heating the system with an external heat source will maintain a constant pressure and cause temperature and volume to increase. As can be seen in **Figure 2**, the system undergoes a change of state, from state 1 to state 2. As long as the temperature (T) is below the saturation temperature ($T_{\text{sat}} \approx 100\,°\text{C}$), vaporization will not occur. This region is the liquid water region ($T < T_{\text{sat}}$).

Additional heating will take the system to T_{sat}, bringing the liquid to the saturated state, state 3. Any more heating causes part of the liquid to vaporize. It is observed that, during this process, the temperature and pressure are constant and dependent variables, and the volume increases.

The heat transferred during the liquid–vapor change is known as the latent heat of vaporization, and the mixture of saturated liquid in equilibrium with saturated vapor is called humid vapor. In this region, state 4 for example, the state of the system may be defined by using the fraction relating the saturated vapor mass, m_{v}, to the total mass of the mixture, $m_{\text{v}} + m_{\text{l}}$:

$$x = \frac{m_v}{m_v + m_1} \tag{1}$$

where m_{l} is the saturated liquid mass.

This parameter changes from 0% (saturated liquid) to 100% (saturated vapor). It is used to determine the specific volume of humid vapor, $v = V/m$ (where V/m is the volume of the system divided by its mass):

$$v = v_1 + x(v_v - v_1) \tag{2}$$

where v_{v} is the specific volume of saturated vapor (some authors prefer v_{g}) and v_{l} is the specific volume of saturated liquid (some authors use v_{f}) (see **Figure 2**). This relationship is valid for other specific properties, such as internal energy, u, enthalpy, $H = u + Pv$, and entropy, S.

The vaporization process ends at state 5, at the instant when all the liquid is completely vaporized and only saturated vapor is present.

Further heating will cause increases in temperature and volume and take the system to a state called the overheated vapor state ($T > T_{\text{sat}}$), state 6 in **Figure 2**.

If the entire heating process described above is repeated for another pressure, achieved by putting weights on the piston, the lines obtained correspond to P_2, P_3 etc. in **Figure 3**.

It can be observed from **Figure 3** that the horizontal part of the isobar (phase change) tends to be smaller as pressure increases, until it disappears at pressure $P_{\text{c}} = 22.1\,\text{MPa}$, the critical point pressure corresponding to $T_{\text{c}} = 374.2\,°\text{C}$, $v_{\text{c}} = 0.00318\,\text{m}^3\,\text{kg}^{-1}$, where instantaneous vaporization of liquid occurs.

Joining the points corresponding to the saturated liquid states in **Figure 3** (i.e. a, b and c) results in the saturated liquid line. Linking the saturated vapor points (d, e and f) gives rise to the saturated vapor line. These two lines meet at the critical point (see **Figure 4**).

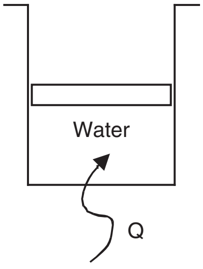

Figure 1 Piston–cylinder arrangement containing water.

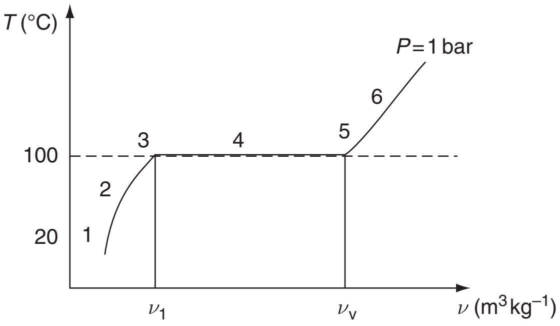

Figure 2 Temperature (*T*) changes for a specific volume of water during isobaric heating.

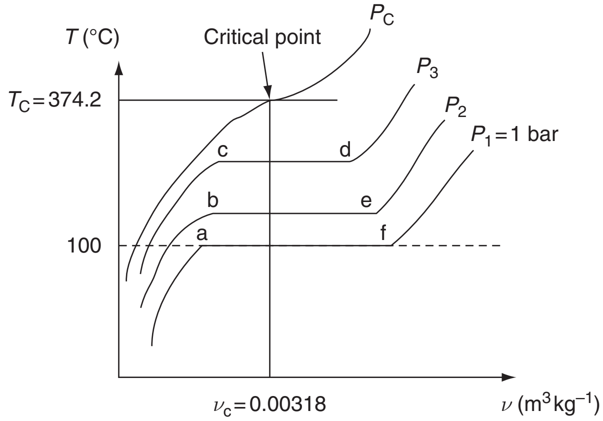

Figure 3 Temperature versus specific volume of water for various pressures.

In problem-solving, the thermodynamic properties of water are frequently obtained from tables: saturated vapor tables represent the saturated line, and overheated vapor tables represent both the overheated vapor and the liquid regions. There are also expressions that establish property relations but these are very awkward to use. In more advanced cases, computer programs generate the property values.

In most of the applications, the water temperature lies between freezing point and the critical point. Using water in this range of temperatures takes advantage of the latent heat. Boilers are the equipment used to convert cold water into hot water, or into steam, by burning fuel that releases heat to the working fluid.

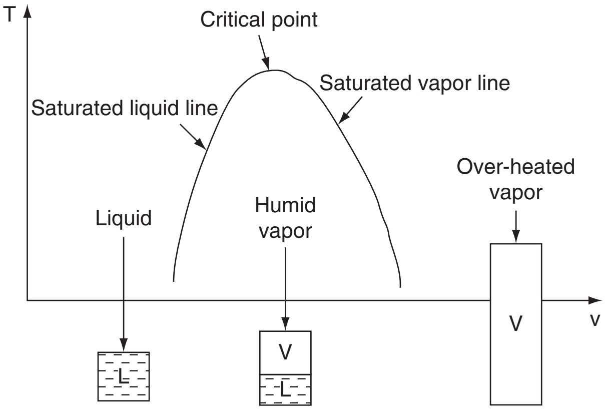

Figure 4 Representation of the saturated line of water in a diagram of temperature versus specific volume.

General Classification of Steam Boilers

Boilers are classified according to different characteristics as follows:

1. Location. The boiler may be moveable or installed in a fixed place.

2. Fuel. The fuel may be fossil, such as coal, fuel oil and natural gas, residual, or there may be none at all. In the latter case, flue gases from industrial processes or internal combustion engines heat the working fluid.

3. Working fluid. Water is the usual working fluid but other fluids may be used, such as thermal fluids. Water is treated to remove impurities. The pH must be controlled to prevent corrosion and should be maintained between 9.5 and 10.5, depending on boiler pressure.

4. Pressure level of the working fluid. The fluid undergoes a phase change and the energy required to vaporize the liquid varies with pressure. Therefore, boilers may be classified according to the working fluid pressure as: low-pressure boilers ($P < 0.15$ MPa), medium-pressure boilers ($0.15\ \text{MPa} < P < 9.0\ \text{MPa}$), high-pressure boilers ($9.0\ \text{MPa} < P < P_{\text{crit}}$ (critical point pressure)) and very high-pressure boilers ($P > P_{\text{crit}}$ – in this case, vaporization is instantaneous).

5. Form of flow promotion. The working fluid may travel through the boiler by natural convection or by forced convection.

6. Flow location. In water-tube boilers, the water flows inside the tubes while the hot gas products of combustion heat the tubes from the exterior. If, instead, the combustion gases flow inside the tubes, heating them internally, and the water surrounds the tubes exteriorly, then the boiler is of the fire tube type.

7. Tubular bank configuration. Tubes may be positioned horizontally, vertically or inclined. The tubes connect to boiler headers that are used to collect steam and water for distribution to other parts of the boiler or users.
8. Furnace position. The boiler is internally fired if the boiler shell contains an internal furnace, or externally fired if the combustion takes place outside the boiler shell and the products of combustion are directed to flow within the tubes inside the shell.
9. Firing arrangements. The firing arrangements may be horizontal (flame travels horizontally into the furnace; used in small- to medium-capacity boilers), vertical (the burner is located at the top of the furnace and the flame travels downward to the bottom of the furnace; used in small-capacity, fire-type tube boilers and also in large-capacity water-tube boilers that burn pulverized coal) or tangential (the furnace has a square or rectangular geometry and, at each of the four corners, the flame travels tangentially to a 'fire ball' where all the flames meet, located at the center of the furnace). The great turbulence favors the mixing of the fuel and air.
10. Number of combustion gas passes. The design may include one, two, three or four passes through the boiler. The latter configuration is the most efficient; however, the greater the number of gas passes, the more fan power is required.

In the dairy industry, small- to medium-capacity unit boilers are used. They may be water-tube or fire-tube boilers, and are equipped with all the boiler auxiliaries, such as water pumps, fans, burners, fittings, controls, etc. The only exterior connections that need to be made are electrical, water, fuel and stack. Stacks, made of steel or concrete, are used to deliver the flue gases to the atmosphere. In this way, dispersion of particles is simplified and has a low impact on the environment.

Prefabricated unit boilers are more advantageous than boilers constructed on site due to ease of installation, compactness of size and lower cost. They are limited to the existing design and produce small steam flow rates, below $250 \times 10^3\,\mathrm{kg\,h^{-1}}$.

The heart of the boiler is the furnace where combustion takes place.

Combustion

Combustion is a process of rapid chemical combination of fuel with air that releases the chemical energy of the fuel. Air and fuel are the reactants in the combustion reaction and the by-product is the flue gases (products of combustion) and heat. It may be represented by the following relation:

$$\text{fuel} + \text{air} \rightarrow \text{products of combustion} + \text{heat} \qquad (3)$$

For this process to occur efficiently, good mixing between the fuel and the air (essentially a mixture of oxygen (O_2) and nitrogen (N_2)) must be accomplished by intensified turbulence, and the ignition temperature of the fuel must be reached. In addition enough time must be allowed for the fuel to burn in the furnace.

Normally, fossil fuels are burnt and these always have in common carbon (C) and hydrogen (H). However, the composition varies greatly with the fuel type:

1. Coal is a solid fuel consisting of carbon, hydrogen, moisture (water), nitrogen, sulfur and ash. It is classified according to the carbon content: anthracite coal has 86–98% carbon (it has a caloric value of approximately $35\,\mathrm{MJ\,kg^{-1}}$, determined experimentally), bituminous coal has 70–86% carbon (and a caloric value of $25–36\,\mathrm{MJ\,kg^{-1}}$), lignite coal has a carbon content up to 70%. Coal needs to be prepared before combustion and its supply to the boiler has to be controlled. It is difficult to burn and produces a high level of ash and sulfur.
2. Fuel oil is a liquid fuel classified according to its ash and moisture content. Its caloric value is the highest of all fossil fuels and may be as high as $46\,\mathrm{MJ\,kg^{-1}}$. Fuel oil has advantages over coal in requiring less storage space, yielding less ash, being easier to control and requiring less equipment. However, it is more expensive because its distribution is not so even around the world.
3. Natural gas is a gaseous fuel with a caloric value of approximately $37\,\mathrm{MJ\,m^{-3}}$. It has advantages over other fossil fuels in requiring the least amount of equipment, being easy to control, mixing well with air and requiring the least amount of excess air, and producing little or no ash (it is the cleanest fuel to burn).

Combustion air is supplied to the reaction in a quantity greater than the theoretically least amount of air needed to burn all of the fuel, so that the combustion reaction is not limited by insufficient air. The amount of excess air depends on the fuel type. Relative to the theoretical amounts, the following are medium values for excess air: large coal particles, 30–40%; pulverized coal particles, 15–20%; fuel oil, 10–15%; and natural gas, 5–10%.

In theory, the fuel is completely burned if the products of combustion are composed mainly of CO_2 and H_2O, and no traces of fuel exist in these products. In practice, this ideal situation does not occur and, due to furnace design, insufficient turbulence, or insufficient residence time of the fuel in the furnace, some traces of fuel, such as CO, always remain in the flue gases, causing incomplete combustion. These gases are undesirable since they are poisonous and explosive and the caloric value is half of the value on complete combustion.

The combustion process may be measured by the combustion efficiency. Among the several notions that exist for this parameter, a simple one is to consider the conversion of carbon to carbon dioxide, given by η_c:

$$\eta_c = \frac{(X_{co_2})\ \text{real}}{(X_{co_2})\ \text{theoretical}} \tag{4}$$

where $(X_{co_2})_{\text{real}}$ is the measured CO_2 molar fraction and $(X_{co_2})_{\text{theoretical}}$ is the CO_2 molar fraction in the off-gas in the case of complete combustion.

The theoretical amount of air needed for combustion is determined by the stoichiometry of the reaction.

Stoichiometry

As mentioned above, normally the fuels burned in boilers are hydrocarbons of the type C_xH_y. The theoretical amount of air needed to burn this fuel completely is given by the following stoichiometric relation:

$$C_xH_y + a(O_2 + 3.76N_2) \rightarrow xCO_2 + \frac{y}{2}H_2O + 3.76aN_2 \tag{5}$$

where $a = x + y/4$.

It must be noted that, since air is a mixture of roughly 21% of O_2 with 79% N_2 by volume (having insignificant traces of other gases), each mole of O_2 is mixed with $79/21 = 3.76$ moles of N_2.

From eqn [5] it can be observed that $(4.76 \times a)$ moles of air are necessary to burn 1 mol of fuel completely. Normally, the stoichiometric air–fuel ratio, $(A/F)_{\text{stoich}}$, represents this relation on a mass basis as:

$$\left(\frac{\text{A}}{F}\right)_{\text{stoich}} = \left(\frac{m_{\text{air}}}{m_{\text{fuel}}}\right)_{\text{stoich}} = \frac{a(1 + 3.76)}{1}\frac{M_{\text{air}}}{M_{\text{fuel}}} \tag{6}$$

where M_{air} and M_{fuel} are the molar masses of air and fuel, respectively, $M_{\text{air}} = 0.21\ M_{O2} + 0.79\ M_{N2} = 28.85\ \text{g mol}^{-1}$, and $M_{\text{fuel}} = xM_C + y\ M_H$ where x and y are the number of carbon and hydrogen atoms in the fuel molecule, and M_C and M_H are the atomic mass of carbon and hydrogen, respectively $12.011\ \text{g mol}^{-1}$ and $1.00794\ \text{g mol}^{-1}$.

If a smaller amount of air is supplied, then the reactant mixture is said to be rich in fuel; if excess air is supplied, it is lean in fuel. The equivalence ratio, φ, is the ratio between the stoichiometric air–fuel ratio and the real air–fuel ratio, $(A/F)_{\text{real}}$:

$$\varphi = \left(\frac{A}{F}\right)_{\text{stoich}} \Big/ \left(\frac{A}{F}\right)_{\text{real}} \tag{7}$$

This parameter allows one to determine whether the combustion is stoichiometric, $\varphi = 1$, the reaction mixture is lean, $\varphi < 1$, or the reaction mixture is rich, $\varphi > 1$, and it is related to the following parameters: the percentage stoichiometric air is given by $100\%/\varphi$, the percentage excess air is equal to $(1 - \varphi)/\varphi \times 100\%$, and the percentage lack in air is given by $(\varphi - 1)/\varphi \times 100\%$.

Combustion and stoichiometry affect the global heat transfer from the fuel to the water in the boiler or, in other words, the boiler efficiency.

Calculation of Boiler Efficiency

Boiler efficiency, η_b, is the ratio between the heat power received by the water, $\dot{Q}_{\text{w}}$, and the heat content of the fuel, $\dot{Q}_{\text{f}}$, since the electrical energy that is necessary to drive the boiler's auxiliary equipment is comparatively much smaller than these values and is normally neglected:

$$\eta_b = \frac{\dot{Q}_w}{\dot{Q}_f} = \frac{\dot{m}_w \Delta h_w}{\dot{m}_f \text{LCV}_f} \tag{8}$$

where $\dot{m}_w$ and $\dot{m}_f$ are, respectively, the water and fuel mass flow rates; Δh_w is the enthalpy difference the water undergoes while it travels through the boiler, and LCV_f is the lower caloric value of the fuel (this is the caloric value usually considered since the water leaves the boiler as a vapor).

Normally, not all the parameters in eqn [8] are easy to determine, so the boiler efficiency must be calculated, by an indirect approach, from the following equation:

$$\eta_b = 100 - \sum L_i \tag{9}$$

This definition considers that the difference between the input and the output energy of the boiler is due to several energy losses. So, from the flue energy content, one subtracts the various energy losses, $\sum L_i$, expressed as a percentage of the LCV_f value, namely:

$$\sum L_i = L_{uf}(\text{unburned fuel}) + L_{ig}(\text{flue gas}) + L_p(\text{purges}) + L_b(\text{heat losses to surroundings})$$

These values may be calculated approximately by the expressions shown in **Table 1**.

The unburned fuel may be present together with the ash, and the energy loss is L_{ufa} (unburned fuel in the ash) because carbon may be carried away by the ash – either the fly-ash, which escapes in the flue gases, and/or the bottom ash, which settles in the boiler.

The flue gases may also contain unburned fuel and the energy loss is L_{uff} (unburned fuel in the flue gases) because CO may exist in the products of combustion due to incomplete combustion.

The energy loss due to the energy content of the fuel gases, besides unburned fuel, is represented by L_{fg} (flue gas). It must be noted that the latent heat of the water in the flue gases is not accounted for because it normally leaves the boiler in the vapor phase.

Periodic removal of debris from the bottom drums is necessary, as well as the removal of water for pH control purposes. The energy content of the water removed represents a loss designated L_p (purges).

Table 1 Boiler energy losses in eqn 9

Energy losses (L_i)	*Expression or value (%LCV_f)*	*Parameters in expressions*
$L_{uf}L_{ufa}$	$= ACLCV_c 100(1-C)LCV_f$	$A =$ mass of ash kg^{-1} of fuel
L_{utt}	$= \frac{F(1-L)_{ufa}COLCV_{co}}{LCV_f}$	$C =$ mass of carbon kg^{-1} of ash
L_{fg}	$= \frac{(1-L_{ufa})FCp_{fg}(T_{fg}-T_{at})}{LCV_1}$	$CO =$ CO mass percentage dry basis
L_p	$= \frac{W\Delta h_1}{LCV_1}$	$Cp_{1g} =$ specific heat of the flue gases
L_h	$= 2.0\%$ for $Q_w \leq 2$ MW	$F =$ fuel gas mass kg^{-1} fuel mass
	$= 1.6$ % for $2MW < Q_w < 5$ MW	$LCV_c =$ lower caloric value of carbon
	$= 1.4\%$ for $Q_w \geq 5$ MW	$LCV_{co} =$ lower caloric value of carbon monoxide
		$LCV_1 =$ lower caloric value of fuel
		$Q_w =$ heat power transferred to water
		$T_{fg} =$ flue gas temperature
		$T_{at} =$ atmospheric temperature
		$W =$ mass of water purged kg^{-1} of fuel
		$\Delta h_1 =$ enthalpy different between leaving and entering boiler liquid

Heat loss to the surroundings, L_h, is very difficult to determine accurately. It is usually obtained by the difference due to all the other losses, so that the energy balance of the boiler is satisfied. Medium values for fire-tube or water-tube boilers at full rate depend on the boiler power, as can be seen from **Table 1**.

By far the most important of the above energy losses is that in the flue gases. For this reason it is usual to recover heat from the flue gases after they leave the boiler, by passing them through economizers (to heat boiler feed water) and air heaters (to preheat combustion air).

To attain good boiler efficiency it is necessary to implement a control strategy that acts on several parameters during boiler operation.

Basic Control Techniques

Basically, the control strategy is implemented as follows: measuring instrumentation detects physical values, such as temperatures, pressures and flow rates; transducers convert these values into electrical signals and send them to data processing systems; these take a controlling action, by comparing the measured values to their preset ones, and send an electrical signal to control systems, e.g. electro-valves, that actuate on the components and regulate the physical values.

By this means it is possible to control the properties of the water vapor produced, the water supply flow and the water level that exists at the boiler's upper drum, the combustion process (regulating the operation of burners or fans), and also ash-removal systems, when applicable.

Obviously, the pressure level of the water vapor produced must be controlled, not only to attain the goal of the boiler, but also to protect it from excessive pressure build-up that may cause boiler explosion. By controlling the pressure, the corresponding saturated temperature is fixed automatically (during vaporization, pressure and temperature are dependent variables). Therefore, temperature control acts on the temperature of overheated vapor that is produced in the boiler. One technique, known as attemperation, may be implemented by regeneration of water vapor or by a spray process. Another technique uses an independent energy source at the boiler exit.

It is also essential to control the water supply flow and the water level that exists at the upper drum in order to guarantee that water in the liquid state is always present inside the boiler (ready to be vaporized) and to avoid a tube explosion due to an excessively high temperature.

The steam produced in the boiler is delivered by a steam piping system (normally made of steel) to the sites where it is used. A return piping system reintroduces the condensed water into the boiler. The design of these piping systems requires great attention because steam leakage losses and hot water losses occurring as a result of deficient design may assume such importance that they may reduce the measures adopted to increase efficiency of the steam generator and of the steam user.

Design of Steam Piping Systems

The design of the steam piping system is normally determined on an economy basis. The total cost of the system is equal to the sum of the capital, installation (an important part of the total cost) and operating costs. These costs depend upon the tube's internal diameter, *D*, as can be seen in **Figure 5** which shows the relationship between cost and *D*.

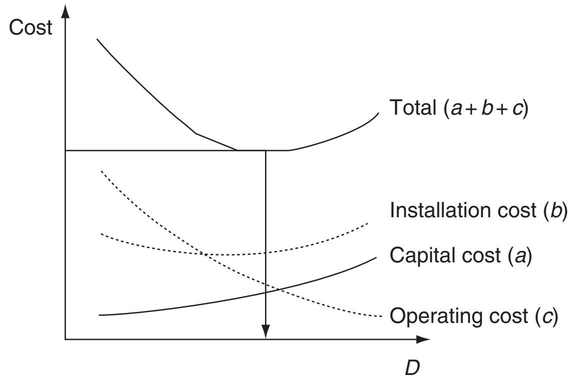

Figure 5 Cost of piping systems as a function of tube diameter (D).

The dependency of the operating cost on diameter results from the pressure drop due to friction between the steam and the tube's internal surface.

Assuming a given flow rate, $\dot{V}$, and considering suggested values for the fluid velocity, ν, saturated steam velocity between 30 and 50 $\mathrm{m\,s^{-1}}$ and overheated steam velocity in the range 50 to 100 $\mathrm{m\,s^{-1}}$, the diameter, D, results from:

$$D\left(\frac{4}{\pi}\frac{\dot{V}}{v}\right)^{1/2} \tag{10}$$

The next step is to select a normalized diameter close to this value, as well as at least two diameter sizes immediately above and immediately below it.

The pressure drop, Δp, is then calculated for each of these diameters from:

$$\Delta p = p\left(f\frac{L}{D} + \sum K\right)\frac{v^2}{2} \tag{11}$$

where ρ is the specific mass given by steam tables ($=1/\nu$); f is the friction factor; L is the tube length (a known quantity); and $\sum K$ is the localized pressure drop due to accessories (some authors convert $\sum K$ to L_{eq}, i.e. an equivalent straight pipe length of the same diameter having the same pressure drop as the accessories).

The friction factor in eqn [11] is given by the Colebrook equation:

$$\frac{1}{\sqrt{f}} = \left[-2.0\log\left(\frac{\varepsilon/D}{3.7} + \frac{2.51}{\mathrm{Re}\sqrt{f}}\right)\right] \tag{12}$$

where Re is the Reynolds number; $\mathrm{Re} = \rho\nu D/\mu$; μ is the steam dynamic viscosity; ε is the tube rugosity; and $\varepsilon = 0.000\ 05\,\mathrm{m}/D$ (m) (for commercial steel tubes).

The pressure drop normally adopted is approximately 5% of the value of the pressure in the main steam pipe. Should the calculated value be greater, it is recommended to redesign the piping system by choosing another value for D.

The outlined procedure allows calculation of the pressure drop, and hence the operating cost shown in **Figure 5**, for a range of possible sizes of tube (such that the steam velocity is within admissible values). Based on the economic criterion, the trend is to favor smaller pipe diameters, i.e. high steam velocities.

An important aspect not to be neglected is that steam at a high temperature flowing in a pipe loses heat to the surroundings (depending on tube insulation), which may cause superheated steam or saturated steam to condense. To prevent damage by erosion or water hammer, the condensed water should be drained by allowing the tube to have a continuous fall in the direction of flow of at least 4–5 mm in every 1 m, and by providing an adequate number of drain points (e.g. in a straight main pipe one every 20–40 m). During operation, condensed water is removed using steam traps; these automatic valves are able to remove liquid but prevent the escape of steam.

The piping system can be optimized using the following parameters:

1. The operating pressure may differ from the design pressure; review the capital cost of the steam generator or steam user due to variation of friction losses.
2. Additional operating costs of returned feed water may be incurred due to an increase in operating pressure; the condensed water is collected and reintroduced into the boiler.
3. Heat loss may differ from the design value; steam enthalpy change affects the steam user and the operating costs.

See also: **Plant and Equipment**: Flow Equipment: Principles of Pump and Piping Calculations; Instrumentation and Process Control: Process Control; Process and Plant Design; **Utilities and Effluent Treatment**: Water Supply.

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Refrigeration

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Vapor Compression Cycle

Principles

Refrigeration is the most common technology used for the conservation of perishable products. It involves the production and maintenance of a level of temperature in a space or object that is lower than ambient temperature.

A consequence of lowering the temperature of perishable products is that the reactions that cause their deterioration, mainly microbial and enzymatic reactions, slow down, enabling the conservation of products for longer periods of time. The lower temperatures needed for conservation of perishable products can be subdivided into two groups: positive temperatures, which are referred to as refrigeration, and negative temperatures, below the freezing point of the product, which are referred to as freezing. Whereas in the former, all water in the product is in the liquid phase, in the latter most can be in solid phase. However, we must bear in mind that the microbial and enzymatic reactions do not cease, they just slow down. As soon as the product is exposed again to ambient temperature, the reactions resume their normal rate. An advantage of freezing is that the products can be stored for much longer periods compared with that for refrigeration. As deteriorative reactions occur in aqueous media, the loss of liquid water to ice substantially reduces water availability, and hence reactions can be severely restricted well beyond the lowering temperature effect. In principle, at temperatures even lower, below the glass transition temperature, all water is in either ice or part of the amorphous vitreous structure, molecular mobility is restricted to mutarotation and vibration, and all reactions cease. Unfortunately, such temperatures depend on water content, and for most foods they are typically well below practical storage applications (-20 to $-50\,^{\circ}\mathrm{C}$ for typical foods).

In the old days, refrigeration was understood as natural refrigeration, that is, the lower temperatures were obtained with ice found in nature. Nowadays, it is understood as artificial refrigeration (i.e., the lower temperatures are obtained by mechanical systems, the most common one being the vapor compression system, shown schematically in **Figure 1**).

The basic system shown is composed of four components, namely the evaporator, which is generally located inside the refrigerated space, the compressor, the condenser, and the expansion valve, connected in series by piping. Inside the system there is a flowing fluid, called refrigerant, which exchanges energy in those components.

In state 1, the refrigerant is in the liquid phase, either saturated or sub-cooled. From state 1 to state 2 the liquid flows through the expansion valve, a device that controls the refrigerant flow rate to the evaporator, where its pressure and temperature are lowered. As in the expansion valve, the refrigerant does not exchange heat or work with the outside, it maintains its total energy – enthalpy (h). In state 2, due to the pressure drop in the expansion valve, the refrigerant has two phases in equilibrium: saturated liquid and vapor. Then it flows through the evaporator where it absorbs heat from the refrigerated space in which the products are stored, lowering or maintaining its temperature. This refrigerant heat gain in the evaporator (increase in enthalpy) causes the boiling of the liquid so that state 3 corresponds with saturated vapor or even super-heated vapor. This process occurs at constant pressure and at constant temperature if there is no super-heating at the outlet of the evaporator. The vapor then enters the compressor where it is compressed to a higher pressure – the same as in state 1 – and with an increase in temperature, and consequently with an increase in enthalpy, state 4. At this point the vapor flows through the condenser, again in a constant pressure process (ideally). In this component, the refrigerant loses heat to the outside (either ambient air or water, or both) with a decrease in enthalpy, changing phase again – condensation – so that at the outlet it is in the same state referred above as state 1.

As can be seen, this cycle operates between two constant pressure levels: a higher one in the condenser and a lower one in the evaporator, the pressure drop and increase being carried out respectively by the expansion valve and by the compressor.

To visualize the refrigerant's evolutions in the vapor compression cycle, different types of thermodynamic diagrams may be used, the most common one in refrigeration being the pressure–enthalpy (p–h) one. **Figure 2** shows a typical p–h diagram of the cycle shown in **Figure 1**. It was considered that no super-heating or sub-cooling exists in the refrigerant at the outlet of the evaporator and condenser.

By applying the first law of thermodynamics to the whole cycle and to each of its components and neglecting changes in kinetic and potential energy, it is possible to calculate the different energy fluxes in the cycle:

$$\dot{Q}_{\mathrm{evap}} + \dot{Q}_{\mathrm{cond}} + \dot{W} = 0$$

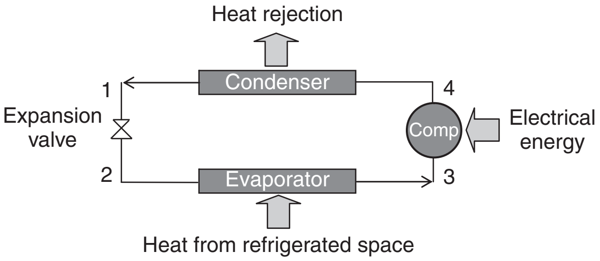

Figure 1 Vapor compression system.

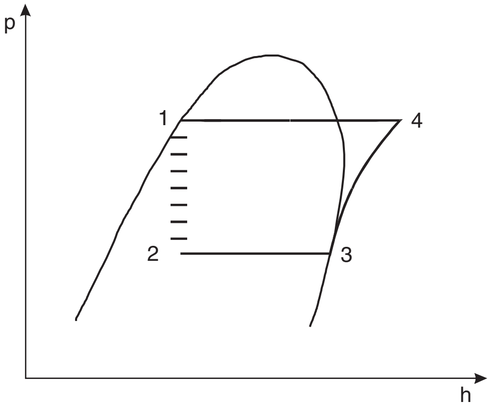

Figure 2 Pressure–enthalpy diagram of the cycle shown in **Figure 1**.

$$\text{evaporator} - \text{refrigeration effect: } Q_{\text{evap.}} = \dot{m}(h_3 - h_2)$$

$$\text{compressor} - \text{compression power: } \dot{W} = \dot{m}(h_4 - h_3)$$

$$\text{condenser} - \text{condensation heat: } \dot{Q}_{\text{cond}} = \dot{m}(h_1 - h_4)$$

$$\text{expansion valve: } h_2 = h_1$$

where $\dot{m}$ is the refrigerant flow rate, Q is the heat exchanged, W is the energy (work) supplied by the compressor, and h_i is the specific enthalpy of the refrigerant at the different points of the cycle.

As can be seen, all the energy fluxes can be easily evaluated if the refrigeration cycle is conveniently plotted in a p–h diagram of the refrigerant used. It is only necessary to read the different enthalpy values and make the above calculations. However, nowadays all the calculations can be performed analytically because the equations of state (equation that enables the evaluation of the different properties of the fluid used) of the different refrigerants are well known. Existing software allows these calculations to be performed easily.

Equipment

There is a wide range of equipment for each component in the vapor compression cycle. The choice of one or another depends mainly on the purpose of the system. In this text, only a general classification of the equipment will be given.

Evaporators are heat exchangers where the refrigerant boils while receiving heat from the surroundings. One possible classification of evaporators is based on their application. In that way, they can be classified as direct expansion or indirect expansion evaporators. In the first type, the coils of the evaporator are in direct contact with the space or body to be refrigerated (i.e., the refrigerant absorbs the heat directly from there). In the second type, the refrigerant takes the latent heat of vaporization from a secondary fluid, usually brine or water. This fluid flows in a closed loop making the connection between the evaporator itself and the objects to be cooled, where it withdraws heat.

The evaporators can be classified into two groups: direct expansion and liquid recirculation type. In the first type, the refrigerant coming from the expansion valve boils completely in the tubes of the evaporator, leaving it as a saturated vapor. The second type is designed so that only part of the liquid boils in the coils. At the outlet of the evaporator there are then two phases in equilibrium, liquid and vapor. The vapor flows to the compressor, while the remaining liquid is recirculated back to the evaporator.

The compressors can be classified as centrifugal compressors, vane compressors, rotary screw compressors, or reciprocating compressors, with the last types used in most refrigeration applications. When the pressure ratio of the compressor is typically above 10 (ratio between condensation pressure and evaporation pressure), the performance of the compressor falls and it is not possible to use only one compressor if it is of the reciprocating type. In this case, it is necessary to either use more than one compressor or choose another type. Usually, the compressors are coupled with electrical motors that provide the necessary running power. However, it is not unusual to find internal combustion engines instead of electrical motors driving the compressors.

Condensers, like evaporators, are heat exchangers, and they are classified as air-cooled, water-cooled, or evaporative. In the first type, the condensing refrigerant loses heat to the ambient air, whereas in the second type the removal of heat is to water that flows in a closed loop, where usually a cooling tower cools the warm water. In the third type, air and water in a packed tower are used in counterflow over the coils of the condenser, inside which the refrigerant condenses. This kind of condenser must be located outside the building and as the refrigerant flows inside it, the length of the pipe carrying it is much longer than the first two types of condensers. Therefore, the pressure drop in the high-pressure part of the system is also higher. Also, as the length of the piping increases, the probability of leakage is increased.

As already mentioned, the expansion valve controls the flow of refrigerant into the evaporator. There are different types of expansion valves, namely, the manually operated, automatic low side float valve, automatic high side float valve, automatic valve, thermostatic valve, and the capillary tube, the last two being very common in most applications. The capillary tube is used in small-capacity refrigeration systems, namely refrigerators and small-size air-conditioning equipment, whereas the application of the thermostatic valve is wider. This valve also controls the degree of refrigerant superheating at the outlet of the evaporator, comparing it with some pre-set value.

Coefficient of Performance

The cycle analyzed so far is an inverse thermal machine. It is therefore possible to evaluate its performance, like in any other thermal machine, as the desirable effect of the system divided by what must be paid for this effect. The performance of refrigeration cycles is expressed through the coefficient of performance (COP), which is the ratio of refrigeration effect (desirable effect) divided by the compression power (what must be paid):

$$\text{COP} = \frac{\dot{Q}_{\text{evap.}}}{\dot{W}} = \frac{h_3 - h_2}{h_4 - h_3}$$

COP values are always positive and usually greater than one, due to the fact that the refrigeration effect is greater than the compression power. Typical values of COP for the vapor compression systems are in the range 2–3. Even if the evaporation temperature is held constant throughout the year, the COP is not constant due to changes in air or water temperature feeding the condenser, which causes changes in the condensing temperature and also in the enthalpies appearing in the COP equation.

Refrigeration Systems

Vapor Compression Systems

The system analyzed so far is the basic vapor compression system that is used in several applications of refrigeration.

However, and keeping in mind this basic system, better performances can be achieved if some modifications are introduced. There are several possible modifications that can be implemented, for specific applications.

A very common modification is the use of multi-stage compression (i.e., the use of more than one compressor), with inter-cooling of the refrigerant between each pair of compressors. Inter-cooling is carried out with the refrigerant at a lower temperature withdrawn from other parts of the system. This technique reduces the system total work. **Figure 3** shows schematically such a system. As can be seen, in this system there are three levels of pressure, a lower one in the evaporator, an intermediate one between the two compressors, and a higher one at the condenser. Multistage systems usually have higher COP values than basic vapor compression systems. This is due to the fact that there is a decrease in compression work and an increase in the refrigerant effect. There are different ways to implement this technique, one of them being to couple the system with several evaporators, each one with a typical operating temperature.

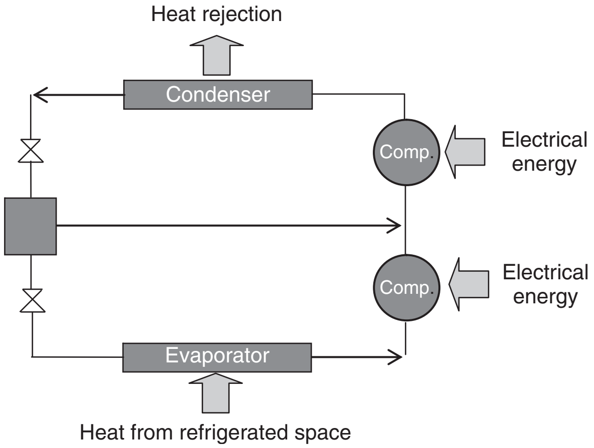

Figure 3 Multistage vapor compression system.

To achieve very low temperatures – much lower than the freezing point of the products – with a good performance, the so-called cascade systems are frequently used. In its simplest form it is composed of two basic vapor compression systems in a way that the evaporator of one cycle is simultaneously the condenser of the second system (see **Figure 4**).

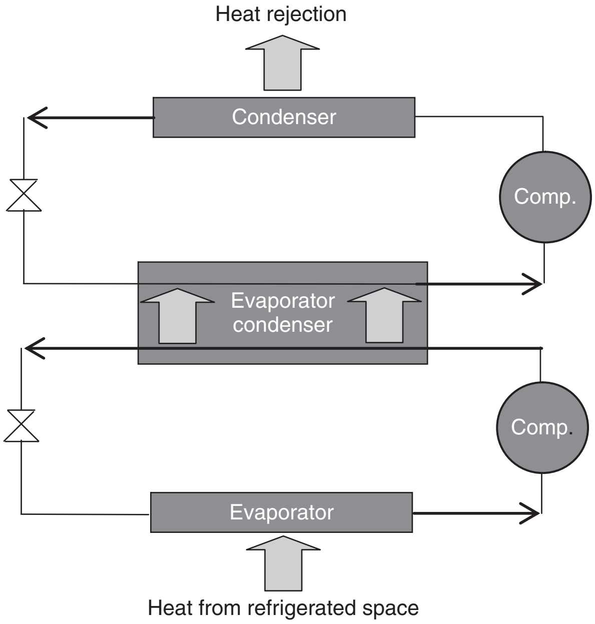

Figure 4 Cascade system.

In that way, the evaporator of the upper system absorbs the heat lost in the condenser of the lower system. Usually two different refrigerants are used, one in each cycle. The refrigerant in the lower cycle should have good characteristics at lower temperatures while the other refrigerant should have good characteristics at higher temperatures. It is also possible for each of the subsystems considered to operate as a multistage system.

Other Systems

One variant of the vapor compression system is the absorption system, also used for refrigeration. This system is as old as the vapor compression system but only recently has its utilization increased, due to the ozone depletion potential of most of the synthetic refrigerants used in the vapor compression system, as will be described later. The absorption system differs from the vapor compression system in that low compression of the refrigerant is carried out, having in common the other three components: the evaporator, the condenser, and the expansion valve. **Figure 5** shows only the part of the cycle that is different. In the absorption system, the compression is done using a secondary fluid that has the capacity of absorbing the main refrigerant flowing in the other three components. At the absorber outlet, where heat is lost to the outside in order to carry out the absorption process, there is a homogeneous liquid solution that is pumped to another component, the generator. Here, it is necessary to supply heat to separate the two fluids, a process opposed to the one in the absorber. The work of compression in the absorption system is much lower than in the vapor compression system due to the fact that a liquid solution is pumped instead of a vapor. But in an opposite way, a large quantity of heat at a higher temperature (typically over 100 °C) must be supplied to the generator. These two combined effects lower the COP value of the absorption system, compared with that of vapor compression systems, to values below 1.0, typically around 0.7. It is however possible to obtain higher COP values if the heat supply in the generator is waste heat (found in many industrial processes) or is complemented with solar energy. Because of the need to supply heat to carry out the compression process, this part of the system (see **Figure 5**) is also called a thermal compressor in opposition to the vapor compression system where a mechanical compressor is used. The absorption system is nowadays very common in household and camping refrigerators as well as in air-conditioning equipment. The most common fluids for the absorption system are H_2O–LiBr (water as refrigerant and lithium bromide as secondary fluid) and NH_3–H_2O (ammonia as refrigerant and water as secondary fluid). The first pair of fluids is used for positive temperatures in the evaporator (water freezes below 0 °C at ambient pressure) while the second one can also be used for negative temperatures. In spite of a fast increase in use, absorption systems are still more expensive than the classic vapor compression systems and are also larger.

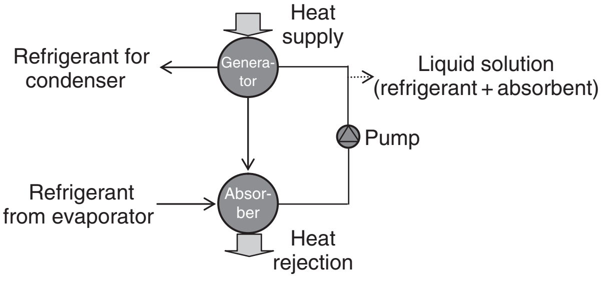
Heat supply
Refrigerant for condenser
Genera-tor
Liquid solution (refrigerant + absorbent)
Pump
Refrigerant from evaporator
Absor-ber
Heat rejection

Figure 5 Compression in the absorption system.

Other types of refrigeration systems are available, some already commercially, and some at the development stage. They can either be operated electrically (like the vapor compression system) or thermally (like the absorption system). An example of electrically operated systems is the one using thermoelectric coolers, where direct current is used to produce a cooling effect. There are more examples of thermally operated systems, namely, adsorption, desiccant, or ejector systems.

The combination of solar energy with refrigeration/cooling equipment is a way of reducing energy consumption and harmful emissions to the environment. Solar thermal collectors can be used with thermally operated cooling equipment, and solar photovoltaic (PV) collectors can be used with electrically operated cooling equipment. Solar cooling systems are interesting, due to the fact that cooling demands in summer are associated with high solar energy availability, which allows operation with maximum collector efficiencies.

Refrigerants

The first refrigerants used in vapor compression systems were inorganic or natural, and some are still widely used, namely NH_3 and H_2O.

However, new refrigerants were produced synthetically from methane (CH_4) and ethane (C_2H_6) being divided into two groups, depending on whether or not chlorine is in the molecular structure. In the first group there are two different kinds of refrigerants: the chlorofluorocarbons (CFCs), namely R-11, R-12, R-113, R-114, R-115, R-500, and R-502, and the hydrochlorofluorocarbons (HCFCs), namely R-22, R-123, R-141b, and R-142b. The second group is hydrofluorocarbons (HFCs), and some refrigerants belonging to this group are R-32, R-134a, R-143a, and R-152a.

Due to the ozone depletion potential (ODP) of CFCs and HCFCs, it was established in 1987 at the Montreal Protocol that the production and use of these refrigerants

Table 1 Characteristics of some synthetic and natural refrigerants

Refrigerant	*R-12 (CFC)*	*R-22 (HCFC)*	*R-134a (HFC)*	*R-717 (NH_3)*	*R-744 (CO_2)*	*R-290 (propane)*	*R-600 (butane)*	*R-718 (H_2O)*	*R-728 (air)*
Natural substanceNo	No	No	Yes	Yes	Yes	Yes	Yes	Yes	
ODP[a]	0.9	0.05	0	0	0	0	0	0	0
GWP[b]	3	0.34	0.29	0	0[c]	<0.03	<0.03	0	0
Toxicity TLV[d] (ppm, volume)	1000	500	1000	25	5000	1000	1000	No	No
Flammability	No	No	No	Yes	No	Yes	Yes	No	No
Critical point temperature (°C)	115.5	96.2	100.6	133	31.1	96.8	152.1	374.2	−140
Critical point pressure (bar)	40.1	49.9	40.7	114.2	73.7	42.6	38.0	221.2	37.2
Normal boiling point (°C)	−30	−40.8	−26	−33.3	−78.4	−42.1	−0.4	100	No
Maximum refrigeration capacity at 0 °C (kJ m^{-3})	2733	4344	2864	4360	22 600	3888	1040	1349[e]	

[a] Ozone depletion potential – compared with R-11.

[b] Global warming potential – compared with R-11.

[c] Zero effective GWP, because more than sufficient quantities of it can be recovered from waste gases.

[d] Threshold limit value (TLV) for exposure of 8 h day^{-1}, 40 h $week^{-1}$, without any adverse effect.

[e] At 100 °C.

should cease gradually. This leaves room for HFCs, the most common one nowadays being R-134a, and to inorganic fluids – air and CO_2 besides those already mentioned. Another problem regarding refrigerants is its global warming potential (GWP), related to the greenhouse effect. In spite of the null ODP of HFCs, they may have a significant GWP, which makes the choice of the refrigerant to be used not easy when taking into account the two parameters simultaneously.

Besides adequate thermodynamic and physical properties, each refrigerant must also have good chemical characteristics. From the safety point of view, these are its flammability and toxicity. Regarding flammability, ammonia is flammable as well as propane and butane, whereas the others are considered as nonflammable. All refrigerants are considered toxic in a small degree, except ammonia, which can be lethal above low concentrations in air.

Table 1 provides a comparison between some characteristics of the most common synthetic and natural refrigerants.

Further Reading

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Compressed Air

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Introduction

Compressed air is a utility that consumes an important share of the energy consumption in industry. Great attention must be paid to the production of this form of energy for it is one of the most expensive. The following are some compressed air applications: driving power for some types of equipment, conveying materials such as chemicals, ventilation of buildings, supplying combustion air for boilers, as a power source for air-operated valves and shutters, and as a medium to transmit signals as in instrumentation.

Air compressors operate on a source of energy (normally electrical energy but other kinds are possible, e.g., internal combustion engines and vapor or gas turbines). Compressors take in air at atmospheric pressure (101 325 N m^{-2} (~100 kPa)) and produce compressed air at a desired pressure. This air is then sent to an air receiver and distributed *via* a piping system to the final points of use.

There are a great variety of compressors and selection depends on the pressure and volumetric flow rate of the compressed air, among other factors.

General Classification of Air Compressors

The general classification of compressors, showing their most important characteristics and applications is summarized in **Table 1**.

Air compressors are normally grouped into two classes: positive displacement and dynamic.

Positive-Displacement Compressors

These compressors operate intermittently, subjecting the air to non-flow processes. An important limitation is that they can handle a small flow rate compared to the dynamic compressors that operate continuously.

The flow proceeds in the same direction as the pressure gradient since these machines have parts that ensure positive admission and delivery of air, preventing undesired reflux.

In this group, a distinction is made between reciprocating and rotating compressors, depending on the motion of the solid boundary.

Reciprocating compressors

A crankshaft is used to transfer the power, e.g., from an electric motor to the compressor (**Figure 1**). A connecting rod joins the crankshaft to the piston, which moves back and forth in the cylinder, with a velocity of between 2 and 4 m s^{-1} at normal crankshaft-rotating velocities. The power received by the air is due to hydrostatic pressure forces acting on a piston during the compression stroke (valves 5a and 5b closed, and the piston moves toward the cylinder head). Consequently, the volume of air decreases and its pressure increases.

Regarding the pressure ratio (Pr, delivery air pressure/admission air pressure), for low values, single-stage compression is sufficient: compression takes place in one or more cylinders of identical size. Multistage (two or more stages) are used when a higher pressure ratio is needed: the air is first compressed in a large low-pressure cylinder, then flows to an intercooler (normally a finned tube), where the air temperature drops, before proceeding next to a small high-pressure cylinder where the final pressure is reached. This arrangement consumes over 15% less power than the single-stage type when producing air at the same volume and pressure.

Rotary Compressors

It is worth noting that rotary compressors are also positive-displacement machines because the fluid is prevented from flowing back in the direction of the pressure gradient by solid boundaries. They have lobes (Roots blowers), vanes or screws to reduce the air volume and increase its pressure (**Figure 2**). Other geometrical configurations exist on the market but, because of their less frequent use in compressed air applications, they are not discussed. The following is a brief reference to these compressors.

Table 1 General classification of compressors

Type		*Pressure ratio, Pr*[a]	*Free air delivery, $\dot{V}_1$ ($m^3\,min^{-1}$)*	*Some applications*
Positive-Displacement				
Reciprocating[b]		<1000	Small <1 Medium 1–10 High >10	Compressed air
Rotating	The Roots blower	<3		Pneumatic transport, vacuum pumps, volumetric flow rate meters
	Vane	<10		Compressed air
	Screw	<4	>3000	Compressed air
Dynamic				
Radial (centrifugal)		<6	>10	Compressed air Small size – as supercharges Large sizes – gas turbine plants
Axial		<10	<500 000	Gas turbine (power plants and aircrafts) Delivery of natural gas in pipelines

[a] =(delivery air pressure)/(admission air pressure).

[b] It is the only technical solution for high pressure ratios.

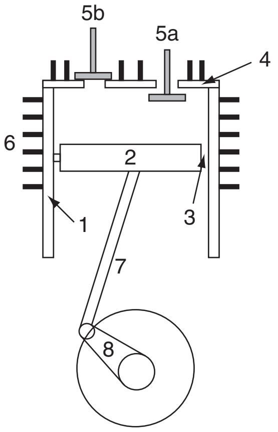

Figure 1 Schematic representation of a reciprocating air compressor: 1, cylinder; 2, piston; 3, piston ring; 4, cylinder head; 5a, admission value; 5b, discharge valve; 6, fins; 7, connecting rod; 8, crankshaft.

Lobe-type compressor (Roots blower)

The Roots blower is one of the simplest and best-known of the rotary compressors. It does not have valves. A gearing system synchronizes the movement of two symmetric rotors, which rotate in opposite directions in the form of a figure 8. Lubrication is not needed because they do not contact each other. This action forces the air to contact the compressed air that exists in the piping system, where oil-free compressed air is produced. Air is the cooling medium.

Compared to the reciprocating compressor, the power required is greater and it has a lower efficiency. For this reason, it is used when low pressure ratios are required, e.g., up to 3 in pneumatic transport, vacuum pumps and volumetric flow rate meters.

Vane-type compressors

The vane compressor action differs slightly from that of the Roots blower. These compressors are said to have internal compression because of the reduction in volume in the initial phase of the compression process (a feature which is absent from the Roots blower).

They have only one rotor, the axis of which is parallel to the stator's axis and is eccentric to it. The eccentric feature is the essence of the principle of operation. Pressure ratios of up to 10 are common. They do not have valves. The cooling medium can be air or water.

Screw-type compressors

These consist of two rotors that move in opposite directions. The rotors have an asymmetric design (made by computer-aided design and manufacture) that makes possible a lower energy consumption, a better gap reduction between rotors and less friction. Practically constant compressed air flow rates are produced.

They can be lubricated or oil-free. When lubricated, the oil and air mix together, so this mixture is compressed and three functions are simultaneously fulfilled: sealing, lubricating and cooling. In the case of oil-free compressors, there is no metallic contact between rotors so a gearing system is used to synchronize the rotor movements.

Dynamic Compressors

Dynamic compressors, or nonpositive displacement compressors, operate continuously, subjecting the air to steady flow processes. An important advantage is that they can handle large flow rates efficiently, as long as

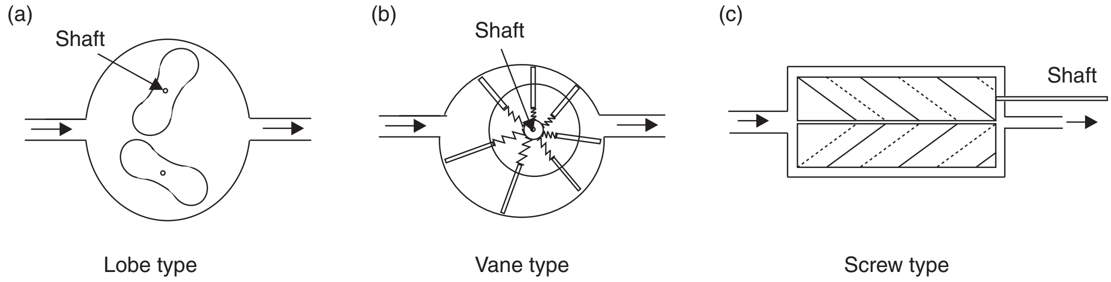

Figure 2 Schematic representation of some rotary compressors: (A) lobe type, (B) vane type and (C) screw type.

the pressure ratio is kept small in comparison to that of alternating and rotary compressors.

These machines have no means of preventing backflow. At a given compressor speed, only a limited range in air flow rate is possible: if it is reduced beyond a certain value, it is impossible to maintain the desired air velocity profile.

In this group, distinction is made between axial and radial compressors, depending on the direction of the air flow.

Radial compressors (centrifugal)

An external source applies an input torque that drives the rotors, which are constructed with recent technology in order to withstand high velocities between 20 000 and 30 000 rpm, accelerating the air in the radial direction (**Figure 3**): the work transfer is due to a change in momentum of the air stream. It then flows into the diffuser and volute casing, suffering a decrease in velocity and an increase in pressure. The combination of the inlet nozzle, impeller, diffuser and volute is called a ‘stage’. Pressure ratios of 4–6 are common. For greater pressure ratios, additional stages are needed.

They are adequate for the production of oil-free compressed air and have been used extensively, both in small sizes as superchargers and in large sizes as compressors for gas turbine plants.

Axial Compressors

In these compressors, the acceleration of the air takes place in the axial direction (parallel to the shaft) (**Figure 3**). Each stage consists of a pair of stationary and moving blade rows (like a turbine): the rotating blades, with velocities in the range 10 000–30 000 rpm, increase the pressure and the stator blades act as a diffuser by converting the air velocity into air pressure. Pressure ratios of 10 or more can be obtained.

Normally, high flow rates are produced (the minimum flow rate is approximately 900 $m^3\ min^{-1}$), making them a good choice for power-plant and aircraft gas turbines, as well as for the conveying of natural gas in pipelines.

Ideal versus Real Reciprocating Cycles

The reciprocating compressor is the most common type used in air compression, followed by the screw compressor. Since both are of the positive-displacement type, the basic theory of reciprocating compressors is also applicable to screw compressors.

The ideal reciprocating compressor cycle is an open cycle that consists of the following evolutions, shown on the *P–V* diagram in **Figure 4** (*P* being pressure and *V* being volume):

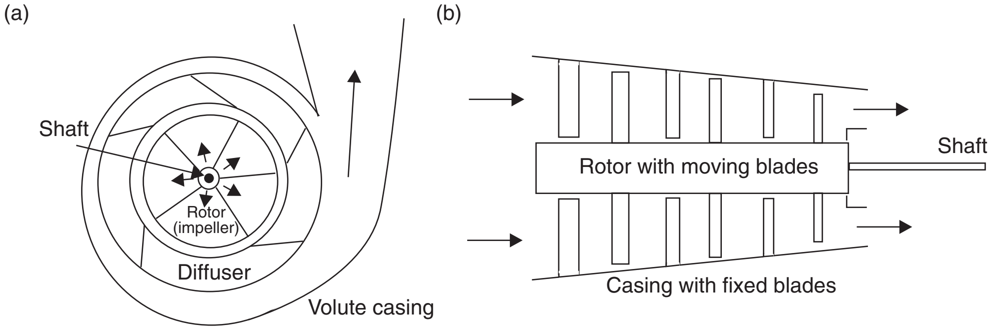

Figure 3 Schematic representation of dynamic compressors: (A) radial type and (B) axial type.

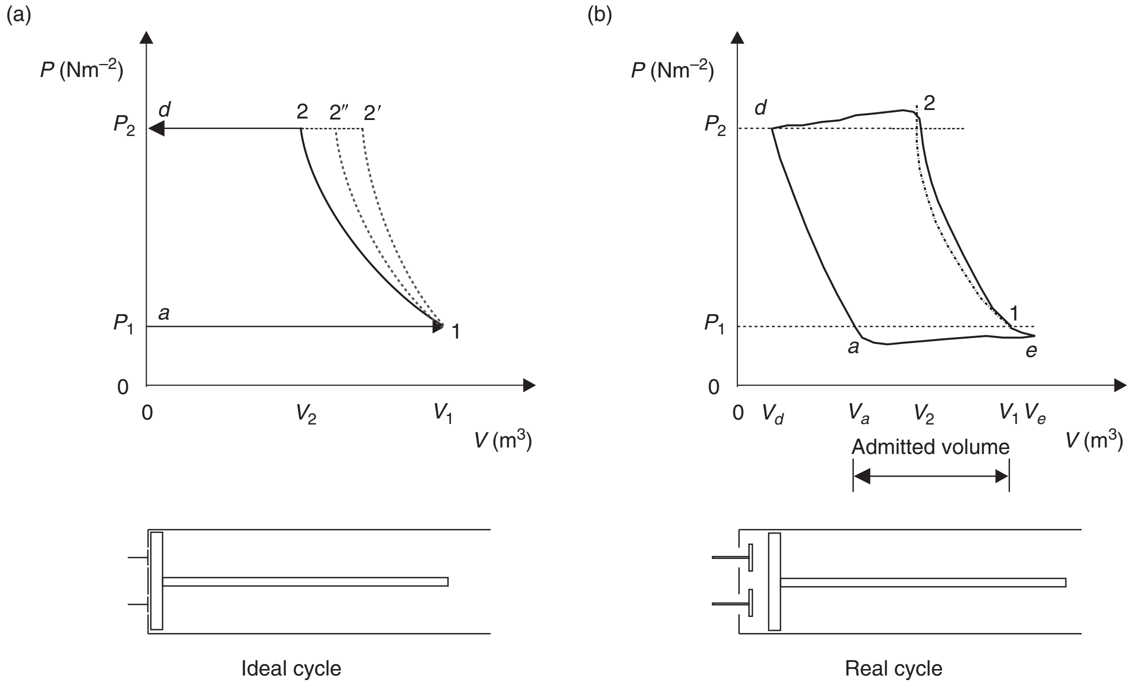

Figure 4 Ideal and real reciprocating compressor cycles: (A) ideal cycle and (B) real cycle.

1. The admission valve opens at a pressure level P_1 and admission of atmospheric air at P_1 and temperature T_1 takes place (state 1). The volume inside the piston cylinder arrangement varies from 0 to V_1.
2. The admission valve closes and the entrapped air suffers a frictionless isothermal compression between state 1 and state 2 (at P_2, T_1). The air volume is reduced from V_1 to V_2.
3. The discharge valve opens when the pressure level is P_2, and the air is discharged to the piping system at state 2. The volume inside the piston cylinder arrangement varies from V_2 to 0.

The work transfer (W) during each step of the cycle is given by $W = -\int PdV$. In terms of the P–V diagram, this is equivalent to the area underneath each evolution, namely:

Admission work performed by the incoming air on the piston: $W_{a\text{–}1} = -P_1V_1$ (area a, 1, V_1, 0, a).
Compression work performed by the piston on the air: $W_{1\text{–}2}$.
Writing the ideal gas equation in terms of P ($P = mRT/V$) gives:

$$W_{1-2} = -\int_{V_1}^{V_2} \frac{mRT_1}{V} dV = -mRT_1 \int_{V_1}^{V_2} \frac{dV}{V} P_1V_1\ln(\mathrm{Pr}) \quad (1)$$

(area 1, 2, V_2, V_1, 1); discharge work performed by the piston on the air: $W_{2\text{–d}} = P_2V_2$ (area 2, d, 0, V_2, 2).

The total ideal cycle work (W_i) is given by:

$$W_1 = -P_1V_1 + P_1V_1\ln(Pr) + P_2V_2 = P_1V_1\ln(Pr) \quad (2)$$

since $P_1V_1 = P_2V_2$.

It can be seen that W_i is equal to the compression evolution work, W_{1-2}. Ideally, this is the minimum work required to increase the pressure from P_1 to P_2.

The ideal power is obtained by dividing W_i by time:

$$W_1 = P_1V_1\ln(Pr) \quad (3)$$

The real compressor cycle is also shown in **Figure 4**. It deviates from the ideal cycle due to a number of differences that cause the flow rate to be smaller and the work/power absorbed by the compressor to be greater, i.e., the compression evolution is not isothermal, there is a clearance volume, there is a pressure drop in the admission and discharge valves, the air temperature increases during the admission stroke, and there are flow rate losses. These causes are analyzed briefly below.

Compression Evolution

If an adiabatic compression is considered (no heat transfer to the surroundings), the compression evolution is characterized by $PV^k = \text{const}$, with $k = 1.4$. Following the same procedure as in the ideal case to calculate the work, the compression work is:

$$W_{1-2} = \frac{P_2V'_2 - P_1V_1}{K-1} \quad (4)$$

and the cycle work, W_a, is given by:

$$W_a = kW_{1-2'} = k\frac{P_2V'_2 - P_1V_1}{K-1} \tag{5}$$

This work is equal to the area *a*, 1, 2′, *d*, *a* (see **Figure 4**). Since this area is greater than that corresponding to the ideal cycle work, $W_a > W_\mathrm{i}$.

In practice, the compression evolution can be considered polytropic, $PV^k = \mathrm{const}$, where normally $k = 1.3$ for low-speed compressors with good refrigeration, and $k = 1.35$ for high-speed compressors.

Clearance Volume

Not all the air is evacuated from the compressor at the end of the delivery stroke: some remains in the clearance volume, $V_d \neq 0$ in **Figure 4**. The air at pressure P_2 enclosed in this space therefore expands from *d* to *a* (**Figure 4**) until it reaches the pressure P_1 when admission commences. At this pressure, it occupies the volume V_a so there is a decrease in aspirated volume represented by $V_a - V_d$.

The clearance volume results in a lower flow rate, and also the work is decreased almost proportionally to the flow rate. This influence is more pronounced for higher pressure ratios; V_a tends to be greater.

This parameter is given as a percentage of the piston displacement (the volume displaced by the piston between the head end and the crank end).

Pressure Drop in the Admission and Discharge Valves

Before the discharge stroke begins, the air pressure must be greater than P_2, allowing the opening of the discharge valve and overcoming the pressure losses due to the flow from the cylinder through the discharge orifice into the piping system. As a consequence, the work increases and the air temperate rises because of its increased pressure.

During the admission stroke, the work increases and the air temperature rises: the air passes isothermally through the admission valve and the pressure depletion that occurs is always greater than that at the end of this stroke. Consequently, the flow rate decreases, causing a decrease in efficiency.

Air Temperature Rises during the Admission Stroke

During compressor operation, the temperature of the cylinder wall will be greater than that of the incoming air. This results in heat transfer to the air and, consequently, a temperature rise, in addition to that caused by pressure changes.

As the flow rate is measured at the inlet, this temperature increase has an important effect on the compressor efficiency and may justify the differences encountered according to their refrigeration modes.

Flow Rate Losses

The effect of these losses on the cycle work is negligible. However, its influence on efficiency must not be ignored because the effective flow rate decreases and the work is fairly constant.

Compressor Efficiency Calculations

The above differences between the real and the theoretical cycles result in a decrease in flow rate and in efficiency.

The volumetric efficiency (η_v) accounts for the decrease in flow rate. It is given by the ratio between the free air delivery ($\dot{V}_1$, the real volumetric air flow rate produced by the compressor measured at the inlet pressure and temperature) and the piston displacement per unit of time (**Figure 4**):

$$\eta_v = \frac{\dot{V}_1}{\dot{V}_e \dot{V}_d} = \Omega\left\{1 - \frac{V_d}{V_e - V_d}\left[\mathrm{Pr}^{1/k} - 1\right]\right\} \tag{6}$$

where Ω depends on Pr, the pressure losses at the valves and on the cooling medium (η_v water-cooled>η_v air-cooled).

An example of the dependency of the volumetric efficiency on clearance volume and pressure ratio is shown in **Figure 5**. It can be understood based on **Figure 4** that, for

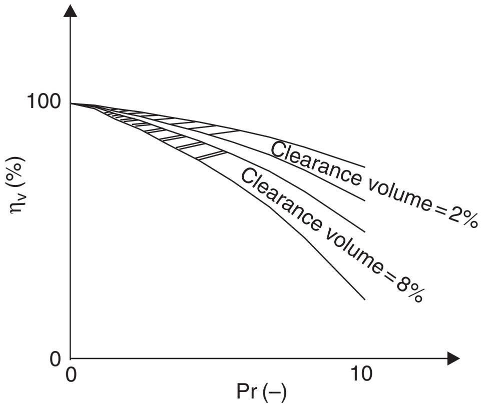

Figure 5 Volumetric efficiency versus pressure ratio with clearance volume as parameter.

a given clearance volume (V_d), as Pr rises ($P>P_2$), it causes the entrapped air to expand to a greater volume ($V>V_a$), reducing the admitted volume and therefore η_v; on the other hand, for a given Pr (e.g. P_2/P_1), if the clearance volume is greater than V_d, the air expands to another volume ($V>V_a$), causing a decrease in η_v.

Normally, η_v ranges between 60% and 90%. The clearance volume causes the greatest decrease of flow rate loss, up to 20% of the piston displacement per time unit.

As mentioned above, another consequence of the differences between the real and theoretical cycles is greater power consumption of the compressor when compared to the ideal power consumption.

The compressor efficiency, also referred to as the isothermal efficiency (η_i), accounts for this effect. It is given by the ratio between the power of a frictionless compressor (with an isothermal evolution) and the shaft power of a real compressor:

$$\eta_{\mathrm{I}} = \frac{\dot{W}_{\mathrm{i}}}{\dot{W}_{\mathrm{r}}} \tag{7}$$

where $\dot{w}_r$ is the shaft power.

This efficiency defines the global compressor efficiency. In order to analyse the compressor operation, one may divide this efficiency into two factors, namely the indicated isothermal efficiency (η_{ii}), and the mechanical efficiency (η_{m}):

$$\eta_{\mathrm{i}} = \eta_{\mathrm{ii}} \ \eta_{\mathrm{m}} = \left(\frac{\text{isothermal power}}{\text{indicated power}}\right) \times \left(\frac{\text{indicated power}}{\text{shaft power}}\right) \tag{8}$$

where η_{ii} is the losses due to cycle imperfections and η_{m} is the friction losses.

Therefore, the ratio $\eta_{\mathrm{i}}/\eta_{\mathrm{m}}$ = (isothermal power/shaft power)×(shaft power/indicated power) shows the power loss due to the fact that the compression evolution deviates from the isothermal evolution.

Another notion of efficiency related to the compressor operation is the polytropic efficiency (η_{p}):

$$\eta_{\mathrm{P}} = \frac{\dot{W}_{\mathrm{P}}}{\dot{W}_{\mathrm{I}}} \tag{9}$$

where $\dot{w}_p$ is the theoretical power of a frictionless compressor undergoing a polytropic evolution such that at the end of the compression the real air temperature is reached.

Relative to the isothermal efficiency, the polytropic efficiency includes the additional work converted to heat the air during compression: $\eta_{\mathrm{p}}-\eta_{\mathrm{i}}$ = losses in percentage of the shaft power.

The referred efficiencies are not the only criteria by which compressors are compared. It must be stated that their characteristics also come into play, namely the relationship between the delivery air pressure, free air delivery and power.

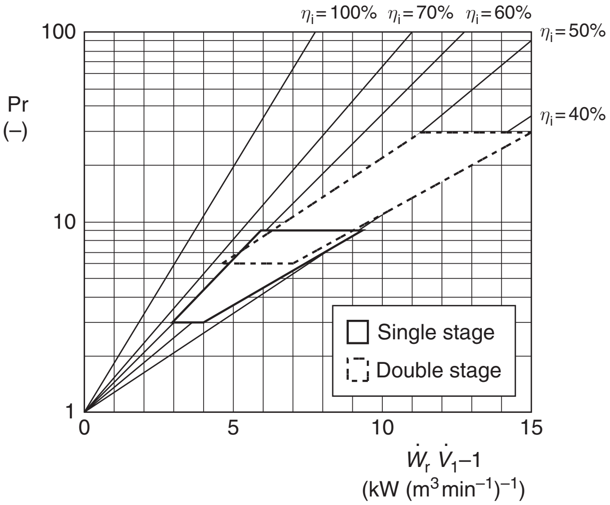

Figure 6 Characteristics of single- and double-stage reciprocating compressors.

It is the operating conditions that establish the delivery air pressure (which must equal the required pressure plus the line pressure losses), the free air delivery (which must equal the required flow rate plus the line flow rate losses), and the power consumption.

The characteristics of reciprocating compressors, Pr versus $W_r/V_I$with η_i as parameter are shown in **Figure 6**. The isoefficiency lines results by combining eqns [3] and [7]:

$$\ln\,(Pr) = \frac{\eta_{\mathrm{I}}}{P_{\mathrm{I}}}\frac{\dot{W}_r}{\dot{V}_{\mathrm{I}}} \tag{10}$$

Two superimposed regions on **Figure 6** show the characteristics of single- and double-stage compressors.

Basic Control Techniques

In general, a flow rate control is necessary because the need for compressed air varies greatly, depending on the number of users connected to the piping system. There are two basic control techniques: motor on/off operation (applied to compressors with shaft power ≤12 kW), and continuous motor operation (shaft power >12 kW).

During the on/off control, the compressor motor operates until the delivery pressure level is reached, turning off at this instant. When the line pressure drops, due to air consumption, below a pre-established value (normally 0.05 MPa below delivery pressure), the compressor turns on again. The maximum number of starts per hour is around 10.

In the continuous motor operation control mode, the motor does not have an intermittent operation, which may cause malfunction considering the powers involved. When the required pressure is reached, the admission valve opens and the discharge valve closes. In this way, the air enters and leaves the compressor without being compressed. The power absorbed is 7–20% of the power needed to produce the normal flow rate.

Treatment of Compressed Air

From the point of view of compressed air production systems, atmospheric air contains undesired constituents or ‘impurities’, mainly water vapour and dust particles. It is essential to treat the resulting compressed air, which may also contain lubricating vapours resulting from compressor operation. The degree of cleaning depends on applications, especially in the dairy and beverage industries.

Several air-intake filters are used before the air enters the compressor:

- viscous filters – as the air passes it impinges on an obstruction that traps the dust
- oil-bath filters – the incoming air agitates the oil in a reservoir, causing the impurities to settle in the bath
- travelling screen filters – air flows through a screen that rotates continuously; the screen is coated with oil to trap the debris, which is then washed out as the screen moves through an oil bath
- dry-type filters – utilizing a medium often made of felt or fibreglass.

It is common to install a silencer integrated with the air inlet filter to reduce the noise level where this is problematic.

Water and lubricant vapours are removed by condensation in cooling systems located at the compressor exit. Purges control the removal of water and oil condensed from the compressed air.

For the majority of applications, compressed air has a dewpoint temperature of 2 °C (below this temperature, water vapour condensation occurs) and the lubricant vapour concentration is $1\,\mathrm{mg\,m^{-3}}$. The concentration of dust particles in air is roughly $5\,\mathrm{mg\,m^{-3}}$, consisting of particles with an average diameter of 5 μm.

Design of Compressed Air Piping Systems

A piping system conveys the compressed air to the consumption points. An advantage of compressed air distribution systems is that no piping system is necessary for the return of the used air because it simply escapes to the atmosphere.

The main distribution system is made of rigid tubes (steel, galvanized iron and plastic are common materials), while flexible tubes join the system to the final users. Dilatation tubes are also used to compensate for temperature differences. Purges must be placed at low-level points (to remove further condensed water and oil), so the slope of the tubes towards these points must be at least 1%.

Large tube diameters favour small pressure drops caused by the air flow. The design of the piping system involves choosing all tube diameters on the basis of the admissible pressure drop through the tubes. However, it is suggested that the air flowing through the longest tube length, from the compressor to the furthest consumption point, should undergo a maximum pressure drop of 0.01 MPa.

Normally, the flow is turbulent and the pressure drop (Δp) is proportional to the square of the air velocity (v), i.e., inversely proportional to the fourth power of the internal tube diameter (D):

$$\Delta p = p\left(f\frac{L}{D}+\sum K\right)\frac{1^2}{2} \tag{11}$$

where ρ is the specific mass given by the ideal gas equation ($=P/(RT)$, $R=287\,\mathrm{J/(kg\,K^{-1})}$), f is the friction factor, L is the tube length, ΣK is the localized pressure drop due to accessories (some authors convert ΣK to L_{eq}, i.e., an equivalent straight pipe length of internal diameter D having the same pressure drop as the accessories) and v is the air velocity (suggested values for v range from $10\,\mathrm{m\,s^{-1}}$ for the main piping system to $20\,\mathrm{m\,s^{-1}}$ for the flexible pipes).

For pressure drop prediction, normally only an order of magnitude is sufficient. A more accurate value requires great calculation effort and it also requires the knowledge of the rugosity (roughness) of the tube’s internal surface and an accurate definition of all the piping system components.

In practice, an abacus is used to determine the pressure drop. The example shown in **Figure 7** was constructed considering smooth pipes, admission air at $P_1=0.1\,\mathrm{MPa}$ and $T_1=293\,\mathrm{K}$ (20 °C). Following the calculus sequence (follow the line with an arrow in **Figure 7**):

- Assume an internal tube diameter D and a delivery pressure P_2.
- For a given free air delivery $\dot{V}_1$, determine the air velocity $v=\dot{V}_1/\pi D^2/4$ and f, from the Colebrook equation:

$$\frac{1}{\sqrt{f}}\rho\left[-2.0\log\left(\frac{\varepsilon/D}{3.7}+\frac{2.51}{\mathrm{Re}\sqrt{f}}\right)\right] \tag{12}$$

where Re is the Reynolds number, $\mathrm{Re}=\rho vD/\mu$, $\mu=1.81\times10^{-5}\,\mathrm{Ns\,m^{-2}}$ (is the dynamic air viscosity at T_1), ε is the tube rugosity ($\varepsilon=0$ (smooth tube)).

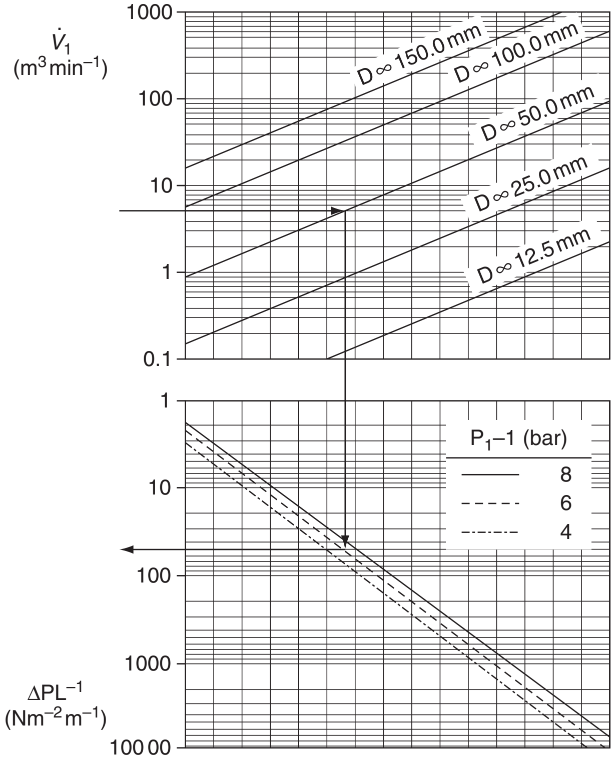

Figure 7 Abacus for determination of the pressure drop in smooth pipes.

- Determine the pressure drop per unit tube length, assuming $\Sigma K = 0$ (no localized pressure drop):

$$\frac{\Delta p}{L} = \rho \frac{f}{D} \frac{1^2}{2} \tag{13}$$

Roughly, one may increase the value of $\Delta P/L$ by 30% to account for the rugosity effect.

The design is confined to determining D since the other variables are known: P_2, the delivery pressure, and $\dot{V}_1$ are imposed by the users of compressed air and the maximum value suggested for $\Delta P/L$ is 0.01 MPa L^{-1}. It must be borne in mind that the equipment consuming compressed air deteriorates with use, so higher pressures and flow rates must be considered, respectively between 0.05 and 0.1 MPa (depending on L) and flow rates between 30% and 40% relative to the operating characteristics when new.

The piping system design is important for it is responsible for energy losses due to pressure drops (caused by the flow) and due to flow rate losses which occur mainly at the tube joints. The latter are the most important (for pipe lengths below 1000 m it may represent 5–10% of the flow rate) so frequent inspection of the piping system is suggested.

To meet the requirements of the piping systems, the choice of the compressor characteristics must be such that it has a small variation in pressure ratio values for a great variation in the flowrate values.

See also: **Plant and Equipment**: Flow Equipment: Principles of Pump and Piping Calculations; Instrumentation and Process Control: Instrumentation; Instrumentation and Process Control: Process Control; Process and Plant Design. **Utilities and Effluent Treatment**: Water Supply.

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Electricity

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Introduction

A well-designed electricity supply system is fundamental to the operation of all processing plants. It is the electricity supply that provides much of the heat, as well as the power to drive pumps, compressors, refrigeration plant, instrumentation, lighting (both standard and emergency), control systems of all types, and computers and communication systems.

It is important, therefore, that dairy scientists and plant operators have at least some understanding of the component parts of the electricity supply system.

Electricity for Power

Electricity is generated primarily in large power stations which are powered by coal, gas, oil or nuclear plants. These are often sited on a river or close to a sea coast because cooling water is a primary requirement for operation. Renewable sources of energy, such as wind, wave, solar and others, will become increasingly relevant as countries attempt to cap their greenhouse gas emissions and increase sustainability. The electricity, which is generated using synchronous generators, is transformed to a higher voltage using transformers and fed into an interconnected grid system, usually at a high voltage of 400–800 kV. This allows for the bulk transfer of power, which can be tapped into, using a transformer, to supply electricity to a city, perhaps at 200 kV or so, or downward to what is known as distribution level, for supply to factories or processing plant, often at a voltage around 11 kV or perhaps 6 kV, depending upon the concentration and size of the plant. Unless very large motors or heaters are involved, this voltage is further reduced to perhaps 415 V three-phase or 240 V single-phase. The exact voltage is determined by the standards of the country in which the plant is operating. Another factor which can vary between countries is the frequency of the supply, which can be either 50 Hz or 60 Hz. Users should be cautious when ordering equipment or instrumentation that is designed for use in plant in another country.

For larger processing plants, a substation containing primarily transformers, switches and circuit-breakers is a feature of the site, often at the roadside. If there are processes or systems within a plant that could be either commercially sensitive or unsafe if the electricity supply should fail, there might also be a diesel generator or some other standby supply of electricity which can be switched on if there is an emergency loss of power from the supply company.

Another feature of processing plant is the existence of a battery room, often associated with an uninterruptible power supply (UPS), which may be used for emergency lighting systems, protection systems, gas alarms, fire alarms, computer networks, or anything that has to be supplied with continuous power in the event of a supply failure.

The interaction of these primary and standby power supplies is determined by commercial and safety considerations. A factor which is becoming increasingly important, as electricity suppliers are becoming more competitive and sophisticated post-deregulation, is making use of, for example, standby generators at times when tariffs are very high due to high peak demand, in order to avoid exceeding agreed maximum demand levels.

Energy Management and Usage

All energy management is a compromise. Energy costs can be saved by use of insulation to prevent heat loss or by the reprocessing of low-grade heat to higher-grade heat. The level of insulation or recovery depends very much on the size of the plant and also on the operating temperatures of hot liquids and the refrigeration plant. It is also a function of the system involved and the life expectancy of the plant. The larger the plant, the higher the temperature differences, and the greater the life expectancy the more scope there is for energy management.

An important factor in the usage of electricity is to minimize costs by planning the optimum use of tariffs. This is a subject which gains in complexity with the size of plant because electricity suppliers, in order to reduce their installation and running costs, financially discourage the consumption of power during times of peak demand. It is therefore advisable to carry out periodic energy audits to see whether cost savings can be made by replanning production or by segmentation of plant so that only essential loads are attached during times of peak demand. For smaller systems, the tariffs tend to fall into four categories, depending on location. These are: (1) a flat rate service fee, (2) a fee for electrical usage measured in kW h, (3) a charge (or credit) for running with a power factor outside an agreed limit and (4) peak demand charges.

Apart from the power factor charge, these are largely self-explanatory.

Loads that are connected in a plant and draw a current which is not in phase with the applied voltage are said to have a poor power factor. This will happen where there is a large induction machine load on the system or when power converters are used to convert three-phase AC into DC for whatever purpose. This poor power factor obliges the supply company to install extra generating capacity and burn extra fuel.

The inductive loads can be balanced at plant level by installing power factor-correction capacitors that will pull the wave shape of the current into phase with the voltage. Depending upon the expected lifetime of the plant and the extent of the inductive load, power factor correction often turns out to be a good investment.

Another factor which sometimes has to be taken into consideration is the effect that installed plant can have on the power quality. If large numbers of AC-to-DC power converters are used, they may distort the incoming supply. If the supply is distorted beyond a certain level (known as the harmonic content) at the point of common coupling with other users, it may be necessary to fit tuned harmonic filters. These will remove the offending distortion close to the source.

Distribution and Safety Issues

The power distributed within a plant is fed from the incoming substation or switching room, primarily using cables. These are normally installed in ducts, using wall- or floor-mounted cable trays, or in the wall, floor or ceiling in spaces created during construction.

In designing the electrical supply to a plant, safety is paramount. Safety considerations fall into three categories: (1) the prevention of personal accidents due to electric shock or contact with rotating machinery, (2) the prevention of damage to plant and the process and (3) the prevention of fire.

The safety of personnel is catered for at several levels. All electrical connections should be covered or positioned inside enclosures to which entry is forbidden except with a permit. All rotating machinery should be fitted with a guard so that it cannot be touched inadvertently and it is impossible to fall onto rotating shafts. Shock prevention at low voltage is enhanced by the use of ‘residual current’ or ‘earth-leakage circuit-breakers’. These trip at very low levels of fault current imbalance and were a major advance when first introduced. Older plant should be retrofitted with such devices during plant maintenance.

Damage to plant and the prevention of electrically induced fires are prevented by using circuit-breakers and fuses. These either trip or blow if excess current is generated by a fault in a piece of equipment or if, for example, a cable is inadvertently cut by maintenance personnel or a vehicle within the plant. These will isolate the electrical supply until the fault is corrected.

Electric Motors

The problem of selecting the most suitable type of motor was at one time a significant factor in the design of new plant. Recent events have, however, simplified the choice.

In the past, processing plant tended to have a mixture of different types of motors, e.g. the DC motor was used widely where a variable shaft speed was required and induction (or asynchronous motors) were used where a fixed speed of operation was deemed necessary.

With the development of larger-powered, pulse width modulated (PWM) converters, the induction motor can now be used to power most variable frequency applications as well. The PWM drive, as the combination is known, is widely used for all applications from a small part of a kilowatt (low horsepower) up to the megawatt region.

Previously, the choice of motor type, which was based on the detail of the starting torque and speed range required, was a matter of much debate. The various grades of motors are based on the National Electrical Manufacturers Association (NEMA) classification A to F, from which different ratios of maximum torque compared to rated torque can be selected. Increasingly, the Class B general-purpose motor is used for all directly connected and variable-speed applications, making selection easier. In the author’s opinion, variable-speed drives should not be bought as a separate converter and motor because this can lead to coordination problems.

There is an exception to this new simplification of the selection process: such motors should not be used in conditions where there is a potential danger of explosions, e.g. in an environment where there are dust particles in the air or where distillation products are escaping into the air. Under these conditions, it may be desirable to purchase totally enclosed motors of the type developed for mining and offshore oil-platform applications. These can also be found in the NEMA standards.

See also: **Hazard Analysis and Critical Control Points**: Processing Plants. **Plant and Equipment**: Process and Plant Design. **Utilities and Effluent Treatment**: Heat Generation.

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Dairy Plant Effluents

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Introduction

Dairies are considered as a 'wet industry' since they consume much water. Water is used for very different purposes (**Table 1**). Therefore, dairies discharge large volumes of wastewater. Recent mean values vary between 0.5 and 2.0 m^3 wastewater per 1 Mg (tonne) treated milk. However, there are large differences according to different production lines:

milk and desserts: 1.0–12.9 $m^3\,Mg^{-1}$ final product;
cheese: 0.5–6.0 $m^3\,Mg^{-1}$ final product; and
milk powder: 0.9–10.0 $m^3\,Mg^{-1}$ final product.

Among other things, variations depend on the equipment and its maintenance, the working method, and the environmental engagement of the management in the dairy, but there is no influence of the amount of milk processed. The figures given include the volumes of cooling water.

As it is common for the food industry, pollution of the wastewater is the most important contribution to the pollution of the environment from dairies in both quality and quantity; contamination by solid waste and waste gas is less serious.

The pollution of wastewater must be considered under the following aspects:

- kind and quantity;
- ecological evaluation; and
- means for reduction.

Analytical Indices for Wastewater Pollution

Dairy effluent mainly contains milk components: organic substances are dissolved or suspended in the wastewater; their quantity is determined analytically using summary parameters. Adsorbable organic halogen (AOX) represents an unspecific value for a group of substances similar in chemical composition (**Table 2**).

Important values for quantification of the organic load of dairy effluent are the biochemical oxygen demand (BOD_5) and the chemical oxygen demand (COD). The quotient COD/BOD_5 indicates the biodegradability of the organic material under aerobic conditions. When the ratio is close to 1, the biodegradability is said to be good. The natural organic constituents of milk are very biodegradable but the aerobic degradation of milk fat requires much oxygen – for instance, the COD of skim milk is about 90 000 $mg\,l^{-1}$ but for cream (30% fat) it is 400 000 $mg\,l^{-1}$. Besides milk fat, the wastewater may contain other lipophilic substances, for example, mineral oils or grease, that are less biodegradable. Due to a relatively high protein content in dairy wastewaters, the nitrogen content is often used as a pollution index.

When comparing effluent loads from different dairies, the indices referred to are expressed in volumetric percent, as shown in **Table 2**. Thus, if a dairy discharges cooling water together with polluted wastewater, the indices will decrease. Therefore, low pollution indices do not conclusively indicate diligent working practice. For this purpose, the complete size of contamination must be considered, that is, the concentration of pollutants and the total volume of wastewater.

Other pollutants come from the regular hygienic operations; detergents represent the biggest portion of chemicals used in dairies. In some cases, NaOH or an acid alone is used, but mixtures of several chemical substances are advantageous to obtain really clean surfaces; **Table 3** lists the most important components of dairy detergents.

Besides, many cleaning solutions also contain residues of dairy products or other kinds of soil (**Table 1**) that have been displaced in the cleaning process. Often, the product residues are altered by the cleaning solution: proteins may be partially hydrolyzed by alkalies or enzymes or may be coagulated in acid solutions. Fats may be emulsified and in strong hot alkalies even soaps may be formed. Soaking alkalies from bottle-washing machines contain dissolved starch or protein glues from removed paper labels.

In contrast to most cleaning solutions, disinfecting solutions can be used only once, and then they must be discharged. Commonly, they contain only very small amounts of soil but always a surplus of the active disinfectant and of potential reaction products also, for instance halogenics and AOX.

Conditioning of boiler feed water demands some chemicals, that is, complexing agents to sequester the residual hardness of the pretreated water and to give an alkaline pH. Other additives, for instance starch, tannins, or synthetic polymers like polyacrylate or polystyrene derivatives, serve to improve the blowdown of the sludge from the boiler into the wastewater. Furthermore, sodium sulfate may appear in the effluent formed from sodium

Table 1 Consumption of water in dairies in view of effluent generation

Area of use	*Application*	*Examples*	*Main effluent pollutants*
Production	Manufacture	Washing of butter or cheese curd	Product streams like buttermilk or whey
		Electrodialysis of whey	Mineral salts
Hygienic operations	Prerinsing	Removal of product residues from equipment after use	Portions of raw materials or products
	Solvent	Solutions of detergents or disinfectants	Portions of raw materials or products, constituents of detergents and disinfectants
	Rinsing	Rinsing off residues of cleaning and disinfecting solutions	Constituents of detergents and disinfectants
Others	Solvent	Regeneration of plants for water conditioning	Ions of chloride, alkali earth metals, or hydrogen
	Cleaning	Washing the outside of transporters	Soil, mineral oil, any detergent used
	Rinsing	Rinsing of sludge from boilers	Alkali earth metals, organic dispersing agents

Table 2 Most important pollution indices for wastewater

Index	*Brief definition*	*Unit*	*Standard methods*
BOD_5	Biochemical oxygen demand; quantity of oxygen used for aerobic biodegradation of organic matter in the sewage during 5 days	mg O_2 l^{-1}	ISO 6060 (1989)
COD	Chemical oxygen demand; quantity of oxygen needed for the chemical oxidation of organic matter in the sewage by potassium dichromate	mg O_2 l^{-1}	ISO 6060 (1989)
TKN	Total Kjeldahl nitrogen; mass of bound nitrogen in the sewage, determined by the Kjeldahl method	mg N l^{-1}	ISO 5663 (1984)
AOX	Mass of adsorbable organic halogen compounds in the sewage	mg Cl l^{-1}	ISO 95625 (2005)
SS	Volume of sedimentable matter in the sewage	ml l^{-1}	ISO 11926 (1997)

Table 3 Common components of detergents and disinfectants for dairies

Range of application	*Kind of components*	*Substances*
Cleaning	Alkalies	Sodium hydroxide
		Sodium carbonate
		Sodium silicates
		Trisodium phosphate
	Acids	Nitric acid
		Phosphoric acid
		Sulfamic acid
		Gluconic acid
	Complexing agents	Phosphonates
		Nitrilotriacetate
		Ethylenediaminetetraacetate
	Surfactants	Linear alkylsulfonates (anionic)
		Alcohol sulfates (anionic)
		Alcohol ethoxylates (nonionic)
	Enzymes	Proteases[a]
Disinfection	Halogens	Iodophores
		Sodium hypochlorite
		Sodium trichloroisocyanurate
		Chloramine T
	Surfactants	Quaternary ammonium compounds (cationic)
	Peroxy compounds	Hydrogen peroxide
		Peracetic acid

[a] Technical grade, especially for cleaning of membranes.

sulfite, which is added to the boiler feed water in order to bind the residual oxygen in the pretreated water.

Quantities of Pollutants

Product Losses

First of all, product losses into the wastewater and discharged whey cause the organic load of dairy effluent and thus the BOD_5 as well as the COD.

Compared with other food industries, for example, starch or meat factories, a specific BOD_5 not higher than 4.0 kg O_2 per Mg processed milk indicates a relatively low organic loading of the wastewater. But the BOD_5 of dairy sewage markedly exceeds the average for domestic wastewater (300 mg $O_2\,l^{-1}$). In contrast, the dairy wastewater contains a very low quantity of sedimentable substances (**Table 4**); this is only a small fraction of the common content in domestic sewage.

Different equipment and production methods strongly influence the degree of pollution by organic matter and explain the varying indices in **Table 4**. Even for a single product, like spray-dried milk, BOD_5 values between 0.152 and 22.4 kg O_2 per Mg final product are reported.

Extremely high organic loads are probable from manufacturing specialities like cacao drinks or processed cheese. As a consequence of their viscosity, they leave more residues on each contact surface, which are discharged into the effluent together with soiled cleaning solutions.

Bottle washing has to remove not only product residues from the bottles but also the labels and glue from the outside. The quantity of glue depends on the gluing technology. The additional organic pollution of the soaking lye may be as high as 1–5 kg glue per 10 000 bottles.

Auxiliary Chemicals

Little is known about the consumption of chemicals for hygienic operations because exact documentation is lacking; only estimated quantities are available, and the data from 1990 are given in **Table 5**. Since dairies tend to reduce the chemical pollution of their wastewater, recycling of soiled alkaline solutions is commonly practiced nowadays. Thus, the consumption of alkaline detergents, NaOH included, should have decreased since 1990.

In the dairy industry, normally alkaline and less frequently acid solutions are used for cleaning. Therefore, wastewater usually contains a surplus of alkali, which must be neutralized to pH 6–9 before it is discharged into the public sewage system. Only in special cases is there a surplus of acid in the sewage. Therefore, more acid than alkali is needed to neutralize the effluent from cleaning operations (**Table 5**).

Figures for the consumption of auxiliary chemicals for purposes other than hygienic care are hardly available. Thus, it is nearly impossible to give any data on their quantity in dairy wastewater. The demineralization of whey by ion exchange demands NaOH and inorganic acids. Increasing degree of demineralization increases

Table 4 Indices for untreated dairy wastewater

	Average value		
		(over the day)	
Index[a]	*Unit*	*Doedens*[1]	*Bertsch/Doedens*[2]
Quantity of polluted effluent	$m^3\,Mg^{-1}$	0.8–2.0	1.0–2.0
BOD_5 load	$kg\,Mg^{-1}$	0.8–2.0	0.8–4.0
Specific BOD_5	$mg\,l^{-1}$	500–2000	500–2000
COD load	$kg\,Mg^{-1}$	-	0.8–4.0
Specific COD	$mg\,l^{-1}$	-	500–4500
COD/BOD_5	-	1.5–2.2	-
TKN	$mg\,l^{-1}$	30–50	30–250
NO_3-N	$mg\,l^{-1}$	20–130	10–100
BOD_5/ total N	-	-	3–14
Total P	$mg\,l^{-1}$	10–100	20–250
Lipophilic substances	$mg\,l^{-1}$	20–250	80–250
SS	$mgl\,l^{-1}$	1.0–2.0	1.0–2.0
pH	-	9–10.5	6–11

[a] For abbreviations, see **Table 2**.

[1] Doedens H (2000) Verarbeitung von Milch und Milchprodukten. In: ATV (Abwassertechnische Vereinigung, Hrsg.) (ed.) *Industrieabwasser Lebensmittelindustrie*, 4. Aufl., pp. 259–277. Berlin: Ernst u. Sohn

[2] Bertsch R and Doedens H (2003) Abwasserentsorgung. In: Verband der Deutschen Milchwirtschaft (VDM) (ed.) *Richtlinien für Wasser und Abwasser*, pp. 120–213. Berlin: Selfpublisher.

Table 5 Specific consumption of cleaning and disinfecting chemicals (kg dry substance per Mg processed milk) in different countries

Country	*Alkaline detergents (including pure alkalis)*	*Acid detergents (including pure acids)*	*Disinfectants*
Czechoslovakia	0.3–0.8	0.1–0.3	0.001–0.7
Switzerland	1.12 (1.23)[a]	0.66 (1.08)[a]	0.065
Finland	1.13	0.71	0.07
Germany	2.4	0.5	-

[a] Including wastewater neutralization.

IDF (1993) Environmental influence of chemicals used in the dairy industry that can enter the waste water. *Bulletin of the International Dairy Federation*, Vol. 288, pp. 17–31. Brussels: IDF.

the required amounts of these chemicals disproportionately and also the chemical load of the sewage.

The quantity of chemicals needed for water conditioning depends primarily on the hardness of the available water and on the applied process; generally, demineralization needs more chemicals than softening would require.

Environmental Evaluation

General Remarks

Dairy effluent often blends with sewage from other industries or from households. This complicates the evaluation of the sewage pollution from dairies. Compared with the effects of product components in dairy wastewater, it is much more difficult to estimate the risks from auxiliary chemicals used. Ecological evaluation has to be based on the knowledge of the concentration of a chemical substance that is expected in the effluent or river. Moreover, fundamental knowledge of the resulting damaging potential is necessary for each applied substance. However, for cleaning and disinfection, dairies often apply mixtures of several chemical agents for increased effectiveness and not a single substance. This complicates environmental evaluation further, because the single components of such a mixture act very differently and may even develop antagonistic or synergistic effects in an aquatic system. The user cannot have the necessary knowledge. Therefore, only the manufacturer is responsible for the selection of suitable components for detergents and/or disinfectants. He has to combine sufficient effectiveness with environmental compatibility in his products as far as possible. But it is the responsibility of the user to apply detergents and disinfectants carefully and to follow the advice of the producer.

All these facts explain that the ecological importance of only single chemical substances and not ready-to-use products can be discussed here.

Product Residues

If untreated dairy effluent is discharged directly into rivers or lakes, the oxygen consumption by aerobic degradation of product residues would disturb the aquatic ecosystem considerably. Besides, such water is unsuitable for the production of potable water. The higher the organic load of the sewage, the more expensive the treatment in the sewage plant will be and the more sludge that will be produced. Milk proteins contribute to the phosphorus and nitrogen load of the effluent (**Table 4**) as well as solutions of detergents and some disinfectants (**Table 3**). Both elements support an unwanted growth of algae in lakes and slowly running waters. For the Federal Republic of Germany, an estimation of phosphorus and nitrogen content of dairy effluents has shown that the mass of nitrogen from product residues and losses and that from chemicals used for hygienic operations are nearly equivalent. But the main sources for phosphorus are detergents. It is possible to eliminate both elements and thus to prevent eutrophic effects (*see* **Utilities and Effluent Treatment**: Design and Operation of Dairy Effluent Treatment Plants). Nowadays, more and more effluent plants are equipped with these additional stages.

Auxiliary Chemicals

High hygienic standards make the application of chemicals for cleaning and disinfection inevitable. The chemical components in these products show very different effects in sewage, rivers, and lakes.

Alkalies and acids strongly change the pH of the wastewater and increase the salt load of running waters, because they pass through the sewage plant unchanged. About one-third of the phosphorus in untreated sewage is used by microorganisms in the biological stage of the effluent plant. The remaining phosphorus, similar to nitrogen, supports the growth of algae, especially in lakes. But nitrogen is not normally as critical as phosphorus because the latter is mostly the limiting factor for the development of algae.

Surface-active agents tend to form a foam on the surface of water and thus impede the uptake of oxygen into water or activated sludge systems. As a consequence of a low oxygen concentration in the water, fish may die. Besides, several surfactants or metabolites from these may impede the reproduction of fish by damaging sperm, eggs, or spawn. Other aquatic animals, like daphnia, are inhibited, too. Therefore, surfactants must be biodegradable. This does not mean total demineralization but at least loss of their surface activity by biodegradation in the effluent plant.

Special attention must be paid to disinfectants, because generally discharged solutions still contain a considerable portion of the active microbicidal agent. However, they are inactivated by the organic load in dairy wastewater. Only traces of reactive disinfectants may reach the treatment plant and do not inhibit the biodegradation of organic material. But exceptionally high concentrations of a disinfectant as a consequence of an accident or inattentive handling can markedly inhibit biodegradation processes. A more important problem results from the reaction of active chlorine with organic material in the sewage to form undesired AOX. They are persistent, accumulate in the food chain, and are more or less toxic. If there are organic substances with a free amino group, active chlorine in the wastewater may form chloramines, which still have a limited bactericidal effect.

Complexing agents can sequester metal ions and some of these compounds can also mobilize undissolved heavy metals from sludge or sediments in rivers or lakes. Especially in the case of ethylenediaminetetraacetate (EDTA), this effect seems dangerous, because it is scarcely biodegradable in contrast to nitrilotriacetate. EDTA passes through the effluent plant unchanged. Therefore, low concentrations of EDTA have been detected in rivers; possible metabolites are aquatoxic. Complexing phosphonates that have substituted for polyphosphates show a 'threshold effect': This means that very low quantities are sufficient to sequester alkaline earth ions in hard water; thus, the danger of eutrophication by phosphonates is considerably lower than by phosphates.

In addition to the quantities of alkalies and acids from cleaning operations, chemicals used for the regeneration of ion exchange plants increase the salt concentration in dairy sewages. Further pollution by salt results from brine bath overflow in cheese factories.

Steps Toward a Reduced Effluent Pollution

Each dairy should attempt to minimize effluent pollution; it saves costs for internal and public effluent treatment. Thus, losses of raw material, products, and auxiliary chemicals must be avoided as far as possible. Moreover, the auxiliary chemicals should be used as sparingly as possible. It is the task of management to detect critical points of sewage pollution, to create the technical conditions for reduced pollution, and to consistently motivate the employees to follow the special instructions.

The following general remarks may help to reduce effluent pollution or to reduce water consumption. These hints are examples; each dairy has to identify its own weak points and resolve them.

Equipment:

- Have short routes for flow and transportation of raw materials and products, because it minimizes the adhering residues after use.
- Ensure that all plants are easy to clean. Therefore, avoid blind pipes, which need additional volumes of cleaning and disinfecting solutions.
- Ensure by automatic control of the flow ways that products and fluids for cleaning and disinfection do not contaminate each other.

Production:

- Avoid losses of raw materials, products, additives, and auxiliary chemicals into the wastewater by splashing, leaking valves or pipe connections, or overflowing containers.
- Optimize operations aiming to have a minimum of residues on product-contacting surfaces. For instance, preheating for the production of ultra-high temperature milk reduces the deposits on heat exchange surfaces.
- Try to utilize whey fully.
- Never discharge solid sludge from centrifugal processing or residues from microfiltration by membranes into the wastewater.

Hygienic operations:

- Remove product residues from product-contacting surfaces. For instance, blowing out by filtered compressed air or rinsing with a small volume of water may be useful; rinsing with warmed skimmed milk is advisable for cream residues, because it makes it easier to recover and to utilize the cream rinsed off.
- Avoid needless dilution of chemical solutions by rinse water; it increases the consumption of detergents or disinfectants.
- Use mixed detergents instead of pure chemicals. Suitable additives can markedly improve the efficiency of alkalies and acids in many cases. Subsequently, the chemical pollution of the wastewater by used solutions decreases.
- Regenerate used cleaning solutions by sedimentation, centrifugation, or membrane techniques in order to extend their useful life whenever it promises success.

The sludge may be disposed or utilized for biogas production.

- Disinfect closed systems by heating instead of applying chemicals, if it is safe and the consumption of energy seems acceptable.
- Avoid chemicals that are dangerous for aquatic systems or may disturb wastewater treatment. If there are any doubts, ask the producer or the distributor of the detergent/disinfectant.

Conditioning of water and sewage:

- Reduce the water hardness only as far as necessary for the intended use. Water softening should be done by ion exchange or reverse osmosis. Other physical methods cannot be advised since at present too little is known about their effectiveness.
- Use as much condensate from evaporators as possible in order to reduce water hardness, but do not overlook possible microbiological risks.
- Use condensates also for cleaning purposes. The condensates from the production of evaporated milk or milk powder contain only small amounts of organic substances. Therefore, it is not necessary to discharge them into the general wastewater. Condensates may be used for cleaning of floors in uncritical areas, like offices, or for cleaning of cars and trucks. Following pollution with mineral oil or grease, they must be eliminated before this wastewater may be discharged into the general sewage. Condensate that is not used should be discharged with rainwater.
- Neutralize the surplus of alkalies from cleaning lyes by carbon dioxide or boiler flue gas but not by mineral acids. This reduces inorganic pollution of the wastewater. Further advantages of using boiler flue gas are less exhausted SO_2 and low running costs but it needs special installations.
- Separate the fat from the wastewater. This is normally not necessary for all the wastewater but essential for the part of the sections producing butter or cream. The separation may be done in gravity traps or by flotation. However, the separation will not work in case of emulsified fat.
- The separation of fat, oil, and grease from the wastewater by the dairy itself not only reduces the BOD_5 and COD, but it also prevents deposits in the drain. Moreover, these pollutants can cause severe problems in the biological wastewater treatment process on-site and in public sewage treatment facilities.
- Collect and discharge the normally unpolluted rainwater separately from the common wastewater. It saves money and avoids overflow of the wastewater plant in case of heavy rainfall.
- Further recommendations for minimizing product losses into the wastewater and also for reducing the cost of freshwater production as well as of wastewater treatment are given in the IDF document no. 382 (2003).

See also: **Milking and Handling of Raw Milk**: Milking Hygiene. **Milk Protein Products**: Membrane-Based Fractionation. **Utilities and Effluent Treatment**: Design and Operation of Dairy Effluent Treatment Plants; Water Supply.

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Design and Operation of Dairy Effluent Treatment Plants

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Glossary

Biochemical oxygen demand (BOD) It is an important measure of water quality. It is a measure of the amount of oxygen needed (in mg l^{-1}) by bacteria and other microorganisms to fully oxidize the organic matter present in a water sample. It is also called the biological oxygen demand.

Chemical oxygen demand (COD) It is defined as the oxygen equivalent of the organic portion of the sample that is susceptible to oxidation by a strong chemical oxidant. COD does not distinguish between refractory and inert organic matter. COD tests require approximately 3 h.

Five-day biochemical oxygen demand (BOD_5) It is defined as the amount of oxygen required by bacteria to decompose organic matter for a specified time (5 days) under aerobic conditions. The amount of oxygen reported with this method represents only the carbonaceous oxygen demand (CBOD) or the easily decomposed organic matter. BOD_5 is commonly used to measure natural organic pollution. The BOD_5 of drinking water should be less than 1, while that of raw sewage may run to several hundreds. The BOD_5 of dairy waste may run from several hundreds to hundreds of thousands.

Total dissolved solids The weight of solids in solution per unit volume of water, measured by evaporating a known volume of filtered water and weighing the residue.

Total solids The weight of all solids, dissolved or suspended, and organic or inorganic, per unit volume of water, measured by evaporating a known volume of water and weighing the residue.

Total suspended solids The measure of particulate matter suspended in a sample of water or wastewater. After filtering a sample of a known volume, the filter is dried and weighed to determine the residue retained.

Effluent Characteristics

Dairy effluents contain dissolved sugars and proteins, fats, and sometimes the residues of additives used in production. The effluent treatment plant designer is primarily interested in the following characteristics of the effluent: biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), total dissolved solids (TDS), phosphorus (P), nitrogen (N), and pH. **Table 1** shows typical losses from some of the principal activities in a dairy.

The typical characteristics of dairy effluent are given in **Table 2**.

The waste-load equivalents of specific milk constituents are

1 kg milk = 3 kg COD
1 kg lactose = 1.13 kg COD
1 kg protein = 1.36 kg COD

The wastewater may also contain pathogens from contaminated materials, grit or other particulates from truck washing, and paper and other packaging materials.

Discharge Standards

The design of the treatment plant depends on the discharge standards set by the licensing authority. A British Royal Commission in 1912 determined that where a receiving water body could provide a dilution of 8:1, a discharge standard of 20 mg l^{-1} BOD and 30 mg l^{-1} Suspended Solids (SS) was appropriate. However, much higher standards are now frequently required. **Table 3** summarizes the license requirements of a number of dairy facilities in Ireland.

Prevention and Control of Pollution

Given the very high capital and operating costs associated with wastewater treatment, not to mention the cost of water and chemicals and the value of lost product, it makes good sense for producers to initiate and maintain a program of pollution prevention. Prevention practices in the dairy industry include

Table 1 Typical losses in milk processing operations (in kg BOD m^{-3} milk)

	Operation	*Average*	*Range*
1	Milk reception, churn washing, cleaning up	0.26	0.11–0.66
2	Cooling raw milk, storage, washing tanks and pipelines	0.19	0.07–0.31
3	Washing road tankers	0.25	0.10–0.40
4	Separation, storage of skim milk and cream	0.14	0.09–0.24
5	Separation, storage of skim milk and cream plus cream pasteurization	0.66	0.46–1.25
6	Churning and washing butter	0.46	0.25–0.80
7	Evaporating skim milk to low total solids	0.23	0.16–0.30
8	Evaporating skim milk to high total solids and spray-drying	0.74	0.14–1.50
9	Roller drying	0.53	0.25–1.30
10	Pasteurizing milk and storage, bottling milk, bottle washing	0.85	0.49–1.70
11	Clotted cream	1.20	
12	Cheesemaking (hard pressed)	0.89	0.23–2.00
13	Cottage cheese (washed curd)	12.00	
14	Condensing fresh whey (to low total solids)	0.25	
15	Condensing sweetened separated condensed milk	1.40	1.20–1.70
16	Full-cream evaporated milk, with canning	0.75	0.50–1.00

Table 2 Typical characteristics of dairy effluent

BOD	*0.8–2.5 kg tonne^{-1} milk*
COD	1.5 times BOD
TSS	100–1000 mg l^{-1}
TDS	10–100 mg l^{-1}
Phosphorus	10–100 mg l^{-1}
Nitrogen	~6% of BOD level
pH	2–12
Flow	1–2 m^3 tonne^{-1} milk

- reduction of product losses by better production control;
- use of disposable packaging (or bulk dispensing of milk) in lieu of bottles where feasible;
- collection and reuse of waste product (where feasible) in lower-grade products such as animal feeds;
- optimization of water and chemical use; use of high-pressure nozzles; recirculation of cooling waters;
- use of condensates for cleaning;
- energy recovery; and
- avoidance of phosphorus-based cleaning agents.

Unit Processes for the Treatment of Dairy Effluent

The series of unit processes chosen for the treatment of any particular effluent will depend upon the characteristics of the wastewater, the location and space available, the outlets for residual products, and the final effluent quality required.

- Pretreatment and preliminary treatment processes
  - Coarse and fine screening
  - Removal of fats, oils, and grease
  - Grit removal
  - pH control
  - Nutrient balancing
  - Flow and load balancing
- Biological treatment processes
  - Activated sludge process
  - Biological filtration
  - Anaerobic treatment
- Clarification
- Sludge treatment
  - Solid–liquid separation
  - Stabilization

Pretreatment and Preliminary Treatment Processes

Coarse and Fine Screening

Screening is designed to remove suspended particles from the wastewater, in order to protect the remainder of the treatment plant from damage by gross solids and to protect subsequent treatment stages from solids overload. Usually, the screening process is divided into two stages:

- coarse screening – to remove solids of nominal size 20 mm and above; and
- fine screening – to remove solids of nominal size 0.25 mm and above.

Coarse screens can be either static, comprising inclined bars at a spacing of approximately 25 mm, or mechanically raked. In either case, it is important that the velocity of flow through the chamber is maintained at values between 0.3 and 0.8 m s^{-1} to ensure that grit or other

Table 3 Comparison of treated effluent discharge standards

Facility	*BOD mg l^{-1}*	*SS mg l^{-1}*	*P_{tot} mg l^{-1}*	*Ammonia as N mg l^{-1}*	*Nitrate mg l^{-1}*	*N_{tot} mg l^{-1}*	*Temperature °C*	*Flow m^3 day^{-1}*	*Discharge to*
A	20	30	2	1	20		25	10 000	River
B	15	15		10	10		21	900	Spray irrigation
C	20	30	2	0.5		15	25	4000	River
D	20	30	2	10		15	25	4500	River
E	20	30	2	2		15	25	8900	Sewer
F	25	35	2	5		15	25	18000	Stream (dilution 6:1)
G	20	30	1	1		15	25	1400	Lake
H	10	15	1	2		10	25	9000	River
J	40	40	2			15		2800	Estuary

detritus does not settle out and that the intercepted screenings are not dislodged and carried forward.

Fine screens can be installed directly after the coarse screens, except where the wastewater has a high content of fat or grease. Fine screens can be

- static – parabolic wedge-wire screens – sometimes called 'sidehill' screens;
- brushed – where the wastewater is screened through a curved perforated plate, which is brushed intermittently or continuously; a typical rotary brushed fine screen is shown in **Figure 1**; and
- rotating drum screens – where the wastewater is led to the center of the drum and flows via wedge wires to the next stage. This type of screen cannot be used where there are high levels of fat in the wastewater.

Provision should always be made for the high-pressure or steam cleaning of fine screens, particularly where the screens are subjected to fatty wastewaters.

Removal of Fat and Grease

Fat and grease will solidify and float to the surface of the liquid given time, temperate ambient conditions, and a quiescent flow pattern. On small plants it is still not unusual to find static grease traps designed on the basis of flow rate. Typically, a retention period of 30 min or more is provided and the accumulated fat/grease is removed manually. The principal drawback of such systems is the possibility of the accumulated fats being subjected to a higher temperature and becoming emulsified.

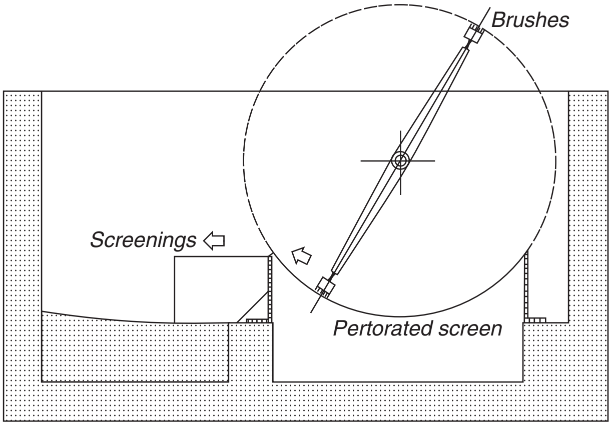

Figure 1 Rotary brushed fine screen.

Recently, however, flotation, particularly dissolved air flotation (DAF), has become the most frequently used process for the removal of fat/grease. In this process, air is dissolved in water under pressure and then the supersaturated air–water mixture is injected into a flotation tank. The process is shown schematically in **Figure 2**. The air comes out of solution in the form of microbubbles, which attach themselves to suspended matter, including fat/grease, which then float to the surface. The floating material forms a scum on the surface of the tank and is removed intermittently by a mechanical skimmer. Typical design parameters for a DAF unit are

- upward flow velocity: up to 7.2 m h^{-1};
- volumetric retention time at maximum inflow: 20–30 min;
- recycle rate: 20–35% of inflow; and
- air/solids ratio: 0.005–0.06 kg air kg^{-1} solids to be removed.

Where the efficiency of the process can be improved by the use of flocculating agents, it has also been found that 50% or more of the COD load can be removed by the DAF process.

Grit Removal

Grit particles, which can include sand, gravel, clay, and other detritus, generally enter the waste stream from the truck and tanker washing area or through the corrosion of concrete or paved surfaces. If allowed to pass through the process, grit could cause serious damage to pumps and other mechanical equipment in addition to combining with sludge to cause pipe obstructions.

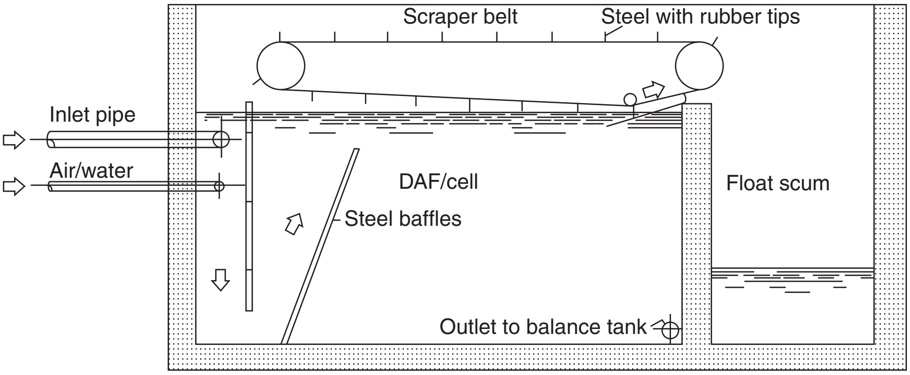

Figure 2 Dissolved air flotation.

Grit will settle readily, provided the velocity of flow is reduced to approximately 0.3 m s^{-1}. It is quite important that the velocity is not permitted to fall below 0.15 m s^{-1}, as this could result in the settlement of organics. Grit settles at about 30 mm s^{-1}, so where the length of the grit channel is 15–20 times the depth, the grit removal efficiency is high.

The maintenance of constant velocity in the grit channels is usually achieved either by the formation of parabolic channels or by using a Sutro Weir. This is a special type of weir that ensures that the velocity of flow is directly proportional to depth.

pH Control

The influence of pH within a treatment plant is both chemical and biological. Control of pH is necessary to ensure that the wastewater does not damage the structures, equipment, or pipework. Most biological processes operate best within the range 6.5–8.5; however, it has been found that process efficiency can be maintained even where the resulting pH is not optimal, provided the pH is reasonably constant and not subject to sudden change.

Nutrient Balancing

Biological treatment processes can be inhibited if the balance of available nutrients is insufficient to ensure that the microbes can break down the organic matter in an efficient manner. Frequently, dairy wastewater may have an excess of phosphorus and a deficiency of nitrogen or potassium. It is generally accepted that the ratio BOD:nitrogen:phosphorus should be 100:5:1 to facilitate microbial breakdown.

Nutrient deficiency can be overcome by the addition of urea (or any other source of N) and phosphoric acid. In addition, there are several proprietary products available that can provide nutrients in different proportions to meet the specified demand. It is important to ensure that the available N and P are measured at the entry to the biological treatment and not prior to other physical/chemical processes.

Hydraulic and Load Balancing

Biological treatment processes operate best under constant and consistent organic load, with only minimal, gradual variations in the substrate. Most physical and physiochemical processes are flow-dependent, as are pumps, pipework, and other items of mechanical equipment. It is therefore essential that adequate provision be made for balancing both pollution load and flows. Balancing can be effected by a combination of provision of adequate storage capacity and control of the forward flow. The theoretical capacity of a balancing tank can be determined as follows:

> buffer required to minimize substrate variations + provision for variations in inflow over day/week/month as appropriate + provision for equipment malfunction + freeboard

Provision must be made for mixing the balancing tank contents thoroughly, and consideration should be given to aerating the contents where the potential for biodegradation of the waste exists.

Biological Treatment Processes

Biological treatment processes have generally been classified as aerobic (where the degradation takes place in the presence of oxygen) and anaerobic (where oxygen is excluded). Processes classified as aerobic include activated sludge process and biological filtration, although in the case of the latter, both aerobic and anaerobic systems coexist on the surface of the media.

Activated Sludge Process

The activated sludge process, discovered in the early 1900s, is a biological wastewater treatment method in which microorganisms are bunched together to form sludge flocs. The flocs develop spontaneously when the wastewater is aerated.

The wastewater and the sludge flocs are mixed in the aeration tank. Most of the impurities in the wastewater are suitable nutrients for the bacteria in the flocs; these bacteria take up the nutrients in their cells. An activated sludge floc is a conglomerate of

- living and dead bacterial cells;
- protozoa and higher organisms;
- trapped inorganic particles and organic fibers; and
- precipitated salts.

The floc is held together by chemical forces and a slime matrix surrounding the cells. The composition of the floc is dynamic, not static, and can be changed through alterations in the process conditions.

The general processes that occur are

- stabilization – slow breakdown of adsorbed materials;
- mineralization – conversion of nutrients to substances like carbon dioxide;
- assimilation – conversion of nutrients to cell material; and
- endogenous respiration – microbial mass converted to new cell material for new cells.

The operating principle of the activated sludge process is that wastewater containing biodegradable organics is fed to a reactor containing a well-mixed, well-aerated population of microbes (biomass, in the form of a flocculent suspension). The resulting mixture of biomass and water is separated, with the solids (sludge) being returned to the reactor (**Figure 3**).

The key parameters used in the design of the activated sludge process are

- food (F), usually expressed in kg BOD;
- microbial population, usually expressed as kg mixed liquor suspended solids (MLSS); it is also referred to as mass (M); and
- the ratio of food to mass, which is usually called F/M ratio and is a measure of the loading rate.

Typical design data are shown in **Figures 4** and **5**.

Activated sludge is generally categorized based on the F/M ratio as follows:

high-rate activated sludge	F/M ratio in the range 0.6–1.8;
conventional activated sludge	F/M ratio in the range 0.2–0.5; and
extended aeration sludge	F/M ratio in the range 0.04–0.1.

Several configurations of the aeration tank have become popular in the treatment of dairy wastewaters, including

- plug flow – the wastewater and sludge are introduced into one end of an aeration basin where the ratio length:width is >12:1;
- the oxidation ditch (developed by Paasver in 1953) – the aeration tank is laid out as a racing track, and oxygen transfer and mixing are effected by horizontal rotors (**Figure 6**);
- the carousel – this is similar to the oxidation ditch; however, the oxygen transfer and mixing duties are frequently split. This configuration allows the establishment of an anoxic zone; and
- the sequencing batch reactor – aeration and clarification take place in the same tank (**Figure 7**).

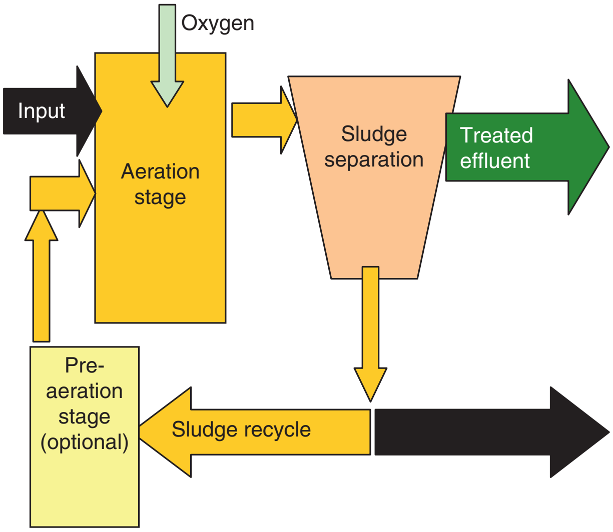

Figure 3 The basic activated sludge process.

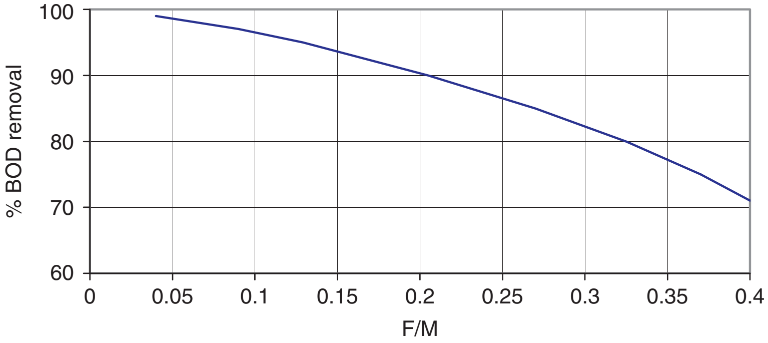

Figure 4 Percentage BOD removal versus food-to-mass (F/M) ratio for the activated sludge process.

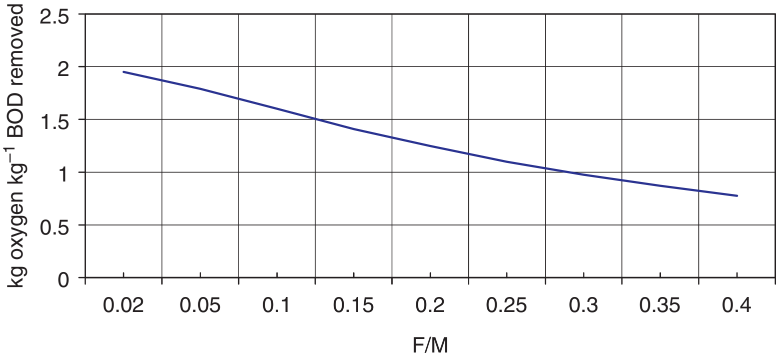

Figure 5 Oxygen demand (kg O_2 kg^{-1} BOD removed) versus food-to-mass (F/M) ratio for the activated sludge process.

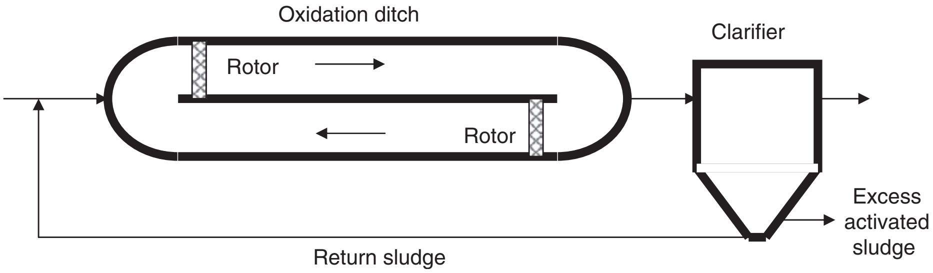

Figure 6 Typical oxidation ditch plant.

Biological Filtration

The principle of the biofiltration process is similar to that of the activated sludge process. In this process, the organic matter (food) is brought into contact with high numbers of microbes (film adhering to media) in the presence of oxygen (**Figure 8**).

Biological filters are not normally mechanically aerated, as the heat generated during the microbial degradation process is usually sufficient to maintain a temperature gradient between the wastewater and the surrounding air, ensuring an adequate draught.

The most common form of biofilter used in the treatment of dairy wastewater is the high-rate biofilter. The biofilter media, which are usually in the form of open-textured plastic, can be either random-packed or modular. High-rate biofilters are normally loaded above 0.6 kg BOD m^{-3} and generally remove 50–70% of the applied BOD (**Figure 9**).

The wastewater is distributed over the surface of the media at a minimum irrigation rate of 1.5 m^3 per m^2 plan area per h, and this ensures that no clogging of the media occurs and discourages insect life (**Figure 10**).

The most critical parameters in the operation of a biofilter are

- irrigation rate – it is essential that the irrigation rate is maintained at all times to ensure that the filter media do not become clogged;

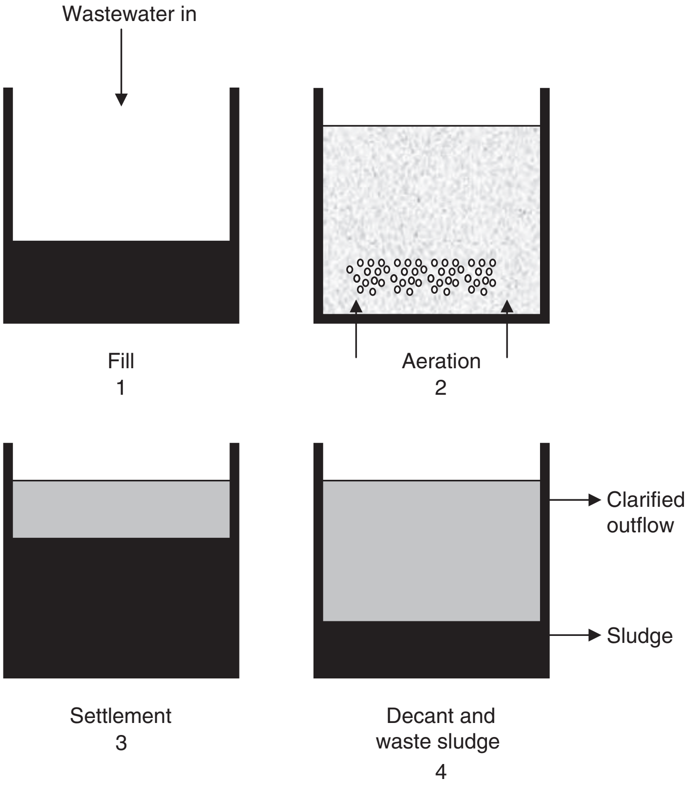

Figure 7 Sequencing batch reactor.

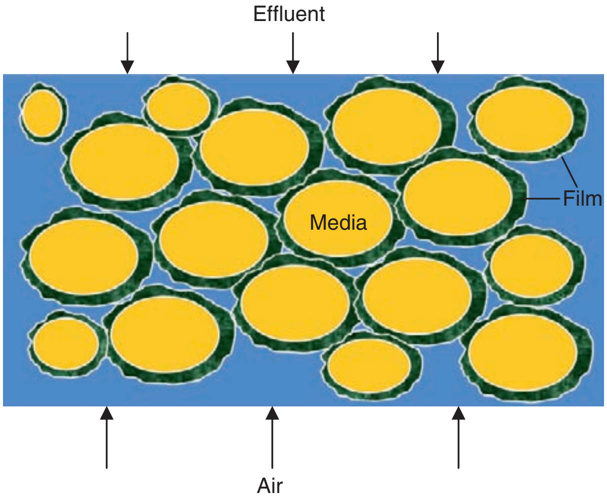

Figure 8 Activity on biofilter media.

- BOD applied – the application of excessive loading rates (shock loads) can also result in clogging of the media and ponding of the surface; prolonged BOD loading can give rise to odor problems;
- pH – inadequate control of the pH will reduce the efficiency of the biofilter and may even result in damage to the media and support structures;
- fats/grease – the presence of fats and grease in concentrations above 50 mg l^{-1} can result in the coating of the biological film; this can lead to uncontrolled anaerobic activity and significant odors in extreme cases; and
- temperature – a reduction in efficiency will occur when the temperature within the biofilter drops below 8 °C.

Usually, the outflow from high-rate biological filters, even after settlement, is not of sufficiently high quality to be discharged to watercourses and will require further treatment; activated sludge process is frequently used.

Nutrient Removal

Nitrogen

This is a two-stage process: nitrification, which is carried out under strongly aerobic conditions, and denitrification, which is carried out under anoxic conditions.

In the nitrification stage, ammonia is converted to nitrite and nitrate. For each kilogram of ammonia oxidized

- ~4.18 kg O_2 is consumed;
- 14.1 kg alkalinity as $CaCO_2$ is destroyed;
- 0.15 kg of cells is created (extra sludge); and
- 0.09 kg of inorganic carbon is consumed.

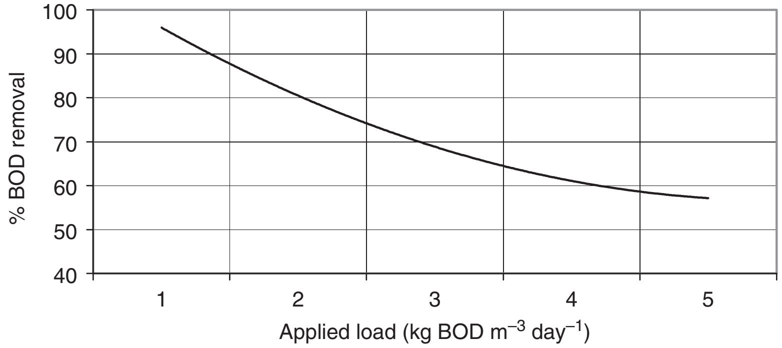

Figure 9 Distribution of wastewater over modular plastic media.

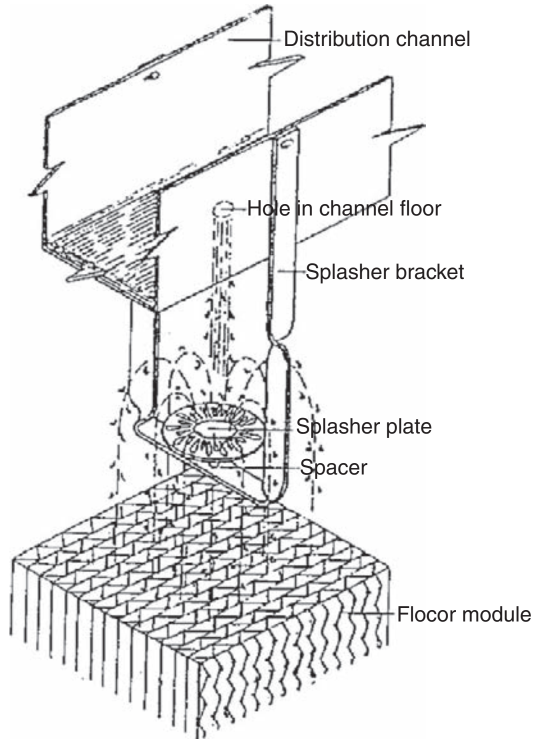

Figure 10 Biofiltration efficiency.

In the denitrification stage, the nitrate and nitrite are converted to nitrogen gas (N_2). For each kilogram of nitrate reduced

- 2.86 kg O_2 is recovered;
- 3.0 kg alkalinity as $CaCO_2$ is recovered; and
- ~0.4 kg of cells is created (extra sludge).

Biological phosphorus removal

Biological phosphorus removal is dependent mainly on the ability of the *Acinetobacter* spp. to release phosphorus under anaerobic conditions and to absorb it under aerobic conditions. The mechanism can be summarized as follows:

- under anaerobic conditions, readily degradable organic matter (BOD) is fermented to short-chain fatty acids (SCFA), which are stored in the cell as polyhydroxyl butyrates (PHB);
- under aerobic conditions, the stored PHB is oxidized and energy is released, allowing the assimilation of soluble orthophosphate; and
- the orthophosphate is metabolized by the cell and excess quantities are stored in the cell as polyphosphate. The storage of excess phosphorus is known as 'luxury uptake' of phosphorus, and it is this ability of the cell that is exploited in the biological phosphorus removal process.

A number of biological processes have been developed, many based on the activated sludge process, that are very effective in the biological removal of nitrogen and phosphorus from wastewater.

The A^{2}O® process

This continuous process (**Figure 11**), developed in the United States, is a refinement of the activated sludge process and takes advantage of the ability of denitrifying bacteria (abundant in the anoxic denitrification (DN) tanks) to convert the nitrate (which is recirculated from the nitrification (N) tanks) to nitrogen gas and phosphorus-accumulating bacteria in the anaerobic (AN) tanks to take up the available P. This process is capable of producing an effluent with $N_{TOT} < 10\,mg\,l^{-1}$ and $P_{TOT} < 1.0\,mg\,l^{-1}$.

The BIODENIPHO® process

This process (**Figure 12**), which was developed in Denmark, is based on the oxidation ditch configuration and utilizes the ability of a special culture of phosphorus-accumulating bacteria, which is cultivated in an anaerobic tank. This process is then incorporated into a nitrification–denitrification system. This process is capable of producing an effluent with $N_{TOT} < 10\,mg\,l^{-1}$ and $P_{TOT} < 1.0\,mg\,l^{-1}$.

The BIOSTYR® process

The BIOSTYR® process (**Figure 13**) was developed in France and is based on upflow filtration through

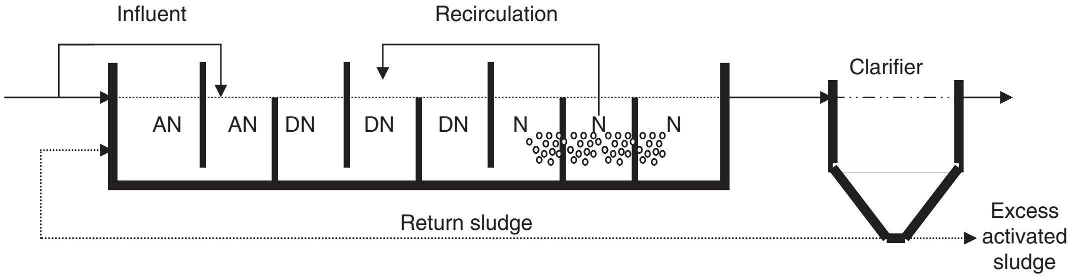

Figure 11 Biological N and P removal.

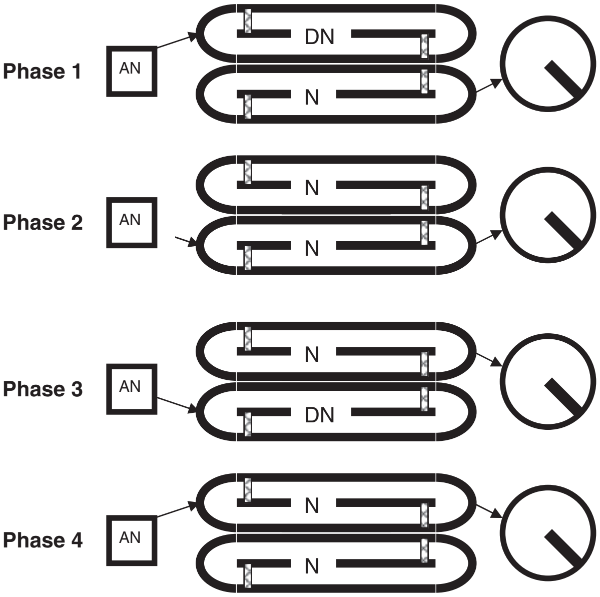

Figure 12 Phased oxidation ditch process for biological nitrogen and phosphorus removal.

submerged and floating fine granular media. Air can be injected at the base of the bed or into the media itself. In the latter case, the filter can simultaneously nitrify and denitrify. The BIOSTYR® process does not require separate clarification.

Special characteristics of the method are

- three processes in one tank, that is, no final settling tank;
- high treatment efficiency;
- compact, space-saving unit;
- can often be built into existing plants;
- low temperature dependency; and
- no odor nuisances, as the surface water is oxygen-saturated, treated water.

Anaerobic Processes

Anaerobic digestion is one of the many microbial processes that have existed since the earliest times for the recycling of organics within the natural environment. It occurs naturally in river and lake sediments, marshes, and so on, and although it has been used since the nineteenth century, it is only in recent times that the biochemistry and microbiology of the process have been understood.

The production of methane – for reuse – is the prime reason for using anaerobic processes in the treatment of dairy wastes. The anaerobic digestion of complex organic waste can be summarized as follows:

1. the breakdown of large molecules such as cellulose, proteins, and fats to simpler molecules such as sugars, alcohols, peptides, amino acids, and other products. This stage is accomplished largely by enzymes released by the microbes (bacteria, fungi, and protozoa);
2. the utilization of simpler molecules by the acid-forming bacteria to form volatile acids, carbon dioxide, and hydrogen; and
3. the production of methane from the products of the second stage by methane-generating bacteria.

The microbial population within an anaerobic digester comprises many different types of bacteria.

1. Non-methanogenic stage
   The non-methanogenic bacteria are obligate anaerobes and facultative bacteria. These bacteria feed on the primary substrate and produce a variety of organic acids and alcohols as end products, and are adaptable and could survive in the progressively decreasing pH.
2. Methanogenic stage
   The methanogenic bacteria are obligate anaerobes and include various methanobacterium, methanosacina, and methanobacilli. These use the acids and alcohols produced in the non-methanogenic stage as their growth substrate and produce a mixture of carbon dioxide and methane:

$$CH_3COOH = CH_4 + CO_2$$

If the process is balanced, the production of acids should be equal to the utilization of acids; however, the production of methane is the rate-limiting process. Methanogenic bacteria are very susceptible to pH

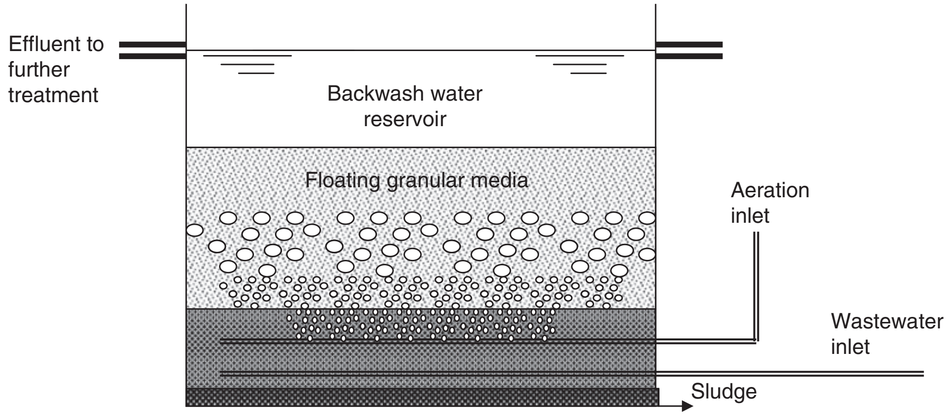

Figure 13 Upward flow submerged filter.

changes (6.8–7.8), so it is critical that the substrate load to the system be strictly controlled and that the system retains a significant buffering capacity.

Buffering

Buffering in an anaerobic process is provided largely by the presence of ammonium bicarbonate formed by anaerobiosis:

$$CO_2 + H_2O + NH_3 = NH_4HCO_3$$

If the waste being treated is mainly carbohydrate, with few proteins, it may be necessary to add some nitrogenous material.

Temperature

Anaerobic digestion generally takes place within two temperature ranges:

- mesophilic 30–37 °C and
- thermophilic 52–60 °C.

Although most anaerobic digestion plants operate in the mesophilic range, the thermophilic range offers the advantages of greater gas production, faster reaction rates, greater breakdown of organics, and, especially, pasteurization of product. The operation and control of thermophilic digesters is much more difficult. For this reason, thermophilic digestion is generally used only where the wastewater is already quite hot and the COD is very high (>5000 mg l^{-1}).

Clarification

An integral part of most biological treatment processes is the clarification of the wastewater to remove the sludge generated. In the activated sludge process, the sludge is generally returned to the aeration tank. Success in meeting the discharge standards is dependent on the efficiency of clarification, which, in turn, is dependent on the settleability of the sludge. In order to ensure that the sludge settles in the clarifier, conservative design parameters should be chosen:

surface overflow rate: 21–27 $m^3\ m^{-2}\ day^{-1}$
sidewall depth: >2.5 m
solids loading rate: 0.25–5 kg $m^{-2}\ h^{-1}$
sludge retention time: <1 h

Sludge Treatment

The on-site treatment of the biological sludge generally comprises a number of elements, some focused on solid–liquid separation and others on stabilization. The choice of the process is heavily influenced by the available disposal routes.

Solid–Liquid Separation

Solid–liquid separation usually comprises two distinct elements: thickening (see **Table 4**) and dewatering (which need not be sequential). Thickening is used to increase the solids content to 3–5% and generally removes the unbound water from the sludge. Dewatering is used to further increase the solids content. Filter belt pressing and centrifugation can increase the solids content to 18–25% (depending on sludge characteristics) and plate presses can increase it to ~40%, while drying can produce sludge granules with a moisture content of <10%.

Stabilization of Sludge

Stabilization of sludge is carried out to ensure that the sludge is suitable for long-term storage and ultimate

Table 4 Comparison of thickening processes

Method	*Advantages*	*Disadvantages*
Picket fence thickener	Simple to operate Low operating cost Low operator attention Minimal power consumption	Odor potential during stirring Thickened sludge concentration low (3–5%) High space requirements
Dissolved air flotation	Effective for excess activated sludge Will work without conditioning chemicals Relatively simple equipment components	Relatively high power consumption Thickened solids concentration limited Odor potential due to air-stripping effects Operator attention High space requirements
Centrifuge	Low space requirements Effective for excess activated sludge Minimum housekeeping and odor considerations High thickened sludge concentrations	Relatively high capital cost and power consumption Sophisticated maintenance requirements Best suited for continuous operation
Gravity belt thickener	Low space requirements Relatively low capital cost Relatively low power consumption requirements High thickened sludge concentrations and solids capture with minimum polymer consumption	Housekeeping Polymer dependent Moderate operator attention Odor potential Building corrosion potential, if enclosed
Rotating drum thickener	Low space requirements Low capital cost Relatively low power consumption High solids capture	Polymer dependent Sensitivity to polymer type Housekeeping Odor potential Moderate operator attention requirements

Table 5 Comparison of biological stabilization processes

Process	*Operating temperature °C*	*Retention period days*	*Comments*
Aerobic digestion	∼20	20–30	Can reduce organic content by up to 40%
ATAD (auto-thermal aerobic digestion)	45–60	<20	Kills most viruses and reduces organic matter by ∼50%
Mesophilic anaerobic digestion	∼36	10–18	Can reduce organic matter by up to 30% and produces about 1 Nm^3 of biogas for every kg of matter degraded
Thermophilic anaerobic digestion	∼55	<6	Can reduce organic matter by up to 30% and produces about 1 Nm^3 of biogas for every kg of matter degraded Process can be very unstable and difficult to operate
Composting	∼60	10–20 + maturation	Can be either in windrows with forced ventilation or regular mechanical turning, or in tanks with forced ventilation

Table 6 Comparison of chemical stabilization processes

Process	*Operating pH*	*Retention period*	*Comments*
Lime stabilization	∼12	Min 3–5 days Material normally stored for 20–30 days	Produces a product with a solids content in excess of 50% and highly desirable as an additive on lime-deficient soils
N-Viro	>11	3–5 days Material normally stored for 20+ days	Uses an alkaline admixture (cement kiln dust and quicklime). Produces a granular product with a solids content in excess of 50% and highly desirable as an additive on acidic soils
Chemfix	11.5–12.5	1–2 days	Produces a soil-like, odorless product with 50% dry solids

disposal without having adverse environmental or safety impacts. A well-stabilized sludge will be free of odors and pathogens, and will generally be free of biological activity. Stabilization can be achieved biologically (aerobic or anaerobic digestion, or composting), chemically (using alkaline admixtures), or thermally (drying) (see **Tables 5** and **6**). Pasteurization is normally required to eliminate pathogens.

See also: **Utilities and Effluent Treatment**: Dairy Plant Effluents.

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Reducing the Negative Impact of the Dairy Industry on the Environment

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Introduction

The dairy industry is important for providing a nutritious food source for millions of people and contributing to a major portion of the economical structure of many communities, states, and countries. Besides providing the livelihood of the family involved at the farm in the production of milk, there is about $2 generated elsewhere in the sector for each $1 earned on the farm and about 2.25 jobs generated elsewhere for every job on a dairy farm. Although the number of dairy farms has decreased (less than 2% of the population today produces food to feed the world), milk production in the United States and worldwide has been increasing. According to the UN Food and Agricultural Organization, the world milk production from cattle expanded by 1.2% from 2007 to 2008 (578 billion tonnes). India produces the most milk in the world, but in terms of total annual milk production from cattle, the United States ranks first, followed by India and China. From 1970 to 2009, the world population increased by about 1.7 times, and the population growth is expected to be about 1.8% per year. For a variety of reasons, the landscape of farms has changed significantly through the years. For example, the number of dairy cows in the United States has declined from about 12 million in 1970 to about 9 million in 2009. However, milk production has increased from 52.5 billion kg in 1970 to 85.8 billion kg in 2009. Thus, the production per cow has substantially increased during the same period from 4432 to 9353 kg $\text{cow}^{-1}\,\text{yr}^{-1}$, a 2.1-fold increase over a 40-year period. Although the total production and efficiency of milk production has increased, the increase in the world population, number of cows per farm, and farming methods and human population densities has necessitated the development of new best management practices (BMPs) and environmental policies for the production and processing sectors of the dairy industry.

Federal and local agencies in several countries, including the United States, closely regulate the environmental practices for dairy farms in order to protect the land, water resources, and air. Although the regulations may be different from country to country or from state to state, farmers employ a wide variety of BMPs to reduce the risk of environmental contamination by manure (animal manure, bedding, and wastewater) and to conserve the quantity of water used.

The manufacturing of fluid milk and other dairy products leads to the production of wastes. The various handling steps include product processing, storage, transportation, and distribution, with each generating waste that has a potential impact on the environment. More than 1000 dairy processing plants in the United States manufacture fluid milk, different types of cheese, butter, yogurt, ice cream, evaporated milk, condensed milk, nonfat dry milk, and other related products.

Approaches are being taken in each sector of the dairy industry to minimize the environmental impact of milk production, transport, and processing (**Figure 1**). This article focuses primarily on practices used to minimize this risk at the production and processing stages. However, it is also important to acknowledge that direct loading of tanker trucks on large farms and the use of ultrafiltration equipment to concentrate milk prior to shipment help to reduce the energy cost of transporting milk from the farm to the processor.

Farm Level

Manure

Manure consists of animal feces, urine, bedding, and any water coming in contact with these materials. The composition of manure varies depending on the type of bedding material used, types and amounts of feed used, and whether the manure is being mixed with parlor wastewater. Application of manure to land provides valuable nutrients for crop growth; however, it can be detrimental if it enters water resources.

One of the fundamental approaches to improving environmental compatibility at the farm level is by reducing the excretion of nitrogen (N) and phosphorus (P) in manure. This is done by improving the diet formulation of these nutrients to meet the animal's requirements for specific nutrients (i.e., amino acids and P). In addition, management practices and improved facilities for animal comfort have increased production per cow, resulting in greater efficiency of nutrient utilization. The increased milk yield of cows while reducing resource use aids in mitigating the dairy industry's environmental impact.

Adequate storage of manure is essential for facilitating land application at suitable times of the year, depending on weather conditions and cropping system used. At least

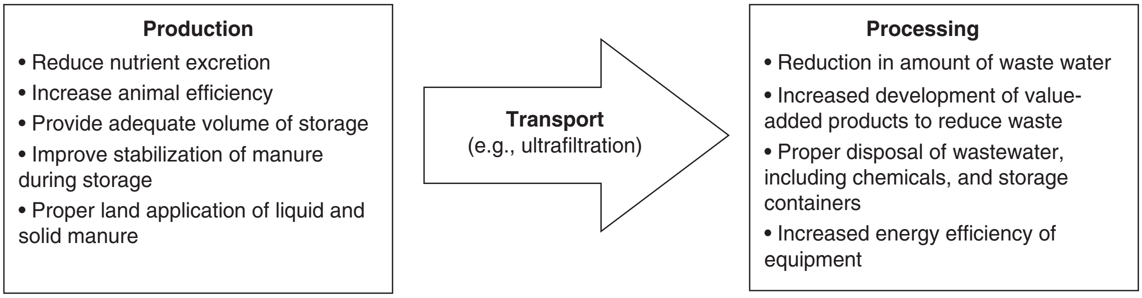
Production
• Reduce nutrient excretion
• Increase animal efficiency
• Provide adequate volume of storage
• Improve stabilization of manure during storage
• Proper land application of liquid and solid manure
Transport
(e.g., ultrafiltration)
Processing
• Reduction in amount of waste water
• Increased development of value-added products to reduce waste
• Proper disposal of wastewater, including chemicals, and storage containers
• Increased energy efficiency of equipment

Figure 1 Approaches to minimize the environmental impact of milk production, transport, and processing.

6 months of storage is needed, but at least 12 months is suggested. Farm manure is generally applied to croplands as a fertilizer, which reduces the dependence on commercial fertilizers. The manure provides nutrients for crops and improves soil quality if applied properly. Land application of manure, especially liquid manure, can cause problems if the land surface cannot absorb the manure or if a major rain event occurs soon after application. In addition, drainage tiles that are buried in fields should be plugged while liquid manure is applied in the event that manure leaches through the soil and enters the drainage tile. Adhering to established setbacks during application, using vegetative buffer zones near waterways, and not applying manure to frozen ground reduce the risk of runoff. In addition, injecting liquid manure and tillage after surface application reduce the risk of runoff, control odor emission, and prevent flies from populating the manure.

There are several techniques and treatments available to improve the utilization of manure and reduce the risk of manure contaminating the environment. Solid–liquid manure separation equipment allows for wastewater to be used in flushing feed alleys and for irrigating fields. The solids can be used as animal bedding (composted or air dried) and/or land applied. Other manure storage and treatment systems include lagoons, anaerobic digestion, activated sludge, and constructed wetlands. Lagoons must be constructed in such a manner that they have a very low permeability, or with certain highly permeable soil types an impermeable liner is needed. Lagoons may be constructed to be aerobic or anaerobic in function; a top-crusted, supplemental thatch (e.g., straw) or synthetic covering can help to reduce odors. In some countries, lagoon covers are required. Alternative farm waste treatment techniques, such as anaerobic digestion and activated sludge techniques, have been developed to recycle and reuse organic wastes more efficiently. The anaerobic fermentation of manure to produce methane allows farmers to produce enough electricity not only to run their farm operations but also to produce a surplus that can be sold to the local utility supplier. However, the cost of construction is quite high, and the price provided by utility companies is often low. The price for excess electricity is higher in some countries and areas within a country than other comparable areas. Constructed wetlands can improve water quality by intercepting the farm wastewater and retaining contaminants and nutrients through a series of vegetative ponds. New practices and innovative technologies to improve manure and odor management are being researched and incorporated in many dairy operations. Some dairy farms are using alternative natural energy sources (e.g., solar and wind). In addition, using plate coolers on dairy farms for heat exchange between the milk and water improves the quality of the milk because the milk is cooled more rapidly. Plate coolers reduce the energy required to operate the refrigerated bulk tank.

Milking Parlor Wastewater

Milking parlor wastewater contains cow feces, urine, chemicals, and wash water used in cleaning the milking units, pipelines, receiver, bulk tank, and the floor and wall surfaces. The volume of wastewater generated from cleaning the milking system can be up to 30% of the total farm wastewater. The wastewater from a toilet or employee comfort area in a milk house must, in most cases based on local and state health codes, be handled according to domestic sewage disposal regulations.

The parlor wastewater may be stored in a separate containment from manure or in the same vessel (e.g., lagoon). Land application is the most common method for discarding wastewater. Some pre-treatments being used prior to land application include facultative ponds, wetlands, anaerobic ponds, and fabricated reactors. Facultative ponds retain wastewater from 5 to 30 days. This retention time allows for some biological degradation, making the wastewater more suitable for land application. Anaerobic ponds utilize microbes to oxidize organic matter, especially to treat such wastes as oils and greases. Fabricated reactor systems, such as anaerobic reactors and sand filters, are also biological treatments for wastewater. A horizontal flow biofilm reactor can be effective for the removal of carbon and nitrogen from wastewater. The treatment process used for wastewater depends on the operating cost and the composition of the wastewater, for example, filtration for ammonium-nitrogen, potassium, and other dissolved solids, precipitation for phosphorus, and nitrification for nitrogen.

Environmental Impact of Dairy Processing

Processing Wastewater

Wastewater from food processing may contain acids or bases, organic chemicals that are toxic and cause depletion of dissolved oxygen, suspended solids, phosphorus, nitrogen, heavy metals (e.g., cadmium, chromium, copper, lead, mercury, nickel, and zinc), cyanide, oily materials, and volatile compounds. Effluents from dairy plants contain dissolved or suspended compounds, such as proteins, fats, sugars, residues of additives, and cleaning and sanitation chemicals. High concentrations of sodium, potassium, calcium, and chloride may also exist. Most of the water consumed in dairy processing plants is for the clean-in-place (CIP) operation, which is responsible for 50–90% of the overall volume of the waste stream. During milk processing, 0.2–11 l of wastewater may be generated per liter of processed milk. The water used for CIP contributes to the high pH (9–11) in wastewater purification stations. Wastewaters from the cleaning of equipment may contain milk or milk products, whey, brines, cleaning agents, and various acid and alkaline detergents, including caustic soda, nitric acid, phosphoric acid, and sodium hypochlorite. Due to the high concentration of dissolved solids ($\sim$1800 mg l^{-1}), dairy wastewater has a high biological oxygen demand (BOD) ($\sim$2000 mg l^{-1}).

Wastewater from dairy processing has been managed by direct land application, discharging wastewater to a nearby sewage treatment plant, removing semi-solids and special wastes through a waste disposal contractor, or operating an on-site wastewater treatment plant. However, most small dairy plants dispose of their wastewater by irrigation onto land, which causes a potential threat to the environment. Improper treatment and disposal may cause odors produced by biological decomposition of milk-derived organic matter.

Biological treatments (aerobic and anaerobic biological systems) are the most promising and cost-effective methods for the removal of organic compounds from dairy processing wastewater compared to other removal systems. Aerobic systems include conventional activated sludge processes, aerobic filter treatment, sequencing batch reactor treatment, rotating biological contactor treatment, lagoons, and the wetland system. Anaerobic biological systems include anaerobic digestion by conventional digester, anaerobic lagoon, completely stirred tank reactors, up-flow anaerobic filter, expanded bed, up-flow anaerobic sludge blanket reactor, fixed-bed digester, down-flow fixed-film digester, membrane anaerobic reactor system, and separated phase digesters. Specific separation methods that are widely used in the food processing industry have been incorporated into the purification, recycling, and treatment of wastewater. The most commonly used separation methods in the dairy industry are membrane technologies, such as ultrafiltration, nanofiltration, reverse osmosis, and microfiltration.

Whey

Whey is a liquid by-product generated from cheese manufacturing. It is composed of approximately 0.3% fat, 0.8% protein, 4.9% lactose, and 0.5% minerals. More than 145×10^6 tonnes of whey wastewater is produced per year worldwide; it is another potential source of environmental contamination. The major components mentioned above can be separated using advanced technologies to reduce the environmental risk of whey. However, approximately half of the world production of whey is not processed, and it is disposed of into waterways or loaded onto land. Whey has high BOD ($\sim$40–60 g l^{-1}) and chemical oxygen demand (COD) values ($\sim$50–80 g l^{-1}) due to the concentrations of lactose, proteins, and mineral salts.

Approximately 35% of the total production of liquid whey in the United States is converted to whey powder, whey protein for human food, and animal feed. An additional 10–15% of the total liquid whey is converted to other products, such as blends of whey with other protein sources (e.g., casein or soybean), lactose, and partially delactosed whey. The rest of the unprocessed whey is directly utilized for animal feed or mixed with wastewater. Serious waste volume is caused by the mixture of unprocessed whey and wastewater from cleaning and sanitizing in the cheese industry.

Whey can be converted through various technologies and processes to different products, such as condensed whey, dry whey, dry modified whey, whey protein concentrate, isolates, and dried lactose. However, separation technologies and storage of whey require high-energy processes that add to the cost and maintenance of cheese plants. Small cheese plants are usually not able to afford such equipment, so they may sell the whey to larger processors. Anaerobic systems are used to treat organic wastewater in the cheese industry, resulting in the production of methane (CH_4) gas. Ethanol can be produced by fermentation of lactose in whey. Ireland started producing fuel ethanol from whey in 1985, and New Zealand started using fuel ethanol produced from whey in August 2007.

Fats, Oil, and Grease

Milk, cheese, butter, whey separation, milk bottling, and plant machinery are the main sources of fats, oil, and grease. These materials in wastewater are a major concern in dairy industries when processing the wastewater. These materials may cause clogging of wastewater treatment systems, holding tanks, and septic tanks; cause sludge flotation; cause the formation of films on the surface of reactors; and result in malodorous wastewater.

Common pre-treatment systems for removing fats, oil, and grease are gravity traps, air flotation systems, or enzymatic hydrolysis. Gravity traps can remove fats, oil, and grease by trapping within a series of cells when wastewater flows through the cells. Despite their high efficiency, there are some disadvantages as the need for constant cleaning and monitoring to prevent variation in pH. Anaerobic digesters for oil and grease residues increase fat reduction rates in the range of 88–94%. Air flotation systems that apply air bubbles in wastewater at a pressure of 400–600 kPa allow for fat globules to attach to the air bubbles and become suspended. High-density solids are then settled at the bottom of the containment, whereas the bubbles are eliminated through the effluent, possibly improving the efficiency of fat removal by 35–60% in waste streams. Enzymatic hydrolysis, which hydrolyzes fat in dairy processing wastewaters by lipases, results in high COD removal efficiencies. A hydrolytic enzyme pretreatment system in grease traps can produce hydrolysis rates greater than 90% for lipid degradation in wastewater. Another alternative method that facilitates biodegradation of lipid concentrations is the use of surfactants. For example, the application of 500 mg l^{-1} of a surfactant derived from cactus to wastewater has been observed to improve the anaerobic biodegradability and reduce the COD level. Dairy industry wastewater pretreated with lipases in an up-flow anaerobic sludge blanket reactor may improve oil and grease removal efficiency by 90% of the initial value.

Energy

Operating dairy processing plants requires significant amounts of energy, which contributes to processing costs. The dairy industry uses about 80% of the energy for thermal processing to generate steam and hot water for process applications (e.g., pasteurization, evaporation, and milk drying) and cleaning purposes, and 20% for electricity to operate refrigeration, air compression, ventilation, and lighting. The combustion of wood, gas, fuel oil, diesel, or coal in turbines, boilers, compressors, and other engines to produce power and heat generates gas emission, such as carbon dioxide, nitrogen oxide, and carbon monoxide, which has a negative environmental impact.

The US Department of Energy estimates that 67.5% of the external energy is lost by electric power generation, transmission, and distribution, including piping for steam, hot water, chilled water, cooling water, and compressed air, steam condensate return piping, chilled water return piping, fuel piping, and wires for electric power transmission. Energy losses from boilers vary from 10 to 45%, and are mainly due to equipment aging, type of fuel, and lack of proper maintenance. The largest energy transmission losses are typically 20% in steam pipes. Equipment energy losses are typically 80% compressor, 35–45% pumps and fans, and 5–10% motors.

Improving energy efficiency in dairy processing plants reduces the risk to the environment and reduces reliance on natural resources. Therefore, improving energy efficiency also reduces energy use, operating costs, and greenhouse gas emission into the atmosphere. Many practices are currently being used to improve energy efficiency in the dairy processing sector, including solar or wind power; continuous pasteurizers instead of batch pasteurizers; multistage evaporators; insulating the refrigerated area; a centralizing CIP system; installation of more efficient heating, ventilation, and air-conditioning (HVAC) systems; or recovering and reusing waste heat. An example of heat recovery equipment in the dairy processing industry is a plate heat exchanger, commonly used for liquid–liquid heat exchange. Combined heat and power (CHP) system increases energy efficiency through on-site production of thermal energy (typically steam) and electricity from a single fuel source. Replacing or retrofitting existing equipment (e.g., lighting and HVAC) improves energy efficiency for on-site heat or power generation and distribution, and manufacturing processes. A CHP system, commonly operated by coal, natural gas, biomass, and fuel oil, increases energy efficiency 70–90% in providing electricity, hot water, and chilled water. Steam system improvement, such as replacing or relocating the steam systems, stabilizing steam pressure, or improving overall boiler efficiency, may save 10–20% in fuel cost per year.

Packaging

In 2005, dairy packaging demand in the United States was $3.5 billion, resulting from packaging of milk (30%), cheese (23%), frozen products (19%), cultured products (12%), and all other applications (16%). It is anticipated that packaging in the dairy industry will increase 4.1% annually from $2.7 billion in 2000 to $4.3 billion in 2010. The demand for pouches used in cheese, small containers of frozen dairy and cultured dairy products, and bottles with milk and drinkable yogurt is expected to grow.

The main packaging materials for dairy products are paper and paper-based products, glass, tin plate, aluminum foil, wood, plastic, and laminates. Most plastic milk bottles are made from high-density polyethylene, which is not biodegradable, taking about 500 years to decompose. Other plastic packaging materials are composed of polystyrene and polyvinyl. Milk containers made from laminated cardboard cartons coated with different layers of plastic or aluminum are extremely slow to biodegrade, and thus, this contributes to a negative impact on the environment. The degradation process of polymers depends on different environmental factors, such as sun rays, moisture and humidity, temperature, chemical compounds, bacteria, and mold. In 2008, the United States recycled about 1 billion kg of plastic bottles, a 27% increase since 1990. Current management of packaging wastes include recycling, combustion for energy recovery, and disposal through land filling.

New packaging materials and new processes are being developed in the dairy industry to reduce the environmental impact of dairy packaging materials. A modification of plastic's chemical structure by organic ketones can enhance its biodegradability. Biodegradable polylactic acid produced from renewable resources is a new packaging material developed to replace petroleum-based packaging films for cheese and yogurt. A new bottle system from molded recycled paper and low-density plastic liners has been developed to reduce waste and plastic usage. Manufacture of new bottles for fluid milk, juices, smoothies, and drinkable yogurt consumes about a third of the energy required to make a plastic bottle and has a 48% lower carbon footprint than plastic. Other new technologies include the use of an electron-beam emitter that sterilizes pouches without use of chemicals and water, and dry sterilization that is conducted with vaporized hydrogen peroxide, eliminating water consumption for sterilizing a lightweight polyethylene terephthalate bottle, additionally providing sustainability benefits by virtually eliminating the need for rinse water in this system.

Greenhouse Gas Emission

Global warming has been a focus for many years, with the focus especially on carbon dioxide (CO_2) and methane (CH_4) absorbing and trapping the heat in the atmosphere. Other gases of concern are nitrous oxide, chlorofluorocarbons, and hydrofluorocarbons, which are generated by the dairy processing industry. The contribution of reactive nitrogen to the atmosphere, primarily from ammonia but also from volatile organic compounds (VOCs; ozone concern), nitrous and nitrogen oxides are the focus of environmental regulators. In addition, the malodorous nature of farms, manure, and wastewater continues to generate many complaints from local residents.

The most notable odorous compounds detectable on dairy farms are ammonia, hydrogen sulfide and other sulfurous compounds, amines, organic acids, and heterocyclic nitrogenous compounds; however, with the exception of manure storage pits and covered silos, the maximum allowable concentrations are not normally found on or near the farm. Therefore, the most common concern with air quality in relation to animal agriculture is the nuisance of odors. Odor from manure on dairy farms can be minimized with proper installation of wind breaks and proper storage, handling, and application of manure to soils. Also, building positive relations with neighbors about the practices at the farm and informing them of major manure application events results in fewer complaints.

The US Department of Agriculture estimates that dairy waste output per billion kg of milk produced is 7.91 million kg nitrogen, 3.31 million kg phosphorus, 1.91 billion kg manure, 26.8 million kg methane, 230 000 kg nitrous oxygen, and 1.35 billion kg carbon dioxide, including CO_2 equivalents from CH_4 and N_2O. In dairy processing plants, gas emissions originate from the use of electricity (75%) and fuel (23%), and refrigerant leakage (2%). System improvements on steam and boiler systems reduce the equivalent of 616 tonnes of CO_2 per year. Optimization of compressed air reduces greenhouse gas emissions to the equivalent of 762 tonnes of CO_2 per year.

There are several ways to reduce greenhouse gas emissions. For example, optimizing processes with new technologies, better process control systems, and renewable energy sources can reduce CO_2 emissions. At the farm level, improvement in the efficiency of microbial fermentation in dairy cattle can lower CH_4 production, for example, feeding supplemental fat or an ionophore (e.g., Rumensin®, Elanco, Greenfield, Indiana, USA). Alternatives for stabilizing ammonia in manure continue to be investigated, including keeping pH low for a greater concentration of NH_4 versus NH_3. Manure can be converted into electricity through anaerobic digester technology, providing for a reduction in fossil fuel consumption and maximizing environmental benefits. This technology is currently being used by about 2% of the US dairy farms, but its use will likely increase in the future.

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V

VITAMINS

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General Introduction

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Historical Background

Vitamin deficiencies are as old as mankind; for example, as early as 3500 years ago people knew about treating night blindness by eating liver. In the centuries that followed, people learned to avoid numerous symptoms of diseases by choosing specific items of food without knowing anything about the vitamins they contained. However, by the end of the nineteenth century, feeding experiments with animals showed that specific deficiency symptoms could be caused by certain diets, which led to the hypothesis that organic micronutrients must be part of a healthy diet. Chemical identifications during investigations on beriberi (a disease caused by vitamin B_1 deficiency) disclosed that an amino group must be part of the unknown substance, and therefore, in 1912, Funk proposed the name 'vitamins' for these substances. This term is still in use today, although many of the known vitamins now do not contain an amino group.

By 1941, all the 13 known vitamins – vitamins A, D, E, K, C, and eight B-vitamins – had been described, and many scientists involved in the research were honored by a Nobel Prize for chemistry or medicine. It became more and more easy to understand vitamin deficiency-derived diseases and to treat these symptoms by optimal nutrition or supplementation. A number of recommendations were published in various countries, and recent investigations aim to understand the preventive effect of specific vitamins on a number of widespread 'modern' diseases like coronary heart disease, atherosclerosis, diabetes, and even cancer.

Today, vitamins are defined as organic compounds that cannot (or not in sufficient amounts) be synthesized by an organism but are indispensable for its life. Thus, vitamins have become essential parts of the diet, either as they are or as provitamins that can be converted to the respective vitamins (e.g., β-carotene = provitamin A). It must be mentioned that not all vitamins are vitamins for all species as per the above definition, owing to the fact that some vitamins can be synthesized by some species, whereas others cannot. For example, vitamin C cannot be synthesized by humans, monkeys, and guinea pigs; vitamins K and B_{12} cannot be synthesized by humans but they can be synthesized by microorganisms in the human intestine (though not in amounts sufficient for the respective person).

Although vitamins fulfill numerous functions, they are divided into only two groups, not based on their functions but based on their resorption, transport, storage, or excretion: fat-soluble vitamins (A, D, E, K) and water-soluble vitamins (C and B group) (for a brief overview, see **Tables 1** and **2**; details are given in the respective articles on each vitamin).

Sources of Vitamins

Most of the vitamins can be synthesized only by microorganisms or plants; hence, we have to consume them in fruits/vegetables or in animal nutrients (meat, fat, milk, eggs), the latter containing vitamins stored as such or as part of coenzymes. However, the animals in their turn have to obtain these vitamins either from vegetables or by resorption after synthesis by, for example, intestinal microorganisms in ruminants. The latter method is not a real possibility in humans, as in the colon – where most of the bacteria live – only vitamin K can be resorbed in detectable amounts and it is still under debate whether these amounts are adequate. Disturbing the colonic flora by long-lasting treatment with antibiotics leads to vitamin K deficiencies.

Table 1 A brief overview of fat-soluble vitamins, A, D, E, K

Vitamin	*RDA*	*Sources*	*Functions*
A (Retinol)	1 mg	Liver, milk, fish, egg yolk, fruits, vegetables	Vision, embryogenesis, cell proliferation and differentiation
D (Calciferol)	5 μg	Cod liver oil, milk, fish. About 90% are synthesized in the skin under UV light (sun!)	Ca^{2+} and P homeostasis, insulin release, inhibition of tumor cell growth, immune functions (?)
E (Tocopherol)	12 mg	Plant oil, germs, nuts	(Membrane-protective) antioxidants
K (Phyllochinon)	65 μg	Green vegetables, liver, wheat germ	Blood clotting

RDA, recommended dietary allowance.

Table 2 A brief overview of water-soluble vitamins

Vitamin	*RDA*	*Sources*	*Functions*
B_1 (Thiamin)	1–1.2 mg	Brewer's yeast, wheat germ, sunflower seed	Coenzyme for enzymes of the intermediary metabolism
B_2 (Riboflavin)	1.2–1.4 mg	Brewer's yeast, liver, almonds, dried whole milk, Camembert	Enzymes in the metabolism of, e.g., glucose, fatty acids, amino acids, drugs
B_3 (Niacin)	13–17 mg[a]	Wheat bran, liver, peanuts, salmon, halibut, Limburger, Brie, Camembert	NAD and NADP are coenzymes of dehydrogenases, and hydrogen and electron carriers
B_5 (Pantothenic) acid	6 mg	Liver, pea, soybean, lentils, dried whole milk, Blue cheese	Numerous functions as coenzyme A
B_6 (Pyridoxine)	1.2–1.6 mg	Soybean, salmon, liver, maize, Camembert	Coenzyme for transaminases
B_7 (Biotin)	30–60 μg	Brewer's yeast, liver, soybean, peanut, dried whole milk	Carboxylation and decarboxylation processes
B_9 (Folate)	400 μg	Brewer's yeast, liver, soybean, egg, Brie	Cofactor to carry one-carbon units
B_{12} Cobalamine	3 μg	Liver, kidney, mackerel, herring, Emmentaler, Camembert	Homocysteine metabolism
C (Ascorbic acid)	100 mg	Pepper, black currant, green cabbage, kiwi, oranges	Antioxidants, collagen synthesis

[a] 1 mg niacin-equivalent = 60 mg tryptophan.
RDA, recommended dietary allowance.

As we do not have any single food that contains all vitamins in optimal concentrations or in optimal relative proportions, we have to rely on a mixed healthy diet containing fruits, vegetables, cereals, dairy products, fish, and also meat to be on the safer side. For special risk groups (e.g., elderly, pregnant women, adolescents, competitive athletes; see below) supplementation of single vitamins might be inevitable. Good sources of the various vitamins and recommended daily allowances are given in **Tables 1** and **2** and in more detail in subsequent articles.

There are some reasons why vitamin deficiencies can occur even in developed countries; these include malnutrition, undernourishment, extreme diets, or destruction of vitamins while food preparation (e.g., cooking or heat-treatment of dairy products; storage under light). In addition, an insufficient intestinal absorption owing to chronic diarrhea, atrophic intestinal mucosa, or resections of the small intestine also leads to deficiencies, as does an enhanced need because of diseases accompanied by fever or cross-reactions with pharmaceuticals, alcohol, or nicotine, or an enhanced loss due to hemodialysis.

Risk Groups

Two of the major risk groups are pregnant women and breast-feeding women. Especially in the second half of pregnancy, large amounts of vitamins are transferred to the fetus via the placenta, in general independent of the vitamin status of the mother; that is, even under developing deficiencies in the mother, the fetus receives the vitamins. Unfortunately, the need for vitamins during pregnancy increases not in parallel with the enhanced energy needs (13%), but can reach levels of up to 50% (folate) or 58% (pyridoxine). In cases of pregnancies with twins or triplets, or short-interval births, the risk of deficiencies is even higher. During breast-feeding, for example, deficiency of vitamin A can occur, and the risk for the neonate is higher than that for the mother, especially in premature babies and babies lacking vitamins from the last weeks or months of pregnancy. In the case of sufficient maternal vitamin stores, no risk exists for the breast-fed suckling, except perhaps of vitamin K deficiency, which therefore is routinely supplemented during the first weeks of life. Strictly vegetarian mothers normally have very low vitamin B_{12} stores and produce almost vitamin B_{12}-free milk, which can lead to irreversible brain damage of the child.

Children and adolescents are other potential risk groups, due to an enhanced demand by the growing body or due to (additional) smoking or malnutrition (e.g., regular intake of fast foods). Also, alcohol and slimming diets can lead to vitamin deficiencies in adolescents as well as in adults.

Elderly people are also at risk of developing vitamin deficiencies due to a reduced food intake on account of reduced energy demand and changes in metabolism and lifestyle. Especially males who live alone have been shown to develop combined deficiencies, as they often use canned food and omit fresh vegetables or fruits.

Finally, ill people must be controlled regarding their vitamin status, but this depends on the kind and degree of their illness.

Prevention of Deficiencies

For healthy people in developed countries with an average workload and under European climatic conditions, a balanced and mixed diet is regarded as sufficient for supplying the necessary vitamins. However, nutrition organizations have tabulated (see **Tables 1 and 2**) the recommended daily allowances of vitamins, almost independent of their sources. Recently, it is being debated whether these recommendations are really sufficient or should be enhanced (and if so, by how much) for a better prevention of diseases like coronary heart disease, cancer, metabolic syndrome, and others. Hopefully we will find out from ongoing and future studies how we have to handle our vitamin stores and which recommendations, under which conditions, should be followed.

Fat-Soluble and Water-Soluble Vitamins

A brief overview of the most relevant data for the single fat-soluble vitamins and single water-soluble vitamins is given in **Tables 1** and **2**, respectively. Further details can be found in the respective articles dealing with each of the vitamins.

See also: **Vitamins:** Biotin (Vitamin B_7); Folates; Niacin; Pantothenic Acid; Riboflavin; Thiamine; Vitamin A; Vitamin B_6; Vitamin B_{12}; Vitamin D; Vitamin E; Vitamin K.

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Vitamin A

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Introduction

Vitamin A is an essential fat-soluble vitamin involved in multiple critical biological functions such as embryonic development, growth, vision, and regulation of gene expression. Because most mammals cannot carry out its synthesis *de novo*, vitamin A must be provided by the diet. In human nutrition, plant products (like carrots or spinach) provide provitamin A (i.e., provitamin carotenoids, like α-carotene, β-carotene or β-cryptoxanthin), which may, after specific cleavage, yield retinal and then retinol. By contrast, animal products (such as cod liver oil or liver, but also ruminant milk and dairy products) provide retinol or its esters directly, and also serve as provitamin A. In this way, dietary provitamin A carotenoids participate actively in the supply of vitamin A to the organism. Because vitamin A is absolutely necessary for embryonic development, it should be provided to the fetus through the placenta. And because vitamin A is also greatly involved in growth after delivery, it must be provided to the newborn through milk consumption.

In the past, studies on the status of β-carotene and vitamin A in bovine plasma and milk have focused on the objective of achieving suitable plasma levels during the peripartum period to maintain the cow's health, to prevent reproductive disorders, and to ensure an adequate vitamin supply to calves via colostrum. More recently, the role of carotenoids and fat-soluble vitamins in the nutritional and sensorial properties (via antioxidant activity or the yellow color of fat) of dairy products and their potential use as biomarkers for the traceability of cows' feeding management have led to a renewed interest at the INRA research center at Clermont–Ferrand. In order to better characterize how the feed can influence the appearance and nutritional properties of milk and other dairy products, new studies that follow variations in vitamin A, carotenoids, and color in cow's plasma and milk during lactation have been carried out.

General Features of Vitamin A and Carotenoids

Vitamin A or retinoids are generic compounds other than carotenoids, which exhibit biological activities like those of retinol, the main component of this family. The International Union of Pure and Applied Chemistry–International Union of Biochemistry (IU-PAC–IUB) defines retinoids as compounds containing a β-ionone ring, substituted with an alkyl chain made of four isoprenoid units ended by a functional group R. The nature of this end-substitution forms the basis of the classification of retinoids (**Figure 1**). Nowadays, because many chemical compounds not based on this structure have been shown to have a retinol-like activity, a new definition based on the ability of retinoids to bind and activate their nuclear receptors (retinoic acid receptors (RARs) and retinoid X receptors (RXRs)) has been proposed. According to this definition, the retinoid family includes all the natural or synthetic derivatives of retinol in terms of structure or biological functions.

It is generally recognized that vitamin A is essential for vision. Retinal acts as the chromophore of vertebrate visual pigment, and vitamin A deficiency results in the loss of structural integrity of photoreceptors, alteration of night vision, and finally blindness. In addition, vitamin A exerts pleiotropic effects on vertebrate development, vitamin A homeostasis, epithelial integrity, gene regulation, and cell differentiation. Recently, it has been shown that these effects are mediated largely through interactions of retinoic acid bound to specific nuclear receptors with retinoic acid response elements (RAREs). Two main classes of receptors were identified: the RARs, which bind both all-*trans* retinoic acid and its 9-*cis* isomer, and RXRs, which respond exclusively to 9-*cis* retinoic acid. Excess of vitamin A supply, dietary or supplementary, can result in embryogenetic defects and fetus malformation.

Vitamin A, or more precisely retinyl esters, is present in animal products. Generally, mammals producing milk

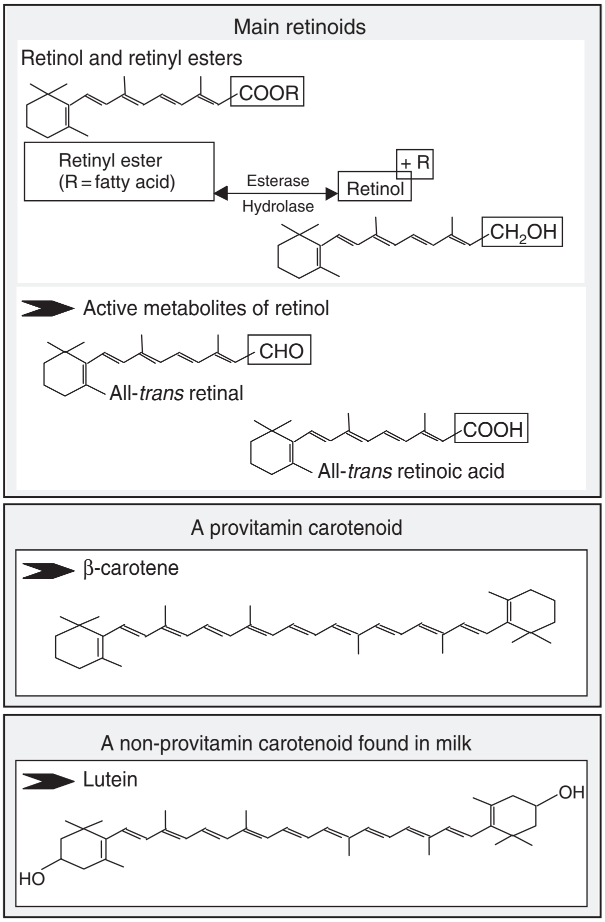

Figure 1 Retinoid family and examples of the main carotenoids found in milk (a provitamin A carotenoid: β-carotene; a non-provitamin A carotenoid: lutein).

consumed by humans (cow, goat, etc.) are usually herbivorous. They acquire vitamin A principally by the conversion of carotenoids from the diet into vitamin A. Preformed vitamin A is sometimes added, often together with other vitamins and minerals, as a supplement to the ration to meet the requirements of the animal and to improve the performance of lactating cows. Carotenoid is also a generic term used to describe plant pigments corresponding to the general formula $C_{40}H_{56}O_n$. In mammals, only 50–60 carotenoids, of the 700 discovered until now, exhibit a provitamin A activity. To be provitaminic A, these pigments should have at least one non-substituted β-ionone ring and an alkyl chain containing at least four conjugated double bonds. For example, among the carotenoids frequently found in cows' milk, lutein (because of its substitution on the two β-ionone rings) is not a provitamin A carotenoid, whereas β-carotene (the main carotenoid found in milk) exhibits the highest provitamin A activity (**Figures 1** and **3**). This conversion occurs mainly in the intestine and to a lesser extent in the liver, the *corpus luteum*, but may also occur in the mammary gland. The enzyme involved in this conversion, and identified as $\beta\beta$-carotene 15-15′ monooxygenase (E C 1.14.99.36), has been cloned in *Drosophila*, chicken, mice, and humans.

β-Carotene is an essential pigment of plants, associated primarily with chloroplasts and chromoplasts. Its primary function is to protect the plant against photooxidation and to contribute along with chlorophylls in collecting light energy. In vertebrates, β-carotene, and to a lesser extent, α-carotene and β-cryptoxanthin, can be converted to vitamin A mainly in the intestine and liver. Thus, β-carotene would contribute 40–60% of dietary vitamin A intake in humans and 100% in non-vitamin A-supplemented herbivores. Carotenoids have also been shown to have antioxidant activity through quenching of activated species of oxygen ($^1O_2$) and scavenging of free radicals. However, at a high concentration and under a high oxygen pressure, carotenoids can act as prooxidants. They might be active in the inhibition of neoplastic cellular transformation by inducing cell–cell communications. They have also been shown to stimulate the immune system.

In cattle, many studies have been focused on the role of β-carotene in reproduction, and the suggestion that it may be essential for normal reproduction in cattle is still a matter of debate. Moreover, studying the absorption and metabolism of β-carotene in cattle is particularly relevant because β-carotene is the main source of vitamin A in milk.

Metabolism of Vitamin A and Carotenoids in Dairy Cattle

Cattle differ from most farmed animals in that they have a significant concentration of circulating β-carotene in their blood. The concentration of β-carotene in blood is dependent on the concentration in feeds, and is particularly high in animals grazing fresh forage. A high blood β-carotene concentration results in yellow coloration of both body fat and milk, and this can influence consumer acceptance of the product.

Both β-carotene and vitamin A are fat-soluble compounds, and consequently, they will be distributed in the lipid globules in milk. When fresh forage is ingested by cattle, about 60% of plant cells are ruptured by chewing (the first step of digestion), releasing cellular contents. Then, the intracellular contents are processed first in the rumen where negligible loss of β-carotene (in contrast to vitamin A) occurs during fermentation. Probably, most of the carotenoids consumed will pass unchanged to the acidic conditions of the abomasum. Lipids and β-carotene are then emulsified with bile salts in the small intestine to form micelles, and maximum lipid absorption occurs when the pH reaches 6.5. After this step of solubilization,

β-carotene is absorbed by enterocytes, and transported with other lipids as β-carotene or linked to specific protein carriers (the retinol-binding proteins) as vitamin A. The efficiency of β-carotene absorption in the cow has not yet been published. However, considering the respective levels of its ingestion with the diet (0.1 to 3 g day^{-1}) and secretion *per se* in milk (1 to 10 mg day^{-1}), the transfer rate of β-carotene from diet to milk could be estimated to be 0.1 to 1.2%. However, this value is underestimated since part of the absorbed β-carotene is converted to retinol to meet the cow's requirements for vitamin A, the latter being stored in the liver of the cow, used by the peripheral tissues, or secreted in the milk (3 to 4 mg day^{-1}) (**Figure 2**). Lactating cows producing 10, 20, or 30 l milk day^{-1} are estimated to require, respectively, 300, 500, and 700 mg β-carotene day^{-1}. These quantities are based on a daily requirement of 100 mg day^{-1} for biological functions and a supplement of 20 mg l^{-1} of produced milk.

The mechanism by which β-carotene is metabolized to retinaldehyde in the intestinal mucosa, or to a lesser extent in the liver, is well defined in humans and rats. This process involves the enzyme called 15-15′-β-carotene monooxygenase. Two theories have been proposed to explain the formation of vitamin A (retinol or retinoic acid) from carotenoids: (1) the central cleavage theory and (2) the eccentric cleavage theory. The first theory is based on the fact that the addition of one molecule of oxygen at the central double bond of carotenoids can lead to the formation of two molecules of retinaldehyde, which are then processed into molecules of retinol or retinoic acid. The second theory has been developed by Krinsky and Russel, who found apocarotenals after incubation of carotenoids with intestine or liver homogenates of rabbit or ferret. Indeed, the system of conjugated double bonds contained in the carotenoid could lead to a resonance phenomenon in which the central double bond of carotenoids is more stable than the others. Consequently, the eccentric cleavage of carotenoids occurs, leading to the formation of only one molecule of retinaldehyde, which is then processed into only one molecule of retinol or retinoic acid. However, today, most authors favor the central cleavage as the most prevalent physiological mechanism. The eccentric oxidation might be an artifact explained by a chemical and nonspecific degradation (nonenzymatic degradation) of carotenoids (**Figure 3**).

Because retinoic acid, the active metabolite of vitamin A, is a potent regulator of gene expression, its precursor (i.e., retinol) concentration has to be regulated precisely in circulating blood to avoid any deleterious effect due to a high concentration of vitamin A. The main plasma form of vitamin A is retinol, which circulates bound to retinol-binding protein (RBP) and transthyretin (TTR).

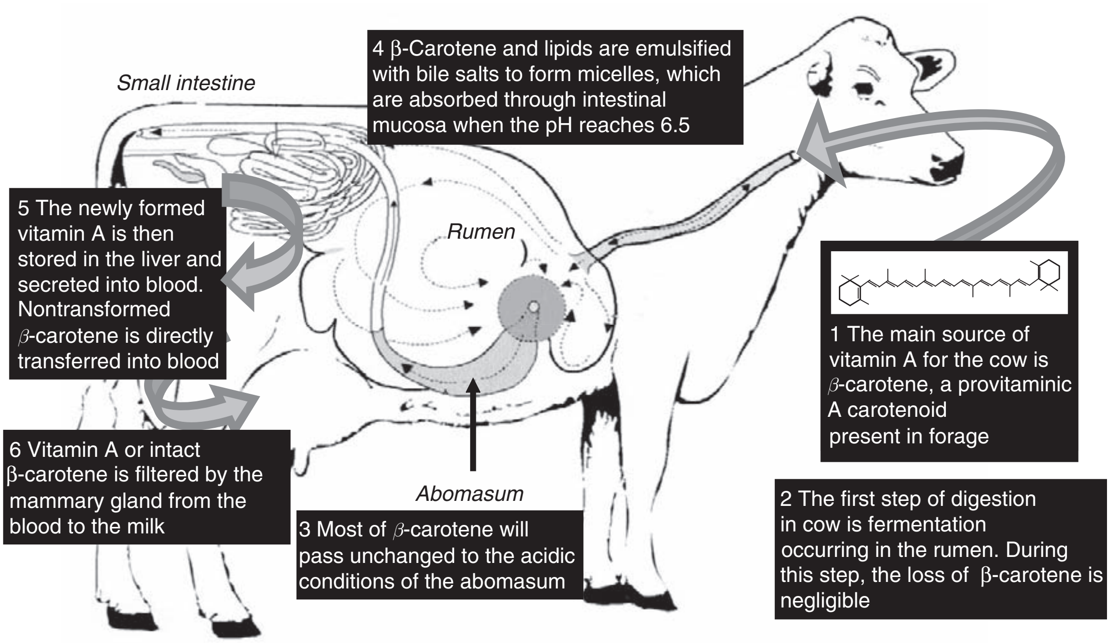
Small intestine
4 β-Carotene and lipids are emulsified with bile salts to form micelles, which are absorbed through intestinal mucosa when the pH reaches 6.5
5 The newly formed vitamin A is then stored in the liver and secreted into blood. Nontransformed β-carotene is directly transferred into blood
Rumen
1 The main source of vitamin A for the cow is β-carotene, a provitaminic A carotenoid present in forage
6 Vitamin A or intact β-carotene is filtered by the mammary gland from the blood to the milk
Abomasum
3 Most of β-carotene will pass unchanged to the acidic conditions of the abomasum
2 The first step of digestion in cow is fermentation occurring in the rumen. During this step, the loss of β-carotene is negligible

Figure 2 Metabolism of vitamin A and β-carotene in cow.

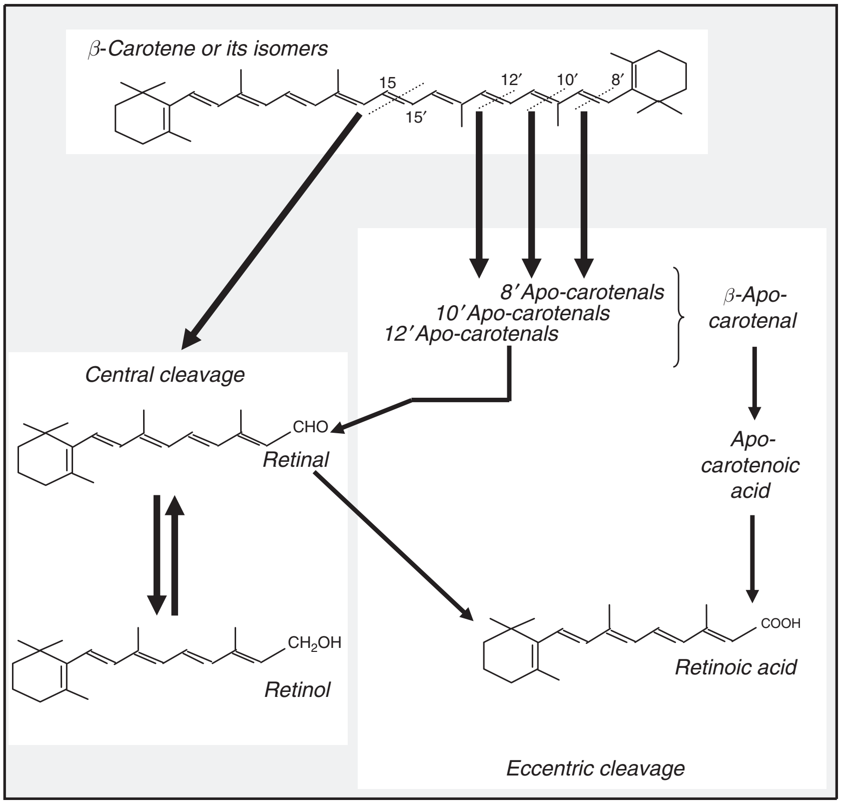

Figure 3 Different cleavage pathways for β-carotene.

Milk is produced by the mammary gland through filtration of blood. An increase in dietary α-tocopherol and β-carotene supply has been shown to lead to higher plasma concentrations of these nutrients in lactating cows but not of circulating retinol. However, in the milk of the same animals, α-tocopherol concentration increased accordingly, whereas β-carotene secretion seemed to saturate near $0.13\,\mathrm{mg\,ml^{-1}}$ and retinol content was stable ($0.19\,\mathrm{mg\,ml^{-1}}$). Elsewhere, the quantitative secretion of α-tocopherol and β-carotene from blood to milk has been shown to follow the Michaelis–Menten kinetics for active transport across membranes ($V_{\max}$ was, respectively, 32.4 and $2.5\,\mathrm{mg\,day^{-1}}$). These results suggest that the daily secretion of vitamin E and β-carotene is limited in quantity. Because retinol concentration is stable in plasma, retinol concentration in milk is also relatively stable.

Factors that Influence the Concentration of Vitamin A and β-Carotene in Milk

The composition of milk is a key factor in its economical value, as well as a relevant criterion in terms of nutrition. Factors that influence milk composition, especially β-carotene concentration, are the breed, the genetics and the age of the animal (mainly parity), the stage of lactation, and the composition of the diet. Channel Island cows, Jerseys and Guernseys, have a higher β-carotene and a lower vitamin A content in the milk fat than other breeds (Friesians). Genetic correlations between carotene yield and the production traits are positive. No significant differences have been observed between mastitic and non-mastitic cows for vitamin A and β-carotene levels in milk. The levels of fat-soluble vitamins (A, E, and β-carotene) in milk are also very much dependent on the amounts of these compounds consumed by the cow. The highest levels are normally found during spring and summer, when the cows are fed on fresh carotene-rich pasture. In forages, a gradient of β-carotene concentration is found from maize silage (1 to $4\,\mathrm{mg\,kg^{-1}}$ DM), hay (5 to $10\,\mathrm{mg\,kg^{-1}}$ DM), haylage (5 to $20\,\mathrm{mg\,kg^{-1}}$ DM), grass silage (25 to $100\,\mathrm{mg\,kg^{-1}}$ DM depending on direct cut or wilting intensity), and fresh grass (up to $400\,\mathrm{mg\,kg^{-1}}$ DM). In milk obtained under experimental conditions, the same trend was observed according to diet composition (from 1 to 2.5 and $2.8\,\mathrm{\mu g\,g^{-1}}$ fat for β-carotene and retinol, respectively, in milk from maize silage-fed cows, to more than $5\,\mathrm{\mu g\,g^{-1}}$ fat for both in milk from cows at pasture). However, under the real conditions at the farm, animal variability and the effect of the nature of forage are limited due to mixing of individual milks in the tank and to specific vitamin supplementations (A and E) through the feed concentrates. Such is not the case for β-carotene for which lower dietary intakes in winter result in a decrease of milk fat color.

Plasma carotene and fat color are generally correlated, with increases in plasma carotene being associated with increases in the concentration of carotene in the milk. In addition, plasma carotene level is well correlated with the amount of ingested carotene. So, β-carotene concentration in milk is usually quite low during the period when the cattle are kept in sheds, as compared with the grazing period. At the end of the grazing period (autumn), when the grass turns yellow, the concentration of carotenoids in forage decreases and, as a consequence, the level of β-carotene both in the blood serum and in milk decreases. Therefore, there is a clear seasonal effect on milk β-carotene content, essentially due to the β-carotene concentration in the pasture eaten by the cow. β-Carotene concentration in milk has been measured in very few studies (**Tables 1** and **2**).

Effects of Processing Conditions on Vitamin A and β-Carotene Content of Milk and Dairy Products

Milk or some of its components are raw materials for a great number of dairy products after various technological processes. These processes are, essentially, designed to improve the stability of milk.

To partially or totally eliminate the microorganisms found in raw milk, dairy industries use thermal treatment. Pasteurization (72 to 76 °C for 15 s) is a mild treatment, whereas ultra-high temperature (UHT) (130 to 140 °C for 3 to 20 s) treatment is more drastic. Under low oxygen tension, retinol is considered to be heat resistant. However, reports on retinol stability are sometimes inconsistent, which may be related to the analytical method used. Several studies in which total vitamin A and total carotenoid levels were measured after different thermal treatments showed that these levels are not affected by heat processing, especially because these treatments are carried out under a closed atmosphere (without removal of air). Nevertheless, using higher-resolution techniques, some authors have demonstrated that during heat treatment, isomerization of retinol may occur. Indeed, in raw milk, the main form of vitamin A is all-*trans* retinol, whereas the level of the 13-*cis* isomer is increased in heat-treated milk. This *cis–trans* isomerization can also be promoted directly by exposure to light, the relative amount of different *cis* isomers depending on the wavelength of the light and the light permeability of the container. A comparison among glass, plastic, and paperboard containers showed no significant loss of all-*trans* retinol in milk contained in paperboard boxes, while the loss was significantly lower in plastic containers than in glass. From a nutritional point of view, it is fundamental to evaluate the pattern of isomers produced in milk after heating or storage. Hence, isomerization of all-*trans* retinol reduces its vitamin activity: 13-*cis* retinol has the highest vitamin A biological activity (75%) relative to all-*trans* retinol (100%), while 9-*cis* retinol exhibits a biological activity as low as 19%. The other isomers have even lower vitamin A activity.

In order to stop the growth of microorganisms in milk, other thermal treatments, such as chilling and freezing,

Table 1 Influence of technical treatments and fat content on vitamin A and carotenoid concentrations in milk

		Retinol	*Carotenoids*	*Total vitamin A*	*Fat*
	Different types of cow's milk	*(μg per 100 g of milk)*	*(μg per 100 g of milk)*	*(μg RE per 100 g of milk)*	*(%)*
Influence of technical process	Consumer milk	28 (25–32)	17 (13–21)	31 (27–36)	3.57 (3.50–3.62)
	Ultra-high temperature-heated milk	30 (27–34)	18 (10–20)	33 (29–37)	3.78 (3.60–3.88)
	Sterilized milk	30 (27–34)	18 (10–20)	33 (29–54)	3.78 (3.60–3.88)
	Condensed milk	64 (61–72)	45 (37–51)	72 (67–81)	10.1 (10.0–10.3)
	Milk powder	230 (220–240)	140 (nd)	253 (243–263)	26.2 (24.6–26.8)
Influence of skimming	Cow's milk, reduced fat (1.5 and 1.8%)	13 (11–14)	8.0 (6.0–9.0)	14 (12–16)	1.6 (1.50–1.80)
	Skimmed milk	2.4 (1.8–3.0)	–	2.4 (1.8–3.0)	0.07 (0.02–0.12)
	Condensed skimmed milk	1.2 (nd)	0.9 (nd)	1.4 (nd)	0.2 (0.10–0.30)
	Dried skimmed milk	5.3 (nd)	21 (nd)	8.8 (nd)	0.97 (0.50–1.50)

Retinol equivalent (RE) is based on an old definition with RE = amount (retinol + (β-carotene/6) + (other carotenoids/12)).
Data are expressed as the average and in parentheses the range of variation is given.
nd, not determined.
Data are obtained from Souci SW, Fachmann W, and Kraut H (2000) *Food Composition and Nutrition Tables*, 6th edn. Stuttgart: Medpharm, Scientific Publishers; Boca Raton, FL: CRC Press.

Table 2 Influence of fat content on the concentrations of vitamin A and carotenoids in commonly consumed dairy products

	Different types of dairy products	*Retinol* *(μg per 100 g of products)*	*Carotenoids* *(μg per 100 g of products)*	*Total vitamin A* *(μg RE per 100 g of products)*
Influence of fat percentage in cheese	Camembert cheese (30% fat content in dry matter)	200 (140–250)	100 (80–150)	217 (153–275)
	Camembert cheese (40% fat content in dry matter)	300 (nd)	170 (130–230)	328 (322–338)
	Camembert cheese (45% fat content in dry matter)	330 (240–420)	190 (140–250)	362 (263–462)
	Camembert cheese (50% fat content in dry matter)	380 (280–480)	220 (160–290)	417 (307–528)
	Camembert cheese (60% fat content in dry matter)	503 (nd)	290 (nd)	552 (nd)
	Yogurt low fat (max 0.3% fat content)	0.8 (0.7–0.9)	0.5 (0.4–0.6)	0.8 (0.7–1.00)
	Yogurt reduced fat (max 1.8% fat content) (min 1.5% fat content)	13 (11–14)	8.0 (6.0–9.0)	14 (12–16)
	Yogurt (min 3.5% fat content)	29 (26–33)	18 (14–22)	32 (28–37)
	Butter (83.2% fat content)	590 (520–670)	380 (300–460)	653 (570–747)
	Butterfat (99.5% fat content)	850 (nd)	200 (nd)	883 (nd)

Retinol equivalent (RE) is based on an old definition with RE = amount (retinol + (β-carotene/6) + (other carotenoids/12)).
Data are expressed as the average and in parentheses the range of variation is given.
nd, not determined.
Data obtained from Souci SW, Fachmann W, and Kraut H (2000) *Food Composition and Nutrition Tables*, 6th edn. Stuttgart: Medpharm, Scientific Publishers; Boca Raton, FL: CRC Press.

may be used. These treatments do not modify vitamin A or carotenoid content. Because vitamin A and carotenoids are fat-soluble compounds, their concentration in milk is increased after elimination of water, by concentration (375 IU 100 g^{-1} of milk) or by dehydration (1150 IU 100 g^{-1} of milk). These differences observed in the concentration of vitamin A during these treatments are due mainly to the degree of concentration occurring during each treatment (×2.5 and ×8, respectively, for concentrated and dehydrated milk).

Fresh, dehydrated, and condensed milk represent the principal sector (~46%) of the world's trade in dairy products and the most consumed dairy products. In these products, the amounts of vitamin A and carotenoids are not modified when compared to those in whole milk. The other main sectors are cheese (~36%), butter (~11%), and ice cream, which represent only 4% of the world sales of milk. Because vitamin A and carotenoids are located exclusively in milk fat, their concentrations decrease proportionally with the degree of skimming of milk (see **Tables 1** and **2**).

For cheeses, recent data have shown that the rates of transfer of retinol and β-carotene from milk fat to cheese fat average 66 and 95%, respectively, considering four different cheesemaking technologies (three from cow's milk: Abondance, Tomme de Savoie, and Cantal; one from goat's milk: Rocamadour) and with the original milk covering a large range of concentration of these micronutrients. Cow's milk cheeses were found to be richer in β-carotene (3.27 mg kg^{-1} fat) and poorer in retinol (5.17 mg kg^{-1} fat) than goat's milk cheeses (0 and 6.81 mg kg^{-1} fat, respectively). Moreover, these values did not vary according to the cheesemaking process (acidification level, heating temperature, ripening time) in agreement with previous reports. Elsewhere, large retinol losses have been reported during heating and ripening of Gruyère cheese, which would result mainly from retinol instability due to exposure to light during cheesemaking technology (photoisomerization into 13-*cis* isomer).

Nutritional Issues

In cows, as in all mammals, vitamin A homeostasis in blood is very well regulated by the liver, avoiding any deleterious effects related to an excess of vitamin A. Inversely, the level of carotenoids in blood is strongly influenced by the nature of the diet. Because milk is the result of a specific synthesis of proteins (caseins, etc.), glucides (lactose, etc.), and lipids (short-chain fatty acids, etc.) and of blood transfusion for vitamin A and carotenoids, the vitamin A content of milk is quite stable, whereas β-carotene concentration (the main provitamin A carotenoid in milk) follows the concentration of β-carotene in the forage consumed by the cow. Because the concentration in forage is highly dependent on season and altitude, milk composition also varies according to these conditions.

Vitamin A and β-carotene are relatively stable during food processing and storage. The main factors that may influence the stability of these compounds are exposure to light and the skimming process. Because milk is stored in opaque containers, their levels are not changed significantly during storage. Because vitamin A and β-carotene are fat-soluble, their concentrations are closely related to the fat percentage, and are dependent largely on the skimming process. This consideration is nutritionally important, in view of the recommendation to reduce the consumption of a fat-rich diet in an attempt to reduce the risk of cardiovascular disease. For example, in France, most of the milk consumed is half-skimmed milk, which results in a reduction in the intake of fat-soluble vitamins.

When the reduction of fat intake in the whole diet remains limited, the consequent reduction in the level of fat-soluble vitamins generally has little nutritional outcomes, especially for vitamin A, which can be obtained from β-carotene from fruits and vegetables. However, some special diets, including the slimming diets, can have a critically low nutritional density and may create infraclinical nutritional deficiencies. This problem is real in some population categories, and restoration (addition of the vitamins unavoidably lost because of the technological process) or enrichment (addition of vitamins above the level contained in the raw materials) is a good means to cope with it. With or without restoration or enrichment, milk and dairy products are excellent sources of vitamin A, a vitamin involved in several key physiological functions in animals and humans.

See also: **Vitamins**: General Introduction.

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Vitamin D

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Introduction

Vitamin D is the generic descriptor for all steroids having similar biological activity as cholecalciferol or vitamin D_3. Important forms of vitamin D in this context are the following:

- ergocalciferol (vitamin D_2): is sparsely present in natural sources, but it is the major synthetic form of vitamin D;
- cholecalciferol (vitamin D_3): is widely distributed in animals, in which its provitamin D form, 7-dehydrocholesterol, is a normal metabolite, but in contrast it has an extremely limited distribution in plants;
- calcidiol (25-hydroxyvitamin D (25(OH)D)): is produced in the liver from D_2 or D_3 and circulating calcidiol is a good indicator of the vitamin D status in humans;
- calcitriol (1,25-dihydroxyvitamin D ($1,25(OH)_2D$)): is the result of further hydroxylation of calcidiol in the kidney; it is the most active form of vitamin D.

In human beings, the main source of vitamin D is cutaneous formation when the skin is exposed to adequate sunlight. Therefore, vitamin D might be considered a hormone and not a vitamin. Deficiencies of vitamin D lead to structural lesions of bone. The role of vitamin D in the increasing prevalence of osteoporosis and related fractures in elderly people has led to new research in recent decades. The discovery that most tissues and cells have a vitamin D receptor (VDR) and that several possess the enzymes necessary to convert the primary circulating form of vitamin D, 25(OH)D, to the active $1,25(OH)_2D$ has provided new insights into the vitamin. Of great interest is the role it can play in reducing the risk of many chronic illnesses. The results of studies have led to ongoing discussions about daily requirements of vitamin D.

Historical Perspective and Discovery of Vitamin D

Vitamin D is a unique vitamin and hormone the origin of which dates back about 750 million years when it was first produced in ocean-dwelling phytoplankton and zooplankton while these were being exposed to sunlight. It is likely that most plants and animals exposed to sunlight have the capacity to produce vitamin D. In the nineteenth century, Bennett (1812–75) of Edinburg recognized the nutritional value of cod liver oil in the treatment of rickets. In the same century, Mellanby showed in experiments with puppies that rickets is a nutritional disease which responded to a fat-soluble vitamin present in cod liver oil. Rickets is a bone disease characterized by deformities of the skeleton, shortened stature, and muscle weakness. The disease was first identified in children living in industrialized cities of northern Europe. The relationship between lack of sunshine and rickets was demonstrated by Sniadecki in Poland in 1822, and McCollum and associates showed that the antirachitic activity of cod liver oil was due to vitamin D rather than vitamin A.

In the early twentieth century, Steenbock and Black exposed a variety of foods such as wheat, lettuce, vegetable oils, and animal feed to ultraviolet (UV) radiation. They found that this radiation gave antirachitic activity to these foods. It was in the early 1930s that Askew's and Windaus's groups isolated and identified 'ergocalciferol' or vitamin D_2 by irradiation of ergosterol. A few years later, after a suggestion of Wadell, Windaus's group also isolated 'cholecalciferol' or vitamin D_3 by irradiation of 7-dehydrocholesterol. Once the structure of vitamin D was established in the 1960s and a simple process was developed for its synthesis, vitamin D could be used as a supplement and was added directly to milk in the United States. In Europe, other supplementation programs were developed for the prevention of rickets.

In the 1970s, intensive research on vitamin D revealed that vitamin D is a hormone rather than a vitamin.

Chemistry

Vitamin D is the generic descriptor for all steroids exhibiting qualitatively the biological activity of cholecalciferol. These compounds contain the intact 'A', 'C', and 'D' steroid rings being ultimately derived *in vivo* by photolysis of the 'B' ring of 7-dehydrocholesterol (see **Figure 1**).

These compounds have either of two types of isoprenoid side chains attached to the steroid nucleus at C-17 of the 'D' ring. Ergocalciferol and derivatives have one side chain containing nine carbons and a double bond. Cholecalciferol and derivatives have a side chain with eight carbons and no double bond. Cholecalciferol (D_3) and ergocalciferol (D_2) are white to yellowish powders and are insoluble in water, moderately soluble in fats, oils,

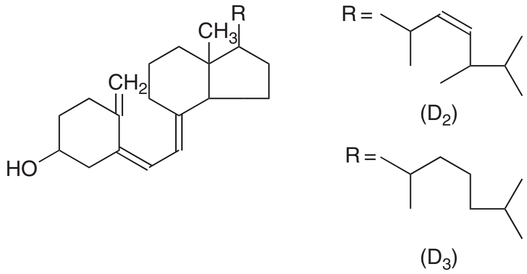

Figure 1 Structure of vitamin D (calciferol).

and ethanol, and freely soluble in acetone, ether, and petroleum ether. Each shows a strong UV absorption, with a maximum at 264 nm. Vitamin D is sensitive to oxygen, light, and iodine. Heating and mild acidity can convert it to an inactive form. Whereas the vitamin is stable in dry form, in organic solvents and most plant oils (due to the presence of α-tocopherol, which serves as a protective antioxidant), its thermal- and photolability can result in losses during such processes as saponification with refluxing. However, storage and processing of foods in general do not affect vitamin D activity.

Food Sources and Endogenous Synthesis

The main food sources of vitamin D are fish and fish products, many of which contain 5–15 μg per 100 g, and foods to which vitamins have been added. **Table 1** shows the main sources in Europe.

This list does not include dairy products, because the amount is only 0.7–1.0 $\mu g\, l^{-1}$ whole milk. Even a high-fat dairy product like butter does not contain more than 0.75 μg per 100 g. Also, human milk does not contain much vitamin D, at least 75% of which is available as calcidiol (vitamin D_2). The total activity in human milk is about 0.4–0.6 $\mu g\, l^{-1}$, which does not meet the daily requirement of infants; an extra supply is required either via exposure to sunlight or by vitamin D supplements.

Photosynthesis of pre-cholecalciferol occurs in the skin during exposure to sunlight. The high-energy UVB photons with wavelength between 290 and 320 nm penetrate into the skin, where they are absorbed by epidermal and dermal stores of 7-dehydrocholesterol (D_3). When the sky is clouded, exposure to this radiation is only 30% and via glass it is reduced to about zero. Only 2 h after pre-cholecalciferol is formed in the skin, it is converted to cholecalciferol. Once formed, cholecalciferol exits the skin into the dermal capillary bed where it is bound to a vitamin D-binding protein (DBP). In 1967, Loomis estimated that the pink cheeks of a European infant (area 20 cm^2) can synthesize daily about 10 μg of vitamin D if adequately exposed; this is sufficient to prevent rickets.

Absorption and Metabolism

Dietary vitamin D is predominantly in the D_3 form and is passively absorbed from the small intestine. The absorption is dependent upon micelle solublization and, hence, the presence of bile salts. Vitamin D enters the lymphatic circulation mainly with chylomicrons and is predominantly associated with the α-globulin fraction. The efficiency of this absorption process for vitamin D appears to be about 50%. After absorption, vitamin D is transported first to the liver and converted to 25-hydroxycalciferol (calcidiol), and then it is converted in the kidney to 1,25-dihydroxycalciferol (calcitriol), the metabolically active form of the vitamin.

Table 1 Important dietary sources of vitamin D

Source		*Vitamin D content*
Fish		The vitamin D content depends on where fish is caught
	Pike, perch	9–12 μg per portion (150 g)
	Salmon	20 μg per portion (150 g)
	Sardines, canned	2.2 μg per can (70 g fish)
	Tuna, canned	1.2–2.0 μg per can (70 g fish)
Wild mushrooms	Wild chanterelles	13 μg per 100 g
Meat products		The vitamin D content of meat products probably depends on the feed of the animals
	Liver	1.8–2.7 μg per 100 g
	Chicken	1.7 μg per 100 g
Eggs		1.4 μg per 110 g
Margarine		Vitamin D is added to margarine in most European countries
Milk	Whole	0.7–1.0 μg per 100 ml
	Low fat	0

1 μg vitamin D = 40 IU.

From European Commission (1999) Report on Osteoporosis in the European Community. Luxemburg: Directorate Employment: Social affairs and Health.

Vitamin D, like other steroids, is transported in the plasma in association with the protein called DBP. The appearance of vitamin D in the blood is short-lived, as it is either stored in the fat or metabolized in the liver. The half-life of 25(OH)D in the human circulation is approximately 10 days to 3 weeks. It has been suggested that the efficiency of endogenously produced vitamin D_3 is greater than that given orally because the former enters the circulation strictly via DBP, whereas the latter enters as complexes with DBP as well as in chylomicrons. This indicates that oral vitamin D remains longer in the liver and is therefore catabolized more quickly to excretory forms. Furthermore, cholecalciferol (D_3) is very photolabile and when it does not escape in the circulation after it is formed in sunlight, it will be converted efficiently into nontoxic substances.

DBP has a higher affinity for 25(OH)D than for $1,25(OH)_2D$, most likely because 25(OH)D is present in the circulation at concentrations about 1000 times higher than that of $1,25(OH)_2D$, and DBP binds 99% of it.

Metabolic Functions

The most clearly elucidated function of vitamin D is the maintenance of homeostasis of Ca^{2+} and phosphate in the extracellular fluid. Three organ systems are involved in the regulation: intestinal absorption; bone accretion; and mobilization and renal excretion. In these systems, control of the blood levels of Ca^{2+}, parathyroid hormone (PTH), and calcitonin (CT) is important (see **Figure 2**).

For example, when serum Ca^{2+} falls below the target level of $10\,mg\,dl^{-1}$, PTH is secreted by the parathyroid glands, which function to detect hypocalcemia. The kidney responds in two ways to increased PTH level: diuresis of phosphate and stimulation of 25(OH)D-1α-hydroxylase. The latter effect increases the production of

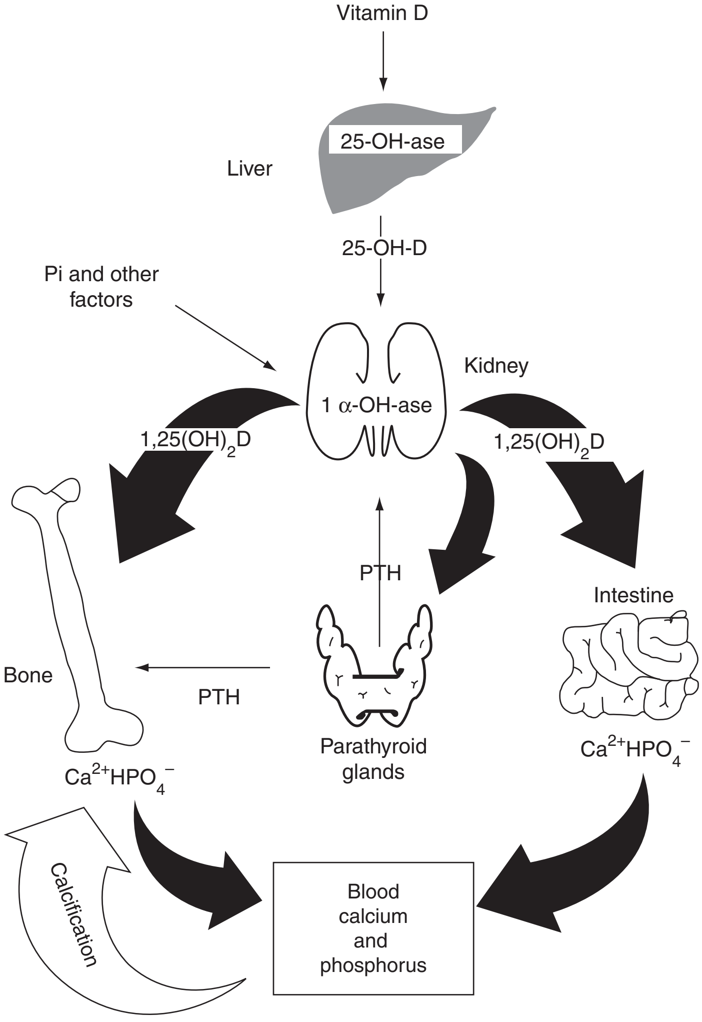

Figure 2 Metabolism of vitamin D and the biological actions of 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$). PTH, parathyroid hormone.

$1,25(OH)_2D$, which will stimulate the enteric absorption of both Ca^{2+} and phosphate. Also, calcitriol acts jointly with PTH in bone to promote the mobilization of Ca^{2+} and phosphate. The combined result of these responses is an increase in the concentration of Ca^{2+} and phosphate in the plasma. Clearly, in the long run, this will lead to demineralization of bone and consequently to an increased risk of fractures.

In addition to maintaining homeostasis of Ca^{2+} and phosphate in the extracellular fluid, $1,25(OH)_2D$ exerts its effects by binding to a specific nuclear receptor (VDR), a ligand-dependent transcription factor that belongs to the superfamily of steroid–thyroid hormone–retinoid nuclear receptors. This VDR when activated switches on the gene that induces synthesis of a calcium transport protein (calbindin) in the epithelium of the small intestine. Until now, the main effects of VDR have been found in the small intestinal epithelium and the cells in the bone, osteoblasts (that form new bone), and osteoclasts (that break down bone). However, VDR appears to be present not only in these target cells, but also in a range of tissues. This observation will lead to promising research on the functional role of vitamin D in areas such as type I diabetes, some cancers, autoimmune disease (e.g., multiple sclerosis), and infectious diseases (e.g., tuberculosis).

Reference Intakes

The Institute of Medicine of the US National Academy of Sciences has estimated adequate intakes of vitamin D for those with no sun-mediated synthesis in the skin. For those aged 0–50 years (including pregnant and lactating women), the adequate intake is $5\,\mu g\,day^{-1}$; for those 51–70 years of age, $10\,\mu g\,day^{-1}$; and for those over 70 years of age, $15\,\mu g\,day^{-1}$. The European Community has an ongoing project (EURRECA) to harmonize dietary reference intakes. First results show that the median vitamin D intake recommendations for the European population vary from $5\,\mu g\,day^{-1}$ for people aged 5–50 years to 10–15 μg for younger and older age groups and for pregnant and lactating women. But note that the reference intake is already up to 20 μg according to EURRECA. With a few exceptions, in most countries the median recommendations are the same for males and females.

Factors Affecting Vitamin D Requirements

Availability of Calcium

Vitamin D requirement is inversely related to the intake of calcium. Calcium is absorbed by active transport, which is vitamin D-dependent, and passive diffusion, which does not depend on the availability of vitamin D. When the intake of calcium is high, the percentage absorbed is low, and is mostly by passive absorption. This makes the requirement for vitamin D also lower. The vitamin D requirements mentioned are based on an adequate intake of calcium and other nutrients.

Dietary Fiber

Studies have shown that increasing dietary fiber in a daily diet from 20 to 40 g shortens the biological half-life of calcidiol by about 30% and therefore may increase the requirements for vitamin D by 40%.

Skin Pigmentation

Melanin skin pigmentation determines the color of the skin. Increased melanin pigmentation reduces the efficiency of sun-mediated photosynthesis of pre-cholecalciferol. This is particularly important for dark-colored immigrants who live in Northern latitudes and who ingest little dietary vitamin D.

Effect of Latitude, Season, and Time of the Day

Latitude, season, and time of the day have a dramatic effect on the cutaneous production of vitamin D_3. Above and below latitudes of approximately 40° N and 40° S, respectively, vitamin D_3 synthesis in the skin is absent during most of the 3–4 winter months. This also holds for the middle of the day, when in summer production of vitamin D via sunlight is highest. Far-Northern and far-Southern latitudes extend this period to 6 months.

Sunscreen Use, Clothing, and Glass

Sunscreens protect against the damaging effect of high-energy UVB radiation; however, it is this radiation that produces pre-cholecalciferol in the skin. It is therefore not surprising that studies show that young adults covered with a sunscreen with a sun protection factor of 8 or higher are unable to increase circulating vitamin D above baseline after exposure to simulated sunlight. Similarly, clothing absorbs most UV radiation and exposure of the skin to sunlight that has passed through windowpane glass or Plexiglas will not increase circulating calcidiol levels.

Aging

In adults over 65 years, there is a fourfold decrease in the production of vitamin D compared with younger adults aged 20–30 years. This is well documented. It is not known whether the absorption of physiological amounts of vitamin D is altered. However, after the age of 20 years, skin thickness decreases linearly with age. This thickening reduces the synthesis of vitamin D. Nevertheless, it has been shown that 5 min exposure daily to UV light to

$0.1\ \mathrm{m}^2$ (about hands and face) has the same effect as a daily supplement with 10 μg vitamin D. Clearly, important additional risk factors in elderly people are immobility and protection against sunlight. Since dietary intake for most people will not exceed $5\ \mu\mathrm{g\,day}^{-1}$, unless sufficiently fortified foods are used, elderly people above the age of 70 (and in some countries even earlier) are advised to use a vitamin D supplement.

Obesity

Obesity is associated with vitamin D insufficiency and secondary hyperparathyroidism. It has been demonstrated that in obese individuals (body mass index (BMI) $>30\ \mathrm{kg\,m}^{-2}$), bioavailability of cutaneously synthesized vitamin D_3 is reduced by >50%. Obesity-associated vitamin D insufficiency is most likely due to the deposition of vitamin D_3 from cutaneous and dietary sources in body fat compartments.

Malabsorption Disorders

Patients suffering from various intestinal malabsorption syndromes, such as steatorrhea, sprue, Whipple's disease, Crohn's disease, and severe liver failure, often suffer from vitamin D deficiency, due to their inability to absorb vitamin D via the intestine; they should receive vitamin D either via exposure to UV light or intravenously.

Vitamin D Deficiency and the Relationship with Chronic Disease

Two forms of bone disease, caused by inadequate mineralization or demineralization of the skeleton, may accompany vitamin D deficiency. Severe deficiency results in rickets in children and osteomalacia in adults. Rickets in children is characterized by widening at the end of the long bones, rachitic rosary, and deformations in the skeleton including frontal bossing and outward or inward deformities of the lower limbs causing bowed legs or knocked knees, respectively. Signs of osteomalacia are more generalized than those of rickets, for example, muscle weakness and bone tenderness, especially in the spine, shoulders, ribs, or pelvis. Patients with osteomalacia are at increased risk to fractures of all types, but particularly to those of the wrist and pelvis.

In addition, the secondary hyperparathyroidism associated with vitamin D deficiency enhances mobilization of calcium from the skeleton, resulting in osteoporotic bones. It is one of the causes of osteoporosis, the etiology of which is not fully understood but appears to involve impairment of vitamin D metabolism and/or function associated with decreasing estrogen levels.

Vitamin D deficiency causes muscle weakness. This weakness and the susceptibility to infections in rickets or osteomalacia may reflect roles for VDR in the muscles and the immune system.

Causes of a vitamin D-deficient state can be any alteration in the cutaneous production of vitamin D_3, absorption in the intestine, or the metabolism of vitamin D to its active form, $1{,}25(OH)_2D$. Furthermore, an alteration in the recognition of $1{,}25(OH)_2D$ by its receptor can also cause vitamin D deficiency, metabolic bone disease, and accompanying biochemical abnormalities. Biochemical characteristics of vitamin D deficiency are

- reduced plasma calcium and phosphate concentrations,
- an elevated level of serum PTH ($>30\ \mathrm{ng\,ml}^{-1}$) in conjunction with a low level of serum 25(OH)D,
- a serum level of 25(OH)D below $20\ \mathrm{ng\,ml}^{-1}$ or $50\ \mathrm{nmol\,l}^{-1}$,
- an elevated level of serum alkaline phosphate, and
- urinary excretion of bone collagen by-products and an increase in their level, including hydroxyproline, pyridinoline, deoxypyridinoline, and *N*-telopeptide.

There is increasing evidence that in addition to osteoporosis, vitamin D deficiency may be associated with other chronic diseases. For instance, in epidemiological surveys, colon, breast, and prostate cancers seem to be less prevalent in people living at sunny (lower) latitudes and so is multiple sclerosis. A likely explanation is that colon, breast, prostate, and other tissues express 25(OH)D-1α-hydroxylase and produce $1{,}25(OH)_2D$ locally to control genes that help to prevent cancer by keeping cellular proliferation and differentiation in check. It has been suggested that if a cell becomes malignant, $1{,}25(OH)_2D$ can induce apoptosis and prevent angiogenesis, thereby reducing the potential for the malignant cell to survive.

It has been shown in culture experiments that $1{,}25(OH)_2D$ modulates T and B lymphocytes. In Finland, a 20-year trial including treatment of children with vitamin D reduced the risk of developing type I diabetes. Furthermore, psoriasis has long been known to improve in the summer and is now treated with $1{,}25(OH)_2$ derivates. Currently, a relationship between vitamin D and cardiovascular diseases, mental health, lung function, and other autoimmune diseases has also been found. How these findings will affect vitamin D requirements is not yet known.

Hypervitaminosis D

Hypervitaminosis D is characterized by raised circulating 25(OH)D plasma levels up to more than $160\ \mathrm{ng\,ml}^{-1}$, accompanied by thirst, nausea, and anorexia. Hypervitaminosis D involves increased enteric absorption and bone resorption of calcium, producing hypercalcemia, with attendant decreases in PTH and glomerular filtration rate and, ultimately, loss of calcium homeostasis. The result is calcinosis, expressed in various organ systems including kidney, bone, central nervous system, and cardiovascular system.

The upper level of intake per day for adults set by Institute of Medicine of the US National Academy of Sciences is 50 μg and for infants this is 25 μg.

For most people, vitamin D intake from food and supplements is unlikely to exceed the tolerable upper intake levels. However, persons who are at the upper end of the ranges for both sources of intake, particularly those who use many supplements and those with high intakes of fish or fortified milk, may be at risk for vitamin D toxicity.

Under some conditions, people are more sensitive to vitamin D (e.g., sarcoidosis and a rare condition in infants with elfin facial appearance, Williams syndrome).

Vitamin D and Dairy Fortification

As part of a nutrition policy to combat vitamin D deficiency in infants and children, many countries have a vitamin D supplementation program. In some countries, one of the items of this program is fortification of dairy products. Since there is a growing number of elderly people at risk for insufficient vitamin D supply, this might be one of the options to solve this problem. However, since the margin between an adequate vitamin D supply and the upper tolerable level of intake is rather small for elderly people (15–50 $\mu g\,day^{-1}$), caution should be taken to avoid an overdose. In the United States and Canada, where fortification is a long-standing practice, studies have demonstrated that the amount of vitamin D added to milk is variable and does not amount to that stated on the label.

Concluding Remarks

Vitamin D is considered a hormone rather than a vitamin. Nevertheless, as a dietary component it can prevent deficiency diseases, in concert with other sources such as exposure to sunlight and supplements. Undiagnosed vitamin D deficiency is not uncommon and 25(OH)D is the barometer for vitamin D status. Serum 25(OH)D is not only a predictor of bone health, but, according to recent research, is also an independent predictor of risk for cancer and other chronic diseases. In the Western world particularly, elderly people are at risk of an insufficient vitamin D supply. Also immigrants and particularly pregnant and lactating women, with a colored skin and who have moved to countries at a greater distance from the equator, are at increased risk for vitamin D deficiency. In these countries, a sensible and reliable supplementation program including fortification of dairy products may contribute to the prevention of vitamin D-related diseases.

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Vitamin E

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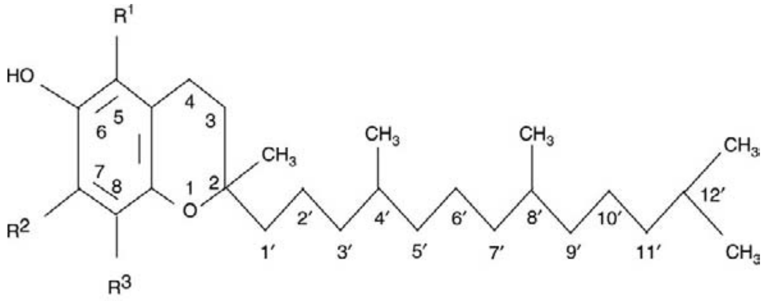
Introduction

In 1922, Evans and Bishop discovered a fat-soluble dietary constituent that was essential for the prevention of fetal death and sterility in rats fed a diet containing rancid lard. This was originally called 'factor X' or 'antisterility factor', but was later named vitamin E. Subsequently, the multiple nature of the vitamin began to appear when two compounds with vitamin E activity were isolated from wheat germ oil and characterized. These compounds were designated α- and β-tocopherol, derived from the Greek 'tokos' for childbirth, 'phorein' meaning to bring forth, and 'ol' for the alcohol portion of the molecule. Later, two additional tocopherols, γ- and δ-tocopherol, as well as four tocotrienols were isolated from edible plant oils. After the initial discovery, more than 40 years passed before it was proved that vitamin E deficiency could cause disease in humans. Vitamin E has been linked to several distinct clinical problems in humans, including hemolytic anemia in premature infants, reduced erythrocyte stability in patients with cystic fibrosis, and 'short bowel syndrome'. Other studies demonstrated that vitamin E plays an essential role in maintaining the integrity of neuromuscular systems and retina. Studies on children and adults with specific causes of fat malabsorption and patients with familial isolated vitamin E deficiency syndrome have conclusively shown that neurological dysfunction is associated with vitamin E deficiency and that vitamin E is an essential nutrient for the optimal development and maintenance of the integrity and function of the human nervous system. One of the more significant developments in the history of vitamin E was the identification that it is an effective lipid-soluble scavenger of lipid peroxyl radicals and, while present at very low concentrations, it is extremely efficient in protecting membranes against lipid peroxidation.

This article reviews the chemistry of the tocopherols and their dietary sources, absorption, transport, and storage mechanisms, and metabolic function. In addition, the potential role of dietary or supplemental tocopherol intake in the prevention of chronic diseases and possible mechanisms for the observed protective effects are discussed. Finally, a summary of the assessment of tocopherol status in humans, intake requirements, and an overview of the safety of high intakes is provided.

Chemistry

The chemistry of vitamin E is rather complex because there are eight structurally related forms – four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ) – that are produced at various levels and in different combinations by all plant tissues and in some cyanobacteria. All are amphipathic molecules with the general structures shown in **Figure 1**. The polar head group is derived from aromatic amino acid metabolism and the hydrophobic tail is derived from phytyl-diphosphate (phytyl-DP) or geranylgeranyl diphosphate (GGDP) in tocopherols and tocotrienols, respectively. α-Tocopherol is methylated at C-5, C-7, and C-8 on the chromonol ring, whereas the other homologues (β, γ, and δ) differ in the number and positions of the methyl groups on the ring (**Figure 1**). Tocopherols have a fully saturated 20-carbon phytyl side chain attached at C-2 and have three chiral centers that are in the R configuration at positions C-2, C-4^1, and C-8^1 in the naturally occurring form, which are given the prefix 2R, 4^1R, and 8^1R (designated RRR). They are more biologically active than their synthetic counterparts, which are mixtures of all eight possible stereoisomers and are given the prefix all-*rac*. Tocotrienols differ from the corresponding tocopherols in that the 20-carbon isoprenoid side chain at C-3^1, C-7^1, and C-11^1 is unsaturated and they possess one chiral center at C-2 in addition to two sites of geometric isomerism at C-3^1 and C-7^1. Natural tocotrienols have the 2R, 3^1-*trans*, 7^1-*trans* configuration. The phenolic hydroxyl group is critical for the antioxidant activity of vitamin E, as donation of hydrogen from this group stabilizes free radicals. The presence of at least one methyl group on the aromatic ring is also critical. The biological activity of vitamin E is defined in terms of α-tocopherol equivalents (α-TE) whenever possible. RRR-α-tocopherol has an activity of 1 mg α-tocopherol equivalents (α-TE mg^{-1} compound). The activities of RRR-β, RRR-γ, and RRR-δ-tocopherol are 0.5, 0.1, and 0.03, respectively. Synthetic all-*rac*-α-tocopheryl acetate has an activity of 0.74 mg α-TE mg^{-1}. Of the tocotrienols, only α-tocotrienol has significant biological activity (0.3 mg α-TE mg^{-1}). Lengthening or shortening of the side chain results in a progressive loss of vitamin E activity.



Compound	R^1	R^2	R^3
α-Tocopherol	CH_3	CH_3	CH_3
β-Tocopherol	CH_3	H	CH_3
γ-Tocopherol	H	CH_3	CH_3
δ-Tocopherol	H	H	CH_3

Figure 1 The four major forms of vitamin E (α-, β-, γ-, and δ-tocopherol) differ by the number and position of methyl groups on the chromanol ring. In α-tocopherol, the most biologically active form, the chromanol ring is fully methylated. In β- and γ-tocopherol, the ring contains two methyl groups, while in δ-tocopherol, it is methylated at one position. The corresponding tocotrienols have the same structural arrangement except for the presence of double bonds on the isoprenoid side chain at C-3^1, C-7^1, and C-11^1.

Dietary Sources

The composition and content of the different tocopherol components in plant tissue vary considerably, ranging from the extremely low levels found in potato tubers to the high levels found in oil seeds. α-Tocopherol is the predominant form in photosynthetic tissues and is localized mainly in plastids. The particular enrichment in the chloroplast membranes is probably related to the ability of tocopherols to quench or scavenge reactive oxygen species (ROS) and lipid peroxy radicals by physical or chemical means. In nonphotosynthetic tissues, γ-tocopherol frequently predominates and can be involved in the prevention of autoxidation of polyunsaturated fatty acids (PUFAs).

Most of the tocopherol content of wheat germ, sunflower, safflower, canola, and olive oils is in the form of α-tocopherol and these oils contain about 1700, 500, 350, 200, and 120 mg α-TE kg^{-1}, respectively. Vegetable oils such as of corn, cottonseed, palm, soybean, and sesame and nuts such as Brazil nuts, pecans, and peanuts are rich sources of γ-tocopherol. Corn and soybean oils contain 5–10 times as much γ-tocopherol as α-tocopherol-rich sources and each contains about 200 mg α-TE kg^{-1}. Because of the widespread use of these plant products, γ-tocopherol is considered to represent ~70% of the vitamin E consumed in the typical US diet. The level of vitamin E in nuts ranges from 7 mg α-TE kg^{-1} in coconuts to 450 mg α-TE kg^{-1} in almonds. Cereals are moderate sources of vitamin E, providing between 6 (barley) and 23 (rye) mg α-TE kg^{-1}. Fresh fruit and vegetables generally contain about 1–10 mg α-TE kg^{-1}. Different authors have reported concentrations of α-tocopherol between 0.2 and 0.7 mg l^{-1} in bovine milk; γ-tocopherol has also been found in addition to the presence of trace amounts of some other vitamers. Colostrum contains about 1.9 mg l^{-1} of α-tocopherol, and the level decreases in approximately 4 days to the level in fresh milk (0.3 mg l^{-1}). γ-Tocopherol is also present in small amounts. The concentration of vitamin E in milk appears to be dependent principally on the amount consumed by the cows. Even in the case of human milk, the concentration of vitamin E in colostrum is much higher than that in mature milk. Human colostrum contains 14.4 ± 2.3 mg α-TE l^{-1} compared to 3.1 ± 0.5 mg α-TE l^{-1} in mature milk. Ingestion of colostrum is therefore important to provide mammalian neonates with an adequate source of vitamin E to protect against oxidative stress and enhance the immune response. Following birth, colostrum intake induces a sharp increase in the circulating and tissue levels of vitamin E in the young.

Mean dietary intakes of 6.3–13.0 mg α-TE day^{-1} have been reported in various European and American population studies. Data from the Third National Health and Nutrition Examination Survey (NHANES III) (1988–94) in the United States indicate a median total intake (including supplements) of α-TE of 12.9 mg day^{-1} and a median intake from food only of 11.7 mg day^{-1} in men aged 31–50 years. In women in this age range, the median total intake (including supplements) of α-TE was 9.1 mg day^{-1} and the median intake from food only was 8.0 mg day^{-1}. In the United States, fats and oils used in spreads and other products contribute 20.2% of the total vitamin E intake; vegetables, 15.1%; meat, poultry, and fish, 12.6%; desserts, 9.9%; breakfast cereals, 9.3%; fruit 5.3%; bread and grain products, 5.3%; dairy products, 4.5%; and mixed main dishes, 4.0%.

The North/South Ireland Food Consumption Survey, published in 2001, reported that the median daily intake of vitamin E from all sources was 6.3 mg for men and 6.0 mg for women aged 18–64 years. The largest contributors of vitamin E to the diet were vegetables and vegetable dishes (18.9%) and potatoes and potato products (12.4%), most likely as a result of the oils used in composite dishes. Nutritional supplements contributed 5.5% of the vitamin E intake of men and 11.9% of women overall. In the subgroup that regularly consumed nutritional supplements (23% of total), vitamin E was the nutrient most frequently obtained in supplemental form by men (78%) and women (73%). In these people, supplements made a larger contribution to total vitamin E intake than did food.

Absorption, Metabolism, and Excretion

Following ingestion, fats are emulsified into smaller particles, first in the stomach and then in the small intestine, where they are mixed with pancreatic and biliary secretions. Pancreatic esterases convert triglycerides to monoglycerides and free fatty acids, which together with bile acids form micelles into which vitamin E and other hydrophobic molecules become solubilized. Vitamin E is absorbed in the proximal part of the small intestine, where transport across the brush border is thought to occur by passive diffusion. There are no selective differences in absorption between α- and γ-tocopherols, but β- and δ-tocopherols are absorbed poorly and are excreted in the feces. Together with triglycerides, phospholipids, and apolipoproteins, the tocopherols (α and γ) are reassembled to chylomicrons by the Golgi of the mucosal cells. The chylomicrons are stored as secretory granula and eventually excreted by exocytosis to the lymphatic compartment from which they reach the bloodstream via the ductus thoracicus.

The transfer of vitamin E from chylomicrons is regulated by complex mechanisms that control lipid and lipoprotein metabolism. Chylomicrons are degraded to remnants by lipoprotein lipase (LPL) and some α- and γ-tocopherols are transferred to peripheral tissues such as muscle, adipose tissue, skin, and brain by this enzyme-mediated mechanism. The resulting chylomicron remnants are then taken up by the liver, where most of the remaining α-tocopherol and only small amounts of γ-tocopherol are reincorporated into nascent very low-density lipoproteins (VLDLs) by a specific 32-kDa α-tocopherol transfer protein (α-TTP), which enables further distribution of α-tocopherol throughout the body.

Approximately 50% of the dietary intake of γ-tocopherol appears to be degraded in the liver by a cytochrome P450-dependent process and is then excreted primarily in the urine. Catabolism of α-tocopherol by this route occurs only when the daily intake of α-tocopherol exceeds 150 mg or plasma concentration of α-tocopherol is above a threshold of 30–40 μmol l^{-1}. Plasma or serum concentrations of α-tocopherol are typically around 20–35 μmol l^{-1}. γ-Tocopherol concentrations are approximately 5–15% of those of α-tocopherol and generally remain around 1 μmol l^{-1} even after supplementation. The highest concentrations of α-tocopherol in the body are in the adipose tissues and adrenal glands. Adipose tissues are also the major stores of the vitamin, followed by liver and skeletal muscle. The rate of uptake and turnover of α-tocopherol by different tissues varies greatly. Uptake is most rapid in the lungs, liver, spleen, kidney, and red blood cells (in rats $t_{1/2} < 15$ days) and slowest in the brain, adipose tissues, and spinal cord ($t_{1/2} < 30$ days). Likewise, depletion of α-tocopherol from plasma and liver during times of dietary deficiency is rapid, whereas adipose tissues, brain, spinal cord, and neural tissues are much more difficult to deplete.

A considerable, but variable proportion (typically 30–70%) of ingested vitamin E is unabsorbed and therefore excreted in the feces, making this the main route of elimination, and is influenced by experimental conditions and a variety of luminal and physiological factors. When large doses of vitamin E are administered, much of it is secreted in the bile, which may account for the relative safety of vitamin E compared to vitamins A and D. Both bile acids and pancreatic juices are important for the absorption of vitamin E. This has been established clearly in patients where secretion of either, or both, is severely diminished as in patients with cystic fibrosis or pancreatitis. The simultaneous intake of fat is necessary to stimulate bile flow and the secretion of pancreatic enzymes to allow micelle formation. However, the amount of fat necessary to ensure absorption may be small.

Vitamin E as an Antioxidant

Under normal physiological conditions, cellular systems are incessantly challenged by stressors arising from both internal and external sources. The most important potential stressors are reduced derivatives of oxygen, which are classified as ROS. ROS are toxic as they can oxidize biomolecules, leading to cell death and tissue injury, and are associated with the onset of a variety of chronic disease conditions in humans. ROS include the superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$, and oxygen-centered radicals of organic compounds (peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$)) together with other nonradical reactive compounds, such as hydrogen peroxide (H_2O_2). In addition, reactive nitrogen species (RNS) such as nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), peroxynitrite ($ONOO^-$), and hypochlorous acid are involved.

Cell systems have evolved a powerful and complex antioxidant defense system to limit inappropriate exposure to these stressors. Much of the work on antioxidant defense has been confined to studies on the chain-breaking antioxidants vitamins C and E and the carotenoids. α-Tocopherol is quantitatively the most important antioxidant in plasma and biological membranes. α-Tocopherol is an indispensable component of biological membranes and has membrane-stabilizing properties. The molecule is anchored in the highly hydrophobic hydrocarbon part of the membrane layer by the isoprenoid chain (**Figure 2**). The chromanol nucleus lies at the surface of lipoprotein or at the membrane–water interface, and it is the phenolic group that quenches free radicals ($X^{\cdot}$) (**Figure 2**). In this position, the chromanol ring has considerable mobility; it is able to quench peroxyl radicals that partition to the water–membrane interface and can be regenerated by harvesting the antioxidant capacity of other lipid-soluble antioxidants (e.g., ubiquinols) and water-soluble reductants, such as ascorbate and glutathione. The overall mechanism of lipid peroxidation and antioxidant protection in biological systems is outlined in **Figure 3**. Steps a and b show the abstraction of a labile hydrogen from a methylene group adjacent to the double bond of an unsaturated fatty acid (RH) by an oxidizing radical ($X^{\cdot}$) and subsequent oxygenation to form a peroxyl radical. Step c shows how the peroxyl radical moiety diffuses out of the autooxidizable, nonpolar interior region of the bilayer and into the non-autooxidizable polar membrane–water interface. Step d shows how the proximity to the interface of the anchored phenol on the chromanol ring allows for rapid formation of the lipid hydroperoxide (ROOH) and tocopheroxyl radical ($TO^{\cdot}$). The $TO^{\cdot}$ can be regenerated, thereby recycling tocopherol. The remarkable antioxidant properties of vitamin E may be explained by its ability to be efficiently re-reduced from its radical form to its native state by ascorbate and other intracellular reductants. The ascorbate radical can be regenerated and the potentially toxic lipid hydroperoxide can be cleaved, detoxified, and repaired.

The antioxidant activities of chain-breaking antioxidants are determined primarily by how rapidly they scavenge peroxyl radicals, thereby preventing the propagation of free radical reactions. When the chromanol phenolic group of α-tocopherol (TOH) encounters a peroxyl radical ($ROO^{\cdot}$), it forms hydroperoxide (ROOH), and in the process a tocopheroxyl radical ($TO^{\cdot}$) is formed:

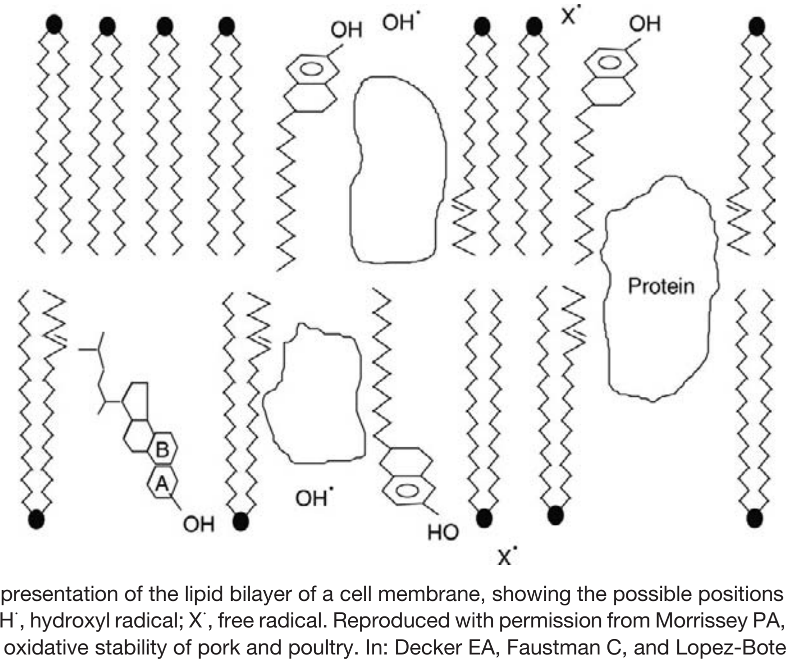


Figure 2 Schematic representation of the lipid bilayer of a cell membrane, showing the possible positions of the tocopherol and cholesterol molecules. $OH^{\cdot}$, hydroxyl radical; $X^{\cdot}$, free radical. Reproduced with permission from Morrissey PA, Buckley DJ, and Galvin K (2000) Vitamin E and the oxidative stability of pork and poultry. In: Decker EA, Faustman C, and Lopez-Bote CJ (eds.) *Antioxidants in Muscle Foods: Nutritional Strategies to Improve Quality*, pp. 263–287. New York: John Wiley.

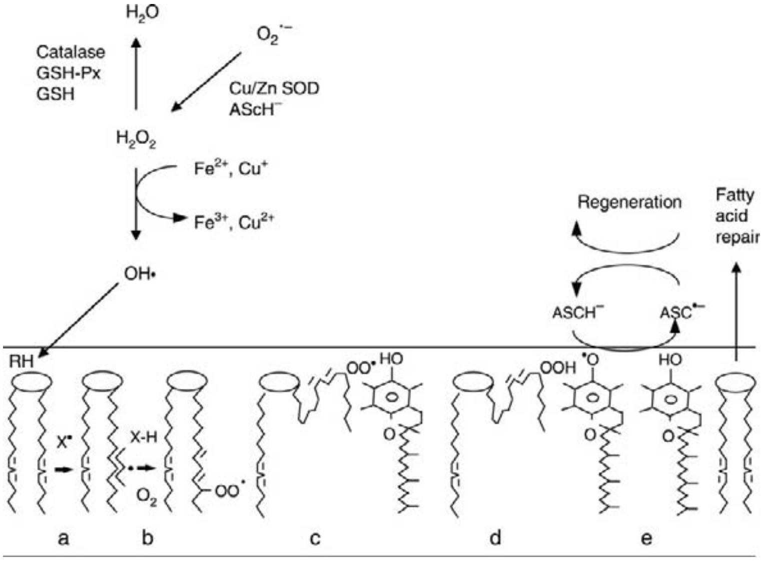


Figure 3 Membranal lipid oxidation. Details on a–e are explained in text. RH, unsaturated fatty acid; GSH-Px, glutathione peroxidase; GSH, glutathione; SOD, superoxide dismutase; $ASCH^-$, ascorbate monoanion; ASC^-, ascorbate free radical; X°, free radical. Reproduced with permission from Morrissey PA, Buckley DJ, and Galvin K (2000) Vitamin E and the oxidative stability of pork and poultry. In: Decker EA, Faustman C, and Lopez-Bote CJ (eds.) *Antioxidants in Muscle Foods: Nutritional Strategies to Improve Quality*, pp. 263–287. New York: John Wiley.

$$\mathrm{TOH} + \mathrm{ROO}^{\cdot} \xrightarrow{k_1} \mathrm{ROOH} + \mathrm{TO}^{\cdot}$$

The rate constant (k_1) for hydrogen abstraction from α-tocopherol is $2.35 \times 10^6\,\mathrm{mol\,l^{-1}\,s^{-1}}$, which is higher than that for the other tocopherols and related phenols. Because the rate constant (k_2) for the chain propagation reaction between $\mathrm{ROO}^{\cdot}$ and an unsaturated fatty acid (RH) ($\mathrm{ROO}^{\cdot} + \mathrm{RH} \rightarrow \mathrm{ROOH}$) is much lower than k_1, at approximately $10^2\,\mathrm{mol\,l^{-1}\,s^{-1}}$, α-tocopherol outcompetes the propagation reaction and scavenges the $\mathrm{ROO}^{\cdot}$ $\sim 10^4$ times faster than RH reacts with $\mathrm{ROO}^{\cdot}$. The concentration of α-tocopherol in biological membranes is approximately 1 mol per 1000–2000 mol phospholipids (i.e., $\sim$1:10^3). This effectively means that about 90% of $\mathrm{ROO}^{\cdot}$ are scavenged by tocopherol before they can attack another RH. Thus, the kinetic properties of antioxidants, and in particular α-tocopherol, require that only relatively low concentrations are required for them to be effective. Because γ-tocopherol lacks one of the electron-donating methyl groups on the chromonal ring, it is less hydrophobic and is somewhat less potent in donating electrons than α-tocopherol, and is, thus, a slightly less powerful antioxidant. However, the unsubstituted C-5 position on γ-tocopherol allows it to trap lipophilic electrophiles such as RNS. Excessive generation of RNS is associated with chronic inflammation in humans and animals.

Vitamin E Deficiency

Vitamin E deficiency is seen rarely in humans. However, there may be a risk of vitamin E deficiency in premature infants because the placenta does not transfer α-tocopherol to the fetus in adequate amounts. When it occurs in older children and adults, it is usually a result of lipoprotein deficiencies or a lipid malabsorption syndrome. Patients with abetalipoproteinemia or homozygous hypobetalipoproteinemia, those with cholestatic disease, and patients receiving total parenteral nutrition suffer from vitamin E deficiency. There is also an extremely rare disorder in which primary vitamin E deficiency occurs in the absence of lipid malabsorption. This disorder is a rare autosomal recessive neurodegenerative disease, and is caused by mutations in the gene for α-TTP. This disorder is known as ataxia with vitamin E deficiency (AVED). Patients with AVED have extraordinary low plasma vitamin E concentrations ($<5\,\mu\mathrm{g\,ml^{-1}}$) and have an onset between 4 and 18 years, with progressive development of peripheral neuropathy, spinocerebellar ataxia, dysarthria, absence of deep tendon reflexes, and vibratory and proprioceptive sensory loss. Therapeutic and prophylactic vitamin E supplementation (up to $2000\,\mathrm{mg\,day^{-1}}$) prevents the onset of the disease before irreversible neurological damage develops.

Vitamin E and Low-Density Lipoprotein Modification

It is generally accepted that low-density lipoprotein (LDL) undergoes oxidation *in vivo* when challenged by a variety of ROS and RNS and that oxidized LDL is the component central to the initiation and/or progression of atherogenesis at the molecular and cellular level. The typical LDL particle is not only rich in cholesterol, but also contains approximately 1300 molecules of RH, which are very sensitive to oxidation. Vitamin E, mainly as α-tocopherol, is quantitatively the most important lipophilic antioxidant present in LDL particles. On average, each LDL particle is protected by ~10 mol of α-tocopherol (range 3–15 mol), 1 mol of γ-tocopherol, and smaller amounts of carotenoids.

All major cells of the artery wall, such as monocytes–macrophages, endothelial cells, and smooth muscle cells, can modify LDL oxidatively *in vitro*. Monocytes have been shown to induce peroxidation of lipids such as those in LDL by the generation of reactive species, including superoxide anion, hydrogen peroxide, and hydroxyl radicals. Other oxidants have been implicated, including 15-lipoxygenase, myeloperoxidase-generated hypochlorous acid, and RNS such as peroxynitrite. *In vivo*, oxidized LDL particles are recognized by macrophage scavenger receptors and taken up by macrophages, forming lipid-laden foam cells in the fatty streak lesions. The free radical oxidation of LDL results in numerous structural changes that all depend on a common event, that is, the peroxidation of PUFAs in the LDL particle.

In vitro studies have indicated that increasing the vitamin E content of LDL particles increases LDL resistance to oxidation and decreases their uptake by macrophages. A number of research groups have concluded that vitamin E is the most important variable that determines oxidative resistance of LDL. This mechanism of protecting LDL may be significant when γ-tocopherol constitutes a major portion of vitamin E intake, as in the United States. It is worth noting that the ability of γ-tocopherol to attenuate oxidative damage produced by RNS may prevent or delay the progression of other diseases, in which inflammation plays a role, such as cancer, rheumatoid arthritis, inflammatory bowel disease, and neurodegenerative disorders, as well as cardiovascular disease (CVD).

Vitamin E and Other Metabolic Function

Vitamin E, in addition to having a protective role in the oxidative modification of LDL, may affect or limit the progression of atherosclerosis and a number of other conditions in ways that are unrelated to its antioxidant activity, as outlined below:

1. Vitamin E acts as a negative regulator of smooth muscle cell proliferation via modulation of protein kinase C activity and by activating protein phosphatase 2A. Protein kinase C is an important element in the signal transduction cascade mediated by growth factors such as platelet-derived growth factors, which are necessary for the progression and completion of the cell proliferation cycle.
2. Vitamin E may stabilize the atherosclerotic plaque and prevent its rupture and subsequent clot formation. This could be an important contributor to the prevention of ischemic heart disease, because plaque types that are most subject to rupture present the greatest threat.
3. Vitamin E reduces the expression of adhesion molecules that can cause neutrophils to stick to the endothelial cells lining the artery, leading to platelet aggregation.
4. Vitamin E may inhibit vitamin K-dependent clotting factors by the action of vitamin E quinone.
5. Vitamin E supplementation reduces the level of thromboxane A_2 *in vivo*, supporting the role of vitamin E as an antithrombic agent.
6. Vitamin E reduces the uptake of oxidized LDL by endothelial cells, preserves the barrier function, increases the activity of cytosolic phospholipase A_2 and cyclooxygenase, and augments the release of prostaglandin I_2 by endothelial cells. Thus, vitamin E may act both directly, by affecting the uptake of oxidized LDL by endothelial cells, and indirectly, by modulation of oxidized LDL formation and activity.
7. Vitamin E regulates mitochondrial production of ROS, changes the redox state and/or redox controlling factors, and in turn modulates redox-sensitive signaling pathways and transcriptional factors (e.g., activator protein 1, nuclear factor κB, mitogen-activated protein). High levels of ROS are likely to increase the expression of a number of 'atherogenic' genes and signal transduction pathways leading to inflammation and vascular dysfunction. At low levels of ROS, 'atheroprotective' genes are expressed, anti-inflammatory factors are produced, and the vascular system is protected. Thus, by regulating the production of ROS, vitamin E modulates the expression and activation of signal transduction pathways and other redox-sensitive biological modifiers, and thereby may prevent or delay the onset of degenerative diseases.

Vitamin E and Cardiovascular Disease

The effects of dietary vitamin E have been examined in several studies, and many have reported a clear association between reduction in the relative risk of CVD and high intakes of vitamin E from foods or supplements of vitamin E, although some have shown no such association. The Vitamin Substudy of the WHO/MONICA Project showed that in European populations whose classical risk factors for CVD were very similar, the sevenfold differences in CVD mortality could be explained at least to about 60% by differences in the plasma level of vitamin E and up to 90% by the combination of vitamins E, A, and C. The Edinburgh Case Control Study and Basel Prospective Study consistently revealed an increased risk of ischemic heart disease and stroke for low plasma level of vitamin E.

However, other European population studies have not found an association between blood levels of vitamin E and end points of CVD. In the European Community Multicentre Study on Antioxidants, Myocardial Infarction and cancer of the Breast (EURAMIC) study, the adipose levels of vitamin E did not correlate with the relative risk of myocardial infarction.

A number of prospective studies examined the association between vitamin E intake and risk of CVD. The Nurses' Health Study, conducted on 87 245 women, showed a 34% reduction in CVD in women who had consumed vitamin E supplements containing more than 67 mg α-TE daily for more than 2 years. However, there was no significant effect of vitamin E obtained from food sources. The Established Populations for Epidemiologic Studies of the Elderly (EPESE) trials showed that the use of vitamin E supplements significantly reduced the risk of all-cause mortality and mortality from heart disease. Another prospective study, performed in Canada, reported a consistent inverse association between CVD and vitamin E supplement usage. The Health Professionals Study, conducted on 39 910 men aged 40–75 years, also showed that dietary intakes of vitamin E were not significantly correlated with reduced risk of CVD or death. A protective effect was seen in those who took 67–160 mg supplemental α-TE daily for more than 2 years. In contrast, the Iowa Women's Health Study reported that dietary vitamin E (mainly γ-tocopherol) was inversely associated with the risk of death from CVD. This association was particularly striking in the subgroup of women who did not consume vitamin supplements. There was little evidence that the intake of vitamin E from supplements (mainly α-tocopherol) was associated with a reduced risk of death from CVD. The reasons for the differences between dietary and supplemental vitamin E are not clear. Other studies point to the potential importance of γ-tocopherol in preventing heart disease. High dietary intake of nuts, an excellent source of γ-tocopherol, lowered serum cholesterol, improved plasma lipid profiles, and was inversely associated with the risk of death from heart disease. γ-Tocopherol may also function in nitric oxide formation, which suppresses the expression of pro-inflammatory cytokines and maintains the integrity of the arterial wall. Several other human clinical trials have shown an improvement in markers of atherosclerosis by vitamin E supplementation. These findings have been contradicted by several vitamin E supplementation trials. It is important to note that, in general, women develop fewer cardiovascular events than do men. Thus, in studies in which many women are enrolled, the low incidence of CVD may weaken the statistical power of the overall trial. However, overall evidence from cell culture, as well as animal and human clinical and observational studies, strongly supports the contribution of dietary vitamin E in the maintenance of vascular function and health, in particular when used in combination with other foods containing antioxidants.

Vitamin E and Cancer

The capacity of vitamin E, particularly α-tocopherol, to quench free radicals damage, induce apoptosis, and impact expression of oncogenes makes it a strong candidate for chemotherapeutic strategies. Clinical and epidemiological data, together with evidence from experimental models, support a role for the involvement of free radicals throughout the cancer process. Several studies of oral, pharyngeal, and cervical cancer found a relationship between vitamin E status and cancer risk. The evidence for stomach and pancreatic cancers has not been consistent, and no association with breast cancer has been found.

Evidence for the role of vitamin E in cancer prevention was derived from a study conducted in Linxian, China, in a population with persistently low micronutrient intakes and having one of the world's highest incidences of esophageal/stomach cancer, where an overall reduction in cancer mortality, particularly mortality from stomach and esophageal cancer, was observed. The efficacy of supplementation with α-tocopherol on the prevention of certain cancers in male smokers was investigated in the Finnish ATBC study. There was no decrease in the incidence of lung cancer among men supplemented with synthetic α-tocopherol compared to those who were not fed supplements. In contrast, prostate cancer incidence and mortality rates were reduced by 32 and 41%, respectively, among the vitamin E-supplemented group. The overall results of epidemiological studies relating to vitamin E and colon cancer have been inconsistent and mixed.

Recent epidemiological experiments and mechanistic evidence suggest that γ-tocopherol may be a more potent cancer chemopreventive agent than α-tocopherol. A nested case–control study examined the association of α-tocopherol, γ-tocopherol, and selenium with the incidence of prostate cancer. The most striking finding was that men in the highest quintile of plasma γ-tocopherol concentrations had a five-fold reduction in the risk of prostate cancer compared with those in the lowest quintile. Other studies have shown that only the plasma levels of γ-tocopherol served as a biomarker of CVD and cancer.

In summary, inverse associations between dietary and supplemental vitamin E intakes and the incidence of several common chronic diseases have been noted in many observational studies, whereas results from studies using blood concentration of vitamin E have been limited and inconsistent. Randomized trials using supplemental vitamin E have not shown substantial effects on mortality end points. Recently, a prospective cohort study of 29 092 Finnish male smokers, aged 50–69 years, who participated in the ATBC study was carried out where the prospective association between circulating concentrations of α-tocopherol and total and cause-specific mortality in the group was evaluated. Higher circulating concentrations of α-tocopherol within the normal range were associated with significantly lower total and cause-specific mortality in older male smokers. The lower total and cause-specific mortality rates in older male smokers were observed as the serum α-tocopherol values increased from 9.1 mg l^{-1} (21 μmol l^{-1}) up to ~13–14 mg l^{-1} (~30– 33 μmol l^{-1}), after which no further benefit was noted.

Vitamin E and Other Diseases

Vitamin E appears to act as an immunosuppressant due to its ability to suppress both humoral and cellular immune responses. Tocopherol supplementation significantly enhances lymphocyte proliferation, interleukin-2 production, and delayed-type hypersensitivity skin response and decreases prostaglandin E_2 production by inhibiting cyclooxygenase activity. There appears to be compelling evidence that intervention with dietary antioxidants, such as vitamin E, may help maintain the well-preserved immune function of 'very healthy' elderly, restore the age-related decrease in immune function, and reduce the risk of several age-associated chronic diseases. Epidemiological evidence suggests an association between the incidence of cataract and vitamin E status. In a prospective study, the sum of serum α- and γ-tocopherol, but neither tocopherol alone, was inversely associated with the incidence of age-related nuclear cataracts.

Among the most common neurological diseases are neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, which may be caused by oxidative stress and mitochondrial dysfunction leading to progressive neural death. An increasing number of studies show that antioxidants (vitamin E and polyphenols) can block neuronal death *in vitro*. In a 2-year, double-blind, placebo-controlled, randomized trial in patients with moderately severe impairment from Alzheimer's disease, treatment with 1340 mg α-TE day^{-1} significantly slowed the progression of the disease. Clinical treatment of Alzheimer's patients with large doses of vitamin E (670 mg α-TE twice daily) is one of the key therapeutic guidelines published by the American Academy of Neurology. In a multicenter, double-blind trial, vitamin E (1340 mg α-TE day^{-1}) was not beneficial in slowing functional decline or ameliorating the clinical features of Parkinson's disease. Administration of vitamin E significantly relieved symptoms in patients suffering from several types of acute or chronic inflammatory conditions such as acute arthritis, rheumatoid arthritis, and osteoarthritis.

Vitamin E Status and Requirements

Interest in the role of vitamin E in disease prevention has encouraged the search for reliable indices of vitamin E status. Most studies on human subjects make use of static biomarkers of status, usually α-tocopherol concentrations in plasma, serum, erythrocytes, lymphocytes, platelets, lipoproteins, adipose tissues, buccal mucosal cells, and LDL, and the α-tocopherol:γ-tocopherol ratio in serum or plasma. Other markers of vitamin E status include susceptibility of erythrocyte or plasma LDL to oxidation, breath hydrocarbon exhalation, and the concentration of α-tocopherol quinone in the cerebrospinal fluid. There is no consensus as to the threshold concentration of plasma or serum α-tocopherol at which a person can be defined as having inadequate tocopherol status, but values of <11.6, 11.6–16.2, and >16.2 μmol l^{-1} are normally regarded as indicating a deficient, low, and acceptable vitamin E status, respectively. It is now recommended that plasma or serum α-tocopherol concentrations be lipid-corrected (i.e., expressed relative to either the sum of cholesterol and triacylglycerol or cholesterol alone). The α-tocopherol (μmol):cholesterol (mmol) ratio is the simplest to obtain and probably the most useful, with values <2.25 indicating a risk or deficiency and values >5.2 optimal values. It has been estimated that an average daily dietary intake of 15–30 mg of α-tocopherol would be required to maintain this plasma level, an amount that could be obtained from dietary sources if a concerted effort was made to eat foods rich in vitamin E.

The US Institute of Medicine set an estimated average requirement (EAR) of 12 mg of α-tocopherol for adults >19 years on the criterion of vitamin E intakes that were sufficient to prevent hydrogen peroxide-induced hemolysis in men. The same value was set for men and women on the basis that although body weight is smaller on average for women than men, fat mass as a percentage of body weight is higher on average for women. As information is not available on the standard deviation of the requirement for vitamin E, the recommended dietary allowance (RDA) was established for men and women as the EAR (12 mg) plus twice the coefficient of variation (assumed to be 10%), rounded up, giving a value of 15 mg day^{-1}. The RDA values set by the US Institute of Medicine are estimates that meet the requirements of practically all healthy people and the values differ by life stage (**Table 1**). In the case of lactation, the average vitamin E secreted in human milk is calculated at 4 mg day^{-1} of α-tocopherol. For this reason, 4 mg α-tocopherol is added to the RDA for non-lactating women, giving an RDA for lactation of 19 mg day^{-1} of α-tocopherol (**Table 1**). In the case of infants aged 0–12 months, the recommended intakes of vitamin E are based on an adequate intake (AI) that reflects a calculated mean

Table 1 Recommended dietary allowance

Age (years)	*RDA*
Children	
1–3	6
4–8	7
Boys & Girls	
9–13	11
14–18	15
Men & Women	
19–70	15
>70	15
Lactation	19

RDA, recommended dietary allowance.
Institute of Medicine (2000) *Dietary Reference Intakes of Vitamin C, Vitamin E, Selenium and Carotenoids*. Washington, DC: National Academic Press.

vitamin E intake of infants fed principally with human milk. In Europe, the Scientific Committee for Food did not set a population reference intake (PRI) for vitamin E on the basis that there is no evidence for deficiency from low intakes, and the frequency of distribution of intakes is skewed to the right, making it difficult to set a PRI that is not inappropriately high, especially for those with a low consumption of PUFAs, whose requirements is lower than those with a high consumption of PUFAs.

It has been suggested that the optimum concentration of α-tocopherol in plasma for protection against CVD and cancer is $>30\,\mu\text{mol}\,\text{l}^{-1}$, given normal plasma lipid levels and in conjunction with a plasma vitamin C concentration $>50\,\mu\text{mol}\,\text{l}^{-1}$ and a β-carotene level $>0.4\,\mu\text{mol}\,\text{l}^{-1}$. This has not been proven in large-scale human intervention trials, but even in the absence of conclusive evidence for a prophylactic effect of vitamin E on the prevention of chronic diseases, some experts believe that a recommendation of a daily intake of 87–100 mg of α-tocopherol is justifiable based on the current evidence. Realistically, these levels can be achieved only by using nutritional supplements. The tolerable upper intake level (UL) for vitamin E is $1000\,\text{mg}\,\text{day}^{-1}$, based on studies showing hemorrhagic toxicity in rats, in the absence of human dose–response data. The Scientific Committee for Food proposed that the intake should not exceed $2000\,\text{mg}\ \alpha\text{-TE}\,\text{day}^{-1}$.

See also: **Milk Lipids**: Lipid Oxidation; Nutritional Significance. **Vitamins**: Vitamin C.

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Vitamin K

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Introduction

Vitamin K (the coagulation vitamin) was discovered in the 1930s as a result of investigations into the cause of an excessive bleeding disorder in chickens fed on a fat-free diet. Its isolation and structural determination were accomplished in 1939 and its metabolic function was defined only after a new amino acid, γ-carboxylglutamic acid, was discovered in bovine prothrombin in 1974. Vitamin K is essential for the blood clotting process, where it serves as an essential cofactor for the specific carboxylation of a number of vitamin K-dependent coagulation proteins.

Chemistry

The term 'vitamin K' is a group name for a number of related compounds that have in common a 2-methyl-1,4-naphthoquinone ring system, but differ in the length and degree of saturation of their isoprenoid side chain at the 3-position. Three vitamin K compounds have biological activity (**Figure 1**).

Phylloquinone, vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), is found in green leafy vegetables and represents the main dietary source of vitamin K in Western diets. Menaquinones (MKs), vitamin K_2 (2-methyl-3-1,4-naphthoquinone), are synthesized by the gut microflora, and have fully or partially unsaturated isoprenoid side chains of various length at the 3-position. The predominant forms of the MK compounds contain between 6 and 10 isoprenoid units, but Makes containing up to 13 units have been isolated. The parent structure of the vitamin K group of compounds is 2-methyl-1,4-naphthoquinone, commonly called menadione (vitamin K_3). This compound is not found in nature but is a synthetic form that can be metabolized to phylloquinone or MK and thus may be regarded as a provitamin. Menadione is also used as an animal feed supplement and in this way may indirectly enter the human food chain as preformed MK-4.

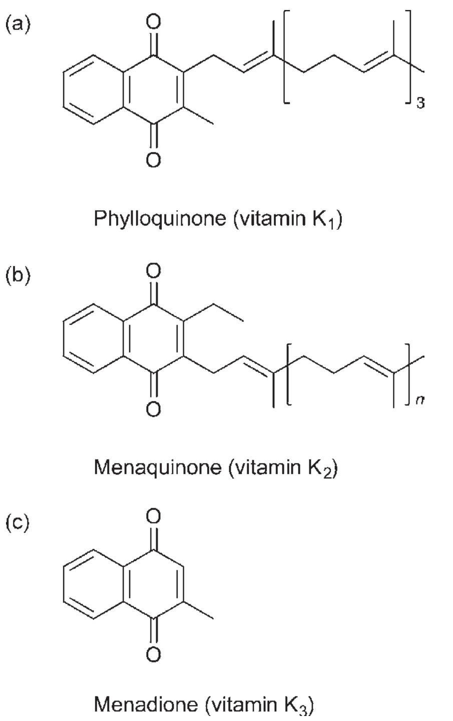
Previous analytical techniques to measure vitamin K compounds, such as the chick bioassay, were cumbersome and tended to overestimate the vitamin K content of foods. However, at present, the method of choice for vitamin K analysis in foodstuffs is high-performance liquid chromatography (HPLC) separation after lipid extraction. Electrochemical or fluorescence detection (after reduction to the hydroquinone form) offers the sensitivity and selectivity needed for quantification of the small amounts of vitamin K compounds. Food composition data for vitamin K derived from HPLC are generally lower than earlier data derived from the chick bioassay. The use of these HPLC-derived data on the vitamin K content of foods allows for a more accurate determination of the phylloquinone content of a typical Western diet.

Dietary Sources of Vitamin K

Green leafy vegetables are the best dietary source of vitamin K (as phylloquinone) (see **Table 1**). Some plant oils such as soybean oil and rapeseed oil are good dietary sources, containing 173 and 123 μg of phylloquinone per 100 g, respectively. Some vegetable oils, such as peanut, corn, sunflower, and safflower oils, have much lower phylloquinone content ($1–10\,\mu g\,100\,g^{-1}$). In general, meat, cereals, fish, and milk are poor sources of phylloquinone. MKs seem to have a more restricted distribution in the diet than does phylloquinone. In the Western diet, nutritionally significant amounts of long-chain MKs have been found in animal livers and fermented foods such as cheeses. The Japanese food 'natto' (fermented soybeans) has an MK content higher than the content of phylloquinone in green leafy vegetables.

Mean dietary phylloquinone intakes were reported for a nationally representative sample of US consumers ($n = 3967$), aged 13+ years, at levels of 81 and 73 $mg\,day^{-1}$ in men and women. There are limited data on dietary phylloquinone levels in European populations and, of those that are available, the use of different dietary tools precludes comparison in some cases. For example, mean intake estimates of phylloquinone in the United Kingdom, Ireland, and Norway of 60–85 $mg\,day^{-1}$ were obtained using food records, while estimates in the Netherlands (250 $mg\,day^{-1}$) were based on a food frequency questionnaire.

Phylloquinone intake decreases with age, especially for adults over the age of 65 years and, more notably, over the age of 85 years. However, consistently higher intakes for older adults (>40 years) than younger adults are reported, most probably due to lower green vegetable consumption by younger adults.



Phylloquinone (vitamin K_1)

Menaquinone (vitamin K_2)

Menadione (vitamin K_3)

Figure 1 Structure of vitamin K_1 (a), K_2 (b), and K_3 (c).

Table 1 Vitamin K_1 (phylloquinone) concentration in commonly consumed vegetables

Vegetables	*Phylloquinone content (μg 100g^{-1} raw food)*
Kale	816
Spinach	483
Broccoli	102
Brussels	139–193
Onions	207
Lettuce	23–173
Cabbage, savoy	69
Cauliflower	16
Celery	29
Carrots	13
Green pepper	7

Source: USDA National Nutrient Database for Standard Reference. http://www.nal.usda.gov/fnic/foodcomp/Data/SR17/wtranks/sr17a430.pdf

Absorption, Metabolism, and Excretion

Dietary vitamin K, mainly as phylloquinone, is absorbed into the lymphatic system from the proximal intestine after solubilization into mixed micelles composed of bile salts and the products of pancreatic lipolysis. In healthy adults, the efficiency of absorption of phylloquinone is about 80%. Intestinal bacteria can synthesize a variety of MKs, which are absorbed to a limited extent from the large intestine, transported into the lymphatic system, cleared by the liver, and released in very low-density lipoprotein (VLDL). However, it is not fully clear to what extent intestinal MK contributes to the vitamin K requirement. Approximately 50% of vitamin K is carried in the plasma in the form of VLDL, about 25% in low-density lipoprotein (LDL), and about 25% in high-density lipoprotein (HDL). Once in the circulation, phylloquinone is cleared rapidly at a rate consistent with its continuing association with chylomicrons. Vitamin K is extensively metabolized in the liver and excreted in the urine and bile. It has been demonstrated in tracer experiments that about 20% of an injected dose of phylloquinone is recovered in urine, whereas about 40–50% is excreted in the feces via the bile and the proportion excreted was the same regardless of whether the injected dose was 1000 or 45 μg. It seems likely, therefore, that about 60–70% of the amount of phylloquinone absorbed from each meal will ultimately be lost to the body by excretion. These results suggest that the body's stores of phylloquinone are constantly replenished. Vitamin K itself is too lipophilic to be excreted in the bile and is excreted as side chain-shortened carboxylic acid metabolites.

There is no evidence that phylloquinone and MK are toxic. However, high intakes of phylloquinone can negate the effects of the anticoagulant warfarin. The synthetic form of vitamin K, menadione, can interfere with the function of glutathione, one of the body's natural antioxidants, resulting in oxidative damage to cell membranes. Menadione given by injection has been shown to induce liver toxicity, jaundice, and hemolytic anemia (due to the rupture of red blood cells) in infants, and is no longer used for the treatment of vitamin K deficiency. No tolerable upper level (UL) of intake has been established for vitamin K.

Metabolic Function of Vitamin K

Vitamin K acts as a cofactor for a specific carboxylation reaction that transforms selective glutamate (Glu) residues to γ-carboxyglutamate (Gla) residues. The reaction is catalyzed by the microsomal enzyme vitamin K-dependent γ-glutamyl carboxylase, which, in turn, is linked to a cyclic pathway known as the vitamin K epoxide cycle. The resultant Gla residues are common to all vitamin K-dependent proteins and these have increased affinity for calcium. Prothrombin and other proteins of the blood clotting system, as well as certain bone matrix proteins, contain Gla.

The vitamin K epoxide cycle serves to recycle the nutrient via a cyclic interconversion. In this cycle, the vitamin K quinone form is reduced by the FAD-containing enzyme DT-diaphorase (NAD(P)H:quinone oxidoreductase) into the vitamin K hydroquinone (KH2), which then serves as a cofactor for vitamin K carboxylation of Gla proteins and, in so doing, is oxidized to vitamin K epoxide. Vitamin K epoxide is then recycled back to the quinone form by the enzyme vitamin K epoxide reductase (VKOR), completing the cycle. At a molecular level, vitamin K epoxide is reduced in two steps: first to the quinone form by VKOR and then to KH2 by DT-diaphorase.

Vitamin K-Dependent Proteins

Vitamin K-Dependent Coagulation Proteins

There are seven vitamin K-dependent proteins involved in blood coagulation, namely, prothrombin (factor II), factors VII, IX, and X, and proteins C, S, and Z, all of which are synthesized in the liver and contain between 10 and 12 Gla residues (**Table 2**). The Gla residues enable Ca^{2+}-mediated binding of the proteins to the negatively charged phospholipid surfaces provided by blood platelets and endothelial cells at the site of injury. Prothrombin and factors VII, IX, and X possess procoagulant activity and participate in the cascade that results in the formation of the fibrin clot. A key element in the formation of fibrin is the conversion of prothrombin to thrombin by activated factor X (which is, in turn, activated by activated factors VII and IX). In contrast, proteins C and S act as anticoagulants. Protein C inhibits coagulation by inactivating activated factors V and VIII and enhancing fibrinolysis, with protein S as a cofactor.

Bone Vitamin K-Dependent Proteins

There are two bone matrix proteins that contain Gla: osteocalcin (OC) and matrix Gla protein (MGP) (**Table 2**). OC is an osteoblast-derived, specific vitamin K-dependent protein that also contains hydroxyproline and is the most abundant of all the noncollagenous bone matrix-bound proteins. It has a molecular mass of 5700 Da and contains three Gla residues, which give this protein a high affinity for hydroxyapatite, in fact much higher than its affinity for calcium. The synthesis of OC is under the regulatory control of the active vitamin D metabolite, 1,25 dihydroxyvitamin D (1,25OHD), and its release into the circulation provides a sensitive index of vitamin D action. While a high proportion of newly synthesized OC is incorporated into bone, approximately 30% of it is released into the circulation and serum levels of the protein are used widely as an indicator of the rate of bone formation. The precise physiological function of OC remains unclear. The less well characterized MGP has a molecular mass of 9600 Da and contains five Gla residues and in contrast to OC, which is exclusively associated to mineralized tissues, MGP is present in cartilage and is expressed at a high rate in many soft tissues (heart, kidney, lungs), in addition to bone.

Vitamin K and Health

Deficiency

Newborn infants are at serious risk of hemorrhaging because of poor placental transfer of vitamin K, lack of

Table 2 The main vitamin K (Gla)-dependent proteins and their physiological function

Gla protein	*Tissue*	*Physiological function*
Blood coagulation		
Prothrombin (factor II), factors VII, IX, and X	Liver (then plasma)	Procoagulants
Protein C	Liver (then plasma)	Anticoagulant
Protein S	Liver (then plasma), endothelium	Cofactor for protein C
Protein Z	Liver (then Plasma)	Exact function unknown
Bone		
Osteocalcin (bone Gla protein)	Bone	Unknown, may be a matrix signal for osteoclasts
Matrix Gla protein	Bone, cartilage, and most soft tissues	Inhibitor of calcification
Protein S	Bone	Unknown
Others		
Nephrocalcin	Renal tissue	Undetermined, may inhibit the growth of calcium oxalate monohydrate crystals
Plaque Gla protein	May be present in artherosclerotic plaque	Undetermined
Growth arrest specific gene 6 (Gas 6)	Detected in cartilage and numerous soft tissues	Cellular growth regulation factor
Proline-rich Gla protein 1,2 (PRGP 1,2)	Broad tissue distribution	Undetermined

Data from Ferland G (1998) The vitamin K-dependent proteins:an update. *Nutrition Reviews* 56: 223–230; Shearer MJ and Bolton-Smith C (2000) *Food Chemistry* 68: 213–218.

intestinal bacteria, and the low vitamin K content in breast milk. For this reason, vitamin K is routinely administered prophylactically at birth in many countries. The risk of bleeding is greatest in prematurely born infants, in breast-fed infants, and in those with gastrointestinal conditions that impair vitamin K absorption. In normal infants, plasma prothrombin concentrations and those of the other vitamin K-dependent factors are approximately 20% of adult values at birth.

Normal or near-normal blood coagulation is usually maintained in older children and adults. Several factors protect adults from a lack of vitamin K and these include widespread distribution of vitamin K in plant and animal tissues, the vitamin K cycle, which conserves the vitamin, and the microbiological flora of the normal gut, which synthesizes MKs. The causes of the reduced levels of vitamin K-dependent coagulation factors in adults are largely secondary to diseases such as cystic fibrosis, celiac disease, ulcerative colitis, and short-bowel syndrome. Biliary obstruction and liver disease may also lead to vitamin K deficiency. There are numerous reports of bleeding episodes in patients treated with anticoagulant drugs and broad-spectrum antibiotics.

In children and adults, 'clinical' vitamin K deficiency in terms of blood coagulation is rare. However, 'subclinical' vitamin K deficiency in extrahepatic tissues, particularly in bone, is not uncommon in the adult population. From the multitude of proteins that require carboxylation of Glu to Gla residues for proper functioning, it is clear that poor vitamin K status may contribute to certain chronic vascular and skeletal diseases. Furthermore, it has been suggested that dietary phylloquinone levels that are sufficient to maintain normal blood clotting (which forms the basis of the recommended dietary allowance) may be suboptimal for adult bone health.

Vitamin K and Bone Health

The identification of γ-carboxyglutamyl-containing proteins in bone, notably OC and MGP, has generated considerable interest in the role of vitamin K in bone metabolism and bone health. In addition, another functional index of vitamin K status in bone metabolism is the level of undercarboxylated osteocalcin (ucOC). The extent to which OC is uncarboxylated has been assessed with respect to age, bone status, and risk of hip fracture. A high concentration of circulating ucOC has been associated with low bone mineral density and increased risk of hip fracture. The percentage of uncarboxylated OC is high (by 40%) in post-menopausal women compared with pre-menopausal women. The post-menopausal women responded to phylloquinone supplementation with an increase in total and carboxylated OC and a decrease in urinary calcium and hydroxyproline. The incidence of hip fractures in aged women correlates directly with the increase in ucOC and bone mineral density correlates negatively with the rise in ucOC. The relationship between ucOC and bone health in young growing teenagers has also received attention recently. For example, a significant inverse association has been reported between ucOC and bone mineral content of the total body and lumbar spine in peripubertal Danish girls.

Vitamin K intake has been associated with bone health in epidemiological studies. A cohort of elderly men and women from the Framingham Study in the United States showed an association of vitamin K intake with the incidence of hip fracture. In addition, there was evidence that phylloquinone intakes $<109\,\mu\text{g}\,\text{day}^{-1}$ are associated with an increased risk of hip fracture in 72 327 women participating in the Nurse's Health Study in the United States. In addition, among post-menopausal Scottish women, phylloquinone intake has been positively associated with bone mineral density of the femoral neck and lumbar spine as well as biochemical markers of bone turnover.

Based on the intervention studies to date that have investigated the effect of phylloquinone supplementation on bone loss in later life (generally >50 years), it appears that phylloquinone supplementation does not protect against loss of bone mineral in some skeletal sites (lumbar spine, total body, mid-distal radius). Furthermore, the evidence base for bone health benefits at the femoral neck from phylloquinone supplementation is mixed and may require further research. In particular, the inconsistent findings in relation to the effects of phylloquinone supplementation on bone mineral density of the hip do not explain the mechanism underpinning the protective effect of high phylloquinone intake/status against hip fracture observed in a number of prospective cohort studies. More research is needed on whether phylloquinone supplementation may be lowering the risk of fractures through other mechanisms such as effects on bone quality parameters. The finding that relatively low-dose phylloquinone supplementation improved bone mineral density of the forearm (ultradistal radius) of post-menopausal women is interesting even though it was investigated in only one study. It has been suggested that ultradistal forearm has a higher metabolic turnover rate than predominantly cortical bone and thus may be more responsive to dietary treatment.

Vitamin K and Cardiac Health

A role for vitamin K in atherosclerosis was hypothesized when proteins containing Gla residues were isolated from hardened atherosclerotic plaque, which were later identified as OC and MGPs. Increasing evidence is emerging suggesting a role for vitamin K in the calcification of arteries and atherogenesis. Moreover, the therapeutic potential of vitamin K_2 as an antihepatoma drug has been recently highlighted.

Results from human observational studies investigating relations between vitamin K intake and cardiovascular diseases are inconsistent. The Nurses' Health Study showed a modest risk reduction of coronary heart disease (CHD) for high phylloquinone intakes, while no significant associations were observed in the Health Professionals Follow-up Study and the Rotterdam Study. On the other hand, in the Rotterdam Study, a strong inverse association between MK intake and CHD mortality and severe aortic calcification was observed. These inconsistencies may relate to different effects of phylloquinone and MK on coronary calcification.

In a large study on 16 057 women, MK intake was inversely associated with coronary events, while phylloquinone intake was not related to CHD. Similarly, in animals, MK-4-but not phylloquinone-appears to inhibit warfarin-induced coronary calcification. The different effects of MK and phylloquinone probably reflect differences in metabolism as a result of different distributions over plasma lipoproteins.

Vitamin K Status and Requirements

Defining reliable indicators of vitamin K status has proven to be a difficult task. The serum concentration of ucOC is a more sensitive indicator of vitamin K status than the traditional blood coagulation tests, and a high serum level of ucOC is indicative of low vitamin K status and vice versa. UcOC has been reported to have a negative association with plasma phylloquinone concentrations. The difference between the vitamin K-dependent coagulation factors (all synthesized in the liver) and the bone Gla protein OC suggests that different tissues (at least bone and liver) may have different vitamin K requirements; hence, bone tissue may be more prone to vitamin K deficiency than liver. If this is the case, impaired synthesis of some vitamin K-dependent proteins may be far more prevalent in the human population than coagulation assays previously indicated, potentially resulting in an increase in dietary recommendations for vitamin K, especially for the elderly. A number of clinical trials have shown that high circulating ucOC levels are common in postmenopausal women as well as healthy young and elderly adults but levels are reduced significantly with vitamin K supplementation. Even in healthy newborns, whose vitamin K status is known to be precarious, very low levels of undercarboxylated prothrombin are detectable, whereas all babies tested exhibited high concentrations of serum ucOC. These data together with other evidence suggest that circulating OC is the most sensitive known marker for vitamin K status.

Until recently, the only widely accepted criterion for vitamin K sufficiency was the maintenance of plasma prothrombin concentration. It has been estimated that 0.5–1.0 $\mu g\,kg^{-1}\,day^{-1}$ was required to correct induced clotting changes. In adults, primary vitamin K-deficient states that resulted in bleeding were almost unheard of, except in a hospital setting. This is due to the widespread distribution of vitamin K in foods, the ability of the vitamin K cycle to conserve vitamin K, and endogenous bacterial syntheses of MKs. Therefore, a healthy population is not at risk of dietary vitamin K deficiency as the recommendation for optimal blood clotting is readily achievable. However, recent attention has focused on the importance of vitamin K for optimizing bone health and it has been proposed that vitamin K supplies believed sufficient to maintain normal blood coagulation may be suboptimal for bone health.

The Food and Nutrition Board (2001) has recently established an adequate intake (AI) value for vitamin K. The recently discovered indicators sensitive to vitamin K intake, although useful to describe relative diet-induced changes in vitamin K status, were not used for establishing an estimated average requirement (EAR) because of the uncertainty surrounding their true physiological significance and the lack of sufficient dose–response data. Therefore, the AI for adults was based on reported vitamin K dietary intake in apparently healthy populations. A large review, including 11 different studies, reported that phylloquinone intake ranged from 60 to 210 $\mu g\,day^{-1}$ with an average intake of approximately 80 $\mu g\,day^{-1}$ for younger adults (<45 years) and approximately 150 $\mu g\,day^{-1}$ for older adults (>55 years). Healthy individuals with a phylloquinone intake approaching 80 $\mu g\,day^{-1}$ have been investigated and showed no signs of deficiency, suggesting that this level is probably adequate for the majority of the adult population. Because dietary assessment methods tend to underestimate the actual daily intake of foods, the highest intake value reported for four adult age groups was used to set the AI for each gender rounding up to the nearest 5 μg. Therefore, the most recent guideline (AI) for vitamin K intake in the United States for adults (aged 19 years and older) is 120 and 90 $\mu g\,day^{-1}$, for men and women, respectively.

To date, no adverse effect has been reported for individuals consuming greater than the AI for vitamin K. However, the data on adverse effects from high vitamin K intake are not sufficient for a quantitative risk assessment and a tolerable UL of intake has not been established by the Institute of Medicine in the United States or by the Scientific Committee of Food in the European Union.

See also: **Nutrition and Health**: Nutritional and Health-Promoting Properties of Dairy Products: Bone Health; **Vitamins**: General Introduction.

Further Reading

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Vitamin C

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Introduction

Among specific nutritional deficiency diseases, scurvy was the dreaded disease of seamen and explorers forced to subsist for months on diets of dried beef and biscuits. The symptoms of scurvy are rather characteristic and consist of bleeding and rotting gums, swollen and inflamed joints, dark blotches on the skin, and muscle weakness. Scurvy afflicted nineteenth-century populations on land, including armies of the Crimean and United States Civil wars and the California gold rush communities. In 1907, scurvy was produced experimentally in the guinea pig and from 1928 to 1930, Albert Szent-Gyorgy and Glen King independently published their isolation of vitamin C or hexuronic acid. It was later named ascorbic acid for its antiscorbutic properties and its molecular structure was determined in 1933.

Ascorbate, also known as ascorbic acid (AA) or vitamin C, is synthesized *de novo* from glucose in the liver of most adult mammals. D-Glucose is converted into L-ascorbic acid via D-glucuronic acid, L-gulonic acid, L-gulonolactone, and L-gulono-γ-lactone as intermediates. However, humans and non-human primates, guinea pigs, the Indian fruit bat, several species of birds, and some fish have lost the ability to synthesize ascorbate *de novo*. As a result of a gene mutation, they lack a key ascorbate-oxidizing enzyme, L-gulono-γ-lactone oxidase, an essential oxidizing enzyme in the liver for the conversion of L-gulono-γ-lactone into 2-oxo-L-gulono-γ-lactone, a tautomer of L-ascorbic acid, which transforms spontaneously into the vitamin. In plants, the biosynthesis of ascorbate is more complicated than in animals. The vitamin is synthesized from guanosine diphosphate (GDP)-mannose, and the pathway shares GDP-sugar intermediates with the synthesis of cell wall polysaccharides and those glycoproteins that contain D-mannose, L-fucose, and L-galactose. Ascorbate is quantitatively the predominant antioxidant in plant cells and is found in all subcellular compartments, including the apoplast, and has an average cellular concentration of 2–25 mmol l^{-1} or more in the chloroplast stroma. This article discusses the chemistry of vitamin C. In addition, the role of vitamin C as a biological antioxidant, specific functions in humans, and role in health and disease are highlighted.

Chemistry

Ascorbic acid is the enolic form of an α-ketolactone (2,3-didehydro-L-threo-hexano-1,4-lactone). The molecular structure (**Figure 1**) contains two ionizable –OH groups at C_2 and C_3 that give the compound its acidic character, and since pK_{a1} at C_3 is 4.17 and pK_{a2} at C_2 is 11.79, a monoanion is the favored form at physiological pH where 99.95% of AA is present as ascorbate monoanion ($AscH^-$), 0.05% as AA ($AscH_2$), and 0.004% as ascorbate dianion (Asc^{2-}). Thus, the antioxidant chemistry of vitamin C is the chemistry of $AscH^-$. The asymmetric carbon 5 atom allows two enantiomeric forms, of which the L-form is naturally occurring. Oxidation of AA takes place as either two one-electron transfer processes or as a single two-electron reaction without detection of the intermediate ascorbyl radical. In the two one-electron oxidation processes, the first step involves loss of one electron from $AscH^-$ to form the neutral ascorbyl radical ($AscH^{\bullet}$), which is not protonated in biological systems and is present as the resonance-stabilized tricarbonyl ascorbate free radical ($Asc^{-\bullet}$), which is relevant in biology. $Asc^{-\bullet}$ is a weakly reactive radical, and *in vivo* it is likely that reducing enzymes are involved in its removal, resulting in the recycling of ascorbate. Loss of an additional electron yields L-dehydroascorbic acid (DHA). The oxidation of AA to DHA is reversible via the same intermediate radical process, and for this reason DHA also exhibits biological activity, since it can be easily converted to AA in the human body. However, DHA is highly unstable because of the susceptibility to hydrolysis of the lactone bridge. DHA has a half-life, in aqueous solutions at 37 °C, of approximately 6–20 min as a function of concentration, and catabolism beyond DHA is enhanced by alkaline pH and metals, especially copper and iron. Hydrolysis of DHA irreversibly forms 2,3-diketogulonic acid and leads to the loss of vitamin C activity (**Figure 1**). Further catabolism leads to the formation of a wide array of other nutritionally inactive products such as L-xylonic acid, L-lyxonic acid, L-xylose, oxalic acid, and L-threonic acid. The rate of oxidative degradation of the vitamin is a nonlinear function of pH because the various ionic forms of AA differ in their susceptibility to

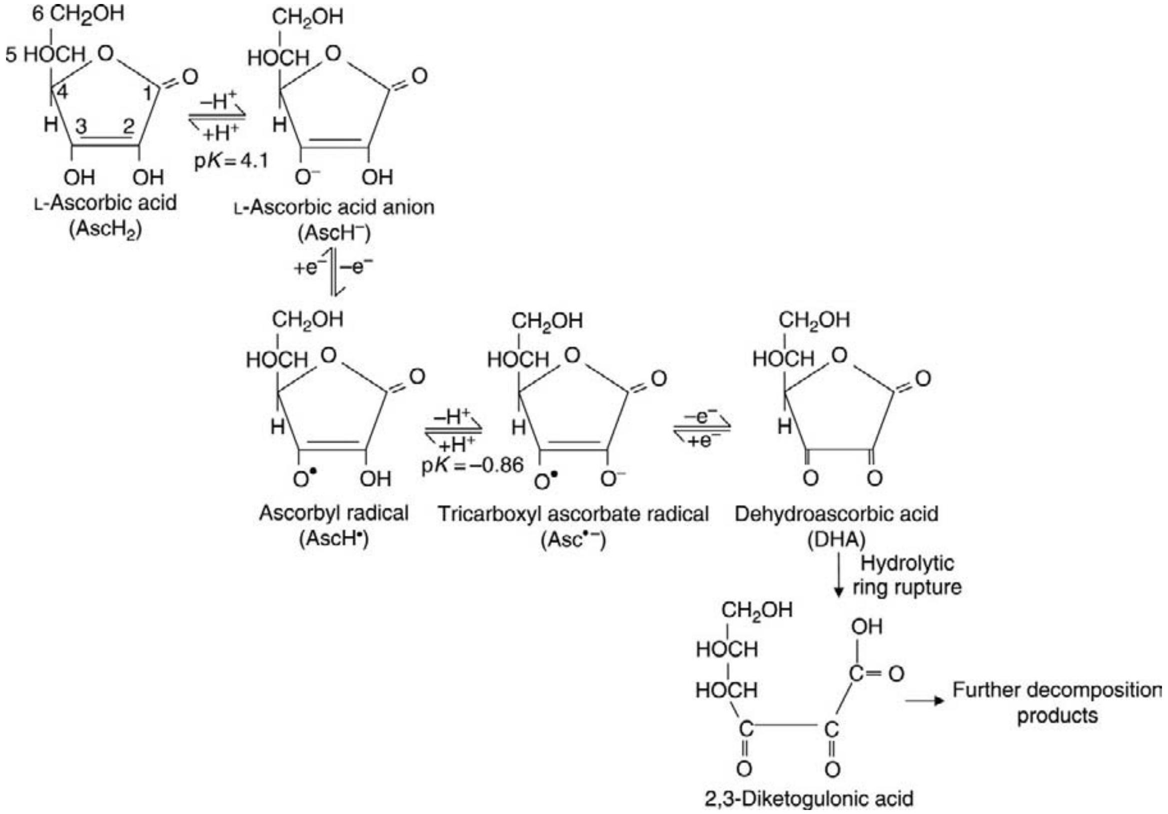
6 CH2OH
5 HOCH
O
4
1
O
H
3
2
OH
OH
L-Ascorbic acid
(AscH2)
−H+
+H+
pK = 4.1
CH2OH
HOCH
O
O
H
O−
OH
L-Ascorbic acid anion
(AscH−)
+e−
−e−
CH2OH
HOCH
O
O
H
O•
OH
Ascorbyl radical
(AscH•)
−H+
+H+
pK = −0.86
CH2OH
HOCH
O
O
H
O•
O−
Tricarboxyl ascorbate radical
(Asc•−)
−e−
+e−
CH2OH
HOCH
O
O
H
O
O
Dehydroascorbic acid
(DHA)
Hydrolytic
ring rupture
CH2OH
HOCH
HOCH
C
O
C
O
OH
C=O
Further decomposition products
2,3-Diketogulonic acid

Figure 1 Ascorbic acid and its oxidation products. Dehydro-L-ascorbate may exist in multiple forms. Formation of 2,3-diketogulonic acid by hydrolytic cleavage is probably irreversible.

oxidation: fully protonated $AscH_2 < AscH^- < Asc^{2-}$. Under conditions relevant to most biological systems, the pH dependence of oxidation is governed mainly by the relative concentrations of $AscH_2$ and $AscH^-$ species and this, in turn, is governed by pH (pK_{a1} 4.17). The rate of oxidation of ascorbate is generally observed to be first order with respect to the concentration of $AscH^-$, molecular oxygen, and the metal ion.

Dietary Sources

More than 80% of the vitamin C in western diets comes from fruits and vegetables, with citrus fruits, tomatoes and tomato juice, and potatoes being major contributors. A minor portion comes from enriched or fortified products, meats, fish, poultry, eggs, and dairy products, and essentially none from grains. The mean content of vitamin C is 2.11 mg per 100 g (range 1.65–2.75 mg per 100 g) in cow's milk, 5.48 mg per 100 g in goat's milk, 3.9 mg per 100 ml in summer human milk, and 3.02 mg per 100 ml in winter human milk. There is some evidence that the concentration of vitamin C in cow's and goat's milk changes with season. It has been observed that in raw milk sampled in March or August the concentration of vitamin C was higher (2.0–2.7 mg per 100 ml) than in samples collected in October (1.2 mg per 100 ml). The mean concentration of vitamin C in human milk also appears to be affected by the stage of lactation and declines from 6.18 mg per 100 ml in colostrum to 4.68 mg per 100 ml at 9 months. The influence of maternal vitamin C intake and its effect on the vitamin C content of human milk have not been clearly defined. It has been observed that the vitamin C level in human milk did not increase significantly in response to increasing maternal intake (up to 10-fold). It appears that a regulatory mechanism may be present in mammary cells to prevent an elevation in the concentration of vitamin C in milk beyond a certain saturation level. On the other hand, when the intake of vitamin C is low, breast milk levels are sensitive to supplementation.

In the United States, the median dietary intake of vitamin C by adult men from 1988 to 1994 was about 105 mg day^{-1} and the median total intake (including supplements) was about 120 mg day^{-1}. For women, the median intake was estimated to be 90 mg day^{-1} and median total intake (including supplements) was about 108 mg day^{-1}. The average consumption for children was 84 mg day^{-1}. The recent North/South Food

Consumption Survey in Ireland (2001) showed that mean daily intake of vitamin C was not significantly different in men (116 mg) and women (108 mg). The primary sources of vitamin C for the total population were potatoes and potato products (25.9%); fruit juices; nuts and seeds; herbs and spices (25.6%); and vegetable and vegetable products (22.1%). The contribution from supplements was 5.8% for men and 8.6% for women.

Ascorbic acid is also added to some processed foods for its antioxidant or functional properties and consequently the mean total vitamin C intake may be considerably higher than indicated above. Ascorbic acid is the nutrient taken most frequently as a supplement, particularly among the elderly population. The Boston Nutritional Status Survey of the Elderly (1992) estimated that 35 and 44% of males and females, respectively, took some form of vitamin supplements, with a median supplemental intake of 300 mg day^{-1}. Clinical signs of vitamin C deficiency are rarely seen in developed countries. The content of vitamin C in foods may be reduced significantly because of thermal destruction that occurs during cooking, losses in cooking water, and subsequent holding prior to consumption.

Absorption, Metabolism, and Excretion

In rats and hamsters (for which AA is not a vitamin), intestinal absorption is passive. In the case of guinea pigs and humans, both of which have an absolute requirement for exogenous AA in their diet, there is a sensitive sodium-dependent active transport system for AA in the brush border of the duodenum and upper ileum, and another sodium-independent transfer process in the basolateral membrane. There is also a passive transport mechanism, which in humans is predominant only at high intake levels. Ascorbate transport has been specifically shown to require metabolic energy, with a stoichiometry for Na^{+} from 1.1 to 2.1. Intestinal absorption of AA and its entry into cells are facilitated by conversion to DHA, which is transported across cell membranes more rapidly than ascorbate. Because DHA is structurally similar to glucose, its transport across membranes is facilitated by glucose transporters (GLUTS). Transport of DHA is primarily Na^{+}-independent in animal and human tissues, and does not require metabolic energy. Upon cell entry, DHA is reduced immediately to AA, which produces an effective gradient of DHA across the membrane. Intracellular reduction of DHA to AA is mediated by two major pathways: chemical reduction by glutathione and enzymatic reduction. The flux of ascorbate in and out of the cell via facilitated diffusion and active transport is mediated by distinct classes of proteins such as facilitative glucose transporters and sodium–vitamin C cotransporters, respectively.

Information on the bioavailability of vitamin C in foods is limited. It is generally agreed that at relatively low intakes (less than 30 mg day^{-1}), ascorbate is nearly completely absorbed, and 70–90% of the usual dietary intake of ascorbate (30–180 mg day^{-1}) is absorbed. Similar levels of absorption (~80%) have been reported for pure ascorbate, ascorbate in orange juice, and ascorbate in cooked broccoli, which suggests that the absorption of vitamin C is almost complete. However, absorption falls to approximately 50% or less with increasing doses above 1.5 g day^{-1}. Following absorption, ascorbic acid circulates freely in plasma, leukocytes, and red cells, and enters all tissues, with maximum concentrations of 68–86 μmol l^{-1} plasma being achieved with an oral intake of 90–150 mg day^{-1}. Excess is excreted by the kidney, which conserves the vitamin at plasma levels of up to 46–86 μmol l^{-1} by a saturable, sodium-dependent reabsorption process. The upper limit of plasma ascorbic acid concentration is controlled by the gastrointestinal absorption and renal reabsorption mechanisms, and fasting plasma concentration rarely exceeds 100 μmol l^{-1}, even with dietary supplementation. Specific proteins mediate the entry and exit of vitamin C in cells by facilitated diffusion or active transport. These cellular transport systems are responsible for high intertissue ascorbate levels found in the pituitary and adrenal glands (30–400 mg per 100 g tissue), followed by the brain, spleen, pancreas, kidney, liver, and tissues of the eye with 10–50 mg per 100 g of tissue. Vitamin C concentration also varies widely in different blood cell types. About 70% of blood-borne ascorbate is in plasma and erythrocytes. The remainder is in white cells, which have a marked ability to concentrate ascorbate; mononuclear leukocytes achieve 80-fold concentration, platelets 40-fold, and granulocytes 25-fold, compared with plasma concentration. Tissue-specific cellular mechanisms of transport and metabolism allow for wide variation of tissue ascorbate concentration in order to enhance its function as an enzyme cofactor and antioxidant. Intracellularly, and in plasma, vitamin C exists predominantly in the free form as AscH^{-}. DHA is either not detectable or found at only very low levels in the circulation of healthy people.

The total body pool of ascorbate is affected by limited intestinal and renal tubular absorption. It reaches a maximum value of about 20 mg kg^{-1} body weight or about 1500 mg for the average-size man when the ascorbate intake is increased from 30 to 180 mg day^{-1}; above this level of intake, excretion of ascorbate in the urine rises rapidly. Unabsorbed ascorbate is degraded in the intestine, a process that may account for the diarrhea and intestinal discomfort sometimes reported by persons ingesting large doses.

Antioxidant Activity of Ascorbic Acid

Ascorbate is often called the outstanding antioxidant. In chemical terms, this is simply a reflection of its redox properties as a reducing agent. In physiological terms, this means that ascorbate provides electrons for enzymes, for chemical compounds that are oxidants, or for other electron acceptors in biological systems. In addition to its redox potential, other properties of ascorbate make it an excellent electron donor in biological systems. Ascorbate undergoes two consecutive, reversible, one-electron oxidation processes forming the ascorbate radical ($Asc^{-\bullet}$) as an intermediate. The $Asc^{-\bullet}$ has an unpaired electron, making it a relatively unreactive free radical, especially with oxygen, and the ascorbate oxidation product, DHA, is reduced by cells to ascorbate, which then becomes available for reuse. These properties make ascorbate an excellent biological donor system. Thus, ascorbate is a reversible biological reductant and, as such, it provides reducing equivalents for a variety of biochemical reactions, is essential as a cofactor for reactions requiring a reduced metal ion (Fe^{2+}, Cu^{+}), and serves as a protective antioxidant that operates in the biological aqueous phase and can be regenerated *in vivo* when required.

Ascorbate is thermodynamically close to the bottom of the list of one-electron reducing potentials of oxidizing free radicals ($E^{\circ\prime} = +282\,\text{mV}$). For this reason, ascorbate is considered to be the most important antioxidant in extracellular fluids and is the first line of defense against reactive oxygen species (ROS) and reactive nitrogen species (RNS) (e.g., nitric oxide, $NO^{\bullet}$, and nitric dioxide, $NO_2^{\bullet}$) in plasma. It efficiently scavenges all oxidizing species with a greater one-electron potential (higher $E^{\circ\prime}$ values), which include the superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$), and oxygen-centered radicals of organic compounds (peroxyl, $LOO^{\bullet}$, and alkoxyl, $LO^{\bullet}$) can be repaired by ascorbate as follows:

$$AscH^{-} + X^{\bullet} \rightarrow Asc^{-\bullet} + XH$$

where $X^{\bullet}$ is any of the oxidizing radicals. Although ascorbate itself forms a radical in the reaction, a potentially very damaging radical ($X^{\bullet}$) is replaced by the relatively unreactive $Asc^{\bullet}$. Overall, ascorbate is reactive enough to affectively interrupt oxidants in the aqueous phase before they can attack and cause detectable oxidative damage to DNA and lipids. In aqueous solutions, ascorbate also scavenges RNS efficiently, preventing nitrosation of target molecules. Consequently, both thermodynamically and kinetically, ascorbate can be considered to be an excellent aqueous antioxidant.

Ascorbate may also regenerate α-tocopherol (α-TOH) from the tocoperoxyl radical ($TO^{\bullet}$), which is formed upon inhibition of lipid oxidation by α-tocopherol. Ascorbate has a lower redox potential ($E^{\circ\prime} = +282\,\text{mV}$) than α-TOH ($E^{\circ\prime} = +500\,\text{mV}$) and, in addition, the α-$TO^{\bullet}$ is at the membrane–water interface, thereby allowing water-soluble ascorbate access to membrane-bound α-$TO^{\bullet}$ for the repair reaction and recycling of α-tocopherol:

$$AscH^{-} + \alpha\text{-}TO^{\bullet} \xrightarrow{\Delta E^{\circ\prime} = +200\,\text{mV}} Asc^{\bullet -} + \alpha\text{-}TOH$$

The rate constant for the reaction is $1.5 \times 10^{6}\,\text{l}\,\text{mol}^{-1}\,\text{s}^{-1}$.

Thus, in cellular membranes, ascorbate plays an indirect antioxidant role to reduce the α-tocoperoxyl radical (α-$TO^{\bullet}$) to α-tocopherol (α-TOH). Recycling of α-tocopherol by ascorbate has been demonstrated in liposomes and cellular organelles and may also spare and recycle α-tocopherol in erythrocyte membranes and intact erythrocytes (*see* **Vitamins**: Vitamin E).

The $Asc^{-\bullet}$ formed in the above reaction dismutates to DHA and is then regenerated to ascorbate at the expense of glutathione, dihydrolipoate, thioredoxin, and other enzyme systems. This process allows for the transportation of a radical load from a lipophilic compartment to an aqueous compartment where it is taken care of by efficient enzymatic defenses.

It should be noted that as a reducing agent, ascorbate has the ability to reduce Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+}, thereby increasing the prooxidant activity of the metals and generating $HO^{\bullet}$, $O_2^{\bar{\bullet}}$, and H_2O_2 that initiate lipid peroxidation in biological systems. It is considered unlikely that ascorbate shows prooxidant properties *in vivo* since the concentrations of 'free' transition metals in healthy biological systems are very small because they are effectively bound by metal ion storage and transport proteins.

Biological Functions

Many of the biological functions of ascorbic acid are based on its ability to provide reducing equivalents for a variety of biochemical reactions. The vitamin can reduce most physiologically relevant reactive species and, as such, functions primarily as a cofactor for reactions requiring a reduced iron or copper metalloenzyme and as a protective antioxidant that operates in the aqueous phase both intra- and extra-cellularly. Ascorbate is known to be a specific electron donor for eight human enzymes; three enzymes participate in collagen hydroxylation, two in carnitine biosynthesis, and three in hormone and amino acid biosynthesis. Evidence also suggests that ascorbate plays a role in or influences collagen gene expression, cellular procollagen secretion, and the biosynthesis of other connective tissue components, including elastin, proteoglycans, bone matrix, and elastin-associated fibrillin. Ascorbate is also involved in the synthesis and modulation of some hormonal components of the nervous system.

Collagen Formation

Scurvy, the classical disease of severe ascorbate deficiency, is characterized by symptoms related to connective tissue defects. Clinically, signs of scurvy are seen when the total body pool of ascorbate is below ~300 mg. Clinical features of scurvy include skin bruises, perifollicular hemorrhages, bleeding gums, joint pain and swelling, and fatigue. Oxidative degradation of some blood coagulation factors due to a low concentration of plasma ascorbate may contribute to hemorrhagic symptoms. Ascorbate-dependent aspartate β-hydroxylase is known to be required for the postsynthetic modification of protein C, the vitamin K-dependent protease that hydrolyses activated factor V in the blood-clotting cascade.

Ascorbic acid affects the biosynthesis of collagen at several levels from collagen transcription, to expression, including the regulation of the processing enzymes. It acts as a cofactor for several metal-dependent oxidative reactions, catalyzed by both monooxygenases and dioxygenases. Other cofactors required by the dioxygenases are Fe^{2+}, α-ketoglutarate, and O_2, whereas the monooxygenase requires Cu^{+} and O_2 for activity. Ascorbate functions as a reductive cofactor for posttranslational hydroxylation of peptide-bound proline and lysine residues during the formation of collagen. The hydroxyproline is required for normal triple-helical backbone structure and the hydroxylysine cross-linkages are needed for normal collagen fiber formation. The enzyme involved in proline hydroxylation, prolyl hydroxylase, requires molecular oxygen, ascorbic acid, iron, and α-ketoglutarate. The first step in the reaction is the attack on peptide-bound proline by oxygen, followed by condensation with α-ketoglutarate, the release of the hydroxylated substrate, and decarboxylation to release succinate. During the hydroxylation reaction, the enzyme-bound iron is oxidized to Fe^{3+}, which is catalytically inactive. The ascorbate is involved in reactivating the enzyme by reduction of Fe^{3+} back to the loosely bound ferrous form.

In an analogous reaction, ascorbate participates as a cofactor in the hydroxylation of lysine residues catalyzed by copper-dependent lysyl hydroxylase. Hydroxylysine cross-linkages are central for normal collagen fiber formation. Prolyl and lysyl hydroxylases are also called dioxygenases, referring to the ability of the enzymes to provide two oxygen atoms to the same or separate substrates. Ascorbate may also serve as a reductant for other metal-dependent polymerization and cross-linking reactions of connective tissue and as a carrier for sulfate groups needed for the production of glycosaminoglycans (e.g., chondroitin).

A deficiency of ascorbate results in a weakening of collagenous structures, causing tooth loss, joint pains, bone and connective tissue disorders, and poor wound-healing, all of which are characteristic of scurvy. This disease is now rare in developed countries, but is occasionally seen in individuals in classes with exceptionally poor or restricted diets, such as low socioeconomic groups and those who have a near total lack of fruit and vegetables, or those who abuse alcohol or drugs. Low ascorbate levels and scurvy are most often noted in men who live alone and eat a diet frequently low in fruit and vegetables. Because breast milk provides adequate ascorbic acid, infantile scurvy is seen more often after weaning, between 6 and 12 months. Modern infant formulae are fortified with sufficient ascorbic acid such that infantile scurvy is now almost nonexistent.

Neurotransmitter Synthesis

Ascorbic acid appears to be involved in catecholamine metabolism in two mixed-function oxidases, dopamine β-hydroxylase and *para*-hydroxyphenylpyruvate oxidase. Ascorbic acid is required as a cofactor for the copper-containing dopamine-β-monooxygenase enzyme, which catalyzes hydroxylation of the dopamine side chain to form norepinephrine. Ascorbate provided electrons for reduction of molecular oxygen, transferred by copper to dopamine, and hydrogen atoms to reduce the other oxygen to water. The active enzyme contains Cu^{+}, which is oxidized to Cu^{2+} during hydroxylation of the substrate: reduction back to Cu^{+} specifically requires ascorbate, which is oxidized to $AscH^{\bullet}$. Depression, hypochondria, and mood changes frequently occur during scurvy and could be related to deficient dopamine hydroxylation. Ascorbic acid also appears to be involved in the hydroxylation of tryptophan to form serotonin in the brain and in the degradation of tyrosine by *p*-hydroxyphenylpyruvate hydroxylase.

Carnitine Biosynthesis

Carnitine plays a central role in transporting long-chain fatty acids across the mitochondrial membrane wherein β-oxidation provides energy to cells, especially for cardiac and skeletal muscles. Esterification with carnitine appears to provide a mechanism for transport, storage, and excretion of long-chain fatty acid acyl groups. The biosynthesis of carnitine involves the methylation of lysine, with methionine as methyl donor, and requires ascorbate, ferrous iron, vitamin B_6, and niacin as cofactors for various enzymes of the pathway. The loss of fatty acid-based energy production because of limited carnitine biosynthesis may explain the fatigue and muscle weakness observed in humans with ascorbic acid deficiency.

Other Functions of Ascorbate

Ascorbate is also involved in the hepatic microsomal hydroxylation of cholesterol in the conversion and excretion of cholesterol as bile acids via 7α-hydroxycholesterol. These reactions require the microsomal enzymatic system containing cytochrome P-450 hydroxylase. Impaired cholesterol transformation to bile acids causes cholesterol accumulation in the liver and blood, and atherosclerotic changes in coronary arteries. Hydroxylation and demethylation of aromatic drugs and carcinogens by hepatic cytochrome P-450 appear to be enhanced by reducing agents such as ascorbate. Limited data suggest that ascorbate modulates prostaglandin synthesis and thus exerts bronchodilatory and vasodilatory function as well as anticlotting effects. Vitamin C has been shown to affect various components of the human immune response, including antimicrobial and natural killer cell activity and lymphocyte proliferation. The ability of phagocytes and lymphocytes to concentrate vitamin C at levels up to 100 times higher than in plasma may indicate that the vitamin has a physiological role in these immune cells. Cataracts which appear to be due to oxidation of lens proteins in the eye may also be protected by ascorbate. One report has shown that the use of vitamin C supplements (ranging from 400 to 700 mg day^{-1}) for 10 years or more reduced the number of lens opacities by about 80%. Women who consumed vitamin C supplements for less than 10 years were not protected. Data from other studies suggest that dietary measures to increase plasma ascorbate may be an important public health strategy for reducing the prevalence of diabetes. Ascorbic acid is a potent enhancer of nonheme iron absorption, both in its natural form in fruit and vegetables, and when added as the free compound. In addition, ascorbic acid increases the bioavailability of all iron fortification compounds. The mechanism of ascorbate action is believed to involve the reduction of intraluminal iron by ascorbate to the more absorbable ferrous state and/or the formation of soluble iron complexes in the duodenum. Generally, the enhancement of iron absorption is proportional to the amount of ascorbic acid in the meal, although observed differences in the effect of ascorbic acid may result from varying the amounts of substances in the food that promote or inhibit iron absorption. Ascorbate reacts with nitrite and other nitrosating agents, forming nitric oxide and nitrous oxide and thereby preventing the formation of carcinogenic nitrosamines by reaction between nitrites and amines present in foods in the acid conditions in the stomach.

Ascorbate and Cardiovascular Disease

It is generally accepted that the oxidation of low-density lipoprotein (LDL) particles and the accumulation of oxidized LDL in the vessel wall are key early events in the progression of atherosclerosis. Studies have shown that high plasma concentrations of ascorbate not only correlate with lower concentrations of oxidized LDL, but also function to protect endothelial cells against the detrimental effects of oxidized LDL once this is formed. Since ascorbate is water soluble and is not incorporated in LDL particles, it has been proposed that it may prevent oxidation of LDL particles by scavenging aqueous ROS and RNS in the aqueous milieu. Ascorbate is also capable of regenerating α-TOH from α-TO$^{\bullet}$, which is formed on inhibition of lipid peroxidation by vitamin E. Ascorbyl radicals formed in this process may be reduced to ascorbate by dismutation, chemical reduction, or enzymatic reduction.

Several epidemiological studies have examined the association between vitamin C concentration in blood and the risk of cardiovascular disease. A prospective study of 1605 Finnish men showed that those with increased plasma vitamin C (greater than 11.4 μmol^{-1}) had a 60% decreased risk of coronary heart disease. The Basel Prospective Study of 2974 Swiss men reported that plasma vitamin C concentrations greater than 23 μmol^{-1} were associated with nonsignificant reduction in the risk of coronary artery disease and stroke. In a 20-year follow-up study of elderly adults ($n = 730$) in Britain, plasma concentrations greater than 28 μmol^{-1} were associated with a 30% decreased risk of death from stroke compared with concentrations less than 12 μmol l^{-1}. The Second National Health and Nutrition Examination Survey (NHANES II) reported that the relative risk of coronary heart disease and stroke was reduced by about 26% with serum vitamin C concentrations of 63 – 153 μmol l^{-1} (1.1–2.7 mg dl^{-1}) compared with concentrations of 6–23 μmol l^{-1} (0.1–0.4 mg dl^{-1}). However, supplementation with vitamin C did not reduce the risk of major cardiovascular events. The EPIC–Norfolk Prospective Study in the United Kingdom showed that plasma ascorbate levels were inversely related to mortality from all causes and from cardiovascular disease and ischemic heart disease in men and women. A 20% fall in the risk of all-cause mortality, independent of other risk factors, was associated with a 20 μmol l^{-1} rise in plasma ascorbate, approximately equivalent to a 50 g day^{-1} increase in fruit and vegetable intake. It was also noted that a high plasma concentration of ascorbate was inversely related to various cardiovascular risk factors. Compared with people in the lowest quartile of the ascorbate distribution, those in the highest quartile had a 33% lower risk of coronary artery disease, independent of other known risk factors, including age, blood pressure, plasma lipids, cigarette smoking, body mass index, and diabetes.

Several prospective cohort studies have shown that vitamin C intakes between 45 and 113 mg day^{-1} are associated with reduced risk of cardiovascular disease. Results from the First US National Health and Nutrition Examination Survey (NHANES I) showed that cardiovascular mortality rates were 50% lower than average among participants with the highest vitamin C intake, defined as 50 mg or more per day from the diet plus regular supplements. A Finnish study on 5000 men and women found that women who consumed more than 91 mg day^{-1} vitamin C had a lower risk of coronary artery disease than those who consumed less than 61 mg day^{-1}. However, a similar association was not found for men. The NHANES I Epidemiologic Follow-up Study cohort of more than 11 000 adults showed a reduction in cardiovascular disease of 45% in men and 25% in women whose vitamin C intake from both food and supplements was approximately 300 mg day^{-1}. However, in contrast to the above, other studies reported no association between vitamin C intake and risk of cardiovascular disease. It is important to emphasize that much of these data were obtained from well-nourished populations.

Vitamin C and Cancer

Early epidemiological evidence indicated that high intakes of vitamin C-rich fruit and vegetables and a high vitamin C concentration in serum are inversely associated with the risk of certain cancers. Of 46 such studies in which a dietary vitamin C intake index was calculated, 33 found a statistically significant protective effect, with high intakes conferring approximately a two-fold protective effect compared with low intakes. The evidence for a risk-reducing role of vitamin C is not as strong as for fruit and vegetables. However, an extremely strong and consistent protective effect of vitamin C was found in 17 of 19 studies of stomach, esophageal, and pharyngeal cancers. The Iowa Women's Health Study found a 20% decrease in breast cancer risk with greater than 500 mg day^{-1} of vitamin intake from supplements; in contrast, the Nurses' Health Study, which used the same dietary assessment instrument, found no decreased risk of breast cancer at intake greater than 357 mg day^{-1}.

In a large case–control study in New York, the data showed that increased intake of vitamin C from food and supplements was associated with a reduced risk of rectal cancers. In contrast, the Iowa Women's Cohort Study found no association between vitamin C intake from fruit and supplements of approximately 300 mg day^{-1} and colon cancer risks. However, in women who consumed more than 60 mg day^{-1} vitamin C from supplements compared to no supplements, the risk was reduced by 30%. The association between vitamin C intake and the risk of lung cancer is generally weak, but still in a protective direction in several studies.

Epidemiological and experimental evidence has suggested that vitamin C may protect against the development of gastric cancer by several potential mechanisms, including the following: vitamin C reduces gastric mucosal oxidative stress, DNA damage, and gastric inflammation by scavenging ROS; it inhibits gastric nitrosation reaction for the formation of *N*-nitroso compounds by reducing nitrous acid to nitric oxide and producing dehydroascorbic acid in the stomach; it enhances host immunologic functions; it has a direct effect on *Helicobacter pylori* growth and virulence; and it inhibits gastric cell proliferation and induces apoptosis.

Recent reports on the NHANES II survey in the United States and the EPIC–Norfolk Prospective Survey in the United Kingdom showed that men with a low serum ascorbate concentration may have an increased risk of mortality, probably because of an increased risk of dying from cancer. In contrast, serum ascorbate concentrations were not related to mortality among women. The EPIC–Norfolk report concluded that increases in dietary foods rich in ascorbic acid might have benefits for all-cause mortality in men and women. A report from the World Cancer Research Fund and the American Institute of Cancer Research rated the anticancer effect of ascorbate as 'probable' only for stomach; 'possible' for prostate, mouth, pharynx, esophagus, lung, pancreas, and cervical cancer; and 'insufficient data' for cancers of the colon, rectum, larynx, breast, and bladder.

Vitamin C Status and Requirements

In setting values for average population requirements and individual nutrient intakes, the important question is how do we differentiate between preventing deficiency symptoms, ensuring an adequate intake, and promoting optimal intake for the prevention of disease? The recommended dietary allowance (RDA) of 60 mg day^{-1} in the United States, the reference nutrient intake (RNI) of 40 mg day^{-1} in the United Kingdom, and the population reference intake (PRI) of 45 mg day^{-1} in the European Union were aimed at prevention of the clinical deficiency state, scurvy. However, no obvious deficiency does not necessarily indicate adequacy, and subclinical or marginal deficiency of vitamin C owing to insufficient intake and/or to increased utilization may be common in many disease situations. Increased risk of chronic disease, including cancer, cataract, and coronary heart disease, is associated with low intake or plasma concentrations of vitamin C. However, the contribution of high intake or plasma levels of vitamin C to lowered risk of disease is difficult to assess, as other health-promoting habits generally accompany high vitamin C intake, and clinical trials have shown inconsistent and inconclusive results. The Institute of Medicine in the United States (2000) established an estimated average requirement (EAR) for vitamin C, which is the nutrient intake value that is estimated to meet the requirements of half of a specific gender and life-stage group and was based on evidence that 75 mg day^{-1} vitamin C can maintain near-maximal neutrophil concentration with minimal urinary loss. Thus, the EAR for men aged 19–50 years is 75 mg day^{-1}, with a value of 60 mg day^{-1} for women, based on women having less lean body mass and body water, and a smaller body size than men. There are no data on the distribution of vitamin C requirements in healthy adults; therefore the US RDA for vitamin C, which is the intake value considered to meet the requirements of 97.5% of the relevant life-stage and gender population group, is set at 90 mg day^{-1} for men and at 75 mg day^{-1} for women ($\text{RDA} = \text{EAR} + 2\text{CV}$), assuming a coefficient of variation (CV) of 10%. In Japan, Germany, Austria, and Switzerland, an uptake of 100 mg day^{-1} is recommended for both men and women. There is evidence to show that an average intake of 90 mg day^{-1} of vitamin C can maintain a plasma ascorbate concentration at 50 μmol l^{-1} and for this reason a 'potential protective plasma level' of 50 μmol l^{-1} has also been proposed. This concentration has been shown to inhibit plasma LDL oxidation *in vitro* and may have relevance for the prevention of heart disease *in vivo*. Smokers have been recommended by the United States to consume an additional 35 mg over and above the RDA value, but this recommendation has not been made explicit in other countries. Excessive consumption of vitamin C is unusual, and the upper intake level (UL) set by the United States is 2000 mg day^{-1}, which is achievable only by using chronic megadoses of concentrated vitamin C supplementation.

See also: **Milk Lipids**: Lipid Oxidation. **Vitamins**: Vitamin E.

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Vitamin B_{12}

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Cobalamin or Vitamin B_{12}

The terms vitamin B_{12} and cobalamin, represent a group of several cobalt-containing corroids. A corrin ring with four reduced pyrrole rings and cobalt as central atom, a nucleotide-like compound, and an additional variable compound are their common features (**Figure 1**). B_{12} is the only vitamin containing a metal ion. In biological systems, hydroxo-, aquo-, methyl-, and 5′-deoxyadenosylcobalamin occur, while cyanocobalamin is a decomposition product which, however, is used for therapeutic purposes as also is hydroxycobalamin.

Only microorganisms are able to synthesize vitamin B_{12}. Thus, some animal species have sufficient supply from their intestinal microorganisms. In humans, however, the synthesizing organisms are localized in the colonic part of the intestine which is too distal from the small intestine (ileum), where vitamin B_{12} must be taken up. Consequently, humans obtain B_{12} exclusively from their diet and only animal-derived foods contain sufficient amounts of vitamin B_{12} (**Tables 1** and **2**). Another prerequisite for the uptake of vitamin B_{12} is an intrinsic factor which is secreted from gastric parietal cells and facilitates ileal uptake of cobalamin.

While storage has only minor effects on the concentration of cobalamin in milk (~30–40% in sterilized milk after 90 days at room temperature) and radiation also has small effects, heat destruction plays a major role. Losses in cow milk caused by heat treatment are, sterilization: 20–100%; evaporation: ~50%; boiling: 20%; pasteurization: <10%; ultra-high temperature (UHT): 5–10%. In cheese, an overall loss of 10–50% can be assumed, although there are differences between cheese types, for example, in Gruyère cobalamin concentration even increases due to vitamin B_{12}-synthesizing microorganisms.

Functions of Cobalamin

Adenosylcobalamin (in the cytosol) and methylcobalamin (in the mitochondria) are the coenzyme forms of cobalamin. In humans, these coenzymes are involved in three metabolic reactions (for details see textbooks):

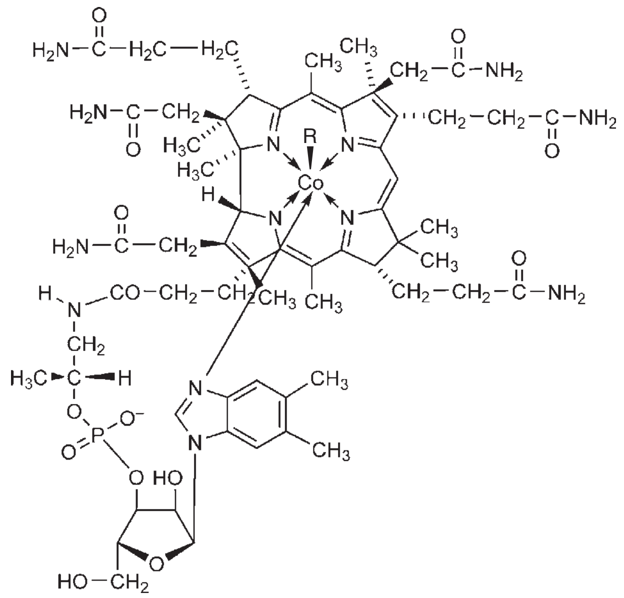
1. Leucine 2,3-amino-mutase reversibly changes α-leucine into β-leucine (3-aminocaproic acid), thus starting the degradation of this amino acid.
2. Methionine synthetase (*N*5-methyltetrahydrofolate homocysteine methyltransferase) reaction. Methionine synthetase needs methylcobalamin as a cofactor for the remethylation of homocysteine to methionine. During this process, the methyl group of 5-methyltetrahydrofolate is transferred to homocysteine, resulting in methionine and tetrahydrofolate. The latter is converted to *N*5,10-methylenetetrahydrofolate, a cofactor of thymidylate synthetase, finally ending up in DNA synthesis.
3. Adenosylcobalamin is needed by methylmalonyl-CoA-mutase for the isomerization of methylmalonyl-CoA to succinyl-CoA during the degradation of propionic acid, thus offering the entrance to the citric acid cycle.

Sources of Cobalamin

Cobalamin can be synthesized only by microorganisms and does not occur in plant-derived food. Therefore, animal-derived foods containing cobalamin are essential for humans; more or less good sources are listed in **Tables 1** and **2**. In cow's milk, the cobalamin content is very constant regarding feed, breed, season, or stage of lactation, except colostrum which has a very high level. In contrast, concentrations in human milk are markedly lower than in cow's milk and vary depending on the above-mentioned parameters. Concentrations in the milk of cow, human, and other species and in some dairy products are given in **Table 2**.

Cobalamin Deficiencies

Although normally in developed countries the vitamin B_{12} uptake meets the recommendations (**Table 3**), cobalamin deficiencies are the most numerous vitamin deficiencies requiring clinical treatment. This is due mainly to a reduced uptake in the intestine that can have various causes. One major prerequisite is a sufficient amount of intrinsic factor



R	Vitamer
–CN	Cyanocobalamin
–OH	Hydroxocobalamin
–5′-Deoxyadenosyl	5′-Deoxyadenosylcobalamin
–CH_3	Methylcobalamin
–H_2O	Aquacobalamin
–NO_2	Nitritocobalamin

Figure 1 Structure of the different vitamin B_{12} vitamers.

Table 1 Vitamin B_{12} concentration in foods

Food	*Concentration (ng 100 g^{-1})*
Ox liver	65 000
Pig liver	39 000
Ox kidney	33 000
Kipper	9700
Mackerel	9000
Herring	8500
Beef	5000
Red perch	3800
Salmon	2900
Egg	1900

From Souci *et al*. (2008).

which is produced by parietal cells of the stomach and binds vitamin B_{12}. This molecule is resistant to intestinal proteolysis and binds under neutral pH conditions to specific mucosal receptors on the microvilli of the enterocytes mostly in the distal ileum to be taken up either as a complex

Table 2 Vitamin B_{12} concentration in milk, dairy products, cheese, and milk from different species

Food	*Concentration (ng 100 g^{-1})*
Emmental cheese (45% fat in dry matter)	3000
Camembert (45% fat in dry matter)	2800
Brie (50% fat in dry matter)	1700
Dried whole milk	1500
Condensed milk (min. 10% fat)	540
Cream cheese (min. 60% fat in dry matter)	530
Yogurt (min. 3.5% fat)	420
Consumer milk (min. 3.5% fat)	410
Cream (min. 30% fat)	400
UHT milk	380
Skim milk	300
Buttermilk	200
Sweet whey	200
Sterilized milk	100
Milk from	
Sheep	510
Cow	420
Buffalo	300
Horse	300
Donkey	110
Goat	70
Human	50

From Souci *et al*. (2008).

Table 3 Recommended daily uptake of vitamin B_{12}

	Vitamin B₁₂		
		μg MJ^{-1} (nutrient density)[a]	
Age	*μg day^{-1}*	*Male*	*Female*
Sucklings <4 months[b]	0.4	0.20	0.21
Sucklings 4–12 months	0.8	0.27	0.28
Children 1–4 years	1.0	0.21	0.23
Children 4–7 years	1.5	0.23	0.26
Children 7–10 years	1.8	0.22	0.25
Children 10–13 years	2.0	0.21	0.24
Children 13–15 years	3.0	0.27	0.32
Adults 15–25 years	3.0	0.28	0.36
Adults 25–51 years	3.0	0.29	0.38
Adults 51–65 years	3.0	0.33	0.41
Adults >65 years	3.0	0.36	0.43
Pregnant[c] women	3.5		0.38
Breast-feeding[d] women	4.0		0.37

[a] Calculated for adolescents and adults, mostly sedentary (PAL-value 1.4).
[b] Estimated value.
[c] 0.5 μg more for filling up body stores and to maintain nutrient density.
[d] Approximately 0.13 μg vitamin B_{12} added per 100 ml secreted milk.
From Deutsche Gesellschaft für Ernährung (DGE) (2007) Die Referenzwerte für die Nährstoffzufuhr. http://www.dge.de/modules.php?name=St&file=w_referenzwerte (accessed May 2009).

or as cobalamin alone. After absorption, cobalamin is bound to transcobalamin and transported to the liver in the portal blood. Reasons for vitamin B_{12} deficiency can be

- chronic atrophic gastritis,
- defects of the gastric mucosa (loss of parietal cells),
- atrophic gastritis (chronic),
- gastrectomy (partial or total),
- malabsorption in the ileum,
- intestinal stasis,
- sucklings when the mother suffers from vitamin B_{12} deficiency,
- congenital disturbances of cobalamin metabolism,
- intestinal parasites, and
- age.

If normal stores in humans are sufficiently filled (2–5 mg) and enterohepatic recirculation occurs, it takes 10–15 years for deficiency symptoms to appear after stopping any B_{12} intake.

Characteristic vitamin B_{12} symptoms are macrocytic hyperchromic anemia (CAVE: this can also appear due to folic acid deficiency) and funicular myelitis, that is, neurological disorders like symmetrical paraesthesias in feet and fingers, disturbances of proprioception and vibratory senses, spastic ataxia, and degeneration of the spinal cord. Vitamin B_{12} delays the onset of signs of dementia (and blood abnormalities), provided it is administered before the onset of the first symptoms. Supplementation with cobalamin improves cerebral and cognitive functions in the elderly; it frequently improves the functioning of factors related to the frontal lobe, as well as the language function of those with cognitive disorders. Adolescents who have a borderline level of vitamin B_{12} develop signs of cognitive changes. Although the homocysteine-lowering effect of vitamin B_{12} and folate supplementation is well known, a protective effect on the development of vascular diseases by this supplementation can be seen as a tendency; however, the final results of some ongoing trials are required before making a final recommendation.

While, in former times, the ingestion of about 1 kg of raw liver per week was used to treat vitamin B_{12} deficiency, nowadays pernicious anemia is treated by a multistep therapy: 1 mg day^{-1} hydroxycobalamin intramuscularly for 7 days followed by the same dose applied weekly for up to 6 weeks, followed by the same dose every 2 months for life. Funicular myelitis is treated by a 2-week therapy with 250 µg day^{-1} to replenish stores followed by lifelong monthly injections of 100 µg if a disturbed absorption was the cause of cobalamin deficiency. Elderly people with reduced absorption due to gastric problems (reduced intrinsic factor) should be supplemented with 500 µg of crystalline vitamin B_{12} daily. Side effects have not been observed, even with pharmacological doses up to 5 mg. An overview of the varying strategies and the discussion of the different methods in different countries can be found in 'Further reading'.

See also: **Vitamins**: General Introduction; Vitamin B_6.

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Folates

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Introduction

Folate is a B-vitamin present *inter alia* in milk and dairy products. The term folate is generic, referring to a class of compounds having similar chemical and nutritional properties as pteroyl-L-(mono)glutamic acid (folic acid) (**Figure 1**). Food-borne folates mostly have reduced pteridine moieties as dihydro- and tetrahydrofolates with different C1-substituents linked to the N_5 and/or N_{10} position (**Figure 1**). Up to seven glutamyl residues are attached, via γ-peptide linkages, to the *para*-aminobenzoic group. Especially reduced folates are labile to interconversion and oxidative degradation. The stability is pH-dependent, with the reduced folates being most stable between pH <2 and >8. Oxidative cleavage of the molecule at the C_9–N_{10} bond results in loss of biological activity. For fortification purposes, synthetic fully oxidized folic acid is used, which is not naturally present in foods, but shows higher stability during food processing and storage.

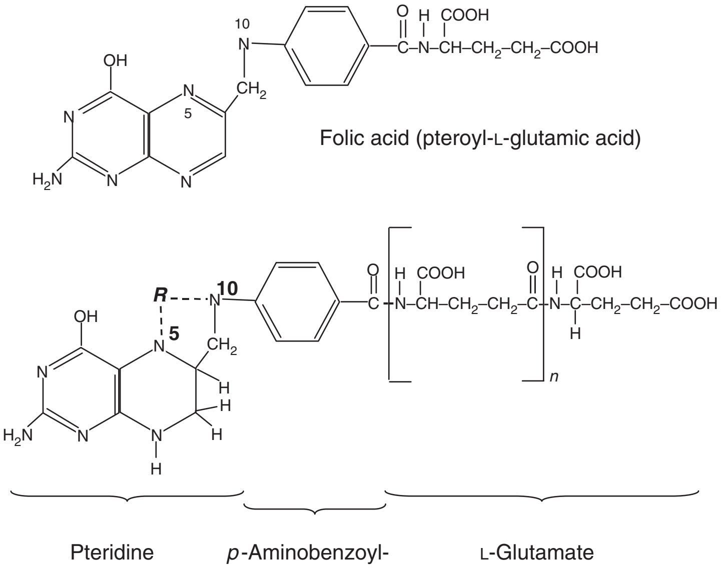
For mammals, folate is an essential micronutrient that has to be obtained from the diet. Folates are involved as a cofactor in one-carbon reactions in the cell, for example, during nucleotide synthesis, supplying carbon units for purine and pyrimidine bases (**Figure 2**).

They are also coenzymes in the methylation cycle, for example, for protein methylation and synthesis of neurotransmitters and phospholipids. Therefore, a good folate status is essential for normal cell division, and the requirement for folate is increased during, for example, growth, pregnancy, and lactation. A suboptimal folate status during pregnancy has been shown to increase the risk of birth defects, for example, neural tube defects (NTD). Deficiency leads to megaloblastic anemia, which is nowadays seldom observed in the United States and European countries.

Dietary folates occur mainly in their polyglutamate forms and are absorbed at physiological concentrations by an active carrier-mediated process in the small intestine after hydrolysis of the polyglutamate chain by the intestinal brush-border enzyme γ-glutamyl hydrolase. When present at higher concentrations, folate monoglutamates also pass through the cell membrane by passive diffusion. During transit through the intestinal mucosa cell, folates are reduced and converted to 5-methyl tetrahydrofolate monoglutamate, the transport form of the vitamin, which is released thereafter into the portal vein. It is estimated that, depending on hepatic folate status, during first pass through the liver, up to 20% of the portal folate is retained. The remaining folate is transported via peripheral blood circulation into the body tissues. In tissues or red blood cells, folates are retained as polyglutamates.

A good folate status is linked to several health benefits, for example, a reduced risk for NTD and other malformations during pregnancy. Furthermore, folate is assumed to play a key role regarding prevention of coronary heart disease by lowering serum homocysteine concentration, which is suggested as an independent risk factor. Less consistent is the evidence regarding the health-protective role and mechanisms with respect to the prevention of certain cancers. Unclear is, for example, the impact of folate status on initiation, progression, and growth of subclinical cancers, as well as on neuropsychiatric disorders, such as dementia and Alzheimer's disease. Consequently, this vitamin remains still today in research focus with the demand to further elucidate and evaluate the health-protective role of natural dietary folate as well as folic acid fortificant.

In the past, nutrient intake recommendations had the aim to prevent deficiency diseases, while today it is rather focused on health benefits in connection with a good nutritional status. Already a decade ago, the US Food and Nutrition Board included the concept of potential health-protective effects when publishing dietary reference intakes for folate by drastically doubling intake recommendations for adults to 400 μg per day. Similar recommendations were made in many European countries. However, food intake surveys in most Western countries show that the average intake of folate – from natural food sources – ranges between 200 and 300 μg day^{-1}, much below recommendations. Fortification policies were discussed as efficient ways to increase the average folate intake. The United States and Canada in 1989 introduced mandatory fortification of cereal-grain products with synthetic folic acid to reduce prevalence of NTD. This aim was achieved and epidemiologic data state that the US population improved their folate status by an increased average intake of folic acid fortificant.


Folic acid (pteroyl-L-glutamic acid)
OH
H2N
10
5
CH2
COOH
C–N–CH–CH2–CH2–COOH
R
n
Pteridine
p-Aminobenzoyl-
L-Glutamate

Folate form	Abbreviation	Substituent	Position
5-Methyl tetrahydrofolate	5-CH_3–H_4folate	–CH_3	N-5
5-Formyl tetrahydrofolate	5-HCO–H_4folate	–HCO	N-5
10-Formyl tetrahydrofolate	10-HCO–H_4folate	–HCO	N-10
5-Formimino tetrahydrofolate	5-CHNH–H_4folate	–CHNH	N-5
5,10-Methylene tetrahydrofolate	5,10-CH_2–H_4folate	–CH_2–	N-5 and N-10
5,10-Methenyl tetrahydrofolate	5,10-CH^+–H_4folate	–C^+H=	N-5 and N-10

Figure 1 Structure of folic acid and folates.

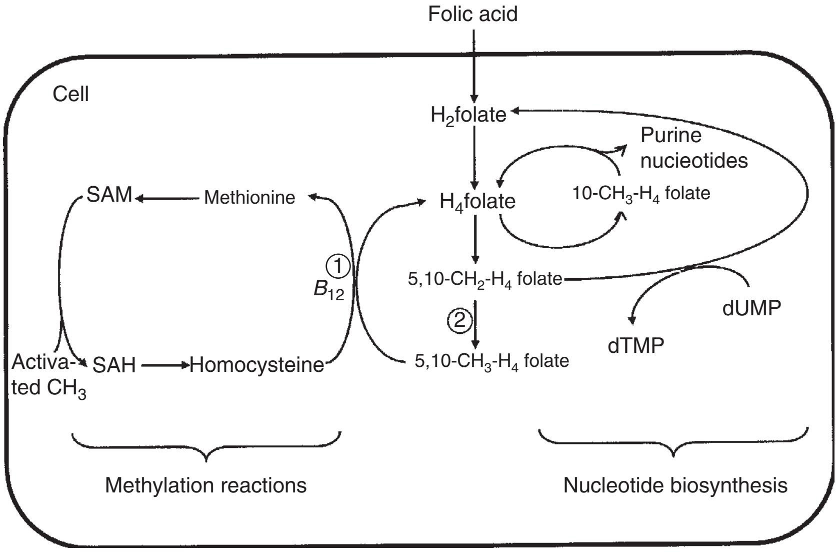

Folic acid
Cell
H_2folate
Purine nucieotides
H_4folate
10-CH_3-H_4 folate
SAM
Methionine
1
B_{12}
5,10-CH_2-H_4 folate
2
5,10-CH_3-H_4 folate
dUMP
dTMP
Activa-ted CH_3
SAH
Homocysteine
Methylation reactions
Nucleotide biosynthesis

Figure 2 Some metabolic function of folates in the cell. H_2folate – dihydrofolate, H_4folate – tetrahydrofolate, –5-CH_3-H_4folate–5-methyl tetrahydrofolate, 5,10-CH_2-H_4folate – 5,10-methylene tetrahydrofolate, 10-HCO-H_4folate – 10-formyl tetrahydrofolate, SAH – *S*-adenosyl homocysteine, SAM – *S*-adenosyl methionine, dTMP – thymidine monophosphate, dUMP – uridine monophosphate, 1 – methionine synthase (vitamin B_{12}-dependent), 2 – methylene tetrahydrofolate reductase.

Analysis

Folate food composition data derive traditionally from microbiological analyses with *Lactobacillus rhamnosus* (ATCC7469) (**Table 1**). The assay principle is based on estimation of growth of the organism, which is dependent on folate with up to three glutamate residues. This assay does not discriminate between the different folate forms and therefore 'total folate' is quantified. In order to differentiate between the individual folate forms, HPLC methods are used, for which an important prerequisite is commercially available standards of the numerous folate forms. After chromatographic separation, various detection modes can be used for quantification of folates. In the past, UV spectrophotometric or fluorescence detection was used widely, but in recent years, the use of LC–MS or MS–MS techniques resulted in improved sensitivity and specificity of the determination. For clinical routine purposes, (radio-) protein-binding assays (RPBAs) are used, which are based on the affinity of folate for bovine folate-binding proteins (FBP). RPBAs have also been used by some investigators for food applications.

Depending on the chosen method for folate quantification, the sample pretreatment (**Figure 3**) is more or less laborious, but it is always a crucial step. The extraction is usually carried out, in a stabilizing buffer with antioxidants, by means of heat, for example, in a boiling water bath or by autoclaving. The next step is an enzyme treatment, where samples are incubated with preparations of γ-glutamyl hydrolase in order to deconjugate the folate polyglutamate chain. Depending on the method of quantification, different sources for γ-glutamyl hydrolase can be chosen. Deconjugation with enzyme preparations from hog kidney or human plasma generates folate monoglutamates as usually required for HPLC quantification because most commercial folate standards are available only in their monoglutamate forms. For microbiological assays, incubation with chicken pancreas enzymes is usual, leading to folate di- and triglutamates. Depending on the food matrix, monoenzyme extraction may not be sufficient to release all folates from the matrix. Di- or trienzyme extraction methods have been developed, using, in variable order, additional enzyme preparations containing thermostable α-amylase or protease. The final step is the purification of the food extract, for example, by centrifugation and filtration. Especially in connection with HPLC quantification, solid-phase extraction methods or affinity chromatography using agarose gel immobilized bovine FBP is used. Different approaches for sample preparation were developed, omitting or replacing individual steps, for example, by sonication or microwave treatment. Thorough quality control and documentation during all steps of sample preparation, however, is required.

Folates in Dairy Products

Dairy products are considered to be moderate folate sources, but are reported to contribute between 10 and 15% to the dietary folate intake. The folate content of milk and fermented dairy products is in the range of a

Table 1 Common methods for folate analyses – an overview

Method	*Microbiological assay*	*Competitive binding assay*	*Biosensor*	*HPLC*
Principle	Estimation of growth of *Lactobacillus rhamnosus* (ATCC7469), responding almost equally to reduced and oxidized folate mono- to triglutamates	Competitive binding of the analyte to a protein or antibody, often using radio-labeled standard	Biosensor-based inhibition-immuno assay with monoclonal antibodies (surface plasmon resonance principle)	Chromatographic separation of folates, identification/ quantification by FLD, DAD, UV, EC, MS, MS-MS
Application	Food samples	Clinical samples	Folic acid-fortified food samples	Food samples
+ Advantages – Limitations	+ High sensitivity, high throughput when automated, accredited methods for food analysis – Quantification of total folate, no discrimination of individual folates	+ Kit commercially available, high throughput – Not suitable for food samples containing different folate forms due to varying affinity of folate binder	+ Kit commercially available, label-free, real-time quantification, suitable for analysis of binding kinetics – Quite expensive, no discrimination of individual folates	+ Determination of individual folates – Dependent on (commercial) availability of folate standards, usually only available as folate monoglutamates

FLD, fluorescence detector; DAD, diode array detector; EC, electrochemical detector; MS, mass spectrometer; MS-MS, tandem mass spectrometer.

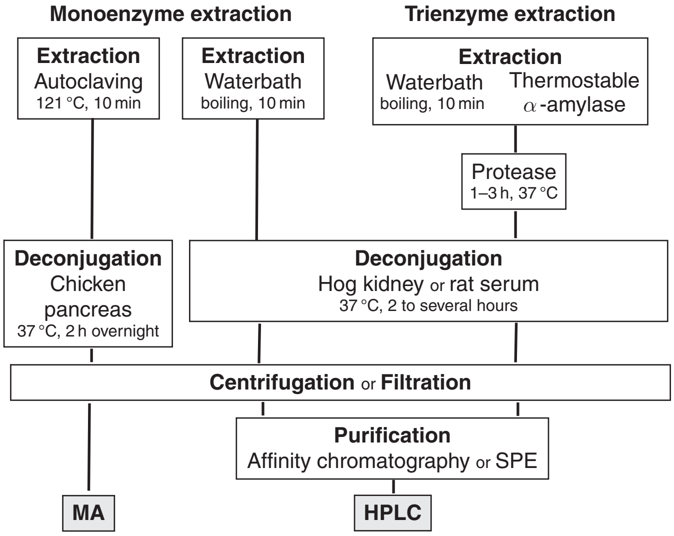


Figure 3 Principles of sample pretreatment prior to folate quantification. HPLC – high-performance liquid chromatography, MA – microbiological assay, SPE – solid-phase extraction.

few micrograms to around 40 µg per 100 g (**Table 2**). Typical folate sources, with rich or high folate content, are leafy green vegetables, pulses, liver, yeast, citrus fruits, and some berries, containing up to several 100 µg per 100 g folate. Fermentation of bovine milk with *L. rhamnosus* (ATCC7469), however, results in a three- to fourfold increase of the initial folate content from 4–8 µg per 100 g to around 20 µg per 100 g. The folate increase during fermentation seems to be dependent on the type of starter culture. Whey products and dairy products containing whey show a slightly higher folate content than milk, for example, up to 30 µg per 100 g folate was found in Cottage cheese. Hard cheeses, for example, Cheddar, Edam, and Gouda, are reported to contain 20–45 µg folate per 100 g and ripened soft cheeses like Brie and Camembert, around 100 µg folate per 100 g and more. The relatively high folate content in cheeses is assumed to be a result of both the reduction of water and biosynthesis of folate by microorganisms during ripening. The folate content of milk seems to undergo minor seasonal variation, which could be caused by feeding with folate-rich green pasture during summer.

The dominant folate derivative in milk is reported to be 5-methyl tetrahydrofolate amounting to about 90%, but some other folate forms like 5-formyl tetrahydrofolate and tetrahydrofolate were also found by some investigators using HPLC methods. Bovine milk contains folate in both monoglutamate and polyglutamate forms. Human breast milk contains similar amounts of folate to bovine milk, with around 5 µg per 100 g, while the folate content in goat's milk is only half as high and in buffalo's milk, for example, below 1 µg per 100 g.

Fortification

Food fortification is defined as the addition of a nutrient to a food above the level that is normally present. This practice is governed differently in countries, and both mandatory and voluntary fortification policies exist, with respect to folates. A prerequisite is the availability of a stable form of the nutrient, which survives food processing and storage and can be synthesized at low cost. For fortification purposes, commonly the synthetic and fully oxidized vitamer folic acid is used.

Risk and safety aspects are of importance with respect to fortification, but safety issues focus exclusively on synthetic folic acid, because natural folates ingested via food are considered safe over a wide intake range. The upper safe level for folic acid intake was set by the US Food and Nutritional Board in 1998 at 1 mg day^{-1}. In the United States, folic acid levels in fortified foods of 140 µg per 100 g are far below doses reported to show adverse epileptogenic and neurotoxic effects in animal models.

However, a potential risk of folic acid fortification is the masking of an undiagnosed vitamin B_{12} deficiency. Vitamin B_{12} deficiency is fairly common in the elderly population due to age-related atrophy of the gastric mucosa and can lead to irreversible neurological dysfunction and pernicious anemia. Due to the metabolic interrelationship of both vitamins, vitamin B_{12} deficiency can induce a secondary folate deficiency. According to the methylfolate-trap hypothesis, the activity of the vitamin B_{12}-dependent enzyme, methionine synthase, is reduced in B_{12} deficiency (**Figure 2**). This results in accumulation of methyl groups in the form of 5-methyl tetrahydrofolate

Table 2 Folate content of milk and dairy products

Dairy product		*Total folate content* (μg *per 100 g)*
Milk	Pasteurized, bovine	4–10
	UHT, bovine	4–8
	Condensed milk, bovine	8–11
Goat		1–3
Sheep		2
Buffalo		1
	Camel	4
Fermented milk	Filmjölk	8–11
	Yogurt	7–18
	Buttermilk, bovine, cultured	2–15
Cottage cheese	Plain	9–12
	Average	9–27
Soft curd cheese	Plain	4–12
Soft cheese	Brie	38–150
	Camembert	44–102
	Feta	18–62
Hard cheese	Average	10–45
	Edam	16–40
	Gouda	43
	Emmental	40
	Grevé®	16–18
	Herrgård®	18–21
	Cheddar	16–33
	Västerbotten®	13
Blue cheese	Average	24–94
	Kvibille ädel	30–50
Whey products	Whey	6
	Whey (cream) cheese	5–50
Milk powder	Bovine, skimmed	21–60
Whipping cream	Average	4–7

Data given as ranges, as compiled from numerous publications and food composition tables, analyzed by microbiological assay or HPLC.

which is no further converted into tetrahydrofolate. Synthetic folic acid bypasses this reaction and is converted via dihydrofolate into tetrahydrofolate, thereby masking the B_{12} deficiency and reducing methionine synthase activity.

In 1998, the United States and Canada introduced nationwide mandatory fortification of cereal-grain products and ready-to-eat cereals, to reduce the incidence of pregnancies affected by NTD. The folic acid fortification level of 140 μg per 100 g, aiming to provide an additional 100 μg folic acid to the folate intake from the diet, was chosen to increase the average intake of women of childbearing age while preventing excessive intake by other population groups. Folate status of the US population improved in the post-fortification period, resulting in less than 1 and 5% being at risk from too low serum and erythrocyte folate levels, respectively. However, smaller concentrations of (unmetabolized) folic acid were found in peripheral blood of a certain group of the US population in addition to the normal plasma folate form 5-methyl tetrahydrofolate.

Also several other countries, for example, Chile and Costa Rica, introduced folic acid fortification of staple foods with the aim of reducing births complicated by NTD. Most European countries, however, have voluntary fortification policies, as to date no final agreement has been reached regarding potential risks from long-term exposure to folic acid from fortification. An alternative approach to the current fortification practice with folic acid, which was discussed with respect to potential risks of folic acid fortification, is the fortification with synthetic (6*S*)-5-methyl tetrahydrofolate, a natural food folate form. However, synthesis of the nonracemic (biological) diasteromer (6*S*)-5-methyl tetrahydrofolate is not cost-efficient and this form of folate is less stable than folic acid fortificant during food processing and storage.

While the protective role of folates with respect to the prevention of NTD is commonly accepted, no conclusive recommendations are made regarding the effect of folic acid fortification on initiation, progression, and growth of subclinical cancers.

Folic acid-fortified foods are, like in the United States and Canada, staple foods and foods of cereal-grain character. Other fortified products – offered in some countries by voluntary fortification – are beverages (juices, soft drinks, and mineral water), snacks, and table salt. Also, dairy products have been considered as vehicles for folic acid fortification.

Processing

Food composition data for milk and dairy products are usually presented as total folate content. Only few systematic data are available regarding the pattern of individual folate forms in milk and processed dairy products, but the folate forms, tetrahydrofolate, 5-methyl tetrahydrofolate, 5-formyl tetrahydrofolate, and 10-formyl folic acid, were reported to be present in fermented dairy products and cheese.

The chemical reactivity of reduced food folates makes the vitamin one of the most vulnerable with respect to losses during food processing. Following a number of different processes, considerable losses have been reported due to degradation enhanced by oxygen, light, and heat. Folates are also easily lost by leakage into water during processing.

Fermentation of milk increases folate content by 40–50%, as reported for yogurt and a Swedish fermented milk product, called filmjölk. The organism of the starter culture seems to have influence on the resulting folate content and pattern, with respect to species and strains. *Streptococcus thermophilus* species were reported to lead to a three- to fourfold folate increase, while fermentation with *Lactobacillus bulgaricus* resulted in only a minor increase or even a decrease in folate content in the final product. An increase of folate content was reported after fermentation with *S. thermophilus*, *Lactobacillus acidophilus, Bifidobacterium longum*, and *L. bulgaricus*, which thereafter during storage at 4 °C over a 3-week period decreased, suggesting folate utilization by the lactic acid bacteria.

The increase of folate content in hard cheeses can be explained mainly by reduction of the water content. The different starter cultures and length of ripening period influence folate concentrations in the final product.

Mild heat treatment seems to cause minor losses of milk folate amounting to less than 10%, while folate retention during ultra-high temperature (UHT) treatment is substantially lower. Folate retention is affected not only by the amount of soluble oxygen present in the milk before processing, but also by the amount of protective ascorbic acid.

Storage of pasteurized milk in the refrigerator during shelf-life does not affect folate content, while folate retention was lower during storage of UHT milk at ambient room temperature. Inconsistent data are available regarding refrigerated storage of fermented dairy products and cheeses, and most probably effects on folate content are also affected by the lactic acid bacteria strain.

Bioavailability of Dairy Folates

Bioavailability is defined as the absorption and metabolic utilization of a nutrient, thereby referring to the proportion of the ingested nutrient being absorbed and metabolized or stored. A nutrient’s bioavailability is dependent on host-related factors, for example, folate status of body stores, gastrointestinal function, health status, genetic factors, age, and possibly sex. Also extrinsic factors like properties of the ingested folate (oxidation status, substituent, and length of the polyglutamate chain), the food matrix (presence of pro- and antioxidants, fibers, binding proteins), and processing and storage were reported to influence bioavailability.

Different methods can be used to assess folate bioavailability (**Table 3**). *In vitro* methods, using everted sacs or Caco cell culture models, allow the study of mechanisms during absorption and affecting factors, but do not reflect the complexity of *in vivo* folate absorption. An advanced computer-controlled *in vitro* model simulating the gastrointestinal tract is the so-called TNO gastroIntestinal Model, TIM. The TIM model has been applied successfully to study effects of food processing on folate bioaccessibility in dairy products and other foods. Animal models were used to investigate the biological role of folate in growth and reproduction, but how relevant is the information from animal trials to predict folate metabolism in humans is questionable. Human *in vivo* trials are time- and cost-intensive and limited by ethical restrictions. However, as short-term protocols, they can supply information regarding folate absorption. Intervention trials can be used to study long-term effects on folate status. Several trials have been carried out with the aim of investigating effects of processing and fortification of dairy foods on folate bioavailability (*see* section ‘Bioavailability Studies with Dairy Products’).

Folate-Binding Proteins

Folate in milk is bound to an excess of FBP. At saturation, FBP binds approximately 1 mol of folate per mol protein. The function of FBP is to sequester folate from the blood in order to secure an adequate folate supply to the neonate although the concentration of folate in milk is relatively low. FBP occurs in two forms, whereby the major part exists in a soluble form and a very small amount as particulate FBP. The transport of folate in, for example, serum or milk has been suggested as the physiological function of soluble FBP. Particulate FPB is found in cell membranes and plays a role during membrane transport.

Table 3 Models used to study folate bioavailability

Model/assay	In vitro	*Animal*	*Human short-term*	*Human long-term*
Example	• Everted sacs/ dissected loop/cell culture model • TIM	Chicken, rat, pig, monkey	Investigator-controlled feeding trials	Random-controlled intervention trials (CRT)
Principle/studied effect	• Use of human cell culture model (Caco-2 cells) to study uptake and transport/ flux • Simulation of the human GIT to determine stability and bioaccessibility of nutrient	• Study biological role of folate on growth and reproduction • Study effects on tissue folate distribution and concentrations • Depletion/repletion models	Random application of supplements, foods, meals (with labeled compounds) as • single doses • multiple doses. Determination of folate concentrations and metabolites in body fluids, absorption kinetics by plasma AUC.	Effect of (ideally blind) intervention with supplements, foods, meals on folate status parameters/end points
+ Advantages − Limitations	+ No ethic restrictions − No reflection of complexity of *in vivo* absorption	+ Information on folate metabolism and deficiency − Limited (quantitative) information due to physiological differences between species	+ Information on absorbed folate − Problems with compliance and nonphysiological doses, expensive	+ Evidence of effect from intervention on status − Problems with compliance, choice of relevant intervention period, expensive

AUC, area under the curve; GIT, gastrointestinal tract; TIM TNO, gastroIntestinal Model.

Pasteurized milk contains between 160 and 250 nmol l^{-1} FBP. Data regarding survival of FBP in processed dairy products are conflicting; some investigators reported reduction of the FBP content while others observed reduction of the folate-binding capacity. While 80–90% of the initial FBP content of untreated milk survives pasteurization at 72 °C, it is almost completely destroyed by intense heat treatment, for example, UHT treatment or heat treatment before fermentation of milk. FBP concentrations of <15 nmol l^{-1} are found in yogurt, other fermented dairy products, and UHT milk. Skimmed milk powder and Cottage cheese, however, contain up to 2000 nmol kg^{-1} and 500 nmol l^{-1} FBP, respectively. During cheese-making, half of the FBP from the milk, around 100 nmol l^{-1}, is recovered in the whey fraction. Hard cheeses contain around 13 nmol kg^{-1} FBP. FBP was shown to enhance folate stability under certain processing conditions.

Initially, FBP was considered to stimulate folate absorption in general, as it could be shown that breast-fed babies had a better folate status than bottle-fed babies. This was attributed to the presence of intact FBP in human breast milk, while in infant formula FBP was destroyed by heat treatment. However, most of these studies investigating the effect of FBP on folate bioavailability were animal experiments. FBP was also suggested to prevent folate uptake by intestinal bacteria. Recent *in vitro* and *in vivo* studies, however, indicate reduced folate bioavailability by complex building with FBP.

Bioavailability Studies with Dairy Products

Only a few bioavailability trials have been carried out on dairy products fortified with synthetic folic acid or the alternative fortificant (6*S*)-5-methyl tetrahydrofolate.

Using the *in vitro* model TIM, folate bioaccessibility was estimated from pasteurized milk and yogurt samples which were fortified with folic acid or (6*S*)-5-methyl tetrahydrofolate with and without FBP in equimolar concentrations (ca. 900–1000 nmol l^{-1}). Unexpectedly, folic acid in both milk and yogurt was less bioaccessible in the presence of FBP (**Figure 4**). For added (6*S*)-5-methyl tetrahydrofolate, the folate form naturally present in dairy products, a decrease of folate bioaccessibility from FBP was observed only in yogurt, but not in milk. Both folate forms were more than 80% bioaccessible in yogurt without added FBP. Yogurt usually contains almost no FBP. A similar negative effect of FBP on short-term folate absorption was observed in human volunteers, who consumed, in random order, pasteurized milk or fermented milk (filmjölk) fortified with (6*S*)-5-methyl tetrahydrofolate with or without FBP. Absorbed folate was assessed by post-dose plasma concentrations (area under the plasma curve, AUC),

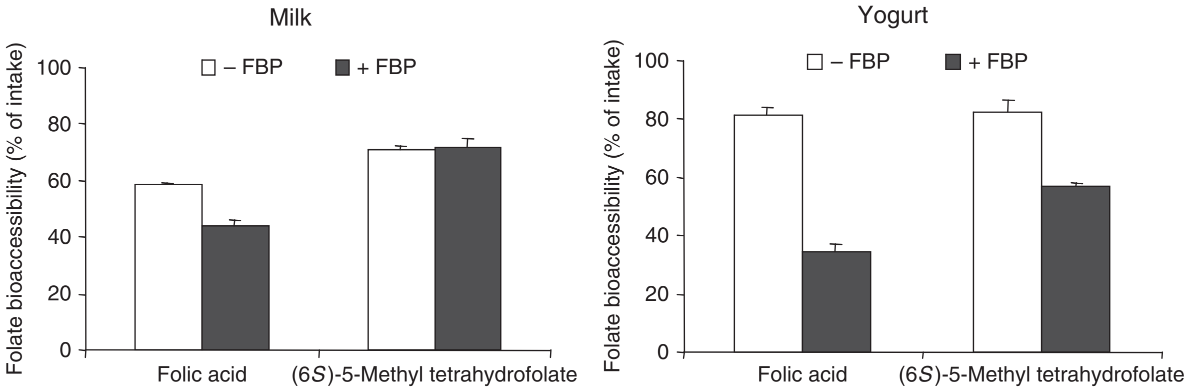


Figure 4 Effect of folate-binding proteins on the bioaccessibility of folate fortificants in dairy products as studied in dynamic *in vitro* TNO gastroIntestinal Model, TIM. TIM – TNO gastroIntestinal Model, FBP – folate-binding proteins, panels: left – milk, right – yogurt, -FBP (white bars) – no FBP added, +FBP (grey bars) – 1100–1200 nmol l^{-1} FBP added; added fortificants: 900–1200 nmol l^{-1} folic acid or 5-methyl tetrahydrofolate. Compiled from Arkbåge K, Verwei M, Havenaar R, and Witthöft C (2003) Bioaccessibility of folic acid and (6S)-5-methyltetrahydrofolate decreases after addition of folate-binding protein to yogurt as studied in a dynamic in vitro gastrointestinal model. *The Journal of Nutrition* 133: 3678–3683.

which were corrected for ingested doses (**Figure 5**). For seven of nine volunteers, FBP reduced short-term folate absorption, as shown by significantly greater AUCs after consumption of the fermented dairy product without FBP. Median apparent absorption was estimated to be 86% of the ingested dose of the FBP-free fermented milk and 62% and 55% for fermented milk and pasteurized milk containing added FBP, respectively. However, after a 12-week intervention with folic acid-fortified (400 μg day^{-1}) milk (resolubilized from powder), female volunteers improved their folate status. In a Dutch intervention trial with pasteurized and UHT milk, both fortified with folic acid (200 μg day^{-1}), the folate status of volunteers improved after 4 weeks. The Dutch investigators concluded that milk was a suitable matrix for folic acid fortification, and that the endogenous FBP content in pasteurized milk did not affect the bioavailability of folic acid fortificant significantly.

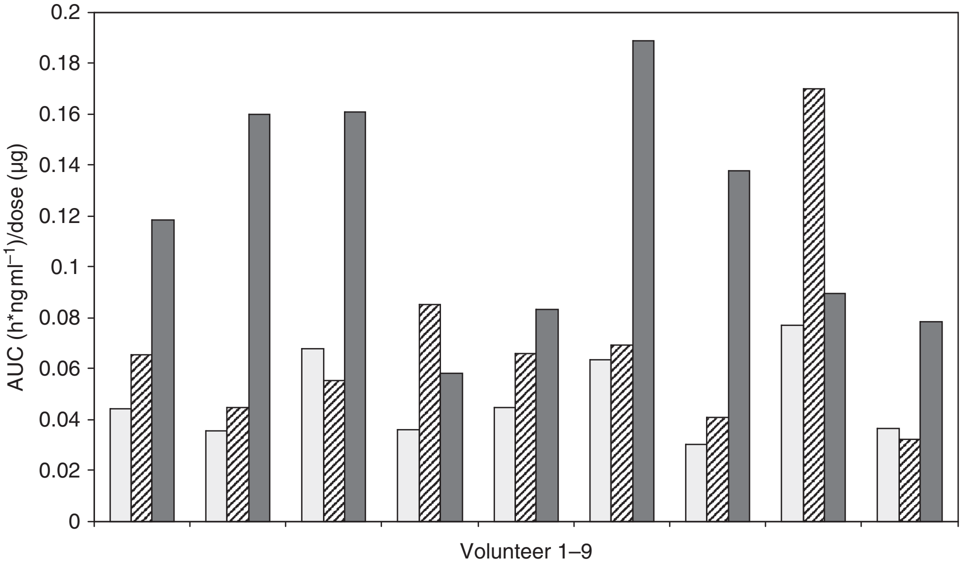


Figure 5 Relative absorption of (6*S*)-5-methyl tetrahydrofolate fortificant from dairy products with and without folate-binding protein in nine human volunteers. Relative absorption estimated by the area under the plasma folate concentration curve (dose-corrected AUC) for: light-grey bars – pasteurized milk with 250 μg (6*S*)-5-methyl tetrahydrofolate and 260 nmol FBP, striped bars – fermented milk with 180–205 μg (6*S*)-5-methyl tetrahydrofolate and 156–442 nmol FBP, dark grey bars – fermented milk with 187–234 μg (6*S*)-5-methyl tetrahydrofolate, no FBP. Test foods were ingested after overnight fasting on independent days in random order; blood was collected until 10 h postdose. Compiled from Witthöft CM, Arkbåge K, Johansson M, *et al*. (2006) Folate absorption from folate-fortified and processed foods using a human ileostomy model. *British Journal of Nutrition* 95: 181–187.

Summary and Future Trends

This article summarized data on the folate content of milk and processed dairy products. Systematic studies are, however, not many. Dairy products are 'moderate sources' of folate, but they provide between 10 and 15% of the average dietary folate intake in Western populations.

Folates have been recognized regarding their health beneficial effects, for example, with respect to their protective role against NTD. With regard to the gap between actual dietary folate intake and nutritional recommendations, mandatory folic acid fortification has been discussed or introduced in several European countries; here, cereal-grain staple foods are targeted. Milk and dairy products, however, were also discussed as suitable matrices for folic acid fortification. In order to develop folate-rich functional foods with benefit for the consumer, more research is required regarding effects of processing on fortified products and their physiological effects on humans.

See also: **Fermented Milks**: Nordic Fermented Milks; Yoghurt: Types and Manufacture.

Further Reading

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Biotin (Vitamin B_7)

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Introduction

The chemical structure of biotin (hexahydro-2-oxo-1H-thieno (3,4-D) imidazol-4-valeric acid) is shown in **Figure 1**. Of the eight isomers that exist, only D-(+)-biotin is biologically active and occurs naturally. Although in milk (cow's as well as human) biotin occurs in the free form, most foods of animal origin or cereals contain it in an enzyme-bound form named biocytin (ε-N-biotinyl-L-lysine).

Biotin is essential for many microorganisms and numerous animals, including humans, and it can be synthesized by the colonic microflora. Although recent findings demonstrate an uptake of water-soluble vitamins (including biotin) by colonocytes, it remains unclear whether the amount produced is sufficient to fulfill all physiological functions or it is only a kind of fine-tuning of body homeostasis. The loss of biotin during processing or storage of food is generally small or negligible. There was no loss of biotin observed in milk kept in frozen state for some weeks or in dried milk stored at room temperature for 1 year or subjected to 2 h of sunlight or 10 Gray gamma radiation. Even UHT sterilization did not lead to biotin loss, whereas pasteurization and/or sterilization caused a <10% loss. In evaporated, condensed, or dried whole milk, the loss is <15%.

Functions of Biotin

Carboxylation and decarboxylation processes are the main reactions in which biotin is involved. It is linked to the enzymes by an amide bond between the amino group of a specific lysyl residue in the active center of the respective apo-carboxylase and its valeric acid side chain. During the (ATP-dependent) carboxylase reaction, a CO_2 molecule is attached to biotin at the ureido nitrogen, which is opposite to the side chain. The activated CO is then transferred from carboxybiotin to the substrate. The four representative biotin-dependent enzymes (as a prosthetic group) of the intermediary metabolism are

- Acetyl-CoA-carboxylase (catalyzes the first step in fatty acid synthesis)
- Propionyl-CoA-carboxylase (catalyzes the carboxylation of propionyl-CoA to form methyl-malonyl-CoA)
- Pyruvate carboxylase (catalyzes the carboxylation of pyruvate to form oxaloacetate)
- 3-Methylcrotonyl-CoA-carboxylase (plays an important role in the catabolism of leucine) which can be metabolized via alternative pathways as well. Such metabolites detected in urine suggest biotin depletion at the tissue level in individuals without congenital metabolic disorders.

Recently, some additional functions of biotin have been found: it induces dermal differentiation and has been used to treat lameness in animals and brittle nails in humans.

Sources of Biotin

Tables 1 and **2** give an overview of dietary sources of biotin and its appearance especially in the milks of various species and in dairy products; the recommended daily intake is given in **Table 3**. In cheese, changes in the concentration of biotin depend on processing procedures or maturation (microbiological synthesis, e.g., in Limburger or Brie); the highest concentrations are often found in the outer layers but can also extend throughout the cheese.

Biotin Deficiencies

One outstanding biotin deficiency is egg white injury, caused by extensive consumption of raw egg white, which contains the glycoprotein avidin, which binds biotin and is resistant to intestinal digestion. Symptoms of biotin deficiency are severe dermatitis, hair loss, and neuromuscular dysfunction. In several other species (mouse, rat, hamster), subclinical biotin deficiency has been shown to be teratogenic, and this may be the case also in humans.

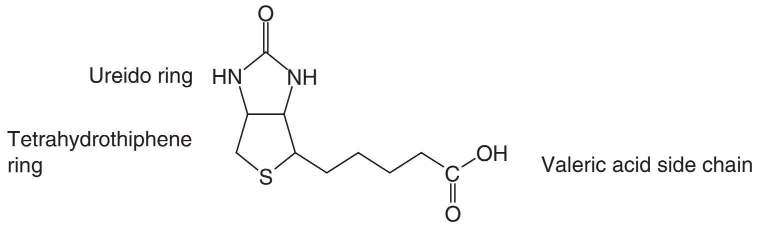

Figure 1 Structure of biotin.

Table 1 Biotin concentration in foods

Food	*Concentration ($\mu g\ 100\ g^{-1}$)*
Brewer's yeast	115
Ox liver	100
Calf liver	75
Soy bean, dry seed	60
Peanut	34
Egg	25
Oat flakes	20
Wheat germ	17
Rice, unpolished	12
Wheat, wholemeal flour	8

From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 6th edn. Stuttgart: Medpharm Scientific Publishers.

Table 2 Biotin concentration in milk, dairy products, cheese, and milk from different species

Food	*Concentration ($\mu g\ 100\ g^{-1}$)*
Dried whole milk	24
Limburger (40% fat in dry matter)	9
Condensed milk (min. 10% fat)	8
Quark/fresh cheese, from skim milk	7
Brie (50% fat in dry matter)	6
Camembert (45% fat in dry matter)	5
Cream cheese (min. 60% fat in dry matter)	4
Consumer milk (min. 3.5% fat)	4
Sterilized milk	4
UHT milk	4
Yogurt (min. 3.5% fat)	4
Cream (min. 30% fat)	3
Buttermilk	2
Skim milk	2
Sweet whey	1
Milk from	
Buffalo	11
Sheep	9
Goat	4
Cow	4
Human	1

From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 6th edn. Stuttgart: Medpharm Scientific Publishers.

Table 3 Recommended daily uptake of biotin

Age	*Biotin ($\mu g\ day^{-1}$)*
Sucklings <4 months	5
Sucklings 4–12 months	5–10
Children 1–4 years	10–15
Children 4–7 years	10–15
Children 7–10 years	15–20
Children 10–13 years	20–30
Children 13–15 years	25–35
Adults 15–25 years	30–60
Adults 25–51 years	30–60
Adults 51–65 years	30–60
Adults >65 years	30–60
Pregnant women	30–60
Breast-feeding women	30–60

From Deutsche Gesellschaft für Ernährung (DGE) (2007) Die Referenzwerte für die Nährstoffzufuhr. http://www.dge.de/modules.php?name=St&file=w_referenzwerte (accessed April 2009).

In humans, biotin deficiency is very rarely detected in industrial countries with an average biotin intake of 35–70 $\mu g\ day^{-1}$ which exceeds the recommended daily intake (see **Table 3**). However, it must be recognized that such recommendations are difficult to calculate, as the sources of biotin (diet, microorganisms, bioavailability) are quite variable. In general, biotin uptake seems to be adaptively regulated and the sodium-dependent multivitamin transporter hSMVT is involved in this regulation.

Besides those who consume raw egg white in excessive amounts, persons on long-term parenteral nutrition with insufficient biotin supplementation, people suffering from congenital biotinidase deficiency ('secondary biotin deficiency'; see below), or patients on a long-term anticonvulsant therapy are at risk of developing biotin deficiency symptoms, although only after biotin deprivation for months or even years. Symptoms like scaly/seborrheic and red/eczematous skin rash around the eyes, nose, and mouth, and anorexia, and also neurological symptoms like depression, lethargy, hallucinations, and paraesthesiasis of the extremities have been described. In children, generally comparable symptoms appear under parenteral nutrition, but earlier (3–6 months).

Biotinidase is an enzyme that catalyzes the hydrolysis of biocytine to biotin and lysine in the intestine, making biotin bioavailable; in addition, biotinidase plays a role in biotin recycling. Deficiency of this enzyme is therapeutically treated by a daily supplementation with 50–150 μg of free biotin. In this context, it is interesting to note that in such patients intestinal biotin production is insufficient.

See also: **Milk Proteins**: Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins. **Vitamins**: General Introduction.

Further Reading

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Niacin

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Introduction

Niacin is the common name for a group of vitamers with a biological activity associated with nicotinamide. These include nicotinamide itself (pyridine-3-carboxamide), nicotinic acid (pyridine-3-carboxylic acid), and a number of pyridine nucleotide structures. Nicotinamide and nicotinic acid are white, crystalline structures. When dissolved in water, they have a maximum UV-absorbance at 263 nm. Each of these vitamers can be converted to the other; their structures are shown in **Figure 1**. Two coenzymes also exist, nicotinamide-adenine dinucleotide (NAD^+) and nicotinamide-adenine dinucleotide phosphate ($NADP^+$), and the structures of these are shown in **Figure 2**. These coenzymes are most prominent in animal food sources and possess a high bioavailability, in contrast to nicotinic acid which has a lower bioavailability and is present in lower concentrations, mainly in plants. Niacin in cereals is found mainly in the outer layer, bound to the protein niacytin and thus having a bioavailability of only 30%. In milk, almost all niacin appears in the free form.

Niacin is stable to sunlight, to various storage conditions, and also to heat used in dairy processes. High pressure-low temperature treatment leads to a significant loss from raw milk. Exposing liquid milk to gamma radiation (1 megarad) leads to a loss of about 30%, whereas milk powder resists even higher doses of radiation. During cheese production, most of the niacin passes to the whey, which can partly be compensated by an increase of the niacin content in the outer layers during maturation (~10–25-fold).

Functions of Niacin

Most important are the roles of NAD and NADP as coenzymes of dehydrogenases. The C4 position on the pyridine ring of the nicotinamide part of the molecule participates in oxidation as well as in reduction reactions by taking up the hydrogen ion. Thus, NADH and NADPH are intermediate hydrogen and electron carriers.

NADP-dependent coenzymes are involved in cytosolic syntheses (reductive biosynthetic processes), while NAD-dependent enzymes are located in the mitochondria, delivering H_2 to the respiratory chain for oxidation and energy metabolism. NADH is used in the respiratory chain and can be delivered by the tricarboxylic acid cycle, glycolysis, the β-oxidation of fatty acids, and the degradation of amino acids. NADPH is used for the synthesis of fatty acids, cholesterol, and steroids, and for hydroxylations; it can be delivered from the pentose phosphate pathway, photosynthesis, malic enzyme, and extramitochondrial isocitrate dehydrogenase.

In addition, NAD has non-redox functions: the energy provided by breaking the high-energy bond of the glycosidic linkage between nicotinamide and ribose allows the addition of ADP-ribose to quite a number of nucleophilic acceptors. NAD serves also as a substrate in poly(ADP-ribose) synthesis (important for DNA repair processes) and in mono(ADP-ribosyl)ation reactions (involved in endogenous regulation of various aspects of signal transduction and membrane trafficking in eukaryotic cells).

NADP serves as the substrate for the formation of nicotinic acid adenine dinucleotide phosphate (NAADP), which is involved in the regulation of intracellular calcium stores. Recent publications describe additional functions of nicotinic acid, which exhibits vasodilatory and antilipolytic effects, and of niacin, which lowers plasma levels of C-reactive protein (CRP), a general marker for inflammation, which is also involved in cardiovascular disease. The nicotinic acid receptor is also discussed as a target for the development of dyslipidemic drugs for the prevention and treatment of cardiovascular disease.

Sources for Niacin

Tables 1 and **2** give an overview of the dietary sources of niacin and specifically the milks of various species and dairy products.

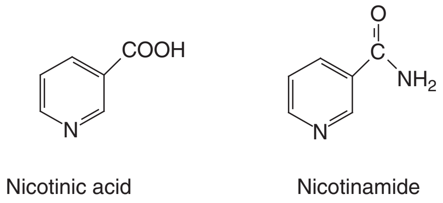

Figure 1 Structure of nicotinic acid and nicotinamide.

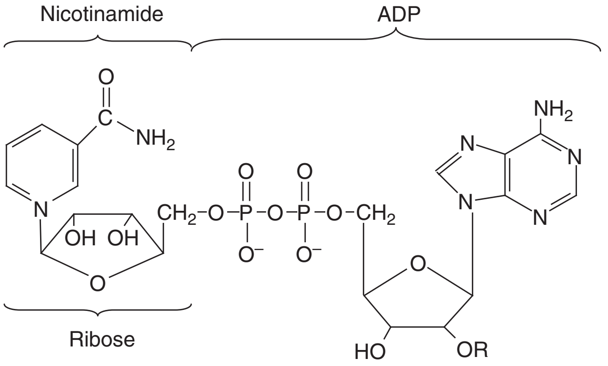

Figure 2 Structure of nicotinamide-adenine dinucleotide (NAD^+; R = H) and nicotinamide-adenine dinucleotide phosphate ($NADP^+$; R = PO_3H_2).

Table 1 Niacin concentration in foods

Food	*Concentration ($\mu g\ 100\,g^{-1}$)*
Wheat bran	18 000
Pig's liver	16 000
Ox liver	15 000
Peanuts	15 000
Coffee, roast	14 000
Beef	7500
Salmon	7500
Ox heart	7200
Chicken	6800
Pig heart	6600
Halibut	5900
Rice (unpolished)	5200
Mushroom	5200
Pork	5000
Wheat flour (wholemeal)	5000
Sunflower seeds	4100
Herring	3800
Trout	3400

From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart: Medpharm Scientific Publishers.

Table 2 Niacin concentration in milk, dairy products, cheese, and milk of different species

Food	*Concentration ($\mu g\ 100\,g^{-1}$)*
Limburger (40% fat in dry matter)	1200
Brie (50% fat in dry matter)	1100
Camembert (45% fat in dry matter)	1100
Blue cheese (50% fat in dry matter)	870
Dried whole milk	700
Condensed milk (min. 10% fat)	260
Sweet whey	190
Cream cheese (min. 60% fat in dry matter)	110
Buttermilk	100
Skim milk	95
Consumer milk (min. 3.5% fat)	90
Sterilized milk	90
UHT milk	90
Yogurt (min. 3.5% fat)	90
Cream (min. 30% fat)	80
Milk from	
Sheep	450
Goat	320
Human	170
Horse	140
Cow	90
Buffalo	80
Donkey	74

From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart: Medpharm Scientific Publishers.

Niacin Deficiencies

Owing to the coenzymes' major role in energy metabolism, the requirement is related to energy intake. The recommended dietary allowance in the United States has been set at 6.6 niacin equivalents (NE; see below) per 1000 kcal per day; the requirements have been established experimentally for adults and were set at a minimum of 8 mg NE day^{-1}. However, the overall calculation is difficult because the intestinal utilization rates are unknown and, in addition, NAD can be synthesized from tryptophan when the latter is available in sufficient amounts. Therefore, the niacin content of the diet is often given as niacin equivalents, that is, 1NE = 1 mg niacin = 60 mg tryptophan. The tryptophan content of some selected foods as a percentage of total protein is eggs 1.5%; milk 1.4%; animal products >1.1%; cereals, fruits, and vegetables ~1%; and maize ~0.6%. A 'normal, mixed diet' in industrial countries includes about 13 mg NE day^{-1}, thus providing more than the required amount. Milk, for example, is regarded as a good pellagra-preventing food (see below), not because of its niacin concentration, which is relatively low, but because of its high concentration of tryptophan. Other good sources of niacin are shown in **Table 1**. Niacin content of milk and other dairy products are listed in **Table 2**. The recommended daily uptake is shown in **Table 3**.

Table 3 Recommended daily uptake of niacin

	Niacin	
	(mg-equivalent day^{-1}) *1 mg niacin equivalent* *= 60 mg tryptophan*	
Age	*Male*	*Female*
Sucklings <4 months	2 (estimated)	
Sucklings 4–12 months	5	
Children 1–4 years	7	
Children 4–7 years	10	
Children 7–10 years	12	
Children 10–13 years	15	13
Children 13–15 years	18	15
Adults 15–25 years	17	13
Adults 25–51 years	16	13
Adults 51–65 years	15	13
Adults >65 years	13	13
Pregnant (>4 month)		15
Breast-feeding		17

From Deutsche Gesellschaft für Ernährung (DGE) (2007) Die Referenzwerte für die Nährstoffzufuhr. http://www.dge.de/modules.php?name=St&file=w_referenzwerte (accessed April 2009).

The main disease caused by niacin deficiency is pellagra (pellagrosis; maidism; mal de la rosa, Saint Ignatius itch, erythema endemicum; Jolliffe syndrome). It is characterized by the three Ds dermatitis, diarrhea, and dementia. The name pellagra is derived form pelle agra = rough skin, that is, the prominent symptom is a rough skin in light-exposed areas with a symmetric appearance and with a distinct border to normal skin (glove-like appearance on the hands). Disorders of the gastrointestinal tract (GIT) can include nausea, abdominal pain, increased salivation, soreness of the mouth, inflammation of the mucosa, and diarrhea. Early neurologic disorders are depression, anxiety, and poor concentration; prolonged symptoms are disorientation, confusion, and delirium. The prevalence of pellagra is sporadic in USA and Europe but higher in poor and malnourished people, alcoholics, patients undergoing long-time parenteral nutrition with insufficient niacin supplementation, and also in some psychiatric patients. Pellagra is endemic in a few regions of Africa and Asia where maize (corn) represents a major part of the common food together with very low amounts of meat, fruits, and vegetables. Pellagra-like symptoms can also appear during tryptophan deficiency (cf. Hartnup syndrome).

The clinical diagnosis of pellagra is that of the (rough) skin, preferably in context with GIT symptoms. The laboratory diagnosis is still unsatisfactory in terms of a specific measurement to estimate niacin. Thus, fluorometric assays for urinary metabolites N′-methyl-nicotinamide (NMN) and N′-methyl-2-pyridone-5-carboxamide (2-pyridone) are used. Urinary NMN levels of <0.8 mg or a combined excretion of <1.5 mg per 24 h indicates niacin deficiency.

Acute therapy is by oral application of 100–300 mg day^{-1} niacinamide or niacin in three doses, niacinamide having less intense side effects (flushing). Mental changes disappear within 24–48 h, and skin lesions, within several weeks. Concomitant administration of riboflavin and pyridoxine, and a diet rich in calories and protein are reasonable.

Nicotinic acid is used as a supplement in the treatment of hyperlipidemia; high doses of up to 5 g day^{-1} resulted in the reduction of total serum cholesterol (−20%), serum triglycerides (−40%), and high-density lipoprotein (LDL; +15%). Such high doses ingested over longer periods can lead to some side effects: besides the immediate symptoms (mainly flushing and itching), hepatotoxicity, including elevated liver enzymes and jaundice, appears mostly after long-term treatment (3–9 g day^{-1}) for months or years, but has also been reported with 750 mg day^{-1} for 2 months. Nicotinamide potentiates the effects of chemotherapy and radiation treatment fighting tumor cells, perhaps attributable to increased blood flow and oxygenation. Interestingly, supplementation with 2 g day^{-1} led to a decreased insulin sensitivity in adults at high risk of insulin-dependent diabetes.

See also: **Milk**: Human Milk. **Nutrition and Health**: Effects of Processing on Protein Quality of Milk and Milk Products; Nutritional and Health-Promoting Properties of Dairy Products: Contribution of Dairy Foods to Nutrient Intake. **Vitamins**: General Introduction.

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Pantothenic Acid

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Pantothenic Acid or Vitamin B$_5$

The natural form of pantothenic acid (**Figure 1**) and the only stereoisomer with biological activity is D(+)-pantothenic acid. However, the alcohol (D)-panthenol can be converted to pantothenic acid, thus having – although indirectly – also biological activity. The major portion of pantothenic acid in the diet occurs as coenzyme A (CoA) or pantetheine (**Figure 2**).

Pantothenic acid is a highly hygroscopic, light-yellowish viscous oil that is soluble in water as well as in ethanol. It is stable to heat and light, but otherwise is unstable; thus in pharmaceutical preparations, the Na$^+$ or Ca^{2+} salts of panthenol are usually used.

About 50–95% of pantothenic acid occurs as CoA or pantetheine (fatty acid synthetase complex) in the overall diet. In milk, about 25% of pantothenic acid is protein-bound, but this value rises to 40–60% in cheese, depending on the type of cheese.

Function of Pantothenic Acid

The main physiological activity of pantothenic acid is related to that of CoA, pantetheine being a part of both. The HS group of cysteamine in CoA and pantetheine represents the active site for the binding of acyl or acetyl residues. Furthermore, there is a pantothenate-dependent step in the synthesis of arginine, leucine, and methionine.

For the detailed synthesis of pantetheine and CoA and their numerous functions in the intermediate metabolism of animal cells (carbohydrates, fatty acids, nitrogenous compounds), the reader is referred to relevant textbooks on biochemistry or physiology or recent reviews (*see* Further Reading). In brief, CoA plays a role in

- acylation of proteins
- internal acetylation of proteins
- N-terminal acetylation of proteins
- transfer of C2-units released in β-oxidation of fatty acids and oxidative degradation of amino acids
- transfer of C2-units required for fatty acid synthesis
- introduction of C2-units in the tricarboxylic acid cycle
- transfer of fatty acids required for the synthesis of triglycerides and phospholipids
- synthesis of isoprenoid-derived compounds
- synthesis of hemoglobin, cytochromes, acetylcholine, taurine, and acetylated sugars

In summary, pantothenic acid, especially as part of CoA, plays numerous roles in animal and human cellular metabolism.

Pantothenic Acid Sources

Tables 1 and **2** give an overview of dietary sources of pantothenic acid, especially in the milk of various species and in dairy products; the recommended daily intake is given in **Table 3**. In cheese, amounts of pantothenic acid depend on the degree of proteolysis. While Cheddar and Cottage cheese lose large amounts (mainly of the free form) during manufacture, the concentration in some types of cheese (e.g., Limburger, Camembert, Brie) increases due to microbiological synthesis.

Pantothenic Acid Deficiencies

When suffering from a deficiency of pantothenic acid, chickens develop keratitis, dermatitis, degenerations of the spinal cord, and a fatty liver, while rats show retarded growth. For humans, only effects that occur under experimental conditions are known (e.g., application of an antagonist (ω-methyl pantothenic acid); severe undernutrition), as pantothenic acid occurs in almost all kinds of food and true requirements are hard to assess. The first symptoms of a deficiency are headache, fatigue, gastrointestinal tract (GIT) disturbances, palpitation of the heart, burning feet syndrome (first described in prisoners during World War II in Burma, Japan, and the Philippines). Prolonged deficiency leads to retarded wound healing, hypotonia, and uncoordinated movements. In general, all symptoms are reversible.

Due to large individual variations, both the level in blood and the amount in urine excretion are not good indicators of the vitamin status. However, a blood level between 1 and 4 mg l^{-1} is regarded as sufficient. A daily uptake of 4–6 mg is recommended by several nutritional societies because of epidemiological data: 1 mg day^{-1} does not lead to deficiency symptoms in humans. Interestingly, some intestinal bacteria can synthesize pantothenic acid, but this source seems to be ineffective in humans.

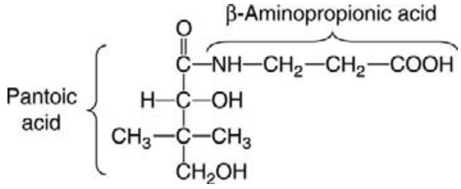

Figure 1 Structure of pantothenic acid.

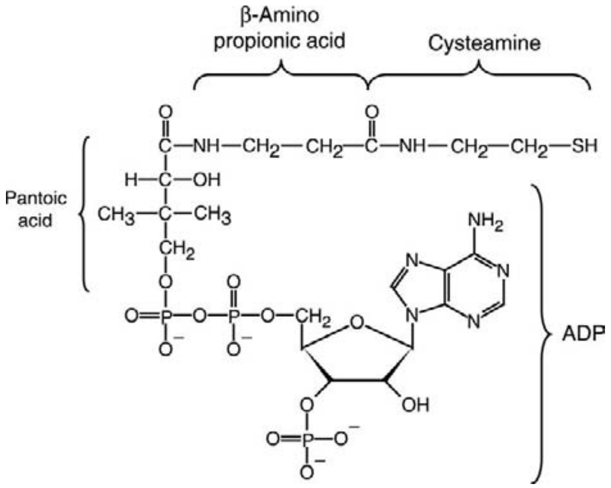

Figure 2 Structure of coenzyme A (CoA). ADP, adenosine diphosphate.

Table 1 Pantothenic acid concentration in foods

Food	*Concentration (μg per 100 g)*
Ox liver	7300
Pig's liver	6800
Ox kidney	3900
Pea, dry seed	2000
Soybean, dry seed	1900
Rice, unpolished	1700
Egg	1600
Watermelon	1600
Lentil, dry seed	1600
Rye, whole grain	1500
Broccoli	1300
Wheat flour, wholemeal	1200
Herring	940
Oats	710
Pork	700

From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart, Germany: Medpharm Scientific Publishers.

Pantothenic acid is used therapeutically in doses up to 5 mg day^{-1} to treat burns (sunburn), anal fissures, rhagades, and conjunctival inflammation without any signs of hypervitaminosis. It is required as a supplement by patients on

Table 2 Concentration of pantothenic acid in milk, dairy products, cheese, and milk of different species

Food	*Concentration (μg per 100 g)*
Dried whole milk	2700
Blue cheese (50% fat in dry matter)	2000
Limburger (40% fat in dry matter)	1200
Condensed milk (min. 10% fat)	840
Camembert (45% fat in dry matter)	800
Quark/fresh cheese, from skim milk	740
Brie (50% fat in dry matter)	690
Parmesan	530
Cream cheese (min. 60% fat in dry matter)	440
Consumer milk (min. 3.5% fat)	350
Sterilized milk	350
UHT milk	350
Yogurt (min. 3.5% fat)	350
Sweet whey	340
Buttermilk	300
Cream (min. 30% fat)	300
Skim milk	280
Milk from	
Buffalo	370
Sheep	350
Cow	350
Goat	310
Horse	300
Human	270

UHT, ultra-high temperature treated.
From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart, Germany: Medpharm Scientific Publishers.

Table 3 Recommended daily uptake of pantothenic acid

Age	*Pantothenic acid (mg day^{-1})*
Sucklings	
<4 months	2
4–12 months	3
Children	
1–4 years	4
4–7 years	4
7–10 years	5
10–13 years	5
13–15 years	6
Adults	
15–25 years	6
25–51 years	6
51–65 years	6
>65 years	6
Pregnant	6
Breast feeding	6

From DGE (Deutsche Gesellschaft für Ernährung) (2007) Die Referenzwerte für die Nährstoffzufuhr. http://www.dge.de/modules.php?name=St&file=w_referenzwerte (accessed April 2009).

total parenteral nutrition or those who regularly undergo dialysis. In addition, administration of pantothenic acid is used to counteract the inhibitory effects of some drugs on respiratory metabolism (e.g., valproic acid). The vitamin leads to improved wound healing following surgery.

See also: **Vitamins**: General Introduction.

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Vitamin B_6

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Pyridoxine or Vitamin B_6

The term vitamin B_6 represents a group of substances with vitamin B activity and these substances are derivatives of 3-hydroxy-2-methylpyridine: pyridoxine (PN, alcohol), pyridoxal (PL, aldehyde), and pyridoxamine (PM, amine; **Figure 1**), and their 5′-phosphorylated forms (**Figure 2**).

PN and PM and their phosphorylated forms are the predominant forms in plant-derived foods, while PL and pyridoxal-5′-phosphate (PLP) predominate in animal-derived foods. In cow's milk, 14% of vitamin B_6 is in the bound form and 86% is in the free form. It is sensitive to light and heat, partly depending on the pH of the medium. PM and PN are more stable than PL, particularly to light. In neutral and alkaline solutions, B_6 is destroyed by UV light. PL, PM, and PN are generally heat stable in acidic surroundings but heat sensitive in an alkaline medium. The amount of reported loss differs between different publications, maybe due to different procedures. However, some approximations can be made:

Heat treatment leads to a loss:

- Drying: insignificant effects
- Pasteurization: 0–8%
- Ultra-high temperature (UHT) treatment: <10%
- Boiling: 10%
- In-container sterilization: 20–50%
- Evaporation: 35–50%

UV light leads to a reduction of 10–45%, depending on the intensity and duration of exposure, and gamma radiation (10 Gy) causes a loss of ~90%.

Functions of Vitamin B_6

In general, PLP serves mainly as a coenzyme for about 100 enzymes in amino acid metabolism. It is covalently bound to its enzyme by a Schiff base linkage to the ε-amino group of lysine in the enzyme. During the enzymatic reaction, the amino group of the substrate and the aldehyde group of PLP form a Schiff base. All subsequent reactions can occur at the α-, β-, or γ-C of the respective substrate. In the following, a brief list of common reaction types is given; for further details, the reader is referred to specific literature (see Further Reading):

- Transamination (transfer of the amino group of one amino acid to the keto analogue of another amino acid)
- Decarboxylation (deletion of a CO_2-group from a molecule; e.g., resulting in biogenic amines)
- Elimination (deletion of 2 subsituents from neighbored (C-)atoms; e.g., by Serine dehydratase)

Pyridoxamine-5′-phosphate (PMP) acts exclusively as a coenzyme for transaminases:

1. Transferase
   a. Serine hydroxymethyl transferase (C1 metabolism)
   b. D-Aminolevulinate synthase (porphyrin biosynthesis)
   c. Glycogen phosphorylase (glycogen mobilization)
   d. Aspartate aminotransferase (transamination)
   e. Alanine aminotransferase (transamination)
2. Oxidoreductase
   a. Lysyl oxidase (collagen biosynthesis)
3. Hydrolase
   a. Kynureninase (niacin biosynthesis)
4. Lyase
   a. Glutamate decarboxylase (γ-aminobutyric acid (GABA) synthesis)
   b. Tyrosine decarboxylase (tyramine biosynthesis)
   c. Serine dehydratase (β-elimination)
   d. Cystathionine β-synthase (methionine metabolism)
   e. Cystathionine γ-lyase (γ-elimination)

Due to its role as a coenzyme in amino acid metabolism (see below), vitamin B_6 has a broad range of functions in many systems of the body, including the immune system, the nervous system, gluconeogenesis, lipid metabolism, erythrocyte function, hormone modulation, gene expression, and niacin formation. In the immune system, for example, vitamin B_6 increases the immune response of critically ill patients and suppresses the nuclear factor-kappa B (NF-κB) reaction in lipopolysaccharide (LPS)-stimulated mouse macrophages, a well-established laboratory method to challenge the immune system. In the nervous system, PLP may cause neurological dysfunction, particularly epilepsy. On the other hand, there is no direct evidence for an influence on cognitive functions in people with either normal or impaired cognitive functions. An intensively investigated role is that of vitamin B_6 (with B_{12} and folate) in regulating homocysteine levels, with homocysteine levels playing a major role in

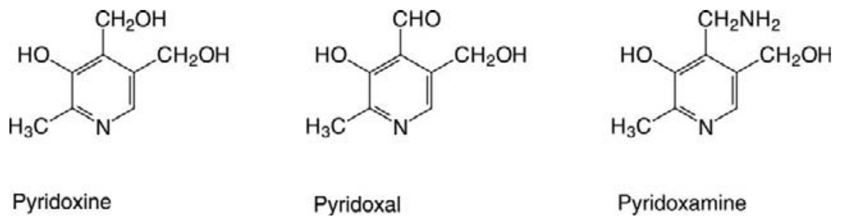

Figure 1 Structures of pyridoxine, pyridoxal, and pyridoxamine.

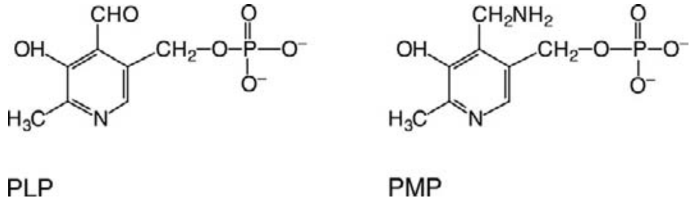

Figure 2 Structure of the coenzymes pyridoxal-5′-phosphate (PLP) and pyridoxamine-5′-phosphate (PMP).

cardiovascular diseases like atherosclerosis or endothelial dysfunction, and is also associated with the risk of hip fractures in the elderly. The benefits of vitamin B_6 supplementation during pregnancy (e.g., higher birth weight or reduced incidence of preeclampsia and preterm birth) could not be confirmed by a meta-analysis. Additional effects of B_6 include decarboxylation and transamination, inhibition of DNA polymerases and a couple of steroid receptors, and usefulness as an adjunct in cancer chemotherapy (see Further Reading).

Sources of Vitamin B_6

Tables 1 and **2** give an overview of the concentrations of vitamin B_6 in selected foods and dairy products, including the milk of different species that is used for human consumption. Regarding vitamin B_6 in cheese, most of the vitamin passes into the whey and the concentration decreases further during early maturation, while in later phases the concentration increases, especially on the surface (yeast and molds). Cheese types like Camembert and Brie have the highest vitamin B_6 concentration, followed by very hard, hard, semi-hard, and soft unripened cheese. **Table 3** presents recommended dietary uptakes.

Table 1 Vitamin B_6 concentration in foods

Food	*Concentration (μg per 100 g)*
Soybean, dry seed	1000
Salmon	980
Oats	960
Ox liver	877
Mackerel	630
Pig's liver	590
Lentil, dry seed	575
Pork	565
Chickpea, dry seed	550
Millet	519
Wheat germ	492
Wheat, wholemeal flour	460
Herring	450
Maize	400
Rice, unpolished	275

From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart, Germany: Medpharm Scientific Publ.

Table 2 Vitamin B_6 concentration in milk, dairy products, cheese, and milk from different species

Food	*Concentration (μg per 100 g)*
Camembert (45% fat in dry matter)	250
Brie (50% fat in dry matter)	230
Dried whole milk	200
Emmental cheese	111
Condensed milk (min. 10% fat)	77
Cream cheese (min. 60% fat in dry matter)	60
Skim milk	50
Yogurt (min. 3.5% fat)	46
Sweet whey	42
UHT milk	41
Buttermilk	40
Consumer milk (min. 3.5% fat)	36
Cream (min. 30% fat)	36
Sterilized milk	23
Milk from	
Cow	36
Horse	30
Goat	27
Buffalo	25
Human	14

UHT, ultra-high temperature treated.
From Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart, Germany: Medpharm Scientific Publ.

Table 3 Recommended daily uptake of vitamin B_6

	Vitamin B_6 (mg day^{-1})	
Age	*Male*	*Female*
Sucklings <4 months	0.1 (estimated)	
Sucklings 4–12 months	0.3	
Children 1–4 years	0.4	
Children 4–7 years	0.5	
Children 7–10 years	0.7	
Children 10–13 years	1	
Children 13–15 years	1.4	
Adults 15–25 years	1.6	1.2
Adults 25–51 years	1.5	1.2
Adults 51–65 years	1.5	1.2
Adults >65 years	1.4	1.2
Pregnant (>4 months)		1.9
Breast feeding		1.9

From DGE (Deutsche Gesellschaft für Ernährung) (2007) Die Referenzwerte für die Nährstoffzufuhr. http://www.dge.de/modules.php?name=St&file=w_referenzwerte (accessed April 2010).

Vitamin B_6 Deficiencies

The vitamin is essential for humans, most animals, and some microorganisms. Some recommendations concerning uptake relate the concentration of B_6 to protein uptake; the German Society for Nutrition, for example, recommends a minimum intake as shown in **Table 3** based on a quotient of 20 μg g^{-1} recommended protein uptake. Bioavailability is negatively correlated with the amount of glycosylated forms of vitamin B_6 in the respective food. The glycosylated form mainly appears in plant-derived foods but not animal-derived foods. As an estimation, the bioavailability of vitamin B_6 in a 'normal, mixed diet' is about 75%.

A specific vitamin B_6 deficiency in humans can hardly be detected, as the first symptoms resemble the symptoms of niacin and riboflavin deficiency (stomatitis, dermatitis like pellagra). Sometimes, in children, neurological problems occur, maybe due to changes in neurotransmitter metabolism (PLP functions as a coenzyme of an amino acid decarboxylase). Longer-lasting deficiency might lead to peripheral neuropathy (nerve demyelination and hypochromic anemia that cannot be cured by iron supplementation (vitamin B_6 functions in heme synthesis)), and also the risk of dementia is recently under discussion.

Some drugs, hydrazines, chelators, antibiotics, oral contraceptives, L-DOPA (L-3,4-dihydroxyphenylalanine), and alcohol, reduce vitamin B_6 concentration, especially when they are taken over an extended period of time (then vitamin B_6 status should be monitored.

Vitamin B_6 status is normally measured by

- PLP in plasma
- 4-Pyridoxic acid excretion in 24 h urine (short-term)
- Assessment of the activation coefficient of erythrocyte transaminase (long term)

PN is highly toxic when taken over an extended period of time. A dose of 150 mg day^{-1} over several months leads to (reversible) peripheral neuropathy with dysreflexia and insensibility. However, therapies with megadoses of vitamin B_6 showed high positive potential in the treatment of PN dependency (2–11 mg day^{-1}), cystathioninuria (400 mg day^{-1}), homocystinuria (250–1250 mg day^{-1}), primary oxalosis type I ('spine syndrome', 150 mg day^{-1}), and also isoniazid intoxication (1 g PN g^{-1} isoniazid). In some cases, beneficial effects have been described for carpal tunnel syndrome, premenstrual syndrome, and rheumatic diseases, although for the latter it is still unclear.

See also: **Vitamins**: Folates; Vitamin B_{12}.

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Thiamine

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Introduction

Thiamine or vitamin B_1 is a water-soluble vitamin and is unstable and loses its biological activity in alkaline solutions (pH >7) as well as in the presence of oxidants and radiation. The chemical name of thiamine is 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]- 5-(2-hydroxyethyl)-4-methylthiazolium; its coenzyme form is thiamine pyrophosphate (TPP; **Figure 1**).

In pharmaceutical and other preparations, thiamine is used in the form of water-soluble thiazolium salts (thiamine chloride hydrochloride, thiamine mononitrate); synthetic lipophilic derivatives (allithiamins) also exist. The latter can pass through biological membranes more easily and in an almost dose-related manner, thus offering a possibility to develop thiamine stores by supplementation, which are normally low and last for only 4–10 days.

In the presence of oxidizing agents and in strongly alkaline solutions, thiamine is converted into thiochrome, a fluorescent substance used to determine the thiamine content of feeds, foods, or pharmaceutical preparations.

Functions of Thiamine

A number of enzymes (pyruvate dehydrogenase complex; α-ketoglutarate dehydrogenase complex; branched-chain α-keto acid dehydrogenase complex) involved in intermediary metabolism and playing a role in the oxidative decarboxylation of α-keto acids require TPP as a coenzyme. Thus, metabolites from carbohydrate metabolism and keto analogues from amino and fatty acid metabolism are made available for energy metabolism. In addition, a TPP-dependent transketolase is involved in the formation of NADPH and pentose in the pentose phosphate pathway. Both metabolites play important roles in several other synthetic pathways. There are hints that the above-mentioned enzymes are also involved in neural functions; however, the exact mechanisms of action need to be elucidated further. Interestingly, a decrease of glutamate uptake in the prefrontal cortex of thiamine-deficient mice is described.

Sources of Thiamine

Tables 1 and **2** show the thiamine content of various foods. **Table 2** focuses on dairy products, including milk from different species, that are consumed by humans.

It has to be taken into account that heat treatment, as well as storage conditions, can lead to losses of the thiamine content of the foods:

- low pasteurization 3–4%
- boiling 4–8%
- spray-drying 10%
- roller-drying 15%
- pasteurization 9–20%
- condensed milk 3–75%
- sterilization 20–45%
- evaporated milk 20–60%

Fresh milk in dark bottles loses 24% of its initial thiamine content on storage for 24 h at 4 °C, 14% on storage at 12 °C, and 16% on storage at 20 °C. Evaporated milk loses 15–50% over periods >12 months; spray-dried whole milk shows no changes up to 12 months. Thiamine is lost during cheese manufacture mainly during drawing of the first whey; no significant changes occur during maturation.

UV light-induced inactivation of thiamine can, under certain conditions (cheese, fresh milk), be counterbalanced by thiamine-synthesizing microorganisms. Modern high-pressure-assisted thermal sterilization methods result in almost stable vitamins, although the decay in model solutions (acetate-buffered, pH 5.5) was about 30 times higher than in minced pork. Thus, a general deduction of the test results to routine food preparation needs further investigations.

The recommended daily uptake of thiamine given by the DGE (German Nutrition Society) is shown in **Table 3**.

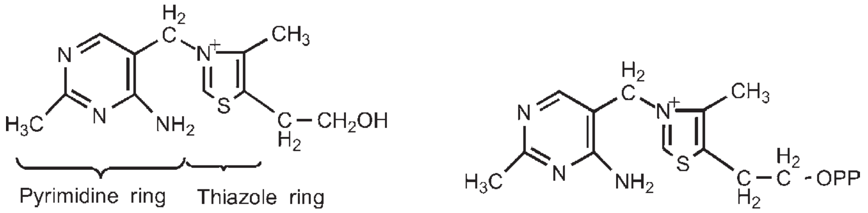

Figure 1 Structure of thiamine (left) and thiamine pyrophosphate (TPP, right).

Table 1 Thiamine concentration in selected foods

Food	*Concentration ($\mu g\ 100 g^{-1}$)*
Brewers' yeast, dried	12 000
Wheat germ	2000
Sunflower seed, dry	1900
Soybean, dry	999
Pork	900
Pea seed, dry	765
Oat flakes	590
Wheat, wholemeal flour	470
Hazelnut	390
Pig, kidney or liver	310–340
Ox, kidney or liver	290–300
Eel	180
Potato	110

Data from Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart, Germany: Medpharm Scientific Publ.

Table 2 Thiamine concentration in dairy products and milk

Food	*Concentration ($\mu g\ 100 g^{-1}$)*
Dried whole milk	270
Condensed milk (min. 10% fat)	88
Camembert (45% fat in dry matter)	45
Cream cheese (min. 60% fat in dry matter)	45
Quark/fresh cheese, from skim milk	43
Skim milk	38
Consumer milk (min. 3.5% fat)	37
Sweet whey	37
Yogurt (min. 3.5% fat)	37
Buttermilk	34
UHT milk	33
Gouda	30
Cottage cheese	29
Cream (min. 30% fat)	25
Sterilized milk	24
Parmesan	20
Milk from	
Buffalo	50
Goat	49
Sheep	48
Donkey	41
Cow	37
Horse	30
Human	15

UHT, ultra-high temperature.
Data from Souci SW, Fachmann W, and Kraut H (2008) *Food Composition and Nutrition Tables*, 7th edn. Stuttgart, Germany: Medpharm Scientific Publ.

Table 3 Recommended daily uptake of thiamine

	Thiamine ($mg\ day^{-1}$)	
Age	*Male*	*Female*
Sucklings		
<4 months	0.2	
4–12 months	0.4	
Children		
1–4 years	0.6	
4–7 years	0.8	
7–10 years	1.0	
10–13 years	1.2	1.0
13–15 years	1.4	1.1
Adults		
15–25 years	1.3	1.0
25–51 years	1.2	1.0
51–65 years	1.1	1.0
>65 years	1.0	1.0
Pregnant		1.2
Breast feeding		1.4

From DGE (Deutsche Gesellschaft für Ernährung) (2007) Die Referenzwerte für die Nährstoffzufuhr. http://www.dge.de/modules.php?name=St&file=w_referenzwerte (accessed April 2009).

Thiamine Deficiencies

Because of the relatively small and short-lasting thiamine stores, marginal deficiencies are quite common, but early symptoms are rarely recognized.

Symptoms of thiamine deficiency are cardiac failure, muscle weakness, peripheral and central neuropathy, and gastrointestinal malfunction. Reasons for deficiency besides a thiamine-free diet (e.g., parenteral nutrition) might be reduced absorption (gastrointestinal diseases), impaired transport, increased requirements (pregnancy, lactation, infancy, childhood, adolescence, increased physical activity, infections, trauma, surgery), or increased losses and impaired biosynthesis of TPP.

Clinically manifest deficiency appears in several forms of an illness called beriberi, which is nowadays mostly a problem in some regions of Southeast Asia, mainly because of the consumption of thiamine-free rice or raw fish (which contains thiaminase) or chewing of betel nuts or fermented tea leaves (which contain 'antithiaminic' tannins). Another risk group is chronic alcoholics who often consume low-quality meals, have poor appetite, and suffer from gastrointestinal problems and malabsorption. One can differentiate infantile beriberi (often lethal in sucklings fed by thiamine-deficient mothers) from two forms of adult beriberi: dry beriberi is characterized by peripheral neuropathy ('burning feet syndrome', exaggerated reflexes, diminished sensation, and weakness in all limbs, muscle pain, problems rising from squatting position, and, in severe cases, eventually seizures). Wet beriberi is characterized by cardiovascular symptoms (rapid heart rate, enlargement of the heart, edema, breathing problems, and ultimately congestive heart failure). 'Cerebral' beriberi mostly leads to Wernicke's encephalopathy and Korsakoff's psychosis, both together appearing as the Wernicke–Korsakoff syndrome, which is, however, not easily diagnosed but can be treated by thiamine supplementation.

Thiamine Supplementation

For the therapeutic treatment of diseases of the central (CNS) and the peripheral nervous system (PNS) and of exhaustion and during cytostatic treatment, doses of 50–200 mg thiamine day^{-1} are administered orally. Clinically manifest beriberi is treated by administering 50–100 mg day^{-1} subcutaneously or intravenously for several days, followed by the same dose orally for several weeks. Other than single cases of anaphylactic shock after intravenous application, no side effects of higher doses of thiamine (e.g., up to 200 mg day^{-1}) are known.

See also: **Vitamins**: General Introduction.

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Riboflavin

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Riboflavin or Vitamin B$_2$

The chemical name for riboflavin is 7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine; riboflavin exists in an oxidized and a reduced form (**Figure 1**), from which two coenzymes are formed: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD; **Figure 2**).

The ending 'flavin' refers to its yellowish color (in Latin *flavus* means yellow).

Free as well as protein-bound riboflavin occurs in the diet, and milk in general is the best source. In cow's milk, the free form, with a higher bioavailability, is the major one (61% riboflavin, 26% FAD, 11% hydroxyethyl form, and others), whereas the protein-bound, and thus less bioavailable, form predominates in other foods. In human breast milk, approximately one- to two-thirds of riboflavin occurs as FAD.

Riboflavin is very heat stable but it is extremely photosensitive. It is photodegraded to lumiflavin (under alkaline conditions) or lumichrome (under acidic conditions), both of which are biologically inactive. Concentrations are significantly reduced in high-pressure low-temperature treated milk as compared to raw milk. UV light excites riboflavin to a high degree of natural fluorescence, which is used for its detection and determination in yogurt or non-fat dry milk.

Functions of Riboflavin

Riboflavin-dependent enzymes are called flavoproteins or flavoenzymes, because of their yellowish appearance. They catalyze hydroxylations, oxidative decarboxylations, dioxygenations, and reduction of oxygen to hydrogen peroxide, serving as electron carriers, mediators of electron transfer from pyridine nucleotides to cytochrome *c* or to other one-electron acceptors, and as catalysts of electron transfer from a metabolite to molecular oxygen. The two flavoenzymes, FMN and FAD, play major roles in the metabolism of glucose, fatty acids, amino acids, purines, drugs and steroids, folic acid, pyridoxine, vitamin K, niacin, and vitamin D.

The FAD-dependent enzyme, glutathione reductase, plays a major role in the antioxidant system by restoring reduced glutathione (GSH) from oxidized glutathione (GSSH). GSH is important in protecting lipids from peroxidation and in stabilizing the structure and function of red blood cells; it is the most important antioxidant in erythrocytes and in keeping lens proteins in solution (thus preventing cataracts).

The formation of FMN and FAD is ATP dependent and takes place mainly in the liver, kidney, and heart. All enzymatic steps are under the control of thyroid hormones.

- Flavokinase: riboflavin + ATP → r/FMN + ADP
- FAD pyrophosphorylase: FMN + ATP → /FAD + PP
- FAD + apoenzyme/protein → covalently bound flavins

Sources of Riboflavin

Tables 1 and **2** summarize dietary sources of riboflavin and its concentration, especially in the milk of various species and in dairy products.

Heat treatment has only negligible effects on riboflavin concentrations, whereas exposure of milk to sunlight results in the loss of 20–80% of riboflavin. Thus, storage in dark bottles, light-tight wax cartons, or special polyethylene terephthalate (PET) bottles is recommended.

Photo-degradation of riboflavin catalyzes photochemical oxidation and loss of ascorbic acid. Gamma radiation of 10 Gy destroys about 75% of riboflavin in liquid milk, whereas milk powder shows no losses even at higher doses.

Storage influences riboflavin concentration as follows: condensed milk loses 28% (33%) of its initial riboflavin content when stored at 8–12 °C for 2 years (10–15 °C for 4 years), ice cream loses 5% when stored at –23 °C for 7 months. No losses were found in fresh milk stored at 4–8 °C for 24 h or in milk powder stored for 16 months.

In cheese, most losses (66–88%) of the original riboflavin content of the milk appear to occur during whey

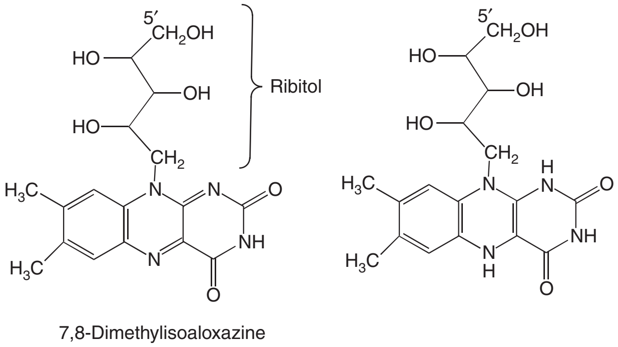

Figure 1 Structures of oxidized (flavoquinone, left) and reduced (flavohydroquinone, right) forms of riboflavin (vitamin B$_2$).

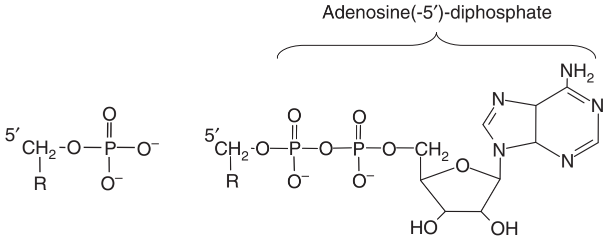

Figure 2 Structure of flavin mononucleotide (FMN, left) and flavin adenine dinucleotide (FAD, right). R: riboflavin.

Table 1 Riboflavin concentration in food

Food	*Concentration (μg per 100 g)*
Brewers' yeast	3800
Pig's liver	3200
Ox liver	2900
Wheat germ	720
Almonds	620
Wheat bran	510
Soybean, seed, dry	460
Mushroom	436
Egg	408
Mackerel	360
Eel	320
Lentil, seed, dry	262
Beef	260
Pork	230
Herring	220
Maize	200

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drainage, while ripening has almost no effects. However, in some cheese varieties, the concentration is higher in the outer layers due to microbial synthesis. High-pressure tests for thermal sterilization processes led to different results concerning the decay of the vitamin, depending on the matrix of the food tested.

Table 2 Riboflavin in milk, dairy products, and cheese

Food	*Concentration (μg per 100 g)*
Dried whole milk	1400
Parmigiano	620
Camembert (45% fat in dry matter)	600
Blue cheese (50% fat in dry matter)	500
Condensed milk (min. 10% fat)	480
Limburger (40% fat in dry matter)	350
Quark/fresh cheese (from skim milk)	300
Cream cheese (min. 60% fat in dry matter)	230
Consumer milk (3.5% fat)	180
UHT milk	180
Yogurt (min. 3.5% fat)	180
Skim milk	170
Buttermilk	160
Cream (min. 30% fat)	150
Sweet whey	150
Sterilized milk	140
Milk from	
Sheep	230
Cow	180
Goat	150
Buffalo	100
Donkey	64
Human	38

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Riboflavin Deficiency

Riboflavin is essential for humans, animals, and some microorganisms. Among humans, seniors and adolescents seem to be at particular risk of deficiency; the recommended uptake is given in **Table 3**. In some cases, recommended

Table 3 Recommended daily uptake of riboflavin

	Riboflavin (mg day^{-1})	
Age	*Male*	*Female*
Sucklings <4 months	0.3	
Sucklings 4–12 months	0.4	
Children 1–4 years	0.7	
Children 4–7 years	0.9	
Children 7–10 years	1.1	
Children 10–13 years	1.4	1.2
Children 13–15 years	1.6	1.3
Adults 15–25 years	1.5	1.2
Adults 25–51 years	1.4	1.2
Adults 51–65 years	1.3	1.2
Adults >65 years	1.2	1.2
Pregnant		1.5
Breast feeding		1.6

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uptake is related to energy intake, and 0.6 mg riboflavin per 1000 kcal is considered adequate. Milk and milk products (without butter) can contribute about 30% of the total riboflavin supply.

A major portion of riboflavin is bound to proteins and these flavoproteins have to be hydrolyzed before absorption by specialized transporters in the upper gastrointestinal tract. The amount that can be stored depends on the availability of proteins providing binding sites. Although a limited uptake makes sense in preventing accumulation in tissues, it increases the body's dependence on dietary supply. Under normal conditions, riboflavin stores last for 2–6 weeks, but in cases of protein deficiency, they last significantly shorter.

Symptoms of a marginal deficiency are often nonspecific: weakness, fatigue, mouth pain, glossitis, stomatitis, burning and itching of the eyes, and personality changes. Signs of increased deficiency are cheilosis; angular stomatitis; seborrheic dermatitis at the mouth, nasolabial sulcus, and ears (later extending to the trunk and extremities); desquamative dermatitis with itching in genital regions; opacity of the cornea; cataract; and brain dysfunction. The major reasons for riboflavin deficiency are

- Insufficient dietary intake by seniors and adolescents (especially girls)
- Endocrine abnormalities, insufficient adrenal and thyroid hormones
- Drugs (psychotropic, anti-depressant, cancer therapeutics, anti-malarial)
- Alcohol intake interfering with the digestion and absorption of food flavins
- Agents that chelate or form complexes with riboflavin or FMN, affecting their bioavailability: copper, zinc, iron, caffeine, theophylline, saccharine, nicotinamide, ascorbic acid, tryptophan, urea.

As riboflavin (via FAD-dependent glutathione reductase) is involved in antioxidant mechanisms, riboflavin deficiency may considerably affect erythrocyte metabolism. However, several studies have reported protective effects of a deficiency against malaria infection. A study in the United States showed that the uptake of yogurt, milk, cereals, and also riboflavin was inversely correlated with homocysteine levels in plasma, which, in turn, seem to be positively correlated with a higher risk of developing atherosclerosis.

Assessment of the riboflavin (mainly by HPLC methods) status uses the following parameters:

- Erythrocyte glutathione reductase activity coefficient,
- Excretion in urine ($\mathrm{mg\,g^{-1}}$ creatinine to assess short-term effects), and
- Riboflavin in erythrocytes ($\mathrm{mg\,g^{-1}}$ hemoglobin).

Concerning supplementation, no case of intoxication has been described. Thus, riboflavin is regarded as safe even at high doses. Supplements are usually given to reverse deficiency symptoms or to support high-risk groups:

- Regular intake of drugs (e.g., anti-depressants, oral contraceptive)
- Malnutrition
- Patients after trauma
- Malabsorption
- Chronic alcoholics

Hyperbilirubinemia can be treated much quicker by phototherapy when 0.5 mg riboflavin per kg of bodyweight is given. Finally, persons with congenital methemoglobinemia might benefit from 20–40 $\mathrm{mg\,day^{-1}}$.

See also: **Milk Proteins**: Minor Proteins, Bovine Serum Albumin, Vitamin-Binding Proteins. **Vitamins**: General Introduction.

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W

WATER IN DAIRY PRODUCTS

Contents
Water in Dairy Products: Significance
Analysis and Measurement of Water Activity

Water in Dairy Products: Significance

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Properties of Water and Water Activity

Water is a well-characterized compound that exhibits physical and chemical properties that differ significantly from those of other compounds with a corresponding molecular structure. These include relatively high phase transition temperatures, heats of changes in phase, and other thermodynamic quantities. The latent heat of melting of ice at 0 °C, ΔH_m, is 334 J g^{-1} (6.012 kJ mol^{-1}), the latent heat of vaporization of water, ΔH_v, at 100 °C is 2255 J g^{-1} (40.63 kJ mol^{-1}), and the heat of sublimation of ice at 0 °C is 2826 J g^{-1} (50.91 kJ mol^{-1}). The solid, liquid, and gaseous states of water may coexist in equilibrium at the triple point, which is located at 0.0099 °C and a pressure of 610.4 Pa. Water may solidify in various forms of ice depending on the pressure. Water may also solidify as an amorphous glass. Vapor-deposited glassy water undergoes the glass transition with onset temperature, T_g, at −138 °C. The fundamental physical properties of water in dairy products are the main determinants of energy and temperature requirements as well as economics of all heat treatments and the evaporation and dehydration processes, in particular, in the dairy industry.

The purest forms of water in dairy products are crystalline ice and gaseous water vapor. The vapor pressure of water is lower in solutions as well as in dairy products than the vapor pressure of pure water at the same temperature. In ideal dilute solutions, vapor pressure is defined by Raoult's law:

$$p = xp_0 \qquad [1]$$

where p is the vapor pressure of water in the solution, p_0 is the vapor pressure of pure water at the same temperature, and x is the mole fraction of water.

Raoult's law defines that water in a solute–solvent system has a lower vapor pressure than that of pure water. This results in a lower freezing temperature and a higher boiling temperature than those of pure water at the same pressure. Real solutions, such as milk, do not obey Raoult's law, but an 'effective' mole fraction for the solutes can be defined. The effective mole fraction of water is often referred to as water activity, a_w. Water activity is equal to the equilibrium relative vapor pressure (RVP) of water in the surrounding atmosphere, i.e., a_w as the ratio of the vapor pressure in a solution to that of pure water at the same temperature:

$$a_w = \frac{p}{p_0} \qquad [2]$$

Therefore, the equilibrium or steady-state water activity is related to equilibrium relative humidity (ERH) corresponding to the equilibrium RVP of the surrounding atmosphere by

$$a_w = \frac{\text{ERH}}{100} \qquad [3]$$

Although water activity is a measure of water availability, it should be emphasized that water activity is a temperature-dependent property of water in a material, such as food. In most dairy products, temperature and pH are more important factors that control rates of deteriorative changes and the growth of microorganisms than water activity. This is because the average water content of milk is very high, 87.1%, and milk contains only about 8.9% of non-fat solids. The water activity of milk is, therefore, very high, 0.993. The pH of milk is close to neutral, about 6.7, which allows the growth of almost all microorganisms. Thus, in high-water, non-fermented dairy products, temperature is the main variable controlling microbial growth. In fermented milk products and in the water phase of butter, the pH is reduced, for example, to about 4.6 in ripened cream butter, which significantly reduces the growth of spoilage microorganisms. Water activity is, however, an important factor controlling the microbial flora in ripening cheese, and quality changes of some cheeses and dairy powders during storage. The reduction of water activity in these products affects the predominant microbial culture, and, in some cases, an increase in water activity improves shelf life due to reduced availability of water for microbial growth. The water activity of evaporated milk is 0.986 and a relatively rapid removal of water by dehydration or freezing results in supersaturation of soluble compounds. Evaporation of water also reduces the pH of milk to about pH 6, depending on the extent of concentration.

Water Sorption

Water sorption characteristics, as well as most other interactions of solids with water, are defined by the composition of the non-fat solids of dairy products. The water sorption properties are affected mainly by the component carbohydrates and proteins, which represent most of the non-fat fraction of milk solids, as well as by the physical state. The sorption properties may also be affected by time-dependent phenomena as a result of structural transformations and solute crystallization. Water sorption in low-water dairy products results from the difference between the vapor pressure of water in the material and the vapor pressure of water in the surrounding atmosphere. Water sorption occurs when the solids are exposed to conditions where the vapor pressure of water is higher than that within the solids. Therefore, the solids may sorb water until an equilibrium vapor pressure within the food and the surrounding atmosphere is reached. The sorption properties are strongly dependent on temperature. Moreover, sorption properties of low-water solids and desorption of high-water solids may differ resulting in water sorption hysteresis.

Sorption isotherms, which show the water content as a function of water activity at a constant temperature, are useful tools in describing the relationships between water content and steady-state RVP. Typical sorption isotherms of dairy powders, such as that of skim milk solids in **Figure 1**, with amorphous components, and milk and whey proteins are sigmoid curves and exhibit hysteresis. However, the amorphous lactose in dairy powders is unstable and it tends to crystallize during the storage of powder above a critical water content or water activity. Such crystallization is observed from time-dependent loss of sorbed water and a break in the sorption isotherm. The water sorption properties of the nonhygroscopic, crystalline lactose differ significantly from the water sorption properties of amorphous lactose. Therefore, crystallinity and crystalline forms of lactose may greatly affect sorption properties and one of the most significant differences between dairy powders with glassy or precrystallized lactose is the water sorption behavior. Amorphous lactose is very hygroscopic and it may sorb high amounts of water at low relative humidities. Crystalline lactose shows little sorption of water at low humidities and its water sorption becomes significant only at the higher relative humidities as a result of partial solubilization. In addition, differences in salt content may affect water sorption; for example, the presence of salts may increase water sorption by cheese and milk proteins.

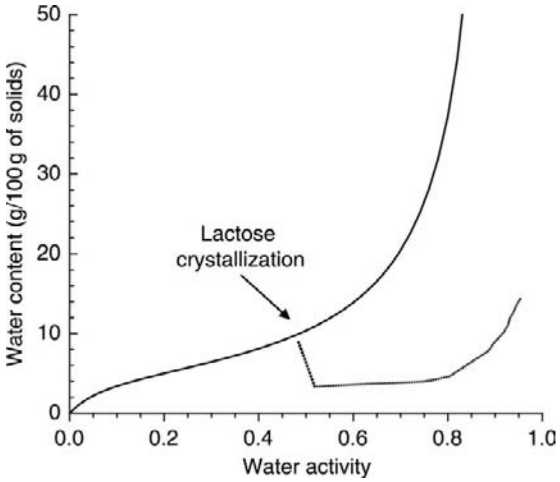

Figure 1 Sorption isotherm of skim milk solids (——). The break in the sorption isotherm resulting from crystallization of amorphous lactose is shown schematically (...).

Phase and State Transitions

Water-soluble milk solids often form amorphous, supercooled liquids or glasses as a result of dehydration or freeze concentration, for example in dairy powders and frozen desserts. The dominant component in defining the physical state is lactose. The formation of the non-crystalline, amorphous state of lactose results either from the rapid removal of solvent water by dehydration or from freeze concentration in freezing of water. In the amorphous solids, water behaves in a manner similar to plasticizers in synthetic polymers. Water plasticization is observed from a softening of the amorphous material, which is accompanied by increasing rates of quality changes. Plasticization may also result from an increase in temperature, or an increase in both temperature and water content. At a sufficient level of plasticization by temperature or water, the amorphous solids exhibit the glass transition. The glass transition occurs over a temperature or water content (water activity) range, and it can be observed from changes in heat capacity, dielectric properties, various mechanical properties, volume, and molecular mobility. The effect of water on the physical state of milk solids can be observed from decreasing glass transition with increasing water content (**Figure 2**) and structural changes that occur above a critical temperature, water activity, or water content, as the material suffers the glass transition.

The temperature–water combinations that support the various states or that result in state or phase transitions of amorphous solids and freeze-concentrated solutions can be described using state diagrams. State diagrams are simplified phase diagrams that describe the concentration dependence of the glass transition of solutes and relationships between ice formation and solute concentration at low temperatures. State diagrams are useful in the characterization of the physical state and physical properties of milk solids at various temperatures and water contents. The state diagram, as shown for lactose in **Figure 3**, shows the glass transition of the solids and the decrease in the glass transition temperature, T_g, with increasing water content. At sufficiently high water contents, ice formation before vitrification cannot be avoided, separating ice from the material with concurrent freeze concentration of solutes in an unfrozen water-solute phase. Therefore, the state diagrams often show the effect of ice formation on the phase and state behavior. A maximally freeze-concentrated solute has the glass transition at T'_g, which corresponds to a solute concentration of C'_g. Moreover, a full state diagram shows the onset temperature for ice melting in the maximally freeze-concentrated solution, T'_m, equilibrium ice melting temperature, T_m, curve, and the solubility curve. The solute concentration of maximally freeze-concentrated solute matrices, including non-fat milk solids, has been found to be about 80% (w/w).

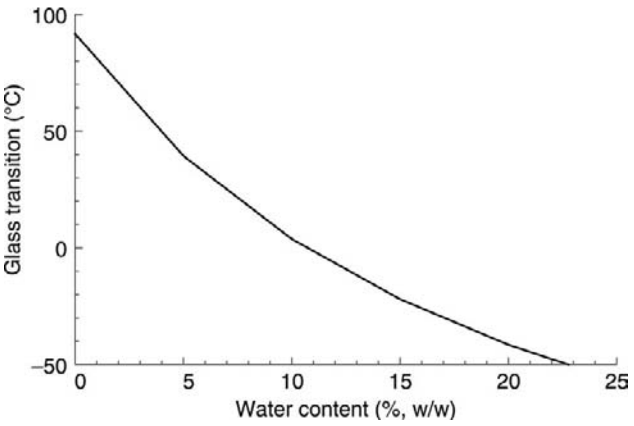

Figure 2 Glass transition temperature, T_g, of skim milk solids as a function of water content.

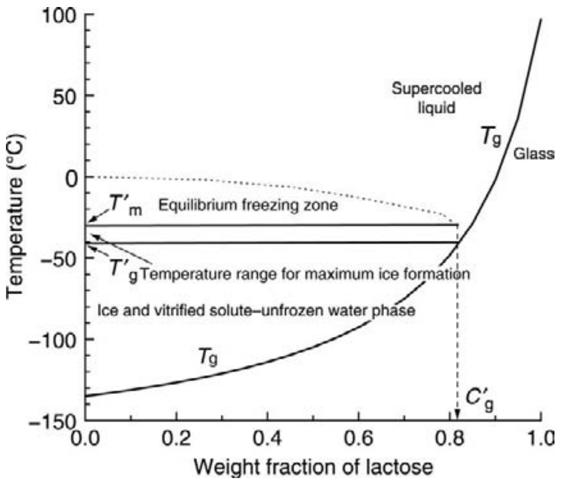

Figure 3 State diagram of lactose showing the decrease of the glass transition temperature, T_g, with increasing water content (—), the glass transition temperature of maximally freeze-concentrated lactose solutions, T'_g, and onset temperature for ice melting in the maximally freeze-concentrated state, T'_m. C'_g shows lactose concentration in the maximally freeze-concentrated, unfrozen solution. The equilibrium ice melting temperature, T_m, curve is shown schematically (---).

Water in Milk Solids and Dairy Powders

Stickiness and Caking

Various time-dependent structural transformations or changes in mechanical properties may occur in dairy powders at temperatures or water contents resulting in the glass transition. These transformations include stickiness and caking of powders, plating of particles on amorphous granules, and structural collapse of dehydrated structures, which are related to a rapid decrease in viscosity and increase in flow above the glass transition.

The main cause of stickiness is water or thermal plasticization of particle surfaces, which allows a sufficient decrease of surface viscosity for adhesion. Since viscosity is extremely high in the glassy state, the contact time must be very long for the occurrence of stickiness. A dramatic decrease in viscosity above T_g reduces the contact time and causes stickiness that can be related to the timescale of observation. A contact time of 1–10 s is sufficient at a surface viscosity less than 10^6–10^8 Pa s to cause stickiness. The decrease in viscosity is orders of magnitude over a fairly narrow water activity range, which results from the transformation of the solid material into the free-flowing liquid state. Obviously, water activity or storage relative humidity is often a more important indicator of stability than water content. The most common caking mechanism in food powders is plasticization due to water sorption and subsequent interparticle fusion. Caking of amorphous powders results from the change of the material from the glassy to the less viscous liquid-like state, which allows liquid flow and the formation of interparticle liquid bridges. The close relationships between collapse phenomena and glass transition suggest that the former occur above T_g with rates that are defined by the temperature difference, T–T_g.

Agglomeration is an important step in achieving instant solubility properties for dairy powders. The process is based on controlled thermal and water treatment of fine particles. Common agglomeration methods are based on rewetting of fine powders or on agglomeration during and after spray drying using a straight-through process. The straight-through process is accomplished by producing plasticized particles with a temperature and water content that allow sufficient plasticization of particle surfaces and the formation of interparticle liquid bridges. The plasticized agglomerates enter a vibrating fluidized bed dryer, which completes dehydration and allows sufficient cooling of the product with concurrent solidification and vitrification of the particle surfaces. Agglomeration by both the rewetting and straight-through processes requires that amorphous solids are allowed to exist for a sufficiently long time in the plasticized state at appropriate temperature–humidity conditions allowing controlled stickiness. The proper agglomeration conditions are defined by the T_g of the particles and their water plasticization properties.

Lactose Crystallization

Drying of milk and whey by spray drying or roller drying produces a glass that is composed of a noncrystalline mixture of α- and β-lactose. The existence of lactose in the glassy state and lactose crystallization due to increased molecular mobility have been confirmed by several studies, which have determined the physical state using polarized light microscopy, electron microscopy, differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) spectroscopy, and X-ray techniques. Crystallization of amorphous lactose in dairy powders may accompany the glass transition due to thermal or water plasticization and the increase in molecular mobility. Such crystallization is often detrimental to powder quality and it may significantly alter rehydration characteristics and reduce shelf life.

In general, the crystallization time of amorphous sugars above T_g depends on temperature and water content. The crystallization behavior of amorphous lactose is also temperature dependent. An increase in storage temperature shifts the break in the sorption isotherm, indicating loss of sorbed water, to a lower relative humidity. Typical DSC heating scans of milk powders with amorphous lactose show a glass transition followed by a crystallization exotherm. Lactose crystallization during water sorption may occur either into the anhydrous β-form or into α-lactose monohydrate. The crystalline form produced depends on relative humidity and temperature. Lactose crystallization occurs into the anhydrous β-form at relatively low water activities and the α-lactose monohydrate form often crystallizes at water activities above 0.57 a_w at room temperature. Crystallization into the anhydrous β-lactose crystals releases water associated with the amorphous lactose while the α-lactose monohydrate contains 5% water in the crystalline lactose phase. At higher temperatures, crystallization behavior may change according to the stability of the crystalline form at the crystallization temperature.

The kinetics of crystallization at a constant temperature above glass transition can be related to water content and water activity, which define the T–T_g. Therefore, lactose crystallization may occur above a critical water content or water activity at a constant temperature with a rate defined by T–T_g. An increasing relative humidity increases water sorption by amorphous lactose, which causes water plasticization and increases the temperature difference, T–T_g. Combined T_g and water sorption data have suggested that a water content of 7.6 g per 100 g non-fat solids depresses the glass transition to room temperature. The corresponding water content for pure lactose is 6.8 g per 100 g solids and the critical a_w is 0.37. This water activity or storage relative humidity of 37% RH is empirically known as critical to the stability of dairy powders, including milk and whey powders.

Milk powders with lactose hydrolyzed to galactose and glucose show no break in the sorption isotherm. Both the water sorption and crystallization behavior of these sugars differ significantly from that of lactose, and the behavior of the sugar mixture differs from that of typical skim milk solids. For example, component crystallization in the protein–glucose–galactose mixture is delayed in comparison to lactose crystallization in common dairy powders. Skim milk powders containing hydrolyzed lactose show a glass transition well below that of amorphous lactose. The glass transition of lactose-containing anhydrous skim milk powder has an onset at 92 °C. Anhydrous powder produced from skim milk with lactose hydrolyzed to galactose and glucose has the glass transition onset at 49 °C, and the critical water content that depresses the glass transition to room temperature is as low as 2.0 g per 100 g. The decrease in the T_g and the low critical water content are responsible for difficulties in the production of lactose-hydrolyzed dairy powders. These powders are extremely sensitive to temperature and water and they show hygroscopicity and stickiness during processing and storage.

Chemical Stability

Rates of deteriorative changes at reduced water activities are often related to water content and molecular mobility. Water as a plasticizer has a significant effect on molecular mobility above a critical, temperature-dependent water activity or water content. Molecular mobility is governed by the physical state and water plasticization of solids, and rates of several deteriorative changes are probably affected by diffusion. Diffusion below T_g may be assumed to be restricted, and a chemical reaction may become diffusion limited in a glassy matrix. Although diffusion of water occurs in glassy systems, the diffusion of larger reactant molecules is most likely to be affected by the glass transition. A significant increase in a reaction rate may occur as the material is transformed into the supercooled liquid state as a result of the glass transition. The temperature dependence of chemical changes often follows the Arrhenius equation, but kinetics may show deviations from Arrhenius kinetics at reduced water contents due to diffusional limitations.

Non-enzymatic browning is one of the most important, water content-related deteriorative reactions in low-moisture dairy foods. The non-enzymatic browning reaction is a series of condensations, but it may be considered as a bimolecular reaction. Browning rates in non-fat milk powder below the critical water activity (water content) are low, but the rate of browning is dependent on water content and it increases substantially above the critical water activity. In general, non-enzymatic browning occurs very slowly in glassy dairy products. However, above the glass transition, the rate of the reaction increases and a further increase often results from lactose crystallization and release of the sorbed water. Lactose crystallization must be prevented to avoid caking and impaired solubility. The loss of lysine is most rapid at water activities that allow lactose crystallization. It should also be taken into account that crystallization of amorphous lactose in closed containers or packages is more detrimental as the water released from amorphous lactose remains in the system, accelerating deterioration of the noncrystalline solids.

Diffusion of reactants is probably the main requirement for the occurrence and increasing rates of chemical reactions above some critical temperature or water content. In some cases, flow through pores may increase reaction rates. Such exceptions include oxidation of free fat in dairy powders. Oxygen may diffuse in the material and enhance oxidation on the pore membranes. Crystallization of lactose coincides with an increase in free fat, which presumably facilitates lipid oxidation. In powders containing amorphous lactose, milk fat is encapsulated within the amorphous lactose–protein matrix and it is protected from oxidation. Exceeding the T_g and subsequent crystallization releases the encapsulated lipids, which become accessible to atmospheric oxygen and undergo oxidation rapidly.

Frozen Dairy Products and Ice Cream

The freezing temperature of milk, about −0.53 °C, is relatively constant. At the freezing temperature, all ice formed at lower temperatures is melted into water. The initial ice formation in milk, dairy products, and in foods, in general, requires supercooling to below the equilibrium melting temperature and it is followed by further crystallization of water as the temperature is decreased. Freezing behavior of water, for example, in ice cream is significantly affected by the component compounds, and by sugars in particular. The main component affecting freezing behavior is lactose. Ice formation in a lactose solution, provided that no lactose crystallization is taking place, occurs at temperatures above −30 °C. At −30 °C, the maximum ice formation in the solution can be achieved and a highly viscous, freeze-concentrated lactose solution with approximately 80% lactose and 20% unfrozen water remains unfrozen, as described by the state diagram. During further cooling, this unfrozen solution suffers the glass transition. Such nonequilibrium ice formation is a typical phenomenon of carbohydrate solutions and probably the most common form of ice formation in frozen foods, including frozen dairy products.

The viscosity of a freeze-concentrated solute phase is an important factor that may affect time-dependent crystallization phenomena, ice formation and recrystallization, and material properties. At a sufficiently low temperature, the viscosity of a freeze-concentrated solute matrix becomes high enough to retard diffusion and delay ice formation. The ice formation in real time ceases at the T'_g, since the high viscosity of the freeze concentrated solute matrix prevents diffusion of water molecules to the surface of ice crystal lattice and crystal growth. Ice formation and the extent of freeze concentration are dependent on temperature according to the melting temperature depression of water caused by the solute phase. Maximum freeze concentration may occur at temperatures slightly below the onset temperature of ice melting, T'_m, in the maximally freeze-concentrated material.

The size of ice crystals that are formed during freezing depends on the freezing method and freezing rate. Rapid freezing at a low temperature produces a large number of small ice crystals, while slow freezing at a higher temperature results in the formation of relatively few large ice crystals. The ice crystals, which form during freezing, are not stable, and recrystallization is common at typical storage temperatures of frozen dairy products. Recrystallization is a temperature-dependent process, which is enhanced by temperature fluctuations. Recrystallization with a decrease in the number of crystals and an increase in the average crystal size is referred to as Ostwald ripening. Recrystallization by fusion of smaller crystals resulting in the formation of large ice crystals is also an important recrystallization mechanism in ice cream. Melt–refreeze crystallization, which involves melting of ice and refreezing of unfrozen water, may occur under fluctuating temperatures, especially at relatively high temperatures. An average ice crystal size of 40 μm with a distance of 6–8 μm between the crystals is acceptable. The critical size, which produces a grainy texture, is 40–55 μm. Recrystallization of ice in ice cream and other products that are consumed in the frozen state produces a coarse, icy, undesirable texture. In addition, solute crystallization in freeze-concentrated products can be retarded by the use of sugar blends and syrups.

Evaluation of kinetic data on ice recrystallization should consider the effect of ice melting above T'_m. The viscosity of the unfrozen matrix is increased by stabilizers, which decrease the rate of recrystallization. Recrystallization of ice, as well as lactose crystallization during frozen storage, can be reduced by using hydrocolloids, such as carrageenans, guar gum, or locust bean gum, as stabilizers. Polysaccharide stabilizers do not significantly alter the T'_g of ice cream mixes, but ice crystal growth in stabilized ice cream above T'_g is a function of kinetic properties of the unfrozen solute matrix and the mobility of water within the unfrozen matrix. An increase in the amount of unfrozen water increases the recrystallization rate, but stabilizers reduce the recrystallization rate.

Water and Microbiological Stability

Microbial growth requires a minimum amount of water in an environment supporting the growth of microorganisms. In dairy products, the effect of water on the growth of microorganisms is probably most important in the ripening, texture, and quality of cheeses. Water availability may also be an important factor in controlling mold growth in low-fat dairy spreads and butter. Water activities of milk products vary widely, from 0.1 to 0.3 for dried dairy products to above 0.99 for liquid milk and whey. Sweetened condensed milk with 0.77–0.85 a_w has an intermediate water activity, but most other dairy foods have high water activities supporting the growth of bacteria and other microorganisms.

The minimum requirement for microbial growth is a_w 0.62, which allows the growth of xerophilic yeasts. An increasing a_w allows the growth of molds, other yeasts, and finally bacteria at high water activities. The most important water activity value for the safety of food materials is 0.86, which is the limit for the growth of *Staphylococcus aureus* (**Table 1**). The water activity of various cheeses and processed cheeses varies between 0.86 and 0.99 (**Table 2**). There are also critical a_w values for the multiplication of bacteria used in cheese manufacture. For example, propionibacteria, which are responsible for eye formation in Swiss cheese, are very

Table 1 Minimum water activities (a_w) for the growth of selected pathogenic bacteria in dairy products

Pathogen	*Minimum* a_w
Bacillus cereus	0.930
Campylobacter jejuni	0.990
Clostridium perfringens	0.945
Escherichia coli	0.935
Listeria monocytogenes	0.920
Salmonella spp.	0.940
Shigella spp.	0.960
Staphylococcus aureus	0.860
Vibrio parahaemolyticus	0.936
Yersinia enterocolitica	0.960

Table 2 Typical water activity (a_w) of common cheeses at 25 °C

Cheese type	*Water activity*
Appenzeller	0.96
Brie	0.98
Blue	0.94
Camembert	0.98
Cheddar	0.95
Cottage cheese	0.99
Edam	0.96
Emmentaler	0.97
Gouda	0.95
Mozzarella	0.99
Parmesan	0.92
Tilsiter	0.96

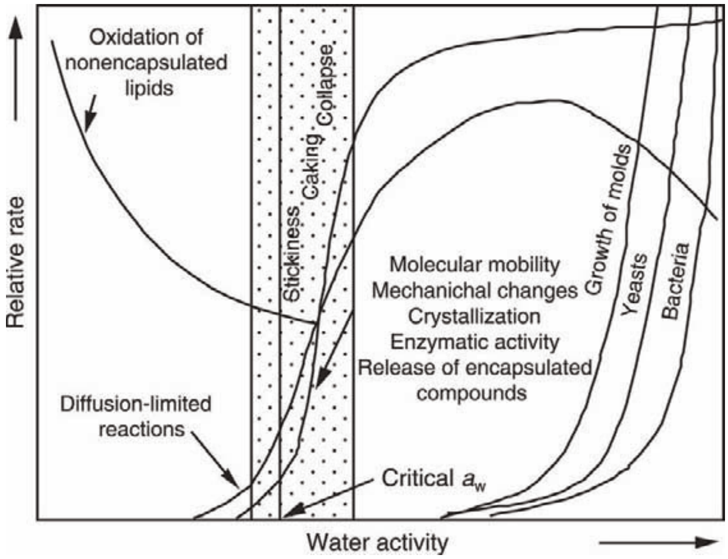

Figure 4 Food stability map showing the effect of water activity on the relative rates of various changes in food systems. The critical a_w refers to the water activity at which the glass transition occurs at the storage temperature.

sensitive to changes in a_w over the range 0.95–0.99. Water activity in cheese ripening may affect growth and CO_2 production.

Inhibition of the growth of most microbes in salted butter may be accounted for by the high concentration of salts in the water fraction and reduced water activity. The water activity of salted butter is 0.91–0.93, while that of unsalted butter is >0.99. In low-fat dairy spreads, a low pH with a reduced water activity is necessary to prevent the growth of pathogens and molds.

Stability Maps

The relative rates of deteriorative changes in food materials are traditionally related to water content and water activity on the assumption that stability at common storage temperatures can be maintained at a low water content. Structural transformations in milk solids may occur at temperatures above the glass transition, which corresponds to a water content that can be quantified. Such transformations include collapse of the physical structure, which reduces diffusion through pores and crystallization of amorphous lactose. Crystallization of amorphous lactose may also release encapsulated fat, which becomes susceptible to oxidation.

The effect of water activity on the relative rates of deteriorative changes is often described by stability maps, which are used to show the relative rate of enzymatic changes, non-enzymatic browning, lipid oxidation, microbial growth, and overall stability as a function of water activity (**Figure 4**). Various reactions rates may also be related to the physical state, molecular mobility, and water plasticization and glass transition of amorphous food solids. It is obvious that structural transformations, as well as diffusion-limited deteriorative reactions and those affected by lactose crystallization, occur at increasing rates with increasing water activity above the critical a_w.

See also: **Butter and Other Milk Fat Products**: Anhydrous Milk Fat/Butter Oil and Ghee; Fat Replacers; Milk Fat-Based Spreads; Modified Butters; Properties and Analysis; The Product and its Manufacture. **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of milk Powders; Milk Powder: Types and Manufacture. **Ice Cream and Desserts**: Dairy Desserts; Ice Cream and Frozen Desserts: Manufacture; Ice Cream and Frozen Desserts: Product Types. **Plant and Equipment**: Milk Dryers: Dryer Design; Milk Dryers: Drying Principles.

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Analysis and Measurement of Water Activity

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Definition and Significance of Water Activity

Definition

Water activity of a system is a way of characterizing the potential energy of the contained water, which is thought to be related to the difficulty to remove it, for example, in drying, and to its availability to allow the functioning of living cells. It can be viewed, for instance, as the difference between the water vapor pressure measured over pure water and that measured over a food product (**Figure 1**(**a**)), or as the energy necessary to compensate for the osmotic pressure of a solution (**Figure 1**(**b**)). **Figures 2** and **3** show schematic views of water activity of dairy products as compared to their water content.

The chemical potential (μ_i, J mol^{-1}) of the component i in a mixture is defined as the partial derivative of the free energy, G, when the number of molecules of i (n_i) is varied, whereas the temperature, pressure, and total number of molecules in the system are kept constant.

$$\left(\frac{\partial G}{\partial n_i}\right)_{T,P,n} = \mu_i \qquad [1]$$

If the component considered is water, the subscript w will be used. For a process where the water concentration (expressed as the mole fraction X_w) is changed from X_w^0 to X_w, the change in free energy, ΔG, is

$$\Delta G = \mu_w - \mu_w^0 \qquad [2]$$

where X_w^0 and μ_w^0 concern a state of reference, namely, pure water under the temperature and pressure conditions of the system. As we will see later, ΔG can be calculated for various processes, for example, as the work corresponding to the upward motion of the piston if water is allowed to enter the concentrated solution in the system shown in **Figure 1**(**b**), or as the change in free energy as the pressure is changed from p_w^0 to p_w in the system shown in **Figure 1**(**a**).

If the system is a dilute solution, ΔG is observed to be proportional to the logarithm of the solvent molar fraction (Raoult's laws and van't Hoff's law):

$$\Delta G = \mu_w - \mu_w^0 = RT \ln X_w \qquad [3]$$

Solutions obeying eqn [3] are called 'ideal' solutions. For nonideal systems, X_w is replaced by a new characteristic named 'water activity' (a_w):

$$\Delta G = \mu_w - \mu^0{}_w = RT \ln a_w \qquad [4]$$

Comparing eqns [3] and [4], $a_w = X_w$ for ideal solutions. For nonideal solutions, a parameter, γ (activity coefficient), was introduced to represent the deviation from ideality:

$$a_w = \gamma X_w \qquad [5]$$

Water activity is a colligative property; that is, it depends on the number of solute molecules in the solution. The presence of solutes induces a disorder in the water structure, that is, an increase in entropy, which results in a reduced chemical potential. With small solutes, a_w is controlled mainly by X_w, that is, by the number of solute molecules. In the presence of macromolecules or hydrophilic surfaces (cell membranes), the mixing entropy plays a minor role in the depression of a_w (X_w remains high); low values of μ_w (and, in turn, of a_w) are attributed to interactions of the water molecules with the other constituents. When interactions occur between water and solutes (dipole–dipole, ionic interactions, or hydrogen bonds), or when the size of the solute is much larger than the size of the water molecule, $\gamma < 1$. When solute–solute interactions dominate over water–solute interactions, $\gamma > 1$ (remember, however, that a_w is always <1). **Figure 4** shows some examples of the a_w-lowering effect of dairy components.

If pure water is contained in a cylindrical capillary space (diameter d), the wall of which is perfectly wetted by water, the difference in chemical potential defined as in eqn [2] can be shown to be (Kelvin's law)

$$\mu_w - \mu^0{}_w \cong -V_w \sigma \frac{4}{d}$$

$$\ln a_w = -\frac{V_w \sigma}{RT}\frac{4}{d} \qquad [6]$$

where V_w and σ are, respectively, the molar volume and the surface tension of water. For a descending pore diameter range of 100–1 μm (and a surface tension of ~35 mN m^{-1} for a caseinate solution–air interface), eqn [6] predicts a_w in the range 1–0.999, that is, a very small lowering of a_w by capillary effects. If Kelvin's law can be assumed to be valid for a pore size approaching molecular dimensions ($d \approx 10$ nm), a_w could be lowered to ~0.90.

In products with intermediate or low water content, it is generally considered that water is retained by adsorption processes. The model most often referred to is the BET multilayer adsorption process, according to which a

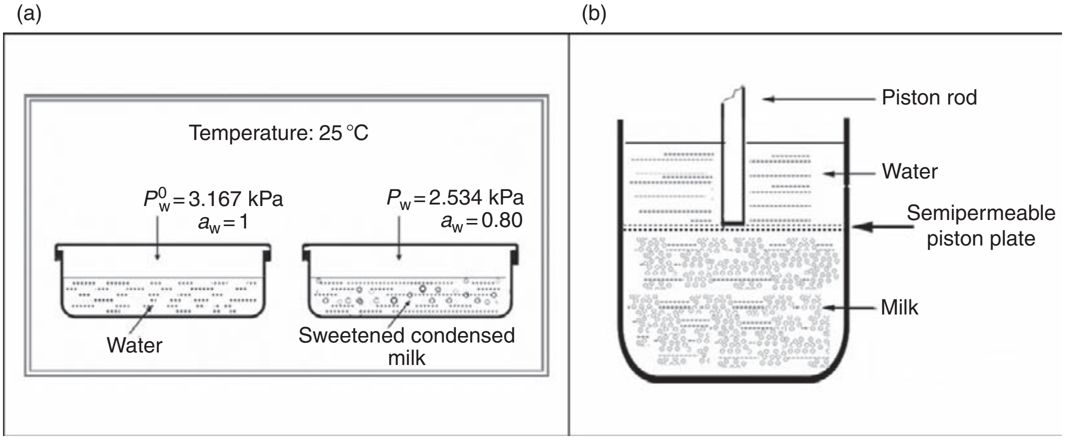

Figure 1 (a) Schematic representation of the difference in water vapor pressure above pure water and above a food product, measured at the same temperature (*T*). This difference may be known, either from a direct measurement of the partial water vapor pressures ($P^0{}_w$ (*T*), the saturated water pressure at *T*, above pure water, and P_w above sweetened condensed milk) or from the relative humidity (RH) in the headspaces. (b) Since the chemical potential of water in the concentrated milk is less than in pure water, there is a tendency for water to move into milk through the semipermeable plate. A downward pressure, $\prod$, on the piston is required to maintain it in place. Reproduced from Simatos D, Champion D, Lorient D, and Roudout G (2009) Water in dairy products. In: McSweeney PLH and Fox PF (eds.) *Advanced Dairy Chamistry-3. Lactose, Water Salts and Minor Constituents*, 3rd edn. In press.

first layer of sorbate (water) molecules would be formed on the surface of the solid, followed by a multilayer condensation of the sorbate onto the monolayer.

The relations between a_w and water content are represented by sorption isotherms (SI) for dairy products and components, example of which are shown in **Figures 5** and **6**. SI have a sigmoid shape for products with a high content of biopolymers. Small-molecular-weight solutes, such as lactose, have a higher a_w than biopolymers at low water contents, and a lower a_w than biopolymers at high water contents. As illustrated in **Figures 5** and **6**, it is important to remember that important variations in SI may result not only from variations in chemical composition, but also from variations in the conditions of determination such as water content measurement, equilibration time, variations in structure resulting from the drying process, or from evolution during the sorption experiment (crystallization of lactose, physical aging of polymers). SI found in the literature should therefore be used only as a first approximation.

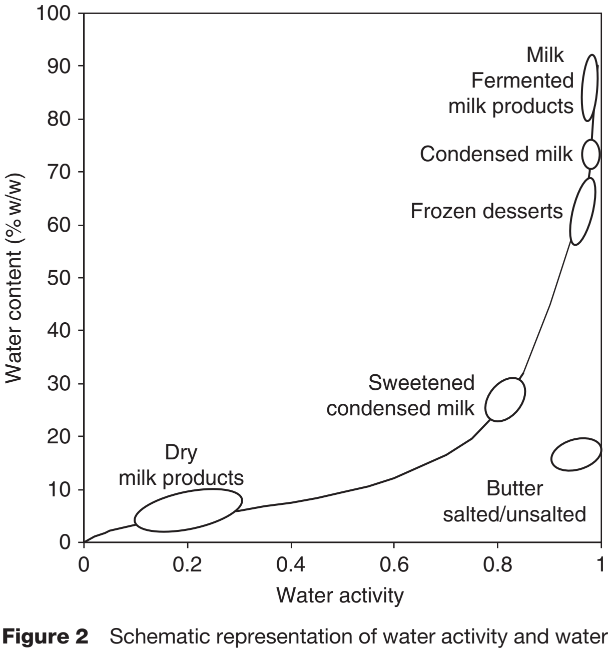

Figure 2 Schematic representation of water activity and water content of various dairy products (for cheese, see **Figure 3**).

One interesting aspect of a_w in food science and technology is that chemical potential must be the same in all phases of the system at equilibrium; this is important because food products most often comprise regions with different chemical compositions, physical states, or structures. A particular example of a multiphase system is frozen food products. As will be seen below, in a frozen product $a_w = p_{ice}/p_{supercooled\ water}$ and is, therefore, a function of temperature only. For instance, a food product with a water content high enough for the food to contain ice at −20 °C has (if at equilibrium) $a_w = 1.035/1.257 = 0.82$.

It must be remembered, however, that the definition of a_w and the derived expressions for its determination assume that the system is in a state of thermodynamic equilibrium. Hence, it is sensible to question the validity of their applicability to food products (Expert Panel, ISOPOW, 2000). Actually, solutions of small solutes, even if concentrated, can be considered to be at thermodynamic equilibrium, whereby the a_w concept is valid. For low-moisture or semi-moist solid food products, it is difficult to predict the actual status of the product, which will depend on (1) its position in comparison with glass

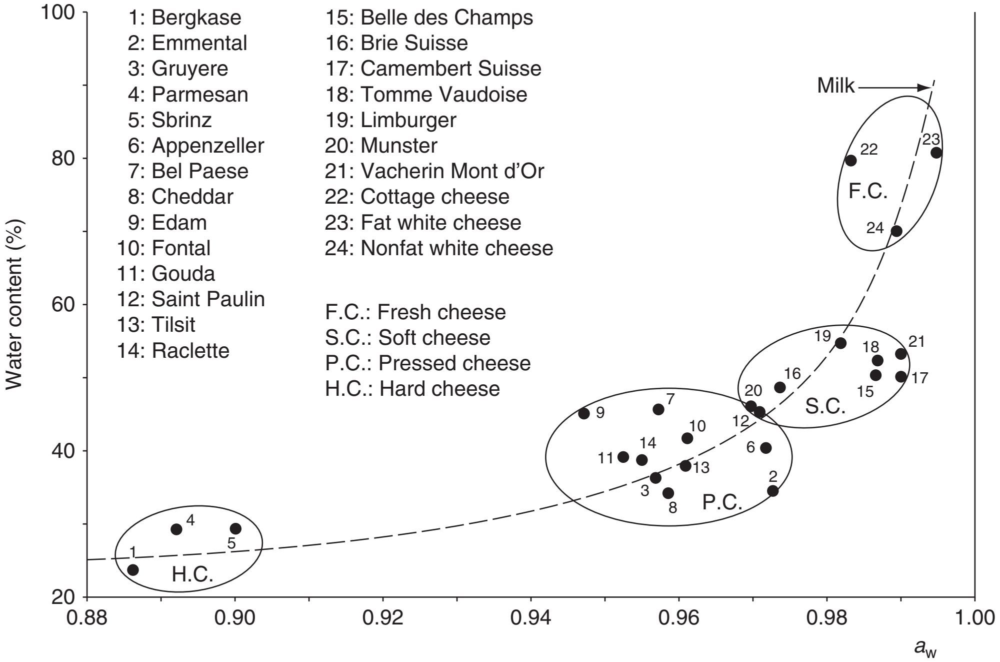

Figure 3 Water content and a_w of cheeses. Reproduced from, Banon S and Hardy J (2002), eau dans les produits laiters. In: LeMeste M, Lorient D, and Simatos D (eds.) *L'Eau dans les Aliments*. pp. 235–238. Paris: Lavoisier.

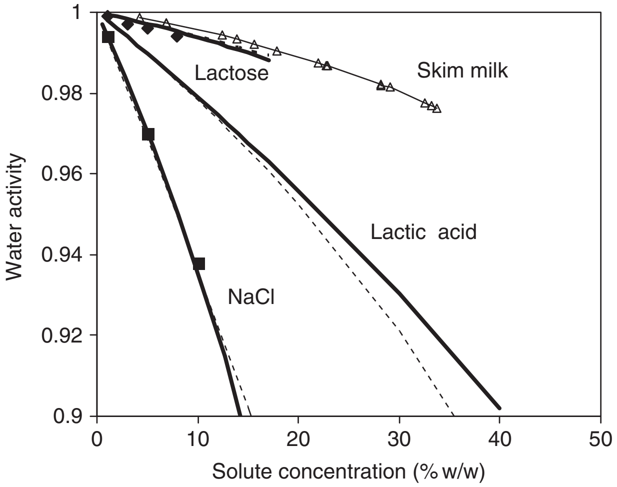

Figure 4 Water activity versus concentration for some dairy components:

- experimental values from freezing point depression: △, spray-dried milks (solute concentration as non-fat solids) (Chen P, Chen XD, and Free KW (1996) Measurment and data interpretation of the freezing point depression of milk. *Journal of Food Engineering* 30: 239–253) ◆, lactose and ■, NaCl (Lerici CR, Piva M, and Dalla Rosa M (1983) Water activity and freezing point depression of aqueous solutions and liquid foods. *Journal of Food Science* 48: 1667–1669).
- bold lines calculated from Norrish equation: lactose (Miracco JL, Alzamora SM, Chirife J, and Ferro Fontan C (1981) On the water activity of lactose solutions. *Journal Food Science* 46: 1612–1613); lactic acid (Chirife J and Ferro Fontan C (1980) The prediction of water activity in aqueous solutions in connection with intermediate moisture foods. V. Experimental investigation of the a_w lowering behaviour of sodium lactate and some related compounds. *Journal of Food Science* 45: 802–804).
- bold line for NaCl: *CRC Handbook of Chemistry and Physics*.
- dotted lines: values calculated from Raoult's law.

Note that the a_w of skim milk is very similar to that of lactose solutions in this a_w range. The large depression of a_W by NaCl is due to its low molecular weight and due to its dissociation into two 'active particles' (ions). Lactic acid has been considered as nondissociated by the authors.

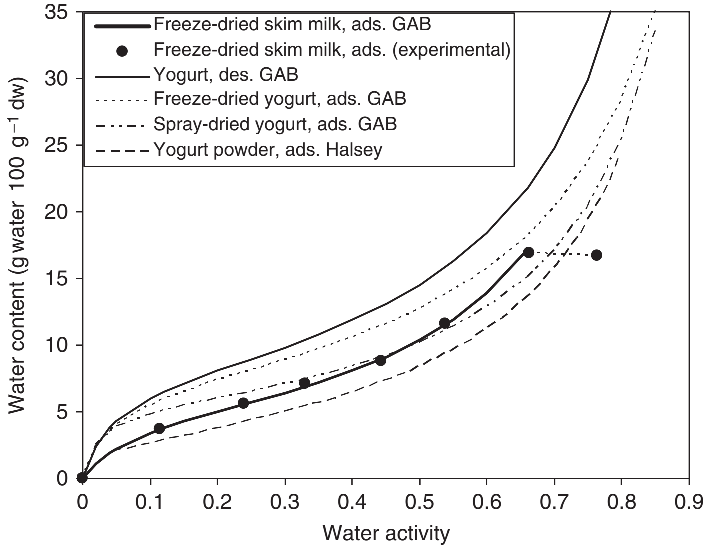

Figure 5 Water sorption isotherms for skim milk powder and yogurts. The experimental data for milk showing that the sorbed water amount remains constant (and even decreases) for a_W >0.66 indicate that lactose is crystallizing (crystals of β anhydrous lactose are able to adsorb much less water than amorphous lactose). Lines are from GAB or Halsey models, as indicated in the figure. (ads, adsorption; des, desorption). Freeze-dried skim milk: Jouppila K and Roos YH (1994) Glass transitions and crystallization in milk powders. *Journal of Dairy Science* 77: 2907–2915; yogurt, freeze-dried and spray-dried yogurt: Kim SS and Bhowmik SR (1994) Moisture sorption isotherms of concentrated yogurt and microwave vacuum dried yogurt powder. *Journal of Food Engineering* 21: 157–175; yogurt powder: Wolf W, Spiess WEL, and Jung G (1973) cited by Iglesias HA and Chirife J (1982) *Handbook of Food Isotherms: Water Sorption Parameters for Food and Food Components*. New York: Academic Press.

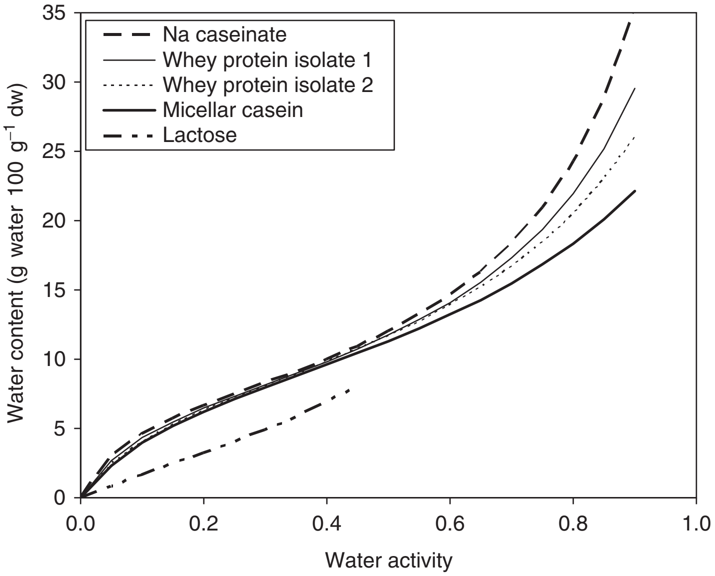

Figure 6 Water sorption isotherms for lactose (adsorption, 20–38 °C, Bronlund J and Paterson T (2004) Moisture sorption isotherms for crystalline, amorphous and predominanthy crystalline lactose powders. *International Dairy Journal* 14: 247–254), micellar casein and whey protein isolate 2 (adsorption, 4–37 °C, Foster KD, Bronlund JE, and Paterson T (2005) The prediction of moisture sorption isotherms for dairy powder. *International Dairy Journal* 15: 411–418), whey protein isolate 1 (adsorption, 23 °C, Zhou P and Labuza TP (2007) Effect of water content on glass transition and protein aggregation of whey protein powders during short-term storage. *Food Biophysics* 2: 108. DOI: 10.1007/s11483-007-9037-4), and Na caseinate (adsorption, 25 °C, Weisser H (1985) Influence of temperature on sorption equilibria. In: Simatos D and Multon J-L (eds.) *Properties of Water in Foods*, pp. 95–118. Dordrecht: Martinus Nijhoff Publishers). The lines are from the GAB model.

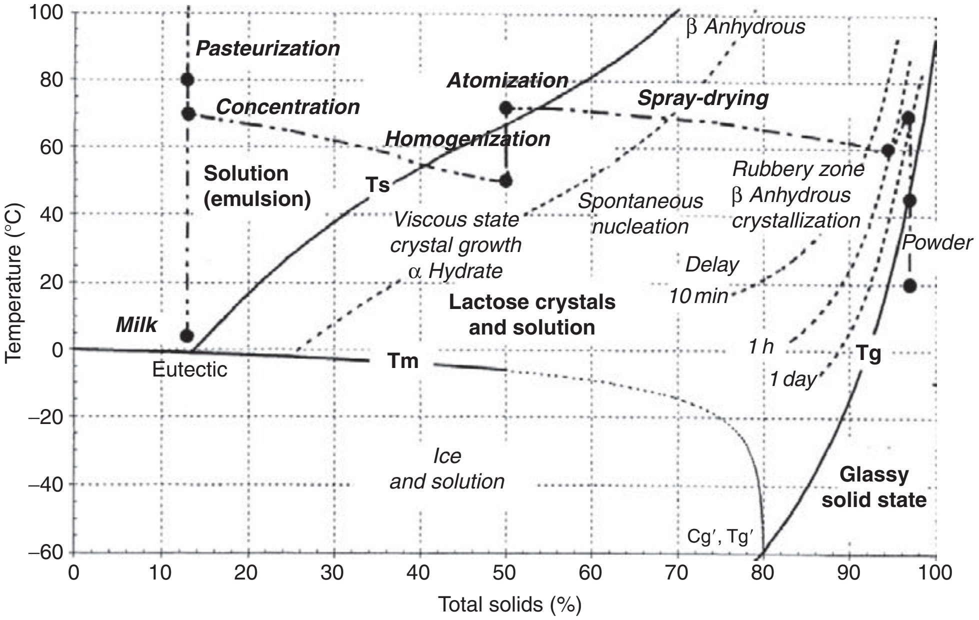

Figure 7 State diagram for milk, based on lactose (Vuattaz G (2002) The phase diagram of milk: A new tool for optimising the drying process. *Lait* 82: 485–500). **Ts**, solubility curve; **Tm**, freezing point versus concentration; **Tg**, temperature of glass transition versus concentration. The dry product (with a water content below ≈5%) is a glass at room temperature. If the ambient temperature is increased, or if T_g is decreased as a consequence of an increase in water content, the product becomes 'rubbery'. An increase in molecular mobility allows crystallization of lactose. The larger the distance of water content/temperature conditions to T_g, the shorter the delay, as indicated on the graph.

transition, as determined by its temperature and water content and (2) the duration of its stay under these conditions. A food material such as skim milk powder in the dry state is in a glassy state at room temperature (its glass transition temperature T_g is ~100 °C). When water is sorbed, the material is plasticized, that is, its T_g decreases to ~20 °C for a critical water content of ~8%. For a higher water content, the product is transformed into a viscoelastic material (supercooled liquid or rubber depending on the composition) (**Figure 7**). In the rubbery state, the product is in a metastable state: amorphous constituents (e.g., lactose) may crystallize; the more distant the product is from its glass transition, the faster the crystallization. In the glassy state, the product is out of equilibrium: it may undergo some evolution of its structure (physical aging); the closer the temperature is to T_g, the faster the evolution, but in practice it is always very slow. In both the rubbery and glassy states, the mobility of water remains rather high, and one may expect that equilibration of a_w will be usually accomplished within times of interest to the food technologist, as suggest some observations. In some situations, however, it must be recognized that what is measured is the relative humidity of the atmosphere in contact with the product, at best in a pseudo-equilibrium state. Hence it would be safer to use the term 'apparent water activity' (Expert Panel, ISOPOW, 2000).

Water Activity versus Bound/Free Water

Water activity is a way of measuring the energy status of water in a product. It gets depressed as a result of water structure being perturbed and due to interactions between water and solute molecules. Nevertheless, it does not allow one to define a fraction of bound water. The first point is the broad range of interaction energies between water and solutes: from the van der Waals interactions (~1 kJ mol^{-1}) to hydrogen bonds (10–40 kJ mol^{-1}) and ion–water interactions (50–100 kJ mol^{-1} for univalent ions and even more for multivalent ions). It can be noted that water–water and water–solute interactions that occur through H bonds have strength values of the same order; water molecules cannot therefore be viewed as strongly bound to solutes. Actually, spectroscopic observations and molecular dynamics simulations show that water molecules remain highly mobile, even when in direct contact with solute molecules. In liquid water at room temperature, water molecules tumble about with a reorientation time of ~2 ps (2×10^{-12} s). Although the properties (orientation, mobility) of some water molecules belonging to the primary hydration shell of ions are strongly modified, they still exchange with bulk water, albeit more slowly (e.g., lifetime ~10^{-9} s for Na^+). Similarly, molecular dynamics simulations of a sugar molecule in an aqueous solution show water molecules located at specific sites on the sugar molecule; however, within a few picoseconds, these molecules escape into the bulk water and are replaced by other water molecules. From spectroscopic methods, the ratio $\tau_s/\tau_{\mathrm{bulk}}$ of the rotational correlation time of water molecules in direct contact with the surface of solutes and that of bulk water is found to be in the range 1.0–2.5 at room temperature for free amino acids and other small organic molecules. For proteins in solution, only water molecules in direct contact with the protein surface are significantly perturbed, although they are still highly mobile, with mean residence times in the range 10–100 ps and the ratio $\tau_s/\tau_{\mathrm{bulk}}$ averaging around 5.5. Only water molecules buried within the protein have residence times longer than 1 ns (in the range 10^{-8}–10^{-4} s at room temperature). It is estimated that reduction of the mobility of water molecules in contact with proteins is not due to the interaction with the protein *per se* but rather due to their physical entrapment within the protein matrix.

Even in low-moisture solid products, water molecules appear to have a relatively high freedom of motion. The translational diffusion coefficient of water in a hydrated polysaccharide (pullulan with 20% water) has been estimated at $5 \times 10^{-12}\,\mathrm{m}^2\,\mathrm{s}^{-1}$ at 0 °C, that is, 1000 times lower than in liquid water, whereas the viscosity of the system in this glassy state (~50 °C below the glass transition temperature) would be 10^{15} times higher.

Based on the multilayer adsorption process, mathematical expressions have been derived to describe sorption isotherms (**Table 1**). The BET expression correctly describes the experimental curves of food materials for $0.2 < a_w < 0.5$; the GAB expression, which is derived from the BET expression, can be fitted satisfactorily to experimental data up to a_w ~0.9. The fitting parameters of these expressions are commonly used to calculate the water content corresponding to the monolayer and sorption energies, which are supposed to give information about the interactions of water with the material components. However, this use of the BET and GAB expressions is very questionable. First, the hysteresis observed between sorption and desorption points to the non-equilibrium character of the isotherms. Moreover, it is increasingly being admitted that the basic assumptions of the BET model are not fulfilled in the case of water sorbed on polar materials (energy equivalence of all sites on the sorbing solid surface). In the end, the plastifying action of water on the solid certainly plays a role in determining the form of the sorption curves. A plausible view would be that the sorption process changes at glass transition: in the glassy state, where the conformation is 'frozen', an adsorption model (such as the Freundlich model, assuming a distribution of independent sorption sites with different energies) would be suitable, whereas in the supercooled state, the isotherm could be based on the Flory–Huggins theory of polymer solutions.

Water Activity versus Food Quality and Food Processing Operations

In the 1960s, water activity became the favored parameter to characterize the availability of water to control the physical, chemical, or biological evolutions in foods. This originated in observations showing that the a_w values of media generally correlated well with the potential for growth and metabolic activity of microorganisms. Stability maps were produced, indicating a_w thresholds and a_w ranges corresponding to the maximal rates of chemical and biological evolutions. In the late 1980s, the value of a_w as a predictive index of food stability was questioned, first because most food products are not in a state of thermodynamic equilibrium, as discussed before; moreover, emphasis was placed on the importance of molecular mobility, namely in connection with the glass transition phenomenon. The respective relevance of both approaches, a_w and glass transition, has been discussed vigorously. Currently, a consensus appears to have become established, to recognize that both may have an essential role, in a particular domain, depending on the type of product or the objectives.

Microbial cells cannot be considered to behave as true osmometers; other parameters, such as pH, nature of the solutes in the medium, and mobility of the metabolites, must also be taken into account. Glass transition concepts, however, do not provide any better alternatives than a_w as a predictor of microbial behavior. Water activity is now generally recognized as an essential parameter for all aspects of microbial activity: germination and growth, and production of toxins and aroma. As regards texture, water content seems a better predictor than a_w; for instance, the texture of cheese was found to be better correlated to water content (coefficient of correlation with an extrusion force = −0.867) and fat content than to a_w (coefficient of correlation = −0.548). Crystallization (e.g., of lactose in milk powder) is a good example of evolution, the kinetics of which are controlled by water content and temperature, in connection with glass transition. With regard to chemical and biochemical reactions, the implication of water is complex: besides being a reactant for many reactions in foods and being necessary for the establishment of the appropriate conformation of enzymes, it constitutes the usual solvent for reactants and imparts the necessary mobility to reactants and reaction products. Water activity does not seem to be directly involved in the control of reaction kinetics; the observation that a reaction (e.g., non-enzymatic browning) occurs, in various food products, with a maximum rate in a characteristic a_w range most likely can be explained by these products having similar sorption isotherms and similar T_g values (e.g., because of a high content of biopolymers). Actually, if mobility is increased in these products, through the addition of a liquid fat or of a

Table 1 Expressions to describe or predict relations between water activity and water content

Model	*Equation*	*Parameters*	*Range*	*References (for dairy products)*
Raoult's law	$a_W = X_w = \dfrac{m/18}{m/18 + (100/M_s)\nu}$	X_w = mole fraction of water M_s = molecular weight of the solute ν = dissociation constant of electrolyte	Dilute solutions	
Norrish	$a_W = X_w \exp(-KX_s^2)$	X_s = mole fraction of solute K = empirical constant for the solute[a]	Nonelectrolyte solutions	Chirife and Ferro Fontan (1980), Miracco *et al*. (1981)
Pitzer	$\ln a_w = -0.180\,2\Phi \sum_i \mu_i$ $\Phi = 1 + \lvert z_M z_X \rvert F + 2\mu\left(\dfrac{n_M n_X}{n}\right) B_{MX} + 2\mu^2\left(\dfrac{n_M n_X}{n}\right)^{0.5} C_{MX}$ $F = -0.392\dfrac{I^{0.5}}{1 + 1.2I^{0.5}} \qquad I = 0.5 \sum_i \mu_i z_i^2$ $\lvert z_M z_X \rvert = \dfrac{\sum_i \mu_i z_i^2}{\sum_i \mu_i} \qquad B_{MX} = B_{MX}(0) + B_{MX}(1)\exp(-2I^{0.5})$	Φ = osmotic coefficient $\mu_{\mathbf{i}}$ = molality of ion i Z_M, $Z_{\mathbf{X}}$, n_M, n_X = charges and numbers of ions M and X $n = n_M + n_X$ I = ionic strength $B_{MX}(0)$, $B_{MX}(1)$, C_{MX} = Pitzer coefficients for the electrolyte MX[b]	Electrolyte solutions	Ferro Fontan *et al.* (1980)
Flory–Huggins	$\ln a_w = \ln(1 - \Phi_2) + \left(1 - \dfrac{1}{n}\right)\Phi_2 + \chi\Phi_2^2$	Φ_2 = volume fraction of the polymer n = number of polymeric segments χ = fitting interaction parameter	$a_w > 0.90$	
Freundlich	$m = Ca_w^{1/n}$	n > 1	Glassy state	
BET	$m = \dfrac{m_{1B} C_B a_w}{(1 - a_w)[1 + (C_B - 1)a_w]}$	m_{1B} = water content of the « monolayer »	0.2 < a_w < 0.5	Ruegg amd Blanc (1979)
GAB	$m = \dfrac{m_{1G} C_G K a_w}{(1 - K a_w)[1 + (C_G - 1)K a_w]}$	m_{1G} = water content of the « monolayer »	0.2 < a_w < 0.9	Bronlund and Paterson (2004), Jouppila and Ross (1994), Kim and Bhowmik (1994), Lin *et al*. (2005), Lomauro *et al*. (1985), Weisser (1985)
Kuhn	$m = \dfrac{K_1}{\ln a_w} + K_2$			Jouppila and Rose (1994)
Halsey	$\ln a_w = -\dfrac{P_1}{mP_2}$			Iglesias and Chirife (1982), Kim and Bhowmik (1994), Miracco *et al*. (1981)
Peleg	$m = k_1 a_w^{n_1} + k_2 a_w^{n_2}$			
Lewicki	$m = A(\dfrac{1}{a_w} - 1)^{b-1}$			

(Continued)

Table 1 (Continued)

Model	*Equation*	*Parameters*	*Range*	*References (for dairy products)*
Ross	$a_{\text{wmix}} = (a_{\text{w}1})\ldots(a_{\text{w}i})\ldots(a_{\text{w}n})$	$a_{\text{wmix}} = a_{\text{w}}$ of a mixture of n solutes $a_{\text{wi}} = a_{\text{w}}$ of a solution where the solute i would be dissolved in all the water of the mixture		
Salwyn-Slawson	$a_{\text{wmix}} = \dfrac{\sum \text{M}_i a_{wi} \tan_i}{\sum \text{M}_i \tan_i}$	$a_{\text{wmix}} = a_{\text{w}}$ of a mixture of n components for which SI are known M_{i} = dry weight of the component i a_{wi} = initial a_{w} of component i tani = average slope of the SI of component i in the range of a_{wi}		
Additive model	$m(a_{\text{w}}) = \sum_{1}^{n} M_i m_{i(a_w)}$	m=predicted water content at a_{w} M_{i} = mass fraction of component *i* (db) $M_i(a_w)$ = water content of i at a_{w}		4

[a] $K = 10.2$ for lactose; $K = -1.59$ for lactic acid.

[b] For NaCl, $B_{\text{MX}}(0) = 0.076\,5$, NaCl, $B_{\text{MX}}(1) = 0.266\,4$, $C_{\text{MX}} = 0.001\,27$.

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Table 2 Water activity of cheese vs. chemical composition

Equation	*Range*	*Std error of estimate*	*Concentration units*	*References*
$a_w = 0.94 - 0.005\,6(\mathrm{NPN}) - 0.005\,9(\mathrm{NaCl}) - 0.001\,9(\mathrm{Ash\text{-}NaCl}) + 0.015\mathrm{pH}$	$a_w > 0.90$	0.009	g per 100 g water	Ruegg (1985)
$a_w = 1.004\,8 - 0.038\,6(\mathrm{NaCl})$	Fresh cheeses (moisture > 40% ≈ no proteolysis)	0.01	mol kg^{-1} water	Esteban and Marcos (1990)
$a_w = 1.023\,4 - 0.007\,0(\mathrm{Ash})$	Bacterial-ripened cheeses		g per 100 g water	
$a_w = 0.980\,8 - 0.005\,8(\mathrm{Ash})$	Blue cheeses		g per 100 g water	
$a_w = 0.99\,0 - 0.936(\mathrm{NaCl}) + 0.951(\mathrm{water})(\mathrm{NaCl})$	French Emmental after brining	0.006 0.006	kg$_{\mathrm{NaCl}}$ kg^{-1} water kg$_{\mathrm{water}}$ kg^{-1} dry solids	Saurel *et al.* (2004)
$a_w = 1.066 - 0.194(\mathrm{water}) - 3.490(\mathrm{NaCl}) - 0.331(\mathrm{NH_2}) + 6.509(\mathrm{water})(\mathrm{NaCl}) + 0.571(\mathrm{water})(\mathrm{NH_2})$	after ripening		mol eq glycine kg^{-1} cheese	

From Ruegg M (1985) Water in dairy products related to quality, with special reference to cheese. In: Simatos D and Multon J-L (eds.) *Properties of water in Foods*, pp. 603–625. Dordrecht: Martinus Nijhoff Publishers; Esteban MA and Marcos A (1990) Equations for calculation of water activity in cheese from its chemical composition: A review. *Food Chemistry* 36: 179–186; Saurel R, Pajonk A, and Andrieu J (2004) Modelling of French Emmental cheese water activity during salting and ripening periods. *Journal of Food Engineering* 63: 163–170.

plasticizer such as glycerol, the a_w range for maximal reaction rate can be shifted widely. However, a_w continues to serve as a useful guide for chemical stability because it allows prediction of the water content of the product in a given environment (so long as the specific sorption isotherm of the product is known). More generally, a_w is an essential tool in food technology, because it allows description of the gradient that will determine the transfer of water between two compartments having different initial relative vapor pressures in a multidomain food system, or between a food product and its environment during drying or osmotic dehydration, or during storage.

Principles of Measurement

Physical Properties to Be Measured

Water activity cannot be measured directly, but eqn [4] allows derivation of relations between a_w and some physical properties (**Table 3**), which lead to measurement methods.

Water vapor pressure

When the pressure of water vapor is changed from p^0 to p (**Figure 1(a)**), the change in free energy can be calculated from basic thermodynamics (and assuming that water vapor behaves as a perfect gas) to be $\Delta G = RT\ln p/p^0$. Then

$$a_w = \frac{p}{p^0} \qquad [7]$$

$100 \times p/p^0$ (%) defines the relative humidity (RH) of the atmosphere in equilibrium with the product, and a_w can be obtained from the measurement of p by various methods and calculation using the known p^0 values at the temperature of measurement.

- *Direct measurement with a manometer*: The air must be evacuated from the apparatus; drying of the sample during this operation must be kept at a negligible

Table 3 Relations between water activity and physical properties

(1) Equilibrium relative humidity	$a_w = \dfrac{p}{p^0} = \dfrac{\mathrm{ERH}}{100}$	p = partial water vapor pressure (Pa) p^0 = saturated water vapor pressure (Pa) at temperature T ERH = equilibrium relative humidity
(2) Freezing point depression	$\ln a_w = \dfrac{\Delta H_m}{R}\dfrac{T - T_0}{TT_0} + \dfrac{\Delta C_p (T - T_0)^2}{2RT^2}$	T_0, T = temperature of freezing of pure water and of the sample (K) ΔH_m = melting enthalpy of ice at T_0 (= 6 kJ mol^{-1}) ΔC_p = difference in the specific heat of ice and liquid water (= 37.697 J K^{-1} mol^{-1}) R = 8.314 J K^{-1} mol^{-1}
(3) Osmotic pressure	$\ln a_w = -\Pi \dfrac{V_w}{RT}$	$\prod$ = osmotic pressure (Pa) V = molar volume (8^{10-6} m^3 mol^{-1}) T = temperature (K) R = 8.314 J K^{-1} mol^{-1}

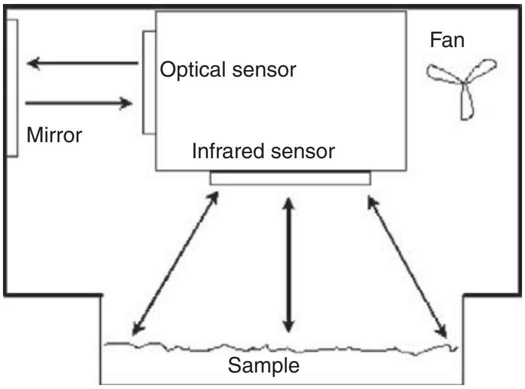

Figure 8 Schematic diagram of a dew point cell. The mirror is cooled progressively (e.g., via a Peltier device). The optical sensor emits light onto the mirror and detects the reflected light. When condensation occurs, the temperature of the mirror is recorded (giving the value of p_W). The temperature of the sample is recorded via the infrared sensor (giving the value of $p^0{}_w$). Diagram from Decagon.

level, for instance, by freezing it. This method is not suitable for products containing volatiles. Moreover, because of its technical requirements, its use is restricted to the laboratory as a standard method.

- *Dew point temperature*: **Figure 8** shows the experimental setup for measuring dew point temperature. As the mirror is progressively cooled, condensation occurs when its temperature is that for which the saturated vapor pressure is equal to p. Commercial instruments claim a measurement range of 0.030–1.000 a_w with an accuracy of ±0.003 a_w.

Freezing point

The second equation in **Table 3** is derived from thermodynamics. ΔC_p is assumed to be independent of temperature in the range T–T_0. This assumption does not result in any significant loss of accuracy. Considering that chemical potential must be equal in both the phases present in a frozen solution, that is, ice and the concentrated solution resulting from the separation of ice, the vapor pressure at equilibrium with the frozen product is p_{ice}. The reference vapor pressure (p^0) is the vapor pressure of supercooled water at the same temperature (T). Then

$$a_w = \frac{p_{ice}}{p^0} \qquad [8]$$

Both expressions give very similar results, confirming the validity of both. The measurement of T, where the first ice crystals are formed, by a classical cryoscopic measurement, gives accurate values of a_w (up to ±0.001 a_w) in the range 0.80–1. Actually, what is measured is a_w of the product at T. The differences with a_w at 25 °C are shown to be not more than ~0.01 a_w. The values may be corrected to obtain more accurate ones at the desired temperature.

Mechanical/electrical properties varying with RH

Unlike the preceding methods, which can be absolute measurements, these methods require calibration of the sensor. The sensor can be a thread-like material (a hair or a synthetic polymer) the length of which, when exposed to a force of given strength, depends on its water content and is measured. The sensor can also be a material the electrical conductivity or dielectric constant of which depends on the water content and which is measured.

Sorption isotherms

Water activity can be determined from SI of the product after measurement of the water content. In this case, it is more practical to have a mathematical expression of the SI, allowing interpolations. Besides the ones cited before, many expressions have been proposed to describe SI, a few examples of which are given in **Table 1**. For milk products, as for other foods, GAB expression was shown to be fitted satisfactorily to experimental data up to $a_w \approx 0.90$. To create SI, representative food samples that are initially dried (for adsorption isotherms) or hydrated (for desorption) are placed in controlled humidity chambers at constant temperature and are weighted periodically until a constant weight is reached. In the static desiccators method (**Figure 9**), different levels of RH are obtained using

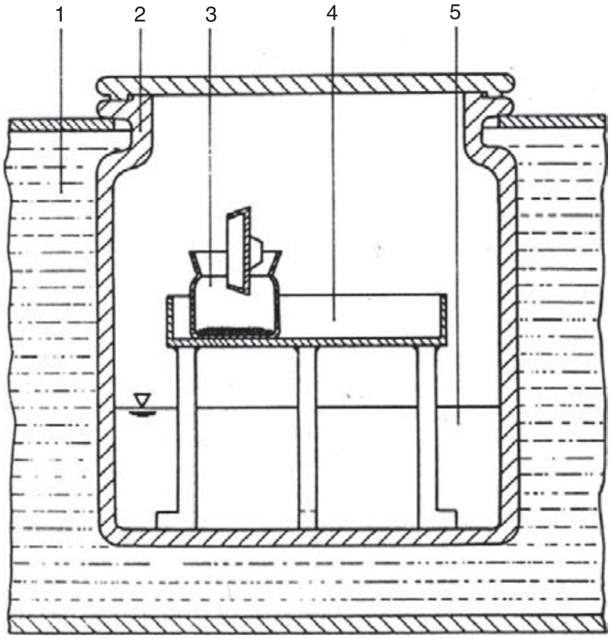

Figure 9 Sorption device as standardized for the COST Project 90 (Wolf W, Spiess WEL, and Jung G (1985) Standardization of isotherm measurement (COST Project 90 and 90 bis). In: Simatos D and Multon J-L (eds.) *Properties of Water in Foods*, pp. 661–679. Dordrecht: Martinus Nijhoff Publishers). 1. water bath, 2. sorption container (1-l glass jar, with a vapor-tight lid), 3. weighing bottle with a ground-in stopper, 4. Petri dish on trivet, 5. saturated salt solution.

saturated salt slurries that have known a_w values. Commercial devices control RH by mixing wet and dry gas streams and continuously monitor weight changes of the samples.

Equilibrium

The issue of equilibrium has already been mentioned concerning the internal moisture equilibrium of the product; equilibration of water vapor pressure between the sample, headspace of the measurement chamber, and the probe is also an important practical problem. Theoretically, the equilibration process is slowed as vapor pressures come closer, and equilibration time is infinite. Practically, therefore, the rate of change of RH is monitored continuously and the measurement is ended when the rate of change falls below a chosen limit. Equilibration of most products typically requires 45–60 min and can take as long as a couple of hours. Gentle ventilation in the measuring chamber may reduce equilibration time by 50%. For the generation of SI by the desiccators method, reducing the total pressure in the containers also reduces the equilibration time by a factor of 2–3. The volume and geometry of the sample are important parameters. Increasing its surface area (possibly by grinding) will reduce equilibration time; care should also be taken to ensure that the amount of sample is large enough, as compared to the headspace volume of the chamber (and to the surface area of the dew point mirror), so that the water lost by the sample will not significantly decrease its water content.

Several commercial instruments designed to generate SI change the controlled RH in a stepwise progression; some another instruments uses only air saturated with water (for adsorption) or dry air (for desorption), and continuously monitor weight changes and RH in the chamber with a dew point sensor. While the SI obtained with the former devices may be considered to represent equilibrium states, the latter are expected to provide information on the evolutions (glass transition, crystallization) occurring in the sample during sorption (**Figures 10** and **11**).

Temperature

Water activity of a product shows only small variations with temperature. For ideal solutions, a_w, being identical to X_w, is independent of temperature; even for nonideal systems, changes are small. SI of various dairy powders show no obvious temperature dependence between 4 and 38 °C; only at 50 °C the amounts of sorbed water are lower. Similarly, the a_w measured for six different cheeses between 5 and 30 °C showed no significant temperature dependence.

The temperature of a sample, however, is an important concern for a_w measurements. For methods relying on the measurement of p_w (direct measurement of p_w, dew point), an error in the sample temperature measurement will result in an error in $p^0{}_w$ and consequently in a_w. Between 20 and 25 °C, a 1 °C error in the sample temperature measurement represents a 6% error in $p^0{}_w$ and a_w. Similarly, to achieve an accuracy of ±0.003 a_w within the range of 0.800–1.000 a_w, the temperature of a dew point sensor is to be measured with an accuracy of ±0.05 °C. Because the temperature dependence of a_w is small, the actual temperature need not to be known precisely with apparatus measuring RH, so long as both temperatures of sample and of sensor are the same.

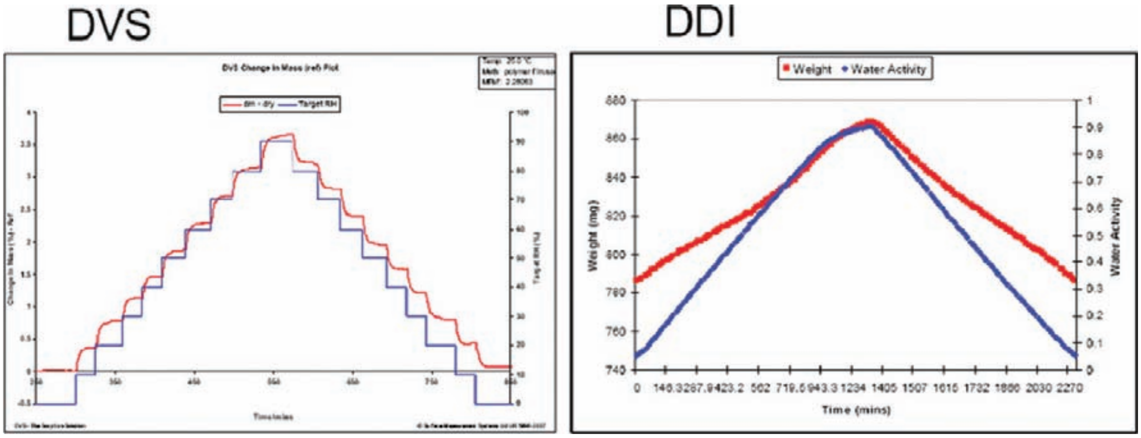

Figure 10 Two dynamic methods of sorption isotherm generation: dynamic vapor sorption (DVS): change in mass (blue) and change in target RH (red) versus time; dynamic dewpoint isotherm (DDI): sample weight (red) and measured RH (blue) versus time. Carter B (Decagon), personal communication.

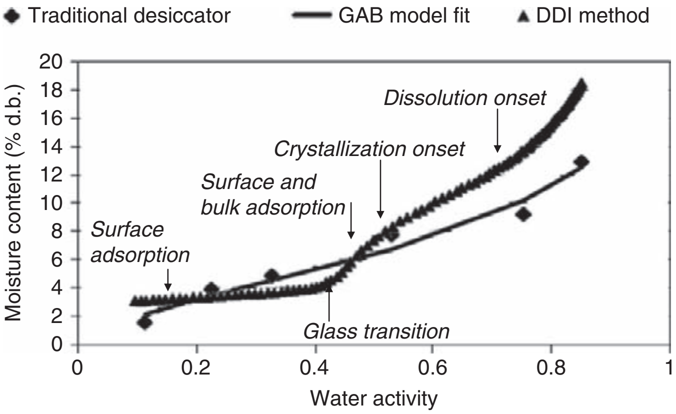

Figure 11 Sorption isotherm of milk powder (adsorption) obtained by continuously changing RH above the sample (DDI method), as compared to that obtained with the desiccators method. As water is adsorbed by the dry product, the temperature of glass transition is lowered to the working temperature; the glassy product is plasticized, water is then more easily adsorbed in the bulk of the sample. With the increase in molecular mobility,crystallization of lactose develops. Redrawn from Carter B (Decagon), personal communication.

See also: **Cheese**: Microbiology of Cheese. **Concentrated Dairy Products**: Sweetened Condensed Milk. **Dehydrated Dairy Products**: Milk Powder: Physical and Functional Properties of Milk Powders. **Water in Dairy Products**: Water in Dairy Products: Significance.

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WELFARE OF ANIMALS, POLITICAL AND MANAGEMENT ISSUES

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Introduction

Since World War II, a major revolution in social concern with the welfare and moral status of agricultural animals has emerged. The animal rights movement has arisen from old ideas but with new philosophies, emphasizing moral and ethical standards for how human beings should treat animals.

Public policy establishing the animal welfare movement began in the United Kingdom with the passage of an act in 1835 to "consolidate and amend the several laws relating to the cruelty and improper treatment of animals". In 1911 the UK Parliament passed the Protection of Animals Act which is still in force. It was established on the principle that, although human beings are free to subjugate animals, it is wrong for people to cause animals to suffer unnecessarily. The cultural and social evolution of animal protection in Europe has led to changes in the United States.

Terms Defined

Animal protection refers to all efforts to prevent cruelty, improve humane treatment, reduce stress and monitor research with animals.

Animal welfare generally describes the philosophy espoused by those who support the humane treatment of all animals without concern for their ultimate use. An 'animal welfarist' believes that human beings have the right to use animals so long as suffering is reduced or eliminated. Those who believe in animal welfare work for the reform of abusive or neglectful situations to alleviate animal suffering. Farmers have historically been perceived as strong supporters of animal welfare because they believed that animals raised under humane conditions and practices would be the most productive and profitable.

The animal rights philosophy, encompassing animal liberation, includes some fundamental differences from animal welfarism. It involves the idea that nonhuman animals are sentient beings – that they have the capacity consciously to experience pain and pleasure, among other things. Accompanying this belief is the notion that animals have certain inalienable moral rights which humans should not violate.

Philosophers, Activists and Political Action

Ruth Harrison, an English homemaker, initiated much of the public concern for the welfare of farm animals under modern production methods when her book *Animal Machines* was published in 1964. Following publication of her book, the UK Parliament called for an investigation. In 1965, the Brambell Committee, a group of scientists and concerned citizens, issued their report calling for certain mandatory standards that would conform to good husbandry in agricultural/animal production systems.

The political dimension of the animal rights and animal welfare movements involves individual and group efforts supporting or opposing specific issues. The methods include campaigns to influence legislation through letter-writing and other direct contacts; seminars and media events to influence members of legislative bodies and public opinion; demonstrations to draw public attention to what activists see as improper treatment; inviting sympathetic legislators and government officials to speak or receive awards at meetings and other special events; and securing sponsorship of bills in various legislative bodies. The participating activists may be classified as reformists or abolitionists.

Political action by animal rights and animal welfare advocates covers many issues from many different perspectives. However, four phases in these movements to influence political action can be identified: (1) identifying the problems of animal mistreatment; (2) developing appropriate ideology to cover the principles and concerns; (3) understanding how change occurs; and (4) developing explicit standards of ethics for advocating change.

Although intensive methods of animal production, which use more capital and less labor than traditional methods do, have improved production efficiency, they have at times put milk and meat producers in defensive positions because animal activists have branded certain ones of these contemporary methods as 'factory farming'. Producers adopted high-technology, intensive production systems because they would allow them to produce more product by substituting capital for labor to achieve lower cost per unit product.

Critics, however, see intensive animal agriculture differently because their views are based on philosophical thinking, feelings or opinions, often with little exposure to or understanding of the economics, the science or the actual practice of agricultural/animal production.

Dairy Cattle, Animal Welfare and Management Issues

A British report in 1983 identified animal protectionists' major animal welfare concerns for dairy animals:

- reduction of quality and quantity of individual attention in larger herds
- dehorning of calves with caustic chemicals with or without anesthetic
- prolonged stanchion tying of cows, especially without exercise
- need for separation of cow and calf
- neglect of unwanted bull calves
- raising replacements in individual hutches rather than in groups
- confinement of veal calves in small crates
- failure to employ welfare-related research knowledge
- production-related susceptibility to disease and metabolic disorders
- transportation of injured and sick animals to slaughter.

All of these issues continue to be mentioned to this day (but much progress has been made in correcting those issues that deserved attention). In addition another issue has come on the scene – docking cows' tails.

Several of these issues have been addressed by scientists and government officials around the western hemisphere in the intervening two decades. The UK Ministry of Agriculture, Fisheries and Food (now the Department of the Environment, Food and Rural Affairs) issued its codes of recommendations for the welfare of cattle emphasizing avoiding discomfort or distress and allowing the animals to fulfil their 'basic needs'. The recommendations called for these provisions to be considered: comfort and shelter, readily accessible fresh water and a diet to maintain full health and vigor; freedom of movement; company of other animals, particularly of like kind; and opportunity to exercise most normal patterns of behavior. Specific recommendations were identified for buildings, fire and other emergency precautions, ventilation and temperature, lighting, mechanical equipment and services, space allowance, feed and water and management.

Recommended codes of dairy cattle and veal calf husbandry practices also have been published in Canada.

Guidelines for the care of dairy cattle and veal calves have been published by dairy-industry stakeholders and scientists in the United States.

With respect to some of the issues listed above, much progress has been made, and general consensus currently stands as follows:

1. Cow behavior and care in large groups can be satisfactory.
2. Dehorning is beneficial, and its conduct has been refined to make it more humane.
3. Very few dairy cows are stanchioned for long periods nowadays.
4. Separating calf from cow very soon after birth is justifiable in terms of the dam's udder health so long as the calf receives an adequate dose of appropriate colostrum the first day after delivery. If a calf is to be weaned early, in terms of minimizing the stress of separation and loss it should be done as soon after birth as possible.
5. Dairy farmers are being educated as to the necessity of ensuring that surplus bull calves (to be finished as veal calves or dairy beef) be cared for just as heifer calves being kept as herd replacements, especially with respect to receiving an adequate dose of colostrum the first day after birth.
6. Individual outdoor hutches – in terms of calf sanitation, health and performance – are preferable to group housing for calves for the first 2 months after birth. This is so, regardless of the nature of the local climate. Benefits of the opportunity to socialize with other calves are outweighed by vices and other practical problems associated with group-rearing of young calves.
7. Although there have been numerous studies in many places seeking a suitable alternative to the conventional veal-calf stall, no alternative has emerged. Applied discovery research continues along this line.
8. There remains in the industry a strong tendency to follow economic dictates rather than ethical ones when the two are in conflict. Some of the reasons for this seem to be that, unfortunately, the correspondence of high animal state-of-being with high animal performance rate has not been adequately demonstrated by scientists, so the whole economic situation in such cases may be neither understood nor considered. Ironically, criticism and charges by animal protection advocates have probably dampened the support resources the industry and the governments of the various nations have devoted to such research, the results of which would probably for the most part be 'win–win' for agriculturists and animal protectionists alike.

9. Simply put, with modern animal production systems come the smouldering, multifactorial production diseases characterized by long-term, moderate morbidity and low mortality. But on balance the overall health of the animals is roughly the same in intensive production systems as in extensive systems. In the latter systems, the animals are more likely to suffer from parasitic diseases and the acute infectious diseases characterized by short-term, severe morbidity and high mortality.
10. Most abattoirs nowadays refuse to permit nonambulatory animals to be off-loaded at their docks, so most nonambulatory cows (which became nonambulatory at the farm) are not on-loaded and transported in the first place.
11. A consensus regarding tail-docking of dairy cows is now forming. On balance the practice seems to have few, if any, advantages but several disadvantages.

Policy and Legal Aspects

Calls for regulation of agricultural/animal care practices have been more successful in western Europe than in North America. The US House of Representatives did conduct hearings on the issues of veal calf husbandry and handling and transportation of nonambulatory animals in the late 1980s and early 1990s, but so far no legislation regulating these matters has been passed and signed into law. Some European countries, however, have set legal standards for animal husbandry on farms, although these standards often differ among nations.

In 1986 the European Communities Council issued its directive for protection of animals used for 'experimental and other scientific purposes'. The directive was designed to provide guidelines for uniform laws in the member countries. The objectives were to reduce the use of animals for experimental purposes to a minimum, ensure that they were adequately cared for, and avoid or minimize pain, suffering, distress and harm.

The farm animal welfare policies and regulations in the United Kingdom are developed in line with European Union (EU) directives and have led the way for other European countries. The major issues around which all European animal welfare policies have evolved focus on housing, rearing, feeding, transporting, marketing and killing. In 1996 the EU Commission proposed that French, Italian and Dutch farmers could continue to use crates for raising veal calves until the year 2008, but new crates would be banned after 1998.

The UK Department of the Environment, Food and Rural Affairs (DEFRA) recognizes that both ethical and scientific issues play a part in this issue. For advice and counsel on animal welfare matters, DEFRA looks first to the Farm Animal Welfare Council (FAWC), comprised of scientists, educators and producers.

The FAWC is expected to combine the use of appropriate new technology with efficient use of available resources and adequate provision for the welfare and behavioral needs of animals. More practices requiring a veterinary surgeon are spelled out. Citizens who believe a livestock owner is not following the welfare guidelines can file a complaint, and government inspectors then determine whether violations have occurred.

In Sweden the 1988 Animal Protection Act established the most detailed and comprehensive laws dealing with animal welfare in any country. Livestock buildings had to have windows and provide space so all animals could lie at once and be able to move freely. Milk cows had to be sent out to pasture in the summer.

In Switzerland, veal calves must receive iron and roughage in their rations.

Differences within the EU exist on animal welfare policies. The northern countries tend to be more sympathetic to welfare policies than the southern countries. Eastern European countries are in transition, and animal welfare policies have much lower priority than other economic and social concerns.

Producer attitudes toward animal welfare regulations in the western hemisphere have changed in recent years. Many now recognize that public opinion cannot be ignored if they are to maintain a market for their products.

The development of welfare-oriented regulations for production practices is only part of the evolution of government influence. Along with concerns for the humane treatment of animals is the concern for the quality and safety of products and the environment. Production regulation also involves pollution controls, manure disposal, dead-animal disposal and the use of medicines and feed additives that could affect the safety of the processed animal product.

Producers are consulted as new animal welfare regulations are developed, but in their minority position they must accept the final policy decision. Many of the regulatory guidelines simply represent good management practices that any considerate producer would follow.

In Europe the policies and regulations established are primarily welfare-oriented, with less noticeable activism for animal rights *per se* than is observed in the United States. There is a growing consumer interest and awareness in Europe and North America about how food is produced. Although the primary concern is food safety, the humane treatment of animals is second and interest is growing. The rising profile of animal welfare in public awareness is contributing to growing demands for food that is labeled as having been produced under certain standards.

The legal framework regarding animals in the United States has focused on concerns that they should be treated humanely. Successful legislative efforts fall into these categories: (1) humane treatment of animals in slaughter plants, in research facilities and in transit; (2) protection of endangered animal species; (3) protection of fish, marine mammals and wildlife; (4) establishment of standards for conducting research with laboratory and other animal species; (5) protection of pets; and (6) control of terrorism. Dairy cattle owners have the most concern with categories (1), (4) and (6).

One of the oldest federal laws deals with livestock transportation. The act applies to the transport of animals and requires a respite period unless the vehicle itself provides feed, water and space.

In the United States, many bills dealing with animal protection have been introduced but none has been enacted. Those that would target dairy animals included bills attempting to identify humane animal husbandry practices for livestock, to establish a farm animal husbandry committee to investigate all aspects of intensive farm animal husbandry and to mandate diets and accommodations for veal calves.

In 1989 the US House of Representatives Agriculture Subcommittee on Livestock, Dairy and Poultry held a hearing on a bill (HR 84) that attempted to prohibit certain practices in raising veal calves. Testimony revealed support for the bill from the Humane Society of the United States, Humane Farming Association and other animal rights/welfare groups. Veal producers, the US Secretary of Agriculture, and members of Congress with major producer constituencies opposed the bill. The bill failed to receive a favorable vote in committee.

In the United States, advocates of a humane ethic for animals are gaining momentum based on a philosophy regarding the sacredness of life. Animal-welfare advocates emphasize that animals are sensing, living beings capable of feeling fear and pain and that they must be respected as such. Some members of Congress recognize the emotional commitment of animal-welfare advocates.

However, the US animal industry's strong public support, the close ties between trade associations and government agencies and the rapport between producers, state legislators and members of the US Congress provide major advantages over organizations and individuals' philosophies that would disrupt the economically sound management practices used on dairy, livestock and poultry farms.

See also: **Office of International Epizooties**: Mission, Organization and Animal Health Code.

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WHEY PROCESSING

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Utilization and Products

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Introduction

Whey, the greenish translucent liquid obtained from milk after precipitation of casein, has been viewed until recently as one of the major disposal problems of the dairy industry. The biological oxygen demand (BOD) of whey is very high ($40\,000\,\mathrm{mg\,kg^{-1}}$ or more), constituting a major ecological burden if disposed off as a waste material. Thus, the disposal practices of the past, including drainage into waste treatment facilities or spraying onto fields, are currently seldom practiced. Use of whey as cattle or pig feed is still one of the significant alternatives to utilization in the human food chain, now being predominantly favored due to the economic opportunities provided by some of the milk nutrients contained in the whey.

Whey Types and Composition

There are several types of whey, depending mainly on the processing sequence resulting in casein removal from fluid milk. The type of whey most often encountered originates from the manufacture of cheese or certain industrial casein products, where the processing is based on coagulating the casein by rennet, an industrial casein-clotting preparation containing chymosin or other casein-coagulating enzymes. Since the rennet-induced coagulation of casein and the subsequent whey drainage occur at a pH value of approximately 6.5–6.0, this type of whey is referred to as sweet whey. The second basic whey type, acid whey, results from processes using fermentation or addition of organic or mineral acids to coagulate the casein as in the manufacture of fresh acid-coagulated cheeses (e.g., Cottage cheese or quark) or most industrial acid casein.

The main components of both sweet and acid wheys, after water (which constitutes approximately 93% of the whey on an ‘as is’ basis), are lactose (approximately 70–72% of the total solids), whey proteins (approximately 8–10%), and minerals (approximately 12–15%). **Table 1** gives a more detailed breakdown of these components of the two basic whey types. The main differences between the two whey types are in the mineral content, the acidity, and the composition of the whey protein fraction (WPF). Although these differences are relatively minor on an ‘as is’ basis, they can have a profound effect on the technological as well as nutritional properties of the wheys and must be taken into consideration in applications of the various whey processing technologies now available to whey processors. The acid coagulation approach (using conversion of some of the lactose in milk to lactic acid by lactic acid bacteria and/or addition of acidulants such as glucono-δ-lactone or various acids such as sulfuric, phosphoric, hydrochloric, citric, or lactic acid) results in substantially increased acidity (final pH approximately 4.5) necessary for casein precipitation. At this low pH, the colloidal calcium contained in the casein micelles in normal milk is solubilized and partitioned into the whey. On the other hand, rennet clotting produces a fragment of the κ-casein molecule, termed glycomacropeptide (GMP), which ends up in the whey. Thus, the GMP constitutes approximately 20% of the WPF of sweet, rennet-based wheys but is not found in acid wheys unless use of rennet was included in the fresh cheese manufacturing process (as sometimes happens in the Cottage cheese manufacture for increased firmness) in addition to the acid coagulation.

Various technological steps used in the pretreatment of milk before the main processes (such as various thermal treatments before the casein-clotting operation) may also influence the composition of the whey resulting from such

Table 1 Typical composition of sweet and acid whey (g l^{-1} whey)

Component	*Sweet whey*	*Acid whey*
Total solids	63.0–70.0	63.0–70.0
Lactose	46.0–52.0	44.0–46.0
Protein	6.0–10.0	6.0–8.0
Calcium	0.4–0.6	1.2–1.6
Phosphate	1.0–3.0	2.0–4.5
Lactate	2.0	6.4
Chloride	1.1	1.1

Illustrative data compiled from various sources.

milk. Typically, the composition of the mineral fraction may be altered slightly and the content of heat-labile whey proteins may be reduced; these changes may result in further alterations in the technological properties of such wheys.

New technological alternatives for processing of dairy fluids, including membrane processing by ultrafiltration (UF) of milk in cheese manufacture or fractionation of the various wheys into various whey-based products, produce a whey-like residue termed UF permeate. The main difference between UF permeates and the various whey types is typically the virtual absence of whey proteins from the permeate. Although technically UF permeate does not fit the definition of whey, it is referred to in this article where appropriate, as its processing and utilization often present similar challenges and opportunities as for whey.

Industrial Technologies Used in the Processing of Whey and UF Permeates

As a general rule, about 9 l of whey is obtained for every kg of cheese produced; thus, the volume of whey to be processed, originating from just one typical large-scale cheesemaking operation, can exceed $1 \times 10^6 \, l \, day^{-1}$. Most of the technological alternatives used in specialized whey-processing plants are thus large-scale operations, some with a capacity to handle up to 10^7 l of whey daily.

The simplest technology for the conversion of whey to industrially valuable products is drying. Typical traditional whey-drying operations consist of evaporation in multistage vacuum evaporators, followed by spray-drying. The equipment used does not differ greatly from other such dairy plant installations but the evaporation and drying conditions must be adjusted to accommodate the specific properties of the whey. In particular, the differences between evaporation or spray-drying of skim milk and whey include the need to precrystallize the lactose in whey before the drying step to minimize the problems of hygroscopicity, as well as careful manipulation of the heat conditions to minimize problems related to heat sensitivity of whey proteins. Dried whey powders can differ rather substantially in composition and technological properties, depending on various pretreatment operations used to handle the original milk or the original whey. Some of the typical dried whey products are listed in **Table 2**. Partial (70%) or almost complete (90%) demineralization of the whey is an important pretreatment process differentiating many of the whey powders (*see* **Whey Processing**: Demineralization).

Table 2 Typical composition of major types of dried whey products (%, w/w)

Product type	*Total protein*	*Lactose*	*Minerals*
Regular whey powder	12.5	73.5	8.5
Demineralized (70%) whey powder	13.7	75.7	3.5
Demineralized (90%) whey powder	15.0	83.0	1.0
Ultrafiltration permeate powder	1.0	90.0	9.0
Whey protein 'concentrate' (skim milk replacer)	35.0	50.0	7.2
Whey protein concentrate	65.0–80.0	4.0–21.0	3.0–5.0
Whey protein isolate	88.0–92.0	< 1	2.0–3.0

Illustrative data compiled from various sources including data from manufacturers.

Dried whey powder can be produced also by roller (drum)-drying; although roller-drying of whey is not easy, the process is much cheaper than spray-drying and the lower quality of the resulting powder may not be detrimental for all applications. Whey, or nowadays more importantly UF permeate from milk or whey, is the principal raw material for the crystallization of lactose (*see* **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Production, Applications); the residual, partially or more substantially delactosed whey ('mother liquor') constitutes yet another dried whey product differing in composition from the basic dried unmodified whey. Finally, UF permeates are also being dried with increasing frequency, giving yet another modified dried whey product consisting primarily of lactose and whey minerals but almost devoid of protein. Until recently, spray-drying was the only technique used for the production of dried permeates; however, due to the very low market value of these powders, the spray-drying of permeates is uneconomical. Several novel technological approaches to drying of milk or whey permeates have been described in the literature and are available to the industry. In principle, these technologies are based on the production of concentrates with very high total solids content (about 76%) combined with additional moisture removal by alternative means, for example, by specially designed screw conveyors allowing additional evaporation of water while inducing lactose crystallization. The final drying step is accomplished in a fluidized bed, thus avoiding the need for the costly spray-drying.

The levels and forms of whey proteins are important factors differentiating the various dried whey protein products. In contrast to the processes described above, the WPF can be removed (selectively or totally) from raw whey and concentrated by using various membrane processes (see Membrane-based fractionation) giving rise to whey protein concentrate (WPC), whey protein isolate (WPI), or WPF products (*see* **Milk Protein Products**: Whey Protein Products). Although products with as little as 35% protein (produced by partial removal of lactose through crystallization or by using simple UF and intended for replacement of skim milk powder in certain applications) are included under the term WPC, the more valuable WPC products have at least 65% protein and the production technology does not involve any step involving lactose precrystallization. The highest quality WPI and WPF products are manufactured using various technologies including diafiltration, electrodialysis, ion exchange, nanofiltration, or their combinations. For the production of almost all whey protein products, the final step is spray-drying, which should be controlled carefully to minimize heat damage of the thermally sensitive whey proteins. Both spray-drying and especially evaporation can cause heat damage resulting in loss of solubility and other functionality defects. Thus, as a preconcentration step, especially for the production of WPC, WPI, and WPF products, reverse osmosis or freeze concentration can be viewed as viable alternatives to traditional thermal evaporation. The use of reverse osmosis to increase the capacity of conventional whey evaporators, or even for preconcentrating large amounts of whey before transportation to a central whey processing facility, is quite common in the whey processing industry.

Utilization of Whey in Industrially Processed Foods

The preceding discussion focused on industrial large-scale processes resulting in an array of technological and functional whey ingredients or whey protein products, used commonly in many processed foods such as spreads, sauces, dry soups, beverage and similar mixes, cookies and other bakery products, ice cream, and a myriad of other industrial food items. Dried whey is a very suitable 'bulking' food ingredient due to its bland taste compatible with many food processing applications. The heat sensitivity of whey proteins could pose problems in heated liquid food applications, but in most solid foods this is of no concern. Similarly, the limited solubility of lactose must be kept in mind when using dried whey in applications leading to development of supersaturated lactose solutions, for example, the unfrozen fraction of ice cream. The well known – but nowadays rarely seen – sensory defect called sandiness can occur more readily when dried whey is used as a portion of nonfat milk solids in ice cream formulation. The various higher value dry whey-based products, listed in **Table 2**, are increasingly being used as nutritionally important components in special dietary products for infant, geriatric, or sport nutrition. Large amounts of whey are being processed for use in the various infant nutrition products, with demineralized whey, lactose, and modified whey protein products being especially important in this application. Some of the unique technological properties of whey protein (in particular the acid solubility, heat-gelling, and foaming) make the various whey-based high-protein products ideal for use in acid beverages, foamed dairy desserts, yogurts, and similar dairy and nondairy products. A heat-denatured whey protein powder termed 'traditional lactalbumin' has a very high water-holding capacity and thus has been shown to be preferable to the undenatured whey protein in applications such as protein enrichment of pasta dough. In general, it has been estimated that of about 10 million tonnes of dried whey solids produced annually worldwide, over 50% is being utilized in human foods, with the rest finding less attractive valorization in animal feeds.

However, liquid unprocessed whey can also be a raw material for the production of some traditional foods destined for direct consumer markets. Two such product classes, whey cheeses and whey beverages, represent the traditional uses of whey on a small scale, practiced long before the industrial approach became feasible, and are still important in some parts of the world.

Whey Beverages

The drinking of whey for therapeutic applications was already advocated in ancient Greece by Hippocrates. This and other similar anecdotal comments concerning the use of whey as a beverage (including even in the rhyme Little Miss Muffet...) illustrate one of the most obvious, but least industrially advantageous, uses of whey as a drink, logically paralleling the use of milk, buttermilk, and other fluid milk products as beverages.

In general, whey beverages have not been overly successful with the sophisticated modern consumer, save for a few rather exceptional instances. Minimally processed unflavored or modified raw whey is sold in health-food stores in various countries, especially in Europe, where the current organic food movement may result in increased opportunities in this regard. Occasional reports on whey-based beverages marketed for special occasions (e.g., an 'official Olympic Games drink' in 1984 in Sarajevo) can be found in the literature. Local markets often feature whey beverages produced by dairy companies looking for new outlets for their surplus whey; unfortunately, these attempts are typically short-lived, often due to the lack of any serious product development effort that is necessary preceding the launch of any such new product. The rather unpleasant flavor of raw whey, the origins of which have never been satisfactorily explained, is a major deterrent limiting the acceptance of these products by contemporary consumers, especially in view of the fierce competition of other flavorful and inexpensive thirst-quenching beverages.

The only whey beverage with a record of lasting success is the Swiss product Rivella, which in fact uses highly modified whey as only a minor ingredient at 33% total volume (the remaining 67% being added water). Other locally successful products have existed on some European national markets (Austria, Finland, The Netherlands, and Switzerland) for some time; however, several known international marketing attempts with these products have failed. The most typical approach to whey beverage development is combination with fruit juices, especially citrus fruits, which are most compatible with the flavor characteristic of whey. This is especially true in the case of acid whey, which is more suitable for this application due to its high content of lactic acid and calcium in comparison to sweet whey. Some of the fruit flavors used in commercial whey beverages include mango, passion fruit, grapefruit, lemon, orange, pear, or their combinations. Other approaches documented in the literature include the production of yogurt drinks containing a substantial whey component, or fermentation of liquid WPCs from sweet whey to produce a 'thin sour milk', as the stability of the whey protein at low pH does not lead to the clotted appearance of traditional sour milk products. Milk or whey UF permeates are suitable for the production of isotonic sport drinks or beverages destined for replenishment of the mineral balance after vigorous physical activity; several such products have been described in commercial product surveys.

Attempts to produce whey beverages with high whey protein content have been recorded in the literature and are often seen in various food trade exhibitions, with little or no indication of commercial viability. It may be that with the presently increasing reputation of whey proteins as nutraceutically important food components, the development of such products will be intensified. The heat sensitivity of whey proteins is one important problem encountered in manufacturing these products with a demand for extended shelf life. However, since the resistance of whey proteins to heat-induced coagulation in the absence of casein increases dramatically at pH below 3.9, it may be possible to formulate high whey protein drinks, even for ultra-high temperature (UHT) processing. Alternatively, various nonthermal processes now being studied actively for various food processing uses can be considered. Whey beverages offered as liquid concentrates or as dry powders for home reconstitution can be occasionally found on regular market shelves, including even suitably flavored plain dry whey powder. Various "miracle protein" milk shake formulae based on isolated – even hydrolyzed – whey proteins are commonly marketed as products for body builders, active sport enthusiasts, and other health-food applications.

Whey Cheeses

Whey cheeses have suffered a somewhat similar lack of international marketing success; however, in a few localities, whey cheeses belong among the most traditional and most important foods. Two types of whey cheeses are recognized in the textbooks and by the International Dairy Federation. The main differences between the production technologies for these two products are illustrated schematically in **Figure 1**. The more widespread of the two is the Italian-type whey cheese Ricotta; similar products are also popular in Portugal, Turkey, and other localities. These products are essentially a heat–acid-coagulated whey protein paste, sometimes referred to as 'whey quark' (*see* **Cheese**: Acid- and Acid/Heat Coagulated Cheese). The processing technology is quite simple, consisting of heating whey (often mixed with up to 25% added skim milk) to at least 90 °C for a few minutes, resulting in heat-induced coagulation of whey proteins (and any caseins if present due to the added milk); the coagulum is then separated by suitable mechanical means. The traditional batch manufacturing procedures are cumbersome and involve much

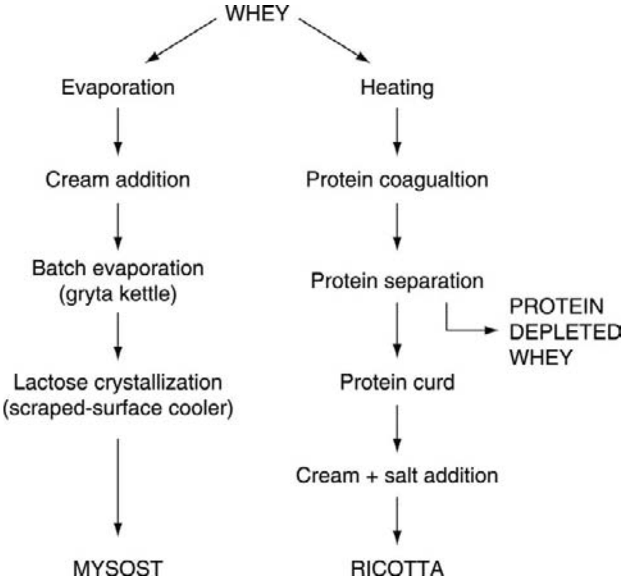

Figure 1 Alternative processes for manufacturing whey cheeses.

hand labor, in particular in the separation of the heat-coagulated, fragile whey protein curd. Mechanized and automated continuous systems are now in existence and these result in increased economy, improved shelf life, and better sensory qualities of the final product.

In contrast to the Ricotta-type whey cheese involving primarily the protein fraction of whey (and thus not offering a solution to the whey disposal problem, which is especially pressing for the small-scale cheese manufacturers), the Norwegian-style whey cheese Mysost utilizes all the whey components and leaves no residue other than water vapor. The principle of the Mysost cheese process is even simpler than that for the manufacture of Ricotta, as Mysost is essentially highly concentrated whey to which some other components (such as milk fat, cream, or goats' milk in the most traditional version) have been added (**Table 3**). The main technological problem encountered in the manufacture of Mysost cheese is the crystallization of lactose in the highly concentrated whey-based paste related to the low solubility of lactose. Thus, a 'controlled crystallization' step, consisting of rapid cooling of the hot paste with intensive stirring, from about 95 °C (the temperature of the last phase of the evaporation process) to below 65 °C, is the most essential aspect of the otherwise routine evaporation process carried out in two steps. After preconcentration in traditional dairy evaporators, the final operation involves a special kettle, 'gryta', in which batch evaporation is carried out at an elevated temperature needed to reduce the viscosity of the thick paste. This high temperature tends to minimize the uncontrolled crystallization of lactose and is the main reason for the typical brown color of the product due to the pronounced Maillard reaction. The subsequent rapid cooling step in scraped-surface agitators/coolers promotes formation of very small lactose crystals, thus minimizing the development of pronounced grittiness/sandiness of the final product. There are two basic types of Mysost – sliceable or spreadable – available in Norway, its country of origin and still the only significant market for these products; the two main types are differentiated principally by the moisture content and encompass further variations differing in the fat content, inclusion of some goats' milk for a stronger taste, intensity of the brown color development, and sometimes inclusion of sweetening agents or other nondairy ingredients such as hazelnuts or chocolate.

Table 3 Compositional characteristics of whey cheeses (%, w/w)

Cheese	*Moisture*	*Fat*	*Protein*	*Lactose*
Ricotta				
Whole milk	72.2	12.7	11.2	3.0
Whey	82.5	0.5	11.3	1.5
Mysost				
Sliceable	17.4	28.3	11.5	36.2
Spread	26.6	3.6	7.7	46.2

Illustrative data compiled from various sources.

Because the relatively simple Mysost process technology leaves no residue other than the evaporated water, its principle is much more useful than that of the Ricotta process as a means of whey disposal, particularly for small cheese manufacturers. However, significant product development efforts would be necessary to modify the sensory profiles of the basic Norwegian products to suit the tastes of markets outside Norway, as demonstrated in several consumer studies conducted in Canada and elsewhere.

Utilization of Whey as a Fermentation Substrate for Food or Nonfood Applications

Although whey and whey-like UF permeates originate from milk and contain about 50% of valuable milk nutrients, the profitable utilization of these materials for human nutrition continues to be a problem because of the sheer volume of these by-products of conventional or modern cheesemaking. In numerous research reports and several major industrial projects, whey – due to its suitable content of a fermentable carbohydrate, lactose – was used as a medium for the production of various food-grade or nonfood products using microbial fermentations. Among the most successful current uses of whey in this regard are the plants producing food-grade or industrial alcohol in New Zealand, the United States, Ireland, and possibly other countries; one of the best examples of such use is the Original Bailey's Cream Liqueur from Ireland or most of the liqueurs produced in New Zealand. In the past, whey was an important substrate for conversion into *Torula* spp. yeast biomass used in animal feeds, or for other fermentation-based, lactose-derived products such as antibiotics, lactic acid, or other microbial metabolites. The current preoccupation with organically produced foods and natural food ingredients may signal a possible opportunity for revitalization of some of these processes, abandoned in the past due to unfavorable economic feasibility in comparison to direct chemical synthesis or using other fermentable substrates. Use of whey for the propagation of lactic cultures for cheese manufacturing is well established in many countries. Whey can be used also for the generation of biogas; several such installations where biogas is used as an energy source in the same cheese plant at which whey is produced are in operation in Switzerland and possibly other countries. More recently, whey or whey permeates have been shown to be suitable fermentation substrates for the production of bacteriocins such as nisin, or for other valuable food ingredients using specific strains of various lactic acid bacteria.

Lactose is not fermented by many microorganisms, necessitating careful selection of the cultures for many of the possible fermentation applications. Uses of whey as a raw material for the production of alcoholic beverages, such as whey wine or whey beer, have been described in the literature, but because lactose is not fermented by common yeasts without special pretreatments, as well as other reasons, these attempts at profitable whey utilization have not resulted in lasting success. However, since the key to new approaches to utilization of whey is to find new avenues for conversion or other utilization applications of lactose, the fermentation route will undoubtedly continue to be explored. One of the currently active industrial research topics is the conversion of lactose to oligosaccharides, either by fermentation with whole microorganisms or by applications of crude enzymatic extracts of suitable bacterial cultures. Similarly, production of exopolysaccharides from lactose is increasingly being used in the production of yogurt; it is likely that this route could be explored in large-scale whey fermentations as well.

Nutritional and Nutraceutical Aspects of Whey Utilization

The new approach to developing technically and economically feasible uses of whey and whey-like products lies in finding some unique properties of at least some of the main whey components. The current trend in the food field, focusing on the health-promoting aspects of traditional or novel foods, has opened up new possibilities for whey-based products. Whey contains many minor milk components that are known (or thought) to have physiologically important functions. Some of these compounds are found especially among whey proteins; these include minor whey protein components such as lactoferrin or lactoperoxidase, the immunoglobulins, and even major constituents of the WPF such as the GMP or bovine serum albumin. Some of the mineral compounds, especially the calcium phosphate complex, are now also being marketed as 'natural' milk-based food ingredients. Even lactose is being reexamined for its unique nutritional properties, including the purported enhancement of calcium absorption or its unique disaccharide composition, thus serving as a raw material for the production of prebiotic compounds such as galacto-oligosaccharides (*see* **Lactose and Oligosaccharides**: Lactose: Galacto-Oligosaccharides) or heterooligosaccharides. The limited sweetness of lactose can be enhanced by hydrolysis into the two lactose monosaccharides, glucose and galactose. Most of the industrial lactose hydrolysis processes developed for the production of sweetening syrups for food uses (such as in ice cream) using immobilized β-galactosidase enzyme reactors have failed mainly because of the high cost of such technology. Alternative low-cost processes, based, for example, on the use of mechanically disrupted common dairy bacteria producing high amounts of β-galactosidase, or on other principles, continue to be investigated. In general, the lactose hydrolysis process, which seems to be grossly underutilized by the industry today, could have a major impact on expanding the dairy markets for lactose- intolerant consumers not only for the processing and utilization of whey, but also for uses in other dairy and especially nondairy foods (*see* **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase, **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Production, Applications).

The main focus of interest regarding the nutritional/nutraceutical properties of whey is currently centered on the WPF. Today, whey protein products are the main ingredients in most protein formula supplements used by bodybuilders and active athletes worldwide. Whey protein products have been one of the principal ingredients in infant feeding formulae for some time, often requiring additional treatments such as enzymatic hydrolysis to minimize the allergenicity potential of some of the components such as β-lactoglobulin. Most recently, whey protein and/or its main fractions have been investigated for many unique health-enhancing or disease-combating properties, including immunopotentiation through increased intracellular glutathione synthesis; role in reducing cancer cell proliferation; counteracting the wasting syndrome in HIV-positive individuals; antimicrobial properties of some of the minor whey proteins; and other effects. WPIs advertised to supply some of these benefits are now being marketed even as dietary supplements at highly inflated prices. Hydrolysates of whey proteins have been shown to contain bioactive peptides effective in blood pressure reduction, and several industrial products are now on the market.

Extraction of some functional components from whey has become a major industrial activity for specialized whey processors, although in most cases, the physiological functionality in human subjects still awaits confirmation from well-controlled clinical trials. Of course, manufacture of any such isolated functional product from the liquid whey will not alleviate requirements for further processing of the residual by-product. As many of the components with documented or proposed unique functionalities are present in the whey in very minute amounts, the need to process the bulk of the residual whey stream will remain a major challenge accompanying any such novel development.

Surprisingly, by far the most abundant whey component, water, seems to have been neglected in the innumerable research attempts to find new profitable uses of whey and/or its components. The only well-known use of whey water is as a condensate from the evaporators, mainly for rinsing and equipment washing purposes, but not for productive economically advantageous applications. As one potential such use suggested in the past, the adjustment of the mineral content of the whey water by suitable membrane processing could lead to various pharmaceutical uses for cleansing solutions mimicking the human body fluids.

The approach to whey processing and utilization has changed in the recent past, from considering whey a bothersome waste to capitalizing on the opportunities that the whey and whey-like products present for the innovative technically advanced processor. However, for traditional cheese manufacturers, the problems of dealing with large volumes of whey or whey permeate continue to pose difficulties, determining the overall success of their main industrial activity. Nowadays, industrial processing of whey is a highly specialized, technologically advanced segment of the dairy industry requiring up-to-date knowledge and focused attention.

See also: **Cheese**: Acid- and Acid/Heat Coagulated Cheese. **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase. **Lactose and Oligosaccharides**: Lactose: Chemistry, Properties; Lactose: Galacto-Oligosaccharides; Lactose: Production, Applications. **Milk Protein Products:** Membrane-Based Fractionation; Whey Protein Products. **Whey Processing**: Demineralization.

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Demineralization

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Introduction

In order to make nonhygroscopic whey powder as well as to make whey powder a suitable ingredient for certain foods, a reduction of the mineral content is needed. Typically, whey contains 8–10% minerals on a dry weight basis. This can be problematic when processing whey, especially when concentrating, crystallizing lactose, and spray-drying whey. It is also a problem when using whey and whey powders as food, especially for infant formulas, where a 90–95% reduction in minerals is needed. This is necessary in order to mimic the mineral composition of human milk. Critical ions for the preparation of infant formulas are Na^+, K^+, Cl^-, and PO_4^{3-}. For example, for ice cream applications, in order to reduce the salty taste of ordinary whey powder, a 50–70% overall reduction in minerals is often enough. Addition of less demineralized whey powder is always a possibility. It should be remembered that instead of using a specific demineralization process, like ion exchange or electrodialysis, ultrafiltration in combination with pure lactose is an alternative for the manufacture of infant formulas. The necessary composition of macrocomponents can thus be reached in many different ways. This article will focus on three main technologies for whey demineralization, that is, electrodialysis, ion exchange, and nanofiltration (NF). Combination of these processes will also be mentioned.

Electrodialysis

Electrodialysis is defined as the transport of ions through semipermeable membranes under the driving force of an electric field caused by an applied direct current (DC) source. The membranes used have both anion and cation exchange functions, making electrodialysis capable of reducing the mineral content of a process liquid, for example seawater or whey. **Figure 1** is a schematic representation of an electrodialysis unit. It consists of a number of compartments separated by alternate cation and anion exchange membranes, which are spaced about 1 mm apart. The end compartments contain electrodes. There can be as many as 200 cell pairs between each pair of electrodes. The two electrodes at each end of the cell stack have separate rinse channels, through which a separate acidified stream is circulated to protect the electrodes from chemical attack. For whey treatment, the whey feed and acidified brine pass through alternate cells in the stack.

Operating Principle

Alternate cells in the electrodialysis stack act as concentration and dilution cells, respectively. Whey is circulated through the dilution cells and a 5% brine carrier solution through the concentration cells.

When DC is applied across the cells, cations attempt to migrate to the cathode and anions to the anode, as shown in **Figure 1**. However, completely free migration between the cells is not possible because the membranes act as barriers to ions of like charge. Anions can pass through an anion membrane but are stopped by a cation membrane. Conversely, cations can pass through a cation membrane but not an anion membrane. The net effect is depletion of ions in the whey (depletion) cells. The whey is thus demineralized, to an extent determined by the ash content of the whey, residence time in the stack, current density, and flow viscosity.

The electrodialysis plant can be run either continuously or in batches. A batch system, which is often used for demineralization levels above 70%, can consist of one membrane stack over which the process liquid, for example, whey, is circulated until a certain ash level is reached. This is indicated by the conductivity of the process liquid. The holding time in a batch system can be as long as 5–6 h for 90% demineralization at 30–40 °C. Preconcentration of the whey to 20–30% dry matter (DM) is desirable for maximum utilization of installed membrane area and low electric power consumption. The whey concentrate should be clarified before it enters the electrodialysis unit.

The high process temperature means that there is a risk of bacterial growth in the product. A bacteriostatic compound such as hydrogen peroxide is therefore often added to the whey, when allowed. The process liquid heats up during the process, so cooling is needed if it is necessary to maintain process temperature. In a

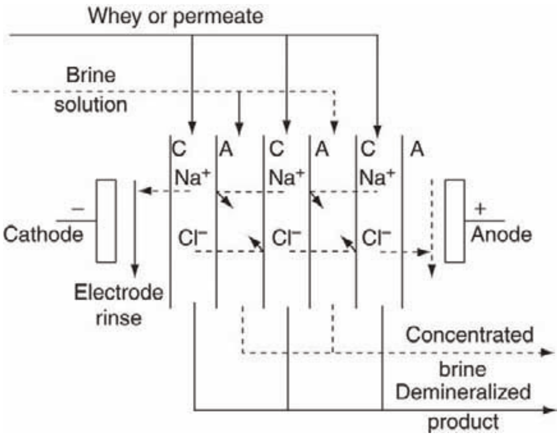

Figure 1 Schematic layout of an electrodialysis stack.

continuous plant, consisting of, for example, five membrane stacks in series, the holding time can be reduced to 10–40 min. The maximum demineralization level of such a plant is 60–70%. In relation to capacity, the installed membrane area is much larger in a continuous plant than in a batch plant.

An electrodialysis plant can easily be automated and furnished with a programmed clean-in-place (CIP) system. The cleaning sequence normally includes water rinse, cleaning with an alkaline solution (maximum pH 9), water rinse, cleaning with hydrochloric acid (pH 1), and a final water rinse. A typical cleaning program takes about 100 min.

Power Supply and Automation

DC is used in the electrodialysis plant, which should have facilities for regulating current in the range of 0–185 A and voltage in the range of 0–400 V. Flow rates, temperatures, conductivity, pH of process water and product, product inlet pressure, pressure difference between the stacks, and current and voltage over each membrane stack are monitored and controlled during production.

Limiting Factors in Electrodialysis and Processing Costs

A major limiting factor for using electrodialysis in dairy processing is the cost of replacing membranes, spacers, and electrodes, which constitute about 35–40% of the total running costs of the plant. Replacement is necessary due to fouling of the membranes, which in turn is caused by (1) precipitation of calcium phosphate on the cation exchange membrane surfaces and (2) deposition of protein on the anion exchange membrane surfaces.

The first problem can be handled by proper flow design over the membrane surface and regular acid cleaning. The second problem, with adsorption of protein, is the main factor shortening the lifetime of the anion membrane. The theoretical background to this problem is as follows. At the normal pH of whey, the main whey proteins are negatively charged (anionic character) and move as such under the influence of the electric field in the stack. These molecules, which are too big to pass through the anion exchange membranes, are deposited as a thin protein layer on the face of the anion exchange membranes in the whey compartments. Techniques such as polarity reversal can be used to dislodge these deposits.

Although frequent cleaning at high pH removes most of the deposits, at least in older plants, disassembly of the stack for manual cleaning is recommended at intervals of 2–4 weeks. The processing cost of electrodialysis depends very much on the degree of demineralization. Increasing the demineralization level in steps from 50 to 75 to 90% doubles the processing cost per step, as a rule of thumb.

Water treatment, electric power, chemicals, and steam account for the operating costs of a demineralization plant. Wastewater treatment is a particularly expensive item. During production, lactose leaks through the membranes to an extent of as much as 5–8% at 90% demineralization. The phosphate removed from the whey accumulates in the waste stream. The cost of electric power amounts to 10–15% of the processing cost, while the chemicals used in the process, mainly hydrochloric acid, account for 5%. The cost of steam used for preheating the product and cost of cooling for controlling the process temperature are 10–15%, depending on the demineralization level.

Ion Exchange

Industrial application of ion exchange often means pumping the process liquid to be treated through a fixed-bed column filled with polymeric beads loaded with ions that are exchangeable with the ions in the process liquid. The capacity of the process is limited by the amount of ions on the resin in the fixed bed. After the ion exchange capacity has been used up, the adsorbed ions must be removed by regeneration of the column by an appropriate regeneration solution. After that, the flow of process liquid through the column can be resumed.

Modern ion exchange resins are macromolecular porous plastic materials formed into beads with a diameter in the range of 0.3–1.2 mm for technical applications. Chemically, they act as insoluble acids or bases, which when converted to salts remain insoluble. The main characteristic of ion exchange resins is their capacity to exchange the mobile ions (counterions) that they contain for ions of the same charge sign contained in the solution to be treated. The exchange reactions are equilibrium reactions governed by a constant. The concentration of ionic species is an important factor for the driving force in the exchange reaction. The functional groups of ion exchange resins for demineralization purposes vary. First of all, a distinction between anion exchangers and cation exchange resins can be made and within these groups there are both weak or strong base and acid varieties, respectively. The group to which the specific ion exchange resin belongs depends on the functional group. Strong cation exchange materials often have sulfonic groups bound to the matrix. This group is ionized throughout the pH range 0–14, and therefore active for exchange reactions to take place. The most common weak acid exchange material has a carboxylic acid functionality. This group is ionized at pH values above the isoelectric point, typically above pH 5–6. Strong base functionality is based on quaternary amine groups. These groups are dissociated throughout the pH range. Weak base ion exchange functionality is often based on tertiary amine groups, which are active in the pH range 0–7.

From the point of view of ease of regeneration, it is beneficial to use weak resins whenever possible. They can be regenerated with acid or alkali in excess in comparison with the theoretical need of just 10–50%. Strong resins need 200–400% excess of regenerant to be fully converted to the active form.

For demineralization according to the classical procedure, a strong cation exchanger in the hydrogen ion form is often combined with a weak base anion exchanger working in the free base (hydroxyl) form. The whey passes through the cation exchange column before the anion exchange column. It is not possible to use a weak acid cation exchange resin instead of a strong one, because of pH and the carboxyl functionality of the weak cation exchange resin. The equilibrium constant for the exchange reaction to occur is unfavorable.

Other important characteristics of ion exchangers that are not further discussed are (1) ion exchange capacity, (2) swelling properties, (3) mechanical strength, (4) fluidization during backwashing of the bed, (5) pressure drop, (6) flow velocity restrictions, and (7) water rinse requirement after regeneration.

Conventional Ion Exchange for Demineralization

A simple demineralization plant based on ion exchange is shown in **Figure 2**. The whey enters the strong cation exchanger, loaded in the hydrogen ion form, and continues to the weak base anion exchanger in its free base form. The ion exchange columns are rinsed and regenerated separately with dilute hydrochloric acid and sodium hydroxide (or ammonia). Once a day, the columns are disinfected by rinsing with water containing active chlorine. The following net reactions take place during demineralization (NaCl is used to illustrate the salts of whey and R represents the insoluble resin exchange site):

$$\text{cation exchange:} \text{R-H} + \text{Na}^+ + \text{Cl}^- \rightleftharpoons \text{R-Na} + \text{H}^+ + \text{Cl}^-$$

$$\text{anion exchange:} \ \text{R-OH} + \text{H}^+ + \text{Cl}^- \rightleftharpoons \text{R-Cl} + \text{H}_2\text{O}$$

The above reactions illustrate that by the ion exchange process the whey salts are exchanged for water.

The flow program in the ion exchange process includes the following steps:

1. exhaustion: 10–15 bed volumes of whey can be treated per regeneration cycle. The bed volume refers to the volume of the cation exchanger;
2. regeneration;
3. displacement of whey from the columns by water;
4. backflushing;
5. contact with regeneration solution; and
6. water rinse.

A typical cycle time is about 6 h: 2 h for exhaustion and 4 h for regeneration. The ion exchange vessels are often

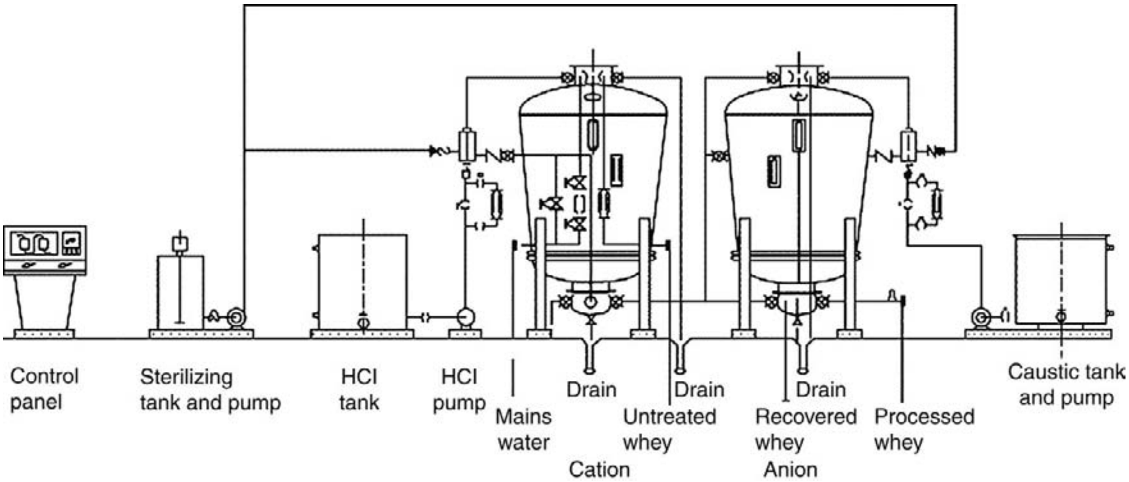

Figure 2 Schematic diagram of an automatic whey demineralization plant.

made of rubber-lined mild steel to avoid corrosion problems. A conical shape is used specially for the anion exchanger to allow for swelling of the bed during transformation from the free base to the salt form. It is common practice to regenerate the cation column in upflow (countercurrent to exhaustion). This system reduces the consumption of regeneration chemicals by as much as 30–40%. The plant can easily be automated. Two or three parallel ion exchange systems are needed for a continuous flow of whey.

Process Limitations and Costs

Whey is a liquid with a high ash content, which means short runs between regenerations with high consumption of regeneration chemicals. These facts lead to a high salt load in the wastewater from both the whey and from the surplus of regeneration chemicals. Consumption of rinse water is also high, especially from washing out excess sodium hydroxide from the weak anion exchange resin.

Losses of whey proteins occur due to adsorption phenomena, primarily on the anion exchange resin. Consumption of regeneration chemicals accounts for 60–70% of the operating costs. The process is primarily designed for 90–95% demineralization but any level can be chosen if a bypass system is used.

An Alternative Ion Exchange Process

In order to reduce the consumption of regeneration chemicals and thus also create a better waste situation for a demineralization plant, the R&D Department of the Swedish Dairies' Association, SMR, developed an alternative ion exchange process. In this process, several different unit operations are linked together, namely, ion exchange, evaporation, distillation, and absorption in order to recover the regenerant, NH_4HCO_3, as illustrated in **Figure 3**. In this process, the whey is first treated with the anion exchange resin regenerated in the bicarbonate form. During anion exchange, the anions of whey are exchanged for HCO_3^-. After this, the whey enters the cation exchange column regenerated in the ammonium form. During the passage of the whey through this column, the cations in the whey are exchanged for NH_4^+. Thus after the process, the whey salts are exchanged for ammonium bicarbonate, NH_4HCO_3. The reactions can be summarized as follows:

$$\text{anion exchange: R-HCO}_3 + \text{Na}^+ + \text{Cl}^- \rightleftharpoons \text{R-Cl} + \text{Na}^+ + \text{HCO}_3{}^-$$

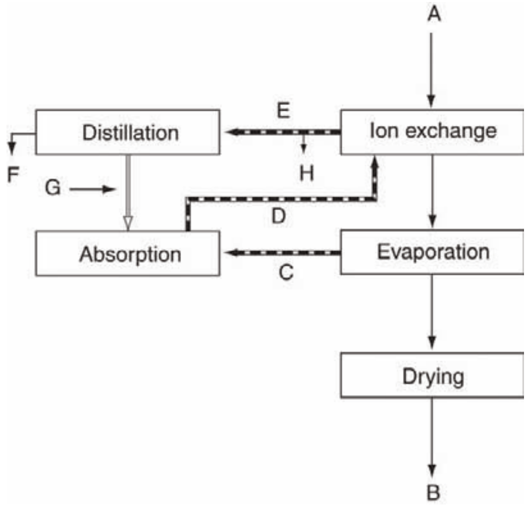

Figure 3 The SMR process for demineralization. A, whey; B, demineralized whey powder; C, condensate with NH_3 and CO_2; D, new regeneration solution; E, spent regeneration solution; F, whey salts; G, NH_3 and CO_2; H, precipitate of $MgNH_4PO_4$.

$$\text{cation exchange: R-NH}_4 + \text{Na}^+ + \text{HCO}_3{}^- \rightleftharpoons \text{R-Na}^+ + \text{NH}_4{}^+ + \text{HCO}_3{}^-$$

NH_4HCO_3 is a thermolytic salt (sometimes used as baking powder), which decomposes to NH_3, CO_2, and H_2O when heated. This happens during the subsequent evaporation of the whey, offering the possibility of recovering the NH_3 and CO_2 vaporized from the whey to make new regeneration solution (NH_4HCO_3). Part of the used regeneration solution after passing the columns, containing surplus of regeneration salt (about 100% of theoretical need is used during regeneration), is collected for vaporization in a distillation tower. **Figure 4** shows the industrial layout of the SMR process. The description of the flow arrangement is given below. The whey first enters the anion exchange column regenerated in bicarbonate form and then the cation exchange column in ammonium form. This is in reverse order compared with the classical ion exchange demineralization procedure.

In practical design, the ion exchange systems are paired, one working on whey while the other is being regenerated. With two pairs of columns, an uninterrupted flow of whey is obtained. The cycle time is 4 h (2 h for ion exchange and 2 h for regeneration). After passing through the ion exchange unit (1) the cool whey is used for heat recovery in the absorption tower and as cooling medium in the condenser (9). Then, the whey enters the evaporator (3) and finally the demineralized whey concentrate is spray-dried (10). The condensate from evaporator stage 2, which is especially rich in ammonia, is separated from the other condensate streams and continues to the absorption

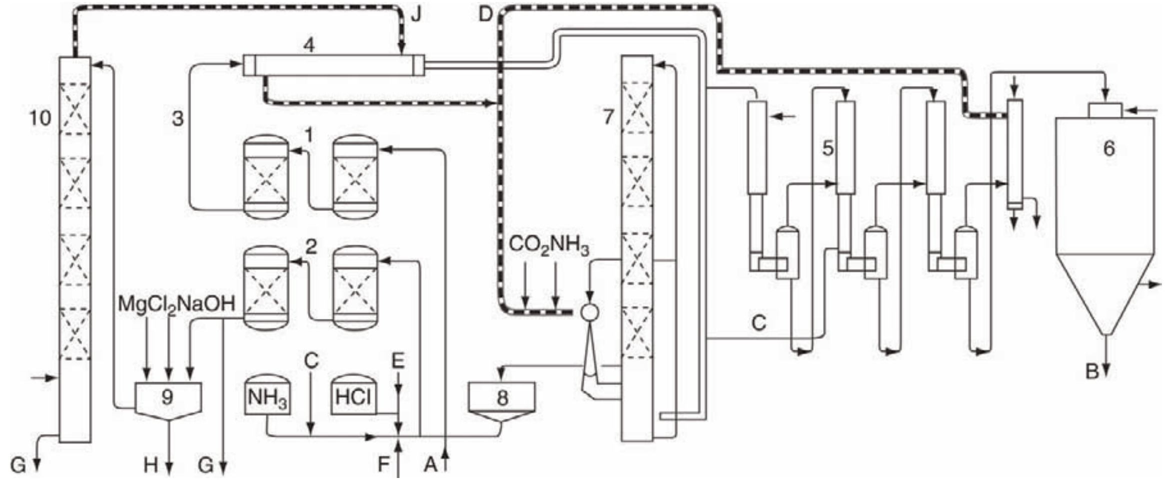

Figure 4 Flow sheet of a full-scale production plant based on the SMR process. A, whey; B, whey powder; C, condensate; D, CO_2 and NH_3; E, water; F, disinfectant; G, wastewater; H, phosphate salt; J, CO_2 and NH_3; 1, ion exchanger; 2, ion exchanger; 3, heat recovery; 4, condenser; 5, evaporator; 6, spray tower; 7, adsorption column; 8, fresh regeneration solution; 9, spent regeneration solution; 10, stripping solution.

tower (4), where it forms the liquid base for the new regeneration solution. The condensates from evaporator stages 1, 3, and 4 are used to rinse off the ion exchangers, giving further recovery of ammonia in these condensates. The recovery of ammonia is 75–80% in the process. Most of the CO_2 stripped off during evaporation is recovered in gaseous form from the mechanical vacuum pump of the evaporator. This gas flows directly into the bottom of the absorption tower, where it is almost completely absorbed in the synthesis of NH_4HCO_3. Overall recovery of CO_2 is more than 90%. To compensate for losses of NH_3 and CO_2 in the process, fresh quantities of NH_4HCO_3 are injected into the circulation flow of the absorption tower (4). The part of the regeneration solution which is rich in NH_4HCO_3 is collected in a tank (8), where the phosphate from whey is precipitated by addition of $MgCl_2$. When the precipitate of magnesium ammonium phosphate ($MgNH_4PO_4$) has settled, the supernatant liquid is pumped to the top of the distillation tower (9) and at the same time preheated in a plate heat exchanger (not shown in the figure) using the bottom liquid in the distillation tower as the heating medium. About 10% of the liquid is stripped off as vapor, which in turn is condensed by using the whey after ion exchange as cooling medium (2).

In summary, the SMR process has the following characteristics:

1. low running costs due to recovery of the regeneration chemicals;
2. low losses of whey solids and only half of the salt discharge compared to the classical ion exchange process;
3. small variations in pH of the whey (6.5–8.2), resulting in minimum losses of denatured whey proteins due to adsorption on the columns;
4. high demineralization efficiency (more than 90%);
5. low operating temperature (4–6 °C), enhancing the microbiological status of the end product;
6. high yield of whey solids compared to classical ion exchange and electrodialysis; and
7. optimum heat recovery.

Process Limitations and Costs

In most cases, depending on the cost of chemicals, the operating cost of the SMR process is 30–70% lower than that of the classical ion exchange process. The equipment for plant design of an SMR plant includes more components than the classical ion exchange process. Therefore, the capital costs are somewhat higher. For optimum profitability, a plant greater than 100 m^3 is needed.

Nanofiltration

An interesting alternative to electrodialysis and ion exchange is NF. NF is a membrane pressure-driven process in between reverse osmosis and ultrafiltration. It is named after the mean pore diameter, which is approximately 1 nm. Thus, the separation area for molecules, especially charged monovalent ions like Na^+, K^+, and Cl^-, lies in the molecular range of 100–1000 Da. The normal operating pressure is typically 2–3 MPa.

Apart from steric exclusion and considering the low value of pore diameter, the membrane separation characteristic is to a large extent determined by electrostatic forces in the membrane matrix because of the electric charges of its carboxyl groups; this causes ions to avoid regions of low dielectric constant. Moreover, the role of the retention of multivalent co-ions (salts and/or proteins) in the facilitated transmission of monovalent ions has been demonstrated.

In the case of sweet whey (membrane negatively charged), the retention of polyvalent anions leads to the presence of higher amounts of negative charges in the retentate, which results in an increased transmission of Cl^- and an increased retention of Na^+ and K^+ and Ca^{2+} in order to maintain the electroneutrality. The demineralization efficiency is thus almost restricted to removal of monovalent ions.

For a volume reduction ratio of 4–5 during filtration of whey, an ash removal from whey of 40–60% is obtainable. It corresponds to 70–80% for monovalent co-ions (which are Na^+, K^+, and H^+ for acid whey and Cl^- and OH^- for sweet whey) and 40–70% for monovalent counterions (which are Na^+, K^+, and H^+ for sweet whey and Cl^- and OH^- for acid whey). Divalent ions are reduced in the range of 3–20%.

Partial demineralization is often needed in various situations when manufacturing dairy ingredients, for example, various whey protein concentrate products, in order to adjust the mineral composition.

Another effect of NF is the concomitant concentration of the whey up to a DM of 20–22%, which helps to economize the evaporation costs.

By combining with water dilution of the NF retentate and renewed filtration (diafiltration), the ash reduction can be driven from 35–50 up to 60–70% but at the expense of increased cost, water utilization, and by-product (nanofiltrate) production. In contrast, in newer types of NF membranes, the loss of organic molecules has been improved, especially lactose.

Indeed the loss of lactose and the loss of nonprotein N and protein in NF are today lower than those found in electrodialysis or ion exchange, making retentate more valuable and leading to a permeate with lower biological oxygen demand (BOD).

Urea does leak quite extensively. Also, organic acids like lactic and acetic acid can pass through the membrane to a large extent, presenting the possibilities of deacidification of acid whey. Corresponding salts of these acids are strongly retained by the membrane.

The benefits of NF are low investment costs and simple installations, which are easy to run. Moreover, the amount of effluents is greatly reduced in comparison with the other demineralization processes, and the generated effluents have a lower BOD. Demineralization by electrodialysis and ion exchange is usually known to generate high amounts of effluents. Moreover, it has been demonstrated that the running costs of these demineralization techniques are 25–55% higher than NF, due to their combination with evaporation concentration.

NF is a fast-growing technology in the dairy world today for different application purposes.

Combination of Processes

For large demineralization installations, that is, those processing more than $400\,m^3\ day^{-1}$ of whey, investment in combined technologies may be of interest. The combinations used are electrodialysis or NF in combination with the classical ion exchange process. The benefits are reduced costs of chemicals but the capital costs are higher and more complicated processes are more difficult to run.

Many modern demineralization plants are combinations of classical ion exchange with NF. By doing this, the ionic load on the ion exchangers is reduced by about 30% in combination with lower volumes to treat. The size of the columns can be reduced by the same figure, in principle.

When electrodialysis and classical ion exchange are combined, the whey first passes the electrodialysis step to about 50% demineralization level. After that, the whey passes on to the ion exchange plant.

See also: **Dehydrated Dairy Products**: Dairy Ingredients in Non-Dairy Foods; Infant Formulae. **Milk Protein Products**: Whey Protein Products. **Milk Salts**: Distribution and Analysis; Interaction with Caseins. **Whey Processing**: Utilization and Products.

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YEASTS AND MOLDS

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Yeasts in Milk and Dairy Products

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Introduction

Yeasts are widespread microorganisms that are able to colonize many different types of habitats. Food with high contents of nutrients such as sugar, organic nitrogen sources, minerals, and vitamins in general presents an ideal substrate for yeast growth. However, the influence of yeasts in dairy products can be either beneficial or detrimental. Some species play an important role in the production of traditional fermented milk products and cheese. They generate specific aroma ingredients, for example, ethanol and carbon dioxide in kefir and kumys, and contribute to the growth of bacterial starter cultures on surface-ripened soft cheeses and semihard cheeses. Nevertheless, growth of yeasts is in most instances undesirable in milk and dairy products, because these microorganisms harbor a high risk of spoilage. Yeasts are able to grow in a broad range of pH environments. Hence, they are normally capable of spoilage of dairy products with a low pH fermented by lactic acid bacteria (LAB), whereas the frequently occurring organisms in milk, belonging to the bacterial families Bacillaceae, Enterobacteriaceae, and Pseudomonadaceae, cannot proliferate at such conditions. Acid dairy products formed with the addition of fruit mixes, honey, cereals, chocolate, and so on are at maximum risk. These products contain, in addition to lactose and small amounts of galactose, considerable amounts of fructose and sucrose providing excellent conditions for growth and fermentation of many yeast species. Consequently, lactose-utilizing species are not the only yeast species that spoil these products.

Raw and Market Milk

During milking, yeast contamination originates in most cases from the floors, litter, feed, and air, and only less frequently from the milking machine or udders affected with mastitis. The total yeast count in raw milk is negligible at 10^1–10^3 cfu ml^{-1}. The species most often found are

Candida intermedia, *C. parapsilosis*, *Cryptococcus curvatus*, *Debaryomyces hansenii*, *Galactomyces geotrichum*, *Issatchenkia orientalis*, *Kluyveromyces marxianus, K. lactis* (both sometimes referred to as *K. marxianus* subsp. *marxianus*, *K. marxianus* subsp. *lactis*, *K. marxianus* subsp. *bulgaricus*, *Candida kefyr*), *Pichia farinosa*, *P. fermentans*, *P. membranifaciens*, *P. anomala*, *Trichosporon beigelii*, and *Yarrowia lipolytica* (**Table 1**). There is a significant relationship between udder health, and general hygiene during milking, and the yeast count in raw milk (*see* **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects).

Even after homogenization at 55–65 °C and heat treatment at pasteurization conditions, milk can get recontaminated with yeasts and can develop moldiness, mustiness, and yeast odor. Amino acids and peptides in spoilt milk can stimulate the growth of starter cultures. During fermentation, this can lead to overacidification or to an undesirable population displacement. Also, inhibitory substances can be produced. Consequently, product defects like soft curd, separating and stripping, flavor defect or lack of aroma can occur.

During pasteurization of milk, vegetative cells of yeasts, blastospores, chlamydospores of *Candida albicans* and other species, teliospores, ballistospores, basidiospores, endospores, ascospores of ascosporogenous yeasts, and arthrospores of the Endomycetaceae (e.g., *Galactomyces geotrichum*) are destroyed. For the most heat-resistant species, *Zygosaccharomyces bailii*, the following inactivation values were determined: vegetative cells: $D_{56\,^\circ\mathrm{C}} = 1$ min, $z = 4\,^\circ$C; ascospores: $D_{64\,^\circ\mathrm{C}} = 1$ min, $z = 3\,^\circ$C (*see* **Heat Treatment of Milk:** Sterilization of Milk and Other Products). Under low water activity (a_{w}) conditions, for example, when yeast cells are pressed in porous gaskets, the heat inactivation can be insufficient. Areas in the processing plant that pose a risk of recontamination are heat exchangers, cooling water, filling equipment, air, and packaging materials.

Generally, recontamination with yeasts is less important for the spoilage of pasteurized milk and neutral-pH dairy products. *Acinetobacter* spp. and many Gram-positive bacteria, including spores of *Bacillus* spp. and *Clostridium* spp., survive pasteurization. Moreover, because the filling operation is not sterile, the milk is most frequently recontaminated with Gram-negative organisms. Hence, these bacteria are responsible for the shelf life limit of 7–14 days for pasteurized milk.

Various milk products are heat treated at 90–110 °C for a short time. Under these conditions, all bacteria as well as ascospores from thermophilic molds are destroyed. But it has no effect on spores of bacilli and clostridia. Hence, these spore-forming bacteria can cause microbiological problems. Only in case of recontamination can yeasts contribute to or cause spoilage. Under high-temperature pasteurization (e.g., 125 °C for 5 s) and sterilization (e.g., 109–115 °C for 20–40 min) conditions, all microorganisms except for some heat-resistant bacterial endospores are inactivated. Nevertheless, it has been observed that the occurrence of spoilt milk is connected with the presence of heat-stable proteases and lipases, which originate from the high numbers of microorganisms present in milk before heating (*see* **Microorganisms Associated with Milk**). However, these enzymes originate from *Pseudomonas* spp. as enzymes produced by yeasts are completely inactivated during these heat treatments.

Butter and Natural Buttermilk

Butter made in a dairy plant and stored at a low temperature seldom undergoes microbial spoilage within its shelf life. The fine dispersion of water, low pH value (in cultured butter), and low solubility of oxygen in fat hinder the growth of microorganisms. The product surface is exposed to limited hygienic risk. Yeasts can grow there and damage the butter, for instance, by lipolysis or discoloration. Improper production conditions or unsterile storage can facilitate spoilage caused by yeasts. Homemade butter contains many serum droplets with lactose as a convenient nutrient for microorganisms; its hygienic status leaves much to be desired. Here yeasts can reach high counts. Addition of NaCl reduces the risk of spoilage.

Natural buttermilk should at the most contain 200 cfu ml^{-1} yeast cells. A large number of microbiological defects can be traced to yeasts, as they can grow even at pH <3.5 and at 0 °C. The spoilage is caused by proteolysis, lipolysis, and carbohydrate fermentation with the formation of gas. Overacidification as well as consistency and sensory problems can arise and packages can blow. The yeast species most often found in industrially produced buttermilk are *Debaryomyces hansenii*, *Candida tropicalis*, *Galactomyces* spp., *Geotrichum* spp., *Issatchenkia* spp., *Kluyveromyces* spp., *Pichia anomala*, *P. kluyveri*, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*. In buttermilk made from traditional Jordanian butter, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia kluyveri*, *Galactomyces geotrichum*, *Issatchenkia orientalis*, *Torulaspora delbrueckii*, *Candida tropicalis*, and *Trichosporon ovoides* were the most prevalent yeast species.

Ice Cream and Frozen Yogurt

Milk products marketed in the frozen state cannot be spoiled by yeasts. The growth rate and metabolic activity at temperatures <0 °C are irrelevant. However, the finished product should not be more than very minimally contaminated; the product cannot be permitted to thaw out in between.

Table 1 Yeast species frequently isolated from milk and dairy products

Species[a]	*Habitat*[b,c]
Bullera variabilis	en
Candida atmosphaerica	en
Candida auringiensis[d]	
Candida boidinii	fr, yo
Candida butyri	bu
Candida catenulata	**ch**, en, ma, mi
Candida diddensiae	br, bu, en
Candida etchellsii	br, yo
Candida ethanolica	en, fr, yo
Candida fennica[d]	
Candida glabrata	fr, **yo**
Candida glaebosa	mi
Candida haemulonii	fr, **yo**
Candida intermedia[d]	**br**, **ch**, co, **en**, **fr**, **yo**
Candida lactis-condensi	ch, co, yo
Candida magnoliae	fr, yo
Candida parapsilosis	**br**, bu, **ch**, **en**, **fr**, ma, **yo**
Candida peltata	ma
Candida rugosa	**br**, bu, **ch**, en, ke, mi, se
Candida sake	bu, ch, en, fr, mi, yo
Candida santamariae	fr, en
Candida savonica	fr, en
Candida sorbophila	**en**
Candida tenuis[d]	**br**, ke, ma
Candida tropicalis	**bm**, **br**, bu, **ch**, **fr**, ke, mi, **yo**
Candida versatilis[d]	**br**, ch, en, ke, mi
Candida vini	bu, ch, se
Candida zeylanoides	**br**, **ch**, se
Citeromyces matritensis (*Candida globosa*)	co, fr, mi
Clavispora lusitaniae (*Candida lusitaniae*)	**br**, **ch**, **en**, **fr**, ma, **yo**
Cryptococcus albidus/curvatus/humicolus/laurentiae[d]	br, ch, **en**, fr, **mi**, yo
Debaryomyces hansenii (*Candida famata*)[d]	bm, **br**, bu, **ch**, **en**, **fr**, ke, mi, re, **yo**
Dekkera anomala (*Brettanomyces anomalus*)[d]	ch, ke
Dekkera bruxellensis/custersiana (*Brettanomyces* spp.)	ke, mi, yo
Filobasidiella neoformans (*Cryptococcus neoformans*)	ma
Filobasidium floriforme	en
Galactomyces geotrichum (*Geotrichum candidum*)	**br**, **bm**, **ch**, **en**, ke, **mi**, yo
Geotrichum fragrans/klebahnii	**bm**, **br**, **ch**, **en**, mi, yo
Hanseniaspora uvarum (*Kloeckera apiculata*)	en, **fr**, mi, **yo**
Hanseniaspora vineae (*Kloeckera africana*)	en, fr, mi, **yo**
Issatchenkia occidentalis (*Candida sorbosa*)[e]	**br**, bm, ch, **en**, fr, ke, **mi**, yo
Issatchenkia orientalis (*Candida krusei*)[e]	**br**, bm, ch, **en**, fr, ke, **mi**, **yo**
Kloeckera lindneri	fr, yo
Kluyveromyces lactis (*Candida sphaerica*)[d]	br, bm, bu, **ch**, **en**, fr, **ke**, **mi**, **yo**
Kluyveromyces marxianus (*Candida kefyr*)[d]	br, bm, bu, **ch**, **en**, fr, **ke**, **mi**, **yo**
Leucosporidium scottii (*Candida scottii*)	en
Metschnikowia pulcherrima (*Candida pulcherrima*)	fr, yo
Metschnikowia bicuspidate/reukauffii	fr, yo
Pichia angusta	ch, fr, mi, ma, yo
Pichia anomala (*Candida pelliculosa*)[e]	bm, ch, co, en, **fr**, **mi**, ma, **yo**
Pichia burtonii (*Candida chodati*)	ch, mi, yo
Pichia cactophila	bu, fr, yo
Pichia etchellsii	bu
Pichia fabianii (*Candida fabianii*)[e]	fr, en
Pichia farinose (*Candida cacaoi*)	mi, en
Pichia fermentans (*Candida lambica*)	bm, ch, ke, **mi**, yo
Pichia guilliermondii (*Candida guilliermondii*)	bm, bu, ch, en, fr, yo
Pichia jadinii (*Candida utilis*)[e]	**br**, bm, **ch**, fr, ma
Pichia kluyveri	**bm**
Pichia membranifaciens (*Candida valida*)	**ch**, **fr**, ke, **yo**

(*Continued*)

Table 1 (Continued)

Species[a]	*Habitat*[b,c]
Pichia norvegensis (*Candida norvegensis*)	bu, en, yo
Pichia pini	fr, yo
Pichia pseudocactophila	ch, en, yo
Pichia sorbitophila[d]	-
Pichia triangularis (*Candida polymorpha*)	**br**, ch
Rhodotorula glutinis/graminis/minuta/mucilaginosa[d]	**br**, bu, ch, **en**, fr, mi, ma, yo
Saccharomyces cerevisiae (*Candida robusta*)	**br**, **bm**, **ch**, en, **fr**, **ke**, mi, **yo**
Saccharomyces dairensis/kluyveri	bm, **ke**
Saccharomyces exiguus (*Candida holmii*)	bm, ke, yo
Saccharomyces servazzii	**ke**
Saccharomyces unisporus	ch, **ke**
Saccharomyces turicensis	ke
Saccharomycodes ludwigii	yo
Sporobolomyces roseus/salmonicolor	fr, en
Sterigmatomyces halophilus	br
Torulaspora delbrueckii (*Candida colliculosa*)	**br**, **bm**, **ch**, **en**, **fr**, **ke**, mi, **yo**
Trichosporon asteroids/ovoides/pullulans	bm, bu, ch
Trichosporon beigelii/cutaneum	**bm**, **ch**, **en**, se, **mi**, yo
Trichosporon capitatum (*Geotrichum capitatum*)	ch, ma
Williopsis californica/saturnus[e]	ch, yo
Yarrowia lipolytica (*Candida lipolytica*)	**br**, bu, **ch**, en, ke, yo
Zygoascus hellenicus (*Candida hellenica*)[d]	ma
Zygosaccharomyces rouxii	ch, fr, yo
Zygosaccharomyces bailii/bisporus	ch, fr, yo
Zygosaccharomyces florentinus/mellis	ke

[a] In parentheseis: imperfect state.

[b] Often found in: bu, butter; bm, natural buttermilk; ch, cheese; br, cheese brine; co, condensed milk; en, dairy environment; se, dairy sewage; fr, fruit mix for fruit-supplemented yogurt and quark; ke, kefir; mi, milk; ma, mastitis milk; re, rennet; yo, yogurt.

[c] Bold print: very frequently found.

[d] Lactose assimilation and fermentation.

[e] New classification according to Kurtzman CP (2008):
formerly *Issatchenkia occidentalis*: *Pichia occidentalis*,
formerly *Issatchenkia orientalis*: *Pichia kudriavzevii*,
formerly *Pichia anomala*: *Wickerhamomyces anomalus*,
formerly *Pichia fabianii*: *Lindneri fabianii*,
formerly *Pichia jadinii*: *Lindnera jadinii*,
formerly *Williopsis californica*: *Barnettozyma californica*,
formerly *Williopsis saturnus*: *Lindnera saturnus*.

Data compiled from Barnett JA, Payne RW, and Yarrow D (1990) *Yeasts: Characteristics and Identification*. Cambridge: Cambridge University Press.

A health risk from yeasts exists when facultative pathogens appear in food or on food-contaminated equipment. Facultative pathogens are known to cause infections in susceptible individuals such as infants, seniors, immunocompromised persons, persons with AIDS, diabetics, alcoholics, and pregnant women. In young children, oral thrush and nappy rash are not unheard of; allergies can also be involved. Immunosuppressed individuals can suffer from a serious mycosis of the organs. More than 50% of all fungal infections are caused by *Candida* spp. like *C. albicans, C. dubliniensis, C. glabrata, C. krusei* (=*Issatchenkia orientalis*), *C. parapsilosis* (group I), *C. metapsilosis* (formerly *C. parapsilosis* group III), *C. orthopsilosis* (formerly *C. parapsilosis* group II), *C. stellatoidea* (a taxonomic synonym *C. albicans* but with differences in the pathogenesis), and *C. tropicalis*. But also species of other genera that can appear in food are regarded as facultative pathogens: *Filobasidiella neoformans* subsp. *neoformans*, *F. neoformans* subsp. *bacillisporus,* and *F. neoformans* subsp. *gattii*; *Cryptococcus* spp.; *Debaryomyces hansenii*; *Pichia guilliermondii*; *Rhodotorula* spp.; *Sporobolomyces* spp.; and *Trichosporon beigelii.* An attack by *Filobasidiella* (teleomorph of *Cryptococcus*) leads to the dreaded *Cryptococcus* mycosis of the brain, lungs, bone marrow, kidneys, respiratory tract, digestive tract, eyes, skin, central nervous system, and nails. These yeasts are ubiquitously distributed, but *Candida albicans* and *Filobasidiella neoformans*, the species with the highest potential risks, are very seldom found in milk and dairy products. In food, potentially dangerous concentrations of toxins are not produced. Opportunistically pathogenic yeasts are generally found in milk from cows with mastitis.

Cultured Milk Products

Cultured milk products (fermented milk, sour cream, yogurt, drinking yogurt, cottage cheese, cream cheese, etc.) should be free from yeasts. These foods are ideal media for the propagation of yeasts, as they exhibit a low pH of 4–6, which is optimal for yeast growth. The approximate decimal doubling time in fruit yogurt without shaking for *Saccharomyces cerevisiae* is $t_d = \sim 5$ h (30 °C), ~10 h (20 °C), ~62 h (10 °C), and ~84 h (4 °C), and for *Galactomyces geotrichum* is $t_d = \sim 6$ h (30 °C), ~12 h (20 °C), ~96 h (10 °C), and ~7 days (4 °C).

Due to the acidic environment (pH ~3.8–4.5), there is limited competition from bacteria in yogurt. Most of those that can still grow alongside the yeasts and molds are LAB (i.e., *Lactobacillus*, *Lactococcus*, *Weissella*, and *Leuconostoc* spp.), streptococci, enterococci, bifidobacteria, propionic acid bacteria, acetic acid bacteria, *Micrococcus kristinae*, and *Zymomonas mobilis*. The enterobacteria, for example, die off quickly in yogurt. Spores of bacilli and of most clostridia cannot germinate. For the acid-tolerant anaerobic *Pediococcus* spp., *Pectinatus cerevisiiphilus*, and *Megasphaera cerevisiae*, this environment contains too much oxygen. Therefore, next to contamination with molds, contamination with yeasts is the largest microbial problem in these types of products. The annual economic losses to the dairy industry are substantial. Fruit-containing fermented milk products spoil quickly, owing to the high fructose and sucrose content of the fruit preparations, which encourage yeast growth and fermentation. A collapse (with nonfermenting yeasts) or swelling (with fermenting yeasts) of the cups, a change in texture, product discoloration, off-flavors, off-tastes, or visible microbial colonies on the product surface are the evidence of this spoilage.

The fruit preparations are delivered to the dairy in large containers. As a rule, even negligible contamination with yeasts in these containers can lead to immense losses. An entire day's yogurt production can be affected. These products cannot be offered for sale. In Germany, for example, such contaminated products are declared as hazardous waste and consequently have to be discharged on a special rendering facility. The risk of damage can be reduced by maintenance of the filling temperature of the fruit mix at < 15 °C, consistent chilled storage of the container, avoidance of a stepwise emptying of the container, a high sugar concentration in the fruit preparation, and prompt processing. Large dairies increasingly produce the fruit preparations themselves. This increases the microbiological safety considerably, as the processes of portioning and transport are eliminated. The fruit preparation is pumped directly from the cooking boiler or tubular heat exchanger through a cooler into the storage tank, from where the portions for the fruit yogurt are drawn directly.

In the preparation of heat-treated yogurt, the mixture of fruit preparation and cultured milk is heated to 70–80 °C in a scraped-surface or tubular heat exchanger (immediately before filling into the cups). This type of yogurt has a shelf life of 1–5 months longer than yogurt with viable LAB cultures. This product generally does not undergo changes due to the activity of LAB and fruit mix contaminants such as other acid-tolerant bacteria as well as yeasts and molds.

With the exception of the 'white mold' *Galactomyces geotrichum* (anamorphic state = *Geotrichum candidum*; formerly *Oidium lactis* or *Oospora lactis*), no fungi that are limited only to the fruit preparations or to the yogurt portion are found. The xerophilic species *Zygosaccharomyces* spp., *Citeromyces matritensis*, *Candida versatilis*, *Pichia etchellsii*, *P. ciferrii*, and *P. sorbitophila* are considerably more common in fruit preparations than in products made only from milk. On the other hand, the typical cheese yeast *Debaryomyces hansenii* is found more often in milk than in the fruit mix. The fermented milk portion is occasionally sweetened with sucrose syrup. In such cases, it is not possible to distinguish between yeast species originating from the production environment of the dairy and that of the fruit mix processor.

In the dairy plant environment (floors, walls, equipment) the yeast species identified most commonly are *Debaryomyces hansenii*, *Clavispora lusitaniae*, *Rhodotorula* spp., *Cryptococcus* spp., *Candida intermedia*, *C. parapsilosis*, *C. sorbophila*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Issatchenkia* spp., *Trichosporon* spp., and *Galactomyces geotrichum*. The dominant yeast species from fruit preparations and contaminated fruit-containing cultured milk products are *Saccharomyces cerevisiae*, *Pichia anomala*, *P. fabianii*, *P. membranifaciens*, *Hansenispora vineae*, *H. uvarum*, *Debaryomyces hansenii*, *Candida parapsilosis*, *C. tropicalis*, *C. intermedia*, *Torulaspora delbrueckii*, and *Clavispora lusitaniae*. The molds *Mucor* spp. and *Aureobasidium* spp., as well as the unpigmented algae *Prototheca* spp., show yeast-like growth at submerged cultivation; hence a risk exists of mistaking them for yeasts. Moreover, *Mucor* spp. and *Prototheca* spp. produce large quantities of carbon dioxide and are opportunistic pathogens. *Prototheca* spp. have a physiological reaction pattern similar to that of *Saccharomyces cerevisiae*. A yeast-killing toxin designated mycocin HMK (*Hansenula mrakii* killer strain) produced by *Williopsis mrakii* (reclassified) was reported to have potential application in controlling yogurt spoilage caused by yeasts (*see* **Fermented Milks**: Yoghurt: Types and Manufacture).

Neutral-pH Fruit-Containing Milk Products

In milk products with a pH near neutrality, for example, milk rice, semolina pudding, or milk pudding, with additions based on fruit, cocoa, nuts, vanilla extracts, vitamin mixes, or cereals, yeasts are (next to bacilli and molds) the most common spoilage organisms. All yeast species that can be found in the added preparations, in the dairy-based component, and in the dairy environment are possible contaminants. The spoilage organisms are strictly oxidative as well as fermentative yeasts. The usual appearances of spoilage are blowing or sucking in of containers and a conspicuous change in product consistency or in flavor and taste. In most cases of spoilage, the fault lies with the added preparations, owing to the favorable nutrient conditions (glucose, fructose, sucrose, organic acids) as well as to the occasional long storage time of the product containers at ~20 °C; in such situations, a considerable increase in yeast count can occur.

In addition, underlaid products are often produced, which means that first the fruit preparation is filled in the cup and then the hot dairy portion at a temperature of 70–80 °C is added on top. No yeasts appear in the milk portion. Yeasts are also seldom found in toppings made from whipping cream or vegetable oil foams, as these have been heated and there is little possibility for yeasts to multiply.

Fermented Products Containing Yeasts

A Japanese patent describes milk fermentation with *Bifidobacterium longum* together with yeasts under not strictly anaerobic conditions. The growth of these fastidious bacteria is positively influenced by the change in the milk environment brought about by the yeast growth.

In Finland, viili is a popular set-curd milk product. It is consumed pure, or sweetened with jam and raisins, or with cereals. The milk is inoculated with a mesophilic, aroma-producing LAB culture and with *Galactomyces geotrichum*. Taxonomically, the yeast-like fungi *Galactomyces* spp. and also *Geotrichum* spp. and *Dipodascus* spp. are seen to be somewhere between yeasts and molds. Different authors will place the genera in one taxonomic order or the other. Because of the growth of the strictly oxidative white mold on the product–air interface, viili has a matte, velvety, white to light yellow surface. *Galactomyces geotrichum* works to develop the flavor and also hinders autooxidation of fats as well as contamination of the product surface by wild molds. The strains used are only those that have minimal lipase activity. *Galactomyces geotrichum* and similar species can produce a nutlike flavor. Similar products are the Norwegian tettemelk and the Swedish långfil (*see* **Yeasts and Molds**: *Geotrichum candidum*).

Leben is a fermented milk product from Arab countries and is similar to kefir. This product is made from fresh milk using a mesophilic LAB and thermophilic yogurt culture as well as yeasts. Correspondingly, one also finds ethanol, acetoin, and diacetyl in leben. Again *Kluyveromyces marxianus* becomes dominant within the yeast species. Generally, however, the microflora is not homogeneous, because leben is mostly homemade. Coliform bacteria, black and green molds, and enterococci are also regularly found in this product (*see* **Fermented Milks**: Middle Eastern Fermented Milks).

Similar yeast-containing products are yaghurt (Middle East); dahi and misti dahi (India); acidophilus yeast milk, busa, kuban, kurunga, prohlada, and salomat (Russia); rob (Egypt); omaere (Africa); skyr (Iceland); samokisselis (Yugoslavia); airan and arsa (Asia); aker (Tibet); airag, khoormog, tschigan, and umdaa (Mongolia); matzoon (Armenia); brano milk (Bulgaria); felisowka (Poland); galazyme (France); cellarmilk (Norway); hooslanka, urda, and zhentitsa (Carpathian Mountains) (*see* **Fermented Milks:** Types and Standards of Identity).

Kefir and Kumys

Kefir is a type of fermented milk produced by a mixed flora consisting of yeasts, various LAB, and acetic acid bacteria (*see* **Fermented Milks**: Kefir). This flora forms nodules known as ‘kefir grains’. Typical kefir contains ethanol and predominantly L-(+)-lactic acid and has effervescence owing to the presence of CO_2. Under German regulations, kefir must contain at least 0.05% (w/v) ethanol and CO_2 produced by yeast fermentation. According to the Swiss food manual, there should be a minimal yeast count of 10^5 cfu g^{-1}. The International Dairy Federation standard proposes a minimal yeast count of 10^4 cfu g^{-1}, but without being species specific.

Kumys (also kumiss, koumyss, koumiss, coomys, kimiz) is a fermented beverage made from mare’s milk (*see* **Fermented Milks**: Asia). Its origins are in central Asia. The flora of kumys includes yeasts and various LAB. Owing to a relatively high lactose concentration in mare’s milk, alcohol content in kumys can reach ~3.5%.

Cheese and Brine

White Cheese

The yeast species most frequently isolated from acid-curd cheeses (quark, Gervais, cottage cheese, cream cheese) are *Galactomyces geotrichum*, *Kluyveromyces marxianus*, *K. lactis*, *Pichia membranifaciens*, *P. guilliermondii*, *Debaryomyces hansenii*, *Trichosporon beigelii*, *Issatchenkia* *orientalis*, and *Yarrowia lipolytica*. The yeast count thresholds for defects such as slightly versus strongly 'yeasty, fermenting, fruity, old, musty, bitter' are ~10^4–10^5 and ~10^5–10^6 cfu ml^{-1}, respectively. The effect on sensory profile depends upon the species. The lowest count value to cause a sensory change was shown by *Galactomyces geotrichum*, followed by *Kluyveromyces* spp., *Pichia membranifaciens, Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Issatchenkia orientalis*, *Yarrowia lipolytica*, and *Saccharomyces exiguus*. A separator curd that had been initially contaminated with ~100 cfu g^{-1} showed the first signs of sensory defects after 5–7 days at 10 °C; after 10 days, it was spoiled. Therefore, a good product should have <100 cfu g^{-1} contaminants. The generation times of yeasts in curd at 2, 4, 6, and 10 °C are ~100, 50, 20, and 10 h, respectively. Yeasts have a direct correlation with the quality of acid-curd cheese, and their absence is an important indicator of good manufacturing practice (GMP). With the presence of yeasts the shelf life of products at 10–6 °C is limited to 10–15 days.

Soft, Semihard, and Hard Cheese

Positive aspects

Yeasts are important in the development of the micoflora of surface-ripened cheese (*see* **Cheese**: Smear-Ripened Cheeses). It is commonly known that there exists a synergistic relationship among yeasts, *Brevibacterium linens*, *Microbacterium* spp., micrococci, and LAB. The survival of lactobacilli is enhanced by the presence of yeasts. The low pH resulting from the metabolism of the LAB is raised through lactate utilization by yeasts as well as the formation of alkaline products through proteolysis, so that aerobic, acid-sensitive bacteria such as *Brevibacterium linens* and micrococci can develop. For the growth of this bacterial flora, vitamins and amino acids are provided by yeasts. At the same time, yeasts are a protective culture against undesired enterobacteria, *Clostridium tyrobutyricum*, *Staphylococcus aureus*, or wild molds.

Through the development of gas in Gorgonzola and other blue cheeses, an open doughy structure is promoted. Aromatic components are formed through lipolysis and proteolysis. Some yeast species or single strains that hydrolyze specific casein fractions have a positive effect on the growth of LAB and *Penicillium roqueforti* in blue-veined cheeses. Aminopeptidases and carboxypeptidases from *Galactomyces geotrichum* are essential contributors to the breakdown of bitter peptides. Extracellular and intracellular peptidases with varying pH optima are found, respectively, in *Yarrowia lipolytica* and *Candia catenulata*, and in *Trichosporon beigelii* and *Debaryomyces hansenii*. The concentration of soluble nitrogen increases; glutamate and aspartate as well as tryptophan, leucine, methionine, phenylalanine, and other amino acids are deaminated. Sensorially important alcohols and aldehydes are produced by utilization of amino acids and carbohydrates. The intracellular enzymes are found after yeast autolysis, that is, in the late stages of ripening. In cheese ripening studies done with aseptic curd slurries, *Debaryomyces hansenii* produced an acidic, fruity, and cheesy aroma. Other cheese isolates did not reveal similar tendencies. The species *Yarrowia lipolytica*, *Trichosporon beigelii*, and *Galactomyces geotrichum* had a positive influence on the aroma as a result of the breakdown of proteins and peptides. The species *Clavispora lusitaniae, Pichia jadinii*, and *Williopsis californica* showed weak proteolytic activity, but caused an increase in pH owing to their ability to utilize lactate. The strongest lipolytic and proteolytic activity was demonstrated by *Yarrowia lipolytica. Debaromyces hansenii* and other yeast species are able to assimilate the amines cadaverine, histamine, putrescine, and tyramine.

Negative aspects

Strong yeast growth on cheese can lead to defects in aroma and flavor (yeasty, moldy, putrid, overripe, alcoholic, musty, fermented, earthy, spicy, ammonia, pungent, rancid, sweet, and gassy). Sometimes, *Pichia jadinii* causes blowing in young cheese. The rind can become slimy and even semifluid owing to the growth of *Cryptococcus* spp., because this genus produces an extracellular starch-like capsule. The yeast species *Pichia anomala* reduces the growth of *Penicillium roqueforti*. Some DOPA-positive strains of *Debaryomyces hansenii* and *Yarrowia lipolytica* with high levels of metabolic activity produce an undesirable brownish pigment. With acid-curd cheeses, an overdominance of *Galactomyces geotrichum* is an indicator of spoilage; *Yarrowia lipolytica* was found to generate the strongest odor, from overripe to putrid, whereas *Trichosporon beigelii* was judged to produce a rancid odor. In mixed cultures, *Issatchenkia orientalis* inhibited the growth of *Lactococcus lactis*, whereas *Lactococcus lactis* stimulated the growth of *Issatchenkia orientalis*. In pickled cheese brine, acid-consuming yeasts increased the pH of the brine to a level that enabled the development of *Staphylococcus aureus*. This indicates the need to monitor yeast contamination in cheeses preserved by a combination of acid and high salt content. In Brick cheese, a low temperature during ripening, a high salt content, or drying of the cheese surface suppresses yeast growth, thereby prolonging the ripening process. Interactions among the various LAB and yeast organisms take place in the overall product formation. Because of this, one must specify the yeast count, species, and strains favorable to the respective cheese type. In some countries, the antifungal agents natamycin (pimaricin), propionate, or sorbate are used to inhibit the growth of yeasts on the surfaces of hard and semihard cheeses.

Starter cultures

Yeasts, together with conidia of *Penicillium camemberti*, can be added as a starter culture directly to the milk (for white surface-ripened cheeses such as Camembert and Brie), or sprayed or brushed onto the rind together with the red smear flora (red cheese, acid-curd cheese, Croute Mixte cheese). Culture suppliers offer preparations with *Debaryomyces hansenii* and *Pichia jadinii* for the production of Brick cheese. For manufacturing acid-curd cheeses, a preparation with 'mycoderma' species is offered. Mycoderma were previously known as the pellicle-forming fungi *Galactomyces geotrichum*, *Dipodascus capitatus*, *Geotrichum* spp., *Trichosporon beigelii*, *Issatchenkia orientalis*, *Pichia membranifaciens*, and *P. fermentans*. The cultures contain selected strains with a defined ability to metabolize lactate, citrate, and acetate (deacidifying activity), as well as galactose and lactose (growth rate). They should also have proteinase, peptidase, and esterase activity (flavor, taste), and lipase and phospholipase activity (aroma), and be able to produce CO_2 (doughy structure); tolerate anaerobiosis, acid, cold, and NaCl; have a high growth rate; and have an effect on cheese surface appearance, as well as inhibit wild molds (*see* **Cheese**: Starter Cultures: Specific Properties. **Yeasts and Molds**: *Geotrichum candidum*).

Interior of cheeses

In the interior of cheeses made under strict standards of hygiene, the yeast count is low at 10^1–10^3 cfu g^{-1}. Metabolic products of importance are not expected in these cases. The process of diffusion between the rind and the core brings about deacidification. In the interior of raw-milk cheeses the counts and the diversity of the flora are higher than those in cheeses made from pasteurized milk. The species identified inside the raw-milk cheese Tête de Moine were *Clavispora lusitaniae*, *Debaryomyces hansenii*, *Pichia pseudocactophila*, *Kluyveromyces marxianus*, *Rhodotorula mucilaginosa*, *Debaryomyces capitatus*, and *Pichia jadinii*. Owing to the large differences in the yeast counts of the surface, middle, and core layers of the cheese, distinct variations in the metabolic products are observed. For example, in Taleggio cheese after 35 days of ripening at 3–10 °C, the three layers mentioned above had pH values 6.5, 5.5, and 5.2, respectively. In cheeses that should have an adequate yeast flora in the interior to soften the cheese structure through CO_2 production, the yeasts have to be added to the milk vat. Here, a naturally high yeast count can be established. The speed of deacidification of the core area will depend upon the size of the cheese and its dry weight.

Yeast species and counts on cheese surface

In the first few days of the ripening period the yeast count on the surface increases rapidly and after 8–10 days it reaches a maximum of 10^6–10^9 cfu g^{-1} or $\sim 10^8$ cfu cm^{-2}. This count decreases slightly during further ripening. The softer the rind, the higher the initial yeast count, and, therefore, the smaller the decrease in viable count. On soft, semihard, and hard cheeses, diverse yeast species are seldom found, but various species of the flora may dominate. The predominant species are *Debaryomyces hansenii*, *Trichosporon beigelii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Candida zeylanoides*, *C. catenulata*, *Torulapsora delbrueckii*, and *Galactomyces geotrichum*.

In well-ripened Mozzarella cheese, *Saccharomyces cerevisiae* and *Kluyveromyces* spp. were primarily found; in cheeses with a yeasty flavor defect, the species *Yarrowia lipolytica*, *Issatchenkia orientalis*, and *Candida parapsilosis* were found in addition to *Saccharomyces cerevisiae* and *Kluyveromyces* spp. On surface-ripened, acid-coagulated skim milk cheeses, *Debaryomyces hansenii* and *Pichia membranifaciens* were the predominant yeast species. On Brick cheese, the dominant yeasts were *Debaryomyces hansenii* and *Galactomyces geotrichum*. The surfaces of blue-veined cheese overwhelmingly had *Debaryomyces hansenii*, and on the inside *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Pichia anomala*, *Torulaspora delbrueckii*, and *Galactomyces geotrichum* were found. In Harzer cheese, a shift in the flora was observed. At the beginning, those primarily found in dry quark were *Kluyveromyces marxianus*, *Trichosporon beigelii*, *Issatchenkia orientalis*, and *Dekkera anomala*, while in the early stages of ripening *Trichosporon beigelii* and *Candida catenulata* were predominant. At the end, only *Yarrowia lipolytica* could be identified. *Kluyveromyces marxianus*, *K. lactis*, and *Saccharomyces cerevisiae* contribute to the characteristic open structure of Gorgonzola. In foil-ripened Raclette model cheese, a combined yeast starter culture of *Debaryomyces hansenii*, *Pichia jadinii*, *Yarrowia lipolytica*, and *Galactomyces geotrichum* was found to be advantageous.

In general, different population spectra are observed depending on milk quality, water and salt content of the cheese, production hygiene, possible addition of yeast culture, storage temperature, stage of ripening, competing flora, and location of cheese sampling. Microbial flora is also affected by the geographic region, manufacturer, the range of products made on site, production lot or batch, age of the brine bath, and season of the year, as well as the methods of isolation, enumeration, and identification of yeasts used.

As a rule, *Debaryomyces hansenii* is the prevailing species identified. Its role in the deacidification of surface-ripened cheeses is essential. This is due to its favorable metabolic capacity in the specific environment (fermentation of glucose, galactose, etc.; metabolism of lactose, lactate, citrate, etc.; proteolysis), high rate of sodium expulsion, potassium uptake potential, and its high salt tolerance (a_w minimum 0.85). To protect against plasmolysis caused by NaCl and dehydration, osmotolerant yeasts produce large quantities of polyols such as glycerol, arabitol, xylitol, erythritol, and mannitol. *Debaromyces hansenii* still shows growth to ~0.3 OD after 100 h incubation in a broth medium containing yeast extract, malt extract, glucose, and ~18% (w/v) NaCl. It is followed by *Kluyveromyces marxianus* (~15% NaCl), *Torulaspora delbrueckii* (~14%), *Yarrowia lipolytica* (~14%), *Pichia farinosa* (~14%), *Candida versatilis* (~14%), *Saccharomyces unisporus* (~14%), *Candida zeylanoides* (~13%), *Candida catenulata* (~13%), *Saccharomyces cerevisiae* (~9%; a_w minimum 0.94), *Galactomyces geotrichum* (~2%), and *Trichosporon beigelii* (~2%). For *Debaryomyces hansenii*, the optimum for growth lies between 0 and 11% NaCl, and the inhibitory concentration of NaCl is ~24%. Distinctly lower are the optimum concentrations for *Clavispora lusitaniae* (0–6%) and *Saccharomyces cerevisiae* (0–2%). The xerotolerant *Zygosaccharomyces* spp. and *Citeromyces matritensis* have a high tolerance to low a_w values (minimum 0.65−0.60) in an environment with high sugar concentrations, yet are relatively sensitive to NaCl.

In values of osmotic tolerance reported in the literature the specific test substance is generally named. For example, for a strain of *Zygosaccharomyces rouxii* the following a_w values are given: glucose/fructose ~0.71, ammonium sulfate ~0.82, xylose ~0.89, KCl ~0.87, PEG 400 (polyethylene glycol with an average molecular weight of 400) ~0.88, NaCl ~0.89, PEG 200 (polyethylene glycol with an average molecular weight of 200) ~0.95. As a rule, different values are observed depending on whether the strains were cultivated on agar plates (colony-forming capability as the criterion) or in broth (OD as the criterion); whether gas production was used as a parameter (fermentation as the criterion); and whether the ability to bud could be determined under a microscope (initiation of reproduction as the criterion). Also of diagnostic importance is what incubation temperature and time were used, as well as which factor is limiting their ability to grow (OD), ferment (amount of gas), or bud (number of budding cells). These values therefore allow differentiation between various strains of a species on the basis of differing test conditions and defined positive metabolic activities. Because of this, no general values can be given. Strains of the osmotolerant to osmophilic species *Candida etchellsii*, *Sterigmatomyces halophilus*, *Pichia triangularis*, and *Candida halonitratophila* are more salt tolerant than *Debaryomyces hansenii.* They do not grow at low NaCl concentrations (optimum is 11–13%) and they generally grow more slowly than *Debaryomyces hansenii.*

Brine

In brines for surface-ripened soft, semihard, and hard cheeses with ~12–22% NaCl, the dominant species are *Debaryomyces hansenii* and *Candida versatilis.* Also worth mentioning are the species *Trichosporon beigelii*, *Candida parapsilosis*, *C. tropicalis*, *C. polymorpha*, *C. zeylanoides*, *C. rugosa*, *C. intermedia*, *C. tenuis*, *Kluyveromyces marxianus*, *K. lactis*, *Clavispora lusitaniae*, *Issatchenkia orientalis*, *Pichia jadinii*, *Geotrichum* spp., *Yarrowia lipolytica*, *Saccharomyces* spp., and *Torulaspora delbrueckii.* Several other species are less frequently isolated. In the brine of Nabulsi cheese, a Jordanian traditional boiled white cheese usually made from sheep's milk and kept in brine with ~20% NaCl, the halotolerant species *Debaryomyces hansenii* and *Candida parapsilosis*, and the halophilic species *Pichia triangularis, P. etchellsii*, and *Sterigmatomyces halophilus* were found. The yeast counts were between 10^2 and 10^6 cfu ml^{-1}. For the determination of halophilic species, one needs an isolation medium with salt (e.g., 15% NaCl). In the brines of Feta made in Germany and Italy, the yeast species found were *Debaryomyces hansenii*, *Pichia membranifaciens*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Zygosaccharomyces bicuspidata*, *Candida magnoliae*, *C. zeylanoides*, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii.* The yeast counts were mostly between 10^5 and 10^7 cfu ml^{-1}. Two products were free of yeasts because they had been pasteurized. From the consumer's point of view, packages should be used up quickly after opening, so that yeasts do not multiply with the introduction of oxygen and develop a musty, yeasty, soapy, or rancid flavor in the product.

See also: **Cheese**: Smear-Ripened Cheeses; Starter Cultures: Specific Properties. **Fermented Milks**: Asian Fermented Milks; Kefir; Middle Eastern Fermented Milks; Types and Standards of Identity; Yoghurt: Types and Manufacture. **Heat Treatment of Milk**: Sterilization of Milk and other Products. **Liquid Milk Products**: Liquid Milk Products: Pasteurized Milk; Pasteurization of Liquid Milk Products: Principles, Public Health Aspects. **Microorganisms Associated with Milk. Yeasts and Molds**: *Geotrichum candidum*; *Kluyveromyces* spp.

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Kluyveromyces spp.

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Introduction

The genus *Kluyveromyces* is constituted by yeasts isolated from a wide range of environments such as fruit flies, trees, seawater, and dairy products. The ubiquity of the genus has resulted in a high variability in the morphological, physiological, and molecular characteristics of the yeast strains, hence making very difficult the classification of yeast strains in the different species.

In the first monograph on the genus *Kluyveromyces*, 18 species were included in the genus. Presently, a multisequence-based approach has reduced the number of species in the genus to six. Species that are most important for the dairy industry are *K. lactis* and *K. marxianus*, whose strains contribute to the ripening process of different cheeses and to the production of kefir. Several studies have exposed the exceptional heterogeneity in the physiology and genetics of these yeasts, which has led to their division into several ecological and geographical 'populations'. The most interesting population is constituted by the dairy yeast pertaining to the variety *K. lactis* var. *lactis*, characterized by the presence of the lactose regulon in their genome. The recent completion of the whole-genome sequence has made *K. lactis* one of the best-known 'nonconventional' yeasts.

Despite the taxonomical problems created by the heterogeneity of the *Kluyveromyces* yeasts, their metabolic diversity has led to numerous biotechnological applications such as production of enzymes, single-cell proteins, bioingredients from cheese whey, and metabolites with biological activity.

Recent History of the Genus *Kluyveromyces*

The last edition of *The Yeasts: A Taxonomic Study* included 15 species in the genus *Kluyveromyces*. The diagnosis of the genus and key characters for species delineation were based on several indistinct morphological traits such as cell shape, which may be ovoidal, ellipsoidal, cylindrical to elongate; absence of formation of true mycelium although pseudomycelium may be formed; sexual reproduction by ascus formation preceded or not by conjugation; formation of 1–4 (or more in several species) ascospores per ascus, with the ascospores being smooth, reniform, bacilliform, ellipsoidal, or spheroidal and tending to agglutinate after liberation.

Molecular-genetic characterization of the species in the genus *Kluyveromyces* showed a very heterogeneous portrait of the genus. Karyotype analysis revealed that the species in the genus *Kluyveromyces* can be divided into two major groups. One group includes the species *K. aestuarii*, *K. blattae*, *K. dobzhanskii*, *K. lactis*, *K. marxianus*, *K. thermotolerans*, *K. waltii*, and *K. wickerhamii*, which exhibit chromosomal patterns with less than 10 chromosomes. The second group comprises the species *K. africanus*, *K. bacillisporus*, *K. delphensis*, *K. lodderae*, *K. phaffii*, *K. polysporus*, and *K. yarrowii*, composing the so-called '*Saccharomyces*-like' group, because their karyotypes exhibited more than 12 chromosomes resembling that of the *Saccharomyces* species.

This division of the genus *Kluyveromyces* was in accordance with the position of the species in phylogenetic reconstructions of the family Saccharomycetaceae. In the phylogenetic reconstructions based on the partial sequences of the nuclear 26S rRNA and the mitochondrial COXII genes, the species displaying the *Saccharomyces*-like karyotype appeared intermixed with species of the genus *Saccharomyces*, whereas the rest of species in the genus *Kluyveromyces* constituted a clearly separated monophyletic group (see **Figure 1**).

A more detailed examination of the evolutionary tree of Saccharomycetaceae shows that the *Kluyveromyces* species are distributed into six clades, demonstrating that the morphological definition of the genus has no phylogenetic basis. Accordingly, a new genus *Kluyveromyces* based on phylogenetic relationships determined from multigene sequence analysis was proposed.

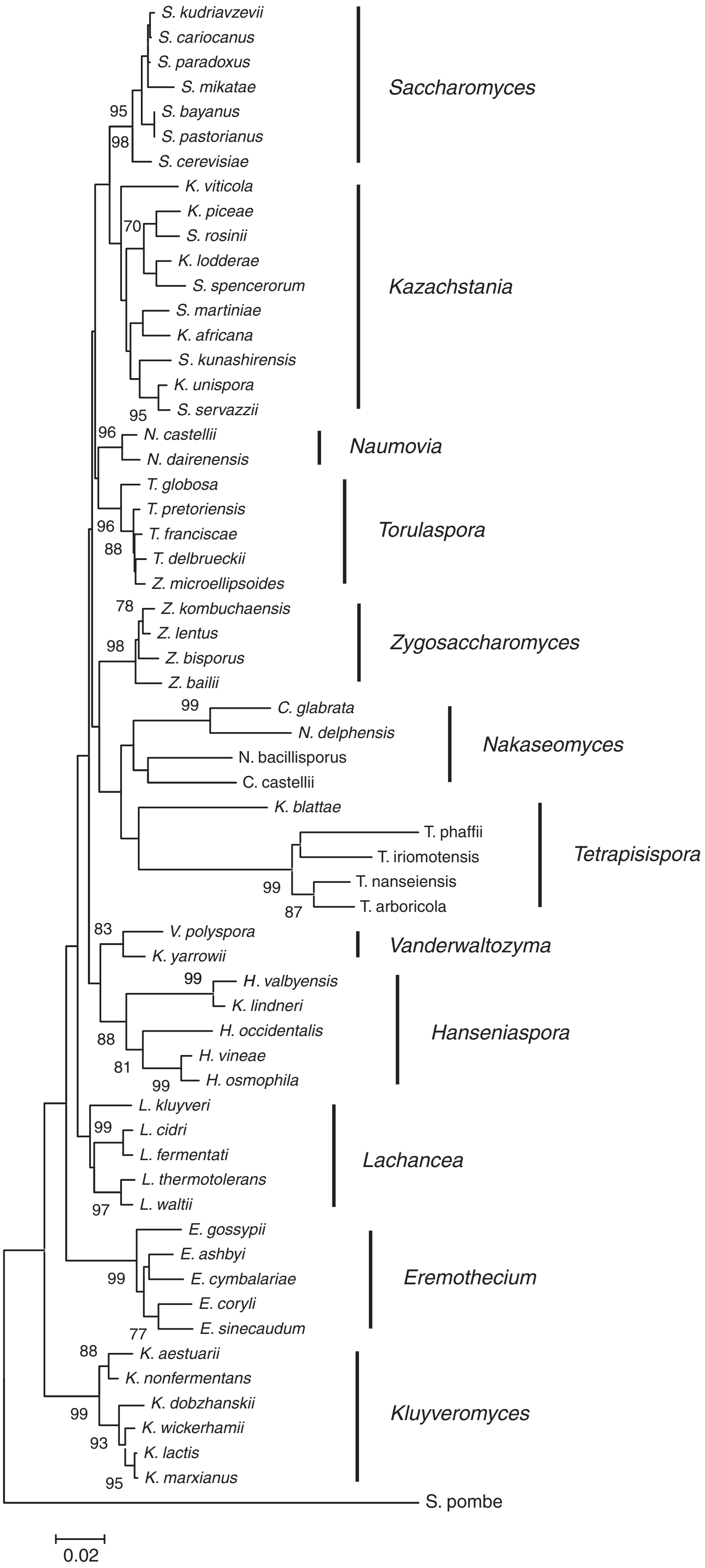
S. kudriavzevii
S. cariocanus
S. paradoxus
S. mikatae
Saccharomyces
95
S. bayanus
98
S. pastorianus
S. cerevisiae
K. viticola
K. piceae
70
S. rosinii
K. lodderae
Kazachstania
S. spencerorum
S. martiniae
K. africana
S. kunashirensis
K. unispora
S. servazzii
95
96
N. castellii
Naumovia
N. dairenensis
T. globosa
T. pretoriensis
Torulaspora
96
T. franciscae
88
T. delbrueckii
Z. microellipsoides
78
Z. kombuchaensis
Z. lentus
Zygosaccharomyces
98
Z. bisporus
Z. bailii
99
C. glabrata
N. delphensis
Nakaseomyces
N. bacillisporus
C. castellii
K. blattae
T. phaffii
Tetrapisispora
T. iriomotensis
99
T. nanseiensis
87
T. arboricola
83
V. polyspora
Vanderwaltozyma
K. yarrowii
99
H. valbyensis
K. lindneri
H. occidentalis
Hanseniaspora
88
81
H. vineae
99
H. osmophila
L. kluyveri
99
L. cidri
L. fermentati
Lachancea
L. thermotolerans
97
L. waltii
E. gossypii
E. ashbyi
99
E. cymbalariae
Eremothecium
E. coryli
77
E. sinecaudum
88
K. aestuarii
K. nonfermentans
K. dobzhanskii
99
Kluyveromyces
K. wickerhamii
93
K. lactis
95
K. marxianus
S. pombe
0.02

Figure 1 Phylogenetic tree showing the distribution of *Kluyveromyces* species in several genera of the family Saccharomycetaceae. The neighbor-joining tree is based on 26S rRNA gene sequences. Bootstrap values higher than 70% are given. *Schizosaccharomyces pombe* is the outgroup species.

Current Status of the Genus *Kluyveromyces*

The new genus *Kluyveromyces* has been reduced to six species, *K. marxianus*, *K. lactis*, *K. dobzhanskii*, *K. aestuarii*, *K. nonfermentans*, and *K. wickerhamii*. In addition, the species *K. lactis* is presently divided into the varieties *lactis* and *drosophilarum*. The remaining species in the former *Kluyveromyces* genus have been assigned to the new or revised genera *Kazachstania*, *Nakaseomyces*, *Tetrapisispora*, *Vanderwaltozyma*, and *Lachancea* (see **Figure 1**).

The new description of the genus *Kluyveromyces* is consistent with previous studies regarding ascospore shape and number and the sexual compatibility to form stable hybrids, as well as with genetic studies such as DNA base composition, DNA–DNA relatedness, and isoenzyme analysis. Furthermore, the six species in the genus *Kluyveromyces* display similar morphological and physiological characteristics. Sexual reproduction occurs by ascus formation and the asci contain only 1–4 ascospores. Physiological traits common to all species are galactose assimilation and utilization of cadaverine, L-lysine, and ethylamine as nitrogen sources. Physiological key characters for differentiation of the species are presented in **Table 1**.

In contrast, the monophyletic nature of the new genus *Kluyveromyces* does not conceal the significant genetic heterogeneity within the species when large numbers of strains are compared. Characterization of *K. dobzhanskii*, *K. lactis*, and *K. marxianus* yeasts by means of restriction analysis of their mitochondrial DNA (mtDNA) showed a high level of intraspecific pattern heterogeneity (see **Figure 2**). Restriction

Table 1 Physiological key characters for identification of species in the genus *Kluyveromyces*

	Assimilation						
Species	*αGL*	*Tre*	*Lac*	*In*	*Ce*	*Xy*	*Suc*
K. aestuarii	−	−	+	−	+	−	+
K. dobzhanskii	+	+	−	−	+	−	+
K. lactis var. *lactis*	v	+	+	v	v	v	+
K. lactis var. *drosophilarum*	v	v	−v	v	v	+	
K. marxianus	−	−	v	+	v	+	+
K. nonfermentans	−	−	+	−	v	−	−
K. wickerhamii	−	−	+	−	+	+	v

Abbreviations: αGL, α-methyl-D-glucoside; Tre, trehalose; Lac, lactose; In, inulin; Ce, cellobiose; Xy, D-xylose; Suc, succinate.

Data compiled from Lachance MA (1998) *Kluyveromyces* van der Walt emend. van der Walt. In: Kurtzman CP and Fell JW (eds.) *The Yeasts, a Taxonomic Study*, 4th edn. New York: Elsevier; Belloch C, Fernandez-Espinar T, Querol A, Garcia MD, and Querol A (2002) An analysis of inter- and intraspecific genetic variabilities in the *Kluyveromyces marxianus* group of yeast species for the consideration of the *K. lactis* taxon. *Yeast* 19: 257–268; Nagahama T, Hamamoto M, Nakase T, and Horikoshi K (1999) *Kluyveromyces nonfermentans* sp. nov., a new yeast species isolated from the deep see. *International Journal of Systematic Bacteriology* 49: 1899–1905.

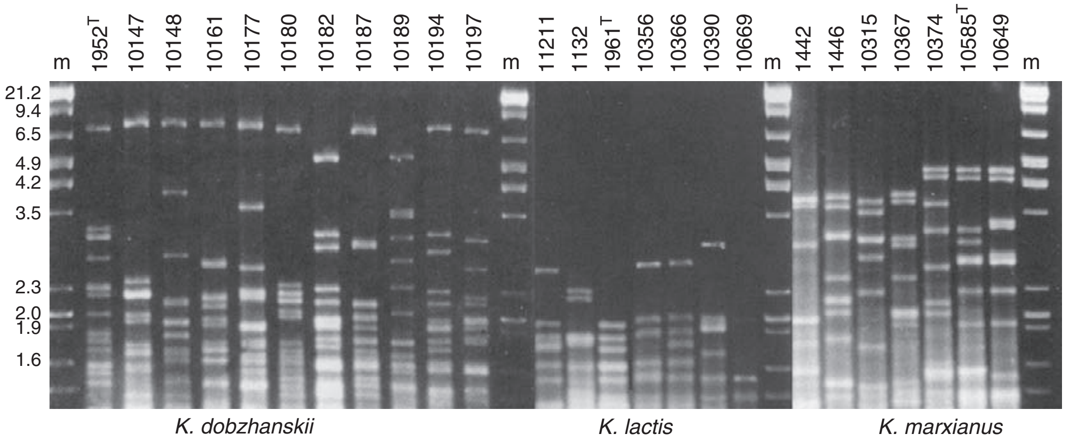

Figure 2 Mitochondrial restriction analysis of *Kluyveromyces dobzhanskii*, *K. lactis*, and *K. marxianus* with the endonuclease *Hin*fI. Lanes m correspond to molecular size markers. Strain numbers are CECT (Spanish Type Culture Collection) numbers. From Belloch C, Barrio E, Uruburu F, Garcia MD, and Querol A (1997) Characterisation of four species of the genus *Kluyveromyces* by mitochondrial DNA restriction analysis. *Systematic and Applied Microbiology* 20: 397–408.

analysis of the mtDNA of 46 strains revealed 34 unique restriction patterns and 8 common patterns shared by the remaining 12 strains. mtDNA restriction using the enzyme *Hin*fI revealed the highest genetic variability among strains in all species. Most of the total genetic diversity was attributable to variation among subgroups of strains within *K. lactis* and *K. marxianus*, indicating restriction of gene flow due to ecological or biogeographical barriers, asexual reproduction, or self-fertilization.

Intraspecific analysis of chromosomal patterns by pulsed-field gel electrophoresis (PFGE) showed a rich polymorphism in the species *K. dobzhanskii*, *K. lactis*, and *K. marxianus* (see **Figure 3**). The most common karyotype in the species *K. lactis* is constituted by six chromosomes distributed in five bands (the second band is a doublet), which is present in all strains in the variety *K. lactis* var. *lactis*. The homogeneity in the chromosomal patterns in this variety is in accordance with the heterothallism of the strains. On the contrary, the homothallic strains in the

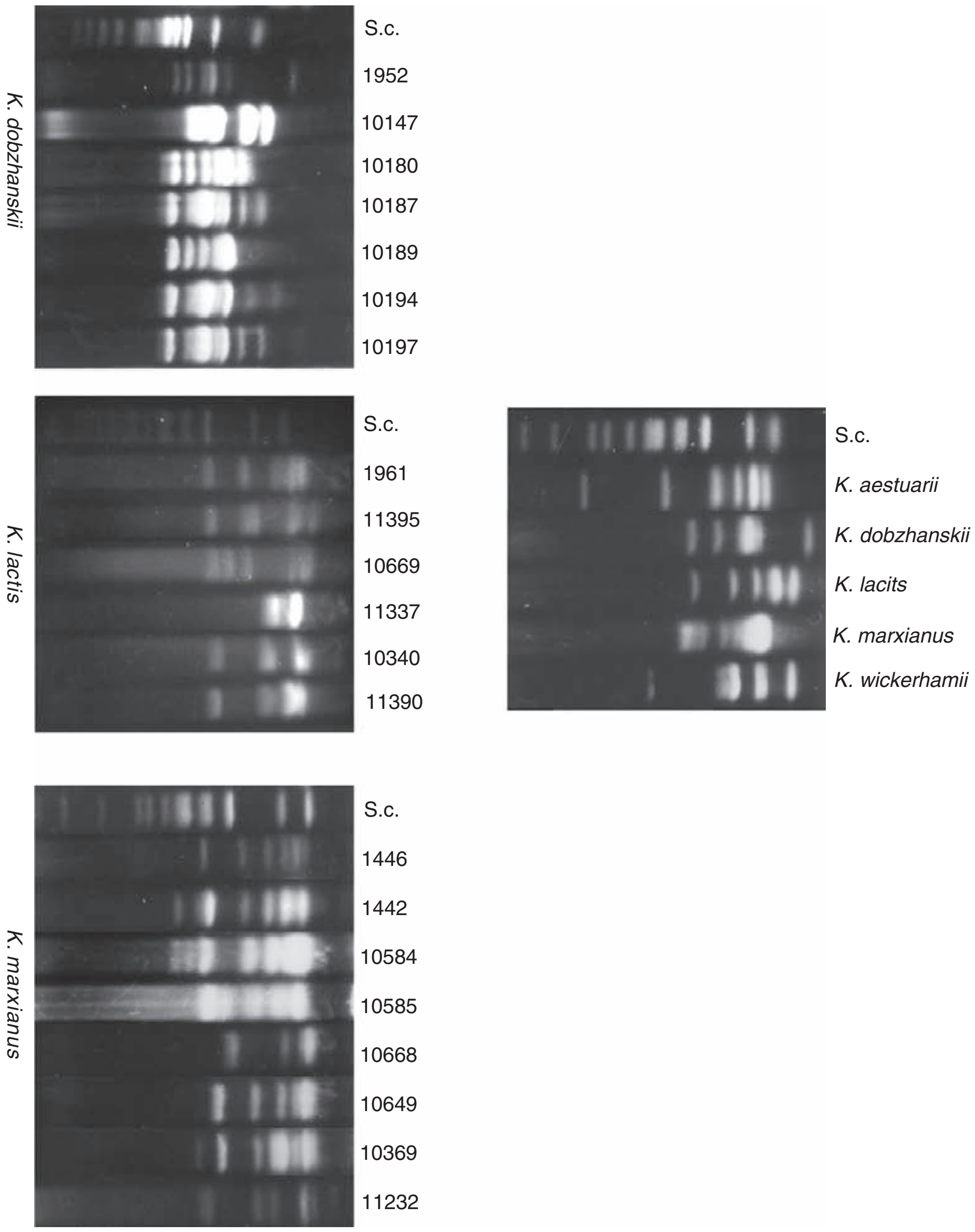
K. dobzhanskii
S.c.
1952
10147
10180
10187
10189
10194
10197
K. lactis
S.c.
1961
11395
10669
11337
10340
11390
S.c.
K. aestuarii
K. dobzhanskii
K. lacits
K. marxianus
K. wickerhamii
K. marxianus
S.c.
1446
1442
10584
10585
10668
10649
10369
11232

Figure 3 Chromosomal profiles of strains in the new genus *Kluyveromyces* after pulsed-field gel electrophoresis (Contour-clamped homogeneous electrical field) under the following conditions: 600–1200 s, 75 V for 48 h; 120–400 s, 80 V for 48 h; and 60–120 s, 100 V for 18 h at 14 °C in 0.8% chromosomal grade agarose gel. Lanes S.c. correspond to the karyotype of *Saccharomyces cerevisiae*. Data extracted from Belloch *et al*. (1998a; unpublished results).

variety *K. lactis* var. *drosophilarum* display very different chromosomal patterns. The electrophoretic karyotype of the strains in the species *K. marxianus* also shows a common set of eight chromosomes. This species-specific pattern can be recognized in almost all strains. Variations from the basic species-specific pattern include the presence of one or two extra bands with different mobility in some strains. The species showing the most different karyotypes was *K. dobzhanskii.* The strains analyzed exhibited such different karyotypes that a common chromosomal pattern could not be found. Five different karyotypes could be distinguished, some of them shared by more than one strain.

The intra- and interspecific genetic variability among the species in the new genus *Kluyveromyces* examined by sequence analysis of the 5.8S rRNA gene and comparisons of the two internal transcribed spacers (5.8S-ITS rRNA) of 59 strains revealed a complex sequence heterogeneity. **Figure 4** shows the maximum parsimony (MP) tree, which minimizes the number of nucleotide substitutions required to connect the different 5.8S-ITS sequences from the strains in the genus *Kluyveromyces.* The highest

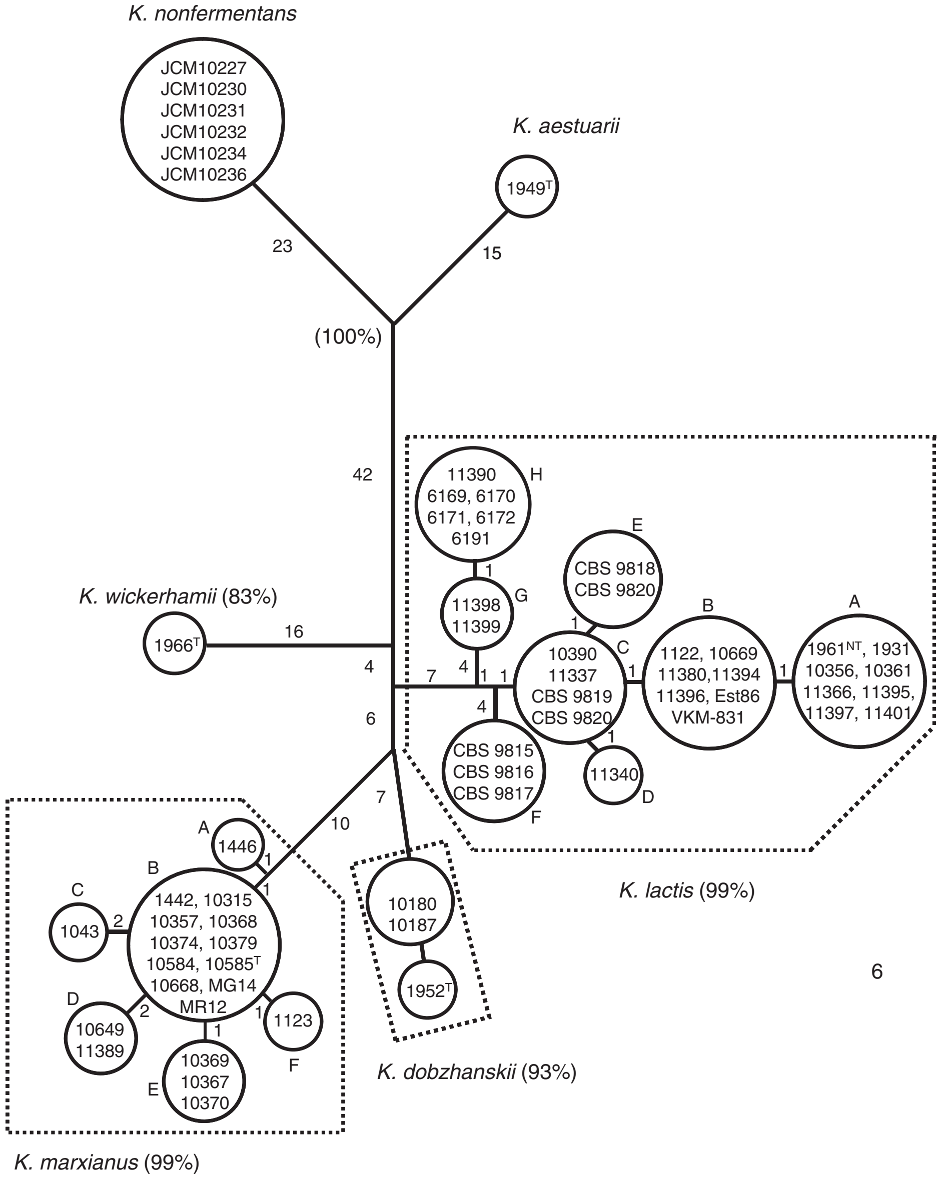
K. nonfermentans
JCM10227
JCM10230
JCM10231
JCM10232
JCM10234
JCM10236
K. aestuarii
1949T
23
15
(100%)
42
K. wickerhamii (83%)
1966T
16
4
6
7
10
11390
6169, 6170
6171, 6172
6191
H
1
11398
11399
G
E
CBS 9818
CBS 9820
1
4
1 1
10390
11337
CBS 9819
CBS 9820
C
1
B
1122, 10669
11380,11394
11396, Est86
VKM-831
1
A
1961NT, 1931
10356, 10361
11366, 11395,
11397, 11401
4
CBS 9815
CBS 9816
CBS 9817
F
1
11340
D
K. lactis (99%)
A
1446
1
B
1
C
1043
2
1442, 10315
10357, 10368
10374, 10379
10584, 10585T
10668, MG14
MR12
D
10649
11389
2
1
10369
10367
10370
E
1
1123
F
K. marxianus (99%)
10180
10187
1952T
K. dobzhanskii (93%)
6

Figure 4 Maximum parsimony tree that minimizes the number of nucleotide substitutions (indicated on the branches) required to connect the different 5.8S-ITS sequences of the strains within the circles. The alignment of the 5.8S-ITS sequences showed 120 variable positions out of 642, out of which 78 were phylogenetically informative. Numbers given in parentheses are the percentage of bootstrap values. CBS, Centraalbureau voor Schimmelcultures; JCM, Japanese Culture Collection; VKM, All-Russian Collection of Microorganisms. Strain numbers without collection acronym are CECT (Spanish Type Culture Collection) numbers.

variability in the sequence of the 5.8S-ITS rDNA region was found within the species *K. lactis* and *K. marxianus*, both represented by a large number of strains. The species *K. aestuarii* and *K. nonfermentans* isolated from marine environments in the United States and Japan appear as sister taxa. The first species to diverge is the type strain of *K. wickerhamii* isolated from *Drosophila montana* in the United States. The remaining three species *K. lactis*, *K. dobzhanskii*, and *K. marxianus* appear more closely related than the others although they are clearly separated. The next species to diverge is *K. lactis*. The most heterogeneous strains, constituting the variety *drosophilarum*, appear separated into seven different groups. These strains have been isolated from natural environments such as water, insects, and trees or fruits in Europe, Far East, Africa, and North America. The last group of strains appearing at the end of the branch constitute the variety *lactis*; most of the strains in this group have been isolated from dairy products. Finally, *K. dobzhanskii* and *K. marxianus* are the last species to diverge. The strains in the species *K. dobzhanskii* have been isolated exclusively from insects in Europe and the United States. The species *K. marxianus* is constituted by strains isolated from very different environments such as plants, beer, wine, clinical sources, and dairy products in Europe, Africa, and the United States. Several groups of strains can also be recognized in the species *K. marxianus*, but none of them is constituted exclusively by dairy strains.

Sequence comparisons of the 5.8S-ITS rDNA established the existence of nucleotide substitutions involving restriction site gains or losses in the sequences of strains belonging to the different species. This was found very useful to formulate strategy for discrimination among the species in the genus *Kluyveromyces*.

Key to Species in the Genus *Kluyveromyces*

PCR amplification of the 5.8S-ITS rDNA and restriction with specific endonucleases produces different band patterns useful for discrimination of the species in the genus *Kluyveromyces* (see **Table 2**). The species *K. aestuarii*, *K. nonfermentans*, *K. marxianus*, *K. dobzhanskii*, and *K. lactis* var. *lactis* produce species-specific patterns after digestion with *Hin*fI.

The strains within the variety *K. lactis* var. *drosophilarum* yield three different patterns. Pattern I is identical to the pattern of *K. lactis* var. *lactis*. *In silico* restriction of the 5.8S-ITS sequences generates no discriminative restriction bands. However, strains displaying this pattern can be distinguished easily by their lactose utilization, which is positive in the variety *lactis* and negative in the variety *drosophilarum*. Pattern II is identical to the pattern of *K. wickerhamii*; however, they can be distinguished by their different restriction bands with the enzyme *Alu*I. Pattern III is different from the other two patterns in the variety *drosophilarum* and it is also different from the rest of restriction patterns displayed by the species of the genus.

Taxonomy of the Dairy Yeast Species *Kluyveromyces lactis* and *Kluyveromyces marxianus*

The strains in the genus *Kluyveromyces* inhabit very different environments both natural and man-made; however, strains isolated from dairy products belong only to the species *K. marxianus* and *K. lactis*.

The ability to use lactose as the sole carbon source as well as the dairy origin of strains within

Table 2 Key for species differentiation in the genus *Kluyveromyces*

		Fragment size	
Species	*PCR product*	*Hin*fI	*Alu*I
K. aestuarii	725	350 + 170 + 160 + 40	580 + 150
K. dobzhanskii	725	240 + 180 + 115 + 100 + 80	395 + 180 + 130
K. lactis var. *lactis*	725	285 + 180 + 115 + 100 + 60	395 + 180 + 130
K. lactis var. *drosophilarum* I	725	285 + 180 + 115 + 100 + 80	395 + 180 + 130
II	725	240 + 180 + 115 + 80 + 60 + 50	395 + 180 + 150
III	725	240 + 190 + 180 + 60 + 50	395 + 180 + 130
K. marxianus	725	240 + 180 + 115 + 60 + 60 + 50	395 + 180 + 150
K. nonfermentans	725	350 + 170 + 115 + 50 + 40	500 + 150 + 70
K. wickerhamii	725	240 + 180 + 115 + 80 + 60 + 50	520 + 130 + 70

Differences in the size of the restriction fragments obtained with the enzymes *Hin*fI and *Alu*I of the 5.8S-ITS PCR product are used for differentiation among the strains in the different species.

Data compiled from Belloch C, Barrio E, Garcia MD, and Querol A (1998b) Phylogenetic reconstruction of the genus *Kluyveromyces*: Restriction map analysis of the 5.8S rRNA gene and the two ribosomal transcribed spacers. *Systematic and Applied Microbiology* 21: 266–273; Belloch *et al*., unpublished results.

the species *K. lactis* was the basis for the division of this species into the varieties *K. lactis* var. *lactis* and *K. lactis* var. *drosophilarum.* The strains in the variety *lactis* are homogeneous in their karyotypes and 5.8S-ITS sequences although they display variability in their sugar assimilation patterns (see **Table 3**). The strains in the variety *drosophilarum* are very heterogeneous and combinations of karyotypes, 5.8S-ITS sequences, and assimilation patterns produce no clear groups of strains. However, a specific population of *K. lactis* var. *drosophilarum* strains isolated from European natural habitats showed the same karyotypes and 5.8S-ITS sequences as the strains in the variety *K. lactis* var. *lactis.* This specific population of strains was named 'krassilnikovii'. Other populations of strains within the variety *drosophilarum* separated on the basis of 5.8S-ITS sequencing were named 'aquatic', 'oriental', 'drosophilarum', 'phaseolosporus', 'pseudovanudenii', 'vanudenii', and 'new' (see **Table 3**).

Kluyveromyces marxianus is the only inulin-assimilating *Kluyveromyces* species that does not assimilate or ferment α-glucosides and grows well at 37 °C. Molecular-genetic studies indicate that the strains in this species are very heterogeneous in their karyotypes and 5.8S-ITS sequence types. The *K. marxianus* species was formerly represented by five populations denominated 'fragilis', 'bulgaricus', 'cicerisporus', 'wikenii', and 'marxianus' on the basis of morphological and physiological characters (see **Table 4**). However, only the group constituted by the population 'bulgaricus' isolated from yogurt (type E in **Figure 4**) shares similar karyotype profile and 5.8S-ITS sequence.

Recent Advances in Genomic Studies of *Kluyveromyces lactis* and *Kluyveromyces marxianus*

The complete genome sequencing of a *K. lactis* strain was achieved by the 'Genolevures 2' consortium in 2004. The genome of the *K. lactis*-type strain has a size of 10.6 Mb distributed into six chromosomes. Comparison of the *K. lactis* genome with other yeast genomes revealed that *K. lactis* has a very compact genome (71.6 gene density), with less redundancies than other species, as well as 38 unique open-reading frames (ORFs). Comparison of the *K. lactis* and *Saccharomyces cerevisiae* genomes revealed that 78%

Table 3 Summary of assimilation tests, electrophoretic karyotyping, and 5.8S-ITS sequence type displayed by strains in the species *Kluyveromyces lactis*

	Assimilation							
Strain numbers	*Lac*	*Mel*	*Cell*	*Sal*	*Mal*	*αGL*	*ITS type*[a]	*Population of* K. lactis
CECT 1121	+	−	−	−	+	−	A	lactis
CECT 1961	+	+	+	+	+	+	A	lactis
CECT 10356	+	+	+	+	+	+	A	lactis
CECT 10361	+	−	+−	+	−	A	lactis	
CECT 10364	+	+	+	−	+	−	A	lactis
CECT 10366	+	+	+	−	+	+	A	lactis
CECT 11401	+	−	−	−	−	−	A	lactis
CECT 1122	−	−	−	−	+	−	B	krassilnikovii
CECT 11380	−	+	+	+	+	+	B	krassilnikovii
CECT 11394	−	+	+	+	+	+	B	krassilnikovii
CECT 11395	−	−	+	+	−	+	B	krassilnikovii
CECT 11397	−	+	+	+	+	+	B	krassilnikovii
CECT 10669	−	+	+	+	+	+	B	vanudenii
CECT 10390	−	−	−	−	−	−	C	drosophilarum
CECT 11337	−	+	+	+	+	+	C	drosophilarum
CBS 9821	−	nd	nd	nd	−	nd	C	New
CECT 11340	−	−	−	−	−	−	D	phaseolosporus
CBS 9818	−	nd	nd	nd	−	nd	E	pseudovanudenii
CBS 9815	−	nd	nd	nd	−	nd	F	Oriental
CECT 11398	−	+	−	−	+	+	G	aquaticus
CECT 11390	−	+	+	+	+	+	H	aquaticus

[a] Correspond to the 5.8S-ITS sequence types in Figure 4.

Abbreviations: Cell, cellobiose; Lac, lactose; Mal, maltose; Mel, melibiose; nd, not determined; Sal, salicin; αGL, α-methyl-D-glucoside.

Data compiled from Belloch C, Fernandez-Espinar T, Querol A, Garcia MD, and Querol A (2002) An analysis of inter- and intraspecific genetic variabilities in the *Kluyveromyces marxianus* group of yeast species for the consideration of the *K. lactis* taxon. *Yeast* 19: 257–268; CBS, www.cbs.knaw.nl.

Table 4 Summary of assimilation tests and 5.8S-ITS sequence type displayed by strains in the species *Kluyveromyces marxianus*

	Assimilation							
Strain numbers	*Lac*	*Mel*	*Cell*	*Suc*	*Mal*	*Tre*	*ITS type*[a]	*Population of* K. marxianus
CECT 1446	+	−	−	−	nd	−	A	marxianus
CECT 10585	+	−	+	+	−	−	B	marxianus
CECT 1043	+	−	+	+	−	−	C	marxianus
CECT 10584	+	−	+	+	−	−	B	fragilis
CECT 10668	−	−	+	+	−	−	B	cicerisporus
CECT 10649	−	−	−	+	−	−	D	wikenii
CECT 10369	+	−	−	+	−	v	E	bulgaricus

[a] Correspond to the 5.8S-ITS sequence types in Figure 4.

Abbreviations: Lac, lactose; Mel, melibiose; Cell, cellobiose; Suc, sucrose; Mal, maltose; Tre, trehalose.

Belloch *et al*., unpublished data.

of the genes belonging to clusters of syntenic genes correspond to intermingled series in which one region of the *K. lactis* genome corresponds to two (and sometimes more) distinct chromosomal regions in *S. cerevisiae*. Compared with the 56 duplicated blocks scattered throughout the genome map in *S. cerevisiae* (plus 21 blocks in subtelomeric regions), only 8 blocks (1 tandem) are duplicated in *K. lactis*. However, none of the eight duplicated blocks in *K. lactis* coincide with the duplicated blocks in *S. cerevisiae*, indicating a distinct formation, unrelated to the major duplication event that occurred in an ancestor of *S. cerevisiae*. Apparently, the eight duplicated blocks observed in *K. lactis* may have originated from independent segmental duplications because they are too few to indicate massive genome duplications. In addition to the duplication events, the genome of *K. lactis* lacks five genes, including the genes for sterol uptake, which may explain the lack of anaerobic growth by this yeast.

Presently, only a small part (approximately 17%) of genome sequence of *K. marxianus* is available. The type strain *K. marxianus* var. *marxianus* contains 10 chromosomes with an estimated genome size of 14 Mb. Comparison of *K. marxianus* and *S. cerevisiae* genomes revealed that several genes that are adjacent in *K. marxianus* have homologues that are distributed in two different chromosomal regions in *S. cerevisiae*. Such series of genes may reflect the chromosomal map of the common ancestor of *S. cerevisiae* and *K. marxianus*.

According to the ‘Genolevures’ sequencing data, an expression microarray for the yeast *K. lactis* consisting of 482 genes involved in central metabolism facilitated transport, and stress response was developed. Using this partial-genome microarray a comparison of the expression patterns of two dairy strains was achieved. The study revealed unexpected differences in expression of genes involved in the respiratory and fermentative metabolic pathways of different carbon sources. Presently, a commercial microarray containing ORFs of the whole *K. lactis* genome has been made available.

Lactose Metabolism, a Salient Characteristic of *Kluyveromyces* spp.

Yeast species that assimilate lactose aerobically are widespread, but those that ferment lactose are very rare. The species *K. lactis* and *K. marxianus* include strains that are lactose-fermenting yeasts. Lactose utilization in *K. lactis* is an inducible system triggered by lactose or galactose. The lactose regulon in *K. lactis* consists of *LAC4* (encoding β-galactosidase) and *LAC12* (encoding lactose permease) genes. Both genes are contiguously placed in the genome of *K. lactis* sharing a divergent promoter that might have been cotransferred from external origin.

The presence of lactose regulon in *K. lactis* var. *lactis* was decisive for its survival in milk products and, therefore, to explain the adaptation of this yeast to man-manipulated environments. The lactose-negative strains within *K. lactis* var. *drosophilarum* have also been investigated for the presence of lactose regulon genes. Complementation of strains with functional *LAC4* and *LAC12* genes produced lactose-negative strains; therefore, the locus containing nonfunctional genes is absent in natural *K. lactis* var. *drosophilarum* strains. In the case of *K. marxianus*, most of nondairy strains are able to assimilate lactose. Similar studies complementing lactose-negative *K. marxianus* with functional *LAC12* genes yielded lactose-positive strains, indicating that these strains are mutant for the permease gene. Furthermore, comparison of *LAC4* gene sequences of *K. marxianus*- and *K. lactis* var. *lactis*-type strains revealed a very high sequence similarity. Additionally, these facts indicate that *LAC4* gene might have originated from *K. marxianus* and then transferred to *K. lactis* var. *lactis*, while both concur in dairy products.

Role of *Kluyveromyces* spp. in Dairy Products

Kluyveromyces marxianus and *K. lactis* (*Kluyveromyces* spp.) are the only lactose-fermenting species regularly found in milk and dairy products. Their main role in dairy products is lactose metabolism, but they also possess weak proteolytic and lipolytic activities. The ability of *Kluyveromyces* spp. to metabolize milk constituents (lactose, proteins, and fat) makes them very important in cheese ripening and fermented milk products such as kefir, as they contribute to maturation and aroma formation. However, despite the importance of yeasts in dairy products, commercial yeast starters are not commonly used and the yeast flora developing in cheeses and other dairy products appears as a result of spontaneous contamination. The occurrence of yeasts in cheese is not unusual as they tolerate low pH, low water activity (a_w) (moisture), elevated salt concentration, and low storage temperatures. Investigation of yeast microbiota of cheese brines, dairy utensils, raw milk, and smear water revealed the presence, among other yeasts, of *Kluyveromyces* spp.

Kluyveromyces marxianus can grow in milk at 25 °C, reaching populations of 10^8 cfu ml^{-1} in 3 days. Salt concentration affects growth rate; at 15% salt concentration, the maximum growth reached was 10^5 cfu ml^{-1}. Temperature affects lactose fermentation rate; lactose utilization at 10 °C is 70% of the total utilization at 25 °C. Utilization of lactose produces galactose, glucose, ethanol, and glycerol. Proteolytic activity of *K. marxianus* in milk is of medium importance when compared with other proteolytic yeasts such as *Debaryomyces hansenii* and *Yarrowia lipolytica*. Concentration of total free amino acids in milk increased very little after 12 incubation days and the main amino acids present were leucine, valine, alanine, isoleucine, and phenylalanine. Similarly, *K. marxianus* excretes intermediate levels of lipases in milk. Increase in free fatty acids in milk was significant after 12 days growth at 25 °C. However, the different free amino acids and fatty acids produced by the growth of *K. marxianus* in milk indicate that the proteases and lipases are different from those produced by other dairy yeasts.

The lactose-fermenting *Kluyveromyces* spp. yeasts produce ethanol but also aromatic esters of fatty acids and acetaldehyde. In experimental hard cheeses, the presence of *Kluyveromyces* spp. increased the production of ethanol, isoamyl alcohol, and ethyl acetate. Sensory analysis of the slurries after incubation at 25 °C for 7 days associated the growth of *K. marxianus* with acidic, cidery, alcoholic, fermented, and fruity flavors as a result of volatile fermentation products such as formic or acetic acid.

Kluyveromyces spp. are part of the microbiota of the surface and interior of the cheese, and they play an important role in the ripening process of several cheese varieties. The growth of *Kluyveromyces* spp. on the surface of blue-veined cheese contributes to the open structure of the cheese. Production of CO_2 due to lactose fermentation generates small holes in the cheese curd, helping the growth of aerobic *Penicillium* during the ripening process. After the first days of cheese maturation, *Kluyveromyces* yeasts disappear from the surface but appear in high counts in the cheese interior. The lactose-fermenting ability of *Kluyveromyces* spp. promotes their growth in the interior of the cheeses, where other dominant yeasts such as *D. hansenii* and *Y. lipolytica* are scarce.

Kluyveromyces strains are constituents of the yeast microbiota in traditional kefir. The kefir grain is produced by the synergic growth of yeasts such as *Kluyveromyces* spp. and *Saccharomyces* spp. and several lactic acid bacteria. Ethanol and CO_2 production due to lactose fermentation by *Kluyveromyces* spp. gives kefir its particular alcoholic aroma.

In recent years, several microorganisms have been selected by starter-producing companies for their functionalities in the dairy industry. These microorganisms are mainly lactic acid bacteria, molds, and yeasts. Presently, specific dairy cultures with application for cheese ripening and kefir production containing strains of *Kluyveromyces* spp. are commercially available.

The Potential Biotechnological Applications of *Kluyveromyces* Yeasts

The broad spectrum of metabolic activities displayed by several strains of *Kluyveromyces* has led to the investigation of different biotechnological applications of these yeasts. In earlier times, the dairy *Kluyveromyces* yeasts were mainly investigated for production of β-galactosidase. Lactase (β-galactosidase from *K. lactis*) was produced commercially by several enzyme-producing companies for hydrolyzing lactose into the sweeter-tasting monosaccharides (glucose and galactose) in the manufacture of ice cream, fermented milk, and milk drinks. Presence of lactose in milk makes milk not suitable for the majority of world's adult population due to lactose intolerance. Problems of lactose intolerance are especially severe in African and Asian populations; therefore, low-lactose milks are very important in food-aid programs. However, lactase usage has not reached its full potential at present because lactase production is expensive and the enzyme can be effective only at low temperatures. Recent studies have reported the easy and cost-effective production of β-galactosidase by *K. marxianus* growing on cheese whey; also the growth of *K. marxianus* on cheese whey is effective in reducing whey disposal problems.

Other enzymes of industrial interest produced by *K. marxianus* are β-glucosidases for hydrolysis of cellulose-containing materials, inulinase for production of fructose syrups from inulin-containing feed stocks, and polygalacturonases for reduction of viscosity in fruit processing products.

One of the major achievements of the biotechnological industry in the 1990s was the use of *K. lactis* as expression host for production of the milk clotting enzyme bovine chymosin. This protein was the first heterologous enzyme originating from a higher eukaryote that was produced in a microorganism. Presently, more than 40 proteins have been expressed and produced using *K. lactis*. Some of the recombinant proteins secreted by *K. lactis* are α-amylase, cellulase, endoxylanase, glucoamylase, insulin precursor, interferon, interleukin beta, invertase, lipase, and xylanase.

In recent years, *Kluyveromyces* spp. yeasts have been investigated for their production of peptides derived from whey fermentation with potential bioactive sites. Milk whey contains water-soluble milk proteins such as β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulins IgG, IgA, and IgM. Proteolysis of whey proteins by *K. marxianus* can result in bioactive peptides with potential applications in food products; short peptides produced during proteolysis of whey proteins are among the most potent pharmacologically active agents. As an example, peptides derived from whey protein proteolysis have been reported to show angiotensin-I-converting enzyme (ACE) inhibitory activity. Furthermore, extensive research has been done on the antitumor activity of kefir and kefir grains due to the ability of specific cultures isolated from kefir to bind to mutagenic substances such as imidazole and indole.

Another recent application of *Kluyveromyces* is the production of oligosaccharides used as components of functional foods or nutraceuticals. These food bioingredients inhibit the growth of pathogenic Gram-negative bacteria and stimulate the growth of *Bifidobacterium* sp. in the human and animal intestines. Specifically, production of fructooligosaccharides (FOSs) and galactooligosaccharides (GOSs) by *Kluyveromyces* is presently investigated. Several studies have shown that in the presence of high concentrations of lactose and galactose, the β-galactosidase enzyme of the dairy yeasts *K. lactis* and *K. marxianus* shows transferase activity and produces galactosyl-oligosaccharides. Similarly, the inulinase activity shown by *K. marxianus* can be used for production of FOSs.

In the next future, products containing bioingredients may play an important role in improving human health and well-being; therefore, current research on the production of metabolites from *Kluyveromyces* spp. has a promising prospect in the future.

See also: **Cheese**: Microbiology of Cheese. **Enzymes Exogenous to Milk in Dairy Technology**: β-D-Galactosidase; **Fermented Milks**: Kefir; Koumiss. **Lactose and Oligosaccharides:** Lactose: Galacto-Oligosaccharides. **Yeasts and Molds**: Yeasts in Milk and Dairy Products.

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Geotrichum candidum

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Introduction

Geotrichum candidum is an acid-tolerant, yeast-like fungus, often characterized as an intermediate between molds and yeasts but classified as a yeast for more than 25 years. It is the best-known species of the genus and very common, occurring on moist substrates rich in nutrients. Apart from milk and milk products, the habitat of this worldwide-distributed species encompasses soil, water, air, maize and other cereals, rice grain, grapes, citrus fruits, bananas, tomatoes, cucumber, frozen fruitcake, fruit juices, bread, animals, and humans. *Geotrichum candidum* is used in dairies as a culture for cheesemaking and in some traditional fermented milks, especially those from northern Europe, but is also frequently recorded as a spoilage organism, called 'machinery mold' because the undesired contamination is mainly caused by deficiencies in equipment hygiene. Furthermore, this species is weakly pathogenic for plants, animals, and also humans. The main human disorders caused by *G. candidum* are bronchial or pulmonary infections known as geotrichosis (*see* **Cheese**: Secondary Cultures. **Yeasts and Molds**: Yeasts in Milk and Dairy Products).

The taxonomic position of *G. candidum* was revised in 2004 by de Hoog and Smith. The current taxonomy for the teleomorphic state (sexual form) is as follows:

Ascomycota (phylum), Hemiascomycetes (class), Saccharomycetales (order), Dipodascaceae (family), *Galactomyces* (genus). For the anamorphic state (asexual form) the taxonomy is Candidaceae (family) and *Geotrichum* (genus). The genus *Geotrichum* is composed of 22 species (including 10 sp. nov.). The ecology of the species of the genus *Geotrichum* shows a rather unexpected degree of consistency given the large phylogenetic distances between the species.

For several years, the taxonomic position of *G. candidum* was unclear. DNA–DNA reassociation experiments with *Galactomyces geotrichum* and its anamorph *G. candidum* showed that representative strains were not homogenous. Four groups were found, and all the studied strains originating from dairy products were found to be in the same group, including the type culture of *Geotrichum javanense*, which is an isolate from yogurt. On the basis of its rDNA sequences, nDNA–DNA reassociation data, mol% G+C of nDNA, and physiological characters, de Hoog and Smith, in 2004, split up this complex into four species (**Table 1**).

A standardized protocol developed through a series of intra- and interlaboratory trials was proposed by Gente *et al.* in 2006 to identify *G. candidum* at the species and strain level. In contrast to the teleomorphic species, the anamorphic species (*G. candidum*) is commonly found in food products and is an important organism in food technology. It is therefore proposed to conserve the name *G. candidum* throughout this article.

Morphology and Physiology

Two main morphotypes have been described, with differences depending only partly on cultural conditions. The yeast morphotype is characterized by smooth, yeast-like, and cream-colored colonies that produce abundant asexual spores named arthrospores. The mold morphotype corresponds to hairy, felting, and white-colored colonies with a predominance of vegetative hyphae.

The hyphae are dichotomously branched (forked) and 7–11 μm across. Arthrospores form by the breaking up of fertile hyphae (**Figure 1**) and not by budding as in most of the yeasts. They are cylindrical, barrel-shaped, or ellipsoidal, and are mostly 6–12 × 3–6 μm in size (**Figure 2**). Formation of blastospores does not occur.

Geotrichum candidum grows very rapidly on media that are commonly used. On malt extract agar at 25 °C, it forms colonies with a diameter of 7 cm within 7 days. The species grows at temperatures ranging from 5 to 35 °C (variable response at 37 °C). Among the four species previously mentioned, *G. candidum* is the only one able to grow at 35 °C. The temperature optimum lies at 25 °C. Furthermore, a broad range of pH values is tolerated; values between 5.0 and 5.5 are optimal. For the dairy industry, the organism's low salt tolerance is worth mentioning. Contrary to the case of the majority of other dairy yeasts, the growth of *G. candidum* is limited by salting. Concentrations of 1% NaCl lead to a slight suppression of

Table 1 Teleomorph and anamorph species of the *Galactomyces*/*Geotrichum* complex

Teleomorph	*Anamorph*
Galactomyces candidus sp. nov.[a]	*Geotrichum candidum*[b]
Galactomyces geotrichum	Unnamed *Geotrichum* species
Galactomyces pseudocandidus sp. nov.	*Geotrichum pseudocandidum*
Unknown	*Geotrichum europaeum* sp. nov.

Types:
[a]CBS 178.71 exholotype strain of teleomorph.
[b]CBS 615.84 neotype of anamorph.
From de Hoog GS and Smith MTh (2004) Ribosomal gene phylogeny and species delimitation in *Geotrichum* and its teleomorphs. *Studies in Mycology* 50: 489–515.

growth. NaCl concentrations of 5–6% have an inhibitory effect.

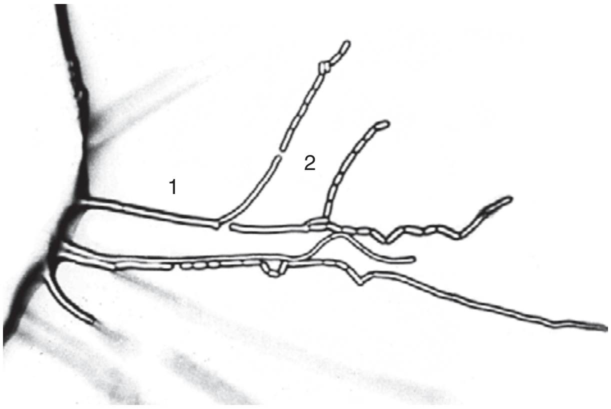

Figure 1 Microculture of *Geotrichum candidum* at 25 °C for 17 h on malt extract medium. (1) Vegetative hyphae slightly septated. (2) Sporulating hyphae corresponding to future arthrospores. Photograph MILA Laboratory, University of Caen Basse–Normandie, France.

The species of the genus *Geotrichum* can assimilate only a few carbon compounds. These tests are usually remarkably stable within the species, which can be explained by the rather strict ecological preferences of a number of species. The ability to ferment sugar is rare in *G. candidum*, except for a weak or delayed fermentation of D-glucose or D-galactose; fermentation of lactose does not occur. Nearly all *G. candidum* isolates from the cheese environment assimilate glucose, galactose, sorbose, xylose, glycerol, and succinate. Lactate is used as a good carbon and energy source; use of citrate is strain-dependent; and lactose is not metabolized. Like the other three species, *G. candidum* does not require a supply of exogenic vitamins.

Table 2 shows the percentage of assimilation results of selected isolates from milk products compared with the standard description for the species according to de Hoog and Smith (2004).

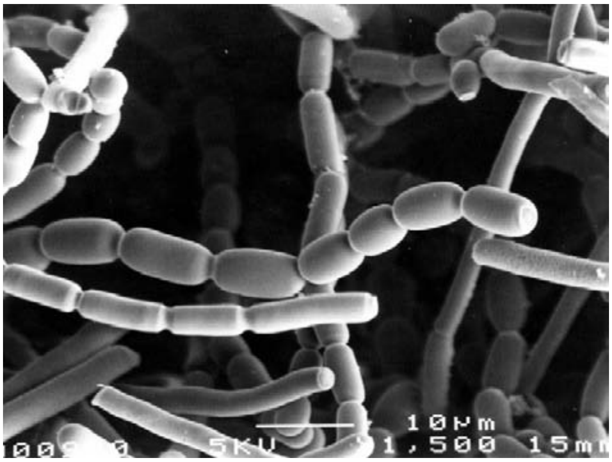

Figure 2 Arthrospores of *Geotrichum candidum* formed by breaking up of hyphae after 48 h in YEG broth. Scanning electronic microscopy (scale = 10 µm). Photograph MILA Laboratory and Microscopy Center Applied to Biology, University of Caen Basse–Normandie, France.

Physiological Adaptation to Freezing Stress

Like other microorganisms, *G. candidum* is able to generate a biotic stress against pathogenic bacteria such as *Listeria monocytogenes*. Conversely, as a ripening starter, this micromycete is itself subjected to numerous anthropogenic stresses generated by food processing, including cheesemaking. Among those stresses, freezing/thawing has been the focus of many studies. It has been shown that *G. candidum* can be physiologically adapted to this lethal challenge by homologous (positive cold temperature) or heterologous pretreatment. Heterologous adaptation can be stimulated by osmotic stress agents (NaCl, $MgCl_2$, $CaCl_2$) and by nystatin, an antifungal compound that targets the plasma membrane and can act as a thermomimetic. Currently, freezing stress is considered to be a combination of both osmotic and mechanical stresses, leading to cryoinjury of cellular structures and

Table 2 Physiological characteristics of *G. candidum* isolates from milk products compared to the standard description of *Galactomyces candidus*

	Standard description[a]	*% Positive (n = 135)*[b]
D-Glucose	+	100
D-Galactose	+	100
L-Sorbose	+	93
D-Glucosamine	−	0
D-Ribose	−	4
D-Xylose	+	100
L-Arabinose	−	0
D-Arabinose	−	0
L-Rhamnose	−	0
Sucrose	−	0
Maltose	−	0
α,α-Trehalose	−	0
Me α-D-glucoside	−	0
Cellobiose	−	0
Salicin	−	0
Arbutin	−	0
Melibiose	−	0
Lactose	−	1
Raffinose	−	0
Melezitose	−	0
Inulin	−	nd
Sol. starch	−	nd
Glycerol	+	100
Erythritol	−	0
Ribitol	v	4
Xylitol	−	0
L-Arabinitol	−	0
D-Glucitol	+	nd
D-Mannitol	+	44
Galactitol	−	nd
myo-Inositol	−	nd
D-Glucono-1,5-lactone	+	nd
D-Gluconate	w	21
D-Glucuronate	−	nd
D-Galacturonate	+	nd
DL-Lactate	+	99
Succinate	+	nd
Citrate	v	24
Methanol	−	nd
Ethanol	+	nd
Propane-1,2-diol	+	nd
Butane-2,3-diol	+	nd
Nitrate	−	10
Ethylamine	+	60
L-Lysine	+	99
Cadaverine	+	99
Without vitamins	+	86
At 25 °C	+	100
At 30 °C	+	100
At 35 °C	+	49
At 37 °C	v	1
At 40 °C	−	0
Mol% G + C low	38.4	
Mol% G + C high	41.6	

+, positive; −, negative; v, variable; w, weak; nd, not determined.

[a] Adapted from de Hoog GS and Smith MTh (2004) Ribosomal gene phylogeny and species delimitation in *Geotrichum* and its teleomorphs. *Studies in Mycology* 50: 489–515.

[b] F. Eliskases-Lechner, unpublished results.

macromolecular damage. The adaptation of *G. candidum* to freezing stress by pretreatment with osmotic chemicals is in accord with this dichotomous approach. Cryopreservation is an important technology used to produce food starters and to improve the *ex situ* preservation of microbial biodiversity. Therefore, *G. candidum*, which is characterized by a significant intraspecies diversity, is an interesting model to examine the relation between morphotype, arthrospore-to-hyphea ratio, and freezing sensitivity. The phenomenon of homologous or heterologous adaptation in *G. candidum* allows us to consider a substantial development of studies on the adaptation caused by abiotic (physical and chemical) stresses, which may lead to an active response by the cell, in parallel with the usual modifications of physical (cooling and warming rate) and chemical (addition of cryoprotectants and nucleators) freezing parameters, which may lead mainly to a passive response by the cells.

Biochemical Characteristics

Geotrichum candidum produces cellulolytic enzymes, glycerol dehydrogenases, gluconases, lipases, proteinases, and peptidases. Proteolytic and lipolytic activities, as well as catabolism of amino acids and free fatty acids, and deacidification activity are of primary importance when selecting suitable strains as a culture for use in cheesemaking (*see* **Cheese**: Microbiology of Cheese).

Proteolytic Activity

Geotrichum candidum plays an important part in the degradation of proteins in many soft cheeses and in some semihard cheeses. It synthesizes extracellular and intracellular proteinases (pH optima 5.5–6.0), which catabolize α_{s1}- and β-caseins. Peptide degradation to amino acids is achieved through aminopeptidases and carboxypeptidases. Enzyme production varies widely from one strain to another and can be attributed to the strain's origin. Commercially available cultures have a relatively low proteolytic activity as compared to wild strains isolated from diverse cheese varieties.

Lipolytic Activity

Fatty acids released by microbial lipases contribute to the flavor of cheeses. In mold-ripened cheeses of the Camembert type, hydrolysis of fat by *G. candidum* is of particular importance. *Geotrichum candidum* produces extra- and intracellular lipolytic enzymes, some of these being highly specific with regard to the action and not the position. The particularly high proportion of free oleic acid in Camembert has been attributed to *G. candidum* lipases, which preferentially release this fatty acid. Furthermore, *G. candidum* lipases show specificity for palmitoleic, linoleic, and linolenic acids. The lipolytic activity is strain specific. Strains producing a slight lipolysis are preferentially used as ripening organisms for diverse soft-cheese varieties.

Flavor-Forming Activities

Geotrichum candidum produces several enzymes (amino and carboxypeptidases, decarboxylases, deaminases, thiolase, β-keto-acyl-decarboxylase) for the degradation of amino acids and fatty acids resulting in important aroma compounds. The catabolism of amino acids by *G. candidum* leads to many compounds important for cheese flavor development, such as 2-methylpropanol, 3-methylbutanol, and especially sulfur compounds (methanethiol, sulfides, dimethyl disulfide, *S*-methylthioesters), which are catabolized from L-methionine. From free fatty acids, *G. candidum* produces various volatile compounds or precursors of aromatic compounds such as methyl ketones (pentan-2-one, heptan-2-one, nonan-2-one, undecan-2-one).

Deacidification

Deacidification plays a major role in the cheese ripening process. Lactic acid produced by the starters may be utilized by yeasts. In addition, yeasts produce alkaline metabolic products, which further increase the pH value. The pH first increases at the surface of the cheese, then, later, in the inner part because of the migration of lactic acid toward the surface. Deacidification favors the activity of various ripening enzymes whose pH optima are often close to neutrality and enhances the development of acid-sensitive microflora. A rapid decrease of lactate levels coincides with the growth of the mold cultures and coryneforms. Therefore, the deacidification process is extremely important for the appearance and the organoleptic characteristics of mold- and smear-ripened cheeses (*see* **Cheese**: Smear-Ripened Cheeses).

The deacidification properties of *G. candidum* are not only strain-dependent but also vary significantly according to the growth medium and incubation conditions. *Geotrichum candidum* can neutralize a calcium lactate yeast extract medium within 24 h, producing ammonia values of ~290 mg kg^{-1} but without lactate reduction. In contrast, when a cheese medium is used, *G. candidum* reduces lactic acid from 150 to 5 mmol kg^{-1} within 4 days, while the ammonia values remain stable. Therefore, for the purpose of testing deacidification ability, it is necessary to distinguish between the pH increase due to lactate utilization and that due to ammonia production.

Applications

Development of *G. candidum* is typical for quite a large number of cheese varieties. It is a part of the surface flora of mold-ripened soft cheeses from cow's milk (e.g., Camembert), goat's milk (e.g., Chabichou), or ewe's milk (e.g., Perail); smear-ripened soft cheeses (e.g., Livarot); smear-ripened semihard cheeses (e.g., Tilsit); and acid-coagulated cheeses (e.g., Quargel, Harzer), and plays an active part in the formation of the characteristic grayish-white crust found on the surface of Saint-Nectaire. Depending on the type of cheese, *G. candidum* appears either only at the very early stages of cheese ripening or during the entire ripening period. A recent application is its use in cow's milk cheese made without *Penicillium camemberti*.

Knowledge about the direct contribution of *G. candidum* to cheese ripening continues to grow. Even if little is known about gene expression, DNA sequencing will be completed in the near future. From the controlled production of Camembert-type cheeses inoculated with *Kluyveromyces lactis*, *G. candidum*, *P. camemberti*, and *Brevibacterium linens*, Leclercq-Perlat *et al.* (2004) have established relationships between the different microbiological and biochemical changes during cheese ripening.

Though the presence of *G. candidum* has always been desirable in ripened soft cheese manufactured from raw milk, its relevance to the cheese-ripening process is a subject of controversy. Metabolism of lactic acid, which is responsible for the increase in pH as well as for the production of aroma substances and proteolytic and lipolytic enzymes, and the ability to reduce bitterness in cheeses, is one of the main reasons for the use of *G. candidum* as a culture. Nevertheless, *G. candidum* is feared by cheesemakers because of the risk of overgrowth, which leads to the 'slippery rind' defect, and the appearance of off-flavors due to contamination. These originate from the raw milk, air, utensils, brine, and the smear water during the manufacturing and ripening processes.

Cultures

Properties of the strains can vary widely, and different specifications are required depending on the cheese variety. The applications of *G. candidum* cultures may vary according to the cheese type produced:

- addition to milk in the vat is only suitable for cheeses of the Camembert type, where a limited growth of the culture is desired
- the culture can be sprayed onto the surface of the cheese, for soft cheeses after brining and for acid-coagulated cheeses after molding
- for smear-ripened cheeses, the culture should be added to the smear water; a single application at the first smearing step may be sufficient

Smear-Ripened Cheeses

The surface smear is a viscous coating consisting of microorganisms and abraded particles of the cheese surface. On the surface of young, still acidic cheeses, yeasts are dominant and the contribution of *G. candidum* resembles that of other yeasts. They deacidify the surface and consequently permit the development of acid-sensitive bacteria. Since the bacterial flora is composed of a salt-tolerant population of coryneforms, salting can be used to control their development. Due to the lower salt tolerance of *G. candidum* as compared to other yeasts, the relationships within the yeast flora can be influenced by salting, thus preventing the overgrowth of *G. candidum*. Depending on the cheese variety, the counts normally vary between 10^6 and 10^7 cfu g^1 if *G. candidum* is a desired part of the surface flora. This species contributes to the flavor of those cheeses by the production of sulfur compounds, which have very low threshold values. Furthermore, *G. candidum* influences the texture of the smear. The white film of *G. candidum* on the cheese surface dries up the cheese surface and thereby reduces the risk of a sticky smear (*see* **Cheese**: Smear-Ripened Cheeses).

Mold-Ripened Cheeses

The formation of aroma substances by *G. candidum* is of great importance for mold-ripened cheeses. *Geotrichum candidum* contributes notably to the taste of Camembert, especially that of pasteurized cheeses, and to the production of the typical Camembert aroma. Typical substances produced by *G. candidum* are secondary alcohols, methyl-ketones, and sulfur compounds.

Geotrichum candidum appears at the same time as the other two yeasts (*Debaryomyces hansenii*, *K. lactis*) and develops during the first week of ripening. Later, counts remain more or less unchanged until the time of consumption. The rapid growth on the cheese surface leads to growth interactions. While *P. camemberti* subsp. *caseicolum* (named *P. candidum* by cheesemakers) is stimulated, undesirable molds such as *Mucor* may be suppressed by certain strains. On the other hand, abundant growth of *G. candidum* hinders the implantation of *Penicillium*, leading to defective cheeses. This defect is called 'toad skin', where the rind of the cheese does not adhere to the inner part. In addition, uncontrolled development of *G. candidum* produces defects in the appearance of the cheeses and may also affect the taste.

Furthermore, *G. candidum* can contribute to improving the organoleptic properties of cheese by reducing bitterness. *Penicillium camemberti* plays a leading role in the appearance of bitterness in Camembert; an excessive growth of the mycelium can lead to this defect. Because of the high activity of the proteinases of *P. camemberti*, an accumulation of bitter peptides may occur in the cheese. However, if the growth of *Penicillium* is limited by the presence of *G. candidum* or by incubating the cheese in the presence of ammonia, proteolysis is reduced and this defect does not occur. The effect of ammonia can be explained by higher pH values in the cheese rind, which delay the growth of *P. camemberti* and consequently reduce the intensity of proteolysis. Some strains of *G. candidum* produce such high amounts of ammonia that a pronounced increase in pH can be attained. Ammonia values as high as 50–300 mg kg^{-1} were reached when *G. candidum* strains were incubated in a sterile cheese curd medium. Therefore, for the purpose of reducing bitterness in soft cheeses, strains have to be carefully selected according to their ammonia-producing capacity.

Since the optimal relationship between *P. camemberti* and *G. candidum* is of such great importance, a combined culture of both microorganisms is advisable. The portion of *G. candidum* in the total inoculum can vary between 0.5 and 5%. The advantage of the *G. candidum* culture lies in the more rapid covering of the cheese surface, which leads to a suppression of undesired molds. During culture selection, strains that produce the typical aroma combined with a low lipolytic activity should be given preference.

Acid-Coagulated Cheeses

Acid-coagulated cheeses include some smear-ripened cheeses as well as cheeses without a smear surface. On the surface of the smear-ripened varieties, *G. candidum* is regarded as a contaminant, whereas it gives all other varieties their typical ammonia odor and characteristic texture. The cultures used have a relatively high lipolytic and proteolytic activity (*see* **Cheese**: Acid- and Acid/Heat Coagulated Cheese).

Safety Assessment

Regarding the safety assessment of *G. candidum*, only 11 yeasts, which do not include *G. candidum*, and no filamentous fungi, have obtained the qualified presumption of safety (QPS) status delivered by the European Food Safety Authority (EFSA). Nevertheless, *G. candidum* infections are very rare, with fewer than 100 cases having been reported between 1842 and 2006. Less than one case per year of disease is possibly caused by *G. candidum*, and this never includes dairy products or foodborne infections. The species can be unambiguously differentiated from the two species most frequently described in human pathology: *Geotrichum clavatum* (reclassified *Saprochaete clavata*) and *Geotrichum capitatum* (reclassified *Magnusiomyces capitatus*/*Saprochaete capitata*). The risk of developing an infection due to *G. candidum* in connection with its technological use and consumption in dairy products is therefore virtually nil.

Geotrichum candidum as a Spoilage Organism

As in the case of other yeasts, the presence of *G. candidum* leads to product spoilage of fermented milks and fresh cheeses, and limits the shelf life of these products. Besides the visually observable change due to colonies on the product surface, degradation of protein or fat results in smell and taste defects in cottage cheese, quark, yogurt, buttermilk, and fermented cream. Further, growth of *G. candidum* on the surface of butter causes flavor defects and surface discoloration.

Enumeration

Detection and identification of fungal contamination in dairy products are important in assessing hygiene practices during the manufacture and distribution of foods. Especially in ripened cheeses, where *G. candidum* is a part of the ripening flora, other yeasts and molds must be counted separately in order to interpret the results correctly.

Direct Microscopic Method

Microscopic examination permits rapid estimation of the *G. candidum* content when relatively high levels are present. The presence of a fungus with holothallic spore production named arthrospores can also be microscopically determined.

In Situ Quantification

Polymerase chain reaction (PCR)-based methods can rapidly identify and quantify yeast species in complex microbial ecosystems without isolation. A real-time PCR method was developed by Larpin *et al.* (2006), to quantify the main yeasts, including *G. candidum*, that composed the microbial communities of many cheeses.

Culture Method

Yeast extract-dextrose-chloramphenicol agar and yeast extract-dextrose-oxytetracycline agar were found to be among the most satisfactory media available for the enumeration of *G. candidum* and the other yeasts in milk products (ISO 6611/IDF94). These media contain chloramphenicol or oxytetracycline as selective substances to suppress the accompanying bacterial flora. Plates are incubated aerobically at 25 °C for 5 days; however, *G. candidum* can be counted after 48–72 h of incubation. The incubation temperature should not exceed 25 °C because the growth of some strains is restricted at higher temperatures. For the examination of ripened cheeses that have a mold coat, it may be desirable to split the sample into the surface part and the inner part, depending on the purpose of the investigation.

For special purposes, several modifications may lead to better results:

1. Addition of dyes, for example, bromophenol blue ($0.01\ \mathrm{g\,l^{-1}}$), is advantageous in distinguishing between *G. candidum* and colonies of other yeasts and molds. Furthermore, bacteria resistant to chloramphenicol or oxytetracycline are generally easier to distinguish from yeast colonies.
2. Generally, for the quantification of *G. candidum*, the spread-plate method is preferable to the pour-plate method because higher counts are obtained in the former. In addition, surface plating facilitates the characterization of the morphology of the colonies and their isolation for further investigation. It is also easier to differentiate between *G. candidum* and the accompanying mold colonies. Whereas the surface colonies of *G. candidum* can be easily removed from the agar when using a loop, mold colonies are intimately bound within the agar.
3. For certain samples, for example, raw milk or farm house products, lowering the pH to 4.6 may be necessary to suppress the accompanying flora of chloramphenicol- or oxytetracycline-resistant bacteria. The pH value of the medium is lowered by an aseptic addition of tartaric acid, after sterilization.
4. Quantitative analysis of *G. candidum* can be difficult in cheeses made with mold cultures. Mold cultures usually exceed the *G. candidum* counts and overgrow the plates. The antibiotic oligomycin is particularly useful in suppressing the mold cultures usually used for cheesemaking. Addition of 0.1 ml oligomycin solution ($100\ \mu\mathrm{g}$ oligomycin $\mathrm{ml^{-1}}$ ethanol) on the agar surface before plating suppresses mold growth, while growth of *G. candidum* and other yeasts is not inhibited.

See also: **Cheese**: Acid- and Acid/Heat Coagulated Cheese; Microbiology of Cheese; Secondary Cultures; Smear-Ripened Cheeses. **Yeasts and Molds**: Yeasts in Milk and Dairy Products.

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Penicillium roqueforti

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Introduction

Penicillium roqueforti is a saprophytic fungus that is commonly found in nature and can be isolated from soil or from decaying organic matter. It is used as a fungal starter culture for the production of blue-veined cheeses such as Danablu, Gorgonzola, Roquefort, and Stilton. The mold is primarily responsible for ripening the cheese, involving the production of both proteolytic and lipolytic enzymes. The proteolytic enzymes produced by the fungus act to soften the curd and produce the desired body in the cheese. These proteolytic enzymes involve both extra- and intracellular proteinases and peptidases, with the extracellular aspartic proteinases being particularly important for the ripening process. The water-soluble lipases produced by the fungus hydrolyze the milk fat to free fatty acids, such as butyric caprylic, caproic, and capric acids, which contribute to the flavor of Blue cheeses. Other important components of Blue cheese flavor are also produced by the mold, such as the ketone heptan-2-one, which is produced from caprylic acid, as well as other ketones such as pentan-2-one and nonan-2-one. In addition, *P. roqueforti* is also known to reduce methyl ketones to form secondary alcohols such as heptan-2-ol, pentan-2-ol, and nonan-2-ol, which also contribute to cheese flavor.

Due to its resistance to organic acids and weak acid preservatives together with an ability to grow at low pH, particularly at water activity (a_w) values >0.95, *P. roqueforti* also commonly spoils processed foods such as bread, beer, olives, and hard cheeses. In addition, it is commonly found in silage and grains stored under microaerophilic conditions.

Morphology

Penicillium roqueforti is a rapidly growing fungus and it produces low and velutinous dark green colonies, which on malt extract agar (MEA) grow rapidly to 40–70 mm in diameter. These colonies are characterized by moderate to heavy conidial production with either grayish turquoise or dull green color at the margins of the colony, with olive brown color sometimes being observed in the center of the colony. The reverse of the colony is pale, brown, or green to deep blue green (almost black). Conidiophores are borne from subsurface hyphae with stipes (hyphae supporting the fruiting structure, forming the conidiophore) of between 100 and 200 μm long. These stipes have very rough walls, which bear large terminal penicillia, which are rarely biverticillate, typically terverticillate, and occasionally quaterverticillate.

Physiological Factors Affecting Growth of the Fungus

Penicillium roqueforti appears to have the lowest oxygen requirements for growth of any *Penicillium* species. It can grow at only 10% of standard atmospheric O_2 partial pressure. In reduced oxygen atmospheres, the fungus is largely unaffected until the oxygen concentration drops below 4.2%. In addition, it is known that growth of the fungus is stimulated by carbon dioxide concentrations of up to 15% in air. In an atmosphere containing 80% CO_2, 4.2% O_2, and 15.8% N_2, growth of *P. roqueforti* has been reported to be 30% of that in air at 20 °C. The fungus has also been reported to grow at lower oxygen concentrations, such as 0.05%, and in the presence of 20% CO_2 at 25 °C, while growth and sporulation also occur in atmospheres containing 20% O_2 and 80% CO_2. These properties are the main reason for the dominant growth of this fungus in ripening cheese.

Penicillium roqueforti is a psychrophile and grows vigorously at temperatures as low as 4 °C, but not above 35 °C. It is tolerant to both acid and alkaline conditions and can grow in the pH range of 3–10. Many *P. roqueforti* strains are known to be very tolerant to weak acid preservatives, being able to grow in the presence of 0.5% acetic acid. This property can be used to selectively grow *P. roqueforti* as other *Penicillium* species are unable to grow under these conditions. *Penicillium roqueforti* is also resistant to sorbate. Sorbate-resistant isolates of the fungus isolated from sorbate-treated cheeses are able to metabolize and grow in the presence of 9000 ppm sorbate. Tolerance to sorbate is accompanied by degradation of the preservative and the development of a ‘kerosene’ taint in cheese, through the formation of 1,3-pentadiene. In addition, at a_w values >0.97, growth of *P. roqueforti* isolates is stimulated by propionate, another commonly used weak acid preservative. This resistance to weak acid preservatives, which are routinely used to prevent fungal spoilage of foods, coupled with its ability to grow at refrigeration temperatures, makes the fungus a common cause of spoilage in cool-stored preserved commercial and domestic foods.

The microenvironment of Blue cheese is characterized by profound NaCl gradients from the core to the surface of the cheese, which reach equilibrium slowly during ripening. These differences are known to affect the growth, germination, and sporulation of *P. roqueforti*. *Penicillium roqueforti* has an optimum water activity (a_w) value of 0.998 for growth at 25 °C, and a colony growth rate of 13.4 mm day^{-1}. The lag phase of growth for *P. roqueforti* is relatively stable at $a_w > 0.92$, but increases for $a_w < 0.94$. This is advantageous for the use of the fungus as a starter culture, as the final a_w values of Blue cheeses are in the range of 0.91–0.94, which allows *P. roqueforti* to germinate quickly and grow through the entire cheese processing and ripening process. The pH and NaCl concentrations of the cheese are also known to influence the proteolytic activity of the fungus, with proteolysis typically being less pronounced in the high salt environment in the outer parts of the cheese.

Growth of *P. roqueforti* strains is stimulated at low salt concentrations, with 1% salt (NaCl) having the highest stimulating effect. In addition, while it is known that *P. roqueforti* strains can grow at low temperatures, the rate of growth at 10 °C is around 2–3 times lower than that at 25 °C, the optimum temperature for the species. At 25 °C, *P. roqueforti* strains have been reported to produce around 10% more mycophenolic acid (MPA) at an a_w value of 0.97 when compared to that at an a_w value of 0.95. This effect does not appear to be significantly affected within the pH range of 4.7–7.4.

At a_w values >0.97, growth of *P. roqueforti* is stimulated by propionate, another commonly used weak acid preservative. There are a number of conflicting results reported in the scientific literature with respect to the effects of preservatives on growth and mycotoxin production by *P. roqueforti*. Some reports appear to indicate that at subinhibitory levels preservatives inhibit mycotoxin production, whereas the opposite has been reported by other groups. Thus, it is likely that the mechanisms of mycotoxin regulation are quite complex and not readily generalized and are most probably not only species-dependent but also affected by the growth medium and by the concentration of the preservative.

In a study involving 30 *P. roqueforti* strains, the effects of various physiological conditions on both esterase and lipase activities were monitored, using diffusion assays on tributyrin and olive oil agars, and growth at either 10 or 25 °C in butterfat emulsions containing up to 7% NaCl was also monitored. This study reported that extracellular lipase production is stimulated at low NaCl concentrations and that lipases show a higher activity against short-chain fatty acids while triolein is hydrolyzed at a much lower rate. Mathematical models combining the effects of temperature and salt concentration have been developed to predict their effects on the growth rate of *P. roqueforti*, in an attempt to prevent food spoilage by the fungus. Other approaches to prevent growth of the fungus have involved the use of an antifungal compound produced by a *Bacillus subtilis* strain and which has been reported to inhibit the germination of *P. roqueforti* conidiospores. This iturin-like compound is believed to act by permeabilizing the fungal spores, thereby inhibiting germination. The addition of essential oils has also been shown to inhibit the growth of *P. roqueforti*, with the addition of eugenol, caryophyllene, *p*-cymene, and thymol being reported to be particularly effective.

Production of Volatiles

A number of methods have been developed to study the volatile compounds produced by *Penicillium* species, including *P. roqueforti*. Three popular methods include diffusive sampling from headspace on carbon black adsorbent in glass tubes, purging and trapping of headspace gases with carbon black adsorbent tubes, and simultaneous distillation extraction (SDE) with diethyl ether solvent. The diffusive sampling method is regarded as the most appropriate method because with the purge-and-trap method purge flow significantly determines the quantitative volatile metabolite profile and SDE causes formation of lipid oxidation products. Such an approach has been successfully employed to profile volatile metabolites to allow the differentiation of species from the *P. roqueforti* group. It has also been shown that *P. roqueforti* strains that produce PR toxin (7-acetoxy-5,6-epoxy-3,5,6,7,8,8*a*-hexahydrocarboxaldehyde) produce the volatile metabolite (+)-aristolochene, which is considered a biomarker for *P. roqueforti* within the *Penicillium* genus.

Genetics

Penicillium roqueforti is quite a heterogeneous species and has recently been divided, based on differences in its internal transcribed spacer (ITS) regions and its secondary metabolite patterns, into three distinct species, namely, *P. roqueforti*, *P. carneum*, and *P. paneum*. While all three species are morphologically very similar, there are marked differences in their ability to produce secondary metabolites. *Penicillium roqueforti* can produce PR toxin, marcfortines, and fumigaclavine A, *P. carneum* can produce patulin, MPA, and penitrem A, while *P. paneum* produces patulin and botryodiploidin.

DNA-based molecular techniques have been developed and applied in the detection and identification of

Penicillium species. Polymerase chain reaction (PCR) primers based on the ITS region have been developed to monitor *P. roqueforti* in a variety of foods. These PCR primer pairs, which specifically amplify a 300-bp fragment, not only specifically identify all members of *Penicillium* subgenus *Penicillium*, but also specifically recognize *P. roqueforti* and *P. carneum*. Even though many of the *P. roqueforti* strains that have been isolated from Blue cheeses are known to produce both PR toxin and roquefortine and these secondary metabolites have been shown to be present in cheese, they are not thought to pose a significant health risk to consumers as they are very unstable in cheese. Notwithstanding this, *P. roqueforti* strains that do not produce secondary metabolites or mycotoxins would be preferable as starter cultures for cheese manufacture, from a food safety perspective. Thus, several groups have set out to develop DNA-based methods to identify *P. roqueforti* starter strains that do not produce toxic secondary metabolites. In this respect, Geisen and coworkers targeted the *ari1* gene encoding aristolochen synthase, one of the key enzymes in the PR toxin biosynthetic pathway, in a PCR-based approach to screen for PR toxin-free strains of *P. roqueforti*. Using the *ari1*-specific PCR primers, a product of the expected length was observed in many of the 21 strains tested. However, some of the strains that were PCR-negative were also toxin producers. These were subsequently shown to be positive following dot-blot hybridization using an *ari1*-specific gene probe, indicating the presence of *ari1* genes in some *P. roqueforti* strains with altered PCR primer binding sites. Another potential problem with this method was also identified whereby *ari1* gene homologues were observed in *Penicillium* species, such as *P. italicum* and *P. nalgiovense*, which are known not to produce PR toxin. Thus the group advise that care should be taken when using a monomeric PCR reaction, which targets only one mycotoxin biosynthetic gene, as the primers may not be sufficiently specific to detect the mycotoxin-producing fungus. More recently, the group have successfully performed random amplified polymorphic DNA (RAPD) analysis, using three primers (*ari1* (CTGCTTGGCA CAGTTGGCTTC), *nor1* (ACCGCTACGCCGGCAC TCTCGGCAC), and *omt1* (GTGGACGGACCTAGT CCGACATCA)), of 76 *P. roqueforti* starter culture strains and reported a correlation between RAPD patterns and the production of MPA. In addition, they reported on one fungal genotype, which was distinguishable with the *ari1* primer, that produced fewer secondary metabolites than other genotypes and which did not produce PR toxin. Thus, this strain may be a good candidate for use as a safe starter culture. The group advocate that before being used as starter cultures in the dairy industry *P. roqueforti* strains should be checked for their inability to produce toxins in the cheese, and suggest the approach they employed as a reliable method for achieving this goal.

Mycotoxins Produced by *Penicillium roqueforti*

PR Toxin

PR toxin is one of the most acutely toxic metabolites produced by the fungus and is frequently detected in Blue cheese. PR toxin produces acute toxic effects in animals via an increase in capillary permeability and due to direct damage to lungs, heart, liver, and kidneys. PR toxin also inhibits RNA and protein synthesis, DNA polymerase activity as well as mitochondrial respiration and oxidative phosphorylation in animal cells. It has also been reported to result in gene alterations and gene conversions in *Saccharomyces cerevisiae* and *Neurospora crassa* strain N24, respectively.

A number of *P. roqueforti* strains have been isolated from Blue cheese, which when grown under different culture medium produce PR toxin, with levels of toxin production being highly dependent on environmental conditions. For example, when *P. roqueforti* is grown in yeast extract–sucrose medium, which favors the production of PR toxin, levels of between 82 and 770 $\mathrm{mg\,l^{-1}}$ are produced. It has also been reported that toxin production is highest in stationary cultures at temperatures ranging from 20 to 24 °C and at pH 4; the addition of octanoic acid to *P. roqueforti* cultures growing on wheat kernel medium has recently been reported to inhibit PR toxin biosynthesis.

However, despite the ability of *P. roqueforti* strains to produce the toxin, no PR toxin or at most very low levels can be detected in cheese. Researchers believe that the microaerophilic conditions that prevail in most cheeses appear not to favor the production of PR toxin. In addition, the toxin appears to be very unstable in cheese, where it is believed to react with ammonia and free amino acids, which are present in high concentrations in Blue cheese, to form PR, which is unstable and is subsequently degraded to PR acid.

Roquefortine

This is an indole mycotoxin and is identical to roquefortine C. It has been assigned the structure 10*b*-(1,1-dimethyl-2-propenyl)-3-imidazol-4-methylene-5*a*,10*b*,11,11*a*-tetrahydro-2*H*-pyrazino-[1′,2′:1,5]pyrrol[2,3,*b*]indole-1,4-(3*H*,6*H*)-dione. Roquefortine is a relatively weak neurotoxin and in studies in ruminants that have consumed contaminated silage, clinical symptoms include muscle weakness and lack of coordination. Roquefortine is also reported to cause convulsive seizures in mice when injected intraperitoneally. *Penicillium roqueforti* strains isolated from Blue cheeses have been reported to produce between 0.18 and 8.44 mg l^{-1} of roquefortine in culture medium containing yeast extract, while in experiments in which cheese was inoculated with a toxigenic strain of the fungus, levels of between 2.1 and 2.4, and 2.1 and 3.8 mg kg^{-1} have been reported in cheese ripened at 5 and 12 °C, respectively.

Roquefortine C levels ranging from 0.05 to 12 mg kg^{-1} have been reported in cheeses, while in a recent study roquefortine at concentrations of 0.8–12 mg $kg^{-1}$was detected in all of the 10 blue mold cheese samples obtained from Finnish supermarkets. Although roquefortine is produced by most strains of *P. roqueforti* that have been isolated from Blue cheese or which are used as starter cultures, the low levels of the toxin that are present in Blue cheese together with the relatively low toxicity of roquefortine are such that roquefortine is believed not to present a major health hazard to the consumer and make the consumption of Blue cheese safe.

Mycophenolic Acid

This is a mycotoxin and is reported to be produced by many strains of *P. roqueforti* that have been tested and by some other *Penicillium* strains, particularly *P. brevicompactum* and *P. paneum*. For example, in a study where 80 *P. roqueforti* strains were tested, of which 62 of the strains were starter culture strains from western Europe, only 20 were able to produce up to 600 mg of the toxin in 2% yeast extract–5% sucrose broth. MPA has antibiotic activity against bacteria and dermatophytic fungi and is also known to interfere with viral multiplication. For mammals, the toxicity of MPA is low. There are reports of toxicity in rats, with oral administration of daily doses of 30 mg kg^{-1} resulting in anemia and death. Interestingly, MPA is routinely used in the treatment of psoriasis and in addition both MPA and MPA derivatives have been reported to have both antitumor and immunosuppressive effects. MPA is the active ingredient in mycophenolate mofetil (MMF), which is widely used to prevent rejection after solid organ transplantation. It acts as a reversible, noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in *de novo* purine biosynthesis in T and B lymphocytes.

Penicillium roqueforti strains isolated from baled grass silage have been reported to produce MPA, and thus subsequent consumption of this silage by livestock should be a concern for livestock producers. In one study, all 16 strains of *P. roqueforti* isolated from Blue cheeses have been reported to produce MPA, at levels of between 0.8 and 4 mg g^{-1} dry culture, with the highest levels of the toxin being reported following 10 days of incubation of fungal cultures at 15 °C. However, the yeast *Geotrichum candidum* has been reported to inhibit the growth of *P. roqueforti* when cocultured on agar medium at both 18–25 °C and in addition to inhibit the production of MPA.

See also: **Yeasts and Molds**: *Penicillium camemberti*.

Further Reading

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Penicillium camemberti

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Introduction

Penicillium camemberti was first described by Thom and is thought to be a domesticated form of *P. commune.* A number of synonyms exist for the species including *P. rogeri*, *P. candidum*, *P. album*, and *P. caseicolum.* The fungus is mainly (almost exclusively) found either on cheese or in the cheese factory environment and is rarely found away from this environment. *Penicillium camemberti* is used in the production of Camembert and Brie cheeses, on which colonies of the fungus form a white crust. It is also used as a starter culture for fermented meat products and is often found as a spontaneous colonizer of fermented sausages originating from the mycobiota of the production facility. There have been reports on the wider exploitation of *P. camemberti* especially in the decontamination of softwood bleach effluents, which contain high levels of ecologically undesirable phenolic and chlorinated phenolic compounds. Therefore, *P. camemberti*, which to date has been predominantly used in the dairy industry, may also find future utility in other nondairy-related areas.

Growth Characteristics of *Penicillium camemberti*

Colony diameter on Czapek yeast extract agar (CYA) is typically 19–27 mm at room temperature (24–26 °C) after 10 days. Colonies on CYA appear yellow/orange or green to fawn to pale brown/blue in color. The reverse sides of the colonies on CYA typically appear either yellow/orange or green brown in color. Colony diameter on malt extract agar (MEA) is typically 12–27 mm. Colonies on MEA also appear yellow/orange or green in color, with the reverse sides of the colonies being red, olive, green, or brown in color depending on the growth medium. The optimum growth temperature range is 20–25 °C, with growth being recorded at 5 °C but not at 37 °C. With respect to pH, growth can take place in the pH range of 3.5–6.5. *Penicillium camemberti* has similar water activity (a_w) limits for growth as *P. roqueforti* with an optimum a_w value of 0.998 for growth at 25 °C and an ability to grow in the a_w range from 0.91 to 0.94. Thus, from a pH standpoint, *P. camemberti* is ideal as a starter culture given that the pH of Camembert and related types of cheese reaches about 4.6 during the first 24 h and eventually following maturation increases to around 5.5 in the center of the cheese and around 7.0 in the outer part of the cheese. Similarly, it is suitable as a starter culture from an a_w standpoint given that the a_w of the surface and center of Camembert cheese has been recorded as 0.93 and 0.97, respectively. The salt tolerance of the fungus coupled with its ability to grow at an a_w of 0.93 results in it growing on the surface of Camembert cheese during the cheese maturation process. However, it is only after 1 week of ripening that *P. camemberti* is observed, and within 2–3 weeks it covers the entire surface of the cheese. During this process, the fungus also metabolizes lactate to CO_2 and H_2O at the surface of the cheese to establish a pH gradient, which is a key factor in the maturation process, and results in a higher pH. This effect is pronounced on the surface of the cheese, with a pH gradient becoming established toward the center of the cheese, resulting in lactate migrating toward the surface, where it is assimilated as a carbon source by the fungus. This depletion of lactate in the center of the cheese results in casein being degraded primarily by enzymes from the rennet, the plasmin from the milk, and by enzymes from the lactic acid starter cultures. Ammonia is formed at the surface of the cheese from amino acids, the consequence of which is a further increase in pH. The proteinases from *P. camemberti* are activated by the increasing pH and they migrate only slowly into the cheese.

During ripening, the dynamics of sporulation of *P. camemberti* is affected by the concentration of CO_2 in the atmosphere. For example, the number of *P. camemberti* spores present in the rind is fairly constant at around 10^4 cfu g^{-1} during the first 6 days of ripening at 6% CO_2. However, at 2% CO_2, the fungus is known to sporulate at a faster rate and spore counts can reach levels as high as 10^6 cfu g^{-1} on the 6th day of growth. After day 11 and until day 40, sporulation remains stationary, close to 10^6 cfu g^{-1}.

Regardless of CO_2 concentration, the mycelium of *P. camemberti* begins to grow from day 4 onward with both mycelium and aerial mycelia being visible. Between days 5 and 12, the mycelia grow and uniformly cover the entire cheese surface. From day 10 to 16, if the cheese is wrapped, the rind color remains white and is around 3 mm thick. Increases in CO_2 concentration above 2% negatively affect the growth of *P. camemberti* on cheese. Because in Camembert-type cheese, *P. camemberti* is generally inoculated in a mixed culture with *Geotrichum candidum*, CO_2 is known to alter the equilibrium between these two strains, with higher CO_2 concentrations favoring *G. candidum* and resulting in poorer development of *P. camemberti* mycelium.

Enzymes Produced by *Penicillium camemberti*

Penicillium camemberti produces a variety of different proteinases including two extracellular endopeptidases. One of the two extracellular endopeptidases is a metalloprotease and is the principal proteolytic enzyme active at close to neutral pH values (pH 6.5). It is similar to the metalloprotease produced by *P. roqueforti.* At acidic pH (pH 4.0), *P. camemberti* produces an acid protease. Other proteolytic enzymes produced by *P. camemberti* include an aminopeptidase and a carboxypeptidase. These proteolytic enzymes play an important role in cheese ripening. There are some differences between strains with respect to the production of different types of proteinases. There is, however, greater variation between *P. camemberti* strains in their ability to produce extracellular lipolytic enzymes. The lipase system is active within broad pH (5.5–9.5) and temperature (1–35 °C) ranges.

Penicillium camemberti as a Biocontrol Agent in Cheese

Starter cultures are known to contribute to the inhibition of the undesirable growth of fungal contaminants and mycotoxin production in fermented foods. When *P. camemberti* is used as secondary starter culture, it exerts a powerful inhibitory effect on many common cheese contaminants such as *Cladosporium herbarum*, *P. roqueforti*, *P. caseifulvum*, and *P. commune.* The antagonistic power of *P. camemberti* is strain dependent in that the growth inhibition of *C. herbarum* is not affected by the choice of the strain of *P. camemberti*, whereas the *Penicillium* contaminants are very sensitive to the choice of strain. The antagonistic activity is much stronger when *P. camemberti* is used as pure culture, with the inhibitory activity being reduced considerably if the fungus is used in a mixed culture, for example with *G. candidum.*

Secondary Metabolism in *Penicillium camemberti*

A number of *Penicillium* toxins have been identified in contaminated cheese, including roquefortin C, isofumigaclavine A, cyclopiazonic acid (CPA), mycophenolic acid, and, much less frequently, ochratoxin A and PR toxin. A few strains of *P. camemberti* are known to produce a number of secondary metabolites such as cyclopaldic acid, rugulovasine A, and rugulovasine B as well as palitantin. However, CPA, which is a neurotoxic and immunosuppressive compound, remains the most significant toxin produced by *P. camemberti,* particularly at higher storage temperatures. The toxicity of CPA results in a large part from its specific inhibition of calcium-dependent ATPase in the sarcoplasmic reticulum, leading to altered cellular (Ca^{2+}) levels and resulting in increased muscle contractions.

CPA is almost exclusively found in the rind but not in the core of the cheese. This is due to the inability of *P. camemberti* to grow in the cheese core. CPA does not appear to constitute a major threat to the consumer with the highest levels in cheese being reported to date at >2 ppm, which would constitute <4 μg CPA in a portion of the most highly contaminated cheeses. It has also been reported that CPA can be produced by *P. camemberti* in submerged culture, at levels <4 ppm following a 96 h fermentation. Production of CPA is known to be strain-specific and appears to be unrelated to spore yield.

No noticeable mutagenic activity was detected when crude extracts of several strains of *P. camemberti* containing a pool of metabolites were assessed, suggesting that undesired long-term effects from the consumption of *P. camemberti*-ripened cheese are unlikely. This may explain the fact that despite many *P. camemberti* strains possessing the ability to produce CPA, no acute toxicity associated with the consumption of food produced by the fungus has been reported to date. The possibility exists that this may also be due to the fact that many other metabolites are likely to be produced at the same time as CPA and that these metabolites may in some way have an antagonistic effect that could neutralize or negate the toxicity of CPA. However, no clear scientific evidence exists to support this hypothesis and further work is required to substantiate this theory.

Penicillium camemberti and Cheese Flavor

The production of numerous flavor compounds in Camembert cheese can be directly attributed to the enzymatic activity of *P. camemberti.* While the presence of the fungus at the surface of the cheese gives the cheeses their characteristic appearance, it is known that low-molecular-weight compounds produced by the fungus contribute significantly to taste. Volatile compounds are an important component of these low-molecular-weight molecules, with volatile fatty acids being the most abundant compounds within the volatile fraction. In fact, lipolysis is particularly important in soft cheeses such as Camembert where free fatty acids can reach up to 10% of the total fatty acids present. As previously mentioned, *P. camemberti* produces a lipase, which is similar to the alkaline lipase produced by *P. roqueforti*, and proteases. Lipase and protease activities are involved in the degradation of short-chain fatty acids and peptides in the cheese. The resulting products are subsequently transformed into important taste and aroma compounds such as ammonia, methyl ketones, primary and secondary alcohols, esters, aldehydes, lactones, and sulfur compounds. The methyl ketones are by far the most abundant neutral compounds in the volatile fraction of mold-ripened cheeses. Such methyl ketones include 2-nonanone, 2-undecanone, 2-heptanone, and 2-pentanone and their corresponding secondary alcohols, and contribute to the musty flavor of the cheese. Due to their typical odors and their low odor thresholds as well as their concentration in cheese, ketones and methyl ketones play a key role in the overall flavor of surface mold-ripened cheeses. The secondary alcohol 1-octen-3-ol in particular is responsible for the characteristic mushroom flavor of Camembert-type cheese. Most esters have floral and fruity notes and are believed to contribute to the overall aroma by minimizing the sharpness and bitterness imparted by fatty acids and amines, respectively.

Types of Cheese Involving *Penicillium camemberti*

Camembert Cheese

In the traditional manufacture of the surface mold-ripened Camembert cheese, whole pasteurized milk is warmed to 29–33 °C and ripened using a lactic acid bacteria starter culture. The high acidity of the milk assists in whey drainage and suppresses the growth of undesirable organisms. Coagulating enzyme is added to allow the formation of a firm curd within a 1–2 h period. The resulting curd is then dipped into small, perforated forms and allowed to drain for 1–2 days, with frequent turning. The cheese is then removed and salted, and is typically inoculated with a culture containing both mold and bacteria. The curing of Camembert cheese is quite a complex process and involves not only the uniform and progressive development of certain ripening agents, but also the gradual drying out of the curd. To help achieve this, the curing rooms are usually maintained at temperatures of around 13 °C and at a relative humidity of 90%. The creamy, semiliquid interior consistency characteristics of Camembert are largely due to the activity of *P. camemberti*. The mold can be mixed with the milk, sprinkled on the curd, or rubbed on the cheese along with salt. After 2 weeks, the primary surface of mold growth forms a thin, gray-white, felt-like rind but does not penetrate the cheese. The cheese is then wrapped in parchment and foil, and boxed. The cheese is regarded as being in prime condition after a 4- to 5-week period at which time it should be consumed.

Ammonia, which has a low odor threshold (5 mmol kg^{-1}), is associated with a ripened aroma when its concentration is within the accepted limit. However, overripened Camembert cheeses can develop a strong ammonia odor as a result of the intense deamination activity of *P. camemberti*. Thus, a pronounced aroma of ammonia is indicative of overripening of Camembert. Flavor defects characterized by a typical celluloid flavor originating from the production of styrene by the mold sometimes appear during ripening or storage of mold-ripened cheese. It has recently been shown that *P. camemberti* can produce styrene from phenylalanine by phenylalanine ammonia lyase activity followed by a decarboxylation reaction catalyzed by a cinnamic acid decarboxylase.

Brie Cheese

Brie, a cheese that is surface ripened by mold, is very similar to Camembert. Differences exist, however, in the internal ripening and in the characteristic flavor and aroma of the cheeses. The traditional manufacture of Brie cheese involved initially warming the milk to 32 °C and then adding coagulating enzyme to initiate curd development within 2–3 h. The curd is then dipped into small forms and hoops and allowed to drain for about 24 h. The hoops are removed, and the cheese is turned and dry-salted. Initial ripening for about 8 days occurs in a well-ventilated drying room maintained at 13–16 °C. During this time, the curd softens rapidly and becomes slightly yellow and translucent in color, and a felt-like layer of white mold appears on the surface. The cheese is then moved to a dark, moist room or cellar that is maintained at 11 °C and at a relative humidity of 85% for 2–4 weeks. The initial white mold layer formed by *P. camemberti* eventually changes to a yellow color and is subsequently overgrown with Gram-positive organisms similar to those found on smear-ripened cheese appearing red in color. The cheese becomes less acidic and the curd is yellow and creamy. The surface growth of both *P. camemberti* and smear organisms during ripening is responsible for the characteristic flavor of Brie. Like Camembert, Brie ripens rapidly, is perishable, and must be consumed soon after ripening.

Penicillium camemberti in Other Food-Related Applications

Penicillium camemberti has successfully been used to improve the quality of sausage meat, through the superficial inoculation and growth of an atoxigenic strain of *P. camemberti* on the surface of the sausage. This has been reported to result in strong proteolysis and lipolysis, which produce an intense increase in the diglyceride, monoglyceride, phospholipid, and free fatty acid concentrations and in volatile compounds and a corresponding decrease in triglyceride levels. Compounds such as branched aldehydes and the corresponding alcohols, acids, and esters, derived from the catabolism of amino acids, are responsible for the ripened flavor. The development of the fungal mycelia on the surface of the sausages not only protects lipids from oxidation, resulting in lower 2-thiobarbituric acid values and lipid oxidation-derived compounds, such as aliphatic aldehydes and alcohols, but also completely eliminates the growth of undesirable naturally occurring mold contaminants. Thus, the use of *P. camemberti* results not only in the protection of the sausage from fungal contamination but also in an improvement in the odor and flavor of the sausage.

Advanced Methods for the Identification of *Penicillium camemberti*

Numerous industries are currently employing electronic nose (e-nose)-based detection systems to monitor food quality control, storage, and spoilage by both bacteria and fungi. E-nose involves an analysis of the chemicals contained in an extract using gas chromatography coupled to mass spectrometry (GC–MS) and liquid chromatography coupled to mass spectrometry (LC–MS). A fungal taxon can be identified by the metabolites it produces. Thus, profiling the pool of volatile and non-volatile metabolites produced by *Penicillium* species, in defined conditions, can be used as a chemotaxonomic tool to differentiate efficiently even closely related species and to distinguish between cheese-related fungi. Karlshøj and colleagues analyzed the pool of volatile metabolites produced by several fungi species, including *P. camemberti*, using the e-nose approach and showed an increasing difference between fungal species throughout time. These authors indicated that *P. camemberti* can be unambiguously identified after 3 days of growth on yeast extract glucose medium, with no CPA being detected. Thus, they clearly demonstrated the ability of e-nose to identify correctly closely related fungi, grown on given conditions, to a species level. Because these species have also been shown to differ in mycotoxin production, it also demonstrates the potential use of e-nose as a powerful tool for the identification of mycotoxigenic fungi in food and feedstuffs.

See also: **Yeasts and Molds**: *Penicillium roqueforti*.

Further Reading

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Spoilage Molds in Dairy Products

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Introduction

Molds are present in air, water, and soil and are regularly found on production equipment; hence, they can contaminate milk and dairy products. Molds are important in dairy products because they are involved in fermentation, spoilage, and mycotoxin production. Thus, molds may, even in the same product, contribute to desirable flavors or off-flavors, cause cheese coloring or discoloring, result in high-quality product structure or disintegration, and even produce toxins if not controlled. Several types of cheese are manufactured with molds either as veins throughout the cheese or as exterior crusts covering soft-cheese interiors. Some mold genera, dominated by *Penicillium* and *Cladosporium*, have species that are involved in the spoilage of cheese, yogurt, and other fermented or concentrated dairy products. Spoilage molds are mainly characterized by growth at low temperature, at low $a_{w,}$ and in atmospheres of low oxygen tension. Some of these molds also have an advantage because they resist some preservatives. Species of mainly *Aspergillus* and *Penicillium* can produce low concentrations of mycotoxins or other toxic metabolites on cheese as a result of growth during maturation, distribution, or storage in homes. Overall, the presence of molds in dairy products can be either acceptable or unacceptable depending on why, when, and where the molds have grown. Control of undesirable mold infection and growth in or on dairy products puts emphasis on hygiene, clean air practice, and the use of preservatives and controlled atmosphere packaging.

Molds Involved in the Spoilage of Dairy Products

Cheese

Although molds can be isolated from many dairy products, spoilage by molds is mainly associated with cheeses. The susceptibility depends on several conditions, namely, sanitation during manufacture and ripening, length and degree of ripening, storage conditions (temperature, relative humidity, type and extent of packaging), water activity (a_w), and composition. During the ripening of cheese, species from genera such as *Penicillium*, *Cladosporium*, as well as *Phoma* and other minor molds are found on cheeses because they grow in the refrigerator at temperatures as low as 1–5 °C. *Penicillium* species can grow at a_w levels that approach 0.80; however, *Cladosporium* species only grow down to a_w of only 0.86. Some *Penicillium* species, particularly *P. roqueforti*, can grow in low oxygen (at 1%); however, carbon dioxide at levels of 40% or more can prevent growth. In various surveys of molds in commercial packages of cheese (hard, semihard, semisoft), 50 to > 90% of the isolates were *Penicillium* species, with *P. commune*, *P. nalgiovense*, and *P. roqueforti* dominating the spoilage microflora and other *Penicillium* species (*P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. cyclopium*, *P. expansum*, *P. glabrum*, *P. granulatum*, *P. palitans*, *P. solitum*, *P. verrucosum*, *P. viridicatum*) being isolated less often. Although exceptions may occur, the above appear to be representative of the results that have been reported internationally from several countries including Australia, New Zealand, South Africa, Argentina, Norway, Turkey, Spain, Italy, Denmark, Greece, France, Germany, Belgium, Japan, the Netherlands, the Czech Republic, Switzerland, Malta, Costa Rica, Azores, the United Kingdom, and the United States.

In the literature, many names are used for *Penicillium* species that are no longer recognized; the currently accepted names are listed in **Table 1**. In addition, species of *Aspergillus* (*A. versicolor*), *Cephalosporium*, *Cladosporium*, *Geotrichum*, *Mucor*, *Scopulariopsis*, and *Syncephalastrum* have been isolated at percentages less than 10% of the total mold isolates. In vacuum-packaged Cheddar cheeses, *Cladosporium cladosporioides*, *C. herbarum*, *P. commune*, *P. glabrum*, and *Phoma* species produce a condition termed 'thread mold' because these molds grow in the refrigerator and tolerate low levels of oxygen. Shredded cheese is particularly susceptible to spoilage by yeasts and molds. Modified atmosphere packaging in CO_2/N_2 (e.g., 73%/27%) has been explored and may be necessary to control mold growth.

Some molds can produce bitter peptides in surface-ripened or blue-veined cheeses, with strains of *P. camemberti*, *P. roqueforti*, and *G. candidum* being among those identified. In highly acidic Cottage cheese, molds may cause spoilage because many bacteria cannot grow. Besides causing

Table 1 Names of molds that have been either misidentified in the literature or renamed based on new taxonomic tools

Names of molds as cited in the literature	*Accepted name of molds*
Penicillium candidum, *Penicillium caseicolum*, *Penicillium caseicola*	*Penicillium camemberti*
Penicillium cyclopium, *Penicillium puberulum*, *Penicillium verrucosum* var. *cyclopium*	*Penicillium aurantiogriseum*[a]
Penicillium patulum, *Penicillium urticae*	*Penicillium griseofulvum*

[a] *Penicillium commune* is similar to *Penicillium aurantiogriseum* and either may be misidentified in the literature citations.

discoloration due to mold spore pigments and 'fuzzy' appearance on the surface, molds can produce numerous off-flavors that have been described as bitter, earthy, kerosene, musty, mushroom, plastic, rancid, and related flavors. Resistant species of *Penicillium*, such as *P. roqueforti*, decarboxylate the preservative sorbic acid to 1,3-pentadiene, which causes the kerosene off-flavor in chemically preserved cheese spreads. Other *Penicillium* species reduce sorbic acid to 4-hexanoic acid and 4-hexanol. Molds can grow in cheese because they can overcome several conditions that are unfavorable to other microorganisms, namely, low temperature, low oxygen levels, reduced a_w, lack of carbohydrates, and presence of chemical preservatives and free fatty acids.

Other Dairy Products

Although molds are normally associated with the spoilage of cheeses, spoilage of yogurt, butter, sweetened condensed milk, cream, and other dairy products is occasionally caused by mold growth. Species of *Absidia*, *Alternaria*, *Aspergillus*, *Monilia*, *Mucor*, *Penicillium*, and *Rhizopus* can grow on the surface of yogurt; however, mold growth is usually secondary to yeast growth because they grow more slowly than yeasts. Fruit preparations added to yogurts may be a source of molds and yeasts and should be considered; for example, some *Mucor* species grow well at refrigeration temperatures and also under conditions of very low oxygen tension. Heat-resistant molds may be introduced in the fruit preparations; however, such molds are often not of the psychrotrophic strains and may not grow at all at refrigeration temperatures. Improved sanitation and control of dairy plant air have reduced the level of mold spoilage of butter by lipase-producing species of *Aspergillus*, *Cladosporium*, *Geotrichum*, and *Penicillium*. Low-salt margarine is a product that invites increased fungal spoilage. *Penicillium* and *Cladosporium* species have been observed; these molds are lipolytic and also produce off-flavors including 2-methylisoborneol and geosmin, thus contributing to an undesirable earthy flavor. In sweetened condensed milk, species of *Aspergillus* and *Penicillium* can grow on the surface if there is poor sanitation in the processing plant that allows entry of mold spores and a large enough headspace in the can to provide oxygen for growth. Sometimes, cream that is stored for long periods at temperatures close to 0 °C can have *Penicillium* species growing on the top. *Geotrichum candidum* and yeasts can grow on cream containing added sucrose for sale to bakeries because they produce lipases. Heat-resistant fungi, which produce ascospores, do not normally spoil dairy products; however, there are reports of *Byssochlamys nivea*, *Eupenicillium brefeldianum*, *Neosartorya fischeri*, and *Talaromyces avellaneus* causing spoilage in products such as ultra-heat-treated (UHT) custard and cream cheese. In addition, *Fusarium oxysporum* has been isolated from UHT flavored milks in Australia, possibly due to the production of thick-walled chlamydoconidia and the ability to tolerate low oxygen tensions. Strawberry yogurt may also harbor relatively heat-resistant *Talaromyces*. Under some conditions, molds can occasionally grow in or on these different dairy products and cause spoilage.

Control of Mold Growth in Dairy Products

Several methods can be used to control the growth of molds on dairy products. One important way to prevent contamination of dairy products by molds is to use good practices to clean and sanitize dairy plants and processing equipment to reduce the level of mold spores in the environment. Areas that may be improperly cleaned and allow mold buildup include conveyor belts, pumps, and valves. The choice of sanitizers can be critical because chlorine seems to be more effective against molds than either peracetic acid or peroxides. Molds can grow in moist environments found in dairy plants and establish themselves on ceilings, floors, walls, and even in floor drains if these areas are not properly cleaned and sanitized. Mold spores can also enter the processing plant in raw ingredients, on packaging materials, and on people; therefore, all potential sources of molds need to be considered when determining whether contamination can occur.

Another source of contamination by mold spores is the air in the processing, ripening, and packaging areas; therefore, good air-filtration systems are essential in dairy processing plants to reduce the level of mold spores. Recommendations of fewer than 50–100 molds and yeasts per cubic meter have been suggested for cheese processing plants. One way to reduce molds in the air is to use high-efficiency particle air (HEPA) filters because they are designed to remove 90–99% of particles $\geq 0.3\,\mu m$ in size, which should help to reduce the level of mold spores in the air.

As with all filtration systems, proper maintenance and regular filter replacement are critical for good air quality. In addition, the use of positive air pressure in critical areas, such as rooms for ripening and packaging of cheese, can significantly decrease the level of mold contamination. The air intake and exhaust systems in the plant need to be properly separated from each other to prevent recontaminated air from coming back into the processing plant. In an effort to improve air quality, 'cleanroom technology' has been introduced into some cheese processing plants, especially in the ripening rooms. The design of the rooms is carefully monitored to control air filtration and circulation, movement of people who are dressed in cleanroom attire, and microbial contamination by using footbaths and airlocks before entering the area. Generally, cleanrooms have air zones that limit particles $>0.5\,\mu m$ per $0.3\,m^3$ of air, providing a room that is considered to be bacteria-free, because there are <0.1 bacteria per $0.3\,m^3$ of air. The humidifiers in these areas also operate by using sterilized and even demineralized water. In addition to humidity, control of the temperature of the ripening and storage rooms can help to slow down the growth of molds in cheeses.

Mold spoilage of cheeses has been considerably reduced by the use of new packaging technologies. Packaging material can be coated with antimycotic agents, such as sorbates, propionates, or natamycin, or, alternatively, antimycotic agents can be incorporated directly into the packaging material. Excluding oxygen by the use of vacuum and modified atmosphere packaging is also used to limit growth of molds on cheeses sold commercially. Usually, the use of more than 50% carbon dioxide and less than 0.5% oxygen prevents spoilage molds from growing on cheeses in modified atmosphere packages. However, package leakage and pinhole defects can be problems with these types of packages, allowing molds to grow and cause spoilage.

Toxic Metabolites Produced by Molds in Dairy Products

Mycotoxins and other toxic metabolites can be produced in cheese; however, many of these metabolites are produced in low concentrations or their toxicity for humans is slight or unknown at the present time. Generally, lower concentrations of mycotoxins are produced in cheese than in laboratory media; therefore, caution needs to be exercised when assessing the importance of these toxic metabolites to human health when reviewing laboratory research. The major fungal metabolites produced in cheese are listed in **Table 2**. *Aspergillus flavus* and *A. parasiticus*, which produce aflatoxins, rarely grow on cheese that is held at a temperature below 10 °C because these species have a minimum temperature for growth above 7 °C and, for aflatoxin production, usually above 15 °C. If aflatoxin M_1 is present in the milk, then it may still be present in dairy products made from that milk. A concern about the presence of mycotoxin-producing fungi in cheese is that several fungi reported in the literature have been misidentified; hence, the mycotoxins attributed to them have been inaccurate. One example is that *P. viridicatum* has been incorrectly identified as

Table 2 Mycotoxins produced in cheese by molds and potential health effects

Mycotoxin	*Mold producers*	*Effect on humans*
Citrinin	*Penicillium citrinum*, *Penicillium verrucosum*	Unknown, may affect kidneys
Cyclopiazonic acid	*Penicillium camemberti*, *Penicillium commune*	No current evidence for human toxicity
Ochratoxin A	*Penicillium verrucosum*,[a] *Aspergillus ochraceus*	Kidney disease (Balkan endemic nephropathy)
Patulin	*Penicillium expansum*, *Penicillium roqueforti* var. *carneum*	Low toxicity[b]
Penitrem A	*Penicillium crustosum*	Potential neurotoxicity in humans not well understood
Penicillic acid	*Penicillium aurantiogriseum*, *Penicillium cyclopium*, *Penicillium viridicatum*	Unknown
Roquefortine C	*Penicillium* species (*P. chrysogenum*, *P. crustosum*, *P. roqueforti*)	Unknown for humans because of low toxicity
Rugulovasine A and B	*Penicillium commune*	Unknown
Sterigmatocystin[c]	*Aspergillus versicolor*	Potential for liver cancer but 1/150 strength of aflatoxin

[a] *Penicillium viridicatum* has been misidentified as producing ochratoxin A (incorrectly cited in many journal articles).
[b] Reports of carcinogenicity have not been documented.
[c] Precursor of aflatoxin.

producing ochratoxin A and citations to this still occur in the literature.

Although several mycotoxins are listed in **Table 2**, the ones that may be of importance because of potential human health effects are ochratoxin A and sterigmatocystin. Since *P. verrucosum* and *A. ochraceus*, which produce ochratoxin A, are not frequent isolates from cheese, the presence of ochratoxin A may be of minor importance. *Aspergillus versicolor* produces sterigmatocystin and both the mold and its mycotoxin have been isolated from cheese in temperate climates. Since *A. versicolor* is a mesophile that does not grow below 10 °C and can be controlled by modified atmosphere packaging, both refrigerated ripening and storage of cheese plus packaging to reduce oxygen prevent its growth and toxin production. Molds that produce mycotoxins grow on the surface of cheese. However, studies on the migration of mycotoxins into cheese rarely show greater than a 2 cm movement into the cheese; therefore, cutting more than 2 cm under the moldy portions of the cheese should remove the mycotoxin as well as the mold.

Much research has been done on the growth of molds on cheeses and the production of various mycotoxins. When studies have been done to detect mycotoxins in naturally contaminated cheeses, the results have varied from the detection of moderate amounts of the mycotoxins to absence of mycotoxins. The effects of mycotoxins on human health still need to be evaluated. The importance of mycotoxins in cheese also needs further study; however, the use of low ripening and storage temperatures (<10 °C) and vacuum or modified atmosphere packaging can greatly reduce the potential for mycotoxin production in cheese.

Enumeration of Molds from Dairy Products

The methods for the enumeration of molds from dairy products vary from country to country; however, the International Commission on Food Mycology has made recommendations for enumeration media and techniques over the past two decades. These recommendations are beginning to become the methods of choice in many countries and are now appearing as the standards in some manuals and governmental publications on official microbiological methods. For general mold enumeration in dairy foods with high a_w (>0.95), dichloran rose bengal chloramphenicol (DRBC) agar is the medium of choice because the dichloran (2,6-dichloro-4-nitroaniline) and rose bengal prevent the spreading of molds and thereby allow easier counting of colonies and the chloramphenicol inhibits bacterial growth. Spread or surface plates are prepared because molds need plenty of air for growth. The plates are incubated upright at 25 °C for 4–5 days for most molds. Additional incubation time may be needed for slower-growing molds. In cheeses where the a_w is below 0.95, dichloran 18% glycerol (DG18) agar is recommended because, in this medium, the final a_w is 0.955. Most *Penicillium* and *Aspergillus* species that are associated with cheese grow on this medium with lowered a_w. Chloramphenicol is used as the sole bacterial inhibitor in these media because it can be autoclaved and remains stable in the prepared media. A different medium, creatine sucrose dichloran agar (CREAD), was developed for selection of mold species occurring on foods particularly high in lipids and protein. Molds commonly detected on cheese, *Penicillium* and *Aspergillus* species, grow well on this medium; however, certain airborne penicillia, for example, *P. brevicompactum*, do not thrive on this medium or on the cheese. The use of acidified media is discouraged because acid-sensitive molds can be prevented from growing on the media. Although potato dextrose agar has been used previously for enumeration of molds, this medium is low in nutrients, and this can prevent some species of *Penicillium* and *Aspergillus* from growing. The use of plate count agar or similar media does not prevent the spreading of rapidly growing molds, and colonies could be hard to count. The development of selective media for the isolation of toxigenic molds has been done for *A. flavus/parasiticus* and some toxigenic *Penicillium* species; however, these media have been mainly used for foods other than dairy products. Newer methods to enumerate and detect molds in foods are being developed using enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and other molecular biological techniques. In the future, there may be rapid and more precise methods for the enumeration and identification of molds in dairy products.

See also: **Analytical Methods**: DNA-Based Assays; Immunochemical Methods; Microbiological. **Cheese**: Acid- and Acid/Heat Coagulated Cheese; Starter Cultures: General Aspects; Starter Cultures: Specific Properties. **Fermented Milks**: Starter Cultures. **Nutrition and Health**: Effects of Processing on Protein Quality of Milk and Milk Products. **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds; Mycotoxins: Classification, Occurrence, and Determination; *Penicillium camemberti*; Yeasts in Milk and Dairy Products.

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Aspergillus flavus

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Introduction

Aspergillus flavus is a member of the *Aspergillus* genus, which contains more than 100 recognized species, most of which grow well on common synthetic or semisynthetic media and around 50 of which have been shown to produce toxic metabolites. *Aspergillus flavus* is a long-established and well-defined species dating from 1806 and can be classified in *Aspergillus* sect. *Flavi.* It is closely related to *A. parasiticus* and to *A. oryzae* and *A. sojae*, the latter two species being particularly important in the manufacture of fermented foods in Asia. The taxonomy of *Aspergillus* has suffered for many decades by the incorrect application of the rule of the International Code of Botanical Nomenclature (ICBN), leading in many cases to the inaccurate identification of many species as *A. flavus*, when in fact they were *A. parasiticus* or *A. nomius* species. *Aspergillus flavus* and *A. parasiticus* species, while very similar, can be differentiated on mycotoxin production profiles with *A. flavus* isolates usually producing B aflatoxins, with fewer than 50% of isolates being toxigenic, while *A. parasiticus* isolates produce G as well as B aflatoxins and are all invariably toxigenic. *Aspergillus nomius* morphologically resembles *A. flavus*, but differs by producing smaller, more elongated sclerotia than those of *A. flavus,* which are more globose, and by the production of B and G mycotoxins, and in the production of a unique metabolite nominine, which exhibits activity against *Helicoverpa zea* (corn earworm) larvae in dietary assays at $100\,\mu g\,g^{-1}$.

Aflatoxin B1 (AFB_1) is a potent human carcinogen that is produced by *A. flavus*. If AFB_1 is ingested by dairy animals in contaminated feedstuffs or forage, the metabolite is biotransformed at the hepatic level by microsomial cytochrome P450 into aflatoxin M1 (AFM_1). It has been estimated that approximately 1–3% of the AFB_1 initially present in animal feedstuff appears as AFM_1 in milk. Given the evidence that AFM_1 is a genotoxic carcinogen and that milk has the greatest demonstrated potential for the introduction of AFM_1 into the human diet, consumption of AFM_1 contaminated infant milk and milk products by infants and young children in particular should be avoided. Thus, very low AFM_1 limits have been set ($0.01–0.05\,\mu g\,kg^{-1}$) for infant foods, given the relatively high consumption rate of these products by infants, their low body weight, and the potential higher susceptibility of young children to aflatoxins.

Morphology

Aspergillus flavus can be readily distinguished from other *Aspergillus* species, by lack of growth at 5 °C, by rapid growth at both 25 and 37 °C, and by the production of a bright yellow-green conidial color, when cultured on malt extract agar (MEA) or Czapek yeast extract agar (CYA). Colony growth on CYA can vary, from rapid growth reaching around 60–70 mm in diameter to slower growth of 30–40 mm in diameter at room temperature (24–26 °C) in 10 days. Colonies usually consist of a thin, close-textured, basal mycelium. Most strains produce abundant conidial structures directly from the mycelium. The sclerotia, which are produced by many strains, particularly in fresh isolates, can dominate the colony appearance. They first appear as white mycelial tufts that are characteristically globose to subglobose, before gradually changing from white through dark reddish-brown to black in color and appearing spherical, from 400 to 800 μm in diameter. Following 42–48 h growth on *A. flavus* and *A. parasiticus* agar (AFPA), colonies of *A. flavus* exhibit a brilliant orange-yellow reverse coloration. Few other colonies produce this coloration on AFPA medium. However, prolonged incubation on AFPA, beyond 4 days, is not recommended, because *A. ochraceus* and other closely related species may also produce yellow reverse color after this time. This aside, AFPA is recommended for the detection and enumeration of *A. flavus* strains in nuts, corn, spices, and other commodities.

Aspergillus flavus produces conidial heads that are typically radiate and highly variable in both shape and size, usually possessing relatively thin, finely roughened, or, rarely, smooth walls.

Conidiophores are borne from either subsurface or surface hyphae. Stipes (hyphae supporting the fruiting structure, forming the conidiophore) can be 400 μm to 1 mm or more in length. The vesicles are spherical to subspheroidal in shape and are usually 20–45 μm in diameter, but can be up to 50 μm in diameter. They usually bear both metulae and phialides, but occasionally in some isolates a fraction or even a majority bear phialides alone.

Various isolates of *A. flavus* appear to have a requirement for $0.2\,ng\,g^{-1}$ molybdenum for growth and conidial formation. Molybdenum deficiency appears to depress growth, conidial formation dry weight, soluble protein, and the specific activities of nitrate reductase, succinic dehydrogenase, and aconitase in the fungus.

Ecology

Members of the *A. flavus* group are distributed widely in nature and have been routinely isolated from soils, particularly in tropical and subtropical areas, and from forage and decaying vegetation. Some of them are pathogenic to insects, and much less commonly to higher animals, including humans. They are commonly isolated from plant materials undergoing microbial decomposition, from grains and stored seeds, and from a variety of different food products. They contribute to the decomposition process at moisture levels above those tolerated by the *A. candidus* and *A. glaucus* groups. *Aspergillus flavus* can invade maize, peanuts, and cottonseed in the field, while in other types of agricultural crops it behaves as other storage fungi do, and does not invade seeds before harvest. Due to the potential aflatoxin problems associated with *A. flavus,* its presence has been sought in many different types of foodstuffs. This, coupled with the relative ease of identification, has led to *A. flavus* becoming the most widely reported foodborne fungus. It appears to be particularly prevalent in the tropics with many reports of its growth on oilseeds and nuts, in particular peanuts. In the United Sates and Europe, *A. flavus* has been reported to occur in a variety of other nuts besides peanuts, from time to time. These include coconut, copra, hazelnuts, kola nuts, pecans, pistachios, and walnuts. *Aspergillus flavus* is one of the dominant species found on stored products, particularly grains, as it is able to thrive in low-water activity (a_w), high-temperature environments. Cereals are also commonly spoiled by the fungus, with maize and maize-based products being particularly susceptible to spoilage. Contamination has also been reported in wheat, wheat flour, and flour products, including bread. Growth on wheat results in the production of methylfuran, 2-methylpropanol, and 3-methylbutanol, and the presence of these compounds is often regarded as an indication of deterioration in grain due to fungal growth. In addition, growth on cotton fibers, for example, results in the production of a bright greenish-yellow fluorescence, thought to be due to kojic acid, which is produced by the fungus and then converted to the fluorescent substance by plant tissue peroxidases. This fluorescence is also visible in maize and other grains in which the fungus has grown. *Aspergillus flavus* has also been found to contaminate pasta and bran, barley, paddy, milled and parboiled rice and rice bran, sorghum, and pearl millet. Unlike in the case of crops high in oil, spoilage in small-grain cereals is usually a result of poor handling. Interestingly, *A. flavus* has been shown to produce anti-insectan metabolites. Following the initial observation that the sclerotia of *A. flavus* were avoided by the common detritivorous beetle *Carpophilus hemipterus*, an insect that feeds on the conidia and mycelium of the fungus, a number of secondary metabolites of the sclerotia were isolated and shown to have anti-insect properties. The most potent of these metabolites is also nontoxic to vertebrates at 300 mg kg^{-1}. Some of these compounds have also been shown to be active against *Helicoverpa zea.* Whether these metabolites are produced as a type of 'defense mechanism' by the fungus is open to debate.

Aspergillus flavus has also been reported to be present in many different kinds of spices together with green coffee beans and herbal drugs. Other reported sources of the fungus include chickpeas, pigeon peas, soybeans, olives, and rapeseed; and other seeds such as mustard seeds, sesame seeds, amaranth seeds, sunflower seeds, and betel seeds. *Aspergillus flavus* has been reported to be present in a variety of food products, including processed and smoked meats, bacon, milk, and cheese, particularly in countries where refrigerated storage facilities are not always available. Processed cheese is a very good growth substrate for *A. flavus*. It has also been isolated from smoked and dried fish, dry-cured hams, and Italian-type salami. Finally, the fungus is also capable of spoiling fruit and vegetables such as citrus, peppers, pineapples, and tomatoes, but spoilage of these particular types of materials is not usually of great economic importance. Interestingly, a green fluorescent protein (GFP) reporter expressing *A. flavus* strain has recently been developed to monitor fungal growth, mode of entry, colonization of cottonseeds, and production of aflatoxins by the fungus.

Physiological Factors Affecting Growth of the Fungus

Water Activity

Aspergillus flavus is xerophilic, being capable of growth down to around a_w 0.78, with an optimum a_w of 0.99. Reported data for growth at various a_w values show some variation, from a low of 0.78 at 33 °C to 0.84 at 25 °C, with other reports of a minimum of 0.82 at 25 °C, 0.81 at 30 °C, and 0.80 at 37 °C. Conidia of *A. flavus* can germinate at a_w of 0.75 a_w and 29°C, but do not grow, while at a_w less than 0.75 conidia remain dormant but viable. Lag times before germination increase with decreasing a_w, where at high water activities (>0.98), lag times vary from a few hours to several days, and they can even extend to several months at lower a_w. The salinity and osmotic pressure of the growth medium affect the production of conidia. The vegetative growth of *A. flavus* increases with an increase in the NaCl content up to 9% NaCl, but at higher salt concentrations inhibitory effects are observed on the production of conidia. However, *A. flavus* growth and aflatoxin production on processed cheese have been shown to be reduced through the addition of 6% NaCl. The lower limit of moisture for growth of *A. flavus* on cereal grains such as maize, wheat, sorghum, and rice is 18.0–18.5%; for soybeans it is 17–17.5%; and for peanuts, sunflower seeds, and copra it is 11.0–12.0%. Survival of conidia of *A. flavus* in a variety of dried foods (a_w 0.32–0.78) at 21 °C is reduced at high a_w and low pH. The effect of a_w on colony growth rate for each species has been employed to quantify the 'relatedness' of four species belonging to *Aspergillus* sect. *Flavi* (*A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. nomius*). A linear model was subsequently proposed in which *A. oryzae* and *A. parasiticus* are very close to each other, placed between *A. nomius* and *A. flavus* and closer to the latter species.

Temperature

Aspergillus flavus grows at temperatures as low as 10–12 °C and as high as 50–55 °C, with optimal growth occurring at temperatures near 33 °C. At optimal growth temperatures specific growth rates can reach 500 μm h^{-1} (or approximately 25 mm day^{-1}). While most storage fungi have a minimum temperature for growth of 0–5 °C, optimum of 25–30 °C, and a maximum of 40–45 °C, *A. flavus* has been reported to grow on Cheddar cheese at 15, 18, or 25 °C and to produce aflatoxin on the cheese at 25 °C. *Aspergillus flavus* can grow vigorously at 50–55 °C and can raise the temperature of the materials in which it is growing to that level, maintaining it there for some weeks. The fungus is not very heat resistant, with a D_{45} value of more than 160 h, a D_{50} of 16 h, a D_{52} of 40–45 min, and a D_{60} of 1 min, at neutral pH and high a_w, with *z*-values from 3.3 to 4.1 °C being reported. a_w provides a degree of protection. At 52 °C, the *D*-values for conidia increase from 44 to 54 min with increase in level of sucrose from 0 to 60% (a_w 0.99–0.89). In addition, high sucrose concentrations reduce the effect of preservatives on *D*-values. Thus, in general, preservatives act synergistically with heat at low a_w values to reduce heat resistance in *A. flavus*. The combined and independent effects of sucrose, sodium chloride, potassium sorbate, and sodium benzoate on heat inactivation of conidia of *A. flavus* have shown that increasing concentrations of sucrose results in increased tolerance to heat by the fungus, while low concentrations (3 and 6%) of sodium chloride also protect *A. flavus*. Potassium sorbate and sodium benzoate acted synergistically with heat to reduce sensitivity to preservatives and reduced a_w, whether achieved by the presence of sucrose or sodium chloride, thus demonstrating heat-induced injury. At the same concentration, potassium sorbate is more inhibitory than sodium benzoate to colony formation by *A. flavus*, and the presence of sucrose and sodium chloride enhances this inhibition. Conidia of *A. flavus* have been reported to be resistant to freezing in water at −73 °C. It is believed that this survival may be partially due to a very low water content such that little or no ice formation occurs, which can affect the integrity of the spore. *Aspergillus flavus* is also extremely tolerant to freezing injury, remaining viable for over 20 years in liquid nitrogen vapor.

pH

Several reports have singled out the importance of pH on mold growth and indicated that the pH effect may vary with mold type, acid, and levels of other variables. Growth of *A. flavus* is largely unaffected by pH; it can grow over the entire pH range from 2.1 to 11.2, although growth rates are slower at pH <3.5. Some reports have shown complete inhibition at pH <3 and a 50% reduction of mycelium production at pH <4. In six strains of *A. flavus* that were tested, a pH change from 6.0 to 4.0 reduced the mycelium production by 13%.

Preservatives, even at low concentrations, reduce the heat resistance of *A. flavus* conidia markedly, particularly at low pH, with sodium benzoate being more effective in this regard than potassium sorbate. It has been reported that 1500 μg g^{-1} vanillin (4-hydroxy-3-methoxybenzaldehyde) is inhibitory at pH 3.5 for *A. flavus*. In addition, it has been shown that on potato dextrose agar at an a_w of 0.98 mold germination times and radial growth rates are affected by vanillin and pH, with increases in lag time being observed with increasing vanillin concentration and reduced pH.

Studies on the growth of *A. flavus* under modified atmosphere packaging (MAP) conditions, where the combined effect of a_w, pH, storage temperature, headspace oxygen, and CO_2 concentration on the growth of *A. flavus* on synthethic media using response surface methodology (RSM) were analyzed, indicated that *A. flavus* can grow in a CO_2 enriched atmosphere if headspace oxygen is present. In addition a_w, pH, storage temperature, and initial concentration of headspace oxygen in the gas mix are all highly significant factors ($p < 0.01$) in controlling the growth of the organism on synthetic media.

Genetics

DNA-based techniques have been developed and applied in the molecular identification and detection of *A. flavus*, primarily in an effort to distinguish between aflatoxin-producing and nonproducing strains. Research groups have targeted genes that are involved in aflatoxin biosynthesis in *A. flavus,* such as *ver-1*, *omt-1*, and *apa-2* and designed PCR (polymerase chain reaction) primers based on these genes. These PCR primers have then been used to detect aflatoxigenic strains of the fungus in grains and foods. Geisen and coworkers, targeting these three genes in a multiplex PCR-based approach, succeeded in differentiating *A. flavus* from two other *Aspergillus* species from section *Flavi*, namely, *Aspergillus sojae* and *Aspergillus oryzae*. In addition, they were able to distinguish between toxigenic and atoxigenic *A. flavus* strains. In follow-on studies using sequences of the *nor-1* gene, primers have been set up together with a probe for a TaqMan^{TM} real-time PCR assay, in which *A. flavus* has been quantified in contaminated food samples and in cereals. A PCR-based method targeting the *aflR* gene has also been developed, with specific primers being designed to generate a PCR fragment, and restriction fragment length polymorphism (RFLP) of the PCR product being performed, which allowed the differentiation between *A. flavus* and *A. parasiticus* in spiked samples of sterile maize flour.

Reverse transcription-polymerase chain reaction (RT-PCR) has been applied to differentiate aflatoxin-producing from aflatoxin-nonproducing strains of *A. flavus*. Specific primers were employed, which were based on the conserved regions of the nine structural genes, *aflD*, *aflG*, *aflH*, *aflI*, *aflK*, *aflM*, *aflO*, *aflP*, and *aflQ,* and two regulatory genes, *aflS* and *aflR,* of the aflatoxin B1 biosynthetic pathway. Expression of the *aflD*, *aflO*, and *aflP* genes in particular was shown consistently to correlate with aflatoxin production by the fungus. Amplified fragment length polymorphism (AFLP-) based techniques have also been employed in an attempt to distinguish *A. flavus* from other *Aspergillus* species from section *Flavi,* but have proven to be problematic. In one study, 500 potentially polymorphic fragments were identified following AFLP analysis involving the use of 12 different primer combinations on 24 of *A. sojae, A. parasiticus*, *A. oryzae,* and *A. flavus* isolates. Subsequent analysis of the AFLP data allowed the separation of the *A. sojae*/*A. parasiticus* isolates from the *A. oryzae*/*A. flavus* isolates. However, despite the presence of many polymorphisms between isolates within the *A. oryzae*/*A. flavus* subgroup, no markers that distinguish between the two species could be identified. Subsequent sequencing of the ribosomal DNA ITSs (internal transcribed spacers) from selected isolates from the *A. oryzae*/*A. flavus* subgroup resulted in the identification of some ITS variation between isolates within this subgroup, but did not correlate with the species classification, indicating that it is difficult to use molecular data to separate the two species.

Other DNA-based techniques have, however, been successfully employed in the genetic identification of *Aspergillus* section *Flavi* fungal isolates. A novel method for heteroduplex panel analysis (HPA), which utilizes fragments of the ITS regions (ITS1-5.8S-ITS2) of the rRNA gene that can be PCR amplified with universal primer has been developed. The method involves formation of heteroduplexes with a set of reference fragments amplified from *A. flavus*, *A. parasiticus*, *A. tamarii*, and *A. nomius*, and subsequent comparison with species-specific standard panels generated by pairwise reannealing among reference fragments. This HPA approach appears to be a useful identification method that may be particularly suitable for rapid and inexpensive screening of large numbers of *A. flavus* isolates.

In addition, ITSs, inter-simple sequence repeats (ISSRs), and random amplified polymorphic DNA (RAPD) molecular markers have also successfully been used to characterize *A. flavus* strains genetically. In one study a high degree of genetic diversity was revealed by RAPD and by ISSR, using the primer (GACA)4, which generated ISSR and RAPD profiles that allowed strain identification. Recently, the *A. flavus* genomics program has been launched with the major objective being the identification of genes involved in aflatoxin biosynthesis and regulation, as well as in pathogenicity. The *A. flavus* genome has been sequenced and initial annotation has revealed genes that potentially encode for enzymes involved in secondary metabolite production in the fungus. Genome-wide analysis of *A. flavus* will provide a better understanding of not only the mechanism of aflatoxin formation in the fungus and the factors affecting production of the mycotoxin, but will also allow strategies to be developed to control aflatoxin contamination of preharvest agricultural crops and postharvest grains during storage.

Thus, these molecular-based techniques represent significant progress toward the detection and identification of *A. flavus* strains and in increasing our understanding of the physiological parameters involved in mycotoxin production in the fungus. These techniques will prove useful not only in the detection of aflatoxigenic and nonaflatoxigenic strains in Hazard Analysis Critical Control Point systems, but also in *A. flavus* species identification, where they will ultimately be employed together with traditional techniques, resulting in a higher efficiency of isolate characterization and in differentiating *A. flavus* species and strains.

Preservatives

A large number of both naturally occurring metabolites and chemical preservatives affect the growth of *A. flavus*.

Naturally Occurring Preservatives

Lactococcus lactis subsp. *lactis* CHD-28.3 has been shown to exert antifungal activity against different *A. flavus* species. This antifungal activity is due to a proteinaceous compound, and given the generally regarded as safe (GRAS) nature of lactococcal species, this compound may prove useful in the preservation of different milk-based foods. The antifungal properties of extracts from plants and plant parts used as flavoring agents in foods and beverages have been well documented. Citrus oils when added to grapefruit juice or glucose-yeast extract medium at a concentration of 3000–3500 mg kg^{-1} suppress growth of

A. flavus, while orange oil added to either medium at concentrations up to 7000 mg kg^{-1} also results in reduced growth rates. Volatile compounds derived from maize have been found to affect growth and aflatoxin production in *A. flavus*. Five different concentrations of aqueous extracts of the plants *Lupinus albus*, *Ammi visnaga,* and *Xanthium pungens* (2, 4, 6, 8, and 10 mg ml^{-1}) inhibited the mycelial growth of *A. flavus* in a chemically defined medium. The inhibitory effect has been shown to be proportional to the applied concentration. Extracts from seven *Allium* plants (garlic, bakeri garlic, Chinese leek, Chinese chive, scallion, onion bulb, and shallot bulb) also exhibit antifungal activity against *A. flavus*. With the exception of scallion, the inhibitory effect decreased with increasing incubation and heating temperature ($p < 0.05$). Acetic acid treatment of the extracts increases the inhibitory effect for all *Allium* plants against the three fungi than heat treatment alone ($p < 0.05$). Treatment of the extracts with NaCl, at concentrations of 0.2 and 0.4 mol l^{-1}, does not affect the inhibitory effect of the plant extracts. Thus, a combination of acetic acid plus *Allium* plants appears to be an effective way to inhibit growth of the fungus. Essential oils and methanol extracts from the plant of *Satureja hortensis* have recently been reported to have strong antifungal activity against *A. flavus*, while essential oils extracted from the leaves of *Chenopodium ambrosioides* Linn. (Chenopodiaceae) have also been reported to inhibit completely the growth of aflatoxigenic strains of the fungus.

A number of reports have cited the inhibitory effects of onion extracts on *A. flavus* growth. Thiopropanal *S*-oxide, a compound in the ether extract of onion, has been demonstrated to inhibit the growth of *A. flavus*. The activity is apparently lost, however, by heating, freeze-drying, dehydration, aeration, agitation, and storage. Ethanol extracts of Welsh onion also appear to have an inhibitory effect on *A. flavus* growth. The mycelial growth of *A. flavus* cultured on yeast extract-sucrose broth is completely inhibited in the presence of ethanol extracts of the Welsh onions at a concentration of 10 mg ml^{-1} during 30 days of incubation at 25 °C. These extracts showed more pronounced inhibitory effects against *A. flavus* than did the same added levels of the preservatives sorbate and propionate at pH values near 6.5. The survival of spores of *A. flavus* depends on both the extract concentration and the exposure time of the spores to the onion extracts. Other reports indicate that natural nontoxic materials including extracts of eugenol and garlic can inhibit the mycelial growth of *A. flavus*, with garlic extract being particularly effective (approximately 62% growth inhibition). Base-soluble proteins (BSPs) and methanol-soluble polysaccharides (PSs) from *A. flavus*-susceptible (Huffman) genotypes of maize have also been shown to possess antifungal activity, with microgram quantities of the protein and polysaccharides being sufficient to retard fungal growth.

Aqueous extracts from three Egyptian plants, namely, *Ammi visnaga* (Umbellliferae), *Lupinus albus* (Leguminosae), and *Xanthium pungens* (Compositae), at varying concentrations ranging from 2 to 10 mg ml^{-1}, have been shown to inhibit *A. flavus* growth in a dose-dependent manner. The radial growth of *A. flavus* in solid culture was inhibited when exposed to atmospheres containing various cotton-leaf-derived volatiles. While 3-methyl-1-butanol and 3-methyl-2-butanol inhibited *A. flavus* growth by 20%, the most bioactive compounds were the C6–C9 alkenals, which completely inhibited fungal growth. Propolis ethanolic extract (PEE) at 3 and 4 g l^{-1} and ultra-griseofulvin (UG) at 0.75 and 1 g l^{-1} have been shown to reduce the percentage of conidia germination in *A. flavus* isolates, with PEE at 1–4 g l^{-1} decreasing the mycelial dry mass of *A. flavus* isolates by 11–80% and UG concentrations of 0.25–1 g l^{-1} reducing the growth of the isolates by 16–88%. At equal concentration, UG is about 4 times more effective than PEE. Essential plant oils and their components have been shown to be effective in protecting maize kernels from infection by *A. flavus*. Essential oils of *Cinnamomum zeylanicum* (cinnamon), *Mentha piperita* (peppermint), *Ocimum basilicum* (basil), *Origanum vulgare* (oregano), *Teloxys ambrosioides* (the flavoring herb epazote), *Syzygium aromaticum* (clove), and *Thymus vulgaris* (thyme) are known to cause a total inhibition of *A. flavus* development on maize kernels. In addition, the plant components thymol and *O*-methoxycinnamaldehyde have been shown to significantly reduce maize grain contamination. The optimal dosage for protection of maize varies from 3 to 8%, with residual effects in some cases being detected up to 4 weeks after kernel treatment. An essential oil from *Cymbopogon citratus* has also been shown to exhibit fungitoxicity toward *A. flavus* with an MIC of 1000 mg l^{-1}. The fungitoxic potency of the oil remains unaltered for 7 months of storage upon introduction of high doses of inoculum of the test fungus. It is thermostable in nature at temperatures ranging between 5 and 100 °C. Essential oils and methanol extracts from the plant *Satureja hortensis* have recently been reported to have strong antifungal activity against *A. flavus*, while essential oils extracted from the leaves of *Chenopodium ambrosioides* Linn. (Chenopodiaceae) have also been reported to completely inhibit the growth of aflatoxigenic strains of the fungus.

Herbs and spices have been shown to inhibit the growth of *A. flavus*, with water-soluble extracts of garlic bulbs, green garlic, and green onions showing an inhibitory effect. In some cases, increases in temperature from 60 to 100 °C significantly decreased this inhibitory effect, while acid treatments at pH 2, 4, or 6, or salt treatments at concentrations of 0.1, 0.2, 0.3, and 0.4 mol l^{-1} have no effect.

Finally, volatile metabolites of *Rhizopus arrhizus,* such as ethanol, isobutyl alcohol, and 3-methyl butanol, or of their mixtures when present in the vapor phase at levels ranging between 3 and 6% (v/v of headspace), have been shown to inhibit competitively the growth of *A. flavus*. This growth retardation can be synergistically enhanced by decreases in the a_w. The physical state of the alcohols, that is, their partition between solid and gaseous phases, seems to be one of the determinants of their antifungal activity. The mycoparasite *Humicola fuscoatra* has been reported to produce natural antifungal metabolites, namely, monorden (MIC >28 μg ml^{-1} of growth medium) and monocillin IV (MIC >56 μg ml^{-1}), and a new monorden analog that is active against *A. flavus* has been developed.

Chemical Preservatives

Sorbic acid (1000 mg l^{-1}) and monolaurin (750 mg l^{-1}) reduce the mycelial growth of *A. flavus*, with monolaurin being 2.4 times more active on a mole-per-mole basis than sorbic acid against the fungus. Formic acid at concentrations of 60 mmol l^{-1} has been shown to reduce the growth of *A. flavus* on barley. Polyunsaturated fatty acids have been reported to have sporogenic effects on the development of *A. flavus*, with the development of cleistothecia and sclerotia in the fungus affected by linoleic acid and light. Linoleic acid specifically induces precocious and increased asexual spore development and alters sclerotium production in *A. flavus* strains in which sclerotium production decreases in the light but increases in the dark. These sporogenic effects suggest that these factors may be significant environmental signals in the development of the fungus. Sodium diacetate has been shown to inhibit *A. flavus* at 0.1–0.5% in potato dextrose agar (pH 3.5 and 4.5) and in animal feeds and silage. Diethyl dicarbonate (DEDC), commonly called diethyl pyrocarbonate, has been shown to be fungicidal to *A. flavus* resulting in a 100% kill at concentrations of 1 g l^{-1}.

Phenolics act as antioxidants inhibiting the growth of *A. flavus*. A number of research groups have reported that butylated hydroxyanisole (BHA) at concentrations ranging from 100 mg l^{-1} through 200 to –750 mg l^{-1} results in inhibition of *A. flavus* growth. However, BHA is less effective in the presence of corn oil. BHA incorporated at 400 mg l^{-1} in processed cheese spread inhibits the growth of *A. flavus*, while lower concentrations of 150–200 ppm are effective when BHA is sprayed on the surface of the cheese. The pH of the product may also affect the activity of BHA, and there are reports stating that outgrowth of *A. flavus* conidia is inhibited to a greater extent at pH 3.5 than at pH 5.5. Phenolic compounds isolated from olive cake are also known to inhibit the growth of *A. flavus*. Vanillic and caffeic acids at concentrations 0.2 mg ml^{-1} and hydroxybenzonic, protocatechuic, syringic, *para*-coumaric acids, and quercetin at concentrations 0.3 mg ml^{-1} completely inhibit the growth of *A. flavus*. In addition, four compounds, three of which were phenolic in nature, which were extracted with acetone from cotyledons of freshly harvested peanut seeds, have been reported to inhibit the growth of *A. flavus*.

Benzoic acid derivatives also inhibit *A. flavus* growth. Benzoic acid (10 mg g^{-1}), sodium benzoate (24 mg g^{-1}), and salicylic acid (2 mg g^{-1}) completely inhibit the mycelial growth of *A. flavus* in groundnut. In a separate study, methyl benzoate and ethyl benzoate, at concentrations of 2.5 and 5.0 mg per 25 ml of medium, respectively, have also been shown to reduce the mycelial growth of *A. flavus*. A 96% formulation of gentian violet has been shown to be fungistatic to *A. flavus*, when incorporated into corn meal agar at 6.5, 12.8, 26.6, 39.0, and 156.0 mg kg^{-1} of gentian violet.

Studies on the effect of chloroperoxidase (EC 1.1.1.10) and hydrogen peroxide on the viabilities of quiescent and germinating conidiospores of *A. flavus* have shown hydrogen peroxide to be moderately lethal and chloroperoxidase to produce a 30-fold increase in the lethality of hydrogen peroxide to germinating conidia, which were 75-fold more susceptible to chloroperoxidase than were quiescent conidia. Mortality occurs due to oxidation rather than peroxidative chlorination.

Fungicides are known to inhibit the growth of *A. flavus*. In yeast extract-sucrose media, dicloran, iprodione, and vinclozolin fungicides significantly inhibit mycelial growth of *A. flavus* at 250 mg kg^{-1}. Sensitivity to fungicides increased approximately fivefold in a yeast extract-starch medium with an appreciable reduction in sugar uptake and α-amylase activity.

In a separate study, pyridazinone herbicides at concentrations of 20, 40, or 60 μg ml^{-1} herbicide have been shown not to affect mycelium production in *A. flavus*. Other agents, such as phosphine (insecticide), when added to grain at a_w 0.80 or 0.86 reduce growth of *A. flavus* without affecting survival of conidia. Finally, polyamines appear to play a role as modulators of microcycle conidiation in *A. flavus*, with putrescine being essential for vegetative growth of the fungus, while spermidine is involved in microcycle conidiation. A low putrescine/spermidine ratio is important for spore differentiation to microcycle conidiation.

Effects of Physical Agents

Near-UV (NUV) irradiation (10 and 20 min) has been shown to stimulate protein synthesis in *A. flavus*, with concomitant decreases in DNA synthesis, while far-UV (FUV) irradiation induces protein synthesis in *A. flavus* with no effect on DNA synthesis and reversal of RNA synthesis. UV irradiation produces no effect on the process of lipid synthesis. Total soluble carbohydrates increase markedly in *A. flavus* (240 min NUV), with FUV irradiation resulting in an increase in total soluble carbohydrates. Ozone treatments inactivate the fungus, with *D*-values for conidia of *A. flavus* exposed to 1.74 μg g^{-1} ozone in 1 mmol l^{-1} potassium phosphate buffer (pH 7.0 and 5.5) at 25 °C being 1.72 and 1.54 min at pH 5.5 and 7.0, respectively. Microwave energy also affects *A. flavus*, with germination of fungal spores on slides directly exposed to 6, 9, and 18 kJ for periods of 0–7 min being significantly reduced. *Aspergillus flavus* is also inactivated by doses of gamma irradiation between 0.6 and 1.7 kGy, with, for example, reports of complete growth inhibition and toxin production in ground beef samples treated with 1.5 kGy.

See also: **Contaminants of Milk and Dairy Products**: Environmental Contaminants. **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds; Mycotoxins: Classification, Occurrence and Determination.

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Mycotoxins: Classification, Occurrence and Determination

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Introduction

Since the discovery of carcinogenic aflatoxins from *Aspergillus flavus* isolated from the feed was connected to the death of a large number of turkeys (turkey X disease) in Great Britain in 1960, many studies on mycotoxins have been carried out worldwide. Such studies, however, had already begun in the 1950s in Japan, where it had been found that a yellow pigment, (−)-luteoskyrin, isolated from *Penicillium islandicum* grown on rice caused hepatopathy in experimental animals. Today, such toxic fungal metabolites as aflatoxins and (−)-luteoskyrin are called 'carcinogenic mycotoxins'.

Many toxic fungal metabolites cause paralysis, tremor, or convulsion in experimental animals. For example, citreoviridin isolated from *Penicillium citreoviride*, which infects rice, causes paralysis in experimental animals, and fumitremorgins isolated from *Aspergillus fumigatus* grown on miso (bean paste) causes tremor or convulsion in mice. These fungal metabolites, which have a toxic effect on the nervous system, are called 'neurotropic mycotoxins'.

There are many other mycotoxins, for example, trichothecenes isolated from some *Fusarium* species cause immunomodulation in experimental animals, and sporidesmins isolated from *Pithomyces chartarum* cause photohypersensitive eczema in sheep.

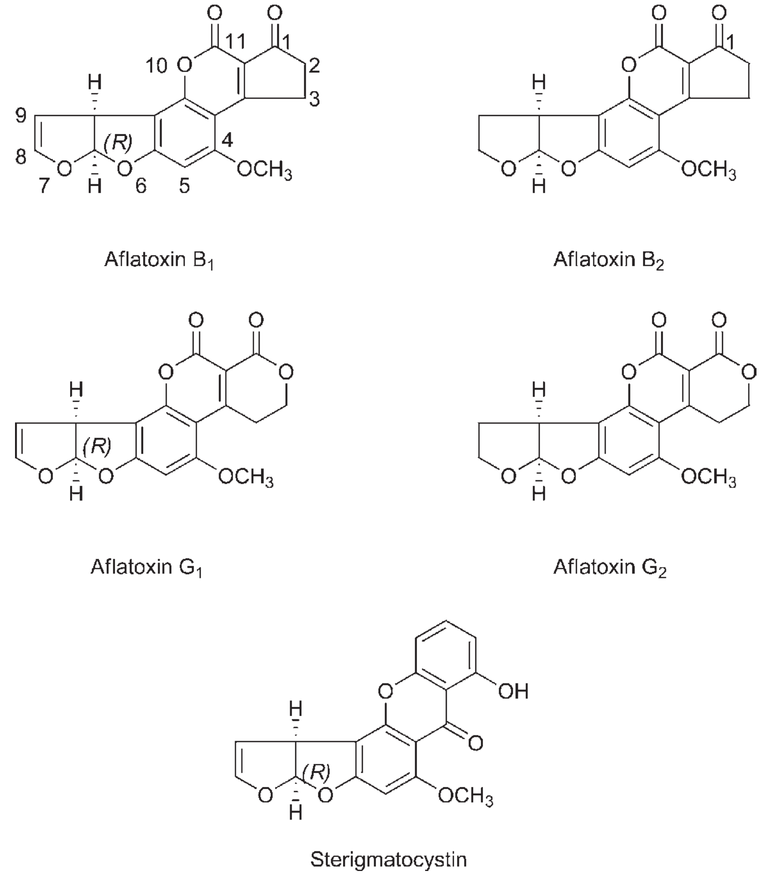
The important features of mycotoxins may be summarized as follows:

1. Mycotoxins are secondary metabolites of fungi (Fungi Imperfecti and Ascomycetes) that exhibit toxic effects called 'mycotoxicoses' when food or feed contaminated with them is ingested by humans or domestic animals.
2. Though mycotoxicoses often spread regionally and seasonally, they are not infectious diseases; therefore, mycotoxicoses are clearly distinguishable from mycoses, which are infectious diseases caused by infectious fungi like *Trichophyton* spp.
3. Almost all mycotoxicoses are chronic diseases, which result from a prolonged consumption of food or feed contaminated with tiny quantities of mycotoxins, and acute forms of mycotoxicoses are comparatively rare.
4. A large number of mycotoxins showing diversity in structure and in mode of action are known, because of which it is not easy to classify mycotoxins on the basis of their characteristics. In this article, mycotoxins are classified into three categories, carcinogenic, neurotropic, and other mycotoxins.
5. For qualitative and quantitative analyses and identification of mycotoxins, various chromatographic techniques, including column chromatography (CC), thin-layer chromatography (TLC), gas liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and various spectroscopic techniques such as nuclear magnetic resonance (NMR), infrared (IR), ultraviolet (UV), mass (MS), optical rotation, optical rotatory dispersion (ORD), and circular dichroism (CD) spectroscopy are employed, and various *in vitro* and *in vivo* bioassay methods are used on the basis of the biological properties of mycotoxins.
6. Among a large number of mycotoxins, polyketides synthesized through the acetate–malonate pathway, amides and peptides formed through the amino acid pathway, terpenoids formed through the mevalonate pathway, and complex metabolites formed through mixed-type pathways are of major importance (*see* **Yeasts and Molds**: *Aspergillus flavus*).

Carcinogenic Mycotoxins

Bisfuranoids: Aflatoxins and Sterigmatocystin

Aflatoxins are at present considered to be among the strongest natural carcinogens. These are produced mainly by the three fungi belonging to the *A. flavus* group, *A. flavus*, *A. parasiticus*, and *A. nomius*, which have been isolated from many types of food and feed, such as peanuts and cereals including maize, spices, nuts, beans, and sugarcanes. There are four congeners of aflatoxins: B_1, B_2, G_1, and G_2. Structurally, coumarino dihydro- and tetrahydro-bisfuran, a five-membered α,β-unsaturated cyclic ketone, is condensed to build up aflatoxins B_1 and B_2, and the six-membered α,β-unsaturated lactone is condensed to form aflatoxins G_1 and G_2 (**Figure 1**). Biosynthetically, all aflatoxins are formed from decaketide through the acetate–malonate pathway. Aflatoxins cause acute hepatopathy and chronic hepatic cancer in many species of mammals, fowl, and fish (the order of the peroral toxicity against ducklings is: aflatoxin $B_1 > G_1 > B_2 > G_2$). In an *in vitro* experiment, the dihydrobisfuran moiety in aflatoxin B_1 easily formed a covalent by bonded adduct with the base part of nucleic acid by way of an 8,9-epoxide type intermediate, suggesting that the carcinogenicity of aflatoxins B_1 and G_1 is the result of the dihydrobisfuran



Aflatoxin B_1

Aflatoxin B_2

Aflatoxin G_1

Aflatoxin G_2

Sterigmatocystin

Figure 1 Structures of aflatoxins B_1, B_2, G_1, and G_2, and sterigmatocystin.

moieties in their molecules, which inhibit normal protein biosynthesis by the formation of an adduct with the base part of nucleic acid (**Figure 2**).

Sterigmatocystin was first isolated as a yellow pigment from *A. versicolor* in 1954, and the structure was established in 1962 (**Figure 1**). Sterigmatocystin has also been isolated from *Aspergillus aurantio-brunneus*, *Aspergillus amstelodami*, *Aspergillus chevalieri*, *A. flavus*, *Aspergillus multicolor*, *Aspergillus nidulans*, *A. parasiticus*, *Aspergillus quadrilineatus*, *Aspergillus ruber*, *Aspergillus unguis*, and *Aspergillusustus*. Sterigmatocystin, the dihydrobisfuran moiety, which has the same configuration as that of aflatoxins, has already been shown to be a natural biosynthetic precursor of aflatoxin produced by *A. parasiticus*. Although sterigmatocystin has a dihydrobisfuran moiety quite similar to that of aflatoxin B_1, the carcinogenicity of sterigmatocystin is only about one-hundredth of that of aflatoxin B_1 because the solubility of sterigmatocystin is so much lower than those of aflatoxins. Sterigmatocystin has been reported in Gouda and Edam cheese contaminated by *A. versicolor* (*see* **Yeasts and Molds**: Mycotoxins: Aflatoxins and Related Compounds).

Bisanthraquinonoids: (–)-Luteoskyrin and (+)-Rugulosin

Rice contaminated with *P. islandicum*, *P. rugulosum*, *P. citrinum*, and *P. citreoviride* becomes yellow. (−)-Luteoskyrin has been isolated as a yellow pigment from rice infected with *P. islandicum*; it possesses a unique cage-type dimeric bisanthraquinonoid structure and shows levorotatory optical activity (**Figure 3**). The bisanthraquinonoid structure of (−)-luteoskyrin is formed from two molecules of anthraquinone, which are synthesized from octaketide through the acetate–malonate pathway. (−)-Luteoskyrin causes hepatopathy, including liver necrosis, fatty degeneration, and hepatic cancer. Hepatoma was induced by (−)-luteoskyrin in a dose-dependent manner when administered to mice for 216 days at 16.7, 68.8, and 84.6% at 50, 150 and 500 μg day^{-1}, respectively. This tumorigenic effect on the livers of mice was greater in males than in females.

Another yellow pigment, (+)-rugulosin, has been isolated from *P. rugulosum*. The structure of (+)-rugulosin is also a cage-type bisanthraquinonoid very similar to that of (−)-luteoskyrin, but this compound is dextrorotatory (**Figure 3**). (+)-Rugulosin also causes hepatic necrosis, fatty degeneration, and hepatic cancer in mice, but the

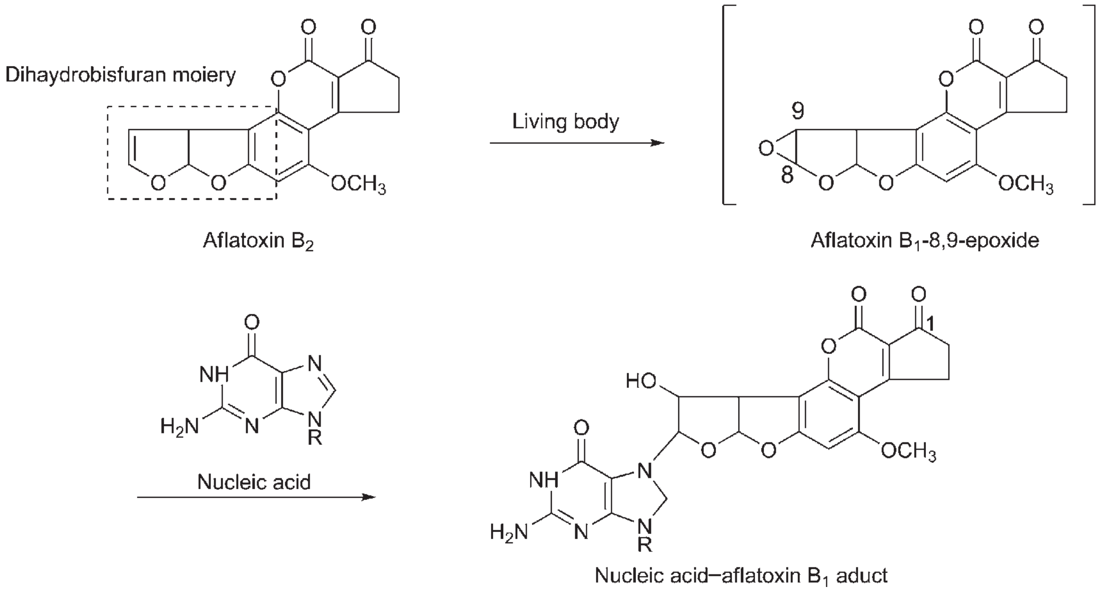

Dihaydrobisfuran moiery
Aflatoxin B$_2$
Living body
Aflatoxin B$_1$-8,9-epoxide
9
8
Nucleic acid
Nucleic acid–aflatoxin B$_1$ aduct
1
HO
NH
H$_2$N
R
OCH$_3$

Figure 2 Suggested role of the dihydrobisfuran moiety of aflatoxin B$_1$ in its carcinogenicity.

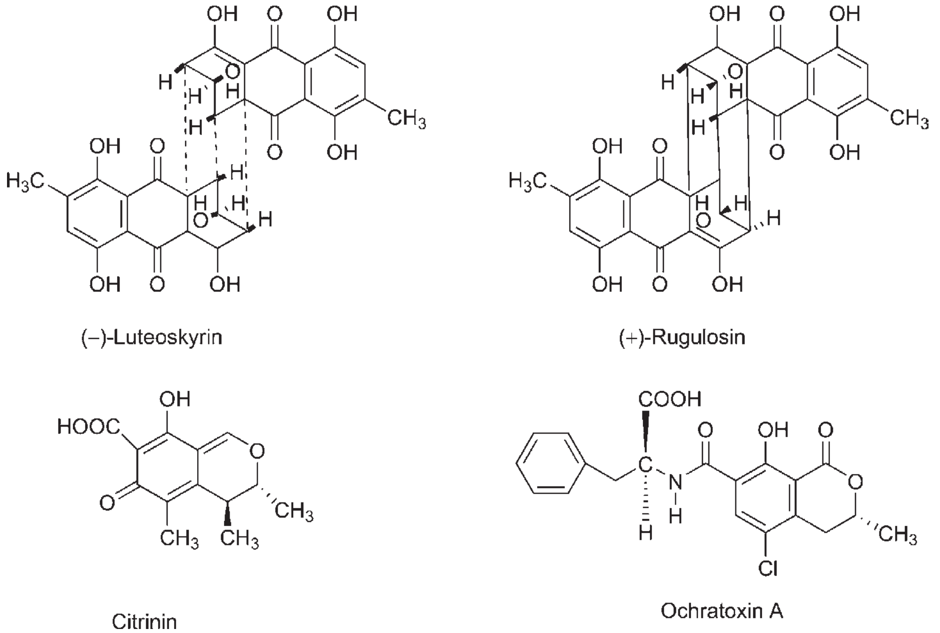

(−)-Luteoskyrin
(+)-Rugulosin
Citrinin
Ochratoxin A
OH
H$_3$C
CH$_3$
HOOC
COOH
Cl

Figure 3 Structures of (−)-luteoskyrin, (+)-rugulosin, citrinin, and ochratoxin A.

toxicity of this compound is about one half of that of (−)-luteoskyrin.

Citrinin and Ochratoxin A

The yellow pigment, citrinin, has been isolated from *P. citrinum* found on yellow rice called ‘citrinum yellow rice’. Citrinin, which is biosynthesized from a pentaketide through the acetate–malonate pathway with three C$_1$-sources, causes renal damage in swine. It has also been shown to possess antibacterial, antifungal, and antiprotozoal activity. Citrinin was previously used as an antibiotic, but was later banned because of its nephrotoxicity.

Ochratoxin A has been isolated from *A. ochraceus*, which grows on many types of farm produce. It is an amide formed from a bicyclic carboxylic acid synthesized from a pentaketide with a C_1-source and L-phenylalanine, and has been shown to cause kidney necrosis and cancer. It is now known that a renal inflammation (nephropathy), which sometimes appears in swine in Northern Europe, results from poisoning by citrinin and ochratoxin A produced by *Penicillium viridicatum*, which contaminates feed (**Figure 3**). There is some evidence that ochratoxin A can be produced in cheese contaminated by *Penicillium* spp.

Fumonisins

Fumonisins have been isolated from a fungal contaminant of maize, *F. verticillioides* (formerly *Fusarium moniliforme*), which occurs worldwide (the teleomorphic state: *Gibberella fujikuroi*), and *Fusarium proliferatum*. It has been shown that fumonisins cause leukoencephalomalacia in horses and pulmonary edema in swine. Several congeners of fumonisins, that is, fumonisins A_1, A_2, B_1, B_2, B_3, and B_4, are known (**Figure 4**). It has been established that fumonisin B_1 causes hepatocarcinoma in male rats fed with feed containing $50\,\mathrm{mg\,kg^{-1}}$ for prolonged periods, and also causes nephrosis in male rats fed with $\geq 9\,\mathrm{mg\,kg^{-1}}$. Fumonisins, which have a long carbon chain aminoalcohol structure as their basic skeleton, are structurally similar to sphingosines (sphingoids). In fact, it has been demonstrated that fumonisins inhibit sphingolipid metabolism, and consequently, disrupt critical sphingolipid-mediated cell signaling pathways or sphingolipid-dependent physiological functions.

Other Carcinogenic Mycotoxins

A cyclic chlorine-containing pentapeptide named 'cyclochlorotine' has been isolated together with (−)-luteoskyrin from *P. islandicum* growing on rice (**Figure 4**). It has been shown that cyclochlorotine causes hepatopathy in mice, which results in hepatic cancer. Cyclochlorotine also has a cytotoxic effect on cultured cells. Patulin, which has been isolated from *P. patulum* and *Aspergillus clavatus*, and penicillic acid, which has been isolated from *P. cyclopium*, *P. puberulum*, and many other *Penicillium* and *Aspergillus* fungi, are the compounds possessing an α,β-unsaturated γ-lactone structure, which is formed via opening of an aromatic ring from tetraketide through the acetate–malonate pathway (**Figure 4**). Subcutaneous injection of patulin or penicillic acid causes sarcoma in experimental animals.

The presence of patulin has been reported in cheese contaminated with *Penicillium* spp.

Neurotropic Mycotoxins

Citreoviridin

In 1940, an extract of *Penicillium toxicarium* (the synonym of *P. citreoviride*), which contaminated Formosan rice called 'toxicarium yellowed rice', was found to cause ascending paralysis, hypothermia, and breathing

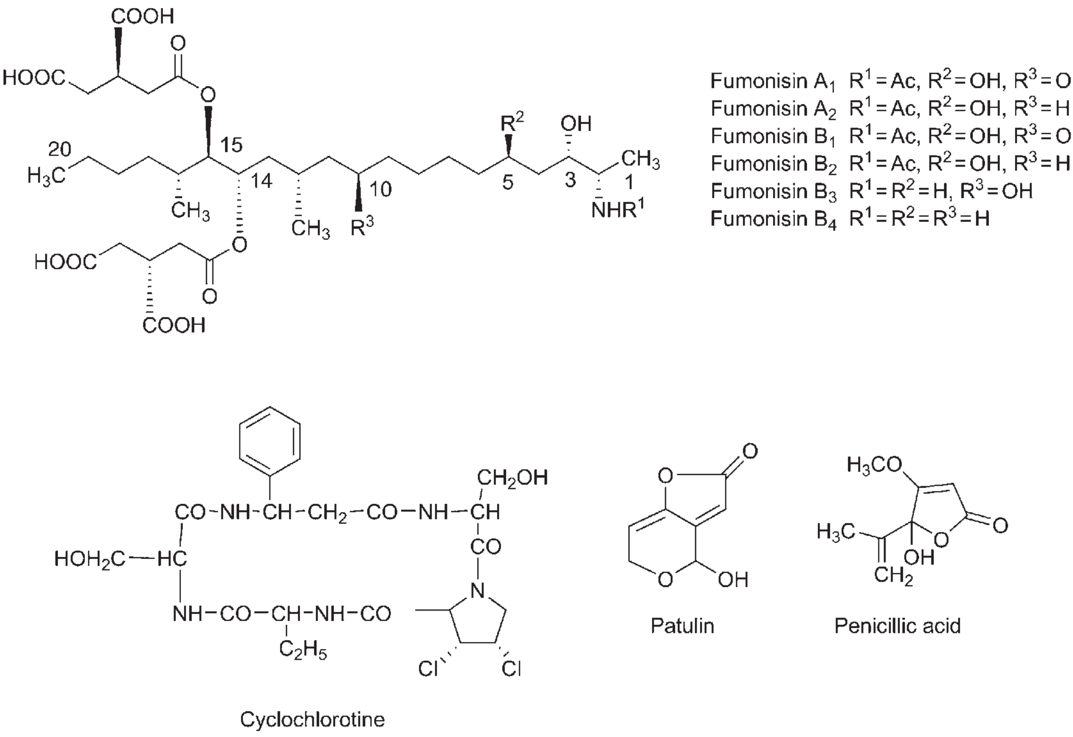

COOH
HOOC
O
O
20
H_3C
15
14
CH_3
10
CH_3
R^3
R^2
5
OH
3
CH_3
1
NHR^1
O
HOOC
O
COOH
Fumonisin A_1 R^1 = Ac, R^2 = OH, R^3 = OH
Fumonisin A_2 R^1 = Ac, R^2 = OH, R^3 = H
Fumonisin B_1 R^1 = Ac, R^2 = OH, R^3 = OH
Fumonisin B_2 R^1 = Ac, R^2 = OH, R^3 = H
Fumonisin B_3 R^1 = R^2 = H, R^3 = OH
Fumonisin B_4 R^1 = R^2 = R^3 = H
CH_2OH
CO-NH-CH-CH_2-CO-NH-CH
CO
HOH_2C—HC
N
NH-CO-CH-NH-CO
C_2H_5
Cl
Cl
Cyclochlorotine
O
O
O
OH
Patulin
H_3CO
H_3C
O
O
OH
CH_2
Penicillic acid

Figure 4 Structures of fumonisins, cyclochlorotine, patulin, and penicillic acid.

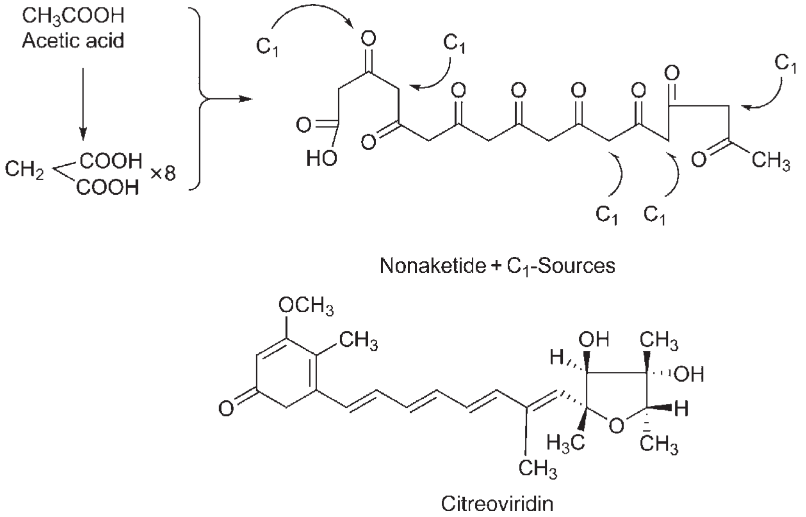


Figure 5 Biosynthesis of citreoviridin.

difficulties in mice. These symptoms were thought to be similar to those of cardiac beriberi, which was widespread until about 1925 in Japan and later disappeared. At that time, vitamin B_1 was ineffective against cardiac beriberi. In 1947, a yellow pigment named 'citreoviridin' was isolated from *P. citreoviride*, and some time later, this compound was found to be the toxic factor of this fungus. Citreoviridin is formed from 2-pyrone, a conjugated polyene chain, and a tetrahydrofuran moiety is synthesized from nonaketide and C_1-sources through the acetate–malonate pathway (**Figure 5**). Citreoviridin causes neural damage including ascending paralysis in mice, suggesting that the conjugated polyene system in this compound may affect the electron transport system in mice. It is now being suggested that cardiac beriberi, a disease of the past, may have resulted from ingestion of rice contaminated with citreoviridin.

Tremorgenic Dioxopiperazines

In 1971, it was discovered that the extract of *A. fumigatus* grown on miso (bean paste) and rice caused marked tremor in mice and rats. Subsequently, tremorgenic constituents named 'fumitremorgins A and B' were isolated from the extract. Fumitremorgins A and B are composed of a basic skeleton of 2,5-dioxopiperazine formed from L-tryptophan and L-proline, with three and two isoprenyl (C_5) units in fumitremorgins A and B, respectively (**Figure 6**). The ED_{50} values of fumitremorgins A and B needed to cause tremor in mice are 0.18 and 3.5 mg kg^{-1} i.p., respectively. The tremor induced by fumitremorgin A increases with a high level of serotonin, which is an excitatory neurotransmitter in the central nervous system in the brain of mice, and decreases at a high level of γ-aminobutyric acid (GABA), which is a suppressive neurotransmitter.

Verruculogen isolated from *P. verruculosum* obtained from peanuts has the structure of fumitremorgin A except that the isopentenyl ether group is replaced with a hydroxyl group in verruculogen (**Figure 6**). Verruculogen shows tremorgenic activity similar to that of fumitremorgin A. Both fumitremorgin B and verruculogen are produced by *Aspergillus caespitosus* and *Penicillium piscarium*. Verruculogen is also produced by *Penicillium paraherquei*, and both fumitremorgins A and B are produced by *Neosartorya fischeri* (the teleomorphic state of *A. fumigatus*). Roquefortine (the synonym: roquefortine C) was isolated in 1976 from *P. roqueforti*, which is a mold used in the production of blue cheese. Roquefortine, which possesses a dioxopiperazine skeleton composed of tryptophan and histidine with an isopentenyl unit, also exhibits tremorgenic activity (**Figure 6**). Roquefortine has also been isolated from *Penicillium crustosum*.

Tremorgenic Indoloditerpenes

Paxilline, a tremorgenic compound, was isolated from *P. paxilli* grown on pecans in 1974, and the structure was determined by X-ray crystallographic analysis in the following year (**Figure 7**). This was the first tremorgenic indoloditerpene (meaning: indole + diterpene) to reveal its structure. Paspalinine was isolated from *Claviceps paspali* as its tremorgenic factor in 1977. *Claviceps paspali* was suspected to be the causative mold of a neuroataxia of cattle in the United States called 'paspalum staggers'; its structure was determined in 1980 (**Figure 7**). Metabolites such as paxilline and paspalinine are thought to be synthesized from

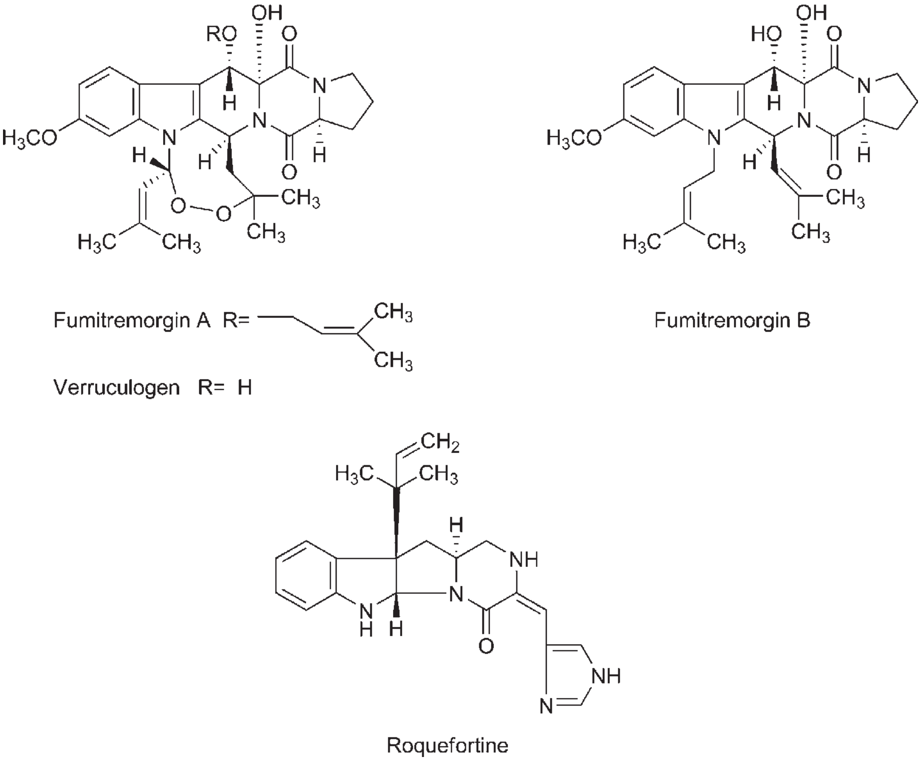

Fumitremorgin A R=
Verruculogen R= H
Fumitremorgin B
Roquefortine

Figure 6 Structures of fumitremorgins A and B, verruculogen, and roquefortine.

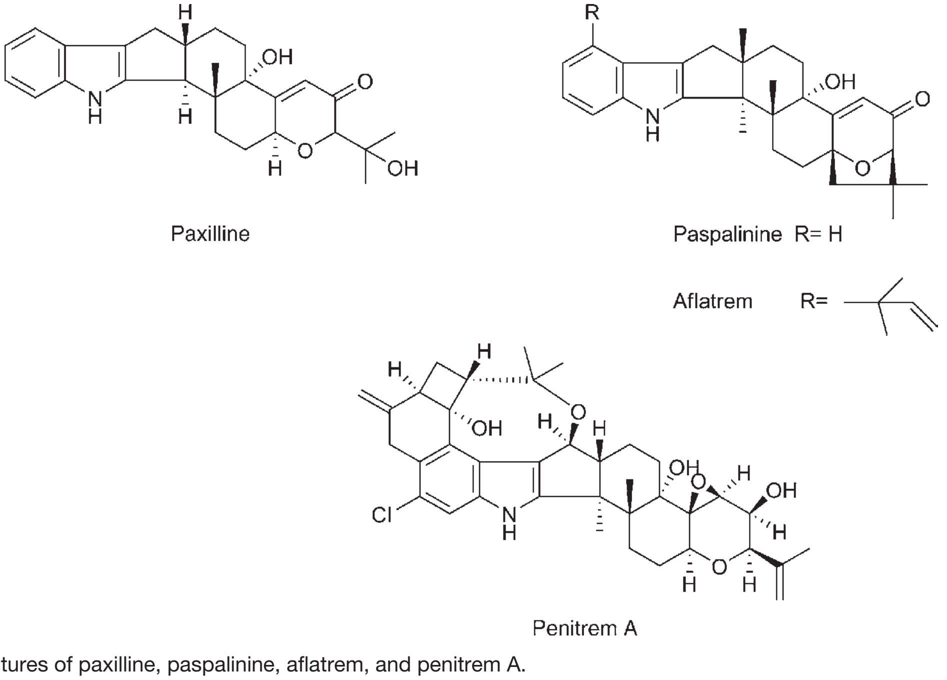

Paxilline
Paspalinine R= H
Aflatrem R=
Penitrem A

Figure 7 Structures of paxilline, paspalinine, aflatrem, and penitrem A.

tryptophan and geranylgeraniol through the pathway shown in **Figure 8**.

Aflatrem was isolated from *A. flavus* in 1964 as the tremorgenic agent in this fungus (probably the first tremorgenic metabolite to be isolated from fungi), and its structure was established in 1980 (**Figure 7**). In 1968, penitrem A was isolated from *P. cyclopium* obtained from peanuts implicated in a case of sheep poisoning; it was later also isolated from *Penicillium palitans* and *P. crustosum.* The structure of penitrem A was established in 1981

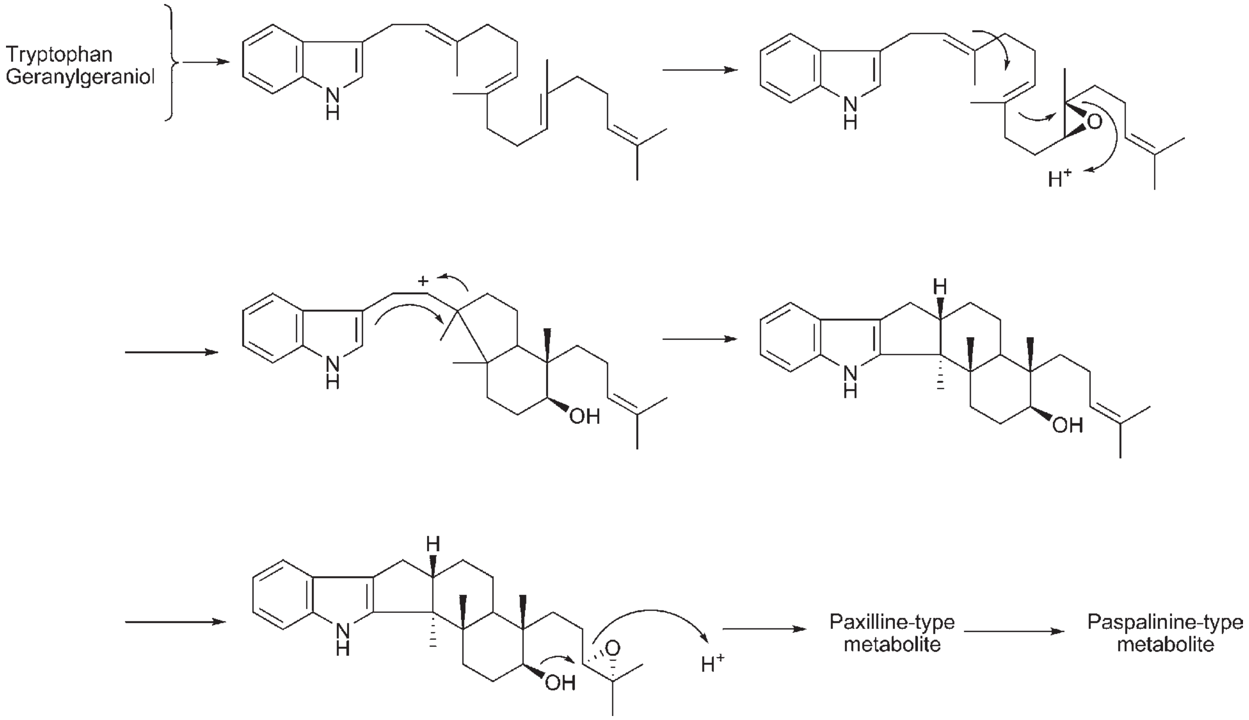


Figure 8 Suggested biosynthetic route for indoloditerpenes. Adapted from Turner WB and Aldridge DC (1983) *Fungal Metabolites II*. London: Academic Press.

(**Figure 7**). It is a derivative of tremorgenic indolodite rpenes; the skeleton of this metabolite is formed from tryptophan, geranylgeraniol, and two further isoprenyl units. This compound is believed to be one of the substances that causes a neuroataxia of cattle named 'ryegrass staggers', which occurs in New Zealand and Australia.

Other Mycotoxins

Trichothecenes

Fusarium toxicosis results from toxic metabolites of *Fusarium nivale* and other *Fusarium* spp. isolated from wheat and pasture. The causative agents of this toxicosis are nivalenol, deoxynivalenol, T-2 toxin, fusarenon-X, and related compounds, which belong to a sesquiterpene group named 'trichothecenes' (**Figure 9**). Trichothecenes, which possess the unique sesquiterpene skeleton named 'trichothecane', cause hemorrhage, vomiting, diarrhea, anorexia, and malfunction of hematopoietic organs, resulting in decreased lymphocyte production and, consequently, immunodeficiency in mice, rats, and swine. The key target cells of trichothecenes are leukocytes, and the toxicity of trichothecenes is complicated because they are immunostimulatory at low doses, but immunosuppressive at high doses. Many compounds belonging to trichothecenes, including various macrocyclic-type trichothecenes such as verrucarin A isolated from *M. verrucaria*, are known (**Figure 9**). These macrocyclic-type trichothecenes are particularly toxic. Various trichothecenes are produced by some species in the genera *Fusarium*, *Trichothecium*, *Trichoderma*, and *Myrothecium*.

Sporidesmins

In New Zealand, a photohypersensitive exudative eczema called 'facial eczema' occurs sometimes in sheep. Sporidesmins have been isolated from the fungus *Pithomyces chartarum* (the synonym of *Sporidesmium bakeri*) found in the feed associated with this disease of sheep. This disease is characterized by both photohypersensitive eczema and hepatopathy, which ultimately result in death several weeks later. Sporisdesmins are composed of many congeners, that is, sporidesmin (synonym: sporidesmin A), and sporidesmins B–J. Each sporidesmin possesses a 2,5-dioxopiperazine skeleton formed from tryptophan and alanine as the basic common structure. The dioxopiperazine ring is bridged with a disulfide chain in sporidesmin and sporidesmin B, with a trisulfide chain in sporidesmin E, and with a tetrasulfide chain in sporidesmin G to form epidithio-, epitrithio-, and epitetrathio-dioxopiperazine structures, respectively. These sulfide bridges are eliminated or modified in sporidesmins C, D, and F (see **Figure 9**). The relative ratio of

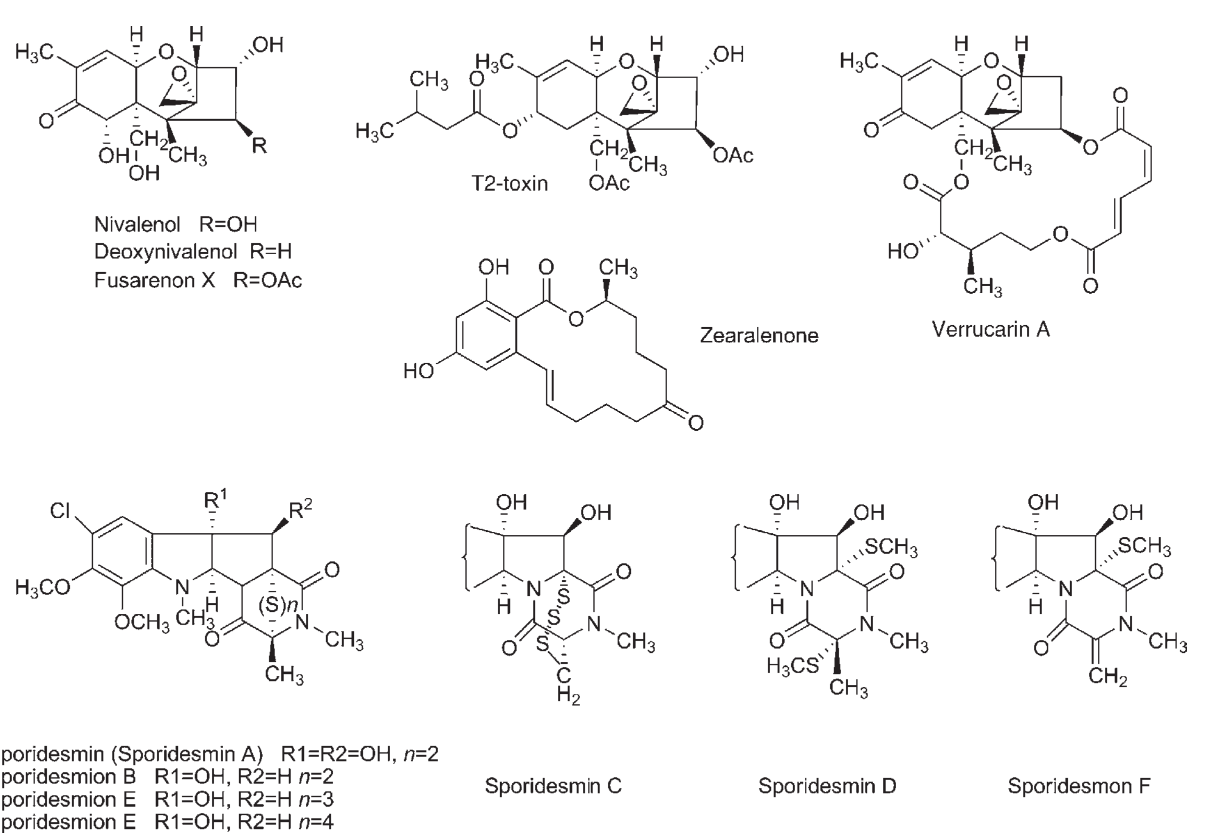

Nivalenol R=OH
Deoxynivalenol R=H
Fusarenon X R=OAc
T2-toxin
Zearalenone
Verrucarin A
Sporidesmin (Sporidesmin A) R1=R2=OH, n=2
Sporidesmion B R1=OH, R2=H n=2
Sporidesmion E R1=OH, R2=H n=3
Sporidesmion E R1=OH, R2=H n=4
Sporidesmin C
Sporidesmin D
Sporidesmon F

Figure 9 Structures of five trichothecenes, seven sporidesmins, and zearalenone.

cytotoxic activity of di-, tri-, and tetrasulfides against HeLa epithelial cells is 1:4:1. Sporidesmins whose sulfide bridges have been eliminated or modified show no activity.

Zearalenone

Zearalenone was isolated from *Fusarium roseum* (*F. graminearum*, teleomorphic state: *Gibberella zeae*), which grows on the maize fed to swine. Zearalenone exhibits estrogenic activity, enlarging the uterus and mammary glands, and causing swelling of the vulva (vulvovaginitis) in sows. Hyperestrogenism resulting from zearalenone has also been reported in other animals (and in humans), but swine is perhaps the species most sensitive to this compound. Zearalenone is thought to be synthesized from nonaketide through the acetate–malonate pathway (**Figure 9**).

Miscellaneous Mycotoxins

There are many other important mycotoxins known today. Lysergic acid amides isolated from *Claviceps purpurea* (ergot), which contaminate rye, are notorious as the causative agents of ergotism. Rubratoxins isolated from *Penicillium rubrum* obtained from grains and other feedstuffs have a cytotoxic effect. Phomopsin A isolated from *Phomopsis leptostromiformis*, which infects the lupin, interferes with tubulin function. Cytochalasins isolated from *Helminthosporium dematioideum* and chetoglobosins isolated from *Chaetomium globosum* show inhibition of cytoplasmic cleavage in mammalian cell culture to form large multinuclear cells.

For information on the occurrence and significance of mycotoxins in milk and dairy products, *see* **Contaminants of Milk and Dairy Products**: Environmental Contaminants.

See also: **Contaminants of Milk and Dairy Products**: Environmental Contaminants. **Yeasts and Molds**: *Aspergillus flavus*; Mycotoxins: Aflatoxins and Related Compounds.

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Mycotoxins: Aflatoxins and Related Compounds

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Introduction

Aflatoxins (AFs) are very important mycotoxins due to their extremely high toxicity, carcinogenic activity for animals (including humans), and frequent occurrence in various foods and feedstuffs.

AFs found in 1961 in Brazilian groundnut meal were the source of the toxicity associated with 'turkey X' disease; more than 100 000 turkeys died in the United Kingdom. Since then, many researchers have vigorously investigated AFs, particularly AFB_1, in various fields.

Structure and Chemical Properties

More than 10 types of AFs and related compounds have been identified. Among them AFB_1, AFB_2, AFG_1, and AFG_2 are especially important because they are highly toxic and often occur in foods and feeds. The structures of the major AFs are shown in **Figure 1**.

AFB_1 is representative of the AFs and contains a dihydrobisfuran and coumarin nucleus fused to cyclopentanone. AFB_2 is 8,9-dihydro-AFB_1. In the AF-G group, six-membered lactone is substituted by the cyclopentanone of AF-B group. The origins of the names AF-B and AF-G lie with the 'b' and 'g' of the blue and green fluorescent colors produced under ultraviolet (UV) light on thin-layer chromatography (TLC). AF-M group are 9a-hydroxy derivatives of the AF-B group and are found in cows' milk as metabolites of the AF-B group in their feeds. Ingested AFB_1 is converted to AFM_1 in the cows' liver, and approximately 0.9% of ingested AFB_1 is found in the milk as AFM_1. AFM_1, a major animal metabolite of AFB_1, is found in the urine of AFB_1-exposed animals at levels of up to 20% of the ingested oral dose. AFB_{2a} and AFG_{2a} are 8-hydroxy AFB_1 and AFG_1, respectively, formed under acidic conditions (below pH 3) from parent AFs. Aflatoxicol (AFL)-I is a major metabolite of AFB_1 formed by microorganisms, and AFL-α is the stereoisomer of AFL-I. These are reduced to AFB_1; the keto moiety on the terminal cyclopentanone of AFB_1 is reduced to a hydroxy group.

Most of the other AFs known are hydroxylated metabolites of AFs, such as AFP_1 (*O*-demethylated AFB_1), AFQ_1 (3-hydroxy AFB_1), $AFGM_1$ (10a-hydroxy AFG_1), AFL-M_1 (9a-hydroxy AFL), and AFL-H_1 (3-hydroxy AFL).

AFs are slightly soluble in water, insoluble in nonpolar solvents, and soluble in moderately polar or polar organic solvents such as chloroform, acetonitrile, and methanol. Most AFs have intense blue or green fluorescence (emission wavelength: 420–450 nm) under UV light (excitation wavelength: 350–370 nm).

AF-Producing Fungi

AFs are produced in nature only by some strains of *Aspergillus flavus*, most strains of *Aspergillus parasiticus*, and *Aspergillus nomius*. *Aspergillus flavus*, the origin of the name of aflatoxin, is the main source of AFs, but not all strains produce AFs. It has recently been reported that *Aspergillus tamarii* also produces the AF-B group.

Generally, *A. flavus* produces only the AF-B group, whereas *A. parasiticus* and *A. nomius* produce the AF-B and AF-G groups. In most strains, AFB_1 is produced in the largest quantities. AFB_2 and AFG_2 are produced at one-tenth to one-third of the amount of AFB_1 and AFG_1, respectively.

Aspergillus oryzae, the domesticated form of *A. flavus*, adapted by centuries of use in fermented food manufacture, never produces AFs.

Condition Favoring Production of AFs

The limiting temperature and relative humidity for AF production vary slightly depending on the kind and quality of food. The lower limiting temperature for AF production is approximately 12 °C, whereas the upper limiting temperature is 41 °C at 99% relative humidity. The limiting relative humidity is approximately 83% or higher at 30 °C, varying with the type of growth medium and length of the incubation period.

Reducing oxygen concentration generally leads to a reduction in the amount of AF produced, notably so at an oxygen concentration of less than 1%. The presence of certain amino acids, fatty acids, and zinc ions stimulates the formation of AFs.

Biosynthesis

The intermediates in the biosynthetic pathway of AFB_1, AFB_2, AFG_1, and AFG_2 have been determined, and the synthetic steps were revealed by feeding studies

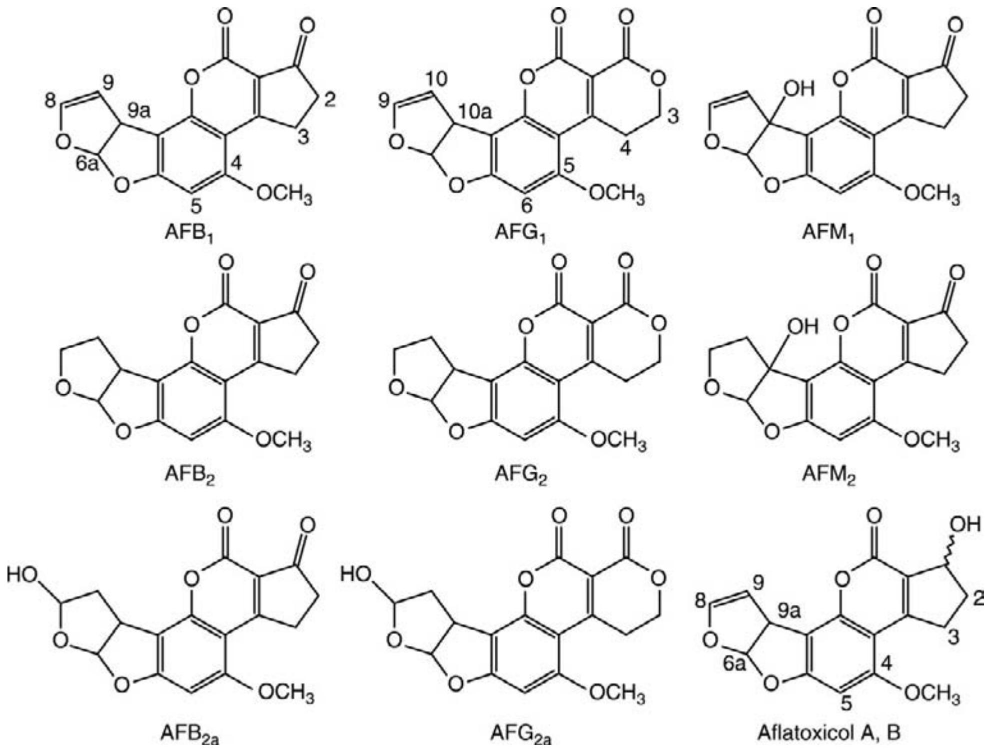

Figure 1 Structures of major aflatoxins.

with radioactive precursors, pathway-blocked mutant strains, and metabolic inhibitors. AFs are formed by head-to-tail condensation of acetyl units to form a cyclized polyketide, which is enzymatically altered through a series of intermediates. At least 18 enzymatic steps are required for conversion of acetyl coenzyme A (acetyl-CoA) and malonyl coenzyme A (malonyl-CoA) to its final product, AFB$_1$. The generally accepted pathway for the production of AFB$_1$ and AFG$_1$ is as follows: acetyl-CoA + malonyl-CoA → hexanoyl-CoA → norsolorinic acid → averantin → 5′-hydroxyaverantin → averufin → versiconal acetate → versiconal → versicolorin B → versicolorin A → demethylsterigmatocystin → sterigmatocystin → *O*-methylsterigmatocystin → AFB$_1$ and AFG$_1$. The enzymatic reactions in the synthesis of AFB$_2$ and AFG$_2$ are the same as AFB$_1$ and AFG$_1$, except for the formation of dihydrodemethylsterigmatocystin from versicolorin B.

Acute Toxicity in Animals

AFs are toxic to many forms of life, including animals, birds, and fish. LD$_{50}$ values of AFB$_1$ are shown in **Table 1**.

The sensitivity toward AFs differs with animal species. Mice and hamsters are relatively resistant to acute AFB$_1$, whereas ducks, rabbits, and rainbow trout are relatively sensitive.

Structure–activity relationship has been studied for four major AFs: AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$. Their acute toxicity in rats and ducklings followed the order AFB$_1$ > AFG$_1$ > AFB$_2$ > AFG$_2$. AFs containing an unsaturated terminal furan (AFB$_1$ and AFG$_1$) are much more potent than AFs containing a saturated terminal furan (AFB$_2$ and AFG$_2$). These results indicate that the presence of the double bond in the terminal furan is an important determinant of potential for acute toxicity, and that AFs containing cyclopentanone are more acutely toxic than AFs containing six-membered lactone.

Mutagenicity

AFB$_1$ is potently mutagenic for *Salmonella* strains (TA100 and TA98) at a low dose level (0.1 μg plate^{-1}) in the presence of S-9 mix, coenzymes and buffer. It is known that activated AFB$_1$ induces guanine-cytosine to thymine-adenine transistion in genes. The activated K-ras gene detected in AF-induced primary liver tumor contained a guanine to adenine transition in codon 12.

Table 1 Oral LD_{50} of aflatoxin B_1

Animal	*LD_{50} (mg kg^{-1})*	*References*
Rabbit	0.3–0.4	Butler (1974), Pier (1992)
Duck	0.3–0.6	Butler (1974), Pier (1992), Robens and Richard (1992)
Cat	0.5	Butler (1974)
Dog	0.5–1.0	Butler (1974), Robens and Richard (1992)
Pig	0.6–1.0	Butler (1974), Pier (1992)
Horse	0.6–1.0	Pier (1992)
Rainbow trout	0.8	Bauer *et al.* (1969)
Calf	1.0–1.5	Pier (1992)
Sheep	1.0–2.0	Butler (1974), Pier (1992)
Turkey	1.4	Pier (1992)
Guinea pig	1.4	Butler (1974)
Monkey	2.2–7.8	Campbell and Stoloff (1974)
Rat	1.0–17.9	Butler (1974), Robens and Richard (1992)
Mouse	9.0	Butler (1974)
Hamster	10.2	Butler (1974), Robens and Richard (1992)

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Among AFs and related compounds, AFB_1 is the most potent, followed by AFL, AFG_1, AFM_1, AFB_2, AFG_2, and AFB_{2a}.

Carcinogenicity

AFB_1 is one of the most potent carcinogens known. The major target organ of AFB_1 is the liver. One hundred percent (12/12) of male rats given 15 μg kg^{-1} dietary AFB_1 for 68 weeks, and 100% (13/13) of female rats fed the same diet for 82 weeks developed hepatocellular carcinomas. The carcinogenicity of AFs has been demonstrated in a variety of animals such as ducks, rats, monkeys, and rainbow trout. Other AFs (AFB_2, AFG_1, AFL, AFM_1, and AFQ_1) have also been proved to be carcinogenic. The order of carcinogenic potency in rainbow trout was $AFB_1 > AFL > AFM_1 > AFQ_1 > AFG_1$, whereas AFB_2 and AFG_2 were inactive. Overall, these results indicate that the presence of the double bond in the terminal furan ring is the most important determinant for toxic and carcinogenic activity. The importance of the substitutes on the lactone portion of the molecule is also illustrated by the difference in the potency of AFB_1 and AFG_1 in all systems studied.

The International Agency for Research on Cancer (IARC) evaluated the carcinogenic risk of AFs and classified AFs under group 1 carcinogens, which means that they are carcinogenic to humans.

Metabolism and Mechanism of Toxicity

The metabolic pathways for AFB_1 in animals are shown in **Figure 2**. After intake, AF is metabolized by cytochrome p450 in the liver to several compounds; most of them are hydroxylated derivatives, such as AFM_1 and AFP_1, and are less toxic than AFB_1. AFM_1 is a major animal metabolite of AFB_1. Ingested AFB_1 is converted to AFM_1 in the cows' liver, and approximately 0.9% of ingested AFB_1 is found in the milk as AFM_1. AFM_1 is also found in the urine of AFB_1-exposed animals at levels of up to 20% of the ingested oral dose.

Among the metabolites, AFB_1-8,9-epoxide is the source of the potent mutagenicity and carcinogenesis of AFB_1. This intermediate binds to cellular macromolecules such as DNA, RNA, and protein (**Figure 3**) because of which the presence of the double bond in the terminal furan in AFs is such an important determinant of acute toxicity, mutagenicity, and carcinogenicity.

The existence of the intermediate, AFB_1-8,9-epoxide, was confirmed by the isolation and identification of the absolute structure of 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 (AFB_1-N^7-Gua), formed *in vitro.* It is considered that binding of AFB_1 to DNA causes mutation

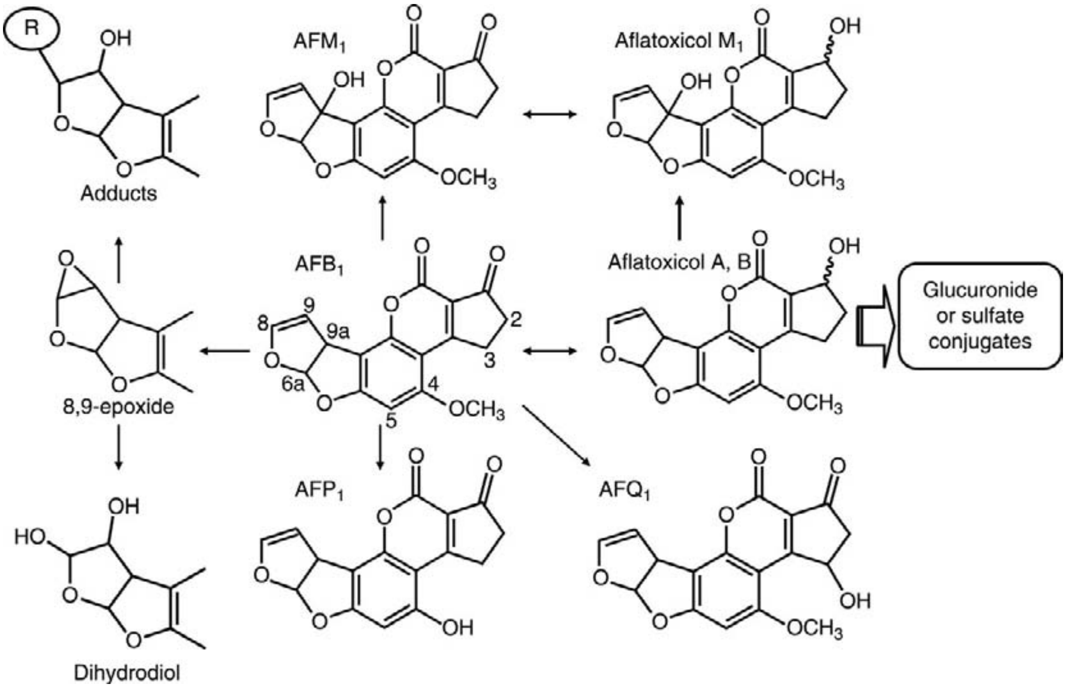
R
OH
Adducts
AFM$_1$
OH
OCH$_3$
Aflatoxicol M$_1$
OH
OH
OCH$_3$
8,9-epoxide
AFB$_1$
8
9
9a
2
3
4
5
6a
OCH$_3$
Aflatoxicol A, B
OH
OCH$_3$
Glucuronide or sulfate conjugates
HO
OH
Dihydrodiol
AFP$_1$
OH
AFQ$_1$
OH
OCH$_3$

Figure 2 Metabolic pathways of aflatoxin B_1.

in genes, resulting in the activation of *ras* oncogene and inactivation of p53 tumor suppressor gene.

Effects on Cattle

The general effect of AFs in cattle is liver disease. High levels of AF cause acute aflatoxicosis, such as liver lesions, reduced feed consumption, weight loss, and reduction in milk production. The chronic effects of low-level consumption of AFs in cattle are reduced reproductivity, immunosuppression, and reduced feed efficiency. Dairy cattle convert ingested AFB_1 in their liver to AFM_1, which is secreted in milk. When calves consume milk contaminated with AFM_1, they may contract aflatoxicosis.

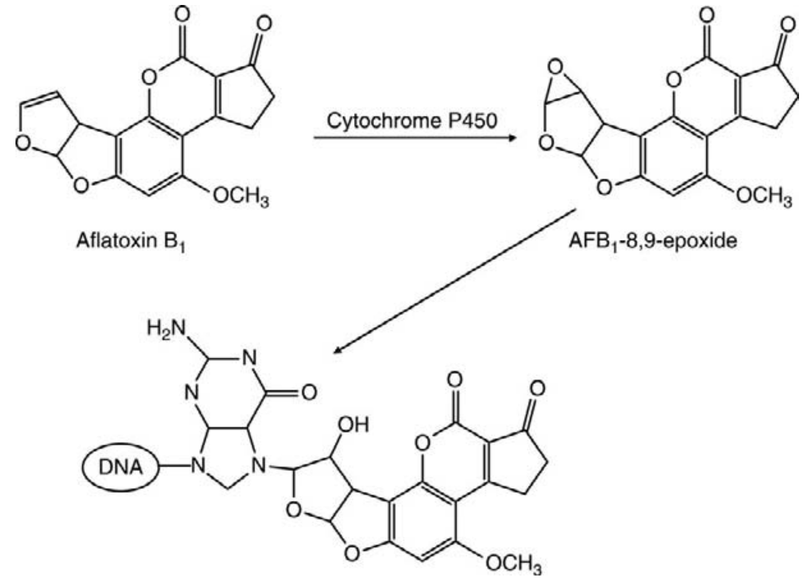
Aflatoxin B$_1$
OCH$_3$
Cytochrome P450
AFB$_1$-8,9-epoxide
OCH$_3$
H$_2$N
N
N
O
DNA
N
N
OH
OCH$_3$

Figure 3 Mechanism of the toxicity of aflatoxin B_1.

Effects on Human

Acute Toxicity

Most of the recorded outbreaks of acute aflatoxicosis have occurred in tropical countries. In India (1974–75), a total of 397 patients were affected, and 106 died. The disease was characterized by jaundice, rapidly developing ascites, portal hypertension, and a high mortality rate and was associated with the consumption of maize contaminated with AF; the AF concentration ranged from 6250 to 15 600 μg kg^{-1}, which means the affected people consumed 2–6 mg of AF daily over a month. In Kenya (1981), 12 out of 20 patients died. They ingested maize that contained 12 000 μg kg^{-1} of AFB$_1$. The liver tissue at necropsy showed centrobulbar necrosis and contained up to 89 μg kg^{-1} of AFB$_1$. In 2004, more than 100 people died following consumption of maize highly contaminated with AFs.

Reye's syndrome, manifested by a rapid onset of vomiting, convulsions, coma, and a high mortality rate, was considered to be a kind of aflatoxicosis, because autopsy specimens of the children who died from the syndrome contained AFB$_1$. However, many researchers have recently reported that Reye's syndrome is caused by other factors, concluding that it is likely to be caused by a combination of factors; AFB$_1$ is probably not an important etiological agent of this disease in the United States.

Cancer

In tropical areas, such as Southeast Asia, India, and Africa, the incidence of primary hepatocellular carcinoma (PHC) is high. Epidemiological surveys carried out over the past 25 years in Asia and Africa have revealed a strong statistical association between AF ingestion and PHC incidence. A high rate of mutation at codon 249 of the human p53 tumor suppressor gene has been reported in these tumors.

Exposure to AFB$_1$ and infection with human hepatitis B virus (HBV) are considered to be the major risk factors in the development of PHC. The G to T transversion was found in p53 tumor suppressor gene of hepatocellular carcinomas from patients at high risk of exposure to AFs. The combined experimental and epidemiological evidence has led to designation of AFs as human carcinogens. Collectively, current evidence strongly suggests that PHC is of multifactorial origin, with probable interactions between viral and chemical agents in populations concurrently exposed to both classes of risk factors.

Regulation

Because AFs are highly toxic to humans and animals and are frequently found in various foods and feeds, they are of worldwide concern. Regulations concerning AFs have been established in many countries to protect people from the harmful effects of AFs. More than 79 countries regulate the permissible levels of AFs in foods and feeds. The maximum permitted levels have been set for AFB$_1$ alone or total AFs (the sum of AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$) (**Table 2**). The maximum levels range from 1 to 20 μg kg^{-1} for AFB$_1$ and from 0 to 35 μg kg^{-1} for total AFs.

More than 60 countries set limits on AFM$_1$ in milk by the end of 2003. The maximum permitted levels are 0 (not detectable) to 15 μg kg^{-1} (**Table 3**). The levels of the limits for AFM$_1$ in milk are much lower than those for AFB$_1$ in food, because babies or infants are considered to be highly sensitive. AFM$_1$ is a metabolite of AFB$_1$. Therefore, regulation on AFB$_1$ in feed for cattle is most effective for controlling levels of AFM$_1$ in milk. Regulations for AFB$_1$ in feed for dairy cattle exist in at least 39 countries. Although the maximum limits range from 5 to 50 μg kg^{-1}, most of these countries set the limit at the level of 5 μg kg^{-1}.

Table 2 Regulation for aflatoxins in food

Country	*Aflatoxin*	*Limit* (μg kg^{-1})	*Commodity*
Codex	$B_1+B_2+G_1+G_2$	15	Peanut, raw
EU	B_1	2–8	Foods (nut, cereals, spices, dry fruits)
	$B_1+B_2+G_1+G_2$	4–15	
Mercosur	$B_1+B_2+G_1+G_2$	20	Peanut, maize and products
United States	$B_1+B_2+G_1+G_2$	20	All foods
China	B_1	20	Maize, peanut (products), rice, edible oil
	B_1	10	
Thailand	$B_1+B_2+G_1+G_2$	20	All food products
Japan	B_1	10	All foods

Extracted from FAO (2004). In: *FAO Food and Nutrition Paper 81*, Worldwide regulations for mycotoxins in food and feed in 2003. Rome, Italy: FAO.

Table 3 Regulation for aflatoxin M_1 in food

Country	*Limit* (μg kg^{-1})	*Commodity*
Codex	0.5	Milk
EU	0.05	Milk
Mercosur	0.5	Fluid milk
	5	Powdered milk
United States	0.5	Milk
China	0.5	Milk and milk products
Indonesia	5	Milk, cheese
Vietnam	0.5	Milk and milk products

Extracted from FAO (2004) Worldwide regulation for mycotoxins in food and feed in 2003. *FAO Food and Nutrition Paper 81*. Rome, Italy: FAO.

Determination

Standards

Standards of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, AFB_{2a}, AFG_{2a}, AFP_1, AFQ_1, AFL-A, and AFL-B are commercially available. AFs are unstable in some polar solvents, such as methanol; therefore, the storage solvent system must be carefully selected. AFs are stable in chloroform and benzene:acetonitrile (9:1) in the dark and at low temperatures.

Sampling

Sampling is one of the most important steps in AF determination, because the distribution of AFs in naturally contaminated samples is extremely heterogeneous. Usually only a few percentage of kernels in a sample lot are highly contaminated with AFs, whereas other kernels are free of AFs. For example, it was reported that only 0.03% of peanut kernels were contaminated with AFs, the mean concentration was 5 μg kg^{-1}, and the content in a single kernel was 1100 μg kg^{-1}. Therefore, it is very difficult to collect a sample that actually represents the mean concentration. An inappropriate sampling plan leads to wrong results, even if the analytical method is very precise.

Several theoretical distributions for AFs have been reported. Among them negative binomial distribution is usually applied to determine the sample size and sampling procedure.

Extraction

Samples are comminuted, and AFs are extracted by shaking or homogenizing with organic solvents, such as methanol–water, acetonitrile–water, or chloroform. Generally, one portion of sample is extracted with 4–5 volumes of solvent. For dry samples, a small amount of water is necessary to extract naturally contaminated AFs, although AFs are rarely dissolved with water.

Purification

AFs are purified using an immunoaffinity column, or solid phase extraction, such as florisil or a multifunctional column. The most effective purification is obtained by an immunoaffinity column; the shortcomings of this type of column are high cost and low sample capacity. After purification with an immunoaffinity column, few peaks of ingredient are found in a HPLC (high-performance liquid chromatography) chromatogram. Although multifunctional columns have the same shortcomings as immunoaffinity columns, the process is easy and speedy. With a florisil column, AFs are effectively purified at low cost and high sample capacity. The disadvantage of this column is the necessity to use chloroform.

Detection

As AFs have intense fluorescence under UV light, they are determined quantitatively by the measurement of their fluorescence intensity. AFs are usually determined by HPLC or TLC. In HPLC analysis, usually an ODS (octadecyl silane) column and polar mobile phase are used. Generally, the reverse phase mode HPLC is used. The fluorescence of AFB_1 and AFG_1 is quenched in the polar solvent used as mobile phase; therefore, these AFs cannot be determined without making derivatives with trifluoroacetic acid (TFA) or using a photochemical reactor. It is at times difficult to determine AFs in spice samples such as red pepper, paprika, and white and black pepper by HPLC methods because they contain many impurities, which are difficult to remove by the purification methods.

Reliable results are obtained by two-dimensional TLC, with a high-performance TLC (HPTLC) plate and two kinds of developing solvents. Chloroform:acetone (9:1) and diethyl ether:methanol:water (94:4.5:1.5) are commonly used. Because AFs are intensely fluorescent on TLC under UV light, the sensitivities of AFs by TLC method are so high that they enable detection of AFs at the level of 0.1–0.2 ng/spot. The shortcoming of the TLC method is that it needs a densitometer for quantitative analysis.

Enzyme-linked immunosorbent assay (ELISA) has been employed for AF screening, but the method should be applied to limited samples because matrices of the samples often give false positive and negative results. Immunochromatography, which is useful for screening for AFs, has been recently developed for AF analysis.

Confirmation

When AFs are detected, it is necessary to confirm their presence by another analytical method, because some interfering substances remain in the sample solution despite the various purification steps. The comparison of the peak with or without TFA treatment in reverse mode HPLC is not sufficient. The most popular and reliable confirmatory method for AFs with unsaturated terminal furan, such as AFB_1, AFG_1, and AFM_1, is two-dimensional TLC following derivatization with TFA. After the first development, a small amount of TFA is dropped on the spot presumed to be AF and developed in the second dimension. The AFs that have a double bond in the terminal furan ring react with TFA to form their hemiacetals (AFB_{2a}, AFG_{2a}, and AFM_{2a}), which have a lower Rf value than their parent AFs on TLC.

Presently, AFs are sometimes confirmed by liquid chromatography-tandem mass chromatography (LC/MS/MS).

Occurrence in Foods and Feedstuffs

Many reports about AF contamination in foods and feedstuffs are available. AFs are frequently detected in various foods and feeds produced in hot, humid climates. AF contamination of corn is considered to be the greatest health risk to humans and animals throughout the world because the incidence and level of AF contamination of corn are high, and a large amount of corn is consumed worldwide.

AF-contaminated commercial foods and feedstuffs are listed in **Table 4**.

AFs are found in nuts and seeds (e.g., peanut, pistachio nut, Brazil nuts, and sesame), cereals (e.g., corn, rice, buckwheat, and Job's tears), spices (e.g., nutmeg, red pepper, paprika, and white pepper), beans (butterbean), and dairy products (cheese).

AFB_1 is the most frequent type present in contaminated samples and is usually found in the greatest quantity. AFB_2, AFG_1, and AFG_2 are never detected in the absence of AFB_1.

Comparing these results by year, aflatoxin contamination in foods was variable (**Figure 4**).

Aflatoxin M1 in dairy foods is a metabolite of aflatoxin B1 in dairy cattle. Therefore, these results indicated that aflatoxin contamination in feed for dairy cattle decreased after 1985. The reason for this seems to be that the number of countries with legislation controlling aflatoxin in feedstuffs increased from 22 in 1981 to 35 in 1986. Also, the European Community directive was tightened in 1984, when the tolerance for aflatoxin B1 in feedstuffs for dairy cattle was reduced from 20 to 10 $\mu g\,kg^{-1}$.

Aflatoxin contamination in buckwheat was found in 1982–85. The highest incidence was 46%, found in 1985. Since then, no aflatoxin has been detected, possibly because buckwheat from Brazil has not been imported to Japan after 1985.

Until 1992, the incidence of aflatoxins in white pepper was over 30%, but was low in recent years. A high incidence was found in nutmeg throughout the period, reaching over 80% during 1985–90. The contamination level and incidence were then reduced by the efforts of trading companies that collected only good-quality nutmeg from the country of origin.

Some causes of the change in AF contamination in commercial food in Japan were factors in the country of origin, including its weather and regulation for mycotoxins. Other causes were factors in Japan such as examination of mycotoxins at port of entry for imported foods, choice of county of origin, and provision of education about mycotoxins to farmers.

AF in Dairy Products

AFM_1 is sometimes found in dairy products, such as milk and natural cheese (**Table 5**). The level of AFM_1 in dairy products is usually not more than 1 $\mu g\,kg^{-1}$.

Table 4 Aflatoxin contamination in commercial foods (Japan)

			Range ($\mu g\,kg^{-1}$)				
Foods	*No. of samples*	*No. of positive samples*	*AFB_1*	*AFB_2*	*AFG_1*	*AFG_2*	*AFM_1*
Peanut	459	35	0.4–21.7	0.1–5.3	0.3–22.1	0.1–6.8	ND[a]
Pistachio nut	481	9	0.8–1380	0.1–260	306	48.3	ND
Brazil nut	8	1	10.2	0.8	3.2	0.3	ND
Sesame seed	47	5	0.6–2.4	0.2–0.5	ND	ND	ND
Job's tears	212	48	0.1–14.9	0.1–1.8	0.3–0.7	ND	ND
Buckwheat	252	23	0.1–8.8	0.1–0.9	0.2–0.8	0.1	ND
White pepper	220	21	0.1–2.3	0.1–0.3	ND	ND	ND
Red pepper	81	31	0.2–27.7	0.1–1.2	0.1–2.1	0.1–0.2	ND
Paprika	44	26	0.2–6.5	0.1–0.3	ND	ND	ND
Nutmeg	257	155	0.2–60.3	0.1–6.5	0.1–0.4	0.1–0.4	ND
Natural cheese	354	44	ND	ND	ND	ND	0.1–1.2

[a] Not detected (detection limit: 0.1 $\mu g\,kg^{-1}$).

Adopted from Tabata S (1998). Aflatoxin contamination in foods and foodstuffs *Mycotoxins*; **47**: 9–14.

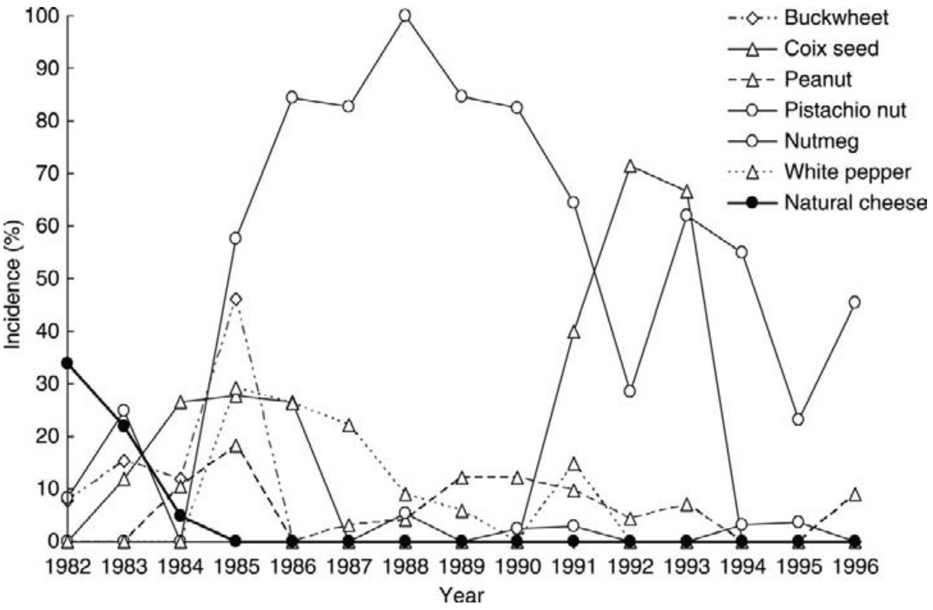

Figure 4 Change in the incidence of aflatoxins.

AFM_1 in cheese is not produced in the fermentation process because of the AF-contaminated feedstuffs consumed by cows. Ingested AFB_1 is converted to AFM_1 in the cows' liver and approximately 0.9% of ingested AFB_1 is found in the milk as AFM_1. Feedstuffs for cows often contain imported materials. Therefore, AFM_1 is also found in dairy products produced in areas not normally associated with AF contamination.

AFM_1 is stable in the fermentation or heating process in cheesemaking, and its levels are not reduced on storage.

Detoxification or Elimination of AFs from Foods and Feeds

For the purpose of reducing the human and animal risk of exposure to AFs, various approaches, including physical, chemical, and biological ones, have been attempted to degrade or eliminate AFs from foods and feeds.

It is fairly easy to degrade pure AFs by various methods, such as UV irradiation, heating, boiling, and treatment with chemical reagents. However, AFs in foods are very stable and the mechanisms of their stability are unknown. Cooking processes, such as roasting, boiling, and frying, cannot reduce AFs. To degrade AFs, many procedures have been proposed, such as gamma irradiation, extraction with solvents, and treatment with ozone, hydrogen peroxide, sodium hypochlorite, and alkali. Most procedures are neither practical nor very effective in reducing AFs to safe levels without damaging the quality of the foods. Before AFs are destroyed, foods are damaged by the treatments. Ammoniated corn may be used for animal feed but not for human food.

An exception is the edible oil refining process. In a normal commercial procedure, all AFs in crude oil are removed by washing with water after adding alkali.

Attempts at biological degradation of AFs have not been satisfactory. AFB_1 is enzymatically converted to AFL, or chemically converted to AFB_{2a}, under acidic conditions of the media by various mycelia. Another biological method, using fungi that do not produce AF to compete with AF-producing fungi, has not succeeded.

Detoxification or elimination of AFs in foods without damage to the quality of the product is hardly possible. Therefore, the efforts to avert the occurrence of AFs in foods seem to be the most effective protection against AFs.

Sterigmatocystin

Sterigmatocystin is a precursor of AFs in their biosynthesis and is also toxic and carcinogenic.

Structure and Chemical Properties

Sterigmatocystin consists of a xanthone nucleus attached to a bisfuran structure (**Figure 5**), similar to AFs.

Sterigmatocystin is soluble in acetone, benzene, ethyl acetate, and chloroform, slightly soluble in ethanol, methanol, and diethyl ether, but insoluble in petroleum ether and water.

Table 5 Aflatoxin M_1 contamination in dairy products

Country	*Period of sampling*	*Type of product*	*No. of samples*	*No. of positive samples*	*Range ($ng\,g^{-1}$)*	*Detection limit ($\mu g\,kg^{-1}$)*	*References*
USA	1979	Cottage cheese	209	1	0.3	0.08	Stoloff and Wood (1984)
		Cheddar cheese	190	0		0.08	Stoloff and Wood (1984)
		Nonfat dry milk	121	0		0.4	Stoloff and Wood (1984)
		Ice cream	328	0		0.08	Stoloff and Wood (1984)
		Yogurt	144	0		0.08	Stoloff and Wood (1984)
Japan (imported)	1982–85	Natural cheese	272	44	0.1–1.2	0.1	Tabata *et al.* (1987)
	1986–96	Natural cheese	82	0		0.1	Tabata *et al.* (1987)
USA (imported)	Before 1985	Cheese	118	8	0.1–1.0	0.05	Trucksess and Page (1986)
Spain	1985	Milk	47	14	0.02–0.1[a]	0.02[a]	Blanco *et al*. (1988)
Brazil	1992	Milk	52	4	0.073–0.37		de Sylos *et al.* (1996)
Japan	2001	Milk	208	207	0.001–0.029	0.001	Nakajima *et al.* (2004)
Taiwan	2005	Milk	144	100	0.001–0.055	0.001	Peng and Chen (2009)

[a] μg/L

Stoloff L and Wood G (1981). Aflatoxin M_1 in manufactured dairy products produced in the United States in 1979. *J.Dairy Science* **64**:2426-2430.

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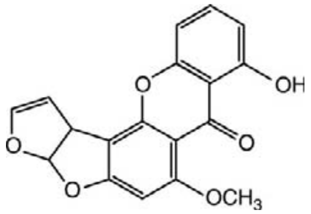

Figure 5 Structure of sterigmatocystin.

Producing Fungi

Sterigmatocystin is produced by several species of *Aspergillus*, including *Aspergillus versicolor, Aspergillus nidulans, Aspergillus sydowii*, and some species of *Bipolaris*. Among them, *A. versicolor* is the major producer of sterigmatocystin, and almost all the isolates produce sterigmatocystin.

Toxicity

The biological activity of sterigmatocystin is much like that of AFB_1, but it is much less toxic than AFB_1. The LD_{50} value of ST for male rats is 60–800 $mg\,kg^{-1}$, whereas that of AFB_1 is 5.5 $mg\,kg^{-1}$. Sterigmatocystin is a potent mutagen. ST is mutagenic at 10 μg/plate; this potency is 1/100 of AFB_1s, which is mutagenic at 0.1 μg/plate. Sterigmatocystin is a primary hepatotoxic agent. All male rats given 150 μg day^{-1}/rat of dietary sterigmatocystin for 58 ± 4 weeks developed hepatocellular carcinomas. The heopatotoxigenic activity of sterigmatocystin is approximately 1/10 to 1/1000 of that of AFB_1. The IARC classified sterigmatocystin as a group 2B carcinogen, which means that it is possibly carcinogenic to humans. No outbreak of the disease in humans and domestic animals attributed to sterigmatocystin has been reported.

Regulation

Although sterigmatocystin is highly toxic, no country has set maximum permitted levels for sterigmatocystin owing to the low incidence of natural occurrence.

Determination

Sterigmatocystin is extracted from ground samples with acetonitrile:4% potassium chloride (9:1). After solvent partition, sterigmatocystin is cleaned up with column chromatography and determined by TLC, HPLC, or LC/MS.

Contamination in Foods

Although sterigmatocystin-producing fungi are widely distributed in the world, reports concerning natural occurrence of sterigmatocystin are few. Sterigmatocystin has been found in stored grains or cheese, but not in the field.

See also: **Yeasts and Molds**: Mycotoxins: Classification, Occurrence and Determination.

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GLOSSARY

Abomasum The 'true stomach' of the ruminant animal with digestive functions similar to the stomach of monogastric species. The abomasum is preceded by the forestomach compartments, the rumen, the reticulum, and the omasum. *See also* **Rumen**.

Absorption The movement of ions, metabolites or a chemical substance through a body membrane. In the case of magnesium it is the movement of Mg ions from the digestive tract into the bloodstream, either by passive diffusion down a concentration gradient, or active transfer requiring an energy source and usually against a concentration gradient.

Acaricide A chemical that kills ticks and mites. It may be mixed with water and put in a dip tank, spray race or used in a knapsack sprayer; there are also pour-on versions available.

Acid detergent fiber (ADF) A method for determining the relative digestibility of fibrous feeds.

Acidulation The process in which cooked acid-precipitated casein curd and whey are gently agitated in a holding vessel (e.g. a vat) to 'condition' the curd. During this period (usually up to 15min), the minerals, especially calcium, in the curd and whey come to equilibrium.

Acrosome reaction A change in the membrane at the apical end of the sperm head in a matured spermatozoon which results in release of enzymes needed for the sperm to penetrate the ovum during fertilization.

Activation energy (E_a) The minimum energy required for a reaction to occur; expressed in Joules (J). It is independent of temperature or concentrations.

Ad libitum A term (literally, 'according to pleasure') that refers to the consumption of food at will. In experimental animal studies, *ad libitum* refers to the provision of food in a manner that allows the animal to consume as much of the food, and at any time, as it desires.

Adhesion The surface reaction between a surface and a particle due to intermolecular attraction forces such as van der Waals' forces or electrostatic forces.

Adjunct culture An adventitious non-starter lactic acid bacteria culture consisting mainly of *Lactobacillus* spp. used in addition to a standard mesophilic starter to improve and to enhance the flavour of cheese. In order to maximize the role of the adjuncts in cheese ripening, the intracellular enzymes must be released from the cells into the cheese matrix. This fact explains the great deal of attention given to cell autolysis during ripening. It is believed that attenuated adjunct cultures with enhanced autolytic properties provide a more controlled and consistent ripening resulting in flavour and texture improvement, particularly in lower fat cheese. Adjunct cultures are modified or attenuated to enable them to play an appreciable role during cheese ripening without producing excess lactic acid. Physical methods of sublethal treatments such as freeze-shocking, heat-shocking and spray-drying are the most studied techniques for the attenuation of the adjunct cells. These treatments lead to varying levels of the cell viability, modification of the ability to produce acid and intracellular proteinase or esterase activities.

Agricultural agreement The agreement within the framework of the World trade organization (WTO). With the establishment of the WTO the regulation of the trade in food and agricultural produce was finally incorporated into the international trading system. This agreement covers export subsidies and competition, market access and imports, and internal/domestic support. All major agricultural trading countries have been forced into changing their agricultural policy according to the agreement.

Alkaline phosphatase An indigenous milk enzyme which is denatured by pasteurization. It is used to demonstrate that milk is adequately pasteurized.

Allergy An abnormal immune response to an allergen, causing adverse clinical reactions. Allergens may be from the environment, e.g. pollens, or from food, e.g. milk proteins. Symptoms are manifest on the skin (e.g. pruritus and urticaria), or the gastrointestinal (e.g. abdominal pain and diarrhea) or respiratory tracts (e.g. asthma). *See also* **Intolerance**.

AM system An automated milking machine that can milk cows without human supervision. The AM system has electronic cow identification, robotic teat cleaning and teat-cup attachment systems and a milking machine to milk the cow. The cow visits the AM system voluntarily, with the inducement of the supply of concentrate. Computer controlled sensors are present to detect any abnormalities in the milking process or the milk.

Aminopeptidases Enzymes, highly conserved in dairy lactic acid bacteria, that release N-terminal amino acids from dipeptides (except for those containing proline), tripeptides and oligopeptides.

Animal model A statistical model used in genetic evaluation in which an animal's estimated genetic merit is a function of its own performance and the genetic merit of its parents and offspring. All relationships among animals are considered, and males and females can be evaluated simultaneously.

Anionic salts Inorganic salts (usually chloride-based) added to close-up dry cow rations to lower urine pH, increase calcium mobilization and raise blood calcium levels to prevent milk fever or hypocalcemia.

Anestrus Absence of cyclicity in a mature intact female. The condition can be caused by seasonal factors, severe underfeeding and suckling of offspring.

Anestrus, lactational The generalized situation in which an animal that is lactating has diminished, delayed or absent reproductive cyclicity. This is usually exacerbated by decreased energy intake.

Anthelmintic A medication used to expel or destroy parasitic worms found in the digestive system.

Antibody An immunoglobulin molecule synthesized in response to a foreign substance which provides an animal with means of protection against that substance by combining specifically with it.

Artificial insemination The introduction of fresh, chilled or frozen-thawed semen into the female reproductive tract using specific devices. Undiluted or diluted semen can be deposited either into the uterus or the oviduct.

Artificial neural network (ANN) A highly interconnected computational structure of elementary processing units (termed neurons) and parameters (termed weights) that are adjusted by an optimization procedure, known as network training. ANNs are implemented for data processing and information storage with its main application in pattern recognition, process modelling, signal filtering and control structure design.

Asthma A reaction involving wheezing and breathing difficulty (or sometimes coughing) caused by reversible narrowing of the lung's airways and often connected with allergic problems.

Atopic dermatitis Eczema, an allergic skin reaction, most commonly seen in small children and often affecting the groin, the creases of the elbows and knees, and the hands and face.

Atopy A genetic predisposition to produce immunoglobulin E against common antigens in the environment with atopic symptoms, e.g. bronchial asthma, allergic rhinitis and atopic dermatitis.

Azadirachtin A compound that exhibits effective insect repellent and sterilization properties. It works on the tick's hormonal system and does not lead to development of resistance in future generations. Generic name: tetranortriterpenoid.

Bacteriocins Antimicrobial ribosomally synthesized peptides that kill species other than the producer species, bacteriocins usually kill closely related bacteria. The three classes of bacteriocins produced by lactic acid bacteria include the lantibiotics, the small heat-stable peptides not containing lanthionines, and the large heat-labile bacteriocins.

Bacteriophage (or phage) A virus that infects a bacterial cell. While the *virulent* phages usually kill the cells they infect, the *temperate* phages do not cause cell lysis but exist in a state called lysogeny where most virus genes are not expressed. Bacteriophages are used as a vector for DNA cloning. Phage infection can rapidly destroy the acid-producing activity of starter cultures.

Bactofugation A technique in which milk is treated in a type of centrifuge with a continuous separation of a small amount of milk that contains dense particles. This heavy phase contains most of the spores of the anaerobic bacterium *Clostridium tyrobutyricum* which have a higher density than those of most other bacteria.

Bifidus factors Compounds of natural origin able to pass intact to the colon, and which are able to enhance the growth of species of *Bifidobacterium* spp. Examples are the complex carbohydrates containing *N*-acetyl glucosamine and L-fucose attached to galacto-oligosaccharide chains in human milk. Other identified bifidus factors include some casein and whey protein digests, lactulose, and cell extracts from *Propionibacterium* spp.

Bioavailability The proportion of a dietary constituent that is utilized for normal body functions.

Biochemical oxygen demand (BOD) An important measure of water quality. It is a measure of the amount of oxygen needed (in milligrams per liter) by bacteria and other microorganisms to fully oxidize the organic matter present in a water sample. It is also called the biological oxygen demand. A five-day biochemical oxygen demand (BOD_5) is commonly determined. The amount of oxygen reported with this method represents only the carbonaceous oxygen demand (CBOD) or the easily decomposed organic matter. BOD_5 is commonly used to measure natural organic pollution. The BOD_5 of drinking water should be less than one, while that of raw sewage may run to several hundred. The BOD_5 of dairy waste may run from several hundred to hundreds of thousand. *See also* **Chemical oxygen demand (COD)**.

Biofilm A life community based on the capability of microorganisms to adhere to solid surfaces, to proliferate at the surface and to form a microenvironment characterized by the excretion of exopolysaccharides (called glycocalyx). Biofilms growing on stainless steel surfaces, particularly in heat exchangers, can be an important source of contamination of dairy products.

Biogas A mixture of gases resulting from anaerobic fermentation of whey, or any other biological matter, and containing methane, carbon dioxide, hydrogen sulfide and other minor gaseous components.

Biohydrogenation the process by which unsaturated fatty acids in the diet are converted to *trans* fatty acids and stearic acid by microorganisms in the rumen.

Biopsy A technique in which small amounts of tissue, such as a single cell, can be removed from a tissue or embryo for examination of the genetic makeup of the particular tissue or embryo from which it was derived and/or examination of changes in cell morphology.

Biosecurity Management practices designed to prevent transmission of disease agents into, or within, a livestock operation.

Biosensor An analytical device including a biological recognition component and a signal transducer. The biological material undergoes a physicochemical change in the presence of the analyte(s). This change is detected by the transducer, amplified and then reported to the operator.

Blastocyst An early embryonic stage represented by a spherical mass of cells with a fluid-filled cavity which forms from the cleavage of a fertilized ovum and exists from approximately 8 to 12 days after fertilization in cattle.

Blitz therapy An antibiotic therapy technique used against mastitis. All infected cows are treated in all quarters simultaneously in an attempt to maximize treatment success.

Bloat A serious and sometimes fatal disorder of ruminants. It is characterized by extreme distension or inflation of the animal's rumen or first stomach, due to the accumulation of gases.

Body condition A general term referring to the relative amount of body fat and muscle on an animal.

Body condition score (BCS) A scale for assessing the level of body fat on an animal. It runs from 1 = very thin to 5 = obese, and may be expressed in decimal values, e.g. 3.5.

Boiler efficiency (η_b) Ratio between the heat received by the water and the heat content of the fuel. The electrical energy that drives the boiler's auxiliary equipment is comparatively much smaller than these values, and is normally neglected.

Bovine lymphocyte antigen (BoLA) genes Genes restricted to the genus *Bos* that are responsible for tissue compatibility between individuals and function in cell-to-cell signalling between lymphocytes and antigen-expressing cells.

Brown mid rib (BMR) A group of mutants in maize, sorghum and millet with higher plant digestibility. Lignin reduces and cellulose and hemicellulose increases BMR. Leaf midrib, stem sheath and pith show a brown coloration in BMR plants.

Browse To feed on buds, leaves or twigs, as distinct from grass (grazing). Goats are typical browsing animals.

Buttermilk, cultured (fermented) A product made from fresh pasteurized skimmed milk or homogenized, pasteurized low-fat milk by fermentation with flavor-producing mesophilic lactic acid bacteria.

Buttermilk, natural (conventional) A byproduct of buttermaking. Depending on the processing conditions, either sour cream or sweet cream buttermilk is obtained. Sweet cream buttermilk can be processed further to fermented buttermilk by flavor-producing mesophilic lactic acid bacteria. Traditionally, buttermilk was the fresh serum that was separated during buttermaking on farms after churning cream ripened with naturally occurring lactic acid bacteria.

Byproduct feed A feed generated during the production of food and fiber products for human consumption. Usually not of the quality or composition for human use, they provide economical sources of feed for cattle.

Calcium-induced precipitation An isoionic precipitation mechanism caused by progressive addition of calcium which decreases the net negative charge of caseins to such an extent that the caseins have little, if any, net charge. Electrostatic repulsive forces are at a minimum and precipitation occurs.

Calf hutches Shelters designed to provide individual housing for calves. Hutches can be made of wood, plastic or fiberglass and are intended to provide adequate ventilation.

Calving interval The time between calving and reimpregnation of the cow.

Calving rate The number of calves born as a percentage of cows mated over a 12-month period.

Canonical transformation A procedure commonly used to reduce computational requirements for simultaneous genetic evaluation of more than one trait. Correlated traits are 'transformed' to uncorrelated traits, which can be evaluated separately and then retransformed, thus reducing computer processing.

CAP genes A group of genes activated at the onset of labor. Contraction-associated protein (CAP) gene expression is increased as estrogen concentration rises at term. CAPs include oxytocin and prostaglandin receptors, Na^+ and Ca^{2+} channels and gap junction proteins (connexin-43), which when activated, increase spontaneous activity of the myometrium.

Capillary electrophoresis (CE) A technique that resolves analytes based on net charge, their mass and Stokes's radius under the infuence of an electric field in a buffer-filled capillary. Capillary electrophoresis is a relatively new technique with the first applications in the early 1980s. One of the major advantages of this technique over traditional electrophoretic techniques is the ease with which quantitative data may be obtained.

Casein The acid-insoluble proteins of milk, which occur as large colloidal aggregates called micelles.

Casein solubilization (1) The process in which, after casein hydrolysis, the peptides become water soluble. (2) The process in which intact casein molecules become dissociated from casein micelles due to an alteration in pH, electrostatic charge or temperature.

Cataract An opacity in the crystalline lens of the eye, which may be partial or complete.

Cation exchange capacity (**CEC**) A measure of a soil's capacity to hold the plant nutrient cations calcium (Ca^{2+}), potassium (K^+), sodium (Na^+) and aluminum (Al^{3+}) to surfaces of negatively charged particles of clay and/or organic matter. Extracts containing ammonium ions displace cations into solution. Individual exchangeable cation concentration is measured in the extract as milliequivalents 100 g^{-1} (meq%) and added to estimate the CEC.

Cheese slurry A semi-solid paste containing about 40% solids and possessing the characteristic flavor of the particular cheese used in its preparation.

Chemical oxygen demand (**COD**) The oxygen equivalent (in milligrams of O_2 per liter) of the organic portion of the sample that is susceptible to oxidation by a strong chemical oxidant. COD does not distinguish between refractory and 'inert' organic matter. COD tests require approximately 3 hours. *See also* **Biochemical oxygen demand** (BOD).

Chocolate bloom Fat or sugar on the surface of chocolate giving a white 'mold-like' appearance. It can be caused by heat damage or by the crystallization of cocoa butter in the wrong form.

Chocolate conche A machine for coating the solid particles in the chocolate with fat and at the same time producing the final flavor. The latter is achieved by removing some acidic volatile components and/or developing other flavors by means of heating.

Chymosin A milk-clotting enzyme produced in the glandular cells of the ruminant abomasum (fourth stomach). Chymosin is an aspartic (acid) proteinase (EC 3.4.23.4) and has a high specifc milk-clotting activity; it primarily hydrolyzes the peptide bond between Phe105–Met106 in bovine κ-casein. Chymosin dominates the milk-clotting activity of calf rennets. *See also* **Rennet**.

Cleanroom A room that is constructed to minimize the introduction of airborne microorganisms or particles and where the concentration of those microorganisms, or particles, is controlled.

Closed flock A flock where all female and some male breeding replacements are produced on the same farm as the breeding flock. This system significantly reduces the risk of introducing new diseases into the farm from purchased replacement stock.

Coagulant A preparation of milk-clotting enzymes of nonruminant origin. Most often they are milk-clotting enzymes derived from different fungi or plants. Coagulants are considered to give a lower yield of cheese and a different cheese flavor compared to calf rennet.

Coagulum *See also* **Gel, Coagulum**.

Celiac disease A disorder caused by a reaction to the gluten of wheat and other cereals in the diet and accompanied by (most notably) bowel disturbances and anemia.

Coffee cream A cream product that usually contains 10% or 12% fat and is manufactured for a long shelf-life either by in-bottle sterilization or, more frequently, by UHT sterilization, followed by aseptic filling. Storage stability (prevention of creaming and sedimentation) and coffee stability (resistance against coagulation or 'feathering') are most important for the quality of the product.

Cold housing A system of housing for cattle in which barn indoor temperature fluctuates with outdoor temperature. Ventilation maintains indoor temperature within 3–6 °C of outdoor temperatures in winter. Usually, the barn is not insulated and ventilation is largely unregulated, except to adjust for seasonal changes.

Coliform bacteria Bacteria that produce acid and gas from lactose. Many, but not all, are of enteric origin. They are killed by mild heat, but occasionally recontaminate pasteurized products.

Colloid A state of matter in which the particles are in the size range of 10 to 1000 nm. Colloidal particles are approximately of the same size as the wavelength of light and therefore strongly scatter light; they are largely unaffected by gravity.

Colloidal calcium phosphate Calcium phosphate that is attached through electrostatic interactions to serine phosphate residues on casein molecules. It is the portion of calcium and (inorganic) phosphate that can potentially be removed from the casein when milk or cheese is acidified. In contrast, the noncolloidal or organic phosphate is directly attached to serine (covalently linked) and can be removed only by enzymatic activity, i.e. phosphatases.

Colostrum Mammary secretions during the early period (3-4 days) post-partum.

Combustion A rapid chemical combination process of fuel with air that releases the chemical energy of the fuel. Air and fuel are the reactants in the combustion reaction, and the byproducts are the flue gases (products of combustion) and heat.

Communal area An area where animals from different herds are communally grazed but may be housed as individual herds at night. Usually, there are no fences in the grazing areas but they may have paddocks.

Concentration polarization An increase in the concentration of a component in the boundary layer of a membrane as a result of its rejection. The phenomenon is characterized by a decrease in permeate flux through a membrane to a constant value, irrespective of increasing transmembrane pressure.

Conception rate The proportion of cows maintaining pregnancy beyond three weeks.

Confocal microscopy A light microscopy technique which greatly reduces out-of-focus blur, enabling optical sectioning of bulk materials. This is particularly useful for shear-sensitive, opaque food materials requiring minimal sample preparation. The most common configuration used in dairy research is the confocal scanning laser microscope.

Conjugated linoleic acid A family of 18-carbon fatty acids with conjugated double bonds. These fatty acids are produced in the rumen during biohydrogenation and by action of the Δ^9-desaturase enzyme within the mammary gland. Isomers can have important effects on human health.

Consumer nominal assistance coefflicient The ratio of the Consumer support estimate (CSE) to the total value of consumption expenditure on farm commodities produced domestically and valued at world market prices, excluding budgetary support to consumers.

Consumer support estimate (CSE) An indicator of the annual monetary value of gross transfers to (from, if negative) consumers of agricultural commodities, arising from policy measures.

Contemporary group A group of animals that are subjected to the same environmental influences (e.g. same age, same herd, same calving season, same location). Comparison of an animal with its closest contemporaries allows a more accurate determination of its genetic merit.

Continuous ice cream freezer A swept-surface heat exchanger, jacketed with a refrigerant, through which ice cream (or frozen dairy dessert) mix is pumped in order to freeze a portion of its water and incorporate small air bubbles.

Cooking of casein Heating of precipitated casein by means of steam injection, or through a heat exchanger, from precipitation temperature to a temperature at which the individual particles of casein agglomerate to form curd of sufficient strength to withstand subsequent wet processing.

Copolymer a polymer made up of monomers of two or more types.

Corpus luteum An ovarian structure that forms following ovulation. It is responsible for the secretion of progesterone during the luteal phase of the estrous cycle and pregnancy. Luteinizing hormone is the major luteotropic hormone that stimulates luteinization of the theca and granulosa cells of the preovulatory follicle into luteal cells.

Cottonseed Seed separated from cotton lint during ginning. It contains a moderate concentration of oil and protein and a high concentration of fiber from lint remaining on hull. Meal is produced from protein and hull as a byproduct when whole seeds are crushed to extract cottonseed oil.

Cow comfort A general term that implies that animals are provided with an environment that minimizes stress, illness, mortality, injury and behavioral problems; an environment that permits them to grow, mature, maintain health, reproduce and produce.

Cream liqueur A cream product combining the flavor of an alcoholic drink with the texture of cream, and expected to have a shelf-life of several years at ambient temperature. Besides a sufficient amount of alcohol and sugar (for microbiological stability), a very fine milk fat dispersion, non-fat milk solids (from cream), water, sodium caseinate and trisodium citrate are the main ingredients.

Cream The part of milk, rich in fat, that can be separated by centrifugation of milk. The fat content of the different liquid and cultured products ranges from 10% to 50%. The special 'creaminess' results from the fine dispersion of the fat globules protected by a special membrane against de-emulsification.

Cross-flow microfiltration A pressure-driven membrane separation process. It could be described as a more porous form of ultrafiltration where instead of molecular weight cut-off criterion, membranes are defined by their pore size in μm. The selective permeation of protein may be facilitated by choice of membrane and optimization of processing conditions.

Cryopreservation A system whereby live cells are preserved using an ultra-low temperature freezing process that allows most of the cells to recover after thawing.

Cultured cream A cream product with various applications as an ingredient in sauces or dressings. The fat content of cultured creams ranges from 10% to more than 40%. The manufacturing process is similar to that for other fermented products. Fermentation may take place in retail packages or in a fermentation tank.

Cytotoxin A toxin that kills mammalian cells.

D value Time in minutes at a defined temperature, required to reduce the microbial population by one log (i.e. to cause destruction of 90% of microorganisms); the temperature is indicated in a subscript, i.e. D_{70} for 70 °C. *See also* **z value**.

***de novo* fatty acid synthesis** The synthesis of fatty acids within the mammary gland, primarily from acetate. It can also be initiated from β-hydroxybutyrate. Key enzymes include acetyl-CoA carboxylase and fatty acid synthase complex. The resulting fatty acids have an even carbon chain length between 4 and 16 carbon atoms.

Dewatering of casein curd The final separation of casein curd and water before the curd is conveyed to the drier. It involves mechanical means for expressing the maximum amount of water from the curd consistent with a friable texture for maximum drying efficiency.

Dewheying of casein curd The separation of casein curd and whey before the curd is washed in water. This may be effected by means of inclined stationary screens and/or by mechanical separation, such as a roller press or a decanter centrifuge.

Dip tank A long, narrow, deep tank into which acaricide solution is poured and through which cattle are herded. The tank should be deep enough so that cattle have to swim through and become covered in acaracide. Also called plunge dip.

Direct government payment A subsidy to producers in the form of transfers from taxpayers rather than through import barriers or government-set minimum prices.

Discounts for multiples of maintenance A method used by the US National Research Council (NRC) to account for decreased digestibility as animals increase energy intake at multiples above the energy required for maintenance. The NRC system for dairy cattle uses discount factors of approximately 8% at 3× and 12.5% at 4× maintenance.

Dispute settlement body A part of the machinery of the WTO. The Dispute Settlement Body and Dispute Settlement System together form the core legal institution within the WTO when solving bilateral disputes concerning trade. Member countries within the WTO are obliged to implement the rulings of the Dispute Settlement Body and no single country has a veto right over the dispute rulings.

DNA array A rigid slide or flexible membrane containing a series of up to tens of thousands of single- or double-stranded DNA polymers used to qualitatively or quantitatively evaluate complementary DNA present in a sample using nucleic acid hybridization.

Domestication The process of genetic adaptation of a species so that it supplies and receives benefits to and from a human population.

Downer cow A cow that lies down and cannot get up. This condition may be due to several causes, such as severe lameness, temporary loss of nerve function after calving, or an acute metabolic disease such as milk fever (calcium deficiency) or magnesium deficiency.

Drench To dose an animal orally with a solution using a bottle, syringe or specifically designed drenching apparatus (drench gun).

Dry matter (DM) The forage component remaining after water has been removed by oven-drying at a controlled temperature (80 °C) until constant weight is attained. Heating to 105 °C will remove all water (oven dry). Pasture and crop yield are often reported as kilograms of dry matter per hectare (kg DM ha $^{-1}$).

Dry matter intake (DMI) The weight (kg) of dry matter consumed by an animal each day after feed refusals have been subtracted.

Dry period The days during which pregnant cows are not being milked. The recommended dry period is the 60-day period preceding calving.

Dynamic compressor A compressor that operates continuously, subjecting air to steady-flow processes. Such a machine has no means of preventing backflow. Distinction is made between axial and radial compressors, depending on the direction of the air flow.

Dystocia A prolonged or difficult delivery. It can be due to either fetal factors (size, birth position) or maternal factors (pelvic size).

Early embryo loss Loss of embryo during the first three weeks of pregnancy.

Edometry The measurement of the physical locomotion of a cow by means of a device attached to its leg. Pedometry is reported to identify 70–80% of the cows in estrus; their activity increases approximately 4 h prior to the onset of standing estrus. The predicted optimum time for artifcial insemination is between 6 and 17 h after increased activity.

Effective fiber Fiber (neutral or acid detergent fiber) provided by plants that are effective at stimulating rumination.

Effective population size The size of a population relative to the amount of inbreeding which is expected to accumulate in that population, frequently smaller than the census number of animals in the population.

Electrochemical process The process by which chemical change is introduced into a system with electricity, e.g. an electrolytic cell, or by which electricity is produced though a chemical change in a system, e.g. a galvanic cell.

Electron microscopy A technique of microscopy in which accelerated electrons, rather than photons, produce images. This offers a much higher resolution than light microscopy. A beam of electrons interacts with the sample to reveal fine structural detail. The two main electron microscopy techniques are scanning electron microscopy (SEM) which reveals topographic features with a high depth of field and transmission electron microscopy (TEM), which is capable of revealing two-dimensional macromolecular structures.

Electrophoresis The migration of charged particles under the infuence of an electric field. Electrophoresis is commonly applied for separation, identification, purification and characterization of a variety of proteins and peptides.

Electrophoretic mobility (*m*) The rate of migration of a particle in an electric field. It is dependent on the conditions under which electrophoresis is conducted. Electrophoretic mobility is equal to the vector sum of the driving force and a number of resisting forces.

Endocrine A term to describe the secretion of a hormone by an internal gland; the hormone is transported (usually via the blood) and received by a distant gland to exert an effect (i.e. stimulation or inhibition).

Enterohemorrhagic Causing hemorrhage in the gastrointestinal tract of the host.

Enzyme A protein formed in cells that acts as a catalyst in initiating or speeding up specific chemical reactions.

Equine chorionic gonadotropin (eCG) A protein hormone produced by the placenta of pregnant mares from about 40 days post mating until mid-pregnancy. This hormone has biological actions similar to follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Also called pregnant mare serum gonadotropin (PMSG).

Equivalence ratio (ϕ) Ratio between the stoichiometric air–fuel ratio and the real air–fuel ratio, $A/\mathrm{F}_{\mathrm{real}}$. This parameter allows one to determine if the combustion is stoichiometric, $\phi = 1$, if the reaction mixture is lean, $\phi < 1$, or if it is rich, $\phi > 1$.

Essential amino acids Amino acids that cannot be synthesized by an organism at a sufficient rate and must be supplied in the diet.

Estrous behavior The behavior expressed by female animals during the period when they are receptive to mating by males. In heifers and cows, the definitive sign of estrus is standing to be mounted by herdmates or a bull. Estrus typically lasts for 8 to 24 hours in cattle and occurs at 18-to 24-day intervals.

Estrus The period of sexual receptivity and behavior in the female brought about by a high systemic concentration of estradiol-17β produced by the preovulatory follicle which stimulates behavior coincident with the ovulatory surge of luteinizing hormone.

Ethylene vinyl alcohol (EVOH) A compound formed by reacting ethylene vinyl acetate with methanol in the presence of catalysts. It is a packaging material with high strength, clarity and good odor and oxygen barrier characteristics and is used as an oxygen barrier in multilayer coextruded plastic containers.

Eutectic The term used to describe the situation in which two dissimilar materials are blended so that they can combine in such a way that the resulting melting point is lower than the melting point calculated from those of the individual components.

Eutherians *See also* **Placental mammals**.

Evaporative cooling The transfer of heat from the body of an animal to the environment by sweating and/or panting.

Export subsidy A government payment conditioned on the export of a commodity that may allow exports even when the domestic market price is higher than the price in export markets. Such subsidies are still used to a small extent by the United States and quite extensively by the EU.

Extended shelf-life (ESL) milk A product processed in such a manner that the shelf-life is extended to 60 to 90 days. The milk still must be held at refrigeration temperature (<7 °C) throughout distribution and storage and will spoil readily once exposed to the environment.

Extensive management Management of animals under range conditions, frequently with migration to seasonal pastures.

Extrusion of animal feeds A process used to reduce the degradability of protein in a feed, involving forcing feed through a die using pressure. Heat generated during the process coupled with pressure alters protein structure and susceptibility to degradation by rumen microorganisms.

Fat cow syndrome A condition in which a cow enters late pregnancy and early lactation with excessive body fat. This can lead to depressed feed intake, ketosis, displaced abomasum and other metabolic or reproductive problems, as well as reduced milk production.

Fatty liver The accumulation of excessive fat in the liver (usually over 10% but can be much higher) which can occur when the rate of lipolysis of adipose tissue exceeds the rate at which peripheral tissues and liver use fatty acids. It can result in diminished functioning of the liver for metabolism of key nutrients.

Feedback control A control procedure that operates by measuring the controlled process outputs and feeding back this information to the controller. The control action is based on comparing the actual (measured) output with the desired one (the reference signal). This is by far the most widely used process control strategy because the feedback makes the closed-loop system robust against perturbations and noise.

Feedforward control A control procedure that operates by measuring important process disturbances (instead of the controlled process outputs) and feeding forward this information to the controller. The control action aims at compensating these measurable disturbances in such a way that process outputs are not affected by them. Feedforward control is usually used in combination with feedback control.

Feedwater A blended composite of make-up and returned condensate waters specifc to boiler installations; the quality of boiler water improves as the proportion of the condensate return increases.

Fertilization rate The proportion of ovulated oocytes that are fertilized.

Final control element (FCE) A device, driven by controller signals, employed to directly manipulate process variables. Usually, the controller output (digital) signal cannot be applied directly to the process. It is first converted by a transducer to a standard (analogue) current signal, which activates the FCE. The most widely used FCEs are flow regulator valves.

Flock health plan (sheep) Written preventative health maintenance strategy agreed by the owner and a veterinary adviser. The plan covers the implementation and timing of routine prophylactic interventions such as vaccination, antimicrobial and antiparasitic treatments, footcare and lambing management. It may also extend to cover other issues such as nutrition, housing, shearing and pasture management as necessary.

Flow cytometer A device whereby individual cells in suspension flow, in single file, through a beam of light with which they interact individually by emitting a measurable response or florescent signal. A cell sorter has, in addition to the analytical system of the flow cytometer, the ability to vibrate the stream exiting the nozzle of the system to form droplets containing individual cells. This cell sorter system utilizes electronics to charge droplets containing selected cells, either positively or negatively, so that charged droplets can be separated physically from other droplets by the electrical attraction of a plate exhibiting the opposite charge. The charged droplets containing selected cells can be collected into a vessel separate from uncharged droplets or droplets of the opposite charge.

Fluazuron The first acarine growth inhibitor to have become commercially available for the control of ticks on cattle. Chemical name: benzoylphenyl urea.

Foil-ripening Method of ripening in which, after brining, the cheese is packed in a foil practically impermeable to gases and water vapor. It is the opposite of naturally ripening cheese that is covered with a permeable coating layer. As a result, foil-ripened cheese develops no rind by drying and the ripening of the outer zone is similar to that of the centre.

Folate Generic name of a native form of one of the B vitamins (synthetic form; folic acid). Folates carry different one-carbon units (methyl, formyl, methenyl, methylene, formimino), produced from the catabolism of certain amino acids, and used for the biosynthesis of purines and pyrimidines for DNA and RNA. Folates regenerate methionine by methylation of homocysteine. *See also* **Milk folates**.

Folate deficiency A condition in humans indicated by low concentrations of folates in erythrocytes and plasma. Severe folate deficiency rarely occurs in Western countries. It causes megaloblastic anemia and a decrease in cell replication and growth. Major causes of folate deficiency are malnutrition, malabsorption and increased demands (during pregnancy).

Follicle-stimulating hormone (FSH) One of the two gonadotropin hormones secreted by the anterior pituitary gland. Its secretion pattern occurs as recurrent transient increases at 7–10 day intervals that stimulate each wave of ovarian follicle growth. A surge in follicle-stimulating hormone secretion also occurs coinciding with the luteinizing hormone surge in the luteal follicular phase (preovulation).

Follow-on formulae Foodstuffs intended for particular nutritional use by infants aged over four months and constituting the principal liquid element in a progressively diversified diet of this category of persons.

Foods for special medical purposes A category of foods for particular nutritional uses, specially processed or formulated and intended for the dietary management of patients and to be used under medical supervision.

Footrot A bacterial infection of the hoof most frequently caused by *Fusobacterium necrophorum* with frequent mixed infections resulting from *Dichelobacter nodosus, Porphyromonas asaccharolytica* and other organisms. Footrot is a leading cause of lameness in sheep, goats, cattle and other even-toed ungulates.

Forage quality A general term for forage nutritive value which often includes plant protein, fiber and energy content as well as other intrinsic factors that may affect consumption and animal performance.

Free air delivery The real volumetric air flow rate produced by a compressor, measured at the admission pressure and temperature.

Freeze-shocking A means for damaging the cell wall and membrane, resulting in cell lysis; it also leads to loss of the ability to produce acid. The range of temperature used has varied from −20 °C to −30 °C for 20–36 hours.

Functional property A term generally used to describe the physical effects of an ingredient in a food. Functional properties may include water sorption, fat emulsification, viscosity, gelation and texturization, but may also refer to solubility, color, flavour and nutrition.

Furosine A relatively stable derivative of the fructose-lysine moiety, formed during hydrolysis with concentrated hydrochloric acid. It is an important marker for the early stage of the Maillard reaction of lysine with glucose (fructoselysine), lactose (lactuloselysine) or maltose (maltuloselysine) and for calculation of blocked lysine, especially in milk products.

Galacto-oligosaccharides Nondigestible carbohydrates which are resistant to gastrointestinal digestive enzymes, but are fermented by specific colonic bacteria. They are produced commercially by transgalactosylation from lactose using β-galactosidases. They are usually sold as a mixture of tri-, tetra- and penta-galacto-oligosaccharides.

Galactose cataract A cataract induced experimentally in animals by an excess of galactose in the diet.

Gametes The mature haploid germ cell lines, spermatozoa or ova, capable of transmitting genetic information to the next generation.

Gel, coagulum A gel is an ordered network of macromolecules or particles that has solid-like properties (i.e. can support its own weight at rest) and has little tendency to synerese, e.g. heat-coagulated egg white or a gelatin gel.

A coagulum is also a network of macromolecules or particles but is less structurally organised and has a greater propensity to synerese than a gel, e.g. yogurt, rennet-coagulated milk. Both a coagulum and a gel exhibit viscoelastic rheological properties, with a preponderence of the elastic (solid-like) modulus. *See also* **Syneresis**.

Genetic evaluation An estimation of the genetic merit of an animal, usually through the use of statistical models, with results expressed as breeding value or predicted transmitting ability, which is half of breeding value.

Glass transition A change between solid and liquid states of a noncrystalline, amorphous substance. For example, sugars can be cooled from melt or dehydrated from solution to noncrystalline solids that exhibit the glass transition.

Gluconeogenesis The synthesis of glucose from new sources (genesis of new glucose). Mammals can synthesize glucose only from propionic acid produced by ruminal or hindgut fermentation, or from several amino acids.

Glycocalyx Exopolysaccharides synthesized by microorganisms adhering to a surface that form a three-dimensional matrix with a sticky character, allowing microorganims to be protected from the environment and to be supplied with nutrients through microchannels and diffusion.

Gonadotropin-releasing hormone (GnRH) A decapeptide hormone secreted by the hypothalamus. It is secreted by hypothalamic nuclei into the hypophyseal portal blood system, which carries it to its site of action in the anterior pituitary. It is responsible for stimulating the secretion of luteinizing hormone and follicle-stimulating hormone.

Gossypol A polyphenolic compound concentrated in the pigment glands of cottonseed. It is toxic to animals without a functioning rumen or when cottonseed or cottonseed meal is fed in large quantities.

Granuloma A mass or nodule of a collection of modified macrophages resembling epithelial cells surrounded by a rim of mainly lymphocytic mononuclear cells and sometimes a center of giant multinucleate cells, either of the Langhan's (epitheloid) or foreign body type, due to a chronic inflammatory response associated with an infectious disease.

Gravimetric analysis Chemical analysis based on the measurement of mass.

Growing degree days (GDD) An index of crop maturity which accumulates from sowing and is calculated from daily temperatures.

Heat stability (objective) The time elapsed between heating a sample of milk (140 °C for unconcentrated milk or 120 °C for concentrated milk) and coagulation as denoted by a sharp increase in viscosity or amount of nitrogenous material sedimentable at low gravitational forces.

Heat stability (subjective) The time elapsed between heating a sample of milk (140 °C for unconcentrated milk or 120 °C for concentrated milk) and the flocculation of solid material.

Heat-induced acidification Decrease in the pH of milk when heated at an elevated temperature (>100 °C) due to thermal oxidation of lactose, precipitation of primary and secondary phosphate as tertiary phosphate.

Heat-shocking A means for reducing the acid-producing capability of bacterial cells without a significant decrease in their proteolytic activities, where the optimum heat treatment varies from 56 to 70 °C with heating time varying from 15 to 22 seconds.

Helminths A broad term that includes many parasitic worms and flukes that are parasites of animals, and thus of veterinary importance, belong to four phyla: (1) Nematoda or roundworms and (2) Platyhelminthes, which include Trematoda (flukes) and Cestoda (tapeworms), (3) Acanthocephala or thorny-headed worms and (4) Annelida or leeches. Phyla (1) and (2) are most important in dairy animals.

Heritability The percentage of phenotypic superiority or inferiority of parents transmitted to offspring. Also, the percentage of phenotypic differences that are explained by additive genetic effects, which ranges from 0 (no genetic control) to near 1 for traits unaffected by the environment.

Heterofermentative *See also* **Lactic acid bacteria**.

Heterosis The average advantage of crossbred animals in comparison to the average of purebred animals from the component breeds. Also known as 'hybrid vigour'.

High-density polyethylene (HDPE) A compound of which the basic building unit is polymerized ethylene. It is a high molecular weight packaging material of great strength, good gas barrier properties, and low clarity. It is generally of low cost.

High-performance liquid chromatography (HPLC) A widely used, highly developed analytical technique for separation of analytes in a complex mixture. The basic theory is the partitioning of the analyte between two phases, one mobile and the other stationary.

High-temperature, short-time pasteurization (HTST) A continuous heat treatment process which destroys all pathogenic bacteria and most spoilage bacteria. The heat treatment is sufficient to denature alkaline phosphatase. Products are heated to a minimum of 72 °C for at least 15 seconds.

Homofermentative *See also* **Lactic acid bacteria**.

Hydrocyanic or prussic acid (HCN) An acid contained in cyanogenic plants, particularly sorghum species, which causes cyanide poisoning and death of livestock. Glucosides in the plant combine sugar and HCN. Glucoside concentration increases when plant growth is restricted. Enzymes released in plants that are damaged (e.g. by chewing, frost or wilting) break down the glucoside and release HCN.

Hydrolytic rancidity Enzymatically catalysed release of free fatty acids from triglycerides. It leads to soapy, goaty or bitter off-flavours in milk.

Hydrophilic Term (literally 'water-loving') used to describe those segments or parts of a protein molecule that prefer to be in water.

Hydrophobic Term (literally 'water-fearing') used to describe those segments of a protein molecule that prefer to interact with the oil phase or other hydrophobic groupings.

Hyperglycemia Elevated blood glucose concentration. This finding is typical in dairy cows, sheep and goats with milk fever.

Hypersensitivity A form of allergy; this term is sometimes also applied to nonimmune-mediated reactions such as intolerance due to an enzyme deficiency.

Hypocalcemia (nonparturient) A depression in blood calcium concentration in dairy cows, sheep and goats that does not occur around parturition. Hypocalcemia in these cases is usually secondary to some other disease problem.

Hypocalcemia (subclinical) A depression in blood calcium concentration around parturition in dairy cows, sheep and goats that causes no apparent clinical signs. Affected animals are at risk of reduced milk yield, ketosis, retained placenta and displaced abomasum.

Hypomagnesemia Decreased blood magnesium concentration. Clinical signs include hyperesthesia and tetany. Also known as grass tetany. Hypomagnesemia can also be a cause of milk fever.

Hypostome The central mouthpart of a tick which serves as the feeding apparatus.

Hypothalamic–pituitary–adrenal axis The endocrine axis that controls the regulation and release of cortisol, which involves the release of hypothalamic (CRH) and pituitary (ACTH) hormones to stimulate the release of cortisol by the adrenal cortex.

Hypothalamic–pituitary–gonadal axis The endocrine system controlling reproduction in animals. It comprises gonadotropin releasing hormone neurons in the hypothalamus, gonadotroph cells in the pituitary (responding to gonadotropin releasing hormone by secreting luteinizing hormone and follicle-stimulating hormone), and the ovary or testis (responding to luteinizing hormone and follicle-stimulating hormone).

Ice cream mix A combination of liquid and solid ingredients, including sources of fat, milk solids-not-fat, sugars, stabilizers, emulsifiers and water, that is blended together, pasteurized and homogenized from which ice cream (or frozen dairy dessert) is manufactured by whipping and freezing.

Immune system The scattered bodily cells and tissues that react to foreign and potentially harmful agents (such as bacteria and viruses) but can sometimes react inappropriately (and unpleasantly) to harmless substances such as foods or pollens, causing allergic reactions.

Immunoglobulins Proteins of the immune system produced by B lymphocytes. They augment phagocytosis and cell-mediated cytotoxic reactions by leucocytes, activate complement system and agglutinate and neutralize microbes and toxins. Immunoglobulins in milk and colostrum protect the offspring against microbial pathogens and toxins and the mammary gland against microbial and viral infections. Immunoglobulins (Ig) occur in five classes: IgM, IgG, IgA, IgD and IgE.

Import barrier Any of a set of policy tools that inhibit imports and protect domestic producers. Examples include import tariffs or duties, limits on the quantity of imports or import quotas, and tariff-rate quotas which comprise a combination of tariffs and quotas.

***In vitro* dry matter digestibility (disappearance) (IVDMD)** A laboratory estimate of feed digestibility. A mixture of rumen fluid, enzymes and feed is incubated at body temperature (39 °C). In a second stage, the incubated mixture is acidifed and digested with pepsin. The residue is dried and weighed. The weight loss is expressed as a percentage of the feed dry matter.

In vitro A term (literally, 'in glass') that refers to an experiment conducted using isolated tissues, cells or biochemical reactions outside of a living animal. Such experiments can be performed, for example, in cell culture dishes or test tubes. *See also* ***In vivo***.

In vivo A term (literally, 'in life') that refers to scientifc experiments performed in a whole, living animal, as opposed to using isolated tissues or cells. *See also* ***In vitro***.

In-line measurement The set of real-time measurements used in process control. Process control can only be based on the measurement of the quantity of interest. Beside physical quantities (temperature, pressure, etc.), the in-line measurement of chemical quantities (constituent concentrations) becomes more important. Infrared spectroscopy offers the possibility to determine the constituent concentrations directly in the production line.

Inbreeding The mating of relatives.

Incidence The new cases of a disease in a specifed population of humans or animals. Incidence may be measured as the proportion or percentage of new cases over a particular time interval, or as the rate of new cases as a function of the length of time that each individual in the population is at risk of becoming a new case.

Infant formulae Foodstuffs intended for particular nutritional use by infants during the first 4–6 months of life and satisfying by themselves the nutritional requirements of this category of persons.

Infrared instrument calibration Procedure for validating infrared absorption data. The infrared absorption measurement is an indirect method, i.e. one has to correlate the measured absorption with the chemically obtained constituent concentration value by using multivariate statistical methods (calibration procedure).

Infrared instrument network Any of several networks, having in common the aim of reducing the amount of calibration work, to check the calibration and to harmonize the results.

Infrared spectroscopy A technique that excites vibrational states of specifc groups of atoms in molecules. It measures the absorption of infrared radiation as a function of the wavelength. The information is used for identification of the substance or quantitative determination of constituent concentrations in the product.

Infusion heating A method of heat transfer in which the target medium is infused into a high temperature steam environment as a fine mist; it effects a rapid increase in temperature with minimal nutrient and flavour degradation. Condensed steam is removed in a subsequent vacuum chamber. *See also* **Injection heating**.

Injection heating A method of heat transfer wherein the target medium is injected with culinary-quality steam under pressure and at sufficient velocity through a specialized port. It effects a rapid increase in temperature with minimal nutrient and flavour degradation. Condensed steam is removed in a subsequent vacuum chamber. *See also* **Infusion heating**.

Intensive management Management of animals in a confined area with permanent housing and equipment for feeding, watering, milking and other activities.

Intolerance An adverse, reproducible reaction to a food or a food component, which is not mediated by the immune system. Food intolerance may be related to an enzyme deficiency (e.g. lactose intolerance) or may have other underlying mechanisms. *See also* **Allergy**.

Ion exchange A process by which ions present in a solution (e.g. in milk or whey) are exchanged for ions that are electrostatically bound to an ion exchanger. The molecules bound to the ion exchange resin can be eluted with buffers of different ionic strength, pH or composition. Ion exchange process is often used to soften hard water. *See also* **Water hardness**.

Ion-selective electrodes (ISEs) Potentiometric analysers that measure the activities of ions in solution. Activity differs from concentration by the activity coefficient and depends on the overall ionic strength of the analyte solution. The most common type is the glass membrane pH electrode.

Isoelectric focusing (IEF) A technique involving separation of analytes based on differences in their isoelectric point, exploiting the fact that each protein has a unique isoelectric point. Analytes migrate through a gel medium containing a pH gradient and cease to migrate at a pH value corresponding to their isoelectric point.

Kelvin model A model of linear viscoelastic behavior. The Kelvin model comprises a Hookean spring and a Newtonian dashpot connected in parallel. *See also* **Maxwell model**.

Kishk A dried fermented milk product made from mixed 'burghol' (preboiled dried wheat grains) and low-fat yogurt or laban zeer (concentrated fermented buttermilk). The mixture is fermented naturally, shaped into balls or nuggets and sundried or dried in warm shade. It is consumed mainly as a porridge-like product.

Knowledge-based hybrid modelling A combination of different modelling techniques based on available process knowledge sources in an integrated hybrid model. The main sources of process knowledge are: (1) classical mechanistic models, (2) heuristic (empirical) knowledge expressed by fuzzy rules and expert systems and (3) data-driven knowledge hidden in the acquired process data.

Kraal An enclosure where animals are housed. Also known as corral.

Kumys Fermented horse milk, containing at mean 2% alcohol, due to the action of bacteria and yeast species. It is frequently consumed in some parts of Russia, West Asia and Mongolia.

Laban kad (rob) A low-fat fermented buttermilk obtained by direct churning of sour milk in goatskin bags called 'kerbah'. Laban kad is made into either Kariesh cheese or concentrated in earthenware jars ('zeer') as laban zeer.

Laban rayeb A low-fat fermented milk obtained after removal of the top sour cream layer from naturally fermented milk. Laban rayeb is consumed either directly, in salads or used for the manufacture of Kariesh cheese.

Labneh A concentrated fermented milk made by straining full cream yogurt or zabady. Addition of table salt (0.2–0.5%) to stirred yogurt before whey removal is optional. Labneh has a soft, smooth, spreadable and creamy texture, with a clean acid taste.

Lactase An enzyme occurring in the small intestine of mammals, as well as in some bacteria and yeasts, which hydrolyzes lactose, yielding a mixture of glucose and galactose; also called β-galactosidase.

Lactic acid bacteria Bacteria that generate lactic acid as the primary product of fermentation and most are associated with dairy fermentations. They are Gram-positive, non-sporeforming and do not produce catalase. They can be either homofermentative (lactic acid makes up over 90% of the end products) or heterofermentive (lactic acid makes up less than 50% of the end products). The main species are from six genera: *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus*. *Lactobacillus, Lactococcus* and *Streptococcus* are typical genera found and used in fermented milk products.

Lactoferrin An iron-binding glycoprotein composed of a single-chain polypeptide sequence of about 700 amino acids with a molecular weight of about 78 kDa. Lactoferrin is found at much higher (>10 times) concentrations in human than in bovine colostrum or milk. It has, among other things, antimicrobial and immunomodulatory functions, and may thus play an important role in the natural non-specifc defense of the body.

Lactose A disaccharide present in milk made up of galactose and glucose.

Lantibiotic A class of bacterially-derived inhibitory peptides characterized by signifcant posttranslational modifications. In particular, lantibiotics possess internal rings formed by the condensation of a dehydrated hydroxy amino acid and a cysteine to form crosslinking lanthionine residues. *See also* **Bacteriocins**.

Leptin A recently discovered protein hormone which is made in the adipose tissue and functions in the brain to partially regulate food intake and reproductive function. Defects or mutations in the gene for this hormone, or for its receptor in the brain, result in genetic obesity in rodents and perhaps in humans.

Limiting amino acids The essential amino acids in digested protein that are in shortest supply relative to body requirements for absorbed amino acids. The first-limiting amino acid is the essential amino that is provided in shortest supply relative to body need. The second limiting amino acid is the essential amino acid that is in the second shortest supply relative to body need.

Lipases enzymes that hydrolyze esters at an oil/water interface.

Lipolysis The enzymatic breakdown of triglycerides into mono-and diacylglycerols and free fatty acids. The enzymes involved are lipases that act at the fat–water interface and esterases that act on water-soluble acylglycerols. The release of fatty acids from the adipose tissue supplies energy to tissues and milk fat precursors to the mammary gland. It increases during glucose or fat deficit as that associated with early lactation.

Lipolytic bacteria Bacteria that are able to produce lipases necessary for splitting fats into partial acylglycerols and fatty acids.

Liposomes An assemblage of phospholipids and other lipids sustaining a biomolecular configuration and not requiring mechanical support for their stability; they are now established as a useful model membrane system. Attempts have been made to use enzymes entrapped in liposomes for the acceleration of cheese ripening and the enhancement of cheese flavor.

Lodging The falling over of certain forage species due to weak stems. Lodging occurs more frequently as the plants mature and may be precipitated by wind or rain.

Low birth weight (LBW) infants Infants born prematurely with a gestational age (GA) of less than 36 weeks or infants born small for GA, i.e. which weigh <2500 g. Infants born with a gestational age of less than 32 weeks are generally referred as 'very low birth weight' infants (VLBW) and are generally less than 1000 g.

Low-temperature, long-time pasteurization (LTLT) A batch heat treatment process which destroys all pathogenic bacteria and most spoilage bacteria. The heat treatment is sufficient to denature alkaline phosphatase. Products are heated to a minimum of 63 °C for at least 30 minutes.

Lower caloric value of a fuel (LCV_f) The chemical energy content of the fuel, per unit fuel mass, when the products of the combustion reaction contain water in the vapor state.

Lower critical temperature (LCT) The temperature at which an animal needs to increase metabolic heat production to maintain body temperature.

Luteinizing hormone (LH) One of the two gonadotropin hormones secreted by the anterior pituitary gland. It is secreted in a pulsatile manner and is stimulated by gonadotropin-releasing hormone pulses. It is secreted as a preovulatory surge in the late follicular phase, which is responsible for stimulating ovulation. Its main functions include final maturation and ovulation of ovarian follicles and acting as a luteotropin that stimulates the corpus luteum formation and maintenance.

Lysine damage (1) derivatization (mainly fructose-lysine) or crosslinking (e.g. lysinoalanine); it results in a loss of bioavailability of lysine and reduced protein digestibility. (2) Complete destruction of lysine by severe heating (total lysine loss).

Lysinoalanine A compound formed by the reaction of lysine with dehydroalanine, which itself is produced under alkaline conditions from cystine and especially phosphoserine (casein). This crosslinking reaction impairs the utilization of the amino acids involved and reduces protein digestibility.

Lysogenic bacteriophage A bacterial virus the genome of which is integrated into the host bacterial genome.

Lysozyme An enzyme found in egg white, milk and many other sources, which digests the cell wall structure of certain bacteria.

Maillard reaction The reaction of free amino groups with reducing sugars forming various compounds that are colorless in the initial and advanced stages and brown-colored in the final stages of the reaction. In proteins, predominantly lysine, with its reactive ε-amino group, is involved, thereby losing its bioavailability.

Major histocompatibility complex A series of genes found in all mammals that are primarily responsible for the compatibility or rejection of grafted tissues and organs between individuals, and also function in the signalling between lymphocytes and antigen-expressing cells.

Make-up water Process water used to replace water losses incurred by overflow or, more especially, evaporation in boilers and cooling systems.

Mammals Class of animals that, 1. possess mammary glands (which may secrete milk for the nutrition of the neonate), 2. can control body temperature (homothermic), 3. have hair or wool.

Management intensive grazing (MIG) A grazing system characterized by high animal concentration per unit of land area and rapid pasture defoliation (usually within 12–72 h), followed by a prolonged rest period (often 14–28 days) for plant regrowth.

Marsupials Mammals with a pouch found in Australasia, South, Central and southern North America. Young are born at a very early stage of development and climb unaided to the pouch where they obtain milk, initially attached to a nipple. The intestine joins the cloaca at its outer end.

Mastitis Infammation of the milk gland, usually caused by bacterial or viral infection.

Maxwell model A model of simple linear viscoelastic behavior. The Maxwell mechanical model (mechanical analogue) comprises a Hookean spring and a Newtonian dashpot connected in series. More complex models are built up by connecting Maxwell models, Kelvin models and individual springs and dashpots in various configurations. *See also* **Kelvin model**.

Melatonin A hormone secreted by the pineal gland mainly during darkness that alters gonadotropin-releasing hormone and gonadotropin secretion. Melatonin secretion controls annual photoperiodic changes in mating activity of, among other things, seasonal sheep breeds.

Mesophilic starter A starter culture with an optimum growth temperature of approx. 30 °C, used for the majority of cheeses.

Metabolic disease A general term to describe metabolic disorders such as ketosis, milk fever, fatty liver and others that most frequently occur during late pregnancy or early lactation. These disorders may be caused by a specific nutrient deficiency or by a blockage of a metabolic pathway.

Metabolite A substance that is produced by living organisms.

Microencapsulation technology A technology recognized as a means for improving the flavor, stability, nutritive value and appearance of foods, offering a means for duplicating some of the protective and selective properties of natural membranes. A more recent application of microencapsulation technology in the dairy industry is the use of enzymes, entrapped with their substrates, for controlled and faster production of flavor compounds in cheese.

Micromineral Any of the minerals required in the diet in very small quantities, usually less than $100\,\text{mg kg}^{-1}$ diet.

Microstructure A general term describing structuring units, or the visual appearance, of materials of microscopic dimensions, from macromolecules to large phases of up to 500 μm. Microstructures may also be inferred by other physical measurement techniques such as rheometry.

Milk fat depression A reduction in milk fat yield with corresponding changes in fatty acid composition of milk fat that is related to dietary conditions with low effective fiber, high fermentable carbohydrate and unsaturated fatty acids. Milk fat composition refects a reduction in *de novo* fatty acid synthesis with greater proportional use of performed fatty acids.

Milk fever An acute drop in blood calcium around the time of parturition in dairy cows, sheep and goats. Clinical signs typically include paralysis and loss of consciousness. Also known as parturient paresis, hypocalcemia, paresis puerperalis and parturient apoplexy.

Milk folates The folates found in milk, comprising mainly methyltetrahydrofolates in mono- and polyglutamate forms. In unprocessed and pasteurized milk, the folates are bound to a folate-binding protein (FBP) which is destroyed upon heating above low-temperature pasteurization. FBP is believed to stabilize the folate and improve folate absorption. *See also* **Folate**.

Milk marketing order A government-sponsored milk-pricing system that sets minimum prices for farm milk delivered within a specifed region in order to raise the average price received by producers of that milk. Milk marketing orders in the United States set minimum prices according to the use of the milk and then specify pooling of revenue across participating producers.

Milk protein coprecipitate The product derived from skim milk that has been heat-treated sufficiently to cause interaction between the caseins and the whey proteins. Precipitation of the caseins by acidification or by addition of calcium chloride causes most of the whey proteins to coprecipitate with them.

Milk protein hydrolysate The products of enzymatic hydrolysis of milk proteins.

Modifed environment housing Usually with minimal insulation and manual control of ventilation, this barn type may have less than adequate ventilation in winter as ventilation openings are blocked during extreme weather to keep manure from freezing or for other reasons. Not increasing ventilation during severe weather leads to excess moisture build-up and high relative humidity; unhealthy conditions for animals.

Monophyletic Term to describe a group derived from a common ancestor. It is applied to taxomonic groupings that are derived from and include a single founder species. The term monophyletic contrasts with polyphyletic, having origin in several different lines of descent, and paraphyletic, which applies to groups such as reptiles which have evolved from and include a single ancestral species (known or unknown), but do not contain all the descendants of that group.

Monotremes The most primitive group of living mammals, found only in Australasia and New Guinea. Embryos develop inside a shelled egg. Once laid, the egg is carried in a pouch (echidnas) or protected in a nest (duck-billed platypus). Young lick milk from mammary glands without nipples. Like reptiles, monotremes have only one opening, the cloaca, for the genital, urinary and digestive tracts.

Morula An early embryonic stage represented by a spherical mass of cells which have resulted from cleavage of a fertilized ovum. In cattle, the cell numbers range from approximately 16 cells on day 4 to 64 cells on day 7 after fertilization.

Mold A fungus that grows by producing filamentous or threadlike structures.

Mold colony A group of fungal hyphae that grow on microbiological media from one spore or cell and appear as velvet, cotton, granular or other textures.

Mycotoxin A metabolite produced by molds that is toxic to other organisms, especially vertebrates.

Mysost whey cheese A cheeselike product manufactured from a whey base (with added cream and/or other ingredients) by removal of most of the water through evaporation.

N^{ε}**-carboxymethyllysine (CML)** A compound formed by oxidative release of erythronic acid from fructoselysine or lactuloselysine; a useful indicator of advanced heat damage in milk products.

Nanofiltration A pressure-driven membrane separation process similar to reverse osmosis, except that its specially formed membranes are also partially permeable to mineral ions and other low molecular weight constituents. Thus, a limited level of product demineralization may be obtained in addition to dewatering/concentration.

Natamycin An antimycotic agent produced by *Streptomyces natalensis*. It is added in small amounts to the coating dispersion before application on the outside of cheese in order to inhibit mold and yeast growth.

Necrotoxigenic Causing death in host tissue.

Nematoda A phylum of worms that are elongate with a cylindrical body pointed at both ends, possessing an alimentary tract; both female and male worms are found. They are an important group of parasites of vertebrates. The most important cattle nematodes are *Haemonchus placei, Ostertagia ostertagi* (abomasal nematodes), *Cooperia oncophora* (intestinal nematode) and *Dictyocaulus viviparus* (lung nematode).

Net positive suction head (NPSH) The difference between the pressure head at a given point and the vapor pressure head.

Neuro-fuzzy control A control procedure that combines the learning and structural properties of an artificial neural network (ANN) with the rule-based explanations associated with fuzzy systems. Knowledge of the process extracted from experienced process operators is formulated as a set of language-based fuzzy rules. This knowledge is complemented by an ANN for modeling and state estimation.

Neuroendocrine Term to describe the stimulation of sensory neurons that results in the release of a neurohormone which then acts in an endocrine fashion. *See also* **Endocrine**.

Neutral detergent fiber (NDF) Plant tissue (total cell walls: structural carbohydrates) not soluble in neutral detergent solution (hemicellulose, cellulose, lignin, and a variable portion of pectins).

Nondigestible oligosaccharides (NDOs) A term to distinguish those oligosaccharides that are not digested in the stomach and small intestine by acid conditions or by hydrolytic enzymes, and which reach the colon largely intact, from oligosaccharides which are digested such as malto-oligosaccharides. NDOs include galacto-, fructo-and xylo-oligosaccharides.

Nonesterified fatty acids (NEFA) Free fatty acids that have been mobilized from adipose tissue and are circulating in the blood. NEFA can be used as a source of energy, ketone bodies, and/or milk fat.

Nonfiber carbohydrates (NFC) An element of the diet of cattle consisting primarily of sugars and starch. It is calculated using the following equation: 100% −% neutral detergent fiber −% crude protein −% fatty acids −% ash. Nonstructural carbohydrates (NSC) consist of the same types of carbohydrates but are determined by a wet chemistry method in a laboratory.

Nonprotein nitrogen The trichloroacetic acid soluble nitrogen remaining in the supernatant following precipitation of the protein in milk. It contains many components including peptides, urea, uric acid, ammonia, free amino acids, creatine, creatinine, nucleic acids, nucleotides, polyamines, carnitine, choline, amino sugars, hormones and other biologically active compounds such as growth factors.

Non-seasonal dairying A commercial dairy production system that employs year-round milking as opposed to seasonal dairying in which all cows are nonlactating for a portion of the year.

Nonstructural carbohydrates *See also* **Nonfiber carbohydrates**.

Nucleic acid hybridization A process by which two strands of DNA (or DNA and RNA) bind. The complementary nature of nucleic acid bases makes this process exquisitely sensitive to small changes in nucleic acid sequence and thus an extremely accurate measure of the identity of the two strands.

Nucleosides *N*-glycosides of pyrimidines and purines.

Nucleotides *o*-phosphoric acid esters of nucleosides.

Oligosaccharides Short chains of monosaccharide units (single sugars) joined together by covalent bonds. Most oligosaccharides that have three or more units do not exist in a free form; rather they are joined as side chains to polypeptides in glycoproteins, proteoglycans or glycolipids.

Oocyte The female gamete (egg) that is released into the duct system during ovulation. It has the potential for fertilization through interaction with a spermatozoon resulting in a one-cell embryo.

Organoleptic A term which concerns, regarding food use, the senses of taste and smell. Organoleptic properties of foods contribute to the enjoyment of the foods rather than to nutritional or other technological properties such as preservation.

Osteoporosis A bone disease characterized by a reduced bone mass, leading to enhanced bone fragility.

Ovarian follicle The structure that nurtures the oocyte (female gamete) in the ovary. Follicles grow in response to stimulation by follicle-stimulating hormone and at later stages of development by luteinizing hormone. There are two, three or four recurrent waves of follicle growth during the estrous cycle in heifers and cows.

Overrun A measure of the air content of ice cream (or frozen dairy desserts), calculated as the percent increase in the volume of ice cream (or frozen dairy dessert) compared to the initial starting mix.

Ovulation The rupture of a mature follicle from the ovary resulting in the release of an oocyte (i.e. female gamete or egg).

Palmar A term referring to the caudal or rear aspect or surface of the forelimb.

Partial coalescence The agglomeration of emulsifed fat into clusters as a result of applied shear, which forms three-dimensional networks due to the presence of both fat crystals and liquid oil in the emulsion droplets. Partial coalescence is responsible for structure formation in whipped cream and ice cream.

Pasteurization *See* **High-temperature, short-time pasteurization (HTST); Low-temperature, long-time pasteurization (LTLT)**.

Pasture A population of herbaceous plants, usually bounded by a fence, considered as a functional unit for grazing.

Pasture-based nutrition Nutrition system in which the majority of the forage consumed is from grazed pasture.

Pasture utilization The percentage of available forage consumed by cattle and other farm animals.

Peak milk The highest level (kg day^{-1}) of milk production achieved during the lactation; typically 40 to 60 days after calving. If fat-corrected milk is used to define peak milk, it may occur 2–3 weeks earlier in the lactation.

Pedometry The measurement of physical locomotion of a cow by means of a device attached to its leg. Pedometry is reported to identify 70–80% of the cows in estrus, whose activity increases approximately 4 h prior to the onset of standing estrus. The predicted optimum time for artificial insemination is between 6 and 17 h after increased activity.

Phenomenological rheology A type of rheology, also called macrorheology or continuum rheology, that treats a material as a continuum, without explicit consideration of microstructure.

Placental mammals The largest suborder of living mammals, giving birth to live young. Exchange of gas and nutrients between mother and fetus takes place via the placenta developed at implantation site(s) in the uterus and formed from maternal and fetal tissues. The large intestine, bladder and uterus open separately to the exterior.

Plantar A term referring to the caudal or rear aspect of the hindlimb.

Plasmids DNA molecules, generally composed of double-stranded, circular or linear DNA, that replicate independently of the bacterial host chromosome, with a size less than 20 times that of the chromosome. Plasmids carry genes that are nonessential for cell survival under non-selective conditions. Many plasmids are used as cloning vectors.

Plasmin An alkaline serine proteinase; the major indigenous protease in milk.

Plasminogen The inactive form of plasmin, converted to active plasmin by proteolytic action of plasminogen activators.

Plasminogen activators (PAs) Serine proteinases that convert plasminogen to plasmin. There are two major types, urokinase-type PA and tissue-type PA.

Plasticization The softening of amorphous substances by solvents. For example, amorphous lactose is softened by water. Such plasticization decreases the glass transition to a lower temperature.

Pliability The ability of cheese to be bent or deformed. If a cheese can be bent or deformed without breaking or splitting, it is said to have a 'long body'. This is often used in the context of eye development in Swiss cheese or in the stretch behavior of Mozzarella cheese.

Polyacrylamide gel electrophoresis (PAGE) A technique involving the use of a polyacrylamide gel as a medium in which to stabilize the electrophoresis buffer. Early PAGE was performed in long cylindrical (pencil) gels, while slab gel PAGE systems are now used more commonly for separation of proteins.

Polycarbonate A polyester formed by reacting phosgene and bisphenol A. It is a packaging material with high strength, rigidity, impact resistance and inertness to food components, used in reusable milk bottles.

Polyethylene terephthalate (PET) A compound formed by low pressure melt polymerization of ethylene glycol and either dimethyl terephthalate or terephthalic acid. It is a high-clarity packaging material with high strength, and excellent gas barrier properties.

Polymerase chain reaction (PCR) An *in vitro* laboratory procedure for detection of DNA that relies on the specificity of DNA–DNA hybridization with a pair of reagent primers for exponential amplification of product. The procedure can be designed to be extremely specific and sensitive.

Polymerization The process by which many molecules are joined together to form a few larger ones.

Polyestrous Term to describe a female animal that exhibits multiple estrous cycles continuously throughout the year or seasonally (i.e. seasonally polyestrous).

Positive-displacement compressor A compressor that operates intermittently, subjecting the air to flow in the same direction as the pressure gradient since it has members that ensure positive admission and delivery of air, preventing undesired reflux. Distinction is made between reciprocating and rotating compressors, depending on the motion of the solid boundary.

Preformed fatty acids Fatty acids in milk fat that originate from dietary lipids absorbed from the digestive tract and from mobilized body fat reserves. These fatty acids are greater than or equal to 16 carbons in length.

Pregnancy toxemia A metabolic disorder of advanced pregnancy in ewes due to inadequate supply of glucose. Incomplete oxidation of body fat in the liver results in hyperketonemia and acidosis. Multiparous ewes and ewes carrying multiple fetuses are more susceptible to pregnancy toxemia that often causes death of the pregnant ewe, hence the alternative name 'twin lamb disease'.

Pressure head Pressure expressed in m of fluid height, i.e. the value of pressure in Pa divided by the density of the fluid in kg m^{-3} and the acceleration of gravity (9.8 m s^{2}).

Pressure ratio (Pr) The ratio between the delivery air pressure of a compressor and the admission air pressure.

Prevalence The existing cases of a disease in a specified population at a given point in time. Prevalence is a function of the incidence of a disease, and the mean duration of disease cases.

Price support A government-set minimum producer price for a commodity. In the United States, the government is ready to purchase manufacturing milk products at a stated minimum price, thus ensuring that any commercial buyer of the manufactured products must pay a price at least as high as the government purchase price.

Primary ripening The term often applied to the hydrolytic breakdown of milk proteins to small peptides and free amino acids and milk lipids free fatty acids. Also included in this term is the fermentation of milk lactose, usually to lactic acid. These compounds form the substrates for secondary ripening reactions. *See also* **Secondary ripening**.

Probiotics Microorganisms, mostly bacteria, that beneficially infuence the host by improving its microbial balance. Well-documented health-related effects of probiotics are, for instance, alleviating symptoms of lactose intolerance, shortening the duration of rotavirus diarrhea and immune enhancement.

Process water Water for general use and circulation within a processing unit. This will normally have been produced by appropriate treatment of ground or surface water ('raw' water).

Processing adjustment factor (PAF) A factor used in the US National Research Council's 2001 calculations that accounts for the effect that particle size, heat, and steam have on the digestibility of nonfiber carbohydrates.

Producer nominal assistance coefficient The ratio of the Producer support estimate (PSE) and the total value of total gross farm receipts valued at world market prices, excluding budgetary support to producers.

Producer support estimate (PSE) An indicator of the annual monetary value of gross transfers from consumers and taxpayers to agricultural producers, arising from policy measures.

Progesterone A steroid hormone produced by the corpus luteum during the diestrous stage of the estrus cycle and pregnancy.

Progesterone releasing intravaginal device (PRID) A silicon coil containing progesterone that is inserted intravaginally into cows and some other species for the purpose of synchronization of estrus.

Protected fat Fat from sources specifically designed to resist biohydrogenation by ruminal microbes and modify fatty acid profile of body tissues and milk.

Proteinase Enzyme that catalyses hydrolytic cleavage of the peptide bonds in proteins and large peptides.

Proteolysis The enzymatic breakdown of protein to smaller peptides and, eventually, to amino acids. There are many different types of proteolytic enzymes (proteases and peptidases) that have different specificities, presenting difficulties in classification and consistency in nomenclature. Common generic names are proteinases (endopeptidases), aminopeptidases (exopeptidases with specificity for N-terminal residues of proteins and peptides), and carboxy-peptidases (exopeptidases with C-terminal specificity).

Proteomics Study of the complete protein profile of cells or secretions.

Protocol A set of rules and formats that govern the way in which devices communicate with each other.

Pseudoplastic Term used to describe fluids that exhibit decreasing viscosity with increasing shear rate. The term preferred now is shear-thinning.

Psychrotropic bacteria Bacteria that are cold tolerant and can grow at refrigeration temperatures, with an optimum usually between 25 and 30 °C and a maximum growth temperature of 35 °C. Their growth rate is much reduced as the temperature approaches freezing.

Puberty The acquisition of reproductive competence in morphologic, behavioral and endocrine terms such that the animal (individual) is capable of reproducing.

Pulsed-field gel electrophoresis (PFGE) A gel electrophoresis method used to separate large DNA fragments (~10–100 kb), frequently used to distinguish between bacterial strains.

Pump A device that promotes the circulation of liquids between and through pieces of equipment.

Pumping efficiency The ratio between the energy absorbed by the fluid in a pump and the energy used by the pump from the electricity supply.

Q_{10} value The fold change in a reaction rate with a 10 °C alteration in temperature.

Quarantine Isolation of newly purchased stock to limit the risk of spreading new diseases to the existing stock on the farm. Quarantined stock will usually have to undergo a series of agreed quarantine treatments set out in the flock health plan before being mixed with existing stock.

Radiotelemetry Radio frequency data communications.

Random amplified polymorphic DNA (RAPD) A technique that utilizes a single short synthetic primer (about 10 bp) of arbitrary sequence, serving as both forward and reverse primers, in a low stingency amplification reaction by the polymerase chain reaction (PCR) in order to generate anonymous DNA fragments from genomic DNA.

Range The interval defined by the upper and lower limit of values of an input variable over which a device can be calibrated.

Real-time Term pertaining to a data-processing system that controls an ongoing process and delivers its output or controls its inputs not later than the time these are needed for effective control.

Recombined milk products Foods that are made by the rehydration of dry milk products and their subsequent processing with or without milk fat.

Reconstitution Term used to describe the process of rehydration of dry milk products into water.

Refrigerant The working fluid that flows inside a refrigeration system. Generally, it is designated by the letter, e.g. R, followed by a number, e.g. R-22, R-134a.

Refrigeration vapor compression system A system designed to fulfill the aims of refrigeration. It is composed of two heat exchangers (one condenser and one evaporator), a compressor and an expansion valve.

Rennet An extract from ruminant abomasa (fourth glandular stomach) containing the milk-clotting enzymes, chymosin and pepsin, in different proportions. Chymosin dominates in extracts from young ruminants (calf rennet). Rennet has been used for the coagulation of milk for cheese since time immemorial and most cheese varieties are produced by use of rennet. *See also* **Chymosin**.

Reverse osmosis The pressure required to reverse the transport phenomenon (osmosis) that prevails when water migrates across a semipermeable membrane in order to equilibrate the osmotic effect created by solutes present in the solution on the other side of the barrier. Hence, it provides an opportunity to concentrate solutes by means of dewatering.

Rheology Science of the flow or deformation of matter.

Ribotyping A nucleic acid hybridization based identification system that uses probes complementary to ribosomal genes to identify the species or even provide subspecies strain information.

Ricotta cheese A cheeselike product manufactured from whey by heating and separation of the coagulated protein paste from the deproteinated whey residue.

Roasting A process used to reduce degradability of protein in a feed, consisting of exposing a feed to heat using a direct or indirect heat source for a fixed time. After heating, the feed is typically steeped for additional time to achieve the desired reduction in protein degradability.

Rumen The largest of the forestomach compartments of the ruminant animal. Ingested forages and other feedstuffs enter the rumen from the esophagus and undergo fermentative digestion by the action of bacterial and protozoal enzymes, a process that permits utilization of cellulose which cannot be digested by endogenous digestive enzymes found in mammals. Products of this fermentative digestion are either absorbed by the rumen as volatile fatty acids or transported via the reticulum to the omasum, abomasum, and small intestines where additional digestive action occurs.

Rumen degradable protein (RDP) The fraction of ingested protein that is degraded by microbes in the rumen.

Rumen undegradable protein (RUP) The fraction of ingested protein that is not degraded in the rumen and passes intact to the abomasum providing amino acids directly from feed for absorption in the lower digestive tract.

Rumen-active fat A fat supplement having the potential to interfere with microbial fermentation in the rumen and reduce feed digestibility when fed to dairy cattle or other ruminant species.

Rumen-inert fat A commercial fat specifically designed to have little, if any, negative effects on feed digestibility when fed to dairy cattle or other ruminant species.

Ruminally protected amino acids Free amino acids that have been encapsulated or coated with materials that will allow most of the amino acids to escape ruminal degradation. At present, commercial forms are limited to methionine. Selective use of ruminally protected amino acids and protein supplements allow dairy nutritionists to more adequately match the profile of absorbed amino acids with the profile as required by the animal. This increases the efficiency of use of absorbed amino acids for growth and milk protein production.

Salmonellosis A disease caused by infection with *Salmonella* bacteria that may affect all animal species and humans.

Secondary ripening The term often applied to the process by which the products of primary ripening, such as amino acids, fatty acids and lactic acid are converted into flavor compounds by a number of different enzyme (and chemical) reactions. *See also* **Primary ripening**.

Sensing element Generic name for a device that detects either the absolute value of a physical quantity or a change in value of the quantity and converts the measurement into a useful input signal for an indicating or recording instrument. Also known as a primary detector.

Sensitivity (process control) The input signal variation required to produce the minimum detectable output signal of a device.

Sensitivity (animal health) The ability of a diagnostic assay to detect true cases of the target disease. An assay with a 65% sensitivity is known to identify a mean of 65% of the truly diseased individuals in a population. Lower assay sensitivities lead to higher probabilities of false-negative assay results.

Sensor A generic name for a device composed of a sensing element and a transmitter, also termed measuring device. It is used in acquiring information on the current status of the process output variables.

Serovar A strain of bacteria that has a characteristic antigenic structure that differs from other strains of a particular genus of bacteria. (e.g. *Leptospira pomona* and *L. hardjo* are serovars of *Leptospira interogens*).

Shelf-life The predicted time at which a product will change from acceptable to unacceptable quality. It is infuenced by such factors as raw ingredient quality, processing conditions, packaging practices, and storage conditions. Typically, shelf-life is determined by a combination of microbial, sensory and chemical methods.

Shubat A fermented product made from camels' milk, produced mainly in Kazakhstan. It is of snow-white color. The fat content reaches 8%. It can be kept for some time without losing its properties.

Silent ovulation Ovulation (release of the ovum) without the expression of estrous behavior.

Soluble fiber Nonstarch polysaccharides not included with hemicellulose and cellulose in neutral detergent fiber. Not digestible by mammalian enzymes, they are typically readily fermented by microorganisms. Includes pectic substances and mixed linkage β-glucans.

Soya bean A legume containing moderate concentrations of oil and protein. Meal is a byproduct produced on extraction of soya oil. Protein concentration is standardized through the addition of hulls.

Span (in process control) The difference between the highest input value and the minimum input value set for a measurement device within its work range.

Specificity of diagnosis The ability of a diagnostic assay to detect individuals that are truly free of the target disease. An assay that has a 60% specifcity will correctly identify a mean of 60% of the disease-free individuals in a population. The remaining mean 40% of disease-free individuals can be expected to have false-positive assay results.

Spirochaete A type of long and slender bacterium, usually only a fraction of a micrometre in diameter but 5–500 μm long. They are tightly coiled, and so look like miniature springs or telephone cords. Members of this group are also unusual among bacteria for the arrangement of axial filaments, which are otherwise similar to bacterial flagella. These filaments run along the outside of the protoplasm, but inside an outer sheath; they enable the bacterium to move by rotating in place. *Treponema* is the only genus to lack the outer sheath.

Spoilage Lowering of eating quality of food due to degradation by microbial, chemical or physical means. Spoiled food is generally not harmful to eat, but is of lower eating quality.

Spontaneous recovery The ability of an animal to cure herself of an infection without the aid of therapy.

Spores Metabolically inactive forms produced by bacteria that allow the cells to survive heat and other stresses. Fungi (yeasts and molds) produce spores as part of their sexual reproduction cycle and provide a means for the microorganism to disseminate in the environment. Fungal spores are not as heat-resistant as bacterial spores.

Standardization A process by which a content of a specific component (protein, fat) is kept at a desired predetermined level in a final product, ingredient or material used in further processing.

Starter Generally, an inoculum of microorganisms used to initiate (start) fermentation in the manufacture of fermented foods. In the manufacture of fermented milk products, a portion of the previous batch was traditionally used to seed the milk. Today, most large-scale manufacture relies on the use of pure cultures of defined or mixed strains. Either mesophilic (e.g. *Lactococcus lactis*) or thermophilic (e.g. *Streptococcus thermophilus, Lactobacillus helveticus*) starter cultures are added to milk, usually at high numbers and primarily to ferment lactose rapidly to lactic acid. *See also* **Lactic acid bacteria**.

Steric A term used to describe any effect that is caused simply by a group physically getting in the way, rather than by any particular properties of the group.

Stocking rate The number of grazing animals per unit of pasture area.

Stoichiometric air–fuel ratio $(A/F)_{\text{stoich}}$ Ratio between the theoretical air mass needed to completely burn a fuel and the fuel mass.

Strain (in animals) The animal's response to stress that usually represents a cost to the animal. The level of strain will vary from animal to animal. Infertility is a strain that is caused by the stress of high milk production in dairy cattle. Stressors may be environmental, physical, physiological or psychological.

Stress *see also* **Strain.**

Subsistence management The rearing of a small number of animals (such as goats) for family food. They may be kept in the family dwelling at night and are frequently tethered around the borders of fields, on road banks, or other limited grazing areas.

Substitution rate The reduction of herbage intake associated with the intake of 100 g of a supplement (both in terms of dry matter).

Subunit vaccines Vaccines generated by biotechnology and genetic engineering. They use only part of a bacterium or virus and produce a potent immune response without stirring up separate and potentially harmful immune reactions to the many antigens carried on a microbe.

Sugars Low molecular carbohydrates, including mono- and oligosaccharides, soluble in 80% ethanol. Typically rapidly fermented by ruminal microbes, they include glucose, fructose, sucrose, lactose, stachyose and raffinose.

Superovulation Ovulation of an abnormally high number of ova as a result of exogenous administration of gonadotropins. Superovulation treatments are conducted mainly as part of embryo transfer programs.

Supersaturation Saturation of a solution with a solute beyond the solubility of the solute.

Syneresis The process by which a network of macromolecules or particles (gel or coagulum) contracts through the formation of new bonds and/or the rearrangement of existing inter-molecular or interparticle bonds, which results in the expression/exudation of serum, i.e. liquid phase. *See also* **Gel, Coagulum**.

Taxonomy The study of the classification of organisms according to their similarities and differences.

Teat canal keratin Protein deposit formed by the surface cells of the epithelium of the teat canal desquamating continuously. Keratin plays an important role in the prevention of mastitis.

Teat-end callosity A change in teat-end tissue as a result of mechanical forces exerted by vacuum and the collapsing liner during machine-milking.

Temperature-humidity index (THI) A single numerical rating of an environment based upon the temperature and humidity. It is often used to measure the degree of animal stress due to heat stress conditions. Equation is: THI $= (0.81 \times$ dry bulb temperature, °C) $+$ (relative humidity $\times$ (dry bulb temperature $-14.4)) + 46$.

Temperer A machine for crystallizing the fat in chocolate in the correct form. It operates by cooling to produce several different crystalline states. Heating and intense mixing are then used to remove all but the required stable form.

Tempering (in casein manufacture) The process in which, after fluid-bed drying, in particular, the particles of warm casein are transferred between bins for a period of 8–24 h. This allows transfer of moisture from the larger, wetter particles to the smaller, drier particles, producing casein of uniform moisture ready for milling.

Tetany A state of increased neuromuscular irritability caused by reduced serum magnesium concentrations and manifested by twitching of the extremities, intermittent muscular spasms, loss of consciousness and convulsions.

Tetraploid A plant that contains four sets of individual chromosomes within a cell. This is double the more usual diploid two sets per cell. Tetraploids develop naturally or are induced by treating dividing diploid cells with colchicine. They will breed true, producing tetraploid offspring. Tetraploid cells are larger than diploid cells to accommodate the additional chromosomes.

Thermization Process for heating milk with a heat load below pasteurization level before storage. The indigenous enzyme alkaline phosphatase is not fully inactivated but the result is that psychrotropic bacteria are practically killed without substantial denaturation of whey proteins. After storage, the milk is pasteurized before further processing.

Thermoduric bacteria Bacteria that can survive exposure to temperatures higher than the maximum temperatures for their growth. In the dairy industry, the term refers to microorganisms that survive pasteurization but do not grow at pasteurization temperatures.

Thermoneutral zone (TZ) The range of environmental temperatures in which normal body temperature is maintained and heat production is at the basal level.

Thermophilic bacteria Bacteria that grow at elevated temperatures (55 °C or higher) with an optimum temperature of about 60 °C.

Thermophilic starter A starter culture with an optimum temperature above about 40 °C used for Swiss-type cheeses and for yogurt.

Thermophoresis Movement of substances due to a thermal gradient.

Thixotropic Term used to describe materials exhibiting time-dependent shear-thinning, i.e. decreasing viscosity with time at a given shear rate.

Total dissolved solids (TDS) The weight of solids in solution per unit volume of water, measured by evaporating a known volume of filtered solution and weighing the residue. *See also* **Total solids**.

Total mixed ration (TMR) A feeding system in which all feed ingredients are blended together in specific proportions or amounts and offered to dairy cattle as one feed resource. Also known as complete diet.

Total potentially available nucleosides (TPAN) All potential sources available in milk for the generation of nucleosides via digestion and metabolism.

Total solids (TS) The weight of all solids, dissolved or suspended, organic or inorganic, per unit volume of a liquid, measured by evaporating a known volume of the liquid and weighing the residue. *See also* **Total dissolved solids**.

Total support estimate (TSE) An indicator of the annual monetary value of gross transfers from taxpayers and consumers to the agricultural sector, arising from policy measures that support agriculture.

Total suspended solids (TSS) The measure of particulate matter suspended in a sample of water or wastewater. After filtering a sample of a known volume, the filter is dried and weighed to determine the residue retained.

Transconjugant A strain that has acquired genetic information (and an associated trait) through the conjugal transfer of a plasmid or a transposon from a donor to a recipient cell.

Transducer Any device or component that converts an input signal of one form to an output signal of another form. Devices such as sensing elements, transmitters and signal transducers are considered as transducers.

Translucent color A semi-transparent appearance to cheese, i.e. allowing some light to penetrate. It is typically seen in reduced-fat cheeses. If translucent cheese is sliced very thin, shadows of objects can be seen behind the cheese.

Transmitter (1) A transducer that responds to a measured variable by means of a sensing element and converts it to a standardized transmission signal, which is a function only of the measurement. (2) A device that converts a variable into a form suitable for transmission to another location.

Ultrafiltration A membrane filtration process that separates components on the basis of molecular 'sieving'. Macromolecular material, such as protein (and fat), is readily recovered as retentate from solutions such as whey, while lower molecular weight material (lactose, minerals, other solutes and water) permeate the membrane. Ultrafiltration operates at a much lower pressure than reverse osmosis and nanofiltration.

Upper critical temperature The highest temperature at which the normal body temperature can be maintained without altering basal metabolic rate.

Urticaria Hives, the itching weals that can come and go on the limbs and trunk, associated in some cases with allergy and the local release of histamine.

Uterotonic agents Hormones that induce uterine (myometrial) contractions. For example, oxytocin is a potent uterotonic stimulant.

Vector A living organism that transmits a pathogen and causes a disease.

Ventilation A process of air exchange, which serves to dilute inside barn air and all of its components, many of which at high concentrations lead to animal health problems.

Vernalization The process of exposing plants to low temperature for a defined period. Some plants require exposure to a period of low temperature before they will initiate flowering. Without the temperature stimulus, the plants remain vegetative. For example, winter cereals require vernalization to flower while spring cereals flower without a cold stimulus.

Virial coefficients The coefficients in the second and higher-order terms of a virial equation. Such an equation consists of a limiting law as the first term with further terms added to account for effects ignored by the limiting law. For example, the virial equation for osmotic pressure is based on the van't Hoff equation as the limiting law, and the coeffcient of the second term is called the second osmotic virial coeffcient.

Viscoelastic Term used to describe materials exhibiting both liquid-like (viscous) and solid-like (elastic) properties.

Vitamin An organic compound found in the nonenergetic part of food. They are essential for the normal functioning of the body. Vitamins are necessary for growth, vitality and general well-being. With few exceptions the body cannot synthesize vitamins, so they must be supplied by the diet.

VO_{2max} The level of exercise recorded when maximum oxygen uptake has occurred despite additional increases in exercise; VO_{2max} indicates an individual's capacity for aerobically resynthesizing ATP. Exercise performed above VO_{2max} can only take place by energy transfer predominantly from anaerobic glycolysis with lactate formation.

Voltammetry A group of widely used electrochemical techniques, where the current flowing through an electrochemical cell is measured as a function of the applied potential. Examples of this kind of technique are polarography, amperometry and cyclic voltammetry.

Volumetric effciency (ηv) A measure of the decrease in compressor flow rate, given by the ratio between the free air delivery and the piston displacement per unit of time.

Warm housing A system of housing for cattle in which barns are well insulated and, by necessity, have a well-controlled ventilation system. These barns are designed to provide a relatively uniform environment throughout the winter. Tie-stall dairy barns are an example. Ventilation must be regulated to compensate for changing outside climatic conditions.

Water activity (a_w) The ratio of water vapor pressure in a system to that of pure water at the same temperature.

Water absorption (farinograph method) The amount of water absorbed during a standard test, using an instrument called farinograph. The product under test (such as milk protein coprecipitate) is added to a standard formula consisting of a standard four and water.

Water hardness A term describing the inability of some waters to rinse clean natural fibers of oil and fatty substances. Hardness of water is caused by large concentrations of calcium, magnesium and other multivalent metallic ions. A hard water reduces the effects of detergents, because it forms precipitates with the soap and forms troublesome scales on heating equipment.

Wet chemistry (1) Chemistry involving the use of water and/or other solvents. (2) Classical gravimetric, titrimetric or colorimetric analysis.

Whey Liquid phase of milk that remains after removal of fat and casein; *also*, The liquid byproduct of cheesemaking or manufacture of industrial casein products.

Whey beverage Any whey-based drink prepared from liquid whey or whey permeate, usually sweetened and containing fruit juices or other compatible ingredients.

Whey protein nitrogen index (WPNI) An index used as a means to classify milk powders as low, medium and high heat milk powders, based on the reduction in solubility of whey proteins due to heat denaturation.

Whipping cream A typical cream product that has a fat content of 30–40% and is processed without (or with low-pressure) homogenization. Originally, it required no complicated preparation, just careful handling before whipping. The demand for a considerable prolongation of shelf-life may lead to the demands for a premium product with a unique flavour.

World trade organization (WTO) An organization established in 1995 as the successor to the General Agreement on Tariffs and Trade (GATT). It aims to ensure the development of a nondiscriminating, smooth, predictable, and free trade between member countries. The WTO evolves around trade negotiations between the 144 member countries.

***z* value** the change in temperature (in degrees Celsius) required to bring about a one log (i.e. tenfold) change in the D value. *See also* **D value**.

Zabady A traditional plain set yogurt, which is made mainly from nonhomogenized buffaloes' milk. It is characterized by a surface skin high-fat top layer and cooked flavour. Zabady from the previous batch is usually used as starter.

Zoonosis An infectious disease of animals that can be transmitted to humans under natural conditions. Also known as zoonotic disease.

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NOTES:

Cross-reference terms in *italics* are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space). Readers are also advised to refer to the end of each article for additional cross-references – not all of these cross-references have been included in the index cross-references.

The index is arranged in set-out style with a maximum of three levels of heading. Major discussion of a subject is indicated by **bold** page numbers. Page numbers suffixed by t and f refer to Tables and Figures respectively. *vs.* indicates a comparison.

This index is in letter-by-letter order, whereby hyphens and spaces within index headings are ignored in the alphabetization. For example, 'milk protein' is alphabetized after 'milking'. Prefixes and terms in parentheses are excluded from the initial alphabetization.

Where index subentries and sub-subentries pertaining to a subject have the same page number, they have been listed to indicate the comprehensiveness of the text.

ABBREVIATIONS:

HPLC – high-performance liquid chromatography
LAB – lactic acid bacteria
NMR – nuclear magnetic resonance
NSLAB – non-starter lactic acid bacteria
PAGE – polyacrylamide gel electrophoresis
PCR – polymerase chain reaction

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